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# Emerging trends in the quality check of herbal medicines, supplements and 'botanicals'

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# Emerging trends in the quality check of herbal medicines, supplements and 'botanicals'

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# Editorial: Emerging trends in the quality check of herbal medicines, supplements, and "botanicals"

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**botanicals, quality assessment and control, emerging technologies, metrology, data management**

Editorial on the Research Topic
Emerging Trends in the Quality Check of Herbal Medicines, Supplements and 'Botanicals'

## 1 Introduction

This Research Topic aims to explore and discuss emerging trends in the quality assessment of herbal medicines, supplements, and botanicals.

To gather further insights in the quality assessment of herbal products the following topics were addressed: comparative analysis of chemical and spectrophotometric techniques for quality assessment; identification and validation of quality markers in herbal products; development of sustainable and innovative analytical methods; challenges and solutions in standardizing quality checks for diverse herbal matrices; and management and utilization of data and databases in herbal medicine research. In this context, the present Research Topic, Emerging Trends in the Quality Check of Herbal Medicines, Supplements, and "Botanicals", brings together 10 contributions that address these matters well from different points of view.

Some authors presented the development of new methodologies or applications and case studies of research. For instance, Wang et al. presented a quality evaluation of *Polygonatum sibiricum* slices from different regions based on appearance traits and multi-index metabolites combined with a technique for order preference by similarity to ideal solution—TOPSIS—and gray relation analysis. Zhou et al. aimed to authenticate Renshen Jianpi Wan (RSJPW), a classical CCPP composed of 11 prescribed botanical drugs, using DNA metabarcoding to overcome challenges in species-level identification of processed biological ingredients.

Xin et al. investigated metabolomic and lipidomic profiling of traditional Chinese medicine *Testudinis Carapax et Plastrum* and its substitutes.

Luis and Schneider reported a large variability in the alkaloid content of *Corydalis yanhusuo* dietary supplements available online in the United States.

Xu et al. studied the efficacy of Hongjing I granule, an herbal medicine, in patients with mild to moderate erectile dysfunction in a randomized controlled trial.

Bai and Dong presented the development and validation of the HPLC–MS/MS method and its application in the pharmacokinetic study of the Mongolian drug Sendeng-4 in rat blood plasma.

Lenssen and de Boer studied, through an analysis of EU and national case law from the Netherlands, including self-regulatory decision-making, the implications of case law on botanical health claims.

It is worth mentioning the article by Harnly et al. that carried out a review focused on one-class modeling for verification of botanical identity.

Hao et al. presented a review of botany, application, processing, phytochemistry, quality control, pharmacology, and toxicology of *Euodiae Fructus*.

Al-Sammarraie et al., by carrying out an artificial intelligence-aided scoping review of medicinal plant research in the Fertile Crescent, showed that the number of ethnobotanical studies was limited, suggesting an urgent need to prevent the loss of ancestral knowledge by formalizing it through evidence-based research and policy guidelines. Al-Sammarraie et al. suggested that to address these gaps through interdisciplinary collaboration and improved data-sharing, mechanisms will be crucial for advancing Traditional Arabic and Islamic Medicine research and medicinal plants.

## Author contributions

NP: Conceptualization, Writing – original draft, Writing – review and editing. DDRA: Conceptualization, Writing – original draft, Writing – review and editing. ML: Conceptualization, Writing – original draft, Writing – review and editing. AD: Conceptualization, Writing – original draft, Writing – review and editing.

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# Efficacy of Hongjing I granule, an herbal medicine, in patients with mild to moderate erectile dysfunction in a randomized controlled trial

Run-Nan Xu[1,2†], Jun Guo[3†], Chun-He Zhang[4†], Qing Zhou[5†], Qiang Gen[6,7†], Fu Wang[3], Yu Zhao[6,7], Xin-Yun Luo[5], Yan-Feng Li[4], Yi-Jia Fu[1,2], Xin Zhang[1,2], Wen-Zhi Wang[8], Jian-Xiong Ma[1], Jian Wang[1,2], Xiao-Jun Huang[1], Wen-Jie Huang[1] and Bo-Dong Lv[1,2]*

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**Background:** HJIG is a potential treatment for erectile dysfunction (ED) that has been used in China for over 20 years. We conducted a multi-center, double-blind, randomized, placebo-controlled trial to evaluate the effectiveness and safety of the Chinese Herbal Medicine, Hongjing I granule (HJIG), in patients with mild to moderate erectile dysfunction (ED).

**Methods:** This study is structured as a randomized, double-blind, placebo-controlled trial, executed across multiple centers. The recruitment strategy is primarily oriented towards patients demonstrating a pronounced preference for solely leveraging traditional Chinese medicine (TCM) interventions, a preference that is widely observed within TCM healthcare settings. A total of 100 patients, presenting with mild to moderate ED, specifically linked to the traditional diagnostic criteria of qi deficiency and blood stasis, will be enrolled. These participants will be randomly distributed between the HJIG (N = 50) and placebo (N = 50) arms. The designated treatment period is set at 8 weeks. Primary outcome measures encompass the International Index of Erectile Function-Erectile Function domain (IIEF-EF) score, the Sexual Encounter Profile (SEP), and scores derived from the traditional Chinese medicine symptom evaluation.

**Abbreviations:** HIJG, Hongjing I granule; ED, Erectile dysfunction; TCM, traditional Chinese medicine; IIEF-EF, International Index of Erectile Function-Erectile Function domain; SEP, Sexual Encounter Profile; EF, erectile function; EHS, erection hardness scale; AEs, Adverse events; CRF Case report form.

**Results:** Of the 122 men enrolled, the baseline IIEF-EF score averaged 16.00 [IQR: 13.00, 18.00]. Eight weeks post-randomization, the HJIG group demonstrated a mean change in IIEF-EF scores of 7.80 (±3.25), compared to 3.33 (±3.90) in the placebo group, signifying a marked difference ($P < 0.001$). The median alterations in SEP3 scores were 0.50 [IQR: 0.36, 0.75] for the HJIG group and 0.50 [0.20, 0.67] for the placebo group, revealing a statistically relevant distinction ($P = 0.05$). In both primary outcomes, HJIG proved superior to the placebo. Additionally, improvements in TCM symptom scores were notably greater in the HJIG group relative to the placebo, with no adverse events reported across both groups.

**Conclusion:** The Hongjing I granule significantly improved symptoms in patients with mild to moderate ED. However, to validate these findings, further extended randomized trials are warranted.

**Clinical Trial Registration:** The study has been registered in the Chinese Clinical Trial Registry (ChiCTR) and the registration number was ChiCTR2000041127.

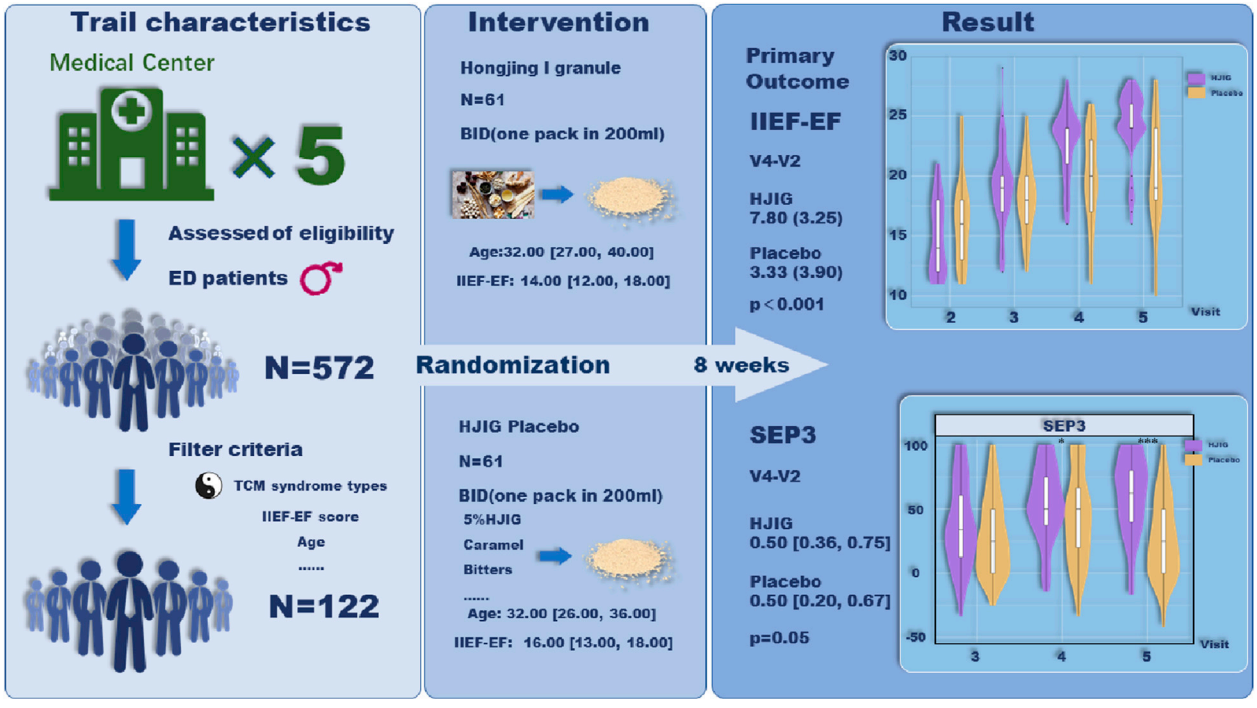
KEYWORDS

**Chinese herbal formula, randomized control trial, Hongjing I granule, erectile dysfunction, traditional Chinese medicine (TCM)**

## Introduction

Erectile Dysfunction (ED) stands as a predominant sexual dysfunction in men, manifesting as a consistent inability to achieve or maintain an erection conducive to satisfactory sexual intercourse (Najari and Kashanian, 2016). Current estimates indicate that over 150 million men globally grapple with varying intensities of ED (Travison et al., 2007). Utilizing assessment tools such as IIEF, IIEF-5, and their related variants, there's a discernible year-on-year escalation in ED incidence, mirroring the global aging demographic trend. Projections suggest that by 2025, the worldwide ED-afflicted populace might approach 322 million (Costa and Potempa, 2012).

The mainstay therapeutic intervention remains the Type 5 Phosphodiesterase inhibitors (PDE5i), yet its efficacy hovers between 60% and 70% (Ji et al., 2023). This has driven a substantial fraction of patients towards alternative medicine, predominantly herbal therapies (Muncey et al., 2021). Herbal formulations have a long-standing history and are widely used across Asia, particularly in China, Korea, and Japan, where they form an integral part of traditional medicinal practices (Bailly, 2024). The efficacy of herbal remedies or herbal formulations in




GRAPHICAL ABSTRACT

treating erectile dysfunction has been corroborated by numerous studies (Qi et al., 2004; Kamenov et al., 2017; Borrelli et al., 2018; Choi et al., 2019; Chung et al., 2023). A burgeoning corpus of research intimates that Traditional Chinese Medicine (TCM) offers tangible benefits for ED (Wang et al., 2020; Wu et al., 2023), though some studies proffer equivocal conclusions (Xiong et al., 2014). Hence, there's a pressing advocacy for rigorously orchestrated Randomized Controlled Trials (RCTs) to delve deeper into the subject.

Hongjing I granule (HJIG) is a traditional Chinese medicine prescribed by experienced physicians from the Second Affiliated Hospital of Zhejiang University School of Medicine and Dongzhimen Hospital of Beijing University of Chinese Medicine, with nearly 20 years of clinical application.

The traditional Chinese medicine theoretical basis for its application is elucidated as follows: The theory of Qi and Blood originates from the "Huangdi Neijing," where the "Su Wen·Tiao Jing Lun" states, "Diseases arise and transform when the Qi and Blood are in disharmony." Based on this, the harmony of Qi and Blood is closely associated with the onset and progression of diseases. Zhang Zhongjing, in his creation of the Six Meridian Diagnosis and Treatment System, also reflected the academic notion that all diseases are ultimately governed by the regulation of Qi and Blood. The "Zheng Zhi Gai Yao" mentions, "The penis, being constituted by sinews, relies on the nourishment of Qi and the moistening of Blood to become strong and vigorous." This indicates that the penis is "structured by sinews and functions through the utility of Qi and Blood." (Zhang G. et al., 2017). In a clinical study, 80 patients with mild to moderate ED were randomized into two groups: one receiving Tadalafil (5 mg qd) and the other a combination of Tadalafil and HJIG (1 pack bid) for 3 months. Evaluations of penile length, circumference, rigidity, IIEF-5 scores, partner satisfaction, and CT cavernosography showed post-treatment improvements in both groups ($P < 0.05$), with the combination therapy outperforming Tadalafil alone. Specifically, in the Tadalafil group, 33 cases showed venous leakage improvement with 7 cases completely resolved; the combination group had 17 cases improved and 23 cases fully resolved, indicating a statistically significant difference ($\chi2 = 13.65$, $P < 0.05$) (Ping et al., 2021).

Additionally, 76 patients with Qi deficiency and blood stasis type mild to moderate ED received either Tadalafil (5 mg nightly for 12 weeks) or a combined Tadalafil and HJIG treatment (45 cases) with a step-down Tadalafil regimen. Both treatments significantly enhanced IIEF-5 and EHS scores ($P < 0.05$). The combined treatment group showed more substantial improvements in IIEF-5 and EHS scores after 4 weeks of medication and 4 weeks post-discontinuation compared to the Tadalafil only group ($P < 0.01$) (Zhang G. et al., 2017). Animal models have corroborated HJIG's potential to promote nerve regeneration, attenuate hypoxia, curtail phenotype transformation, and stymie tissue fibrosis (Yang et al., 2014; Ye et al., 2019; Ma et al., 2020).

However, the evidence supporting the efficacy of HJIG in treating ED remains limited, and many studies on Chinese herbal formulations suffer from a lack of correlation assessment with their traditional use. It is essential to conduct high-quality clinical research to evaluate the therapeutic effectiveness of HJIG for ED and the validity of its application within traditional Chinese medicine frameworks.

HJIG is comprised of nine traditional Chinese plants, including Rhodiola crenulata (Hook. f. et Thoms.) H. Ohba [Crassulaceae; Rhodiola crenulata (Hook. f. et Thomas.) H. Ohba] (红景天), Astragalus mongholicus Bunge [Fabaceae; astragali radix praeparata cum melle] (黄芪), Codonopsis pilosula (Franch.) Nannf. [Campanulaceae; Codonopsis pilosula (Franch.) Nannf.] (党参), Salvia miltiorrhiza Bunge [Lamiaceae; Salvia miltiorrhiza Bge.] (丹参), Angelica sinensis (Oliv.) Diels [Apiaceae; Angelica sinensis (Oliv.) Diels] (全当归), Paeonia lactiflora Pall. [Paeoniaceae; Paeonia lactiflora Pall.] (白芍), Cyathula officinalis K.C.Kuan [Amaranthaceae; Cyathula officinalis Kuan] (川牛膝), Lycium chinense Mill. [Solanaceae; Lycium chinense Mill.] (枸杞), and Epimedium brevicornu Maxim. [Berberidaceae; Epimedium brevicornu Maxim.] (淫羊藿). The pharmacopoeial names are all referenced from the Chinese Pharmacopoeia 2015. The botanical names have been verified with MPNS (http://mpns.kew.org) (Access date: January 4, 2024).

Therefore, we designed this multicenter, double-blind, placebo-controlled randomized study. In addition to strict inclusion and exclusion criteria, we have also incorporated Traditional Chinese Medicine (TCM) syndrome scoring to evaluate whether the traditional medical basis of using HJIG (TCM syndrome) aligns with improvements in patient erectile function.

# Materials and methods

## Study design

This study is a multicenter, double-blind, placebo-controlled trial conducted in China, enrolling 122 ED patients seeking traditional Chinese medicine treatment from five distinct hospitals. Participants will be equally allocated to the HJIG herbal treatment group and a placebo group. The trial strictly adheres to the SPIRIT guidelines (Calvert et al., 2018), the Helsinki Declaration (Goodyear et al., 2007), and Good Clinical Practice (Guideline, 2001). In reporting details of the trial, we followed the recommendations of CONSORT Extension for Chinese Herbal Medicine Formulas 2017 (Cheng et al., 2017). Ethical approval for this study was obtained from the Ethics Committee of the Second Affiliated Hospital of Zhejiang Chinese Medical University (Approval ID: 2019-KL-012-01; see Supplementary Data Sheet S2). The trial is registered with ChiCTR (registration number: ChiCTR2000041127) and is coordinated by the Second Affiliated Hospital of Zhejiang Chinese Medical University, in partnership with four renowned institutions: The First Affiliated Hospital of Hunan University of Chinese Medicine, the First Affiliated Hospital of Tianjin University of Traditional Chinese Medicine, Yunnan Provincial Hospital of Traditional Chinese Medicine, and Xiyuan Hospital of the China Academy of Chinese Medical Sciences.

All participants provided written informed consent before randomization.

The more detailed research process of this trail can be retrieved from PubMed (Xu et al., 2023) (Supplementary Data Sheet S1).

## Selection criteria

### Inclusion criteria

The predefined inclusion criteria were as follows:

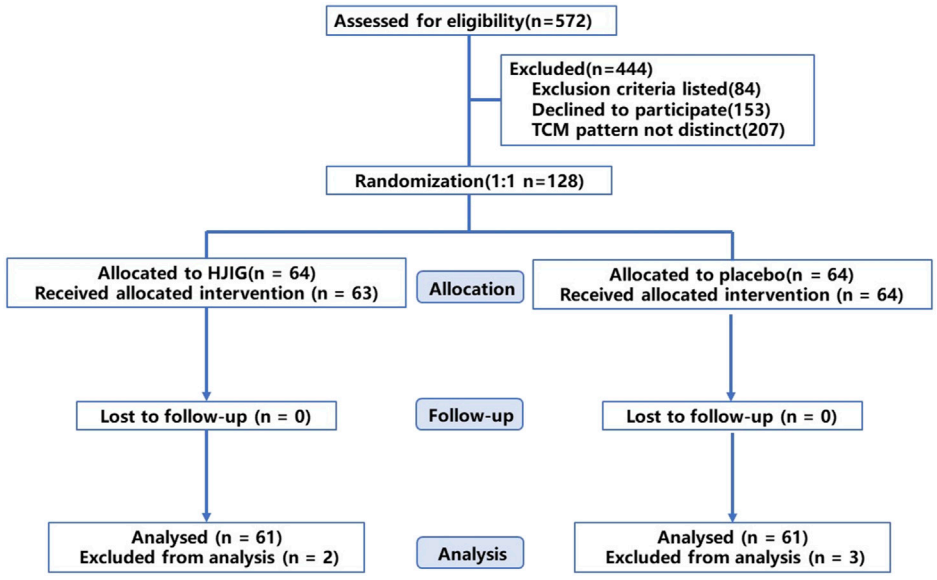



FIGURE 1
Patient flow chart.

(1) Diagnosis of erectile dysfunction based on traditional Chinese medicine standards: Erectile dysfunction is defined as an adult male's inability to achieve or maintain an erection suitable for intercourse, or the rapid loss of an erection after achieving one. This condition must persist for a duration exceeding 3 months, as referenced by the T/CACM 2015-BZ076 standard from the China Association of Chinese Medicine.
(2) Diagnosed with "Qi deficiency" and "Blood stasis" in accordance with traditional Chinese medicine criteria, as outlined in the "Clinical Terminology of Traditional Chinese Medicine" publication by the National Technical Supervision Bureau, National Standard GB/16751.2-1997.
(3) Age ranging between 22 and 65 years.
(4) Demonstrated mild to moderate erectile dysfunction with an IIEF-5 score of >7 but ≤21.
(5) Engaged in a consistent heterosexual relationship for a minimum of 3 months.
(6) Willingness to attempt sexual intercourse a minimum of four times within each 4-week span throughout the study.
(7) At the preliminary assessment, made at least four sexual intercourse attempts, and recorded an IIEF-EF score between 11 and 25 during the second visit (a score of 17–25 indicates mild dysfunction, 11-16 points to moderate dysfunction, and ≤10 signifies severe dysfunction).
(8) Voluntary participation in the study with provided informed consent.

## Exclusion criteria

Exclusion criteria included:

(1) Diabetes that's uncontrolled with fasting glucose >120% of the normal upper limit.
(2) Erectile dysfunction resulting from spinal/neural injuries or surgeries related to prostate cancer.
(3) Penile deformities or risk of priapism (e.g., from sickle cell anemia).
(4) Presence of penile implants.
(5) Severe psychological disorders and impulse control problems.
(6) Untreated significant endocrine disorders, such as hypogonadism.
(7) Receipt of testosterone therapy within the last 3 months.
(8) History of significant cardiovascular events/issues within the past 6 months.
(9) Levels of AST, ALT, or creatinine that are more than twice the normal upper limit.
(10) History of bleeding disorders or active ulcers.
(11) Extreme blood pressure measurements (<90/50 or >170/100 mmHg).
(12) History of alcohol or drug abuse in the past 6 months, exceeding >14 units/week.
(13) An inability to maintain required study records.
(14) Presence of severe comorbid diseases.

## Drugs

Investigational Product: "Hongjing I Granules", manufactured by Zhejiang Jolly Pharmaceutical Co., Ltd. It has been prescribed for the treatment of male erectile dysfunction by the Department of Urology at the Second Affiliated Hospital of Zhejiang Chinese Medical University. The details of the herbal formula are elaborated in Supplementary Data Sheet S3. The preparation process of HJIG is as follows: (1) Extraction: Qualified medicinal materials are pre-processed, and the prepared herbs and slices are extracted according to the specified process to obtain an extract; (2) Concentration: The extract filtrate is vacuum-concentrated to the required specific gravity to produce a clarified paste; (3) Spray Drying: The clear paste is sieved and spray-dried, with the powder being promptly collected to yield an intermediate product; (4) Sieving and

TABLE 1 Baseline characteristics of participants.

| | Total | HJIG | placebo | *P* |
|---|---|---|---|---|
| n | 122 | 61 | 61 | |
| Age | 32.00 [26.00, 38.00] | 32.00 [27.00, 40.00] | 32.00 [26.00, 36.00] | 0.159 |
| Height | 173.00 [170.00, 176.00] | 173.00 [170.00, 175.00] | 173.00 [170.00, 177.00] | 0.994 |
| Weight | 72.00 [68.00, 77.50] | 73.00 [68.00, 75.00] | 71.00 [68.00, 78.00] | 0.873 |
| History of ED | 12.00 [6.00, 33.00] | 12.00 [6.00, 36.00] | 12.00 [6.00, 24.00] | 0.485 |
| IIEF-EF-Total | 16.00 [13.00, 18.00] | 14.00 [12.00, 18.00] | 16.00 [13.00, 18.00] | 0.027 |
| IIEF-EF-q1 | 3.00 [2.00, 3.00] | 3.00 [2.00, 3.00] | 3.00 [2.00, 3.00] | 0.456 |
| IIEF-EF-q2 | 3.00 [2.00, 3.00] | 3.00 [2.00, 3.00] | 3.00 [2.00, 3.00] | 0.060 |
| IIEF-EF-q3 | 3.00 [2.00, 3.00] | 3.00 [2.00, 3.00] | 3.00 [2.00, 3.00] | 0.093 |
| IIEF-EF-q4 | 3.00 [2.00, 3.00] | 2.00 [2.00, 3.00] | 3.00 [2.00, 3.00] | 0.159 |
| IIEF-EF-q5 | 3.00 [2.00, 3.00] | 3.00 [2.00, 3.00] | 3.00 [2.00, 3.00] | 0.336 |
| IIEF-EF-q6 | 2.00 [2.00, 3.00] | 2.00 [2.00, 2.00] | 2.00 [2.00, 3.00] | 0.001 |
| Primary Symptoms of Qi Deficiency | 12.00 [8.00, 14.00] | 12.00 [10.00, 16.00] | 12.00 [8.00, 14.00] | 0.057 |
| Secondary Symptoms of Qi Deficiency | 4.00 [2.25, 5.00] | 4.00 [2.00, 5.00] | 4.00 [3.00, 5.00] | 0.600 |
| Primary Symptoms of Blood Stasis | 18.00 [12.00, 20.00] | 18.00 [12.00, 20.00] | 14.00 [12.00, 20.00] | 0.848 |
| Secondary Symptoms of Blood Stasis | 4.00 [2.25, 5.00] | 4.00 [3.00, 6.00] | 4.00 [2.00, 5.00] | 0.603 |
| SEP1 | 1.00 [0.75, 1.00] | 1.00 [0.75, 1.00] | 1.00 [0.75, 1.00] | 0.110 |
| SEP2 | 0.55 [0.40, 0.75] | 0.50 [0.29, 0.75] | 0.75 [0.50, 1.00] | 0.106 |
| SEP3 | 0.25 [0.00, 0.50] | 0.25 [0.00, 0.40] | 0.25 [0.00, 0.50] | 0.953 |
| SEP4 | 0.00 [0.00, 0.16] | 0.00 [0.00, 0.12] | 0.00 [0.00, 0.25] | 0.527 |
| SEP5 | 0.00 [0.00, 0.25] | 0.00 [0.00, 0.25] | 0.00 [0.00, 0.25] | 0.689 |
| Severity Distribution (%) | | | | 0.268 |
| Mild<br>Moderate | 49 (40.2%)<br>73 (59.8%) | 21 (34.4%)<br>40 (65.6%) | 28 (45.9%)<br>33 (54.1%) | |
| History of diabetes (%) | 3 (2.5%) | 0 (0%) | 3 (5%) | |
| Hyperlipidemia (%) | 2 (1.64%) | 2 (3.2%) | 0 (0%) | |

Age: Patient's age (years), median [interquartile range]; Height: Patient's height (cm), median [interquartile range]; Weight: Patient's weight (kg), median [interquartile range]; Duration of Erectile Dysfunction History: Length of erectile dysfunction history (years), median [interquartile range]; IIEF-EF-Total: International Index of Erectile Function - Erectile Function Domain total score, median [interquartile range], IIEF-EF-q1~6: IIEF-EF, item 1~6 score, median [interquartile range]; Primary Symptoms of Qi Deficiency: Main symptoms of Qi deficiency, median [interquartile range]; Secondary Symptoms of Qi Deficiency: Secondary symptoms of Qi deficiency, median [interquartile range]; Primary Symptoms of Blood Stasis: Main symptoms of blood stasis, median [interquartile range], Secondary Symptoms of Blood Stasis: Secondary symptoms of blood stasis, median [interquartile range]; SEP1~5: Percentage of "yes" responses to SEP, question 1~5 after sexual encounters, median [interquartile range]; Severity Distribution (%): Percentage of patients with mild/moderate ED, Number of patients (percentage).

Mixing: The intermediate product is sieved and mixed for 30 min to ensure uniformity; (5) Granulation: The mixture is dried and granulated into pellets. Packaging is conducted using blank aluminum foil bags with quality control reports and batch numbers of each herbal granule provided in Supplementary Data Sheet S3.

Placebo: A corresponding placebo of the herbal granules, also produced by Zhejiang Jolly Pharmaceutical Co., Ltd. It has been designed to mimic the appearance, color, and aroma of the "Hongjing I Granules" as shown in Supplementary Data Sheet S3.

We hereby confirm that the collection and processing of plant materials for this study fully comply with the Nagoya Protocol, CITES, all associated treaties including phytosanitary regulations, as well as the laws and regulations of China and the requirements of the Chinese Pharmacopoeia.

## Randomization and intervention

Patients with erectile dysfunction seeking traditional Chinese medicine treatment at five centers were invited to participate in this study. Those who expressed interest underwent comprehensive medical and sexual history evaluations, physical examinations, and the required laboratory tests. Four weeks post-evaluation, qualifying patients were stratified and block-randomized. Initially, stratification was based on centers, each hosting 20 participants subdivided into a control and trial

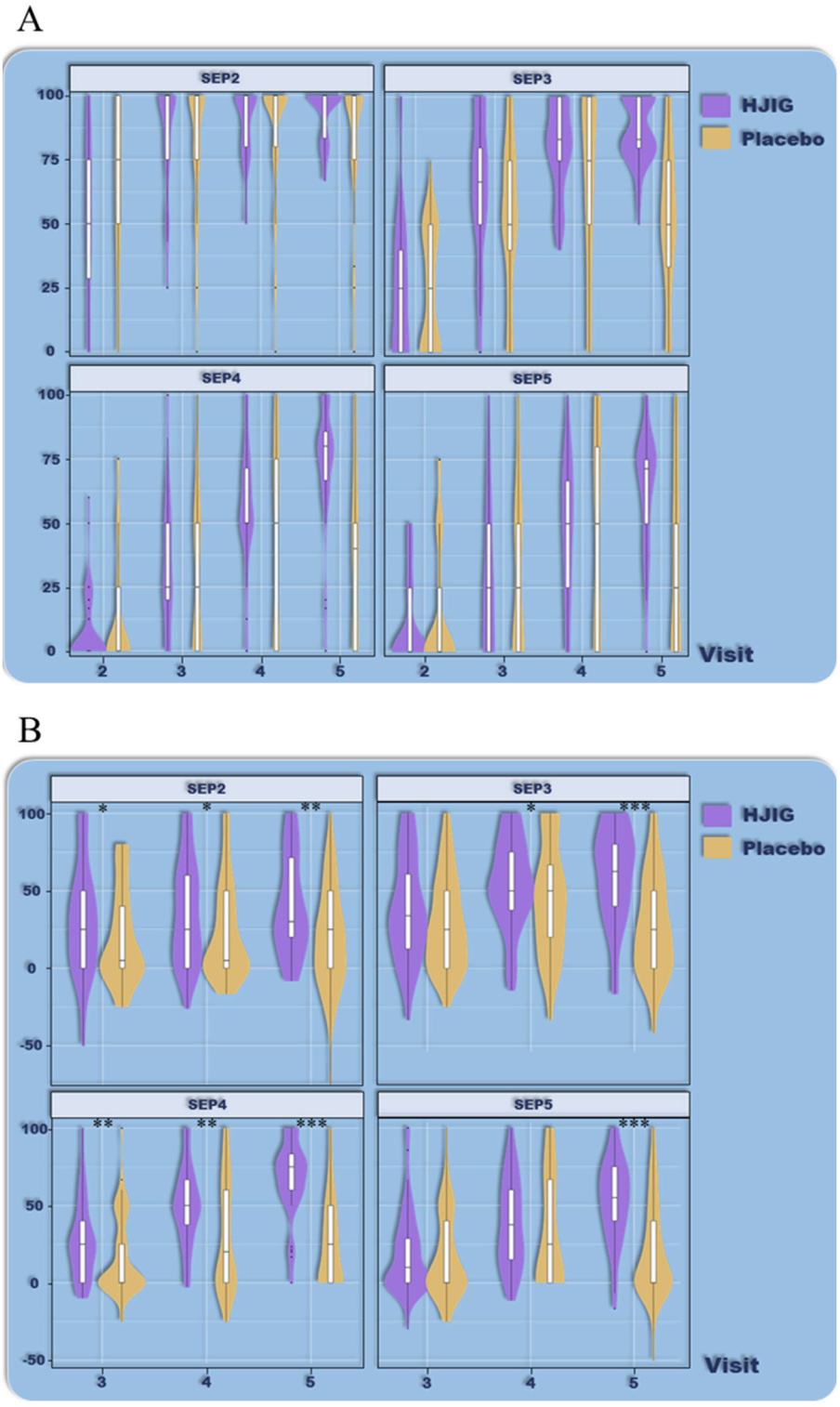



FIGURE 2
**(A)** Percentage of "Yes" responses to SEP questions 2–4 at each Visit point. **(B)** Difference in percentage of "Yes" responses to SEP questions 2–4 between visits 3–5 and visit 2.

group. Subsequent block randomization took into account appointment timing; every four consecutive patients were grouped.

The specific steps included: (1) determining block size and potential permutations for both groups, (2) allocating sample numbers to each permutation, and (3) randomly organizing block assignment numbers through a computer-generated random number table. These steps were followed to ensure that each participant had an equal chance of being assigned to either the treatment group or the placebo group. Upon inclusion confirmation and assignment of participant numbers, medication numbers based on statistical unit-provided random digits

TABLE 2 Changes in scores when comparing visits 3, 4, and 5 to visit 2.

| | HJIG | placebo | *P* |
|---|---|---|---|
| n | 61 | 61 | |
| IIEF-EF (v3-v2) | 3.00 [2.00, 6.00] | 2.00 [0.00, 3.00] | <0.001 |
| Severity Distribution (%) | | | 0.266 |
| Normal | 1 (1.6%) | 0 (0.0%) | |
| Mild | 50 (82.0%) | 45 (73.8%) | |
| Moderate | 10 (16.4%) | 16 (26.2%) | |
| SEP1 (v3-v2) | 0.00 [0.00, 0.25] | 0.00 [0.00, 0.25] | 0.132 |
| SEP2 (v3-v2) | 0.25 [0.00, 0.50] | 0.00 [0.00, 0.50] | 0.022 |
| SEP3 (v3-v2) | 0.35 [0.14, 0.75] | 0.25 [0.00, 0.50] | 0.062 |
| SEP4 (v3-v2) | 0.25 [0.00, 0.40] | 0.00 [0.00, 0.25] | 0.006 |
| SEP5 (v3-v2) | 0.10 [0.00, 0.33] | 0.00 [0.00, 0.40] | 0.694 |
| IIEF-EF (v4-v2) | 7.80 (±3.25) | 3.33 (±3.90) | <0.001 |
| Severity Distribution (%) | | | 0.001 |
| Normal | 7 (11.5%) | 1 (1.6%) | |
| Mild | 52 (85.2%) | 46 (75.4%) | |
| Moderate | 2 (3.3%) | 14 (23.0%) | |
| SEP1 (v4-v2) | 0.00 [0.00, 0.25] | 0.00 [0.00, 0.25] | 0.128 |
| SEP2 (v4-v2) | 0.25 [0.08, 0.58] | 0.05 [0.00, 0.50] | 0.023 |
| SEP3 (v4-v2) | 0.50 [0.36, 0.75] | 0.50 [0.20, 0.67] | 0.050 |
| SEP4 (v4-v2) | 0.50 [0.35, 0.67] | 0.20 [0.00, 0.62] | 0.004 |
| SEP5 (v4-v2) | 0.29 [0.14, 0.60] | 0.25 [0.00, 0.67] | 0.232 |
| IIEF-EF (v5-v2) | 9.00 [6.00, 13.00] | 4.00 [0.00, 6.00] | <0.001 |
| Severity Distribution (%) | | | 0.001 |
| Normal | 24 (39.3%) | 8 (13.1%) | |
| Mild | 36 (59.0%) | 41 (67.2%) | |
| Moderate | 1 (1.6%) | 9 (14.8%) | |
| Severe | 0 (0.0%) | 3 (4.9%) | |
| SEP1 (v5-v2) | 0.00 [0.00, 0.25] | 0.00 [0.00, 0.25] | 0.153 |
| SEP2 (v5-v2) | 0.30 [0.20, 0.71] | 0.25 [0.00, 0.50] | 0.003 |
| SEP3 (v5-v2) | 0.63 [0.40, 0.80] | 0.25 [0.00, 0.50] | <0.001 |
| SEP4 (v5-v2) | 0.75 [0.60, 0.83] | 0.25 [0.00, 0.50] | <0.001 |
| SEP5 (v5-v2) | 0.55 [0.40, 0.75] | 0.00 [0.00, 0.40] | <0.001 |

IIEF-EF (v3~5-v2): The difference in the total score of the 6 questions of the IIEF-EF, between each visit point (V3~V5) and visit 2, median [interquartile range](Data not normally distributed), Mean (standard deviation) (Data normally distributed); SEP1~5 (v3~5-v2): The difference in the percentage of "yes" responses to each SEP, question after sexual encounters between each visit point (V3~V5) and visit 2, median [interquartile range]; Severity Distribution (%): Percentage of patients with normal/mild/moderate/severe ED, Number of patients (percentage).

were designated. Throughout the trial, participants consistently used medication with identical numbers. Any medication with a specific number, if not wholly utilized, was not given to other participants.

Granules (either active drug or placebo, depending on the group assignment) provided by Zhejiang Jolly Pharmaceutical Co., Ltd., were dissolved in 200 mL hot water and taken twice daily. Both the primary medication, HJIG, and its placebo were indistinguishable in appearance, smell, and taste. They were packaged in identical bags and boxes. Consequently, physicians, data collectors, and patients remained blind to group allocations throughout the study. Treatment compliance was monitored through patient self-reports, regular follow-ups, and medication return checks, with detailed records of medication usage, treatment responses, and any adverse events to ensure data integrity and accuracy.

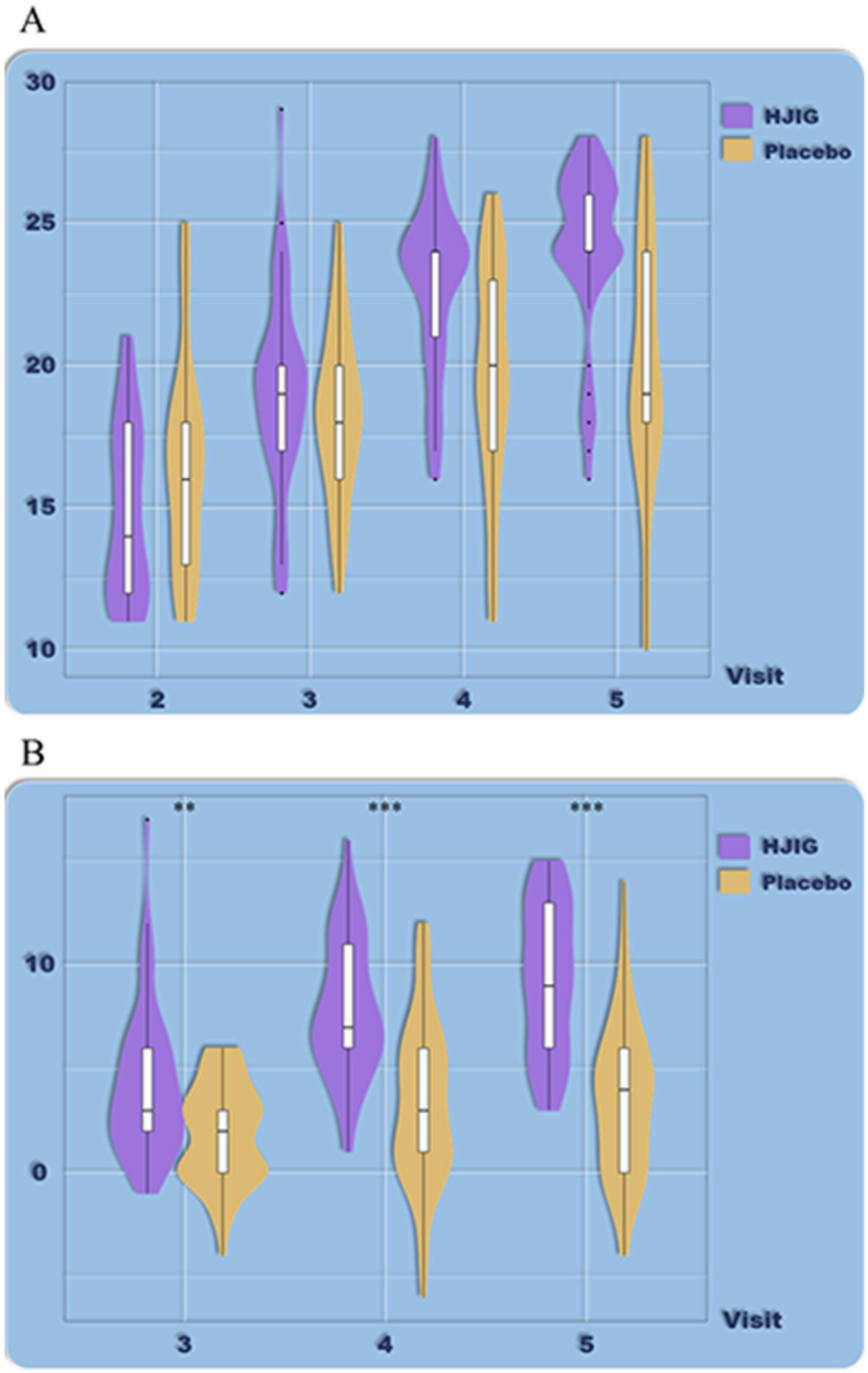



FIGURE 3
**(A)** Total IIEF-EF scores at each visit. **(B)** Differences in IIEF-EF scores between visits 3–5 and visit 2.

## Outcomes

All participants underwent an 8-week treatment, followed by a 12-week follow-up. Five visits were scheduled: at Week −4 (run-in), Week 0 (baseline), Week 4 (mid-treatment), Week 8 (end of treatment), and Week 12 (end of follow-up).

The primary outcome was designed to evaluate the improvements induced by HJIG in two composite endpoints. These composite endpoints reflected changes between Visit 2 and Visit 4 in:

(1) The percentage change in successful erections leading to ejaculation throughout all sexual encounters (Sexual Encounter Profile question 3, SEP3).
(2) The variation in IIEF-EF (International Index of Erectile Function - Erectile Function) specific scores. Differences

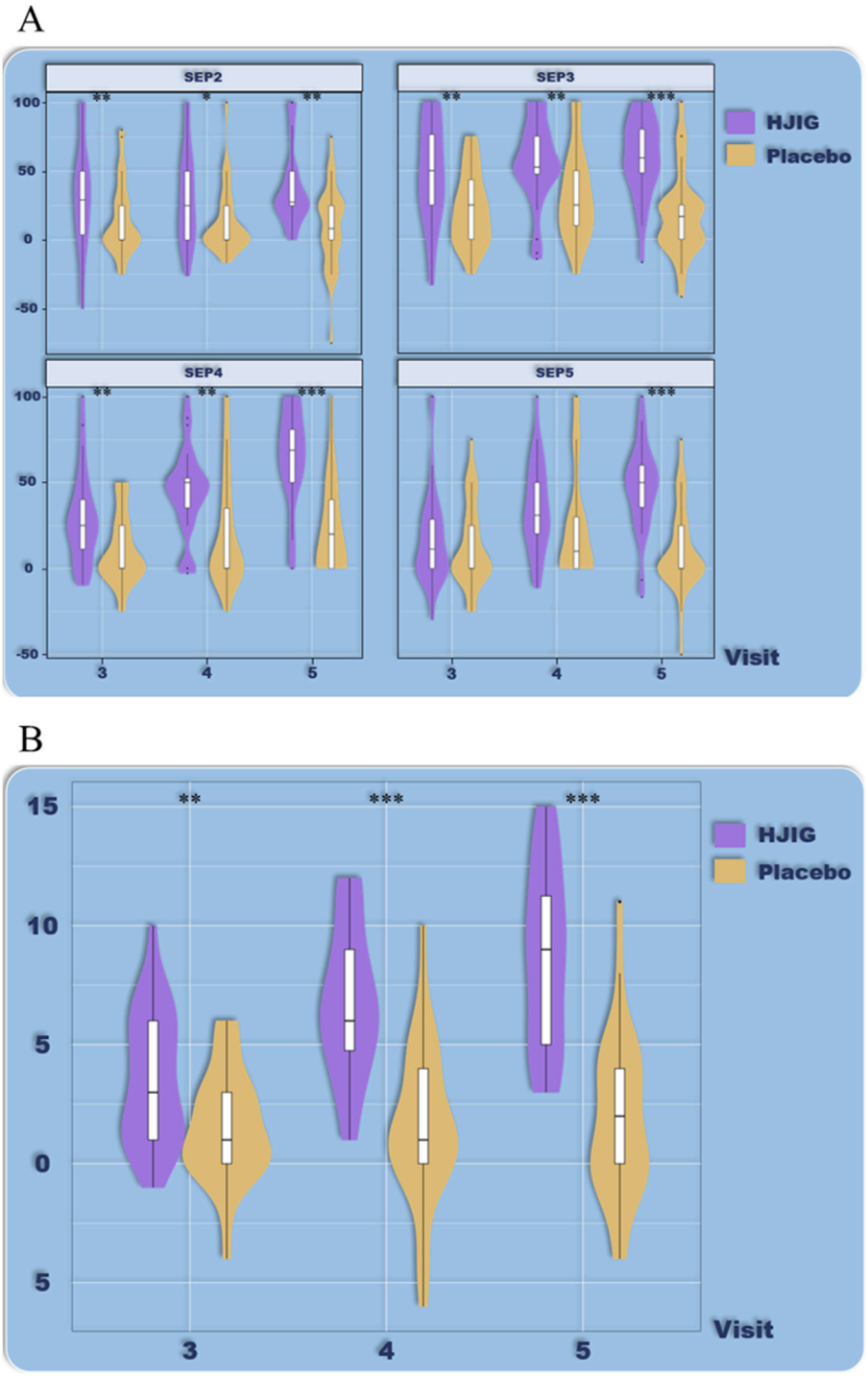



FIGURE 4
Age-stratified subgroup analysis (age >32), **(A)** Percentage of "Yes" responses to SEP questions 2–4 at each visit point, **(B)** Differences in IIEF-EF scores between visits 3–5 and visit 2.

between the HJIG group and the placebo group were assessed separately, and the primary outcome was considered achieved if both endpoint measures in the HJIG group were superior to those in the placebo group.

Secondary outcomes included:

(1) Stratified subgroup analysis of IIEF-EF and SEP results at Visit 4 based on participants' baseline characteristics, such as age (>32 years old, ≤32 years old) and duration of ED (<12 months, ≥12 months but <36 months, and ≥36 months).

(2) Percentage change in successful erections that led to vaginal penetration at Visits 3 and 4 compared to Visit 2 (SEP2).

(3) Percentage variation in SEP3 and IIEF-EF scores from Visit 3 to Visit 2.

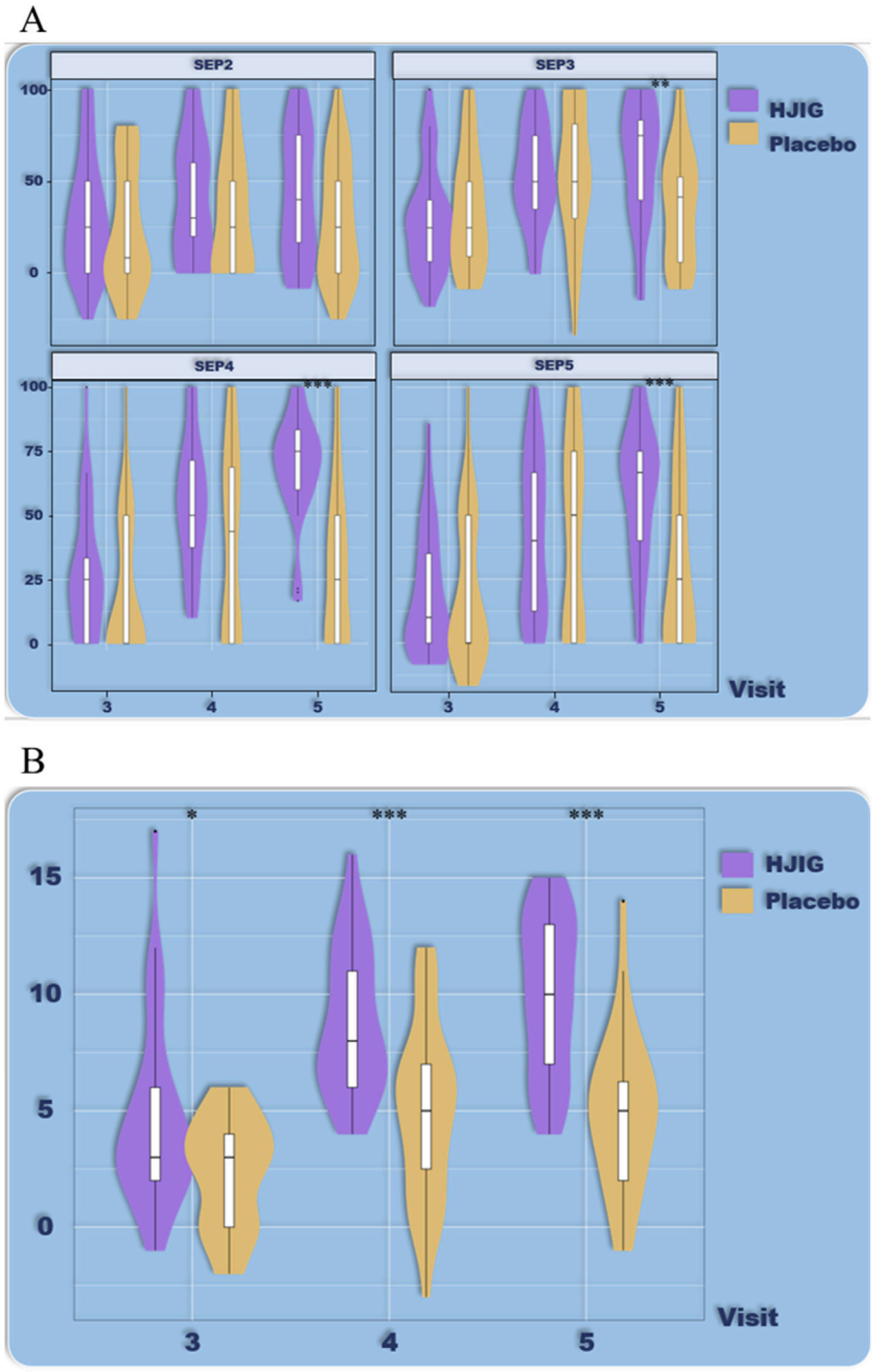



FIGURE 5
Age-stratified subgroup analysis (age ≤32), **(A)** Percentage of "Yes" responses to SEP questions 2–4 at each visit point, **(B)** Differences in IIEF-EF scores between visits 3–5 and visit 2.

(4) Shift in Traditional Chinese Medicine symptom scores between Visit 3 or Visit 4 and Visit 2.
(5) Alteration in scores reflecting sexual desire (frequency of sexual activities) and overall satisfaction with sexual life between Visit 3 or Visit 4 and Visit 2.
(6) The proportion of participants with normalized IIEF-EF scores at Visit 4 relative to Visit 2.

The safety of HJIG was gauged by noting adverse events (AEs) and analyzing clinical laboratory results.

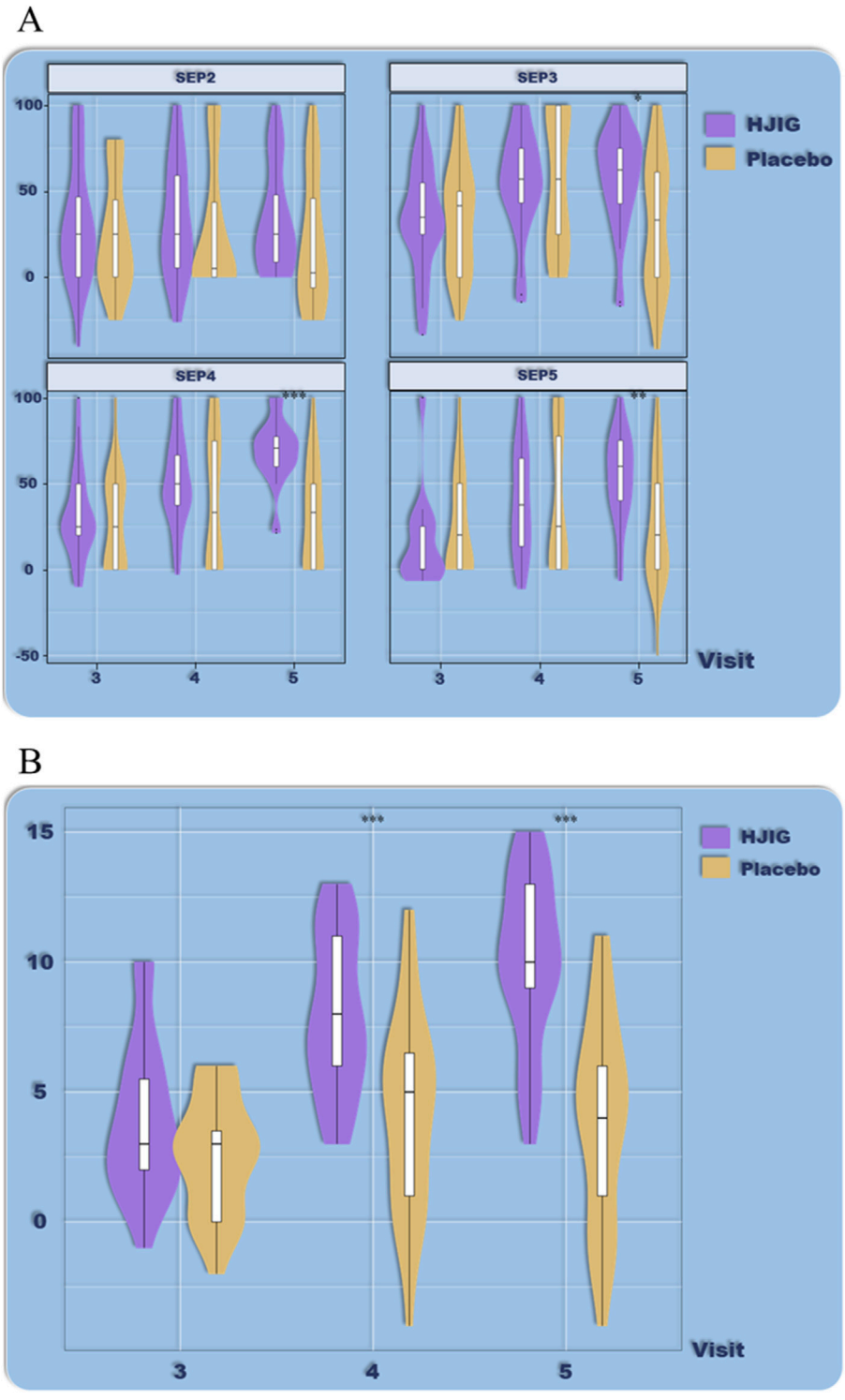



FIGURE 6
ED Duration-stratified subgroup analysis (≤12 months), **(A)** Percentage of "Yes" responses to SEP questions 2–4 at each visit point, **(B)** Differences in IIEF-EF scores between visits 3–5 and visit 2.

## Sample size calculation

This trail represents the first randomized controlled trial (RCT) to explore the efficacy of the traditional Chinese medicine formula, Hongjing I granule (HJIG), as a sole therapeutic agent for erectile dysfunction (ED). In 2017, we conducted a pilot trial involving patients seeking traditional Chinese medicine remedies for mild to moderate ED at the urology outpatient clinic of Zhejiang Province Xinhua Hospital. Upon diagnosis of Qi deficiency and Blood stasis by the primary physician, a secondary specialist in Chinese medicine

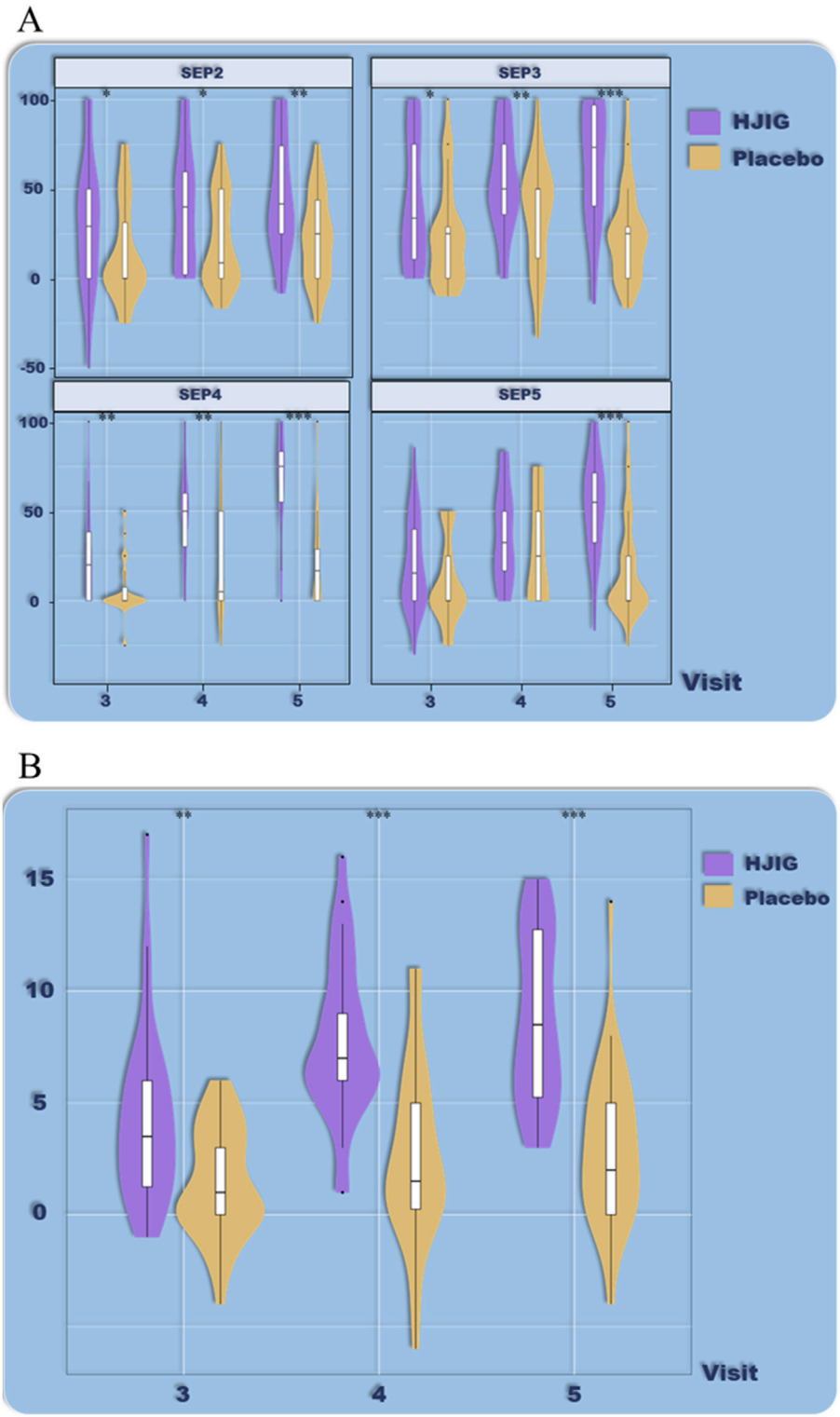



FIGURE 7
ED Duration-stratified subgroup analysis (>12 months), **(A)** Percentage of "Yes" responses to SEP questions 2–4 at each visit point, **(B)** Differences in IIEF-EF scores between visits 3–5 and visit 2.

was consulted to corroborate the diagnosis. If confirmed, the patient's case number was documented, and an assessment using the International Index of Erectile Function - Erectile Function (IIEF-EF) was executed. Patients who then consumed Hongjing I granule for over 8 weeks underwent a follow-up IIEF-EF scoring. Those achieving a Minimal Clinically Important Difference of 5 points (Rosen et al., 2011) were deemed responsive. The effectiveness rate, reported by three physicians, ranged between

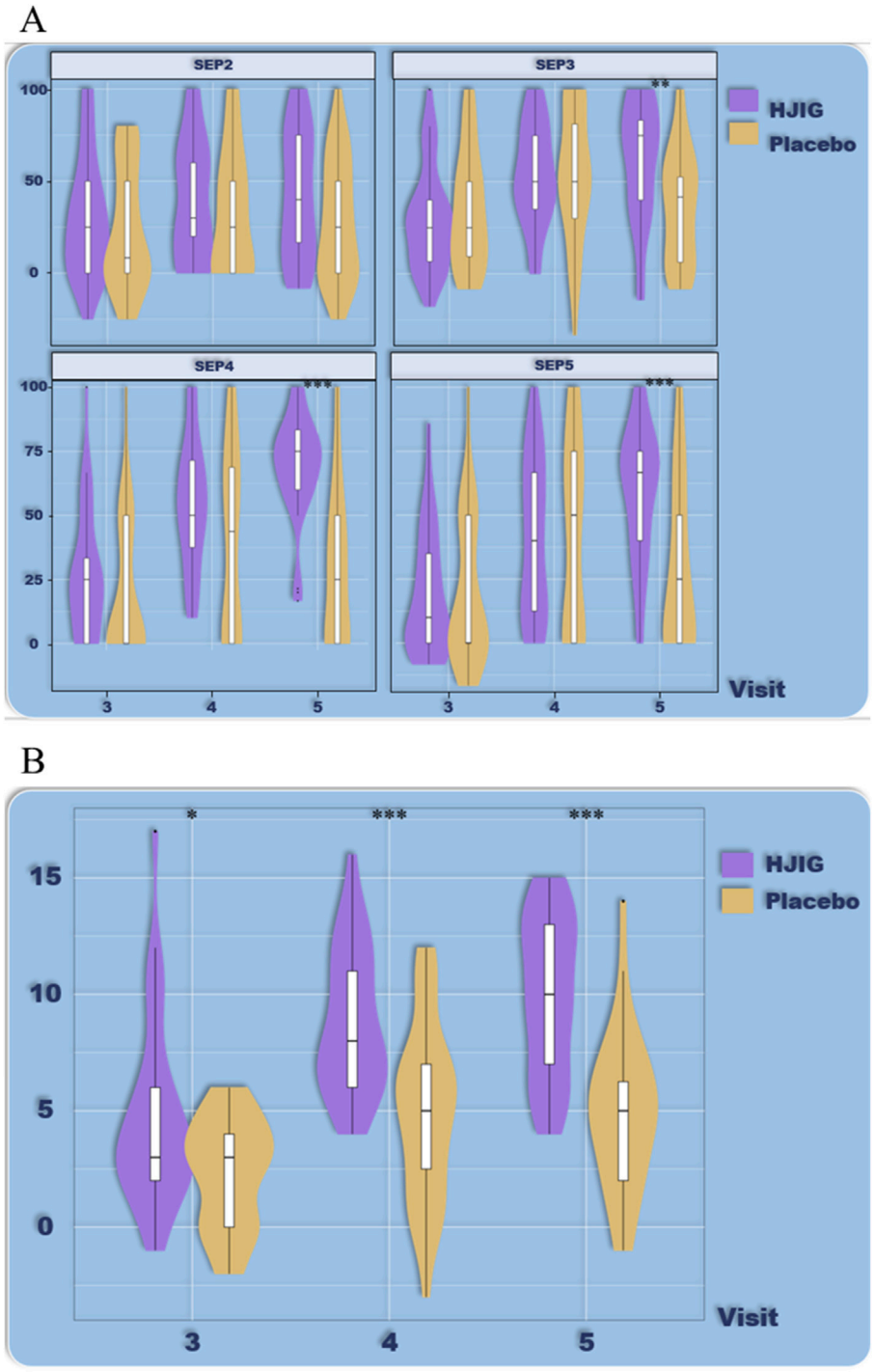



FIGURE 8
ED Duration-stratified subgroup analysis (≥36 months), **(A)** Percentage of "Yes" responses to SEP questions 2–4 at each Visit point, **(B)** Differences in IIEF-EF scores between visits 3–5 and visit 2.

60% and 70% for their initial visit patients. This is congruent with the efficacy rate deduced by our andrology experts based on combined treatment clinical research findings and empirical observations. Thus, we postulated a 65% efficacy rate for the HJIG monotherapy. The core hypothesis for this investigation is the superiority of the HJIG cohort over the placebo cohort. The metrics for assessing this superiority encompass: 1. Changes in the Sexual Encounter Profile Question 3 (SEP3) and 2. Variations in specific scores of IIEF-EF. All superiority assessments are statistical, devoid of a defined superiority benchmark. As per pertinent literature, the test level is pegged at 0.05, with a test assurance degree of 0.9. The calculation for superiority, applicable for a 1:1 allocation between the test and control cohorts, is outlined below:

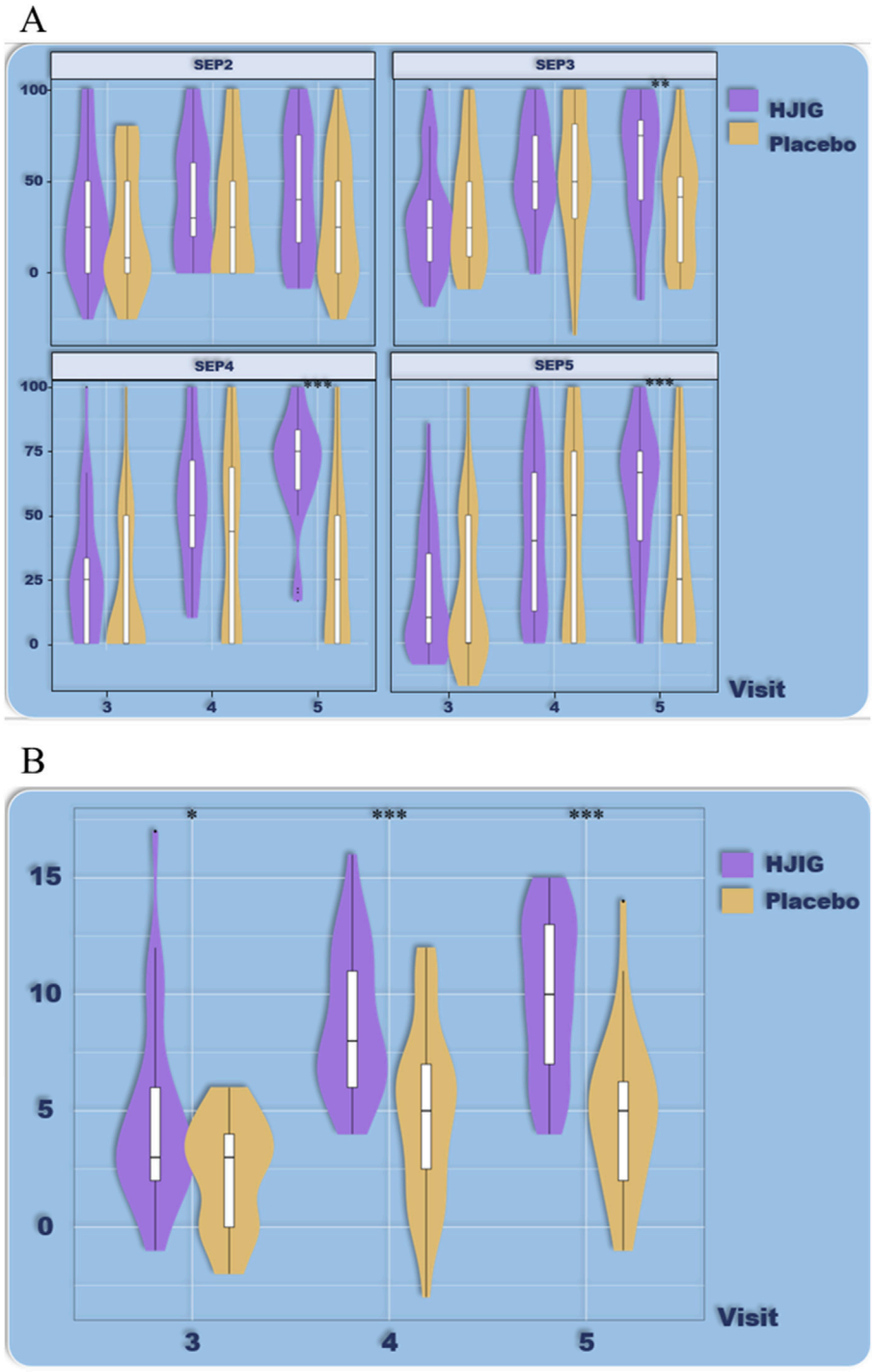



FIGURE 9
ED Duration-stratified subgroup analysis (<36 months), **(A)** Percentage of "Yes" responses to SEP questions 2–4 at each visit point, **(B)** Differences in IIEF-EF scores between visits 3–5 and visit 2.

$$n = \frac{\left(Z_\alpha + Z_\beta\right)^2 \left[P_C\left(1 - P_C\right) + P_T\left(1 - P_T\right)\right]}{\left(P_T - P_C\right)^2}$$

In this equation, PC denotes the efficacy rate of the control cohort, and PT represents the efficacy rate of the test cohort. α signifies a Type I error, conventionally set at α = 0.05, yielding $Z_\alpha$ = 1.645; β stands for a Type II error, typically marked at β = 0.10, leading to $Z_\beta$ = 1.282. Drawing from available research datasets, the clinical efficacy rate of a placebo in ED studies stands at PC = 0.33 (Feys et al., 2014). Based on our projected efficacy rate of HJIG at PT = 0.65, and considering α = 0.05 and β = 0.10, the relevant data was integrated into the superiority equation, producing an n ≈ 40. Factoring in a 20% attrition rate, an estimated 50 subjects per group are required, summing to a

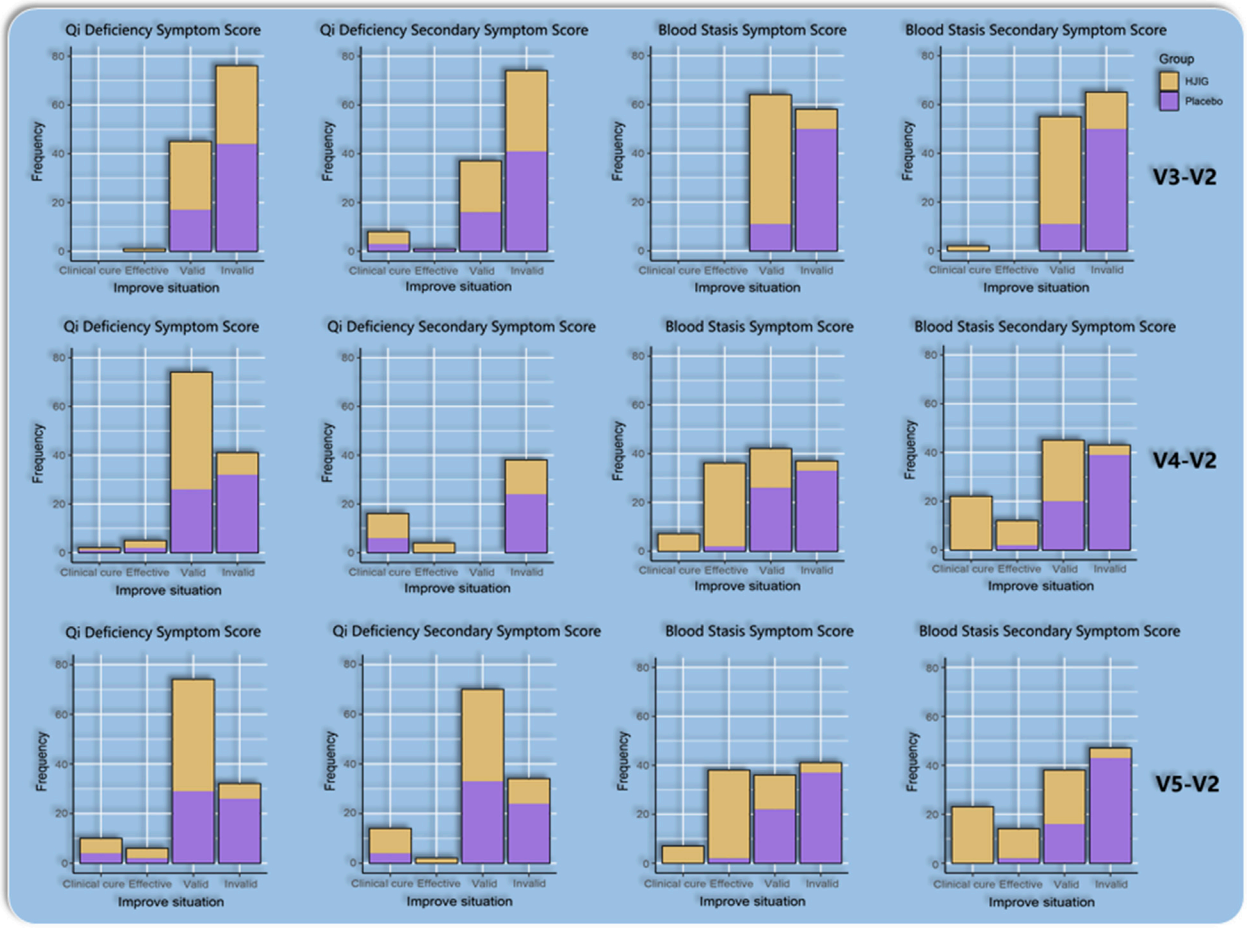



FIGURE 10
Improvement in traditional Chinese medicine symptom patterns.

minimum of 100 subjects altogether. Given potential variations between the pilot trial and actual therapeutic outcomes, we petitioned the ethics committee for a potential enlargement of the sample size during the study duration (2021.1–2022.12). This was contingent on available funds to counteract dropouts and study discontinuations. We preemptively arranged for 200 trial drug allocations (100 for placebo and 100 for HJIG) to offset potential attrition. In the end, a total of 122 patients were enrolled.

## Statistical analysis

For continuous variables, if the data do not follow a normal distribution, they will be presented using the median [interquartile range]; if they are normally distributed, they will be expressed as the mean (standard deviation). Categorical variables will be presented as the number of cases (percentage). Before comparing differences in various indicators between the experimental and control groups, the Shapiro-Wilk test will be applied to check the normality of continuous variables. If the variables are normally distributed, a t-test will be applied; otherwise, the Wilcoxon test will be used. For categorical variable comparisons, the Chi-square test will be employed. All analyses will be conducted using R software (version 4.2.3), and a $P$-value <0.05 will be considered statistically significant (Zhang et al., 2023).

# Results

## Patient characteristics

Between January 2021 and December 2022, we screened a total of 572 potential participants. Of these, 128 were randomly assigned to either the HJIG or placebo groups, with 64 participants in each group. Six participants withdrew from the study: one due to dissatisfaction with the results, and five due to self-administration of PDE5i prior to engaging in sexual activity. Among those who completed the study, 100% (122/122) adhered to at least 80% of the prescribed dosage, as determined by calculating the returned medication. The participant flow is illustrated in Figure 1. Baseline characteristics were well-balanced between the two groups ($P > 0.05$), as detailed in Table 1.

TABLE 3 Changes in traditional Chinese medicine symptom scores at visits 3, 4, and 5 compared to visit 2 Qi Deficiency Symptom Score (v3~5-v2).

| | HJIG | placebo | *P* |
|---|---|---|---|
| n | 61 | 61 | |
| Qi Deficiency Symptom Score (v3-v2) | −4.00 [−4.00, −2.00] | −2.00 [−4.00, 0.00] | <0.001 |
| Qi Deficiency Secondary Symptom Score (v3-v2) | −1.00 [−2.00, 0.00] | −1.00 [−1.00, 0.00] | 0.661 |
| Blood Stasis Symptom Score (v3-v2) | −8.00 [−10.00, −6.00] | −2.00 [-4.00, −1.00] | <0.001 |
| Blood Stasis Secondary Symptom Score (v3-v2) | −2.00 [−2.00, −1.00] | 0.00 [−1.00, 0.00] | <0.001 |
| Qi Deficiency Symptom Score (v4-v2) | −6.00 [−8.00, −4.00] | −4.00 [−6.00, 0.00] | <0.001 |
| Qi Deficiency Secondary Symptom (v4-v2) | −2.00 [−3.00, −1.00] | −1.00 [−2.00, −1.00] | 0.034 |
| Blood Stasis Symptom Score (v4-v2) | −14.00 [−18.00, −10.00] | −4.00 [−8.00, −2.00] | <0.001 |
| Blood Stasis Secondary Symptom Score (v4-v2) | −3.00 [−4.00, −2.00] | −1.00 [−2.00, 0.00] | <0.001 |
| Qi Deficiency Symptom Score (v5-v2) | −6.00 [−8.00, −6.00] | −4.00 [−6.00, −2.00] | <0.001 |
| Qi Deficiency Secondary Symptom Score (v5-v2) | −2.00 [−3.00, −1.00] | −1.00 [−2.00, 0.00] | 0.018 |
| Blood Stasis Symptom Score (v5-v2) | −14.00 [−18.00, −10.00] | −4.00 [−6.00, −2.00] | <0.001 |
| Blood Stasis Secondary Symptom Score (v5-v2) | −3.00 [−4.00, −2.00] | −1.00 [−2.00, 0.00] | <0.001 |

The difference in Qi deficiency primary symptom scores between visit 3–5 and visit 2, median [interquartile range]; Qi Deficiency Secondary Symptom Score (v3~5-v2): The difference in Qi deficiency secondary symptom scores between visit 3 and visit 2, median [interquartile range]; Blood Stasis Symptom Score (v3~5-v2): The difference in Blood stasis primary symptom scores between visit 3–5 and visit 2, median [interquartile range]; Blood Stasis Secondary Symptom Score (v3~5-v2): The difference in Blood stasis secondary symptom scores between visit 3 and visit 2, median [interquartile range].

## Primary outcome assessment

At Visit 4, the percentage change in SEP3 for both patient groups was evaluated relative to Visit 2 (Figure 2). For the HJIG group, the median change was 0.50 [IQR: 0.36, 0.75], whereas for the placebo group, it was 0.50 [0.20, 0.67]. The statistical difference between the two groups is significant ($P = 0.05$) (Karppinen et al., 2001; Romano, 2002), HJIG treatment led to an increase in the proportion of successful ejaculations during sexual encounters compared to the placebo group; further details are provided in Table 2. The IIEF-EF scores for both groups demonstrated an increasing trend with prolonged treatment. Notably, the HJIG group showed a more pronounced increase and higher scores than the placebo group subsequent to Visit 2 (Figure 3). Eight weeks post-randomization, the mean change in the IIEF-EF score for the HJIG group was 7.80 (±3.25) compared to 3.33 (±3.90) for the placebo group. The difference between the two groups was statistically significant ($P < 0.001$), HJIG treatment resulted in a significant improvement in erectile function, which was superior to the placebo group.

Refer to Table 2 for a detailed depiction.

## Secondary outcome assessment

### IIEF-EF and SEP results in subgroup analysis

#### Age-stratified subgroup analysis (>32 years old, ≤32 years old)

Based on the IIEF-EF scores, there was no significant difference in efficacy between patients older than 32 (Figure 4) and those younger than 32 (Figure 5). The improvements in IIEF-EF scores for patients treated with HJIG from visits 3–5 were consistently higher than those in the placebo group, suggesting that HJIG treatment effectively enhances erectile function. Additionally, a higher proportion of patients achieved normalization in the HJIG group. The results of the SEP questions exhibited certain differences after stratifying by age. For patients older than 32 years, the percentage difference in SEP3 "yes" responses per total sexual encounters was significantly higher in the treatment group compared to the control group by visit 3, indicating that HJIG treatment increased the number of times patients were able to maintain an erection until the completion of intercourse. For patients 32 years or younger, the percentage difference in SEP3 responses only showed a significant difference between the HJIG group and the placebo group by visit 5. A similar trend was observed in the other SEP questions.

#### ED Duration-stratified subgroup analysis (≤12 months, >12 to <36 months, ≥36 months)

**Analysis based on a 12-month ED history revealed.** Using 12 months as the cut-off point for disease duration, the results indicate that for patients with a disease history longer than 12 months, HJIG significantly improved overall erectile function. At visit 4, the improvement in IIEF-EF scores in the HJIG group was significantly higher than that in the placebo group and was maintained until visit 5 (Figure 6). Regarding sexual completion as assessed by the SEP3 percentage difference, there was no significant difference between the two groups at visit 4, but a significant difference emerged at visit 5 (Figure 6).

For patients with a disease history of 12 months or less, HJIG also significantly improved overall erectile function. At visit 3, the improvement in IIEF-EF scores in the HJIG group was significantly higher than that in the placebo group and was maintained until visit 5 (Figure 7). In terms of sexual completion based on the

TABLE 4 Improvement in traditional Chinese medicine symptom patterns.

| Visit/index | Group | Recover | Significant | Effective | Ineffective |
|---|---|---|---|---|---|
| V3 Qi Deficiency | HJIG | 0 | 1 | 28 | 32 |
| Symptom | PLACEBO | 0 | 0 | 17 | 44 |
| V4 Qi Deficiency | HJIG | 1 | 3 | 48 | 9 |
| Symptom | PLACEBO | 1 | 2 | 26 | 32 |
| V5 Qi Deficiency | HJIG | 6 | 4 | 45 | 6 |
| Symptom | PLACEBO | 4 | 2 | 29 | 26 |
| V3 Qi Deficiency | HJIG | 5 | 0 | 21 | 34 |
| Secondary Symptom | PLACEBO | 3 | 1 | 16 | 41 |
| V4 Qi Deficiency | HJIG | 10 | 4 | 31 | 15 |
| Secondary Symptom | PLACEBO | 6 | 0 | 31 | 24 |
| V5 Qi Deficiency | HJIG | 10 | 3 | 33 | 14 |
| Secondary Symptom | PLACEBO | 4 | 1 | 28 | 28 |
| V3 Blood Stasis | HJIG | 0 | 0 | 53 | 8 |
| Symptom | PLACEBO | 0 | 0 | 11 | 50 |
| V4 Blood Stasis | HJIG | 7 | 34 | 16 | 4 |
| Symptom | PLACEBO | 0 | 2 | 26 | 33 |
| V5 Blood Stasis | HJIG | 7 | 36 | 14 | 4 |
| Symptom | PLACEBO | 0 | 2 | 22 | 37 |
| V3 Blood Stasis | HJIG | 2 | 0 | 44 | 15 |
| Secondary Symptom | PLACEBO | 0 | 0 | 11 | 50 |
| V4 Blood Stasis | HJIG | 22 | 10 | 25 | 4 |
| Secondary Symptom | PLACEBO | 0 | 2 | 20 | 39 |
| V5 Blood Stasis | HJIG | 23 | 12 | 22 | 4 |
| Secondary Symptom | PLACEBO | 0 | 2 | 16 | 43 |

SEP3 percentage difference, at visit 3, the improvement in the SEP3 percentage in the HJIG group was significantly higher than in the placebo group, and this improvement became more pronounced with the continuation of treatment and observation time (Figure 7). In summary, using 12 months as a threshold for stratifying disease history, HJIG was effective in improving overall erectile function and the ability to maintain an erection until the completion of sexual intercourse in both patients with a disease history longer than 12 months and those with a disease history of 12 months or less. However, for patients with a disease history of 12 months or less, the therapeutic effects manifested earlier than in those with a disease history longer than 12 months.

**Considering a 36-month ED history boundary.** Using 36 months as the cut-off point for disease duration, the results indicate that for patients with a disease history of 36 months or longer, HJIG significantly improved overall erectile function. At visit 4, the improvement in IIEF-EF scores in the HJIG group was significantly higher than that in the placebo group and was maintained until visit 5 (Figure 8). Regarding sexual completion as assessed by the SEP3 percentage difference, there was no significant difference between the two groups at visit 4, but significant differences emerged at visits 3 and 5 (Figure 8).

For patients with a disease history of less than 36 months, HJIG also significantly improved overall erectile function. At visit 3, the improvement in IIEF-EF scores in the HJIG group was significantly higher than that in the placebo group and was maintained until visit 5 (Figure 9). In terms of sexual completion based on the SEP3 percentage difference, the improvement in the SEP3 percentage in the HJIG group was significantly higher than in the placebo group only at visit 5 (Figure 9).

In summary, using 36 months as a threshold for stratifying disease history, HJIG was effective in improving overall erectile function and the ability to maintain an erection until the completion of sexual intercourse in both patients with a disease history of 36 months or longer and those with a disease history of less than 36 months. However, patients with a disease history of 36 months or longer may require a longer treatment period and

TABLE 5 Frequency of sexual activity at different visit time points.

| Visit | Overall | | HJIG | | placebo | |
|---|---|---|---|---|---|---|
| | $M(Q_1,Q_3)$ | *p* | $M(Q_1,Q_3)$ | *p* | $M(Q_1,Q_3)$ | *p* |
| V2 | 4 (4,4) | | 4 (4,5) | | 4 (4,4) | |
| V3 | 4 (4,5) | 0.0022 | 5 (4,6) | 0.0062 | 4 (4,5) | 0.1055 |
| V4 | 4 (4,6) | 0.0002 | 6 (4,7) | 0.0001 | 4 (4,5) | 0.1302 |
| V5 | 4 (4,5) | 0.0287 | 5 (4,6) | 0.0004 | 4 (4,4) | 0.5531 |

recovery time to achieve significant improvements in overall erectile function. For SEP3 percentage evaluation of sexual completion, both disease history stratification results showed a significant difference between the HJIG group and the placebo group at visit 5. However, for patients with an ED history of 36 months or longer, a significant difference between the HJIG group and the placebo group was observed as early as visit 3. At visit 4, this significance diminished and was near the margin of significance. Considering the high dispersion of the SEP percentage difference data and the relatively small number of patients with an ED history of 36 months or longer, as well as the substantial difference in the number of patients between the two groups, the interpretation of this result should be approached with caution.

### Percentage of erections achievable for vaginal insertion (SEP2)

Comparing the percentage of successful erections suitable for vaginal insertion (SEP2) between Visits 3 and 2, the median change in the HJIG group was 0.25 [IQR: 0.00, 0.50], whereas for the placebo group, it was 0.00 [IQR: 0.00, 0.50]. For the comparison between Visits 4 and 2, the median change in the HJIG group was 0.25 [IQR: 0.08, 0.58], while in the placebo group, it was 0.05 [IQR: 0.00, 0.50]. The differences between the groups were statistically significant at both time points ($P < 0.05$), this indicates that HJIG treatment can effectively improve the erectile firmness of patients.

### Percentage of IIEF-EF scores

IIEF-EF: When comparing results from Visit 4 to Visit 2, the HJIG group had an improvement rate of 50.00% [IQR: 33.33, 83.33], while the placebo group reported a rate of 18.75% [IQR: 4.17, 40.00]. The HJIG group's performance was notably superior to the placebo group ($P < 0.001$).

### Traditional Chinese medicine symptom scores

At Visit 4, the effectiveness rate of the HJIG group was 85.2% for the primary symptom of Qi Deficiency and 75.0% for its secondary symptom. For the primary symptom of Blood Stasis, the effectiveness rate was 93.4%, and the rate was the same for its secondary symptom. This marked a significant improvement in the cure rate compared to Visit 3. Meanwhile, in the placebo group, the effectiveness rate for the primary symptom of Qi Deficiency stood at 47.5% and was 60.7% for its secondary symptom. For the primary symptom of Blood Stasis, the effectiveness rate reached 45.9%, while it was 36.1% for its secondary symptom. The difference in change values between the two groups, based on each visit point, was significant for all results, except for the secondary symptom score of Qi Deficiency at Visit 3 ($P < 0.05$ for all other outcomes) (Table 3; Table 4; Figure 10), this indicates that HJIG treatment improved the patients' TCM symptoms.

### Frequency of sexual activity and sexual satisfaction

Sexual Activity Frequency: From visits 2–5, the distribution of sexual activity frequency among the subjects did not follow a normal distribution. Using the Wilcoxon rank-sum test for comparison between visit 2 and visits 3–5, there was a significant increase in the sexual activity frequency in the HJIG group during visits 3–5 compared to visit 2. In contrast, no significant difference was observed in the placebo group across these visits (Table 5; Figure 11).

Sexual Satisfaction: At visit 4, the improvement level in sexual satisfaction for the HJIG group was 0.29 [IQR: 0.14, 0.60], while it was 0.25 [IQR: 0.00, 0.67] for the placebo group, showing no statistical significance between the groups. By visit 5, the median difference in the HJIG group was 0.55 [IQR: 0.40, 0.75] versus 0.00 [IQR: 0.00, 0.40] in the placebo group, highlighting a marked superiority of sexual satisfaction improvement in the HJIG group ($P < 0.001$) (Table 2).

This indicates that HJIG treatment can enhance patients' sexual desire and improve their sexual experience.

### Safety and adverse events

There were no significant differences in renal and liver function between and within the HJIG, and placebo groups after treatments. Most subjects well tolerated the research medication, and no serious adverse events were reported. The accusation report of HJIG can be found in Supplementary Data Sheet S3, and the metabolites of HJIG detected through LC-MS/MS of HJIG can be found in Supplementary Data Sheet S4. Using rat/human coefficients of 25.2, doses per kilogram of body weight of the rats were calculated based on the ratio of rat to human surface area: 11.34 g/kg. The rats (n = 50) were gavaged with this dose for 60 days, followed by an additional 14 days of observation. Over the total of 74 days, apart from some rats having diarrhea, no significant side effects were observed.

## Discussion

Herbal remedies and natural products have gained traction worldwide for treating erectile dysfunction (ED) (Magheli and Burnett, 2009; Feng et al., 2022), with substantial research underpinning their utility in recent years (Ciocanel et al., 2019;

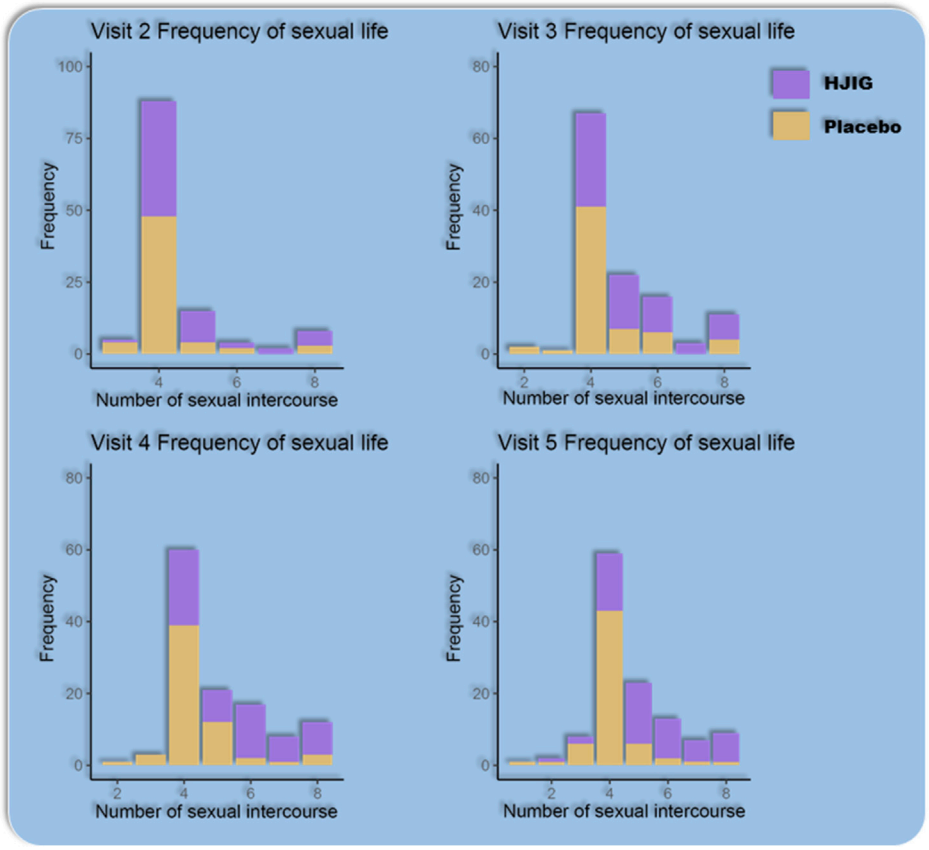



FIGURE 11
Frequency of sexual activity at different visit time points.

Wang et al., 2020; Wu et al., 2023). Traditional Chinese Medicine (TCM) originates from ancient China, with its primary therapeutic approach focusing on symptom differentiation and improving the overall health of the patient. Herbal formulation treatments constitute a significant component of TCM. Meta-analyses suggest that coupling Chinese herbs with PDE5i (phosphodiesterase-5 inhibitors) enhances efficacy without amplifying side effects (Wang et al., 2020). This corroborates the conclusions drawn from our preliminary studies on HJIG (Zhang G. et al., 2017; Ping et al., 2021). Another analysis indicates that singular Chinese herbal treatments can elevate IIEF (International Index of Erectile Function) scores and amplify clinical recovery rates (Wu et al., 2023). However, previous studies have touted the need for more rigorous clinical trials for Chinese herbal medicine (Xiong et al., 2014). Recognizing the pivotal role of accurate symptom differentiation in TCM and its association with ED, we advocate for randomized controlled trials (RCTs) targeting explicit symptom patterns to genuinely gauge Chinese medicine's potency.

Our study indicates that exclusive use of HJIG can improve erectile function in patients with mild to moderate ED and enhance sexual completion (the ability to maintain an erection until ejaculation). Secondary outcomes also show that HJIG has the potential to increase sexual desire, as evidenced by an increase in sexual activity frequency. Clinical trail on herbal or herbal formula treatments for ED have yielded positive results (Kam et al., 2007; Shah et al., 2012; Xiong et al., 2014; Park et al., 2019).

A double-blind randomized controlled trial of a Thai herbal formulation composed of four medicinal plants showed improvements in IIEF-5 scores and sexual desire in patients with mild to moderate ED (Lin et al., 2022). Guojun et al.'s study indicated that acupuncture could improve IIEF-EF and SEP3 results in patients with psychogenic erectile dysfunction, although there was no significant difference in SEP2 between the treatment and placebo groups (Wang et al., 2023). A multicenter randomized controlled study provided a more detailed classification of patients and applied a TCM herbal formulation for those with liver depression and kidney deficiency symptoms related to psychogenic or mild arterial ED. By evaluating sexual success rates and erectile hardness in 500 patients, it was found that the herbal formulation Shugan Yiyang Capsule effectively improved erectile function and sexual completion (Qi et al., 2004). Nam Cheol Park et al. used a formula composed of five herbal granules for an 8-week intervention in ED patients. The results showed significant improvement in IIEF-EF scores in the treatment group compared to the placebo group, although there were no differences in SEP2 and SEP3 (Park et al., 2019). Gaurang R Shah used a mixture of eight herbal formulations to treat patients with mild to moderate ED, significantly improving their IIEF-EF scores. It is evident that the IIEF score remains a primary method for evaluating ED patients,

especially in preliminary efficacy studies of new therapies (Shah et al., 2012).

Previous studies suggest that drugs like tadalafil are insufficient in alleviating auxiliary ED-related symptoms, whereas herbal interventions may fill this therapeutic gap (Zixue and Pengchao, 2018). This is a key advantage of herbal formulations. Therefore, in high-quality, large-scale studies focusing on other diseases, clinical research on traditional Chinese herbal formulas often incorporates TCM syndromes as criteria for inclusion, exclusion, or as a basis for evaluating treatment efficacy (Zhong et al., 2019). We adopted this model, and our results indicate significant improvement in patients' specific TCM symptoms. Additionally, we used difference scores to evaluate treatment efficacy, which significantly reduces the impact of individual differences. By calculating the pre- and post-treatment differences, we can more accurately assess treatment effects, minimizing the influence of individual variability such as baseline levels. Furthermore, stratification results indicate that differences in patient age and duration of ED history may influence treatment efficacy. Older patients and those with a longer duration of ED may require longer treatment and recovery periods to achieve optimal therapeutic outcomes.

From a pharmacological perspective, the efficacy of HJIG may stem from the synergistic properties of its botanical drug metabolites. The main active metabolite in Rhodiola crenulata (Hook. f. et Thoms.) H. Ohba., salidroside, has been proven to improve hypoxic conditions (Zhao et al., 2020), inhibit fibrosis (Ma et al., 2020), and reduce phenotypic transformation of smooth muscle cells (Zhang X. et al., 2017). Astragali radix praeparata cum melle, a traditional herb used in ED treatment, though not fully explored mechanistically, has shown potential in improving cardiac diastolic function in postmenopausal women with hypertension, suggesting a similar beneficial effect on cavernous smooth muscle cells (Li et al., 2018). Salvia miltiorrhiza Bge. has been shown to inhibit oxidative stress and reduce apoptosis, thereby improving erectile function in diabetic rats (Zhang et al., 2018). Angelica sinensis (Oliv.) Diels has been found to enhance nitric oxide synthase (NOS) activity in rats with damaged cavernous nerves (Li et al., 2017). Cyathula officinalis Kuan has demonstrated efficacy in improving erectile function in diabetic rats (Wang et al., 2021). Lycium Chinense Miller. through its antioxidative stress properties improves erectile function in aged rats (Moon et al., 2017). Epimedium brevicornu Maxim. has been proven to induce erection in rats (Chen and Chiu, 2006), and its metabolites exhibit PDE5 inhibitory effects. Although not every herbs in the HJIG formulation has been extensively studied for its mechanism, most herbs have been proven beneficial in improving erectile function.

Our study, being exploratory in nature, still presents a range of aspects that necessitate further refinement and improvement. We lack bolstering through objective measures like NPTR (Nocturnal Penile Tumescence and Rigidity) or CT (Computed Tomography) tests. Additionally, even though five participants admitted to intermittent PDE5i usage and subsequently exited the study, there remains a latent ambiguity regarding undisclosed PDE5i usage, potentially skewing our results. The self-selection bias, where participants more inclined toward herbal treatment remain, while skeptics drop out, may also tilt outcomes.

In summary, HJIG demonstrates significant enhancements in sexual functionality for ED patients, complemented by improvements in their TCM symptomatology. We are planning broader real-world TCM studies to eliminate biases, reaffirm the therapeutic efficacy of HJIG, and extend the follow-up duration. Incorporating HJIG into a structured treatment regimen offers promise for a more favorable prognosis for patients. In future research endeavors, we are considering incorporating effectiveness parameters, such as time-efficiency and cost-effectiveness. The reduction in time and financial costs can alleviate patients' burdens, increasing their engagement in the treatment, exemplifying one of the inherent benefits of herbal intervention.

We acknowledge that the sample size in this study is relatively small compared to large-scale clinical trials. However, given the exploratory nature of this research and the promising preliminary results, we believe the findings provide valuable insights into the efficacy of HJIG for mild to moderate ED. It is also important to note that collecting patients who meet the criteria for using Chinese herbal medicine alone, without involving ethical concerns, is particularly challenging. This recruitment difficulty further limits the sample size, but we are confident that the data gathered offers a solid foundation for future research. Larger studies with more extended follow-up periods are necessary to validate these findings and explore HJIG's full therapeutic potential.

## Data availability statement

The datasets presented in this article are not readily available. In consideration of patient privacy and the agreements signed, the data from this study is not permitted to be shared on public platforms. Researchers with relevant needs can contact the corresponding author of this article for access.

## Ethics statement

The studies involving humans were approved by the Ethics Committee of the Second Affiliated Hospital of Zhejiang Chinese Medical University. 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

R-NX: Methodology, Visualization, Writing–original draft, Writing–review and editing, Software. JG: Data curation, Supervision, Writing–review and editing. C-HZ: Data curation, Supervision, Writing–review and editing. QZ: Data curation, Supervision, Writing–review and editing. QG: Data curation, Supervision, Writing–review and editing. FW: Conceptualization, Data curation, Supervision, Validation, Writing–review and editing. YZ: Data curation, Supervision, Writing–review and editing. X-YL: Data curation, Supervision, Writing–review and editing. Y-FL: Data

curation, Supervision, Writing–review and editing. Y-JF: Visualization, Writing–review and editing. XZ: Visualization, Writing–review and editing. W-ZW: Data curation, Supervision, Writing–review and editing. J-XM: Data curation, Supervision, Writing–review and editing. JW: Data curation, Supervision, Writing–review and editing. X-JH: Data curation, Funding acquisition, Supervision, Writing–review and editing. W-JH: Data curation, Funding acquisition, Supervision, Writing–review and editing. B-DL: Conceptualization, Funding acquisition, Methodology, Resources, 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.

## Publisher's note



## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2024.1367812/full#supplementary-material

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# Large variability in the alkaloid content of *Corydalis yanhusuo* dietary supplements

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**Introduction:** Extracts from the rhizome of the traditional Chinese medicinal plant *Corydalis yanhusuo* (CY) mediate a number of biologic effects that are associated with its content of isoquinoline alkaloids. CY alkaloids have shown analgesic, cardioprotective, and anti-addictive effects in animal models of disease. Since CY alkaloids are available to consumers as dietary supplements we analyzed the content of alkaloids in 14 products including open powders, capsules, and liquid formulations, capturing a majority of the products available online in the US.

**Methods:** Nineteen alkaloids were quantified using HPLC analyses with diode array detection after extraction using a weak cation exchange column.

**Results:** Total alkaloid content was highly variable among the products, ranging from below quantifiable in some to ≈11 mg/g in others. Five of the products had comparable content of alkaloids (9.5 ± 1.6 mg/g), equaling about the amount of alkaloids of an extract prepared from CY rhizome (12.7 mg/g). The other samples had much lower content (1.8 ± 0.9 mg/g), or alkaloids were below quantifiable. One of the products was highly enriched in tetrahydropalmatine (≈5 mg/g), suggesting adulteration from the natural product, and raising concerns about possible toxicologic liability.

**Discussion:** Considering alkaloid content as a key quality criterium for CY supplements, the large variability among the products seems unacceptable and makes it difficult for consumers to select products with an appropriate content of alkaloids.

KEYWORDS

**isoquinoline alkaloid, corydalis rhizome, tetrahydropalmatine, botanical extract, dietary supplement, consumer safety**

## 1 Introduction

The plant *Corydalis yanhusuo* W.T. Wang (CY) is listed in the Chinese Pharmacopeia (Tian et al., 2020) and has been used in Traditional Chinese Medicine (TCM) to promote blood circulation, reinforce vital energy, and alleviate pain (Ma et al., 2008; Zhang et al., 2020). Biological effects of CY are mediated by its content of isoquinoline alkaloids of the

**Abbreviations:** CY, Corydalis yanhusuo; DHCB, dehydrocorybulbine; HR-MS, high resolution-mass spectrometry; LOQ, limit of quantification, MeN, methyl nicotinate, ODS, Office of Dietary Supplements; WCX, weak cation exchange.

protoberberine and tetrahydroprotoberberine structural classes. CY alkaloids were shown to have analgesic (Alhassen et al., 2021), cardioprotective and antiarrhythmic (Wu et al., 2007; Han et al., 2012; Li et al., 2022; Han et al., 2022), as well as anti-addictive effects (Chu et al., 2008; Wang and Mantsch, 2012; Nesbit and Phillips, 2020). For example, analgesic properties of CY have been associated with the alkaloids tetrahydropalmatine (Guo et al., 2014; Zhou et al., 2016; Kang et al., 2016; Liu et al., 2019) and dehydrocorybulbine (Zhang et al., 2014; Wang et al., 2016). A systematic analysis of a CY alkaloid extract showed effective attenuation of acute, inflammatory, and neuropathic pain in animal models without causing tolerance (Wang et al., 2016). Mechanistic studies showed that CY alkaloids were able to affect several pathways in the transmission of pain by targeting dopamine receptors (Wang et al., 2016), voltage gated sodium channels (Xu et al., 2021), NMDA and mGlu1/5 receptors (Dai et al., 2017), and the spinal sigma-1 receptor (Kang et al., 2016). The ability to modulate a number of different pain-related pathways in the absence of alkaloids with addiction potential makes CY an attractive candidate for a complementary-alternative approach towards pain management.

Toxicological and case studies indicate that CY alkaloids may not only mediate desirable effects but can also cause toxicity, mostly upon oral overdosing (Wu et al., 2021; Du et al., 2022). Overdosing with tetrahydropalmatine, one of the most abundant alkaloids of CY, in adult and pediatric populations resulted in depression of neurological, respiratory, and cardiac function as acute effects and chronic hepatitis after regular use (Lai and Chan, 1999). Case reports on overdosing, however, appeared related to consumption of products highly enriched in tetrahydropalmatine rather than using CY botanical extracts (Lai and Chan, 1999; Horowitz et al., 1996), indicating that toxicological consequences of consuming CY botanical extracts are unlikely yet not impossible.

Consumers might use CY dietary supplements as a source of bioactive alkaloids in order to address various ailments. The alkaloid content of CY dietary supplements is an important criterium for the quality of a product and its possible beneficial as well as toxicological effects. In order to understand what products are available to consumers and for researchers interested in using CY for animal or clinical research, we quantified alkaloids in CY dietary supplements, focusing on 19 abundant out of 84 reported alkaloids in CY (Feng et al., 2023).

# 2 Materials and methods

## 2.1 Materials

The following alkaloids were purchased from Cayman Chemical: scoulerine (item no. 35140; batch no. 0626850-3), isocorypalmine (item no. 29886; batch no. 0694012-1), glaucine (item no. 17338; batch no. 0481648-13), tetrahydropalmatine (item no. 20535; batch no. 0491304-7), tetrahydroberberine (item no. 33157; batch no. 0605003-10), corydaline (item no. 27654; batch no. 0570888-1), tetrahydrocoptisine (item no. 34603; batch no. 0620069-2), protopine (hydrochloride) (item no. 23366; batch no. 0514853-5), columbamine (item no. 35032; batch no. 0626484-3), coptisine (chloride) (item no. 28424; batch no. 0568598-14), palmatine (chloride) (item no. 30318; batch no. 0585590-3) and dehydrocorydaline (chloride) (item no. 30972; batch no. 0594357-5). CY dietary supplements and the CY rhizome sample (Plum Dragon Herbs) were ordered from online retail markets. Chemicals and HPLC solvents were from Sigma Aldrich or Fisher.

## 2.2 Large-scale alkaloid isolation and identification

CY dietary supplement sample 7 (2 g) was extracted with hot water (20 mL) by vortex mixing followed by centrifugation at 4,650 x g for 10 min. The supernatant was collected and the pellet was re-extracted with 10 mL of hot water. The supernatants were combined, centrifuged again (4,650 x g for 12 min) and loaded on a Waters Oasis WCX 6 cc cartridge (500 mg, 60 µm) that had been preconditioned with methanol and water. Two 2 g-aliquots of CY powder were extracted and processed in parallel. After loading the WCX cartridges were washed with 5% $NH_4OH$ (6 mL) and eluted with methanol (6 mL) followed by a solvent of acetonitrile/methanol/formic acid (80/20/2; by vol.; 6 mL) to give the WCX "methanol" and "formic acid" eluates, respectively. Preparation and elution of the WCX cartridge was performed according to the manufacturer's instructions. The eluates were evaporated under a stream of $N_2$ and dissolved in ca. 3 mL of methanol for the methanol eluate and ca. 150 µL of methanol for the formic acid eluate.

Semi-preparative RP-HPLC used an Agilent 1,200 Series HPLC system with a diode array detector and a Waters Symmetry C18 7 µm column (7.8 × 150 mm) eluted at 2 mL/min flow rate. For isolation of alkaloids of the methanol eluate the gradient used a solvent of water/acetonitrile/acetic acid 90/10/0.01 (by vol.) to 75/25/0.01 (by vol.) in 30 min, then changed to 20/80/0.01 (by vol.) in 2 min, hold 2 min, and return to starting solvent in 2 min. For the formic acid eluate the gradient used a solvent of 50 mM $NH_4OAc$ pH 8/acetonitrile 90/10 (by vol.) changed to 60/40 (by vol.) in 40 min, then changed to 30/70 (by vol.) in 2 min, hold 2 min, and return to starting solvent in 2 min. Use of a basic HPLC solvent for alkaloid purification was suggested by ref. (Zhang et al., 2009). Peaks from the formic acid eluate were collected in tubes that contained 50 µL of a mixture of water/acetic acid (4/1, by vol.) in order to prevent isolation artifacts occurring at basic pH (Marek et al., 2003).

The alkaloids collected from the methanol eluate were extracted from RP-HPLC solvent by evaporating acetonitrile under a stream of $N_2$, adding water, and loading on a pre-conditioned Waters Oasis HLB 3 cc cartridge (60 mg). The cartridge was washed with water and alkaloids were eluted with methanol. Alkaloids from the formic acid eluate were extracted from RP-HPLC solvent by evaporation of acetonitrile and adding water and loading on a pre-conditioned Waters Oasis WCX 3 cc cartridge (60 mg). The cartridge was washed with water, and alkaloids were eluted with acetonitrile containing 1% acetic acid (750 µL) followed by acetonitrile (750 µL).

Collected alkaloids were checked for purity using a Waters Symmetry C18 5 µm column (4.6 × 250 mm) eluted at 1 mL/min flow rate with a gradient of water/acetonitrile/acetic acid 90/10/0.01 (by vol.) to 60/40/0.01 (by vol.) in 18 min. When isolated compounds had a purity (RP-HPLC UV 205 nm) less than 95% they were repurified. Re-purification was achieved using the same column and gradient as in the original purification or isocratic elution with a solvent of water/acetonitrile/acetic acid 85/15/0.01 (by

vol.). Dehydrocorybulbine was re-purified using a gradient of 50 mM $NH_4OAc$ pH 8/acetonitrile 70/30 (by vol.) to 60/40 (by vol.) in 20 min; collected fractions were acidified using a mixture of water/acetic acid (4/1, by vol.) as described above.

## 2.3 Alkaloid extraction from dietary supplements and quantification

CY dietary supplements (25 mg) were weighed into a 2 mL Eppendorf tube. Hot water (ca 80°C; 1 mL) was added, spiked with internal standards (methyl nicotinate (MeN): 40 µg; nitidine chloride: 20 µg), and samples were vortex mixed for 1–2 min. After centrifugation (2 min at 17,000 x g) the supernatant was loaded on a pre-conditioned (3 mL of methanol followed by 3 mL of water) Waters Oasis WCX 3cc cartridge (60 mg, 30 µm). The cartridge was washed with 1 mL of 5% $NH_4OH$ and eluted with 1 mL of methanol followed by 1 mL of acetonitrile/methanol/formic acid (80/20/2, by vol.). Each supplement sample was extracted in two independent repeats. For the generation of calibration curves, different amounts of alkaloids were mixed with a fixed amount of standard (MeN or nitidine), and injected on RP-HPLC. Glaucine, tetrahydropalmatine, scoulerine, and corydaline standard curves were used to quantify alkaloids in the methanol eluate. Protopine, coptisine, and palmatine standard curves were used to quantify the alkaloids in the formic acid eluate.

For quantification an aliquot of 20 µL of each eluate (≈1 mL) was injected on RP-HPLC using an Agilent 1200 SL HPLC system equipped with a Water Symmetry Shield C18 5 µm column (4.6 × 250 mm) and a diode array detector with monitoring at 205, 220, 235, 270, 340, and 430 nm wavelength. The flow rate was 1 mL/min and compounds were eluted using a linear gradient of water/acetonitrile/acetic acid from 90/10/0.01 (by vol.) to 70/30/0.01 (by vol.) within 28 min followed by a wash step using water/acetonitrile/acetic acid 20/80/0.01 (by vol.). Alkaloids were quantified based on their peak area at 205, 220, or 340 nm.

## 2.4 CY rhizome extraction

CY rhizome was obtained in slices of 2–3 mm thickness and about 1 cm in diameter. Three slices were ground into a fine powder using a mortar and pestle. The powder (25 mg) was weighed into a 2 mL Eppendorf tube, spiked with internal standards, and alkaloids were extracted using 1 mL of hot water followed by WCX cartridge fractionation into methanol and formic acid eluates as described above.

## 2.5 HR-MS

High-resolution LC-MS of alkaloids was performed using a Q Exactive HF Hybrid Quadrupole-Orbitrap instrument (Thermo Fisher Scientific) operated in the electrospray ionization (ESI) positive mode. Prior to analysis, the instrument was calibrated with an ESI-positive ion calibration solution. A Waters Symmetry C18 column (2.1 × 50 mm, 1.8 µm) was eluted at room temperature with a gradient of acetonitrile in water/0.1% formic acid changed from 10% acetonitrile to 95% in 3 min at a flow rate of 0.4 mL/min.

## 2.6 NMR

Purified alkaloids were dissolved in deuterated solvents ($CD_3OD$, $CD_3CN$, $CDCl_3$, or mixtures thereof) for NMR analysis. NMR spectra were recorded using a Bruker AV-II 600 MHz spectrometer equipped with a cryoprobe. Chemical shifts (δ value) are given relative to the residual non-deuterated solvent and are reported in parts per million (ppm). Coupling constants (*J*) are given in Hertz (Hz). Pulse frequencies were taken from the Bruker library.

# 3 Results

## 3.1 Selection of CY dietary supplements

A survey of three local health food and supplement stores in Nashville, TN conducted in March 2024 did not find any CY products available. Therefore, all CY dietary supplements were purchased online. Products containing CY in combination with other extracts or bioactive ingredients were excluded from the analysis. Fourteen different products were selected, capturing a majority of the products available. By comparison, the NIH/Office of Dietary Supplements Dietary Supplement Label Database (https://dsld.od.nih.gov/, accessed March 2024) listed about 13 different products on the market that contain CY as the only bioactive ingredient. Thus, the selected products provided a meaningful survey of CY dietary supplements available to consumers in the United States at the time.

The 14 selected products included open powders or granules (4), capsules (8), and two liquid formulations. Table 1 provides an overview of the product names, serving sizes, and supplement facts information retrieved from the labels. The "supplement facts" labels of samples 8, 10, and 12 identified the products as "Corydalis" yet not explicitly as "Corydalis yanhusuo". Sample 8 was referred to as "Yan Hu Suo" elsewhere on the label, implying that the product contained an extract of CY. The two other samples (10 and 12) were assumed to contain CY, or might be mistaken by consumers to do so, and thus were included in the analysis.

## 3.2 Alkaloid identification

We isolated and identified 19 alkaloids from one of the CY dietary supplements (sample 7 in Table 1) that had shown high alkaloid content in a preliminary analysis. Alkaloids were solubilized using hot water and separated from other material by binding to a weak cation exchange (WCX) cartridge. Alkaloids were eluted from the WCX cartridge using methanol to obtain tertiary amine alkaloids (i.e., tetrahydroprotoberberines; "methanol eluate") followed by elution with a solvent of acetonitrile/methanol/formic acid (80/20/2, by vol.) to provide mostly quaternary amine alkaloids (i.e., protoberberines and others; "formic acid eluate"). Alkaloids were purified using semi-preparative RP-HPLC; structural

TABLE 1 CY dietary supplements included in the analysis.

| Sample no. | Serving size | Form | "Supplement facts" label |
|---|---|---|---|
| 1 | 1,000 mg | capsule | Corydalis root P.E. 10:1 *(Corydalis Yanhusuo)* |
| 2 | 1,000 mg | powder | Corydalis Extract *(Corydalis Yanhusuo)* (Root) |
| 3 | 500 mg | capsule | Corydalis Extract (Corydalis yanhusuo) (Root) (A 20:1 extract equivalent to 10,000 mg of Corydalis root powder) |
| 4 | 1,000 mg | capsule | Corydalis Root P.E. 10:1 (Corydalis Yanhusuo) |
| 5 | 400 mg | capsule | *Corydalis yanhusuo* (rhizome) |
| 6 | 500 mg | capsule | Corydalis (15:1 Extract) (Corydalis Yanhusuo) (Root) |
| 7 | 1,000 mg | powder/ granules | Corydalis yanhusuo (tuber) (100 g of the concentrated granules extracted from 500 g of the raw herbs) |
| 8 | 1,000 mg | capsule | Corydalis Extract (4:1 Hot Water Extract)[a] |
| 9 | 1,000 mg | capsule | Corydalis Extract *(Corydalis yanhusuo)* (rhizome) (DHCB dehydrocorybulbine) |
| 10 | 1,000 mg | capsule | Corydalis Root 10:1 (Root) Extract (equivalent to 10,000 mg) |
| 11 | 1,000 mg | powder | Corydalis root (yan hu suo) |
| 12 | not given | powder | Corydalis poppy powder[b] |
| 13 | 0.7 mL | liquid | Corydalis tuber (*Corydalis yanhusuo*) (purity verified) (extract 665 mg) Extraction rate 233 mg herb per 0.7 mL. Dry herb:menstruum ratio 1:3 |
| 14 | 5 drops | liquid | Corydalis Root (Corydalis yanhusuo)[c] |

[a]Elsewhere on the label there is a reference to "Yan Hu Suo".
[b]Package does not have a "Supplement Facts" label.
[c]Labeled as "Product Information" instead of "Supplement Facts".

identification of isolated alkaloids was achieved using NMR and HR-MS analyses and/or comparison to authentic standards when available (see Supplementary Material). The structures of nine alkaloids (M1-M9) isolated from the methanol and 10 alkaloids (F1-F10) from the formic acid WCX eluates, respectively, are shown in Figure 1.

## 3.3 Analytical approach for alkaloid quantification

Alkaloids were solubilized from the dietary supplements using hot water and applied to a WCX column to provide a "methanol" and "formic acid" eluate for separate RP-HPLC analysis. The method used for quantification of alkaloids is illustrated in Figure 2, showing RP-HPLC analysis with UV/Vis diode array detection of sample 7 as example. Both the tertiary (methanol eluate; Figure 2A) and quaternary (formic acid eluate; Figure 2B) amine alkaloids obtained from the WCX cartridge were well resolved. Resolution and peak shape of alkaloids was enhanced by using a Symmetry Shield C18 column (Waters) that provides embedded polar carbamate groups at the base of each ligand. The embedded polar groups bind water molecules that "shield" charged silanol groups and therefore decrease the amount of tailing with charged basic compounds. Regardless of what HPLC column was used we noticed that alkaloids showed a tendency to switch elution order and change retention times beyond what was expected upon variations in chromatographic conditions, even when variations were minor. Similar effects were observed when different amounts of alkaloids were injected. Thus, caution should be used in peak identification especially since the UV spectra of many of the alkaloids are similar. Two internal standards, methyl nicotinate and nitidine, were added to the supplements for quantification of alkaloids in the WCX methanol and formic acid eluates, respectively. The standards were present only in the intended WCX eluates, were well resolved from the alkaloids of interest, had suitable UV/Vis spectra, and were absent from a list of known alkaloids of CY (Feng et al., 2023).

Alkaloids were grouped by their UV/Vis spectra for quantification. Alkaloids had one of two general types of UV/Vis spectra, based on the degree of saturation in the third ring. Protoberberines are yellow in color with several maxima around 230 nm, 270 nm, 340 nm, and 430 nm whereas the saturated tetrahydroprotoberberines have mostly end absorbance in the UV and are lacking color (Supplementary Figures 1, 2). Accordingly, alkaloids were quantified using the absorbance of the chromatographic peaks at 205 nm, 220 nm, or 340 nm as appropriate. For the methanol eluate, a calibration curve for scoulerine (M1) was used to quantify M1, M2, M3, and M4 at 205 nm. Glaucine (M5) and tetrahydropalmatine (M6) calibration curves were used to quantify M5 and M6, respectively, at 220 nm. A calibration curve for corydaline (M8) was used to quantify M7, M8, and M9 at 205 nm. For alkaloids in the formic acid eluate, a calibration curve for protopine (F4) was used to quantify F1, F2, F3, F4, and F5 at 205 nm. A calibration curve for coptisine (F6) was used to quantify F6, F7, and F8 at 340 nm. A calibration curve for palmatine (F9) was used to quantify F9 and F10 at 340 nm (see Supplementary Material). The LOQ was between 5 and 6 ng per injection (20 µL) on HPLC.

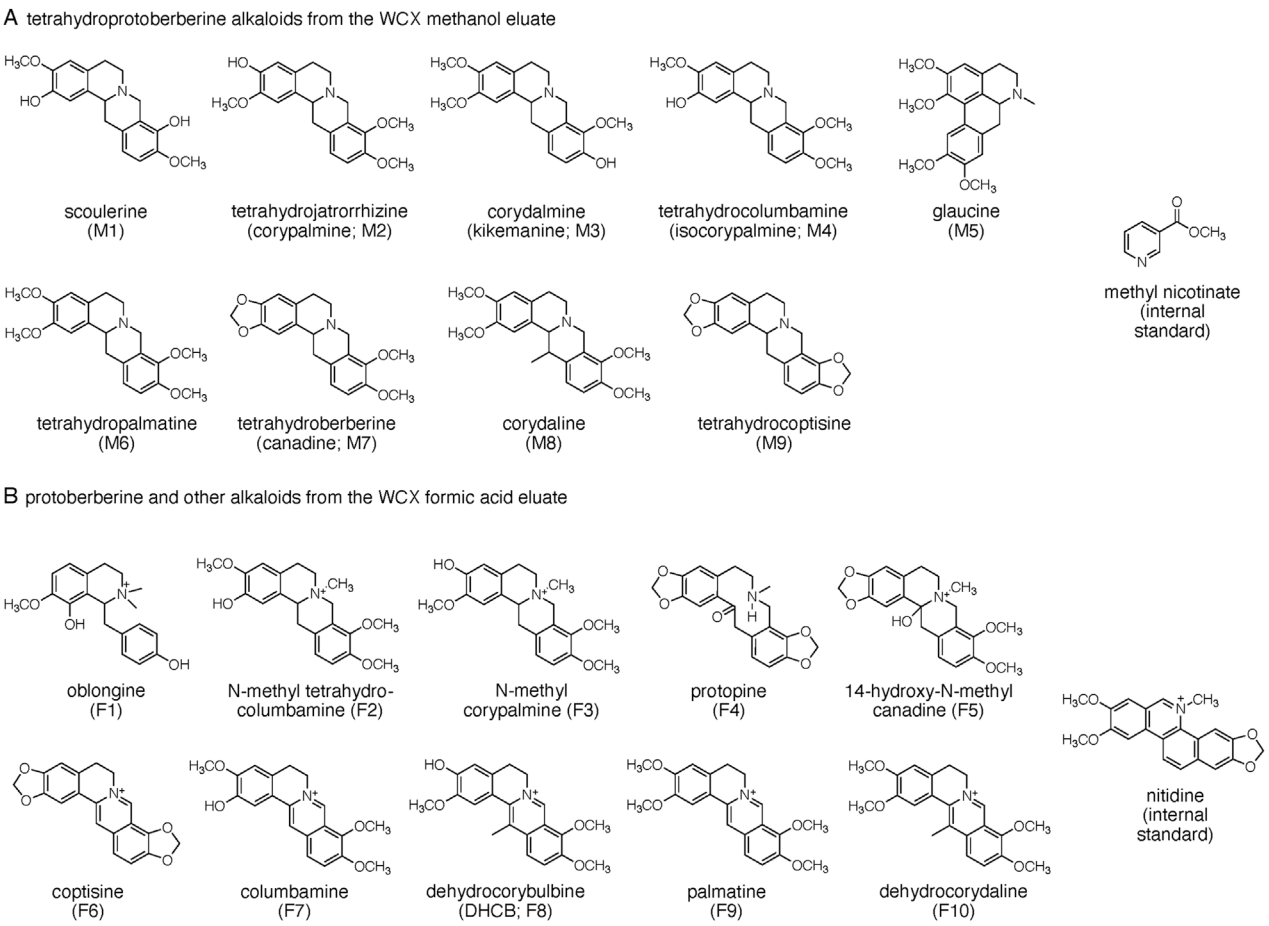


FIGURE 1
Structures of alkaloids quantified in the CY dietary supplements. Alkaloids are grouped into **(A)** tetrahydroprotoberberines and **(B)** protoberberines and others as obtained by differential elution from the weak cation exchange (WCX) cartridge. Methyl nicotinate and nitidine were used as internal standards for quantification of the respective alkaloid groups.

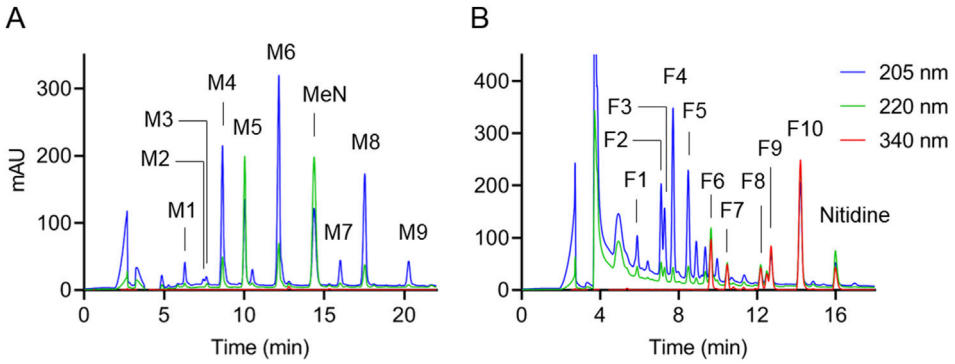


FIGURE 2
Analysis of alkaloids in CY dietary supplement 7, representative of a CY dietary supplement with high content of alkaloids. RP-HPLC analysis with UV/Vis diode array detection of the **(A)** methanol and **(B)** formic acid eluates from the WCX cartridge used to enrich alkaloids after hot water extraction of the sample. The sample was spiked with internal standards methyl nicotinate (MeN) and nitidine prior to extraction. Peak labels refer to the numbering of alkaloids in Figure 1. The chromatograms were recorded at 205 nm (blue), 220 nm (green), and 340 nm (red).

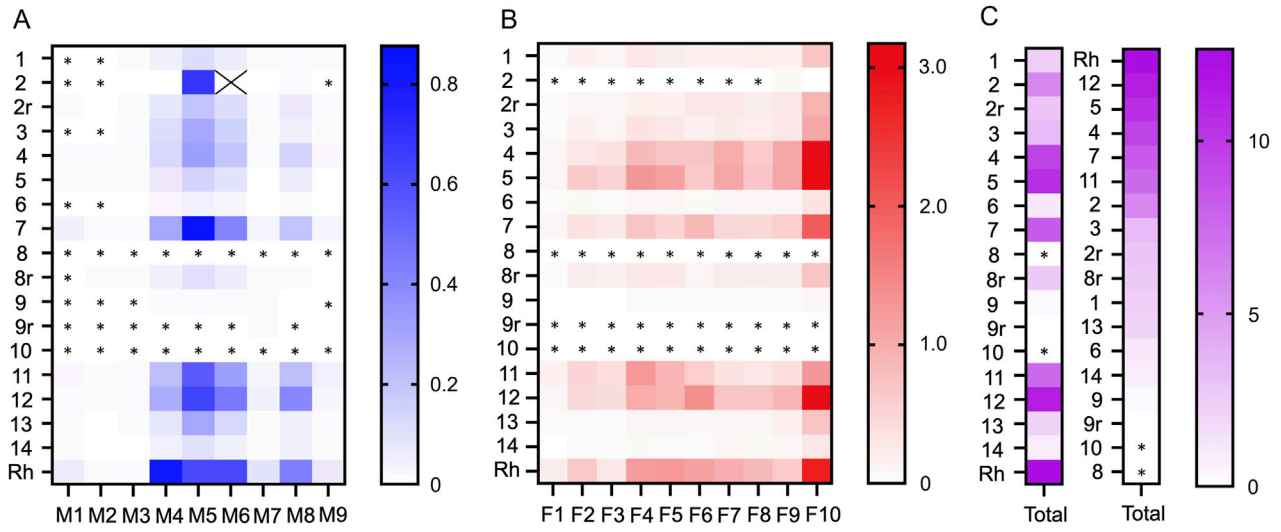


FIGURE 3
Graphic representation of the alkaloid content in the CY dietary supplements. The colored matrices show the content of alkaloids (mg/g) of the **(A)** methanol eluate (M1-M9) and **(B)** formic acid eluate (F1-F10) for dietary supplement samples 1-14 and *Corydalis yanhusuo* rhizome (Rh). **(C)** The total sum of alkaloids (mg/g) for each supplement is shown, sorted by sample number in the left column and by amount in the middle column. Samples marked with a lowercase "r" were repurchased. Alkaloid content in the supplements is coded by color intensity. The "X" in panel A denotes tetrahydropalmatine in sample 2 that was omitted from the graph because the concentration was 6-fold higher than the next most abundant alkaloid. Alkaloids that were below LOQ are marked with an asterisk (*).

## 3.4 Content of alkaloids in CY dietary supplements

A graphical summary of the quantification results is shown in Figure 3. The content of alkaloids in the methanol and formic acid eluates of the CY dietary supplements is listed in Tables 2, 3, respectively. The most abundant alkaloids in the CY dietary supplements were tetrahydrocolumbamine (M4), glaucine (M5), tetrahydropalmatine (M6), corydaline (M8), protopine (F4), 14-hydroxy-N-methylcanadine (F5), coptisine (F6), and dehydrocorydaline (F10) (Figures 3A, B). Based on their content of alkaloids the CY dietary supplements roughly fell into three groups (Figure 3C). A group of five supplements (samples 4, 5, 7, 11, 12) had an overall high and similar content of alkaloids (9.5 ± 1.6 mg/g). The remaining samples either had a much lower content of alkaloids (samples 1, 3, 6, 13, 14; 1.8 ± 0.9 mg/g), or the alkaloids were near or below the lower limit of quantification (samples 8, 9, 10). When a 4-fold higher amount of the latter three samples was extracted the results were unchanged.

The near absence of the alkaloids of interest is illustrated for sample 9 (Figures 4A, B), showing RP-HPLC chromatograms of the methanol and formic acid eluates of the WCX column. No other peaks with UV/Vis spectra characteristic of isoquinoline alkaloids were detected, indicating that the sample was largely devoid of this type of bioactive alkaloids. For sample 2 the unusually high content of tetrahydropalmatine together with an overall low to absent content of other alkaloids is shown in Figures 4C, D. Tetrahydropalmatine was present in sample 2 at about 5 mg/g (i.e., per suggested serving size), which was >10-times the amount of tetrahydropalmatine in the next highest sample. The fact that only one alkaloid was of high abundance in the sample suggested it was added as a pure compound (of synthetic or natural origin) and was not included as an extract from another botanical which would likely have provided additional alkaloids.

Three of the four samples with unusual or low to absent alkaloid content (samples 2, 8, and 9; sample 10 was no longer available) were repurchased in order to test a different batch for consistency of the findings. In repurchased sample 2 the excessive content of tetrahydropalmatine was no longer present while other alkaloids were increased. Sample 8, which did not contain any alkaloids in the original sample, showed an increased though low amount in the repurchased sample. For sample 9, which had very low content of alkaloids, the amount was even further decreased, and the repurchased sample contained only three alkaloids that were barely above the LOQ. Even though the product label explicitly mentioned "DHCB" (i.e., dehydrocorybulbine) sample 9 had the lowest content of dehydrocorybulbine of all samples, and the amount was decreased to below LOQ in the repurchased sample.

## 3.5 Comparison to CY rhizome

The alkaloids of the CY dietary supplements were compared to an alkaloid extract prepared from the rhizome of CY. The alkaloids in the supplements with high alkaloid content and in the CY rhizome were similar regarding type and their relative abundance (Figure 3). Quantitative analysis showed that the absolute amount of alkaloids in the supplements with high content of alkaloids (9.5 ± 1.6 mg/g) was similar to the rhizome (12.7 mg/g) (Tables 2, 3).

TABLE 2 Alkaloid content (mean ± SD in ng/mg) in the WCX methanol eluate of the CY dietary supplements. Alkaloids are identified by name and numbering used in Figure 1 as well as the wavelength used for quantification. Missing entries indicate values were below the LOQ. Samples marked with a lowercase "r" were repurchased. Rh, *Corydalis yanhusuo* rhizome.

| Sample ID | Scoulerine (M1; 205 nm) | Tetrahydro-jatrorrhizine (M2; 205 nm) | Corydalmine (M3; 205 nm) | Tetrahydro-columbamine (M4; 205 nm) | Glaucine (M5; 220 nm) | Tetrahydro-palmatine (M6; 220 nm) | Tetrahydro-berberine (M7; 205 nm) | Corydaline (M8; 205 nm) | Tetrahydro-coptisine (M9; 205 nm) | Total |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | | | 4.3 ± 0.12 | 46.4 ± 2.49 | 103.2 ± 4.66 | 56.8 ± 2.01 | 4.3 ± 0.02 | 16.3 ± 1.41 | 4.3 ± 0.00 | 236 ± 10.73 |
| 2 | | | 2.5 ± 0.27 | 0.9 ± 1.26 | 686.3 ± 27.70 | 5,217.1 ± 247.86 | 2.1 ± 0.31 | 6.9 ± 0.63 | | 5,916 ± 275.51 |
| 2 r | 6.2 ± 0 | 3.2 ± 0.12 | 3.8 ± 0.10 | 77.8 ± 0.60 | 196.1 ± 1.03 | 110.0 ± 1.23 | 8.2 ± 0.87 | 68.6 ± 0.73 | 12.0 ± 0.80 | 486 ± 4.04 |
| 3 | | | 7.3 ± 0.05 | 118.5 ± 6.80 | 293.4 ± 12.42 | 149.3 ± 5.77 | 6.1 ± 0.99 | 51.6 ± 2.98 | 4.7 ± 1.26 | 631 ± 30.17 |
| 4 | 9.3 ± 0.09 | 9.3 ± 2.95 | 5.6 ± 0.22 | 128.6 ± 1.14 | 325.6 ± 10.86 | 188.1 ± 6.04 | 16.1 ± 1.08 | 142.9 ± 3.87 | 18.6 ± 1.24 | 844 ± 21.15 |
| 5 | 5.7 ± 0.07 | 4.2 ± 0.12 | 3.5 ± 0.99 | 69.4 ± 2.23 | 142.1 ± 6.55 | 86.0 ± 4.81 | 2.7 ± 0.17 | 54.6 ± 3.06 | 1.2 ± 1.05 | 369 ± 16.59 |
| 6 | | | 1.9 ± 0.12 | 17.8 ± 0.82 | 39.1 ± 1.26 | 23.7 ± 0.83 | 1.3 ± 0.07 | 5.7 ± 0.08 | 1.4 ± 0.11 | 91 ± 2.53 |
| 7 | 36.1 ± 1.00 | 9.3 ± 1.04 | 16.4 ± 0.00 | 299.5 ± 1.40 | 876.9 ± 11.71 | 421.0 ± 13.85 | 31.3 ± 0.22 | 201.3 ± 1.26 | 34.2 ± 0.30 | 1926 ± 28.35 |
| 8 | | | | | | | | | | |
| 8 r | | 4.3 ± 6.10 | 3.7 ± 0.10 | 37.0 ± 1.19 | 102.2 ± 1.84 | 51.8 ± 1.64 | 3.5 ± 0.19 | 4.3 ± 0.58 | 1.6 ± 0.02 | 208 ± 0.79 |
| 9 | | | | 8.6 ± 0.19 | 16.3 ± 0.23 | 7.3 ± 0.23 | 3.6 ± 0.19 | 3.4 ± 0.10 | | 39 ± 0.56 |
| 9 r | | | | | | | 3.7 ± 0.02 | | 2.0 ± 0.28 | 6 ± 0.30 |
| 10 | | | | | | | | | | |
| 11 | 18.2 ± 0.44 | 6.6 ± 1.74 | 9.7 ± 0.90 | 219.5 ± 5.56 | 558.9 ± 11.11 | 323.3 ± 13.58 | 29.3 ± 0.29 | 219.9 ± 4.16 | 37.9 ± 0.76 | 1,423 ± 34.17 |
| 12 | 14.6 ± 0.00 | 5.6 ± 0.90 | 9.3 ± 0.58 | 279.9 ± 3.41 | 646.4 ± 0.53 | 451.7 ± 21.26 | 34.7 ± 0.34 | 404.0 ± 5.42 | 24.0 ± 0.71 | 1870 ± 28.09 |
| 13 | 4.9 ± 0.19 | 2.4 ± 0.19 | 3.6 ± 0.41 | 81.1 ± 3.07 | 300.5 ± 1.28 | 127.4 ± 1.46 | 6.0 ± 0.17 | 50.8 ± 0.21 | 8.4 ± 0.16 | 585 ± 5.67 |
| 14 | 4.0 ± 0.23 | 1.0 ± 1.40 | 2.8 ± 0.05 | 40.3 ± 0.99 | 97.1 ± 4.94 | 47.7 ± 4.16 | 4.3 ± 0.58 | 42.5 ± 1.88 | 4.0 ± 0.09 | 244 ± 13.86 |
| Rh | 54.1 ± 2.11 | 6.6 ± 2.40 | 12.2 ± 0.53 | 820.1 ± 35.78 | 616.4 ± 24.41 | 621.3 ± 31.76 | 85.6 ± 3.87 | 432.6 ± 20.21 | 70.9 ± 2.22 | 2,720 ± 123.29 |





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TABLE 3 Alkaloid content (mean ± SD in ng/mg) in the WCX formic acid eluate of the CY dietary supplements. Alkaloids are identified by name and numbering used in Figure 1 as well as the wavelength used for quantification. Missing entries indicate values were below the LOQ. Samples marked with a lowercase "r" were repurchased. Rh, *Corydalis yanhusuo* rhizome.

| Sample ID | Oblongine (F1; 205 nm) | N-Methyltetrahydrocolumbamine (F2; 205 nm) | N-Methylcorypalmine (F3; 205 nm) | Protopine (F4; 205 nm) | 14-Hydroxy-N-methylcanadine (F5; 205 nm) | Coptisine (F6; 340 nm) | Columbamine (F7; 340 nm) | Dehydrocorybulbine (F8; 340 nm) | Palmatine (F9; 340 nm) | Dehydrocorydaline (F10; 340 nm) | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 48 ± 0.9 | 142 ± 4.9 | 112 ± 2.2 | 272 ± 12.8 | 192 ± 6.6 | 165 ± 4.6 | 147 ± 4.0 | 175 ± 3.9 | 158 ± 4.3 | 677 ± 15.8 | 2088 ± 55.5 |
| 2 | | | | | | | | | 61 ± 1.4 | 10 ± 0.1 | 71 ± 1.5 |
| 2 r | 19 ± 0.3 | 93 ± 3.4 | 73 ± 2.9 | 179 ± 0.5 | 132 ± 1.6 | 249 ± 1.9 | 269 ± 2.2 | 209 ± 1.3 | 296 ± 1.8 | 922 ± 4.2 | 2,440 ± 13.3 |
| 3 | 47 ± 5.2 | 149 ± 15.8 | 120 ± 11.6 | 318 ± 20.2 | 249 ± 30.1 | 171 ± 11.4 | 237 ± 22.4 | 236 ± 23.1 | 260 ± 27.7 | 1,049 ± 108.3 | 2,835 ± 275.8 |
| 4 | 83 ± 23.6 | 287 ± 64.1 | 317 ± 89.6 | 842 ± 224.4 | 715 ± 184.1 | 685 ± 102.7 | 1,020 ± 155.1 | 618 ± 97.5 | 1,058 ± 162.2 | 3,079 ± 477.4 | 8,703 ± 1,452.7 |
| 5 | 124 ± 12.1 | 639 ± 76.8 | 505 ± 78.3 | 1,272 ± 150.8 | 1,102 ± 131.7 | 649 ± 93.5 | 1,076 ± 144.5 | 665 ± 88.5 | 1,094 ± 146.3 | 3,173 ± 416.0 | 10,301 ± 1,338.5 |
| 6 | 16 ± 1.2 | 58 ± 1.7 | 47 ± 1.6 | 108 ± 3.3 | 94 ± 1.1 | 53 ± 0.7 | 84 ± 1.9 | 74 ± 0.3 | 92 ± 0.5 | 321 ± 2.8 | 948 ± 8.2 |
| 7 | 108 ± 9.7 | 341 ± 10.7 | 261 ± 7.5 | 693 ± 16.0 | 523 ± 17.4 | 867 ± 32.1 | 482 ± 10.3 | 463 ± 4.7 | 571 ± 7.2 | 1994 ± 28.8 | 6,303 ± 144.4 |
| 8 | | | | | | | | | | | |
| 8 r | 21 ± 7.8 | 196 ± 49.2 | 154 ± 40.7 | 254 ± 57.8 | 262 ± 61.4 | 124 ± 14.0 | 216 ± 24.8 | 193 ± 22.2 | 218 ± 24.9 | 695 ± 81.3 | 2,334 ± 368.4 |
| 9 | 4 ± 0.1 | 12 ± 0.3 | 10 ± 0.3 | 20 ± 0.1 | 17 ± 0.2 | 16 ± 0.2 | 18 ± 0.5 | 13 ± 0.3 | 20 ± 0.3 | 64 ± 0.9 | 194 ± 2.9 |
| 9 r | | | | | | | | | | | |
| 10 | | | | | | | | | | | |
| 11 | 159 ± 41.8 | 491 ± 119.7 | 374 ± 98.6 | 1,252 ± 268.4 | 919 ± 200.2 | 600 ± 6.0 | 340 ± 0.2 | 287 ± 0.2 | 377 ± 0.2 | 1,265 ± 3.3 | 6,067 ± 718.8 |
| 12 | 82 ± 10.3 | 461 ± 78.4 | 405 ± 72.3 | 1,021 ± 159.7 | 932 ± 151.5 | 1,356 ± 216.0 | 690 ± 124.7 | 697 ± 131.3 | 883 ± 167.8 | 3,032 ± 569.2 | 9,558 ± 1,681.2 |
| 13 | 28 ± 0.4 | 45 ± 0.0 | 36 ± 0.4 | 106 ± 1.2 | 87 ± 1.1 | 68 ± 1.0 | 124 ± 1.0 | 103 ± 0.6 | 140 ± 1.4 | 715 ± 6.1 | 1,451 ± 12.4 |
| 14 | 9 ± 0.1 | 26 ± 0.2 | 20 ± 0.3 | 53 ± 0.9 | 43 ± 0.5 | 44 ± 0.8 | 75 ± 2.2 | 52 ± 1.9 | 67 ± 2.5 | 279 ± 9.7 | 668 ± 18.5 |
| Rh | 188 ± 1.2 | 657 ± 28.3 | 286 ± 14.7 | 1,228 ± 36.1 | 1,249 ± 58.2 | 1,127 ± 69.8 | 982 ± 63.9 | 854 ± 69.7 | 611 ± 46.8 | 2,758 ± 200.8 | 9,939 ± 587.1 |





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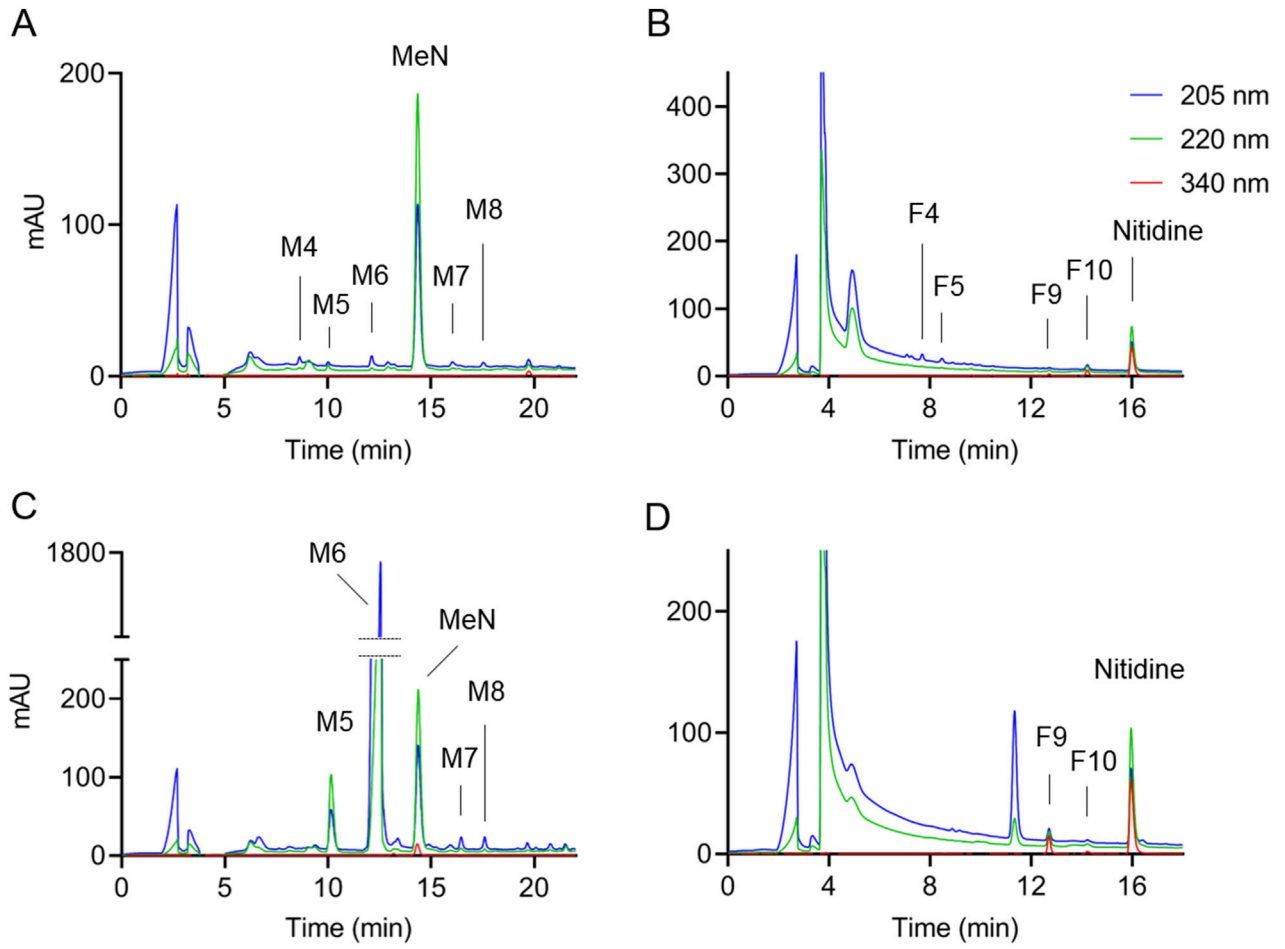


FIGURE 4
Analysis of CY dietary supplements with low or unusual content of alkaloids. RP-HPLC analysis of samples 9 (top) and 2 (bottom) of the **(A, C)** methanol and **(B, D)** formic acid WCX eluates, respectively. Peak labels refer to the numbering of alkaloids in Figure 1. The chromatograms were recorded at 205 nm (blue), 220 nm (green), and 340 nm (red). MeN, methyl nicotinate.

## 4 Discussion

*In vitro* and animal studies testing the biologic effects of CY alkaloids most often use a lab-prepared extract from the rhizome or chemically synthesized or isolated compounds. For consumers interested in exploring the potential health benefits of CY these sources are not readily accessible and they are instead likely to rely on CY dietary supplements as a source of the respective alkaloids. CY dietary supplements are labeled as containing extracts from CY rhizome, but which alkaloid and how much of each is present in a given CY dietary supplement is not listed on the product label (cf. Table 1). To our knowledge, the alkaloid content of CY dietary supplements has not been analyzed and made publicly available, and it is not known whether CY dietary supplements provide an adequate source of bioactive alkaloids.

We have developed a targeted analytical method to quantify CY alkaloids based on their UV/Vis absorbance upon resolution of the compounds by RP-HPLC following extraction on a WCX column. The HPLC-UV/Vis analysis (cf. Figures 2, 4) also provides a visual impression of the relative abundance of alkaloids, normalized by the internal standards, that is representative of the actual content since the molar absorption coefficients are similar within the groups of saturated and unsaturated alkaloids, respectively, (Shammna et al., 1969). The approach was suitable to clearly identify and quantify 19 of the most abundant alkaloids in the CY supplements, and to document their (near) absence in others. While it is possible that the CY supplements contained additional alkaloids not assessed via this approach, these were low in abundance and thus less likely to be biologically relevant. Our quantification method using WCX extraction combined with HPLC and diode array detection provides a simple, sensitive, reliable, and affordable approach for the analysis of alkaloids in CY dietary supplements that can be readily implemented for product control.

A limitation to our approach is that the developed analytical method was not validated. While we are convinced that the lack of validation does not compromise the overall findings on the alkaloid content in the dietary supplements, we want to emphasize a key aspect for the method to be reproduced by other laboratories. We found that especial attention needs to be placed on the correct identification of alkaloid peaks when even seemingly minor modifications are made to chromatographic parameters (solvent composition, gradient), or when switching between HPLC columns

of the identical type. For the alkaloids with closely related UV/Vis spectra changes in retention times or elution order may be difficult to spot, and ideally should be confirmed by comparison to authentic standards.

We found an unexpectedly large range in the content of alkaloids of different CY dietary supplements. Only about one-third of the CY dietary supplements (5 out of 14; samples 4, 5, 7, 11, 12) provided what can be considered an acceptable amount of alkaloids when compared to the type and amount of alkaloids obtained from a CY rhizome sample. A second group of supplements (samples 1, 3, 6, 13, 14) had a considerably lower content of alkaloids, and in the remaining four samples (2, 8, 9, 10) alkaloids were either below quantifiable, low, or inconsistently present when a different batch was analyzed. There was no correlation between the form of the supplement (powder, capsule, liquid) and the content of alkaloids.

Type and relative amount of alkaloids in the supplements with high alkaloid content were similar to what was present in a CY rhizome sample analyzed here and as described in refs. (Ma et al., 2008; Ding et al., 2007). Variability of alkaloid content in response to differences in growing conditions appears to be small relative to the variability in the supplement products observed here (Lu et al., 2020; Chen et al., 2020). It does not seem technically justified then that so many of the supplements had a very low alkaloid content, considering that the process for making CY dietary supplements most likely involves grinding of the rhizome into a powder followed by a simple extraction of the ground rhizome with hot water.

One of the products with an overall low amount of alkaloids (sample 2) stood out due to its high content of tetrahydropalmatine, a cause for concern regarding adulteration as well as potential toxicologic liability (Du et al., 2022; Lai and Chan, 1999). This sample provided about 5 mg tetrahydropalmatine per serving which is about 5-fold below the content of tablets that were causing acute poisoning as a result of unregulated use of biopharmaceuticals containing purified tetrahydropalmatine (Lai and Chan, 1999). In these cases of overdosing, two of the patients were assumed to have consumed up to 1,500 mg and 2000 mg of tetrahydropalmatine, respectively, while the recommended safe daily oral doses were quoted as 60–180 mg (Wang and Mantsch, 2012; Lai and Chan, 1999). While it appears unlikely that consumers would ingest an amount of sample two that might result in toxic exposure to tetrahydropalmatine, it cannot be safely excluded. The high content of tetrahydropalmatine in sample two was most likely due to adulteration whereas the content in the repurchased sample was within the range of other samples. It should be noted that due to safety concerns, certain preparations containing tetrahydropalmatine are banned by the US FDA for human consumption in the US (Horowitz et al., 1996; Woolf et al., 1994).

Two of the three samples that were essentially devoid of alkaloids (samples 8 and 10) were labeled as "Corydalis" – i.e., not as "Corydalis yanhusuo". This could indicate the particular botanical present in the supplement was not CY but possibly another *Corydalis* species, e.g., *Corydalis ambigua*. While this might explain the (near) absence of typical CY alkaloids, there was no indication from our analyses that the samples contained other alkaloids nor did the label suggest that another *Corydalis* species was present. It seems unlikely that consumers would pick up on the difference in the name and change expectations accordingly. Most likely, consumers expect supplements labeled as "Corydalis" to contain a CY extract, similar to us when we selected the products for analysis. This rationale is supported by the fact that sample 12, labeled as "Corydalis poppy powder", had among the highest content of typical CY alkaloids even though the label did not include the term "yanhusuo". Thus, the supplements containing a nearly undetectable or inconsistent amount of alkaloids appear fraudulent even though some of them may not be in strictly legal terms due to the incomplete naming.

The content of alkaloids in CY dietary supplements is a key measure for quality of the products besides any markers of safety–that were not assessed in the current study. The large range in alkaloid content between the products makes it difficult for consumers to find one that meets their expectations regarding product quality, i.e., alkaloid content. A large fraction of the available products may not meet consumer expectations, and when a product does not deliver an expected biologic effect it might be due to the lower than expected content of bioactive alkaloids. Consumers unsure about which CY dietary supplement to select may want to try different products or might consider using the rhizome since our analyses suggested that the content of alkaloids in the ground CY rhizome exceeds that of any supplement, and in addition there is less opportunity for adulteration. For researchers interested in testing CY in animal or human studies it is advisable to conduct a thorough chemical analysis of the product intended for use, a requirement that extends to all clinical and non-clinical studies with botanical products (Hosbas Coskun et al., 2021; Funk and Schneider, 2021; Sorkin et al., 2022; Heinrich et al., 2022).

We have previously participated in a study aimed at analyzing turmeric dietary supplements with regard to curcuminoid content and safety parameters (Skiba et al., 2018). The study found that turmeric dietary supplements in the US marketplace largely contained the declared amount of curcuminoids and were safe with respect to residual solvents from the extraction process and free from lead contamination. Turmeric/curcumin is a top selling dietary supplement in the US and around the world (Panknin et al., 2023; Skiba et al., 2020), and the size of the market may contribute to an overall consistent quality as well as efficient monitoring. CY dietary supplements, in contrast, are more of a niche product, and this may underlie current deficiencies in content and control of available products. An overview on the current status of adulteration of botanical products focusing on five major ingredients including turmeric can be found in ref. (Orhan et al., 2024).

Our analysis of alkaloid content in CY dietary supplements has established a baseline profile and amount of alkaloids equivalent to the natural product that was present in about one-third of the products. We have uncovered substantial deficiencies in this product segment available to US customers online. The large variability in alkaloid content between and within different products, with some appearing fraudulent or substantially adulterated from the natural product, seems unacceptable. The excessive amount of tetrahydropalmatine in one of the products was alarming and possibly a health concern. The shortcomings are unlikely due to variability in the natural product used as source. In the current marketplace consumers

and researchers alike should be aware that not all CY dietary supplements may meet expectations regarding key quality criteria.

## Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: https://osf.io/b68ps/.

## Author contributions

PL: Conceptualization, Data curation, Formal Analysis, Investigation, Writing–review and editing. CS: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, 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.

## Publisher's note



## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2024.1518750/full#supplementary-material

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# Euodiae Fructus: a review of botany, application, processing, phytochemistry, quality control, pharmacology, and toxicology

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Euodiae Fructus (EF) is the dried and nearly ripe fruit of *Euodia rutaecarpa*, first recorded in *Shen Nong's Herbal Classic*. EF is a versatile Traditional Chinese Medicine (TCM) known for the effects of dispelling colds and alleviating pain, suppressing adverse qi to relieve vomiting, and boosting yang to mitigate diarrhea. However, it should be noted that EF possesses mild toxicity. In TCM prescriptions, EF is employed to treat various ailments, including abdominal pain, diarrhea, chronic non-atrophic gastritis, irritable bowel syndrome, and primary dysmenorrhea. This review collected the literature published before September 2024 on EF. An exhaustive analysis of EF literature was conducted utilizing multiple sources, namely classic TCM books and various scientific databases like Web of Science, PubMed, Elsevier, ACS, ResearchGate, Google Scholar, and Chinese National Knowledge Infrastructure. So far, more than 300 metabolites have been extracted and identified from EF, exhibiting various pharmacological effects, such as cardiovascular protection, gastrointestinal protection, neuroprotection, anti-inflammation, analgesia, anti-tumor, glucose and lipid metabolism regulation, etc. It also exhibits diverse toxicological properties and poses specific toxic risks to the liver, heart, and kidney. Nonetheless, research is scarce regarding the toxicology of EF, especially on its cardiotoxicity and nephrotoxicity. Further in-depth research is necessary to explore the mechanisms underlying EF's pharmacological and toxicological mechanisms and to develop strategies for quality control and toxicity mitigation. The toxicity of EF can be reduced by processing, but this aspect is rarely discussed, and the quality control needs to be further standardized. Evodiamine, rutaecarpine, and limonin are the effective metabolites of EF and are also one of the causes of EF toxicity. The pharmacological effects of evodiamine and rutaecarpine have been intensely studied, but there are few studies on limonin and other metabolites of EF. Therefore, this paper focuses on the botanical characteristics, traditional applications, processing methods, phytochemistry, quality control, pharmacology, and toxicology of EF. We hope this paper provides a theoretical basis for the future high-value and high-connotation development of EF.

KEYWORDS

**Euodiae Fructus, traditional uses, processing, phytochemistry, quality control, pharmacology, toxicology**

# 1 Introduction

EF is a dry, nearly ripe fruit of the genus *Euodia rutaecarpa*, first recorded in the top grade of *Shen Nong's Herbal Classic* (Dong Han Dynasty, A.D. 25–220). It relieves cold and pain, suppresses adverse qi to relieve vomiting, and enhances yang to stop diarrhea. Nonetheless, it is essential to acknowledge that EF has a slight toxic effect. EF ranks among the most prevalent botanical drugs clinically in TCM, boasts a history of more than 2000 years, and has been formally listed in various editions of Chinese Pharmacopoeia (ChP) (https://www.nmpa.gov.cn/). Lately, numerous studies have concentrated on examining the metabolites, pharmacological effects, clinical function, and toxicology of EF. Up to this point, more than 300 metabolites have been extracted and pinpointed from EF (Xiao et al., 2023). Contemporary research indicates that EF's primary active elements comprise alkaloids, terpenoids, flavonoids, volatile oils, and other compounds (Liu L. et al., 2020). Among them, evodiamine, rutaecarpine, and limonin are characteristic metabolites (Tian et al., 2019). Research in pharmacology reveals that EF, along with its raw extract and refined form, offers a range of pharmacological effects, such as cardiovascular protection, gastrointestinal protection, neuroprotection, anti-inflammation, analgesia, anti-tumor, and glucose and lipid metabolism regulation. In clinical settings, this medication serves as both a supplement and a substitute treatment for conditions like abdominal pain, vomiting, diarrhea, indigestion, hypertension, eczema, and oral ulcers (Huang et al., 2021). EF, combined with various botanical drugs, is effective in gastrointestinal diseases, headaches, vomiting, skin diseases, dysentery, menorrhagia, and postpartum hemorrhage (Li D. et al., 2022).

However, it is important to recognize that excessive use of EF may lead to stomach pain, vomiting, blurred vision, and other toxic symptoms (Cai et al., 2006; Ma et al., 2018). As EF's clinical application has expanded, its toxicity has become increasingly apparent. It is widely accepted among scholars that the toxicity of EF may be attributed to reactive metabolites (RMs) produced by the metabolic activation of evodiamine, rutaecarpine, and limonin (He et al., 2024). Studies have demonstrated that different parts of EF can induce varying degrees of hepatic injury in rats (Liu et al., 2015), and EF has obvious toxic damage to the human liver (Teschke et al., 2014). Some studies have also highlighted the heart and kidney as possible focal points for EF toxicity. Yet, investigations into EF cardiotoxicity and nephrotoxicity processes are scarce and insufficient for the specific toxic risks and potential disadvantages of EF. Exploring the toxic metabolites of EF and methods for mitigating its toxicity is crucial to guiding safe clinical use. This paper reviews the plant morphology, traditional application, processing, phytochemistry, quality control, pharmacology, toxicology, monitoring, and prevention of EF. Particular emphasis is placed on discussing the mechanisms of EF-induced cardiotoxicity and nephrotoxicity, strategies for reducing and controlling EF's toxicity, and preventive measures for clinical monitoring.

# 2 Botany

The ChP recorded the dried and nearly ripe fruit of three plants of the genus *Euodia rutaecarpa* (Juss.) Benth. (ER), *Euodia rutaecarpa* (Juss.) Benth. var. *officinalis* (Dode) Huang (ERO), *Euodia rutaecarpa* (Juss.) Benth. var. *bodinieri* (Dode) Huang (ERB). *Euodia rutaecarpa* is also divided into large grains and small grains. ER primarily supplies large grains, categorized into large EF flowers (LEF) and medium EF flowers (MEF). LEF has reached full ripeness, the fruit shows cracks, and its effectiveness is subpar. Approximately seven mature MEFs, characterized by a yellowish-green and potent odor, are commonly utilized in medical treatments. ERO and ERB primarily supply diminutive grains, predominantly consisting of small EF flowers (SEF), often immature and green. The diminutive size of ERO and ERB fruits typically classifies them as ER varieties, distinct from ER due to their unique, strong smells. The botanical characteristics of ER, ERO, and ERB are similar to those of dried fruits, as shown in Figure 1.

The *Euodia rutaecarpa* are shrubs or trees, standing 3–5 m high, and are thickly adorned with grayish yellow, rust-red downy hair or have few hairs and dark purplish-red shoots. The leaves have 5–11 leaflets, ovate, elliptic, or lanceolate, 6–18 cm long and 3–7 cm wide. Inflorescences are terminal and dioecious. Male inflorescence flowers are separated from each other, with petals measuring 3–4 mm long. Female inflorescences are dense or distant, and petals measure 4–5 mm long. Most of the sepals and petals are 5 pieces, occasionally 4 pieces, arranged in a pincer pattern. The fruit is spherical or slightly pentagonal oblate, and the surface is dark greenish, yellow, or brown. The outer pericarp has oil spots. The inner pericarp is a thin shell or woody, waxy yellow or brown, and the ovary can be seen as 5-located with 1 seed per mericarp. At its peak is a star-shaped fissure with five points, while its base features a calyx and a fruit stalk and is adorned with yellow hairs. The quality is hard and crisp, with a full green color and rich aroma is better. However, the botanical characteristics of ER, ERO, and ERB are different in growth form, maturity period, ecological environment, and resource distribution, as shown in Table 1.

*Euodia rutaecarpa* cultivation began at the end of the Eastern Han Dynasty. *Miscellaneous Records of Famous Physicians* (Wei and Jin Dynasties, A.D. 220–450) first recorded that "*Euodia rutaecarpa* is grown in the valley, picked on September 9, kept cool and dry, and kept as long as possible". EF had become a widely used medicine during the Tang Dynasty, with renowned poet Wang Wei noting that *Euodia rutaecarpa* could be seen throughout mountains during the Double Ninth Festival. Contemporary research indicates that *Euodia rutaecarpa* thrives in sunlit, warm environments, typically flourishing in thinly spread forests or shrubs in mountainous areas ranging from flat to 1,500 m above sea level, predominantly on sunlit inclines. It is relatively cold resistant, but in cold, windy, and dry areas in winter, and in areas with many diseases, the results are low, and the growth is poor. *Illustrated Classic of Materia Medica* (Song Dynasty, A.D. 960–1,279) recorded that "*Euodia rutaecarpa* can be found everywhere, especially in Jiangsu, Zhejiang, and Sichuan. Jiangxi is EF's the authentic origin, rich in high-quality MEF. It is distributed in the north and south of Jiangxi Province, mainly in urban Zhangshu, Fengcheng, Gaoan, Xingan, Xiajiang, Xinyu, and Jishui. As clinical needs swiftly rose, numerous provinces introduced *Euodia rutaecarpa*. Now, it is distributed in the south of the Qinling Mountains in China, mainly in Guizhou, Guangxi, Hunan, and Yunnan Provinces (Figure 2). Nowadays, *Euodia rutaecarpa* is predominantly thrived in Asia, East Africa, and Oceania, with extensive cultivation in ancient Japan and Korea. *Euodia*

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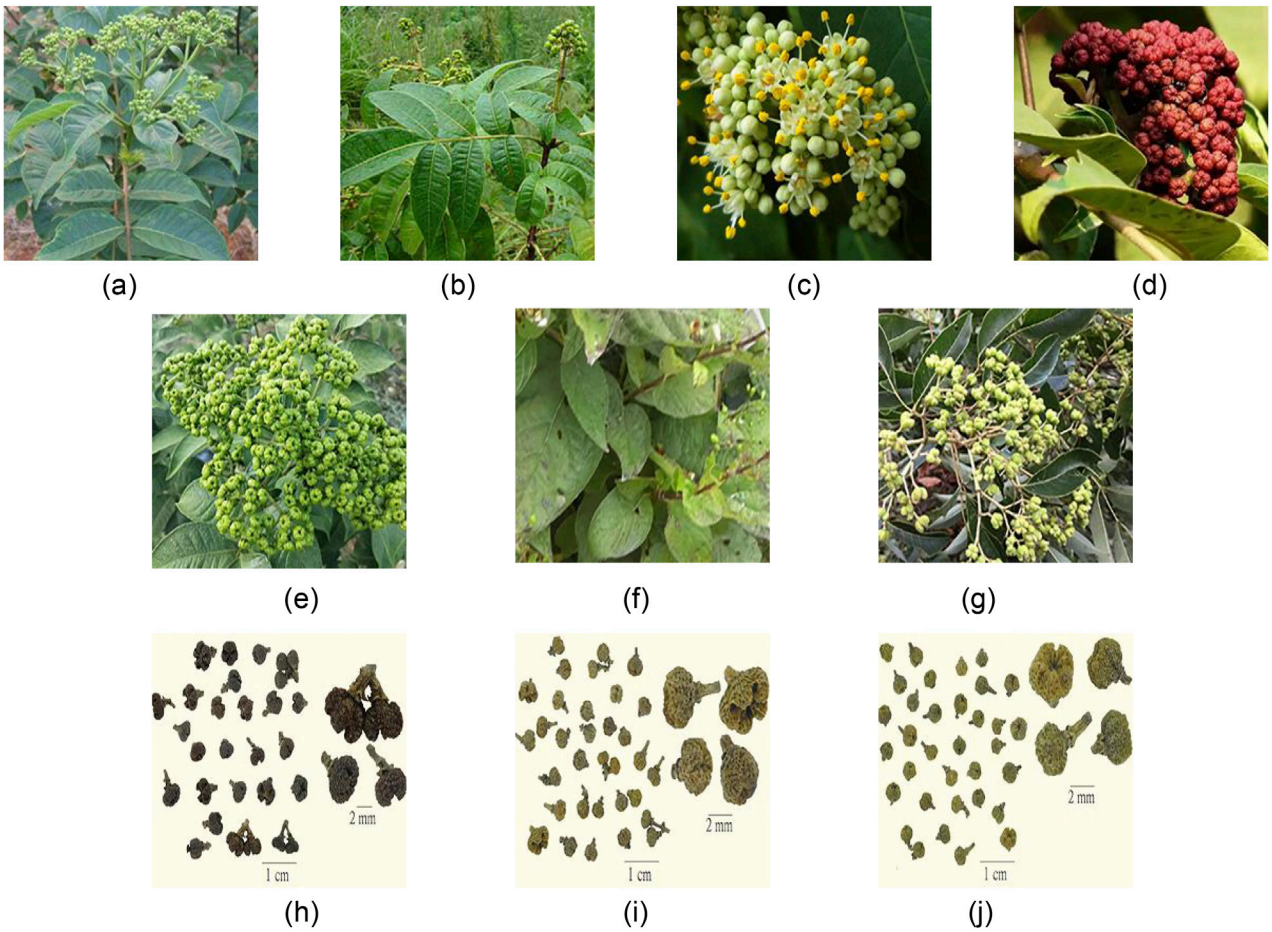


FIGURE 1
The above-ground portion **(A)**, leaves **(B)**, flowers **(C)**, fruits **(D)**, ER **(E)**, ERO **(F)**, ERB **(G)**, LEF **(H)**, MEF **(I)** and SEF **(J)** of *Euodia rutaecarpa*.

*rutaecarpa* was introduced in Korea during The Three Kingdoms Period, and the *Goryeo Master Fang* (Wei and Jin Dynasties, A.D. 220–420) recorded the treatment of beriberi with EF (Nam et al., 2016). *Euodia rutaecarpa* was introduced in Japan during the Edogawa period. EF and its namesake, Goshuyuto (known as Wuzhuyu decoction in Chinese), are frequently utilized in clinical settings (Hibino et al., 2009a).

# 3 Traditional applications

## 3.1 The drug application of EF

EF was first recorded in *Shen Nong's Herbal Classic*, which records the nature, taste, meridian tropism, and efficacy of EF, laying the foundation for the modern clinical application of EF. Over time, the therapeutic impact of EF has evolved through extensive research. It is pointed out that EF can treat many diseases, such as Jueyin headache, hernia, abdominal pain, beriberi, vomiting blood, acid regurgitation, and diarrhea (Table 2). Concurrently, *Miscellaneous Records of Famous Physicians* initially documented EF's mild toxicity, with ongoing research enhancing EF's toxicology. *Illustrated Classic of Materia Medica* recorded that EF harms the eyes and hair. *Amplified Herbology* (Song Dynasty, A.D. 960–1,279) recorded that EF damages the intestines and stomach. *Correlation between Materia Medica Companion* (Ming Dynasty, A.D. 1,368–1,644) recorded that EF damages the healthy atmosphere. Mouth ulcers, tongue sores, and dizziness caused by excessive consumption of EF are recorded in *Compendium of Materia Medica* (Ming Dynasty, A.D. 1,368–1,644). The ChP recorded the minor toxicity of EF and stipulated that the dosage of EF was 2–5 g.

## 3.2 The classic prescription application of EF

EF has been a staple in clinical prescriptions since antiquity (Table 3). Wuzhuyu decoction, named after the monarch medicine EF in *Treatise on Febrile Diseases* (Dong Han Dynasty, A.D. 25–220), has the effect of warming the middle to replenish deficiency, lowering qi and stopping vomiting. It was the earliest record of the use of EF in clinical treatment. *Synopsis of the Golden Chamber* (Dong Han Dynasty, A.D. 25–220) mentioned twice the EF of warming channels, dispelling cold, and stopping vomiting, which is an important medicine for tonifying the spleen and stomach. Among the over 5,000 prescriptions in *Thousand-Gold Essential Formula for Emergency* (Tang Dynasty, A.D. 618–907),

TABLE 1 The differences in botanical descriptions between three plants.

| Plant | Botanical morphology | Mature period | Ecological environment | Resource distribution |
|---|---|---|---|---|
| ER | Odd-pinnate compound leaves opposite, leaflets thin to thick papery, ovate or elliptic, apex abruptly narrowed into mucronate, base cuneate to broadly cuneate or rounded, entire margin or indistinctly serrate, lateral veins indistinct, both sides are yellowish brown puberulent, especially on veins, with distinct large and numerous oil spots. The fruit is single or several together, spherical or slightly pentagonal and oblate, about 3–6 mm in diameter | The flowering period is from June to August, and the fruiting period is from September to October | It grows at low elevations under or on the margins of open forests facing the sun | It is mainly distributed in Shaanxi, Gansu, Anhui, Zhejiang, Fujian, Taiwan, Hubei, Hunan, Guangdong, Guangxi, Sichuan, Guizhou, and Yunnan |
| ERO | The leaves are narrow, oblong to narrowly lanceolate, apex acuminate or long acuminate, and the leaflets are distant. Both sides are densely villous, the veins are the densest, and the oil glands are thick. The inflorescence rachis is often covered with yellowish villous hairs. The ripe inflorescence is not as dense as the orthodox. The fruit is smaller and less than 3.5 mm in diameter. The seeds are bluish-black | The flowering period is from July to August, and the fruiting period is from September to October | Born in the hillside grass | It is mainly distributed in Zhejiang, Jiangxi, Hubei, Hunan, Guangxi, Sichuan, and Guizhou |
| ERB | Branchlets are sparsely hirsute with yellow rust or silky color, leaf rachis villous. The leaf shape is oblong, lanceolate, ovate-lanceolate; the upper surface midvein is slightly sparsely pubescent, the lower vein is pubescent, the lateral vein is precise, and the oil gland is small. The fruit is small, mung bean-colored in appearance, and less than 3.5 mm in diameter | The flowering period is from July to August, and the fruiting period is from September to October | Born on the village side of the road and hillside grass | It is mainly distributed in Jiangxi, Hunan, Guangdong, Guangxi, and Guizhou |

there were 143 prescriptions mentioned EF. The Essential Secrets from the *Imperial Library* (Tang Dynasty, A.D. 618–907) had over 6,000 prescriptions, and the number of prescriptions contained EF reached 176. *Formula of Peaceful Beneuolence Pharmacy* (Song Dynasty, A.D. 1,078–1,085) was the first official preparation standard. This book recorded a total of 788 prescriptions, and 13 referred to EF. In the clinical realm, essential formulas featuring EF encompass the Wuzhuyu Decoction, Zuojin Pill, Wenjing Decoction, and Sishen Pill, among others.

In addition to examining EF in traditional medical texts, the study of EF in botanical drugs has been extensively explored in contemporary medical settings. Wuzhuyu Decoction can treat chronic non-atrophic gastritis (Hu et al., 2023), chronic migraine (Pan et al., 2015; Nan et al., 2022), alcoholic gastric ulcer (Wang X. et al., 2023), and atherosclerosis (Li C. et al., 2022). Zuojin Pill has pharmacological effects such as anti-tumor (Peng et al., 2015), protection of gastric mucosa, anti-inflammation, anti-ulcer, and so on (Wang et al., 2015; Zhang J. et al., 2022). It can treat bile reflux gastritis (Li Y. Y. et al., 2022) and septic lung injury (Yin et al., 2021). Wenjing Decoction can treat primary dysmenorrhea (Gao et al., 2017) and endometriosis (Huang et al., 2023). Sishen Pill has anti-inflammatory and anti-tumor pharmacological effects (Zhang B. et al., 2024), can treat abdominal pain, diarrhea (Li et al., 2024), irritable bowel syndrome (Zhang X. Y. et al., 2021; Zhao et al., 2024), and insomnia (Wang L. X. et al., 2023). EF is recommended as the primary treatment in Japan for ailments like cold headaches, dysmenorrhea, and inflammatory pain in rheumatoid joints (https://www.mhlw.go.jp/index.html). Certain EF-containing prescriptions, including Changkang Tablets, Huatuo Zaizao Wan, Compound Berberine Tablets, and Jiawei Zuo Jin Wan, have undergone extensive research and clinical application. Changkang Tablets are used in the treatment of dysentery, abdominal pain, and tenesmus (Shi et al., 2013; Guan et al., 2020), Huatuo Zaizao Wan can treat Alzheimer's disease (Jiang et al., 2023) and stroke (Duan et al., 2017).

# 4 Phytochemistry of EF

Presently, about 300 metabolites of EF have been isolated and purified, which are mainly divided into alkaloids (1–148), terpenoids (149–184), flavonoids (185–213), volatile oils (214–283), and others (284–299). These metabolites are summarized in Supplementary Table S1, and their structures are shown in Supplementary Figures S1–S8. The alkaloids are primarily categorized into indoles and quinolones (Li D. W. et al., 2020). Evodiamine and rutaecarpine in indole are the index metabolites of EF. The limonin in terpenoids plays a crucial role in EF.

## 4.1 Alkaloids

Alkaloids are the metabolites of EF, mainly composed of indoles (Supplementary Figure S1) and quinolones (Supplementary Figure

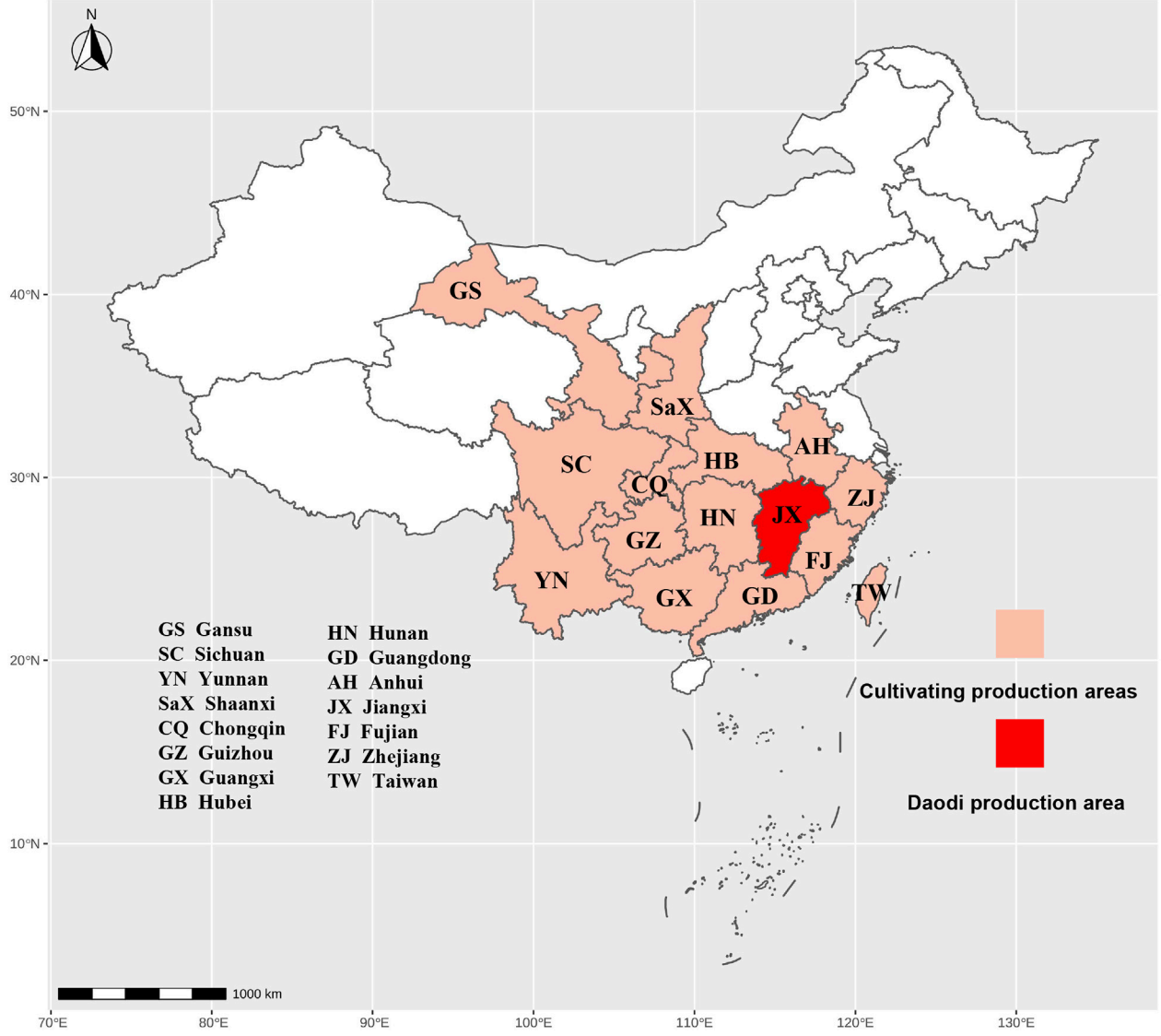


FIGURE 2
Cultivation and distribution of *Euodia Rutaecarpa* in China.

S2). Alkaloids fundamentally possess a circular form and are non-soluble in water, resulting in a higher concentration of EF alkaloids in ethanol. Indoles are synthesized mainly through methanesulfonic acid and amino acids. Over ten different compounds, including evodiamine, rutaecarpine, and dehydroevodiamine, were extracted from EF (Zuo et al., 2000; Wang Q. Z. et al., 2010; Wang T. Y. et al., 2010; Wang X. X. et al., 2013; Li et al., 2014; Zhao N. et al., 2015; Ma et al., 2021; Qin et al., 2021; Zhao X. M. et al., 2021). Evodiamine and rutaecarpine are the most important metabolites, and their contents are also the highest in EF (Huang et al., 2019; Li D. W. et al., 2020). Dihydroevocarpine and evocarpine are the crucial metabolites of quinolones found in EF (Li Y. H. et al., 2020). Furthermore, the group includes quinolines, organic amines, acridone, and purines (Minh et al., 2003; Kim et al., 2022) (Supplementary Figure S3). Research revealed a reduction in the levels of evodiamine, rutaecarpine, and carpine in EF correlating with the fruit's diminution. Some commercial evodiamine and rutaecarpine in SEF are below the content specified in ChP (Cao et al., 2019).

## 4.2 Terpenoids

Typically, terpenoids (Supplementary Figure S4) originate from methylpentanedioic acid, with isoprene forming the fundamental structural component of the molecular framework (Zhao H. et al., 2021; Qian et al., 2014). Limonin is an oxidized tetracyclic triterpene with a distinctive furan ring, and it is a terpenoid extracted and recognized from EF. They constitute the material basis of the bitter properties of EF (Bae et al., 2020). Its representative metabolites are limonin and rutaevine, and limonin is another index metabolite of EF. In addition, there are high contents of metabolites, such as evodol, obacunone, rutaevine acetate, 6β-acetoxy-5-epillimonin,

TABLE 2 The traditional uses of EF in China.

| Traditional uses | Dynasty/ Years | References |
|---|---|---|
| Warming the middle to dispel colds, relieve cough, remove dampness, and stop arthralgia | Eastern Han Dynasty | *Shen Nong's Herbal Classic* 《神农本草经》 |
| Dispelling phlegm, relieving heartache, and reconciling the five internal organs | Wei and Jin Dynasties | *Miscellaneous Records of Famous Physicians* 《名医别录》 |
| Killing insects and warming Yang | Tang Dynasty | *A Supplement to Materia Medica* 《本草拾遗》 |
| Warming the spleen, promoting digestion and defecation. Treatment of cold pain in the heart and abdomen, vomiting and diarrhea | Tang Dynasty | *Theory of Medicinal Properties* 《药性论》 |
| Strengthening the spleen, promoting joint function, eliminating phlegm, dispelling wind, treating abdominal pain, athlete's foot, edema, and postpartum blood stasis | Five Dynasties | *Rihuazi Bencao* 《日华子本草》 |
| Relieving sore throat and chest-relaxing | Jin and Yuan Dynasties | *Properties and Actions of Medicinals* 《药类法象》 |
| Relieving heart, abdominal pain and alcohol | Jin and Yuan Dynasties | *Medical Origins* 《医学启源》 |
| Treatment of acid regurgitation, abdominal pain, hernia, dysentery, mouth sores | Ming Dynasty | *Compendium of Materia Medica* 《本草纲目》 |
| Relieving cough, expelling wind, eliminating food, dispelling arthralgia | Ming Dynasty | *Explain of Medicinal Properties* 《药性解》 |
| Moistening the liver, invigorating the spleen, relieving depression, removing phlegm, killing insects, and dispelling cold. Treatment of Jueyin headache, hemorrhoids, and dysentery | Qing Dynasty | *Bencao Beiyao* 《本草备要》 |
| Descending qi, dispelling cold, eliminating abdominal distension. Treatment of acid regurgitation, diarrhea, abdominal pain, beriberi, edema, and aphtha | Qing Dynasty | *Bencao Qiuzhen* 《本草求真》 |
| Dispelling cold, tonifying the lung, relieving pain, activating blood circulation, and dispelling arthralgia | Qing Dynasty | *Notes on Shen Nong's Herbal Classic* 《神农本草经读》 |
| Warming the stomach, dispelling cold, relieving pain, stopping cough, promoting blood circulation | Qing Dynasty | *Record of One Hundred Species of Shen Nong's Herbal Classic* 《神农本草经百种录》 |
| Treatment of Jueyin headache, hernia, abdominal pain, beriberi, hematemesis, acid regurgitation, and diarrhea | 2020 | Chinese pharmacopoeia 《中国药典》 |

jangomolide, and shihulimonin A (Lacroix et al., 2011). Due to their significant solubility in fats, terpenoids are typically processed using an ethanol solvent. Research indicates that limonin levels in SEF and MEF are notably elevated, approximately 0.74% and 0.65%, respectively, in contrast to LEF's mere 0.24% limonin content (Zhang et al., 2021c).

## 4.3 Flavonoids

Flavonoids (Supplementary Figure S5) generally refer to a series of metabolites formed by connecting two benzene rings with three carbon atoms. EF additionally has a higher content of flavonoids. It mainly includes these three metabolites: flavonoids and their glycosides, flavonols and their glycosides, and flavonones and their glycosides. Flavonoids and their glycosides are predominantly associated with *O*-glucose, *O*-xylose, *O*-galactose, *O*-rhamnose, *O*-rue sugar, and *O*-mulberry disaccharide. Flavonols and their glycosides mainly include quercetin, isorhamnetin, limocitrin glycosides, and aglycones (Xiao et al., 2023; He et al., 2024). Flavonones and their glycosides are phellodensin F, catechin, and hesperidin, respectively (Zhao Z. et al., 2015; Li and Wang, 2020).

## 4.4 Volatile oil

EF has a strong and fragrant smell because of its high content of volatile oil (Supplementary Figure S6). The volatile oil metabolites are monoterpene, sesquiterpene, aliphatic, and aromatic. Within the isolated volatile oil, the proportions of sesquiterpenes exceed 38%, monoterpenes surpass 35%, and esters exceed 13% (Liu S. S. et al., 2019). Furthermore, while EF volatile oil exhibits significant pharmacological properties, it simultaneously constitutes a toxic metabolite of EF. Its metabolites are monoterpenoids, such as myrcene and (*E*)-ocimene, and sesquiterpenes, such as β-caryophyllene and β-elemene.

## 4.5 Others

EF also contains some organic acids (Supplementary Figure S7), including caffeic acid, citric acid, isocitric acid, trans-caffeoylgluconic acid, and feruloylgluconic acid. EF also contains phenylpropanoids, mainly divided into simple phenylpropanoids, coumarins, and lignans, most of which belong to simple phenylpropanoids. Simple phenylpropanoids include

TABLE 3 Traditional application of EF in classic prescriptions.

| Classification | Formula name | Main compositions | Dosage form | • Traditional efficacy ▪ Clinical applications | EF's function | References |
|---|---|---|---|---|---|---|
| Classic prescription | Wuzhuyu Tang | Euodiae Fructus, Ginseng Radix Et Rhizoma, Zingiberis Rhizoma Recens, Jujubae Fructus | Decoction | • Tonifying deficiency in warming and stopping vomiting<br>▪ Chronic gastritis, nervous vomiting, and otogenic vertigo | Warming liver for dispelling cold | *Treatise on Febrile Diseases* 《伤寒论》 |
| | Wenjing Tang | Euodiae Fructus, Cinnamomi Ramulus, Angelicae Sinensis Radix, Chuanxiong Rhizoma, Moutan Cortex, Asini Corii Colla, Paeoniae Radix Alba, Ophiopogonis Radix, Ginseng Radix et Rhizoma, Glycyrrhizae Radix et Rhizoma, Pinelliae Rhizoma, Zingiberis Rhizoma Recens | Decoction | • Warming channels and dispelling cold<br>▪ Functional uterine bleeding and chronic pelvic inflammatory | Disperse cold and alleviate pain | *Synopsis of the Golden Chamber* 《金匮要略》 |
| | Wu Ji Wan | Coptidis Rhizoma, Euodiae Fructus, Paeoniae Radix Alba | Pill | • Soothing liver and regulating spleen<br>▪ Abdominal pain, stomachache, and chronic diarrhea | Harmonizing stomach for descending adverse qi | *Formula of Peaceful Beneuolence Pharmacy* 《太平惠民和剂局方》 |
| | Ai Fu Nuan Gong Wan | Artemisiae Argyi Folium, Cyperi Rhizoma, Euodiae Fructus, Cinnamomi Cortex, Angelicae Sinensis Radix, Chuanxiong Rhizoma, Paeoniae Radix Alba, Rehmanniae Radix, Astragalus Radix, Dipsaci Radix | Pill | • Warming uterus and regulating menstruation<br>▪ Irregular menstruation, and dysmenorrhea | Warming channel, dispelling cold and promote blood circulation | *Standards for Diagnosis and Treatment* 《仁斋直指方论》 |
| | Zuo Jin Wan | Coptidis Rhizoma, Euodiae Fructus | Pill | • Purging fire and soothing the liver<br>▪ Esophagitis, gastritis and peptic ulcer | Lowering adverse flow of qi and arresting vomiting | *Danxi's Mastery of Medicine* 《丹溪心法》 |
| | Sishen Wan | Psoraleae Fructus, Euodiae Fructus, Myristicae Semen, Schisandrae Chinensis Fructus, Jujubae Fructus | Pill | • Warming the kidney and dispelling cold<br>▪ Chronic diarrhea and irritable bowel syndrome | warming spleen and stomach for dispelling cold | *Standards for Diagnosis and Treatment* 《证治准绳》 |
| | Ji Ming San | Arecae Semen, Citri Reticulatae Pericarpium, Chaenomelis Fructus, Euodiae Fructus, Platycodonis Radix, Zingiberis Rhizoma Recens, Perillae Folium | Decoction | • Regulating the qi flowing in the channels<br>▪ Beriberi and rheumatoid arthritis | dissipating cold and eliminating dampness | *Standards for Diagnosis and Treatment* 《证治准绳》 |
| TCM preparation | Changkang Tablets | Berberine hydrochloride, Euodiae Fructus, Aucklandiae Radix | Pill | • Clearing heat and dampness, regulating qi and relieving pain<br>▪ Diarrhea, dysentery and abdominal pain | dissipating cold and eliminating dampness | Chinese pharmacopoeia 《中国药典》 |
| | Huatuo Zaizao Wan | Concentrated water-honeyed pill composed of Chuangxiong Rhizoma, Euodiae Fructus, Borneolum Syntheticum, etc | Pill | • Promoting blood circulation, resolving phlegm and dredging collaterals<br>▪ Stroke and its sequelae | Warming channel, dispelling cold and move qi to relieve pain | Chinese pharmacopoeia 《中国药典》 |
| | Jiawei Zuo Jin Wan | Coptidis Rhizoma, Euodiae Fructus, Scutellariae Radix, Bupleuri Radix, Aucklandiae Radix, Cyperi Rhizoma, Curcumae Radix, Paeoniae Radix Alba, Citri Reticulatae Pericarpium Viride, Aurantii Fructus, Citri Reticulatae Pericarpium, Corydalis Rhizoma, Angelicae Sinensis Radix, Radix Glycyrrhizae Preparata Radix et Rhizoma | Pill | • Soothing the liver and relieving depression to relieve pain<br>▪ Acute or chronic hepatitis or gastritis | Lowering adverse flow of qi and arresting vomiting | Chinese pharmacopoeia 《中国药典》 |

(Continued on following page)

TABLE 3 (*Continued*) Traditional application of EF in classic prescriptions.

| Classification | Formula name | Main compositions | Dosage form | • Traditional efficacy<br>▪ Clinical applications | EF's function | References |
|---|---|---|---|---|---|---|
| | Compound Berberine Tablets | Berberine hydrochloride, Euodiae Fructus, Aucklandiae Radix, Paeoniae Radix Alba | Pill | • Heat-clearing, damp-drying, stopping dysentery and diarrhea<br>▪ Acute gastroenteritis, dysentery and chronic diarrhea | Warming channel, dispelling cold and promote blood circulation | Chinese pharmacopoeia《中国药典》 |


FIGURE 3
Flow chart of processing EF with licorice.

p-hydroxycinnamic acid, ferulic acid, coniferin, chlorogenic acid, etc. Besides the metabolites mentioned above, EF also contains anthraquinones, such as chrysophanol, emodin, and physcion, and steroids, such as β-sitosterol, β-daucosterol, and β-stigmasterol (Supplementary Figure S8).

# 5 Processing

## 5.1 Traditional processing methods

Processing is the essence of TCM application, which can increase the efficacy and reduce the toxicity of drugs. EF's processing boasts an extensive historical background, with raw EF typically exerting a significant influence in heating the spleen and eliminating cold. The long traditional technology of processing EF with licorice is pointed out in the *Synopsis of the Golden Chamber. Master Lei's Treatise on Drug Processing* (Northern and Southern Dynasties, A.D. 420–479) recorded that EF is processed with salt to enhance the analgesic effect and vinegar to correct the taste. *Materia Medica for Dietotherapy* (Tang Dynasties, A.D. 618–907) recorded that EF could enhance the antiemetic effect after processing ginger and the analgesic effect after being processed with yellow rice wine. *General Records of Holy Universal Relief* (Song Dynasties, A.D. 1,078–1,085) recorded that EF can reduce toxicity when processed with soybean products. *Prescriptions for Universal Relief* (Ming Dynasty, A.D. 1,368–1,644) recorded that EF fried with Psoraleae Fructus can enhance the antidiarrheal effect. *Wonderful Well-Tried Recipes* (Ming Dynasty, A.D. 1,368–1,644) recorded that EF processed with Coptidis Rhizoma can enhance the antiemetic effect. The process and method of processing EF with licorice are described in detail in ChP (Figure 3). In addition, different doses of licorice can affect EF's chemical composition and pharmacological effects (Xiao et al., 2012). Certain academics have experimented with varying the EF to licorice dosage ratios, discovering the optimal 100:6.

## 5.2 Enhance efficiency and reduce toxicity

In its extended clinical application, TCM has developed distinct theories and techniques for detoxifying and improving its healing impact, encompassing both processing and compatibility (Li R. L. et al., 2021). Special processing techniques such as licorice, salt, ginger, and vinegar can reduce the toxicity of EF. The ChP stipulates that EF ought to be fried alongside licorice. Some scholars have found that processing EF with licorice can reduce the toxicity of alkaloids (Ren et al., 2023a). The cytochrome P450 (P450, CYP) enzyme activates evodiamine in EF during metabolism, which leads to liver injury and inflammation. Licorice can reduce the toxicity of EF by inhibiting the P450 enzyme and blocking the metabolic activation of EF (Ren et al., 2024). Licorice can obstruct EF protein coupling by suppressing the P450 enzyme, elevating GSH levels in human liver cells, and mitigating the GSH reduction induced by EF (Ren et al., 2023b). Salt-processed EF can introduce drugs into the kidney channel, reduce toxicity, and ensure the safety of clinical drug use (Hou et al., 2023). When EF is combined with ginger, its antiemetic properties can be amplified. Some studies found that EF processed with ginger, licorice, and salt had better antitoxic effects (Zhang M. et al., 2021). Other studies found that the three processing methods of ginger, licorice, and vinegar could reduce the content of rutaecarpine in EF, with vinegar processing increasing the content of evodiamine, and licorice processing had the most significant effect on reducing toxicity (Li H. et al., 2021). The metabolites of EF obtained by different processing methods, such as stir-frying, roasting, and steaming, are also different (Xiao et al., 2023). A comparative study of various EF-processed products revealed that the

TABLE 4 Changes of content indexes and limits of EF in ChP.

| Edition | Fluidity | Wavelength | Quality markers | Content requirements |
|---|---|---|---|---|
| 2000 | Acetonitrile-water-tetrahydrofuran-acetic acid (51:48:1:0.1) | 225 nm | Evodiamine | ≥0.20% |
| | | | Rutaecarpine | ≥0.20% |
| 2005 | Acetonitrile-0.04% sodium octane sulfonate (43:57) | 225 nm | Evodiamine | ≥0.15% |
| | | | Rutaecarpine | ≥0.15% |
| 2010 | Acetonitrile-water-tetrahydrofuran-glacial acetic acid (41:59:1:0.2) | 225 nm | Evodiamine | ≥0.15% |
| | | | Rutaecarpine | ≥0.15% |
| | | | Limonin | ≥1.00% |
| 2015/2020 | [Acetonitrile-tetrahydrofuran (25:15)]-0.02% phosphoric acid (35:65) | 215 nm | Evodiamine | ≥0.15% |
| | | | Rutaecarpine | ≥0.15% |
| | | | Limonin | ≥0.20% |

combined amounts of evodiamine, rutaecarpine, and evodol in EF for stir-frying exceed those in baking and cooking.

EF combined with other drugs can also counteract its toxicity. For example, the toxicity of EF significantly decreased when EF was used in combination with licorice and jujube (He et al., 2024). EF with Coptidis Rhizoma can enhance the anti-inflammatory effect and inhibit the inflammatory reaction in RAW264.7 cells by significantly reducing the levels of IL-6, TNF-α, and IL-1β (Wang J. et al., 2024). Additionally, it is capable of markedly reducing apoptosis and enhancing the defensive role of the gastric mucosa through the suppression of gastric acid release (Zhang Z. et al., 2024). Berberine is an important metabolite of Coptidis Rhizoma, which can counteract the side effects of evodiamine and reduce the risk of evodiamine in treating gastric cancer (Shi et al., 2013). The combination of berberine and evodiamine can synergistically inhibit the proliferation of human breast cancer cells by inducing cell cycle arrest and apoptosis (Du et al., 2017). The researchers found that EF combined with ginger, Citri Reticulatae Pericarpium, Paeoniae Radix Alba, and Angelicae Sinensis Radix can improve the efficacy of EF (Wu et al., 2021).

# 6 Quality control

## 6.1 Quality standard of EF in ChP

With the continuous updating of the edition of ChP, the identification, content evaluation, testing technology, and quality standards of EF are constantly improving (Table 4). EF was first recorded in the 1963 edition of ChP, and it was clearly recorded that EF was processed with licorice. Then, the identification method of EF appeared for the first time in the 1973 edition of ChP. In the 1985 edition of ChP, the EF identification method was officially determined as hydrochloric acid filtration, potassium mercuric iodide de-precipitation, and the formation of a reddish-brown ring zone at the interface between dimethylaminobenzaldehyde and EF solution. The ChP has been improving the quality control of EF since 2000. For the first time, the determination method and the lowest value of EF appeared in the 2000 edition of ChP, which stipulates that the total amount of evodiamine and rutaecarpine should not be less than 0.2%. Next, the total amount of evodiamine and rutaecarpine should not be less than 0.15% in the 2005 edition of ChP. Limonin content is adjusted to no less than 1.0% in the 2010 edition of ChP. Limonin content is again adjusted to no less than 0.20% in the 2015 edition of ChP. The 2015 edition of ChP is basically consistent with the 2020 edition of ChP, indicating that the metabolites of EF are relatively stable and can better represent the efficacy of EF. However, the maximum reference dose of metabolites is not specified in ChP, and the toxicity of EF has not been reasonably controlled.

## 6.2 Exploration of modern quality control

TCM's effectiveness can differ significantly based on the type of plant, its source, and yield. Diverse environmental factors like soil, air quality, precipitation, and sunlight play a crucial role in shaping EF growth, with each EF metabolite varying in content across regions (Liu Y. et al., 2019). Some studies have shown that the metabolite of EF varies greatly in different locations, and there is little change in the metabolite of EF in different years in the same location (Huang et al., 2008; Zhou et al., 2010). By comparing the EF of different batches of LEF, MEF, and SEF, it was found that the limonin content in SEF was higher than that of MEF and LEF, while the alkaloid content was higher in MEF and LEF. The toxicity of EF is related to the content ratio of limonin and alkaloid. The higher the ratio, the lower the toxicity of EF. In other words, MEF is more toxic than other categories, and SEF is less toxic than other categories (Zhang et al., 2021b). Furthermore, certain academics analyzed the four vital metabolites of ER, ERO, and ERB, namely evodiamine, evodiamine, dehydroevodiamine, and narcissoside, discovering comparable levels of dehydroevodiamine. The content of evodiamine, rutaecarpine, and narcissoside was the highest in ERO and the lowest in ERB (Li C. H. et al., 2021).

The ChP stipulated the detection method of EF and pointed out that evodiamine, rutaecarpine, and limonin are the crucial metabolites of EF. However, these three metabolites do not represent the overall pharmacological effects of EF. Consequently, sophisticated detection techniques are essential for qualitative and quantitative analysis of metabolites in EF. The leading analytical

technologies include TLC, HPLC, HPLC-MS, GC-MS, CE, and CCC (Xia et al., 2023). At present, there are many studies on the metabolites of EF. Scholars have isolated three metabolites from EF: rutaecarpine, evodiamine, and evodiamide (Zhou et al., 2006). Then, two new-lactone derivatives, evodinoids A and B, and a new volatile oil, are separated from EF (Xin et al., 2022). In addition, five metabolites of EF were found. They are limonin, 1-methyl-2-undecyl-4(1H) quinolone, evocarpine, 1-methy-2-[(6Z,9Z)]-6,9-pentadecadienyl-4-(1H)-quinolone, and dihydroevocarpine (Zhang et al., 2013). It has been proven that evodiamine is closely related to the hepatotoxicity of EF (Zhang et al., 2021b). Limonin serves as the primary liver-protective metabolite of EF, while evodiamine is the chief liver-damaging metabolite of EF. Subsequently, eleven critical metabolites of EF underwent analysis using non-specific metabonomics and *in vitro* functional techniques (Yong et al., 2024). Up to this point, a total of 17 metabolites have undergone screening from EF, including neochlorogenic acid, caffeic acid, chlorogenic acid, 3-*O*-feruloylquinic acid, hyperoside, quercetin-3-*O*-sambubioside, rutin, dehydroevodiamine, isorhamnetin-3-*O*-β-*D*-galactoside, narcissin, isorhamnetin-3-*O*-β-*D*-glucopyranoside, diosmin, rutaevine, limonin, evodiamine, rutaecarpine, and evocarpine.

# 7 Pharmacological effects

EF is a classical plant medicine with various pharmacological effects, such as cardiovascular protection, gastrointestinal protection, neuroprotection, anti-inflammation, analgesia, anti-tumor, glucose and lipid metabolism regulation, etc. (Supplementary Table S2). Evodiamine, rutaecarpine, and limonin serve as the indicator metabolites of EF but are also abundant in phytochemistry and pharmacology, which are often used to treat diseases of the immune, nervous, digestive, circulatory, and endocrine systems. Elucidating the pharmacological effects of EF is the key to guiding the rational clinical application of drugs and ensuring the curative effect.

## 7.1 Cardiovascular protection

EF has a cardiovascular protective effect, and its aqueous extract can contract the aorta (Hibino et al., 2009b). In managing cardiovascular conditions, evodiamine, rutaecarpine, and limonin serve as crucial metabolites of EF, offering protection against myocardial ischemia-reperfusion (I/R), anti-myocardial fibrosis, anti-arrhythmia, safeguarding vascular endothelial damage, altering vascular tension, and so on. Studies have shown that evodiamine has an anti-atherosclerotic effect, can regulate energy by inhibiting the expression of the β1-adrenergic receptor, and prevents cardiac I/R injury (Xue et al., 2015). Subsequently, evodiamine has the ability to control the growth and movement of vascular smooth muscle cells by blocking the PI3K/AKT axis activation in the traditional route, thereby preventing atherosclerosis onset and progression (Zha et al., 2023). What's more, evodiamine can prevent isoproterenol-induced cardiac fibrosis by regulating endothelial-to-mesenchymal transition (Huang et al., 2017). Moreover, rutaecarpine promotes endothelial nitric oxide synthase (eNOS) phosphorylation and NO synthesis via the $Ca^{2+}$/calmodulin-dependent protein kinase II (CaMKII) and calmodulin-dependent protein kinase kinase β (CaMKKβ)/AMP-activated protein kinase (AMPK) signaling pathways through transient receptor potential vanilloid type 1 (TRPV1), and effectively prevent endothelial dysfunction (Lee et al., 2021). Rutaecarpine can also reduce the damage to myocardial cells caused by myocardial infarction by enhancing vascular smooth muscle calcification (Zhan et al., 2021). Some studies have shown that limonin can inhibit adriamycin-induced cardiotoxicity by activating Nrf2 and SIRT2 signal pathways (Li X. H. et al., 2022). Limonin can also inhibit the ubiquitination and degradation of SIRT6, stabilize the level of SIRT6 protein, promote its expression, reduce cardiac hypertrophy, and improve cardiac function (Liu et al., 2022).

## 7.2 Gastrointestinal protection

EF is an effective botanical drug for treating gastrointestinal diseases, especially evodiamine, rutaecarpine, and dehydroevodiamine (Chen et al., 2023). Evodiamine can act as an antioxidant by blocking the Rho/NF-κB pathway and alleviating gastric mucosal injury (Zhao Z. et al., 2015). It can also inhibit gastritis caused by *Helicobacter pylori* infection by inhibiting the NF-κB pathway (Yang et al., 2021). Then, evodiamine can effectively improve the imbalance of intestinal microflora and relieve the symptoms of ulcerative colitis by increasing the level of *lactobacillus* acidophilus and the production of acetate (Wang et al., 2020). In addition, it can suppress gastrointestinal hyperactivity caused by stress via cholecystokinin (CCK) and the CCK1 receptor (Ren et al., 2018). Moreover, rutaecarpine is effective in mitigating gastric damage caused by ethanol through the suppression of NF-κB pathway anti-inflammation, Nrf2 pathway antioxidation, stimulation of Bcl-2, suppression of Bax and caspase-3 expression, and prevention of gastric cell apoptosis (Ren et al., 2020). Some studies have shown that TRPV1/calcitonin gene-related peptide (CGRP) pathway is an important therapeutic target for gastric mucosal injury (Luo et al., 2013). Rutaecarpine can stimulate the TRPV1 receptor to release CGRP, inhibit the excessive secretion of gastric acid, and improve the symptoms of gastric ulcers (Liu et al., 2008). Moreover, Dehydroevodiamine can reduce the inflammatory injury of gastric mucosa by reducing the ERK/p38 signal pathway, down-regulating the expression of myeloperoxidase (MPO), TNF-α and IL-6, upregulating the expression of IL-10, regulating gastric pH and mucosal thickness (Wei et al., 2021). Then, dehydroevodiamine can also improve MNNG-induced gastric mucosal injury and GES-1 migration in chronic atrophic gastritis rats and treat atrophic gastritis by inhibiting hypoxia-inducible factor 1α/vascular endothelial growth factor angiogenesis pathway (Wen et al., 2021). Furthermore, EF polysaccharides have a protective effect on gastric mucosa and can alleviate the symptoms of gastric ulcers. By increasing the expression of Nrf2 and HO-1 protein, reducing the expression of Keap1 protein, activating Keap1/Nrf2/HO-1 signal pathway, and reducing oxidative stress in the stomach (Luo et al., 2023).

## 7.3 Neuroprotection

The application of EF in neurological disorders is becoming increasingly widespread. EF and its metabolites have neuroprotective effects on neurodegenerative diseases such as ischemic injury, neuropathic pain, neuroinflammation, Alzheimer's disease (AD), and so on. Studies have shown that EF methanol extract (200 mg/kg) protects neurons and prevents ischemia-induced cognitive impairment (Lee et al., 2011). Then, evodiamine can reduce peripheral hypersensitivity and anxiety in nerve-injured mice (Zhang et al., 2020). Evodiamine can also repair memory and cognitive impairment, protect neurons *in vitro*, and inhibit glial cell activation and neuroinflammation (Lima and Hamerski, 2019). In the experimental AD mice induced by intracerebroventricular injection of streptomycin, evodiamine (50 and 100 mg/kg) was orally given daily for 21 days, the ability to recognize new targets and the score of water maze test was improved in AD mice (Wang et al., 2018a). In addition, evodiamine can improve AD mice's learning and cognitive impairment (Wan et al., 2024) and treat AD through antioxidation and anti-apoptosis (Zhang et al., 2018). Moreover, rutaecarpine ameliorates neuronal injury in rats with cerebral I/R by regulating the expression of ERK1/2 and Nrf2/HO-1 pathway (Han et al., 2019). Rutaecarpine can affect $Ca^{2+}$ influx and activate PI3K/AKT signal pathway by activating specific capsaicin receptor TRPV1, inhibit intracellular oxidative stress and apoptosis protease activity, and protect neurons from apoptosis induced by hypoxia-reoxygenation (Yang Y. et al., 2018). Additional research has shown that limonin and its variants are versatile in combating neuroinflammation and neuronal apoptosis by activating PI3K/AKT and reducing TLR4/NF-κB pathway activity and are effective in treating AD (Panda S. P. et al., 2024). Limonin plays a neuroprotective role by inhibiting neuronal autophagy and microglial activation in rats injected with 6-hydroxydopamine (Gao et al., 2023). Furthermore, dehydroevodiamine (10 mg/kg) can improve the symptoms of memory impairment induced by scopolamine in mice. Dehydroevodiamine has a strong protective effect on cognitive impairment through its antioxidant activity, inhibition of neurotoxicity, and intracellular calcium. Therefore, Dehydroevodiamine may be an important drug for treating memory disorders (Shin et al., 2017). Tg2576-induced AD mice were treated with dehydroevodiamine (0.5 mg/kg) for 4 months, which improved the memory impairment of Tg mice and decreased the levels of soluble amyloid-β 40 (Aβ40), soluble Aβ42 and total Aβ peptide in the cortex of Tg mice. Dehydroevodiamine can inhibit the activity of β-secretase in a dose-dependent manner, which is related to the production of Aβ and the formation of neuritis plaques. Dehydroevodiamine may have a therapeutic effect on AD as a β-secretase inhibitor (Shin et al., 2016).

## 7.4 Anti-inflammation

The anti-inflammatory activity of EF has been widely recognized. Research indicates that the 70% ethanol extract of EF can inhibit the inflammatory response in HaCaT cells. It exerts its anti-inflammatory effects by modulating the JAK-STAT and MAPK signaling pathways. This regulation suppresses inflammatory mediators, cytokines, and chemokines, alleviating symptoms associated with atopic dermatitis (Jin et al., 2024). EF has a potent anti-inflammatory and uric acid-lowering effect. The EF water extract can significantly improve the production of serum inflammatory cytokines IL-1β and TNF-α and inhibit the activation of renal NLRP3 inflammatory signal (Wang Z. et al., 2024). In addition, rutaecarpine can significantly reduce the inflammatory response induced by pseudotype severe acute respiratory syndrome coronavirus 2 by blocking the activity of 3C-like protease (Lin et al., 2023). It has been proved that rutaecarpine can inhibit inflammation by inhibiting the NF-κB signal pathway mediated by PI3K/AKT and MAPK and reduce lipopolysaccharide (LPS)-induced cell migration and number by inhibiting Src/FAK pathway (Jayakumar et al., 2021). Rutaecarpine has also reduced inflammatory responses by downregulating interferon-α, IL-23 p19, and IL-17A protein. This anti-inflammatory effect is mediated through the NF-κB and TLR7 signaling pathways (Li Y. et al., 2019). Moreover, evodiamine is anti-inflammatory by inhibiting IL-1β, IL-2, IL-6, IL-8, TNF-α, and other inflammatory factors mediated by NF-κB (Zhang Y. et al., 2022). Evodiamine can also significantly reduce the pathological damage of breast tissue, inhibit the activation of inflammation-related pathways such as AKT, NF-κB p65, ERK1/2, p38, and JNK, and significantly reduce the production of pro-inflammatory cytokines (Yang Y. et al., 2022). Evodiamine can also improve ulcerative colitis by down-regulating NF-κB signal pathway and NLRP3 inflammatory bodies (Shen et al., 2019). Furthermore, limonin can reduce hepatic steatosis, lipid accumulation, and the expression of p-STAT3/STAT3, caspase-8, and prostaglandin-endoperoxide synthase 2, and improve the inflammatory response of non-alcoholic fatty liver (Wang W. et al., 2023). Limonin can effectively regulate the inflammation mediated by $CD4^+$T cells and inhibit the proliferation of $CD4^+$T cells by inhibiting the nuclear translocation of NF-κB p65 in activated $CD4^+$T cells (Kim W. et al., 2009). Limonin also participates in the regulation of inflammatory pathways by effectively inhibiting p38 MAP. Limonin can also counteract hypertension and vascular damage associated with metabolic syndrome by reducing inflammation and fibrosis (Hassan et al., 2018). Additionally, limonin significantly decreased TNF-α, IL-1β, and IL-6 and inhibited the expression of inflammatory factors in lipopolysaccharide LPS-induced acute lung injury in mice (Wang et al., 2018b).

## 7.5 Analgesia

The analgesic effect of EF is closely related to its anti-inflammatory effect. Oral 50% or 70% methanol extract of 200 mg/kg EF has an analgesic effect on writhing induced by acetic acid (Matsuda et al., 1997). The analgesic effect of EF is related to its metabolites, including evodiamine, rutaecarpine, dehydroevodiamine, rutin, and limonin. Evodiamine exerts analgesic effects through various mechanisms, such as inhibiting ion channels, directly suppressing pain signals, reducing neuronal inflammation, restoring the balance between excitatory and inhibitory neurotransmission, and modulating neurotransmitter release (Jiang et al., 2022). *In vitro*, evodiamine can significantly reduce capsaicin-induced current and thermal hyperalgesia in rats

by activating TRPV1 and neuron desensitization (Iwaoka et al., 2016). Evodiamine can also inhibit migraine-like pain response, which may be due to the regulation of nNOS and the inhibition of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor glutamate A1 (Lin et al., 2020). Evodiamine has been shown to inhibit neuropathic pain, improving paclitaxel-induced neuropathic pain by suppressing inflammatory responses and maintaining mitochondrial antioxidant function (Wu and Chen, 2019). In addition, limonin exhibits significant pain-relieving effects at 30 or 100 mg/kg doses. This analgesic effect is likely associated with its anti-inflammatory properties (Matsuda et al., 1998).

## 7.6 Anti-tumor

EF exhibits potent anti-cancer properties and significant healing properties against various cancers, including lung, liver, stomach, breast, and cervical. Studies have shown that 70% ethanol extract of EF can significantly reduce the vitality of human cervical cancer HeLa cells at 20–60 μg/mL and show a certain concentration correlation (Park et al., 2017). Evodiamine has anti-tumor effects by inducing apoptosis, blocking the cell cycle, regulating autophagy, and inhibiting tumor cell metastasis (Luo et al., 2021; Panda et al., 2023). Evodiamine can treat non-small cell lung cancer (NSCLC) by down-regulating the expression of SOX-9 and β-catenin and significantly inhibiting cell migration by inhibiting epithelial-mesenchymal transition (EMT) (Panda M. et al., 2024). Then, evodiamine can induce cell cycle arrest in the G2/M phase, inhibit cell migration, and inhibit the Notch3 signal pathway against NSCLC by inhibiting γ-secretase (Yang X. et al., 2020). Evodiamine can increase the expression of cleaved-caspase-3, decrease the activity of TSGF and alpha-fetoprotein, induce AKT-mediated apoptosis, and exert an anti-hepatoma effect (Yang F. et al., 2017). Moreover, evodiamine inhibits PI3K/AKT, ERK1/2, and p38 MAPKs and promotes apoptosis of ovarian cancer cells by activating caspase-9/8/3 and poly ADP-ribose polymerase cleavage (Wei et al., 2016). Evodiamine activates retinoblastoma protein through p53 and p21, which selectively inhibits breast cancer stem cells in the G1/S phase, resulting in cancer cell death (Han et al., 2016). Evodiamine inhibits the proliferation and induces apoptosis of cholangiocarcinoma cells, inhibits the migration and invasion of cholangiocarcinoma cells, suppresses IL-6/STAT3 signal transduction by upregulating the expression of SHP-2, and treats cholangiocarcinoma (Zhu et al., 2019).

Beyond that, rutaecarpine can inhibit CYP1A1 and exert an anti-tumor effect by inhibiting the binding of 2,3,7,8-Tetrachlorodibenzo-p-dioxin to its receptor (Rannug et al., 1992). Rutaecarpine can also significantly inhibit human CYP1A2 and CYP3A4 (Don et al., 2003; Iwata et al., 2005). Moreover, limonin can inhibit the cell activity of colorectal cancer cells, block STAT3 signal transduction, and inhibit the proliferation, migration, invasion, and colony formation of colorectal cancer cells (Zhang W. F. et al., 2024). Limonin exhibits properties that combat breast cancer. It has been reported that limonin has cytotoxicity on estrogen receptor-positive or negative human breast cancer cells, which may inhibit proliferation by activating caspase-7 dependent pathway (Kim et al., 2013). What's more, dehydroevodiamine pancreatic is the activator of DNA damage-inducible transcript 3 (DDIT3) and has the ability to inhibit the AKT/mammalian target of rapamycin (mTOR) pathway. It can effectively inhibit the proliferation of pancreatic ductal adenocarcinoma cells, and the growth of prostate cancer stem cells *in vitro* and *in vivo* (Zhu et al., 2024).

## 7.7 Glucose and lipid metabolism regulation

EF can warm the stomach, invigorate the spleen, and be used in glucose and lipid metabolism. In fat metabolism, evodiamine reduced the food intake rate and weight gain rate of rats after growth by downregulating the expression of neuropeptide Y (NPY) and agouti-gene-related protein (AgRP) mRNA and peptide expression in hypothalamic arcuate nucleus (Shi et al., 2009). Evodiamine, a new non-irritant vanillic acid receptor agonist, can simultaneously induce heat loss and heat production, dissipate food energy, and prevent visceral fat accumulation and weight gain (Kobayashi et al., 2001). Then, evodiamine can also activate AMPK and adiponectin polymerization in 3T3-L1 adipocytes, which is related to the activation of $Ca^{2+}$-dependent PI3K/Akt/CaMKII signal pathway (Liu et al., 2014). Treatment with evodiamine over 13 weeks has been shown to lower serum total cholesterol, low and high-density lipoprotein cholesterol, and triglycerides in obese rats on a high-fat diet, decrease blood lipids, and mitigate obesity-related symptoms (Zhang et al., 2017). The combination of berberine and evodiamine can affect the protein expression of PPARγ and liver X receptor α in hyperlipidemic rats and reduce the level of blood cholesterol in hyperlipidemic rats (Zhou et al., 2017b). Furthermore, evodiamine acts as a berberine promoter, and the mechanism of synergistic reduction of serum cholesterol in rats involves inhibiting the expression of Acetyl-CoA Acetyltransferase 2 (ACAT2), Niemann-Pick C1-like 1 (NPC1L1) and apolipoprotein B-48 (apoB-48) to reduce blood lipids (Zhou et al., 2017a). Moreover, ruteacarpine inhibited the expression of NPY and AgRP in the hypothalamic arcuate nucleus and the expression of these two neuropeptides in N29-4 neuronal cells. This method effectively lowers blood cholesterol, non-fasting glucose, insulin, and leptin and ameliorates obesity (Kim S. J. et al., 2009).

On top of that, EF can also contribute to glucose metabolism and improve the symptoms of diabetes. Studies have found that low-dose evodiamine can prevent increased body weight and improve glucose tolerance in mice. Enhanced phosphorylation of AMPK and reduced mTOR signal transduction, a regulator of energy metabolism, are observed in white adipose tissue and are known to avert obesity and insulin resistance (Yamashita et al., 2015). Evodiamide inhibits insulin-stimulated mTOR-S6K activation and IRS1 serine phosphorylation in adipocytes and improves glucose tolerance in obese/diabetes mice (Wang T. et al., 2013). Evodiamine and rutaecarpine can inhibit gluconeogenesis and adipogenesis by activating constitutive androstane receptor (CAR) *in vitro* and *in vivo* and have therapeutic potential for the treatment of hyperglycemia and diabetes mellitus type 2 (Yu et al., 2016). Rutaecarpine can regulate the IRS1/PI3K/AKT signal pathway in the liver and AMPK/acetyl-CoA carboxylase 2 signal pathway in skeletal muscle to improve hyperlipidemia and hyperglycemia in fat-fed and streptozotocin-treated rats (Nie et al., 2016). Beyond that, EF polysaccharides extracted by water solvent have strong antioxidant

activity and α-glucosidase inhibition. It is a promising natural antioxidant and α-glucosidase inhibitor.

## 7.8 Protective effect of liver and kidney

EF can warm the liver and kidney, protect the liver and kidney, and is closely related to evodiamine, rutaecarpine, and limonin. In the liver, evodiamine can promote the translocation of Nrf2 into the nucleus, thereby reducing reactive oxygen species (ROS) levels and oxidative stress in grass carp hepatocytes. It also downregulates the MAPK pathway, alleviating DEHP-induced apoptosis and restoring the expression of antioxidant genes. By blocking the Nrf2/MAPK pathway, evodiamine inhibits DEHP-induced apoptosis in grass carp hepatocytes (Lei et al., 2023; Xiong et al., 2022). Evodiamine (15 and 25 mg/kg) has an anti-fibrotic effect on CCl4-induced hepatic fibrosis and reduces the proliferation and collagen metabolism of hepatic stellate cells *in vitro* by down-regulating the relative expressions of TGF-β1, p-Smad2/3, and α-smooth muscle actin (Yang D. et al., 2018). Additionally, rutaecarpine upregulates antioxidant enzymes through CaMKII-Akt and Nrf2/antioxidant response element (ARE) pathways, enhances the expression of HO-1 in hepatocytes, and has a protective effect on hepatotoxicity induced by TBHP (Jin et al., 2017). Rutaecarpine protects mice from acute acetaminophen-induced liver injury by activating antioxidant enzymes. It could significantly reduce the activity of serum ALT/AST and MDA induced by acetaminophen and prevent liver GSH depletion induced by acetaminophen (Choi et al., 2021). Moreover, limonins have a furan ring structure and are easily activated to form RMs, which are crucial in induced hepatotoxicity (Liu Y. et al., 2020). CYP3A4 inducer aggravated the hepatotoxicity induced by large flower germ, while limonin reduced its hepatotoxicity (Zhang et al., 2021c). Limonin can also diminish the liver toxicity caused by acetaminophen by activating the Nrf2 antioxidant signal and suppressing NF-κB inflammation by increasing SIRT1 levels. Limonin shows potential as a treatment for liver damage caused by acetaminophen (Yang R. et al., 2020).

In renal metabolism, I/R injury can lead to acute kidney failure. Owing to its antioxidant, anti-inflammatory, and anti-apoptotic properties, evodiamine can reduce the biochemical and pathological tissue of renal I/R injury in rats (Eraslan et al., 2019). Studies have shown that evodiamine can also protect against LPS-induced acute renal injury and cytotoxicity by regulating ROS NF-κB-mediated inflammation (Shi et al., 2019). What is more, rutaecarpine can prevent and treat renal I/R injury by inhibiting JNK/p38 MAPK signal pathway and interfering with oxidative stress (Wang et al., 2017). Moreover, limonin, acting as a natural ERK2 agonist, plays a role in averting ischemic acute renal damage, primarily through the activation of the ERK signal pathway, which aids in the growth of renal tubular cells and diminishes apoptosis following acute kidney injury (AKI) (Zhou et al., 2023). Limonin regulates arachidonic acid metabolism by inhibiting CYP3A4 activity, thus improving cisplatin-induced acute renal injury and ultimately protecting renal function (Zeng et al., 2023).

## 7.9 Insecticidal and antibacterial

Ancient records indicate that EF possesses insecticidal properties. The extracts and metabolites of EF showed specific insecticidal properties. Findings indicated superior deworming effects of ethyl acetate, petroleum ether, and methanol extract on Goldfish-*Gyrodactylus kobayashii* in living organisms, with EF ethyl acetate extract emerging as the most efficient and secure (Lian et al., 2019). Subsequently, EF volatile oil exhibits insect-killing properties against maize weevils, *Sitophilus zeamais*, and red flour beetle *Tribolium castaneum*, showing $LC_{50}$ values of 36.89, 24.57, and 57.31 mg/L air, in that order (Liu and Du, 2011). In addition, evodiamine and rutaecarpine have insecticidal activity against larvae of *Drosophila melanogaster* with $LC_{50}$ values of 0.30 and 0.28 μmol/mL diet, respectively, among which rutaecarpine has the strongest activity (Miyazawa et al., 2002). Evodiamine and rutaecarpine had strong insecticidal activity against the fourth instar larvae of *Aedes albopictus* with $LC_{50}$ values of 12.51 and 17.02 μg/mL, respectively. EF ethanol extract, limonin, and evodiol also had insecticidal activity against Asian tiger mosquitoes, and the $LC_{50}$ values were 43.21, 32.43, and 52.22 μg/mL, respectively (Liu et al., 2012). Moreover, the nematicidal activity of evodiamine and rutaecarpine against *Meloidogyne incognita* was stronger than the crude EF ethanol extract, and the $LC_{50}$ values were 73.55, 120.85, and 131.54 μg/mL, respectively. Both evodiol and limonin demonstrated their ability to kill *Meloidogyne incognita*, evidenced by $LC_{50}$ values of 155.02 and 197.37 μg/mL, respectively, yet they were less potent than the raw EF ethanol extract (Liu et al., 2013). Other studies found that limonin had effective biological activity against larvae and adults of *Schistosoma mansoni*, and its antiparasite activity was enhanced in a dose-dependent manner (Eraky et al., 2016).

EF's long-term use in treating diarrhea and beriberi is due to its antibacterial and antifungal properties. The EF volatile oil has the strongest activity against *Bacillus subtilis* and *Staphylococcus aureus*, the maximum inhibitory zone diameter is 17.9 and 12.2 mm, respectively, and the MIC is 3.2–6.4 mg/mL (Liu S. S. et al., 2019). Furthermore, three novel quinazoline alkaloids, specifically evodiamine A, evodiamine B, and evodiamine C, were extracted from EF methanol. They prohibited excellent inhibition against *Xanthomonas oryzae* pv. *oryzae*, *Xanthomonas oryzae* pv. *oryzicola*, and *Xanthomonas campestris* pv. *campestris*, with respective $EC_{50}$ values of 3.13, 14.32, and 32.72 nmol (Su et al., 2018). Moreover, evodiamine can enhance the activation of NLRP-3 inflammatory bodies by inducing acetylation of α-tubulin lysine 40 residues, thus enhancing innate immunity to bacterial infection (Li C. G. et al., 2019).

## 7.10 Anti-osteoporosis

Rutaecarpine, evodiamine, and limonin demonstrated obvious anti-osteoporotic effects. Rutaecarpine significantly inhibits osteoclast production and bone resorption of bone marrow-derived macrophages osteoclasts by reducing the protein level of nuclear factor of activated T cells 1 (NFATc-1) and phosphorylation of other signal pathways during osteoclast differentiation (Fukuma et al., 2018). In addition, evodiamine has been reported to inhibit osteoclast formation by blocking receptor activators for NF-κB ligand (RANKL)-induced ERK and c-Fos activation and NFATc-1 induction in a dose and time-dependent manner (Jiang et al., 2017). Evodiamine can inhibit osteoclast formation induced by

RANKL through NF-κB and calcium signaling pathways and reduce bone loss in ovariectomy and ovariectomized mice by inhibiting osteoclast production (Jin et al., 2019). Subsequently, evodiamine is capable of mitigating osteoporosis in zebrafish caused by dexamethasone, by counteracting the disproportion in bone development and resorption and triggering the matrix metalloproteinase 3-osteopontin-MAPK pathway signal (Yin et al., 2019). Beyond that, limonin can increase the calcium concentration of the femur and fifth lumbar vertebra in ovariectomized rats, and the mechanism may be related to promoting bone formation (Mandadi et al., 2009). Research has found that the loss of ovarian function can lead to a lack of ovarian-related hormones, resulting in rapid loss of ovarian-related bones (Renno et al., 2006). Limonin can efficiently prevent bone mass reduction and enhance bone mineral density in rats post-ovariectomy. Moreover, limonin stimulates the activity of ALP in osteoblast MC3T3-E1 and enhances the expression of osteoblast differentiation gene markers by regulating extracellular signal-regulated kinase and P38 signal (Lee et al., 2016).

## 7.11 Other activity

EF not only offers attractive pharmacological effects but also provides antiallergic, antioxidant, antidepressant, and protection for the prostate, among others.

### 7.11.1 Antiallergic

*In vitro* and *in vivo*, evodiamine and rutaecarpine may inhibit the biosynthesis of allergy-related cytokines (TNF-α and IL-4) in mast cells and basophils, suggesting that they may be effective against IgE-induced allergic diseases such as atopic dermatitis and rhinitis (Shin et al., 2007). Subsequently, limonin effectively manages allergies induced by IgE. This substance can significantly reduce IgE production in the peripheral blood mononuclear cells (PBMC) and B cell lines of children allergic to food, potentially owing to the suppression of ε-germ line transcript expression in PBMC (Yang et al., 2014).

### 7.11.2 Antioxidant

In natural aging rats, limonin decreased the levels of MDA and lipofuscin in serum and brain tissue, increased the activities of superoxide dismutase (SOD) and GSH-Px in serum and brain tissue, and enhanced the total antioxidant capacity in brain tissue (Li et al., 2016). Notably, when altered by the structure of limonin, limonin glycosides can attain antioxidant properties by neutralizing free radicals (Poulose et al., 2005). However, some researchers question whether limonin has antioxidant activity (Breksa and Manners, 2006). There is a lack of research on the antioxidant mechanism of limonin, and this natural antioxidant is worthy of further exploration.

### 7.11.3 Antidepressant

The antidepressant effect of evodiamine on chronic, unpredictable stress rats may be achieved by regulating monoamine transmitters and BDNF-TrkB signal transduction in the hippocampus (Jiang et al., 2015).

### 7.11.4 Prostate protection

The EF ethanol extract has a strong 5α-reductase inhibitory activity. The treatment of EF ethanol extract in benign prostatic hyperplasia-1 cells inhibits cell viability through caspase-8 and cystatin-3-dependent apoptosis and effectively inhibits the growth of benign prostatic hyperplasia-1 cells (Park et al., 2018). In addition, EF volatile oil has obvious anti-inflammatory effects and inhibits the growth of prostate cancer-3 cells by directly and indirectly regulating the cytokine secretion profile of spleen cells (Yeh and Lin, 2021). Not only this, evodiamine can also inhibit prostate hyperplasia and migration through the PI3K/AKT/NFκB signal pathway, indicating that it may be a potential lead drug in the treatment of prostate cancer (Lei et al., 2022).

# 8 Toxicity

Ancient TCM texts have chronicled EF's mild toxic effects, potentially causing eye harm, hair fall, and gastrointestinal issues if misused. In recent years, many studies have shown that overuse of EF can cause toxic symptoms such as nausea, vomiting, abdominal pain, diarrhea, and blurred vision (Ma et al., 2018) and cause liver and kidney toxicity to the human body (Teschke, 2014). EF and its metabolites have been reported to cause liver damage at high doses and induce arrhythmias, leading to cardiotoxicity (Teschke et al., 2014). *In vitro* and *in vivo* studies have shown that EF has hepatotoxicity, cardiotoxicity, and nephrotoxicity. However, there are few studies on the cardiotoxicity and nephrotoxicity of EF. The toxicity of EF is summarized as follows (Table 5).

## 8.1 Hepatotoxicity

EF causes hepatocyte cytotoxicity, attributed to oxidative stress, mitochondrial damage, endoplasmic reticulum stress, liver metabolic disorder, and apoptosis (Cai et al., 2014). The toxicological mechanisms are peroxidation, inflammatory factors, mitochondrial damage, and the formation of drug-protein adducts. Research revealed that various EF extracts might lead to sudden liver damage, with volatile oil exhibiting the highest level of hepatotoxicity, succeeded by total extract and ethanol extract, and water extract showing minimal hepatotoxicity.

### 8.1.1 Oxidative damage

EF may cause oxidative harm by impacting vital elements of the body's oxidation-antioxidant mechanism. Studies have shown that EF aqueous extraction can cause liver injury in mice after continuous intragastric administration for 21 days. It was found that the content of MDA in liver tissue increased, the ratio of SOD/MDA and the activity of GSH-Px decreased significantly, and the pathomorphology showed focal hepatocyte necrosis (Zhou L. et al., 2013). The hepatotoxicity of EF is related to the oxidative stress in the liver and has a certain dose-effect relationship. Following 15 days of orally administering EF via aqueous extraction, there was a notable reduction in SOD activity in the mice's liver tissues, with SOD levels rising in each dosage group as the dose was increased (Cai et al., 2014). In addition, liver injury occurred after continuous intragastric administration of EF volatile oil for 7 days, resulting in

TABLE 5 Toxicity of EF and its metabolites.

| Toxicology | Toxicity mechanisms | Chemical compounds | Type of study | Experimental subject | Dose range | Duration | Toxic manifestations | References |
|---|---|---|---|---|---|---|---|---|
| Hepatotoxicity | Oxidative damage and inflammatory response | EF aqueous extract | *In vivo* | KM mice | 10, 20, 30 g/kg | 21 days | (−): The level of SOD/MDA, GSH-Px and NOS.<br>(+): The level of ALT, AST, GSH, MDA, IL-1β, IL-6 and TNF-α | Zhou et al. (2013a) |
| | Oxidative damage and inflammatory response | EF aqueous extract | *In vivo* | Male SD rats | 6, 12, 24 g/kg | 15 days | (−): The level of MnSOD, GSH, ATP, and mitochondrial potential<br>(+): The level of MDA. | Cai et al. (2014) |
| | Oxidative damage | EF essential oil | *In vivo* | KM mice | 0.25, 1, 1.25 mL/kg | 7 days | (−): The level of SOD, GSH, and GSH-Px<br>(+): The level of MDA, NO, and NOS. | Zhang et al. (2011) |
| | Oxidative damage | Evodiamine and rutaecarpine | *In vitro* | HLM and P450s | 10 and 50 mmol/L, respectively | 60 min | (−): The level of GSH; the activation of CYP3A4 | Wen et al. (2014) |
| | Inflammatory response | EF aqueous extract | *In vivo* | KM mice | 0.65, 3.25, 6.5 g/kg | 15 days | (−): Liver mitochondrial ($Ca^{2+}$)-ATP activity; the expression of SOD and Bcl-2 protein<br>(+): The level of ALT, AST, MDA, TNF-α, and IL-1β; the expression of Bax protein | Liu et al. (2018) |
| | Inflammatory response | EF aqueous and 70% ethanol extract | *In vivo* | KM mice | 0.01 mL/g | 15 days | (−): The expression of STAT3 and Src proteins<br>(+): The expression of ERK, cyclin-dependent kinase 8, and casein kinase 1 ε proteins | Liao et al. (2014) |
| | Inflammatory response | EF aqueous extract | *In vivo* | Male C57BL/6 mice | 7.5 g/kg | 1 h | (−): The level of SOD and GSH-PX.<br>(+): The level of ALT, AST, ALP, LDH, MPO, MDA, TNF-α, IL-6 and IL-1 | Ren et al. (2024) |
| | Mitochondrial damage | EF aqueous and 70% ethanol extract | *In vivo* | KM mice | 0.2 mL/10 g | 14 days | (−): Mitochondria function and the ratio of AST/ALT. | Yang et al. (2024b) |
| | Mitochondrial damage | Evodiamine | *In vitro* | L-02 cells | 6.25–200 μmol/L | 14 days | (−): The cell counts and MMP. | Yang et al. (2024b) |
| | Mitochondrial damage | Evodiamine | *In vitro* | HepG2 cells | 0.2, 1, 5 μmol/L | 48 h | (−): The SOD activity and MMP.<br>(+): The ALT, AST, LDH, ALP activities; the content of total bilirubin and MDA; cells apoptosis | Gao et al. (2021) |
| | Formation of drug-protein adducts | Rutaecarpine | *In vitro* | Primary male SD rat hepatocyte | 10, 30, 100, 300 mmol/L | 24 h | (−): Hepatocyte survival; mitochondrial membrane potential; the acitivity of CYPs<br>(+): The level of ROS and LDH; cellular stress and membrane damage | Cai et al. (2014) |
| | Formation of drug-protein adducts | Rutaecarpine | *In vivo* | KM mice | 10, 20, 30 mg/kg | 7 days | (+): The expression of hepatic transporters, CYP, and phase-2 enzyme genes | Labbe et al. (2008) |
| | Formation of drug-protein adducts | Evodiamine | *In vivo* | Male SD rats | 50 mg/kg | 7 days | (−): The expression of CYP1A2, CYP2C9 and CYP2D6 | Zhang et al. (2016) |

(Continued on following page)





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TABLE 5 (*Continued*) Toxicity of EF and its metabolites.

| Toxicology | Toxicity mechanisms | Chemical compounds | Type of study | Experimental subject | Dose range | Duration | Toxic manifestations | References |
|---|---|---|---|---|---|---|---|---|
| Cardiotoxicity | Oxidative damage | Evodiamine | *In vitro* | SD Rat cardiomyocytes | 31.3, 62.5, 125, 250 μg/mL | 24 h | (−): Cardiomyocyte viability; heart rate; the level of SOD.<br>(+): The level of MDA and LDH. | Yang et al. (2017b) |
| | Oxidative damage | Evodiamine | *In vivo* | Wild-type zebrafish and the transgenic strain zebrafish | 31.3, 62.5, 125, 250 μg/mL | 24 h | (+): The straight-line distance between the venous sinus and arterial bulb | Yang et al. (2017b) |
| | Oxidative damage | Evodiamine and rutaecarpine | *In vitro* | H9C2 and NRCMs | 5, 10, 25, and 60, 80, 100 μmol/L, respectively | 24 h | (+): The levels of LDH and CK; MMP. | Zhang et al. (2022a) |
| | Oxidative damage | EF aqueous extract | *In vivo* | Male SD rats | 0.525 g/mL | 15 days | (−): The protein expression of the cyclic guanosine monophosphate-protein kinase G pathway; frequency of spontaneous beat in NRCMs<br>(+): The intensity of calcium fluorescence | Zhang et al. (2022a) |
| | Oxidative damage | EF aqueous extract | *In vivo* | Wild-type zebrafish and the transgenic strain zebrafish | 0.4 mg/mL | 48 h | (+): The morphological abnormalities in the liver, myocardial concentrations, and pericardial edema | Fan et al. (2024) |
| | Inhibition of the cardiac hERG channel | Hydroxyrutaecarpine | *In vitro* | HEK 293 cells | 10 μmol/L | 24 h | (−): The expression of transcription factor Sp1 and hERG protein; the activation of hERG channel | Li et al. (2022c) |
| | Inhibition of the cardiac hERG channel | Rutaecarpine | *In vitro* | HEK 293 cells | 1, 10 μmol/L | 24 h | (−): The expression of transcription factor Sp1 and hERG protein | Zhan et al. (2021) |
| | Inhibition of the cardiac hERG channel | Rutaecarpine | *In vivo* | Male guinea pigs | 25 mg/kg/d | 2 weeks | (+): The QT/QTc intervals; the induction rate of ventricular fibrillation | Zhan et al. (2021) |
| | Inhibition of the cardiac hERG channel | Dehydroevodiamine and hortiamine | *In vitro* | HEK 293 cells | 0.01, 0.1, 1, 10 μmol/L | – | (+): The action potential duration and early afterdepolarizations | Baburin et al. (2018) |
| | Inhibition of the cardiac hERG channel | Dehydroevodiamine and hortiamine | *In vivo* | Anesthetized rabbits and CAVB dogs | 0.05, 0.5, and 0.33 mg/kg, respectively | 5 min | (+): The QT interval | Baburin et al. (2018) |
| Nephrotoxicity | Renal apoptosis | EF 70% ethanol extract | *In vivo* | SD rats | 2.5, 6.6, 20.83 g/kg/d | 28 days | (+): Glomerular mesangial curvature, swelling of renal podocytes and glomerular vascular endothelial cells | Liu et al. (2015) |
| | Renal apoptosis | Evodiamine | *In vitro* | HK-2 cells | 0.1, 0.2, 0.4, 0.8, 1.6, 3.2 μmol/L | 48 h | (+): The calcium overload caused by activation of the TRPV1 protein; PI3K pathway-mediated apoptosis | Yang et al. (2024a) |
| | Renal apoptosis | Evodiamine | *In vitro* | HK-2 cells | 0.1, 0.2, 0.4, 0.8, 1.6, 3.2 μmol/L | 24 h | (−): The mitochondrial membrane potential<br>(+): The cell membrane permeability and Cytochrome C release; the level of LDH; the expression of apoptosis-related proteins Bax and Bcl-2 | Yang et al. (2022a) |
| | Renal apoptosis | Evodiamine and evodine | *In vitro* | HEK 293 cells | 4.15, 8.3, 16.6, 33.2, and 25, 50, 100, 200 μg/mL, respectively | 24 h | (+): Varying degrees of shrinkage, reduction, and even death of renal cells | Zhou et al. (2013b) |





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increased activities of MDA and NOS in blood and liver tissue, decreased GSH content, SOD, and GSH-Px activities (Zhang et al., 2011). Research indicates that the presence of 3-alkylindoles in evodiamine and rutaecarpine leads to the creation of highly electrophilic intermediates, namely iminoquinone and 3-methyleneindolenine, via P450-driven oxidation in liver microsomes (primarily driven by CYP3A4 and to a smaller degree by CYP1A2 and CYP2D6), causing harmful effects on hepatocytes when GSH is depleted (Wen et al., 2014).

### 8.1.2 Inflammatory response

Inflammatory injury is one of the causes of liver injury caused by EF. IL-1β, IL-6, and TNF-α are inflammatory transmitters closely related to inflammatory response. These inflammatory transmitters can further amplify the signal of inflammatory response and promote apoptosis and necrosis of hepatocytes. Findings indicate that mice experienced liver damage and elevated levels of TNF-α and IL-1β in their liver tissue 15 days following the EF aqueous extraction (Liu et al., 2018). After continuous intragastric administration of EF aqueous extraction for 21 days, the high, middle, and low dose groups of EF could significantly increase the contents of TNF-α, IL-1 β, and IL-6 in liver tissue of mice, with a certain dose-effect relationship (Zhou L. et al., 2013). After continuous intragastric administration of EF aqueous and ethanol extraction for 15 days, the expression of phosphorylated ERK1/2 in the liver of mice was significantly upregulated. Activation of ERK1/2 can induce cells to produce TNF-α, which mediates inflammatory response and apoptosis-related transcriptional regulatory factors (Liao et al., 2014). During metabolic processes, the P450 enzyme activates evodiamine in EF aqueous extraction, resulting in liver damage and inflammation, primarily due to elevated levels of ALT, AST, ALP, LDH, MPO, MDA, TNF-α, IL-6, and IL-1 (Ren et al., 2024).

### 8.1.3 Mitochondrial damage

Mitochondria are the main sites of cell biological oxidation, which mainly synthesize ATP. Mitochondria are essential targets of drug toxicity in drug-induced liver injury. Studies have shown that intragastric administration of EF aqueous extracts of 6, 12, and 24 g/kg for 15 days can cause hepatocyte mitochondrial swelling and vacuolation, and eventually lead to apoptosis due to ATP depletion and cytochrome C release (Cai et al., 2014). Moreover, both ethanol and aqueous extracts of EF have hepatotoxicity, and the cytotoxicity of EF ethanol extract is stronger. In addition, evodiamine has the strongest toxicity among the extracts of EF, which can significantly reduce the number of cells and increase the mitochondrial membrane potential (MMP) *in vitro* (Yang et al., 2024b). Evodiamine (0.04–25 μmol/L) decreased the survival rate of HepG2 cells, increased MMP, and induced apoptosis in a time- and dose-dependent manner (Gao et al., 2021). Mitochondrial permeability transition plays an important role in mediating hepatocyte injury (Labbe et al., 2008; Pessayre et al., 2010). Limonin has hepatotoxicity, which can cause oxidative damage to rat mitochondria, lead to mitochondrial swelling, mitochondrial permeability transition pore opening, mitochondrial potential decrease, and finally trigger cell death signal pathway (Fan et al., 2019).

### 8.1.4 Formation of drug-protein adducts

The alkaloids easily combine with proteins to form drug-protein adducts. Drug-protein adducts may cause toxicity by damaging the physiological function of the modified protein or through an immune-mediated mechanism (Zhou et al., 2005). The role of RMs is significant in liver damage caused by drugs. The secondary amine configuration of Rutaecarpine enables its activation into RMs via the CYPs enzyme, leading to a covalent bond with CYPs and proteins in rat liver cells, resulting in drug-protein complexes and subsequent liver damage (Zhang et al., 2015). RMs can consume GSH, leading to excessive ROS production, respiratory chain dysfunction, cell stress, mitochondrial damage, cell membrane damage, and hepatocyte damage (Akbulut et al., 2014). CYPs are the primary enzymes involved in drug metabolism within the human body. Certain medications transform into RMs via the biological actions of CYPs (Yan et al., 2023). It has been found that rutaecarpine can inhibit many types of CYP activity, such as CYP1A2, CYP2C9, CYP2C19, and CYP2E1 (Zhang et al., 2015). The induction of cytochrome P450 enzyme gene, liver transport protein, and phase 2 enzyme gene are involved in the interaction between evodiamine and drugs (Zhu et al., 2013). Evodiamine and rutaecarpine can cause toxicity through P450-mediated dehydrogenation, produce highly electrophilic intermediates, and lead to drug-drug interaction mainly through the inactivation of CYP3A4 (Wen et al., 2014). In addition, evodiamine can inhibit CYP1A2, CYP2C9, and CYP2D6 in rats (Zhang et al., 2016). Evodiamine is easily oxidized to an epoxy structure that binds to GSH. When GSH is depleted, some liver damage will occur (Zhang et al., 2015).

## 8.2 Cardiotoxicity

The cardiotoxicity of EF is mainly caused by oxidative damage and inhibition of human ether-a-go-go-related gene (hERG) channels in the heart. Its primary connections are in alkaloids with evodiamine, rutaecarpine, dehydroevodiamine, and hydroxyrutaecarpine.

### 8.2.1 Oxidative damage

The heart is the most oxygen-consuming organ, and many basic studies have confirmed the cardiotoxicity mediated by oxidative stress (Shen et al., 2024). Oxidative damage is closely related to evodiamine and rutaecarpine. Studies have shown that evodiamine at the concentration of 31.3–250 μg/mL for 24 h can significantly reduce the level of MDA and the activity of superoxide dismutase, resulting in oxidative stress injury of cardiomyocytes. Subsequently, evodiamine could induce oxidative stress by generating free radicals, potentially harming the architecture and functionality of cardiomyocytes. After being treated with 28.44 μg/mL evodiamine for 24 h, cardiomyocytes reached 50% inhibitory concentration, which significantly decreased the activity of SOD in rat cardiomyocytes. In the zebrafish model, the mortality rate of zebrafish treated with 354 ng/mL evodiamine was 10%, causing cardiac dysfunction and pericardial malformations (Yang W. et al., 2017). The findings imply that evodiamine could lead to heart-related side effects, including oxidative stress. Determining LDH and creatine kinase (CK) activity is one of the biochemical indexes for

evaluating and diagnosing heart disease. The level of LDH in serum reflects the injury of myocardial cell permeability. The activity level of CK is directly related to the consumption and supply of myocardial oxygen and energy, muscle contraction, and mitochondrial function (Zervou et al., 2016; Bak and Schousboe, 2017; Klein et al., 2020). Evodiamine and rutaecarpine have toxic effects on rat cardiomyocytes H9c2 and neonatal rat cardiomyocytes (NRCMs), mainly by reducing the protein expression of cyclic guanosine monophosphate-protein kinase G pathway in H9c2 cells and changing the spontaneous beating frequency in NRCMs (Zhang D. et al., 2022). What is more, a high dose of evodiamine will lead to severe morphological abnormalities of the liver, pericardial edema, and increased myocardial concentration.

### 8.2.2 Inhibition of cardiac hERG channel

The hERG channel is the ion channel on the myocardial cell membrane, which is very important to maintain the normal electrophysiological activity of the heart. Abnormal opening or closing of the hERG channel will lead to arrhythmia (Liao et al., 2024). *In vitro*, rutaecarpine can reduce the threonine/tyrosine phosphorylation of Sp1 and the expression of the hERG channel through the PI3K/AKT pathway in HEK 293 cells. Subsequently, administering rutaecarpine for 2 weeks may extend the QT/QTc interval intervals and enhance the rate of ventricular fibrillation induction in the hearts of guinea pigs (Zhan et al., 2021). In addition, dehydroevodiamine can inhibit the hERG channel, change the myocardial excitation process, and lead to arrhythmia and even ventricular fibrillation in severe cases (Zhang et al., 2023). Depending on the dose, dehydroevodiamine, and hortiamine can prolong action potential duration and early afterdepolarizations of cardiomyocytes, eventually leading to arrhythmias (Baburin et al., 2018). There are also studies indicating that hydroxyrutaecarpine inhibits hERG current by binding to F656 and Y652 sites in the hERG channel. It can shorten the inactivation time constant, accelerate the process of channel inactivation, and inhibit the function of the hERG channel (Li X. H. et al., 2022).

## 8.3 Nephrotoxicity

EF's nephrotoxic effects primarily stem from renal cell death and oxidative stress. Its similarity to evodiamine and limonin in EF is notable. Mice were administered the EF ethanol extract in groups of low, medium, and high dosages. In the group receiving a high dosage, there was a noticeable flexing of the glomerular Mesangium and an enlargement of both glomerular podocytes and endothelial cells (Liu et al., 2015). *In vivo* experiments showed that evodiamine could induce renal cell death and regulate the PI3K/AKT/mTOR pathway by inducing intracellular calcium overload (Yang et al., 2024a). Rutaecarpine can significantly reduce the level of cortisol and regulate glucocorticoid metabolism. Renal injury may be related to the induction of apoptosis-related protein Bax and Bcl2 expression (Yang C. Q. et al., 2022). Evodiamine and evodiolide in alkaloids can damage mitochondria, lead to mitochondrial dysfunction, produce a large number of free radicals from mitochondria, further aggravate oxidative stress, induce apoptosis, and promote renal damage (Zhou Q.j. et al., 2013). In addition, limonin also has a certain toxicity to kidney cells. Comprehensive analysis of animal experiments and chromatographic analysis showed that hydroxyl or acetoxy limonoid derivatives and coumarin in EF may be the leading causes of toxicity (Shan et al., 2021). Studies have shown that limonin (50–200 μg/mL) can significantly inhibit the viability of HEK 293 cells in a dose-dependent manner. A concentration ranging from 100–200 μg/mL may lead to atrophy, reduction, or even fatality of renal cells (Fan et al., 2019).

# 9 Monitoring and prevention of toxicity of EF

## 9.1 Surveillance and prevention of hepatotoxicity

The typical oral dose of EF is 2–5 g, taken by water decoction or pill powder. It is generally non-toxic within a reasonable dose range, but it needs to be monitored if it is overused. The liver function test, coagulation function test, serum bilirubin level test, liver ultrasound, and liver biopsy can monitor the hepatotoxicity of EF. Liver function tests can detect transaminase, bilirubin, alkaline thrombin, cholinesterase, and other indexes in blood and evaluate the functional status of the liver (Shiihara et al., 2024). Coagulation function tests can determine prothrombin time, thrombin time, and activated thrombin time and help to judge whether the liver is abnormal (Kumagai et al., 2023). A high serum bilirubin level may indicate liver injury or biliary obstruction disease (Poynard et al., 2023). Liver ultrasound can observe liver structural abnormalities or steatosis (Vardar et al., 2022). Liver biopsies help to confirm whether there is hepatocyte injury or inflammation (Palmer et al., 2024). Therefore, patients using EF should regularly check their liver function and blood coagulation function, pay close attention to the indexes of transaminase, bilirubin, alkaline thrombin, and cholinesterase, and adjust the dosage of EF in time to avoid drug-induced liver injury.

## 9.2 Surveillance and prevention of cardiotoxicity

The cardiotoxicity of EF can be evaluated by physical monitoring and measurement of specific biomarkers, including electrocardiogram changes, blood electrolyte levels, and myocardial enzyme activity (Qiu et al., 2023). An electrocardiogram is a direct indicator of the heart's electrophysiological function, and irregular waveforms could signal the presence of an arrhythmia (Henriksen et al., 2023). Disproportionate levels of electrolytes in the blood, particularly irregular potassium ion concentrations, frequently lead to arrhythmias (Sauer and Porter, 2021). Increased myocardial enzyme activity, such as creatine kinase and lactate dehydrogenase, may damage heart tissue (Gai et al., 2021). Therefore, patients using EF should have regular ambulatory electrocardiogram monitoring. The global cardiac activity was measured for 24 h, and the heart rate variability and myocardial perfusion indexes were observed. It assists patients in adjusting their medication dosage promptly to reduce the occurrence of cardiotoxicity.

### 9.3 Surveillance and prevention of nephrotoxicity

The nephrotoxicity of EF can be monitored by urinalysis, renal function tests, biochemical assays, and renal biopsy (Canki et al., 2024). Urine tests can reflect proteinuria, hematuria, hemoglobinuria, cylindruria, urinary calcium, and alkaline urine (Wu and Fenton, 2018). Renal function tests assess the endogenous creatinine clearance rate, blood urea nitrogen, creatinine, and uric acid levels and evaluate the glomerular filtration rate through a radionuclide renogram (Nieto et al., 2020). Biochemical tests can monitor serum liver and renal function-related enzyme levels (Sumer et al., 2020). A renal biopsy is capable of directly monitoring the pathological alterations in kidney tissue, including the deterioration of tubular epithelial cells, necrosis, congestion in the interstitial space, swelling, infiltration of inflammatory cells, fibrosis in the renal interstitial area, and the expansion or shrinkage of the renal tubules (Xu et al., 2024). Therefore, patients taking EF should have regular urinalysis and blood tests and pay attention to the levels of urinary protein, red blood cells, serum creatinine, and urea nitrogen to ensure timely detection of issues and implementation of appropriate treatment measures, thereby reducing the incidence of renal toxicity. Concurrently, it is crucial to rigorously regulate the dosage while administering EF to prevent excessive use. Regular monitoring of kidney function during usage is crucial for early identification of kidney toxicity.

## 10 Conclusion and prospect

EF ranks among the most prevalent and extensively utilized TCM, with a history of use in China spanning millennia. The latest research achievements of EF in standardized cultivation, traditional application, processing methods, quality control, phytochemistry, pharmacology, and toxicology were reviewed in this paper. EF has cardiovascular protection, anti-inflammation, analgesia, gastrointestinal protection, anti-tumor, neuroprotection, glucose and lipid metabolism regulation, and other pharmacological effects. Frequently, it serves as a treatment for abdominal discomfort, vomiting, diarrhea, dyspepsia, high blood pressure, eczema, and oral ulcers. Despite EF's diverse pharmacological effects, ancient texts document its mild toxicity. The harmful effects of TCM have persistently been a worry in both its clinical usage and formulation. Therefore, it is suggested that the following areas should be considered in future research.

From a botanical perspective, it is evident that ER, ERO, and ERB are the trio of EF sources, with nearly mature fruits primarily categorized into LEF, MEF, and SEF. MEF produced in Jiangxi is a genuine medicinal material mainly distributed in Sichuan, Guizhou, and other places south of the Qinling Mountains in China. However, most current studies only distinguish the varieties of *Euodia rutaecarpa*, and few studies have demonstrated the effect of EF size on pharmacological action. From a conventional standpoint, EF's initial documentation appears in *Shen Nong's Herbal Classic*, known for its customary properties of alleviating cold and pain, diminishing nausea and vomiting, enhancing yang, and halting diarrhea. Currently, numerous clinical prescriptions and formulations have been developed, primarily using EF, for treating conditions like abdominal pain, diarrhea, chronic non-atrophic gastritis, irritable bowel syndrome, primary dysmenorrhea, and more. However, the specific mechanism is not completely clear, and the clinical application of EF needs more extensive verification. Moreover, modern pharmacological studies mainly focus on EF or its metabolites, seriously ignoring the effects caused by drug interaction. According to the traditional application, exploring the synergistic effect of drugs to improve clinical efficacy and safety is of great significance. In terms of processing methods, ancient classical works have recorded the processing of EF with licorice, salt, ginger, vinegar, and other methods, which can increase efficiency and reduce toxicity. Modern research has proved that the best result can be obtained when the ratio of EF to licorice is 100: 6. However, few articles currently study this aspect. The metabolites of EF were widely studied. About 300 metabolites were isolated and identified from the plant, including alkaloids, terpenoids, flavonoids, volatile oils, and others. Indole alkaloids, especially evodiamine, and rutaecarpine, have been studied for many years. However, studying terpenoids, flavonoids, and volatile oils in EF is not deep enough, which seriously limits people's understanding of the pharmacological and toxicological mechanisms. Therefore, further study of other metabolites is a priority for the future. The quality assurance of EF is closely related to evodiamine, rutaecarpine, and limonin. It is stipulated that the content of evodiamine and rutaecarpine is not less than 0.15%, and the content of limonin is not less than 0.20%. However, there is no maximum limit for using these three ingredients. According to modern research, the content of rutaecarpine and limonin should not exceed 100 mg/kg/d, and the content of evodiamine should not exceed 300 mg/kg/d. Although researchers have done a lot of research on the minimum and maximum dose, a unified standard has not been formed, so it is necessary to conduct an in-depth study.

The research on toxicology and its mechanisms of EF *in vivo* and *in vitro* is insufficient, limiting its more extensive application. Contemporary studies extensively focus on EF's hepatotoxic effects, with lesser emphasis on cardiotoxicity and nephrotoxicity and a lack of comprehensive and clear understanding of the toxicity of EF. After analyzing the metabolites of EF, it is concluded that evodiamine, rutaecarpine, limonin, and dehydroevodiamine are related to cardiotoxicity and nephrotoxicity. In a specific range, the toxicity of EF to the heart and kidney is proportional to the dose, and long-term overuse will aggravate the toxicity. Attention must be paid to the dosage of EF in clinical application. The research on drug dosage is also very scarce, and the dosage of 2–5 g stipulated in ChP is not exactly the same as the actual clinical dose. Therefore, the research on the dose of EF should be further strengthened. *In vitro* and vivo experiments showed that EF may be toxic to the liver through peroxidation injury, inflammatory response factor mediation, mitochondrial damage, and the formation of drug-protein adducts. EF may cause cardiotoxicity through oxidative damage and inhibition of hERG channels in the heart. EF could react to kidney toxicity by inducing apoptosis and oxidative stress. However, EF's specific toxic risks and potential disadvantages must be further studied, especially cardiotoxicity and nephrotoxicity. The existing studies mainly focus on the preliminary stage of animal experiments and clinical trials. The specific application effect of EF

in different diseases needs to be further verified, and its safety needs to be evaluated more comprehensively. Therefore, the clinical use of EF should be closely monitored to ensure its safety.

TCM stands as a repository of abundant resources and distinctive processing techniques. However, the phytochemistry, pharmacology, and toxicology of TCM are complex. Therefore, we should further strengthen the research on the pharmacological and toxicological mechanisms of EF, further expand and optimize its application in medicine, and better meet the clinical needs. EF is a valuable plant medicine with various uses, pharmacological activities, and reliable clinical efficacy. It has significant medicinal value and potential to develop new therapeutic drugs. Therefore, systematic and comprehensive research on its metabolism and pharmacological and toxicological mechanisms is a hot spot.

## Author contributions

YH: Conceptualization, Data curation, Formal Analysis, Methodology, Software, Visualization, Writing–original draft, Writing–review and editing. JQ: Data curation, Formal Analysis, Methodology, Software, Writing–original draft. XH: Software, Validation, Visualization, Writing–original draft. CL: Methodology, Project administration, Supervision, Writing–review and editing. YL: Funding acquisition, Investigation, Project administration, Resources, Supervision, 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.

## Generative AI statement

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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.2025.1509032/full#supplementary-material

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# Glossary

| | |
|---|---|
| **ACAT2** | Acetyl-CoA Acetyltransferase 2 |
| **AD** | Alzheimer's disease |
| **AgRP** | Agouti-gene related protein |
| **AKI** | Acute kidney injury |
| **apoB-48** | Apolipoprotein B-48 |
| **ARE** | Antioxidant response element |
| **Aβ40** | amyloid-β 40 |
| **CaMKII** | Ca2+/calmodulin-dependent protein kinase kinase II |
| **CAR** | Constitutive androstane receptor |
| **CCK** | Cholecystokinin |
| **CGRP** | Calcitonin gene related peptide |
| **CK** | Creatine kinase |
| **CP** | Chinese Pharmacopoeia |
| **CYP** | Cytochrome P450 |
| **DDIT3** | DNA damage inducible transcript 3 |
| **DSS** | Dextran Sulfate Sodium Salt |
| **EF** | Euodiae Fructus |
| **EMT** | Epithelial-mesenchymal transition |
| **eNOS** | Endothelial nitric oxide synthase |
| **ER** | *Euodia rutaecarpa* (Juss.) Benth. |
| **ERB** | ER var. *bodinieri* (Dode) Huang |
| **ERO** | ER var. *officinalis* (Dode) Huang |
| **hERG** | Human ether-a-go-go related gene |
| **I/R** | Ischemia/reperfusion |
| **LEF** | Medium flowers of EF |
| **LPS** | Lipopolysaccharide |
| **MAP** | Mitogen-activated protein |
| **MEF** | Large flowers of EF |
| **MMP** | Mitochondrial membrane potential |
| **MPO** | Myeloperoxidase |
| **MPT** | Mitochondrial permeability transition |
| **mTOR** | Mammalian target of rapamycin |
| **NFATc-1** | Nuclear factor of activated T cells 1 |
| **Notch3** | Notch receptor 3 |
| **NPC1L1** | Niemann-Pick C1-like 1 |
| **NPY** | Neuropeptide Y |
| **NRCMs** | Neonatal rat cardiomyocytes |
| **NSCLC** | Non-small cell lung cancer |
| **PBMC** | Peripheral blood mononuclear cells |
| **RANKL** | Receptor activator for nuclear factor-κB ligand |
| **RMs** | Reactive metabolites |
| **ROS** | Reactive oxygen species |
| **SEF** | Small flowers of EF |
| **SOD** | Superoxide dismutase |
| **TCM** | Traditional Chinese Medicine |
| **UC** | Ulcerative colitis |

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# Bridging regulation and practice: CJEU and Dutch case law on botanical health claims

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**Introduction:** Even though botanicals are increasingly popular ingredients for food supplements, health claims related to their putative bene ts remain unclearly regulated.

**Methods:** Through an analysis of EU and national case law from the Netherlands, including self-regulatory decision-making, we have studied the implications of case law on botanical health claims.

**Results:** Our analysis reveals that there are multiple issues related to claims on botanical-containing products: whether it should be classi ed as food or medicine; what statements should be understood as health claims; what type of evidence should underlie health claims and, more specically, botanical health claims; and how to deal with online commercial communication. The case law analysis highlights rst that a gray area will continue to exist when classifying products as foods or medicinal products, particularly when it comes to products that contain botanical ingredients. Most importantly, our study also reveals that claims—even when they are on hold, like botanical claims—need a certain scienti c foundation before they can be used on products. Finally, the courts believe that even though on-hold claims will continue to give a certain level of uncertainty for food business operators, this is not a legal but rather a regulatory issue.

**Discussion:** The findings from our case law analysis highlight that even though case law is useful in further interpretation of legislation, it does not provide any policy advancement. In the case of botanicals, a political decision regarding their substantiation is highly desired.

KEYWORDS

**botanicals, European food law, food information, voluntary food information, risk analysis**

## 1 Introduction

The use of herbal or botanical food supplements is widespread in the European Union (EU) (Garcia-Alvarez et al., 2014; Papatesta et al., 2023). These products, made from one or more botanical species and sold in dosed form, are used by consumers for their alleged beneficial effects on health (Kloosterman et al., 2022). Botanicals are defined as products derived from plants, algae, fungi, or lichens (EFSA, 2018). The use and sales of botanical food supplements have been increasing for years and are expected to continue to grow (Euromonitor International, 2018).

Botanical supplements are classified and regulated as food products in the EU. The communication of health benefits of these products is regulated by Regulation (EC) No

1924/2006 on Nutrition and Health Claims (NHCR), which requires that health claims are substantiated with generally accepted or newly developed scientific evidence (European Parliament and the Council of the European Union, 2006). After being subjected to an authorization procedure in which the underlying scientific evidence is reviewed by the European Food Safety Authority (EFSA), health claims may be authorized for use on food products by the European Commission (Commission). An ongoing debate on the substantiation of botanical health claims prevents the full implementation of the NHCR for the health claims on botanicals (Lenssen et al., 2020). The authorization procedure for these botanical health claims is on hold: the proposed health claims on botanicals have not yet been subjected to the full authorization procedure. Until the procedure is completed, the botanical health claims are subject to transitional measures, as described in Art. 28.5 of the NHCR (European Parliament and the Council of the European Union, 2006). In its check of the regulatory fitness of the NHCR, the Commission concluded that, although the NHCR is still relevant, the current situation regarding botanical health claims has a negative impact on food business operators (FBOs) and consumer protection (European Commission, 2020).

The authorization procedure of the botanical health claims has been on hold for more than 10 years and is not expected to be resolved soon. The inconclusive and unclear status of botanical health claims has affected the work of enforcement authorities, resulting in national court cases on the potential violation of relevant provisions in the NHCR. For the implementation of EU regulations in national courts, the Court of Justice of the European Union (CJEU) can be requested to provide preliminary rulings, which have happened in various instances. As a result, these CJEU rulings have significantly shaped the implementation of the NHCR, especially in relation to the application of the transitional measures. The main aim of this study is to understand how CJEU case law on health claims and botanicals shaped the development of the NHCR and, more specifically, these transitional measures. To study this question, the interpretation of the NHCR in CJEU cases was reviewed. To further understand the implications of this EU case law, the practical translation of the CJEU cases in Dutch national court cases and Dutch advertisement committee cases were analyzed. Together, this review shows the implications of CJEU cases issued because of the on-hold status of the botanical health claims and how these impacted the interpretation of the NHCR.

# 2 Regulatory and conceptual framework

Botanical supplements are regulated as food supplements, making them subject to the regulatory framework of food products (European Parliament and the Council of the European Union, 2002a). The basis of EU food law can be found in Regulation (EC) No178/2002, also known as the general food law (GFL) (European Parliament and the Council of the European Union, 2002b). The general aim of this regulatory framework for foods is to ensure the effective functioning of the internal market and to protect consumers (European Parliament and the Council of the European Union, 2002b). The NHCR, implemented to regulate communication on the health benefits of food products, defines information on nutritional value and health attributes of food products as nutrition and health claims. Such claims must be substantiated by generally accepted scientific evidence (Art. 6), which is evaluated in the pre-market authorization procedure (European Parliament and the Council of the European Union, 2006). The evaluation constitutes an assessment of the scientific evidence compiled in a scientific dossier by EFSA (European Parliament and the Council of the European Union, 2006). EFSA evaluates the evidence in three distinct steps: (i) the bioactive substance must be sufficiently characterized, (ii) the claimed effect must be a beneficial physiological effect, and (iii) there must be a cause-and-effect relationship between the bioactive substance and the beneficial physiological effect (de Boer et al., 2014; EFSA NDA Panel et al., 2017; van Steenwijk et al., 2021). The evaluation of the criteria is published in a Scientific Opinion. After weighing the outcome of the assessment and other relevant considerations, the Commission decides upon the authorization of a health claim.

## 2.1 Health claims on botanicals

Upon implementation of the NHCR in 2006, FBOs could submit the scientific dossiers for putative general function health claims, article 13.1 claims, until 31 January 2008, after which EFSA was asked to evaluate these dossiers (European Parliament and the Council of the European Union, 2006). Many claims, including several reviewed on botanicals, received a negative opinion from EFSA's Panel on Nutrition, Novel Foods and Food Allergens (NDA Panel) and were subsequently not authorized (European Commission, 2020). Quickly thereafter, discussions commenced on the comparability of botanicals in food and medicinal products and the seemingly stricter review process for the health benefits of foods, for which different assessment criteria apply (Lenssen et al., 2020).

The EU regulatory frameworks for food and medicinal products are mutually exclusive: a product is categorized as either a food or a medicinal product (Melcher and Timmermans, 2009). Any statement that refers to the treatment, cure, or prevention of a disease would classify a product as a medicinal product (Verma, 2013). For food products, including botanicals and botanical supplements, health effects must be substantiated with scientific studies, including blinded, randomized human intervention trials (EFSA NDA Panel et al., 2017). For botanicals in medicinal products, however, an alternative authorization is possible in the category of traditional herbal medicinal products (European Parliament and the Council of the European Union, 2004). For these products, safety and efficacy can be established by sources that indicate a long tradition of use of 30 years, of which 15 are within the EU (Committee on Herbal Medicinal Products, 2011; 2017). If such traditional use can be established, a botanical product can be authorized for placement on the market as a traditional herbal medicinal product (European Parliament and the Council of the European Union, 2004). As different stakeholders and member states started to question the seemingly arbitrary differential treatment under the food regulatory regime, it was deemed necessary that the Commission investigate this further. The evaluation of the botanical health claims was therefore put on

hold, allowing for exploring the potential role of evidence on traditional use as substantiation for botanical health claims.

Because the assessment of botanical health claims is still on hold, these claims are subject to the transitional measures of the NHCR (European Parliament and the Council of the European Union, 2006). These transitional measures are usually temporary measures that provide FBOs with a timeframe to implement a new regulation and authorities to inplement procedures. Formerly, the use of trade names with reference to health also fell under such transitional measures (European Parliament and the Council of the European Union, 2006). Given that the procedures as laid down in the NHCR are not fully implemented, the transitional measures still apply for these approximately 2000 claims. This means these claims can be used in the communication of products under two conditions: (1) their application was submitted before 31 January 2008, and (2) the claim and its substantiation meet the general requirements of the NHCR and relevant national provisions of the EU member states. Based on Article 6 of the NHCR, these claims must thus be substantiated with generally accepted scientific evidence. It is, however, up to the member states what this evidence should entail and how it is evaluated (European Parliament and the Council of the European Union, 2006).

## 2.2 The role of case law in regulatory frameworks

Different consultations (EHPM and EBF, 2012; Anton et al., 2013), as well as the most recent regulatory fitness check (European Commission, 2019b; 2020), have not led to a solution for resuming the assessment of botanical health claims. Various CJEU cases were filed on health claims as such and addressed botanical health claims specifically. In the EU, cases from the CJEU can be used to further interpret legislation and ensure its consistent application throughout the European Union (The Member States, 2007). Any natural or legal person is allowed to start a proceeding against a regulatory act. A successful challenge must meet five conditions: (i) the body must be amenable to judicial review; (ii) the type of act in question must be open to challenge; (iii) the claimant must have a standing to act in that position (*locus standi*); (iv) the illegality must be in the scope of Article 263 TFEU; and (v) the time limits set in the treaty must be respected. An applicant is considered not to have *locus standi* if they are to no extent concerned with the request that is raised. A ruling of the CJEU is legally binding for the EU member states. It remains, however, up to national courts to apply and enforce the CJEU interpretations of EU law.

The rulings on botanical health claims have been used in national courts to interpret the legality of actions on the Dutch market. The CJEU rulings are furthermore used by self-regulatory institutions in the Netherlands, like the "Keuringsraad" or the Dutch Advertising Committee (DAC). These self-regulatory bodies must ensure a level playing field in advertising communications in the Dutch market. The advertising code on health products of the Keuringsraad has been reviewed and approved by the formal enforcement authority in the Netherlands, the Dutch Food and Consumer Product Safety Authority (NVWA) (NVWA & Keuringsraad, 2022). As such, self-regulatory institutes play an important role in regulating the health communications of food products in the Netherlands.

Although the status of botanical health claims has been unchanged since 2010, the CJEU case law and consequential national interpretation in national courts and self-regulatory actions have thus led to further clarification and interpretation of this specific group of claims falling under the transitional measures.

# 3 Methodology

The main aim of this study was to understand the role of CJEU case law on the implementation of the NHCR and, more specifically, the botanical health claims. It may furthermore highlight emerging issues from the implementation of this legislative act relevant to food products in general.

The impact of CJEU case law was analyzed in four steps. At first, case law of the CJEU was reviewed to understand the most pressing issues forwarded to the CJEU in relation to the NHCR, botanical health claims, and risk assessment, respectively. Second, national cases addressing (botanical) health claims and transitional measures were analyzed to clarify how national courts interpreted and applied the rulings from the CJEU and how this was connected to enforcement. Third, the decisions from DAC were subsequently reviewed to obtain a more practical interpretation and application of the CJEU case law.

Finally, academic literature on the topics emerging from the reviewed cases was reviewed to gain deeper insights into the implications of the NHCR and case law.

## 3.1 Case law selection

Judgments of the CJEU were retrieved via the Eur-lex and Curia databases using the following search terms: "health claims" and "botanicals." Cases that concerned the risk assessment process or the practical use of specific health claims toward consumers were included. Cases concerning general health claims, claims used prior to the implementation of the NHCR, or cases that concerned information outside the scope of this review were excluded. One additional case was excluded as this concerned the annulment of recitals of Commission Regulation (EC) No 432/2012 and the register of health claims, which are not binding legislative acts. A list of excluded cases can be found in Supplementary Table 1.

All relevant cases were analyzed to understand the case subject, the formulated requests, the CJEU's arguments, and the final ruling. The CJEU's reasoning was used and extrapolated to the implications of that case for the interpretation and application of the NHCR. Relevant cases included requests for preliminary rulings, appeals from rulings of the General Court, claims on failure to act, and claims on actions for annulment. A total of 14 individual cases and one joint case were analyzed, including preliminary rulings, appeals, actions for failure to act, and actions for annulment.

The national case law was retrieved from the Dutch public court decision databases, accessed in June 2024. The cases were accessed using the following search strategies: "health claims AND botanicals," "Health claims AND food supplement," and

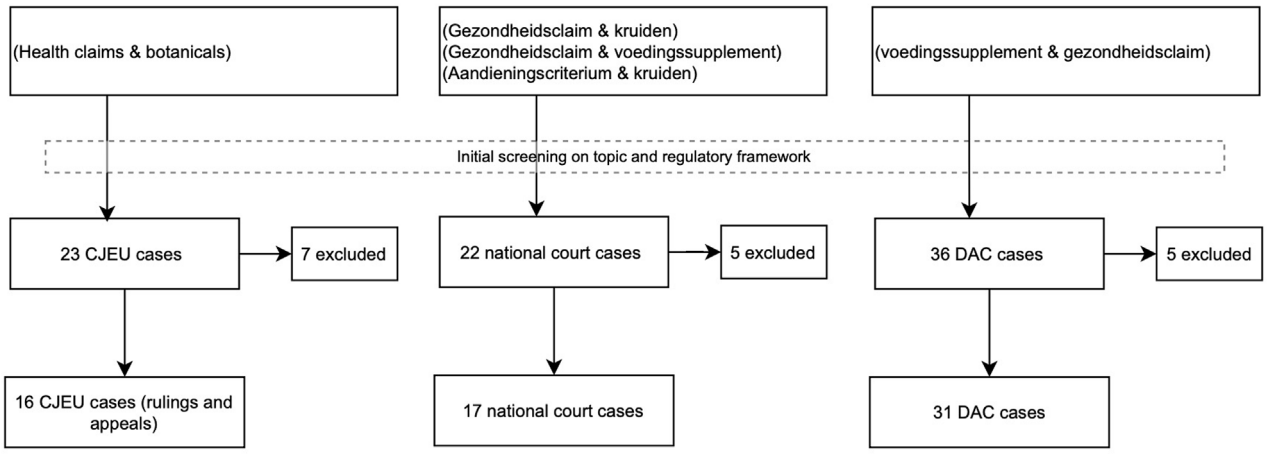


FIGURE 1
Cases included after the search and initial review and following the full case review.

"aandieningscriterium AND botanicals." Only cases that took place after the publication of the permitted list in Commission Regulation No 432/2012 were included. There were no limitations as to the regulatory framework of the cases, which included food and medicinal law, commercial practice law, customs law, and tax law. Cases were included when they referred to or were ruling on similar issues as identified in the CJEU cases or when new emerging issues were found. Cases were excluded when they concerned specialized food or regulatory frameworks outside the scope of this review (e.g., military cases or business disputes). The included and excluded cases are displayed in Supplementary Table 2. Both search strategies resulted in the inclusion of 17 cases.

The decisions from the Dutch Advertising Committee were retrieved from their website, which includes a database with all past cases. The database was searched using the following search terms: "food supplement and health claim." Similarly to the national case law, only decisions from after 2012 were included in the analysis. Like the Dutch national court cases, inclusion followed when the cases concerned CJEU or new emerging issues on the risk assessment of (botanical) health claims. Cases falling under other regulatory frameworks (e.g., veterinary products) or referring to health claims prior to the implementation of the NHCR were excluded. Supplementary Table 3 provides the in- and excluded DAC cases. The search resulted in 31 included decisions of the Dutch Advertising Committee.

Cases were excluded for inclusion after initial review when they were referring to substances, such as feed, cosmetics, or others.

## 3.2 Case law analysis

The analysis commenced with the review of the cases from the CJEU that provided (preliminary) rulings on health claims, risk assessment, and botanicals. The main issues were identified and used for the further analysis of the national and DAC cases.

National cases were included in the review when they either used CJEU case law in argumentation or when similar topics were addressed in the argumentation or decision. Following inclusion, cases were analyzed in-depth to understand the argumentation and regulatory framework applied to the dispute. To what extent the argumentation by the CJEU and the national courts were similar was assessed and, if different, what the reasoning was to deviate from existing case law. In line with national cases included, rulings from the self-regulatory authority, although not legally binding, also provide insight into the use of the regulatory framework and the case law for the self-regulation of foods. These cases were reviewed to gain further insight into the more practical consequences of these cases. Several cases were used in this review as illustrations to allow for an understanding of the line of argumentation followed by the Dutch national court and the DAC.

Finally, the topics and argumentation were reviewed with literature from, amongst others, legal scholars, nutritional sciences, and economic scholars. The literature was searched based on the five emerging issues identified from the case law analysis.

# 4 Results

An overview of the reviewed cases that contributed to the understanding of the emerging issues is illustrated in Figure 1.

The analysis of the CJEU cases led to four emerging issues: the classification of food and medicinal products, the definition of health claims, general evidence requirements, and evidence requirements for claims falling under the transitional measures. One emerging issue from the national and DAC cases was identified in commercial communication in the online environment.

## 4.1 Is it a food or a medicine?

CJEU *Case C-140/07 Hecht-Pharma GmbH v Staatliches Gewerbeaufsichtsamt Lüneburg* is mainly known for its impact on the classification of medicinal products and food products (Court of Justice of the European Union, 2009). This preliminary ruling, requested by the German federal administrative court, concerned

the classification of red yeast rice, which is known to contain small amounts of monacolin K. Monacolin K is known for its effect on the reduction of cholesterol, which is a known pharmacological effect. In this case, the Court ruled medicinal products by function are those products eliciting effects designed to make a medical diagnosis or to restore, correct, or modify physiological functions. The Court also specifies that the dose matters, as not every substance that can elicit a physiological response should be considered a medicinal product by function. Products that are presented as having properties for the prevention or treatment of disease are known as medicinal products by presentation and also fall within the definition of medicinal product as specified in Directive 2001/83/EC Art. 1.2.a (Court of Justice of the European Union, 2009).

In *Case C-177/15 Nelsons GmbH v Ayonnax Nutripharm GmbH and bachblütentreff Ltd.*, the classification of products under the transitional measures was addressed (Court of Justice of the European Union, 2016a). In this case, a product was previously considered a medicinal product but was now, under the NHCR, considered a food product. This classification issue was a result of the interpretation of medicinal products in case C-140/07. In this case, the Court ruled that because it was now categorized as a food product, the NHCR applies to the product (Court of Justice of the European Union, 2016a).

Although the Hecht-Pharma case further defined the classification of foodstuffs and medicinal products, three national cases further explained the concept of medicinal products by function: two specifically on products containing melatonin and one on a product containing glucosamine and chondroitin.

In the Netherlands, the Healthcare Inspectorate (IGZ) and the NVWA issued a general classification for all products containing 0.3 mg melatonin in a single dose as medicinal products in 2011. The authorities consider it sufficiently substantiated in the literature that melatonin significantly influences physiological functions. In the two national cases, the Dutch industry association for food supplements questioned the validity of the Dutch government taking this general stance. The first case, no 2788332, was predominantly content related and addressed the legality of imposing this general measure on a product category (Rechtbank Gelderland, 2015). For this, the decision was reviewed on several criteria. The court ruled that it was not recent, the case was dated 2015, but authorities had regarded doses of above 0.3 mg melatonin as medicinal since 2011. Additionally, the authorities were judged to have sufficient evidence to defend their viewpoint and enforcement measures based on this view. Finally, the measure was also not considered to be too general: the authorities differentiated between products based on the dose. Not all products containing melatonin are considered to be medicinal products; only those containing 0.3 mg of melatonin or more (Rechtbank Gelderland, 2015).

In the second case, no C/09/489402/HA ZA 15-635, another industry association claimed that the Hecht-Pharma case required the appraisal of products on a case-by-case basis and that consequently, the general policy instated here for all products containing melatonin was against this arrest. The court saw differently, as this stance would not allow for any general policies on products (Rechtbank Den Haag, 2016).

A third national court case, no AWB-21_2502 and 21_5482, appealed a decision to classify glucosamine- and chondroitin-containing supplements as medicinal products (Rechtbank Zeeland-West-Brabant, 2022). The court ruled here that a product is only classified as a medicinal product by presentation if there is any doubt about the functionality of a product in an individual's diet. In this case, the product was labeled as a food supplement, and a statement that the product should not be considered a substitute for varied nutrition was sufficient indication that the product is used to supplement the diet and should thus be classified as a food product. The claims that were considered medicinal were false and not allowed but did not lead to the conclusion that the product is a medicinal product (Rechtbank Zeeland-West-Brabant, 2022).

The use of medicinal claims, and thus the sales of medicinal products by presentation, was also observed in the DAC cases (Reclame Code Commissie, 2018; 2023b).

### 4.1.1 Classification because of customs tariff

In the EU, businesses need a specific license to produce and sell medicinal products. As a consequence, the classification of a food supplement as a medicinal product can become economically negative for an FBO because they can either not sell the product if they do not have a license or need to invest in obtaining the license. National case law, however, unveiled that importing products classified as medicinal products is preferred over importing them as food supplements. Medicinal products are also defined by the Customs Administration as products having a therapeutic or prophylactic effect. Different import rules and customs tariffs are enforced for these products.

Two national cases on the import of products with vitamins illustrate this different classification, where one product was classified as a food supplement and the other product as a medicinal product, although the bioactive ingredients were partially similar. These products were initially considered to be food supplements. It was up to the business to prove that the effects of these products were considered therapeutic or prophylactic. This must be substantiated by literature or otherwise.

The first case, no AWB-21_6600, was about supplements with vitamins D and K, which were presented as prevention products against deficiencies (Rechtbank Noord-Holland, 2024c). According to the FBO, a deficiency in vitamin D can lead to osteoporosis or sarcopenia in older people or rickets disease in children. The court decided that merely the prevention of vitamin deficiency would not classify a product as a medicinal product, as vitamin status says something about an individual's general health. The therapeutic or prophylactic effect must be indicated in the label, literature, or otherwise. For this specific product, there was no such information on the label. Literature and otherwise did not provide evidence to support the necessity of the consumption of this product in the treatment or prevention of disease (Rechtbank Noord-Holland, 2024c).

In the second case, no AWB-21_6599, the FBO was able to provide sufficient evidence for the claim that vitamin C had to be regarded as a medicinal product (Rechtbank Noord-Holland, 2024b). As in the previous case, the prevention of vitamin deficiency was also not considered to be sufficiently valid to classify it as a medicinal product. A sufficient vitamin level was considered a general health status and not necessarily a disease outcome. The decisive information that led to the classification of the product as a medicinal product was the role of vitamin C in the

prevention of scurvy and Barlow disease (Rechtbank Noord-Holland, 2024b).

One national court case, no AWB-21_6598, was an appeal on the import of ubiquinol (Rechtbank Noord-Holland, 2024a). The appellant indicated the product is a medicinal product that can be used to prevent ubiquinol deficiency and in the prevention and treatment of cardiovascular disease, chronic inflammation, diabetes type II, and neurodegenerative diseases. The product packaging, however, stated the product is a food supplement. The packaging did not provide any information on the therapeutic or prophylactic effects. A medicinal product by presentation must have the therapeutic or prophylactic effects visible on the "label, literature or otherwise." As this was not the case for this product on the label and the appellant was not able to provide evidence in "literature or otherwise," the appeal was dismissed, and the product was classified as a food supplement (Rechtbank Noord-Holland, 2024a).

It is important to note here that the court considered several aspects: the trade license obtained for the sales, distribution, or advertising of a medicinal product, the pharmacy-only status of the product in some jurisdictions, or the product classification already seen on the market. The inclusion of a leaflet or any other form of communication alongside a multivitamin product where reference was made to disease was also mentioned specifically. The court regarded these, however, to be indicative and not decisive. DAC cases were not found in this specific topic among the commercial cases evaluated.

## 4.2 Defining health claims and their scope

Three CJEU cases contributed to the further interpretation and definition of health claims within the context of the NHCR. The most well known and often-cited case is *Case C-544/10 Deutsches Weintor eG v Land Rheinland-Pfalz*, in which the statement "gentle acidity/easily digestible" was reviewed to determine whether it would fall under the definition of a health claim under Art. 2 (2) (5) of the NHCR (Court of Justice of the European Union, 2012). The FBO argued that the statement refers to general well-being and not health, whereas the national court considered that the definition of health claims should be broadly interpreted, leading to the used statement being considered a claim. The CJEU confirmed a broad interpretation of the definition of health claims, including references to products or substances as "less harmful" and both long-term and short-term health effects. The stated positive effect could persuade a consumer to purchase a product and should thus be considered as a statement falling within the scope of the NHCR (Court of Justice of the European Union, 2012). In *Case C-299/12 Green Swan Pharmaceuticals CR, a.s. v Státní zemědělská a potravinářská inspekce, ústřední inspektorát,* the CJEU was asked to determine whether claims fall under the definition of a reduction of disease risk claim when the reduction of a risk factor of the disease is not explicitly mentioned (Court of Justice of the European Union, 2013). The CJEU reasoned that the suggestion or implication of an effect on a risk factor makes it a health claim, irrespective of whether it is indicated how significant the reduction may be (Court of Justice of the European Union, 2013). In *Case T-17/12 Hagenmeyer & Hahn v European Commission*, the decision to reject the authorization of a disease risk reduction claim related to drinking water and the reduction to the risk of dehydration was appealed (Court of Justice of the European Union, 2014). The proposed claim was rejected as it was not considered to be sufficiently linked to the reduction of an actual risk factor for disease development. The CJEU stated that the risk factor component of the definition in Art. 14 (1) (a) cannot be ignored. Any claim authorized under this Article requires a designated risk factor in the development of disease, which also allows for distinguishing such disease risk reduction claims from medical claims on the treatment, prevention, and cure of disease (Court of Justice of the European Union, 2014).

The definition of health claims and the scope in which the NHCR applies has been the subject of two national court cases and five DAC cases. The argumentation in these cases often follows the conclusions of the Weintor case and the subsequent broad interpretation of the definition of health claims (Reclame Code Commissie, 2016a; 2016b, 2020c).

Two national court cases, no ROT 22/4631 and no 19/463, addressed the definition of health claims related to glucosamine-chondroitin products and the effect they have on joint health (College van Beroep voor het Bedrijfsleven, 2021; Rechtbank Rotterdam, 2023b). In both cases, not necessarily the products but the substances in the products were used in information on the maintenance of joint health. The court here argues that although no direct claim was made, the NHCR also covers the implication or suggestion of a connection between a food constituent and health. Given the Weintor case and the judgment that the definition of a health claim should be broadly interpreted, the indirect implication was considered a health claim in this case (College van Beroep voor het Bedrijfsleven, 2021; Rechtbank Rotterdam, 2023b).

The definition of health claims mainly focused on the distinction between general information on health and health claims in the DAC cases. In one DAC case on joint health, a complaint was raised about the product name, "joint support" (Reclame Code Commissie, 2021). The product name was believed to be a health claim, but it was not sufficiently clear how this product would lead to a positive health effect. In this specific case, the DAC concluded that although there are approved claims for vitamin C, vitamin D, and zinc in relation to joint health, which are also in the product, the product name was not sufficiently related to these nutrients. This could be confusing for consumers and thus mislead them. The FBO was consequently requested not to advertise the product in this way anymore (Reclame Code Commissie, 2021).

## 4.3 Evidence requirements for health claims

In *Case T-296/12 Health Food Manufacturers' Association and Others v Commission*, the annulment of Commission Regulation (EU) 432/2012 and the list of on-hold claims was requested as it was deemed to be based on "improper assessment criteria" (Court of Justice of the European Union, 2015a). The CJEU ruled that there was sufficient legal ground, specifically the term "relationship" in article 2.5.5, to allow for the use of assessment criteria by the risk assessor. The specific requirements regarding the identification of the particular food that causes the effect are deemed necessary to fully understand the scientific substantiation and assess its relevance (Court of Justice of the European Union, 2015a).

*Case T-334/12 Plantavis GmbH and NEM v Commission* described several requests, of which one was on the annulment of the opinions published by EFSA (Court of Justice of the European Union, 2015b). However, the Court stipulated that the opinions of EFSA merely represent "intermediary" steps in the procedure and do not have any legal effects. Thus, they cannot be part of an action for annulment within the scope of Article 263 TFEU (Court of Justice of the European Union, 2015b).

One specific unauthorized health claim was subject in *Case C-296/16 Dextro Energy GmbH&Co.KG v Commission,* which appealed *Case T-100/15 Dextro Energy v Commission* (Court of Justice of the European Union, 2016c; Court of Justice of the European Union, 2017a). In the case, the annulment of Commission Regulation 2015/8 on the refusal to authorize five health claims, including a claim on glucose and the positive effects on the energy-yielding metabolism, was requested. This specific claim was positively assessed by EFSA but not authorized by the Commission on the grounds of general nutrition and health considerations. It was believed that such claims would pose a conflicting message with the general recommendation to reduce the consumption of sugar. The court ruled that messages that are potentially misleading cannot be protected under the freedom of speech. The principles of proportionality were not infringed as the NHCR specifically allows the Commission to weigh aspects of political, economic, and social perspectives in their decision-making. This case thus confirmed that the opinion issued by EFSA is merely one element that is considered by the Commission and not a legal act that can be disputed in court (Court of Justice of the European Union, 2016c; Court of Justice of the European Union, 2017a). The practical interpretation of the evidence requirements was addressed in several DAC cases in which particular studies were reviewed in line with the assessment criteria and CJEU rulings (Reclame Code Commissie, 2012). These cases followed the EFSA criteria, and no additional evidence types were deemed sufficient to substantiate health claims.

## 4.4 Claims falling in the transitional measures

In *Case C-363/19 Konsumentombudsmannen v Mezina AB*, a preliminary ruling was requested to determine whether specific function claims and related general health claims should be substantiated with scientific evidence and, were that to be the case, where the burden of proof would lie (Court of Justice of the European Union, 2020). The CJEU here ruled that claims falling under the transitional measures, including botanical claims, must fulfill the applicable general requirements laid down in the NHCR and national provisions. It is up to the users of the claim to justify them, meaning the burden of proof lies with the FBOs. The Court furthermore stipulates that the NHCR does not provide any clarification as to what the scientific evidence, which is consequently dealt with in national law, should entail (Court of Justice of the European Union, 2020).

Other court actions have also touched upon the transitional measures. In *Case T-296/12 Health Food Manufacturers' Association and Others v Commission*, the applicant requested the annulment of Commission Regulation (EU) No 432/2012 and Commission Decision of 16 May 2012 that resulted in a list of permitted claims and a list of on-hold claims (Court of Justice of the European Union, 2015a). The applicants argued that the Commission failed to achieve the compiling of one list because of the on-hold claims on botanicals. However, the court ruled that a list being incomplete and composed gradually was not against the requirements laid down in Article 13 of the NHCR. It did not lead to any legal uncertainty, and thus, no action was required on either the positive list or the on-hold botanical claims. Another request for the annulment of Commission Regulation (EU) 432/2012 was made in Case T-334/12. The applicants were, however, not concerned by Commission Regulation (EU) 432/2012, and the request was consequently denied (Court of Justice of the European Union, 2015a).

Lastly, two appeal cases also referred to the transitional measures for health claims. *Case C-637/15P VSM Geneesmiddelen BV v Commission* appealed General Court decision *Case T-578/14* (Court of Justice of the European Union, 2015c), in which VSM claimed that the Commission failed to act as it had not asked EFSA to continue the assessment of the botanical health claims (Court of Justice of the European Union, 2016b). The Court ruled, however, that the "act" in this sense referred to taking a position or defining a position as per Article 265 TFEU. Although the Commission did not satisfy the wishes of the applicant, it did take a decision that led to a sufficiently equal condition and no legal uncertainty. In the appeal in Case C-637/15 P, the appellant requested to set aside the previous ruling and used the same claims. The Court concluded all grounds of appeal were inadmissible: on-hold claims do not lead to legal uncertainty, and the current situation may be more advantageous than a situation in which the claims are assessed (Court of Justice of the European Union, 2016b). The appeal in Joined *Cases C-596/15 P and C-597/15 P* (Court of Justice of the European Union, 2017b) appealed the General Court decision in *Cases T-619/14 and T-620/14,* which the appellants also viewed as an infringement of the Commission because it had not requested EFSA to resume the assessment (Court of Justice of the European Union, 2015d; 2015e). The actions were deemed inadmissible because the applicants were not sufficiently concerned with transitional measures. They were not producers or sellers of botanical products and thus lacked locus standing. Furthermore, they could not argue how the adoption of a positive list, and thus resuming the evaluation, would benefit them. All grounds for appeal were thus deemed inadmissible (Court of Justice of the European Union, 2015d; 2015e).

No national cases addressed the botanical health claims under the transitional measures, but the evidence requirements for on-hold claims have been discussed in multiple DAC cases. The decisions in these cases follow the conclusions that health claims on the on-hold list can be used but also unveil the national provisions that can apply. All DAC cases follow a similar line of reasoning. FBOs must have scientific evidence available that justifies the claimed effect. If this cannot be sufficiently achieved, claims can only be made under certain conditions. In practice, this overall condition is translated into the use of a disclaimer for such products (Keuringsraad, 2019). By using the disclaimer, for example, "evaluation health claim is pending" or "health claim is awaiting European approval," FBOs put a reserve on their claim and inform consumers about the on-hold status of the claim, thus avoiding deception. FBOs must still be able to provide scientific evidence upon request.

In one of the cases, 2015/00916 – CVB, the board of appeal refers to a published scientific opinion (Reclame Code Commissie, 2015). In this case, a claim is made about green tea and the stimulation of fat metabolism. In the initial case, the DAC concluded that the FBO did not provide sufficient evidence to substantiate the claim. In the appeal, this conclusion was maintained, and the board referred to the scientific opinion from EFSA on green tea, which had previously indicated that there was no cause-and-effect relationship established between green tea and weight management or fat metabolism. The board, therefore, concluded that this claim may be on hold but can "apparently not be proven" (Reclame Code Commissie, 2015). The use of this published scientific opinion to not allow for the use of a specific claim illustrates how national provision can differ from EU policies. Even though the claim has been assessed, it still falls under the transitional measures and is thus allowed to be used following EU standards. The Board of Appeal, however, concludes that the claim cannot be used based on this assessment.

### 4.5 Commercial communication in the online environment

An emerging issue from the included national and self-regulatory case law was the provision of information in the online environment (Reclame Code Commissie, 2020a; Rechtbank Amsterdam, 2022). In the Dutch court case, a drug store was given a fine for breaching medicinal law by presenting and selling a product as a medicinal product whilst not having a license for it (Rechtbank Rotterdam, 2023a). Another case concerned the reviews of a product (Rechtbank Amsterdam, 2022). In those reviews, reference was made to medicinal effects, whilst other information did not make sufficiently clear that the product is a food supplement (Rechtbank Amsterdam, 2022). The applicant in this case, who was initially fined for the information in the reviews, argued that she could not be held responsible for reviews that were not written by her. The Court reasoned differently and stated that an FBO is responsible for ensuring the information on a website is in line with applicable rules and regulations. Reviews are considered product information, and it is an FBO's responsibility to moderate the reviews to ensure compliance with existing legislation (Rechtbank Amsterdam, 2022).

Several DAC cases concerned the use of unauthorized health claims and medicinal claims in testimonials that were accessible on the website (Reclame Code Commissie, 2023a) or in the product reviews on a website (Reclame Code Commissie, 2020a). Unauthorized statements and third-party webpages, such as those of independent distributors (Reclame Code Commissie, 2016a) or a forum linked to a product's page (Reclame Code Commissie, 2013b), have also been brought to the DAC. Similarly, printed information on a food supplement, including statements on its benefits, provided alongside a medicinal product has been the topic of discussion in a case brought to the DAC (Reclame Code Commissie, 2013a). In all cases, the DAC ruled that the information on the leaflet was considered a claim, either an unauthorized health claim or a medicinal claim. This is in line with the broad interpretation of health claims from the CJEU cases and the CJEU case on the communication of health claims by healthcare providers (Reclame Code Commissie, 2013a).

Two DAC cases requested a decision on the provision of information via an advertorial (Reclame Code Commissie, 2020b; 2023c). The *Oxford Learner's Dictionary* defines an advertorial as "an advertisement that is designed to look like an article in the newspaper or magazine in which it appears" (Oxford Learner's Dictionaries, 2024). For one of the advertorials, the complaint was about general false and misleading information. The article described the difference between synthetic and natural vitamin C and the general necessity to consume vitamin C (Reclame Code Commissie, 2023c). In the other case, a product containing vitamin A was promoted as a product for maintaining healthy mucous membranes (Reclame Code Commissie, 2020b). Although the information was provided in the form of an article, it was still sufficiently linked to a specific product for it to be regarded as a health claim. The DAC believed both advertorials to be subject to the NHCR and the subsequent advertising guidelines issued by the DAC. In the case of vitamin C, the DAC ruled that the information in the advertorial was against both specific and general premises of the NHCR (Reclame Code Commissie, 2023c). The advertorial, consequently, did not follow the advertising code. In the second case on vitamin A and healthy mucous membranes, the claim was allowed, although the wording was not in line with the allowed translations of the authorized health claim (Reclame Code Commissie, 2020b). Other claims in the advertorial were based on the on-hold claims that were not sufficiently substantiated by the FBO, and no disclaimer was provided alongside the claims (Reclame Code Commissie, 2020b). These claims were thus deemed unallowed and cannot be used again by the FBO.

## 5 Discussion

The main aim of this study is to understand how CJEU case law on health claims and botanicals shaped the interpretation and application of the NHCR and, more specifically, the transitional measures. As shown in the results section above, rulings by the CJEU, national courts, and self-regulatory bodies in the Netherlands have provided a clarification on the interpretation of the classification of food and medicinal products, the definition of health claims, the evidence requirements for health claims in general and more specifically for claims falling under the transitional measures and the commercial communication in the online environment. The effect of CJEU rulings is highlighted in the usage of their conclusions in the argumentation of the more practical national cases and self-regulatory DAC cases. These rulings have thus contributed to both a clarification of the regulatory framework and shaped the food information environment for voluntary food information.

### 5.1 Foods or pharmaceuticals in products and claims

As shown by the rulings in Cases C-140/07 and C-544/10, case law has further established the classification of food and medicinal products as well as health claims and medicinal claims. The classification of a product is based on the effect a product (by function for a medicinal product) has or the effects it presents to have (by presentation for a medicinal product) (Nicoletti, 2012). The Dutch national court case further clarified that presenting the medicinal effects of a product, even though its physiological effect is known to not be related to curing, treating, or preventing disease, would classify a product as a food product

(Rechtbank Gelderland, 2024). Hence, not every presented medicinal effect immediately leads to a classification of a product as a medicinal product; it can also merely be an illegal claim. In other cases, in which the wording referred to the treatment, prevention, or cure of a disease and thus referred to the medicinal or pharmacological effects of a product, the court or DAC ruled that the product would classify as a medicinal product by presentation.

Although the court cases provided additional insights into the argumentation to classify a product as a food or medicinal product, classification still remains somewhat of a gray area: it remains the responsibility of individual member states. In their decisions, other aspects, such as culture, can be weighed in this classification, which may lead to differences among member states in product status (Silano et al., 2011). This also became apparent in the Dutch cases on melatonin. Following Commission regulation No 432/2012 on the authorized health claims, a 0.5 mg dose of melatonin would be considered a food that can support in relieving subjective feeling of jetlag, and for a dose of 1 mg of melatonin, a claim can be made to reduce the amount of time it takes to fall asleep (European Parliament and the Council of the European Union, 2012). These are authorized health claims based on doses over 0.3 mg, which may be considered foods in certain countries, but products containing this amount of melatonin would be regarded as medicinal products in the Netherlands. The different court rulings indicated that the Dutch authorities have rightfully established the maximum amount of melatonin in foodstuffs (Rechtbank Gelderland, 2015). Products with higher amounts of melatonin are thus medicinal products by function in the Netherlands, even though they may be classified as foodstuffs in other member states. Clarification of the definition of food and medicinal products by CJEU rulings has thus not fully resolved these classification differences.

The classification of food products and medicinal products and their claims have been criticized before. Whereas these two product categories seem to be strictly separated, the evidence requirements, including the research methodology to show the beneficial effects, are highly similar (de Boer et al., 2014; Todt and Luján, 2017; Lenssen et al., 2022). Food products, including botanicals, can contain multiple bioactive substances, of which synergistic effects cause beneficial effects, or one substance that has multiple but subtle positive effects on health (Heaney, 2006; Bast et al., 2013). Short-term intervention trials would not show these effects, although long-term consumption may be beneficial.

The classification of products, although further clarified in case law, will remain subject to discussion because of differences among member states and the complexity in differentiating between pharmacological and physiological effects. The analysis of case law did show that communicating effects a product cannot elicit, such as claims on preventing, treating, or curing disease, results in the statements being considered illegal health claims or making the product a medicinal product by presentation.

## 5.2 Scientific evidence is mandatory to substantiate claims, including those falling under the transitional measures

In CJEU case C-363/19, the evidence requirements for claims falling under the transitional measures were further clarified: on-hold claims must be substantiated with scientific evidence. That general requirement applies to all claims that fall within the definition of a health claim. The assessment of the scientific evidence by EFSA is not a legal act on which legal actions via the CJEU are possible. The assessment is considered an intermediary step in the process in which the final authorization decision published in a commission regulation is the formal legislative act. The assessment criteria used by EFSA are appropriate, given that the scientific evidence must establish a relationship between food and health.

The NHCR requires, per Art. 6, that health claims are substantiated with generally accepted scientific evidence (European Parliament and the Council of the European Union, 2006). In one of the CJEU cases on the transitional measures, this was further interpreted as evidence that cannot be limited to "beliefs, hearsay derived from popular wisdom, or the observations or experiences of persons outside the scientific community" (Court of Justice of the European Union, 2020). Although this ruling does further clarify the requirements for claims falling under the transitional measures, it still does not provide any insights into the way forward regarding the risk assessment of the claims that are currently on hold. Especially with the individual member states being responsible for the assessment of the scientific substantiation of these claims falling within the transitional measures (Court of Justice of the European Union, 2020), member states may approach this differently. This is exemplified by the Dutch authorities introducing a disclaimer that is currently not implemented in other member states (Keuringsraad, 2019). It could be argued that, based on these CJEU conclusions, evidence of traditional use alone cannot be considered generally accepted scientific evidence. However, it does also not fully dismiss its use. Hence, traditional use evidence could be derived from scientific disciplines other than nutritional sciences, such as history. The risk assessment in its current form, however, requires the establishment of a cause-and-effect relationship (de Boer et al., 2014; Lenssen et al., 2018). Such a strong scientific base for a statement cannot be derived from historical research but would require human intervention trials (Lenssen et al., 2020). Given this requirement, evidence based solely on traditional use would not be sufficient substantiation of botanical health claims.

A tiered evidence approach has been suggested by the European industry association for health products in 2021 (European Federation of Associations of Health Product Manufacturers, 2021). In this approach, it is suggested that different tiers of evidence lead to different types of assessment, subsequentially leading to authorization of differently phrased claims. One of these tiers could be authorization based on traditional use evidence using the wording "x is traditionally used for y." This would require a different approach for both risk assessment and risk management. Currently, EFSA uses a relatively clear approach, and health claims with a negative opinion from EFSA are not authorized by the Commission. In order to implement a graded evidence approach, the evaluation and the authorization would need to be adjusted: new assessment criteria are required for studies other than those from nutritional sciences, and risk management would need to establish how these evaluations are to be used in the authorization process.

The botanical claims have already been on hold for more than 10 years, and the needed political decision on how to move forward is eagerly awaited in the field. Although some direction is provided by case law, a more thorough decision is required to fully resume the evaluation of botanical health claims.

### 5.3 Sufficient legal certainty yet regulatory uncertainty for botanical health claims

Several CJEU cases have addressed requests for the Commission to ask EFSA to continue the evaluation and rulings on the Commission's failure to act on the on-hold status of the botanical health claims. There is, however, no legal ground upon which the CJEU could force action from the Commission to request EFSA to finalize the assessment of the botanical health claims. Therefore, these cases did not change the status of the botanical health claims.

The Commission did a regulatory fitness check of, amongst others, the General Food Law and the NHCR (European Commission, 2019a; 2020). One of the aims of this review was to understand whether the regulations are meeting their objectives. For the review of the NHCR, the botanical health claim was one of two main subjects. The fitness check concluded that as long as all botanical health claims fall under the transitional measures, the objectives are not fully met. Differences in the substantiation requirements among member states may negatively impact the internal market. Additionally, there may be claims on the market that cannot be substantiated with the required level of evidence. Hence, consumers may be exposed to misleading claims. This was also concluded by AG Bobek, who considers claims from the permanent regime different from those under the transitional regime (Bobek, 2017). The NHCR's objectives, ensuring the optimal functioning of the internal market and creating the highest level of consumer protection, may thus not be met. Although the CJEU repeatedly concluded that there is sufficient legal certainty created by the transitional measures—as business operators know that these transitional measures exist and the measures as such are clear—there is uncertainty on the effectiveness of the regulation. Not knowing when EFSA will resume its assessment of the health claims on hold and under what conditions leads to uncertainty, which may negatively impact innovation in the long run (Lenssen et al., 2018).

For botanicals in food specifically, another aspect of interest in different member states is the safety of these supplements. Although the NHCR regulates the communication of food product benefits, some botanicals have known side effects or risks (Greeson et al., 2001; de Heer, 2024). For medicinal products, information on potential adverse events must be provided in a leaflet, and a pharmacovigilance system must be in place to monitor the side effects of medicinal products (European Parliament and the Council of the European Union, 2001). This has led to an overall ban on certain botanicals, such as kava. The Dutch authority for public health and the environment recently looked into several botanicals to understand their benefits and risks (Chen, 2024; de Heer, 2024; de Heer and de Wit-Bos, 2024; de Heer et al., 2024). They concluded that some botanicals, like ashwagandha, may pose risks to consumers. To optimize not only the communication of health benefits but also improve the communication of risks, the Dutch authority has suggested a vigilance system for botanicals to gather information, monitor side effects, and ensure the provision of information (Bureau Risicobeoordeling & onderzoek, 2024). In Belgium, France, and Italy, the BELFRIT program aimed to create positive or negative lists of botanicals that can or cannot be part of food products (Cousyn et al., 2013). These lists were also mainly based on the safety of these products.

The current status of botanical health claims currently suffices for FBOs in terms of legal certainty. At the same time, the regulatory fitness check concluded that the objectives of the NHCR may not be met: it remains uncertain how the field will be impacted by measures that are still expected to be taken. It is particularly questioned to what extent companies are affected in their innovation plans due to the uncertainty on how this regime will be continued (European Commission, 2020). This again emphasizes the clear need for a decision to be made on the substantiation of botanical health claims. This decision should follow both the objectives of the NHCR and the interpretation of the CJEU of the NHCR. Simultaneously, the protection of consumers should also be viewed in light of the risks of botanicals, raising the possibility for dedicated legislation for this product category dealing with safety and efficacy.

## 6 Conclusion

The CJEU rulings, national court cases, and self-regulatory DAC output have clarified how the NHCR needs to be interpreted and implemented. They have shed light on multiple issues, including what statements we understand as being a health claim and how the transitional measures for botanical claims need to be seen from a legal perspective, including the necessity of scientific evidence to substantiate claims falling under these measures. The court rulings, together with the Regulatory Fitness and Performance (REFIT) evaluation of the NHCR, have particularly highlighted that having a substantial group of on-hold claims negatively affects the market and impacts innovation. Although there is no legal uncertainty as such, the internal market may currently not function optimally as two different types of claims are found on the market: those that are scientifically sound and those that have not been assessed on their scientific merit. Consumers may, therefore, be exposed to claims for which no objective scientific evidence is available. The CJEU confirms that the assessment criteria for scientific evidence used by EFSA are appropriate and even indicates that merely non-scientific evidence, like experiences or hearsay, cannot sufficiently substantiate botanical health claims. However, this does not fully close the door to other types of scientific evidence, such as historical or anthropological studies on the health benefits of botanicals.

The analysis of case law on botanical health claims highlights that case law does lead to further interpretation of the NHCR. Its effects are limited to legal interpretation, whereas a broader policy perspective is required to resume the assessment of the botanical health claims. Only a formal decision by the risk manager can resolve the impasse with these claims and lift the uncertainty faced by producers of products using these claims.

## Data availability statement

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

## Author contributions

KL: conceptualization, formal analysis, methodology, and writing–original draft. AdB: conceptualization, formal analysis, methodology, supervision, and writing–review and editing.

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## Publisher's note



## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2025.1523904/full#supplementary-material

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# Development and validation of the HPLC–MS/MS method and its application to the pharmacokinetic study for the Mongolian drug Sendeng-4 in rat blood plasma

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**Abstract:** Sendeng-4 is a Mongolian drug. The Mongolian people have been using it to treat rheumatoid arthritis. At present, an increasing number of Han people are paying attention to the anti-rheumatoid effect of Sendeng-4. However, information on the pharmacokinetics of Sendeng-4 is limited, which limits its wide application in China.

**Objective:** Liquid chromatography–tandem mass spectrometry (LC–MS/MS) was established to study the pharmacokinetics of Sendeng-4.

**Method:** MS/MS with a negative ionization mode (ESI-) and multiple reaction monitoring at m/z 300.95→193.09 and 317.08→192.10 were detected for (2R, 3R)-dihydromyricetin and myricetin, respectively. The pharmacokinetic parameters were analyzed by DAS 2.0.

**Result:** The results showed that the plasma concentration time (C–T) curves of (2R, 3R)-dihydromyricetin and myricetin showed double peaks. The $T_{max}$ value of (2R, 3R)-dihydromyricetin and myricetin in both groups was 3 h. In absorption, the $AUC_{(0-\infty)}$ values of (2R, 3R)-dihydromyricetin and myricetin in the normal group and the arthritis model group were 16.151 ± 2.670 mg·h/L vs. 11.331 ± 0.749 mg·h/L and 2.626 ± 0.400 mg·h/L vs. 2.213 ± 0.388 mg·h/L, respectively. In the distribution, the Vz/F values of (2R, 3R)-dihydromyricetin and myricetin in the normal group and the arthritis model group were 8.212 L/kg vs. 1.744 L/kg and 5.252 L/kg vs. 10.568 L/kg, respectively. In metabolism, the MRT (0-∞) values of (2R, 3R)-dihydromyricetin and myricetin in the normal group and the arthritis model group were 6.848 h vs. 3.476 h and 5.661 h vs. 8.959 h, respectively. In excretion, the CLz/F values of (2R, 3R)-dihydromyricetin and myricetin in the normal group and the arthritis model group were 0.021 vs. 0.024 L/min/kg and 0.018 vs. 0.021 L/min/kg, respectively. There were significant variations in the absorption levels, distribution levels, and elimination rate of (2R, 3R)-dihydromyricetin and myricetin after the administration of Sendeng-4.

**Conclusion:** The study laid the foundation for the subsequent study of pharmacokinetics of Sendeng-4 in humans. The results of this study will contribute to a better understanding of the activity and clinical application of Sendeng-4 and other related traditional Mongolian drug prescriptions.

KEYWORDS

**Mongolian drug, Sendeng-4, myricetin, LC–MS/MS, pharmacokinetic, (2R, 3R)-dihydromyricetin**

# 1 Introduction

Rheumatoid arthritis (RA) is a common systemic autoimmune disease characterized by chronical, symmetrical, and erosive damage of the synovium of joints. Sendeng-4, a traditional Mongolian drug, is commonly used as an anti-rheumatic therapy in the clinic, which is recorded in "The People's Republic of China Ministry of Health Standards for drug (Mongolian branch)" (National Pharmacopoeia Committee, 1998). This formula includes four herbs, namely, *Xanthoceras sorbifolium* Bunge (Yu et al., 2012; Wang et al., 2016) (Sapindaceae; Xanthoceras; *Xanthoceras sorbifolium* stem), *Melia azedarach* L (He et al., 2010; Liu et al., 2016; He et al., 2011) (Meliaceae; Melia; *Melia azedarach* fructus), *Gardenia jasminoides* (Lin et al., 2015; Sung et al., 2014) (Rubiaceae; Gardenia; *Gardenia jasminoides* fructus), and *Terminalia chebula* Retz (Manosroi et al., 2013; Eshwarappa et al., 2015) (Combretaceae; Terminalia; *Terminalia chebula* fructus).

The Mongolian drug is a type of ethnic medicine and is a Chinese herbal medicine used by the Mongolian people in the Inner Mongolia region. The Mongolian drug is similar to traditional Chinese drugs. It consists of a variety of herbs, which contain complex chemical ingredients. Despite containing the same ingredients, it also shows different effects in different formulations, for example, (2R, 3R)-dihydromyricetin and myricetin (Figure 1) of Sendeng-4 in this study, which mainly have anti-inflammatory and antioxidant activities in the Sendeng-4 formula. According to the literature reported, (2R, 3R)-dihydromyricetin has anti-inflammatory, anti-oxidative, hypoglycemic, hepatoprotective, anti-browning, and antibacterial effects (Liang et al., 2014; Zhang et al., 2007), and myricetin has anti-inflammatory, anti-oxidative, antianaphylaxis, and anti-tumor effects (Li and Ding, 2012; Ko, 2012; Wu et al., 2016).

In pre-project studies, (2R, 3R)-dihydromyricetin and myricetin were isolated from Sendeng-4 in the extraction and separation experiment. In addition, through cell biological activity tests, it was proven that (2R, 3R)-dihydromyricetin and myricetin showed anti-inflammatory biological activity *in vitro*. We also established a HPLC–MS/MS method for simultaneously detecting the blood concentration of (2R, 3R)-dihydromyricetin and myricetin (Bai et al., 2020). Although plasma concentrations of (2R, 3R)-dihydromyricetin and myricetin were detected, the distribution, metabolism, and excretion of dihydromyricetin and myricetin in the body were not clear. Therefore, it is necessary to study the pharmacokinetic characteristics of (2R, 3R)-dihydromyricetin and myricetin. So, the established HPLC–MS/MS method (Bai et al., 2020) was applied to study the pharmacokinetics in normal and arthritis model rats.

# 2 Materials and methods

## 2.1 Chemicals and reagents

Myricetin (purity, >98%) was isolated in our laboratory (Department of Natural Medicinal Chemistry, Inner Mongolia Medical University, Hohhot, PR China), which was identified by combination NMR, mass spectrometry, and infrared spectroscopy, and purity was determined by HPLC chromatography. (2R, 3R)-

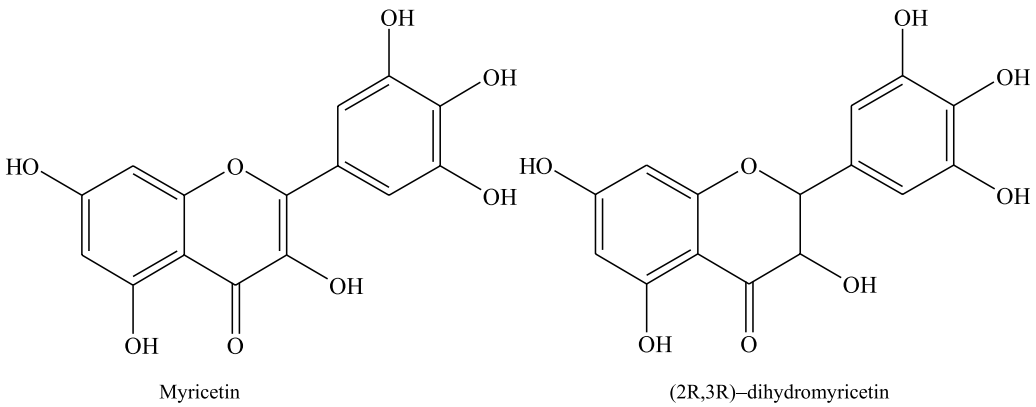



FIGURE 1
Structures of myricetin and (2R, 3R)-dihydromyricetin in Sendeng-4.

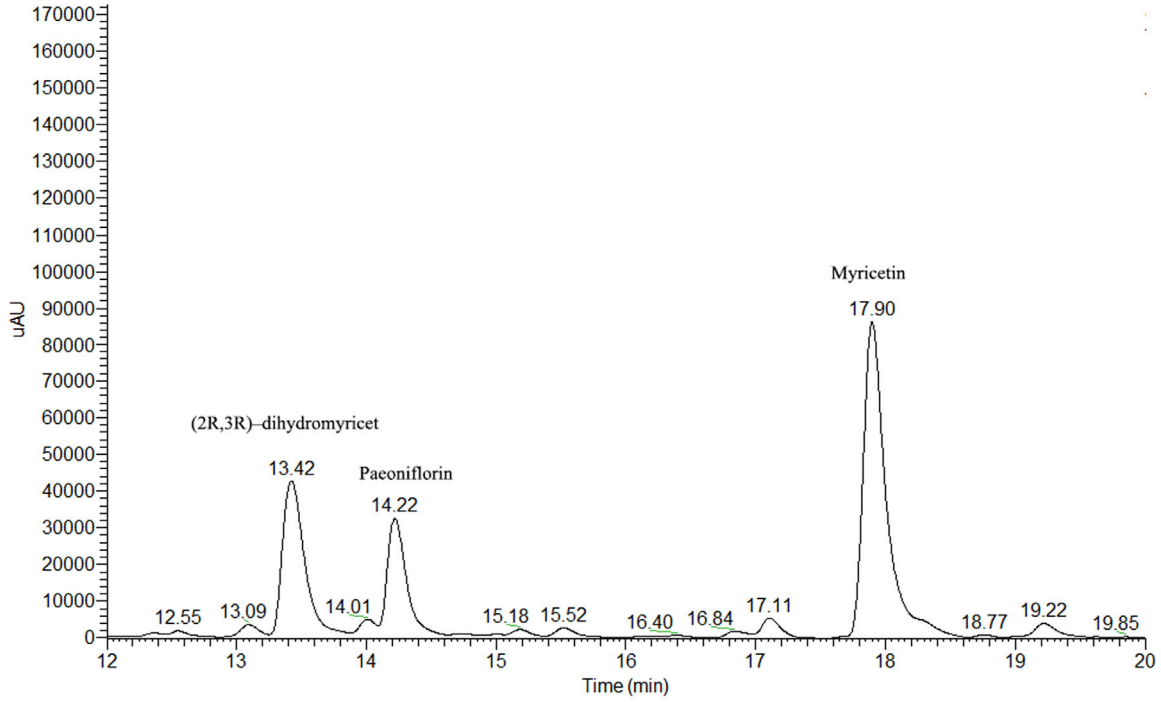



FIGURE 2
Chromatography of myricetin, (2R, 3R)-dihydromyricetin, and IS in rat blood plasma.

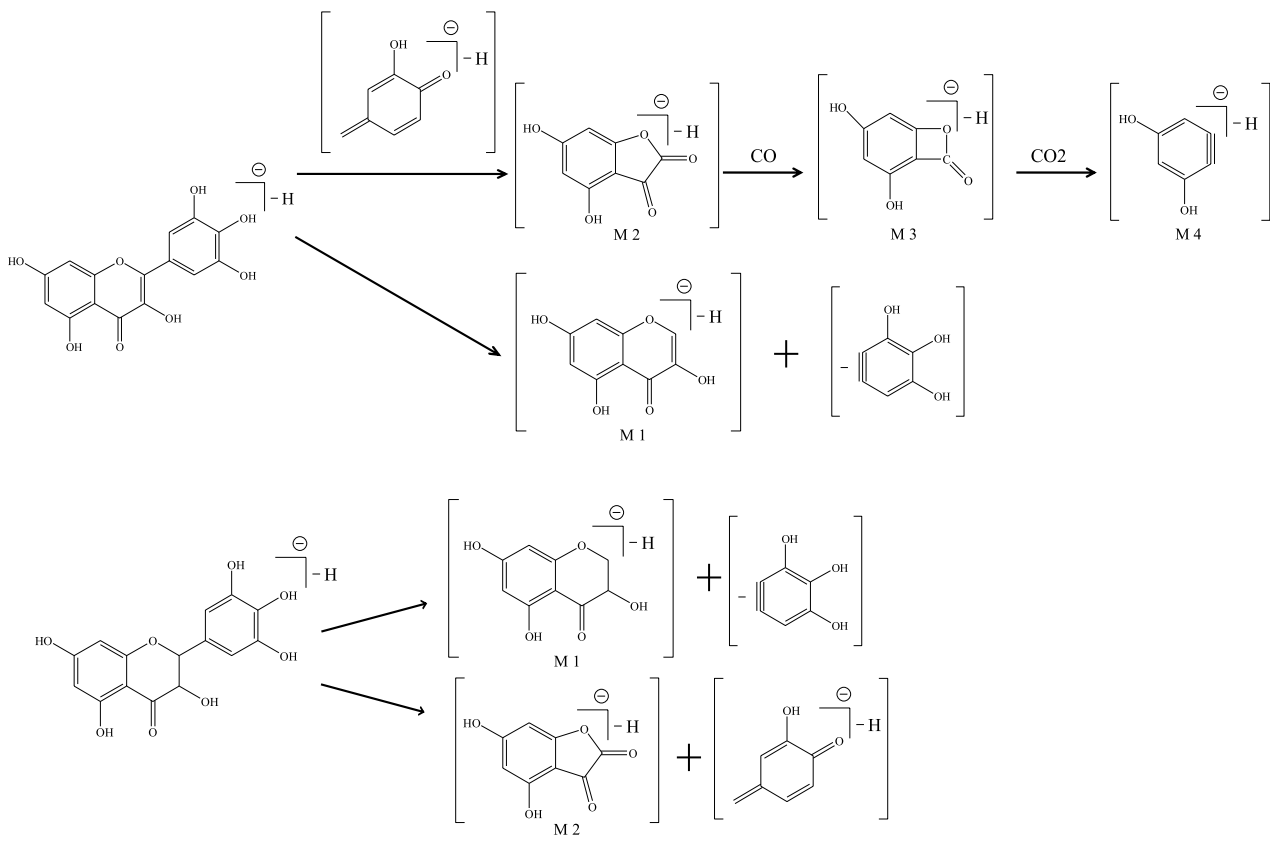
**TABLE 1** Molecular formula and fragment information on myricetin, (2R, 3R)-dihydromyricetin, and paeoniflorin (IS).

| Analyte | Molecular formula | Molecular weight | Pathway | MS (*m/z*) | Fragment (*m/z*) | Retention time (min) |
|---|---|---|---|---|---|---|
| (2R, 3R)-Dihydromyricetin | $C_{15}H_{12}O_8$ | 320 | [M−H]− | 319 | 300.95, 256.98, 193.09, and 179.03 | 10.58 |
| Myricetin | $C_{15}H_{10}O_8$ | 318 | [M−H]− | 317 | 317.08, 192.10, 179.02, 151.03, 137.09, 125.01, and 106.92 | 15.23 |
| Paeoniflorin (IS) | $C_{23}H_{28}O_{11}$ | 480 | [M−H]− | 479 | 478.84, 448.90, and 326.97 | 11.71 |

Dihydromyricetin (purity, >98%) was purchased from J&K Scientific LTD. (Beijing, PR China), and the batch number is 110749–201316. Acetic acid was purchased from Sigma-Aldrich (St. Louis, MO, United States). Acetonitrile and methanol of HPLC grade were purchased from Fisher Scientific Co., Ltd (St. Louis, MO, United States). Ultrapure water was purified using a Milli-Q Reagent Water System (Millipore, Burlington, MA, United States). Collagen type II and Freund's complete adjuvant were purchased from Sigma-Aldrich (St. Louis, MO, United States). All other chemicals and reagents used were of HPLC grade.

## 2.2 Animal and animal grouping

A total of 30 healthy Sprague–Dawley rats, weighing 220 ± 20 g, were purchased from the Experimental Animal Center of Inner Mongolia University (Hohhot, China). The rats were randomly divided into two groups: 15 rats in the normal group and 15 rats in the model group. Randomized grouping procedures are as follows: (1) Number: all rats were numbered from 1 to 30. (2) Obtain random numbers: 30 numbers were selected from rows 10 and columns 15 on the right side of the random number table to correspond to 30 numbered rats. (3) Find the remainder: 30 random numbers were divided by 2 to get the remainder. (4) Group: the rats corresponding to a random number with remainder 0 were assigned to the normal group. The rats corresponding to a random number with remainder 1 were assigned to the model group. The rats were kept in an air-conditioned animal room with a relative humidity of 50% ± 10% and temperature of 22°C ± 2°C. All rats had free access to water and food (the Experimental Animal Center of Inner Mongolia University, Hohhot, China). The rats were acclimatized to the environment for 15 days, and then, they were fasted for 12 h before the experiment. The animal studies were approved by the Animal Ethics Committee of Inner Mongolia Medical University (Hohhot, China).




SCHEME 1
Mass spectrum cracking pathway of myricetin and (2R, 3R)-dihydromyricetin.

## 2.3 Rat model of rheumatoid arthritis preparation

A model of collagen-induced arthritis was first reported by Trentham in 1977 (Myers et al., 2008). Based on the study of domestic and foreign experts and scholars over the years, this model is similar to human rheumatoid arthritis in pathology, immunology, and genetics (Griffiths et al., 1981; Wooley et al., 1981). So, it is recognized as an ideal animal model for screening and studying the treatment or prevention of rheumatoid arthritis drugs.

An emulsion (0.2 mL) made of Freund's complete adjuvant and Collagen type II was injected into the hind feet of each rat. Twenty days later, the emulsion (0.1 mL) was injected at the same place of the rats. After two injections, the arthritis model was successfully induced.

## 2.4 Preparation of Sendeng-4 extracts

The *Xanthoceras sorbifolium* Bunge (Sapindaceae; Xanthoceras; *Xanthoceras sorbifolium* stem), *Melia azedarach* L (Meliaceae; Melia; *Melia azedarach* fructus), *Gardenia jasminoides* (Rubiaceae; Gardenia; *Gardenia jasminoides* fructus), and *Terminalia chebula* Retz (Combretaceae; Terminalia; *Terminalia chebula* fructus) samples were purchased from Inner Mongolia Hohhot Tianli Chinese herbal medicine company. These herbs were identified and authenticated by Professor Li Xiao in appearance, microscopic identification, and physical and chemical identification and stored in the Engineering Technology Research Center of Pharmacodynamic Substance and Quality Control of Mongolian Medicine in Inner Mongolia.

The four herbs were dried in the shade. In accordance with the proportion, the crude powder (2,000 g) was soaked in 70% ethanol (v/w, 10:1) for 1 h, followed by reflux extraction for 2 h. After filtration, the extracts were reflowed again in 70% ethanol (v/w, 10:1) for 2 h. The filtrate was pooled together and concentrated by a rotary evaporator to no alcohol at 45°C.

The solution was adsorbed by AB-8 macroporous resin and eluted with distilled water and ethanol. The ethanol elution was collected and concentrated to no alcohol. Finally, the dry extract powder of Sendeng-4 was obtained. The dry extract powder of Sendeng-4 (1.62 g) was weighed in a beaker. It was then dissolved in 0.5% CMC-Na (Qingyun et al., 2018) to acquire the Sendeng-4 extract solution at the concentration of 0.2025g/mL.

## 2.5 Preparation of blood samples

In this study, protein precipitation and liquid extraction were applied to extract (2R, 3R)-dihydromyricetin, myricetin, and IS from blood samples. A measure of 200 μL Plasma, 200 μL HCl (2 M), 50 μL 10% L-ascorbic acid, and 100 μL internal standard (IS)

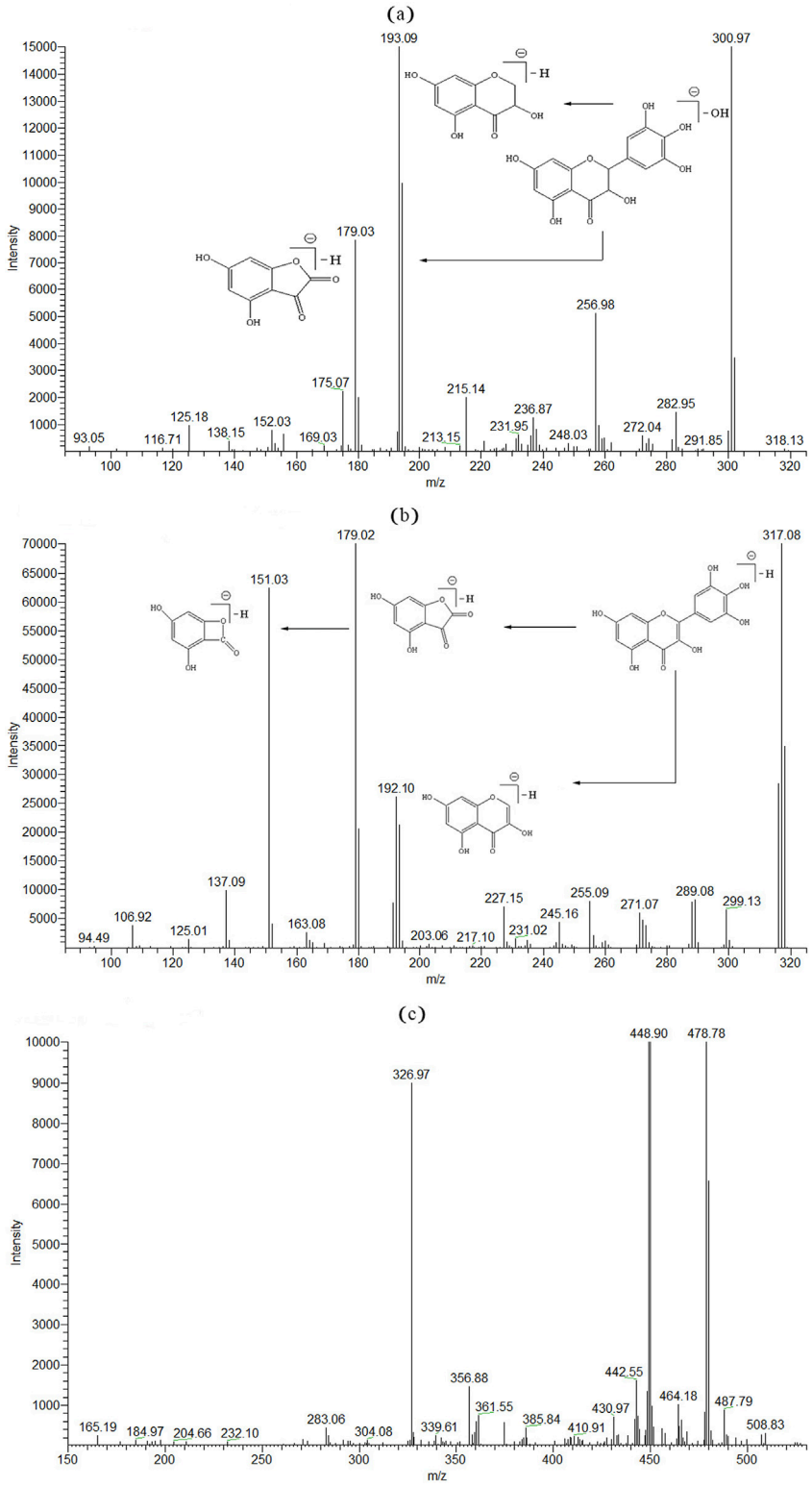



FIGURE 3
MS/MS spectrum of (2R, 3R)-dihydromyricetin **(A)**, myricetin **(B)**, and paeoniflorin **(C)**.

paeoniflorin solution (0.8 μg/μL) was added to a 5.0-mL Eppendorf tube. The mixture was vortex-mixed for 1 min at room temperature and placed for 30 min in an 80°C water bath. After 30 min, the sample was cooled to room temperature, 1 mL ethyl acetate was added, and centrifuged at 3,000 rpm for 10 min. Then, the supernatant was carefully removed and transferred to a new 1.5-mL Eppendorf tube and evaporated to dryness at 40°C under nitrogen gas. The residue was dissolved in 500 μL

TABLE 2 Mass spectral data on myricetin, (2R, 3R)-dihydromyricetin, and its fragmentation.

| Flavonol type and flavanone type | Molecular formula | Precursor ion (*m/z*, $[M-H]^-$) | | Product ions (*m/z*) |
|---|---|---|---|---|
| | | Calculated | Observed | |
| Myricetin | | | | |
| $[M-H]^-$ | $C_{15}H_{10}O_8$ | 317 | 317.08 | — |
| M1 (M-$C_6H_4O_3$) | $C_9H_5O_5$ | 192 | 192.10 | 124 |
| M2 (M-$C_7H_5O_2$) | $C_8H_3O_5$ | 179 | 179.02 | 109 |
| M3 (M2-CO) | $C_7H_3O_4$ | 151 | 151.03 | 28 |
| M4 (M3-$CO_2$) | $C_6H_3O_2$ | 107 | 106.92 | 44 |
| (2R, 3R)-Dihydromyricetin | | | | |
| $[M-H_2O]^-$ | $C_{15}H_9O_7$ | 301 | 300.95 | 18 |
| M1 (M-$C_6H_4O_3$) | $C_9H_5O_5$ | 193 | 193.09 | 124 |
| M2 (M-$C_7H_6O_2$) | $C_8H_3O_5$ | 179 | 179.03 | 109 |

methanol–water (50:50, v/v). A portion of the supernatant (10 μL) was injected into the HPLC/MS/MS system for analysis.

## 2.6 Instruments and analytical conditions

The analysis was performed on a Thermo Finnigan Surveyor Plus HPLC tandem LCQ Advantage MAX Multi-Stage Ion Trap Mass Spectrometer (Thermo Fisher Scientific, Finnigan, United States). The high-performance liquid chromatography (HPLC) system consisted of a quaternionic pump (Thermo Fisher Scientific, United States), an autosampler (Thermo Fisher Scientific, United States), and a UV detector (Thermo Fisher Scientific, United States). LCQ Advantage MAX was equipped with an electrospray ionization (ESI) probe.

The specific parameters of the MS analysis are as follows: samples were detected by the multiple reaction monitoring (MRM) mode. The capillary voltage was 4.5 kv. The gas temperature was 250°C. The sheath gas was 30 L/min, and the aux/sweep gas flow rate was 5 L/min. The capillary temperature was 250°C.

The specific parameters of HPLC are as follows: the mobile phase consisted of acetonitrile (solvent A) and 0.1% acetic acid water (solvent B) at a flow rate of 1.0 mL/min. The gradient elution conditions are as follows: 0–5 min, 12%–14% A; 5–15 min, 14%–40% A, 15–20 min, and 40%–100% A. The injection volume was 10 μL. The detection wavelength was at 254 nm. The column temperature was 25°C.

We evaluated the following columns: Waters XBridge C-18 column (250 × 4.6 mm, 5.0 μm, Waters, Milford, United States), Extend ODS C-18 column (250 × 4.6 mm, 5.0 μm, Agilent, United States), Hypersil ODS-2 C-18 column (250 × 4.6 mm, 5.0 μm, Thermo Fisher Scientific, United States), and ShimNex CS C-18 column (250 × 4.6 mm, 5.0 μm, Shimaizumi, Japan). The column with the best separation was selected. Other instruments used are as follows: a SIGMA 3–18 K centrifugal machine, a HH-ZK4 Intelligent digital display thermostatic bath, and a G560E Vortex machine.

## 2.7 Stock solution, standard solution, and quality control sample solution preparation

The stock solutions of (2R, 3R)-dihydromyricetin and myricetin were prepared at the concentration of 71.8 ng/μL and 5.32 ng/μL, respectively. The standard solutions were obtained from the stock solution by dilution with methanol. The calibration sample solutions were prepared by adding 100 μL standard solutions to 200 μL blank plasma in 1.5 mL Eppendorf tubes. Therefore, the final calibration sample solutions were obtained at the concentrations of 0.399–39.900 ng/μL (2R, 3R)-dihydromyricetin and 0.050–5.026 ng/μL myricetin, respectively. The quality control (QC) sample solutions were prepared in the same way as the calibration samples.

The final prepared low-, medium-, and high-quality control concentrations of (2R, 3R)-dihydromyricetin were 0.399 ng/μL (LQC), 3.990 ng/μL (MQC), and 39.900 ng/μL (HQC), respectively. The concentrations of myricetin LQC, MQC, and HQC were 0.050 ng/μL, 0.503 ng/μL, and 5.026 ng/μL, respectively. The stock solution of the internal standard (IS), paeoniflorin, was prepared in methanol at the concentration of 2 mg/mL. The IS working solution of 0.8 μg/μL was obtained by diluting the stock solution with methanol.

## 2.8 Method validation

The method was validated in compliance with the FDA bioanalytical method validation guidelines (Fda, 2018).

### 2.8.1 Specificity and sensitivity

The plasma chromatograms of (2R, 3R)-dihydromyricetin and myricetin were compared to determine whether the corresponding retention times of (2R, 3R)-dihydromyricetin, myricetin, and IS had impurity peaks and whether there was any interference between them.

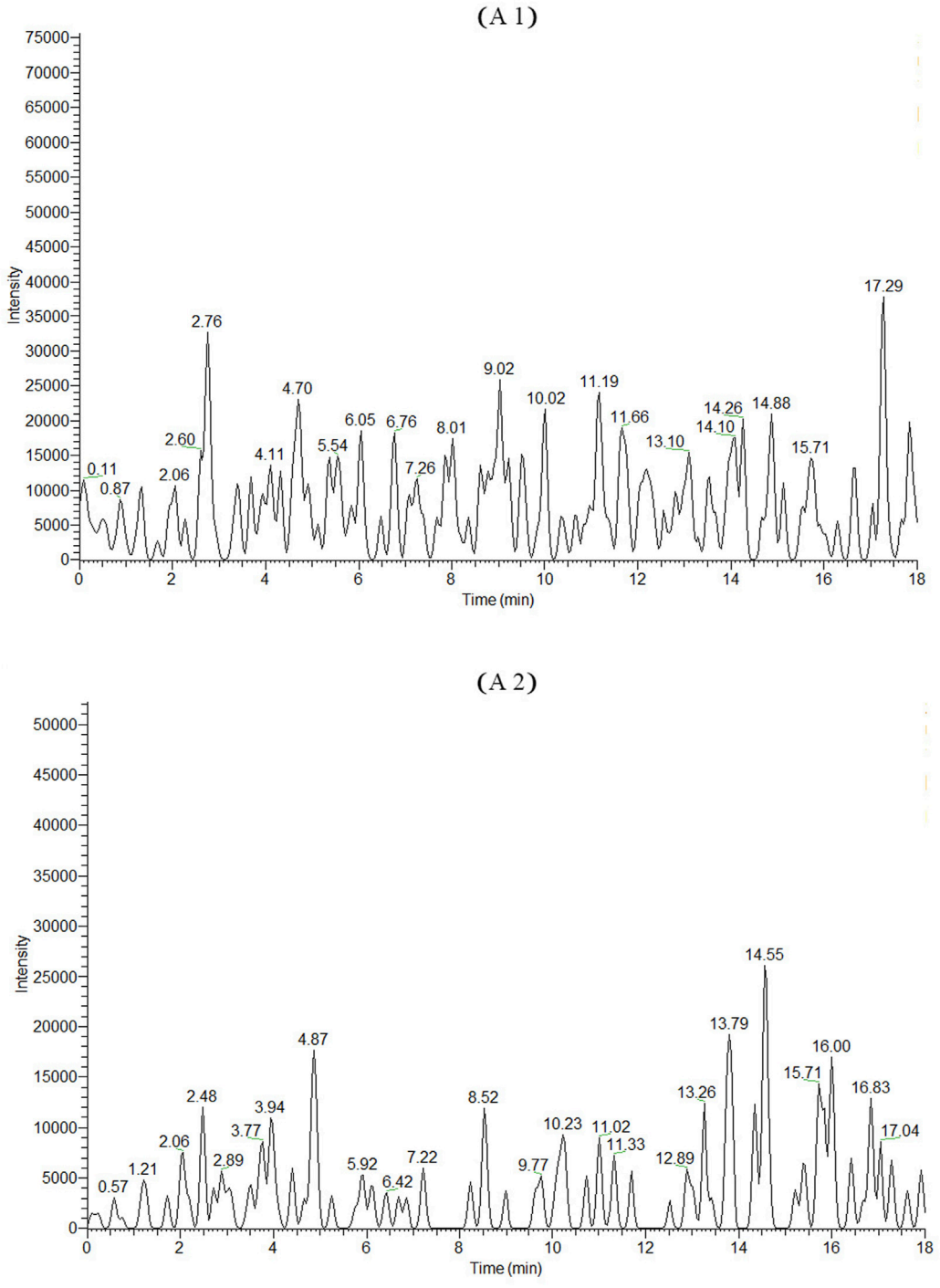



FIGURE 4
(Continued).

## 2.8.2 Linearity and LLOQ

The linear equation (y = ax + b) of (2R, 3R)-dihydromyricetin and myricetin was established by using the ratio of the peak area of (2R, 3R)-dihydromyricetin to the peak area of IS as the *y* value and the concentration of (2R, 3R)-dihydromyricetin and myricetin as the *x* value, and the linearity of the method of (2R, 3R)-dihydromyricetin and myricetin was verified. The lower limit of quantitation (LLOQ) value was established at the lowest concentration of the linear range, while the upper limit of quantification value was established at the highest concentration of the linear ranges.

## 2.8.3 Precision and accuracy

The intra- and inter-day accuracy and precision were assessed by analyzing three levels of QC samples three times on three consecutive days. The accuracy and precision were evaluated as

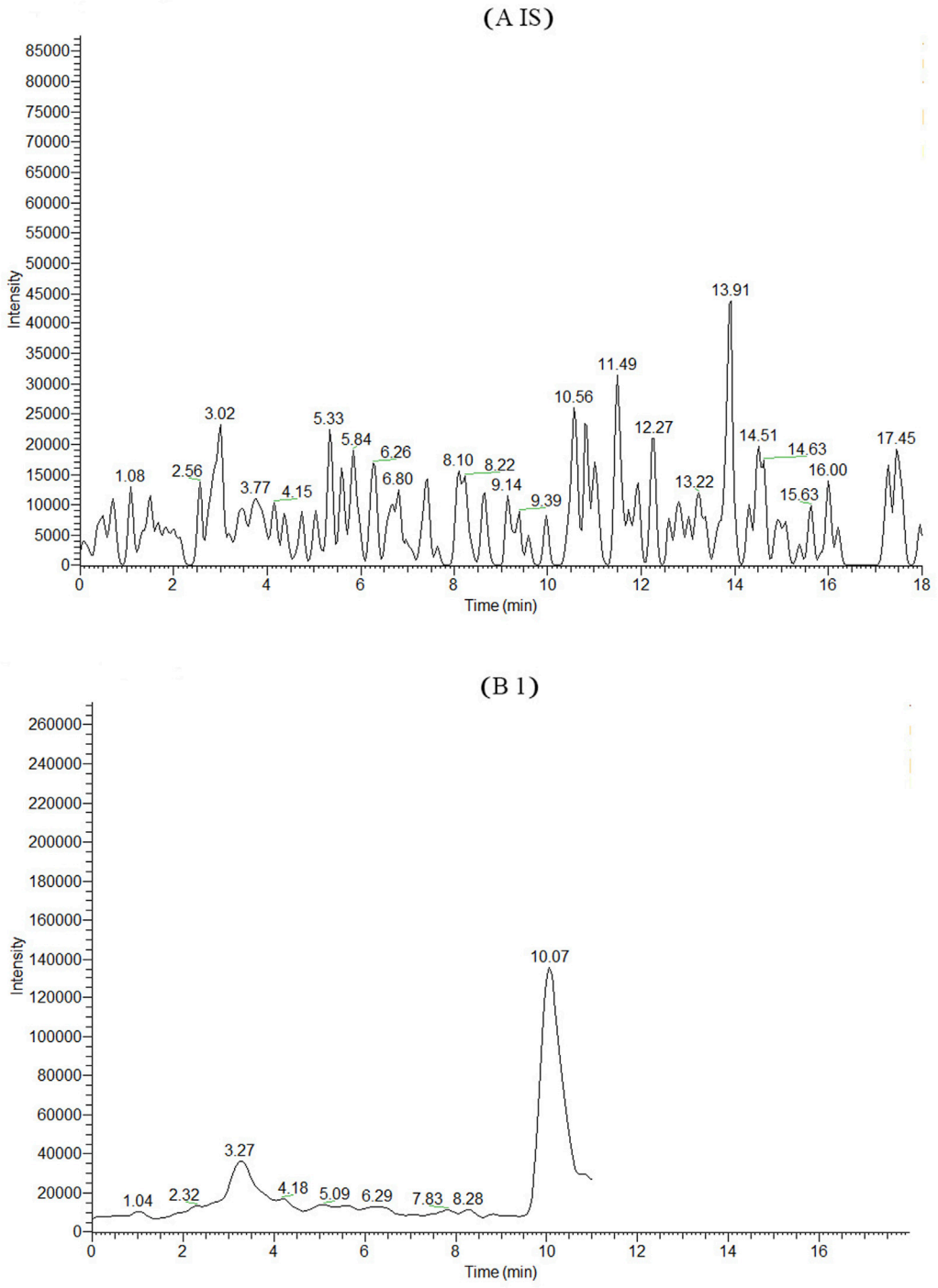



FIGURE 4
(Continued).

the relative error (RE) and the relative standard deviation (RSD), respectively. The RE was within ±15%, and the RSD could not exceed 15%.

### 2.8.4 Extraction recovery and matrix effect

The extraction recovery and the matrix effect were evaluated by analyzing three levels of QC concentrations three times. The extraction recovery was calculated by comparing the plasma drug concentrations of plasma samples with (2R, 3R)-dihydromyricetin and myricetin added before extraction and with the equivalent amount of (2R, 3R)-dihydromyricetin and myricetin added after extraction. The matrix effect was evaluated by the post-extraction quantitative method. The matrix effect was calculated by comparing plasma drug concentrations of (2R, 3R)-dihydromyricetin and

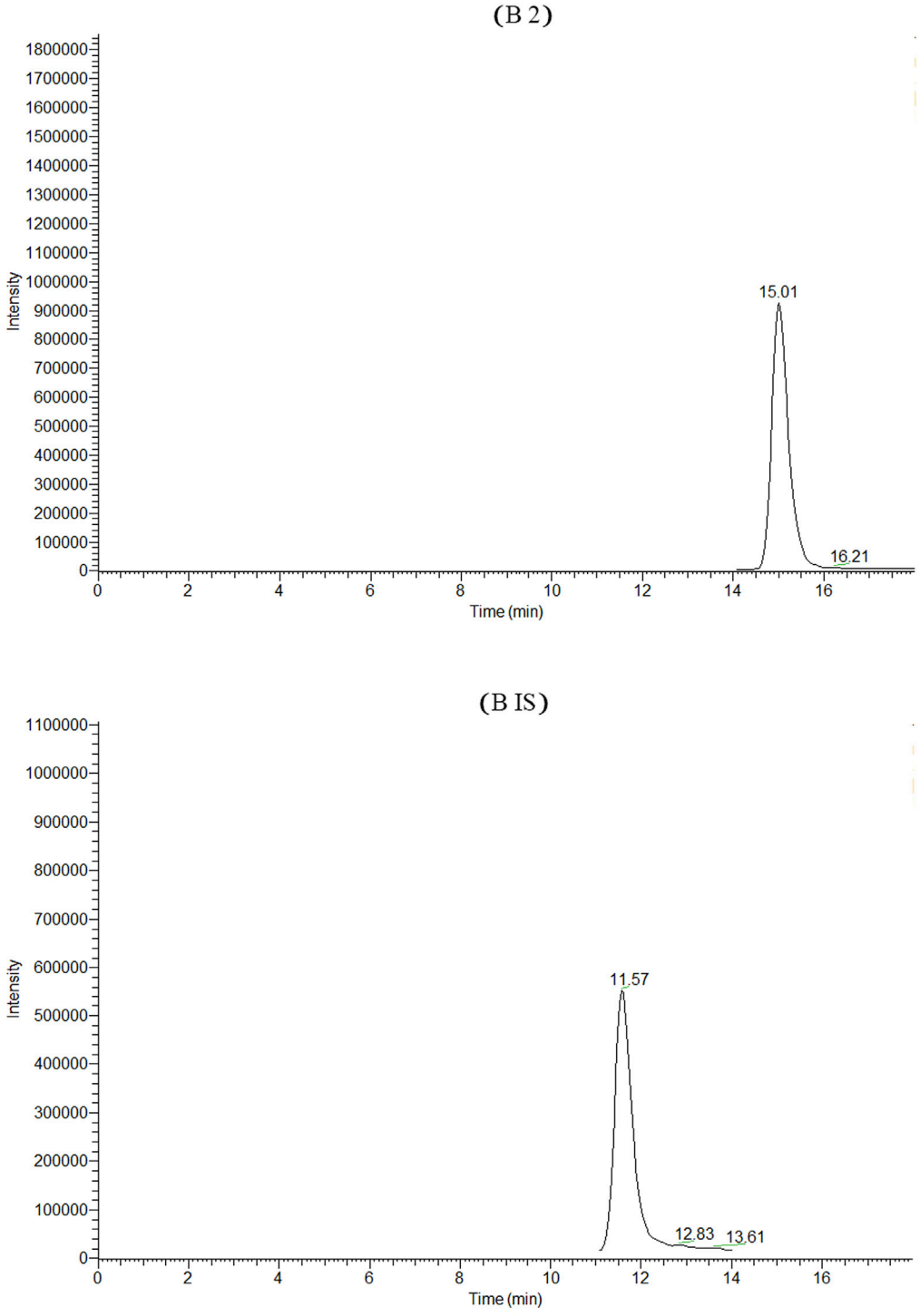



FIGURE 4
(Continued).

myricetin added to plasma samples after extraction with the plasma drug concentrations actually added.

### 2.8.5 Stability

The stability was studied at different conditions analyzing three levels (LQC, MQC, and HQC) of QC sample solutions three times. The room temperature stability study analyzed the stability of the sample at room temperature over a period of 24 h. The freeze–thaw stability study was to detect the stability of samples after three freeze–thaw cycles at −75°C–25°C. The stability was evaluated by relative standard deviation (RSD). The RSD was within ±15%.

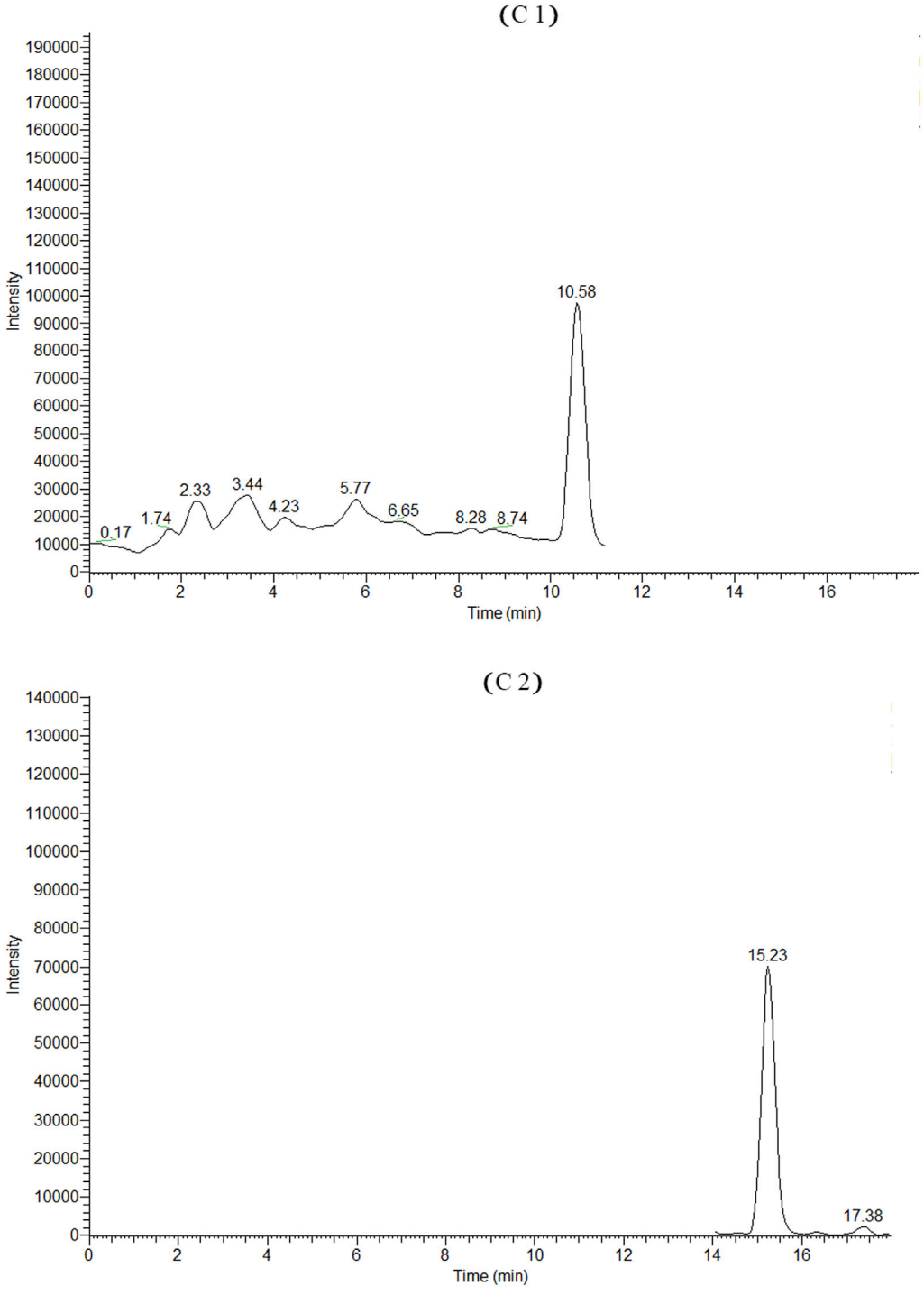



FIGURE 4
(Continued).

## 3 Application to a pharmacokinetic study

The pharmacokinetics of RA model rats and normal rats were investigated in the study. Rats were fed under standard environmental conditions (12 h light and 12 h dark cycle) for a few days before the pharmacokinetic study. After the rats were fasted for at least 12 h, they were orally administered (using the gavage technique) 5.4 g/kg Sendeng-4, which was suspended in 0.5% g/mL sodium carboxymethyl cellulose (CMC-Na). Under light anesthesia, blood samples (200 μL) were collected from orbital sinus at 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0 h after oral administration. Plasma samples were centrifuged at 12,000 rpm for 10 min. The concentrations of (2R, 3R)-dihydromyricetin and

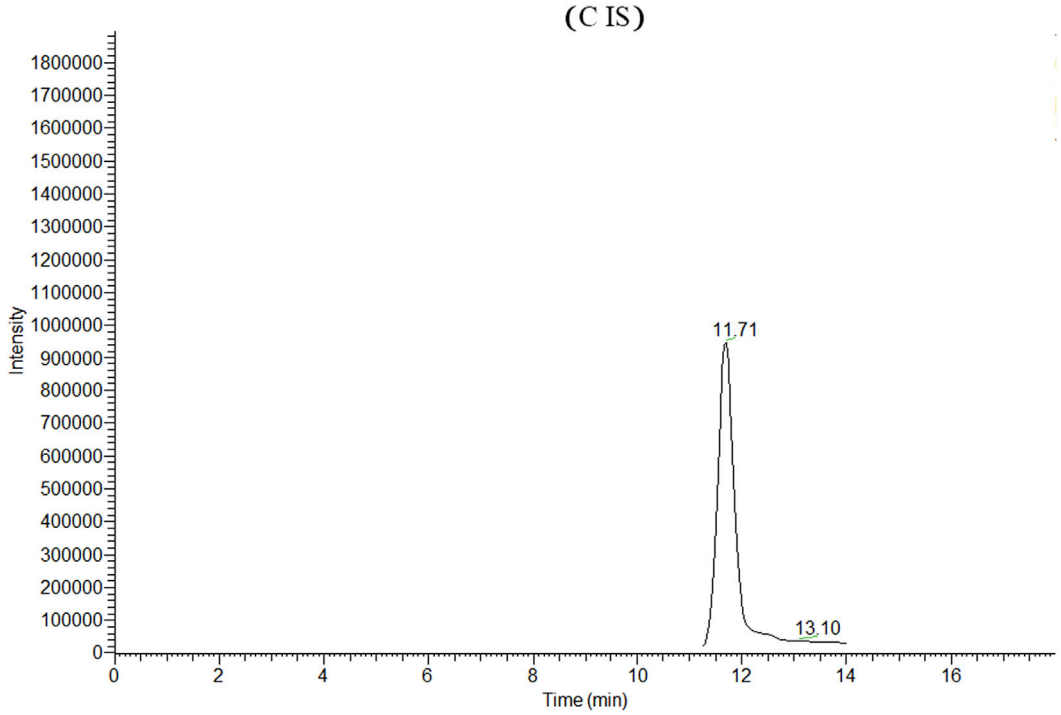



FIGURE 4
(Continued). Extracted ion chromatogram (EIC) of the two ingredients and paeoniflorin (IS) in rats. **(A)** Blank plasma. **(B)** Blank plasma spiked with standard substances. 1: myricetin, 2: (2R, 3R)-dihydromyricetin, and IS: paeoniflorin. **(C)** Rat blood plasma after oral administration of Sendeng-4.

myricetin were measured by the developed LC–MS/MS method. The parameters of pharmacokinetics ($t_{1/2}$, $t_{max}$, and $C_{max}$) were calculated by DAS 2.0.

The C–T curve can reflect the qualitative characteristics of the pharmacokinetics, while the area under the curve (AUC), mean residence time (MRT), maximal plasma concentration ($C_{max}$), and T1/2 can quantitatively reflect the systemic exposure level of the drug. The PK results of (2R, 3R)-dihydromyricetin and myricetin of Sendeng-4 were analyzed and discussed from the above two aspects.

# 4 Statistical analysis

DAS 2.0 software (Mathematical Pharmacology Professional Committee of China, Shanghai, China) was used for the pharmacokinetic studies. All data were shown as mean ± standard deviation (SD).

# 5 Results

## 5.1 Method development

In terms of HPLC column selection, several types of HPLC columns were evaluated, such as Agilent Extend ODS C-18, Thermo Hypersil ODS-2 C-18, SHIMADZU ShimNex CS C-18, and Waters XBridge C-18 column. The data revealed that the Waters C-18 column exhibited better separation of peaks. Thus, the Waters C-18 column was chosen. During the HPLC run time of 20 min, (2R, 3R)-dihydromyricetin, myricetin, and IS (paeoniflorin) were well-separated with retention times of 13.42, 14.22, and 17.90 min, respectively (Figure 2).

The rat blood plasma samples were pretreated by organic solvent precipitation protein method. A variety of organic solvents were evaluated, including acetone, methanol, acetonitrile, and trichloroacetic acid, among others. The matrix effect of acetonitrile was the lowest, and the recovery rate of acetonitrile was the highest. So, we chose acetonitrile protein precipitation.

## 5.2 Mass spectral study of myricetin and (2R, 3R)-dihydromyricetin

The mass spectrometry detection conditions were optimized and modified. The full ion mode scanning procedure was used to find the most optimum precursor-to-product ion pairs. Both positive and negative ionization ESI modes were tested for (2R, 3R)-dihydromyricetin, myricetin, and paeoniflorin (IS). The negative mode (ESI-) of (2R, 3R)-dihydromyricetin and myricetin had better sensitivity than the positive mode. (2R, 3R)-dihydromyricetin and myricetin were detected and quantified in the negative ionization mode (ESI-). The selective ion monitoring mode was used for quantitation by the $[M-H]^-$ molecular ions of the analytes (Table 1). Under ionic bombardment, (2R, 3R)-dihydromyricetin and myricetin were split into different fragment ions through a cleavage reaction (Scheme 1) (Fabre et al., 2001). Among them, (2R, 3R)-dihydromyricetin and myricetin showed the

TABLE 3 Calibration curves, linear ranges, correlation coefficients, and LLOQ of (2R, 3R)-dihydromyricetin and myricetin.

| Analyte | Linear regression equation | $R^2$ | Linear range (ng/μL) | LLOQ (ng/μL) |
|---|---|---|---|---|
| (2R, 3R)-Dihydromyricetin | y = 2E-05x+0.0083 | 0.9998 | 0.399–39.900 | 0.199 |
| Myricetin | y = 0.0004x-0.0052 | 0.9997 | 0.050–5.026 | 0.025 |

TABLE 4 Precision and accuracy of (2R, 3R)-dihydromyricetin and myricetin in rat blood plasma.

| Analyte | Spiked concentration (ng/μL) | Calculated concentration (ng/μL) | Intra-day precision (RSD, %) | Accuracy (RE, %) | Calculated concentration (ng/μL) | Inter-day precision (RSD, %) | Accuracy (RE, %) |
|---|---|---|---|---|---|---|---|
| (2R,3R)-Dihydromyricetin | 0.399 | 0.375 ± 0.017 | 4.530 | 6.047 | 0.387 ± 0.012 | 3.005 | 3.058 |
| | 3.990 | 3.941 ± 0.126 | 3.185 | 1.277 | 3.873 ± 0.273 | 7.062 | 2.935 |
| | 39.900 | 41.874 ± 0.831 | 1.983 | 4.947 | 39.580 ± 1.088 | 2.749 | 0.803 |
| Myricetin | 0.050 | 0.050 ± 0.002 | 3.444 | 0.514 | 0.050 ± 0.003 | 6.601 | 0.095 |
| | 0.503 | 0.542 ± 0.010 | 1.906 | 7.671 | 0.482 ± 0.036 | 7.392 | 4.084 |
| | 5.026 | 5.149 ± 0.257 | 4.997 | 2.457 | 4.928 ± 0.344 | 6.979 | 1.943 |

same m/z 179.02 characteristic MS fragment in the MS spectra due to the similar molecular structure (Figure 3; Table 2).

## 5.3 Method validation

### 5.3.1 Specificity and selectivity

The chromatograms of rat blank blood plasma, rat blank blood plasma spiked with standard solutions and IS, and rat blood plasma after oral Sendeng-4 administration are shown in Figure 4. The retention times of myricetin, (2R, 3R)-dihydromyricetin, and the IS (paeoniflorin) were 15.23 min, 10.58 min, and 11.71 min, respectively. By comparison with the chromatogram of rat blank plasma, it was found that the endogenous substance did not interfere with (2R, 3R)-dihydromyricetin, myricetin, and IS. These results showed that the method developed has good selectivity without endogenous substance interferences.

### 5.3.2 Linearity and lower limit of quantitation (LLOQ)

The calibration curves for (2R, 3R)-dihydromyricetin and myricetin exhibited good linearity, with coefficients of correlation (r) within the range of 0.9998–0.9999. Linear ranges, slopes, intercepts, LLOQs, and correlation coefficients of calibration curves are listed in Table 3. The correlation coefficients (r) were more than 0.9999. It indicated that our method has good linearity.

### 5.3.3 Precision and accuracy

The intra-day and inter-day precision and accuracy were evaluated three times. The results are summarized in Table 4. The relative standard deviations of intra- and inter-day were less than 15%. These results showed that the precision and accuracy was accurate and reliable.

### 5.3.4 Extraction recovery and matrix effect

The extraction recovery and matrix effect of (2R, 3R)-dihydromyricetin and myricetin are shown in Table 5. The extraction recovery of (2R, 3R)-dihydromyricetin and myricetin were within the range of 91.609%–110.180% and 92.111%–103.933%, respectively. The matrix effect of (2R, 3R)-dihydromyricetin and myricetin were within the range of 94.289%–99.919% and 97.091%–99.820%, respectively. The results indicated that endogenous substances did not have a significant effect on the quantification of all analytes.

### 5.3.5 Stability

As shown in Table 6, the relative standard deviations (RSDs) of room temperature stability and freeze–thaw stability were within 15% ((2R, 3R)-dihydromyricetin: <3.287% vs. < 2.865 and myricetin: <7.709 vs. < 5.275). These results indicated that (2R, 3R)-dihydromyricetin and myricetin were stable and applicable.

## 5.4 Results of pharmacokinetic (PK) studies

DAS 2.0 software was used to fit the data to the compartmental model, and the AIC values of different models were compared. The one-compartment model, two-compartment model, three-compartment model, and different weights were used to fit the curve of the data, and the results showed that the one-compartment model with non-intravenous injection (weight 1/cc) had a good fit with the measured blood concentration, and the AIC value of the fitted model was the lowest.

### 5.4.1 Pharmacokinetics of Sendeng-4 in normal rats

The plasma C-T curves of (2R, 3R)-dihydromyricetin and myricetin after oral Sendeng-4 administration are shown in Figure 5, and the corresponding PK parameters are listed in Table 7.

TABLE 5 Recovery of (2R, 3R)-dihydromyricetin and myricetin in rat blood plasma.

| Analyte | Spiked concentration (ng/μL) | Extraction recovery | | Matrix effect | |
|---|---|---|---|---|---|
| | | Mean ± SD (%) | RSD (%) | Mean ± SD (%) | RSD (%) |
| (2R, 3R)-Dihydromyricetin | 0.399 | 93.939 ± 4.684 | 4.986 | 99.919 ± 1.523 | 1.524 |
| | 3.990 | 110.180 ± 3.487 | 3.164 | 94.289 ± 3.281 | 3.480 |
| | 39.900 | 91.609 ± 5.470 | 5.971 | 99.051 ± 4.780 | 4.826 |
| Myricetin | 0.050 | 93.921 ± 2.443 | 2.601 | 97.933 ± 5.886 | 6.011 |
| | 0.503 | 92.111 ± 1.652 | 1.793 | 97.091 ± 2.743 | 2.825 |
| | 5.026 | 103.933 ± 4.806 | 4.624 | 99.820 ± 0.907 | 0.908 |

TABLE 6 Stability of (2R, 3R)-dihydromyricetin and myricetin in rat blood plasma under various storage conditions.

| Analyte | Spiked concentration (ng/μL) | Room temperature stability | Freeze–thaw stability |
|---|---|---|---|
| | | RSD (%) | RSD (%) |
| (2R, 3R)-Dihydromyricetin | 0.399 | 3.287 | 1.281 |
| | 3.990 | 2.904 | 2.865 |
| | 39.900 | 3.257 | 2.847 |
| Myricetin | 0.050 | 1.909 | 5.275 |
| | 0.503 | 7.709 | 2.144 |
| | 5.026 | 3.577 | 1.048 |

In Figure 5, the C–T curve showed a double peak after oral Sendeng-4 administration. Two plasma concentration peaks were observed at 1h and 3 h in the C–T curves of (2R, 3R)-dihydromyricetin and myricetin, and the maximum plasma concentration peak was at 3 h. The C–T curve approximates the one-compartment model (calculated by using DAS 2.0 software, Table 7). In absorption, the $AUC_{(0-\infty)}$ values of (2R, 3R)-dihydromyricetin and myricetin were 16.151 ± 2.670 mg·h/L and 2.626 ± 0.400 mg·h/L, respectively. In distribution, the Vz/F of (2R, 3R)-dihydromyricetin and myricetin were 8.212 L/kg and 5.252 L/kg, respectively. In metabolism, the $MRT_{(0-\infty)}$ of (2R, 3R)-dihydromyricetin and myricetin were 6.848 h and 5.661 h, respectively. In discharge, the CLz/F values of (2R, 3R)-dihydromyricetin and myricetin were 0.021 L/min/kg and 0.018 L/min/kg, respectively.

### 5.4.2 Pharmacokinetics of Sendeng-4 in arthritis-induced rats

The arthritis-induced rats' plasma C–T curves of (2R, 3R)-dihydromyricetin and myricetin are shown in Figure 5, and the corresponding PK parameters are listed in Table 7. Similarly, the C–T curves showed double peaks after oral Sendeng-4 administration in Figure 5, and the C–T curves approximated the one-compartment model (calculated by using DAS 2.0 software, Table 7). Two plasma concentration peaks were observed at 1h and 3 h in the C–T curves of (2R, 3R)-dihydromyricetin and myricetin. The $T_{max}$ values of (2R, 3R)-dihydromyricetin and myricetin in both groups was 3 h. In absorption, the $AUC_{(0-\infty)}$ of (2R, 3R)-dihydromyricetin and myricetin were 11.331 ± 0.749 mg·h/L and 2.213 ± 0.388 mg·h/L, respectively. In distribution, the Vz/F values of (2R, 3R)-dihydromyricetin and myricetin were 1.744 L/kg and 10.568 L/kg, respectively. In metabolism, the $MRT_{(0-\infty)}$ values of (2R, 3R)-dihydromyricetin and myricetin were 3.476 h and 8.959 h, respectively. In discharge, the CLz/F values of (2R, 3R)-dihydromyricetin and myricetin were 0.024 L/min/kg and 0.021 L/min/kg, respectively.

## 6 Discussion

Comparing other traditional botanical drug therapies across the world, the most important characteristic of the Mongolian drug is the multi-herbs treatment based on the theory and unique treatment mode of Mongolian medicine. Due to the interaction of various ingredients in the Mongolian drug, the pharmacokinetic study of the Mongolian drug is complicated.

Through the study of network pharmacology, it was found that (Tian and Dong, 2015) (2R, 3R)-dihydromyricetin and myricetin, respectively, act on tumor necrosis factor-α and nuclear transcription factor Kappa B, and xanthine dehydrogenase/oxidase exerts anti-inflammatory effects. It is proven that Sendeng-4, a Mongolian drug, has a treatment effect on rheumatoid arthritis. Pre-project studies of cell biological activity tests proved that (2R, 3R)–dihydromyricetin and myricetin are active ingredients of Sendeng-4.

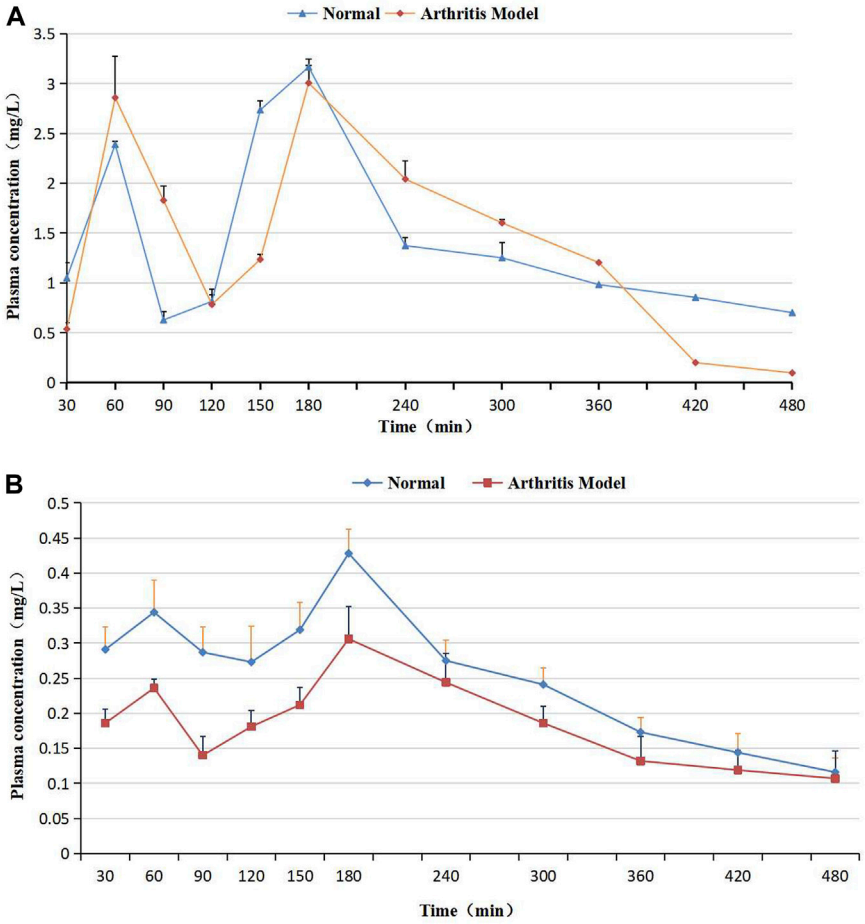



FIGURE 5
**(A)** Plasma concentration–time curves of (2R, 3R)-dihydromyricetin after oral Sendeng-4 administration in normal and arthritis model rats. **(B)** Plasma concentration–time curves of myricetin after oral Sendeng-4 administration in normal and arthritis model rats.

**TABLE 7** Pharmacokinetic parameters of (2R, 3R)-dihydromyricetin and myricetin after oral administration of Sendeng-4 in normal and arthritis model rats.

| Parameter | | (2R, 3R)-Dihydromyricetin | | Myricetin | |
|---|---|---|---|---|---|
| | | Normal | Arthritis model | Normal | Arthritis model |
| $C_{max}$ | mg/L | 3.166 ± 0.083 | 3.507 ± 0.174 | 0.428 ± 0.084 | 0.306 ± 0.046 |
| $T_{max}$ | h | 3 | 3 | 3 | 3 |
| $T_{1/2z}$ | h | 4.686 | 0.843 | 3.556 | 5.948 |
| $AUC_{(0\rightarrow t)}$ | mg·h/L | 10.938 ± 0.096 | 11.219 ± 0.752 | 1.971 ± 0.221 | 1.386 ± 0.259 |
| $AUC_{(0-\infty)}$ | mg·h/L | 16.151 ± 2.670 | 11.331 ± 0.749 | 2.626 ± 0.400 | 2.213 ± 0.388 |
| $MRT_{(0-t)}$ | h | 3.630 | 3.414 | 3.511 | 3.818 |
| $MRT_{(0-\infty)}$ | h | 6.848 | 3.476 | 5.661 | 8.959 |
| CLz/F | L/min/kg | 0.021 | 0.024 | 0.018 | 0.021 |
| Vz/F | L/kg | 8.212 | 1.744 | 5.252 | 10.568 |

The LC–MS method was used to detect the drug concentration in biological samples with the advantages of good selectivity and high sensitivity. At present, it is increasingly used in the detection of pharmacokinetics and drug concentrations (Fang et al., 2016; Tao et al., 2013; Huisi et al., 2016; Qian et al., 2017; Tong et al., 2006; Yu et al., 2007). However, individual differences in rats, drug

dissolution, and biological sample processing methods make it difficult to detect the tested ingredients. The concentration of the ingredients detected by the LC–MS/MS technique used the selected ion monitoring mode scan and molecular ion ionization mode.

As shown in Figure 5, the plasma C–T curves of (2R, 3R)-dihydromyricetin and myricetin showed double peaks. The reasons for the double peaks were summarized as follows: (a) Enterohepatic circulation (Okusanya et al., 2007): after oral administration, the drug entered into the liver through the portal vein, and a part of the drug accumulated in the gallbladder. When the gallbladder contracted, this part of the drug was rapidly released to the intestinal tract and reabsorbed. If the reabsorption of the drug is large enough to cause two increases in blood concentration, the C–T curve appears as double peaks. (b) Time of gastric emptying: the drug reached the small intestine two times. This causes the drug to enter the blood two times. Double peaks come into being in C–T curves (Chang and Shojaei, 2004; Metsugi et al., 2008). (c) Multiple site absorption in the gastrointestinal tract: there are multiple absorption sites in different parts of the gastrointestinal tract. However, the permeability of different sites in the inner membrane is different. Therefore, the absorption time and absorption rate of different parts were not consistent after oral administration, and double peaks came into being in C–T curves (Lennernäs and Regårdh, 1993; Piyapolrungroj et al., 2000). (d) Multi ingredient characteristics of the traditional Chinese drug. The multi-peak phenomenon of pharmacokinetics is increasingly complex. This is closely related to the multi-ingredient characteristics of the traditional Chinese drug. Since most traditional Chinese drugs contain a large number of ingredients with the same parent nucleus, the ingredients easily transform each other under environmental conditions in the body. In the mutual transformation of ingredients, the concentration of some ingredients increases and results in a multi-peak phenomenon.

## 7 Conclusion

This study established a simple, rapid, and stable HPLC–MS/MS method. The LC–MS/MS method satisfies all of the validation criteria suggested in the bioanalytical method validation guidelines from the FDA (Food and Drug Administration, 2020) and proves the specificity, reliability, and repeatability of the established quantitative method. This method improves the specificity and sensitivity of detection. In addition, the pretreatment method is simple with the high recovery rate, and the endogenous substances and impurities in the biological samples have no interference with the determination of substance. This method is applicable to pharmacokinetic studies of the Mongolian drug Sendeng-4 in rat blood plasma. The plasma concentration of normal rats was higher than that of arthritis model rats. There were significant differences in the absorption level, distribution level, and elimination rate of (2R, 3R)-dihydromyricetin and myricetin between arthritis model rats and normal rats. This result indicates that arthritic rats significantly consume (2R, 3R)-dihydromyricetin and myricetin, which indirectly proves that (2R, 3R)-dihydromyricetin and myricetin are active ingredients of Sendeng-4. The study lays the foundation for the subsequent study of pharmacokinetics of Sendeng-4 in humans and contributes to the wide application of Sendeng-4 in clinical practice.

## 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 animal study was approved by the Ethics Committee of Inner Mongolia Medical University. The study was conducted in accordance with the local legislation and institutional requirements.

## Author contributions

PB: data curation, methodology, software, writing–original draft, and validation. YD: funding acquisition, project administration, resources, supervision, writing–review and editing, and conceptualization.

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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 authors declare that no generative AI was used in the creation of this manuscript.

## Publisher's note



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# Metabolomic and lipidomic profiling of traditional Chinese medicine *Testudinis Carapax et Plastrum* and its substitutes

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**Introduction:** *Chinemys reevesii* (Gray) species–sourced *Testudinis Carapax et Plastrum* (TCP) is an animal-based traditional Chinese medical material, and its decoction or extract possesses multiple pharmacological effects. However, other species-sourced substitutes are sometimes used in the market, potentially impairing the quality and effectiveness of TCP medications. To address this issue, it is very necessary to develop applicable approaches that can accurately differentiate genuine TCP from its counterfeit counterparts.

**Methods:** In this study, liquid chromatography–tandem mass spectrometry (LC-MS/MS)–based metabolomic and lipidomic analyses were performed to comprehensively detect water-soluble metabolites and organic-soluble lipids in water decoctions of genuine TCP and its substitutes, such as *Trachemys scripta elegans* (Wied)– and *Ocadia sinensis* (Gray)–sourced tortoise shells. Differential analyses based on fold change (FC), principal component analysis (PCA), and Orthogonal partial least squares–discriminant analysis (OPLS-DA) were performed to assess the differences among TCP decoctions from different origins, as well as between decoctions of TCP samples and the two substitutes. Further, Kyoto Encyclopedia of Genes and Genomes (KEGG) database–based pathway enrichment analysis was performed for differential metabolites and lipids among them. Besides, LC-MS/MS–based absolute quantitative method was used to quantify the amino acid–relevant metabolites in decoctions of TCP and substituted tortoise shell samples.

**Results:** All told, 1117 water-soluble metabolites (including amino acids, organic acids, nucleotides and their metabolites or derivatives, etc.) and 574 organic-soluble lipids (including glycerolipids, sphingolipids, glycerophospholipids, fatty acids, and sterol lipids) were detected in decoctions of TCP and two substitutes. Comparative analyses revealed that there were significantly differential metabolites and lipids among TCP decoctions from different origins, as well

as between decoctions of TCP samples and the two substitutes. Of particular interest, the content of N-methyl-4-aminobutyric acid was lower in the substituted samples than TCP samples. Furthermore, the content of 27 amino acids, 22 amino acid derivatives, and 18 small peptides in the decoctions of TCP and two substitutes were absolutely quantified, constituting up to tens of milligrams per 10 g of tortoise shell.

**Discussion:** In conclusion, our study provides comprehensive metabolomic and lipidomic information of TCP decoction. However, the current results represent preliminary data, and further extensive research is required to validate these findings.

KEYWORDS

***Testudinis carapax et Plastrum*, liquid chromatography-tandem mass spectrometry, metabolomic and lipidomic analyses, metabolites, lipids**

# 1 Introduction

*Testudinis Carapax et Plastrum* (TCP), often referred to as "guijia" in Chinese, is the dried carapace and plastron from *Chinemys reevesii* (syn. *Mauremys reevesii Gray*, 1831) (Commission, 2020). The *Pharmacopoeia of the People's Republic of China* (2020 edition) lists that TCP is salty, sweet in taste and slightly in nature; and passes through the liver, kidney, and heart channels. TCP's main indications include nourishing yin and latent yang, invigorating the kidneys and strengthening bone, nourishing blood and tonifying the heart, and reinforcing the meridian and stopping collapse. Current studies have demonstrated that the extracts of *Plastrum Testudinis* (i.e., the dried plastron of *Chinemys reevesii*) have protective effects against bone diseases (e.g., senile osteoporosis, intervertebral disc degeneration) (Liang et al., 2016; Ren et al., 2017; Chen et al., 2021; Zhang et al., 2021; Zhang et al., 2023), acute promyelocytic leukemia (Chen et al., 2024), Parkinson's disease (Zhong et al., 2020; Ye et al., 2021; Yi et al., 2024), beta-thalassemia and sickle cell anemia (Qian et al., 2013), Alzheimer's disease (Hui et al., 2017), and skin wounds (Tang et al., 2018).

TCP materials are typically ground into a powder and then decocted in water for therapeutic use. TCP medications can be taken internally or externally. Water decoctions of TCP can be further made into *Testudinis Carapacis et Plastri Colla* (named as "guijia jiao" in Chinese) (Su et al., 2018; Commission, 2020). Various TCP-associated Chinese patent drugs are available on the market, such as Gulu Erxian Gao, Yangyin Jiangya Jiaonang, and Jianbu Qiangshen Wan. These processed TCP products have lost their original morphological characteristics (including shape, color, texture, etc.), thereby creating critical vulnerabilities for economically motivated adulteration within pharmaceutical supply chains. For instance, TCP powders are sometimes mixed with low-cost substitutes from other species-sourced tortoise shells, such as carapace- or plastron-derived materials from *Ocadia sinensis* (Gray) and *Trachemys scripta elegans* (Wied). Such adulteration may compromise the bioactive composition of TCP decoctions, ultimately diminishing their therapeutic efficacy. It is therefore urgent to develop applicable approaches allowing accurate distinguishment of TCP samples from counterfeits.

Currently, polymerase chain reaction (PCR)-based DNA molecular strategies, such as quantitative PCR and multiplex PCR, have been leveraged to identify species of tortoise shells (Jiao et al., 2020; Lv et al., 2020). Species-specific primers have been designed for genomic DNA and mitochondrial DNA of TCP (Li et al., 2018; Yang et al., 2018). However, such approaches are limited to assessing highly processed TCP samples with trace amounts of degraded DNA. Metabolites that are small molecules (typically <2,000 Da) are chemically transformed during metabolism, reflecting the direct signature of biochemical activity in the organism (Sarmad et al., 2023). The chemical constituents detected in TCP extracts include fatty acids, steroids, amino acids, peptides, etc. (Wang et al., 2012; Ren et al., 2024). Thus, system-wide profiling of metabolites, including lipids, in TCP samples and counterfeits may reveal the characteristic metabolites that may be used as novel indicators to discriminate adulteration in processed TCP samples. With advances in mass spectrometry (MS)-based metabolomics and lipidomic analyses, thousands of metabolites and lipids that span a large dynamic range can now be quantitatively measured in a single sample (Guo et al., 2020; Alseekh et al., 2021; Perez de Souza et al., 2021). Gas chromatograph–MS (GC-MS)– and liquid chromatography–MS (LC-MS)–based metabolomic and lipidomic analyses have been performed to authenticate traditional Chinese medicine materials, such as cordyceps (Zhang et al., 2020; Lin et al., 2022; Hou et al., 2025). In comparison to GC-MS, which is primarily limited to volatile and thermally stable compounds, LC-MS demonstrates superior analytical capability for a wider spectrum of analytes, particularly non-volatile and thermally labile compounds.

In this study, we used LC-MS/MS–based widely targeted metabolomic and lipidomic approaches to comprehensively analyze both water-soluble metabolites and organic-soluble lipids in decoctions of *Chinemys reevesii* (Gray) species–sourced TCP and other species-sourced substitutes. Univariate and multivariate statistical analyses were carried out to assess differences in decoctions of TCP samples collected from different places and those in decoctions of TCP samples and two substitutes, including *Trachemys scripta elegans* (Wied)– and *Ocadia sinensis* (Gray)–sourced tortoise shells collected from different places. Furthermore, targeted quantification was conducted to evaluate the contents of amino acids and their metabolites across specimen groups.

# 2 Materials and methods

## 2.1 Tortoise shell samples and their decoction preparation

*Chinemys reevesii* (Gray) species–sourced TCP samples (including CR1, CR2, and CR3), along with *Trachemys scripta elegans* (Wied)– and *Ocadia sinensis* (Gray)–sourced tortoise shells (including TS1, TS2,

TABLE 1 Information of genuine and counterfeit TCP samples.

| Tortoise shell samples | Species | Collection site | Identification |
|---|---|---|---|
| CR1 | *Chinemys reevesii* (Gray) | Jingshan, Hubei, China | Genuine TCP |
| CR2 | *Chinemys reevesii* (Gray) | Hanshou, Hunan, China | Genuine TCP |
| CR3 | *Chinemys reevesii* (Gray) | Jingzhou, Hubei, China | Genuine TCP |
| OS1 | *Ocadia sinensis* (Gray) | Jingshan, Hubei, China | Counterfeit TCP |
| OS2 | *Ocadia sinensis* (Gray) | Hanshou, Hunan, China | Counterfeit TCP |
| TS1 | *Trachemys scripta elegans* (Wied) | Jingshan, Hubei, China | Counterfeit TCP |
| TS2 | *Trachemys scripta elegans* (Wied) | Hanshou, Hunan, China | Counterfeit TCP |

OS1, and OS2), were provided by the Hubei Institute for Drug Control (Wuhan, China) (Table 1). The authentication of CR, TS, and OS samples was performed by Professor Ling Xiao, Chief Pharmacist at the Hubei Institute for Drug Control (Wuhan, China). CR1, OS1, and TS1 samples were sourced from Jingshan, Hubei, China. CR2, OS2, and TS2 samples were collected from Hanshou, Hunan, China. CR3 samples were collected from Jingzhou, Hubei, China.

To prepare decoctions of CR, TS, and OS samples, 100 mL of distilled water was added to 10 g of each sample, which was subsequently soaked for 1 h. Then, the mixture was heated under reflux conditions and kept boiling slightly for 8 h. Finally, the mixture was cooled to room temperature and filtered. The filtered solution was stored at −80°C for later use.

## 2.2 Chemical Reagents

Acetonitrile, methanol, isopropanol, and methyl-tert-butyl ether (MTBE) were purchased from Merck (Darmstadt, Germany). Formic acid, ammonium hydroxide, and ammonium acetate were purchased from Sigma-Aldrich (St. Louis, MO, United States), while ammonium formate was purchased from Thermo Fisher Scientific (Waltham, MA, United States). Ultrapure water was obtained using a Milli-Q system (Millipore, Billerica, MA, United States). Standards for quantitative analysis of amino acid–relevant metabolites are shown in Supplementary Table S1.

## 2.3 LC-MS/MS–based metabolomic and lipidomic analyses of tortoise shell decoctions

### 2.3.1 Analysis of water-soluble metabolites in decoctions

To extract water-soluble metabolites, 150 μL of acetonitrile/methanol (1:4, v/v) was added to 50 μL of each decoction sample and vortexed for 3 min (Want et al., 2006). Then, the mixture was centrifuged at 12,000 rpm for 10 min at 4°C, and 150 μL of supernatant was collected. To precipitate the proteins in the samples as far as possible, the supernatant was stored at −20°C for 30 min. Finally, the supernatant was centrifuged again at 12,000 rpm for 3 min at 4°C, and 120 μL of supernatant was collected for LC-MS/MS analysis.

For LC separation, an ExionLC AD UPLC system (SCIEX, Framingham, MA, United States) was used to separate water-soluble metabolites. The injection volume was 2 μL. A Waters ACQUITY UPLC HSS T3 C18 column (2.1 mm × 100 mm, 1.8 μm) was used and its temperature was maintained at 40°C. The mobile phases were composed of (A) water with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid. The flow rate was 0.4 mL/min. The gradient elution was performed as follows: 0–11 min, 5%–90% B; 11–12 min, 90% B; 12–12.1 min, 90%–5% B; and 12.1–14 min, 5% B.

MS data acquired in the multiple reaction monitoring (MRM) mode were performed on a QTRAP® 6500+ MS system (SCIEX, Framingham, MA, United States) equipped with an electrospray ionization source. Parameters were set as follows: source temperature 500°C; positive ion spray voltage, 5,500 V; negative ion spray voltage, −4,500 V; GSI, 55 psi; GSII, 60 psi; curtain gas, 25 psi; and collision gas, high.

### 2.3.2 Analysis of organic-soluble lipids in decoctions

To extract organic-soluble lipids, 1 mL of MTBE/methanol (3:1, v/v) was added to 200 μL of each decoction sample, which was vortexed for 15 min (Matyash et al., 2008). Then, 100 μL of water was added to the mixture, which was followed by vortexing for 1 min and then centrifugation at 12,000 rpm for 10 min at 4°C to achieve phase separation. Subsequently, 500 μL of upper organic phase was collected, concentrated, and reconstituted in 200 μL of acetonitrile/isopropanol (1:1, v/v). Finally, the reconstituted sample was centrifuged at 12,000 rpm for 3 min at 4°C, and the supernatant was collected for LC-MS/MS analysis.

For LC separation, an ExionLC AD UPLC system (SCIEX, Framingham, MA, United States) was used to separate lipids. A Thermo Accucore™ C30 column (2.1 mm × 100 mm, 2.6 μm) was used, and its temperature was maintained at 45°C. The injection volume was 2 μL. The mobile phases were composed of (A) acetonitrile/water (60:40, v/v) with 0.1% formic acid and 10 mmol/L of ammonium formate and (B) acetonitrile/isopropanol (10:90, v/v) with 0.1% formic acid and 10 mmol/L of ammonium formate. The flow rate was 0.35 mL/min. The gradient elution was performed as follows: 0–2 min, 20%–30% B; 2–4 min, 30%–60% B; 4–9 min, 60%–85% B; 9–14 min, 85%–90% B; 14–15.5 min, 90%–95% B; 15.5–17.3 min, 95% B; 17.3–17.5 min, 95%–20% B; and 17.5–20 min, 20% B.

MS data acquired in MRM mode were also performed on a QTRAP® 6500+ MS system (SCIEX, Framingham, MA, United States). Parameters were set as follows: source temperature 500°C; positive ion spray voltage, 5,500 V; negative ion spray voltage, −4,500 V; GSI, 45 psi; GSII, 55 psi; and curtain gas, 35 psi; collision gas, medium.

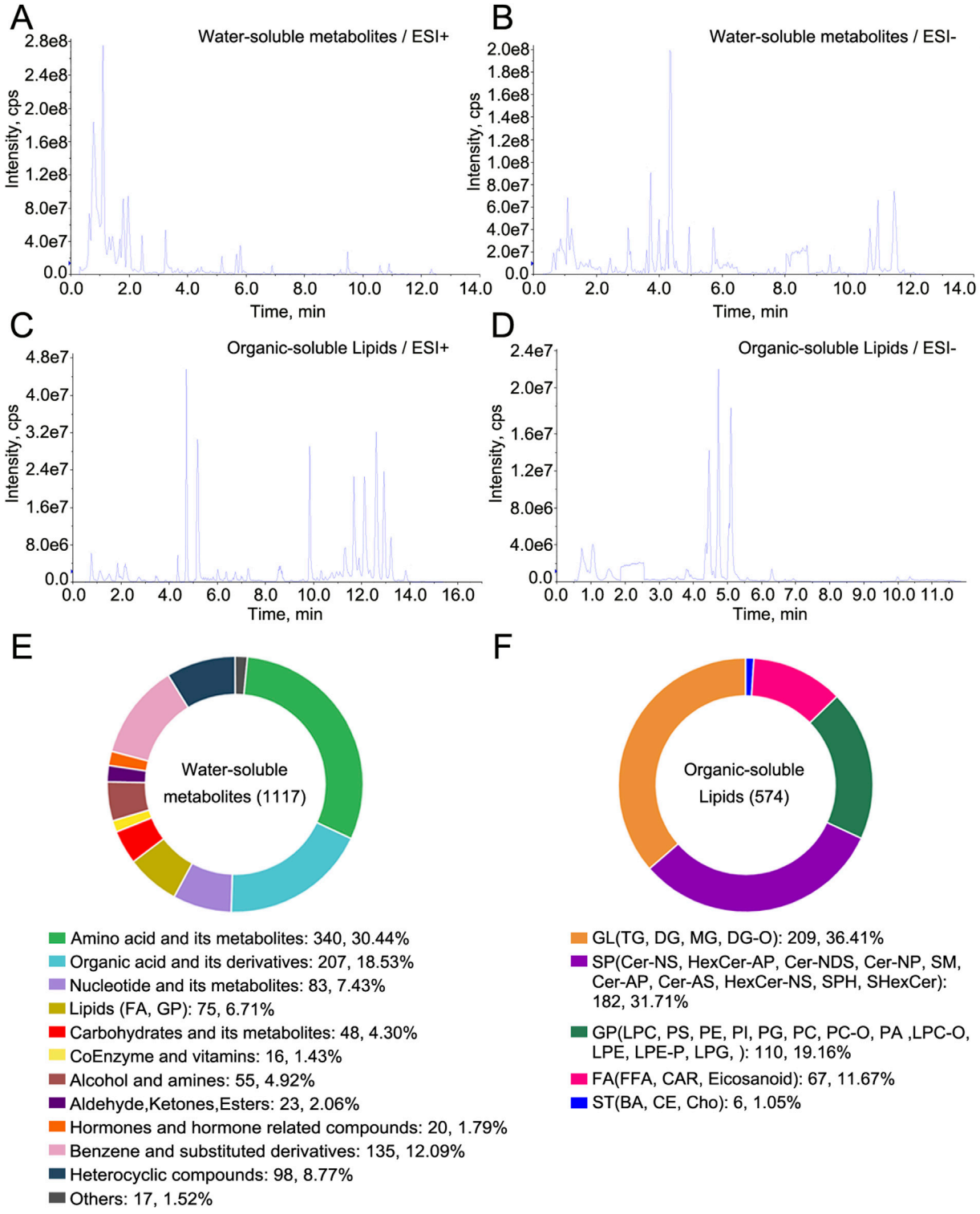


FIGURE 1
Metabolomic and lipidomic profiling of water-soluble metabolites and organic-soluble lipids in decoctions of TCP and substituted tortoise shells. **(A–D)** Total ion chromatograms of metabolomic and lipidomic analyses of water-soluble metabolites and organic-soluble lipids in ESI+ and ESI− modes. **(E)** Water-soluble metabolites detected in decoctions of tortoise shell samples. **(F)** Organic-soluble lipids detected in decoctions of tortoise shell samples.

### 2.3.3 Data processing

Water-soluble metabolites and organic-soluble lipids were identified in Analyst 1.6.3 (SCIEX, Framingham, MA, United States) based on retention time, precursor ion/product ion information, and MS/MS spectrum patterns from a self-compiled database (Metware Biotechnology Co., Ltd., Wuhan, China) and public databases including

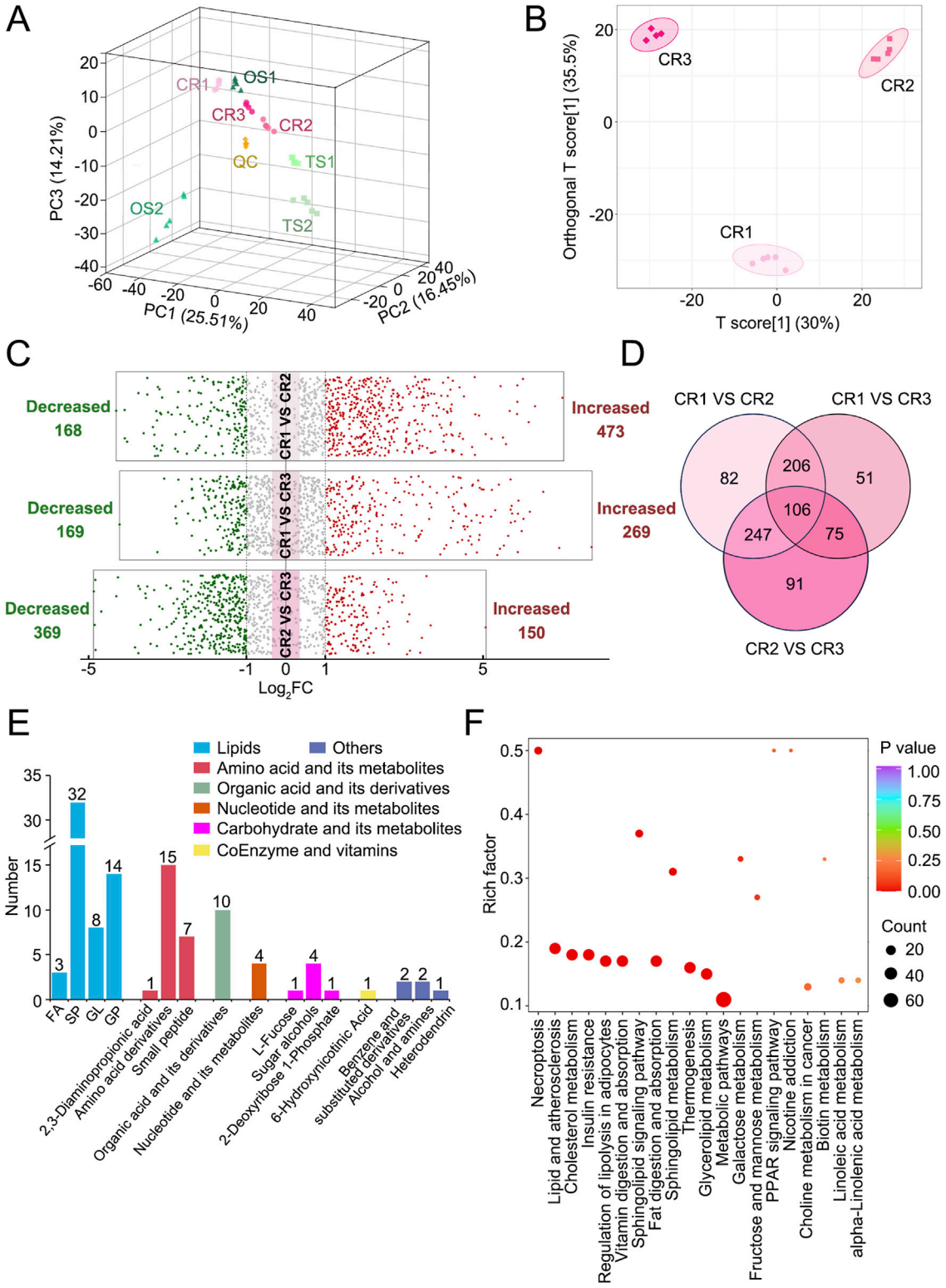


FIGURE 2
Differential analyses of metabolites and lipids detected in decoctions of genuine TCP samples from different origins, including CR1–3 samples. **(A)** PCA results of CR1–3, OS1–2, and TS1–2 samples. **(B)** OPLS-DA score plot of CR1–3 samples. **(C)** Volcano plots of differential analyses among CR1–3 samples. The filter criteria were $|Log_2FC| \geq 1.0$ and VIP ≥1. **(D)** Venn diagram of differential metabolites and lipids among CR1–3 samples. **(E)** Diagram of common differential metabolites and lipids found among CR1–3 samples. **(F)** Pathway enrichment analysis of common differential metabolites and lipids found among CR1–3 samples. The top 20 enriched pathways as ranked by *p*-value are displayed.

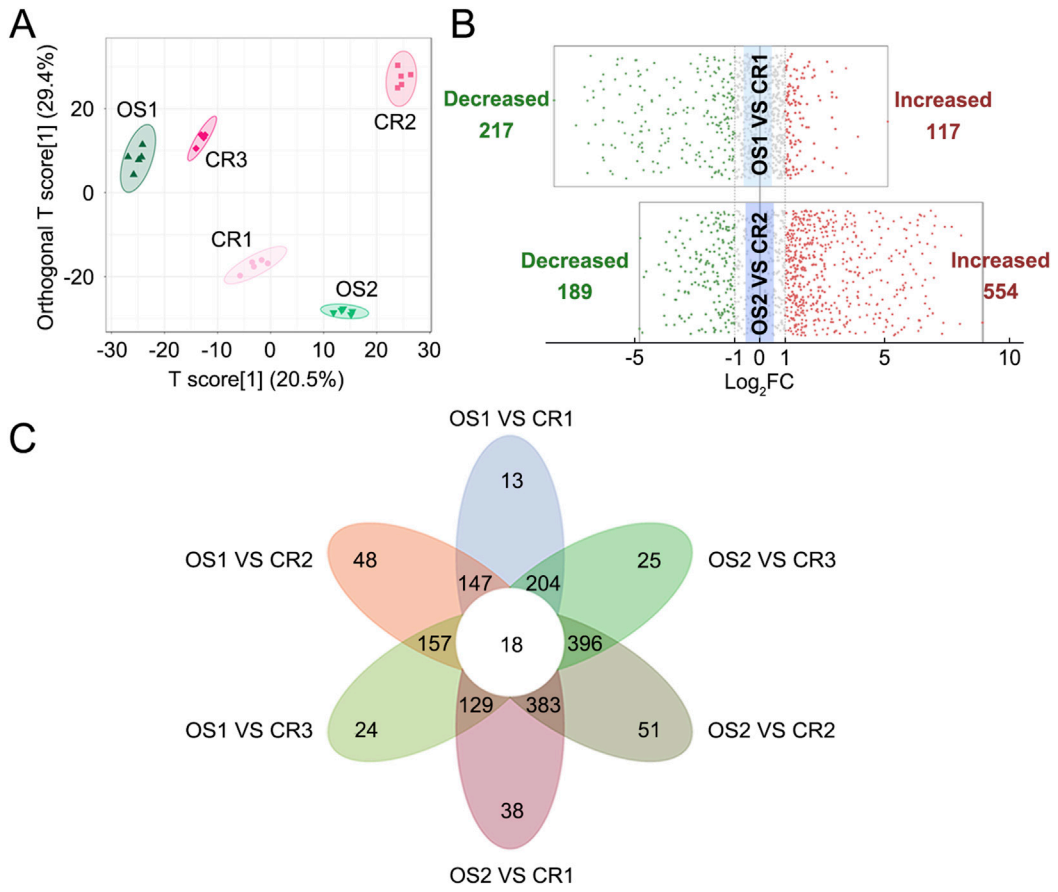


FIGURE 3
Differential analyses of metabolites and lipids detected in decoctions of CR samples and OS samples. **(A)** OPLS-DA score plot of CR1–3 and OS1–2 samples. **(B)** Volcano plots of differential analyses between CR and OS samples from the same origins. The filter criteria were $|Log_2FC| \geq 1.0$ and VIP ≥1. **(C)** Venn diagram of differential metabolites and lipids found between CR1–3 and OS1–2 samples.

the Metabolite Link database (METLIN; https://massconsortium.com/) and Human Metabolome Database (HMDB; https://hmdb.ca/). Integration and calibration of the chromatographic peaks were performed in MultiQuant 3.0.3 (SCIEX, Framingham, MA, United States). Data of water-soluble metabolites and organic-soluble lipids detected in quality control (QC) samples with a peak area coefficient of variation of ≥30% were filtered out and not analyzed. QC samples were prepared by mixing small aliquots of each decoction from tortoise shells.

Principal component analysis (PCA) was performed in R (base package, version 4.1.2; R Foundation for Statistical Computing, Vienna, Austria). Orthogonal partial least squares–discriminant analysis (OPLS-DA) was performed by using the R package MetaboAnalystR (version 1.0.1). Differential metabolites and lipids were screened based on the combination of a statistically significant threshold of variable importance in projection (VIP) ≥ 1 and $|Log_2FC| \geq 1.0$.

The identified water-soluble metabolites and organic-soluble lipids in decoctions were annotated using the Kyoto Encyclopedia of Genes and Genomes (KEGG) Compound database (http://www.kegg.jp/kegg/compound/). Annotated metabolites and lipids were then mapped to the KEGG Pathway database (http://www.kegg.jp/kegg/pathway.html). Pathways containing significantly regulated metabolites and lipids were subsequently subjected to metabolite set enrichment analysis.

## 2.4 LC-MS/MS–based quantitative analysis of amino acid–relevant metabolites in decoctions

Here, 250 μL of acetonitrile/methanol (1:4, v/v) was added to 50 μL of each decoction sample, followed by vortexing for 3 min. Then, the mixture was centrifuged at 12,000 rpm for 10 min at 4°C. In addition, 250 μL of supernatant was collected and kept at −20°C for 30 min to further precipitate the proteins. Finally, the supernatant was centrifuged at 12,000 rpm for 10 min at 4°C, and 180 μL of supernatant was collected for LC-MS/MS–based absolute quantitative analysis. The external standard method was used to quantify 27 amino acids, 22 amino acid derivatives, and 18 small peptides in decoctions of each tortoise shell sample.

LC separation was performed on an ExionLC AD UPLC system (SCIEX, Framingham, MA, United States) with two types of columns, including a Waters ACQUITY HSS T3 C18 column (100 mm × 2.1 mm, 1.8 μm) and a Waters ACQUITY UPLC BEH Amide column (100 mm × 2.1 mm, 1.7 μm) (Supplementary Table S1).

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TABLE 2 Common differential metabolites and lipids found between CR and OS samples.

| Number | Class | Subclass | Metabolites and lipids |
|---|---|---|---|
| 1 | Lipids | FA | FFA (20:5) |
| 2 | | | (±)5-HEPE |
| 3 | | GL | DG (O-19:2_20:0) |
| 4 | | | TG (14:0_18:0_20:2) |
| 5 | | | TG (16:0_18:0_20:1) |
| 6 | | | TG (16:0_18:1_22:1) |
| 7 | | | TG (18:1_18:1_22:1) |
| 8 | | GP | LPC(O-14:0) |
| 9 | | | LPC(O-18:0) |
| 10 | | | LPC(O-18:1) |
| 11 | | | LPC(O-18:2) |
| 12 | | SP | Cer(d16:0/18:0) |
| 13 | | | Cer(t17:2/22:0) |
| 14 | Amino acids and metabolites | Amino acid derivatives | N-lactoyl-phenylalanine |
| 15 | | | N-methyl-4–aminobutyric acid |
| 16 | Organic acids and derivatives | Organic acids and derivatives | 3-Methylcrotonyl glycine |
| 17 | Others | Benzene and substituted derivatives | 3-Sulfocatechol |
| 18 | | Heterocyclic compounds | Imidazoleacetic acid |

The injection volume was 2 μL. The column temperature was maintained at 40°C. For C18 column–based LC separation, the flow rate was 0.35 mL/min. Mobile phases included (A) water with 0.05% formic acid and (B) acetonitrile with 0.05% formic acid. The gradient elution was performed as follows: 0–8 min, 5%–95% B; 8–9.5 min, 95% B; 9.5–9.6 min, 95%–5% B; and 9.6–12 min, 5% B. For amide column–based LC separation, the flow rate was 0.4 mL/min. Mobile phases included (A) water with 0.3% ammonium hydroxide and 10 mM of ammonium acetate and (B) acetonitrile/water (90:10, v/v). The gradient elution was performed as follows: 0–8 min, 5%–95% B; 8–9.5 min, 95% B; 9.5–9.6 min, 95%–5% B; and 9.6–12 min, 5% B.

MS data acquired in MRM mode were performed on a QTRAP® 6500+ LC-MS/MS system (SCIEX, Framingham, MA, United States). Parameters were set as follows: ESI source temperature, 550°C; positive ion spray voltage, 5,500 V; negative ion spray voltage, −4,500 V; GSI, 50 psi; GSII, 60 psi; curtain gas, 35 psi; and collision gas, medium.

# 3 Results

## 3.1 Metabolomic and lipidomic profiling of water-soluble metabolites and organic-soluble lipids in decoctions of genuine TCP and two substitutes

In this work, water-soluble metabolites and organic-soluble lipids in decoctions of *Chinemys reevesii* (Gray) species–sourced tortoise shells (i.e., TCP) and two common substitutes (i.e., *Ocadia sinensis* (Gray) species–sourced and *Trachemys scripta elegans* (Wied) species–sourced tortoise shells) were analyzed by LC-MS/MS–based widely-targeted metabolomic and lipidomic profiling (Table 1). These three species belong to the *Emydidae* family, Testudinate order, Reptilia class (http://museum.ioz.ac.cn/). Genuine TCP samples included CR1, CR2, and CR3. *Ocadia sinensis* (Gray) species–sourced tortoise shell samples included OS1 and OS2. *Trachemys scripta elegans* (Wied) species–sourced tortoise shell samples included TS1 and TS2 (Table 1).

Total ion chromatograms of metabolomic and lipidomic analyses of water-soluble metabolites and organic-soluble lipids in the QC sample in ESI+ and ESI− modes are shown in Figure 1A–D. A total of 1,117 water-soluble metabolites and 574 organic-soluble lipids were measured in decoctions of tortoise shell samples, including CR1–3, OS1–2, and TS1–2 (Figures 1E, F; Supplementary Tables S2, S3). No unique metabolites or lipids were found in decoctions of genuine or substituted tortoise shells. The classes of detected water-soluble metabolites consisted of amino acids and their metabolites, organic acids and their derivatives, and nucleotides and their metabolites (Figure 1E). The classes of detected organic-soluble lipids included glycerolipids (GLs), sphingolipids (SPs), glycerophospholipids (GPs), fatty acids (FAs), and sterol lipids (STs) (Figure 1F). Among them, the number of lipids, amino acids, organic acids, and their metabolites or derivatives detected in decoctions was more than 200; in particular, the number of lipids was the highest (649) (Figures 1E, F). These

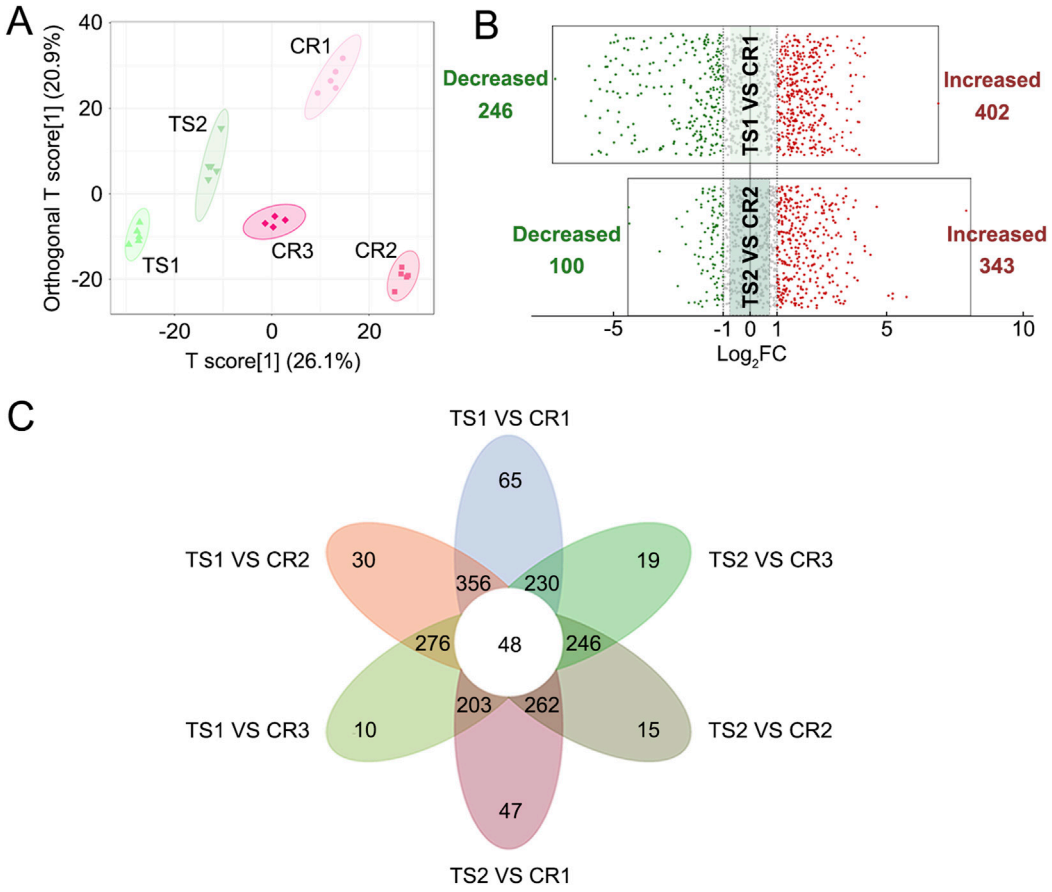


FIGURE 4
Differential analyses of metabolites and lipids detected in decoctions of CR1–3 samples and TS1–2 samples. **(A)** OPLS-DA score plot of CR1–3 and TS1–2 samples. **(B)** Volcano plots of differential analyses between CR and TS samples from the same origins. The filter criteria were $|Log_2FC| \geq 1.0$ and VIP ≥1. **(C)** Venn diagram of differential metabolites and lipids found between CR1–3 and TS1–2 samples.

results show that decoction samples of both genuine and substituted tortoise shells contain multiple chemical constituents, providing chemical evidence for parsing functions of tortoise shells.

## 3.2 Differential analyses of metabolites and lipids detected in decoctions of genuine TCP samples from different origins

Multivariate statistical analyses, such as PCA and OPLS-DA, clearly showed that CR1–3 sample clusters were separate, suggesting that different water-soluble metabolites and organic-soluble lipids exist in decoctions of genuine TCP samples from different origins (Figures 2A, B). Based on $|Log_2FC| \geq 1.0$ and VIP ≥1, more than 400 differential metabolites and lipids were screened out between CR1 and CR2, between CR1 and CR3, and between CR2 and CR3, respectively (Figure 2C; Supplementary Tables S4–S6). Among them, the number of common differential metabolites and lipids was 106 (Figure 2D). The classes of common differential substances included lipids, amino acids and their metabolites, organic acids and their derivatives, nucleotides and their metabolites, carbohydrates and their metabolites, coenzymes, and vitamins (Figure 2E), which are highlighted in bold in Supplementary Tables S4–S6. More common differential metabolites and lipids were significantly enriched into metabolic pathway and lipid metabolism–related pathways, such as those relating to cholesterol metabolism, glycerolipid metabolism, sphingolipid signaling, fat digestion and absorption, and regulation of lipolysis in adipocytes (Figure 2F). Taken together, there were significant differences in decoctions of genuine TCP samples from different origins.

## 3.3 Differential analyses of metabolites and lipids detected in decoctions of genuine TCP samples and *Ocadia sinensis* (Gray) species–sourced substituted tortoise shell samples

PCA and OPLS-DA results revealed five distinct sample clusters for CR1, CR2, CR3, OS1, and OS2 (Figure 3A), indicating that decoctions of substituted tortoise shell samples OS1–2 from different origins differ from those of genuine TCP samples CR1–3. Based on $|Log_2FC| \geq 1.0$ and VIP ≥1, 334 and

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TABLE 3 Common differential metabolites and lipids found between CR and TS samples.

| Number | Class | Subclass | Metabolites and lipids |
|---|---|---|---|
| 1 | Lipids | FA | PGF2α |
| 2 | | | 12-Hydroxyoctadecanoic acid |
| 3 | | SP | Cer(t18:0/24:0(2OH)) |
| 4 | | | Cer(t20:0/24:0(2OH)) |
| 5 | | | Cer(t18:0/24:1) |
| 6 | | | Cer(d16:1/24:2) |
| 7 | | | Cer(d20:1/28:2) |
| 8 | | | HexCer(t22:2/14:1(2OH)) |
| 9 | | | HexCer(d18:1/18:0) |
| 10 | | | HexCer(d18:1/18:1) |
| 11 | | | HexCer(d18:1/20:0) |
| 12 | | | HexCer(d18:1/20:1) |
| 13 | Amino acids and metabolites | Amino acid derivatives | 2–Aminoisobutyric acid |
| 14 | | | Aminoisobutyric acid |
| 15 | | | N, N-dimethylglycine |
| 16 | | | N-methyl-4–aminobutyric acid |
| 17 | | Small peptides | Ala-Tyr |
| 18 | | | Arg-Ile |
| 19 | | | Glu-Phe |
| 20 | | | Leu-Asp |
| 21 | | | Met-Phe |
| 22 | | | Phe-Glu |
| 23 | | | Tyr-Ser |
| 24 | Organic acids and derivatives | Sulfonic acids | p-Tolyl sulfate |
| 25 | | Carboxylic acids and derivatives | Cinnamic acid |
| 26 | | | Fumaric acid |
| 27 | | | L-2–aminobutyric acid |
| 28 | | | Maleic acid |
| 29 | | | Shikimic acid |
| 30 | Nucleotides and metabolites | Purines and purine derivatives | 1-Methyladenosine |
| 31 | | | 2-Aminopurine |
| 32 | | Purine nucleosides | 2′-Deoxyadenosine |
| 33 | | | 2′-Deoxyinosine |
| 34 | | | 3-Deoxyadenosine |
| 35 | | | 5′-Deoxy-5′-fluoroadenosine |
| 36 | | | 5′-Deoxyadenosine |
| 37 | | | Adenosine |
| 38 | | | Arainosine |

(Continued on following page)

TABLE 3 (*Continued*) Common differential metabolites and lipids found between CR and TS samples.

| Number | Class | Subclass | Metabolites and lipids |
|---|---|---|---|
| 39 | | | Guanosine |
| 40 | | | N6-methyl-2′-deoxyadenosine |
| 41 | | | N6-methyladenosine |
| 42 | Carbohydrates and metabolites | Sugar derivatives | 2,4-diacetamino-2,4,6-triphenoxy-D-mannopyranose |
| 43 | Coenzymes and vitamins | Coenzymes and vitamins | Riboflavin |
| 44 | Others | Alcohols and amines | Putrescine |
| 45 | | Heterocyclic compounds | Carbendazim |
| 46 | | | Ectoine |
| 47 | | | N'-methyl-2-pyridone-5-carboxamide |
| 48 | | Others | Furfural diacetal |

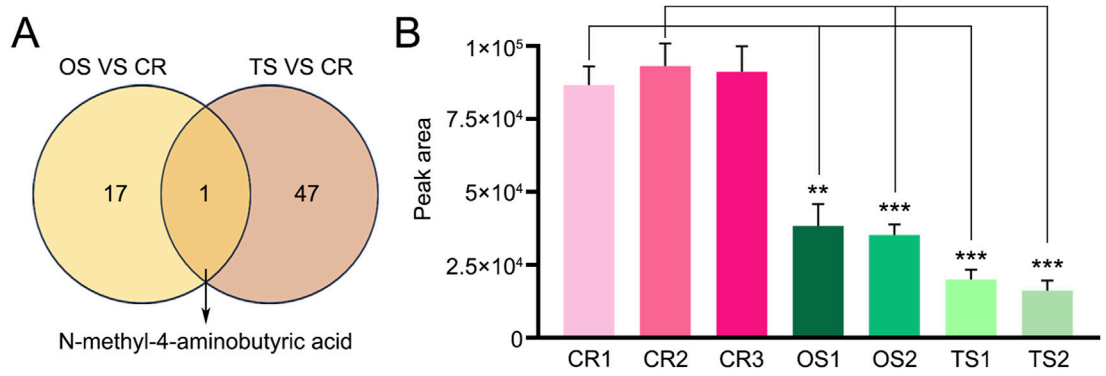


FIGURE 5
Display of the only common differential (N-methyl-4–aminobutyric acid) found in decoctions of genuine TCP samples and substituted OS and TS samples. **(A)** Venn diagram of differential metabolites or lipids found between CR, OS, and TS samples. **(B)** Levels of N-methyl-4–aminobutyric acid in decoctions of CR, OS, and TS samples. Data are presented as mean ± SD, n = 5. **: $p < 0.01$; ***: $p < 0.001$.

743 differential metabolites and lipids were sifted out between CR1 and OS1 and between CR2 and OS2, respectively (Figure 3B; Supplementary Tables S7, S8). Besides, decoctions of genuine TCP and substituted OS tortoise shell samples from different origins also demonstrated different metabolites and lipids (Figure 3C). In all, 18 common differential metabolites and lipids were screened out between CR and OS samples, including 13 lipids (i.e., 2 FAs, 1 DG, 4 TGs, 4 LPCs, and 2 Cers), two amino acids and their metabolites (N-lactoyl-phenylalanine and N-methyl-4–aminobutyric acid), one organic acid and its derivatives (3-methylcrotonyl glycine), one benzene and substituted derivatives (3-sulfocatechol), and one heterocyclic compound (imidazoleacetic acid) (Figure 3C; Table 2), which are highlighted in bold in Supplementary Tables S7, S8. KEGG pathway enrichment analysis showed that 18 differential metabolites and lipids were significantly enriched in cell growth and death, lipid metabolism, and endocrine and metabolic disease pathways ($p < 0.05$) (Supplementary Figure S1A). These results indicate that metabolic and lipidomic profiling of decoctions of genuine TCP samples from different origins are different from those of substituted OS tortoise shell samples.

## 3.4 Differential analyses of metabolites and lipids detected in decoctions of genuine TCP and *Trachemys scripta elegans* (Wied)–sourced substituted tortoise shell samples

PCA and OPLS-DA results also showed significant differences between TCP samples (CR1–3) and substituted tortoise shell samples (TS1–2) (Figures 2A, 4A). Based on $|Log_2FC| \geq 1.0$ and VIP ≥1, more than 400 differential metabolites and lipids were found between CR and TS samples from the same origins (Figure 4B; Supplementary Tables S9, S10). All told, 48 common differential metabolites and lipids were found between CR1–3 and TS1–2 samples, including 12 lipids, 11 amino acids and their metabolites, 6 organic acids and their derivatives, 12 nucleotides and their metabolites, 1 carbohydrate and its metabolites (2,4-diacetamino-2,4,6-triphenoxy-D-mannopyranose), 1 coenzyme or vitamin (riboflavin), and 5 other compounds (putrescine, carbendazim, ectoine, N′-methyl-2-pyridone-5-carboxamide, and furfural diacetal) (Figure 4C; Table 3), which are highlighted in bold in Supplementary Tables S9, S10. KEGG pathway enrichment analysis revealed that 48 differential metabolites and lipids were

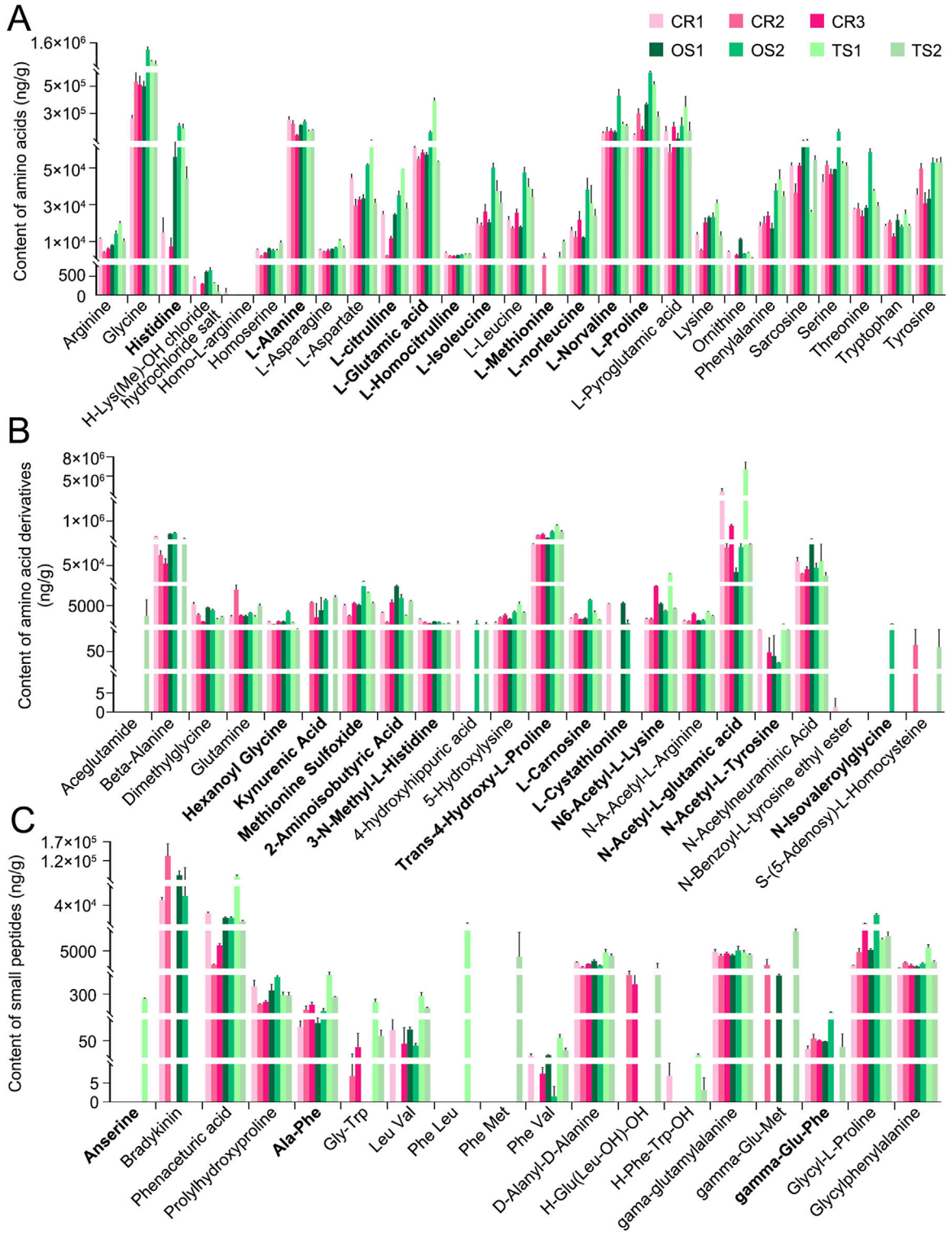


FIGURE 6
Absolute quantitative analysis results of some amino acids and their metabolites in decoctions of CR, OS, and TS samples. **(A)** Amino acids. **(B)** Amino acid derivatives. **(C)** Small peptides. Data are presented as mean ± SD, n = 3.

significantly enriched in multiple metabolic pathways, such as those associated with nicotinate and nicotinamide metabolism, purine metabolism, nucleotide metabolism, sphingolipid metabolism, and butanoate metabolism ($p < 0.05$) (Supplementary Figure S1B). Furthermore, it was found that the N-methyl-4–aminobutyric acid content was significantly lower in decoctions of substituted tortoise shell samples (OS1–2 and TS1–2) compared to in genuine TCP samples ($p < 0.01$) (Figures 5A, B). These results further suggest

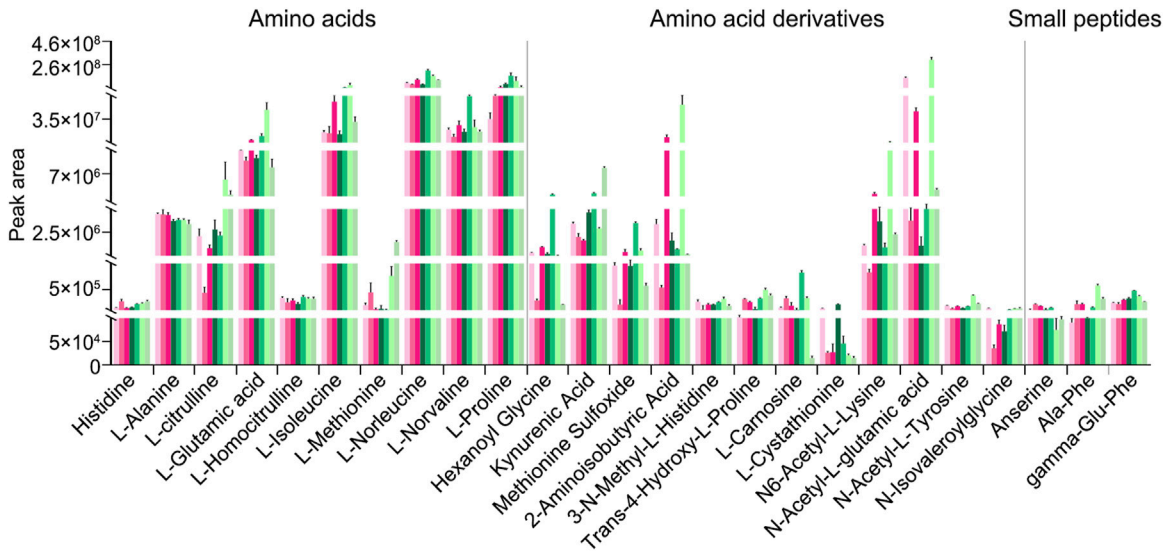


FIGURE 7
Peak areas of some amino acids and their metabolites that were also absolutely quantified in decoctions of CR, OS, and TS samples; they are also highlighted in bold in Figure 6. Data are presented as mean ± SD, n = 5.

that metabolic and lipidomic profiling of decoctions of genuine TCP samples from different origins differ from that of substituted TS tortoise shell samples.

## 3.5 Quantitative analyses of amino acids and their metabolites in decoctions of genuine and substituted tortoise shell samples

In addition to peak area–based semi-quantitative analysis of metabolites and lipids detected in the decoctions of genuine TCP samples (including CR1, CR2, and CR3) and substituted samples (including OS1, OS2, TS1, and TS2) from different origins, absolute quantitative analyses of 27 amino acids (Figure 6A), 22 amino acid derivatives (Figure 6B), and 18 small peptides (Figure 6C) were carried out. Quantitative results clearly showed that (1) the content of these amino acids, amino acid derivatives, and small peptides in decoctions of 10 g of powdered tortoise shell reached up to 0–69.6 mg, and (2) the content of small peptides was less than that of amino acids and amino acid derivatives by at least an order of magnitude (Figure 6; Supplementary Table S10). Figure 7 shows peak areas of 10 amino acids, 12 amino acid derivatives, and three small peptides that were also quantified and are highlighted in bold in Figure 6. Among them, several low-abundance amino acids and their metabolites, including L-methionine, N-isovaleroylglycine, and anserine, were not well quantified across all tortoise shell decoction samples (Figures 6, 7). Both semi-quantitative and absolute quantitative analyses showed that the content of these amino acids and their metabolites did not differ in decoctions of CR, OS, and TS samples (Figures 6, 7), suggesting the reliability of peak area–based semi-quantitative analysis for screening out differential metabolites and lipids between CR, OS, and TS samples.

# 4 Discussion

*Chinemys reevesii* (Gray) species–sourced traditional Chinese medicine TCP decoction is medicinal and edible. However, what ingredients are contained in this TCP decoction and its substitutes have not yet been comprehensively analyzed. In this study, we integrated LC-MS/MS–based metabolomic and lipidomic profiling to identify water-soluble metabolites and organic-soluble lipids in water decoction samples of genuine TCP and substituted tortoise shell samples from different origins, then screened out differential metabolites and lipids among them. Compared to previous studies focusing on certain classes of TCP chemical components (Wang et al., 2012; Ren et al., 2024), our study for the first time employed high-throughput metabolomic and lipidomic strategies to comprehensively detect hundreds to thousands of metabolites and lipids in TCP decoctions, providing rich chemical information for future exploration of bioactive constituents and optimal utilization.

Additionally, our results revealed remarkable metabolomic and lipidomic differences not only between TCP (CR1–3) and substituted tortoise shell samples (OS1–2 and TS1–2) but also among TCP samples (CR1–3) from different origins. Such findings indicate that the quality of TCP samples collected from different places might vary, and further suggest that potential adulteration with substituted tortoise shell materials may compromise the therapeutic efficacy of TCP preparations. Enrichment analyses revealed that differential metabolites and lipids between CR1–3, OS1–2, and TS1–2 were mainly localized to various metabolism-related pathways. However, N-methyl-4–aminobutyric acid was the only statistically significant ($p < 0.01$) common differential metabolite between CR1–3, OS1–2, and TS1–2. Accordingly, N-methyl-4–aminobutyric acid may be a new indicator for TCP decoction quality evaluation. Furthermore, since the chemical compositions in the decoctions of different

substitutes vary, the impact of mixing different substitutes on the efficacy of TCP decoctions may also differ.

To date, studies on the chemical components of TCP and its substitutes are scarce. Our preliminary results demonstrate the feasibility of using metabolomic and lipidomic strategies to evaluate the quality of genuine TCP and substituted tortoise shells. However, this study has several limitations, as follows: (1) the sample size and diversity of TCP and substituted tortoise shells were limited, and the differential metabolites and/or lipids identified among CR, OS, and TS samples may not be generalizable to other substituted samples; (2) the potential effects of rearing conditions and cultivation periods of *Chinemys reevesii* (Gray), *Ocadia sinensis* (Gray), or *Trachemys scripta elegans* (Wied) on the chemical profiles of their respective tortoise shell decoctions have not been validated; and (3) the compositional differences between the carapace and plastron were not investigated.

## 5 Conclusion

In summary, our work for the first time employed LC-MS/MS–based metabolomic and lipidomic analyses to provide a comprehensive overview of chemical constituents in decoctions of genuine TCP and its substitutes. A total of 1,691 metabolites and lipids were detected in decoctions of tortoise shell samples from *Chinemys reevesii* (Gray), *Ocadia sinensis* (Gray), or *Trachemys scripta elegans* (Wied) species. Furthermore, our preliminary results revealed significant compositional differences among *Chinemys reevesii* (Gray) species–sourced TCP samples from different places and between TCP and substituted tortoise shell samples from *Ocadia sinensis* (Gray) or *Trachemys scripta elegans* (Wied) species. N-methyl-4–aminobutyric acid exhibited a statistically significant difference ($p < 0.01$) between TCP and substituted tortoise shell samples, suggesting its potential as an indicator for TCP quality assessment. Further studies are warranted to validate and expand upon these findings, particularly focusing on elucidating the compositional profile and functional components of TCP, to enhance its therapeutic and preventive potential in disease management.

## 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

Ethical approval was not required for the study involving animals in accordance with the local legislation and institutional requirements because In our study, *Chinemys reevesii* (Gray) species-sourced TCP samples (including CR1, CR2, and CR3), *Trachemys scripta* elegans (Wied)- and Ocadia sinensis (Gray)-sourced tortoise shells (including TS1, TS2, OS1, and OS2) were provided by the Hubei Institute for Drug Control (Wuhan, China). CR, TS, and OS samples were authenticated by the deputy chief pharmacist, Ling Xiao (Hubei Institute for Drug Control, Wuhan, China), and conformed to the Pharmacopoeia of the People's Republic of China (2020 edition). According to the "Notice of National Health Commission of the People's Republic of China on the further regulation of raw material management for health foods" (http://www.nhc.gov.cn/wjw/gfxwj/201304/e33435ce0d894051b15490aa3219cdc4.shtml), the tortoise shell samples belong to the items that can be used as a health tonic and could be traded on the market. Thus, tortoise shell samples used in our study for analysis of their chemical constituents do not require ethical approval.

## Author contributions

MX: Writing–original draft, Writing–review and editing, Data curation, Investigation, Validation. YP: Supervision, Writing–original draft. YiZ: Funding acquisition, Writing–original draft. WZ: Formal Analysis, Methodology, Writing–original draft. LZ: Project administration, Writing–original draft. YoZ: Software, Writing–original draft. WS: Visualization, Writing–original draft. LW: Supervision, Writing–original draft. WM: Project administration, Writing–original draft. LX: Conceptualization, Resources, Writing–original draft. SG: Data curation, Methodology, Validation, Writing–original draft, Writing–review and editing. HH: Conceptualization, Funding acquisition, Writing–original draft.

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## Conflict of interest

Authors WS and HH were employed by Hubei Shengchang Aquatic Products Co., Ltd. Authors LW and HH were employed by Hubei Laozhongyi Pharmaceutical 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.

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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.2025.1549834/full#supplementary-material

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# Glossary

| | |
|---|---|
| **TCP** | Testudinis Carapax et Plastrum |
| **FC** | fold change |
| **KEGG** | Kyoto Encyclopedia of Genes and Genomes |
| **MRM** | multiple reaction monitoring |
| **MTBE** | methyl-tert-butyl ether |
| **PCA** | principal component analysis |
| **PCR** | Polymerase chain reaction |
| **QC** | quality control |
| **FA** | fatty acid |
| **GL** | glycerolipid |
| **GP** | glycerophospholipid |
| **SP** | sphingolipid |
| **ST** | sterol lipid |
| **DG** | diacylglycerol |
| **TG** | triacylglycerol |
| **MG** | monoglyceride |
| **FFA** | free fatty acid |
| **LPC** | lysophosphatidylcholine |
| **LPE** | lysophosphatidylethanolamine |
| **LPG** | lysophosphatidylglycerol |
| **PA** | phosphatidic acid |
| **PC** | phosphatidylcholine |
| **PE** | phosphatidylethanolamine |
| **PG** | phosphatidylglycerol |
| **PI** | phosphatidylinositol |
| **PS** | phosphatidylserine |
| **SM** | sphingomyelin |
| **SPH** | sphingosine |
| **Cer** | ceramide |
| **CAR** | carnitine |
| **HexCer** | hexosylceramide |
| **BA** | bile acid |
| **CE** | cholesteryl ester |
| **Cho** | cholesterol |
| **Ala** | alanine |
| **Gly** | glycine |
| **Tyr** | tyrosine |
| **Phe** | phenylalanine |
| **Asp** | aspartic acid |
| **Glu** | glutamic acid |
| **Arg** | arginine |
| **Ile** | isoleucine |
| **Met** | methionine |
| **Leu** | leucine |
| **Ser** | serine |

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# One-class modeling for verification of botanical identity: a review

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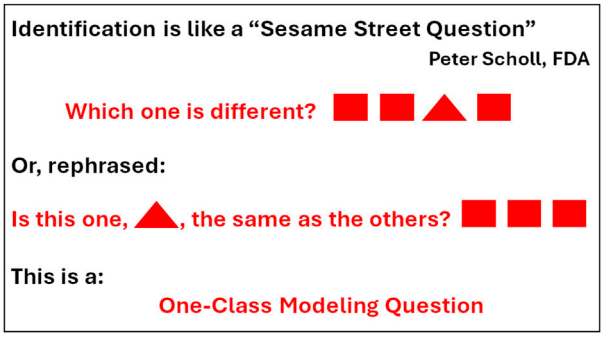
One-class modeling is a supervised multivariate botanical identification method based on principal component analysis (PCA) that constructs a model based only on the characteristics of the reference samples and uses the Q statistic as a combined metric. Test samples are judged to be similar (authentic) if their combined metric falls within the model limits or different (adulterated or contaminated) if the metric falls outside the model limits. This review initially considers three major factors affecting identification: the number of variables (univariate versus multivariate), the number of classes (one-class versus multi-class), and the type of analysis (quantitative versus qualitative). Multivariate analysis is commonly used for identification, providing a broader coverage of the identity specifications of the samples. With a combined metric, multivariate methods are analogous to univariate methods. One-class modeling and multi-class modeling employ different approaches for identification with one-class modeling being more flexible. While most methods to date have had a quantitative basis, qualitative methods are possible. This review focuses on multivariate, one-class modeling based on PCA. Examples are presented for the application of one-class modeling to identification of American ginseng (*Panax quinquefolius*), *Echinacea purpurea*, Black Cohosh (*Actaea racemosa*), and Maca (*Lepidium meyenii*). These examples demonstrate the utility and flexibility of one-class modeling.

KEYWORDS

**PCA, one-class modeling, review, authentication, multivariate analysis**




GRAPHICAL ABSTRACT

## Introduction

The goal of botanical identification is conceptually simple as shown by the graphical abstract; determine whether a test sample has features that are similar to those for a set of reference samples. This approach assumes that valid reference materials exist, and that identification can be made through a direct comparison of the test sample with a set of reference materials. The features used for a comparison can be sensory, morphological, microscopic, genetic, or chemical. The reference samples must be authenticated botanical reference materials or vouchered materials obtained from reliable sources. The authenticity of the reference materials is critical to the validity of the comparison. Multiple reference samples are needed to account for genetic, environmental, and processing variability. A single reference material from a metrological source can indicate similarity but fails to provide any information on biological variability.

Classically, herbalists, botanists, and taxonomists have authenticated plant materials based on monographs that describe a series of observations and tests developed using vouchered samples. In general, this approach requires access to the full plant material and flower. At one time, all commercial botanical materials were wild crafted by an expert and supplied directly to the user or to a local distributor. Today, botanical supplements are a multi-billion dollar industry and the link between the supplier and the manufacturer is much more tenuous. Manufacturers of botanical supplements are often faced with the problem of verifying that the barrel of brown powder delivered on the back dock by their supplier is really *ginkgo biloba*.

Commercial botanical supplements are classified as a food and are only regulated by FDA's current good manufacturing practices (cGMPs) which require the manufacturer to describe the steps they took to test the purity of their ingredients and products (Food and Drug Administration and Health and Human Services, 2024). Since the ingredients have often lost their morphological integrity (e.g., they may be sold as powders or extracts), chemical methods are most frequently used for identification although genetic methods are becoming more popular with recent advances in technology. Chemical methods provide quantitative multivariate data in the form of chromatograms or spectra that can be used for targeted or non-targeted analysis (Nichani et al., 2023a; Nichani et al., 2023b). However, these methods can only be used for identification by comparison to appropriate reference materials.

The need for identification methods led AOAC International to develop "Guidelines for Validation of Botanical Identification Methods" in 2012 (Harnly, 2012). The Guidelines describe a probability of identification (POI) method, establish a well defined nomenclature (Table 1), and describe several basic principals. First, the guidelines recognize the use of both quantitative chemical methods and qualitative morphological methods. Second, the guidelines assume a multivariate analysis and specify that the chosen method must reduce multiple observations or measurements to a combined metric. Next, the metric is used to generate a binary response, "yes" the sample is authentic or 'no" it is adulterated. Finally, the POI method is a two-class analysis method that requires comparison of an authentic and an adulterated sample (Table 2) (Brereton, 2009). Since the Guidelines are not method specific, 60 analyses of the authentic and adulterated samples are required to guarantee 95% confidence in discriminating between the two populations (Table 3). A companion paper to the Guidelines presented an example of a quantitative chemical analysis using mass spectrometric data (LaBudde and Harnly, 2012). Unfortunately, an example based on a qualitative morphological analysis was not given and has not been forth coming.

It is recognized that the POI method is philosophically and statistically defensible but not practical. There have been very few applications of the POI method to identification problems (Harnly et al., 2013). Major obstacles are the need to identify each potential adulterant and to test each with 60 analyses. A daunting task. AOAC International has recently charged an expert panel with the development of new guidelines.

A new method has recently been proposed based on one-class modeling using principal component analysis (PCA) (Harnly et al., 2013; Harnly, 2023). This approach simplifies the identification process. It builds a single model based on the reference samples and treats every other sample as a potential adulterant. If an unknown sample falls within the model, the sample is judged to be authentic. If it falls outside the model it is deemed to be adulterated. The method is compatible with any multivariate data set and the Q statistic (Brereton, 2009) of PCA provides an inherent combined metric that can be used to determine the confidence limit of the model (Harnly et al., 2013). The validity of the model can be established by the cross validation. Thus, one-class modeling simplifies identification by eliminating the need to identify every potential adulterant, provides an inherent combined metric for each sample, provides confidence limits based on the number and quality of the reference samples, and provides a binary authentic/non-authentic output based on a statistical analysis.

This review will consider how chemical identification can be based on one or more features (univariate or multivariate), one or more classes of samples (one-class or two-class modeling), and quantitative or qualitative data. Regardless the number of features, classes, or nature of the data, the method generates a combined metric that produces a binary result, "yes" or "no," with regards to authenticity (Harnly, 2012). This review will focus on the use of one-class modeling of multivariate quantitative data using PCA (Harnly, 2023). One-class modeling offers an inherently different approach from the POI model and two-class modeling and requires only the identification of the reference samples. In the last 10 years, this approach has been used to authenticate numerous raw botanicals and botanical supplements (Harnly et al., 2013; Harnly et al., 2016; Harnly and Upton, 2024; Harnly et al., 2017; Geng et al., 2020). Several examples of the application of one-class modeling to identification problems will be presented.

## Univariate versus multivariate analyses

Identification methods, as stated, generally employ multivariate analysis. Qualitative morphological methods are inherently multivariate since all the features of a plant are involved in identification. Quantitative chemical methods offer non-targeted multivariate analyses based on many chromatographic and spectral methods such as gas and liquid chromatography (GC and LC) and infrared spectrometry (IR), near IR spectrometry (NIR), mass

TABLE 1 Glossary (LaBudde and Harnly, 2012).

| |
|---|
| Botanical: Of, or relating to, plants or botany. May also include algae and fungi. May refer to the whole plant, a part of the plant (e.g., bark, woods, leaves, stems, roots, rhizomes, flowers, fruits, seeds, etc.), or an extract of the parts. |
| Botanical identification method (BIM): A method that establishes identity specifications for a botanical material and determines YES, the test material is a true example of the target botanical material or NO, it is not the target botanical. |
| Combined Metric: (analytical parameter in previous papers) A measured or computed analytical value used to determine whether the test material matches the target material. The combined metric may be based on morphological features, genetic sequences, chromatographic patterns, spectral patterns, or any other metric appropriate for the target material. |
| Exclusivity Panel: A list of practically obtainable botanical materials that that are expected to give a negative result when tested by the BIM. |
| Identity specification (IS): The morphological, genetic, chemical, or other characteristics that define a target botanical material. Specifications may include, but are not limited to, data from macroscopic, microscopic, genetic (e.g., DNA sequencing), chromatographic fingerprinting (e.g., capillary electrophoresis, gas chromatography, liquid chromatography, or thin-layer chromatography), and spectral fingerprinting (e.g., infrared, near-infrared, nuclear magnetic resonance, ultraviolet/visible absorbance, or mass spectrometry) methods. |
| Inclusivity Panel: A list of practically obtainable botanical materials that are expected to give a positive result when tested by the BIM. |
| Non-target botanical material: Any botanical material that does not meet the identity specification. |
| Probability of identification (POI): The expected or observed fraction of test portions at a given concentration that give a positive result when tested by the BIM. |
| Sample: A small portion or quantity, taken from a population or lot that is ideally a representative selection of the whole. |
| Sensitivity: Ability of a BIM to correctly identify variants of the target material that meet the identity specification. |
| Specificity: Ability of a BIM to correctly reject nontarget botanical materials. |
| Standard inferior test material (SITM): A botanical material mixture that has the maximum concentration of target material that is considered unacceptable, as specified by the SMPRs. The BIM must reject this material. |
| Standard method performance requirements (SMPRs): Performance requirements based on the fitness-for-purpose statement for each method. For BIMs, the SMPRs should include the physical form of the sample, the ISF, the ESF, the SSTM, the SITM, the number of samples for the inclusivity/ exclusivity panels, and the desired probability and confidence limits for the method. |
| Standard superior test material (SSTM): A botanical material mixture that has the minimum acceptable concentration of the target material, as specified by the SMPR. The BIM must accept this material. |
| Target botanical material: The botanical material of interest as described in the identity specification. |

TABLE 2 Chemometric methods (Brereton, 2009).

| Approach | Method[a] | Supervision | Classes |
|---|---|---|---|
| Exploratory | PCA | None | None |
| One Class Modeling (soft modeling) | PCA | ID 1 class | 1 |
| | SIMCA | ID all classes | 2 or more |
| Two Class Classification (hard modeling) | LDA | ID all classes | 2 or more |
| | QDA | ID all classes | 2 or more |
| | PLS-DA | ID all classes | 2 or more |
| | SVM | ID all classes | 2 or more |
| | ANN | ID all classes | 2 or more |

[a]ANN, Artificial neural networks; LDA, Linear discriminate analysis; PLS-DA, Partial least squares-discrimination analysis; PCA, Principal component analysis; QDA, Quadratic discriminate analysis; SIMCA, Soft independent modeling of class analogy; SVM, Support vector machines.

spectrometry, and nuclear magnetic resonance spectrometry (NMR) (Nichani et al., 2023a; Nichani et al., 2023b). These methods may offer hundreds, even thousands (depending on resolution), of variables to characterize a sample. The many variables improve the chances of including important components of chemical identity or observing components that are not present (adulterants or contaminants) in the reference samples. Multivariate methods are less susceptible to fraud. Ideally, a botanical can be characterized with respect to hundreds of components with known structural and concentration variations that will allow the user to discriminate between similar species, samples with intentionally altered compositions, or material substitutes. However, incorporating a large number of variables in a binary decision (authentic or not authentic) is a challenge. Reduction of multiple variables into a combined metric offers the advantage of using classic univariate statistics to make this binary decision.

There are two excellent examples that illustrate the use of univariate statistics for class analysis. First, the limit of detection (LOD) is a classic one-class model (Figure 1A) aimed at determining if a sample signal is a member of the blank signal population (Long and Winefordner, 1983). In the univariate mode, multiple measurements of the method blank establish the "baseline" (blank mean) and the standard deviation (distribution). Assuming a normal distribution, it is possible to statistically determine whether a signal is not simply due to random variation of the blank signal. For this purpose, a threshold is usually set at 3 times the baseline standard deviation (3s). This provides 99% confidence that any signal observed above this level is due to the presence of the analyte. Inversely, this threshold confirms that the test signal is not a member of the blank population. This is a form of one-class modeling.

Second, the students t test is a well established univariate approach to determining if the means of two populations are similar (Figure 1B) (Moore and McCabe, 1999). Multiple measurements of the value of interest are used to establish the mean and standard deviation of each population. Assuming both

TABLE 3 Sample size required for proportion (Harnly, 2012).

| Minimum Probability | Number tests | Number failures | 1-Sided LCL | 2-Sided LCL | 2-Sided UCL | Effective AOQL |
|---|---|---|---|---|---|---|
| 50% | 10 | 2 | 54.1% | 49.0% | 94.3% | 71.7% |
| 50% | 20 | 6 | 51.6% | 48.1% | 85.5% | 66.8% |
| 50% | 40 | 14 | 52.0% | 49.5% | 77.9% | 63.7% |
| 50% | 80 | 32 | 50.8% | 49.0% | 70.0% | 59.5% |
| 55% | 10 | 1 | 65.2% | 59.6% | 100% | 79.8% |
| 55% | 20 | 5 | 56.8% | 53.1% | 88.8% | 71.0% |
| 55% | 40 | 12 | 57.1% | 54.6% | 85.8% | 68.2% |
| 55% | 80 | 28 | 55.9% | 54.1% | 74.5% | 64.3% |
| 60% | 10 | 1 | 65.2% | 59.6% | 100% | 79.8% |
| 60% | 20 | 4 | 62.2% | 58.4% | 91.9% | 75.2% |
| 60% | 40 | 10 | 62.4% | 59.8% | 85.8% | 72.8% |
| 60% | 80 | 24 | 61.0% | 59.2% | 78.9% | 69.1% |
| 65% | 10 | 1 | 65.2% | 59.6% | 100% | 79.8% |
| 65% | 20 | 3 | 67.8% | 64.0% | 94.8% | 79.4% |
| 65% | 40 | 9 | 65.1% | 62.5% | 87.7% | 75.1% |
| 65% | 80 | 21 | 65.0% | 63.2% | 82.1% | 72.7% |
| 70% | 10 | 0 | 78.7% | 72.2% | 100% | 86.1% |
| 70% | 20 | 2 | 73.8% | 69.9% | 97.2% | 83.6% |
| 70% | 40 | 7 | 70.7% | 68.0% | 91.3% | 79.7% |
| 70% | 80 | 17 | 70.4% | 68.6% | 86.3% | 77.4% |
| 75% | 10 | 0 | 78.7% | 72.2% | 100% | 86.1% |
| 75% | 20 | 1 | 80.4% | 76.4% | 100% | 88.2% |
| 75% | 40 | 5 | 76.5% | 73.9% | 94.5% | 84.2% |
| 75% | 80 | 13 | 75.9% | 74.2% | 90.3% | 82.2% |
| 80% | 20 | 1 | 80.4% | 76.4% | 100% | 88.2% |
| 80% | 40 | 3 | 82.7% | 80.1% | 98.6% | 88.8% |
| 80% | 80 | 10 | 80.2% | 78.5% | 93.1% | 85.8% |
| 85% | 20 | 0 | 88.1% | 83.9% | 100% | 91.9% |
| 85% | 40 | 2 | 86.0% | 83.5% | 98.6% | 91.1% |
| 85% | 80 | 6 | 86.1% | 84.6% | 96.5% | 90.6% |
| 90% | 40 | 0 | 93.7% | 91.2% | 100% | 95.6% |
| 90% | 60 | 2 | 90.4% | 88.6% | 99.1% | 93.9% |
| 90% | 80 | 3 | 91.0% | 89.5% | 98.7% | 94.1% |
| 95% | 60 | 0 | 95.7% | 94.0% | 100% | 97.0% |
| 95% | 80 | 0 | 96.7% | 95.4% | 100% | 97.7% |
| 95% | 90 | 1 | 95.2% | 94.0% | 100% | 97.0% |
| 98% | 130 | 0 | 98.0% | 97.1% | 100% | 98.6% |

(Continued on following page)

TABLE 3 (*Continued*) Sample size required for proportion (Harnly, 2012).

| Minimum Probability | Number tests | Number failures | 1-Sided LCL | 2-Sided LCL | 2-Sided UCL | Effective AOQL |
|---|---|---|---|---|---|---|
| 98% | 240 | 1 | 98.2% | 97.7% | 100% | 98.8% |
| 99% | 28/0 | 0 | 99.0% | 98.6% | 100% | 99.3% |
| 99% | 400 | 1 | 99.1% | 98.8% | 100% | 99.4% |

Assume: 1. Binary outcome (occur/not occur).
2. Constant probability of event occurring
3. Independent trials
4. Fixed number of trials
Inference: 95% confidence interval lies entirely at or above the specified minimum.
Desired: Sample size N.
Notes: 1. Based on modified Wilson score 1-sided confidence limit.
2. AOQL = Average Outgoing Quality Level

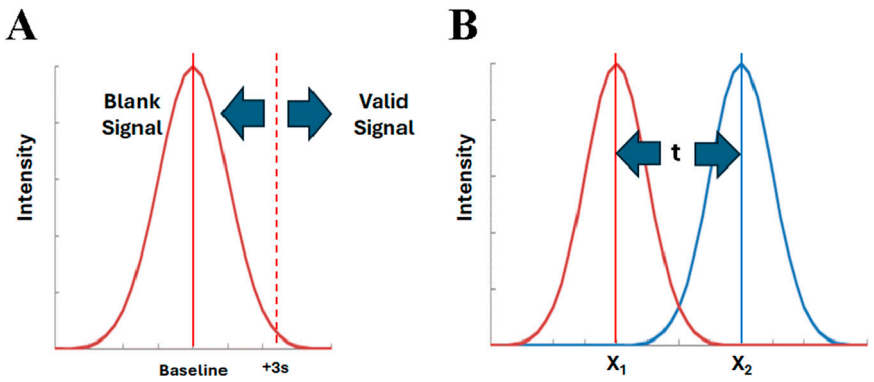



FIGURE 1
One class modeling and two class classification, **(A)** Detection limit and **(B)** Students' t-test. Applied to univariate tests, the intensity is the measure for a single variable. For multivariate tests, the intensity is the combined metric computed from all variables.

populations are normally distributed, the difference between the means of the populations is evaluated in terms of their standard deviations to establish the statistical significance. Similarly, the F-test can be used for establishing the difference for three or more populations (Moore and McCabe, 1999). These are forms of two-class or multi-class modeling. This approach is used as the basis of the POI method. However, in that case, the standard deviation is not known for either population, and the reference and adulterated samples must each be run 30 times to establish their distribution.

The diagrams in Figure 1 are the same for univariate and multivariate analyses, if the multivariate data is represented by a combined metric (Nichani et al., 2023a; Harnly, 2012; LaBudde and Harnly, 2012; Harnly et al., 2013). That is, all the variables from a multivariate analysis are used to compute a single, combined value or metric for each sample. This combined metric is then processed in exactly the same manner as a univariate value. The baseline value in Figure 1A corresponds to the combined metric mean of the reference samples. In this case, a test sample is judged to be authentic if it falls within the pre-determined level of uncertainty (e.g., within ±2s). In Figure 1B, the means correspond to the combined metrics for the reference population and the test (potentially adulterated) population. Classic statistics can be used to compute the confidence with which the two populations can be distinguished. When analyzed, the combined metric for the test sample will be judged to belong to either the authentic or test population.

For both the LOD and t-test, the number of measurements, n, for reference and test sample populations is critical to establishing the confidence with which similarity can be determined (Long and Winefordner, 1983; Moore and McCabe, 1999). The calculated mean and standard deviation of a population are only estimates of the true mean and standard deviation. As n increases, the level of confidence in the two values increases as does the decision regarding similarity.

## One-class modeling versus multi-class classification

There are numerous chemometric methods for processing multivariate data sets (Table 2). Unsupervised analysis requires no user input and reveals naturally occurring patterns. The most widely used unsupervised methods are PCA and hierarchical clustering analysis (HCA). Both serve to reveal sample patterns that may not be obvious. Supervised methods require identification of the classes of samples in the data set. Brereton has divided

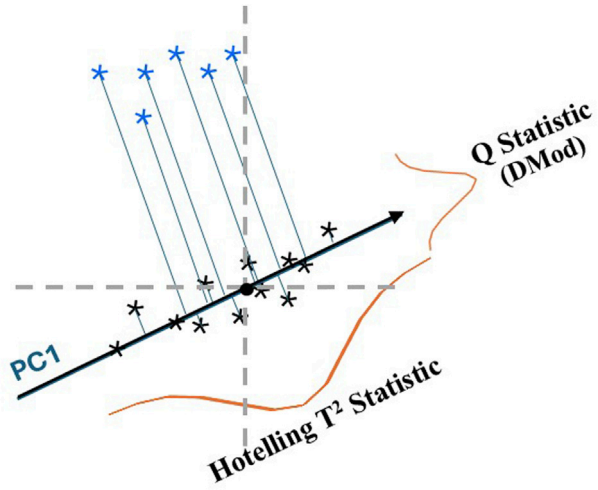



FIGURE 2
Illustration of the Hotelling $T^2$ statistic and Q statistic for the first principal component (PC1) fit to a bivariate data set (★) with test samples (★). The Q statistic characterizes the variance outside the model, the distance from the sample perpendicular to the model, PC1. The Hotelling $T^2$ statistic characterizes the variance within the model, the distance on PC1 from the sample intercept to the origin (mean centered data). The normal distribution of the Q statistic and the Hotelling T2 statistic are shown in red. A plot of the Q statistic versus the Hotelling T2 statistic is known as an influence plot.

supervised multivariate analysis into the one-class classifiers and the two- (or multi-) class classifiers and described their fundamental differences (Brereton, 2009). Both approaches require a priori identification of the sample classes making them supervised methods.

One-class classifiers or one-class modeling constructs a model for each class based only on the characteristics of that class (Brereton, 2009). Comparison between sample classes requires comparison of the models for each class through soft independent modeling class analogy (SIMCA). SIMCA is considered a soft modeling technique as samples can be assigned to a single class, multiple classes, or no class. This is an excellent approach for recognizing outliers. Two-class classifiers or classification builds a single model for all the classes based on the features of all the classes (Brereton, 2009). Two-class classification is considered a hard modeling technique since samples are forced into one of the pre-designated classes. For example, partial least squares-discriminant analysis (PLS-DA) of *Gingko biloba* and *Echinacea purpurea* will require a sample of *Actaea racemosa* to be classified as either *Gingko* or *Echinacea*. Two-class classification methods have difficulty dealing with outliers and require re-calculation when additional classes are added.

One-class modeling has historically been used for process control (Brereton, 2009). Samples from key locations/times in a successful process are used to build models which can track the fidelity of succeeding processes. The one-class models tell the operator whether the process is within the limits established in previous runs.

There are numerous ways to compute a combined metric from multivariate analyses (Nichani et al., 2023a; Nichani et al., 2023b; Harnly, 2012; Brereton, 2009). The two statistical measures for evaluating a multivariate PCA model are shown in Figure 2 where a single principal component (PC1) is fit to a set of bivariate data (Brereton, 2009; LaBudde and Harnly, 2012). In this case PC1 is the model, it is a vector which intercepts the greatest variance of the data set (black symbols). The Q statistic is the variance outside the model, the distance of the sample from the model determined by a line from the sample perpendicular to the model. The Hotelling $T^2$ statistic characterizes the sample variance within the model, the distance from the perpendicular intercept to the model center. This latter distance is analogous to the distance of a sample to the center of a normal distribution for univariate data. Figure 2 shows that the Hotelling $T^2$ statistic would place the test samples (blue symbols) in the same class as the reference samples while the Q statistic shows that they belong in different classes. In general, the Q statistic is much more sensitive to compositional differences and detection of outliers.

One-class modeling using PCA is ideally suited for identification. A model can be built using reference samples, the Q statistic provides an excellent combined metric, confidence limits can be established, and a test sample can be determined to lie within (authentic) or outside (adulterated) the model. One-class modeling is flexible since only the reference data is identified. In the case of two classes of samples, both can be modeled and the models compared or either class can be modeled and the other treated as unknowns (see the example for black cohosh below).

## Quantitative versus qualitative

The facility of computing a combined metric from quantitative data using one-class modeling is readily seen in the preceding section. Both the Hotelling $T^2$ statistic and the Q statistic are readily derived from chemometric analyses (Brereton, 2009) and provide a combined metric for all variables for each sample. Deriving such a metric for qualitative data is much more challenging (Harnly, 2012; Sudberg, 2024). The AOAC

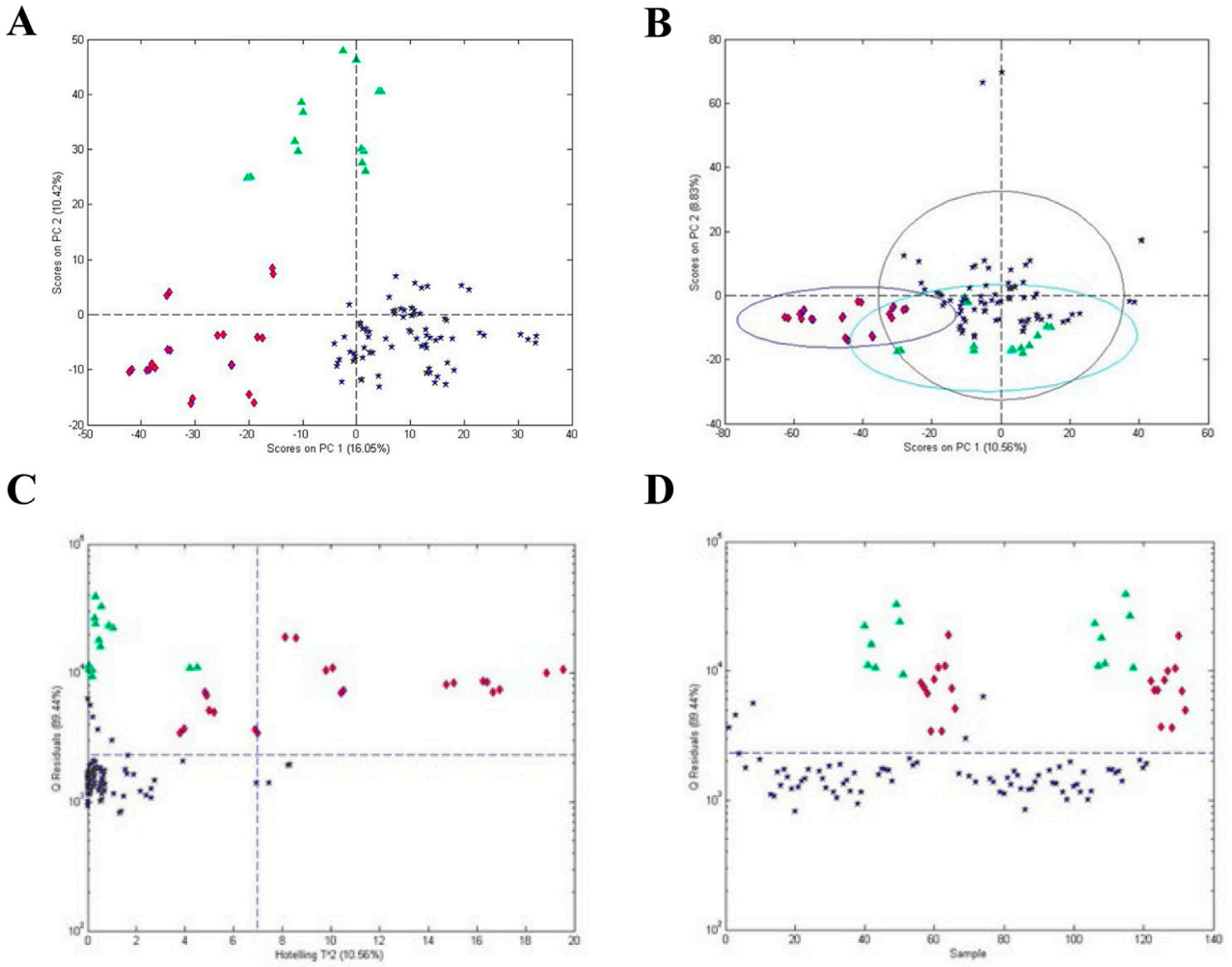



FIGURE 3
Analysis of processed Maca (*Lepidium meyenii*): **(A)** unsupervised PCA scores plot for all materials, **(B)** PCA scores plot based on one-class modeling of processed Maca from Peru, **(C)** influence plot (Q residuals versus Hotelling T2 residuals) based on one-class modeling of processed Maca from Peru, and **(D)** Q statistic versus sample based on one-class modeling of processed Maca from Peru. Symbols: (★) processed Maca from Peru, (◆) Maca root from Peru, and (▲) Maca root from China.

Guidelines state that they are intended for all candidate botanical identification methods and that identity specifications can be based on morphological, genetic, chemical, and/or other defining features of the botanical material. At that date it was envisioned that morphological or microscopic methods could be reduced to an algorithm or check list that would provide a suitable combined metric for judging authenticity. The companion paper (LaBudde and Harnly, 2012) illustrating the application of the POI method was based on quantitative analysis (mass spectral data) and, unfortunately, no example of a qualitative method was included.

There have not been any reports of qualitative identification in the literature. However, Sudberg (2024) at Alkemist Labs recently described a qualitative method for discriminating between spearmint (*Menta spicata* L.) and peppermint (*Mentha* x *piperita*) at the International Conference on Natural Products Research (Krakow, Poland, 2024) based on Bayes' Theorem. This report nicely incorporated the POI principles which are discussed below.

## Probability of identification (POI)

The AOAC International POI method is based on two-class classification (Brereton, 2009). The glossary (Table 1) shows that the key feature of the method is to identify an authentic sample population that would always give a positive result (inclusivity sampling frame) and a population that would always give a negative result (exclusivity sampling frame). From their respective populations, a standard superior test material (SSTM, representing the minimum acceptable concentration of the target material) and standard inferior test material (SITM, representing the maximum unacceptable concentration of target material) are chosen to be run 30 times each to establish the precision of the method (Table 3).

In more general terms, an authentic (acceptable) and an adulterated (not acceptable) sample are chosen to characterize the resolution of the method, i.e., to determine if the method can discriminate between the two levels of adulteration. The adulterated

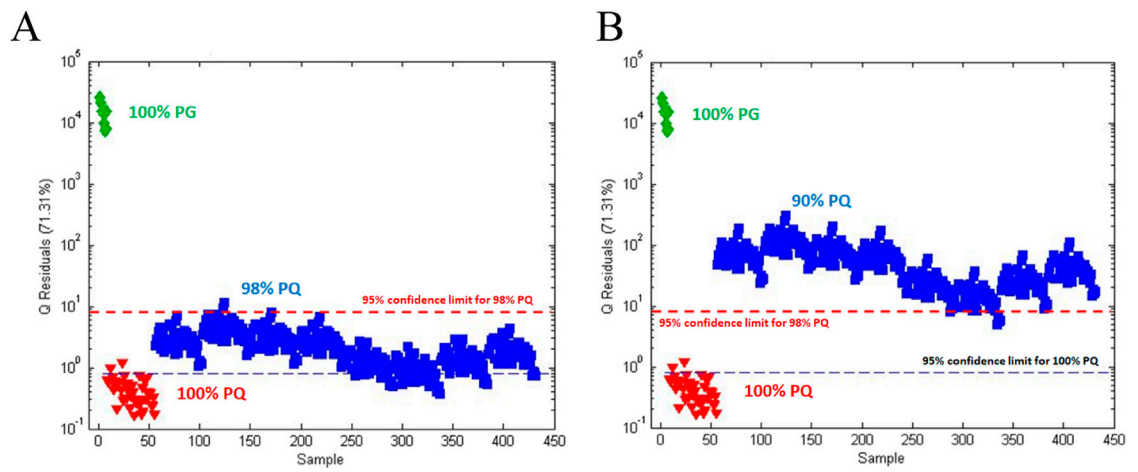



FIGURE 4
Q statistics plot of American ginseng (PQ) adulterated with Asian ginseng (PG) based on one-class modeling of 100% PQ: **(A)** Q statistic plot for (▼) 100% PQ, (◆) 100% PG, and (■) 98% PQ; **(B)** Q statistic plot for (▼) 100% PQ, (◆) 100% PG, and (■) 90% PQ. The black dashed line is the 95% confidence limit for a one-class model of 100% PQ. The red dashed line is the 95% confidence limit for the one-class model of 98% PQ. Sensitivity for 100% and 98% PQ is 95% and 98%, respectively. Specificity for 100% and 90% PG is 100% and 99%, respectively.

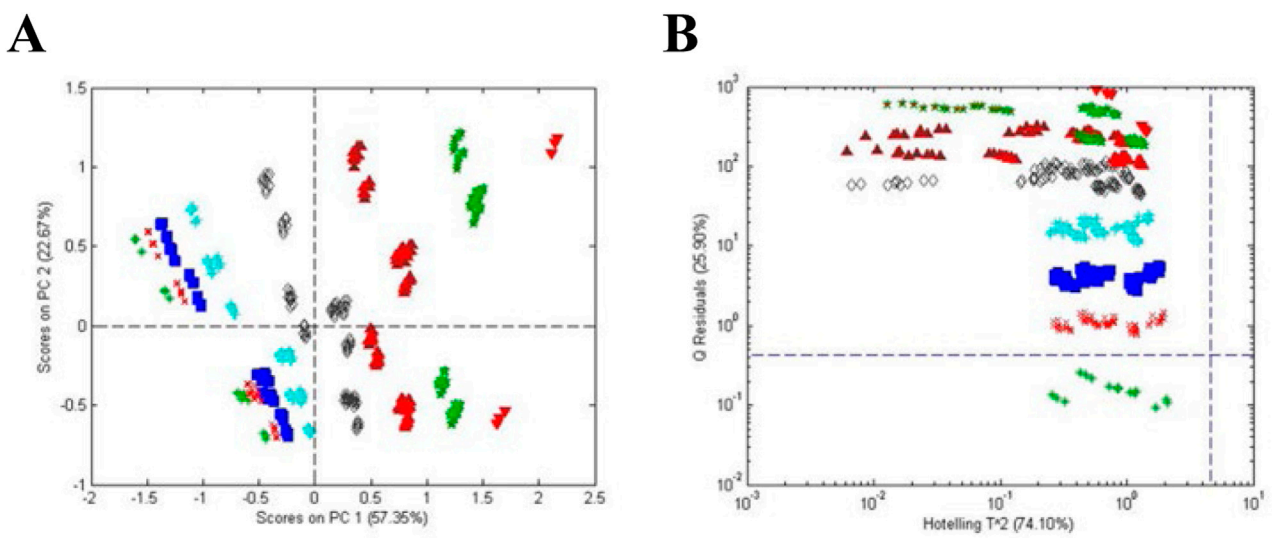



FIGURE 5
Adulteration of American ginseng (*Panax quinquefolius*) with Asian ginseng (*P. ginseng*): **(A)** Unsupervised PCA and **(B)** influence plot (Q residuals versus Hotelling $T^2$ residuals) based on one-class modeling of 100% *P. quinquefolius*. Symbols: (★) 100%, (X) 95%, (■) 90%, (+) 80%, (◇) 60%, (▲) 40%. (★) 20%, and (▼) 0% PQ.

sample is frequently an aliquot of the authentic sample spiked with a known level of adulterant or a different species from the same genus of the authentic sample (Nichani et al., 2023b; Harnly et al., 2013; Harnly, 2023; Harnly et al., 2016; Harnly and Upton, 2024; Harnly et al., 2017). Without specifying the method, the 30 repeats of each are used to establish the mean and distribution of the two populations (Harnly, 2012). The ability to perform 60 analyses with no false positives or negatives suggests a close to baseline separation of the two populations as shown in Figure 1B. Table 3 shows that 2 failures out of 60 translates to identification at the 90% confidence level 93.9% of the time, whereas no failures in 60 attempts corresponds to separation at the 95% confidence level 97.0% of the time.

The requirement of identifying an SITM for each potential adulterant and analyzing both the SSTM and SITM materials 30 times made the POI method unpopular. Today there is considerable interest in reducing the number of required analyses and expanding qualitative applications. However, it should be recognized that the number of samples analyzed, and the variance associated with the reference samples will always determine the confidence level that can be achieved by the method.

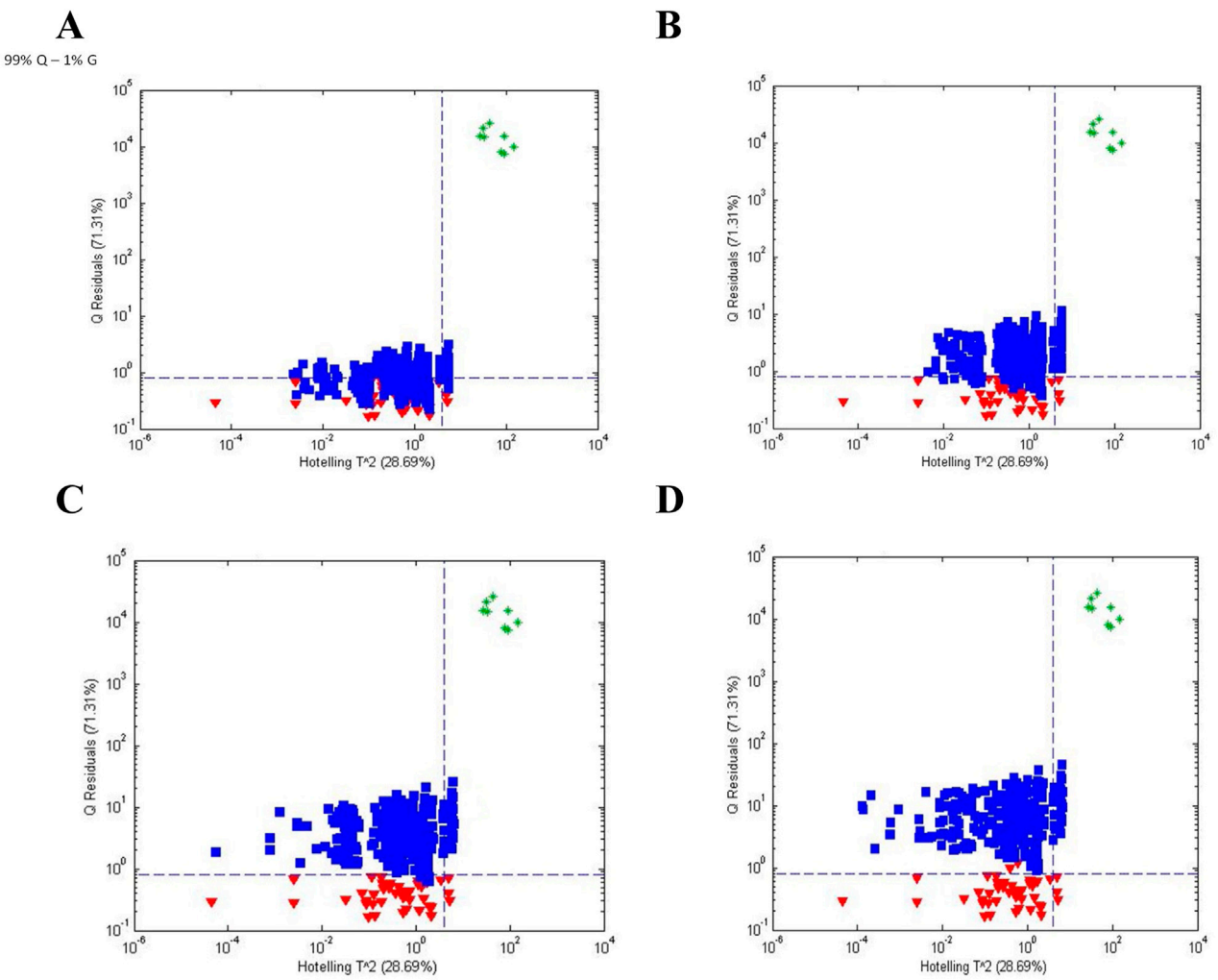



FIGURE 6
Influence plots (Q residuals versus Hotelling T^2 residuals) for **(A)** PQ:PG = 99:1, **(B)** PQ:PG = 98:2, **(C)** PQ:PG = 97:3, and **(D)** PQ:PG = 96:4 based on one-class modeling of 100% PQ. Symbols: (▼) 100% PQ and (■) 99%, 98%, 97%, and 96% PQ.

## One-class modeling

One-class modeling has been described in detail in a previous paper (Harnly et al., 2013) and in the previous section on "One-class modeling versus multi-class classification." It makes use of the original requirement of the POI method to acquire an inclusivity panel (Table 2) of samples, requires establishing a PCA model, and uses the Q statistic as a combined metric to determine whether any test sample lies within or beyond the specified confidence limit of the model. There is no exclusivity panel, and any combination of unknown samples can be tested against the model. The false positive and negative rate are determined by the confidence level specified by the user. Validation of the model is established using either cross validation or an external validation sets of samples.

Like all statistical evaluations, the degree of confidence is dependent on n, the number of reference samples. In general, the more reference samples used, the more confidence the user has in the average Q value and the standard deviation. However, with respect to botanical materials, the biggest source of variability is the biological variability of the samples. Significant variability has been observed between sources, growing location, growing year, harvests, and from plant-to-plant. The more varied the plant meta-data, the greater the population distribution. As will be shown for the example of *A. racemosa* below, the variance covering three classes of identical BRMs from different sources is greater than the variance for each class.

Figure 3 presents an example of the application of one-class modeling to a collection of Maca (*Lepidium meyenii*) samples consisting of roots collected in Peru and China and Processed Maca supplements from Peru (Geng et al., 2020). Processed root supplements are heated, extruded, powdered, and sold commercially. The question to be answered was could the processed Maca be distinguished from the root samples. Figure 3A shows an unsupervised PCA score plot for the Maca samples. The three clusters suggest the classes can be distinguished from each other but offer minimal statistics with respect to their differences. Figure 3B shows the results for supervised one class modeling of the processed Peruvian Maca samples. The variable

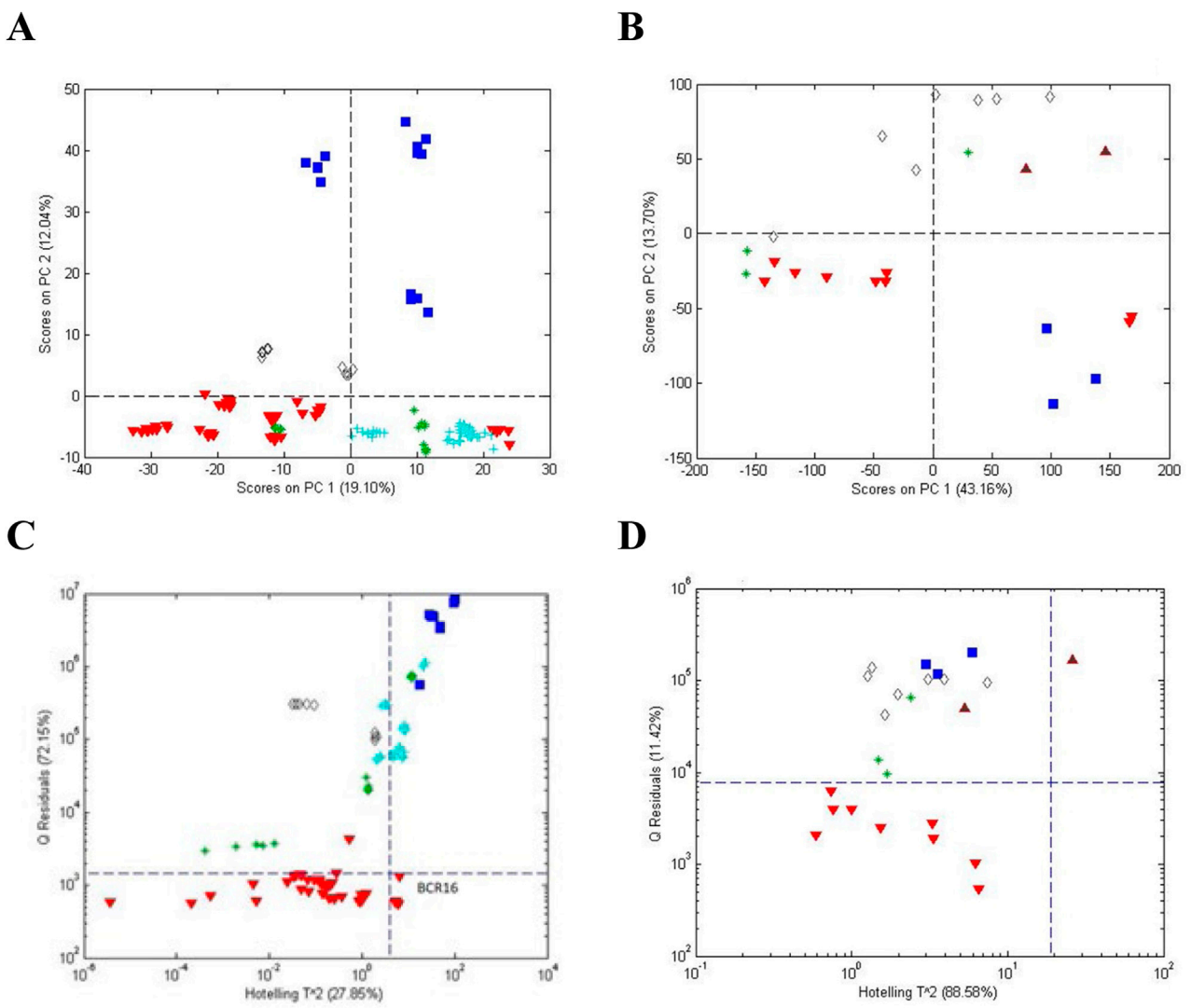



FIGURE 7
Unsupervised PCA scores plots **(A, B)** and influence plots (Q residuals versus Hotelling $T^2$ residuals) **(C, D)** for one-class modeling of *A. racemosa* analyzed by FIMS **(A, C)** and NMR **(B, D)**. Symbols: (▼) *A. racemosa*, (◇) *A. rubra*, (▲) *A. cimicifuga*, (✶) *A. pachypoda, and* (■) *A. podocarpa*.

loadings for the processed Maca samples were used to compute scores for the other samples (Peruvian and Chinese roots). Figure 3B shows ellipsoidal 95% confidence limits for each of the classes but it is again obvious that the score plot is a poor display for statistical differences of the classes.

Figure 3C presents a influence plot that displays the Q residuals (vertically) versus the Hotelling $T^2$ residuals (horizontally) for a one class model of the processed Maca data, i.e., a influence plot for the data in Figure 3B. The data in Figure 3C were acquired using only one principal component, minimizing the possibility of over fitting. The statistics for the model can be seen much more clearly in the influence plot as can the difference between the Q and Hotelling $T^2$ statistics. For the Hotelling $T^2$ statistic, the sensitivity for the processed Maca is 97% and the specificity for the Peruvian and Chinese roots are 64% and 0%, respectively. For the Q statistic, the sensitivity is 93% and the specificity for both roots is 100%. Thus, the Q statistic is much more sensitive to differences in the sample composition. Plotting only the Q value versus individual samples (Figure 3D) presents a further simplified plot. This presentation can also be rotated to view the sample data as a frequency plot as will be shown below for one of the examples.

Choosing the processed Maca as the reference samples is arbitrary. Selection of the reference samples is dependent on the question being asked. The Peruvian or the Chinese root samples could also be chosen as the reference samples. The number of non-reference samples is also arbitrary. In Figure 3, two sets of non-reference samples (Peruvian and Chinese Maca roots) were chosen. Since the one-class model is based solely on the reference samples, the average and distribution of the non-reference samples is not a concern.

In essence, one-class modeling examines the difference associated with every variable in the data set (8). Simplistically, a model based on a mass spectrum of 205 ions requires that all 205 variables for the test material lie within the statistical limits

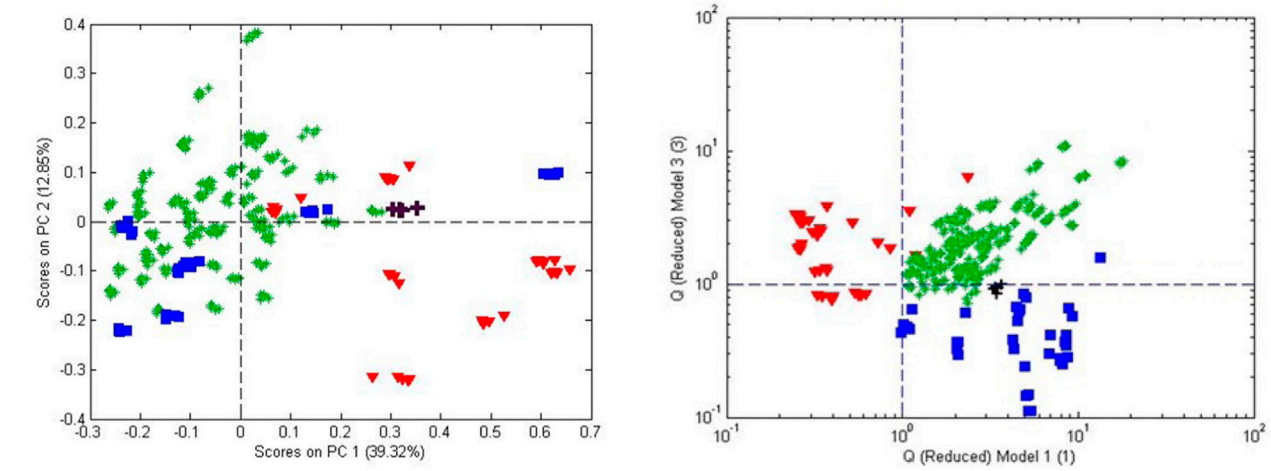



FIGURE 8
Comparison of *A. racemosa* reference samples from 4 sources (AHP, SS, NCA, and NIST). **(A)** Unsupervised PCA scores plot and **(B)** influence plot based on one-class modeling of *A. racemosa* from AHP (vertically) and SS (horizontally). Symbols: (■) AHP, (▼) SS, (+) NIST, and (+) NCA. AHP and SS provided clusters below and to the left of the 95% confidence limits, respectively. NIST samples lay barely below the confidence limit with the AHP samples and NCA samples were outliers (upper right quadrant) for both one-class models.

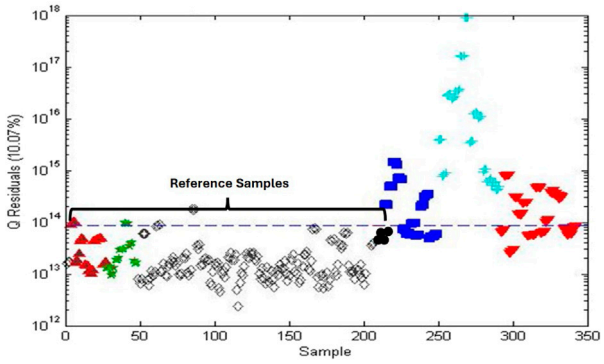



FIGURE 9
Q residuals plot based on one-class modeling of *A. racemosa* reference samples obtained from (▲) American Herbal Pharmacopoeia, (+) Strategic Sourcing, (◇) North Carolina Arboretum, and (✱) the National Institutes of Health. Non-modeled samples are: (▼) other *Actaea* species from AHP, (■) commercial *Actaea* roots, and (+) commercial *Actaea* supplements. The horizontal dashed line provides the 95% confidence limit, p = 0.05).

determined for each variable of the reference samples. Practically, some deviation of variable(s) can be tolerated as determined by the limits set by the user. If a test material is to be judged different or adulterated, one (or more) of the variables in the data set must be significantly different. As shown in a previous study (Harnly, 2023), variables that are autoscaled will have a variance of 1.0. The 205 MS variables will have a total variance of approximately 205. The signal necessary for a variable to have a significantly different variance and have a significant statistical impact on the combined metric can be predicted from the average intensity of the variable (Harnly, 2023).

An added factor of considerable importance is that the plots in Figure 3 can be constructed by anyone using any commercial chemometric platform. Starting with the same data set and using the same pre-processing steps, any commercial platform will produce the same plots. Identification using one-class modeling can be done in any lab without the need for a chemometrics expert.

## Examples of one-class modeling

### American Ginseng (*Panax quinquefolius*)

American Ginseng (PQ) adulterated with varying levels of Asian Ginseng (*Panax ginseng*, or PG) was used to illustrate an application of the POI (Harnly et al., 2013). For the study, 44 PQ samples were obtained from the Wisconsin Ginseng Board (harvested over 3 years from 20 different farms in Wisconsin) and 8 PG samples grown in

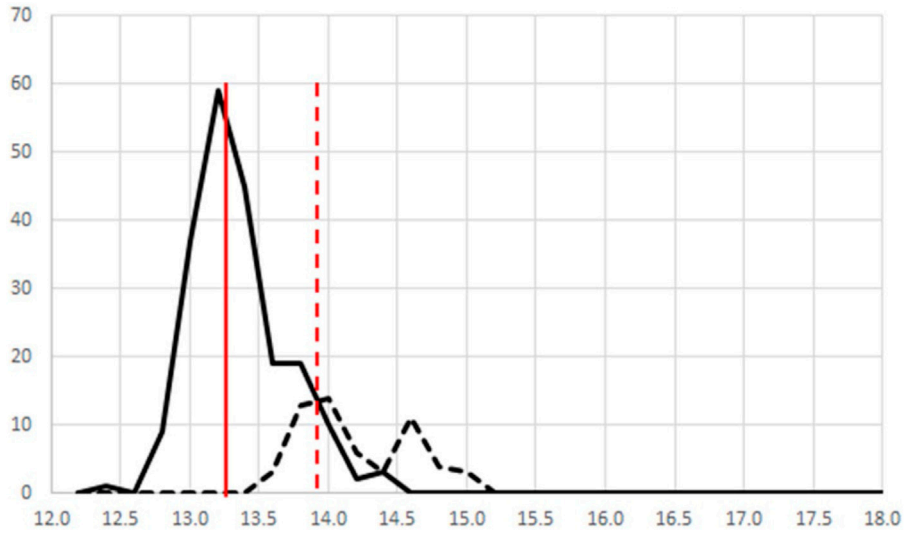



FIGURE 10
A side view of Figure 6 showing the frequency plot for (—) all 4 *A. racemosa* reference samples and (----) other *Actaea* species. The solid vertical line presents the mean for the *A. racemosa* reference samples and the dashed vertical line presents the +2s limit.

China and acquired from American Herbal Pharmacopoeia and commercial sources. Samples were analyzed by flow injection mass spectrometry (FIMS) which yielded spectra with more than 1,000 ions.

The SSTM and SITM (Table 1) for the POI analysis were chosen as 98% and 90% PQ, respectively. Spectra for the test materials were synthesized mathematically using appropriate ratios of the 100% PQ and 100% PG spectra. In all, 344 (43 PQ × 8 PG) spectra were generated for each test material. The unsupervised PCA score plot (not shown) showed visual separation but the computed confidence limits did not allow detailed statistical analysis. Ironically, one-class modeling was used to provide detailed statistical analysis of the data although it was not the focus of the paper. The Q statistic served as the combined metric (Figure 4).

Figure 4A shows the Q statistic plot for 100% PQ, 100% PG, and 98% PQ with confidence limits based on one-class modeling for both 100% PQ and 98% PQ. The one-class model for 100% PQ has a sensitivity of 95% (42/44) and a specificity of 100%. The one-class model for 98% PQ, the SSTM, has a sensitivity of 98% (342/344) and a specificity of 100%. Figure 4B shows a similar plot with 90% PQ, the SITM. Based on the one-class model for 98% PQ, the 90% PG has a specificity of 99% (341/344).

A follow up study verified the previous numerical dilution model with physical dilution of 100% PQ with 100% PG (Harnly et al., 2013). The more labor intensive nature of the physical dilutions restricted the number of samples. Five samples of 100% PQ (arbitrarily selected from the samples in the previous study) were diluted with two samples of PG at ratios of 95:5, 9:1, 8:2, 6:4, 4:6, and 2:8. Figure 5A shows that unsupervised PCA provided a near linear progression from 100% PQ to 100% PG on the X-axis indicating that PQ concentration was the primary source of variance. Figure 5B shows the influence plot (Q statistic versus Hotelling $T^2$) based on a one-class model of 100% PQ. While the Hotelling T2 residuals failed to discriminate between the different PQ purities, the Q statistic provided excellent separation of the five PQ concentrations. A plot

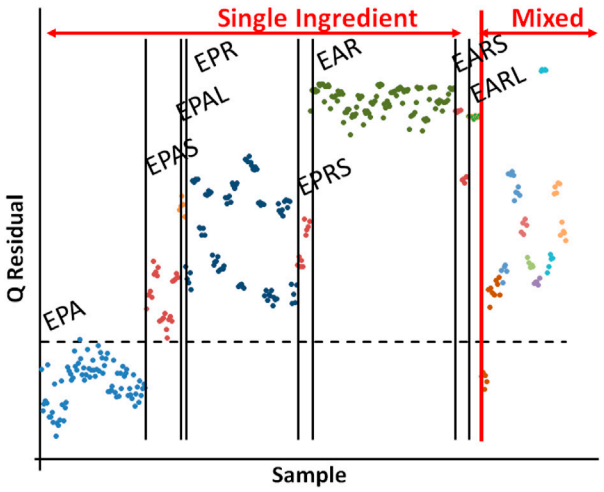



FIGURE 11
A Q statistics plot based on one-class modeling of *Echinacea purpurea* aerial samples (lower left). Labels for single ingredient samples: EPA – *E. purpurea* aerial samples, EPAS – EPA solid supplements, EPAL - EPA liquid supplements, EPR - E. purpurea root samples, EPRS - *E. purpurea* root solid supplements, EAR – *E. angustifolia* root samples, EARS - *E. angustifolia* root solid supplements, and EARL - E. angustifolia root liquid supplements. Mixed supplements (far right) are not individually labeled.

of the square root of the Q statistic showed a linear relationship with the PQ concentration (plot not shown).

An additional follow up study examined the ability of FIMS to discriminate between PQ:PG ratios of 99:1, 98:2, 97:3, and 96:4 (unpublished data) to determine the level of adulteration of PQ that could be detected. Figure 6 shows the influence plots (Q statistic versus Hotelling $T^2$) for the 4 purity levels of PQ. Once again, Q statistic was more useful than Hotelling $T^2$ for discriminating

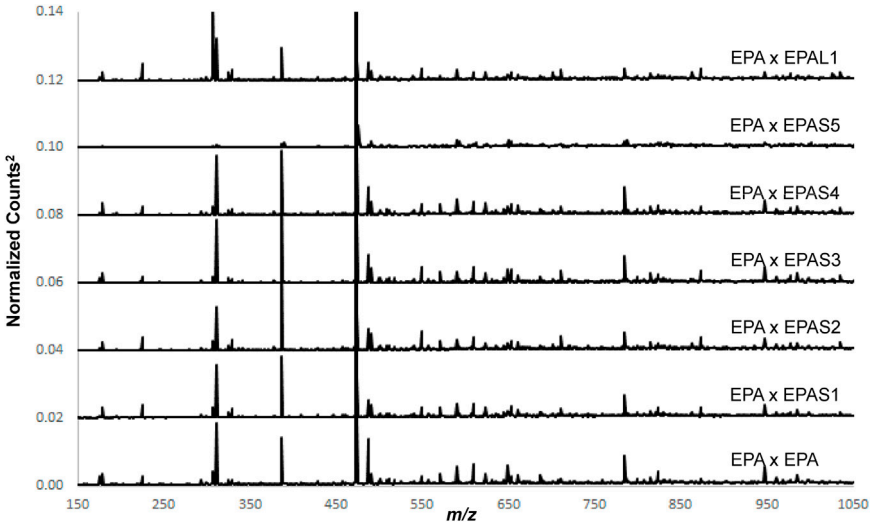



FIGURE 12
Spectra of *E. purpurea aerial* (EPA) cross-correlated with itself, *E. purpurea* aerial solid supplements 1–5 (EPAS1-EPAS5), and *E. purpurea* aerial liquid supplement sample (EPAL1).

between populations. The sensitivity for 100% PQ was 95% and the specificity was 57%, 90%, 99%, and 100% for PG contamination of 1%, 2%, 3%, and 4%, respectively.

This study demonstrates the utility of one-class modeling for detecting adulteration. The Q values for physical and mathematical dilution showed excellent agreement. The digital model, based on the pure spectra (100%) of the authentic material and the adulterant, provides a much simpler means of evaluating the ability of the method to detect adulteration.

The POI studies used the term SIMCA (soft independent modeling of class analogy) instead of the one-class modeling. SIMCA, as explained earlier, consists of a series of one-class models. In all the studies reported in this paper, only a single class was used for modeling, a single PCA model. For the ginseng studies and all those that follow, the 95% confidence limit was chosen as the statistical limit. Other statistical limits can be selected by researchers. The Q and Hotelling $T^2$ statistics were based on only one principal component, thus mitigating the possibility of over fitting. In addition, pre-processing for all the PCA calculations consisted of unit vector normalization of the samples (i.e., the sum of the squares of the sample intensities are set to 1.0) and autoscaling (normalization of each variable by its standard deviation) with mean centering of each variable.

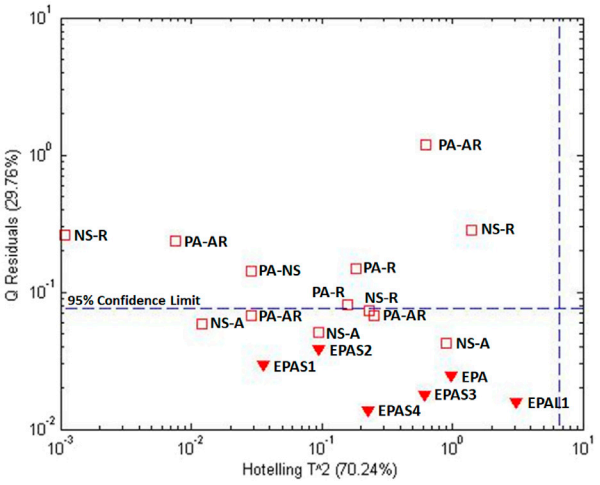



FIGURE 13
Influence plot of mixed supplements spectra cross correlated with the average *E. purpurea* single ingredient spectra based on EPA, EPAS, and EPAL (Figure 12). Labels: EPA – *E. purpurea* aerial, EPAS1-EPAS4 – EPA solid supplements, EPAL - EPA liquid supplement. Mixed labels have two factors. First factor abbreviations are NS - species no specified, P – *E. purpurea*, A- *E. angustifolia*. Second factor abbreviations are NS – plant part not specified, A – aerial, and R – root.

## Black cohosh (*Actaea racemosa*)

One-class modeling was used to distinguish *A. racemosa* L. (Ranunculaceae) from other *Actaea* species and commercially available roots and supplements (Harnly et al., 2016). FIMS and proton nuclear magnetic resonance spectrometry (NMR), two metabolic fingerprinting methods, and DNA sequencing were used to identify and authenticate the *A. racemosa* species. For this study, authentic *A. racemosa* botanical reference materials were acquired from four sources: American Herbal Pharmacopoeia (AHP), Strategic Sourcing (SS), North Carolina Arboretum (NCA), and the National Institutes of Standards and Technology (NIST). The NCA samples were triplicate samples collected from 22 sites across the eastern US.

Initial analyses of the *Actaea* species furnished by AHP using FIMS and NMR gave similar result as shown in Figure 7. PCA score plots (Figures 7A, B) for both methods showed a pattern differentiating *A. racemosa* from the other *Actaea* species. This differentiation was statistically verified by one-class modeling based on *A. racemosa* (Figures 7C, D). DNA sequencing using two independent gene regions (ITS and psbA-trnH) confirmed the metabolic fingerprinting results. Although not the point of this

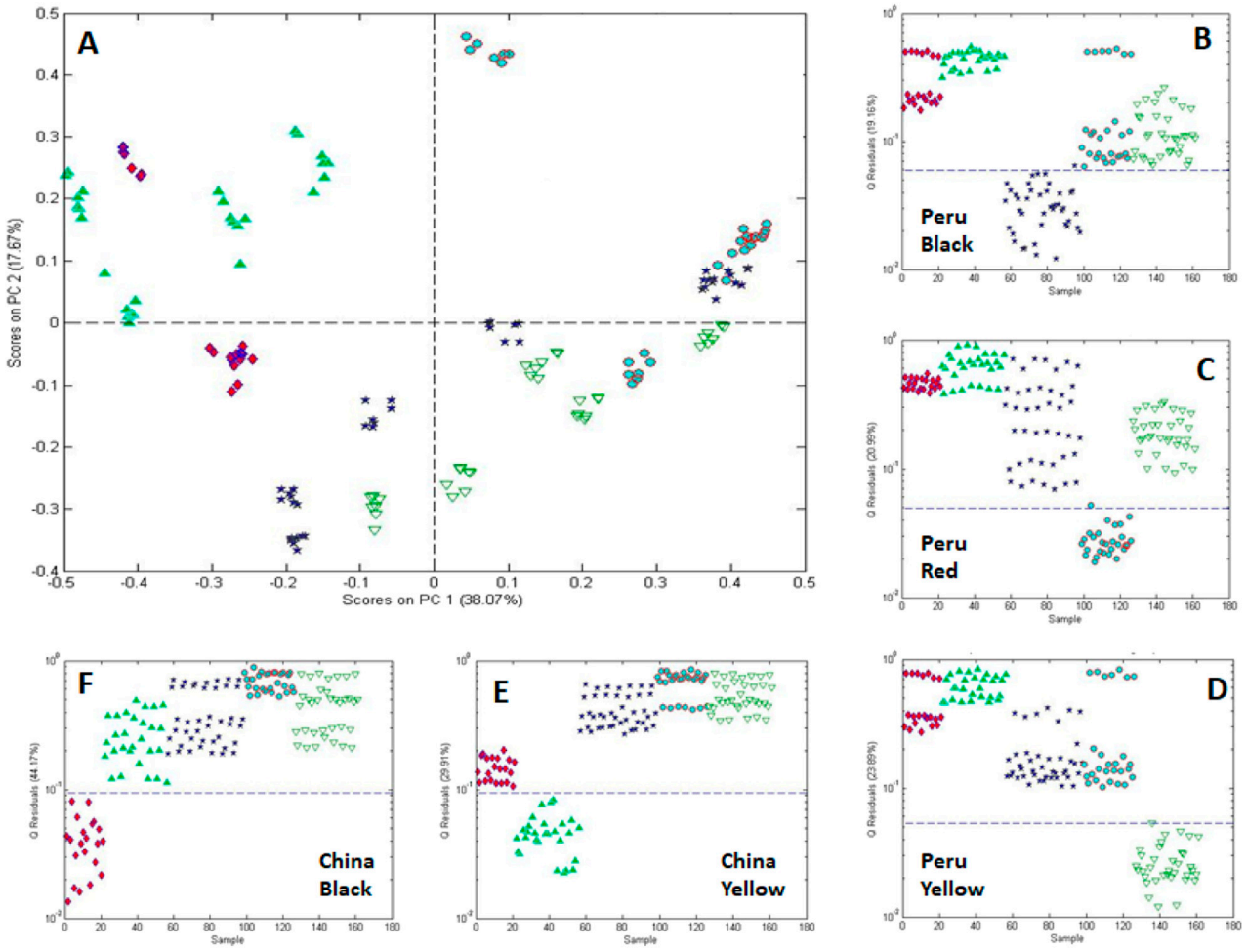



FIGURE 14
One-class modeling of FIMS fingerprints acquired by negative ionization after processing by multivariate ANOVA showing **(A)** the score plot for black, red, and yellow maca acquired from Peru and China surrounded by **(B–F)** single-class models for each of the 5 country-color combinations. Sample icons: ★ Peru black, ● Peru red, ▽ Peru yellow, ▲ China yellow, and ◆ China black.

review, DNA sequencing provided identification for root materials but was inconsistent for the supplements. It was assumed that processing of the supplements destroyed the DNA in some cases.

The simplicity of categorization in Figure 7 was complicated when *A. racemosa* reference samples from the four sources were compared. A PCA score plot (Figure 8A) and a influence plot (combined one-class model plots for AHP and SS reference materials) (Figure 8B) demonstrated that the reference materials were statistically different. The plots show three separate groups with the NIST samples in the same quadrant as those from SS. Sensitivity for the AHP and SS samples was 94% and 91%, respectively, and 91% for the un-modeled NCA samples in the upper right quadrant, exceeding the 95% confidence limits for AHP and SS, i.e., outliers compared to the other reference materials. Interestingly, the NCA samples collected from 22 sites across the US showed a similar lack of uniformity. These data suggested that sample handling and storage, local environmental conditions, and/or genetic drift influenced the plant metabolic profiles. It was also suggested that endophytic bacteria might have a significant influence on the metabolic profile.

A follow-up study took a closer look at the differences between the reference samples and the effect of pre-processing on PCA and one-class modeling patterns (Harnly and Upton, 2024). In Figure 9, samples from all four sources were combined to serve as reference samples and used for one-class modeling. The variance of the combined reference materials exceeded that of each modeled individually, i.e., the model for AHP in Figure 7C. Sensitivity was 97% for the combined *A. racemosa* samples and the specificity was 56% for other *Actaea* species, 55% for commercial *Actaea* roots, and 100% for commercial *Actaea* supplements. These data demonstrated the difficulty of differentiating between the *Actaea* species and that some of the commercial root samples are correctly identified as *A. racemosa*. None of the commercial *Actaea* supplements exhibited metabolic profiles similar to the reference samples, most likely due to the influence of sample preparation.

Figure 10 shows an alternate perspective for one-class modeling data. If the modeled data in Figure 9 are viewed from the side, they

can be displayed as frequency plots. This is a more intuitive view, providing the mean and distribution of the populations. Figure 10 shows only the frequency plots for *A. racemosa* and the other *Actaea* species. Alternate pre-processing methods had little impact on the separation of the two populations. Use of only variables with a low variance provided improved overlap of the *A. racemosa* from the different sources (data not shown) but reduced the ability to discriminate between species (data not shown). Use of variables that enhanced discrimination between species (based on an f-test) also failed to enhance differentiation as the differences between the reference samples from the 4 different sources were also increased, i.e., the frequency plot for *A. racemosa* broadened (data not shown).

This study demonstrated that not all reference materials are created equal, use of multiple sources for reference materials increases the variance of the model, discriminating between species is difficult because the same metabolites are present but in a slightly different ratios, and finally, there is no simple pre-processing for improving discrimination between species.

## Echinacea (*Echinacea purpurea*)

Preparation of commercial supplements from botanical ingredients results in changes in the chemical composition of the supplement. This makes it difficult to authenticate supplements based on the raw ingredients. One approach to comparing the composition of different samples is cross-correlation of the two spectra (Harnly et al., 2017). Ions found in both the reference samples and supplements spectra will provide an enhanced numerical product while those missing from either will yield a product close to zero. Comparison of the cross-correlated spectra can be achieved using PCA and eliminates the need for a normalization factor. As usual, however, one-class modeling provides a clearer analysis with statistical evaluation.

*E. purpurea* (L.) Moench aerial samples were chosen as the test material and cross-correlation and one-class modeling were used to distinguish *E. purpurea* (L.) aerial samples from *E. purpurea* and *Echinacea angustifolia* roots and supplements. Authentic *E. purpurea* aerial and root samples and *E. angustifolia* root samples were obtained from Missouri Botanical Gardens, American Herbal Pharmacopoeia, and commercial sources. Twenty-three liquid and solid (tablets and capsules) supplements were purchased locally; 11 were single-ingredient supplements containing only *E. purpurea* and *E. angustifolia* aerial or root material and 13 were mixed or unknown *Echinacea* supplements labeled to contain mixtures of *E. purpurea* with *E. angustifolia* and/or *Echinacea pallida* (Nutt.) and consisting of either aerial or root material. Samples were analyzed by FIMS.

One-class modeling based on *E. purpurea* aerial samples showed that all other species and plant parts were significantly different from *E. purpurea* aerial (Figure 11). Thus, the compositional profiles of *E. purpurea* aerial could be differentiated from that of the *E. purpurea* roots and *E. angustifolia* roots and from any of the single ingredient or mixed supplements. The sensitivity for the model in Figure 11 was 99% and the specificity was 98%. These data reaffirm previous observations that most raw materials are not suitable for authenticating supplements. The preparation process for commercial samples (e.g., extraction, back extraction, drying, powdering, and possible addition of other materials) can result in significant differences in the chemical composition and their levels. It should be noted that his does not necessarily reflect on the supplements efficacy.

Figure 12 shows the average EPA spectrum cross-correlated with itself (auto-correlation), each of the EPA solid supplements (EPAS) (EPAS1-EPAS5), and EPA liquid supplements (EPAL). Only EPAS5 appears to be significantly different. One-class modeling of the cross-correlated spectra of the average EPA with each of the individual EPA samples (plot not shown) showed that the EPAS and EPAL spectra, with the exception of EPAS5 were the same as the cross-correlated EPA population. These results provide some interesting conclusions. First, since the correlation spectra for EPAS1-EPAS4, and EPAL were similar, the supplements contained similar extracted components. This means that the starting ingredients and the processes used by the manufacturers were similar. Second, since the auto-correlation spectrum of EPA was similar to the correlation spectra of EPA with EPAS1-EPAS4 and EPAL, the reference samples in this study were similar to the raw ingredients used by the manufacturers. Finally, since the extracted compounds were the same, the analytical extraction process used in this study was similar to the preparation method employed by the manufacturers. These data also indicate that supplement EPAS5 was produced using either a different starting ingredient or a different extraction process.

Figure 13 shows a one-class model for mixed supplements based on the cross-correlated spectra for EPA, EPAS1-EPAS4, and EPAL. All mixed supplements that were known to contain aerial plant material fell within the 95% confidence level, even if the species were not specified. All supplements with root material, even if the species were not specified, fell outside the 95% confidence limit. The exceptions falling outside the 95% confidence limit were supplements composed of both species and both plant parts. This suggests that perhaps the aerial portion was of insufficient concentration to provide a clear cross-correlated spectrum.

The data in Figures 12, 13 indicate that cross correlation of supplements with the raw ingredient accurately identifies ions found in both spectra. Ions common to both spectra provide an enhanced multiplicative product whereas ions found in only one spectrum provide a product close to zero. Thus, cross-correlation and one-class modeling can identify commonalities of the raw ingredients and supplements.

## Maca (*Lepidium meyenii* walpers)

Maca data were presented earlier for the general description of one-class modeling. This study was a collaboration with the American Botanical Council; Gaia Herbs; Hong Kong Baptist University; Charles Sturt University & Therapeutic Research, TTD International Pty Ltd; and NSF International Authenticity Laboratory (Geng et al., 2020). There is some question as to whether the plant material is accurately identified as *L. meyenii* Walpers or *Lepidium Peruvianum* Chacon (Meissner et al., 2015). Samples consisted of 39 commercial maca supplements from 11 manufacturers, 31 unprocessed maca roots grown in Peru and China, and an historic non-tuber maca sample from Peru. Samples were analyzed using FIMS and DNA next-generation sequencing (NGS).

Initial, untargeted PCA placed all the maca samples in 3 classes: commercial maca samples, roots grown in Peru, and roots grown in China (Figure 3A). With one-class modeling the commercial Maca

was readily differentiated from the raw root materials (Figures 3C, D). A similar approach, selecting either the Chinese or Peruvian samples for the model, showed that either could be differentiated from the other two classes (data not shown).

One-class modeling was also combined with analysis of variance (ANOVA) to differentiate roots based on country (China and Peru) and color (black, red, and yellow) (Figure 14). ANOVA was used to isolate the mean variance for each experimental factor (country, processing, and color) and cross factor to provide residuals free of the variance of the other factors (Harnly et al., 2014). One-class modeling of the individual factor residuals provided plots in Figures 14B–F. These data show, not surprisingly, that the metabolite composition correlates with country and color.

Metabolite profiling using ultra-high performance liquid chromatography-high resolution mass spectrometry (UHPLC-HRMS) in combination with PCA loadings was used to annotate the compounds responsible for differentiating between country and color. Genetically, all samples were confirmed to be similar and to be L. meyenii Walpers based on NGS at 3 gene regions (ITS2, psbA, and trnL) and comparison to recorded sequences of vouchered standards.

The results of this study show that one-class modeling can be used in combination with ANOVA to determine the statistical significance of differences arising from experimental factors associated with the samples. The metadata associated with genetics, country of origin, year of origin, climate, and handling can have a major impact on a plant's metabolite profile and one-class modeling provides a versatile in deconvoluting the interaction of the factors.

## Conclusion

One-class modeling is a versatile method that can be readily applied to any set of multivariate data (targeted or non-targeted) data. This is a supervised method (reference samples must be identified) that develops a model based on the characteristics of a single class of samples, the reference samples. Using PCA, the Q statistic offers an inherent combined metric which can be used to determine if the reference and test samples belong to the same class, i.e., the test sample is authentic or adulterated. It can also be used as an effective tool for determining the similarity of sample metabolites for different species and, with cross-correlation, the similarity of raw ingredients and supplements. Finally, in combination with ANOVA, one-class modeling can be used to determine the significance of experimental factors. The beauty of one-class modeling is that it can be implemented on any commercial chemometrics platform and is applicable to any data file, i.e., chromatograms, spectra, or database, targeted or non-targeted.

## Author contributions

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

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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.

## Generative AI statement

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

## Publisher's note



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# DNA metabarcoding unveils authenticity and adulteration in commercial Chinese polyherbal preparations: Renshen Jianpi Wan as a critical case study

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**Objectives:** Ensuring quality and authenticity of traditional medicines is crucial, particularly for multi-ingredient formulations like commercial Chinese polyherbal preparations (CCPPs). This study aims to authenticate Renshen Jianpi Wan (RSJPW), a classical CCPP composed of 11 prescribed botanical drugs, using DNA metabarcoding to overcome challenges in species-level identification of processed biological ingredients.

**Methods:** We analyzed 56 commercial RSJPW products from different manufacturers and production batches, alongside eight laboratory-prepared reference samples serving as authentic controls. A dual-marker protocol combining ITS2 and psbA-trnH regions was employed, with optimized DNA extraction and PCR protocols to mitigate degradation issues.

**Results:** Detection rates varied across samples, with the highest detection being 10 out of 11 prescribed ingredients in a single sample. The key fungal ingredient *Poria cocos* (茯苓) was consistently undetectable, likely due to DNA degradation during processing and challenges in extracting fungal DNA from complex matrices. Multiple high-abundance non-prescribed species from Fabaceae, Apiaceae, Brassicaceae, and other families were frequently detected as potential contaminants.

**Conclusions:** This study establishes a systematic framework for molecular authentication of complex herbal formulations, providing technical support for reliable identification of botanical drugs. While DNA metabarcoding offers valuable insights into CCPP composition, authentication of heavily processed ingredients remains a significant technical limitation. The integration with complementary analytical methods such as metabolomics could provide more

comprehensive quality assessment in future studies, demonstrating the necessity of multi-analytical approaches in ensuring the authenticity of traditional medicine.

KEYWORDS

commercial Chinese polyherbal preparations, DNA metabarcoding, ITS2, PSBA-TRNH, quality control

# 1 Introduction

Traditional herbal medicine has been a cornerstone of global healthcare systems for millennia, offering natural therapeutic approaches deeply rooted in diverse cultural and theoretical frameworks. The COVID-19 pandemic has renewed global interest in these remedies, particularly for their potential roles in symptom management and immune system modulation, highlighting their adaptability and accessibility during public health crises (Lyu et al., 2021). This resurgence of interest coincides with the substantial growth of traditional Chinese medicine (TCM) market, which was valued at $231.3 billion in 2023 and is projected to reach $420.7 billion by 2032, with a compound annual growth rate (CAGR) of 6.87% (Business Research Insights, 2024). This expansion is driven by increasing consumer preference for natural healthcare products, greater awareness of alternative medicine, and a broader shift toward preventive health strategies (Emergen Research, 2024). Despite this market growth, significant challenges persist regarding the safety, efficacy, and authentication of traditional herbal products. These concerns are particularly acute in regions where herbal medicines serve as primary healthcare options, such as China, Bangladesh, India, Vietnam, and South Africa (Zhang B. et al., 2022). The lack of standardized regulation and comprehensive scientific validation of potential adverse effects continues to impede their global acceptance and integration into modern healthcare systems (You et al., 2022).

China, as the largest producer and consumer of herbal medicines, faces particular challenges in the authentication of Commercial Chinese Polyherbal Preparations (CCPPs) (Xia et al., 2022)—standardized pharmaceutical preparations derived from traditional herbal prescriptions. The accurate authentication of medicinal material sources is particularly critical for quality control, as the therapeutic efficacy of CCPPs directly depends on using the correct plant species in their preparation. Recent incidents have highlighted critical issues in product safety and authenticity. Documented cases include the substitution of *Isotrema manshuriensis* (Kom.) H. Huber with *Akebia quinata* (Houtt.) Decne. in Longdan Xiegan Wan (Xin et al., 2018a), or the detection of undeclared toxic aconite in Bisset (1981). Additionally, products such as Simotang (Yi et al., 2012) have raised public health concerns due to the presence of potentially carcinogenic substances like betel nut. These incidents not only jeopardize consumer trust but also reveal systemic vulnerabilities in authentication methods.

Traditional authentication methods, including microscopic identification (Zhao et al., 2005) and thin-layer chromatography (TLC) (Zhang et al., 2018), have long been employed in the quality control of botanical drugs. However, these methods face significant limitations in dealing with the complexity of CCPPs, which often involve processed and multi-ingredient formulations. As herbal formulations become more sophisticated and diverse, there is a growing need for more advanced approaches to ensure product quality and regulatory compliance. DNA metabarcoding, introduced by Taberlet et al. (2012) in 2012, has emerged as a transformative tool for species identification by enabling the simultaneous detection of multiple species within complex samples. This method uses genetic markers to amplify specific regions of DNA, allowing for the comprehensive analysis of species composition in herbal preparations. Coghlan et al. (2012) utilized high-throughput sequencing to analyze herbal preparations and identified a wide range of plant and animal species, including toxic and endangered species. Since then, DNA metabarcoding has been increasingly adopted for biodiversity biomonitoring and environmental assessments (Miya, 2022; Pawlowski et al., 2022), and its application in CCPP authentication has gained increasing recognition for its accuracy and efficiency (Arulandhu et al., 2017; Gao et al., 2019; Liu et al., 2019; Seethapathy et al., 2019; Yu et al., 2021; Shah et al., 2023).

This study focuses on Renshen Jianpi Wan (RSJPW), a representative traditional CCPP with extensive clinical application. The formula, first recorded in *Zhengzhi Zhunsheng Leifang* by Kentang Wang during Ming Dynasty, includes 11 botanical drugs with distinct therapeutic roles. These botanical drugs, including Ginseng Radix et Rhizoma (Renshen), Atractylodis Macrocephalae Rhizoma (Baizhu), Poria (Fuling), and Dioscoreae Rhizoma (Shanyao) *etc.*, are commonly used to strengthen the spleen and stomach, promote digestion, regulate qi, and alleviate various gastrointestinal disorders (Zu et al., 2023). Its widespread use is evidenced by its current market presence. According to the National Medical Products Administration database, 141 pharmaceutical companies hold production licenses for RSJPW, with over 40 manufacturers actively selling their products. However, quality concerns have emerged alongside its increasing market demand. For instance, the National Medical Products Administration reported quality deficiencies in five batches of RSJPW from three manufacturers in 2010 (The Central People's Government of the People's Republic of China, 2010). Current quality control methods, as specified in the Chinese Pharmacopoeia Committee (2020), only provide authentication protocols for 6 out of the 11 prescribed botanical drugs and rely primarily on operator-dependent techniques. Moreover, these methods cannot effectively detect unauthorized substitutions or adulterations. The complex manufacturing process of RSJPW, which involves pulverization, drying, and high-temperature processing, poses additional challenges for quality control by potentially degrading DNA and other chemical markers. These factors make RSJPW an ideal candidate for exploring advanced authentication approaches.

In this study, we developed and validated a DNA metabarcoding-based method for systematic authentication of the

TABLE 1 Composition and Botanical Sources of prescribed ingredients in RSJPW (Chinese Pharmacopoeia, 2020).

| Herbal ingredients | Dosage (g) | Ratio (%) | Medicinal part | Botanical source | Family |
|---|---|---|---|---|---|
| Ginseng Radix et Rhizoma (Renshen) | 25 | 4 | Root and Rhizome | *Panax ginseng* C.A. Mey | Araliaceae |
| Atractylodis Macrocephalae Rhizoma (Baizhu) | 150 | 24 | Rhizome | *Atractylodes macrocephala* Koidz | Asteraceae |
| Poria (Fuling) | 50 | 8 | Sclerotium | *Poria cocos* (Schw.) Wolf | Polyporaceae |
| Dioscoreae Rhizoma (Shanyao) | 100 | 16 | Rhizome | *Dioscorea polystachya* Thunb | Dioscoreaceae |
| Citri Reticulatae Pericarpium (Chenpi) | 50 | 8 | Fruit | *Citrus reticulata* Blanco | Rutaceae |
| Aucklandiae Radix (Muxiang) | 12.5 | 2 | Root | *Aucklandia costus* Decne | Asteraceae |
| Amomi Fructus (Sharen) | 25 | 4 | Fruit | *Amomum villosum* Lour | Zingiberaceae |
| | | | | *A. villosum* var. *Xanthioides* T.L. Wu et Senjen | |
| | | | | *A. longiligulare* T.L. Wu | |
| Astragali Radix (Huangqi) | 100 | 16 | Root | *Astragalus membranaceus* var. *Mongholicus* (Bye.) Hsiao | Fabaceae |
| | | | | *A. membranaceus* (Fisch.) Bge | |
| Angelicae Sinensis Radix (Danggui) | 50 | 8 | Root | *Angelica sinensis* (Oliv.) Diels | Apiaceae |
| Ziziphi Spinosae Semen (Suanzaoren) | 50 | 8 | Seed | *Ziziphus jujuba* var. *Spinosa* (Bunge) Hu ex.H.F. Chou | Rhamnaceae |
| Polygalae Radix (Yuanzhi) | 25 | 4 | Root | *Polygala tenuifolia* Willd | Polygalaceae |
| | | | | *P. sibirica* L. | |
| **Panacis Quinquefolii Radix (Xiyangshen)** | 12.5 | 2 | Root | *Panax quinquefolius* L. | Araliaceae |

biological composition of RSJPW. By analyzing both reference materials and commercial samples, we aimed to establish a reliable authentication strategy that addresses the technical challenges in authenticating complex herbal formulations. Our method was specifically designed to detect both legitimate botanical drugs and potential adulterants, with particular attention to discriminating between *Panax ginseng* C. A. Mey. and its common adulterant (Chen et al., 2013), *Panax quinquefolius* L. This research provides not only a practical tool for RSJPW species authentication but also demonstrates a systematic approach to DNA-based authentication of CCPPs.

# 2 Materials and methods

## 2.1 Materials

### 2.1.1 Collection and identification of raw materials

The RSJPW formula comprises 11 medicinal ingredients (Table 1): Ginseng Radix et Rhizoma (Renshen), Atractylodis Macrocephalae Rhizoma (Baizhu, stir-fried with wheat bran), Poria (Fuling), Dioscoreae Rhizoma (Shanyao), Citri Reticulatae Pericarpium (Chenpi), Aucklandiae Radix (Muxiang), Amomi Fructus (Sharen), Astragali Radix (Huangqi, honey-processed), Angelicae Sinensis Radix (Danggui), Ziziphi Spinosae Semen (Suanzaoren, stir-fried), and Polygalae Radix (Yuanzhi, processed). Raw materials were collected from authorized traditional Chinese medicine pharmacies and certified online pharmaceutical platforms during 2022–2023. Additionally, Panacis Quinquefolii Radix (*P. quinquefolius*, Xiyangshen), a common adulterant of Ginseng Radix et Rhizoma, was included as a positive control. All materials were stored in airtight bags at room temperature (20–25°C) with relative humidity maintained below 60% until analysis.

The morphological identification of botanical drugs was performed by Dr. Jing Zhou according to the macroscopical identification criteria specified in the Chinese Pharmacopoeia (2020), including examination of characteristic features such as shape, size, color, surface, texture, fracture (cross-sectional appearance), odor, and other distinctive physical properties of the medicinal parts. For molecular identification, DNA was extracted from each botanical drug separately. The internal transcribed spacer 2 (ITS2) and *psb*A-*trn*H intergenic spacer regions were amplified following the guidelines in the Chinese Pharmacopoeia Committee (2020). However, as Poria (Fuling), a fungal medicinal material, cannot be amplified using the ITS2 and *psb*A-*trn*H primers specified in the Pharmacopoeia, we used ITS primers ITS1 (5′-TCCGTAGGTGAA CCTGCGG-3′) and ITS4 (5′-TCCGCTTATTGATATGC-3′) for its identification (Qin et al., 2023). The sequencing data were analyzed using BLAST against the GenBank database (NCBI), with a sequence similarity threshold of ≥99% and query coverage ≥95% for species-level identification. This molecular approach, combined with morphological identification, ensured the accurate identification of the collected raw materials for subsequent quality analysis.

### 2.1.2 Collection of commercial samples

A total of 56 commercial RSJPW samples from 12 manufacturers were collected in 2022–2023. Each manufacturer contributed 2 to 6 batches of samples, which were purchased from major online pharmaceutical platforms and licensed brick-and-mortar pharmacies across China (Supplementary Table S1). All samples were within their shelf life and stored according to the manufacturer's instructions until analysis.

### 2.1.3 Preparation of reference materials

Two sets of reference materials were prepared to validate the detection method. The authenticated raw materials (including *P. quinquefolius*) were individually ground and sieved. For the standard formula reference set (RF05–RF08), the 11 medicinal ingredients were weighed and mixed according to the proportions specified in the Chinese Pharmacopoeia (2020) (Table 1). The mixture was then processed into pills (10 g each) according to standard procedures. For the positive control set (RF01–RF04), *P. quinquefolius* powder was incorporated at 1.96% w/w (equivalent to the proportion of Aucklandiae Radix) into the standard formula before pill formation.

## 2.2 Development of DNA metabarcoding method for authentication

### 2.2.1 DNA extraction

After preliminary comparison of two extraction methods (CTAB and commercial kit), the plant genomic DNA extraction kit (Beijing Biomed Gene Technologies Co., Ltd.) was selected and optimized for CCPPs. The protocol was optimized through the following modifications: (1) cleaning samples with 75% ethanol; (2) adding Tris-HCl buffer (pH 8.0) during grinding; and (3) extending the water bath duration. To maximize DNA recovery, extracts were processed in triplicate with subsequent combination during AC column adsorption.

DNA quality and concentration were assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, United States).

### 2.2.2 PCR amplification

ITS2 and *psb*A-*trn*H regions were selected as complementary markers following recommendations from the Consortium for the Barcode of Life (CBOL) and previous studies demonstrating their effectiveness in botanical drug authentication (Kress and Erickson, 2007; Chen et al., 2010; Yao et al., 2022). ITS2 provides superior species-level resolution for medicinal plants, and *psb*A-*trn*H offers robust discriminatory power even in processed materials. This dual-marker approach has been validated in multiple studies as particularly effective for traditional medicine authentication, providing complementary identification power when analyzing complex botanical formulations (Arulandhu et al., 2017). Universal primers were modified with sample-specific 6-bp tags at their 5′ ends (Supplementary Tables S1, S2). PCR was performed using TransStart Fastpfu DNA Polymerase (TransGen AP221-02) in a 20 μL reaction system: 10 μL of 2× Pro Taq buffer, 0.8 μL of forward primer (5 μM), 0.8 μL of reverse primer (5 μM), 4 μL of template DNA (10 ng/μL), and dd$H_2O$ added to a final volume of 20 μL. Specific amplification conditions for each marker are detailed in Supplementary Table S2.

### 2.2.3 Library construction and sequencing

The PCR products were visualized on 2% agarose gel electrophoresis and purified using the AxyPrep DNA Gel Extraction Kit (AXYGEN). DNA quantification was performed using the QuantiFluor™-ST Blue Fluorescence Quantification System (Promega). Libraries were constructed using the TruSeq™ DNA Sample Prep Kit according to the manufacturer's protocol. Paired-end 300 bp sequencing was performed on the Illumina MiSeq platform by Majorbio Bio-pharm Technology Co., Ltd. (Shanghai, China). The raw sequence data has been submitted to NCBI wth the accession number of PRJNA1242227.

### 2.2.4 Bioinformatic analysis

Raw sequence data processing and analysis were performed using QIIME two software v. 2021.2 (Hall and Beiko, 2018). After quality assessment using q2-demux, primers and sample-specific tags were removed with q2-cutadapt. Quality filtering parameters were set as follows: minimum quality score of 25, trimming of first 10 bases, and maximum expected errors of 2.0. The filtered sequences were processed using q2-dada2 for denoising, paired-end read merging and chimera removal. Feature tables and representative sequences were generated, and their statistics were analyzed using the feature-table summarize command. To minimize potential sequencing artifacts and improve data reliability, Amplicon Sequence Variants (ASVs) with fewer than 10 reads per sample were filtered out, a threshold determined based on the complexity of botanical drug matrices and sequencing depth.

For accurate species identification, each representative sequence was manually compared against the NCBI GenBank database using BLAST, with stringent criteria optimized for RSJPW authentication (sequence similarity ≥98% and query coverage ≥95%). To ensure specificity in the context of traditional Chinese medicine, only plant species documented in Chinese pharmacopoeia were considered as valid matches to avoid false positives from closely related species. For diversity analysis, alpha diversity (Shannon index) was calculated using the q2-diversity plugin to evaluate species richness and evenness across samples. Beta diversity analysis was performed using Bray-Curtis distances, and the results were visualized through Principal Coordinates Analysis (PCoA) to examine compositional differences between samples. The analysis was optimized to address the unique challenges of RSJPW, focusing on comparing species composition and relative abundances across different commercial samples to evaluate formula consistency and detect potential adulterations in this complex preparation.

### 2.2.5 Method validation

The reproducibility and reliability of the established workflow was validated through parallel reference samples. Two sets of quadruplicate reference samples were prepared: RF01–RF04 (spiked with *P. quinquefolius*) and RF05–RF08 (without *P. quinquefolius*) serving as positive controls and quality control materials, respectively. The validation protocol included: (1) Technical reproducibility through consistency analysis of species detection across quadruplicate samples; (2) Method specificity by comparing species profiles between samples with and without *P.*

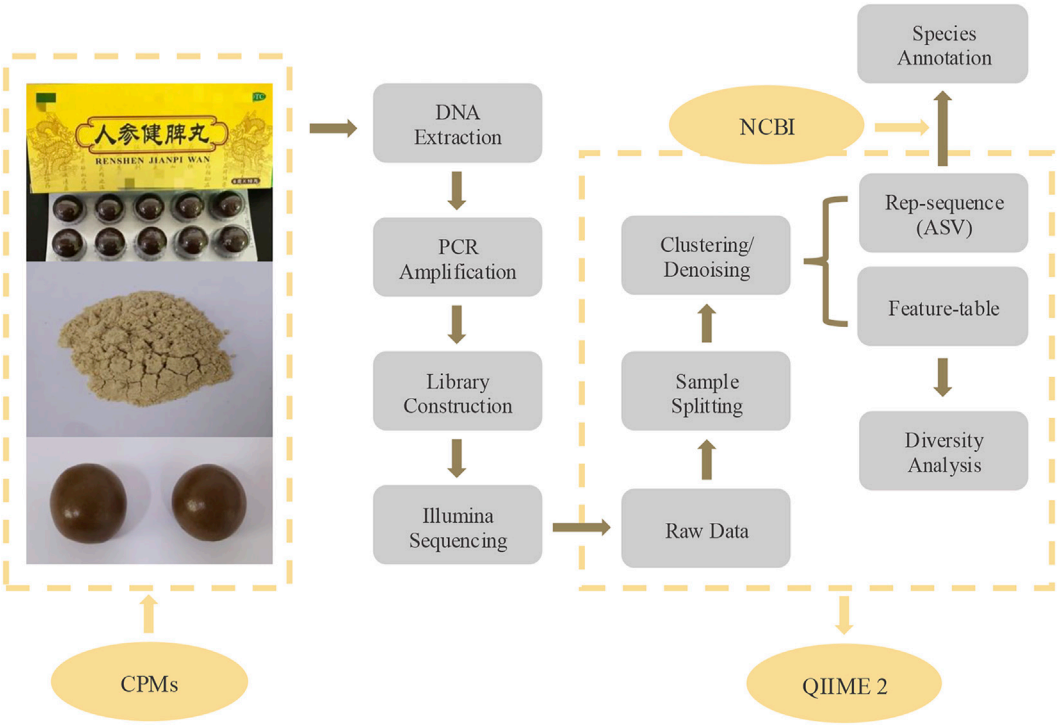


FIGURE 1
Workflow for biological ingredient monitoring of RSJPW using DNA metabarcoding.

*quinquefolius*; (3) Cross-validation of species detection between ITS2 and *psb*A-*trn*H markers. Sixteen unique 6-bp tags were designed and appended to the 5′ end of the universal primers for both ITS2 and *psb*A-*trn*H sequences (Supplementary Table S1) to distinguish PCR amplicons from different samples.

# 3 Results

## 3.1 Authentication of RSJPW raw materials

All 11 medicinal ingredients used in the RSJPW formula, along with the positive control *P. quinquefolius*, were verified through both morphological and molecular approaches. Morphological characteristics of all materials matched their corresponding authentic descriptions in Chinese Pharmacopoeia (2020). For molecular authentication, ITS2 and *psb*A-*trn*H sequences showed ≥99% similarity to their corresponding authentic species in reference databases, except for the fungal material *Poria cocos* (Schw.) Wolf (Poria) which was authenticated using ITS region due to its taxonomic classification. All raw materials were authenticated as genuine species documented in Chinese Pharmacopoeia (2020).

## 3.2 Development and validation of DNA metabarcoding workflow for botanical ingredient authentication

A stepwise DNA metabarcoding workflow was established to authenticate and profile the biological ingredients of RSJPW (Figure 1), which comprises four main steps: sample processing, molecular amplification, high-throughput sequencing, and bioinformatic species annotation.

The sequencing quality assessment showed robust data generation, with a total of 291,339 and 439,257 reads obtained for ITS2 and *psb*A-*trn*H regions, respectively, all of which achieved a mean Q30 quality score exceeding 95% (Table 2). The Shannon rarefaction curves (Figures 2A, B) reached clear plateaus, indicating sufficient sequencing coverage to capture species diversity within RSJPW samples.

The workflow validation was conducted using eight laboratory-prepared RSJPW reference samples, including four spiked with *P. quinquefolius* (RF01–RF04) and four standard formula samples (RF05–RF08). After quality filtering, ITS2 and *psb*A-*trn*H regions yielded 66 and 59 ASVs, respectively (Table 2; Supplementary Table S3). ITS2 marker detected seven prescribed ingredients with relatively consistent detection across replicates, including *P. ginseng* C. A. Mey., *Aucklandia costus* Decne., *Amomum villosum* Lour., *Astragalus membranaceus* (Fisch.) Bge., *Angelica sinensis* (Oliv.) Diels, *Z. jujuba* var. *Spinosa* (Bunge) Hu ex.H.F. Chou, and *Polygala tenuifolia* Willd. In the cross-validation analysis using *psb*A-*trn*H marker, *P. ginseng* and *Ziziphus jujuba* var. *Spinosa* were consistently detected, while *Dioscorea polystachya* Thunb. Was uniquely identified by this marker, demonstrating complementary detection capabilities of the two markers. The positive control *P. quinquefolius* was specifically identified in spiked samples (RF01–RF04) while absent in standard formula samples (RF05–RF08), demonstrating the workflow's sensitivity and its ability to identify formula adulteration. However, despite their successful authentication in raw materials, *Citrus reticulata*

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TABLE 2 Overview of sequencing metrics for reference and commercial samples.

| Parameters | Marker | Amplicon size (bp) | Sequencing length (bp) | Raw reads | Total bases | Average Q30 score (%) | Clean reads | Final reads | Recovery rate (%) | Initial ASVs | Final ASVs |
|---|---|---|---|---|---|---|---|---|---|---|---|
| References samples | ITS2 | 500 | 300 | 291,339 | 131,490,894 | 95.73 | 216,384 | 216,008 | 74.27 | 146 | 66 |
| | *psb*A-*trn*H | 500 | 300 | 439,257 | 160,418,602 | 97.43 | 370,110 | 369,815 | 84.26 | 116 | 59 |
| Commercial samples | ITS2 | 500 | 300 | 1,764,844 | 786,454,412 | 94.76 | 1,265,805 | 1,263,271 | 71.72 | 1,477 | 941 |
| | *psb*A-*trn*H | 500 | 300 | 1,642,373 | 588,937,401 | 96.71 | 1,482,386 | 1,480,963 | 90.26 | 1,125 | 842 |

Note: Clean reads represent the remaining reads after Qiime two software quality control; Final reads represent the number of remaining reads after ASVs, with fewer than 10 reads are removed.

Blanco, *P. cocos*, and *Atractylodes macrocephala* Koidz. Were not detected in the prepared formula samples by either marker (Table 3; Figure 3).

## 3.3 Analysis of commercial RSJPW samples

### 3.3.1 Sequencing results and prescribed ingredients detection

High-throughput sequencing of 56 commercial RSJPW samples using ITS2 and *psb*A-*trn*H markers yielded high-quality data (Q30 > 94%, Table 2). Shannon rarefaction curves also plateaued for all samples, indicating sufficient sequencing depth for species diversity assessment (Figures 2C, D). Post-quality filtering, ITS2 sequencing generated 941 ASVs across commercial samples (13–175 ASVs per sample), while *psb*A-*trn*H produced 842 ASVs (3–151 ASVs per sample) (Figure 4; Supplementary Tables S4, S5). These values considerably exceeded those from reference samples (66 and 59 ASVs for ITS2 and *psb*A-*trn*H, respectively), indicating enhanced biological complexity in commercial formulations.

The two markers exhibited complementary detection patterns for prescribed ingredients. ITS2 demonstrated complete detection (100%) for *Z. jujuba* var. *spinosa*, with high detection rates for *P. ginseng* and *A. membranaceus* (94.64%), as well as *A. sinensis* (91.07%). However, ITS2 failed to detect *P. cocos* and *D. polystachya* across all samples. For *psb*A-*trn*H analysis, *A. sinensis* showed complete detection (100%), followed by *Z. jujuba* var. *spinosa* (92.86%). Notably, *psb*A-*trn*H uniquely identified *D. polystachya* (78.57%) but showed limited detection of *P. tenuifolia* (3.57%). Five prescribed ingredients (*A. macrocephala*, *P. cocos*, *C. reticulata*, *A. costus*, and *A. villosum*) remained undetected by *psb*A-*trn*H. When combining both markers, 10 out of 11 prescribed ingredients were successfully detected in the commercial samples, with detection frequencies varying from 3.57% to 100%. Thus, the combined use of both markers enabled the detection of a broader range of species, with each marker contributing unique detection capabilities.

The relative abundance of detected species, inferred from sequencing reads, showed substantial variation among samples (Supplementary Tables S6, S7), suggesting potential differences in ingredient proportions across manufacturers. Due to the variable detection patterns across the 56 batches and incomplete detection of all prescribed ingredients, direct correlation analysis between read abundance and ingredient proportions in the original RSJPW formula was not feasible.

### 3.3.2 Non-prescribed species identification

Metabarcoding analysis using dual markers (ITS2 and *psb*A-*trn*H) revealed substantial non-prescribed species contamination in commercial RSJPW samples. These species can be classified into three categories: non-formula medicinal plants, food crops, and wild plants.

A total of 120 non-prescribed species (Tables 4, 5; Supplementary Table S8) were identified using the ITS2 region, representing 43 different families. Species from the Leguminosae and Apiaceae families were the most prevalent. *Verbena officinalis* L. had the highest relative abundance with 23,911 reads across 36 ASVs, and was detected in 25% of samples. Other frequently

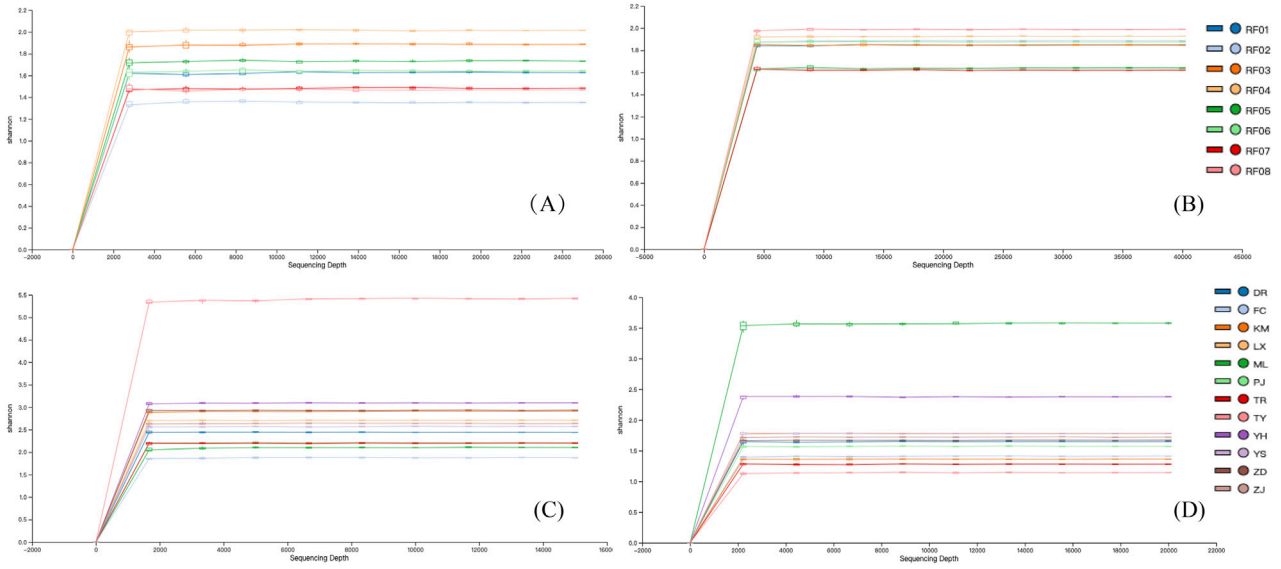


FIGURE 2
Rarefaction curves based on Shannon index. **(A)** ITS2 sequences from reference samples; **(B)** *psb*A-*trn*H sequences from reference sanples; **(C)** ITS2 sequences from commercial samples; **(D)** *psb*A-*trn*H sequences from commercial samples.

detected species included *Alnus nepalensis* D. Don (44.64%), *Cucurbita moschata* Duchesne (39.29%), and *Triticum aestivum* L. (42.86%). Moreover, we also identified closely related species or potential substitutes of the prescribed ingredients, such as *Hedysarum polybotrys* Hand.-Mazz., a substitute for *A. membranaceus*, and several confusable varieties of *P. ginseng*, including *P. quinquefolius*, *P. japonicus* (T. Nees) C. A. Meyer and *Codonopsis pilosula* (Franch.) Nannf., *etc*. Beyond plant species, two fungi- *Bacillus altitudinis* and *Dimargaris bacillispora*, were also detected by the ITS2 sequence, both exclusively in samples from the manufacturer TY.

Parallel analysis using the *psb*A-*trn*H sequence verified the findings of ITS2 in terms of species composition patterns and revealed an additional 55 non-prescribed species (Supplementary Table S9) from 26 different families. Species from the Leguminosae and Salicaceae families were predominant. *Arachis hypogaea* L. had the highest relative abundance, with a total of 62,146 reads across 9 ASVs, and a detection frequency of 64.29%. In addition, *Salix alba* L. (48.21%), *Polygonum multiflorum* Thunb. (16.07%), and *Ziziphus mauritiana* Lam., a known adulterant of *Z. jujuba* var. *spinosa*, (16.07%) were also frequently detected. Notably, *Paeonia rockii* (S.G. Haw and Lauener) T. Hong and J.J. Li, a first-class protected plant in China, was detected in 10.71% of the samples, with a total read count of 3,135.

### 3.3.3 Batch-to-batch and manufacturer variation

Metabarcoding analysis successfully detected all prescribed species except *P. cocos*. However, distinct inter-manufacturer and inter-batch variations in both ASV abundance and species detection profiles were revealed (Supplementary Tables S4, S5).

ITS2 sequencing demonstrated marked manufacturer-specific variation in ASV diversity. *Ziziphus jujuba* var. *spinosa* exhibited the most substantial variation, with manufacturer TY samples yielding 52–82 ASVs compared to <12 ASVs from other manufacturers. *Amomum villosum* displayed moderate variation (1–18 ASVs). In contrast, core prescribed species, including *P. ginseng*, *A. macrocephala*, *C. reticulata*, *A. costus*, *A. membranaceus*, *A. sinensis*, and *P. tenuifolia*, maintained consistent ASV profiles (1–4 ASVs) across all manufacturers. The *psb*A-*trn*H marker analysis corroborated these variation patterns, with notably high ASV counts in *Z. jujuba* var. *spinosa* samples from manufacturer ML (77–94 ASVs). Additionally, *D. polystachya* and *A. sinensis* showed elevated diversity in specific manufacturers, while the remaining prescribed species maintained relatively stable profiles across all sources.

Species detection rates exhibited substantial heterogeneity across manufacturers and batches. In ITS2 analysis, manufacturers showed varying levels of consistency in species detection: YH and TR demonstrated reliable performance by consistently detecting 6 out of 11 prescribed species across all batches, while other manufacturers displayed more variable detection patterns. Detection capability ranged from high-performing batches, such as KM02 which detected up to 9 species, to notably poor performance in some cases, with certain TY batches detecting only a single species in *psb*A-*trn*H analysis. This heterogeneity in detection rates is consistent with the observed variations in ASV profiles and is further supported by PCoA analysis (Figure 5), which revealed manufacturer-specific clustering patterns ranging from highly cohesive (as seen in TY samples) to broadly dispersed distributions, suggesting varying levels of standardization and quality control among manufacturers.

## 4 Discussion

### 4.1 DNA metabarcoding for botanical drug authentication in CCPP: advantages and challenges

Commercial Chinese polyherbal preparations (CCPPs) are typically composed of multiple biological drugs with complex

TABLE 3 Detection of prescribed ingredients in RSJPW reference samples using ITS2 and *psb*A-*trn*H markers.

| Herbal ingredients | ITS2 | | | | | | | | *psb*A-*trn*H | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | RF01 | RF02 | RF03 | RF04 | RF05 | RF06 | RF07 | RF08 | RF01 | RF02 | RF03 | RF04 | RF05 | RF06 | RF07 | RF08 |
| Ginseng Radix et Rhizoma (Renshen) | √ | √ | √ | √ | √ | √ | √ | √ | √ | √ | √ | √ | √ | √ | √ | √ |
| Atractylodis Macrocephalae Rhizoma (Baizhu) | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| Poria (Fuling) | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| Dioscoreae Rhizoma (Shanyao) | -- | -- | -- | -- | -- | -- | -- | -- | √ | √ | √ | √ | √ | √ | √ | √ |
| Citri Reticulatae Pericarpium (Chenpi) | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| Aucklandiae Radix (Muxiang) | √ | √ | √ | √ | √ | √ | √ | √ | -- | √ | √ | -- | -- | -- | -- | √ |
| Amomi Fructus (Sharen) | √ | √ | √ | √ | √ | √ | √ | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| Astragali Radix (Huangqi) | √ | √ | √ | √ | √ | √ | √ | √ | -- | -- | -- | -- | -- | -- | √ | √ |
| Angelicae Sinensis Radix (Danggui) | √ | √ | √ | √ | √ | √ | √ | √ | -- | -- | -- | -- | -- | -- | -- | -- |
| Ziziphi Spinosae Semen (Suanzaoren) | √ | √ | √ | √ | √ | √ | √ | √ | √ | √ | √ | √ | √ | √ | √ | √ |
| Polygalae Radix (Yuanzhi) | √ | √ | √ | √ | √ | √ | √ | √ | -- | -- | -- | -- | -- | -- | -- | -- |
| **Panacis Quinquefolii Radix (Xiyangshen)** | √ | √ | √ | √ | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |

Note: -: the ingredient was not detected for the sample. Boldface indicates positive control herbal ingredients.





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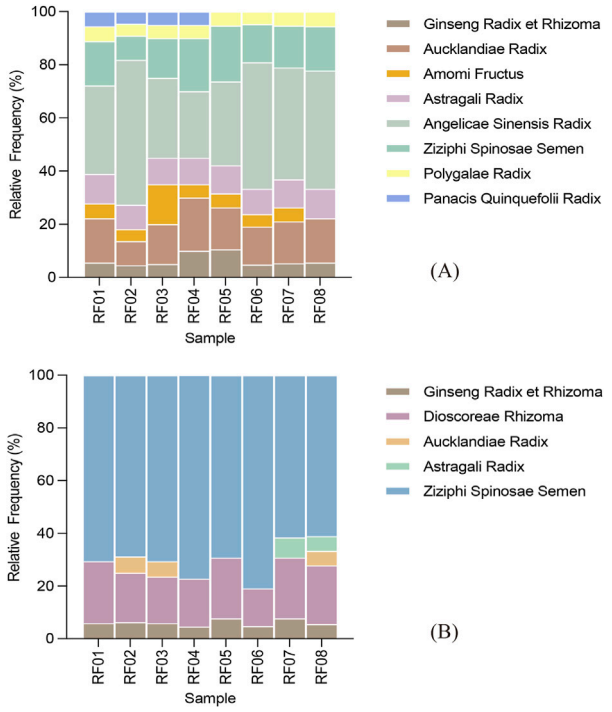


FIGURE 3
ASV abundances for detected species in reference samples. **(A)** ASV abundances through ITS2 analysis; **(B)** ASV abundances through *psb*A-*trn*H analysis.

sources. The frequent inconsistencies between pharmacopoeia-specified materials and their substitutes present unique challenges in quality assessment, which directly impact therapeutic reliability and safety. While current analytical methods, including chromatography and mass spectrometry, are valuable for specific aspects of quality assessment, they lack the capability for simultaneous multi-ingredient authentication (Wang et al., 2021). DNA metabarcoding overcomes this limitation by enabling comprehensive detection of multiple species in complex mixtures (Taberlet et al., 2012; Urumarudappa et al., 2020; Pandit et al., 2021; Raclariu et al., 2021; Travadi et al., 2023). This approach demonstrated high efficiency in our study by successfully identifying 10 out of 11 prescribed ingredients in RSJPW samples. The complementary use of ITS2 and *psb*A-*trn*H markers enhanced detection comprehensiveness through their distinct molecular characteristics (Lv et al., 2020; Zhu et al., 2022). ITS2's broad taxonomic coverage enables wide species identification, while *psb*A-*trn*H's specific amplification efficiency compensates for ITS2's limitations in certain taxa. For instance, while ITS2 successfully identified most botanical materials, *psb*A-*trn*H specifically enabled the detection of *D. polystachya*, likely due to its conserved chloroplast genome regions that remain intact during processing.

The method's enhanced sensitivity revealed both environmental contamination and authentication issues that conventional TLC (Raclariu et al., 2017b) and HPLC-MS (Raclariu et al., 2017a) techniques might overlook. Multiple high-abundance non-prescribed species from Fabaceae, Apiaceae, and Brassicaceae were frequently detected, raising significant quality control concerns. The primary sources may include field contamination during harvesting where non-target plants grow alongside medicinal plants, cross-contamination during processing on shared production lines, possible storage contamination as well as challenges in completely removing environmental DNA from raw materials. The presence of related species suggested potential cross-contamination or deliberate substitution during manufacturing (Liu et al., 2021b). Fungi were detected in the samples of some manufacturers (TY), suggesting improper preservation of botanical drugs in the production process. These contaminants could potentially affect therapeutic efficacy or safety through unexpected biological activities or allergenicity. These findings provide crucial insights into critical control points in the production chain that require strengthened monitoring (Liu et al., 2018). Notably, the identification of a nationally protected Class I species (*P. rockii*) in commercial samples demonstrated the technique's value in conservation monitoring and regulatory compliance, highlighting the need for systematic oversight in raw material sourcing. Our findings thus emphasize the importance of implementing more rigorous quality control measures throughout the supply chain, including stricter source material authentication, improved cleaning procedures, dedicated production lines to prevent cross-contamination, and regular DNA metabarcoding screening as part of quality control protocols.

However, like any analytical method, DNA metabarcoding faces specific technical challenges, particularly regarding DNA integrity and processing effects. The failure to detect *P. cocos* and variable detection rates of *A. macrocephala* and *C. reticulata* revealed distinct DNA degradation patterns related to processing methods and taxonomic differences. *Poria cocos*, being a fungal ingredient, represents a taxonomic limitation of our plant-optimized markers. The ITS2 and psbA-trnH markers selected for this study are plant-specific, with the primers designed to preferentially amplify plant DNA. Fungal ingredients would require different marker regions and primers specifically optimized for fungal DNA, such as the full ITS region (ITS1-5.8S-ITS2) with fungal-specific primers. This highlights the need for multi-marker approaches when analyzing complex formulations containing ingredients from diverse taxonomic origins. For plant ingredients, processing-related DNA degradation presented variable challenges. The high-temperature processing of *A. macrocephala*, which typically involves stir-frying at 180–220°C, likely leads to DNA fragmentation, as thermal treatment is known to cause DNA degradation through denaturation (Karni et al., 2013). Additionally, oxidative metabolites in aged *C. reticulata* peel directly interfere with DNA stability (Li et al., 2024). These processing-specific DNA degradation mechanisms emphasize the importance of considering molecular integrity in quality control protocols (Li et al., 2023).

DNA metabarcoding should be integrated with other analytical approaches for comprehensive quality assessment. Its unique ability to detect both intended and unexpected ingredients makes it valuable for CCPP botanical drug authentication, despite limitations with processed materials. The integration with chemical analysis methods, particularly metabolomics, could

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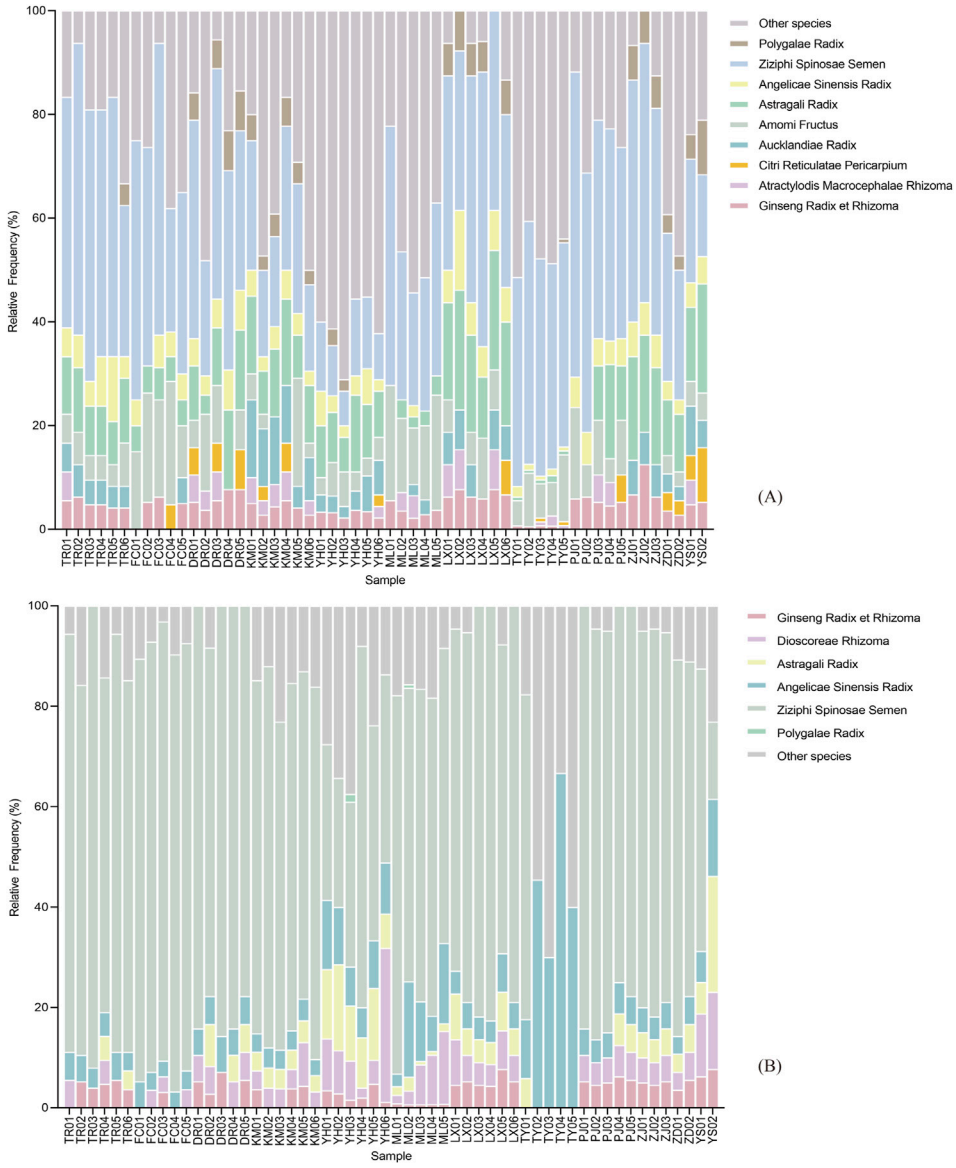


FIGURE 4
ASV Abundances for detected species across commercial RSJPW samples. **(A)** ASV abundances through ITS2 analysis; **(B)** ASV abundances through *psb*A-*trn*H analysis.

provide both qualitative authentication and quantitative composition assessment, offering a more complete quality control solution (Coghlan et al., 2015; Gao et al., 2023). This integrated approach would provide complementary authentication perspectives, i.e., DNA metabarcoding identifies the biological origins, while metabolomics characterizes the bioactive metabolites. By detecting characteristic metabolites specific to each botanical ingredient, metabolomic profiling can confirm the presence of medicinally relevant compounds even when DNA is heavily degraded, enabling authentication at both the species level (DNA) and functional level (metabolites). Combined datasets could also help establish correlations between botanical ingredients and their metabolite profiles, potentially creating more robust authentication frameworks for complex formulations.

## 4.2 Technical optimization and methodological considerations for CCPP analysis

Previous research has demonstrated that modified extraction protocols can significantly improve DNA recovery from complex CCPP formulations (Arruda et al., 2017). Drawing on these findings, our protocol incorporated several refinements to address the complex nature of processed materials. The application of 75% ethanol pretreatment effectively reduced interference from polysaccharides and other processing-derived metabolites, while extended water bath incubation (1.5 h) enhanced DNA recovery from recalcitrant materials. These modifications were essential for improving DNA yield from highly processed botanical drugs,

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TABLE 4 Non-prescribed species detected in commercial RSJPW samples based on ITS2 sequences.

| Family | Latin name | Reads no. | ASV no. | Sample no. | Detection frequency (%) | Possible source category |
|---|---|---|---|---|---|---|
| Verbenaceae | *Verbena officinalis* L. | 23,911 | 36 | 14 | 25.00 | Medicinal plant |
| Brassicaceae | *Brassica napus* L. | 18,182 | 58 | 15 | 26.79 | Food crop |
| Cucurbitaceae | *Cucurbita moschata* (Duch. Ex Lam.) Duch. Ex Poir | 11,870 | 15 | 22 | 39.29 | Food crop |
| Apiaceae | *Peucedanum caespitosum* H. Wolff | 9,926 | 17 | 6 | 10.71 | Medicinal plant |
| Brassicaceae | *Raphanus sativus* L. | 5,067 | 9 | 14 | 25.00 | Food crop |
| Apiaceae | *Peucedanum praeruptorum* Dunn | 4,776 | 11 | 8 | 14.29 | Medicinal plant |
| Asteraceae | *Artemisia argyi* H. Lév. and Vaniot | 4,707 | 10 | 1 | 1.79 | Medicinal plant |
| Poaceae | *Setaria viridis* (L.) P. Beauv | 4,356 | 2 | 6 | 10.71 | Wild plant |
| Betulaceae | *Alnus nepalensis* D. Don | 4,351 | 10 | 25 | 44.64 | Wild plant |
| Convolvulaceae | *Cuscuta australis* R. Br | 3,977 | 7 | 17 | 30.36 | Medicinal plant |
| Poaceae | *Triticum aestivum* L. | 3,938 | 9 | 24 | 42.86 | Food crop |
| Cucurbitaceae | *Cucumis sativus* L. | 3,122 | 12 | 3 | 5.36 | Food crop |
| Pinaceae | *Pinus tabuliformis* Carrière | 1,438 | 6 | 12 | 21.43 | Wild plant |
| Paeoniaceae | *Paeonia* × *suffruticosa* Andrews | 1,040 | 3 | 3 | 5.36 | Medicinal plant |
| Amaryllidaceae | *Hymenocallis littoralis* (Jacq.) Salisb | 987 | 20 | 6 | 10.71 | Wild plant |

Note: This table shows the 15 species with the highest sequencing read counts. Detection frequency = (Number of samples where the species is detected/Total number of samples) × 100%.

TABLE 5 Related species of prescribed ingredients detected in commercial RSJPW samples based on ITS2 sequences.

| Family | Latin name | Reads no. | ASV no. | Sample no. | Detection frequency (%) | Related prescribed species |
|---|---|---|---|---|---|---|
| Fabaceae | *Hedysarum polybotrys* Hand.-Mazz | 343 | 3 | 5 | 8.93 | *Astragalus membranaceus* |
| Rhamnaceae | *Ziziphus mauritiana* Lam | 551 | 8 | 2 | 3.57 | *Ziziphus jujuba* |
| Araliaceae | *Panax quinquefolius* L. | 95 | 1 | 2 | 3.57 | *Panax ginseng* |
| Araliaceae | *Panax japonicus* (T. Nees) C. A. Meyer | 11 | 1 | 1 | 1.79 | *Panax ginseng* |
| Campanulaceae | *Codonopsis pilosula* (Franch.) Nannf | 116 | 1 | 2 | 3.57 | *Panax ginseng* |
| Rutaceae | *Citrus sinensis* (L.) Osbeck | 16 | 1 | 2 | 3.57 | *Citrus reticulata* |
| Apiaceae | *Angelica acutiloba* (Siebold and Zucc.) Kitag | 12 | 1 | 1 | 1.79 | *Angelica sinensis* |
| Zingiberaceae | *Amomum compactum* Soland ex Maton | 65 | 1 | 1 | 1.79 | *Amomum villosum* |

Note: Detection frequency = (Number of samples where the species is detected/Total number of samples) × 100%.

particularly those containing heavily processed or fungal materials. Though DNA recovery efficiency varies among ingredients due to their distinct processing methods and chemical compositions, the utilization of the AxyPrep DNA Gel Extraction Kit, combined with triplicate processing and pooling strategy, enabled consistent DNA isolation from complex CCPP matrices.

PCR bias emerged as a critical methodological challenge in our analysis, particularly evident in the dramatic fluctuations of *Z. jujuba* var. *spinosa* ASV counts. This phenomenon, well-documented in amplicon sequencing studies (Berry et al., 2011; Peng et al., 2015), manifested in our analysis as ASV count variations ranging from less than 10 to more than 90, suggesting substantial amplification preferences. These variations likely stem from differences in template GC content and secondary structure, factors known to influence PCR efficiency (Coissac et al., 2012). Recent advances in PCR optimization have suggested several promising approaches for bias mitigation. Modification of thermal cycling protocols and careful adjustment of reaction parameters have shown potential in reducing preferential amplification (Kanagawa, 2003). The development of

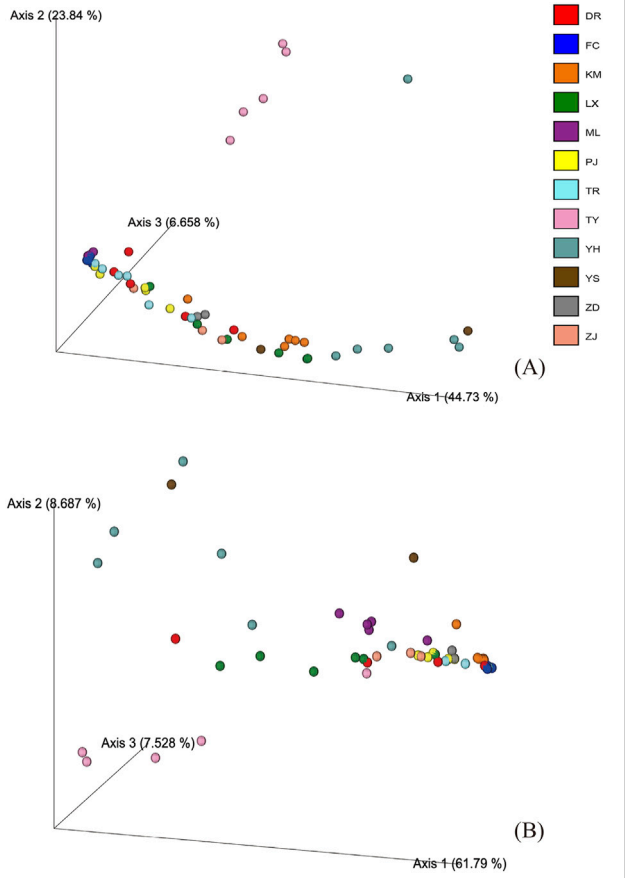


FIGURE 5
PCoA plot of species detected in commercial samples based on Bray-Curtis distance. **(A)** Analysis results of ITS2 sequences. **(B)** Analysis results of *psb*A-*trn*H sequences.

standardized controls using mock communities has also demonstrated value in quantifying and correcting for amplification bias (McLaren et al., 2019).

The systematic validation of our methodology through standard controls and *P. quinquefolius*-spiked samples demonstrated robust qualitative detection capabilities, particularly for low-abundance ingredients. However, our findings revealed that quantitative applications require careful consideration of species-specific amplification efficiencies, highlighting the importance of appropriate controls and standardization procedures. The validation process provided crucial insights into both the strengths and limitations of our approach, establishing a foundation for method optimization.

Recent advancements have significantly expanded the analytical toolkit for CCPP authentication beyond conventional DNA barcoding. Although DNA barcoding has provided a foundational approach for botanical identification, its application to complex or processed formulations presents numerous challenges. Our metabarcoding approach demonstrated significantly greater sensitivity than conventional barcoding for detecting botanical ingredients in complex CCPP mixtures. However, alternative methodologies offer additional advantages for specific analytical challenges. For instance, shotgun metagenomics can eliminate amplification-related distortions while providing broader genomic coverage (Liu et al., 2021a), particularly beneficial for complex mixtures where PCR bias is problematic. For analyzing degraded DNA in processed materials, Single-Molecule Real-Time (SMRT) sequencing (Jia et al., 2017; Xin et al., 2018b) shows particular promise, though cost considerations currently limit its widespread adoption. TaqMan probe-based quantitative real-time PCR has emerged as another valuable technique for specific taxa like *Panax notoginseng* in complex CCPP formulations, offering greater sensitivity and quantitative capabilities (Lou et al., 2022). Unlike standard DNA barcoding, qPCR methods can detect target species at concentrations as low as 0.1%, making them particularly valuable for quality control in highly processed products where DNA is degraded. Complementary analytical approaches include multi-omics integration frameworks combining genomic, metabolomic, and chemical profiling data for holistic authentication (Wang et al., 2022). These integrative approaches have shown superior discriminatory power compared to single-method authentication, particularly for processed formulations where molecular integrity is compromised. Advanced computational methodologies have similarly transformed CCPP authentication. Machine learning algorithms, including deep learning and ensemble methods, have significantly improved pattern recognition capabilities for complex botanical mixtures (Chen and He, 2022; Magdas and Berghian-Grosan, 2023; Wang et al., 2024). These approaches can process multi-dimensional data from diverse analytical platforms, potentially overcoming the limitations of individual methods while providing more robust authentication frameworks. Recent innovations in molecular authentication include nucleotide signature-based identification strategies specifically optimized for processed materials (Zhang T. et al., 2022; Niu et al., 2024) and isothermal amplification methods for rapid authentication of complex materials (Sheu et al., 2023). While many of these technologies have primarily been validated in experimental settings, their translation to routine CCPP quality control represents a promising direction for future applications.

## 4.3 Future perspectives: Database development and bioinformatic integration

While DNA metabarcoding has become a powerful tool in environmental microbiome research with established specialized databases (SILVA, GreenGenes, and RDP) (McDonald et al., 2012; Quast et al., 2013; Cole et al., 2014), its application in TCM authentication faces significant database limitations. Currently, specialized platforms like the TCM DNA Barcode Identification System (Chen et al., 2014) and the DNA barcode databases from the Institute of Medicinal Plant Development (Chen et al., 2012) represent the primary resources for TCM identification. However, as demonstrated in our RSJPW analysis, these systems' heavy reliance on public databases like NCBI limits their effectiveness for processed botanical materials. To enhance the practical application of DNA metabarcoding in CCPP quality control, several strategic advancements are crucial, such as development of comprehensive, validated reference databases specifically tailored for traditional medicinal plants and their common adulterants, iImproved bioinformatic pipelines optimized for highly processed materials with degraded DNA, standardized authentication protocols considering the unique challenges of complex formulations and integration of automated identification

systems with regulatory databases. The processing of high-throughput sequencing data in CCPP analysis presents unique bioinformatic challenges beyond conventional environmental DNA studies. While QIIME 2 provides a robust analytical framework (Bolyen et al., 2019), our study revealed limitations in taxonomic assignment due to the scarcity of CCPP-specific reference databases. This issue is particularly evident in processed materials where DNA modifications can affect sequence matching accuracy. Recent studies have demonstrated improved authentication accuracy through specialized reference databases incorporating both raw and processed material sequences (Xin et al., 2018b).

The integration of bioinformatic tools with tiered analytical approaches represents a promising development direction in CCPP authentication. Recent work by Mück et al. (2024) demonstrated how combining DNA metabarcoding data with chemical profiles achieves improved authentication accuracy for complex formulations. This multi-analytical strategy provides both qualitative and quantitative insights, overcoming the limitations of conventional DNA barcoding which often proves insufficient for processed herbal materials. Furthermore, the development of automated analysis pipelines can streamline workflows and minimize reliance on high-performance computing resources (Fung et al., 2021; Dubois et al., 2022). These advances, coupled with expanding reference databases, will be crucial for improving the reliability and accessibility of DNA metabarcoding in CCPP authentication and could potentially create more robust authentication frameworks for complex formulations.

## 5 Conclusion

This study applied DNA metabarcoding to authenticate RSJPW, demonstrating both the capabilities and limitations of this approach in CCPP botnical drug authentication. Our dual-marker strategy successfully identified most prescribed ingredients while revealing authentication issues, including contamination and potential substitution in commercial products. However, processing-induced DNA degradation significantly affected detection rates for certain ingredients, particularly evident in *P. cocos* and heat-processed materials. These findings highlight the importance of considering processing effects in molecular authentication protocols.

While DNA metabarcoding offers advantages in multi-ingredient authentication, our results indicate that comprehensive CCPP quality assessment requires integration with complementary analytical methods. The systematic validation approach and optimized protocols developed in this study contribute to the methodological framework for complex botanical drug formulation analysis. Future improvements in CCPP-specific reference databases and bioinformatic tools, combined with chemical analysis methods (e.g., metabolomics), will be crucial for enhancing the practical application of DNA metabarcoding in CCPP botanical drug authentication.

## Data availability statement

The raw sequencing data presented in this study are deposited in the NCBI Sequence Read Archive (SRA) repository under BioProject accession number PRJNA1242227.

## Author contributions

SZ: Conceptualization, Data curation, Formal Analysis, Methodology, Software, Writing – original draft. TZ: Formal Analysis, Investigation, Resources, Writing – original draft. YZ: Investigation, Methodology, Resources, Writing – original draft. BY: Investigation, Methodology, Software, Writing – original draft. JN: Investigation, Resources, Validation, Writing – original draft. WL: Formal Analysis, Visualization, Writing – original draft. YW: Data curation, Investigation, Resources, Writing – original draft. FL: Data curation, Investigation, Writing – original draft. ZL: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review and editing. JZ: Conceptualization, Funding acquisition, Supervision, 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 authors declare that no Generative AI was used in the creation of this manuscript.

## Publisher's note



## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2025.1584065/full#supplementary-material

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# Quality evaluation of *Polygonatum sibiricum* slices from different regions based on appearance traits and multi-index metabolites combined with TOPSIS and gray relation analysis

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**Introduction:** Traditional Chinese medicine quality control faces challenges, lacking multidimensionality and reliable quantitative evidence. Comprehensive evaluation models based on external characteristics and multiple indicator metabolites are the future research direction. This study focuses on *Polygonatum sibiricum* slices, aiming to establish a method for its quality evaluation.

**Methods:** The appearance traits of *P. sibiricum* slices were quantified, and the contents of six functional metabolites were determined. With eight traits and six metabolite contents as variables, principal component analysis (PCA) and orthogonal partial least-squares discrimination analysis (OPLS-DA) were performed. A weighted TOPSIS-GRA fusion model was established by combining the technique for order preference by similarity to ideal solution (TOPSIS) and gray relation analysis (GRA).

**Results:** The six metabolites showed good linear relationships ($R^2 > 0.9992$) within their respective ranges, with an average recovery rate of 98.54% - 103.07% (relative standard deviation less than 1.64%). Precision, stability, and repeatability met the relevant standards. There were significant differences in traits and metabolite contents among slices from different habitats. OPLS-DA identified differential quality-affecting markers. PCA showed that the first three principal components contributed over 80% of the cumulative variance, and 16 batches of slices were clustered into three categories by origin. The weighted TOPSIS-GRA fusion model indicated significant quality differences among slices from different regions, consistent with PCA and OPLS-DA clustering results.

**Discussion:** The established multi-index content determination method is accurate and reliable for detecting metabolites in *P. sibiricum* slices. The PCA, OPLS-DA, and weighted TOPSIS-GRA fusion models are scientifically reliable.

The correlation between appearance traits and product quality can be used to evaluate *P. sibiricum* slices from different regions, which is of great significance for quality control and standardization of traditional Chinese medicine.

KEYWORDS

***P. sibiricum* slices, appearance characteristics, indicator metabolites, weighted TOPSIS-GRA fusion model, quality evaluation**

# 1 Introduction

Traditional Chinese medicine (TCM) is the foundation for innovative developments in the medical industry in China and abroad, and forms the material basis for ensuring the clinical safety of associated treatments. The quality standards of TCM are key to the high-quality development of the industry. However, limitations in research ideas and methods mean that current research on TCM quality control lacks multidimensionality, chain continuity, and integration. TCM evaluation methods are based on the concept of "identifying patterns and discussing quality" (Kang et al., 2024), which makes it difficult to assess the internal metabolites and their efficacy, and results in a lack of reliable quantitative evidence. Modern physical and chemical evaluation methods are based on quantifying the content of a single or group of metabolites. Although accurate quantification can be achieved, the results are difficult to relate to clinical efficacy and safety. Moreover, the inherent characteristics of TCM mean that quality cannot be judged solely by metabolites. Although the external characteristics of medicinal materials are closely related to their internal metabolites and efficacy, the correlation and internal mechanisms acting between numerous characteristics and complex metabolites present several research challenges. Therefore, comprehensive evaluation models based on external characteristics and multiple indicator metabolites have become the mainstream research direction for future TCM quality control.

The technique for order preference by similarity to ideal solution (TOPSIS) assigns weights to multiple indicators, using the degree of proximity to the idealized target as a benchmark for the comprehensive evaluation of samples. This eliminates subjective factors brought about by "identifying patterns and discussing quality", and ensures objectivity, accuracy, and a scientific basis (Xu et al., 2024). Gray relation analysis (GRA) has been used to identify complex relationships between multiple indicators, and can analyze and compare the factors that have the greatest impact on samples based on the degree of interrelation among multiple factors. In this way, GRA intuitively reflects the comprehensive value of each indicator (Chen et al., 2018).

*Polygonatum sibiricum*, a historically revered tonic botanical drug, is traditionally used to nourish vital energy (Qi), dispel rheumatism, and harmonize visceral functions. Modern pharmacological studies reveal its rich metabolites of polysaccharides, saponins, flavonoids, alkaloids, and phenylpropanoids, which collectively contribute to hypoglycemic, lipid-lowering, antioxidant, anti-aging, and antitumor activities (Yang et al., 2021; Zhang et al., 2024a). Despite polysaccharides being recognized as primary active metabolites in *P. sibiricum* (Chinese Pharmacopoeia, 2020), emerging evidence underscores the synergistic roles of secondary metabolites in its therapeutic efficacy. To address regional quality disparities and align with the "Quality Marker (Q-Marker)" concept (Yuan and Wang, 2024), this study integrates morphological traits (long diameter, short diameter, single weight, thickness, chromaticity values) with quantification of five bioactive metabolites: baicalein, liquiritigenin, neoliquiritin, 3′-methoxydaidzin, and diosgenin. The selection of baicalein, liquiritigenin, neoliquiritin, 3′-methoxydaidzin, and diosgenin was driven by a hierarchical rationale rooted in pharmacological relevance, alignment with TCM synergy principles, and methodological rigor. First, the pharmacological significance of these metabolites directly mirrors *P. sibiricum*'s traditional and modern therapeutic applications. For instance, baicalein, a flavonoid, was prioritized for its NF-κB-mediated anti-inflammatory and antioxidant properties, which align with the botanical drug's historical use in rheumatism management (Gao et al., 2024; Mabalirajan et al., 2013). Moreover, liquiritigenin and its glycoside neoliquiritin were included due to their immunomodulatory and neuroprotective effects, supporting the botanical drug's role in Qi replenishment and immune enhancement (Yao et al., 2022; Xu et al., 2024). Furthermore, 3′-methoxydaidzin, an isoflavone with estrogenic activity, reflects *P. sibiricum*'s yin-nourishing applications in alleviating menopausal symptoms (Hou et al., 2024), while diosgenin, a steroidal saponin, was selected for its antitumor and lipid-lowering mechanisms via apoptosis induction and metabolic regulation (Xu et al., 2022; Zhang et al., 2021). Critically, these metabolites collectively embody the TCM paradigm of multi-metabolite synergy, wherein therapeutic efficacy arises from interconnected interactions rather than isolated constituents (Xiao et al., 2025). Finally, methodological coherence was ensured by integrating prior Q-Marker predictions (Jiang et al., 2017; Wang et al., 2024) with TOPSIS-GRA frameworks, enabling robust correlations between morphological traits and metabolite levels to harmonize traditional and modern quality standards.

By synthesizing morphological and multi-metabolite data, this work establishes a scientifically robust framework for evaluating *P. sibiricum* quality, offering insights for authenticity verification, origin tracing, and standardized industrial practices. The findings advance TCM quality standardization and underscore the necessity of integrating traditional wisdom with contemporary analytical methodologies.

# 2 Materials and methods

## 2.1 Test drugs

The reference substances of baicalein (batch No. A1119L021, purity 98.5%), liquiritigenin (batch No. A105J021, purity 98.5%), neoliquiritin (batch No. 2230816001, purity 98.5%), 3′-methoxydaidzin (batch No. Y14F10w79687, purity 98.5%), and diosgenin (batch No. A922E024, purity 98.5%) were procured

TABLE 1 Origins of *P. sibiricum* slices.

| No. | Source |
|---|---|
| S1 | Tongnan, Chongqing |
| S2 | Shizhu, Chongqing |
| S3 | Changshou, Chongqing |
| S4 | Hechuan, Chongqing |
| S5 | Mianyang, Sichuan |
| S6 | Luzhou, Sichuan |
| S7 | Jianyang, Sichuan |
| S8 | Yibin, Sichuan |
| S9 | Emei, Sichuan |
| S10 | Zhongjiang, Sichuan |
| S11 | Anshun, Guizhou |
| S12 | Kaili, Guizhou |
| S13 | Bijie, Guizhou |
| S14 | Zunyi, Guizhou |
| S15 | Jindong, Yunnan |
| S16 | Jinggu, Yunnan |

from Beijing Solarbio Science & Technology Co., Ltd. High-performance liquid chromatography-grade acetonitrile, methanol, ethanol, and glacial acetic acid were supplied by Tianjin Fuyu Fine Chemical Co., Ltd. The P. sibiricum slices were authenticated as the dried rhizomes of Polygonatum by Professor Cairang Nanjia from the School of Pharmacy at Qinghai Minzu University. The *P. sibiricum* specimens used in this study are preserved in the Specimen Laboratory of the School of Pharmacy, Qinghai Minzu University. Table 1 details the information regarding the sample collection sites. As shown in Figure 1, the slices of *P. sibiricum* are presented, which can provide a visual reference for the morphological characteristics of this TCM.

## 2.2 Instruments

To prepare, examine, and characterize the samples, we used a Shimadzu LC-20A High Performance Liquid Chromatograph (Shimadzu, Japan); Analytical High-Performance Liquid Chromatography Column (Hypersil ODS-C18: Elite Suzhou Analytical Instruments Co., Ltd., China); KQ-50DA CNC Ultrasonic Cleaner (Kunshan Hechuang Ultrasonic Instrument Co., Ltd., China); XS105DU Electronic Balance (Shanghai Mettler Toledo Co., Ltd., China); SHE-D Vacuum Pump (Gongyi Yuhua Instrument Co., Ltd., China); UPH-IV Ultra Pure Water Machine (Chengdu Ultra Pure Science & Technology Co., Ltd., China); and 3nh Color Difference Meter (Guangdong Sannshi Intelligent Technology Co., Ltd., China).

## 2.3 Appearance characteristic measurement

Slices of *P. sibiricum* from diverse origins were thoroughly homogenized and arranged to form a square configuration. Subsequently, a partition board was employed to divide the square into four congruent sections along the diagonal. Two diagonally opposed sections were selectively retained, while the remaining two sections were discarded. The two selected portions were then re-combined, and this procedure was iteratively repeated until the number of samples per batch attained 20. A precision balance and a vernier caliper were utilized to measure the mass, thickness, major diameter, and minor diameter of the samples. Subsequently, the average values of these measured parameters were calculated.

## 2.4 Decoction pieces chromaticity measurement

A colorimeter was employed to conduct measurements under the D65 light source, with a measurement wavelength range spanning from 180 to 740 nm and a measurement field of 10°. Prior to sample measurement, black and white calibration was performed on the colorimeter. Each batch, consisting of 20 samples, was placed within the test chamber, ensuring that the colorimeter's measuring spot was precisely aligned at the center of the test chamber. The colorimetric values $L^*$, $a^*$, and $b^*$ were then recorded. Here, $L^*$ denotes the brightness of the sample color, $a^*$ represents the red - green gradient axis, and $b^*$ represents the yellow - blue gradient axis, where larger absolute values correspond to more intense colors (Yang et al., 2020). Each measurement was replicated three times, and the average values were calculated. Subsequently, the comprehensive color difference

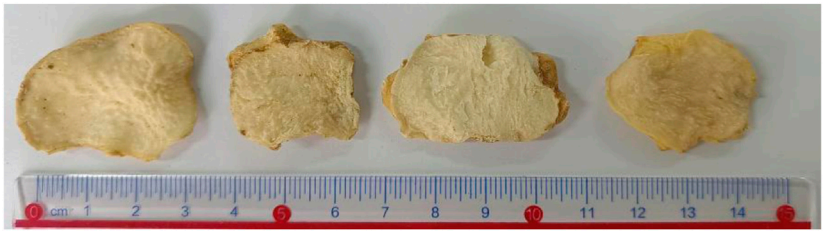


FIGURE 1
Typical photographs of sliced *P. sibiricum*

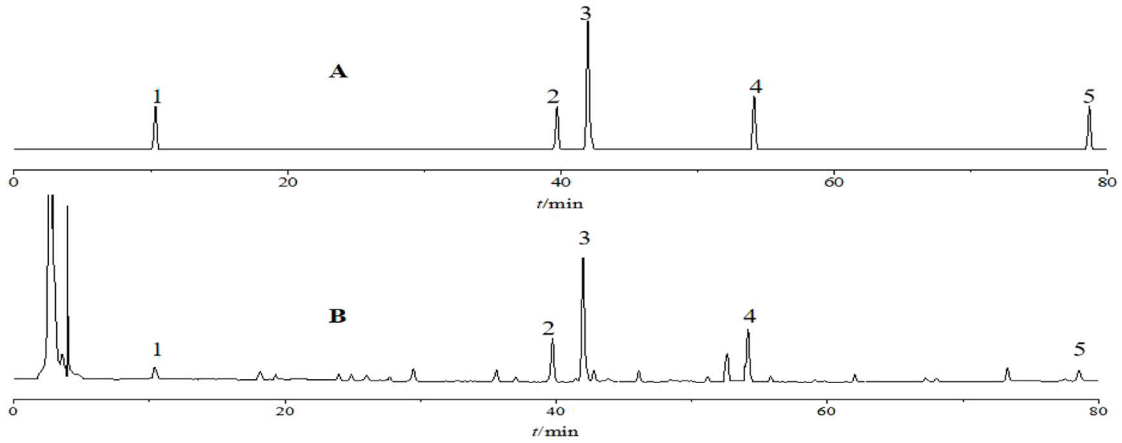


FIGURE 2
Chromatograms of standard solution **(A)** and sample solution **(B)** obtained by high-performance liquid chromatography. Note: 1-Neoliquiritin; 2-Liquiritigenin; 3–3′-Methoxydaidzin; 4-Baicalein; 5-Diosgenin.

value $\Delta E$ for each batch of samples was computed according to Equation 1.

$$\Delta E = \left(L^{*2} + a^{*2} + b^{*2}\right)^{1/2} \quad (1)$$

## 2.5 Determination of indicator metabolite contents

### 2.5.1 Preparation of standard solution

The requisite amounts of the reference substances, namely, baicalein, liquiritigenin, neoliquiritin, diosgenin, and 3′-methoxydaidzin, were accurately weighed. These substances were then dissolved in chromatographic-grade methanol and transferred to a 25 mL volumetric flask. The volume was adjusted to the mark with the same solvent, thereby preparing a mixed reference substance solution. The resulting mass concentrations of baicalein, liquiritigenin, neoliquiritin, diosgenin, and 3′-methoxydaidzin in the solution were 0.0816, 0.094, 0.0828, 0.098, and 0.0796 mg·mL$^{-1}$, respectively.

### 2.5.2 Preparation of test Sample solution

Appropriate quantities of *P. sibiricum* samples were meticulously pulverized and sieved. Subsequently, precisely 2.00 g of the resultant powder was weighed and transferred into a conical flask. To this, 40 mL of 70% ethanol was added, and the mixture was subjected to sonication for a duration of 30 min. After sonication, the mixture was cooled and then filtered. A portion of the filtrate was collected, and the above-mentioned procedures were reiterated for the filter residue. The filtrates were then pooled together and concentrated under reduced pressure to yield a paste. This paste was re-dissolved in methanol to a final volume of 10 mL. Subsequently, 100 μL of a 10 g·L$^{-1}$ chitosan-glacial acetic acid solution was added dropwise, and the resulting solution was left to stand overnight. Finally, the solution was filtered through a 0.22 - μm filter membrane to obtain the test solution.

### 2.5.3 High-performance liquid chromatography conditions

High-performance liquid chromatography (HPLC) analysis was performed using a Hypersil ODS-C18 column (250 mm × 4.6 mm, 5 μm particle size). The detection wavelength was set at 230 nm. The mobile phase, consisting of acetonitrile (A) and water (B), was applied for gradient elution with the following program: 0–20 min, 5%–30% A; 20–30 min, 30%–40% A; 30–40 min, 40%–60% A; 40–80 min, 60%–100% A. The injection volume was 10 μL, the column temperature was maintained at 30°C, and the flow rate was 1.0 mL·min$^{-1}$. Chromatograms of the reference and test samples of *P. sibiricum* slices under these conditions are shown in Figure 2.

### 2.5.4 Investigation of the linear correlation

In accordance with the chromatographic conditions described in Section 2.5.3, injection volumes of 4, 8, 12, 16, 20, and 24 μL were prepared. The corresponding chromatographic peak areas were recorded. Linear regression equations were established, with the injection volume as the independent variable (X, abscissa) and the peak area as the dependent variable (Y, ordinate).

### 2.5.5 Precision test

An appropriate volume of the mixed standard solution was subjected to six consecutive injections (10 μL per injection) under the chromatographic conditions specified in Section 2.5.3. The chromatographic profiles were recorded and the relative standard deviation (RSD) values of the peak areas for five target analytes - baicalein, liquiritigenin, neoliquiritin, diosgenin, and 3′-methoxydaidzein - were systematically calculated to evaluate the precision of the analytical method.

### 2.5.6 Stability test

A sample (S1) solution of P. sibiricum slices was extracted and detection was performed every 4 h over a 24-h period. The baicalein, liquiritigenin, neoliquiritin, diosgenin, and 3′-methoxydaidzin peak areas were recorded.

### 2.5.7 Repeatability test

Six samples of *P. sibiricum* powder (S1) were prepared in parallel according to the method described in Section 2.5.2. The samples were injected according to the chromatographic conditions detailed in Section 2.5.3, and the peak area was determined.

### 2.5.8 Sample recovery test

Nine aliquots of S1 powder containing known amounts of the five target analytes (baicalein, liquiritigenin, neoliquiritin, diosgenin, and 3′-methoxydaidzin) were prepared. Three aliquots each of low-, medium-, and high-mass-fraction control solutions (three replicates per concentration level) were then spiked. The test solution was prepared according to the method in Section 2.5.2, and chromatographic analysis was performed under the conditions described in Section 2.5.3. Finally, the average recoveries and RSD were calculated by comparing the measured amounts with the spiked amounts.

### 2.5.9 Content determination

Sixteen batches of samples were pulverized and sieved, and then test solutions were prepared according to the method detailed in Section 2.5.2, after which the prepared test solutions were diluted with methanol at a ratio of 1:10, vortex-mixed for 30 s, and allowed to stand for 5 min.The prepared samples were injected under the chromatographic conditions specified in Section 2.5.3, and the corresponding peak areas were recorded. The contents of baicalein, liquiritigenin, neoliquiritin, diosgenin, and 3′-methoxydaidzin were calculated by applying the pre-established linear regression equations.

## 2.6 Determination of polysaccharide content in *P. sibiricum* slices

### 2.6.1 Drawing of standard curve

The anhydrous glucose was dried to constant weight at 105°C, and precisely 33 mg was taken and added to a 100-mL volumetric flask. Then, 0, 0.2, 0.5, 1, 1.5, and 2 mL of glucose solution was added to six 10-mL test tubes, with distilled water added up to 2 mL where necessary, followed by the addition of 0.2% anthrone–sulphuric acid solution in an ice-water bath up to 10 mL. The test tubes were left at room temperature before being subjected to a boiled water bath for 10 min, followed by a room temperature water bath for 10 min. The absorbance was then measured at 620 nm. The standard curve was plotted, with the concentration c of glucose standard solution as the horizontal coordinate and the absorbance A as the vertical coordinate. The results showed that there was a good linear relationship between absorbance and concentration, with the regression equation A = 34.661c+0.8079 achieving $R^2$ = 0.9998.

### 2.6.2 Determination of polysaccharide content

The sample powder was dried at 60°C to constant weight. Subsequently, 0.25 g of the dried powder was accurately weighed and transferred into a round-bottomed flask. Then, 150 mL of 80% ethanol was added. Ultrasonic extraction was performed for 15 min, followed by reflux extraction in a boiling water bath for 1 h. The hot solution was filtered, and the residue was washed three times with 10 mL of 80% ethanol each. The residue and filter paper were transferred to another flask, and 150 mL of distilled water was added. Reflux extraction in a boiling water bath was repeated for 1 h, followed by hot filtration. The filtrate and washings were combined and further washed four times with 10 mL of distilled water each (filtered while hot). The residue and flask were also washed four times with 10 mL of hot distilled water. All filtrates and wash solutions were combined, cooled to room temperature, and transferred quantitatively to a 250 mL volumetric flask. The volume was adjusted to the mark with distilled water, and the flask was shaken thoroughly to obtain the test solution.

Exactly 1 mL of the test solution was pipetted into a 10 mL stoppered test tube, and 2 mL of distilled water was added. After thorough mixing, 0.2% anthrone-sulfuric acid solution was slowly added to the marked volume in an ice-water bath. The mixture was shaken gently, cooled, and then placed in a boiling water bath for 10 min. After cooling in a room-temperature water bath for 10 min, the solution was equilibrated at ambient conditions. Using distilled water as the blank, absorbance was measured at 620 nm. The absorbance value was substituted into the pre-established linear regression equation to calculate the polysaccharide content.

## 2.7 Statistical analysis

In the SIMCA 14.1 software, the orthogonal partial least-squares discrimination analysis (OPLS-DA) function was used to analyze the variable importance in the projection (VIP) of the indicator metabolites and polysaccharides. Duncan's multiple range test ($P < 0.05$) was applied to determine the level of statistical significance after principal component analysis (PCA) and one-way analysis of variance in IBM's SPSS Statistics 27.0 software. All data (mean ± standard deviation) were determined independently in triplicate.

## 2.8 Weighted TOPSIS-GRA fusion modeling

### 2.8.1 Weighted TOPSIS data normalization

The analysis described in the previous subsections suggests that larger values of the mass, thickness, long diameter, short diameter, baicalein, liquiritigenin, neoliquiritin, diosgenin, 3′-methoxydaidzin, and polysaccharides of *P. sibiricum* slices are preferable. These variables were normalized using Equation 2. In contrast, smaller values of $L^*$, $a^*$, $b^*$, and $\Delta E$ are desirable. These variables were normalized using Equation 3 (Liao et al., 2024).

$$Y_{ij} = \frac{X_{ij} - \min(x_j)}{\max(x_j) - \min(x_j)} \quad (2)$$

$$Y_{ij} = \frac{\max(x_j) - X_{ij}}{\max(x_j) - \min(x_j)} \quad (3)$$

Note: $Y_{ij}$ is the normalization result and $X_{ij}$ is the value of each indicator measurement.

### 2.8.2 Weighted TOPSIS Calculation for each evaluation indicator

The variable importance in projection (VIP) values of the 14 indicators derived from orthogonal partial least-squares

discriminant analysis (OPLS-DA) were assigned as the weighting coefficients ($Q_j$). The weighting matrix values ($Z_{ij}$) for each indicator were then computed using Equation 4. Based on this matrix, the positive and negative ideal samples ($Z^+$) and ($Z^-$) were defined. Finally, the distances from each evaluation indicator to the ideal samples ($d_i^+$) and ($d_i^-$) were calculated via Equations 5, 6 (Zheng et al., 2024).

$$Z_{ij} = Y_{ij} \times Q_j \tag{4}$$

$$d_i^+ = \sqrt{\sum_{j=1}^{n} \left(Z_{ij} - Z_j^+\right)^2} \tag{5}$$

$$d_i^- = \sqrt{\sum_{j=1}^{n} \left(Z_{ij} - Z_j^-\right)^2} \tag{6}$$

### 2.8.3 GRA standardization

Because of the significant differences in the results of the 14 indicators measured in the 16 batches of *P. sibiricum* slices, data standardization was required prior to GRA (Li et al., 2024). The data were standardized using Equation 7.

$$M_{ij} = X_{ij} / X_{mj} \tag{7}$$

Note: $M_{ij}$ is the result of the standardization process for each indicator and $X_{mj}$ is the average of the results for each indicator.

### 2.8.4 Calculation of GRA correlation coefficients

The GRA method evaluates the interrelationships among research objects by quantifying their degrees of relevance. Higher GRA correlation values indicate stronger associations and superior quality of the objects (Fu et al., 2019). Among the 16 batches of *P. sibiricum* slices, parameters including mass, thickness, long diameter, short diameter, and the contents of baicalein, liquiritigenin, neoliquiritin, diosgenin, 3′-methoxydaidzin, and polysaccharides were classified as positive indicators. For these positive indicators, the maximum values observed across all batches were selected as the optimal reference sequence, while the minimum values were designated as the worst reference sequence during GRA standardization. Conversely, the color parameters $L^*$, $a^*$, $b^*$, and $\Delta E$ were considered negative indicators. For these, the maximum values from GRA standardization were assigned as the worst reference sequence, and the minimum values as the optimal reference sequence. The correlation coefficients between each evaluation index and both reference sequences (optimal and worst) were calculated using Equations 8, 9.

$$E_{jb}^i = \frac{\Delta_{min} + \alpha\Delta_{max}}{|M_{ij} - M_{bj}| + \alpha\Delta_{max}} \tag{8}$$

$$E_{js}^i = \frac{\Delta'_{min} + \alpha\Delta'_{max}}{|M_{ij} - M_{sj}| + \alpha\Delta'_{max}} \tag{9}$$

Note: $E_{jb}^i, E_{js}^i$ are the correlation coefficients of the best and worst reference sequences; $M_{bj}, M_{sj}$ are the best and worst reference sequences; $\Delta_{min} = min|M_{ij} - M_{bj}|$, $\Delta_{max} = max|M_{ij} - M_{bj}|$, $\Delta'_{min} = min|M_{ij} - M_{sj}|$, $\Delta'_{max} = max|M_{ij} - M_{sj}|$; α is the resolution coefficient, which takes a value of 0.5 (Zhang et al., 2020).

### 2.8.5 Calculation of relative correlation degree of GRA

Using the process explained in Section 2.8.4, the relative correlation of each batch was calculated. The relative correlation is the mean value of the correlation coefficients of all evaluation indicators of the research object. Thus, the correlation of *P. sibiricum* slices with respect to the optimal and the worst reference sequences was calculated using Equations 10, 11 (Qu et al., 2019), respectively.

$$q_i^+ = \frac{1}{14}\sum_{j=1}^{14} E_{jb}^i \tag{10}$$

$$q_i^- = \frac{1}{14}\sum_{j=1}^{14} E_{js}^i \tag{11}$$

### 2.8.6 Weighted TOPSIS-GRA fusion Modeling

Using Equations 12–15, the Euclidean distance and relative correlation of the reference sequence for 16 batches of *P. sibiricum* slices were subjected to dimensionless processing. Subsequently, post-processing by the weighted TOPSIS-GRA fusion model was applied using Equations 16, 17. $D_i^+$ and $Q_i^-$ denote the extent to which samples deviate from the ideal sample; smaller values indicate closer proximity to the ideal sample. Conversely, $D_i^-$ and $Q_i^+$ signify the extent to which samples approach the ideal sample; larger values suggest a closer resemblance to the ideal sample. $G_i^+$ offers a comprehensive assessment of how closely all samples approximate the ideal sample; higher values equate to superior sample quality. $G_i^-$ provides a comprehensive evaluation of the distance of all samples from the ideal sample; higher values correspond to lower sample quality. The relative closeness κ was determined using Equation 18; higher values signify higher comprehensive sample quality.

$$D_i^+ = d_i^+ / max\, d_i^+ \tag{12}$$

$$D_i^- = d_i^- / max\, d_i^- \tag{13}$$

$$Q_i^+ = q_i^+ / max\, q_i^+ \tag{14}$$

$$Q_i^- = q_i^- / max\, q_i^- \tag{15}$$

$$G_i^+ = \alpha D_i^- + \beta Q_i^+ \tag{16}$$

$$G_i^- = \alpha D_i^+ + \beta Q_i^- \tag{17}$$

$$\kappa = G_i^+ / (G_i^+ + G_i^-) \tag{18}$$

Note: $\alpha$ and $\beta$ are correlation coefficients, both taking a value of 0.5 (Yan et al., 2024).

## 3 Results

### 3.1 Appearance characteristics of *P. sibiricum* slices

The measurements of appearance-characteristic indices are presented in Table 2. As shown in the table, the 16 batches of samples exhibit significant differences among different origins based on eight evaluated traits.

TABLE 2 Appearance traits of samples of *P. sibiricum* slices from different producing areas ($x \pm s$, $n = 3$).

| No. | Mass/g | Thickness/mm | Long diameter/mm | Short diameter/mm | Chromaticity values | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | *L** | *a** | *b** | *ΔE* |
| S1 | 4.028 ± 2.251c | 2.104 ± 1.501de | 45.818 ± 3.601c | 17.590 ± 2.021cde | 72.468 ± 0.020f | 2.302 ± 0.005f | 29.906 ± 0.016ab | 78.430 ± 0.051 d |
| S2 | 7.816 ± 3.052a | 3.188 ± 1.103abc | 78.940 ± 2.835a | 20.734 ± 0.857b | 74.332 ± 0.016ef | 3.064 ± 0.030de | 10.752 ± 0.007f | 75.168 ± 0.652e |
| S3 | 2.248 ± 2.057g | 2.046 ± 1.106de | 41.302 ± 3.006de | 19.118 ± 1.503bc | 75.362 ± 0.059cdef | 3.628 ± 0.018bcd | 27.628 ± 0.020bcd | 80.349 ± 0.509bcd |
| S4 | 3.400 ± 2.967 d | 2.556 ± 1.214bcd | 39.528 ± 2.809ef | 16.886 ± 2.557cdef | 80.412 ± 0.036a | 1.130 ± 0.029g | 24.986 ± 0.021de | 84.212 ± 0.209a |
| S5 | 3.464 ± 2.908cd | 1.948 ± 0.869def | 41.820 ± 3.290de | 23.918 ± 1.836a | 74.456 ± 0.059ef | 2.770 ± 0.010eff | 30.444 ± 0.069ab | 80.487 ± 0.093bcd |
| S6 | 3.235 ± 3.907de | 1.890 ± 0.680def | 42.380 ± 3.309 d | 17.068 ± 2.083cdef | 76.596 ± 0.068bcde | 5.588 ± 0.009a | 28.158 ± 0.019abc | 81.799 ± 0.092abc |
| S7 | 3.406 ± 1.208 d | 3.206 ± 0.830abc | 45.570 ± 2.075c | 15.944 ± 1.153def | 74.366 ± 0.016ef | 2.658 ± 0.035ef | 29.074 ± 0.082ab | 79.892 ± 0.021cd |
| S8 | 7.846 ± 4.085a | 3.280 ± 1.410ab | 78.558 ± 4.510a | 20.882 ± 2.59b | 74.262 ± 0.005ef | 3.106 ± 0.027de | 12.384 ± 0.017f | 75.351 ± 0.013e |
| S9 | 2.726 ± 1.570efg | 1.378 ± 0.570ef | 43.800 ± 3.917cd | 19.078 ± 2.907bc | 75.694 ± 0.019bcde | 3.754 ± 0.076bc | 30.940 ± 0.038a | 81.859 ± 0.043abc |
| S10 | 2.388 ± 2.037fg | 1.262 ± 0.029f | 42.556 ± 4.162 d | 19.780 ± 1.277bc | 75.266 ± 0.030cdef | 3.932 ± 0.020b | 29.036 ± 0.065ab | 80.768 ± 0.045bcd |
| S11 | 1.540 ± 0.970h | 1.792 ± 0.561def | 36.940 ± 3.009fg | 15.680 ± 2.309ef | 78.168 ± 0.089abc | 1.056 ± 0.029g | 24.768 ± 0.057de | 82.005 ± 0.065abc |
| S12 | 2.496 ± 1.095fg | 1.898 ± 0.751def | 53.348 ± 4.068b | 18.264 ± 3.005bcde | 78.520 ± 0.037ab | −0.606 ± 0.015h | 24.046 ± 0.025e | 82.122 ± 0.028abc |
| S13 | 2.218 ± 1.305g | 2.082 ± 1.073de | 34.414 ± 3.507g | 14.454 ± 2.801f | 77.798 ± 0.034abcd | 2.710 ± 0.011ef | 25.820 ± 0.079cde | 82.015 ± 0.044abc |
| S14 | 7.214 ± 1.808b | 3.272 ± 1.283ab | 78.974 ± 5.207a | 20.804 ± 3.051b | 74.130 ± 0.022ef | 3.068 ± 0.073de | 11.882 ± 0.035f | 75.139 ± 0.053e |
| S15 | 2.896 ± 1.099def | 2.432 ± 1.677cd | 43.618 ± 2.033cd | 18.728 ± 2.006bcd | 77.452 ± 0.031bcd | 4.044 ± 0.016b | 28.652 ± 0.039abc | 82.681 ± 0.025ab |
| S16 | 7.478 ± 2.331ab | 3.950 ± 1.922a | 78.460 ± 3.367a | 20.938 ± 3.303b | 74.920 ± 0.088def | 3.170 ± 0.075cde | 11.446 ± 0.076f | 75.856 ± 0.077e |

Note: Different letters in the same column indicate $P < 0.05$.

TABLE 3 Linear relationships for the five metabolites.

| Metabolite | Linear equation | $R^2$ | Linear range/μg |
|---|---|---|---|
| Baicalein | Y = 8872.4X-53014 | 0.9992 | 0.9792~2.6112 |
| Liquiritigenin | Y = 14645X-14574 | 0.9996 | 0.3760~2.2560 |
| Neoliquiritin | Y = 189982X-49663 | 0.9994 | 0.3312~1.9872 |
| Diosgenin | Y = 341895X-216226 | 0.9993 | 0.3920~2.3520 |
| 3′-Methoxydaidzin | Y = 14128X-22450 | 0.9995 | 0.3184~1.9104 |

## 3.2 Methodological review

### 3.2.1 Linear range Inspection

The standard curve of five quality metabolites was plotted, and the linear regression equation, correlation coefficient, and linear range of each reference substance was calculated. The results are presented in Table 3.

### 3.2.2 Precision test results

The RSD values for baicalein, liquiritigenin, neoliquiritin, diosgenin, and 3′-methoxydaidzin were determined to be 1.75%, 0.65%, 0.31%, 1.56%, and 1.06%, respectively. These findings indicate that the precision of the analytical instrument meets the requirements of the test.

### 3.2.3 Stability test results

The RSD values of baicalein, liquiritigenin, neoliquiritin, diosgenin, and 3′-methoxydaidzin were found to be 1.78%, 1.71%, 1.96%, 1.10%, and 1.98%, respectively. These results show that the solutions of P. sibiricum slices attain good stability within 24 h.

### 3.2.4 Repeatability test results

The RSD values of baicalein, liquiritigenin, neoliquiritin, diosgenin, and 3′-methoxydaidzin were found to be 1.90%, 1.86%, 1.96%, 1.66%, and 0.92%, respectively. These results indicate that the method has good repeatability.

### 3.2.5 Sample recovery test results

The baicalein, liquiritigenin, neoliquiritin, diosgenin, and 3′-methoxydaidzin average recoveries were 99.17%, 100.54%, 98.54%, 103.07%, and 99.08%; the RSD values were 1.51%, 0.87%, 1.64%, 1.09%, and 1.62%, respectively. These results demonstrate that the method satisfies the requirements of the spiked sample recovery test, indicating a high degree of accuracy.

## 3.3 Determination of indicator metabolites and polysaccharide content

The contents of the five indicator metabolites in *P. sibiricum* slices were quantified using the method detailed in Section 2.5. The polysaccharide content was assayed by the technique described in Section 2.6. The resultant data are presented in Table 4.

The results in Table 4 indicate that *P. sibiricum* slices of different origins exhibit significant differences in all indicator metabolites, except for diosgenin. The highest contents of baicalein, liquiritigenin, neoliquiritin, 3′-methoxydaidzin, and polysaccharides originated from samples S16, S5, S16, S6, and S8, respectively.

TABLE 4 Determination results of five metabolites and polysaccharide in *P. sibiricum* slices from different origins and batches ($n$ = 3).

| No. | Mass fraction/mg·g$^{-1}$ | | | | | |
|---|---|---|---|---|---|---|
| | Baicalein | Liquiritigenin | Neoliquiritin | Diosgenin | 3′-methoxydaidzin | Polysaccharide |
| S1 | 37.286f | 10.457bc | 2.091cde | 3.899a | 8.690fg | 186.3c |
| S2 | 68.295a | 14.871a | 2.339abc | 3.894a | 26.089b | 194.0b |
| S3 | 54.156c | 9.208d | 2.242abcd | 3.96a | 9.911de | 159.6e |
| S4 | 33.917g | 11.058b | 1.629e | 3.919a | 7.916gh | 118.0g |
| S5 | 39.032e | 7.937e | 2.479abc | 4.010a | 8.725fg | 172.7d |
| S6 | 42.454d | 13.87a | 2.390abc | 3.902a | 11.6934c | 116.2f |
| S7 | 38.501e | 10.796bc | 2.516abc | 3.890a | 9.638def | 184.0c |
| S8 | 68.648a | 14.888a | 2.727ab | 3.869a | 26.577b | 209.5a |
| S9 | 55.529b | 10.368bc | 2.140cde | 4.400a | 10.105d | 157.3e |
| S10 | 54.805bc | 11.143b | 2.218abcd | 4.013a | 9.431ef | 156.1e |
| S11 | 30.177i | 11.226b | 1.637e | 3.827a | 7.464h | 117.4g |
| S12 | 34.609g | 11.268b | 1.705de | 3.814a | 8.950ef | 115.0g |
| S13 | 32.508h | 14.527a | 2.001cde | 3.970a | 8.828fg | 114.8g |
| S14 | 68.634a | 14.387a | 2.463abc | 4.338a | 46.944a | 196.1b |
| S15 | 55.345b | 9.852cd | 2.158bcde | 3.912a | 9.349ef | 154.2e |
| S16 | 68.003a | 14.324a | 2.781a | 4.429a | 26.71b | 181.0c |

Note: Different letters in the same column indicate $P < 0.05$.

TABLE 5 PCA results.

| Principal component | Initial eigenvalue | | | Extract the Sum of squares load | | |
|---|---|---|---|---|---|---|
| | Summation | Variance contribution/% | Cumulative contribution/% | Summation | Variance contribution/% | Cumulative contribution/% |
| 1 | 8.011 | 52.219 | 52.219 | 8.011 | 52.219 | 52.219 |
| 2 | 2.324 | 16.601 | 73.820 | 2.324 | 16.601 | 73.820 |
| 3 | 1.100 | 7.858 | 81.678 | 1.100 | 7.858 | 81.678 |
| 4 | 0.897 | 6.409 | 88.087 | | | |
| 5 | 0.762 | 5.439 | 93.526 | | | |
| 6 | 0.415 | 2.967 | 96.493 | | | |
| 7 | 0.225 | 1.607 | 98.101 | | | |
| 8 | 0.120 | 0.855 | 98.955 | | | |
| 9 | 0.063 | 0.453 | 99.408 | | | |
| 10 | 0.045 | 0.318 | 99.726 | | | |
| 11 | 0.032 | 0.227 | 99.954 | | | |
| 12 | 0.004 | 0.031 | 99.985 | | | |
| 13 | 0.002 | 0.014 | 99.999 | | | |
| 14 | $9.597 \times 10^{-5}$ | 0.001 | 100.000 | | | |

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TABLE 6 Composition matrix of 14 indicators in *P. sibiricum* slices.

| Principal component | Payloads | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mass | Thickness | Long diameter | Short diameter | *L** | *a** | *b** | Δ*E* | Baicalein | Liquiritigenin | Neoliquiritin | Diosgenin | 3′-methoxydaidzin | Polysaccharide |
| 1 | 0.962 | 0.670 | 0.946 | 0.619 | −0.646 | 0.288 | −0.843 | −0.955 | 0.866 | 0.601 | 0.789 | 0.146 | 0.878 | 0.843 |
| 2 | 0.190 | 0.363 | 0.218 | −0.365 | 0.623 | −0.587 | −0.490 | 0.040 | −0.163 | 0.493 | −0.411 | 0.619 | 0.208 | −0.371 |
| 3 | −0.044 | −0.061 | −0.059 | −0.484 | 0.085 | 0.580 | −0.031 | 0.038 | 0.104 | 0.575 | 0.131 | −0.292 | 0.060 | −0.251 |

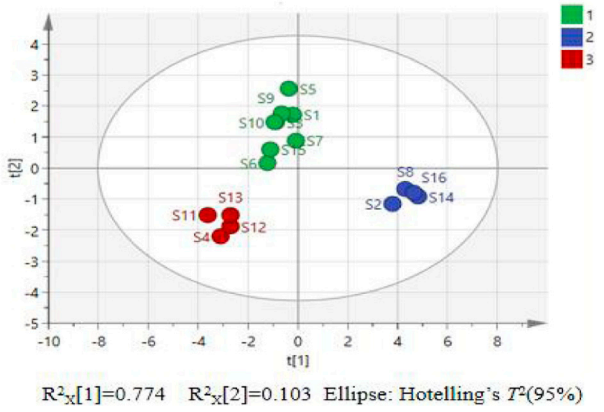


FIGURE 3
PCA score chart for 16 batches of *P. sibiricum* slices.

## 3.4 PCA and OPLS-DA

### 3.4.1 PCA

PCA was applied to the 16 batches of *P. sibiricum* slices, with the appearance index data (mass, thickness, long diameter, short diameter, and chromaticity values *L**, *a**, *b**, Δ*E*) and the contents of baicalein, liquiritigenin, neoliquiritin, diosgenin, 3′-methoxydaidzin, and polysaccharides as the variables. The analysis was performed using SPSS27.0 software. The results are presented in Tables 5, 6.

As shown in Table 5, the first three principal components exhibit eigenvalues greater than 1 (8.011, 2.324, and 1.100) (Zhang et al., 2024b), contributing variances of 52.219%, 16.601%, and 7.858%, respectively. Collectively, these components account for 81.678% of the total variation in *P. sibiricum* slices. Table 6 demonstrates that mass, thickness, long diameter, short diameter, *L**, *b**, Δ*E*, baicalein, liquiritigenin, neoliquiritin, 3′-methoxydaidzin, and polysaccharides exhibit higher loadings on the first principal component, while *a** and diosgenin are more strongly associated with the second principal component. A PCA model was constructed using SIMCA 14.1 software to explore the clustering patterns of *P. sibiricum* slices. The results are presented in Figure 3.

As shown in Figure 3, the 16 batches of *P. sibiricum* slices can be divided into three categories with significant clustering and dispersion, indicating that there are large differences in quality between the categories. Samples S4 and S11–S13 are clustered into one category; S2, S8, S14, and S16 are clustered into another category; and the remaining samples are clustered into a third category.

### 3.4.2 OPLS-DA

To further investigate the factors influencing the quality differences between categories, the data matrix of 14 indicators in the 16 batches of *P. sibiricum* slices was imported into SIMCA 14.1 and OPLS-DA was applied. The results are presented in Figures 4, 5. The model parameters $R^2_X = 0.986$, $R^2_Y = 0.971$, and $Q^2 = 0.853$ indicate that the model has good stability, reliability, and predictive ability.

Figure 4 reveals that the 16 batches of *P. sibiricum* slices are categorized into three distinct groups, consistent with the PCA results.

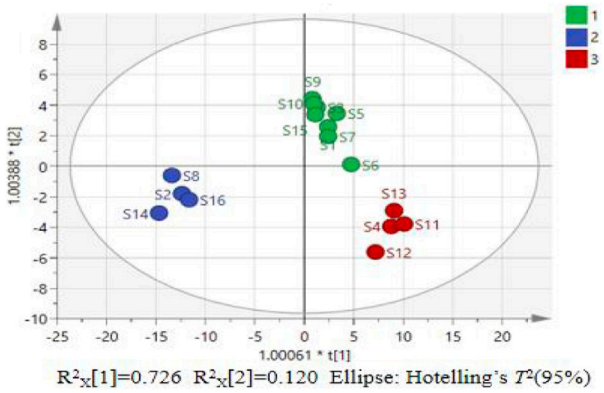


FIGURE 4
Score chart of OPLS-DA model for 16 batches of *P. sibiricum* slices.

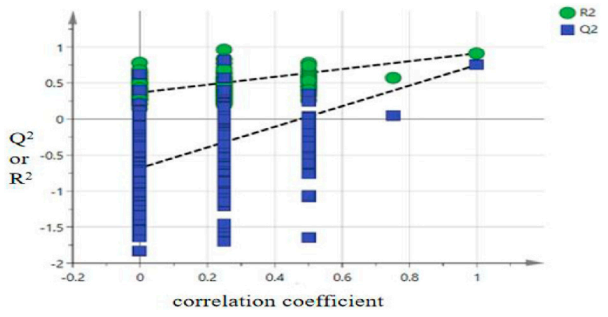


FIGURE 6
OPLS-DA replacement detection results.

Figure 5 presents the VIP values. Applying a VIP threshold >1 as the screening criterion (Gan et al., 2021), polysaccharides, long diameter, baicalein, $b^*$, and 3′-methoxydaidzin were identified as key markers for quality classification of *P. sibiricum* slices. These parameters exhibit substantial contributions to the overall quality evaluation and can effectively discern quality variations. The OPLS-DA model was validated through 200 permutation tests, with results displayed in Figure 6.

Figure 6 shows that the resultant $R^2$ fitted curve intercepts the Y-axis below Y = 0.3 and the $Q^2$ fitted curve intercepts the Y-axis below Y = 0. The results of cross validation analysis of variance (CV-ANOVA) indicated that the F value was 135.76 ($P < 0.01$), suggesting that the model was significantly effective. Therefore, the OPLS-DA model can be applied to evaluate the quality of *P. sibiricum* slices from different regions.

## 3.5 Matrix calculation of weighted TOPSIS

Table 7 presents the TOPSIS weighting matrix for each index, and Table 8 lists the distances between each evaluation index and the positive/negative ideal samples ($d_i^+$, $d_i^-$).

The data presented in Tables 7,8 demonstrate that the distances between the 16 batches of samples and the positive ideal samples span from 0.5138 to 3.4690, while the distances to the negative ideal samples range from 0.6597 to 3.4573. Samples S2, S8, and S14 exhibit relatively higher quality, whereas S4, S11, and S13 show markedly lower quality. These findings indicate that *P. sibiricum* slices sourced from diverse geographical origins exhibit a heterogeneous quality profile, with statistically significant disparities.

## 3.6 Relative correlation degree of GRA

As shown in Table 9, the correlation coefficients of the 16 batches of *P. sibiricum* slices range from 0.3895 to 0.8094 for the optimal reference sequence and 0.3811 to 0.8085 for the worst reference sequence. Notably, samples S2, S8, S14, and S16 exhibit significantly stronger correlations with the optimal sequence (coefficients >0.7000), indicating superior quality. In contrast, samples S4, S11, S12, and S13 demonstrate higher correlations with the worst sequence (coefficients >0.7000), suggesting markedly inferior quality. These findings are consistent with the results of the weighted TOPSIS model.

## 3.7 Calculation of relative closeness κ

The results of the weighted TOPSIS-GRA fusion model demonstrate that the average relative closeness values of the 16 batches of *P. sibiricum* slices range from 0.2526 to 0.7635 (Table 10), indicating substantial quality variation across different geographical origins. The top four samples (S14, S8, S16, and S2) exhibit relative closeness values exceeding 0.7000, while the remaining samples fall below 0.6000. Notably, *P.*

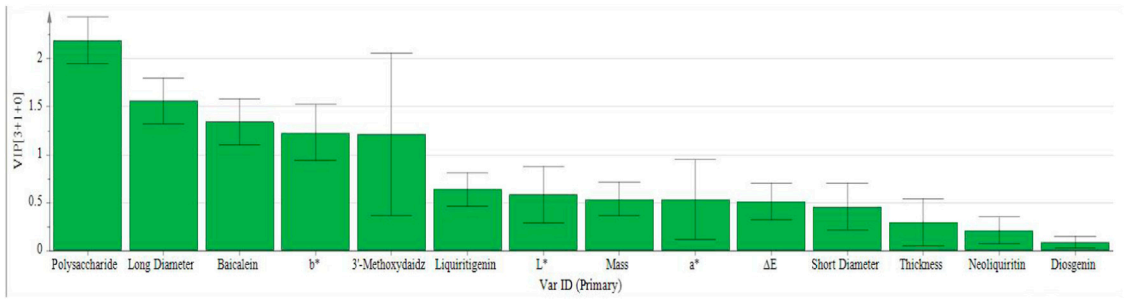


FIGURE 5
Variable importance in projection images of 16 batches of *P. sibiricum* slices.

TABLE 7 Weighting matrix.

| No. | $Z_{ij}$ | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mass | Thickness | Long diameter | Short diameter | *L** | *a** | *b** | Δ*E* | Baicalein | Liquiritigenin | Neoliquiritin | Diosgenin | 3′-methoxydaidzin | Polysaccharides |
| S1 | 0.2126 | 0.0938 | 0.3999 | 0.1529 | 0.5865 | 0.2839 | 0.0631 | 0.5147 | 0.2485 | 0.2322 | 0.0857 | 0.0123 | 0.0378 | 1.6544 |
| S2 | 0.5363 | 0.2145 | 1.5615 | 0.3063 | 0.4489 | 0.2180 | 1.2320 | 0.3871 | 1.3325 | 0.6390 | 0.1317 | 0.0115 | 0.5735 | 1.8325 |
| S3 | 0.0605 | 0.0873 | 0.2416 | 0.2274 | 0.3728 | 0.1693 | 0.2022 | 0.2439 | 0.8383 | 0.1172 | 0.1137 | 0.0211 | 0.0754 | 1.0367 |
| S4 | 0.1590 | 0.1441 | 0.1794 | 0.1186 | 0.0000 | 0.3851 | 0.3633 | 0.0000 | 0.1307 | 0.2876 | 0.0000 | 0.0151 | 0.0139 | 0.0741 |
| S5 | 0.1644 | 0.0764 | 0.2597 | 0.4615 | 0.4397 | 0.2435 | 0.0303 | 0.2913 | 0.3096 | 0.0000 | 0.1577 | 0.0283 | 0.0388 | 1.3397 |
| S6 | 0.1449 | 0.0699 | 0.2794 | 0.1275 | 0.2817 | 0.0000 | 0.1698 | 0.1585 | 0.4291 | 0.5467 | 0.1412 | 0.0127 | 0.1376 | 0.0324 |
| S7 | 0.1595 | 0.2165 | 0.3913 | 0.0726 | 0.4464 | 0.2531 | 0.1138 | 0.3746 | 0.2910 | 0.2634 | 0.1639 | 0.0110 | 0.0670 | 1.6011 |
| S8 | 0.5389 | 0.2248 | 1.5483 | 0.3135 | 0.4541 | 0.2144 | 1.1324 | 0.4166 | 1.3449 | 0.6405 | 0.2037 | 0.0079 | 0.5885 | 2.1912 |
| S9 | 0.1014 | 0.0129 | 0.3291 | 0.2255 | 0.3483 | 0.1584 | 0.0000 | 0.2819 | 0.8863 | 0.2240 | 0.0948 | 0.0846 | 0.0813 | 0.9834 |
| S10 | 0.0725 | 0.0000 | 0.2855 | 0.2597 | 0.3799 | 0.1431 | 0.1162 | 0.2032 | 0.8610 | 0.2954 | 0.1093 | 0.0287 | 0.0605 | 0.9556 |
| S11 | 0.0000 | 0.0590 | 0.0886 | 0.0598 | 0.1657 | 0.3915 | 0.3766 | 0.0468 | 0.0000 | 0.3031 | 0.0015 | 0.0019 | 0.0000 | 0.0603 |
| S12 | 0.0817 | 0.0708 | 0.6640 | 0.1858 | 0.1397 | 0.5351 | 0.4207 | 0.1369 | 0.1549 | 0.3069 | 0.0141 | 0.0000 | 0.0457 | 0.0046 |
| S13 | 0.0579 | 0.0913 | 0.0000 | 0.0000 | 0.1930 | 0.2486 | 0.3124 | 0.0460 | 0.0815 | 0.6073 | 0.0690 | 0.0225 | 0.0419 | 0.0000 |
| S14 | 0.4849 | 0.2239 | 1.5628 | 0.3097 | 0.4638 | 0.2177 | 1.1630 | 0.4148 | 1.3443 | 0.5943 | 0.1547 | 0.0756 | 1.2157 | 1.8811 |
| S15 | 0.2211 | 0.1303 | 0.3229 | 0.2084 | 0.2185 | 0.1334 | 0.1396 | 0.1568 | 0.8798 | 0.1765 | 0.0981 | 0.0141 | 0.0580 | 0.9118 |
| S16 | 0.5074 | 0.2994 | 1.5448 | 0.3162 | 0.4054 | 0.2089 | 1.1896 | 0.3942 | 1.3223 | 0.5886 | 0.2137 | 0.0887 | 0.5926 | 1.5316 |





10.3389/fphar.2025.1547398

TABLE 8 Values of $d_i^+$ and $d_i^-$ in TOPSIS evaluation of *P. sibiricum* slices.

| No. | $d_i^+$ | $d_i^-$ |
|---|---|---|
| S1 | 2.4680 | 1.9474 |
| S2 | 0.8911 | 3.2666 |
| S3 | 2.5621 | 1.4831 |
| S4 | 3.3408 | 0.6916 |
| S5 | 2.6619 | 1.6030 |
| S6 | 3.1906 | 0.8812 |
| S7 | 2.4437 | 1.8429 |
| S8 | 0.7534 | 3.4573 |
| S9 | 2.6008 | 1.4903 |
| S10 | 2.5972 | 1.4601 |
| S11 | 3.4302 | 0.6597 |
| S12 | 3.1416 | 1.0532 |
| S13 | 3.4690 | 0.7700 |
| S14 | 0.5138 | 3.4376 |
| S15 | 2.6030 | 1.4046 |
| S16 | 1.0022 | 3.0753 |

TABLE 9 Values of $q_i^+$ and $q_i^-$ in GRA evaluation of *P. sibiricum* slices.

| No. | $q_i^+$ | $q_i^-$ |
|---|---|---|
| S1 | 0.5191 | 0.5989 |
| S2 | 0.7502 | 0.4266 |
| S3 | 0.4470 | 0.6259 |
| S4 | 0.4072 | 0.7625 |
| S5 | 0.4988 | 0.6277 |
| S6 | 0.4352 | 0.6847 |
| S7 | 0.5157 | 0.5972 |
| S8 | 0.7951 | 0.4186 |
| S9 | 0.4783 | 0.6219 |
| S10 | 0.4457 | 0.6405 |
| S11 | 0.3895 | 0.8085 |
| S12 | 0.4400 | 0.7084 |
| S13 | 0.4219 | 0.7506 |
| S14 | 0.8094 | 0.3811 |
| S15 | 0.4346 | 0.6390 |
| S16 | 0.7685 | 0.3872 |

*sibiricum* slices from Zunyi (Guizhou Province), Yibin (Sichuan Province), Jinggu (Yunnan Province), and Shizhu (Chongqing Municipality) show significantly higher quality compared to other regions within the same provinces. These findings align with historical records of *P. sibiricum*'s traditional Dao-di producing areas (Ma et al., 2019), thereby validating the reliability of the TOPSIS-GRA fusion model.

TABLE 10 Quality sequencing of 16 batches of *P. sibiricum* slices.

| No. | $D_i^+$ | $D_i^-$ | $Q_i^+$ | $Q_i^-$ | $G_i^+$ | $G_i^-$ | $k$ | Sort |
|---|---|---|---|---|---|---|---|---|
| S1 | 0.7114 | 0.5633 | 0.6413 | 0.7408 | 0.6023 | 0.7261 | 0.4534 | 5 |
| S2 | 0.2569 | 0.9448 | 0.9269 | 0.5276 | 0.9359 | 0.3923 | 0.7046 | 4 |
| S3 | 0.7386 | 0.4290 | 0.5523 | 0.7741 | 0.4907 | 0.7564 | 0.3935 | 9 |
| S4 | 0.9630 | 0.2000 | 0.5031 | 0.9431 | 0.3516 | 0.9531 | 0.2695 | 15 |
| S5 | 0.7673 | 0.4637 | 0.6163 | 0.7764 | 0.5400 | 0.7719 | 0.4116 | 7 |
| S6 | 0.9197 | 0.2549 | 0.5377 | 0.8469 | 0.3963 | 0.8833 | 0.3097 | 13 |
| S7 | 0.7044 | 0.5330 | 0.6371 | 0.7387 | 0.5851 | 0.7216 | 0.4478 | 6 |
| S8 | 0.2172 | 1.0000 | 0.9823 | 0.5177 | 0.9912 | 0.3675 | 0.7295 | 2 |
| S9 | 0.7497 | 0.4311 | 0.5909 | 0.7692 | 0.5110 | 0.7595 | 0.4022 | 8 |
| S10 | 0.7487 | 0.4223 | 0.5507 | 0.7922 | 0.4865 | 0.7705 | 0.3870 | 10 |
| S11 | 0.9888 | 0.1908 | 0.4812 | 1.000 | 0.3360 | 0.9944 | 0.2526 | 16 |
| S12 | 0.9056 | 0.3046 | 0.5436 | 0.8762 | 0.4241 | 0.8909 | 0.3225 | 12 |
| S13 | 1.0000 | 0.2227 | 0.5213 | 0.9284 | 0.3720 | 0.9642 | 0.2784 | 14 |
| S14 | 0.1481 | 0.9943 | 1.000 | 0.4714 | 0.9972 | 0.3089 | 0.7635 | 1 |
| S15 | 0.7504 | 0.4063 | 0.5369 | 0.7904 | 0.4716 | 0.7704 | 0.3797 | 11 |
| S16 | 0.2889 | 0.8895 | 0.9495 | 0.4789 | 0.9195 | 0.3839 | 0.7055 | 3 |

# 4 Discussion

## 4.1 Relationship between morphological traits and quality attributes in *P. sibiricum* slices

Morphological traits are critical yet undervalued in *P. sibiricum* quality assessment. Single-marker methods are insufficient; integrating morphological and metabolic perspectives is essential (Chinese Pharmacopoeia, 2020; Yuan and Wang, 2024). Processed *P. sibiricum* quality links to traits like slice long diameter and chromatic parameter $b^*$, reflecting regional environments and bioactive metabolite accumulation (Liang et al., 2025; Shao et al., 2025). Longer diameters indicate better-developed tissues (potentially higher active ingredients), while $b^*$ correlates with specific metabolites, signaling environmental impacts on biosynthesis. These traits are indispensable for quality evaluation, offering insights into metabolic processes and therapeutic efficacy.

## 4.2 Regional differences in polysaccharide and metabolite profiles of *P. sibiricum* slices

Geographical differences significantly shape *P. sibiricum* polysaccharide and metabolite profiles. OPLS-DA revealed distinct patterns: Sichuan and Guizhou samples exhibited elevated polysaccharides and region-specific metabolites (e.g., flavonoids,

saponins) (Chen et al., 2024). Polysaccharides, a key pharmacopeial metabolite, underpin pharmacological effects, while metabolites like 3′-methoxydaidzin and baicalein—regionally variable—exhibit anti-inflammatory activities. Aligned with the "Dao-di botanical drugs" concept, these differences facilitate standardized sourcing and quality control in pharmaceutical applications.

### 4.3 Multi-model integrated approach for quality evaluation of *P. sibiricum* slices

This study introduces a TOPSIS-GRA methodology weighted by OPLS-DA-derived VIP values, providing a robust framework for *P. sibiricum* quality assessment. By incorporating VIP values—quantifying variable importance in sample differentiation—the model mitigates subjectivity, enabling objective, comprehensive evaluations. Industrially, it supports quality prioritization, raw material selection, and origin traceability—critical for pharmaceutical compliance. Consistent with empirical evidence, this approach ensures safety and quality in *P. sibiricum*-based products by integrating multi-dimensional factors.

In summary, this research establishes a morphology-metabolism linkage paradigm, advancing TCM quality control. Future studies should dissect biosynthetic pathways underlying regional traits to optimize cultivation, ensuring clinical consistency and enhancing TCM product quality.

## 5 Conclusion

We demonstrated that high-quality *P. sibiricum* slices are typically distinguished by their elongated, yellowish-white appearance, and contain elevated levels of polysaccharides, baicalein, and 3′-methoxydaidzin. Manufacturers should concentrate on managing the five quality-determining factors identified above, while sellers and consumers can perform preliminary selection based on the length and color attributes of the slices. Our group intends to further investigate the correlation between the regional characteristics of *P. sibiricum* slices and their appearance, chemical composition, and pharmacological activities. This will lay the groundwork for the development of a quality control system for botanical drugs that embody the three excellences of shape, quality, and superior effect.

## 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

CW: Conceptualization, Writing – original draft, Writing – review and editing. JY: Conceptualization, Funding acquisition, Writing – original draft. SJ: Writing – review and editing. XH: Writing – review and editing. MM: Supervision, Writing – original draft. LY: Supervision, 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 authors declare that no Generative AI was used in the creation of this manuscript.

## Publisher's note



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# An artificial intelligence-aided scoping review of medicinal plant research in the Fertile Crescent

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Traditional Arabic and Islamic Medicine (TAIM) originated in the seventh century, but unlike Chinese and Ayurvedic knowledge, TAIM has not evolved through evidence-based research and commercialization. Today, while global interest in traditional medicine is growing, TAIM ancestral knowledge remains unknown and unexplored. The purpose of this study is to provide baseline information on the status of TAIM research to guide future research and contribute to the growth of the sector. The focus of the study is the Fertile Crescent, a region of the Arab World endowed with a rich and diverse eco-geography. The method adopted was a scoping review following the Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA) guidelines. The databases used included the Arab World Research Source: Al Masdar, CAB Direct, Iraqi Academic Scientific Journals, MEDLINE, Scopus, Web of Science and Google Scholar. The timeline of the search spanned from the database inception date to June 2024. The search led to 10,171 records which were subsequently reduced to 1,990 publications after deleting duplicates and performing a two-stage screening. Artificial intelligence (AI) technology was used to analyze the data focusing on reported plant species, treatment applications, study types and countries. The Generative Pretrained Transformer 4 (GPT-4) Turbo, a large language model, was used to extract the key features and the results were validated by the researchers. The findings revealed that the types of studies were mostly laboratory-based (86%), while few studies (14%) were field based. The top five treatment applications include cancer (29%), bacterial infections (22%), inflammation (12%), fungal infections (9%), and diabetes (8%). The most notable plant species that were under investigation in the various studies were *Nigella sativa* L. (Ranunculaceae), *Rosmarinus officinalis* L. (Lamiaceae), *Salvia fruticosa* Mill (Lamiaceae), *Teucrium polium* L. (Lamiaceae), and *Thymus vulgaris* L. (Lamiaceae). In this review we discuss our findings which suggest potential avenues for further developing TAIM research and exploring the development of botanical drugs. Our findings also revealed that the number of ethnobotanical studies was limited suggesting an urgent need to prevent the loss of ancestral knowledge by formalizing it through evidence-based research and policy

guidelines. Addressing these gaps through interdisciplinary collaboration and improved data-sharing mechanisms will be crucial for advancing TAIM research and medicinal plants.

KEYWORDS

**medicinal plants, Arab medicine, Islamic medicine, Fertile Crescent, pharmacology, artificial intelligence, herbal medicine**

# 1 Introduction

Traditional Arabic and Islamic Medicine (TAIM) is unique in that Islamic scholars in the seventh century integrated Graeco-Roman, Chinese, Persian, and Ayurvedic medical knowledge with Arab knowledge (Alrawi and Fetters, 2012). However, today while TAIM remains informal and fragmented, traditional plant-based medicine originating in other parts of the world are widely investigated, commercialized and practiced, especially in rural areas and developing economies (Azaizeh et al., 2006; Manaf et al., 2017). The need to strengthen and formalize TAIM stems from the fact that traditional and complementary medicine plays a significant role in healthcare in the Arab World, with a projected market growth of 23% annually from 2020 to 2027 (Grand View Research, 2023). Yet, TAIM research does not seem to be strengthening the sector. Several reasons may explain the stagnation of the sector, these probably include poor collaboration between traditional healers, ethnobotanists, pharmacists, and biomedical researchers as well as limited access to standardized, consolidated databases on medicinal plant usage in the region (Azaizeh et al., 2006). To advance the TAIM sector, collaboration, data sharing, and robust, evidence-based research on the identity, safety, toxicity, and dosage of used medicinal plants are required (AlRawi et al., 2017; Chaachouay and Zidane, 2024; Naja et al., 2015; Zbeeb et al., 2024). Focusing on the Fertile Crescent region of the Arab World, this review seeks to fill a gap by presenting a comprehensive overview of TAIM research.

The Fertile Crescent is a region of the Arab World which today includes Iraq, Syria, Lebanon, Jordan, and Palestine. It strategically bridges Asia, Africa, and Europe and has played a pivotal role in the development of ancient civilizations due to its fertile soil, access to water sources, and proximity to trade routes connecting these continents. In fact, the Fertile Crescent is often referred to as the "cradle of civilization" because of its ancient and rich cultural and historical heritage (Lawrence et al., 2016). Ecologically, the Fertile Crescent presents a unique combination of fertile plains, mountains, and deserts (Hekmat and Al-Obeidi, 2019) that have led to a diverse range of climates and ecosystems that support a wide range of flora and fauna. Agriculturally, the Fertile Crescent's fertile soil and favorable climate have made it ideal for the cultivation of crops, including medicinal plants, which have been used for centuries by ancient civilizations to treat various ailments and diseases (Zeder, 2024). The identification and analysis of 1,990 studies spanning nearly a century (1933–2024) in this review offers the largest compiled dataset on medicinal plants research in the Fertile Crescent. To ensure a timely publication of the studies collected, we resorted to AI-based text analysis, specifically GPT-4 Turbo, because manual data extraction is time-consuming and inconsistent. AI technology enables rapid processing of large datasets, ensuring a comprehensive and standardized approach to documenting collected data.

We expect the compilation of articles would enhance understanding of research trends and therapeutic applications making it a foundational resource for future studies. Additionally, by highlighting the most frequently studied species, such as *Nigella sativa* L. (Ranunculaceae), *Rosmarinus officinalis* L. (Lamiaceae), *Salvia fruticosa* Mill. (Lamiaceae), *Teucrium polium* L. (Lamiaceae), and *Thymus vulgaris* L. (Lamiaceae), we provide insights into which plants species have been prioritized for research. Furthermore, by categorizing dominant therapeutic uses, including cancer, bacterial infections, inflammation, diabetes, and fungal infections, we offer a roadmap for prioritizing future research collaborations across the region. The low number of ethnobotanical field studies (14%) we found compared to laboratory-based research (86%) underscores the need for more extensive documentation of traditional healing practices before they are lost. This finding should encourage future collaborations between researchers and local communities, ensuring that traditional knowledge is preserved and scientifically validated. Moreover, by identifying medicinal plants at risk due to overharvesting, such as *T. polium* L. (Lamiaceae) and *Origanum syriacum* L. (Lamiaceae), our findings play a supporting role for advocating for conservation policies and sustainable cultivation programs to protect biodiversity and maintain the availability of these valuable resources. On a policy level, we provide actionable recommendations for integrating traditional medicinal plants into modern healthcare systems. This includes developing standardized guidelines for safe medicinal plant use, implementing pharmacovigilance programs to monitor herb-drug interactions, and promoting community-based conservation programs to ensure sustainability. These efforts are essential for ensuring that medicinal plant research translates into practical healthcare applications while safeguarding regional biodiversity. Furthermore, our research is pioneering in its use of AI (GPT-4 Turbo) for large-scale text extraction in ethnopharmacology. By demonstrating the potential of AI to accelerate literature reviews and trend analysis, we pave the way for more efficient and systematic data processing in future medicinal plant research.

# 2 Methodology

The scoping review was designed and conducted following the Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA) guidelines (Figure 1). A comprehensive literature search was initiated on June 14–16, 2023, across multiple electronic databases, including Arab World Research Source: Al Masdar® (EBSCO), CABI Digital Library®, Iraqi Academic Scientific

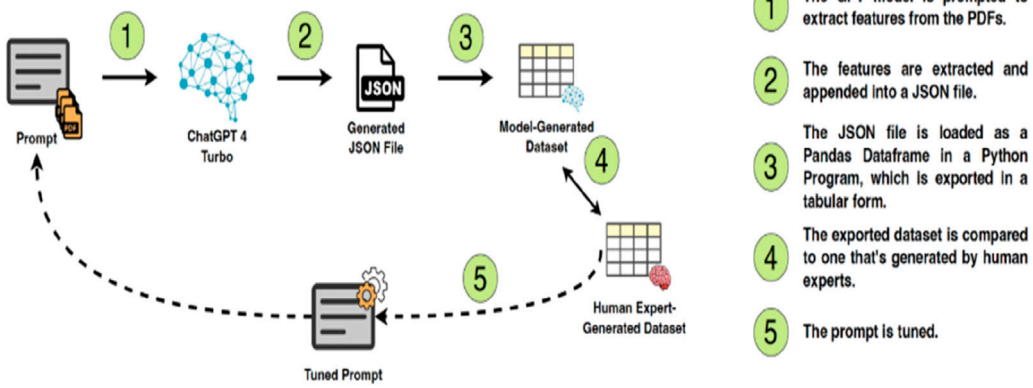


FIGURE 1
Extraction process roadmap.

Journals®, MEDLINE® (Ovid), Scopus®, and Web of Science Core Collection® and Google Scholar®. To capture new publications, email alerts were set up in databases supporting this feature, covering updates until June 2024. The search strategy incorporated keywords related to medicinal plants and their associated concepts (e.g., herbal medicine, traditional medicine, phytotherapy), as well as geographic terms relevant to the Fertile Crescent (Iraq, Jordan, Lebanon, Palestine, and Syria). Full search strategies for each database are detailed in Supplementary File 1. Eligible sources comprised peer-reviewed journal articles, conference papers, and dissertations on medicinal plants used in the Fertile Crescent. Only publications in English or Arabic were considered, while studies lacking scientific plant names or containing incomplete data were excluded. No restrictions were applied to publication year, and all relevant documents available up to June 2024 were included. A total of 10,067 records were retrieved across all sources and were imported into reference management software (EndNote) for deduplication and screening. After removing duplicates, 6,038 unique records remained. An additional 104 records were identified through e-mail alerts, bringing the final dataset to 6,142 records for screening. Two investigators (Al-Sammarraie RN and Naalbandian S) independently reviewed titles and abstracts for relevance, followed by a full-text assessment of potentially eligible studies based on predefined criteria. Any discrepancies were resolved through discussion or consultation with a third reviewer (Talhouk SN). The two-stage screening process identified a total of 1,990 publications deemed relevant for data extraction. Quality assessment was performed based on relevance to the research scope, study rigor, and completeness of reported findings. Scientific names were standardized using the Medicinal Plant Names Services (MPNS) and Plants of the World Online (POWO) to ensure taxonomic accuracy.

To process a large volume of research efficiently, data extraction was performed using Generative Pretrained Transformer 4 (GPT-4) Turbo, a large language model (LLM) developed by OpenAI. The model was not fine-tuned on a custom dataset but was instead prompted iteratively using a structured approach to ensure optimal performance (Huang et al., 2024). LLMs leverage attention, deep learning architectures, and massive datasets that are in conversational AI applications and can generate human-like natural language text based on prompts. It is one of the largest and most powerful language models available, with 175 billion parameters (Jupudi, 2018; Sharma, 2024). In this study, OpenAI's ChatGPT 4 Turbo is employed to extract key features from the comprehensive dataset of research publications using identifying key features, including scientific names of plants, reported therapeutic applications, type of research (laboratory-based *versus* field-based), and any pharmacological properties mentioned.

After applying the inclusion/exclusion criteria, the dataset comprised 1,990 research papers, ranging in length from 1 to 705 pages, all written in English, with publication dates spanning from 1958 to 2024. The extracted features included the title of the paper, the country of origin of the plant, the type of research conducted, the name of the medicinal plant discussed, whether the plant is traditional or non-traditional, its status as native or indigenous, its toxicity, and whether the plant was used as a treatment for various conditions such as skin disorders, respiratory problems, liver function improvement, and snake bites. The roadmap of the PDF feature extraction process is visualized in Figure 1.

In the initial phase, features were extracted from 300 PDFs. Each PDF was processed individually using a unified prompt. The extracted features were then compared with those manually determined by human experts. This comparison enabled several iterations of prompt refinement and tuning to ensure the model's outputs closely aligned with the human-generated features. The extracted features were then loaded into a Pandas DataFrame in Python program for data analysis. Python is a high-level, interpreted, multi-purpose programming language that can be used for many applications that include statistical computing with various packages and functions (Kiranbala Nongthombam, 2021). The program was designed to process the extracted information, generate interpretations and analyses aligned with the objectives of the scoping review, and create visual representations of the data. These analyses provided valuable insights into research trends and the characteristics of medicinal plants in the Fertile Crescent. The final results were reviewed by human experts before inclusion in the database.

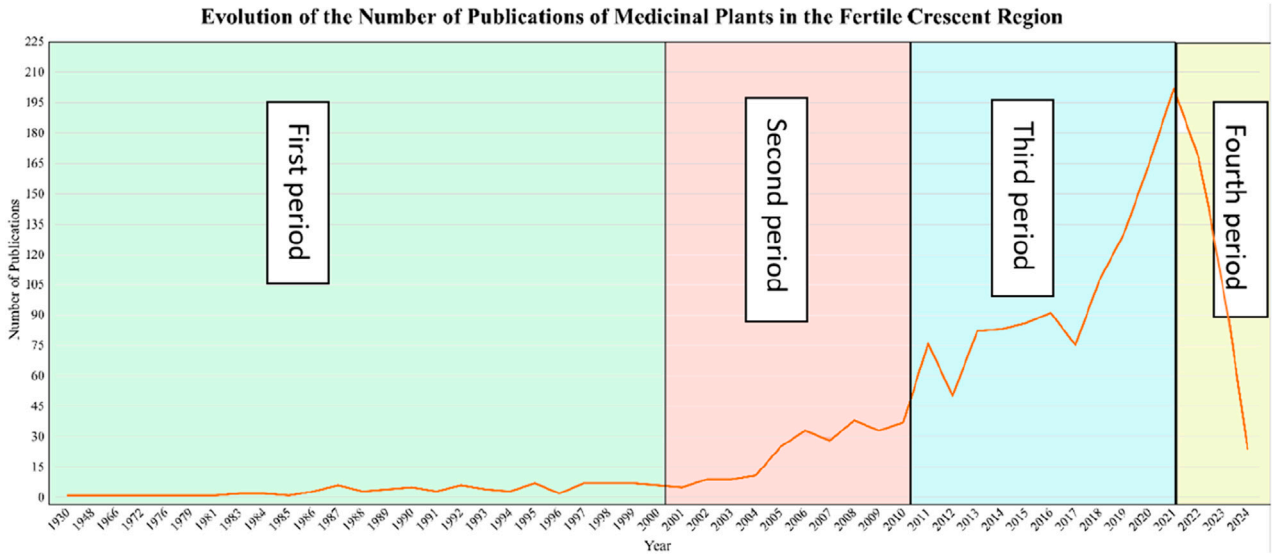


FIGURE 2
Temporal evolution of medical plants publications in the Fertile Crescent.

## 2.1 Potential biases and limitations

The integration of AI into literature reviews offers notable advantages, such as increased efficiency in data processing and synthesis. However, it also introduces several challenges that researchers must navigate to maintain the integrity and quality of their work. A prominent concern is the phenomenon known as "hallucination," where AI systems generate information that appears plausible but is incorrect or misleading (Mostafapour et al., 2024). This occurs because AI models, despite their advanced capabilities, lack genuine understanding and rely solely on patterns learned from data. Therefore, despite the advantages of integrating AI into literature review, human validation is still needed to correct minor errors in plant classification or therapeutic claims. Moreover, AI models are limited by their training data and may struggle with nuanced ethnobotanical terminology or context-specific meanings. In addition, LLMs struggle in handling non-English languages that is why studies in non-English languages were excluded potentially omitting relevant findings.

# 3 Results and discussion

Figure 2 illustrates the temporal evolution of research publications on medicinal plants in the Fertile Crescent region from 1930 to 2024. We identified four distinct periods and explored the possible scientific, historical, and socio-political drivers behind the observed trends in research output.

From 1930 to 1999, medicinal plant research in the Fertile Crescent was low with fewer than 15 publications per year. This is probably because medicinal plant knowledge in the region was transmitted through oral traditions or written as Arabic compendiums rather than formal academic Eurocentric publications (Azaizeh et al., 2006). In addition, this period witnessed the end of European colonialism and was faced with economic instability, and political conflicts (Qassrawi, 2024). Additionally, during much of the 20th century, Western biomedicine dominated healthcare systems, undermining the value of traditional medicine and ethnobotanical research (World Health Organization, 2002).

A slight increase in publication numbers is observed after 2000, indicating a growing but still moderate interest in medicinal plant research. This shift aligns with the global rise of ethnopharmacology as an academic discipline and the World Health Organization's renewed efforts to integrate traditional medicine into healthcare systems (World Health Organization, 2002). The establishment of research institutions focusing on medicinal plants, such as the Lebanese University's Faculty of Pharmacy and Jordan University of Science and Technology's medicinal plant research programs, may have played a role in increasing publications during this time. Technological advancements also contributed to this trend. The early 2000s saw increased accessibility to scientific journals through digital platforms, enabling greater knowledge exchange (Haleem et al., 2022). Additionally, increased interest in biodiversity conservation, such as the Global Strategy for Plant Conservation (GSPC) initiated in 2002, encouraged more research on medicinal flora (CBD, 2002). Despite this progress, the political instability in Iraq, Syria, and Palestine limited scientific advancements in the region. The Iraq War (2003) and ongoing conflicts in Palestine and Lebanon resulted in funding cuts and displacement of researchers, which may have slowed the growth of medicinal plant research.

Between 2010 and 2020, the number of publications surged, reflecting a strong global and regional shift toward natural product research and integrative medicine. Several factors contributed to this trend. These include scientific advancements in Medicinal Plant Research, the development of high-performance liquid chromatography (HPLC), mass spectrometry (MS), and DNA barcoding allowed for more precise identification of medicinal plant compounds, leading to an increase in phytochemical and pharmacological studies (Ganzera and Sturm, 2018). The rise of

TABLE 1 The number of medicinal plant species in India, China, and the Fertile Crescent.

| Countries | Number of medicinal plant species | Unit area in square kilometers | Number of medicinal plant species per unit area (square kilometer) |
|---|---|---|---|
| India | 7,500 | 3,287,263 | 438 |
| China | 11,146 | 9,596,961 | 861 |
| Fertile Crescent | 1,141 | 1,021,452 | 895 |

bioinformatics tools and network pharmacology further enabled researchers to explore plant-based drug discovery (Hopkins, 2008). Research funding for alternative medicine and biodiversity conservation increased significantly in the 2010s, as organizations such as the International Union for Conservation of Nature (IUCN) and United Nations Development Programme (UNDP) supported projects on medicinal plant sustainability in the Fertile Crescent (International Union for Conservation of Nature, 2017). The Convention on Biological Diversity (CBD) Nagoya Protocol (2010) also encouraged fair and equitable sharing of benefits from medicinal plant resources, further driving research efforts (Convention on Biological Diversity, 2010). A growing global interest in natural therapies, dietary supplements, and herbal medicine-based treatments led to increased market demand, prompting more scientific investigations (Ekor, 2014). This period saw an expansion of herbal medicine industries in Jordan, Lebanon, and Iraq, where pharmaceutical companies began incorporating plant-based compounds into commercial products (Azaizeh et al., 2006). The recognition of habitat loss and climate change impacts on medicinal plants became a pressing issue, resulting in more ecological studies focused on plant conservation in Lebanon's Bekaa Valley, Jordan's Dana Biosphere Reserve, and Iraq's Mesopotamian wetlands (IUCN, 2018). These efforts translated into higher research output during this period.

A sharp spike in publications between 2020 and 2022 coincides with the COVID-19 pandemic, during which interest in plant-based antiviral compounds skyrocketed. Studies explored medicinal plants with antiviral, immune-boosting, and anti-inflammatory properties, leading to a surge in research output (Benarba and Pandiella, 2020). Countries in the Fertile Crescent, particularly Iraq, Jordan, and Lebanon, saw increased government and institutional funding for herbal medicine research aimed at identifying potential plant-derived treatments for COVID-19 (Ang et al., 2020). However, a steep decline after 2022 suggests a shift in research priorities post-pandemic. As COVID-19 research funding subsided, scientists may have redirected their focus toward other pharmaceutical and medical research areas. Another possible explanation for this decline is the saturation of herbal medicine publications during the pandemic, leading to fewer novel studies being conducted. The economic downturn in several Fertile Crescent countries post-pandemic may have also reduced funding for medicinal plant research, contributing to the sharp drop. The drop in 2023–2024 may also be due to incomplete data collection or publication delays, as the research only includes publications up until June 2024.

With respect to plants, the review recorded 1,141 medicinal plant species in the Fertile Crescent. This number is significant when compared with the number of medicinal plant species reported in China and India, the two leading countries in terms of traditional knowledge and research on medicinal plants. Both India and China have exceptionally rich medicinal plant diversity, with India having around 7,500 medicinal plant species and China having over 11,000 species (Huang et al., 2024; Singh et al., 2022; Xiong et al., 2020). In Table 1 we show how the Fertile Crescent compares favorably with these two countries showing the richness in diversity of medicinal plants in the region.

TABLE 2 Most investigated medicinal plant species in the Fertile Crescent and their reported traditional uses.

| Medicinal plant species | Country | Reported uses |
|---|---|---|
| *Nigella sativa* L. (Ranunculaceae) | Iraq | Immune system effects<br>Skin disorders<br>Respiratory problems |
| *Rosmarinus officinalis* L. (Lamiaceae) | Jordan | Cardiovascular system effects<br>Skin disorders<br>Respiratory problems<br>Liver function<br>Kidney function<br>Lipid panel |
| *Teucrium polium* L. (Lamiaceae) | Palestine | Urinary tract infections<br>Gastrointestinal problems |
| *Peganum harmala* L. (Nitrariaceae) | Syria | Nervous system effects |
| *Origanum syriacum* L. (Lamiaceae) | Lebanon | Gastrointestinal problems |

Globally, it is estimated that there are between 72,000 and 77,000 medicinal plant species, which represent around 17%–18% of the world's flora (Chekole et al., 2015; Hazarika et al., 2023; Tshabalala et al., 2022). China, Japan, India, and the USA are among the top countries contributing to research on traditional herbal medicine (Hazarika et al., 2023; Pironon et al., 2024).

Despite the large number of recorded medicinal plant species in the Fertile Crescent, the review sheds light on the fact that each country seems to focus on the most popular medicinal plant species locally. So, although the top research plants species are native to all five countries the priorities do not seem aligned between countries. Instead, researchers in Iraq focus on *Nigella sativa* L. (Ranunculaceae), *Rosmarinus officinalis* L. (Lamiaceae) is Jordan's focus, *Teucrium polium* L. (Lamiaceae) in Palestine, *Peganum harmala* L. (Nitrariaceae) in Syria, and *Origanum syriacum* L. (Lamiaceae) in Lebanon (Table 2).

Further elaboration about published research on these species (Table 2) shows that *Nigella sativa* L. (Ranunculaceae) is investigated in Iraq for its effects on the immune system, skin disorders, and respiratory problems. *Nigella sativa* L. (Ranunculaceae) is well known to the Arab World and to Islam

where it is described as a "*cure for every disease, except death.*" (Sahih al-Bukhari, Imam Muhammad al-Bukhari (870 AD). Numerous studies have demonstrated its ability to enhance the immune system by modulating cytokine production, increasing phagocytic activity, and stimulating the proliferation of immune cells (Abdullah et al., 2022; Al Turkmani et al., 2015). The second most reported uses are for skin disorders and respiratory problems, which can also be attributed to the plant's anti-inflammatory, antioxidant, and anti-microbial activities that are beneficial for these conditions ((Alharchan and Ashor, 2010) Aqel, 1991; Osman and Methil Kannan, 2013). *Nigella sativa* L. (Ranunculaceae) contains active compounds including thymoquinone, thymohydroquinone, dithymoquinone, thymol, and carvacrol. These compounds exhibit immunomodulatory, anti-inflammatory, and anti-microbial properties, which could explain the reported research on *N. sativa* L. (Ranunculaceae) in Iraq (Niu et al., 2021; Wei et al., 2022).

In Jordan, *Rosmarinus officinalis* L. (Lamiaceae) has been traditionally used to improve blood circulation and heart health (Khatib et al., 1998), and research has demonstrated that it possesses cardioprotective properties due to its ability to modulate lipid profiles, reduce blood pressure, and improve endothelial function. Other frequently reported uses of *R. officinalis* L. (Lamiaceae) are for skin disorders, respiratory problems, liver function, kidney function, and lipid panel, which could be explained by the plant's rich pharmacological activities, including anti-inflammatory, antioxidant, hepatoprotective, and hypolipidemic effects (Abu-Al-Basal, 2010; Umran et al., 2013). Carnosic acid, carnosol, rosmarinic acid, ursolic acid, and caffeic acid are active compounds found in *R. officinalis* L. (Lamiaceae) (Borrás-Linares et al., 2014; Vieira et al., 2022). These compounds possess antioxidant, anti-inflammatory, and cardioprotective properties, which align with the reported uses of *R. officinalis* L. (Lamiaceae) for reported research in Jordan (Sharma et al., 2020; Vieira et al., 2022).

*Teucrium polium* L. (Lamiaceae) in Palestine is mainly reported for its urinary tract effects. This plant has been traditionally used in the Middle East to treat various urinary tract disorders, including urinary tract infections, kidney stones, and bladder problems (Al-Bahtiti, 2012). Another reported use is for gastrointestinal problems, which is also a common traditional application of this plant due to its anti-inflammatory, antispasmodic, and gastroprotective properties (Al-Bahtiti, 2012). *Teucrium polium* L. (Lamiaceae) contains flavonoids, terpenoids, and phenolic compounds. These compounds have anti-inflammatory, antioxidant, and antimicrobial properties, which could contribute to the reported research on *T. polium* L. (Lamiaceae) in Palestine (Alali and Khazem, 2020; Jaradat et al., 2017; Suleiman et al., 1988).

In Syria, the focus is on *Peganum harmala* L. (Nitrariaceae) a plant species that has been used in traditional medicine to treat a variety of neurological and psychiatric conditions, such as anxiety, depression, and epilepsy, due to its ability to modulate neurotransmitter systems and exert neuroprotective effects (Asgarpanah and Ramezanloo, 2012; Shatarat et al., 2020). Harmine, harmaline, harmalol, harmol, and peganine are active compounds found in *P. harmala* L. (Nitrariaceae). These compounds have been associated with neuroprotective, anti-depressant, and anxiolytic effects, which might explain the reported research on *P. harmala* L. (Nitrariaceae) in Syria (Doskaliyev et al., 2021b; Wang et al., 2022).

Lastly, *Origanum syriacum* L. (Lamiaceae) in Lebanon is investigated for its traditional use in the Fertile Crescent to treat various gastrointestinal disorders, such as indigestion, diarrhea, and stomach ulcers, which may be because of its anti-microbial, anti-inflammatory, and antioxidant properties (Al Hafi et al., 2016; AlKahlout et al., 2022; Daouk et al., 1995; Mesmar, Abdallah, Hamade, et al., 2022). *Origanum syriacum* L. (Lamiaceae) contain carvacrol, thymol, rosmarinic acid, and quercetin. These compounds possess anti-microbial, anti-inflammatory, and antioxidant properties, which could underline the reported research on *O. syriacum* L. (Lamiaceae) in Lebanon (El-Moneim et al., 2014; Mesmar, Abdallah, Badran, et al., 2022).

The review revealed 29 medical conditions under investigation in the Fertile Crescent with the two most investigated are cancer (573 mentions) and bacterial infections (440 mentions). These were followed by 27 health conditions including inflammation (237), fungal infections (176), diabetes (154), skin disorders (151), gastrointestinal (149), respiratory (112), urinary tract (85), nervous system (85), cardiovascular (83), lipid panels (76), analgesics (63), kidney function (62), liver function (61), hypertension (51), obesity (48), immune system (44), mental health (42), parasites (40), insecticidal (37), viral (35), reproductive health (33), Alzheimer's (26), snake bite (19), anticoagulant (14), detoxification (8), tuberculosis (7), and COVID-19 (8) (Figure 3).

## 3.1 Anti-cancer properties

The exploration of plants species with anti-cancer properties in all biomes and ecosystems, including the Fertile Crescent, is still needed as it offers potential alternatives to conventional cancer treatments, which can have significant side effects and may not be effective for all types of cancer (Newman and Cragg, 2020; Yan et al., 2023). The anti-cancer properties of medicinal plants under investigation in the Fertile Crescent countries are thought to be due to their ability to modulate multiple cellular pathways involved in cancer development and progression. Some plant species were reported to help prevent cancer initiation and progression through their anti-inflammatory and antioxidant effects, while others report anti-proliferative and pro-apoptotic effects, which can help inhibit cancer cell growth and induce cell death (Samaha et al., 2019).

Nine plant species have been investigated *in vitro* and *in vivo* for their anticancer properties in the Fertile Crescent; these include *R. officinalis* L. (Lamiaceae), *N. sativa* L. (Ranunculaceae), *Salvia fruticosa* Mill. (Lamiaceae), *T. polium* L. (Lamiaceae), *Thymus vulgaris* L. (Lamiaceae), *Crocus sativus* L. (Iridaceae), *O. syriacum* L. (Lamiaceae), *Lavandula stoechas* L. (Lamiaceae), and *Satureja thymbra* L. (Lamiaceae). The most investigated species is *N. sativa* L. (Ranunculaceae), which contains in its seeds thymoquinone, a metabolite that has been shown to inhibit the growth of cancer cells and induce apoptosis in various cancers, including breast, lung, and colon cancer (Alabdallat and Alanazi, 2023; Dabrowski et al., 2024; Yaghi et al., 2023). Thymoquinone has also been shown to inhibit the expression of genes involved in cancer cell proliferation and survival, including cyclin D1 and Bcl-2. Similarly, *Salvia viscosa* Jacq. (Lamiaceae), which contains in its essential oils a metabolite

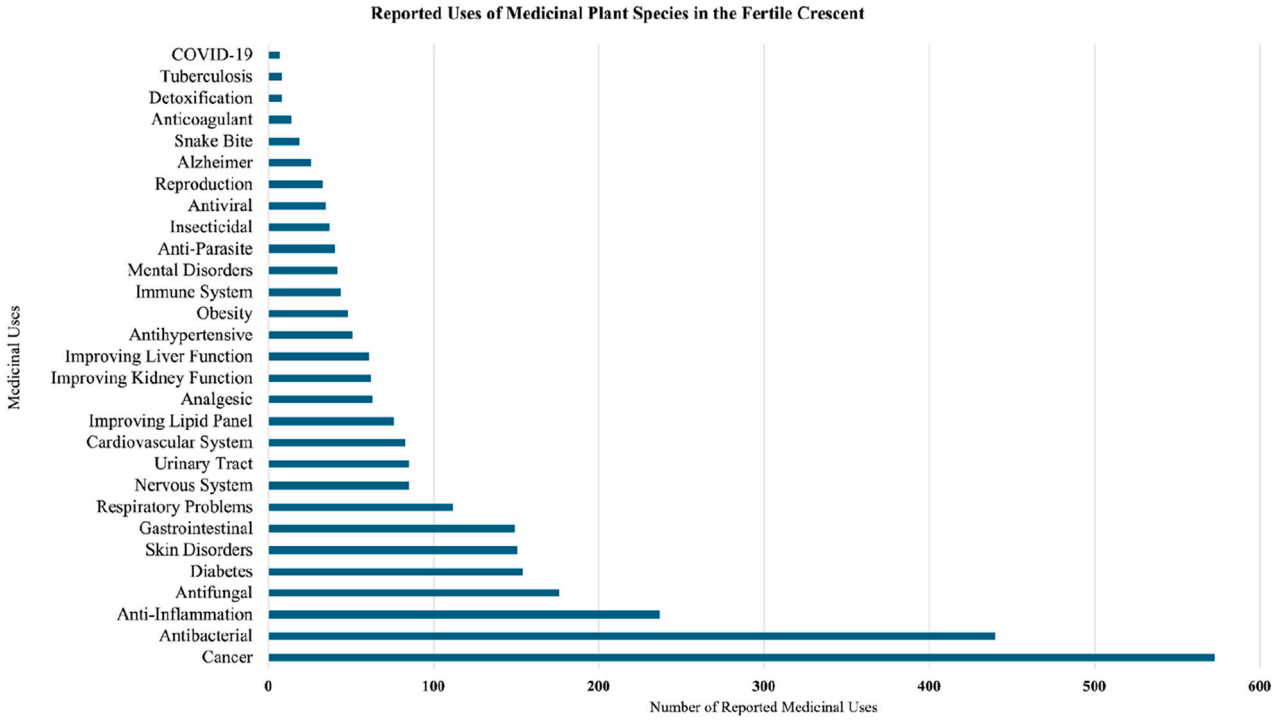


FIGURE 3
Potential pharmacological uses of medicinal plants in the Fertile Crescent.

called caryophyllene oxide, has been shown to inhibit cancer cell growth and induce apoptosis in various types of cancer, including colon and prostate cancer by inhibiting gene expression linked to cancer cell proliferation and survival, including cyclin D1 and Bcl-2 (Russo et al., 2018). Plant extracts with anti-proliferative and pro-apoptotic effects were also reported for *T. polium* L. (Lamiaceae) on human prostate cancer cells, *O. syriacum* L. (Lamiaceae) on human ovarian cancer cells, and *S. thymbra* L. (Lamiaceae) on human stomach cancer cells (Atoum et al., 2023; Tabaza and Aburjai, 2024).

Essential oils from various species were also found to inhibit cancer cell growth and induce apoptosis including *R. officinalis* L. (Lamiaceae) on breast cancer cells, *S. fruticosa* Mill. (Lamiaceae) on colon cancer cells, *L. stoechas* L. (Lamiaceae) on liver cancer cells, and *T. vulgaris* L. (Lamiaceae) on skin cancer cells.

## 3.2 Anti-bacterial effects

The Arab region is facing a significant rise in anti-bacterial resistance (Alkheraije, 2024; Ballouz et al., 2021) due to the availability of antimicrobials over the counter, inadequate infection prevention and control programs, and the presence of poor-quality antibiotics in the market, especially in conflict zones (Abdallah et al., 2023; Ballouz et al., 2021; Hamarsheh et al., 2021; Rizk et al., 2021). More specifically, studies conducted in countries of the Fertile Crescent have noted an alarming increase in multidrug-resistant bacteria, including strains resistant to last-line antibiotics (Abdel-Massih and El Beyrouthy, 2022) like ESKAPE organisms (*Acinetobacter* baumannii, *Pseudomonas aeruginosa*, and *Klebsiella* pneumonia) (Morel et al., 2021; Selvarajan et al., 2022; Wang et al., 2022). On the other hand, medicinal plants in the Arab world, including the Fertile Crescent region, offer a vast array of bioactive metabolites that may combat bacterial infections effectively (Darwich et al., 2022; Roula and El Beyrouthy, 2022). Our review revealed that the anti-bacterial properties of plant species investigated in the Fertile Crescent are attributed to their ability to inhibit bacterial growth, prevent biofilm formation, and induce bacterial cell death. One of the most studied species for its anti-bacterial properties is *T. vulgaris* L. (Lamiaceae) (Hudaib and Aburjai, 2007; Shalaal et al., 2019). This plant's essential oil contains high levels of a metabolite called thymol, which has been shown to inhibit the growth of various types of bacteria, including *Staphylococcus aureus* and *Escherichia coli*. Thymol has also been shown to prevent biofilm formation and induce bacterial cell death. The essential oil of *O. syriacum* L. (Lamiaceae) contains a metabolite called carvacrol which has been shown to inhibit the growth of various types of bacteria, including *P. aeruginosa* and *Klebsiella pneumonia* (Mesmar et al., 2022; Mohamad et al., 2021; Shehadeh et al., 2019). Carvacrol has also been observed to prevent biofilm formation and induce bacterial cell death.

The over-representation of cancer and bacterial infection studies (51% combined) may reflect global funding priorities as well as the pressing healthcare needs of the region. While antimicrobial resistance (AMR) is a well-documented global crisis (Ballouz et al., 2021), conditions such as cardiovascular diseases and respiratory disorders, which are prevalent in the Middle East, remain under-researched in ethnopharmacology. This suggests an opportunity for future research to realign priorities with regional health burdens.

## 3.3 Anti-inflammatory properties

The anti-inflammatory properties of medicinal plants investigated in countries of the Fertile Crescent region are thought to be due to their ability to modulate multiple cellular pathways involved in inflammation. For example, some plant species have been found to have antioxidant effects, which can help reduce oxidative stress and inflammation. Others have been revealed to have immunomodulatory effects, which can help regulate the immune response and prevent excessive inflammation. Plant species found to possess anti-inflammatory properties, include *N. sativa* L. (Ranunculaceae), *R. officinalis* L. (Lamiaceae), *S. fruticosa* Mill. (Lamiaceae), *T. polium* L. (Lamiaceae), *T. vulgaris* L. (Lamiaceae), *O. syriacum* L. (Lamiaceae), *Lavandula coronopifolia, S. thymbra* L. (Lamiaceae), and *Achillea Fragrantissima* (Forssk.) Sch. Bip. (Asteraceae) (Abdel-Massih and Abraham, 2014; Aburjai et al., 2005; Al-Tawarah, 2022; Kharma and Hassawi, 2006; Marrelli et al., 2018; Merajuddin et al., 2019; Naseef et al., 2022; Sadeq et al., 2021). These plant species contain various bioactive metabolites, including flavonoids, phenolic acids, and terpenes, which have been demonstrated to have anti-inflammatory activities *in vitro* and *in vivo* (Bahramikia and Yazdanparast, 2012; Rafieian-Kopaei et al., 2014). Evidence of anti-inflammatory effects using animal models of inflammation was attributed to the inhibition of inflammatory cytokines. This was reported for plant extracts of *N. sativa* L. (Ranunculaceae), *S. fruticosa* Mill. (Lamiaceae), *O. syriacum* L. (Lamiaceae), *Achillea millefolium* L. (Asteraceae), *Melissa officinalis* L. (Lamiaceae), and *T. polium* L. (Lamiaceae) (Aldal'in, 2018; Salamon et al., 2019). Essential oils of *R. officinalis* L. (Lamiaceae), *T. vulgaris* L. (Lamiaceae), *Cinnamomum verum* J. Presl (Lauraceae), *Punica granatum* L. (Lythraceae), and *L. coronopifolia* poir. (Lamiaceae) were found to inhibit the production of inflammatory mediators (Bakkour et al., 2011; Naseef et al., 2022; Hekmat and Al-Obeidi, 2019). In addition, plant species with anti-inflammatory properties in their underground organs (roots and rhizomes) include *Glycyrrhiza glabra* L. (Fabaceae) roots, which contain glycyrrhizin, and *Zingiber officinale* Roscoe (Zingiberaceae) rhizomes, which contain gingerol. Both metabolites have been reported to inhibit the production of pro-inflammatory cytokines and enzymes, stimulate the production of anti-inflammatory cytokines, and suppress the activation of inflammatory cells, such as macrophages and T cells (Abu-Al-Basal, 2010; Umran et al., 2013).

## 3.4 Anti-fungal characteristics

One of the most studied medicinal plant species in the Fertile Crescent for anti-fungal properties is *Allium sativum* L. (Amaryllidaceae) whose bulbs contain allicin, a metabolite shown to prevent biofilm formation, induce fungal cell death, and inhibit the growth of various types of fungi, including *Candida albicans* and *Aspergillus fumigatus* (Abdulkhaleq et al., 2022; Abdulla and Ismael, 2023; Fahed et al., 2021). Studies have also investigated the bark of *C. verum* J. Presl (Lauraceae) which contains cinnamaldehyde, a metabolite that has also been observed to prevent biofilm formation and induce fungal cell death (Ubaid et al., 2022) and to inhibit the growth of various types of fungi, including *C. albicans* and *A. fumigatus* (Al-Zereini et al., 2022). Other plant species extracts reported to possess anti-fungal properties include extracts of *T. vulgaris* L. (Lamiaceae) which was tested against *C. albicans* and *Aspergillus flavus* (Al-Saidy et al., 2012; Azeez, 2020), *Teucrium Polium* L. which was tested against *C. albicans* and *Candida tropicalis* (Abdullah et al., 2022; Al-Bahtiti, 2012; Darwish and Aburjai, 2011; Rahmouni et al., 2021), *Origanum* syriacum L. (Lamiaceae) which was tested against *C. albicans* and *Candida krusei* (Al Hafi et al., 2016; Daouk et al., 1995; Kassaify et al., 2008; Mesmar et al., 2022), *S. thymbra* L. (Lamiaceae) which was tested against *C. albicans* and *Candida glabrata* (Al Hafi et al., 2017; Beyrouthy et al., 2013), and *M. officinalis* L. (Lamiaceae) which was tested against *C. albicans* and *C. tropicalis* (Aldal'in, 2018).

## 3.5 Anti-diabetic properties

Many plant species in the Fertile Crescent were shown to possess anti-diabetic properties because of their ability to increase insulin secretion, improve insulin sensitivity, and reduce glucose absorption in the gut. For example, extracts of *N. sativa* L. (Ranunculaceae), *T. polium* L. (Lamiaceae), and *A. millefolium* L. (Asteraceae) reduced blood glucose levels and improved insulin sensitivity in streptozotocin-induced diabetic rats (Fadheel, 2019; Mosleh et al., 2022; Raziani et al., 2022). Extracts of *C. verum* J. Presl (Lauraceae) reduced blood glucose levels and improved insulin sensitivity in alloxan-induced diabetic rats (Alkubaisy et al., 2019). The metabolite 4-hydroxyisoleucine found in *Trigonella foenum-graecum* L. (Fabaceae) seeds was shown to inhibit glucose absorption, stimulate insulin secretion, improve insulin sensitivity, and reduce blood glucose levels in diabetic patients (Abdel-Barry et al., 1997; Abdel-Barry et al., 2000).

## 3.6 Other medicinal plant properties

This section tackles the smaller number of studies conducted to address various ailments and disorders. Research on the beneficial effects of plant species extracts on ***skin health*** is reportedly related to the plants' abilities to inhibit inflammation, prevent bacterial growth, and improve skin hydration. Aloin found in the gel of *Aloe vera* (L.) Burm. f. (Asphodelaceae), and calendulin which is present in the flowers of *Calendula officinalis* L. (Asteraceae) have both been shown to inhibit inflammation, prevent bacterial growth, improve skin hydration, and reduce skin disorders such as acne and eczema (Hasan and Abdullah, 2022). Research has also confirmed traditional uses of plant species for skin health, these include extracts of *Myrtus communis* L. (Myrtaceae), *P. granatum* L. (Lythraceae), and *S. fruticosa* Mill. (Lamiaceae) (Abu-Darwish et al., 2013). Studies reported that these plants possess anti-inflammatory, anti-bacterial, and antioxidant properties, attributing their effect on the skin by reducing inflammation, preventing infection in skin wounds, and promoting wound healing (Al-Mariri et al., 2016; Barzani et al., 2014; Hamidi et al., 2023).

Many plant species have been traditionally used by peoples of the Fertile Crescent for centuries to treat various types of ***gastrointestinal disorders*** including irritable bowel syndrome,

inflammatory bowel disease, and gastroesophageal reflux disease. More specifically, glycyrrhizin found in the roots of *G. glabra* L. (Fabaceae) and gingerol found in the rhizomes of *Z. officinale* Roscoe (Zingiberaceae) have both been shown to inhibit inflammation, prevent bacterial growth, improve gut motility, and reduce the severity of gastrointestinal disorders such as irritable bowel syndrome (Abbas, 2020; Al-Mousawi et al., 2022; Shawarb et al., 2021). Cuminum *cyminum* L. (Apiaceae) and *Foeniculum vulgare* Mill. (Apiaceae), were reported to alleviate symptoms associated with irritable bowel syndrome by reducing inflammation (Bouhenni et al., 2021; Karik et al., 2021; Kashamar et al., 2018). Other medicinal plants in the Fertile Crescent, such as *Trachyspermum ammi* (L.) Sprague (Apiaceae) and *Carum carvi* L. (Apiaceae), were reported to have carminative and anti-spasmodic properties, making them effective in the treatment of digestive disorders such as bloating, cramps, and diarrhea (Abd Al-Behadili et al., 2019; Anwar et al., 2016; Naquvi et al., 2022). Furthermore, the following species, *Pimpinella anisum* L. (Apiaceae), *Coriandrum sativum* L. (Apiaceae), and *Achillea* L. (Asteraceae) (Kharma and Hassawi, 2006), traditionally used for gastrointestinal disorders were observed to have anti-inflammatory and antioxidant properties, reducing inflammation and promoting gut health, and modulate the gut microbiota (Al-Bayati, 2008; Al-Daody and Al-Ta'ee, 2018; Al-wendaw et al., 2021; Sihoglu Tepe and Tepe, 2015; Sulaiman and Ahmed, 2018).

Many plant species in the Fertile Crescent are aromatic, a trait that is essential for their survival and defense during hot dry summers. These same species have also been traditionally used by people in the region to treat ***respiratory illnesses***. For example, studies indicated that the essential oil of *T. vulgaris* L. (Lamiaceae) which contains thymol, inhibits inflammation and prevents bacterial growth (Al-Assaf et al., 2023; Al-Saidy et al., 2012; Aziz et al., 2022; Shalaal et al., 2019). Thymol has also been demonstrated to improve lung function, have expectorant properties, help relieve coughs and congestion, and reduce the severity of respiratory disorders such as asthma (Al Hafi et al., 2017; Alimari et al., 2023). Similarly, *Eucalyptus globulus* Labill. (Myrtaceae) and *R. officinalis* L. (Lamiaceae), have decongestant and anti-inflammatory properties, making them effective in the treatment of respiratory disorders such as colds, flu, and sinusitis (Abdel-Massih and Abraham, 2014; Al-Taai et al., 2022; Aqel, 1991; Dheyab et al., 2022; Qabaha et al., 2016). *Mentha* × *piperita* L. (Lamiaceae), was reported to reduce inflammation and alleviate symptoms associated with bronchitis and asthma (Al-Saidy et al., 2012; Shawarb et al., 2021). *Glycyrrhiza glabra* L. (Fabaceae), and *P. granatum* L. (Lythraceae) were reported to have anti-inflammatory and antioxidant properties, reducing inflammation and promoting lung health (Al-Laham and Al-Fadel, 2013; Al-Mousawi et al., 2022; Husin et al., 2015; Upadhyay et al., 2020; Hekmat and Al-Obeidi, 2019). *Cinnamomum verum* J. Presl (Lauraceae) was reported to have bronchodilatory properties, relieving bronchospasms and improving lung function (Al-Zereini et al., 2022), while *Origanum majorana* L. (Lamiaceae), *and Lavandula angustifolia* Mill. (Lamiaceae), were reported as having expectorant and anti-inflammatory properties, relieving coughs and congestion, and reducing inflammation in the lungs (Abdel-Massih and Abraham, 2014; Ahmed et al., 2022; Koleilat et al., 2017; Marrelli et al., 2016; Raafat et al., 2013).

Most plant species traditionally used in the Fertile Crescent to treat ***urinary tract disorders***, have been found to have diuretic, anti-inflammatory, and anti-microbial properties, and were reported to inhibit bacterial growth, prevent stone formation, and improve urinary tract function (Ahmed et al., 2021; Akour et al., 2021; Allami et al., 2020). For example, Urticin found in *Urtica dioica* L. (Urticaceae) leaves inhibits bacterial growth, prevents urinary tract infections, prevents stone formation, and improves urinary tract function (Ahmed et al., 2021). Several other species investigated include *U. dioica* L. (Urticaceae), *Petroselinum sativum* Hoffm. (Apiaceae) (Kashamar et al., 2018), *Arctium lappa* L. (Asteraceae) (Al-Shammaa et al., 2013), *Taraxacum officinale* F.H.Wigg. (Asteraceae) (Ali et al., 2021), *Cichorium intybus* L. (Asteraceae) (Abdullah et al., 2019), *Juniperus phoenicea* L. (Cupressaceae) (Abu-Darwish et al., 2013), *Cynara scolymus* L. (Asteraceae) (Nasser, 2012), *Achillea fragrantissima* (Forssk.) Sch. Bip (Alsohaili, 2018), *F. vulgare* Mill. (Apiaceae) (Kashamar et al., 2018), and Rhus *coriaria* L. (Anacardiaceae) (Marouf et al., 2022).

People in the Fertile Crescent traditionally used an array of medicinal plant species with beneficial effects on ***nervous system health***. Research has shown that these plant species have anxiolytic, sedative, and neuroprotective properties thought to be due to their ability to modulate the activity of neurotransmitters, reduce oxidative stress and inflammation, and improve cognitive function (Abbas, 2020; Alsmadi et al., 2018). For example, *M. officinalis* L. (Lamiaceae) was reported to help reduce anxiety and promote relaxation through its anxiolytic and sedative properties (Aldal'in, 2018; Rasool and Muhammad, 2013). *Lavandula angustifolia* Mill. (Lamiaceae) has neuroprotective activity as it reduces oxidative stress and inflammation in the nervous system through aromatherapy (Koleilat et al., 2017). Bacoside, a metabolite found in the leaves of *Bacopa monnieri* (L.) Wettst. (Plantaginaceae) was shown to inhibit inflammation and prevent oxidative stress (Alwash, 2018). Bacoside has also been shown to improve neurotransmitter function and reduce the severity of nervous system disorders such as anxiety and depression (Al-Snafi, 2015). *Crocus sativus* L. (Iridaceae) was reported to have neuroprotective and anti-inflammatory properties, making it effective in the treatment of neurodegenerative diseases such as Alzheimer's and Parkinson's (Makhlouf et al., 2011; Samaha et al., 2021). *Ferulago angulata* (Schltdl.) Boiss. (Apiaceae) (Bagci et al., 2016), *Salvia fructicosa* Mill. (Lamiaceae) (Abu-Darwish et al., 2013), and *T. vulgaris* L. (Lamiaceae) (Al-Assaf et al., 2023) were reported to have antioxidant, anti-inflammatory, anxiolytic, and sedative properties, which helped improve cognitive function and memory, reduce anxiety and promote relaxation. Other medicinal plant species, namely, *P. harmala* L. (Nitrariaceae), *O. syriacum* L. (Lamiaceae), were reported to have antidepressant and anxiolytic properties, making them effective in the treatment of depression and anxiety (Jasim, 2019; Kanaan et al., 2014).

Our review shows that the number of publications resulting from laboratory research dominates investigations (86%) while only a smaller proportion (approximately 14%) addresses field research. This noted lack of attention to traditional knowledge holders by researchers in the Fertile Crescent has been also observed in global

ethnopharmacological research, where experimental studies often dominate over traditional knowledge documentation (Pirintsos et al., 2022). This lack of alignment between traditional and modern research may hinder the translation of findings into practical applications for local communities. Our findings are confirmed by a recent scoping review by Alzweiri et al. (2011); Abu-Odeh et al. (2023). The authors who focused on Jordanian medicinal plants and analyzed 124 articles published between 2000 and 2022 noted a predominance of laboratory-based studies over field research. In contrast, Astutik et al. (2019) reported a higher prevalence of field-based studies in South Asian countries, indicating stronger preservation of traditional knowledge transmission. Similarly, da Silva et al. (2018) found that greater collaboration exists between ethnobotanists and medical researchers in Latin America, leading to a higher percentage of clinical studies. This finding underscores the need for a more integrated research approach in the Fertile Crescent, bridging laboratory work with field-based ethnobotanical surveys.

### 3.7 Implications for practice and policy

Based on our analysis, we propose three key policy recommendations that are likely to be most effective in addressing the challenges of medicinal plant research and conservation in the Fertile Crescent region: First, for TAIM to become part of integrative medicine in the region, national regulatory bodies should establish guidelines for the safe use of traditional remedies, similar to efforts in China's Traditional Chinese Medicine modernization initiative (Zhang et al., 2022; Wang et al., 2021). Pharmacovigilance programs should be developed to monitor potential herb-drug interactions. Second, ethnopharmacologists should engage with traditional healers in Fertile Crescent countries to document oral knowledge before it disappears by collectively creating a database of regional medicinal plants. Third, overharvesting of medicinal plants in the Fertile Crescent threatens biodiversity. Our review identified several species (e.g., *T. polium* L. (Lamiaceae), *O. syriacum* L. (Lamiaceae)) that are at risk due to unsustainable harvesting. Policymakers should implement community-based conservation programs to promote sustainable cultivation practices and share with communities the commercial benefits when they arise.

### 3.8 Limitations and future research directions

Despite its comprehensive scope, this study has several limitations. Only English studies were included, potentially omitting valuable research published in Arabic, Kurdish, Persian, or French. While GPT-4 Turbo improved efficiency, AI models may misinterpret context or fail to differentiate between ambiguous plant names. Manual validation reduces errors, but some misclassifications may persist. Additionally, a significant proportion of studies lacked phytochemical standardization, and only 20% included clinical or *in vivo* trials, affecting the generalizability of findings. Future research should aim to expand language coverage, implement human-AI hybrid screening processes, and prioritize clinical validation of promising plant extracts.

## 4 Conclusion

This review sheds light on the state of TAIM with a focus on the Fertile Crescent region. The findings revealed that the region harbors a rich diversity of medicinal plants and a clear inclination towards scientific validation of the medicinal properties of plants. This trend underscores the region's commitment to evidence-based medicine and the rigorous examination of plant-derived metabolites for their potential therapeutic benefits (Dafni and Böck, 2019; Talib et al., 2020). The high percentage of laboratory research also highlights regional interest in phytochemical analysis, signaling a nuanced understanding of the bioactive metabolites present in medicinal plants and their mechanisms of action (Talib et al., 2020). On the other hand, research on traditional use of medicinal plants accounted for approximately 14% of the total research conducted on medicinal plants in the Fertile Crescent. This suggest that scientific research does not seem to be harnessing traditional knowledge and practices which remain integral to the region's healthcare landscape, there is a notable shift towards incorporating scientific methodologies and experimental approaches in understanding and harnessing the medicinal properties of indigenous flora (Dar-Odeh and Abu-Hammad, 2020; Dehyab et al., 2020). Although the predominance of experimental research in medicinal plant studies aligns with global trends in evidence-based healthcare practices and fostering innovation in natural product research (Baydoun et al., 2015; Saad et al., 2005), the lack of field research may lead to loss of traditional plant knowledge that would go unrecorded. The documentation of the traditional distribution and uses of medicinal plants is crucial, given the reliance on complementary and alternative medicine in developing countries and the increasing threats to the natural habitats and conservation status of these valuable species. The establishment of a systematic database to record the medicinal plants of the Fertile Crescent is a step towards preserving this important aspect of the region's collective cultural and ecological heritage. Given these insights, the review points to the need for supporting collaborations between traditional healers, ethnobotanists, pharmacologists, and healthcare professionals to open an information exchange corridor between all stakeholders for the purpose of creating standardized methodologies and regulatory frameworks that facilitate the integration of traditional medicine into healthcare systems and support the documentation and preservation of traditional knowledge before it disappears. Future research focus should also support the development of the sector by expanding clinical trials to validate the efficacy and safety of commonly studied medicinal plants and developing pharmacovigilance programs to monitor herb-drug interactions and ensure consumer safety.

## Author contributions

RA: Data curation, Formal analysis, Methodology, Writing – original draft. HA: Formal analysis, Methodology, Software,

Writing – original draft. MA: Conceptualization, Software, Supervision, Validation, Formal analysis, Writing – review and editing. SN: Conceptualization, Data curation, Resources, Writing – review and editing. ND: Validation, Writing – reviewing and editing. RZ: Validation, Writing – reviewing and editing. MR: Validation, Writing – reviewing and editing. SNT: Conceptualization, Funding acquisition, Project administration, Supervision, Validation, Writing – original draft, Writing – reviewing 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 Generative AI was used in the creation of this manuscript. Generative AI was used for editing the text.

## Publisher's note



## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2025.1542709/full#supplementary-material

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