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# Food derived bioactive metabolites: unlocking their potential health benefits and medical potential

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# Food derived bioactive metabolites: unlocking their potential health benefits and medical potential

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# Editorial: Food derived bioactive metabolites: unlocking their potential health benefits and medical potential

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Editorial on the Research Topic
Food derived bioactive metabolites: unlocking their potential health benefits and medical potential

## 1 Introduction

The "food and medicine continuum" is commonly seen in different geographic and biocultural backgrounds, as many food-derived metabolites also have health-promoting functions (Yao et al., 2023). Two decades ago, the study of edible medicines was reported as "an ethnopharmacology of food" by Etkin, (2006). In this context, the present Research Topic aims at investigating the intricate relationship between medicines and foods across various cultures and traditions. Components isolated from these food-medicines can serve preventative roles, support physiological functions, or act as targeted treatments due to their interaction with specific receptors. Given these overlapping domains, a comprehensive understanding of the scientific potential of food-derived metabolites is crucial. The present Research Topic, titled "Food-Derived Bioactive Metabolites: Unlocking their Potential Health Benefits and Medical Potential", brings together several contributions. Articles were focused on the study of food-based metabolites and their bioactivities, highlighting their health-promoting potentials.

This Research Topic starts with a study on two vegetable species from Asia, namely, *Colocasia affinis* Schott and *Colocasia gigantea* Hook. f. by Alam et al. The authors isolated six bioactive phytochemicals from these two *Colocasia* species and elucidated their structures employing the NMR technique; tested their health functions, including antidiarrheal, analgesic, and anti-inflammatory, were then tested. Tian et al. identified 85 components from the fruit *Sinopodophyllum hexandrum* (Royle) Ying, which is mainly distributed in the Himalayan alpine region, and explored the *in vivo* transformation of their chemical components. Their anti-tumor activities were studied with network pharmacology and provided new insights into developing natural anti-tumor agents. Olas presented a mini

review on an African species, marula [*Sclerocarya birrea* (A. Rich.) Hochst.], and its products, with a special emphasis on their chemical composition, biological activity, and health-promoting potential. Jakimiuk et al. reported the *ex vivo* biotransformation of lady's mantle extracts via the human gut microbiota, with focus on the formation of phenolic metabolites and their impact on human normal and colon cancer cell lines. The genus *Elsholtzia* Willd. comprises fragrant food-medicinal species, and Inta et al. reported on the species found in Northern Thailand, using ethnobotany, chemical analysis, and anti-glycation activity, providing important clues for the development of food-medicinal uses of this taxon.

Several globally popular species are also studied in this Research Topic. The genus *Citrus* L. includes many of the common edible fruits, and their peels are a rich source of flavonoids; Xu et al. summarizes the types, bioactivities, and mechanisms of action of *Citrus* flavonoids, providing scientific evidence for their research and development. Du et al. reviewed the bioactivity and biomedical applications of pomegranate (*Punica granatum* L.) peel, which is rich in various bioactive metabolites such as polyphenols, tannins, and flavonoids, showing high medicinal and nutritional value. Liu et al. conducted a systematic review on the pharmacology and mechanism of action of *Monascus purpureus* Went, providing a reference for future study. From a perspective of sustainability, Marrone et al. discussed the important role of plant-based diets in human health, with an emphasis on chronic kidney disease prevention and treatment.

Moreover, bioactivities of specific compounds have also been studied. Luteolin is a flavonoid widely found in food plants; Lv et al. summarized the research advancements in improving the solubility and bioavailability of luteolin, and discussed its therapeutic effects in the treatment of pulmonary diseases. In the study by Enkhbat et al., grifolin and grifolic acid were isolated from the edible mushroom *Albatrellus confluens* (Alb. and Schwein.) Kotl. and Pouzar and structurally confirmed, their inhibitory activity was validated *in vitro*, and their metabolic effects were tested in a mouse model of diet-induced obesity. It was found they can act as sphingomyelin synthase inhibitors, prevent weight gain, and improve vitamin D homeostasis. Resveratrol is a natural polyphenolic compound. Lv et al. presented a comprehensive and systematic review on resveratrol intervention in animal models of retinal diseases, providing preclinical support for its possible therapeutic uses in the management of retinal diseases. Formononetin is a common natural metabolite; Jin et al. presented a review on its sources, pharmacological activities and molecular mechanisms, co-administration, toxicity, derivatives, and drug delivery systems over the last 5 years. Royal jelly acid (10-HDA) is an unsaturated fatty acid unique to royal jelly—Zhi et al. contributed a review on its preparation, metabolism, and potential pharmacological activities in managing cancer, inflammatory disorders, and glucolipid metabolic diseases.

In the area of traditional Chinese medicine, Liu et al. present the development and preliminary mechanistic analysis of compound ganoderma lucidum hepatoprotective effervescent granules. Liu et al. reviewed the natural metabolites from medicinal plants used in traditional Chinese medicine for cardiovascular diseases by highlighting pharmacological mechanisms, evidence, and future directions; specifically, they suggest how traditional Chinese medicine-derived therapies could be integrated into cardiovascular care as a novel multi-target approach.

## 2 Concluding remarks

People from different cultural backgrounds and biogeographic regions have different strategies for sustaining their lives and avoiding hunger and illness. Plants have proven to be common substances for food and/or medicine. In this Research Topic, contributors report the plants used for medicinal purposes, reveal their active compounds, and indicate their potential health uses. Moreover, several species used on a global scale are also presented. This shows that the framework of cross-cultural assembly of traditional plant knowledge will benefit global health. Ethnopharmacology is still a reliable way to demystify the health-promoting mechanism of food-medicinal plants and is thus proof of the 'food and medicine continuum'.

## Author contributions

RY: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Writing – original draft, Writing – review and editing. ML: Conceptualization, Data curation, Formal Analysis, Investigation, Resources, Software, Validation, Writing – original draft, Writing – review and editing. AD: Conceptualization, Data curation, Formal Analysis, Investigation, Project administration, Resources, Visualization, Writing – original draft, Writing – review and editing.

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# First-time report on compound isolation from two *Colocasia* species: vegetable-derived bioactive metabolites and their medicinal potential

Safaet Alam [1,2], Fahmida Tasnim Richi [2], Nazim Uddin Emon [3], Abu Asad Chowdhury [2], Choudhury Mahmood Hasan [2] and Mohammad Rashedul Haque [2]*

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**Background:** *Colocasia affinis* Schott and *Colocasia gigantea* Hook.f. are two commonly found vegetable species of the genus *Colocasia*, found mainly in the Asian region.

**Objectives:** The objective of this study was to isolate bioactive phytochemicals from *C. affinis* and *C. gigantea* and elucidate their structure employing the NMR technique followed by bioactivity evaluation.

**Methodology:** Column chromatography was utilized to isolate phytochemicals, followed by NMR analysis for characterization. An *in vivo* analgesic test was performed through an acetic acid-induced writhing test, an anti-inflammatory test was performed through a formalin-induced licking test, and an antidiarrheal test was performed through a castor oil-induced diarrhea model. The *in vitro* antimicrobial test was executed through the disc diffusion method. Computer-aided simulation was also implemented to validate the wet laboratory results.

**Results:** Six compounds from *C. affinis* and *C. gigantea* were isolated and characterized from the dichloromethane (DCM)-soluble fractions of the methanolic extracts of these two species. Three of the compounds were from *C. gigantea* and proposed as penduletin **(C1)**, a mixture of α-amyrin **(C2a)**, β-amyrin **(C2b)**, and monoglyceride of stearic acid **(C3),** while the remaining three compounds were from *C. affinis* and proposed as penduletin **(C4)** (which was also isolated from *C. gigantea*), 7,8-(3″,3″-dimethyl-pyrano)-4′-hydroxy flavonol **(C5)**, and lastly a mixture of 7,8-(3″,3″-dimethyl-pyrano)-4′-hydroxy flavonol **(C5)** and 4′,7,8-trihydroxy flavonol **(C6)**. These compounds showed promising zones of inhibition against Gram-positive and Gram-negative bacteria and fungi. In the antidiarrheal test, **C5** demonstrated the highest reduction in castor oil-induced diarrhea (44.44%) at a dose of 20 mg/kg compared to loperamide's 77.78% reduction. However, the analgesic assessment showed a noteworthy inhibition of acetic acid-induced writhing by **C1/C4** and **C2** (56.52%) at a dose of 20 mg/kg compared to the 76.09% by diclofenac sodium. In comparison, **C2** showed pronounced anti-inflammatory action by 68.15% and 52.06% reduction,

respectively, in the early and later phases compared to the ibuprofen's outcomes of 73.54% and 74.68%. Plausible targets such as dihydrofolate reductase (DHFR) for antimicrobial, kappa opioid receptor (KOR) for antidiarrheal, and cyclooxygenase 2 (COX-2) for anti-inflammatory and analgesic activities showed a noteworthy binding affinity with isolated compounds, and ADME/T studies displayed these phytochemicals' drug-likeness profiles.

**Conclusion:** To the best of our knowledge, this is the first report on compound isolation from these plants, which demands further extensive research for more absolute findings.

KEYWORDS

***Colocasia gigantea*, *Colocasia affinis*, vegetable, NMR, antibacterial, antidiarrheal, analgesic, anti-inflammatory**

## Introduction

Since time immemorial, mankind has used plant extracts from different medicinal plants to cure many diseases and thus relieve physical agony, playing an essential role in the world's healthcare (Yadav et al., 2006). A wide array of phytoconstituents, called secondary metabolites, which do not appear to contribute directly to plant growth and reproduction, are responsible for the pharmacological and therapeutic effects. Thus, purification, isolation, and bioactive assessment of such secondary metabolites from medicinal and vegetative plants are widely practiced (Duraipandiyan et al., 2006).

Araceae plants (family Arum ) are commonly known as "aroids" and are distributed all over the world, and are abundant in tropical and sub-tropical regions (Ara and Hassan, 2019). *Colocasia* is one of the 27 genera of the Araceae family available in Bangladesh. It is also native to southeastern Asia and the Indian subcontinent (Wagner et al., 1990; Ara and Hassan, 2019). In Bangladesh, this genus of flowering plants is known to contain the following nine species: *C. affinis* Schott, *C. esculenta* (L.) Schott, *C. fallax* Schott, *C. gigantea* (Blume) Hook. f., *C. heterochroma* H. Li et Z.X. and Wei, *C. lihengiae* C.L. Long et K.M. Liu, *C. mannii* Hook. f., *C. oresbia* A. Hay, and *C. virosa* Kunth (Ara and Hassan, 2012). *Colocasia* leaves have demonstrated antidiabetic, antihypertensive, immunoprotective, neuroprotective, and anticarcinogenic activities. Detailed assessment of the phytochemical compounds present in various extracts of the leaves has shown the presence of active chemical compounds like anthraquinones, apigenin, catechins, cinnamic acid derivatives, vitexin, and isovitexin, which are possibly responsible for the exhibited biological properties (Gupta et al., 2019). Phytochemical extraction and structural elucidation of *Colocasia* leaves also yield notable bioactive chemical compounds, such as isoorientin, orientin, isoschaftoside, Lut-6-C-Hex-8-C-Pent, vicenin, alpha-amyrin, beta-amyrin, monoglycerol stearic acid, penduletin anthraquinones, apigenin, catechins, cinnamic acid derivatives, vitexin, and isovitexin (Gupta et al., 2019).

Pathogenic bacteria are one of the leading causes of morbidity and mortality in humans, driving pharmaceutical companies to develop numerous new antibacterial agents to address infections, which have become a global concern. Clinical microbiologists are particularly interested in plant-derived antimicrobials for two primary reasons: the potential of phytochemicals to serve as effective antimicrobial agents prescribed by healthcare professionals, and the need to increase awareness of the risks associated with the misuse of conventional antibiotics. Additionally, diarrhea is a common condition marked by frequent bouts of watery bowel movements and abdominal pain. It is a major problem in developing countries, often leading to malnutrition and even death (Workneh et al., 2024). Plant extracts have shown promise in treating diarrhea by helping the body reabsorb water, preventing electrolyte loss, and slowing gut movements (Agbor et al., 2004; Alam et al., 2020). Furthermore, inflammation manifests through a multitude of pathways and different mediators, leading to a spectrum of adverse effects like necrosis, degeneration, and various forms of exudation (Medzhitov, 2008). To alleviate pain and inflammation, analgesic medications such as opioids (like morphine and fentanyl), NSAIDs, and emerging treatments such as gabapentin, carbamazepine, and ketamine are commonly employed. Glucocorticoids exert their effects by binding to receptors, resulting in enhanced transcription of anti-inflammatory proteins (such as IL-1 antagonists) and inhibition of activated transcription factors (e.g., NF-κB) (Barnes, 1998). NSAIDs also inhibit cyclooxygenase enzymes (COX-1 and COX-2), which are responsible for synthesizing various inflammatory mediators. Despite the array of NSAIDs available, they pose significant side effects, including gastrointestinal ulceration, liver toxicity, and kidney disease, particularly with prolonged usage (Sostres et al., 2010). More specifically, COX-2 selective inhibitors run the risk of harming the heart, while non-selective inhibitors tend to wreak havoc on the gut and kidneys (Emon et al., 2021a). Hence, exploring novel phytochemicals holds promise for the development of more effective alternatives (Liu, 2007).

In addition to *in vitro* and *in vivo* studies, in today's scientific realms, computational biology plays a pivotal role, in facilitating the generation and validation of vast amounts of data used by today's molecular and experimental biologists. Presently, this paradigm enables the meticulous exploration and verification of drug design endeavors for nascent molecules. The adoption of computer-aided drug discovery (CADD) techniques alongside molecular docking stands as an efficacious and expedient *in silico* approach (Parmar et al., 2023; Parmar et al., 2020). Prudent molecular docking methodologies must adeptly discern the positioning of the native ligand within the three-dimensional confines of the binding site of the protein structure while also taking into account their intricate physicochemical interactions (Parmar et al., 2022; Guedes et al., 2014).

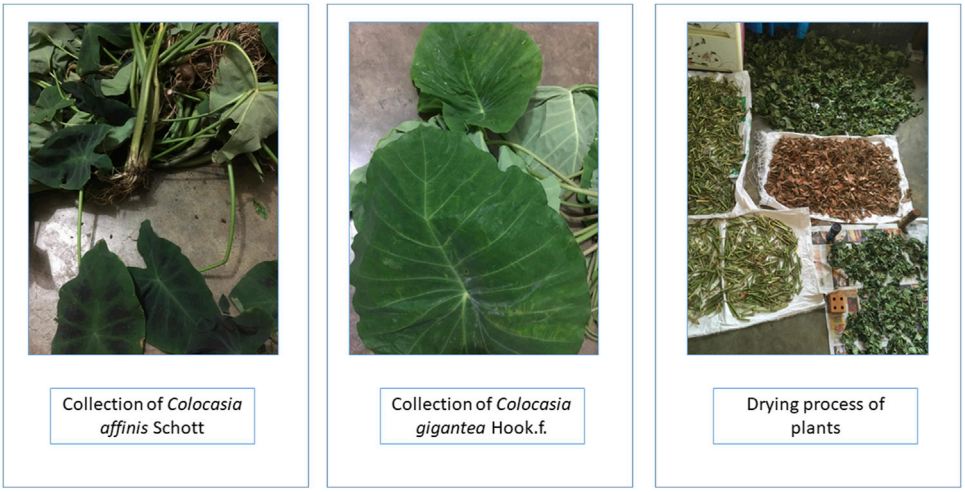



FIGURE 1
Plant parts of *C. gigantea* and *C. affinis*.

The present work was carried out for the isolation and identification of bioactive secondary metabolites from the species *C. gigantea* and *C. affinis*, along with *in vitro*, *in vivo,* and *in silico* investigation of the bioactivities of the identified compounds. Isolation and identification were carried out by chromatographic separation, followed by $^{1}$H-NMR analysis. Then, the identified compounds were employed for the assessment of their antibacterial antidiarrheal and analgesic potentials. Furthermore, the potential of the identified compounds for these activities was also assessed by molecular docking. An ADME/T study was done for their pharmacokinetic properties.

# Materials and methods

## Plant collection

Whole plants of *C. gigantea* and *C. affinis* were collected from Bandarban and Moulovibazar, Bangladesh, in May 2019 (Figure 1). The plants were identified by the experts of Bangladesh National Herbarium, Mirpur, Dhaka, and voucher specimens (DACB; Accession numbers 57,065 and 57,066, respectively) were deposited for these collections.

## Extraction of plant material

After a proper wash, whole plants were sun-dried for several days, followed by grinding to a coarse powder using a high-capacity grinding machine. Two clean 5-L round bottom flasks were each filled with 800 g of powdered plant material soaked in 2.4 L of methanol. The containers were kept for 30 days with daily shaking and stirring. The total mixture obtained from the two containers was then filtered through a fresh cotton plug and finally through a Whatman No.1 filter paper. The volume of two filtrates was reduced by using a Buchi Rotavapor at low temperature and reduced pressure. The total weight of the crude extracts of *C. gigantea* and *C. affinis* was found to be 60.82 gm and 71.25 gm, respectively.

## Drugs and chemicals

Analytical-grade medicines and substances were employed in this investigation. Methanol and Tween-80 were bought from Merck (Darmstadt, Germany). Diclofenac sodium, loperamide, ibuprofen, azithromycin, amoxicillin, ciprofloxacin, and fluconazole were purchased from Square Pharmaceuticals Ltd., Bangladesh.

## Test microorganisms

For the antimicrobial assay, Gram-positive bacteria (*Sarcina lutea, Bacillus megaterium, Staphylococcus aureus*, *Bacillus cereus,* and *Bacillus subtilis*), Gram-negative bacteria (*Pseudomonas aeruginosa, Salmonella typhi, Salmonella paratyphi, Escherichia coli*, *Shigella dysenteriae*), and fungal strains (*Aspergillus niger*, *Saccharomyces cerevisiae*, and *Candida albicans*) were utilized, provided by the University of Dhaka, Bangladesh.

## Experimental animal models

To conduct the *in vivo* experiment, 4–5 week-old Swiss albino mice of both sexes were acquired from the Animal Resource Branch of the International Centre for Diarrheal Diseases and Research, Bangladesh (ICDDR,B). The mice were kept in standard polypropylene cages with a 12-h light-dark cycle. Other optimal conditions, including controlled room temperature of 24°C ± 2°C and relative humidity of 60%–70%, and feeding with formulated rodent food and water (*ad libitum*), were also maintained. During the experiments, all the guidelines regarding the use and care of laboratory animals, ethical rules, and regulations were implemented

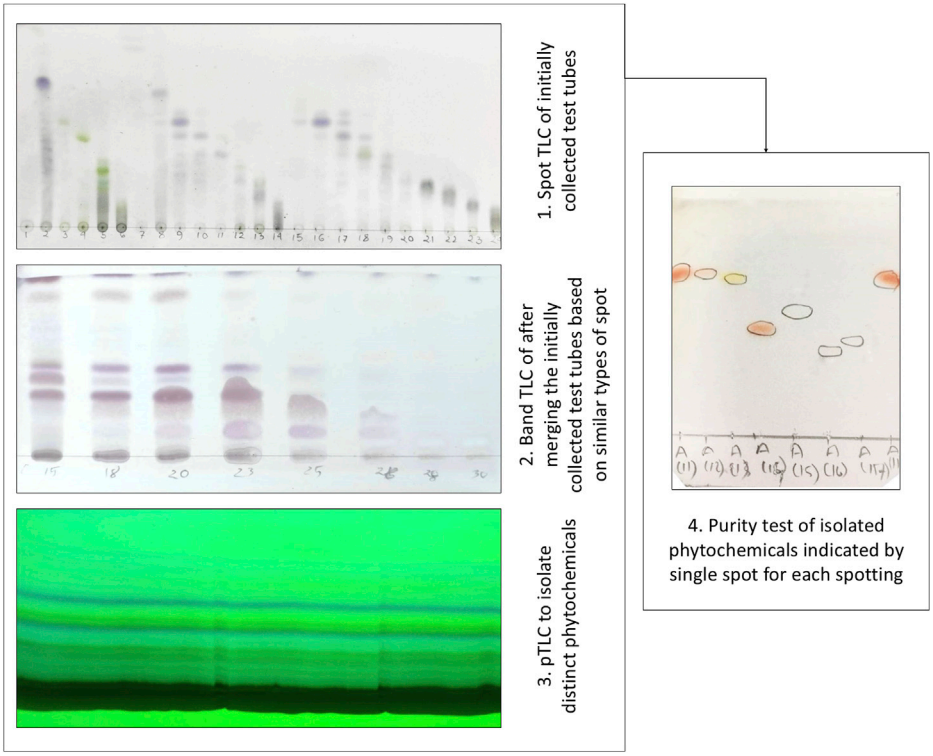



FIGURE 2
Isolation and purity testing of phytochemicals employing thin layer chromatographic (TLC) techniques.

while designing the research and experiments. An intraperitoneal anesthetic overdose of ketamine HCl (100 mg/kg) and xylazine (7.5 mg/kg) was administered to the mouse models at the end of the experiment, followed by euthanasia. All experiments were conducted following the guidelines for the care and use of laboratory animals, which were approved by the institutional ethics committee (Zimmermann, 1983). The Animal Ethics Number for the experimental animal models of this work is 2023-01-04/SUB/A-ERC/002, indicating approval by the Animal Ethics Committee, State University of Bangladesh. This ethical certificate was issued by Prof. Dr. Mohammed Ibrahim, Chairman, Animal Ethics Committee, State University of Bangladesh (Date: 04/01/2023).

## General experimental procedures for compound isolation

Solvent–solvent partitioning was done by using the protocol designed by Kupchan and modified by VanWagenen et al. (1993) to avail four fractions: *n*-hexane, dichloromethane, ethyl acetate, and aqueous fraction. Gel permeation chromatography (GPC/SEC) was performed on Sephadex (LH-20) (Sigma-Aldrich) along with PTLC and TLC conducted on a silica gel 60 $F_{254}$ on aluminum sheets with a thickness of 0.25 mm (Merck, Germany), which were observed under a UV lamp (UVGL-58, United States) at 254 nm and 365 nm. Visualization of the developed plates was done after spraying the vanillin-sulfuric acid mixture, followed by heating for 5 min at 100°C. Thus, pure compounds were isolated by the PTLC method, and the purities of the compounds were analyzed by the subsequent spot TLC method (Figure 2). The isolated compounds were then subjected to $^1$H-NMR. The $^1$H-NMR techniques were performed on a Bruker VNMRS 500 and Bruker Ascend 400 instrument using $CDCl_3$ as a solvent, and the chemical shifts were documented in the δ ppm scale, keeping TMS as a reference (Ashrafi et al., 2022).

## Experimental design

### *In vitro* tests

#### Antibacterial assay: disk diffusion test

The disk diffusion technique was used to evaluate the antibacterial properties of various fractionates of the crude extract (Huys et al., 2002). In this conventional approach, test samples were evenly distributed on nutrient agar medium that had been pre-inoculated with test bacteria using sterilized and desiccated filter paper discs (6 mm). Three commercially available antibiotic discs (azithromycin, amoxicillin, and ciprofloxacin) were considered positive standard blank discs, while fluconazole was used against fungal strains. The plates were kept for approximately 24 h upside down at a low temperature (4°C) to enable maximum diffusion of the test samples into the medium. After being flipped over, the plates were kept in an incubator set at 37°C for a whole day. Samples with antibacterial potential were deeply diluted in the medium and exhibited growth inhibition.

## *In vivo* tests

### Antidiarrheal bioassay: castor oil-induced diarrhea test

To assess the antidiarrheal potential of the isolated and identified compounds from *C. gigantea* and *C. affinis*, they were tested on castor oil-induced diarrheal mice. In this study, the mice were divided into twelve groups: control, positive control, and ten test groups, each containing three mice. The control group was administered with a dose of 10 mL/kg of 1% Tween 80 in a water vehicle orally. The positive control or standard group was administered with a dose of 5 mg/kg of loperamide orally (Rudra et al., 2020). Simultaneously, the two test groups were administered orally with the test compounds C1, C2, C3, and C5, and the mixture of C5 and C6 each at doses of 10 mg/kg and 20 mg/kg, respectively. One hour following the administration of the test samples, 1 mL of highly pure analytical-grade castor oil was fed to each mouse to induce diarrhea. After that, each mouse was individually placed on the box floor lined with transparent paper, and during an observation period of 5 h, the number of diarrheal feces excreted by each animal was recorded.

Throughout the treatment, each mouse was placed in an individual cage, and the floor lining was changed every hour. To evaluate the antidiarrheal activity of the test compounds, the observations of the test groups were compared against those of the control group. During the observational period of 4 h, the number of fecal stool spots was documented for each mouse. The percent inhibition of diarrhea was calculated by the following equation.

$$\%\,inhibition\,of\,defecation = \frac{Mean\,number\,of\,defecations\,by\,control - Mean\,number\,of\,defecations\,by\,test\,samples\,or\,standard}{Mean\,numbers\,of\,defecation\,by\,control} \times 100$$

### Analgesic bioassay: acetic acid-induced writhing test

The peripheral analgesic activity of the isolated and identified compounds from *C. gigantea* and *C. affinis* was investigated in the acetic acid-induced writhing test in mice. In this study protocol, mice were divided into twelve groups: control, positive control, and ten test groups, each containing three mice. The mice in the negative control group were administered 0.1 mL of acetic acid intraperitoneally. The positive control group received standard diclofenac sodium at an oral dose of 5 mg/kg b.w. (Ahmad et al., 2010).

The two test groups were administered orally with the test compounds C1, C2, C3, C5, and the mixture of C5 and C6 each at a dose of 10 mg/kg and 20 mg/kg (b.w.; p.o.), respectively. Following the administration of the test samples, the count of writhing movements was recorded 5 min after the injection of acetic acid and documented over a period of 25 min. The percentage of writhing inhibition was subsequently calculated using the following formula:

$$\%\,Inhibition\,of\,writhing = \frac{Control\,\text{of}\,writhing\,response - Test\,\text{of}\,writhing\,response}{Control\,of\,writhing\,response} \times 100$$

### Anti-inflammatory bioassay: formalin-induced paw-licking test

Four groups of mice, each consisting of five mice, with weights ranging from 20 g to 25 g, were subjected to the formalin-induced licking test following the protocol of Emon et al. (2021b), Alam et al. (2021a), and Sultana et al. (2022). Subcutaneous injections of 20 μL of 1% formalin solution in 0.9% saline were administered to the dorsal side of each mouse's hind paw. The mice were transferred to a transparent observation space immediately following the injection. Then the authors measured the duration of licking time of the injected paw in rodents. Doses of 10 mg/kg and 20 mg/kg of the test compounds C1–C5 and a combination of C5 and C6 extracted from *C. affinis* and *C. gigantea* were administered orally to the groups participating in the experiment. As a standard control, a separate group was administered ibuprofen (10 mg/kg, intraperitoneally, i.p.). Additionally, a control group was administered normal saline (0.1 mL/10 g) as a baseline to compare the analgesic effects of the test extracts.

$$\%\,Inhibition\,of\,licking = \frac{Mean\,licking\,by\,the\,control - Mean\,licking\,by\,the\,test\,sample}{Mean\,licking\,by\,the\,control} \times 100.$$

## *In silico* tests

### Molecular docking

**Software.** In the *in silico* studies, the docking scores of the identified six compounds from *C. affinis* and *C. gigantea* were evaluated against the three selected biologically active target enzyme/receptor macromolecules. Various software programs, including Discovery Studio 4.5, Swiss-PDB viewer, PyRx, and PyMOL 2.3, were employed to assess the molecular interactions comprehensively (Jiko et al., 2024; Shahriar et al., 2024).

**Ligand preparation.** The structures of compounds **1**–**4**, along with the standard drug molecules, were downloaded from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/), and the structures of compounds **5** and **6** were drawn using ChemDraw Ultra 12. 0 software. The structures of the identified compounds and standard drugs are presented in Figure 3. The ligands were downloaded in 3D SDF format and serially loaded into Discovery Studio 4.5. To improve the docking accuracy, the semiempirical technique was used to optimize all the phytoconstituents (Bikadi and Hazai, 2009).

**Target protein preparation.** The target macromolecules were obtained as 3D crystal structures from the RCBS Protein Data Bank (https://www.rcsb.org/structure) in the PDB format. For antibacterial, antidiarrheal, and peripheral analgesic activity assessment, dihydrofolate reductase (DHFR) enzyme [PDB ID: 4M6J] (Khatun et al., 2021), kappa opioid receptor (KOR) [PDB ID: 6VI4] (Alam et al., 2021b), and cyclooxygenase-2 (COX-2) enzyme [PDB ID: 1CX2] (Muhammad et al., 2015) were employed. After that, all water molecules and heteroatoms were removed from the proteins using Discovery Studio 2021, and Swiss-PDB Viewer's energy minimization tool was used to optimize biomolecules by arranging nonpolar hydrogen atoms.

**Ligand–protein interaction.** To gain insight into how molecules interact at the molecular level, the potential binding patterns and binding affinities were predicted through a computer-aided ligand–protein interaction diagram. The software PyRx was employed for the molecular drug–protein binding procedure. A

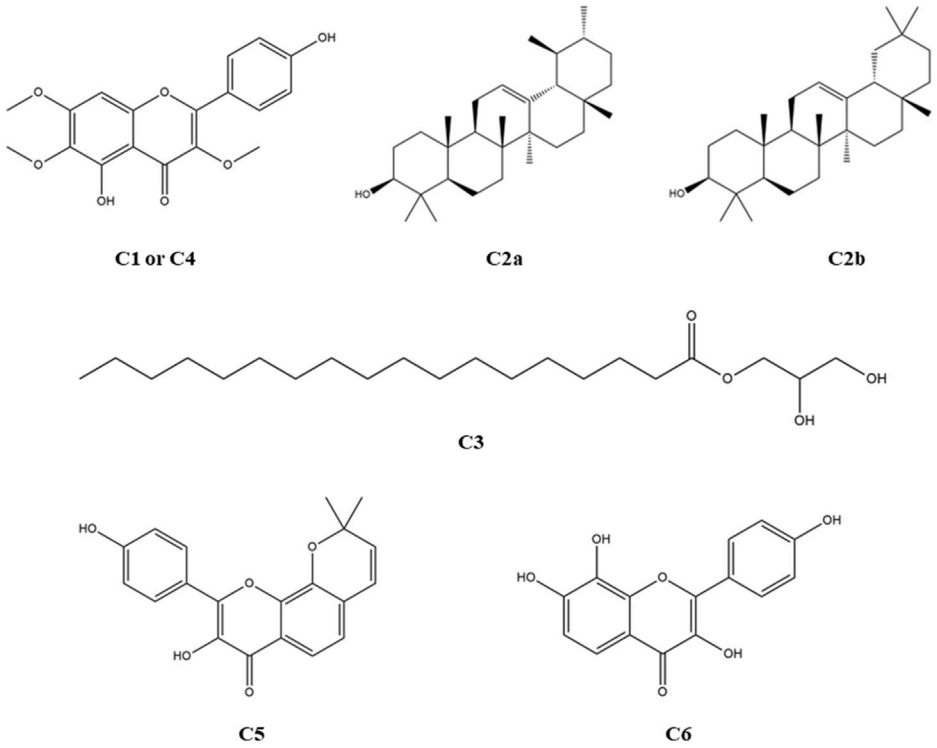



FIGURE 3
Structures of the identified phytocompounds from *C. affinis*, *C. gigantea*, and the standard drug molecules considered for the respective bioactivities.

careful selection of particular amino acids, along with their corresponding IDs, was undertaken from the scientific literature for each enzyme/receptor individually, aiming for precise target docking (Khatun et al., 2021; Alam et al., 2021b; Muhammad et al., 2015). Subsequently, the protein was prepared by loading and formatting as the necessary macromolecule, guaranteeing specific ligand binding to the intended targets.

To enhance the docking process with the chosen macromolecules, ligand SD files were brought in and transformed into pdbqt format using the Open Babel tool within the PyRx software. Grid mapping identified the active amino sites within specific grid boxes, following the predetermined center and dimensional axes outlined in Table 1. During this stage, basic supportive default functions remained unchanged (Muhammad et al., 2015). The last step entailed interpreting the results and employing BIOVIA Discovery Studio version 4.5 to forecast the optimal 2D and 3D models.

**ADME/T study.** For the evaluation of pharmacokinetic parameters like absorption, distribution, metabolism, excretion, and toxicity (ADME/T), pkCSM (http://structure.bioc.cam.ac.uk/Pkcsm) was employed (Pires et al., 2015). Concurrently, the Swiss ADME (http://www.swissadme.ch), which predicts drug-likeness based on Lipinski's rules and pharmacokinetic parameters (Daina et al., 2017; Shompa et al., 2024), was used to determine the drug-likeness and bioavailability score of selected compounds.

### Statistical analysis

The statistical analysis was done through the presentation of mean values accompanied by the standard error of the mean (SEM). These values were meticulously compared to those of the control group and discerned to be statistically significant (***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$), following a rigorous one-way analysis of variance (ANOVA) supplemented by Dunnett's test. All statistical manipulations were conducted utilizing GraphPad Prism Version 5.2 (San Diego, CA).

## Results

### Identification of compounds

A total of six compound structures, shown in Figure 3, were isolated and identified from the *Colocasia gigantea* and *Colocasia affinis*.

**Penduletin (C1 or C4):** Pale yellow crystals, $1^H$ NMR (500 MHz, $CDCl_3$): δH 6.59 (1H, s, H-8), 7.90 (1H, d, $J$ = 9.0 Hz, H-2′), 7.04 (1H, d, $J$ = 9.0 Hz, H-3′), 7.04 (1H, d, $J$ = 9.0 Hz, H-5′), 7.90 (1H, d, $J$ = 9.0 Hz, H-6′), 3.90 (3H, s, 3-OCH3), 4.02 (3H, s, 6-OCH3), 4.04 (3H, s, 7-OCH3), 12.79 (1H, s, 5-OH), 6.37 (1H, s, 4′-OH). The corresponding $^1$H-NMR spectrum is depicted in Figure 4.

**A mixture of α-amyrin and β-amyrin (C2) α-Amyrin (C2a):** White powder, $^1$H NMR (400 MHz, $CDCl_3$): $\delta_H$ 5.28 (1H, t, H-12), 3.24 (1H, dd, $J$ = 4.4, 4.8 Hz, H-3), 1.16 (3H, s, H-27), 1.11 (6H, s, H-26), 1.01 (6H,s, H-28), 0.98 (6H, s, H-25), 0.93 (3H, d, $J$ = 3.2 Hz, H-30), 0.82

TABLE 1 Target site selection and grid mapping of target receptors.

| Receptor | Standard | Target binding sites | References | Grid box | |
|---|---|---|---|---|---|
| DHFR (4M6J) | Ciprofloxacin | Ala 9, Ile 16, Lys 54, Lys 55, Thr 56, Leu 75, Ser 76, Arg 77, Glu 78, Arg 91, Ser 92, Leu 93, Gly 117, Ser 118, Ser 119, and Val 120 | Khatun et al. (2021) | Center | x = 3.20179912784 |
| | | | | | y = −3.79824570941 |
| | | | | | z = −18.5444224326 |
| | | | | Dimension | x = 25.2409863514 |
| | | | | | y = 30.9244686771 |
| | | | | | z = 28.5216876816 |
| KOR (6VI4) | Loperamide | Leu 103, Leu 107, Ser 136, Ile 137, Try 140, Ile 180, Trp 183, Leu 184, Ser 187, Ile 191, Leu 192 Ile 194, and Val 195 | Alam et al. (2021a) | Center | x = 53.9115751709 |
| | | | | | y = −50.1152995766 |
| | | | | | z = −16.2408511962 |
| | | | | Dimension | x = 18.2799964783 |
| | | | | | y = 30.7770137609 |
| | | | | | z = 20.3958600148 |
| COX-2 (1CX2) | Diclofenac sodium | His 90, Gln 192, Val 349, Leu 352, Ser 353, Tyr 355, Tyr 385, Ala 516, Phe 518, Val 523, Ala 527, and Ser 530 | Muhammad et al. (2015) | Center | x = 23.2515878129 |
| | | | | | y = 21.7831246177 |
| | | | | | z = 16.1794397334 |
| | | | | Dimension | x = 28.7572839296 |
| | | | | | y = 22.8801400278 |
| | | | | | z = 30.7900966105 |

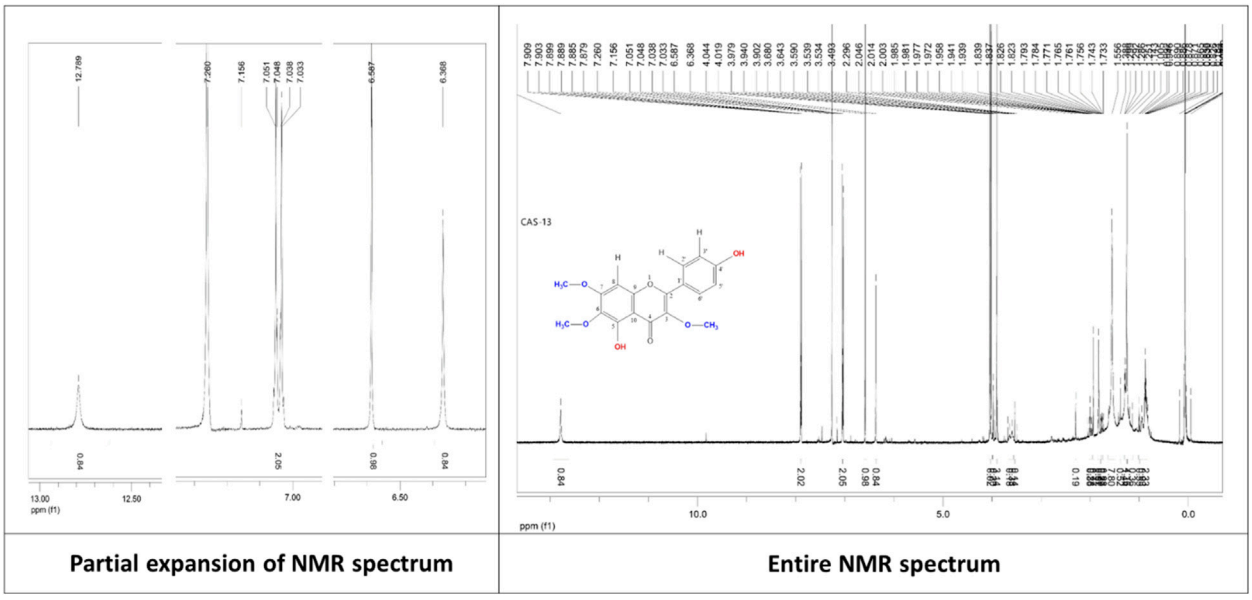



FIGURE 4
$^1$H-NMR spectrum of the compound penduletin isolated from *C. gigantea* and *C. affinis*.

(3H, d, $J$ = 3.2 Hz, H-29), 0.80 (s, 6H, H-23), 0.79 (s, 6H, H-24). The corresponding $^1$H-NMR spectrum is depicted in Figure 5.

**β-Amyrin (C2b):** White powder, $^1$H NMR (400 MHz, $CDCl_3$): $\delta_H$ 5.31 (t, 1H, H-12), 3.24 (dd, 2H, $J$ = 4.4, 4.8 Hz, H-3), 1.28 (s, 3H, H-27), 1.11 (s, 6H, H-26), 1.01 (s, 6H, H-28), 0.98 (s, 6H, H-25), 0.95 (s, 6H, H-29,30) 0.80 (s, 6H, H-23), 0.79 (s, 6H, H-24). The corresponding $^1$H-NMR spectrum is depicted in Figure 5.

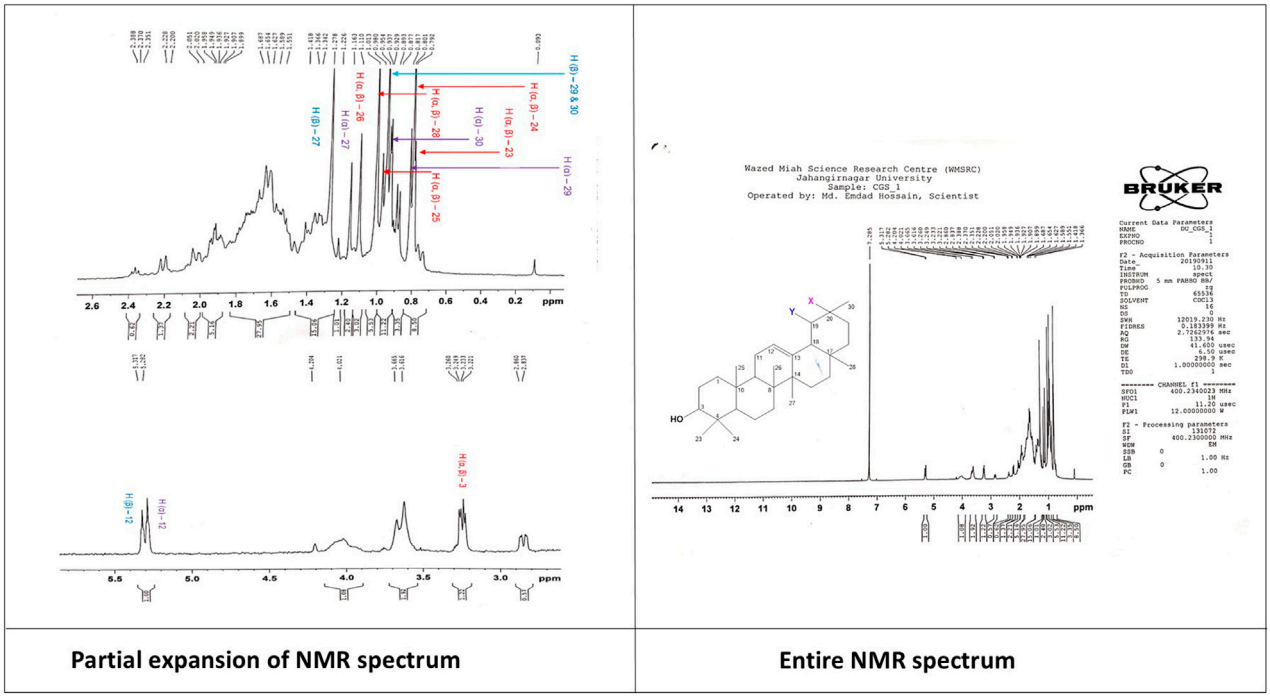



FIGURE 5
$^1$H-NMR spectrum of the compound mixture of α-amyrin and β-amyrin isolated from *C. gigantea*.

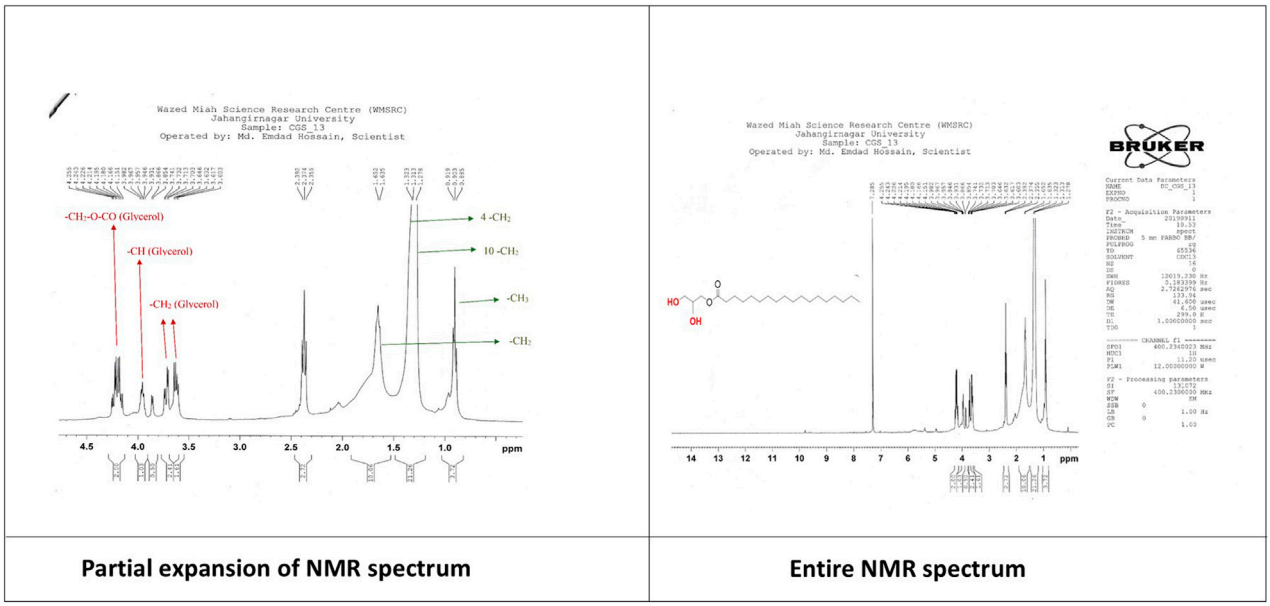



FIGURE 6
$^1$H-NMR spectrum of the compound monoglyceride of stearic acid isolated from *C. gigantea*.

**Monoglyceride of stearic acid (C3):** Colorless crystals, $^1$H NMR (400 MHz, $CDCl_3$); **Glycerol backbone:** -$CH_2$-O-CO: 4.195 (2H, dd, *J* = 13.6, 5.6 Hz), -CH: 3.957 (1H, m), -$CH_2$: 3.732 (2H, dd, *J* = 11.6, 4.0 Hz), 3.617 (1H, dd, *J* = 11.6, 5.6 Hz). **Stearic acid part:** -$CH_2$-CO-O: 2.37 (t, 2H), -$CH_2$: 1.64 (d, 2H), 4-$CH_2$: 1.31 (d, 8H), 10-$CH_2$: 1.25 (s, 20H), -CH3: 0.90 (t, 3H). The corresponding $^1$H-NMR spectrum is depicted in Figure 6.

**7,8 -(3′′,3′′-dimethyl-pyrano)-4′-hydroxy flavonol (C5):** White powder, $^1$H NMR (500 MHz, $CDCl_3$): $\delta_H$ 7.46 (1H, d, *J* = 8.5 Hz, H-5), 6.86 (1H, d, *J* = 8.5 Hz, H-6), 7.68 (1H, d, *J* = 8.5 Hz, H-2′), 7.68 (1H, d, *J* = 8.5 Hz, H-6′), 6.96 (2H, d, *J* = 12.5 Hz, H-1′′), 6.84 (1H, d, *J* = 9.0 Hz, H-3′), 6.84 (1H, d, *J* = 9.0 Hz, H-5′), 5.87 (1H, d, *J* = 12.5 Hz, H-2′′), 1.55 (6H, s, H-4′′, H-5′′). The corresponding $^1$H-NMR spectrum is depicted in Figure 7.

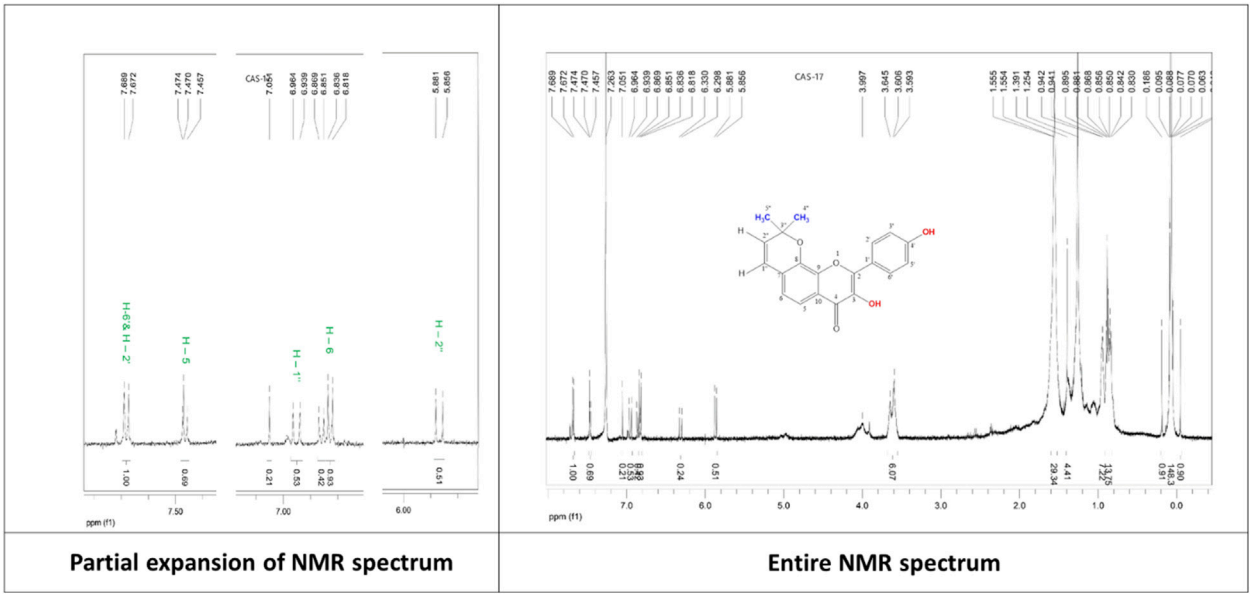



FIGURE 7
$^{1}$H-NMR spectrum of the compound 7,8-(3″,3″-dimethyl-pyrano)-4′-hydroxy flavonol isolated from *C. affinis*.

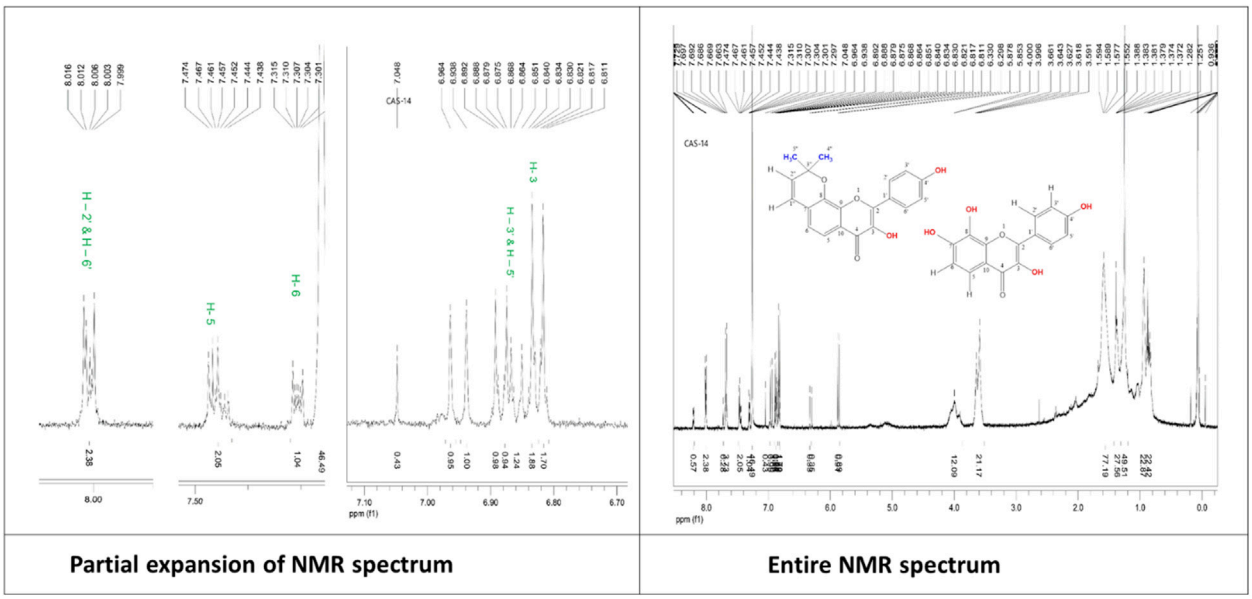



FIGURE 8
$^{1}$H-NMR spectrum of the compound mixture of 7,8-(3″,3″-dimethyl-pyrano)-4′-hydroxy flavonol and 4′,7,8-trihydroxy flavonol isolated from *C. affinis*.

**Mixture of 7,8-(3′′,3′′-dimethyl-pyrano)-4′-hydroxy flavonol (C5) and 4′,7,8-trihydroxy flavonol (C6):** White powder, $^{1}$H NMR (500 MHz, $CDCl_3$): $\delta_H$ 6.81 (1H, s, H-3), 7.46 (1H, d, $J$ =9.0 Hz, H-5), 7.30 (1H, d, $J$ = 9.0 Hz, H-6), 8.00 (1H, d, $J$ = 8.5 Hz, H-2′), 6.88 (1H, d, $J$ = 8.5 Hz, H-3′), 6.88 (1H, d, $J$ = 8.5 Hz, H-5′), 8.00 (1H, d, $J$ = 8.5 Hz, H-6′). The corresponding $^{1}$H-NMR spectrum is depicted in Figure 8.

## *In vitro* testing

### Effect of the identified test compounds on the disc diffusion assay

The antibacterial activity of all the partitions was tested against five strains of gram-positive and gram-negative bacteria and three strains of fungi. As a reference standard, azithromycin, amoxicillin,

10.3389/fphar.2024.1474706

TABLE 2 Antibacterial activity of the isolated compounds from *C. gigantea* and *C. affinis* against Gram-positive and Gram-negative bacteria.

| Test microorganisms | Zone of inhibition (mm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Azithromycin (30 µg/disc) | Amoxicillin (30 µg/disc) | Ciprofloxacin (30 µg/disc) | C1/C4 (100 µg/disc) | C2 (100 µg/disc) | C3 (100 µg/disc) | C5 (100 µg/disc) | Mixture of C5 and C6 (100 µg/disc) |
| Gram-positive bacteria | | | | | | | | |
| *Bacillus cereus* | 37 | 35 | 31 | 15 | 12 | 8 | 11 | — |
| *Bacillus megaterium* | 35 | 32 | 30 | 14 | 11 | 5 | 12 | — |
| *Bacillus subtilis* | 34 | 28 | 32 | 9 | 10 | 6 | 7 | — |
| *Staphylococcus aureus* | 41 | 39 | 33 | 18 | 17 | 14 | 8 | 8 |
| *Sarcina lutea* | 37 | 35 | 29 | 16 | 13 | 15 | 11 | — |
| Gram-negative bacteria | | | | | | | | |
| *Escherichia coli* | 38 | 36 | 34 | 17 | 14 | 11 | 7 | — |
| *Pseudomonas aeruginosa* | 41 | 37 | 38 | 20 | 13 | 10 | 8 | 6 |
| *Salmonella paratyphi* | 30 | 31 | 27 | 15 | 8 | 7 | 6 | — |
| *Salmonella typhi* | 39 | 31 | 36 | 18 | 16 | 8 | — | 8 |
| *Shigella dysenteriae* | 37 | 32 | 33 | 17 | 15 | 12 | 6 | — |
| Fungi | | | | | | | | |
| Test Microorganisms | Fluconazole (30 µg/disc) | | | C1/C4 (100 µg/disc) | C2 (100 µg/disc) | C3 (100 µg/disc) | C5 (100 µg/disc) | Mixture of C5 and C6 (100 µg/disc) |
| *Aspergillus niger* | 45 | | | 12 | 5 | 9 | 6 | — |
| *Candida albicans* | 38 | | | — | — | — | — | — |
| *Saccharomyces cerevisiae* | 41 | | | 9 | — | 7 | — | — |

ciprofloxacin, and fluconazole were taken to test the respective antimicrobial activity. The zone of inhibition (ZOI) of the test samples ranged from 6 mm to 20 mm and is summarized in Table 2. C1/C4, C2, C3, and C5 showed considerable antibacterial activity, whereas C1/C4 and C3 exhibited promising antifungal attributes. As per ZOI, the fractionated extracts exerted notable antimicrobial activities against *B. cereus*, *B. megaterium*, *B. subtilis*, *S. aureus*, *S. lutea*, *E. coli*, *P. aeruginosa*, and *S. dysenteriae* and relatively lower ZOI against *S. paratyphi*, *S. typhi*, *A. niger*, *C. albicans*, and *S. cerevisiae*. In addition, all the standard drugs exhibited the expected pronounced ZOIs against all the tested strains, ranging from 27 mm to 45 mm.

## *In vivo* study

### Effect of the identified test compounds on castor oil-induced diarrhea

Compounds C2, C5, and the mixture of C5 and C6, each at doses of 10 mg/kg and 20 mg/kg, and compound C1 at a dose of 20 mg/kg exhibited significant ($p < 0.05$, $p < 0.01$) reduction in the number of feces (Table 3). In terms of wet feces number, C2, C5, and the mixture of C5 and C6 demonstrated percentages of diarrhea inhibition of 29.63%, 37.04%, and 25.93%, respectively, at the 10 mg/kg dose, while at the 20 mg/kg dose, C1, C2, C5, and the mixture of C5 and C6 exhibited reductions of 33.33%, 40.74%, 44.44%, and 37.04%, respectively. The value of the standard loperamide was 77.78%.

### Effect of the identified test compounds on acetic acid-induced writhing in the mice model

All the test compounds C1, C2, C3, C5, and the mixture of C5 and C6 each at 10 and 20 mg/kg doses exhibited significant ($p < 0.01$, $p < 0.001$) analgesia with a considerable percentage reduction of acetic acid-induced writhing compared to the standard diclofenac sodium (Table 3). Among them, C1 and C2 exhibited the highest percentage reduction of writhing with 56.52% at the 20 mg/kg dose when compared to the standard of 76.09%.

### Effect of the identified test compounds on formalin-induced licking in the mice model

The administration of test compounds C1/C4, C2, C3, and C5, and the combination of C5 and C6 at doses of 10 mg/kg and 20 mg/kg resulted in significant analgesic effects, as evidenced by a marked reduction in formalin-induced paw licking behavior. The observed anti-inflammatory effect was statistically significant ($p < 0.01$, $p < 0.001$) (Table 4), demonstrating a marked reduction in formalin-induced writhing relative to the control group receiving

TABLE 3 Antidiarrheal and analgesic activities of isolated compounds from *C. gigantea* and *C. affinis,* respectively, on castor oil-induced diarrhea and acetic acid-induced writhing test in mice.

| Animal group with corresponding doses (ml/kg or mg/kg, b.w.; p.o.) | Number of diarrheal feces (Mean ± SEM) | % reduction in diarrhea | Number of writhing episodes (Mean ± SEM) | % reduction in writhing |
|---|---|---|---|---|
| CTL | 9 ± 0.58 | — | 15.33 ± 0.33 | — |
| STD (loperamide/diclofenac sodium) | 2 ± 0.58*** | 77.78 | 3.67 ± 0.67*** | 76.09 |
| Compound 1 or 4 (10 mg/kg b.w.) | 7.33 ± 0.88 | 18.52 | 10.33 ± 0.67** | 32.61 |
| Compound 1 or 4 (20 mg/kg b.w.) | 6 ± 0.58* | 33.33 | 6.67 ± 0.68** | 56.52 |
| Compound 2 (10 mg/kg b.w.) | 6.33 ± 0.67* | 29.63 | 9.33 ± 0.33*** | 39.13 |
| Compound 2 (20 mg/kg b.w.) | 5.33 ± 0.33** | 40.74 | 6.67 ± 0.33*** | 56.52 |
| Compound 3 (10 mg/kg b.w.) | 7.67 ± 0.33 | 14.81 | 11.67 ± 0.33** | 23.91 |
| Compound 3 (20 mg/kg b.w.) | 7 ± 0.001 | 22.22 | 11.33 ± 0.33** | 26.09 |
| Compound 5 (10 mg/kg b.w.) | 5.67 ± 0.33* | 37.04 | 11 ± 0.58** | 28.26 |
| Compound 5 (20 mg/kg b.w.) | 5 ± 0.58** | 44.44 | 8.33 ± 0.88** | 45.65 |
| Mixture of compounds 5 and 6 (10 mg/kg b.w.) | 6.67 ± 0.33* | 25.93 | 10.67 ± 0.33*** | 30.43 |
| Mixture of compounds 5 and 6 (20 mg/kg b.w.) | 5.67 ± 0.33* | 37.04 | 7.67 ± 0.33*** | 50.0 |

Values are expressed as mean ± SEM (n = 3); CTL, negative control; STD, positive control; ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ compared to negative control.

ibuprofen. C5 and C2 demonstrated the highest anti-inflammatory efficacy among the compounds evaluated, resulting in a 64.50% and 68.15% reduction in licking during the early phase and a 66.66% and 52.06% reduction during the later phase, respectively, at a dosage of 20 mg/kg. On the other hand, the standard drug ibuprofen led to a decrease of 73.54% and 74.68% in early and late phase subsequently. The findings indicate that compounds C5 and C2 demonstrate substantial potential for development as anti-inflammatory agents, primarily due to their marked reduction in pain responses in the examined mice.

## *In silico* study

### Molecular docking

The docking scores of the identified compounds from the methanolic extracts of *C. affinis* and *C. gigantea* to selected targets are depicted in Table 5. For the enzyme DHFR, **C5** showed the highest docking score of −9.1 kcal/mol, followed by **C2a**, **C2b,** and **C1/C4** with promising docking scores of −8.8 kcal/mol, −8.8 kcal/mol, and −7.9 kcal/mol, respectively, when compared to the standard drug ciprofloxacin with a docking score of −8.1 kcal/mol. The 3D and 2D graphical representations of the molecular interactions of these compounds and the standard drug ciprofloxacin with the DHFR enzyme are depicted in Figures 9, 12, respectively. In the case of the KOR receptor, the standard drug loperamide showed a docking score of −9.1 kcal/mol, while the test compound **C2b** showed the highest docking score of −10.5 kcal/mol. The other compounds also showed significant scores of −9.7 kcal/mol **(C5)**, −9.4 kcal/mol **(C2a)**, −8 kcal/mol **(C6)**, and −7.7 kcal/mol **(C1/C4)**. The 3D and 2D graphical representations of the molecular interactions of these compounds and the standard loperamide with the KOR receptor are depicted in Figures 10, 12. Additionally, with the COX-2 enzyme, the compound C2b showed a promising docking score of −8.7 kcal/mol, along with other notable docking scores: −7.3 kcal/mol **(C6)**, −7.2 kcal/mol **(C5)**, and −7 kcal/mol **(C1 and C3)**. The docking score of the standard drug diclofenac sodium to the COX-2 enzyme was −7.8 kcal/mol. The 3D and 2D graphical representations of the molecular interactions of these compounds and the standard diclofenac sodium with COX-2 enzyme are depicted in Figures 11, 12. The corresponding binding interactions and the binding sites of highly active compounds against the targets, including DHFR, KOR, and COX-2, are presented in Table 6.

### ADME/T study

The *in silico* study also analyzed the ADME/T parameters of the identified compounds. The bioavailability score and Lipinski's rule of five data were also considered for the assessment of drug-likeness of the compounds, which are demonstrated in Table 7.

## Discussion

This study revealed the first-time report on compound isolation ascertained by structure elucidation employing NMR technology from two *Colocasia* species, namely, *C. gigantea and C. affinis*. Previous reports on these plants only focused on phytochemical profiling through HPLC-DAD and GC-MS (Zilani et al., 2021; Alam et al., 2024). Thus, phytochemical isolation and the elucidation of their structures are the novelty of this current research. These revealed three bioactive flavonoids along with two triterpenoids and one monoglyceride in total, which sheds light on the polyphenol-rich candidacy of these plants, which may result in promising lead compounds for future drug discovery and development. Dichloromethane fractions of both plants were used in phytochemical screening and isolation of compounds. A total of four compounds were elucidated from the dichloromethane

TABLE 4 Anti-inflammatory effect of the isolated compounds from *C. gigantea* and *C. affinis* on the formalin-induced mouse model.

| Animal group with respective doses (ml/kg or mg/kg, b.w; p.o) | Time of licking in seconds (Ealy Phase; 0–5 min) | Time of licking in seconds (Late Phase; 15–30 min) |
|---|---|---|
| CTL | 60.38 ± 2.20 | 73.68 ± 1.34 |
| STD (Ibuprofen, 10 mg/kg, b.w) | 15.98 ± 1.62*** | 18.65 ± 2.52*** |
| Compound 1 or 4 (10 mg/kg, b.w.) | 32.54 ± 3.12*** | 38.87 ± 1.09*** |
| Compound 1 or 4 (20 mg/kg, b.w.) | 31.38 ± 1.12*** | 27.61 ± 1.02*** |
| Compound 2 (10 mg/kg, b.w.) | 44.32 ± 1.52*** | 51.49 ± 0.71*** |
| Compound 2 (20 mg/kg, b.w.) | 19.23 ± 3.34*** | 35.32 ± 1.77*** |
| Compound 3 (10 mg/kg, b.w.) | 38.04 ± 4.19** | 45.68 ± 1.95*** |
| Compound 3 (20 mg/kg, b.w.) | 28.43 ± 2.38*** | 30.56 ± 2.16*** |
| Compound 5 (10 mg/kg, b.w.) | 40.38 ± 1.51*** | 53.29 ± 1.43*** |
| Compound 5 (20 mg/kg, b.w.) | 21.43 ± 0.53*** | 24.56 ± 0.98*** |
| Mixture of compound 5 and 6 (10 mg/kg, b.w.) | 44.28 ± 2.20** | 56.21 ± 2.40** |
| Mixture of compound 5 and 6 (20 mg/kg, b.w.) | 30.44 ± 1.05*** | 36.43 ± 4.81** |

Values are expressed as mean ± SEM (n = 5); CTL, negative control; STD, positive control; ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ compared to negative control.

TABLE 5 Binding affinities of the identified compounds from *C. gigantea* and *C. affinis* and standards against three macromolecules, namely, DHFR, KOR, and COX-2, representing antibacterial, antidiarrheal, and peripheral analgesic activities, respectively.

| Compound code | Compound name | Molecular formula | Molecular weight (g/mol) | Binding affinities (kcal/mol) | | |
|---|---|---|---|---|---|---|
| | | | | Antibacterial | Antidiarrheal | Analgesic |
| | | | | DHFR (4M6J) | KOR (6VI4) | COX-2 (1CX2) |
| C1/C4 | Penduletin | $C_{18}H_{16}O_7$ | 344.3 | −7.9 | −7.7 | −7 |
| C2a | α-Amyrin | $C_{30}H_{50}O$ | 426.7 | −8.8 | −9.4 | −5.3 |
| C2b | β-Amyrin | $C_{30}H_{50}O$ | 426.7 | −8.8 | −10.5 | −8.7 |
| C3 | Monoglyceride of stearic acid | $C_{21}H_{42}O_4$ | 358.6 | −5.4 | −6.3 | −7 |
| C5 | 7,8-(3″,3″-Dimethyl-pyrano)-4′-hydroxy flavonol | $C_{20}H_{16}O_5$ | 336.34 | −9.1 | −9.7 | −7.2 |
| C6 | 4′,7,8-Trihydroxy flavonol | $C_{15}H_{10}O_6$ | 286.24 | −8.1 | −8 | −7.3 |
| Standards | Ciprofloxacin | | 331.34 | −8.1 | — | — |
| | Loperamide | | 477 | — | −9.1 | — |
| | Diclofenac sodium | | 318.1 | — | — | −7.8 |

soluble fraction of *C. gigantea* as a result of consecutive chromatographic separation and purification. The compounds isolated from *C. gigantea* are penduletin (**1**), a mixture of α-amyrin (**2a**) and β-amyrin (**2b**), and a monoglyceride of stearic acid (**3**). Another three compounds were isolated from the dichloromethane-soluble fraction of *C. affinis* following the same technique: penduletin (**4**) (which was also isolated from *C. gigantea*), 7,8 -(3″,3″-dimethyl-pyrano)-4′-hydroxy flavonol (**5**), and a mixture of 7,8 -(3″,3″-dimethyl-pyrano)-4′-hydroxy flavonol (**5**) and 4′,7,8-trihydroxy flavonol (**6**).

The $^1$HNMR spectrum (500 MHz, CDCl3) of compounds **1** and **4** (Figure 4) in $CDCl_3$ indicated the presence of three methoxy groups at $\delta_H$ 4.04 (3H, s), $\delta_H$ 4.02 (3H, s), and $\delta_H$ 3.90 (3H, s). The two pairs of *ortho*-coupled ($J$ = 9.0 Hz) doublets at $\delta_H$ 7.04 and $\delta_H$ 7.90 showed that ring B is monosubstituted at C-4'. The peak at $\delta_H$ 12.79 belongs to 5-OH. There is a one-proton singlet at $\delta_H$ 6.59. There are three possible positions for this singlet: C-3, C-6, and C-8. Another proton singlet at $\delta_H$ 6.37 could be assigned to the -OH group at C-4'. These $^1$H NMR data are in close agreement with the published value of penduletin (Wang et al., 1989; Makhmoor and Choudhary, 2010), where the singlet at $\delta_H$ 6.59 is at the C-8 position, and the three methoxy groups are at the C-3, C-6, and C-7 positions. Thus, the structure of compounds **1** and **4** can be concluded as penduletin.

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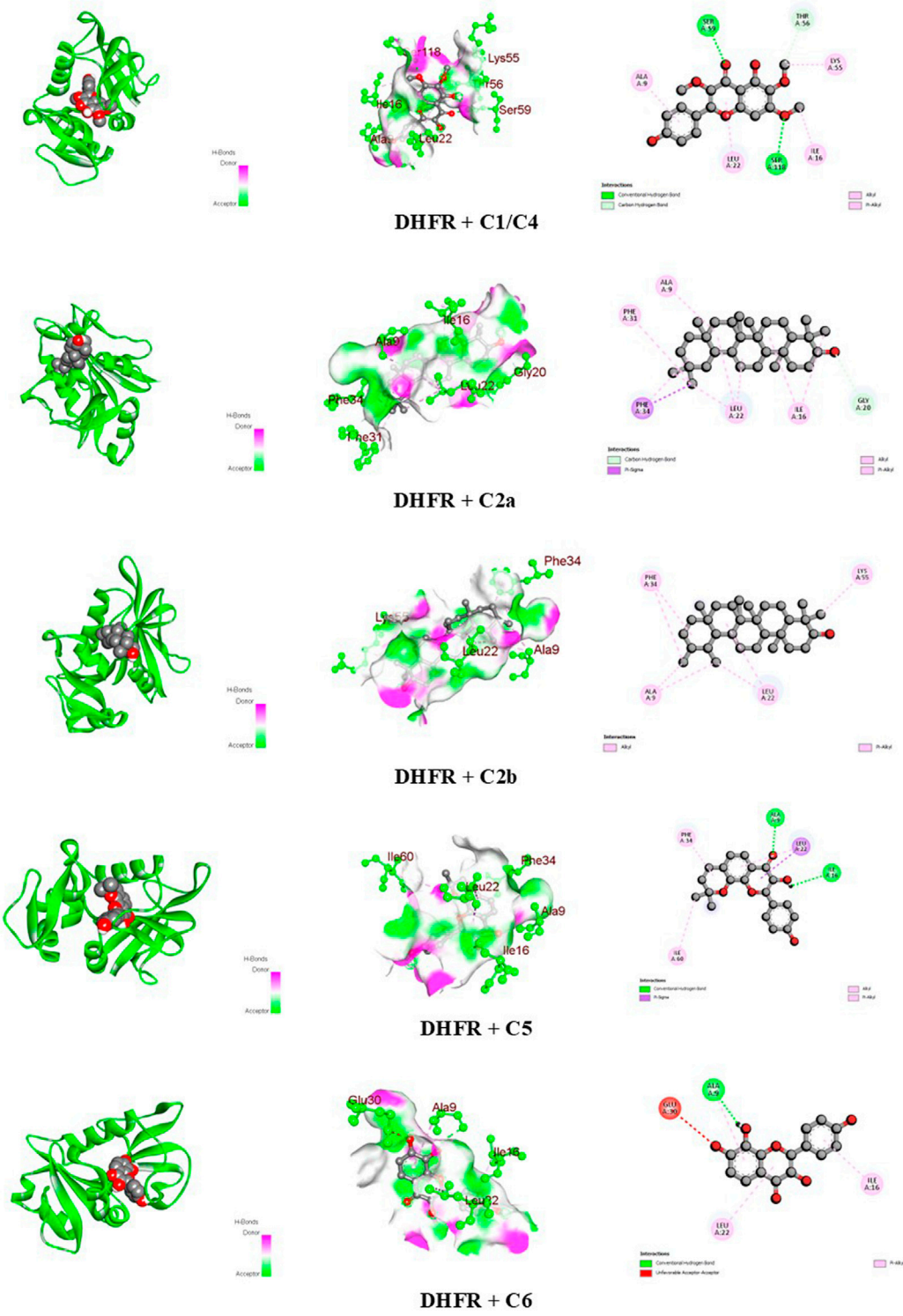



FIGURE 9
Graphical representation of the molecular interactions of the isolated phytocompounds with the DHFR enzyme with 3D and 2D visualization.

The $^{1}$H NMR spectrum (400 MHz, $CDCl_3$) of compound **2** (Figure 5) showed two triplets at $\delta_H$ 5.32 ppm and $\delta_H$ 5.28 ppm characteristic of the olefinic proton (H-12) of α- and β-amyrin, respectively. In addition, eight singlets and two doublets of n$CH_3$ protons were identified in the range $\delta_H$ 1.28-0.79 ppm. The singlet at $\delta_H$ 0.89 (3H) and $\delta_H$ 0.88 ppm (3H) indicated the presence of $CH_3$-28. This means that the analyzed component was not an acid with the carboxyl group at C-17. The peaks of the methyl groups of α-amyrin were identified at $\delta_H$ 1.16 ppm (singlet; $CH_3$-27), $\delta_H$ 0.93 ppm (doublet-doublet; $J$ = 3.2 Hz; $CH_3$-30), and $\delta_H$

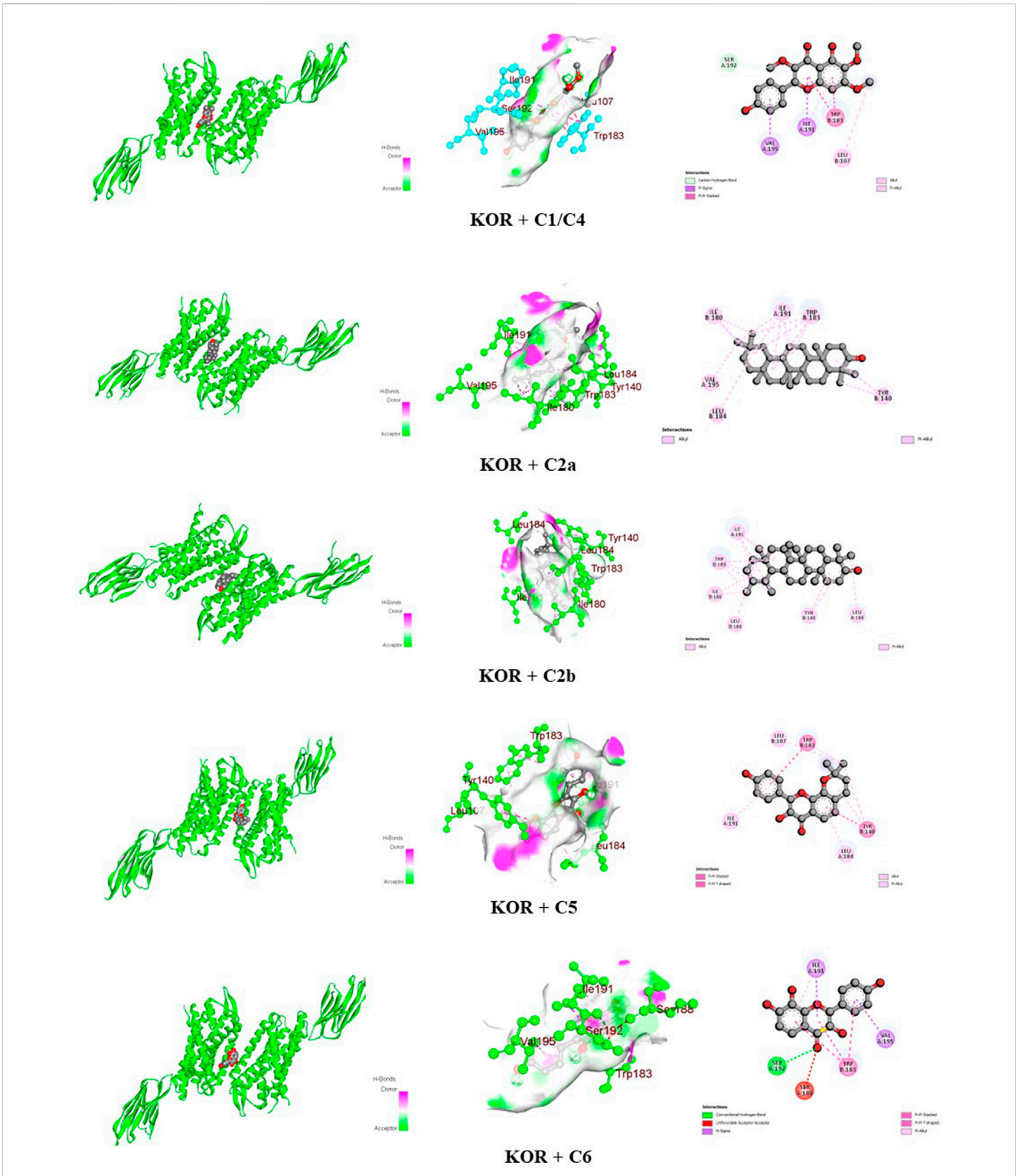



FIGURE 10
Graphical 3D and 2D visualization of the molecular interactions of the isolated phytocompounds with the KOR receptor.

0.82 ppm (singlet; $CH_3$-29). The protons of the $CH_3$-27, $CH_3$-29, and $CH_3$-30 groups of β-amyrin had peaks at $\delta_H$ 1.28 ppm (singlet; 3H; $CH_3$-27) and $\delta_H$ 0.95 ppm (singlet; 6H; $CH_3$-29 and 30). The other signals were identical for both amyrins: $\delta_H$ 1.11 (6H, s; $CH_3$-26), $\delta_H$ 1.01 (6H, s; $CH_3$-28), $\delta_H$ 0.98 (6H, s; $CH_3$-25), $\delta_H$ 0.82 (6H, s; $CH_3$-23), and $\delta_H$ 0.80 (6H, s; $CH_3$-24). The above data identified compound **2** as a mixture of α-amyrin (**2a**) and β-amyrin (**2b**) with a ratio of 4:3 based on the $^1$H NMR peak heights. This identity was further confirmed by a direct comparison of its $^1$H NMR spectrum with that recorded for a

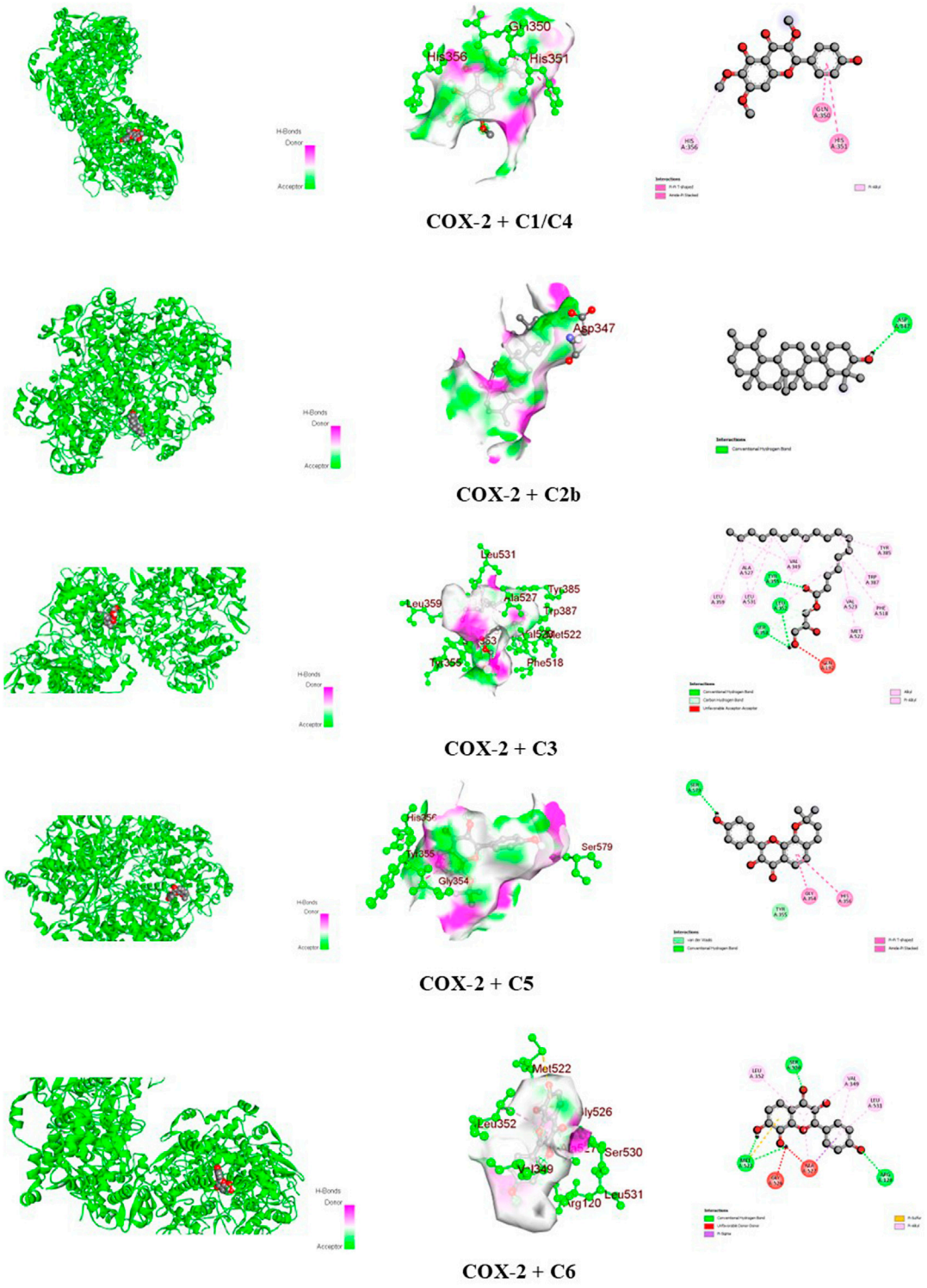



FIGURE 11
Graphical 3D and 2D visualization of the molecular interactions of the isolated phytocompounds with the COX-2 enzyme.

mixture of α-amyrin and β-amyrin in $CDCl_3$ (400 MHz, $CDCl_3$) (Migas et al., 2005).

The structure of compound **3** can be proposed as a monoglyceride of saturated fatty acid. The $^1$H NMR spectrum (400 MHz, $CDCl_3$) showed signals at $\delta_H$ 4.195 (2H, dd, $J$ = 13.6, 5.6 Hz), $\delta_H$ 3.957 (1H, m), and $\delta_H$ 3.732 (2H, dd, $J$ = 11.6, 4.0 Hz). This group of signals can be attributed to the protons of the (-$CH_2$-CH-$CH_2$-) backbone (glycerol structure). The upfield peak at $\delta_H$ 0.903 with three proton intensities corresponds to one –$CH_3$ proton. Two additional distinct groups of upfield signals at $\delta_H$ 2.374 (2H, t,

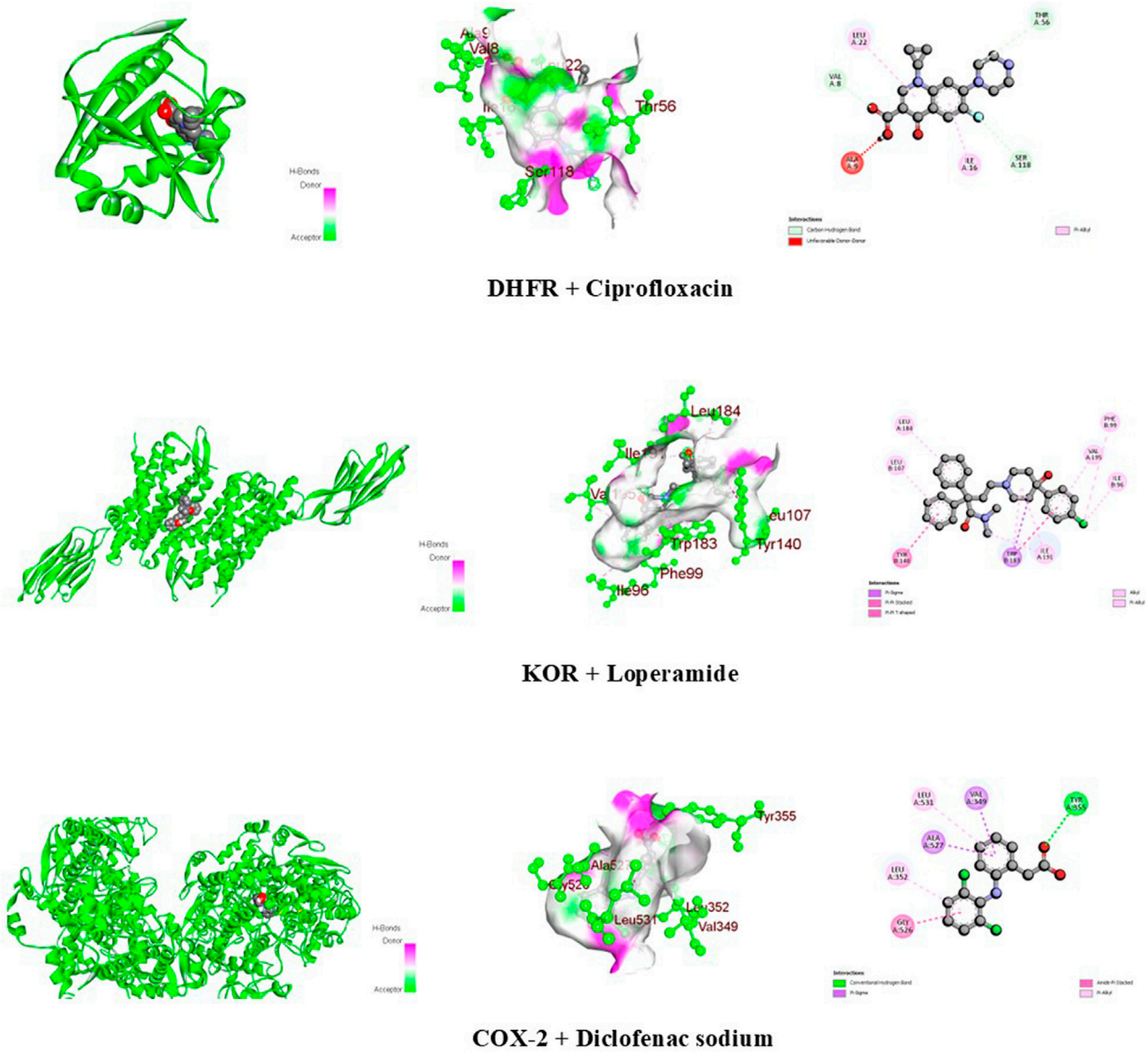



FIGURE 12
Graphical 3D and 2D visualization of the molecular interactions of the standard drug molecules with their respective enzymes.

$J$ = 7.6 Hz), $\delta_H$ 1.64 (2H, m), $\delta_H$ 1.313 (8H, m), and $\delta_H$ 1.25 (20H, m) could correspond to 16 (sixteen) methylene groups. The presence of an additional oxymethine proton in the carbon chain can be justified by the signal at $\delta_H$ 3.617 (1H, dd, $J$ = 11.6, 5.6 Hz). Comparing all the spectral data on the structure of compound **3** with the previously documented study (Kavadia et al., 2017) supports identifying this compound as a monoglyceride of stearic acid with the structure drawn in Figure 6.

The $^1$H NMR spectrum (CDCl3, 500 MHz) of compound **5** (Figure 7) showed two aromatic systems, including one 1,4-disubstituted benzene ring with the doublet at $\delta_H$ 7.68 (2H, d, $J$ = 8.5 Hz), one tetra-substituted benzene ring with the $^1$H signals at $\delta_H$ 6.86 (1H, d, $J$ = 8.5 Hz), $\delta_H$ 7.46 (1H, d, $J$ = 8.5 Hz), and a 3″,3″-dimethyl-pyrano group. The presence of 3″, 3″-dimethyl-pyrano group can be proved by two tertiary methyl signals at $\delta_H$ 1.55 (6H, s) and two olefinic proton signals at $\delta_H$ 5.87 (1H, d, $J$ = 12.5 Hz) and $\delta_H$ 6.96 (1H, d, $J$ = 12.5 Hz). All these chemical shift values are in close agreement with the published values of citrusinol except for the presence of an extra aromatic proton signal that could be assigned to H-5. Another change in the chemical shift of the H-6 proton at $\delta_H$ 6.84 differs largely in values, which is at $\delta_H$ 6.21 for citrusinol (Qing-Hui et al., 2017). This high value of the chemical shift can be justified only if the position of the oxygen atom of the pyran ring is at C-8 rather than C-7. Thus, the structure of compound **5** can be proposed as 7, 8-(3″,3″-dimethyl-pyrano)-4′-hydroxy flavonol.

The $^1$H NMR spectrum ($CDCl_3$, 500 MHz) of compound **6** shows signals that exactly match those of compound **5** (7,8-(3″,3″-dimethyl-pyrano)-4′-hydroxy flavonol). But the presence of additional peaks suggest the presence of another compound as a mixture. The additional signals include two para-coupled doublets with $J$ = 8.5 Hz at $\delta_H$ 8.00 and $\delta_H$ 6.88, each integrating for two protons, which were assigned to the coupled H-2′ and H-6′, and H-

TABLE 6 Bonds and binding sites of the identified best-binding compounds from *C. gigantea* and *C. affinis* against different targets, including DHFR, KOR, and COX-2.

| Receptor | Compound | Docking score (kcal/mol) | Bond type | Amino acids |
|---|---|---|---|---|
| DHFR (4M6J) | C1/C4 | −7.9 | Conventional hydrogen | Ser 59 and Ser 118 |
| | | | Carbon-hydrogen | Thr 56 |
| | | | Alkyl | Ile 16 and Lys 55 |
| | | | Pi-alkyl | Ala 9 and Leu 22 |
| | C2a | −8.8 | Carbon-hydrogen bond | Gly 20 |
| | | | Pi-sigma | Phe 34 |
| | | | Alkyl | Ala 9, Ile 16, Leu 22, and Phe 31 |
| | C2b | −8.8 | Alkyl | Ala 9, Leu 22, Phe 34, and Lys 55 |
| | C5 | −9.1 | Conventional hydrogen | Ala 9 and Ile 16 |
| | | | Pi-sigma | Leu 22 |
| | | | Alkyl | Ile 60 |
| | | | Pi-alkyl | Phe 34 |
| | C6 | −8.1 | Conventional hydrogen | Ala 9 |
| | | | Unfavorable acceptor–acceptor | Glu 30 |
| | | | Pi-alkyl | Ala 9, Ile 16, and Leu 22 |
| | Ciprofloxacin | −8.1 | Carbon-hydrogen | Val 8, Thr 56, and Ser 118 |
| | | | Unfavorable donor-donor | Ala 9 |
| | | | Pi-alkyl | Ile 16 and Leu 22 |
| KOR (6VI4) | C1/C4 | −7.7 | Carbon-hydrogen | Ser 192 |
| | | | Pi-sigma | Ile 191 and Val 195 |
| | | | Pi-pi stacked | Trp 183 |
| | | | Alkyl | Leu 107 |
| | | | Pi-alkyl | Ile 191 |
| | C2a | −9.4 | Alkyl | Tyr 140, Ile 180, Trp 183, Leu 184, Ile 191, and Val 195 |
| | C2b | −10.5 | Alkyl | Tyr 140, Ile 180, Trp 183, Leu 184, and Ile 191 |
| | C5 | −9.7 | Pi-pi stacked | Tyr 140 and Trp 183 |
| | | | Alkyl | Leu 107, Leu 184, and Ile 191 |
| | C6 | −8 | Conventional hydrogen | Ser 192 |
| | | | Unfavorable acceptor-acceptor | Ser 188 |
| | | | Pi-sigma | Ile 191 and Val 195 |
| | | | Pi-pi stacked | Trp 183 |
| | | | Pi-alkyl | Ile 191 |
| | Loperamide | −9.1 | Pi-sigma | Trp 183 |
| | | | Pi-pi stacked | Tyr 140 and Trp 183 |
| | | | Alkyl | Ile 96, Phe 99, Leu 107, Leu 184, Trp 183, Ile 191, and Val 195 |

(Continued on following page)

TABLE 6 (*Continued*) Bonds and binding sites of the identified best-binding compounds from *C. gigantea* and *C. affinis* against different targets, including DHFR, KOR, and COX-2.

| Receptor | Compound | Docking score (kcal/mol) | Bond type | Amino acids |
|---|---|---|---|---|
| COX-2 (1CX2) | C1/C4 | −7 | Pi-pi T-shaped | Gln 350 and His 351 |
| | | | Pi-alkyl | His 356 |
| | C2b | −8.7 | Conventional hydrogen | Asp 347 |
| | C3 | −7 | Conventional hydrogen | Leu 352, Ser 353, and Tyr 355 |
| | | | Carbon-hydrogen | Leu 352 and Ser 353 |
| | | | Unfavorable acceptor-acceptor | Gln 192 |
| | | | Alkyl | Val 349, Leu 359, Tyr 385, Trp 387, Phe 518, Met 522, Val 523, Ala 527, and Leu 531 |
| | C5 | −7.2 | Van der Waals | Tyr 355 |
| | | | Conventional hydrogen | Ser 579 |
| | | | Pi-pi T-shaped | Gly 354 and His 356 |
| | C6 | −7.3 | Conventional hydrogen | Arg 120, Met 522, and Ser 530 |
| | | | Unfavorable donor-donor | Gly 526 and Ala 527 |
| | | | Pi-sigma | Ala 527 |
| | | | Pi-sulfur | Met 522 |
| | | | Pi-alkyl | Val 349, Leu 352, Ala 527, and Leu 531 |
| | Diclofenac sodium | −7.8 | Conventional hydrogen | Tyr 355 |
| | | | Pi-sigma | Val 349 and Ala 527 |
| | | | Amide-pi stacked | Gly 526 |
| | | | Pi-alkyl | Leu 352 and Leu 531 |

3′ and H-5′, respectively. A typical ortho-coupled signal for protons is found at C-5 and C-6 at $\delta_H$ 7.46 (1H,d, $J$ = 9.0 Hz) and $\delta_H$ 7.30 (1H,d, $J$ = 9.0 Hz). All these additional proton signal values are in close agreement with published values (Ponce et al., 2009) of the structure (Figure 8) and suggested as 4′,7,8-trihydroxy flavonol. Thus, the structure of compound **6** appeared as a mixture of 4′,7,8-trihydroxy flavonol with 7,8-(3″,3″-dimethyl-pyrano)-4′-hydroxy flavonol (compound **5**) in a ratio of 1:2, according to the peak heights in the $^1$H NMR spectrum.

Diarrhea, characterized by disrupted intestinal movements and fluid accumulation coupled with increased peristalsis, occurs due to disruptions in the electrolyte permeability of the intestinal membrane (Bristy et al., 2020). Infectious diarrhea primarily arises from the invasion and spread of pathogens, particularly *Salmonella* and *Shigella* (Panda et al., 2012), against which traditional remedies have proven effective (Koné et al., 2004). The study of phytochemicals extracted from medicinal plants showcased promising antidiarrheal effects attributed to their various phytoconstituents, notably flavonoids, which are known for their antidiarrheal properties (Otshudi et al., 2000). Flavonoids are thought to achieve this by lessening motility in both the small and large intestine and suppressing bowel contractions (Capasso et al., 1991; Meli et al., 1990). All of the identified phytochemicals exhibited more pronounced antidiarrheal activity at higher doses (20 mg/kg b.w.) than at lower doses (10 mg/kg b.w.) by reducing the average weight of total and wet feces, as well as decreasing the total number of feces at a dose of 20 mg/kg, akin to the efficacy of loperamide, the standard drug used to treat diarrhea in this investigation, despite not displaying quite a dose-dependent response. However, unlike flavonoids and amyrin, the ester did not exert noteworthy antidiarrheal activity in mouse models.

The human body orchestrates a myriad of chemical reactions through various catabolic and anabolic processes, yielding a plethora of substances. These reactions, which can induce pain, inflammation, and oxidative stress, are often triggered by inflammatory mediators and reactive oxygen and nitrogen species (RONS) (Wilhelm et al., 2016). Inflammatory cascades, spurred by triggers such as microbial invasion or tissue distress, prompt the mobilization of defense cells like leukocytes to the affected site, a phenomenon orchestrated by receptors such as toll-like receptors (TLRs) and NOD-like receptors (NLRs) (Abdel Motaal and Abdel Maguid, 2005). Furthermore, an array of inflammatory mediators such as eicosanoids, cytokines, chemokines, and vasoactive amines, displays the inflammatory milieu (Sun et al., 2014). Flavonoids have demonstrated the capacity to curtail the abundance of inflammatory cells and the synthesis of MMP-9 (matrix metalloproteinase) and other inflammatory mediators (Li et al., 2012). Moreover, several flavonoids have demonstrated anti-inflammatory prowess by

TABLE 7 ADME/T study of the identified best-binding compounds from *C. gigantea* and *C. affinis* against DHFR, KOR, and COX-2 macromolecules.

| Property | Model name (Unit) | C1/C4 | C2a | C2b | C3 | C5 | C6 |
|---|---|---|---|---|---|---|---|
| Absorption | Water solubility (log mol/L) | −3.386 | −6.499 | −6.531 | −6.044 | −3.975 | −3.114 |
| | $CaCo_2$ permeability (log Papp in 10–6 cm/s) | −0.045 | 1.227 | 1.226 | 0.362 | 0.91 | 0.389 |
| | Intestinal absorption (human) (% absorbed) | 88.746 | 94.062 | 93.733 | 90.234 | 94.821 | 84.522 |
| | Skin permeability (log Kp) | −2.741 | −2.814 | −2.811 | −2.814 | −2.744 | −2.735 |
| | P-glycoprotein substrate | Yes | No | No | No | Yes | Yes |
| | P-glycoprotein I inhibitor | No | Yes | Yes | Yes | No | No |
| | P-glycoprotein II inhibitor | Yes | Yes | Yes | Yes | Yes | No |
| Distribution | VDss (human) (log L/kg) | −0.104 | 0.266 | 0.268 | −0.288 | 0.375 | 0.201 |
| | Fraction unbound (human) (Fu) | 0.083 | 0 | 0 | 0.123 | 0.112 | 0.067 |
| | BBB permeability (log BB) | −0.788 | 0.674 | 0.667 | −0.911 | −0.234 | −1.192 |
| | CNS permeability (log PS) | −3.135 | −1.773 | −1.773 | −3.365 | −1.816 | −2.434 |
| Metabolism | CYP2D6 substrate | No | No | No | No | No | No |
| | CYP3A4 substrate | Yes | Yes | Yes | Yes | No | No |
| | CYP1A2 inhibitor | Yes | No | No | Yes | Yes | Yes |
| | CYP2C19 inhibitor | Yes | No | No | No | Yes | No |
| | CYP2C9 inhibitor | Yes | No | No | No | Yes | Yes |
| | CYP2D6 inhibitor | No | No | No | No | Yes | No |
| | CYP3A4 inhibitor | No | No | No | No | Yes | Yes |
| Excretion | Total clearance (log mL/min/kg) | 0.569 | 0.119 | −0.044 | 2.04 | 0.076 | 0.458 |
| | Renal OCT2 substrate | No | No | No | No | No | No |
| Toxicity | AMES toxicity | No | No | No | No | No | No |
| | Max. tolerated dose (human) (log mg/kg/day) | −0.048 | −0.571 | −0.56 | 0.193 | 0.286 | 0.82 |
| | hERG I inhibitor | No | No | No | No | No | No |
| | hERG II inhibitor | No | Yes | Yes | No | Yes | No |
| | Oral rat acute toxicity (LD50) (mol/kg) | 2.283 | 2.467 | 2.478 | 1.734 | 2.318 | 2.563 |
| | Oral rat chronic toxicity (LOAEL) (log mg/kg_bw/day) | 2.152 | 0.856 | 0.873 | 2.897 | 0.992 | 1.516 |
| | Hepatotoxicity | No | No | No | No | No | No |
| | Skin sensitization | No | No | No | Yes | No | No |
| | *Tetrahymena pyriformis* toxicity (log ug/L) | 0.371 | 0.384 | 0.383 | 0.617 | 0.434 | 0.323 |
| | Minnow toxicity (log mM) | 1.32 | −1.309 | −1.345 | −0.969 | −0.102 | 1.498 |
| Drug-likeness | Bioavailability score (%) | 0.55 | 0.55 | 0.55 | 0.55 | 0.55 | 0.55 |
| | Lipinski's Rule of Five | Yes; 0 violation | No; 2 violations: MW > 350, XLOGP3>3.5 | No; 2 violations: MW > 350, XLOGP3>3.5 | No; 3 violations: MW > 350, Rotors>7, XLOGP3>3.5 | No; 1 violation: XLOGP3>3.5 | Yes; 0 violation |

inhibiting the expression of the COX-2 gene (Chen et al., 2000). In the present study, the isolated flavonoids exhibited significant inhibition of writhing and licking compared to the standard. Hence, it can be conjectured that the secondary metabolites, notably flavonoids, may exert an analgesic effect by quelling the inflammatory cascade. Interestingly, other compounds, especially a mixture of α- and β- amyrin, also showed noteworthy activity. Previous studies also support the candidacy of the analgesic activity of amyrin through a prospective association of prostaglandins and TNF-alpha inhibition (Aragao et al., 2008). A monoester extracted from the plant showed relatively mild analgesic activity compared to other compounds, which invalidates its strong candidacy as a lead compound for further drug development exploration.

DHFR, an enzyme present in all living organisms, plays a vital role in cellular metabolic pathways. It facilitates the conversion of dihydrofolate to tetrahydrofolate using reduced nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor. Tetrahydrofolate is further utilized in the synthesis of thymidylate, purines, and amino acids through biosynthetic processes (Canh Pham and Truong, 2022). Thus, binding to DHFR and inhibition of this enzyme leads to the disruption of nucleic acid synthesis, ultimately causing cell death (Kalogris et al., 2014). Although ciprofloxacin primarily targets GyrA and ParC, docking ciprofloxacin with DHFR would be an exploratory computational exercise to see if there are any meaningful interactions between the drug and the enzyme (Dhillon et al., 2013). In the *in silico* study, ciprofloxacin showed a docking score of −8.1 kcal/mol with the DHFR enzyme, including carbon-hydrogen bond, pi-alkyl, and unfavorable donor-donor interactions with the respective amino acids (Table 6; Figure 12). Compounds **C5, C2a,** and **C2b** surpassed this value and gave docking scores of −9.1 kcal/mol, −8.8 kcal/mol, and −8.8 kcal/mol, respectively (Table 5). Among all the compounds, **C5** showed the highest binding affinity with interactions of pi-sigma, alkyl, pi-alkyl, and conventional hydrogen bonds (Table 6; Figure 9). The binding interactions for the other compounds are briefly depicted in Table 6. These binding interactions in molecular docking are important as they determine the strength and stability of the ligand binding to the target protein, which directly influences the efficacy of a potential drug or inhibitor (Morris and Lim-Wilby, 2008).

μ, κ, and δ are the opioid receptors that modulate the activity of the enteric nervous system and the release of neurotransmitters, thereby influencing both stimulatory and inhibitory motor pathways. This sequence of events influences gastrointestinal motility and stool consistency by slowing colonic transit, reducing the sensitivity of enteric nerves, and altering the secretion and transportation of fluids (Pannemans and Corsetti, 2018). Loperamide is an opioid agonist and serves as a potential antidiarrheal agent (Kopsky et al., 2019). In computational docking studies, for antidiarrheal activity, the standard loperamide drug molecule showed a binding affinity of −9.1 kcal/mol with the KOR receptor, including pi-sigma, pi-pi stacked, and alkyl bond interactions (Tables 5, 6; Figure 12). Compounds **C2b**, **C5,** and **C2a** had higher docking scores of −10.5 kcal/mol, −9.7 kcal/mol, and −9.4 kcal/mol, respectively, compared to the standard drug (Table 5). The compound β-amyrin **(C2b)** exhibited the highest affinity among all the compounds, including alkyl bond interactions with the amino acids Tyr 140, Ile 180, Trp 183, Leu 184, and Ile 191 (Table 5; Figure 10). Considering these binding interactions is for in predicting the binding affinity and stability of a ligand–protein complex, which ultimately affects the design of more effective drugs. Effective docking must consider these interactions to identify strong binders with good pharmacological potential (Morris and Lim-Wilby, 2008).

The identified compounds were docked against the COX-2 enzyme to investigate the peripheral analgesic properties. The prostaglandins synthesized from arachidonic acids by the COX-2 enzyme are responsible for the sensation of pain and inflammation (Lee et al., 2005). Therefore, COX-2 inhibitors are considered to work in chronic pain relief and post-surgical analgesia by reducing the production of prostaglandins responsible for inflammation, pain, and fever, along with the minimization of gastrointestinal side effects (Camu et al., 2003). Diclofenac sodium is a well-established COX-2 inhibitor, and it interferes with the production of prostaglandin G2, which is the precursor to other prostaglandins (Kaur and Sanyal, 2011). In computational docking modeling, this standard drug showed a binding affinity of −7.8 kcal/mol against the COX-2 enzyme, including pi-sigma, amide-pi stacked, pi-alkyl, and conventional hydrogen bond interactions (Tables 5, 6; Figure 12). The compound β-amyrin **(C2b)** showed a higher docking score of −8.7 kcal/mol than the standard and demonstrated a strong conventional hydrogen bond interaction with the amino acid Asp 347 (Tables 5, 6; Figure 11). Compounds **C5** and **C6** also exhibited notable binding affinities of −7.2 kcal/mol and −7.3 kcal/mol, respectively. These interactions are crucial for specific binding, as they enhance the affinity and selectivity of the ligand toward the target. The interactions improve docking scores, lead to more stable complexes, and play a key role in fine-tuning the fit of the ligand within the binding pocket, ensuring proper spatial alignment (Morris and Lim-Wilby, 2008).

The ADME/T study of the identified compounds also exhibited promising results, which are listed in Table 7. According to Lipinski, if the following requirements, such as molecular weight <500 amu, hydrogen bond acceptor sites <10, hydrogen bond donor sites <5, and lipophilicity value LogP ≤5 are met, then that particular substance is likely to be orally active (Zhang and Wilkinson, 2007). Compounds penduletin **(C1/C4)** and 4′,7,8-trihydroxy flavonol **(C6)** did not violate the five Lipinski principles. Additionally, 7,8-(3″,3″-dimethyl-pyrano)-4′-hydroxy flavonol **(C5)** violated only one of Lipinski's rules. However, the compounds α- and β-amyrin **(C2a and C2b)** each had two violations, and the monoglyceride of stearic acid **(C3)** compound had three violations. The results suggest that the compounds **C1/C4** and **C5** conform to Lipinski standards, signifying their oral safety and potential as promising candidates for medicinal purposes.

pkCSM is a novel approach to predicting pharmacokinetic and toxicological consequences that use graph-based signatures to represent the chemistry and topology of small compounds (Pires et al., 2015). Table 7 shows that all the test compounds possessed negative water solubility values (log mol/L), suggesting that they are lipophilic, which promotes effective absorption (Kuentz and Arnold, 2009). The bioavailability scores of all compounds were found to be 0.55%. None of the identified test compounds exhibited any hepatotoxicity. Moreover, none of the compounds was found to inhibit hERG I, and except for **C2a**, **C2b,** and **C5**, none of them

inhibited hERG II, indicating that these compounds may not be cardiotoxic (Muster et al., 2008).

Finally, from all the hypotheses of the *in silico* study, the identified compounds that gave promising docking scores with the respective receptors, adhered to Lipinski's rule, and conformed satisfactorily with pharmacokinetic and toxicological assumptions should be considered for their drug candidacy and further investigation. Compared with existing therapeutic agents, the identified compounds exhibited remarkable effects in bioassays as well as remarkable binding affinities with the corresponding biomolecules.

## Conclusion

Vegetables are a source of nutrition and disease management and can be crucial as nutritional strategies for integrative healthcare. The discovery of promising pharmacological activities and bioactive phytochemicals in locally consumed vegetables can add a new dimension to therapeutics, pharmacological research, and development. The present study represents the first-time report on compound isolation from *C. gigantea* and *C. affinis*, employing NMR technique. The outcome reveals three bioactive flavonoids, two triterpenoids, and a monoglyceride, underscoring these species' polyphenol-rich attributes. Upon biological investigations, these compounds showed prospective antimicrobial, analgesic, and antidiarrheal potentials, justifying the tribal use of these taro vegetables. Further extensive scientific research on the isolated compounds in dosage form on a clinical trial basis is highly recommended to ascertain their safety and efficacy profile and mode of action to ease the process of future drug discovery and development.

## Data availability statement

The original contributions presented in the study are included in the article/supplementary material; further inquiries can be directed to the corresponding author.

## Ethics statement

The Animal Ethics Number for the experimental animal models of this work is 2023-01-04/SUB/A-ERC/002, which indicates approval by the Animal Ethics Committee, State University of Bangladesh. The study was conducted in accordance with the local legislation and institutional requirements.

## Author contributions

SA: conceptualization, data curation, formal analysis, investigation, methodology, writing–original draft, and writing–review and editing. FR: conceptualization, data curation, investigation, methodology, software, writing–original draft, and writing–review and editing. NE: Investigation, software, writing–original draft, and writing–review and editing. AC: supervision, writing–original draft, and writing–review and editing. CH: data curation, supervision, writing–original draft, and writing–review and editing. MH: funding acquisition, supervision, writing–original draft, and writing–review and editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Corrigendum: First-time report on compound isolation from two *Colocasia* species: vegetable-derived bioactive metabolites and their medicinal potential

Safaet Alam [1,2], Fahmida Tasnim Richi [2], Nazim Uddin Emon [3], Abu Asad Chowdhury [2], Choudhury Mahmood Hasan [2] and Mohammad Rashedul Haque [2]*

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KEYWORDS

***Colocasia gigantea*, *Colocasia affinis*, vegetable, NMR, antibacterial, antidiarrheal, analgesic, anti-inflammatory**

A Corrigendum on
First-time report on compound isolation from two *Colocasia* species: vegetable-derived bioactive metabolites and their medicinal potential

by Alam S, Richi FT, Emon NU, Chowdhury AA, Hasan CM and Haque MR (2024). Front. Pharmacol. 15:1474706. doi: 10.3389/fphar.2024.1474706

In the published article, there was an error in the **Footnote** for "Table 3" as published. An incorrect expression was used; "[n = 5]" was used when this should have been "[n = 3]". The corrected footnote appears below.

"Values are expressed as mean ± SEM (n = 3); CTL, negative control; STD, positive control; ***p < 0.001, **p < 0.01, *p < 0.05 compared to negative control."

In the published article, there was an error in Table 4 as published. Columns 1-3 of "Table 3" were incorrectly replicated as Table 4. The corrected Table 4 and its caption appear below.

In the published article, there was an error. The number of mice included in the control and test groups for pharmacological assessment was incorrect.

A correction has been made to **Materials and Methods**, *Experimental design*, *In vivo* tests, *Antidiarrheal bioassay: castor oil-induced diarrhea test*, Paragraph 1. This sentence previously stated:

"In this study, the mice were divided into four groups: control, positive control, and two test groups, each containing five mice."

The corrected sentence appears below:

TABLE 4 Anti-inflammatory effect of the isolated compounds from *C. gigantea* and *C. affinis* on the formalin-induced mouse model.

| Animal group with respective doses (ml/kg or mg/kg, b.w; p.o) | Time of licking in seconds (Ealy Phase; 0–5 min) | Time of licking in seconds (Late Phase; 15–30 min) |
|---|---|---|
| CTL | 60.38 ± 2.20 | 73.68 ± 1.34 |
| STD (Ibuprofen, 10 mg/kg, b.w) | 15.98 ± 1.62*** | 18.65 ± 2.52*** |
| Compound 1 or 4 (10 mg/kg, b.w.) | 32.54 ± 3.12*** | 38.87 ± 1.09*** |
| Compound 1 or 4 (20 mg/kg, b.w.) | 31.38 ± 1.12*** | 27.61 ± 1.02*** |
| Compound 2 (10 mg/kg, b.w.) | 44.32 ± 1.52*** | 51.49 ± 0.71*** |
| Compound 2 (20 mg/kg, b.w.) | 19.23 ± 3.34*** | 35.32 ± 1.77*** |
| Compound 3 (10 mg/kg, b.w.) | 38.04 ± 4.19** | 45.68 ± 1.95*** |
| Compound 3 (20 mg/kg, b.w.) | 28.43 ± 2.38*** | 30.56 ± 2.16*** |
| Compound 5 (10 mg/kg, b.w.) | 40.38 ± 1.51*** | 53.29 ± 1.43*** |
| Compound 5 (20 mg/kg, b.w.) | 21.43 ± 0.53*** | 24.56 ± 0.98*** |
| Mixture of compound 5 and 6 (10 mg/kg, b.w.) | 44.28 ± 2.20** | 56.21 ± 2.40** |
| Mixture of compound 5 and 6 (20 mg/kg, b.w.) | 30.44 ± 1.05*** | 36.43 ± 4.81** |

"In this study, the mice were divided into twelve groups: control, positive control, and ten test groups, each containing three mice."

In the published article, there was an error. The number of mice included in the control and test groups for pharmacological assessment was incorrect.

A correction has been made to **Materials and Methods**, *Experimental design*, *In vivo* tests, *Analgesic bioassay: acetic acid-induced writhing test*, Paragraph 1. This sentence previously stated:

"In this study protocol, mice were divided into four groups: control, positive control, and two test groups, each containing five mice."

The corrected sentence appears below:

"In this study protocol, mice were divided into twelve groups: control, positive control, and ten test groups, each containing three mice."

In the published article, there was an error.

A correction has been made to **Results**, *In vivo* study, *Effect of the identified test compounds on formalin-induced licking in the mice model*, Paragraph 1. These sentences previously stated:

"C5 and C2 demonstrated the highest anti-inflammatory efficacy among the compounds evaluated, resulting in a 44.44% and 40.74% reduction in licking respectively, at a dose of 20 mg/kg. On the other hand, the standard drug ibuprofen led to a decrease of 77.78% at a dose of 10 mg/kg."

The corrected sentences appear below:

"C5 and C2 demonstrated the highest anti-inflammatory efficacy among the compounds evaluated, resulting in a 64.50% and 68.15% reduction in licking during the early phase and a 66.66% and 52.06% reduction during the later phase, respectively, at a dosage of 20 mg/kg. On the other hand, the standard drug ibuprofen led to a decrease of 73.54% and 74.68% in early and late phase subsequently."

The authors apologize for these errors and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

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# *Ex vivo* biotransformation of lady's mantle extracts via the human gut microbiota: the formation of phenolic metabolites and their impact on human normal and colon cancer cell lines

Katarzyna Jakimiuk[1]*, Aleksandra Kruk[2], Marta Kinga Lemieszek[3], Jakub W. Strawa[1], Sebastian Granica[4], Adrian Wiater[5] and Michał Tomczyk[1]

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**Introduction:** For centuries, various species from the genus *Alchemilla* have been utilized in traditional medicine worldwide. Among them, *Alchemilla vulgaris* L. (Rosaceae) stands out as a promising herbal drug candidate due to its phytochemicals displaying anti-inflammatory and antioxidant properties.

**Methods:** In our study, we investigated the interaction between the human gut microbiota and lady's mantle herb extract (AV) following the biotransformation of the extract's constituents and their impact on colorectal cancer cells (HT-29) and normal CCD 841 CoN epithelial cells. The *A. vulgaris* herb metabolites were obtained by incubating the extract (AV) with human fecal slurries from three healthy donors (D1, D2, and D3).

**Results:** After incubating the AV extract with the human gut microbiota (AVD1-AVD3 samples), thirty-three metabolites were detected and characterized by LC-MS. Among them, one was identified as urolithin C. The AV and AVD1-AVD3 extracts and their metabolites exhibit various levels of antiproliferative and cytotoxic activities against cancer cells. Their biological effect might be linked to the changes and direct activity of bioavailable metabolites. Samples from AVD1, AVD2, and AVD3 increase the lactate dehydrogenase (LDH) released from damaged colon cancer cells in a dose-dependent manner. At 250 µg/mL, AVD1, AVD2, and AVD3 elevated the LDH level by 12.6%, 25.3%, and 30.0%, respectively. The biotransformed samples also showed significantly higher antiproliferative activity than the AV extract. The most active sample from donor 3 (AVD3) reached $IC_{50}$ = 471 µg/mL.

**Discussion:** The differences in anticancer effect might be linked to the changes and direct activity of bioavailable metabolites. The non-transformed AV extract affected neither normal nor cancer colon cells, indicating the beneficial effect of

the biotransformation procedure on the anticancer properties of the evaluated extracts. The above results clearly indicate that microbial metabolism is a crucial factor that is potent in altering the biological activity of lady's mantle extract.

KEYWORDS

***Alchemilla*, microbiota, biotransformation, colorectal cancer, deep eutectic solvent**

# 1 Introduction

Since the Middle Ages, people have searched for natural drugs to cure their diseases. Plant-derived products play an imperative biological role against certain diseases and are a major source of modern drugs (Petrovska, 2012). Lady's mantle (*Alchemilla vulgaris* L., Rosaceae) is a well-known plant used in folk medicine and ethnopharmacology to treat gynecological disorders such as menorrhagia, dysmenorrhea, or menstrual pain. It is distributed across temperate regions of the Northern Hemisphere, mainly in low-temperature and subarctic regions of Europe and Asia (Shilpee et al., 2021). Our recent review regarding the *Alchemilla* genus points out that *A. vulgaris* possesses a broad phytochemical composition, e.g., phenolic acids, flavonoids, anthocyanins, coumarins, and triterpenes (Jakimiuk and Tomczyk, 2024). In addition, tannins are a typical feature of the Rosaceae family, especially ellagic acid, casuarictin, pedunculagin, and agrimoniin, with documented antitumor activity (Fedotcheva et al., 2021).

A primary limitation of research investigating the pharmacological activity of polyphenols is their pharmacokinetics. Numerous scientific reports have proven that substances introduced into the body undergo metabolic changes under the influence of bacteria in the digestive tract. The produced metabolites differ in their structures and, consequently, in their biological activity from orally ingested xenobiotics (Kamada et al., 2013; Kruk et al., 2022). It is assumed the primary galenic form prepared from *A. vulgaris* is orally administered as an infusion. To prepare a single portion of the infusion, 2–4 g of the dried plant material should be added to 150 mL of hot water and left for 10 min. The recommended dosage is administered three times a day (Czygan, 2004). As mentioned, lady's mantle extracts are a rich source of flavonoid glycosides and tannins. Currently, it is generally accepted that flavonoid glycosides are not active *per se*. Rather, these molecules are passed through further gut microbiota-mediated modification and transformation to develop their bioavailability and biological activities (Walle, 2004; Luca et al., 2020). Unabsorbed in the upper gastrointestinal tract, approximately 90% of flavonoid glycosides are subjected to enzymatic oxidation, reduction, and decarboxylation in the small intestine. Subsequently, the colon microbiota enzymatically metabolizes glycosides to aglycones, which are further transformed into ring fission products with lower molecular weights. Sulfate derivatives of these catabolites such as glucuronides produced in the liver are excreted through bile and urine (Murota et al., 2018). However, tannins are another abundant class of compounds present in *A. vulgaris*. The bioavailability of orally administrated ellagitannins and gallotannins is low mainly due to the high molecular weights of condensed polymers and interactions with other dietary elements (Piwowarski et al., 2014). The bioavailability of tannins and resulting pharmacological actions are significantly influenced by their absorption rate, possible metabolism by the gut microbiota, or liver enzymes (Serrano et al., 2009; Sallam et al., 2021). Under the influence of many gut microbiota enzymes, hydrolyzable tannins are metabolized to gallic acid, glucose (gallotannins), and ellagic acid (ellagitannins). The subsequent bacterial metabolism of ellagic acid in the colon leads to the production of urolithins which are ultimately responsible for the pharmacological effects (Espín et al., 2013).

In addition to research dedicated to identifying polyphenolic metabolites present in plants and determining their ADME parameters (absorption, distribution, metabolism, and excretion), another direction of research has been devoted to identifying the biological activity of metabolites. Due to the key role played by the gut microbiome in the metabolism of polyphenolics, colon disease therapy provides a broad spectrum for studying the pharmacological activity of secondary metabolites after the intestinal biotransformation process. Cancer of the large intestine is one of the leading causes of cancer-related death. Polyphenols have shown significant efficacy in preventing cancer development and have exhibited anticancer properties (Aiello et al., 2019). Many herbal extracts may be able to inhibit the growth and proliferation of colon cancer cells through apoptosis; cell cycle arrest in the S phase; the reduction in PI3K, P-Akt protein, and MMP expression; inhibition of DNA biosynthesis; or increase in the expression of both cell cycle inhibitors (e.g., p53, p21, p27, BAD, Bax, caspases 3, 7, 8, and 9 proteins) (El-Najjar et al., 2007; Zorofchian Moghadamtousi et al., 2014; Aiello et al., 2019). Although plant extracts display multiple anticancer effects, the clinical usage of these results requires more studies on these compounds in *in vivo* models, including bioavailability, solubility, and metabolic alteration compounds administrated *per os* or *per rectum*.

The primary objective of this study was to establish the interaction between the human gut microbiota and lady's mantle herb extract, observing the biotransformation of the extract's constituents and their impact on human colorectal cancer cell line HT-29 and human colon epithelial CCD841 CoN cells. Our studies create further scientific hypotheses to describe a new pharmaceutical formulation used for safe phytotherapy of gastrointestinal diseases.

# 2 Materials and methods

## 2.1 Materials

The CCD841 CoN cells were obtained from ATCC (American Type Culture Collection, Manassas, VA, United States), and the HT-29 cells were purchased from the European Collection of Cell Cultures (ECACC, Centre for Applied Microbiology and Research, Salisbury, United Kingdom). DMEM (Dulbecco's

Modified Eagle's Medium, 41966029) supplemented with 10% FBS (fetal bovine serum, 10270106), PBS (phosphate-buffered saline, 14190136), and 100 U/mL penicillin, 100 U/mL streptomycin (15140122) were obtained from ThermoFisher Scientific (Waltham, MA, United States). The positive control 5-fluorouracil (5-FU) (343922), *In Vitro* Toxicology Assay Kit, Lactate Dehydrogenase Based (TOX7), MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) (M5655), ellagic acid (E2250), and brevifolincarboxylic acid (PHL83841) were purchased from Sigma-Aldrich (Saint Louis, MO, United States). 3-*O*-caffeoylquinic and pedunculagin used as standards were isolated in the Department of Pharmacognosy Medical University of Bialystok (Poland) (Grochowski et al., 2016; Strawa et al., 2020). Methanol (MeOH) was obtained from POCH (Gliwice, Poland). Ultra-pure water was obtained in-house using a POLWATER DL3-100 deionizer (Kraków, Poland). Acetonitrile Optima (ACN) was purchased from Fisher Chemical (Loughborough, United Kingdom). The mobile phase modifier formic acid (HCOOH) was purchased from POCH (Gliwice, Poland). LC-MS analyses were conducted using an Agilent Technologies 1,260 Infinity chromatography system connected to a 6,230 time-of-flight mass spectrometer (TOF/MS) (Santa Clara, CA, United States). Experiments in anaerobic conditions were performed using a Bactron 300 Anaerobic Chamber (Sheldon Manufacturing, Inc. Cornelius, Oregon, United States).

## 2.2 Plant material

The plant material was obtained from a commercial source, Dary Natury company (Koryciny, Poland) (batch number 5902741001733). Authentication of the plant material was performed in the Department of Pharmacognosy at the Medical University of Białystok (Poland) by Michał Tomczyk, according to monography in the 11th edition of the European Pharmacopoeia (European Directorate for the Quality of Medcines and HealthCare, 2019). The examination of plant material was carried out using microscopic and thin layer chromatography (TLC) methods (European Directorate for the Quality of Medcines and HealthCare, 2019).

## 2.3 Cell cultures

The human colon epithelial cell line CCD841 CoN and the human colon adenocarcinoma cell line HT-29 were grown in Dulbecco's Modified Eagle′s Medium/Nutrient Mixture F-12 Ham supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 µg/mL). Cells were maintained in a humidified atmosphere of 95% air and 5% $CO_2$ at 37°C.

## 2.4 Preparation of plant extract (AV)

The plant extract (AV) was obtained using a choline chloride-based deep eutectic solvent (DES) according to the previously described method with a slight modification (Kovač et al., 2022). In short, the DES was prepared as follows: in choline chloride/urea at a molar ratio of 1:2% and 30% of ultra-pure water (*m*/*v*). The plant material was extracted using an ultrasonic water bath (40°C, 30 min), resulting in a 20 mg/mL stock solution for evaluating the metabolism of the gut microbiota.

## 2.5 Gut microbiota metabolism of AV extract

Human fecal samples were received from three (D1, D2, and D3) healthy volunteers (28–38 years old, 2 men and 1 women) without a history of gastrointestinal disease who did not use antibiotics in the last 6 months before sample collection. All donors followed a low polyphenol diet for 3 days prior to collection. The investigations were carried out in accordance with the Declaration of Helsinki and the Ethical Committee of the Medical University of Białystok (Poland), approval no. APK.002.455.2023, allowing for feces collection from healthy human volunteers used in *ex vivo* studies. The BHI medium (brain heart infusion) was produced according to the manufacturer's instructions by dissolving 37 g of BHI in 1 L of distilled water and then sterilizing it via autoclaving at 121°C for 15 min. All experiments with human feces were conducted under anaerobic conditions in a Bactron 300 Anaerobic Chamber. Firstly, fecal slurries were prepared by suspending human feces in BHI (1:10, *m*/*v*; 37°C) in triplicate of a particular donor. Then, 3 mL of the fecal slurries and 12.5 mL of the extract solution (20 mg/mL) were mixed with 234.5 mL of the BHI. As a control, incubations of the AV extract without fecal slurries (with BHI) and a blank without the extract in the BHI (fecal slurries with the BHI) were carried out. Prepared samples were incubated in an anaerobic condition for 24 h. After incubation, the metabolic reactions were terminated by adding MeOH–0.1% HCOOH (1:1, *v*/*v*) to 1 mL of each sample before being sent for LC/MS analysis of the obtained metabolites. The remaining mixtures were centrifuged and fractionated via solid phase extraction (SPE) using 30% $H_20$% and 100% MeOH. The obtained fractions (AVD1-AVD3) were evaporated, lyophilized, and used for biological analysis. All samples from which we could obtain biological material but which were not used for further analysis were destroyed.

## 2.6 LC-MS analysis of AV and AVD1-AVD3

The AV extract and AVD1-AVD3 samples were analyzed using an adapted and slightly modified method developed by Duckstein and co-workers (Duckstein et al., 2012). A Poroshell 120 EC-C18 column measuring 250 mm × 2.1 mm, and 4 mm was used to adapt the conditions of chromatographic separation. Chromatographic analyses were carried out using an Agilent Infinity 1260 LC system equipped with a vacuum degasser, a binary pump, an autosampler, a thermostatic column compartment, and a PDA detector (Agilent, USA). An analytical reverse-phase column was used at a temperature of 25°C with detection wavelengths of 280 and 360 nm to identify tannins and flavonoids, respectively. The eluents were 0.1% HCOOH (eluent A) and ACN/$H_2O$ (9:1, *v*/*v*; eluent B) used for the following gradient with a constant flow rate of 0.350 mL/min: 0–5 min, 0% B; 5–40 min, 0%–12.5% B; 40–105 min, 12.5%–25% B; 105–110 min, 25%–100% B; 110–115 min, 100% B; and then re-equilibration to starting conditions. The injection

volume was 10 μL. TOF/MS analyses were performed using an Agilent 6,230 mass spectrometer with an ESI ion source. MS acquisition was performed under the following conditions: negative and positive ionization mode with capillary voltages of 2,500 and 4000 V, respectively; dry gas flow (N2), 9 L/min; nebulizer pressure, 35 psi; capillary temperature, 365°C. Mass spectra were recorded between m/z 50 and 2000. Peaks were identified according to their specific fragmentation patterns, UV spectra, and retention times compared with literature data and commercial reference standards. The MassHunter Qualitative Analysis V 10.0 software (Agilent) was used for LC-MS control and data processing.

## 2.7 Preparation of samples for anticancer activity evaluation

Stock solutions of investigated samples (AV, AVD1-AVD3) (20 mg/mL), were prepared by dissolving them in the DMSO/$H_2O$ (1:1, *v*/*v*) mixture and were stored in the fridge. Working solutions of the investigated samples were prepared by dissolving an appropriate stock solution in a culture medium. The working solutions were prepared to contain the same amount of the solvent mixture. As a control of the experiment, 25 μg/mL 5-fluorouracil (5-FU) was used and dissolved in the DMSO/$H_2O$ (1:1, *v*/*v*) mixture.

## 2.8 Assessment of compounds cytotoxicity–lactate dehydrogenase (LDH) assay

The cells were seeded in 96-well microplates at a density of 5 × $10^4$ cells/mL. The following day, the culture medium was removed, and the cells were exposed to the investigated samples (AV, AVD1-AVD3) prepared in a fresh medium supplemented with 2% FBS. After 96 h of incubation under standard conditions (5% $CO_2$, 37°C), the culture supernatants were collected in new 96-well microplates, which were used to perform the LDH assay following the manufacturer's instructions (*in vitro* Toxicology Assay Kit Lactate Dehydrogenase Based). The test was based on measuring the LDH released into the culture medium upon damage to the cell plasma membrane. Absorbance readings were recorded using a microplate reader (BioTek ELx800, Highland Park, Winooski, Vermont, United States) at a 450 nm wavelength. The results are presented as the percentage of LDH released from cells treated with the tested compound versus cells grown in the control medium (indicated as 100%).

## 2.9 Assessment of cell proliferation–MTT assay

The cells were seeded on 96-well microplates at a density of 5 × $10^4$ cells/mL. On the following day, the culture medium was removed, and the cells were exposed to the investigated samples (AV, AVD1-AVD3) prepared in a fresh medium supplemented with 10% FBS. After 96 h of incubation, under standard conditions (5% $CO_2$, 37°C), the MTT solution (5 mg/mL in PBS) was added to the cells for 3 h. The resultant crystals were solubilized overnight in SDS buffer with a pH of 7.4 (10% SDS in 0.01 N HCl), and the product was quantified spectrophotometrically by measuring the absorbance at a 570 nm wavelength using a microplate reader (BioTek ELx800, Highland Park, Winooski, Vermont, United States). The results are presented as a percentage of the metabolic activity of cells treated with the investigated compound versus cells grown in the control medium (indicated as 100%).

## 2.10 Statistical analysis

The data were presented as mean ± SEM. One way ANOVA with Tukey's *post hoc* test and column statistics were used for comparisons. Significance was accepted at $p < 0.05$. The $IC_{50}$ value (concentration leading to 50% inhibition of proliferation compared to the control) was calculated using GraphPad PRISM.

# 3 Results

## 3.1 Composition of the AV extract and metabolized samples (AVD1-AVD3)

In the first step of our experiment, we conducted LC-PDA-ESI-TOF/MS analysis of the raw AV extract. As shown in Figure 1 and Table 1, the use of a eutectic mixture led to the extraction of thirty three compounds, including flavonoid derivatives (e.g., quercetin and kaempferol *O*-glucuronides) and tannins (e.g., agrimoniin, pedunculagin α or β, and sanguiin H-10 isomer). The study of postbiotic metabolites resulted in the detection of twelve metabolites, where three were identified as brevifolincarboxylic acid (**11**), ellagic acid (**19**), and urolithin C (**24**) (donor D1) (Figure 1; Table 1). It is worth noting that both the crude extract and the metabolized samples contained **11** and **19**. The presence of **19** among the products of biotransformation by the intestinal gut microbiota is proof of the degradation of ellagitannins and the gradual breakdown process before the formation of urolithins. Urolithin C (**24**), absent from the AV extract, was detected in the metabolized sample (AVD1). The presence of several unclassified metabolites (**3**, **5**, **7**, **16**, **20**, **23**, **27**, **29**) in the donor samples and their absence in the AV extract indicate that a biotransformation process of plant secondary metabolites had occurred.

## 3.2 Anticancer activity evaluation

In the first set of anticancer experiments, the cytotoxicity of samples was examined in both normal and cancer colon cell lines. As shown in Figure 2, these all investigated extracts (AV, AVD1-AVD3) in the whole range of tested concentrations (25–250 μg/mL) did not affect the membrane integrity of the human colon epithelial cell line CCD841 CoN, while, when used as a positive control of the experiment, 25 μg/mL 5-FU increased the LDH level to an average of 121.8%. Studies on the human colon adenocarcinoma cell line HT-29 revealed that the AV extract in the whole range of tested concentrations was not cytotoxic. On the

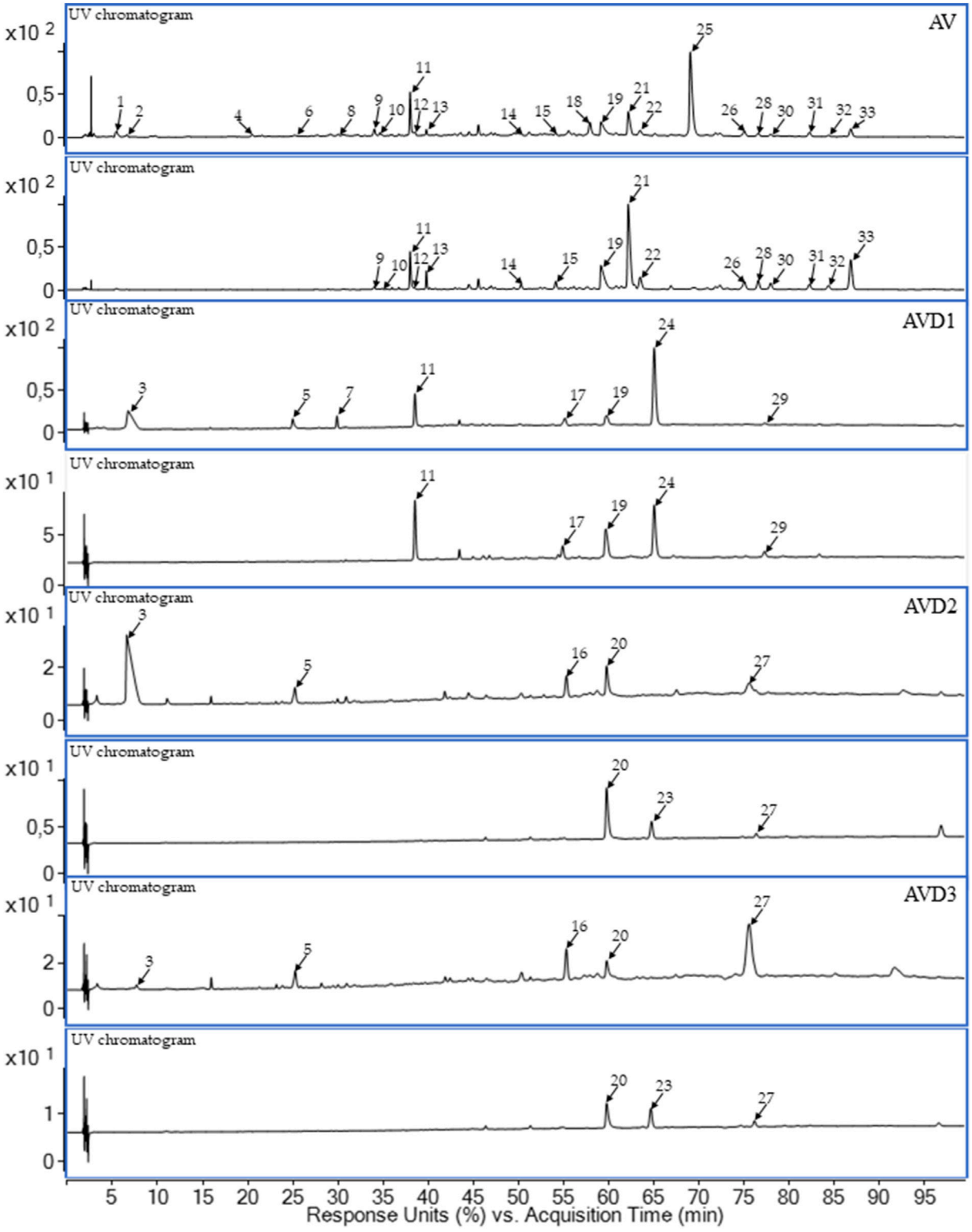



FIGURE 1
The LC-PDA-ESI-TOF/MS extracted ultra-violet chromatograms (UV) of representative samples of Lady's mantle herb DES extract (AV) donors' samples (AVD1-AVD3) after 24 h of incubation with human gut microbiota. Upper line – 280 nm, lower line – 365 nm; D1–D3 – faecal donor number.

contrary, AVD1, AVD2, and AVD3, in a dose-dependent manner, increased LDH released from damaged colon cancer cells. Of the samples that underwent biotransformation, the strongest cytotoxic effect induced AVD3, which, at the higher tested concentration, elevated the LDH level by 30.0%, while AVD1 and AVD2 decreased the membrane integrity of cancer cells by 12.6% and 25.3%,

TABLE 1 The UV–Vis and MS data of compounds identified in AV and AVD1–AVD3 by LC-PDA-ESI-TOF/MS.

| No. | Rt [min] | UV spectra [λ max nm] | Observed[a] | Δ [ppm] | Formula | Fragmentation | | Tentatively predicted compounds | Extract/samples | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Negative ionisation | Positive ionisation | | AV | AVD1 | AVD2 | AVD3 |
| 1 | 5.36 | 316 | 182.99276 | 3.3 | $C_7H_4O_6$ | 366, **182**, 139 | - | 2-Pyrone-4,6-dicarboxylic acid isomer[c] | + | - | - | - |
| 2 | 6.31 | 316 | 182.99422 | 3.86 | $C_7H_4O_6$ | 366, **182**, 139 | - | 2-Pyrone-4,6-dicarboxylic acid isomer[c] | + | - | - | - |
| 3 | 6.38 | 275 | 233.10194 | −4.71 | $C_{10}H_{18}O_6$ | **233**, 153 | **235** | Unknown | - | + | + | + |
| 4 | 20.46 | 260*sh* | 783.0718 | 4.05 | $C_{34}H_{24}O_{22}$ | **783**, 481, 301 | - | Pedunculagin α or β (s)[c] | + | - | - | - |
| 5 | 24.94 | 272 | 834.41302 | 2.35 | $C_{36}H_{67}O_{21}$ | **834**, 203 | **836**, 205 | Unknown | - | + | + | + |
| 6 | 26.17 | 295*sh*, 325 | 353.08747 | −0.96 | $C_{16}H_{18}O_9$ | **353**, 191, 179 | 355, **163** | 5-*O*-caffeoylquinic acid (s) | + | - | - | - |
| 7 | 29.83 | 225*sh*, 275 | 529.25755 | −3.58 | $C_{33}H_{37}O_6$ | **529**, 259, 243 | - | Unknown | - | + | - | - |
| 8 | 30.00 | 260*sh* | 783.072 | 4.26 | $C_{34}H_{24}O_{22}$ | **783**, 481, 301 | - | Pedunculagin α or β (s)[c] | + | - | - | - |
| 9 | 33.98 | 295*sh*, 325 | 353.0892 | 4.01 | $C_{16}H_{18}O_9$ | **353**, 191 | 355, **163** | 3-*O*-caffeoylquinic acid (s)[c] | + | - | - | - |
| 10 | 34.52 | 230, 315 | 325.09416 | 3.92 | $C_{15}H_{17}O_8$ | **325**, 145 | **250**, 121 | Unknown | + | - | - | - |
| 11 | 34.63 | 278, 360 | 291.016 | 4.55 | $C_{13}H_8O_8$ | **291**, 247 | 293 | Brevifolincarboxylic acid (s) | + | + | - | - |
| 12 | 35.09 | 275 | 633.07359 | 0.39 | $C_{27}H_{22}O_{18}$ | **633**, 463, 301 | - | Galloyl-HHDP-glucose[c] | + | - | - | - |
| 13 | 39.73 | 270, 350 | 639.12498 | 5.63 | $C_{27}H_{28}O_{18}$ | **639**, 463, 301 | **641**, 465, 303 | Quercetin *O*-hexoso *O*-uronic acid derivative | + | - | - | - |
| 14 | 50.22 | 254, 268sh, 348 | 637.10943 | −1.69 | $C_{20}H_{29}O_{23}$ | 637 | **639**, 287 | Flavonol derivatives | + | - | - | - |
| 15 | 54.11 | 255, 268*sh*, 295*sh*, 355 | 623.12565 | 0.45 | $C_{27}H_{27}O_{17}$ | **623** | **625**, 303 | Quercetin derivatives | + | - | - | - |
| 16 | 55.22 | 280 | 1445.72706 | 0.33 | $C_{78}H_{109}O_6$ | **1,445**, 929, 282 | **1,447**, 931, 284 | Unknown | - | - | + | + |
| 17 | 55.38 | 274 | 596.33914 | −3.74 | $C_{28}H_{53}O_{13}$ | **596** | **598**, 284 | Unknown | - | + | - | - |
| 18 | 57.91 | 255 | 1567.15152 | −0.56 | $C_{68}H_{48}O_{44}$ | 1,567, 1,265, **783**, 301 | - | Sanguiin H-10 isomer[c] | + | - | - | - |
| 19 | 59.20 | 254, 370 | 300.99841 | −1.6 | $C_{14}H_6O_8$ | **301**, 271 | **303** | Ellagic acid (s)[c] | + | + | - | - |
| 20 | 60.36 | 254, 367 | 1090.5504 | −0.76 | $C_{60}H_{82}O_{18}$ | **1,090**, 300 | **1,092** | Unknown | - | - | + | + |
| 21 | 62.19 | 256, 354 | 477.06713 | −0.6 | $C_{21}H_{18}O_{13}$ | **477**, 301 | **479**, 303 | Quercetin *O*-glucuronide[c] | + | - | - | - |
| 22 | 63.40 | 254, 266*sh*, 348 | 461.0742 | 3.57 | $C_{21}H_{18}O_{12}$ | **461**, 285 | **463**, 287 | Kaempferol *O*-glucuronide | + | - | - | - |
| 23 | 64.81 | 264, 352, 400*sh* | 836.42674 | 0.77 | $C_{36}H_{68}O_{21}$ | **836**, 299 | **838**, 301 | Unknown | - | - | + | + |
| 24 | 65.05 | 258, 306, 338 | 243.02980 | −0.09 | $C_{13}H_8O_5$ | **243** | **245** | Urolithin C | - | + | - | - |

(Continued on following page)





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TABLE 1 (*Continued*) The UV–Vis and MS data of compounds identified in AV and AVD1–AVD3 by LC-PDA-ESI-TOF/MS.

| No. | Rt [min] | UV spectra [λ max nm] | Observed[a] | Δ [ppm] | Formula | Fragmentation | | Tentatively predicted compounds | Extract/samples | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Negative ionisation | Positive ionisation | | AV | AVD1 | AVD2 | AVD3 |
| 25 | 68,99 | 230, 260 | 1870.15790 | −0.41 | $C_{82}H_{54}O_{52}$ | 1870, 1,567, 1,246, **934**, 783, 301 | - | Agrimoniin[c] | + | - | - | - |
| 26 | 75.00 | 231, 266, 336 | 445.07909 | 3.28 | $C_{21}H_{18}O_{11}$ | **445**, 269 | **447**, 271 | Flavone derivative | + | - | - | - |
| 27 | 75.55 | 270 | 687.41612 | −1.61 | $C_{32}H_{64}O_{15}$ | **687** | **689**, 461, 345 | Unknown | - | - | + | + |
| 28 | 76.55 | 252, 356 | 491.08441 | 2.64 | $C_{22}H_{20}O_{13}$ | **491**, 315 | **493**, 317 | Methyl quercetin glucuronide[c] | + | - | - | - |
| 29 | 77.31 | 252, 360 | 1077.60771 | 0.89 | $C_{50}H_{94}O_{24}$ | 1,077 | 1,079, 758, 689, 345 | Unknown | - | + | - | - |
| 30 | 84.33 | 266, 350 | 477.10284[b] | 0.02 | $C_{22}H_{20}O_{12}$ | 951, **475**, 299 | **477**, 300 | Flavonol derivative | + | - | - | - |
| 31 | 82.18 | 258, 273, 343 | 523.10790 | 1.23 | $C_{23}H_{22}O_{14}$ | **521**, 345 | **523**, 431, 347, 269 | Flavonoids derivative | + | - | - | - |
| 32 | 84.33 | 266, 350 | 477.10284[b] | 0.52 | $C_{22}H_{20}O_{12}$ | 951, **475**, 299 | **477**, 300 | Flavonol derivative | + | - | - | - |
| 33 | 86.76 | 254, 273*sh*, 355 | 505.09952 | 2.08 | $C_{23}H_{21}O_{13}$ | 1,011, **505**, 329 | **507**, 331 | Unknown | + | - | - | - |

[a]Exact mass of [M-H]- ion.

[b]Exact mass of [M-H]+ ion; sh, peak shoulder; bold, most aboundantion; s, reference substance; HHDP, hexahydroxydiphenoyl group.

[c]Correlated with published data (Duckstein et al., 2012).





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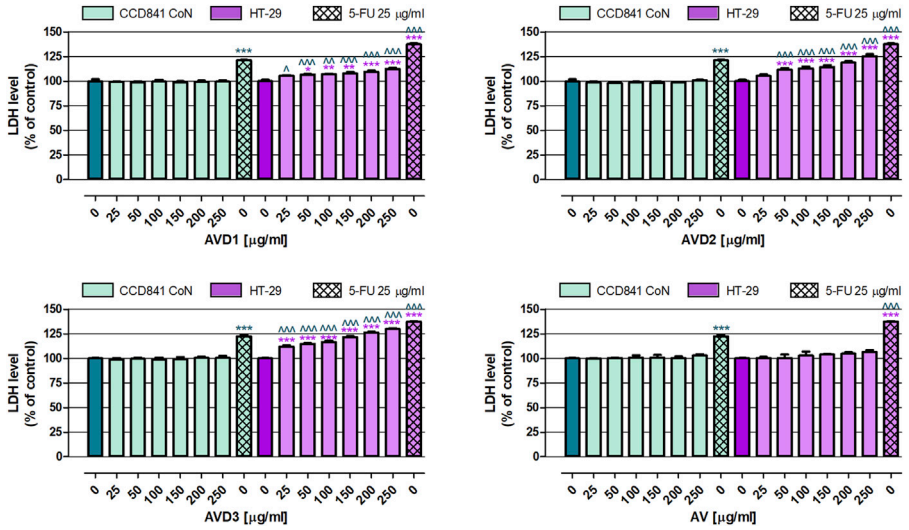



FIGURE 2
Influence of the *Alchemilla vulgaris* extract (AV) and samples after biotransformation (AVD1-AVD3) on cell membrane integrity of human colon epithelial cell line CCD841 CoN and human colon adenocarcinoma cell line HT-29. The cells were exposed to the culture medium alone (control) or extracts at concentrations of 25, 50, 100, 150, 200 and 250 μg/mL; or 25 μg/mL, 5-fluorouracil (5-FU; positive control) for 96 h. Samples cytotoxicity (level of LDH released into the cell culture medium from damaged cell membranes) was measured using an LDH assay. Results are presented as mean ± SEM of 6 measurements. *p < 0.05; **p < 0.01; ***p < 0.001 vs. control; ^p < 0.05; ^^p < 0.01; ^^^p < 0.001 colon cancer cells treated with extract/5-FU vs. colon epithelial cells exposed to the extract/5-FU at the corresponding concentration; one-way ANOVA test; post-test: Tukey.

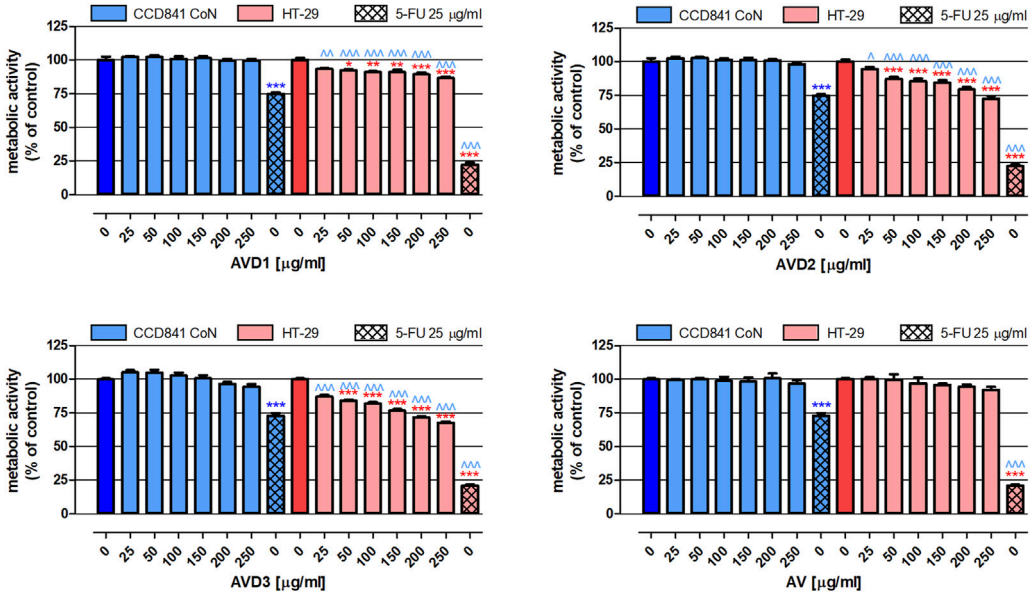



FIGURE 3
Influence of the *Alchemilla vulgaris* extract (AV) and samples after biotransformation (AVD1-AVD3) on the proliferation of human colon epithelial cell line CCD841 CoN and human colon adenocarcinoma cell line HT-29. The cells were exposed to the culture medium alone (control) or extracts at concentrations of 25, 50, 100, 150, 200 and 250 μg/mL; or 25 μg/mL 5-fluorouracil (5-FU; positive control) for 96 h. The metabolic activity of investigated cells in response to tested compounds was examined photometrically by means of the MTT assay. Results are presented as mean ± SEM of 6 measurements. *p < 0.05; **p < 0.01; ***p < 0.001 vs. control; ^p < 0.05; ^^p < 0.01; ^^^p < 0.001 colon cancer cells treated with extract/5-FU vs. colon epithelial cells exposed to the extract/5-FU at the corresponding concentration; one-way ANOVA test; *post hoc* test: Tukey.

respectively. On the contrary, 25 μg/mL 5-FU increased the LDH level to an average of 137.3%. It must be highlighted that AVD1 and AVD2 have shown cytotoxic properties against HT-29 in concentrations ranging from 50 to 250 μg/mL, while AVD3 in the whole range of tested concentrations significantly damaged the membranes of colon cancer cells. Comparison data collected from normal and cancer cells treated with biotransformed extracts revealed their great selectivity, indicating that compounds were not

toxic against CCD841 CoN cells and, at the same time, significantly decreased the viability of HT-29 cells. The non-transformed AV extract did not affect either cancer or normal colon cell lines.

In the next step of our study, the extracts' impact on the proliferation of both normal and cancer colon cells was examined. As presented in Figure 3, none of the tested extracts altered the metabolic activity of human colon epithelial cell line CCD841 CoN. Moreover, AV did not impact the proliferation of human colon cancer HT-29 cells. On the contrary, AVD1- AVD3 in concentrations ranging from 50 to 250 μg/mL revealed significant antiproliferative properties in HT-29 cells, and the observed changes were dose-dependent. Among biotransformed extracts, the strongest inhibition (by 32.2%) of cancer cell proliferation was observed after treatment with 250 μg/mL AVD3 ($IC_{50\ HT\text{-}29}$ = 471 μg/mL), while the weakest effect was observed for AVD1 ($IC_{50\ D1\ HT\text{-}29}$ = 1,440 μg/mL), which, at the concentration of 250 μg/mL, reduced metabolic activity by 13.4%. Used as a positive control, 25 μg/mL 5-FU decreased the proliferation of CCD841 CoN and HT-29 cells by 26.1% and 78.4%, respectively.

## 4 Discussion

*Alchemilla vulgaris* is one of the most well-known medicinal plants in terms of its phytochemical content. According to recent research, deep eutectic solvents (DESs) have been adapted for new methods to extract and isolate polyphenols (Zainal-Abidin et al., 2017). The main benefits of the use of DESs are the low cost of the starting components and their biodegradable properties with no or low toxicity. The selection of the DES for our experiments was based on an examination conducted by Kovač and co-workers. According to their article, choline chloride/urea (1:2, molar ratio) was the most favorable mix of components due to the highest yields of gallic acid, ellagic acid, and hydrolyzable tannins. As the authors mentioned, the level of desired components was also affected by the amount of added water due to the impact on the viscosity that affects mass transfer in the extraction process (Kovač et al., 2022). The following phytochemicals are prevalent in the literature: pedunculagin isomers (**4**, **8**), brevifolincarboxylic acid (**11**), sanguiin H-10 isomer (**18**), ellagic acid (**19**), and agrimoniin (**25**). These phytochemicals are found in ethanol and acetone/water extracts from *A. vulgaris* and are also present in DES extracts (Duckstein et al., 2012). Thus, in the crude *A. vulgaris* extract (AV), numerous compounds from the polyphenol group were present, contains specific molecules belonging to a large group of tannins (Table 1).

Ten compounds were detected, which were not observed in the crude extract (AV). Even though most of the reported examinations agreed on the resulting products of the digestion of plant secondary metabolites, the qualities and quantities of these metabolites may vary among individuals. These differences may relate to variations in the composition of the colonic microbiota, dietary habits, and health status among individuals. Ion fragmentation analysis results show that they are formed by ellagitannins. In addition to the pattern-compliant retention time, the ion distribution confirms the presence of the alpha and beta isomers of pedunculagin (**4**, **8**) in the extract. We have observed ions at m/z 783 and m/z 481 in the MS spectra, indicating the loss of HHDP (hexahydroxydiphenoyl unit), and at m/z 301, indicating the loss of hexose + O. It is worth noting that in the ion distribution of compound **25**, common elements were observed. The initial phase of MS fragmentation involves the cleavage of two HHDP units at m/z 1,567 (loss of HHDP) and m/z 1,265 (loss of HHDP), which are distinguished by a GOG-type bond between the two galloyl groups, comprising compound **25** (with two galloyl-bis-HHDP hexose units). Subsequently, **25** has been cleaved at m/z 783 (loss of 132 Da + loss of HHDP) and m/z 301 (loss of hexose + O). The loss of 132 Da from a galloyl moiety without oxygen after cleavage showed that the type of linkage was a GOG. In the present investigation, the fragmentation of the dimeric ellagitannin structure (m/z 1,567 → m/z 783 → m/z 301) led to the conclusion that this ellagitannin was sanguiin H-10 (**18**).

Traditionally, lady's mantle is administered orally and thus undergoes intestinal digestion. Although this plant material occurs in the pharmacopeial monograph, there are no reports on its interaction with the human gut microbiota (ESCOP, 2003). Data from the literature indicate that ellagic acid can be transformed by the gut microbiota into urolithin C (Kujawska and Jodynis-Liebert, 2020). According to the information in Table 1, ellagic acid (**19**, m/z = 302) present in the AV extract underwent biotransformation, as it is not present in the samples from donors. This fact is also confirmed by the occurrence of urolithin C (**24**, m/z = 244) in the donor D1 sample (AVD1 after the biotransformation process). Another large group of phytochemicals present in the AV extract but missing in donor samples (AVD1-AVD3) are flavonoids and their derivatives. Their gut microbiota-mediated biotransformation depends on the attached moiety (Murota et al., 2018; Al-Ishaq et al., 2021). As flavonoid derivatives, when they reach the colon, they can be hydrolyzed to aglycons and into ring fission products (Pei et al., 2020; Xiong et al., 2023). Thus, quercetin and kaempferol *O*-glucuronides occur in the AV extract but they are lacking in the metabolized donor samples (AVD1-AVD3). The compounds present in the AV extract may have undergone complete degradation or may be the compounds marked as unidentified (Table 1).

As mentioned in the introduction, biotransformation through the microbiota and modifications in the composition of lady's mantle may influence its therapeutic properties and qualities. At this point, we evaluated the anticancer potential of samples after biotransformation compared to the crude extract (AV). So far, the anticancer potential of *A. vulgaris* extracts has been proven in the murine melanoma cell line B16, human ovarian cancer cell line A2780, human prostate cancer cell line PC-3, and human breast cancer cell lines 4T1 and MCF-7 (Vlaisavljević et al., 2019; Jelača et al., 2024; Al-Zharani and Abutaha, 2023). There is also a scientific report presenting the efficacy of a water/methanol extract (2:8, *v*/*v*) of *A. vulgaris* against human colorectal adenocarcinoma cell line Caco-2 (Ibrahim et al., 2022). In the presented study, we also focused on human colorectal cancer cells; however, the investigation also included an assessment of the extracts' impact on normal human colon epithelial cells, which, unfortunately, is not a very common procedure. The performed analysis revealed that the significant chemopreventive potential of *A. vulgaris* extracts after biotransformation (AVD1, AVD2, and AVD3) manifested via the effective elimination of human colon cancer HT-29 cells. This caused both damage to their cell membranes (LDH assay) and inhibition of their proliferation (MTT assay), with the simultaneous lack of cytotoxicity and antiproliferative impacts on normal colon epithelial CCD841 CoN cells (Figures 2, 3). The obtained data indicate the high selectivity of

the tested extracts, which is crucial for their potential therapeutic use. An extremely valuable observation is that the AV extract did not cause any changes in both normal and cancer cell lines, indicating that biotransformation is required to reveal the beneficial health-promoting properties of the examined extracts. In addition, the donor samples are a post-fermentation mixture of various compounds created from the culture medium and microbial metabolism, which can mutually interact with the used model.

## 5 Conclusion

*Alchemilla vulgaris* extract contains phytoconstituents from the group of polyphenols, the largest group of which are flavonoids and tannins. After gut microbiota biotransformation, there were changes in the chemical composition of the donors' samples compared to the native extract. Our study revealed the great anticancer potential of biotransformed *A. vulgaris* extracts, which selectively eliminated human colon cancer HT-29 cells without any negative changes in human colon epithelial CCD841 CoN cells. The lack of changes in the viability and proliferation of the investigated normal and cancer cell lines in response to non-transformed *A. vulgaris* extract clearly indicates the beneficial effect of the biotransformation procedure on the anticancer properties of evaluated extracts. However, the exact mechanism of these interactions remains undiscovered and requires further investigations of microbiota composition changes and direct action of bioavailable metabolites. Our finding indicated new therapeutic possibilities for *A. vulgaris* extract in the context of gastrointestinal diseases and interesting concepts of the composition of potential drugs of natural origin used in safe phytotherapy.

## Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## Ethics statement

The studies involving humans were approved by Ethical Committee of Medical University of Białystok (Poland). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

## Author contributions

KJ: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Visualization, Writing–original draft. AK: Investigation, Methodology, Writing–review and editing. ML: Investigation, Methodology, Writing–original draft. JS: Investigation, Methodology, Writing–original draft. SG: Methodology, Writing–review and editing. AW: Investigation, Writing–review and editing. MT: Supervision, Writing–original draft, Writing–review and editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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# Luteolin: exploring its therapeutic potential and molecular mechanisms in pulmonary diseases

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Luteolin is a flavonoid widely found in plants, including vegetables, botanical drugs, and fruits. Owing to its diverse pharmacological activities, such as anticancer, oxidative stress protection, anti-inflammatory, and neuron-preserving effects, luteolin has attracted attention in research and medicine. Luteolin exhibits therapeutic effects on various pulmonary disease models through multiple molecular mechanisms; these include inhibition of activation of the PI3K/Akt-mediated Nuclear Factor kappa-B (NF-κB) and MAPK signaling pathways, as well as the promotion of regulatory T cell (Treg) function and enhancement of alveolar epithelial sodium channel (ENaC) activity (alleviating inflammation and oxidative stress responses). Luteolin has therapeutic effects on chronic obstructive pulmonary disease (COPD), acute lung injury/acute respiratory distress syndrome (ALI/ARDS), pulmonary fibrosis, allergic asthma, and lung cancer. Luteolin, a naturally occurring polyphenol, is poorly water-soluble. The oral route may be ineffective because the gut poorly absorbs this type of flavonoid. Therefore, although luteolin exhibits significant biological activity, its clinical application is limited by challenges associated with its poor water solubility and low bioavailability, which are critical factors for its efficacy and pharmacological application. These challenges can be addressed by modifying the chemical structure and enhancing pharmaceutical formulations. We summarized the research advancements in improving the solubility and bioavailability of luteolin, as well as the effects of luteolin on various pulmonary diseases and their related mechanisms, with the aim of providing new ideas for researchers.

KEYWORDS

**luteolin, pulmonary diseases, molecular mechanisms, bioavailability, pharmacokinetics, pharmacological activities**

## 1 Introduction

Pulmonary diseases are one of the major health issues worldwide, with high incidence and mortality rates (Chen and Li, 2024; Meng et al., 2025; GBDCRD, 2023). According to the World Health Organization, COPD, asthma, pulmonary fibrosis, lung cancer, and other pulmonary diseases lead to millions of deaths each year (Roh, 2024; Wang et al., 2025; Podolanczuk and Raghu, 2024; Lu et al., 2025). With the aging of the population and the

intensification of environmental issues, the burden of pulmonary diseases is expected to increase further (Faniyi et al., 2022). Although there are various drugs available for the treatment of pulmonary diseases, there are still many challenges, such as drug side effects, drug resistance, and the lack of effective treatments for certain diseases (Mazumder et al., 2024; Dorababu and Maraswami, 2023; Bandyopadhyay and Mirsaeidi, 2023).

Against this backdrop, the potential of natural products in the treatment of pulmonary diseases is increasingly gaining attention. Natural products, with their rich chemical diversity and biological activity, offer valuable resources for drug development. Compared with traditional chemical drugs, natural products often have better biocompatibility and lower toxicity, and have shown unique advantages in regulating the body's immune system, anti-inflammatory, and antioxidant aspects (Li C. et al., 2024; Wang et al., 2023). From traditional Chinese medicine to modern botanical drugs, the application of natural products in the treatment of pulmonary diseases has a long history, and new research results are constantly emerging (Rahman et al., 2022).

Currently, there are numerous narrative reports about luteolin in various diseases (Zhang et al., 2024; Shi et al., 2024; Huang L. et al., 2023; Diniz et al., 2022), but most of the research on pulmonary diseases focuses on describing its therapeutic effects, with few exploring and summarizing its potential mechanisms. This study summarizes the effects of luteolin on various pulmonary diseases and related mechanisms, aiming to provide new insights for researchers and to promote further research and application of luteolin in the field of pulmonary disease treatment.

# 2 Plant sources of luteolin and its physicochemical properties

Over the last 2 decades, there has been mounting emphasis on utilizing metabolites derived from medicinal plants to treat and manage a diverse array of diseases (Xiong et al., 2021; Ahmad et al., 2020; Singh et al., 2023; Ramadaini et al., 2024). Luteolin, an active flavonoid metabolite isolated from *Lonicera japonica*, exhibits many biological activities. It is also known for its potent antioxidant and anti-inflammatory characteristics (Xiong et al., 2024; Jia et al., 2023). Plants abundant in luteolin have been used in traditional Chinese medicine for treating a wide array of illnesses, including hypertension (Gao et al., 2023), various inflammatory conditions (Attiq et al., 2021), and cancer (Jiang et al., 2021). In addition to its medicinal use, luteolin is also present in various vegetables, such as fresh *Spinacia oleracea*, *Petroselinum crispum*, and *Brassica oleracea* var. *Capitata,* and a range of fruits and botanical drugs (Manzoor et al., 2019; Almatroodi et al., 2024). Flavonoids—prevalent polyphenolic metabolites within the plant kingdom—have demonstrated health benefits in humans at particular concentrations (Wen et al., 2021). These metabolites are biosynthesized via the phenylpropanoid pathway in plants (Cocuron et al., 2019). Luteolin has a classic flavonoid structure featuring four hydroxyl groups at 5′, 7′, 3′, and 4′positions. The structural hallmark of luteolin includes two aromatic rings, A and B, which are linked to an oxygenated pyran ring labeled as C. The biological activities of luteolin are potentially contingent upon the presence of its hydroxyl groups and the double bond that exists between carbons 2 and 3. Typically, luteolin is encountered in a glycosylated form, where one or more of its hydroxyl groups have been replaced by diverse glycosyl groups (Huang L. et al., 2023). Similar to other flavonoids, luteolin is an antioxidant and can prevent damage to DNA, lipids, and proteins induced by reactive oxygen species (ROS) (Zou et al., 2021). Molecules such as hydrogen peroxide ($H_2O_2$), singlet oxygen, and hydroxyl radicals are highly reactive with numerous biological targets and play crucial roles in neuromodulation, immunomodulation, ion transport, and apoptosis (Mittler, 2017). Luteolin influences the activities of various enzymes associated with oxidative stress. Among these, oxidases responsible for the generation of ROS, such as xanthine oxidase, are inhibited by luteolin (Liu X. et al., 2022). Luteolin also inhibits the activity of enzymes that catalyze the oxidation of cellular constituents, including cyclooxygenases and lipoxygenases (Han et al., 2024; Ren et al., 2021). Additionally, luteolin can be oxidized and serves as a scavenger of ROS—an activity that stems from the chemical structure it shares with other flavonoids, enabling it to provide hydrogen atoms from the hydroxyl groups present on its aromatic rings (Ramesh et al., 2021). Despite the promising outlook on the therapeutic characteristics of luteolin, it is imperative to consider its possible toxic effects and broader health implications (Galati and O'Brien, 2004; Khan et al., 2021). Understanding the full spectrum of its effects on human biology is crucial for its effective use, as shown in Figure 1.

## 2.1 Pharmacokinetics of luteolin

### 2.1.1 Bioavailability

The *in vivo* bioavailability of luteolin is relatively low owing to its poor stability and low absorption rate, which consequently diminishes its therapeutic effectiveness. The bioavailability of total luteolin, including free and bound forms, has been reported to be 53.9%. Despite an apparent good absorption rate, the level of free luteolin present in the systemic circulation is lower than that of its metabolites, with the actual bioavailability of free luteolin being merely 17.5%. This suggests that luteolin is promptly absorbed upon entering the rat intestinal system and undergoes comprehensive metabolism in the intestinal and/or hepatic cellular structures, which is the reason for its low bioavailability (Yao et al., 2023; Deng et al., 2017). Following oral administration, luteolin achieves its peak plasma concentration within 1–2 h and is retained in circulation for several hours thereafter. Its metabolism is sufficiently slow to allow the metabolite to exhibit biological activity *in vivo* (Ali and Siddique, 2019). When comparing the absorption efficiency of a single oral dose of pure luteolin or luteolin derived from peanut hull extract, the jejunum and duodenum had higher absorption rates than the colon and ileum. Furthermore, the bioavailability of luteolin from the peanut hull extracts significantly surpassed that of pure luteolin (Zhou et al., 2008). Upon absorption, luteolin is rapidly taken up, and can be detected in the plasma as luteolin, luteolin monoglucoside, and luteolin glucuronide, along with sulfate conjugates of luteolin aldehyde and O-methylated luteolin (Yasuda et al., 2015; Shimoi et al., 1998). Certain co-occurring metabolites, such as eriodictyol, enhance the absorption of luteolin in the intestine, thereby improving its bioavailability (Zhou et al., 2008). Moreover, various pharmaceutical techniques, including cyclodextrin complexation, luteolin-loaded nanocarriers, polymeric micelles, phospholipid complexation, RA (rosmarinic acid)-SS-mPEG, and combined hydroxyethyl starch luteolin nanocrystals have been used to augment the solubility and bioavailability of luteolin. These methods slow the degradation of luteolin in the bloodstream, thereby prolonging

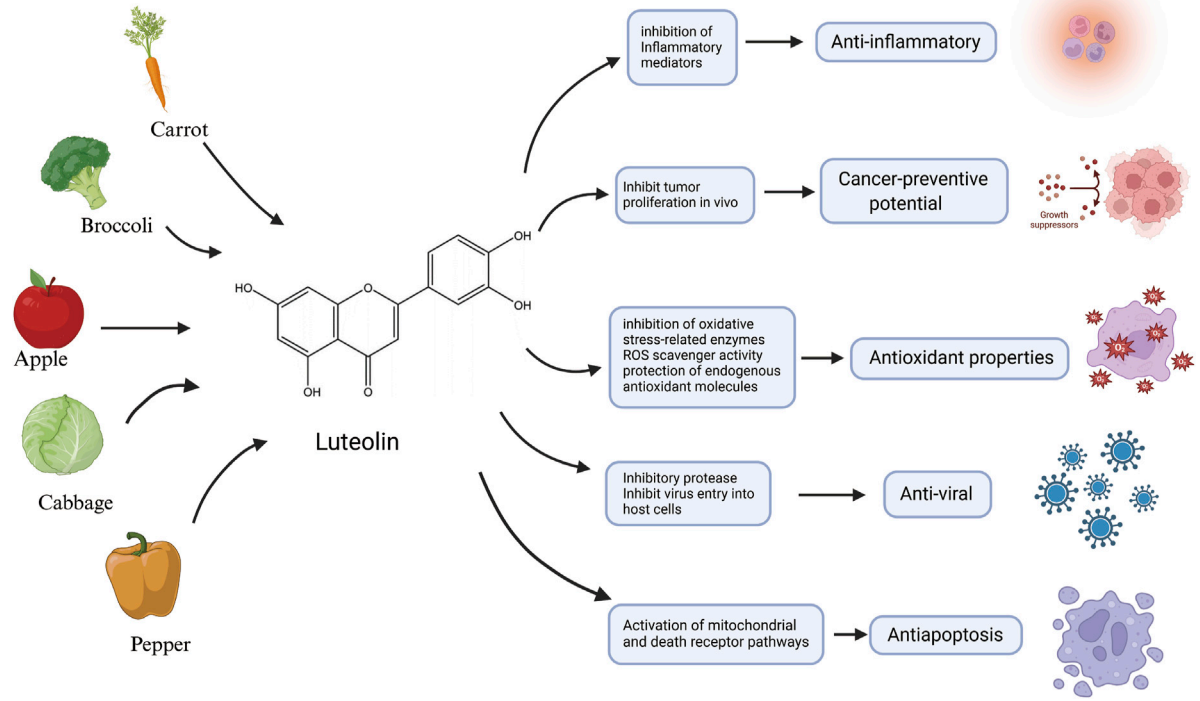



FIGURE 1
An illustration of the main source and anti-inflammatory, anticancer potential, antioxidant properties, antiviral activity and antiapoptotic characteristics of luteolin, created using Biorender.com.

its circulation time (Liu et al., 2013; Khan et al., 2016; Qing et al., 2017; Kar et al., 2024; Luo et al., 2024; Lu et al., 2023; Pittol et al., 2022). Recently, Imam and colleagues prepared luteolin-loaded nanovesicles (NVs) using the solvent evaporation method, and antioxidant activity results demonstrated that luteolin-NVs exhibited greater activity than pure luteolin. Cytotoxicity studies have shown that the IC50 value of luteolin NVs was lower than that of pure luteolin (Imam et al., 2022). Miao et al. formulated luteolin-loaded methoxy poly (ethylene glycol)-polylactide micelles (luteolin/MPEG-PLA) to enhance the bioavailability of luteolin in pulmonary infectious diseases (Miao et al., 2021). Ren et al. improved the bioavailability of luteolin four-fold using a cyclodextrin MOF-modified dry powder inhaler to treat fibrotic interstitial lung disease (Ren et al., 2023). These studies illustrate that strategies for improving the solubility and bioavailability of luteolin through structural modifications and formulation advancements are likely to facilitate its use in the treatment of diseases in the future.

### 2.1.2 Distribution

In the plant kingdom, luteolin is commonly found in a glycosylated state. During absorption, these glycosides are hydrolyzed to yield the free form of luteolin (Wang Z. et al., 2021). Glycosylation of the aglycone moiety primarily occurs via two distinct mechanisms: free hydroxyl groups to form O-glycosides and forming a carbon-carbon bond to create C-glycosides (Figure 2) (Hostetler et al., 2017; Punia Bangar et al., 2023). Following absorption, luteolin and its metabolites are rapidly and widely distributed throughout the major organs and tissues of the body. Throughout the rat circulatory system, luteolin is largely present in conjugated forms, with luteolin-glucuronide being the most prevalent metabolite circulating in most tissues (Hayasaka et al., 2018). In the current study, luteolin was highly concentrated in the liver, kidneys, gastrointestinal tract, and lungs in conjunction with its multiple metabolites. Importantly, luteolin can cross the blood-brain barrier, suggesting its potential for treating central nervous system diseases (Daily et al., 2021). These findings support the action of luteolin on these medicinal targets (Chen et al., 2010; Domitrović et al., 2009; Lu et al., 2015; Xu et al., 2015). Because of their ability to cross the placenta, these substances may be toxic to the fetus in the womb (Skibola and Smith, 2000).

### 2.1.3 Elimination

After absorption, flavonoids undergo metabolic processes in the liver, including glucuronidation, sulfation, and methylation. In addition, they can be metabolized into smaller phenolic metabolites (Bravo, 1998). Specifically, luteolin is first metabolized through glucuronidation or methylation and converted into a methylated glucuronide form. The principal Phase II metabolite has been confirmed to be luteolin-3′-O-glucuronide (Boersma et al., 2002). Luteolin-3′-O-glucuronide is the predominant metabolite in these metabolic pathways (Kure et al., 2016). Furthermore, two novel diglucuronide conjugates of luteolin were separated by incubation with uridine diphosphate glucuronosyltransferase (UGTs). Methylation has also been identified as a significant metabolic pathway for luteolin. This methylation process produces chrysoeriol and diosmetin, a reaction facilitated by catechol-O-methyltransferase in rats (Wu

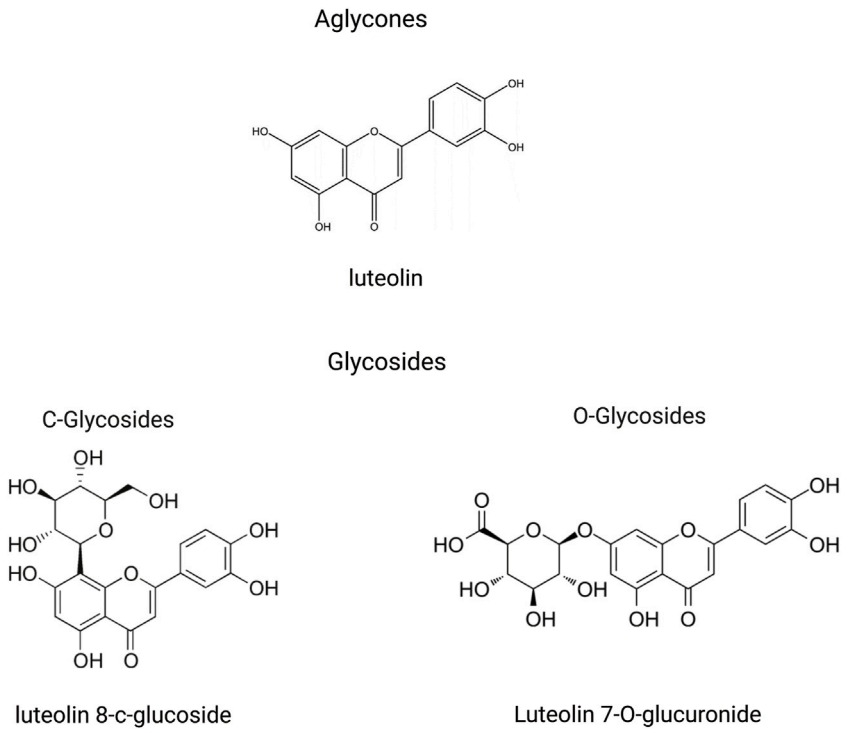



FIGURE 2
An illustration of structural formulas (through the free hydroxyl groups to form O-glycosides and through the formation of a carbon-carbon bond to create C-glycosides) of metabolites related to luteolin, created using Biorender.com.

et al., 2015; Chen et al., 2011; Wang et al., 2017). Upon oral ingestion, luteolin is quickly absorbed into the systemic circulation and undergoes extensive metabolic processing within the rat body. The recovered metabolites included five isolates from the urine and two from the bile. Luteolin is rapidly metabolized, likely in the intestinal lining and/or the liver cells throughout the first-pass metabolism after oral administration (Boersma et al., 2002). Its metabolite, luteolin-3′-O-glucuronide is primarily excreted in the urine. The feces showed no evidence of conjugated metabolites, whereas approximately 5.8% of the starting dose was recovered as free luteolin. A second peak of luteolin and most metabolites occurred between 2 and 8 h after administration, suggesting the possible involvement of enterohepatic recirculation. Biliary excretion is the main route of elimination of orally administered luteolin. In particular, metabolites with 7′-O-glucuronidation show widespread metabolism and extensive distribution of luteolin in rats (Figure 3) (Shi et al., 2024; Wu et al., 2022).

## 3 Pharmacological and therapeutic effects of luteolin

Luteolin has demonstrated a variety of pharmacological influences in both *in vitro* and *in vivo* studies (Huang L. et al., 2023), possessing characteristics of antioxidant (Seelinger et al., 2008a), anti-tumor (Ganai et al., 2021; Seelinger et al., 2008b), anti-microbial (El-Shiekh et al., 2023), anti-viral (Men et al., 2023), anti-inflammatory (Conti et al., 2021; Aziz et al., 2018), anti-apoptotic (Han et al., 2023), anti-allergic (Liang et al., 2020), anti-diabetic (Pradhan and Kulkarni, 2024), chemopreventive (Nguyen et al., 2021), renoprotective (Diniz et al., 2022; Oyagbemi et al., 2021; Xiong et al., 2020), cardioprotective (Liu Z. et al., 2022; Xu et al., 2012) and neuroprotective (Nabavi et al., 2015; Ashaari et al., 2018) properties. Additionally, luteolin has shown anti-anxiety (Gadotti and Zamponi, 2019), antidepressant-like (Mokhtari et al., 2023), antipruritic (Ueda et al., 2005), hepatoprotective (Yao et al., 2023) and antithrombotic (Choi et al., 2015). Numerous studies have indicated that the toxicity of luteolin is minimal, showing no significant harmful effects in clinical trials (Terzo et al., 2023; Gelabert-Rebato et al., 2019; Taliou et al., 2013) (Alzaabi et al., 2022). This included a study by Terzo et al., where 50 obese individuals completed the research protocol. Studies have shown that luteolin's effect in pre-obesity is safe and has no significant toxic side effects at normal dosages (Terzo et al., 2023). Therefore, the safety of luteolin provides a foundation for further exploration of its use in treating human pulmonary diseases. Some scholars have studied various aspects of luteolin, such as luteolin concentration, lowest toxic dose, and determination of interference effects (Rameshbabu et al., 2024). In terms of Lipinski's rule of drug-likeness, luteolin meets Lipinski's Rule of Five, demonstrating drug-like properties (Rameshbabu et al., 2024).

Interestingly, several studies have explored the potential of luteolin to protect lungs across various models. Luteolin also activates the Treg function (Zhang ZT. et al., 2021) and enhances transepithelial sodium transport (Hou et al., 2022) to alleviate inflammation-associated ALI. Here, we discuss the various roles of luteolin in improving dysfunction caused by pulmonary diseases.

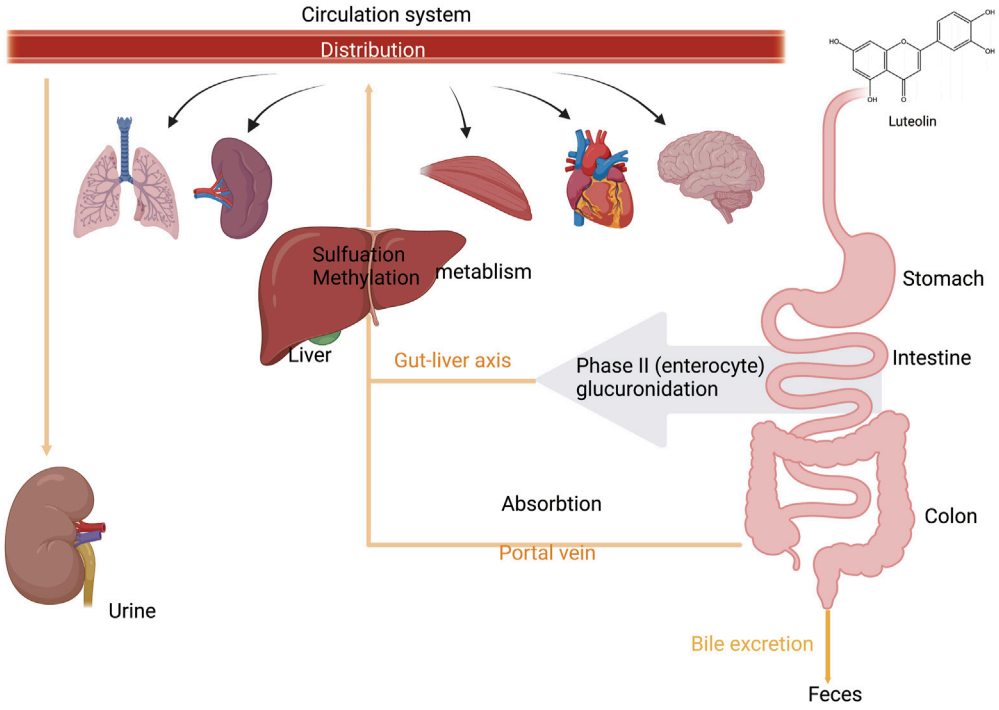



FIGURE 3
An illustration of absorption, distribution, and metabolism of luteolin. Luteolin is absorbed from the intestine and enters the liver via the portal vein. During the first phase of metabolism, it is metabolized in the intestinal wall and/or by liver cells. In the second phase of metabolism, it undergoes enterohepatic recirculation. It is then distributed to various organs and tissues throughout the body via the bloodstream and is finally excreted through urine and bile, created using Biorender.com.

## 3.1 Impact of luteolin on ALI/ARDS

ALI/ARDS has consistently been a primary cause of acute respiratory exhaustion in seriously ill patients, with high incidence and mortality rates over the past 20 years (Fielding-Singh et al., 2018). According to the "International Sepsis Consensus 5.0 Guidelines" (fifth edition, 2021) (Evans et al., 2021), sepsis is a multi-organ dysfunction caused by a dysregulated host inflammatory response to infection (Cavaillon and Chrétien, 2019; Cecconi et al., 2018). The lungs are the most affected and vulnerable target organs in the process of multiple organ dysfunction caused by sepsis (Fan and Fan, 2018). The pathophysiological process of sepsis-associated acute lung injury (SALI) involves disruption in epithelial and endothelial cell function, excessive accumulation and activation of immune cells, inflammatory response, oxidative stress, programmed cell death, and mobilization of coagulation pathways (Sun et al., 2023). Given the relatively high mortality rate and medical burden, ARDS interventions have always been at the forefront of critical care. Because current treatments for ALI are limited and face additional challenges, the introduction of alternative or complementary treatment methods is necessary (Patel et al., 2018). Fortunately, several "conventional" pharmacological therapies for ALI have been found to reduce mortality. Considering that luteolin is expected to have good effects on ALI, numerous studies have utilized luteolin to explore various cell and animal models induced by Lipopolysaccharide (LPS), demonstrating that luteolin exerts its effects on LPS-induced ALI by acting on multiple pathways involved in the pathogenesis of LPS-induced ALI (Figure 4).

### 3.1.1 Anti-inflammatory action of luteolin in ALI/ARDS

Studies using animal models of severe sepsis have shown that inflammation plays a key role in the pathophysiology of sepsis. Sepsis is associated with the activation of pro-inflammatory mediators, among which, NF-κB is an important pro-inflammatory transcription factor that mediates the upregulation of several pro-inflammatory cytokines and chemokines, such as tumor necrosis factor (TNF-α), interleukin-6 (IL-6), interleukin-8 (IL-8), and IL-1β, leading to further amplification of inflammatory damage. Luteolin exhibits anti-inflammatory effects by blocking the activity of Hsp90 in macrophages (Malhotra et al., 2001; Yeo et al., 2004; Chen et al., 2014). Thus, luteolin reduces the triggering of LPS-induced MAPK and NF-κB pathways. IL-10, an anti-inflammatory cytokine, can increase sepsis scores and mortality by diminishing inflammatory defenses and dampening immune responses (Levy et al., 2001; Sabat et al., 2010). The anti-inflammatory effects of luteolin are associated with reduced IL-10 levels. Luteolin protects lung tissue from SALI and reduces the excessive accumulation of IL-10 in the lungs during sepsis (Rungsung et al., 2018; Wang X. et al., 2021). Luteolin is a protective antagonist against LPS-induced ALI in mice. Pretreatment of septic mice significantly reduced the mRNA expression of ICAM-1. There is a protective impact on mice with SALI by curbing the ICAM-1, NF-κB, and specific

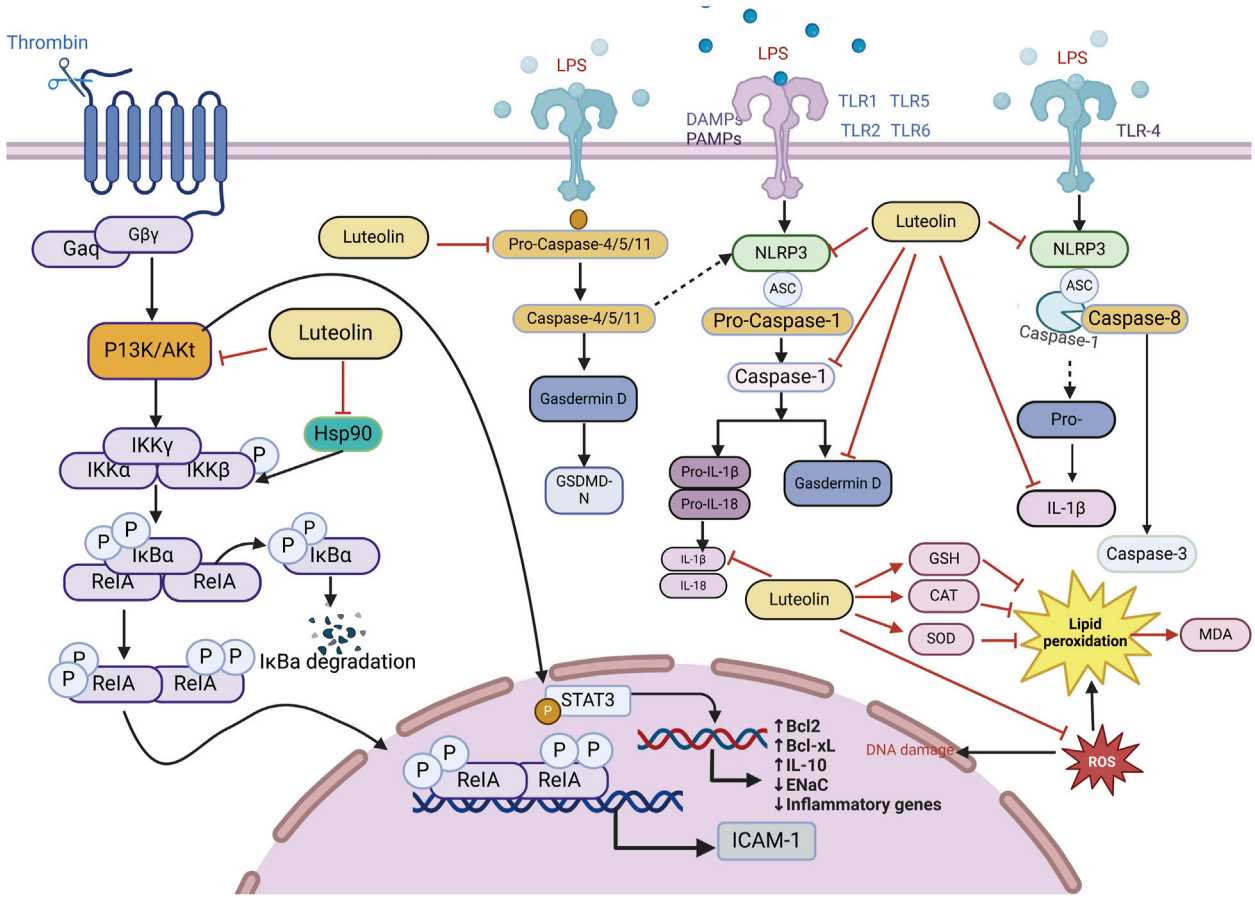



FIGURE 4
An illustration of the signaling pathways by which luteolin affects ALI/ARDS, created using Biorender.com. The symbol "——|" represents inhibition, and "——>" represents promotion, "-----→" represents indirect promotion (PI3K, phosphatidylinositol 3-kinase; GSH, Glutathione, Reduced; STAT3, signal transducer and activator of transcription 3; AKT, Protein kinase B; IL-18, interleukin-18; IL-1β, interleukin-1 beta; IL-10, interleukin-10; TNF-α, tumor necrosis factor; MDA, malondialdehyde; CNP, C-type natriuretic peptide; NLRP3, NOD-like receptor protein 3; SOD, serum superoxide dismutase; CAT, catalase; NF-κB, nuclear factor kappa-B; GSDMD-N, gasdermin D-N).

inducible nitric oxide synthase (iNOS) signaling cascades and reducing the levels of pulmonary IL-6, IL-1β, and TNF-α (Rungsung et al., 2018). Luteolin activates Tregs to promote IL-10 expression and alleviates caspase-11-dependent pyroptosis in SALI, thereby significantly inhibiting inflammation and mitigating cecal ligation and puncture (CLP)-induced lung injury (Zhang Z. T. et al., 2021). Luteolin also regulates AKT1/iNOS levels to promote pyroptosis (Zhang et al., 2023). Therefore, luteolin has immense potential for treating ALI, and the pathways above can serve as therapeutic targets for reducing the levels of inflammatory cytokines induced by sepsis (Table 1).

### 3.1.2 Antioxidant role of luteolin in ALI/ARDS

Oxidative stress, characterized by an imbalance between ROS production and the antioxidant defense system, plays a critical role in inflammation-related diseases. Excessive ROS can induce cellular damage, impair normal physiological functions, trigger the release of inflammatory mediators, exacerbate inflammatory responses, and ultimately lead to irreversible tissue damage. Gu et al. coordinated Ce ions with luteolin to synthesize cerium-luteolin nanocomplexes (CeLutNCs). The prepared CeLutNCs can effectively eliminate excess ROS, prevent apoptosis, downregulate the levels of important inflammatory cytokines, modulate the response of inflammatory macrophages, and inhibit the activation of the NF-κB pathway. This confirmed the therapeutic potential of CeLutNCs in an ALI model (Gu et al., 2024). Luteolin alleviates ALI in a mouse model of CLP-induced sepsis through its antioxidant effects (Rungsung et al., 2018; Liu and Su, 2023). Elevated levels of TNF-α can increase free radicals (Cinar et al., 2019; Kutlu et al., 2020), and early studies have shown that luteolin can reduce TNF-α levels in sepsis and other inflammatory experimental models (Rungsung et al., 2018; Basu et al., 2016; Móritz et al., 2024). Celebi and colleagues found that luteolin mainly reduces the expression of TNF-α mRNA, decreases oxidative stress, and reduces the biochemical and histopathological damage occurring in lung tissue of CLP-induced septic rats, which is attributed to the antioxidant properties of luteolin. An increase in malondialdehyde (MDA) caused by free radicals formed during sepsis was normalized by luteolin treatment, which improved glutathione (GSH) levels, CAT, and superoxide dismutase (SOD) activity, and reduced MDA levels. Luteolin is a potent antioxidant even under intense free radical production conditions, such as sepsis (Rungsung et al.,

TABLE 1 Experimental studies on the anti-inflammatory effects of luteolin on ALI/ARDS.

| Dose range | Minimum active concentration | Model | Control | Duration | Extract type | Effect | References |
|---|---|---|---|---|---|---|---|
| 0.5 mg/kg and 1 mg/kg Luteolin | 1 mg/kg | *in vivo* BALB/c mice | The negative control: LPS treatment of mice | 7days | Purchased from Sigma and dissolved in ethyl alcohol at 10 mM stock solutions | Hsp90↓; c-Jun↓; Akt↓; HMGB1 mediated inflammatory cascade (NF-αB and NO)↓ | Chen et al. (2014) |
| 6.25–100 μM Luteolin | 50 μM | *in vitro* RAW 264.7 cells | The positive control: HA-Hsp90 plasmids RAW 264.7 cells the negative control: cells untreated or treated only with the vehicle (ethanol) | 90 min | | | |
| 0.2 mg/kg Luteolin | | *in vivo* adult Swiss Albino male mice (25–30 g) | The negative control: Group-I, Sham operated; the positive control: Group- III sepsis (CLP) | 1 h before CLP surgery | s purchased from Cayman Chemicals, United States of America. Luteolin stock was prepared in absolute alcohol and further diluted with isotonic normal saline | Wet weight/dry weight ratio ↓; ICAM-1 mRNA and NF-kappa B protein expression ↓; Improved lung tissue structure (IL-6 and IL-1β ↓); MDA↓; SOD and CAT↑; PMN infiltration ↓ | Rungsung et al. (2018) |
| 20 mg/kg | | In Vivo Eight-week-old male C57BL/6 mice | The positive control: CLP group mice; the negative control: Sham group mice | Injected 1 h before CLP. | Purchased from MedChemExpress | (IL-1β, TNF-α)↓; pyroptosis↓; AKT1; iNOS protein↑ | Liu and Su (2023) |
| 0–140 μM Luteolin | 80 μM | In Vitro RAW264.7 macrophage cells | The negative control: Control group, the cell culture medium contains 0.1% DMSO; the positive control: LPS group, cells stimulated with 1 μg/mL LPS for 6 h | 6 h | | | |

2018; Xiong et al., 2017). Through the mechanisms above, luteolin can affect pulmonary oxidative stress and enhance the activity of antioxidant enzymes, including CAT, glutathione peroxidase, and superoxide dismutase, thereby counteracting lipid peroxidation.

### 3.1.3 Effect of luteolin on alveolar sodium channels in ALI/ARDS

The main characteristic of ALI/ARDS is pulmonary edema. Rungsung et al. treated mice with luteolin, which not only reduced the wet-to-dry weight ratio and protein concentration in the bronchoalveolar lavage fluid (BALF) but also alleviated lung edema in LPS-challenged ALI mice [77]. ENaC, located on the epithelial cell membrane, is a major participant in sodium transport across the alveoli and a primary determinant of alveolar fluid clearance (AFC). ENaC is crucial in effectively clearing excess alveolar edema fluid, which is vital for restoring gas exchange and reducing damage to peripheral tissues. Luteolin enhances alveolar sodium transport, which is reduced by LPS in H441 cell monolayers, strongly suggesting that luteolin increases sodium transport associated with ENaC activity. Inhibition of cyclic guanosine monophosphate (cGMP) or PI3K can counteract the luteolin-induced increase in ENaC protein expression in the plasma membrane, indicating that the regulation of ENaC by luteolin is related to the cGMP/PI3K pathway. The downstream signaling cascade of PI3K is associated with several effector proteins, including AKT, serum, and glucocorticoid-induced kinase (SGK),

both of which can upregulate cellular ENaC by inhibiting the ubiquitination of Nedd4-2 (Hou et al., 2022; Lamothe and Zhang, 2013). Therefore, luteolin alleviates SALI/ARDS by activating alveolar ENaCs via the cGMP/PI3K pathway. Additionally, Chen et al., by knocking down STAT3, demonstrated that luteolin decreases phosphorylation in the JAK/STAT pathway while elevating SOCS3 levels, thereby eliminating the suppressive effect of LPS-induced ENaC expression, indicating that luteolin can alleviate ALI/ARDS by enhancing transepithelial sodium transport through the JAK/STAT pathway (Chen et al., 2023). In summary, luteolin can exert ameliorative effects on LPS-induced ALI through various pathways, such as acting as an anti-inflammatory drug (that inhibits the activity of Hsp90, reduces IL-10 levels, and inhibits NF-κB and MAPK pathways) and an antioxidant (that eliminates excess ROS and enhances antioxidant enzyme activity), as well as improving the function of the pulmonary alveolar ENaC (that activates alveolar ENaC and regulates JAK/STAT pathway) in LPS-induced ALI. Research on luteolin in the treatment of ALI/ARDS provides new insights into the pathophysiology of ALI/ARDS and offers a scientific basis for developing new therapeutic strategies. As our research progresses, we hope to identify luteolin as a novel treatment option for patients with ALI/ARDS.

## 3.2 Impact of luteolin on pneumonia

Respiratory viruses are a substantial threat to human wellbeing, and respiratory syncytial virus (RSV) is the primary cause of respiratory infections. Studies by Weifeng Li et al. have shown that luteolin exerts therapeutic effects on RSV pneumonia through various mechanisms. Luteolin mitigates lung inflammation in mice, curbs RSV proliferation in the lungs, elevates pulmonary IFN-β and interferon-stimulated genes (ISG) expression, and modulates murine glucose metabolism. AMs constitute the chief producers of IFN-β in the lungs at the beginning of RSV infection, and the absence of AMs can exacerbate RSV-induced lung tissue damage. Luteolin predominantly targets AMs. By safeguarding the mitochondria in AMs and curbing oxidative stress within cells, luteolin can prevent RSV-induced apoptosis and stimulate IFN-β secretion by AMs (Li W. et al., 2024). Luteolin exerts anti-inflammatory effects on lung tissue in rat models of acute pneumonia. Results from the study by Kong et al. indicated that luteolin inhibits inflammation by suppressing cAMP-phosphodiesterase (cAMP-PDE) activity and the expression of microvascular endothelial cell adhesion molecules (Kong et al., 2019). *S. aureus* infections can range from mild skin infections to acute diseases such as pneumonia (David and Daum, 2010). Yuan et al. showed that the expression of genes involved in virulence and biofilm formation in *S. aureus* strains was downregulated after treatment with luteolin, confirming the potential anti-virulence effects of luteolin (Yuan et al., 2022). Additionally, Yuan et al. found that luteolin treatment mitigated the damage to human alveolar epithelial A549 cells caused by the wild-type (WT) strain and protected mice from pneumonia induced by the WT strain. They suggested that luteolin is a promising metabolite that can interfere with the agr system and can be developed as a novel therapeutic agent for *S. aureus* infections (Lv et al., 2023). Moreover, docking studies with methicillin-resistant *Staphylococcus aureus* (MRSA) strains showed that luteolin exhibits good pharmacokinetics, drug-likeness, and high binding energy with MRSA strains. Ligands with good binding energy values, pharmacokinetics, and drug-likeness have been proven to be potential ligands for treating MRSA infections, and luteolin may serve as a potential therapeutic inhibitor of pneumonia caused by MRSA (Nandhini et al., 2023). At the end of 2019, an outbreak of novel acute viral pneumonia caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) triggered a severe medical and economic crisis. SARS-CoV-2 uses the receptor-binding domain (RBD) of its spike protein to bind to angiotensin-converting enzyme 2 (ACE2) in host cells (Zhu et al., 2023). Therefore, metabolites that bind to human ACE2 or the SARS-CoV-2 spike protein may block the SARS-CoV-2-ACE2 receptor interaction (Day et al., 2021). Luteolin has been shown to bind and significantly inhibit SARS-CoV infection (Stalin et al., 2022). Stalin et al. used computational simulations and virtual screening methods to demonstrate that luteolin engages in uniquely strong interactions with hotspots in the RBD of the SARS-CoV-2 RBD/ACE2 complex, inhibiting the infection of SARS-CoV-2 (Figure 5) (Stalin et al., 2022). In summary, luteolin and its derivatives demonstrate therapeutic potential through multiple mechanisms, such as inhibiting cAMP-PDE activity and the expression of adhesion molecules on microvascular endothelial cells, enhancing pulmonary IFN-β and ISG expression while regulating glucose metabolism in mice, protecting AMs mitochondria and suppressing intracellular oxidative stress to prevent RSV-induced cell apoptosis and stimulate AMs secretion of IFN-β, enhancing the blockade of the RBD/ACE2 complex to inhibit SARS-CoV-2 infection, and downregulating gene expression in *S. aureus* strains. These actions collectively contribute to anti-inflammatory effects, protection of AMs, inhibition of pathogen proliferation, metabolic regulation, and direct interference with virus-host cell receptor interactions, showing promise in treating respiratory diseases. This not only provides a scientific basis for the development of luteolin as a new type of anti-pneumonia drug but also deepens our understanding of the potential of plant-derived metabolites in modern medicine. We look forward to future clinical trials that can further verify these findings and explore new uses for luteolin in treating pneumonia.

## 3.3 Impact of luteolin on asthma

Asthma is a common chronic airway disease characterized by airway narrowing, thickening of the airway walls, and increased airway mucus secretion, which leads to restricted airflow. The clinical features include dyspnea, wheezing, chest tightness, and cough. The prevalence and incidence of asthma has increased significantly. Asthma affected approximately 262 million people in 2019 and was responsible for 455,000 deaths that year. The asthma mortality rate is highest in low and middle sociodemographic index (SDI) countries, whereas its prevalence is highest in high-SDI countries (GBD 2019 Diseases and Injuries Collaborators, 2020). During the sensitization process and after sensitization in a model of ovalbumin (OVA)-sensitized airway hyperresponsive male BALB/c mice aged 8–9 weeks, the

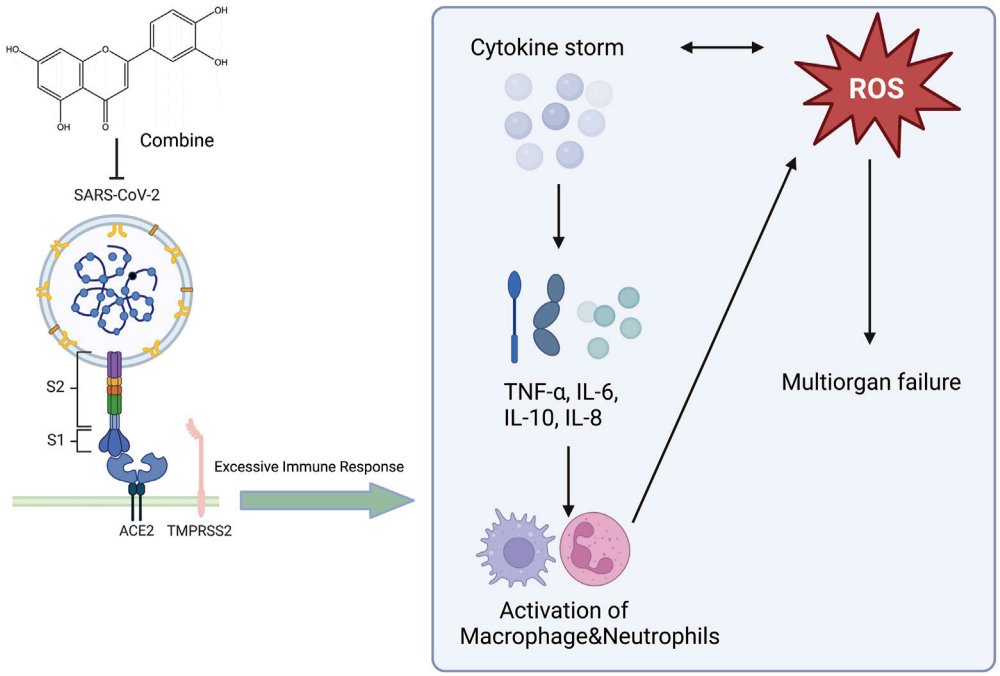



FIGURE 5
An illustration of the effect of luteolin on the novel coronavirus, created using Biorender.com. The symbol "——|" represents inhibition, and "——>" represents promotion (SARS-CoV-2, Severe Acute Respiratory Syndrome Coronavirus 2; ROS, Reactive Oxygen Species; TNF-α, Tumor Necrosis Factor-alpha; IL-6, Interleukin-6; IL-10, Interleukin-10; IL-8, Interleukin-8; ACE2, Angiotensin-Converting Enzyme 2; TMPRSS2, Transmembrane Protease Serine 2).

administration of luteolin (0.1 mg/kg body weight led to a marked reduction in OVA-induced constriction of the airways and bronchial hyperreactivity, in contrast to the control group. Luteolin also reduced the levels of OVA-specific IgE in the serum, increased the levels of interferon-gamma (IFN-γ), and decreased the levels of interleukin-4 (IL-4) and interleukin-5 (IL-5) in the BALF, attenuating the asthmatic characteristics of the experimental mice (Das et al., 2003). Luteolin alleviates bronchoconstriction and airway hyperresponsiveness in OVA-sensitized mice. Luteolin exerts anti-asthmatic activity against OVA-induced pulmonary inflammation by downregulating the transcription of $CD4^+$ T cells with Th2 phenotype cytokines and inhibiting the production of Prostaglandin E2 (PGE2) (Jin et al., 2009). Luteolin attenuates the immediate and late asthmatic responses to nebulized OVA exposure in conscious guinea pigs. Compared to the control group, luteolin, and apigenin significantly reduced specific airway resistance in both immediate and late asthmatic responses. They also reduced the recruitment of leukocytes and release of histamines, as well as the activity of phospholipase A2 (PLA2) and eosinophil peroxidase (EPO) in bronchoalveolar lavage fluid (BALF). Still, the anti-asthmatic activity of luteolin was lower than that of sodium cromoglycate and dexamethasone (Lee et al., 2014). Therefore, luteolin can be used not only as a lead molecule for identifying effective anti-asthmatic therapies but also as a means of identifying novel anti-asthmatic targets (Figure 6). In addition, luteolin reduces autophagy in allergic asthma. They observed that luteolin treatment significantly inhibited OVA-induced inflammatory responses and autophagy in the lung tissue. Moreover, luteolin activated the PI3K/Akt/mTOR pathway in the lung tissues of asthmatic mice and inhibited the Beclin-1-PI3KC3 protein complex. This strategy encompasses the prevention of autophagic processes in allergic asthma via upregulation of the PI3K/Akt/mTOR pathway and downregulation of the Beclin-1-PI3KC3 complex (Wang S. et al., 2021). Asthma often manifests as an excessive buildup of mucus in the airway lining, which can lead to severe clinical outcomes. Although general asthma therapies are effective, specialized treatments designed to limit mucus overproduction in patients with asthma are still lacking. Recent studies have revealed that stimulating the gamma-aminobutyric acid type A receptor (GABAAR) is crucial for promoting excessive mucus secretion by pulmonary airway epithelial cells. Shen and colleagues found that in an OVA-induced asthma mouse model, with a 10 mg/kg administration, luteolin markedly reduced the number of goblet cells within pulmonary tissue and suppressed excessive mucus production. Luteolin hindered GABAAR-induced currents in A549 cells, according to patch-clamp data. Additionally, the suppressive effects of luteolin on goblet cell hyperplasia and excessive mucus secretion induced by OVA are reversed by picrotoxin, a GABAAR antagonist (Shen et al., 2016). In summary, luteolin and its derivatives have demonstrated potential in treating asthma by modulating various cellular signaling pathways and immune responses. These include the activation of the PI3K/Akt/mTOR pathway and inhibition of the Beclin-1-PI3KC3 protein complex. The therapeutic potential is reflected in mechanisms such as reducing airway inflammation, decreasing airway hyperresponsiveness, modulating immune cytokines, reducing eosinophil infiltration, inhibiting autophagy,

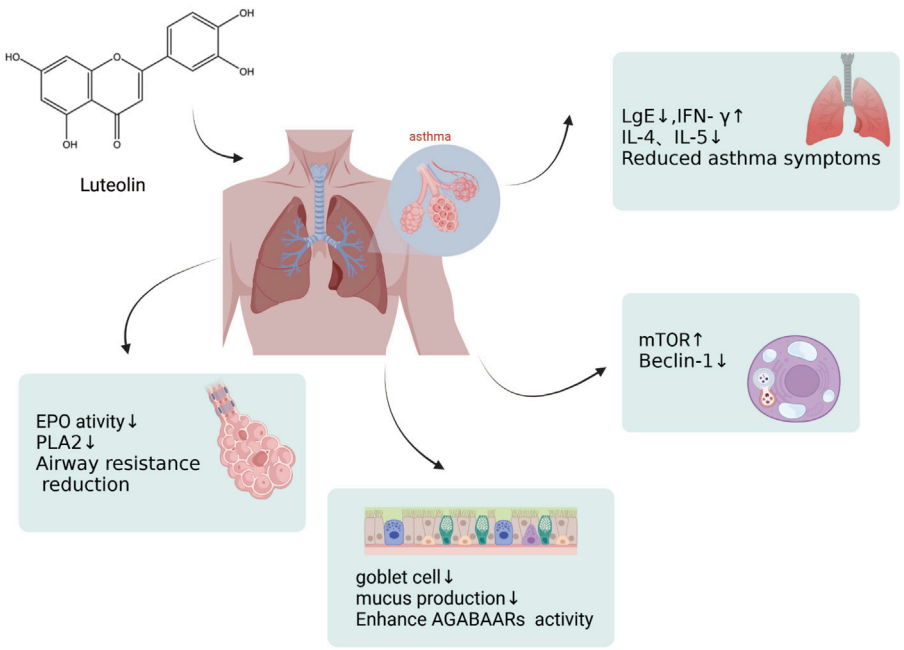



FIGURE 6
An illustration of effects by which luteolin affects asthma, created using Biorender.com. The symbol "↓" indicates downregulation, and "↑" indicates upregulation (mTOR, mammalian Target of Rapamycin; PLA2, Phospholipase A2; GABAAR, γ-Aminobutyric acid sub-type A receptors).

and suppressing excessive mucus secretion. After evaluating the potential impact of luteolin on asthma, we believe that this metabolite shows promise for improving asthma symptoms by modulating various cellular signaling pathways and immune responses. Nonetheless, we should recognize the complexity of asthma, which necessitates further research to determine the optimal dose of luteolin for asthma treatment. We are confident that with a deeper understanding of the mechanisms of action of luteolin, it could become an important metabolite in future asthma treatment strategies.

## 3.4 Impact of luteolin on COPD

COPD is one of the leading causes of morbidity and mortality worldwide (Buhl et al., 2024) and poses a significant economic and social burden (Alupo et al., 2024). Patients experience progressive deterioration until end-stage COPD, which is characterized by severe airflow limitations, severely compromised lung function, chronic respiratory failure, advanced age, multiple comorbidities, and severe systemic manifestations/complications. COPD is often underdiagnosed and undertreated (Viegi et al., 2007). Intensified oxidative stress and body-wide inflammation are critical elements of the complex pathophysiology of COPD (Austin et al., 2016). Studies have shown that matrix metalloproteinases (MMPs) are associated with COPD progression, and luteolin has been identified as an inhibitor of MMP-2 and MMP-9 (Ende and Gebhardt, 2004). Therefore, Yaghi et al. explored the use of luteolin in their study, measuring ciliary beat frequency (CBF) in nasal cilia from COPD patients as an important indicator of ciliary function in COPD. Compared to the high-risk and control groups, CBF was significantly reduced in individuals with moderate-to-severe COPD. The treatment group observed a significant and sustained increase in CBF in COPD cilia tested with luteolin, increasing the CBF of cilia in patients with COPD, favorably enhancing mucociliary clearance, reducing retention of secretions, and decreasing infections (Yaghi et al., 2012). Li et al. proposed that luteolin inhibits inflammation and oxidative stress in COPD by suppressing the NADPH oxidase 4 (NOX4)-mediated NF-κB signaling pathway. Studies have used cigarette smoke (CS) to induce COPD in mice or A549 cells both *in vivo* and *in vitro*. *In vivo*, luteolin mitigated the inhibitory effects of CS on body weight and the activities of SOD and CAT in COPD model mice and reduced the levels of MDA. Similar results have been obtained *in vitro*. Li et al. transfected A549 cells with a NOX4 overexpression plasmid to induce NOX4 overexpression. Compared to the control group, the overexpression of NOX4 reversed the downregulation of the ratio of p-p65/p65 and p-IκB/IκB in the luteolin group, upregulation of oxidative stress-related proteins, and reduction of inflammatory-related factors. Luteolin alleviates inflammation, oxidative stress, and activation of the NOX4-mediated NF-κB signaling pathway induced by CS in A549 cells (Li M. et al., 2023). Zhou and colleagues proposed that luteolin alleviates oxidative stress induced by CS in COPD by modulating the transient receptor potential vanilloid type 1 (TRPV1) and cytochrome P450 family 2 subfamily A member 13 (CYP2A13)/nuclear factor erythroid 2-related factor 2 (NRF2) signaling pathways. They observed that luteolin downregulated the expression of TRPV1 and CYP2A13 proteins and upregulated the expression of sirtuin 6 (SIRT6) and NRF2 proteins in CS + LPS-induced COPD mice and A549 cells treated with CS. CS treatment increased intracellular calcium ions, overall ROS, and mitochondria-

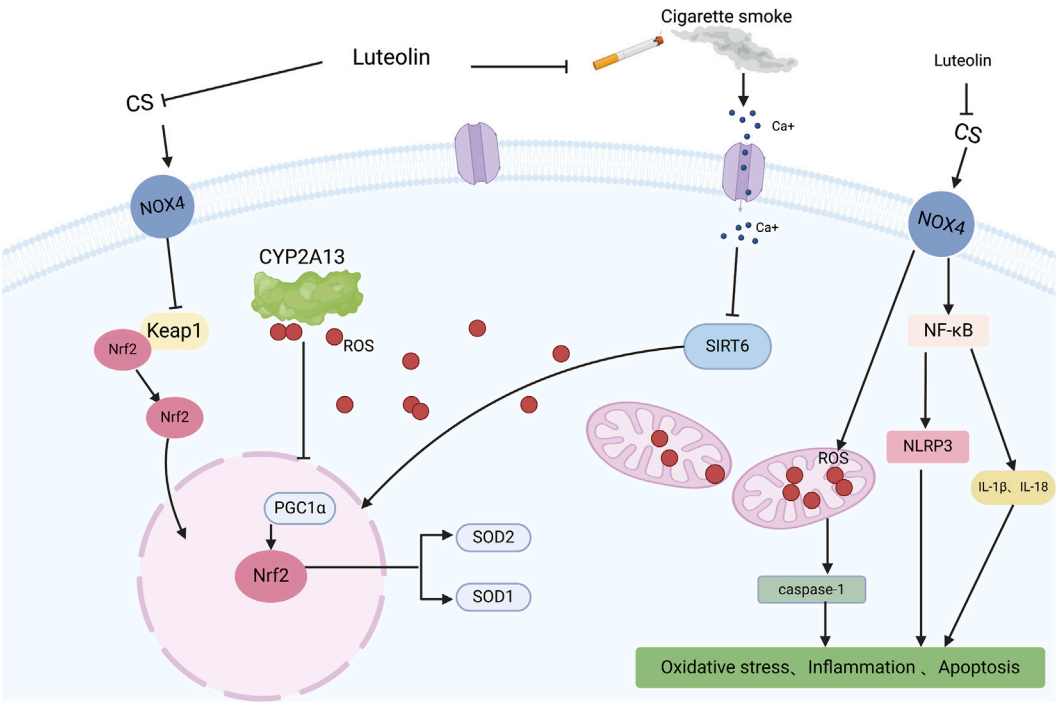



FIGURE 7
An illustration of the protective effect of luteolin in CS-induced COPD, created using Biorender.com. The symbol "——|" represents inhibition, and "——>" represents promotion (CS, Cigarette Smoke; NOX4, NADPH oxidase 4; Ca+, Calcium ions; CYP2A13, Cytochrome P450 family 2 subfamily A member 13; Keap1, Kelch-like ECH-associated protein 1; NF-KB, Nuclear factor kappa B; ROS, Reactive Oxygen Species; SIRT6, Sirtuin 6; Nrf2, Nuclear factor erythroid 2-related factor 2; NLRP3, NLR family pyrin domain containing 3; IL-1β, Interleukin-1 beta; IL-18, Interleukin-18; PGC1α, Peroxisome proliferator-activated receptor gamma coactivator 1-alpha; SOD2, Superoxide dismutase 2; SOD1, Superoxide dismutase 1).

derived ROS levels in A549 cells. Importantly, luteolin inhibited the influx of $Ca^{2+}$ into CS-treated A549 cells and mitigated the excessive production of mitochondrial and intracellular ROS. By modulating the TRPV1/SIRT6 and CYP2A13/NRF2 signaling pathways, luteolin exhibits a protective effect by reducing oxidative stress and inflammation in COPD mice induced by CS-and LPS, as well as in A549 cells exposed to CS (Figure 7) (Zhou et al., 2023). In summary, luteolin has the potential to alleviate inflammation and oxidative stress and improve cilia function in COPD by inhibiting NOX4-mediated NF-κB signaling pathway, regulating TRPV1 and CYP2A13/NRF2 signaling pathways, and modulating various signaling pathways and cytokines. However, we are also aware that COPD is a multifactorial disease requiring a comprehensive treatment strategy. Therefore, future research should explore the combined application of luteolin and other drugs to manage COPD effectively.

## 3.5 Impact of luteolin on pulmonary fibrosis

Idiopathic pulmonary fibrosis (IPF), classified as progressive fibrosing interstitial lung disease (ILD), leads to a gradual decline in lung function (Raghu et al., 2018). The hallmarks of IPF include a progressive deterioration in lung capacity, exacerbation of fibrosis as depicted on high-resolution computed tomography (HRCT) scans, decline in both symptomatology and quality of life for patients, and a propensity for early death (Travis et al., 2013; Wells et al., 2018; Cottin et al., 2019; Kolb and Vašáková, 2019). Aside from pirfenidone and nintedanib, which are specifically used for treating IPF, no other medications have been approved for treating ILDs (Brown et al., 2020). The predominant pharmacological approach for treating other progressive fibrosing ILDs involves immunosuppression (Cottin et al., 2019). When the fibrotic response of an ILD to lung injury advances to a stage where fibrosis becomes self-perpetuating and continues to progress, targeted anti-fibrotic therapy is essential to mitigate the rate of disease progression. Clinical studies have demonstrated that natural substances extracted from plants possess potent antifibrotic properties and can alleviate dyspnea, a common and debilitating symptom experienced by patients with ILD (Chang et al., 2021). Luteolin is an active flavonoid metabolite isolated from *Lonicera japonica* and is known for its broad range of biological activities, particularly its antioxidant and anti-inflammatory effects. Ren and colleagues loaded luteolin into γ-cyclodextrin metal-organic frameworks (CD-MOFs) for pulmonary drug delivery using a dry powder inhaler. *In vitro* lung deposition results showed that luteolin effectively inhibited the progression of ILD in bleomycin (BLM)-induced fibrotic ILD rat model. Compared to the control group, treatment with luteolin significantly protected rats from BLM-induced lung injury. They demonstrated that luteolin possesses potent anti-inflammatory activity and that inhalation of luteolin could be considered a promising new strategy for treating fibrotic ILD (Ren et al., 2023). Oral administration of luteolin (10 mg/kg) in both the early and delayed luteolin treatment groups effectively

TABLE 2 Experimental studies of luteolin in the treatment of pulmonary fibrosis.

| Model | Does | Duration | Type of study | Effect | References |
|---|---|---|---|---|---|
| Male C57BL/6 rats | 10 mg/kg Luteolin | 21 day | in vivo | Collage deposition↓; TGF-β1 mRNA↓; myocardium inflammation (TNF-α, IL-6, NUET) ↓ | Chen et al. (2010) |
| Primary lung fibroblasts and A549 cells | 25 μM Luteolin | 72 h | in vitro | TGF-β1 expression (R-SMA, Collagen 1 and vimentin) ↓; P-Smad 3↓ | |
| BLM-induced fibrosing ILD rats | 400 mg/kg LUT + CDMOF | 20 days | in vivo | weight↑; lung coddicient↑; TGF-β1 and Smad3↓ | Brown et al. (2020) |
| MHS cells | 0.5,1 and 2 ug/mL LUT + CDMOF | 12 h | in vitro | myocardium inflammation (TNF-α, IL-1β and IL-6) ↓ | |

inhibited the infiltration of neutrophils into BALF and increased TNF-R and IL-6 levels in C57BL/6 mice intubated with BLM. Luteolin also mitigated collagen deposition, TGF-β1 expression, and pulmonary fibrosis following BLM injection. Subsequently, *in vitro* studies showed that luteolin inhibited the expression of TGF-β1-induced reactive serum myoglobin (R-SMA), type I collagen, and vimentin in primary cultured mouse lung fibroblasts. Luteolin significantly blocked the downregulation of epithelial markers (E-cadherin) and upregulation of mesenchymal cell markers (fibronectin and vimentin) mediated by TGF-β1 and maintained the epithelial morphology of human alveolar epithelial-derived A549 cells. This indicates that luteolin exerts effective antifibrotic activity by inhibiting pulmonary inflammation and suppressing myofibroblast differentiation and the epithelial-mesenchymal transition (EMT) of cells (Chen et al., 2010). Data analysis in informatics implied that luteolin may offer substantial therapeutic benefits in treating IPF and gastroesophageal reflux disease (GERD). By identifying and analyzing differentially expressed genes (DEGs) between IPF and GERD and predicting drug molecules for IPF and GERD, luteolin was recognized as a potential biomarker and promising therapeutic target for both IPF and GERD (Table 2) (Shen et al., 2016). In summary, luteolin demonstrates potential in the treatment of IPF through various mechanisms, including anti-inflammatory effects, anti-fibrotic actions (inhibition of Smad3 phosphorylation in the TGF-β1 signaling pathway), inhibition of myofibroblast differentiation, suppression of EMT, modulation of key signaling pathways, and potential as a drug delivery vehicle. After analyzing the potential applications of luteolin in treating pulmonary fibrosis, we were encouraged by the antifibrotic potential of the metabolite. We believe that luteolin's anti-inflammatory and anti-fibrotic properties and its role in modulating key signaling pathways make it a strong candidate for treating pulmonary fibrosis. Future studies should focus on determining the optimal route of administration and luteolin dosage to maximize its therapeutic effects.

## 3.6 Therapeutic effect of luteolin on lung cancer

The GLOBOCAN 2020 estimates from the International Agency for Research on Cancer (IARC) regarding cancer incidence and mortality rates show that carcinoma of the lung stands as the most prevalent cause of death from cancer, with projections indicating approximately 2.2 million new instances and 1.8 million deaths worldwide (Sung et al., 2021). In China, the primary treatment options for lung cancer include surgical intervention, radiation treatment, chemotherapy, precision cancer therapies, immune system treatments, and traditional Chinese medicine (Yang et al., 2018; Chaft et al., 2016). Recent reports suggest that luteolin may be a novel and potent agent against lung cancer with the potential to inhibit disease progression (Imran et al., 2019).

KRAS mutations are known to increase the expression of programmed death-ligand 1 (PD-L1) within tumor cells, enabling the tumor to shun the immune system for detection and subsequent destruction. An *in vitro* experiment demonstrated that luteolin inhibits the growth of KRAS-mutated cells in a dose-dependent manner. The expression of PD-L1 is a consequence of STAT3 activity, which engages the PD-L1 gene promoter upon phosphorylation. Luteolin inhibits the STAT3 signaling pathway and the expression of PD-L1, thereby exposing tumor cells to T cells of the immune system (Jiang et al., 2021; Watterson and Coelho, 2023). MiR-34a is commonly downregulated in cancer cells, and elevated levels of miR-34a have been shown to suppress tumor growth (Zhang et al., 2019; Fu et al., 2023). Research has indicated that luteolin upregulates the expression of miR-34a, which may subsequently inhibit the proliferation of non-small cell lung cancer (NSCLC) cells (Shi et al., 2014; Jiang et al., 2018). Circular RNAs (circRNAs) are upregulated in lung cancer tissues, and luteolin suppresses lung cancer progression by targeting the circ_0000190/miR-130a-3p/notch-1 signaling pathway (Zheng et al., 2023). Luteolin may inhibit the proliferation and migration of A549 cells by reducing the expression of AR and modulating the phosphorylation of AR receptor sites (Li X. et al., 2023).

The anticancer properties of luteolin have also been demonstrated through the induction of apoptosis in neoplastic cells (Çetinkaya and Baran, 2023). Luteolin induces mitochondria-dependent apoptosis in human lung adenocarcinoma cells (Chen et al., 2012). Luteolin promotes the activation of caspase-9 and caspase-3, decreases the expression of Bcl-2, increases the expression of BAX, increases the BAX/Bcl-2 ratio, and induces apoptosis by activating intrinsic pathways (Chen et al., 2012). Additionally, the accumulation of ROS induced by luteolin can enliven the extrinsic pathways modeled by TNF-induced signaling (Ju et al., 2007). Luteolin has been

TABLE 3 Experimental studies of luteolin in the treatment of lung cancer cells.

| Model | Does | Duration | Effect | References |
|---|---|---|---|---|
| Human NSCLC A549 and H460 cells | 0,10,20 and 40 μM Luteolin | 24 h | Tumor suppressor P53 and P21 expression↑; apoptosis (Bax, caspase-3, caspase-9) ↑; cancer cells proliferation↓ | Jiang et al. (2018) |
| H358, H460 and H549 cells | 30 μM Luteolin | 24 h | MUC1/STAT3 signaling↓; block PD-1/PD-L1 axis IL-2↑ | Jiang et al. (2021) |
| A549 cells | 80 μM Luteolin | 48 h | AR protein expression↓; cell proliferation↓; lung cancer cell apoptosis↑; migration ofA549 is inhibit | Li et al. (2023b) |
| A459, H1975 cells and Beas-2B cells | 20 μM Luteolin and/or 25 ng/mL TRAIL | 24 h | DR5 expression↑; Drp1↓; apoptosis↑; p-JNK↑ | Wu et al. (2020) |
| NCI-H1975 and NCI-H1650 cells | 5,10,20 and 40 μM luteolin | 24,48 or 72 h | LIMK1 signaling pathway↓; p-LIMK↓; P-cofilin↓; CyclinD1 and D3↓ | Zhang et al. (2021b) |

demonstrated to inhibit EMT and downregulate E-cadherin by suppressing the triggering of key signaling pathways, such as TGF-β1 and the PI3K/AKT-NF-κB-Snail pathways (Chen et al., 2013). Furthermore, Masraksa and colleagues have demonstrated that luteolin can hinder the spread and invasion of lung cancer cells by targeting the signaling pathways of focal adhesion kinase and non-receptor tyrosine kinase (Src/FAK) as well as its downstream pathways involving Ras-related C3 botulinum toxin substrate 1 (Rac1), cell division cycle 42 (Cdc42), and ras homologous gene family member A (RhoA) (Masraksa et al., 2020). Luteolin works synergistically with osimertinib, a third-generation epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI), to overcome acquired resistance in NSCLC cells by suppressing the hepatocyte growth factor (HGF)-MET-Akt signaling cascade, which is involved in MET amplification and hyperactivation (Huang G. et al., 2023). When used alone or in combination with factor-related apoptosis-inducing ligand (TRAIL), a novel and potent anticancer agent, can enhance the expression of death receptor 5 (DR5) and promote dynamin-related protein 1 (Drp1)-dependent mitochondrial fission. By increasing DR5 expression and Drp1-mediated mitochondrial fission, luteolin enhances the sensitivity of NSCLC cells to TRAIL (Wu et al., 2020). Another study indicated that LIM Kinase 1 (LIMK1) is a target of luteolin, which suppresses tumor growth by inhibiting LIMK1 activity (Zhang M. et al., 2021). Beyond its anticancer characteristics, luteolin also possesses the ability to alleviate bone cancer pain (Zhou et al., 2022). Luteolin involves numerous determinants and cascades related to lung cancer progression (Rakoczy et al., 2023). In summary, luteolin has shown potential as a therapeutic agent for lung cancer by affecting multiple key factors in disease progression, and further research into its clinical applications is warranted (Table 3). Luteolin has shown the potential to inhibit lung cancer progression by affecting various key tumor biological pathways. We believe that these mechanisms of action provide a scientific basis for luteolin as a candidate drug for lung cancer treatment. Future research should focus on clinical trials of luteolin and optimizing its efficacy through drug delivery systems.

## 4 Limitations and future perspectives

The multifaceted pharmacological profile of luteolin makes it a potential candidate for further investigation as a natural therapeutic agent for the treatment of pulmonary diseases. However, poor solubility in water and low bioavailability hinder their therapeutic potential. This article emphasizes that strategies to enhance water solubility and bioavailability through chemical modifications and advanced pharmaceutical formulations such as nanovesicles and cyclodextrin complexes hold promise for addressing the challenges associated with luteolin. Balancing the concentration of luteolin, determining the minimum toxic dose, and addressing interference effects in broad-spectrum assays are also issues that deserve attention. While preclinical studies in cellular and animal models are promising, and numerous studies indicate that luteolin has minimal toxicity, this article emphasizes the need for further research, particularly clinical trials in humans and rigorous toxicity experiments that are still required to assess its safety and promote its rational development, to fully understand the efficacy and safety of luteolin in treating pulmonary diseases. Owing to the structural diversity of flavonoids in plants and the inherent limitations of existing technologies, industrial-scale production of flavonoids faces challenges that necessitate the improvement and optimization of current extraction techniques (Li L. et al., 2024). In this context, enhancing the purification efficiency and recovery rate of luteolin is a critical step that is essential for the large-scale production and widespread application of luteolin-based pharmaceuticals. Although there are already commercial luteolin health foods and cosmetic products on the market (Wang Z. et al., 2021), the use of luteolin as a drug for the treatment of other diseases is still in the clinical trial phase (Shi et al., 2024; Di Stadio et al., 2022; Cordaro et al., 2020). Future research should focus on two key areas: optimizing the solvent extraction technology for luteolin and developing advanced pharmaceutical formulations that can improve its solubility and bioavailability. These two tasks are of great significance in overcoming the limitations of luteolin in practical applications, fully exerting its therapeutic effects, and promoting its development for clinical applications.

## 5 Conclusion

In this article, we reviewed the therapeutic potential and molecular mechanisms of action of luteolin in various pulmonary

diseases. Luteolin has shown significant effects in the treatment of ALI/ARDS, COPD, asthma, pulmonary fibrosis, and lung cancer, owing to its pharmacological actions such as anti-cancer, antioxidant, and anti-inflammatory properties. Despite its notable bioactivity, water-solubility and bioavailability of luteolin limit its clinical applications. These limitations can be overcome through chemical structural modifications and pharmaceutical formulation innovations. This article summarizes the research progress in improving the solubility and bioavailability of luteolin, discusses its multifaceted therapeutic effects in the treatment of pulmonary diseases and provides new directions for future research.

## Author contributions

JL: Data curation, Formal Analysis, Project administration, Resources, Supervision, Validation, Writing–original draft. XS: Conceptualization, Methodology, Software, Visualization, Writing–original draft. ZL: Data curation, Formal Analysis, Writing–original draft. DH: Software, Visualization, Writing–original draft. LX: Software, Visualization, Writing–original draft. KZ: Conceptualization, Methodology, Writing–review and editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2025.1535555/full#supplementary-material

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# Glossary

| | |
|---|---|
| PGE2 | Prostaglandin E2 |
| NF-κB | Nuclear Factor kappa-B |
| COPD | Chronic Obstructive Pulmonary Disease |
| ALI | Acute Lung Injury |
| ARDS | Acute Respiratory Distress Syndrome |
| ENaC | Epithelial Sodium Channel |
| AFC | Alveolar Fluid Clearance |
| cGMP | Cyclic Guanosine Monophosphate |
| LPS | Lipopolysaccharide |
| IL-6 | Interleukin-6 |
| TNF-α | Tumor Necrosis Factor alpha |
| CLP | Cecal Ligation and Puncture |
| ROS | Reactive Oxygen Species |
| GSH | Glutathione |
| SOD | Superoxide Dismutase |
| MDA | Malondialdehyde |
| HMGB1 | High-Mobility Group Box 1 |
| iNOS | Inducible Nitric Oxide Synthase |
| GSDMD-N | Gasdermin D-N gasdermin D |
| TRAIL | Tumor Necrosis Factor-related Apoptosis-inducing Ligand |
| DR5 | Death Receptor five |
| NLRP3 | NOD-like Receptor Protein 3 |
| LIMK1 | LIM Kinase 1 |
| NSCLC | Non-Small Cell Lung Cancer |
| EGFR | Epidermal Growth Factor Receptor |
| PD-L1 | Programmed Death-Ligand 1 |
| STAT3 | Signal Transducer and Activator of Transcription 3 |
| miR-34a | MicroRNA-34a |
| circRNA | Circular RNA |
| Tregs | regulatory T cells |
| IPF | Idiopathic pulmonary fibrosis |
| ILD | interstitial lung disease |
| PLA2 | Phospholipase A2 |
| GABAAR | γ-Aminobutyric acid sub-type A receptors |
| CS | Cigarette Smoke |
| NOX4 | NADPH oxidase four |
| Ca+ | Calcium ions |
| CYP2A13 | Cytochrome P450 family 2 subfamily A member 13 |
| Keap1 | Kelch-like ECH-associated protein 1 |
| SIRT6 | Sirtuin 6 |
| Nrf2 | Nuclear factor erythroid 2-related factor 2 |
| PGC1α | Peroxisome proliferator-activated receptor gamma coactivator 1-alpha |
| SOD2 | Superoxide dismutase 2 |
| SOD1 | Superoxide dismutase 1 |
| ACE2 | angiotensin-converting enzyme 2 |
| TMPRSS2 | Transmembrane Protease serine 2 |
| ISG | interferon-stimulated genes |
| MRSA | methicillin-resistant *staphylococcus aureus* |
| SARS-CoV-2 | severe acute respiratory syndrome coronavirus. |

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# Marula [*Sclerocarya birrea* (A. Rich.) Hochst.] products as a food and medicine

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The fruit of *Sclerocarya birrea* (A. Rich.) Hochst., commonly known as the marula, is widely appreciated for its nutritious pulp and edible nuts. The pulp has a higher vitamin C than those of other fruits, including pineapple, guava, and oranges. In addition, fresh marula fruits are often used to produce delicious sweets, wine and flavorings: it is perhaps best known as the flavor of Amarula liqueur. *In vitro* and *in vivo* studies indicate that the various parts of marula have pro-health properties, such as antioxidant, antibacterial, antifungal, antidiabetic activities. This paper reviews the current state of knowledge regarding the marula fruit and its products, with a special emphasis on their chemical composition, biological activity and pro-health potential.

KEYWORDS

**marula, marula fruit, marula juice, marula seed, antioxidant**

## Introduction

While *Sclerocarya birrea* (A. Rich.) Hochst. has many common names, such as morula, jelly plum, cat thorn, cider tree, maroola plum, maroola nut, moroela, elephant tree, mafula, and nkanyi, it is best known as the marula. The species belongs to Anacardiaceae, alongside the sumac, cashew, mango and pistachio. *S. birrea* is divided into three subspecies differentiated by leaf shape and size: subsp. *birrea*, subsp. *afra* and subsp. *multifoliolata*. Subsp. *birrea* is found in northern Africa and subsp. *afra* in southern Africa, while subsp. *multifoliolata* is only found in Tanzania. The generic name *Sclerocarya* is derived from the Ancient Greek words *skleros* meaning *hard*, and *karyon* meaning *nut*, i.e., the hard pit of the fruit. The specific epithet *birrea* comes from the common name *birr*, for this type of tree in Senegal (Masarirambi and Nxumalo, 2012; Nyoko et al., 2015; Mashau et al., 2022; Abdelwahab et al., 2025).

The single-stemmed marula tree is found in 29 countries. Female trees bear up to 500 kg of fruit each year, while the male tree puts on a delicate floral display instead. Marula fruit are cylinder-like plum-sized drupes, and may range from 3 to 4 cm in thickness. They ripen between December and March, and have a light yellow skin with white flesh. Marula fruit is especially appreciated for its nutritious pulp and edible nuts. The pulp has a higher vitamin C (67–403 mg/100 g fresh weight) than the that of other fruits, including pineapples, guava, and oranges (Hiwilepo-van Hal, 2013). The ripe fruit is aromatic with a turpentine taste and

**Abbreviations:** CE, catechin equivalent; DP, degree of polymerization; DPPH, 2,2-diphenyl-1-picrlhydrazyl; GAE, gallic acid equivalent; HDL, high-density lipoprotein; Il, interleukin; LDL, low-density lipoprotein; MIC, minimum inhibitory concentration; TBARS, thiobarbituric acid reactive substances; TNF-α, tumor necrosis factor-α.

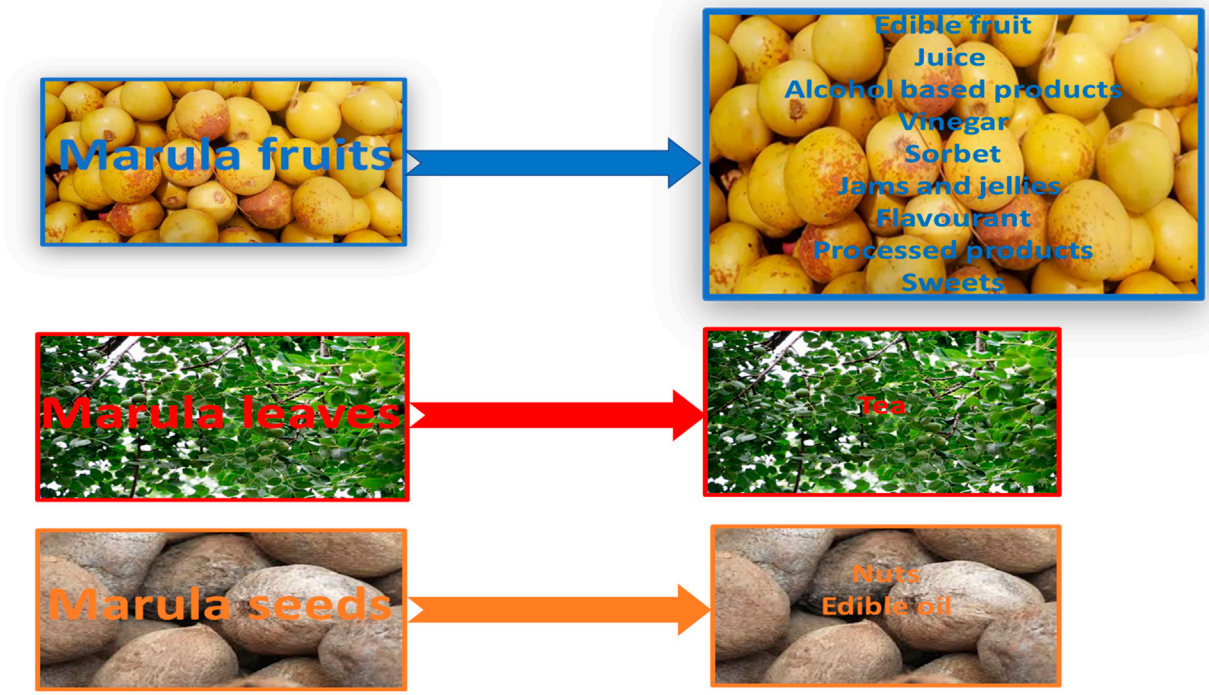



FIGURE 1
Bioactive ingredients of various parts of marula used as food.

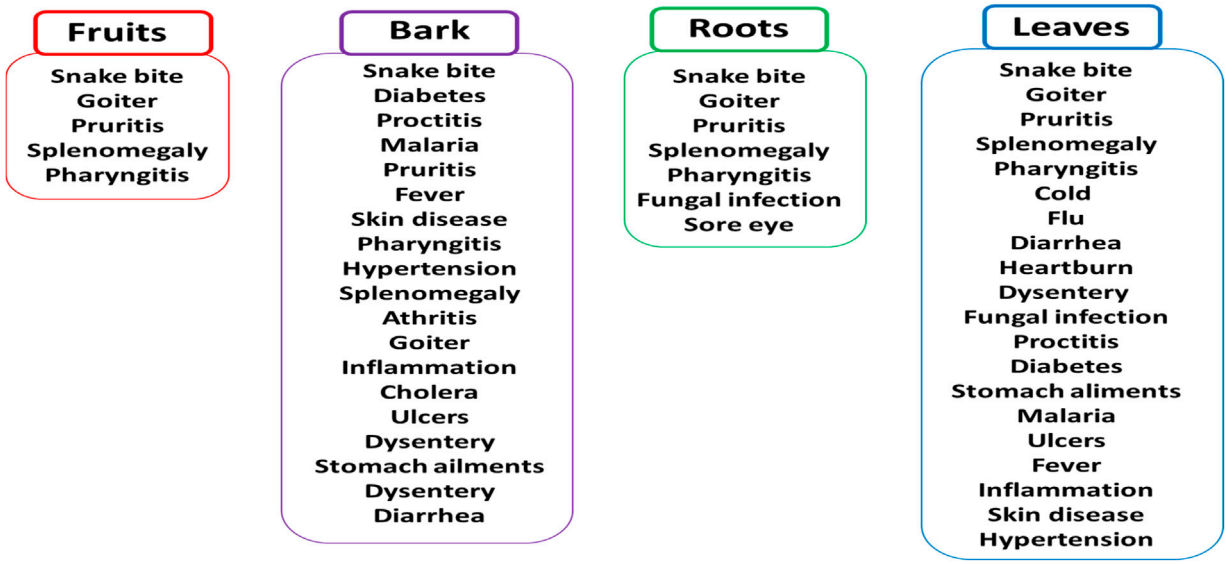



FIGURE 2
Marula parts (fruits, bark, roots, and leaves) used for treatment of pathology in folk medicine.

the white succulent pulp sticks tightly to the nut (2–3 cm in diameter). The nut itself is divided into three or four hollows, each with one seed. The fruit has a single seed, which has a delicate nutty flavor (Mashau et al., 2022).

The marula tree is also regarded as the "king of African trees". It is a green, leafy plant standing between 9 and 18 m tall and demonstrates considerable drought resistance, with each tree still managing to produce large amounts of fruit during dry seasons. All

of its parts, viz. the fruits, bark, leaves, stem, and nuts, are used extensively, with their precise use varying with location and tribe. The parts used for food are summarized in Figure 1. The fresh fruits can also be incorporated in various other products including wine, known locally as *mokhope*, *omagongo* or *ubuganu*, and flavorings, most famously Amarula liqueur. It is also used to produce delicious sweets similar to fruit rolls (Van Wyk, 2011; Pfukwa et al., 2020; Mashau et al., 2022).

Marula fruits have demonstrated various antioxidant, antibacterial, antifungal and antidiabetic activities, as noted in *in vitro* and few *in vivo* studies (Pfukwa et al., 2020; Mashau et al., 2022; Fakudze et al., 2023; Abdelwahab et al., 2025); as such, they are often used in folk medicine (Figure 2). Although relatively few review papers have explored the medicinal potential of marula fruits (Mashau et al., 2022; Fakudze et al., 2023; Abdelwahab et al., 2025), their findings generally suggest the fruits have little medicinal value; however, these papers generally do not include the other parts of the marula plant and their products (Mashau et al., 2022; Lekhulenie et al., 2024). For example, a review by Mashau et al. (2022) only describes the biological activities of marula fruits.

The aim of the present mini-review is to provide an overview of the beneficial potential of marula fruits, and to highlight that other parts of the marula tree, and their products, also have beneficial potential.

## Research methods

ScienceDirect, Web of Science, SCOPUS, Web of Knowledge, PubMed, and Sci Finder were searched for papers examining potentially beneficial functional components of the various parts of the marula plant. The search terms comprised the terms "marula"; "*S. birrea*", "marula tree", "marula seed", and "marula fruit" and their combinations. No time criteria were applied to the search, but recent papers were evaluated first. The last search was run on 15 December 2024. Papers were first selected based on the relevance to the title of the present manuscript, and the identified articles were screened by reading the abstract. Any relevant identified articles were summarized. About 88 articles were obtained from the searches, and only 43 were included in this review. The molecular mechanisms underpinning the biological action of various parts of marula were also analyzed and discussed as part of the search.

## Bioactive compounds of various parts of marula and marula products

Marula fruits contain considerable amounts of protein, dietary fiber, fatty acids, amino acids (threonine, tyrosine, methionine, valine, phenylalanine, isoleucine, leucine, lysis, and histidine), minerals (sodium, potassium, calcium, magnesium, copper, manganese, iron, and other), and vitamins (A, $B_3$, C, E, and carotene) (Sibiya et al., 2020; Lekhulenie et al., 2024). The fruits also contain various other bioactive compounds, including phenolic compounds, phytosterols and triterpenoids, which determine their various biological properties (Mawoza et al., 2010; Hiwilepo-van Hal, 2013; Mashau et al., 2022; Lekhuleni et al., 2024).

Marula fruits may be eaten fresh or fermented into beer. The preparation of marula beer does not require additional substances such as sorghum, maize, yeast, and sugar (Molelekoa et al., 2018). The alcoholic concentration increases from 0.9% v/v, in the initial stage of fermentation, to 5.5% v/v in the final stage (Hiwilepo-van Hal, 2013). The fruit is also used to make a popular wine with high vitamin C and alcohol content, with the latter reaching strengths of 15%, depending on the tree and the length of fermentation (Hailwa, 1998; Hillman et al., 2008). The marula fruit is also, most famously, the base of the alcoholic Amarula cream liqueur, and milk chocolate with amarula syrup filling (Mashau et al., 2022; Lekhuleni et al., 2024). Marula fruits may be used to produce vinegar, which is often added to salad dressing and mayonnaise (Mashau et al., 2022).

Marula jams and jellies with an attractive natural waxy yellow color are also popular with consumers (Hiwilepo-van Hal, 2013; Mashau et al., 2022; Lekhuleni et al., 2024). Marula jam and ice cream manufactured according to industrial protocols were found to be rich in ascorbic acid 45 days post-production (Hillman et al., 2008). Various other marula products also exist, such as chutneys and pie fillings, fruit teas and supplements, i.e., powder capsules, as well as coffee substitute made from the burnt skin (Mawoza et al., 2010; Hiwilepo-van Hal, 2013; Mashau et al., 2022).

Marula juice prepared from fresh and fully-ripe pulp has high levels of vitamin C and various minerals, including manganese, potassium, zinc, calcium, and magnesium. Its sugar content is 22 mg/mL fructose, 4.9 mg/mL sucrose, and 4.9 g/mL glucose (Eromosesle et al., 1991; Hillman et al., 2008; Mashau et al., 2022). Hiwilepo-van Hal et al. (2013) report that the vitamin C in marula fruit pulp demonstrated considerably low thermal degradation. It was found to be 15 times more stable compared to guava and mango pulp.

Marula juice is also a good source of phenolic compounds including tannins, hydroxycinnamic acid derivatives and catechins (Borochov-Neori et al., 2008).

Marula seeds may be eaten dried, fresh or milled. In addition, they may be also incorporated to soups, boiled meat, and vegetables to enhance the flavor. Fresh marula seeds are also often mixed with porridge. De-kernelled marula seeds, including the shells, can be served as a tea or extract as a nutritional supplement (Pfukwa et al., 2020).

Importantly, marula seeds also contain various vitamins and minerals. They also contain oil which is a rich source of protein and mono-unsaturated fatty acids, especially oleic acid (Mariod et al., 2008; Mariod and Abdelwahab, 2012; Sybille et al., 2012; Mashau et al., 2022). However, Eromosele and Paschal (2003) indicate that marula oil has low concentration of vitamin E compared to other nut oils. A recent study comparing the effects of decoction and maceration on the phytochemical content and antioxidant properties of de-kernelled marula seeds by Rama et al. (2023) found decoction to achieve the highest concentrations of chemical compounds.

The varied uses of marula in food are presented in more detail in Figure 1. Table 1 also compared the nutritional content of edible marula fruit (pulp) and its products (nuts, jam and alcohol). It can be seen that pulp has higher fiber content (4.25–10.5 g/100 g) than the nut (2.47 g/100 g), lipids and protein levels are lower in the pulp than the nut. Again, it is important to note that marula pulp has a high concentration of vitamin C (0.062–0.179 g/100 g). In addition,

TABLE 1 Concentrations of chemical compounds in marula fruit (pulp) and its products; and concentrations of phenolic compounds in various parts of marula (based on (Mashau et al., 2022); modified).

| Chemical compound | Concentration of chemical compound (g/100 g) | | | | References |
|---|---|---|---|---|---|
| | Marula pulp | Marula nut | Marula alcohol | Marula jam | |
| Proteins | 12.5–30.1 | 26.5–28.4 | — | — | Attiogbe and Abdul-Rozak (2016), Mashau et al. (2022), Chiesa et al. (2020) |
| Lipids | 9.7–25.3 | 28.4–57.2 | — | — | Attiogbe and Abdul-Rozak (2016), Mashau et al. (2022) |
| Carbohydrates | 25.3 - 61.7 | 6.4–7.3 | — | — | Hillman et al. (2008), Attiogbe and Abdul-Rozak (2016), Mashau et al. (2022) |
| Fiber | 4.2–10.5 | 0 - 2.5 | — | — | Eromosele et al. (1991), Lekhuleni et al. (2024) |
| β- caroten | 0–0.1 | - | — | — | Lekhuleni et al. (2024) |
| Vitamin C | 0.062–0.179 | - | — | — | Lekhuleni et al. (2024) |
| Calcium | 0.006–0.052 | 0.106–0.156 | — | — | Lekhuleni et al. (2024) |
| Magnesium | 0.010–0.167 | 0.193–0.467 | — | — | Lekhuleni et al. (2024) |
| Potassium | 0.044–0.133 | 0–0.677 | — | — | Lekhuleni et al. (2024) |
| Sodium | 0.003–0.015 | - | — | — | Lekhuleni et al. (2024) |
| Copper | 0–0.001 | 0–0.002 | — | — | Lekhuleni et al. (2024) |
| Iron | 0–0.009 | 0.264–0.677 | — | — | Lekhuleni et al. (2024) |
| Zinc | 0–0.003 | 0.003–0.006 | — | — | Lekhuleni et al. (2024) |

| Part of marula | Concentration of phenolic compounds | |
|---|---|---|
| | Total phenolic compounds (mg gallic acid equivalent (GAE)/100 g) | Flavonoids (mg quercetin equivalent (QE)/ 100 g) |
| Pulp | 2.5–872.3 | 2.0–33.9 |
| Leaves | 13.9–304.5 | 0.47 |
| Bark | 23.0–593.0 | No data |
| Roots | 26.5–78.6 | No data |

marula pulp and nut are rich in various saturated fatty acids, such as tetradecanoic and hexadecanoic acid, and unsaturated fatty acids, such as oleic acid, linoleic acid, α-linolenic acid, and eicosanoid acid; the latter have pro-health properties, such as cardioprotective potential (Mariod and Abdelwahab, 2012; Sybille et al., 2012; Mashau et al., 2022).

The phenolic profile of marula varies with the part of the plant. For example, higher levels of flavonoids are noted in marula pulp than the leaves, bark, and roots (Table 1). The polyphenolic content of marula fruit is described in more detail by Mashau et al. (2022) and Lekhuleni et al. (2024).

## Chemical anti-oxidant assays for marula products

Hillman et al. (2008) noted that fresh marula fruit juice has antioxidant capacity ranging from 141–440 mg/100 mL ascorbic acid equivalent (*in vitro* model). The total antioxidant and polyphenol content of the juice varied among clones and with time post-abscission, as did the activity of superoxide dismutase. In addition, ascorbic acid content was found to be high in all the clones with significant differences among clones and time post-abscission. Clone no 12 at 1 week post-abscission had the higher ascorbic acid content, 21.177 mg/g dry weight and clone no four at the abscission day had the lower ascorbic acid content, 7.142 mg ascorbic acid/g dry weight. It also has around four times more antioxidant potential than the juice of either orange or pomegranate. In addition, marula ice cream and jam manufactured according to industrial protocols were rich in ascorbic acid 45 days post-production.

Mariod et al. (2008) studied radical scavenging capacities and antioxidant properties of various methanolic extracts from *S. birrea* leaves, roots, barks, and kernel oil cake *in vitro*. The total phenolic compounds were found as 304.5 mg/g of dry product (for the extract from leaves), 367.5 mg/g of dry product (for the extract from roots), 593.0 mg/g of dry product (for the extract from barks), and 258.0 mg/g of dry product (for the extract from kernel oil cake). All tested extracts were markedly effective in inhibiting the oxidation of linoleic acid and subsequent bleaching of β-carotene in comparison with the control. However, based on oxidation of β-carotene/ linoleic acid, the extract from kernel oil cake was the most

effective followed by extracts from roots, leaves, and barks. The antioxidant properties determined by the DPPH (1,1-diphenyl-b-picrylhydrazyl) method also revealed that the extract from kernel oil cake had the highest antioxidant activity on DPPH free radicals followed by extracts from barks, roots, and leaves.

Other research found young stem extract of marula to contain the highest levels of total phenolic compounds (14.15 ± 0.03 mg gallic acid equivalent (GAE)/g), flavonoids (1.21 ± 0.01 mg catechin equivalent (CE)/g) and gallotannins (0.24 ± 0.00 mg GAE/g) compared to other parts. The $EC_{50}$ value of the stem extract was found to be 5.02 μg/mL (6.86 μg/mL for ascorbic acid) in the DPPH free radical test (Moyo et al., 2010). Also, the antioxidant properties of extracts from marula leaves, bark and roots have been associated with their phenolic compound content (Mariod et al., 2008; Moyo et al., 2010; Misihairabgwi and Cheikhyoussef, 2017).

In addition, Ndhlala et al. (2006a) and Ndhlala et al. (2006b) report that marula juice has antioxidant potential associated with its phenolic compounds (total phenolics: 2262 μg GAE/g, flavonoids: 202 μg catechin/g, and 6.0% condensed tannins). Other studies attribute the antioxidant properties of marula juice to the degree of polymerization (DP) of phenolic compounds, when DP is less than 10 (Zhou et al., 2014).

A recent study of the effect of freezing on the chemical content (total phenolic compounds: 196.42 mg catechin equivalent/mL; 0 weeks) and antioxidant properties of marula fruit juice by Nthabising et al. (2023) found an approximate 37% decrease in total phenolic compounds, and 36% decrease in vitamin C compared to fresh, unfrozen controls as a result of the freezing process; this data indicates that freezing is not a good method of preservation. Freezing also reduced the antioxidant properties of the fruit juice, measured by various methods.

## Biological activity of marula

Marula products and supplements have commercial, cultural and ethnomedicinal value in Africa and in other parts of the world. However, studies indicate that the different parts of the plant have different biological properties.

### Antioxidant potential (*in vitro* and *in vivo* models)

Borochov-Neori et al. (2008) indicate that marula juice (1 and 2 μg/mL) showed radical scavenging capacity by causing a 32% and 62% reduction, in optical density of DPPH solution. In a model of oxidative stress caused by copper ions, marula juice also decreased the production of lipid peroxide ($IC_{50}$–0.055 μg/mL) and thiobarbituric acid reactive substances (TBARS) ($IC_{50}$–0.050 μg/mL) caused by LDL oxidation. Moreover, supplementation of this juice (100 and 200 mL/kg b.w./day; for 3 weeks) significantly reduced the level of triglyceride (7%), LDL (17%), and total cholesterol (8%), and increased the level of high-density lipoprotein (HDL) by 10% in healthy individuals. The juice contained a significant amount of phenolic compounds (56 mg of pyrogallol equivalence) and its antioxidant capacity was found to be high (382 mg of ascorbic acid equivalent). Most importantly, its antioxidant activity was not destroyed by pasteurization; however, after 4 weeks of low temperature storage (at – 18 °C), the antioxidant potential was reduced by 14%. In addition, tested marula juice was found to contain high vitamin C and potassium levels and low sugar concentration (267 mg/dL, 328 mg/dL, and 7.3 g/dL, respectively).

### Antidiabetic potential (*in vivo* models)

Various studies have reported that marula bark extract has antidiabetic activity in animal models. For example, Gondwe et al. (2008) observed that 5-week supplementation modulates blood glucose and glomerular filtration rate in diabetic rats. The extract was found to have the same effects as methormin treatment. The action of used extract (120 mg/kg p.o., daily) was monitored for 5 weeks in animals. This extract exhibited dose-dependent reduction in blood glucose concentration. In addition, the hypoglycemic effect of this extract treatment was associated with increased hepatic glycogen synthesis. Moreover, the extract treatment reduced blood pressure in animals.

Dimo et al. (2007) also noted that administration of marula bark extract reduces the level of blood glucose in diabetic rats. Experimental animals using by Dimo et al. (2007), were treated by oral administration of plant extract (150 and 300 mg/kg body weight) and metformin (500 mg/kg; control - reference drug) for 21 days. They observed that marula stem bark extract exhibited a significant reduction in blood glucose and increased plasma insulin levels in diabetic rats. Moreover, used extract also prevented body weight loss in diabetic rats. The effective dose of this extract (300 mg/kg) tended to reduce plasma cholesterol, triglyceride and urea levels toward the normal levels.

### Antibacterial and antifungal potential (*in vitro* models)

Eloff (2001) report that marula bark and leaf extracts have antibacterial properties against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia* faecais, and *Escherichia* cola. The minimum inhibitory concentration (MIC) values ranged from 0.15 to 3 mg/mL.

Interestingly, various parts of the marula also have antifungal activity. For example, Masoko et al. (2008) found acetone, ethanol, and methanol marula bar extracts to have antifungal properties against *Candida* parapsilosis, Cryptococcus albidus, and Rhodotorula mucilaginosa. Of the tested extracts, the greatest activity was found for methanol. Hamza et al. (2006) also found marula root methanolic extract to have antifungal activity against Cryptococcus neoformans, *Candida* albicans, *Candida* kruseii, *Candida* glabrata, *Candida tropicalis*, and *Candida* parapsilosis with a MIC value of 0.5 mg/mL. However, authors (Eloff, 2001; Hamza et al., 2006; Masoko et al., 2008) did not describe the phytochemical profile of used extracts.

### Other biological potential (*in vitro* and *in vivo* models)

The anti-inflammatory effects of aqueous and methanolic stem-bark extracts of S. birrea (500 mg/kg p.o.) were also examined on rat paw oedema induced by subplantar injections of fresh egg albumin (0.5 mL/kg). The typical anti-inflammatory agent - acetylsalicylic acid (100 mg/kg p.o.) was used for comparison. Both, the aqueous and methanolic extracts progressively and time-dependently reduced rat paw oedema induced by subplantar injections of fresh egg albumin. But, the methanolic extract produced relatively greater and more pronounced anti-inflammatory effect

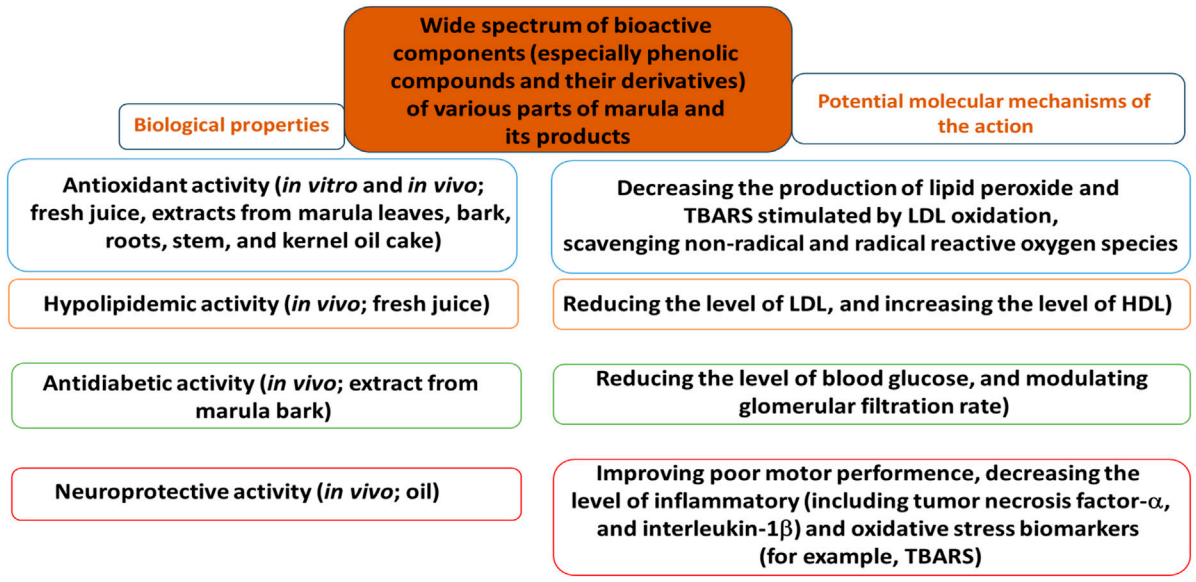



FIGURE 3
Main biological properties of various parts of marula and its products; their potential molecular mechanims

TABLE 2 Biological properties of various marula products.

| Marula product | Biological activity | References |
|---|---|---|
| Fruit juice (100 and 200 mL/kg b.w., for 3 weeks; amount of phenolic compounds – 56 mg of pyrogallol equivalent) | Hypolipidemic activity (*in vivo* – healthy humans) | Borochov-Neori et al. (2008) |
| Extract from stem bark (500 mg/kg p.o.; non data – chemical content) | Anti-inflammatory activity (*in vivo* - rat paw oedema induced by subplantar injections of fresh egg albumin (0.5 mL/kg)) | Ojewole (2003) |
| Extract from roots (0.5 mg/mL; non data – chemical content) | Antifungal activity (*in vitro*) | Hamza et al. (2006) |
| Extract from bark (120 mg kg(-1) p.o., daily), for 5 weeks, non data – chemical content) | Antidiabetic activity (*in vivo* – diabetic rats) | Gondwe et al. (2008) |
| Extract from bark (150 and 300 mg/kg body weight, daily, for 21 days); non data – chemical content) | Antidiabetic activity (*in vivo* – diabetic rats) | Dimo et al. (2007) |
| Extract from bark (0.15–3 mg/mL; non data – chemical content) | Antibacterial activity (*in vitro*) | Eloff (2001) |

than its aqueous extract counterpart in the experimental animal model (Ojewole, 2003).

A combination of marula stem bark extract and those of other medicinal trees was tested against Plasmodium falciparum and Plasmodium berghei. The extracts demonstrated both anti-plasmodial activity *in vitro* and anti-malarial potential *in vivo*. However, phytochemical profile of the tested extract was not described (Gathirwa et al., 2008).

A recent study found marula oil nanoemulsion to have neuroprotective potential in a mouse model of experimental Parkinson's disease induced by rotenone (Alshaman et al., 2023). Treatment with marula oil or marula nanoemulsion improved poor motor performance; it also downregulated the elevated expression of protein inflammatory parameters, such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) in the mice, as well as biomarkers of oxidative stress (e.g., TBARS).

Recently, Tientcheu et al. (2023a) and Tientcheu et al. (2023b) also investigated the neuroprotective and antidiabetic properties of a mixture of Piper longum, Nauclea latifolia, and S. birrea (75, 150, and 300 mg/kg) in a rat model of type 2 diabetes and memory impairment. The tested mixture (150 and 300 mg/kg) showed neuroprotective and memory-strengthening potential by protecting the hippocampus neurons and normalized blood glucose levels. Other studies by these authors indicate that this mixture may protect the striatal neurons and movement-associated functionalities in a rat model of locomotion dysfunction caused by diabetes. However, authors only describe that tested mixture contains active metabolites, mainly flavonoids, and fatty acids.

Wide spectrum of active components, especially phenolic compounds of various parts of marula and its products have been found to demonstrate different biological properties and these are summarized in Figure 3 together with their potential molecular mechanisms. For example, marula juice and extracts from various parts of marula have been noted to effectively inhibit generation of non-radical and radical reactive oxygen species and lipid peroxidation. In addition, marula oil

demonstrates neuroprotective activity, which may be associated with its anti-inflammatory effect by regulating signaling pathways such as TNF-α and IL-1β.

## The safety of marula extracts

Some *in vitro* and animal studies have examined the safety of marula extracts, but with varying results. For example, Muhammad et al. (2014) report in in vivo model that marula fruit peel extract (oral administration: 3000 and 4,000 mg/kg body weight) has a toxic effect on kidneys and liver in rats, manifested in significantly higher uric acid, urea, serum total protein, creatinine, transaminase, bilirubin, and albumin content.

An *in vitro* study found that exposure to high concentrations (600–1000 μg/mL) of bark-stem extract decreases the viability of kidney cell lines (distal and proximal tubules) (Gondwe et al., 2008), but the same extract (120 mg/kg) has no effect on renal fluid in nondiabetic and diabetic rats.

The cytotoxicity of marula was tested against VERO cells (*in vitro*), evaluated as the concentration required to cause visible alterations in 50% of intact cells ($CC_{50}$). The analysis found the methanolic extract to have a $CC_{50}$ of 361.2 μg/mL and the aqueous extract 3375.2 μg/mL. However, the authors did not write which part of marula was used (Gathirwa et al., 2008).

It has also been found *in vivo* that aqueous, methanolic, and hexane extracts from marula bark and stem (10–1000 mg/mL) did not demonstrate toxic potential against brine shrimp (McGaw et al., 2007). However, Ojewole (2023) report that methanolic and aqueous marula bark-stem extracts (500 mg/kg) are safe in rats. Unfortunately as there is not yet any unequivocal clinical evidence for the safety of marula products and supplements, further research is needed to confirm this. More details about the toxicity of marula extracts are described by Fakudze et al. (2023). However, this review paper only reports research data *in vitro* models.

# Conclusion

Marula is consumed in a variety of forms, including raw fruits, alcohol-based products, jams and jellies. Marula seeds are surrounded by a delicate white kernel high in arginine and glutamic acid, as well as myristic, oleic, and palmitic acids. Marula preparations (especially extracts) have high levels of total phenolic compounds, with various biological properties (Table 2). However, many of these studies are limited to *in vitro* and animal models, and demonstrate considerable heterogeneity, making it difficult to compare results. In addition, authors often do not describe the phytochemical profile of used preparations, including extracts. Often biological activities of marula preparations reported very naively. Moreover, such papers about biological properties of marula products are sometimes common in journals with no or poor peer-review. In addition, full taxonomic validity of the plant material under investigation was not always ascertained.

Although the food products derived from marula fruits and seeds may be valuable sources of bioactive compounds, further *in vivo* studies are needed to clarify their exact mechanism (Hiwilepo-van Hal et al., 2012; Hiwilepo-van Hal et al., 2014; Sibiya et al., 2020; Mashau et al., 2022; Lekhuleni et al., 2024). Such studies should also examine the health potential of fresh marula fruits, and their food products and supplements, as well as their long-term effects. Moreover, only few papers (Ojewole, 2003; Gathirwa et al., 2008; Gondwe et al., 2008; Muhammad et al., 2014; McGaw et al., 2007) describe safety of extracts from various parts of marula in in vitro and animal models. There are no information about the safety of food products derived from marula fruits and seeds in animal and human models. Such studies should examine the safety of these food products, as well as their long-term safety.

The biological activity of marula fruits and their products is doubtlessly influenced by their chemical composition, with vitamin C, phenolic compounds, and unsaturated fatty acids playing key roles. However, again, their mechanisms of action are unclear and require further study.

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# Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Bioactive flavonoids metabolites in citrus species: their potential health benefits and medical potentials

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Citrus flavonoids are naturally occurring phytochemicals widely present in the peels and pulps of citrus fruits. They exhibit a wide range of biological activities, including antioxidant, anti-inflammatory, hypoglycemic, lipid-lowering, antimicrobial, and gut-protective effects. These metabolites show great potential in improving metabolic syndromes such as diabetes, non-alcoholic fatty liver disease (NAFLD), and cardiovascular diseases. Additionally, citrus flavonoids have demonstrated significant effects in inhibiting pancreatic lipase activity, regulating lipid metabolism, and enhancing intestinal barrier function. Advances in extraction and purification techniques have further promoted their applications in the fields of food, medicine, and functional materials. This review systematically summarizes the types, bioactivities, and mechanisms of action of citrus flavonoids, providing scientific evidence for their research and development.

KEYWORDS

**citrus, flavonoids, bioactivities, mechanisms of action, extraction techniques, health benefits**

## 1 Introduction

Citrus fruits, belonging to the subfamily *Aurantioideae* of the *Rutaceae* family, include oranges, grapefruits, lemons, limes, mandarins, and tangerines. They are considered one of the largest groups of plant species and are widely distributed across tropical, subtropical, and temperate regions of the world (Sharma et al., 2017). Research has confirmed that south-central China is a major origin center for the genus Citrus (Huang et al., 2023). Citrus fruits are rich in phytochemicals, particularly flavonoids, which represent an essential group of dietary flavonoids. The primary methods for extracting citrus flavonoids include solvent extraction and subcritical water extraction technology. These methods efficiently yield high-purity flavonoid metabolites from citrus, providing a solid foundation for subsequent research.

A substantial body of research indicates that citrus flavonoids possess a variety of biological activities, including antioxidant, anti-inflammatory, antibacterial, lipid-lowering, blood sugar-lowering, and intestinal protective effects. These natural plant chemicals show particular promise in improving metabolic syndromes such as diabetes, non-alcoholic fatty liver disease (NAFLD), and cardiovascular diseases. Moreover, the application of citrus flavonoids in the food industry is expanding, with the development of novel food packaging

materials and their use as antioxidants in meat products, enhancing food safety and quality. In the field of pharmacology, citrus flavonoids like naringin and hesperidin demonstrate potential in treating various diseases, including overcoming drug resistance in cancer therapy. Therefore, in-depth research on citrus flavonoids is expected to provide more scientific evidence and therapeutic options for humanity.

## 2 Extraction methods of citrus flavonoids

### 2.1 Organic solvent extraction method

The organic solvent extraction method is based on the physical properties of flavonoids, selecting appropriate solvents to isolate different types of flavonoid metabolites. Commonly used solvents include methanol, ethanol, acetone, and ethyl acetate, with methanol and ethanol being the most commonly used. Methanol generally provides better extraction efficiency, followed by ethanol, but both solvents have higher extraction efficiency compared to other organic solvents. However, due to methanol's toxicity and ethanol's more environmentally friendly nature, ethanol is more commonly used. Phucharoenrak et al. (2022) used discarded sour orange peels from an acid orange juice factory as raw material, employing an ethanol-water extraction technique to obtain hesperidin and limonene. The study found that the optimal conditions for the highest yield were extracting with 80% ethanol at a solid-to-solvent ratio of 0.01 g/mL, pH 7, and 50°C for 100 min. Jeong et al. (2023) selected citrus junosas the raw material and employed solvent extraction combined with enzyme treatment techniques. The optimal conditions identified were 5% pomelo powder, 50% ethanol as the solvent, and 0.05% rhamnosidase treatment for 24 h. The mixture was purified using an HP-20 chromatography column, with 50% ethanol as the elution solvent, resulting in the highest concentrations of naringin and hesperidin. The organic solvent extraction method is cost-effective, has low toxicity, good reproducibility, and is suitable for industrial production (Bajkacz and Adamek, 2018).

### 2.2 Subcritical water extraction

Subcritical water extraction (SWE) is a novel extraction technology developed after supercritical fluid extraction. It uses subcritical fluids as the extraction solvent. Under certain pressures, extraction is carried out at temperatures ranging from 100°C to 374°C. This method offers several advantages, including easy solvent recovery with no residue, prevention of oxidation of the extracted products, preservation of the bioactivity of the extract, and low cost (Cheng et al., 2021). Kim and Lim (2020) employed a semi-continuous subcritical water extraction technique to extract flavonoids from citrus peel. Their study showed that under conditions of 145.3°C–165.6°C and a water flow rate of 2.25 mL/min, using water as the solvent, semi-continuous extraction yielded 87.8%–98.9% of bioactive flavonoids from the peel. Hwang et al. (2021) used discarded citrus peels as raw material to investigate whether combining subcritical water extraction with pulsed electric field (PEF) treatment could enhance the extraction efficiency of flavonoids from citrus. The results indicated that the extraction temperature and time significantly affected flavonoid extraction, with higher temperatures improving efficiency. The PEF treatment time also had a significant impact on the extraction of hesperidin. Compared to traditional extraction methods, subcritical water extraction is an efficient technique for extracting bioactive metabolites.

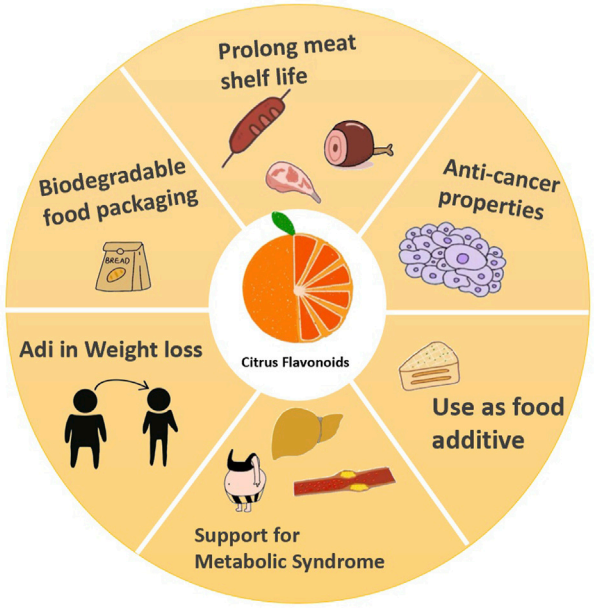



FIGURE 1
Health benefits of citrus flavonoids.

## 3 Types of flavonoid metabolites in citrus

To date, over 250 citrus flavonoid metabolites have been identified (Wang et al., 2019). Flavonoids are natural phenolic metabolites composed of two benzene rings and 15 carbon atoms. They can be broadly classified into six categories: flavanones, flavanols, flavones, flavonols, isoflavones, and anthocyanins (Peterson and Dwyer, 1998). Flavanones are the most abundant flavonoid metabolites found in citrus fruits, primarily existing in the forms of rutin glycoside and neo-hesperidoside. The flavonoids formed by the former are tasteless, such as naringin, hesperidin, pomelosidin, and melittoside, while those formed by the latter have a distinctly bitter taste, such as neo-naringin, neohesperidin, neo-poncirin, and rutinoside (Tripoli et al., 2007). Naringin, primarily found in grapefruit, may account for 30%–40% of the citrus flavonoids, while hesperidin, mainly present in the peels of oranges and other citrus fruits, typically makes up 20%–30%. In total, approximately 4,000 flavonoid metabolites have been isolated, with most of them found in fruits and vegetables. The concentration and distribution of citrus flavonoids vary significantly between species (Benavente-García and Castillo, 2008).

# 4 Health benefits of citrus flavonoids

Citrus flavonoids are widely found in citrus fruits and offer significant health benefits. In the food industry, they serve as natural antioxidants to extend shelf life, while in pharmacology, they exhibit anti-inflammatory, anti-diabetic, and anti-cancer effects, playing a crucial role in human health (Figure 1).

## 4.1 Applications in the food industry

Food safety is the foundation of human health and is crucial to everyone's daily life. With the development of technology, consumers' demand for high-quality food has increased, making food packaging and quality hot topics.

There is widespread controversy and concern about the chemicals added to traditional plastics. Naringin is a flavonoid metabolite naturally found in citrus fruits, and it is widely distributed in the peels of fruits like grapefruit and oranges. Guzman-Puyol et al. (2022) developed a novel, transparent, UV-resistant, high-barrier, and biodegradable food packaging material made from a mixture of cellulose andnaringin. These bioplastics were prepared by dissolving the metabolites in trifluoroacetic acid (TFA) and trifluoroacetic anhydride (TFAA), followed by solvent removal via casting. Experiments showed that when 20% hesperidin was added (CN-20), the transparency remained over 85%, while the UV-B barrier reached 100%. In terms of mechanical properties, CN-20 exhibited a 25% increase in elongation at break, a 27% increase in toughness, and a reduction in Young's modulus to approximately 1,500 MPa, demonstrating a significant plasticizing effect. The water vapor transmission rate (WVTR) decreased to 3,000 g/m$^2$·day, and the oxygen transmission rate (OTR) dropped to 55 mL/m$^2$·day, which is comparable to petroleum-based plastics such as polyester. Biodegradation testing in seawater showed that CN-20 had a biochemical oxygen demand (BOD) of 10 mgO2/L and a weight loss of 19%, indicating its suitability as a biodegradable packaging material in marine environments.

Free radical oxidation is one of the main mechanisms leading to the deterioration of food, especially meat products. Goliomytis et al. (2015) studied the addition of hesperidin or naringin to chicken feed, significantly improving the oxidative stability of breast and thigh meat. The study showed a dose-dependent decrease in the oxidative marker MDA levels, with the antioxidant effect of naringin approaching that of vitamin E, significantly extending the shelf life of the meat. These natural bioflavonoids may improve the antioxidant properties of meat by inhibiting lipid oxidation, providing a sustainable additive option for both consumers and the poultry industry. However, the extraction and purification costs of hesperidin and naringin are relatively high compared to common additives, which may impact their application in large-scale production.

## 4.2 Applications in pharmacology

Diosmin is a flavonoid glycoside derived from hesperidin. Huwait and Mobashir (2022) demonstrated that diosmin reduces oxidative stress by altering the activity of specific enzymes and promotes apoptosis in various cancer cell lines through multiple mechanisms. Its anti-inflammatory properties are attributed to its ability to lower the levels of several inflammatory markers. Diosmin also helps alleviate complications of diabetes, such as neuropathy and dyslipidemia. When combined with other flavonoids, especially hesperidin, diosmin is highly effective in treating chronic venous insufficiency and hemorrhoids. However, diosmin is a central nervous system depressant, and long-term use may trigger excitatory reactions, such as irritability, restlessness, dizziness, and headaches. Naringin has the ability to promote the conversion of white fat into beige fat, thereby enhancing the thermogenic capacity of fat and glucose, which aids in weight and blood glucose control. Studies have found that naringin activates genes that improve blood glucose control and increase calorie burning, making it a promising strategy for treating obesity and diabetes. Beige fat has thermogenic functions, accelerating metabolism, breaking down white fat, reducing fat storage, and improving glucose and cholesterol metabolism (Chen, 2025). Naringin is also an effective inhibitor of breast cancer resistance protein (BCRP), helping to overcome BCRP-mediated multidrug resistance, and is used as a P-glycoprotein (P-gp) inhibitor. The combination of naringin with paclitaxel enhances anticancer activity. The results showed better cytotoxicity against breast cancer cell lines, offering an effective approach to combating drug resistance (Jabri et al., 2019). Naringin also inhibits the expression of STAT-3, AKT, and Bcl-2, playing a role in resistance to chemotherapy. Treatment with naringin reduces tumor size in mice bearing tumors.

# 5 Medical potential of citrus flavonoids

Citrus flavonoids possess various biological activities, primarily including antioxidant, anti-inflammatory, antimicrobial, antidiabetic, lipid-lowering, cardiovascular protective, and gut protective effects (Table 1).

## 5.1 Improvement of diabetes

Ji (2019) used the HepG2 cell-glucose consumption model to demonstrate significant differences in the hypoglycemic activity of different parts of citrus fruits. The active metabolites were primarily enriched in the oil vesicle and white peel layers of the fruit skin. Potential high-efficiency hypoglycemic citrus varieties such as "Ougan," "Ehime 30," "Chazhi Gan," and "Amakusa" were identified, with the fruit peel oil vesicle layers being rich in polymethoxyflavones (PMFs). PMFs isolated from the peel of "Ougan" citrus significantly promoted glucose consumption and regulated the expression of glucose metabolism genes through the Wingless-integrated, inhibiting certain gene expressions and upregulating key regulatory factors. The physiological benefits of PMFs from "Ougan" fruit peel were further validated in the KK-Ay diabetic mouse model. These metabolites not only effectively improved persistent hyperglycemia and glucose tolerance but also showed positive effects in lipid metabolism regulation and liver function protection.

Xiao et al. (2013) explored the hypoglycemic effects and potential mechanisms of citrus peel flavonoids in alloxan-induced diabetic mice. Treatment with citrus peel flavonoids significantly reduced fasting

TABLE 1 Medical potential of citrus flavonoids.

| Author (Reference) | Application fields | Major active metabolites | Mechanism of action | Specific effects |
|---|---|---|---|---|
| Ji (2019)<br>Xiao et al. (2013) | Improvement of diabetes | Citrus flavonoids | Enhance antioxidant capacity, eliminate free radicals, improve lipid metabolism disorders, and regulate the expression of glucose metabolism genes through the Wnt signaling pathway | Reduce fasting blood glucose, improve insulin resistance, and regulate lipid levels |
| Guan et al. (2021)<br>Zhang et al. (2021)<br>Wu et al. (2017) | Improvement of NAFLD | Naringin | Activate autophagy pathways, promote lipid degradation, and reduce hepatic lipid accumulation | Significantly reduce TG levels, inhibit *de novo* lipogenesis, and improve liver function |
| Nakajima and Ohizumi (2019)<br>He et al. (2021)<br>Kim et al. (2016) | Neuroprotective | Citrus flavonoids | Inhibit β-secretase activity, promote amyloid protein degradation, activate the cAMP/PKA signaling pathway, enhance synaptic function, and reduce oxidative stress to alleviate neuroinflammation | Reduce amyloid plaque accumulation in Alzheimer's disease (AD) and improve motor dysfunction and dopamine signaling in Parkinson's disease (PD) |
| Heidary Moghaddam et al. (2020)<br>Guo et al. (2022) | Cardiovascular protective | Naringin、Nobiletin | Inhibit oxidative stress, reduce cardiomyocyte apoptosis, activate the AMPK-mTOR pathway, decrease myocardial hypertrophy, and improve lipid profiles | Reduce atherosclerotic plaque formation, decrease myocardial damage, and inhibit the expression of inflammatory factors |
| Ferlazzo et al. (2016) | Antioxidant | Nobiletin、Naringenin | Increase the expression of antioxidant enzymes (SOD) and inhibit free radical generation | Reduce oxidative stress, protect cell membranes and mitochondria from damage |
| Chen et al. (2022)<br>He et al. (2018) | Anti-inflammatory | Naringin、Naringenin | Inhibit the NF-κBpathway,reduce the expression of pro-inflammatory factors,and suppress cox-2andiNOS activity | Alleviate inflammatory damage and improve the tissue microenvironment |
| Dey et al. (2020) | Antimicrobial | Naringin | Enhance the efficacy of antibiotics (e.g., ciprofloxacin) and inhibit bacterial biofilm formation | Significantly clear *Pseudomonas aeruginosa* biofilm, reduce its extracellular matrix thickness, and protect healthy cells |

blood glucose levels in diabetic mice while increasing the activity of the antioxidant enzyme SOD, decreasing lipid peroxidation product MDA levels, and enhancing spleen and thymus function. These findings suggest that the hypoglycemic effects are closely related to the enhancement of antioxidant capacity, free radical scavenging, and immune system enhancement. Furthermore, diabetes is often accompanied by lipid metabolism disorders, including elevated serum TG, TC, and LDL-C levels, and decreased HDL-C levels. These metabolic disturbances are key contributors to severe cardiovascular complications such as coronary heart disease in diabetic patients. Citrus peel total flavonoids significantly reduced TC, TG, and LDL-C levels while increasing HDL-C levels, improving lipid metabolism disorders in diabetic mice. This further suggests that the hypoglycemic effects may be related to the regulation of lipid metabolism. By enhancing antioxidant capacity, scavenging free radicals, improving immune function, and ameliorating lipid metabolism disorders, citrus peel flavonoids not only effectively lower blood glucose but also show significant potential in the prevention and treatment of diabetes and its complications. However, the hypoglycemic effect of citrus flavonoids is relatively weak and cannot be used alone as the primary antidiabetic medication. They generally need to be combined with other antidiabetic drugs to achieve better blood sugar control.

## 5.2 Improvement of NAFLD

Non-alcoholic fatty liver disease (NAFLD) is a common chronic liver disease characterized by fat accumulation in the liver, but its onset is not due to excessive alcohol consumption. It is closely associated with metabolic syndrome, type 2 diabetes, obesity, and insulin resistance (Byrne and Targher, 2015; Pouwels et al., 2022). The disease can progress over time, increasing the risk of cirrhosis, end-stage liver disease, and hepatocellular carcinoma (Dufour et al., 2020). Globally, the prevalence of NAFLD is approximately 25% (Younossi et al., 2019). Naringin has shown significant protective effects in alleviating non-alcoholic fatty liver disease (NAFLD). The autophagic degradation of lipid droplets, known as lipophagy, is the primary mechanism of lipid consumption in hepatocytes. Guan et al. (2021) demonstrated that naringin significantly reduced hepatic fat accumulation and inflammation induced by a high-fat diet in mice by restoring autophagic flux and enhancing lipophagy. *In vitro* experiments also showed that naringin enhanced lipid breakdown by promoting transcription factor EB (TFEB)-mediated lysosome biogenesis and the fusion of autophagosomes with lysosomes, thereby reducing lipid accumulation. However, in TFEB knockout mice and hepatocyte models, the aforementioned effects of naringin were abolished, indicating the critical role of TFEB in naringin's action against NAFLD. Zhang et al. (2021) showed that naringin significantly improved lipid metabolism in a NAFLD model. Naringin reduced intracellular triglyceride (TG) levels by 52.7% (from 0.36 ± 0.05 mM/g protein to 0.17 ± 0.03 mM/g protein, $P < 0.05$) and decreased very low-density lipoprotein (VLDL) secretion by 24.7% (from 10.24 ± 1.006 nmol/mL to 7.71 ± 0.576 nmol/mL, $P < 0.05$). Additionally, naringin downregulated key proteins associated with fatty acid uptake and *de novo* lipogenesis, such as cluster of differentiation 36 and acetyl-CoA carboxylase, and enhanced fatty acid β-oxidation by

upregulating carnitine palmitoyltransferase 1 (CPT-1) and peroxisome proliferator-activated receptor alpha (PPAR-α). Molecular docking analysis revealed that naringin directly interacts with CD36 and PPAR-α, further supporting its role in reducing lipid accumulation and promoting fat oxidation. However, the TEF model may not fully reflect the dynamic changes in the human liver during long-term metabolic disorders. Citrus total flavonoids (PTFC) have been shown to alleviate liver inflammation and lipid deposition by regulating the TLR/CCL signaling pathway, providing a new perspective for the treatment of NAFLD (Wu et al., 2017).

## 5.3 Neuroprotective activity

Alzheimer's disease (AD) is the most common age-related neurodegenerative disease, characterized by amyloid plaques, neurofibrillary tangles, and neurodegenerative lesions. Parkinson's disease (PD) is the second most common, characterized by the loss of dopaminergic neurons in the substantia nigra. Nakajima and Ohizumi (2019) studied the effects of Nobiletin, a metabolite extracted from citrus peel, on Alzheimer's and Parkinson's diseases. They found that Nobiletin could improve neurodegenerative lesions through multiple mechanisms. The study indicated that Nobiletin reduces amyloid plaque accumulation in AD models by inhibiting β-secretase activity and promoting amyloid protein degradation, thereby alleviating its toxic effects on neurons. It also promotes synaptic plasticity and neuronal function recovery by regulating the cAMP/PKA/ERK/CREB signaling pathway. Additionally, its antioxidant, anti-inflammatory, and endoplasmic reticulum stress-relieving effects significantly reduced oxidative stress and neuroinflammation levels in both AD and PD models, protecting neurons from inflammatory damage. In PD models, Nobiletin restored dopamine signaling, reduced neuronal loss, and improved motor dysfunction.

The incidence of Parkinson's disease is steadily increasing, but its etiology and pathogenesis are not yet fully understood. However, excessive neuronal apoptosis is thought to be closely related to the disease. Naringin and naringenin have shown significant neuroprotective potential in the treatment of Parkinson's disease. He et al. (2021) demonstrated that naringin and naringenin regulate the expression of long-chain non-coding RNA SNHG1 and further modulate its downstream molecular mechanisms. For example, downregulation of small nucleolar RNA host gene (SNHG) can reduce its binding to specific microRNAs (such as miR-7 or miR-124), thereby releasing the activity of these microRNAs and inhibiting the expression of pro-inflammatory factors and apoptosis-related genes. This regulatory effect not only alleviates neuroinflammation and mitochondrial damage but also promotes neuronal survival and inhibits apoptosis by modulating the PI3K/AKT signaling pathway. However, research on other potentially involved signaling pathways, such as NF-κB or Nrf2-ARE, is still insufficient. Kim et al. (2016) used 8-week-old C57BL/6 mice to establish a Parkinson's disease neurotoxic model by unilaterally injecting 6-hydroxydopamine (6-OHDA) into the striatum. They investigated whether naringin had neuroprotective and neurorestorative effects on the nigrostriatal dopamine projection. The experiment included both pre-treatment and post-treatment approaches, where naringin was administered before the 6-OHDA injection and 3 weeks after. The study found that naringin significantly alleviated the neurotoxicity induced by 6-OHDA, including reducing neuronal death and fiber loss in the nigrostriatal dopamine projection. Moreover, naringin exhibited both anti-inflammatory and neuroprotective effects by activating mammalian target of rapamycin complex 1 (MTORC1) and inhibiting microglial activation. However, current research has many limitations, and further experimental validation and clinical studies are needed to fully assess its translational medical value.

## 5.4 Antioxidant, anti-inflammatory and antimicrobial effects

### 5.4.1 Antioxidant

Naringin and hesperidin are the primary flavonoids in the citrus family, and they can exert effective antioxidant effects by being converted into naringenin and hesperetin, which then influence various signaling pathways in the body (Razavi and Hosseinzadeh, 2019). Madeira and Macedo (2015) used hesperidin and naringin as raw materials and employed enzymatic extraction and biotransformation (cellulase, pectinase, and tannase) to obtain hesperetin and naringenin. The study found that their antioxidant activity *in vitro* was significantly stronger than that of their glycoside forms (Xie et al., 2022). Guo et al. (2020) confirmed through several *in vitro* studies using antioxidant evaluation methods that citrus flavonoids extracted with ethyl acetate (EtOAcE) are effective natural antioxidants. Ferlazzo et al. (2016) investigated the antioxidant effects of citrus extracts rich in flavonoids in an Fe2(SO4)3-induced A549 cell model. The study showed that citrus extracts exhibited significant antioxidant activity. The levels of ROS induced by ferric sulfate increased 6–13 times, and lipid peroxides and DNA damage were significantly elevated, while mitochondrial function was impaired. However, citrus extracts significantly reduced ROS production (by 50%–70%), lipid peroxides (by 55%–73%), and DNA damage (by 65%–70%). The mechanism of action may be related to the chelating properties of iron and the increased antioxidant enzyme activity, including catalase, in the flavonoid extracts.

### 5.4.2 Anti-inflammatory and antimicrobial

Naringin is a potential antibiofilm agent that can be used in combination with antibiotics to develop drugs for the treatment of biofilm-related infections. Naringin has been shown to enhance the antibacterial effects of ciprofloxacin and tetracycline against *Pseudomonas aeruginosa* biofilms. Dey et al. (2020) demonstrated that Naringin exhibited significant biofilm clearance effects at sub-minimum inhibitory concentrations (sub-MIC), with the combination of naringin and ciprofloxacin achieving up to 89.35% biofilm clearance, as shown in crystal violet and MTT assays. Furthermore, Congo red binding assays and microscopic imaging confirmed that the naringin combination significantly reduced the extracellular polymeric substance (EPS) matrix and thickness of the biofilm. Compared to antibiotics alone, the combination therapy showed better biofilm clearance on indwelling catheter surfaces and exhibited lower toxicity to mouse macrophages, with cell viability exceeding 95%.

Naringin can significantly counteract the detrimental effects of TNF-α on NP cells and demonstrate strong anti-inflammatory activity in an *in vitro* intervertebral disc degeneration (IDD) model. Chen et al. (2022) found that naringin could significantly reduce the TNF-α-induced inflammation in human NP cells. After 24 h of TNF-α treatment, the level of COX-2 in NP cells was significantly elevated, while in cells pretreated with naringin, COX-2 levels were effectively suppressed (Wang et al., 2021). In an acidic environment, which is typical of localized inflammation, TNF-α-treated cells exhibited lower pH compared to untreated or DMSO-treated control groups, while naringin pretreatment significantly increased the pH. He et al. (2018) found that naringenin, nobiletin, and hesperetin exert anti-inflammatory effects on LPS-induced RAW264.7 cells, and the mechanism may be related to the TNF-α-induced NF-κB pathway, as well as COX-2 and iNOS regulation.

## 5.5 Cardiovascular protective effects

In recent decades, cardiovascular diseases have been one of the leading causes of death worldwide. In the past 2 years, it has been estimated that approximately 330 million people globally suffer from cardiovascular diseases, and there is still a large population in China with cardiovascular risk factors. Both the incidence and mortality rates of cardiovascular diseases are continuously rising (Wang et al., 2023). Naringin and naringenin have potential roles in the prevention and treatment of cardiovascular diseases. Both are flavonoid metabolites widely found in citrus fruits, especially grapefruits. Heidary Moghaddam et al. (2020) demonstrated that in a high-cholesterol diet rabbit model, naringin and naringenin could downregulate the gene expression of monocyte chemoattractant protein-1 (MCP-1) and vascular cell adhesion molecule-1 (VCAM-1), reducing atherosclerotic plaque formation. In a high-glucose environment, naringin effectively reduced cardiomyocyte apoptosis by inhibiting oxidative stress and the mitogen-activated protein kinase (MAPK) signaling pathway. Additionally, naringenin alleviated cardiac hypertrophy by activating the AMPK-mTOR signaling pathway and improved endothelial function. In rat experiments, naringenin also showed protective effects against myocardial ischemia-reperfusion injury by regulating mitochondrial-related signaling pathways to improve heart function. Naringin improved myocardial ischemia-reperfusion injury through the miR-126/GSK-3β signaling pathway. Guo et al. (2022) demonstrated that in a rat myocardial ischemia/reperfusion (I/R) model, treatment with naringin or miR-126 agomir combined significantly alleviated cardiac inflammation, reduced cardiomyocyte apoptosis, and notably suppressed the expression of inflammatory factors (IL-6, IL-8, and TNF-α). Moreover, naringin or miR-126 agomir regulated the GSK-3β signaling pathway by lowering the phosphorylation levels of GSK-3β, promoting β-catenin entry into the nucleus, thereby exerting a protective effect on the myocardium.

Atherosclerosis (AS) is a chronic inflammatory cardiovascular disease characterized by lipid deposition in the vessel wall and immune cell recruitment, and it is a major risk factor for cardiovascular diseases (Liu et al., 2023). Yu et al. (2022) found that in mice fed a high-fat diet, oral administration of naringin significantly reduced total cholesterol and triglyceride levels while increasing high-density lipoprotein cholesterol levels. Li et al. (2014) showed that naringin upregulated the expression of superoxide dismutase (SOD), downregulated malondialdehyde (MDA) levels, and protected endothelial cells from TNF-α-induced damage by blocking oxidative stress, thereby improving the development of atherosclerosis.

## 5.6 Regulation of lipid metabolism

Lipid metabolism refers to the series of biochemical reactions involved in the digestion, absorption, transport, storage, and breakdown of lipid substances in the body, such as triglycerides, cholesterol, phospholipids, and free fatty acids. Imbalance in lipid metabolism can lead to diseases such as obesity, fatty liver, hyperlipidemia, and atherosclerosis.

The digestion and absorption of dietary fats primarily occur in the small intestine, and this process depends on the activity of pancreatic lipase (PL) in the duodenum and jejunum. Pancreatic lipase is the key enzyme responsible for triglyceride hydrolysis in the gastrointestinal tract. Therefore, inhibiting PL activity is considered a potential strategy to reduce fat absorption and restore lipid homeostasis. Among them, natural product lipase inhibitors such as naringin and hesperidin have gradually become a research focus due to their safety and diversity (Liu et al., 2020; Rajan et al., 2020). Huang et al. (2020) analyzed eight citrus peel extracts and found that hesperidin exhibited the most significant pancreatic lipase inhibition and antioxidant activity. It interacts with lipase through hydrogen bonds and van der Waals forces, making it a potential natural ingredient for free radical scavenging and controlling obesity. Rotimi et al. (2018) used a high-fat, low-chain uric acid rat T2DM model and found that naringin showed significant anti-diabetic effects and improved lipid metabolism in the diabetes model. Naringin dose-dependently reduced plasma glucose, DPP-IV levels, and renal ACE activity, while significantly increasing paraoxonase (PON) activity, particularly in plasma and liver. Naringin also lowered cholesterol levels, inhibited the increase in PON activity in VLDL and VLDL 3, and reversed the decline in HDL3 cholesterol. Additionally, it restored the decrease in Carnitine Palmitoyltransferase 1(CPT -1) activity caused by diabetes and regulated the gene expression of liver genes such as Scarb1 and Ahr.

## 5.7 Gut protective effects

Inflammatory bowel disease (IBD) is an uncontrollable, non-specific chronic immune-mediated intestinal inflammation, which can be divided into Crohn's disease (CD) and ulcerative colitis (UC) (Kaplan, 2015). The pathogenesis of UC is not yet fully understood. The general consensus is that UC is closely associated with immune system dysfunction, intestinal mucosal damage, and dysbiosis in genetically susceptible populations. Naringin has shown significant therapeutic effects on UC. Cao et al. (2021) used a dextran sulfate sodium (DSS)-induced mouse model of ulcerative colitis and found that naringin reduced the Disease Activity Index (DAI) score, alleviated colon tissue damage, and exerted effects through oxidative stress and

inflammatory factors. Additionally, naringin increased the expression of tight junction proteins in the mucosa, improved the relative abundance of *Firmicutes/Bacteroidetes*, and reduced the levels of Proteobacteria, thus improving gut microbiota dysbiosis. The integrity of the intestinal barrier is crucial for defending against pathogen invasion. When the body is exposed to various environmental factors, the intestinal barrier may be compromised, allowing the entry of foreign pathogens and endotoxins, thereby jeopardizing health Zhang et al. (2022). Naringin can alleviate lipopolysaccharide (LPS)-induced intestinal barrier damage in mice. Luo et al. (2023) demonstrated that naringin effectively reduced LPS-induced intestinal barrier damage by inhibiting inflammatory factors, improving antioxidant function, and enhancing the expression of intestinal tight junction proteins. This protective effect may be mediated by the activation of the Nrf2 signaling pathway and inhibition of the TLR4/p38 MAPK/NF-κB signaling pathway. Experimental results showed that LPS significantly increased serum diamine oxidase (DAO) activity, D-lactic acid (D-LA) concentration, and malondialdehyde (MDA) levels, while reducing the activity of antioxidant enzymes (SOD, Gpx, CAT) and causing damage to intestinal tissue morphology and tight junction protein expression (ZO-1, Occludin, Claudin). LPS also upregulated the expression of inflammatory factors (TNF-α, IL-1β, IL-6) and genes related to TLR4/p38 MAPK/NF-κB signaling. However, naringin significantly reversed these changes, thereby protecting the intestinal barrier (Carroll et al., 2003). Nevertheless hesperidin, as a natural product, has a low bioavailability, meaning its absorption, distribution, metabolism, and excretion in the body may be limited. This can result in a decreased therapeutic efficacy in clinical treatment.

## 6 Conclusion

Citrus flavonoids, as natural phytochemicals, have demonstrated a wide range of health benefits, particularly in the areas of antioxidant, anti-inflammatory, anti-diabetic, lipid-lowering, and protection of the gut and cardiovascular system. In recent years, significant progress has been made in the study of the biological activities and molecular mechanisms of citrus flavonoids, providing new strategies for the prevention and treatment of metabolic diseases such as diabetes and NAFLD. At the same time, advancements in extraction and purification technologies have facilitated the development and application of citrus flavonoids in the fields of food, medicine, and functional materials. Future research should focus on further elucidating the mechanisms of action of citrus flavonoids and their safety and efficacy in clinical applications, ultimately contributing to greater benefits for human health.

## Author contributions

YX: Writing–original draft. PH: Writing–original draft. BH: Writing–original draft. ZC: Validation, Writing–review and editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Formononetin: a review of its source, pharmacology, drug combination, toxicity, derivatives, and drug delivery systems

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Formononetin (FMN) is a common natural metabolite that can be extracted and isolated from some common botanical drugs. In recent years, FMN has garnered increasing attention due to its beneficial biological activities. In this paper, we systematically summarize the sources of FMN and provide a comprehensive review of its pharmacological activities and molecular mechanisms, co-administration, toxicity, derivatives, and drug delivery systems in the last 5 years. The study results found that FMN has a wide range of pharmacological activities in neurological disorders, organ damage and cancer, showing great potential for clinical application and broad prospects. Researchers are exploring various types of delivery systems, including nanoparticle carriers, ligand modifications and polymer microspheres. These advanced delivery systems can enhance the stability of FMN, prolong its release time *in vivo*, and improve targeting, thereby optimizing its therapeutic efficacy and reducing side effects, and greatly improving its bioavailability. In conclusion, FMN is a natural metabolite with considerable research value, and its diverse biological activities make it a promising candidate for drug development and medical research.

KEYWORDS

**formononetin, pharmacology, drug combination, toxicity, derivatives, drug delivery systems**

## 1 Introduction

Flavonoids are a class of natural metabolites widely found in plants and foods, belonging to an important branch of polyphenolic compounds, which have a wide range of biological activities, including anti-inflammatory, antidiabetic, and anticancer (Billowria et al., 2024). Because of their different structural characteristics, they are divided into different types, thus displaying various functions. Among them, isoflavones are a unique large class of flavonoids, mainly found in soybeans and legumes, which have estrogenic activity and can be combined with estrogen receptors. Phytoestrogens are not hormones in the traditional

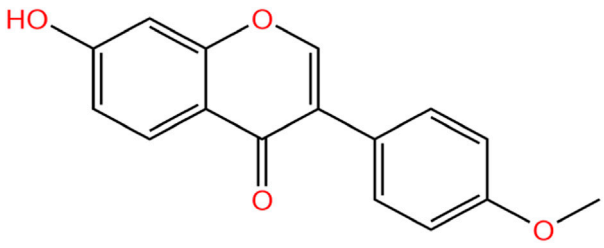



FIGURE 1
Chemical structures of FMN.

sense, but a secondary metabolite product of plants. It is similar to animal estrogen in structure and function, and can have many effects on health, which may help to prevent certain hormone-related diseases, such as breast cancer and osteoporosis (Patra et al., 2023).

FMN is a kind of isoflavone phytoestrogen, which is widely found in plants and common diets. It has important biological activities, and has become the current research hotspot in the field of hormones. Modern research has shown that FMN is one of the active ingredients in some commonly used botanical drugs, such as *Astragalus mongholicus, Pueraria montana*, and *Trifolium pratense*. With the deepening of modern pharmacological research, the medicinal value of FMN has been further developed. Currently, the research on the pharmacological effects, combination application and drug delivery system of FMN has been carried out in-depth, indicating that FMN has a variety of pharmacological activities and clinical application potential as a single-component drug, and is expected to become a promising drug. In the past few years, the pharmacological effects of FMN have been widely reported. However, most of the previous reports were scattered and lacked systematic summarization and generalization. Therefore, this article aims to summarize the reports on FMN in the past 5 years, which will provide a basis for the future development and clinical application of FMN.

## 2 Chemical properties and plant origin of FMN

FMN is a well-known flavonoid with the parent structure of isoflavones, so it is also known as 7-hydroxy-4′-methoxy isoflavone, with the molecular formula $C_{16}H_{12}O_4$, relative molecular weight 268.26 (Křížová et al., 2019), and the chemical structure is shown in Figure 1. Structurally, FMN has only one phenolic hydroxyl group, so it is poorly soluble in water and easily soluble in organic solvents such as methanol, ethyl acetate, and ether. FMN is a secondary metabolite produced by plants, predominantly found in legumes, as shown in Table 1. In addition, it is also present in normal diets, such as milk (Andersen et al., 2009), beer (Lapcík et al., 1998), and coffee (Alves et al., 2010).

## 3 Pharmacological activities and molecular mechanisms of FMN

### 3.1 Neurological diseases

#### 3.1.1 Alzheimer's disease

Alzheimer's disease (AD) is a progressive neurodegenerative disease and has been listed as a global priority public health problem (Ren and Qu, 2023), and its pathogenesis is related to the hyperphosphorylation of tau protein and Aβ deposition. It had been found that FMN, isolated from *Sophora secundiflora*, could

TABLE 1 Plant origin of FMN.

| Plant species | Family | Present site | Ref. |
|---|---|---|---|
| *Butea superba Roxb* | Leguminosae | Tuberous root | Ngamrojanavanich et al. (2007) |
| *Cicer arietinum* L | Leguminosae | Seed | Gao et al. (2015) |
| *Dalbergia tonkinensis* | Leguminosae | Leaves, Heartwood | Cuong et al. (2022) |
| *Glycyrrhiza glabra* | Leguminosae | Root | Vaya et al. (1997) |
| *Pueraria montana var. lobata* (Willd.) | Leguminosae | Root | Cui et al. (2018) |
| *Spatholobus suberectus* | Leguminosae | Stem | Park et al. (2017) |
| *Trifolium subterraneum* L. | Leguminosae | Whole plant | Sakakibara et al. (2004) |
| *Trifolium pratense* L. | Leguminosae | Whole plant | Sakakibara et al. (2004) |
| *Astragalus mongholicus* Bge. | Leguminosae | Root | Pan et al. (2021) |
| *Dalbergia odorifera* | Leguminosae | Heartwood | Zhao et al. (2020) |
| *Sophora flavescens* | Leguminosae | Root | Hwang et al. (2005) |
| *Sophora secundiflora* | Leguminosae | Leaves | Aly et al. (2020) |
| *Amorpha fruticosa* L. | Leguminosae | Leaves | Cui et al. (2017) |
| *Andira inermis* | Leguminosae | Stem, Leaves | Kraft et al. (2000) |

improve memory problems by restoring the level of oxidative stress in brain tissues and modulating acetylcholinesterase activity. It was a potential neuroprotective agent for further research and development of AD treatment in the pharmaceutical industry (Aly et al., 2020; Sugimoto et al., 2021). Inflammatory responses could lead to vascular damage in the brain, disrupting the blood-brain barrier, promoting Aβ deposition and aggregation, and accelerating the development of AD (Silva et al., 2019). Therefore, inhibition of cerebral vascular inflammation plays a crucial role in the pathogenesis of AD. Fan et al. (2022) treated human brain microvascular endothelial cells (HBMECs) with $A\beta_{25-35}$ for 12 h and performed quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR). The results of western blot analysis showed that the expression of intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin protein increased, and nuclear factor-κB [NF-κB (p65)] was translocated to the nucleus of HBMECs. But after the administration of FMN, the expression levels of the above three decreased and NF-κB activation was inhibited, which may be related to the release of FMN blocking kelch-like ECH-associated protein-1 (Keap1) and activating the nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway. Similarly, FMN exerted anti-neuroinflammatory effects by targeting peroxisome proliferator-activated receptor coactivator-1α (PGC-1α) and bidirectionally regulating NF-κB signaling pathway and Nrf2/Heme oxygenase-1 (HO-1) signaling pathway, thereby inhibiting tau protein hyperphosphorylation. Significantly FMN improve cognitive dysfunction in mice caused by high-fat feeding (Fu et al., 2019).

### 3.1.2 Depression and anxiety

Neurotransmitters are chemicals in the nervous system that regulate the transmission of signals between neurons. Disruptions in their function can lead to alterations in emotional and cognitive processes, potentially contributing to conditions such as depression and anxiety (Lee, 2013). In mice models of depression established by chronic corticosterone (CORT) injection, FMN significantly increased sucrose preference and shortened the resting time of forced swimming. Using ELISA kits and Western blot analysis, researchers found that FMN decreased serum corticosterone levels and upregulated the protein expression levels of the glucocorticoid receptor (GR) and brain-derived neurotrophic factor (BDNF) in the hippocampus. Additionally, FMN mitigated CORT-induced neuronal damage in the CA1 and CA3 regions of the hippocampus and promoted neurogenesis in this brain area (Zhang et al., 2022). In addition, the inflammatory response could indirectly affect the development of depression by influencing body system diseases. Yang J. et al. (2023) demonstrated for the first time that FMN improved depressive behaviors in mice with myocardial infarction-associated depression. The underlying mechanism involves the reduction of interleukin-6 (IL-6) and IL-17A by inhibiting glycogen synthase kinase-3β (GSK-3β) activity and downregulating downstream signaling molecules, including Notch homolog 1 (Notch1) and CCAAT/enhancer binding protein α (C/EBPα). This, in turn, promoted macrophage/microglia polarization toward the M2 phenotype, reduced neuroinflammation, increased BDNF and 5-hydroxytryptamine (5-HT) levels, and mitigated the progression of depression in mice. To investigate the anxiolytic effect of FMN, Wang X. S. et al. (2019) used a complete Freund's adjuvant (CFA)-induced anxiety mice model in mice and administered FMN continuously for 14 days. The results showed that FMN significantly increased the open-arm time in the elevated plus maze test and the central area time in the open-field experiment in mice, and had a favorable anxiolytic effect. Molecular mechanism studies showed that FMN may alleviate the inflammation and neuronal hyperexcitability in the basolateral amygdala and alleviate the anxiety-like behavior in mice by inhibiting the NF-κB and N-methyl-D-aspartate (NMDA)/cAMP-response element binding protein (CREB) signaling pathways. Thus, FMN can be used as a new candidate drug for the treatment of depression and anxiety disorders.

### 3.1.3 Cerebral ischemia-reperfusion injury

Cerebral ischemia-reperfusion (I/R) injury refers to the phenomenon that the injury is further aggravated and deteriorates during reperfusion after reversible ischemic brain injury, which has a high mortality and disability rate. FMN could target Janus kinase 2 (JAK2)/signal transducers and activators of transcription 3 (STAT3) and phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/extracellular regulated protein kinases (ERK) signaling pathways. It could significantly reduce the level of inflammatory factors, increase the number of dendritic spines in neurons, and increase the expression of βIII-tubulin, growth-associated protein 43 (GAP-43), nerve growth factor (NGF) and BDNF. FMN could protect the neurological dysfunction and pathological changes of brain tissue in rats with arterial occlusion from both anti-inflammatory and neuroprotective aspects (Wu Y. et al., 2020; Yu et al., 2022). Other studies showed that poly(ADPribose) polymerase-1 (PARP-1) and poly (ADP-ribose) glycohydrolase (PARG) played key roles in ischemic neuronal cell death and disease progression. When the activities of both are inhibited, they could effectively reduce the area of cerebral infarction, eliminate the inflammatory response, and restore neurological function in stroke patients (Cuzzocrea and Wang, 2005; Liu et al., 2022). On this basis, Luo et al. (2024) used PARP-1 inhibitor PJ34 and PARG inhibitor ethacridine lactate to investigate whether FMN's mechanism of action involves PARP-1 and PARG. The result found that FMN significantly reduced PARP1, PARG, apoptosis-inducing factor (AIF), cysteinyl aspartate-specific protease 3 (caspase-3) and p53 protein in rats with cerebral ischemia-reperfusion injury, and increased the expression of endogenous neuroprotective gene Iduna, effectively reducing the area of cerebral infarction and neuronal apoptosis in rats. These findings suggested that FMN may serve as a potential inhibitor of PARP-1 and PARG for the treatment of cerebral I/R injury.

### 3.1.4 Spinal cord injury

Spinal cord injury (SCI) is a devastating neurological state that leads to impaired sensory and motor functions and has a significant impact on the global healthcare system (Anjum et al., 2020). FMN can improve neurological function and exert neuroprotective effects in spinal cord injury by reducing local apoptosis and inflammatory cell infiltration. Zhou and Zhang (2023) treated PC12 cells with lipopolysaccharide (LPS) and different concentrations of FMN (50 μM, 100 μM, 200 μM). They found that FMN decreased

tumor necrosis factor-α (TNF-α), IL-1β, IL-6, p-p65 NF-κB, nucleotide-binding oligomerization domain-like receptor protein 3 (NLRP3) and lactate dehydrogenase (LDH) levels, increased proliferating cell nuclear antigen (PCNA) expression, increased cell viability, inhibited apoptosis, and significantly ameliorated LPS-induced inflammatory injury in neuronal cells. In another report, Fu et al. (2023) demonstrated *in vivo* and *in vitro* that FMN attenuated microglial cell inflammatory response to spinal cord injury by inhibiting the epidermal growth factor receptor (EGFR)/mitogen-activated protein kinase (p38 MAPK) signaling pathway, thereby promoting nerve injury repair in rat spinal cord.

#### 3.1.4.1 Peripheral neuropathy

Oxaliplatin, a platinum-based chemotherapeutic agent, is commonly used in the treatment of metastatic rectal cancer but is limited in clinical application due to its severe neuropathy. Fang et al. (2020) found that FMN had a favorable ameliorative effect on oxaliplatin-induced peripheral neuropathy and did not affect the chemotherapeutic function of oxaliplatin. Molecular mechanism studies demonstrated that FMN had this effect because it could target the activation of the Nrf2 pathway, increase the activity of the phase II metabolizing enzyme glutathione S-transferase pi 1 (GSTP1), and inhibit oxaliplatin-induced peripheral neuropathy by protecting mitochondrial function.

Diabetic peripheral neuropathy is one of the most common chronic complications of diabetes. The main symptoms include spontaneous refractory pain, which may last for weeks to months. Currently, there are no drugs specifically for the treatment of diabetic peripheral neuropathy on the market. Drugs for the prevention and treatment of diabetic peripheral neuropathy also have strong side effects, which make clinical application difficult (Yang et al., 2022). In both *in vivo* and *in vitro* experiments, FMN was shown to be a potential therapeutic agent for diabetic peripheral neuropathy. It promoted the release of NGF by activating silenced information regulatory factor 3 (SIRT3), inhibited oxidative stress, enhanced neuronal survival, restored mitochondrial function, and significantly increased the pain threshold in neuropathic rats, as demonstrated in the hot-plate and tail-dip experiments (Oza and Kulkarni, 2020; Jiang et al., 2023). The neuroprotective mechanism of FMN is shown in Figure 2.

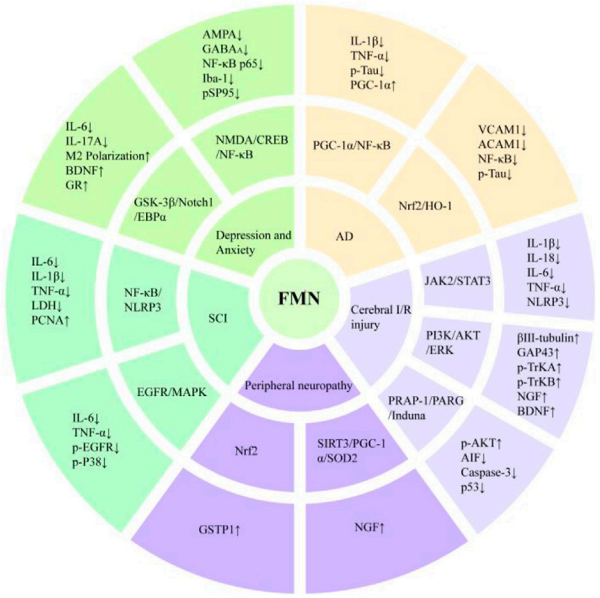



FIGURE 2
Neuroprotective mechanism of FMN.

## 3.2 Organ injuries

### 3.2.1 Lung injury

FMN has good therapeutic effects on pulmonary arterial hypertension (PAH), hyperoxia-induced acute lung injury (ALI), and chronic obstructive pulmonary disease (COPD). Cai et al. (2019) used monocrotaline (MCT) to induce PAH in rats and administered FMN. The results indicated that FMN may provide a protective effect against MCT-induced PAH in rats by inhibiting the PI3K/AKT and ERK pathways, decreasing the expression of α-smooth muscle actin (α-SMA), PCNA, and B-cell lymphoma 2 (Bcl-2), thereby preventing the excessive proliferation of pulmonary artery smooth muscle cells (PASMCs) and pulmonary vascular remodeling. Similarly, Wu J. et al. (2020) found that FMN inhibited pulmonary vascular remodeling in MCT-induced PAH rats by down-regulating the expression of transforming growth factor-β1 (TGF-β1), matrix metalloproteinase-2 (MMP-2), MMP-9, collagen types I and III, and fibronectin. It also reduced the dense focal collagen deposition induced by MCT. In addition, FMN alleviated MCT-induced lung inflammation by decreasing the expression of inflammatory cytokines, including TNF-α, IL-1β, and monocyte chemotactic protein-1 (MCP-1). Chen et al. (2021) exposed C57BL/6 mice to hyperoxia for 72 h to establish an ALI model. Intraperitoneal injection of FMN significantly reduced hyperoxia-induced increases in lung water content, pro-inflammatory cytokine levels, and lung neutrophil infiltration in mice. Western blot analysis showed that FMN increased the expression and activity of Nrf2, HO-1, and superoxide dismutase (SOD), thereby promoting the polarization of macrophages to the M2 phenotype. In another report, Li et al. (2024a) established the COPD mice model by exposing mice to cigarette smoke (CS) for 24 weeks and treated bronchial epithelial BEAS-2B cells with CS extract for 24 h to investigate the *in vivo* and *in vitro* effects of FMN on COPD. The results showed that FMN inhibited the activation of aryl hydrocarbon receptor (AhR)/CYP1A1 and AKT/mechanistic target of rapamycin (mTOR) signaling pathways, reduced CS-induced inflammatory response, endoplasmic reticulum stress and apoptosis, and effectively improved lung function and airway remodeling in COPD mice, both *in vivo* and *in vitro*.

### 3.2.2 Liver injury

Liao et al. (2021) found that FMN may be a potential active ingredient in the prevention or treatment of liver injury by network pharmacology combined with biochemical assays, which could reduce hepatotoxicity and improve liver function through inflammatory molecular pathways. In the concanavalin A (ConA)-induced autoimmune hepatitis (AIH) mice model, FMN-treated mice showed reduced hepatocyte swelling and inflammatory cell infiltration (Liu G. et al., 2021). Further molecular mechanism

studies showed that FMN inhibited the activation of the NF-κB signaling pathway and NLRP3 inflammasome, and reduced the serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in mice. At the same time, FMN upregulated the expression of Bcl-2, downregulated the expression of Bcl-2-associated X protein (Bax), cleaved caspase 9, cleaved caspase 3 expression in liver tissue, and inhibited ConA-induced apoptosis in mice hepatocytes.

The process of hepatic ischemia-reperfusion injury involves the regulation of multiple factors, among which mitochondrial damage is one of the causative factors (Cannistrà et al., 2016). Ma et al. (2022) found that FMN could protect against I/R-induced hepatic injury by restoring the function of mitochondrial autophagy. Compared with the model group, the FMN group could effectively improve sinus congestion and cell swelling in rat liver tissue, and reduce serum AST, ALT, TNF-α and IL-1β levels. In addition, through the prohibitin-2 (PHB2)/PTEN-induced putative kinase protein 1 (PINK1)/Parkin signaling pathway, the levels of LC3II, Beclin1, p62, cyclooxygenase-2 (COX2), COX4, MMP and adenosine triphosphate (ATP) were increased, and the liver mitochondrial autophagy function and energy metabolism failure were restored. In addition, during this process, the activity of antioxidant proteins glutathione (GSH), catalase (CAT), GSH-PX in the FMN treatment group recovered, and the levels of reactive oxygen species (ROS) and malondialdehyde (MDA) decreased. These findings suggest that FMN can inhibit oxidative stress in the liver and restore mitochondrial function.

Liver injury is not only a direct lesion at the liver, but other pathologies can also indirectly lead to the occurrence of liver injury, such as cholestasis. Cholestasis is characterized by intracellular bile acid (BA) overload in hepatocytes, based on which FMN can improve hepatic/systemic BA metabolism and protect the liver from liver injury caused by intracellular retention of BA. Yang et al. (2019) induced cholestasis in the mice model using α-naphthylisothiocyanate (ANIT) and used FMN by gavage for 10 days. The results showed that FMN significantly improved gallbladder enlargement and bile color change in the cholestasis model mice. qRT-PCR and Western blot analyses revealed that FMN targeted sirtuin 1 (SIRT1) and peroxisome proliferator-activated receptor alpha (PPARα), reducing the levels of inflammatory markers, promoting bile flow from hepatocytes to the bile ducts, improving liver and portal vein bile acid transport, and inhibiting ANIT-induced liver injury and cholestasis. These findings suggest that FMN may serve as a potential therapeutic strategy for cholestatic liver disease.

### 3.2.3 Kidney injury

Kidney disease has become an significant threat to human death. There are about 850 million patients with kidney disease worldwide, and the global prevalence of chronic kidney disease (CKD) is more than 10% (Feng et al., 2023). Regulation of cell death is a necessary condition for maintaining normal physiological homeostasis. Various forms of cell death, including apoptosis, autophagy, necrosis, and pyroptosis, are involved in the pathogenesis of kidney disease. Iron ptosis is the accumulation of iron-dependent lipid peroxides, which is a new mode of cell death and is associated with acute kidney injury under a variety of stimuli, such as ischemia-reperfusion, sepsis, or toxins, and CKD (Bayır et al., 2023; Li et al., 2024). FMN increased the expression of SLC7A11, glutathione peroxidase 4 (GPX4) and Nrf2, reduced the level of 4-hydroxy-2-nonenal (4-HNE), and negatively regulated iron sagging by inhibiting the translocation of Smad3 from the cytoplasm to nucleus. At the same time, it reduced the expression of fibrosis genes including α-SMA, Col1a1 and fibronectin, and hindered iron apoptosis-related fibrosis, thereby alleviating CKD (Zhu et al., 2023). In addition, FMN also had a good repair effect on drug-induced renal injury. In the rat model of kidney injury induced by gentamycin and methotrexate, FMN prevented kidney tissue damage and renal function reduction caused by gentamycin and methotrexate administration to a large extent (Aladaileh et al., 2019; Althunibat et al., 2022). This was all related to the enhancement of Nrf2, HO-1, GSH and SOD expression, reduction of MDA, TNF-α, IL-1β, COX-2, and inducible nitric oxide synthase (iNOS) levels, and enhancement of renal antioxidant and anti-inflammatory capacity. Hao et al. (2021) demonstrated the protective effect of FMN against renal injury due to cisplatin treatment by *in vivo* and *in vitro* experiments. PPARα is a ligand-activated nuclear hormone receptor transcription factor that regulates the expression of genes related to inflammation and cellular lipid metabolism and contributes to the maintenance of renal function (Iwaki et al., 2019). FMN upregulated PPARα expression in a ligand-dependent manner to increase the levels of Nrf2, HO-1, NAD(P)H: quinone oxidoreductase 1 (NQO1), and CAT, decreased the levels of MDA, TNF-α, and IL-1β, inhibited myeloperoxidase (MPO) activity, reduced renal proximal tubular cell apoptosis, oxidative stress, and inflammatory response. Thus, FMN is a promising drug to prevent drug-induced organ damage and can be used as a first-line treatment for acute kidney injury in future studies.

Diabetic nephropathy (DN) is one of the most common and serious complications of diabetes, which is related to the increased morbidity and mortality of diabetic patients (Samsu, 2021). In a high-glucose pathological state, elevated glucose levels stimulate cells to produce excessive ROS, activating various downstream inflammatory signaling pathways. This process induces and accelerates the accumulation of renal inflammatory fibrosis markers, such as fibronectin (FN) and ICAM-1, ultimately leading to renal fibrosis characterized by glomerulosclerosis (Li Y. et al., 2022). Therefore, oxidative stress is considered to be a major pathogenic factor in diabetic nephropathy. *In vivo* and *in vitro* experiments demonstrated that FMN could reduce inflammation and fibrosis within the glomerulus by increasing sirtuin-1 (Sirt1) protein levels in renal tissues, activating the Nrf2/ARE signaling pathway, promoting the expression of downstream antioxidant enzymes, inhibiting oxidative stress, and then down-regulating the levels of FN and ICAM-1. Restoring renal function indexes in mice with DN (Zhuang et al., 2020). In addition, FMN could reduce mitochondrial superoxide production and attenuate the loss of mitochondrial membrane power by increasing the expression of PGC-1α, a downstream target of Sirt1, and Mfn2 in the kidney, and decreasing the expression of Drp1 and Fis1, thus improving mitochondrial homeostasis and restoring renal function in rats (Huang et al., 2022). Based on oxidative stress injury, Smad3 directly binds to the promoter region to activate COl-I and COl-III, which promotes extracellular matrix (ECM) synthesis, leading to fibroblast activation and enhancing the pathological process of renal fibrosis (Dan Hu et al., 2023). FMN reduced Smad3, COl1a1, and COl3a1 mRNA and protein

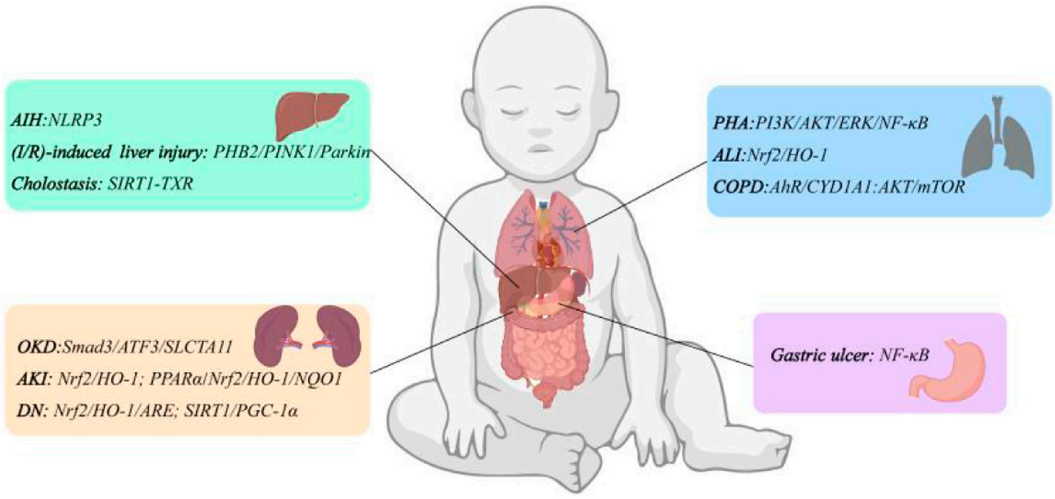



FIGURE 3
Organ protective mechanism of FMN.

expression, attenuated α-SMA protein overactivation, reduced collagen production and deposition, and improved renal fiber structure (Lv et al., 2020). The above studies have shown that FMN can be used as a potential drug for the treatment of diabetic nephropathy.

### 3.2.4 Gastric ulcer

Gastric ulcer is a disease characterized by chronic inflammation and barrier damage of the gastric mucosa, affecting 5%–10% of the population. Its etiology is closely related to excessive drinking, use of non-steroidal anti-inflammatory drugs and infection with *helicobacter pylori* (Mousa et al., 2019). Due to the side effects and resistance to conventional medications such as cimetidine, amoxicillin, and omeprazole, there is a need to find new therapeutic approaches. Rubab et al. (2022) used ethanol-induced gastric ulcer rats model to explore whether *Caragana ambigua* had anti-gastric ulcer effect and used liquid chromatography-mass spectrometry for component analysis. The results showed that FMN in *C. ambigua* significantly reduced the gastric ulcer index in the gastric tissue of model rats, and increased the pH value of gastric juice, gastric parietal protein and gastric mucus content. The results of histopathological observation showed that FMN could also significantly reduce gastric mucosal injury, submucosal edema and leukocyte infiltration in rats. In the rats model of gastric ulcers stimulated by non-steroidal anti-inflammatory drugs, FMN also played the same role (Mendonça et al., 2020). Yi et al. (2022) found that FMN could significantly improve the phenomenon of exfoliation necrosis and a large number of inflammatory cell infiltration in some areas of gastric mucosa induced by acetic acid in rats. At the same time, FMN upregulated the levels of CD34 protein, tight junction protein, vascular endothelial growth factor (VEGF) and nitric oxide (NO) in a dose-dependent manner, downregulated the level of human endothelin-1 (ET-1), and promoted gastric mucosal angiogenesis. In addition, compared with the model group, FMN could effectively reduce the levels of TNF-α, IL-1β, IL-6, p-P65/P65 and MPO activity, and increase the level of p-IκBα/IκBα, suggesting that FMN could play an anti-gastric ulcer role by inhibiting the activation of NF-κB signaling pathway. The above studies have shown that FMN can treat gastric ulcers through damage repair and anti-inflammatory effects. The organ protective mechanism of FMN is shown in Figure 3.

## 3.3 Cardio vascular diseases

### 3.3.1 Atherosclerosis

Atherosclerosis (AS) is a chronic inflammatory disease characterized by lipid deposition and persistent inflammation within the arterial wall (Zhu et al., 2018). In the early stage of AS, the primary trigger of AS is vascular endothelial dysfunction, which is caused by vascular endothelial cell damage and hemodynamics (Wang et al., 2023). Zhang et al. (2021) discovered through network pharmacology and *in vitro* experiments that FMN alleviated inflammation, oxidative stress, and apoptosis responses in HUVECs induced by oxidized low-density lipoprotein by activating peroxisome proliferator-activated receptor-γ (PPAR-γ) signaling. He et al. (2024) further demonstrated that FMN upregulated the expression of α7nAChR, CD206, IL-10, SHIP1, and Arg-1, while down-regulating the expression of CD68, iNOS, COX-2, miR155-5p, IL-6, and IL-1β. FMN also promoted macrophage polarization to the M2 phenotype, thereby reducing inflammation and atherosclerosis in ApoE−/− mice by inhibiting the JAK/STAT signaling pathway. In addition, foam cells play a key role in the development of AS, composed of macrophages, vascular smooth muscle cells, and other types of cells that adhere to endothelial cells, ingesting accumulated lipids and lipid products as foci for growing lesions (Galindo et al., 2023). Ma et al. (2020) found that FMN reduced vascular smooth muscle cell and macrophage-derived foam cell formation and their accumulation in the arterial wall, which may be associated with decreased SRA expression and reduced monocyte adhesion. Therefore, foam cells may serve as a potential breakthrough for FMN in the treatment of AS.

### 3.3.2 Protecting blood vessels

Zhou et al. (2022) found that FMN can reduce vascular endothelial injury caused by deep vein thrombosis in rats by increasing the expression of endothelial nitric oxide synthase (eNOS) and NO production. On this basis, Wu et al. (2020) further determined through *in vivo* and *in vitro* experiments that the mechanism by which FMN exerted its effects was due to the activation of the ERK1/2 and AKT signaling pathways, which in turn significantly enhanced endothelial function. In addition, FMN dose-dependently increased the levels of insulin-like growth factor 1 (IGF-1), ICAM-1, and VEGF, and promoted the proliferation, migration, and angiogenesis of HUVECs (Liang et al., 2019).

Diabetes induces vascular endothelial dysfunction, a critical and initiating factor in the development of diabetic vascular complications (Shi and Vanhoutte, 2017). The JAK/STAT signaling pathway is involved in the development of diabetic vascular complications (Banes-Berceli et al., 2007). FMN acted as an inhibitor of JAK, significantly reducing the phosphorylation and mRNA expression of JAK2 and STAT, as well as down-regulating the protein and mRNA levels of IL-1β and ICAM-1. Additionally, FMN restored NO synthesis and phenylephrine-induced contraction and acetylcholine-induced relaxation in the aortic tissues of rats on a high-glucose diet in a dose-dependent manner (Zhou et al., 2019). These findings suggested that FMN may be a new candidate natural metabolite for preventing and treating diabetic vascular complications.

### 3.3.3 Protect the heart

FMN was protective against both cardiac I/R injury and cardiac fibrosis. For cardiac I/R injury, FMN significantly attenuated cardiac dysfunction, infarct size, and release of cardiac markers, and inhibited the elevation of inflammatory factors TNF-α, IL-1β, and IL-6 in rats with cardiac I/R injury, primarily by inhibiting the activation of the ROS-TXNIP-NLRP3 pathway (Wang et al., 2020). Regarding cardiac fibrosis, FMN improved mitochondrial function by downregulating MOAB mRNA expression and upregulating the mRNA expression of aldehyde dehydrogenase 2 (ALDH2) and hydroxyacyl-CoA dehydrogenase (HADH). Additionally, it significantly inhibited isoproterenol-induced cardiac fibrosis and the expression of fibrosis-related proteins and genes in mice (Qian et al., 2024).

## 3.4 Joint diseases

### 3.4.1 Osteoporosis

Osteoporosis is a common clinical pathologic bone disease, which is the result of the imbalance between the functions of osteoblasts and osteoclasts and is most common in the elderly and postmenopausal women. Estrogen levels decline in postmenopausal women, leading to disruptions in the differentiation and activation of osteoblasts, reduced synthesis and deposition of new bone, and ultimately osteoporosis. FMN belonged to isoflavones and had estrogen-like effects. It can promote osteoblast differentiation by activating the p38 MAPK/Smad/bone morphogenetic protein (BMP) signaling pathway (Soundharrajan et al., 2019). In addition, enhanced osteoclast resorption is another important mechanism in the development of osteoporosis. Under the stimulation of inflammatory factors, mouse bone marrow-derived macrophages (BMMs) can differentiate into osteoclasts, increasing osteoclast bone phagocytosis capacity and leading to increased bone loss (de Villiers, 2023). The joint inflammatory response caused by CoCrMo particles produced by the implanted prosthetic biomaterials will also induce the formation, differentiation, and maturation of osteoclasts, and ultimately transform the homeostasis of bone metabolism into osteolysis (Yin et al., 2023). In response to this process, FMN could prevent this process by inhibiting the activation of the NF-κB/MAPK signaling pathway and inhibit osteolysis triggered by osteoclast over-resorption (Yu et al., 2023), making it a potential therapeutic agent for the prevention and treatment of periprosthetic osteolysis. It can be seen that FMN can improve osteoporosis disease by reducing the inflammatory response, inhibiting BMMs differentiation to osteoclasts, and reducing osteoclast resorption, which is instructive for the treatment of osteoporosis.

### 3.4.2 Osteoarthritis

Osteoarthritis (OA) is a degenerative joint disease characterized by cartilage degeneration and inflammatory responses (Abramoff and Caldera, 2020). Studies had shown that FMN reduces the expression of cartilage-degrading enzymes, such as MMP-1, MMP-3, and MMP-13, in primary rat chondrocytes, effectively counteracting IL-1β-induced catabolism and enhancing chondrocyte viability (Cho et al., 2019). Furthermore, FMN inhibited the inflammatory response by blocking the phosphorylation of related components in the NF-κB/MAPK signaling pathway. It also reduced the levels of cartilage catabolic markers, including MMP-3, MMP-13, and thrombospondin motifs 5 (ADAMTS5), in a concentration-dependent manner, while increasing the level of the cartilage-specific marker COL2A1 and improving bone and joint damage in OA mice (Ni et al., 2023). In addition to targeting chondrocyte degradation, FMN also reduced NO production and PGE2 expression, upregulated the levels of type II collagen and Aggrecan, and thus improved chondrocyte ECM synthesis and inhibited cartilage surface destruction and bone mineral formation in OA rats by modulating the PTEN/AKT/NF-κB signaling pathway, and this change was dose-dependent (Jia et al., 2022).

## 3.5 Skin diseases

### 3.5.1 Atopic dermatitis

Atopic dermatitis is a recurrent, chronic, non-infectious inflammatory skin disease characterized by persistent itching. It is the largest component of non-fatal diseases worldwide and mainly occurs in infants and children (Sroka-Tomaszewska and Trzeciak, 2021). Previous studies showed that FMN inhibited the expression of intracellular murine double minute 2 (MDM2), hypoxia-inducible factor 1 alpha (HIF-1α), and NF-κB, while reducing the levels of thymic stromal lymphopoietin (TSLP) and VEGF (Han et al., 2023). This results in decreased ear swelling and thickness in mice with acute ear edema, suggesting that FMN had potential as a treatment for inflammatory skin diseases. On this basis, Yuan et al. (2021) demonstrated that FMN could effectively alleviate AD symptoms *in vivo* and *in vitro* experiments. The underlying

mechanism appeared to involve the activation of the G protein-coupled estrogen receptor (GPER), which upregulated A20 protein and mRNA expression. This, in turn, affected the ubiquitination of signal transducer and activator of transcription 1 (STAT1) and STAT3, thereby inhibiting TSLP production. Thus, TSLP is identified as a key target of FMN in the treatment of AD.

### 3.5.2 Psoriasis

Psoriasis is a common chronic inflammatory skin disease, which is mainly characterized by distinctive erythematous plaques with white scales (Kamiya et al., 2019). Currently, mild psoriasis is mainly treated by topical therapy with steroids, vitamin D analogs, calcium-modulated phosphatase inhibitors, keratolytic agents, and phototherapy, while moderate and severe psoriasis is mainly treated by systemic therapy (Calabrese et al., 2023). Although these therapies are effective in the short term, they are associated with potential side effects and contraindications, limiting their suitability for long-term use. Therefore, there is an urgent need for affordable, safe, and effective treatments. *In vitro* experiments found that FMN could significantly inhibit the growth of HaCaT cells, and 20 μM of FMN could minimize the levels of TNF-α and IL-6, which had good anti-inflammatory activity (Xu et al., 2024). This result was confirmed *in vivo* experiments, and it was found that the interferon (IFN) signaling pathway was inhibited, which could effectively reduce the expression of related inflammatory chemokines, and significantly improve the erythema, scales and thickness of skin lesions in the psoriasis mice model.

## 3.6 Diabetes

Diabetes is a metabolic disease characterized by hyperglycemia and is mainly classified as Type I and Type II, of which Type 1 diabetes mellitus (T1DM) is caused by the destruction of pancreatic β-cells, resulting in an almost complete loss of, or a severe deficiency in, insulin secretion (American Diabetes Association, 2011). Pancreatic cell damage caused by oxidative stress stimulation is one of the key factors promoting the development of T1DM. Based on this, Chen et al. (2024) demonstrated through *in vivo* and *in vitro* experiments that FMN binds to Keap1, activating the Keap1/Nrf2 signaling pathway. This promoted the translocation of Nrf2 from the cytoplasm to the nucleus, enhanced the expression of antioxidant proteins HO-1 and NQO1, and prevented excessive ROS production in pancreatic cells. As a result, FMN effectively mitigated alloxan-induced pancreatic β-cell and DNA damage, lowered blood glucose levels, and increased insulin content.

## 3.7 Lipid metabolism

Non-alcoholic fatty liver disease (NAFLD) is one of the most common liver diseases worldwide, characterized by steatosis caused by the accumulation of triglycerides in hepatocytes (Sheka et al., 2020). Non-alcoholic steatohepatitis (NASH) is a progressive form of NAFLD, which can further develop into cirrhosis and hepatocellular carcinoma with the development of time. Liao et al. (2024) established the mice model of NASH using a methionine-choline-deficient diet. Following intervention with FMN, liver function and hepatocyte steatosis were significantly improved. This effect was attributed to FMN's ability to increase the expression and activity of SIRT1, promote the deacetylation of PGC-1α, and enhance the transcriptional activity of PPARα. These effects led to the upregulation of fatty acid oxidation (FAO) capacity, as well as increased levels of carnitine, acyl-CoA dehydrogenase medium-chain (ACADM), and carnitine palmitoyl transferase 1A (CPT1A), thus promoting liver fatty acid β-oxidation and regulating lipid metabolism. Finally, the steatosis of hepatocytes in NASH mice was improved. Furthermore, if it developed into NASH, FMN promoted the nuclear translocation of transcription factor EB (TFEB) by activating adenosine monophosphate-activated protein kinase (AMPK). This activation enhanced lysosome biogenesis and the fusion of autophagosomes with lysosomes, alleviating autophagic flux blockage and inducing lipid droplet degradation to prevent cellular lipid accumulation (Wang et al., 2019). In addition, FMN could act as a prebiotic to regulate intestinal microbial flora, thereby improving host metabolism and preventing obesity (Naudhani et al., 2021).

## 3.8 Antioxidation

Oxidative stress is a disorder of cellular function and environmental homeostasis caused by the accumulation of overproduction of ROS, which can trigger multiple cellular pathways and induce organismal damage (Tang et al., 2022). Nrf is known as the"main regulator"of the antioxidant response and is a key redox-sensitive transcription factor. When Nrf is activated, it will promote the expression of phase II detoxification enzymes and antioxidant enzymes, improve oxidative stress, promote cell survival, and maintain cellular redox homeostasis (Hybertson et al., 2011). Flap necrosis is a common postoperative complication, and hypoxia leads to secondary damage to the flap tissue. By increasing the expression of Nrf2, FMN could activate the expression of downstream target antioxidant/phase II detoxification enzymes to play a transcriptional function, thereby increasing the activity of SOD and GSH-Px. At the same time, the expression of VEGF was increased and angiogenesis was enhanced (Li et al., 2022). It could be seen that FMN exerted a strong antioxidant effect through the Nrf2-driven antioxidant defense system to restore flap tissue damage. In addition, FMN could also inhibit the sustained production of ROS through the Nrf2/HO-1 pathway, alleviate acute kidney injury due to oxidative stress and improve kidney function in rats (Aladaileh et al., 2019). The main pharmacological activity mechanism of FMN is shown in Table 2.

## 3.9 Anti-inflammation

FMN has good therapeutic effects against inflammatory diseases, such as rhinitis, mastitis, and keratitis. Huang et al. (2021) reported that FMN could inhibit IL-13-induced inflammation and mucus formation in JME/CF15 cells by activating the SIRT1/Nrf2 signaling pathway, suggesting that FMN may be a promising drug for the treatment of allergic

TABLE 2 The main pharmacological activity mechanism of FMN.

| Disease type | Cell/Animal models | Test concentration | Signal pathway | Molecular target | Ref. |
|---|---|---|---|---|---|
| AD | HBMECs, THP-1 | 2, 5, 7.5 and10 μM | Nrf2/HO-1 | ↑Nrf2; ↓NF-κB, ICAM-1, VCAM-1, eselectin | Fan et al. (2022) |
| | HFD mice | 20 and 40 mg/kg | PGC-1α/NF-κB | ↑PGC-1α,p-GSK-3β(Ser9), Nrf2, HO-1; ↓IL-1β, TNF-α, p-NF-κB, TC, TG, Tau | Fu et al. (2019) |
| Depression | depressive mice | 20 mg/kg | - | ↑GR, BDNF; ↓CORT | Zhang et al. (2022) |
| | RAW264.7, BV2; depression mice | 20 μM; 20 or 40 mg/kg | GSK-3β/ Notch1/EBPα | ↑BDNF, 5-HT, Bcl-2/Bax, IL-10, PPARγ, Arg1, Fizz1, Ym1; ↓GSK-3β, cleaved caspase-3, caspase-3, IL-6, IL-17A, iNOS, CXCL10, TNF-α, IL-1β, Notch1, C/EBPα | Yang et al. (2023a) |
| Anxiety | chronic inflammatory pain mice | 25 mg/kg | NMDA/CREB, NF-κB | ↓NMDA, AMPA, CBP, p-GluN2B-t1472, p-GluA1-s831, p-GluA1-s845, p-GluN2B-s1303, GluN2B, GluN2A, PSD95, GABAA α2, GABAA γ2, Iba-1 | Wang et al. (2019a) |
| Cerebral I/R injury | MCAO rats | 30 mg/kg | JAK2/STAT3 | ↓IL-18, TNF-α, IL-6, IL-1β, p-JAK2, p-STAT3, NLRP3, ASC, cl-Caspase-1, cl-IL-1β | Yu et al. (2022) |
| | MCAO rats | 30 mg/kg | PI3K/AKT/ERK | ↑β III-tubulin, GAP-43, NGF, BDNF, p-Trk A, p-Trk B, p-AKT, p-ERK 1/2 | Wu et al. (2020a) |
| | MCAO rats | 10 mg/kg | PARP-1/PARG/ Iduna | ↑*Iduna*, p-AKT; ↓PARP-1, PARG, Caspase-3, p53, AIF | Luo et al. (2024) |
| SCI | PC12 | 50, 100 and 200 μM | NF-κB/NLRP3 | ↑PCNA; ↓TNF-α, IL-1β, IL-6, p-p65 NF-κB, NLRP3, LDH | Zhou and Zhang (2023) |
| | Primary microglia, HMC3; SCI rats | 2.5, 5, and 10 nM; 20 and 40 mg/kg | EGFR/ p38 MAPK | ↓TNF-α, IL-6, p-EGFR, p-p38 | Fu et al. (2023) |
| OIPN | ND7/23, CT-26, Caco-2, DLD-1, HCT-116, PC9, A649, H1975, HCC8827, H520, BxPC3, Panc1, HUVECs, OIPN mice | 0.1, 1, 10 and 25 μM; 10 mg/kg | KEAP1-Nrf2-GSTP1 | ↑Nrf2, GSTP1; ↓ROS | Fang et al. (2020) |
| Diabetic perineuropathy | type 2 diabetic neuropathy rats | 10, 20 and 40 mg/kg | - | ↑SIRT1, NGF, GSH, SOD, catalase; ↓cholesterol, triglyceride, insulin, MDA, ROS | Oza and Kulkarni (2020) |
| | RSC96 | 5, 10 and 25 μM | SIRT3/PGC-1α/ SOD2 | ↑SIRT1, *Ho-1, Sod1, Sod2*, NGF, PGC-1α, *Tfam, Tfb2 m*; ↓MDA, ROS | Jiang et al. (2023) |
| PAH | PAH rats | 10, 30 and 60 mg/kg | PI3K/AKT/ERK | ↑Bax/Bcl-2, cleaved caspase-3; ↓IL-1β, IL-6, MDA, α-SMA, PCNA, p-AKT, p-ERK | Cai et al. (2019) |
| | PAH rats | 10, 30 and 60 mg/kg | ERK/NF-κB | ↓TGF-β1, MMP-2, MMP-9, p-ERK, p-NF-κB, TNF-α, IL-1β, MCP-1, type I collagen, type III collagen, FN | Wu et al. (2020b) |
| ALI | PMVECs; ALI mice | 10, 20, 60, 80 and 160 μM; 10 and 100 mg/kg | Nrf2/HO-1 | ↑Nrf2, HO-1, SOD, Arg1, CD163; ↓MDA, IL-1β, IL-6, MCP1, CXCL10 | Chen et al. (2021) |
| COPD | BEAS-2B; COPD mice | 50 μM; 50 mg/kg | AhR/CYP1A1, AKT/mTOR | ↑Bcl-2, CYP1A1; ↓TNF-α, CXCL1, IL-10, IL-1β, IL-8, CCL22, Bax, cleaved caspase-3, GRP78, CHOP, ATF6, p-ERK, p-EIF2α, p-AKT, p-mTOR | Li et al. (2024a) |
| AIH | AIH mice | 50 and 100 mg/kg | NLRP3 | ↑Bcl-2; ↓Bax, cleaved caspase 9, cleaved caspase 3, TNF-α, IL-6, IL-1β, p-NF-κB p65, IκBα, NLRP3, ALT, AST | Liu et al. (2021a) |
| (I/R-) induced liver injury | I/R-induced liver injury rats | 30, 60, and 90 mg/kg | PHB2/PINK1/ Parkin | ↑PHB2, COX2, COX4, GSH, CAT, GSH-Px, MMP, Parkin, PINK1, LC3 II, P62, Beclin1, ATP; ↓PARL, PGAM5, AST, ALT, TNF-α, IL-1β, ROS, MDA | Ma et al. (2022) |

(Continued on following page)

TABLE 2 (*Continued*) The main pharmacological activity mechanism of FMN.

| Disease type | Cell/Animal models | Test concentration | Signal pathway | Molecular target | Ref. |
|---|---|---|---|---|---|
| Cholestasis | HepG2; acute and chronic hepatic cholestasis mice | 10, 20 and 50 mg/kg | SIRT1-FXR | ↑SIRT1, BSEP, MRP2, ACOX1, OATP1, OATP4, NTCP, PPARα, CYP7A1, CYP27A1, CYP8B1, CYP7B1; ↓ALT, AST, ALP, γ-GT, TBIL, IL-6, TNF-α, IL-1β, OSTα, OSTβ, MRP3, MRP4, p-JNK | Yang et al. (2019a) |
| CKD | pTECs, CKD rats | 20, 40 and 80 μM; 40 mg/kg | Smad3/ATF3/ SLC7A11 | ↑SLC7A11, GSH, Nrf2, GPX4; ↓α-SMA, Col1a1, 4-HNE, KIM-1, MDA, p-Smad3, ATF3, FN | Zhu et al. (2023) |
| AKI | AKI rats | 60 mg/kg | Nrf2/HO-1 | ↑GSH, SOD, CAT, Nrf2, HO-1, Bcl-2; ↓MDA, NF-κB p65, IL-1β, IL-6, TNF-α, Bax, caspase-3, creatinine, urea, protein carbonyl | Althunibat et al. (2022) |
| | AKI rats | 10, 20 and 40 mg/kg | Nrf2/HO-1 | ↑GSH, SOD, GSH, ATP, CAT, Nrf2, HO-1, Bcl-2; ↓Kim-1, MDA, NF-κB p65, IL-1β, IL-6, TNF-α, Bax, caspase-3, creatinine, urea, protein carbonyl | Aladaileh et al. (2019) |
| | HK-2, AKI rats | 10 and 25 μM; 75 mg/kg | PPARα/Nrf2/ HO-1/NQO1 | ↑PPARα, Nrf2, HO-1, NQO1, CAT; ↓BUN, creatinine, MDA, MPO, TNF-α, IL-1β | Hao et al. (2021) |
| DN | GMCs, DN mice | 5, 10 and 20 μM; 25 and 50 mg/kg | Nrf2/ARE | ↑Sirt1, HO-1, SOD-1, Nrf2; ↓FN, ICAM-1, Keap1, ROS, FBG, TG, TC, Cr, BUN | Zhuang et al. (2020) |
| | HK-2, DN rats | 10 and 20 μM; 20 mg/kg | Sirt1/PGC-1α | ↑Sirt1, Bcl-2, Mfn2, PGC-1α; ↓Bax, cleaved-caspase-3, Drp1, Fis1, ROS | Huang et al. (2022) |
| | DN mice | 25 and 50 mg/kg | - | ↑ISI, SOD, GSH-Px, CAT; ↓FBG, FINS, IRI, TG, TC, Ucr, BUN, Scr, ACR, MDA, COL-III, α-SMA, COl1a1, COl3a1, smad3 | Lv et al. (2020) |
| gastric ulcer | gastric ulcer rats | 25, 50 and 50 mg/kg | NF-κB | ↑VEGF, NO, CD34, ZO-1, p-IκBα/IκBα, occludin; ↓TNF-α, IL-1β, IL-6, MPO, ET-1, p-P65/P65 | Yi et al. (2022) |
| AS | HUVECs | 40 μM | PPAR-γ | ↑SOD, eNOS, PPAR-γ; ↓TNF-α, IL-1β, COX-2, MDA, cleaved caspase-3, ROS | Zhang et al. (2021) |
| | AS mice | 15, 30 and 60 mg/kg | JAK/STAT | ↑CD206, IL-10, SHIP1, Arg-1, α7nAChR; ↓CD68, iNOS, COX-2, miR155-5p, IL-6, IL-1β, p-JAK2, p-STAT3, LDL-C, ox-LDL, TC, TG | He et al. (2024) |
| | AS mice | 10 mg/kg | - | ↑VSMCs, α-SMA, KLF4, Arg1; ↓SRA, CD68, VCAM-1, ICAM-1, PECAM-1, iNOS, ROS, TNF-α, IL-1β, IL-6 | Ma et al. (2020) |
| Vascular injury | DVT rats | 10, 20 and 40 mg/kg | - | ↑p-eNOS; ↓IL-1β, IL-18, D-dimer, F1+2, TF, TM, Iκκβ, p-NF-κB p65/NF-κB p65 | Zhou et al. (2022) |
| | HUVECs; dermal wound healing mice | 10, 20 and 40 μM | Erk1/2/Akt | ↑p-eNOS, NO, p-ERK1/2, p-AKT | Wu et al. (2020c) |
| | HUVECs; high-glucose diet rats | 2, 20 and 200 μM; 4, 40 and 400 mg/kg | JAK/STAT | ↑NO; ↓p-JAK2, p-STAT3, caspase-3, IL-6, IL-1β, ICAM-1 | Zhou et al. (2019a) |
| I/R cardiac injury | NRCMs, MIRI rats | 1 and 10 μM; 10 and 30 mg/kg | ROS-TXNIP-NLRP3 | ↑Bcl-2/Bax; ↓TNF-a, IL-1β, IL-6, ROS, NLRP3, LDH, AST, cTnT, INF/AAR, ROS, cleaved caspase-1, IL-1β, cleaved GSDMD, TXNIP | Wang et al. (2020a) |
| cardiac fibrosis | cardiac fibrosis mice | 20 and 40 mg/kg | - | ↑ALDH2, HADH; ↓TGF-β1, type I collagen, ROS, MOAB, α-SMA, vimentin, Col1a1, Col3a1, Acta2, Tgfb1 | Qian et al. (2024) |
| Osteoporosis | C2C12 | 1.25 and 2.5 μM | p38MAPK/ Smad/BMP | ↑myogenin, myosin heavy chains, MyoD, BMP-2, BMP-7, BMP-4, ALP, RUNX2, OCN, BMPs, p38MAPK, Smad1/5/8; ↓JAK1-STAT1 | Soundharrajan et al. (2019) |

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TABLE 2 (*Continued*) The main pharmacological activity mechanism of FMN.

| Disease type | Cell/Animal models | Test concentration | Signal pathway | Molecular target | Ref. |
|---|---|---|---|---|---|
| | BMMS; C57BL/6 J mice | 10 μM; 10 mg/kg | NF-κB/MAPK | ↓Nfatc1, c-Fos, Ctsk, TRAP, F-actin rings, NF-κB p65, IκBα | Yu et al. (2023) |
| OA | Primary Rat Chondrocytes | 25 and 50 μM | - | ↓MMP-13, MMP-1, MMP-3, NO, iNOS, COX-2, PGE2, CINC-2, CINC-3, fractalkine, GM-CSF, IL-1α, IL-1β, IL-4, IL-6, IL-10, LIX, MCP-1, MIP-3α, β-NGF, TIMP-1, TNF-α, VEGF | Cho et al. (2019) |
| | BMMs, MCPCs; C57BL/6J mice | 5, 10, 20, and 40 μM | NF-κB/MAPK | ↓NFATc1, c-fos, DC stamp, TRAP, ctsk, IkBα, p65, p-ERK, p-JNK, MMP-3, MMP-13, adamts5 | Ni et al. (2023) |
| | first-passage chondrocytes, OA rats | 25, 50 and 100 μM; 10 mg/kg | PTEN/AKT/ NF-κB | ↑type II collagen, ECM, Aggrecan; ↓NO, PGE2, COL-II, TNF-α, IL-6, iNOS, COX-2, ADAMTS5, MMP-3, MMP-13, IκBα, p65, p-AKT | Jia et al. (2022) |
| Atopic dermatitis | HaCaT; Atopic dermatitis mice | 0.1, 1 and 10 μM; 10 mg/kg | - | ↑A20, GPER; ↓TSLP | Yuan et al. (2021) |
| Psoriasis | HaCaT, psoriasis mice | 10, 20 and 40 uM; 60 mg 2% FMN | IFN | ↓IL-6, IL-17, IFN-β, IFN-γ, TNF-α, pSTAT1, p-STAT3, CD3, IRF1, Cxcl9, Cxcl10, Cxcl11, Cxcr3 | Xu et al. (2024) |
| T1DM | MIN6; T1DM mice | 3.125, 6.25 and 12.5 μM; 2.5, 5 and 10 mg/kg | Keap1/Nrf2 | ↑Nrf2, HO-1, NQO1; ↓ROS, Keap1, p-H2A | Chen et al. (2024) |
| NASH | L02; NASH mice | 20, 40 and 80 μM; 25, 50 and 100 mg/kg | SIRT1/PGC-1α/ PPARα | ↑FAO, carnitine, ACADM, SIRT1, CPT1A; ↓IL-1β, Il-6, TNF-α, PGC-1α, TG, ALT, AST | Liao et al. (2024) |
| NAFLD | HepG2; NAFLD mice | 10 and 20 μM; 100 mg/kg | TFEB | ↑LC3B, LAMP1, ATP6V1A, AMPK, Beclin1, S6K1, PGC1α, LC3B-II, p62, TFEB, PPARα, CPT1α; ↓TG, TC, LDL-C, ALT, AST | Wang et al. (2019b) |
| Obesity | WSD mice | 20, 60 and 100 mg/kg | - | ↑occludin; ↓glucose, insulin, IL-6, IL-22, TNF-α, Muc-2, HDL, LDL | Naudhani et al. (2021) |
| Flap necrosis | Flap necrosis mice | 25 and 50 mg/kg | PI3K/Akt/Nrf2 | ↑HO-1, NQO1, GCLc, GCLm, TrxR, SOD, GSH-Px, VEGF, Nrf2; ↓Keap1, TNF-α, IL-1β | Li et al. (2022b) |

rhinitis. Xiang et al. (2022) found that FMN could protect the breast by inhibiting the inflammatory response and enhancing the integrity of the blood-milk barrier. Specifically, FMN significantly reduced the production of inflammatory factors TNF-α and IL-1β by inhibiting the NF-kB signaling pathway and concentration-dependently increased the expression of AhR and inhibited the phosphorylation of Src, thus exerting an anti-mastitis effect. Additionally, FMN increased the expression of mammary epithelial tight junction proteins (claudin-3, occludin, and ZO-1), which enhanced the integrity of the blood-milk barrier and provided protection to the mammary gland. In the model of fungal keratitis induced by Aspergillus fumigatus in mice, researchers observed that TSLP and TSLPR were overexpressed in epithelial cells and infiltrating immune cells. After treatment with FMN, the expression of TSLP, TSLPR, and inflammatory factors was reduced, ROS production decreased, and the migration of macrophages and neutrophils was inhibited, alleviating the cornea's inflammatory response (Feng et al., 2024). In addition, FMN enhanced the migration of HCECs from human corneal cells and promoted corneal epithelial repair. Thus, it could be seen that FMN had the potential to be an inhibitor of TSLP to treat fungal keratitis.

## 3.10 Antitumor

### 3.10.1 Inhibition of tumor cell proliferation

#### 3.10.1.1 COX-2/cyclin

COX-2 is expressed in many types of tumor cells, which regulates cell growth and participates in tumorigenesis and carcinogenesis through mitosis (Cheng et al., 2012). There is increasing evidence suggesting that COX-2 could serve as a potential target to reduce cancer risk (Hashemi Goradel et al., 2019). The continuous program of mitosis is known as the cell cycle, which consists of the G0, G1, S, G2, and M phases. The G1 phase is an important period that affects cell division and development. It is mediated by cyclin-dependent kinases (CDKs) and regulatory cyclin subunits, which determine whether cells further self-renew and develop (Massagué, 2004). Therefore, the G1 phase of the cell cycle can be used as a key target to control the proliferation of tumor cells. Chen et al. (2023) demonstrated that FMN effectively inhibited the proliferation of KYSE170 and KYSE150 cells by significantly reducing the mRNA and protein expression levels of COX-2 and cyclin D1, while inducing G1 phase arrest. The incidence of esophageal cancer in mice treated with FMN

was significantly reduced at 18 weeks (0/15 vs. 2/15) and 24 weeks (6/15 vs. 13/15). In addition, FMN also exhibited a strong anti-proliferative effect on MCF-7 and MDA-MB-468 cells, where it significantly increased the proportion of G0/G1 phase cells after treatment (Liu et al., 2024).

#### 3.10.1.2 EGFR

EGFR is a tyrosine kinase receptor that is often overexpressed or mutated in patients with non-small cell lung cancer (NSCLC), leading to uncontrolled cell proliferation and tumor formation (Riely et al., 2024). Tyrosine kinase inhibitors, such as gefitinib, erlotinib and osimertinib, have become the first-line treatment for patients with EGFR activation mutations. However, its clinical application is limited due to drug resistance. FMN was found to have the potential to become a new EGFR inhibitor and show a significant inhibitory effect on both osimertinib-sensitive and resistant non-small cell lung cancer cells, which could greatly reduce the generation of drug resistance (Yu et al., 2020). Further *in vitro* and *in vivo* studies demonstrated that FMN binds to both WT and mutant EGFR, reducing EGFR kinase activity and inhibiting downstream signaling. This, in turn, activated GSK-3β and decreased the expression of myeloid leukemia sequence 1 (Mcl-1), without causing significant toxicity to the vital organs of mice. These findings highlight the importance of developing targeted therapies for the inhibition of tumor proliferation.

### 3.10.2 Anti-tumor angiogenesis

HIF-1 is a heterodimeric protein consisting of HIF-1α protein and HIF-1β protein, which can activate the expression of target genes involved in angiogenesis, such as VEGF (Semenza, 2003). During cancer cell proliferation, tumor cell growth is promoted by increasing VEGF expression, enhancing vascular permeability, and stimulating tumor angiogenesis (Ahmad and Nawaz, 2022). Therefore, VEGF can be used as a target to inhibit tumor proliferation. Zhang et al. (2019) established Balb/c nude mice model of cervical cancer by inoculating HeLa cells and gavaged FMN every 7 days for a month. The results showed that during the drug intervention, the mice in the FMN group had no obvious adverse reactions and were in good condition, while the mice in the cisplatin group had poor appetite, listlessness, and reduced activity. The tumor inhibition rate in the FMN group was comparable to that in the cisplatin group, and the tumor inhibition rate was 50.17%. Further analysis using RT-qPCR and western blotting showed that the expression levels of HIF-1α, VEGF mRNA, and protein in the cervical cancer tissues of mice in the FMN group were significantly lower than those in the positive control group. It could be seen that FMN inhibited the proliferation and growth of cervical cancer cells by inhibiting the expression of HIF-1-α and VEGF. In addition, FMN also had a significant inhibitory effect on the proliferation of bladder cancer cells. Zhou H. et al. (2023) used two bladder cancer cell lines, TM4 and 5,637, to explore the mechanism of action of FMN in the treatment of bladder cancer through transcriptomics. After 24, 48, and 72 h of FMN treatment, the growth of bladder cancer cell lines T24 and 5,637 was inhibited in a dose-dependent manner, without affecting the viability of non-tumor cells. Further analysis revealed that the above phenomenon was attributed to the fact that FMN mediated VEGF expression through the regulation of fibroblast growth factor receptor (FGFR) and its receptor, which in turn impaired tumor angiogenesis and inhibited cancer cell proliferation.

### 3.10.3 Inhibition of tumor migration invasion

#### 3.10.3.1 ERK

The ERK pathway is a classical and important pathway in the MAPK signaling pathway, which transmits extracellular signals to the nucleus and regulates the expression of related genes, and is highly expressed in aggressive cancer cells (Krueger et al., 2001; Guo et al., 2022), such as nasopharyngeal carcinoma. In addition to the compounds clinically used for the treatment of nasopharyngeal carcinoma, FMN is also considered to be a therapeutically promising compound, which can achieve antiproliferative and invasive effects through effective inhibition of the oncogenic ERK1/2 pathway and the Lamin A/C signaling pathway, thereby modulating the nasopharyngeal carcinoma tumor microenvironment and inhibiting the migration and invasion of cancer cells (Ying et al., 2019).

### 3.10.4 Inducing tumor cell apoptosis

Apoptosis is a form of programmed cell death that plays a crucial role in normal embryonic development, maintenance of homeostasis in adult tissues, and suppression of carcinogenesis (Willis et al., 2003). Mitochondrial function is closely related to cancer development, and mitochondrial dysfunction, induced by aberrant oncogenic and tumor suppressor signals, alters cellular metabolic pathways, disrupts redox homeostasis, and contributes to resistance to apoptosis (Luo et al., 2020). The Bcl-2 family of proteins, mainly located in the outer membrane of the mitochondrion, is a key factor in regulating cellular death and an upstream regulator of the caspases cascade, which can be disrupted by disrupting the inner membrane of the mitochondrion. It can effectively promote the release of pro-regulatory factors from mitochondria by disrupting the inner mitochondrial membrane and drive apoptosis (Bruckheimer et al., 1998). FMN, as a candidate anticancer drug, could release cytochrome C (cyto C) directly through the mitochondrial pathway and activate the cascade reaction of caspase-9, caspase-3 and PARP, which ultimately lead to FaDu cell death (Oh et al., 2020). In addition, both *in vivo* and *in vitro* experiments demonstrated that FMN upregulated the levels of LDH, Bax, Apaf-1, and caspase-3 in a dose-dependent manner, and downregulated the levels of ERα, p-PI3KCA$^{Tyr317}$, p-AKT$^{Ser473}$ (Hu et al., 2019). Based on these findings, we concluded that FMN induced osteosarcoma cell death via a mitochondria-dependent signaling pathway.

Mitochondrial autophagy is a critical cellular process and a key regulatory mechanism for the removal of damaged mitochondria, essential for maintaining cellular homeostasis (Qiu et al., 2021). In addition, more and more data suggest that mitochondrial autophagy plays an important role in the occurrence, development, and treatment of many tumors. Li et al. (2023) collected clinical samples from triple negative breast cancer (TNBC) patients and explored the effect of FMN on TNBC. The results demonstrated that FMN inhibited mitosis by inactivating the BACH1/p53 signaling pathway, promoted the release of cyto C and cysteine asparaginase, and mediated mitochondrial autophagy, resulting in mitochondrial dysfunction. In this process, FMN delivered ROS to mitochondria to release cyto C and activated caspase-3 and caspase-9 cascade reactions to induce apoptosis in MCF7 cells. These findings

suggest that FMN may exert its anti-tumor function by hindering mitochondrial function in breast cancer cells.

### 3.10.5 Regulation of microRNAs

MicroRNAs are small non-coding regulatory RNAs ranging from 17 to 25 nucleotides. In the past few years, research on microRNA-based therapies for cancer has begun to emerge. The expression of microRNAs can reflect either carcinogenic or tumor-suppressive effects, making them useful for tumor diagnosis and prognosis (Lee and Dutta, 2009). TNBC is the most refractory breast cancer type and the second leading cause of cancer-related deaths worldwide (Li S. et al., 2022). Currently, therapeutic options for TNBC are limited to surgery and chemotherapy. However, because it is a highly heterogeneous cancer with specific mutational signaling, targeted therapy is a promising option for the treatment of TNBC. FMN demonstrated targeted potential in the treatment of TNBC. By up-regulating the expression of miR-195 and miR-545, it could inhibit the expression of CDK4 and Raf-1 in the development of TNBC, thereby inhibiting the proliferation, migration and invasion of MDA-MB-231 and BT-549 cells (Wu et al., 2021). However, the experiment has not been validated by *in vivo* experiments, so it lacks the comprehensiveness and reliability of the experimental results, and the follow-up needs to be supplemented with *in vivo* experiments to verify the actual effect and safety in organisms. Li et al. (2020) found that FMN could inhibit the viability of osteosarcoma MG-63 cells and induce apoptosis. It was found by dual luciferase assay, western blot and qRT-PCR that FMN targetedly increased the expression of PTEN by reducing the expression of miR-214-3p. In addition, FMN also showed anti-gastric cancer activity (Wang and Zhao, 2021). *In vitro* experiments, FMN inhibited the proliferation of gastric cancer cells SGC-7901 and MGC-803 in a dose-dependent manner and significantly reduced cell colony formation, which was related to the decreased expression of miR-542-5p. The anti-gastric cancer potential of FMN *in vivo* was confirmed using an SGC-7901 xenograft model. Compared with the control group, the xenograft tumor volume of the FMN group was smaller, the tumor weight was lighter, and the expression of miR-542-5p was decreased. The results were consistent with *in vitro* experiments. In summary, natural metabolites combined with molecular targets have great prospects in treating cancer.

### 3.10.6 Immune checkpoint inhibitor therapy

Programmed death 1 (PD-1) is a protein expressed on T-cells. When it binds to the PD-L1 receptor on immune cells, it disrupts the effector function of lymphocytes and inhibits T-cell proliferation, thereby facilitating tumor escape from immune system attack (Safi et al., 2021). Liu et al. (2024), Wang et al. (2022) found that FMN could inhibit the occurrence of breast cancer and cervical cancer by interfering with the activation of PD-L1, but their mechanisms were different. For breast cancer, FMN mainly interfered with PD-L1 activation by inhibiting the STING-NF-κB signaling pathway. For cervical cancer, FMN mainly inhibited MYC protein expression and STAT3 activation through RAS/ERK and JAK1/STAT3 signaling pathways to inhibit PD-L1 synthesis. At the same time, it also promoted lysosomal biogenesis and induced PD-L1 degradation. Consequently, FMN could block the binding of PD-1 to PD-L1, and restore T cell activity, thereby enhancing the ability of the immune system to attack tumors. These findings suggest that FMN has the potential to serve as a PD-1/PD-L1 inhibitor for clinical use. The anti-tumor mechanism of FMN is shown in Figure 4.

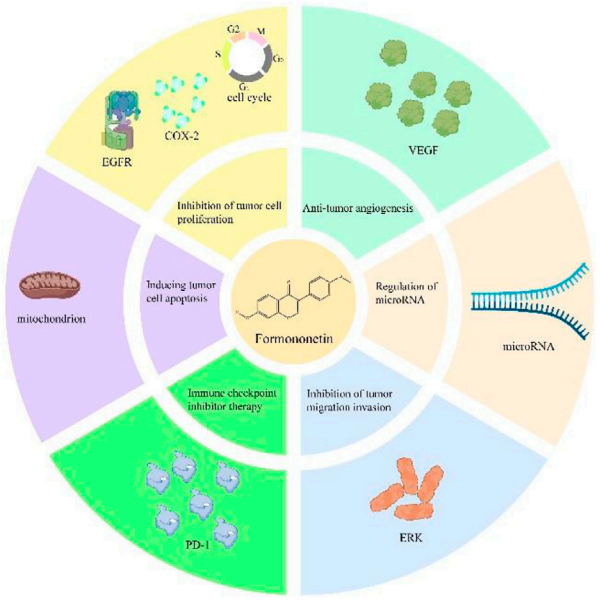



FIGURE 4
Anti-cancer mechanism of FMN.

## 3.11 Others

FMN has been reported to possess other pharmacological activities, such as antibacterial, antiviral, and antiallergic. Feng et al. (2024) found that FMN inhibited the growth of aspergillus fumigatus at concentrations ranging from 8–512 μg/mL, and impeded the formation of biofilm at a concentration range of 256 μg/mL. In addition, FMN also showed good inhibitory effects against *streptococcus* suis (Wang et al., 2020). Cui et al. (2021) reported that FMN had anti-cat calicivirus effect. When combined with icariin, it could play a synergistic effect. Zhou et al. (2023) suggested that FMN may be a novel anti-allergic drug with great potential to alleviate pseudoallergic reactions. In models of C48/80-stimulated BMMCs and RBL-2H3 cells, FMN inhibited immunoglobulin E-independent degranulation of dependent mast cells by reducing the release of β-hexose and histamine and the expression of inflammatory factors. Moreover, in the 2,4-dinitrobenzene-induced AD mice model, FMN reduced the local allergic reaction of AD model mice by increasing the expression of filaggrin and loricrine. Liu et al. (2021) used CKD rats and TNF-α-induced C2C12 myotubes to establish *in vivo* and *in vitro* muscle atrophy models, and found that FMN improved muscle atrophy caused by chronic renal dysfunction. This was related to FMN increasing the phosphorylation of PI3K/Akt/FoxO3 a pathway and the level of myogenic differentiation factor D (MyoD) and myogenin, thereby improving the proliferation and differentiation dysfunction of satellite cells. In the mice model of caerulein-induced pancreatitis, the researchers used FMN for continuous intragastric administration for 7 days. The results showed that FMN

TABLE 3 The mechanism of combination therapy.

| FMN concentration | Combined drugs and their concentration | Treatment outcome | Cell/Animal models | Signal pathway | Molecular target | Ref. |
|---|---|---|---|---|---|---|
| 200 μM | 20 μM cisplatin | Reduce toxicity | Cochlear hair cell | PI3K/AKT/Nrf2 | ↑Bcl-2, Nrf2, Gclc, Gpx2, Txnrd1, HO-1, p-PI3K/PI3K, p-AKT/AKT; ↓MDA, ROS, GSSG, Bax, c-caspase-3/caspase-3 | Li et al. (2024c) |
| 40 μM; 18 mg/kg | 160 μM Calycosin, 125 μM TMZ; TMZ: 30 mg/kg, Calycosin: 40 mg/kg | Synergistic effect; Increase the sensitivity of TMZ | C6; C6 xenograft mice | - | ↑Bax, Cleaved Caspase-3, Cleaved Caspase-9, GFAP; ↓Bcl-2, MMP-2, MMP-9 | Ni et al. (2019) |
| 40 μM; 12.6 mg/kg | 160 μM Calycosin, 125 μM TMZ; TMZ: 21 mg/kg, Calycosin: 28 mg/kg | Synergistic effect; Increase the sensitivity of TMZ | C6; C6 xenograft mice | - | ↓NOS2, TNF-α, GFAP, ribitol, glutamic acid, aspartic acid, phosphonic acid, serine, ribose, methionine | Li et al. (2022d) |
| 30 μM | 20 μM Sulforaphane | Synergistic effect; PI3K/AKT/mTOR inhibitors | HeLa | PI3K/AKT/mTOR | ↑ROS; ↓cyclin-D1, PCNA, CDK6, p-PI3K, p-AKT, p-mTOR | Jiang et al. (2024) |
| - | Cantharidin, Isofraxidin | Synergistic effect | Hep 3B2.1–7, Li-7; zebrafish | EGFR/PI3K/AKT | ↓EGFR, p-PI3K/PI3K, p-AKT/AKT, BIRC5, FEN1, EGFR | Lu et al. (2024) |
| 150 μmol/L; 50 mg/kg | 100 nmol/L everolimus; everolimus: 2 mg/kg | Synergistic effect | MDA-MB-468; MDA-MB-468 xenograft mice | mTOR | ↑PTEN, p-4EBP-1; ↓p-mTOR, p-P70S6K, p-Akt | Zhou et al. (2019b) |
| 40 μM or 80 μM | 150 μM MET | Synergistic effect | MCF-7 | ERK1/2 | ↓Bcl-2, p-ERK1/2 | Xin et al. (2019) |

significantly improved pancreatic edema in mice by activating the Keap1/Nrf2 signaling pathway and reducing the levels of ROS, NLRP3 and inflammatory factors (Yang et al., 2023). In addition, FMN could also upregulate the expression of colonic tight junction proteins, restore intestinal mucosal barrier function, improve the intestinal flora environment, and provide a new solution for the treatment of pancreatitis. Park et al. (2023) believed that FMN could be used as a non-hormone treatment option for endometriosis. Through *in vivo* and *in vitro* experiments, FMN was found to achieve therapeutic effects on endometriosis by decreasing the expression of p27, p-STAT3, and p-ERK within the endometrium.

# 4 Drug combination

For many cancers, the combined treatment of drugs is superior to the effect of single treatment. This is due to the uniqueness of each drug treatment, when combined, synergistic effects can be exerted (Plana et al., 2022). The mechanisms of combination therapy for FMN are shown in Table 3.

## 4.1 Combination with chemotherapeutic agents

Cisplatin is a common chemotherapeutic drug in the clinic, and ototoxicity is an unavoidable side effect during its use, which can damage the inner ear and impair both hearing and balance functions in patients (Wang et al., 2023). Currently, no effective treatment exists to repair the hair cell damage induced by cisplatin. Based on this, Li et al. (2024) used cisplatin to culture cochlear hair cells *in vitro* and treated them with FMN, and found that FMN effectively reduced the accumulation of ROS and mitochondrial damage in hair cells by activating the PI3K/AKT-Nrf2 signaling pathway, restored the balance of GSH/GSSG. Therefore, FMN was a potential therapeutic agent for cisplatin-induced ototoxicity.

Glioma is a common malignant tumor of the brain, and temozolomide (TMZ) is currently the first-line drug used in clinical practice for postoperative or non-surgical chemotherapy of gliomas, which can easily penetrate the lesion site through the blood-brain barrier, but the acquired and intrinsic resistance of TMZ limits its application (Xu et al., 2020). In addition to using FMN alone, the combination of FMN and mullein isoflavones may enhance the antitumor effects of TMZ. *In vivo* and *in vitro* experiments showed that the IC50 of TMZ was significantly reduced from 1000 μM to 125 μM when the low effective concentration of calycosin (160 μM) and FMN (40 μM) were combined with TMZ. The combination of the three greatly improved the sensitivity of TMZ, significantly inhibited the growth and migration of C6 cells, and the toxicity of the drugs to the heart, liver, spleen, lung and kidney of mice was small (Ni et al., 2019). In addition, based on metabolomics and molecular biology studies, nitric oxide synthase 2 (NOS2) was a key target for FMN and calycosin to regulate TMZ in the treatment of glioma. FMN and calycosin reduced the secretion of TNF-α in the tumor area by inhibiting the expression of NOS2, and regulated amino acid metabolism, especially glutamic acid, ornithine, aspartic acid, proline and arginine, thereby increasing the cytotoxicity of TMZ on malignant glioma, inhibiting tumor growth and infiltration in C6 glioma rats, and reducing cell aggregation and GFAP expression in C6 glioma area (Li et al., 2022). The above studies showed that cancer cells acquire drug resistance because multi-gene abnormalities in tumor cells allow them to evade the effects of

these drugs when patients receive treatment for a single pathway. Therefore, combination therapy had better control effects on multiple targets and a lower risk of drug resistance, which had great application prospects for treating cancer.

## 4.2 Combination with natural medicines

The PI3K/Akt/mTOR signaling pathway plays a critical role in the apoptosis and proliferation of various cancer cells. The activation of Akt/mTOR can initiate a signaling cascade that regulates apoptosis, survival, growth and proliferation, thus promoting tumorigenesis (Li et al., 2017). The combination of FMN (30 μM) and sulforaphane (20 μM) exhibited a significant synergistic effect (CI = 0.57), and inhibited the expression of cyclin-D1, PCNA and CDK6 in HeLa cells by down-regulating the PI3K/Akt/mTOR signaling pathway, reducing the proportion of cells in G0/G1 phase, hindering the entry of cells into S phase, and leading to the death of HeLa cells (Jiang et al., 2024). In addition, the combination of the two could promote the generation of ROS in HeLa cells, leading to oxidative damage, which was also one of the causes of HeLa cell death. It could be seen that the combination of FMN and sulforaphane could be used as an inhibitor of PI3K/AKT/mTOR, which had important clinical significance in the treatment of cervical cancer. Aidi injection is a widely used Chinese patent medicine for the treatment of hepatocellular carcinoma. Lu et al. (2024) used Hep3B2.1-7, Li-7 cells and zebrafish to explore the anti-hepatocarcinoma mechanism of Aidi injection. The results showed that the strong anti-hepatocarcinoma effect of Aidi injection was the result of the combined treatment of cantharidin, FMN and isofraxidin. Meta-analysis showed that BIRC5, FEN1 and EGFR could be the key targets of Aidi injection against hepatocellular carcinoma, and the three chemical components exerted synergistic inhibitory effects through their targets, respectively, which revealed the mechanism of anti-hepatocellular carcinoma of Aidi injection, and also provided a typical example for the pharmacological evaluation of other proprietary Chinese medicines.

## 4.3 Combination with targeted drugs

In another report, FMN could enhance the anti-tumor effect of everolimus on MDA-MB-468 cells, increasing the apoptosis rate by 27.9%. There was no significant loss of appetite in mice with the MDA-MB-468 cell xenograft model, and the tumor volume was reduced to half (Zhou et al., 2019). It could be seen that FMN combined with everolimus can safely inhibit tumor growth. RT-qPCR and western blot analysis revealed that both FMN and everolimus inhibited the expression of p-mTOR and p-P70s6k and increased the expression of PTEN and p-4EBP-1. However, FMN alone inhibited p-Akt expression, whereas everolimus had no inhibitory effect on this. When Akt siRNA was used to silence Akt, everolimus did not affect the expression of mTOR, and FMN still reversed the expression of p-4EBP-1 and p-P70s6k. It could be seen that the synergistic effect of FMN on everolimus plays a role by inhibiting Akt activity and inhibiting the mTOR signaling pathway. In addition, Xin et al. (2019) also found that FMN-assisted metformin (MET) in the treatment of breast cancer, which had great application prospects. The combination of 40 μM FMN and 150 μM MET or the combination of 80 μM FMN and 150 μM MET could reduce the proliferation rate of MCF-7 cells. Compared with the monotherapy, the levels of Bcl-2 and p-ERK1/2 were greatly reduced, and the apoptosis rate was increased.

# 5 Toxicity of FMN

FMN had been reported to be cytotoxic to human-immortalized epidermal cells HaCaT with an IC50 of 40.64 μM (Xu et al., 2024). Yu et al. (2023) treated BMMs with FMN at concentrations up to 20 μM without any significant cytotoxicity. However, Ni et al. (2023) further found that FMN had a greater effect on the viability of BMMs when the concentration reached 40 μM or higher, with an IC50 of 29.61 μM. Cho et al. (2019) evaluated the effect of FMN on the viability of rat primary chondrocytes using the MTT assay, and the results showed that, even at a concentration of 100 μM, FMN did not affect rat primary chondrocyte viability. In addition, FMN had no toxic effect on the growth of normal ovarian cell lines (Zhang et al., 2018). In another report, Pingale and Gupta (2023) carried out acute toxicity tests and subacute toxicity tests of FMN by intraperitoneal injection in mice. The results showed that the acute dose of FMN was 300 mg/kg, the LD50 was 103.6 mg/kg, and the NOAEL was 50 mg/kg. During the subacute toxicity experiment, the body weight, food intake, water intake and behavior of the animals did not change, and the organs did not have any toxic effects and pathological damage. Therefore, it was proved that FMN was safe and non-toxic and could be used for pharmacological and therapeutic purposes. In summary, FMN may be a relatively safe natural compound component. However, there is still a lack of subchronic toxicity, genotoxicity and reproductive toxicity experiments, and the follow-up should be expanded to carry out clinical trials of FMN.

# 6 Derivatives

FMN has low water solubility, which limits its application in the pharmaceutical industry. To solve the water solubility problem of FMN, Zhao et al. (2021) succinylated FMN using *Bacillus* amyloliquefaciens FJ18 to form the compound FMN-7-O-β-D (6″-O-succinyl)-D-glucoside (FMP), which compared to FMN, the water solubility was increased more than 106-fold. Moreover, in the mice model of isoprenaline-induced acute ischemia, the compound significantly attenuated cell membrane damage during myocardial ischemia by scavenging free radicals in serum, increasing CAT and SOD activities, and decreasing lactate dehydrogenase (LDH) activity. In another study, Yan et al. (2024) prepared seven derivatives of FMN from FMN. Among them, 9-butyl-3-(4-methoxyphenyl)-9,10-dihydro-4H,8H-chromeno[8,7-e][1,3]oxazin-4-one(2) and 9-(furan-3-ylmethyl)-3-(4-methoxyphenyl)-9,10-dihydro-4H,8H-chromeno-[8,7-e][1,3]oxazin-4-one possessed the ability to promote bone formation and inhibit osteoclastogenesis, which was superior to the effect of ipliflavone, suggesting that this compound could be a patentable candidate for anti-osteoporosis. In addition, Zuo et al. (2022) used structural modification to replace the methoxy group at position 40 of FMN with the phenoxy group to obtain a new compound that greatly improved the antihypertensive effect of FMN. Kim et al. (2019) utilized microbial transformation technology to synthesize a novel compound, FMN 7-O-phosphate.

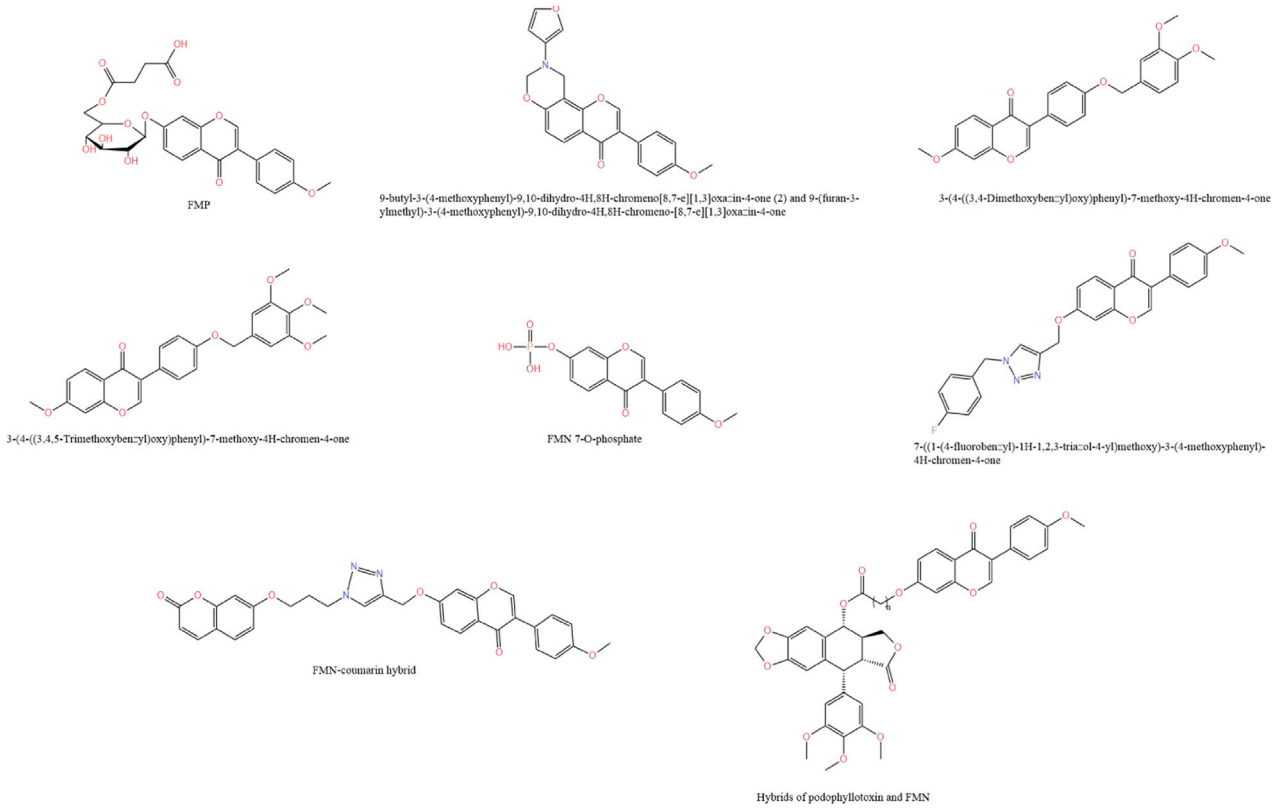



FIGURE 5
Chemical structure of FMN derivatives.

Compared with FMN, FMP showed lower cytotoxicity in RAW264.7 cells and significantly improved cell viability. At the same time, it inhibited the production of pro-inflammatory factors in a dose-dependent manner and decreased the mRNA expression of inducible iNOS and COX-2, which could be used as a promising candidate compound for new anti-inflammatory drugs.

FMN can act as a Bax agonist to exert anti-cancer effects. To improve its bioavailability, Jia et al. (2023) modified its structure and obtained a new compound 7-((1-(4-fluorobenzyl)-1H-1,2,3-triazol-4-yl)methoxy)-3-(4-methoxyphenyl)-4H-chromen-4-one. The compound could significantly inhibit the growth of the A549 cell line, downregulate the expression of Bcl-2 in cells, upregulate the expression of Bax, and promote apoptosis. Its efficacy was 40 times and 6.94 times greater than that of FMN and adriamycin, respectively, indicating that the derivative had strong potential as an anti-cancer lead compound. Based on the natural FMN, Yao et al. (2019) designed and synthesized a FMN-coumarin hybrid product by molecular hybridization strategy. Compared with FMN, the hybrid product could significantly inhibit the growth and migration of gastric cancer SGC7901 cells through the wnt/β-Catenin and AKT/mTOR pathways, and could be used as an inhibitor of SIRT1 to inhibit its expression, thereby inducing cancer cell death. *In vivo*, the hybrid effectively inhibited the growth of SGC7901 xenograft tumors in nude mice without significantly affecting their body weight. Similarly, Yang et al. (2019) inserted the FMN fragment into the C4 hydroxyl position of podophyllotoxin to form a new hybrid product, which had a stronger ability to resist A549 tumor growth than podophyllotoxin. This was because FMN could be inserted into the αβ-interface to form hydrogen bonds, destroy the cytoskeleton of tubulin, and enhance anti-tumor ability. The chemical structure of FMN derivatives is shown in Figure 5.

## 7 Drug delivery systems

Drug delivery system is the use of carriers or technologies to effectively and safely deliver drugs to specific targets in the body, which has the characteristics of optimizing the therapeutic effect of drugs, high solubility and long retention time in biological systems (Prakash, 2023). Since FMN is characterized by low water solubility and low bioavailability, limiting its application in various areas. Therefore, research based on nano-delivery systems has emerged in recent years. The following text should be added: The FMN delivery systems is shown in Figure 6.

### 7.1 Carbon nanotubes

Carbon nanotubes (CNs) are small, flexible, robust, inert, and electrically conductive materials that have been investigated for treating spinal cord injuries due to their electrical properties and nano-size. However, CNs have low hydrophilicity and poor dispersibility, which tend to lead to aggregation, so it is usually necessary to functionalize the CNs to avoid its aggregation (Imani et al., 2016). de Vasconcelos et al. (2020) used FMN to functionalize

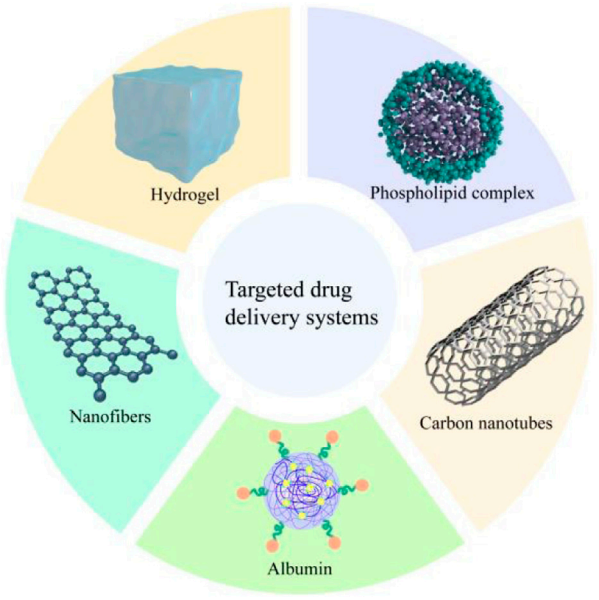



FIGURE 6
FMN's delivery system.

CNs, which not only improved the stability of CNs, but also further stimulated neuronal growth and cell membrane fusion through FMN, and even reduced inflammatory response. On this basis, in order to enhance the bio-interface with nerve cells, gelatin methacryloyl hydrogel was used as a scaffold, which could retain the nanocomposite of multi-walled carbon nanotubes and FMN at the inflammation site, and was directly used for the repair of neural tissues in the diseased site.

## 7.2 Protein nanoparticles

Ouyang et al. (2023) used bovine serum albumin (BSA) as raw material, and nanoprecipitation with 20:1 as the optimal ratio of albumin to FMN to prepare FMN-containing nanoparticles FMN@BSA. The nanoparticles improved the bioavailability of FMN by promoting the preferential accumulation of albumin's affinity for SPARC protein overexpressed in lung inflammation and pulmonary fibrosis, and alleviated bleomycin-induced lung inflammation and interstitial deposition in mice by blocking NLRP3 inflammasome to slow down the pyroptosis process of macrophages. It could be seen that the nanoparticles could be used as a macrophage pyroptosis inhibitor. Compared with the commonly used first-line therapeutic drug pirfenidone, FMN@BSA was a natural product as the active ingredient, which had lower toxicity and stronger clinical transferability.

## 7.3 Cyclodextrin inclusion complexes and phospholipid complexes

It has been previously shown that β-cyclodextrin can improve the solubility of FMN (Bhardwaj and Purohit, 2023). On this basis, Wang et al. (2021), in order to improve the oral bioavailability of FMN, prepared instantly soluble nanofibers containing FMN/methyl-β-cyclodextrin (FMN/Me-β-CD) inclusion complex, which increased the solubility of FMN by about 50-fold in 20 mM Me-β-Cd aqueous solution. After that, polyvinyl alcohol nanofiber webs containing FMN/Me-β-CD inclusion complex was prepared by electrostatic spinning method, which could be dissolved in artificial saliva in about 2 s. The rapid dissolution of FMN in artificial saliva was greatly improved, which would be beneficial to the application of FMN in oral diseases. In addition, Agarwal et al. (2024) had also made efforts to improve the oral bioavailability of FMN. The researchers used phospholipid (PL) as a carrier to prepare FMN phospholipid complex (FNT-PC) using the solvent evaporation method and characterized it. The results showed that FNT-PC exhibited amorphous geometry, and the water solubility was 4 times higher than FMN. In addition, *in vivo* pharmacokinetic studies in rats by gavage showed that FNT-pc also reduced first-pass metabolism, increased gastric mucosal permeability, and significantly improved oral bioavailability.

## 7.4 Dual ligand-modified

As mentioned earlier, FMN has excellent antitumor activity, and to further enhance its clinical application value and therapeutic effect, the use of the nano-delivery system to effectively improve the bioavailability and targeting of FMN is a promising strategy. It had been reported that some researchers used epidermal growth factor receptor-targeting peptide (GE11)-modified nanoparticles (GE-NPs) loaded with paclitaxel and hyaluronic acid (HA)-modified nanoparticles (HA-NPs) encapsulated with FMN, and self-assembled both of them to construct binary nanoparticles modified by GE11 and HA dual ligands (HAGE-DTX/FMN-NPs). The binary nanoparticles showed a stronger inhibitory effect on PC3 cells with a cellular uptake rate of 59.6% compared with the single ligand-modified nanoparticles and free drug. PC3 cells were injected into the right side of Balb/c nude mice to prepare cancer xenografts by subcutaneous injection and treated by tail vein injection, and it was found that HAGE-DTX/FMN-NPs were highly aggregated at the tumor site, whereas the single ligand-modified nanoparticles and the free drug had more distribution of the drug in the liver and kidneys, which showed that the binary nanoparticles modified with dual ligands have better targeting and bioavailability and can avoid unwanted side effects (Dong et al., 2022).

## 7.5 Microspheres

Cao et al. (2023) used microfluidic technology to prepare loaded FMN microspheres, which could encapsulate and release the drug in a controlled manner. *In vitro* experiments showed that the microspheres inhibited the proliferation, migration, and apoptosis of A549 cells better than FMN, and had good anti-tumor selectivity and low toxicity to normal cells. In addition, the microspheres could induce intracellular ROS production, leading to oxidative damage and better antitumor effects.

# 8 Conclusion and future perspectives

In recent years, more and more attention has been paid to developing natural metabolite, mainly because they are derived from

plants with low toxicity and high efficiency. FMN is one of the important discoveries of novel natural metabolite with considerable medicinal value. It is widely found in plants and daily diets, and is also detected in some chinese patent medicines, such as Qiliqiangxin capsule (Zhang et al., 2018), Zhenqi Fuzheng capsules (Liu et al., 2015), Guanjiekang (Wu et al., 2016) and Naoxintong capsule. This paper reviews the source, chemical properties, pharmacological activities and molecular mechanisms, co-administration, toxicity, derivatives and drug delivery system of FMN, which can provide a basis for in-depth research and clinical application of FMN.

FMN is involved in various disease processes, such as neurological disorders, organ damage and cancer, by regulating various factors, enzyme activities and signaling pathways, and it is evident that FMN has multiple pharmacodynamic effects. However, the current study still has certain flaws and limitations. First, studies on FMN have mainly focused on cellular and animal models, and the multiple pharmacological mechanisms have not been fully clarified. Although the treatment of the nervous system, organ injury and cancer is outstanding, it is still in the preliminary stage, without in-depth study, and lacks large-scale and long-term clinical trials to verify its safety and effectiveness. And the lack of FMN metabolic process, can not fully understand the drug circulation in the body. Therefore, in the future, in addition to the use of traditional *in vivo* and *in vitro* experimental analysis, other research methods should be strengthened, such as metabolomics, proteomics and genomics analysis, in order to better study the absorption, distribution and metabolic process of FMN, and how to act on the target to play a therapeutic effect. Through these studies, we can better understand the regulatory role and overall effect of FMN at the cellular molecular level. Second, to realize the maximum potential of its clinical application, further exploration of its quantitative relationship, side effects, and toxicity is needed, and this information is critical for assessing its safety and feasibility in clinical application. Finally, Although toxicity studies have been conducted on FMN, most of the tests have been at the cellular level, and more comprehensive toxicity tests such as subchronic toxicity, genotoxicity and reproductive toxicity are lacking. It is recommended to use different animal models to validate the toxicity results and extend the experimental period to obtain more data. Finally, systematic clinical trials, including randomized controlled trials, long-term efficacy observation and adverse reaction evaluation, are needed to fully understand the actual effect and safety of FMN in different diseases.

As previously mentioned, FMN has the characteristics of poor water solubility, low bioavailability and insufficient targeting. To solve those problems, structural modification and nano-delivery systems can be used as a promising solution. Numerous scholars have utilized nano-delivery systems to modulate the particle size, surface properties and carrier materials of drugs, which can significantly improve the bioavailability and targeting of FMN, and show good application prospects. At present, although studies have confirmed that the nano-delivery system has a significant improvement on the shortcomings of FMN, there is a lack of long-term safety and toxicological evaluation, and further research is needed in the future to ensure its reliability and safety in clinical applications. Meanwhile, the design of the delivery system should be further optimized to achieve higher drug loading capacity and more precise targeted release. In addition to the use of drug delivery systems alone, FMN can be combined with other treatments, such as immunotherapy, to enhance the therapeutic effect and improve the prognosis of patients; gene editing technology can be used to solve the disease directly from the root cause by precisely modifying the disease-causing genes. In the future, with the gradual enrichment of knowledge, the pharmacological activity of FMN and its molecular mechanism will be further elucidated, and the study of its structural modification and delivery system will be further optimized and innovated, which will have a new impact on the future pharmaceutical industry.

## Author contributions

MJ: Writing–original draft. LW: Writing–review and editing. JW: Software, Writing–review and editing. YuS: Software, Writing–review and editing. LG: Data curation, Writing–review and editing. FZ: Data curation, Writing–review and editing. QG: Formal Analysis, Writing–review and editing. YM: Formal Analysis, Writing–review and editing. YoS: Supervision, Writing–review and editing. YL: Supervision, Writing–review and editing. GJ: Writing–review and editing. PC: Writing–review and editing. RY: Funding acquisition, Writing–review and editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

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# Bioactivity and biomedical applications of pomegranate peel extract: a comprehensive review

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Pomegranate peel is a by-product generated during the processing of pomegranate (*Punica granatum* L.) fruit, accounting for approximately 50% of the total mass of the fruit. Although pomegranate peel is usually regarded as waste, it is rich in various bioactive metabolites such as polyphenols, tannins, and flavonoids, demonstrating significant medicinal and nutritional value. In recent years, Pomegranate peel extract (PPE) has shown broad application prospects in the biomedical field due to its multiple effects, including antioxidant, anti-inflammatory, antibacterial, anti-apoptotic properties, and promotion of cell regeneration. This review consolidates the major bioactive metabolites of PPE and explores its applications in biomedical materials, including nanodrug carriers, hydrogels, and tissue engineering scaffolds. By synthesizing the existing literature, we delve into the potential value of PPE in biomedicine, the challenges currently encountered, and the future directions for research. The aim of this review is to provide a scientific basis for optimizing the utilization of PPE and to facilitate its broader application in the biomedical field.

KEYWORDS

**pomegranate peel extract, bioactivity, biomedical applications, nanodrug carriers, hydrogels, tissue engineering scaffolds**

## 1 Introduction

Pomegranate (*Punica granatum* L.), belonging to the family Lythraceae and the genus Punica, is one of the oldest edible fruits, originating in Persia (modern-day Iran) and surrounding areas. It was later spread to Asia, North Africa, and the Mediterranean regions through activities such as seafaring and wars, and is now widely cultivated around the world (Benedetti et al., 2023; Langgut, 2024). It is estimated that the total global cultivation area of pomegranate exceeds 300,000 ha, with an annual production of approximately 3 million tons (Melgarejo-Sánchez et al., 2021). Pomegranate peel is one of the main components of the fruit, accounting for about 50% (Arun et al., 2022; Derakhshan et al., 2018). In current pomegranate product processing, pomegranate peel is usually discarded as waste, resulting in around 1.6 million tons of waste annually (Bertolo et al., 2021; Giri et al., 2023). In

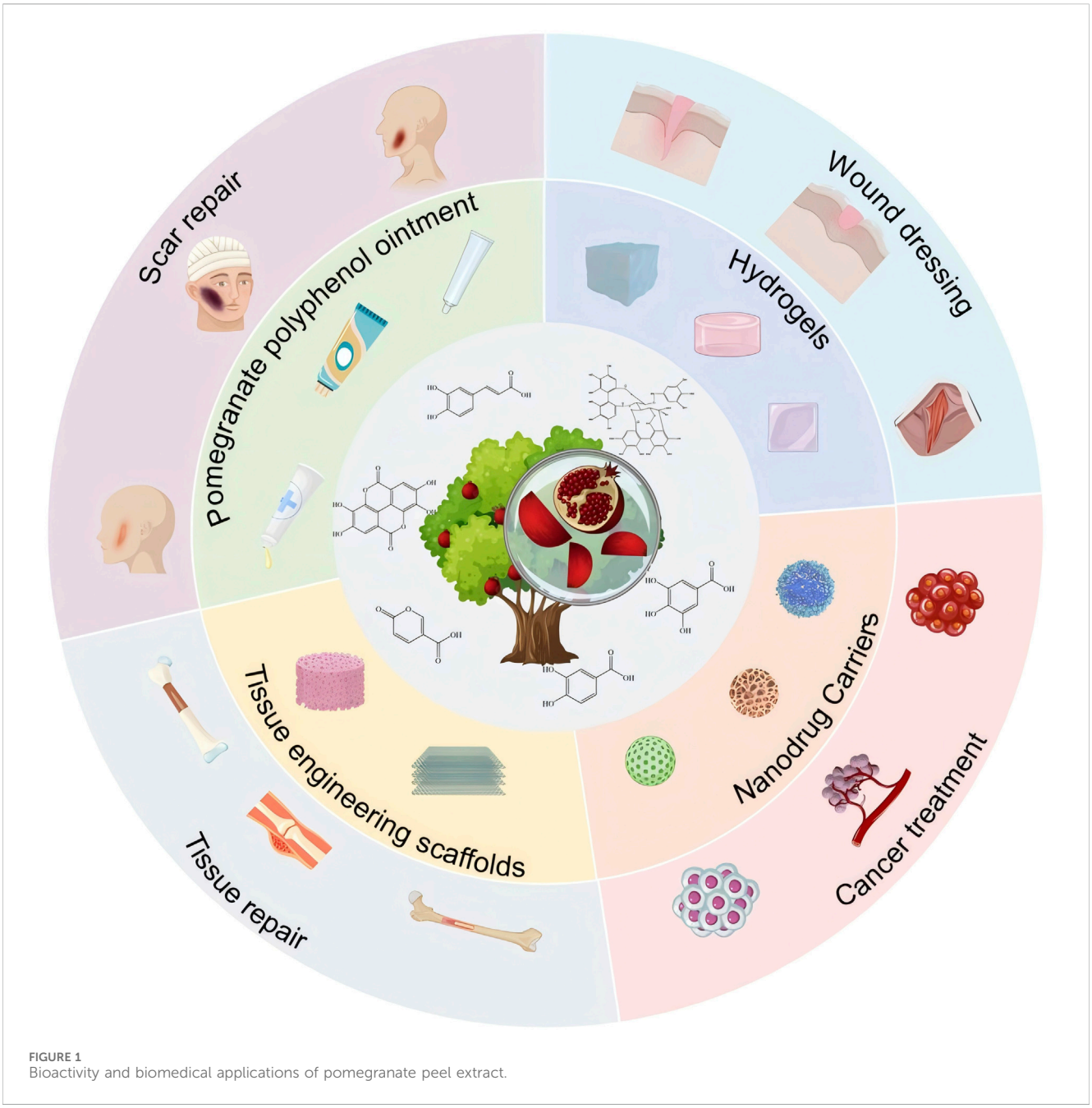
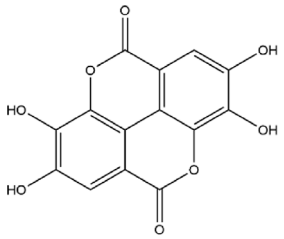
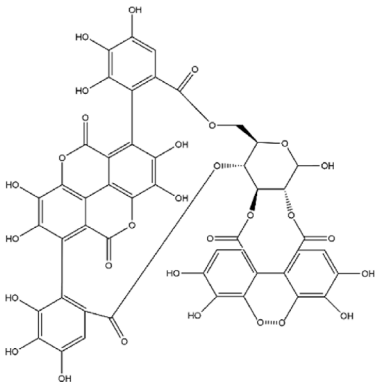
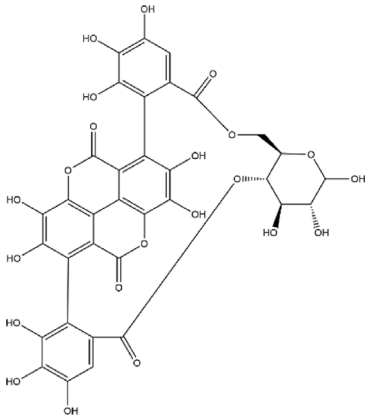
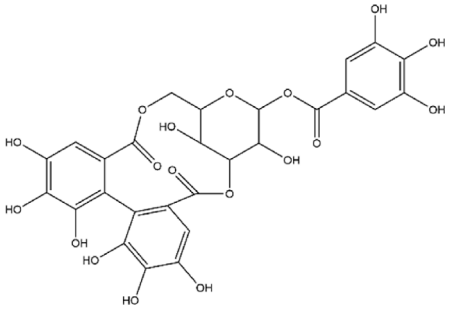



FIGURE 1
Bioactivity and biomedical applications of pomegranate peel extract.

addition to resource wastage, pomegranate peel waste negatively impacts natural resources such as land and water, further exacerbating environmental issues (Sagar et al., 2018).

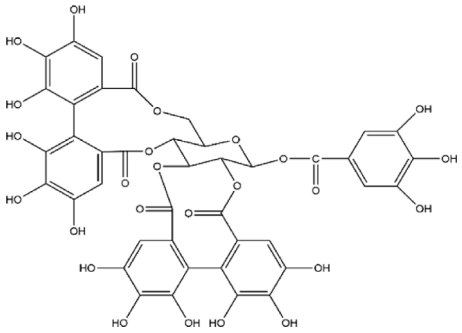
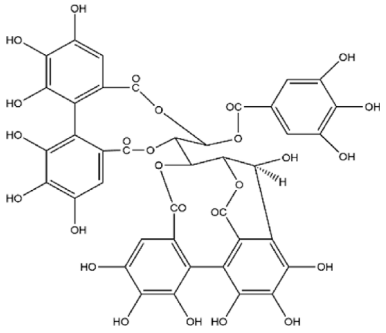
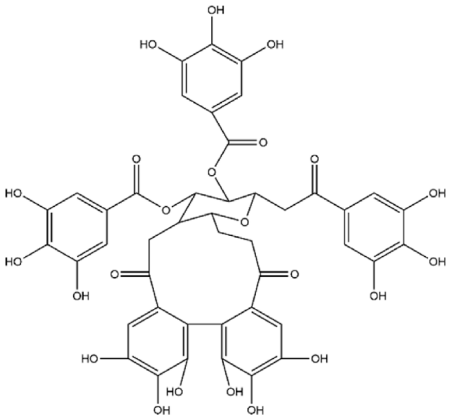
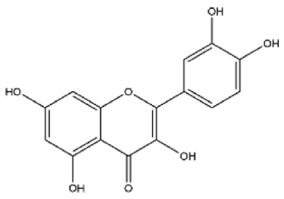
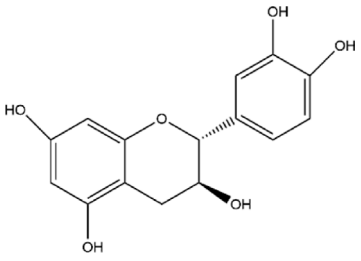
Pomegranate peel is not without value but possesses unique medicinal properties. Thousands of years ago, pomegranate peel was widely used in traditional medicine across many cultures (Li et al., 2006; Liu et al., 2012). Historically, pomegranate peel has been utilized in various cultures for medicinal purposes. The Romans employed it as an anthelmintic, while some ethnic groups in the Middle East and South America have used boiled pomegranate peel for treating dysentery (Ge et al., 2021; Longtin, 2003). In traditional Chinese medicine, pomegranate peel is regarded as a potent astringent and anti-inflammatory agent, applied to treat traumatic bleeding, infections, and gastrointestinal disorders such as diarrhea (Mo et al., 2013). In Ayurvedic medicine, it is similarly valued as an effective remedy for oral infections (Rafey et al., 2021). In modern times, the medicinal efficacy of pomegranate peel has been scientifically validated. Studies reveal that pomegranate peel is rich in bioactive metabolites such as tannins, phenolic acids, and flavonoids, which impart a range of pharmacological effects, including antioxidant, anti-inflammatory, antibacterial, and anticancer activities (Ain et al., 2023; Ozkan, 2023; Siddiqui et al., 2024). Research has demonstrated that the antioxidant capacity of pomegranate peel extract (PPE) is nearly ten times greater than that of other parts of the pomegranate fruit, a property closely linked to its polyphenolic structure (Hanafy et al., 2021). Furthermore, PPE is capable of modulating several critical molecular pathways, including mitogen-activated protein

TABLE 1 Metabolites in PPE.

| | No. | Metabolite | Molecular formula | Structure | References |
|---|---|---|---|---|---|
| Ellagitannins | 1 | Ellagic acid | $C_{14}H_6O_8$ |  | Gaya et al. (2018) |
| | 2 | Punicalagin | $C_{48}H_{28}O_{30}$ |  | Lyu et al. (2017) |
| | 3 | Punicalin | $C_{34}H_{22}O_{22}$ |  | Sun et al. (2017) |
| | 4 | Corilagin | $C_{27}H_{22}O_{18}$ |  | Adili et al. (2020) |

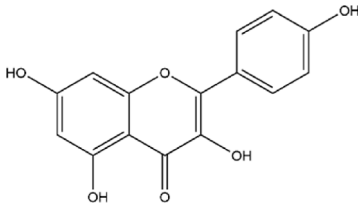
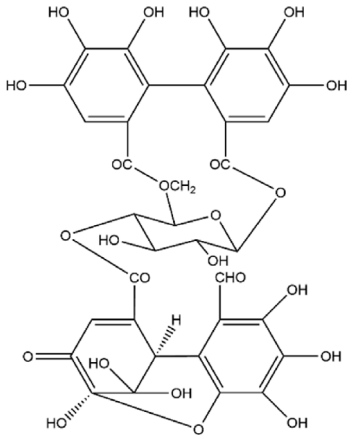
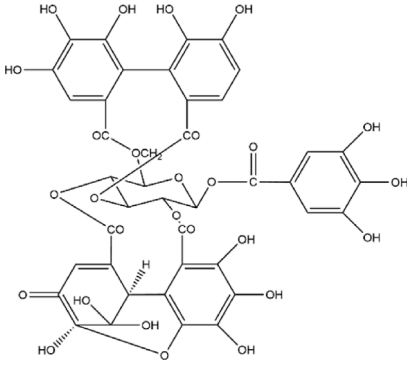
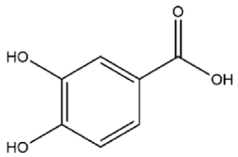
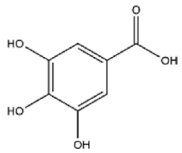
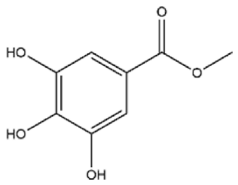
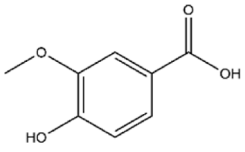
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TABLE 1 (*Continued*) Metabolites in PPE.

| | No. | Metabolite | Molecular formula | Structure | References |
|---|---|---|---|---|---|
| | 5 | Pedunculagin | $C_{41}H_{28}O_{26}$ |  | Khan et al. (2017) |
| | 6 | Casuarinin | $C_{41}H_{28}O_{26}$ |  | Satomi et al. (1993) |
| | 7 | Tellimagrandin | $C_{41}H_{30}O_{26}$ |  | Satomi et al. (1993) |
| Flavonoids | 8 | Quercetin | $C_{15}H_{10}O_7$ |  | Adili et al. (2020) |
| | 9 | Catechin | $C_{15}H_{14}O_6$ |  | Adili et al. (2020) |

(Continued on following page)

TABLE 1 (*Continued*) Metabolites in PPE.

| | No. | Metabolite | Molecular formula | Structure | References |
|---|---|---|---|---|---|
| | 10 | Kaempferol | $C_{15}H_{10}O_6$ |  | Hussain et al. (2024) |
| | 11 | Granatin A | $C_{34}H_{24}O_{22}$ |  | Satomi et al. (1993) |
| | 12 | Granatin B | $C_{41}H_{32}O_{27}$ |  | Satomi et al. (1993) |
| Phenolic Acids | 13 | Protocatechuic acid | $C_7H_6O_4$ |  | Xiao et al. (2021) |
| | 14 | Gallic acid | $C_7H_6O_5$ |  | Satomi et al. (1993) |
| | 15 | Methyl gallate | $C_8H_8O_5$ |  | Satomi et al. (1993) |
| | 16 | Vanillic acid | $C_8H_8O_4$ |  | Kumari et al. (2021) |

kinases, nuclear factor-kappa B (NF-κB), cysteine-aspartic acid-specific proteases, and peroxisome proliferator-activated receptors, thus providing theoretical support for its use in the treatment of a variety of diseases (Andishmand et al., 2025). In addition to its notable pharmacological properties, PPE can be processed into nanodrug carriers, hydrogels, and other biomedical materials through chemical reduction, complexation, emulsification, and hydration methods, offering potential therapeutic applications for diseases such as diabetes and breast cancer (Andishmand et al., 2023; Askari and Nikoonahad Lotfabadi, 2020; Baccarin and Lemos-Senna, 2017; Ganeshkumar et al., 2013; Savekar et al., 2024; Tiyaboonchai et al., 2015). Thus, utilizing pomegranate peel, a by-product, can achieve resource recycling and create new economic value.

This paper aims to explore the main bioactive metabolites of PPE and its applications in biomedicine (Figure 1), while providing an in-depth analysis of its limitations in practical applications and its development potential. The goal is to offer a theoretical foundation and scientific support for the future development and utilization of pomegranate peel.

## 2 Retrieval methods

The literature review was conducted using several databases, including Web of Science (https://webofscience.clarivate.cn), ScienceDirect (www.sciencedirect.com), and PubMed (https://www.ncbi.nlm.nih.gov/). The search keywords included "pomegranate peel extract," "Punica granatum L.," "bioactivity," "hydrogels," "nanodrugs," "tissue engineering," "biomedical materials," and their combinations. All duplicate articles identified during the search were excluded. The species names were verified using the taxonomic database (http://mpns.kew.org/mpns-portal/).

## 3 Bioactive metabolites in pomegranate peel

The content of polyphenolic metabolites in pomegranate peel is significantly higher than in the flesh and seeds, containing ellagic acid, punicalagin, punicalin, and other ellagitannins and their derivatives, as well as gallic acid, coumaric acid, and other phenolic acids, along with flavonoids such as catechin and epicatechin (Table 1). These polyphenolic metabolites exhibit a wide range of bioactivities and can exert certain physiological or pharmacological effects on organisms, including antioxidant, anti-inflammatory, and antibacterial activities (Table 2).

### 3.1 Ellagitannins

Ellagitannins are derived from esters of hexahydroxydiphenic acid, which undergo hydrolysis to form ellagic acid, and bind with polyols. Through oxidative coupling reactions of ellagic acid moieties, ellagitannins form hexahydroxydiphenyl groups, which are dehydrodimeric ellagic acid esters with aromaticity (Molnar et al., 2024). This property gives ellagitannins a more complex structural characteristic compared to ordinary tannic acids. Ellagitannins in PPE include ellagic acid, punicalagin, punicalin, corilagin, and pedunculagin, among others. Punicalagin, characterized by a high total phenolic content, is the primary contributor to the antioxidant properties of pomegranate products (Nuncio-Jáuregui et al., 2015; Valero-Mendoza et al., 2023). Its antioxidant capacity is remarkably high, approximately 50%, whereas ellagic acid demonstrates a weaker antioxidant capacity, with only 3% per molecule (Shabir et al., 2024). Punicalagin contains galloyl groups esterified with glucose, which undergo hydrolysis to form ellagic acid and punicalin (Yamada et al., 2018). Punicalin retains significant antioxidant activity, effectively scavenging free radicals and reducing oxidative damage to cells, thereby contributing to the prevention of aging and related diseases (Magangana et al., 2020; Ranjha et al., 2023; Sharma et al., 2022).

The role of ellagitannins and their derivatives in disease prevention is closely tied to their antioxidant potential, which increases with the number of hydroxyl groups in their molecular structure (Gupta et al., 2019; Ismail et al., 2016). Pedunculagin, the most abundant and biologically active ellagitannin in pomegranate, has therapeutic effects on inflammatory diseases (Shabir et al., 2024; Xu et al., 2022). Seo et al. used a lupus-prone mouse model with spontaneously occurring severe rheumatoid disease to investigate the effects of different doses of pedunculagin (Seo et al., 2020). As the concentration of pedunculagin increased, the severity of renal damage in the mice significantly decreased. The study demonstrated that pedunculagin could alleviate lupus nephritis in mice by inhibiting the protease-activated receptor 2 pathway. Future research could further explore the role of potential pathways such as NF-κB or MAPK, and evaluate the potential toxic side effects of pedunculagin on the liver and kidneys. Nguyen et al. stimulated human placental, visceral fat, and subcutaneous fat tissue explants with tumor necrosis factor (TNF), and the study showed that punicalagin significantly inhibited the TNF-induced expression of pro-inflammatory factors (IL-1β, IL-6) in human placenta and adipose tissue (Nguyen-Ngo et al., 2020). A strength of this study was the use of human-derived tissues *in vitro*, providing greater clinical relevance. However, the limitation of this research lies in the lack of *in vivo* studies with animal models, leading to insufficient validation of its *in vivo* efficacy and mechanisms. Furthermore, other ellagitannins such as corilagin, casuarinin, and tellimagrandin, which contain multiple hydroxyl groups, exhibit significant antioxidant activity. Corilagin inhibits the reverse transcriptase activity of RNA tumor viruses, thereby demonstrating anticancer effects (Li et al., 2018). Casuarinin exhibits antiviral activity by inhibiting the adhesion and penetration of herpes simplex virus type 2 (Cheng et al., 2002). Chang et al.'s study showed that tellimagrandin inhibits the activity of methicillin-resistant *Staphylococcus aureus* with a minimum inhibitory concentration of 128 μg/mL (Chang et al., 2019). Overall, ellagitannins and their derivatives possess multiple bioactivities, including antioxidant, anti-inflammatory, antibacterial, and antitumor effects, demonstrating broad application potential in disease prevention and treatment.

The metabolic pathways of ellagitannins *in vivo* show significant differences from those of tannic acids. Ellagitannins are not directly absorbed into the bloodstream but are metabolized through hydrolysis processes (Shabir et al., 2024). For example,

TABLE 2 The biological activities of the chemical constituents of PPE.

| NO. | Biological activity | PPE | Models | Dosage/ Concentration | Conclusion | References |
|---|---|---|---|---|---|---|
| 1 | Antioxidant | Ellagic Acid | Cisplatin-Induced Nephrotoxicity Model On day 1, adult male Sprague-Dawley rats were intraperitoneally injected with cisplatin (7 mg/kg body weight (mg/kg BW)) | From days 1–10, ellagic acid dissolved in corn oil was administered intraperitoneally at a dose of 10 mg/kg BW daily. At the end of the experiment, blood samples were collected from the rats | Ellagic acid exhibited protective effects against cisplatin-induced nephrotoxicity and oxidative stress in rats, potentially due to its potent scavenging activity against superoxide anions and hydroxyl radicals | Ateşşahín et al. (2007) |
| 2 | | | 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD)-Induced Nephrotoxicity Model From days 11–13, male Wistar albino rats were injected daily with 15 μg/kg BW of TCDD dissolved in 0.15 mL of corn oil. At the end of the experiment, blood was collected *via* the jugular vein | From days 1–10, ellagic acid was administered orally at a dose of 10 mg/kg BW daily, dissolved in 0.15 mL of 50% dimethyl sulfoxide | Ellagic acid enhanced antioxidant capacity and prevented oxidative stress by maintaining $Ca^{2+}$ homeostasis in HepG2 cells and inhibiting CYP1A1 activity, thereby providing protection against TCDD-induced nephrotoxicity | Padma et al. (2014) |
| 3 | | | High-Fat and High-Cholesterol Diet Model From weeks 1–8, 4-6-week-old New Zealand white rabbits with an average body weight of 1.5 kg were fed a diet enriched with 10% corn oil and 0.5% cholesterol | During the same period, rabbits were supplemented with 1% (w/w) ellagic acid. At the end of week 8, blood samples were collected *via* cardiac puncture | Ellagic acid demonstrated the ability to scavenge RO· or ROO· free radicals, inhibit the production of hydrogen peroxide and singlet oxygen, and suppress lipid peroxidation, thereby improving atherosclerosis | Yu et al. (2005) |
| 4 | | Punicalagin | Lipopolysaccharide-induced testicular oxidative stress injury model: Day 1–7, adult male ICR mice were injected daily with lipopolysaccharide (600 μg/kg BW) | Day 1–7, mice were orally administered punicalagin at a dose of 9 mg/kg BW daily *via* gavage. On the last day of treatment, male mice were exposed to female mice for 4 days, followed by dissection and examination | Punicalagin significantly reduced the production of the strong oxidant NO and mitigated oxidative stress in the testes by activating nuclear factor erythroid 2-related factor 2 (Nrf2), reducing oxidative damage, and increasing sperm count | Rao et al. (2016) |
| 5 | | | $CCl_4$-induced liver injury model: In the 5th week, mice were intraperitoneally injected with 0.07% CCl4 (v/v, 0.1 mL/100 g), and blood and liver tissue samples were collected 16 h later | After a 1-week adaptation period, mice were gavaged daily with punicalagin dissolved in distilled water (80 mg/kg BW) for 4 consecutive weeks | Punicalagin alleviated oxidative stress by increasing liver superoxide dismutase (SOD), glutathione peroxidase (GPx) activity, and Nrf2 protein expression, thereby protecting against $CCl_4$-induced liver injury | Luo et al. (2019) |
| 6 | | Quercetin | 3-Nitropropionic acid (3-NPA)-induced oxidative stress model: Day 1–42, SPF-grade female mice were injected daily with 3-NPA (20 mg/kg BW) | Day 1–42, mice were intraperitoneally injected with quercetin (200 mg/kg BW) | Quercetin alleviated oxidative stress in this model by promoting SIRT1 expression, activating the SIRT1/ROS/AMPK signaling pathway, inhibiting ROS accumulation induced by oxidative stress, regulating autophagy levels, reducing cell apoptosis, and ultimately restoring ovarian function | Duan et al. (2024) |
| 7 | | | Xenoestrogen bisphenol S (BPS)-induced behavioral change model in zebrafish: From day 1 to day 21, adult zebrafish were placed in a 15-L aquarium with a BPS concentration of 20.52 M | From day 1 to day 21, quercetin was added to the aquarium water at a concentration of 2.96 M | Quercetin alleviated oxidative stress and behavioral changes induced by BPS by increasing GSH levels and superoxide dismutase activity, while reducing elevated lipid peroxidation in the zebrafish brain | Ragunath et al. (2022) |

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TABLE 2 (*Continued*) The biological activities of the chemical constituents of PPE.

| NO. | Biological activity | PPE | Models | Dosage/ Concentration | Conclusion | References |
|---|---|---|---|---|---|---|
| 8 | | Catechin | Chronic unpredictable mild stress (CUMS) depression model constructed by external stressors: From week 1 to week 8, male Sprague Dawley rats were subjected daily to various stressors such as food deprivation, water deprivation, tail clipping, electric shock, and forced swimming | From week 1 to week 8, catechin hydrate (50 mg/kg BW) was orally administered daily | After catechin treatment, antioxidant parameters such as catalase, glutathione, and superoxide dismutase levels were restored, reversing CUMS-induced depression in rats by alleviating oxidative stress | Rai et al. (2019) |
| 9 | | Protocatechuic acid | Isoproterenol-induced heart failure model in mice: From day 1 to day 14, C57BL/6NTac mice were intraperitoneally injected with isoproterenol (80 mg/kg BW) to construct a heart failure model | From day 6 to day 14, mice were injected daily with protocatechuic acid (100 mg/kg BW) | Kynurenine-3-monooxygenase (*Kmo*) was identified as a potential target of protocatechuic acid. Protocatechuic acid prevented heart failure by reducing ROS production induced by isoproterenol both *in vivo* and *in vitro* through *Kmo* downregulation | Bai et al. (2023) |
| 10 | | Gallic acid | 3-Nitropropionate acid (3-NP)-induced ovarian oxidative stress model in mice: From day 7 to day 16, female ICR mice were intraperitoneally injected with 3-NP (12.5 mg/kg BW) twice daily | Mice were administered gallic acid *via* oral gavage | Gallic acid exerted its protective effects by increasing antioxidant enzyme activity and reducing the mRNA expression levels of pro-apoptotic genes (*Bim* and *Caspase-3*), thereby inhibiting apoptosis in ovarian granulosa cells | Li et al. (2017) |
| 11 | Anti-inflammatory | Ellagic acid | Acute liver injury model induced by Lipopolysaccharide/ d-Galactosamine (LPS/GalN) in Mice: Mice were intraperitoneally injected with GalN (800 mg/kg BW) and LPS (50 μg/kg BW), dissolved in PBS. After treatment, the mice were sacrificed | One hour prior to LPS/GalN administration, ellagic acid was injected intraperitoneally | Ellagic acid significantly suppressed the production of pro-inflammatory cytokines such as TNF-α and effectively inhibited Nuclear Factor-kappa B (NF-κB) activation by preventing the phosphorylation of IκB-α and NF-κB p65. This reduced inflammation and prevented liver injury | Gu et al. (2014) |
| 12 | | Punicalagin | Pelvic inflammatory disease (PID) model in rats: A PID model was constructed by implanting a mixed microbial solution of *Escherichia coli* and *Staphylococcus aureus* into the rat cervix. On days 1–7, female Sprague Dawley rats were injected with 10 mg of progesterone. On days 9, 11, 13, and 15, gelatin sponges soaked in a nutrient broth containing *E. coli* and *S. aureus* were implanted into the cervical region | Rats were divided into preventive and therapeutic groups. The preventive group was administered punicalagin *via* gavage 1 day before PID induction, while the therapeutic group received punicalagin *via* gavage 1 day after confirming the PID model | In both groups, punicalagin significantly reduced IL-1β levels, lipid peroxidation, and catalase activity. It also decreased local leukocyte infiltration and oviduct fibrosis, demonstrating its potential as a safe and effective treatment for PID. | Zoofeen et al. (2024) |

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TABLE 2 (*Continued*) The biological activities of the chemical constituents of PPE.

| NO. | Biological activity | PPE | Models | Dosage/ Concentration | Conclusion | References |
|---|---|---|---|---|---|---|
| 13 | | | Collagen-induced arthritis (CIA) model in mice: On day 1, DBA/1 male mice were injected intradermally at the base of the tail with 200 mg of bovine type II collagen emulsified in a 1:1 ratio with complete Freund's adjuvant to induce an initial immune response. On day 22, an immunization booster was administered with bovine type II collagen emulsified in incomplete Freund's adjuvant (1:1) | From day 23 to day 37, punicalagin (50 mg/kg BW) was intraperitoneally injected daily | Punicalagin significantly reduced serum levels of IL-6 and TNF-α in CIA mice, alleviated cellular infiltration, synovial hyperplasia, cartilage destruction, and limb swelling, showing strong anti-arthritis effects | Huang et al. (2021) |
| 14 | | Corilagin | Acute lung injury (ALI) model induced by Lipopolysaccharide (LPS) in mice: C57BL/6 mice were intratracheally instilled with LPS (2.5 μg/g BW) dissolved in PBS. | After 30 min of LPS stimulation, corilagin (10 mg/kg BW) was administered, and the mice were anesthetized and sacrificed 6 h later | Corilagin significantly reduced inflammatory cell infiltration in lung tissues and inhibited the production of pro-inflammatory cytokines TNF-α, IL-6, and IL-1β, thereby providing effective protection against LPS-induced ALI. | Liu F. C. et al. (2022) |
| 15 | | Quercetin | Osteoarthritis (OA) model induced by monosodium iodoacetate (MIA) in rats: On day 1, male Sprague-Dawley rats were given a single intra-articular injection of 1 mg MIA into the right knee joint | From day 1 to day 28, rats were gavaged with quercetin daily | Quercetin significantly reduced serum levels of inflammatory cytokines, including IL-5, IL-6, IL-7, IL-10, and TNF-α, in OA rats. It also inhibited the expression of MMP-3, MMP-13, ADAMTS4, and ADAMTS5, reducing cartilage matrix degradation and demonstrating excellent anti-inflammatory and chondroprotective effects | Wang et al. (2023) |
| 16 | | Catechin | Establishment of a coronary heart disease (CHD) model in rats using a high-fat diet and pituitrin injection: From weeks 1–10, 10-week-old SPF-grade Wistar rats were fed a high-fat diet. Forty-eight hours after the last feeding, the rats were intraperitoneally injected with pituitrin (30 U/kg BW) once daily for two consecutive days | Following the pituitrin injections, the rats were administered catechin *via* gavage once daily for 4 weeks | catechin significantly reduced levels of inflammatory biomarkers and cytokines such as C-reactive protein, Lp-PLA2, IL-6, and TNF-α. It also activated anti-inflammatory and protective signaling molecules such as Farnesoid X Receptor, STAT-3, and PKB/Akt, effectively improving the pathological state of CHD. | Tu et al. (2018) |
| 17 | | Kaempferol | Establishment of a mouse asthma model using ovalbumin (OVA): On days 1 and 15, BALB/c mice were subcutaneously injected with 20 μg of OVA to induce sensitization. From days 29–31, the mice were exposed to 5% OVA aerosol for 20 min daily to establish the asthma model | During days 29–31, the mice were orally administered 0.1 mL of kaempferol solution (10 or 20 mg/kg BW) daily | Kaempferol inhibited the production of macrophage inflammatory protein-2 and the overexpression of its receptor CXCR2. It also modulated the Tyk2-STAT1/3 signaling pathway in response to IL-8, thereby alleviating airway inflammation | Gong et al. (2013) |
| 18 | | Methyl gallate | Establishment of a hyperuricemia nephropathy (HN) model in mice using potassium oxide (PO): From days 1–28, male C57BL/6 mice were intraperitoneally injected daily with 300 mg/kg BW of PO. | Thirty minutes prior to each PO injection, the mice were administered methyl gallate *via* gavage | Methyl gallate significantly reduced levels of NLRP3, ASC, IL-1β, and caspase-1 proteins in HN mice. It effectively inhibited the activation of the NLRP3 inflammasome, thereby exerting renal protective effects against PO-induced HN. | Liu et al. (2021) |

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TABLE 2 (*Continued*) The biological activities of the chemical constituents of PPE.

| NO. | Biological activity | PPE | Models | Dosage/ Concentration | Conclusion | References |
|---|---|---|---|---|---|---|
| 19 | Antibacterial | Ellagic acid | Establishment of a mouse *H. pylori* Infection Model using *H. pylori Strain* (Sydney Strain 1): To establish the model, male C57BL/6 mice were orally administered 0.2 mL of bacterial suspension ($10^8$ CFU/mL) three times at 2-day intervals using a gavage tube | Two weeks after bacterial infection, the mice were administered ellagic acid (10 mg/kg BW) daily for 1 week *via* gavage | Ellagic acid restored and repaired gastric mucosal damage caused by *H. pylori*, demonstrating significant anti-*H. pylori* effects | De et al. (2018) |
| 20 | | | Establishment of a mouse *Aeromonas hydrophila* infection model: Male MF1 albino mice were fed a bacterial suspension of *Aeromonas hydrophila* (0.2 mL, $2 \times 10^8$ CFU) once a week for 2–4 weeks | The mice were administered ellagic acid *via* a stainless-steel gavage tube three times weekly (total dose 150 mg/kg BW) for 2–4 weeks | Ellagic acid treatment significantly increased levels of anti-LPS and anti-ECP IgM in infected mice, maintained the integrity of intestinal villi, and demonstrated antibacterial activity against *Aeromonas hydrophila* | Abuelsaad et al. (2013) |
| 21 | | Kaempferol | An infection model was established by intraperitoneally inoculating C57BL/6 female mice with $5 \times 10^6$ CFU of the wild-type *Listeria monocytogenes EGD* strain | After infection, the mice were treated with kaempferol (100 mg/kg BW) | Kaempferol treatment significantly reduced the bacterial load in major target organs, such as the spleen, and improved the survival rate of infected mice by more than 20% on the 6th day post-infection, demonstrating strong anti-*Listeria monocytogenes EGD* activity | Wang et al., 2022) |
| 22 | | Quercetin | *Citrobacter* rodentium-induced colitis model in mice: The colitis model was constructed by orally administering $2 \times 10^8$ CFU of *Citrobacter rodentium* culture to C57BL/6 mice, which were sacrificed on the 7th day post-infection | During weeks 1–2, quercetin (30 mg/kg BW) was supplemented in the mice's basal rodent diet. | The mice showed no signs of colitis, and the colon's bacterial populations, including *Fusobacterium* and *Enterococcus*, were suppressed, indicating that Quercetin exhibits antibacterial activity against *Citrobacter rodentium* | Lin et al. (2019) |
| 23 | | Gallic acid | *S. Typhimurium* peritoneal infection model in mice: Mice were peritoneally infected with $1 \times 10^6$ CFU of *S. Typhimurium* | Starting 3 days before infection and continuing for 10 days after infection, mice were orally administered 100 μL of gallic acid (100 μg/mL BW) daily | Gallic acid treatment significantly increased the levels of cytokines such as IFN-γ, indicating that it might stimulate the host immune response. Infected mice showed a significant reduction in bacterial loads in organs such as the liver and a decrease in mortality, suggesting that the virulence of lethal-dose *Typhimurium* was attenuated. These results indicate that gallic acid effectively protects macrophages and mice from *Typhimurium* infection | Reyes et al. (2017) |
| 24 | | | *B. abortus* peritoneal infection model in mice: Mice were peritoneally infected with $2 \times 10^4$ CFU of *B. abortus* | Starting 3 days before infection and continuing for 14 days after infection, mice were orally administered 100 μg/mL BW of gallic acid daily | Gallic acid interfered with *B. abortus* invasion by inhibiting F-actin polymerization and downregulating mitogen-activated protein kinases (MAPKs). Additionally, it induced the secretion of protective cytokines such as IL-12, triggering a protective immune response and inhibiting bacterial proliferation in the spleen, demonstrating its antibacterial activity | Reyes et al. (2018) |

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TABLE 2 (*Continued*) The biological activities of the chemical constituents of PPE.

| NO. | Biological activity | PPE | Models | Dosage/ Concentration | Conclusion | References |
|---|---|---|---|---|---|---|
| 25 | | | *Galleria mellonella* (*G. mellonella*) infection model with multidrug-resistant *escherichia coli*: A multidrug-resistant *Escherichia coli* infection model was established by injecting 10 μL of a $2 \times 10^6$ CFU/mL bacterial suspension into the right fourth leg of *G. mellonella* larvae | One hour after infection, 10 μL of gallic acid was injected into the left fourth leg of the larvae | Gallic acid disrupted the bacterial outer and inner membranes and inhibited the mRNA expression of membrane permeability-related genes (e.g., acrA, acrB, tolC, acrD, and acrF), significantly improving the survival rate of *Galleria mellonella*. This demonstrates its antibacterial activity against multidrug-resistant *Escherichia coli* | Tian Q. et al. (2022) |
| 26 | | Methyl gallate | An infection model was constructed by injecting $5 \times 10^5$ CFU of extensively drug-resistant *P. aeruginosa* into the hind leg of *Galleria mellonella* larvae | Two hours post-infection, the larvae were injected with methyl gallate | Methyl gallate significantly downregulated the expression of quorum-sensing-related genes in *P. aeruginosa* and dose-dependently improved the survival rate of the larvae. This demonstrates its antibacterial activity against extensively drug-resistant *P. aeruginosa* | Flores-Maldonado et al. (2024) |

metabolites such as punicalagin and corilagin are hydrolyzed under acidic or alkaline conditions to generate hexahydroxydiphenoyl (HHDP) groups, which spontaneously lactonize into ellagic acid (Evtyugin et al., 2020). Ellagic acid, a major metabolite of pomegranate peel (Kumar et al., 2022), is a polyphenolic dilactone that has been proven to exhibit various bioactivities, including antioxidant, anti-inflammatory, and antibacterial effects (Cuevas-Magana et al., 2022; Yu et al., 2023). After ingesting ellagitannins or ellagic acid, the human body metabolizes these metabolites through intestinal microbiota, producing 6H-dibenzo [b,d]pyran-6-one derivatives, also known as urolithins (Uros) (Figure 2), which are beneficial to health (Cerdá et al., 2003a; Cerdá et al., 2003b). Depending on the number and position of hydroxyl groups, Uros can be further classified into different types, such as urolithin A, urolithin B, urolithin C, *etc.* Long-term intake of ellagitannin-rich foods can increase the concentration of Uros in bodily fluids such as plasma and urine (Singh et al., 2022; Zhang et al., 2021; Zhang et al., 2020), as well as in tissues like the breast and bones (Andreux et al., 2019; Angeles Avila-Galvez et al., 2019). Research has shown that urolithins play a significant role in the biological activities within the body. For instance, in an inflammatory bowel disease model (Larrosa et al., 2010), following the administration of pomegranate extract, inflammation markers such as inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and prostaglandin E synthase (PTGES) were significantly reduced in the inflammatory rats. Additionally, oxidative stress levels in the plasma and colonic mucosa decreased, and the concentration of urolithins in fecal extracts was significantly lower than in the healthy rat group. In a Parkinson's disease model, after the administration of pomegranate extract, urolithins were detected in the brains of the affected rats, and improvements were observed in their olfactory and motor impairments (Kujawska et al., 2021). Furthermore, the levels of α-synuclein oligomers were also reduced (Kujawska et al., 2020). Additionally, urolithins can regulate cholesterol metabolism by reducing the abundance of Coriobacteriaceae bacteria and altering the bile acid pool, thereby promoting lipid absorption in the intestines and providing cardiovascular protection (Cortes-Martin et al., 2024). These findings highlight that foods rich in ellagic acid can positively impact health both in the gastrointestinal tract and systemically through urolithins.

Upon entering the human body, ellagitannins undergo bidirectional modulation with the gut microbiota. The gut microbiota can metabolize ellagitannins into urolithin metabolites through hydrolysis, reduction, and other reactions, while ellagitannins and their metabolites can also selectively regulate the composition of the microbiota (Caballero et al., 2022; Henning et al., 2022; Sanchez-Terron et al., 2024). For example, punicalagin intervention significantly enriched short-chain fatty acid-producing bacteria such as *Akkermansia muciniphila* and *Ruminiclostridium 9* in the intestines of high-fat diet mice (Gao et al., 2024), while also increasing the abundance of Lachnospiraceae *NK4A136* and *Bifidobacterium*, which have bile acid metabolism functions (Liu et al., 2024). The gut microbiota, as a "metabolic regulatory organ," plays a crucial role in the bile acid metabolic cycle. Approximately 95% of primary bile acids are reabsorbed through the terminal ileum sodium-dependent bile acid transporter and enter the enterohepatic circulation, while the remaining 5% are metabolized by bile salt hydrolases and 7α-dehydroxylases secreted by *Bacteroides*, *Clostridium*, *Bifidobacterium*, and *Lactobacillus* into secondary bile acids such as deoxycholic acid and lithocholic acid (Begley et al., 2005; Ridlon et al., 2016). Secondary bile acids play a pivotal role in inflammation regulation. Studies have shown that the levels of secondary bile acids in the intestines of patients with inflammatory bowel disease are lower compared to healthy controls (Lloyd-Price et al., 2019). Supplementation with secondary bile acids can alleviate inflammation in acute and chronic colitis models in mice (Sinha et al., 2020), and remodeling the gut microbiota to improve bile acid metabolism can reduce colitis (Zhou et al., 2023). These findings

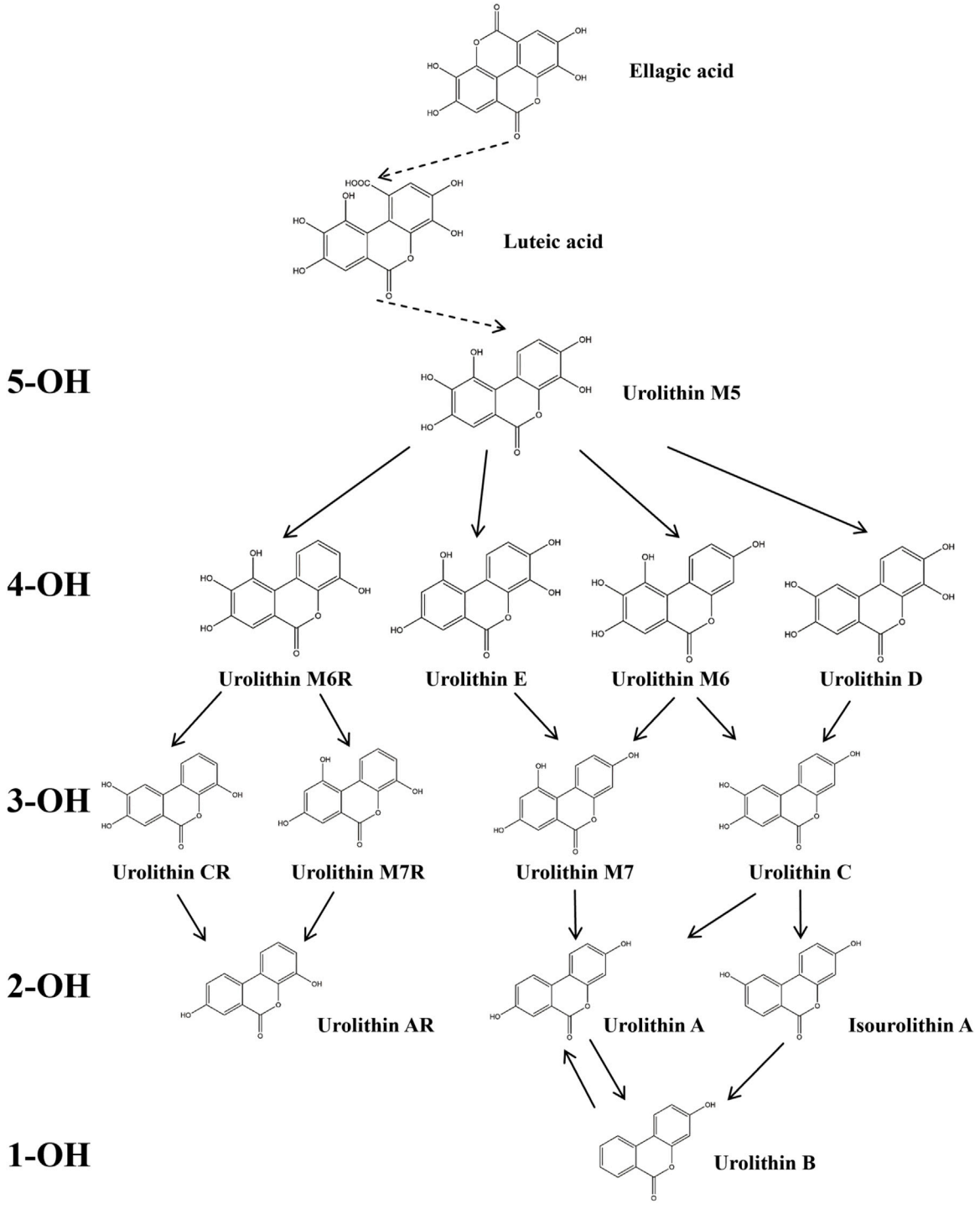



FIGURE 2
Degradation Pathway of ellagic Acid Metabolism into Uros. In the figure, Uros are classified based on the number of hydroxyl groups (5-OH, 4-OH, 3-OH, 2-OH, 1-OH), and the final products include urolithin A, urolithin B, and Isourolithin A (Xiang et al., 2022).

suggest that ellagitannins may modulate disease development through the gut microbiota-bile acid axis. The bidirectional regulation of ellagitannins and the gut microbiota offers a new perspective on their potential health benefits. However, current research still faces certain limitations, particularly the lack of systematic studies on the combined administration of ellagitannins with specific probiotics. Future in-depth exploration in this area may not only help elucidate their synergistic mechanisms but also open new avenues for the application of PPE in functional nutritional supplements.

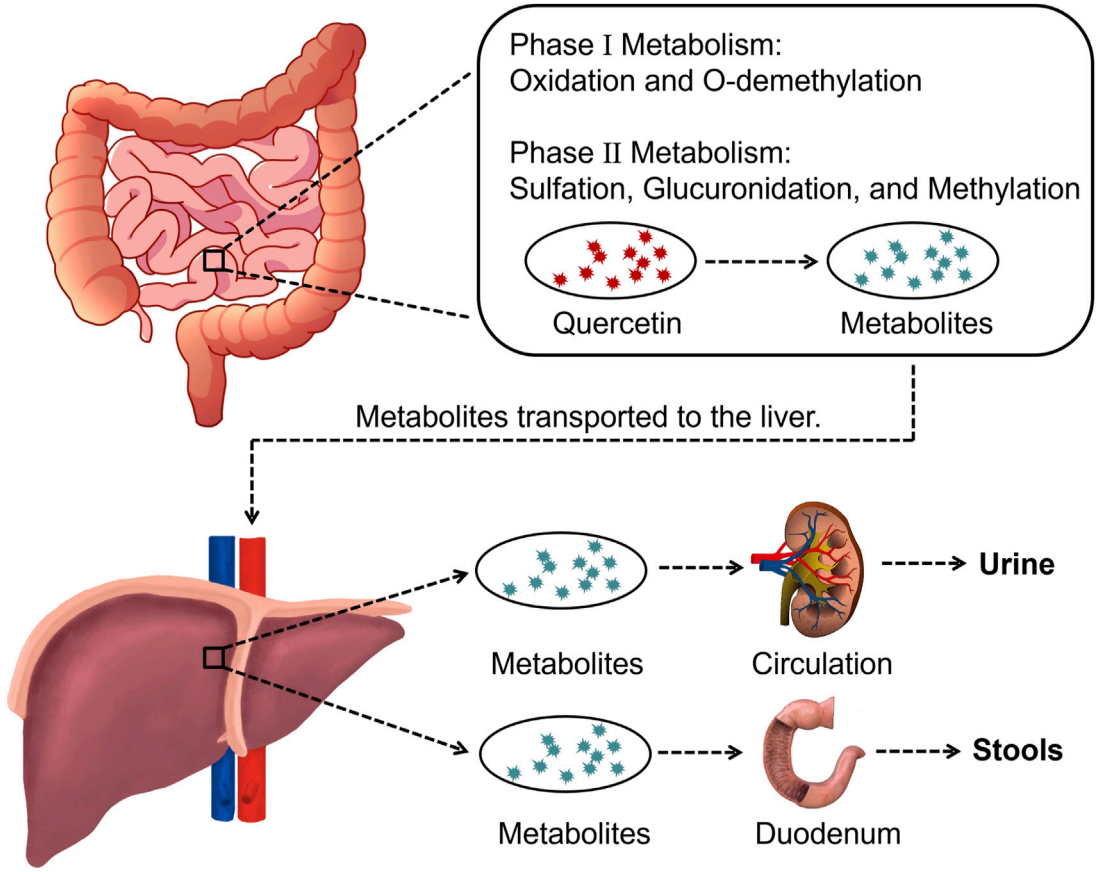



FIGURE 3
Overview of quercetin metabolization in the body.

## 3.2 Flavonoids

Pomegranate peel also contains flavonoids such as quercetin, catechin, and kaempferol. Among them, quercetin is one of the most common flavonoids, though its content in pomegranate peel is relatively low (Kumar et al., 2022). After oral administration, quercetin is metabolized into derivatives such as O-glycosides and C-glycosides. These metabolites are absorbed in the small intestine, bind to serum albumin, and are transported to the liver, where they undergo sulfation, glucuronidation, and methylation to form stable metabolites, which are then distributed throughout the body (Figure 3) (Manach et al., 1995; Nijveldt et al., 2001; Zhang et al., 2023). Quercetin exerts antioxidant effects by directly scavenging reactive oxygen species (ROS) and chelating metal ions (Min et al., 2019; Tang et al., 2014). It also improves cardiovascular health by modulating the hsp70/ERK/PPAR signaling pathway (Oyagbemi et al., 2018; Pereira et al., 2018).

Catechin is one of the most studied flavonoids in pharmacology and has been reported to possess diverse pharmacological activities. Catechin exhibits anticancer properties through mechanisms such as inducing apoptosis, inhibiting cancer cell proliferation, arresting the cell cycle, and regulating tumor-related signaling pathways (e.g., inhibiting AKT phosphorylation) (Shikha et al., 2024; Shukla et al., 2018; Sun et al., 2020). Manikandan et al. utilized HCT 15, HCT 116 (human colon adenocarcinoma cells), and Hep G-2 (human laryngeal carcinoma cells) as experimental models to investigate the synergistic anticancer activity of curcumin and catechin (Manikandan et al., 2012). The results indicated that both curcumin and catechin alone, as well as their combination, effectively inhibited the proliferation of HCT 15, HCT 116, and Hep G-2 cells. However, when combined, the cytotoxicity was more pronounced, significantly disrupting the monolayer of the cells. This suggests a synergistic effect between catechin and other anticancer agents like curcumin, enhancing the suppression of cancer cells. Notably, HCT 15, HCT 116, and Hep G-2 cells exhibited significant pathological differences, making it difficult to universally interpret the applicability of the synergistic effect. Furthermore, the study did not validate the shared targets of curcumin and catechin through molecular docking or protein interaction experiments. Therefore, future research should strengthen these aspects to clarify their molecular mechanisms. Additionally, catechin is an effective molecule for treating inflammation. It exerts anti-inflammatory effects by inhibiting the gene expression of pro-inflammatory cytokines such as IL-1α, IL-1β, IL-6, and IL-12p35, downregulating the activity of inflammatory enzymes like iNOS and COX-2, and suppressing key factors in inflammation signaling pathways such as NF-κB, FOXO3a, and SIRT1(Cheng et al., 2019). Moreover, catechin has been found to exhibit antibacterial activity

against various bacterial strains by disrupting bacterial membranes, inhibiting spore and vegetative cell growth, and inducing oxidative stress (Hara-Kudo et al., 2005; Parvez et al., 2019). However, despite these studies showcasing the bioactivity of catechins, their clinical efficacy may be limited due to their low bioavailability *in vivo*.

Kaempferol is another important flavonoid metabolite found in pomegranate peel, exhibiting multiple bioactivities such as antiviral, anti-inflammatory, anti-metabolic disorder, and cardiovascular protective effects (Al-Numair et al., 2015; Hussain et al., 2024; Huwait et al., 2022; Zhuang et al., 2017). In terms of antiviral effects, kaempferol effectively inhibits hepatitis B virus activity by suppressing the synthesis of hepatitis B surface antigen and hepatitis B e antigen (Parvez et al., 2022). Studies have revealed that increasing kaempferol concentration significantly reduces intracellular viral DNA levels (Li et al., 2008). Additionally, kaempferol demonstrates neuroprotective properties in neurodegenerative diseases. In Alzheimer's disease models of *Drosophila* expressing wild-type human amyloid-beta 42, kaempferol alleviates symptoms by reducing oxidative stress in the brain (Han et al., 2019). Li et al. induced Parkinson's disease in C57BL/6 mice by injecting MPTP (neurotoxin) and treated the mice with different doses (25, 50, and 100 mg/kg) of kaempferol for 14 consecutive days prior to MPTP injection (Li and Pu, 2011). The results showed that kaempferol could effectively protect dopaminergic neurons by increasing the activity of endogenous antioxidants (such as superoxide dismutase and glutathione peroxidase) and decreasing malondialdehyde levels. These studies confirmed the neuroprotective effects of kaempferol *in vitro* models. However, the MPTP model primarily simulates the motor symptoms of Parkinson's disease but does not fully replicate pathological features such as α-synuclein aggregation. Additionally, current research has not compared the efficacy of kaempferol with commonly used neuroprotective drugs. Future studies could consider using α-synuclein overexpressing transgenic mice to better simulate the pathological characteristics of Parkinson's disease, combined with comparative experiments, to further assess the relative efficacy and application potential of kaempferol in neuroprotection.

Granatin A and Granatin B are two flavonoids predominantly found in pomegranate peel, differing mainly in the type and number of glycosides they contain. Their content in pomegranate peel is relatively low, with yields of 0.003% and 0.013%, respectively, obtained *via* fractionation techniques such as column chromatography (Lee et al., 2010). Despite structural differences, Granatin A and Granatin B generally exhibit similar bioactivities, primarily in anti-inflammatory and anticancer applications. Research has shown that they selectively inhibit microsomal prostaglandin E synthase-1 expression in non-small cell lung cancer A549 cells, downregulate tumor necrosis factor-α, inducible nitric oxide synthase, and anti-apoptotic factor B-cell lymphoma 2, and induce apoptosis in A549 cells (Toda et al., 2020). Additionally, these metabolites effectively suppress NO production and nitric oxide synthase expression in RAW 264.7 macrophages, thereby exerting anti-inflammatory effects (Lee et al., 2010). These findings highlight the potential applications of Granatin A and Granatin B in anti-inflammatory and anticancer research. However, due to their low yields, this may limit their future application in the biomedical field.

## 3.3 Protocatechuic acid

Phenolic acids are an important subclass of polyphenolic metabolites, characterized by the presence of one or more hydroxyl (-OH) and carboxyl (-COOH) groups attached to a benzene ring (Sun and Shahrajabian, 2023; Wani et al., 2023). Protocatechuic acid (PCA), or 3,4-dihydroxybenzoic acid, is one of the main phenolic acids found in pomegranate peel and is also a metabolite of flavonoid metabolites such as quercetin (Azzini et al., 2010; Crozier et al., 2010; Singh et al., 2016). PCA exhibits various bioactivities, including antioxidant, anti-inflammatory, and antibacterial properties, playing an important role in the prevention and treatment of diseases such as cancer, diabetes, and Alzheimer's disease.

ROS are byproducts of aerobic metabolism and primarily include superoxide anion ($O_2^-$), hydrogen peroxide ($H_2O_2$), and hydroxyl radical (·OH). When ROS become free radicals due to unpaired electrons, if not neutralized by antioxidants in a timely manner, they can cause oxidative damage to biological macromolecules such as lipids, proteins, and DNA. This oxidative damage can lead to cellular dysfunction, aging, and the onset of various diseases (Song et al., 2020). As a natural polyphenolic metabolite, PCA exerts its antioxidant effects through multiple mechanisms. PCA enhances the activity of intracellular antioxidant enzymes, such as superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), promoting the clearance of ROS and alleviating oxidative stress in cells (Figure 4) (Cadena-Iniguez et al., 2024; Laskar and Mazumder, 2020). PCA also inhibits the Fenton reaction catalyzed by metal ions (e.g., $Fe^{2+}$, $Cu^+$), reducing the production of hydroxyl radicals and mitigating oxidative stress from the source (Shahidi and Ambigaipalan, 2015). Additionally, compared to the standard antioxidant Trolox, PCA has demonstrated stronger *in vitro* antioxidant activity in both lipid and aqueous environments (Kakkar and Bais, 2014). Therefore, with its multiple antioxidant mechanisms, PCA serves as an effective natural antioxidant, aiding in the prevention of aging and various diseases related to oxidative stress.

Inflammatory processes are regulated by various cytokines, chemokines, and inflammatory mediators. Pro-inflammatory cytokines such as TNF-α can bind to corresponding receptors and activate signaling pathways like NF-κB and MAPK, which in turn induce the expression of pro-inflammatory factors like IL-1 and IL-6, thereby exacerbating the inflammatory response (Al-Khayri et al., 2022; Hirano, 2021; Ren et al., 2020). In vitro experiments with RAW 264.7 cells, PCA was shown to inhibit the expression of TNF-α by modulating the activation of the NF-κB and MAPK pathways (Min et al., 2010). Wang et al. used AIN-93G diet containing PCA or standard AIN-93G diet to feed apoE knockout mice for 20 weeks (Wang et al., 2010). The animal experiment results showed that the PCA supplement reduced the area of aortic sinus plaques by 42%. PCA exerted anti-inflammatory effects by lowering the expression of Vascular Cell Adhesion Molecule 1 (VCAM-1) and Intercellular Adhesion Molecule 1 (ICAM-1) in the aorta, inhibiting NF-κB activity, and reducing the levels of soluble VCAM-1 and ICAM-1 in plasma, thereby inhibiting the development of atherosclerosis. These findings suggest that PCA, as a natural metabolite, can regulate inflammation-related signaling pathways, showing

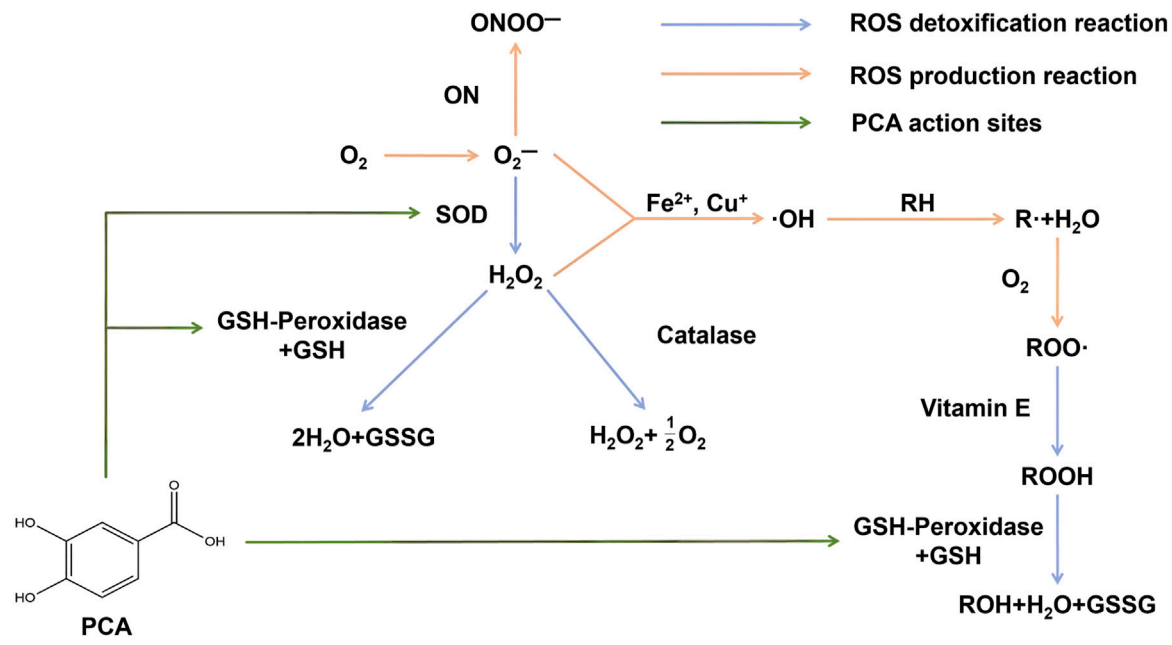



FIGURE 4
PCA can enhance the activity of SOD and GSH-PX enzymes, thereby playing a regulatory role in maintaining redox balance.

potential for anti-inflammatory applications and the treatment of inflammation-related diseases.

Among plant-derived active metabolites, antimicrobial potency generally decreases in the order of phenols, aldehydes, ketones, alcohols, ethers, and hydrocarbons, making polyphenolic extracts ideal candidates to replace traditional antimicrobial agents (Nguyen et al., 2018). As a polyphenolic metabolite derived from plants, PCA exhibits significant antibacterial activity, and has been shown to specifically inhibit the growth of *Pseudomonas aeruginosa*, *Listeria monocytogenes*, *Aeromonas hydrophila*, *Pasteurella multocida*, *Escherichia coli*, and *certain Salmonella strains*, while also reducing the pathogenicity of other bacteria such as *Proteus mirabilis* (Aldulaimi, 2017; Alvarado-Martinez et al., 2020; Bernal-Mercado et al., 2020; López-Romero et al., 2018; Torzewska and Rozalski, 2014). Research has indicated that the antimicrobial mechanism of PCA primarily involves disrupting bacterial membrane integrity, interfering with bacterial metabolism, and inhibiting biofilm formation (Tian L. et al., 2022; Wu et al., 2022; Yuan et al., 2024). Additionally, when used in combination with other antibiotics, PCA demonstrates synergistic effects, further enhancing its antibacterial efficacy (Fifere et al., 2022). These multiple mechanisms make PCA a promising candidate for antimicrobial applications.

Furthermore, PCA also possesses anti-apoptotic and anti-tumor activities. Abdelrahman et al. induced chronic liver injury and hepatic encephalopathy in male BALB/c mice (7–9 weeks old) through intraperitoneal injection of thioacetamide (200 mg/kg, 3 times per week) (Abdelrahman and El-Tanbouly, 2022). Starting from the fourth week, the mice were treated with oral PCA (100 mg/kg and 150 mg/kg). The results showed that PCA significantly restored the normal histological structure of the liver and brain tissues of the treated mice by downregulating the expression of Caspase-3 in the liver and the expression of tumor protein p53 in both the liver and brain, showing a significant anti-apoptotic effect. However, the PCA dosage used in the study was much higher than the normal dietary intake, which creates a gap in the clinical translational application. PCA's anti-apoptotic mechanisms are closely related to its regulation of the abnormal expression of mitochondrial pathway-related proteins such as Bcl-2-associated X protein and cytochrome c, and its ability to modulate the Nrf2/p62 pathway, reducing oxidative stress and apoptotic signaling in cells (Zhao et al., 2021). In addition to its anti-apoptotic effects, PCA exerts anti-tumor activity through various mechanisms. PCA significantly reduces the expression of matrix metalloproteinase 2 (MMP2) by inhibiting signal transduction in the RhoB/PKCε and Ras/Akt pathways, thereby preventing tumor cell migration and invasion (Cadena-Iniguez et al., 2024; Lin et al., 2011). PCA also inhibits the Heme Oxygenase-1 (HO-1) mediated activation of p21, reducing colorectal cancer cell viability and inducing apoptosis (Acquaviva et al., 2021). Moreover, when combined with anticancer drugs such as 5-fluorouracil, PCA enhances the efficacy of chemotherapy while minimizing drug-induced side effects (Motamedi et al., 2020). Through these various mechanisms, PCA shows promising anti-tumor activity and potential for use in combination with other anticancer drugs.

Gallic Acid is one of the major phenolic acid metabolites in pomegranate, particularly abundant in the peel. The concentration of gallic acid in every 100 g of pomegranate peel is approximately 128 mg (Singh et al., 2023). Gallic acid exhibits various biological properties, with its antioxidant capacity being the most notable (Wianowska and Olszowy-Tomczyk, 2023). For instance, gallic acid is used as a standard antioxidant in the DPPH method (Pyrzynska and Pękal, 2013), and its antioxidant activity has been shown to surpass common antioxidants such as uric acid, water-soluble vitamin C, and ascorbic acid (Antolovich et al., 2002). Methyl gallate, an ester derivative of gallic acid, also exhibits significant

antioxidant activity (Liang et al., 2023). Regarding anti-inflammatory activity, gallic acid can inhibit inflammation induced by lipopolysaccharides, including the release of nitric oxide, prostaglandin E2, interleukin-6, and cyclooxygenase-2 (BenSaad et al., 2017; Marinov et al., 2019). Its anti-inflammatory mechanism may involve the suppression of pro-inflammatory signaling pathways such as NF-κB, thereby alleviating inflammatory responses and exerting protective effects (Zhu et al., 2019). In terms of antitumor activity, Sun et al. investigated the effect of gallic acid on the HepG2 and SMMC-7721 human hepatocellular carcinoma (HCC) cell lines (Sun et al., 2016). The results showed that gallic acid effectively inhibited the growth of liver cancer cells, with IC50 values of 28.5 ± 1.6 μg/mL and 22.1 ± 1.4 μg/mL, respectively. Gallic acid suppressed the proliferation of both HepG2 and SMMC-7721 human liver cancer cells in a time- and dose-dependent manner by activating caspase-3 and caspase-9 and promoting the generation of reactive oxygen species. For antibacterial activity, gallic acid can inhibit the growth of pathogenic bacteria such as *Escherichia coli*, *S. aureus*, and *Campylobacter jejuni* by reducing membrane permeability and regulating pH levels (Kang et al., 2008; Sarjit et al., 2015). Moreover, gallic acid demonstrates anti-apoptotic activity by counteracting apoptosis mediators such as 6-hydroxydopamine (Erol-Dayi et al., 2012). Similarly, vanillic acid, which contains a phenolic hydroxyl group, can scavenge ROS and exhibits antioxidant properties (Parvin et al., 2020). Additionally, numerous studies have highlighted the pharmacological potential of vanillic acid in cardiovascular diseases (Khoshnam et al., 2018), inflammatory pain (Calixto-Campos et al., 2015), and asthma (Bai et al., 2019).

# 4 Biomedical applications of PPE

Pomegranate peel is rich in bioactive metabolites, exhibiting multiple health benefits, including antioxidant, anti-inflammatory, antibacterial, anti-apoptotic, and anticancer effects. The bioactive substances and biocompatibility of pomegranate peel make it an ideal candidate for the preparation of biomedical materials such as nanodrug carriers and hydrogels, offering promising applications for PPE in drug delivery, tissue repair, and regenerative medicine. Specifically, the antioxidant and anti-inflammatory properties of PPE help mitigate oxidative stress and inflammation caused by biomedical materials during their application, thereby enhancing the stability and therapeutic efficacy of these materials. Furthermore, PPE's inherent antibacterial activity provides infection resistance to biomedical materials, significantly improving their potential applications in wound healing and infection control. For instance, in the context of tissue engineering scaffolds, PPE can significantly enhance the bioactivity and antioxidant properties of scaffolds, thereby promoting cell adhesion, proliferation, and tissue repair. The bioactive molecules in PPE regulate cellular signaling pathways, effectively improving the inflammatory microenvironment and further accelerating tissue regeneration. Consequently, PPE demonstrates significant advantages in drug delivery, tissue repair, and regenerative medicine, paving the way for the development of novel biomedical materials with multifunctional properties.

## 4.1 Nanodrug carriers

Drug carriers can improve therapeutic outcomes by altering the pharmacokinetics and biodistribution of conventional drugs (bare drugs) or by acting as reservoirs and slow-release systems for conventional drugs (Cheng et al., 2021; Guo et al., 2021; Tenchov et al., 2021). To summarize, drug carriers must exhibit good biocompatibility and efficiently and safely deliver drugs to target sites. By providing sustained-release characteristics, these carriers can prolong drug action, reduce dosing frequency, and improve therapeutic outcomes. Nanodrug carriers can be broadly categorized into three types based on the materials used for their preparation: inorganic nanodrug carriers, lipid-based nanodrug carriers, and polymer-based nanodrug carriers.

Inorganic nanodrug carriers are typically made from metals, metal oxides, or carbon-based materials (such as gold nanoparticles, silver nanoparticles, silica nanoparticles, carbon nanotubes, *etc.*), which often possess unique optical, electrical, and magnetic properties, making them highly advantageous in cancer treatment, imaging, diagnostics, and other fields (Du et al., 2023; Khan et al., 2022; Khizar et al., 2023; Raza et al., 2021; Shang et al., 2022; Song et al., 2021). Taherian et al. prepared nanoparticles BPPE-CCMNPs containing PPE through a two-step precipitation method and evaluated their anticancer capabilities (Taherian et al., 2021). These nanoparticles, with a size of 37 nm, demonstrated stability in biological environments and showed a favorable release profile in tumor-simulating conditions. Cytotoxicity results indicated that these nanoparticles were toxic to cancer cells but non-toxic to normal cells, suggesting better prospects compared to chemotherapy drugs. Additionally, PPE can serve as a reducing agent and stabilizer for the green synthesis of gold and silver nanoparticles, which are used in antibacterial and cancer treatments (Fu et al., 2023; Ghobadi et al., 2024; Khan et al., 2021). Although researchers have developed various PPE-based inorganic nanodrug carriers using different methods, the potential of inorganic materials, particularly in exploring their optical, electrical, and magnetic properties, has not been fully harnessed. For instance, gold and silver nanoparticles hold significant promise in photothermal therapy and imaging. Further research is needed to combine the physical properties of these nanomaterials with the bioactivity of PPE to achieve dual or multiple therapeutic effects.

Lipid-based nanodrug carriers, composed of natural or synthetic lipid molecules (such as phospholipids, cholesterol, *etc.*), can spontaneously form bilayer structures called liposomes (Liu P. et al., 2022; Nsairat et al., 2022). Lipid-based nanodrug carriers can efficiently encapsulate both hydrophilic and hydrophobic drugs, and they offer high drug-loading capacity and good stability (Albertsen et al., 2022). Okumus et al. used maltodextrin and soybean lecithin to encapsulate PPE, with Zeta potentials of −18.05, −15.51, and −57.6 for PPE and the encapsulated nanoparticles, respectively, indicating that liposomal encapsulation can enhance PPE's stability (Okumus et al., 2021). Wang et al. prepared lipid carriers containing PPE using a warm microemulsion technique, and the nano-liposomes demonstrated sustained release for 12 h, with strong anti-tyrosinase activity (Tokton et al., 2014). However, despite the advantages of lipid-based nanodrug carriers, they still face challenges related to

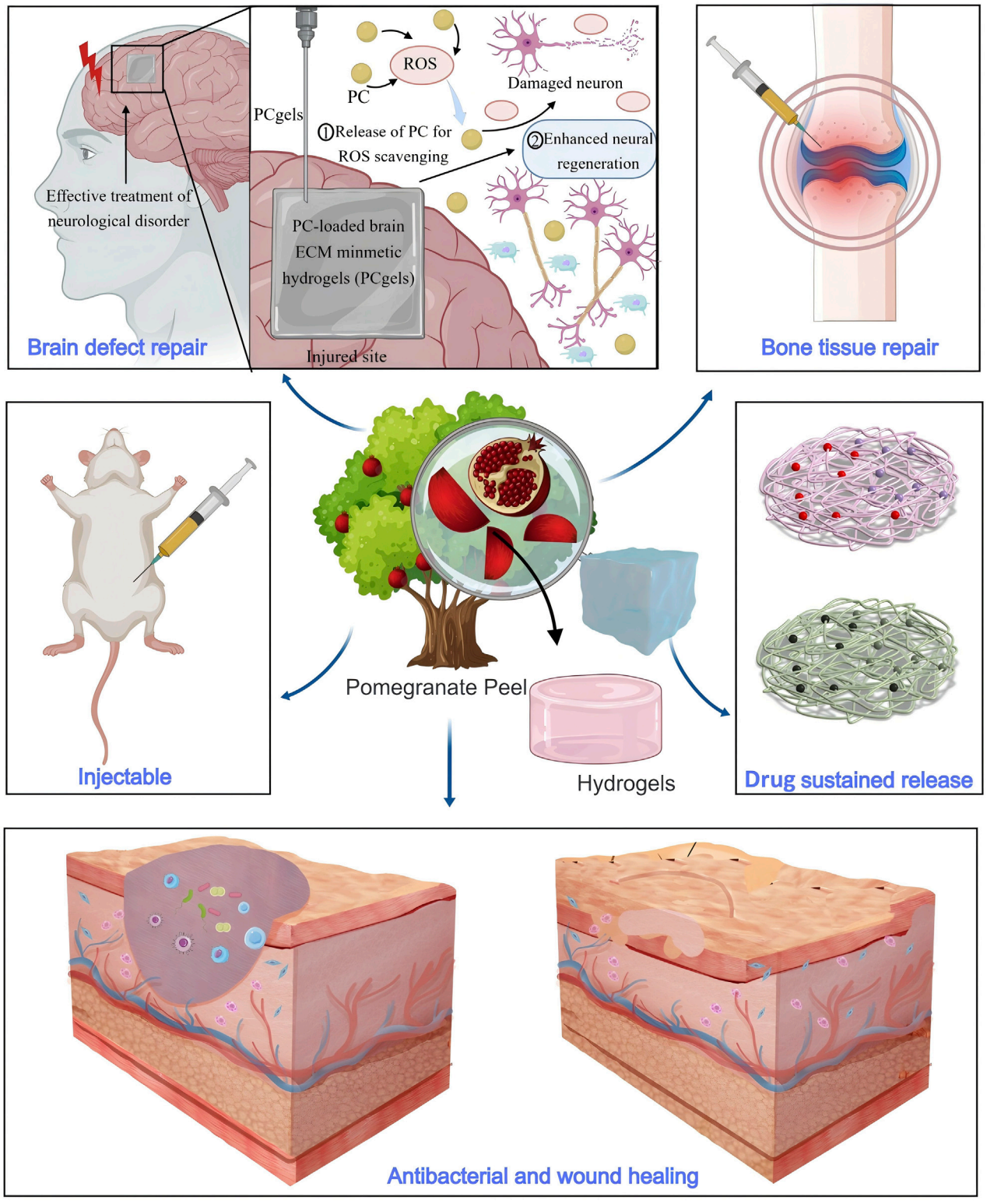



FIGURE 5
Hydrogels containing PPE exhibit properties such as injectable, antibacterial activity, and drug sustained release, making them suitable for applications in wound healing, brain defect repair, and bone tissue repair.

solubility, stability, and aggregation (Andishmand et al., 2023). Future efforts could focus on optimizing formulations or modifying the surface of nanoparticles to enhance the application potential of PPE in cancer therapy and other fields.

Polymer-based nanodrug carriers are another widely used carrier system, typically made from synthetic or natural polymer materials such as polylactic acid (PLA), polyethylene glycol (PEG), and poly (D,L-lactic-co-glycolic acid) (PLGA) (De et al., 2022; Dou

et al., 2020; Du et al., 2020; Ibrahim et al., 2022; Shi et al., 2022). These carriers possess tunable physicochemical properties and excellent biocompatibility, allowing for sustained drug release (Arkaban et al., 2022). Shirode et al. developed PLGA-PEG nanoparticles loaded with PPE (Shirode et al., 2015), while Soltanzadeh et al. employed chitosan nanoparticles to encapsulate PPE (Soltanzadeh et al., 2021), both nanoparticles exhibited good anticancer and antibacterial activity, along with sustained drug release. However, one major advantage of polymer-based nanocarriers is their ability to achieve targeted delivery to specific cells or tissues through surface functionalization (Ghosh and Biswas, 2021; Shi et al., 2021), research on PPE-based polymer nanocarriers remains largely unexplored.

In recent studies, many nano-drug carriers have been prepared using PPE. These studies highlight that PPE's biocompatibility and bioactivities—such as antibacterial and anticancer properties—endow nano-drug carriers with excellent compatibility and the ability to inhibit bacterial and cancer cell growth. However, these studies still face numerous challenges. Inorganic nano-drug carriers exhibit various properties depending on the materials used. For instance, metallic nanoparticles can be utilized to prepare photothermal and imaging systems for photodynamic therapy (Ouyang et al., 2021; Xing et al., 2018; Zhan et al., 2024), offering unique advantages in cancer treatment. Current research has yet to fully explore and leverage these properties. Combining these physical properties with the bioactivities of PPE could lead to more effective therapies. Additionally, targeting is a critical factor in evaluating the performance of drug carrier systems. Most anticancer nano-drug carriers prepared with PPE rely on the enhanced permeability and retention (EPR) effect for passive targeting, which has limitations, such as uneven distribution within tumor tissues or incomplete drug release (Gawali et al., 2023; Takakura and Takahashi, 2022). Future studies could explore strategies such as ligand or antibody modifications to enhance active targeting, ensuring precise delivery of carriers to target regions and effective release of drugs to maximize PPE's bioactivities.

## 4.2 Hydrogels

Hydrogels are a class of soft polymer materials with a three-dimensional network structure that can absorb large amounts of water or biological fluids while maintaining their shape and structure (Norahan et al., 2023; Qiao et al., 2023; Shao et al., 2023). Due to their good biocompatibility, tunable mechanical properties, and ability to mimic natural tissues, hydrogels are widely used in wound dressings and tissue engineering. The polyphenolic metabolites in PPE have antibacterial and antioxidant properties, making it an ideal additive for the preparation of functional hydrogels.

In terms of wound dressings, hydrogels containing PPE offer significant advantages (Figure 5). First, the polyphenolic metabolites in PPE effectively inhibit bacterial growth, reducing the risk of infection. Ul-Islam et al. developed an antibacterial wound dressing based on bacterial cellulose loaded with PPE (Ul-Islam et al., 2023). This dressing demonstrated good water absorption and retention capabilities, with excellent antibacterial performance, achieving 100% inhibition of *S. aureus* growth and 50% inhibition of *Escherichia coli*. Secondly, PPE can accelerate wound healing by regulating signaling pathways. Savekar et al. prepared a hydrogel film from PPE, citric acid, and β-cyclodextrin-carboxymethyl cassava starch, which could prevent wound infection and accelerate the healing process (Savekar et al., 2024). This film is biodegradable and exhibits pH-dependent ellagic acid release within 24 h. The released ellagic acid promotes cell division, proliferation, and migration to the wound site by inhibiting Glycogen Synthase Kinase-3 Beta and Casein Kinase 1, thereby promoting wound healing.

In the field of regenerative medicine, PPE-based hydrogels also show broad application potential. Hosseini et al. developed an injectable nanocomposite hydrogel based on polyethylene glycol and chitosan for sustained PPE release (Hosseini et al., 2023). This hydrogel can steadily release PPE for up to 360 h and effectively repair bone injuries by promoting osteoblast differentiation. Similarly, Garcia et al. developed a hydrogel for bone tissue repair, loaded with extracts from pomegranate peel, grape seed, and jabuticaba peel. The study showed that only the hydrogel containing PPE could form an inhibition zone around *S. aureus*, demonstrating significant antibacterial activity (Garcia et al., 2021). In addition to bone tissue repair, Ju et al. prepared an injectable hydrogel using PPE, hyaluronic acid, and collagen for brain defect repair (Ju et al., 2023). This hydrogel could be precisely injected through a 23-gauge needle (inner diameter 337 μm) to adapt to the deep folds of brain tissue and specific defect areas. The loaded PPE not only exhibited ROS scavenging properties but also promoted neuronal regeneration and differentiation, effectively repairing brain tissue defects.

Hydrogels, with their three-dimensional network structure, provide high hydration capacity, effectively maintaining a moist wound environment and promoting healing, making them ideal wound dressings. Hydrogels prepared with PPE can exhibit antibacterial and antioxidant properties, making them highly effective in preventing infections and accelerating wound healing. The polyphenolic metabolites in PPE effectively inhibit bacterial growth, reducing the risk of wound infection, while its antioxidant properties alleviate oxidative stress and promote cell repair. Additionally, PPE can regulate cellular signaling pathways, facilitating cell proliferation and migration to accelerate the wound healing process. However, despite the significant antibacterial and wound-healing effects of PPE-based hydrogels, challenges remain. For example, the mechanical properties and durability of hydrogels can sometimes be weak, which may affect their performance in long-term applications. Future research could explore combining PPE with cross-linking agents such as polyvinyl alcohol or extracellular matrix metabolites to enhance the mechanical properties and stability of hydrogels in complex wound environments (Chen et al., 2024; Morton et al., 2023). Furthermore, improving the controlled release mechanisms and structural design of hydrogels to introduce responsiveness to stimuli such as temperature and pH (Haidari et al., 2021; Yu et al., 2024) could enhance their effectiveness and reliability in long-term treatments.

## 4.3 Tissue engineering scaffolds

Tissue engineering scaffolds are three-dimensional porous solid materials that typically possess high mechanical strength. Their main purpose is to provide a temporary physical framework for

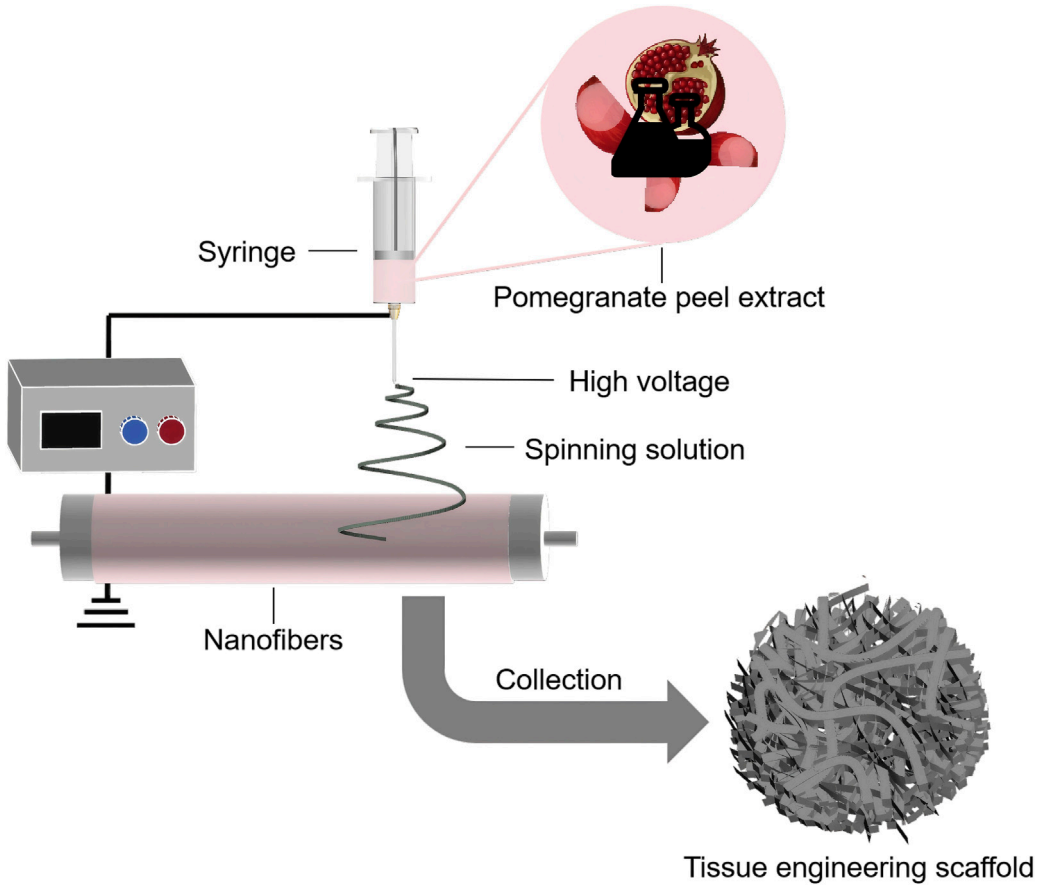



FIGURE 6
Schematic illustration of the preparation of PPE-containing tissue engineering scaffolds using electrospinning technology.

damaged tissues, facilitating cell attachment, migration, and proliferation through their porous structure, thereby offering the necessary physical support for the growth of new tissue (Arif et al., 2022; Janmohammadi et al., 2023; Zhang et al., 2022). PPE promotes osteoblast differentiation and proliferation, inhibits osteoclast activity, and exhibits significant anti-inflammatory and antibacterial properties, enabling it to accelerate tissue repair and reduce inflammatory responses. With these advantages, PPE has been utilized in the design of tissue engineering scaffolds.

PPE has been shown to promote osteoblast differentiation and proliferation, inhibit osteoclast activity, and exhibit significant anti-inflammatory and antibacterial properties, accelerating bone tissue repair and reducing inflammatory responses (Bahtiar et al., 2014; Kim et al., 2009; Siddiqui and Arshad, 2014; Spilmont et al., 2015). Owing to these advantages, PPE has been widely applied in the design of scaffolds for bone tissue repair (Figure 6). Sadek et al. used electrospinning technology to prepare a tissue engineering scaffold containing PPE, which demonstrated a nearly 96% improvement in antioxidant activity compared to scaffolds without PPE, along with significant enhancements in osteoblast attachment and proliferation (Sadek et al., 2021). Oliveira et al. further evaluated the effect of polymer scaffolds with PPE on repairing mandibular defects in rats (de Oliveira et al., 2024). The experiment involved three groups: no graft, polymer scaffolds with Brazilian palm resin, and polymer scaffolds with PPE. The average percentages of bone volume formed in these groups were 17.17% ± 2.68%, 27.45% ± 1.65%, and 34.07% ± 0.64% (mean ± standard deviation), with the PPE-containing polymer scaffolds showing the best bone repair ability. Additionally, Karabulut et al. used 3D printing technology to create dental membrane scaffolds containing PPE, and their study showed that the addition of PPE significantly enhanced the scaffold's biocompatibility and antibacterial activity (Karabulut et al., 2023).

Tissue engineering scaffolds based on PPE have achieved notable results in the field of bone tissue repair, owing to their exceptional bioactivity. Currently, tissue engineering scaffolds have been widely applied in the repair of soft tissue, cardiovascular systems, and skin (Chen et al., 2023; Roacho-Perez et al., 2022; Weems et al., 2021). By incorporating drugs or enabling controlled release of gases (e.g., oxygen, nitrogen, *etc.*) (Augustine et al., 2023; Li et al., 2020; Mo et al., 2023), these scaffolds can provide more personalized therapeutic effects. Future studies could explore the potential of PPE in repairing other types of tissues and facilitating combination therapies.

## 4.4 Other biomedical applications

In addition to being an important component of nanodrug carriers, hydrogels, and tissue engineering scaffolds, PPE is also

widely applied in fields such as skin repair in the form of ointments and films. In skin repair studies, Luo et al. successfully developed a pomegranate polyphenol ointment and applied it to a scarred rat model (Luo et al., 2022). The results showed that the ointment significantly reduced the damaged area on the rats' skin and decreased the expression levels of proteins such as Alpha-Smooth Muscle Actin, Phosphorylated Extracellular Signal-Regulated Kinase 1/2, Phosphorylated Janus Kinase 2, Phosphorylated Signal Transducer and Activator of Transcription 3, and Phosphorylated Protein Kinase B. These effects effectively alleviated inflammatory responses, inhibited scar formation, and promoted the skin repair process. Similarly, Hayouni et al. prepared a skin repair ointment using PPE, which demonstrated outstanding antioxidant and antibacterial activities. The ointment significantly accelerated wound contraction and epithelialization in guinea pigs and exhibited potent antibacterial effects against *P. aeruginosa*, *S. aureus*, and *Escherichia coli* (Hayouni et al., 2011). Additionally, Barbalinardo et al. combined PPE with natural silk fibroin to create a transparent, flexible, and bioactive film (Barbalinardo et al., 2022). This film, when applied to skin repair, effectively reduced oxidative stress-induced cell damage and promoted skin regeneration and repair. Beyond skin repair, Jebahi et al. developed a medical dressing using PPE for the treatment of gastric ulcers (Jebahi et al., 2022). In a rabbit gastric ulcer model, the dressing exhibited high blood compatibility (with a hemolysis rate of only 0.42%) and significant antibacterial activity against *S. aureus* and *Helicobacter pylori*, with inhibition rates of 84.62% and 88.41%, respectively, after 18 h of contact. The dressing effectively reduced the incidence and severity of gastric tissue lesions in rabbits, demonstrating excellent anti-ulcer effects. Furthermore, Silva developed an oral adhesive ointment using PPE to improve canine oral hygiene. This ointment showed significant antibacterial activity against oral microbiota in Labrador dogs (Silva et al., 2020). In summary, PPE, with its excellent antioxidant, antibacterial, and anti-inflammatory properties, provides innovative solutions for skin repair, gastric ulcer treatment, and oral care.

With the continued advancement of biomedical materials research, more natural biomaterials with favorable bioactivity are being developed and applied in emerging fields such as microneedle patches and medical adhesives (Ding et al., 2024; George and Suchithra, 2019; Medlej et al., 2022; Ryall et al., 2022). Although there are currently no studies on the application of PPE in these areas, its natural bioactivity and good biocompatibility suggest tremendous application potential. Future research could focus on further developing PPE for use in microneedle patches, tissue adhesives, and other biomedical materials, opening up new directions for research and application.

## 5 Conclusion

PPE is rich in bioactive metabolites such as ellagic acid, phenolic acids, and flavonoids, which possess multiple functions including antioxidant, anti-inflammatory, antimicrobial, anti-apoptotic, and cell regeneration-promoting properties. These qualities present a wide range of potential applications in biomedical fields such as nanoparticle drug delivery systems, hydrogels, and tissue engineering scaffolds. Through various preparation techniques, PPE can be effectively integrated into medical materials, significantly enhancing their biocompatibility and functionality, promoting wound healing and tissue regeneration, and showing potential in anticancer therapy. The combination of natural extracts with modern technologies not only promotes the development of green biomaterials but also provides new avenues for efficient and safe treatments. However, further research is needed to address issues such as long-term stability, precise delivery, and the mechanisms of action of PPE. In the future, its application techniques should be optimized, and its clinical therapeutic potential should be further explored to achieve broader medical applications.

## Author contributions

JD: Validation, Writing–original draft, Conceptualization, Writing–review and editing. HW: Data curation, Writing–original draft. LZ: Data curation, Writing–original draft. SW: Data curation, Writing–original draft. XM: Visualization, Writing–original draft. HD: Visualization, Writing–original draft. XZ: Visualization, Writing–original draft. MZ: Funding acquisition, Writing–review and editing. YH: Funding acquisition, Writing–review and editing.

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## Conflict of interest

Author MZ was employed by Lanshu Cosmetics Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

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# Sustainability and role of plant-based diets in chronic kidney disease prevention and treatment

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Chronic kidney disease (CKD) affects 10% of the world's population (namely, 800 million of people) and an increase in CKD prevalence has been observed over the years. This phenomenon in developed countries is related to the spread of chronic degenerative non-communicable diseases (CDNCDs), such as diabetes mellitus, arterial hypertension, obesity, etc., while in low-income to middle-income countries, the CKD prevalence is attributable not only to CDNCDs, but also to infection conditions (like HIV, hepatitis, *etc.*). Another important difference lies in the age of onset of CKD, which is about 20 years lower in developing countries compared to developed ones. Therefore, CKD is becoming a public health problem, requiring preventive and treatment strategies to counteract its spread and to slow its progression. Moreover, the healthcare costs for the CKD management increase as the disease progresses. In this regard, the approach to prevent and reduce the CKD progression involves pharmacological and nutritional treatments (like Mediterranean Diet, MedRen diet, Flexitarian Diet, Vegetarian Diet and Plant-dominant Low Protein Diet) in order to improve the patients' quality of life and, at the same time, promote the environmental sustainability. Recent studies have highlighted the benefits of these diets not only for individuals, but also for environment. In particular, plant-based diets have increasingly gained an important role in the prevention and management of chronic diseases, including CKD. In fact, recent scientific studies have highlighted how a greater adherence to predominantly plant-based diets, is associated with a lower risk in developing CKD and also in slowing its progression. With regard to environmental sustainability, it is known how our food choices influence the climate crisis, since the food sector contributes for the 25% to the greenhouse gas emissions. Therefore, to reduce the consumption of animal proteins and to replace them with plant-based proteins are key strategies for sustainability and health, also supported by the European policies. In this context, food industries are starting to increase the offer of plant-based products that have similar characteristics, both sensorial and nutritional, to those of animal origin. This innovation, in fact, presents difficulties due to the perception of taste and the organoleptic appearance of these products. An additional challenge concerns the resistance of the traditional food industry and the lack of awareness of the consumer. The paradigm shift is dictated by the obtained benefits for health

and for environment. Life cycle assessment studies have compared the land footprint, carbon footprint and blue water footprint of plant-based products with those of animal origin and pointed out the lower environmental impact of the former. In conclusion, the adoption of sustainable food models will slow down the spread of CDNCDs, such as CKD, positively impacting both on human health and on planet, significantly reducing the costs and resources of the National Health Systems, since they absorb up to 70%–80% of the healthcare costs.

KEYWORDS

**sustainable diets, natural bioactive compounds, PLADO diet, chronic kidney disease, carbon footprint, Mediterranean diet, renal diets, circular economy**

## 1 Introduction

Chronic kidney disease (CKD) is a progressive and degenerative disease that affects approximately 10% of the world's population (10.4% men and 11.8% women), namely, more than 800 million individuals (Mills et al., 2015; Jager et al., 2019). For example, it is estimated that it affects 6%–7% of the Italian population (Ministero della Salute, 2020) and the 11%–12% of the English population (Hounkpatin et al., 2020).

This high incidence is due to the widespread presence of its main risk factors (such as diabetes mellitus, arterial hypertension and obesity, *etc.*) as they could impact on the CKD onset and worsening, and to the world's population aging (Lv and Zhang, 2019). In detail, diabetes mellitus is considered one of the major causes of CKD, as the presence of chronic hyperglycemia damages the kidney blood vessels, impairing their ability to filter blood. This impairment leads to the diabetic nephropathy, a condition characterized by albuminuria and the progressive reduction in kidney function (Kumar et al., 2023). At the same time, arterial hypertension too is considered one of the major risk factors for CKD development, because of the presence of high blood pressure progressively damages renal microcirculation, consequently reducing the kidney filtration capacity and causing kidney failure (De Bhailis and Kalra, 2022). Moreover, the obesity is able to induce renal dysfunction through direct and indirect mechanisms. The first one is related to the production by adipose tissue of several factors that causes hemodynamic changes, inflammation, oxidative stress, inducing the obesity-related glomerulophaty. The second one is related to obesity complications, such as atherosclerosis, arterial hypertension (due to the hyperactivation of renin-angiotensin-aldosterone system-RAAS), and diabetes mellitus (Nawaz et al., 2023). In low-income to middle-income countries, the CKD prevalence is attributable not only to CDNCDs, but also to infection conditions (like HIV, hepatitis, *etc.*) (George et al., 2017).

Although mortality in the end-stage kidney disease (ESKD), namely, the final stage of CKD, significantly decreased in recent years, CKD continues to represent one of the main causes of death worldwide. In fact, as the Global Burden of Disease (GBD) has estimated, CKD will become the fifth leading cause of death in the world by 2040 (Kovesdy, 2022). This prediction has been formulated according to epidemiological studies that affirms the increasing prevalence of CKD over the years. This enhancement is due to, as already discussed, the aging of the population and the spread of CKD risk factors like diabetes mellitus, arterial hypertension and obesity. Other reasons that will bring to this data is attributable to the underdiagnosis of CKD in the early stages, which contributes to its progression to ESKD and an increased risk of cardiovascular mortality.

The death among CKD patients is mainly related to cardiovascular diseases (Tonelli et al., 2006). CKD patients, especially in the more advanced stages, are at high risk of developing heart failure, arrhythmias, coronary artery disease and sudden cardiac death. The CKD determines the onset of a persistent, chronic and systemic microinflammation that causes the remodeling of blood vessels, stimulating the development of atherosclerotic lesions, the vessels calcifications, the vascular aging, as well as the cardiac muscle fibrosis and heart valves calcifications (Jankowski et al., 2021).

For managing the complex clinical picture of CKD patients, the healthcare costs are substantial. In fact, a CKD patient involves a huge consumption of resources, which increases significantly with the disease progression. These include the direct costs, namely, the managing costs for CKD in the phases of prevention, diagnosis, treatment of the patient (like drugs, medical visits, laboratory tests, instrumental diagnostics, dialytic treatments, hospitalization, *etc.*), and the indirect ones, which refer to the lower productivity of the patient and caregivers, which is further reduced as the disease progresses (Golestaneh et al., 2017; Turchetti et al., 2017).

From a study conducted in 31 Countries, the average costs for the conservative treatment of CKD in the 3a-3b stages, are approximately $3,000–3,500 *per* year, while the costs for the treatment of stage 4 rise up to $5,000 *per* year and they reach more than $8,000 *per* year in the stage 5 (Jha et al., 2023). Moreover, costs for renal replacement treatments (RRTs) increase significantly. On average, hemodialysis costs are $57,000 *per* year, peritoneal dialysis are $49,000 *per* year, and renal transplant approximately are $75,000 in the first year after transplant and $16,000 starting from the second year (Dessi et al., 2014; Jha et al., 2023).

In particular, the Authors observed a four-fold increase in the costs of managing a patient in stage G5, compared to stage G3a (Jha et al., 2023). These healthcare costs increase significantly when the patient is affected by additional CKD-related comorbidities, such as arterial hypertension, CKD mineral bone disorders, *etc.*, (Taccone-Gallucci et al., 2010; Cannata-Andia et al., 2021; Gupta et al., 2023; Li et al., 2023). In detail, the mean annual costs for the management of CKD-related complication are $18,294 for myocardial infarction, $8463 for heart failure, $10,168 for stroke and $5975 for acute kidney injury (Jha et al., 2023). It is estimated that these annual costs are bound to rise, due to the growing CKD incidence and the rampant increase in its risk factors, which accelerate its progression

towards the ESKD. In a recent study, a microsimulation model estimated that in 2027, healthcare costs for CKD diagnosis increase up to 9.3% compared to 2022, while those attributed to kidney transplantation up to 10% (Chadban et al., 2024; Tinti et al., 2019).

In general, it has been demonstrated that the healthcare costs of ESKD correspond to 4.7 times the costs for patients with early-stage CKD, highlighting the enormous importance of an early diagnosis. In fact, the earlier the diagnosis CKD is, the sooner patients are taken into care (implementing strategies aimed at slowing the progression of the disease towards the terminal phase). Consequently, the economic savings for their management is greater (Jommi et al., 2018).

As asserted, it is fundamental to adopt preventive strategies aimed at slowing down the CKD progression towards ESKD and at counteracting the onset of CKD-related comorbidities, due to the enormous healthcare costs necessary for CKD treatment. These preventive strategies involve a combination of pharmacological therapies and non-pharmacological adjuvant strategies, which aim at modifying lifestyle habits through the promotion of healthy diets and a regular physical activity (Dabek et al., 2023). In fact, it has been demonstrated that implementing personalized nutritional therapies, starting from the early CKD stages, allows for huge savings in healthcare costs, mainly because it delays the need for RRTs. Specifically, it has been estimated that in Italy the cost of a patient following a nutritional therapy with controlled protein content is around 700 € *per* month; this is a very small cost compared to those estimated for RRTs, which would allow for savings of around 25% of the annual costs necessary for the treatment of CKD patient covered by the NHS (Mennini et al., 2014).

In this context, the nutritional therapies based on the consumption mainly of plant-based foods play a key role. In fact, these types of diets are rich in natural bioactive compounds (NBCs) with numerous beneficial properties for CKD patient's health. Numerous studies have shown that the consumption of plant-based foods in CKD patients is able to reduce drug treatments, to counteract the development of the main complications of the disease and slow down its progression, and in the meantime to increase the patients survival and improve their quality of life (Joshi et al., 2021; Grazioli et al., 2022; Marrone et al., 2024b).

In this perspective, it is important to underline how these nutritional therapies do not offer important benefits only for the patient himself, but also for the environment. However, it is important to underline that dietary restrictions can be challenging for patients who must follow nutritional plans that limit the intake of protein, sodium, potassium and phosphorus. Symptoms of the disease, such as anorexia, nausea and alterations in taste, can also make it difficult to follow the nutritional plan. Economic factors can also negatively affect adherence to the nutritional plan, since organic foods are more expensive than industrial ones. Finally, managing a personalized diet requires time and the will to be followed, but unfortunately not all patients have the resources to make the prescriptions in practice.

In 2015, the United Nations Organization decided to adopt 17 Sustainable Development Goals (SDGs) that are part of the 2030 Agenda. This is a real call to action that commits all member Countries to achieve a series of goals by 2030, the priority is for: i) wellbeing of the person, ii) protection of the planet and iii) peace and prosperity for man and the planet (2025). The implementation of pant-based diets supports these goals thanks to their sustainability, and thus saving important resources (such as water and land) and reducing greenhouse gas emissions (Kraak and Aschemann-Witzel, 2024).

The aim of this review is to describe the main beneficial effects of plant-based diets with a controlled protein intake for CKD patients and to highlight the importance of their environmental sustainability. In this review, we will analyze the main nutritional diet treatments for CKD patients under conservative therapy, such as Low-Protein diet (LPD), Mediterranean (MD) (MedRen diet and Flexitarian Diet), Plant-Dominant Low-Protein (PLADO) diet and vegetarian diet, giving a particular attention to their environmental sustainability.

## 2 Low-protein diet

In Italy, the origin of LPD can be traced to the Dogma of Giovannetti and Maggiore of the University of Pisa, published on Lancet in 1964 (Giovannetti and Maggiore, 1964). The first half of the 1960s was a historical period in which RRTs were not well developed yet and were scarcely available for CKD patients. For this reason, they often died due to the clinical picture related to terminal uremia.

In developed Countries, the RRTs become available for a greater number of ESKD patients in 1966 (Jacobs, 2009). Therefore, previously, it was necessary to develop a dietary-nutritional therapy (DNT) that allowed CKD patients to survive, slowing down the decline in kidney function and consequently to delay the dialysis treatment. In the study conducted by Giovannetti and Maggiore, eight severe chronic uremic patients have been treated with a LPD, which replaced the protein-deficient diet, characterized by a protein intake of 0.5 g/kg b.w./day and by an adequate caloric intake, up to a 10-month period. The LPD was supplemented with essential amino acids at the dose of 1.74 g per day, fractionated into four or five portions and assumed during the meals, and daily protein intake was achieved by taking a maximum of 2.2 g of high biological value egg proteins *per* day. The Authors concluded that LPD was able to reduce blood urea concentration and the negativity of the nitrogen balance, to maintain for a longer time the residual kidney function and to improve the uremic symptoms. The nitrogen balance became positive or reached the balance when essential amino acids or egg proteins were assumed, without impact significantly on blood urea concentration, thus explaining the re-use of the protein catabolites. Therefore, the LPD is born in 1964 and subsequently it spreads over the years in order to reduce the protein catabolites and to prevent muscle proteolysis. Moreover, in chronic uremia, it has been proved that very low amounts of dietary nitrogen with a high biological value may be sufficient to maintain nitrogen equilibrium (Giovannetti and Maggiore, 1964).

Since the 1980s, the goals of the LPD have changed with the hyperfiltration theory of Barry M. Benner. This theory states that an excessive protein intake causes hyperfiltration and glomerular hypertension, resulting in a faster progression of the kidney damage. Since then, several experimental studies evaluated the LPD effects on preventing glomerular hypertension, on reducing progressive glomerular damage and on the CKD progression

(Brenner et al., 1996; Santoro, 2008). LPD reduces the glomerular hyperfiltration through a nephroprotective hemodynamic mechanism, which can be explained by the induction of vascular tone increases of the afferent arteriole, resulting in its vasoconstriction. The synergism of the renin-angiotensin-aldosterone system inhibitors with the low-sodium intake results in a superior additive effect, compared to single pharmacological treatment, through the dilatation of the efferent arteriole and the reduction of intraglomerular pressure and glomerular damage (Kalantar-Zadeh and Fouque, 2017; Cupisti et al., 2020). Moreover, the LPD further reduces the glomerular hyperfiltration and the consequent CKD progression, through these actions: i) the mitigation the mesangial cell signaling, leading to a lower expression of transforming growth factor β and consequently to a reduced interstitial fibrosis; ii) the reduction of nitrogenous compounds, leading to a less production of ammonia and other uremic toxins; iii) the positively impact the gut microbiota, with a lower production of trimethylamine N-Oxide, p-cresol and indoxyl sulfate (namely, the gut-derived uremic toxins); iv) the decrease of acid load; v) the reduction of advanced glycation end products (Kalantar-Zadeh and Fouque, 2017).

The LPD paradigm evolved with the advent of the sodium-glucose-transporter 2 inhibitors (SGLT2i). In fact, in CKD patients, it can be assumed the synergistic effect of LPD and SGLT2i on glomerular hemodynamics. The antiproteinuric and nephroprotective effects are made possible by the fact that both treatments act at the level of the afferent arterial, causing its vasoconstriction and, in CKD patients with type II diabetes mellitus, also at the level of the efferent arterial, increasing its caliber. The result is a reduction in albuminuria (Giannese et al., 2023).

In 1994, Saulo Klahr et al., published on The New England Journal (NEJ) of Medicine the Modification of Diet in Renal Disease Study Group, conducted on 585 patients with GFR comprised between 25 and 55 mL/min/1.73 $m^2$. The Authors realized for the first time that the LPD (0.58 g of protein/kg b. w./day) initially reduced GFR, compared to the usual-protein diet (1.3 g of protein/kg b. w./day). They argued that the steeper initial decline probably reflected a hemodynamic response to the reduction in protein intake, rather than an effective CKD progression. As the months passed, patients undergoing LPD experimented a lighter GFR decrease, with a beneficial effect on the CKD progression. After 36 months from baseline, GFR was reduced by 10.3 mL/min/1.73 $m^2$ in the LPD group and by 11.2 mL/min/1.73 $m^2$ in the usual protein group, without significant differences (Klahr et al., 1994). Although the results were very encouraging, it is necessary to underline the possible limitations of the study, first of all, the difficulty of adhering to dietary recommendations of CKD patients, especially in the long term. In fact, the dietary restrictions imposed can reduce the quality of life of patients, who may feel frustrated or isolated, and this would lead them, if not sufficiently supported, to have poor adherence.

Thirty years later, it was published the effect of dapagliflozin in CKD patients, with or without type 2 diabetes mellitus, with a GFR comprised between 25 and 75 mL/min/1.73 $m^2$. The initial hemodynamic effect was the same observed with LPD, while the final outcome was more enhanced. After 30 months from baseline, GFR was reduced by 8.58 mL/min/1.73 $m^2$ in the dapagliflozin group and by 11.37 mL/min/1.73 $m^2$ in the standard care group, with a significant difference (Heerspink et al., 2020).

In 1998 Bertram L. Kasiske et al., published a meta-analysis that described the effects of dietary protein restriction on the rate of decline in renal function. The Authors analyzed 13 randomized controlled trials, highlighting that a dietary protein restriction reduced the rate of GFR decline by only 0.53 mL/min/year. They concluded that, although LPD delayed the decline of GFR, the relatively weak magnitude of this effect suggests that combined therapies (pharmacological and dietic) are necessary to slow the rate of CKD progression (Kasiske et al., 1998).

In the 21st century, Denis Fouque and Maurice Laville collected ten studies, conducted from 1987 to 2008. A total of 2000 patients were analyzed; in particular, 1002 had received a LPD and 998 a higher protein diet. They showed that in CKD patients, the compliance to the LPD significantly reduced the number of ESKD patients by about 32%, delaying the need to start dialysis treatment (Fouque and Laville, 2009).

In the same century, Giacomo Garibotto et al., confirmed that CKD patients were able to maintain nitrogen balance despite a significantly lower protein intake, but how and to what extent muscle protein metabolism adapts to a LPD was still unexplored. The Authors demonstrated that the LPD (0.55 g of protein/kg b. w./day), compared to the conventional diet (1.1 g of protein/kg b. w./day), induced i) a decrease in muscle protein degradation, ii) the nitrogen balance, iii) no change in muscle protein synthesis, iv) a slight decrease in whole-body protein degradation and v) an increase in the efficiency of muscle protein turnover (Garibotto et al., 2018), thus promoting a physiological body composition in these patients.

The key elements in the success and safety of the LPD are the proper setting of drug therapy by the nephrologists, the proper setting of DNT therapy by the nutritionists and the appropriate adherence to the DNT by the patients. In this regard, in 2018 Cupisti et al. published a consensus document, defining twenty essential points to highlight several relevant aspects of the nutritional approach in patients with a CKD advanced stage. The number five and the number eleven reiterate how an adequate DNT must provide, in addition to the reduction of protein intake, an adequate calorie intake, a reduced intake of sodium, a reduced or controlled intake of phosphorus and potassium and a limitation of the fixed acids load (Cupisti et al., 2018a). Although the leitmotif of the LPD is the reduced protein intake, The National Kidney Foundation's Kidney Disease Outcomes Quality Initiative (KDOQI) guidelines recommend an energy intake between 25 and 35 kcal/kg b.w./day, adjustable according to age, sex, level of physical activity, body composition and body weight goals. In fact, if patients' daily caloric requirements are not met, the nitrogen balance becomes negative with protein degradation and loss of lean body mass (Cupisti et al., 2018a; Ikizler et al., 2020). To confirm this, in the study conducted by Nanhui Zhang et al., a protein-controlled DNT, characterized by a reduced carbohydrate-derived caloric intake (150 g carbohydrates/day vs. 264 g carbohydrates/day), increases the risk of all-causes mortality (Zhang et al., 2022). Regarding micronutrients, special attention should be paid to an intake less than 3,000 mg/day of potassium, (Cupisti et al., 2018b), 700 mg/day of phosphorus (Cupisti et al., 2018a) and 2.3 g/day of sodium (Ikizler et al., 2020).

The statement number twelve of the consensus document confirms that LPD may delay the need for RRT, while the number sixteen states that an appropriate DNT allows to contain the costs and the resources by National Healthcare Systems, for the

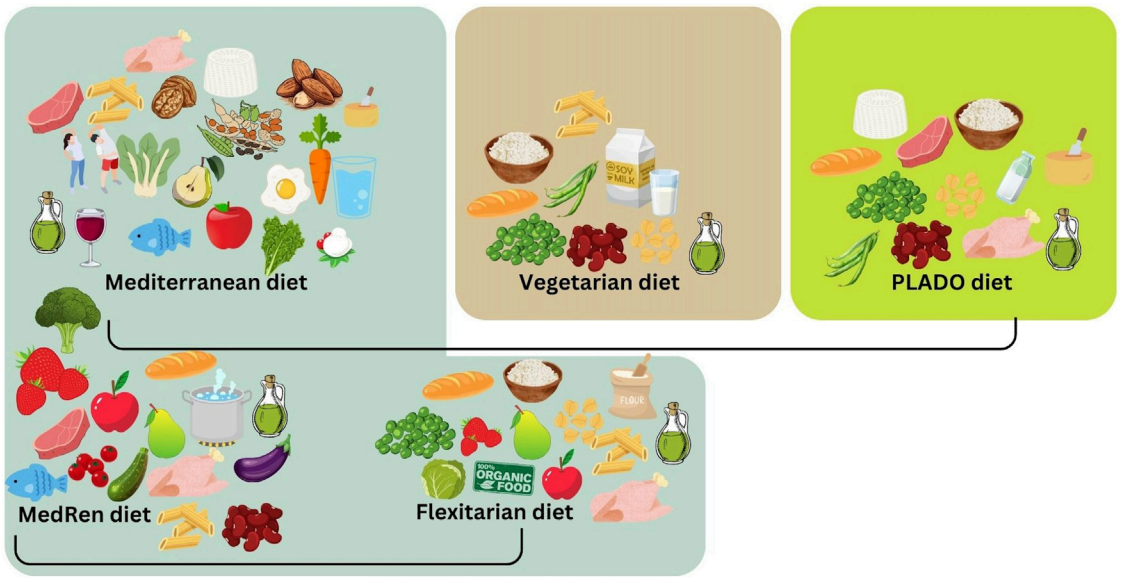



FIGURE 1
Possible sustainable nutritional patterns in chronic kidney disease (CKD) patients under conservative therapy.

clinical management of ESKD patients (Cupisti et al., 2018a). However, patient eligibility for the LPD remains at the discretion of the nephrologists (Ikizler et al., 2020; Kidney Disease: Improving Global Outcomes, 2024). In this regard, the recent publication of the NEJ about an interactive clinical decision inherent to a patient eligible for the LPD collected 2,628 total responses: 38% recommended it, while 61% did not (Chang et al., 2024).

Over time, LPD has also been positively influenced by issues, related to environmental sustainability and how plant-based foods are beneficial to the organism, so much so that predominantly plant-based declinations of LPD were born (Kalantar-Zadeh et al., 2020).

## 3 Possible advantages of plant-based diets in chronic kidney disease

In recent years, increasing evidence has highlighted the advantages of plant-based diets in preventing and managing lifestyle-related diseases, including CKD (Katz and Meller, 2014). Currently, many studies suggest a lot of benefits in using this dietary approach for treating CKD and its common comorbidities (De Angelis et al., 2007; Joshi et al., 2020).

Enhancing the nutrient profile of patients' diets by incorporating more plant-based foods and reducing animal protein may help to decrease the reliance on nephroprotective medications, to counteract CKD complications and potentially to slow the disease progression, thus improving patient survival rates (Patel et al., 2012; Haghighatdoost et al., 2017; Haring et al., 2017).

Among the main criticisms and objections to these diets, there are the hyperkalemia and the protein deficiency. By thoroughly evaluating these potential risks, it emerges that these events may be less severe than believed, while the benefits are substantial (Babich et al., 2023). Overall, the risk-benefit ratio seems to increasingly support the broader adoption of plant-based diets.

More generally, the plant-based diets, if not well balanced, may be deficient in essential amino acids. In order to avoid this condition, it is essential to combine different protein sources (e.g., legumes and cereals). Iron intake must also be closely monitored as iron of plant origin (non-heme iron) is less bioavailable than animal iron. In order to prevent possible deficiencies, it is advisable to take iron-rich foods in conjunction with a source of vitamin C to improve its absorption (Marrone et al., 2021).

Omega-3 fatty acids, particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are mainly contained in the fish and they may be deficient in plant-based diets. In this case, it would be advisable to supplement these nutrients through specific food supplements (Lane et al., 2022). Zinc and iodine may also be deficient as the former is less bioavailable in plants, while the latter may be deficient if iodized salt is not consumed (Bakaloudi et al., 2021).

Another possible limitation of plant-based diets may be a greater tendency to consume refined carbohydrates and sugars than proteins, posing a greater risk for metabolic alterations (Key et al., 2022).

In this context, the dual aspect of plant-based diets in CKD is of notable interest. On one hand, it can serve as an excellent strategy to slow the disease progression, while on the other, it may be considered for the prevention of the disease's onset (Chauveau et al., 2019; Zarantonello and Brunori, 2023). The Tehran Lipid and Glucose Study (TLGS) (Azizi, 2018) and the Multi-Ethnic Study of Atherosclerosis (MESA) (Blaha and DeFilippis, 2021) are the two main cross-sectional studies that investigated the incidence of CKD according to the kind of the protein consumed. TLGS study found that among the 5.000 participants, those who consumed a higher

amount of plant proteins showed a 30% lower risk to develop CKD, compared to those who consumed less plant proteins. In the MESA, the Authors pointed out that those who consumed a higher proportion of whole grains, fruits, vegetables, and low-fat dairy products showed a lower urinary albumin-to-creatinine ratio. However, these interesting studies should be confirmed by other clinical trials conducted on higher number of patients and with a longer observational period.

Regarding pre-existing CKD conditions, the Nurses' Health Study (NHS) showed the potential role of plant-based diets in the secondary prevention.

Plant-based foods are recognized as a key part of a healthy diet across a broad range of eating styles. The "common denominator" of all plant-based diets is the prevalence of plant-based proteins within their eating patterns. Among them, the most significant plant-based dietary patterns are the MD, and its adaptations as the MedRen diet and the Flexitarian diet, the Vegetarian diet and the PLADO diet (Figure 1).

## 3.1 Mediterranean diet

The MD is not just a dietary pattern, typical of countries along the coast of the Mediterranean Sea, including Greece, Italy, Spain, and nations of the Middle East. The MD is rather a real lifestyle that in 2010 was included in the list of the "Intangible Cultural Heritage of Humanity" of United Nations Educational, Scientific and Cultural Organization (UNESCO), which describes it as "a set of skills, knowledge, rituals, symbols and traditions concerning crops, harvesting, fishing, animal husbandry, conservation, processing, cooking, and particularly the sharing and consumption of food" (Pett et al., 2017).

The concept of the "Mediterranean diet" was born from the intuition of the American physiologist Ancel Keys, who in the mid-twentieth century tried to understand the reasons for the lower cardiovascular disease incidence rate in the Mediterranean countries. The first study that investigated the beneficial effects of the MD conducted on a global scale, was the Seven Country Study (SCS), which involved researchers from seven countries (United States, Finland, Holland, Italy, Greece, Former Yugoslavia and Japan) with the aim of exploring in detail the associations between eating habits and some risk factors with the rates of cardiovascular diseases (Keys et al., 1984; Pett et al., 2017). The study, after 25 years (1958–1983) of data collection from 16 cohorts of middle-aged men, has demonstrated, for the first time, that high serum cholesterol levels, high blood pressure levels, diabetes mellitus and cigarette smoking are the main universal risk factors for cardiovascular diseases. Furthermore, a higher intake of animal foods, rich in saturated fats and animal proteins (excluding fish) and of simple sugars was associated with a higher rate of cardiovascular mortality; while, a higher intake of foods rich in flavonoids, therefore plant-based foods (such as fruit, vegetables and legumes), was inversely associated, in a statistically significant manner, with cardiovascular mortality rates (Keys et al., 1984; Menotti and Puddu, 2015; Blackburn, 2017).

The MD as a dietary model is characterized by a rich consumption of plant-based foods (cereals, legumes, dried fruit, fresh fruit, vegetables, *etc.*), a moderate consumption of fish and seafoods products, eggs, white meat and dairy products and a low consumption of red and processed meat. The MD also includes a moderate consumption of alcohol (red wine, to be consumed only during meals) and the use of extra virgin olive oil as the main source of vegetable fats (Bach-Faig et al., 2011).

Numerous studies suggest that the MD principles are able to reduce the risk not only of cardiovascular diseases but also of others chronic-degenerative diseases, including CKD (Riboli and Kaaks, 1997; Palli et al., 2003; Cicero et al., 2008; Martinez-Gonzalez et al., 2015).

In literature, it has been reported that the MD, in the early CKD stages, exerts numerous effects, including the reduction of cardiovascular risk factors, the decrease of chronic systemic micro-inflammation and the oxidative stress, the improvement of the lipid profile, the slowing of the progression towards ESKD, the improvement of blood pressure values, and the positive modulation of the gut microbiota (Chauveau et al., 2018; Cigarran Guldris et al., 2022; Perez-Torres et al., 2022).

As a proof, the study conducted by De Lorenzo et al. demonstrated the potential benefits of the Italian Mediterranean Organic Diet (IMOD) in nephropathic patients. In detail, 50 CKD male patients with stage 2–3 (according to the 2013 K-DIGO guidelines (Lamb et al., 2013), after 14 days of MD with organic products, showed an improvement in homocysteine, phosphorus, blood glucose, lipid profile, and microalbuminuria. Moreover, the results highlighted an amelioration of the body composition, measured with Dual-X absorptiometry (DXA). In detail, the Authors observed an increase in the lean body mass of CKD patients as well as a statistically significant reduction in fat mass in both kilograms and percentage (De Lorenzo et al., 2010).

Among natural bioactive compounds, those that mostly characterize the MD are the polyphenols contained in foods of plant origin and particularly in extra virgin olive oil (EVOO). Of particular importance among these are hydroxytyrosol, oleuropein and oleocanthal (Jimenez-Lopez et al., 2020). Numerous scientific studies have shown their beneficial effects on health as they are able to exert powerful anti-inflammatory actions. Therefore, daily consumption of an EVOO can counteract the microinflammatory environment that promotes the progression of CKD (Noce et al., 2021b).

However, it is worth noting that the NBCs in the EVOO are also able to induce cardiovascular protection through the reduction of oxidative stress and to improve purine and lipid metabolisms and the body composition parameters in nephropathic patients (Romani et al., 2020a; Marrone et al., 2022). The main variations of the MD are the MedRen diet and the Flexitarian diet.

### 3.1.1 MedRen diet

The Mediterranean Renal Diet (MedRen diet) is a modified version of the MD, characterized by a quantitative decrease in the recommended daily allowances (RDA) for protein, salt and phosphate, compared to the general population's standards. In particular, it should be formulated in order to achieve an amount of 0.8 g of proteins/day, 6 g of salt/day and less than 800 mg of phosphorus/day (Naber and Purohit, 2021). This diet is outlined by the presence of a greater number of plant-based foods that allow patients to assume a higher proportion of alkalizing compounds, fiber, and polyunsaturated fatty acids, than foods of animal origin

(D'Alessandro et al., 2023). This dietary pattern also turns out to be easily accepted by patients and thus favoring a higher patients' adherence to the personalized nutritional plan.

The "strength" of this diet is that following the traditional MD and only making minor "adjustments" it preserves the renal function and supports the metabolic patterns, which are often compromised in mild-to-moderate CKD (Narasaki and Rhee, 2020; Noce et al., 2021c; Babich et al., 2023). The main recommendations include: i) to prefer white meat instead of red meat, ii) to increase the consumption of vegetable proteins as legumes, iii) to define portions and cooking methods according to the patient's needs/requirements (D'Alessandro et al., 2023). For example, in case of hyperkalemia it is advisable to select fruit types with less potassium content and eat at maximum two portions of vegetables *per* day, properly boiled before consumption. Hard cheeses should be avoided (maximum two teaspoons of Parmesan *per* day) and fresh cheeses (such as cow ricotta cheese) once every 7–10 days. Hydrate in the correct way, introducing a share of water equal to the sense of thirst. Increase, if necessary, the amount of fluids with infusions and/or hot drinks, paying attention to possible drug interactions.

An interesting study conducted by Cupisti et al. (2015) showed that, after 6 months of nutritional treatment, a group of 93 stage 3 CKD patients compared to 223 stage 3 CKD patients (that did not follow any nutritional treatment), showed lower values of azotemia, phosphorus and parathyroid hormone (PTH), suggesting its potential role also in the management of CKD-mineral bone disorders.

In this context, the Mediterranean Renal Diet can be considered as a "change" from a healthy eating pattern to a diet "appropriate" for mild-to-moderate CKD. This approach, lending itself particularly well to being followed daily by patients, yields excellent results in terms of long-term adherence to the nutritional plan (D'Alessandro et al., 2023).

### 3.1.2 Flexitarian diet

The world of plant-based diets is heterogeneous. Current definitions range from the complete exclusion of all animal products, to the partial inclusion of fish, poultry and yogurt, thus effectively reducing processed foods and saturated fats. In this perspective, a plant-based diet can be declined toward different patterns that do not necessarily frame the vegan or vegetarian diet (Storz, 2022).

The Eat-Lancet Commission describes the flexitarian diet as a plant-based pattern, which favors plant-based foods but may occasionally include small portions of fish, meat and dairy products (Willett et al., 2019; EAT Lancet, 2025).

In more details, Spiringmann et al., in the journal Lancet Planet Health define the flexitarian diet as a dietary pattern that contains no processed meat, modest portions of red meat and sugar, moderate amounts of poultry, dairy, eggs and fish and high amounts of fruits, vegetables and legumes (Springmann et al., 2018).

A distinctive feature of the flexitarian diet is that it takes into consideration the ethical aspects of the food chain. In particular, this dietary pattern is aimed at improving animal welfare, avoiding agricultural intensification, inefficient production of foods, greenhouse gas emissions and issues about dietary and public health problems (including zoonoses and veterinary antibiotic use) (van der Weele et al., 2019). Moreover, the flexitarian diet deters long-term consumption of red and processed meats because of its association with increased risks of mortality, cardiovascular diseases, and cancer (Pighin et al., 2016). An additional aspect that distinguishes the flexitarian diet is that as well as encouraging the consumption of foods of plant origin, it promotes foods of local tradition and suggests their consumption according to its seasonality For these reasons, in the CKD scenario, the flexitarian diet could be a useful dietary model, both in its prevention and in the management of those patients, who have already developed CKD. In fact, this diet implies not to completely exclude foods of animal origin but rather to consider their potential benefits on the health maintenance, paying attention to their qualities and quantities.

A Finnish study suggests that in a flexitarian pattern, the protein intake should be no more than 30% from animal sources and that the main share of calories should come from foods such as legumes, whole grains, fruits and vegetables (Pellinen et al., 2022), indications that fit well with the preventive strategies currently used to combat CKD onset.

A recent study conducted in 2024 by Bruns et al. (2024) evaluated the cardiovascular risk associated with the flexitarian diet compared with the omnivore and vegetarian diets. Their results showed in those who followed a flexitarian diet, better metabolic health (assessed by MetS-score), better body mass index and in particular, waist circumference and better pulse wave velocity, compared with both vegetarians and omnivores. This result lends itself to support the hypothesis that the flexitarian diet is cardioprotective, demonstrating that lower consumption of red and processed meats and the promotion of the consumption of plant-based foods, obtained from a sustainable supply chain, can significantly reduce the risk of developing cardiovascular diseases.

Currently, there are no studies conducted on CKD patients and flexitarian dietary nutritional therapy in the literature. We can speculate that its particular composition may exert a significant impact, due to what was discussed earlier, in terms of prevention and improved therapeutic management of the nephropathic patients. In fact, its peculiar characteristic of not completely excluding foods of animal origin, but rather of selecting their quantity and quality, allows the body to get its fair share NBCs such as vitamins (B12), minerals (zinc, iron, calcium), peptides (essential amino acids) or fatty acids (like omega-3) that combine themselves with those found in plant origin foods (Pogorzelska-Nowicka et al., 2018).

## 3.2 Vegetarian diet

Vegetarian diets are nutritional regimes characterized by a greater consumption of plant-based foods, compared to those of animal origin. There are different types of vegetarian diets: the vegan diet, which completely excludes all foods of animal origin; the lacto-ovo vegetarian diet, which includes the consumption of eggs and dairy products; the pescatarian diet, which allows the consumption of fish and fish products; and, finally, the so-called "flexible" vegetarian diet, which includes moderate consumption of poultry (Gluba-Brzozka et al., 2017). Regardless of the type, vegetarian diets are characterized by a large consumption of legumes, fresh fruits and vegetables, excluding the consumption of ultra-processed foods.

These types of diets, therefore, are a rich source of dietary fibers, vitamins and phytochemicals (Narasaki et al., 2023).

A vegetarian diet has been shown to improve renal filtration in CKD patients and reduce the major risk factors for the disease onset, such as body mass index, blood pressure, fasting glucose, low density lipoprotein-cholesterol and triglycerides levels (Dinu et al., 2017; Swiatek et al., 2023).

One of the main points of debate, in CKD patients, regarding the vegetarian diets, is the serum potassium intake. It should be underlined that serum potassium levels can be elevated in patients with a reduced kidney function mainly after the consumption of foods with potassium-based additives or with a highly concentrated potassium content (such as juices, dried fruit, or purees). Instead, fresh plant-based foods may have properties that help to reduce potassium retention, such as their alkalizing effects, the limited bioavailability of potassium, and the role of dietary fiber in organic plant foods, in promoting potassium excretion through the colon. Additionally, by following the traditional low-potassium "renal diet," CKD patients may miss out on many of the benefits that plant foods offer. For this reason, the latest dietary recommendations for kidney health advocate for patient-focused recipes centered around plant-based foods without restricting them (Babich et al., 2023).

The vegetarian diet specifically designed for CKD patients is the "renal vegan diet". The vegan diet is characterized by a protein intake of 0.7 g/kg b. w/day and provides adequate support of essential amino acids derived from a close combination of cereals and legumes (Barsotti et al., 1996). The vegan diet results in more favorable outcomes, including reduced net acid production, a stronger anti-proteinuric effect, and lower phosphorus intestinal absorption, compared to omnivorous diet with the same protein content (Cupisti and Kalantar-Zadeh, 2013).

Fresh plant-based foods, highly recommended in vegetarian diets, are rich in NBCs with numerous beneficial properties for CKD patients. Among these, the most abundant are polyphenols that positively impact on the slowing of the CKD progression through different mechanisms: by means of an antioxidant action, as scavenger of reactive oxygen species (ROS) and as supporter of the natural antioxidant defenses; by means of an anti-inflammatory action, with the reduction of pro-inflammatory cytokines production; by means of the protection from cells and tissues damage and the attenuation of endothelial dysfunction, typical of CKD patients (Cho et al., 2018; Grazioli et al., 2021; Natesan and Kim, 2025).

## 3.3 Plant-dominant low-protein diet

In 2020, Kalantar-Zadeh delineated a new type of diet suitable for CKD patients, characterized by a prevalent consumption of plant-based proteins (i.e., from legumes) to the detriment of those of animal origin. The Authors defined this diet as plant-dominant LPD, or PLADO diet (Kalantar-Zadeh et al., 2020). The PLADO diet is characterized by a protein intake equal to 0.6–0.8 g/kg/day, of which at least 50% from plant-based sources, thus avoiding ultra-processed foods. The PLADO diet also provides an adequate dietary energy intake (i.e. 30–35 Kcal/kg/day), a low sodium intake (i.e., <3 g/day) and a high fiber intake (at least 25–30 g/day) (Kalantar-Zadeh et al., 2020).

The benefits that this type of diet exerts on slowing the progression of renal damage are carried out through numerous mechanisms. First of all, there is a reduction in glomerular hyperfiltration. Moreover, an amplification of the therapeutic effect of RAAS and SGLT2 inhibitors, with the reduction of intraglomerular pressure, has been demonstrated (Koppe and Fouque, 2019). Furthermore, the reduction of nitrogenous compounds intake, leads to a lower production of urea and uremic toxins, so improving the control of uremia and delaying the start of dialysis.

The lower bioavailability of phosphorus in vegetal proteins, compared to those of animal origin, allows a better control of serum phosphorus. The higher fibers intake, in addition to reducing the acid load of the diet (Rodrigues Neto Angeloco et al., 2018), is able to reduce the production of advanced glycation end-products (AGEs), which intervene in the renal damage progression (Demirci et al., 2019). An increased consumption of plant-based foods leads to a higher production of antioxidant and anti-inflammatory molecules, thus reducing the chronic micro-inflammation and oxidative stress, typical of CKD patients (Ko and Kalantar-Zadeh, 2021; Kalantar-Zadeh et al., 2022; Sakaguchi et al., 2023).

Finally, the consumption of plant-based foods, rich in fiber, allows to the positive modulation of gut microbiota. Recent studies have shown that CKD patients are characterized by gut dysbiosis. This is typified by an increase in bacteria responsible for proteolytic fermentation, which leads to the increased production of gut-derived uremic toxins (such as indoxyl sulfate, oxidized trimethylamine and p-cresyl sulfate) and a reduction in bacteria responsible for saccharolytic fermentation, which, instead, produce beneficial molecules, including short-chain fatty acids (SCFAs) (Noce et al., 2022). The former is able to impair the barrier function and deplete the intestinal tight junctions, allowing intestinal bacteria to reach the bloodstream and exacerbate inflammation and multi-organ damages. The latter, on the other hand, help maintaining the intestinal health, ensuring good functionality, exerting an anti-inflammatory action and positively modulating the immune system (Lau et al., 2015; Lau et al., 2018; Noce et al., 2022). The consumption of foods rich in fiber, such as plant-based foods, in CKD patients, is able to restore the balance of the gut microbiota, leading to an increase in saccharolytic bacterial species (such as the Prevotellaceae and Lactobacillaceae families) and a concomitant reduction in proteolytic bacterial species (such as the Enterobacteriaceae family) (Wiese et al., 2021; Koppe and Soulage, 2022).

The main source of vegetable protein in the PLADO diet is represented by legumes. The latters, in addition to being an excellent source of protein (mainly rich in lysine), show a high quality of complex carbohydrates and soluble fibers (with a low glycemic index), a low-fat content (mainly represented by linoleic acid) and an excellent content of vitamins (A, E, and B) and minerals (poor in sodium and rich in potassium, zinc, calcium and iron). Moreover, legumes are an important source of phytochemicals that exert numerous beneficial effects on health, including renal ones. In particular, they are phytosterols, tocopherols, alkaloids, carotenoids,

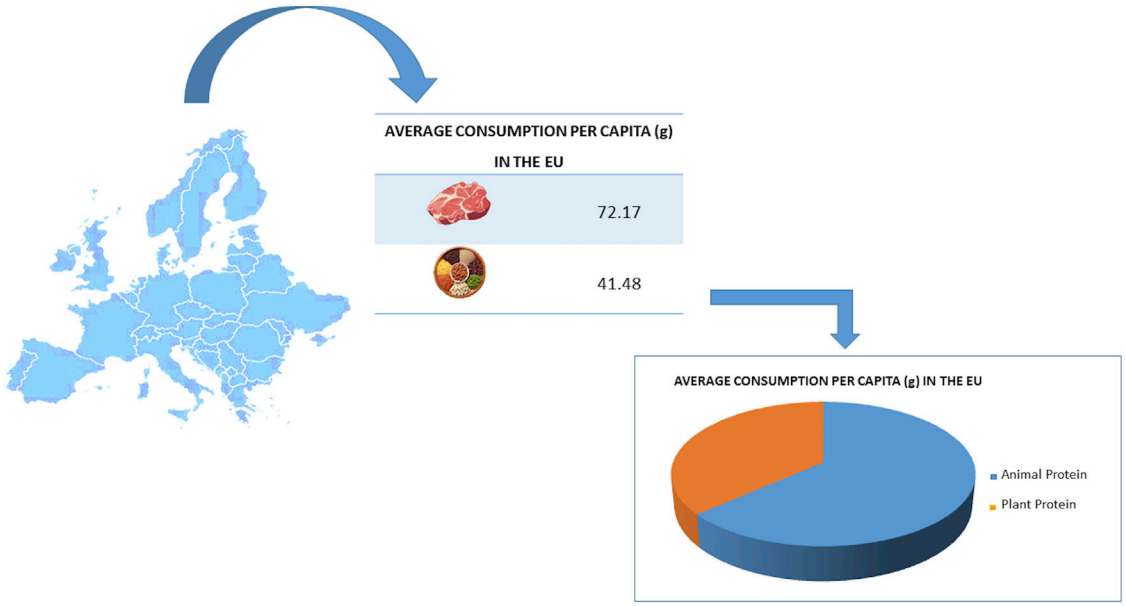



FIGURE 2
The average consumption *per capita* (g) of different protein sources in the European Union (EU).

TABLE 1 kg $CO_2$-equivalents per kg of product for animal proteins.

| | kg $CO_2$-equivalents for kg of product |
|---|---|
| Beef | 60 |
| Pork meat | 12 |
| Chicken | 6 |
| Fish (e.g., salmon) | 4.5 |
| Dairy products (milk and cheese) | 7.5 |

TABLE 2 kg $CO_2$-equivalents per kg for plant-based proteins.

| | kg $CO_2$-equivalents for kg of proteins |
|---|---|
| Soy (protein isolate) | 1.9 |
| Lentils | 0.9 |
| Peas | 1.3 |
| Rice (protein isolate) | 2.5 |
| Wheat (protein isolate) | 2.5 |

flavonol, flavone and isoflavone (Tor-Roca et al., 2020; Yin et al., 2023).

## 4 Plant-based diets sustainability

Our food choices can influence the course of the climate crisis, especially with regard to the issue of global warming, as the food sector is responsible for approximately 25% of greenhouse gas emissions worldwide (FAO, 2019). To address current sustainability challenges and reduce greenhouse gas emissions, the food system as a whole must also be changed by acting both on waste reduction and on changes in dietary habits, replacing animal proteins with plant based-proteins. At this regard, the current political agendas of the European Union (EU) are focused on the increase consumption of plant-based proteins (EuropeanCommission, 2022).

One of the most frequently proposed dietary modification measures is to reduce the consumption of foods of animal origin, especially meat, together with the advice to increase the consumption of plant-based foods. The current scenario in the EU sees an average meat consumption *per capita* of 72,17 g, while plant protein provide an average consumption of 41,48 g (FAO, 2023) (Figure 2).

In recent years, there has been an emerging trend towards a greater consumption of plant-based proteins, such as plant-based hamburgers, milk or yogurt, as healthy substitutes for animal-based products (Jaeger et al., 2023).

Nowadays, the demand for sustainable, nutritious and healthful foods has greatly increased and the market has been enriched with processed plant-based products that are alternatives to animal products, especially meat products. The goal of the food industries is to obtain foods with sensory, structural and nutritional characteristics similar to those of animal origin.

At this regard, comparative studies of life cycle assessment (LCA) were applied to both plant and animal proteins. LCA is a method used to evaluate the impact of a product on the environment, taking into account all inputs and outputs in the various phases of the product's life cycle, that is from the extraction

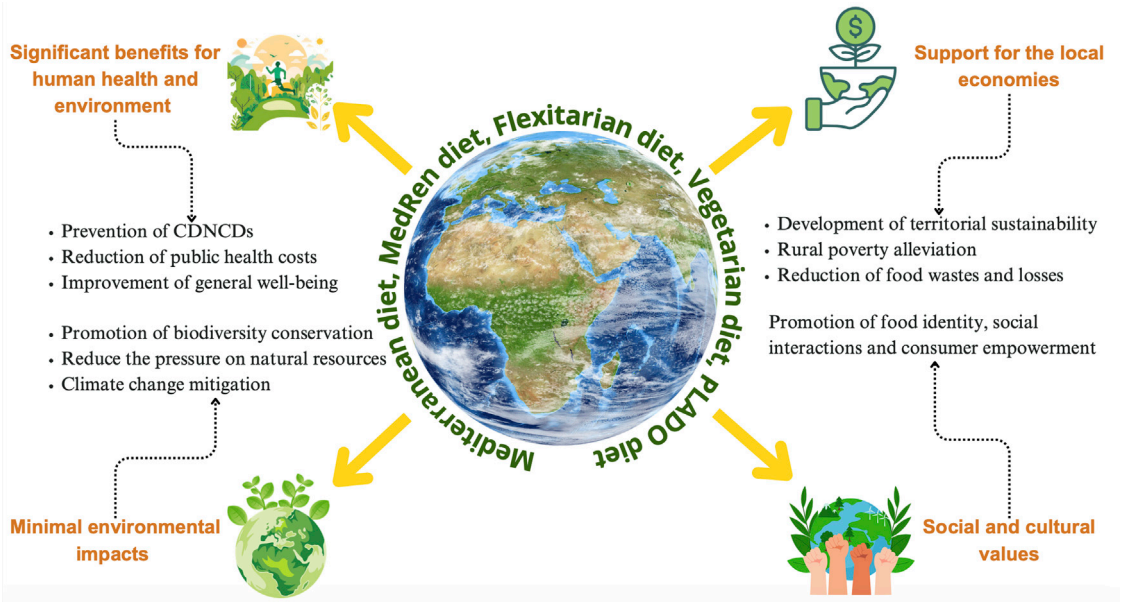



FIGURE 3
Plant-based diets: key aspects of their sustainability.

of raw materials to the production, transport, use and disposal (ISO 14040:2006).

The evaluation parameters for the comparison between proteins of animal and plant origin were based on land footprints, carbon footprints and blue water footprints (CarbonTrust, 2021). Comparative carbon footprint data between animal and plant proteins clearly show that plant proteins tend to have a lower environmental impact than animal proteins.

Poore and Nemecek (2018) were the first to conduct a comprehensive analysis of the ecological footprint of various foods, comparing animal and plant proteins in detail. The results are summarized in Tables 1, 2.

As shown in the data, plant-based proteins have a significantly lower environmental impact than animal-based proteins, especially when compared to the red meat (e.g., beef), which owns the highest carbon footprint. On the contrary, plant-based proteins such as soy, peas and lentils are particularly sustainable, with a carbon footprint ranging from 0.9 to 2.5 kg $CO_2$-equivalents per kg of proteins.

Plant-based meat alternatives (such as pea-based veggie burgers) are significantly less impactful than traditional meat, making them an attractive option for reducing greenhouse gas emissions in the environment. The sustainability of some plant-based foods is twofold. For example, legumes, present a very low greenhouse gas (GHG) intensity (known as "carbon footprint") per unit of nutritional density compared to other foods (Williams et al., 2020). Another very important aspect relating to the sustainability of legumes cultivation concerns their characteristic of being nitrogen-fixing soil. In fact, their cultivation does not require the application of nitrogen fertilizers, by virtue of their ability to biologically fix atmospheric nitrogen, improving the structure and presence of micronutrients in the soil (Meena et al., 2017).

In this perspective, consumers prefer plant protein-based products because of their potential health benefit, while can be observed negative health effects related to diets high in animal proteins.

Moreover, an increased focus on sustainable foods and ethical issues regards the treatment of the animals (Hertzler et al., 2020). We need to underline, it was estimated that globally the food production is the largest contributor to biodiversity loss and is responsible for 80% of deforestation, >70% of freshwater use and 30% of GHG emissions (Nelson et al., 2016).

In the 2010, the FAO defined as sustainable diet "diets with low environmental impacts which contribute to food and nutrition security and to healthy life for present and future generation". In particular, sustainable diets should: i) have a low environmental impact (minimizing the use of natural resources, thereby reducing the strain on ecosystems and protecting biodiversity; promoting food and nutritional safety); ii) ensure access to sufficient, safe, and nutritious food sources for a healthy living now and in the future; iii) be culturally acceptable and accessible (align with different cultural norms and practices, remaining at the same time, economically and physically available to all); iv) be economically fair and affordable (support equitable access to food, while being cost-effective for producers and consumers) (Burlingame et al., 2011).

These advantages make plant-based proteins a key component in promoting sustainable diets, as they align with the FAO's goal which tends to reduce environmental impact and to support food safety and nutritional quality (Medina-Vera et al., 2024).

The shift towards plant-based foods reflects the growing awareness for environmental sustainability, animal welfare and human health benefits. Plant-based foods are rich in dietary fibers and natural bioactive compounds, which contribute to increase their nutritional value. Their production and development copes with sustainable practices, reducing the environmental impact associated with animal-based food production.

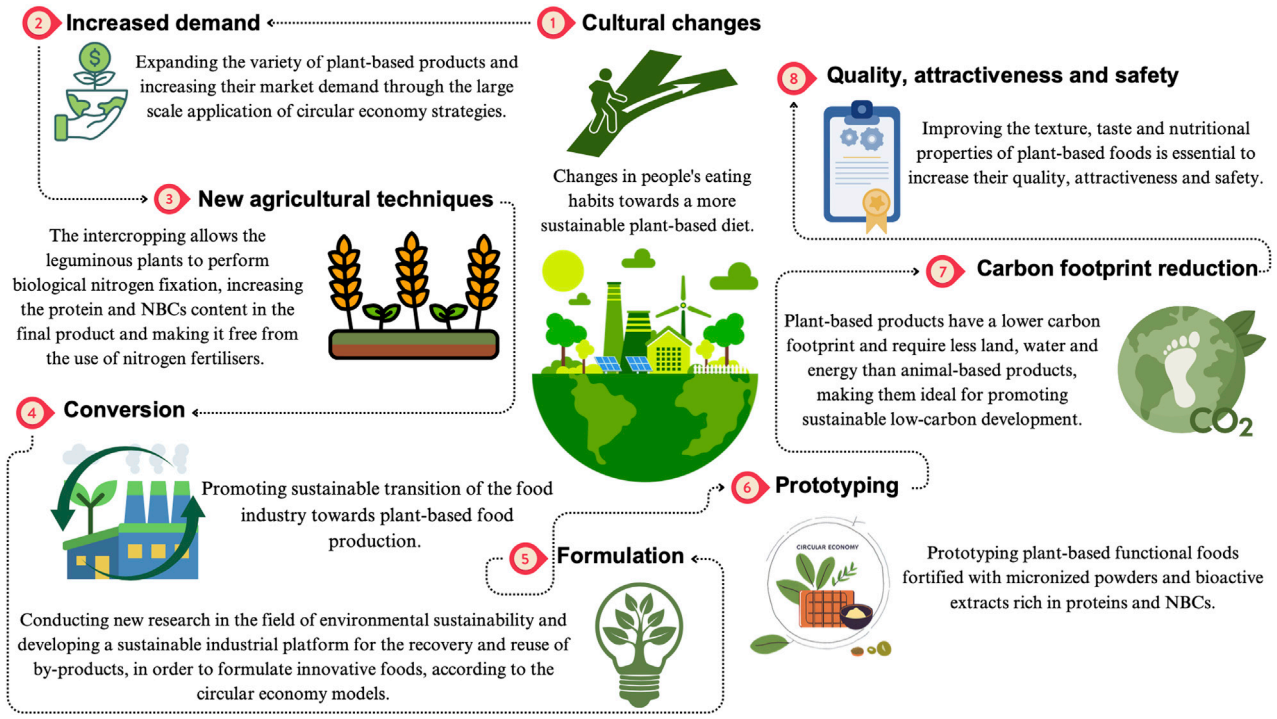



FIGURE 4
Plant-based products: challenges for the European sustainable food system.




FIGURE 5
Representation of sustainable nutritional treatment for chronic kidney disease thorough the present Colosseum. As shown in the figure, at the first level of the clinical management of chronic kidney disease patients, there is the clinical staff (nephrologists, nutritionists and/or dietitians, clinical kinesiologists and nurses). The levels two, three and four focus on the dietary nutritional therapy, to define these diets as completely or predominantly plant-based. The second level can be implemented by a legislative part (Food and Agriculture Organization with the implementation of the Agenda 2030 for Sustainable Development Goals, European Food Safety Authority, circular economy models and European Food Information Council), in order to achieve the maximum sustainability from the plant-based dietary nutritional therapy. The combination of the second and third levels outlines a predominantly plant-based dietary nutritional therapy. The fourth level summaries the foods to be avoided in completely or predominantly plant-based dietary nutritional therapy. Abbreviation: DNT, dietary-nutritional therapy.

This trend has driven to some innovations in the food processing technologies in order to enhance the quality, safety, and appeal of the plant-based products. These advancements focus on improving texture, flavour and nutritional properties. The increasing demand for plant-based alternatives underscores the need of a ceaseless research and development in this field.

In terms of ecological and environmental protection, the development and the utilization of plant protein-based products go along with the goals of resources recycling. Plant-based foods production generally requires fewer resources compared to those required for the animal products, making it in accordance with the principles of the low-carbon sustainable development. The production of plant-based products is characterized by a lower carbon footprint, so it requires less land, water, and energy compared to the production of animal-based products. This makes them an excellent choice for supporting a low-carbon sustainable development.

Plant protein-based products show also some limitations due to their functional and nutritional properties, to allergens and antinutritional factors and to processing challenges and market limitations (Figure 3).

In order to overcome these limits, it is required a combination of innovative researchers, technological advancements and market diversifications. Refining processing techniques, expanding product ranges and enhancing quality stability will be the pivotal solution for unlocking the full potentiality of the plant protein-based products for food, medical and commodity applications (Fu et al., 2023).

The rising global population has created an urgent demand for economical, abundant, and sustainable foods with the aim to meet nutritional needs. At the same time, the food and agriculture sectors generate massive wastes and by-products at various stages of the supply chain. However, from many of these by-products, with specific and innovative processes, nutrients can be extracted, enhancing their functionality and nutritional values. This approach not only reduces food wastes but also contributes to a sustainable and circular economy by turning wastes into high-value products. Furthermore, it provides a potential strategy for the formulation and prototyping functional foods with healthy beneficial effects, useful in the nutritional treatment of CKD patients (Marrone et al., 2024b; Marrone et al., 2024c). We should also consider that by converting agro-industrial wastes into functional foods with antioxidant and antioxidant capacity, the food industry can reduce its environmental impact, improve food security and create innovative solutions to allow either wastes recycle or the creation of new functional foods, rich in NBCs with healthy benefits (Noce et al., 2021a; Catalfamo et al., 2022; Marrone et al., 2024a; Marrone et al., 2024c; Marrone et al., 2024d).

The concept of environmental sustainability can be applied not only to plant-based proteins but also to plant-based diets.

The already discussed dietary patterns, namely, MD, MedRen diet, Flexitarian diet, Vegetarian diet and PLADO diet are recognized as a sustainable food consumption pattern, encompassing several key aspects: i) significant healthy and nutritional benefits, including the prevention of chronic degenerative non-communicable diseases (CDNCDs), reducing public health costs for the CDNCDs management and enhancing the overall wellbeing; ii) minimal environmental impact, promoting the biodiversity conservation, reducing the pressure on natural resources and contributing to the mitigation of climate changes; iii) support for local economies, developing the territorial sustainability, alleviating the rural poverty and reducing the food wastes and losses; iv) social and cultural values, fostering food-related identity, social interactions and consumer empowerment (Figure 4) (Dernini et al., 2017).

The already examined diets, if promptly applied and tailored on the clinical characteristics of the patients, represent a useful instrument, combined with the pharmacological therapy, in order to delay the need of RRT (Naber and Purohit, 2021). As shown in Figure 5, the clinical management of CKD patients can be represented by the present Colosseum. The first level is depicted by the clinical staff (nephrologists, nutritionists and/or dietitians, clinical kinesiologists and nurses) required for the treatment, psycho-physical wellbeing and medical care of nephropathic patients. The second, third and fourth levels focus on the DNT. The second plant-based level is represented by fruits and vegetables (with indications on cooking techniques) (Cupisti et al., 2018b), non-whole cereals, legumes, EVOO (containing at least 250 mg of hydroxytyrosol and derivatives *per* kg of product) and foods obtained by circular-economy model. EVOO characterized by high content in minor polar compounds and sustainable and antioxidant functional foods, rich in NBCs, exert innumerable beneficial effects in CKD patients (Noce et al., 2021b; Marrone et al., 2022; Marrone et al., 2024b). This mandatory level can be implemented by a legislation part of European Food Safety Authority, which defines the EVOO health claim able to reduce oxidative stress, to exert antioxidant properties and to protect human body cells and low-density lipoproteins from oxidative damages (EFSA, 2011). Another part of the legislation comes from the European Food Information Council which defines the functional foods and finally, from the 2030 Agenda which sustains "...prevention, reduction, recycling and reuse" (SDG number twelve) of NBCs-rich supply chain by-products useful to formulate functional foods (Romani et al., 2020b). The DNT sustainability reaches its peak if the third level is not considered in the nutritional plan. In fact, a protein-controlled and high-calorie content DNT does not require the implementation of animal-based proteins, as those deriving from red and white meats (with particular attention to those with a medium-high phosphorous content), white fishes and egg whites. The fourth level is mandatory for a proper setting of the DNT, whether it is completely or predominantly plant-based, because it gathers the foods to be avoided.

This is a further strength-point for the plant-based diets sustainability in the clinical management of the CKD patients, as dialysis itself negatively impacts on the environment. At this regard, dialysis leaves a heavy environmental footprint due to water and energy consumption, greenhouse gas emissions and wastes production (Barraclough and McAlister, 2022).

The environmental problems associated with hemodialysis (HD) include high water and energy consumption, $CO_2$ emissions, and plastic waste (Vanholder, 2022). Annually, hemodialytic treatments produce around 3.8 tons of $CO_2$ *per* patient, primarily from biomedical equipment (37%), energy use (21%), and patient transport (20%). The transport emissions are due to patients' mobility to reach the dialysis centre from home *and back*, which require vehicles that release $CO_2$, CO, $NO_x$, and particulate matter (Pelliccia et al., 2011).

HD is a water-intensive treatment that requires purified water to prepare the dialysis fluid of appropriate quality. In the world, for HD, it is assumed that the annual consumption of water is approximately 265 million $m^3$. Up to two-thirds of this wastewater consists of the reject water by the reverse osmosis system (176 million $m^3$) and by the dialysis machine (Ben Hmida et al., 2023). Water is the main component of dialysate, which establishes the concentration gradient for the diffusive removal of solutes during HD, through filtration steps, ion exchange and reverse osmosis. Additional water is used during the preparation and disinfection cycle of the HD machines (Gauly et al., 2022). In fact, dialysis water treatment should remove chemical and microbial contaminants, since the dialysate effluent is mainly water that contains byproducts of human metabolism (like organic compounds and minerals), possible bacteria, viruses and drugs (Coulliette and Arduino, 2013). The reject water must satisfy all potable water criteria defined by the World Health Organization. In fact, this water is often considered a wastewater, despite being of high quality after the filtering and treatment with activated carbon (Ponson et al., 2014).

Energy consumption is also driven by the facilities' lighting, cooling and ventilation systems, as well as by heating (using diesel or gas) and air conditioning. The environmental impact also depends on sanitation and laundry services that require water, energy, and cleaning agents (Pelliccia et al., 2011).

Focusing on the huge water consumption in dialysis patients, it is important to reduce the environmental burden for the renal care by implementing virtuous strategies, aimed not only at eco-friendly respect but above all at a healthy lifestyle and an appropriate nutrition.

## 5 Conclusion

CKD is a worldwide spread disease that involves expensive healthcare costs for its management. These costs increase significantly when comorbidities arise or when the disease progresses. A valid strategy to reduce healthcare costs, as well as to increase patients' survival and to improve their quality of life, is to implement a targeted therapy (pharmacological and non-pharmacological), that aims at slowing the disease progression.

In this context, among the non-pharmacological adjuvant therapies, a key role is played by the nutritional therapy, characterized by a controlled protein intake and by a prevalence of plant-based foods, like MD (in particular the MedRen diet and the Flexitarian diet), the Vegetarian diet and the PLADO diet. Numerous studies have shown that these DNTs exert numerous beneficial properties for CKD patient's health and for the environmental sustainability.

In fact,

- The NBCs contained in plant-based foods exert antioxidant and anti-inflammatory effects, positively modulate the gut microbiota, improve purine and lipid metabolisms and the body composition of the nephropathic patient. The consumption of plant-based foods in CKD patients is able to reduce drug treatments, to counteract the development of the main complications of the disease and slow down its progression.
- Plant-based foods represent a pivotal strategy for addressing the global challenges of environmental sustainability, human health and food security. Due to their lower environmental impact, superior nutritional profile, and alignment with sustainable practices, plant-based foods offer a valid alternative to traditional animal-based products.
- Innovations in foods processing through the adoption of industrial and sustainable platforms, based on circular economy models, allow the recovery and reuse of supply chain wastes for the formulation and the prototyping of functional foods, rich in NBCs. In this way, food industry can create plant-based functional foods, formulated *ad hoc* for specific patient populations, such as those nephropathic, diabetics, *etc.*, maximising the resource efficiency, reducing the environmental impact and improving food security, concepts that are part of the SDGs of the 2030 Agenda.

In view of what has been stated, the adoption of sustainable food models will allow the containment of the spread of CDNCDs, such as CKD, positively impacting both human health and planet, significantly reducing the costs and resources of the National Health Systems. Moreover, only changes in people's eating habits towards plant-based diets, by raising awareness among patients, will allow the increasing market demand of plant-based foods and expanding their variety. Therefore, the farmers will be incentivized to adopt new farming techniques that will enable them to create foods rich in NBCs and free of nitrogen fertilizers. At the same time, the existing food companies will be incentivized to convert toward the production of plant-based food. Conducting new research in the fields of nephrology, nutrition and environmental sustainability is the right direction to go, in order to encourage the consumption of a plant-based diet and to formulate *ad hoc* functional foods for specific patient populations, including those with CKD.

## Author contributions

GM: Conceptualization, Visualization, Writing – original draft, Writing – review and editing. MD: Writing – original draft, Writing – review and editing. KC: Visualization, Writing – original draft, Writing – review and editing. CM: Writing – original draft, Writing – review and editing. GV: Writing – review and editing. CV: Visualization, Writing – original draft, Writing – review and editing. AN: Conceptualization, Writing – original draft, Writing – review and editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

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# Exploring the transformation of chemical components and the discovery of anti-tumor active components in the fruit of *Sinopodophyllum hexandrum*

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**Introduction:** The fruit of *Sinopodophyllum hexandrum* (FSH) is derived from *Sinopodophyllum hexandrum* (Royle) Ying, a plant belonging to the family Berberidaceae of the order Ranunculaceae. It is mainly distributed in the Himalayan alpine region, and born in the understory of forests, and wetlands at the edge of forests, thickets or grasses. FSH grows at an altitude of 2,200–4,300 meters above sea level. Its main pharmacological activities include anti-tumor, anti-inflammation, analgesia, heat clearing and detoxification. In the current experiment, ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF-MS) was adopted for investigating the chemical components contained in FSH, their transformation patterns *in vivo* and the potential anti-tumor components, so as to provide an experimental basis for the utilization and development of the resources of FSH.

**Methods:** The chemical components of FSH and their transformation patterns *in vivo* were investigated by UPLC-Q-TOF-MS, and the potential anti-tumor active components were predicted from the *in vivo* transformed components of FSH by using a network pharmacology approach.

**Results:** Totally 85 chemical components were identified in FSH, among which, 61 were flavonoids and 24 were lignans. The above components were transformed *in vivo*, including 36 prototype components and 13 transformed products. As revealed by the results of network pharmacology on the prediction of anti-tumor components of FSH, 17 compounds such as Kaempferol, Uralenol, and 8-Prenylquercetin in FSH were used as the potential anti-tumor components.

**Conclusion:** In this study, the chemical composition, *in vivo* transformed components of FSH and their metabolites are investigated, and the *in vivo* transformed components are predicted to have potential anti-tumor pharmacological activities. This study provides the experimental bases for the utilization and development of the resources of FSH.

KEYWORDS

**fruit of *Sinopodophyllum hexandrum* (FSH), network pharmacology, composition analysis, anti-tumor, active ingredient**

# 1 Introduction

The fruit of *Sinopodophyllum hexandrum* (FSH) is derived from *Sinopodophyllum hexandrum* (Royle) Ying, a plant belonging to the Berberidaceae family. First officially included in the 2010 edition of the "Chinese Pharmacopoeia" (1), FSH is a perennial herb with a height of 20–50 cm. Its rhizome is short, stout, and nodding, with many fibrous roots, whereas the stem is erect, simple, longitudinal, and glabrous, and the base is covered with large brown scales. Its leaves are thinly papery, peltate, base cordate, glabrous, and pilose abaxially, with a coarsely serrate margin. Its petioles are longitudinally ribbed and glabrous. Moreover, the flowers are large, solitary, pink, bisexual and open before leaves. It has 6 sepals, 6 petals, 6 stamens, and the filaments are slightly shorter than anthers, which are linear, longitudinally lobed, and apex rounded, with non-extended connectives. There is one pistil, the ovary is ellipsoid, with one locule and laterally membranous placentation, which contained numerous ovules, style short, and stigma capitate. The berry is ovoid, with the length of 4–7 cm, the diameter of 2.5–4 cm, and is orange-red when ripe. Additionally, the seeds are ovate-triangular and brownish red, without fleshy aril. The flowering period is from May to June, and the fruiting period is from July and September (2). *Sinopodophyllum hexandrum* is mainly distributed in the Himalayan alpine region, including China, India, Nepal, Pakistan and the nearby understory, forest edge wetland, scrub or grassland and other cold and wet areas at an altitude of 2,200–4,300 m. In China, it is mainly distributed in Xizang, Sichuan and Yunnan.

FSH has long been used in Tibetans. At present, there are many reports that FSH has health care functions, and it has been developed into health care wine, health care tea and other products. Local people have the habit of picking and eating this fruit directly (3). Furthermore, as documented in the Jingzhu Bencao, FSH can be used to treat venous disease and uterine disease (4). Besides, it is recorded in the Ruyi Baoshu that, FSH has a special effect on the treatment of blood disease and gynecological disease (4). According to modern pharmacology, FSH exhibits anti-tumor, anti-viral, anti-microbial, immunomodulatory, and anti-inflammatory effects (5, 6).

Existing studies on the chemical composition of FSH have proved that FSH mainly contains flavonoids and lignans (4, 7–10), of which, flavonoids mainly comprise quercetin-3-methyl ether-3′/4′-O-glucoside, citrusinol, 6′-prenylquercetin-3-methyl ether, quercetin and other compounds, whereas lignans mostly encompass podophyllotoxne isomer, demethyldesoxypo-dophyllotoxin isomer diglucoside, $\alpha$-peltatin or isomer, desoxypodophyllotoxin and other compounds. Based on a comprehensive review of the relevant literature, flavonoids and lignans present in FSH exhibit inhibitory effects on various tumor cell lines (6) and demonstrate significant anti-tumor activity (4, 11). Among the lignans, podophyllotoxin and its derivatives, such as etoposide and teniposide, are extensively utilized as anti-tumor agents in clinical settings (9, 10, 12). The mechanism underlying their action is associated with the modulation of the PI3K/AKT/mTOR signaling pathway, leading to the induction of apoptosis and autophagy in tumor cells (13). Furthermore, podophyllotoxin exhibits anti-herpes simplex virus and immunosuppressive properties, demonstrating significant efficacy in the clinical management of HPV. Flavonoids in FSH also possess antibacterial, anti-inflammatory, and immunomodulatory activities, making them applicable in the treatment of hepatitis B and bronchitis. These pharmacological properties not only underpin the use of FSH in traditional Tibetan medicine but also offer promising avenues for the development of modern therapeutics, particularly highlighting its potential in anti-tumor clinical applications.

Currently, relevant studies have reported the chemical components of FSH, but the process of how these components are transformed in organisms has not been reported. And there are also few reports on which components have potential anti-tumor activities. To solve the above problems of FSH, this experiment first identified 87 chemical components of FSH by ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF-MS), and subsequently explored the transformation process of these chemical components *in vivo*. Meanwhile, combined with the network pharmacology approach, 17 potential anti-tumor active components were identified among the transformed chemical components *in vivo*. The above results offer a preliminary basis for the development and utilization of FSH plants and the research and development of new anti-tumor drugs.

# 2 Materials and methods

## 2.1 Chemicals and reagents

FSH samples were harvested from five random locations within the range of Chamdo Lado Township (S1: longitude E: 97°40′, latitude N: 31°50′; S2: longitude E: 97°41′, latitude N: 31°56′; S3: longitude E: 97°40′, latitude N: 31°64′; S4: longitude E: 97°34′, latitude N: 31°55′; S5: longitude E: 97°45′, latitude N: 31°54′) in the Tibet Autonomous Region, and dried in the shade after harvesting. All FSH samples were identified as mature fruit of *Sinopodophyllum hexandrum* (Royle) Ying by Prof. Zhang Jiankui from Liaoning University of Traditional Chinese Medicine. Quercetin-3-O-glucoside, kaempferol-3-O-rutinoside, quercetin-3-methyl ether, uralenol, podophyllotoxne and podophyllotoxin control products (purity≥98%, Baoji Weibin District Xinyi Scientific Instruments Business Department), methanol (MERCK, Germany; batch no. l1143835114), acetonitrile (MERCK, Germany; batch no. l1165729132), formic acid (Thermo Fisher Scientific (China) Co., Ltd.; batch no. 214911), and sodium heparin were also utilized in this experiment. DMEM, L-15 and 1,640 medium (Dalian Meilun Biotechnology Co., Ltd., China), human hepatocellular carcinoma cells (HepG2) (Qi's (Shanghai) Biotechnology Co., Ltd., China), human colon cancer cells (SW620) (Saibaikang Biotechnology Co., Ltd., China), human non-small cell lung cancer cells (A549) and mouse mammary carcinoma cells (4T1) (Cell Bank of the Chinese Academy of Sciences, China).

## 2.2 Thin layer chromatography identification of FSH

In accordance with the method of "Identification (2)" of FSH under the item of "Medicinal Materials and Drinking Tablets" in Part I of the 2020 edition of the Chinese Pharmacopoeia. FSH samples collected from five locations were identified by TLC, so as to determine the correct source of the fruit. The specific experimental process is as follows: accurately weigh 5.00 g of FSH powder, then accurately add 10 mL of methanol and perform ultrasonic extraction at 40 kHz for

20 min. After extraction, filter the solution and dry the filtrate, add 2 mL of methanol to dissolve the residue, which will serve as the FSH sample solution. A methanol solution of podophyllotoxin with a concentration of 0.5 mg•mL$^{-1}$ is used as the control solution. Pipette 4 μL of each of the above test solutions and control solutions onto the same silica gel G thin-layer plate. Use a mixture the upper solution of cyclohexane, water-saturated n-butanol, and formic acid (6.5, 2.5, 0.8) as the unfolding agent. Allow the solutions to unfold and dry, then spray with a 1% vanillin sulfuric acid solution, and heat until the spots are clear.

## 2.3 Preparation of the decoction for UPLC-Q-TOF-MS analysis and oral administration

First of all, 2.00 g of the powdered FSH (Sample S1-S5: passed through the No.4 sieve) was weighed precisely, then 50 mL of methanol was added precisely for 30 min of sonication at 40 KHz, and the resultant sample was later passed through the 0.22 μm microporous filtration membrane to acquire the test solution. Thereafter, the obtained test solution was analyzed by UPLC-Q-TOF-MS and the results were imported into MassHunter Qualitative Analysis B.06.00 software for analysis and processing.

Approximately 30 g of the powdered FSH (Sample S1: passed through the No.4 sieve) was weighed, later 10 times the amount of water was supplemented to soak the sample for 15 min. Later, the resultant sample was heated and reflux extracted twice for 1 h each. After filtration, the two filtrates were combined and concentrated under the reduced pressure to a solution with a concentration of 1.0125 g mL$^{-1}$ (with 1 mL solution containing 1.0125 g of the raw drug), thus the FSH solution of water was obtained for oral administration.

## 2.4 Animals handing and plasma samples preparation

Twelve SPF-grade healthy male SD rats weighing (200 ± 20) g (Liaoning Changsheng Biotechnology Co., Ltd.; Certificate of Conformity No.: SCXK (Liao) 2020-0001) were used in this experiment. Our experimental protocol was approved by the Ethics Committee of Liaoning University of Traditional Chinese Medicine (no. 2020059).

Altogether 12 healthy male SD rats (200 ± 20 g) were taken and randomly classified into the blank group and the drug administration group ($n$ = 6 each). These rats were raised in an environment at the temperature of (25 ± 2)°C and relative humidity of (55 ± 5)%, under the natural day and night condition, with free access to water and standard maintenance feed (Liaoning Changsheng Biotechnology Co., Ltd.). The experiment was initiated after 1 week of adaptive feeding.

The rats in each group were weighed before the experiment, and after fasting without water for 12 h, the drug group was given FSH extract at 4.86 g•Kg$^{-1}$ by gavage according to 6 times the highest adult clinical dosage recommended by the Pharmacopoeia. Meanwhile, the rats in the blank group were given an equal amount of distilled water by gavage. On the third day of gavage, blood was collected from the orbital venous plexus of rats into the 2 mL centrifuge tube containing sodium heparin, slightly shaken, and allowed to stand at 4°C for 30 min. Then, the blood sample was subject to centrifugation at 4°C for 15 min at 3000 r•min$^{-1}$, and the supernatant was aspirated to obtain the plasma of FSH.

By optimizing the plasma sample preparation method of rats in the FSH administration group (14), the optimal plasma sample preparation process was shown as follows. In brief, 100 μL of plasma sample was collected from the FSH administration group, then 300 μL of the pre-cooled methanol was added at the plasma: solvent ratio of 1:3, and the resultant sample was subject to centrifugation at 4°C for 15 min at 12,000 r·min$^{-1}$. Afterwards, the supernatant of the plasma sample was obtained after centrifugation, and then blown dried by the nitrogen gas. The residue was reconstituted by adding 50 μL of the pre-cooled methanol at the plasma: solvent ratio of 1:0.5, vortexed at 3000 r•min$^{-1}$ for 2 min, centrifuged at 4°C and 12,000 r•min$^{-1}$ for 15 min, and the supernatant was aspirated to obtain the plasma test solution of FSH. The plasma of rats in the blank group was treated in an identical way.

## 2.5 Instrumentation and chromatographic conditions

### 2.5.1 Ultra performance liquid chromatography

The Agilent poroshell 120 SB-C18 column (100 mm × 4.6 mm, 2.7 μm) was applied in UPLC. The mobile phases were consisted of A water (0.1% formic acid) and B methanol with gradient elution (0 ~ 30 min, 5% ~ 100% B) in the positive ionization mode; whereas A water and B acetonitrile with gradient elution (0 ~ 30 min, 5% ~ 100% B) in the negative ionization mode. The injection volume in both modes was 2 μL, the flow rate was 0.4 mL•min$^{-1}$, the equilibrium column time was 8 min, and the column oven temperature was 30°C.

### 2.5.2 Mass spectrometry

The electrospray ion source was used. In the positive ion mode, the capillary voltage was 4,000 V, sheath gas temperature was 350°C, dry gas temperature was 250°C, sheath gas flow rate was 11 L/min, dry gas flow rate was 13 L/min, nebulizer pressure was 45 psig, fragmentation voltage was 125 V, and mass range was 50 ~ 1,500 m/z. In the negative ion mode, all the MS conditions were the same as those in the positive ion mode, except for the capillary voltage of 3,500 V.

## 2.6 Data analysis

Through a review of the literature pertaining to the chemical composition of FSH, a comprehensive chemical composition information library was constructed using PCDL Manager B.07.00 software. This library was subsequently imported into MassHunter Qualitative Analysis B.06.00 software to facilitate the search for potential compounds, employing +H and + Na as positive adducts and -H and -COOH as negative adducts. The retention times and precise molecular weights of pertinent compounds were determined by analyzing the FSH mass spectra in both ionization modes. Secondary mass spectrometry analysis of the target compounds was conducted to detect their chemical compositions, utilizing retention time, mass-to-charge ratio, and fragmentation ion data from the

secondary mass spectra. This information was then compared and analyzed against control products and relevant literature to complete the identification of the compounds. For the identified compounds, an allowable mass error of 10 ppm was established (15, 16).

Based on the identified *in vitro* chemical composition of FSH, a comprehensive database detailing the chemical composition of FSH and its potential *in vivo* metabolites was established, including products of oxidation, glucuronidation, and other reactions involving the prototype components. Utilizing this database, a comparative analysis of plasma mass spectra from both blank and FSH-administered rat groups was conducted. Compounds detected in the plasma of the administered group, but absent in the blank group, were further analyzed using MS/MS secondary mass spectrometry. The *in vivo* transformation of FSH constituents was inferred from retention times, secondary mass spectral fragmentation patterns, and additional pertinent data (17, 18).

## 2.7 Network pharmacology analysis

### 2.7.1 Search for the main chemical components and target sites of FSH

Based on the above *in vivo* analysis results of the absorbed and transformed components of FSH, the derived chemical components were adopted to obtain the Canonical SMILES of the chemical components through PubChem.[1] Besides, the Swiss target prediction[2] was used for predicting the targets of pharmacodynamic components.

### 2.7.2 Acquisition and screening of tumor-related targets

The GeneCards database[3] and OMIM database[4] were searched with the keyword "tumor," and the disease-related targets were obtained after de-emphasis.

### 2.7.3 Intersection of FSH-and tumor-related targets

The above disease targets were intersected with the screened gene targets for the roles of the absorbed and transformed components of FSH in tumor treatment. Venn diagrams were generated using the microbiological letter platform.

### 2.7.4 Construction of the drug-component-target-disease network

To create a drug-component-target-disease network model, the active components of FSH and the gene targets and targets of FSH for tumor treatment were imported into Cytoscape 3.9.1 software.

### 2.7.5 Construction of the protein–protein interaction network and screening of core targets

The intersected targets of FSH for tumor treatment were imported into the STRING database to construct the PPI network. After removing the free nodes, the network topology analysis was performed using Cytoscape 3.9.1 software. Thereafter, the topological parameter analysis of the network nodes was implemented with the Network Analyzer plug-in. Using the three parameters of betweenness centrality (BC), closeness centrality (CC) and degree value (degree) as indicators, the medians of the three indicators were calculated, respectively. When the target indicator was greater than the corresponding median, it was predicted as the core target. Finally, core targets were ranked in accordance with the degree value (degree).

### 2.7.6 Gene ontology functional annotation and Kyoto encyclopedia of genes and genomes pathway enrichment analysis

GO and KEGG enrichment analyses were performed on the potential targets using the DAVID 6.8 database, with the species being defined as "*Homo Sapiens*," and the screening criterion being set to $p \leq 0.05$. The results of the top 10 GO and 25 KEGG enrichment analyses meeting the criteria were visualized in the bar charts.

## 2.8 Anti-tumor efficacy of chemical components in FSH

### 2.8.1 Tumor cells and drug delivery components selection

Based on the screening results of the core components in network pharmacology, there were 10 chemical components, including 6'-Prenylquercetin-3-methyl ether, Uralenol, Kaempferol-4′-methyl ether, Kaempferol and so on, each exhibiting a Degree value of ≥40. Among these core constituents, Quercetin-3-methyl ether and others were only detected as metabolites in rat plasma, which led to their exclusion from further analysis. Consequently, Uralenol and Kaempferol were selected for validation of cellular efficacy. Following the classification of KEGG pathway screening results, tumor-related pathways were prioritized, encompassing prostate cancer, gastric cancer, non-small cell lung cancer, hepatocellular carcinoma, breast cancer, colorectal cancer, among others. Considering the pathways through which Uralenol and Kaempferol both exert their effects, human liver cancer cells (HepG2), human non-small cell lung cancer cells (A549), human colon cancer cells (SW620), and mouse mammary carcinoma cells (4T1) were ultimately selected for the cellular assays. For the selection of positive control drugs, Adriamycin, which is widely used in the treatment of hepatocellular carcinoma, breast cancer, and non-small cell lung cancer, and 5-fluorouracil, commonly used for colon cancer, were chosen as the positive controls for the cell tests. These drugs were used to demonstrate the inhibitory effects of the aforementioned components on the growth of different tumor cells.

### 2.8.2 Anti-tumor efficacy evaluation

Different tumor cells were routinely cultured in 5% $CO_2$ and 37°C. And HepG2 cells were cultured in DMEM, SW620 in L-15, and 4T1 and A549 in 1640 medium. Logarithmic-phase tumor cells were seeded at $1 \times 10^5$ cells/mL in 96-well plates, 100 μL per well, and cultured for 12 h post-adhesion. Set up administration groups with different concentrations. HepG2 cells received Uralenol and Kaempferol (1, 10, 100, 200, 300 μmol/L) and 1 μmol/L doxorubicin; SW620 cells were treated with Kaempferol (1, 10, 100, 200, 300 μmol/L), Uralenol (1, 25, 50, 75, 100 μmol/L), and 50 μmol/L 5-fluorouracil; A549 cells received Kaempferol (1, 10, 100, 200, 300 μmol/L), Uralenol (1, 25, 50, 100, 200 μmol/L), and 1 μmol/L doxorubicin; 4T1 cells were given Uralenol and Kaempferol (1, 10,

1 https://pubchem.ncbi.nlm.nih.gov/
2 http://swisstargetprediction.ch/
3 https://genealacart.genecards.org/
4 https://www.omim.org/

100, 200, 300 μmol/L) and 5 μmol/L doxorubicin. The control group of all the cells mentioned above was treated with equal volumes of complete culture medium corresponding to the cells. After 24 h of administration, CCK-8 assay was used to measure the absorbance (OD) at 450 nm using an enzyme labeling instrument. The proliferation inhibition rate of the administered monomer on different tumor cells was calculated [cell proliferation inhibition rate = (1-OD value of the treated group cells/OD value of the control group cells) × 100%], as well as the IC50 value.

# 3 Results

## 3.1 TLC results

The TLC results are presented in Figure 1. According to the thin-layer chromatographic identification method of FSH in the Chinese Pharmacopoeia, it can be observed that after the color development was complete, the test solution (FSH) and the control solution (Podophyllotoxin) exhibited the same color spots at the same position on the same silica gel plate. The Rf values for the different FSH samples were calculated as follows: $Rf_{control}$: 0.224; $Rf_{S1}$: 0.224; $Rf_{S2}$: 0.224; $Rf_{S3}$: 0.224; $Rf_{S4}$: 0.224; $Rf_{S5}$: 0.224 (calculated as: Specific Shift Value (Rf) = Distance traveled by the solute / Distance traveled by the solvent front). Suggesting that the above FSH samples were authentic according to the Pharmacopoeia testing methods. S1-S5 represent five different samples.

## 3.2 Analysis on the chemical composition and cleavage pattern of some compounds of FSH

Through UPLC-Q-TOF-MS analysis, it was found that the chemical components present in the five samples of FSH (S1-S5) showed no differences in their types, except for slight variations in mass spectrometry response values. Therefore, sample S1 was selected as an example for further explanation. Based on the retention time of each chemical component in the FSH extract, MS data, extracted total ion-flow diagrams, and relevant literature reports, the chemical composition of the FSH test solution was analyzed, as shown in Figure 2 and Table 1. A total of 85 chemical components were obtained.

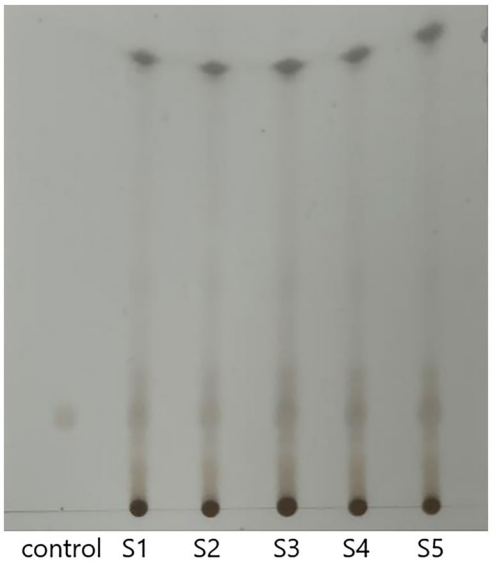



FIGURE 1
TLC results of FSH at different locations. *Control: Podophyllotoxin control solution; S1-S5: FSH samples from different locations.

Since FSH mainly contain flavonoids and lignin compounds, the cleavage patterns of certain compounds in the above two classes were investigated.

### 3.2.1 Partial cleavage patterns of flavonoids

Peaks 5, 12 and 16: the $t_R$ of peak 5 was 6.838 min, while those of peaks 12 and 16 were 7.883 min and 9.223 min, respectively. In addition, the quasimolecular ion peaks in the negative ion mode were m/z 477.1053$[M-H]^-$, m/z 477.1024$[M-H]^-$ and m/z 477.1045$[M-H]^-$, respectively. The molecular formula was $C_{22}H_{22}O_{12}$, and the relative molecular mass was 478.1111, indicating that the three were isomers. In the secondary MS of the negative ions, the fragment ion peaks m/z 315.0523, m/z 315.0520, and m/z 315.0495 were observed, all of which were the glycosidic fragment ions obtained by removing one molecule of glucose. The ion fragment peaks m/z 300.0276, m/z 300.0277 and m/z 300.0226 obtained by removing one molecule of $CH_3$ on the top of the glycoside fragment ion indicated the presence of methoxy substitution in the molecular backbone of the glycoside. Besides, a series of ion fragments m/z 271.0251 and m/z 255.0227 were found in the secondary mass spectra of the negative ions as the typical mass spectral cleavage features of the flavonoid glycosidic skeleton. The three fragment ions were roughly the same, with relative differences in their respective ion abundances. The above fragmentation information of the flavonoid glycoside element basically agreed with the mass spectral cleavage pattern of quercetin. According to the relevant reference (19), peak 5 was presumed to be quercetin-3-methyl ether-3′/4’-O-glucoside, peak 12 was quercetin-3-methyl ether-3′/4’-O-glucoside, and peak 16 was quercetin-3-methyl ether-7-O-glucoside.

Peak 6: The $t_R$ of peak 6 was 6.981 min, and the quasimolecular ion peak in the negative ion mode was m/z 609.1458$[M-H]^-$, which was deduced to have a molecular formula of $C_{27}H_{30}O_{16}$ and a relative molecular mass of 610.1534. In the secondary MS of the negative ions, the ion fragment m/z 463.0088 was the fragmented ion formed by the detachment of rhamnose from the compound, while the ion fragments m/z 301.0326 and m/z 300.0278, as well as m/z 271.0282 and m/z 255.0291 were the fragmentation of glycosides obtained from the continued removal of glucose by this ion. The above fragment ion information was basically consistent with the MS cleavage pattern of rutin, meanwhile, according to the related reference (20), peak 6 was presumed to be rutin.

Peak 8: Its $t_R$ was 7.363 min, and the quasimolecular ion peak in the negative ion mode was m/z 463.0872$[M-H]^-$, which was inferred to have a molecular formula of $C_{21}H_{20}O_{12}$ and a relative molecular mass of 464.0955. In the secondary MS of the negative ions, the ion fragment m/z 301.0326 was obtained through stripping off a molecule of $C_6H_{10}O_5$, and the compound was suspected to be flavonoid glucoside. At the same time, ion fragments m/z 271.0282 and m/z 255.0291 were also found in the secondary spectrum, consistent with the mass spectral cleavage pattern of quercetin (Figure 3). Combined with the ion fragments of the

control product and the relevant references (4, 7), it was hypothesized that peak 8 was attributed to quercetin-3-O-glucoside.

Peaks 18 and 57: Their $t_R$ values were 9.917 min and 16.922 min, respectively, and the quasimolecular ion peaks in the negative ion mode were m/z 367.0810[M-H]$^-$ and m/z 367.0843[M-H]$^-$, respectively, which inferred that both of them had a molecular formula of $C_{20}H_{16}O_7$ and a relative molecular mass of 368.0896, suggesting that the two were isomers. In the negative ion secondary mass spectra, the ion fragments m/z 352.0520, 293.0688, 266.0580, and 193.0146 for peak 8 were observed. Meanwhile, the ion fragments m/z 312.0269, 296.0304, 283.0258, 242.0209, 217.0521, and 173.0590 of peak 31 were seen. Based on the secondary MS fragment ion data of the above two compounds and combined with the related literature reports (4), it was hypothesized that peak 18 was 2′,3′-(2″,2″-dimethyl pyrane)-3,7,5,4′-tetrahydroxy flavone or isomer, whereas peak 57 was 7,8-(2″,2″-dimethyl pyrane)-3,5,3′,4′-tetrahydroxy flavone or isomer.

Peak 25: The $t_R$ was 10.928 min, and the quasimolecular ion peak in the negative ion mode was m/z 315.0502[M-H]$^-$, which was inferred to have a molecular formula of $C_{16}H_{12}O_7$ and a relative molecular mass of 316.0583. In the negative ion secondary MS, fragment ions were found, such as m/z 271.0288, 255.0311, and 227.0365, which represented the typical fragments of the quercetin backbone structure. Also, based on the m/z 300.0293 plasma fragment, the control fragment ions and the related literature reports (21), it was hypothesized that peak 25 was quercetin-3-methyl ether.

Peak 30: Its $t_R$ was 12.393 min, and the quasimolecular ion peak in the negative ion mode was m/z 285.0407[M-H]$^-$, which was inferred to have a molecular formula of $C_{15}H_{10}O_6$ and a relative molecular mass of 286.0477. In the negative ion secondary MS, the fragment ion m/z 255.0256 was formed from the parent ion by removing the CO from the C-ring, and the fragment ion m/z 239.0342 was obtained by continuing to remove O after the removal of CO from the parent ion, while the fragment ion m/z 211.0385 was formed through removing $CO_2$ from the parent ion and then removing CO again. In addition, the fragment ion m/z 133.0299 formed after the cleavage of RDA was also found. Combined with the relevant literature reports (4), it was hypothesized that peak 30 was associated with the compound kaempferol.

Peaks 37 and 39: Their $t_R$ values were 14.6 min and 14.875 min, respectively, and the quasimolecular ion peaks in the negative ion mode were 369.0967 [M-H]$^-$ and 369.0949 [M-H]$^-$, separately, which were predicted to have a molecular formula of $C_{20}H_{18}O_7$ and a relative

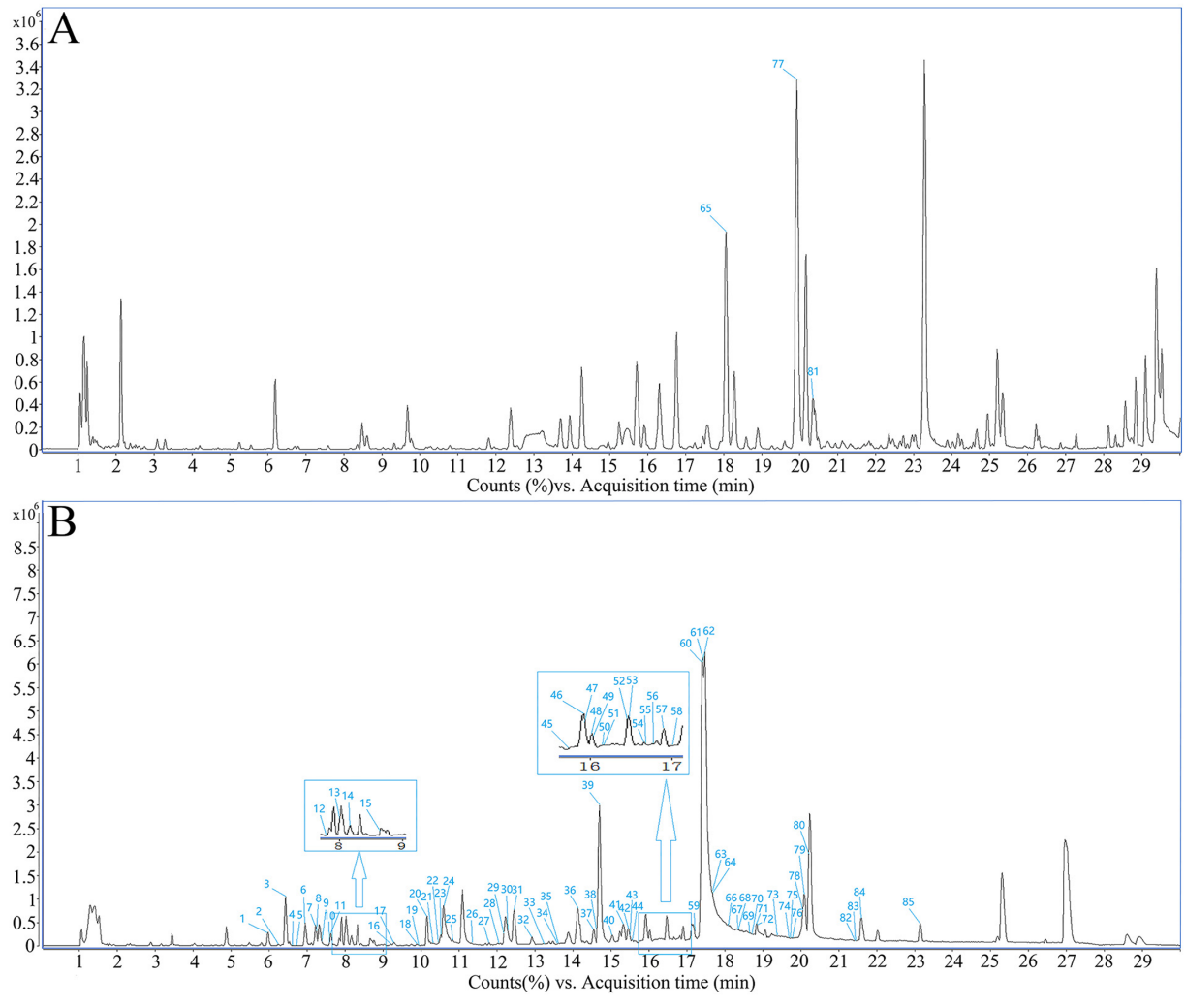



FIGURE 2 (Continued)

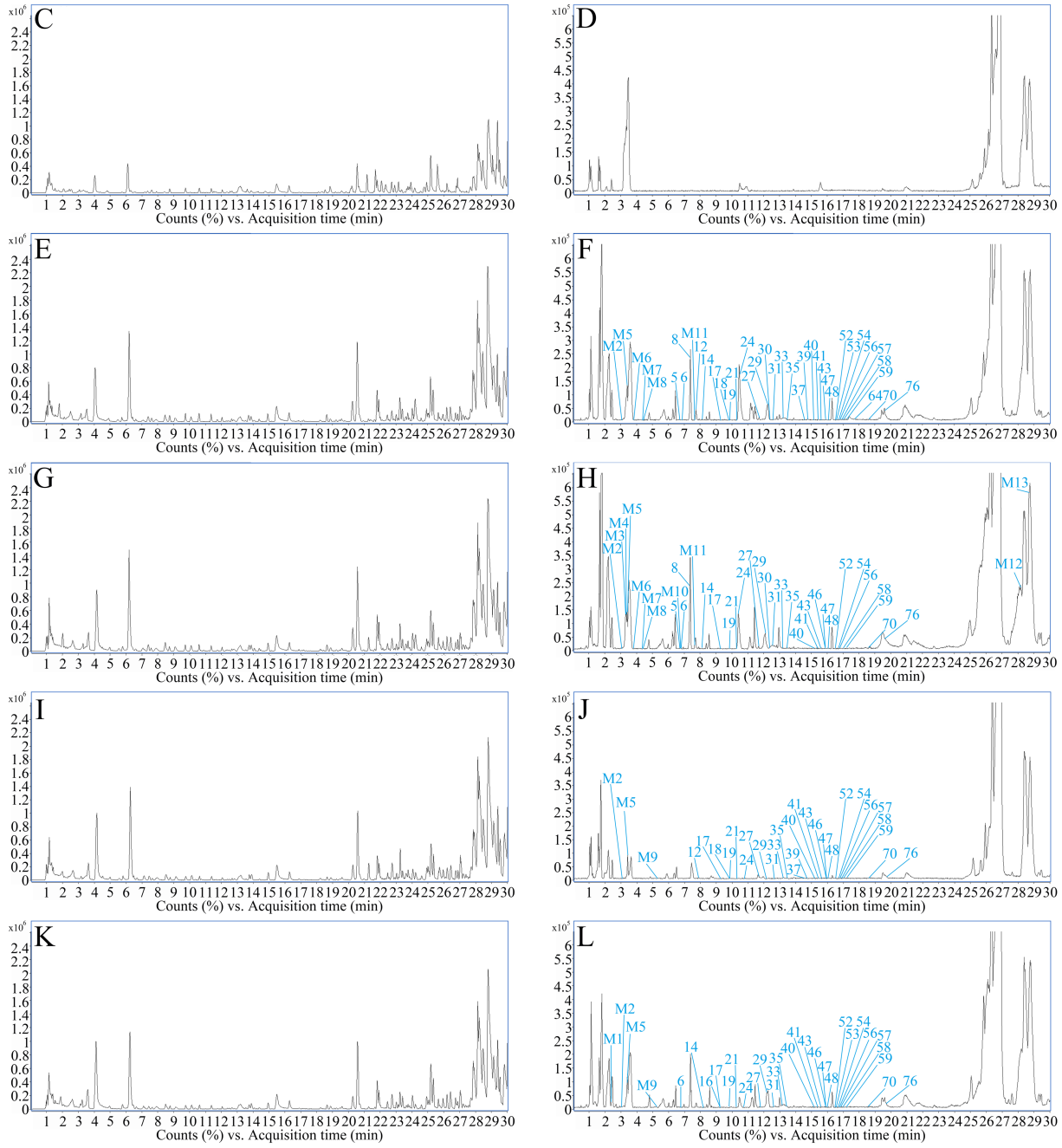



FIGURE 2
TIC profiles of FSH. **(A)** TIC profile in positive ion mode. **(B)** TIC profile in negative ion mode. **(C)** Positive ions in blank group rats. **(D)** Negative ions in blank group rats. **(E)** Positive ions in treatment group rats for 30 min. **(F)** Negative ions in treatment group rats for 30 min. **(G)** Positive ions in treatment group rats for 1 h. **(H)** Negative ions in treatment group rats for 1 h. **(I)** Positive ions in treatment group rats for 2 h. **(J)** Negative ions in treatment group rats for 2 h. **(K)** Positive ions in treatment group rats for 3 h. **(L)** Negative ions in treatment group rats for 3 h.

molecular mass of 370.1053, indicating that the two were isomers. In the negative ion secondary MS, fragment ions m/z 351.0850, 323.0866, 299.0530, and 255.0265 were observed in peak 19, which, combined with the relevant literature report (4), were presumed to be uralenol in peak 37. Fragment ion m/z 219.0688, which could be found in peak 39, was typical of the flavonoid glycoside element RDA. The fragment ion m/z 300.0257 was obtained from the parent ion by removing the isopentenyl group. Additionally, the characteristic fragment ions m/z 313.0386, 247.0572, 219.0688, 202.9968, 191.0014, 179.0004, and 151.0779 were also found (Figure 4). Combined with the relevant literature report (4), peak 39 was hypothesized to be 8-prenylquercetin.

Peaks 40, 47 and 48: Their $t_R$ were 15.084 min, 15.961 min and 16.025 min, respectively. The quasimolecular ion peaks in the negative ion mode were m/z 383.1150[M-H]$^-$, m/z 383.1156[M-H]$^-$, m/z 383.1152[M-H]$^-$, and m/z 383.1152[M-H]$^-$. It was deduced that the three were isomers with a molecular formula of $C_{21}H_{20}O_7$, and a relative molecular mass of 384.1209. In the secondary sub-spectrum of the negative ion of peak 40, the fragment ion m/z 368 was obtained

TABLE 1 Results of the chemical composition of FSH.

| No. | $t_R$/min | Measured value (m/z) | Theoretical value (m/z) | ppm | Fragment (m/z) | Molecular formula | Chemical compound | Ionization mode | *In vivo* conversion or not |
|---|---|---|---|---|---|---|---|---|---|
| 1[a] | 5.976 | 369.0979 | 369.0980 | −0.27 | 247.0683, 220.0716, 219.0637, 175.0828 | $C_{20}H_{18}O_7$ | gancaonin P (23) | $[M-H]^-$ | - |
| 2[a] | 6.242 | 625.1397 | 625.1410 | −2.08 | 464.4814, 463.0792, 302.0378, 301.0340 | $C_{27}H_{30}O_{17}$ | quercetin-3,7-di-O-*β*-D-glucoside (23) | $[M-H]^-$ | - |
| 3[a] | 6.449 | 479.0813 | 479.0831 | −3.76 | 316.0247, 271.0720, 214.8611 | $C_{21}H_{20}O_{13}$ | myricetin-3-O-glucoside (38) | $[M-H]^-$ | - |
| 4[a] | 6.626 | 533.1652 | 533.1664 | −2.25 | 371.1191, 356.6958, 341.4304, 257.9164 | $C_{26}H_{30}O_{12}$ | kaempferol,8-(3-hydroxy-3-methylbutyl)-7-glucoside (23) | $[M-H]^-$ | - |
| 5[a] | 6.838 | 477.1053 | 477.1038 | 3.14 | 316.0548, 315.0523, 300.0276, 271.0252, 255.0227 | $C_{22}H_{22}O_{12}$ | quercetin-3-methyl ether-3′/4’-O-glucoside (19) | $[M-H]^-$ | √ |
| 6[a] | 6.981 | 609.1458 | 609.1461 | −0.49 | 343.0441, 301.0344, 271.0240, 255.0352, 151.0058 | $C_{27}H_{30}O_{16}$ | rutin (20) | $[M-H]^-$ | √ |
| 7[a] | 7.335 | 463.0891 | 463.0882 | 1.94 | 301.0318, 300.0287, 271.0301, 178.9964, 151.0055 | $C_{21}H_{20}O_{12}$ | quercetin-7-O-glucoside (4, 7) | $[M-H]^-$ | - |
| 8[a]* | 7.363 | 463.0872 | 463.0882 | −2.16 | 301.0326, 300.0278, 271.0282, 255.0291 | $C_{21}H_{20}O_{12}$ | quercetin-3-O-glucoside (4, 7) | $[M-H]^-$ | √ |
| 9[b] | 7.549 | 607.1655 | 607.1668 | −2.14 | 561.0267, 399.1068, 397.1258, 381.0992, 307.1403, 323.0176 | $C_{27}H_{30}O_{13}$ | 4′-demethylepipodophyllotoxin glucoside (20) | $[M + HCOO]^-$ | - |
| 10[a]* | 7.637 | 593.1507 | 593.1512 | −0.84 | 286.0446, 285.0405, 255.0224, 229.6993, 211.0364 | $C_{27}H_{30}O_{15}$ | kaempferol-3-O-rutinoside (39) | $[M-H]^-$ | - |
| 11[a] | 7.795 | 369.0971 | 369.0980 | −2.44 | 235.0603, 283.0694, 178.9958 | $C_{20}H_{18}O_7$ | A-ring-monohydroxylated-8-prenylkaempferol (39) | $[M-H]^-$ | - |
| 12[a] | 7.883 | 477.1024 | 477.1038 | −2.93 | 315.0520, 300.0277, 271.0185 | $C_{22}H_{22}O_{12}$ | quercetin-3-methyl ether-3′/4’-O-glucoside (19) | $[M-H]^-$ | √ |
| 13[a] | 8.057 | 447.0929 | 447.0933 | −0.89 | 285.0382, 284.0326, 255.0285, 229.0509, 163.0036 | $C_{21}H_{20}O_{11}$ | kaempferol-3-O-glucoside (23) | $[M-H]^-$ | - |
| 14[b] | 8.179 | 399.1077 | 399.1085 | −2.00 | 384.0878, 314.0450, 284.0400, 257.0464, 241.0428 | $C_{21}H_{20}O_8$ | α-peltatin or isomer (40) | $[M-H]^-$ | √ |
| 15[b] | 8.771 | 707.2173 | 707.2193 | −2.83 | 545.9067, 383.1150, 325.0169, 179.0004, 151.0049 | $C_{33}H_{40}O_{17}$ | demethyldesoxypo-dophyllotoxin isomer diglucoside (23) | $[M-H]^-$ | - |
| 16[a] | 9.223 | 477.1045 | 477.1038 | 1.47 | 315.0495, 300.0226, 299.0172 | $C_{22}H_{22}O_{12}$ | quercetin-3-methyl ether-7-O-glucoside (19) | $[M-H]^-$ | √ |

*(Continued)*





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TABLE 1 (Continued)

| No. | $t_R$/min | Measured value (m/z) | Theoretical value (m/z) | ppm | Fragment (m/z) | Molecular formula | Chemical compound | Ionization mode | *In vivo* conversion or not |
|---|---|---|---|---|---|---|---|---|---|
| 17[b] | 9.288 | 399.1088 | 399.1085 | 0.75 | 383.0747, 366.0762, 355.0910, 340.0970, 270.0442 | $C_{21}H_{20}O_8$ | α-peltatin or isomer (40) | $[M-H]^-$ | √ |
| 18[a] | 9.917 | 367.0810 | 367.0823 | −3.54 | 352.0520, 293.0688, 266.0580, 193.0146 | $C_{20}H_{16}O_7$ | 2′,3′-(2″,2″-dimethylpyrane)-3,7,5,4′-tetrahydroxy flavone or isomer (4) | $[M-H]^-$ | √ |
| 19[b] | 9.963 | 399.1080 | 399.1085 | −1.25 | 384.0862, 355.0810, 399.0855, 329.0855, 314.0440 | $C_{21}H_{20}O_8$ | α-peltatin or isomer (40) | $[M-H]^-$ | √ |
| 20[b] | 10.176 | 621.1823 | 621.1825 | −0.32 | 575.1790, 413.1256, 383.1156, 355.1458, 189.6925 | $C_{28}H_{32}O_{13}$ | β-peltatin-O-β-D-glucoside (23) | $[M + HCOO]^-$ | - |
| 21[b] | 10.263 | 399.1090 | 399.1085 | 1.25 | 383.0758, 369.0643, 355.0809, 341.0674 | $C_{21}H_{20}O_8$ | α-peltatin or isomer (40) | $[M-H]^-$ | √ |
| 22[a] | 10.484 | 301.0351 | 301.0354 | −1.00 | 193.0159, 179.0009, 151.0039 | $C_{15}H_{10}O_7$ | Quercetin (4) | $[M-H]^-$ | - |
| 23[b] | 10.545 | 399.1079 | 399.1085 | −1.50 | 384.0868, 339.0840, 297.0416, 279.0353, 175.0056 | $C_{21}H_{20}O_8$ | α-peltatin or isomer (40) | $[M-H]^-$ | - |
| 24[b] | 10.761 | 399.1085 | 399.1085 | 0.00 | 384.0862, 369.0624, 325.0715, 298.0438 | $C_{21}H_{20}O_8$ | α-peltatin or isomer (40) | $[M-H]^-$ | √ |
| 25[a]** | 10.928 | 315.0502 | 315.0510 | −2.54 | 300.0293, 271.0288, 255.0311, 227.0365 | $C_{16}H_{12}O_7$ | quercetin-3-methyl ether (21) | $[M-H]^-$ | - |
| 26[b] | 11.483 | 399.1121 | 399.1085 | 9.02 | 384.0858, 312.0275, 284.0304, 240.0414 | $C_{21}H_{20}O_8$ | α-peltatin or isomer (40) | $[M-H]^-$ | - |
| 27[b] | 11.878 | 399.1103 | 399.1085 | 4.51 | 384.0860, 356.0945, 173.0630, 159.0491 | $C_{21}H_{20}O_8$ | α-peltatin or isomer (40) | $[M-H]^-$ | √ |
| 28[b] | 12.039 | 399.1101 | 399.1085 | 4.01 | 384.0464, 314.0443, 285.0436 | $C_{21}H_{20}O_8$ | α-peltatin or isomer (40) | $[M-H]^-$ | - |
| 29[b] | 12.131 | 399.1097 | 399.1085 | 3.01 | 382.1045, 381.0979, 369.0942, 178.9998, 152.0114 | $C_{21}H_{20}O_8$ | α-peltatin or isomer (40) | $[M-H]^-$ | √ |
| 30[a] | 12.393 | 285.0407 | 285.0405 | 0.70 | 239.0342, 229.0549, 187.0479, 185.0589, 163.0022, 151.0040 | $C_{15}H_{10}O_6$ | kaempferol (4) | $[M-H]^-$ | √ |
| 31[b] | 12.599 | 399.1104 | 399.1085 | 4.76 | 384.0860, 312.0249, 285.0436, 283.0211 | $C_{21}H_{20}O_8$ | α-peltatin or isomer (40) | $[M-H]^-$ | √ |
| 32[b] | 12.930 | 399.1099 | 399.1085 | 3.51 | 384.0858, 367.0857, 284.0304, 179.0015 | $C_{21}H_{20}O_8$ | α-peltatin or isomer (40) | $[M-H]^-$ | - |
| 33[b] | 13.250 | 399.1096 | 399.1085 | 2.76 | 339.0872, 323.0926, 281.0429, 233.0448 | $C_{21}H_{20}O_8$ | α-peltatin or isomer (40) | $[M-H]^-$ | √ |

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TABLE 1 (Continued)

| No. | $t_R$/min | Measured value (m/z) | Theoretical value (m/z) | ppm | Fragment (m/z) | Molecular formula | Chemical compound | Ionization mode | *In vivo* conversion or not |
|---|---|---|---|---|---|---|---|---|---|
| 34[a] | 13.675 | 367.0826 | 367.0823 | 0.82 | 323.0190, 312.0269, 295.0289, 284.0312, 255.0276, 251.0338 | $C_{20}H_{16}O_7$ | 7,8-(2″,2″-dimethyl pyrane)-3,5,3′,4′-tetrahydroxy flavone or isomer (4) | $[M-H]^-$ | - |
| 35[b] | 13.699 | 399.1094 | 399.1085 | 2.26 | 381.0995, 367.0857, 325.0345, 178.9966 | $C_{21}H_{20}O_8$ | α-peltatin or isomer (40) | $[M-H]^-$ | √ |
| 36[a] | 14.255 | 469.1845 | 469.1868 | −4.90 | 395.1165, 265.0747, 247.0620, 219.0690 | $C_{26}H_{30}O_8$ | Sinoflavonoids K (4) | $[M-H]^-$ | - |
| 37[a]* | 14.600 | 369.0967 | 369.0980 | −3.52 | 351.0850, 323.0866, 299.0530, 255.0265 | $C_{20}H_{18}O_7$ | Uralenol (49) | $[M-H]^-$ | √ |
| 38[a] | 14.720 | 383.1128 | 383.1136 | −2.09 | 368.0906, 327.0416, 284.0334, 267.0308, 256.0385 | $C_{21}H_{20}O_7$ | 2′-prenylquercetin-3-methyl ether (41) | $[M-H]^-$ | - |
| 39[a] | 14.875 | 369.0949 | 369.0980 | −8.40 | 313.0386, 247.0572, 219.0688 | $C_{20}H_{18}O_7$ | 8-prenylquercetin (4) | $[M-H]^-$ | √ |
| 40[a] | 15.084 | 383.1150 | 383.1136 | 3.65 | 368.0916, 369.0947, 326.0401, 314.0385, 176.0136 | $C_{21}H_{20}O_7$ | 6-prenylquercetin-3-methyl ether (4) | $[M-H]^-$ | √ |
| 41[a] | 15.309 | 467.1705 | 467.1711 | −1.28 | 449.1621, 437.1601, 247.0621, 219.0667 | $C_{26}H_{28}O_8$ | dysosmaflavone F (4) | $[M-H]^-$ | √ |
| 42[a] | 15.582 | 453.1554 | 453.1555 | −0.22 | 435.1427, 383.1177, 247.0614, 219.0635, 175.0733 | $C_{25}H_{26}O_8$ | 8-prenyl-3′,4′-(2″,2″-dimethyl-4″-hydroxy-3″,4″-dihydropyran-e)-3,5,7,4′-tetrahydroxy flavone or isomer (4) | $[M-H]^-$ | - |
| 43[a] | 15.670 | 299.0555 | 299.0561 | −2.01 | 284.0350, 255.0308, 227.0363, 183.0473, 167.0518 | $C_{16}H_{12}O_6$ | kaempferol-3-methyl ether (23) | $[M-H]^-$ | √ |
| 44[a] | 15.777 | 367.0823 | 367.0823 | 0.00 | 311.0915, 173.0600, 131.0505 | $C_{20}H_{16}O_7$ | 7,8-(2″,2″-dimethyl pyrane)-3,5,3′,4′-tetrahydroxy flavone or isomer (4) | $[M-H]^-$ | - |
| 45[a] | 15.811 | 453.1549 | 453.1555 | −1.32 | 435.1427, 395.1150, 367.1198, 351.1219, 247.0630, 219.0688 | $C_{25}H_{26}O_8$ | 8-prenyl-3′,4′-(2″,2″-dimethyl-4″-hydroxy-3″,4″-dihydropyran-e)-3,5,7,4′-tetrahydroxy flavone or isomer (4) | $[M-H]^-$ | - |
| 46[a] | 15.944 | 299.0562 | 299.0561 | 0.33 | 284.0365, 227.0364, 164.0098, 151.0046 | $C_{16}H_{12}O_6$ | kaempferol-4′-methyl ether (22) | $[M-H]^-$ | √ |

*(Continued)*





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TABLE 1 (Continued)

| No. | $t_R$/min | Measured value (m/z) | Theoretical value (m/z) | ppm | Fragment (m/z) | Molecular formula | Chemical compound | Ionization mode | *In vivo* conversion or not |
|---|---|---|---|---|---|---|---|---|---|
| 47[a] | 15.961 | 383.1156 | 383.1136 | 5.22 | 369.0950, 368.0913, 339.0899, 323.0925, 284.0276, 283.0244 | $C_{21}H_{20}O_7$ | 6′-prenylquercetin-3-methyl ether (4) | $[M-H]^-$ | √ |
| 48[a] | 16.025 | 383.1152 | 383.1136 | 4.18 | 368.0917, 325.0364, 284.0354, 175.0053 | $C_{21}H_{20}O_7$ | 8-prenylquercetin-3-methyl ether (4) | $[M-H]^-$ | √ |
| 49[a] | 16.072 | 451.1771 | 451.1762 | 1.99 | 451.1797, 247.0630, 219.0672, 177.0204 | $C_{26}H_{28}O_7$ | 6,2′-diprenylquercetin-3-methyl ether (42) | $[M-H]^-$ | - |
| 50[a] | 16.144 | 383.1126 | 383.1136 | −2.61 | 368.0288, 312.0267, 284.0276 | $C_{21}H_{20}O_7$ | 5′-prenylquercetin-3-methyl ether (4) | $[M-H]^-$ | - |
| 51[b]* | 16.158 | 411.1087 | 411.1085 | 0.49 | 379.0825, 351.0835, 219.0652 | $C_{22}H_{20}O_8$ | podophyllotoxne (43) | $[M-H]^-$ | - |
| 52[a] | 16.501 | 467.1704 | 467.1711 | −1.50 | 449.1621, 393.0994, 247.0621, 175.4930 | $C_{26}H_{28}O_8$ | dysosmaflavone E (4) | $[M-H]^-$ | √ |
| 53[a] | 16.596 | 353.1024 | 353.1031 | −1.98 | 298.0455, 253.0451, 225.0525, 219.0689, 163.0048, 136.0178 | $C_{20}H_{18}O_6$ | 8-prenylkaempferol (4) | $[M-H]^-$ | √ |
| 54[a] | 16.700 | 383.1147 | 383.1136 | 2.87 | 339.1289, 324.1010, 173.0610 | $C_{21}H_{20}O_7$ | dimethoxyflavonol (4) | $[M-H]^-$ | √ |
| 55[a] | 16.750 | 437.1595 | 437.1606 | −2.52 | 368.0913, 247.0609, 219.0668, 176.0129, 133.0663 | $C_{25}H_{26}O_7$ | broussonol E (4) | $[M-H]^-$ | - |
| 56[a] | 16.838 | 383.1145 | 383.1136 | 2.35 | 325.0369, 308.0334, 254.0474, 152.0116, 151.1032 | $C_{21}H_{20}O_7$ | sinoflavonoid F (4) | $[M-H]^-$ | √ |
| 57[a] | 16.922 | 367.0843 | 367.0823 | 5.45 | 312.0269, 296.0304, 283.0258, 242.0209, 217.0521, 173.0590 | $C_{20}H_{16}O_7$ | 7,8-(2″,2″-dimethyl pyrane)-3,5,3′,4′-tetrahydroxy flavone or isomer (4) | $[M-H]^-$ | √ |
| 58[a] | 17.054 | 383.1120 | 383.1136 | −4.18 | 353.0888, 351.0892, 178.9993, 151.0038 | $C_{21}H_{20}O_7$ | 4′,5′-(2″,2″-dimethyl-3″,4″-dihydropyran)-5,7,3′-trihydroxy-3-methoxy flavone (4) | $[M-H]^-$ | √ |
| 59[a] | 17.237 | 383.1133 | 383.1136 | −0.78 | 315.0455, 314.0407, 300.0256, 299.0202 | $C_{21}H_{20}O_7$ | 5,7,3′-trihydroxy-3-methoxy flavone-4′-prenyl ether or isomer (4) | $[M-H]^-$ | √ |
| 60[a] | 17.416 | 451.1756 | 451.1762 | −1.33 | 247.0623, 248.0685, 219.0675, 177.0204, 151.0770 | $C_{26}H_{28}O_7$ | 8,2′-diprenylquercetin-3-methyl ether (4) | $[M-H]^-$ | - |
| 61[a] | 17.465 | 367.0837 | 367.0823 | 3.81 | 339.0904, 312.0313, 217.0516, 173.0612 | $C_{20}H_{16}O_7$ | 7,8-(2″,2″-dimethyl pyrane)-3,5,3′,4′-tetrahydroxy flavone or isomer (4) | $[M-H]^-$ | - |

*(Continued)*





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TABLE 1 (Continued)

| No. | $t_R$/min | Measured value (m/z) | Theoretical value (m/z) | ppm | Fragment (m/z) | Molecular formula | Chemical compound | Ionization mode | *In vivo* conversion or not |
|---|---|---|---|---|---|---|---|---|---|
| 62[a] | 17.482 | 451.1766 | 451.1762 | 0.89 | 393.0994, 247.0629, 219.0672 | $C_{26}H_{28}O_7$ | 6,6′-diprenylquercetin-3-methyl ether (4) | [M-H]$^-$ | - |
| 63[a] | 17.837 | 351.0862 | 351.0874 | −3.42 | 323.0950, 295.0990, 217.0524, 189.0544, 173.0609, 161.0616 | $C_{20}H_{16}O_6$ | citrusinol (4) | [M-H]$^-$ | - |
| 64[a] | 17.847 | 367.1171 | 367.1187 | −4.36 | 352.0973, 309.0425, 297.0380, 267.0333 | $C_{21}H_{20}O_6$ | 2′-prenylkaempferol-3-methyl ether (4) | [M-H]$^-$ | √ |
| 65[b]* | 18.048 | 415.1384 | 415.1387 | −0.72 | 247.0671, 189.0633, 229.0257 | $C_{22}H_{22}O_8$ | podophyllotoxin (4) | [M + H]+ | - |
| 66[a] | 18.078 | 367.1185 | 367.1187 | −0.54 | 352.0963, 323.0918, 309.0416, 297.0434, 295.0979, 239.0347 | $C_{21}H_{20}O_6$ | 6-prenylkaempferol-3-methyl ether (44) | [M-H]$^-$ | - |
| 67[a] | 18.211 | 367.0815 | 367.0823 | −2.18 | 352.0953, 309.0353, 297.0333, 245.0475, 173.0606 | $C_{20}H_{16}O_7$ | 7,8-(2″,2″-dimethyl pyrane)-3,5,3′,4′-tetrahydroxy flavone or isomer (4) | [M-H]$^-$ | - |
| 68[b] | 18.275 | 397.1273 | 397.1293 | −5.04 | 313.0726, 188.0849, 179.0039, 165.0177, 151.0032 | $C_{22}H_{22}O_7$ | desoxypodophyllotoxin (23) | [M-H]$^-$ | - |
| 69[a] | 18.605 | 367.1175 | 367.1187 | −3.27 | 352.0978, 309.0414, 253.0514 | $C_{21}H_{20}O_6$ | 3′-prenylkaempferol-3-methyl ether (9, 10) | [M-H]$^-$ | - |
| 70[b] | 18.707 | 411.1072 | 411.1085 | −3.16 | 379.0803, 327.0136, 180.0059, 152.0122 | $C_{22}H_{20}O_8$ | podophyllotoxne isomer (4) | [M-H]$^-$ | √ |
| 71[a] | 18.868 | 451.1763 | 451.1762 | 0.22 | 247.0620, 219.0674, 177.0202, 151.0768, 133.0663 | $C_{26}H_{28}O_7$ | 8,6′-diprenylquercetin-3-methyl ether (4) | [M-H]$^-$ | - |
| 72[a] | 18.880 | 367.1183 | 367.1187 | −1.09 | 309.0398, 297.0402, 253.0500, 225.0581 | $C_{21}H_{20}O_6$ | 8-prenylkaempferol-3-methyl ether (45) | [M-H]$^-$ | - |
| 73[a] | 19.458 | 451.1759 | 451.1762 | −0.66 | 368.0840, 247.0626, 219.0672, 177.0205, 163.0026 | $C_{26}H_{28}O_7$ | 6,5′-diprenylquercetin-3-methyl ether (4) | [M-H]$^-$ | - |
| 74[a] | 19.732 | 435.1432 | 435.1449 | −3.91 | 417.1320, 287.1008, 219.0671 | $C_{25}H_{24}O_7$ | 8,2′-diprenylkaempferol-3-methyl ether (46) | [M-H]$^-$ | - |
| 75[b] | 19.771 | 379.0844 | 379.0823 | 5.54 | 364.0566, 335.0573, 319.1958, 199.0734 | $C_{21}H_{16}O_7$ | diphyllin (23) | [M-H]$^-$ | - |
| 76[a] | 19.823 | 435.1446 | 435.1449 | −0.69 | 349.0341, 326.0420, 325.0352 | $C_{25}H_{24}O_7$ | 7,8-(2″,2″-dimethyl pyrane)-6′-prenyl-3,5,3′,4′-tetrahydroxy flavone (4) | [M-H]$^-$ | √ |

*(Continued)*





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TABLE 1 (Continued)

| No. | $t_R$/min | Measured value (m/z) | Theoretical value (m/z) | ppm | Fragment (m/z) | Molecular formula | Chemical compound | Ionization mode | *In vivo* conversion or not |
|---|---|---|---|---|---|---|---|---|---|
| 77[b] | 19.918 | 399.1444 | 399.1438 | 1.50 | 231.0681, 203.0779, 187.0812 | $C_{22}H_{22}O_7$ | desoxypodophyllotoxin (4) | $[M + H]^+$ | - |
| 78[a] | 20.006 | 449.1600 | 449.1606 | −1.34 | 218.0583, 217.0517, 203.0355 | $C_{26}H_{26}O_7$ | 7,8-(2″,2″-dimethyl pyrane)-2′-prenyl-5,3′,4′-trihydroxy-3-methoxy flavone (9, 10) | $[M-H]^-$ | - |
| 79[a] | 20.170 | 451.1753 | 451.1762 | −1.99 | 436.1535, 393.0982, 367.0831, 325.0345 | $C_{26}H_{28}O_7$ | 8,5′-diprenylquercetin-3-methyl ether (4) | $[M-H]^-$ | - |
| 80[a] | 20.235 | 449.1610 | 449.1606 | 0.89 | 218.0587, 217.0515, 173.0613, 131.0504 | $C_{26}H_{26}O_7$ | 8-prenyl-2′,3′-(2,2-dimethyl pyrane)-5,7,4′-trihydroxy-3-methoxy flavone (9, 10) | $[M-H]^-$ | - |
| 81[b] | 20.398 | 385.1283 | 385.1283 | 0.00 | 231.0621, 173.0638, 187.0511 | $C_{21}H_{20}O_7$ | 4′-demethyldeox Ypodophyllotoxin (4) | $[M + H]^+$ | - |
| 82[a] | 21.578 | 449.1603 | 449.1606 | −0.67 | 375.0838, 332.0329, 321.0394, 320.0328, 247.0628, 219.0674 | $C_{26}H_{26}O_7$ | 6-prenyl-3′,4′-(2,2-dimethyl pyrane)-5,7,5′-trihydroxy-3-methoxy flavone (4) | $[M-H]^-$ | - |
| 83[a] | 21.587 | 367.0820 | 367.0823 | −0.82 | 339.0887, 312.0284, 311.0930, 284.0355, 283.0232, 217.0525, 173.0624 | $C_{20}H_{16}O_7$ | 7,8-(2″,2″-dimethyl pyrane)-3,5,3′,4′-tetrahydroxy flavone or isomer (4) | $[M-H]^-$ | - |
| 84[a] | 21.761 | 449.1607 | 449.1606 | 0.22 | 375.0890, 320.0316, 219.0673 | $C_{26}H_{26}O_7$ | 8-prenyl-3′,4′-(2,2-dimethyl pyrane)-5,7,5′-trihydroxy-3-methoxy flavone (47) | $[M-H]^-$ | - |
| 85[a] | 23.211 | 447.1434 | 447.1449 | −3.35 | 217.0491, 216.0444, 215.0358, 188.0499, 187.0381 | $C_{26}H_{24}O_7$ | 7,8-(2″-isopropenyl-furano)-2′-prenyl-5,3′,4′-trihydroxy-3-methoxy flavone (9, 10) | $[M-H]^-$ | - |

[a]Represents flavonoids.
[b]Represents lignans.
"*" indicates that the changed compound has been compared with the control product.





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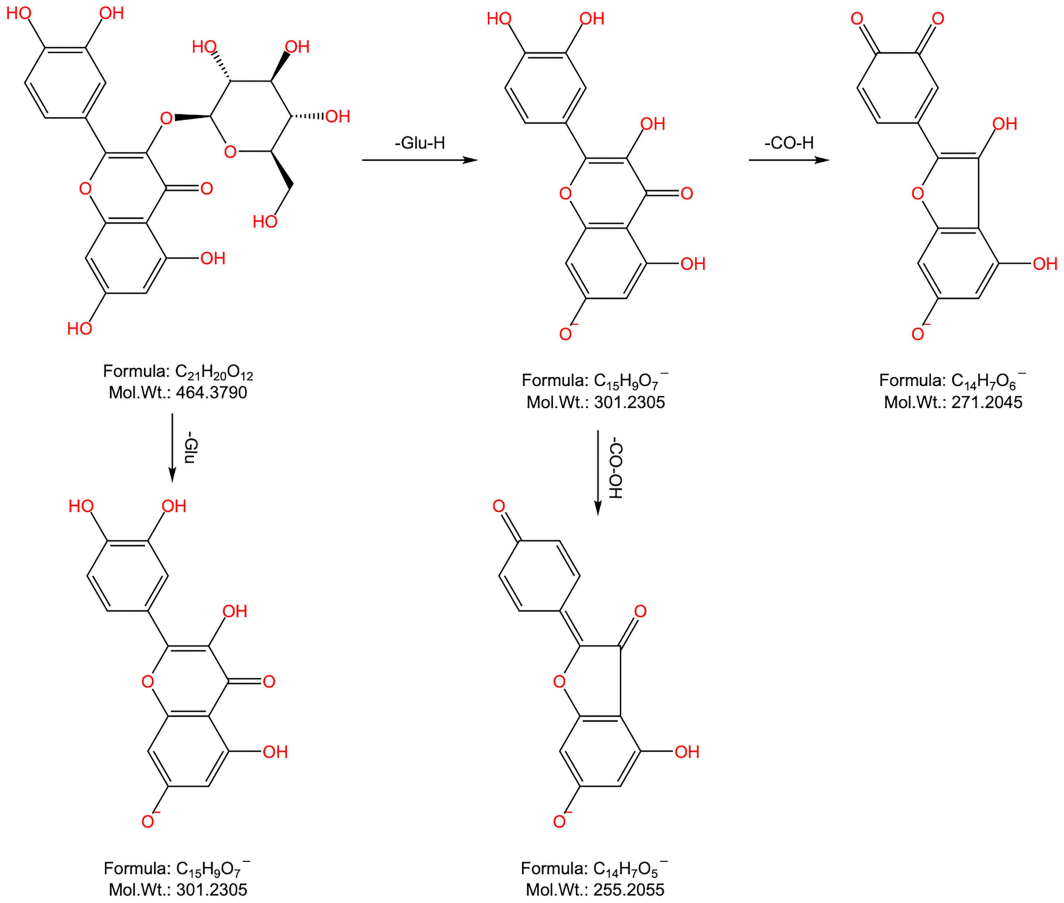



FIGURE 3
Chemical structure and major cleavage fragments of quercetin-3-O-glucoside.

from the parent ion stripped off $CH_3$, the fragment ion m/z 339 was derived from the parent ion by removing $CO_2$, the fragment ion m/z 325 was acquired from the parent ion by eliminating $C_4H_{10}$, and the fragment ion m/z 175 was obtained from the parent ion. Moreover, the fragment ions m/z 369.0947, 326.0401, 314.0385, and 176.0136 were also found, which were presumed to be 6-isopentenylquercetin-3-methyl ether by combining with the related literature report (4). In the secondary MS of the negative ion of peak 47, the fragment ion m/z 368.0913 was obtained from the parent ion by removing one molecule of methyl ether, and the fragment ion m/z 325 was acquired from the parent ion by removing $C_4H_{10}$, whereas the fragment ion m/z 175 was derived from the parent ion by removing $C_{13}H_{20}O_2$. The fragment ion m/z 368.0913 was produced from the parent ion by removing one molecule of methyl radical, and the fragment ion m/z 339.0899 was generated from the parent ion by removing one molecule of $CO_2$. Additionally, the fragment ions m/z 369.0950, 323.0925, 284.0276, and 283.0244 were also detected, which, combined with the relevant literature report (4), were presumed to be 6′ -isopentenylquercetin-3-methyl ether. In the secondary MS of the negative ion of peak 48, the fragment ion m/z 368.0917 was formed from the parent ion by removing the methyl group, while the fragment ion m/z 325.0364 was obtained from the parent ion by eliminating the $C_4H_{10}$ group, and the fragment ion m/z 175.0053 was acquired from the parent ion by removing the $C_{13}H_{20}O_2$ group. By comparison with other fragment ions and combination with literature report (4), peak 48 was presumed to be 8-isopentenylquercetin-3-methyl ether.

Peaks 43 and 46: Their $t_R$ were 15.67 min and 15.944 min, respectively, and the quasimolecular ion peaks in the negative ion mode were m/z 299.0555$[M-H]^-$ and m/z 299.0562$[M-H]^-$, separately. It was deduced that the two were isomers with a molecular formula $C_{16}H_{12}O_6$ and a relative molecular mass of 300.0634. In the two negative ion secondary mass spectra, the fragment ions m/z 284.0350 and m/z 284.0365 were found to be obtained by removing $CH_3$ from the corresponding parent ions. Meanwhile, the same fragment ions m/z 227.0363 and m/z 227.0364, as well as the related ion fragments m/z 255.0308, 167.0518, 164.0098, 151.0518 164.0098, and 151.0046 were also found, which, combined with relevant literature reports (22, 23), speculated that peak 43 was kaempferol-3-methyl ether and peak 46 was kaempferol-4′-methyl ether.

Peak 64: The $t_R$ was 17.847 min, and the quasimolecular ion peak in the negative ion mode was m/z 367.1171$[M-H]^-$, and peak 64 was inferred to have a molecular formula of $C_{21}H_{20}O_6$ and a relative molecular mass of 368.1260. In the negative ion secondary MS, the fragment ion m/z 352.0973 was obtained from the parent ion by removing $CH_3$, and the fragment ion m/z 309.0425 was generated by the continuous removal of $C_3H_7$ from the parent ion, while the fragment ion m/z 297.0380 was produced from the parent ion by continuing to remove $C_4H_7$ after the removal of $CH_3$. Additionally, the fragment ion

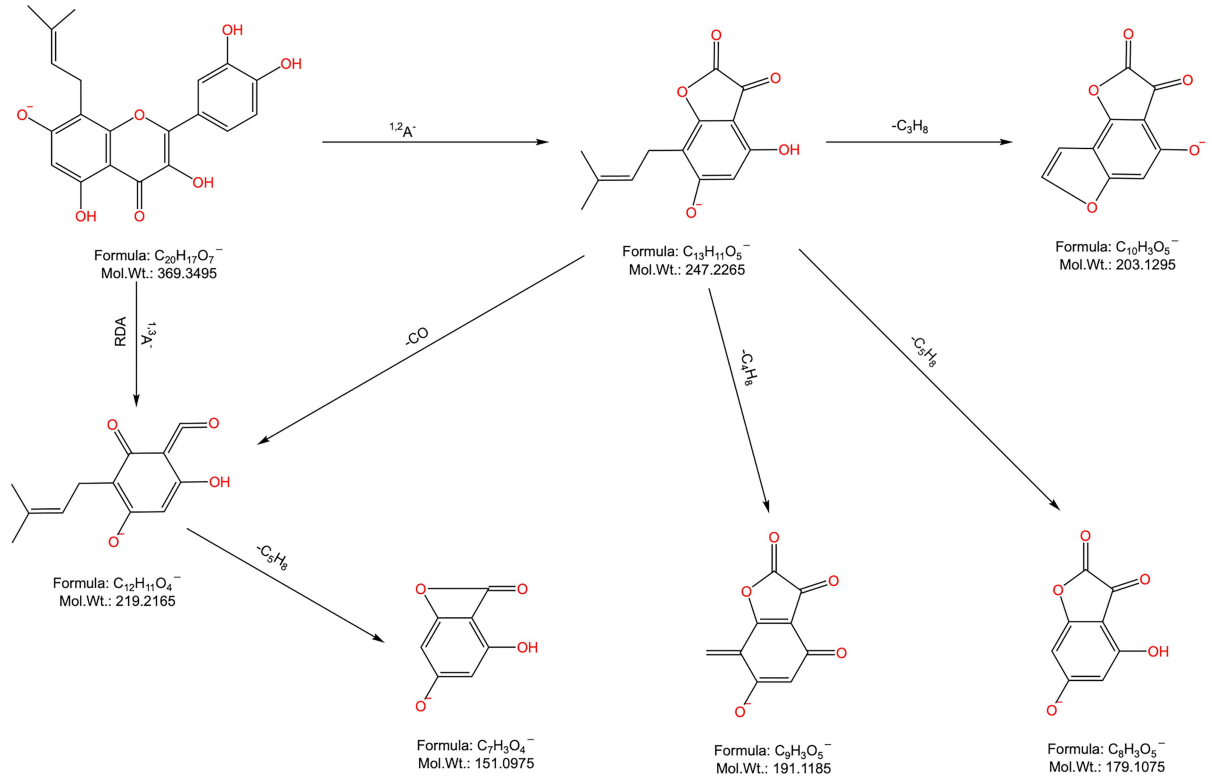



FIGURE 4
Chemical structure and major cleavage fragments of 8-prenylquercetin.

m/z 309.0425 was obtained when the parent ion continued to remove $C_3H_7$, and the fragment ion m/z 297.0380 was acquired when the parent ion further removed $C_4H_7$ after removing $CH_3$. Combined with the above ion fragmentation information and related literature report (4), it was hypothesized that peak 64 was 2′-prenylkaempferol-3-methyl ether.

### 3.2.2 Partial cleavage patterns of lignin analogs

Peak 70: The $t_R$ was 18.707 min, and the quasimolecular ion peak in the negative ion mode was m/z 411.1072[M-H]$^-$, which was inferred to have a molecular formula of $C_{22}H_{20}O_8$ and a relative molecular mass of 412.1158. In the negative ion secondary MS, the characteristic fragment ion m/z 379.0803 and fragment ion m/z 297.0345 formed by the cleavage rearrangement of the bynyl group were also detected in addition to fragment ions m/z 327.0136, 180.0059, and 152.0122. Combined with the related literature report (4), it was hypothesized that peak 70 was the podophyllotoxne isomer.

## 3.3 Analysis of the *in vivo* absorbed and transformed chemical components of FSH

The mass spectra of rat drug-containing plasma samples and blank plasma samples were compared. Combined with the MS analysis patterns of the chemical components of FSH, a total of 35 prototype components and 13 *in vivo* transformed products were identified in the rat plasma based on MS data. The mass spectra of blank plasma and drug-containing plasma samples are shown in Figures 2C–L. More details of the *in vivo* absorbed components are displayed in Table 1, and the *in vivo* transformed products are presented in Table 2.

Compound M1: The $t_R$ was 2.446 min, the quasimolecular ion peak in the negative ion mode was m/z 409.0914, and its molecular formula was inferred to be $C_{22}H_{18}O_8$, which differed from the quasimolecular ion peak in the negative ion mode of compound 51 of m/z 411.1087 by 2 Da. It was suggested that compound 51 was dehydrogenated from $H_2$ in rats, therefore, compound M1 was inferred to be the dehydrogenated product of compound 51, as shown in Figure 5A.

Compound M2: The $t_R$ was 3.094 min, and the quasimolecular ion peak in the negative ion mode was m/z 577.1538, which was inferred to have a molecular formula of $C_{27}H_{30}O_{14}$. In the meantime, the quasimolecular ion peak in the negative ion mode of compound 10 was m/z 593.1507, with a difference of 16 Da compared with compound M2. Consequently, compound M2 was speculated to be the dehydrogenated product of compound 10 in rats, as observed from Figure 5B.

Compound M3: The $t_R$ was 3.310 min, and the quasimolecular ion peak in the negative ion mode was m/z 607.1650. Its molecular formula was deduced to be $C_{28}H_{32}O_{15}$, which was 14 Da different from the quasimolecular ion peak in the negative ion mode of compound 10, m/z 593.1507. It was speculated that compound 10 increased $CH_3$ in rats, therefore compound M3 was assumed to be the methylated product of compound 10, as shown in Figure 5C.

Compound M4: Its $t_R$ was 3.409 min, its quasimolecular ion peak in the negative ion mode was m/z 671.1980, and it was inferred to have a molecular formula of $C_{32}H_{34}O_{13}$, which differed by 221 Da from the quasimolecular ion peak of compound 78 in the negative ion mode (m/z 449.1600). Consequently, it was presumed that compound M4

TABLE 2 The *in vivo* absorbed and transformed chemical components of FSH.

| No. | $t_R$/min | Measured value (m/z) | Theoretical value (m/z) | ppm | Molecular formula | Chemical compound | Ionization mode |
|---|---|---|---|---|---|---|---|
| M1 | 2.446 | 409.0914 | 409.0929 | −3.67 | $C_{22}H_{18}O_8$ | Dehydrogenation products of podophyllotoxne | $[M-H]^-$ |
| M2 | 3.094 | 577.1538 | 577.1563 | −4.33 | $C_{27}H_{30}O_{14}$ | Oxygen-depleted product of kaempferol-3-O-rutinoside | $[M-H]^-$ |
| M3 | 3.310 | 607.1650 | 607.1668 | −2.96 | $C_{28}H_{32}O_{15}$ | Kaempferol-3-O-rutinoside methylation product | $[M-H]^-$ |
| M4 | 3.409 | 671.1980 | 671.1981 | −0.15 | $C_{32}H_{34}O_{13}$ | 7,8-(2″,2″-Dimethylpyrane)-2′-prenyl-5,3′,4′-trihydroxy-3-methoxy flavone glucuronidation product | $[M + COOH]^-$ |
| M5 | 3.487 | 575.1382 | 575.1406 | −4.17 | $C_{27}H_{28}O_{14}$ | α-peltatin or isomer glucuronidation product | $[M-H]^-$ |
| M6 | 3.891 | 461.0718 | 461.0725 | −1.52 | $C_{21}H_{18}O_{12}$ | Kaempferol glucuronide product | $[M-H]^-$ |
| M7 | 4.569 | 491.0809 | 491.0831 | −4.48 | $C_{22}H_{20}O_{13}$ | Quercetin-3-methyl ether glucuronide product | $[M-H]^-$ |
| M8 | 4.660 | 559.1443 | 559.1457 | −2.50 | $C_{27}H_{28}O_{13}$ | 4′-demethyldeoxypodophyllotoxin glucuronide product | $[M-H]^-$ |
| M9 | 5.216 | 545.1288 | 545.1301 | −2.38 | $C_{26}H_{26}O_{13}$ | 8-Prenylquercetin glucuronide product | $[M-H]^-$ |
| M10 | 6.879 | 627.2069 | 627.2083 | −2.23 | $C_{32}H_{36}O_{13}$ | 8, 6'-Diprenylquercetin-3-methyl ether glucuronide product | $[M-H]^-$ |
| M11 | 7.751 | 447.0918 | 447.0933 | −3.36 | $C_{21}H_{20}O_{11}$ | Quercetin-3-methyl ether-7-O-glucoside hydroxymethylene loss products | $[M-H]^-$ |
| M12 | 28.118 | 395.1498 | 395.1500 | −0.51 | $C_{23}H_{24}O_6$ | 2'-Prenylkaempferol-3-methyl ether dimethylated product | $[M-H]^-$ |
| M13 | 28.799 | 421.1651 | 421.1657 | −1.42 | $C_{25}H_{26}O_6$ | Broussonol E Eoxygen loss products | $[M-H]^-$ |

was the methylated product of compound 10, namely, the dimethylpyrane-2′-prenyl-5,3′,4′-trihydroxy-3-methoxy flavone glucuronidation product (Figure 5D).

Compound M5: The $t_R$ was 3.487 min, the quasimolecular ion peak in the negative ion mode was m/z 575.1382, and it was inferred with a molecular formula of $C_{27}H_{28}O_{14}$. Besides, the quasimolecular ion peak in the negative ion mode of compound 4′-demethylprenyl glucuronide had was m/z 399.1085, which differed from compound M5 by 176 Da. In this regard, it was inferred that compound M5 was the 4′-demethyldauricin glucuronidated product, as observed from Figure 5E.

Compound M6: The $t_R$ was 3.891 min, the quasimolecular ion peak in the negative ion mode was m/z 461.0718, and its molecular formula was inferred to be $C_{21}H_{18}O_{12}$, which was 176 Da different from the quasimolecular ion peak in the negative ion mode of compound 30 (m/z 285.0407). As a result, it was presumed that compound M6 was a kaempferol glucuronidated product (Figure 5F).

Compound M7: The $t_R$ was 4.569 min, and the quasimolecular ion peak in the negative ion mode was m/z 491.0809, which suggested that the molecular formula of compound M7 was $C_{22}H_{20}O_{13}$, with a difference of 176 Da compared with the quasimolecular ion peak in the negative ion mode of compound 25 (m/z 315.0502). Consequently, compound M7 was presumed to be the quercetin-3-methyl ether glucuronidated product (Figure 5G).

Compound M8: The $t_R$ was 4.660 min, the quasimolecular ion peak in the negative ion mode was m/z 559.1443, and its molecular formula was inferred to be $C_{27}H_{28}O_{13}$, which differed from the quasimolecular ion peak in the negative ion mode of compound 4′-demethyldeoxypodophyllotoxin by 176 Da. Therefore, compound M8 was presumed to be the 4′-demethyldeoxypodophyllotoxin glucuronide product, as observed from Figure 5H.

Compound M9: The $t_R$ was 5.216 min, and the quasimolecular ion peak in the negative ion mode was m/z 545.1288, inferring that compound M9 had a molecular formula of $C_{26}H_{26}O_{13}$. Additionally, there was a difference of 176 Da compared with the quasimolecular ion peak in the negative ion mode of compound 39 (m/z 369.0980). So it was speculated that compound M9 was the 8-prenyl quercetin glucuronidated product (Figure 5I).

Compound M10: The $t_R$ was 6.879 min, and the quasimolecular ion peak in the negative ion mode was m/z 627.2069, which was deduced to have a molecular formula $C_{32}H_{36}O_{13}$. There was a difference of 176 Da compared with the quasimolecular ion peak in the negative ion mode of compound 60 (m/z 451.1762). In this regard, it was hypothesized that compound M10 was the 8,6′-diprenylquercetin-3-methyl ether glucuronidated product (Figure 5J).

Compound M11: The $t_R$ was 7.751 min, and the quasimolecular ion peak in the negative ion mode was m/z 447.0918, which was inferred to have a molecular formula of $C_{21}H_{20}O_{11}$. A difference of 30 Da was found compared with the quasimolecular ion peak of compound 16 in the negative ion mode (m/z 477.1038). Therefore, compound M11 was assumed to be the quercetin-3-methyl

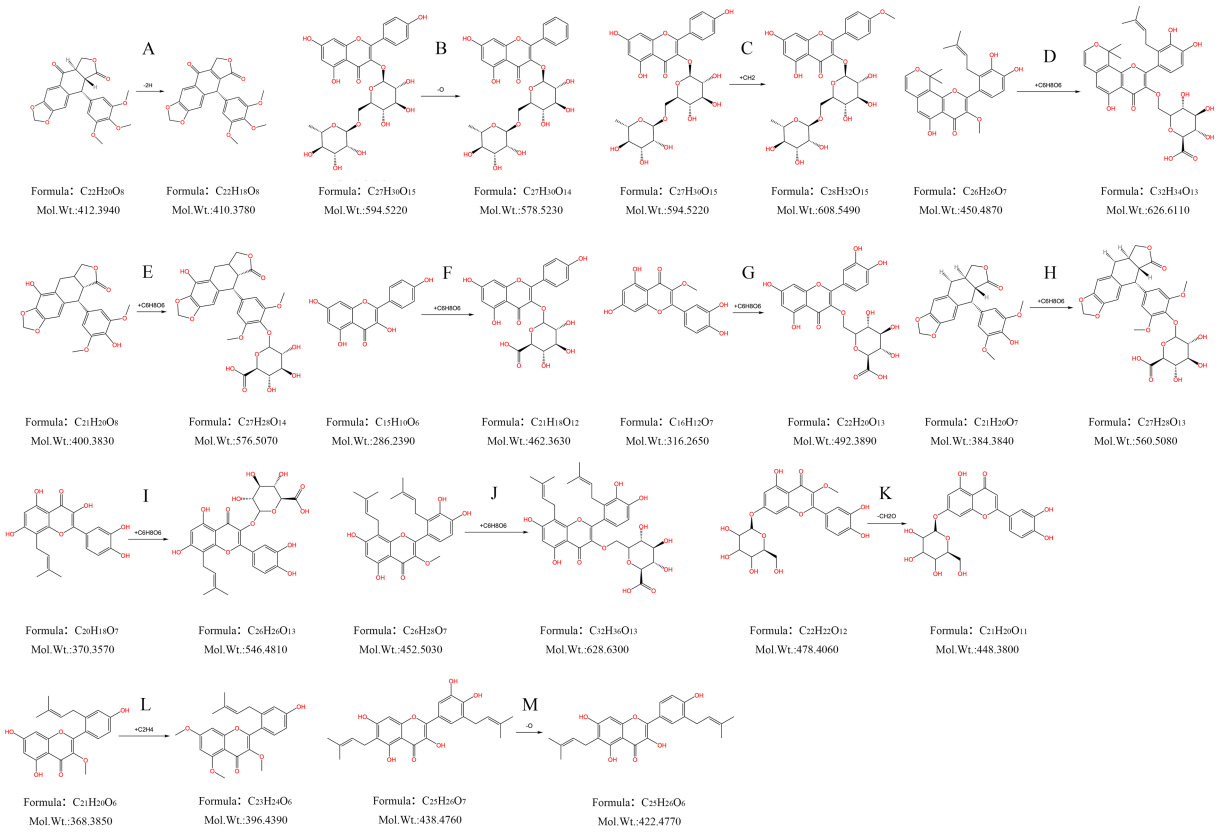



FIGURE 5
Metabolism pattern of metabolites in FSH. **(A)** Dehydrogenation products of podophyllotoxne. **(B)** Oxygen-depleted product of kaempferol-3-O-rutinoside. **(C)** Kaempferol-3-O-rutinoside methylation product. **(D)** 7,8-(2″,2″-Dimethylpyrane)-2′-prenyl-5,3′,4′-trihydroxy-3- methoxy flavone glucuronidation product. **(E)** α-peltatin or isomer glucuronidation product. **(F)** Kaempferol glucuronide product. **(G)** Quercetin-3-methyl ether glucuronide product. **(H)** 4′-demethyldeoxypodophyllotoxin glucuronide product. **(I)** 8-Prenylquercetin glucuronide product. **(J)** 8, 6′-Diprenylquercetin-3- methyl ether glucuronide product. **(K)** Quercetin-3-methyl ether-7-O-glucoside hydroxymethylene loss products. **(L)** 2′-Prenylkaempferol-3- methyl ether dimethylated product. **(M)** Broussonol E Eoxygen loss products.

ether-7-O-glucoside loss product of hydroxymethylene, as observed from Figure 5K.

Compound M12: The $t_R$ was 28.118 min, and the quasimolecular ion peak in the negative ion mode was m/z 395.1498. Its molecular formula was inferred to be $C_{23}H_{24}O_6$, which was 28 Da different from the quasimolecular ion peak in the negative ion mode of compound 64 (m/z 367.1171). Thus, compound M12 was inferred to be the 2′-prenylkaempferol-3-methyl ether dimethylated product, as shown in Figure 5L.

Compound M13: Its $t_R$ was 28.799 min, and its quasimolecular ion peak in the negative ion mode was m/z 421.1651, which was inferred to have a molecular formula of $C_{25}H_{26}O_6$. Meanwhile, a difference of 16 Da was detected compared with the quasimolecular ion peak in the negative ion mode of compound 55 (m/z 437.1595). Consequently, compound M13 was inferred to be the oxygen-loss product of broussonol E (Figure 5M).

## 3.4 Network pharmacology of the anti-tumor effect of FSH

### 3.4.1 Acquisition of the *in vivo* transformed components and targets of FSH

Based on the molecular structures of the 35 prototypical absorbed components and 13 prototypical components of *in vivo* transformed metabolites identified in FSH, their potential targets were predicted based on their chemical structural formulas by swiss target prediction (see text footnote 2), and finally after de-weighting, a total of 319 drug-related targets for 25 components were obtained.

### 3.4.2 Acquisition of the tumor disease-related targets

Using "tumor" as the keyword, the Gene Cards database and OMIM database were searched, respectively, which obtained 1,567 disease-related targets after taking the concatenated set.

### 3.4.3 Acquisition of the intersected targets between FSH and the tumor disease

The tumor-related targets were mapped with the transformed components of FSH by adopting the microbiology data analysis platform, and 118 intersected targets were obtained, as shown in Figure 6A.

### 3.4.4 Construction of the drug-component-target-disease network

The transformed components and drug-disease intersected targets of FSH were imported into Cytoscape 3.9.1 software, and the "drug-component-target" map was plotted in Figure 6B. In this visualization, green nodes represent drug-disease intersected targets, cyan nodes

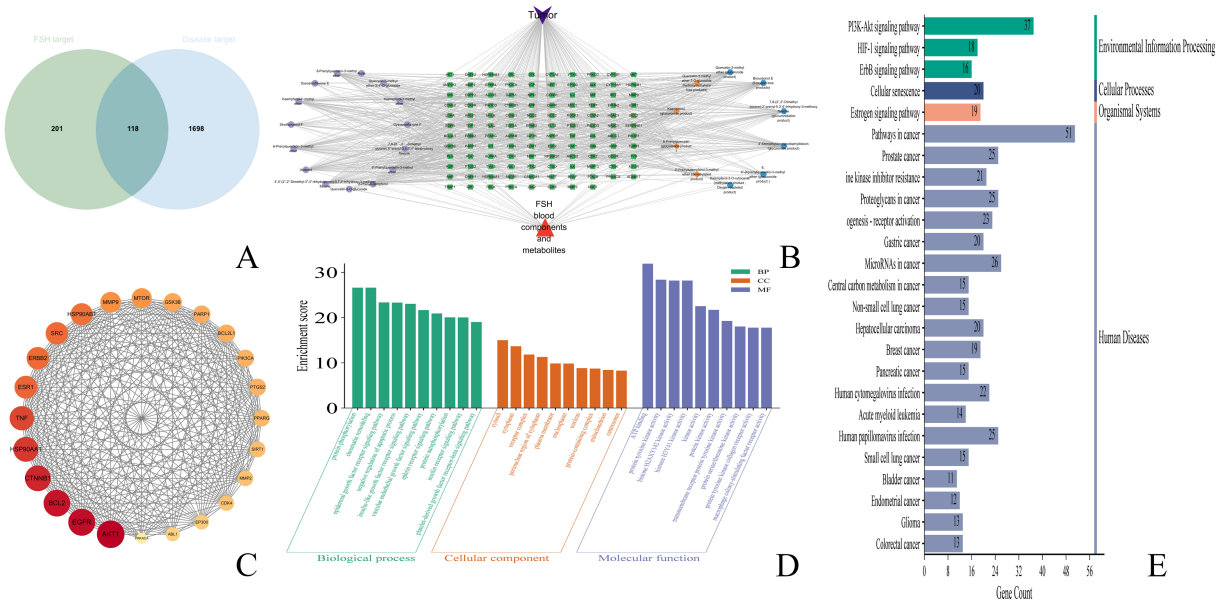



FIGURE 6
Anti-tumor network pharmacological results of FSH. **(A)** veen plot. **(B)** drug-component-target plot. **(C)** Protein–protein interaction (PPI) network plot. **(D)** GO pathway analysis plot. **(E)** KEGG pathway analysis plot.

denote the prototype component of the transformed component of FSH *in vivo*, orange nodes indicate components where both the prototype component and the metabolite were observed, and blue nodes signify components where only the metabolite was detected. Subsequently, utilizing the predicted core targets, the associated chemical components were quantified within Cytoscape 3.9.1 software. The top ten core components were identified based on a Degree value threshold of ≥40, as detailed in Table 3.

### 3.4.5 Protein–protein interaction network analysis

The 113 intersected targets of FSH and tumor disease were imported into the String database to construct the PPI network. After removing the free nodes, the network topology was analyzed using Cytoscape 3.9.1 software, and then the topological parameters of the network nodes were analyzed by the Network Analyzer plug-in. Using the three parameters of BC, CC and Degree as the indicators, the medians of these three indicators were calculated. When the target indicator was larger than the corresponding median, it was predicted as a core target. Finally, the core targets were ranked in accordance with the Degree value. There were 24 nodes and 255 edges in the network, as observed from Figure 6C and Table 4.

### 3.4.6 GO and KEGG enrichment analyses

Upon GO functional annotation, totally 612 biological processes (BP) were analyzed, including protein phosphorylation, chromatin remodeling, epidermal growth factor receptor signaling pathway, and negative regulation of apoptotic process. Meanwhile, 100 cellular components (CC) were obtained, including cytosol, cytoplasm, receptor complex, perinuclear region of cytoplasm, and plasma membrane. There were also 179 molecular functions (MF) being acquired, including ATP binding, protein tyrosine kinase activity, histone H2AXY142 kinase activity, and histone H3Y41 kinase activity.

TABLE 3 Core components of FSH for tumor therapy.

| No. | Degree | Core component |
|---|---|---|
| 1 | 53 | 6'-Prenylquercetin-3-methyl ether |
| 2 | 49 | 8-Prenylquercetin (glucuronide product) |
| 3 | 49 | Quercetin-3-methyl ether (glucuronide product) |
| 4 | 48 | Uralenol |
| 5 | 46 | Kaempferol-4′-methyl ether |
| 6 | 45 | Kaempferol (glucuronide product) |
| 7 | 44 | 6-Prenylquercetin-3-methyl ether |
| 8 | 44 | Kaempferol-3-methyl ether |
| 9 | 43 | 4'-Demethyldeoxypodophyllotoxin (glucuronide product) |
| 10 | 43 | Broussonol E (Eoxygen loss products) |

TABLE 4 Core targets of FSH for tumor therapy.

| No. | Degree | Core targets |
|---|---|---|
| 1 | 95 | AKT1 |
| 2 | 93 | EGFR |
| 3 | 92 | BCL2 |
| 4 | 89 | CTNNB1 |
| 5 | 86 | HSP90AA1 |
| 6 | 84 | TNF |
| 7 | 79 | ESR1 |
| 8 | 78 | ERBB2 |
| 9 | 78 | SRC |
| 10 | 77 | HSP90AB1 |

The data were analyzed by the Microbiology Data Analysis Platform (MDA). The bar chart of GO functional enrichment analysis was produced by the Microbiotics data analysis platform (Figure 6D).

As suggested by the KEGG pathway enrichment analysis results, there were 146 pathways enriched by the tumor treatment with FSH. The top 25 KEGG pathways were input into the MDA platform, and the bubble diagram showing the KEGG pathway enrichment analysis results was produced (Figure 6E). Obviously, the main signaling pathways engaged in the targets of FSH for tumor treatment were Pathways in cancer, Prostate cancer, PI3K-Akt signaling pathway, EGFR tyrosine kinase inhibitor resistance, and Proteoglycans in cancer. The results of the top 25 KEGG pathways enriched were summarized with their associated chemical compositions, and the results are presented in Supplementary Table S1.

After combining the top 25 KEGG results with PPI data, it was observed that AKT1 was present in 23 pathways, with the exceptions of "MicroRNAs in cancer" and "Bladder cancer." EGFR was identified in 22 pathways, excluding "Cellular senescence," "Acute myeloid leukemia," and "Small cell lung cancer." BCL2 appeared in 11 pathways, including "Pathways in Cancer" and "Prostate Cancer," among others. CTNNB1 was involved in 10 pathways, such as "Prostate Cancer" and "Proteoglycans in Cancer." HSP90AA1 was found in "Pathways in Cancer," "Prostate Cancer," and five additional pathways. TNF was associated with 3 pathways: "Proteoglycans in Cancer," "Human Cytomegalovirus infection," and "Human Papillomavirus infection." ESR1 was included in 5 pathways, notably "Pathways in Cancer" and "Proteoglycans in Cancer." ERBB2 was involved in 15 pathways, including "Pathways in Cancer" and "Prostate Cancer." SRC was present in "EGFR tyrosine kinase inhibitor resistance," "Proteoglycans in Cancer," and seven other pathways. Lastly, HSP90AB1 was included in five pathways, such as "Pathways in Cancer" and "Prostate Cancer".

## 3.5 Anti-tumor efficacy of chemical components in FSH

The inhibition results of Uralenol and Kaempferol on different tumor cells, as detected by the CCK-8 method, are shown in Tables 5–8. The results showed that Uralenol and Kaempferol exert good inhibitory effects on human hepatoma cell line HepG2, human non-small cell lung cancer cell line A549, human colon cancer cell line SW620, and mouse breast cancer cell line 4T1, which verified the reliability of the network pharmacology results.

TABLE 5 The inhibitory effect of different monomers on HepG2 cells.

| Ingredient name | Concentration (μmol/L) | Inhibition rate (%) | IC50 (μmol/L) |
|---|---|---|---|
| Adriamycin | 1 | 50.01 ± 0.48 | — |
| Kaempferol | 1 | 0.24 ± 1.11 | 158.57 |
| | 10 | 3.11 ± 2.38 | |
| | 100 | 32.33 ± 3.00 | |
| | 200 | 53.41 ± 5.40 | |
| | 300 | 83.53 ± 1.84 | |
| Uralenol | 1 | 0.14 ± 0.08 | 117.47 |
| | 10 | 10.43 ± 2.28 | |
| | 100 | 36.17 ± 1.33 | |
| | 200 | 86.60 ± 0.41 | |
| | 300 | 96.88 ± 3.69 | |

TABLE 6 The inhibitory effect of different monomers on SW620 cells.

| Ingredient name | Concentration (μmol/L) | Inhibition rate (%) | IC50 (μmol/L) |
|---|---|---|---|
| 5-Fluorouracil | 50 | 48.38 ± 3.57 | — |
| Kaempferol | 1 | 0.67 ± 0.15 | 144.27 |
| | 10 | 7.65 ± 3.22 | |
| | 100 | 34.02 ± 4.03 | |
| | 200 | 58.54 ± 2.53 | |
| | 300 | 92.15 ± 3.34 | |
| Uralenol | 1 | 1.10 ± 0.15 | 51.47 |
| | 25 | 11.18 ± 2.69 | |
| | 50 | 43.79 ± 1.64 | |
| | 75 | 88.61 ± 1.72 | |
| | 100 | 96.04 ± 5.14 | |

TABLE 7 The inhibitory effect of different monomers on A549 cells.

| Ingredient name | Concentration (μmol/L) | Inhibition rate (%) | IC50 (μmol/L) |
|---|---|---|---|
| Adriamycin | 1 | 43.03 ± 0.28 | — |
| Kaempferol | 1 | 0.43 ± 0.28 | 171.83 |
| | 10 | 4.65 ± 1.15 | |
| | 100 | 27.43 ± 3.81 | |
| | 200 | 55.07 ± 1.23 | |
| | 300 | 75.51 ± 2.55 | |
| Uralenol | 1 | 4.43 ± 1.46 | 53.85 |
| | 25 | 9.87 ± 1.39 | |
| | 50 | 41.08 ± 2.74 | |
| | 100 | 94.32 ± 1.09 | |
| | 200 | 98.15 ± 6.51 | |

# 4 Discussion

The FSH is derived from *Sinopodophyllum hexandrum* (Royle) Ying, a plant of the Berberidaceae family, and is a Tibetan habitual plant. Local people have the habit of picking and eating this fruit directly. Modern studies have found that FSH contains various chemical components such as podophyllotoxin, kaempferol, and rutin (4, 23). As a health food and medicine, it possesses health functions such as strengthening the spleen and stomach, and diverse pharmacological effects including anti-tumor, lipid-lowering, and bacteriostatic activities.

In this experiment, according to the Part I, 'Herbs and Drinking Pieces', 'Xiaoyeolian' herbs, 'Identification (2)' in the 2020 edition of the "Chinese Pharmacopoeia," FSH at five different locations was subjected to TLC analysis, which revealed that TLC from the five different locations were all authentic. The chemical components of FSH from five different locations were investigated and analyzed, and it was shown that there existed no significant difference in the

TABLE 8 The inhibitory effect of different monomers on 4T1 cells.

| Ingredient name | Concentration (μmol/L) | Inhibition rate (%) | IC50 (μmol/L) |
|---|---|---|---|
| Adriamycin | 5 | 56.70 ± 4.17 | — |
| Kaempferol | 1 | 1.20 ± 0.86 | 162.83 |
| | 10 | 9.95 ± 1.31 | |
| | 100 | 33.56 ± 3.82 | |
| | 200 | 55.89 ± 4.29 | |
| | 300 | 68.60 ± 1.93 | |
| Uralenol | 1 | 7.88 ± 2.33 | 46.24 |
| | 10 | 15.29 ± 3.00 | |
| | 100 | 64.41 ± 1.04 | |
| | 200 | 98.82 ± 0.64 | |
| | 300 | 98.57 ± 1.08 | |

chemical composition of FSH among different locations. Therefore, Sample S1 of the 5 samples was selected for subsequent experiments.

At present, the studies on FSH are basically confined to the chemical composition, but the *in vivo* transformation patterns of its components have not been reported. In this study, the *in vivo* transformation patterns of the chemical components of FSH were investigated by UPLC-Q-TOF-MS analysis. Among the 85 chemical components of FSH, including gancaonin P, β-peltatin-O-β-D-glucoside, and kaempferol, 35 were detected as the prototype components, such as quercetin-3-methyl ether-3′/4'-O-glucoside, rutin, quercetin-3-O-glucoside, quercetin-3-methyl ether-3′/4'-O-glucoside, *α*-peltatin and isomer. Among the 35 *in vivo* transformed prototype components, 24 were flavonoids, accounting for 68% of the total transformed prototype components, and the remaining 12 components were lignans. There were also 13 *in vivo* transformed metabolites being found, of which, 10 had flavonoids as the prototype compounds, and the remaining metabolites had lignans as the prototype components, indicating that flavonoids have a better absorption and conversion efficiency in rats. The above flavonoids were all based on kaempferol and quercetin, and there were altogether 20 flavonoids in the form of prototypes and metabolite that could be converted into each other *in vivo*, indicating the good absorption and conversion efficiency *in vivo* with kaempferol and quercetin as the mother nuclei.

Network pharmacology was conducted to predict the potential active compounds among the *in vivo* transformed components of FSH for the treatment of tumors. It was found that the *in vivo* transformed components of FSH were related to a variety of anti-tumor pathways. Totally, 25 significantly enriched molecular pathways were screened out by KEGG enrichment analysis. The pathways in cancer of which including 6'-Prenylquercetin-3-methyl ether, 8-Prenylquercetin, Uralenol, Kaempferol other 23 compounds were enriched into this pathway. Meanwhile, 19 compounds, including 6'-Prenylquercetin-3-methyl ether, Uralenol, Kaempferol-4′-methyl ether, Kaempferol and 4'-Demethyldeoxypodophyllotoxin were enriched into the prostate cancer pathway. In PI3K-Akt signaling pathway, 24 compounds, consisting of 6'-Prenylquercetin-3-methyl ether, 8-Prenylkaempferol, Uralenol and Kaempferol were enriched in this pathway. While 20 compounds, including 6-Prenylquercetin-3-methyl ether, 8-Prenylkaempferol, Dysosmaflavone E, Kaempferol and Kaempferol, were enriched in the EGFR tyrosine kinase inhibitor resistance. The above pathway analysis indicates that 6'-Prenylquercetin-3-methyl ether, Uralenol, Kaempferol, 8-Prenylquercetin, Broussonol E and other compounds of FSH can be used as the potential anti-tumor active ingredients, which lays a certain basis for the use and development of FSH.

In this study, network pharmacology was performed to predict the effective active ingredients, targets and pathways of FSH for the treatment of tumors. Our Protein–protein interaction (PPI) network analysis results demonstrated that the key target genes of FSH for anti-tumor treatment were AKT1, EGFR, BCL2, CTNNB1 and HSP90AA1. Of them, BCL2 is a key protein in the cancer pathway, the activation of BCL2/BAX protein in cells can promote the apoptosis of liver cancer cells (24). Down-regulation of BCL-2 accelerates the apoptosis of prostate cancer cells, and exerts an anti-prostatic cancer effect (25). The PI3K/AKT/mTOR pathway is aberrantly activated in oesophageal cancer cells (26, 27), and the related proteins downstream of the PI3K/AKT/mTOR pathway (P70S6K and 4EBP1) can regulate cell proliferation- and cell cycle-related proteins (28–30), as well as apoptosis-related proteins (31) in human oesophageal cancer cells. EGFR is a key protein in the cancer pathway, prostate cancer pathway (Prostate cancer) and PI3K-Akt signaling pathway. Besides, EGFR, Ki-67 and P53 are the common immunohistochemical markers of breast tumors in the clinical practice, and some studies have reported that EGFR expression in breast tumor tissues is correlated with clinical stage, tumor size and regional lymph node metastasis (32). Meanwhile, EGFR plays a promotional role in tumor angiogenesis, which provides nutrients for tumor cell growth and accelerates tumor cell invasion and metastasis (50). Also, CTNNB1 is highly expressed in cancer tissues of children with hepatoblastoma (HB), which is closely related to the initial methotrexate, POST-TEXT staging, tumor diameter, and the presence of tumor invasion or metastasis (48). ESR1 is a key protein in the cancer pathway, which is closely associated with the adhesion and migration of tumor cells, and the suppression of EAR1 expression can inhibit the estrogen receptor (ER) breast cancer cells. Patient-derived tumor-like HSP90AA1 is a key protein in the cancer pathway, prostate cancer pathway and PI3K-Akt signaling pathway, and it also belongs to the HSP90 family, which interacts with various co-chaperones to regulate its substrate recognition, ATPase cycle and chaperone function (33, 34). Typically, the high HSP90AA1 expression levels have been detected in numerous malignant growth and invasive tumor samples (35). HSP90AA1 mRNA expression is up-regulated in breast cancer tissues, and its high expression is strongly correlated with the short overall and progression-free survival of patients (36). The elevated level of TNF-*α* in rat serum can improve the body's immune function (37), which in turn inhibits tumor growth and exerts its anti-tumor effect.

Meanwhile, this experiment also found that there were several groups of isomers in the absorbed and transformed components of FSH *in vivo*. Among them, the compounds with the molecular formula of $C_{21}H_{20}O_8$ had a total of 10 prototype components in the blood, which are all α-peltatin or its isomers; followed by the compounds with the molecular formula of $C_{21}H_{20}O_7$, which had 7 prototype components in the blood, including 6-prenylquercetin-3-methyl ether and 6′-prenylquercetin-3-methyl ether. Although it was impossible to accurately resolve the specific chemical structures corresponding to each of these isomers by liquid-mass spectrometry, a large number of isomers were detected in the form of *in vivo* uptake, suggesting that this component may be an important part of the medicinal substances in FSH. Follow-up experiments should be designed to separate the above multiple isomers for further identification.

Based on the identification of the anti-tumor monomer components Uralenol and Kaempferol through network pharmacology, which are

implicated in the pathways of non-small cell lung cancer, hepatocellular carcinoma, breast cancer, and colorectal cancer, it is proposed that these two components may serve as potential active ingredients in the anti-tumor activity of FSH. To test this hypothesis, *in vitro* cell experiments were conducted using human hepatocellular carcinoma cells (HepG2), human non-small cell lung cancer cells (A549), human colon cancer cells (SW620), and mouse mammary carcinoma cells (4T1). The results demonstrated that Uralenol and Kaempferol exert inhibitory effects on various tumor cell lines. These findings validate the anti-tumor potential of the components identified through network pharmacology, thereby confirming the reliability of the network pharmacology results and providing experimental support for the anti-tumor efficacy of FSH.

## 5 Conclusion

In conclusion, the current experiment was carried out to analyze the chemical components of FSH and their *in vivo* absorption and transformation patterns by UPLC-Q-TOF-MS technology. The detected *in vivo* absorbed and transformed components were predicted to be the anti-tumor pathways by network pharmacology, and the potential anti-tumor active components of FSH were identified. Moreover, the above study provides a certain foundation for the development and use of the resources of FSH and the development of anti-tumor drugs.

## Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

## Ethics statement

The animal study was approved by Ethics Committee of Liaoning University of Traditional Chinese Medicine (no. 2020059). The study was conducted in accordance with the local legislation and institutional requirements.

## Author contributions

X-mT: Data curation, Methodology, Software, Validation, Writing – original draft, Formal analysis, Writing – review & editing. SW: Writing – original draft, Data curation, Formal analysis, Methodology, Software, Validation, Writing – review & editing. T-jL: Writing – review & editing, Data curation, Methodology. X-xY: Supervision, Writing – review & editing, Formal analysis, Validation, Writing – original draft. Y-rB: Formal analysis, Supervision, Validation, Writing – original draft, Writing – review & editing. X-sM: Supervision, Validation, Writing – review & editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2025.1555318/full#supplementary-material

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# Royal jelly acid: preparation, metabolism and therapeutic potential

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Royal jelly acid (10-HDA), an unsaturated fatty acid unique to royal jelly, exhibits a diverse range of biological activities, including hypoglycemic, anti-inflammatory, and anticancer properties. In recent years, the increasing demand for natural product supplements as health enhancers has led to a significant rise in the consumption of royal jelly products for daily wellness. Consequently, the market for royal jelly supplements has expanded considerably. Understanding the mechanisms underlying the biological activities of royal jelly acid is crucial for optimizing its therapeutic applications and guiding the development of innovative royal jelly-based products. This review consolidates current research on the preparation, metabolism and potential pharmacological roles of royal jelly acid in managing cancer, inflammatory disorders, and glucolipid metabolic diseases and explores the molecular mechanisms driving these effects. Future research should leverage advanced analytical techniques to uncover the intricate mechanisms of royal jelly acid's actions, paving the way for its broader integration into healthcare and clinical settings.

KEYWORDS

**royal jelly acid, 10-HDA, fatty acids, function, pharmacological effects**

## 1 Introduction

Natural products have long captured the interest of scientists due to their potent therapeutic benefits and relatively low risk of side effects (Atanasov, et al., 2021). Among these, royal jelly acid—scientifically known as 10-hydroxy-trans-2-decenoic acid (10-HDA, Figure 1)—stands out as a bioactive unsaturated fatty acid with the chemical structure HO-CH2-(CH2)6CH = CH-COOH (C10H18O3), is a unique unsaturated fatty acid found in royal jelly (Hattori, et al., 2007). It is a primary active compound in royal jelly and plays a pivotal role in regulating genetic pathways associated with its biological effects (Spannhoff, et al., 2011). Numerous studies have highlighted the substantial pharmacological potential of royal jelly acid, including its ability to regulate glycolipid metabolism, exert anti-inflammatory effects, and offer neuroprotection (Yu, et al., 2023). Additionally, with its antimicrobial properties (Melliou and Chinou, 2005; Šamšulová, et al., 2023), royal jelly acid has been shown to inhibit a wide range of bacterial strains, making it a promising candidate for combating microbial infections. These attributes have prompted its exploration as a treatment for various conditions, including diabetes (Hu, et al., 2022), colitis (Huang M. et al., 2022), and cancer (Lin, et al., 2020). This review systematically synthesizes research advancements in the preparation methods, metabolic

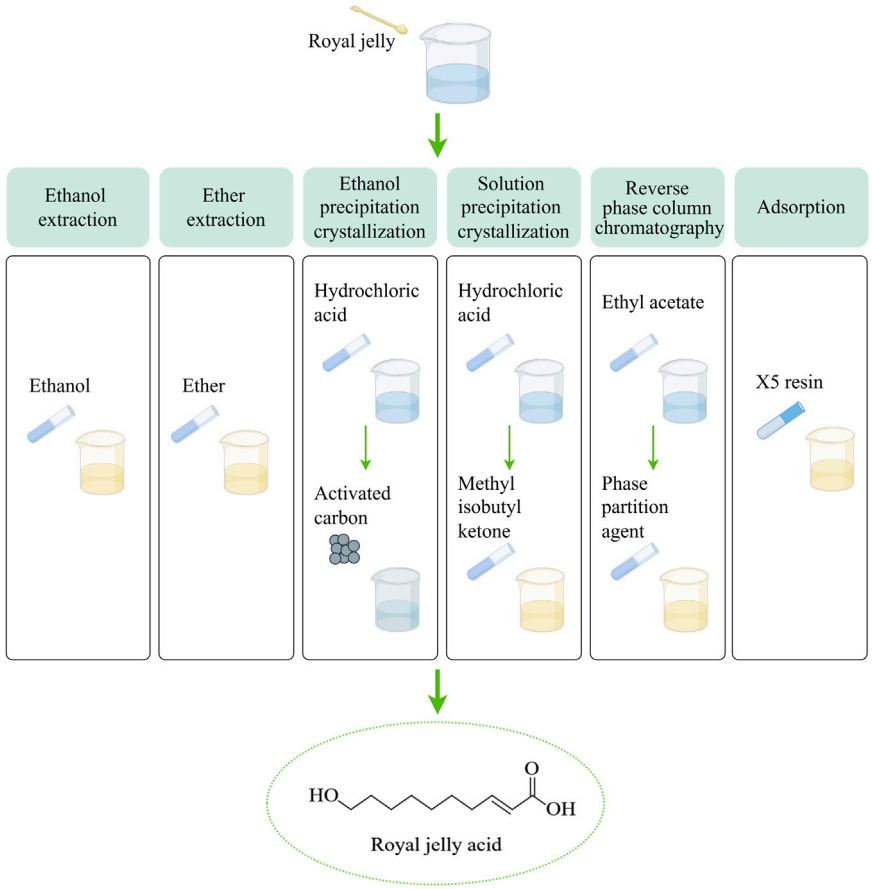



FIGURE 1
Six methods are used to extract royal jelly acid from royal jelly. In the ethanol extraction method, The lyophilized royal jelly is dissolved in ethanol, and centrifuged, the supernatant is collected and subjected to reverse extraction, crystallization, and drying to isolate royal jelly acid. The ether extraction method builds upon the ethanol extraction method by introducing ether to enhance the extraction process and isolate royal jelly acid more effectively. In the ethanol precipitation crystallization method, first, royal jelly is dissolved in ethanol and centrifuged to obtain the supernatant. The supernatant is acidified with hydrochloric acid, filtered, and dissolved in boiling ethanol. Activated carbon is then added, followed by cooling and crystallization to isolate royal jelly acid. Based on the ethanol precipitation crystallization method, the solution precipitation crystallization method involves melting the solution acidified with hydrochloric acid to form methyl isobutyl ketone. The subsequent steps, including crystallization, follow the ethanol precipitation process. In the reverse phase column chromatography method, first, royal jelly is fractionated using ethyl acetate. ethyl acetate fraction is dissolved in dichloromethane and methanol, the dried and precipitation crystallization in reverse phase column chromatography. In adsorption method, X-5 resin is used to selectively adsorb royal jelly acid from the royal jelly solution. Created by Figdraw.

pathways, and therapeutic applications of royal jelly acid through a comprehensive analysis of data curated from multiple scientific databases. It also examines the underlying mechanisms that mediate its pharmacological effects, offering valuable insights into its potential as a treatment for various diseases. Moreover, it underscores the importance of incorporating royal jelly acid into daily healthcare regimens and calls for further investigation into its biological functions and therapeutic potential.

# 2 Preparation methods

## 2.1 Extraction methods

Royal jelly acid can be extracted from royal jelly through several methods, including ethanol extraction (Iegaki, et al., 2020), ether extraction (Antinelli, et al., 2002), ethanol precipitation crystallization (Wang, et al., 2006), solution precipitation crystallization (Wang, et al., 2006), reverse phase column chromatography (Pandeya, et al., 2019) and adsorption method (Zhang and Yang, 2014). The extraction methods are shown in Figure 1. The extraction efficiency of ethanol extraction, ether extraction, ethanol precipitation crystallization and solution precipitation crystallization is generally inefficient, which leads to their gradual phase-out. Reverse phase column chromatography method and adsorption method have garnered attention because of their efficiency and scalability. The X-5 resin demonstrates an adsorption capacity of 9.7 mg/g, with a purity level of approximately 86% (Zhang and Yang, 2014). Its reusability makes this method particularly promising for large-scale industrial applications.

## 2.2 Synthesis methods

Royal jelly acid can also be synthesized using chemical synthesis and microbial synthesis. Additionally, it is naturally produced in the

TABLE 1 Chemical synthesis methods of royal jelly acid.

| Methods | Raw material | Mechanism | Yield (%) | References |
|---|---|---|---|---|
| Wittig reagent alkylene synthesis | 8-acetoxyoctanal | The Wittig reagent effectively addresses issues related to cis-trans isomerism and double bond isomerism in alkene reactions | - | Fray, et al. (1961a) |
| | 1,8-octanediol | Acylation, oxidation, Wittig-Horner reaction, alkaline hydrolysis, and acidification | 80 | Zong and Wu (2014) |
| | 8-hydroxyoctanal | Reaction with the Wittig-Horner reagent in the presence of a phase transfer catalyst | 77 | Villiéras, et al. (1985) |
| Oxydic ozone synthesis | Cyclic dimer of butadiene | Oxidation, acetylation, hydrolysis, hydrogenation, acetal formation, esterification, reduction, and enylation | 49 | Odinokov, et al. (1983) |
| | Oleic acid | The key intermediate methyl dimethyl acetal pelanoate is formed | 15 | Kharisov, et al. (2002) |
| Bromination elimination alkylene synthesis | 10-acetoxy-decanoic acid | Hell-Vothard-Zelinsky reaction | 9.7 | Fray, et al. (1961b) |
| | Undecylenic acid | Hilditch reaction | - | Robert (1960) |
| Knoevenagel condensation synthesis | 1,8-suberic acid | Reduction, oxidation, acetylation, condensation, and hydrolysis | 63 | Quan, et al. (1992) |
| | Oleic acid | The key intermediate, 8-oxooctyl acetate, is formed | 90 | Feng, et al. (2016) |
| | 1,6-hexandiol | Bromination, oxidation, Grignard reaction, and Knoevenagel condensation | 75.3 | Li, et al. (2007) |
| Increased carbon chain synthesis | Hexamethyleneglycol | Monochlorination and Grignard reaction | 50 | Kenneth and Sommers (1948) |
| | Hydrocyanic acid | Aldehyde reaction | - | Ishmuratov, et al. (2002) |

The synthesis methods of royal jelly acid are categorized into six distinct classes based on their underlying chemical principles, and describe its main mechanism.

mandibular glands of worker bees, conclude hydroxylation of stearic acid at the 18th (w) or 17th (o-1) position, then β-oxidation to obtain royal jelly acid (Plettner, et al., 1996). The process thought to be closely linked to the *KAT* gene (Liu, et al., 2016).

### 2.2.1 Chemical synthesis method

Six primary chemical synthesis methods are used for royal jelly acid: Wittig reagent alkylene synthesis, ozonolysis, bromination-elimination alkylene synthesis, Knoevenagel condensation, and increased carbon chain elongation synthesis. The principles or processes of these methods are summarized in Table 1. Although these chemical synthesis methods are well-studied, their industrial application remains limited due to several challenges, including generation of multiple by-products, purification complexity, and relatively low yields.

### 2.2.2 Microbial synthesis method

Microbial synthesis has recently emerged as a promising alternative for producing royal jelly acid, overcoming some of the limitations associated with traditional production methods (Li, et al., 2022). This approach primarily involves the use of *Escherichia coli* (Fang, et al., 2024; Li, et al., 2022; Wang, et al., 2022) and *Pichia pastoris* GS115 (Zhang and Yang, 2014) as microbial systems. The first method begins with the use of *E. coli* to synthesize royal jelly acid from decanoic acid. The mechanism involves the conversion of decanoic acid into decanoyl-CoA by acyl-CoA synthetase. Subsequently, decanoyl-CoA is transformed into trans-dec-2-enoyl-CoA by acyl-CoA dehydrogenase. The intermediate is further converted into trans-2-decenoic acid through the action of acyl-CoA thioesterase. Finally, the hydroxylation of trans-2-decenoic acid at the terminal position, catalyzed by the P450 enzyme, results in the production of 10-hydroxy-2-decenoic acid (royal jelly acid) (Li, et al., 2022). The second method involves the catalytic conversion of 10-hydroxycapric acid into royal jelly acid by using the yeast species *P. pastoris* GS115 (Zhang and Yang, 2014).

## 3 Metabolic pathways

Fat metabolism plays a crucial role in maintaining homeostasis within the body (Hansen, et al., 2013). Royal jelly acid undergoes a series of metabolic transformations. Initially, it is metabolized into sebacic acid (SA), which exhibits various physiological activities (Liao, et al., 2024). Subsequently, SA is further broken down into short-chain dicarboxylic acids. These dicarboxylic acids can enter the tricarboxylic acid cycle to release substantial amounts of energy or participate in further metabolic reactions with other bodily substances (Bharathi, et al., 2020). It has been reported that the oxidation of sebacic acid can yield 61 ATP (445.3 kcal/mol), while royal jelly acid may generate over 61 ATP (Liao, et al., 2024). In addition to energy provision, succinate, oxaloacetate, and citrate produced via the tricarboxylic acid cycle play crucial roles in maintaining physiological homeostasis. For instance, citrate synthesis contributes to reduced ketogenesis (Iaconelli, et al., 2010). Furthermore, certain metabolites may continue to release energy through gluconeogenesis (Roden, et al., 2000). Figure 2 presents the

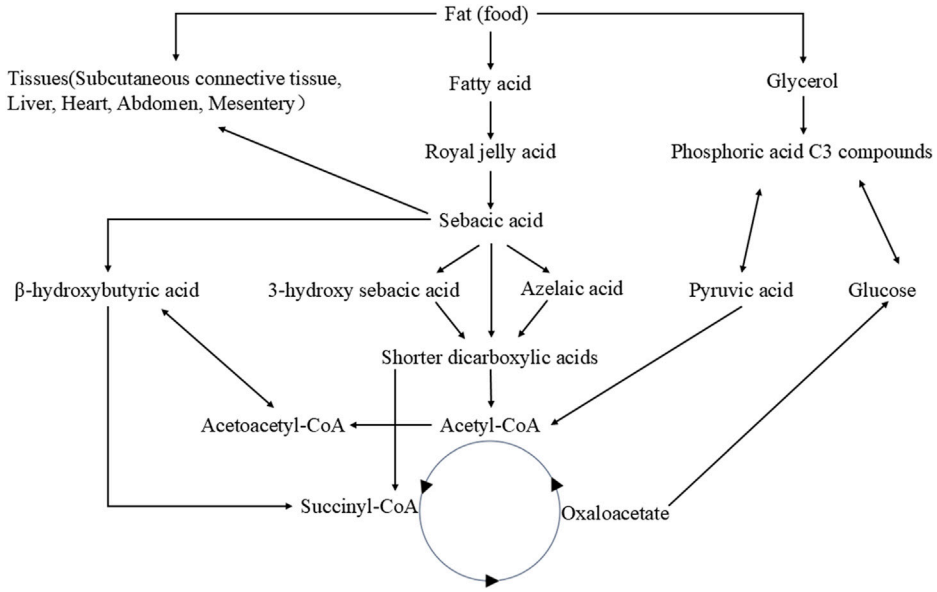



FIGURE 2
Metabolic pathways of royal jelly acid. The food-derived royal jelly acid is initially oxidized via β-oxidation and ω-oxidation pathways to yield SA. Subsequently, sebacic acid metabolic conversion into shorter-chain dicarboxylic acids. These intermediates are further metabolized through acetyl-CoA to enter the tricarboxylic acid (TCA) cycle, where they undergo complete oxidation to $CO_2$ and $H_2O$, accompanied by substantial energy release. Created by Figdraw.

metabolic pathways of royal jelly acid. It provides a detailed visualization of royal jelly acid's metabolic journey.

# 4 Therapeutic potential

Royal jelly acid has demonstrated a broad spectrum of pharmacological effects, including antibacterial activity (Gao, et al., 2022; Šedivá, et al., 2018), regulation of cancer (Saad Al Shehri, et al., 2023), glucolipid metabolic diseases (Hu, et al., 2022), and inflammatory diseases (Huang S. et al., 2022). Studies have recently begun to unravel the molecular mechanisms that underlie these remarkable bioactivities. This section provides a summary of its pharmacological potential.

## 4.1 Anti-bacterial potential

In Gram-positive bacteria, *Paenibacillus larvae*, the causative agent of American foulbrood (AFB), are one of the most devastating bacterial diseases affecting honeybee larvae and pupae (Hansen and and Brødsgaard, 1999). The antibacterial effect of royal jelly acid against *P. larvae* is strongly pH-dependent, with its activity significantly enhanced under acidic conditions (Šamšulová, et al., 2023; Šedivá, et al., 2018). *Staphylococcus aureus* is a common human pathogen responsible for various infectious diseases and a major contributor to foodborne illnesses (Bergin, et al., 2017). Royal jelly acid exhibits a minimum inhibitory concentration of 2.25 mg/mL against *S. aureus* ATCC25923. It inhibits the production of extracellular polymeric substances in *S. aureus* biofilms, prevents biofilm formation. Moreover, royal jelly acid significantly reduces the hemolytic activity of *S. aureus* (Gao, et al., 2022). Royal jelly acid has demonstrated antimicrobial activity against a range of Gram-positive bacteria (Melliou and Chinou, 2005; Yang, et al., 2018), including a cause of sepsis and meningitis, *Streptococcus alactolyticus* (Mylonas, et al., 2020), a cause of skin abscesses, *S. intermedius B* (Biberstein, et al., 1984), linked to various infections, *Staphylococcus xylosus* (Battaglia and Garrett-Sinha, 2023), and a major contributor to dental plaque formation, *Streptococcus mutans* (Forssten, et al., 2010). These findings collectively underscore that royal jelly acid's potent antibacterial activity, particularly against Gram-positive bacteria, making it a promising candidate for addressing bacterial infections in both humans and animals.

Royal jelly acid demonstrates strong inhibitory effects against several Gram-negative bacteria. *Salmonella choleraesuis*, a pathogen causing systemic infections and potentially fatal mycotic aneurysms (Chiu, et al., 2004), *Vibrio parahaemolyticus*, a significant contributor to acute gastroenteritis in humans (Letchumanan, et al., 2014), and *Escherichia coli (hemolytic)*, a strain associated with bacterial pathogenicity (Griffin and Tauxe, 1991), are all strongly inhibited by royal jelly acid (Yang, et al., 2018). Additionally, *Salmonella typhimurium* induces congestion, edema, and bleeding in the digestive tract (Dar, et al., 2017) and is effectively inhibited by royal jelly acid. Notably, the diHDA-glycerol derivative of royal jelly acid exhibits substantial nonspecific resistance against *S. typhimurium* (Pollet, et al., 2002). Despite these findings, research on the antibacterial effects of royal jelly acid against a broader range of pathogenic Gram-negative bacteria remains limited. Future studies should focus on exploring its potential against diverse pathogenic bacterial strains and pathogenic microorganisms.

Royal jelly acid also exhibits antifungal properties, effectively inhibiting the growth of various pathogenic fungi. *Candida tropicalis* is an opportunistic pathogen causing infections under immunocompromised conditions or altered vaginal environments

(Chai, et al., 2010). *Candida albicans* is responsible for infections of the skin, mouth, mucosa, and internal organs (Henriques and Silva, 2021). *Candida glabrata* causes significant systemic infections (Duggan and Usher, 2023). Royal jelly acid inhibits the growth of these pathogenic fungi (Melliou and Chinou, 2005). By contrast, *Saccharomyces cerevisiae*, widely used in the production of bread, wine, and other food products (Parapouli, et al., 2020), is unaffected by royal jelly acid, which shows no inhibitory activity against this yeast (Wang, 2008).

## 4.2 Anti-pest potential

Royal jelly acid demonstrates notable anti-pest properties, targeting a range of vectors and parasites. *Aedes aegypti* is a primary vector for several significant diseases, such as malaria, dengue fever, chikungunya, and Zika virus (Matthews, 2019). *Plasmodium falciparum* is the most common and deadly malaria-causing parasite (Maier, et al., 2019). *Leishmaniasis* is a tropical infection caused by the parasite *Leishmania major* (Sacks and Noben-Trauth, 2002). Royal jelly acid effectively inhibits *Aedes aegypti*, *P. falciparum* K1-strain, and *L. major amastigotes*. The median lethal concentrations for these three pathogens were 31.4, 2.41, and 3.8 μg/mL, respectively. Royal jelly acid exhibited no toxicity to normal human HEK239T cells, suggesting its safety for human applications (Alkhaibari and Alanazi, 2022).

## 4.3 Anti-cancer potential

Royal jelly acid has shown promising anticancer effects in liver, lung, colon, and other types of cancer. In liver cancer research, royal jelly acid significantly inhibited ANGPTL8 expression in HepG2 liver cancer cells (Inoue, et al., 2022). HepG2 cells viability were markedly reduced in a dose-dependent matter. Additionally, it promoted apoptotic pathways by increasing the gene expression of *Caspase-3, Bax* and *miR-34a*, increasing the protein expression of Caspase-3, PARP, and Bax (Saad Al Shehri, et al., 2023). In another experiment, royal jelly acid markedly reduced Hep3B and HCCLM3 liver cancer cell viability, migration and proliferation, and increased the apoptosis process. Multi-omics analysis determined that royal jelly acid interferes with glycolytic metabolism pathway by reducing lactate production, providing a good antitumor effect in both *in vitro* and *in vivo* models (Xu, et al., 2023).

In lung cancer, royal jelly acid inhibited the proliferation of A549, NCI-H460 and NCI-H23 human lung cancer cells, causing nonsignificant toxicity to normal cells. It caused apoptosis in A549 cells through increased the levels of reactive oxygen species. and causes cell cycle arrest at the G0/G1 phase in a time-dependent manner. Furthermore, royal jelly acid modulated key signaling molecules, including increased levels of phosphorylated c-Jun N-terminal kinase (p-JNK), p-p38, and I-κB, whereas reducing the levels of phosphorylated extracellular signal-regulated kinase (p-ERK), p-STAT3, and NF-κB. Moreover, royal jelly acid regulated TGF-β1, SNAI1, GSK-3β, E-cadherin, N-cadherin, and vimentin, inhibiting lung cancer cell migration (Lin, et al., 2020). It suggests that royal jelly acid could potentially be used to treat human lung cancer.

In HCT-116 and SW-480 human colon cancer cells, royal jelly acid has displayed remarkable migratory and invasive effects by inducing significant suppression of promigratory/proinvasive markers, N-cadherin, vimentin and Snail on protein and gene levels (Jovanović, et al., 2022). In WiDr human colon cancer cells, the growth was inhibited by royal jelly acid, and it exhibited anti-inflammatory properties by decreasing NF-κB, IL-8, IL-1β, and TNF-α, and activating IL-1ra (Yang, et al., 2018). Moreover, a combination treatment of royal jelly acid and royal jelly significantly inhibited the growth of CaCo-2 human colon cancer adenocarcinoma cells with decreasing the levels of glutathion and increasing malondialdehyde (Filipič, et al., 2015). While these findings are promising, further *in vitro* studies are needed to confirm the efficacy of royal jelly acid against colorectal cancer.

In Ehrlich solid tumors, royal jelly acid, either alone or when combined with cyclophosphamide, significantly reduced tumor volume and increased the inhibition rates in mice. The treatment also decreased the expression levels of alpha-fetoprotein and carcinoembryonic antigen tumor, tumor necrosis factor alpha, tumor lipid peroxidation, nitric oxide. Furthermore, it enhanced the activity of antioxidant enzymes, including glutathione peroxidase, catalase enzyme, Superoxide dismutase and the apoptotic genes *Caspase-3* and *Bax* (Albalawi, et al., 2021). Additionally, royal jelly acid may also act as a toll-like receptor four antagonist, which could contribute to its tumor-suppressing effects (Eslami-Kaliji, et al., 2022; Eslami-Kaliji, et al., 2021). Research needs to focus on the combination of royal jelly acid.

Royal jelly acid demonstrated a dose-dependent inhibitory effect on SU-DHL-2 lymphatic cancer cells by modulating key proteins involved in the complement and coagulation cascade pathways, including prothrombin, plasminogen, plasminogen activator, and carboxypeptidyl B2 (Tian, et al., 2024). In HCT116 and MDA-MB-231 breast cancer cells, royal jelly acid reduced histone deacetylase enzyme levels, promoting chromatin lysine residue acetylation and triggering an increase in gene expression levels (Aparecida Dos Santos France, et al., 2024). Moreover, when combined with other royal jelly lipids, royal jelly acid exhibited inhibitory activity on the proliferation of various mammalian cell lines, namely, immortalized C2C12 mouse myoblast cells, PC3 human prostate cancer cells, and SH-SY5Y human neuroblastoma cells. Royal jelly acid also significantly inhibited the proliferation, migration, and tube formation of human umbilical vein endothelial cells (Izuta, et al., 2009), thereby limiting tumor angiogenesis and progression. Ultimately, royal jelly acid has important anti-cancer effects, and more robust *in vitro* experiments are required. The anti-cancer action of royal jelly acid is represented in Figure 3.

## 4.4 Anti-glucolipid metabolic diseases potential

Royal jelly acid exhibits potent therapeutic effects in the treatment of glycolipid metabolic disorders through its regulatory, anti-inflammatory, and antioxidant properties (Hu, et al., 2022). In hyperlipidemic rats, royal jelly acid significantly reduced the levels of triglycerides, total cholesterol, and β-lipoproteins, whereas increased high-density lipoprotein levels (Xu, et al., 2002). A total of 41 key metabolites played key roles

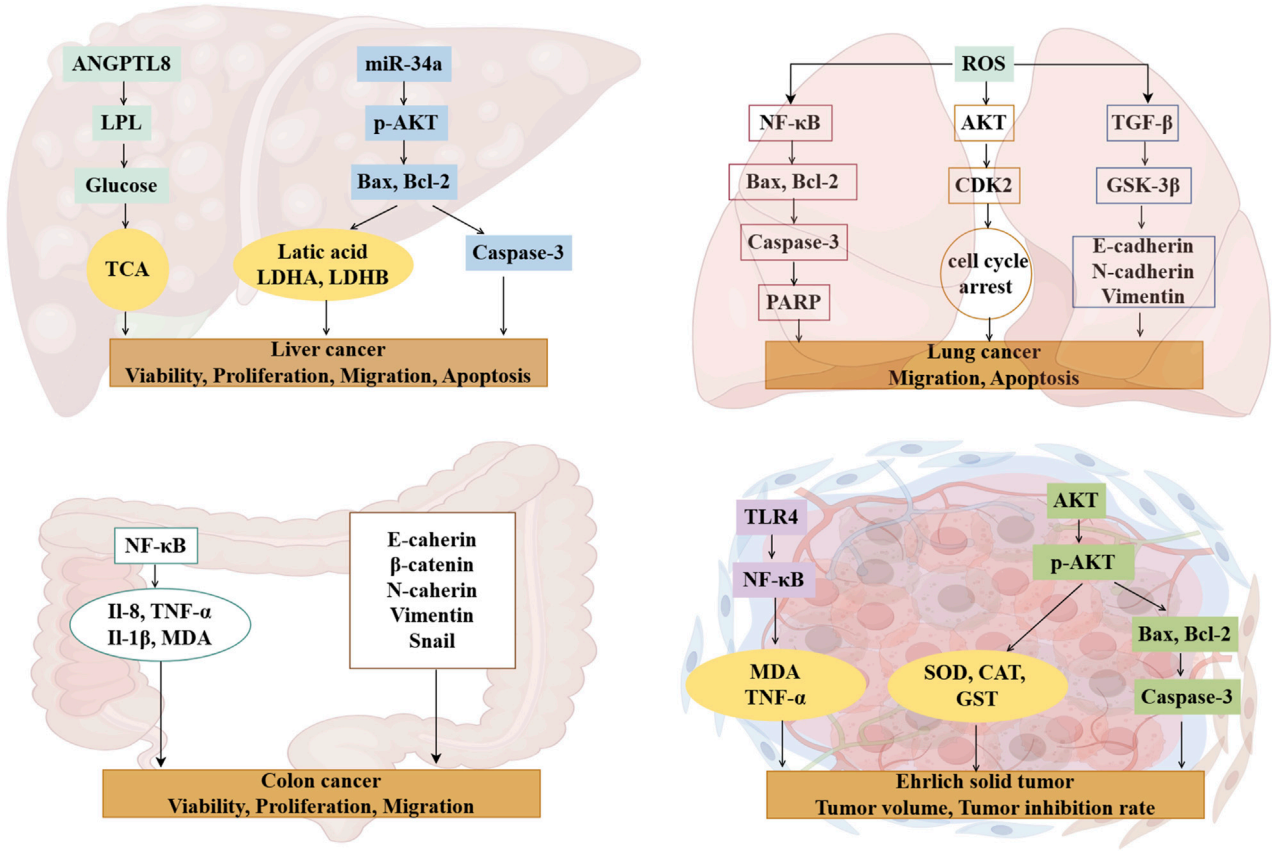



FIGURE 3
Mechanisms of royal jelly acid on cancer diseases. Royal jelly acid exhibits potential therapeutic benefits against various cancers. In liver cancer, it demonstrates therapeutic potential by modulating key regulators including lactic acid, Caspase-3, and Bax to improve liver cell viability, proliferation, migration, and apoptosis. In lung cancer, royal jelly acid regulates apoptosis-related factors (Bax, Bcl-2, Caspase-3), antioxidant factor (ROS), and anti-inflammatory factor (NF-κB), thereby influencing lung cancer cell migration and apoptosis. For colon cancer, royal jelly acid modulates inflammatory factors (NF-κB, IL-8, IL-1β, TNF-α) and junctional proteins (E-cadherin, β-catenin, N-cadherin), which collectively regulate colorectal cancer cell viability, proliferation, and migration. In Ehrlich solid tumors, royal jelly acid exerts anticancer effects by regulating antioxidant factors (SOD, MDA, CAT) and apoptosis-related factors (Caspase-3, Bax, Bcl-2), leading to reduced tumor volume and enhanced inhibition rate. These findings highlight that royal jelly acid exhibits therapeutic potential against cancer. Created by Figdraw.

in the exertion of antihyperlipidemic effects, including glutamylaspartic acid, caproic acid and tryptophyl-glutamine (Zhi, et al., 2025). In studies involving type 2 diabetes mice, royal jelly acid displayed strong hypoglycemic effects by modulating inflammatory markers such as IL-6 and TNF-α, and by enhancing antioxidant enzymes, including SOD, CAT, and GPx. The compound improved glucose metabolism through activation of the PI3K/AKT signaling pathway and upregulated the expression of PGC-1α, a key regulator of mitochondrial biogenesis in mice (Hu, et al., 2022). In HepG2 liver cells, royal jelly acid increased phosphorylated AMP-activated protein kinase levels, inhibited aquaporin-9 gene expression, and facilitated glycerol uptake by liver tissues (Watadani, et al., 2017). According to animal studies, royal jelly acid enhances pAMPK phosphorylation and facilitates GLUT4 translocation to the cell membrane, thereby improving glucose uptake in skeletal muscle cells (Usui, et al., 2019). In 3T3-L1 adipocytes, royal jelly acid downregulated oxidative stress markers such as ROS and suppressed the expression of key lipogenic transcription factors, including PPARγ, FABP4, C/EBP-α, SREBP-1c, and leptin (Pandeya, et al., 2019). The compound also scavenged DPPH radicals (Kolayli, et al., 2016) and activated TRPA1 and TRPV1 channels, inducing thermogenesis and augmenting energy expenditure (Terada, et al., 2011). Royal jelly acid evidently has a more beneficial effect on glycolipid metabolic disease improvement. The primary mechanism is depicted in Figure 4.

## 4.5 Anti-inflammatory diseases potential

Inflammation plays a pivotal role in the development and progression of various diseases (Okin and Medzhitov, 2012). Lipopolysaccharide (LPS), commonly used to induce immune responses in mammalian cells, triggers the release of pro-inflammatory cytokines, and serves as a standard model for studying inflammation (Zhang, et al., 2024). In LPS-induced inflammation models generated using RAW264 cells, royal jelly acid exhibits anti-inflammatory effects by inhibiting IκB-ζ and IL-6 expression, although it does not influence TNF-α levels (Spannhoff, et al., 2011). By contrast, in another study, royal jelly acid significantly downregulated TNF-α expression while markedly

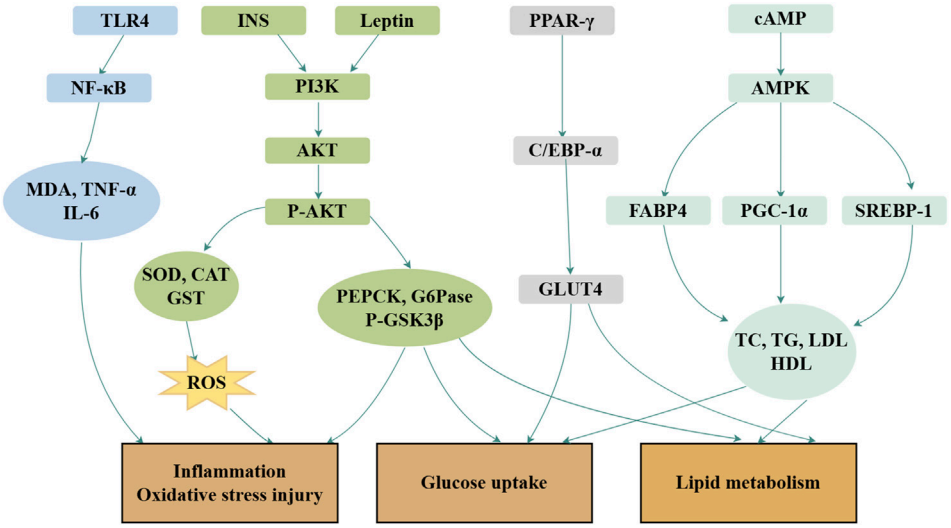



FIGURE 4
Mechanisms of royal jelly acid on glucolipid metabolic diseases. In glucolipid metabolic diseases, royal jelly acid demonstrates therapeutic potential through multi-target regulatory effects. It modulates lipid factors (TC, TG, LDL, HDL), anti-inflammatory factors (TNF-α, IL-6), and antioxidant factors (SOD, CAT, GST), thereby ameliorating inflammation, oxidative stress injury, glucose uptake, and lipid metabolism dysregulation. Created by Figdraw.

inhibiting IL-6 and IL-1β production (Huang M. et al., 2022). Further research revealed that royal jelly acid alleviates inflammation by suppressing JNK1/2 and p38 MAPK phosphorylation (Chen, et al., 2016), resulting in reduced IL-6 (Chen, et al., 2016; Huang S. et al., 2022; Sugiyama, et al., 2012) and IL-1β (Huang M. et al., 2022) levels. In BV-2 microglial cells exposed to LPS-induced inflammation, royal jelly acid mitigated inflammation by directly inhibiting the NLRP3 inflammasome or indirectly promoting autophagy (You et al., 2020a). Metabolomic and transcriptomic analyses further highlighted that royal jelly acid modulates pathways involved in amino acid metabolism, antigen processing and presentation, and NOD receptor signaling, which are critical to its anti-inflammatory effects (Huang, et al., 2023). Royal jelly acid has exhibited major protective effects against inflammation both *in vitro* and *in vivo*.

Nitric oxide (NO) is a pro-inflammatory mediator that contributes to various inflammatory diseases by generating peroxynitrite anions (Litvinova, et al., 2015). In a model of NO-induced inflammation in RAW264 cells treated with IFN-γ, royal jelly acid significantly reduced NO production and suppressed the activation of the NO synthase promoter. It also inhibited the expression of IRF-8, TNF-α, and NF-κB, while leaving IRF-1 expression unaffected (Takahashi, et al., 2012). Similar findings were observed in another inflammatory RAW264 cell model, where LPS- or IFN-β-mediated NO production was significantly reduced by royal jelly acid, which concurrently increased TNF-α and NF-κB levels (Sugiyama, et al., 2012).

Several *in vivo* studies have further validated the anti-inflammatory properties of royal jelly acid. In a mouse model of colitis, royal jelly acid significantly reduced the expression of key inflammasome components, including TXNIP, NLRP3, ASC, Caspase-1, GSDMD, N-GSDMD, IL-1β, and IL-18, while in THP1 cells, it inhibited LPS/ATP-induced inflammasome-mediated pyroptosis, thereby alleviating inflammation and associated colitis (Huang S. et al., 2022). Additionally, in chicken models of intestinal mucosal injury, royal jelly acid reduced the levels of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6), reversed the upregulation of TLR4 and NF-κB, and ameliorated intestinal mucosal injury (Han, et al., 2023). In rheumatoid arthritis synovial fibroblast cells, royal jelly acid modulated the p38 kinase and c-Jun N-terminal kinase (JNK)-AP-1 signaling pathways, thereby alleviating symptoms of rheumatoid arthritis (Yang, et al., 2010). Although royal jelly acid has demonstrated effective anti-inflammatory properties *in vitro* and *in vivo*, more studies are necessary to explore its potential mechanisms in different inflammatory and related conditions. Figure 5 illustrates the main mechanism.

## 4.6 Anti-neurological diseases potential

Royal jelly acid has demonstrated potential neuroprotective and antidepressant effects across various studies (Dubey, et al., 2024; Gong, et al., 2023; Ito, et al., 2012). It enhances neuronal development, especially when used in conjunction with TAU protein, and displays strong binding affinity for TAU protein kinase and phosphatase (Dubey, et al., 2024). Royal jelly acid activates TRPA1 (Terada, et al., 2011), acetylates histones H3 and H4, and inhibits the expression of active caspase-3 and PARP-1 (Koc, et al., 2025), thus contributing to neuroprotection. In microglial BV2 cells, royal jelly acid alleviates neuroinflammation by promoting the FOXO1-mediated autophagy pathway (You et al., 2020b) It also enhances neurogenesis in cultured neural stem/progenitor cells (Hattori, et al., 2007) and preserves blood–brain barrier integrity by inhibiting the degradation of tight junction proteins via the AMPK/PI3K/AKT signaling pathway (You, et al., 2019). Likely in APP/PS1 mouse models of Alzheimer's disease, royal jelly significantly improved memory deficits by stimulating the cAMP/PKA/CREB/BDNF pathway and reducing neuronal apoptosis (You, et al., 2018). Moreover, when combined with aspirin to treat memory impairment and neuroinflammation, royal jelly acid synergistically reduced pro-inflammatory mediator levels, inhibited glial

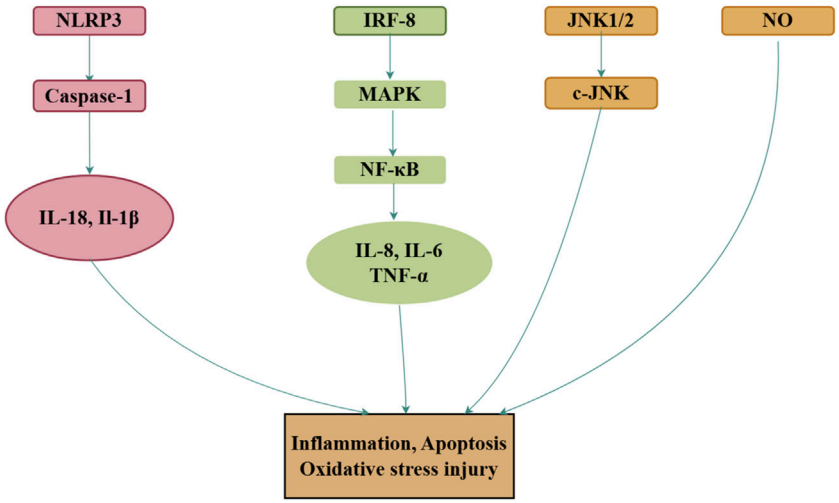



FIGURE 5
Mechanisms of royal jelly acid on inflammatory diseases. In inflammatory diseases, royal jelly acid demonstrates therapeutic potential by modulating anti-inflammatory factors (TNF-α, IL-17, IL-1β) to regulate inflammation, apoptosis, and oxidative stress injury. Created by Figdraw.

cell activation, and alleviated both memory deficits and neuroinflammation (You, et al., 2022). Incorporating royal jelly and royal jelly acid as supplements could be a hopeful therapeutic strategy for neurological ailments.

## 4.7 Other beneficial potentials

In life extension and immune enhancement, The queen bee, which eats royal jelly for its entire life, lives about ten times longer than the worker bee. Studies have shown that royal jelly acid, a special fatty acid in royal jelly, can also prolong lifespan and boost immunity in various models. Studies on *Caenorhabditis elegans* revealed that lipid mixtures containing royal jelly acid increased lifespan by activating the FOXO transcription factor DAF-16 and reducing insulin/IGF-1 signaling (IIS) (Honda, et al., 2011). Interestingly, it also extended lifespan through mechanisms IIS-independent mechanisms involving dietary restriction and TOR signaling pathways (Honda, et al., 2015). Additionally, in cyclophosphamide-induced immunocompromised mice, royal jelly acid restored body weight and increased thymus and spleen mass. It enhanced DNA/RNA/protein activity, while stimulating pathways that support B lymphocyte affinity maturation, antigen presentation, and macrophage activity (Fan, et al., 2020). These effects indicate its immune regulatory potential. Furthermore, royal jelly acid inhibited T cell proliferation, downregulated IL-2, CD86, MHC II, and IL-12 production, and upregulated IL-10 production, demonstrating its ability to modulate immune responses effectively (Vucevic, et al., 2007). These properties contribute to its role in improving immunity and addressing immune-related diseases.

Royal jelly acid exhibits protective effects in a range of conditions, including testicular toxicity, osteoporosis, and dry eye disease in mice. In studies on rats exposed to PbAc, royal jelly acid improved testicular function. A combination of royal jelly acid and ZnO nanoparticles reduced inflammatory and apoptotic markers (Caspase-3 and Bax); enhanced semen quality; and improved the levels of pituitary and gonadal hormones, antioxidants, and testicular tissue structure (Maher, et al., 2024). Regarding osteoporosis, royal jelly acid interacts with FFAR4 to inhibit NF-κB signaling, which contributes to its anti-osteoporotic effects in mice (Tsuchiya, et al., 2020). While it did not prevent bone loss following ovariectomy, it improved bone density and reduced femoral bone hardness, suggesting potential benefits in bone health (Hanai, et al., 2023). Moreover, royal jelly acid stabilizes acetylcholine levels, which may help alleviate symptoms of dry eye disease, improving tear production and ocular surface health (Yamaga, et al., 2021). Given the anti-inflammatory, anti-bacterial, and anti-cancer properties of royal jelly acid, more research is needed to understand the specific mechanisms of its effects on various diseases.

# 5 Conclusion

As a natural product, royal jelly acid has exhibited therapeutic potential against various diseases and pathological conditions. Both *in vivo* and *in vitro* studies have demonstrated its pharmacological versatility, but much remains to be explored regarding its efficacy and safety in humans. Key research areas include exploring the pharmacokinetics of royal jelly acid, that is, how royal jelly acid is absorbed, metabolized, and excreted in the human body, and its potential for inducing drug resistance; conducting rigorous studies to evaluate its efficacy and safety in treating specific human diseases, including liver cancer, hyperlipidemia and colitis; elucidating the mechanisms underlying anti-inflammatory, antioxidant, and lipid metabolism-regulating effects of royal jelly acid; exploring age-stratified studies, including adolescents, pregnant individuals, and geriatric cohorts; accelerating the clinical research; assessing the safety profile, including potential side effects and interactions with other drugs or conditions; enhancing bioavailability by developing optimized drug formulations to augment its absorption and therapeutic action; and utilizing advanced technologies such as single-cell sequencing, spatial transcriptomics, and gut

microbiome analysis to uncover its molecular pathways. Concomitantly, intensified research efforts should prioritize the investigation of royal jelly acid derivatives, particularly diHDA-glycerol derivative of royal jelly acid. Strengthen the development of royal jelly acid-based functional products, including nutraceutical supplements and bioactive beverages. While royal jelly acid holds significant promise for daily healthcare and therapeutic applications, a comprehensive understanding of its mechanisms, safety, and effectiveness remains a significant challenge for future research.

## Author contributions

DZ: Conceptualization, Writing – original draft. XH: Supervision, Writing – review and editing. YX: Writing – review and editing. WZ: Writing – review and editing. XG: Conceptualization, Writing – review and editing. YG: Conceptualization, Investigation, Writing – review and editing. XL: Conceptualization, Writing – review and editing. YT: Investigation, Supervision, Writing – review and editing. KD: Data curation, Funding acquisition, Validation, Visualization, Writing – review and editing.

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## Conflict of interest

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# A comprehensive and systematic review on resveratrol supplementation as a promising candidate for the retinal disease: a focus on mechanisms of action from preclinical studies

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**Background:** Resveratrol is a natural polyphenolic compound that shows great potential in neuroprotection, anti-inflammation,and antioxidation. Previous studies have demonstrated that resveratrol can effectively treat various animal models of retinal diseases.

**Purpose:** The aim of the research was to use an animal experimental model to assess the effectiveness of resveratrol in treating retinal-related diseases in various animal models of retinal diseases such as ischemia-reperfusion injury, diabetic retinopathy, glaucoma, chronic ocular hypertension, optic neuritis, age-related macular degeneration, and retinopathy of prematurity. Furthermore, this study aims to reveal the underlying mechanisms of resveratrol related to the treatment of retina-related diseases.

**Methods:** A search was conducted across several databases, including PubMed, EMBASE, the Cochrane Central Register of Controlled Trials, Web of Science, and OVID. The search time was from the establishment of the database to October 2024 to collect studies on resveratrol intervention in animal models of retinal diseases. The studies included in this paper adopted the SYRCLE's risk of bias tool. Stata 16.0 and RevMan 5.4 software were used to analyze and visualize the results.

**Results:** Our meta-analysis comprises 26 studies and 365 animals demonstrates the following effects of resveratrol compared to the control group: a significant increase in the number of retinal ganglion cells (SMD = 3.91, 95% CI = [2.97, 4.86], $p < 0.00001$) and superoxide dismutase activity (SMD = 3.14, 95% CI = [0.96, 5.33], $p = 0.005$). Moreover, a decrease in malondialdehyde (SMD = −9.29,95% CI = [−12.84, −5.74], $p < 0.00001$), reactive oxygen species level (SMD = −4.29,95% CI = [-6.25, −2.32], $p < 0.0001$), cyclooxygenase-2 (SMD = −2.66, 95% CI = [−4.01, −1.30], $p = 0.0001$), tumour necrosis factor-α(SMD = −3.96,95% CI = [−6.27, −1.65], $p = 0.0008$) and interleukin-6 (SMD = −3.32,95% CI = [−4.20, −2.44], $p < 0.00001$) was observed. The A-wave amplitude and B-wave amplitude showed an increase respectively (MD = 105.92,95% CI = [58.99, 152.84], $p < 0.00001$); (MD = 158.00,95% CI = [86.35, 229.65], $p < 0.0001$), along with an increase in inner retinal thickness (SMD = 6.33, 95% CI = [5.10, 7.56], $p < 0.00001$) and total retinal thickness (SMD = 2.70, 95%CI =

[0.77, 4.83], $p = 0.01$). Subgroup analysis showed that different doses of resveratrol were associated with an increase in the number of RGCs ($p < 0.05$). Resveratrol improves retinal diseases through multiple mechanisms: i) Neuroprotection: it activates the SIRT1/NF-κB and Nrf2 pathways, inhibits Caspase-3 expression, and promotes the survival of RGCs and ii) Antioxidation: it upregulates SOD activity, reduces the levels of MDA and ROS, and alleviates oxidative damage and iii) Anti-inflammation: it inhibits the COX-2, TNF-α, IL-6, and NF-κB pathways, alleviating the inflammatory response. These mechanisms resulted in enhanced amplitude of A/B waves, improved retinal thickness and visual function.

**Conclusion:** Resveratrol has neuroprotective, anti-inflammatory and antioxidant effects through multiple mechanisms, thereby reducing retinal damage and maintaining the structure and function of the retina. This provides preclinical support for its possible therapeutic uses in the management of retinal diseases.

**Systematic Review Registration:** https://www.crd.york.ac.uk/PROSPERO/myprospero.

KEYWORDS

**resveratrol, retinal disease, animal models, systematic review, mechanisms**

## 1 Introduction

The retina, a vital tissue in the human eye, plays a crucial role in light perception and the transmission of visual signals. However, due to inflammatory responses, oxidative stress and neurovascular dysfunction, retinopathy ensues and led to the occurrence of various retinal diseases (Wang et al., 2022). In ischemia-reperfusion (I/R) injury, the recovery of blood flow after ischemia produces reactive oxygen species (ROS) which intensifies the apoptosis of ganglion cells and endothelial cells (Qin et al., 2022). Diabetic retinopathy (DR) is characterized by persistent hyperglycemia that induces mitochondrial dysfunction, generates continuous oxidative stress, promotes the production of cytokines (such as VEGF and IL-6), and leads to retinal neurodegeneration (Yue et al., 2022). Glaucoma and chronic ocular hypertension (COH) are mainly related to elevated intraocular pressure, causing gradual ischemia of the retina and damaging retinal ganglion cells and optic nerve axons (Sim et al., 2022). Age-related macular degeneration (AMD) is caused by degenerative damage to the macula, leading to dysfunction of the retinal pigment epithelium and neovascularization, resulting in irreversible vision impairment. Optic neuritis, characterized by optic nerve inflammation, frequently associates with autoimmune disorders like multiple sclerosis, disrupting signal transmission between the retina and brain (Petzold et al., 2017). Retinopathy of prematurity (ROP) is caused by the retina of premature infants being exposed to a hyperoxic environment after birth, resulting in oxidative reactions and abnormal development of retinal blood vessels (Dammann et al., 2023). Due to the limited treatment methods, the incidence of various retinal diseases continues to rise, imposing a significant burden on both individual quality of life and global healthcare systems (Fisher, 2021). This emphasizes the importance of exploring new treatment strategies.

Resveratrol (RSV) is a naturally occurring polyphenol and the main bioactive compound in red wine, which has been widely studied at present. Beyond red wine, resveratrol is abundantly present in various plants, including grapes (particularly the skin and seeds), peanuts, berries, mulberries, and blueberries (Tian and Liu, 2020). Studies have demonstrated that resveratrol, as a flavonoid drug, has anti-cancer, anti-inflammatory, anti-hypertensive, anti-thrombotic, and anti-addictive effects (Yunusoğlu et al., 2025). Regarding its pharmacological effects in retinal diseases, it also shows multiple targets and has received more attention. The inflammatory response in retinopathy is manifested as the overexpression of various inflammatory factors, such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and cyclooxygenase-2 (COX-2). These factors can cause damage and death to retinal cells. Resveratrol can significantly reduce the expression levels of inflammatory factors and inhibit the activation of inflammatory signaling pathways (Kovoor et al., 2022). Resveratrol effectively inhibits oxidative stress responses and protects retinal cells from oxidative damage by enhancing the activity of superoxide dismutase (SOD) and reducing the levels of malondialdehyde (MDA) and reactive oxygen species (ROS) (Dziedziak et al., 2021). The damage and death of retinal ganglion cells (RGCs) are among the key factors causing vision loss due to retinal diseases. Resveratrol can significantly increase the number of RGCs and promote survival and functional recovery by activating the neurotrophic factor signaling pathway. Furthermore, resveratrol can enhance the structure and function of the retina as well as its electrical function (Liu et al., 2017).

Despite numerous studies that have explored the role of resveratrol, different researchers have focused on different retinal diseases and studied different indicator outcomes. So far, no researchers have conducted a meta-analysis of the ability of resveratrol to treat retinal diseases. This study aims to systematically investigate the feasibility and potential of resveratrol as a therapeutic agent for retinal diseases through animal experiments. Additionally, it endeavors to comprehensively summarize the various mechanisms by which resveratrol exerts its effects on retinal diseases, promote the translation of animal experimental findings into clinical applications, and provide novel treatment strategies.

# 2 Materials and methods

## 2.1 Protocol and registration

This study was based on a meta-analysis (PRISMA 2020). The detailed procedures and analysis results are listed in Supplementary Table 2. In addition, the study protocol for this review has been registered with the International Prospective Register of Systematic Reviews (PROSPERO) under the registration number CRD42025621096.

## 2.2 Search strategy

In this study, researchers conducted a comprehensive search across five electronic databases (PubMed, EMBASE, Cochrane Central Register of Controlled Trials, Web of Science, and OVID) from the establishment of the database to October 2024. The search strategy included both subject terms and free terms. Two authors (XM Lv and N Li) used the subject terms: "resveratrol", and "retina" (Supplementary Table 1). The detailed search strategy is shown in Table 1 (PubMed is provided as an example).

## 2.3 Inclusion and exclusion criteria

The studies included in this analysis met the following criteria: (1) evaluation of resveratrol effects on an animal models; (2) the intervention group was given resveratrol, and the dose, route or treatment time were not limited; (3) randomized controlled trials; (4) assessment of outcome measures including the number of RGCs, SOD, MDA, ROS,TNF-α, IL-6, COX-2,the inner retinal thickness, the total retinal thickness, the A-wave and B-wave amplitudes.

The studies excluded based on the following criteria: (1) non-rat or non-mouse animal species; (2) include other interventions or drugs; (3) research forms: clinical case reports, reviews and other non-randomized controlled trials.

## 2.4 Study selection and data extraction

Two researchers (XM Lv and N Li) conducted the literature search and data extraction of this study respectively. Disagreements were resolved through discussion with a third party (LW Chen). Duplicate content was removed using EndNote 20 software. Titles and abstracts of the literature were screened to exclude irrelevant studies. Subsequently, a thorough evaluation against the exclusion criteria was performed to identify articles meeting the criteria. Data extracted included the following: (1) baseline content of included studies; (2) rat characteristics including sex, species, age, body weight, and sample size; (3) interventions in the treatment and control groups; (4) study outcomes. Outcome indicator data were reported as mean ± standard deviation. In cases where data were solely presented in graphical format, Engauge Digitizer commercial software was utilized to extract the data from the charts.

TABLE 1 Search strategy on PubMed.

| #1 | Resveratrol [MeSH terms] |
|---|---|
| #2 | (resveratrol) OR (3,4′,5-Stilbenetriol) OR (3,5,4′-Trihydroxystilbene) OR (3,4′,5-Trihydroxystilbene) OR (trans-Resveratrol) OR (trans Resveratrol) OR (Resveratrol-3-sulfate) OR (Resveratrol 3 sulfate) OR (SRT 501) OR (SRT-501) OR (SRT501) OR (cis-Resveratrol) OR (cis Resveratrol) OR (Resveratrol, (Z)-) OR (trans-Resveratrol-3-O-sulfate) OR (trans Resveratrol 3 O sulfate) |
| #3 | (#1) OR (#2) |
| #4 | Retina [MeSH Terms] |
| #5 | (retina) OR (Ora Serrata) |
| #6 | (#4) OR (#5) |
| #8 | #3 AND #6 |

## 2.5 Assessment of risk of bias in individual studies

This study was evaluated by the SYRCLE's risk of bias tool. Two authors (XM Lv and N Li) analyzed seven items in the assessment tool, including randomized sequence generation, allocation concealment, blinding of participants and personnel, blinding of outcome assessments, incomplete outcome data, selective reporting, and bias from other sources.

## 2.6 Statistical analysis

In this study, RevMan 5.4 and Stata 16.0 software were used for data analysis. The effect values selected were standardized mean difference (SMD) and mean difference (MD) based on the result units, with a 95% confidence interval (CI) utilized. In cases of minimal heterogeneity ($p > 0.1$, $I^2 < 50\%$), a fixed-effect model was applied for statistical combination. Conversely, in instances of substantial heterogeneity ($p \leq 0.1$, $I^2 \geq 50\%$), a random-effect model was employed. Forest map was used to analyze the combined effect values of each index. Subgroup analysis was conducted on the experimental groups with varying doses of resveratrol intervention, and sensitivity analysis was carried out to confirm the meta-analysis's stability. Egger's test and funnel plots were used to analyze potential publication bias in the results.

# 3 Results

## 3.1 Study selection

A total of 559 articles were identified. Using EndNote 20 software, 211 duplicates were removed. Following this, a review of the titles and abstracts led to the exclusion of 285 articles. Upon full-text review, an additional 37 articles were eliminated. Finally, 26 articles were included in the meta-analysis (Figure 1).

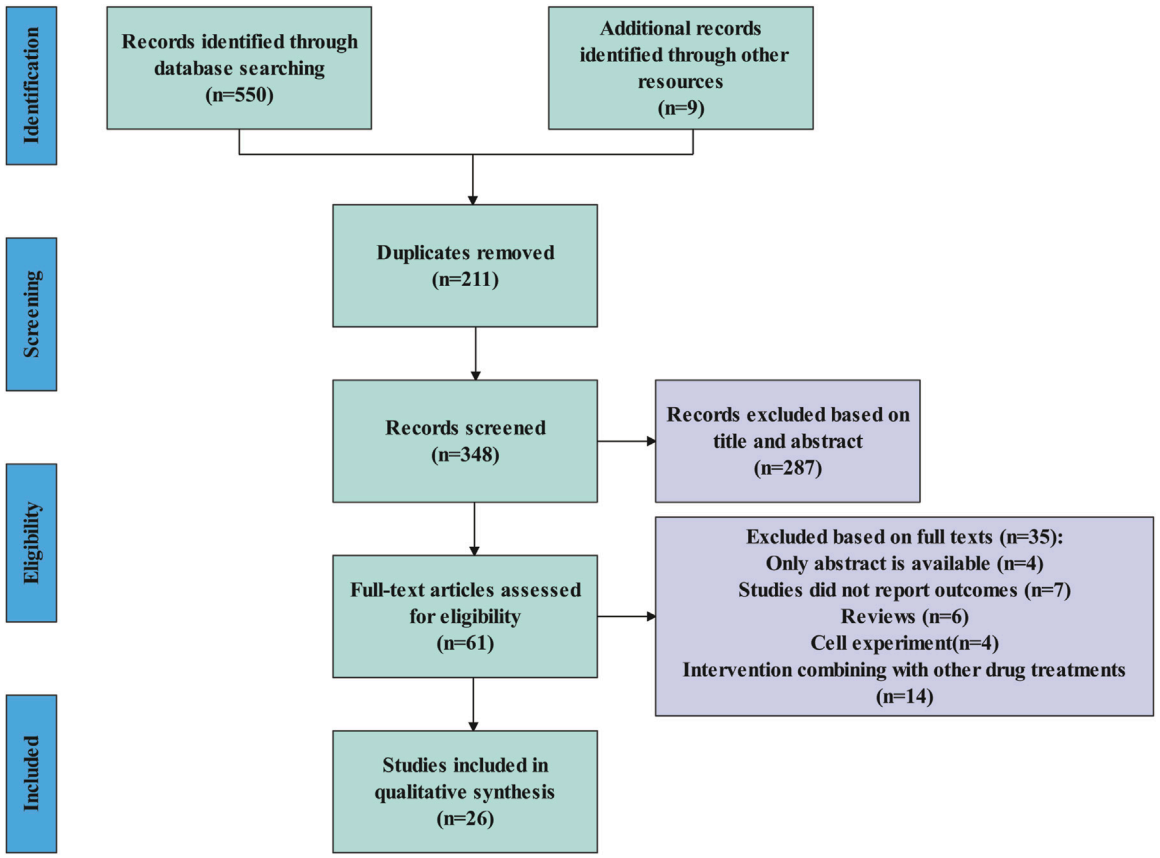



FIGURE 1
Flow diagram of literature selection.

## 3.2 Characteristics of the included studies

This meta-analysis included 26 articles, involving 10 studies with Sprague-Dawley rats, 11 with C57BL/6J mice, 4 with Wistar rats, and 1 with Brown Norway rats. The control group consisted of 183 animals, while the experimental group included 182 animals. This study encompasses animal models of retinal injury, comprising 8 models of I/R, 8 models of DR, 3 models of COH, 4 models of glaucoma, 1 model of optic neuritis,1 model of AMD and 1 model of ROP. The number of RGCs was assessed as an outcome in 18 studies, retinal thickness in 8 studies, A-wave and B-wave amplitude in 5 studies, anti-inflammatory markers in 8 studies and antioxidant markers in 8 studies. The characteristics of the studies included in Table 2.

## 3.3 Quality evaluation

A total of 26 relevant articles were systematically and comprehensively evaluated. While all studies mentioned randomization, 7 did not specify the randomization methods used. Information regarding allocation concealment, blinding of participants and personnel, and blinding of outcome assessment was not provided in any of the studies, resulting in an overall classification of "unclear" for these domains. None of the studies mentioned any other bias, leading to a classification of "low risk" across all studies. The SYRCLE's risk of bias evaluation is depicted in Figure 2.

## 3.4 Meta analysis of primary outcomes

### 3.4.1 The number of RGCs

Eighteen of the included studies (Huang et al., 2013; Ghadiri Soufi et al., 2015; Pirhan et al., 2015; Seong et al., 2017; Chen et al., 2018; Luo et al., 2018; Zhang et al., 2018; Cao et al., 2020; Pang et al., 2020; Ji et al., 2021; Seong et al., 2021; Zeng et al., 2021; Chronopoulos et al., 2023; Xiao et al., 2023; Feng et al., 2024; Ji et al., 2024; Shamsher et al., 2024; Yuan et al., 2024) reported the number of RGCs in animal models with retinal disease (experimental group, n = 117; control group, n = 118). The results showed that resveratrol significantly increased the number of RGCs in the retina when compared to the control group (SMD = 3.91, 95% Cl = [2.97, 4.86], $p < 0.00001$) (Figure 3).

A subgroup analysis conducted according to dosage categories (0 mg/kg/d ≤ dosage ≤ 10 mg/kg/d, 10 mg/kg/d < dosage ≤ 20 mg/kg/d, and dosage > 20 mg/kg/d) revealed the following results: for 0 mg/kg/d ≤ dosage ≤ 10 mg/kg/d (SMD = 3.80, 95% Cl = [2.50, 5.10], $p < 0.00001$), for 10 mg/kg/d < dosage ≤ 20 mg/kg/d (SMD = 4.54, 95%Cl = [2.86, 6.23], $p < 0.00001$), and for dosage > 20 mg/kg/d (SMD = 3.58, 95%Cl = [−2.14, 9.31], $p = 0.22$) (Figure 4). Dose-based subgroup analysis showed that although there was

TABLE 2 The characteristics of the included studies.

| Study | Country | Animals | Weight (g) | Sample size (n) | Type of retinal disease | Treatment group | Control group | Outcome |
|---|---|---|---|---|---|---|---|---|
| Yuan et al. (2024) | China | Male C57BL/6J mice (8 weeks old) | 25–30 | T:5 C:5 | DR | RSV 10 mg/kg/d, OG, 1month | No treatment | RGCs↑, MDA↓ SOD↑ Total retinal thickness↑ |
| Shamsher et al. (2024) | United Kingdom | Female C57BL/6J mice (6 weeks old) | NM | T: 5 C: 6 | Optic neuritis | RSV 8.44 mg/kg/d, intranasal, 30d | Drug solvent | RGCs↑ |
| Ji et al. (2024) | China | C57BL/6J mice (6–8 weeks old) | NM | T: 4 C: 4 | Glaucoma | RSV 20 mg/kg/d, IP, 5d | No treatment | RGCs↑, A-Wave↑ B-Wave↑, IL-6↓ |
| Xiao et al. (2023) | China | Male C57BL/6J mice (8 weeks old) | NM | T: 8 C: 8 | DR | RSV 10 mg/kg/d, OG, 12weeks | No treatment | RGCs↑ |
| Chronopoulos et al. (2023) | Germany | Male C57Bl/6J mice (5–6 months old) | NM | T: 8 C: 8 | I/R | RSV 30 mg/kg/d, OG, 7d | Drug solvent | RGCs↑, ROS↓ |
| Zeng et al. (2021) | China | SD rats (14weeks old) | 180 ± 20 | T: 17 C: 17 | DR | RSV 10 mg/kg/d, OG, 36weeks | No treatment | RGCs↑ |
| Seong et al. (2021) | Korea | Male C57BL/6J mice (8 weeks old) | 20–25 | T: 4 C: 4 | I/R | RSV 20 mg/kg/d, IP, 5d | No treatment | RGCs↑, ROS↓ |
| Hu et al. (2022) | China | C57BL/6 mice (7 days old) | NM | T: 6 C: 6 | ROP | RSV 50 mg/kg/d, intravitrea, 5d | No treatment | MDA↓, SOD↑ |
| Ji et al. (2021) | China | Male C57BL/6 mice (6–8weeks old) | NM | T: 5 C: 5 | I/R | RSV 20 mg/kg/d, IP, 5d | No treatment | RGCs↑ A-Wave↑, B-Wave↑, inner retina thickness↑ |
| Pang et al. (2020) | China | Male SD rats (2–3 months old) | NM | T: 3 C: 3 | I/R | RSV 25 mg/kg/d, IP, 3d | Drug solvent | RGCs↑, inner retina thickness↑ |
| Deng et al. (2020) | China | Male SD rats (2–3 months old) | NM | T: 4 C: 4 | I/R | RSV 250 mg/kg/d, IP, 3d | No treatment | Total retinal thickness↑, COX-2↓ |
| Cao et al. (2020) | Japan | male C57BL/6 mice (14 weeks old) | 25–30 | T: 6 C: 6 | Glaucoma | RSV 0.004 mg/kg/d, IVT, 21d | No treatment | RGCs↑, ROS↓ |
| Dong et al. (2019) | China | Male Wistar rats (8–10weeks old) | 200–250 | T: 8 C: 8 | DR | RSV 300 mg/kg, OG, 3months | No treatment | Total retinal thickness↑, TNF-α↓, IL-6↓ |
| Chen et al. (2018) | China | Male SD rats (14 weeks old) | 200–230 | T: 5 C: 5 | DR | RSV 0.05 mg/kg/d, IV, 12weeks | No treatment | RGCs↑, TNF-α↓, IL-6 |
| Zhang et al. (2018) | China | Male SD rats (4–6 weeks old) | 100–150 | T: 6 C: 6 | COH | RSV 20 mg/kg/d, OG, 4weeks | Drug solvent | RGCs↑, ROS↓ |
| Luo et al. (2018) | China | SD rats (2–3 months old) | NM | T: 6 C: 6 | I/R | RSV 250 mg/kg/d, IP, 3d | No treatment | RGCs↑ Total retinal thickness↑, COX-2↓ |
| Feng et al. (2024) | China | C57BL/6J mice (6–8 weeks old) | NM | T: 4 C: 4 | I/R | RSV 20 mg/kg/d, IP, 3d | No treatment | RGCs↑ A-Wave↑, B-Wave↑ |
| Seong et al. (2017) | Korea | Male C57BL/6J mice (8 weeks old) | 20–25 | T: 7 C: 7 | I/R | RSV 20 mg/kg/d, IP, 5d | No treatment | RGCs↑, inner retina thickness↑ |

(Continued on following page)

TABLE 2 (*Continued*) The characteristics of the included studies.

| Study | Country | Animals | Weight (g) | Sample size (n) | Type of retinal disease | Treatment group | Control group | Outcome |
|---|---|---|---|---|---|---|---|---|
| Zeng et al. (2015) | China | SD rats (14 weeks old) | 180 ± 20 | T: 5<br>C: 5 | DR | RSV 10 mg/kg/d, OG, 7months | No treatment | B-Wave↑ |
| Razali et al. (2016) | Malaysia | SD rats | NM | T: 24<br>C: 24 | COH | RSV 0.8 mg/kg/d, IVT, 3weeks | Drug solvent | SOD↑, inner retina thickness↑ |
| Pirhan et al. (2015) | Turkey | Wistar rats (adult) | 367 ± 12 | T: 12<br>C: 12 | Glaucoma | RSV 10 mg/kg, IP, 6weeks | No treatment | RGCs↑ |
| Ghadiri Soufi et al. (2015) | Iran | Male Wistar rats (12 weeks old) | 320–350 | T: 6<br>C: 6 | DR | RSV 5 mg/kg/d, OG, 4months | No treatment | RGCs↑, COX-2↓, TNF-α↓, IL-6↓ |
| P. Vin et al. (2013) | United States | Male SD rats (adult) | 200 | T: 6<br>C: 6 | COH | RSV 30 mg/kg/d, IP, 5d | Drug solvent | A- Wave↑<br>B- Wave↑ |
| Huang et al. (2013) | United States | Male Brown Norway rats | 300–450 | T: 6<br>C: 6 | Glaucoma | RSV 20 mg/kg/d, IP, 3d | Drug solvent | RGCs↑ |
| Soufi et al. (2012) | Iran | Wistar rats | 320–350 | T: 6<br>C: 6 | DR | RSV 5 mg/kg/d, OG, 4months | No treatment | SOD↑, TNF-α↓, IL-6↓ |
| Nguyen et al. (2022) | China | SD rats | 250–400 | T: 6<br>C: 6 | AMD | RSV 0.01 mg/kg/d, IVT, 2months | No treatment | ROS↓, IL-6↓ |

Abbreviations: T, treatment group; C, control group; NM, not mentioned; RSV, resveratrol; RGCs, retinal ganglion cells; OG, oral gavage; IP, intraperitoneal injections; IV, intravenous injection; IVT, intravitreal injection; DR, diabetic retinopathy; ROP, retinopathy of prematurity; I/R, Ischemia-reperfusion; COH, chronic ocular hypertension; AMD, age-related macular degeneration; COX-2, cyclooxygenase-2; TNF-α, tumour necrosis factor-α; IL-6, interleukin-6; ROS, reactive oxygen species.

significant heterogeneity, different doses of resveratrol could significantly increase the number of RGCs when compared to the control group (SMD = 3.91, 95%Cl = [2.97, 4.86], $p < 0.00001$). However, when comparing the different dosage groups (low, medium, and high doses) with each other, no significant difference was observed in their ability to increase the number of RGCs ($p = 0.78$).

### 3.4.2 Markers of oxidative stress

Four of the included studies (Soufi et al., 2012; Razali et al., 2016; Hu et al., 2022; Yuan et al., 2024) reported the effects of resveratrol on SOD activity in animal models with retinal disease (experimental group, n = 41; control group, n = 41). The results showed that resveratrol led to a significant increase in SOD activity in the retina when compared to the control group (SMD = 3.14, 95% Cl = [0.96, 5.33], $p = 0.005$) (Figure 5A).

Two of the included studies (Hu et al., 2022; Yuan et al., 2024) reported the effects of resveratrol on MDA levels in animal models with retinal disease (experimental group, n = 11; control group, n = 11). The results showed that resveratrol significantly reduced the MDA levels in the retina compared with the control group (SMD = −9.29, 95% Cl = [−12.84, −5.74], $p < 0.00001$) (Figure 5B).

Five of the included studies (Zhang et al., 2018; Cao et al., 2020; Seong et al., 2021; Nguyen et al., 2022; Chronopoulos et al., 2023) reported the effects of resveratrol on ROS levels in animal models with retinal disease (experimental group, n = 32; control group, n = 32). The results showed that resveratrol led to a significant reduced in ROS levels in the retina when compared to the control group (SMD = −4.29, 95% Cl = [−6.25, −2.32], $p < 0.0001$) (Figure 5C).

### 3.4.3 Markers of inflammation

Three of the included studies (Ghadiri Soufi et al., 2015; Luo et al., 2018; Deng et al., 2020) reported the effects of resveratrol on COX-2 levels in animal models with retinal disease (experimental group, n = 13; control group, n = 13). The results showed that resveratrol led to a significant reduced in COX-2 levels in the retina when compared to the control group (SMD = −2.66, 95% Cl = [−4.01, −1.30], $p$ =0.0001) (Figure 6A).

Four of the included studies (Soufi et al., 2012; Ghadiri Soufi et al., 2015; Chen et al., 2018; Dong et al., 2019) reported the effects of resveratrol on TNF-α levels in animal models with retinal disease (experimental group, n = 25; control group, n = 25). The results showed that resveratrol led to a significant reduced in TNF-α levels in the retina when compared to the control group (SMD = −3.96,95% Cl = [−6.27, −1.65], $p = 0.0008$) (Figure 6B).

Six of the included studies (Soufi et al., 2012; Ghadiri Soufi et al., 2015; Chen et al., 2018; Dong et al., 2019; Nguyen et al., 2022; Ji et al., 2024) reported the effects of resveratrol on IL-6 levels in animal models with retinal disease (experimental group, n = 34; control group, n = 34). The results showed that resveratrol led to a significant reduced in IL-6 levels in the retina when compared to the control group (SMD = −3.32, 95% Cl = [−4.20, −2.44], $p < 0.00001$) (Figure 6C).

### 3.4.4 Electroretinography

Four of the included studies (Vin et al., 2013; Ji et al., 2021; 2024; Feng et al., 2024) reported the effects of resveratrol on A-wave amplitudes in animal models with retinal disease (experimental group, n = 21; control group, n = 21). The results showed that resveratrol significantly increased the A-wave amplitudes in the

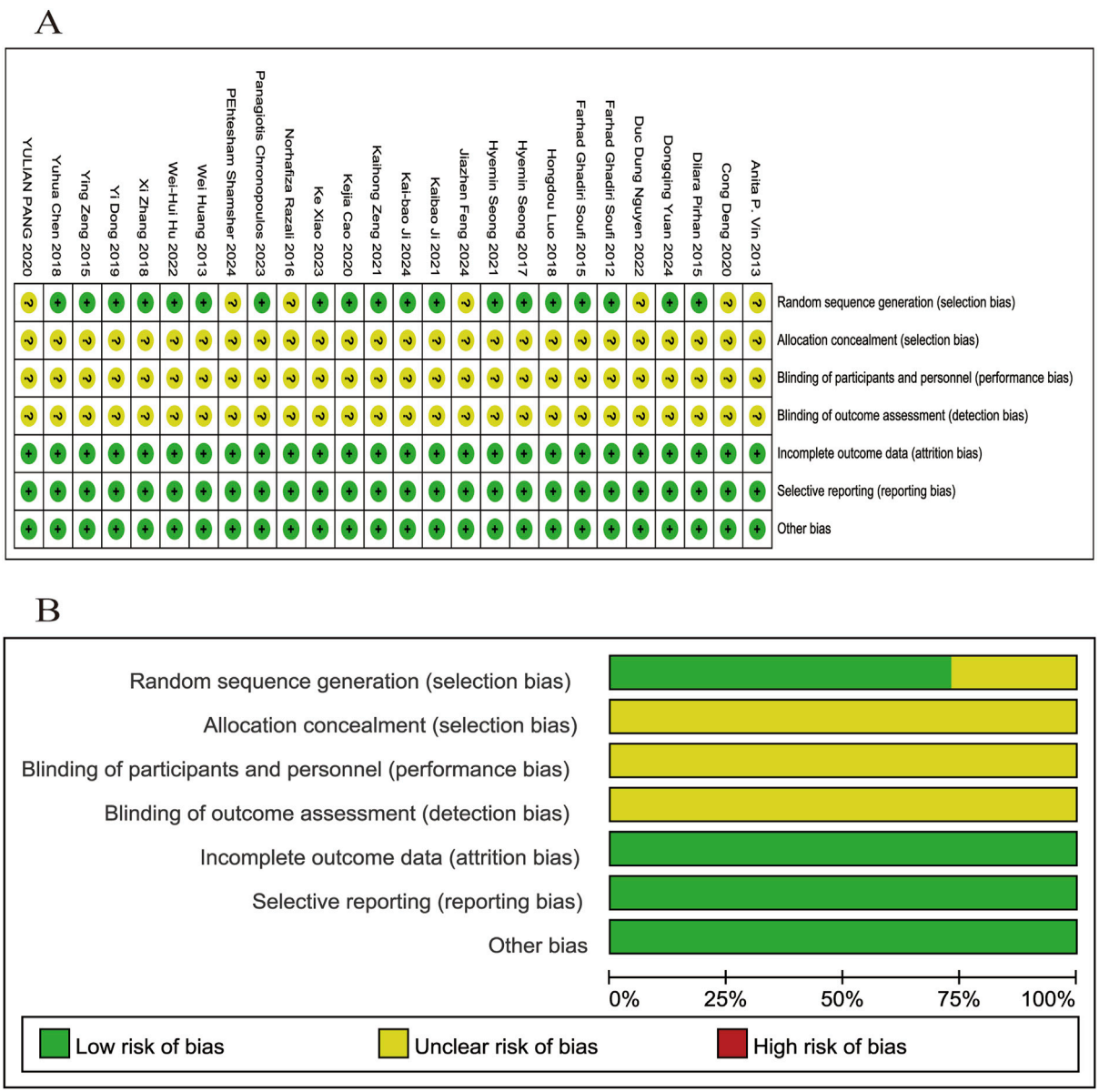



FIGURE 2
Evaluation of literature quality outcomes derived from SYRCLE's Risk of Bias utilizing the Cochrane tool. **(A)** Risk of bias summary: the review authors' assessments of each risk of bias item for each included study; **(B)** Risk of bias graph: review authors' judgments about each risk of bias item displayed as a percentage for all included studies.

retina compared with the control group (MD = 105.92, 95% Cl = [58.99, 152.84], $p < 0.00001$) (Figure 7A).

Five of the included studies (Vin et al., 2013; Zeng et al., 2015; Ji et al., 2021; 2024; Feng et al., 2024) reported the effects of resveratrol on B-wave amplitudes in animal models with retinal disease (experimental group, n = 26; control group, n = 26). The results showed that resveratrol significantly increased the B-wave amplitudes in the retina compared with the control group (MD = 158.00, 95% Cl = [86.35, 229.65], $p < 0.0001$) (Figure 7B).

### 3.4.5 Retinal thickness

Four of the included studies (Razali et al., 2016; Seong et al., 2017; Pang et al., 2020; Ji et al., 2021) reported the effects of resveratrol on inner retinal thickness in animal models with retinal disease (experimental group, n = 38; control group, n = 38). The results showed that resveratrol significantly increased inner retinal thickness compared with the control group (SMD = 6.33, 95% Cl = [5.10, 7.56], $p < 0.00001$) (Figure 8A).

Four of the included studies (Luo et al., 2018; Dong et al., 2019; Deng et al., 2020; Yuan et al., 2024) reported the effects of resveratrol on total retinal thickness in animal models with retinal disease (experimental group, n = 22; control group, n = 22). The results showed that resveratrol significantly increased the total retinal thickness in the retina compared with the control group (SMD = 2.70, 95% Cl = [0.57, 4.83], $p = 0.01$) (Figure 8B).

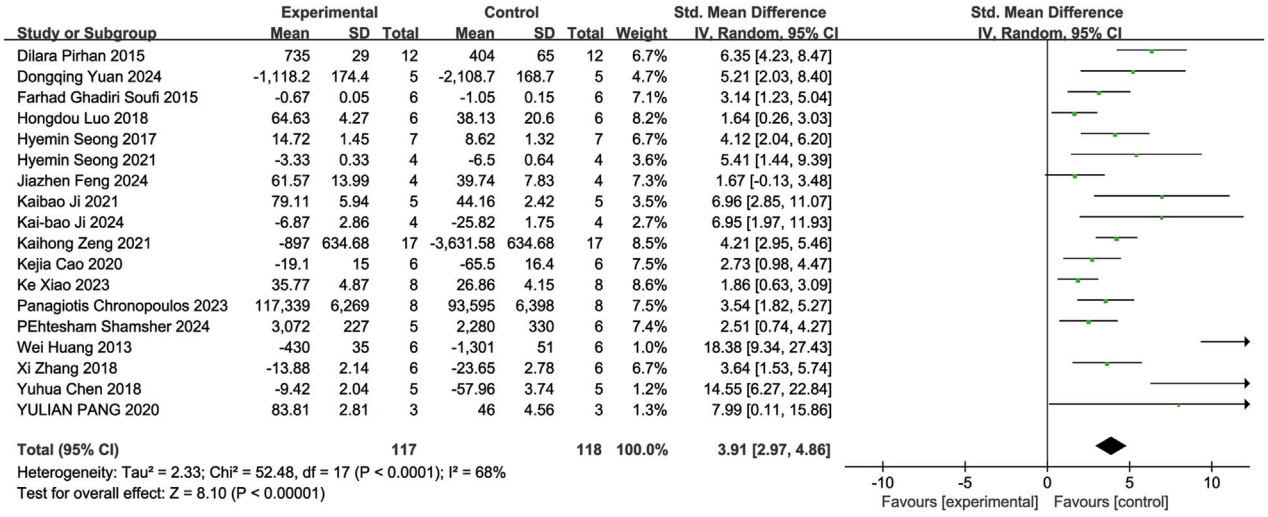



FIGURE 3
Forest plot of RGCs.

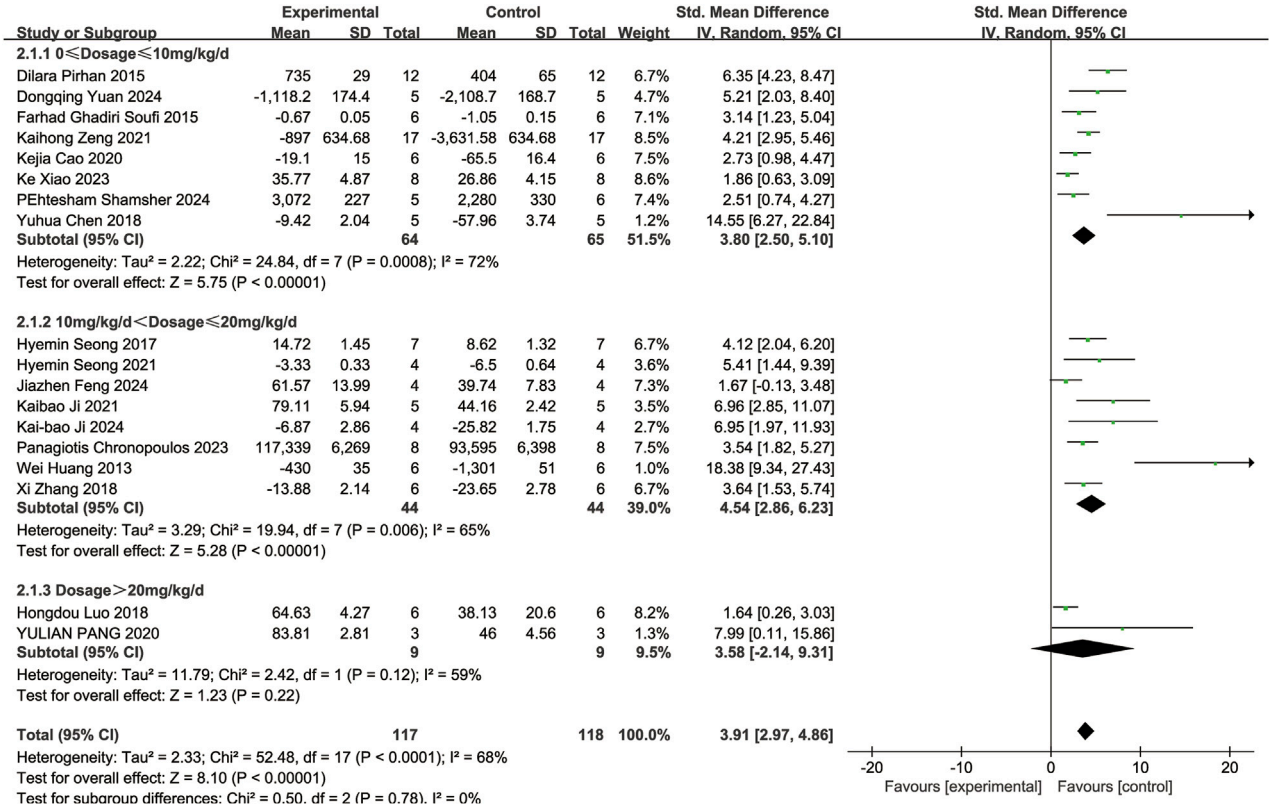



FIGURE 4
Subgroup analysis of RGCs based on dosage.

## 3.5 Publication bias analysis

To assess publication bias in the meta-analysis of the outcome indicators (Figures 9A–K), the results showed asymmetry in the funnel plot. The scatter of the funnel plot of the number of RGCs (A), SOD (B), ROS(D), COX-2 (E), TNF-α (F) and total retinal thickness (J) deviated from the axis of symmetry, which may be related to the small sample study, the type of animal, and the difference in detection methods. The scatter distributions of IL-6 (G), A-wave (H), B-wave (I), and inner retinal thickness (K) were

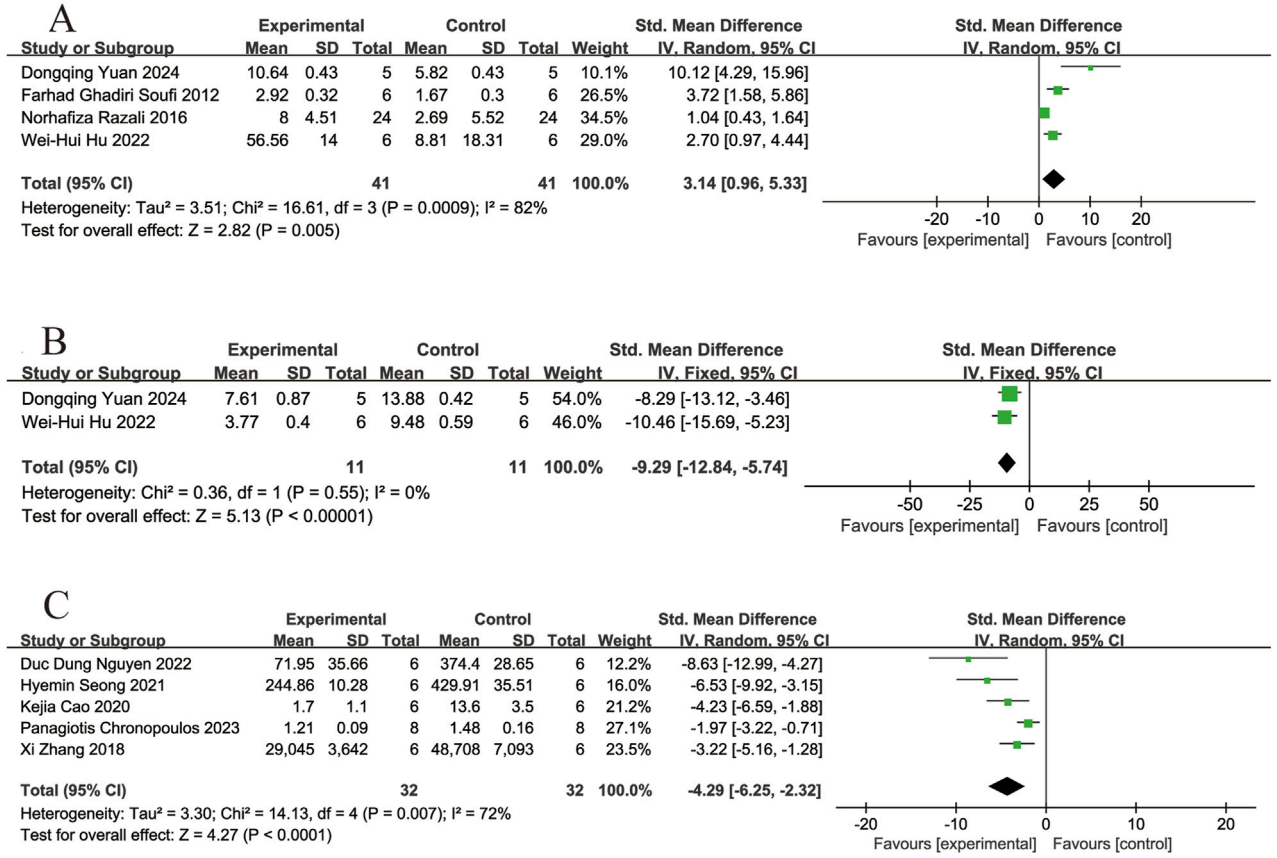



FIGURE 5
Forest plot. **(A)** The levels of superoxide dismutase; **(B)** The levels of malondialdehyde; **(C)** The levels of reactive oxygen species.

relatively scattered with weak asymmetry. Egger's test were used to quantitatively analyze publication bias (Table 3). The results indicate the presence of publication bias for the number of RGCs, SOD, ROS, COX-2, TNF-α and total retinal thickness ($p < 0.05$).

## 3.6 Sensitivity analysis

To evaluate the consistency and reliability of the meta-analysis results, Outcome measures included the number of RGCs (A), SOD (B), ROS (C), COX-2 (D), TNF-α (E), IL-6 (F), A-wave amplitudes (G), B-wave amplitudes (H), inner retinal thickness(I), and total retinal thickness (J), sensitivity analysis was performed. By recalculating the pooled effect size after excluding individual studies one by one, the pooled effect size of each of the above indicators was not significantly changed by the exclusion of individual studies. The conclusion of this meta-analysis is robust (Figures 10A–J).

# 4 Discussion

This systematic review mainly investigated the therapeutic effects of resveratrol on retinal diseases in animal models from multiple aspects, such as retinal neuroprotection, structural function, oxidative stress,and inflammatory factors. The findings demonstrated a beneficial impact of resveratrol in enhancing the evaluated indices of retinal injury post-treatment. Retinal diseases can result in reduced RGC numbers, consequently affecting visual function (Yuan et al., 2024). Excessive generation of ROS can lead to oxidative stress. SOD is the primary enzyme for scavenging oxygen free radicals in biological systems, whereas MDA serves as a crucial marker reflecting the extent of damage caused by free radicals. In retinal diseases, a reduction in SOD levels and an elevation in MDA and ROS levels signify heightened oxidative stress, impacting the standard function and metabolism of retinal cells, consequently contributing to the occurrence of retinal diseases (Dziedziak et al., 2021). As an inflammatory marker, TNF-α mainly causes retinal damage through inflammatory and apoptotic pathways. The level of IL-6 is related to the severity of retinal diseases, while COX2 participates in disease progression by regulating inflammation and angiogenesis. Therefore, these factors and their signaling pathways may be important targets for the treatment of retinal diseases. The A-wave in the electroretinogram predominantly mirrors the functionality of retinal photoreceptors, while the B-wave indicates the signal transmission process between the inner retinal nerve cells. Therefore, abnormal changes in the amplitudes of A and B waves can reveal different mechanisms of retinal damage (Xiao et al., 2023). There is a significant correlation between changes in retinal thickness and visual function, with variations in inner retinal

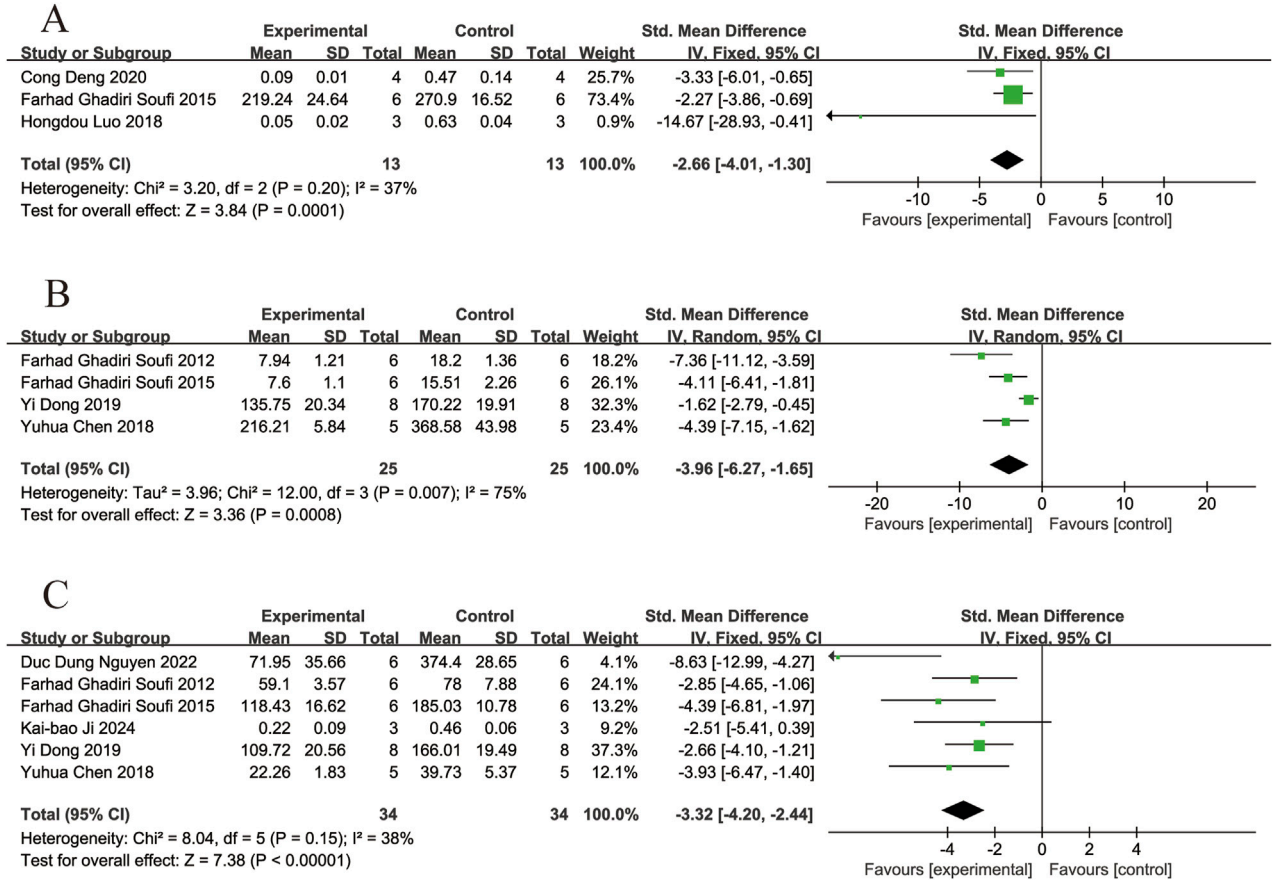



FIGURE 6
Forest plot. **(A)** The levels of cyclooxygenase-2; **(B)** The levels of tumor necrosis factor-α; **(C)** The levels of interleukin-6.

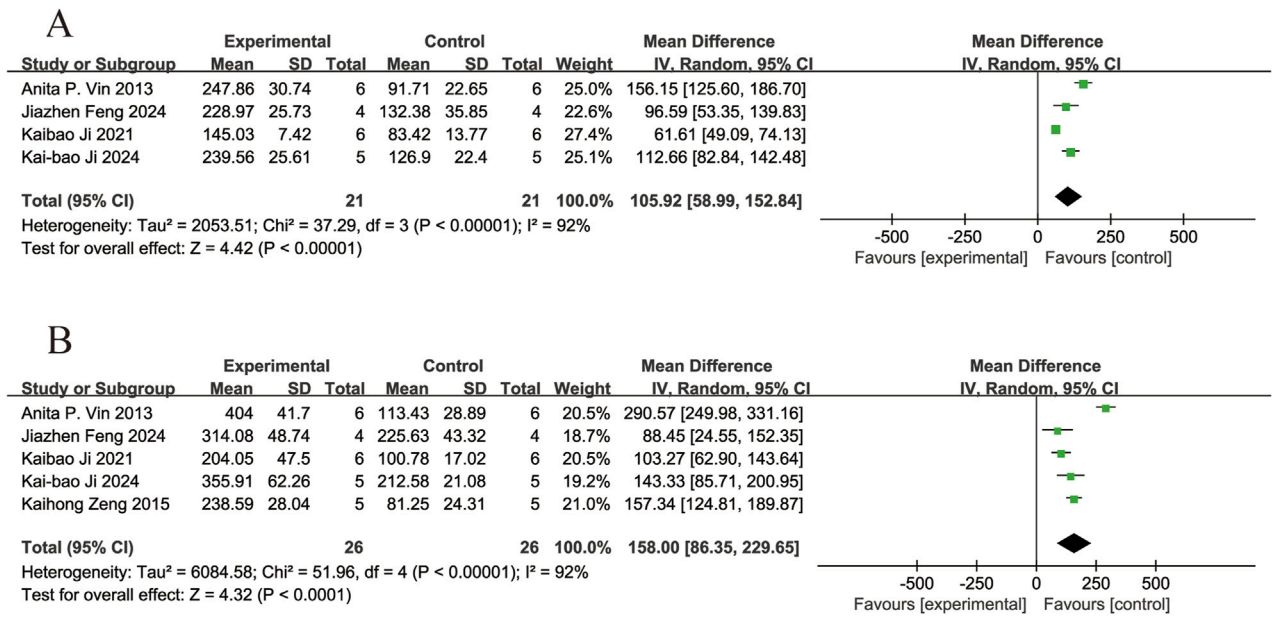



FIGURE 7
Forest plot. **(A)** The amplitudes of A-wave; **(B)** The amplitudes of B-wave.

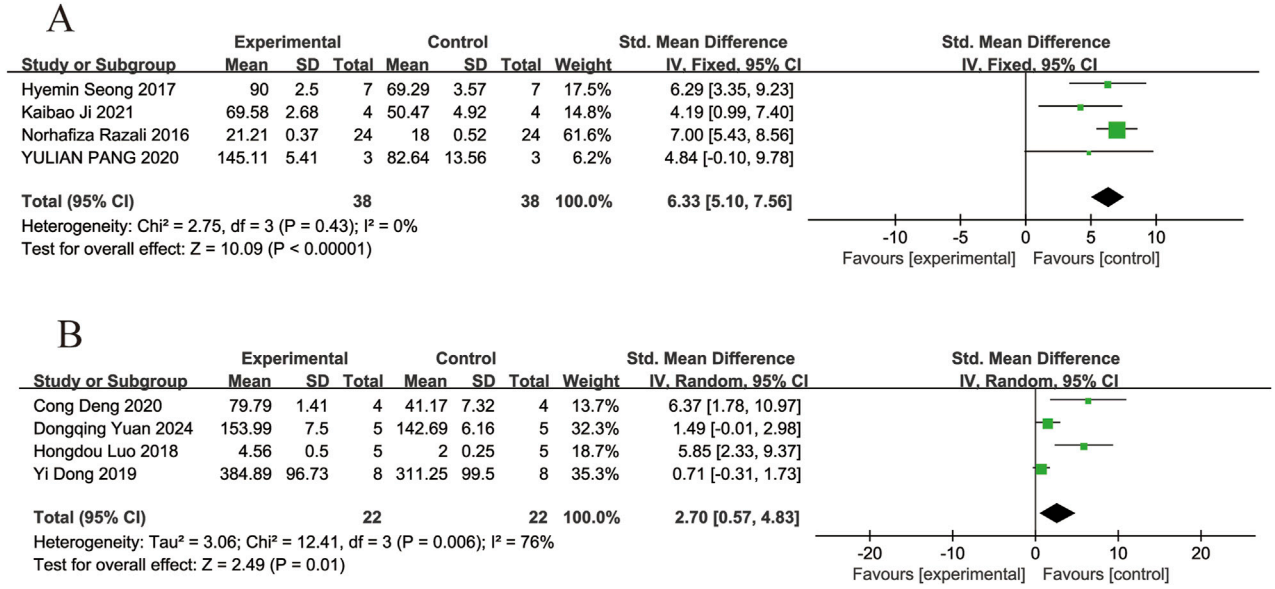



FIGURE 8
Forest plot. **(A)** The thickness of inner retinal; **(B)** The thickness of total retinal.

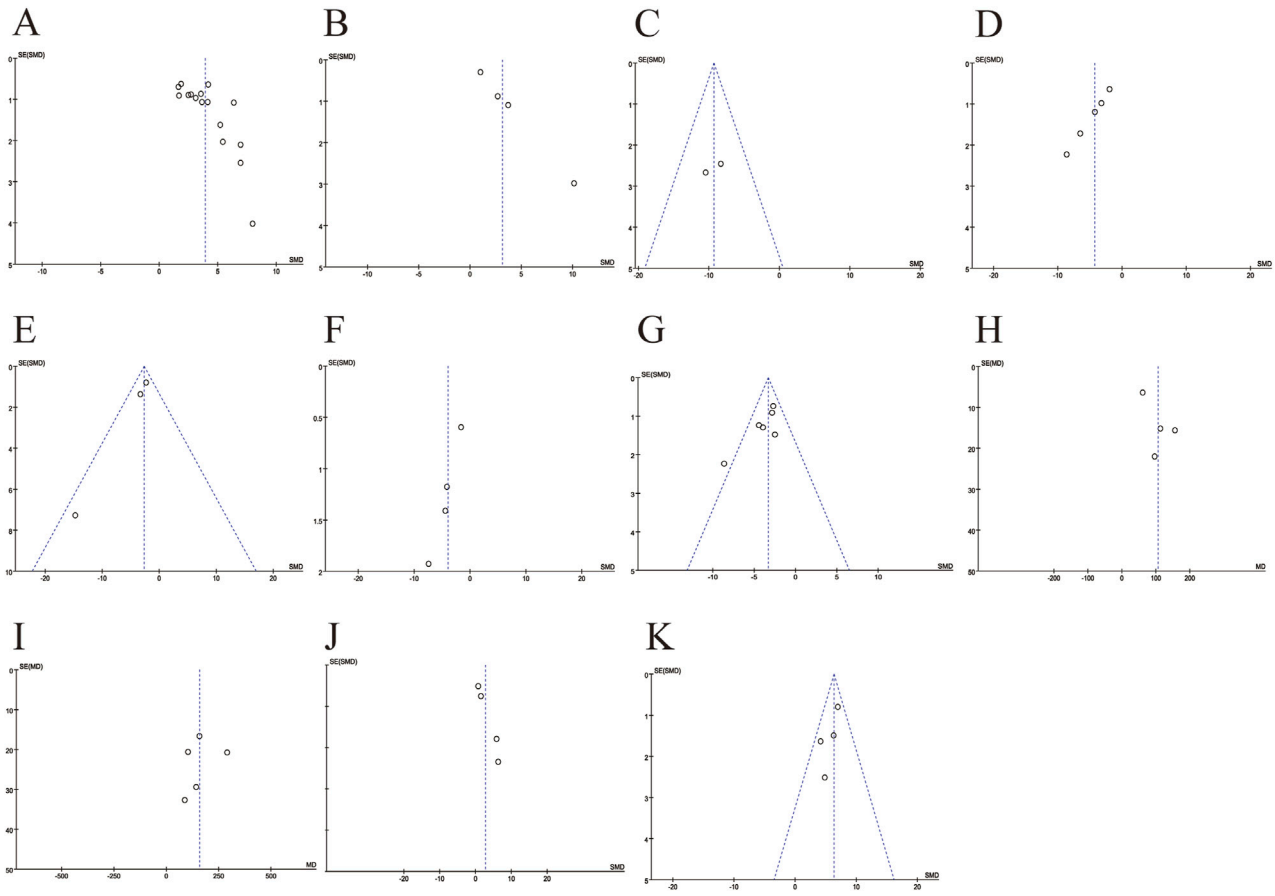



FIGURE 9
Funnel plots for evaluating publication bias. **(A)** The number of retinal ganglion cells; **(B)** The levels of superoxide dismutase; **(C)** The levels of malondialdehyde; **(D)** The levels of reactive oxygen species; **(E)** The levels of cyclooxygenase-2; **(F)** The levels of tumor necrosis factor-α; **(G)** The levels of interleukin-6; **(H)** The amplitudes of A-wave; **(I)** The amplitudes of B-wave; **(J)** The thickness of total retinal; **(K)** The thickness of inner retinal.

TABLE 3 Egger's test for evaluating publication bias.

| Outcome | Egger's test |
|---|---|
| | *p*-Value |
| The number of RGCs | 0.000 |
| SOD | 0.003 |
| MDA | — |
| ROS | 0.000 |
| COX-2 | 0.003 |
| TNF-α | 0.008 |
| IL-6 | 0.059 |
| A-wave | 0.195 |
| B-wave | 0.571 |
| Inner retinal thickness | 0.181 |
| Total retinal thickness | 0.010 |

thickness serving as an early indicator of retinopathy (Chronopoulos et al., 2023; Feng et al., 2024). Therefore, we collected and analyzed the published high-quality experimental data to investigate the application of resveratrol on retinal diseases.

This study showed that resveratrol significantly increased the number of RGCs in animal models treating retinal diseases. Dose-based subgroup analyses showed similar results, although high heterogeneity was observed at lower doses. Researcher have studied the neuroprotective effect of resveratrol on RGC survival in ischemic and reperfusion retinal injury (I/R injury). Their findings revealed that resveratrol regulates a variety of signaling pathways, including SIRT1/NF-κB axis and SIRT1-JNK pathway, by activating SIRT1, thereby decreasing the programmed cell death and axonal deterioration of retinal ganglion cells (Wu et al., 2020). The study demonstrated that upon activation of the Nrf2 signaling pathway, resveratrol can significantly reduce the expression of apoptosis protein Caspase-3. Caspase-3 serves as a pivotal effector protein in the apoptosis cascade, and its reduced expression signifies a decrease in apoptosis, thereby exerting a protective role on retinal ganglion cells and improving the pathological process of diabetic retinopathy (Yuan et al., 2024). In conclusion, the current study suggests that resveratrol has a significant neuroprotective effect in the protection of retinal ganglion cells by regulating and reducing protein expression through signaling pathways.

The retina may sustain structural and functional harm as a result of prolonged oxidative stress. The results of this study show that resveratrol can significantly increase the activity of SOD and reduce the levels of MDA and ROS, thereby reducing the damage of the retina caused by oxidative stress. Additionally, although heterogeneity was

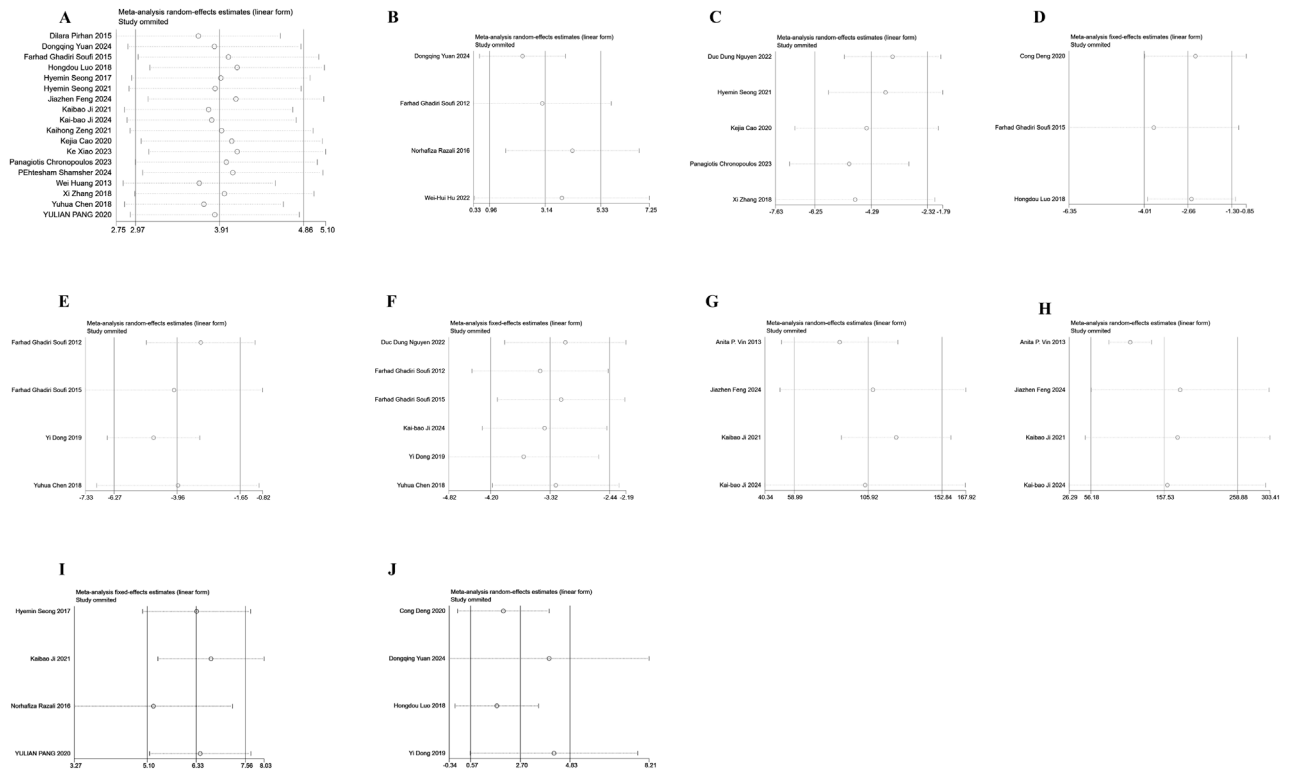



FIGURE 10
Sensitivity analysis chart. **(A)** The number of retinal ganglion cells; **(B)** The levels of superoxide dismutase; **(C)** The levels of reactive oxygen species; **(D)** The levels of cyclooxygenase-2; **(E)** The levels of tumor necrosis factor-α; **(F)** The levels of interleukin-6; **(G)** The amplitudes of A-wave; **(H)** The amplitudes of B-wave; **(I)** The thickness of inner retinal; **(J)** The thickness of total retinal.

observed in the SOD and ROS studies, subgroup analysis could not be performed due to the insufficient number of articles. Meanwhile, only two studies examined the MDA index. SOD is considered to be an important antioxidant that safeguards cells by neutralizing free radicals, thereby preserving the oxidative/antioxidant balance in the body during oxidative stress. As an important product in the oxidative damage process, MDA is an important index to measure the degree of oxidative damage (Pisoschi and Pop, 2015). Nrf2 functions as a transcription factor capable of modulating the expression of numerous antioxidant enzymes. There are studies further explored and found that resveratrol can elevate SOD expression by activating the Nrf2 signaling pathway (Wang et al., 2025). Furthermore, through the augmentation of antioxidant enzyme activity such as SOD, resveratrol can effectively clear intracellular ROS, reduce the occurrence of lipid peroxidation, and thus decreas the generation of MDA. In conclusion, resveratrol can effectively protect retinal cells from oxidative stress by regulating the levels of SOD, MDA and ROS. This protective mechanism has significant preventive and management significance for retinal diseases caused by oxidative stress.

The results of this study demonstrate that resveratrol can significantly decrease the levels of inflammatory markers. Resveratrol reduces the expression of matrix metalloproteinases and inhibits the production of IL-6 and TNF-α (Fisher, 2021). Furthermore, it has been shown that resveratrol effectively suppresses NF-κB (nuclear factor-kappa B) signaling by inhibiting NF-κB activity and suppressing the phosphorylation of JAK/STAT signaling pathways. Numerous polyphenols inhibit the expression of COX genes, and it has been established that resveratrol inhibits the activity of COX-1 and COX-2 in a dose-dependent manner (Li et al., 2018). It is reported that resveratrol may further exert neuroprotective effects by activating Peroxisome Proliferator-Activated Receptors (PPARs) and inhibiting the production of NO to resist oxidative stress and inflammatory damage (Yunusoğlu, 2021). These mechanisms of action provide a theoretical basis for the potential application of resveratrol in the treatment of retinal diseases (Wu et al., 2020).

The results of this study show that resveratrol can significantly enhancing the amplitude of A and B waves in electroretinogram. A wave and B wave are waveforms in electroretinogram (ERG), with the A wave predominantly reflecting the functionality of retinal photoreceptors. Resveratrol enhances the A-wave amplitude by safeguarding photoreceptor cells. In particular, under conditions such as light damage or diabetic retinopathy, resveratrol can protect photoreceptor cells and reduce their apoptosis to enhance the a-wave signal intensity (Luo et al., 2018). The B-wave primarily correlates with the function of retinal bipolar cells. Research has demonstrated that resveratrol enhances signal transmission between bipolar cells and retinal ganglion cells by suppressing retinal inflammation and oxidative stress, consequently elevating the B-wave amplitude (Wu et al., 2020). Therefore, resveratrol has a protective effect on the A and B-wave amplitudes in electroretinogram through various mechanisms, effectively enhancing retinal function and showcasing potential application value in the treatment of retinal diseases.

Inner retinal thickness refers to the thickness of each layer of the innermost layer of the retina, encompassing structures such as the ganglion cell layer and the inner core layer. Progressive pathological conditions often lead to a notable reduction in inner retinal thickness, detectable at early stages. Total retinal thickness represents the cumulative thickness of all retinal layers, serving as an indicator of overall retinal health. The outcomes of this study demonstrated a significant enhancement in both inner retinal thickness and total retinal thickness following resveratrol treatment. The study demonstrated that resveratrol mitigates retinal ganglion cell apoptosis by regulating SIRT1-JNK pathway (Luo et al., 2018). Moreover, resveratrol inhibits retinal neuronal cell apoptosis by upregulating Bcl-2 expression and downregulating Caspase-3 expression, thereby preserving inner retinal structure. Furthermore, resveratrol diminishes retinal damage through the inhibition of inflammatory factors, specifically TNF-α and IL-6,that helps preserve both the overall structure and thickness of the retina. Therefore, resveratrol exerts a protective influence on inner retinal thickness and total retinal thickness through diverse mechanisms, effectively enhancing retinal structure, which has potential application value in the treatment of retinal diseases.

This study represents the first comprehensive investigation into the impact of resveratrol on animal models of retinal diseases. To provide more reliable evidence for the protection of resveratrol on retinal disease models by multidimensional evaluation of funnel plot, Egger's test and sensitivity analysis. Despite the meticulous screening and assessment, there are still deficiencies. Firstly, detailed information regarding the characteristics of resveratrol, such as content and properties, was not provided in the study, potentially introducing certain discrepancies in the results. Secondly, the imbalance observed in Egger's test and the funnel plot suggests the presence of publication bias, which may affect the interpretation of the results. The high heterogeneity may result from different study designs, including differences in animal models, methods, doses, and durations. It is recommended that future studies consider these potential sources of heterogeneity to improve the consistency of study designs. Finally, the studies that were included lacked descriptions of assignment concealment, randomization of animals during experiments, and blinding of investigators.

## 5 Conclusion

This meta-analysis included 26 articles that summarized resveratrol's influence on retinal diseases while also offering significant preclinical mechanistic studies in terms of neuroprotection, structural function, and oxidative inflammatory markers. Our meta-analysis results show that resveratrol affects several markers of retinal diseases, implying positive effects on animal models of retinal diseases and establishing it as a prospective adjuvant that can improve retinal diseases. Nonetheless, given the heterogeneity, potential for bias, and other limitations of the included studies, more high-quality studies are needed to confirm resveratrol's effectiveness in treating retinal diseases.

## Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

## Author contributions

XL: Formal Analysis, Data curation, Conceptualization, Methodology, Writing – original draft, Software. NL: Formal Analysis, Writing – review and editing, Software, Visualization. LC: Supervision, Visualization, Writing – review and editing. CS: Supervision, Investigation, Writing – review and editing, Visualization.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2025.1615910/full#supplementary-material

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# The pharmacology and mechanism of action of *Monascus purpureus* Went: a scoping review

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**Objective:** The aim of this study is to review the recent studies on the pharmacology and mechanism of action of *Monascus purpureus* Went, analyze its medicinal value, and explore future research directions.

**Method:** A scoping review was conducted by searching the China National Knowledge Infrastructure (CNKI), Wanfang database, VIP database, SinoMed, and PubMed from inception until September 2024. The basic information of the included studies, such as study types, disease types, main components, outcomes, and efficacy, was reviewed and summarized. Methodological quality was assessed using the SYRCLE's risk of bias assessment tool for animal studies and the Cochrane risk of bias assessment tool for clinical trials.

**Results:** We identified 251 studies from the five databases. Among them, 153 were experimental studies, 70 were reviews, and 28 were clinical trials. Of the experimental studies, molecular studies accounted for the largest portion, totaling 80 (52%). Among the reviews, research progress accounted for the most, totaling 41 (59%). The clinical trials studied the effects of *Monascus purpureus* Went and its related Chinese patent medicines and preparations. Of these, 17 (61%) used *Monascus purpureus* Went-related Chinese patent medicines and preparations as interventions and 11 (39%) used traditional Chinese medicine (TCM) formulations of *Monascus purpureus* Went as interventions. In terms of methodological quality, both animal studies and clinical trials related to *Monascus purpureus* Went showed deficiencies in randomized allocation sequence generation, allocation concealment, and blinding methods.

**Conclusion:** We summarized existing studies on the active ingredients and effects of *Monascus purpureus* Went and found that it is necessary to improve the generation of random allocation sequences and the application of the blinding method in *Monascus purpureus* Went-related animal studies and clinical trials. When similar studies are conducted in the future, the specific methods of random assignment should be more clearly described, and blinding methods should be applied to improve the objectivity and accuracy of the studies, thereby providing a reference for selecting future research directions and establishing supporting evidence.

KEYWORDS

***Monascus purpureus* Went, pharmacological effects, pharmacological mechanisms, scope review, evidence**

# 1 Introduction

*Monascus purpureus* Went is a traditional Chinese medicine (TCM) with a long history, produced through the fermentation of ordinary rice using Monascus species (Chen et al., 2000). It is mainly produced in Fujian, Zhejiang, and Jiangxi. Additionally, *Monascus purpureus* Went is sweet in taste, warm in nature, and belongs to the liver, spleen, stomach, and large intestine meridians, according to TCM theory. It is recorded in *the Supplement to Augmented Materia Medica* that *Monascus purpureus* Went has the effects of "promoting blood circulation and helping digestion, strengthening the spleen and warming the stomach, treating dysentery, and bringing down water." Clinically, *Monascus purpureus* Went is mainly used to treat postpartum lochia, abdominal pain with stagnation, food accumulation and fullness, dysentery, and bruises (Song et al., 1999a).

In 2024, Japan's Kobayashi Pharmaceutical Co., Ltd. experienced a safety incident involving "health products containing *Monascus purpureus* Went ingredients." After investigation, the issue was potentially caused by contamination of the *Monascus purpureus* Went fermentation raw materials with penicillic acid or by inadequate cleanliness in the production environment, lead to the presence of penicillium mixed. This incident once again brought the efficacy and safety of red yeast into the global social hot spot (Ma 2025; Sun, 2025; Zhu et al., 2025).

Modern pharmacology has discovered that *Monascus purpureus* Went has lipid-lowering, anti-tumor, antioxidant, anti-osteoporosis, antibacterial, and other effects (Wei et al., 2023). In addition, *Monascus purpureus* Went has a wide range of applications in brewing, fermented foods, food coloring, and other fields. In recent years, new application fields have been gradually developed, such as animal husbandry and veterinary medicine, feed fermentation, healthy fermented foods, healthy drinks, and healthy seasonings (Xie, 1996). In recent years, the extraction of the lipid-lowering component *lovastatin* from *Monascus purpureus* Went (Endo, 1980) has further enhanced the research value of *Monascus purpureus* Went, attracting great attention from scholars both at home and abroad. The significant lipid-lowering effect and medicinal potential of *lovastatin* have inspired many scholars to conduct studies on the pharmacology and mechanism of action of this new component in *Monascus purpureus* Went, resulting in many remarkable findings, such as studying the lipid-lowering mechanism and content determination method of *lovastatin* (Wen et al., 2001), optimizing the extraction process of *lovastatin* from *Monascus purpureus* Went (Wang et al., 2024a), and producing drugs mainly composed of *lovastatin*, such as Xuezhikang (Kong et al., 2005). At present, the pharmacological research on *Monascus purpureus* Went still needs to be improved to further understand and develop its medicinal value and expand its application range.

A large number of studies on *Monascus purpureus* Went have been published, including reviews (Wang et al., 2024b). However, these published reviews lacked a comprehensive literature search, which led to limited references and unreliable evidence evaluation. Such limitations may introduce certain biases into the review, which is not conducive to the reference of other studies. In contrast, this study conducts a scoping review based on evidence-based medicine methods to systematically summarize the recent studies on the pharmacology and mechanism of action of *Monascus purpureus* Went, analyze its medicinal value, and explore future research directions.

# 2 Data and methods

## 2.1 Search strategy

Five databases, namely, China National Knowledge Infrastructure (CNKI), Wanfang database, VIP database, SinoMed, and PubMed, were searched from inception to September 2024. The search terms consisted of *Monascus purpureus* Went, Monascus, *Monascus purpureus* Went, pharmacology, pharmacological effect, pharmacological mechanism, biological activity, and active ingredient. The full search terms of all databases are shown in Supplementary Add S1.

## 2.2 Inclusion and exclusion criteria

Inclusion criteria: Studies focused on *Monascus purpureus* Went; study types were not limited, and the language was English or Chinese.

Exclusion criteria: Manuscripts with unavailable full text and duplicate publications were excluded. News reports, conference papers, or dissertations were also excluded.

## 2.3 Study screening and data extraction

The screening and extraction process was as follows: 1) NoteExpress software was used to exclude duplicate studies, 2) two reviewers performed an initial screening after reading the titles and abstracts based on the inclusion and exclusion criteria, 3) the full text was reviewed when additional information was needed for screening, and 4) any disagreements were resolved through discussion with a third researcher.

The basic information in the included studies, such as title, authors, year of publication, type of study, subject of study, study population, study method, intervention, duration of treatment, dosage, control measure, measurement index, method of measurement, result, and conclusion, was extracted.

**Abbreviations:** AA, automatic amino acid analyzer; AMPK, AMP-activated protein kinase; ASA, anthrone–sulfuric acid; CE, capillary electrophoresis; CNKI, China National Knowledge Infrastructure; DNS, dinitrosalicylic acid; FAAS, flame atomic absorption spectroscopy; GC, gas chromatography; HDL, high-density lipoprotein; HPLC, high-performance liquid chromatography; LDL, low-density lipoprotein; NPC, nasopharyngeal carcinoma; PSA, phenol–sulfuric acid; QuEChERS-UPLC-MRM-IDA Criteria-EPI; quick, easy, cheap, effective, rugged, safe–ultra-performance liquid chromatography–multiple reaction monitoring–ion-dependent acquisition–criteria-enhanced product ion; RAPD, random amplified polymorphic DNA; SMF, secondary metabolite fingerprint; SPSS CA, statistical package for the social sciences cluster analysis; SPSS PCA, statistical package for the social sciences principal component analysis; TC, total cholesterol; TCM, traditional Chinese medicine; TG, triglyceride; TLCS, thin-layer chromatography scan.

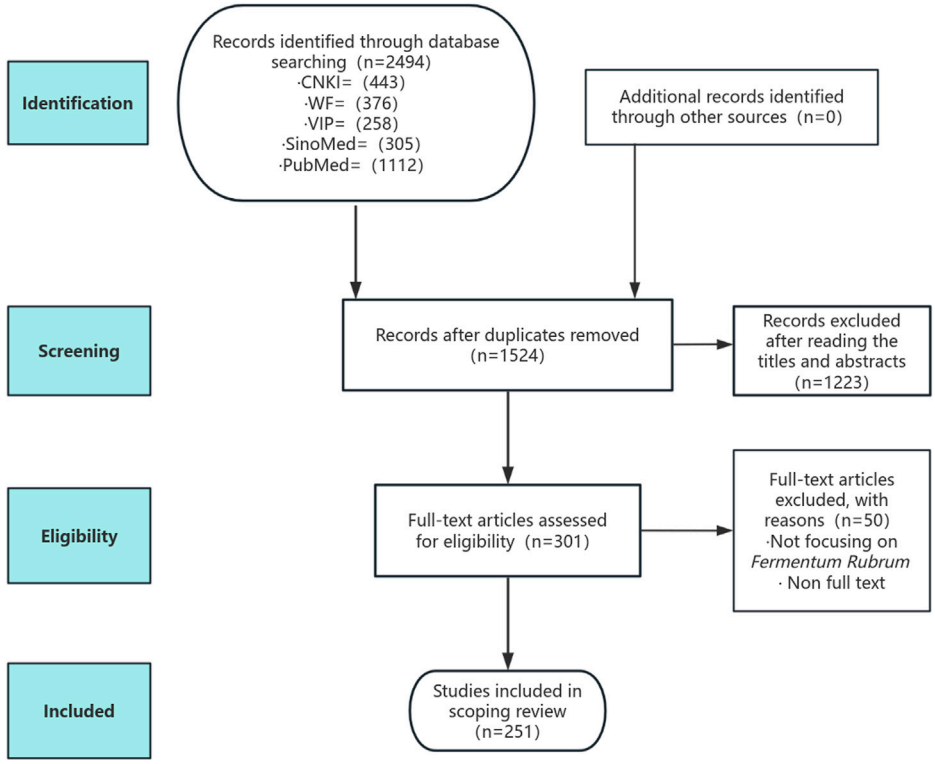



FIGURE 1
Flowchart of study screening.

## 2.4 Data analysis

We analyzed all extracted data fields, including bibliometric statistics, visual data analysis, and evidence graph analysis. We also systematically organized and comprehensively summarized study evidence information.

## 2.5 Quality assessment

Two reviewers assessed the methodological quality of the included studies, including animal studies and clinical trials, as this is an integral part of evidence-based research. The SYRCLE's risk of bias assessment tool was used to assess the quality of animal studies, and the Cochrane risk of bias assessment tool was used to assess the quality of randomized controlled trials (RCTs). The evaluation results were indicated as "low risk," "high risk," or "unclear risk."

# 3 Results

## 3.1 Search results

A total of 2,494 studies were initially searched. Among them, 970 were duplicates, and 1,223 were excluded after reading the title or abstract. Among 301 studies assessed in full text, 50 were excluded for the following reasons: not focusing on *Monascus purpureus* Went (n = 41) and lack of full text (n = 9). Finally, 251 studies were included in the final review.

The study screening process is presented in Figure 1.

## 3.2 Characteristics of included studies

A total of 251 literature studies were included, comprising 242 Chinese publications (96%) and 9 English publications (4%). There were 153 experimental studies (61%), 70 reviews (28%), and 28 clinical trials (11%). The included studies were published between 1988 and 2024, with the highest number published in 2007 (n = 18). The number of studies published each year is presented in Figure 2.

We summarized and generalized evidence from studies on *Monascus purpureus* Went, including its definition, origin, fermentation strain, morphological features, production process, acquisition methods, ingredients, identification methods, clinical efficacy, safety evaluation, and applications. The chart of evidence from studies on *Monascus purpureus* Went is presented in Figure 3.

Among the experimental studies, molecular studies accounted for most of them, totaling 80 (52%). Among the reviews, research progress accounted for most of the studies, totaling 41 (59%). Clinical trials studied the effects of *Monascus purpureus* Went and its related Chinese patent medicine in the treatment of diseases, with 17 (61%) using *Monascus purpureus* Went-related Chinese patent medicines and preparations as interventions and 11 (39%) using TCM formulations of *Monascus purpureus* Went as

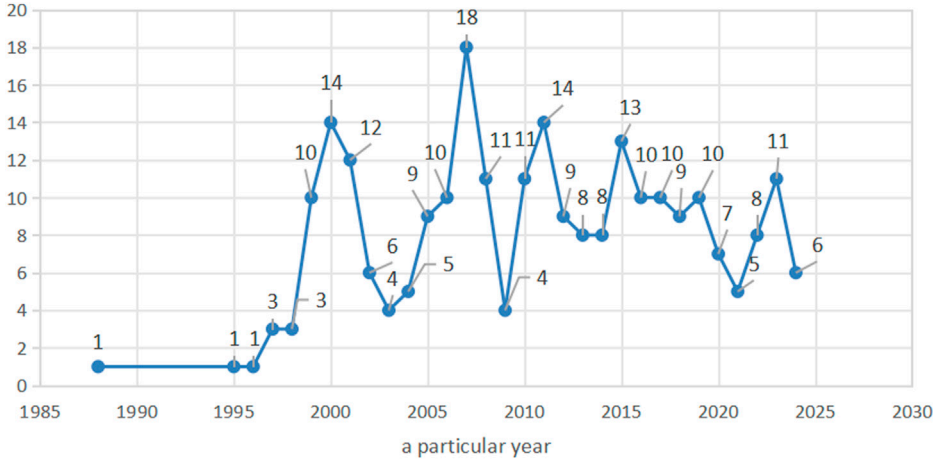



FIGURE 2
Number of studies related to *Monascus purpureus* Went each year.

interventions. The details of the research topics of the included studies are shown in Table 1.

## 3.3 Experimental study

### 3.3.1 Molecular study

Eighty molecular studies related to *Monascus purpureus* Went were included.

#### 3.3.1.1 Content determination

Forty studies determined the content of active ingredients in *Monascus purpureus* Went, with *lovastatin* and citrinin (*orange mold*) being the most common ingredients. The methods for content determination included high-performance liquid chromatography (HPLC) (n = 29), thin-layer chromatography scan (TLCS) (n = 2), capillary electrophoresis (CE) (n = 2), automatic amino acid analyzer (AA) (n = 2), gas chromatography (GC) (n = 1), quick, easy, cheap, effective, rugged, safe–ultra-performance liquid chromatography–multiple reaction monitoring–ion-dependent acquisition–criteria-enhanced product ion (QuEChERS-UPLC-MRM-IDA-Criteria-EPI) (n = 1), phenol–sulfuric acid (PSA) (n = 1), anthrone–sulfuric acid (ASA) (n = 1), dinitrosalicylic acid (DNS) (n = 1), flame atomic absorption spectroscopy (FAAS) (n = 1), and statistical analyses including statistical package for the social sciences principal component analysis and cluster analysis (SPSS PCA and SPSS CA) (n = 1).

#### 3.3.1.2 Chemical property

Fourteen studies examined the chemical properties of the active ingredients in *Monascus purpureus* Went, including antioxidant activity (n = 5), chemical structure (n = 5), lipid-lowering activity (n = 2), poisoning mechanism (n = 1), and protease and amylase activities (n = 1).

#### 3.3.1.3 *Monascus purpureus* Went identification method

Thirteen studies examined the methods for identifying different *Monascus purpureus* Went strains using chromatography (n = 6), spectroscopy (n = 4), random amplified polymorphic DNA (RAPD) (n = 2), and secondary metabolite fingerprint (SMF) (n = 1).

#### 3.3.1.4 Component extraction process

Eight studies investigated the extraction process of active ingredients in *Monascus purpureus* Went.

(1) *Lovastatin*: One study used 88% ethanol in an amount 13 times that of the raw material, with reflux extraction for 1.3 hours (n = 1); another study used six times the amount of 95% ethanol, with reflux extraction performed twice, each for 0.5 hours (n = 1); and a third study used 250 mL of ethanol, with reflux for 5 hours (n = 1).
(2) Monacolin K: One study used 70% ethanol (pH 7.5) with a material-to-solvent ratio of 1:30, an extraction time of 1.5 hours, and an extraction temperature of 50°C (n = 1); another study used pure methanol with an extraction temperature 60°C, a liquid-to-solid ratio of 20:1, and ultrasonic extraction for 1 hour (n = 1); and a third used 65% ethanol with a material-to-liquid ratio of 1:20, an extraction temperature of 70°C, and an extraction time of 3 hours (n = 1).
(3) Total flavonoid: One study used analytically pure ethanol and ultrasonic (50 kHz, 250 W) extraction for 20 min, followed by adsorption using 1 g of polyamide powder. The sample was then transferred to a chromatography column (inner diameter 1.0 cm) and eluted with methanol elution (0.5 mL/min) (n = 1).
(4) *Monascus purpureus* Went pigment: One study used a 70% ethanol aqueous solution with an extraction temperature of 60°C and an extraction time of 2 hours (n = 1).

#### 3.3.1.5 Strain identification and screening

Five studies examined the identification and screening of *Monascus purpureus* Went strains based on molecular biology.

### 3.3.2 Animal study

Among the 58 animal studies, 54 used rat models (93%), 3 used rabbit models, and 1 used the quail model. A total of 55 studies

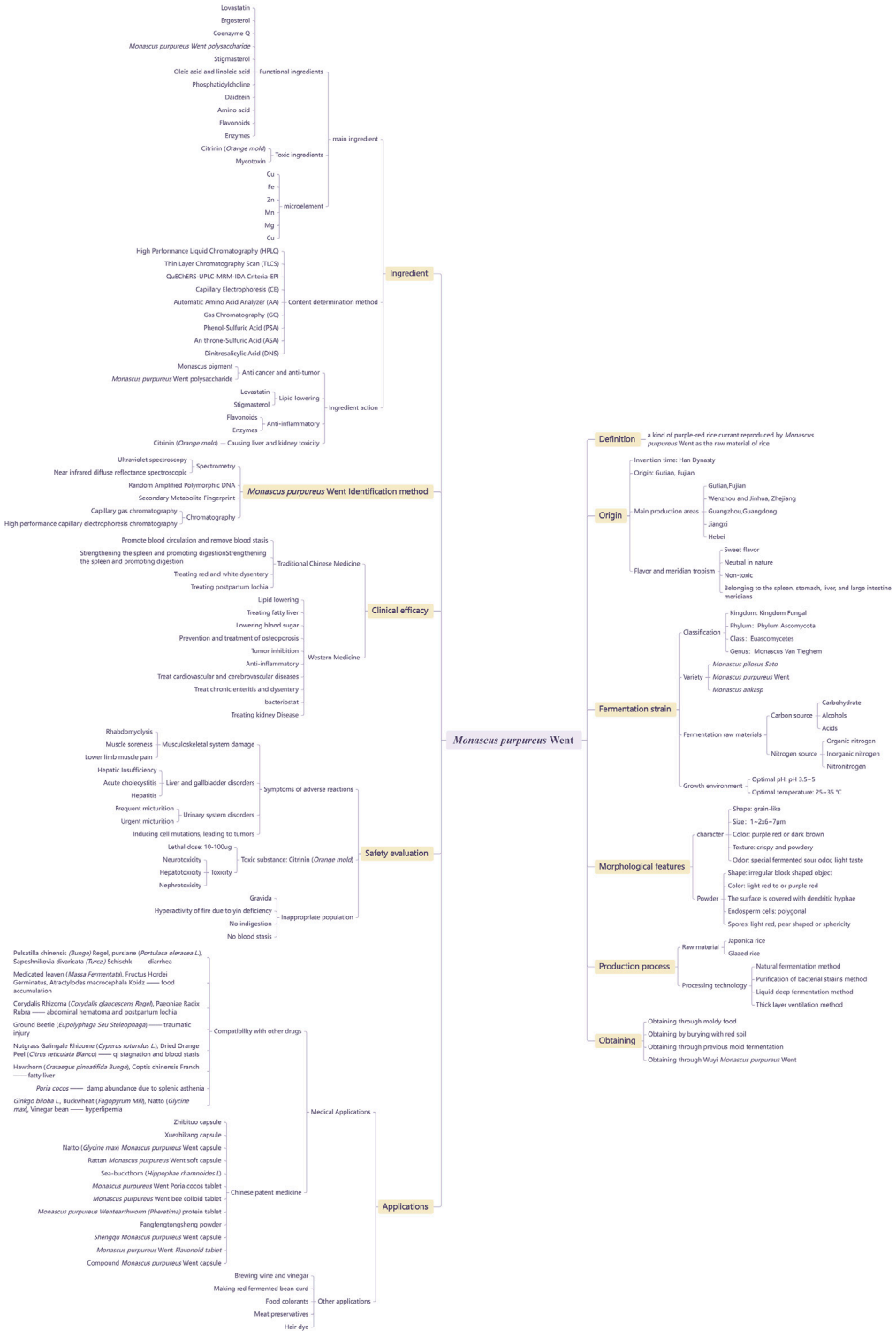



FIGURE 3
Chart of evidence from studies on *Monascus purpureus* Went.

determined the efficacy of *Monascus purpureus* Went, including double-armed studies (n = 6), three-armed studies (n = 10), and multi-armed studies (n = 39). Three studies determined the safety of erythromycin, including a two-armed study (n = 1), a three-armed study (n = 1), and a multi-armed study (n = 1). Fifty-four studies contained a blank control, and four studies did not contain a blank control.

Among them, 26 studies showed that *Monascus purpureus* Went had lipid lowering effects, involving TCM *Monascus purpureus* Went (n = 5), *Monascus purpureus* Went compound preparation

TABLE 1 Study topics.

| Study | Type | Study topic |
|---|---|---|
| Experimental studies | Molecular study (Wu and Luo, 2013) | Content determination (Luo et al., 2010)<br>Chemical property (Wang et al., 2000)<br>*Monascus purpureus* Went identification method (Yan, 1999)<br>Component extraction process (Endo, 1980)<br>Strain identification and screening (Zhu et al., 2025) |
| | Animal study (Huang et al., 2019) | Lipid lowering (Lu et al., 2006)<br>Effect on bones (Wang et al., 2024b)<br>Improvement of fatty liver (Zhu et al., 2025)<br>Lowering blood pressure (Sun, 2025)<br>Anti-inflammatory effect (Ma, 2025)<br>Safety evaluation (Ma, 2025)<br>Tumor inhibition (Song et al., 1999a)<br>Cerebrovascular disease (Chen et al., 2000)<br>Regulation of gastrointestinal diseases (Chen et al., 2000)<br>Kidney disease (Chen et al., 2000) |
| | Cell study (Yu et al., 2000) | Identification and screening of bacterial strains (Xie, 1996)<br>Efficacy (Wei et al., 2023)<br>Physiological morphology of bacterial strains (Song et al., 1999a) |
| Reviews | Research progress (Luo and Zhang, 2011) | Pharmacological mechanisms (He, 2004)<br>Active ingredients (Wang et al., 2024b)<br>Clinical efficacy (Wen et al., 2001)<br>Classification criteria (Chen et al., 2000) |
| | Popular science (Chen et al., 2005) | Pharmacological mechanisms (Wang et al., 2024a)<br>*Monascus purpureus* Went-related food and drug use (Wei et al., 2023)<br>Origin (Sun, 2025)<br>Safety evaluation (Song et al., 1999a) |
| | Other (Zhu et al.) | Production (Song et al., 1999a)<br>Industry standard (Song et al., 1999a)<br>Selective breeding (Chen et al., 2000) |
| | Meta-analysis/SR (Song et al., 1999a) | Clinical efficacy (Chen et al., 2000)<br>Safety evaluation (Chen et al., 2000) |
| Clinical trials | Randomized controlled trial (Chen et al., 2005) | TCM *Monascus purpureus* Went (Xie, 1996)<br>Xuezhikang capsule (Wei et al., 2023)<br>Shengqu *Monascus purpureus* Went capsule (Song et al., 1999a)<br>Lipid-lowering *Monascus purpureus* Went micro-powder (Song et al., 1999a)<br>Zhibituo capsule (Chen et al., 2000)<br>Compound *Monascus purpureus* Went capsule (Chen et al., 2000)<br>*Monascus purpureus* Went flavonoid tablet (Chen et al., 2000)<br>Rattan *Monascus purpureus* Went soft capsule (Chen et al., 2000)<br>*Coptis chinensis* Franch.*Monascus purpureus* Went medicine (Chen et al., 2000) |
| | Controlled trial (Wei et al., 2023) | TCM *Monascus purpureus* Went (Song et al., 1999a)<br>*Monascus purpureus* Went compound preparation (Chen et al., 2000)<br>Zhibituo capsule (Chen et al., 2000)<br>*Monascus purpureus* Went capsule for reducing sugar (Chen et al., 2000)<br>Danxi *Monascus purpureus* Went wine (Chen et al., 2000) |

(n = 3), vinegar bean lipid-lowering capsule (n = 1), *Monascus purpureus* Went earthworm (*Pheretima*) protein tablet (n = 1), *Monascus purpureus* Went *Allium sativum* L. fermentation extract (n = 1), *Monascus purpureus* Went *Poria cocos* (Schw.) Wolf tablet (n = 1), compounded *Monascus purpureus* Went capsule (n = 1), a mixture of *Monascus purpureus* Went and grape seed anthocyanidin (n = 1), *Monascus purpureus* Went bee glue tablet (n = 1), Xuezhikang capsule (n = 1), *lovastatin* (n = 1), natto (*Glycine max*) *Monascus purpureus* Went (n = 1), *Ginkgo biloba* L. *Monascus purpureus* Went vitamin grouping (n = 1), *Monascus purpureus* Went combined with Fang Feng Tong Sheng powder (n = 1), *Fagopyrum esculentum* Moench *Monascus purpureus* Went powder (n = 1), *Monascus purpureus* Went–phytosterol ester compound preparation (n = 1), Yunnan *Monascus purpureus* Went powder (n = 1), compounded *Monascus purpureus* Went extract (n = 1), yellow Monascus pigment (n = 1), and sea-buckthorn (*Hippophae rhamnoides* L.) *Monascus purpureus* Went capsule (n = 1). Twelve studies showed that *Monascus purpureus* Went had an effect on repairing bones, involving TCM *Monascus purpureus* Went (n = 11) and *Monascus purpureus* Went capsule-containing coenzyme Q10 (n = 1); five studies showed that *Monascus purpureus* Went had an effect on improving fatty liver, involving TCM *Monascus purpureus* Went (n = 3), *Monascus purpureus* Went *Crataegus pinnatifida* Bunge (n = 1), and *Coptis chinensis* Franch. *Monascus purpureus* Went medicine (n = 1); four studies showed that *Monascus purpureus* Went had an effect on lowering blood pressure, involving TCM *Monascus purpureus* Went (n = 4); three studies showed that *Monascus purpureus* Went had an effect on

TABLE 2 Information on animal studies related to *Monascus purpureus* Went.

| Inclusion of studies | Disease | Model | Method of administration | Intervention | Dosage | Course of treatment | Outcome indicator |
|---|---|---|---|---|---|---|---|
| Yan (1999) | Hyperlipemia | Rat | Irrigation | Distilled water vs. vinegar lipid-lowering capsule low dose (Chinese patent medicine) vs. vinegar lipid-lowering capsule medium dose (Chinese patent medicine) vs. vinegar lipid-lowering capsule high dose (Chinese patent medicine) | 0.3 g/kg.bw, 0.6 g/kg.bw, and 1.2 g/kg.bw | 28 days | ①②③ |
| Wang et al. (2000) | Nephrosis | Rat | Irrigation | TCM *Monascus purpureus* Went vs. blank control | 0.8 $g{\cdot}kg^{-1}{\cdot}day^{-1}$ | 20 days | ①②③④⑥⑦ |
| Yu et al. (2000) | Hyperlipemia | Rabbit | Irrigation | Yunnan *Monascus purpureus* Went powder low dose (traditional TCM preparation) vs. Yunnan *Monascus purpureus* Went powder medium dose (traditional TCM preparation) vs. Yunnan *Monascus purpureus* Went powder high dose (traditional TCM preparation) vs. *lovastatin* | 4 $mg{\cdot}kg^{-1}{\cdot}day^{-1}$, 6 $mg{\cdot}kg^{-1}{\cdot}day^{-1}$, 10 $mg{\cdot}kg^{-1}{\cdot}day^{-1}$, and 6 $mg{\cdot}kg^{-1}{\cdot}day^{-1}$ | 42 days | ①②③④⑥⑦ |
| Sun et al. (2001a) | Hyperlipemia | Rat | Irrigation | TCM *Monascus purpureus* Went low dose vs. TCM *Monascus purpureus* Went high dose vs. blank control | 0.6 g/kg BW and 1.2 g/kg BW | 28 days | ①②③ |
| Sun et al. (2001b) | Hyperlipemia | Rat | Irrigation | TCM *Monascus purpureus* Went low dose vs. TCM *Monascus purpureus* Went medium dose vs. TCM *Monascus purpureus* Went high dose vs. blank control | 0.4 g/kg BW, 0.8 g/kg BW, and 1.2 g/kg BW | 21 days | ⑧ |
| Wang et al. (2002) | Fatty liver | Quail | Irrigation | TCM *Monascus purpureus* Went low dose vs. TCM *Monascus purpureus* Went high dose vs. Dongbao liver-healthy vs. *lovastatin* vs. blank control | 0.8 $g{\cdot}kg^{-1}$, 1.6 $g{\cdot}kg^{-1}$, and 0.6 $g{\cdot}kg^{-1}$ | 20 days | ①②③④ |
| He (2004) | Hyperlipemia and atherosclerotic lesions | Rabbit | Irrigation | *Monascus purpureus* Went *Allium sativum* L. fermentation extract vs. blank control | 100 g/day | 126 days | ①②③④ |
| Lu et al. (2004) | Osteoporosis | Rat | Irrigation | TCM *Monascus purpureus* Went vs. α-D3 vs. blank control | 10 mL/kg | 98 days | ⑨⑩⑪⑰ |
| Tang et al. (2004) | Hypertension | Rat | Irrigation | TCM *Monascus purpureus* Went vs. blank control | 0.417 g/(kg·day) | 21 days | ⑫⑬ |
| Chen et al. (2005) | Hyperlipemia | Rat | Irrigation | *Lovastatin* low dose vs. *lovastatin* medium dose vs. *lovastatin* high dose vs. *Gynostemma Blume* | 5, 15, and 30 mg/kg and 20 mg/kg | 42 days | ④⑭ |
| Lu et al. (2005a) | Osteoporosis | Rat | Irrigation | TCM *Monascus purpureus* Went vs. α-D3 vs. blank control | 10 mL/kg | 90 days | ⑮⑯ |
| Lu et al. (2005b) | Osteoporosis | Rat | Irrigation | TCM *Monascus purpureus* Went vs. α -D3 vs. blank control | 10 mL/kg | 84 days | ⑪⑰ |
| Wang et al. (2005) | Hyperlipemia | Rat | Irrigation | Compound *Monascus purpureus* Went capsule high dose vs. compound *Monascus purpureus* Went capsule medium dose vs. compound *Monascus purpureus* Went capsule high dose vs. blank control | 167, 333, and 1,000 mg/kg | 28 days | ①②③ |
| Lu et al. (2006) | Osteoporosis | Rat | Irrigation | TCM *Monascus purpureus* Went vs. Pravastatin vs. Premarin vs. blank control | 10 mL/kg | 10 days | ⑯⑱ |

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TABLE 2 (*Continued*) Information on animal studies related to *Monascus purpureus* Went.

| Inclusion of studies | Disease | Model | Method of administration | Intervention | Dosage | Course of treatment | Outcome indicator |
|---|---|---|---|---|---|---|---|
| Wang et al. (2006a) | Air pouch synovitis | Rat | Irrigation | Ibuprofen vs. *lovastatin* high dose vs. *lovastatin* low dose vs. TCM *Monascus purpureus* Went low dose vs. TCM *Monascus purpureus* Went medium dose vs. TCM *Monascus purpureus* Went high dose vs. blank control | 30 mg/kg, 3.6 mg/kg, 1.8 kg/mg, 200 mg/kg, 100 mg/kg, and 50 mg/kg | 5 days | ⑲⑳㉑ |
| Wang et al. (2006b) | Small ear swelling | Rat | Irrigation | TCM *Monascus purpureus* Went low dose vs. TCM *Monascus purpureus* Went medium dose vs. TCM *Monascus purpureus* Went high dose vs. *lovastatin* high dose vs. *lovastatin* low dose vs. blank control | 300 mg/kg, 150 mg/kg, 75 mg/kg, 5.2 mg/kg, 2.6 mg/kg | 5 days | ㉒ |
| Wu et al. (2006) | Osteoporosis | Rat | Irrigation | TCM *Monascus purpureus* Went vs. Pravastatin vs. Premarin vs. blank control | 10 mL/kg | 10 days | ⑮ |
| Ding (2007) | Tumor | Rat | Irrigation | *Monascus purpureus* Went polysaccharide low dose vs. *Monascus purpureus* Went polysaccharide medium dose vs. *Monascus purpureus* Went polysaccharide high dose vs. blank control | 100, 400, and 800 mg/kg | 14 days | ㉓㉔ |
| Lei et al. (2007) | Hypertension | Rat | Irrigation | TCM *Monascus purpureus* Went high dose vs. TCM *Monascus purpureus* Went low dose vs. positive vs. blank control | 0.83 g/kg, 0.42 g/kg, and 10 mg/kg | 28 days | ⑫⑬㉕㉖ |
| Wang et al. (2007) | Hyperlipemia | Rat | Irrigation | *Monascus purpureus* Went compound preparation high dose vs. *Monascus purpureus* Went compound preparation medium dose vs. *Monascus purpureus* Went compound preparation high dose vs. blank control | 0.47 g/kg, 0.23 g/kg, and 0.12 g/kg | 30 days | ①②③ |
| Wu et al. (2007a) | Fracture | Rat | Irrigation | TCM *Monascus purpureus* Went vs. bone-knitting tablet vs. blank control | 10 mL/kg | 42 days | ⑮㉗ |
| Wu et al. (2007b) | Fracture | Rat | Irrigation | TCM *Monascus purpureus* Went vs. blank control | 10 mL/kg | 42 days | ㉘ |
| Zheng et al. (2007) | Hypertension | Rat | Irrigation | TCM *Monascus purpureus* Went low dose vs. TCM *Monascus purpureus* Went medium dose vs. TCM *Monascus purpureus* Went high dose vs. captopril vs. indapamide vs. blank control | 0.25 g/BW/day, 0.42 g/BW/day, 0.84 g/BW/day, 10 mg/BW/day, and 0.21 mg/BW/day | 28 days | ⑧⑫⑬㉕㉖ |
| Jiang et al. (2008) | Hyperlipemia | Rat | Irrigation | Mixture of *Monascus purpureus* Went and grape seed *anthocyanidin* low dose vs. mixture of *Monascus purpureus* Went and grape seed *anthocyanidin* medium dose vs. mixture of *Monascus purpureus* Went and grape seed *anthocyanidin* high dose vs. blank control | 12 mg/mL, 120 mg/mL, and 360 mg/mL | 70 days | ①②③⑤ |
| Lu et al. (2008) | osteoporosis | Rat | Irrigation | TCM *Monascus purpureus* Went vs. pravastatin vs. Premarin vs. blank control | 10 mL/kg | 10 days | ⑯⑱㉙ |

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TABLE 2 (*Continued*) Information on animal studies related to *Monascus purpureus* Went.

| Inclusion of studies | Disease | Model | Method of administration | Intervention | Dosage | Course of treatment | Outcome indicator |
|---|---|---|---|---|---|---|---|
| Wang et al. (2008) | Arthritis | Rat | Irrigation | TCM *Monascus purpureus* Went vs. indomethacin vs. blank control | 500 mg·kg$^{-1}$ and 5 mg·kg$^{-1}$ | 34 days | ㉚㉛㉜ |
| Chen et al. (2010) | Hyperlipemia | Rat | Irrigation | Compounded *Monascus purpureus* Went extract low dose vs. compounded *Monascus purpureus* Went extract medium dose vs. compounded *Monascus purpureus* Went extract high dose vs. lovastatin vs. blank control | 0.6, 1.2, and 2.4 g·kg$^{-1}$ and 0.24 g·kg$^{-1}$ | 21 days | ①②③④ |
| Luo et al. (2010) | Non-alcoholic fatty liver | Rat | Irrigation | TCM *Monascus purpureus* Went low dose vs. TCM *Monascus purpureus* Went medium dose vs. TCM *Monascus purpureus* Went high dose vs. *Gynostemma Blume* vs. blank control | 1 g/(kg · day), 0.33 g/(kg · day), and 0.17 g/(kg · day) | 56 days | ㉟㊱㊲ |
| Luo and Zhang (2011) | Non-alcoholic fatty liver | Rat | Irrigation | TCM *Monascus purpureus* Went low dose vs. TCM *Monascus purpureus* Went medium dose vs. TCM *Monascus purpureus* Went high dose vs. *Gynostemma Blume* vs. blank control | 1 g/(kg · day), 0.33 g/(kg · day), and 0.17 g/(kg · day) | 56 days | ①②④㉝㉞ |
| Zhao and Liu (2011) | Hyperlipemia | Rat | Irrigation | *Monascus purpureus* Went–phytosterol ester compound preparation low dose vs. *Monascus purpureus* Went–phytosterol ester compound preparation medium dose vs. *Monascus purpureus* Went–phytosterol ester compound preparation high dose vs. Xuezhikang capsules vs. blank control | 0.167, 0.333, and 1.0 g/kg | 45 days | ①②③④ |
| Zhou et al. (2011) | Tumor | Rat | Irrigation | *Monascus purpureus* Went polysaccharide low dose vs. *Monascus purpureus* Went polysaccharide medium dose vs. *Monascus purpureus* Went polysaccharide high dose vs. Tremella polysaccharide vs. blank control | 200, 100, and 50 mg/(kg · day) and 50 mg/(kg · day) | 14 days | ㉓㉔㊳ |
| Ou-yang et al. (2012) | Hyperlipemia | Rat | Irrigation | *Fagopyrum esculentum* Moench *Monascus purpureus* Went powder high dose vs. *Fagopyrum esculentum* Moench *Monascus purpureus* Went powder medium dose vs. *Fagopyrum esculentum* Moench *Monascus purpureus* Went powder small dose vs. Xuezhikang capsule vs. blank control | 1,200, 600, and 300 mg/kg and 400 mg/kg | 14 days | ①②③④ |
| Zhai et al. (2012) | Hyperlipemia | Rat | Irrigation | *Monascus purpureus* Went compound preparation low dose vs. *Monascus purpureus* Went compound preparation medium dose vs. *Monascus purpureus* Went compound preparation high dose vs. Xuezhikang capsule vs. blank control | 0.21 g·kg$^{-1}$BW, 0.42 g·kg-1BW, 1.25 g·kg$^{-1}$BW, –0.2 g·kg$^{-1}$BW | 30 days | ①②③④ |
| Du and Chen (2013) | Safety evaluation | Rat | Irrigation | TCM *Monascus purpureus* Went vs. blank control | 15 g/kg | 14 days | ㊴ |

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TABLE 2 (*Continued*) Information on animal studies related to *Monascus purpureus* Went.

| Inclusion of studies | Disease | Model | Method of administration | Intervention | Dosage | Course of treatment | Outcome indicator |
|---|---|---|---|---|---|---|---|
| Liu et al. (2013) | Hyperlipemia | Rat | Irrigation | Compounded *Monascus purpureus* Went capsule low dose vs. compounded *Monascus purpureus* Went capsule medium dose vs. compounded *Monascus purpureus* Went capsule high dose vs. blank control | 167, 333, and 1,000 mg/kg | 30 days | ①②③ |
| Lin et al. (2014) | Hyperlipemia | Rat | Irrigation | TCM *Monascus purpureus* Went vs. blank control | 10 mL/kg | 28 days | ①②③④㊵㊶ |
| Su et al. (2014) | Hyperlipemia | Rat | Irrigation | Xuezhikang capsule vs. blank control | 500 $mg{\cdot}kg^{-1}{\cdot}day^{-1}$ | 14 days | ㊷ |
| Zhou (2014) | Hyperlipemia | Rat | Irrigation | TCM *Monascus purpureus* Went low dose vs. TCM *Monascus purpureus* Went medium dose vs. TCM *Monascus purpureus* Went high dose vs. blank control | 0.5, 1.0, and 2.0 g/kg | 28 days | ①②③④ |
| Gao et al. (2015) | Hyperlipemia | Rat | Irrigation | TCM *Monascus purpureus* Went combined with Fang Feng Tong Sheng powder (Chinese patent medicine) vs. Fang Feng Tong Sheng powder vs. blank control | 1 mL/time and 2 times/day | 30 days | ①②③④ |
| Qian et al. (2015) | Osteoporosis | Rat | Irrigation | TCM *Monascus purpureus* Went low dose vs. TCM *Monascus purpureus* Went medium dose vs. TCM *Monascus purpureus* Went high dose vs. *fluvastatin* vs. blank control | (0.1, 0.5, and 1.0 g/mL) 10 mL/kg and (0.1 g/L) 10 mL/kg | 35 days | ㊸ |
| Lu et al. (2016) | Osteoporosis | Rat | Irrigation | *Monascus purpureus* Went capsule-containing coenzyme Q10 vs. diethylstilbestrol vs. blank control | 0.5 tablet $\cdot kg^{-1}$ and 30 $\mu g{\cdot}kg^{-1}$ | 60 days | ⑰㊹ |
| Lu et al. (2017) | Osteoporosis | Rat | Irrigation | TCM *Monascus purpureus* Went vs. estrogen vs. blank control | 10 mL/kg | 84 days | ⑰㉙㉛ |
| Pang et al. (2017) | Hyperlipemia | Rat | Irrigation | Natto (*Glycine max*) *Monascus purpureus* Went low dose vs. natto (*Glycine max*) *Monascus purpureus* Went medium dose vs. natto (*Glycine max*) *Monascus purpureus* Went high dose vs. blank control | 0.2, 0.4, and 0.8 g/kg BW | 30 days | ①②③④ |
| Zhang et al. (2017) | Safety evaluation | Rat | Irrigation | *Monascus purpureus* Went polysaccharide low dose vs. *Monascus purpureus* Went polysaccharide high dose vs. blank control | 100 mg/(kg · day) and 300 mg/(kg · day) | 21 days | ㉔㉝㉞㉟㊴ |
| Shen et al. (2018) | Osteoporosis | Rat | Irrigation | TCM *Monascus purpureus* Went vs. estrogen vs. blank control | 10 mL/kg | 56 days | ⑪㊺ |
| Zhou et al. (2018) | Hyperlipemia | rabbit | Irrigation | Sea-buckthorn (*Hippophae rhamnoides* L.) *Monascus purpureus* Went capsule high dose vs. sea-buckthorn (*Hippophae rhamnoides* L.) *Monascus purpureus* Went capsule low dose vs. simvastatin vs. blank control | 3.6 g/kg, 1.8 g/kg, and 0.005 g/kg | 15 days | ①②③④ |
| Huang et al. (2019) | Hyperlipemia | Rat | Irrigation | *Monascus purpureus* Went bee glue tablet low dose vs. *Monascus purpureus* Went bee glue tablet medium dose vs. *Monascus purpureus* Went bee glue tablet high dose vs. blank control | 0.17, 0.33, and 1.00 g/kg | 42 days | ①②④ |

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TABLE 2 (*Continued*) Information on animal studies related to *Monascus purpureus* Went.

| Inclusion of studies | Disease | Model | Method of administration | Intervention | Dosage | Course of treatment | Outcome indicator |
|---|---|---|---|---|---|---|---|
| Liu et al. (2019) | Hyperlipemia | Rat | Irrigation | Yellow Monascus pigment low dose vs. yellow Monascus pigment medium dose vs. yellow Monascus pigment high dose vs. SIM vs. blank control | 50, 100, and 200 mg/(kg · day) and 15 mg/(kg · day) | 28 days | ①②㉝㉞ |
| Luo et al. (2019) | Syndrome of food retention due to spleen deficiency | Rat | Irrigation | TCM *Monascus purpureus* Went unleavened vs. TCM *Monascus purpureus* Went fermented vs. domperidone vs. blank control | 1.17 $g{\cdot}kg^{-1}{\cdot}day^{-1}$, 1.17 $g{\cdot}kg^{-1}{\cdot}day^{-1}$, and 3.9 $mg{\cdot}kg^{-1}{\cdot}day^{-1}$ | 10 days | ㊻ |
| Wang (2019) | Hyperlipemia | Rat | Irrigation | TCM *Monascus purpureus* Went low dose vs. TCM *Monascus purpureus* Went medium dose vs. TCM *Monascus purpureus* Went high dose vs. blank control | 5 mg/kg, 30 mg/kg, and 90 mg/kg | 31 days | ①② |
| Liu et al. (2020) | Hyperlipemia | Rat | Irrigation | *Monascus purpureus* Went *Poria cocos* (Schw.) Wolf tablet low dose vs. *Monascus purpureus* Went *Poria cocos* (Schw.) Wolf tablet medium dose vs. *Monascus purpureus* Went *Poria cocos* (Schw.) Wolf tablet high dose vs. Xuezhikang capsule vs. blank control | 0.79 g/kg, 1.58 g/kg, 4.75 g/kg, and 0.2 g/kg | 28 days | ①②③④㊻ |
| Liu et al. (2021) | Hyperlipemia | Rat | Irrigation | *Ginkgo biloba* L. *Monascus purpureus* Went vitamin grouping (low dose) vs. *Ginkgo biloba* L. *Monascus purpureus* Went vitamin grouping (high dose) vs. positive | 25 mg/kg and 50 mg/kg | 32 days | ①②④ |
| Sun et al. (2022) | Cerebral small vessel disease | Rat | Irrigation | TCM *Monascus purpureus* Went low dose vs. TCM *Monascus purpureus* Went medium dose vs. TCM *Monascus purpureus* Went high dose vs. nimodipine vs. blank control | 0.75 $g{\cdot}kg^{-1}$, 1.5 $g{\cdot}kg^{-1}$, 3 $g{\cdot}kg^{-1}$, and 20 $mg{\cdot}kg^{-1}$ | 30 days | ㊼ |
| Ma et al. (2022) | Safety evaluation | Rat | Irrigation | *Panax Notoginseng* (Burk.) F.H.Chen and *Monascus purpureus* Went compound preparation high dose vs. *Panax Notoginseng* (Burk.) F.H.Chen and *Monascus purpureus* Went compound preparation medium dose vs. *Panax Notoginseng* (Burk.) F.H.Chen and *Monascus purpureus* Went compound preparation low dose vs. blank control | 2, 4, and 8 $g{\cdot}(kg{\cdot}day)^{-1}$ and 40 mg/kg | 14 days | ㊽ |
| Zheng et al. (2022) | Hyperlipemia | Rat | Irrigation | TCM *Monascus purpureus* Went low dose vs. TCM *Monascus purpureus* Went medium dose vs. TCM *Monascus purpureus* Went high dose vs. *lovastatin* vs. blank control | 0.09 mg/kg, 0.16 mg/kg, 0.21 mg/kg, and 0.21 mg/kg | 42 days | ①②③④㉝㉞ |
| Ding and Ren (2023) | Hyperlipemia | Rat | Irrigation | *Monascus purpureus* Went earthworm (*Pheretima*) protein tablet low dose vs. *Monascus purpureus* Went earthworm (*Pheretima*) protein tablet medium dose vs. *Monascus purpureus* Went earthworm (*Pheretima*) protein tablet high dose vs. *Simvastatin* vs. blank control | 0.167 g/kg, 0.333 g/kg, 0.666 g/kg, and 0.01 g/kg/d | 28 days | ①②③④ |

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TABLE 2 (*Continued*) Information on animal studies related to *Monascus purpureus* Went.

| Inclusion of studies | Disease | Model | Method of administration | Intervention | Dosage | Course of treatment | Outcome indicator |
|---|---|---|---|---|---|---|---|
| Yu et al. (2023) | Non alcoholic steatohepatitis | Rat | Irrigation | *Monascus purpureus* Went–*Crataegus pinnatifida* Bunge high dose vs. *Monascus purpureus* Went–*Crataegus pinnatifida* Bunge low dose vs. PPC vs. blank control | 1.323 g·(kg·day)$^{-1}$, 2.646 g·(kg·day)$^{-1}$, and 0.086 g·(kg·day)$^{-1}$ | 24 days | ①②④㉛㉝㉞ |
| Zhang et al. (2023) | Non-alcoholic fatty liver | Rat | Irrigation | *Coptis chinensis* Franch. *Monascus purpureus* Went medicine high dose vs. *Coptis chinensis* Franch. *Monascus purpureus* Went medicine low dose vs. obeticholic acid vs. blank control | 2.1 g/kg, 1.1 g/kg, and 0.5 mg/kg | 28 days | ①②④⑥㉝㉚㉟㊲ |

Note: ①, total cholesterol (TC) level; ②, triglyceride (TG) level; ③, high-density lipoprotein cholesterol (HDL-C) level; ④, low-density lipoprotein cholesterol (LDL-C) level; ⑤, plasma oxygen-free radical (SOD, MDA, and GSH Px) level; ⑥, ApoA level; ⑦, ApoB level; ⑧, systolic blood pressure (SBP); ⑨, bone gla protein (BGP) level; ⑩, calcitonin (CT) level; ⑪, bone density; ⑫, endothelin (ET) level; ⑬, calcitonin gene-related peptide (CGRP) level; ⑭, atherosclerotic index (AI); ⑮, BMP-2 level; ⑯, osteoblast number; ⑰, bone biomechanical properties; ⑱, number of mineralized nodules; ⑲, white blood cell (WBC) count; ⑳, C-reactive protein (CRP) level; ㉑, malondialdehyde (MDA) level; ㉒, degree of swelling; ㉓, tumor suppression Rate; ㉔, organ index; ㉕, lung tissue ACE level; ㉖, aortic NOS level; ㉗, Nilsson's histologic score; ㉘, bone tissue morphometric parameters; ㉙, ALP level; ㉚, arthritis index (AI) and pathology score; ㉛, serum TNF-α levels; ㉜, synovial MCP-1 and RANTES levels; ㉝, ALT levels; ㉞, AST levels; ㉟, blood glucose levels; ㊱, GIR levels; ㊲, insulin levels; ㊳, relative growth rate; ㊴, body weight and liver weight; ㊵, serum adiponectin levels; ㊶, AdipoR1/AdipoR2 mRNA expression levels; ㊷, EPC proliferation, adhesion, migration, and *in vitro* angiogenesis ability; ㊸, BMP-4 mRNA and protein expression levels in bone scab tissues; ㊹, bone calcium, bone phosphorus, and hydroxyproline content; ㊺, bone tissue RANKL, OPG protein, and mRNA expression levels; ㊻, serum levels of motilin (MTL), gastrin (GAS), 5-hydroxytryptamine (5-HT), and vasoactive intestinal peptide (VIP); ㊼, PI3K/AKT/mTOR protein expression levels; ㊽, cell micro-nucleus breakage, deletion, reciprocal translocation, circular chromosome, monovalent body, and cell aberration rate.

inflammation, involving TCM *Monascus purpureus* Went (n = 3); three studies showed a good safety profile of *Monascus purpureus* Went, involving *Monascus purpureus* Went polysaccharide (n = 1), *Monascus purpureus* Went extract (n = 1), and *Panax Notoginseng* (Burk.) F.H.Chen *Monascus purpureus* Went compound preparation (n = 1); two studies showed that *Monascus purpureus* Went had an effect on tumor inhibition, involving *Monascus purpureus* Went polysaccharide (n = 2); one study showed that *Monascus purpureus* Went had an effect on cerebrovascular disease, involving TCM *Monascus purpureus* Went (n = 1); one study showed that *Monascus purpureus* Went had an effect on regulating the gastrointestinal tract, involving TCM *Monascus purpureus* Went (n = 1); and one study showed that *Monascus purpureus* Went had an effect on treating renal disease, involving *Monascus purpureus* Went extract (n = 1).

Among these studies, the most commonly used dose was 0.625 g/mL of aqueous *Monascus purpureus* Went solution, administered via irrigation at 10 mL/kg, and the most commonly used course of treatment was 28 days. The specific information on animal studies is presented in Table 2.

All 58 animal studies were randomized controlled studies. Among them, 55 studies only reported "randomization," and 3 used the random number table method. A total of 58 studies reported that the experimental group was consistent with the control group at baseline. Fifty-seven studies did not report allocation concealment and blinding, and only one blinded the investigator and the animal keeper. Forty-two studies did not report whether the environments in which the animals were placed were randomized or not, and 16 studies reported that the animals were grown in the same environments; no missing data appeared. The risk of bias assessment of animal studies is presented in Table 3.

### 3.3.3 Cell study

Fifteen cellular studies related to *Monascus purpureus* Went were included. Seven studies focused on the identification and screening of *Monascus purpureus* Went bacterial strains using cell biology techniques. Six studies determined the efficacy of *Monascus purpureus* Went, including the synthesis and *in vitro* anticancer activity of *Monascus purpureus* Went derivatives (n = 1), the blood pressure-lowering mechanism of *Monascus purpureus* Went (n = 1), the effect of *Monascus purpureus* Went on the secretion of TNF-alpha by peripheral blood single-nucleated cells of ankylosing spondylitis patients (n = 1), the effect of erythrocytes on the cell growth and molecular mechanisms of HCT-116 cells (n = 1), the effect of *Monascus purpureus* Went on the proliferation of myocardial fibroblasts (n = 1), and the role of *Monascus purpureus* Went in inducing apoptosis and autophagy in human colon cancer cells (n = 1).

Two studies examined the physiological characteristics of *Monascus purpureus* Went and *Monascus purpureus* Went (n = 1) and the morphological characteristics of TCM *Monascus purpureus* Went (n = 1).

## 3.4 Clinical trial

A total of 28 clinical trials studied the effects of TCM *Monascus purpureus* Went and its related Chinese patent medicine and

preparation, including double-armed trials (n = 18), single-armed trials (n = 6), three-armed trials (n = 3), and multiple-armed trials (n = 1). One study was set up with a blank control, and four were set up with a placebo. The mode of administration was oral. Patient sources included tertiary hospitals (n = 19), secondary hospitals (n = 7), and other sources (n = 2). Diseases included dyslipidemia (n = 22), carotid atherosclerotic plaque (n = 3), unstable angina (n = 2), abnormal liver function (n = 2), hyperglycemia (n = 1), type 2 diabetes (n = 1), uremia (n = 1), and fatty liver (n = 1).

Among them, 19 studies examined the effects of Chinese patent medicines and preparations related to *Monascus purpureus* Went, including Xuezhikang capsule (n = 6), Zhibituo capsule (n = 2), Shengqu *Monascus purpureus* Went capsule (n = 2), lipid-lowering *Monascus purpureus* Went micro-powder (n = 2), *Monascus purpureus* Went compound preparation (n = 1), compound *Monascus purpureus* Went capsule (n = 1), *Monascus purpureus* Went capsule for reducing sugar (n = 1), *Monascus purpureus* Went flavonoid tablet (n = 1), rattan *Monascus purpureus* Went soft capsule (n = 1), Danxi *Monascus purpureus* Went wine (n = 1), and *C. chinensis* Franch. *Monascus purpureus* Went medicine (n = 1); nine studies determined the clinical therapeutic effect of TCM *Monascus purpureus* Went.

Among them, the most commonly used dose of TCM *Monascus purpureus* Went was 6 g/dose, administered once daily, with a treatment course of 90 days; the most commonly used dose of Xuezhikang capsule was 2 capsules/dose, administered twice daily, and the course of treatment was 56 days; the most commonly used dose of Zhibituo capsule was 1.05 g/dose, administered thrice daily, with a treatment course of 60 or 90 days; the most commonly used dose of Shengqu *Monascus purpureus* Went capsule was 2 capsules/dose or 4 capsules/dose, and the course of treatment was 84 or 168 days; and the most commonly used dose of lipid-lowering *Monascus purpureus* Went micro-powder was 1 capsule/dose, administered thrice daily, with a treatment course of 50 or 180 days.

In terms of indicator domains, physical and chemical testing indicators were applied 28 times, symptom and sign indicators were applied 13 times, and TCM symptom/syndrome indicators were applied six times.

For counting indicators, clinical efficacy was applied 14 times, and TCM evidence efficacy was applied four times. Measurement indicators included lipid levels (n = 25), liver and kidney functions (n = 6), blood routine (n = 2), TCM evidence points (n = 2), blood glucose levels (n = 2), body mass index (n = 2), serum inflammatory factor (n = 2), electrocardiography (n = 1), SGA scores (n = 1), and carotid ultrasound (n = 1). The specific information on clinical trials is presented in Table 4.

A total of 21 studies were randomized controlled trials (79%), and six were self-controlled trials (21%). Among the 22 randomized controlled trials, only 15 reported "randomization"; six used a randomized numeric table method, and one used lottery method. Only one study performed allocation concealment, four studies blinded patients, and one study blinded outcome assessors. The remaining studies did not perform allocation concealment or apply blinding; no missing data were reported. Thirteen studies exhibited other sources of bias, such as not reporting the source of funding or trial enrollment, while nine studies provided complete reporting. The risk of bias assessment of clinical trials is presented in Table 5.

# 4 Discussion and analysis

## 4.1 Content determination method of active ingredients in *Monascus purpureus* Went

### 4.1.1 High-performance liquid chromatography

HPLC is the most commonly used method to determine the content of active ingredients in *Monascus purpureus* Went, particularly *lovastatin* and citrinin (*orange mold*) (Wang and Gao, 2006; Wen et al., 2011; Hao et al., 2017; Tan et al., 2015; Qiu et al., 2012; Luo et al., 2003; Zhang et al., 2016; Lu et al., 2019; Zhang et al., 1997; Liu, 2007; Fan, 2013; Zhang et al., 2001; Chen and Zhao, 2007; Zhu et al., 2023; Huang, 2000; Chen et al., 2008; Wang, 2014; Gao et al., 2022; Li et al., 2011; Wang, 2000; Song et al., 1999b; Xie et al., 2010; Huang et al., 2014; Wang et al., 2020; Li and Li, 2008; Lv et al., 2020; Li et al., 2010; Li et al., 2019; Qi et al., 2021), and is characterized by simplicity, accuracy, reliability, good repeatability, and high sensitivity.

### 4.1.2 Thin-layer chromatography scan

TLCS is a method used to determine the content of phosphatidylcholine and daidzein in *Monascus purpureus* Went and Xuezhikang capsule (Xu et al., 2000; Xu et al., 2001), which is characterized by simplicity and good repeatability and can be used to control the quality of *Monascus purpureus* Went and its preparations.

### 4.1.3 Capillary electrophoresis

CE is a method used to determine the contents of *lovastatin* and citrinin (a compound produced by *orange mold*) in *Monascus purpureus* Went (Chen et al., 2007a; Zhang et al., 2008), which is characterized by simplicity, speed, high sensitivity, and good repeatability in detecting certain charged components.

### 4.1.4 Other methods

AA is a method used to determine the content of amino acids in *Monascus purpureus* Went (Chen et al., 2007b), which is characterized by high sensitivity and accuracy. GC is a method used to determine the contents of oleic acid and linoleic acid in *Monascus purpureus* Went (Zhang et al., 2010). QuEChERS-UPLC-MRM-IDA Criteria-EPI is a method used to determine and quantify the content of *lovastatin* (Wang, 2016). PSA, ASA, and DNS are methods used to determine the content of polysaccharides in *Monascus purpureus* Went (Fang et al., 2021). FAAS is a method used to determine the content of metal trace elements in *Monascus purpureus* Went (Lin et al., 2001). SPSS PCA and SPSS CA were used to analyze trace elements in *Monascus purpureus* Went (Cao and Wu, 2009) to reveal the relationships and distribution patterns between components.

## 4.2 Chemical ingredients of *Monascus purpureus* Went

A variety of active ingredients in *Monascus purpureus* Went provide the material basis for the pharmacological effect of *Monascus purpureus* Went, mainly including Monascus pigment,

monacolin K, ergosterol, stigmasterol, *Monascus purpureus* Went polysaccharide, and a variety of enzymes.

### 4.2.1 Monascus pigment

Monascus pigment is a secondary metabolite of *Monascus purpureus* Went. Monascus pigment not only provides a unique color for *Monascus purpureus* Went but also possesses physiological activities such as antioxidant, antibacterial, and anti-inflammatory properties. So far, as many as 54 types of Monascus pigment have been identified, among which the more intensively studied pigments include yellow Monascus pigment, ankaflavin, rubropunctamine, and monascorubramine. It has been demonstrated that yellow Monascus pigment has a protective effect on the liver of hyperlipemia mice and can regulate blood lipids, and the mechanism of action may be related to the regulation of lipid metabolism and activation of the AMP-activated protein kinase (AMPK) pathway to stimulate fatty acid oxidation (Fang et al., 2021). Monascorubramine can promote the apoptosis of gastric cancer AGS cells, while no obvious inhibitory effect on normal cells was observed, and its therapeutic coefficient is higher than that of paclitaxel, which is a conventional chemotherapeutic drug for gastric cancer (Lin et al., 2001). The safety of Monascus pigment has been proven to be high through acute and chronic toxicity studies, and it has been widely used as an additive ingredient of Monascus pigment in food and cosmetic production processes (Jiang et al., 2021; Xu et al., 2018; Pan et al., 2023).

### 4.2.2 Statin ingredients

The statin component of *Monascus purpureus* Went has a wide range of applications in the field of medicine. In the late 1970s, Japanese scientists discovered and isolated a chemical component called monacolin K from the fermentation of *Monascus purpureus* Went, which can inhibit cholesterol synthesis in the body (Kong et al., 2005). Further studies revealed that the statin component in *Monascus purpureus* Went is similar to chemically synthesized statins in terms of its lipid-lowering effect. Among them, *lovastatin*, the most common statin component in *Monascus purpureus* Went, was formally approved by the FDA in the United States in 1987 and became the first generation of statin lipid-lowering drugs. In addition, *lovastatin* has an anti-tumor effect, which can induce the activation of the key molecule of apoptosis, caspase 7, and its receptor PARP protein cleavage. *Lovastatin* can inhibit the proliferation of PC3 cells and induce apoptosis in prostate cancer and has been shown to be efficacious in common tumors, such as gastric cancer, carcinoma of the bile duct, and nasopharyngeal carcinoma (NPC) (Xu et al., 2018).

### 4.2.3 Sterol composition

*Monascus purpureus* Went produces a variety of sterol components during the fermentation process, such as ergosterol and stigmasterol. Ergosterol is one of the precursor substances of vitamin D2, which can be converted into vitamin D2 after ultraviolet irradiation, and is involved in the metabolism of calcium and phosphorus in the body, which has an obvious effect on the prevention and treatment of rickets in infants and young children and the promotion of calcium and phosphorus absorption in pregnant women and the elderly. Studies have shown that ergosterol can significantly reduce the blood glucose level of diabetic nephropathy model mice, providing a theoretical basis for ergosterol to be used in the clinical treatment of diabetic nephropathy (Xu et al., 2018). Soysterol can competitively inhibit the absorption of cholesterol in the human body and effectively reduce the level of serum cholesterol, which is an important active ingredient in regulating lipid balance and preventing cardiovascular and cerebrovascular diseases (Ge et al., 2012).

### 4.2.4 Other ingredients

*Monascus purpureus* Went contains a variety of other active ingredients, such as *Monascus purpureus* Went polysaccharide, unsaturated fatty acids, a variety of enzymes (e.g., amylase, protease, and lipase), and flavonoids, which also play important roles in the pharmacological effects of *Monascus purpureus* Went.

For example, *Monascus purpureus* Went polysaccharides exhibit various physiological activities, such as immunoregulatory, anti-tumor, and lipid-lowering effects; unsaturated fatty acids help lower blood lipids and prevent cardiovascular diseases; and a variety of enzymes promote digestion and absorption of food in the human body.

## 4.3 Pharmacological mechanism of action of *Monascus purpureus* Went

### 4.3.1 Lipid-lowering ability

*Monascus purpureus* Went has a lipid-lowering effect. This is mainly attributed to the enrichment of statins in *Monascus purpureus* Went, such as monacolin K, which is the active ingredient of *lovastatin.* A number of included clinical trials have shown (Yin et al., 2016; Zhang and He, 2016; Chen et al., 2021; Luo and Zhong, 2020; Zhang et al., 2020; Zhao et al., 2018; Shi et al., 2015; Gao et al., 2016) that *Monascus purpureus* Went has a lipid-lowering effect, generally reducing plasma total cholesterol (TC) levels, low-density lipoprotein (LDL) levels, and triglyceride (TG) levels and also increasing high-density lipoprotein (HDL) levels.

### 4.3.2 Oxidation resistance

Antioxidant components such as polyphenolic compounds and flavonoids in *Monascus purpureus* Went can scavenge free radicals in the body and reduce cellular damage caused by oxidative stress. Studies have shown that the extracellular polysaccharides of *Monascus purpureus* Went have the ability to scavenge DPPH-free radicals, confirming the antioxidant effect of *Monascus purpureus* Went (Cai et al., 2010). This antioxidant effect helps slow down the cellular aging process and protects the cardiovascular system, the liver, and other organs from oxidative damage. In addition, the antioxidant effect of *Monascus purpureus* Went is complemented by its lipid-lowering effect, which works together to maintain the healthy state of the human body. Included clinical trials have shown that *Monascus purpureus* Went can reduce ALT and AST levels in patients with hyperlipemia and liver function abnormalities, thus protecting the liver.

### 4.3.3 Anti-inflammatory action

*Monascus purpureus* Went has an anti-inflammatory effect, which is closely related to the various anti-inflammatory components it contains. Polyphenols and flavonoids in *Monascus*

TABLE 3 Risk of bias assessment of animal studies.

| Included in the study | ① | ② | ③ | ④ | ⑤ | ⑥ | ⑦ | ⑧ | ⑨ | ⑩ |
|---|---|---|---|---|---|---|---|---|---|---|
| Yan (1999) | Unclear | Low risk | Unclear | Unclear | Unclear | Unclear | Unclear | Low risk | Low risk | Unclear |
| Wang et al. (2000) | Unclear | Low risk | Unclear | Unclear | Unclear | Unclear | Unclear | Low risk | Low risk | High risk |
| Yu et al. (2000) | Unclear | Low risk | Unclear | Unclear | Unclear | Unclear | Unclear | Low risk | Low risk | Unclear |
| Sun et al. (2001a) | Unclear | Low risk | Unclear | Unclear | Unclear | Unclear | Unclear | Low risk | Low risk | Unclear |
| Sun et al. (2001b) | Unclear | Low risk | Unclear | Unclear | Unclear | Unclear | Unclear | Low risk | Low risk | Unclear |
| Wang et al. (2002) | Unclear | Low risk | Unclear | Unclear | Unclear | Unclear | Unclear | Low risk | Low risk | Unclear |
| He (2004) | Unclear | Low risk | Unclear | Unclear | Unclear | Unclear | Unclear | Low risk | Low risk | Unclear |
| Lu et al. (2004) | Unclear | Low risk | Unclear | Low risk | Unclear | Unclear | Unclear | Low risk | Low risk | Unclear |
| Tang et al. (2004) | Unclear | Low risk | Unclear | Unclear | Unclear | Unclear | Unclear | Low risk | Low risk | Unclear |
| Chen et al. (2005) | Unclear | Low risk | Unclear | Unclear | Unclear | Unclear | Unclear | Low risk | Low risk | High risk |
| Lu et al. (2005a) | Unclear | Low risk | Unclear | Low risk | Unclear | Unclear | Unclear | Low risk | Low risk | High risk |
| Lu et al. (2005b) | Unclear | Low risk | Unclear | Low risk | Unclear | Unclear | Unclear | Low risk | Low risk | High risk |
| Wang et al. (2005) | Unclear | Low risk | Unclear | Low risk | Unclear | Unclear | Unclear | Low risk | Low risk | Unclear |
| Lu et al. (2006) | Unclear | Low risk | Unclear | Low risk | Unclear | Unclear | Unclear | Low risk | Low risk | High risk |
| Wang et al. (2006a) | Unclear | Low risk | Unclear | Unclear | Unclear | Unclear | Unclear | Low risk | Low risk | Unclear |
| Wang et al. (2006b) | Unclear | Low risk | Unclear | Unclear | Unclear | Unclear | Unclear | Low risk | Low risk | Unclear |
| Wu et al. (2006) | Unclear | Low risk | Unclear | Low risk | Unclear | Unclear | Unclear | Low risk | Low risk | High risk |
| Ding (2007) | Unclear | Low risk | Unclear | Unclear | Unclear | Unclear | Unclear | Low risk | Low risk | High risk |
| Lei et al. (2007) | Unclear | Low risk | Unclear | Unclear | Unclear | Unclear | Unclear | Low risk | Low risk | Unclear |
| Wang et al. (2007) | Unclear | Low risk | Unclear | Unclear | Unclear | Unclear | Unclear | Low risk | Low risk | Unclear |
| Wu et al. (2007a) | Low risk (random digital table method) | Low risk | Unclear | Unclear | Unclear | Unclear | Unclear | Low risk | Low risk | High risk |
| Wu et al. (2007b) | Low risk (random digital table method) | Low risk | Unclear | Unclear | Unclear | Unclear | Unclear | Low risk | Low risk | High risk |
| Zheng et al. (2007) | Unclear | Low risk | Unclear | Unclear | Unclear | Unclear | Unclear | Low risk | Low risk | Unclear |
| Jiang et al. (2008) | Unclear | Low risk | Unclear | Low risk | Unclear | Unclear | Unclear | Low risk | Low risk | Unclear |
| Lu et al. (2008) | Unclear | Low risk | Unclear | Unclear | Unclear | Unclear | Unclear | Low risk | Low risk | Unclear |

(Continued on following page)

TABLE 3 (*Continued*) Risk of bias assessment of animal studies.

| Included in the study | ① | ② | ③ | ④ | ⑤ | ⑥ | ⑦ | ⑧ | ⑨ | ⑩ |
|---|---|---|---|---|---|---|---|---|---|---|
| Wang et al. (2008) | Unclear | Low risk | Unclear | Unclear | Unclear | Unclear | Unclear | Low risk | Low risk | High risk |
| Chen et al. (2010) | Unclear | Low risk | Unclear | Unclear | Unclear | Unclear | Unclear | Low risk | Low risk | Unclear |
| Luo et al. (2010) | Unclear | Low risk | Unclear | Unclear | Unclear | Unclear | Unclear | Low risk | Low risk | Unclear |
| Luo and Zhang (2011) | Unclear | Low risk | Unclear | Unclear | Unclear | Unclear | Unclear | Low risk | Low risk | Unclear |
| Zhao and Liu (2011) | Unclear | Low risk | Unclear | Low risk | Unclear | Unclear | Unclear | Low risk | Low risk | Unclear |
| Zhou et al. (2011) | Unclear | Low risk | Unclear | Unclear | Unclear | Unclear | Unclear | Low risk | Low risk | High risk |
| Ou-yang et al. (2012) | Unclear | Low risk | Unclear | Unclear | Unclear | Unclear | Unclear | Low risk | Low risk | Unclear |
| Zhai et al. (2012) | Unclear | Low risk | Unclear | Unclear | Unclear | Unclear | Unclear | Low risk | Low risk | Unclear |
| Du and Chen (2013) | Unclear | Low risk | Unclear | Unclear | Unclear | Unclear | Unclear | Low risk | Low risk | High risk |
| Liu et al. (2013) | Unclear | Low risk | Unclear | Low risk | Unclear | Unclear | Unclear | Low risk | Low risk | High risk |
| Lin et al. (2014) | Low risk (random digital table method) | Low risk | Unclear | Unclear | Unclear | Unclear | Unclear | Low risk | Low risk | Unclear |
| Su et al. (2014) | Unclear | Low risk | Unclear | Unclear | Low risk | Unclear | Unclear | Low risk | Low risk | High risk |
| Zhou (2014) | Unclear | Low risk | Unclear | Unclear | Unclear | Unclear | Unclear | Low risk | Low risk | Unclear |
| Gao et al. (2015) | Unclear | Low risk | Unclear | Low risk | Unclear | Unclear | Unclear | Low risk | Low risk | High risk |
| Qian et al. (2015) | Unclear | Low risk | Unclear | Low risk | Unclear | Unclear | Unclear | Low risk | Low risk | High risk |
| Lu et al. (2016) | Unclear | Low risk | Unclear | Low risk | Unclear | Unclear | Unclear | Low risk | Low risk | High risk |
| Lu et al. (2017) | Unclear | Low risk | Unclear | Unclear | Unclear | Unclear | Unclear | Low risk | Low risk | Unclear |
| Pang et al. (2017) | Unclear | Low risk | Unclear | Unclear | Unclear | Unclear | Unclear | Low risk | Low risk | Unclear |
| Zhang et al. (2017) | Unclear | Low risk | Unclear | Unclear | Unclear | Unclear | Unclear | Low risk | Low risk | High risk |
| Shen et al. (2018) | Unclear | Low risk | Unclear | Unclear | Unclear | Unclear | Unclear | Low risk | Low risk | High risk |
| Zhou et al. (2018) | Unclear | Low risk | Unclear | Unclear | Unclear | Unclear | Unclear | Low risk | Low risk | High risk |
| Huang et al. (2019) | Unclear | Low risk | Unclear | Low risk | Unclear | Unclear | Unclear | Low risk | Low risk | Unclear |
| Liu et al. (2019) | Unclear | Low risk | Unclear | Unclear | Unclear | Unclear | Unclear | Low risk | Low risk | High risk |
| Luo et al. (2019) | Unclear | Low risk | Unclear | Unclear | Unclear | Unclear | Unclear | Low risk | Low risk | High risk |
| Wang (2019) | Unclear | Low risk | Unclear | Low risk | Unclear | Unclear | Unclear | Low risk | Low risk | Unclear |

(Continued on following page)

TABLE 3 (*Continued*) Risk of bias assessment of animal studies.

| Included in the study | ① | ② | ③ | ④ | ⑤ | ⑥ | ⑦ | ⑧ | ⑨ | ⑩ |
|---|---|---|---|---|---|---|---|---|---|---|
| Liu et al. (2020) | Unclear | Low risk | Unclear | Low risk | Unclear | Unclear | Unclear | Low risk | Low risk | High risk |
| Liu et al. (2021) | Unclear | Low risk | Unclear | Unclear | Unclear | Unclear | Unclear | Low risk | Low risk | Unclear |
| Sun et al. (2022) | Unclear | Low risk | Unclear | Unclear | Unclear | Unclear | Unclear | Low risk | Low risk | High risk |
| Ma et al. (2022) | Unclear | Low risk | Unclear | Unclear | Unclear | Unclear | Unclear | Low risk | Low risk | Unclear |
| Zheng et al. (2022) | Unclear | Low risk | Unclear | Unclear | Unclear | Unclear | Unclear | Low risk | Low risk | High risk |
| Ding and Ren (2023) | Unclear | Low risk | Unclear | Unclear | Unclear | Unclear | Unclear | Low risk | Low risk | Unclear |
| Yu et al. (2023) | Unclear | Low risk | Unclear | Unclear | Unclear | Unclear | Unclear | Low risk | Low risk | High risk |
| Zhang et al. (2023) | Unclear | Low risk | Unclear | Low risk | Unclear | Unclear | Unclear | Low risk | Low risk | High risk |

Note: ① whether the generation or application of the allocation sequence is sufficient; ② whether the baselines are the same; ③ whether allocation concealment is sufficient; ④ whether the animals were randomly placed during the study; ⑤ whether researchers and animal breeders were blinded; ⑥ whether random selection was conducted during result evaluation; ⑦ whether to use blinding methods for the evaluators of the results; ⑧ whether incomplete data were reported; ⑨ whether the research report is not related to selective reporting of results; ⑩ whether there is no other bias present.

*purpureus* Went have antioxidant and free-radical scavenging ability, which can reduce the inflammatory response caused by oxidative stress. Studies have shown that *Monascus purpureus* Went can reduce serum TNF-α and CRP levels in inflammatory mouse models, confirming the anti-inflammatory effect of *Monascus purpureus* Went (Wang et al., 2006a; Wang et al., 2008), which makes *Monascus purpureus* Went potentially useful in the treatment of non-infectious inflammatory diseases, such as arthritis and dermatitis.

### 4.3.4 Anti-tumor activity

In recent years, important progress has also been made in research on the anti-tumor effects of *Monascus purpureus* Went. *Monascus purpureus* Went polysaccharides and Monascus pigments in *Monascus purpureus* Went have an effect on inhibiting the growth and proliferation of tumor cells. These components affect the metabolism and signal transduction pathway of tumor cells through different pathways, thus exerting an anti-tumor effect. By determining the tumor inhibition rate, relative growth rate, and index of each organ in loaded mice, it was found that erythrocyte extracellular polysaccharides had a tumor-inhibitory effect on H22-loaded mice *in vivo* (Zhou et al., 2011). The determination of body weight, tumor weight, tumor suppression rate, and changes in spleen weight and spleen index of the loaded mice indicated that *Monascus purpureus* Went polysaccharides had a good inhibitory effect on tumor growth in loaded mice (Ding, 2007). Although the application of *Monascus purpureus* Went in anti-tumor therapy is still in the research stage, its potential should not be ignored.

## 4.4 Methodological quality

### 4.4.1 Methodological quality of animal studies

The methodological quality assessment of animal studies related to *Monascus purpureus* Went found that most studies only reported "randomization," while a few used the random number table. Assignment concealment and blinding of investigators or animal handlers and evaluators of results were not reported in most studies, which may have led to subjective bias in the expected experimental results, and only a few used assignment concealment and blinding. A number of studies did not report the randomization of the environment in which the animals were placed, which may have influenced objective environmental factors such as temperature, humidity, and light intensity in different locations on the experimental results. The result data were completely reported. In conclusion, it is necessary to improve the generation of random allocation sequences, allocation concealment, application of blinding, and randomness of the environment in animal studies related to *Monascus purpureus* Went.

### 4.4.2 Methodological quality of clinical trials

The methodological quality assessment of clinical trials related to *Monascus purpureus* Went found that most trials only reported "randomization," while a few used the random number table and lottery. Assignment concealment and blinding of patients and outcome evaluators were not reported in most trials, which may increase the risk of measurement bias and evaluator bias, and only a few used assignment concealment and blinding. Some trials did not report registration and conflict of interest, which could lead to inappropriate influence from sponsors. Outcome data were completely reported. In conclusion, it is necessary to improve the generation of random allocation sequences, allocation concealment, application of blinding, and reporting of funding sources in clinical trials related to *Monascus purpureus* Went.

### 4.4.3 Suggestion for the study design

The common problems of methodological quality deficiencies in both animal studies and clinical trials are obvious, and the following suggestions are made: first, it is suggested that suitable random

TABLE 4 Information on clinical trials related to *Monascus purpureus* Went.

| Inclusion of studies | Disease | Source of subjects (hospital level) | Enrollment time | Number of men | Number of women | Total quantity | Age – bracket | Average age | Method of administration | Intervention | Frequency | Course of treatment | Indicator domain | Counting indicator | Measuring indicator |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Cui et al. (2002) | Hyperlipemia | III | August 2000–December 2000 | 52 | 38 | 90 | Did not report | 59 | Profess to be convinced | Compound *Monascus purpureus* Went capsule (Chinese patent medicine) vs Western medicine | 2 grains/time, 3 times/day; 1 grain/time, 1 time/day | 30 days | Symptoms and signs; physical and chemical testing | ① | ③④⑤⑥ |
| Yin et al. (2016) | Primary dyslipidemia | III | December 2013–December 2015 | 22 | 118 | 140 | Did not report | 58.73 ± 10.20 | Profess to be convinced | *Monascus purpureus* Went compound preparation (Chinese medicine preparation) | 1 time/day | 30 days | TCM symptoms/syndromes; physical and chemical testing | Did not report | ③④⑤⑥⑦ |
| Zhang and He (2016) | Hyperlipemia | III | August 2014–January 2016 | 40 | 40 | 80 | Did not report | Did not report | Profess to be convinced | Western medicine vs TCM *Monascus purpureus* Went + Western medicine | 1.5 g/time, 3 times/day; 5 mg/time, 2 times/day | 90 days | Symptoms and signs; physical and chemical testing | ① | ③④⑤⑥ |
| Chen et al. (2021) | Hyperlipemia was associated with abnormal liver function | III | July 2018–December 2019 | 43 | 29 | 102 | 25–75 | 53.48 ± 10.30 | Profess to be convinced | Basic treatment + TCM *Monascus purpureus* Went | 1 time/day | 56 days | TCM symptoms/syndromes; physical and chemical testing | ① | ③④⑤⑥⑦⑧⑨ |
| Luo and Zhong (2020) | Dyslipidemia | II | July–December 2019 | 28 | 32 | 60 | 45–75 | Did not report | Profess to be convinced | Western medicine vs TCM *Monascus purpureus* Went | 1 time/day; 2 times/day | 90 days | Physical and chemical testing | Did not report | ③④⑤⑥⑩ |
| Zhang et al. (2020) | Hyperlipemia | Secondary hospital | May 2016–May 2018 | 25 | 36 | 61 | Did not report | Did not report | Profess to be convinced | TCM *Monascus purpureus* Went | 6 g/day | 56 days | Symptoms and signs; physical and chemical testing | ① | ③④⑤⑥ |

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TABLE 4 (*Continued*) Information on clinical trials related to *Monascus purpureus* Went.

| Inclusion of studies | Disease | Source of subjects (hospital level) | Enrollment time | Number of men | Number of women | Total quantity | Age – bracket | Average age | Method of administration | Intervention | Frequency | Course of treatment | Indicator domain | Counting indicator | Measuring indicator |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Zhao et al. (2018) | Hyperlipemia was associated with abnormal liver function | III | June 2017–March 2018 | 50 | 30 | 80 | 25–75 | Did not report | Profess to be convinced | Basic treatment + TCM *Monascus purpureus* Went vs. basic treatment | 6 g/day | 90 days | Symptoms and signs; physical and chemical testing | ① | ③④⑤⑥⑧⑨ |
| Wang and Li (2018) | Hyperlipemia | III | March 2017–September 2017 | 50 | 34 | 84 | Did not report | Did not report | Profess to be convinced | Western medicine vs. TCM *Monascus purpureus* Went | 10 mg/time, 3 time/day; 3/day | 90 days | Symptoms and signs; physical and chemical testing | ① | ③⑤⑥ |
| Shi et al. (2015) | Dyslipidemia | III | Did not report | 25 | 3 | 28 | 80–92 | 85.6 ± 4.8 | Profess to be convinced | TCM *Monascus purpureus* Went | 6 g/time, 2 times/day | 56 days | Physical and chemical testing | Did not report | ③④⑤⑥⑪⑫ |
| Wang et al. (2022) | Uremia and hyperlipemia | III | September 2021–June 2022 | 31 | 37 | 68 | 62–86 | 68.13 ± 12.53 | Profess to be convinced | Basic treatment + Western medicine vs. basic treatment + TCM *Monascus purpureus* Went | 10 mg/day, 1 time/day; 6 g/day | 56 days | Physical and chemical testing | Did not report | ③④⑤⑥⑬⑭⑮⑯ |
| Wu and Luo (2013) | Carotid atherosclerotic plaque | III | January 2011–December 2012 | Did not report | Did not report | 60 | Did not report | Did not report | Profess to be convinced | TCM *Monascus purpureus* Went vs. Western medicine vs. blank control | 6 g/time, 2 times/day; 1 time/day | 180 days | TCM symptoms/ syndromes; physical and chemical testing | ② | ③④⑤⑥⑰⑱⑲ |
| Liu et al. (1998) | Hyperlipemia | III | July 1995–July 1996 | 41 | 35 | 76 | Did not report | 61.2 ± 3.6 | Profess to be convinced | Zhibituo capsule (Chinese patent medicine) | 3 times/day | 60 days | Symptoms and signs; physical and chemical testing | ① | ③⑥ |
| Qiu et al. (1997) | Hyperlipemia | III | August 1995–February 1996 | 112 | 64 | 176 | Did not report | Did not report | Profess to be convinced | Di'ao Zhibituo capsule (Chinese patent medicine) vs. Western medicine | 3 times/day | 90 days | Symptoms and signs; physical and chemical testing | ① | ③⑤⑥ |

(Continued on following page)





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TABLE 4 (*Continued*) Information on clinical trials related to *Monascus purpureus* Went.

| Inclusion of studies | Disease | Source of subjects (hospital level) | Enrollment time | Number of men | Number of women | Total quantity | Age – bracket | Average age | Method of administration | Intervention | Frequency | Course of treatment | Indicator domain | Counting indicator | Measuring indicator |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Lu et al. (2012) | Hyperlipemia | III | May 2010–October 2011 | 28 | 32 | 60 | 18–75 | Did not report | Profess to be convinced | Basic treatment + Xuezhikang capsule (Chinese patent medicine) vs. basic treatment + placebo | 2 times/day | 56 days | TCM symptoms/ syndromes; physical and chemical testing | ② | ③④⑤⑥ |
| Wang et al. (1995) | Hyperlipemia | III | Did not report | 261 | 185 | 446 | Did not report | Did not report | Profess to be convinced | Xuezhikang capsule (Chinese patent medicine) vs. placebo | 2 times/day | 56 days | Symptoms and signs; physical and chemical testing | ① | ③④⑤⑥ |
| Zhu (2013) | Unstable angina | Secondary hospital | June 2009–November 2012 | 55 | 25 | 80 | 66–89 | Did not report | Profess to be convinced | Basic treatment + Xuezhikang capsule (Chinese patent medicine) vs. basic treatment | 2 grains/ time, 2 times/day | 60 days | Symptoms and signs; physical and chemical testing | ① | ⑳ |
| Tang and Chang (2012) | Hyperlipemia | Secondary hospital | 2009–2011 | 62 | 28 | 90 | 38–65 | 48 | Profess to be convinced | Basic treatment + Xuezhikang capsule (Chinese patent medicine) vs. basic treatment | 2 times/day | 28 days | Physical and chemical testing | Did not report | ③④⑤⑥ |
| Huang and Zhang (2005) | Unstable angina | Secondary hospital | Did not report | 61 | 18 | 79 | Did not report | Did not report | Profess to be convinced | Basic treatment + Xuezhikang capsule (Chinese patent medicine) vs. basic treatment | 2 times/day | 3 days | Physical and chemical testing | Did not report | ⑮⑱⑲ |
| Zheng and Li (2015) | Hyperlipemia | III | July 2013–July 2014 | 23 | 19 | 42 | 39–70 | Did not report | Profess to be convinced | Xuezhikang capsule (Chinese patent medicine) vs. Western medicine | 0.5 g/time, 2 times/day; 1 grain/time, 1 time/day | 50 days | Physical and chemical testing | Did not report | ③④⑤⑥ |

(Continued on following page)





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TABLE 4 (*Continued*) Information on clinical trials related to *Monascus purpureus* Went.

| Inclusion of studies | Disease | Source of subjects (hospital level) | Enrollment time | Number of men | Number of women | Total quantity | Age – bracket | Average age | Method of administration | Intervention | Frequency | Course of treatment | Indicator domain | Counting indicator | Measuring indicator |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Wang et al. (2016) | Carotid atherosclerotic plaque | III | January–December 2015 | 58 | 62 | 120 | 55–73 | 61.4 ± 7.7 | Profess to be convinced | Shengqu *Monascus purpureus* Went capsule (Chinese patent medicine) vs. Western medicine | 4 grains/time, 3 times/day; 20 mg/day | 168 days | Physical and chemical testing | Did not report | ③④⑤⑥ |
| Wang et al. (2014) | Dyslipidemia | III | 2012–2013 | 38 | 82 | 120 | 35–73 | 51.4 ± 7.7 | Profess to be convinced | Shengqu *Monascus purpureus* Went capsule (Chinese patent medicine) vs. Shengqu *Monascus purpureus* Went capsule (Chinese patent medicine) vs. Western medicine | 2 grains/time, 3 times/day; 10 mg/day | 84 days | Physical and chemical testing | Did not report | ③④⑤⑥ |
| Xu et al. (2019) | Hyperlipemia | II | Did not report | 43 | 71 | 114 | Did not report | 44.59 ± 12.32 | Profess to be convinced | Sea-buckthorn (*Hippophae rhamnoides* L.) *Monascus purpureus* Went capsule (Chinese patent medicine) vs. placebo | 2 grains/time, 1 time/day | 90 days | Symptoms and signs; TCM symptoms/syndromes; physical and chemical testing | ①② | ③④⑤⑥ |
| Cao et al. (2007) | Type 2 diabetes | III | September 2006–July 2007 | 14 | 16 | 30 | 28–70 | 55.2 | Profess to be convinced | *Monascus purpureus* Went capsule for reducing sugar (Chinese patent medicine) | 3 grains/time, 1 time/day | 90 days | Symptoms and signs; physical and chemical testing | ① | ⑩ |
| Chen and Liu (2015) | Dyslipidemia and hyperglycemia | II | March 2013–December 2014 | Did not report | Did not report | 70 | Did not report | 50.50 ± 4.20 | Profess to be convinced | Danxi *Monascus purpureus* Went wine (Traditional Chinese medicine preparation) | 50 mL, 150 mL, and 250 mL/time; 2 times/day | 90 days | Physical and chemical testing | Did not report | ③④⑤⑥⑩⑬ |

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TABLE 4 (*Continued*) Information on clinical trials related to *Monascus purpureus* Went.

| Inclusion of studies | Disease | Source of subjects (hospital level) | Enrollment time | Number of men | Number of women | Total quantity | Age – bracket | Average age | Method of administration | Intervention | Frequency | Course of treatment | Indicator domain | Counting indicator | Measuring indicator |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Liu et al. (2011) | Dyslipidemia was associated with carotid atherosclerotic plaque | Other sources | April 2005–April 2006 | 23 | 37 | 60 | Did not report | Did not report | Profess to be convinced | Lipid-lowering *Monascus purpureus* Went micro powder (Chinese patent medicine 1) vs. Xuezhikang capsule (Chinese patent medicine 2) vs. Western medicine | 1 grain/time, 2 times/day; 2 grains/time, 2 times/day; 1 tablet/time, 1 time/day | 180 days | TCM symptoms/syndromes; physical and chemical testing | ② | ③④⑤⑥⑰ |
| Wu et al. (2005) | Hyperlipemia | Other sources | Did not report | Did not report | Did not report | 80 | Did not report | Did not report | Profess to be convinced | Half dose of lipid-lowering *Monascus purpureus* Went micro powder (Chinese patent medicine 1) vs. lipid-lowering *Monascus purpureus* Went micro powder (Chinese patent medicine 2) vs. lipid-lowering *Monascus purpureus* Went crude powder (Chinese patent medicine 3) vs. Xuezhikang capsule (Chinese patent medicine 4) | 1 grain/time, 2 times/day; 0.6 g/time, 2 times/day | 50 days | Symptoms and signs; physical and chemical testing | ① | ③④⑤⑥⑪⑫ |

(Continued on following page)





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TABLE 4 (*Continued*) Information on clinical trials related to *Monascus purpureus* Went.

| Inclusion of studies | Disease | Source of subjects (hospital level) | Enrollment time | Number of men | Number of women | Total quantity | Age – bracket | Average age | Method of administration | Intervention | Frequency | Course of treatment | Indicator domain | Counting indicator | Measuring indicator |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Yang et al. (2024) | Intervention in non-alcoholic fatty liver | III | October 2020–October 2021 | 41 | 39 | 80 | 32–60 | 44.52 ± 6.2 | Profess to be convinced | Western medicine vs. *Coptis chinensis* Franch. *Monascus purpureus* Went medicine (Chinese medicine preparation) + Western medicine | 3 times/day; 1 time/day | 56 days | Physical and chemical testing | Did not report | ③④⑤⑥⑧⑨ |
| He et al. (2007) | Hyperlipemia | III | Did not report | 69 | 21 | 100 | 18–65 | 44.06 ± 9.17 | Profess to be convinced | *Monascus purpureus* Went Flavonoid tablet (Chinese patent medicine) vs. placebo | 2.4 g/day | 30 days | Symptoms and signs; physical and chemical testing | ① | ③⑤⑥ |

Note: ①, clinical efficacy; ②, efficacy of TCM syndrome; ③, total cholesterol (TC) level; ④, low-density lipoprotein cholesterol (LDL-C) level; ⑤, triglyceride (TG) level; ⑥, high-density lipoprotein cholesterol (HDL-C) level; ⑦, TCM syndrome points; ⑧, ALT horizontal; ⑨, AST horizontal; ⑩, blood glucose check; ⑪, ApoB level; ⑫, ApoA level; ⑬, body mass index (BMI); ⑭, serum albumin (ALB); ⑮, C-reactive protein (CRP) level; ⑯, SGA grade; ⑰, carotid artery ultrasound; ⑱, routine blood test; ⑲, liver and kidney function; ⑳, electrocardiogram.



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TABLE 5 Risk of bias assessment of RCTs.

| Included in the study | Generation of the randomly assigned sequences | Allocation concealment | Subjects were blinded | Blinded to the outcome assessors | Resulting data integrity | Selective report | Other bias |
|---|---|---|---|---|---|---|---|
| Cui et al. (2002) | Unclear | Unclear | High risk | Unclear | Low risk | Low risk | High risk |
| Zhang and He (2016) | Unclear | Unclear | High risk | Unclear | Low risk | Low risk | High risk |
| Luo and Zhong (2020) | Low risk (random digital table method) | Unclear | High risk | Unclear | Low risk | Low risk | Low risk |
| Zhao et al. (2018) | Unclear | Unclear | High risk | Unclear | Low risk | Low risk | High risk |
| Wang and Li (2018) | Low risk (random digital table method) | Unclear | High risk | Unclear | Low risk | Low risk | Low risk |
| Wang et al. (2022) | Low risk (lottery method) | Unclear | High risk | Unclear | Low risk | Low risk | Low risk |
| Wu and Luo (2013) | Low risk (random digital table method) | Unclear | High risk | Unclear | Low risk | Low risk | High risk |
| Liu et al. (1998) | Unclear | Unclear | High risk | Unclear | Low risk | Low risk | High risk |
| Qiu et al. (1997) | Unclear | Unclear | High risk | Unclear | Low risk | Low risk | High risk |
| Lu et al. (2012) | Unclear | Unclear | Low risk | Unclear | Low risk | Low risk | High risk |
| Wang et al. (1995) | Unclear | Unclear | Low risk | Unclear | Low risk | Low risk | High risk |
| Zhu (2013) | Unclear | Unclear | High risk | Unclear | Low risk | Low risk | High risk |
| Huang and Zhang (2005) | Unclear | Unclear | High risk | Unclear | Low risk | Low risk | High risk |
| Zheng and Li (2015) | Unclear | Unclear | High risk | Unclear | Low risk | Low risk | High risk |
| Wang et al. (2016) | Unclear | Unclear | High risk | Unclear | Low risk | Low risk | Low risk |
| Wang et al. (2014) | Unclear | Unclear | High risk | Unclear | Low risk | Low risk | Low risk |
| Xu et al. (2019) | Unclear | Low risk | Low risk | Unclear | Low risk | Low risk | Low risk |
| Chen and Liu (2015) | Low risk (random digital table method) | Unclear | High risk | Unclear | Low risk | Low risk | High risk |
| Liu et al. (2011) | Low risk (random digital table method) | Unclear | High risk | Unclear | Low risk | Low risk | Low risk |
| Wu et al. (2005) | Unclear | Unclear | High risk | Unclear | Low risk | Low risk | Low risk |
| Yang et al. (2024) | Low risk (random digital table method) | Unclear | High risk | Unclear | Low risk | Low risk | Low risk |
| He et al. (2007) | Unclear | Unclear | Low risk | Low risk | Low risk | Low risk | High risk |

sequence generation methods, such as the random number table method, should be used to ensure the randomness and fairness of the allocation process. Second, it is suggested that allocation concealment and application of the double-blind method, including blinding of operators and observers, should be implemented. The person in charge of the operation should not know which group each patient is assigned to, in order to eliminate operator-induced subjective bias in the results. The person responsible for data collection and analysis should also not know the specific group to which each patient is assigned. The process of data collection and analysis should be carried out independently to ensure the objectivity and accuracy of the results. In addition, it is suggested that other conditions (e.g., environment and operation) should be strictly controlled for consistency during the study to ensure the reliability and repeatability of results. Finally, during data analysis, care should be taken to control the effect of confounding factors and other biases and to report in detail on the source of funding and registration to ensure the objectivity and accuracy of the results.

## 4.5 Limitation

There are some limitations to this review. First, the English-language literature obtained from the search was relatively limited. Second, the studies mainly focus on molecular and animal studies,

with relatively few clinical trials. Third, some of the studies are of poor quality, and the reference value of their results may therefore be limited. Fourth, excluding studies for which the full texts could not be obtained may lead to data bias. Fifth, some of the clinical trials contained partially unreported information about the subjects, such as the patient source, enrollment time, and age range, which may reduce the strictness of the study. Sixth, only two meta-analyses were obtained and assessed for methodological quality and reporting standards in this study, and the results showed that they were of low quality, but due to their small number, these studies were not examined and described in detail in order to avoid study bias. The assessment of methodological quality and reporting standards for meta-analyses are presented in Supplementary Adds S2, S3.

## 4.6 Research implications

### 4.6.1 Clinical safety

Existing studies have shown that the contraindications for *Monascus purpureus* Went productions mainly include the following: ① patients allergic to monacolin K/lovastatin or any excipients; ② patients with acute liver disease; ③ patients with severe renal impairment (eGFR <30 mL/min); ④ patients with various myopathies; and ⑤ pregnant women, lactating women, and women of childbearing age who have not taken effective contraceptive measures (Banach et al., 2022). It is recommended that its importance be emphasized in clinical practice.

### 4.6.2 Fundamental research challenges

In terms of the bioactive ingredient biosynthetic pathways and regulatory mechanisms of substances produced during *Monascus* fermentation, the current challenges in the development of *Monascus purpureus* Went include improving the content of active ingredients like lovastatin and controlling the content of toxic metabolites like citrinin through methods such as optimizing fermentation parameters, mutagenic breeding, and genetic engineering.

### 4.6.3 Other suggestions

More contamination risks derive from raw materials, microbial metabolism, or processing errors; the suggestions for the production quality control of *Monascus purpureus* Went are as follows: first, the quality control of raw materials should be optimized, the standardized management of fermentation strains should be strengthened, and high-quality fermentation strains should be accurately identified and screened. Second, the quality detection standard system should be improved, the specifications for the determination of active ingredient contents should be clarified, and the detection scope of safety indicators should be expanded. Third, the production process system must be optimized, the operation process must be standardized, fermentation parameters must be improved, and fermentation conditions must be strictly controlled. Fourth, the construction of the regulatory system must be improved, mandatory third-party safety reviews must be implemented, and the post-market supervision of products must be strengthened.

To promote the high-quality development of *Monascus purpureus* Went studies, the suggestions are as follows: first, standards should be set up. Unified quality standards for experimental design and efficacy evaluation should be developed to improve comparability between different studies. The production process and quality control of *Monascus purpureus* Went should be standardized to ensure the stable quality of *Monascus purpureus* Went products used in research and application. Second, multidisciplinary cooperation should be promoted. Experts in the fields of pharmacy, medicine, biology, and other multidisciplinary fields should be encouraged to cooperate in the studies and explore the pharmacological action mechanism of *Monascus purpureus* Went in depth from different perspectives. Third, the research and development of new drugs should be encouraged. Modern preparation technology should be actively used to develop new *Monascus purpureus* Went preparations with more stable efficacy. Fourth, more research on the combination of *Monascus purpureus* Went and other drugs should be encouraged to observe the therapeutic effect and safety. Fifth, human pharmacokinetic studies and clinical trials should be performed to explore the effective dosage range of *Monascus purpureus* Went products, validate safety and prevent adverse events, and provide evidentiary support for clinical practice. Concurrently, key procedures, including ethical review and informed consent, must be strictly implemented throughout the process.

# 5 Conclusion

As a type of traditional Chinese medicine, the pharmacological action mechanism of *Monascus purpureus* Went is extensive and complex. Given the extensive global use of Monascus purpureus Went products, we have gradually revealed its mechanism of action in regulating blood lipids and exerting anti-inflammatory, anti-oxidant, and anti-tumor effects through modern science research and technology. Moreover, we also need to pay attention to the contraindications and safety issues associated with the use of *Monascus purpureus* Went to ensure its safety and effectiveness in our daily lives.

# Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material; further inquiries can be directed to the corresponding authors.

# Author contributions

SL: formal analysis, investigation, visualization, and writing – original draft. YX: formal analysis, investigation, visualization, and writing – original draft. JX: formal analysis, investigation, visualization, and writing – original draft. JH: data curation and writing – original draft. YW: methodology, supervision, and writing – review and editing. JZ: project administration, supervision, and writing – review and editing. SL: conceptualization and writing – review and editing. HH:

conceptualization, project administration, supervision, and writing – review and editing. LA: conceptualization, supervision, and writing – review and editing. ZJ: conceptualization, project administration, supervision, and writing – review and editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2025.1600460/full#supplementary-material

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# Development and preliminary mechanistic analysis of compound effervescent hepatoprotective granules

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**Background:** The causes of acute liver injury (ALI) are complex and diverse, including alcohol, drugs, infections, and toxic substances, and it has become a major global health issue. Traditional Chinese medicine (TCM), with advantages such as broad-spectrum biological activity, low toxicity, and minimal side effects, has been widely used in drug research and development as well as disease management. Some TCMs have shown significant efficacy in treating ALI: Ganoderma lucidum polysaccharides, monoterpene glycosides from Paeonia lactiflora, glycyrrhizic acid saponins, and flavonoids all exhibit liver-protective effects; however, the protective effects of their compound preparations on liver injury remain unclear.

**Methods:** The study optimized the water extraction process using orthogonal experiments with AHP-entropy weight scoring. The preparation process was verified by single-factor experiments, Plackeett–Burman and Box–Bohnken tests. The mechanism of action was validated using network pharmacology methods and a CCl4-induced acute liver injury animal model.

**Results:** The developed extraction and granule formation processes were reliable after validation. The contents of active components in CEHG determined by HPLC were as follows: albiflorin 1.26%, paeoniflorin 5.42%, liquiritin 0.43%, glycyrrhizic acid 1.30%, and ganoderic acid A 0.14%, with batch-to-batch variation coefficients (RSD) of 1.66%, 0.87%, 2.32%, 1.60%, and 4.03%, respectively. The dry extract ratio was 18.23% with an RSD of 2.11%. Network pharmacology revealed that CEHG improved liver injury by regulating the HIF-1, p53, and FoxO signaling pathways. Animal experiments indicated that CEHG granules reduce liver function-related aspartate transaminase (AST), alanine transaminase (ALT), total cholesterol (TC), triglyceride (TG), total bilirubin (TBIL), and LDH levels, decrease the levels of inflammatory factors IL-6, IL-1β, and TNF-α and of oxidative stress-related MDA and ROS, and down-regulated the mRNA and protein levels of P53, Bax, and HIF-1α in rats with liver injury. Meanwhile, CEHG improved liver function-related Total Protein (TP), raised the levels of oxidative stress-related SOD, GSH-Px, and GSH, and up-regulated mRNA and protein expression of Bcl-2.

**Conclusion:** This study successfully optimized the extraction and granule-formation of CEHG and revealed its hepatoprotective mechanism through network pharmacology and animal studies, providing scientific evidence supporting the further development and clinical use of CEHG.

KEYWORDS

**Traditional Chinese medicine, functional food, effervescent granules, liver injury, pharmacology**

# 1 Introduction

Chemical liver injury (CLI) is a major cause of acute liver injury, whose incidence is rising yearly (1). CLI can result from improper drug use, long-term alcohol consumption, and exposure to toxic chemicals in the environment (2–4). These factors can directly poison liver cells, induce metabolic disorders, trigger oxidative stress, and activate immunological responses, all of which can damage liver cells (5). This damage can lead to different liver diseases that not only lower the quality of life of patients but can also be life-threatening. Thus, the development of liver-protective drugs or functional foods is of great practical significance.

Traditional Chinese medicine (TCM), with its multi-component and multi-target approach, offers great potential for modern drug development. Many TCMs and their extracts can protect the liver by regulating hepatic metabolic function, combating oxidative stress, inhibiting inflammation, and enhancing immunity (6, 7). Modern pharmacological studies have shown that *Ganoderma lucidum* contains components such as Ganoderma polysaccharides, Ganoderma triterpenoids, and sterols that exert hepatoprotective effects. Their mechanisms include antioxidation, free radical scavenging, anti-inflammation, immunomodulation, antifibrosis, and metabolic regulation (8–11). *Paeonia lactiflora* contains compounds such as monoterpene glycosides, triterpenoids, flavonoids, and tannins. The total glycosides, are the main hepatoprotective components and include active compounds such as paeoniflorin and albiflorin, which protect the liver through antioxidative stress, anti-inflammation, anti-cell death, immunomodulation, improvement of microcirculation, and anti-hepatic fibrosis (12–15). *Glycyrrhiza glabra* is a widely used medicinal plant and a TCM with medicinal and edible values that are extensively applied in the food and pharmaceutical industries. Its main chemical components consist of triterpenoid saponins, flavonoids, and polysaccharides. These components protect the liver through mechanisms such as antioxidation, anti-inflammation, anti-apoptosis, detoxification, and regulation of lipid metabolism (16–19). In our study, the following three herbs are combined: *Paeonia lactiflora*, which nourishes the blood, soothes the liver, alleviates pain, and protects the liver, serves as the sovereign herb; *Ganoderma lucidum*, which improves overall health, acted as the ministerial herb; and *Glycyrrhiza glabra*, which strengthened spleen function and harmonized the formula, functioned as the assistant and guide herb. Together, the combination was predicted to form a health-promoting formula that strengthened the body, eliminated harmful influences, balanced qi and blood, and coordinated liver and spleen functions.

Compared with ordinary formulations, effervescent granules have a pleasant taste and are easy to carry. They are widely used in health products and can greatly improve treatment compliance. This study aimed to optimize extraction and granule formation processes to develop a hepatoprotective health products with significant effects and easy acceptance (20). Systematic pharmacological studies and animal experiments will be conducted to verify their protective effects on liver injury, providing a theoretical basis and practical support for the development of new liver-protective health products.

# 2 Materials and methods

## 2.1 Materials

*Ganoderma lucidum* originated from Jinghe, Jilin. *Paeonia lactiflora* was purchased from Huiqiao Pharmaceutical Co., Ltd., Bozhou. *Glycyrrhiza glabra* was purchased from the Jingwan Chinese Medicine Slices Factory, Bozhou. Anhydrous citric acid of food grade, sodium bicarbonate, maltodextrin, and mannitol were obtained from Zhongchen Biotechnology Co., Ltd., Henan. Edible alcohol was acquired from Hanyong Alcohol Co., Ltd., Henan.

## 2.2 Reagents and standards

Ganoderic acid A, paeoniflorin, and albiflorin (purity ≥98%) were purchased from Chengdu Puesday Biological Technology Co., Ltd. Ammonium glycyrrhizinate (purity ≥98%) was obtained from Chengdu Chroma Biotechnology Co., Ltd. Liquiritin (purity ≥98%) was sourced from Beijing Zhongke Zhijian Biotechnology Co., Ltd. HPLC-grade methanol and acetonitrile were bought from Beijing J&K Scientific Technology Co., Ltd. Chromatographic-grade formic acid was procured from Shanghai Aladdin Biochemical Technologies Co., Ltd. The ROS detection kit was purchased from Shanghai Beibio Biotechnology Co., Ltd. The apoptosis kit, MDA colorimetric assay kit, SOD colorimetric assay kit, GSH colorimetric assay kit, GSH-PX colorimetric assay kit, BCA protein concentration determination kit, AST/GOT colorimetric assay kit, ALT/GPT colorimetric assay kit, LDH

**Abbreviations:** CEHG, compound ganoderma lucidum hepatoprotective effervescent granules; ALT, alanine transaminase; AST, aspartate transaminase; GSH, glutathione; NF-κB, nuclear factor-kappa B; Nrf2, nuclear factor erythroid 2-related factor 2.

colorimetric assay kit, TC colorimetric assay kit, TG colorimetric assay kit, TBIL colorimetric assay kit, and TP colorimetric assay kit were obtained from Elabscience. The IL-1β kit, IL-6 kit, and TNF-α kit were purchased from Quanzhou Ruixin Biotechnology Co., Ltd. P53, Bax, Bcl-2, and HIF-1α antibodies were obtained from Affinity. β-actin, goat anti-mouse IgG, and goat anti-rabbit IgG antibodies were purchased from Zs-BIO. RIPA lysis buffer (strong) and PMSF were obtained from Biosharp. SDS, glycine, Tris, APS, acrylamide, bis-acrylamide, and PBS buffer powder were purchased from Servicebio. All other reagents were obtained from professional reagent companies.

## 2.3 Animals

SPF-grade SD rats (male, 7–8 weeks old, 200 ± 20 g, $n = 60$) were purchased from Liaoning Changsheng Biotechnology Co., Ltd. in Liaoning, China [SCXK (Liao) 2020-0001]. The Experimental Ethics Committee of Anhui University of Chinese Medicine (Hefei, China, AHUCM-rats-2024049) granted approval for all animal experiments.

## 2.4 Extraction and preparation of CEHG

### 2.4.1 Steps of preparation of the CEHG extract

According to the drug usage standards of the 2020 edition of the Chinese Pharmacopeia, the daily formula dosage of CEHG is as follows: *Ganoderma lucidum* 6 g, *Paeonia lactiflora* 6 g and *Glycyrrhiza glabra* 3 g. Preparation of the extract: Weigh the Chinese herbal slices in proportion, place them in a stainless steel pot, and add water to soak (preliminary experiments show that the water absorption rate of the herbal slices is approximately twice their own weight, so an additional twice the amount of water needs to be added for subsequent extraction). After soaking, heat and decoct the mixture; the filtrate is filtered through 8 layers of medical gauze to obtain the extract. Concentrate the extract in a material-to-liquid ratio of 1:1 using a rotary evaporator, freeze-dry it, then crush it in a mortar and pass through an 80-mesh pharmaceutical sieve to obtain the extract powder.

### 2.4.2 Optimization of extraction process by orthogonal experiment

An orthogonal experimental design with three factors and three levels was adopted to select the levels of the water addition ratio (A), the number of extraction times (B), and the extraction duration (C). The experimental combinations of these factors were arranged according to the orthogonal table, as detailed in Table 1.

TABLE 1 Orthogonal test factors and levels table.

| Level | Factors | | |
|---|---|---|---|
| | Water addition ratio (A, g/ml) | Number of extraction times (B) | Extraction duration (C, h) |
| 1 | 1:10 | 1 | 1 |
| 2 | 1:15 | 2 | 2 |
| 3 | 1:20 | 3 | 3 |

### 2.4.3 Optimization of the extraction process by the combined AHP-entropy weighting method

The critical quality indicators of the Compound Liver-protecting Effervescent Granules, determined by the functions of the product, were identified as the dry extract ratio and the content of albiflorin, paeoniflorin, liquiritin, glycyrrhizic acid and ganoderic acid A. The dry extract ratio was measured by the loss on drying method and the contents of albiflorin, paeoniflorin, liquiritin, glycyrrhizic acid and ganoderic acid A were determined by high-performance liquid chromatography (HPLC). Chromatographic column: caprisil C18-AQ (5 μm, 250 mm × 4.6 mm), mobile phase: 0.1% formic acid water (A) acetonitrile (B); gradient elution: 0–8 min, 90%−86%A; 8–16 min, 86%−75%A; 16–21 min, 75%−74%A; 21–26 min, 74%-68%A; 26–48 min, 68%−42%A; 48–53 min, 42%−12%A; 53–54 min, 12%−90%A; 54–55 min, 90%A; flow rate: 0.8 ml/min; detection wavelength: 254 nm; column temperature: 30 °C; injection volume: 10 μl.

Accurately weigh appropriate amounts of paeoniflorin lactone, paeoniflorin, liquiritin, ammonium glycyrrhizinate, and ganoderic acid A, dissolve them in 80% methanol to prepare reference standard stock solutions with mass concentrations of 1.25, 2.66, 1.38, 1.29, and 0.10 mg/ml respectively. Pipette different volumes of the above reference standard solutions into 5 ml volumetric flasks, dilute to the mark, and obtain a mixed reference standard solution with mass concentrations of 62.50, 532.0, 69.00, 129.0, and 25.00 μg/ml respectively.

Accurately weigh 0.2 g of the main drug into a 50 ml volumetric flask, dilute to the mark with 80% methanol, and weigh the mass. Subject the flask to ultrasonic treatment for 30 min (40 kHz, 200 W), then allow it to cool to room temperature. Replenish the lost mass with 80% methanol, shake well, take 1 ml of the solution, centrifuge it at 12,000 r/min for 10 min, and collect the supernatant to obtain the test sample solution.

According to Table 2, paeoniflorin and albiflorin, act as the main hepatoprotective components with equal importance, both scoring 1 point, but slightly more important than liquiritin and glycyrrhizic acid (both scoring 3 points), and significantly higher than ganoderic acid A and the dry extract ratio (both scoring 5 points); AHP hierarchical analysis, subjective and objective entropy weighting of information were carried out using the SPSSAU online analysis tool (https://spssau.com/indexs.html), in which the combined weights were determined according to formula 1, orthogonal experimental data were standardized according to formula 2. A comprehensive score was calculated by comprehensive weighting according to formula 3, and finally the best process was defined through variance and range hierarchical analysis of AHP.

$$W_j = W_j^S W_j^O / \sum_{j=1}^{n} W_j^S W_j^O \quad (1)$$

$$C_{i,j} = (x_{i,j} - \min(x_{i,j}))/(\max(x_j) - \min(x_{i,j})) \quad (2)$$

TABLE 2 Criteria for AHP construction of judgment matrices.

| Scale | Meaning |
|---|---|
| 1 | Indicates that the two factors are equally important. |
| 3 | Indicates that one factor is slightly more important than the other. |
| 5 | Indicates that one factor is significantly more important than the other. |
| 7 | Indicates that one factor is strongly more important than the other. |
| 9 | Indicates that one factor is extremely more important than the other. |
| 2, 4, 6, 8 | Represent intermediate values between the above judgments. |
| Reciprocal | If the importance of factor $e$ over $f$ is $\alpha_{ef}$, then the importance of factor $f$ over $e$ is $1/\alpha_{ef}$ |

$$\begin{aligned}\text{Comprehensive Score} = {} & C_{i,\text{paeoniflorin}} \times W_{\text{paeoniflorin}} + C_{i,\text{albiflorin}} \\ & \times W_{\text{albiflorin}} + C_{i,\text{glycyrrhizic acid}} \\ & \times W_{\text{glycyrrhizic acid}} + C_{i,\text{liquiritin}} \times W_{\text{liquiritin}} \\ & + C_{i,\text{ganoderic acid A}} \times W_{\text{ganoderic acid A}} \\ & + C_{i,\text{dry extract ratio}} \times W_{\text{dry extract ratio}} \end{aligned} \quad (3)$$

### 2.4.4 Precision test

Take the mixed reference standard solution, inject it continuously for 6 times under the chromatographic conditions specified in "Section 2.4.3", record the peak areas and retention times, and calculate the relative standard deviation (RSD) percentage.

### 2.4.5 Repeatability test

Take the main drug from the same batch, prepare 6 parallel test sample solutions according to the method specified in "Section 2.4.3", and perform injection analysis. Record the peak areas and retention times, then calculate the relative standard deviation (RSD) percentage.

### 2.4.6 Stability test

Accurately pipette the same test sample solution, let it stand at room temperature, and perform injection analysis at 0, 2, 4, 8, 12, 16, and 24 h under the chromatographic conditions specified in "Section 2.4.3". Record the peak areas and retention times, then calculate the relative standard deviation (RSD) percentage.

### 2.4.7 Determination of molding parameters for CEHG

The extract powder was prepared according to the optimal process parameters. For the preparation of CEHG, the extract powder was first mixed with a diluent (mannitol: maltodextrin = 1:1) and divided into two equal parts. Anhydrous citric acid was added to one part, and sodium bicarbonate was added to the other. The ethanol solution was used as the binder for wet granulation of each part separately; after drying, the granules were mixed uniformly and sized. The amount of extract was fixed at 10 g, whereas the amounts of diluent and binder and the volume fraction of ethanol were varied, with the molding rate used as an index to optimize the process parameters. Based on preliminary experiments, the amount of diluent, the volume fraction of ethanol, and the amount of binder were selected as optimization factors for the Box–Behnken experimental design, with 3 levels set for each. The response surface analysis was performed using Design-Expert v.13.0 software, including 12 factorial points and 5 center points, resulting in a total of 17 experiments. The design is shown in Table 3.

TABLE 3 Factors and levels of box—Behnken experiment.

| Level | (a) Amount of diluent/g | (b) Volume fraction of ethanol/% | (c) Amount of binder/% |
|---|---|---|---|
| −1 | 6 | 80 | 10 |
| 0 | 10 | 85 | 12 |
| 1 | 14 | 90 | 14 |

### 2.4.8 Verification of an optimal molding process and quality testing of the pulmonary protective effects of CEHG

After determining the optimal process, the validation experiments were conducted in triplicate. The prepared granules were subjected to quality inspection in accordance with the requirements for the granule agents (0104) specified in Volume III of the 2020 Chinese Pharmacopeia. The tests included measurements of particle size, moisture content, dissolving properties, and angle of repose.

## 2.5 Network pharmacology study of compound liver-protecting effervescent granules

### 2.5.1 Collection of active components and targets

Literature was reviewed to collect active components of Paeonia lactiflora, Glycyrrhiza uralensis, and Ganoderma lucidum. Their SDF (2D) structure files were downloaded from the PubChem (https://pubchem.ncbi.nlm.nih.gov/) platform and imported into the PharmMapper (https://lilab-ecust.cn/pharmmapper/submitfile.html) database to predict potential targets. Data with a normalized fit score ≥0.6 were selected to obtain the UniProt IDs of the targets for each component. These IDs were consolidated and duplicates were removed. Finally, the IDs were converted to gene names via the UniProt (https://www.uniprot.org/) website (21–23).

### 2.5.2 Collection of liver injury disease targets

Targets related to liver injury were collected by searching the GeneCards (https://www.genecards.org/) database using the keyword "Liver injury" (24). The exported data were filtered in

Excel to select target information with a relevance score of at least twice the median value of the relevance scores.

### 2.5.3 Intersection of active components and liver injury targets

Online tool Venny 2.1.0 (https://bioinfogp.cnb.csic.es/tools/venny/index.html) was used to find the intersection of active component targets and liver injury target genes, and to create a Venn diagram. These intersectional genes (or "genes from the intersection"), regarded as potential targets for the granules against liver injury, were used for further analysis.

### 2.5.4 Construction of active component–target–disease network

Cytoscape 3.9.1 was used to construct the target network of active components and visualize the relationships between active components, targets, and diseases in the form of an "Active Component–Target–Disease" network diagram. A network topology analysis was performed, and the network style was set based on node degrees. The importance of nodes in the network was reflected by their degree values: the higher the degree value, the larger the node and the darker its color.

### 2.5.5 PPI network construction and analysis

The intersecting targets were imported into the STRING (http://string-db.org) platform to construct a PPI network, with the organism set to Homo sapiens and a confidence score >0.7 (25). After removing isolated nodes, the network was imported into Cytoscape 3.9.1. Network topology analysis was performed using the CentiScape 2.2 plugin, and key targets were identified based on Degree, Betweenness Centrality (BC), and Closeness Centrality (CC) thresholds.

### 2.5.6 GO and KEGG enrichment analyses

The intersecting targets were imported into the DAVID (https://davidbioinformatics.nih.gov/) database for GO and KEGG pathway enrichment analyses, with the species set to Homo sapiens and a significance threshold of $P < 0.01$ (26). The top 20 entries were selected and visualized as bar and bubble charts using the WeBioinformatics (http://www.bioinformatics.com.cn/) platform, and the results were analyzed (27).

### 2.5.7 Molecular docking

Obtain the SDF files of the 2D structures of the screened core components from the PubChem database, then import the SDF files into Chem3D software for energy optimization and conversion into 3D structures; Download the 3D structures of key targets from the PDB database, perform molecular docking using AutoDock software and related plugins, and plot the results using PyMOL software.

## 2.6 Pharmacological study of CEHG on carbon tetrachloride-induced liver injury in rats

### 2.6.1 Animal grouping and modeling

After 7 days of adaptive feeding in an SPF-grade environment (temperature 23 °C ± 2 °C, 12 h light-dark cycle), 60 rats were randomly divided into 6 groups (10 rats per group). The doses were determined based on the conversion of the human body surface to the rat body area, with low, medium, and high doses set at 5, 10, and 20-fold the human recommended dose, respectively. Carbon tetrachloride (CCl4) (160 mg/kg·BW, dissolved in olive oil) was administered by gavage. Silymarin served as a positive control drug. The experimental groups were as follows: control group (A), model group (B), low-dose group (2.7 g/kg) (C), medium-dose group (5.4 g/kg) (D), high-dose group (10.8 g/kg) (E), and silymarin group (1 g/kg) (F).

The test groups received the samples by gavage daily, whereas the control group and the model group received distilled water. The rats were weighed twice a week to adjust the dose. On day 30, after 16 h of fasting, the model group and the test groups received a single gavage of CCl4, the control group received olive oil, and the test groups continued their respective treatments until the end of the experiment (with a 4-h interval after the gavage with $CCl_4$). Twenty-four hours after CCl4 administration, rats were anesthetized with 20% urethane (0.5 mL/100 g), blood was collected for serum separation, and the rats were euthanized by cervical dislocation before the livers were harvested for further analysis.

### 2.6.2 Pathological histological examination (HE) of the liver

Pathological examination of liver tissue was performed using HE staining of paraffin sections: Paraffin-embedded liver sections were dewaxed with xylene three times (15 min each), then sequentially hydrated with 100%, 95%, and 80% ethanol (5 min each); rinsed under running water until no alcohol remained and the sections were clean and transparent. Sampless were stained with hematoxylin for 3–5 min, rinsed with water, differentiated in 1% hydrochloric acid alcohol for several seconds, then rinsed with water again; treated with dilute lithium carbonate solution for 30 s to restore blue color, rinsed with water, dehydrated with 80% ethanol, and stained with alcohol-soluble eosin for 20 s. The sections were placed in 95% ethanol I and II for about 10 s each for color adjustment, dehydrated in anhydrous ethanol I and II for 1–2 min each, and finally cleared in xylene I and II for 1–2 min each, mounted, and observed under a microscope.

### 2.6.3 Determination of biochemical indicators in rat serum

Rat blood was collected and allowed to stand for 30 min to 1 h for coagulation. The blood was then centrifuged at 4,000 rpm for 10 min to separate the serum, and the supernatant was collected for analysis. Commercial kits were used to measure the levels of ALT, AST, TG, TC, LDH, TP, TBIL, TNF-α, IL-1β, and IL-6 in rat serum.

### 2.6.4 Determination of biochemical indicators in rat liver homogenate

Liver tissue was homogenized using PBS (0.01 M, pH 7.4). The homogenate was centrifuged at 10,000 rpm for 10 min at 4 °C and the supernatant was collected on ice. Commercial kits were used to measure the levels of SOD, MDA, GSH, and GSH-Px in the liver homogenate.

### 2.6.5 Detection of liver tissue ROS levels by flow cytometry

The tissue was homogenized using the homogenate solution according to the ROS kit instructions. After centrifugation, 200 μl of supernatant was retrieved, mixed with 2 μl of probe, and incubated at 37 °C for 30 min. During incubation, cells were mixed to ensure full probe binding. Part of the supernatant was used for protein content determination. After protein measurement, the sample was filtered through a 200-mesh filter and tested using a flow cytometer. The results were analyzed using NovoExpress software.

### 2.6.6 Detection of apoptotic cells in liver tissue by TUNEL assay

Liver tissue sections were dewaxed with xylene three times (15 min each), then sequentially hydrated with 100%, 95%, and 80% ethanol (5 min each); and rinsed under running water until clean and transparent. The sections were treated with 20 μg/ml proteinase K (without DNase) at 37 °C for 20 min, then washed with PBS three times. The TUNEL detection solution (50 μl; TdT enzyme + Biotin-dUTP=5 μl + 45 μl) was added to each sample, and incubated at 37 °C for 60 min. After washing with PBS three times, 100 μl of reaction termination solution was added and the samples were incubated at room temperature for 10 min. Following 3 washes with PBS, 100 μl of Streptavidin-HRP working solution (0.5 μl + 99.5 μl) was added and the samples were incubated at room temperature for 30 min. After the final three washes with PBS, color was developed using DAB at room temperature for 8 min and the sections were rinsed three times with PBS, counterstained with hematoxylin, to differentiate and restore blue color. Finally, the sections were dehydrated and cleared, mounted with neutral gum, and observed under a microscope.

### 2.6.7 Detection of apoptotic cells by flow cytometry

Liver tissue samples were ground on ice, centrifuged at 3,000 rpm for 3 min at 4 °C, and the supernatant was collected. Cells were collected at a density of $1–2\times10^6$ cells, centrifuged at 1,500 rpm for 3 min, and the supernatant was discarded. The cell suspension was then mixed with 2.5 μl of Annexin V-FITC Reagent and 2.5 μl of PI Reagent (50 μg/ml). After gentle vortexing, the mixture was incubated at room temperature in the dark for 15–20 min. Next, 400 μl of diluted 1× Annexin V Binding Buffer was added and thoroughly mixed. The sample was filtered through a 200-mesh filter and analyzed using a flow cytometer. The flow cytometry images of the apoptotic cells were analyzed using NovoExpress software.

TABLE 4 PCR primer sequences.

| Primer | Length (bp) | Forward primer 5′3′) | Reverse primer 5′3′) |
|---|---|---|---|
| Rat-β-actin | 150 | CCCATCTATGAGGGTTACGC | TTTAATGTCACGCACGATTTC |
| Rat-P53 | 85 | GGGAATGGGTTGGTAGTTGC | TTTCACTGTAGGTGCCAGGT |
| Rat-Bax | 85 | TCATCCAGGATCGAGCAGAG | TTCTTGGTGGATGCGTCCTG |
| Rat-Bcl-2 | 95 | GCATGCGACCTCTGTTTGAT | CAGGTATGCACCCAGAGTGA |
| Rat-HIF-1α | 121 | CGCAGTGTGGCTACAAGAAA | AGGCTGTGTCGACTGAGAAA |

TABLE 5 Orthogonal experiment results.

| No. | dry extract ratio/% | Albiflorin/% | Paeoniflorin/% | Liquiritin/% | Glycyrrhizic acid/% | Ganoderic acid A/% |
|---|---|---|---|---|---|---|
| 1 | 13.93 | 1.35 | 5.19 | 0.42 | 1.28 | 0.09 |
| 2 | 21.62 | 0.86 | 4.89 | 0.47 | 0.93 | 0.08 |
| 3 | 22.74 | 1.11 | 5.02 | 0.44 | 1.19 | 0.09 |
| 4 | 16.32 | 0.95 | 4.75 | 0.45 | 1.04 | 0.09 |
| 5 | 20.39 | 1.11 | 5.07 | 0.35 | 0.92 | 0.08 |
| 6 | 21.83 | 0.99 | 5.22 | 0.38 | 0.89 | 0.05 |
| 7 | 18.61 | 1.20 | 5.30 | 0.38 | 1.36 | 0.14 |
| 8 | 17.88 | 1.09 | 5.42 | 0.35 | 1.21 | 0.12 |
| 9 | 22.95 | 0.90 | 4.76 | 0.44 | 1.39 | 0.16 |

### 2.6.8 RT-qPCR detection of P53, Bax, Bcl-2, and HIF-1α mRNA expression

Liver tissue processing: 50–100 mg of liver tissue was ground to powder in liquid nitrogen, and 1 mL of TRIzol was added to lyse the cells. After the addition of 0.2 ml of chloroform, the samples were vortexed for 15 s, and incubated on ice for 5 min; and centrifuged at 12,000 rpm for 10 min at 4 °C.

RNA extraction and cDNA synthesis: the supernatant was collected and the RNA was precipitated with isopropanol. The precipitate was washed with 75% ethanol before resuspending in 20–50 μl of DEPC-treated water. RNA was reverse-transcribed into cDNA using one-step first-strand synthesis.

Gene expression: The reaction system contained 10 μl of Taq SYBR Green qPCR Premix (Universal), 0.4 μl each of forward/reverse primers (10 μm), 3 μl of cDNA, and 6.2 μl of RNase-free water, with a total volume of 20 μl. The cycling conditions were as follows: pre-denaturation at 95 °C for 30 s; 40 cycles (95 °C for 15 s, 60 °C for 30 s). Relative quantification was performed using the $2^{-\Delta\Delta Ct}$ method, and primer sequences are listed in Table 4.

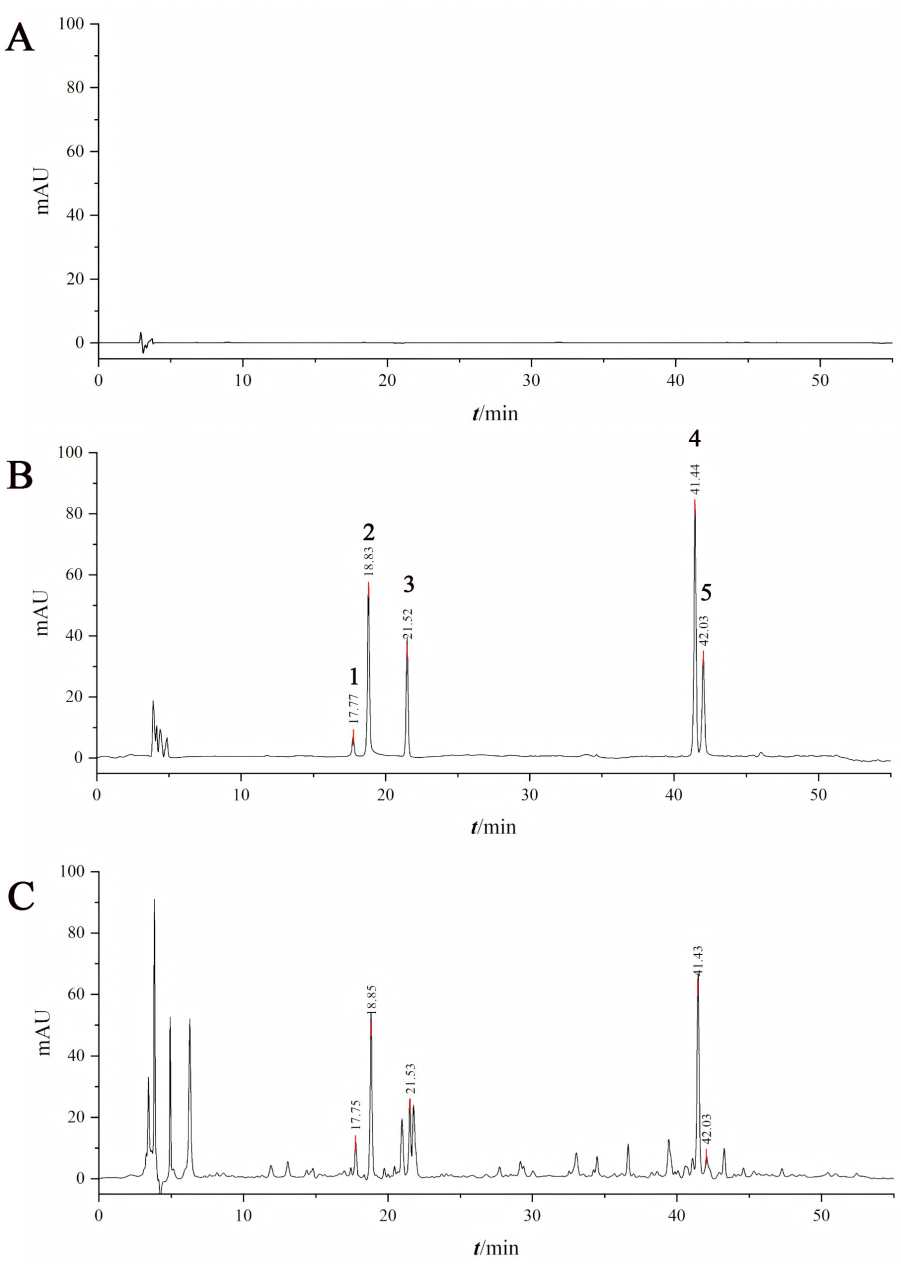



FIGURE 1
Determination of active ingredient content by HPLC. [**A**: 80% methanol; **B**: Mixed reference standards (1: paeoniflorin, 2: paeonolide, 3: liquiritin, 4: ammonium glycyrrhizinate, 5: ganoderic acid A); **C**: Test sample].

### 2.6.9 Western blot (WB) analysis of protein expression of p53, Bax, Bcl-2, and HIF-1α

Liver tissue samples of 50–100 mg were homogenized in RIPA lysis buffer (containing 1 mM PMSF) at a 1:100 ratio and incubated on ice for 30 min. Samples were centrifuge at 12,000 rpm for 15 min at 4 °C to collect the supernatant as total protein. Protein concentration was determined using the BCA assay kit according to the instructions. 5× SDS-PAGE loading buffer was added to protein samples at a 1:4 ratio. Samples were heated in boiling water for 10 min to denature the proteins and then loaded onto a 10% SDS-PAGE gel for electrophoresis for 1 h. The proteins were transferred to a PVDF membrane. The membrane was blocked with 5% skim milk for 2 h, and after discarding the blocking solution, the membranes were washed with TBST. Ther membrane was incubated overnight at 4 °C with primary antibodies diluted as follows: p53 (1:1,000), Bax (1:1,000), Bcl-2 (1:1,000), HIF-1α (1:500), and β-actin (1:1,000). After washing, the secondary antibody (1:10,000) was incubated for 2 h. Proteins were detected using an ECL hypersensitive luminescent reagent kit and the grayscale values of the bands were analyzed with ImageJ software. The relative protein expression levels were determined based on by the grayscale densitometric values of β-actin bands.

TABLE 6 Weight coefficients.

| Indicator | AHP-based subjective weight coefficients | entropy-based objective weight coefficients | Combined weight coefficients |
|---|---|---|---|
| Albiflorin | 31.85% | 17.17% | 33.23% |
| Paeoniflorin | 31.85% | 9.64% | 18.66% |
| Liquiritin | 12.91% | 36.01% | 28.26% |
| Glycyrrhizic acid | 12.91% | 17.18% | 13.48% |
| Ganoderic acid A | 5.24% | 10.33% | 3.29% |
| Dry extract ratio | 5.24% | 9.68% | 3.08% |

TABLE 7 Comprehensive scoring results of the orthogonal test table.

| No. | Factors | | | Comprehensive score |
|---|---|---|---|---|
| | A | B | C | |
| 1 | 1:10 | 1 | 1 | 71.59 |
| 2 | 1:10 | 2 | 3 | 26.21 |
| 3 | 1:10 | 3 | 2 | 54.18 |
| 4 | 1:15 | 1 | 3 | 25.15 |
| 5 | 1:15 | 2 | 2 | 25.62 |
| 6 | 1:15 | 3 | 1 | 38.90 |
| 7 | 1:20 | 1 | 2 | 74.45 |
| 8 | 1:20 | 2 | 1 | 63.52 |
| 9 | 1:20 | 3 | 3 | 46.76 |
| K1 | 151.980 | 171.190 | 174.010 | |
| K2 | 89.670 | 115.350 | 154.250 | |
| K3 | 184.730 | 139.840 | 98.120 | |
| k1 | 50.660 | 57.063 | 58.003 | |
| k2 | 29.890 | 38.450 | 51.417 | |
| k3 | 61.577 | 46.613 | 32.707 | |
| R | 31.687 | 18.613 | 25.297 | A>C>B |

## 2.7 Statistical analysis

The experimental data were analyzed using GraphPad Prism v.9.4.1. Data are presented as mean ± standard deviation ($x \pm s$). For comparisons involving multiple groups, one-way analysis of variance (ANOVA) was used. If homogeneity of variances was confirmed, LSD tests were applied for pairwise comparisons. If the variances were heterogeneous, Dunnett-$t$ tests were used instead. A $P$-value $< 0.05$ was considered statistically significant.

# 3 Results

## 3.1 Optimization of the extraction process based on orthogonal experiments and the AHP-entropy method

### 3.1.1 Optimization of the extraction process

The dry extract ratio and the contents of albiflorin, paeoniflorin, liquiritin, glycyrrhizic acid, and ganoderic acid A in the extract were determined, with the results presented in Table 5 and Figure 1.

The subjective weight coefficients based on AHP, the objective weight coefficients based on entropy, and the combined weight coefficients for the ratio of paeoniflorin, albiflorin, glycyrrhizic acid, liquiritin, ganoderic acid A and dry extract are shown in Table 6.

After standardization of orthogonal experimental data, the significance of the influence of factors on the results was

TABLE 8 ANOVA of orthogonal experiment.

| Source of variance | Sum of squares | Degrees of freedom | Mean square | $F$ value | Significance |
|---|---|---|---|---|---|
| A | 1554.611 | 2 | 777.306 | 443.800 | 0.002 |
| B | 522.299 | 2 | 261.149 | 149.102 | 0.007 |
| C | 1033.370 | 2 | 516.685 | 294.999 | 0.003 |
| Error | 3.503 | 2 | 1.751 | | |

determined through a range and variance analysis. The intuitive analysis of the R range showed that the primary and secondary factors that affected the comprehensive extraction score were A > C > B (the solid-liquid ratio had the greatest influence, followed by the extraction time, and finally the extraction times). As shown in Tables 7A–C all had significant effects on the complete score ($P < 0.05$). The optimal scheme was A3B1C1 (water addition ratio 1:20, extracted once, 1 h each time). The results of the range analysis and variance analysis are shown in Tables 7, 8, respectively.

### 3.1.2 Investigation of precision, repeatability and stability

For the five components (paeoniflorin lactone, paeoniflorin, liquiritin, ammonium glycyrrhizinate, and ganoderic acid A), in the precision investigation, the peak area RSD% values were all in the range of 0.30%−1.63% (all <2%), and the retention time RSD% values were all in the range of 0.01%−0.09% (all <0.10%), indicating good precision of the instrument; in the repeatability investigation, the peak area RSD% values were all in the range of 0.24%−1.24% (all <2%), and the retention time RSD% values were all in the range of 0.03%−0.11% (all <0.20%), indicating good repeatability of the method; in the stability investigation, within 24 h, the peak area RSD% values were all in the range of 0.67%−1.71% (all <2%), and the retention time RSD% values were all in the range of 0.02%−0.30% (all <0.30%), indicating good stability of the test sample within 24 h.

### 3.1.3 Validation of the extraction process

The results of the validation testing are presented in Table 9. Three batches of parallel tests showed similar index component content and dry extract rates, with RSD values below 5%. This indicated that the extraction process was stable and feasible, with excellent reproducibility.

### 3.1.4 Optimization of the molding process

The results and analysis of the Box–Behnken experiment are shown in Tables 10, 11. The Box–Behnken experiment yielded a second order polynomial regression equation for the molding rate $Y = 84.03 - 0.94a - 10.26b + 1.83c - 1.33ab + 0.098ac + 2.13bc - 1.03a^2 - 0.17b^2 - 1.85c^2$. As shown in the table below, the model's $F$ value was 67.36 and $P < 0.05$, indicating that the model terms were statistically significant. The lack of fit item had a $P$-value > 0.05, indicating a good fit of the regression equation. $R^2 = 0.9886$ and $Radj^2 = 0.9739$, which indicated that the model had a good fit. Independent variables effectively explained the changes in the response variable. The similar $R^2$ and $Radj^2$ values suggested that the independent variables introduced were valid. Based on the F value, the significant effects on the formation rate were in the order of $b > c > a$. In the regression model, the linear terms $b$ and $c$, the quadratic term $c^2$, and the interaction term $bc$ were significant, whereas the linear term $a$, the quadratic terms $a^2$ and $b^2$, and the interaction terms $ab$ and $ac$ were not significant. The optimized forming process parameters were as follows: 11 parts of diluent, 12% binder, and 80% ethanol volume fraction.

### 3.1.5 Validation and testing of the optimal molding process of granule quality

Three tests showed a particle formation rate of 95.42%, 96.33%, and 94.76%. The moisture of the CEHG was 5.44%, below 8%. The dissolving time was within 5 min, meeting the Chinese

TABLE 10 Factors and levels table of box—Behnken experimental design.

| No. | (a) Amount of diluent/g | (b) Volume fraction of ethanol/% | (c) Amount of binder/% | Molding rate/% |
|---|---|---|---|---|
| 1 | 14 | 80 | 12 | 94.23 |
| 2 | 10 | 80 | 10 | 91.67 |
| 3 | 10 | 85 | 12 | 83.96 |
| 4 | 10 | 90 | 10 | 67.52 |
| 5 | 6 | 85 | 14 | 83.67 |
| 6 | 10 | 85 | 12 | 85.07 |
| 7 | 6 | 85 | 10 | 81.40 |
| 8 | 14 | 85 | 10 | 78.44 |
| 9 | 10 | 90 | 14 | 76.62 |
| 10 | 6 | 90 | 12 | 74.09 |
| 11 | 14 | 85 | 14 | 81.10 |
| 12 | 10 | 85 | 12 | 84.87 |
| 13 | 14 | 90 | 12 | 70.45 |
| 14 | 10 | 80 | 14 | 92.27 |
| 15 | 6 | 80 | 12 | 92.56 |
| 16 | 10 | 85 | 12 | 84.14 |
| 17 | 10 | 85 | 12 | 82.12 |

TABLE 9 Validation experiment results.

| No. | Albiflorin/% | Paeoniflorin/% | Liquiritin/% | Glycyrrhizic acid/% | Ganoderic acid A/% | Dry extract ratio/% |
|---|---|---|---|---|---|---|
| 1 | 1.25 | 5.44 | 0.42 | 1.28 | 0.14 | 18.63 |
| 2 | 1.28 | 5.37 | 0.44 | 1.31 | 0.15 | 17.86 |
| 3 | 1.24 | 5.46 | 0.43 | 1.32 | 0.14 | 18.21 |
| Mean | 1.26 | 5.42 | 0.43 | 1.30 | 0.14 | 18.23 |
| RSD% | 1.66 | 0.87 | 2.32 | 1.60 | 4.03 | 2.11 |

TABLE 11 Analysis of variance of molding rate.

| Source of variance | Sum of squares | Degrees of freedom | Mean square | F-value | *P*-value | Significance |
|---|---|---|---|---|---|---|
| Model | 920.66 | 9 | 102.30 | 67.36 | <0.0001 | Significant |
| A | 7.03 | 1 | 7.03 | 4.63 | 0.0684 | |
| B | 841.53 | 1 | 841.53 | 554.11 | <0.0001 | * |
| C | 26.75 | 1 | 26.75 | 17.62 | 0.0040 | * |
| AB | 7.05 | 1 | 7.05 | 4.64 | 0.0682 | |
| AC | 0.0380 | 1 | 0.0380 | 0.0250 | 0.8787 | |
| BC | 18.06 | 1 | 18.06 | 11.89 | 0.0107 | * |
| $A^2$ | 4.50 | 1 | 4.50 | 2.96 | 0.1290 | |
| $B^2$ | 0.1160 | 1 | 0.1160 | 0.0764 | 0.7902 | |
| $C^2$ | 14.35 | 1 | 14.35 | 9.45 | 0.0180 | * |
| Residuals | 10.63 | 7 | 1.52 | | | |
| Lack of fit | 5.18 | 3 | 1.73 | 1.27 | 0.3985 | |
| Pure error | 5.45 | 4 | 1.36 | | | |
| Total error | 931.29 | 16 | | | | |

*$P < 0.05$, which means the result is significant.

Pharmacopeia requirements. With a resting angle of <40°, the flow ability of the particles satisfied basic production needs.

## 3.2 Results of the network pharmacology study on CEHG

A total of 121 bioactive components were identified. After importing into the PharmMapper database for summarization, screening, and deduplication, 1,486 potential targets were identified. Through the GeneCards database, 2,764 targets related to "liver injury" were retrieved. The intersection of the active component target and the liver injury disease target, displayed by a Venny diagram, showed 199 overlapping targets (Figure 2a). The active component-target-disease network diagram revealed key components such as palmitic acid ergosta-7,22-diene-3β-ol ester, pentadecanoic acid ergosta-7,22-diene-3β-ol ester, and paeoniflorin, as well as key targets, including F2, PRPS1 and SDHA (Figure 2b). Based on Degree Centrality (DC), Betweenness Centrality (BC), and Closeness Centrality (CC) thresholds, the analysis of the PPI network identified key nodes such as CTNNB1, ALB, and EP300 (Figures 2c, d). GO enrichment analysis yielded 653 entries ($P < 0.01$), including 445 in BP, 73 in CC, and 135 in MF, with the top 20 entries selected (Figures 3a–c). KEGG pathway enrichment analysis identified 114 signaling pathways ($P < 0.01$), and the top 20 were visualized, which involved signaling pathways such as HIF-1, p53, and FoxO (Figure 3d). Molecular docking was performed between three key components (paeoniflorin, ergosta-7,22-dien-3β-yl palmitate, and ergosta-7,22-dien-3β-yl pentadecanoate) and three key target proteins (ALB, EP300, and CTNNB1). The binding energy of each drug molecule to the corresponding protein was <-5 $kJ \cdot mol^{-1}$, indicating that relatively stable binding can be formed between the drug molecules and the proteins. These drug molecules have the potential to bind to the target proteins, possibly regulate their functions, and thereby exert therapeutic effects on diseases. The molecular docking results are shown in Figure 4.

## 3.3 Pharmacological study of CEHG in the CCl4-induced acute liver injury rat model

### 3.3.1 Pathological changes induced by CEHG in rats with acute liver injury

HE staining showed that in the control group, rat liver cells were normal in size and shape, with large round nuclei and a clear structure. The hepatocyte cords were arranged radially around the central vein. In the model group of CCl4-induced acute liver injury, significant necrosis, steatosis, inflammatory infiltration, and hemorrhage was observed in liver tissue. As the dose of the granules increased, the liver damage eased and the morphology of the liver improved visibly. Silybin, a reference drug, also improved CCl4-induced liver injury, nearly restoring the tissue to its normal state (Figure 5).

### 3.3.2 Changes in AST, ALT, TC, TG, TP, TBIL, LDH, IL-6, IL-1β and TNF-α levels in rat serum

Compared with the control group, the model group showed significant increases in ALT and AST, indicating the successful establishment of liver injury models. Compared with the model group, the low, medium, and high dose groups had notable reductions in ALT and AST levels, demonstrating that the effervescent protective granules of compound liver could improve liver function indicators in rats with liver injury in a dose-dependent manner. The positive drug group also had much lower ALT and AST levels than the model group. Compared with the control group, the model group presented higher levels of

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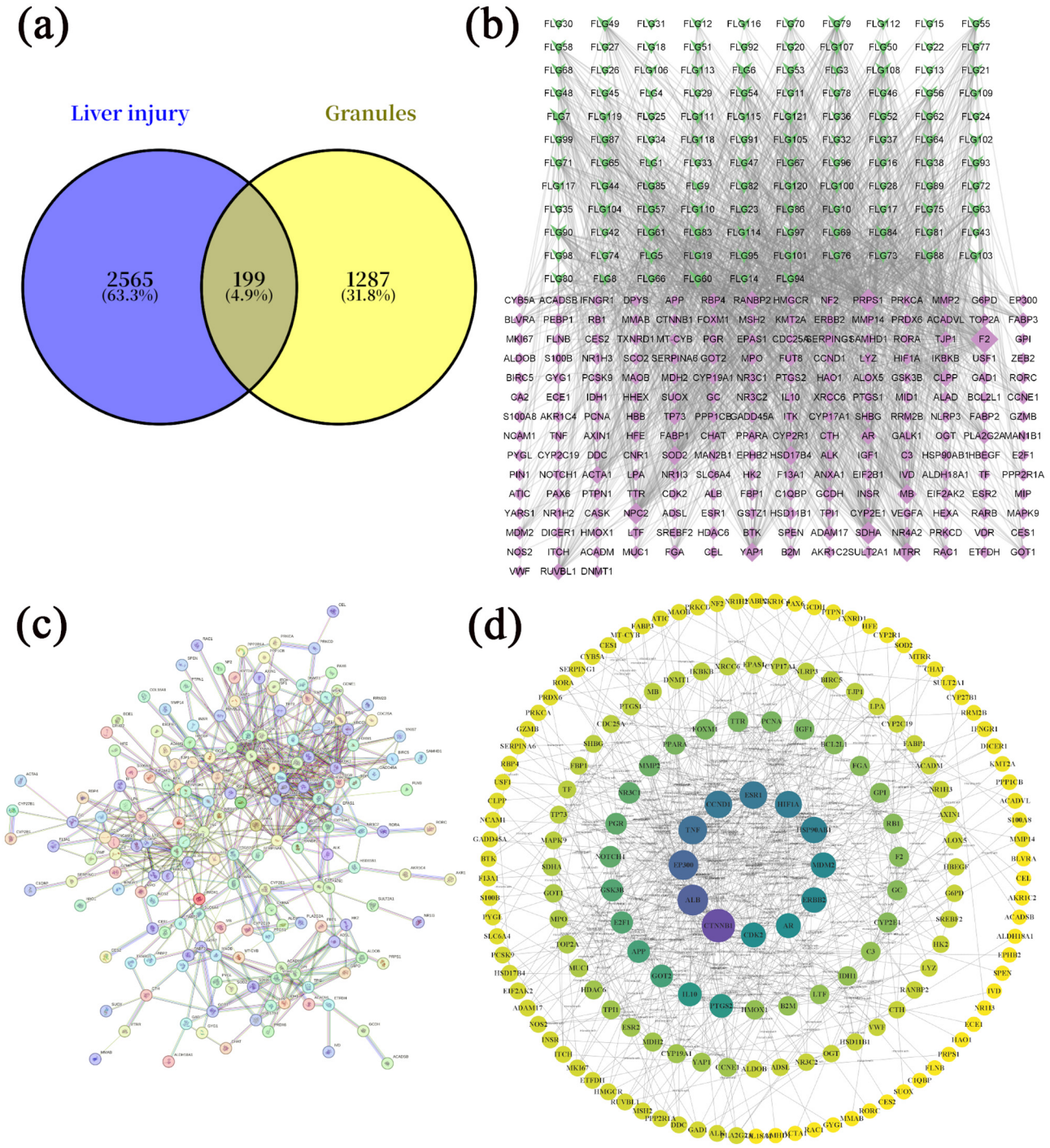



FIGURE 2
Network pharmacology study of compound liver-protecting effervescent granules (CEHG). **(a)** Intersection of active ingredients and target genes of liver injury; **(b)** network analysis of active ingredients-targets-diseases; **(c)** PPI network analysis an of potential targets for CEHG for the treatment of liver injury; **(d)** core targets map showing results of PPI network analysis of potential targets for CEHG.

TC, TG, TBIL, and LDH and significantly lower levels of TP. These changes confirmed the successful establishment of the liver injury model, reflecting lipid metabolic disorders, impaired hepatic synthetic function, and abnormal bilirubin metabolism due to liver injury. Compared with the model group, except for the low-dose group, which showed no significant changes in TP and LDH, all other dosing groups exhibited significantly reduced levels of TC, TG, TBIL, and LDH and significantly increased TP levels. After treatment, TC, TG, TBIL and LDH decreased with increasing doses, whereas TP increased with higher doses. Compared with

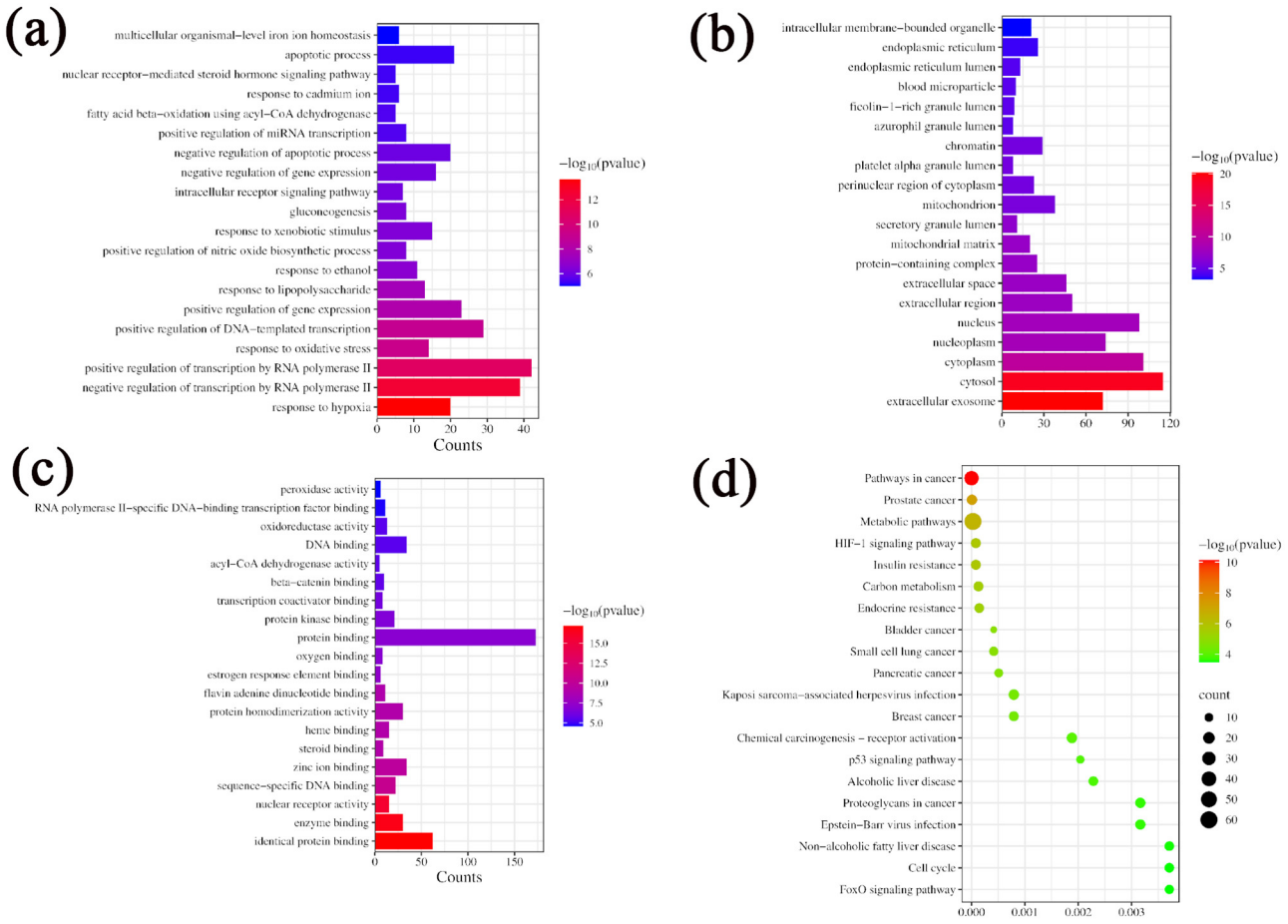



FIGURE 3
GO and KEGG enrichment analysis. **(a)** GO function enrichment map BP; **(b)** GO function enrichment map CC; **(c)** GO function enrichment map MF; **(d)** KEGG Enrichment Analysis.

the control group, the model group showed significantly increased levels of IL-6, IL-1β, and TNF-α. Compared with the model group, all treatment groups, except the low-dose group with non-significant changes in IL-6 levels, exhibited significantly reduced levels of IL-6, IL-1β, and TNF-α. Overall, the liver-protecting effervescent granules of the compound suppressed the release of IL-6, IL-1β, and TNF-α, thus alleviating the inflammatory response (Figure 6).

### 3.3.3 Changes in the contents of SOD, MDA, GSH, GSH-Px and ROS in rat liver tissue

Compared with the control group, the model group showed significantly reduced SOD and GSH-Px activity, increased MDA levels, and decreased GSH levels. This indicates a weakening of hepatic antioxidant capacity, increased oxidative stress, and severe damage to stem cell membranes in the liver injury model. Compared with the model group, all treatment groups (except for GSH content without significant difference) showed significantly increased SOD and GSH-Px activity and significantly decreased MDA levels and increased GSH levels. Thus, CEHG can protect the liver by enhancing antioxidant capacity and reducing oxidative stress (Figure 7a). Compared with the control group, the model group showed a significant increase in ROS levels in rat liver tissue. Compared with the model group, all treatment groups had markedly lower ROS levels. This indicated that the liver-protecting effervescent granules of CEHG reduced ROS in liver tissue from rats with chemical-induced liver injury, thus decreasing damage due to oxidative stress (Figures 7b, c).

### 3.3.4 Analysis of cell apoptosis in rat liver tissue

The results of the TUNEL assay revealed a significantly higher positivity rate in the hepatocytes of the model group than in the control group, indicative of intensified apoptosis. Compared with the model group, the low dose group showed a decrease in positivity rate, albeit not significant, whereas other medicated groups presented markedly lower positivity rates, signaling a substantial reduction in hepatocyte apoptosis. Flow cytometry apoptosis images were analyzed using NovoExpress software as follows: quadrant 1: necrotic cells; quadrant 2: late apoptotic cells; quadrant 3: normal cells; quadrant 4: early apoptotic cells. The

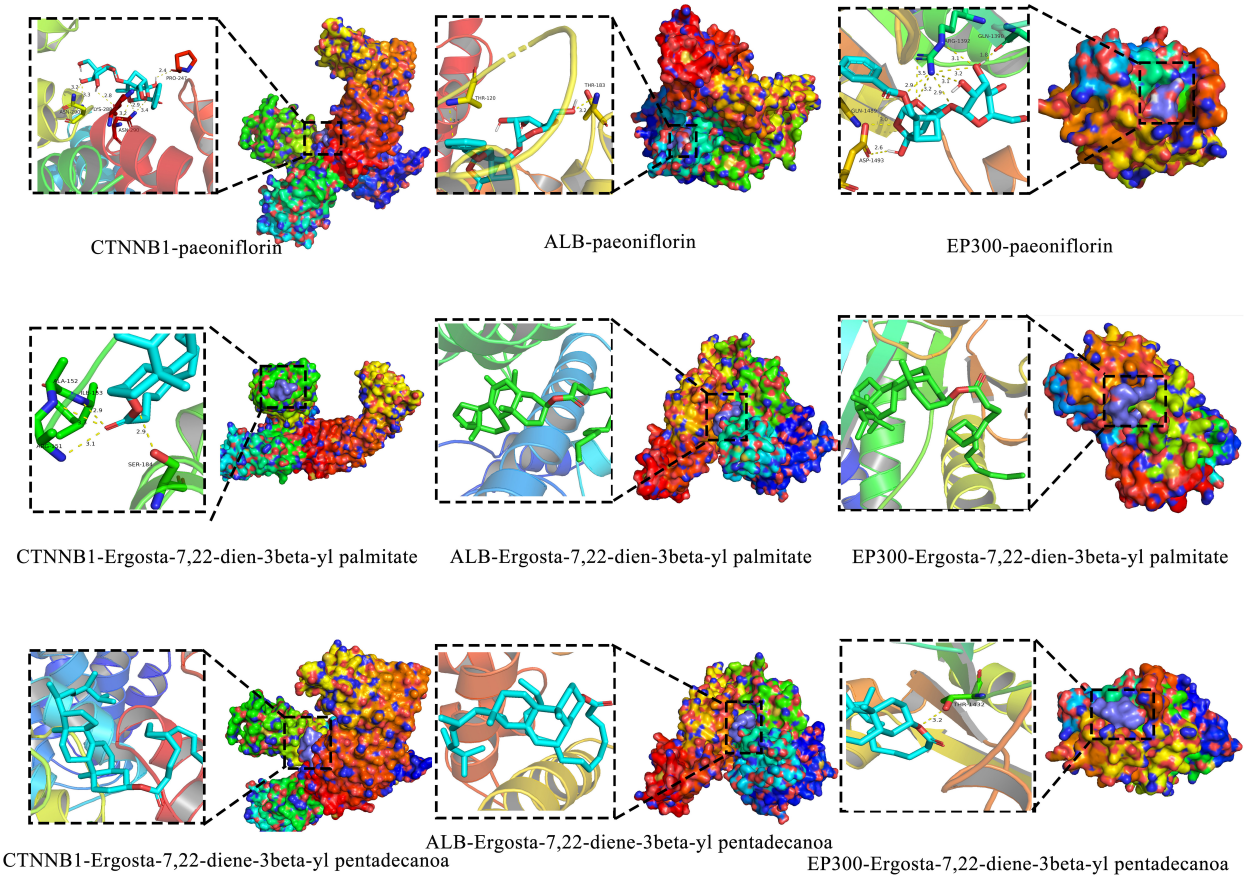



FIGURE 4
Molecular docking.

combined percentage of cells in quadrants 2 and 4 (late and early apoptotic cells) was calculated. The flow cytometry results showed a higher rate of apoptosis in the model group than in the control group (Figure 8). In contrast, the apoptosis rate was significantly lower in the treatment groups than in the model group, which was consistent with the findings of the TUNEL assay. Overall, these results demonstrate that CEHG can inhibit hepatocyte apoptosis in rats with liver injury, fulfilling their liver-protecting function.

### 3.3.5 Effects of CEHG on mRNA and protein expression of P53, Bax, Bcl-2, and HIF-1α

The results of RT-qPCR showed that, compared with the control group, the model group had a significant higher mRNA expression of P53, Bax, and HIF-1α, and a lower expression of Bcl-2 mRNA. Compared with the model group, the treatment groups had a significant decrease in P53, Bax, and HIF-1α mRNA levels and increased Bcl-2 mRNA levels.

The WB results showed that, compared with the control group, the model group had significantly higher expression of P53, Bax and HIF-1α protein, and decreased expression of protein Bcl-2. Compared with the model group, the dosed groups had significantly lower protein levels of P53, Bax, and HIF-1α protein levels but higher Bcl-2 levels (Figure 9).

## 4 Discussion

In this study, CEHG were developed using *Ganoderma lucidum, Paeonia lactiflora,* and *Glycyrrhiza glabra* as raw materials, with the aim of exploring their auxiliary protective effect against CLI. Based on TCM theory and modern pharmacological research, a multi-dimensional study was conducted to systematically optimize the preparation process and clarify the hepatoprotective mechanisms involved, providing a scientific basis for the development of functional hepatoprotective foods.

In the optimization of the extraction process, orthogonal experiments, characterized by advantages of reducing the number of experiments required, thus saving resources and time, combined with the determination of the contents of hepatoprotective active components via HPLC, laid the foundation for process screening. The AHP-entropy weight method (28), by integrating the subjective weights of AHP and the objective weights of the entropy method, balanced subjective and objective factors. The combination of the dry extract rate and active hepatoprotective components was used as the characterization index for the entire formula, and the generalized score was applied to more accurately reflect the

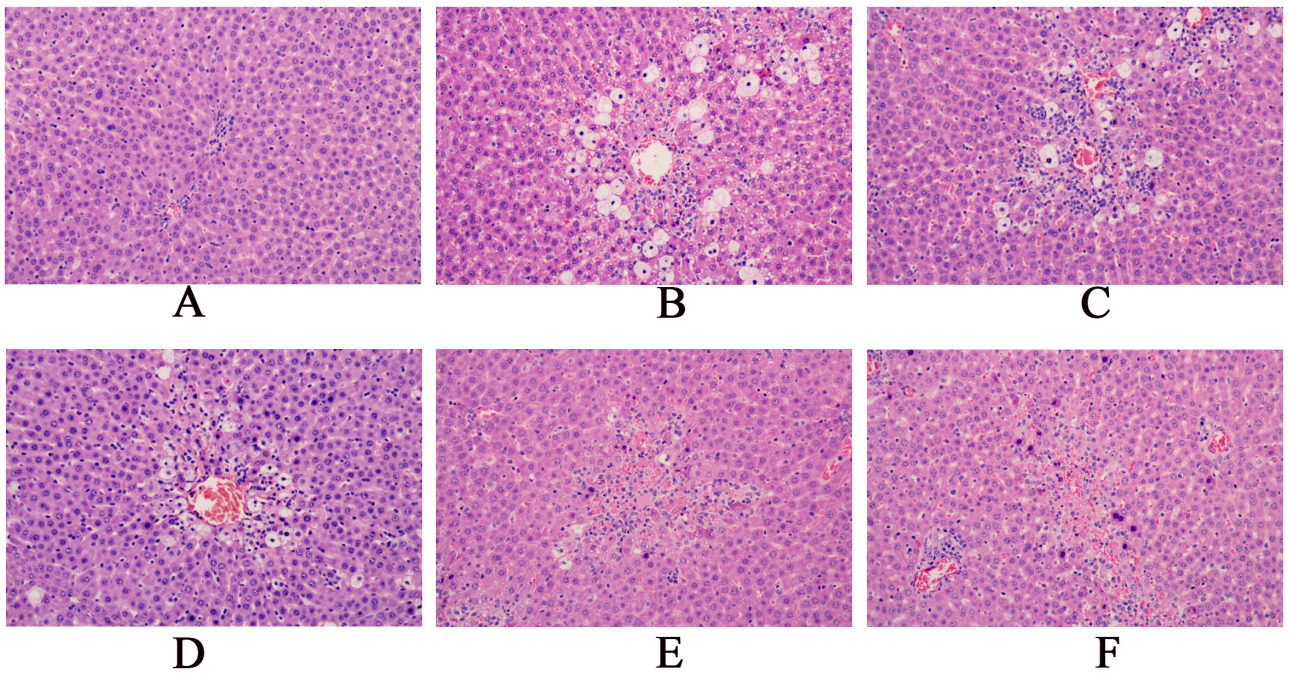



FIGURE 5
Pathological sections of the rat liver (magnification, x200).(**A**: Blank group; **B**: Model group; **C**: Compound Liver-Protecting Effervescent Granules low-dose group; **D**: Compound Liver-Protecting Effervescent Granules medium-dose group; **E**: Compound Liver-Protecting Effervescent Granules high-dose group; **F**: Silybin group).

extraction quality. Eventually, an optimal extraction process was determined as a material-to-liquid ratio of 1:20, with 1 extraction per 1 h. Verification experiments showed that this process was stable, feasible, and reproducible.

The Box–Behnken response surface method was adopted to optimize the granulation process (29). This efficient and flexible three-level experimental design, suitable for quadratic response surface models, enabled sufficient information to be obtained with fewer experiments. The results indicated that the interaction between ethanol volume fraction and binder dosage had a significant impact on granule formation. The optimized granulation parameters (11 parts of diluent, 12% binder, and 80% ethanol volume fraction) were verified to meet the requirements of the Chinese Pharmacopeia, suggesting that in actual production, strict control of the ethanol volume fraction and binder dosage was necessary to avoid quality fluctuations.

Network pharmacology studies provided theoretical clues for the hepatoprotective mechanism of the CEHG: 121 active components were subjected to target prediction and intersection analyses with liver injury disease targets, resulting in 199 overlapping targets involving key signaling pathways such as HIF-1 (30), p53 (31), and FoxO (32). Further analysis of the active component-target-disease network and PPI network identified core components including palmitic acid ergosta-7,22-diene-3β-ol ester and paeoniflorin, as well as key targets such as CTNNB1 and P53, suggesting that the granules may exert their effects through multiple components, multiple targets, and multiple pathways.

Animal experiments were used to verify the aforementioned mechanisms. In the CCl4-induced liver injury model, the granules dose-dependently reduced serum liver function indicators such as AST and ALT and improved pathological damage to liver tissue, indicating their ability to alleviate liver injury. Meanwhile, the granules significantly reduced serum levels of inflammatory factors, including IL-6, IL-1β, and TNF-α, suggesting that they exert protective effects by inhibiting inflammatory responses. In terms of oxidative stress (33), the granules increased the activities of SOD and GSH-Px, as well as the content of GSH in liver tissue, and reduced the levels of MDA and ROS, indicating their capacity to enhance antioxidant capacity and alleviate damage from oxidative stress. Cell apoptosis detection revealed that granules could reduce the proportion of apoptotic cells in liver tissue, down-regulate P53 and Bax mRNA and protein expression, and up-regulate Bcl-2 expression, suggesting that they inhibit excessive hepatocyte apoptosis by regulating apoptosis-related molecules. The down-regulation of HIF-1α indicated that the granules might improve the hypoxic state of the liver, further alleviating oxidative stress.

In summary, this study provided reliable parameters for the large-scale production of CEHG granules through process optimization. Combined network pharmacology and animal experiments revealed that granules exert hepatoprotective effects through multiple pathways such as anti-inflammation, anti-oxidation, inhibition of apoptosis, and regulation of HIF-1α expression, offering solid scientific support for their further development and clinical application. In future studies, we plan to conduct more in-depth research on

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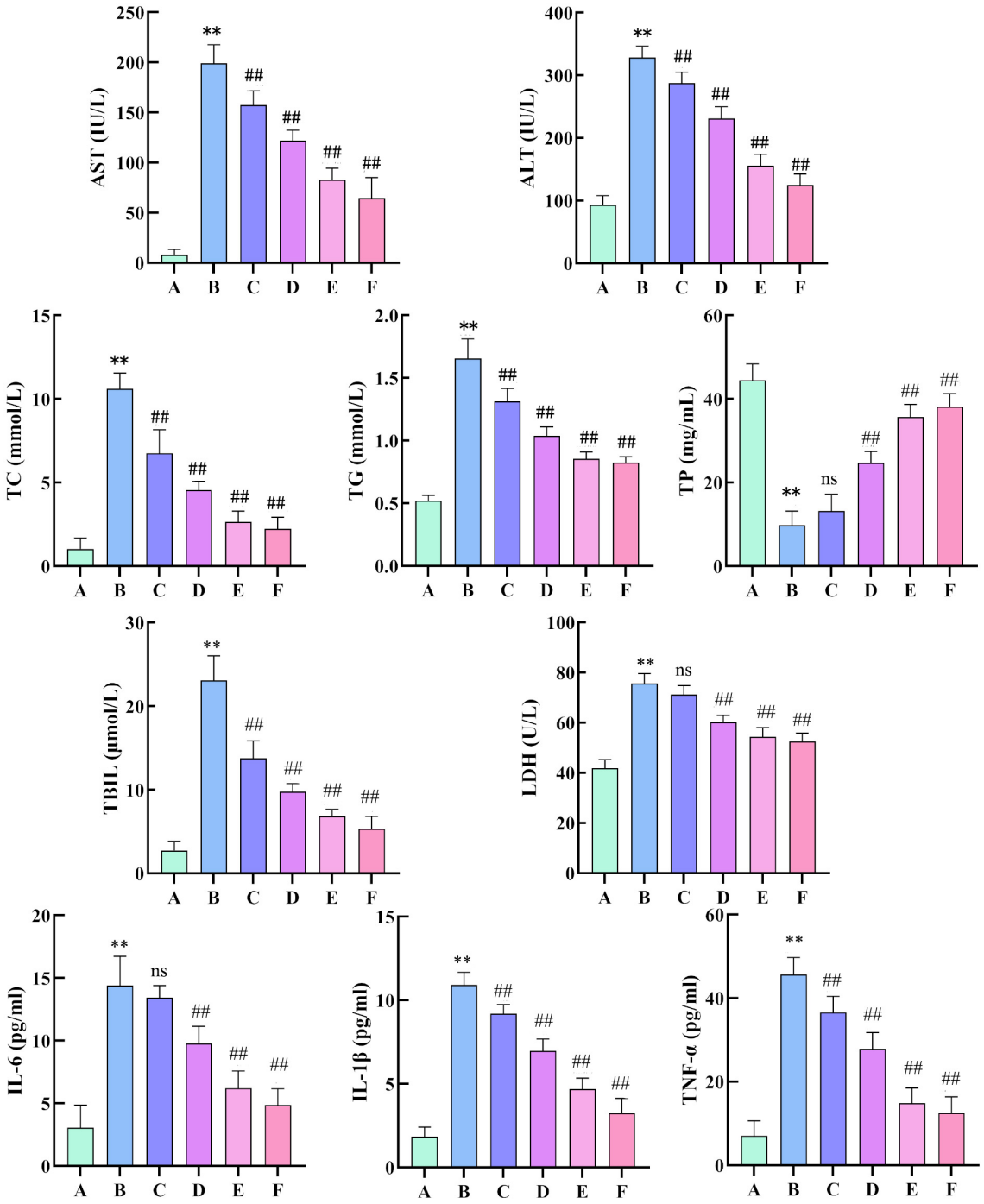



FIGURE 6
Changes in AST, ALT, TC, TG, TP, TBIL, LDH, IL-6, IL-1$\beta$ and TNF-$\alpha$ levels in the serum of rats. ($n = 8$, compared with the control group, $^{**}P < 0.01$, compared with the model group, $^{\#\#}P < 0.01$). (**A**: Blank group; **B**: Model group; **C**: Compound Liver-Protecting Effervescent Granules low-dose group; **D**: Compound Liver-Protecting Effervescent Granules medium-dose group; **E**: Compound Liver-Protecting Effervescent Granules high-dose group; **F**: Silybin group).

the underlying mechanism and increase the number of experimental replicates for the preparation process. This optimization is intended to improve the reproducibility and reliability of the experimental results. Additionally, we will add comparative experiments between effervescent granules and traditional dosage forms in terms of bioavailability, therapeutic efficacy, and other aspects to verify the advantages of effervescent granules.

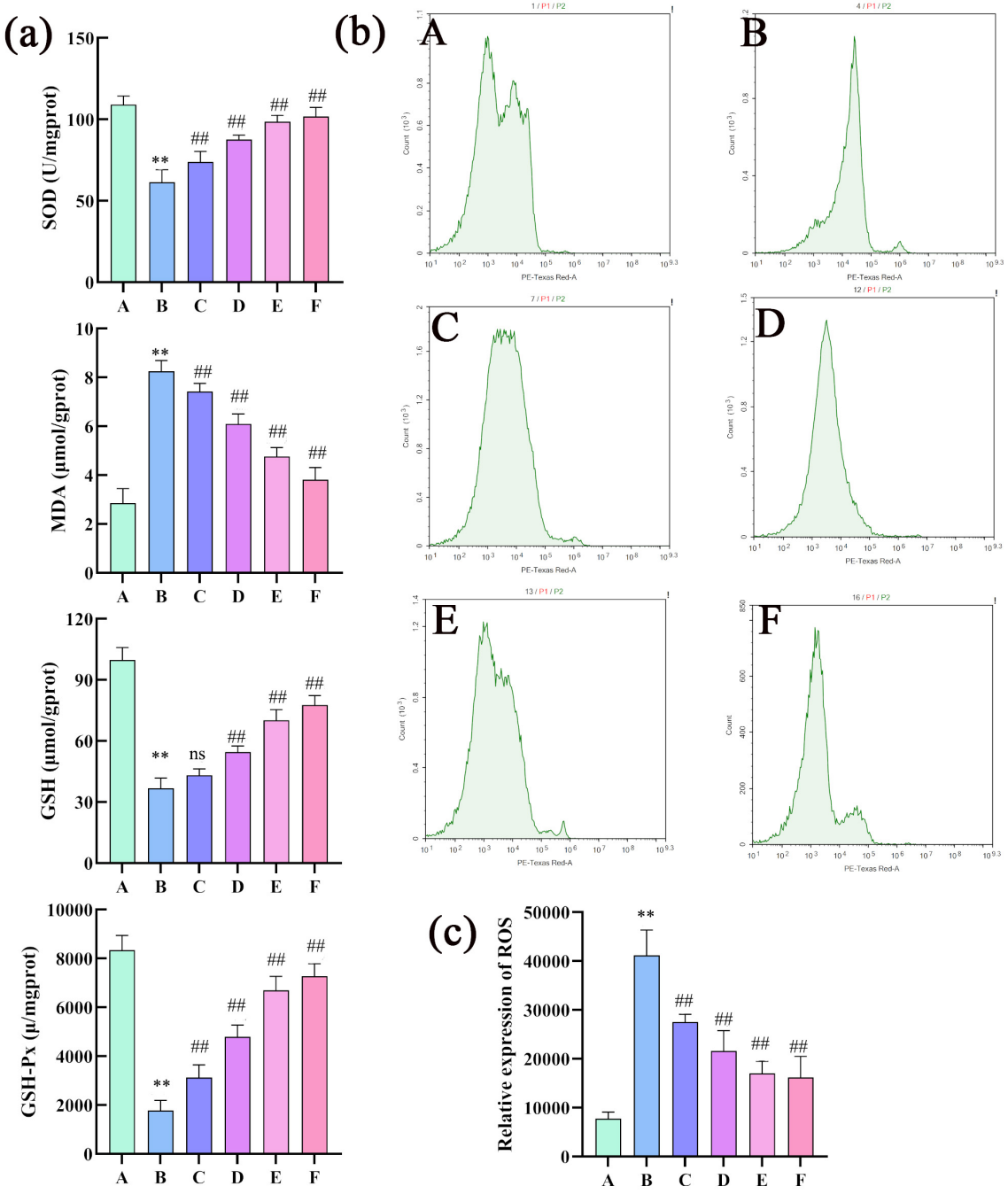



FIGURE 7
Changes in the SOD, MDA, GSH, GSH-Px, and ROS levels in rat liver tissue ($n = 8$; compared with the control group, $^{**}P < 0.01$; compared with the model group, $^{\#\#}P < 0.01$; ns indicates that comparisons were not significant. **(a)** Changes in the SOD, MDA, GSH and GSH-Px levels in rat liver tissues ($n = 8$; compared with the control group, $^{**}P < 0.01$; compared with the model group, $^{\#\#}P < 0.01$; ns indicates not significant); **(b)** changes in ROS levels in rat liver tissues; **(c)** quantitative analysis of ROS ($n = 3$ compared with the control group; $^{**}P < 0.01$, compared with the model group, $^{\#\#}P < 0.01$; ns indicates not significant). Changes in the SOD, MDA, GSH, GSH-Px, and ROS levels in rat liver tissue ($n = 8$); compared with the control group, $^{**}P < 0.01$; compared with the model group, $^{\#\#}P < 0.01$; ns indicates that comparisons were not significant. **(a)** Changes in the SOD, MDA, GSH and GSH-Px levels in rat liver tissues ($n = 8$; compared with the control group, $^{**}P < 0.01$; compared with the model group, $^{\#\#}P < 0.01$; ns indicates not significant); **(b)** changes in ROS levels in rat liver tissues; **(c)** quantitative analysis of ROS (n = 3 compared with the control group; $^{**}P < 0.01$, compared with the model group, $^{\#\#}P < 0.01$; ns indicates not significant).

## 5 Conclusion

This study determined the parameters for the extraction and molding process of CEHG, a hepatoprotective functional food. CEHG improved liver pathology, function indices, oxidative stress, and cell apoptosis of rats with CCl4-induced acute liver injury and regulated P53, Bax, Bcl-2, and HIF-1α expression to exert hepatoprotective effects.

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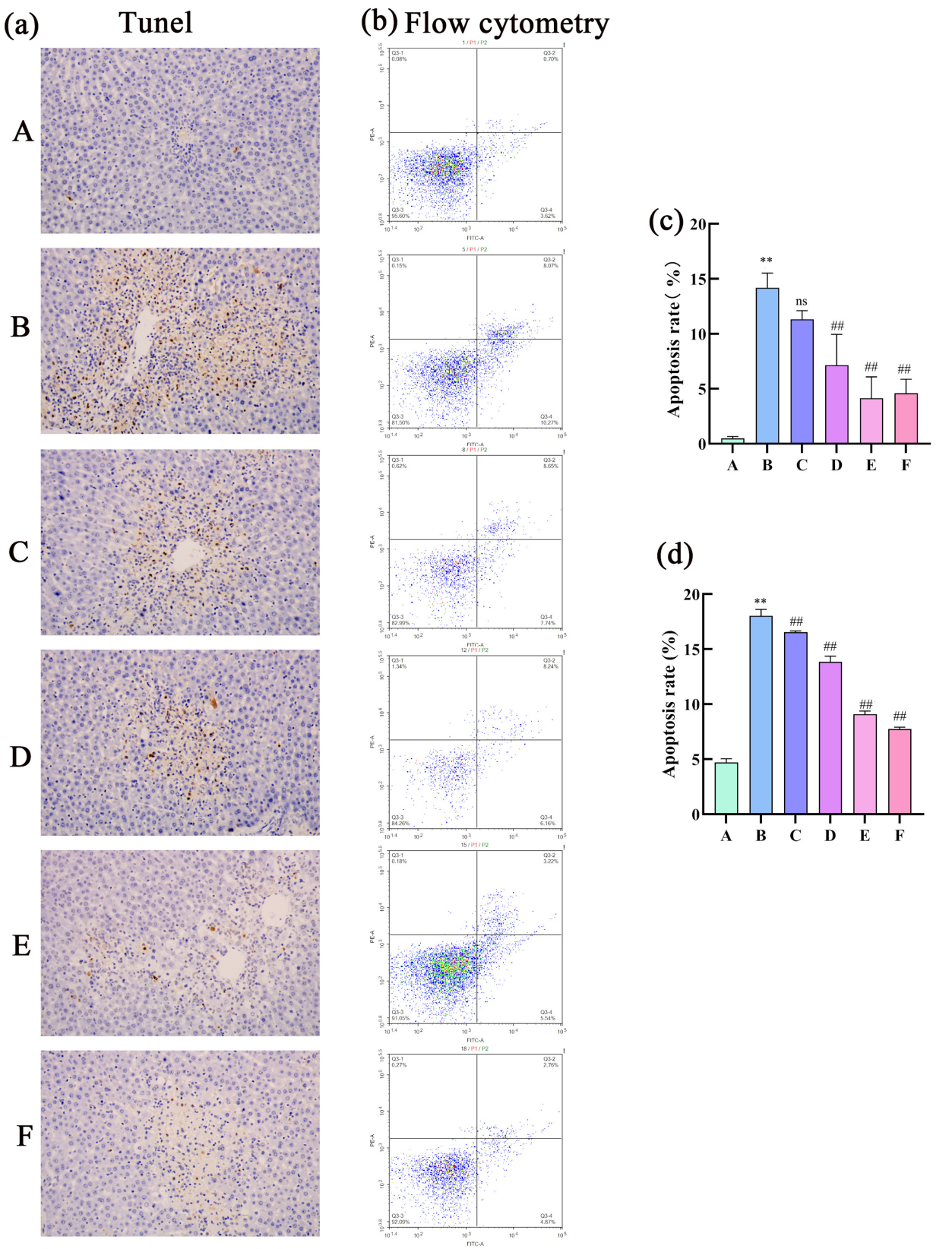



FIGURE 8
Analysis of cell apoptosis in rat liver tissue. **(a)** TUNEL assay; **(b)** flow cytometry detection of apoptotic cells; **(c)** TUNEL quantification of cell apoptosis ($n = 3$; compared with the control group, $^{**}P < 0.01$; compared with the model group, $^{\#\#}P < 0.01$; ns indicates not significant); **(d)** Flow cytometry analysis of apoptotic cells ($n = 3$; compared with the control group, $^{**}P < 0.01$; compared with the model group, $^{\#\#}P < 0.01$).

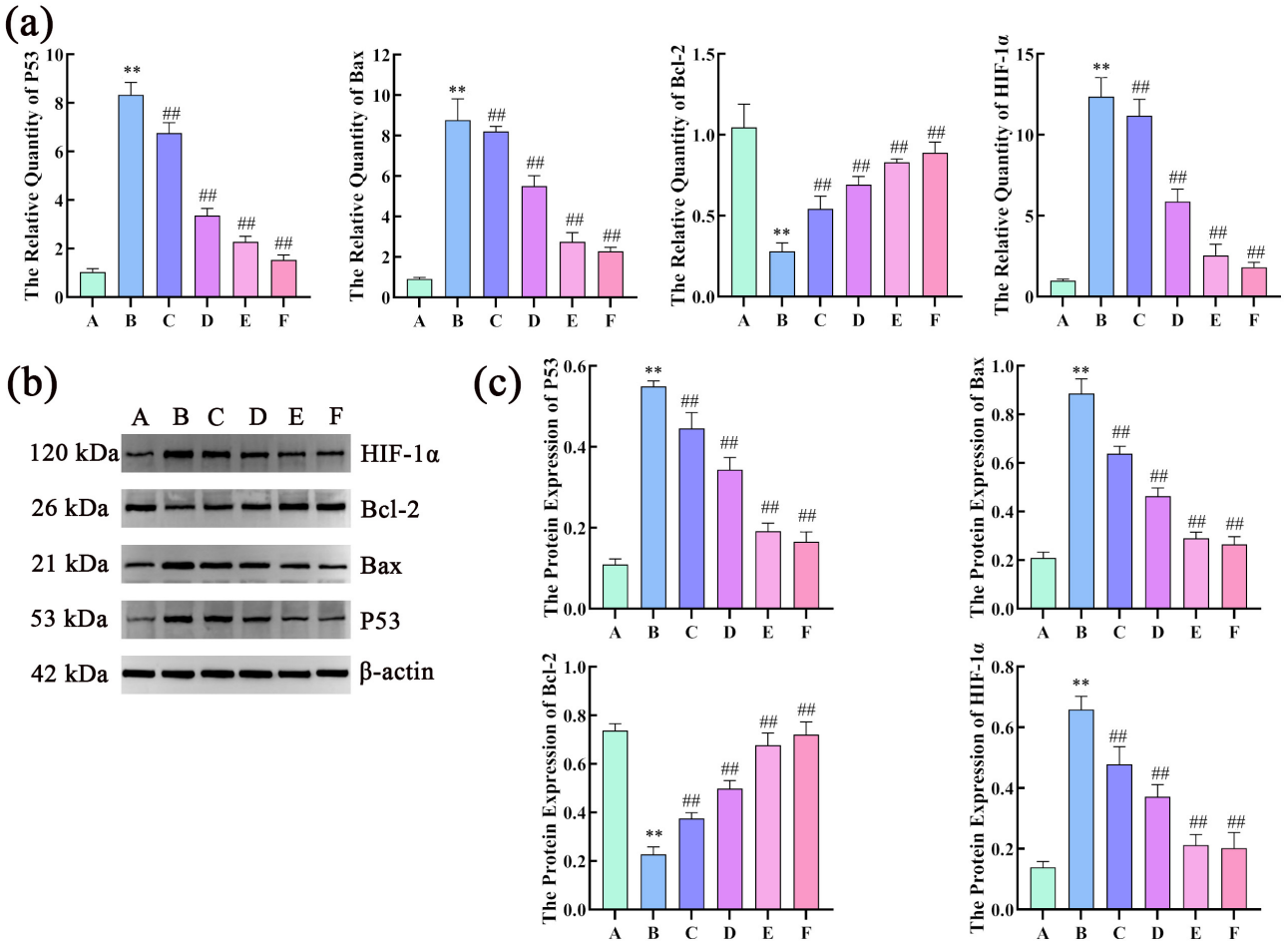



FIGURE 9
Effects of compound liver: protecting effervescent granules on P53, Bax, Bcl-2, and HIF-1α mRNA and protein expression levels. **(a)** Effects of CEHG on the expression of P53, Bax, Bcl-2, and HIF-1 mRNA α in rats with liver injury ($n = 6$, compared with the control group, $^{**}P < 0.01$, compared with the model group, $^{\#\#}P < 0.01$); **(b)** Gel electrophoresis images of P53, Bax, Bcl-2, and HIF-1α; **(c)** Quantification of gel electrophoresis of P53, Bax, Bcl-2, and HIF-1α Proteins ($n = 3$, compared with the control group, $^{**}P < 0.01$, compared with the model group, $^{**}P < 0.01$).

## Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## Ethics statement

The animals were housed in an environment devoid of pathogens. The animal care and use methods were authorized by the Committee on the Ethics of Animal Experiments of Anhui University of Chinese Medicine (Permit Number: AHUCM-rats-2024049). According to the applicable norms and regulations, all techniques were executed. The investigation was conducted in accordance with the ARRIVE standards.

## Author contributions

XL: Conceptualization, Data curation, Formal analysis, Investigation, Validation, Writing – original draft. RW: Conceptualization, Data curation, Formal analysis, Investigation, Visualization, Writing – original draft. YL: Investigation, Resources, Validation, Writing – original draft. JZ: Investigation, Software, Writing – original draft. JL: Data curation, Methodology, Writing – original draft. SL: Formal analysis, Writing – original draft. Methodology. QY: Data curation, Investigation, Writing – original draft. RC: Formal analysis, Writing – original draft. WW: Formal analysis, Writing – original draft. WL: Formal analysis, Writing – original draft. SB: Formal analysis, Writing – original draft. XD: Funding acquisition, Project administration, Supervision, Writing – review & editing. CP: Funding acquisition, Project administration, Supervision, Writing – review & editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Natural metabolites used in traditional Chinese medicine for cardiovascular diseases: pharmacological mechanisms, evidence, and future directions

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Cardiovascular diseases (CVDs) remain the leading cause of death and disability worldwide, highlighting an urgent need for new treatments. Traditional Chinese Medicine (TCM) offers a rich repository of natural metabolites (flavonoids, alkaloids, saponins, etc.) that act on multiple targets to protect the heart and blood vessels. These compounds have demonstrated multiple cardioprotective effects, including anti-inflammatory, antioxidant, anti-atherosclerotic, and blood pressure–lowering activities. They work by reducing oxidative stress, dampening chronic inflammation, improving blood vessel function, correcting abnormal lipid levels, and mitigating cardiac fibrosis. Recent preclinical studies and clinical trials show that TCM-derived metabolites can improve cardiovascular health. For instance, the multi-herb formula Qili Qiangxin and the alkaloid berberine have improved heart failure symptoms and cardiac function in clinical trials when added to standard therapy. These examples underscore the clinical potential of TCM compounds. However, challenges like poor bioavailability, complex multi-component interactions, and lack of standardization still hinder their widespread use. To address these issues, researchers are exploring advanced drug delivery methods and better quality control with modern analytical tools. If these hurdles are overcome, TCM-derived therapies could be successfully integrated into mainstream cardiovascular care, offering a novel multi-target approach to combat CVDs.

KEYWORDS

**Chinese medicine, natural metabolites, CVDs, flavonoids, alkaloids, saponins**

## 1 Introduction

Cardiovascular diseases (CVDs) remain the leading cause of mortality and disability worldwide, imposing an enormous health and economic burden. Globally, CVDs account for roughly one-third of all deaths (approximately 17–18 million per year). The rising prevalence of hypertension, diabetes, obesity, and other risk factors has led to tens of millions of new CVDs cases annually. Low- and middle-income countries are disproportionately affected due to limited healthcare access and resource constraints, but high-income regions also face growing challenges as populations age and lifestyle-related risk factors persist. Despite advances in prevention and acute care, the overall CVDs burden continues to increase, underscoring the urgent need for more effective and

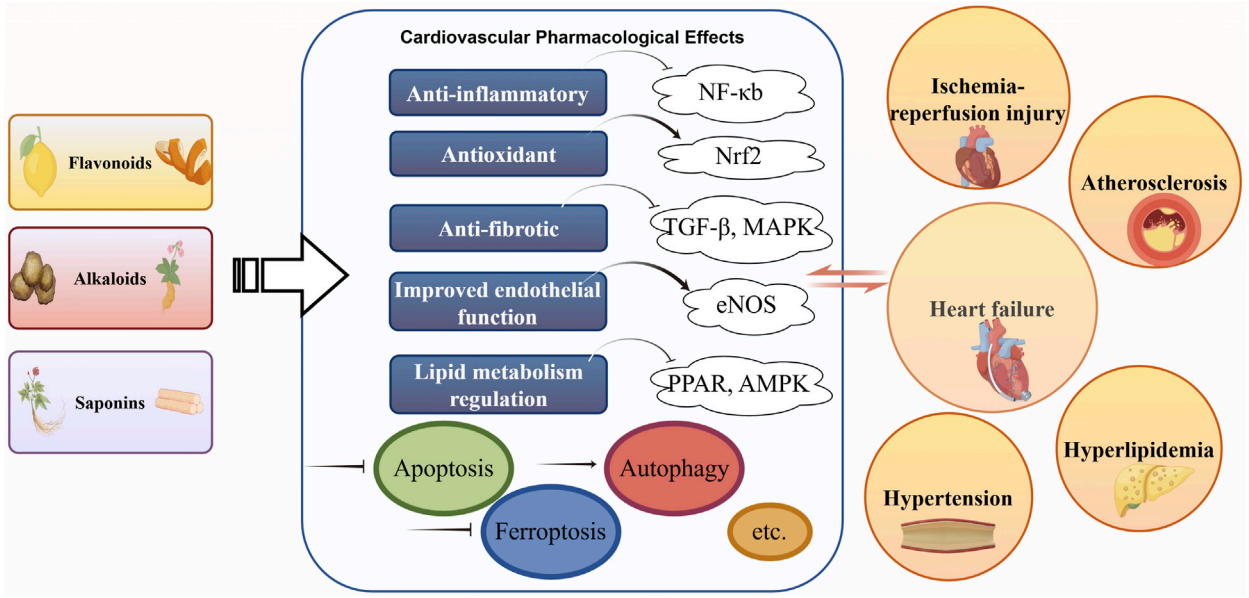



FIGURE 1
Main classes of TCM natural compounds and cardiovascular mechanisms. Explanation: Schematic diagram highlighting three primary classes of Traditional Chinese Medicine (TCM)–derived natural compounds and their cardioprotective mechanisms and disease targets. The figure illustrates how each class exerts beneficial effects on the cardiovascular system. Abbreviations in the figure are defined as follows: TCM (Traditional Chinese Medicine); CVD (cardiovascular disease); MI (myocardial infarction); I/R (ischemia–reperfusion) (Tang et al., 2015a; Feng et al., 2017; Ou-Yang et al., 2018; Shao et al., 2020).

comprehensive strategies to combat these diseases (World Health Organization, 2021; Khan et al., 2024; Centers for Disease Control and Prevention, 2024; Martin Ss Fau - Aday et al., 2025).

Conventional Western therapies for CVDs—such as antihypertensive drugs, statins for dyslipidemia, antiplatelet agents, and neurohormonal blockers for heart failure—have significantly improved patient outcomes. However, these standard treatments have important limitations and unmet clinical needs. Many drugs have a single mechanism of action and are limited in their effects on complex pathological changes. Patients often experience side effects (for example, persistent cough with ACE inhibitors or muscle pain with high-dose statins), issues with adherence, and even drug resistance or tolerance over time. Furthermore, residual risk remains high; even with optimal medical therapy, substantial proportions of patients continue to suffer events such as myocardial infarction or progression of heart failure. These challenges highlight the need for complementary or alternative therapeutic approaches that can safely target additional pathological processes (e.g., inflammation, fibrosis) beyond the reach of current standard-of-care medications.

Many foods have the function of promoting health, so the concept of "dietotherapy" or "medicinal food" is widely known, and this phenomenon is called "continuum of food and medicine" by academic circles; Together with traditional Chinese medicine (TCM) and its natural drug metabolites as potential resources for new CVD therapy, it is attracting increasing academic attention (Chen, 2023; Yao et al., 2023). TCM-derived natural products—isolated from botanical drugs (medicinal plants) long used in TCM practice—offer multi-target and multi-pathway mechanisms that could holistically address the complex pathophysiology of CVDs. Many such metabolites (e.g., flavonoids from *Scutellaria baicalensis* and citrus peels, saponins from *Panax ginseng*, *Astragalus membranaceus* and Dioscorea nipponica Makino, alkaloids like berberine from *Coptis chinensis*) have demonstrated cardioprotective effects with relatively favorable safety profiles. These phytochemicals can concurrently modulate oxidative stress, inflammation, endothelial function, lipid metabolism, and myocardial remodeling, thereby intervening at multiple disease nodes (Tang et al., 2015a; Fan et al., 2016; Feng et al., 2017; Ou-Yang et al., 2018; Zeng et al., 2019; Orgah et al., 2020; Shao et al., 2020; Sadoughi et al., 2021; Jin et al., 2024; Shi et al., 2024). TCM treatments involve multiple components and targets acting concurrently, making their mechanisms difficult to fully elucidate. Furthermore, comprehensive reviews focusing on the active compounds from TCM and their cardiovascular mechanisms remain limited.

This review explores the application of natural metabolites used in TCM in the prevention and treatment of major cardiovascular diseases, highlighting their pharmacological mechanisms and therapeutic potential. We first provide a brief background on the multi-target cardiovascular effects of TCM-derived metabolites (Figure 1), then discuss evidence for their benefits in specific disease contexts (atherosclerosis, hypertension, myocardial infarction, heart failure), integrate findings from clinical studies, and finally consider the challenges and future directions for translating these therapies into mainstream cardiovascular care.

A systematic literature search was performed across the PubMed database for relevant studies published up to June 2025. The search

TABLE 1 Representative Flavonoids and their main sources and pharmacological effects of traditional Chinese medicine.

| Chinese name | Latin name | Main flavonoids | Pharmacological effects |
|---|---|---|---|
| Ban Lan Gen | *Isatis tinctoria* | Luteoli, Quercetin | Anti-inflammatory, antiviral, vascular protection |
| Chen Pi | *Citrus reticulata* | Hesperetin, Naringenin | Lowering blood lipids, anti-atherosclerosis |
| Gan Cao | *Glycyrrhiza uralensis* | Licochalcone A, Isoliquiritigenin | Antioxidant, anti-inflammatory, immunomodulatory |
| Ge Gen | *Pueraria lobata* | Puerarin | Dilate blood vessels, improve microcirculation, and lower blood pressure |
| Hu Pi | *Quercus spp.* | Quercetin | Antioxidant, antiplatelet aggregation, lipid-lowering |
| Hu Zhang | *Polygonum cuspidatum* | Quercetin | Antioxidant, antiplatelet aggregation, anti-atherosclerosis |
| Huang Jing | *Polygonatum sibiricum* | Rutin, Isoquercitrin | Immunomodulation, anti-inflammatory, hypoglycemic |
| Huai Hua | *Sophora japonica* | Rutin, Quercetin | Lowering blood pressure, anticoagulation, anti-oxidation |
| Huang Hua Hao | *Artemisia annua* | Naringenin, Luteolin | Anti-inflammatory, immunomodulatory, anti-vascular sclerosis |
| Huang Qin | *Scutellaria baicalensis* | Baicalin, Baicalein | Anti-oxidation, anti-inflammatory, improve vascular function |
| Jue Ming Zi | *Cassia obtusifolia* | Rutin | Antioxidant, lipid-lowering, anti-inflammatory |
| Ku Qiao | *Fagopyrum tataricum* | Rutin | Lowering blood lipids, anti-diabetes, protecting vascular endothelium |
| Sang Ye | *Morus alba* | Isoquercitrin | Lower blood sugar, anti-oxidation, improve microcirculation |
| Wu Wei Zi | *Schisandra chinensis* | Naringenin | Regulate cardiovascular function, anti-oxidation, protect myocardium |
| Yin Xing Ye | *Ginkgo biloba* | Ginkgoflavones | Protect blood vessels, prevent platelet aggregation, and prevent stroke |
| Zi Su | *Perilla frutescens* | Perilloflavone, Quercetin | Anti-inflammatory, anti-allergic, lipid-lowering |

employed a combination of MeSH terms and free text keywords, including "cardiovascular disease" and the names of specific botanical agents and their bioactive constituents (e.g., "berberine," "ginseng," "flavonoid"). Inclusion criteria were restricted to peer-reviewed articles published in English within the preceding 5 years. Studies encompassing *in vitro* assays, *in vivo* animal models utilizing traditional Chinese medicine (TCM), and clinical trials investigating TCM for cardiovascular diseases [CVDs] were eligible for inclusion. To ensure taxonomic accuracy, all botanical identities were cross-referenced with the Kew Medicinal Plant Names Service. Furthermore, the reporting of plant materials and extracts conformed to the ConPhyMP guidelines. Supplementary Tables S1, S2 provide a synopsis of the included botanical drugs, their preparation methods, and analytical characterization. A summary of key TCM-derived metabolites, along with their sources and molecular targets, is presented in Tables 1–3. Table 4 collates key clinical studies meeting the inclusion criteria. Subsequently, the discussion is organized according to major cardiovascular conditions, featuring representative metabolites from prominent chemical classes (i.e., flavonoids, alkaloids, and saponins) that have demonstrated cardioprotective effects in relevant experimental models.

# 2 Preclinical evidence and mechanistic studies

This section summarizes the key *in vitro* and *in vivo* findings from preclinical studies of major classes of natural metabolites from TCMs with cardioprotective effects, and discusses the key molecular targets and pathways by which these natural metabolites exert their cardioprotective effects. Notably, these natural compounds often act through multiple pathways in parallel, rather than through a single target. Common mechanisms include enhancing antioxidant defenses (typically through activation of the Nrf2/ARE pathway), inhibiting pro-inflammatory signaling (e.g., NF-κB and MAPK pathways), increasing nitric oxide (NO) bioavailability in endothelial cells, regulating lipid metabolism, and alleviating pathological fibrosis.

## 2.1 Multi-target cardiovascular benefits of TCM natural compounds

Natural metabolites from TCM exhibit multifaceted cardioprotective properties that set them apart from single-target conventional drugs. These bioactive molecules often act on numerous molecular pathways simultaneously, conferring broad therapeutic effects across the cardiovascular system. A unifying feature is their ability to mitigate oxidative stress—for example, many flavonoids, alkaloids, and saponins activate the Nrf2/ARE pathway, upregulating endogenous antioxidant enzymes (such as superoxide dismutase and glutathione peroxidase) to neutralize excess reactive oxygen species (Wang Q. W. et al., 2019; Liu et al., 2021; Oluranti et al., 2021; Ren et al., 2021; Shi et al., 2021; Tan et al., 2021; Obeidat et al., 2022; Qi et al., 2022; Wang S. H. et al., 2022; Wei et al., 2022; Fang et al., 2024; Feng et al., 2024; Guo et al., 2024; Liu Z. et al., 2024; Sun et al., 2024; Yao et al., 2024). Through antioxidant action, these metabolites preserve endothelial function and prevent oxidative damage to vessel walls and myocardium. In parallel, Natural metabolites such as flavonoids (e.g. quercetin, luteolin) and alkaloids (e.g. berberine) often exert anti-

TABLE 2 Representative alkaloids and their main sources and pharmacological effects of traditional Chinese medicine.

| Chinese name | Latin name | Major alkaloids | Pharmacological effects |
|---|---|---|---|
| Ban Xia | *Pinellia ternata* | Pinelline | Antiemetic and expectorant, but toxic in raw form, requires processing before use |
| Bei Mu | *Fritillaria thunbergii* | Peimine | Expectorant, anti-inflammatory |
| Chuan Wu | *Aconitum carmichaelii* | Aconitine | Strong analgesic, but highly toxic, requires proper processing before use |
| Huang Lian | *Coptis chinensis* | Berberine | Antibacterial, anti-inflammatory, lipid-lowering, hypoglycemic, gut microbiota regulation, cardiovascular protection, antioxidant |
| Gou Teng | *Uncaria rhynchophylla* | Rhynchophylline | Antihypertensive, sedative, neuroprotective |
| Ku Mu | *Picrasma quassioides* | Quassin | Heat-clearing, detoxifying, antidiarrheal |
| Ku Shen | *Sophora flavescens* | Matrine, Oxymatrine | Anti-inflammatory, antiviral, antitumor, hepatoprotective |
| Ma Huang | *Ephedra sinica* | Ephedrine | Bronchodilator, diuretic, hypertensive |
| Ma Qian Zi | *Strychnos nux-vomica* | Strychnine | Central nervous system stimulant, used for neurological disorders, but highly toxic |
| Man Tuo Luo | *Datura metel* | Scopolamine, Hyoscyamine | Antispasmodic and analgesic effects, but toxic, should be used under medical supervision |
| Nan Tian Zhu | *Nandina domestica* | Nantenine | Heat-clearing, detoxifying, antitussive |
| Shi Song | *Lycopodium japonicum* | Lycopodine, Huperzine A | Neuroprotective, anti-inflammatory, analgesic |
| Wu Tou | *Aconitum carmichaelii* | Acnitine | Anti-rheumatic, analgesic |
| Yan Hu Suo | *Corydalis yanhusuo* | Dehydrocorybulbine | Analgesic, blood circulation improvement |
| Yan Ling Cao | *Trillium* | Colchicine | Anti-inflammatory, cardiovascular protection, antioxidant |
| Yi Mu Cao | *Leonurus japonicus* | Leonurine | Blood circulation improvement, antihypertensive, cardioprotective |

inflammatory effects by inhibiting pro-inflammatory signaling pathways like NF-κB, MAPK, and PI3K/Akt (Shen S. et al., 2023; Chen S. et al., 2024; Tang J. et al., 2024; Tuo et al., 2024). This results in lower expression of cytokines (TNF-α, IL-6, etc.) and adhesion molecules, dampening the vascular inflammation that drives atherosclerosis and hypertensive organ damage (Wang Q. et al., 2020; Muvhulawa et al., 2022; Shao et al., 2022; Barreca et al., 2023; Hassanein et al., 2023; Sun et al., 2023; Yang L. et al., 2023; Fang et al., 2024; Tuo et al., 2024; Wang A. et al., 2024). Many TCM metabolites also improve endothelial function and vasodilation: for instance, flavonoids and saponins promote nitric oxide (NO) production via endothelial NO synthase (eNOS) activation, improving NO bioavailability (Grassi et al., 2013; Martínez-Fernández et al., 2015; Bondonno et al., 2016; Heidary Moghaddam et al., 2020; Lin et al., 2020; Jalili et al., 2024). The resulting vasorelaxation translates into improved blood flow and reduced blood pressure (Adetunji et al., 2023; Li and Zhang, 2023; Harahap et al., 2024; Liu Y. et al., 2024; Miao et al., 2024; Tang F. et al., 2024; Wang Z. et al., 2024). Additionally, several natural metabolites beneficially modulate lipid metabolism – they can inhibit cholesterol absorption, upregulate LDL receptors, and improve lipid profiles by lowering LDL and triglycerides while raising HDL (Assini et al., 2013; Kianbakht et al., 2014; Kuipers et al., 2018; Hou et al., 2020; Wang Q. et al., 2024). By correcting dyslipidemia, they help curb atherosclerotic plaque formation (Panchal et al., 2011; Kwok et al., 2022; Wani et al., 2023; Lv et al., 2024; Mu et al., 2024; Wan et al., 2024; Zhao et al., 2024). Finally, many exhibit anti-fibrotic and cardiomyocyte-protective actions: they interfere with fibrosis-related pathways (TGF-β/Smad, etc.), inhibit cardiac fibroblast activation, and reduce pathological deposition of extracellular matrix in the heart (Xiao et al., 2018; Li et al., 2021; Jia et al., 2022; Wang M. et al., 2023; Lin et al., 2024). Some even promote adaptive autophagy or have positive inotropic effects that support cardiac function (Wang L. et al., 2019; Wang et al., 2021; Qu et al., 2022; Yao et al., 2022; Abudureyimu et al., 2023; Shen F. et al., 2023; Zhan et al., 2023; Erfu et al., 2024). Collectively, the ability of TCM-derived metabolites to address multiple pathogenic factors of CVD concurrently (as summarized in Figure 1) holds promise for treating complex cardiovascular disorders where no single molecular target is sufficient (Zhang et al., 2024). A comprehensive intervention strategy is particularly well-suited for addressing the intricate pathogenesis of complex cardiovascular disease, where targeting a solitary molecule proves inadequate. Within this context, it is noteworthy that various natural compounds interact with essential metabolic nuclear receptors, among them the peroxisome proliferator-activated receptors (PPARs). Specifically, diverse alkaloids and saponins have been shown to activate both

TABLE 3 Representative Saponins and their main sources and pharmacological effects of traditional Chinese medicine.

| Chinese name | Latin name | Major alkaloids | Pharmacological effects |
|---|---|---|---|
| Dan Shen | *Salvia miltiorrhiza* | Danshen saponins | Widely used for cardiovascular diseases; enhances microcirculation, dilates coronary arteries, reduces blood lipid levels, and prevents atherosclerosis |
| Di Huang | *Rehmannia glutinosa* | Catalpol, Rehmanniosides | Impacts heart health by reducing blood pressure and cholesterol, also shown to improve cardiac function in diabetic patients |
| He Shou Wu | *Polygonum multiflorum* | Tuberfleeceflower saponins | Influences lipid metabolism and has been linked to cardiovascular health by potentially reducing the risk of arteriosclerosis |
| Huang Qi | *Astragalus membranaceus* | Astragaloside IV | Protects myocardial cells, reduces hypertension, and acts as a diuretic to treat chronic heart failure |
| Jie Geng | *Platycodon grandiflorus* | Platycodon saponins | Helps to reduce cholesterol levels and improves lung health, which indirectly supports heart function |
| Mai Dong | *Ophiopogon japonicus* | Ophiopogonin | Primarily used for its cardiovascular benefits such as enhancing coronary blood flow and protecting against ischemic injuries |
| Ren Shen | *Panax ginseng* | Ginsenosides | Enhances cardiovascular performance by modulating blood pressure, improving blood circulation, and reducing myocardial oxygen consumption |
| San Qi | *Panax notoginseng* | Notoginsenosides | Promotes blood circulation, reduces blood clot formation, and is used for treating coronary artery diseases and ischemic strokes |
| Shan Zhu Yu | *Cornus officinalis* | Cornus saponins | Known for anti-inflammatory and antioxidant effects that benefit heart health, particularly in ischemic conditions |
| Shan Yao | *Dioscorea opposita* | Diosgenin | Known for its benefits in regulating cholesterol levels, enhancing endocrine function, and its potential in improving cardiovascular health through anti-inflammatory and antioxidant properties |
| Yu Zhu | *Polygonatum odoratum* | Polygonatum saponins | Used for its cardiovascular protective effects, reduces heart rate, and has been shown to decrease blood pressure |

TABLE 4 Comparison of active compound categories in traditional Chinese medicine.

| Category | Key pharmacological effects and mechanisms | Pharmacological characteristics |
|---|---|---|
| Flavonoids | Antioxidant: Activates the Nrf2/ARE pathway, upregulating antioxidant enzymes (SOD, GPx) to reduce oxidative stress. Anti-inflammatory: Inhibits NF-κB signaling, reducing IL-6 and TNF-α expression. Endothelial Function Improvement: Enhances eNOS/NO signaling, promoting nitric oxide synthesis for vasodilation. Lipid Metabolism Regulation: Modulates PPAR-γ expression, reducing LDL oxidation and cholesterol levels. Antiplatelet Activity: Inhibits COX-1/COX-2 pathways to reduce platelet aggregation | Acts on multiple pathways simultaneously, moderate bioavailability, often requiring modifications to improve solubility and absorption |
| Alkaloids | Neuropharmacological Effects: Interacts with cholinergic, adrenergic, and opioid receptors, affecting neurotransmission. Cardiovascular Regulation: Blocks calcium channels and regulates β-adrenergic receptors, reducing hypertension and arrhythmias. Anti-inflammatory: Inhibits Toll-like receptor 4 (TLR4) and NF-κB pathways, reducing inflammatory cytokines. Antibacterial and Antiviral: Targets bacterial DNA gyrase and viral replication enzymes, inhibiting pathogen growth | Lipophilic, easily crosses the blood-brain barrier, significant central nervous system effects, some compounds require toxicity assessment |
| Saponins | Cardioprotective: Enhances eNOS/NO signaling, promoting vasodilation and reducing hypertension. Anti-inflammatory: Inhibits NF-κB and MAPK pathways, reducing cytokine production (TNF-α, IL-6). Lipid Metabolism Regulation: Modulates PPAR-γ and NPC1L1 pathways, improving cholesterol homeostasis. Anti-fibrotic: Suppresses TGF-β/Smad signaling, preventing myocardial and vascular fibrosis. Immunomodulatory: Regulates TLR4/NF-κB, balancing immune responses | Multi-target action with broad physiological effects, poor bioavailability due to extensive metabolism, modifications such as nanoparticle delivery improve absorption and efficacy |

PPARα and PPARγ isoforms. Upon activation, these receptors exert dual functionality by inhibiting NF-κB-driven inflammatory cascades and promoting favorable alterations in lipid metabolism. As such, the engagement of PPAR pathways constitutes a critical mechanism underlying the multi-target cardioprotective properties of TCM metabolites.

## 2.2 Atherosclerosis and coronary artery disease

Atherosclerosis is a chronic arterial disease characterized by the buildup of lipid-rich plaques in the vessel wall, leading to coronary artery disease and other ischemic complications. Endothelial injury

and dysfunction initiate the process, permitting low-density lipoprotein (LDL) particles to infiltrate the intima and become oxidized. Oxidized LDL triggers an inflammatory cascade – monocytes migrate into the intima and differentiate into macrophages, engulfing lipids to form foam cells. A fatty streak evolves as these foam cells accumulate, and ongoing inflammation drives the expansion and instability of plaques. Key contributors to atherogenesis include oxidative stress (excess ROS oxidizing lipids and damaging endothelial cells), vascular inflammation (cytokine and chemokine release recruiting more immune cells), endothelial dysfunction (reduced NO bioavailability and loss of barrier integrity), and dyslipidemia (elevated LDL/triglycerides and low HDL). Over time, plaques can narrow arteries or rupture, causing thrombosis and acute myocardial infarction (Ajoolabady et al., 2024).

Natural products used in TCM have shown significant anti-atherosclerotic activities in preclinical studies by simultaneously counteracting the aforementioned pathogenic mechanisms (Si and Lai, 2024). A prominent example is the class of flavonoids, which includes metabolites from *Scutellaria baicalensis* (Huang Qin; e.g., baicalin, baicalein), *Pueraria lobata* (Ge Gen; puerarin), *Ginkgo biloba* (Yin Xing Ye; quercetin, ginkgoflavones), *Morus alba* (Sang Ye; isoquercitrin, morusin), *Citrus reticulata* (Chen Pi; hesperetin, naringenin), *Perilla frutescens* (Zi Su Ye; perilloflavone, quercetin), *Glycyrrhiza uralensis* (Gan Cao; licochalcone A, isoliquiritigenin), and *Sophora japonica* (Huai Hua; rutin, quercetin), etc. Flavonoids prevent the progression of atherosclerosis by scavenging free radicals and activating antioxidant defenses (Nrf2-mediated), thereby reducing LDL oxidation and endothelial damage. They also inhibit the inflammatory component of plaque formation; *in vitro* studies demonstrate that flavonoids suppress NF-κB signaling and lower pro-inflammatory mediator levels in vascular cells, which in turn mitigates leukocyte adhesion and foam-cell formation (Zhang et al., 2021; Ding et al., 2024; Wang Y. M. et al., 2024). In addition, flavonoid-rich botanical drugs have been noted to improve lipid profiles. For instance, puerarin (from *P. lobata*) and rutin (from *S. japonica*) can upregulate hepatic LDL receptors and promote cholesterol clearance, leading to reduced circulating LDL levels. In some animal studies, increased flavonoid intake has been inversely associated with the development of atherosclerosis (Bondonno et al., 2024).

Saponins (glycosidic metabolites from TCM botanical drugs like *Panax notoginseng*) likewise display broad anti-atherosclerotic effects. *Panax notoginseng* saponins (PNS), for example, have been shown in rats and rabbits to reduce atherosclerotic plaque development via multiple pathways. PNS exert antioxidant and anti-inflammatory actions similar to flavonoids, and notably they inhibit endothelial activation: in one endothelial cell study, notoginsenoside R1 and ginsenoside Rb1 from *Panax* blocked p38 MAPK signaling and downregulated vascular cell adhesion molecule-1 (VCAM-1) expression, thus preventing monocyte adhesion and plaque initiation (Fan et al., 2016). PNS have also been reported to inhibit foam-cell formation and ferroptosis within plaques, stabilizing the lesions. These combined effects led to markedly attenuated plaque progression in PNS-treated animals (Zhao et al., 2024).

Similarly, alkaloids from TCM offer anti-atherogenic benefits. Colchicine is a small-molecule alkaloid drug that has been used in clinical atherosclerosis treatment (as an anti-inflammatory). It is derived from plants such as *Colchicum* and *Trillium* and has anti-inflammatory and antioxidant effects (Buckley and Libby, 2024). In addition, berberine–an isoquinoline alkaloid from *Coptis chinensis* and *Berberis* species–has garnered attention for its lipid-lowering and anti-inflammatory properties. Clinical trials and meta-analyses indicate that berberine significantly reduces total cholesterol, LDL, and triglycerides (while raising HDL) in patients with dyslipidemia, an effect partly due to upregulation of LDL receptor expression in the liver and modulation of AMPK-dependent lipid metabolism. In the arterial wall, berberine also acts as an antioxidant and NF-κB inhibitor, resulting in decreased oxidative modification of LDL and suppression of cytokine-driven vascular inflammation. These actions have been correlated with reduced plaque size and improved plaque stability in preclinical models (Figure 2) (Li et al., 2009; Rui et al., 2021; Ma et al., 2022). Other alkaloids, such as nuciferine (from lotus, *Nelumbo nucifera*), likewise inhibit inflammatory signaling and oxidative stress in vascular cells, contributing to anti-atherosclerotic effects (Xiao et al., 2023).

Empirically, diets or herbal therapies rich in these TCM phytochemicals are associated with lower rates of coronary artery disease (Moss and Ramji, 2016; Penson and Banach, 2021; Centers for Disease Control and Prevention, 2024). As research advances, specific natural metabolites are emerging as promising adjuncts or alternatives for atherosclerosis management alongside standard therapies. However, their exact clinical impact will need confirmation in large-scale trials.

## 2.3 Hypertension and endothelial dysfunction

Hypertension, or chronically elevated blood pressure, results from a combination of increased vascular resistance (often due to arterial constriction or stiffness) and sometimes elevated cardiac output. Endothelial dysfunction is a hallmark of hypertension – the impaired ability of blood vessels to dilate, usually due to reduced NO availability and a pro-inflammatory endothelium. TCM approaches have long included herbal therapies for "calming the liver" and improving circulation in hypertensive patients. Modern pharmacology is beginning to validate some of these botanical treatments.

Flavonoids have demonstrated notable antihypertensive effects attributable to their vasodilatory and antioxidant properties. Many flavonoids stimulate the endothelial production of NO by upregulating eNOS or by enhancing NO release, leading to vasorelaxation. For example, *in vitro* experiments show that quercetin and its glycosides cause endothelium-dependent relaxation via the PI3K/Akt-eNOS pathway (Das et al., 2023; Harahap et al., 2024; Xin et al., 2024). In hypertensive rat models, chronic administration of quercetin, puerarin, or rutin leads to lowered blood pressure, associated with improved endothelial function and decreased oxidative stress markers (Elbarbry et al., 2020; Chen et al., 2021). These effects are comparable to standard antihypertensives in some studies, though achieved through different mechanisms (e.g., antioxidant and anti-inflammatory pathways rather than direct effects on angiotensin or calcium channels) (Zheng et al., 2024; Jomova et al., 2025). Alkaloids like tetrandrine (from *Stephania*

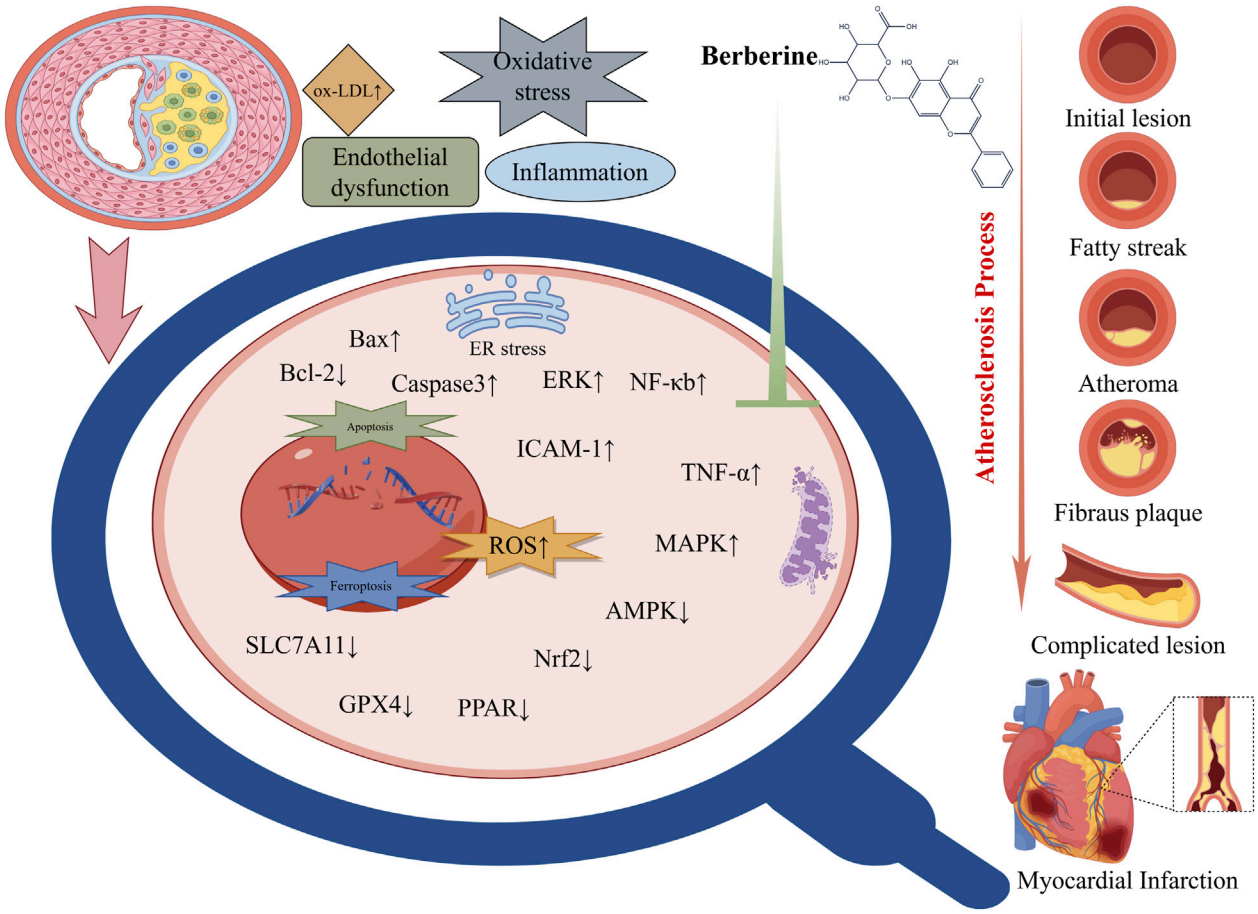



FIGURE 2
Molecular targets and cardioprotective mechanisms of berberine. Explanation: Key molecular actions of berberine include activation of AMPK (AMP-activated protein kinase) and related signaling pathways, which enhances endothelial nitric oxide synthase (eNOS) activity and nitric oxide (NO) bioavailability, leading to improved endothelial function and vasodilation. Berberine also inhibits pro-inflammatory signaling cascades such as NF-κB and MAPK, thereby reducing the production of inflammatory cytokines and oxidative stress in blood vessels. These anti-inflammatory and antioxidant effects help prevent endothelial damage and plaque formation, conferring protection against atherosclerosis and ischemic injury. In cardiac muscle, berberine attenuates cardiomyocyte apoptosis and enhances myocardial energy metabolism, partly through the AMPK–Akt–eNOS and other cardioprotective pathways. Through these combined actions – improving endothelial function, lowering blood lipids, suppressing inflammation, and stabilizing cardiac electrophysiology – berberine provides broad cardioprotection (Abbreviations: BBR, berberine; AMPK, AMP-activated protein kinase; eNOS, endothelial nitric oxide synthase; NF-κB, nuclear factor kappa-B; NO, nitric oxide; MAPK, mitogen-activated protein kinase; all other abbreviations are defined in the text or figure legend).

*tetrandra*) have calcium-channel blocking activity and have been used as antihypertensive agents in China. Berberine also shows benefit in hypertension; beyond its lipid effects, berberine improves endothelial function and induces vasodilation via increased NO and via activating AMPK (Amssayef and Eddouks, 2023). *In vivo* studies indicate that berberine can lower blood pressure in spontaneously hypertensive rats, with effects on the renin-angiotensin system and improved arterial compliance. Similarly, Morus alba leaf extracts (rich in alkaloid DNJ and flavonoids) have demonstrated blood pressure-lowering and endothelial-protective effects in animal models (Cao et al., 2021; Suadoni and Atherton, 2021; Wang Z. et al., 2024). Saponins also contribute to blood pressure reduction. Ginsenosides from Panax ginseng and Panax notoginseng have been found to promote vasodilation by increasing NO bioavailability and endothelial responsiveness. In hypertensive rodent models, ginsenoside Rg1 and Rb1 enhanced endothelium-dependent relaxation and reduced blood pressure, linked to activation of the Akt/eNOS pathway and inhibition of NADPH oxidase-derived ROS. Additionally, notoginsenoside R1 has been reported to improve microvascular endothelial function in animal models of hypertension, partly by reducing inflammation (e.g., lowering VCAM-1 expression as noted earlier). Astragalosides (from Astragalus membranaceus) and glycyrrhizin (from Glycyrrhiza uralensis) have shown vasoprotective effects as well, by ameliorating endothelial dysfunction and oxidative damage (Cao et al., 2021; Chu et al., 2023; Huang et al., 2023; Huang et al., 2024; Wang Y. et al., 2024). Some saponin-rich botanical formulations have historically been used to treat hypertension in TCM, and pharmacological studies now validate their efficacy in modern terms.

Collectively, diverse metabolites isolated from traditional Chinese medicine (TCM) exert a profound beneficial impact on the vasculature, characterized by enhanced endothelial function and diminished peripheral resistance. Their therapeutic action is predicated on a confluence of mechanisms, including increased

nitric oxide (NO) bioavailability, reduced oxidative stress, and the intricate modulation of vasoactive signaling cascades. Consequently, these compounds exhibit significant antihypertensive properties. Given their potential to target the multifaceted pathophysiology of hypertension, either as monotherapies or as adjuncts to conventional treatments, they represent a promising therapeutic strategy. However, translating this substantial preclinical rationale into clinical practice necessitates rigorous, large-scale randomized controlled trials to substantiate their efficacy and safety in human populations.

## 2.4 Myocardial infarction and heart failure

Myocardial infarction (MI) and heart failure (HF) are closely intertwined clinical syndromes. An MI occurs when a coronary artery plaque ruptures or occludes the vessel, causing ischemic death of heart muscle. This often leads to adverse remodeling of the myocardium and can progress to HF. Heart failure, especially with reduced ejection fraction (HFrEF), is characterized by impaired pump function usually following myocardial injury, accompanied by neurohormonal activation and pathological remodeling (fibrosis, hypertrophy). TCM herbal therapies have been used traditionally to promote circulation, resolve blood stasis, and tonify the heart in these conditions. Modern investigations are revealing mechanistic support for some of these approaches in MI/HF models:

Preventing ischemia-reperfusion injury: Several TCM metabolites attenuate myocardial ischemia-reperfusion (I/R) injury – a key component of MI damage – by reducing oxidative stress and cell death. For example, luteolin (a flavonoid from Chrysanthemum and others) decreased infarct size and improved cardiac function in rat I/R models via Nrf2 activation and HO-1 upregulation (Baiyun et al., 2018; Oyagbemi et al., 2018). Salvianolic acid B (from Salvia miltiorrhiza) protects cardiomyocytes during I/R by scavenging ROS and inhibiting apoptosis through PI3K/Akt and ERK pathways (Dawuti et al., 2023). Notoginsenoside R1 also showed cardioprotective effects against I/R injury by modulating inflammation and apoptosis-related signaling (Chen Q. et al., 2024). These compounds essentially help the heart muscle better withstand the oxidative and inflammatory onslaught during reperfusion.

Limiting infarct expansion and fibrosis: After an MI, the reduction of infarct expansion and scar formation is crucial. Tanshinone IIA (a diterpenoid from Salvia miltiorrhiza) has been shown to limit infarct size and fibrosis in rodent MI models by anti-apoptotic and anti-fibrotic mechanisms (Wang N. et al., 2020). It can inhibit cardiac fibroblast activation via TGF-β/Smad pathways. Ferulic acid (from Angelica sinensis and Ligusticum chuanxiong) and astragaloside IV (from Astragalus) have similarly demonstrated reduction in cardiac fibrosis and improved post-MI ventricular function, linked to modulation of inflammatory cytokines and collagen synthesis (Lu et al., 2015; Liao et al., 2021).

Enhancing cardiac repair and function: Some TCM compounds may promote angiogenesis or myocardial repair. Panax notoginseng saponins have been reported to increase capillary density in ischemic myocardium and improve left ventricular function post-MI, potentially via upregulating VEGF and related pathways (Zhu et al., 2021). Erythropoietin-producing hepatocyte (Eph) receptor signaling modulators like icaritin (from Epimedium) showed cardioprotective effects by promoting endothelial repair after MI (Zhang et al., 2015).

In heart failure models, TCM-derived metabolites often exhibit anti-remodeling effects: reducing hypertrophy, fibrosis, and improving calcium handling in cardiomyocytes. For example, berberine was found to inhibit cardiac fibroblast proliferation and myofibroblast differentiation by modulating AMPK/PGC-1α signaling, helping to alleviate cardiac fibrosis in models of ischemic or diabetic cardiomyopathy (Hu et al., 2025). Corynoline (from *Corydalis* species) can increase the interaction between PPAR-α and NF-κB p65, thereby inhibiting NF-κB pro-inflammatory signaling and ameliorating cardiac inflammation (Wang M. et al., 2022). Ginsenosides such as Rg1 and Rd activate Nrf2-driven antioxidant responses in cardiac tissue, leading to higher levels of HO-1 and other antioxidants (Yao et al., 2022). Some saponins also modulate calcium handling and have anti-apoptotic effects in cardiomyocytes (through PI3K/Akt and other pathways) (Ghafouri-Fard et al., 2022). Salvianolic acids from *Danshen* (Salvia) can inhibit NF-κB and p38 MAPK signaling in cardiac fibroblasts, reducing their activation and collagen secretion (Dawuti et al., 2023). These multi-target effects (antioxidant, anti-inflammatory, anti-fibrotic, pro-angiogenic) converge to preserve cardiac structure and function, thereby offering therapeutic benefit in HF models.

In summary, across MI and HF, TCM metabolites work to limit the initial injury (e.g., reducing I/R damage), mitigate adverse remodeling (fibrosis, hypertrophy), and enhance residual function (through improved calcium dynamics and cardiometabolic effects). While these findings in cells and animal models are encouraging, translation to human patients will depend on well-designed clinical trials for post-MI or HF interventions using such compounds.

# 3 Clinical trials

Although much of the evidence for TCM natural metabolites in CVDs comes from preclinical research, a growing number of clinical studies and trials have begun to validate their efficacy and safety in humans. This section highlights key examples of TCM-derived metabolites that have made significant strides toward clinical translation, including berberine, quercetin, and the polyherbal Qili Qiangxin capsule, focusing on trial data, outcomes, and safety profiles (Bellavite et al., 2023).

## 3.1 Clinical evidence and translational successes

As a single-metabolite supplement, berberine has been used in East Asia for metabolic and cardiovascular health for decades, and Western interest has increased in recent years (Song et al., 2020). Multiple randomized controlled trials (RCTs) have evaluated berberine in conditions like dyslipidemia, type 2 diabetes, and heart failure. Some systematic reviews and meta-analyses have found that berberine significantly improves lipid profiles, with a decrease in total cholesterol, LDL, and triglycerides, while slightly increasing HDL. Notably, these improvements were not

accompanied by serious adverse reactions; meta-analyses report no significant difference in overall incidence of adverse events between berberine and placebo, and no serious toxic reactions occurred (Ju et al., 2018; Ye et al., 2021; Blais et al., 2023). This suggests that berberine is generally well-tolerated at the doses used. Clinically, the lipid-lowering effect of berberine is roughly comparable to a low-to-moderate dose statin, but via a different mechanism–berberine upregulates hepatic LDL receptor expression and inhibits PCSK9, rather than inhibiting cholesterol synthesis. In addition, berberine has shown clinical benefits in improving metabolic diseases such as type 2 diabetes and non-alcoholic fatty liver disease, which are often comorbid with CVD. These multi-target effects may provide better cardiovascular risk reduction in patients with metabolic syndrome (Ilyas et al., 2020; Zhang et al., 2020; Zhao et al., 2021; Hu et al., 2022; Zhang et al., 2022; Nie et al., 2024). Overall, berberine stands out as a successful case of a natural metabolite with evidence from Phase II trials and meta-analyses supporting its efficacy (particularly in dyslipidemia) and an encouraging safety profile. It has not yet been incorporated into Western clinical guidelines, but in China berberine is sometimes recommended as an adjunct for dyslipidemia or diabetes management, especially when statins or metformin are insufficient or not tolerated.

Another promising compound is quercetin (a ubiquitous flavonoid, including in Ginkgo and Sophora). Early pilot trials of quercetin supplements in hypertensive patients showed modest reductions in blood pressure and improvements in endothelial function, without major side effects (Bondonno et al., 2016; Zheng et al., 2024). Quercetin's clinical impact needs confirmation in larger trials, but it exemplifies how a well-known dietary phytochemical is being repurposed at higher doses for cardiovascular prevention.

Complex TCM formulas containing a variety of natural metabolites also play an important role in the treatment of CVDs. Qili Qiangxin (QLQX) capsules, which combine extracts of 11 herbs (e.g., *Panax ginseng*, *Astragalus membranaceus*, *Aconitum carmichaelii*, etc.), have been studied in the context of heart failure. Preclinical studies indicated that QLQX reduces myocardial fibrosis, inhibits cardiac hypertrophy, and improves ejection fraction in heart failure models. It acts on pathways like PI3K/Akt and AMPK (reducing inflammation and apoptosis) and enhances energetics in failing hearts (Fan et al., 2022; Wang T. et al., 2023; Wu et al., 2025). QLQX was recently evaluated in a large-scale Phase III clinical trial known as the QUEST study (Li et al., 2013). This multicenter, randomized, double-blind, placebo-controlled trial enrolled 3,110 patients with chronic heart failure with reduced ejection fraction (HFrEF) across 133 hospitals. The primary endpoint was a composite of rehospitalization for worsening heart failure or cardiovascular death. Clinical investigations reported in 2023 revealed compelling evidence for the efficacy of QLQX. When added to standard care, this botanical agent resulted in a significant 22% relative risk reduction for the primary composite endpoint of heart failure hospitalization or cardiovascular mortality compared to placebo (25.0% vs. 30.0%; hazard ratio [HR] = 0.78, 95% CI: 0.68–0.90; $p < 0.001$). Notably, this benefit encompassed a 24% reduction in heart failure hospitalizations and a 17% decrease in cardiovascular fatal events. Crucially, this therapeutic benefit was achieved with a favorable safety profile, as QLQX demonstrated tolerability comparable to placebo, with no significant hepatic or renal toxicity and no increase in arrhythmias or other major adverse events. By meeting stringent Western-style randomized controlled trial (RCT) standards and demonstrating efficacy on hard clinical endpoints, this multi-component traditional Chinese medicine (TCM) formulation challenges the conventional paradigm of single-molecule pharmacotherapy. The successful evaluation of QLQX not only paves the way for its incorporation into Chinese guidelines as an adjunct therapy for heart failure with reduced ejection fraction (HFrEF) but also establishes a robust methodological blueprint for assessing other complex TCM formulas in contemporary cardiovascular clinical trials. Table 3 lists the key clinical studies involving TCM metabolites, including the metabolite name, TCM source, study design, treatment duration, dosage, major outcomes (e.g., improved blood pressure, lipid profile), and any reported adverse effects.

However, not all clinical studies of TCM metabolites have been positive – some trials have yielded neutral results, possibly due to suboptimal dosing, bioavailability issues, or patient population factors. Therefore, continued rigorous research is needed. Overall, the trajectory is clear: TCM natural metabolites are beginning to transition from bench to bedside, supported by growing clinical evidence. The translation pipeline (Figure 3) typically progresses from identifying a promising metabolite or herbal formula, demonstrating efficacy in cellular and animal models, ensuring quality control in formulation, conducting early-phase human safety studies, then larger efficacy trials, and finally seeking guideline inclusion if successful.

## 3.2 Clinical application practice

TCM-derived compounds offer promising adjuncts in cardiovascular care. This section outlines how clinicians can apply these findings in practice – including practical dosing, integration with standard therapies, and safety monitoring – and suggests future research directions.

### 3.2.1 Berberine clinical application and dosing

In clinical trials, berberine typically is administered at 500 mg two to three times daily (1–1.5 g total per day). For example, obese patients given 500 mg thrice daily for 12 weeks achieved significant improvements: ~5 lbs weight loss and a 23% reduction in triglycerides with a 12% drop in total cholesterol. These metabolic effects approach the magnitude of first-line therapies – one trial even noted ~18 mg/dL LDL reduction on average–suggesting berberine can complement conventional agents (e.g. adding to statins or metformin) for additional benefit. Indeed, combination studies report that berberine on top of statin therapy further improves lipid profiles compared to statins alone (Xiong et al., 2020; Zamani et al., 2022; Ge et al., 2024; Kong et al., 2025). Clinicians might consider berberine as an adjunct in patients with metabolic syndrome or hyperlipidemia who require extra risk factor reduction or cannot tolerate higher doses of standard drugs. Importantly, berberine should not replace evidence-based medications like statins but rather be integrated alongside lifestyle modifications and guideline-directed therapies.

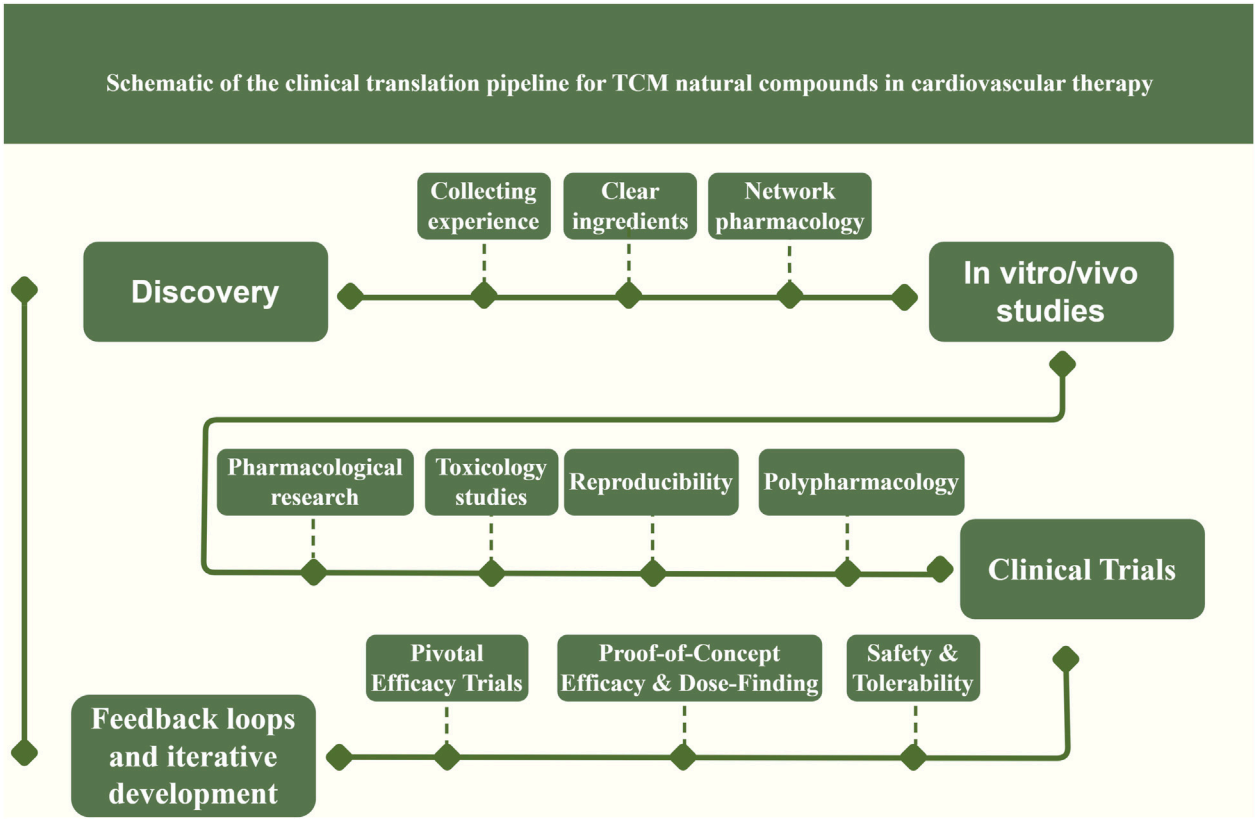



FIGURE 3
Schematic illustration of the clinical translation pipeline for TCM-derived natural compounds in cardiovascular therapy. Explanation: It outlines the sequential steps from initial compound discovery and characterization through mechanistic pharmacological studies, preclinical testing, formulation development, and phased clinical trials. These efforts ultimately culminate in regulatory approval of the new therapy and its integration into clinical practice guidelines. At the mechanistic study stage, emphasis is placed on target identification and pathway validation for key cardioprotective signaling networks (such as Nrf2, AMPK, NF-κB, and PI3K/Akt), utilizing systems biology approaches (omics and network pharmacology analyses) alongside *in vitro* assays and *in vivo* animal models to elucidate molecular mechanisms. Promising lead compounds then undergo rigorous preclinical efficacy and safety testing, concurrently with formulation development to enhance bioavailability and optimize pharmacokinetics. The pipeline's clinical evaluation phase encompasses a series of human trials (Phase I, II, and III) to progressively establish safety and efficacy. Phase I trials focus on initial human safety assessment, tolerability, and pharmacokinetics. Phase II studies refine the dosing regimen and evaluate preliminary efficacy, often using biomarkers as surrogate endpoints to guide therapeutic assessment. Phase III comprises large-scale, endpoint-driven randomized controlled trials designed to confirm definitive clinical efficacy and safety in broad patient populations. In addition, the translation pipeline is iterative: feedback loops are incorporated such that insights from clinical trials (e.g., unexpected biomarker findings or safety signals) can prompt refinement of underlying mechanisms or necessitate reformulation of the compound. This adaptive process helps ensure that the most effective and safe TCM-derived interventions ultimately advance to regulatory approval and guideline-directed cardiovascular therapy.

### 3.2.2 Qili Qiangxin capsule: integration into heart failure therapy

QLQX is a patented Chinese herbal capsule approved in China for chronic heart failure. Clinically, QLQX is used in addition to standard heart failure therapy (beta-blockers, RAAS inhibitors, diuretics, etc.), not as a standalone treatment. QLQX can be a complementary therapy in HFrEF, potentially bridging gaps in symptom relief and disease modification when optimal conventional therapy is insufficient. QLQX is administered orally in capsule form. Each capsule contains 0.3 g of herbal extract. Heart failure studies have used about 1.5–2 g per day of QLQX, typically divided as 4–6 capsules daily. For instance, earlier clinical trials often gave 4–6 capsules total per day (e.g. 2 capsules three times daily) in mild-to-moderate heart failure. In the recent large HFrEF trial, a higher dosage was employed: four 0.3 g capsules three times daily (total 3.6 g/day) alongside standard therapy (Leung et al., 2021; Huang et al., 2022; Nie et al., 2024; Tao et al., 2024). This intensive regimen was well tolerated, though investigators allowed dose reduction to 2–3 capsules TID if adverse. In practice, clinicians should start with the evidence-backed dosing (e.g. 4 capsules twice daily or three times daily, per severity) and adjust based on patient tolerance. Notably, the Chinese pharmacopeia recommendation for QLQX is 4 capsules three times per day, which aligns with the high-dose trial protocol. Integrating QLQX with Conventional Therapy: Patients receiving QLQX must continue on standard heart failure medications. In all major trials, QLQX was an adjunct to guideline-directed medical therapy (GDMT). The add-on of QLQX appears to confer additional benefit (e.g. fewer HF hospitalizations) without impeding standard drugs' actions. The result is a more holistic therapeutic strategy–treating both the "root" (underlying myocardial remodeling) and "branch" (symptoms like edema) in TCM parlance, while fully maintaining modern medical therapy.

### 3.2.3 Other cardiovascular integrations

Beyond heart failure, TCM compounds are being explored in coronary artery disease and hypertension as adjuncts to standard care. Tongxinluo was given as 1.04 g (4 capsules) three times daily for 12 months alongside all guideline-recommended medications, and it produced no increase in adverse events (Yao et al., 2020; Yang Y. et al., 2023; Yang Z. et al., 2023; Ouyang et al., 2025). These outcomes highlight that integrating TCM formulations with conventional therapies can yield additive benefits in ischemic heart disease, potentially by protecting microcirculation and reducing reperfusion injury. The combined use of Chinese herbal medicines and conventional antiplatelet agents represents a promising therapeutic strategy for atherosclerotic cardiovascular diseases (ASCVDs), demonstrating significant clinical benefits. This integrated approach offers dual advantages: enhancing antiplatelet efficacy while simultaneously mitigating bleeding risks. Extensive research confirms that specific herbs, such as Salvia miltiorrhiza, exert a synergistic effect when co-administered with drugs like aspirin or clopidogrel, leading to amplified suppression of platelet aggregation and a reduced thrombotic risk. For instance, extracts from S. miltiorrhiza, rich in tanshinones and salvianolic acids, have been shown to potentiate the effects of these antiplatelet medications. Importantly, this combination also provides superior bleeding risk control compared to alternative anticoagulation regimens. A notable clinical study demonstrated that patients receiving Naoxintong capsules (1.6 g/day, three times daily) plus aspirin (100 mg/day) exhibited a significantly lower incidence of severe bleeding than those on an adjusted-dose warfarin regimen (0% vs. 7.9%; OR = 0.921, 95% CI: 0.862–0.984, p = 0.028), providing robust evidence for the treatment's safety profile. Furthermore, certain herbal formulations can improve vascular endothelial function. Research revealed that pretreatment with Trichosanthis Fructus pellets (1 g/kg/day) and aspirin (0.01 g/kg/day) in rats not only inhibited platelet aggregation but also enhanced endothelial integrity. Mechanistically, this combination therapy modulates key mediators such as thromboxane A2 (TXA2) and prostacyclin (PGI2), including their downstream effector cyclooxygenase-2 (COX-2), thereby contributing to its antithrombotic properties. Collectively, these findings underscore the substantial clinical value and potential of integrating Chinese herbal medicine with antiplatelet therapy for the prevention and management of ASCVDs.

Clinicians can accommodate such integrative approaches cautiously, ensuring that patients continue their ACE inhibitors, calcium blockers, etc. while an herbal remedy is tried. Given the lack of definitive data, TCM for hypertension should be considered complementary and secondary – never a replacement for proven pharmacotherapy.

### 3.2.4 Safety, monitoring, and drug interactions

When integrating TCM-derived treatments, clinicians must monitor safety parameters and be mindful of herb–drug interactions. Both berberine and QLQX have favorable safety profiles in trials, but real-world use requires vigilance:

Hepatic and Renal Function: Berberine has not been associated with liver enzyme elevations or hepatotoxicity in clinical studies. Short-term trials (up to 3 months) reported no significant changes in liver or kidney function tests compared to placebo (Ji et al., 2025). Despite this, experts recommend periodic liver function monitoring if berberine is used long-term, especially in patients with underlying liver disease. This is a precaution to catch any idiosyncratic reaction early. Similarly, QLQX capsules have not shown hepatic or renal toxicity in controlled trials (e.g., adverse event rates were comparable to placebo over 18 months). Still, routine lab monitoring (metabolic panel, renal function) is prudent in heart failure patients adding any new therapy (Xing et al., 2022; Cheang et al., 2024).

Electrolytes and Hemodynamics: Patients on QLQX plus diuretics should have electrolytes closely followed, particularly potassium. The herbal components in QLQX promote diuresis (e.g. Alismatis in the formula) and enhanced natriuresis, which can potentiate the effect of loop or thiazide diuretics. There is a risk of additive hypokalemia when QLQX is combined with aggressive diuretic therapy, so clinicians should check potassium levels and adjust diuretic dose if needed. In practice, co-administration of QLQX with furosemide or hydrochlorothiazide may warrant more frequent electrolyte monitoring during the initial weeks. Conversely, if a patient is on an ACE inhibitor or spironolactone (risking hyperkalemia), the mild diuretic effect of QLQX might actually counterbalance potassium somewhat–but this interaction is not well quantified, so monitoring is still essential. Blood pressure and heart rate should also be observed when starting QLQX. Its vasodilatory and positive inotropic effects could in theory influence hemodynamics. While trials did not find significant hypotension or arrhythmias attributable to QLQX, a patient on multiple vasoactive drugs should be watched for blood pressure changes or palpitations. Notably, aconite (an ingredient in QLQX) is a potent cardiotonic herb; in improper doses aconite can cause arrhythmias, though in QLQX it is present in processed form (Fuzi) at safe dosages. Clinicians should still be alert to any new arrhythmic symptoms in patients on QLQX, especially if also on digoxin or other inotropes. It may be wise to obtain a baseline and follow-up ECG in HF patients when initiating QLQX, particularly if they have a complex arrhythmic history.

### 3.2.5 Drug–herb interactions

In the context of integrated management strategies for cardiovascular and metabolic disorders, Drug–Herb Interactions manifest unique synergistic regulatory mechanisms alongside substantial clinical benefits. For hypertension control, there is robust evidence from randomized controlled trials supporting the efficacy of traditional Chinese medicinal formulations, such as Yiqi Huaju formula and Songling Xuemaikang Capsules. These formulations can modulate blood pressure through distinct pathways. Particularly noteworthy is that Bushen Qinggan demonstrate superior systolic pressure reduction compared to conventional antihypertensive regimens. Moreover, CGSHY herbal mixture exhibits comparable antihypertensive potency to the angiotensin-converting enzyme inhibitor enalapril, while coadministration of Jiangya Capsules with the calcium channel blocker nimodipine markedly enhances therapeutic outcomes in elderly patients with isolated systolic hypertension. Turning to secondary prevention in coronary artery disease, Xuezhikang significantly reduces coronary event rates among hypertensive survivors of myocardial infarction. Shenshao tablets substantially improve quality of life metrics in patients with stable angina

pectoris, whereas Quyu Xiaoban capsules decrease angina frequency by suppressing platelet activation in unstable angina cases. Collectively, these findings underscore how Drug–Herb Interactions operate through multi-targeted mechanisms to offer promising integrated medical approaches for cardiometabolic disease management. Such interactions represent a compelling frontier for developing personalized treatment paradigms that leverage both pharmacological precision and holistic therapeutic principles.

Perhaps the most critical consideration is interactions between TCM compounds and prescription drugs, while potential adverse effects or complexities must also be acknowledged. Berberine is a known inhibitor of P-glycoprotein and certain CYP450 enzymes, which can lead to elevated levels of concurrent medications. A prominent example is the calcineurin inhibitor cyclosporine – coadministration of berberine has been shown to markedly increase cyclosporine blood concentrations, risking toxicity. Similarly, berberine may increase serum levels (and effects) of drugs like tacrolimus, digoxin, diltiazem, or certain statins by reducing their metabolism or efflux (Abushammala, 2021; Adiwidjaja et al., 2022; Nyulas et al., 2024; Zakiev et al., 2024). Clinicians should review each patient's medication list for any narrow-therapeutic-index drugs. If a patient on warfarin, for instance, starts berberine, extra INR checks are indicated, as berberine could potentiate warfarin's effect (reports of enhanced anticoagulation have been noted anecdotally). Berberine also has additive hypoglycemic action; a patient on insulin or sulfonylureas might experience lower blood sugars once berberine is added, so glucose should be monitored and diabetes medications adjusted to avoid hypoglycemia. QLQX being a multi-herb combination, has a lower risk of specific cytochrome-mediated interactions (since it's not a single high-concentration alkaloid), but it can still interact pharmacodynamically with cardiac drugs. Its digitalis-like component (periplocymarin from Periploca) could synergize with prescribed digoxin, potentially increasing inotropic effect–caution is advised to watch for digitalis toxicity signs if patients are on both. However, in the large QLQX trial, many patients were on digoxin and no increased toxicity was reported, suggesting it's generally safe. Nonetheless, prudent practice is to use the lowest effective digoxin dose if combining with QLQX, and monitor serum digoxin levels when available. QLQX's vasodilator herbs (e.g. danshen) could also augment antihypertensive drugs, so blood pressure should be monitored to avoid hypotension when combining, say, QLQX with high-dose ACE inhibitors (Wang et al., 2025). Overall, open communication with patients about herbal and supplement use is essential: clinicians should ask specifically about TCM products, as patients may not volunteer this information, and incorporate that into medication reconciliation to manage interactions.

#### 3.2.6 General tolerability

Both berberine and QLQX are generally well tolerated. The most common side effects of berberine are mild gastrointestinal issues – e.g. constipation, nausea, or diarrhea – occurring in a minority of patients. These often subside with continued use or dose adjustment (starting at 500 mg twice daily with food can improve GI tolerance, then uptitrating to three times daily) (Li et al., 2023). QLQX capsule's side effect profile in trials was similar to placebo. Some patients report dry mouth or gastric discomfort with QLQX, which can be mitigated by taking it after meals. Because QLQX contains multiple ingredients, there is a small risk of allergic reactions; clinicians should discontinue it if any rash or hypersensitivity signs appear. Importantly, any TCM compound should be avoided in pregnancy unless specifically studied, as many herbal components (including berberine) can cross the placenta. Berberine is contraindicated in late pregnancy and neonates due to risk of kernicterus (it can displace bilirubin). While young women are less likely to need these therapies for cardiovascular issues, clinicians should ensure non-pregnant status or discuss contraception if prescribing berberine to women of childbearing potential.

In summary, with appropriate monitoring – periodic labs, tracking vitals, and checking for drug interactions – berberine and QLQX can be safely integrated into practice. The active involvement of clinicians in monitoring is critical to catch any adverse effects early. This includes scheduling follow-ups a few weeks after initiation to reassess blood pressure, volume status, metabolic panel, and therapeutic drug levels as needed. Educating patients to report any unusual symptoms (e.g. dizziness, palpitations, muscle weakness that could indicate hypokalemia, etc.) further enhances safety.

## 4 Challenges and future perspectives

Despite the encouraging advances outlined above, significant challenges remain before TCM-derived therapies can be fully integrated into mainstream cardiovascular medicine. In this section, we discuss key hurdles and future directions: standardization and quality control of natural products, mechanistic complexity and polypharmacology, clinical translation and integration with current therapies, and others.

### 4.1 Standardization and quality control issues

Natural metabolites derived from botanical drugs can have significant variability in content and potency. Factors such as plant species (and potential misidentifications or adulterants), growing conditions, harvest time, and post-harvest processing all influence the concentration of active ingredients. This lack of standardization makes it difficult to ensure consistent efficacy and safety. For instance, the content of ginsenosides in ginseng can vary widely between sources, and flavonoid levels in *Scutellaria* root depend on growth and drying conditions. Without rigorous quality control, one batch of an herbal extract might be far less potent (or conversely, contain more of a toxic constituent) than another.

Furthermore, each botanical drug typically contains multiple active components. Isolating the effect of one specific metabolite can be challenging when others are present, potentially synergizing or antagonizing each other. Reproducibility of research is a concern: if studies do not fully characterize the herbal extract used, results cannot be reliably compared or replicated. Advanced analytical techniques (such as LC-MS metabolite profiling, NMR spectroscopy, and chromatographic fingerprinting) are

increasingly necessary to profile and guarantee the composition of herbal products. Regulatory agencies will need to establish clear standards for botanical drug preparations, similar to Active Pharmaceutical Ingredient (API) standards for single compounds, to facilitate wider acceptance.

An additional complication is the presence of pan-assay interference compounds (PAINS) in some plant extracts. PAINS are promiscuous molecules that can nonspecifically affect multiple assay readouts, leading to false-positive signals (Baell, 2016; Bisson et al., 2016; Baell and Nissink, 2018; Sun et al., 2021). For example, certain polyphenols or quinones can aggregate proteins or generate assay-reactive species, giving an illusion of activity in screening tests. We have to be cautious that some reported "active" natural compounds might actually be PAINS. This highlights the importance of using appropriate controls (such as counter-screens) and applying PAINS filters during drug discovery efforts to avoid being misled by artifacts. By excluding such interference compounds early, researchers can focus on truly promising metabolites.

To ensure reliability, taxonomic authentication of the source plant is essential (e.g., voucher specimens, DNA barcoding) as well as chemical standardization of the extract. For well-known TCM herbs, official pharmacopeias (e.g., Chinese Pharmacopoeia) often specify marker compounds and minimum content levels (see Supplementary Table S2 for examples). Adhering to these standards is critical. For instance, using *Salvia miltiorrhiza* extract standardized to a certain percentage of tanshinones and salvianolic acids helps ensure consistency across studies.

Encouragingly, efforts are underway to improve reporting and reproducibility. A consensus guideline known as ConPhyMP (Consensus on Phytochemical Characterization of Medicinal Plant extracts) has been proposed to standardize how researchers describe plant materials and extracts (Heinrich et al., 2022). We have applied these principles in our review, documenting each botanical drug's scientific name, part used, traditional preparation, and key phytochemical markers (see Supplementary Table S1). Moreover, triple-fingerprinting approaches (combining, for example, TLC, HPLC, and UV-visible spectra) and multi-marker quantification are recommended to fully characterize extracts (Hamburger, 2019; Bārzdiņa et al., 2022; Helmick et al., 2023; Riaz et al., 2023). By following such guidelines and transparently reporting all aspects of the plant material and extraction process, future studies will be more reproducible and easier to evaluate. Adopting the ConPhyMP checklist in peer review (as in this journal) is a positive step toward that goal.

Another critical challenge is the inherently poor bioavailability of many botanical metabolites, which limits their therapeutic consistency. Natural compounds often have low aqueous solubility or are rapidly metabolized, leading to minimal absorption *in vivo*. To address this, researchers are developing advanced delivery systems and chemical modifications. Nanoformulations – such as encapsulating phytochemicals into liposomes, nanoemulsions, or polymeric nanoparticles – can dramatically enhance dissolution, protect labile compounds, and improve membrane permeability. For example, the major Salvia miltiorrhiza diterpenoid tanshinone IIA has very low water solubility and oral absorption; it has been formulated into lipid-based nanocapsules, micelles, and solid lipid nanoparticles to boost its bioavailability (Zhang et al., 2013; Jia et al., 2024; Zou et al., 2024). Another strategy is to create more soluble derivatives or prodrugs. Converting tanshinone IIA into its sodium sulfonate form (sodium tanshinone IIA sulfonate, STS) greatly increases polarity and bioavailability, and indeed STS is used interchangeably with tanshinone IIA in studies and has been approved in China as an injectable drug (Shao et al., 2024; Xie et al., 2025). As a general proof of concept, the alkaloid berberine (another plant-derived drug with <5% oral absorption due to P-gp efflux) showed markedly enhanced uptake when delivered via liposomal nanoparticles (Shan et al., 2013; Zhang et al., 2019; Murakami et al., 2023a, b; Cui et al., 2024). By employing such nano-delivery systems or medicinal chemistry optimizations, researchers can significantly improve the pharmacokinetic profiles of herbal metabolites, ensuring more consistent potency and therapeutic levels *in vivo*.

## 4.2 Mechanistic complexity and polypharmacology

One challenge lies in the very strength of TCM metabolites: their polypharmacology (ability to hit multiple targets). From a scientific standpoint, it can be difficult to pinpoint the primary mechanism of action when an herb or metabolite affects numerous pathways simultaneously. Standard drug development often seeks a single target for a single disease pathway, but these natural agents defy that model. They may exert moderate effects on several pathways rather than a blockbuster effect on one. How do we rigorously demonstrate causality for each effect?

Polypharmacology (multi-target activity) is another double-edged aspect of botanical drugs that demands careful strategy in drug development. On one hand, having multiple targets can produce synergistic efficacy in complex diseases; on the other hand, it complicates pinpointing mechanisms and predicting off-target effects.

Network pharmacology and "omics" approaches are emerging as powerful tools to tackle this complexity. By using transcriptomic or proteomic profiling, researchers can see broad changes induced by a TCM extract, generating a signature of its action. Computational network models can predict which pathways are most crucial. Still, dissecting multi-component mixtures remains arduous. Instead of the old "one drug, one target" paradigm, network pharmacology operates on a "multiple targets, multiple effects" model–essentially replacing the single "magic bullet" with a coordinated "magic shotgun" strategy. In practice, this means computational and experimental methods are combined to identify the network of protein targets and pathways influenced by a given herb or its constituents. High-throughput screening (HTS) and phenotypic assays can be applied to profile natural compounds across many potential targets or cellular pathways, generating data that are then integrated into drug–target networks. This allows researchers to pinpoint which targets are critical for the desired therapeutic effect and which interactions might cause side effects. For instance, network-based analyses of a Danshen (Salvia) multi-component formula identified nine key compounds collectively modulating 42 cardiovascular-related genes, highlighting how a mixture's efficacy arises from multi-target synergy (Li et al., 2011). Such insights guide rational

development: one can prioritize compounds that hit multiple synergistic targets or design combination therapies that harness beneficial polypharmacology while minimizing promiscuous interference. By employing network pharmacology modeling and broad counter-screening early on, developers can manage polypharmacology—leveraging it to enhance efficacy (through synergistic target engagement) and using informatics to avoid trap compounds that bind irrelevant or harmful targets. This integrative strategy improves the chances that a candidate natural drug will have reproducible, mechanism-based effects even with multiple molecular targets, thereby facilitating its progression through the drug development pipeline.

Another issue is that polypharmacology can lead to off-target effects and interactions. While hitting multiple targets can be beneficial (tackling a disease from different angles), it also increases the risk of affecting an unintended pathway, possibly causing side effects. For example, a compound that is antioxidant and anti-inflammatory might also weakly interact with a hormone receptor or ion channel, with unknown implications.

From a research design perspective, carefully deconvoluting the contributions of each component in a herbal formula is valuable. Strategies include testing isolated constituents versus the whole extract, using knockdown of specific pathways in cells or gene knockout models in animals to see if the effect persists, and applying pharmacological inhibitors to block suspected mechanisms. Such approaches have started to be employed (e.g., using Nrf2-knockout mice to confirm a compound's effects are via Nrf2).

Metabolomics has emerged as a promising approach for improving cardiovascular risk stratification, particularly in intermediate-risk asymptomatic populations. Recent studies employing high-performance analytical techniques such as mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy have identified discriminative metabolites including sphingomyelins, lysophosphatidylcholines, and ceramides that correlate with coronary artery disease (CAD) progression. Another team applied ultra-high-performance liquid chromatography quadrupole time-of-flight mass spectrometry (UPLC-QTOF-MS) to analyze the metabolic components of traditional Chinese medicine (TCM) metabolites in rat biological samples (blood, urine, and feces), thereby exploring the mechanisms of natural metabolites' metabolism *in vivo* and their efficacy and effects (Tang et al., 2015b). The researchers also used ultra-performance liquid chromatography coupled with diode array detection and electrospray ionization mass spectrometry (UPLC-DAD-MS) to identify differences in natural metabolites derived from different plant sources (Tang et al., 2013; Yi et al., 2014). This innovative identification method has brought new opportunities for the study of natural metabolites. For instance, a novel risk prediction model based on plasma ceramide profiles was developed, demonstrating significant prognostic value for adverse cardiovascular events in cohorts validated across multiple centers. These metabolic biomarkers exhibit incremental predictive power when integrated with traditional risk assessment tools like the FRS and SCORE2 systems, as evidenced by net reclassification improvements exceeding 15% in meta-analyses involving over 10,000 participants. However, current implementation remains constrained by technical heterogeneity in sample preparation protocols and data interpretation standards. Future research should prioritize multi-center validation studies incorporating standardized metabolomic workflows while exploring synergies between Western biomarkers and therapeutic pathways that modulate lipid peroxidation and inflammation cascades.

In summary, while the multi-target nature of TCM therapies is promising for multi-factorial diseases like CVD, it complicates the mechanistic study. Embracing systems biology and network pharmacology will be crucial in fully understanding and optimizing these interventions.

## 4.3 Clinical translation and integration with western medicine

The ultimate challenge is translating promising natural metabolites into approved, widely adopted therapies. As the clinical evidence section highlighted, there are now successful examples (e.g., the QLQX heart failure trial, and growing data on berberine). But many hurdles remain on the road to regulatory approval and clinical guideline inclusion.

Firstly, robust clinical trials are needed for each candidate. This requires significant funding and logistical support, as well as overcoming any regulatory hesitations about herbal products. Many existing trials of TCM therapies have been small or of variable quality. Future studies must match the standards of pharmaceutical trials – large sample sizes, randomization, placebo-control, and clear endpoints.

Another consideration is botanical drug–drug interactions: many patients will use natural products alongside standard medications, so understanding interactions is critical. For instance, St. John's Wort (a Western herb) famously induces drug-metabolizing enzymes and can reduce the efficacy of warfarin or statins. Similarly, compounds in grapefruit juice (furanocoumarins) inhibit CYP3A4 and raise levels of certain calcium blockers. For TCM herbs, there are examples like licorice (*Glycyrrhiza*) which can cause hypokalemia and affect digoxin toxicity, or ginseng potentially affecting coagulation. As TCM metabolites move into mainstream use, physicians will need clear information on any contraindications or interactions with conventional drugs.

Safety monitoring is also paramount. While many herbal medicines have been used for centuries, rare adverse effects might not be recognized without modern pharmacovigilance. For example, *Aconitum* (aconite) can cause severe arrhythmias if improperly processed – highlighting the need for quality control. Liver and kidney function should be monitored in trials, as there have been instances of herb-induced liver injury (usually idiosyncratic or due to adulterants). So far, most discussed compounds have shown good safety profiles in studies, but ongoing vigilance is required.

On the positive side, regulatory agencies in some countries are increasingly open to natural product drugs. For example, the Chinese FDA (NMPA) has approved certain TCM formula capsules for indication in heart disease (like QLQX). In the West, artemisinin (from *Artemisia annua*, discovered via TCM) has become globally recognized for malaria, and omega-3 fish oil (though not TCM, a natural product) is included in guidelines

for post-MI patients. These cases show that if strong evidence is available, natural metabolites can gain broad acceptance.

Traditional Chinese medicine (TCM) has demonstrated unique advantages in the treatment of cardiovascular diseases, which have been reflected in numerous studies. In terms of antihypertensive effects, Bushen Qinggan granules have shown efficacy comparable to that of Western medicine and can significantly reduce systolic blood pressure, providing a new option for hypertension treatment. The mechanism of action may involve multiple targets and pathways, such as regulating vascular endothelial function and influencing the neuroendocrine system, thereby improving blood pressure regulation. In the treatment of heart failure, Kanlijian can increase left ventricular ejection fraction (LVEF), Shemnai injection can improve the average movement velocity of the mitral ring, and Qili qiangxin can reduce brain natriuretic peptide (BNP) levels, indicating its positive impact on cardiac function. This may be related to the overall concept of TCM in regulating the balance of yin and yang in the body, improving the circulation of Qi and Blood, and thus alleviating the burden on the heart, enhancing myocardial contractility, and ultimately improving symptoms of heart failure. For coronary heart disease treatment, Xuezhikang TCM can reduce coronary events and has shown good effects in multicenter studies involving a large number of patients. Its potential mechanisms of action may include antiplatelet aggregation, anti-inflammation, and lipid metabolism regulation, among others. These mechanisms help protect coronary artery endothelial cells, stabilize plaques, and reduce the risk of cardiovascular events.

Despite robust preclinical data for many TCM-derived metabolites, clinical validation remains limited or preliminary for a majority of these compounds. It is important to acknowledge this gap explicitly: numerous promising agents have not yet been tested in large, high-quality trials, or have only small studies with inconclusive results. For example, tanshinone IIA (from Salvia miltiorrhiza or Danshen) and various flavonoids (e.g. baicalin from Scutellaria or quercetin found in many plants) show cardioprotective effects in animal models, but lack robust randomized controlled trial (RCT) evidence in patients. Many such compounds are supported mainly by preclinical findings or small-scale clinical studies of variable quality. This underscores the need for large-scale, well-designed RCTs to confirm efficacy and safety. To address these gaps, future research should prioritize pharmaceutical-grade clinical trial design for TCM metabolites. Key considerations include: clear inclusion criteria (e.g. enrolling patients with defined cardiovascular conditions – such as chronic heart failure (NYHA II–III) or stable angina – who are already on standard therapy), sufficient treatment duration (e.g. at least 6–12 months of intervention, to capture meaningful clinical outcomes rather than short-term surrogate changes), and meaningful primary endpoints (such as improvement in exercise capacity or cardiac function, reduction in hospitalizations or major cardiovascular events, or other hard outcomes like all-cause mortality). For instance, a multicenter, placebo-controlled RCT could enroll several hundred heart failure patients on optimal therapy, randomizing them to add-on therapy with a purified TCM metabolite (at a standardized dose) versus placebo for 1 year. The primary endpoint might be a reduction in 1-year heart failure hospitalization rates or improvement in left ventricular ejection fraction, with secondary endpoints including quality-of-life scores and biomarkers. Similarly, another trial could test a flavonoid-rich extract in patients with coronary artery disease, with endpoints such as exercise tolerance (e.g. via treadmill test) and incidence of angina or acute coronary events. By designing large, rigorous trials with appropriate populations, durations, and endpoints, researchers can generate the high-level evidence needed for regulatory approval and inclusion of these natural metabolites in clinical guidelines. This approach will not only strengthen the credibility of TCM-derived therapies but also accelerate their integration into mainstream cardiovascular care, guiding future interdisciplinary collaboration in an evidence-based manner.

If we aim for global recognition of a TCM metabolite, it likely has to meet the strictest standards. This means well-characterized chemistry, demonstrated mechanism, clear clinical efficacy, and safety equivalent to current drugs. Developing a natural product into a pill with a defined dose that doctors anywhere can prescribe is a long but feasible process. Some companies and research consortia are now investing in "phytopharmaceutical" development – essentially taking promising herbal compounds through the full drug development pipeline (purification, formulation, trials).

In moving forward, integration between traditional medicine and modern biomedical science will be the key. Bridging the knowledge of experienced TCM practitioners (who can guide which herbs "work" in practice and optimal preparation methods) with cutting-edge research (target validation, medicinal chemistry optimization of natural structures, etc.) will accelerate progress. Although the concept of homology between medicine and food has brought great hope for the future of health and wellbeing, there are still many challenges to be met to fully release its potential. The first challenge is to carry out more rigorous scientific research to verify the safety and effectiveness of homologous substances in medicine and food; Relying on the rich resources of food-borne drugs and traditional Chinese medicine treasure house and using strict scientific verification, we can develop evidence-based and widely recognized new therapies.

Encouraging interdisciplinary collaboration is crucial: teams that include cardiologists, pharmacologists, botanists, and chemists have begun working together on these challenges. Such collaboration is leading to a new paradigm where natural multi-component interventions stand alongside single-target drugs in a complementary fashion. Physicians and patients are increasingly open to integrative approaches, especially as data supporting them become more robust. Ultimately, our goal is to improve cardiovascular health outcomes by whatever means prove effective.

## 5 Conclusion

Natural metabolites used in TCM show great promise as therapeutic agents in the fight against cardiovascular diseases. Over the past decades, substantial progress has been made in understanding their mechanisms and moving them toward clinical use. These metabolites, including flavonoids (e.g., quercetin, baicalin), saponins (e.g., ginsenosides, astragalosides), alkaloids (e.g., berberine, matrine), and others like polyphenols and terpenoids, exhibit a remarkable breadth of cardioprotective actions. They combat oxidative stress, quell inflammation, enhance

endothelial function and nitric oxide bioavailability, modulate lipid metabolism to prevent atherogenesis, and attenuate fibrosis and adverse remodeling in the heart. Such a multi-target approach is particularly advantageous in complex cardiovascular disorders like atherosclerosis, myocardial infarction, and heart failure, where multiple pathological processes co-occur and drive disease progression. These compounds counteract fundamental pathological processes such as oxidative stress, inflammation, endothelial dysfunction, abnormal lipid metabolism, and cardiac remodeling. Recent evidence from preclinical studies and clinical trials underscores their therapeutic potential. For example, the alkaloid berberine improves lipid profiles and heart function, and the multi-herb formula Qili Qiangxin has demonstrated improved outcomes in heart failure patients. However, challenges such as poor bioavailability, complex multi-component formulations, and lack of standardization still hinder widespread use. Emerging strategies like advanced drug delivery systems and modern analytical techniques are being developed to overcome these obstacles.

In light of these findings, further development of cardiovascular therapeutics based on these natural metabolites is highly promising. One approach is to select these active molecules as lead compounds for new drug development, using medicinal chemistry to optimize their efficacy and pharmacokinetic properties. Another approach is to create multi-component formulations that incorporate several active compounds to achieve synergistic effects, akin to traditional herbal combinations. There are already examples of these strategies: for instance, ligustrazine (an active alkaloid from Ligusticum chuanxiong) has been developed into an injectable drug for ischemic stroke, and the Compound Danshen Dripping Pill (composed of Salvia miltiorrhiza and Panax notoginseng extracts) is an approved multi-component medicine for coronary heart disease in China. These cases demonstrate the feasibility of translating natural product compounds into effective single-agent or combination therapies. Therefore, continued efforts to explore chemical derivatives of these components and to design rational compound formulations could yield novel therapeutic options for cardiovascular disease in the future.

Importantly, an increasing body of evidence from *in vitro* experiments, animal models, and clinical studies provides support for the efficacy of these natural metabolites. These positive findings lend credence to the traditional uses of such remedies and provide a scientific rationale for their further development and integration into modern therapy. At the same time, reported safety profiles are generally favorable. With proper standardization and dosing, many natural metabolites appear to cause fewer severe side effects than some synthetic drugs (though vigilance is still required, as any bioactive substance can pose risks at high doses or in susceptible individuals).

The journey of bringing TCM metabolites into mainstream cardiovascular care is underway but not yet complete. Ongoing research is expected to expand the list of TCM-derived metabolites with evidence-based benefits, to optimize their formulations (for example, improving bioavailability of poorly absorbed molecules), and to clarify their roles in practice (whether as adjuncts to standard therapy to address residual risks like inflammation, or as alternatives in specific scenarios such as statin-intolerant patients). Continued high-quality clinical trials will be critical for garnering regulatory approvals and guideline recommendations. In moving forward, integration between traditional herbal medicine knowledge and modern clinical science will be key. By uniting the wisdom of TCM's pharmacopoeia with rigorous scientific validation, we can enrich the therapeutic arsenal against CVD. Collaboration across disciplines and cultures is fostering a paradigm where multi-component natural interventions complement single-target drugs. Physicians and patients are increasingly receptive to these integrative approaches as the evidence grows more robust. Ultimately, the goal is to improve cardiovascular outcomes by all effective means, harnessing the best of both traditional and modern medicine.

In conclusion, the rich pharmacopoeia of traditional herbal medicine offers promising therapeutic candidates for cardiovascular disease, either as standalone agents or in synergy with conventional treatments. Bridging traditional knowledge with modern clinical science not only expands our therapeutic arsenal but also exemplifies a holistic approach to complex diseases that targets multiple pathways to achieve better patient outcomes. The advances reviewed herein provide a strong foundation and impetus for further exploration, ensuring that the future directions of cardiovascular therapeutics will include the wisdom of nature's pharmacy, transformed by and validated through modern science.

## Author contributions

XL: Writing – review and editing, Writing – original draft. WZ: Data curation, Writing – review and editing, Formal Analysis. XM: Writing – review and editing. YH: Supervision, Writing – review and editing, Project administration, Writing – original draft, Funding acquisition.

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## Supplementary material

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# Ethnobotany, chemical analysis, and antiglycation activity of *Elsholtzia* species from Northern Thailand

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Seven species of *Elsholtzia* from upper northern Thailand were ethnobotanically surveyed, and the plant parts utilized by local communities were collected for chemical analysis and antiglycation activity evaluation. The 80% methanol extracts were concentrated and analyzed for total soluble phenolic content using the Folin–Ciocalteu reagent, and phytochemical profiling was performed by high-performance liquid chromatography (HPLC) against standard phenolic compounds. In addition, the extracts were investigated for antiglycation activity using the methylglyoxal (MGO)-BSA method, with aminoguanidine as a positive control. According to the ethnobotanical survey, the seven *Elsholtzia* species were traditionally used as food, medicine, and insect repellents by seven ethnic groups: Akha, Hmong, Karen, Lawa, Lisu, Tai Lue, and Tai Yai. Most species were cultivated and commonly found in agricultural areas, including upland rice fields and home gardens. HPLC analysis identified three major phenolic compounds—rosmarinic acid, ferulic acid, and luteolin—showing species- and plant part-specific variations. The leaf extract of *E. beddomei* contained the highest rosmarinic acid (667.9 mg/100 g), *E. griffithii* exhibited the highest ferulic acid (2.25 mg/100 g), and luteolin was detected exclusively in *E. stachyodes* (15.4 mg/100 g). Consistently, *E. beddomei* also had the highest total phenolic content (36.04 mg GAE/g) and the strongest antiglycation activity, with an $IC_{50}$ value of 1.65 mg/mL. The observed trend suggests that phenolic-rich extracts, particularly those with elevated rosmarinic acid content, are strongly associated with enhanced anti-glycation potential. These findings validate local ethnobotanical practices and highlight the species-specific potential of *Elsholtzia* as sources of bioactive compounds, supporting their further development for pharmaceutical and nutraceutical applications.

KEYWORDS

**antiglycation activity, aromatic plant, phenolic compounds, local vegetables, spices, traditional medicine**

# 1 Introduction

Plants are important sources for natural, nutritional and medicinal compounds (Menichetti et al., 2025; Ulian et al., 2020). Globally, the traditionally used plants are diverse, and associated ethnobotanical knowledge have inspired the discovery of useful secondary metabolites with health benefits (Leonti et al., 2017; Li and Weng, 2017). Thailand is one of the most biologically, ethnically, and culturally diverse countries, located in the Indo-Burma biodiversity hotspot (Myers et al., 2000). This diversity is particularly evident in the wealth of indigenous knowledge passed down through generations within ethnic communities. Such traditional wisdom, particularly in the field of ethnobotany, holds significant potential for scientific study and sustainable development. Previously, the rich food and medicinal plants used in Thailand have been reported (Inta et al., 2025). However, much of this knowledge is at risk of being lost due to socio-economic changes and the lack of systematic documentation (Yao et al., 2024). Preserving and exploring this knowledge are expected to lead to valuable contributions in fields such as medicine, nutrition, and economic development (Gao, 2024; Yao et al., 2021).

The genus *Elsholtzia* belongs to the tribe Elsholtzieae, subfamily Nepetoideae, and family Lamiaceae. It comprises approximately 40 species worldwide, most of which are naturally distributed in the temperate and tropical regions of Asia. Some species are cultivated in Europe and North America. In Southeast Asia, seven species have been reported in Vietnam, five in the Indochina Peninsula, eleven in Myanmar and three in Malaysia. In Thailand, eight species of *Elsholtzia* have been recorded, with most distributed in the northern region of the country, particularly in the open forests or forest edges at elevations starting from 600 m above sea level. Most of these species have also been cultivated by local people (Bongcheewin et al., 2015).

This notable group of plants have remarkable chemical properties and diverse applications in food and traditional medicine in different countries. For instance, in China, 14 species of *Elsholtzia* are used as herbal medicine, among which at least four species are also used for culinary and tea: *E. kachinensis*, *E. communis*, *E. bodinieri* and *E. penduliflora*, and eight species are used as aromatic plants (according to the Scientific Database of China Plant Species https://db.kib.ac.cn/). In India, *Elsholtzia densa* and *E. eriostachya* are used as culinary herbs (Chen et al., 2022), while in Vietnam, the Hmong and Dao ethnic groups utilize essential oils from *E. penduliflora* and *E. blanda* in traditional medicine for treating fever, pain, and insect bites (Dung et al., 2023). These essential oils are also incorporated into soap and spa products, demonstrating their commercial potential. *Elsholtzia* is also important sources in traditional Chinese medicine to treat cold, fever, pneumonia, etc. (Chen et al., 2022). *Elsholtzia* species are well known for their essential oil composition, which includes bioactive compounds with pharmacological properties. Studies have identified key components such as perillene, elsholtzia ketone, α-dehydro-elsholtzione, dehydro-elsholtzia ketone, and humulene (Korolyuk et al., 2002).

Beyond these applications, *Elsholtzia* species may have significant health benefits, particularly in the prevention of diabetes-related complications. Diabetes is a metabolic disorder characterized by impaired glucose regulation and insulin dysfunction, leading to elevated blood sugar levels. High blood sugar is a condition in diabetes which causes free radicals from oxygen leading to various side effects. Glycation is a non-enzymatic reaction between proteins in the body with excess sugar producing advanced glycation end-products (AGEs) which can deposit slowly in the tissues causing structural changes and disfunction. Prolonged hyperglycemia contributes to the formation of AGEs, which play a crucial role in the development of complications such as cardiovascular disease, kidney failure, and retinal damage. AGEs also trigger inflammatory responses through their interaction with receptors (RAGEs), further exacerbating disease progression. Recent studies have indicated that plant-derived phytochemicals, particularly phenolic compounds and flavonoids, exhibit antiglycation properties (Kaewnarin et al., 2013). Bioactive compounds such as procyanidins from cinnamon, caffeic acid and chlorogenic acid from tea, and luteolin, apigenin, and rosmarinic acid from Lamiaceae species have demonstrated potential in mitigating glycation-related damage (Kaewnarin et al., 2013). Given the rich phytochemical diversity of *Elsholtzia*, it is essential to explore their potential antiglycation activity and medicinal applications.

Despite the recognized potential, comprehensive studies on *Elsholtzia* species in Thailand remain limited; many plants in this genus are used by various ethnic groups for their aromatic and medicinal properties, yet their utilization is not widespread. Understanding the distribution and traditional uses of these plants in Thailand can provide insights into their potential development as functional foods and medicinal crops, particularly for health-conscious consumers. Furthermore, these plants may serve as an economic resource for local communities, promoting biodiversity conservation and sustainable agricultural practices.

This study combined an ethnobotanical survey of seven *Elsholtzia* species used by local ethnic communities in upper northern Thailand with chemical analysis of the collected plant parts, including total phenolic content and HPLC profiling, and evaluated their antiglycation activity using the methylglyoxal (MGO)-BSA assay. To the best of our knowledge, this study presents the first evidence of antiglycation activity and quantification of key bioactive compounds (rosmarinic acid, ferulic acid, and luteolin) in *Elsholtzia beddomei*, *E. stachyodes*, *E. communis*, *E. blanda*, and *E. griffithii*, and further records the ethnobotanical uses of *Elsholtzia* species in Northern Thailand. By bridging traditional knowledge with scientific validation, this research contributes to the conservation and sustainable utilization of *Elsholtzia* species, while also supporting their potential development as economic crops that promote community wellbeing and health-conscious consumption.

# 2 Materials and methods

## 2.1 Study sites

The study was conducted across 40 villages representing seven ethnic groups: Akha, Hmong, Karen, Lua, Lisu, Tai Lue, and Tai Yai.

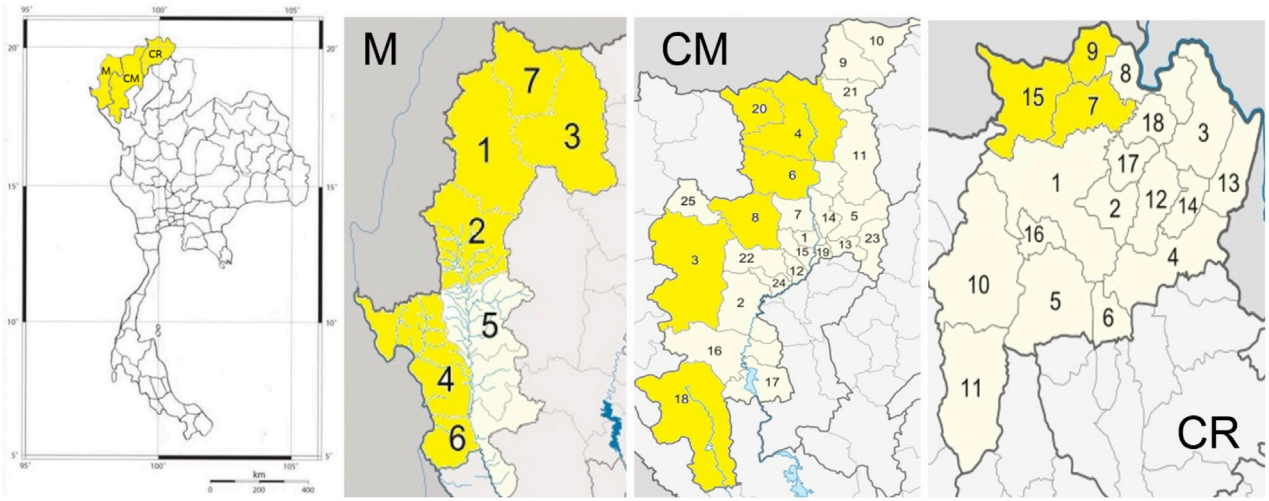



FIGURE 1
The 15 districts across three provinces selected as study sites in upper northern Thailand. [Including Mueng Mae Hong Son (1), Mae Sariang (2), Mae Lanoi (3), Sob Mei (4), Khun Yuam (6) and Pang Ma Pha (7) districts in Mae Hong Son Province (M), Mae Chaem (3), Chiang Dao (4), Mae Taeng (6), Samoeng (8), Omkoi (18) and Wiang Haeng (20) districts in Chiang Mai Province (CM), Mae Chan (7), Mae Sai (9) and Mae Fa Luang (15) districts in Chiang Rai Province (CR)].

These villages are located in 15 districts across three provinces in upper northern Thailand—six districts in Chiang Mai, three in Chiang Rai, and six in Mae Hong Son (Figure 1). The study sites featured predominantly mixed deciduous forest, dipterocarp forest, and dry evergreen forest. Elevation ranged from 345 to 1,800 m above sea level. Despite relocating to new habitats, the communities have preserved their traditional knowledge and way of life.

## 2.2 Ethnobotanical investigation

The ethnobotanical investigation in this study was carried out during 2020–2022 with a focus on an understanding of the local knowledge and practices related to *Elsholtzia* species. The process began by explaining the objectives of the study to the village head, who then advised the key informants on how to collect information from other experienced villagers using a snowball sampling approach. The information gathering process was conducted using a combination of free-listing, semi-structured interviews, group discussion and the walk-to-the-wood method. The questions were designed to collect data on the (i) vernacular name of the plants, (ii) parts used, (iii) methods for preparation, and (iv) characteristics of the plant material.

A total of 80 key informants, aged between 25 and 75 years, participated in the study. They included farmers, local healers, and merchants. The free-listing method was used to gather an initial list of plants used by the informants during group discussion. This was followed by semi-structured interviews, which allowed for a more in-depth exploration of the plants, including the parts used, preparation methods, and purposes of use. The walk-to-the-wood method involved accompanying the informants as they visited their home gardens, nearby forests, and other areas to collect specimens of the plants discussed. This hands-on approach provided an opportunity to observe the plants in their natural habitats and gather additional information from the informants. All participants provided informed consent prior to their participation in the study. The participants were fully informed about the purposes and procedures of the study and were free to withdraw at any time. Those who were literate provided written consent, while those who were illiterate provided verbal consent with a witness present.

## 2.3 Plant identification

The *Elsholtzia* specimens collected during the field survey were deposited as voucher specimens in the Herbarium of Queen Sirikit Botanic Garden (QBG) and were accompanied by photographs taken during the survey. The accepted scientific names and families of the identified plant species were determined based on the Plants of the World Online Database (powo.science.kew.org), which is a comprehensive database that provides information on the current accepted name and family for plant species. In some cases, living specimens were collected to ensure future accurate identification and facilitate propagation efforts.

## 2.4 Chemical analysis

Although seven *Elsholtzia* species were documented during the ethnobotanical, with some samples collected in 2023, chemical analyses were conducted only on species for which sufficient and high-quality plant material was available. Limited specimen availability due to seasonal constraints, small or fragmented populations, and the necessity of preserving voucher specimens precluded the inclusion of some species.

Additionally, time and budgetary constraints restricted the number of samples processed, resulting in five species being selected for chemical analysis. The collected plant materials — *Elsholtzia beddomei* (flowers and leaves), *E. stachyodes* (flowers), *E. communis* (flowers), *E. blanda* (leaves), *E. griffithii* (flowers) — were air dried at room temperature (25 °C ± 2 °C). Each dried plant samples (2.00 g) was accurately weighed and transferred into a pear-shaped flask. The sample was extracted with 100 mL of 80% methanol, sealed, and left to stand overnight at room temperature (25 °C ± 2 °C). The mixture was subsequently subjected to ultrasonication for 60 min and filtered through Whatman No. 1 filter paper. The filtrate was concentrated under reduced pressure using a rotary evaporator and adjusted to a final volume of 5 mL with 80% methanol. The extract was stored at 4 °C until further analysis. The total soluble phenolic content was determined using the Folin–Ciocalteu reagent following a modified method of Thitilertdecha et al. (2008).

Approximately 0.100 g of the extract was accurately weighed and adjusted to a final volume of 1 mL with 80% methanol. The solution was filtered through a 0.45 μm nylon syringe filter prior to analysis. HPLC analysis was performed using a system equipped with a quaternary pump and a UV detector set at 280 nm. Separation was achieved on an Eclipse C18 column (4.6 × 150 mm, 5 μm) with a guard column, at a flow rate of 0.8 mL/min and an injection volume of 20 μL. The mobile phase consisted of solvent A (0.1% formic acid in water) and solvent B (100% acetonitrile) using the following gradient program: 0–10 min, 94% A and 6% B; 10–50 min, 70% A and 30% B; 50–60 min, 50% A and 50% B. Standard compounds—rosmarinic acid, luteolin, apigenin (Sigma-Aldrich), and ferulic acid (Fluka)—were used for the identification and quantification of phenolic compounds in the extracts. Data were expressed as mean ± standard deviation (n = 3) and subjected to statistical analysis.

## 2.5 Antiglycation activity testing

The inhibition of glycation reactions by *Elsholtzia* plant extracts was evaluated using the MGO-BSA method, which was chosen for its established reliability, reproducibility, and physiological relevance in modeling glycation processes. Although alternative techniques, such as fluorometric assays and HPLC–MS/MS, offer higher analytical efficiency, their use was limited by instrument accessibility during the study period. Future investigations will incorporate these advanced methodologies to further substantiate the MGO-BSA–based results (Kaewnarin et al., 2014; Peng et al., 2007). A volume of 300 μL of a mixed solution of BSA (Bovine Serum Albumin) at a concentration of 10 mg/mL was pipetted into a screw tube. Subsequently, 30 μL of MGO (methylglyoxal) at a concentration of 0.5 M was added. Sample extracts at various concentrations were then added, and the final volume was adjusted to 3 mL. The mixture was incubated at 37 °C for 7 days. After incubation, the fluorescence intensity was measured using a luminescence microplate reader (excitation at 370 nm; emission at 440 nm), and the percentage inhibition of the glycation reaction was calculated. Aminoguanidine (AG) was used as the positive control.

$$\%\ inhibition = \frac{(\text{intensity of control} - \text{intensity of sample})}{\text{intensity of control}}\ x\ 100$$

The percentage inhibition values at different concentrations were plotted to generate a dose–response curve, from which the 50% inhibitory concentration ($IC_{50}$) was calculated.

# 3 Results

## 3.1 Surveyed *Elsholtzia* species and associated traditional knowledge

Seven *Elsholtzia* species were used by ethnic groups in upper northern Thailand (Table 1; Figure 2). Most of *Elsholtzia* species was used for food as spice and vegetable. *Elsholtzia penduliflora* and *E. winitiana* was used as medicine for Hmong and Akha ethnic groups, respectively. Both *E. beddomei* and *E. stachyodes* were used as an insect repellent in barns. *Elsholtzia kachinensis* was widely used by most of the ethnic groups including Tai Yai, Tai Lue, Akha, and Lisu. Most *Elsholtzia* species were cultivated plants commonly found in agricultural areas, including upland rice fields and home gardens. Only *E. beddomei* was found in natural habitats (Table 1).

## 3.2 Chemical composition of surveyed *Elsholtzia* species

The total soluble phenolic content of extracts from the five *Elsholtzia* species collected from upper northern Thailand was analyzed. The extracts were reacted with Folin-Ciocalteu reagent, and the absorbance was measured at 760 nm. The values were compared against a standard curve of gallic acid ($R^2$ = 0.9992) (Figure 3), and the results are presented in Table 2. It was found that the total soluble phenolic content ranged from 6.50 to 36.04 mg of gallic acid equivalents per gram of sample (mg GAE/g sample). The highest phenolic content, 36.04 mg GAE/g sample, was observed in the extract from *E. beddomei*, which was obtained from the leaves. The lowest content, 6.50 mg GAE/g sample, was detected in the extract from the *E. communis*.

In *E. beddomei*, which had the highest total phenolic content, a comparison between the flower and leaf parts revealed that the leaves contained more than twice the amount of soluble phenolic compounds compared to that from the flowers of the same species.

The phytochemical content from the five *Elsholtzia* species was analyzed using high-performance liquid chromatography (HPLC) and compared with the standard phenolic compounds (Table 2; Figure 4). Four phenolic compounds were identified: rosmarinic acid (RA), ferulic acid (FA), and luteolin (LU). The highest amount of rosmarinic acid, 667.9 mg per 100 g of sample, was detected in the leaf extract of *E. beddomei*. The highest ferulic acid content, 2.25 mg per 100 g of sample, was observed in the *E. griffithii*. Luteolin was detected only in the *E. stachyodes* (15.4 mg per 100 g of sample).

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TABLE 1 Ethnobotanical use of seven *Elsholtzia* species by ethnic groups in upper northern Thailand.

| Species [voucher specimen no.] | Ethnic group | Vernacular name | Source | | | Use category | Part used: How to use |
|---|---|---|---|---|---|---|---|
| | | | Rice field | Home garden | Forest | | |
| *Elsholtzia beddomei* C.B.Clarke ex Hook.f. [WP5703] | Karen | Hor Prei Pa | | | ✓ | insect repellent | Whole plant: used fresh or dried as an insect repellent in barns |
| [a]*Elsholtzia communis* (Collectt & Hemsl.) Diels [WP7634] | Karen | Hor Wor | ✓ | | | food | Shoot and inflorescences: used fresh or dried as a spice to create the signature taste and aroma in local large cucumber soup or chili paste; fresh parts are sold in local or roadside markets |
| | Lua | Hlan | ✓ | | | food | Shoot and inflorescences: used fresh or dried as a spice to create the signature taste and aroma in local large cucumber soup or chili paste; consumed fresh as a side-dish vegetable |
| | Akha | Yia Pi La Chong | ✓ | | | food | Shoot and inflorescences: used fresh as a spice to create the signature taste and aroma in fermented bamboo soup or chili paste |
| [a]*Elsholtzia griffithii* Hook.f. [WP6522] | Tai Yai | Lum Pum | ✓ | ✓ | | food | Shoot: used fresh as a vegetable in local bean soup; consumed fresh as a side-dish vegetable |
| | Lisu | Lum Pum | ✓ | | | food | Shoot: used fresh as a vegetable in local pumpkin soup; consumed fresh as a side-dish vegetable |
| [a]*Elsholtzia kachinensis* Prain [WP6527] | Tai Yai | Hern | ✓ | ✓ | | food | Shoot: used fresh as a vegetable in bone soup; consumed fresh as a side-dish vegetable |
| | Tai Lue | Lern | ✓ | ✓ | | food | Shoot: used fresh as a vegetable in bone soup; consumed fresh as a side-dish vegetable; fresh parts are sold in local or roadside markets |
| | Akha | Lor Chor | ✓ | | | food | Shoot: used fresh as a vegetable in bone soup; consumed fresh as a side-dish vegetable |
| | Lisu | Xiao Chai | ✓ | | | food | Shoot: used fresh as a vegetable in bone soup; consumed fresh as a side-dish vegetable |
| *Elsholtzia penduliflora* W.W.Sm. [WP2256, WP2209] | Hmong | Yan Chua Tou | | ✓ | | medicine | Leaves: used fresh as a spice in chicken soup consumed for body nourishment; decoction used for bathing to promote good health |
| [a]*Elsholtzia stachyodes* (Link) Raizada & Saxena [WP8235] | Tai Yai | Han | ✓ | ✓ | | food | Shoot: used fresh as a vegetable |
| | Karen | Hor Prei | | ✓ | | food<br><br>insect repellent | Shoot: used fresh as a spice for eliminating fishy smell from offal soup; consumed fresh as a side-dish vegetable<br>Whole plant: used fresh or dried as an insect repellent in barns |
| *Elsholtzia winitiana* Craib [WP6476] | Akha | Ngu Shee | | ✓ | | medicine | Leaves: decoction used for treating cancer, food poisoning, and dog bite (Inta et al., 2008) |

[a]The *Elsholtzia* species was sold in local and roadside markets.

## 3.3 Antiglycation activity of surveyed *Elsholtzia* species

According to the experimental results in Table 3, the leaf extract of *Elsholtzia beddomei* showed the highest antiglycation activity in the MGO-BSA system, with an $IC_{50}$ value of 1.65 mg/mL. In contrast, the leaf extract of *E. blanda* exhibited the lowest antiglycation activity, with an $IC_{50}$ value of 8.10 mg/mL. However, the effectiveness of the extracts in this group was still lower when compared to the standard inhibitor, aminoguanidine, with the $IC_{50}$ value of 0.29 mg/mL.

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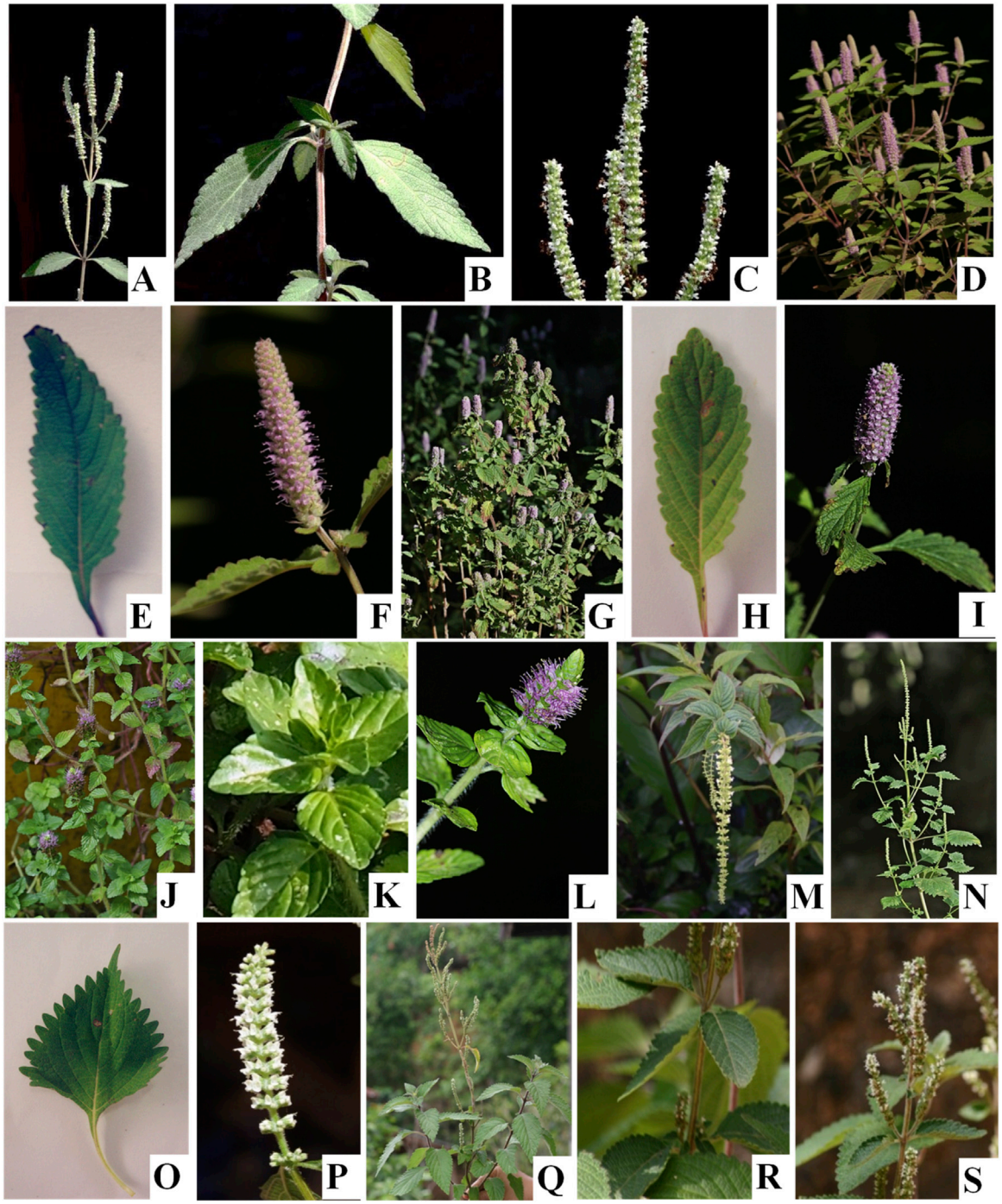



FIGURE 2
Morphological characters of *Elsholtzia* species used by ethnic groups in upper northernThailand. **(A–C)** *E. beddomei* C.B.Clarke ex Hook.f.; **(D–F)** *E. communis* (Collectt & Hemsl.) Diels; **(G–I)** *E. griffithii* Hook.f.; **(J–L)** *E. kachinensis* Prain; **(M)** *E. penduliflora* W.W.Sm.; **(N–P)** *E. stachyodes* (Link) Raizada & Saxena; **(Q–S)** *E. winitiana* Craib.

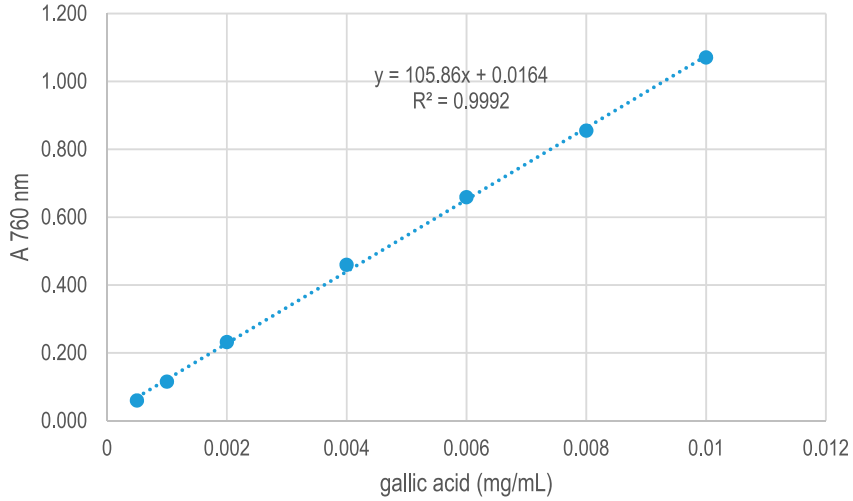



FIGURE 3
Standard curve of gallic acid at various concentrations for the determination of total soluble phenolic compounds.

TABLE 2 The phenolic compounds in five *Elsholtzia* species from upper northern Thailand.

| Species | Sample parts | Content of the phenolic compounds[a] | | | |
|---|---|---|---|---|---|
| | | Total phenolics mg GAE/g sample | Rosmarinic acid (RA) mg/100 g sample | Ferulic acid (FA) mg/100 g sample | Luteolin (LU) mg/100 g sample |
| *Elsholtzia beddomei* | flowers | 15.08 ± 1.13[b] | - | 0.652 ± 0.1 | - |
| | leaves | 36.04 ± 1.06[a] | 667.9 ± 8.7 | - | - |
| *Elsholtzia stachyodes* | flowers | 13.65 ± 0.11[c] | - | - | 15.4 ± 0.8 |
| *Elsholtzia communis* | flowers | 6.50 ± 057[e] | 23.5 ± 0.6 | - | - |
| *Elsholtzia blanda* | leaves | 8.44 ± 0.02[d] | - | - | - |
| *Elsholtzia griffithii* | flowers | 14.51 ± 0.07[c] | 36.7 ± 0.1 | 2.25 ± 0.1 | - |

[a]The mg GAE/g sample refers to milligrams of gallic acid equivalents per gram of crude extract. Experimental results are reported as mean ± standard deviation (n = 3). The [a–e] indicated statistically significant differences at $P < 0.05$.

# 4 Discussion

## 4.1 Ethnobotanical uses of *Elsholtzia* species in upper northern Thailand and other Asian countries

Seven species of *Elsholtzia* were found to be used by ethnic groups in upper northern Thailand, namely, *E. griffithii*, *E. kachinensis*, *E. winitiana*, *E. penduliflora*, *E. communis*, *E. beddomei*, and *E. stachyodes*. Among these, *E. communis* was not previously recorded as part of the *Elsholtzia* (Lamiaceae) flora in Thailand. *E. blanda* was encountered during our field surveys in upper northern Thailand; however, from our ethnobotanical interview it has not been reported as being used by local ethnic groups. *Elsholtzia pilosa* was not found in this study. It has only been collected once, in 1922, and appeared to have disappeared from natural habitats in Thailand (Bongcheewin et al., 2015). Most *Elsholtzia* species documented in this study were found in cultivated areas, such as rice fields and homegardens. Only *E. beddomei* was observed along forest margins.

*Elsholtzia communis* was identified as the most culturally significant seasonal spice among the Karen and Lua peoples (Brikut and Inta, 2019; Inta et al., 2025; Moonjai and Inta, 2016; Panyadee et al., 2021). This species was traditionally planted by nearly every household alongside their rotational upland rice fields each year. It was harvested throughout its growth cycle, from the vegetative to reproductive stages, directly from rice fields for culinary use. Fresh or dried leaves and inflorescences were commonly used as a spice to impart a distinctive taste and aroma to local dishes, such as large cucumber soup and chili paste, or consumed fresh as a side-dish vegetable. Mature dried inflorescences, containing tiny seeds, were typically harvested around November and stored above the cooking stove for use as planting material in the following season. The *E. communis* has been

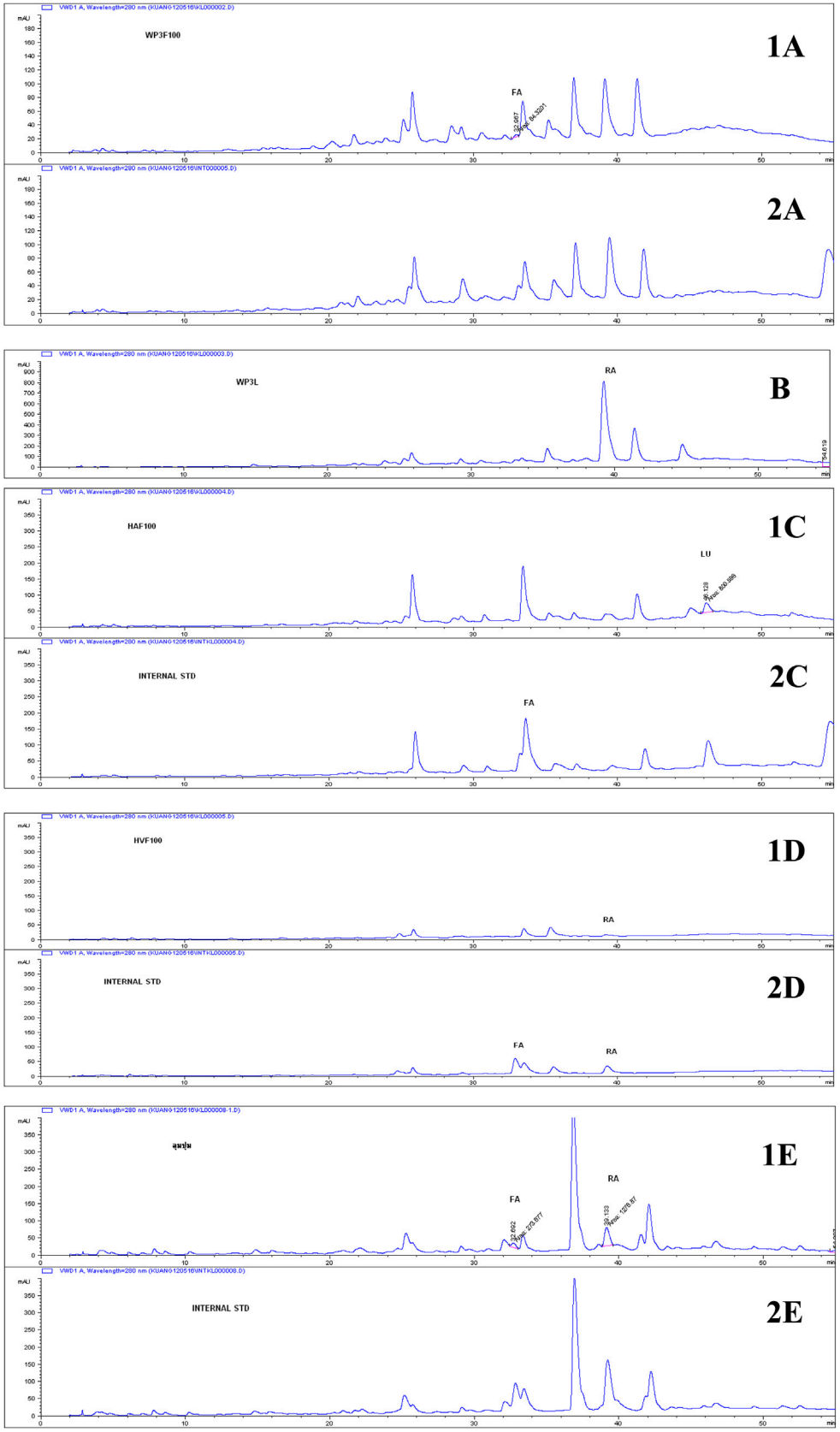



FIGURE 4
Chromatograms of *Elsholtzia* species extracts (1) and chromatograms with internal standards (2) including *Elsholtzia beddomei* (A, flowers; B, leaves), *Elsholtzia stachyodes* (C), *Elsholtzia communis* (D), *Elsholtzia griffithii* (E)

TABLE 3 *In vitro* antiglycation activity of the 80% methanol extracts of *Elsholtzia* species in the MGO-BSA system.

| Species | Sample part | Antiglycation activity $IC_{50}$ (mg/mL)[a] |
|---|---|---|
| *Elsholtzia beddomei* | flowers | 3.46 ± 0.15[c] |
| | leaves | 1.65 ± 0.10[a] |
| *Elsholtzia stachyodes* | flowers | 2.97 ± 0.17[b] |
| *Elsholtzia communis* | flowers | 5.21 ± 0.05[d] |
| *Elsholtzia blanda* | leaves | 8.10 ± 0.31[e] |
| *Elsholtzia griffithii* | flowers | 3.72 ± 0.17[c] |
| Standard inhibitor | | |
| Aminoguanidine | | 0.29 ± 0.02 |

[a]The experimental results are reported as mean ± standard deviation.
The [a–e] indicates statistically significant differences at $P < 0.05$.

traditionally used for both medicinal and culinary purposes in Myanmar, Vietnam, and China. Medicinally, it is particularly employed in the treatment of respiratory ailments such as tonsillitis, fever, and cough, as well as for conditions including nosebleeds and menstrual disorders (Chen et al., 2022).

*Elsholtzia kachinensis* was the most popular among the ethnic groups in Northern Thailand including Tai Yai, Tai Lue, Akha, and Lisu. Fresh shoot was used as a vegetable in bone soup or consumed fresh as a side-dish vegetable. The *E. kachinensis* was planted a small plot in home gardens. It was planted in a big plot in the agricultural area with other commercial vegetables by some household for harvesting and selling in local or roadside markets. It can be propagated by root cutting. The *E. kachinensis* has traditionally been used for culinary purposes in Myanmar and Southern China (Bongcheewin et al., 2015).

*Elsholtzia penduliflora* was specifically used for traditional medicine only in the Hmong communities in upper northern Thailand (Nguanchoo et al., 2022). Fresh leaves were used as a spice in chicken soup consumed for body nourishment or preparing decoction used for bathing to promote good health. The *E. penduliflora* was also used as traditional medicine by Hmong in Southern China (Chen et al., 2022). It seems that when Hmong people migrated to upper northern Thailand, they carried the *E. pendulifkora* with them and planted in their home gardens. The Hmong in Honghe Prefecture in Yunnan Province, southern China used the *E. penduliflora* for bronchitis, influenza, cancer, and human infertility (Chen et al., 2022).

*Elsholtzia winitiana* was found in open places in hill evergreen, deciduous, pine or dipterocarp forests of upper northern Thailand whereas in our study it was found in Akha home garden (Bongcheewin et al., 2015; Inta et al., 2008). The *E. winitiana* was used as traditional medicine by local Akha healers for treating cancer, food poisoning, and dog bite (Inta et al., 2008). The Akha local healers collected this plant and planting it in their home gardens for immediate access. The *E. winitiana* was used as traditional medicine for treating measles, headache, and cold by Akha or Hani people around the Naban River Watershed National Nature Reserve, China (Chen et al., 2022). The *E. winitiana* seems to be a cultural medicinal plant of Akha group.

Among different biocultural backgrounds, ethnobotanical knowledge varies, although using plants as food and medicine is a general tradition (Yao et al., 2023). For example, even in the same ecosystem, people of two cultural backgrounds have different ethnobotanical knowledge (Quave and Pieroni, 2015), while the cross-border circulation of local knowledge facilitates the conservation and utilization of such plants and related traditional knowledge (Prakofjewa et al., 2023). The present work reported the traditional uses of *Elsholtzia* species in upper northern Thailand, showing high diversity in plant species and usages. While this genus is widely distributed in its neighbor regions such as the Great Mekong Subregion (Chen et al., 2022), where the cultural similarity also exists, it is worthy of exploring the traditional knowledge of *Elsholtzia* species in a perspective of comparative ethnobotany.

## 4.2 Chemical composition of some *Elsholtzia* species used by ethnic groups in upper northern Thailand

The phenolic content observed in *Elsholtzia* species studied—particularly in *E. beddomei*—is notably high when compared with several commonly consumed vegetables and medicinal plants. For instance, the total phenolic content in spinach (*Spinacia oleracea*) and broccoli (*Brassica oleracea* var. *italica*), which are known for their antioxidant properties, typically ranges from 5 to 15 mg GAE/g in dried samples (Chu et al., 2002; Zhou and Yu, 2006). In this context, the 36.04 mg GAE/g sample found in *E. beddomei* leaf extract is more than double the phenolic levels in these vegetables, highlighting its potential as a rich source of natural antioxidants. Furthermore, the phenolic content of *Camellia sinensis* (green tea) leaves, one of the most studied medicinal plants, often falls within the range of 20–30 mg GAE/g, depending on processing and cultivar (Balentine et al., 1997; Yen and Chen, 1995). This comparison underscores the significant phenolic richness of *E. beddomei*, making it comparable to or even surpassing established sources of dietary antioxidants. These findings support the ethnobotanical knowledge of local communities in Northern Thailand and point toward the potential for developing *Elsholtzia* species, especially *E. beddomei*, as functional food ingredients or phytopharmaceutical candidates (Balasundram et al., 2006). However, it should be noted that the

chemical composition and yield of phenolic compounds can be affected by multiple factors, including the choice of extraction solvent, the extraction method, and variability in plant material (Pudziuvelyte et al., 2018).

The variation in total phenolic content between different plant parts, such as leaves and flowers, can be attributed to their distinct physiological roles and exposure to environmental stressors (Lattanzio et al., 2006). In the *E. beddomei*, which had the highest total phenolic content, a comparison between the flower and leaf parts revealed that the leaves contained more than double the amount of soluble phenolic compounds compared to that from the flowers of the same species (Balasundram et al., 2006; Sriraksa and Seekhaw, 2024). This difference is likely due to the fact that leaves are the primary site of photosynthesis and are more frequently exposed to ultraviolet (UV) radiation, herbivores, and pathogens. As a result, they tend to accumulate higher levels of phenolic compounds, which function as protective agents against oxidative stress and biotic threats. In contrast, flowers, which have more specialized roles in reproduction, often contain different classes of secondary metabolites such as pigments (e.g., anthocyanins) and volatile compounds that attract pollinators rather than serve as strong antioxidants (Harborne and Williams, 2000). Additionally, biosynthetic pathways for phenolics may be more active in leaf tissues due to greater metabolic activity (Treutter, 2005). These findings highlight the importance of selecting specific plant parts when evaluating phytochemical content and bioactivity, particularly in medicinal and functional plant research.

Three phenolic compounds were identified in the studied *Elsholtzia* species: rosmarinic acid, ferulic acid, and luteolin. Among them, the highest concentration of rosmarinic acid was detected in the leaf extract of *E. beddomei*, supporting its strong antioxidant potential (Alagawany et al., 2021; Petersen and Simmonds, 2003). Ferulic acid was found in the highest amount in *E. griffithii* (Ou and Kwok, 2004; Zhao et al., 2021), while luteolin was uniquely detected only in *E. stachyodes* (Lin et al., 2008; Seelinger et al., 2008). These compounds are well-known secondary metabolites in the Lamiaceae family and are associated with various pharmacological activities, including antioxidant, anti-inflammatory, antimicrobial, and anticancer properties. Their presence reinforces the therapeutic potential of these *Elsholtzia* species and aligns with findings from other members of the Lamiaceae family (Kaewnarin et al., 2013). For example, rosmarinic acid is commonly found in high levels in *Rosmarinus officinalis* and *Ocimum basilicum*, and has been widely studied for its protective effects against oxidative stress and chronic diseases. Ferulic acid, typically present in plant cell walls, contributes to UV protection and pathogen resistance, while luteolin is a flavonoid known for its strong anti-inflammatory and neuroprotective effects.

The selective distribution of these compounds among species suggests that phytochemical composition may vary not only between species but also according to environmental, genetic, and developmental factors. The application of advanced chromatographic techniques, particularly high-performance liquid chromatography (HPLC), enables accurate quantification and profiling of such bioactive compounds. Future studies could employ HPLC in combination with mass spectrometry (LC-MS/MS) (Cuyckens and Claeys, 2004) or metabolomics approaches to monitor chemical variation over time, across habitats, and during different plant growth stages. Such analyses are essential for quality control, pharmacological standardization, and valorization of *Elsholtzia* species in ethnomedicine and potential functional food development.

## 4.3 Exploring the antiglycation potential of *Elsholtzia* species traditionally used by ethnic communities in upper northern Thailand

Local *Elsholtzia* species are rich in phytochemicals with demonstrable biological activities, including the inhibition of protein glycation—a key mechanism underlying many diabetes-related complications. In our study, the crude leaf extract of *E. beddomei* exhibited the strongest antiglycation activity among the species analyzed, though it was less potent than the benchmark inhibitor aminoguanidine. Specifically, the $IC_{50}$ value of aminoguanidine is approximately 0.29 mg/mL (Cushnie et al., 2024; Lee et al., 2017) whereas our crude extract displayed higher (i.e., weaker) $IC_{50}$ values. This is likely due to the complex matrix of inactive or less active compounds present in crude extracts. Our observation aligns with numerous studies demonstrating that crude plant extracts often show lower antiglycation efficacy compared to standard inhibitors, but significantly improve upon purification, which concentrates active phenolic constituents.

Furthermore, there is a strong and consistent correlation between total phenolic content and antiglycation activity in plant extracts. Research on various edible and medicinal plants—including *Punica granatum*, *Mangifera indica*, and *Dimocarpus longan*—has shown that extracts with higher phenolic levels demonstrate stronger inhibitory activity in methylglyoxal-BSA assays, often outperforming aminoguanidine under similar testing conditions (Kaewnarin et al., 2013; Tsai and Lin, 2019). Likewise, investigations into spices and herbs have confirmed that total phenolic content is highly correlated ($r = 0.84$ in BSA-MGO assays) with antiglycation activity (Xie and Chen, 2019).

Given that *E. beddomei* showed both the highest phenolic content (36.04 mg GAE/g sample) and the strongest antiglycation activity among the tested *Elsholtzia* species, our data reinforce the hypothesis that glycation inhibition efficacy is tied to phenolic compound abundance. Future steps such as bioassay-guided fractionation and purification could isolate the specific phenolics driving this activity, potentially yielding compounds with $IC_{50}$ values comparable to or better than aminoguanidine.

If *Elsholtzia* extracts can eventually be used as substitutes for the standard synthetic inhibitor aminoguanidine (commercially known as Pimagedine®), which is currently used to treat complications of diabetes, it could help reduce the side effects associated with synthetic drugs. Aminoguanidine has been reported to cause adverse effects such as fever, chills, muscle weakness, gastrointestinal issues, anemia and in some cases, vitamin B6 deficiency (Reddy and Beyaz, 2006).

In this study, we focused specifically on the antiglycation activity of *Elsholtzia* species to provide a targeted investigation relevant to potential functional food and phytopharmaceutical applications. Owing to time and budget constraints, other biological activities,

including antioxidant, anticancer (Hoang et al., 2025), anti-inflammatory (Zotsenko et al., 2021), and antimicrobial effects, were not assessed. These activities may, however, be addressed in future studies to provide a more comprehensive evaluation of the pharmacological potential of *Elsholtzia* species.

## 5 Conclusion

This ethnobotanical investigation of *Elsholtzia* species in upper northern Thailand reveals the profound traditional knowledge and cultural importance of these aromatic plants among diverse ethnic groups, including the Akha, Hmong, Karen, Lawa, Lisu, Tai Lue, and Tai Yai. For generations, these communities have utilized *Elsholtzia* species for a wide range of purposes–medicinal, culinary, and as natural insect repellents–demonstrating both the versatility of these plants and their vital role in sustaining local health and livelihoods.

The traditional applications of *Elsholtzia* reflect a sustainable human–environment relationship and highlight the value of indigenous knowledge systems in guiding modern scientific discovery. As contemporary pharmacological studies increasingly validate their anti-inflammatory, antimicrobial, and antiglycation properties, *Elsholtzia* species emerge as promising candidates for further development in pharmaceutical and nutraceutical industries.

The observed variation in the quantity of bioactive compounds and antiglycation activity among specimens collected from different localities underscores the influence of cultivation area and plant part on phytochemical composition. Such variability emphasizes the need for optimized cultivation practices and targeted utilization of plant materials to maximize therapeutic potential.

Overall, the integration of ethnobotanical insights with chemical analysis in this study underscores the interconnectedness of cultural and biological diversity. The findings contribute not only to the documentation of traditional knowledge but also to the advancement of sustainable resource management and drug discovery. Future research should expand on these results by examining large-scale cultivation, assessing environmental and seasonal influences on phytochemical profiles, and performing bioassay-guided isolation of active compounds. Furthermore, continued collaboration with local communities will be essential to ensure that the benefits of bioprospecting foster equitable and sustainable development while safeguarding both cultural heritage and native plant biodiversity.

## Data availability statement

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

## Author contributions

AI: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Validation, Visualization, Writing – original draft, Writing – review and editing. PM: Formal Analysis, Methodology, Validation, Writing – review and editing. LS: Formal Analysis, Methodology, Validation, Writing – review and editing. WP: Investigation, Methodology, Resources, Writing – review and editing. YS: Writing – review and editing. YF: Funding acquisition, Writing – review and editing. RY: Conceptualization, Funding acquisition, Writing – review and editing.

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# Sphingomyelin synthase inhibitors from edible mushrooms prevent overweight and improve vitamin D homeostasis in a mouse model of diet-induced obesity

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**Introduction:** Sphingomyelin synthase (SMS) is a key enzyme in sphingolipid metabolism that converts phosphatidylcholine and ceramide into sphingomyelin and diacylglycerol, thereby influencing lipid signaling and metabolic regulation. Identifying natural SMS inhibitors is of interest for the prevention and management of obesity and related disorders.

**Methods:** We screened more than 860 extracts from medicinal plants and mushrooms using a cell-based assay for SMS inhibition. Active fractions were purified, and grifolin and grifolic acid were isolated from the edible mushroom *Albatrellus confluens* (Alb. & Schwein.) Kotl. & Pouzar and structurally confirmed. Their inhibitory activity was validated *in vitro* against sphingomyelin synthase 1 (SMS1) and sphingomyelin synthase 2 (SMS2), and their metabolic effects were tested by dietary supplementation (0.1% in high-fat diet) in male C57BL/6J mice for 9 weeks.

**Results:** Both compounds inhibited SMS activity at low micromolar concentrations. *In vivo*, they significantly reduced body weight gain, improved glucose tolerance, and alleviated hepatic steatosis in high-fat diet–fed mice. Treatment also preserved circulating 25-hydroxyvitamin $D_3$ [25(OH) $D_3$] concentrations and downregulated hepatic expression of the vitamin D–catabolizing enzyme CYP24A1.

**Conclusion:** These findings demonstrate, for the first time, that mushroom-derived metabolites can act as dual-substrate SMS inhibitors with beneficial effects on lipid and vitamin D metabolism. Grifolin and grifolic acid thus represent promising candidates for development as functional food components or nutraceuticals to prevent obesity and metabolic syndrome.

KEYWORDS

**sphingomyelin synthase, grifolin, grifolic acid, vitamin D, obesity**

# 1 Introduction

Overweight and obesity are major global public health challenges. According to the World Health Organization (2024), the prevalence of obesity has more than doubled since 1990. By 2022, approximately 43% of adults worldwide were classified as overweight and 16% as obese.[1] Obesity and insulin resistance are key contributors to metabolic syndrome and increase the risk of cardiovascular disease, type 2 diabetes mellitus (T2DM), fatty liver disease, and certain cancers. Identifying effective strategies to prevent and control these conditions remains a critical public health priority.

Sphingolipid metabolism plays crucial roles in disease pathogenesis and has emerged as a promising therapeutic target. Among the major sphingolipids, ceramide (Cer), sphingomyelin (SM), and sphingosine-1-phosphate (S1P) function as bioactive signaling molecules regulating apoptosis, proliferation, and inflammation (1–3). The balance among these lipids is decisive: ceramide accumulation promotes apoptosis and metabolic dysfunction, whereas S1P and SM support survival and membrane stability. Disruption of this balance contributes to insulin resistance, hepatic steatosis, and chronic inflammation (4–12).

In mammals, sphingolipid metabolism is regulated by approximately 40 enzymes (13), including sphingomyelin synthase (SMS), which catalyzes the transfer of a phosphocholine group from phosphatidylcholine (PC) to ceramide, generating SM and diacylglycerol (DAG). SMS therefore regulates the Cer/SM ratio—a critical determinant of lipid homeostasis. Sphingomyelin synthase 1 (SMS1) and sphingomyelin synthase 2 (SMS2) are the major isoforms, localized to the Golgi and plasma membrane, respectively (14). Dysregulated SMS activity promotes lipid accumulation, insulin resistance, and obesity-related metabolic disorders (15). Notably, SMS2 deficiency in mice reduces adipose mass, alleviates fatty liver, and enhances glucose tolerance, partly through increased energy expenditure and the browning of white adipose tissue. These findings highlight SMS2 as a promising therapeutic target for obesity and metabolic syndrome (16, 17).

Small-molecule SMS inhibitors are therefore of high interest as potential therapeutic agents and functional food components. Natural products, in particular, offer structural diversity, biological compatibility, and favorable safety profile (18). However, despite their therapeutic promise, the precise enzymatic targets of natural compounds often remain poorly characterized (19). The therapeutic properties of mushrooms have been recognized for thousands of years in Eastern countries because they exhibit a wide range of biological activities. Mushrooms are especially attractive in this context: traditionally valued in Eastern medicine for their health-promoting properties, they represent a vast and chemically diverse resource, with ~140,000 estimated species but only ~10% characterized to date (20).

Building on our earlier identification of natural SMS inhibitors such as ginkgolic acid, malabaricones, and daurichromenic acid (21–23), we constructed a methanol-extract library of 212 mushroom species from Japan and screened them using a high-throughput, cell-based SMS assay. Extracts from *Albatrellus dispansus* (Lloyd) Pouzar and the edible *Albatrellus confluens* (Alb. & Schwein.) Kotl. & Pouzar exhibited the strongest activity, leading to the isolation of two phenolic compounds, grifolin and grifolic acid.

Both compounds inhibited SMS1 and SMS2 with the half maximal inhibitory concentration ($IC_{50}$) values ranging from 0.6 to 18.3 μM depending on the assay pathway, with grifolic acid showing consistently greater potency. In HepG2 cells, treatment with 20 μM grifolin or grifolic acid significantly reduced lipid droplet accumulation (by 43 and 48%) without detectable cytotoxicity. In diet-induced obese mice, 0.1% oral administration attenuated body weight gain, improved glucose tolerance, and decreased hepatic steatosis.

To our knowledge, this is the first report identifying grifolin and grifolic acid as natural SMS inhibitors derived from edible mushrooms. These findings reveal a new class of dual-substrate, food-derived SMS inhibitors with potential applications as functional food components or nutraceuticals for preventing obesity and related metabolic disorders.

# 2 Materials and methods

## 2.1 Materials

Fruiting bodies of *A. confluens* and *A. dispansus* were collected in Japan (Mie, Tottori, Okayama, and Hiroshima Prefectures, 2019) and authenticated by mycologists. Additional mushroom specimens (212 species) collected in Hokkaido were cleaned, authenticated, and deposited in the Natural Product Library of Hokkaido University. Samples were extracted with organic solvent, concentrated under reduced pressure, and stored at −20 °C. Crude extracts were dissolved in DMSO (10 mg/mL) for screening, resulting in a mushroom extract library of 212 species.

## 2.2 Cell culture and enzyme assays

Mouse embryonic fibroblasts lacking SMS1 and SMS2 (ZS2), and stable ZS2 lines expressing SMS1 or SMS2, were cultured in Dulbecco's Modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum at 37 °C in a 5% $CO_2$ incubator. Human hepatoma HepG2 cells were used for cytotoxicity and lipid uptake assays.

SMS activity was measured in cell lysates (0.1 μg/μL protein). Lysates were preincubated with test compounds at 37 °C for 30 min, followed by addition of C6-NBD-ceramide (NBD-Cer; N-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl]sphingosine, 5 μM) and C6-NBD-phosphatidylcholine (NBD-PC; 1-acyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl]-sn-glycero-3-phosphocholine, 5 μM) as substrates. The reaction converts ceramide (Cer) and phosphatidylcholine (PC) into sphingomyelin (SM) and diacylglycerol (DAG). Reactions were terminated with methanol/chloroform (1:2, v/v). The fluorescent products C6-NBD–diacylglycerol (C6-NBD-DAG) and C6-NBD–sphingomyelin (C6-NBD-SM) were separated and quantified by HPLC with fluorescence detection (λ_ex = 470 nm, λ_em = 530 nm). Assay linearity was verified with respect to both protein concentration and reaction time. Enzyme activity was calculated from product formation relative to control samples without inhibitor.

1 World Health Organization. (2025). Obesity and overweight. Available from: https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight. [Accessed July 24, 2025].

## 2.3 Cytotoxicity and lipid uptake assays

Cell viability was determined by the CCK-8 assay after 24 h incubation with 0–20 μM grifolin or grifolic acid. For lipid droplet accumulation experiments, HepG2 cells were treated with 1 mM oleic acid in the presence or absence of 20 μM compounds for 24 h. Cells were stained with Nile Red (λ_ex = 545 nm, λ_em = 605 nm) to visualize neutral lipids and with DAPI (λ_ex = 360 nm, λ_em = 460 nm) to label nuclei. Images were acquired using a Keyence BZ-X70 fluorescence microscope (Plan Apochromat 40x objective, fixed exposure times) and analyzed in ImageJ. Lipid droplet content was quantified by Otsu thresholding, expressed as Nile Red fluorescence intensity normalized to DAPI counts, yielding lipid content per cell in arbitrary units (AU/cell). Data are presented from three independent experiments, each including multiple fields of view, and statistical significance was assessed by one-way ANOVA with Tukey's *post hoc* test. Fatty acid uptake assays such as BODIPY-C16 transport were not performed in this study, and our lipid accumulation measurements therefore reflect neutral lipid storage but not uptake kinetics.

## 2.4 Isolation and identification of active compounds

The acetone extract of *A. confluens* fruiting bodies (4.96 kg fresh weight) exhibited strong SMS inhibitory activity. Sequential liquid–liquid extraction with hexane, diethyl ether, and ethyl acetate was followed by silica gel chromatography. The ether fraction showed the greatest activity and yielded two phenolic compounds, grifolin (10.4 g) and grifolic acid (5.8 g). Structural identities were confirmed by NMR spectroscopy and high-resolution mass spectrometry, consistent with reported values.

## 2.5 Animal studies

All animal experiments were approved by the Animal Research Committee of Hokkaido University and performed in accordance with institutional guidelines. Male C57BL/6J mice (5 weeks old, Japan SLC Inc., Shizuoka, Japan) were housed under controlled environmental conditions (24 °C, 50% ± 10% humidity, 12 h light/dark cycle) with ad libitum access to food and water. After a one-week acclimatization period, animals were randomly assigned to four groups ($n = 6$ per group):

1. ND (normal diet; 6.2% kcal fat, AIN-93M, Oriental Yeast Co., Tokyo, Japan)
2. HFD (high-fat diet; 60% kcal fat, HFD-60, Oriental Yeast Co.)
3. HFD + grifolin (HFD supplemented with 0.1% grifolin)
4. HFD + grifolic acid (HFD supplemented with 0.1% grifolic acid).

Grifolin and grifolic acid were incorporated directly into the diet (AIN-93G, Oriental Yeast Co, Tokyo, Japan) and provided as oral dietary supplementation for 9 weeks. Body weight and food intake were monitored weekly.

Glucose tolerance was assessed after overnight fasting by oral gavage of glucose (1 g/kg body weight), and blood glucose was measured at 0, 30, 60, and 120 min. At study termination blood was collected by cardiac puncture under 2% isoflurane inhalation anesthesia.[2] Liver and kidney tissues were excised, snap-frozen in liquid nitrogen, and stored at −80 °C for molecular analyses or processed for histological evaluation (hematoxylin and eosin, Oil Red O staining). Hepatic triglyceride content was measured enzymatically. Lipidomic profiling of ceramide and sphingomyelin species was conducted by Liquid chromatography–tandem mass spectrometry (LC–MS/MS). Serum 25(OH)$D_3$ levels were quantified using a commercial enzyme-linked immunosorbent assay (ELISA) kit, and hepatic and renal expression of CYP24A1, CYP27B1, and CYP2R1 was determined by quantitative reverse transcription PCR (RT–PCR).

Randomization was applied at group assignment, and investigators performing histological and biochemical analyses were blinded to treatment groups.

## 2.6 Statistical analysis

All experiments were performed at least in triplicate unless otherwise specified. Data are presented as mean ± standard deviation (SD). Statistical comparisons were performed using Student's *t*-test or one-way ANOVA followed by Tukey's *post hoc* test (GraphPad Prism 9). Differences were considered significant at $p < 0.05$.

# 3 Results

## 3.1 Screening and identification for SMS inhibitors

To identify SMS inhibitors, we screened methanol and acetone extracts of the fruit bodies, leaves, stems, and roots of approximately 650 medicinal plants and 212 mushrooms from the Hokkaido University natural product library using a cell-based SMS assay (Supplementary Figures 1A,B). The full list of species screened is provided in Supplementary Table S2. We found that the fruiting body extracts of *A. dispansus* and *A. confluens* showed marked inhibitory activity against SMS (Supplementary Figures 1A,B). *A. dispansus* and *A. confluens* are edible and have been used as natural medicines, functional foods, and dietary supplements in China for thousands of years. Bioassay-guided fractionation of the *A. confluens* extract resulted in the identification of grifolin and grifolic acid as inhibitor compounds (Figure 1A). Each was confirmed by NMR spectroscopy, HRMS, and spectroscopic data, similar to previous reports (Supplementary Figure 1D). The dose-dependent inhibitory effects of grifolin and grifolic acid on SMS1 and SMS2 were validated using a cell-based assay, with both compounds exhibiting substantial inhibition across isoforms (Figure 1B).

Grifolin and grifolic acid were identified as major components of *Albatrellus* species. These compounds exhibited strong inhibitory activity against SMS using both PC and Cer substrates. SMS inhibitors

2 American Veterinary Medical Association. Available from: https://www.avma.org/resources-tools/avma-policies/avma-guidelines-euthanasia-animals

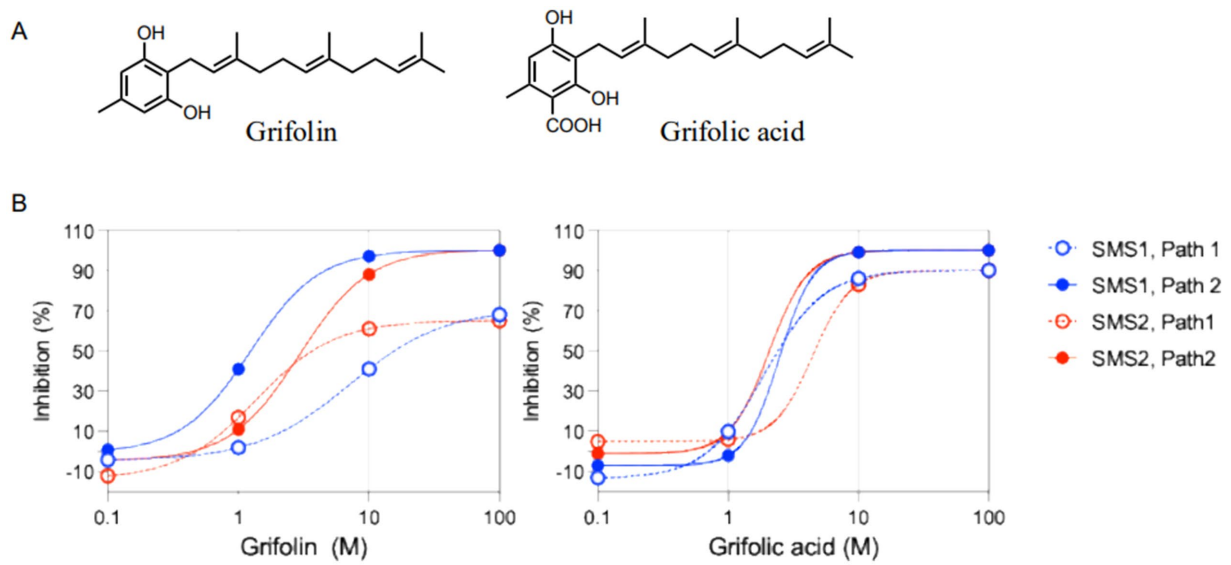



FIGURE 1
Grifolin and grifolic acid inhibit sphingomyelin synthase (SMS) activity *in vitro*. **(A)** Chemical structures of grifolin and grifolic acid isolated from *A. confluens*. **(B)** Dose–dependent inhibition of SMS1 and SMS2. Enzyme assays were performed in ZS2 cells expressing SMS1 or SMS2 using 5 μM C6-NBD-ceramide (NBD-Cer) and 5 μM C6-NBD-phosphatidylcholine (NBD-PC) as substrates. Path 1 represents the conversion of NBD-PC to NBD-diacylglycerol (NBD-DAG), and Path 2 represents the conversion of NBD-Cer to NBD-sphingomyelin (NBD-SM). Products were quantified by HPLC with fluorescence detection. Linearity was verified with respect to protein concentration and incubation time. Data are mean ± SD ($n \geq 3$).

were identified medicinal mushroom and plant extracts library of Hokkaido university, screening assays by using both fluorescence substrates, C6-NBD (N-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino] hexanoyl])-Cer and C6-NBD-PC. The inhibitory activities of the extraction were quantified as follows: against the C6-NBD-PC substrate, both SMS1 and SMS2 showed inhibition at 4.5 μg/mL; against the C6-NBD-Cer substrate, SMS1 was inhibited at 0.5 μg/mL, and SMS2 at 0.3 μg/mL. The inhibitory effects of grifolin and grifolic acid are among the strongest (Supplementary Table 1) natural SMS inhibitors reported to date, targeting both substrates (21–23). The half maximal inhibitory concentration ($IC_{50}$) was defined as the compound concentration required to reduce enzyme activity by 50%. The estimated $IC_{50}$ values for grifolin were: 15.2 μM (SMS1, C6-NBD-PC), 18.3 μM (SMS2, C6-NBD-PC), 0.6 μM (SMS1, C6-NBD-Cer), and 3.0 μM (SMS2, C6-NBD-Cer). For grifolic acid, the $IC_{50}$ values were 3.0 μM (SMS1, C6-NBD-PC), 4.0 μM (SMS2, C6-NBD-PC), 3.0 μM (SMS1, C6-NBD-Cer), and 2.5 μM (SMS2, C6-NBD-Cer) (Table 1).

TABLE 1 $IC_{50}$ values of grifolin and grifolic acid against SMS1 and SMS2.

| Compounds | $IC_{50}$ (μM) | | | |
|---|---|---|---|---|
| | SMS1 | | SMS2 | |
| | Path 1 | Path 2 | Path 1 | Path 2 |
| Grifolin | 15.2 | 0.6 | 18.3 | 3 |
| Grifolic acid | 3 | 3 | 4 | 2.5 |

Assays used ZS2 cell lysates expressing SMS1 or SMS2 with 5 μM NBD-Cer and 5 μM NBD-PC. Path 1 = NBD-PC → NBD-DAG; Path 2 = NBD-Cer → NBD-SM. Values are from nonlinear regression (4-parameter logistic) of concentration–response curves; mean ± SD, $n = 3$ independent experiments. All units are μM.

## 3.2 Cell viability and inhibition of lipid droplet accumulation *in vitro*

We examined cytotoxicity in human hepatoma HepG2 cells. Neither compound showed significant cytotoxicity up to 20 μM (Figure 2A). SMS-deficient cells and mice have previously been reported to exhibit impaired fatty acid uptake and lipid droplet formation (16, 24). To investigate whether the identified SMS inhibitors affected lipid accumulation, we assessed lipid droplet formation in HepG2 cells following treatment with oleic acid (OA). Lipid droplets, which were stained with Nile Red, were remarkably reduced by both grifolin and grifolic acid treatment in a dose-dependent manner (grifolin treated 43% and grifolic acid treated 48% of lipid reduction at 20 μM, Figures 2B,C). Building on a previous report that oral administration of grifolin at 2,000 mg/kg was safe in mammals (25), we conducted an *in vivo* study using a high-fat diet-induced obesity mouse model.

## 3.3 Grifolin and grifolic acid prevent HFD-induced obesity

Oral dietary supplementation with grifolin or grifolic acid (0.1% in HFD, corresponding to ~100 mg/kg/day) for 9 weeks markedly reduced body weight gain in male C57BL/6J mice ($n = 6$ per group). The mean final body weights were: ND – 27.0 g, HFD – 31.2 g, HFD + grifolin – 26.7 g, and HFD + grifolic acid – 25.8 g. When compared to the HFD group, this corresponds to a relative reduction of 14.6% for grifolin and 17.4% for grifolic acid (Figures 3A,C; representative images in Figure 3B).

Feed intake was recorded weekly and differed significantly among groups (Supplementary Figure 2). HFD-fed mice consumed significantly less food (3.02 ± 0.54 g/day) compared with HFD + Gri

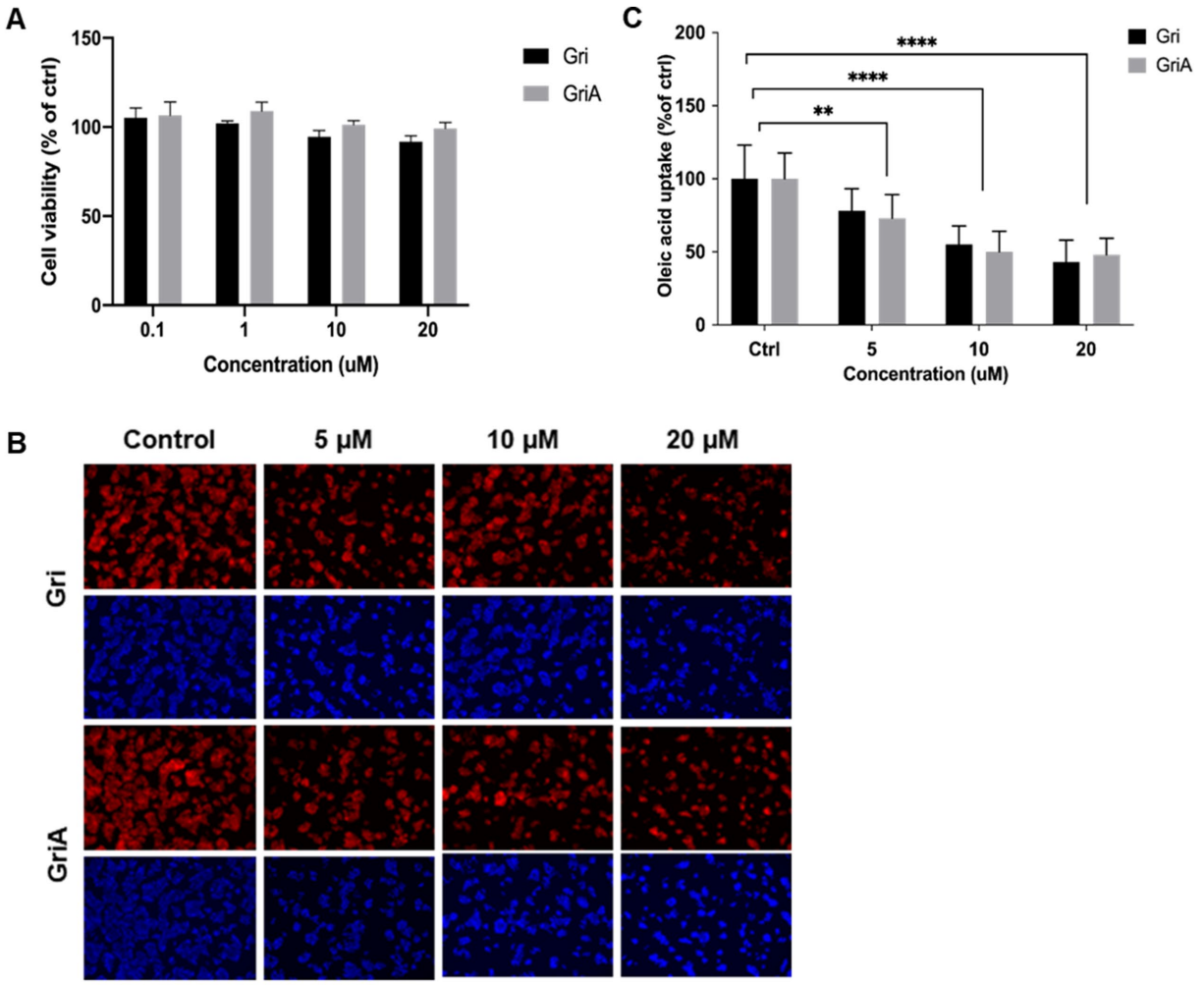



FIGURE 2
Effects of grifolin and grifolic acid on cell viability and lipid droplet accumulation in HepG2 cells. **(A)** Cell viability by CCK-8 after 24 h incubation with 0–20 μM grifolin or grifolic acid. **(B,C)** Nile Red (neutral lipids) and DAPI (nuclei) fluorescence images after 24 h exposure to 1 mM oleic acid (OA) in the absence or presence of 20 μM compounds. Imaging: Keyence BZ-X70, 20 × objective, fixed exposure across conditions; scale bar = 100 μm (applies to all panels). Quantification used ImageJ (Otsu thresholding); lipid content reported as Nile Red intensity per cell (AU/cell; normalized to DAPI counts). Data are mean ± SD, $n = 3$ independent experiments (multiple fields of view per replicate). Statistics: one-way ANOVA with Tukey's *post hoc* test; significance indicated as: (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$, (****) $p < 0.0005$, ns = not significant.

(4.68 ± 1.24 g/day, $p < 0.05$) and HFD + GA (4.82 ± 1.06 g/day, $p < 0.05$), while intake in the treated groups did not differ significantly from ND controls (4.55 ± 0.8 g/day). Thus, the reduced body weight observed in the treated groups was not attributable to reduced caloric intake but rather to the metabolic effects of grifolin and grifolic acid.

We investigated the effects of these inhibitors on glucose tolerance, hepatic TG levels, and lipid droplet accumulation. As expected, after 9 weeks an oral glucose tolerance test (1 g/kg glucose by gavage following overnight fasting) showed that both grifolin- and grifolic acid–treated mice exhibited significantly improved glucose tolerance compared to HFD-group (Figure 3D).

## 3.4 Grifolin and grifolic acid prevent liver steatosis

In agreement with the results obtained in HepG2 cells, the histological study of the Oil Red O staining demonstrated that the treatment with grifolin and grifolic acid prevented lipid accumulation in the liver of HFD-fed mice (Figure 4A). Conversely, the untreated HFD-fed mice showed substantial lipid droplet accumulation, indicative of liver steatosis. In contrast, the treated groups showed improved lipid metabolism and minimal lipid deposition in the liver (Figure 4A). Hepatic triglyceride (TG levels in the HFD group were 30.5 and 38.4% higher than those in the grifolin-treated and grifolic acid-treated groups, respectively Figure 4B).

Subsequently, we measured SM levels in liver tissues collected from control and grifolin-treated HFD mice. As expected, in the liver tissues of grifolin-treated mice, SM levels were significantly reduced, suggesting suppressed SMS activity (Supplementary Figure 3A). Consistent with the SM measurement results, analysis of SMS activity in liver tissues from control and grifolin-treated HFD mice showed that DAG and SM synthesized from PC and Cer were significantly reduced in the grifolin-treated group (Supplementary Figures 3B,C). Grifolic acid treatment also showed a trend toward reduced DAG and SM levels, although the differences were not statistically significant.

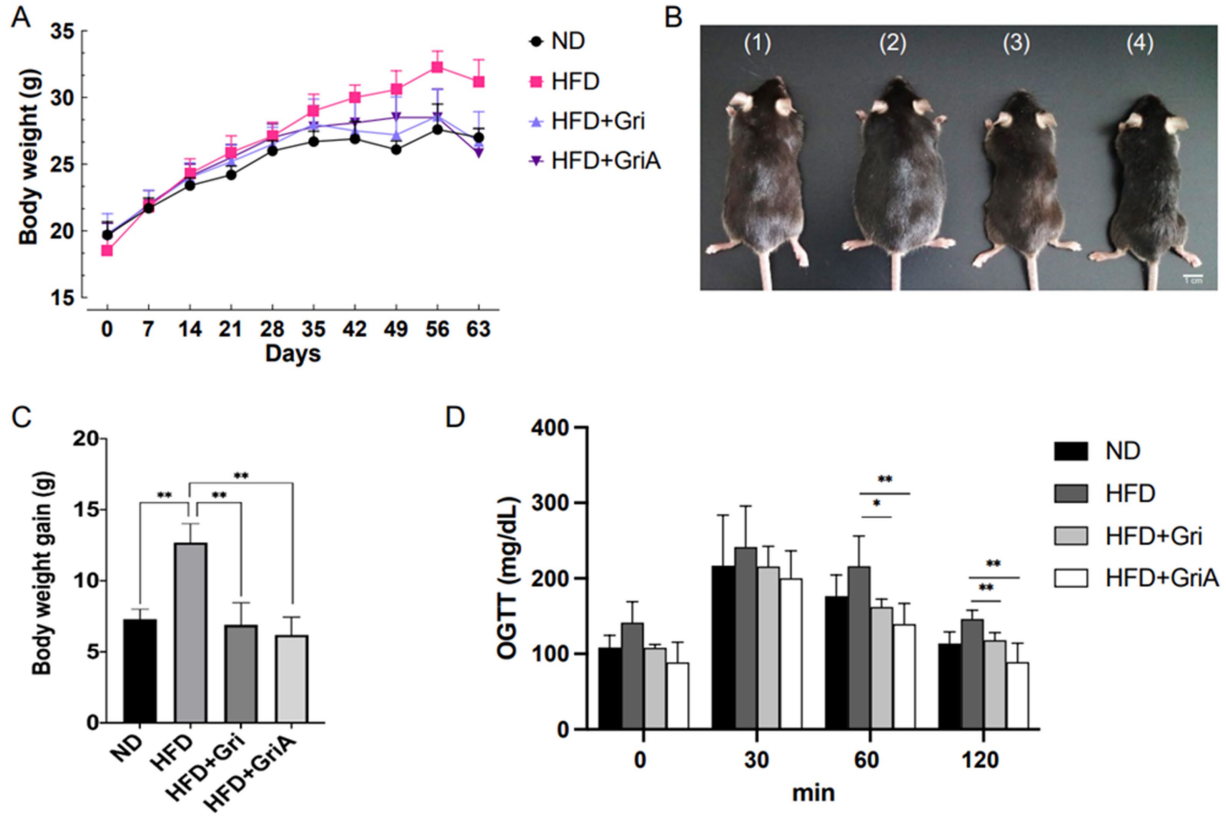



FIGURE 3
*In vivo* effects of grifolin and grifolic acid on body weight and glucose tolerance in diet-induced obese mice. **(A)** Weekly body weight gain; **(B)** Representative images of mice: (1) ND, (2) HFD, (3) HFD + 0.1% grifolin, (4) HFD + 0.1% grifolic acid; **(C)** Final body weight after 9 weeks; **(D)** Blood glucose levels during oral glucose tolerance test (OGTT; 1 g/kg, $n = 6$ per group) at 0, 30, 60, and 120 min; Statistical analysis one-way ANOVA with Tukey's post hoc test: (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$, (****) $p < 0.0005$. Feed intake data are provided in Supplementary Figure 2.

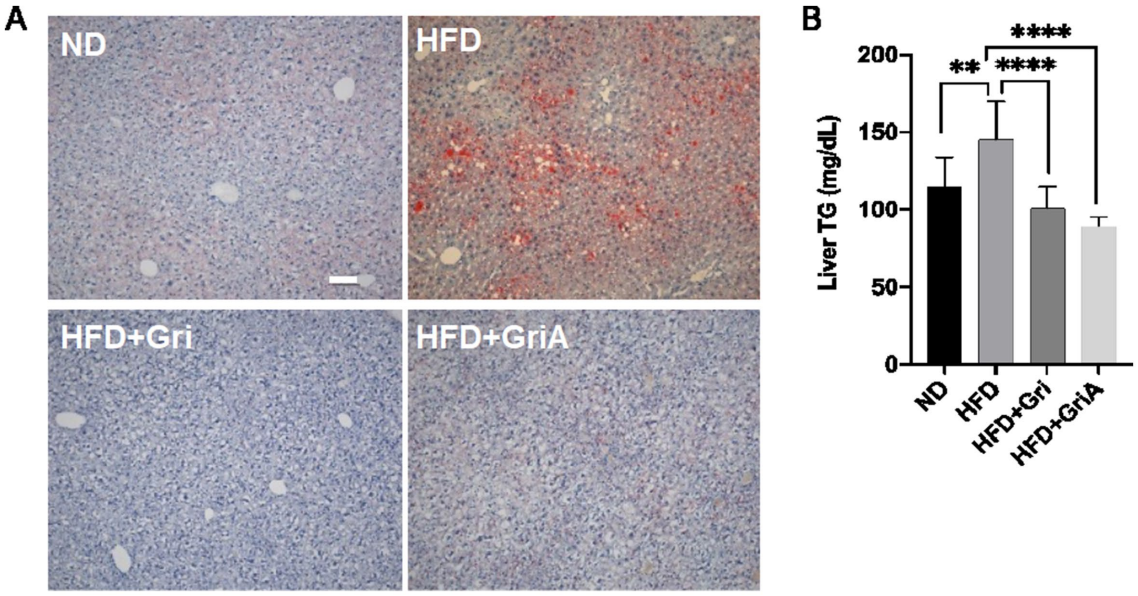



FIGURE 4
Effects of grifolin and grifolic acid on hepatic lipid accumulation in diet-induced obese mice. **(A)** Representative Oil Red O–stained liver sections from ND, HFD, HFD + Gri, and HFD + GA groups ($n = 3$ mice per group, independent samples). Scale bar = 100 μm (applies equally to all panels). **(B)** Hepatic triglyceride levels (mg/dL) in ND, HFD, HFD + Gri, and HFD + GA groups ($n = 6$ mice per group). *T*-test: (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$, (****) $p < 0.0005$, ns = not significant versus control.

Taken together, these results demonstrate that grifolin and grifolic acid suppress weight gain, prevent hepatic lipid droplet formation, and improve glucose tolerance, likely by suppressing SMS activity in HFD-induced obese mice.

## 3.5 Stabilization of vitamin D level in the serum by grifolin and grifolic acid

We selected three genes based on a previous study and performed qRT-PCR on mRNA extracted from liver and kidney tissues of DIO mice after 9 weeks of treatment. The selected CYP24A1, CYP27B1, and CYP2R1 genes showed involvement in 25(OH)$D_3$ regulation. CYP27B1 is involved in the synthesis of active vitamin D, whereas CYP24A1 and CYP2R1 are involved in its catabolism. Serum 25(OH)$D_3$ concentration significantly decreased in the HFD-fed group. In contrast, 25(OH)$D_3$ levels were approximately 2.5-fold higher in the grifolin-fed group ($p < 0.05$) and approximately 6.3-fold higher in the grifolic acid-fed group ($p < 0.001$) than in the HFD-fed group (Figure 5A). The mRNA expression of CYP24A1 was significantly upregulated in the HFD-fed group (Figure 5B). Conversely, no significant differences in the CYP27B1 and CYP2R1 expression levels were observed between the groups (Figures 5C,D). These findings suggest that SMS inhibition by grifolin and grifolic acid may downregulate CYP24A1 expression, thereby reducing the active vitamin D catabolism and stabilizing the physiological levels of active 25(OH)$D_3$.

# 4 Discussion

The exploration of enzyme inhibitors is a key strategy for developing low-molecular-weight drugs and biologically active

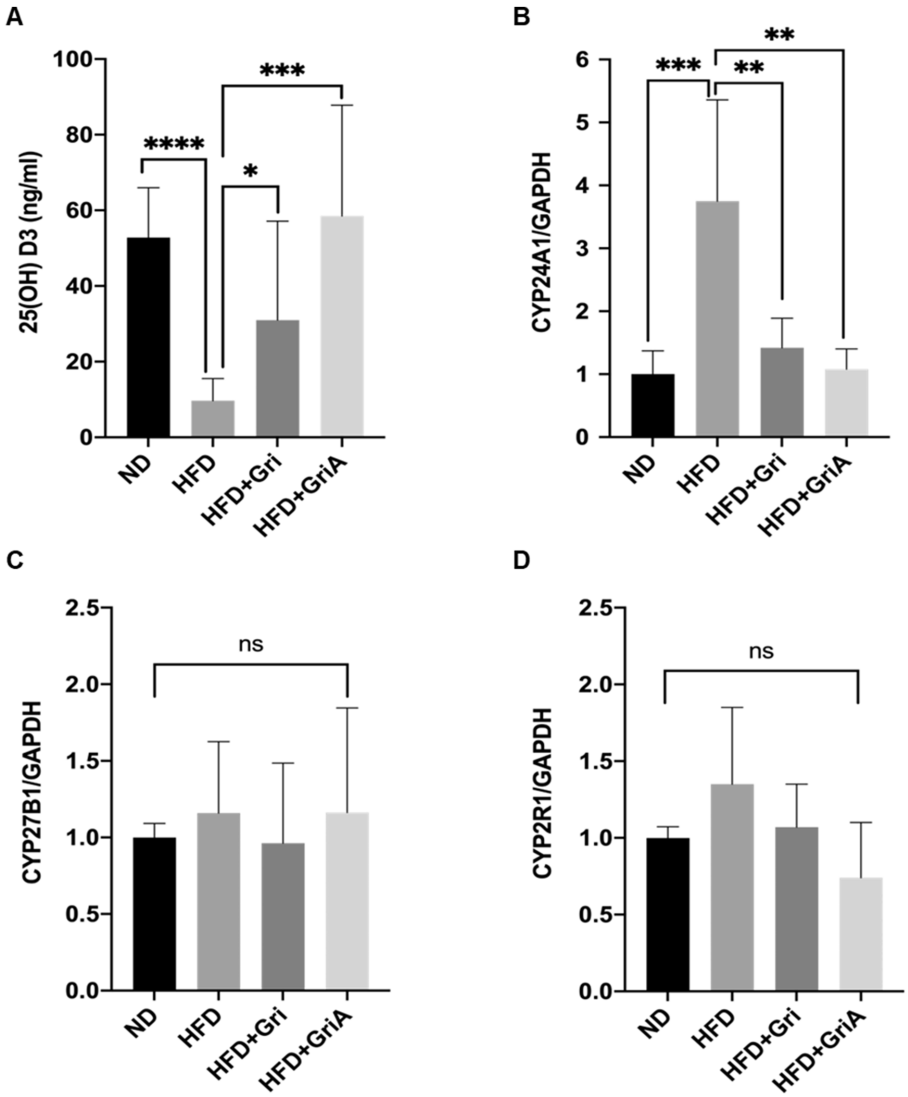



FIGURE 5
Effects of grifolin and grifolic acid on serum vitamin D levels and vitamin D–related gene expression in diet-induced obese mice. **(A)** Serum 25(OH)$D_3$ concentrations. **(B)** Renal CYP24A1 expression. **(C)** Hepatic CYP27B1 expression. **(D)** Hepatic CYP2R1 expression Groups: ND, HFD, HFD + Gri, HFD + GriA ($n$ = 6 mice per group). $T$-test: (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$, (****) $p < 0.0005$, ns = not significant.

compounds, substantially advancing the pharmaceutical field (19). In the present study, we identified two SMS inhibitors from natural sources: grifolin and grifolic acid. Consistent with the findings from studies using SMS-KO mice (16), these compounds were effective in reducing obesity, preventing hepatic steatosis, and improving glucose tolerance in a DIO model. In addition, treatment with grifolin or grifolic acid prevented the decrease in serum 25(OH)$D_3$ levels typically observed in HFD-fed mice. These findings highlight SMS inhibitors as promising candidates for drugs or functional food development targeting obesity and enhance our understanding of the crosstalk between sphingolipid homeostasis and metabolic regulation.

Interestingly, although HFD-fed mice consumed less food than animals receiving grifolin or grifolic acid supplementation, they nevertheless gained more weight and exhibited more severe metabolic impairments. This indicates that the observed benefits cannot be explained by caloric intake alone but instead reflect modulation of lipid metabolism. This conclusion aligns with a growing body of evidence suggesting that obesity is not driven exclusively by energy intake, but also by alterations in lipid signaling pathways and adipose tissue dynamics (26, 27).

To identify SMS inhibitors, we screened a natural product extract library constructed from 650 plants and 212 mushrooms, either grown wild or cultivated in Hokkaido. This library included a local medicinal plant collection and was evaluated using a high-throughput cell-based SMS assay. The most potent inhibitory activity was observed in the preliminary screening of the ether extract from the fruiting bodies of the *A. confluens*. Mushrooms have been used in traditional Chinese medicine since ancient times. Their secondary metabolites such as polysaccharides (mainly $\beta$-D-glucans), heteroglycans, chitinous substances, peptidoglycans, proteoglycans, lectins, RNA components, lectins, lactones, alkaloids, terpenes, flavonoids, terpenoids, steroids, phenols, glycoproteins, nucleotides, fatty acids, vitamins, proteins, amino acids, antibiotics, and minerals, have been reported to exert various beneficial impacts on human health and provide protection against disease (28). Mushrooms have been shown to exert anti-obesity effects. Both long-term (1 year) and short-term (4-day) clinical trials involving obese or diabetic participants were conducted to evaluate the effects of substituting 20% high-energy beef with 20% low-energy white button mushrooms in the diet. These studies demonstrated that mushroom consumers exhibited lower BMI, decreased belly circumference, and increased satiety without compromising palatability (29, 30). Preliminary biological activities of *Albatrellus* species have been reported, including antioxidant (31), antifungal (32), and nitric oxide inhibitory (33) activities; neuroprotective effects in acute cerebral ischemia (25); and inhibition of lipopolysaccharide-induced B-lymphocyte proliferation (34). Chemical studies of *Albatrellus* have identified grifolin and grifolic acid as the major components, along with grifolic acid methyl ester, 3-hydroxyneogrifolin, 1-formylneogrifolin, 1-formyl-3-hydroxyneogrifolin, and a newly identified compound, grifolinol. Their chemical structures were determined using a combination of two-dimensional (2-D) NMR, mass spectroscopy, and chemical reactions (35).

Grifolin and grifolic acid are amphipathic compounds composed of a hydrophilic head group from salicylic acid and a common hydrophobic tail group from the hydrocarbon chain and have physical properties similar to those of sphingosine (Figure 1). Based on their structure, we hypothesized that grifolin and grifolic acid could inhibit SMS by acting as sphingolipid mimics. In this study, we identified for the first time that the SMS inhibitors grifolin and grifolic acids suppress SM and DAG synthesis. Both compounds significantly inhibited the DAG and SM formation *in vitro* (Table 1). We further confirmed that grifolin significantly reduced SM and DAG levels in the liver tissues of treated mice (Supplementary Figure 3). This dual inhibition is noteworthy because DAG synthesized by SMS2 has been reported to act as a second messenger that activates the PKC-JNK signaling pathway, thereby impairing insulin signaling (36). The potency of these compounds can also be placed in the context of other natural SMS inhibitors. As summarized in Supplementary Table 1, their $IC_{50}$ values are comparable to or stronger than previously reported inhibitors, such as ginkgolic acid, malabaricones, and daurichromenic acids (21–23). Grifolic acid, in particular, displayed submicromolar potency against SMS2 in the C6-NBD-ceramide assay, highlighting its potential as one of the most active natural SMS inhibitors identified to date.

A particularly novel observation is the effect of SMS inhibition on vitamin D metabolism. Both grifolin and grifolic acid preserved serum 25(OH)$D_3$ levels and downregulated renal expression of CYP24A1, the enzyme responsible for degrading 1,25$(OH)_2D_3$ (37). This is notable because vitamin D bioavailability is reduced in obesity due to sequestration in adipose tissue (38), and sphingolipid imbalances have been linked to vitamin D deficiency (39–42). Previous studies also suggest that vitamin $D_3$ can stimulate SMase activity (42) and that ceramide and SM levels are associated with dyslipidemia and vitamin D deficiency (39). Taken together, these findings suggest a novel cross-talk between sphingolipid metabolism and vitamin D pathways. Since both systems influence calcium homeostasis, inflammatory signaling, and insulin sensitivity, this interaction may represent an additional mechanism by which SMS inhibition improves metabolic outcomes.

Certain limitations should be acknowledged. Because substrate-varying kinetic analyses were not performed, the precise inhibition mechanism (competitive, mixed, or noncompetitive) with respect to ceramide or phosphatidylcholine remains undefined. Moreover, fatty acid uptake assays (e.g., BODIPY-C16 transport) were not conducted, leaving open the possibility that effects on fatty acid transport contribute to the observed outcomes. Cytotoxicity was assessed only using the CCK-8 assay, without further evaluation of membrane integrity or mitochondrial function. Additionally, lean body mass was not measured, limiting conclusions regarding body composition changes. Addressing these limitations through future studies—particularly kinetic modeling, fatty acid uptake analyses, extended cytotoxicity profiling, and body composition assessment—will provide a clearer mechanistic understanding. Finally, translation to human application will require careful investigation of pharmacokinetics, safety, and effective delivery strategies.

In conclusion, this study demonstrates that grifolin and grifolic acid act as dual-substrate SMS inhibitors with beneficial effects on obesity, glucose tolerance, hepatic lipid accumulation, and vitamin D metabolism in a DIO mouse model. These findings highlight the potential of mushroom-derived natural products as functional food ingredients or nutraceuticals for the prevention and management of obesity and related metabolic disorders. By targeting both lipid signaling and vitamin D pathways, grifolin and grifolic acid represent a unique class of multifunctional

bioactives that warrant further investigation in translational and clinical settings.

## Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

## Ethics statement

The animal study was approved by Hokkaido University, Faculty of Advanced Life Sciences, Committee on Bioethics. The study was conducted in accordance with the local legislation and institutional requirements.

## Author contributions

EE: Writing – original draft, Formal analysis, Data curation, Investigation, Writing – review & editing. YM: Investigation, Supervision, Writing – original draft. KY: Writing – review & editing, Investigation, Writing – original draft, Validation, Supervision, Formal analysis, Visualization. HS: Investigation, Writing – original draft, Data curation, Formal analysis. MS: Data curation, Supervision, Writing – original draft, Formal analysis, Writing – review & editing. YS: Writing – review & editing, Data curation, Formal analysis, Investigation. MA: Validation, Supervision, Writing – original draft. KM: Methodology, Writing – original draft, Resources, Funding acquisition, Validation, Conceptualization, Project administration, Writing – review & editing, Investigation.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2025.1658130/full#supplementary-material

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