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Women in ethnopharmacology 2023

Edited by
Shan-Yu Su, Irene Villasenor, Marilena Gilca, Valentina Echeverria Moran and Pollyanna Francielli de Oliveira

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Women in ethnopharmacology: 2023

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Editorial: Women in ethnopharmacology: 2023

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Editorial on the Research Topic
Women in ethnopharmacology: 2023

Despite the fact that the gender landscape in education and the workforce in terms of women's representation and recognition has improved lately, differences persist in various science-related fields (Charlesworth and Banaji, 2019), with women more likely to experience non-standard professional trajectories and negative health effects (Cabib et al., 2024).

Recognizing the importance of cultivating gender equality for sustainable development, as highlighted by UNESCO, we initiated a Research Topic to provide a collaborative platform to promote the work of women scientists in all fields of Ethnopharmacology. In response to our call for submissions, 21 articles were submitted, of which only 11 met publication standards.

In the following section of this editorial, some important achievements in the field will be highlighted, and also some Research Topic for future research will be identified.

One of the important health Research Topic affecting 10% of the women of reproductive age is endometriosis (Shafrir et al., 2018). In addition to its negative impact on the quality of life, it also represents an economic burden (Swift et al., 2024). A systematic review and meta-analysis performed by Ding et al. concluded that traditional Chinese Herbal Medicines (CHMs) may be a viable postoperative long-term therapeutic strategy for ovarian endometriotic cysts, and may improve pain, pregnancy rate, perimenopausal symptoms, and serum levels of Cancer antigen 125 while reducing recurrence and adverse effects of conventional Western medicine.

Despite the increasing global popularity of CHMs, there are several concerns due to their potential health risks, toxicities, risks of poor quality and adulteration. Difficulties in quality control of CHMs are also due to the complex and diverse processing of Chinese herbal ingredients. Mück et al. addressed these Research Topic and found an undesirably high level of variability in two traditional Chinese herbal formulas used in women's healthcare for the treatment of endometriosis, known as *Gui Zhi Fu Ling Wan* and *Ge Xia Zhu Yu Tang*, which are already available on the European market. Mück et al. showed in another study that conventional DNA barcoding is an insufficient tool for authentication of these samples and proposed a new tiered identification strategy based on HPTLC and DNA metabarcoding, which gives a progressive qualitative and quantitative insight on species diversity, and biological contaminants.

Inflammatory pathways are key components of many pathologies of the female reproductive tract, including endometriosis, polycystic ovary syndrome, etc., and their hyperactivity or dysregulation contributes to the onset of these disorders (Jabbour et al.,

2009). Three articles in our Research Topic identified some potential natural anti-inflammatory agents. Nguyen et al. demonstrated the potential of *Acer tegmentosum* Maxim extract and its fermented product to prevent endothelial inflammation and vascular dysfunction of the retina through the MAPK/NF-κB/SIRT1 signaling pathways. Cebollada et al. evaluated the 5-lipoxygenase inhibitory activity of several essential oils and showed that those derived from clove (*Syzygium aromaticum* (L.) Merr. and L.M.Perry) and wintergreen (*Gaultheria fragrantissima* Wall.) were the most active. Another study conducted by Fabian et al. demonstrated the 15-lipoxygenase and α-glucosidase inhibitory activity of several bioactive fractions obtained from the stem of *Coriaria intermedia* Matsum. and the bark of *Dracontomelon dao* (Blanco) Merr. and Rolfe. *C.*, which were generated, and dereplicated through UHPLC-MS/MS. The authors identified corilagin as a potential anti-inflammatory and anti-diabetic agent that should be prioritized in further studies.

Previous studies have shown that women seem to have a special relationship with traditional medicine and are among its most active consumers. Luo et al. re-confirmed this fact in a cross-cultural study exploring the traditional animal- and mineral-based medicines that are used in the Gansu-Ningxia-Inner Mongolia junction zone, a region of ethnic and cultural diversity. They found that female informants demonstrated greater knowledge of the medicines, probably due to their prevalence among the local herbal practitioners.

Taking into account that women play an essential role in the primary healthcare of children, and, at the same time, that ethnopediatric practices are poorly documented, Petran et al. focused on the study of medicinal plants currently employed in the treatment of childhood illnesses in the southern region of Romania. Higher education correlated not only with the number of plants employed and the variety of ailments treated but, surprisingly, also with the preference for harvesting rather than purchasing plants. The authors also raised concerns regarding the necessity to protect this ethnomedical heritage, since there is a significant reduction in the used taxa when compared to the past.

Although not statistically significant, women with a lower socioeconomic status had a higher cardiovascular risk than men (Ololade et al., 2024). Also, women with polycystic ovary syndrome, a frequent endocrinopathy, have a higher chance of developing a cardiovascular disease (Dutta and Maddukuri, 2024), while the leading cause of mortality in women with breast cancer is represented by the same type of cardiovascular pathology (Jiao et al., 2024).

Given the importance of cardiovascular health to women's wellbeing, some space was given to this area in the present Research Topic. Wang et al. provided a systematic review of the efficacy and safety of anisidine hydrobromide injection for acute ischemic stroke. Anisodine is a tropane alkaloid extracted from the root of *Anisodus tanguticus (Maxim.) Pascher,* family Solanaceae. It can improve significantly cerebral collateral circulation and increase blood flow perfusion in ischemic areas, and has been used for the treatment of ischemic stroke in clinical settings in China for more than a decade.

Blackthorn flower (*Prunus spinosa* L.) is a traditional remedy recommended for treating cardiovascular disease. Flavonol and A-type procyanidin-rich extracts of this medicinal plant exhibited anticoagulant activity through direct thrombin inhibition, without affecting platelet aggregation *in vitro,* according to Marchelak et al.

The studies presented here highlight the diversity of research performed across the breadth of Ethnopharmacology and present some advances in knowledge with applications to compelling women-related health Research Topic, such as endometriosis, and cardiovascular and inflammatory diseases, while identifying problems that require attention, such as quality control of CHMs and preservation of biocultural heritage.

The editors expect that the articles published in this Research Topic will have a significant impact on the readers and believe that more women scientists will be thus encouraged to be involved in ethnopharmacological research, contributing to the development of new effective therapeutic approaches inspired by traditional medicine.

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Efficacy and safety of anisodine hydrobromide injection for acute ischemic stroke: a systematic review and meta-analysis

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Background: Acute ischemic stroke (AIS) is a leading cause of death and disability worldwide. This study aimed to evaluate the efficacy and safety of anisodine hydrobromide (Ani) injection in the treatment of AIS.

Methods: Randomized controlled trials (RCTs) based on Ani injection for the treatment of AIS were retrieved from both Chinese and English databases. The retrieval period was from the databases' inception to May 2023. The Cochrane Collaboration Risk of Bias Tool was used to assess the methodological quality. The outcome indicators were analyzed using RevMan 5.3 software.

Results: We included the findings of 11 RCTs encompassing 1,337 patients with AIS. Our meta-analysis revealed that Ani injection supplementation significantly reduced the National Institutes of Health Stroke Scale [MD = −1.53, 95%CI = (−1.94, −1.12), $p < 0.00001$], modified Rankin Scale [MD = −0.89, 95%CI = (−0.97, −0.81), $p < 0.00001$], and the relative time to peak [SMD = −0.81, 95% CI = (−1.08, −0.55), $p < 0.00001$] significantly. Additionally, Ani injection significantly increased the Barthel Index [MD = 10.65, 95%CI = (4.30, 17.00), $p = 0.001$], relative cerebral blood volume [SMD = 0.28, 95%CI = (0.02, 0.53), $p = 0.03$], and clinical efficacy [RR = 1.2, 95%CI = (1.08, 1.34), $p = 0.001$]. No statistically significant difference in the rate of adverse events was observed between the Ani injection supplemental group and the control group.

Conclusion: Based on currently published evidence, Ani injection was found to be effective and safe in improving AIS outcome. Nevertheless, limitations of the included RCTs still exist, and thus, more multi-center, large-sample, high-quality RCTs are required to further verify the efficacy and safety of Ani injection in patients with AIS.

Systematic Review Registration: [https://www.crd.york.ac.uk/prospero/display_record.php?ID=CRD42023427591], identifier [PROSPERO 2023 CRD42023427591].

KEYWORDS

acute ischemic stroke, anisodine hydrobromide injection, systematic review, meta-analysis, efficacy

1 Introduction

Acute ischemic stroke (AIS) is characterized by ischemia, hypoxic necrosis, and softening of the brain tissue due to a sudden interruption of the cerebral blood supply with inadequate collateral circulation, resulting in a series of symptoms of neurological dysfunction (Zhang et al., 2020). AIS is the most common type of cerebral stroke, accounting for approximately 70% of all strokes. Worldwide, AIS is a leading cause of death and disability (Wang et al., 2019). In China, the mortality rate of hospitalized AIS patients within 1 month of onset is approximately 2.3%–3.2%, the 1-year mortality rate after onset is 14.4%–15.4%, and the disability rate is 33.4%–33.8% (Wang et al., 2013; Wang et al., 2017). The associated socioeconomic burden of AIS is huge; for example, the annual expenditure related to AIS, including long-term rehabilitation and unemployment, is estimated to be £ 25.6 billion in the United Kingdom (Robert et al., 2020). Therefore, AIS has become a major global health concern.

At present, regular treatment of AIS consists of a multidisciplinary approach. Treatment management for AIS includes drug therapy, limb rehabilitation, language training, psychological rehabilitation, and health education (Trialists'Collaboration, 2013). Intravenous thrombolysis with recombinant tissue-type plasminogen activator (rtPA) and endovascular therapy have been the mainstay treatments for AIS in recent years (Powers et al., 2018). Both therapeutic strategies aim to rescue ischemic brain tissue with viable potential by recanalization of occluded cerebral arteries and reperfusion of the ischemic penumbra (Robert et al., 2020). Nevertheless, the number of patients with AIS who are eligible for such reperfusion strategies remains low due to the narrow time window of reperfusion therapy (Rodrigues et al., 2016; Bhaskar et al., 2018). More specifically, the therapeutic effect is heavily time dependent; therefore, the stroke symptom onset should be recorded accurately as a clock time to avoid treatment failure. Intravenous thrombolysis and endovascular thrombectomy for AIS patients with an unclear onset time require further exploration (Qiang et al., 2017). Furthermore, clinical evidence has shown that only patients with large vessel occlusive-type AIS are candidates for endovascular therapy, which accounts for less than 20% of AIS cases (Yasha et al., 2019). Symptomatic intracranial hemorrhage after thrombolysis and endovascular treatment in patients with AIS is a major complication that is associated with a devastating clinical outcome. The high frequency of intracranial hemorrhage poses a huge challenge to the clinical management of AIS (Seet and Rabinstein, 2012; Hao et al., 2017). In addition, as a serious complication of vascular recanalization, ischemia–reperfusion injury in the setting of cerebral ischemia following vascular restoration occurs because of a complex series of events, which can evoke parenchymal brain damage (Nour et al., 2013). Therefore, novel therapeutic strategies are urgently required to improve the efficacy and safety of AIS treatment.

A tenet of traditional folk medicine in China is that herbs possess the ability to treat various diseases. Modern researchers have demonstrated that compounds in these medicinal herbs, which consist of multiple ingredients, have multiple pharmacological actions, which are compatible with the complex pathogenesis of diverse human diseases (Chen et al., 2022). For many years in China, various traditional folk herbs have been applied in the treatment of AIS based on the theory of promoting blood circulation and removing blood stasis (Gong and Sucher, 2002). In-depth studies have elucidated the underlying mechanisms of the therapeutic effect of traditional medicinal herbs, which involve the inhibition of excitotoxicity, inflammation, oxidative damage, ionic imbalances, apoptosis, and so on, in the pathophysiological process of AIS (Sucher, 2006). A meta-analysis including 191 clinical trials involving 22 types of traditional Chinese medicine has demonstrated the improvement of neurological deficits after administration (Wu et al., 2007).

Anisodus tanguticus (Maxim.) Pascher, also named "Tang Chuan Na Bao" in Ethnologue, one of the indigenous Chinese ethnological plants of the Solanaceae, is mainly grown in the Qinghai–Tibet Plateau (Liu et al., 2005). In traditional Chinese medical theory, *A. tanguticus* possesses the traditional characteristics of nature of a warm, bitter flavor and functions to activate the blood to remove stasis (Chen et al., 2022). Anisodine, a tropane alkaloid extracted from the root of *A. tanguticus*, has been used as an ingredient in the compound preparation for treating ischemic stroke in China for more than a decade due to its significant properties of vasoactivity and improvements in microcirculation. To improve the chemical instability, researchers have developed a hydrobromide form of anisodine (Liu et al., 2020). Recently, anisodine hydrobromide (Ani) injection has been used in the clinical setting for the treatment of AIS in China. Multiple clinical studies have demonstrated the neuroprotective effect of Ani in AIS, which can not only alleviate neurological impairment and reduce dependency in activities of daily living but also improve the cerebral collateral circulation and increase cerebral tissue blood flow perfusion in ischemic areas (Zou et al., 2018; Zhang, 2022). Basic research has revealed that, as a central muscarinic cholinergic receptor blocker, the neuroprotective and cerebral circulation-promoting effect of Ani injection in the treatment of AIS can be correlated to the pharmacological actions of anti-oxidative damage, anti-inflammation, inhibition of neuronal apoptosis, and amelioration of hemorheological changes through regulation of the nitric oxide synthase system, preventing Ca^{2+} influx, decreasing IL-6 serum levels, and modulating angiogenic factors. Furthermore, the ability of Ani to activate the ERK1/2 signaling pathway and regulate ATPase activity is also a key underlying mechanism of action (Chen et al., 2017; Wang et al., 2017; Chen et al., 2017d; Xu et al., 2020; Zeng et al., 2021).

The impact of Ani injection on patients with AIS has been investigated in many clinical trials. In 2021, the earliest meta-analysis conducted by Wang et al. (2021) reported that Ani may have a positive effect in the treatment of ischemic stroke. However, the subjects included in Wang's study were patients with ischemic stroke at various stages, including both the acute stage and the convalescent stage. In addition, the study objective of several included randomized controlled trials (RCTs) focused on the synergistic effect of Ani combined with acupuncture or butylphthalide. There were certain limitations without further assessment targeting each specific clinical stage (including the acute stage of ischemic stroke) and the pure effect of Ani injection. Therefore, the present study aimed to systematically collect the current clinical evidence regarding Ani injection in the treatment of the acute stage of ischemic stroke and, more specifically, evaluate its efficacy and safety. We hope this meta-analysis and

systematic review will provide an accurate and reliable evidence-based reference for its rational use in the clinic.

2 Methods

2.1 Study registration

This meta-analysis was performed in strict accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) and was registered in PROSPERO (CRD42023427591).

2.2 Search strategy

Both English databases (including EMBASE, PubMed, Cochrane library, and Web of Science) and Chinese databases (including CNKI, VIP, Wanfang, and Chinese Biomedical Literature Database) were searched comprehensively from the date of their respective inception to May 2023 for the identification of eligible data. The following terms used in the search are a combination of MESH terms and free-text words: ("Anisodine hydrobromide injection" (Text word) OR "Anisodine hydrobromide" (Text word) OR "Anisodine" (Text word) AND ["Acute ischemic stroke" (Text Word) OR "ischemic stroke" (MESH) OR "brain ischemia" (MESH) OR "stroke" (MESH) OR "cerebral infarction" (MESH) OR "Cerebrovascular ischemia" (Text word) OR "Infarction, Anterior Cerebral Artery" (Text word) OR "Infarction, Middle Cerebral Artery" (Text word) OR "Infarction, Posterior Cerebral Artery" (Text word) OR "Apoplexy" (Text word)]. Potential studies in the reference lists of valid studies were also considered as information sources.

2.3 Inclusion and exclusion criteria

The inclusion criteria were as follows: 1) patients with AIS, regardless of age, gender, and disease stage; 2) parallel RCTs of Ani injection for AIS patients published in English or Chinese databases; 3) control group treated with regular therapies, while Ani injection was not applied in the control group; and 4) the trial groups were treated with Ani injection, used alone or in combination with the same regular therapies used in the control groups, regardless of the dose or duration of administration.

The exclusion criteria were as follows: 1) cerebral hemorrhage in patients with AIS; 2) reviews, letters, conference reports, cohort studies, case reports, cross-over studies, and animal studies; 3) duplicate studies or those with no comparison group; 4) literature without essential information or unable to obtain the related data; and 5) the trial group underwent acupuncture.

2.4 Outcome measures

In this systematic review and meta-analysis, the primary outcomes were as follows: National Institutes of Health Stroke Scale (NIHSS), modified Rankin Scale (mRS), and Barthel Index (BI). The secondary outcomes were computed tomography parameters (CTP), effective rate, and adverse events.

2.5 Study selection and data extraction

All electronic bibliographic databases mentioned above were scanned with a pre-designed search strategy. Duplicate articles were removed first. Next, two independent reviewers reviewed the titles and abstracts of the studies to select appropriate studies according to the eligibility criteria. The full texts of the selected studies were downloaded for further assessment. Three initial articles were used as a pilot to establish a standard extraction form, which contains the following domains: study information (title, first author, language, magazine, and year of publication), participant information (e.g., age, sex ratio, sample size, and disease course), intervention information (e.g., type, duration, frequency, and dose of treatment in the trial and control groups), and outcome indexes (primary outcomes and secondary outcomes). Reasons for the exclusion of ineligible studies were identified and recorded. Available data were extracted by two independent reviewers from the full texts. The two reviewers addressed disagreements through discussion or via consultation with a third reviewer.

2.6 Quality assessment

The Cochrane Collaboration Risk of Bias Tool in the Cochrane Handbook for Systematic Reviews of Interventions (Higgins et al., 2022) was used by two independent researchers to evaluate the methodological quality. According to the Cochrane Handbook, the risk of bias assessment was divided into seven domains: random sequence generation (selection bias), allocation concealment (selection bias), blinding of participants and personnel (performance bias), blinding of outcome assessment (detection bias), incomplete outcome data (attrition bias), selective reporting (reporting bias), and other bias. Each domain in the included RCTs was marked according to a low risk of bias, high risk of bias, or an unclear risk of bias. Disagreement between the two researchers was arbitrated by a third researcher.

2.7 Statistical analysis

The RevMan 5.3 software was used for meta-analysis. The relative risk (*RR*) was used as the effect index for the dichotomous variables, and the mean differences (*MD*) or standardized mean difference (*SMD*) were used as the effect index for continuous variables. The confidence interval (*CI*) of each effect index was set to 95%. The I^2 statistic was adopted to assess the heterogeneity. If $I^2 > 50\%$, there was heterogeneity between the studies, and the random-effect model was selected; otherwise, the fixed-effect model was utilized. The heterogeneity was explained by sensitivity analysis or subgroup analysis. In addition, descriptive analysis was performed if the clinical data provided by the included studies were incomplete and could not be

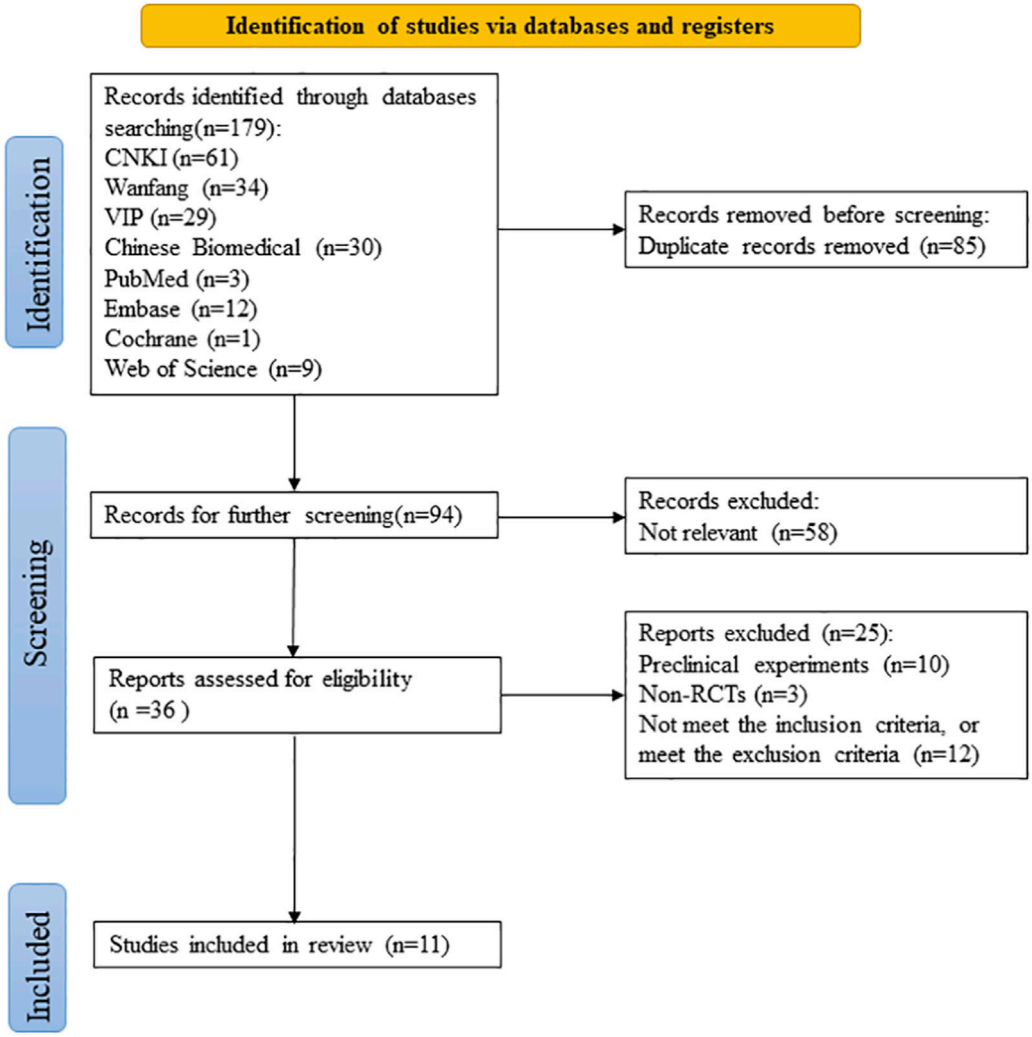

FIGURE 1
PRISMA flowchart for literature screening.

systematically evaluated. Potential publication bias was evaluated through a funnel plot.

3 Results

3.1 Study selection

The PRISMA flowchart of the literature screening process is presented in Figure 1. Initially, according to the search strategy, a total of 179 studies were obtained through retrieval from multiple databases; after the deletion of 85 duplicate publications, the remaining 94 articles were screened. After examination of the titles and abstracts, 58 irrelevant studies were removed. The full texts of the 36 remaining articles were assessed for eligibility. Preclinical studies (n = 10), non-RCTs (n = 3), and studies that did not meet the inclusion or met the exclusion criteria (n = 12) were excluded. Finally, 11 studies were included in the meta-analysis (Zou et al., 2018; Wang et al., 2020; Dong et al., 2021; Li et al., 2021; Zhang et al., 2021; Jiang et al., 2022; Kang, 2022; Zhang, 2022; Zhang et al., 2022; Zhou, 2022; Yan et al., 2023).

3.2 Study characteristics

A total of 1,337 patients with AIS were included in the meta-analysis, including 668 patients in the trial group and 669 patients in the control group. All included studies were conducted in China. The sample size in each included study ranged from 21 to 193. The shortest treatment duration of an Ani injection was 7 days, and the longest was 30 days. The time period from AIS symptom onset to hospital admission was ≤72 h in all studies. Regarding the outcome measurements, the NIHSS was adopted in all studies (Zou et al., 2018; Wang et al., 2020; Dong et al., 2021; Li et al., 2021; Zhang et al., 2021; Jiang et al., 2022; Kang, 2022; Zhang, 2022; Zhang et al., 2022; Zhou, 2022; Yan et al., 2023), five studies used the mRS (Wang et al., 2020; Dong et al., 2021; Kang, 2022; Zhang, 2022; Zhang et al., 2022), four studies used the BI (Zou et al., 2018; Jiang et al., 2022; Zhang et al., 2022; Zhou, 2022), three

TABLE 1 Characteristics of included studies.

Study	Group	Number (M/F)	Age, years	Timeline of onset to initiation of therapy	Intervention and treatment duration	Outcomes
Zou et al. (2018)	Trial	43 (23/20)	65.32 ± 9.12	8.21 ± 1.32 (h)	CT + Ani intravenous, 2 mg daily for 14 days	①③④⑥
	Control	43 (21/22)	66.13 ± 10.31	7.96 ± 1.15 (h)	CT	
Wang et al. (2020)	Trial	35 (14/21)	68.2 ± 11.1	7–72 h	CT + Ani intravenous, 1 mg daily for 7 days	①②
	Control	35 (16/19)	72.7 ± 11.5		CT	
Zhang et al. (2021)	Trial	42 (23/19)	53.86 ± 3.19	NR	CT + Ani intravenous, 2 mg daily for 14 days	①⑥
	Control	42 (20/22)	52.71 ± 3.52		CT	
Li et al. (2021)	Trial	60 (38/22)	59.28 ± 11.82	10.82 ± 6.43 (h)	CT for 14 days + Ani intravenous, 2 mg daily for 14 days	①⑤⑥
	Control	60 (33/27)	60.04 ± 11.96	10.45 ± 6.29 (h)	CT for 14 days	
Dong et al. (2021)	Trial	100	63.22 ± 8.55	18.87 ± 18.95 (h)	CT + Ani intravenous, 2 mg daily for 14 days	①②⑥
	Control	101	64.25 ± 9.54	18.26 ± 17.63 (h)	CT	
Zhou (2022)	Trial	57 (30/27)	71.12 ± 2.74	≤24 h	CT for 90 days + Ani intravenous, 4 mg daily for 14 days	①③④
	Control	57 (31/26)	71.35 ± 2.65		CT for 90 days	
Zhang et al. (2022)	Trial	21	18–80	≤4.5 h	CT + Ani intravenous, 2 mg daily for 7~14 days	①②③④⑥
	Control	21			CT	
Jiang et al. (2022)	Trial	193	63.43 ± 10.09	49.33 ± 27.42 (h)	CT + Ani intravenous, 1~2 mg daily for 10~14 days	①③⑥
	Control	193			CT	
Zhang et al. (2022)	Trial	16 (11/5)	50.14 ± 7.92	≤72 h	CT + Ani intravenous, 2 mg daily for 14 days	①②⑥
	Control	16 (10/6)	51.26 ± 9.35		CT	
Kang (2022)	Trial	71	NR	≤24 h	CT for 30 days + Ani intravenous, 2 mg daily for 30 days	①②⑤
	Control	71			CT for 30 days	
Yan et al. (2023)	Trial	30 (15/15)	62.8 ± 14.8	49.1 ± 27.8 (h)	CT + Ani intravenous, 2 mg daily for 14 days	①⑤
	Control	30 (17/13)	61.7 ± 15.6	48.3 ± 26.9 (h)	CT	

Note: M, male; F, female; NR, not reported; CT, conventional therapy; Ani, anisodine hydrobromide. Outcome indicators (① National Institutes of Health Stroke Scale; ② modified Rankin Scale; ③ Barthel Index; ④ CT, parameters; ⑤ clinical efficacy; ⑥ adverse events).

studies reported the CTP (Zou et al., 2018; Zhang et al., 2022; Zhou, 2022), two studies mentioned the effective rate (Kang, 2022; Yan et al., 2023), and adverse events were described in seven studies (Zou et al., 2018; Dong et al., 2021; Li et al., 2021; Zhang et al., 2021; Jiang et al., 2022; Zhang, 2022; Zhang et al., 2022), while three studies reported the adverse rate (Dong et al., 2021; Li et al., 2021; Zhang et al., 2021). The characteristics of the included studies are presented in Table 1.

3.3 Risk of bias of the included studies

The assessment of bias risk of each eligible study was performed according to the Cochrane bias risk tool. Nine of the included studies mentioned grouping by a random method, three of which specified that a random sequence was generated through the random number table method (Dong et al., 2021; Li et al., 2021; Zhou, 2022). None of these studies referred to information on allocation concealment, blinding of participants and personnel, or blinding of the outcome assessment; therefore, all the studies were rated as having an unclear risk of bias in these three sections. All other bias evaluation risks were unclear. The results of the risk of bias assessment are presented in Figures 2, 3. See the supplementary document of Supplementary Material for rating bias (Supplementary Table S1).

3.4 Outcome measures

3.4.1 National Institutes of Health Stroke Scale

Eleven studies included the NIHSS score, of which one study (Zhang, 2022) did not report the post treatment NIHSS score; this

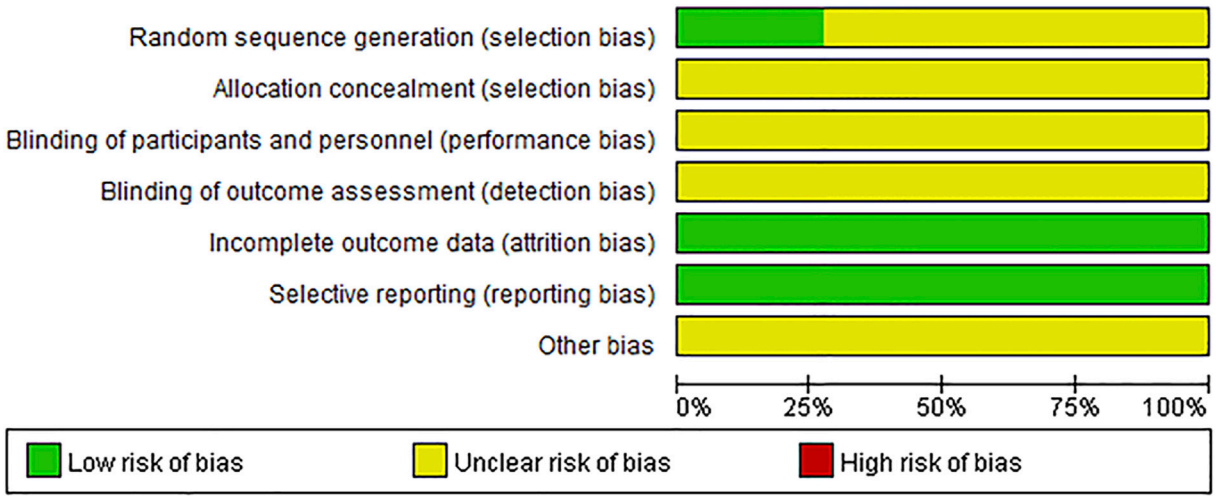

FIGURE 2
Risk of bias graph.

data could not be extracted from the existing information. Thus, ten articles (Zou et al., 2018; Wang et al., 2020; Dong et al., 2021; Li et al., 2021; Zhang et al., 2021; Jiang et al., 2022; Kang, 2022; Zhang et al., 2022; Zhou, 2022; Yan et al., 2023) with 1,305 participants were included in the meta-analysis regarding the NIHSS score. The heterogeneity test results showed that $p < 0.0001$, $I^2 = 75\%$, and there was significant heterogeneity among the studies. Therefore, a random-effect model was adopted. The pooled results of the post-treatment NIHSS score indicated that compared with the control group, Ani injection could significantly reduce the NIHSS score after treatment [MD = −1.53, 95%CI = (−1.94, −1.12), $p < 0.00001$], as shown in Figure 4.

In these studies, the NIHSS score was evaluated at different treatment time periods, which ranged from 7 days to 90 days of treatment. Therefore, we used treatment duration to conduct further NIHSS evaluations (7 days, 14 days, and ≥30 days) as the criteria for the subgroup analysis. The results of the subgroup analysis are shown in Figure 4. It can be clearly seen that at the different time periods of 7 days, 14 days, and ≥30 days for implementing the NIHSS assessment, the NIHSS score of the experimental group was significantly lower than that of the control group [MD = −1.03, 95%CI = (−1.72, −0.33), $p = 0.004$; MD = −1.38, 95%CI = (−1.86, −0.89), $p < 0.00001$; MD = −2.45, 95%CI = (−3.39, −1.52), $p < 0.00001$, respectively]. The result of the subgroup differences ($p = 0.05$, $I^2 = 66.2\%$) indicated that this subgrouping factor might be a source of heterogeneity in the overall meta-analysis regarding the NIHSS score.

3.4.2 Modified Rankin Scale

Five studies reported the mRS score; however, the data could not be extracted from one study (Dong et al., 2021) due to the provided data being dichotomous. Thus, a total of four studies (Wang et al., 2020; Kang, 2022; Zhang, 2022; Zhang et al., 2022) were included. The results of the heterogeneity test demonstrated that $p = 0.81$ and $I^2 = 0\%$; no significant heterogeneity was observed. Using the fixed-effect model, the results of the meta-analysis showed that the mRS score in the experimental group was significantly lower than that of the control group [MD = −0.89, 95%CI = (−0.97, −0.81), $p < 0.00001$], as shown in Figure 5.

3.4.3 Barthel index

Four studies (Zou et al., 2018; Jiang et al., 2022; Zhang et al., 2022; Zhou, 2022) included the BI score. A meaningful increasing effect of Ani treatment was observed with the BI score level from the meta-analysis [MD = 10.65, 95%CI = (4.30, 17.00), $p = 0.001$]. Meanwhile, a significance between heterogeneity was observed ($p < 0.00001$, $I^2 = 96\%$); thus, a random-effect model was adopted for the meta-analysis (Figure 6). The test for subgroup differences between trials that adopted the BI measurement [two RCTs (Zhang et al., 2022; Zhou, 2022)] [MD = 5.82, 95%CI = (4.07, 7.57), $p < 0.00001$] and modified BI measurement [two RCTs (Zou et al., 2018; Jiang et al., 2022)] [MD = 15.10, 95%CI = (2.62, 27.59), $p = 0.02$] was non-significant ($p = 0.15$, $I^2 = 52\%$) (Figure 6).

3.4.4 CT parameters

Three studies (Zou et al., 2018; Zhang et al., 2022; Zhou, 2022) reported the CTP, including relative cerebral blood flow (rCBF), relative cerebral blood volume (rCBV), relative time to peak (rTTP), and relative mean transit time (rMTT). The SMD was used as a summary statistic due to the consistency of the units in these studies being unclear. The pooled results indicated no statistically significant differences in the rCBF [SMD = 0.27, 95%CI = (−0.47, 1.01), $p = 0.48$] and rMTT [SMD = −0.71, 95%CI = (−2.20, 0.79), $p = 0.35$] between the Ani injection and the conventional therapy group and showed large heterogeneity ($p = 0.0006$, $I^2 = 87\%$; $p < 0.00001$, $I^2 = 96\%$, respectively) (Figures 7, 8). The effect of Ani injection on rCBF and rMTT, however, was not substantial. After sensitivity analysis by deleting one study (Zhang et al., 2022), the overall effect of Ani injection on rCBF and rMTT was significantly changed [SMD = 0.68, 95%CI = (0.40, 0.97), $p < 0.00001$; SMD = −1.57, 95% CI = (−1.89, −1.25), $p < 0.00001$, respectively]. Heterogeneity in both outcomes was also significantly reduced to 0%, which suggested that

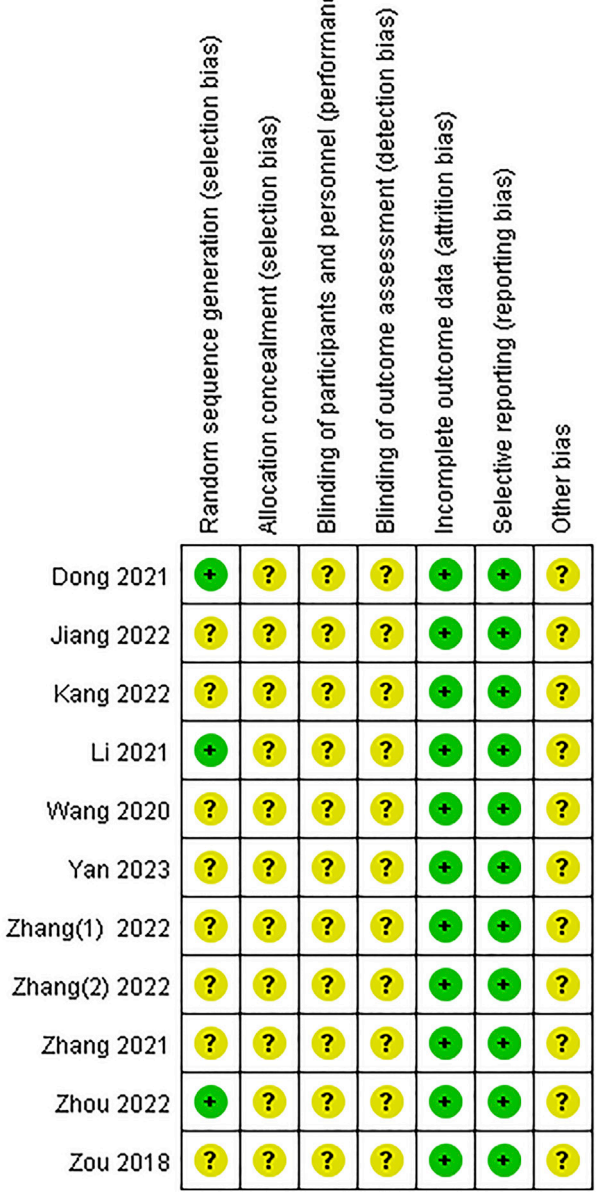

FIGURE 3
Risk of bias summary.

this study (Zhang et al., 2022) might be the source of the heterogeneity of the rCBF and rMTT data. Both the rCBV and rTTP levels were significantly changed by Ani injection therapy [SMD = 0.28, 95%CI = (0.02, 0.53), $p = 0.03$; SMD = −0.81, 95%CI = (−1.08, −0.55), $p < 0.00001$, respectively] without between-study heterogeneity ($p = 0.36$, $I^2 = 3\%$; $p = 0.90$, $I^2 = 0\%$, respectively) (Figures 9, 10).

3.4.5 Clinical efficacy

Two studies (Kang, 2022; Yan et al., 2023) reported the clinical effective rate, which was evaluated according to the NIHSS score for stroke patients. The heterogeneity test results showed that there was no significant heterogeneity among these studies ($p = 0.82$, $I^2 = 0\%$); therefore, the fixed-effect model was adopted. The pooled results showed that the effective rate of the Ani injection-treated group was significantly better than that of the conventional therapy group [RR = 1.2, 95%CI = (1.08, 1.34), $p = 0.001$] (Figure 11).

3.4.6 Adverse events

A total of seven articles (Zou et al., 2018; Dong et al., 2021; Li et al., 2021; Zhang et al., 2021; Jiang et al., 2022; Zhang, 2022; Zhang et al., 2022) recorded adverse reactions, of which two (Zhang, 2022; Zhang et al., 2022) reported that no adverse events occurred during treatment and two (Zou et al., 2018; Jiang et al., 2022) reported mild side effects, including dry mouth and facial flushing; these symptoms had completely disappeared after slowing down the drip rate.

The other three articles (Dong et al., 2021; Li et al., 2021; Zhang et al., 2021) reported the incidence of adverse reactions. Using the random-effect model ($p = 0.12$, $I^2 = 53\%$) for the meta-analysis, the pooled results showed that there were no significant differences between the Ani injection supplemental group and the control group [RR = 1.25, 95%CI = (0.52, 3.03), $p = 0.62$] (Figure 12). Dong et al. (2021) observed 12 cases of dry mouth and facial flushing in the experimental group and 8 cases of nausea and vomiting in the control group. Zhang et al. (2021) reported 6 cases of nausea, vomiting, dry mouth, and facial flushing in the control group and 2 cases of nausea and vomiting in the experimental group. Li et al. (2021) found 2 cases of elevated alanine aminotransferase (ALT), 2 cases of dizziness, 3 cases of weakness, and 6 cases of gastrointestinal reactions in the experimental group and 1 case of elevated ALT, 1 case of weakness, and 4 cases of gastrointestinal reactions in the control group. Table 2 presents the adverse reactions of the involved studies.

3.5 Publication bias

A funnel plot was conducted to assess the publication bias of 10 trials or more. Thus, the 10 included studies that included available NIHSS score data were used for publication bias assessment, as shown in Figure 13. The shape of the funnel plot of the NIHSS showed the moderate symmetry between the included studies, which indicated that the potential of publication bias was low.

4 Discussion

4.1 Summary of findings

A total of 11 RCTs were included in this meta-analysis. Combined with conventional therapy, Ani injection was used to treat 1,337 patients with AIS. The NIHSS, mRS, BI, CTP, effective rate, and adverse events were evaluated in the analysis. According to the findings, the NIHSS score of Ani injection therapy was much lower than that of conventional therapy alone. Other primary indicators showed that Ani injection significantly reduced the mRS score and increased the BI score. The secondary outcome indicators revealed that treatment with Ani injection increased the

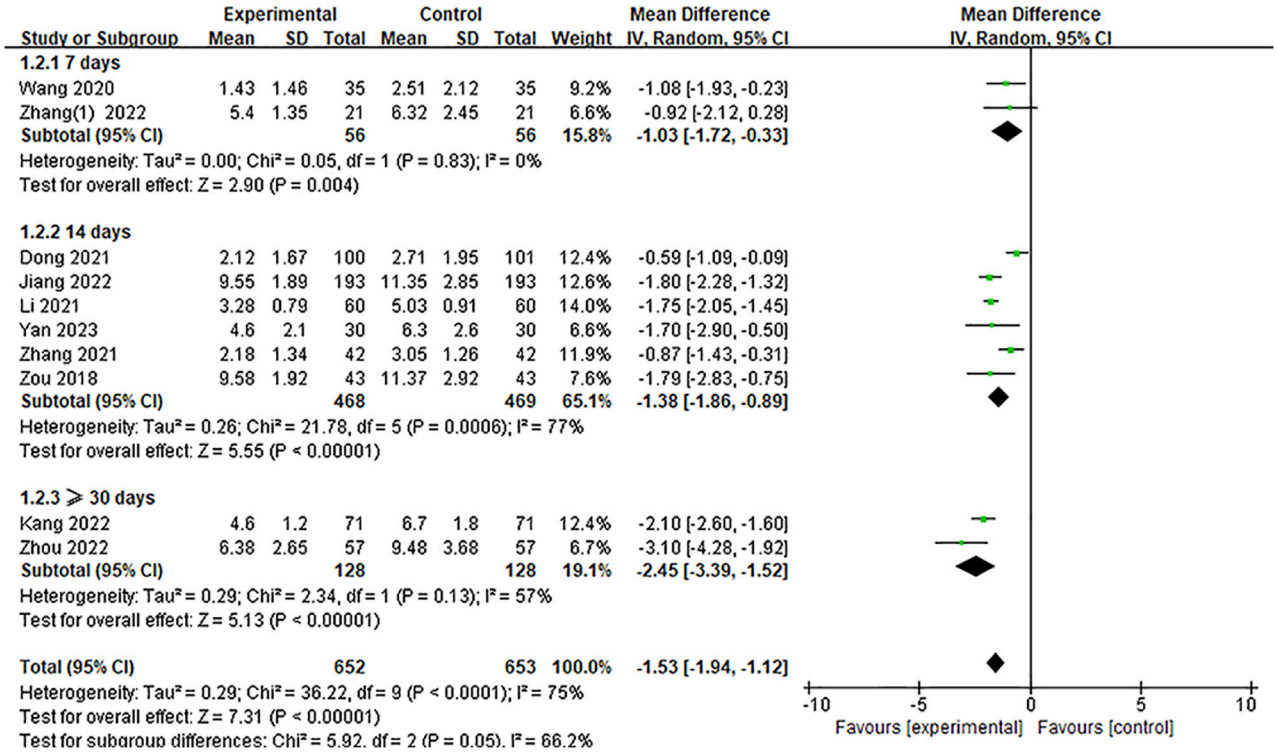

FIGURE 4
A meta-analysis of the NIHSS.

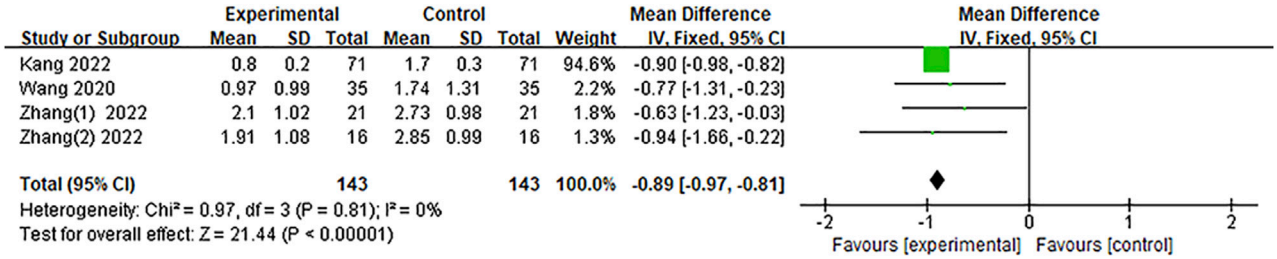

FIGURE 5
A meta-analysis of mRS.

rCBV, reduced the rTTP, and improved the clinical efficacy, with significant differences observed. The pooled analysis of the included studies failed to identify a significant change in the rCBF, rMTT, and rate of adverse reactions.

Subgroup analyses indicated that at the different time periods of 7 days, 14 days, and ≥30 days for implementing the NIHSS assessment, the NIHSS score of the Ani treatment group was considerably decreased. Furthermore, the subgrouping factor might be a source of significant heterogeneity for the NIHSS. Subgroup analyses on the BI score based on the BI assessment method (original BI and modified BI) suggest that regardless of the BI assessment method used, Ani treatment exhibits an advantage in significantly increasing the BI score. However, the subgroup analysis of BI did not identify the source of heterogeneity; significant heterogeneity may be associated with factors such as small sample sizes and few included studies.

A sensitivity analysis of rCBF and rMTT suggested that the pooled results are not robust and that the study of Zhang et al. (2022) might be the source of rCBF and rMTT heterogeneity.

In the risk of bias section, the quality assessment of the current included studies showed that allocation concealment, blinding of participants and employees, and blinding of the outcome assessment, as well as other forms of bias, were not disclosed in any of the included studies, which suggested that the certainty of evidence in the included RCTs was not high. Consequently, the results of the meta-analysis may be influenced, and our findings based on the current evidence should be considered carefully in the clinic. More precise

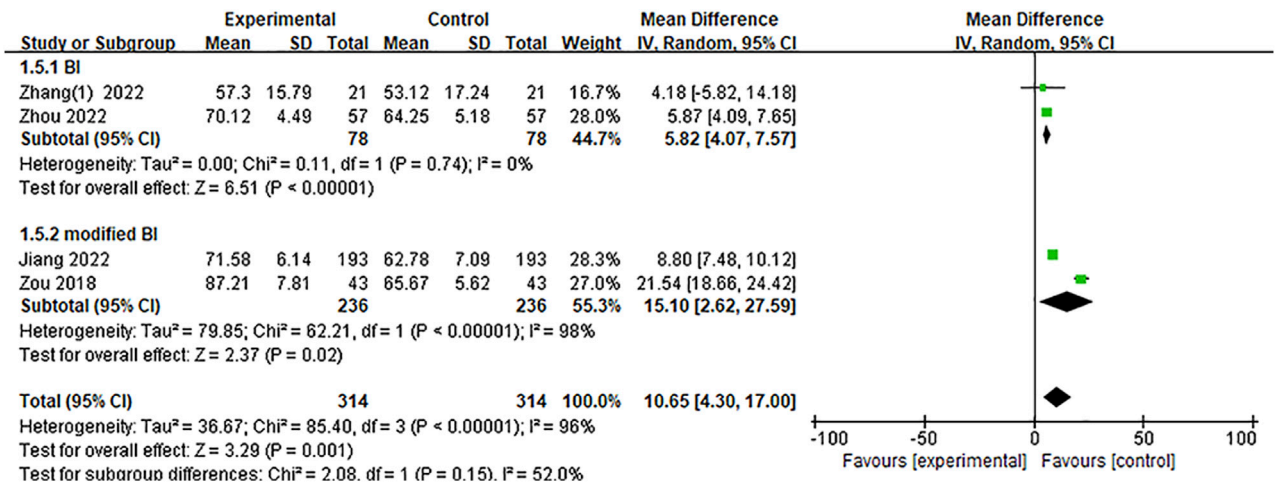

FIGURE 6
A meta-analysis of BI.

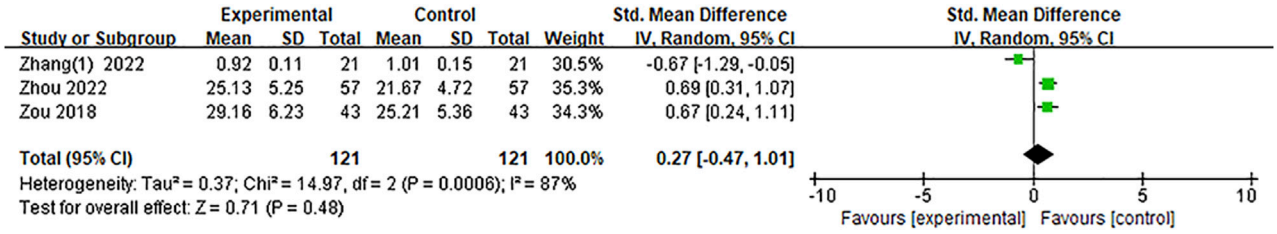

FIGURE 7
A meta-analysis of rCBF.

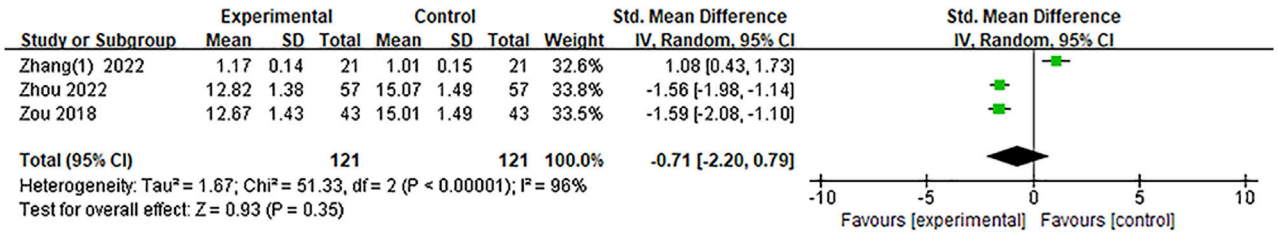

FIGURE 8
A meta-analysis of rMTT.

RCTs are needed to further validate the curative effect of Ani injection in patients with AIS.

4.2 Interpretation

Traditional Chinese herbal medicines have a long history of clinical application in treating various vascular diseases, with distinctive theories and rich practices (Hung et al., 2015; Hao et al., 2017). Products from traditional Chinese medicinal herbs have been widely described in various ancient medicine systems for treating ischemic stroke, myocardial infarction, and so on (Hung et al., 2015). Anisodine is one of the most important ingredients of the tropane-type alkaloids extracted from the traditional folk medicinal herb *A. tanguticus*, with significant biological activities for promoting blood circulation and removing blood stasis (Meng et al., 2023). Pharmaceutical products containing anisodine are frequently used in the clinic for the treatment of vascular diseases, including ischemic stroke (Zou et al., 2018), retinal artery occlusion (Wu et al., 2016), ischemic optic neuropathy

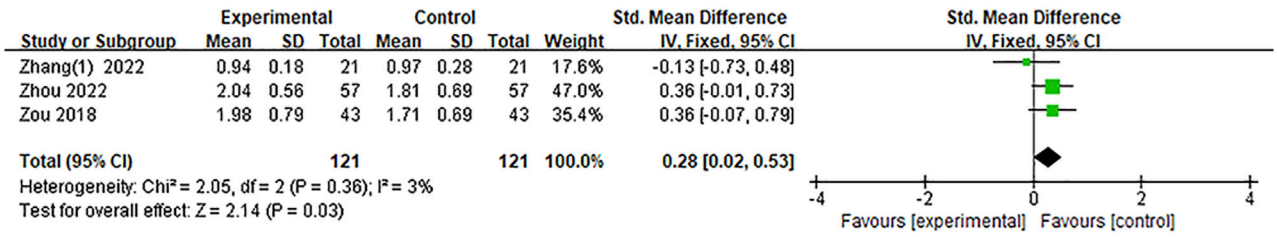

FIGURE 9
A meta-analysis of rCBV.

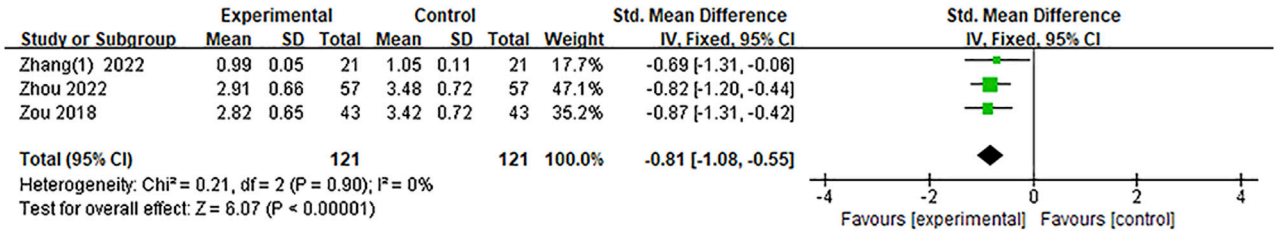

FIGURE 10
A meta-analysis of rTTP.

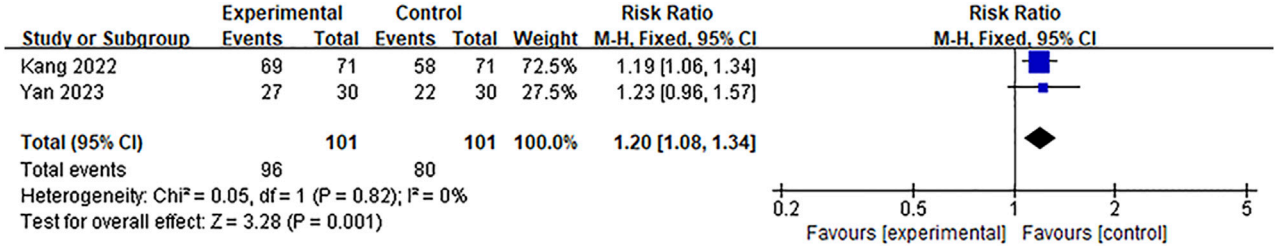

FIGURE 11
A meta-analysis of clinical efficacy.

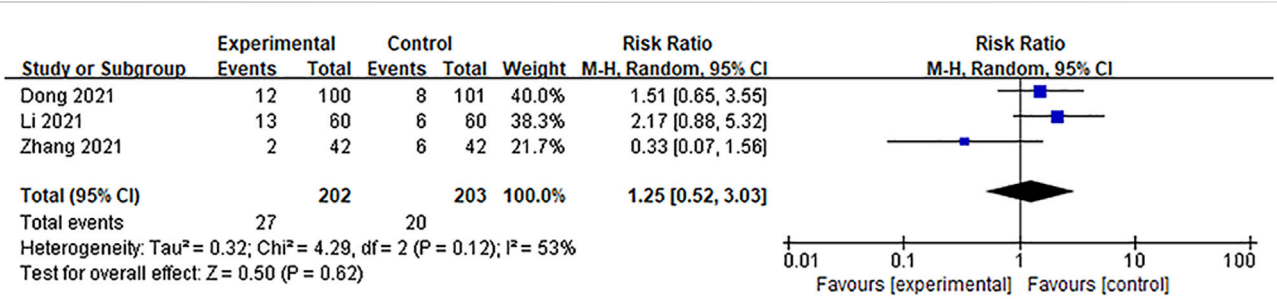

FIGURE 12
A meta-analysis of the rate of adverse events.

TABLE 2 Summary of adverse events of the involved studies.

Study	Group	Number	Number of adverse reactions	Adverse reactions
Zou et al. (2018)	Trial	43	NR	Mild flushed face, dry mouth, dizziness
	Control	43	NR	NR
Zhang et al. (2021)	Trial	42	2	Nausea, vomiting
	Control	42	6	Flushed face, dry mouth, nausea, vomiting
Li et al. (2021)	Trial	60	13	Elevated alanine aminotransferase, dizziness, fatigue, gastrointestinal reactions
	Control	60	6	Elevated alanine aminotransferase, fatigue, gastrointestinal reactions
Dong et al. (2021)	Trial	100	12	Flushed face, dry mouth
	Control	101	8	Nausea, vomiting
Zhang (2022)	Trial	21	NR	No adverse reactions occurred
	Control	21	NR	No adverse reactions occurred
Jiang et al. (2022)	Trial	193	NR	Mild flushed face, dry mouth, bitter mouth, loss of appetite
	Control	193	NR	Mild flushed face, dry mouth, bitter mouth, loss of appetite
Zhang et al. (2022)	Trial	16	NR	No adverse reactions occurred
	Control	16	NR	No adverse reactions occurred

Note: NR, not reported.

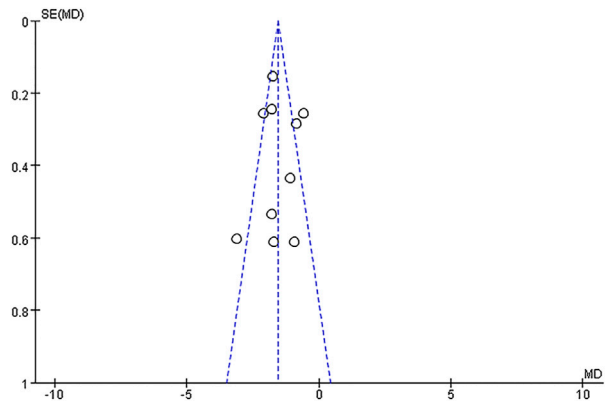

FIGURE 13
Funnel plot for the publication bias of the NIHSS.

(Zhang et al., 2019), and cerebral small vessel disease (Gui et al., 2019). Ani injection has been developed for improved chemical stability and is a promising treatment for AIS.

Poor perfusion of brain tissue caused by the abrupt interruption or reduction of cerebral blood flow is the etiology of AIS, which can then induce ischemic hypoxic necrosis, clinically manifesting as different degrees of neurological impairment (Brott and Bogousslavsky, 2000). The molecular mechanism of AIS can be summarized as a complex series of ischemic cascades, characterized by cellular bioenergetic failure, excitotoxicity, excessive intraneuronal accumulation of Na^+, Cl^-, and Ca^{2+}, oxidative damage, inflammatory reaction, mitochondrial injury, and, finally, cell death (Rosenblum, 1997; Brouns and De Deyn, 2009). Guidelines for the management of AIS have been reported by various countries (Swain et al., 2008; Di et al., 2019; Powers et al., 2019); the fundamental goals of the intervention have been focused on restoring or increasing the blood supply to the brain and blockading or slowing of the cerebral ischemic cascade (Olsen et al., 1983; Escuret, 1995; Brott and Bogousslavsky, 2000). The conventional therapy adopted in the 11 included studies varied across different care settings, including general management (such as respiratory and oxygen intake, cardiac monitoring and cardiac disease management, temperature control, blood pressure control, blood sugar control, and nutritional support, etc.) and specific treatment (thrombolysis, antiplatelet drugs, anticoagulants, statins, defibrase, and diuretics, etc.). Nevertheless, the narrow treatment window and hemorrhagic complications have limited the utilization and therapeutic effect of conventional therapy.

Recent studies have revealed that in addition to the recanalization of the large cerebral vessels, the restoration of normal vasomotor function around the ischemic area, the improvement in micro-perfusion, and neuronal cell protection are crucial for the treatment of cerebral infarction and are closely related to the prognosis of AIS (Tuttolomondo et al., 2009; Shuaib et al., 2011; Bang et al., 2015). As a central muscarinic cholinergic antagonist, Ani can effectively relieve vasospasm, open closed arterioles and the anterior capillary sphincter, and restore the perfusion of brain tissue (Wang et al., 2018). Research has demonstrated that following Ani administration, the microvascular autonomic motion reappears in the small intestinal wall micro-artery ischemia model, with a significant increase in microvascular amplitude, blood velocity, and flow (Zhang et al., 2019). Through the establishment of a hypoxia/reoxygenation (H/R)-induced brain microvascular endothelial cell injury model, Ani injection has been shown to suppress H/R-induced hypoxia-inducible transcription factor 1(HIF-α) over-expression, nitric oxide (NO), and reactive oxygen species (ROS) production, and

all these effects were dependent on M4-AchR (Zeng et al., 2021). Additionally, Ani has multiple non-cholinergic effects, including cell protective effects, autophagy (Chen et al., 2017b), attenuating neuronal cell death and apoptosis (Chen et al., 2017d), alleviating oxidative stress damage and decreasing Ca^{2+} accumulation (Chen et al., 2017; Wang et al., 2017; Chen et al., 2017e), and inhibiting membrane lipid peroxidation (Zhao and Chen, 2010), thereby alleviating cell damage caused by ischemia and hypoxia. Further studies have found that Ani can decrease the Longa rodent stroke scores and cerebral infarction area in middle cerebral artery occlusion (MCAO) rats (Chen et al., 2017c). Moreover, the underlying mechanism of the effect of Ani on AIS can also be attributed to the ability to improve hemorheology and resist platelet aggregation so as to improve cerebral microcirculation disorders (Xu et al., 2020).

4.3 Strengths and limitations

In short, based on current evidence, Ani injection therapy was found to be effective and safe in patients with AIS. The positive effect of Ani injection may be attributed to the ability of Ani to penetrate the blood–brain barrier and act as a non-specific muscarinic cholinergic receptor antagonist, competing with acetylcholine in the central nervous system, resulting in increased cerebral blood supply and neuroprotection effects (Liu et al., 2020; Jiang et al., 2022). The results of this meta-analysis demonstrated that the efficacy of Ani injection in the treatment of AIS was superior to that of conventional therapy; however, several limitations still exist. First, the sample size of the included RCTs was small, so the subgroup analysis and publication bias assessment could not be conducted for all indicators, which may have affected the accuracy and reliability of the results. Second, all trials lacked a precise description of the allocation concealment and blinding methods (for participants, personnel, and outcome assessments); the description of random sequence generation was also missed in some studies. As a consequence, the general methodological quality of the studies was not satisfactory. Third, the longest intervention duration in all the articles was 90 days, and there was a lack of long-term follow-up visits for more than 90 days after treatment, which was insufficient to assess the long-term impact of Ani therapy on the health of patients. Additionally, all the included RCTs were conducted in China; therefore, it is necessary to utilize multi-regional clinical trials for Ani treatment evaluation in different regions of the world in the future to provide strong evidence for the efficacy and safety of Ani treatment in patients with AIS.

5 Conclusion

Taken together, the meta-analysis results from the included RCTs revealed that Ani injection is effective and safe in the treatment of patients with AIS, with positive impacts on the NIHSS, mRS, BI, rCBV, rTTP, and clinical efficacy. However, due to limitations in the number and quality of included studies, more multi-center, large-sample, high-quality RCTs are needed for further verification of the efficacy and safety of Ani injection in treating AIS.

Data availability statement

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

Author contributions

YW: Data curation, Formal Analysis, Methodology, Writing–original draft. FW: Conceptualization, Investigation, Writing–review and editing. PH: Data curation, Investigation, Writing–review and editing. BH: Funding acquisition, Supervision, Writing–review and editing. YH: Funding acquisition, Supervision, Writing–review and editing. YL: Conceptualization, Formal Analysis, Funding acquisition, Methodology, Writing–original draft.

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Conflict of interest

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

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Supplementary material

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Flavonol and A-type procyanidin-rich extracts of *Prunus spinosa* L. flower exhibit anticoagulant activity through direct thrombin inhibition, but do not affect platelet aggregation *in vitro*

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Background: Blackthorn flower (*Prunus spinosa* L.) is a traditional herbal remedy recommended for treating cardiovascular diseases (CVDs).

Aim: This *in vitro* study investigates the effects of flavonol and A-type procyanidin-rich blackthorn flower extracts on the hemostatic system, including the blood plasma coagulation cascade and platelet aggregation.

Methods: Six distinct extracts, characterized through various techniques, including LC-MS/MS, were assessed at *in vivo*-relevant levels (1–50 µg/mL) for their antithrombotic activity. The thrombin, prothrombin, and activated partial thromboplastin times were measured. Additionally, the thrombin enzymatic activity was tested using the chromogenic substrate S-2238 and fibrinogen as the physiological substrate of the enzyme. To gain insights into the mechanism of action, the interactions between the primary extracts' constituents, their potential metabolites, and thrombin were examined *in silico*. The computational analyses were complemented by *in vitro* experiments and circular dichroism spectroscopy. The platelet aggregation in human platelet-rich plasma was assessed after ADP or collagen stimulation. Furthermore, the extracts' biocompatibility was tested on human peripheral blood mononuclear cells (PBMCs) and red blood cells (RBCs).

Results: The extracts slightly prolonged the prothrombin and thrombin times and effectively inhibited the thrombin's enzymatic activity, reducing its amidolytic and proteolytic functions at 50 µg/mL by 91.2% and 74.8%, respectively. *In silico* molecular docking demonstrated a strong binding affinity of the examined polyphenols and their metabolites to thrombin. Most analytes bound exclusively within the enzyme active site; however, afzelin, kaempferitrin, and procyanidin A2 revealed the affinity to additional binding sites, including exosite I.

The structure-activity relationship of flavonols as thrombin inhibitors was studied *in vitro*. Circular dichroism spectroscopy confirmed that the interactions between thrombin and the compounds (even at 1 μg/mL) induce alterations within the α-helices' secondary structure, resulting in noticeable changes in the enzyme's CD spectrum. On the other hand, the extracts did not influence platelet aggregation. Eventually, their cellular biocompatibility with PBMCs and RBCs was confirmed.

Conclusion: The extracts directly inhibit thrombin, a critical serine protease in hemostasis and a prime anticoagulant drug target, and do not exhibit antiplatelet effects. This study enhances the knowledge of the biological activity of blackthorn flowers and supports their traditional use in CVDs.

KEYWORDS

***Prunus spinosa*, blackthorn flowers, polyphenols, hemostasis, thrombin, anticoagulant, blood platelets**

1 Introduction

The physiological balance among platelets, plasma coagulation factors, and fibrinolytic proteins is vital for maintaining blood fluidity, preventing blood loss during injury and controlling the extent of the formed fibrin clot. This equilibrium results from the interplay of procoagulant and anticoagulant elements in the blood, along with the modulatory functions of the vessel wall, particularly the endothelium. However, multiple endogenous and exogenous factors can disrupt this balance, increasing plasma procoagulant activity and reducing fibrinolysis efficiency. Inflammation, elevated plasma fibrinogen levels, platelets hyperactivity, and prothrombotic events are common in various disorders, including cardiovascular diseases (CVDs) (Petäjä, 2011; Stark and Massberg, 2021).

Thrombin (the coagulation factor IIa, EC 3.4.21.5), an executive serine protease of the blood coagulation cascade, plays a central role in forming fibrin clots from plasma fibrinogen. Beyond this, it has various procoagulant and modulatory functions, including activating other coagulation factors and stimulating platelets (Al-Amer, 2022). Due to its critical role in hemostasis, thrombin is a target for anticoagulant medications (Sun et al., 2020). Common thrombin-inhibiting drugs include traditional anticoagulants, which inhibit free thrombin indirectly by binding simultaneously to antithrombin and exosite II, and direct thrombin inhibitors (DTIs). DTIs is a relatively new class of anticoagulants, specifically and potently blocking thrombin by binding to its active site (univalent DTIs) or both active site and exosites (bivalent DTIs), and not dependent on a cofactor such as antithrombin (Lee and Ansell, 2011; Sun et al., 2020). They offer clinical advantages such as more predictable anticoagulant effect, inhibition of thrombin-induced platelet aggregation, and a lower risk of immune-mediated thrombocytopenia. However, they still have limitations and adverse effects, among which the risk of bleeding is the most concerning (Lee and Ansell, 2011). Exploring new, safer thrombin inhibitors, potentially from natural sources, is a promising approach to prevent and treat thromboembolic diseases.

Polyphenols are specialized plant metabolites with pleiotropic activity in cardiovascular system, including antioxidant, anti-inflammatory, antiplatelet and anti-hypertensive effects (Behl et al., 2020). They are able to modulate activity or even inhibit various enzymes, including serine proteases (Xue et al., 2017). Recent studies have noted polyphenols' ability to counteract thrombin action, particularly for isolated compounds like flavonols, anthocyanidins, flavonolignans, and flavan-3-ols (Liu et al., 2010; Bijak et al., 2014). However, there are limited reports on the thrombin-inhibitory effects of polyphenol-rich extracts (Bijak et al., 2013; Sikora et al., 2014; Wei et al., 2019; Rutkowska et al., 2021). Considering the observed synergy between various types of polyphenols and the potential advantages of using extracts over isolated compounds, the search for polyphenol-rich extracts with antithrombotic properties is justified.

Blackthorn (*Prunus spinosa* L.) is a spiny, deciduous shrub or small tree native to Europe, western Asia, and northwest Africa, with naturalized population in New Zealand, Tasmania and North America. In numerous countries, this plant is not only recognized for its edible fruits but also revered for centuries as a medicinal plant. Ethnopharmacological investigations have revealed long-standing use of blackthorn-derived herbal materials (flowers, fruits, leaves, branches, bark) in folk medicine for various purposes, including application in cardiovascular diseases (Vokou et al., 1993; Cavero et al., 2011; Calvo and Cavero, 2014; Ginko et al., 2023). Flowers are especially valued in European tradition for their vasoprotective, anti-inflammatory, diuretic, detoxifying (blood purifying), and spasmolytic properties, and recommended as ingredients of compound herbal prescriptions traditionally applied, e.g., to treat myocarditis, cardiac neurosis and atherosclerosis (Berger, 1949; Wawrzyniak, 1992; Vokou et al., 1993). Our previous research in chemical, enzymatical and biological models *in vitro* documented that polyphenol-rich blackthorn flower extracts exhibit potent antioxidant and anti-inflammatory activity that might be responsible for the effects indicated by traditional medicine (Marchelak et al., 2017; Marchelak et al., 2019; Marchelak et al., 2021). In particular, the extracts were able to scavenge reactive oxygen/nitrogen species (ROS/RNS) considered as the potential inducers of oxidative stress in the circulatory system *in vivo*, they enhanced the total antioxidant status of human plasma, as well as protected the human plasma biomolecules against oxidative and nitrative impairments (Marchelak et al., 2017; Marchelak et al., 2019). Furthermore, significant protection against ROS/RNS-driven structural and functional modification of fibrinogen, a multifunctional protein

essential for hemostasis, was observed (Marchelak et al., 2021). However, the influence of blackthorn flower extracts on the hemostatic system, including the verification of the anticoagulant or antiplatelet properties, has not been investigated.

Therefore, this study aimed to analyze the effects of six distinct *P. spinosa* flower extracts (thoroughly characterized using various phytochemical methods, including LC-MS/MS) on the hemostasis, with a particular emphasis on their potential antithrombotic action. The anticoagulant properties of the extracts were assessed *in vitro* by measuring blood plasma clotting times, as well as thrombin's enzymatic activity, including both amidolytic and proteolytic functions. To gain insights into the molecular-level mechanism of action, *in silico* analyses were conducted to examine the interaction between the primary constituents of *P. spinosa* extracts, their potential plasma metabolites, and thrombin. In addition to computational analyses, this study included *in vitro* experiments and circular dichroism spectroscopy. Furthermore, to gain a deeper understanding of the extracts' influence on the hemostatic system, their antiplatelet effects were investigated. In all analyses, the extracts were used at microgram concentrations (*i.e.* 1-50 μg/mL), providing physiologically achievable levels of their bioactive compounds. The final aspect of our study focused on assessing the safety of the *P. spinosa* extracts on human peripheral blood mononuclear cells (PBMCs) and human red blood cells (RBCs).

2 Materials and methods

2.1 Plant material and preparation of the extracts

The analyses were carried out using dry extracts obtained from the flowers of *P. spinosa* L. Commercial samples of the plant material were bought in 2015 (harvested in April 2015) from a local Polish provider, *Dary Natury* (Koryciny, Poland). The voucher sample (01052015/PSFL_1501_DN) was deposited in the Department of Pharmacognosy, Medical University of Lodz, Poland. The source aqueous extract (AQ) was prepared as follows: the plant material was grounded using an electrical grinder, sieved (0.315 mm), and extracted with chloroform (3 L, 30 h) in a Soxhlet apparatus. Then, the pellet was extracted exhaustively with water (4 × 1 L), and the combined extracts were evaporated *in vacuo* and lyophilized (Alpha 1–2/LD Plus freeze dryer, Christ, Osterode am Harz, Germany). The preparation of the basic methanol-water (7:3, *v/v*) extract (MED) and its concentrated polyphenol-rich fractions of diethyl ether (DEF), ethyl acetate (EAF), *n*-butanol (BF) fractions and water residues (WR), obtained by sequential liquid-liquid partitioning, was described previously (Marchelak et al., 2017).

2.2 Phytochemical standardization of the extracts

The qualitative profiling (UHPLC-PDA-ESI-MS^3 analysis) of AQ was performed according to (Marchelak et al., 2017) using the same equipment and chromatographic procedure. The quantitative profiling of AQ included the measurements of the total phenolic contents (TPC) by the Folin-Ciocalteu method, the total proanthocyanidin contents (TPA) by *n*-butanol-HCl method, as described previously (Marchelak et al., 2017), and the contents of individual analytes by HPLC-PDA assay, performed according to (Marchelak et al., 2020b), using the same equipment and procedure. MED, DEF, EAF, BF and WR were assessed qualitatively and quantitatively in terms of their polyphenolic constituents using a panel of fully validated phytochemical profiling methods, including LC-MS/MS and LC-PDA assays, as was described earlier (Marchelak et al., 2017; Marchelak et al., 2020a; Marchelak et al., 2020b).

2.3 Biological material

The study was approved by the Committee on the Ethics of Research at the Medical University of Lodz (RNN/213/18/KE, RNN/104/20/KE) and the University of Lodz (8/KBBN-UŁ/II/2015). All the experiments were performed in accordance with the Declaration of Helsinki and based on the national legal procedures and the European Union regulations.

Blood plasma was obtained by differential centrifugation of the commercially available buffy coat units, constituting the waste of the blood-derived preparations and purchased from the Regional Centre of Blood Donation and Blood Treatment in Lodz (Poland). Fibrinogen was isolated from human plasma as described previously (Marchelak et al., 2021), and its concentration was established spectrophotometrically at 280 nm using an extinction coefficient of 1.55 for 1 mg/mL solution. For the aggregometry tests, fresh blood was obtained from healthy volunteers and collected into the S-Monovette® 8.5 mL, CPDA1 tubes (Sarstedt AG & Co. KG, Nümbrecht, Germany). PBMCs were isolated from human blood using the Histopaque®-1077 medium, according to the previously described procedure (Matczak et al., 2018). The material for RBCs lysis assay and microscopy studies constituted the blood obtained from the Regional Centre of Blood Donation and Blood Treatment in Lodz (Poland). RBCs were separated from plasma by centrifugation (3000 rpm, 10 min, 20°C), washed three times with 0.9% saline, and used for the studies within 24 h (Markowicz-Piasecka et al., 2015).

2.4 Determination of the hemostatic parameters

When determining the hemostatic parameters, stock solutions of the *P. spinosa* flower extracts (10 mg/mL) were prepared in 30% DMSO. DMSO concentrations in working solutions of the tested extracts (0.1, 0.5 and 5 mg/mL) were 15%. The studies mixed 5 μL of the extracts with 495 μL of blood plasma or thrombin solution. Control samples were untreated with the extracts; however, they contained a vehicle for the plant preparations, i.e., DMSO. The effect of DMSO on the investigated parameters was excluded in preliminary tests. All experiments used argatroban, a reference anticoagulant drug, as a positive control.

2.4.1 Blood clotting times

Thrombin (TT), prothrombin (PT) and the activated partial thromboplastin time (APTT) were measured in blood plasma using a Kselmed K-3002 Optic coagulometer (Kselmed, Grudziadz, Poland) and reagents purchased from Diagon Kft. (Budapest, Hungary), according to the laboratory protocols delivered by the manufacturer. Before the assays, the blood plasma samples were pre-incubated with the examined extracts at the final 1–50 μg/mL concentration for 15 min at 37°C. Control samples were prepared using blood plasma untreated with the extracts.

2.4.2 Amidolytic activity of thrombin

The thrombin enzyme, purchased from Biomed (Lublin, Poland), was diluted with 0.05 M Tris-buffered saline (TBS), pH 7.4, to the concentration of 0.75 U/mL, and pre-incubated with the examined extracts at the final concentration of 1–50 μg/mL for 10 min, at 37°C. In control samples, 20% DMSO instead of the extracts was used. The measurements were conducted using a kinetic protocol, with absorbance recorded at 415 nm, every 10 s, for 15 min. The reaction mixture comprised 280 μL of thrombin (pre-incubated with the extracts or control/untreated enzyme) and 40 μL of 3 mM chromogenic substrate Chromogenix S-2238 purchased from Instrumentation Laboratory (Bedford, MA, United States of America). Thrombin amidolytic activity was estimated using the BMG Labtech software based on the maximal velocity of the reaction (V_{max}). The assays were performed using 96-well microplates and the BMG Labtech SpectroStar Nano spectrophotometer (BMG LABTECH, Offenburg, Germany).

Analogous pre-incubation conditions and experimental systems were used to determine effects of the several components of the extracts (selected based on the results of the phytochemical profiling and molecular docking study), i.e., quercetin (QU), kaempferol (KA), quercetin 3-*O*-*α*-L-arabinofuranoside (avicularin, AV), quercetin 3-*O*-*α*-L-rhamnopyranoside (quercitrin, QC), kaempferol 3-*O*-*α*-L-arabinofuranoside (juglanin, JU), kaempferol 3-*O*-*α*-L-rhamnopyranoside (afzelin, AFZ), kaempferol 3,7-di-*O*-*α*-L-rhamnopyranoside (kaempferitrin, KT), kaempferol 3-*O*-(2″-*O*-E-*p*-coumaroyl)-α-L-arabinofuranoside, *p*-cJU), proanthocyanidin A2 (PA2), and 5-*O*-caffeoylquinic acid (chlorogenic acid, CHA) on the amidolytic activity of the enzyme. High-purity standards of QU and CHA were purchased from Sigma-Aldrich (St. Louis, MO, United States), while KA and PA2 were obtained from Phytolab (Vestenbergsgreuth, Germany). The standards of AV, QC, JU, AFZ, KT, *p*-cJU were isolated previously in the Department of Pharmacognosy, Medical University of Lodz, Lodz, Poland, from the flowers and leaves of *P. spinosa*, with HPLC and NMR purity >98% (Olszewska and Wolbis, 2001; Olszewska, 2002; Olszewska and Wolbiś, 2002).

2.4.3 Proteolytic activity of thrombin

The proteolytic activity of thrombin was determined using fibrinogen isolated from human blood plasma, as was described in section 2.3. The reaction mixture consisted of 100 μL fibrinogen (diluted with TBS to the concentration of 3 mg/mL) and 200 μL thrombin (0.75 U/mL in TBS enriched with 25 mM $CaCl_2$, control or pre-incubated with the tested extracts, analogously to the measurements of the amidolytic activity). Kinetic measurements were started immediately after adding thrombin to the microplate wells with fibrinogen. The absorbance was recorded at 360 nm, every 10 s, for 20 min, at 37°C. The proteolytic activity of thrombin was estimated using BMG Labtech software (SpectrostarNano Mars, Version 3.01. R2) based on the maximal velocity of the polymerization reaction (V_{max}) and lag time. The assays were conducted using 96-well plates, and a microplate reader SpectrostarNano (BMG Labtech, Ortenburg, Germany). The effects of the extracts on the proteolytic activity of thrombin were also evaluated using human blood plasma. The experiments were carried out analogously to the abovementioned procedure, using 100 μL of human blood plasma instead of the isolated fibrinogen.

2.5 *In silico* molecular docking

The selected constituents of *P. spinosa* flower extracts, including CHA, 3- *O*-caffeoylquinic acid (neochlorogenic acid, NCHA), (+)-catechin (CA), PA2, QU, KA, AV, JU, QC, *p*-cJU, AFZ, kaempferol 3-*O*-*α*-L-arabinofuranoside-7-*O*-*α*-L-rhamnopyranoside (KAFR), KT, kaempferol 3-*O*-*β*-D-xylopyranoside (KX), kaempferol 3-*O*-(4″-*O*-*β*-D-glucopyranosyl)-*α*-L-rhamnopyranoside (multiflorin B, MUB), kaempferol 7-*O*-*α*-L-rhamnopyranoside (KR), *p*-coumaric acid (*p*-CA), as well as phenolic compounds considered to be primary metabolites of polyphenols in the human body, including miquelianin (quercetin 3-*O*-*β*-D-glucuronopyranoside, MQ), 3-(3′,4′-dihydroxyphenyl)propionic acid (dihydrocaffeic acid, DCA), protocatechuic acid (PCA), 3-(4′-hydroxyphenyl)propionic acid (PPA), 2-(3′,4′-dihydroxyphenyl) acetic acid (PAA) were prepared for docking to the unliganded (3U69 structure) and liganded (1FPH.PDB structure) macromolecule of human thrombin as described earlier (Kolodziejczyk-Czepas et al., 2017). The docking was accomplished using AutodockVina 1.1.2 (Trott and Olson, 2010), ten times for each ligand. Average and standard deviations (SD) of the predicted ten-fold binding affinity (kcal/mol) were taken for further evaluation. The docked molecular structures were illustrated using UCSF Chimera 1.13 (http://www.cgl.ucsf.edu/chimera/, Pettersen et al., 2004).

2.6 Circular dichroism (CD)

The CD spectra (195–260 nm) were recorded for human thrombin purchased from Sigma-Aldrich (St. Louis, MO, United States), in the presence/absence of the selected constituents of *P. spinosa* flower extracts (PA2, KA, *p*-cJU, QU) on a Jasco J-815 CD spectropolarimeter (Tokyo, Japan) in 5-mm path length quartz cuvettes, with a wavelength step of 1 nm, a response time of 4 s and a scan rate of 100 nm/min, thermostatted at 37°C. Each spectrum was the average of three repetitions. Human thrombin was used at a 15 μg/mL concentration in 10 mM phosphate buffer (pH = 7.4). In determining circular dichroism spectra, stock solutions of *P. spinosa* flower components (10 mg/mL) in 10% tetrahydrofuran were initially prepared. The final concentration of tetrahydrofuran in the sample was less than 0.05%. The selected extract components in phosphate buffer without thrombin at the concentrations used in the experiments were used as a baseline for thrombin/extract complexes spectra.

2.7 Blood platelet aggregation

The blood samples under examination contained a normal range of blood platelets, ranging from 2.15×10^5 to 3.41×10^5 cells/μl. Platelet-rich plasma (PRP) was prepared by differentially centrifuging whole blood according to the method described earlier (Kolodziejczyk-Czepas et al., 2021). Subsequently, freshly obtained PRP samples (495 μL) were pre-incubated for 15 min at 37°C with the tested analytes (5 μL). These mixtures were then transferred into Chrono-Log 490 aggregometer cuvettes (Havertown, United States). Platelet aggregation measurements were conducted after stimulating the samples with either ADP (a final concentration of 10 μM) or collagen (a final concentration of 2 μg/mL), following the manufacturer's instructions.

2.8 Determination of the cellular safety

2.8.1 Influence on PBMCs viability

The cytotoxicity of the examined extracts was performed in an experimental model of PBMCs, according to the procedure described earlier (Matczak et al., 2018). PBMCs were isolated from human blood, suspended in the RPMI-1640 medium (1.5 × 106 PBMCs/mL) and incubated with the extracts at the final concentration of 1–50 μg/mL for 24 h. Cell viability measurements were carried out using the resazurin-based metabolic assay (*In Vitro* Toxicology Assay Kit, Sigma-Aldrich) according to the procedure described by the manufacturer.

2.8.2 Influence on erythrocyte membrane integrity/also hemolysis

Microscopy studies were conducted as follows: a 2% RBCs suspension in 0.9% saline was incubated at 37°C for 1 or 24 h with examined extracts at the final concentration of 1–50 μg/mL. Afterwards, erythrocyte suspension was vigorously mixed, and morphology was evaluated using a phase contrast Opta-Tech inverted microscope (Opta-Tech, Warszawa, Poland), at 400-times magnification, equipped with software (OptaView 7) for image analysis. RBC lysis assay was conducted spectrophotometrically by measuring the amount of hemoglobin released from RBCs, according to (Markowicz-Piasecka et al., 2015). Briefly, RBCs suspension (2% in 0.9% saline) was incubated for 1 and 24 h at 37°C with the examined extracts at the final 1–50 μg/mL concentration. After the incubation, the samples were centrifuged (1000 rpm, 10 min), and the absorbance of the supernatant was measured at 550 nm. A sample of 2% v/v Triton X-100 purchased from Polish Chemical Reagents (Gliwice, Poland) represented 100% of hemoglobin release (positive control), whereas the sample of 0.9% saline represented spontaneous hemolysis of RBCs (negative control).

2.9 Statistical analysis

The results are presented as mean values ±standard error (SE) or standard deviation (SD) for the indicated number of experiments. The significance of the differences between means was evaluated using a one-way ANOVA or one-way ANOVA for repeated measurements, followed by the *post hoc* Tukey's test for multiple comparisons or *post hoc* Dunnett's test. The correlations were determined using an *F*-test. All calculations were performed using the Statistica 12 PL software for Windows (StatSoft Inc., Krakow, Poland). The *p* values lower than 0.05 were considered significant.

3 Results

3.1 Phytochemical profiling

The analyses were performed using primarily the source aqueous (AQ) and hydroalcoholic (MED) extracts prepared from the flowers of *P. spinosa* L. Moreover, the concentrated polyphenol-rich fractions (DEF, EAF, BF) and water residues (WR) obtained by fractionated extraction of MED were also included.

The UHPLC-PDA-ESI-MS^3 analysis of AQ enabled the detection of 43 constituents representing different phenolic classes: flavonols (kaempferol and quercetin glycosides), flavan-3-ols (catechins and A-type proanthocyanidins), and phenolic acids (Figure 1; Supplementary Table S1). The analytes were structurally characterized based on comparing their chromatographic retention and spectral profiles (UV-Vis, ESI-MS^3) with the literature data or reference standards, both commercial and isolated previously in our laboratory from flowers and leaves of *P. spinosa* (Olszewska and Wolbis, 2001; Olszewska, 2002; Olszewska and Wolbiś, 2002). The total phenolic content determined by the Folin-Ciocalteu assay (TPC) was 126.59 mg of gallic acid equivalents (GAE) per g of dry weight (dw), while the total content of the individual analytes determined by the RP-HPLC-PDA method (TPH) amounted to 77.71 mg/g dw. Among the compounds with the highest levels (>5 mg/g dw) are kaempferol diglycosides, such as KT, KAFR, and MUB, as well as kaempferol and quercetin monoglycosides, including AV and JU. The total proanthocyanidin content (TPA) determined by *n*-butanol assay was 24.46 ± mg CYE/g dw (Table 1).

The same panel of phytochemical profiling methods was used previously for the standardization of MED, DEF, EAF, BF and WR (Marchelak et al., 2017; Marchelak et al., 2020a; Marchelak et al., 2020b). The comparison of the recorded UHPLC profiles revealed that qualitative profiles of the source extracts AQ and MED are almost identical (Figure 1; Supplementary Table S1). However, the quantitative profiles of the extracts vary significantly. The concentrations of phenolics and proanthocyanidins were about 1.6–2 times higher for MED than for AQ. Among the individual compounds, KT, KAFR, MUB, AV and JU were the most abundant in both extracts, still reaching 1.6–3 times higher levels in MED than in AQ (Table 1). The summary of the results from the quantitative studies for all the extracts/fractions used in the present investigation was given in Supplementary Table S2.

3.2 Blood clotting tests and determination of the thrombin enzymatic activity

To determine the impact of *P. spinosa* extracts on the hemostatic activity of blood plasma, the well-known diagnostic parameters, i.e., blood clotting times such as TT, PT and aPTT, were used

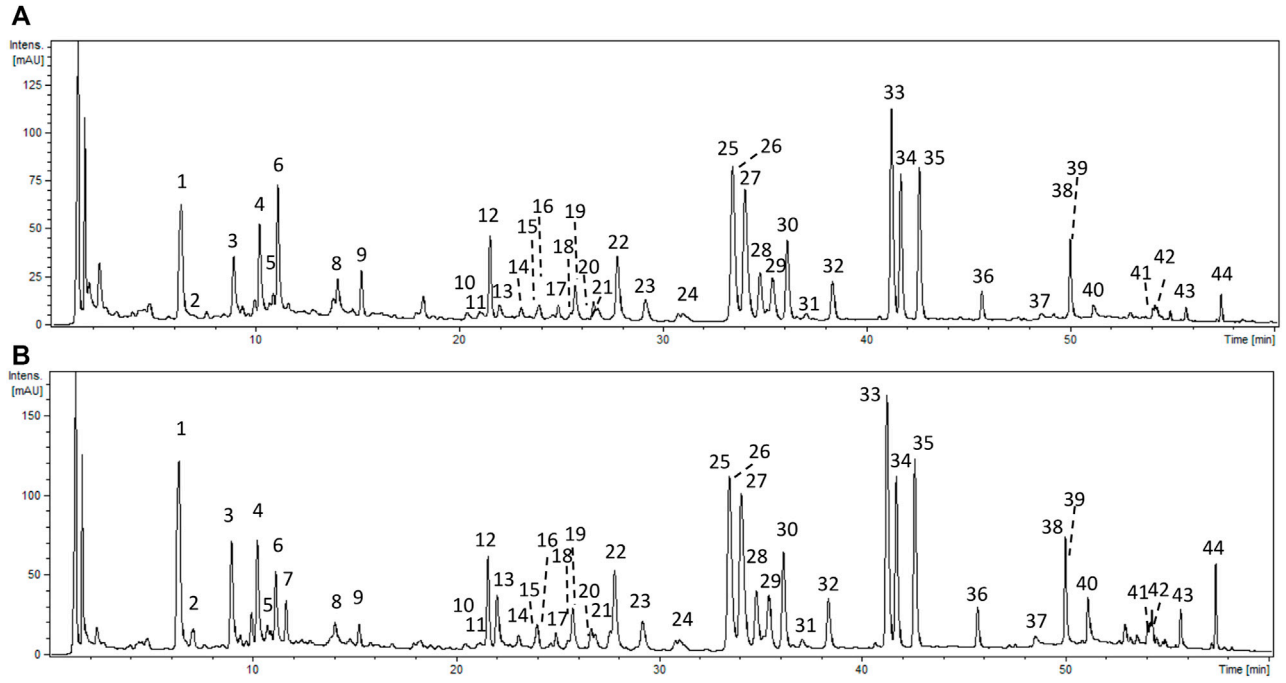

FIGURE 1
Representative UHPLC chromatograms of *P. spinosa* aqueous extract (AQ) **(A)** and hydroalcoholic (methanol-water, 7:3, *v/v*) (MED) **(B)** extracts at 280 nm. Peak numbers refer to those implemented in Supplementary Table S1.

(Table 2). A slight but statistically significant PT prolongation was observed for BF and AQ (about 4.7% and 3.5% at 50 µg/mL, respectively). Moreover, MED, DEF, BF and WR moderately increased TT (by 3.4%–5.1% at 50 µg/mL) with statistical significance.

The influence of the extracts on thrombin enzymatic activity was investigated in different experimental systems *in vitro*, including the measurements of the amidolytic and proteolytic activity of the enzyme. The amidolytic activity of thrombin was measured based on the enzyme's ability to hydrolyze a peptide-*p*NA bond in the chromogenic substrate D-Phe-Pip-Arg-*p*NA. This molecule is patterned after the *N*-terminal portion of the α chain of fibrinogen, the physiological substrate for thrombin. The analyses of the V_{max} parameter revealed that MED and its fractions partly antagonize the reaction in a concentration-dependent manner. DEF and EAF were the most active fractions; even at 1 µg/mL and 5 µg/mL, they inhibited the reaction by about 10% and 60%, respectively, while at 50 µg/mL, the effects reached up to approximately 90%. On the other hand, the efficiency of AQ was lower than MED; at 50 µg/mL, the percentage of inhibition was merely about 10%, and the effect was not statistically significant. (Figure 2A).

The capacity of blackthorn extracts to inhibit the proteolytic activity of thrombin was investigated based on the kinetic studies on the proteolysis of fibrinogen by thrombin and fibrin generation, primarily two key parameters of the polymerization curve: the lag time and the maximum velocity of the process (V_{max}). The lag time corresponds to the thrombin-induced cleavage of fibrinopeptides A and B from the fibrinogen molecule, while the V_{max} describes the rate of fibrinogen polymerization. The studies on the isolated fibrinogen revealed that the extracts reduced the rate of fibrinogen polymerization, which was shown as a decrease in the V_{max} parameter (Figure 2B). Similarly to the case of the amidolytic activity studies of thrombin, DEF and EAF presented the highest effectiveness: the V_{max} values at 5 µg/mL and 50 µg/mL decreased by 27.4%–38.6% and 62.3% and 74.82%, respectively. In addition, the significantly prolonged lag time was noticed: 525.7 s and 545.3 s for DEF and EAF at 50 µg/mL, respectively, *versus* 10 s observed for the control (Supplementary Figure S1A). Moreover, the plasma matrix studies confirmed the extracts' ability to inhibit fibrinogen polymerization (Figure 2C; Supplementary Figure S1B). The decreases in V_{max} for MED and its fraction DEF, EAF and BF at 50 µg/mL were in the range of 35.9%–59.7%; at the same time lag time was prolonged from 13.75 s observed for the control to 78.3–355s. On the other hand, the effects of AQ were negligible.

The correlation studies have been performed to evaluate the contribution of polyphenols to the observed effects of blackthorn flower extracts on the amidolytic and proteolytic activity of thrombin (Table 3). The results revealed that the inhibitory effects of the extracts were firmly phenolic-dependent, with significant correlations ($p < 0.05$) found for total phenolics (TPC and TPH) and total proanthocyanidins (TPA). The effective levels of the phenolic fractions responsible for the thrombin inhibitory effects of the blackthorn flower extracts were estimated from the working extract concentrations of 1, 5, and 50 µg/mL and TPC levels; they

TABLE 1 Quantitative profile of the source extracts obtained from *P. spinosa* flower (mg/g dw).

Analyte	Content (mg/g dw)	
	AQ	MED
Individual compounds		
NCHA	4.89 ± 0.14^{B}	14.46 ± 0.23^{A}
CHA	3.83 ± 0.07^{B}	5.64 ± 0.11^{A}
CA	< LOQ	< LOQ
CCHA	4.51 ± 0.06^{A}	4.26 ± 0.07^{B}
CFA	< LOQ	< LOQ
ECA	< LOQ	< LOQ
KAPR	< LOQ	2.69 ± 0.10
LEP	1.21 ± 0.04^{B}	3.17 ± 0.09^{A}
***p*-CA**	< LOQ	< LOQ
KT	5.65 ± 0.06^{B}	17.42 ± 0.79^{A}
KAFR	5.15 ± 0.10^{B}	15.13 ± 0.21^{A}
RT	2.53 ± 0.14^{B}	4.65 ± 0.15^{A}
QGA	4.80 ± 0.06^{B}	6.28 ± 0.25^{A}
IQ	0.41 ± 0.03^{B}	1.33 ± 0.03^{A}
HY	0.74 ± 0.03^{B}	0.92 ± 0.04^{A}
KRG	1.22 ± 0.12^{B}	3.67 ± 0.14^{A}
RN + GU	2.26 ± 0.09^{B}	4.26 ± 0.15^{A}
MUA	3.71 ± 0.04^{B}	5.38 ± 0.20^{A}
AV	8.59 ± 0.11^{B}	14.89 ± 0.65^{A}
QC	3.37 ± 0.09^{B}	7.41 ± 0.21^{A}
KX	1.73 ± 0.05^{B}	2.97 ± 0.10^{A}
MUB	6.63 ± 0.10^{B}	8.82 ± 0.07^{A}
JU	8.61 ± 0.15^{B}	13.73 ± 0.43^{A}
AFZ	5.65 ± 0.15^{B}	13.33 ± 0.16^{A}
KR	0.60 ± 0.06^{B}	1.78 ± 0.04^{A}
KCAR	< LOQ	1.47 ± 0.02
QU	0.57 ± 0.03^{B}	1.32 ± 0.06^{A}
KA	0.72 ± 0.02^{B}	1.06 ± 0.01^{A}
***p*-cJU**	0.35 ± 0.02^{B}	1.43 ± 0.03^{A}
Phenolic fractions		
TPH	**77.71**	**157.47**
TPC	**126.98 ± 1.61^{B}**	**206.07 ± 10.86^{A}**
TPA	**24.46 ± 1.26^{B}**	**45.13 ± 2.38^{A}**

The results are presented as means ± SD (n = 3). Different superscripts in each row indicate significant differences in the means at $p < 0.05$. The quantitative profile of MED, according to (Marchelak et al., 2017; Marchelak et al., 2020a; Marchelak et al., 2020b).

The bold values refer to the content of groups of compounds not individual constituents.

TABLE 2 Determination of the effects of *P. spinosa* flower extracts on blood clotting times of human plasma. Results are presented as means ± SE ($n = 12$). Statistical differences: $^{**}p < 0.01$, $^{***}p < 0.001$ for samples in the presence of the analytes (50 μg/mL) or the reference inhibitor, argatroban (0.5, 5, 10 μg/mL) *versus* control samples.

P. spinosa extracts or a reference anticoagulant drug	Concentration [μg/mL]	Clothing times [% of the control/untreated human plasma]		
		PT	APTT	TT
MED	50	100.37 ± 0.95	101.85 ± 0.95	103,36 ± 0.84**
DEF	50	100.02 ± 0.75	100.36 ± 0.99	104,03 ± 0.76**
EAF	50	100.56 ± 0.68	100.61 ± 0.98	101,40 ± 0.82
BF	50	104.67 ± 1.33**	98.94 ± 0.61	105,12 ± 1.30***
WR	50	102.29 ± 1.08	99.31 ± 0.74	104,89 ± 1.02***
AQ	50	103.49 ± 1.11**	100.36 ± 1.15	99,27 ± 1.46
Argatroban	0.5	134.01 ± 1.50***	202.58 ± 2.32***	974.60 ± 27.50***
	5	562.65 ± 12.69***	423.72 ± 7.83***	No clot
	10	821.54 ± 46.95***	No clot	No clot

amounted to 0.1–0.6, 0.3–2.9 and 3.2–29.2 μg of the phenolics/mL.

In the abovementioned experiments, the effectiveness of *P. spinosa* flower extracts has been compared to argatroban, the anticoagulant drug that acts by directly inhibiting thrombin. Although the ability of MED and its polyphenol-rich fractions to inhibit thrombin activity was lower than that of argatroban, the results indicated their high potential. For example, the inhibition of the amidolytic and proteolytic activity of the enzyme observed for EAF at 50 μg/mL (29.2 μg of the phenolics/mL) were comparable to argatroban at 10 μg/mL.

3.3 *In silico* molecular docking

The molecular interaction between thrombin and *P. spinosa* polyphenols was analyzed *in silico*. All studied flower constituents (Figure 3) and their potential metabolites *in vivo* (Figure 4) interacted with considerable binding affinity to thrombin within the active site (in unliganded and liganded forms) close to Ser205 and His43 of the catalytic triad (Table 4; Figure 5). Three compounds, i.e., AFZ, KT, and PA2, had additional binding sites outside the catalytic triad of liganded structure; PA2 was bound in exosite I (Figure 5A), while the additional binding of AFZ and KT took place on the opposite side of the thrombin molecule relative to exosite I (Figure 5B).

3.4 Verification of the thrombin inhibitory effects of *P. spinosa* polyphenols *in vitro*

Based on the phytochemical profiling, *in vitro* evaluation of the extracts' effects on thrombin activity and *in silico* data, ten compounds (*i.e.,* QU, KA, AV, JU, AFZ, QC, KT, *p*-cJU, PA2 CHA) were selected to verify their inhibitory efficiency towards thrombin *in vitro*. Among them, QU, KA, *p*-cJU and PA2 significantly inhibited the enzymatic activity of thrombin, with the IC_{50} values ranging from 38.29 to 81.12 μg/mL (Table 5). For the remaining six metabolites, no inhibitory effects on thrombin were found at a concentration range of 1–50 μg/mL (data not presented).

3.5 Circular dichroism (CD)

In order to further verify whether the *P. spinosa* polyphenols affect the secondary structure of thrombin, circular dichroism spectra of thrombin alone and in the presence of selected compounds were performed. The thrombin CD spectrum is typical of the α-helix polypeptide chain structure with two negative bands centered at 208 nm and 222 nm. The addition of all tested compounds to thrombin resulted in changes in CD spectrum intensity, even at the lowest concentration (1 μg/mL). Higher concentrations of compounds significantly altered the shape of the thrombin CD spectrum, indicating variations in the thrombin secondary structure (Figure 6). The most apparent modification of the thrombin α-helix structure has been observed for PA2. Based on the CD spectra, it can be concluded that the selected *P. spinosa* flower constituents changed the secondary structure of thrombin, which may lead to alterations in its enzymatic activity.

3.6 Blood platelet aggregation

To determine the impact of blackthorn flower extracts on platelet hemostasis, the measurements of ADP and collagen-induced aggregation in PRP were conducted. The study focused on the source extract MED and the fractions EAF and DEF, which consistently demonstrated high potential in prior research. Furthermore, AV, JU, and PA2, identified as the primary compounds in the most active extracts, were selected for the investigation. The results revealed that the extracts (5–50 μg/mL) did not affect platelet aggregation significantly ($p > 0.05$) (Figure 7). Among the tested polyphenols, statistically significant inhibition of

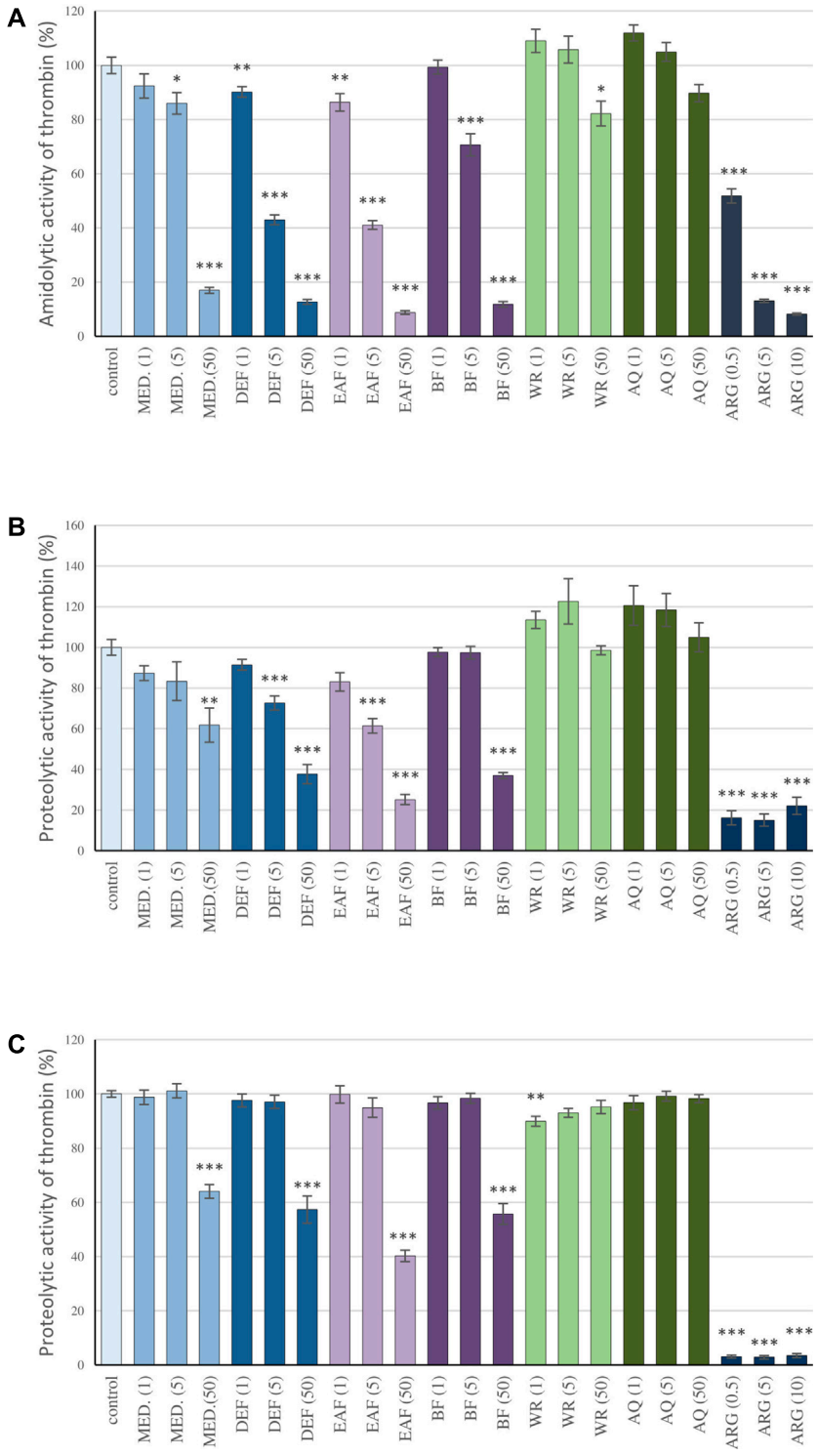

FIGURE 2
Inhibitory effects of the examined *P. spinosa* flower extracts on the amidolytic **(A)** and proteolytic activity of thrombin **(B)** study on the isolated fibrinogen; **(C)** study on the human plasma). The assays were performer using the kinetic protocol and the TH activity was calculated based on the Vmax parameter. Results are presented as means +SE (n = 6). Statistical differences: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ for samples in the presence of the analytes (1, 5, 50 μg/mL) or the reference inhibitor, argatroban (0.5, 5, 10 μg/mL) *versus* control samples.

platelet aggregation was only observed for AV and JU at a concentration of 50 μg/mL, when ADP was used as the agonist; however, the effects not exceed 15%. Indomethacin, employed as a positive control, at 5 μg/mL inhibited platelet aggregation by approximately 23% and 86% when platelets were stimulated by ADP and collagen, respectively.

TABLE 3 Correlation coefficients (*r*) and probability (*p*) values of the linear relationships between phenolic contents of *P. spinosa* flower extracts/fractions and their activity parameters towards thrombin–inhibition of amidolytic activity of thrombin, inhibition of proteolytic activity of thrombin assayed on isolated fibrinogen and on the blood plasma.

r (*p*) for	TPC	TPH	TPA
Amidolytic activity of thrombin	0.8172 (0.000)*	0.8128 (0.000)*	0.7876 (0.000)*
Proteolytic activity of thrombin (isolated fibrinogen)	0.8259 (0.000)*	0.8114 (0.000)*	0.7897 (0.000)*
Proteolytic activity of thrombin (blood plasma)	0.9400 (0.000)*	0.8952 (0.000)*	0.9283 (0.000)*

Phenolic content and the activity parameters of the extracts/fractions are according to Table 1, Supplementary Table S2 and Figure 2. The inhibitory effects of the extracts/fractions were calculated based on V_{max} parameter. Asterisks mean statistical significance of the estimated linear relationships (*$p < 0.05$).

3.7 PBMCs viability

The potential cytotoxicity of *P. spinosa* extracts/fractions was assessed in the model of PBMCs after 24-h incubation with the extracts at the concentration range of 1–50 μg/mL (Figure 8). The viability of PBMCs treated with the extracts constituted 89.6%–98.3% of the control (untreated) samples. The cellular safety of the extracts was evidenced by the lack of significant differences ($p > 0.05$) between the respective results.

3.8 Influence on erythrocyte membrane integrity/also hemolysis

Microscopy studies and hemolysis assay were used to assess the effect of the examined extracts on erythrocyte membrane integrity. At physiological pH 7.4, the erythrocytes suspended in 0.9% saline are primarily observed as discocytes resembling a two-concave disk. After a 1-h co-incubation with the examined extracts/fractions, erythrocytes exhibited a tendency to form echinocytes. These are physiological forms where the discocyte shape is maintained, but the cell membrane folds and forms numerous protrusions (Figure 9A). For instance, DEF, EAF and WR extracts over the entire concentration range contributed to substantial echinocytosis. However, other forms of erythrocytes were detected in the case of other extracts. For example, eryptotic erythrocytes were found after 1-h incubation with MED extract at 5 and 50 μg/mL. In turn, treating RBCs with extract BF at 50 μg/mL resulted in the formation of ovalocytes which are abnormally shaped RBCs. The 24-h incubation of erythrocytes showed that most tested extracts led to extensive echinocytosis (Figure 9B). Furthermore, a trend towards programmed erythrocyte death was observed for all examined extracts (1–50 μg/mL) manifested by a substantial number of eryptotic RBCs. In summary, microscopic analysis showed that examined extracts mainly induce physiological changes in erythrocytes shape, which may indicate that they do not interact strongly or adversely with RBC lipid bilayer.

The results from RBCs lysis assay (Figure 10) showed that AQ over the entire concentration range (1–50 μg/mL) did not contribute to the significant changes in erythrocyte hemolysis after 1-h and 24-h incubation. However, incubation of RBCs with all other extracts at the highest tested concentration (50 μg/mL) significantly increased the percentage of hemolyzed erythrocytes. For instance, DEF at 50 μg/mL contributed to 2.94% ± 0.25% hemolysis after 1-h incubation ($p < 0.001$). More prolonged incubation resulted in 3.53% ± 0.68% of hemolyzed erythrocytes ($p < 0.01$). Notably, in all cases, the degree of RBCs hemolysis accounted for approximately 7%–8%, with BF extract being the most erythrotoxic (6.34% ± 0.57% vs. 2.46% ± 0.34% for control; $p < 0.001$). Since the hemolysis of erythrocytes not exceeding 10% is considered clinically safe (Stasiuk et al., 2009), all examined extracts/fractions can be considered hemocompatible in the 1–50 μg/mL concentration range.

4 Discussion

Thrombin is the ultimate trypsin-like serine proteinase formed from the zymogen prothrombin as the final product of activation of both the intrinsic and extrinsic pathways of the blood coagulation cascade (Marchi and López, 2016). The proteolytically-active enzyme has a molecular weight of 37 kDa. It comprises two polypeptide chains: the light chain, formed by 36 amino acids, and the heavy chain, which has 259 amino acids in its structure (Marchi and López, 2016; Al-Amer, 2022). There are four functional domains of thrombin: the active site, the anion binding exosites I and II, and Na^+ binding site; this unique structure is believed to be responsible for high substrate specificity (Lane et al., 2005; Marchi and López, 2016). The active site of the enzyme, containing the catalytic triad His43, Asp99 and Ser205, is in the central part of the molecule in a negatively charged deep narrow canyon that forms the steric hindrance and restricts access to other molecules. The anion-binding exosites I and II, positively charged regions adjacent to the active site and placed near opposing ends, are also known as the substrate recognition sites as they interact electrostatically with negatively charged groups of the substrates, cofactors, and inhibitors. The Na^+ binding site, through sodium ions binding, allosterically modulates thrombin activity; the access of the small substrates to the active site increases, and procoagulant substrates are preferred over anticoagulant ones (Crawley et al., 2007; Marchi and López, 2016).

The regulation of thrombin generation and activity is essential for hemostatic equilibrium maintenance and, consequently, one of the main therapeutic strategies for preventing and treating thromboembolic disorders involved in the etiology and pathophysiology of severe CVDs such as myocardial infarction, ischemic stroke, and venous thromboembolic disease. Some plants may be promising sources of substances exerting anticoagulant activities through the inhibition of thrombin. In recent years, the direct interaction with this serine protease has been reported for several plant-derived substances, including polyphenol-rich extracts/fractions from black chokeberry (*Aronia melanocarpa*) (Bijak et al., 2013; Sikora et al., 2014), grape seeds (*Vitis vinifera*) (Bijak et al., 2013), rowanberries (*Sorbus aucuparia*) (Rutkowska et al., 2021), St. John's Wort (*Hypericum perforatum*) (Wei et al.,

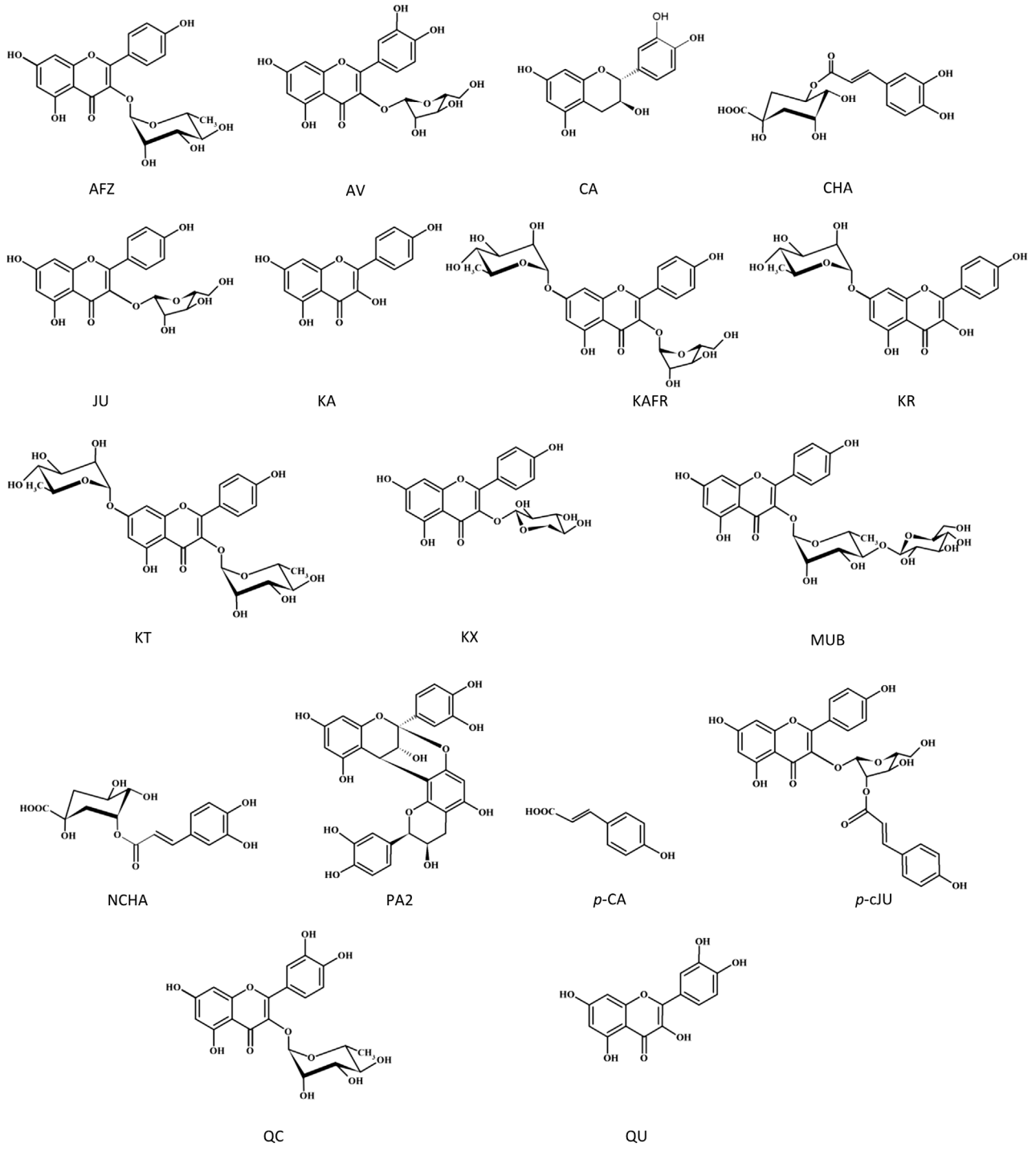

FIGURE 3
Structures of the tested *P. spinosa* flower extracts constituents.

2019), alkaloid and polyphenol-rich extracts from *Uncaria tomentosa* leaves and barks (Kolodziejczyk-Czepas et al., 2021), diterpene-rich extracts from *Salviae Miltiorrhizae* roots (Lu et al., 2015; Yang et al., 2020), and bufadienolide-rich fractions from *Kalanchoe daigremontiana* (Kolodziejczyk-Czepas et al., 2017).

In studies of plant-derived substances, carefully selected and exhaustively standardized extracts are crucial to obtain reliable results and drive meaningful conclusions on their bioactivity. As was pointed out in our previous research, the source defatted methanol-water (7:3, *v/v*) extract of *P. spinosa* flower (MED), and its concentrated phenolic fractions obtained by liquid-liquid fractionation (DEF, EAF, BF), appear to be advantageous for functional application. They exhibited a distinct phytochemical profile with a high content of polyphenols, primarily flavonoids,

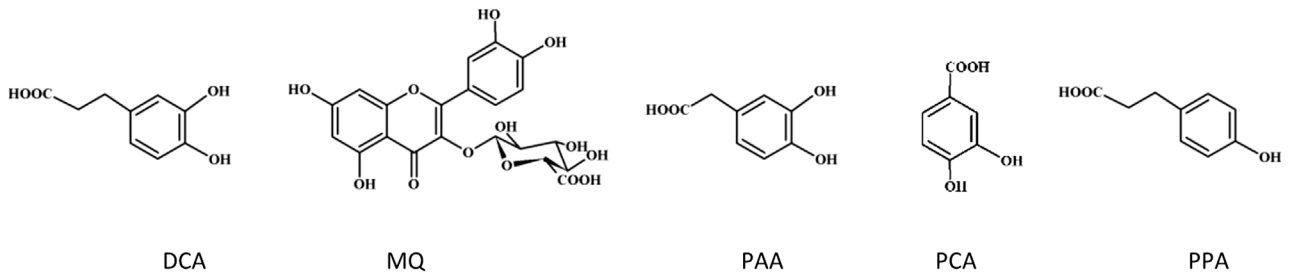

FIGURE 4
Structures of the tested phenolic metabolites.

TABLE 4 Binding affinity calculated for constituents of *P. spinosa* flower extracts and their potential metabolites *in vivo* from molecular docking to unliganded and liganded human thrombin. Visualization of protein-compounds complexes–see Figure 5.

	Unliganded 3U69		Liganded 1FPH	
	Average affinity (kcal • mol-1)	± SD	Average affinity (kcal • mol-1)	± SD
Extracts constituents				
AFZ	−7.9	0.0	−8.2	0.6
AV	−8.9	0.2	−8.5	0.2
CA	−8.4	0.0	−8.3	0.6
CHA	−8.1	0.0	−8.0	0.1
JU	−8.4	0.0	−8.2	0.4
KA	−8.2	0.1	−8.0	0.6
KAFR	−8.1	0.5	−8.0	0.3
KR	−9.4	0.0	−9.4	0.0
KT	−8.7	0.0	−8.4	0.4
KX	−9.2	0.0	−8.9	0.1
MUB	−8.3	0.3	−8.3	0.3
NCHA	−7.8	0.1	−8.7	0.0
PA2	−9.2	0.3	−9.0	0.1
p-CA	−5.3	0.1	−5.0	0.1
p-cJU	−9.9	0.3	−8.7	0.0
QC	−8.0	0.1	−8.6	0.5
QU	−7.4	0.1	−7.5	0.1
Phenolic metabolites				
DCA	−5.6	0.2	−5.3	0.1
MQ	−8.5	0.1	−8.3	0.2
PAA	−5.6	0.2	−5.3	0.1
PCA	−5.5	0.0	−5.3	0.2
PPA	−5.2	0.2	−4.9	0.1

including rare flavonol pentosides, followed by A-type procyanidin dimers, simple phenolic acids, and quinic acid pseudodepsides (Marchelak et al., 2017; Marchelak et al., 2020a; Marchelak et al., 2020b). It is noteworthy that among the substances derived from blackthorn, the flowers appear to be particularly rich in polyphenols. For instance, the total phenolic content (TPC) determined by the

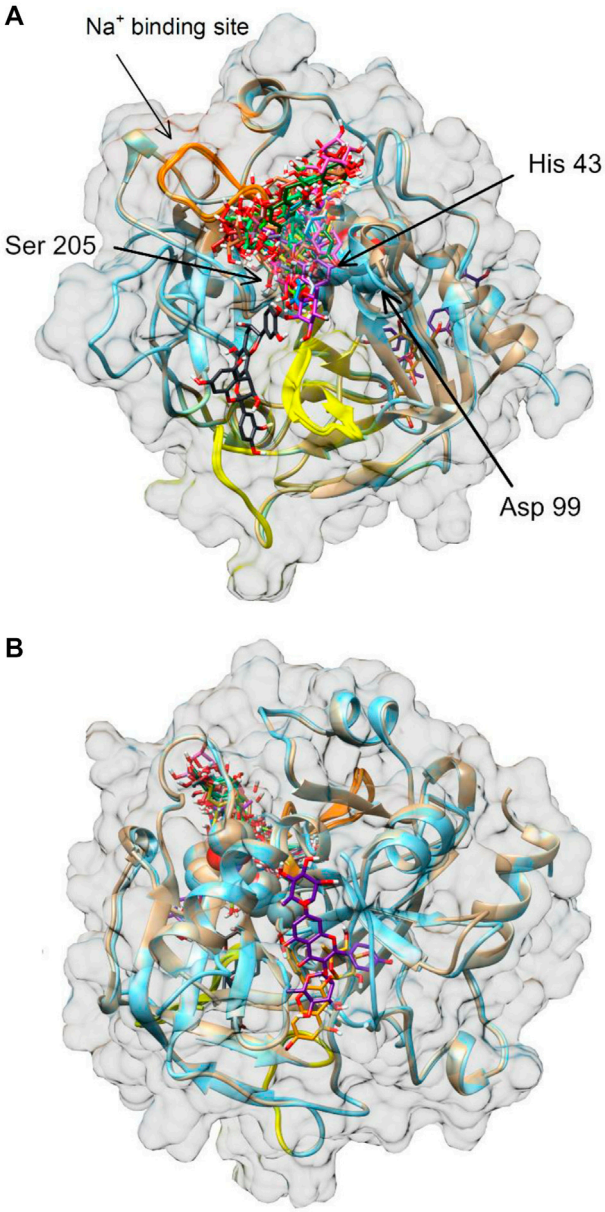

FIGURE 5
Molecular docking of *P. spinosa* compounds and their potential metabolites *in vivo* to human thrombin. Panel **(A)** front view, panel **(B)** back view. Exosites 1 and 2 are marked in yellow and orange, respectively. Amino acid residues Asp99, His43 and Ser205 of the enzyme catalytic domain (triad active site) are shown as spheres in the center of the thrombin molecule representation. Images of thrombin structures 3U69 and 1FPH are shown in brown and blue, respectively.

Folin-Ciocalteu assay for the dry methanol-water (7:3, *v/v*) extract of flowers was more than twice as high as that for the dry methanol-water (7:3, *v/v*) extract of fresh fruits (206.07 ± 10.86 vs. 87.57 ± 3.54 mg GAE/g dw, respectively). Moreover, in contrast to the flower extracts, distinguished by the remarkable diversity of the flavonoid fraction, in the fruit extracts, phenolic acids and aldehydes are the predominant and structurally diverse group of polyphenols, coexisting with flavonoids and anthocyanins (Marchelak et al., 2017; Magiera et al., 2022). As was pointed out in our previous

TABLE 5 The thrombin-inhibitory efficiency of the examined constituents of the *P. spinosa* flower extracts in the amidolytic tests. IC_{50} - the half maximal inhibitory concentration.

Thrombin treated with	IC_{50} [μg/mL]
QU	29.35
KA	81.12
p-cJU	38.29
PA2	52.36
Argatroban	<1

studies blackthorn flower extracts, *i.e.*, MED and its concentrated fractions, exhibited significant biological effects in complementary *in vitro* models (Marchelak et al., 2017; Marchelak et al., 2019; Marchelak et al., 2021). In particular, they were able to maintain the hemostatic balance of human blood due to the significant protective effects against the oxidative stress-induced structural changes in fibrinogen. Such changes might alter the fibrinogen functions and lead to the generation of dysfunctional hemostatic clots, closely linked with numerous CVDs (Marchelak et al., 2021). Therefore, MED and its fractions have been selected for the present investigation into the impact of *P. spinosa* flower on thrombin inhibition. Moreover, the source aqueous extract (AQ) was included in the study as a representative of herbal teas. Herbal teas, defined as the oral aqueous preparations obtained from plant material through infusion, maceration, or decoction, are the most widely used preparation of traditional medicine and a popular global beverage. Since herbal teas/infusions have gained popularity recently, their composition, efficiency, and safety should be thoroughly evaluated (Poswal et al., 2019; Etheridge and Derbyshire, 2020). Therefore, all the extracts/fractions used in the present study were characterized in detail by applying a panel of phytochemical profiling methods, including LC-MS/MS and LC-PDA assays, fully validated for quantitative purposes. According to the results (Table 1, SSupplementary Table S1, 2; Figure 1), a methanol-water (7:3, *v/v*) provided higher recovery of phenolic compounds than pure water, which is consistent with literature data on the best extraction solvents for low-molecular-weight polyphenols (Brglez Mojzer et al., 2016). Considering the quantitative results, we decided not to fractionate AQ and only include the source extract in the activity and cytotoxic studies, which might allow us to illustrate its bioactivity and safety as a traditional herbal preparation.

The present study indicated for the first time that the extracts/fractions of *P. spinosa* flower contain natural inhibitors of the blood coagulation process. The blood clotting tests revealed a slight prolongation of TT (Table 2). TT is the parameter corresponding to the reaction of the conversion of fibrinogen into fibrin monomers catalyzed by thrombin, and its prolongation occurs in the presence of substances that interfere with fibrin polymerization. The observed tendency to increase TT suggests that blackthorn flower extracts/fractions might act as thrombin inhibitors. Thus, their interaction with the enzyme has been investigated in the next step using various experimental systems *in vitro*, including the low-molecular synthetic substrate and fibrinogen–a physiological target of thrombin–isolated and in a

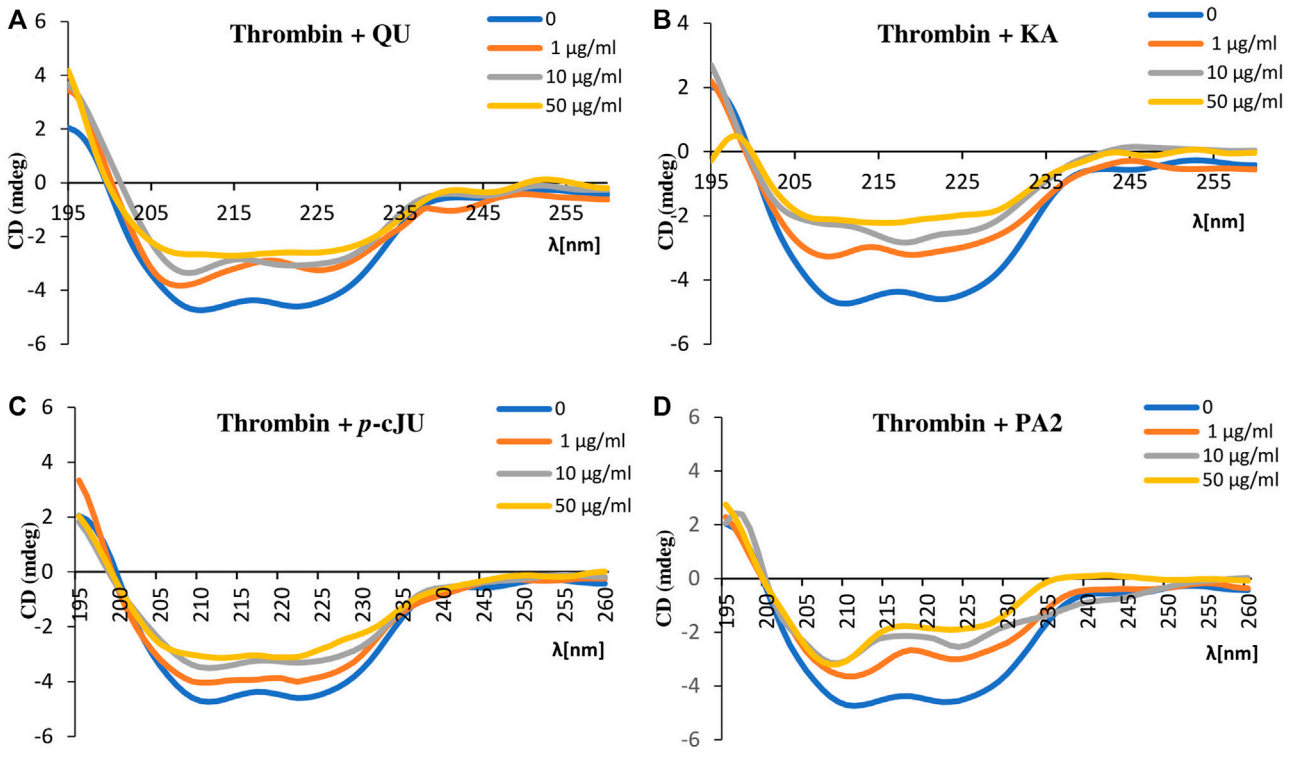

FIGURE 6
Circular dichroism spectra of thrombin (c = 15 µg/mL) in the presence of selected *P. spinosa* flower extracts constituents: **(A)** QU, **(B)** KA, **(C)** *p*-cJU, **(D)** PA2. Each spectrum is the average of three replicates.

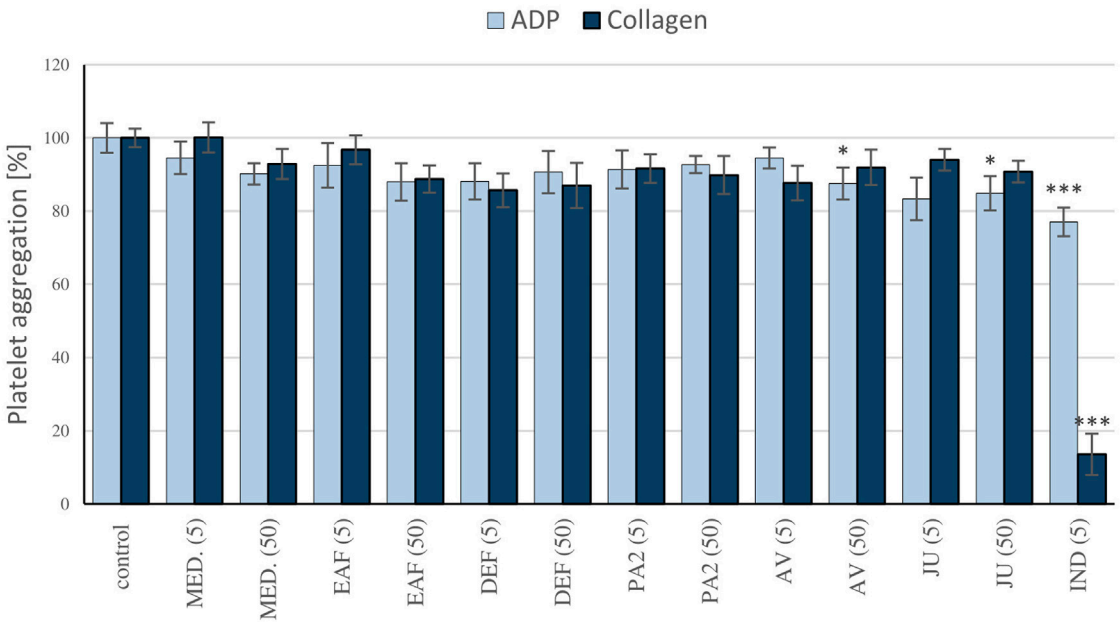

FIGURE 7
Evaluation of the antiplatelet activity of the *P. spinosa* extracts. The effects of the examined plant extracts on blood platelet aggregation were measured in platelet-rich plasma, following the stimulation by ADP (10 µM) or collagen (2 µg/ml). The aggregation of blood platelets in control platelet-rich plasma samples (not treated with the analytes) was assumed as 100%. Results are presented as means ± SE (n = 9). Statistical differences: *$p < 0.05$, ***$p < 0.001$ for samples in the presence of the analytes (5, 50 µg/mL) or the reference inhibitor, indomethacin (5 µg/mL) *versus* control samples.

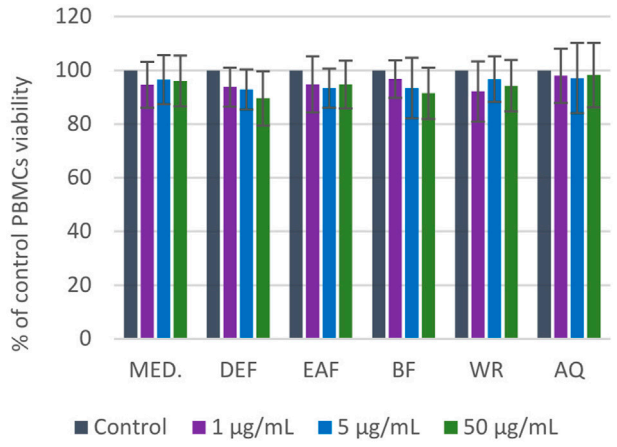

FIGURE 8
Viability of PBMCs after 24 h of incubation with *P. spinosa* flower extracts at 1-50 µg/mL tested in resazurin-based assay. Results are presented as means +SE (n = 12) for repeated measures. All values are not statistically different ($p > 0.05$).

plasma matrix. The tested extracts/fractions have been proven to inhibit amidolytic and proteolytic activity of thrombin even at 1–5 µg/mL (Figure 2; Supplementary Figure S1). MED and its fractions EAF, DEF, and BF revealed the highest capacity of antagonizing thrombin, and they appeared to be the most promising in maintaining the hemostatic balance. On the other hand, the study pointed out that AQ has no anticoagulant effect. These findings show that the extraction from natural matrices is a crucial step in the utilization of phenolic compounds as it affects the composition of the extracts and their biological efficiency. Moreover, as the observed effects strongly correlated with the total levels of phenolics ($p < 0.05$) (Table 3), the studies revealed that the anticoagulant potential of the blackthorn extracts/fractions is phenolic-dependent. What is essential, in all analyses, the extracts/fractions were used in microgram concentrations (1–50 µg/mL), corresponding to 0.1–29.2 µg/mL polyphenols. In case of MED, the concentrations 1, 5, and 50 µg/mL are equivalent to 0.2, 1 and 10 µg of the phenolics/mL, respectively. According to the literature, such levels are physiologically achievable in the blood plasma after oral intake. For instance, the reference plasma level of polyphenols after oral ingestion may reach 3.5–7 µg/mL for quercetin glucosides (Manach et al., 2005) and 2.1–5.2 µg/mL for monocaffeoylquinic acids (Farah et al., 2008).

The results clearly revealed that the extracts/fractions of *P. spinosa* flower, when used at *in vivo*-relevant levels of their active polyphenolic fractions, impact plasma hemostasis. However, they do not significantly affect ADP and collagen-induced platelet aggregation (Figure 7). In the literature, there are several reports on the antiplatelet activity of certain polyphenols, including those present in the investigated extracts. For instance, quercetin has been shown to possess relevant antiplatelet activity by blocking GPIIb/IIIa receptors, suppressing platelet activation, and promoting the pro-aggregate effect of calcium ionophore (Zaragozá et al., 2021). Nonetheless, these effects were observed at a quercetin concentration of 2 mM (approximately 600 µg/mL), which appears unlikely to be achievable *in vivo*. Conversely, the study on kaempferol demonstrated its ability to impair collagen-induced platelet activation through inhibition of NADPH-dependent ROS production at micromolar concentrations (Wang et al., 2015). In the present study, we observed the significant inhibition of blood platelet response to ADP in the presence of quercetin and kaempferol glycosides AV and JU at 50 µg/mL. According to the literature data, glycosylation of flavonols hinders their antiplatelet activity, which might partially explain the only slight anti-aggregatory potential of AV and JU, as well as lack of the effects of the blackthorn extracts rich in flavonols' glycosides.

The association of computational and experimental methods becomes a popular strategy in identifying novel promising compounds from natural matrices. Among several approaches, molecular docking is a powerful tool for studying interactions between small-molecular ligands and macromolecular targets. It allows to accurately predict the conformation of ligands within the binding site of the target and the ligand-receptor binding free energy (Ferreira et al., 2015; Jakhar et al., 2020). Thus, to indicate the most probable mechanism of the thrombin-inhibitory activity of the *P. spinosa* flower extracts, the experimental studies were supported by *in silico* molecular docking. Based on the phytochemical profiles of the extracts, 17 compounds were selected among leading representatives of all groups of blackthorn polyphenols, including flavonoid aglycones–QU, KA; flavonoid monoglycosides–AV, JU, QC, AFZ, KR, KX, *p*-cJU; flavonoid diglycosides–KAFR, KT, MUB; A-type proanthocyanidins–PA2; flavan-3-ols–CA; caffeoylquinic acids–CHA, NCHA; and simple hydroxycinnamic acids–*p*-CA (Figure 3). According to the results (Table 4; Figure 5), most of the tested compounds might act as univalent DTIs, as they bind to unliganded and liganded forms of thrombin only within the active site, near Ser205 and His43 of the catalytic triad. Argatroban, the drug approved as an alternative antithrombotic treatment for patients with heparin-induced thrombocytopenia (HIT), as well as for patients undergoing percutaneous coronary interventions with or at risk for HIT, presents a similar mechanism of action–it interacts reversibly with the catalytic triad of thrombin (Marchi and López, 2016). Some of the tested blackthorn flower constituents, such as PA2, AFZ and KT, have additional binding sites outside the active site, and could be considered bivalent DTIs. For instance, PA2 has been shown to interact with the active site and exosite I. Exosite I, composed of Lys21, Arg 62, Arg 68, Arg70, Tyr71, Arg 73, Lys 106 and Lys 107 residues, is responsible for the binding of the crucial substrate for thrombin: fibrinogen and other molecules such as protease-activated receptor 1 (PAR-1), factor V, factor VIII, thrombomodulin, platelet transmembrane glycoprotein (GPIba), and hirudin (Crawley et al., 2007). Hirudin, a polypeptide derived from the saliva of the leech *Hirudo medicinalis*, forms a biomolecular complex with thrombin via its acidic *C*-terminus and thereby antagonizes this serine proteinase activity. In the last few years, several new synthetic anticoagulants based on the structure of hirudin have been introduced to therapy, for example, bivalirudin, which joins the exosite I, as well as the active site of thrombin (Marchi and López, 2016). Therefore, PA2, with a similar mechanism of action, seems to be a promising molecule in the context of new drug development. Two kaempferol glycosides, AFZ and KT, have additional binding sites located on the opposite side of the thrombin molecule relative to the exosite I. Interestingly, none of the investigated *P. spinosa* polyphenols have been shown to join the

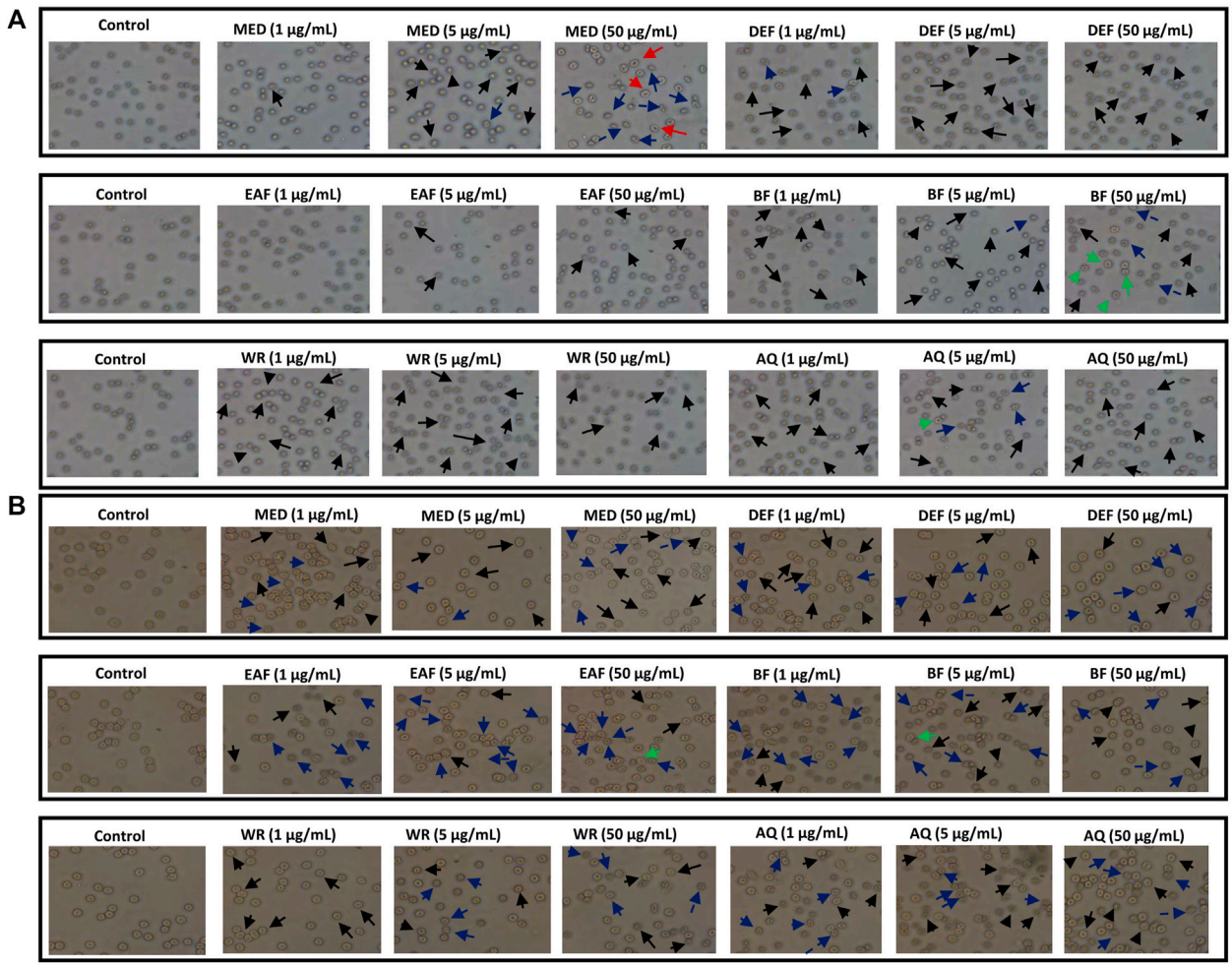

FIGURE 9
Effects of *P. spinosa* flower extracts at 1, 5 and 50 μg/mL on erythrocytes morphology after 1 **(A)** and 24 h **(B)** incubation. Representative phase-contrast images are shown (magnification of 400 times), echinocytes are marked with black arrows, eryptotic erythrocytes are marked with blue arrows, stomatocytes are marked with red arrows, while ovalocytes with green arrows.

exosite II. Exosite II, composed of Arg 89, Arg 98, Arg 245, Lys 248, and Lys 252 residues, binds the anticoagulant polysaccharide heparin and platelet transmembrane glycoprotein (GPIba) and is required for the recognition and cleavage of factor V and factor VIII (Marchi and López, 2016).

Among the *P. spinosa* flower constituents, PA2, QU, KA, and *p*-cJu, represent the most significant potential for thrombin inhibition. They not only bound firmly to the active site of the enzyme as demonstrated by docking calculations with their lowest free binding energy (Table 4) but also independently inhibit thrombin amidolytic activity *in vitro* (Table 5) and showed the most substantial effect in changing the secondary structure of thrombin within the α-helices, as can be seen from CD spectra (Figure 6). CD spectroscopy has a wide range of applications, among which investigating the secondary structure of biomolecules, including proteins, is the most notable. The method quickly analyzes the conformational changes of a protein caused by the addition of ligands and is often used to evaluate the interaction of plant metabolites and enzymes (Oluwagunwa et al., 2021; Yu et al., 2022). Since the vicinity of the catalytic site and exosite I of thrombin contains several α-helices, the changes in CD spectra in the presence of blackthorn flower polyphenols may indicate their actual interaction with the enzyme by binding at these sites. PA2, due to its strong docking binding in the active site and exosite I, and the most extensive changes in CD spectra, may be an essential compound responsible for the thrombin-inhibiting effect observed for the *P. spinosa* flower extracts. Previous studies concerning the influence of PA2 on the hemostatic system established that PA2 slightly increased TT (Owczarek et al., 2021). To the best of our knowledge, the present work is the first report indicating the thrombin-inhibitory activity of PA2 and explaining the molecular mechanism of this action.

The literature data on polyphenols suggests these compounds strongly interact with thrombin. Rodrigues et al. (2015) revealed that flavonol glycosides, i.e., quercetin 3-*O*-arabinoside and quercetin 3-*O*-rhamnoside, isolated from the leaves of white mangrove (*Laguncularia racemosa*) are potent inhibitors of the enzyme. The study conducted by Bijak et al. (2014) pointed out that catechin, epicatechin, cyanidin, cyanidin 3-glucoside and quercetin had an inhibitory effect on the amidolytic activity of

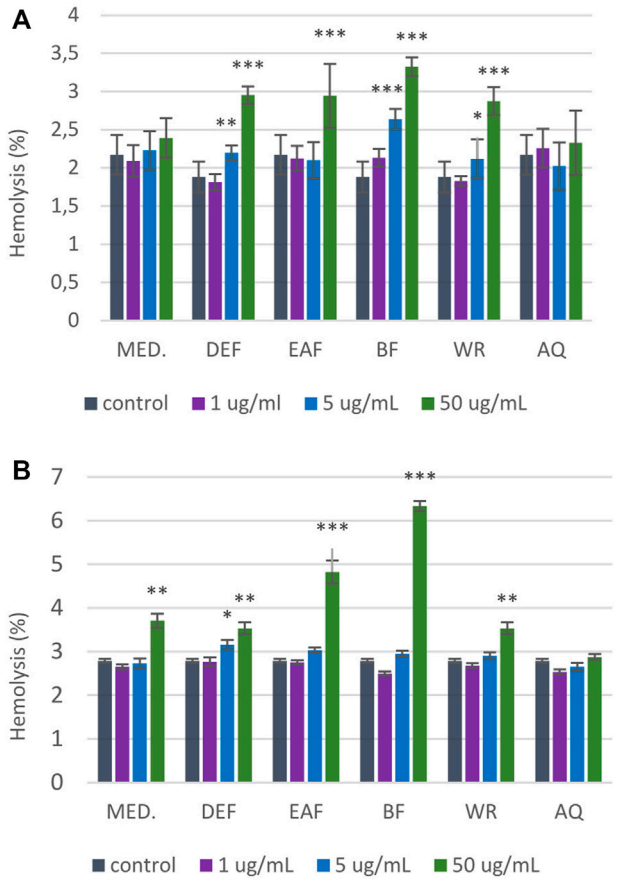

FIGURE 10
Influence of *P. spinosa* flower extracts on the hemolysis of erythrocytes. The results are presented as percentage of RBCs hemolysis obtained from 1-h **(A)** and 24-h **(B)** interaction of studied extracts with 2% RBCs suspension, compared to the positive control Triton X-100 at 0.2% (100% hemolysis). The results are presented as mean ± SE; n = 5, asterisk denotes significant differences compared to control 0.9% NaCl (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

thrombin; however, only cyanidin, quercetin and silybin also inhibited the thrombin-induced polymerization of fibrinogen. Liu et al. (2010) reported a strong interaction between thrombin and gallic acid, catechin, epicatechin, dihydroquercetin, naringenin, apigenin, and baicalein. Choi et al. (2015) revealed that kaempferol significantly inhibited the amidolytic activity of thrombin and fibrin polymer formation. The study performed by Wei et al. (2019) documented that flavonol glycosides rutin and isoquercetin displayed moderate inhibition on the proteolytic activity of thrombin (the IC_{50} 34.08 and 28.76 μM, respectively), while the activity of quercetin and kaempferol was weaker (the IC_{50} 57.77 and 59.99, respectively). Among 42 flavonoids tested by Wang et al. (2020) in terms of their inhibition of amidolytic activity of thrombin, myricetin exhibited the highest inhibitory potential against the enzyme with the IC_{50} value of 56 μM; for comparison, the IC_{50} values of kaempferol and quercetin were 107 and 205 μM, respectively. The results from the present *in vitro* study may lead to some conclusions about the flavonoid structure-activity relationship. Among 8 flavonoid constituents tested for thrombin inhibition were aglycones (QU, KA) and their glycosides (mono- and diglycosides), including compounds with the *p*-coumaroyl group in the sugar moiety. Regarding the aglycones, the presence of the *ortho*-dihydroxyphenyl structure had a positive impact on the investigated activity. With two hydroxyl groups at 3′and 4′of the B ring, QU presented a more potent thrombin inhibition than KA with one hydroxyl group (IC_{50} 29.35 and 81.12 μg/mL, respectively). On the other hand, glycosylation limited the activity of flavonoids, as no inhibitory effects on thrombin were found for all tested glycosides at 1–50 μg/mL. The only exception was *p*-cJU, which, contrary to JU, inhibited the enzyme, and its activity was even more than twice as high as that of KA (IC_{50} 38.29 and 81.12 μg/mL, respectively). Thus, *p*-coumaroyl substitution at the 2″-OH position of arabinofuranose remarkably enhances the anticoagulant effect, and this functionalization in a sugar moiety could be regarded as a potential approach for improving the inhibitory effects of flavonoid glycosides on thrombin. Our results are consistent with that of Liu et al. (2010), which evaluated the thrombin inhibition by flavonoids using the optimized TT assay *in vitro*. As in our study, quercetin was a more potent thrombin inhibitor (the IC_{50} of 35 μM) than kaempferol (the IC_{50} of 109 μM), and the glycosides, i. a., rutin, hyperoside, and kaempferol-3-*O*-glucoside, were inactive in the tests with the IC_{50} above 1000 μM, while kaempferol-3-*O*-(2″-di-*E*-*p*-coumaroyl)-rhamnoside and kaempferol-3-*O*-(2″,4″-di-*E*-*p*-coumaroyl)-rhamnoside displayed moderate activity with the IC_{50} of 83 and 52 μM, respectively. All these data suggest that glycosylation significantly disfavors the thrombin inhibitory effect of flavonoids; however, the presence of other groups in the sugar moiety, for example, *p*-coumaroyl, prompts inhibition of the enzyme.

Polyphenols undergo extensive metabolism in the human body, and their biological *in vivo* effects stem from both their native forms and biotransformation products, such as glucuronidated, methylated, and sulfated forms (Serreli and Deiana, 2019). Some polyphenolic compounds may be absorbed but most of them reach the colon, where gut microbiota contribute to their conversion into low-molecular-weight phenolic acids with potential biological activity (Catalkaya et al., 2020). The primary gut microbiota metabolites of flavonols are DCA, PAA and PCA (Marín et al., 2015). Proanthocyanidins are metabolized into PPA, 3-(3′-hydroxyphenyl) propionic acid, 4-hydroxyphenylacetic acid, and phenylvalerolactone (Ou and Gu, 2014). CHA is metabolized by gut microbiota to caffeic acid, which is then transformed into phenylpropionic acid derivatives like DCA and PPA (Tomas-Barberan et al., 2014). For a thorough assessment of the plant materials' biological effects, our *in silico* docking simulations considered key phenolic compounds representing the main metabolites of polyphenols in the human body and corresponding to the phenolic profile of the blackthorn flower extracts. These included miquelianin, a product of quercetin glucuronidation, as well as DCA, PCA, PPA, and PAA, which serve as model gut microbiota metabolites (Figure 4). The results revealed that the metabolized polyphenols bind to the active site of thrombin and may act as univalent direct thrombin inhibitors. In the last years, the antioxidant, anti-inflammatory and anticancer effects of microbiota metabolites have been revealed in numerous studies (Serreli and Deiana, 2019). Our previous works also revealed the significant activity of the metabolized polyphenols towards multiple *in vivo*-relevant oxidants (Marchelak et al., 2019), as well as adequate protection of human plasma components, including fibrinogen, against $ONOO^-$-induced damage (Marchelak et al., 2021).

5 Conclusion

This paper advances the current understanding of the biological activity of blackthorn flowers by presenting new data on their impact on various aspects of the hemostatic system *in vitro*. For the first time, the results demonstrate that blackthorn flower extracts at *in vivo*-relevant levels might exert anticoagulant effects through direct thrombin inhibition. Among the extracts, MED and its polyphenolic-rich fractions DEF, EAF, and BF exhibit superior activity parameters and appear to be the most promising candidates for functional applications. As indicated by correlation studies, polyphenols significantly contribute to the thrombin-inhibitory effects of the extracts. *In silico* docking simulations suggest that blackthorn flower constituents can act as univalent or bivalent DTIs, as they bind with considerable binding affinity to thrombin only within the active site or have additional binding sites outside the catalytic triad, i.e., exosite I. The interaction of blackthorn polyphenols with the enzyme was confirmed in CD spectroscopy, which revealed changes in the secondary structure of thrombin within the α-helices. Among the individual constituents, PA2, QU, KA, and *p*-cJu demonstrate the most significant potential for antagonizing the enzyme. Notably, PA2 stands out due to strong docking binding in the active site and exosite I, extensive changes in CD spectrum, and significant thrombin inhibition *in vitro*. Additionally, our findings suggest potential structural modifications, such as *p*-coumaroyl substitution at the 2″-OH position in a sugar moiety, as an approach to enhance the thrombin-inhibitory activity of flavonoid glycosides. Moreover, our study indicates that the extracts do not significantly impact platelet hemostasis. These findings contribute to the understanding of the potential medical applications of *P. spinosa* flowers in preventing and treating cardiovascular diseases (CVDs). However, additional research is needed to thoroughly understand their impact on the hemostatic system, including their interactions with other coagulation cascade factors and fibrinolytic proteins. Furthermore, it is essential to complement *in vitro* tests with animal studies and clinical trials to finally assess the therapeutic effectiveness of blackthorn flowers and extracts.

Data availability statement

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

Ethics statement

The studies involving humans were approved by the Committee on the Ethics of Research at the Medical University of Lodz and Committee on the Ethics of Research at the University of Lodz. 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

AM: Conceptualization, Investigation, Methodology, Writing–original draft. JK-C: Conceptualization, Investigation, Methodology, Writing–review and editing. MP: Investigation, Methodology, Writing–review and editing. OL: Investigation, Writing–review and editing. MM-P: Investigation, Methodology, Writing–review and editing. BB: Investigation, Writing–review and editing. KM: Investigation, Methodology, Writing–review and editing. MO: Conceptualization, 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.2023.1307373/full#supplementary-material

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Glossary

AFZ Kaempferol 3-*O*-*α*-L-rhamnopyranoside (afzelin)

APPT activated partial thromboplastin time

AQ aqueous extract

AV Quercetin 3-*O*-*α*-L-arabinofuranoside (avicularin)

BF *n*-butanol

CA (+)-Catechin

CCHA 4-*O*-Caffeoylquinic acid (cryptochlorogenic acid)

CFA Caffeic acid

CHA 5-*O*-Caffeoylquinic acid (chlorogenic acid)

CVDs cardiovascular diseases

DCA 3-(3′,4′-dihydroxyphenyl)propionic acid (dihydrocaffeic acid)

DEF diethyl ether fraction

DTI direct thrombin inhibitors

EAF ethyl acetate fraction

ECA (−)-Epicatechin

GU Quercetin 3-*O*-*α*-L-arabinopyranoside (guaiaverin)

HY Quercetin 3-*O*-*β*-D-galactopyranoside (hyperoside)

IQ Quercetin 3-*O*-*β*-D-glucopyranoside (isoquercitrin)

JU Kaempferol 3-*O*-*α*-L-arabinofuranoside (juglanin)

KA Kaempferol

KAFR Kaempferol 3-*O*-*α*-L-arabinofuranoside-7-*O*-*α*-L-rhamnopyranoside

KAPR Kaempferol 3-*O*-*α*-L-arabinopyranoside-7-*O*-*α*-L-rhamnopyranoside

KCAR Kaempferol 3-*O*-(2″-*O*-*E*-*p*-coumaroyl)-*α*-L-arabinofuranoside-7-*O*-*α*-L-rhamnopyranoside

KR Kaempferol 7-*O*-*α*-L-rhamnopyranoside

KRG Kaempferol 3-O-(6″-O-α-L-rhamnopyranosyl)-β-D-glucopyranoside

KT Kaempferol 3,7-di-*O*-*α*-L-rhamnopyranoside (kaempferitrin)

KX Kaempferol 3-*O*-*β*-D-xylopyranoside

LEP Kaempferol 3-*O*-*β*-D-xylopyranoside-7-*O*-*α*-L-rhamnopyranoside (lepidoside)

MED methanol-water (7:3, v/v) extract

MQ Quercetin 3-*O*-*β*-D-glucuronopyranoside (miquelianin)

MUA Quercetin 3-O-(4″-O-β-D-glucopyranosyl)-α-L-rhamnopyranoside (multinoside A)

MUB Kaempferol 3-*O*-(4″-*O*-*β*-D-glucopyranosyl)-*α*-L-rhamnopyranoside (multiflorin B)

NCHA 3-*O*-Caffeoylquinic acid (neochlorogenic acid)

PA2 proanthocyanidin A2

PAA 2-(3′,4′-dihydroxyphenyl) acetic acid

***p*-CA** *p*-Coumaric acid

***p*-CJU** Kaempferol 3-*O*-(2″-*O*-*E*-*p*-coumaroyl)-*α*-L-arabinofuranoside

PBMCs peripheral blood mononuclear cells

PCA protocatechuic acid

PPA 3-(4′-hydroxyphenyl)propionic acid

PT prothrombin time

RBCs red blood cells

RN Quercetin 3-*O*-*α*-D-xylopyranoside (reinutrin)

ROS/RNS reactive oxygen/nitrogen species

RT Quercetin 3-*O*-(6″-*O*-*α*-L-rhamnopyranosyl)-*β*-D-glucopyranoside (rutin)

QC Quercetin 3-*O*-*α*-L-rhamnopyranoside (quercitrin)

QGA Quercetin 3-*O*-(2″-*O*-*β*-D-glucopyranosyl)-*α*-L-arabinofuranoside

QU Quercetin

TPA total proanthocyanidin content in cyanidin chloride equivalents (CyE) determined by *n*-butanol/HCl assay

TPC total phenolic content in gallic acid equivalents (GAE) determined by Folin-Ciocalteu assay

TPH total phenolic content determined by RP-HPLC-PDA as a sum of individual compounds

TT thrombin time

WR water residues

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Animal-and mineral-based medicines in Gansu-Ningxia-inner Mongolia region, P.R. China: a cross-cultural ethnobiological assessment

Chaoqun Luo[1†], Wenji Zhao[2†], Sha Liu[1], Mingxia Luo[1], Tingting Fan[1], Yongxia Zhao[1], Yan Ren[3], Faming Wu[1,4*] and Jian Xie[1,4*]

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Introduction: Traditional animal- and mineral-based medicines are widely used in the Gansu-Ningxia-Inner Mongolia junction zone, a region with diverse ethnic groups and cultures. This study aims to document, conserve, and explore the potential of these medicines for further research and sustainable development of ethnic medicine.

Methods: We interviewed 56 informants from different ethnic backgrounds and analyzed their responses quantitatively. Additionally, a comparative analysis with adjacent regions was conducted, providing invaluable contextual insights.

Results: The study unveiled a diverse array of traditional medicines in the Gansu-Ningxia-Inner Mongolia junction zone. A total of 47 animal-based medicines were identified, ranging from insects and scorpios to distinctive animal organs. Of notable significance was Moschus, emerging as a pivotal traditional Chinese medicine resource. In parallel, 12 mineral-based medicines were cataloged, procured both locally and from "pharmacies". Female informants, frequently local herbal practitioners, demonstrated broader knowledge of medicines. The analysis of 13 villages revealed varying perceptions of medicine importance, underscoring the wealth of traditional knowledge. Specific medicines, such as Feng-Mi and Xie-Zi, were widely used and valued in local healthcare practices for their cultural and medicinal benefits.

Conclusion: This study provides a comprehensive overview of traditional animal- and mineral-based medicines in the Gansu-Ningxia-Inner Mongolia junction

Abbreviations: NCSI, cultural food significance index; FQI, Frequency of quotation index; AI, Availability index; FUI, Frequency of utilization index; PUI, Parts used index; MFI, Multifunctional use index; CEI, Curative effect index; DSI, Drug safety index.

zone. It highlights the need for preserving and applying these practices in a sustainable manner. It also lays a solid foundation for future research on ethnic medicine, which can contribute to the holistic wellbeing of local communities.

KEYWORDS

traditional ethnic medicine, animal and mineral-based medicine utilization, cultural practices in medicine, sustainable development, traditional knowledge

Introduction

Animal and mineral resources have played pivotal roles in traditional medicine for centuries, constituting integral components of natural remedies (Lu, 2013). The roots of this knowledge can be traced back two millennia, when early practitioners of Traditional Chinese Medicine (TCM) meticulously documented the use of animal and mineral-based medicines (Xie, 2015). "Shennong's Classic of Materia Medica," the earliest extant Chinese pharmacological treatise, meticulously cataloged 67 animal-based and 41 mineral-based medicines (Wu, 1963). Subsequent works in Chinese pharmacology have further expanded upon these records (Zhang et al., 2022b).

Traditional medicine refers to a medical system developed within a specific cultural and historical context, with its theories and practices based on traditional knowledge, experience, and beliefs (Zhang, 2017). Traditional medicine can be classified into TCM, ethnic medicine, and other forms of traditional medicine. TCM, represented by Chinese traditional medicine, includes herbal medicine, acupuncture, and massage therapy. Ethnic medicine encompasses various traditional medical systems practiced by different ethnic groups, such as Tibetan medicine and Mongolian medicine. The characteristics of traditional medicine include comprehensiveness, holism, individualization, and empiricism (Li, 2017).

In China, traditional medicine has been widely applied and has received significant support and attention from the government. China has established a comprehensive legal framework for TCM, promoting its development and inheritance. Currently, traditional medicine has been widely applied and developed both domestically and internationally. In traditional Chinese medicine, medicines derived from animals, often referred to as "medicines with blood, flesh, and emotions," (Sun, 1993), are believed to possess greater potency and efficacy than their plant-based counterparts. The Chinese have also developed unique applications for mineral medicines, particularly in utilizing certain toxic minerals, resulting in distinct knowledge systems and safety protocols. For instance, arsenic trioxide (Pi shuang), a highly toxic mineral, is employed for specific medical purposes (Liu et al., 2008). Extensive modern pharmaceutical research has validated both its effectiveness and safety (Mahwish et al., 2010; Su, 2012; Han, 2015; Huang, 2018).

China, home to 56 diverse ethnic groups, each shaped by distinct geographical and environmental factors, boasts a rich tapestry of traditional ethnic medicine deeply ingrained in its culture and history. Over generations, these ethnic groups have accumulated a wealth of medicinal knowledge through the rigors of daily life, production, and warfare, giving rise to unique medicinal cultures. Varied natural environments and resource availability across different regions have led to diverse choices and applications of medicines. For instance, in the Gansu-Ningxia-Inner Mongolia junction area, the local environment and climate conditions have prompted a greater emphasis on the use of mineral and animal-based medicines (Liang, 2015; Garcia-Hernandez et al., 2021). In contrast, other regions, blessed with abundant herbal resources, lean toward herbal medicines (Luo et al., 2019).

Traditional Tibetan medicine, with its extensive history, places significant emphasis on animal and mineral-based remedies (Demar, 2012). Notable traditional remedies, primarily composed of animal ingredients, include "Gangdi Shi Feng Wan," "Zang Wang Shen Bao Wan," "Er Shi Wu Wei Lv Xue Wan," and "Xiong Dan Gao" (Yutuo, 1983). Mineral-based remedies such as "Er Shi Wu Wei Zhen Zhu Wan," "Er Shi Wu Wei Shan Hu Wan," "Er Shi Wu Wei Song Shi Wan," "Qi Shi Wei Shan Hu Wan," and "Qi Shi Wei Zhen Zhu Wan" are also prominent (Ren and Chen, 2014). The Tibetan Medical Canon meticulously documents up to 258 medicinal substances sourced from animal resources (Dorje, 2007). The preparation of these remedies involves a multistep process, encompassing material selection, cleaning, roasting, grinding, and distillation, culminating in effective medicine (Jia et al., 2015).

Internationally, an increasing number of countries and regions have begun to recognize the value of traditional medicine. Johnson et al. found that kidney diseases may be caused, treated, prevented, improved, or worsened by traditional medicines depending on the setting, the person, and the types, modes, and frequencies of traditional medicine use (Johnson et al., 2022). Grace et al. conducted a structured interview questionnaire with 200 individuals, including patients, hospital visitors and hospital staff, at the main referral hospital in Timor-Leste and found that the use of traditional medicine had become widespread there (Grace et al., 2020). Johnson et al. found through their research that traditional medicine can be a valuable resource for First Nations patients living with diabetes and should be considered a therapeutic modality (Johnson et al., 2022). Additionally, traditional medicine is widely applied in Asian countries. Traditional medicine systems such as Indian Ayurveda (Joshi and Joshi, 2021), Korean medicine (Kim et al., 2016), and Japanese Kampo medicine (Hyun et al., 2019) have deep historical and cultural heritage in various Asian countries. These countries have systematically organized and developed traditional medicine, forming unique medical systems.

The Gansu-Ningxia-Inner Mongolia intersection zone stands as a crucible of Han, Mongolian, and Hui cultures, representing the intersection of ancient nomadic and agrarian traditions in China (Li et al., 2022). This dynamic cultural milieu has engendered a unique system, wherein traditional medicine plays a pivotal role. However, with the march of progress and the influence of both traditional Chinese medicine and modern pharmaceuticals, numerous small-scale medicinal cultures characterized by regional and ethnic

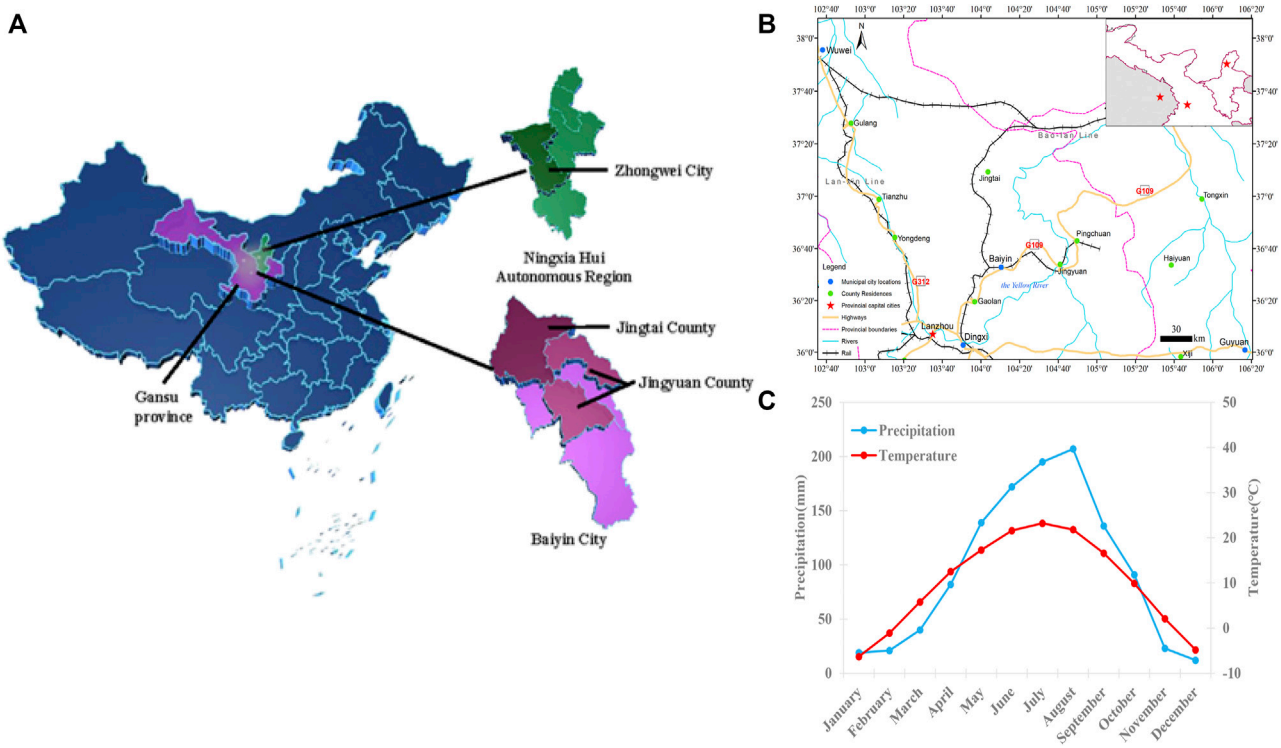

FIGURE 1
Survey area. **(A)** Jingyuan County, Jingtai County and Zhongwei County belong to an intersection zone of Gansu-Ningxia-Inner Mongolia. **(B)** The traffic situation in the study area. **(C)** The annual average temperature and precipitation.

distinctiveness face rapid decline (Ma, 2022). This not only entails the loss of medicinal value but also threatens cultural diversity. Hence, it becomes imperative to explore, document, preserve, and pass on this traditional knowledge. Such endeavors hold significant implications for cultural heritage, ethnic identity preservation, the judicious utilization of medicinal resources, and the safeguarding of ecological integrity. Furthermore, they provide invaluable insights for modern pharmaceutical research and the development of traditional medicinal resources. Consequently, this study undertook a systematic examination of animal and mineral-based medicinal resources utilized in the multiethnic regions of the Gansu-Ningxia-Inner Mongolia intersection zone. It sought to systematically compile and organize the traditional knowledge of local residents regarding these medicines, unearthing valuable insights for their rational utilization and development.

Study area and methods

Study area

This study focuses on the Jingyuan-Jingtai-Zhongwei area located at the intersection of Gansu, Ningxia, and Inner Mongolia. The study area lies between 103°33′E to 106°10′E and longitude 36° N to 37°50′N latitude, with elevations ranging from 1,100 to 3,321 m. The average temperature ranges from 7.3°C to 9.5°C throughout the year, with the coldest season typically occurring in winter and the hottest season occurring in summer. The annual precipitation ranges from 180 to 367 mm, with more rainfall occurring in summer and less in winter. The area experiences almost no rainfall during other times. The annual sunshine hours range from 2,696 to 3,796 h, and the region has a temperate continental climate (Figure 1) (Nan et al., 2004). It is located in the upper reaches of the Yellow River, with high terrain in the west and low terrain in the east. It is a gully area of the Loess Plateau, where the gullies intersect with the river beach. This area is a transitional zone between the Loess Plateau and the Tengger Desert and serves as the gateway to the eastern end of the Hexi Corridor (Table 1) (Xu et al., 2007; Gao, 2017).

This region has a rich history dating back to the Western Zhou period in ancient times. During the Han dynasty, it played a crucial role as one of the key passages along the Silk Road and served as an important military stronghold in ancient Northwest China, functioning as a defensive outpost (June 2016). Presently, the area enjoys robust transportation infrastructure, including major highways such as the Lanzhou-Xinjiang and Ningdong Expressways, as well as railway lines such as the Lanzhou-Xinjiang and Lanzhou-Chongqing Railways, facilitating seamless connectivity both within the region and with neighboring provinces and regions (Gao and Dong, 2020; Cao, 2021). The economy of the region leans predominantly on traditional agriculture and animal husbandry, with Han, Hui, Mongolian, Tibetan, Tujia, and other ethnic groups constituting the primary demographic. The population remains relatively modest, owing to the region's comparatively lower economic development and restricted population mobility, resulting in a pronounced aging population demographic (Bai, 2018). The area's arid climate translates to

TABLE 1 Basic information of the study areas.

County	Location	Population	Main ethnic groups	Main language	GDP/ person	Investigation site	Longitude	Latitude
Jingyuan	E 104°13′-105°15′; N 36°–37°15′	373,100	Han	Chinese	¥26869	Shahe Village Yongxin Township	E 104°35′	N 37°3′
			Hui			Yongxin Township Hassanshan Nature Reserve	E 104°39′	N 37°4′
			Mongolian			Damiao Village	E 104°29′	N 37°12′
			Tibetan			North TanLugou Village	E 104°51′	N 37°12′
						Wulan Town	E 104°41′	N 36°33′
						Dongwan Town Daba Village	E 104°43′	N 36°37′
						Dongsheng Town Xinzhai Village	E 104°58′	N 36°59′
Jingtai	E 103°33′-104°43′; N 36°43′-37°38′	238000	Han	Chinese	¥32142	Caowotan Town Gongjiawan Village	E 104°6′	N 37°17′
			Hui			Luyangshicheng Village	E 104°9′	N 37°8′
			Mongolian			Xiquan Town Santang Village	E 104°3′	N 37°5′
Zhongwei	E 104°17′-106°10′; N 36°06′-37°50′	1075000	Han	Chinese	¥52454	Changshantou Town Pengjian Village	E 105°36′	N 37°21′
			Hui			Dazhanchang Town Dazhanchang Village	E 105°32′	N 37°25′
			Man			Yongkang Town Yongfeng Village	E 105°18′	N 37°29′
			Mongolian					
			Dongxiang					

relatively meagre vegetation resources, predominantly characterized by desert and grassland flora interspersed with patches of forest, shrubbery, and wetland vegetation. Noteworthy plant species include *R.obinia pseudoacacia* L. (*R. pseudoacacia* L.), *Haloxylon ammodendron* (C. A. Mey.) Bunge ex Fenzl (*H. ammodendron* (C. A. Mey.) Bunge ex Fenzl), *Caragana korshinskii* Kom. (*C. korshinskii* Kom.), *Hippophae rhamnoides* L. (*H. rhamnoides* L.) (Du et al., 2016; Wang et al., 2019), among others. While the area is relatively deficient in animal resources, it is home to diverse wildlife, including species such as *Pseudois nayaur* (Hodgson) (*P. nayaur* (Hodgson)), *Cervus elaphus* Linnaeus (*C. elaphus* L.), *Mellivora capensis* (Schreber) (*M. capensis* (Schreber)) (Liu et al., 2022; Xie, 2022; Liu et al., 2023b). However, it boasts a wealth of mineral resources, encompassing coal, *gypsum fibrosum*, limestone, gold, Yin-Zi, copper, and others (Gao, 2022a; Gao, 2022b; Guan et al., 2022).

Ethnobotanical information collection

Ethnobotanical Information Collection During the field survey, we used key informant interviews (Hong et al., 2015), semistructured interviews and participatory rural appraisal (Liu et al., 2014) methods to collect information based on the "5 W+1H" principle (who, what, where when, why, and how) (Pei and Long, 1998). Key informant interviews are a method of interviewing knowledgeable persons as an important part of the research. We selected some traditional healers or herbalists with rich experience and knowledge as key informants and asked them some open-ended questions (Supplementary Table S1) to understand their opinions and suggestions on the use, source, and conservation of animal and mineral medicines. Semistructured interviews are a method of using partly predesigned questions and partly flexible questions to collect qualitative data. In the survey process, we developed an interview guide based on the research objectives and literature review and then asked questions flexibly according to different interviewees and situations to collect information on the use, knowledge, and attitude of animal and mineral medicines. Participatory rural appraisal involves local residents in the research process. We invited local residents to discuss, analyze and evaluate local problems and resources, such as community maps, resource maps, and historical timelines, to express their views and needs on the use, distribution, and threats of animal and mineral medicines and to obtain more local knowledge and experience (Figure 2). During the survey process, we mainly asked the following questions: 1) whether the respondent used any animal or mineral medicine to treat or prevent diseases; 2) if yes, which diseases were the animal or mineral medicine used to treat or prevent; 3) how the animal or mineral medicine was used (including which parts, preparation methods, and use methods); 4) what are the sources of these animal or mineral medicines; 5) how was this knowledge acquired; and 6) what else should be added to this interview. The interviews were conducted in Mandarin (Chinese) or local dialects. During the interview, in addition to

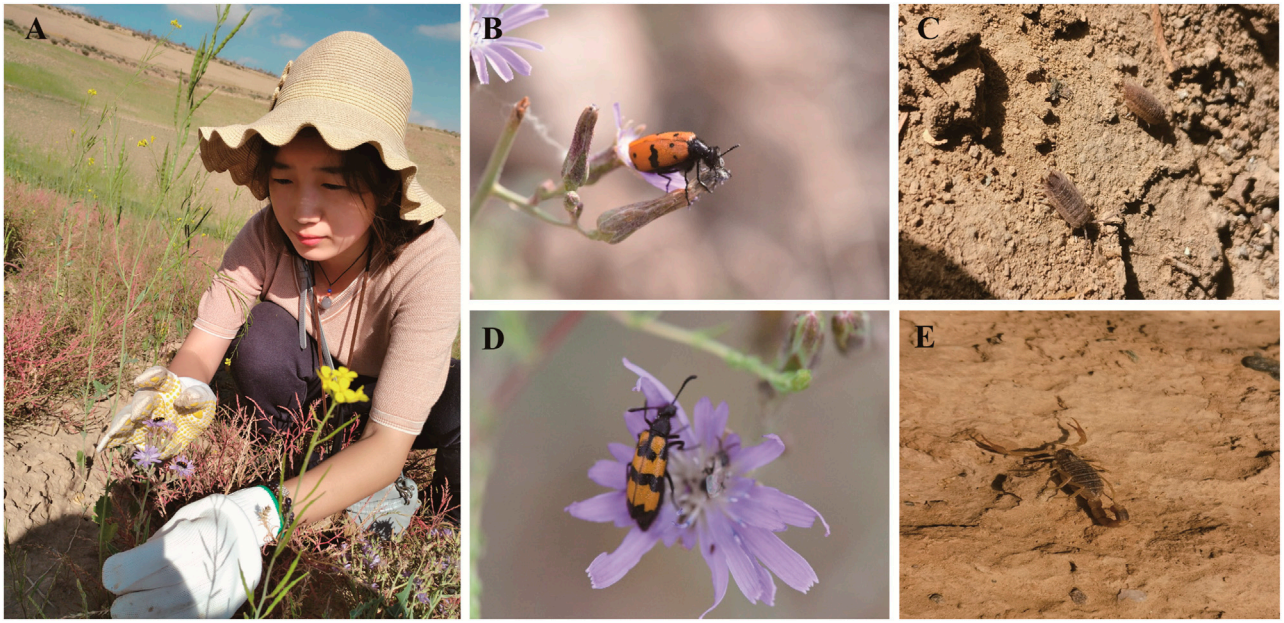

FIGURE 2
Examples of animal-based medicines. **(A, B, D)** *Mylabris phalerata* is widely used in traditional Chinese medicine with anti-inflammatory, analgesic, and anticancer effects. **(C)** Pillbug is also used in traditional Chinese medicine. It has nourishing, tonifying, and hemostatic effects. **(E)** *Scorpios* is a traditional Mongolian medicine with antispasmodic, anticonvulsant, and antitumor effects.

written records, audio recordings and photos were taken with the permission of the other party, and the respondents were asked to take us to the adjacent grassland, farmland, or mountain to identify the animal or mineral medicine that they used and provide local colloquial names in Mandarin (Chinese) or local dialects.

Through these survey methodologies, we systematically accumulated and cataloged the traditional knowledge of animal and mineral-based medicines used by local residents and recorded, organized and analyzed the basic information of the informants and the local names, medicinal parts, processing methods, efficacy, and safety of the medicines.

Data collation and analysis

NCSI analysis

We used the National Plant Cultural Significance Index (NCSI) to evaluate the importance of animal and mineral drugs in the surveyed area.

$$NCSI = FQI \times AI \times FUI \times PUI \times MFI \times CEI \times DSI \times 10^{-2}$$

In this formula, FQI is the frequency of the quotient index, AI is the availability index, FUI is the frequency of utilization index, PUI is the parts used index, MFI is the multifunctional use index, CEI is the curative effect index, and DSI is the drug safety index (Pieroni, 2000). Each index was established and assigned a score according to the guidelines provided in "Research Methods in Ethnobotany" (Wang and Wang, 2017). The frequency of quotation index (FQI) refers to the number of people among all informants who mentioned a particular plant. The availability index (AI) is divided into four categories: very common (4.0), common (3.0), general (2.0), and uncommon (1.0). The frequency of utilization index (FUI) is divided into six categories: used more than 10 times per year (5.0), used 6-10 times per year (4.0), used 2-5 times per year (3.0), used at least once per year (2.0), used once every 2–3 years (1.0), and not used in the past 5 years (0.5). The Parts Used Index (PUI) is divided into three categories: whole plant (3.0), part of the plant (2.0), and special parts or processed products (1.0). The multifunctional use index (MFI) has a base score of 0 and increases by 1 for each additional use. An item with only one use has a score of 1, and an item with five uses has a score of 5. The Curative Effect Index (CEI) is divided into five categories: excellent (5.0), very good (4.0), good (3.0), fair (2.0), and poor (1.0). The Drug Safety Index (DSI) is divided into five categories: very high (dual-use as food and medicine, score of 5.0), high (safe with no toxic side effects, score of 4.0), moderately high (has some side effects, score of 3.0), moderate (slightly toxic, score of 2.0), and low (highly toxic, score of 1.0).

SWOT analysis

SWOT analysis is an analytical framework proposed by Harvard Andrews in 1971 in The Concept of Corporate Strategy. It is a method of systematically identifying strengths, weaknesses, opportunities and threats and proposing strategies to cope with them (Dai, 2000). Among them, strengths are the internal strengths and favorable conditions of the subject of the study, weaknesses are the internal weaknesses and unfavorable conditions of the subject of the study, opportunities are the external opportunities and favorable conditions faced by the subject of the study, and threats are the external threats and unfavorable conditions faced by the subject of the study. An effective strategy should be able to maximize internal strengths and environmental opportunities while minimizing

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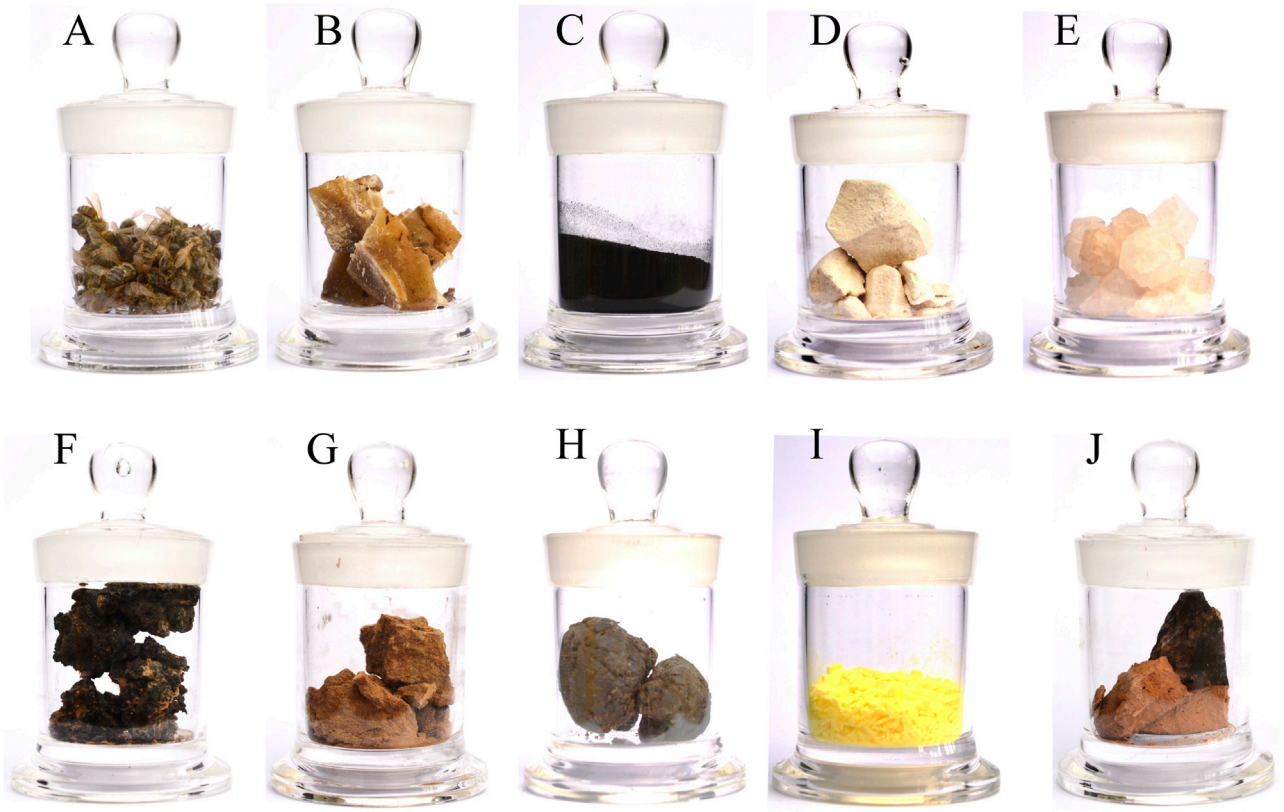

FIGURE 3
Examples of animal- and mineral-based medicinal specimens collected in the study area. **(A)** *Apis cerana* Fabricius. The dried body of *Apis cerana* Fabricius or *Apis mellifera* Linnaeus. **(B)** *Cera Flava*. Wax secreted by *Apis cerana* Fabricius or *Apis mellifera* Linnaeus. The hive is heated in water and filtered, and the wax is condensed or refined. **(C)** Guo Hui. Ashes were stored on the bottom of the pot after the weed was burned. **(D)** *Gypsum Fibrosum*. For the sulfate mineral gypsum group gypsum, debris and sediment were removed after excavation. **(E)** *Halitum*. Halide salt crystals of the rock salt family. **(F)** Kang-Jing. Tar formed by burning the adobe bed. **(G)** *Loess*. A porous yellow powdery soil with columnar joints formed under dry climatic conditions. **(H)** *Propolis*. A viscous solid gel formed by mixing plant resin collected by the worker bees of the honeybee family, *Apis mellifera* Linnaeus, with secretions from their upper frontal glands and wax glands. **(I)** *Sulfur*. Natural sulfur of the sulfur group of natural elemental minerals is mined, heated and melted to remove impurities. **(J)** *Terra Flava Usta*. A scorched yellow clod in the center of the bottom of an earthen stove for burning wood or weeds.

internal weaknesses and environmental threats. Similarly, in traditional medicine, SWOT analysis can help healthcare professionals understand the strengths, weaknesses, opportunities and threats of traditional medicine so that they can formulate appropriate development strategies. Therefore, we use SWOT analysis in this paper (Tang et al., 2018), from the traditional medicine's own internal and external environmental conditions of the two major aspects of the investigation of some of the traditional medicine of the region's strengths, weaknesses, opportunities and threats to identify and analyze, and build SWOT strategy quadrilateral matrix, according to the matrix to focus on the factors and can be biased toward the use of the strategy, put forward the corresponding strategic recommendations, from which to find the future development of the strategic direction.

Specimen identification

The sources of the animal and mineral-based medicines were collected during the investigation by referencing "The Illustrated Book of Chinese Medicinal Animals" (Li et al., 2014), "Mineral Medicine Authenticity Illustration and Application" (Gao, 2014), and "Important Medicinal Insects of China" (Yang, 2015). Voucher specimens (bottle specimens) were meticulously prepared and preserved. Collected data were meticulously sorted and analyzed in alignment with the research objectives, with graphical representations generated. The voucher specimens were archived in the Chinese Medicinal Specimen Museum at the School of Pharmacy, Zunyi Medical University (Figure 3).

This study received ethical approval from the Animal Ethics Review Committee of Zunyi Medical University (ZMU21-2,203-291) and the Medical Ethics Committee of Zunyi Medical University ([2022]1-291).

Results

Characteristics of informants

This study incorporated data from fifty-six informants who provided substantive information. Descriptive analysis was

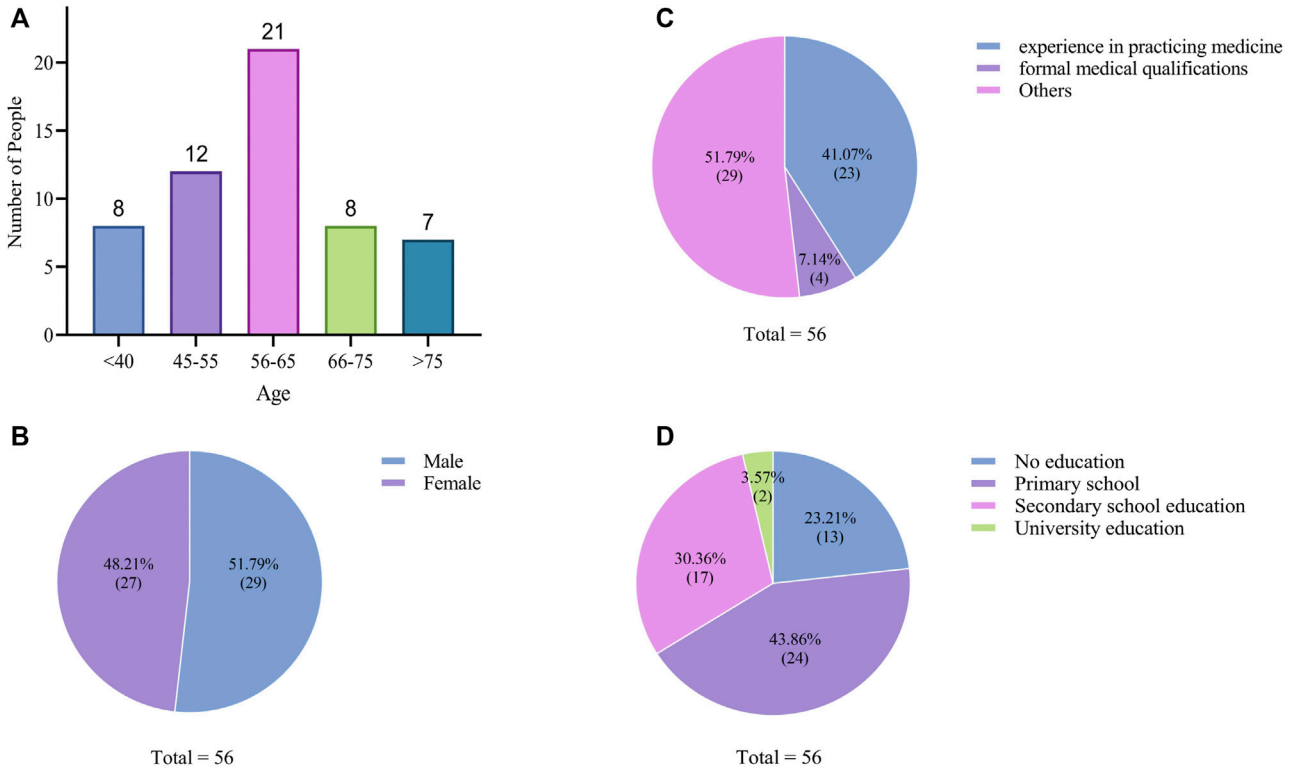

FIGURE 4
Demographic profile of informants. **(A)** Age structure of informants. **(B)** Gender ratio of informants. **(C)** Level of experience of ethnic medicine among informants. **(D)** Education level of informants.

conducted to delineate their age, gender, and ethnic composition. The age range of informants spanned from 35 to 84 years, with eight participants below 40, twelve between 45 and 55, twenty-one between 56 and 65, eight between 66 and 75, and seven above 76 years of age (Figure 4A). The gender distribution was nearly equal, with twenty-nine male and twenty-seven female informants (Figure 4B). Among these participants, twenty-three had prior experience in medical practice, with four holding formal medical qualifications (Figure 4C). All informants had experience using or being treated with animal or mineral medicines (individuals unable to provide valid information were excluded). They were thoroughly interviewed, and their responses were meticulously recorded. Prior to the interviews, informed consent was obtained from all informants, who also affixed their signatures to the consent forms.

According to the survey, women demonstrated an average familiarity with 28.23 types of animal or mineral-based medicines, while men were acquainted with approximately 14.51 kinds. Consequently, in comparison to men, women exhibited broader knowledge of animal or mineral medicines. This phenomenon deviates from findings in other studies, and we posit that it may be attributed to the prevalence of female local traditional herbal doctors (shamans).

Different age groups had different knowledge of animal or mineral medicines. The demographic most inclined to employ these medicines for treating ailments comprised individuals above 45 years old (85.71%). Research has indicated that older individuals tend to possess deeper comprehension and acceptance of traditional medicines (Bouasla and Bouasla, 2017; Lee et al., 2019; Huang et al., 2022). This can be ascribed to the underdevelopment of modern medicine decades ago, where local traditions predominantly relied on ethnic medicine. Furthermore, this cohort might be less inclined to trust in ethnic medicine due to inheritance issues, deeming it superstitious.

With regard to education levels, of the fifty-six informants, thirteen had received no formal education (23.21%), twenty-four had attained primary school education (43.86%), seventeen possessed secondary school education (30.36%), and two had acquired university education (3.57%). The majority exhibited lower educational attainment (Figure 4D). This pattern suggests that knowledge regarding ethnic medicine application primarily circulates among individuals with limited formal education. It is surmised that informants with lower educational backgrounds harbor greater interest in traditional medicine and engage more frequently with the natural environment. Conversely, those with higher education levels evince reduced interest in traditional medicine, owing to prolonged exposure to modern educational systems, resulting in fewer encounters with medicinal plants and local medicine-related knowledge. Analogous results reported in other studies highlight that individuals with lower educational attainment, including illiterate individuals, possess greater proficiency in using medicinal plants compared to intellectuals (Ahmad et al., 2017; Miara et al., 2018).

TABLE 2 Basic information of animal-mineral medicines.

Local name	English name	Source	Use part	Processing method	Ethnopharmacological employment/use	Method of application	Voucher numbers[4]
Zhu-Kudan	Pig's bitter gall	*Sus scrofa* domestica Brisson (*S. scrofa* domestica Brisson)	Guts	Direct use of fresh products	Redness of the eyes/Weeping	Washing/Drops in the eyes/Small amount for internal use	2023-DK-012
Feng-Mi	Honey	*Apis cerana* Fabricius (*A. cerana* Fabricius)/*Apis mellifera* Linnaeus (*A. mellifera* L.)	Products	Direct acquisition	Mouth ulcers/Constipation	Topical application/Internal use	2023-DK-001
Feng-La	Beeswax	*A. cerana* Fabricius/*A. mellifera* L	Products	Direct acquisition	Various inflammatory diseases	Topical application/Decoction with water	2023-DK-021
Feng-Fang	Nidus vespae	*A. cerana* Fabricius/*A. mellifera* L	Products	Direct acquisition	Itchy skin	Decoction with water	2023-DK-022
Feng-Jiao	Propolis	*A. cerana* Fabricius/*A. mellifera* L	Products	Direct acquisition	Skin diseases	Topical application/Decoction with water	2023-DK-029
Mi-Feng	Bee	*A. cerana* Fabricius/*A. mellifera* L	All	Direct use of fresh products	Rheumatoid arthritis	Lure bees to sting the affected area	2023-DK-011
Hua-Banmao	Mylabris	*Mylabris phalerata* Pallas (*M. phalerata* Pallas)/*Mylabris cichorii* Linnaeus (*M. cichorii* L.)	All	Drying after scalding	Mad dog bite	Powdered and applied externally	2023-DK-033
Xi-Querou	Magpie's Meat	*Pica pica* (*P. pica*)	Flesh	Kill and clean feathers and guts after capture	Deficiency syndrome[1]/Diabetes	Stew	——
Wu-Yazui	Crow's beak	*Corvus sp.* (*Corvus sp.*)	Beak	Kill and clean feathers and guts after capture	Cough with deficiency-heat[2]	Stew	——
Tu-Mao	Rabbit hair	*Lepus capensis* Linnaeus (*L. capensis* L.)	Fur	Cutting	Mental illness	Make lotus wear with other medicines	2023-DK-031
Xie-Diban	Pillbug	*Porcellio* sp. (*Porcellio sp.*)	All	Collected and scalded, dried	Traumatic fractures	Grind and swallow	2023-DK-014
She-Dan	Snake gall	*Serpentiformes*	Guts	Fresh use/Shade dried	Redness of the eyes/Weeping	Washing/Drops in the eyes/Small amount for internal use	2023-DK-028
She-Tui	Snake skin cast off during molting	*Serpentiformes*	Skin	Removal of impurities after field collection	Mumps	Fried with eggs/Decoction with water	2023-DK-019
She	Snake	*Serpentiformes*	All	Wild caught and killed to make wine	Various kinds of rheumatoid arthritis	Infusion of wine for internal or external use	2023-DK-010
Tai-Yanggao	Fetal lamb	*Capra hircus* Linnaeus (*C. hircus* L.)/*Ovis aries* Linnaeus (*O. aries* L.)	All	Collection of aborted stillbirths	Deficiency syndrome	Medicinal food/Direct consumption	——
Yang-Nai	Goat's colostrum	*C. hircus* L./*O. aries* L	Breast milk	Direct use of fresh products	Redness of the eyes/Weeping	Washing	——
Yang-Bian	Penis and testis of a goat	*C. hircus* L./*O. aries* L	Penis	Collection at the time of slaughter	Enhance male sexual function/Male infertility	Soup making/Infusion of wine	——
Yang-Shen	Goat's renal	*C. hircus* L./*O. aries* L	Testicles	Collection at the time of slaughter	Enhance male sexual function/Male infertility	Soup making/Infusion of wine	——
Yang-Jiao	Goat's horn	*C. hircus* L./*O. aries* L	Horns	Collection and crushing at the time of slaughter	Eye diseases/Fverish	Decoction with water	2023-DK-015
Tai-Pan	Placenta	*Homo sapiens* Linnaeus (*H. sapiens* L.)	Placenta	The placenta of a woman in delivery, baked in tiles	Female body weakness	Grind and swallow	2023-DK-005

(Continued on following page)

TABLE 2 (*Continued*) Basic information of animal-mineral medicines.

Local name	English name	Source	Use part	Processing method	Ethnopharmacological employment/use	Method of application	Voucher numbers[4]
Zuo-Tu	Loess	*H. sapiens* L	Blood	The blood-sucking soil formed by absorbing the bleeding of women during childbirth with loess	Weakness or blood deficiency due to various diseases	Soak in warm water, strain and drink	——
Ren-Nai	Breast milk	*H. sapiens* L	Breast Milk	Direct use of fresh products	Redness of the eyes/Weeping	Washing/Drops in the eyes	——
Tou-Fa	Hair	*H. sapiens* L	Hair	Cutting	Baby cries	Use female hair for male children and male hair for female children, decoction in water/Making a purse to wear	2023-DK-025
Tong-Ziniao	Children's urine	*H. sapiens* L	Urine	Direct acquisition	Various kinds of deficiency fever	Medicine primer	——
Ban-Mao	Cantharides	*Lytta caraganae* Pallas (*L. caraganae* Pallas)	All	Drying after scalding	Dog bites	Powdered and applied externally	2023-DK-030
Shi-Panniu	Dung beetle	*Geotrupidae*	All	Drying after scalding/ Baking on tiles	Cancer	Grind and swallow	2023-DK-016
Cao-Chong	Paramecium caudatum	*Holotrichia diomphalia* Bates (*H. diomphalia* Bates)	All	Add salt and then dissolve the water	Burns and scalding/Ulcers	Topical application	2023-DK-024
Tuan-Zhu	Honey badger	*M. capensis* (Schreber)	Fat	Collecting fat for storage in clay pots after trapping	Frostbite/Water and fire burns	Topical application	——
Ma-Que	Sparrows	*Passer montanus* (Linnaeus) (*P. montanus* L.))	Flesh	Killed after trapping	Postpartum or post-illness weakness[3]	Cooked or baked and eaten	——
Lü-Fen	Feces of donkey	*Equus asinus* Linnaeus (*E. asinus* L.)	Feces	Direct use of fresh products	Dog bites	Topical application	2023-DK-035
Lü-Rou	Flesh of donkey	*E. asinus* L	Flesh	Direct use of fresh products	Deficiency syndrome	Medicinal food/Direct consumption	——
She-Xiang	Moschus	*Moschus berezovskii* Flerov (*M. berezovskii* Flerov)/*Moschus sifanicus* Przewalski (*M. sifanicus* Przewalski)	Products	Put them into a porcelain vase and put a few sewing needles to seal and raise the needles	Swelling and poisoning	Draw circles with a needle around and on the surface of the lump	2023-DK-036
Lang-Mao	Wolf hair	*Canis lupus* Linnaeus (*C. lupus* L.)	Fur	Collecting in the field	Foreign objects in the eyes	Use Lang-Mao to hook foreign objects out	——
He-Magezao	Tadpoles	*Rana nigromaculata* Hallowell (*R. nigromaculata* Hallowell)	All	Drying after scalding	Cancer	Decoction with water	——
Tui-Louzi	Ant lion larva	*Myrmeleon micans* Mac Lachlan (*M. micans* Mac Lachlan)	All	Sifted through a sieve and scalded to death in hot sand to dry	Cancer	Grind and swallow	2023-DK-013
Ji-Neijin	Membranes of chicken gizzards	*Gallus gallus* domesticus Brisson (*G. gallus* domesticus Brisson)	Stomach	Collected and shade dried	Indigestion	Take directly after mashing/Serve with rice and vegetables	2023-DK-004
Ji-Dan	Eggs	*G. gallus* domesticus Brisson	Egg	Direct use of fresh products	Water and fire burns	External application of egg white	2023-DK-009

(Continued on following page)

TABLE 2 (*Continued*) Basic information of animal-mineral medicines.

Local name	English name	Source	Use part	Processing method	Ethnopharmacological employment/use	Method of application	Voucher numbers[4]
Niu-Bian	Bull's penis	*Bos taurus* domesticus Gmeli (*B. taurus* domesticus Gmeli)	Penis	Collection at the time of slaughter	Enhance male sexual function/ Male infertility	Soup making/Infusion of wine	——
Niu-Shen	Ox kidney	*B. taurus* domesticus Gmeli	Testicles	Collection at the time of slaughter	Enhance male sexual function/ Male infertility	Soup making/Infusion of wine	——
Niu-Jiao	Ox horn	*B. taurus* domesticus Gmeli	Horns	Collection and crushing at the time of slaughter	Eye diseases/Fverish	Decoction with water	2023-DK-026
Gou-Bian	Dog's penis	*Canis lupus* familiaris Linnaeus (*C. lupus* familiaris L.)	Penis	Collection at the time of slaughter	Enhance male sexual function/ Male infertility	Soup making/Infusion of wine	——
Gou-Shen	Dog kidney	*C. lupus* familiaris L	Testicles	Collection at the time of slaughter	Enhance male sexual function/ Male infertility	Soup making/Infusion of wine	——
Gou-Xue	Dog's blood	*C. lupus* familiaris L	Blood	Direct use of fresh products	Mental illness	Internal use	——
Ge-Zi	Pigeons	*Aplopelia* Bonaparte (*A.* Bonaparte)	Flesh	Killed after trapping	Postpartum or post-illness weakness	Soup making	——
Ma-Yi	Ant	*Polyrhachis vicina* Roger (*P. vicina* Roger)	All	Drying after scalding	Male sexual dysfunctions	Infusion of wine for internal use	2023-DK-018
Xie-Zi	Scorpions	*Buthus martensii* (Karsch) (*B. martensii* (Karsch))	All	Drying after capture by boiling water	Rheumatic diseases	Decoction with water/ Infusion of wine for internal or external use	2023-DK-002
Lai-Guazi	Toad	*Bufo bufo* gargarizans Cantor (*B. bufo* gargarizans Cantor)/ *Bufo melanostictus* Schneider (*B. melanostictus* Schneider)	All	Kill after capture and shade dry	Cancer	Decoction with water	——
Zhu-Sha	Cinnabaris	HgS	——	Grinding powder	Mental illness	Decoction with water	2023-DK-020
Xin-Hong	Arsenolite	As_2O_3	——	Grinding powder	Skin diseases	External use	——
Shi-Gao	Gypsum Fibrosum	$CaSO_4{\cdot}2H_2O$	——	Grinding powder/ Cauterize thoroughly and then grind	Fverish/Skin diseases/Water and fire burns	Decoction in water for the treatment of fever/ Apply externally when treating skin diseases and water and fire burns	2023-DK-017
Li-Toutu	Plow soil	SiO_2/Al_2O_3	——	Grinding powder	Mental illness	Decoction with water	2023-DK-023
Tie-Xiu	Rust	Fe_2O_3	——	Grinding powder	Mental illness	Decoction with water	2023-DK-027
Zao-Xintu	Oven earth	$H_2SiO_3/Al_2O_3/Fe_2O_3$	——	Grinding powder	Gastrointestinal dseases	Decoction with water	2023-DK-008
Guo-Hui	Pot bottom ash	H_2SiO_3/Fe_2O_3/ CaO/MgO	——	Grinding powder	Gastrointestinal dseases/ Hemorrhage	Decoction with water/ Topical application	2023-DK-006
Kang-Jing	Tar	——	——	Grinding powder	Parasites/Skin diseases	Decoction with water/ Topical application	2023-DK-032
Huang-Tu	Loess	$H_2SiO_3/Al_2O_3/Fe_2O_3$	——	Grinding powder	Bleeding from trauma	Topical application	2023-DK-003
Da-Qingyan	Carnallite	NaCl	——	Grinding powder	Skin diseases/Mouth ulcers	External use/Dissolve into water for washing	2023-DK-007
Liu-Huang	Sulphur	S	——	Grinding powder	Skin diseases	External use	2023-DK-034

(Continued on following page)

TABLE 2 (*Continued*) Basic information of animal-mineral medicines.

Local name	English name	Source	Use part	Processing method	Ethnopharmacological employment/use	Method of application	Voucher numbers[4]
Yin-Zi	Silver	Ag	——	Forming tools	Scalp skin diseases	Comb your hair regularly with a silver comb	——

Notes: [1]Deficiency syndrome refers to the condition of weakness in the human body, usually caused by insufficient Qi and blood, weak Yang Qi, and other reasons. The manifestations of deficiency syndrome include fatigue, lack of strength, mental fatigue, poor appetite, and pale complexion; [2]Cough with deficiency-heat refers to the symptoms of cough accompanied by deficiency-heat. Deficiency-heat refers to excessive Yang Qi and insufficient Yin fluids in the body, leading to heat syndrome. The manifestations of cough with heat deficiency include dry cough, dry and painful throat, and dry mouth and tongue; [3]Postpartum or postillness weakness refers to the weakened state of the body in women after childbirth or long-term illness. This kind of weakness is usually caused by excessive postpartum bleeding, excessive physical exertion, or depletion of physical strength due to prolonged illness. The manifestations of postpartum or postillness weakness include fatigue, lack of energy, poor appetite, and weight loss. [4]Specimens such as Lang-Mao, Zuo-Tu and Tuan-Zhu are usually difficult to obtain, and specimens such as Ren-Nai, Gou-Xue and Yang-Nai cannot be preserved for a long time, so no specimens have been collected, and are uniformly represented by "-".

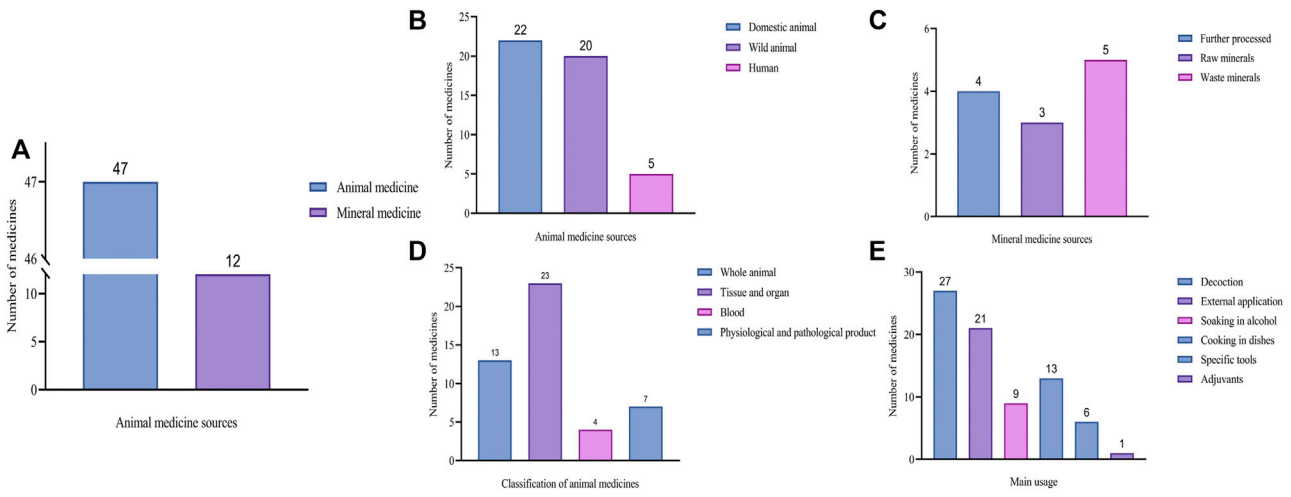

FIGURE 5
Source, classification and use of animal- and mineral-based medicines documented in the Gansu-Ningxia-Inner Mongolia intersection zone. **(A)** Sources of medicines. **(B)** Animal medicine sources. **(C)** Mineral medicine sources. **(D)** Classification of animal medicines. **(E)** The main usage of animal and mineral medicines.

Animal and mineral-based medicine resources

A total of 47 types of animal medicine and 12 types of mineral medicine used by local residents were identified in the surveyed area (Table 2). Among the 20 types of animal medicine derived from wild animals, the majority were sourced from small insects such as Xie-Diban, Tui-Louzi, Shi-Panniu, and *Scorpio*. The most precious among the wild animal medicines was *Moschus*, which was also a valuable traditional Chinese medicine material, but its original wild animal had basically become extinct in the area. The *Moschus* we investigated had been treasured by local residents for at least 50 years. Currently, the *Moschus* used in Chinese medicine and other ethnic groups is mostly synthetic or obtained through artificial breeding. In addition, Wu-Yazui, Lang-Mao, Tu-Mao, and He-Magezao were rarely used by other ethnic groups.

Of the twenty-two animal-based medicines sourced from domestic animals, the majority comprised organs and physiological or pathological products, categorized into organ, bone, and whip subtypes. Notably, Lü-Fen and Gou-Xue stood out among domestic animal-based medicines. Local residents applied heated Lü-Fen externally to treat dog bites, while Gou-Xue was employed to address mental illness, accompanied by an element of superstition (local residents believed Gou-Xue possessed exorcistic properties). Furthermore, human-derived medicines were extensively employed locally. Zuo-Tu was not reported for use in other regions or ethnic groups. While traditional Chinese medicine also incorporates Tou-Fa, it is processed into *Crinis carbonisatus* for application, diverging significantly from local utilization.

A smaller number of mineral-based medicines (12 types) were identified, with *Cinnabaris* and *Realgar* being the most commonly used, despite not being locally produced. These minerals were primarily used for treating mental and skin disorders (Figure 5). *Cinnabaris* and *Realgar* (also called Xin-Hong by local residents) were widely used in various exorcism charms.

Production and use of animal and mineral-based medicine

Local residents primarily rely on gathering animal medicines from insects and other small creatures found in the wild. These

creatures are typically captured and processed in abundance, such as the Tui-Louzi, *Mylabris*, *L. caraganae* Pallas, and *Scorpio*. Additionally, organ, bone, and horn medicines are obtained during animal slaughter or from raised animals, including the widely used digestive aid and stomach tonic, *Galli Gigerii* Endothelium Corneum (*G. Gigerii* Endothelium Corneum) extract. In terms of animal organ use, local residents' understanding is influenced by the traditional Chinese medicine concept of "supplementing form with form," particularly regarding the extensive use of animal sexual organs to address infertility.

These medicines are usually collected, ground into a powder, and then dried for preservation. Among these, *scorpios* are the only animal medicine purchased as a commercial product by locals. However, they are now under strict protection by the Chinese government, and capturing them is strictly prohibited. Despite this, this region boasts a history of being a renowned traditional source of *Scorpios*. Capturing and selling wild *Scorpios* used to be a vital source of income for local residents. Currently, however, wild Scorpio resources are extremely scarce, and attempts at breeding *Scorpios* have yielded unsatisfactory results.

Mineral-based medicines fall into two categories: imported and locally produced. Minerals such as *Cinnabaris*, *Sulfur*, Yin-Zi, and *Gypsum Fibrosum* are predominantly procured from "pharmacies" (these "pharmacies" are not the conventional ones that sell regulated and standardized medicines. Rather, they are clinics run by barefoot doctors in rural China, who are traditional healers who practice ethnic medicine. These "pharmacies" mainly sell the medicines that barefoot doctors collect or obtain from nature and prepare or process according to their own inheritance or experience.) and are usually kept in small reserves within households. Locally produced minerals such as Guo-Hui, Kang-Jing, *Loess*, and *Terra Flava* Usta are commonly integrated into daily life and are typically used immediately after collection. The history of using these medicines is challenging to trace back, as local residents consider it an instinctive habit. For instance, using *loess* to stop bleeding is perceived as a natural response. When injured and bleeding, *loess* is the most readily available and effective remedy found in the wild, and experience has shown that it possesses a significant hemostatic effect. Consequently, this practice is unanimously followed. Kang-Jing, distinct from this ethnic group, is employed for treating skin and parasitic diseases (including toothaches, which local residents attribute to parasites within the teeth). The underlying principle of its efficacy remains unclear. Furthermore, local residents primarily use mineral medicines individually and rarely combine several medicines.

Local residents use animal-based medicines to address diseases characterized by deficiency, cancer, and eye ailments. Nine primary medicines are employed to address deficiencies, embodying the characteristic of animal medicines as "products of flesh and blood with emotions." Locals firmly believe that most chronic conditions can be ameliorated or cured by treating deficiencies with tonics. For instance, they hold that Tai-Yanggao (the deceased fetus of a goat or sheep from childbirth) and human placenta uterina possess potent tonic effects and are frequently used for postpartum and postillness recuperation.

Another category of medicines, referred to as "whip medicine" (involving animal penis or testicles), is used to address male sexual dysfunction and infertility. This category encompasses seven distinct types and reflects the principle of "consuming what you lack." These animal parts, combined with specific plant medicines like *Epimedii Folium* and *Cynomorii Herba*, which are widely employed in treating male sexual dysfunction and infertility, are often stewed and ingested. Some middle-aged and elderly men opt to soak these medicines in wine to enhance or sustain their sexual prowess. The use of penis and testicles aligns with modern pharmacology's bioactivity, while the utilization of kidneys aligns with traditional Chinese medicine's theory that kidneys store essence and govern reproduction.

Mineral-based medicines are mainly used to treat two types of diseases: mental illness and skin diseases or injuries. For example, Li-Toutu or Tie-Xiu may have a magnetic effect on mental illness (a traditional local understanding and use of certain mineral medicines, which are believed to cure mental illness by magnetically attracting and removing pathogenic factors from the body), and *Loess*, *Sulfur*, and Yin-Zi are mainly used for their astringent, hemostatic, and bactericidal properties to treat skin diseases or injuries (Figure 6).

Evaluation of the importance of animal and mineral-based medicines used by local residents

An assessment of the significance of 47 traditional animal medicines and 12 mineral medicines was carried out utilizing the National Plant Cultural Significance Index (NCSI) as the evaluative metric. These 59 traditional medicines were categorized. The first tier of paramount importance (NCSI >100) encompasses 13 medicines, comprising 11 animal-based medicines and 2 mineral-based medicines. Notable representatives in this category encompass Feng-Mi, *Scorpio*, *G. Gigerii* Endothelium Corneum, *Loess*, *Placenta uterina*, and *Halitum*. These medicines are the most widely recognized and easily accessible among local residents.

The second important echelon (100 > NCSI ≥10) includes 21 medicines, with 19 being animal-based medicines and 2 being mineral-based medicines. Prominent medicines in this grouping include Zhu-Kudan, Tui-Louzi, Xie-Diban, and Shi-Panniu. These medicines are primarily sourced from commonplace insects and small creatures in the local environment, as well as domesticated animals that are comparatively easy to obtain.

The third significant tier (10 > NCSI ≥1) encompasses 19 animal and mineral medicines, of which 7 are mineral medicines. This category exhibits the highest prevalence of mineral medicines.

The fourth and least crucial tier (NCSI <1) comprises only 6 medicines. These medicines are infrequently utilized and currently not readily available, such as Moschus and Lang-Mao. This may also be correlated with the reliability of the information (Figure 7).

Among the 59 identified animal and mineral medicines, we did not encounter any relevant literature reports for 5 of them, including Zuo-Tu, Wu-Yazui, Lang-Mao, Li-Toutu, and Kang-Jing. This study may serve as inaugural documentation. While there are reports on some medicines, such as Tou-Fa, Tu-Mao, Tie-Xiu, and He-Magezao, they are seldom observed in clinical practice, and their modes of application differ. The local residents' utilization techniques and the ailments they address vary significantly from

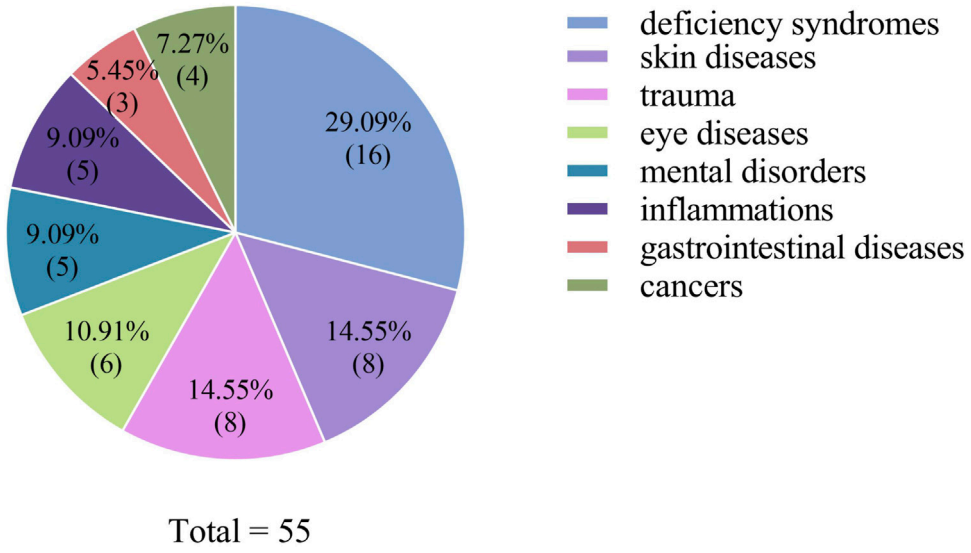

FIGURE 6
Diseases mainly treated by mineral medicines in the Gansu-Ningxia-Inner Mongolia intersection zone.

those of other ethnic groups. Moreover, among the 59 medicines, few share the same efficacies and indications as traditional Chinese medicine or other ethnic medicines. The local residents' application of these medicines is distinctly distinctive. For instance, they employ Feng-Mi to alleviate conditions such as oral ulcers and sores, use heated Lü-Fen for dog bites, and apply *sulfur* through burning for fumigation to address skin disorders, among other practices.

Evaluation of the SWOT of animal and mineral-based medicines used by local residents

We conducted a comprehensive SWOT analysis to assess the potential of traditional medicines and traditional medical science within modern healthcare, presenting the findings in a SWOT matrix (Figure 8). The strengths of ethnic medicine are noteworthy. It employs natural medicinal herbs as primary treatment modalities, capitalizing on abundant and cost-effective resources. Accessibility and user friendliness are further advantages, coupled with minimal associated side effects. Ethnic medicine embraces a holistic approach, systematically dissecting the underlying causes of ailments before tailoring targeted treatments. Additionally, ethnic medicine exhibits diverse pharmacological effects, rendering it efficacious in treating a wide spectrum of diseases. For example, Chinese medicine not only alleviates pain and inflammation (Wang et al., 2018) but also addresses common afflictions such as colds and indigestion (Song et al., 2023; Wu et al., 2023). Tibetan medicine enhances immunity (Pu et al., 2021) and effectively addresses specific conditions, such as altitude sickness (Liu et al., 2018).

However, ethnic medicine confronts several weaknesses and potential threats that impede its advancement and widespread adoption. Foremost among these challenges is the paucity of scientific validation regarding its pharmacological effects and safety profile, prompting certain nations to impose restrictive measures and regulations. Another critical challenge lies in the erosion or incompleteness of certain traditional knowledge associated with ethnic medicine, a consequence of the relentless progress in modern medical science and technology. This has rendered the preservation and transmission of this knowledge an arduous task. Moreover, some patients may favor modern medical technology over ethnic medicine due to its perceived convenience and higher perceived efficacy.

Despite these challenges, ethnic medicine is poised to capitalize on several opportunities for growth and expansion in the current landscape. Notably, there is a discernible surge in the popularity and influence of ethnic medicine, both domestically and on the global stage. More individuals are seeking natural and alternative ways to enhance their overall health and well-being, which also means that we need more professionals to inherit and pass on ethnic medicine, as well as more efforts to scientifically validate its effectiveness. Another significant opportunity lies in the integration of ethnic medicine with modern medical science, augmenting its applicability and overall efficacy. It is therefore our recommendation to seize these opportunities to advance the global promotion of ethnic medicine. Concurrently, addressing its inherent weaknesses and potential threats necessitates concerted efforts in scientific research, policy advocacy, cultural preservation, and widespread public education.

Modern pharmaceutical research

A comprehensive review of the chemical basis, biological activity, and applications of the 59 drugs employed in traditional Chinese medicine and other ethnic minority groups was conducted (Table 3). These medicines predominantly exhibit anti-inflammatory (Hossain et al., 2022), antioxidant (Tang et al., 2023), antibacterial (Zhao and Li, 2017), antitumor (Xia and Zhou, 2019), and analgesic effects (Gu et al., 2021), among others. Within the cohort of 59 traditional animal and mineral-based medicines utilized by local residents, no pertinent literature documentation was found for 5 medicines, including "Zuo-

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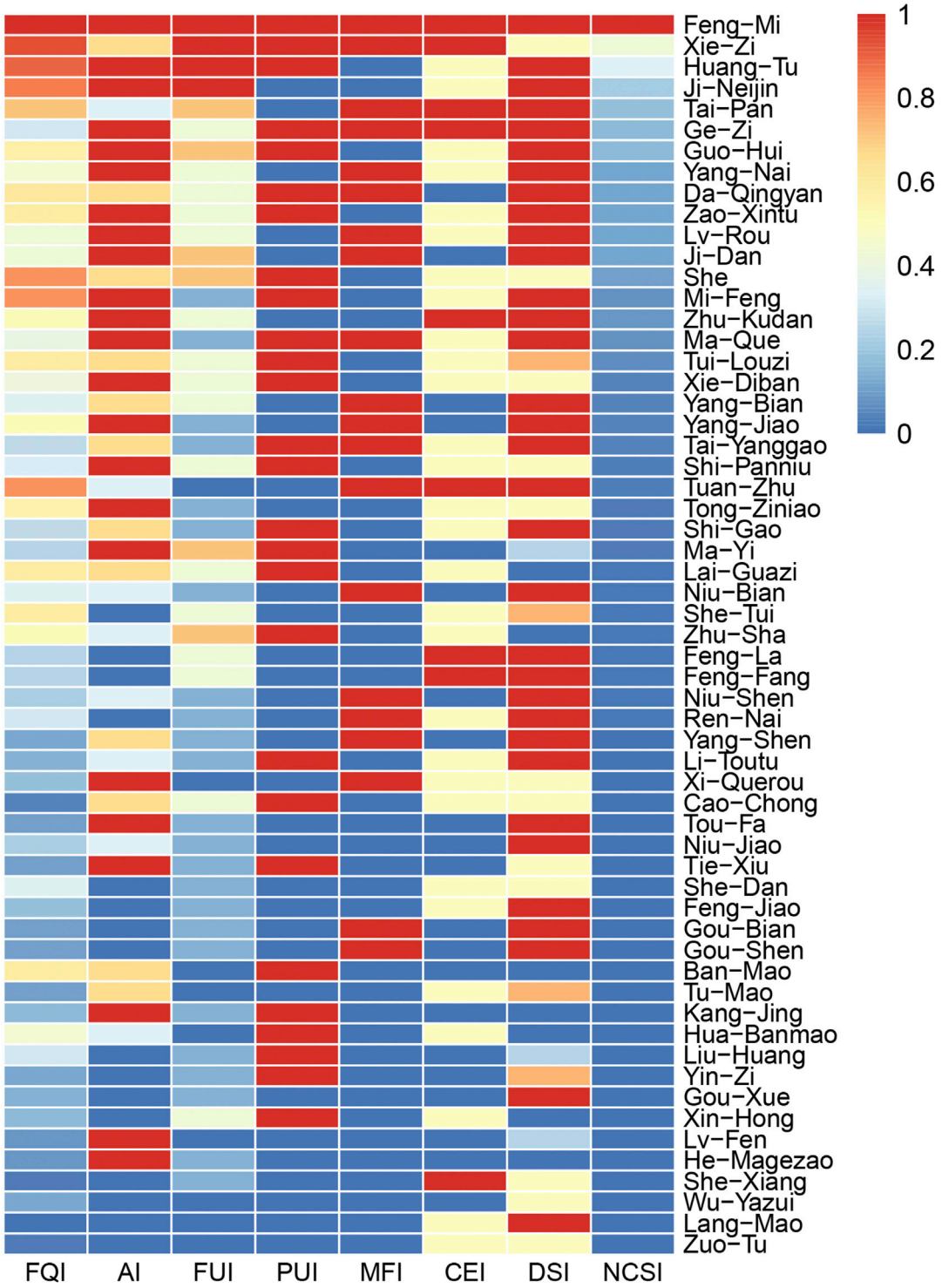

FIGURE 7
Quantitative evaluation of animal and mineral medicines in the study area. (FQI is the frequency of quotation index; AI is the availability index; FUI is the frequency of utilization index; PUI is the parts used index; MFI is the multifunctional use index; CEI is the curative effect index; DSI is the drug safety index; NCSI is the cultural food significance index).

Tu". Additionally, distinctions in application methods were identified between specific drugs and traditional ethnic medicines (including traditional Chinese medicine), such as *Moschus*. Although *Moschus* is a precious medicine employed in traditional Chinese medicine for both internal and external use, local residents predominantly use it for treating various unidentified swellings and lumps. Rather than ingesting *Moschus* directly, they store it in a glass jar containing several sewing needles, known as "yangzhen". To address blood stasis,

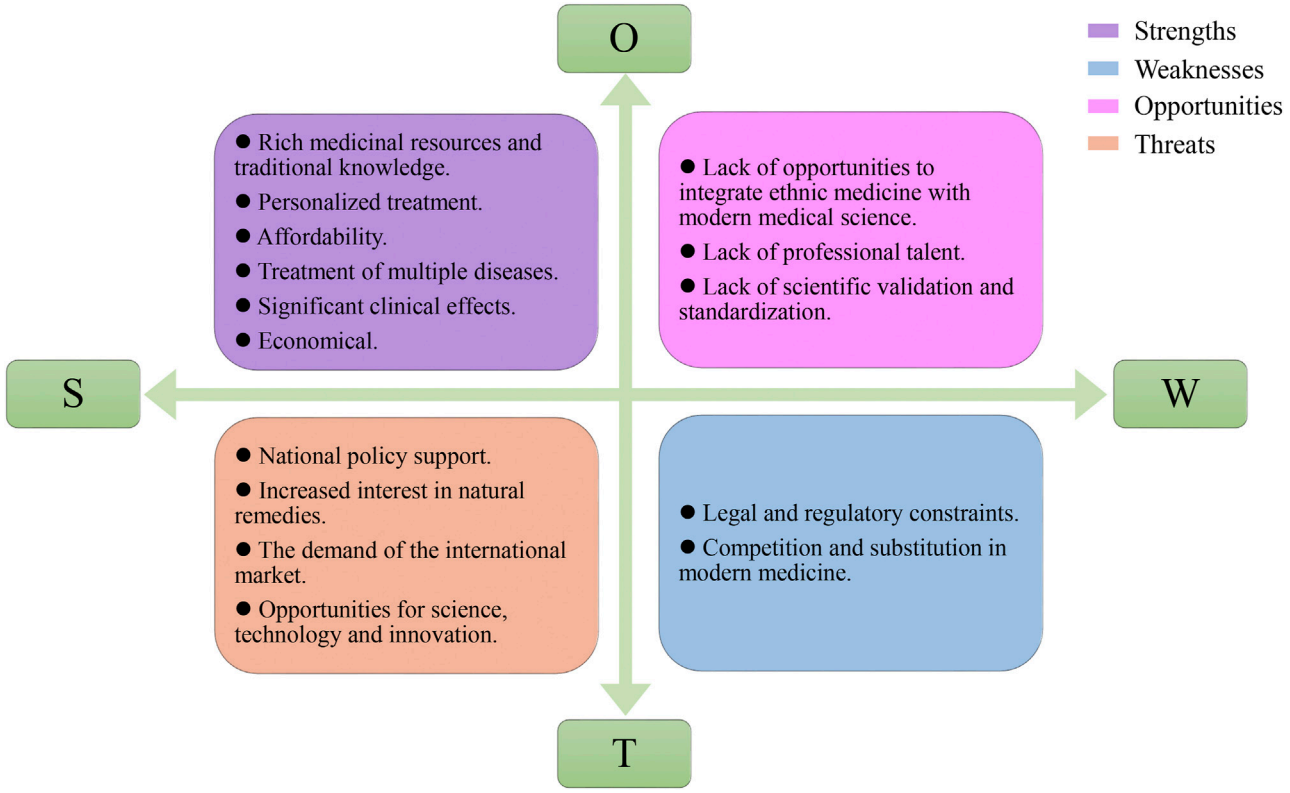

FIGURE 8
SWOT matrix for traditional medicines in the Gansu-Ningxia-Inner Mongolia intersection zone.

pain, and swelling, needles are employed to create circles around the affected area followed by multiple cross-cuts to stimulate surface bleeding. This approach mirrors the efficacy of traditional Chinese medicine in promoting blood circulation, dispelling blood stasis, and alleviating pain, as outlined in the Chinese Pharmacopoeia (Chinese Pharmacopoeia Commission, 2020).

Discussion

Animal and mineral-based medicines, while less commonly utilized than plant-based remedies, possess unique therapeutic properties. In the arid region where the Loess Plateau intersects with the desert in western China, local communities have demonstrated ingenuity in harnessing the medicinal potential of animal waste, emphasizing resourcefulness in an environment with limited resources.

The role of animal and mineral-based medicines in traditional medicine

Traditionally, animal-based medicines constitute approximately one-ninth of the total medicinal inventory, with mineral-based remedies even rarer, accounting for merely one percent of the pharmacopeia (Dong et al., 2023; Lu et al., 2023; Meng et al., 2023; Xu et al., 2023; Zheng et al., 2023). Nonetheless, our research conducted in the ethnically diverse crossroads of Gansu, Ningxia, and Inner Mongolia reveals a distinctive usage pattern. Local inhabitants employ both animal and plant medicines, with mineral medicines comprising approximately one-fourth of the combined total (Xie et al., 2023). This marked departure from traditional ratios underscores the widespread discovery and application of medicinal properties derived from animal resources by local communities. This shift can be attributed, in part, to the region's resource scarcity, as arid environments exhibit significantly lower species diversity and resource reserves in comparison to southern China, thereby constraining access to plant-based resources (Bai et al., 2011; Li, 2013).

Characteristics of medicinal animal and mineral resources

Our investigation uncovered a fascinating aspect of traditional medicine practiced by diverse ethnic groups across various regions (Bai, 1999). Beyond the distinct regional attributes of plant-based remedies, animal-based medicines also exhibit noteworthy regional characteristics. The animal-based medicinal resources employed by local inhabitants are primarily suited for arid climates rather than humid climates. For instance, in the Chinese martial arts literature, the "Five Poison Sect" employs various venomous insects, such as *Scorpios* and *Scolopendra*, cohabiting in the same container to evolve and yield the highly toxic "Gu" insect, aiding them in battle (Jin, 2010). Nevertheless, in reality, we observed that Scorpios are prevalent in the dry northern regions of China, while

TABLE 3 General information on animal and mineral-based medicines in the study area.

Local name	English name	Representative chemical components	Bioactivity	Representative drugs	References[1]
Zhu-Kudan	Pig's bitter gall	Hyocholic acid, Hyodeoxycholic acid, Bile acid, Bilirubin	Antibacterial, Anti-inflammatory, Analgesic	Suis fellis Pulvis, Pig bile Paste	Jiang and Zhu (2019)
Feng-Mi	Honey	Caffeic acid, Quercetin, Bee antimicrobial peptides	Antioxidant, Anti-inflammatory, Antibacterial	Anti-cough and Asthma Oral Liquid, Feng-Mi Mouthwash	Hossain et al. (2022)
Feng-La	Beeswax	Higher fatty acid, Carbohydrate, Esters, Dotriacontanol, Triacontanol	Hypolipidemic, Antioxidant, Anti-thrombotic	Propolis chromium soft Capsules	Theodoraki et al. (2017)
Feng-Fang	Nidus vespae	Amino acid, Peptides, Organic acid, Phenols, Volatile oil	Anti-inflammatory, Analgesic, Antibacterial, Anticancer activity	Nidus Vespae Suppository	Guo et al. (2023)
Feng-Jiao	Propolis	Flavonoids, Phenolic acid, Terpene, Carbohydrate, Amino acid	Anti-inflammatory, Antibacterial, Antiviral	Propolis chromium soft Capsules	Peixoto et al. (2022)
Mi-Feng	Bee	Phospholipase A2, Hyaluronidase, Melittin, Apamin	Anti-inflammatory, Antibacterial, Anti-aging	Bee Venom Plastics	El-Seedi et al. (2020)
Hua-Banmao	Mylabris	Cantharidin, Cyclo-(L-proline-L-alanine), Cyclo-(R-proline-R-leucine)	Antitumor	Compound Banmao Capsules	Xia and Zhou (2019)
Xi-Querou	Magpie's Meat	Protein, Carbohydrates, Inorganic salts, Vitamins, Fat	Improving the body's immunity, Diuresis, Lowering body temperature	Muxiang Shunqi Pills, Niuhuang Shangqing Pills	Peng and Xia (2018)
Wu-Yazui	Crow's beak	——	——	Wujin San	——
Tu-Mao	Rabbit hair	Cellulose, Lignin	Anti-pathogenic microorganism, Anti-inflammatory, Hemostatic	——	——
Xie-Diban	Pillbug	Sterols, Alkaloids compounds, Organic acids, Lipid compounds	Antitumor, Anticoagulation, Analgesic, Anti-inflammatory	Shufu Tablets	Duan et al. (2017)
She-Dan	Snake gall	Bile acid, Bilirubin, Cholesterol	Antitussive, Expectorant, Antitussive effects	Shedan Chenpi Tablets, Niuhuang Shedan Chuanbei Tablets	Chen et al. (2019)
She-Tui	Snake skin cast off during molting	Ossein, Amino acid, Fatty acids, Sterols	Antibacterial, Anti-inflammatory	Snake-shed ash combined with methotrexate Tablets	Zhao and Li (2017)
She	Snake	Cholesterol, Taurine, Oleic acid, Linoleic acid, Arachidonic acid	Anti-inflammatory, Anti-thrombotic, Anti-Cancer	Anticoagulant agents, Zhenzhu Qishe Oral Liquid	Zhang et al. (2021)
Tai-Yanggao	Fetal lamb	Protein, Amino acid, Trace elements, Phospholipids, Lipopolysaccharide, Vitamins	Enhancement of immune function, Anti-aging	Quanyang Pills	Yang (2018)
Yang-Nai	Goat's colostrum	Lactose, Triglycerides, Whey protein	Antioxidant, Immune regulation, Intestinal flora regulation	Goat milk Powder	Zhao et al. (2019)
Yang-Bian	Penis and testis of a goat	Progesterone, Estradiol, Testosterone Propionate	Enhancing sexual function, Strengthens the muscles and bones	Butianling Tablets, Sanbian Capsules	Hu (2000)
Yang-Shen	Goat's renal	Protein, Carbohydrates	Enhancing kidney function, Supplementation, Diuresis	White Lamb Kidney Soup, Acorus calamus Pills	Chen et al. (2023)
Yang-Jiao	Goat's horn	Protein, Peptides, Complex Amino acid, Lipids	Sedation, Hypnosis, Anti-convulsive, Antipyretic	Compound claw Tablets, Compound claw Granules	Zhu et al. (2023)
Tai-Pan	Placenta	Hyaluronidase, Fibrinogen stability factor	Hemostatic, Antiplatelet aggregation, Anti-aging	Placental tissue Injection, Placenta Tablets	Tong et al. (2022)
Zuo-Tu	Loess	——	——	——	——
Ren-Nai	Breast milk	Lecithin, Oxalic acid, Cystine, Tryptophan	Promote hematopoietic function, Relieves dry skin mucous membranes	——	Cimmino et al. (2023)
Tou-Fa	Hair	Calcium ion, Iron ions	Hemostatic, Coagulation	Cuisheng Powder, Huiyan Paste	Liu et al. (2020)
Tong-Ziniao	Children's urine	Urea, Sodium chloride, Potassium, Uric acid, Creatinine	Heat clearing, Sedation	White Plaster, E-ai Pills	Xu (2015)
Ban-Mao	Cantharides	Canthaxanthin, Cantharidin, Chitin	Antibody activity, Anti-cervical cancer	Boyun Ointment	Wang (2019)

(Continued on following page)

TABLE 3 (*Continued*) General information on animal and mineral-based medicines in the study area.

Local name	English name	Representative chemical components	Bioactivity	Representative drugs	References[1]
Shi-Panniu	Dung beetle	Molossusamide A (1), Molossusamide B, Molossusamide C, Dung beetle toxin	Anti-inflammatory, Antitumor, Anticoagulation	Huoxue Xiaoying Tablets	Zhang et al. (2019)
Cao-Chong	Paramecium caudatum	Palmitic acid, Decanoic acid, Cholesterol, Antibacterial peptide	Antitumor, Antibacterial	Grub Eye Drops	Zhang (2021)
Tuan-Zhu	Honey badger	Glyceryl Ester Compounds, Fatty acids	Anti-inflammatory, Antibacterial, Anti-pathogenic microorganism, Promotes skin regeneration	*Meles meles* oil Applicator	Cao (2013)
Ma-Que	Sparrows	Protein, Fat, Inorganic salts	Enhancing immunity function	Maque nourishing Pellets	Wang (2023)
Lü-Fen	Feces of donkey	Protein, Fatty acids, Organic acid, Cellulose, Hemicellulose, Lignin	Diuresis, Sedation, Anti-convulsive, Anti-anxiety, Anticoagulation, Improve blood circulation, Inhibition of gastrointestinal smooth muscle	Erhui Powder, Heilong Pills	——
Lü-Rou	Flesh of donkey	Amino acid, Hexanal, Unsaturated fatty acid	Antioxidant	Sanshen Pill, Colla corii Asini	Wang and He (2021)
She-Xiang	Moschus	Polypeptide from Moschus SXP4, Muscarinone, Cholest-5-en-3β-ol, 5α-androstane-3, 17-dione	Anti-inflammatory, Antibacterial	Shexiang Baoxin Pills	Lv et al. (2022)
Lang-Mao	Wolf hair	——	——	——	——
He-Magezao	Tadpoles	17a, 20a- dihydroxy -4-pregnen-3-one	Anti-Cancer	Guanyin Dew, Tadpole Detoxification Powder	Yang et al. (2003)
Tui-Louzi	Antlion	Long-chain fatty acids, Fatty acid esters, Peptides, Flavonoids, Alkaloids	Anti-thrombotic, Anticoagulation, Analgesic, Anti-inflammatory	Jinshaniu Huashi Tablets	Gu et al. (2021)
Ji-Neijin	Membranes of chicken gizzards	Gastrin, Galli Gigerii Endothelium Corneum polysaccharides, Tyrosine	Promoting the gastrointestinal peristalsis function, Antilithic	Child compound Endothelium Corneum, Galli Gigerii Endothelium Corneum decoction Pieces	Wu et al. (2020)
Ji-Dan	Eggs	Carotenoids, Active peptide	Antioxidant, Bactericidal, Hypoglycemic	Eucommia herbal Eggs, Herbal Eggs	Xue (2010)
Niu-Bian	Bull's penis	Cholesterol, Testosterol, Dihydrotestosterone, Estradiol	Enhancing sexual function, Relieve fatigue	Bullwhip Cream	Xu et al. (2019)
Niu-Shen	Ox kidney	Aldosterone, Adrenalone, Desoxycorticosterone	Enhancing sexual function, Relieve fatigue	Shenrongsanshen Capsule, Jiuwei Shenrong Capsules	Hu and Hao (1989)
Niu-Jiao	Ox horn	Horn Fiber, Sterols, Guanidine	Analgesic, Anti-inflammatory, Anti-infection	Niujiao Dihuang Decoction	Tang et al. (2023)
Gou-Bian	Dog's penis	Androgen, Protein, Fat	Enhancing sexual function	Sanbian Wenyang Capsules	Zhao et al. (2016)
Gou-Shen	Dog kidney	Androgen, Protein, Fat	Enhancing sexual function	Haima qiangshen Pills	Chen et al. (2023)
Gou-Xue	Dog's blood	Protein, Water	Immune Enhancement, Blood enrichment, Sedation, Anti-arrhythmia, Antibacterial, Anti-inflammatory	Yangqi Shengling Dan	——
Ge-Zi	Pigeons	Protein, Lecithin, Calcium	Enhancing immunity function	Compound Pigeon Egg Yigan Huayu Pills	Science (2019)
Ma-Yi	Ant	Acetyldopamine, Trolline, Oleic acid	Anti-inflammatory, Analgesic, Anti-aging, Regulate blood sugar	Compound black ant capsule, Tianyi Astragalus Granules	Ma (2015)
Xie-Zi	Scorpions	Scorpio toxins, Polypeptide, Taurine	Antibacterial	Zaizao Pills, Da-Huoluodan	Xu (2002)
Lai-Guazi	Toad	Bufadienolides, Indole alkaloid, Bufogargarizanines, Steroids	Antitumor, Anti-inflammatory, Immune regulation	Toad Skin Tablets, Toad Cream	Liu et al. (2023a)
Zhu-Sha	Cinnabaris	Mercury Sulfide, Mercury carbonate, Mercury acetate	Sedation, Hypnosis, Antiviral, Antibacterial	Zhusha Anshen Pill, Angong Niuhuang Pills, Bawei Qinpi Pills	Liu et al. (2021)

(Continued on following page)

TABLE 3 (*Continued*) General information on animal and mineral-based medicines in the study area.

Local name	English name	Representative chemical components	Bioactivity	Representative drugs	References[1]
Xin-Hong	Arsenolite	Arsenic trioxide	Antitumor	Ferula Pills	Guo et al. (2021)
Shi-Gao	Gypsum Fibrosum	Calcium sulfate dihydrate ($CaSO_4·2H_2O$), Ulfide and other trace elements	Heat clearing, Sedation, Anti-inflammatory, Promotes wound healing, Anti-inflammatory, Hemostatic	Xiaoqinglong plus gypsum Decoction, Baihu Decoction	Shi et al. (2021)
Li-Toutu	Plow soil	——	——	——	——
Tie-Xiu	Rust	Iron oxide (Fe_2O_3)	Anti-inflammatory, Antibacterial, Antiviral, Sedation	Tiexiu Liuhuang Powder	Tan (2003)
Zao-Xintu	Oven earth	Silicic acid (H_2SiO_3), Aluminum oxide (Al_2O_3), Iron oxide (Fe_2O_3)	Sedation, Anti-emesis, Anti-caries	Fulonggan Pills, Huangtu Decoction	Wang et al. (2021)
Guo-Hui	Pot bottom ash	Calcium oxide (CaO), Magnesium oxide (MgO), Silicic acid (H_2SiO_3), Iron oxide (Fe_2O_3)	Antibacterial, Anti-inflammatory	Baicaoshuang hemorrhoid Ointment	Zhang et al. (2022a)
Kang-Jing	Tar	——	——	——	——
Huang-Tu	Loess	Silicic acid (H_2SiO_3), Aluminum oxide (Al_2O_3), Iron oxide (Fe_2O_3)	Analgesic	Huangtu San, Huangtu Pills	——
Da-Qingyan	Carnallite	Sodium chloride (NaCl)	Antibacterial, Anti-inflammatory	Dahuang Huayu Pills, Qinglin Pills	Zhou and Xu (2016)
Liu-Huang	Sulphur	Sulfur S)	Antibacterial, Stimulation of the intestinal wall, Relieving diarrhea	Sulfur Ointment	Tan (2003)
Yin-Zi	Silver	Silver (Ag)	Antibacterial	Yinxie Capsules, Anshen Dingzhi Pills	Gao et al. (2005)

Notes: [1]Wu-Yazui, Tu-Mao, Zuo-Tu, etc. are mostly used as medicines in local remedies, which have been preserved through oral transmission from generation to generation. And some of them are also widely used in traditional practices and are believed to have certain pharmacological effects. However, the efficacy of these medicines is only based on traditional knowledge and experience, and these views lack the support of scientific research, which is needed to verify their pharmacological activities. Therefore, they are denoted by "-" in Table 3.

Scolopendra thrive in the humid south, making interactions unlikely due to their disparate habitats. Another Chinese folklore, "The Donkey from Guizhou," (Liu, 2021) similarly underscores the regional characteristics of animal-based medicinal resources. This narrative suggests that thousands of years ago, there were no donkeys in the southwestern province of Guizhou, and even today, none were found during our investigation. However, the tiger, another key figure in the tale, once held traditional and esteemed medicinal value for ethnic minorities in Guizhou.

Characteristics of traditional medicine culture of local residents

The utilization of animal and mineral-based medicines by local residents is widespread, and local remedies exhibit both diversity and distinctiveness. The amalgamation of various ethnic cultures has given rise to a distinctive medical culture in the region. However, the transmission of traditional medical knowledge primarily occurs orally and through apprenticeship, emphasizing a blend of practical application and experiential learning.

Broadly, the traditional medicine culture in the region is rooted in traditional Chinese medicine, enriched by an overlay of mystical shamanic practices. Often, treatments integrate both medicines and talismans, particularly in cases of mental health disorders. For instance, employing *Cinnabaris* in talismanic drawings has shown a discernible therapeutic effect in the treatment of mental illnesses. Additionally, there are simpler treatments, such as acupuncture, gua sha, and massage (Samuels et al., 2008; Feng and Dong, 2020).

Conservation of animal resources through traditional practices by local residents

Of the 59 varieties of animal-based medicine employed by local residents, *Moschus* holds the highest regard. Residents can only provide small quantities of samples, carefully preserved for over half a century. Regrettably, local *Moschus moschiferus* Linnaeus (*M. moschiferus* L.), known as "Xiangzhang" in the area, has become extinct. Another prized animal medicine, Scorpio, has faced rampant exploitation due to its soaring market value. In response, local authorities have implemented stringent regulations to safeguard this species (China Food and Drug Administration, 2006), leading to instances of legal action against residents involved in unauthorized capture. It is worth noting that wildlife conservation extends beyond only rare species in the region. In contrast, awareness of mineral medicine conservation among local residents remains limited. On the one hand, the variety of mineral medicines is limited, and valuable specimens such as *Cinnabaris* and *Realgar* are predominantly acquired from Chinese medicine stores. The majority of other mineral medicines are not considered scarce resources (Figure 9).

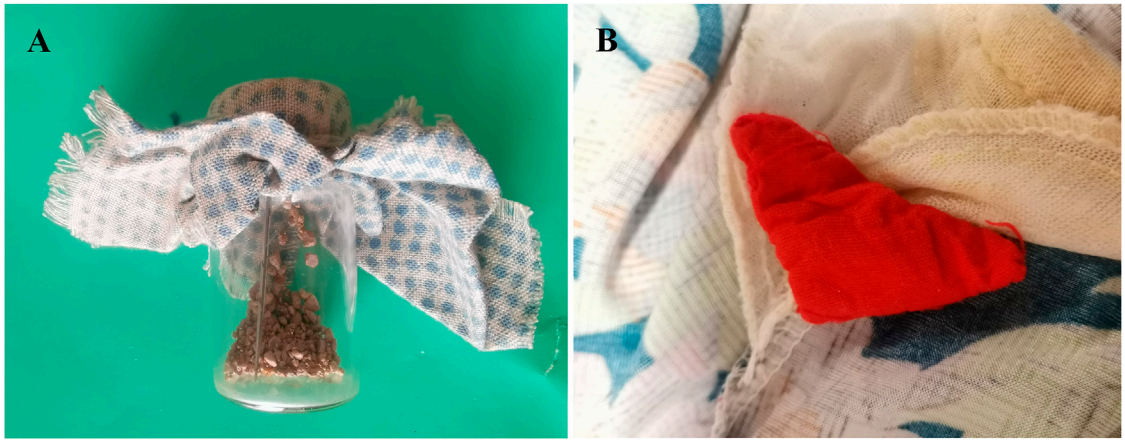

FIGURE 9
Talismans made from mineral medicine by local residents believed to have the effect of exorcism but were actually used for calming and tranquilizing. **(A)** *Moschus* stored in a glass bottle and placed beside the pillow when sleeping. **(B)** *Cinnabaris, Realgar*, and other minerals sewn into cotton cloth and worn on the body.

Current status of local residents' application and inheritance of traditional knowledge of animal and mineral-based medicines

The application of traditional medicine among local residents carries a certain mystique. However, with the advancement of technology and traditional Chinese medicine, this mystique has gradually waned in market appeal (Kiernan, 1978; Xie and Li, 2022). Following the passing of the older generation of rural doctors with distinct shamanic characteristics, the utilization of traditional medicine has seen a sharp decline. This decline is particularly pronounced among young residents who lean toward trust in Chinese and modern medicine. They encounter difficulty in embracing the use of certain traditional remedies, such as Zuo-Tu and Tong-Ziniao. This dynamic, on the one hand, reflects the advancing civilization of local residents. On the other hand, it has also resulted in the erosion of significant longstanding cultural knowledge, including that of traditional medicine (Gao, 2007; Qin et al., 2011).

Suggestions for protecting and promoting local traditional ethnic medicine knowledge

To safeguard and promote traditional ethnic medicine knowledge, we propose the following measures. First, it is imperative to enhance the conservation of wild resources used in animal-mineral medicine while also nurturing the cultivation and development of medicinally valuable plants. Second, there is a pressing need to amplify the promotion and dissemination of traditional ethnic medicine knowledge. This effort is instrumental in fostering a broader understanding and recognition of its cultural significance. Third, we advocate for the reinforcement and continuation of efforts to cultivate talent in the field of traditional ethnic medicine. This ensures the effective transmission and evolution of this vital knowledge. Fourth, harnessing the strengths of modern medicine is paramount. This can be achieved through the integration of traditional ethnic medicine with modern practices, elevating the application and efficacy of animal-mineral medicine. Last, establishing a comprehensive management system for the protection and utilization of medicinal resources is of paramount importance. Such a system will oversee the collection, processing, and utilization of animal and mineral-based medicine, thereby ensuring the sustainable use of these resources.

Ethical and safety issues of animal-and mineral-based medicine in traditional Chinese medicine

In China, TCM has a long history and enjoys wide popularity and governmental support. China has established a comprehensive legal framework to foster the development and inheritance of TCM (Xue et al., 2023). Currently, TCM has been extensively applied and developed both within China and internationally. A large number of animal-based medicines are used in TCM, which inevitably raises animal ethics issues (Chen, 2020). Most of these medicines are derived from dead animals or from legally hunted animals rather than from live animals. These practices, although ethically controversial, do not involve the abuse or torture of live animals. To promote the sustainable practices of local precious animal medicinal resources and to raise the education and awareness of animal welfare, we communicated and exchanged views with the local government officials from the agricultural and forestry departments, as well as the township level, during our investigation process. We aimed to attract the attention of the local government to the animal medicinal resources through various channels, and to improve animal welfare and human health.

Another interesting and important topic is the toxicity issue of mineral medicines. TCM has developed a unique application of mineral medicines, especially using some toxic minerals, forming a distinctive knowledge system and safety procedures (Lu et al., 2017; Wang et al., 2023). For example, arsenic trioxide, a highly toxic mineral, is used for specific medical purposes. Extensive modern pharmacological research has confirmed its effectiveness and safety under certain conditions (Nithyananthan and Thirunavukkarasu, 2020; Guo et al., 2021). Of course, the use of these toxic mineral

medicines also requires strict adherence to the correct dosage, compatibility, preparation, administration and storage methods to avoid adverse reactions or poisoning events.

SWOT strategy formulation

Based on the results of the SWOT analysis, the corresponding strategies are proposed as follows: First, give full play to the strengths, strengthen the education and training of ethnic medicine, cultivate more experts and researchers of ethnic medicine, strengthen scientific research and clinical practice, and improve the scientific validity and credibility of ethnic medicine. Second, overcome the weaknesses, strengthen the scientific verification and standardization of ethnic medicine, establish a scientific evaluation and certification system, strengthen the protection and inheritance of traditional knowledge, improve the clinical application of ethnic medicine, strengthen the cultivation of talent, and improve the talent pool and quality of ethnic medicine. Third, seize opportunities to strengthen international cooperation and exchanges, promote the advantageous features of ethnic medicine, attract more international students and research institutes to participate, and carry out international exchanges and exhibitions to improve the international influence of ethnic medicine. Fourth, cope with threats and strengthen legal and regulatory protection of ethnic medicine to ensure its legal status and rights and interests, enhance public recognition and trust in ethnic medicine and strengthen publicity and education efforts.

Conclusion

In our investigation, we documented and compared the traditional knowledge and use of 47 types of animal medicines and 12 types of mineral medicines among different ethnic groups in the Gansu-Ningxia-Inner Mongolia region of China. Our findings reveal the rich and diverse aspects of the local traditional medicine culture, as well as the current status and challenges of the local animal and mineral resources. One of the major challenges is the use of toxic minerals in traditional medicine, which poses serious health and environmental risks. Therefore, we suggest that a critical appraisal of the safety and efficacy of these substances is urgently needed to ensure the ethical and sustainable use of traditional medicine. Our study provides valuable baseline data for the conservation and development of these traditional medicine cultures, which are part of the global heritage of human civilization. However, we acknowledge that our study is limited by the lack of comprehensive evidence on the clinical value and biological activity of these medicines. Therefore, we propose that future research should focus on the material foundation, pharmacological effects, clinical trials, and safety assessment of these medicines. TCM has become a promising industry in China and the world, thanks to its unique advantages and policy support, but it also faces some internal weaknesses and external threats. In order to integrate TCM into the health service industry, we recommend that more efforts should be made to enhance international cooperation and exchange, learn from international experience and advanced technology, promote the internationalization of ethnic medicine, establish and implement standards to improve the quality and safety of ethnic medicine, introduce modern technology and management methods to promote the modernization of ethnic medicine, and encourage innovative research and practice to promote the innovation of ethnic medicine.

Data availability statement

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

Ethics statement

The animal studies were approved by the Laboratory Animal Welfare and Ethics Committee of Zunyi Medical University. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study. The studies involving human participants were reviewed and approved by the Medical Ethics Committee of Zunyi Medical University. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

CL: Formal Analysis, Writing–original draft. WZ: Writing–original draft, Funding acquisition, Resources. SL: Writing–original draft, Formal Analysis. ML: Writing–original draft, Software, Visualization. TF: Resources, Writing–original draft. YZ: Writing–review and editing, Methodology. YR: Writing–review and editing, Methodology, Resources. FW: Data curation, Funding acquisition, Writing–review and editing. JX: Conceptualization, 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.

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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.2023.1295806/full#supplementary-material

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Complementary authentication of Chinese herbal products to treat endometriosis using DNA metabarcoding and HPTLC shows a high level of variability

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Traditional Chinese Medicine (TCM) is popular for the treatment of endometriosis, a complex gynecological disease that affects 10% of women globally. The growing market for TCMs has yielded a significant incentive for product adulteration, and although emerging technologies show promise to improve their quality control, many challenges remain. We tested the authenticity of two traditional Chinese herbal formulae used in women's healthcare for the treatment of endometriosis, known as *Gui Zhi Fu Ling Wan* (FL) and *Ge Xia Zhu Yu Tang* (GX). Dual-locus DNA metabarcoding analysis coupled with high-performance thin-layer chromatography (HPTLC) were used to authenticate 19 FL and six GX commercial herbal products, as well as three *ad hoc* prepared artificial mixtures. HPTLC was able to detect most of the expected ingredients via comparative component analysis. DNA metabarcoding was able to detect an unexpected species diversity in the products, including 38 unexpected taxa. Chromatography has a resolution for all species indirectly through the identification of marker compounds for the different species ingredients. Metabarcoding on the other hand yields an overview of species diversity in each sample, but interpretation of the results can be challenging. Detected species might not be present in quantities that matter, and without validated quantification, some detected species can be hard to interpret. Comparative analysis of the two analytical approaches also reveals that DNA for species might be absent or too fragmented to amplify as the relevant chemical marker compounds can be detected but no amplicons are assigned to the same species. Our study emphasizes that integrating DNA metabarcoding with phytochemical analysis brings valuable data for the comprehensive authentication of Traditional Chinese Medicines ensuring their quality and safe use.

KEYWORDS

chemical fingerprinting, DNA metabarcoding, endometriosis, pharmacovigilance, Traditional Chinese Medicine, women's healthcare

Introduction

Traditional Chinese Medicine (TCM) is a holistic medical system with a long history of use in healthcare, disease prevention, diagnosis, and treatment, and one of the most popular health resources throughout the world (World Health Organization, 2022). The use of TCMs has gained global prominence and is based on maintaining a balance of vital life force, called *Qi*, which is supposed to surge along meridians in the body and maintain a person's health. Key components of TCM include *inter alia*, acupuncture, herbal medicine, dietary therapy, movement, and concentration exercises (e.g., *Qi Gong*, *Tai Chi*). TCM has been practiced for over 3000 years and is continuously refined through treatment observations and clinical studies (Lu et al., 2012; Gu and Pei, 2017). As part of TCM practice, Chinese Herbal Medicine (CHM) is regarded as effective for various diseases and believed to cause minimal adverse reactions (Tsou et al., 2022). A recent large-scale randomized and placebo-controlled trial confirmed the applicability of specific CHM as a treatment for endometriosis-associated pain and related symptoms (Lin et al., 2022). This opens interesting alternative treatment options, as conventional treatments might not have desirable effectiveness and may have side effects (Ilhan et al., 2019; Meresman et al., 2021; Taylor et al., 2021; Barnard et al., 2023). Today, TCMs are readily available in shops, in TCM stores, sold as food supplements, and are broadly available through online marketing, and increasingly applied as self-care strategies and self-medication for primary disease patterns in women's healthcare (Chen et al., 2019; Barnard et al., 2023). The international market and unregulated trade make TCMs vulnerable to fidelity issues in various stages of the supply chain (Xu et al., 2019; Zhu et al., 2022). Concern has been paid to potential health risks and hazards of poor quality and adulterated herbal medicines associated with TCMs, but also to intrinsic toxicities or extrinsic harmful residues detected in a large number of TCMs (Chen et al., 2023; Coghlan et al., 2015; Coghlan et al., 2012; Liu et al., 2020; Liu et al., 2015; Sakurai, 2011; Xu et al., 2019; Zhu et al., 2021). Chinese herbal remedies are usually complex mixtures of multiple ingredients derived primarily from plants, as well as fungi, animals, and minerals, and the result of manifold processing steps (Guo et al., 2015; Liu, 2015; Engelhardt et al., 2018; Wu et al., 2018). This contributes to implication issues for the standardization of herbal products. Furthermore, juristic and marketing differences among countries contribute to poor regulation and subsequent difficulties in quality assurance of marketed herbal products (Low et al., 2017; Raclariu et al., 2018a; Heinrich et al., 2019; Alostad et al., 2020; Thakkar et al., 2020; Ichim and Booker, 2021; Raclariu-Manolică et al., 2023a).

There is an urgent need for the development of rapid and simple inspection procedures and authenticity testing of Chinese herbal materials (Zhu et al., 2022). DNA barcoding systems adopted by various national pharmacopeias, including the Chinese Pharmacopoeia, British Pharmacopoeia, and Japanese Pharmacopoeia (Chen et al., 2017), are applicable for the identification of raw botanical material, but their efficacy is limited in assessing highly processed polyherbal products (Leong et al., 2020). DNA metabarcoding has shown a high resolution to simultaneously confirm both the presence of target species and off-label ingredients that occur in commercial herbal products, such as CHM remedies (Coghlan et al., 2012; Raclariu et al., 2018b; Seethapathy et al., 2019; Anthoons et al., 2021; Zhu et al., 2022; Raclariu-;Manolică et al., 2023a). Multi-locus DNA barcoding has been used in many studies for the authentication of species in TCMs and other traditional medicines (Arulandhu et al., 2017; Arulandhu et al., 2019; Yao et al., 2022; Zhu et al., 2022). Chromatographic fingerprints are known to provide a high resolution for the detection of target compounds of known ingredients and are a well-applied, basic authentication tool for herbal remedies such as TCMs (Liang et al., 2010; Booker et al., 2016; Raclariu et al., 2017; Fitzgerald et al., 2019; Heinrich et al., 2022). Furthermore, fingerprinting techniques can allow the consideration of the complexity of herbal products by evaluating the whole chemical profile and extracting a common pattern to be used as a criterion for elaborating the individual formulation (Noviana et al., 2022).

In this study, we tested the authenticity of two complex TCM formulae used in the treatment of endometriosis, known as *Gui Zhi Fu Ling Wan* (FL) and *Ge Xia Zhu Yu Tang* (GX). FL is one of the most widely known TCM formulae originally composed by Zhang Zhongjing (150–2019 CE), and once published in the oldest clinical book, *Essentials from the Golden Cabinet*, dedicated to internal, external, gynecological and obstetrical diseases during the Eastern Han dynasty (Zhang et al., 2020). Its efficacy has been tested in clinical practice and it is used to treat conditions like endometriosis, uterine fibroids, and pelvic inflammation among others (Fang et al., 2012; Li et al., 2019a; 2019b; 2019c; Gao et al., 2020; Du et al., 2021). The formula includes five Chinese herbal ingredients: Cinnamomi Ramulus, Poria Cocos, Paeoniae Radix Rubra, Moutan Cortex, and Persicae Semen.

The second formula analyzed, GX, originates from the Wang Qing-ren's dynasty and was first published in 1830, *Correction of Errors among Physicians*. Today, it is also known as the Tangkuei & Corydalis Combination and is specifically used to "drive out stasis below the diaphragm/the Mansion of Blood" (Bensky et al., 2004; Scheid et al., 2009). This formula is more complex and includes 11 to 12 different TCM ingredients, with the 12th being uncommon in products on the European market: Angelica Sinensis Radix, Moutan Cortex, Paeoniae Radix Rubra, Persicae Semen, Linderae Radix, Corydalis Rhizoma, Chuanxiong Rhizoma, Radix et Rhizoma Glycyrrhizae/Glycyrrhiza Radix, Cyperi Rhizoma, Carthami Flos, Aurantii Fructus, and Trogopterori Faeces.

It is worth noting that the official names of TCM ingredients identify the drug through the plant's name and the part of the plant used, but the ingredient name does not often align with official taxonomy. One of the consequences is that, frequently, one ingredient, e.g., Paeonia Radix (translated as "Peony root"), might be associated with materials coming from more than one single species of *Paeonia*, and is traded as a product with a vernacular name only not matching one-to-one with a scientific plant name. The Chinese Pharmacopoeia reports the latest definitions of which species are accepted under the ingredient name with scientific taxonomy (Chinese Pharmacopoeia Committee, 2020). Yet, there are multiple alternate species, local variants, and possible substitutions that are not coherent with the ingredient monographs of the Chinese Pharmacopoeia, and which are cultivated for TCM purposes. These alternatives are mostly traded locally in China and not marketed internationally (Leon and Yu-Lin, 2017).

The aim of this study was to assess the complementarity of results from integrating dual-locus DNA metabarcoding analysis with high-performance thin-layer chromatography (HPTLC) for the authentication of multi-herbal products, like TCMs.

Materials and methods

Sample collection and preparation

Twenty-five samples (n = 19 FL and n = 6 GX) were collected in 2019 from commercial TCM distributors and online retailers in Europe, as well as from China Town, London (United Kingdom). Samples were sold as mixed powdered TCM herbs (n = 3), tablets (n = 14), capsules (n = 1), pills (n = 2), extract and crude powder (n = 1), and granules (n = 4). Three mixtures (AMs) were prepared *ad hoc*, in the laboratory, using raw materials purchased from a German pharmacy and a Chinese health shop in China Town, London. AMs were prepared from crude herbal materials and received the following identification codes: AM1_FL, AM2_FL, and AM_GX. All ingredients were previously studied for substitutions (Bensky et al., 2004; Leon and Yu-Lin, 2017; Scheid et al., 2015; 2009). Accepted binomial names were retrieved from an automated comparison of 36 databases (AnAge, 2023; AskNature, 2023; Biological Library et al., 2012; Collaborative Collection Managment Solutions, 2023; EOL, 2023; EEA, 2019; FADA database, 2023; GBIF Secretariat, 2023; GISD, 2023; iNaturalists, 2023; Index Fungorum, 2023; ION, 2023; IPNI- The International Plant Names Index, 2023; IRMNG, 2023; ITIS, 2023; IUCN, 2022; NCBI, 2023; Noé, 2011; NLBIF, 2023; NYBG.org, 2023; Open Tree of Life Reference Taxonomy, 2023; Papahanaumokuakea Marine National Monument, 2023; P; Mollel, 2011; Qian et al., 2021; Rapid Biological Inventories, 2023; Sequeira, 2015; Shao and Chung, 2022; Teisher and Stimmel, 2023; The Catalogue of Life, 2023; uBio NameBank, 2023; US Wildflower's Database of Wildflowers for Illinois, 2023; USDA NRCS PLANTS Database, 2023; VASCAN, 2023; Wikispecies, 2023; Wilson and Reeder, 2005; WORMS- World Register of Marine Species, 2023). The accepted scientific names of the plant species used as ingredients and names of other putative substitute ingredients were validated using the online platform World Flora Online (WFO) (See Supplementary Tables S1A, B). The TCM products were imported into Norway for scientific analyses under Norwegian Medicines Agency license ref. No 18/13493-2. An overview of the TCM products can be found in Supplementary Table S2.

The sample materials were ground and homogenized using an IKA Tube Mill 100 (IKA-Werke GmbH & Co. KG, Staufen, Germany). Artificial mixtures of FL and GX were prepared according to standard ingredient dosing (Scheid et al., 2015). For FL, we mixed 1 g of crude herbal material for each of the five ingredients from two different sample sets in which all single ingredients are derived from a single distributor of CHMs in Europe. This is equivalent to 10% of the standard prescription. Accordingly, we mixed 10% of the crude material of each plant ingredient for GX. For DNA metabarcoding we used three technical replicates for all three mixtures prepared *ad hoc* (AM1_FL: 1-3FL; AM2_FL: 23FL and 24FL and AM_GX: 7-9GX). (See Supplementary Table S3).

Library preparation and dual locus metabarcoding for TCM authentication

The DNA was extracted using the E.Z.N.A.® SP Plant DNA kit (SKU:D5511, Omega Biotek Inc., Norcross Georgia). The manufacturer's instructions were followed except for a larger quantity of starting material (up to 30 mg), an elongated lysis step, and larger volumes of buffer in all steps before DNA binding to HiBind column (e.g., 1.6 mL SP1 buffer at 65°C for 1 h) as well as a final elution volume of 100 µL per sample. Dual index fusion primers of the internal transcribed spacers nrITS1 and nrITS2, based on 18S-ITS1F and 58S-ITS1R (Omelchenko et al., 2019), and ITS2F and ITSp4 primers (Timpano et al., 2020), were used to create amplicon libraries. Expected amplicon sizes were 400 bp for nrITS1 and 450 bp for nrITS2. Polymerase chain reactions (PCR) were carried out in 25 µL reactions consisting of 5 µL of template DNA, 1X of AccuStart II PCR ToughMix (AccuStart, Quantabio, Massachusetts, United States), and 0.16 µM of each primer. The PCR cycling protocol consisted of initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 10 s, annealing at 52°C for 15 s for ITS1, and at 56°C for 15 s for ITS2, and elongation 72°C for 1 min followed by a final elongation step at 72°C for 2 min. PCRs were conducted using indexed primers as in (Raclariu-Manolică et al., 2023b) following the indexing strategy of (Fadrosh et al., 2014)). All amplicons were visually checked using gel electrophoresis and normalized based on values from the quantity tool in the Image Lab 6.0 software (Bio-Rad Laboratories, Inc., United States). The different genetic markers were kept separate during the normalization and pooling of amplicons. Each pool was cleaned and concentrated using Ampure XP (Beckman Coulter, Inc., United States), and size was selected using a Blue Pippin (Sage Science, Inc., United States) with a size selection window targeting the amplicon size of the respective markers. Then, the pools were visualized on a Fragment Analyzer (Agilent Technologies, Inc.) to verify amplicon length and sent for sequencing on an Illumina MiSeq at the Norwegian Sequencing Centre.

Bioinformatics analysis

Bioinformatic processes for the metabarcoding analysis were conducted as in Raclariu-Manolică et al. (2023b) using the annotated scripts provided in: https://otagomohio.github.io/workshops/eDNA_Metabarcoding. In brief, forward and reverse raw sequencing files obtained from MiSeq sequencing were merged using PEAR 0.9.3 (Zhang et al., 2014) and subsequently demultiplexed using the ngsfilter command from the OBITools software suite (Boyer et al., 2016). Obigrep command was used to choose fragment sizes of respective barcode regions. Quality filtering was conducted to remove sequences <100 bp and >420 bp for ITS1, <100 bp, and >450 bp for ITS2 primers by using the fastq_filter command from USEARCH algorithm (Edgar, 2010). Afterward, sequences were dereplicated using the fastx_uniques command from the USEARCH algorithm (Edgar, 2010). Sequences with less than 10 occurrences in the dataset were removed. By using the UNOISE algorithm (e.g., unoise3 command from USEARCH) (Edgar, 2016), the dataset was denoised and OTUs

(Operational Taxonomic Units) were determined. As a last step, the taxonomic assignment was conducted using the blastn command from the BLAST + application (Camacho et al., 2009). Strict filtering controls were conducted to delete any false positive detections for each sample. For each obtained OTU, we subtracted the highest number of reads that could be found in the corresponding OTU in any of all negative controls (extraction blanks and PCR controls). This standard approach was applied in all PCR replicates of the samples. We used this approach to make sure that potential contaminants or "tag-jumps" will not result in potential false positives. In each unique sample, only OTUs showing ≥ 5 reads were retained for further analysis. For the taxonomic assignment, only the top-scored species were selected as the target species (search tool: BLAST), and all corresponding species with relative abundance below 0.002 were excluded (Yao et al., 2022). OTUs were checked for species delineation with ASAP: assembling species by automatic partitioning (Puillandre et al., 2021). Hence, OTUs corresponding to the same unique species were pooled together in a unique species identifier, and the read numbers were pooled. Thus, overinflation of the observed species range could be avoided. The relative abundance of each plant species per sample was calculated and a complete overview of the detected species and the corresponding number of genetic sequences/reads is provided in Supplementary File S1. Analysis of the results is presented in figures made using the R packages ggplot and gtools.

High-performance thin-layer chromatography (HPTLC)

HPTLC marker compounds and other chemicals were obtained from Merck (Darmstadt, Germany) and Sigma-Aldrich (St. Louis, MA, United States), and botanical reference standards were obtained from ChemStrong Scientific Co., Ltd. China. Marker compounds and botanical reference standards were prepared according to Hong Kong Chinese Materia Medica guidelines (HK Chinese Materia Medica, 2021) unless stated differently. Each botanical reference sample was prepared individually. Each herbal ingredient in the formulae was tested using a plant-specific method (see Supplementary Methods S1). All formulas tested were prepared by dissolving 0.50 g in 10 mL of ethanol, followed by sonication for 20 min and filtration using Merck Millex PES syringe filters (0.22 µm). Each TLC plate (silica gel 60 F_{254} Merck KGaA, Darmstadt, Germany) was visualized under white light and UV 254 and 366 nm prior to the commencement of the analytical procedure. Standard solutions and the extracts obtained from the samples and herbal references were spotted in bands of 8.0 mm width, using a CAMAG Linomat 5 instrument (Camag, Muttenz, Switzerland). The bands were applied at a distance of 8.0 mm from the lower edge of the plate and 20 mm from the left edge. Each plate was developed using a tailored method (see Supplementary Methods S1), using CAMAG Automatic Developing Chamber (ADC 2). For derivatization, CAMAG derivatizer was used when the derivatizing reagent complied with CAMAG's guidelines, otherwise, manual spraying was employed. Following development and derivatization, plates were visualized under white light, UV 254 nm, and 366 nm using CAMAG's Visualizer (Muttenz, Switzerland). All data was acquired and processed using VisionCATS 2.1 software (Camag, Muttenz, Switzerland).

For chemical fingerprints, all ingredients are named with herbal drug names. Each sample was compared to the fingerprint of each single expected ingredient (the Pharmacopoeial botanical standard). To display the results, a Band Intensity Score (BIS) with a scale from zero to five was visually assigned for all ingredients of samples for formulae FL and GX. Each band in the fingerprints was given a score from zero to five based on the intensity perceived by the naked eye, compared to the standard, where zero is "not detectable", and five is the highest intensity. The combination of these scores for one ingredient, was summarized by assigning a Band Intensity Score (BIS) to each ingredient in every sample (see Figure 1 as an example). BISs with scales 1-5 refer to the quality of the ingredients' entire fingerprint with respect to the visibility and positions of bands for the pharmacopoeial reference marker compounds and botanical references in each chromatogram. See Supplementary Figures S1, S2 for ingredients in the FL and GX formula, respectively.

Pharmacopoeial reference standards used are paeoniflorin (expected both in Moutan Cortex and Paeoniae Radix Rubra), paeonol (expected only in Moutan Cortex), and botanical references for each Moutan Cortex and Paeoniae Radix Rubra. Red arrows and frames highlight the bands corresponding to pharmacopoeial reference standards for Moutan Cortex and Paeoniae Radix Rubra. Here, paeonol is visible for Moutan Cortex, while all the multi-ingredient samples do not show this band. Paeoniflorin, the other reference standard, is visible in all samples, but with different intensities. Blue arrows and frames highlight the band patterns of the botanical references, which are the basis for a more representative fingerprint ("holistic") and create, together with the reference standards, the fundament for the evaluation of BISs.

Results

According to their classical formulations, the formula Gui Zhi Fu Ling Wan (FL) incorporates five ingredients, while the formula Ge Xia Zhu Yu Tang (GX) contains 12 ingredients, of which one of animal origin. The five ingredients of FL correspond to six plant species and one fungal species, *Cinnamomum cassia* (L.) J. Presl, *Paeonia lactiflora Pall.*, *Paeonia suffruticosa Andrews*, *Paeonia veitchii* Lynch, *Prunus davidiana Franch*, *Prunus persica* (L.) Batsch and *Poria cocos* (Schw.) Wolf. The 12 ingredients of GX correspond to 15 plant species. The plant species are *Angelica sinensis* (Oliv.) Diels, *Carthamus tinctorius* L., *Citrus aurantium* L., *Corydalis yanhusuo* (Y.H.Chou & Chun C.Hsu) W.T.Wang ex Z.Y.Su & C.Y.Wu, *Cyperus rotundus L.*, *Glycyrrhiza glabra L.*, *Glycyrrhiza inflata Batalin*, *Glycyrrhiza uralensis Fisch.*, *Lindera aggregata* (Sims) Kosterm., *Ligusticum striatum DC.*, *Prunus davidiana Franch.*, *Prunus persica* (L.) Batsch, *Paeonia lactiflora Pall.*, *Paeonia suffruticosa* Andrews, *Paeonia veitchii* Lynch, Species for *Prunus* and *Paeonia* can each be sourced from two plant species, and species for *Glycyrrhiza* can be sourced from three plant species. For each formula, the specific order of herbs according to formulation techniques in TCM (Duan et al., 2018) is described in Supplementary Table S3.

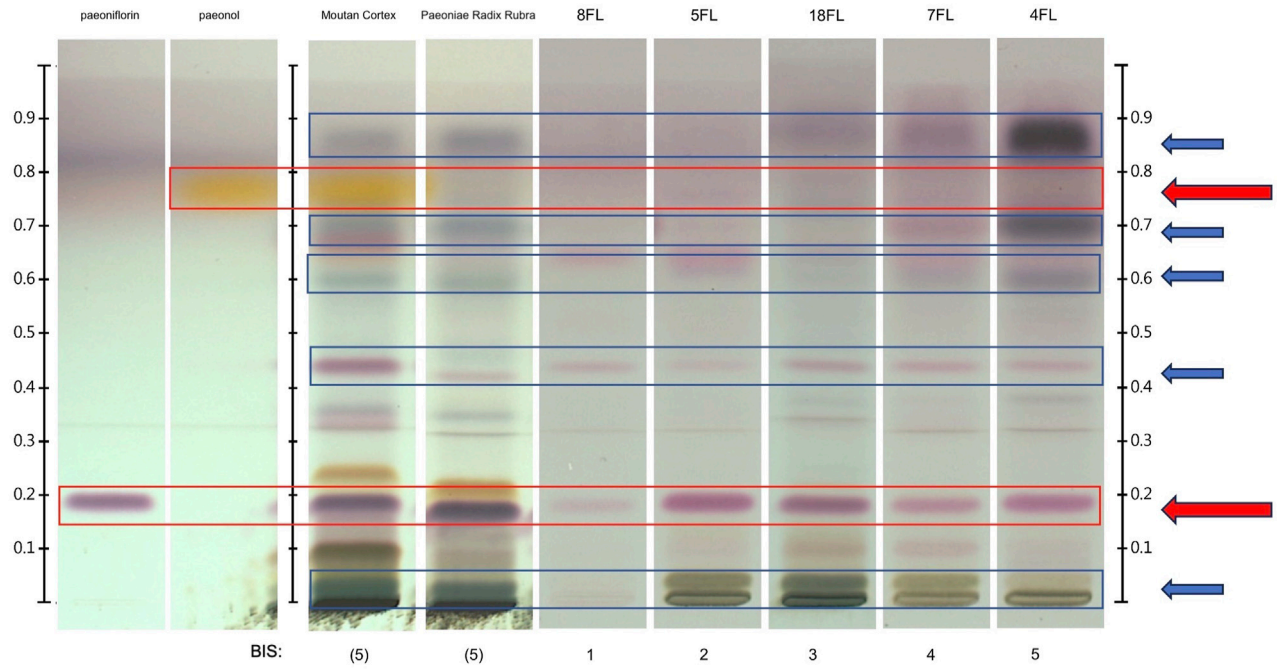

FIGURE 1
Example for the evaluation of Band Intensity Score (BIS) with scores 1-5 for formula FL with the ingredients of Moutan Cortex and Paeoniae Radix Rubra. Pharmacopoeial reference standards used are paeoniflorin (expected both in Moutan Cortex and Paeoniae Radix Rubra), paeonol (expected only in Moutan Cortex), and botanical references for each Moutan Cortex and Paeoniae Radix Rubra. Red arrows and frames highlight the bands corresponding to pharmacopoeial reference standards for Moutan Cortex and Paeoniae Radix Rubra. Here, paeonol is visible for Moutan Cortex, while all the multi-ingredient samples do not show this band. Paeoniflorin, the other reference standard, is visible in all samples, but with different intensities. Blue arrows and frames highlight the band patterns of the botanical references, which are the basis for a more representative fingerprint ("holistic") and create, together with the reference standards, the fundament for the evaluation of BISs.

In total, 39 possible substitutes were recorded as putative ingredients for the expected species (see Supplementary Table S1). For example, *A. sinensis* has seven unofficial substitutes. The foremost are *A. acutiloba* (Siebold & Zucc.) Kitag., which is also categorized as an alternate species or local variant (Scheid et al., 2015), and *Levisticum officinale W.D.J.Koch*. Lesser known substitutes, which are primarily traded locally in China, are *Angelica megaphylla* Diels, *Angelica gigas* Nakai, *Angelica polymorpha* Maxim., *Ligusticum glaucescens Franch.*, and *Hansenia forbesii* (H.Boissieu) Pimenov & Kljuykov (Bensky et al., 2004; Leon and Yu;Lin, 2017). Generally, Paeoniae Radix Rubra (Chi Shao) and Paeoniae Radix Alba (Bai Shao) are differentiated in the ingredient lists (Supplementary Table S2). In all classical texts, their properties are discussed under the single heading of Paeoniae Radix (Shao Yao), though it is recognized that the red and white inflorescences are very distinct in their therapeutic action (Scheid et al., 2015).

Sample 2GX includes *Nelumbo nucifera* Gaertn. (Lian Fang) in the ingredient list. For samples 3GX and 4GX, the resins of frankincense, *Boswellia carterii* Birdw. (Ru Xiang) and myrrh, *Cammiphora myrrha* Engl. or *Balsamodendrum ehrenbergianum* O. Berg (Mo Yao) are included in the ingredient list. Here, frankincense is used as an additional ingredient deviating from the classical formulae as it is common in the treatment of endometriosis (Cho et al., 2023). Sample 1GX and 5GX include the ingredient Trogopterori Faeces (Wu Ling Zhi), a product of animal origin. Other ingredients are listed for 4GX, such as activated carbon, botanical wax, and talcum, and for 8 FL fillers and binders of corn starch (non-GMO), dextrin, activated carbon, and gelatin (pork).

Dual loci metabarcoding for TCM authentication

A dataset consisting of 1 620 141 reads was obtained for nrITS1 with an average of 49 095 reads per sample. Respectively, a dataset with 1 647 249 reads was obtained for nrITS2 with an average of 49 917 reads per sample. Operational taxonomic units (OTUs) were obtained for all sample mixtures for markers nrITS1 and nrITS2. The raw dataset of nrITS1 contained 361 OTUs, and after applying strict quality selection criteria and pooling together OTUs assigned to similar species, 62 unique species were obtained. The raw dataset of nrITS2 contained 291 OTUs, whilst after applying the quality criteria 34 species were obtained. All sample preparations had OTUs that passed the bioinformatic

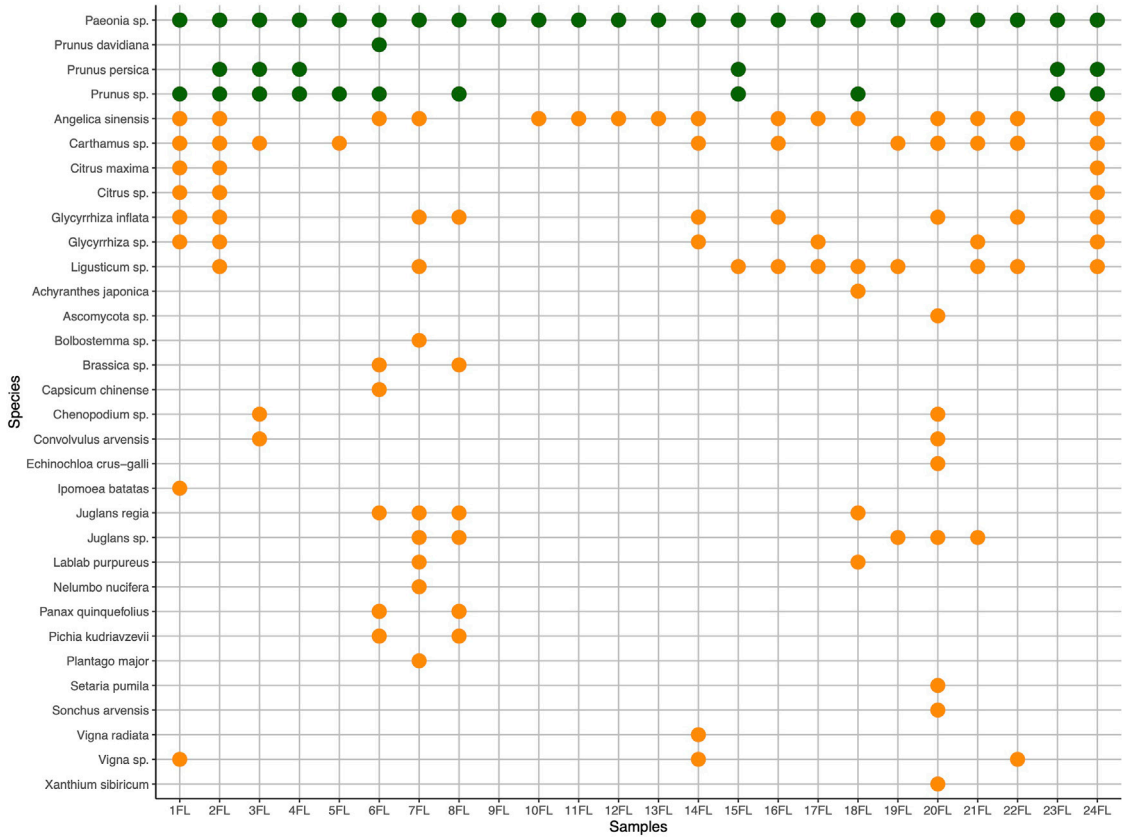

FIGURE 2
Expected (green) and unexpected (orange) species authenticated in products and mixtures of formula Gui Zhi Fu Ling Wan (FL) using DNA metabarcoding. Undetected expected ingredients Cinnamomi Ramulus (*Neolitsea cassia* (L.) Kosterm., syn. *Cinnamomum cassia* Presl.) and Poria Cocos (*Poria cocos* (Schw.) Wolf), have zero identification hits and are not reported in the graphics. *Ad hoc* mixtures (AM) include samples 1-3FL, 23FL, and 24FL.

trimming and filtering criteria and all samples could be included in the results. A complete overview of the detected species per sample mixture and the corresponding number of reads is provided in Supplementary File S1. After applying a threshold for relative species abundance greater than 0.002 to the identified taxa (Yao et al., 2022), a total of 30 ingredients were identified on the species level and 18 ingredients on the genus level for both primers, ITS1 and ITS2. A separate analysis for ITS1 resulted in a total of 24 plant ingredients on the species level and a total of 16 plant ingredients on the genus level with one extra ingredient belonging to the phylum of Ascomycota. A total of 12 plant ingredients were detected at the species level, and 6 ingredients at the genus level for nrITS2, except for one ingredient identified as green algae, *Micractinium* sp. The number of ingredients detected per sample ranged from 1 to 16. A total of 22.4% of the identified species were expected ingredients. Assessing the sources of all unexpected ingredients, we found that 39.5% were crops, such as mung bean and sweet potato, 21.1% were weeds, while another 5.3% may be weeds or crops such as clover or animal feed, 5% were types of grass, while 10.5% are made up of trees, green freshwater algae, fungi and ornamentals. Another 13.2% of ingredients are medicinal botanicals and find use in various traditional East Asian medicinal systems. Amongst those plant species that commonly find use in TCM, *Xanthium sibiricum* Patr. (Pinyin: Can Er) was detected with nrITS1 in two samples, 20FL (see Figure 2) and 2GX (See Figure 3) (Supplementary Table S2).

The results for the authentication of samples from each formula, FL and GX, and barcode ITS1 and ITS2, are discussed separately. For FL, analysis with barcode nrITS1 detected only three potential expected ingredients. *Prunus* species are present, and in most cases specifically associated with the detection of *Prunus persica* L. Batsch and *P. davidiana* Franch. (Pinyin: Tao ren) (both species are accepted as sources for Persicae Semen) (see Supplementary Figure S3A). The detection of *Paeonia* species could account for either or both Paeoniae Radix, which is equivalent to the scientific names *Paeonia lactiflora* Pall. or *P. ostii* T.Hing & J.X.Zhang (Pinyin: Shao yao), and Moutan Cortex, which is *P. suffruticosa* Andrews as both ingredients are sourced from *Paeonia* species, but consequently this results in the impossibility to distinguish between the two ingredients. The two ingredients, Cinnamomi Ramulus (Gui zhi), associated with *Cinnamomum cassia* (L.) J.Presl and other possible substitutes, and Poria cocos, sourced uniquely from *Poria cocos* (Schw.) Wolf, could not be detected across the entire sample range including the *ad hoc* preparations AM1 FL (1-3FL) and AM2 FL (23, 24FL). They are therefore not reported in Supplementary Figure S3A). On the other hand, AM1 FL (1-3FL) and 24FL seem to include plant species from formula GX. Furthermore, Angelica sinensis Radix (Dang gui), sourced uniquely from *Angelica sinensis,* is

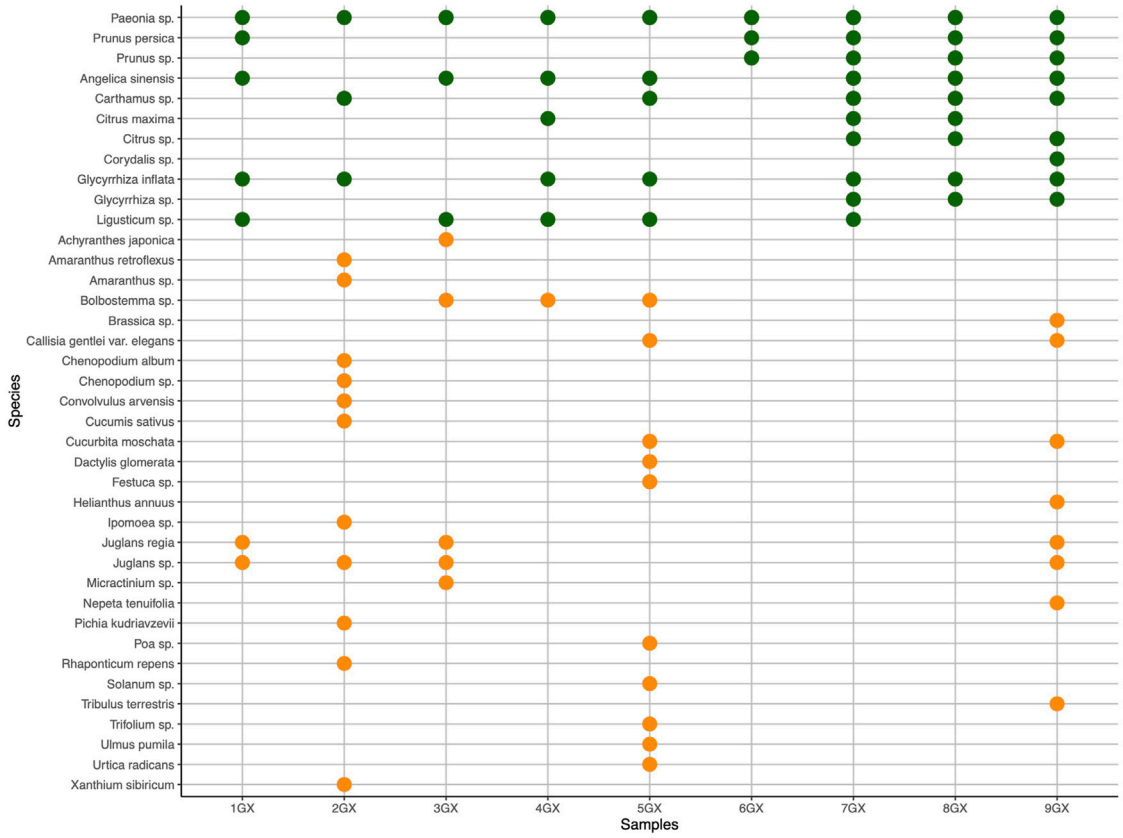

FIGURE 3
Expected (green) and unexpected (orange) species authenticated in products and mixtures of formula Ge Xia Zhu Yu Tang (GX) using DNA metabarcoding. Undetected expected ingredients: Linderae Radix (*Lindera aggregata* (Sims) Kosterm), Cyperi Rhizoma (*Cyperus rotundus* L.), with zero identification hits and are not reported in the graphics. *Ad hoc* mixtures (AM) include samples 7-9GX.

present in 15 samples. It is a prominent ingredient in herbal formulations for women's health. Two samples, 19 and 20 FL, did not show any expected ingredients, again these are tablets purchased online (See Supplementary Table S2).

For FL, analysis using ITS2 barcode managed to identify only two expected ingredients, namely ingredients of the genus *Prunus* and *Paeonia* (see Supplementary Figure S3B). The genus *Paeonia* is found across the whole sample range. AM1 FL (1, 2FL) and 24FL samples seem to include plant species belonging to formula GX, like *A. sinensis* and *Glycyrrhiza inflata* Batalin (Gan cao), which are prominent TCM herbs.

When using nrITS1 on GX samples, seven out of 11 expected ingredients were identified across the sample range (see Supplementary Figures S3C). Samples 1, 3, 4, and 6 GX have only three ingredient hits. Among the analyses of the *ad hoc* preparations, AM 9 GX differs from the other two samples for ingredient abundance. The only expected ingredient, which was possible to identify at the species level, was *A. sinensis*. Sample 5 GX shows several outliers and unexpected ingredients.

Using barcode nrITS2 for the GX formula, it was possible to identify six out of 11 expected ingredients across the sample range (see Supplementary Figures S3D); notably, the presence of *Ligusticum* species could not be detected, as seen with ITS1. The *ad hoc* preparations (7-9GX) show similar scores to each other for the expected ingredient's identity and abundance. The genus of *Juglans* and *Bolbostemma* are found abundantly across the sample range. *A. sinensis*, *G. inflata*, and *Citrus maxima*, the latter most likely a substitute species for *C. aurantium* (Zhi ke) were identified at the species level.

HPTLC

The band positions and visibility of the chemical markers of the single ingredients in the FL and GX formulas appear with characteristic colors and Rf values. All botanical reference materials show clear chromatograms and all marker compounds were identified (Supplementary Figures S1, S2).

With HPTLC, we obtained 130 positive hits for expected ingredients in formula Fl with 20 samples and GX with seven samples, including the AMs. Overall, a total of 70,7% of expected ingredients were identified across the sample range. Single ingredients refer to the accepted species under the ingredients as listed in the Chinese Pharmacopoeia (Chinese Pharmacopoeia Committee, 2020). Moutan Cortex could not be identified with certainty. In the FL samples, the band of paeonol, typical for Moutan Cortex, is not visible in any of the samples. On the other hand, the paeoniflorin band which is typical for both Paeoniae Radix Rubra

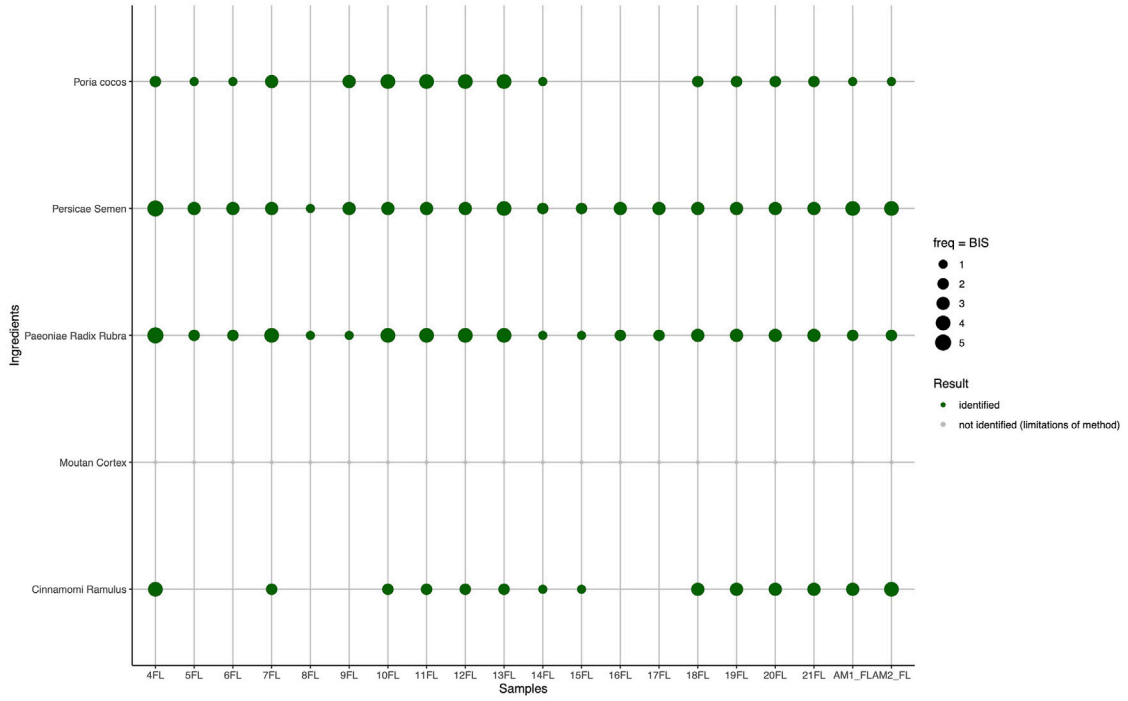

FIGURE 4
HPTLC detection of target ingredients in products and mixtures of formula Gui Zhi Fu Ling Wan (FL) based on BISs. The freq (frequency) of the dots represents the numerical score assigned (0 absent, 1 faintest, 5 most intense). The BIS is directly correlated to the identification success rate of the individual ingredients in the samples. Hence, the green dot size visualizes the scoring level of identification for target ingredients, as described in the method section. Moutan Cortex (gray) could not be determined due to the limitations of the method.

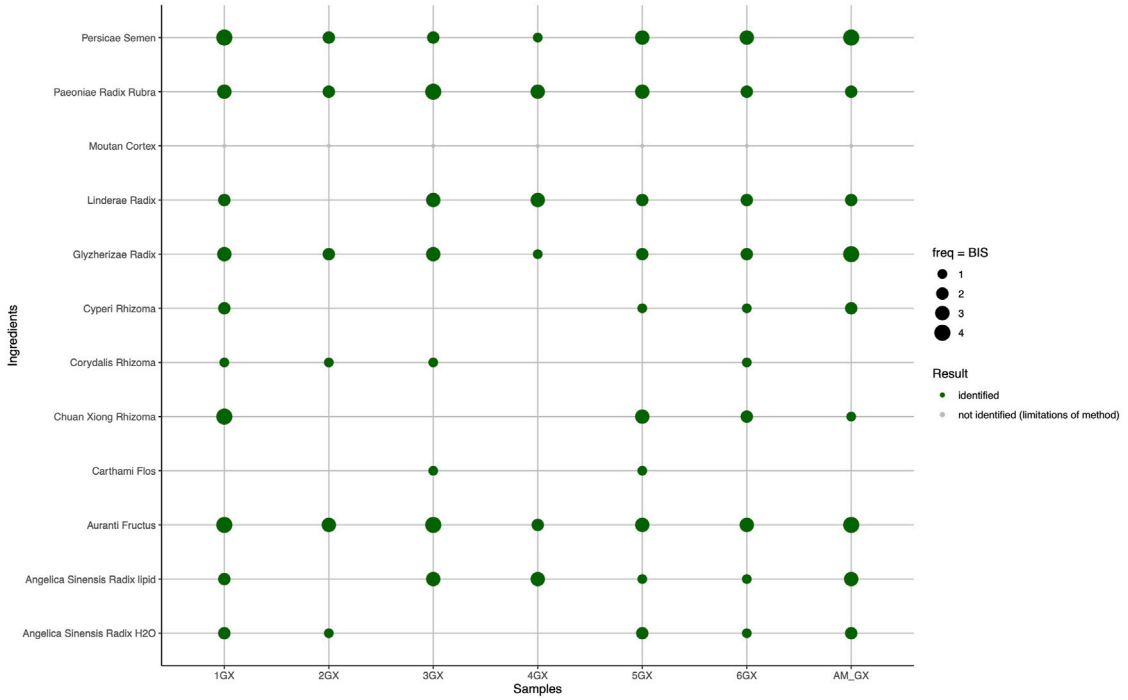

FIGURE 5
HPTLC detection of target ingredients in products and mixtures of formula Ge Xia Zhu Yu Tang (GX) based on BISs.

and Moutan Cortex can be detected in the majority of the samples. This result does not rule out the presence of either, but it means that the sample contains at least one of those two ingredients which is further explored in this discussion.

Among the FL formulae (Figure 4), Cinnamomi Ramulus is the least detected ingredient, showing only in 14 out of 20 samples, followed by Poria cocos, in 16 out of 20 samples. The rest of the sample fingerprints show the presence of all expected ingredients, while Moutan Cortex and Paeoniae Radix Rubra could not be unambiguously distinguished since the paeonol band of Moutan Cortex was not detectable.

In the GX formula samples (Figure 5), Corydalis Rhizoma and Carthami Flos in addition to Moutan Cortex were not identified in the *ad hoc* preparation AM GX, even though the ingredients mixed in these preparations, when analyzed individually, could be clearly identified (see Supplementary Figure S4.) Samples 2GX and 4GX were deemed to be the samples with the poorest ingredient composition, with three undetected ingredients, namely Cyperi Rhizoma, Chuan Xiong, and Carthami Flos. Carthami Flos went undetected in five of seven samples and was detected only with faint bands (BIS = 1) in the last two.

Discussion

Several studies have highlighted the potential of multi-locus DNA barcoding for the identification of TCM ingredients. The biggest advantage of DNA metabarcoding is to simultaneously identify numerous species in complex and processed herbal preparations (Zhu et al., 2022). Nonetheless, the limitations of DNA metabarcoding are framed by the quality, processing state, or product type of extracted material, the DNA purification procedure, choice of primers, markers, amplification protocols for library preparation, sequencing method, bioinformatic filtering and setting for qualitative and clustering thresholds (Raclariu-Manolică et al., 2023a). The accuracy of DNA metabarcoding further requires a comprehensive taxonomic reference library (Taberlet et al., 2007). Shortcomings of barcoding exist due to incomplete data in reference databases for barcodes (Zhu et al., 2022) and challenges with delimitating species with species delimitation models (Taylor and Harris, 2012; Howard et al., 2020; Puillandre et al., 2021). Chemical fingerprinting then complements approaches focused on biological characteristics, i.e., DNA, morphology, etc. Chemical analysis allows us to distinguish between the different parts of the plant used, e.g., the root as opposed to the leaves; this is a non-trivial point, as adulteration can also occur by means of using other (generally cheaper) parts of the plant that are not the ones required for the desired medicinal effect (Ichim and de Boer, 2020). Furthermore, chemical fingerprinting methods enable the assessment of a full qualitative profile, as well as the identification of low-quality aspects of the phytochemical contents, and the detection of significant product-to-product variation (Ichim and Booker, 2021). High-perfomance thin-layer chromatography (HPTLC) is the most advanced form of thin-layer chromatography (TLC) and a robust method for quality control of single,- and poly-herbal formulations (Velho;Pereira et al., 2011; Nicoletti et al., 2013; Booker et al., 2014; Booker et al., 2016; Nandanwadkar et al., 2016; Kandil et al., 2022) and pharmaceutical drugs (Shewiyo et al., 2012). It can be used for the analysis of multiple samples simultaneously and efficiently at a relatively low price (Kunle, 2012; Sonia, 2017; Kandil et al., 2022). Its advantages are improved resolution, detection sensitivity, and enhanced *in situ* densitometric quantification compared to ordinary TLC (Sonia, 2017).

The combination of the internal transcribed spacers regions, nrITS1, and nrITS2 provided the most comprehensive species identification for two short barcode markers as the most sequenced regions for molecular analysis of TCMs at lower taxa levels (Chen et al., 2017; Yao et al., 2022; Zhu et al., 2022). The results from nrITS1 and nrITS2 can be used in an integrative approach to receive an enhanced informative overview of the ingredient profile (Zhu et al., 2022) of samples from FL and GX. NrITS1 and nrITS2 differ in taxonomic identification at species and genus levels. Here, nrITS1 performed generally better in terms of the number of OTUs with taxonomic assignment. However, neither the nrITS1 nor nrITS2 data yielded OTUs for all expected genera in the herbal formulae FL and GX. For example, the presence of Cinnamomi Ramulus (Pinyin: Gui zhi) was confirmed with the HPTLC analysis, but *Neolitsea cassia* (L.) Kosterm (syn. *Cinnamomum cassia* (L.) J.Presl) could not be detected for any of the FL samples. This discrepancy is typical for the nature of TCMs as highly processed herbal remedies. *Pao Zhi*, the traditional processing technique, and other processing methods in TCM are likely to affect the DNA quality through measures like cutting, crushing, roasting, baking, stir-frying, and the application of liquid or solid excipients (Engelhardt et al., 2018; Wu et al., 2018). Furthermore, a methodological challenge for DNA-based identifications in TCMs is that plant compounds including polysaccharides, polyphenols, lipids, essential oils, and alkaloids, can interfere with DNA extraction and PCR amplification. Interference from those compounds or initial processing techniques of source material can yield false-negative results. As such, dominant ingredients with relatively intact DNA compared to other ingredients may yield amplification biases and in turn skew the sequencing results towards those ingredients. For DNA extraction from TCMs and complex, poly-herbal samples it is recommended to employ tailored extraction protocols (Corrado, 2016; Lo and Shaw, 2019). Looking at the *ad hoc* mixtures for FL, where input material for each ingredient was previously thoroughly mixed in equal proportions, we may reveal a common issue of homogeneity for the mixing of different powdered herbal material for consecutive DNA extraction from smaller amounts of the total mix, where some plant ingredients might be overrepresented. Respectively, homogeneity might be an issue in the manufacturing processes of Chinese herbal products, where grand amounts of mixed herbal materials are compressed in small amounts into tablets or capsules. This may have furthermore led to potential false-negative detection of species present at low abundance in the samples. We may also observe false-positive reads from micro contaminations of other species which are common in non-sterile herbal manufacturing processes (weeds, etc. during harvesting, packaging, and handling). These types of contamination, in minute quantities, are common in pharmacy preparation rooms and do not usually affect the quality, safety, and efficacy of these types of preparations. It should be noted that the monographs on “herbal drugs” of the European Pharmacopoeia allow for up to 2% of foreign matter unless differently stated in a specific herb monograph (EMA, 2006; EMA, 2011). Despite this, in

the case of sample 5GX, we have Trogopterori Faeces included in the ingredients list of the product, which may be the reason for ingredient outliers detected with nrITS1. Nonetheless, the analysis detected some use of suspected unreported fillers as well as suspected adulteration. In two samples, in both FL and GX, we detected *Xanthium sibiricum* Patr. This species is toxic if bigger amounts are ingested and can cause serious health risks (Gurley et al., 2010; West et al., 2010; Scheid et al., 2015). Another species detection of interest is *Nelumbo nucifera* Gaertn. in 7FL and 2GX. Here we suspect a label switch for the ingredient. 7FL analyzed contains *Nelumbo nucifera* Gaertn., which is only expected for sample 2GX. Both samples originate from the same company and were custom-made products (See Supplementary Table S2). Following these assumptions, we can notice problems with nomenclature, and correct labeling on the market and we have reasons to assume fraudulent practice for two of the samples.

The HPTLC method presented provides qualitative estimates of the ingredient profiles of each sample, yielding a useful insight into the quality of herbal mixtures. The whole chemical profile of each plant species was considered for the two formulae FL and GX extracting a common pattern as a criterion for elaborating the individual formulations (Noviana et al., 2022). The multi-ingredient chromatographic profiling attempts to separate the individual components and to develop a fingerprint for each sample, which allows for a qualitative evaluation, similar to Nicoletti et al. (2013). Fingerprints of the marketed multi-ingredient botanicals were compared with fingerprints of constituent extracts for each single ingredient and the fingerprint of a respective reference botanical material. The presence of each ingredient in the formulae could be analyzed against their botanical references and their pharmacopeial marker(s). Three *ad hoc* mixtures were prepared and partly served for validation of the method. The *ad hoc* mixtures, AMs, were prepared using single ingredients that were individually tested with HPTLC that successfully confirmed their identity (see Supplementary Figures S4A–M). To compare results for different samples, each fingerprint needed to be quantified. A scoring scheme, the Band Intensity Score (BIS), was further developed for this purpose. Ideally, one would aim for an unequivocal numerical score, but when dealing with multiple ingredients containing multiple chemical constituents, the analysis becomes more complex. The basic points for evaluation remain those typical of HPTLC, which are the presence or absence of desired bands, and their intensity. Therefore, we evaluated each ingredient, first according to the presence of chemical marker compounds, and then for their proximity to other elements present in the botanical references' fingerprints. The method cannot solely rely on the presence or intensity of the marker compound, but needs to be combined with a holistic evaluation of the total fingerprint of an ingredient. Typically, BIS assigns a numerical value to the comparative intensity of bands, but for instance, in the case of Paeoniae Radix Rubra (Figure 1), sample 7FL scored higher than sample 18FL, even though the band for the chemical marker compound is less intense. Looking at the entire fingerprint of the ingredient, in comparison with the botanical reference, is more accurate here. The evaluation of BIS requires an experienced eye, and remains susceptible to subjective interpretation, even though positive or negative detection is quite unequivocal. Generally, we face methodological challenges when analyzing multi-herbal formulae, such as complex TCM preparations with HPTLC and unilateral standardization of methods is not possible. The initial quantity of single ingredients within a preparation can vary, which ultimately leads to a variation of the concentration of chemical marker compounds and other chemical compounds which consequently alters the fingerprint. When the number of ingredients increases, the concentration of each ingredient and its components will consequently be reduced. HPTLC will therefore meet challenges when there are numerous ingredients, as for the GX formula with 12 ingredients. Three ingredients were not detectable in the AM_GX, while signals from only one marker compound were absent for AM_FL. Various chemical compounds of the multi-herbal complex can cause chemical reactions and change or overlay the expression of certain bands. We see this in the case of the band for chemical marker compound paeonol for the detection of Moutan Cortex, which is not visible in the fingerprint for any of the multi-ingredient samples, even though we have a proof of its presence in the single ingredient analysis of the *ad hoc* preparations (see Supplementary Figures S4B). Besides, concentrations for chemical marker compounds can vary for botanical materials of varying origins amongst different plant ingredients and amongst the same ingredients, as well. For instance, paeonol is represented in lower concentrations with 0.9%–2.2% in ingredient Moutan Cortex (He et al., 2006; Tobyn et al., 2011), which is plant species *Paeonia suffruticosa Andrews*, than, for example paeoniflorin with 4.6%–5.1% in ingredient Paeoniae Radix Rubra, which can be plant species *Paeonia anomala* subsp. veitchii (Lynch) D.Y.Hong & K.Y.Pan or *Paeonia lactiflora Pall.* (Cai et al., 1994). Furthermore, it is important to consider natural fluctuations in chemical compounds for different growth cycles, eco-regions, and times of the year. E.g. for *P. lactiflora*, paeoniflorin content was found to be highest in November (Yamamoto, 1988). Some accepted TCM ingredients can be sourced from varying plant species, whilst the standard identification method in the pharmacopeia remains the same for either species. Hence slight differences can be expected for the fingerprints of various samples. According to TCM processing techniques, plant material can further be sourced from differently treated botanical materials (Wang et al., 2019) (fried, boiled, etc.), which can alter the chemistry of chemical compounds. Additionally, In TCM practice, the ratios of different ingredients within a formulation can vary according to individual TCM diagnostics. These factors combined may result in diverse chemical fingerprints of seemingly identical TCM preparations.

Different types of processing techniques have different effects on the content of chemical constituents. For instance, concentrations of paeoniflorin are lower in boiled *Paeonia lactiflora* Pall. due to thermal instability (Cai et al., 1994). From a chemical analytical point of view, we suggest preparing unique standards for each of the accepted ingredients and ultimately multi-herbal formulae *ad hoc*. In practice, for instance, for the ingredient Paeoniae Radix Rubra, this would mean firstly to prepare first standards from vouchered botanical material of both accepted species listed under the ingredient according to the Chinese Pharmacopoeia. Secondly preparing standards from the three possible TCM processing techniques, which are dry-frying, wine-frying, or vinegar-frying of dried slices from the root (Scheid et al., 2015). And thirdly establishing a dictionary for all different fingerprints, which are multi-fold for all possible ingredients and processing techniques of

single ingredients within a formula. Each sample representing a polyherbal formula would then have to be compared to the full spectrum of varying fingerprints in this dictionary. There are some limitations to this method. We have established that, in some cases, both marker compounds and botanical references are not optimal for the methodological approach, e.g., paeonol for Moutan Cortex. Constraints for the method are the use of multiple, possibly expensive chemical marker compounds, and sometimes difficult to retrieve-botanical reference materials. We do not take into account the sensitivity of the type of raw material and manufacturing processes, and limitations in differentiating between plant species (Bansal et al., 2014). The method has limitations in verifying substitutions and adulterations, especially of highly processed multi-herbal products, as merely the presence or absence of marker and reference compounds can be detected.

Conclusion

Our study emphasizes that integrating DNA metabarcoding with chromatography gathers and combines a wider set of data to provide a more representative picture of herbal preparations for a more comprehensive authentication of Traditional Chinese Medicines contributing to their quality and safe use. The study presented here, is one of the few combining dual-loci DNA metabarcoding and HPTLC through an integrated approach, to test for the quality and purity of multi-herbal products, in this case of two TCM formulae (FL and GX), commonly prescribed for the treatment of endometriosis. Dual-loci DNA metabarcoding was able to detect two to three out of five expected FL ingredients, and eight to nine out of eleven GX's expected ones, considering that two ingredients were identified at the same genus level only. In total, 22.4% of the identified species were expected ingredients. Overall, eleven samples of twenty scored higher in identity for FL and six out of nine in GX. Depending on the manufacturing characteristics of each sample, whether they were mixtures *ad hoc*, powders, tablets, granules, capsules, and pill, there are differences in species identification hits. The metabarcoding allowed the identification of 38 unexpected taxa in the sample range. Almost all invested products included other species not listed on the label or known to be an ingredient of the formulae. In contrast, the HPTLC methods were able to detect four out of five expected FL ingredients, and ten out of eleven GX's expected ones, and revealed a higher fidelity of expected ingredients with 70.7% of expected ingredients.

The study highlighted each method's drawbacks and strengths, DNA metabarcoding's sensitivity which allows for the detection of contaminants, and HPTLC's potential to distinguish between quantities of the plant used. DNA techniques are not bound by the quantity and quality of certain chemical compounds but by the presence of viable DNA which is also dependent on the application of varying TCM processing techniques. This study shows that neither genetic barcoding nor chemical analysis alone, as post-quality-control measures, can trace back the complexity of problems when dealing with poly-herbal products.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://doi.org/10.5281/zenodo.8383538.

Author contributions

FM: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Project administration, Resources, Software, Supervision, Visualization, Writing–original draft, Writing–review and editing. FS: Data curation, Formal Analysis, Methodology, Resources, Writing–review and editing. QM: Data curation, Formal Analysis, Software, Writing–review and editing. AR-M: Writing–review and editing. AS-N: Formal Analysis, Writing–review and editing. HW: Conceptualization, Methodology, Project administration, Supervision, Writing–review and editing. HB: Conceptualization, Funding acquisition, Methodology, Project administration, 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.

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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.2023.1305410/full#supplementary-material

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An integrated *in vitro* approach on the enzymatic and antioxidant mechanisms of four commercially available essential oils (*Copaifera officinalis, Gaultheria fragrantissima, Helichrysum italicum,* and *Syzygium aromaticum*) traditionally used topically for their anti-inflammatory effects

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Introduction: Despite the increasing number of essential oils being reported on their potential therapeutic effects, some remain relatively unknown on their biological properties. That is the case of the essential oils obtained from copaiba (*Copaifera officinalis* L.), wintergreen (*Gaultheria fragrantissima* Wall.), everlasting (*Helichrysum italicum* (Roth) G.Don) and clove (*Syzygium aromaticum* (L.) Merr. & L.M.Perry), commonly labelled as being useful on the amelioration of conditions with an inflammatory background.

Methods: To further broaden the current knowledge on the four essential oils, commercially available samples were approached on their effects upon a series of mediators that are involved on the inflammatory and oxidative response, both through in vitro cell-free and cell-based assays (5-lipoxygenase activity, lipid peroxidation, free radical and nitric oxide radical scavenging properties or tyrosinase inhibition).

Results: The four oils proved to be active at some of the concentrations tested in most of the performed assays. Significant differences were found between the essential oils, *S. aromaticum* proving to tbe the most active, followed by *G. fragrantissima* against 5-lipoxygenase (5-LOX) and linoleic acid peroxidation, proving their potential use as antioxidants and anti-inflammatory agents. In fact, the IC_{50} value of *S. aromaticum* in the 5-LOX assay was 62.30 μg mL^{-1}. Besides *S. aromaticum* efficiently scavenged superoxide radicals generated by xanthine/xanthine oxidase, displaying an IC_{50} value of 135.26 μg mL^{-1}. The

essential oil obtained from *H. italicum* exhibited a significant decrease in the nitric oxide levels on BV-2 cells, showing its potential as a cytoprotective agent against toxic damage. Copaiba oil ranked first as the most potent tyrosinase inhibitor, exhibiting an IC_{50} 98.22 μg mL^{-1}.

Conclusion: More studies are needed to describe the essential oils properties, but these results confirm the potential of these essential oils as anti-inflammatory and antioxidant agents.

KEYWORDS

caryophyllene, eugenol, methyl salicylate, pinene, *Syzygium aromaticum*

1 Introduction

Essential oils can be portrayed as a mixture of natural volatile compounds, specifically low molecular weight molecules that include terpenes, alcohols, aldehydes, and ketones, being oftentimes responsible both for the bioactive properties and the aromatic fragrance. Such mixtures can be characterized by the occurrence of 20–60 compounds, with complex quantitative profiles, but with two or three compounds frequently constituting most of the total content. A detailed chemical profiling is required as while the remaining components are usually found in trace amounts, they can also modulate the biological activity of the plant-derived product. In fact, essential oils encompass constituents that exhibit nearly the complete spectrum of chemical functionalities, being usually applied topically, orally or through inhalation (Bakkali et al., 2008; Koyama and Heinbockel, 2020).

Several constituents occurring in essential oils have a wide range of chemo-ecological roles on plants, that range from insecticidal and antimicrobial effects to properties contributing to reduce the effects of abiotic stress. Oftentimes, such biological properties, displayed by the complex compounds in these products, have a translation to the development of medicinal products, cosmetics, preservatives, and perfumery products (Abelan et al., 2022). Essential oils have been playing a pivotal role in Complementary Medicine, namely in Aromatherapy, being progressively becoming popular in Western societies mainly due to the usefulness in the amelioration of pain, inflammation, sleep disorders and anxiety (Sattayakhom et al., 2023).

Specific essential oils have been continuously validated on their anti-inflammatory effects, complementing the therapeutic action of blockbuster non-steroidal anti-inflammatory drugs (NSAIDs) and steroidal anti-inflammatory drugs (SAIDs) (Bacchi et al., 2012). There is an increasing number of reports on essential oils that encompass direct anti-inflammatory effects, impacting signaling cascades, levels of cytokines, regulatory transcription factors, and the expression of pro-inflammatory genes, but also indirect effects through the neutralization of reactive nitrogen species (RNS) and reactive oxygen species (ROS) that play a pivotal role in the development and amplification of inflammatory responses (Ramsey JT et al., 2020). For example, the essential oil obtained from chamomile (*Matricaria chamomilla* L.) has been traditionally used to treat inflammation and irritation symptoms (Qasem et al., 2022) while others have been used in cosmetics and in the development of commercial products such as plant-based supplements and other products, being worth to highlight those obtained from rosemary (*Rosmarinus officinalis* L.) or lavender (*Lavandula* spp.) (Neves et al., 2018; Borges et al., 2019).

Unlike the abovementioned essential oils, some species lack experimental data substantiating their use in Complementary Medicine. Such examples include the essential oils herein investigated, i.e., from clove [*Syzygium aromaticum* (L.) Merr. & L.M.Perry] [Syn. *Eugenia caryophyllus* (Spreng.) Bullock & S.G.Harrison], copaiba (*Copaifera officinalis* L.), wintergreen (*Gaultheria fragrantissima* Wall.) and everlasting [*Helichrysum italicum* (Roth) G.Don]. While these essential oils are commonly used in the pharmaceutical industry, very few reports demonstrate their mechanisms and pharmacological properties, particularly on their ability to interfere with inflammatory mediators and as possible radical scavengers that might indirectly attenuate the exacerbation of the inflammatory response. *S. aromaticum* essential oil is mainly reputed as a food preservative, but also very frequently used in toothache and on the treatment of burns and wounds, with the herbal monograph from the European Medicines Agency validating its use on the symptomatic treatment of minor inflammations in the mouth or the throat (Committee on Herbal Medicinal Products HMPC, 2011). Besides, *S. aromaticum* is traditionally used in the treatment of vomiting, nausea, liver and stomach disorders, while also being used to treat viral, parasitic and bacterial infections (Batiha et al., 2020). Nearly all parts of *C. officinalis* are labelled as having anti-inflammatory and contraceptive effects by the native people from the Brazilian Amazon, the topical application of the essential oil being particularly effective in skin wound healing and used in massages to relief headache (da Trindade et al., 2018). Due to its high content in methyl salicylate, the anti-inflammatory effects of *G. fragrantissima* essential oil is anticipated (Mukhopadhyay et al., 2016). In fact, *Gaultheria* species have garnered growing interest within the scientific community due to the potential antioxidant activity, and therefore, their potential associated health benefits (Pandey et al., 2017). Ethnomedicinal records indicate that *H. italicum* oil soothes inflammation, simultaneously reducing pain with its built-in analgesic properties being also used in cosmetic products (Antunes Viegas et al., 2014a). Despite their wide and popular use, further experimental data is required to elucidate the mechanisms underlying the anti-inflammatory effects of the essential oils from *C. officinalis*, *G. fragrantissima*, *H. italicum* and *S. aromaticum*, which prompted us to carry out the current work. Furthermore, annotation of the anti-inflammatory effects should be accompanied by a standardized chemical profile, as the content in bioactives is dependent on the chemotype, location, season and maturation stage of the plant source.

TABLE 1 Essential oils basic information such as scientific name, batches used, country of recollection, part of the plant and main compounds.

Scientific name	Batch	Country	Part of the plant	Main compounds
Syzygium aromaticum (L.) Merr. & L.M.Perry	OF30342	Madagascar	Flower buds	78.19% Eugenol
				13.56% Eugenyl acetate
				4.97% E-Caryophyllene
Copaifera officinalis (L.)	OF38018	Brazil	Oleoresin	55.89% E-Caryophyllene
				7.51% α-Humulene
				5.91% Germacrene D + Compound Mw = 206
				4.85% α-Copaene
				3.34% δ-Cadinene
				3.30% α-*trans*-Bergamotene
Helichrysum italicum (Roth) G.Don	OF46599	Bosnia and Herzegovina	Flowering tops	22.44% α-Pinene + α-Thujene
				13%–21% γ-Curcumene
				7.09% β-Selinene
				6.11% Neryl acetate
				5.22% E-Caryophyllene
				4.90% Italidione I
				3.87% α-Selinene
				3.34% Italicene
				2.82% α-Curcumene
				2.22% Italidione II
				1.25% Italidione III
Gaultheria fragrantissima Wall	OF47135	Nepal	Leaves	99.58% Methyl salicylate

2 Materials and methods

2.1 Reagents and chemicals

Suppliers of each reagent are indicated on the respective section. In general, enzymes, substrates, free radicals and IFN-γ were acquired from Sigma-Aldrich (St. Louis, MO, United States). Cells were acquired from the American Type Culture Collection (ATCC®) and cell culture reagents from GIBCO, Invitrogen™ (Gran Island, NY, United States). Spectrophotometric determinations were performed in a Multiskan GO plate reader (Thermo Fisher Scientific; Waltham, MA, United States).

2.2 Identification, source and chemical characterization of the essential oils

Description on the four essential oils herein investigated is presented in Table 1, including the geographical location where the plant material was sourced, the plant part, as well as their chemical profiles. Essential oils were provided by Pranarôm International -Inula group (Ghislenghien, Belgium). Their chemical composition, specifically the qualitative profile in volatiles was characterized by GC-FID and provided by Pranarôm (Supplementary Figure S1) (Supplementary Table S1). More than 100 constituents have been identified on the essential oils obtained *C. officinalis* and *H. italicum*, while *S. aromaticum* essential oil is mainly characterized by the occurrence of eugenol. It is also worth to highlight that methyl salicylate encompasses more than 99% of the constituents occurring on the essential oil obtained from *G. fragrantissima*. (Table 1) (Figure 1).

2.3 Inhibition of 5-lipoxygenase

5-Lipoxygenase (5-LOX) is the enzyme that participates on the conversion of arachidonic acid into leukotrienes, its inhibition being assessed by following the oxidation of linoleic acid to 13-hydroperoxylinoleic acid, according to a previously described procedure (Kachmar et al., 2019). Stock solutions of the samples were prepared in methanol 2.5%, absence of a possible interference with the assay read outs being confirmed. Samples were then dissolved in a $Na_2HPO_4{\cdot}2H_2O$ (pH = 9) buffer. A minimum of six concentrations were tested, at concentrations ranging from 7.8125 to 250 μg mL^{-1}. The reaction mixture was prepared in Corning® 96-well clear flat bottom UV-transparent microplates as follows: 180 μL of buffer, following the addition of 20 μL of sample solution and 20 μL (100 U) of soybean 5-LOX (*Glycine max*). After 5 min of incubation at room temperature, 20 μL of a linoleic acid solution, at 4.18 mM in ethanol, were added to

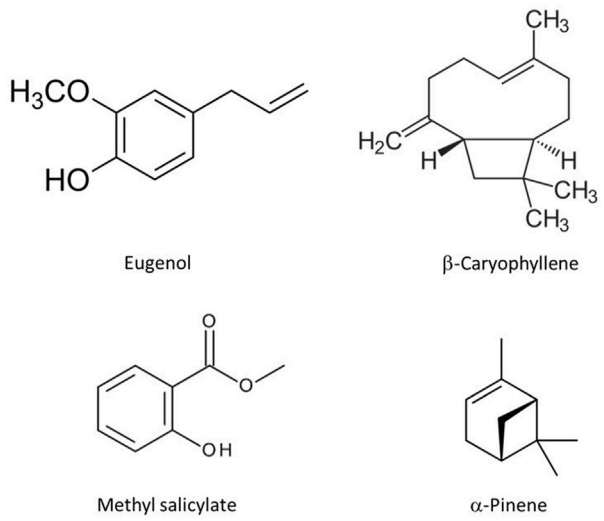

FIGURE 1
Structures of the major components of each essential oil.

the mixture. The absorbance was measured at 234 nm. Three independent assays were performed in triplicate. Quercetin, tested under the same conditions, was used as a positive control at concentrations ranging from 20 to 0.625 μg mL^{-1}.

2.4 Inhibition of lipid peroxidation

Peroxidation of linoleic acid occurs due to the abstraction of a hydrogen proton from an unsaturated lipid and involves the generation and propagation of lipid radicals, the consumption of oxygen and the rearrangement of double bonds of polyunsaturated fatty acids. The rearrangement of the double bonds results in the formation of conjugated dienes (lipid hydroperoxides), which are characterized by an intense absorption at 233 nm (Ferreres et al., 2012; Ayala et al., 2014). Essential oils were prepared in Tris-HCl buffer (100 mM, pH 7.5), six concentrations of each essential oil being prepared. 50 μL of the sample solutions were mixed with 150 μL of buffer and 250 μL of linoleic acid (20 mM in ethanol). 50 μL of a Fe $SO_4 \cdot 7H_2O$ 4 mM solution in ultrapure water and 50 μL of a 5 mM ascorbic acid solution in buffer were also added to the reaction mixture. The reaction mixture was incubated during 60 min at 37°. After incubation 1.5 mL of ethanol-ether (3:1 v/v) were added to stop the reaction and the whole mixture was vortexed. Absorbance was read at 233 nm (Ferreres et al., 2012). Butylated hydroxytoluene (BHT), tested under the same conditions, was used as a positive control. Three independent experiments were performed for each essential oil.

2.5 Determination of NO levels in BV2 microglial cells

2.5.1 Cell culture

BV-2 cells (Microglial cell *Mus musculus* (Mouse) C57BL/6) were maintained in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% heat-inactivated foetal bovine serum (FBS) (GIBCO, Invitrogen Grand Island, NY, United States LOT:2436448) and 1% penicillin/streptomycin (Pen Strep GIBCO, Invitrogen Grand Island, NY, United States) (10000 U mL^{-1}). Cells were kept in an incubator (Toreuse model 2,428 incubator Saint Louis, MO, United States) at 37°C, in a humidified atmosphere of 5% CO_2 (Gomes et al., 2019). Essential oils were dissolved in DMEM and filtrated through a 0.22 μm filter (TRP Spritzen-/syringe-filter 20190479).

2.5.2 Interference with the mitochondrial performance of BV-2 cells

To proceed with the cell assays, it was compulsory to determine a range of working concentrations, ensuring that the effects on NO levels did not outcome from a cytotoxic effect. For that purpose, the MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] assay was performed to investigate the impact on the mitochondrial viability (Gomes et al., 2019).

BV-2 cells were plated in 96-well plates at a cell density of 40,000 cells $well^{-1}$. After 24 h of incubation at 37°C, in a humidified atmosphere of 5% CO_2, the medium was replaced by different concentrations of the extract, cells being cultured in that medium for another 24 h. 100 μL of MTT (0.5 mg mL^{-1}) were added to each well, cells being incubated at 37°C for 90 min, with the resulting formazan crystals being later dispersed in 200 μL of DMSO:isopropanol (3:1) and quantified spectrophotometrically at 560 nm (Gomes et al., 2019).

2.5.3 Impact upon NO levels as assessed by the Griess assay

BV-2 cells were plated in a 96-well microplate at a density of 40,000 cells $well^{-1}$ and cultured for 24 h at 37°C, in a humidified atmosphere of 5% CO_2 (Toreuse model 2,428 incubator Saint Louis, MO, United States). The cells were treated with the essential oils for 2 hours before being stimulated with 20 μL of IFN-γ. After being cultured for 22 h, under the same conditions, aliquots of 75 μL of supernatant from each well were transferred to a 96-well flat bottomed microtiter plate and mixed with 75 μL of Griess reagent (consisting of 0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride and 1% sulfanilamide in phosphoric acid 2%. After 10 min of incubation in the dark, absorbance was determined at 560 nm (Suksungworn et al., 2020).

2.6 •NO scavenging activity assay

The •NO scavenging activity assay is based on the generation of diazonium species from sulphanilamide reacting with NO_2 and 1-naphthylethylenediamine leading to the production of an azo dye, suitable for measuring at 560 nm (Ferreres et al., 2017). Essential oils were prepared in a KH_2PO_4 0.1 M buffer. Assayed concentrations ranged from 15.625 to 500 μg mL^{-1}.100 μL of the samples were added to the 96-well microplate, followed by the addition of 100 μL of SNP (sodium nitroprusside dihydrate) at 20 mM, and incubated for 60 min. 100 μL of the Griess reagent [0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride and 1% sulphanilamide in phosphoric acid 2%] were added to the plate and kept in the dark for 10 min before reading it at 560 nm. Three independent assays were performed in triplicate and the results were compared with those obtained for quercetin, tested under the same conditions, and used as a positive control.

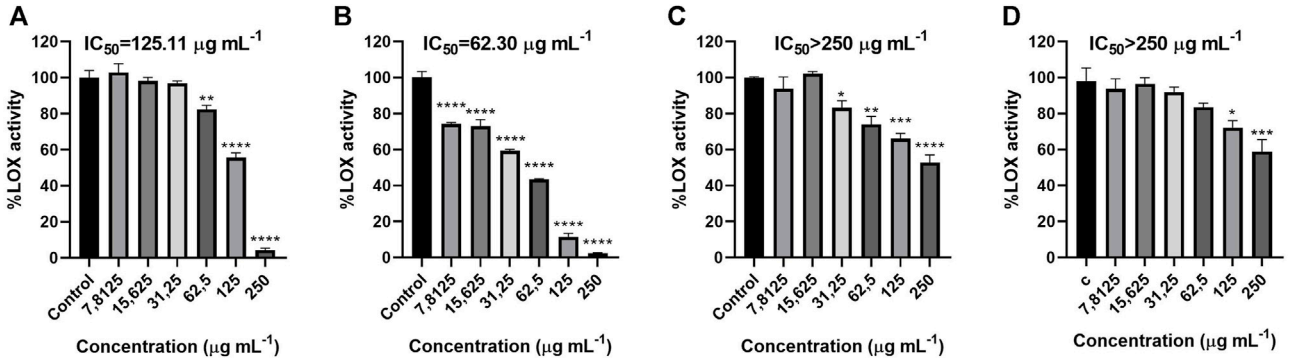

FIGURE 2
Effects of *G. fragrantissima* **(A)**, *S. aromaticum* **(B)**, *H. italicum* **(C)** and *C. officinalis* **(D)** essential oils upon 5- lipoxygenase activity levels in a cell-free assay. Results correspond to the mean ± SEM of a minimum of three independent experiments performed in triplicate (statistical significance: *$p < 0.05$; **$p < 0.01$, ***, $p < 0.001$, ****$p < 0.0001$) IC_{50} (quercetin) = 1.75 μg mL^{-1}.

2.7 Superoxide radical generated by xanthine/xanthine oxidase assay

$O_2^{\bullet -}$ is formed due to the reduction of an oxygen molecule by a single electron, and can be formed via NADH but also via xanthine/xanthine oxidase. $O_2^{\bullet -}$ forms a complex when reacting with NBT which absorbance can be measured allowing to describe the scavenging activity (Rodríguez-Chávez et al., 2015). The reaction mixture consisted of 90 μM xanthine (16 mM Na_2CO_3, and 22.8 μM NBT in phosphate buffer, pH 7.0). The assay was performed in a 96-well microplate, 240 μL of the reaction mixture were added in each well with 30 μL of the essential oil in buffer with 0.5% of methanol as cosolvent.

After, 30 μL xanthine oxidase were added, the plate was incubated in the dark for 7 min at 37°C. The absorbance is read at 560 nm (Plate reader fluorescence BIOTEK SYNERGY HI). Three independent assays were performed in triplicate. Both controls and blanks were taken into account. Gallic acid tested under the same conditions was used as a positive control.

2.8 Inhibition of tyrosinase

Tyrosinase enzyme inhibition was selected as another approach to assay the effects of the essential oils against oxidation. The assay was carried out in a 96-well microplate where the samples were mixed with 80 μL a potassium phosphate buffer (pH 6.8), 40 μL of L-DOPA and 40 μL of tyrosinase enzyme, both dissolved in the buffer. The inhibition of the enzyme was determined at 475 nm. Three experiments were performed in triplicate for each sample. Kojic acid was used as a positive control tested under the same conditions (Farràs et al., 2019).

2.9 Statistical analysis

Statistical analysis was performed with GraphPad Prism 10. On cellular assays, a Grubbs' test was utilized to identify significant outliers. Normality was checked through D'Agostino-Pearson normality test. After, for data following a normal distribution, a one-way analysis of variance (ANOVA) with Dunnett as *post hoc* test was used to compare each experimental condition with the respective control (untreated cells). The level of significance between different groups in cell-free assays was determined by one-way ANOVA with Bonferroni's post-test. Student´s t-test was found to be more appropriate to estimate the statistical significance of the results on the oxidation of the linoleic acid. The different values of IC_{50} for the essentials oils and positive controls were also calculated with GraphPad Prism 10.

3 Results

3.1 Inhibition of 5-lipoxygenase

Figure 2 shows that all the essential oils significantly inhibited the 5-LOX enzyme at certain concentrations. The active concentration range for *G. fragrantissima* was from 250 to 62.5 μg mL^{-1}. *H. italicum* appears to be active in the range between 250 and 31.25 μg mL^{-1} while *C. officinalis* essential oil was active only at 250 and 125 μg mL^{-1}. *S. aromaticum* proved to be active in the whole range of concentrations tested, from 250–7.81 μg mL^{-1}.

However, the IC_{50} value could only be determined for *G. fragrantissima and S. aromaticum.* These essential oils inhibited the enzymatic activity of 5-LOX, at their highest concentration down to 2.45% and 4.30%, respectively. Besides the oils displayed an IC_{50} value of 43.27 and 124.85 μg mL^{-1} respectively, *S. aromaticum* proving to be the most active essential oil.

3.2 Lipid peroxidation assay

As seen in Figure 3, at the highest concentrations tested, both *S. aromaticum* and *G. fragrantissima* were able to significantly decrease the lipid peroxidation down to percentages lower than 50%, 9.5%, and 5% respectively. For *C. officinalis* and *H. italicum,* the highest concentration lead to a percentage of peroxidation of the linoleic acid of 80.16% and 77.11% respectively.

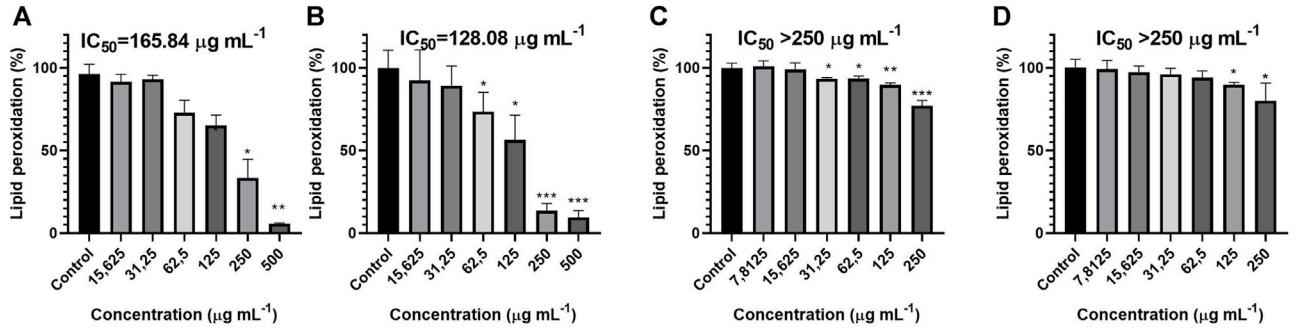

FIGURE 3
Activity of *G. fragrantissima* **(A)**, *S. aromaticum* **(B)**, *H. italicum* **(C)** and *C. officinalis* **(D)** essential oils upon lipid peroxidation levels in a cell-free assay. Results correspond to the mean ± SD of a minimum of three independent experiments (statistical significance: $*p < 0.05$; $**p < 0.01$, $***p < 0.001$). IC_{50} (BHT) 871.74 μg mL–1.

The oil with a lowest IC_{50} value was found to be *S. aromaticum* with an IC_{50} of 128.08 μg mL^{-1} followed by *G. fragrantissima* with an IC_{50} of 165.84 μg mL^{-1}, while the IC_{50} for *H. italicum* and *C. officinalis* were both higher than 250 μg mL^{-1}. *S. aromaticum* and *G. fragrantissima* displayed an IC_{50} lower than the one calculated for the positive control.

G. fragrantissima and *C. officinalis* were active only in the two highest concentrations tested. For *S. aromaticum* the range of concentrations where it proved to be active was from 500 to 62.5 μg mL^{-1} while for *H. italicum* was from 250 μg mL^{-1}–31.25 μg mL^{-1}.

3.3 Cell assay in BV2 microglial cells

As shown in Figure 4, the MTT assay was used to characterize the cell viability of BV2 cells in a range of concentrations from 1000 to 31.25 μg mL^{-1}. *H. italicum* and *G. fragantissima* essential oils did not affect the cell viability in the tested range of concentrations. Concentrations considered safe for the cells for *S. aromaticum* were from 125 to 3.90625 μg mL^{-1} since there was a significant interference on the cellular viability at the range of concentrations from 1000 to 250 μg mL^{-1}. For *C. officinalis* significant cytotoxic effects have been only recorded upon the highest concentration tested 1000 μg mL^{-1} making the select safe range for the cells from 500 to 15.625 μg mL^{-1}.

For the nitrite assay studied in the range of concentrations stablished before, both *S. aromaticum* and *G. fragantissima* proved to be significantly active in the whole established range of concentrations, from 125 to 3.90625 μg mL^{-1} and 1000 to 31.25 μg mL^{-1} respectively. *C. officinalis* was found to be active only at the highest concentration tested, 500 μg mL^{-1}. *H. italicum* proved to have an activity in the range of concentrations from 1000 to 250 μg mL^{-1}. Even if the reduction of the nitric oxide (NO) levels was statistically significant, the IC_{50} values could not be determined since none of the oils reached a 50% reduction *H. italicum* demonstrating to be the most active essential oil on the reduction of NO levels.

3.4 $^{\bullet}$NO scavenging activity assay

C. officinalis essential oil proved to be inactive on the antiradical activity towards nitric oxide radical, but the essential oils obtained from *H. italicum, S. aromaticum* and *G. fragrantissima* significantly reduced the radical levels at 250 and 500 μg mL^{-1} despite their low activity (Figure 5). However, none of the essential oils tested in this case scavenged $^{\bullet}$NO to a percentage lower than 50%. Therefore, the IC_{50} was higher than 500 μg mL^{-1} for all the essential oils. Quercetin was used as a positive control with an IC_{50} of 4.89 μg mL^{-1}.

3.5 Superoxide radical generated by xanthine/xanthine oxidase assay

G. fragrantissima essential oil was not suitable to be assayed due to the inability of the oil to solve in the buffer even with the help of cosolvents. *S. aromaticum* essential oil produced a reduction in the levels of the superoxide radicals generated by xanthine/xanthine oxidase in whole range of concentrations tested. The calculated IC_{50} for *S. aromaticum* was 135.26 μg mL^{-1} (Figure 6).

Both *H. italicum* and *C. officinalis* proved to be active in some of the concentration tested but the slight reduction in the superoxide radical levels did not allow for the IC_{50} to be determined. *H. italicum* was active from 125 μg mL^{-1} while the range displayed by *C. officinalis* was wider, from 250 to 15.625 μg mL^{-1}. For both of the oils the activity at 500 μg mL^{-1} could not be determined due to solving problems (Figure 6).

3.6 Inhibition of tyrosinase

As seen in Figure 7, only *S. aromaticum* and *C. officinalis* displayed a reduction in tyrosinase activity lower than the 50% allowing for the IC_{50} values to be calculated. In particular, *C. officinalis* showed a significant reduction in all of the concentrations tested and the lowest IC_{50} value followed by *S. aromaticum.* For *G. fragrantissima* and *H. italicum* only the reduction for highest concentrations was found statistically significant.

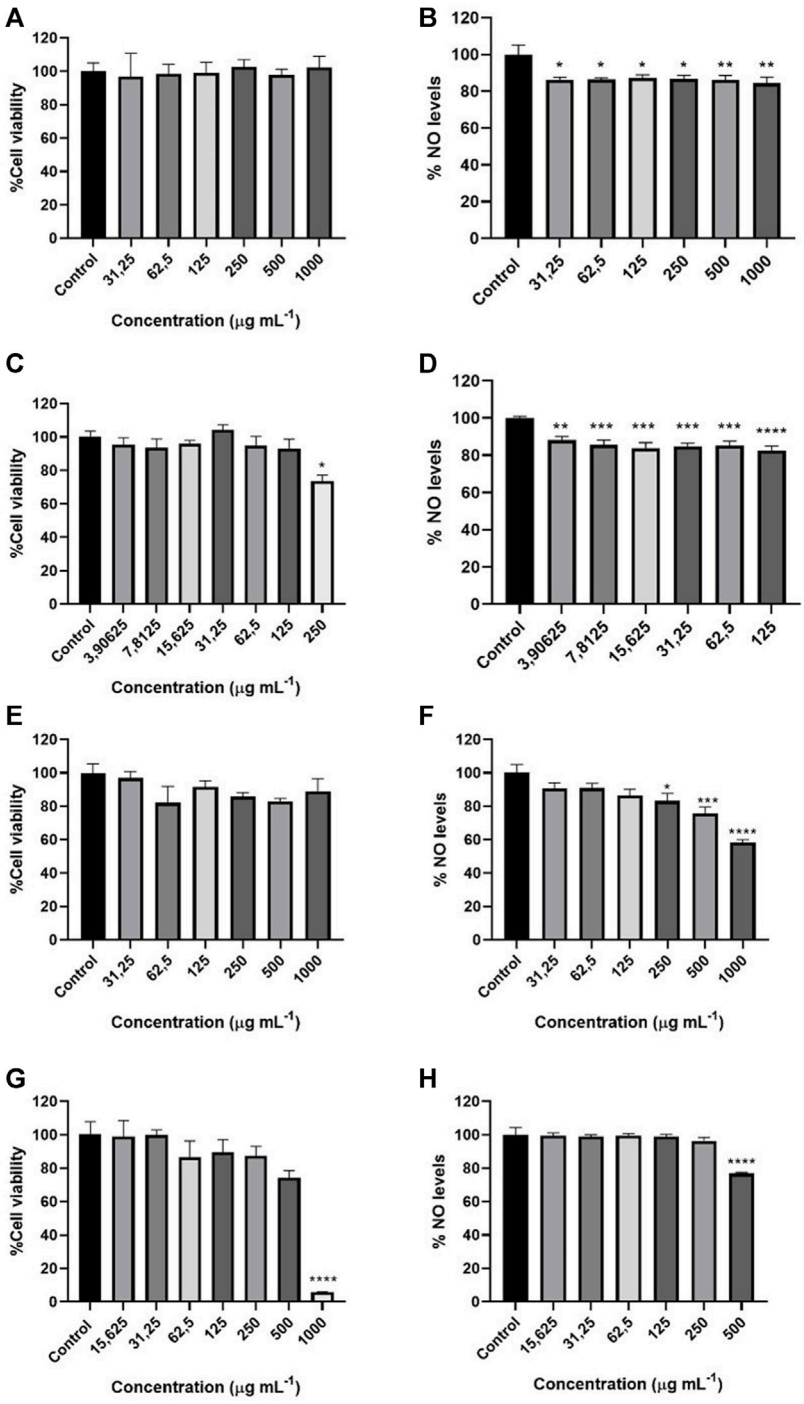

FIGURE 4
Cell viability of *G. fragrantissima* **(A)**, *S. aromaticum* **(B)**, *H. italicum* **(C)** and *C. officinalis* **(D)** essential oils. Effects of *G. fragrantissima* **(E)**, *S. aromaticum* **(F)**, *H. italicum* **(G)** and *C. officinalis* **(H)** essential oils upon $^{\bullet}$NO levels in BV-2 cells assay. Results correspond to the mean ± SEM of a minimum of three independent experiments performed in triplicate (statistical significance: *$p < 0.05$; **$p < 0.01$, ***, $p < 0.001$, ****$p < 0.0001$).

4 Discussion

Wintergreen has been traditionally used as a herbal remedy in America, Canada, and India; recent reports have demonstrated its antioxidant, antibacterial and analgesic activities (Liu et al., 2013). In our work, *G. fragantissima* essential oil showed a significant activity in the inhibition of 5-LOX (Figure 2). The IC_{50} obtained for this essential oil was 124.85 μg mL^{-1}, higher than the obtained for *S. aromaticum* and quercetin, used as positive control (IC_{50} = 1.75 μg mL^{-1}), but still lower than those obtained from *H. italicum* and *C. officinalis* (IC_{50} > 250 μg mL^{-1}). Even if the activity of the *G. fragantissima* essential oil has not been described

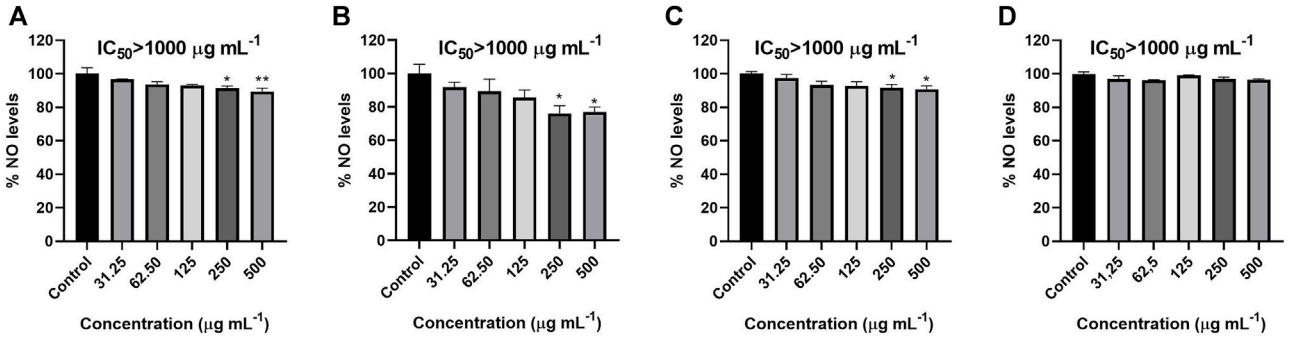

FIGURE 5
Activity of *G. fragrantissima* **(A)**, *S. aromaticum* **(B)**, *H. italicum* **(C)** and *C. officinalis* **(D)** essential oils upon $^{\bullet}$NO levels in a cell-free assay. Results correspond to the mean ± SEM of a minimum of three independent experiments performed in triplicate (statistical significance: $*p < 0.05$; $**p < 0.01$) IC_{50} (quercetin) 4.89 μg mL–1.

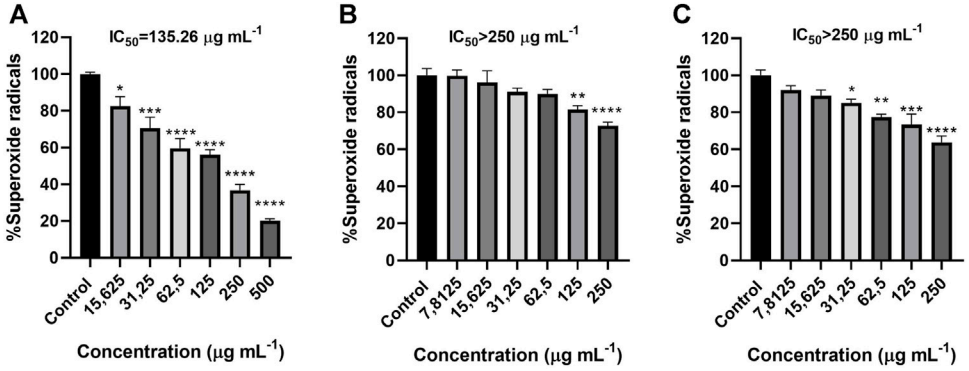

FIGURE 6
Activity of *S. aromaticum* **(A)**, *H. italicum* **(B)** and *C. officinalis* **(C)** essential oils upon superoxide levels in a cell-free assay. Results correspond to the mean ± SEM of a minimum of three independent experiments performed in triplicate (statistical significance: $*p < 0.05$; $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$) IC_{50} (gallic acid) 0.045 μg mL^{-1}.

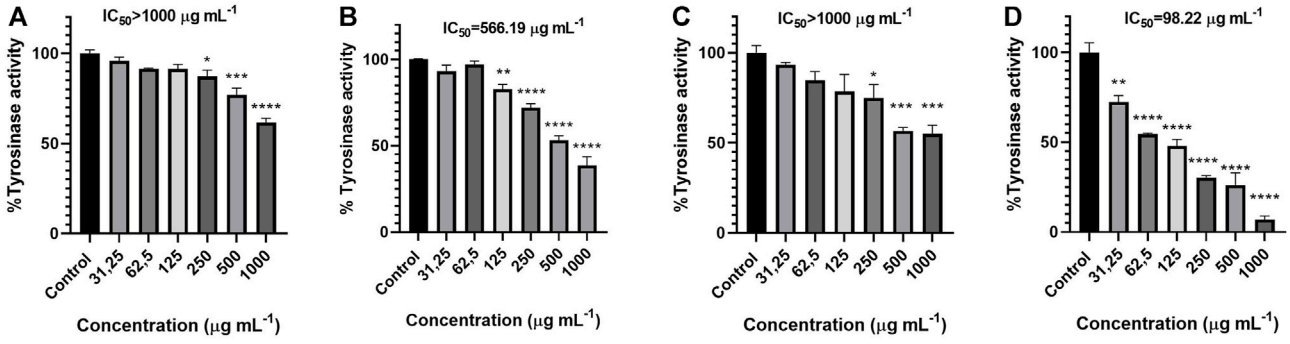

FIGURE 7
Activity of *G. fragrantissima* **(A)**, *S. aromaticum* **(B)**, *H. italicum* **(C)** and *C. officinalis* **(D)** essential oils upon tyrosinase enzyme levels in a cell-free assay. Results correspond to the mean ± SEM of a minimum of three independent experiments performed in triplicate (statistical significance: $*p < 0.05$; $**p < 0.01$, ***, $p < 0.001$, $****p < 0.0001$). IC_{50} (Kojic acid) = 5.94 μg mL^{-1}.

before, Alam et al., in 2017 reported the effects of methanol and DMSO extracts obtained from other *Gaultheria* spp. (*Gaultheria trichophylla*) on the 5-LOX inhibition, obtaining similar but slightly lower activity values than the one obtained in this work (Alam et al., 2017). This seems to indicate that the essential oil has more activity as a 5-lipoxygenase inhibitor than the methanol extract and the

DMSO one, perhaps due to its high content in methyl salicylate (Table 1).

The eicosanoid-metabolizing enzyme 5-LOX acts as a main actor in the process of inflammation as it underlies the synthesis of the major pro-inflammatory lipid mediators leukotrienes (LT). The inflammatory stimuli induce their biosynthesis, namely of LTA4 and further generation of LTB4 and LTC4, which boost the development of diseases such as asthma, allergic rhinitis, atherosclerosis or diabetes (Dennis and Norris, 2015).

Besides, *G. fragantissima* essential oil was found active against lipid peroxidation, decreasing significantly the percentage of linoleic acid peroxidation. Excessive oxidation of lipids alters the physical properties of cellular membranes and can cause covalent modification of proteins and nucleic acids. Peroxides resulting from this process can be classified in lipid hydroperoxides and endoperoxides which act as intermediates in the production of prostaglandins that contribute to the development of inflammation symptoms (Gaschler and Stockwell, 2017). Also, hydroxy eicosatetraenoic acids, which can be generated by lipoxygenases and nonenzymatic lipid peroxidation, can be portrayed as agonists at thromboxane receptors despite having less affinity than thromboxane A2 (Ricciotti and Fitzgerald, 2011). Therefore, the results obtained for *G. fragantissima*, dealing with the peroxidation of the linoleic acid and the 5-LOX assay indicate that the essential oil could be useful in the treatment of inflammatory related pathologies.

The results obtained in the $^{\bullet}$NO scavenging activity assay were similar to those obtained from the other oils, exhibiting a significant activity at the two highest concentrations but with an IC_{50} value quite distant from the one of the positive controls (Figure 5). For the impact upon this radical produced by BV-2 cells (Figure 4), even if *G. fragantissima* essential oil is active in the whole range of concentrations tested, the highest concentration tested only reduced the levels of $^{\bullet}$NO in a moderate way and the activity was lower when compared to the other oils tested in this work. Previous studies had proved a higher reduction in the NO levels on LPS-induced RAW264.7 macrophages mediated by methyl salicylate derivates obtained from *Gaultheria* species (Zhang et al., 2011).

When it comes to the tyrosinase enzyme inhibition assay, even if the reduction in the activity of the enzyme was not significant upon exposure to *G. fragantissima* essential oil, specially compared to the one exhibited by *C. officinalis*, other works have shown higher inhibition levels for other *Gaultheria* species with different composition (Fernández-Galleguillos et al., 2021).

Clove extracts and essential oils have been traditionally used as an analgesic and anti-inflammatory remedy, not coming as a surprise that the essential oil exhibited the best results in the 5-LOX assay as well as in almost all the cell free assays performed. *S. aromaticum* is the essential oil tested in this work with the lowest IC_{50} for the 5- LOX assay and the closest to the calculated IC_{50} for quercetin. Even if there are not other works studying the inhibitory activity of *S. aromaticum* essential oil on 5- LOX activity, the results obtained in this work are similar to those obtained for the clove leaf oil which showed moderate 15-lipoxygenase inhibitory activity (Wei and Shibamoto, 2010). Therefore, both works seem to indicate that the anti-inflammatory activity of *S. aromaticum* is related with the potential inhibition of the arachidonic acid metabolism. Computational analysis also showed eugenol, the major component of *S. aromaticum* essential oil, as an anti-inflammatory compound and an active 5-LOX inhibitor that could replace some NSAIDs in different diseases (das Chagas Pereira de Andrade and Mendes, 2020), being the high occurrence of eugenol a potential reason for the inhibition exhibited by the essential oil tested in this work.

For the lipid peroxidation assay *G. fragantissima* exhibits a greater reduction in the percentage of lipid peroxidation at the highest concentration than *S. aromaticum*; however, the IC_{50} for *S. aromaticum* is still lower than both the one calculated for *G. fragantissima* and the positive control (BHT). The lipid peroxidation products may lead to changes in the regulation of cellular activity and dysfunction. Indeed, some of them may activate NF-κB signaling pathways proving to be related to inflammation (Yadav and Ramana, 2013). Besides, lipid peroxidation has proved to be an important hallmark of Alzheimer's disease cellular pathology, making specially interesting the results obtained from *S. aromaticum* (Ates et al., 2020).

Clove essential oil reduced moderately the nitric oxide levels. These results are not as promising as others obtained in different studies. Pérez-Rosés et al. (2016) described the effects of clove essential oil and eugenol on NO production by human leukocytes stimulated with LPS obtaining a high reduction in the NO levels. The effect of eugenol has also been studied in other works on the LPS-mediated NO production in RAW264.7 macrophages obtaining significant results (Li et al., 2006). That seems to indicate that *S. aromaticum* essential oil could display an higher inhibitory activity towards NO production in other cell lines.

Despite having low scavenging ability upon NO radical, the essential oil proved to have antiradical activity since it also reduced significantly the levels of $O_2^{\bullet -}$, which is also involved in the generation of oxidative stress (Figure 6). The reason behind the inhibition might also be the high concentration of eugenol, has it proved in other works to scavenge the radical generated by this system (Reddy and Lokesh, 1994). Besides, the high activity of the oil could be the result of a synergistic effect between the different phenolic compounds present, even at low concentrations (Radünz et al., 2019). This antiradical and antioxidant activity seems to highlight the *S. aromaticum* essential oil use as preservative in several industries, specially considering its already described antibacterial activity (Teles et al., 2021).

H. italicum has been traditionally used to treat health disorders such as allergies, colds, cough, skin, liver and gallbladder disorders, inflammation, infections, and sleeplessness (Antunes Viegas et al., 2014b). There is compelling evidence that the therapeutic efficacy of this oil changes with the natural variability of the composition. In the case of the essential oil studied in this work, more than 100 constituents have been identified, being α-pinene the component found in a higher concentration (22.44%). The essential oil shows a moderate inhibition of the 5-LOX enzyme, higher than the one exhibited by *C. officinalis* but lower than the ones proved by *S. aromaticum* and *G. fragantissima,* which reduced the activity at the highest concentration to 2.45% and 4.30% respectively. Other plants species such us coriander, rich in α-pinene have also been used for its analgesic and anti-inflammatory properties (Łyczko, 2023). Even if the results were

not as promising as the ones obtained for the two previous oils, the outcome of this work shows that *H. italicum* might have an anti-inflammatory potential inhibiting the arachidonic acid metabolism by 5-LOX despite not acting on the inhibition of lipid peroxidation. When it comes to the antiradical assays, *H. italicum* exhibited the highest decrease in the levels of NO in BV-2 cells within all the essential oils tested in this work. NO radical plays an important role in the regulation of inflammation since it affects every step of the process. Even at low concentrations $^{\bullet}$NO starts exhibiting effects such as inhibit adhesion molecule expression, cytokine and chemokine synthesis and leukocyte adhesion and transmigration. Besides, at higher concentrations the $^{\bullet}$NO radical becomes proinflammatory and toxic (Guzik et al., 2003).

Copaiba essential oil is a highly versatile product utilized across multiple industries, including the cosmetic, food and wellness industries (Urasaki et al., 2020). Even if *C. officinalis* showed the least promising activity in most of the antioxidant assays performed in this work, the essential oil displays significant inhibitory effects upon tyrosinase exhibiting the lowest IC_{50} value amongst the tested samples (Figure 7). Even if there are no works studying *C. officinalis* essential oil activity upon tyrosinase, this results match those obtained for different plant extracts that had E-Caryophyllene as one of their main components (Pintatum et al., 2020). Recent works have shown that the anti-inflammatory effects of *C. officinalis* could be related to an action on the cannabinoid receptor CB2 since E-Caryophyllene, the major component of the essential oil, has proved to selectively bind this receptor, being a functional CB2 agonist (Ferro et al., 2018). This indicates that *C. officinalis* has an anti inflammatory effect not related to lipid peroxidation, 5-LOX inhibition or antioxidant effects, that being, the ones studied in this work.

In the context of increasing demand for natural products, the present study contributes to elucidating the traditional use of these essential oils as anti-inflammatory and antioxidant agents, concurrently shedding light on various mechanisms that may be implicated. Besides, the study delivers preliminary evidence on the medicinal properties of *C. officinalis, G. fragantissima, H. italicum* and *S. aromaticum* in almost all the assays performed. A selective inhibitory effect upon the enzymatic activity of 5-LOX was exhibited by all the essential oils, suggesting the occurrence of anti-inflammatory bioactives, especially in the case of *S. aromaticum* and *G. fragantissima,* which resulted in the lowest IC_{50} values. This seems to indicate that these essential oils could be useful for the treatment of diseases related to inflammation. Future studies using animal studies or alternative models would be interesting in order to confirm the experimental *in vitro* bioactivities.

Data availability statement

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

Author contributions

MC: Data curation, Formal Analysis, Investigation, Writing–original draft. NG: Conceptualization, Investigation, Methodology, Supervision, Writing–review and editing. PA: Funding acquisition, Project administration, Writing–review and editing. VL: Conceptualization, Funding acquisition, Methodology, Resources, 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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2023.1310439/full#supplementary-material

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Three-tiered authentication of herbal traditional Chinese medicine ingredients used in women's health provides progressive qualitative and quantitative insight

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Traditional Chinese Medicine (TCM) herbal products are increasingly used in Europe, but prevalent authentication methods have significant gaps in detection. In this study, three authentication methods were tested in a tiered approach to improve accuracy on a collection of 51 TCM plant ingredients obtained on the European market. We show the relative performance of conventional barcoding, metabarcoding and standardized chromatographic profiling for TCM ingredients used in one of the most diagnosed disease patterns in women, endometriosis. DNA barcoding using marker ITS2 and chromatographic profiling are methods of choice reported by regulatory authorities and relevant national pharmacopeias. HPTLC was shown to be a valuable authentication tool, combined with metabarcoding, which gives an increased resolution on species diversity, despite dealing with highly processed herbal ingredients. Conventional DNA barcoding as a recommended method was shown to be an insufficient tool for authentication of these samples, while DNA metabarcoding yields an insight into biological contaminants. We conclude that a tiered identification strategy can provide progressive qualitative and quantitative insight in an integrative approach for quality control of processed herbal ingredients.

KEYWORDS

chemical fingerprinting, DNA barcoding, endometriosis, pharmacovigilance, Traditional Chinese Medicine, women's healthcare

Introduction

Traditional Chinese Medicine (TCM) is one of the most established traditional medical systems and an increasingly popular health resource throughout the world (Wang et al., 2019; World Health Organization, 2022). TCMs have been widely used in the treatment of acute and chronic diseases over thousands of years (Li et al., 2019). TCM is categorized as a Complementary and Alternative Medicine (CAM) (World Health Organization, 2013), which includes amongst other sub-categories Chinese Herbal Medicines (CHMs).

Gynecology is one of the main branches in TCM and harbors a long history in the treatment and management of gynecological disorders (Zell et al., 2000; Maciocia, 2011; Zhang et al., 2011; Chen et al., 2020). A common health condition for women is severe chronic pain in the pelvic area, and associated disease patterns often fall under the clinical diagnosis of dysmenorrhea, or endometriosis associated with complex symptoms (Reid et al., 2019; Nie et al., 2020; Taylor et al., 2021). A recent large-scale randomized and placebo-controlled trial confirmed the efficacy and safety of specific CHMs as a treatment for endometriosis-associated pain and related symptoms (Lin et al., 2022).

There are unique considerations to be made for the cultivation and processing of herbal ingredients for medicine, as a characteristic part of the ethnopharmacology of TCM (Guo et al., 2015). In the foreground are *Daodi* cultivation, which is linked with high quality material from specific geographical regions, and *Paozhi* processing where raw plant material is processed into decoction pieces which are treated with excipients that fundamentally alter their metabolic profile resulting in changed bioactivity levels, such as enhanced efficacy, modified medicinal properties and reduced toxicity (Shaw, 2010; Guo et al., 2015; Engelhardt et al., 2018; Wu et al., 2018; Wang et al., 2019). Chinese medicinal processing, as an integral part of TCM, is a pharmaceutical technique in order to meet therapeutic, dispensing and preparation requirements (Guo et al., 2015). Depending on wanted individualized medicinal properties from specific plants, a variety of techniques are used in TCM processing. First simple preparation, like cleaning and cutting of herbs, and second exhaustive processing, like stir-frying, stir-frying with liquids or solid adjuvants, steaming with salt water, medicinal juices, vinegar or wine, boiling and calcining with mineral salts, alum or fresh ginger (Guo et al., 2015). Generally, the choice for which processing method is used as a standard for which ingredient remains controversial, and methods for plant specific Chinese medicinal processing are evolving. Nonetheless, decoction pieces produced with ingredient specific processing techniques are indiscriminately prescribed in proprietary TCMs and in prescription handed out by TCM practitioners (Guo et al., 2015). Interestingly, TCM ingredients are described with herbal drug names defining a plant's genus and the part of the plant used. However, the ingredient name is seldom coherent with scientific taxonomy and ingredient names often refer to more than one plant species (Mück et al., 2023). The Chinese Pharmacopoeia states which species are accepted under the ingredient name with scientific taxonomy (Chinese Pharmacopoeia Committee, 2020).

The quality of TCM materials, their safety and therapeutic efficacy are of critical importance. Quality risks in CHM are related to authenticity issues through misidentification, or mislabeling of herbal ingredients, adulterations, and substitutions, or endogenous and exogenous substances, caused by improper processing of herbs, or heavy metal, pesticides and microbial contaminations (He et al., 2015; Wu et al., 2018; Chen et al., 2023). As examples, a study investigating 400 seeds for TCM manufacturing detected that 7.5% of the seeds where incorrectly labeled (Xiong et al., 2018), and in another study by Xu et al. (2019), 166 adulterants were detected from various TCMs. Good manufacturing practice (GMP), good agricultural and collection practices (GACP), good plant authentication and identification practice (GPAIP), and good laboratory practices (GLP) guidelines in TCM are regarded as important tools to meet good quality requirements (Govindaraghavan, 2008; Zhang et al., 2012; Govindaraghavan and Sucher, 2015; He et al., 2015). There are challenges in the "internationalization of TCM" including *inter alia* difficulties in quality control, legislative barriers in marketing TCMs and unclear basis of therapeutic mechanisms (Lin et al., 2018). Current quality control of TCMs including processed herbal preparations and products is in great dispute because, unlike chemical drugs, we lack clear quality standards for TCMs, and analytical methods applying qualitative markers are not integral enough to assess their complex nature. Despite established monographs and standards for quality control, processed products, established preparations in pharmacopeias and directives for identification, tests, assays, and definitions, etc., the information for clear differentiation of closely related, or similar species is not enough (Chan et al., 2009; Bauer and Franz, 2010; Li et al., 2019). The factors for high quality of herbal materials are furthermore very complex leading to technical challenges for regulatory authorities when formulating guidelines, resulting in different regulatory requirements across regions and countries (He et al., 2015; Wu et al., 2018). Besides, juristic and marketing differences among countries contribute to poor regulations and subsequently difficulties on quality assurance of herbal products (Ichim and Booker, 2021).

The development of comprehensive quality standards of CHMs and effective quality control procedures for authenticity testing and standard development of Chinese herbal materials is an ongoing challenge (Li et al., 2019; Leong et al., 2020). To better evaluate the complexity of CHMs, an integrative approach involving effective pharmacological methods, biological and chemical techniques is required. Conventional DNA barcoding systems have been adopted by national pharmacopeias, like the Chinese Pharmacopoeia, British Pharmacopoeia and Japanese Pharmacopoeia (Chen et al., 2017). DNA barcoding techniques, as described in the British Pharmacopoeia (British Pharmacopoeia Commission, 2018), are a valuable screening tool for raw, single species botanical materials. Metabarcoding techniques have been successfully used for ingredient profiling of commercial herbal products, which are composed of varying, processed botanical materials, such as CHMs and provide information about unknown ingredients (Coghlan et al., 2012; Arulandhu et al., 2017; Seethapathy et al., 2019; Anthoons et al., 2021; Zhu et al., 2022; Raclariu-Manolică, 2023a; Raclariu-Manolicăet al., 2023b). Chromatographic authentication gives high resolutions for the detection of target compounds of known ingredients and are basic authentication tools for herbal remedies (Liang et al., 2010; Booker et al., 2016; Heinrich et al., 2022).

The aim of this study was to develop a testing strategy for the authentication of processed herbal ingredients. We investigate different authentication methods looking at the independent CHM ingredients used in two formulae utilized in the context of gynecological health, *Gui Zhi Fu Ling Wan* and *Ge Xia Zhu Yu Tang*. Shared among them these formulae include 13 CHM ingredients corresponding to 17 plant species. We compare the results from authenticating 51 single CHM ingredients, using three different analytical techniques, high-performance thin-layer chromatography (HPTLC), DNA barcoding and DNA metabarcoding.

Materials and methods

Sample material

Fifty-one CHM ingredients were collected in 2019 from commercial TCM distributors and online retailers in Europe,

TABLE 1 Properties[a] of ingredients in Chinese Herbal Medicine formula *Gui Zhi Fu Ling Wan*.

Ingredients	Plant species	Quality indicators for crude materials	Processing method	Known substitutes/ adulterants
Pinyin: Gui Zhi Herbal drug name: Cinnamomi Ramulus	*Neolitsea cassia* (L.) Kosterm., syn.: *Cinnamomum cassia* (L.) J. Presl	Young twigs without leaves or any withered parts	Twigs are dry-fried or stir fried or baked with honey	*Cinnamomum austro-sinensis* H.T.Chang, *Cinnamomum bejolghota* (Buch.-Ham.) Sweet, *Cinnamomum burmanni* (Nees & T.Nees) Blume, *Cinnamomum heyneanum* Nees, *Cinnamomum japonicum* Siebold, *Cinnamomum subavenium* Miq., *Cinnamomum tamala* (Buch.-Ham.) T.Nees & C.H.Eberm., *Cinnamomum wilsonii* Gamble
Pinyin: Fu Ling Herbal drug name: Poriae Cocos	*Poria cocos* (Schw.) Wolf	Hard, solid, white, without inlays of soil, sticks	dry- frying or baking over low heat	Substitution/adulteration not common
Pinyin: Chi Shao Herbal drug name: Paeoniae Radix Rubra	*Peonia lactiflora* Pall. and *Paeonia anomala* subsp. Veitchii (Lynch) D.Y. Hong & K.Y. Pan	Long, thick, powdery roots with pale reddish or yellowish cross sections	Dry-frying, wine-frying, or vinegar- frying of slices	*Paeonia obovata* Maxim., *Paeonia obovata* var. willmottiae, *Paeonia anomala* var. intermedia, *Paeonia anomala* L. subsp. anomala *Paeonia mairei* H. Lev, *Sanguisorba officinalis*
Pinyin: Mu Dan Pi Herbal drug name: Moutan Cortex	*Paeonia suffruticosa* Andrews	Thick, white, starchy quills with xylem removed and strong aroma	Dry-frying, wine-frying, charred moutan (dry-fried or baked at high heat until blackened)	*Paeonia ostii* T. Hong & J. X. Zhang, *Paeonia delavayi* Franch., *Paeonia decomposita* Hand. -Mazz. subsp. decomposita, *Paeonia anomala* L. subsp. Veitchii (Lynch) D. Y. Hong& K. Y. Pan
Pinyin: Tao Ren Herbal drug name: Persicae Semen	*Prunus davidiana* Franch, *Prunus persica* (L.)	Large, flat cut full and closed seeds with white and oily kernels	Stripped peach kernel (clean kernels are boiled and coat rubbed off), ordry-frying, or defatting peach kernel (oils removed from cleaned kernel)	Substitution/adulteration not common

[a]Information retrieved from Bensky et al. (2004); Scheid et al. (2015); Leon and Yu-Lin (2017).

listing: Angelica Sinensis Radix (4), Aurantii Fructus (3), Carthami Flos (3), Chuanxiong Rhizoma (4), Cinnamomi Ramulus (3), Corydalis Rhizoma (4), Cyperi Rhizoma (10), Glycyrrhizae Radix (3), Linderae Radix (3), Moutan Cortex (4), Paeoniae Radix Rubra (4), Persicae Semen (4), and Poriae Cocos (2). Samples were sold as single ingredient TCM decoction pieces. The TCM products were imported into Norway for scientific analyses under Norwegian Medicines Agency license ref. no 18/13,493-2. The sample materials were ground and homogenized using an IKA Tube Mill 100 (IKA-Werke GmbH & Co. KG, Staufen, Germany). The sample materials are CHM ingredients of two formulae utilized in the context of gynecological health, *Gui Zhi Fu Ling Wan* (Table 1) and *Ge Xia Zhu Yu Tang* (Table 2). The tables provide an overview of the species, characteristics, and processing techniques of these CHM ingredients.

High-performance thin-layer chromatography (HPTLC)

Fifty samples were prepared. Sample with the code *Cyperi Rhizoma 10* is not included. HPTLC marker compounds and other chemicals were obtained from Merck (Darmstadt, Germany) and Sigma-Aldrich (St. Louis, MA, United States), and botanical reference standards were obtained from ChemStrong Scientific, China. Marker compounds and botanical reference standards were prepared according to Hong Kong Chinese Materia Medica guidelines (HK Chinese Materia Medica, 2021) unless reported differently. A specific HPTLC method was used for each plant, see Supplementary Data S1. Samples (0.50 g) were dissolved in 10 mL of ethanol, followed by sonication for 20 min and filtration using Merck Millex PES syringe filters (0.22 µm). Each TLC plate (silica gel 60 F_{254} Merck, Darmstadt, Germany) was visualized under white light and UV 254 and 366 nm. Marker compounds, samples and herbal references were spotted in bands of 8.0 mm width, using a CAMAG Linomat 5 instrument (CAMAG, Muttenz, Switzerland). The development distance differed depending on the ingredient being detected, as indicated in each testing method (Supplementary Data S1) and is calculated from the lower edge of the plate using CAMAG Automatic Developing Chamber (ADC 2). For derivatization, CAMAG derivatizer was used when the derivatizing reagent complied with CAMAG's guidelines, otherwise manual spraying was employed. Following development and derivatization, plates were visualized under white light, UV 254 nm and 366 nm using CAMAG's Visualizer (Muttenz, Switzerland). All data was acquired and processed using VisionCATS 2.1 software (CAMAG, Muttenz, Switzerland).

TABLE 2 Properties[a] of Chinese herbal ingredients in Chinese Herbal Medicine formula *Ge Xia Zhu Yu Tang*.

Ingredients	Plant species	Quality indicators for crude materials	Processing method	Known substitutes/ adulterants
Pinyin: Dang Gui Herbal drug name: Angelicae Sinensis Radix	*Angelica sinensis* (Oliv.) Diels	Thick, long main roots, yellowish brown, soft outer bark, yellowish white cross section, dense aroma	Dry-frying, wine-frying, earth-frying (with Terra flava usta), charred fried (dry-fried until surface is blackened) of slices	*Levisticum officinale* W.D. J. Koch *Angelica acutiloba* (Siebold& Zucc.) Kitag., *Angelica megaphylla* Diels, *Angelica gigas* Nakai, *Angelica polymorpha* Maxim., *Ligusticum glaucescens* Franch. and Hansenia forbesii (H.Boissieu) Pimenov & Kljuykov
Pinyin: Wu Yao Herbal drug name: Linderae Radix	*Lindera aggregata* (Sims) Kosterm	Tender roots with yellowish white, powdery cross section, intense aroma	Dry-frying	*Lindera obtusiloba* Blume
				Lindera aggregata var. *aggregata*
				Lindera umbellata Thunb
Pinyin: Yan Hu Suo Herbal drug name: Corydalis Rhizoma	*Corydalis yanhusuo* W.T. Wang	Large, full, hard, and brittle pieces with light yellow, waxy-like cross section	Dry-frying, wine-frying, vinegar- or salt-frying of slices	*Corydalis decumbens* (Thunb.) Pers. *Corydalis glaucescens* Regel
				Corydalis humosa Migo
				Corydalis turtschaninovii Besser
				Curcuma longa L
Pinyin: Chuan Xiong Herbal drug name: Chuanxiong Rhizoma	*Ligusticum stratium* DC.	Large, fleshy, solid, and heavy rhizomes with an intense aroma and a bitter, acrid taste turning slightly sweet	Dry-frying, wine-frying of slices	*Ligusticum chuanxiong* cv. Fuxiong *I*mmature rhizomes sold cheaper as inferior material. Occasional adulteration/ unofficial substitutes of rhizomes from varying plant species recorded in international trade
Pinyin: Gan Cao Herbal drug name: Glycyrrhizae Radix	*Glycyrrhiza inflata* Batalin *Glycyrrhiza uralensis Fisch*	Thin, tight, reddish-brown cork, solid and heavy cortex, yellowish white and powdery surface on cross section	Dry-fried, honey-prepared slices	Substitution/adulteration not common
	Glycyrrhiza glabra L			
Pinyin: Xiang Fu Herbal drug name: Cyperi Rhizoma	*Cyperus rotundus* L	Large, full, hard, and solid rhizomes with intense aroma	Processed (boiled with yellow rice wine/vinegar and dried in the sun, blackened (dry-fried or over heat until inside scorched yellow, outside black), dry-fired, four substance prepared (soaked and mixed with rice vinegar, yellow rice wine, cooked honey and salt/ ginger juice/boy's urine	*Cyperus stoloniferus* Retz
Pinyin: Hong Hua Herbal drug name: Carthami Flos	*Carthamus tinctorius* L	Long, dark, or fresh red soft flowers with intense aroma	Drying	Substitution/adulteration not common
Pinyin: Zhi Ke Herbal drug name: Aurantii Fructus	*Citrus* x *aurantium* L	Large fruit with greenish brown surface, hard soil texture, small thick pulp, fresh, aromatic fragrance	Dry-fried, charred fried (until scorched and blackened externally)	*Citrus maxima (Burm.) Merr*
				Citrus medica L
				Citrus trifoliata L

[a]Information retrieved from Bensky et al. (2004); Scheid et al. (2015); Leon and Yu-Lin (2017)). Overlapping ingredients of the two formulae: Moutan Cortex, Paeoniae Radix Rubra, Persicae Semen are not repeated, see Table 1.

For chemical fingerprints, all ingredients were named with herbal drug names. Each sample was compared to the fingerprint of the pharmacopeial botanical reference standards. To display the results, a band intensity score (BIS) with a scale from zero to five was visually assigned for all ingredients. Each band in the fingerprints was given a score from zero to five, based on the intensity perceived by the naked eye, compared to the standard, where zero is "not detectable", and five is the highest intensity. BISs with scales 0-5 refer to the quality of the ingredients' entire fingerprint with respect to the visibility and positions of bands for the pharmacopoeial reference marker compounds and botanical references in each chromatogram (Mück et al., 2023).

Conventional barcoding with Sanger sequencing

The DNA extraction kit E.Z.N.A SP plant DNA kit (Omega Biotek, Norcross, United States of America) was used according

to the manufacturer's instructions except for a larger quantity of starting material (up to 30 mg) and an elongated lysis with larger volumes of buffer in all steps prior to DNA binding to HiBind columns (e.g., 1.6 mL SP1 buffer at 65°C for 1 h). Samples were mixed frequently during incubation and the final elution volume was 100 µL. Extracted DNA was quantified and polymerase chain reactions were performed to amplify the two internal transcribed spacer regions of the nuclear ribosomal RNA with primers based on ITS1_17SE_F and ITS1_5.8I_R and ITS2_5.8I_F and ITS2_26SE_R (Sun et al., 1994). Expected amplicon sizes were approximately 600 bp for nrITS1 and 100-200 bp for nrITS2. Polymerase chain reactions (PCR) were carried out in 12,5 µL reactions consisting of 2.5 µL of template DNA, 6.25 µL of AccuStart II PCR ToughMix (AccuStart, Quantabio, MA, United States of America), 0.16 µM of each, forward and reverse primer. The PCR cycling protocol consisted of initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 10 s, annealing at 52°C for ITS1 and 59°C for ITS2 for 15 s, and elongation at 72°C for 1 min followed by a final elongation step at 72°C for 1 min. Gel electrophoresis was performed to check amplified DNA products. Trouble shooting was conducted to reduce amplification errors: For missing, - and double bands, DNA templates were diluted 50 times and annealing temperature was lowered for ITS1. PCR products were then treated with illustra ExoProStar 1-STEP (Cytvia, Marlborough, United States of America) with a modified protocol with 10 x dilution and 45 min incubation, and sent for Sanger sequencing (Macrogen, Amsterdam). Visualization and assessment of each obtained sequencing chromatogram was conducted using the software program Geneious by Dotmatics (Boston, MA, United States). Taxonomic assignment was performed via an optimized BLASTn search by selecting base calling score Q > 20 for unique top hits and verifying percent identity with a threshold >95% for identifications at genus level and 98% at species level. The results are presented in an overview of five categories, which include identification at species, genus and family level, such as unexpected identification of ingredients and failed identifications. All ingredients are listed with scientific binomials.

Dual locus metabarcoding

DNA extraction, PCR, normalization and pooling of amplicons, such as library preparation and sequencing was conducted according to Mück et al. (2023). The samples were sequenced and analyzed alongside the sample set presented in Mück et al. (2023). We used dual index fusion primers for amplicon libraries of the internal transcribed spacers nrITS1 and nrITS2, based on 18S-ITS1F and 58S-ITS1R (Omelchenko et al., 2019), and ITS2F and ITSp4 primers (Timpano et al., 2020). PCRs were run using indexing primers as in Raclariu-Manolică et al. (2023b) with applying the indexing strategy of Fadrosh et al. (2014).

Bioinformatic processes related to the metabarcoding analysis were done as described in (Mück et al., 2023). After applying strict filtering controls to delete any false positive detections for each sample, the taxonomic assignment step was conducted by selecting top-scored species as the target species (search tool: BLAST) (Yao et al., 2022). OTUs were checked for species delineation with ASAP, assembling species by automatic partitioning (Puillandre et al., 2021) unique species were pooled together to avoid overinflation of the observed species range. In the final presentation of results, taxonomic identification hits of ingredients are categorized into expected substitutes, expected ingredients at genus and species level, and unexpected ingredients.

Results

We performed a census for detected expected ingredient counts to compare the three methods. Here we pooled the results for nrITS1 and nrITS2 for DNA barcoding and for DNA metabarcoding, gaining a cumulative count which represents the performance of each method. Both identification hits at genus and species level were considered as expected ingredients. HPTLC analysis resulted in 49 positive identifications of expected ingredients, whereas traditional DNA barcoding yielded 16 positive identifications and DNA metabarcoding 33 (See Figure 1A.). In detail, with HPTLC, all ingredients were identified except for one sample of Linderae Radix (Linderae Radix 1), which was not identified across either of the methods. With DNA barcoding ten expected ingredients were identified at species level and another six at genus level. With metabarcoding four expected ingredients were identified at species level, including one, Linderae Radix (sample: Linderae Radix 3), that was not identified with DNA barcoding. Looking at identification hits for expected ingredients using DNA barcoding nrITS1 and metabarcoding nrITS1 separately, five expected ingredients were identified at species level with DNA barcoding nrITS1 and one at species level with metabarcoding nrITS1 (Figure 1B). Looking at identification hits for expected ingredients via DNA barcoding nrITS2 six expected ingredients were identified at species level, and three at species level with metabarcoding nrITS2 (Figure 1C) (For more information see Supplementary Data S2).

HPTLC

The band positions and visibility of the chemical markers of all ingredients appear with characteristic colors and Rf values. All botanical reference materials show clear chromatograms and all marker compounds were identified (Supplementary Data S3). With HPTLC, we obtained 49 positive identifications for expected ingredients across the sample range. One sample with the ingredient of Linderae Radix was not positively identified (see Figure 2). Ingredients refer to the accepted species under the ingredients as listed in (Chinese Pharmacopoeia Committee, 2020).

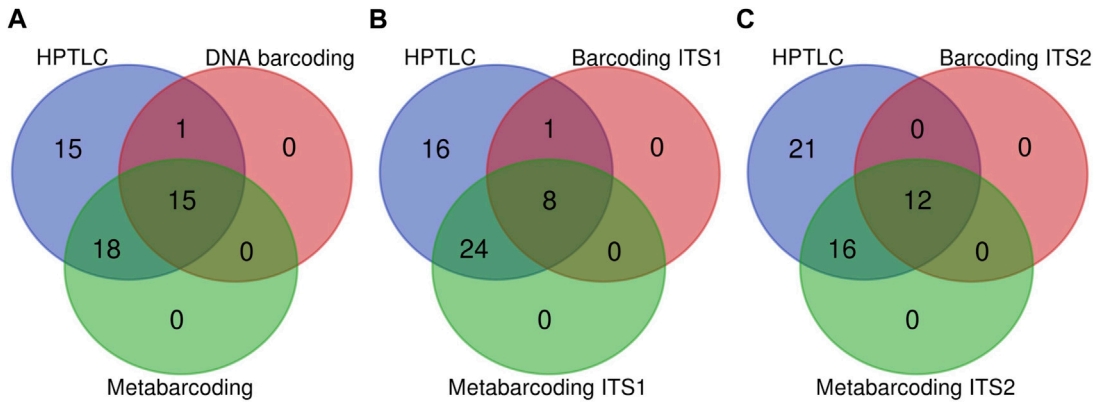

FIGURE 1
Three venn diagrams comparing HPTLC, DNA barcoding and DNA metabarcoding methods. **(A)** Pooled results for nrITS1 and nrITS2 for DNA barcoding and metabarcoding. **(B)** Comparison of identification hits for expected ingredients differentiating DNA barcoding nrITS1 and metabarcoding nrITS1. **(C)** Comparison of identification hits for expected ingredients differentiating DNA barcoding nrITS2 and metabarcoding nrITS2.

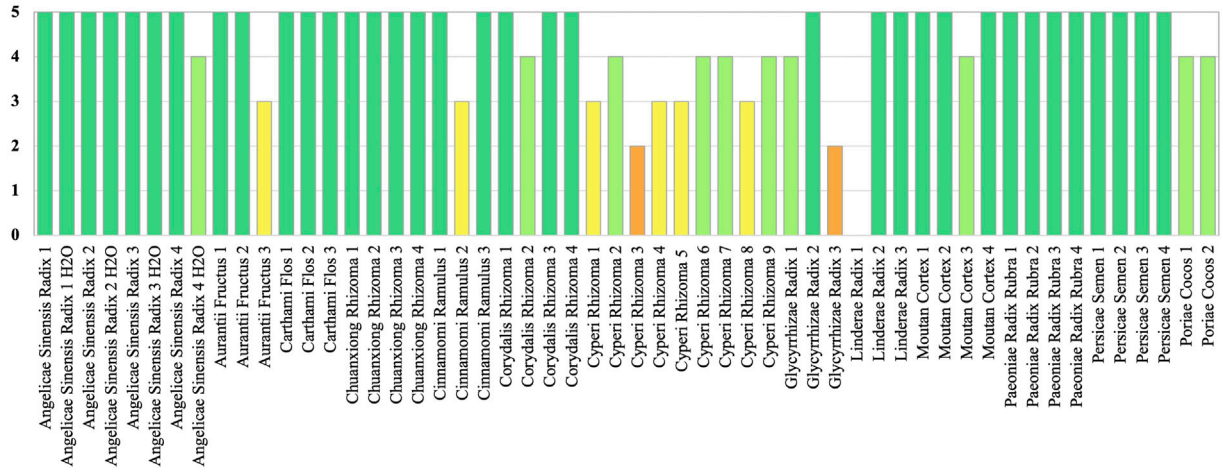

FIGURE 2
HPTLC results are visualized with band intensity scores (BIS, 0-5) for level of identification.

DNA barcoding with nrITS1 and nrITS2

Using traditional identification via DNA barcoding, sample analysis with nrITS1 resulted in nine positive identification hits for the target ingredient representing the sample. The analysis for nrITS2 resulted in 12 positive identification hits (See Supplementary Data S2). Five target ingredients were authenticated at species level with nrITS1, i.e., *Paeonia suffruticosa* (ingredient codes: Moutan Cortex 2), *Glycyrrhiza uralensis/inflata* (Glycyrrhizae Radix 3) and *Angelica sinensis* (Angelica Sinensis Radix 1, 2, 4). Furthermore, we identified *Paeonia suffruticosa* (Moutan Cortex 4), *G. uralensis/inflata* (Glycyrrhizae Radix 1 and 3) and *Ligusticum chuanxiong* (Chuanxiong Rhizoma 4) at genus level. The remaining ingredients yielded unexpected identification hits or failed completely due to poor amplification of primers nrITS1 or poor sequencing chromatograms. We identified six ingredients at species level with nrITS2, i.e., *G. uralensis/inflata* (Glycyrrhizae Radix 2, 3), *Corydalis yanhusuo* (Corydalis Rhizoma 2), *Citrus aurantium* (Aurantii Fructus 2), and *Carthamus tinctorius* (Carthami Flos 2 and 3). Six ingredients could furthermore be identified at genus level: *G. uralensis/inflata* (Glycyrrhizae Radix 1), *L. chuanxiong* (Chuanxiong Rhizoma 4), *Paeonia lactiflora/anomala* (Paeoniae Radix Rubrae 3), and *Paeonia suffruticosa* (Moutan Cortex 1, 2 and 3). In contrast to identification with nrITS1, with nrITS2 no ingredients were identified for *A. sinensis*. For both markers, identifications of ingredients with *Lindera aggregata* (Linderae Radix) failed. Results are illustrated in Figure 3.

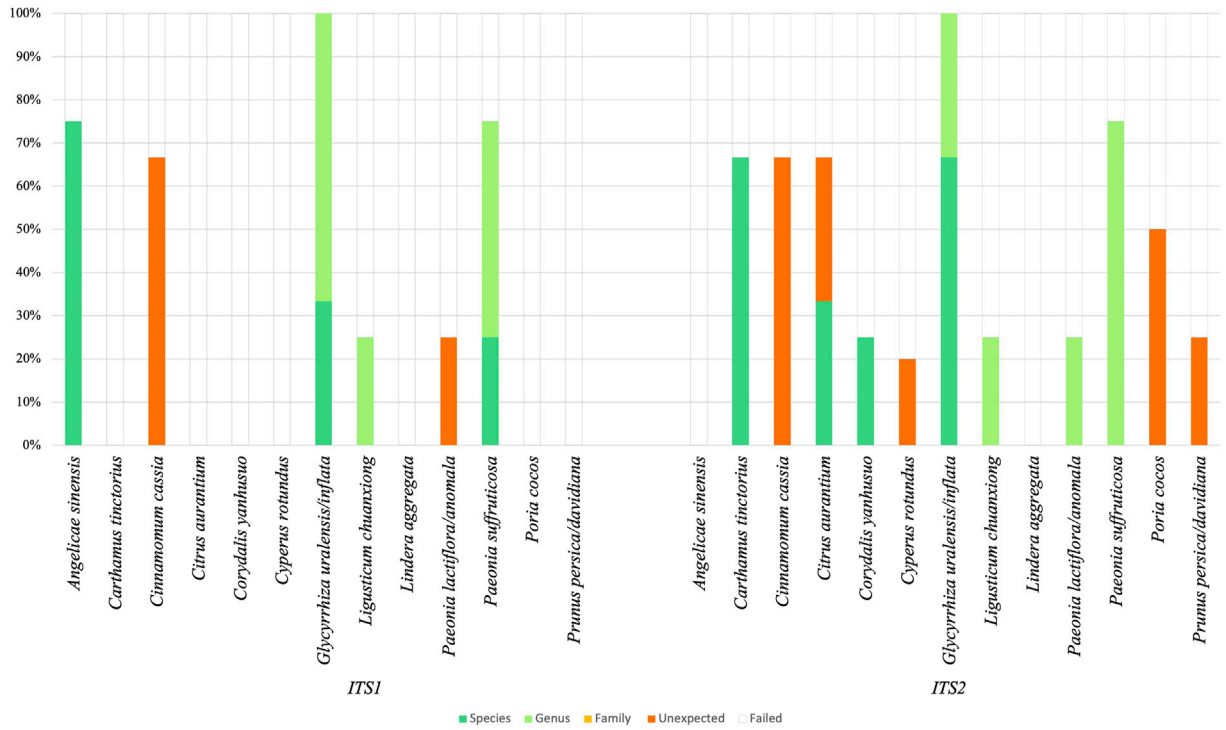

FIGURE 3
DNA barcoding results are presented in an overview of five categories: Identification at species (dark green), genus (light green) and family level (yellow), unexpected identifications (orange) and failed identifications (remainder). Ingredients are listed with scientific binomials.

Metabarcoding with nrITS1 and nrITS2

A dataset with 2 258 042 reads was obtained for nrITS1 with an average of 13,685 reads per sample. Respectively, a dataset with 4 873, 864 reads was obtained for nrITS2 with an average of 29,361 reads per sample. For nrITS1, one sample failed to pass the bioinformatic trimming and filtering criteria, for nrITS2 four samples did not pass the criteria and these samples were excluded from the final results. Operational taxonomic units (OTUs) could be assigned for all samples for markers nrITS1 and nrITS2. The raw dataset of nrITS1 contained 508 OTUs, and after applying strict quality selection criteria and pooling 73 unique species were identified. The raw dataset of nrITS2 contained 347 OTUs and 53 species were identified after applying the quality criteria. The sample analysis using nrITS1 resulted in 18 samples with only unexpected identification hits and 32 samples with at least one positive identification hit for the expected ingredient. The analysis using nrITS2 resulted in 22 samples with only unexpected identifications and 28 samples with at least one positive identification hit (See Supplementary Data S2).

Using nrITS1, ten expected plant taxa and one substitute species could be identified, while three target ingredients were not detected. Proportionally to detected species abundance in one sample, 14.8% of Paeoniae Radix Rubra could be identified at genus level, 26.7% of Moutan Cortex at genus level, 5.6% of Linderae Radix at species level, 50% of Glycyrrhizae Radix at genus level, 8.3% of Cyperi Rhizoma at genus level, 11.1% of Chuanxiong Rhizoma at genus level, 20% of Carthami Flos at genus level, 16.7% of Aurantii Fructus at genus level and 21.4% of Angelica Sinensis Radix at genus level. Fifty-seven percent of Persicae Semen was identified as a substitute species. Poriae Cocos, Corydalis Rhizoma and Cinnamomi Ramulus were not detected in their corresponding samples. The results for nrITS1 are visualized with the total species abundance across all samples in Figure 4.

Using nrITS2, nine target ingredients and one substitute species could be identified, while four target ingredients were not detected. More specifically, each could be identified at genus level at the following percentages, Persicae Semen (63%), Paeoniae Radix Rubra (10.1%), Moutan Cortex (40%), Glycyrrhizae Radix (75%), Corydalis Rhizoma (17.7%), Carthami Flos (33.3%), and Aurantii Fructus (10%). Angelica Sinensis Radix could be identified 20% at genus level and 20% at species level, and Chuanxiong Rhizoma 15.4% at genus level and 7.7% at species level. Aurantii Fructus was revealed as an expected substitute in 5% of Aurantii samples. Poriae Cocos, Linderae Radix, Cyperi Rhizoma and Cinnamomi Ramulus were not detected in their corresponding samples. The results for nrITS1 are visualized with the total species abundance across all samples in Figure 5.

Discussion

Herbal medicines and dietary supplements, as well as their raw ingredients, pose a variety of challenges for quality control. Most pharmacopoeias are focused on analytical chemical methods for

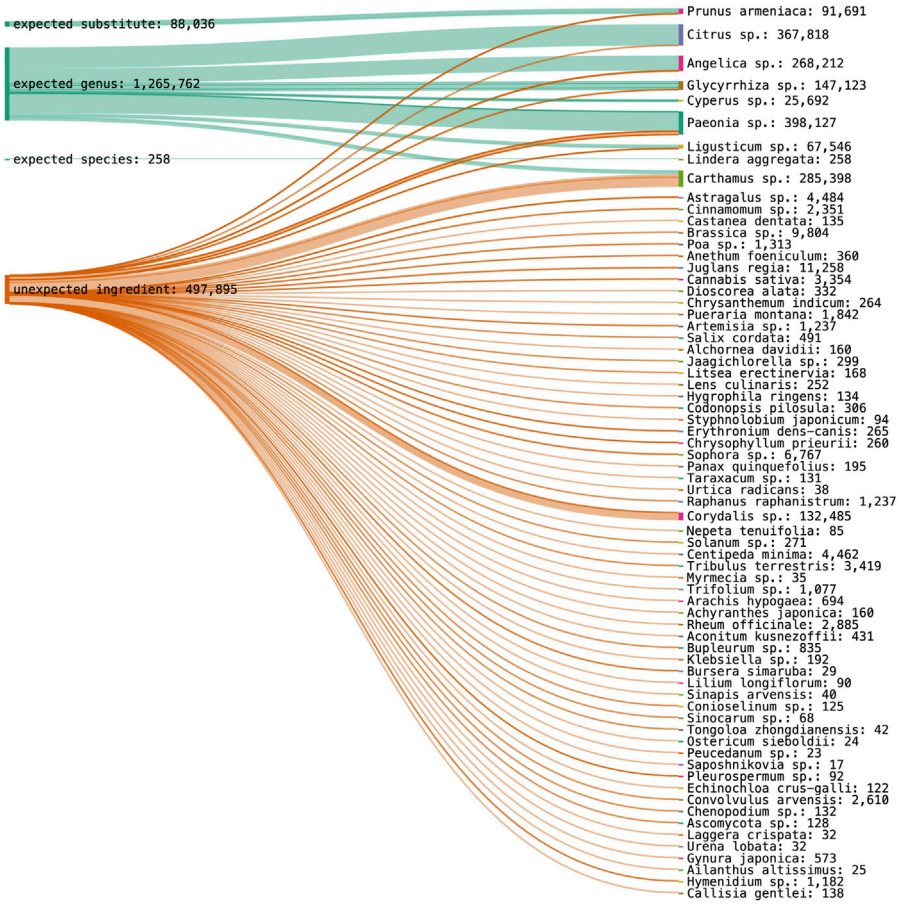

FIGURE 4
DNA metabarcoding results for nrITS1 grouped by identification class (expected substitute, expected genus, expected species, unexpected ingredient) combined with the detected species richness in read abundance.

quality control based on detection of target compounds. More recent studies have shown that other chemical approaches yield more detailed and complementary insights, which also allow for the detection of contaminants and adulterants at low levels. The development of complex chromatographic, spectroscopic and hyphenated methods has contributed to the expansion and development of pharmacopoeial monographs, and responded to regulatory demands and expectations of herbal quality on the market (Fitzgerald et al., 2019). HPTLC is the most advanced and robust form of thin-layer chromatography (TLC) in applied herbal quality control (Velho-Pereira et al., 2011; Nicoletti et al., 2013; Booker et al., 2016; 2014; Nandanwadkar et al., 2016; Raclariu et al., 2018; Kandil et al., 2022). It offers good resolution, detection sensitivity, and enhanced *in situ* densitometric quantification compared to conventional TLC (Ram et al., 2011; Sonia, 2017; Bhusan Champati et al., 2023) and can be applied simultaneously in an efficient and economical manner (Kunle, 2012; Sonia, 2017; Kandil et al., 2022). Furthermore, emerging research trends are centered around gas chromatography (GC), mass spectroscopy (MS), UV/visible spectrophotometric techniques, nuclear magnetic resonance (NMR) and tandem approaches (Booker et al., 2014; 2016; Liang et al., 2018), which allow for a more complex analysis and characterization of single compounds into parts per billion range (MS) and give detailed fingerprints of metabolites across varying polarities (NMR) (Fitzgerald et al., 2019). Recent studies furthermore propose a combination of multidimensional chromatography with chemometric methods, which exhibit stronger capacity for screening and separating bioactive compounds in complex TCM samples (Yang et al., 2023). For the detection of authenticity issues and compound adulteration, specifically chromatography, its tandem technologies and combinations with exploration/classification/regression algorithms, are relatively mature and widely applied (Liu et al., 2023). Additionally, innovative approaches emerge from modern analytical approaches, such as metabolomics (Heinrich, 2015; Emwas et al., 2019; Xiao et al., 2022; Nikolaichuk et al., 2023). Furthermore, new possibilities arise for the establishment of a more elaborate herbal quality control system by developing quality markers for comprehensive fingerprint and multicomponent analysis and novel standardization practices of herbal materials (Zhang, 2016; Liu et al., 2023; 2018; 2017; Liu, 2017; Yang et al., 2017; Bai et al., 2018; Li et al., 2019; He and Zhou, 2021; Noviana et al., 2022; Kaggwa et al., 2023).

In conjunction with chemical fingerprinting, researchers have gradually turned their focus to DNA based molecular methods, which are recognized as techniques to identify edible and medicinal plant species, and also to detect their substitutes and adulterants in crude or processed products (Raclariu et al., 2017; Raclariu-

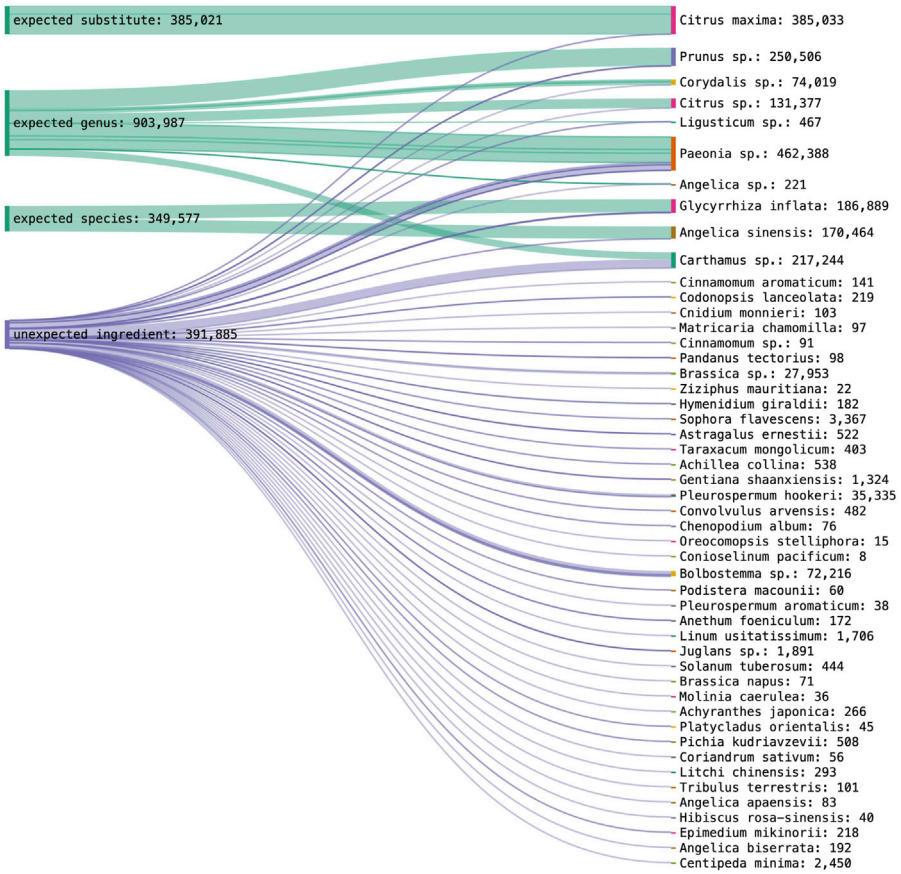

FIGURE 5
DNA metabarcoding results for nrITS2 grouped by identification class (expected substitute, expected genus, expected species, unexpected ingredient) combined with the detected species richness in read abundance.

Manolică et al., 2023b). These methods are independent of species life stage, tissue type, or the physiological conditions of constituents (Techen et al., 2014; Lo and Shaw, 2018b; Hao and Xiao, 2020) and can discriminate botanicals at species level (Besse et al., 2021; Mishra et al., 2016). As such, DNA barcoding is used to qualitatively authenticate herbal medicines by validating the identity of the corresponding species in industrial quality control procedures (Raclariu et al., 2018; Zhu et al., 2022). With most traditional methods, it is difficult to identify crude herbal drug material on species level, but with the help of DNA barcoding, pharmacopoeial monographs of many medicinal plant species could be advanced for accurate, reliable and effective species identification (Song et al., 2009; Chen et al., 2017; Zhu et al., 2022). Conventional DNA barcoding has been adopted by various national pharmacopeias (Chen et al., 2017). User friendly and accessible tools have emerged for correct species assignment with DNA barcoding alongside its establishment as a regulatory post quality control method for herbals, like the Medicinal Materials DNA Barcode Database (MMDBD) (Wong et al., 2018). However DNA barcoding is exclusively fit for unprocessed and single species plant materials, which have not been exposed to processing techniques resulting in DNA degradation (Raclariu et al., 2018). Evolving high-throughput sequencing techniques, like DNA metabarcoding overcome limitations of conventional DNA barcoding and are used for investigation of total species diversity and non-targeted species in processed herbal products (Arulandhu et al., 2017; Raclariu et al., 2018; Omelchenko et al., 2019; Seethapathy et al., 2019; Anthoons et al., 2021; Raclariu-Manolică and de Boer, 2022; Raclariu-Manolică et al., 2023a; Mück et al., 2023). More recent genomics approaches, including genome skimming and shotgun metagenomics, have the potential to overcome limitations of PCR-based methods, like PCR biases due to primer mismatch, limited number of applicable barcodes, limited DNA degradation, and are expected to yield higher discriminatory power (Wu and Shaw, 2022; Raclariu-Manolică, 2023a). High operational costs currently limit wider application of genomics approaches (Manzanilla et al., 2022), and to date only a few metagenomic studies have been conducted in the field of herbal authentication (Xin et al., 2018; Handy et al., 2021). Xin et al. (2018) performed shotgun sequencing of CHMs to obtain the barcode regions ITS2, psbA-trnH, and matK. Barcoding techniques can further be used in conjunction with metabolomics, transcriptomics or proteomics (Mishra et al., 2016; Raclariu-Manolică et al., 2023b). The possibility to process data via multivariate analysis, pattern recognition and metabolomics then gives a broader scope for applications in medicinal plant analysis and the spectrum of compounds found within medicinal plants (Feng et al., 2018; Nöst et al., 2019; Kaigongi et al., 2020; Raclariu-Manolică et al., 2023b). Modern analytical technologies combined

with chemometrics are increasingly used for quality monitoring of medicinal plant matrices, but it is still challenging to choose the adequate type of analysis and statistical method as this is highly dependent on the specific authenticity issue (Liu et al., 2023). Besides, advanced genetic and chemical methods often require high analytical skills, are time consuming and expensive, are not always applicable to all natural compounds or biological materials and may not be suitable for general quality control. Since different quality control methods for herbal products yield different information, an integrated and practical authentication strategy is needed. We find that by establishing a multi-tiered quality control strategy using chemical and genetic methods, such as HPTLC, followed by DNA barcoding and metabarcoding, authentication procedures of processed herbal ingredients can be optimized (Li et al., 2017; Raclariu et al., 2017; Raclariu et al., 2018). First, chemical fingerprinting verifies the presence or absence of analytical marker compounds in sample materials and sheds light on the overall authenticity of plant materials. By evaluating and displaying a band intensity score (BIS) for chromatographically analyzed herbs (Mück et al., 2023), we can furthermore speculate on qualitative information such as cultivation and storage conditions, as chromatographic fingerprinting technique is useful for the evaluation of authentication, quality and investigation of consistency and stability of herbal drugs and can be useful at all stages of the herbal supply chain (Liang et al., 2010; Raclariu et al., 2017). It enables a qualitative profile, such as detection of low-quality aspects of phytochemical contents and significant product-to-product variation (Ichim and Booker, 2021). This is an important consideration for applying authentication procedures, as adulteration and substitution may occur by means of using other, cheaper plant parts (Ichim and de Boer, 2020). By virtue of choosing HPTLC as the primary selective method for a tiered authentication strategy, we optimize inclusion of all samples, including those that have lost their diagnostic microscopic characteristics, or where DNA cannot be recovered (Ichim and Booker, 2021). At the second tier, DNA barcoding reveals the presence or absence of target DNA, and provides a definite answer on the possibility of identification via the quality of genetic sequencing chromatograms (Patel et al., 2018; Raclariu et al., 2018). Thus, it indirectly gives information on the level of processing of herbal materials. At the third tier, with the help of metabarcoding more qualitative aspects can be inferred and to help with creating transparency along the supply chain of herbal products. Detected species diversity with metabarcoding yields an important insight, and the potential to check the integrity of plant ingredients and receive an approximation for qualitative information, like harvesting, storage and processing conditions, as well as conservation issues around wild harvesting of medicinal plants (Staats et al., 2016; Arulandhu et al., 2017; Raclariu et al., 2017; Raclariu et al., 2018; Seethapathy et al., 2019; Turon et al., 2019; Anthoons et al., 2021; Raclariu-Manolică et al., 2023a) (See Figure 6).

Similar applied strategies combining chromatography and DNA barcoding technique for herbal authentication have been previously assessed. A study by Raclariu et al. (2017) shows that HPTLC can be efficiently applied for the detection of target compounds in *Echinacea* products, while DNA metabarcoding complements the analysis by detecting non-targeted species in these herbal products and gives information on species not listed as ingredients. In another study by Seethapathy et al. (2018), DNA barcoding was coupled with NMR and suggested as a regulatory tool for the authentication of *Garcinia* fruit rinds and food supplements. While DNA barcoding gives information on the level of adulteration, NMR provides quantitative information on target chemical constituents. Handy et al. (2021) applied DNA metabarcoding and genome skimming, coupled with HPLC–UV analysis in a more advanced approach to assess 20 dietary supplements of Echinacea. Genome skimming was found to be more effective than DNA metabarcoding for species-level authentication within the Echinacea genus and might be used instead of metabarcoding once its application is more economical for applied herbal quality control. The trend in TCM chemical quality control is towards establishing chemometric applications based on the data gathered from different quality control methods (Liu et al., 2023; Yang et al., 2023). A review by Kandil et al. (2022) on advances in quality control of fenugreek seeds, highlights that chromatography, like HPTLC and DNA-based methods, like DNA barcoding and the NGS when coupled to multivariate analysis, can yield promising results in herbal quality control.

This proposal for a multi-tiered quality control strategy using chemical and genetic methods, such as HPTLC followed by DNA barcoding and metabarcoding, allows for the integration of multifaceted information on the quality of diverse herbal medicine matrices, like CHMs. Its advantage and novelty lies in the accessibility and informative resolution for the applied and regulatory sector. By combining these methods, we take a stride towards establishing an herbal quality control system, which can be further enhanced by developing multivariate indices assessing the combination of analytical outcomes.

In this study, by applying a band intensity score (BIS) (Mück et al., 2023), we were able to grade qualitative characteristics and to differentiate between the quality of identification amongst ingredients with a scale from zero to five (see Figure 2). The variation determination of common analytes in the set of chromatographic fingerprints could provide useful qualitative and quantitative information on the characteristic components of herbal medicines investigated. Nevertheless many analytical chemistry based methods are sensitive to fraud through adulteration of ingredients (Gafner et al., 2023). Furthermore, HPTLC meets challenges when comparing a number of botanicals from different source materials and unilateral standardization of methods for TCM preparations is difficult. This is because concentrations for chemical marker compounds can be different for botanical materials of varying origin and natural fluctuations in chemical compounds can occur for different growth cycles, eco-regions, and times of the year (Yamamoto, 1988; Tobyn et al., 2011). Besides, TCM ingredients can originate from varying accepted plant species, whilst the standard identification method in the pharmacopeia remains the same for either species (Mück et al., 2023). Moreover, the varying processing techniques of CHMs can be different for ingredients of the same plant species and may alter the chemistry of compounds (Wang et al., 2019) (see Tables 1, 2).

Authentication via DNA barcoding resulted in poor identification for most samples. This could be due to the processing state of sample materials, e.g., processing of decoction material resulted in contamination with other plant material and extensive DNA degradation. DNA target amplification (Sun et al., 1994) yielded poor results with messy and or overlaying sequencing chromatograms (Patel et al., 2018). NrITS2 performed better than

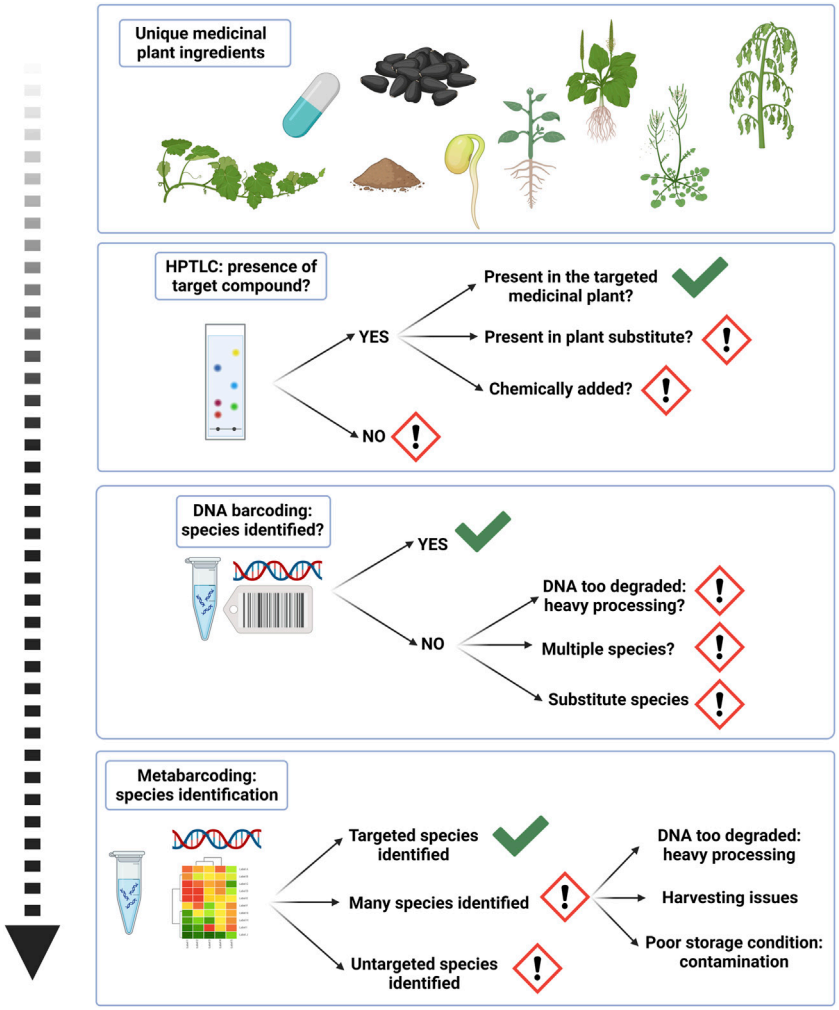

FIGURE 6
A tiered authentication strategy for processed herbal ingredients yields cumulative insight in ingredient quality.

nrITS1 for expected identification hits via Sanger sequencing and for metabarcoding in terms of expected species hits with number of taxon assigned OTUs. The internal transcribed spacer region ITS2 is known as an efficient sequence for taxa identification in comparison to the full-length ITS and has been extensively used for the identification of medicinal plants (Gao et al., 2010; Han et al., 2013). Both ITS1 and ITS2 can provide comprehensive species identification for molecular analysis of TCMs at lower taxa levels (Chen et al., 2017; Yao et al., 2022; Zhu et al., 2022). The combination of nrITS1 and nrITS2 may be used in a cumulative approach to receive enhanced ingredient information (Zhu et al., 2022). Interestingly, for metabarcoding with nrITS1 the genus *Cyperus* could be identified, while it was not detectable with nrITS2. With nrITS2 we could identify two ingredients, *A. sinensis* (Oliv.) Diels and *Glycyrrhiza inflata* Batalin, while with nrITS1, we identified *L. aggregata* (Sims) Kosterm. The species abundance of unexpected ingredients varied between nrITS1 and nrITS2. On another note, this highlights that biological DNA-based assessments are highly dependent on well-curated nucleotide sequence repositories (Taberlet et al., 2007; Howard et al., 2020). Shortcomings of barcoding exist due to gaps in reference databases for DNA markers (Zhu et al., 2022) and challenges with delimitating species through delimitation models (Taylor and Harris, 2012; Howard et al., 2020; Puillandre et al., 2021). Another methodological challenge for DNA-based identifications are several plant compounds including polysaccharides, polyphenols, lipids, essential oils, alkaloids and other secondary metabolites frequently found in medicinal plant species and their processed counter parts, which can interfere with DNA extraction and PCR amplification (Porebski et al., 1997; Shepherd and McLay, 2011; Sudan et al., 2017). Interference from those compounds and processing techniques of source material can lead to false-negative results (Mück et al., 2023). Thus, well-established DNA extraction procedures are crucial when dealing with complex, poly-herbal samples (Corrado, 2016; Lo and Shaw, 2018a; 2018b). Medicine processing techniques, like traditional *Pao Zhi* in TCM, then affects the DNA quality drastically and is a common cause for highly degraded DNA in CHMs. In detail, *Pao Zhi* affect the DNA quality through processes like roasting, baking, stir-frying, and the application of liquid or solid excipients (Engelhardt et al., 2018; Wu et al., 2018) (see Tables 1, 2). Hence, we assume that the main trade-offs for molecular authentication of TCMs are degraded and fragmented DNA, which cannot be amplified and assessed with the common barcoding primers for ITS1 or ITS2. Shorter mini-barcodes can lack

the discriminatory power to identify samples on species level (Di Bernardo et al., 2007; Lo and Shaw, 2019). Our results also suggest false-positive reads from minimal contaminations of other species, which is common in pharmacy preparation rooms and usually don't have a negative impact on quality, safety, and efficacy of the ingredients (Mück et al., 2023). Monographs on "herbal drugs" of the European Pharmacopoeia allow for up to 2% of foreign matter unless differently stated in a specific herb monograph (EMA, 2006; EMA, 2011). Overall, DNA metabarcoding is limited by the quality, processing state, or product type of isolated material, the DNA purification procedure, primer choice, amplification procedure, library preparation, sequencing technique, bioinformatic filtering and qualitative and clustering thresholds (Raclariu-Manolică, 2023a).

Conclusion

Different authentication methods yield different insights into CHM quality. HPTLC is very useful for identification of individual CHM ingredients and was shown to be less affected by heavy processing techniques commonly applied in TCM. DNA barcoding is a suitable method for the identification of raw botanical materials prior to processing, but not equally applicable in assessing processed ingredients. DNA metabarcoding can be used for the authentication of herbal end products, post-marketing control and pharmacovigilance, and determining species composition in botanical medicines, such as TCMs, but yields positives that are hard to interpret without quantitative data. Current authentication, standardization and quality control procedures for herbal products and TCM preparations have shortcomings in inferring aspects of safety, purity and efficacy. In turn, we show that a tiered quality control strategy via HPTLC, followed by DNA barcoding and metabarcoding yields cumulative insights and overcomes limitations of each method. The diversity of standards on scope, requirements, definition and terminology of dietary supplement and herbal medicine categories is a strong argument for transparent science-based quality standards across regulations to increase quality along the growing supply chain. Herbal authentication needs to be expanded based on the standardization and verification of the entire framework for herbal quality control. Advancing and evolving conventional and emerging safety and quality assessment methods for herbal preparations is in the strong interest for both consumers, producers and regulators. A future perspective in TCM quality control may lead to advanced functional network pharmacology studies, where multi-omics, chemical information analysis, data-mining, and network toxicology are included.

Data availability statement

The data presented in the study are deposited in Zenodo repository, accession numbers: https://zenodo.org/doi/10.5281/zenodo.10204233, https://zenodo.org/doi/10.5281/zenodo.10204282, https://zenodo.org/doi/10.5281/zenodo.10204309, https://zenodo.org/doi/10.5281/zenodo.10204326.

Author contributions

FM: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing–original draft, Writing–review and editing. FS: Data curation, Formal Analysis, Investigation, Resources, Software, Writing–review and editing, Methodology. QM: Formal Analysis, Software, Visualization, Writing–review and editing. BT: Formal Analysis, Writing–review and editing. HW: Supervision, Writing–review and editing. HB: Conceptualization, Funding acquisition, Methodology, Project administration, 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.

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2024.1353434/full#supplementary-material

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Anti-diabetic and anti-inflammatory bioactive hits from *Coriaria intermedia* Matsum. stem and *Dracontomelon dao* (Blanco) Merr. & Rolfe bark through bioassay-guided fractionation and liquid chromatography-tandem mass spectrometry

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Women have been found to be at a higher risk of morbidity and mortality from type 2 diabetes mellitus (T2DM) and asthma. α-Glucosidase inhibitors have been used to treat T2DM, and arachidonic acid 15-lipoxygenase (ALOX15) inhibitors have been suggested to be used as treatments for asthma and T2DM. Compounds that inhibit both enzymes may be studied as potential treatments for people with both T2DM and asthma. This study aimed to determine potential anti-diabetic and anti-inflammatory bioactive hits from *Coriaria intermedia* Matsum. stem and *Dracontomelon dao* (Blanco) Merr. & Rolfe bark. A bioassay-guided fractionation framework was used to generate bioactive fractions from *C. intermedia* stem and *D. dao* bark. Subsequently, dereplication through ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) and database searching was performed to putatively identify the components of one bioactive fraction from each plant. Seven compounds were putatively identified from the *C. intermedia* stem active fraction, and six of these compounds were putatively identified from this plant for the first time. Nine compounds were putatively identified from the *D. dao* bark active fraction, and seven of these compounds were putatively identified from this plant for the first time. One putative compound from the *C. intermedia* stem active fraction (corilagin) has been previously reported to have inhibitory activity against both α-glucosidase and 15-lipoxygenase-1. It is suggested that further

studies on the potential of corilagin as an anti-diabetic and anti-inflammatory treatment should be pursued based on its several beneficial pharmacological activities and its low reported toxicity.

KEYWORDS

anti-diabetic, anti-inflammation, enzyme inhibition, dereplication, LC-MS/MS, α-glucosidase, 15-lipoxygenase-1, terrestrial plants

Introduction

Diabetes is a chronic disease that is characterized by abnormally high plasma glucose levels. This is caused by the body's inability to produce insulin or use insulin properly (Kaul et al., 2023). Type II diabetes (T2DM) is the most common form of diabetes, characterized by the body's inability to synthesize and secrete insulin, which prevents the intake of glucose and leads to the subsequent rise of blood glucose levels. On the other hand, asthma is a chronic disease of the lungs affecting people from all age groups, caused by inflammation and the tightening of the muscle around the airways (World Health Organization, 2023). It is characterized by airway obstruction and hypersensitivity, thickening of the airway wall, and increased hypersecretion of mucus (Liu et al., 2023). Combined, diabetes and asthma affect hundreds of millions of people around the world annually (Chowdhury et al., 2021; Sun et al., 2021; Diabetes, 2023).

Women are disproportionately affected by T2DM and asthma compared to men (Klein et al., 1999; Adair et al., 2018; Naeem and Silveyra, 2019; Pennington et al., 2019). There is also a shift observed in asthma prevalence and mortality rates in males and females over time, which suggests a role played by hormonal changes during puberty as well as the interaction of various socioeconomic and health factors (Naeem and Silveyra, 2019; Chowdhury et al., 2021). Therefore, it is crucial to develop new therapeutic agents for the treatment and/or management of T2DM and asthma, especially for women.

Lifestyle modifications are prescribed and several classes of oral medications are used to manage and treat T2DM (Padhi et al., 2020; Reed et al., 2021). One such class of medications used to treat T2DM are called α-glucosidase inhibitors. On the other hand, asthma is most commonly managed using a combination of multiple medications (García-Menaya et al., 2019). In a recent review (Xu et al., 2021), it was mentioned that human arachidonic acid 15-lipoxygenase (ALOX15) inhibitors have the potential to be used as treatments for airway inflammatory diseases, including asthma. ALOX15 and its metabolites have been suggested to also play a role in T2DM and the complications that arise from it (Singh and Rao, 2019; He et al., 2023), implying that ALOX15 inhibitors may be explored as a possible treatment for T2DM and/or its complications. Several compounds and plant extracts that inhibit α-glucosidase or ALOX15 have already been studied (Kumar et al., 2011; Sadeghian and Jabbari, 2015). However, there might be value in exploring compounds/extracts that inhibit both enzymes as possible sources of new treatments for T2DM and asthma.

The immense biodiversity of terrestrial plants makes them a rich source of chemically diverse secondary metabolites with promising biological activities (Atanasov et al., 2015). Given the rich biological niche of the Philippine archipelago, its flora represents an untapped chemical space with the potential to yield novel drug leads. Several Philippine plants, such as *tuway-tuway* (*Bidens pilosa* L.), *takip-kohol* (*Hydrocotyle asiatica* L.), and *gumamela* leaves (*Hibiscus rosa-sinensis* L.), have been used in traditional medicine to treat diabetes and inflammation. The medicinal properties of these plants are likely attributed to a variety of phytochemical constituents, including alkaloids, glycosides, and triterpenoids (Clemen-Pascual et al., 2022).

The current study is part of a government-funded research program called the *Tuklas Lunas* Research & Development program, previously known as the Discovery and Development of Health Products program (Philippine Council for Health Research and Development, Department of Science and Technology, 2024). The program aims to produce medicines from Philippine biodiversity by pursuing two tracks of drug development in parallel: (1) "the development of standardized herbal drugs", and (2) "the identification and characterization of high-value purified active compounds from marine and terrestrial sources for specific therapeutic indications". The initial phase of the program involved pre-screening 2,400 plant extracts from different regions of the Philippines against different enzyme-based bioassays. Cytotoxicity testing on the active extracts was then conducted and only the noncytotoxic extracts were further evaluated.

As part of our ongoing efforts to identify bioactive hits from Philippine plant extracts, we focused on exploring the bioactivity of two Philippine plants: *Coriaria intermedia* Matsum., and *Dracontomelon dao* (Blanco) Merr. and Rolfe. These two plants were chosen to be pursued for further studies because of their significant bioactivity in preliminary studies. The crude methanolic extracts of both *C. intermedia* stem and *D. dao* bark exhibited high levels of activity against α-glucosidase and 15-lipoxygenase-1. The crude extracts from both plants were also shown to be noncytotoxic in preliminary cytotoxicity assays and they were chosen as priority extracts based on the results of the cell-based orthogonal assays conducted by one of the program collaborators.

Coriaria intermedia Matsum., commonly known as *beket* in Tagalog, is a tree native to the Cordillera Central Range of the northern Philippines (Guron and Napaldet, 2020). Plants from this genus are found in a variety of geographic locations, including Western North America and East Asia (Good, 1930). In Taiwan, *C. intermedia* has been traditionally used for treatment of gastrointestinal disturbance, rheumatism, and uterus cancer (Chang et al., 1996). The leaves of the plant were found to contain a variety of natural products, including 20-epibryonolic acid, coriamyrtin, ursolic acid, 3,3′-dimethyl ether, naringenin, β-tutin and phytosterols. Methanolic extracts from the leaves and

flowers of *C. intermedia* exhibited significant antimicrobial activity (San Luis et al., 2014).

Dracontomelon dao (Blanco) Merr. and Rolfe, locally known as *paldao* or *maliyan*, is a deciduous tree under the family Anacardiaceae, and is widely distributed in the Philippines, Thailand, Myanmar, Cambodia, and southern China (Dapar, 2021). It holds significant ethnopharmacological value due to its diverse medicinal applications. In China, its bark is traditionally used to treat skin ulcers and other infectious diseases (Li et al., 2017). Similarly, in the Philippines, its bark is widely used as a traditional medicine to address sore throats, toothaches, and even as a relief for women who underwent labor (Peña et al., 2019). *D. dao* leaves were previously found to contain phytyl fatty acid esters, long-chain fatty alcohols, and long-chain hydrocarbons. Various secondary metabolites have also been isolated from its leaves, including anacardic acid, phytol, and β-sitosterol (Ragasa et al., 2016). In addition, its twigs were found to contain linoleic acid, cardanols, stigmasterol, anacardic acid, and monoacylglycerol, further exemplifying its rich phytochemical diversity (Ragasa et al., 2017). The solvent partition fractions from the methanolic extracts of the leaves, stem, root, and bark of *D. dao* exhibited a significant level of broad-spectrum antimicrobial activity (Khan and Omoloso, 2002). The ethyl acetate partition fraction of the ethanolic extract of *D. dao* leaves was also found to exhibit wound healing effects on bacterially infected wounds in rats (Wen et al., 2022).

Our group's research involves the discovery of bioactive compounds from terrestrial plants using a bioassay-guided fractionation framework. Bioassay or bioactivity-guided fractionation is a common strategy used in natural products research (Hubert, Nuzillard, and Renault, 2015). However, bioassay-guided fractionation is often time- and resource-intensive. To reduce the amount of time required to discover new bioactive natural products, it is suggested that a dereplication procedure be involved in the workflow.

Dereplication refers to the rapid identification of secondary metabolites that have previously been identified (Hubert, Nuzillard, and Renault, 2015). There have been significant improvements in metabolite profiling methods due to the introduction of ultra-high-performance liquid chromatography (UHPLC) and the development of benchtop high-resolution mass spectrometry detectors (Allard et al., 2016) which are able to produce high quality tandem MS data. Coupled with molecular networking platforms such as the Global Natural Products Social Molecular Networking (GNPS), which can efficiently identify secondary metabolites using tandem MS data (Yang et al., 2013), the dereplication of natural products can be expedited.

There have been previous similar studies that have utilized bioassay-guided fractionation and LC-MS/MS analysis coupled with database or library searching to study natural products. In one study, 280 fractioned samples from 35 marine fungal strains from China were evaluated for their acetylcholinesterase (AChE) inhibitory activity and antioxidant activity using a thin layer chromatography array autography-based AChE inhibition assay and 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay (Nie et al., 2020). Their cytotoxicity was also determined using the *Artemia* larval lethality assay. The most bioactive and least cytotoxic fraction was analyzed via bioactivity-coupled LC-MS/MS, and the LC-MS/MS data was also uploaded to GNPS for molecular networking. Twelve compounds were identified to exhibit either AChE inhibitory activity or DPPH radical scavenging activity, with 7 of these compounds being putatively identified, and five compounds were suggested to be new compounds. In another study, the cytotoxic effects of semi-purified fractions from the ethyl acetate extract of *Annona muricata* L. leaves were evaluated against A549 cancer cells using *in vitro* MTS cytotoxicity and scratch/wound healing assays (Salac et al., 2022). Two subfractions (F15-16C and F15-16D) were identified to show the highest anticancer activity, and the fraction F15-F16 was further analyzed using LC-MS/MS, with the obtained data being analyzed using several metabolomics tools, including GNPS. Feature-based molecular networking using GNPS produced 28 hits or putative compounds from the F15-16 fraction.

This study aims to determine potential anti-diabetic and anti-inflammatory bioactive hits from *C. intermedia* stem and *D. dao* bark through a framework utilizing bioassay-guided fractionation, coupled with dereplication using UHPLC-MS/MS and database searching. To the best of our knowledge, this will be the first study which will use this framework for studying bioactive fractions from *C. intermedia* stem and *D. dao* bark.

Materials and methods

General

Distilled technical grade methanol, hexane, ethyl acetate, and ethanol were used to produce the crude plant extracts and the preliminary fractions by vacuum liquid chromatography (VLC). Analytical reagent (AR) grade hexane (JT Baker), ethyl acetate (JT Baker), ethanol (Scharlau, JT Baker), methanol (JT Baker), acetonitrile (RCI Labscan, JT Baker), DMSO (RCI Labscan), and ultrapure water (18.2 MΩ) (OmniaTap, stakpure GmbH) were used for the further fractionation of the VLC fractions via various separation methods, and as needed for the bioassays.

Preparation of plant materials and extraction

C. intermedia stems (accession number UPB-0090) were collected from Halsema Highway, Atok, Benguet. The stems were washed and air-dried for 5–7 days, and were then homogenized in a stainless-steel pulverizer.

D. dao bark (accession number PUH 13423) were collected from the University of the Philippines Diliman Campus, Quezon City. The bark was washed, dried at 40°C in an oven for at least 3 days, and ground in a stainless-steel pulverizer.

The extraction and solvent partitioning procedures used were optimized in the laboratory and adapted from previous protocols (Punzalan and Villaseñor, 2019a). Approximately 1.9 kg of dried *C. intermedia* stems and approximately 1.4 kg of dried *D. dao* bark were soaked in distilled methanol, separately, for at least 3 days. The methanol soakings for both plants were then separately filtered and concentrated using a rotary evaporator (BÜCHI Rotavapor R-100). The water bath was set to 40°C and the chiller (BÜCHI Recirculating

Chiller F-105) was at 2.5°C. The extraction process was repeated at least 7 times for each plant.

Solvent partitioning was performed on the methanolic extracts of *C. intermedia* stem and *D. dao* bark. Approximately 25–26 g of methanolic extract was dissolved in 500 mL ultrapure water and then partitioned with 500 mL distilled hexane. The hexane extract was separated and concentrated using a rotary evaporator, and the addition of distilled hexane to the aqueous mixture was repeated at least 6 times. To extract the slightly polar components, 500 mL distilled ethyl acetate was shaken with the remaining aqueous portion from the initial aqueous mixture. Similarly, extraction of the ethyl acetate extract was performed at least 6 times, and the extract was subsequently concentrated. The remaining aqueous mixture was then filtered to remove insoluble particles and stored at −20°C. A total of 53.30 g and 76.91 g of methanolic extract were partitioned for *C. intermedia* stem and *D. dao* bark, respectively.

Bioassay-guided fractionation

The fractionation procedures used were optimized in the laboratory and adapted from previous protocols (Abaya and Villaseñor, 2019; Punzalan and Villaseñor, 2019b). The ethyl acetate extracts of *C. intermedia* stem and *D. dao* bark were subjected to VLC. The samples were prepared by mixing the extracts with silica gel 60G for thin layer chromatography (Merck) in a 1:1 sample to silica weight ratio. Silica gel 60G was also used as packing material for the stationary phase, using a 1:30 sample to silica weight ratio. Dry silica was loaded and packed with the assistance of a vacuum pump onto a glass column with a radius of 4.25 cm and a medium porosity glass frit. Prior to loading the sample, the stationary phase was equilibrated using distilled hexane. Stepwise gradient elution was employed for both samples using a solvent system of distilled hexane and ethyl acetate, starting with 100% hexane, then changing the concentration of each solvent in 10% increments, until 100% ethyl acetate was reached. Fractions were collected by volume for each solvent system and concentrated using the rotary evaporator.

For *C. intermedia* stem, 5.492 g of ethyl acetate extract was used for VLC with a packed column height of 6.5 cm. The total volume used per solvent system is 500 mL. To recover highly polar compounds, stepwise gradient elution using a solvent system of distilled ethyl acetate and distilled ethanol was performed, starting from 100% distilled ethyl acetate, until 100% distilled ethanol, in 25% increments. The collected fractions were concentrated.

D. dao bark was subjected to VLC twice (5.038 g and 4.681 g), both times with a column height of 5.5 cm and a mobile phase of 250 mL per solvent system. Compounds with higher polarity were recovered by using 50% distilled ethyl acetate:50% distilled ethanol mixture, followed by 100% distilled ethanol. The collected fractions from both runs were pooled and concentrated.

Size-exclusion chromatography (SEC) was performed to fractionate the 13th VLC fraction (which eluted from the 50% ethyl acetate:50% ethanol solvent system) of the *C. intermedia* stem ethyl acetate extract. The packing material used for the stationary phase was Sephadex LH-20 (Sigma-Aldrich, LH20100), made to swell overnight in AR grade methanol. The stationary phase was loaded onto a glass column with a radius of 1 cm and a medium porosity glass frit. The resulting packed stationary phase had a height of 58.5 cm. The sample was prepared by dissolving 105 mg of the 13th VLC fraction in 1.5 mL AR grade methanol, which was then centrifuged prior to loading. AR grade methanol was used as the mobile phase, and the fractions were collected by band and volume. Based on the obtained UHPLC-MS profiles of the SEC fractions, fractions 24 to 31 were pooled together and concentrated using a rotary evaporator. Fractionation of the 13th VLC fraction using SEC was repeated three times. The total weight of sample used for SEC was 315 mg.

The pooled *C. intermedia* stem SEC fraction was then fractionated further through solid phase extraction (SPE). The fraction was dissolved in 100% ultrapure water. The SPE cartridge (Bond Elut C18 500 mg/3 mL; Agilent) was activated using AR grade acetonitrile, then equilibrated with ultrapure water. The sample was loaded onto the cartridge, then washed with ultrapure water. Stepwise gradient elution was performed, starting with 100% ultrapure water until 100% AR grade acetonitrile, in 10% increments. All the fractions were collected per solvent system and concentrated using a refrigerated centrifugal vacuum concentrator (Labconco). The first fraction is henceforth referred to as **CINS**.

Further fractionation of the 12th VLC fraction (eluted from 100% ethyl acetate) of *D. dao* bark ethyl acetate extract was done through gravity column chromatography (GCC). For the stationary phase, silica gel 60 (0.063–0.200 mm) for column chromatography (Merck) was used as packing material. A 1:100 sample to silica weight ratio was followed. The stationary phase was loaded onto a glass column with a radius of 1 cm, and a medium porosity glass frit. The solvent used for column equilibration and as the initial solvent was 30% hexane:70% ethyl acetate. The fraction (142.2 mg) was dissolved with 30% hexane:70% ethyl acetate and loaded onto the column through wet loading. Stepwise gradient elution and collection by band and volume were employed, which resulted in a total of 15 GCC fractions. Based on the profiles of the 15 fractions obtained using UHPLC-MS, similar fractions were pooled together. Pooled GCC fraction 4 (henceforth referred to as **DDAB**) was then subjected to UHPLC-MS/MS analysis.

All the extracts and fractions generated were subjected to α-glucosidase and 15-lipoxygenase-1 inhibition assays.

α-glucosidase inhibition assay

The α-glucosidase inhibitory activities of the extracts and fractions were determined via a spectrophotometric enzyme assay based on the Tuklas Lunas Protocols for Drug Discovery and Development in the Philippines (Naing et al., 2019). The assay was based on the procedure of Shobana et al. (2009) with modifications. Modifications of the assay were established from optimization experiments such as determination of the optimal enzyme concentration, K_m and V_{max} determination, determination of the IC_{50} of the positive control, and solvent tolerance of the enzyme.

The reaction mixture contained phosphate buffer (50 mM NaH_2PO_4 (Loba Chemie Pvt. Ltd.)/Na_2HPO_4 (Loba Chemie Pvt. Ltd.) with 100 mM NaCl (Scharlau) buffer, pH = 6.8), α-glucosidase

from *Saccharomyces cerevisiae* recombinant (EC 3.2.1.20; Sigma-Aldrich, G0660), sample/inhibitor, and p-nitrophenyl-α-D-glucopyranoside (p-NPG) (Merck). The enzyme inhibitory activity was tested by plating 10 µL of the sample (300 ppm), 216 µL of the phosphate buffer, and 24 µL of the α-glucosidase solution (120 mU/mL) into a 96-well quartz microplate (Hellma Analytics). After incubation at 37°C for 10 min, 50 µL of p-NPG (11.34 mM) was added to start the reaction. Using a microplate spectrophotometer (Thermo Scientific Multiskan GO) and the software SkanIt RE v7.0, the absorbance was monitored at 405 nm every 30 s for 30 min. The positive control used was acarbose (Sigma-Aldrich, A8980-1G, SLCF5122), and the negative control used was 5% DMSO in phosphate buffer.

The % inhibition was calculated using the following equation:

$$\%\ Inhibition\ per\ replicate = \frac{Absorbance_{uninhibited} - Absorbance_{inhibited}}{Absorbance_{uninhibited}} \times 100$$

Dose-response curves for CINS, DDAB and acarbose against the α-glucosidase assay were constructed, Supplementary Figures S13–S15 and the IC_{50} values were calculated. For the effective concentrations of CINS, DDAB and acarbose used in the determination of the IC_{50} values, refer to Supplementary Table S1.

15-Lipoxygenase-1 inhibition assay

A spectrophotometric assay for the determination of lipoxygenase inhibitory activity was employed following the Tuklas Lunas Protocols for Drug Discovery and Development in the Philippines (Allanigue and Hernandez, 2019). The assay was based on the procedures of Auerbach et al. (1992) and Axelrod et al. (1981) with modifications. Modifications of the assay were established from optimization experiments such as determination of the optimal enzyme concentration, K_m and V_{max} determination, determination of the IC_{50} of the positive control, and solvent tolerance of the enzyme.

The reaction mixture contained phosphate buffer (0.1 M KH_2PO_4 (Loba Chemie Pvt. Ltd.)/K_2HPO_4 (Loba Chemie Pvt. Ltd.), pH = 7.4), lipoxidase (15-lipoxygenase-1) from *Glycine max* (soybean) Type I-B (EC 1.13.11.12; Sigma-Aldrich L7395), sample/inhibitor, and linoleic acid (Sigma-Aldrich, L1376). The enzyme inhibitory activity was tested by plating 10 µL of the sample (1,000 ppm), 260 µL of the phosphate buffer, and 15 µL of the enzyme solution (1896 U/mL) into a 96-well quartz microplate (Hellma Analytics). After incubation at 25°C for 5 min, linoleic acid (1.6085 mM) was added to start the reaction. Using a microplate spectrophotometer (Thermo Scientific Multiskan GO) and the software SkanIt RE v7.0, the absorbance was monitored at 234 nm every 10 s for 5 min. The positive control used was nordihydroguaiaretic acid (NDGA) (Sigma-Aldrich, 74,540-1G, BCBX4406), and the negative control used was 10% DMSO in phosphate buffer.

The % inhibition was calculated using the following equation:

$$\%\ Inhibition\ per\ replicate = \frac{Absorbance_{uninhibited} - Absorbance_{inhibited}}{Absorbance_{uninhibited}} \times 100$$

Dose-response curves for CINS, DDAB and NDGA against the 15-lipoxygenase-1 assay were constructed, Supplementary Figures S16–S18 and the IC_{50} values were calculated. For the effective concentrations of CINS, DDAB and NDGA used in the determination of the IC_{50} values, refer to Supplementary Table S2.

For both the α-glucosidase and the 15-lipoxygenase-1 assays, extracts and fractions that exhibit ≥50% inhibition were considered to be active against the enzyme.

Statistical analyses

The assays were performed with three trials, with 4 replicates per trial. The enzyme inhibitory activities are presented as average ± standard deviation. IBM SPSS Statistics 26 was used to conduct the statistical analysis of the assay data. One-sample Kolmogorov-Smirnov test was done to check the normality of the distribution of slopes for each replicate, followed by Levene's test to check the equality of the variances from the different test groups. Brown-Forsythe and Welch tests were done afterward, since the variances were unequal ($p < 0.05$). The data was then analyzed using Tamhane T2 - One-way ANOVA with unequal variances to check if the samples are considered active. Grubbs' test (Graphpad outlier calculator) was used to determine any outliers in the replicates. GraphPad Prism 9 was used to calculate the IC_{50} values. The results of the statistical analyses of the assay data can be found in Supplementary Table S3–8.

Dereplication through Ultra-High-Performance Liquid Chromatography–Tandem Mass Spectrometry (UHPLC-MS/MS)

The UHPLC-MS/MS analysis and dereplication procedures were optimized in the laboratory and adapted from previous protocols (Junio et al., 2019).

Fractions were dissolved in LC-MS grade methanol (Merck) at a concentration of 500 µg/mL. The sample solutions were centrifuged, and the supernatants were transferred to LC-MS-certified vials (Waters). UHPLC-MS experiments were conducted on a Waters H-Class series system with a Xevo G2-XS quadrupole time of flight (QToF) mass spectrometer. A 0.3 mL/min flow rate was used with an Acquity UPLC HSS T3 1.8 µm (2.1 × 100 mm) column (Waters). The mobile phase was composed of varying ratios of LC-MS grade acetonitrile (Merck) and water (Merck), both with 0.1% formic acid. The composition of the acetonitrile used was as follows: 10% at 0.00–2.00 min, increasing from 10% to 100% at 2.00–8.00 min, 100% at 8.00–10.00 min, decreasing from 100% to 10% at 10.00–11.00 min, and 10% at 11.00–12.00 min. The injection volumes used for the samples were 1 µL for the positive mode and 3 µL for the negative mode.

Base peak intensity (BPI) chromatograms were obtained for 12 min per sample and spectra were acquired in ESI positive and negative modes within the range of 50–1,200 Da. ESI source parameters for the positive mode were set as follows: capillary voltage = 2.80 kV, sampling cone voltage = 40 V, source offset = 80 V, source temperature = 120°C, desolvation temperature = 450°C, cone gas = 50 L/h, and desolvation gas = 800 L/h. For the negative mode, ESI source parameters were set as follows: capillary voltage = 1.50 kV, sampling cone voltage = 30 V, source offset = 80 V, source

temperature = 120°C, desolvation temperature = 500°C, cone gas = 50 L/h, and desolvation gas = 1000 L/h. Mass spectrometry data were collected using two modes: MS and data dependent acquisition (DDA). For the DDA mode, MS/MS acquisition was done on ions that exceeded an intensity threshold of 1.0×10^5 and 1.0×10^4 for the positive and negative modes, respectively. A maximum of 10 ions were selected for MS/MS acquisition from a 0.5-s MS scan. Three collision energy ramps (15–30 eV, 30–45 eV, and 45–60 eV) were set to acquire extensive data on the small molecules.

MassLynx v4.1 was used to view and obtain the BPI chromatograms of the fractions. Peak alignment and conversion of the RAW data file to mgf file were executed using MSDIAL, v4.9.221218 (Tsugawa et al., 2015). The data were then uploaded to the Global Natural Products Social Molecular Networking (GNPS) platform (Wang et al., 2016) using WinSCP v6.1.1.

The feature-based molecular networking (FBMN) module of the GNPS platform (Nothias et al., 2020) was used to match the data acquired using DDA mode with existing spectral databases to obtain the putative identities of the compounds found in the active fractions. The criteria for matching were also adapted from a previous study (Molino et al., 2021) and optimized in the laboratory. The parameters for the FBMN were set as follows: precursor ion mass tolerance = 0.02 Da and fragment ion mass tolerance = 0.50 Da, a maximum of 5.00 for the ppm error, a minimum cosine score of 0.70, and a minimum of 6 for the matched fragment ions. The mirror matches of the experimental spectra with the reference spectra were also considered as criteria for the determination of the putative hits.

Results

Bioassay-guided fractionation

The preliminary results of the bioassays done on *C. intermedia* stem and *D. dao* bark extracts and fractions can be found in Supplementary Figures S7-12. The methanolic extract of *C. intermedia* stem (12.20% yield) both exhibited α-glucosidase **(AGLUC)** (99.91% ± 0.04%) and 15-lipoxygenase-1 **(LOX)** (91.44% ± 0.11%) inhibitory activities. Solvent partitioning was performed on the methanolic extract to separate the nonpolar, slightly polar, and the polar extracts. *C. intermedia* stem ethyl acetate extract was fractionated using VLC because it exhibited higher inhibition against both AGLUC (99.85% ± 0.03%) and LOX (92.90% ± 0.28%). Moreover, the ethyl acetate extract also had a higher yield (20.63%) compared to the hexane extract (5.29%). A total of 15 fractions were produced from the VLC. Out of the 15 fractions, 5 fractions were active against AGLUC only, and 1 fraction was active against LOX only. Six fractions were found to be active against both AGLUC and LOX and the 13th VLC fraction was selected since it had the highest yield (26.56%).

SEC was used to further fractionate the 13th VLC fraction. From this, 31 fractions were generated through isocratic elution. Thirteen out of the 31 fractions exhibited AGLUC inhibitory activity only. Eight fractions (fractions 24–31) exhibited inhibitory activity against both AGLUC (96.57% ± 0.65% to 99.73% ± 0.11%) and LOX (48.26% ± 2.47% to 91.96% ± 0.53%). Upon UHPLC-MS profiling, it was observed that all eight fractions have almost the same chromatogram. Since all eight fractions contained all the major peaks, they were pooled together into 1 fraction. The pooled fraction was then fractionated using a C18 SPE cartridge, which generated 12 fractions. The first SPE fraction (**CINS**) was chosen for dereplication and IC_{50} screening since it had the highest yield among the 12 fractions.

The AGLUC and LOX inhibitory activities of *D. dao* bark methanolic extract (12.82% yield) were 99.24% ± 0.22% and 99.26% ± 1.16%, respectively. Similar to the *C. intermedia* stem ethyl acetate extract, the *D. dao* bark ethyl acetate extract was pursued for fractionation since it exhibited high inhibitory activities against AGLUC (97.02% ± 1.59%) and LOX (97.73% ± 1.12%). Moreover, the *D. dao* bark ethyl acetate extract also had a higher yield (18.14%) than the hexane extract (8.87%).

The ethyl acetate fraction was fractionated through VLC. Out of the 25 VLC fractions of *D. dao* bark ethyl acetate, 12 fractions were found to be active against AGLUC and LOX. Two fractions exhibited inhibition against AGLUC only. The 12th VLC fraction was prioritized due to its high AGLUC (99.90% ± 0.03%) and LOX (108.22% ± 1.03%) inhibitory activities.

The 12th VLC fraction was further fractionated through GCC. Out of the 15 GCC fractions, 11 fractions showed inhibition against both AGLUC and LOX. One fraction was active against AGLUC only, and 1 fraction was active against LOX only. Based on the BPI chromatograms of the fractions obtained through UHPLC-MS, GCC fractions 6–11 (pooled GCC fraction 4) were pooled together. The pooled GCC fraction 4 **(DDAB)** had the highest yield among all the pooled fractions. DDAB was also selected for dereplication and IC_{50} screening.

Enzyme inhibitory assays

Table 1 summarizes the IC_{50} results from the enzyme assays that were done on the prioritized active fractions. The IC_{50} values reported here for each sample/control are the average of the three trials per sample/control.

It can be observed that both CINS and DDAB exhibited markedly lower IC_{50} values as compared to acarbose in the AGLUC inhibition assay. On the other hand, both CINS and DDAB exhibited greater IC_{50} values compared to NDGA in the LOX inhibition assay. This implies that the compounds present in

TABLE 1 IC_{50} values from enzyme inhibition assays on CINS and DDAB.

Sample	IC_{50} (in μg/mL)	
	AGLUC	LOX
CINS	0.25 ± 0.06	2.57 ± 0.86
DDAB	0.25 ± 0.04	3.58 ± 0.96
Acarbose	129.52 ± 14.01	—
NDGA	—	0.64 ± 0.25

TABLE 2 Putatively identified compounds from CINS using GNPS (GNPS[a], PubChem[b]).

	Compound	Acquisition mode	Retention time (min)	Precursor Adduct	Experimental Mass	Monoisotopic Mass	ppm error	Cosine score
1	ellagic acid	positive	5.908	$[M + H]^+$	303.0129	303.01[a]	9.60[a]	0.89
						303.0141[b]	−3.90[b]	
2	corilagin[c]	positive	5.096	$[M + Na]^+$	657.0693	657.07[a]	−1.00[a]	0.87
						657.0704[b]	−1.59[b]	
		negative	4.932	[M-H]-	633.08362	633.067[a]	25.93[a]	0.81
						633.0728[b]	17.11[b]	
3	(1S,2S,6R,7R,9R)-6-methyl-10,12-dioxatricyclo [7.2.1.0 < 2,7>]dodec-4-en-8-one[c]	positive	9.059	$[M + H]^+$	195.1018	195.102[a]	−1.23[a]	0.86
						195.1021[b]	−1.84[b]	
4	isoscopoletin[c]	positive	5.247	$[M + Na]^+$	215.0331	215.032[a]	4.98[a]	0.77
						215.0320[b]	4.85[b]	
5	2-O-galloylhyperin[c]	positive	5.716	$[M + H]^+$	617.1143	617.113[a]	2.04[a]	0.76
						617.1142[b]	4.86×10^{-4}[b]	
6	oleamide[c]	positive	11.571	$[M + H]^+$	282.2795	282.279[a]	1.91[a]	0.76
						282.2797[b]	−0.53[b]	
7	1,2,3,6-tetra-O-galloyl-beta-D-glucose[c]	negative	5.502	[M-H]-	787.0974	787.1[a]	−3.26[a]	0.75
						787.0994[b]	−2.53[b]	

[c]First report of putative identification in *C. intermedia* stem.

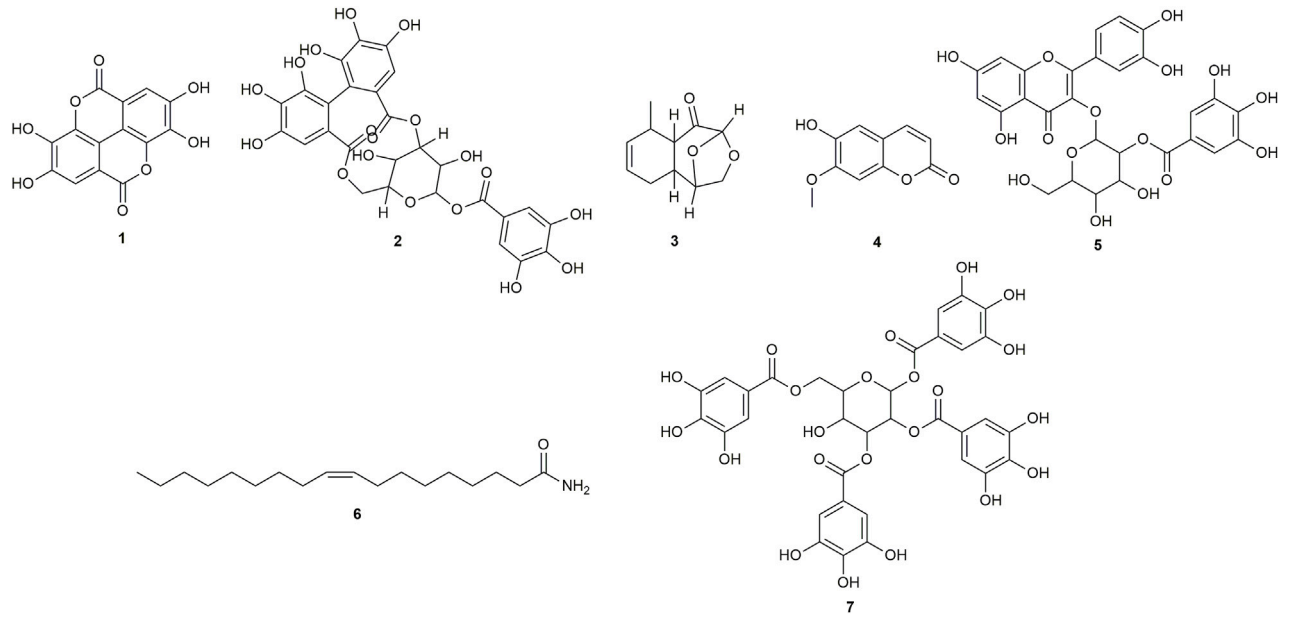

FIGURE 1
Chemical structures of compounds from CINS that were putatively identified by dereplication. Ellagic acid (**1**), corilagin (**2**), (1S,2S,6R,7R, 9R)-6-methyl-10,12-dioxatricyclo [7.2.1.0 < 2,7>]dodec-4-en-8-one (**3**), isoscopoletin (**4**), 2-O-galloylhyperin (**5**), oleamide (**6**) and 1,2,3,6-tetra-O-galloyl-beta-D-glucose (**7**).

TABLE 3 Putatively identified compounds from DDAB using GNPS (GNPS[a], PubChem[b]).

	Compound	Acquisition mode	Retention time (min)	Precursor Adduct	Experimental Mass	Monoisotopic Mass	ppm error	Cosine score
1	ellagic acid	negative	5.708	$[M-H]^-$	301.0005	300.999[a]	4.97[a]	0.82
						300.9984[b]	7.00[b]	
6	oleamide[c]	positive	11.596	[M + H]	282.2795	282.279[a]	1.91[a]	0.81
						282.2797[b]	−0.53[b]	
8	7,2′,3′-trimethoxyflavanone[c]	positive	9.091	$[M + Na]^+$	337.1041	337.105[a]	−2.79[a]	0.91
						337.1052[b]	−3.36[b]	
9	abscisic acid	positive	6.845	$[M + H\text{-}H_2O]$	247.1331	247.133[a]	0.40[a]	0.89
						247.1334[b]	−1.29[b]	
10	pseudoanisatin[c]	positive	6.57	$[M + Na]^+$	321.1318	321.131[a]	2.40[a]	0.83
						321.1314[b]	1.13[b]	
11	1,3,6-tri-O-galloyl-beta-D-glucose[c]	positive	5.418	$[M + Na]^+$	659.0850	659.09[a]	−7.56[a]	0.78
						659.0860[b]	−1.54[b]	
		negative	1.042	$[M-H]^-$	635.0891	635.089[a]	0.19[a]	0.77
						635.0884[b]	1.06[b]	
12	1,2,4,6-tetra-o-galloyl-beta-D-glucose[c]	positive	5.627	$[M + H]^+$	789.1142	789.115[a]	−1.01[a]	0.70
						789.1150[b]	1.07[b]	
13	pyrocatechuic acid[c]	negative	2.952	$[M-H]^-$	153.0184	153.019[a]	−3.89[a]	0.86
						153.0188[b]	2.57[b]	
14	pyrogallol[c]	negative	1.56	$[M-H]^-$	125.0233	125.024[a]	−8.18[a]	0.76
						125.0239[b]	4.71[b]	

[c]First report of putative identification in *D. dao* bark.

these fractions are more potent inhibitors of AGLUC compared to acarbose but are less potent inhibitors of LOX compared to NDGA.

Dereplication

Multiple compounds were putatively identified through the dereplication of CINS and DDAB. These compounds are summarized in Table 2; Figure 1 for CINS, and Table 3; Figure 2, 3 for DDAB.

Based on the dereplication results, a literature search was conducted on the putative hits for both CINS and DDAB to see whether there have been previous studies on their antidiabetic and/or anti-inflammatory activities, which might explain the fractions' bioactivities that were observed in the enzyme inhibition assays.

Ellagic acid (compound **1**) is a phenolic compound that was previously identified in the hexane fraction from the methanolic extract of *C. intermedia* leaves and roots (Chang et al., 1996), and can be found in numerous plants such as the leaves of *Tectaria subtriphylla* (Hook. and Arn.) Copel. (Feng-Lin and Jhy-Yih, 1993), the leaves and bark of *Acer negundo* L. (Saleh et al., 1969), and the bark of *Mallotus japonicus* (L.f.) Müll.Arg. (Yoshida et al., 1982). It also has anti-diabetic and anti-inflammatory properties (Ríos et al., 2018). More specifically, it was found to exhibit inhibitory activity against AGLUC with an IC_{50} value of 2.18 μg/mL (You et al., 2012). Its anti-inflammatory mechanisms include the inhibition of lipopolysaccharide (LPS)-induced nitric oxide (NO), prostaglandin E2 (PGE2), and interleukin-6 (IL-6) production (BenSaad et al., 2017). Compound **1** was putatively identified from both CINS and DDAB fractions.

Corilagin (compound **2**) is a hydrolysable tannin that was previously isolated from the shoot of *Geranium thunbergii* Siebold & Zucc. (Okuda et al., 1975), the roots of *Phyllanthus emblica* L. (Zhang et al., 2000) and the leaves of *Phyllanthus niruri* L. (Colombo et al., 2009). In addition, it was also isolated from the leaves of *Terminalia macroptera* Guill. and Perr. and exhibited AGLUC and LOX inhibitory activities with IC_{50} values of 2.58 ± 0.08 μM and 41 ± 4 μM, respectively (Pham et al., 2014).

For (1S,2S,6R,7R, 9R)-6-methyl-10,12-dioxatricyclo [7.2.1.0 < 2,7>]dodec-4-en-8-one (compound **3**), no previous studies have been conducted which quantified its antidiabetic properties using the AGLUC inhibition assay, and its anti-inflammatory activity using the LOX inhibition assay. Furthermore, this compound has not been previously identified from a plant source.

Isoscopoletin (compound **4**) is a hydroxycoumarin that has been isolated from various plants such as the aerial parts of *Tagetes lucida* Cav. (Céspedes et al., 2006), *Euphorbia hirta* L. (Wu et al., 2012) and

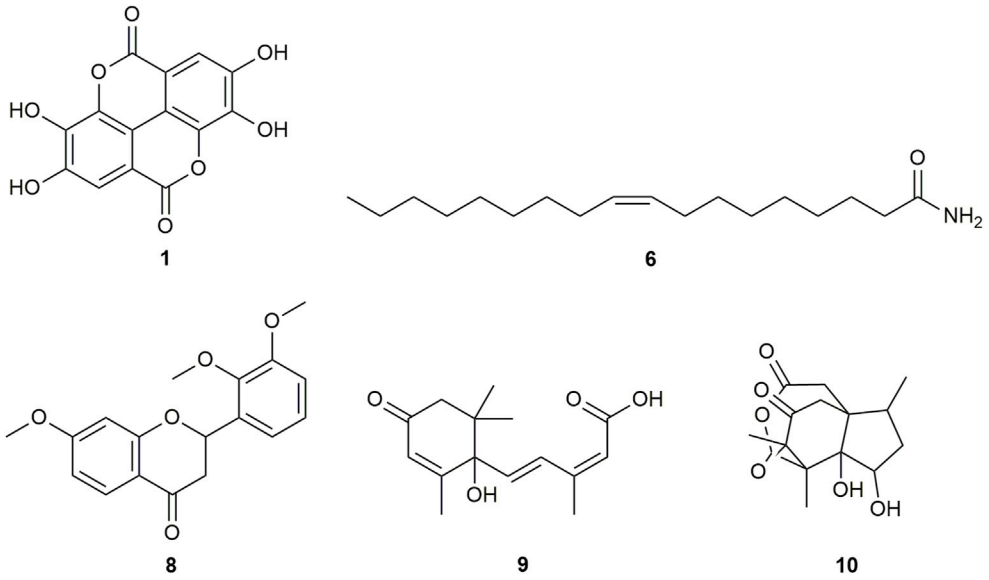

FIGURE 2
Chemical structures of compounds from DDAB that were putatively identified by dereplication. Ellagic acid (**1**), oleamide (**6**), 7,2′,3′-trimethoxyflavanone (**8**), abscisic acid (**9**), and pseudoanisatin (**10**).

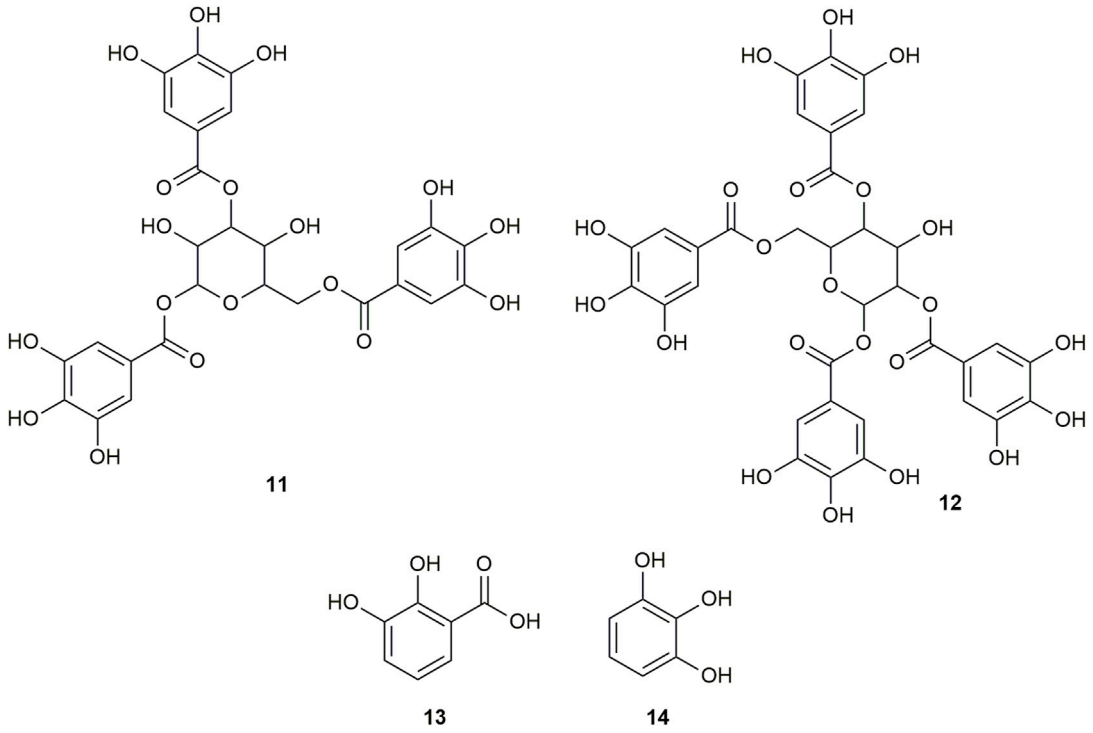

FIGURE 3
Chemical structures of compounds from DDAB that were putatively identified by dereplication. 1,3,6-tri-O-galloyl-beta-D-glucose (**11**), and 1,2,4,6-tetra-O-galloyl-beta-D-glucose (**12**), pyrocatechuic acid (**13**) and pyrogallol (**14**).

from the stem wood of *Zanthoxylum integrifoliolum* (Merr.) Merr. (Chen et al., 2007). Furthermore, it also has antidiabetic activities that is exemplified by a variety of mechanisms such as the inhibition of advanced glycation end products formation (IC_{50} = 18.78 ± 0.50 μM) (Jung et al., 2012). Moreover, it also exhibited inhibitory activities towards α-amylase, maltase, and sucrase with % inhibition values of 0.2%, 32.2%, and 20.9%, respectively, at a concentration of 400 μM (Kato et al., 2008). Its anti-inflammatory

properties can be attributed to its ability to inhibit LOX (IC_{50} = 15.1 μM) (Deng et al., 2007).

2-O-galloylhyperin (compound **5**) is a flavonoid glycoside that was isolated from the aerial parts of *Rubus amabilis* Focke (Chen et al., 2001), *Pyrola incarnata* (DC.) Fisch. ex Freyn (Yazaki et al., 1989), *Euphorbia lunulata* Bunge (Nishimura et al., 2005) and the leaves of *Eucalyptus globulus* Labill. (Park et al., 2023).

Oleamide (compound **6**) is a fatty acid amide that is found in numerous plants such as the stem of *Desmos cochinchinensis* Lour. Sun et al. (1995), the leaves of *Polygonum maritimum* L. (Rodrigues et al., 2017) and the leaves of *Lithocarpus polystachyus* (Wall. ex A. DC.) Rehder (Fang et al., 2022). It was reported with an IC_{50} value of 30.69 ± 1.47 μg/mL against AGLUC (Fang et al., 2022). Moreover, it demonstrated anti-inflammatory effects through various mechanisms such as the inhibition of LPS-induced nuclear factor kappa B (NF-κB) activation (Oh et al., 2010) and the suppression of p38, ERK, and PI 3-kinase/Akt and ROS accumulation (Moon et al., 2018). Compound 6 was putatively identified from both CINS and DDAB fractions.

1,2,3,6-tetra-O-galloyl-beta-D-glucose (compound **7**) is a tannin that was putatively identified in *Corchorus olitorius* L. leaf extract through UHPLC-MS (Alara et al., 2023). It was also isolated from the stem bark of *Bersama abyssinica* subsp. *abyssinica* and *Bersama abyssinica* subsp. *paullinioides* (Nyamboki et al., 2021) and the leaves and branches of *Phyllanthus emblica* L. Zhang et al. (2002).

7,2′,3′-trimethoxyflavanone (compound **8**) is a flavanone. Flavanones are responsible for the bitter taste of citrus peels and fruits, and they exhibit antioxidant, anti-inflammatory, blood lipid-lowering, and cholesterol-lowering properties (Panche et al., 2016).

Abscisic acid (compound **9**) is an isoprenoid phytohormone that is responsible for several regulatory plant functions such as growth. It can be found in numerous plants such as the leaves of *Cinnamomum subavenium* Miq. Kuo et al. (2008) and the epiphyte, *Cuscuta pentagona* Engelm (Kimura et al., 1982). Moreover, this compound can be a promising treatment for diabetes as it regulates glucose uptake *in vitro* and can stimulate insulin production in beta pancreatic cells (Xu et al., 2018). In addition, it was found that abscisic acid intake reduces neuroinflammation (Jeon et al., 2020) and colon inflammation (Guri et al., 2011).

Pseudoanisatin (compound **10)** is a lactone that was isolated from various *Illicium* species. It was isolated from the leaves of *Illicium parviflorum* Michaux ex Ventenat (Schmidt, 1999) and the pericarps of *Illicium oligandrum* Merr. and Chun (Zhu et al., 2009) and *Illicium dunnianum* Tutcher (Jianmei and Chunshu, 1996).

1,3,6-Tri-O-galloyl-beta-D-glucose (compound **11**) is a glycoside which was isolated from the leaves of *Terminalia bellirica* (Gaertn.) Roxb. (Kim et al., 2018; Manome et al., 2022).

Similarly, 1,2,4,6-tetra-O-galloyl-beta-D-glucose (compound **12**) is another glycoside that was isolated from the leaves of *Phyllanthus emblica* L. (Zhang et al., 2000), the fresh pericarps of *Juglans sigillata* Dode (Si et al., 2011) and the leaves of *Melastoma malabathricum* L. (Yoshida et al., 1982).

Pyrocatechuic acid (compound **13)** is a phenolic acid which was isolated from the ethyl acetate extract of *Mangifera casturi* bark (Pardede and Koketsu, 2016). Pyrocatechuic acid was also isolated from the fruit extract of *Flacourtia inermis* Roxb (George et al., 2011).

Pyrogallol (compound **14)** belongs to the class of phenols and has been previously isolated from the stem and bark of *Barringtonia asiatica* (L.) Kurz (Umaru, 2020). It was also isolated from the rhizomes of *Bergenia ciliata* (Haw.) Sternb (Zafar et al., 2019). In a previous study, the interaction of pyrogallol with AGLUC was evaluated through computational simulations and it was found that pyrogallol inhibits AGLUC with an IC_{50} value of 0.72 ± 0.051 mM (Zheng et al., 2018). Pyrogallol was also found to have an IC_{50} value of 8.7 mM against LOX (Yasumoto, Yamamoto, and Mitsuda, 1970).

There have been no previous studies conducted that quantified antidiabetic properties using the AGLUC inhibition assay, and anti-inflammatory activity using the LOX inhibition assay for compounds **3, 5, 7, and 10–13**.

Discussion

Asthma and diabetes are two noncommunicable diseases which affect a significant portion of the global population. Glucose metabolism disorders (which ultimately lead to diabetes) have been identified as potential risk factors for the exacerbation of asthma symptoms and the development of severe asthma (Wu, 2020). These disorders cause changes in the lungs similar to those caused by asthma, mainly through a pathway involving insulin excess. Similarly, chronic airway inflammation has been postulated to increase the risk of T2DM. In a large study of middle-aged and older women in the USA, it was found that women with preexisting asthma had a higher risk of developing T2DM, independent of traditional diabetes risk factors (Song et al., 2010).

It has been identified that there is a significant association between the occurrence of asthma and T2DM (Uppal et al., 2023). The NF-κB signaling pathway is believed to cause asthma. NF-κB regulates the expression of certain proinflammatory molecules which cause low-grade inflammation, indicated by increased levels of IL-6, tumor necrosis factor (TNF), C-reactive protein (CRP), and adhesion molecules (Liu, 2007; Edwards et al., 2009, as cited in Uppal et al. (2023)). Low-grade inflammation has been identified as a major contributor to the development of T2DM. It is speculated that the eventual manifestation of diabetes is due to the development of insulin resistance in the liver, smooth muscle, and vascular endothelium influenced by increased circulating levels of certain inflammatory cytokines, which is in turn caused by chronic airway inflammation.

Human arachidonic acid 15-lipoxygenase (ALOX15 or 15-LOX or 12/15-LOX) is a heme-free dioxygenase which catalyzes the formation of hydroperoxy derivatives via the peroxidation of certain polyunsaturated fatty acids. ALOX15 has been found to promote inflammation by metabolizing linoleic acid to 13(S)-hydroperoxyoctadecenoic acid, which then activates NF-κB (Dwarakanath et al., 2004, as cited in He et al. (2023)). It has also been found that ALOX15 metabolites, such as 12S-hydroxyeicosatetraenoic acid, can stimulate the expression of IL-6 and TNF-α in a dose-dependent manner (Wen et al., 2007, as cited

TABLE 4 Differences between IC_{50} values from enzyme inhibition assays on CINS and DDAB and previously reported IC_{50} values of putative hits.

Fraction/Compound	Putatively identified in	IC_{50} (in μg/mL)	
		AGLUC	LOX
CINS	—	0.25 ± 0.06	2.57 ± 0.86
DDAB	—	0.25 ± 0.04	3.58 ± 0.96
ellagic acid (compound 1)	CINS, DDAB	2.18 You et al. (2012)	N/A
corilagin (compound 2)	CINS	1.63701 ± 0.03 Pham et al. (2014)	26.01 ± 2.5 Pham et al. (2014)
isoscopoletin (compound 4)	CINS	N/A	2.902 Deng et al. (2007)
oleamide (compound 6)	CINS, DDAB	30.69 ± 1.47 Fang et al. (2022)	N/A
pyrogallol (compound 14)	DDAB	N/A	1.097×10^3 Yasumoto et al. (1970)

in He et al. (2023)), which indicates that ALOX15 can induce inflammatory cascades.

ALOX15 is more highly expressed in airway epithelial cells where it regulates the secretion of mucus, and releases chemokines that act on immune cells, ultimately enhancing pro-inflammatory signaling pathways related to airway inflammatory diseases. In particular, ALOX15 promotes eosinophilic inflammation, the migration of immune cells, and the remodeling of the airway (Xu et al., 2021). It has been hypothesized that ALOX15 inhibitors have the potential to be used as treatments for airway inflammatory diseases, including asthma. ALOX15 and its metabolites have also been implicated in the pathology and mechanism of T2DM, which may be related to the effect of ALOX15 on islet cell and macrophage functions, (He et al., 2023), as well as in serious complications that arise from the disease, such as diabetic retinopathy, peripheral neuropathy, and nephropathy (Singh and Rao, 2019). This implies that ALOX15 inhibitors may also be explored as a possible treatment for T2DM and/or its complications. Compounds or extracts that inhibit both ALOX15 and α-glucosidase might be possible new sources of treatments for T2DM and asthma, especially for women.

In this study, a framework utilizing bioassay-guided fractionation combined with dereplication through UHPLC-MS/MS and database searching was used. Bioactive fractions from *C. intermedia* stem and *D. dao* bark were generated, and dereplication of the bioactive fractions through UHPLC-MS/MS analysis and database searching via GNPS were conducted. A literature search on the putatively identified compounds from dereplication was then performed to see whether there have been previous studies on their antidiabetic and/or anti-inflammatory activities, which might explain the bioactivity of the fractions that were observed in the enzyme inhibition assays.

The putatively identified compounds from CINS are presented in Table 2; Figure 1.

This is the first report of compounds **2** to **7** being putatively identified in *C. intermedia* stem. This is also the first report of compound **1** being putatively identified in *C. intermedia* stem, as it was only previously extracted from the leaves and roots of the plant (Chang et al., 1996).

Compounds **1**, **2**, and **6** were previously reported to exhibit inhibitory activity against AGLUC, with reported IC_{50} values of compounds **1**, **2**, and **6** being 2.18 μg/mL, 2.58 ± 0.08 μM (or 1.63701 ± 0.03 μg/mL), and 30.69 ± 1.47 μg/mL, respectively (You et al., 2012; Pham et al., 2014; Fang et al., 2022). Compounds **4** and **5** have not been previously reported to have inhibitory activity against AGLUC. However, compound **4** has been reported to have inhibitory activity against α-amylase, maltase, and sucrase (Kato et al., 2008). A compound closely related to compound **5**, hyperin, differing by the removal of the galloyl moiety, exhibited inhibitory activity against AGLUC with an IC_{50} of 19.26 μg/mL (Zhang et al., 2013). The previously reported IC_{50} values of compounds **1, 2,** and **6** are all greater compared to the observed IC_{50} value of CINS against AGLUC.

Compounds **2** and **4** were previously reported to exhibit inhibitory activity against LOX, with their reported IC_{50} values being 41 ± 4 μM (or 26.01 ± 2.5 μg/mL) and 15.1 μM (or 2.902 μg/mL), respectively (Deng et al., 2007; Pham et al., 2014).

The putatively identified compounds from the dereplication of DDAB are shown in Table 3; Figure 2, 3.

This is the first report of compounds **6, 8**, and **10–14** being putatively identified in *D. dao* bark.

From the compound hits of the dereplication results of DDAB, only compound **6** was previously reported to exhibit inhibitory activity against AGLUC with an IC_{50} value of 30.69 ± 1.47 μg/mL (Fang et al., 2022). It was previously reported that the methanolic extract from *D. dao* bark has been shown to possess AGLUC inhibitory activity, with an IC_{50} value of 3.24 μg/mL (Yusro et al., 2016).

Only one of the putatively identified compounds from DDAB (compound **14**) was found to have previous studies which quantified their anti-inflammatory activity using the LOX inhibition assay, with an IC_{50} value of 8.7 mM (Yasumoto et al., 1970).

Previous studies have reported IC_{50} values of some of the putatively identified compounds in CINS and DDAB. The differences between IC_{50} values obtained in this study for fractions CINS and DDAB, and IC_{50} values from previous studies on the putatively identified compounds are shown in Table 4.

It is observed that the previously reported IC_{50} values of the putative compounds are greater than the obtained IC_{50} values for CINS and DDAB. This implies that the compounds in fractions CINS and DDAB might be acting in synergy to inhibit AGLUC and LOX at lower concentrations. In addition, other compounds present in CINS and DDAB that were unidentified through dereplication may also be responsible for the observed activity of the fractions

against AGLUC and LOX. It can be observed in the chromatogram traces of CINS and DDAB (Supplementary Figures S2–S3, S5–S6) that not all peaks are accounted for when compared to the putative hits from the dereplication results. Further fractionation of these active fractions may lead to more potent bioactivities observed in the enzyme assays since the fractions still contain multiple compounds. Further purification of the bioactive fractions is also necessary to ascertain if the bioactivity observed for the fractions is due to a synergistic effect or if an unidentified compound is responsible for the observed bioactivity.

From the dereplication compound hits and the literature search that was conducted, corilagin (compound **2**) was identified as the only compound having been previously reported to be active against both AGLUC and LOX, having IC_{50} values of 2.58 ± 0.08 μM against AGLUC and 41 ± 4 μM against LOX (Pham et al., 2014). Corilagin (compound **2**) is a gallotannin that has been extensively studied and was shown to exhibit several pharmacological activities (Li et al., 2018).

Corilagin was found to significantly reduce the production of some proinflammatory cytokines and mediators and was also found to reduce cyclooxygenase-2 expression at both the protein and gene level (Zhao et al., 2008, as cited in Li et al. (2018)). Streptozotocin-induced diabetic rats that were treated orally with corilagin were shown to have reduced fasting blood glucose levels compared to the diabetic control rats at the end of the study, similar to the level by which glibenclamide reduced the FBG levels (Nandini and Naik, 2019). Corilagin was found to reduce airway inflammation and collagen deposition in ovalbumin-induced asthmatic mice via the adenosine monophosphate-activated protein kinase pathway (Jin and Yi, 2023). In mice fed with a high fat diet (HFD) to induce nonalcoholic fatty liver disease (NAFLD), treatment with corilagin was found to reduce HFD-induced accumulation of fat in the liver and liver injury, improved plasma lipid concentrations, as well as improve other metabolic disorders associated with NAFLD, such as glucose intolerance and insulin resistance in HFD-fed mice (Liao et al., 2022). Corilagin has been found to exhibit antitumor activities against different cancer cell lines, both *in vitro* and *in vivo* (Li et al., 2018). Corilagin was also found to inhibit the binding of the spike receptor binding domain (spike-RBD) of SARS-CoV-2 virus to human angiotensin-converting enzyme 2 (hACE2) in a dose-dependent manner (Yang et al., 2021). This indicates that corilagin interferes with the fusion of spike-RBD and hACE2 and implies that corilagin may be considered as a potential candidate as an inhibitor for the entry of SARS-CoV-2. In safety tests, corilagin was found to have had almost no toxic effects on normal cells or tissues (Li et al., 2018).

Based on these previous findings, it is strongly recommended that corilagin be further studied as a potential new treatment for people living with diabetes and/or asthma, especially for women, and perhaps for other diseases as well, such as NAFLD, cancer, and COVID-19.

It must be acknowledged that the current study has several limiting factors. First, the samples which were tested (CINS and DDAB) for their bioactivity against AGLUC and LOX are only subfractions from plant extracts, and not purified compounds. The identity of the compound/s from the subfractions which are responsible for the observed bioactivity against the bioassays conducted cannot be confirmed with absolute certainty at this point in time. It is recommended that the active compound/s responsible for the bioactivity are purified and isolated further in future studies.

Second, the study only used *in vitro* enzymatic assays to determine the bioactivity of the samples, in contrast to *in vivo*, *ex vivo*, or *in vitro* cell based assays. In our research group, only *in vitro* enzymatic assays are carried out for all samples. *In vitro* assays, in general, are faster and require less of the sample to work with, but *in vivo* assays are preferred because they more closely mimic clinical conditions while also providing toxicity data (Sarker and Nahar, 2012). It is recommended that the bioactive subfractions that were identified in this study, as well as the putatively identified compounds from these subfractions, should be tested against *in vivo* bioassays (or *in vitro* cell-based assays) for their anti-diabetic, anti-inflammatory, or anti-asthmatic activity. Multiple animal models can be used for *in vivo* testing of the anti-diabetic (King, 2012), anti-inflammatory (Patil et al., 2019), and anti-asthmatic (Kianmeher et al., 2016) activity of a compound or an extract.

Finally, although compounds from CINS and DDAB were identified, the identities of these compounds are only putative at this point. Dereplication by LC-MS/MS does not provide information on the configuration or constitution of a molecule based solely on the molecular ion match or fragmentation pattern match (Zani and Carroll, 2017). Hyphenated techniques also do not consider configurational isomerism within a molecule. The information gathered from dereplication using LC-MS/MS data by database searching is also limited by the compounds and spectra which are actually listed in the database. For example, the compound libraries hosted on GNPS which are used for spectral matching contain a multitude of compounds and reference spectra (221,000 reference library spectra from 18,163 compounds) (Wang, et al., 2016). However, it can still be limiting in the sense that it cannot account for the majority of compounds which may be produced by terrestrial plants. A single plant species is estimated to produce between 5,000 to tens of thousands of compounds, and collectively all plant species are estimated to produce between 100,000 and 1,000,000 compounds (Fang et al., 2019). Again, further purification of the identified bioactive subfractions must be carried out, along with structural identification using other characterization techniques to establish the identity of the bioactive component/s.

Conclusion

This study aimed to determine potential anti-diabetic and anti-inflammatory bioactive hits from *C. intermedia* stem and *D. dao* bark. Bioactive fractions from *C. intermedia* stem and *D. dao* bark were generated, and dereplication through UHPLC-MS/MS and database searching was performed. Seven compounds were putatively identified from the *C. intermedia* stem active fraction, and six of these compounds were putatively identified from this plant for the first time. Nine compounds were putatively identified from the *D. dao* bark active fraction, and seven of these compounds were putatively identified from this plant for the first time. One putative compound from the *C. intermedia*

stem active fraction (corilagin) has been previously reported to have inhibitory activity against both AGLUC and LOX. It is suggested that corilagin should be prioritized in further studies on new therapeutics which target the treatment of T2DM and/or asthma because of its dual inhibitory activity against AGLUC and LOX, as well as its several beneficial pharmacological activities and low reported toxicity.

Data availability statement

The data presented in the study are deposited in Figshare. This data can be found here: https://doi.org/10.6084/m9.figshare.25272313.v1.

Author contributions

MCPF: Formal Analysis, Investigation, Methodology, Project administration, Visualization, Writing–original draft, Writing–review and editing. RMNA: Formal Analysis, Investigation, Methodology, Visualization, Writing–original draft, Writing–review and editing. AAGA: Formal Analysis, Investigation, Methodology, Visualization, Writing–original draft, Writing–review and editing. LAEP: Formal Analysis, Investigation, Methodology, Visualization, Writing–original draft, Writing–review and editing. CCH: 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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2024.1349725/full#supplementary-material

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Current use of medicinal plants for children's diseases among mothers in Southern Romania

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There is a limited number of studies focusing on ethnomedical practices in children, particularly in Eastern Europe. Romania has a rich history of using medicinal plants in ethnopediatric care, and our objective was to identify the medicinal plants currently employed in treating childhood illnesses in the southern region of the country.

Material and methods Our investigation used structured interviews, focusing on respondent demographics, local names of therapeutically employed herbs, the specific plant part(s) utilized, methods of preparation and administration, and local folk indications of taxa. A total of 326 mothers with children aged 0 to 18, hospitalized in the "Grigore Alexandrescu" Children Emergency Hospital Bucharest and residing in Southern Romania, were enrolled in the study. Use Value Citation Index (UVc), Informant Consensus Factor (Fic), and Fidelity Level (FL) were calculated.

Results Twenty-five plants were identified for treating children's diseases in Southern Romania. The majority of informants resided in urban areas, and mothers primarily acquired knowledge from family members and healthcare professionals. The herbs most frequently employed were *Mentha* spp. (UV = 0.509) for diarrhea, *Matricaria spp*. (UV = 0.301) for skin infections (Fic = 0.99) and digestive diseases (Fic = 0.98), and *Calendula officinalis* L. (UV = 0.365) for skin diseases (Fic = 0.99). Less utilized were *Raphanus raphanistrum subsp. sativus* (L.) Domin in respiratory diseases, *Prunus avium* (L.) L. stalks in urinary tract ailments, *Helianthus annuus* L. in ear infections, *Allium sativum* L. in intestinal parasitosis, *Viola tricolor* L. in hives, *Triticum aestivum* L. in dermatitis and *Allium ursinum* L. as a tonic. In 184 cases herbal treatment was used in conjunction with conventional medications. Education level correlated with the number of employed plants and the variety of treated ailments, while residency (rural vs. urban) did not. Both residency and education influenced plant procurement methods: rural background and, surprisingly, higher education were linked to a preference for harvesting rather than purchasing plants.

Conclusion Botanical remedies are still commonly used in the treatment of pediatric diseases in Southern Romania, although the variety of taxa seems reduced compared to the past. Further exploration is essential to unlock the maximum benefits of ethnopediatric practices.

KEYWORDS

children, complementary treatment, medicinal plants, pediatrics, Romania

1 Introduction

Traditional medicine, consisting of a multitude of health-related practices, approaches, knowledge, and beliefs, is an invaluable medical and cultural heritage for humankind. According to the World Health Organization, traditional medicine incorporates plants, animal and mineral-based medicines, spiritual therapies, manual techniques, and exercises (Burton et al., 2015). Among them, herbs represent the main remedies used over the millennia for preventing and treating a large variety of ailments. Since ancient times, people have prepared their own herbal medicines or acquired them from the local traditional healers (Che et al., 2017). It is highly suggestive that about 80% of the populations in developing countries still use herbal medicine to meet their primary healthcare requirements (Zhang et al., 2019). Moreover, in the past few decades, people have been rediscovering more and more traditional, and predominantly herbal medicine (Murgia et al., 2021), either as an alternative to or associated therapy with modern drugs (Santucci et al., 2021)

In traditional medicine, the knowledge is transmitted mainly orally, emphasizing the importance of documentation (Bruhn and Rivier, 2019) to prevent rapid loss. Despite women's contributions to the History of Science often being overlooked or marginalized (Mariath and Baratto, 2023), an increasing number of studies point out the essential role women have played in preserving traditional medical practices over time. From ancient Greek medicine to early modern European herbalism, botanical local knowledge was often held by (more or less) anonymous female herbalists (Bowcutt and Caulkins, 2020). During the medieval period, lettered "middle class and elite" women were often educated in the "domestic arts," encompassing medical subjects and everyday healing based on herbal remedies. Given their significant role in infant care and health, women were trained as empirical practitioners to provide primary medical assistance for children's ailments (Allen, 2016; Soares, 2023).

The recent literature on traditional medicine is mainly focused on the knowledge of experienced practitioners or healers, whereas domestic medicine research focused on the users knowledge, such as the skills and cultural beliefs of women, is rather scarce (Choudhry, 1997; Cabré, 2008), although it is well documented that women and elders are the most active consumers of traditional medicine (Voeks, 2007; Schunko et al., 2012; Jimenez-Fernandez et al., 2023). Women were also related to collecting and drying herbs as a source of botanical drugs (Ishtiaq et al., 2022), as well as to plant preservation efforts (Bowcutt and Caulkins, 2020). Additionally, household strategies using a wide variety of plants and plant parts such as leaves, bark, roots, fruits, seeds, or animal elements like honey, eggs, and fat that are practiced by pauper women have been recognized for yielding satisfactory results. These strategies have successfully coexisted with conventional therapies (Mandu and Silva, 2000). Exploring the contemporary ethnomedical knowledge and practices of mothers holds promise for advancing ethnopediatric research.

Despite the impressive scientific achievements in pediatrics, an unacceptably high number of children suffer from life-threatening conditions (e.g., wasting) or die every year from preventable causes (e.g., acute respiratory infections, diarrhea) ("UNICEF Annual Report, 2021: Protecting Child Rights in a Time of Crises," 2021). For instance, in 2020, 5 million children under the age of 5 lost their lives (equivalent to13,800 children every day), mostly due to the inaccessibility of quality healthcare, affordable treatments, and proper food and water ("UNICEF Annual Report 2021: Protecting Child Rights in a Time of Crises," 2021). The wealth of traditional pediatric knowledge remains underexplored and undervalued, yet it holds the potential to provide accessible therapeutic solutions and inspire scientists in the development of new pharmacological agents. For example, a systematic review suggested that herbal medications can be effective for mild conditions, such as diarrhea, dehydration, and infantile colic (Anheyer et al., 2017).

Unfortunately, there is a relative scarcity of studies that specifically approach the subject of ethnomedical practices in children nowadays, especially in Eastern Europe. We found a few studies evaluating pediatric ethnopharmacological knowledge in Africa (Geissler et al., 2002; Nalumansi et al., 2014; Abubakar et al., 2017; Tugume and Nyakoojo, 2019), South America (Ruysschaert et al., 2009) and Asia (Mahomoodally and Sreekeesoon, 2014). Nevertheless, there is an increasing number of general ethnopharmacological studies in the Balkan area (Serbia, Bulgaria, Montenegro, Albania, Macedonia, etc.), which may provide insight into the pediatric potential of local plants for diseases common to both adults and children or human nutrition. For instance, these studies report the traditional use of plants for wound healing and skin related-problems (Jaric et al., 2018; Tsioutsiou et al., 2022), respiratory, gastrointestinal, urogenital, and cardiovascular diseases (Ivancheva and Stantcheva, 2000; Menković et al., 2011; Šavikin et al., 2013; Zlatkovic et al., 2014; Mustafa et al., 2015; Lumpert and Kreft, 2017; Janaćković et al., 2022; Gradinaru, 2023; Marković et al., 2023; Jarić et al., 2024), as well as the use of edible plants (Redžić and Ferrier, 2014; Dogan et al., 2015).

Romanian people have a valuable ethnomedical and ethnobotanical heritage kept alive through oral transmission, within families of healers, midwives, herb collectors, and monastic communities (Barca and Strachinaru, 1974; Butura, 1979; Pavelescu, 2004; Marian, 2010; 2008; Pop et al., 2010; Drăgulescu, 2013; Mărculescu and Olteanu, 2014; Dragulescu, 2019; 2017; Mattalia et al., 2020). Researchers evaluating the monographs of medicinal plants included in various editions of the Romanian Pharmacopeia (RPh) published between 1862–1993

County	# respondents
Arges	18
Braila	4
Bucuresti	114
Buzau	6
Calarasi	20
Constanta	6
Dambovita	21
Dolj	7
Giurgiu	21
Gorj	6
Ialomita	19
Ilfov	29
Mehedinti	2
Olt	8
Prahova	19
Teleorman	18
Tulcea	3
Valcea	5

FIGURE 1
Map of Romania indicating the study area, which spans three main regions: Muntenia (blue), Oltenia (red), and Northern Dobruja (green), along with the distribution of respondents by counties (top right).

(RPh I 1862; RPh II 1874; RPh III 1893; RPh IV 1926; RPh V 1943; RPh VI 1948; RPh VII 1956; RPh VIII 1965; RPh IX 1976; RPh X 1993) found that over time, 289 medicinal plants were included, with the majority (176, i.e. 60.9%) originating from Romanian ethnomedicine (Mărculescu et al., 2004; Benedec et al., 2017). The first edition of the RPh, consisting of 790 pages and written at the initiative of Dr. Carol Davila, was recognized as one of the most appreciated pharmacopeia in Eastern Europe, with its value corresponding to the scientific standards of that historical period (Besciu et al., 2011; Benedec et al., 2017). Out of the 301 drugs included in RPh I, 207 were of herbal origin (Stancu et al., 2014).

In a previous historical analysis focused on Romanian folk pediatrics practiced during 1860s–1970s, we found 153 medicinal plants with ethnotherapeutic significance for children's diseases (Petran et al., 2020), but the level of their contemporary use was not yet explored. Considering the central role of women as household caregivers, this study aimed to evaluate the current ethnopediatric knowledge and domestic use of medicinal plants by mothers for treating their children in Southern Romania.

2 Materials and method

2.1 Background of study area

Romania is recognized as one of the most biogeographically diverse countries of the European Union, having Alpine, Continental, Pannonic, Pontic, and Steppic regions. These specific geographic features combined with its modified temperate continental climate provide the conditions for a diverse flora (3,829 vascular and 979 non-vascular spontaneous plant taxa) (Hurdu et al., 2022), also characterized by a great abundance of medicinal and aromatic plants. A recent evaluation concluded that in Romania there are more than 750 species of plants with medicinal properties (Dihoru and Boruz, 2014).

Southern Romania includes two regions, known historically as Walachia and Northern Dobruja (Figure 1). Walachia covers two historical regions, Muntenia (Greater Wallachia) and Oltenia (Lesser Wallachia), and has about 10,784,874 million people in an area of 101,248 km^2. It is worth mentioning that the South-Eastern Carpathians alone, partially belonging to Southern Romania, represent one of the major biodiversity hotspots in Europe (Bálint et al., 2011; Dihoru and Boruz, 2014; Hurdu et al., 2022).

Near this area, in Northern Dobruja, there is the Danube Delta, a World Heritage (https://whc.unesco.org/en/list/588/). Danube Delta represents a unique biosphere reserve and wetland ecosystem, quoted as the best-preserved delta in Europe, and the third largest biodiversity area in the world, with over 5500 flora and fauna species (Ciocarlan, 1994; Claudino-Sales, 2019).

2.2 Methods

A survey instrument was developed by a panel of researchers with a medical background and extensive knowledge in phytotherapy and ethnomedicine, in accordance with other studies related to analogous issues (Kennedy, 2005; Raal et al.,

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TABLE 1 Multivariate analysis.

Independent parameters	Estimate	Std.Error	t_value	p-value
Used plant count–1st step				
Age	0.008564	0.008851	0.967	0.33
Urban residency	0.151991	0.162835	0.933	0.35
Higher educational level	0.941605	0.197033	4.779	3e-06
Purchased	−0.265285	0.202679	−1.309	0.19
Used plant count–2nd step				
Age	0.008353	0.008847	0.944	0.35
Higher educational level	0.964837	0.195416	4.937	1e-06
Purchased	−0.226657	0.198369	−1.143	0.25
Used plant count–3rd step				
Higher educational level	1.0090	0.1897	5.319	2e-07
Purchased	−0.2299	0.1983	−1.159	0.25
Ailments treated count–1st step				
Age	0.010236	0.007954	1.287	0.2
Urban residency	0.161682	0.146323	1.105	0.27
Higher educational level	0.632904	0.177053	3.575	0.0004
Purchased	−0.322763	0.182127	−1.772	0.077
Ailments treated count–2nd step				
Age	0.010012	0.007954	1.259	0.21
Higher educational level	0.657617	0.175695	3.743	0.00022
Purchased	−0.281673	0.178351	−1.579	0.12
Ailments treated count–3rd step				
Higher educational level	0.7105	0.1707	4.161	4e-05
Purchased	−0.2855	0.1785	−1.600	0.11
Procurement by purchase–1st step				
Age	−0.008062	0.017718	−0.455	0.65
Urban residency	1.239028	0.350530	3.535	0.0004
Higher educational level	−2.057001	0.615726	−3.341	0.0008
Procurement by purchase#–2nd step				
Urban residency	1.2334	0.3501	3.523	0.0004
Higher educational level	−2.0870	0.6124	−3.408	0.00065

Legend: The dependent variables (Used plant count, Ailments treated count, Procurement by purchase) are bold-typed as are the designations of the various steps of the MVA, for each of these dependent variables The second column contains the estimate, which is the average change in the log odds of the dependent variable associated with a one unit increase in each independent variable.

2013; Wu et al., 2013). The study population encompassed mothers with a domicile in Southern Romania whose children fell in the age category 0–18 years and was conducted between September 2019 and December 2022 in "Grigore Alexandrescu" Children Emergency Hospital, a tertiary hospital in Bucharest, Romania. The project was approved by the Ethics Commission of 'Grigore Alexandrescu' Children Emergency Hospital (no. 3/1736). Ethnobotanical and ethnomedical investigations were carried out following the ethical guidelines outlined by the International Society of Ethnobiology (ISE) Code of Ethics (The ISE code of ethics, International Society of Ethnobiology, 2006).

Data collection was based on structured interviews (Etkin, 1993; Heinrich et al., 2009) applied to mothers of children hospitalized for various diseases. Responses were recorded by a health professional

TABLE 2 Classification of ethnopediatric indications included in the present study, adapted from the International Classification of Primary Care (ICPC).

Body system	Example of disease
General	Weakness, Allergies, Anemia
Musculoskeletal	Trauma
Neuropsychological	Agitation, Sleep disturbances/Insomnia/Nightmares/Weeping during sleep
Respiratory	Acute respiratory diseases, Asthma, Bronchitis, Phlegm in the throat, Cold, Cough, Ear pain
Digestive	Colic, Abdominal cramps, Acute digestive infections, Diarrhea, Flatulence, Intestinal worms, Intestinal parasites
Skin	Burn wounds/Burns, Dermatitis, Diaper (napkin) dermatitis, Eczema, Skin inflammation, Skin infections, Skin lesions, Verruca, Wounds, Eye infections
Urinary	Urinary tract infections, Urolithiasis

TABLE 3 Informant consensus factor.

Disease category	N_t	N_{ur}	F_{ic}
Respiratory diseases	8	169	0.96
Digestive diseases	9	361	0.98
Urinary tract diseases	1	2	1
Skin conditions	4	253	0.99
Nervous system	1	3	1
Trauma/musculoskeletal	1	87	1
General: weakness, allergies, anemia	5	68	0.94

Legend. F_{ic} - Informant Consensus Factor; N_t -number of taxa used in that category; N_{ur}-total number of use citations in each category.

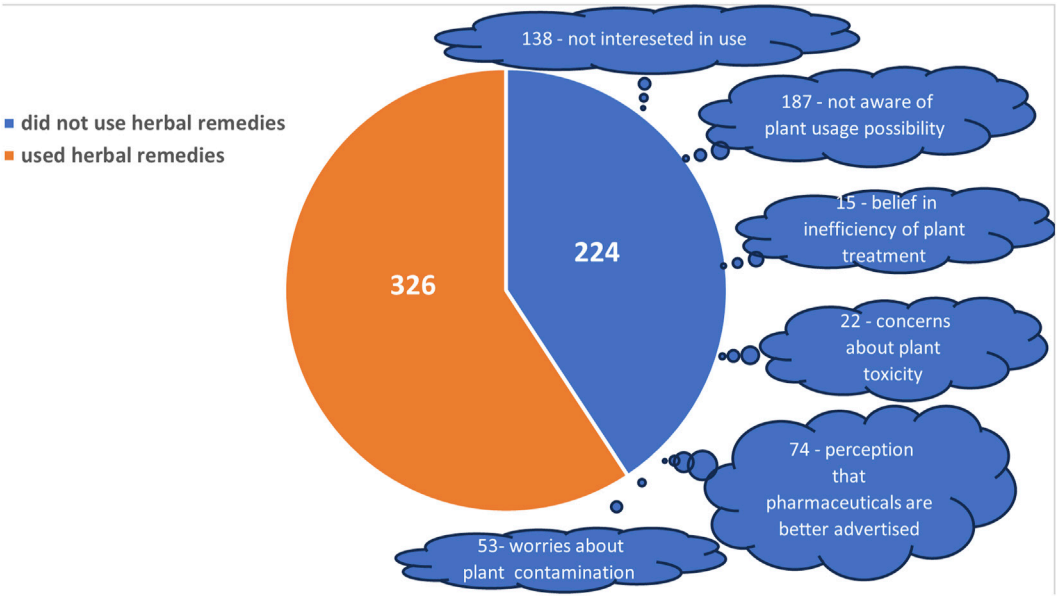

FIGURE 2
Selection of informants for survey participation, with each category representing the number of informants. 326 informants declared the usage of herbal remedies and were included in the study, whereas 224 did not, for reasons depicted in the figure.

TABLE 4 Demographic characteristics of the herbal therapy users.

Characteristic	Description	Percentage (%)	95 (%) CL
Age category	14–18	3	2.06–3.94
	18–24	7.4	5.95–8.85
	25–29	14.3	12.36–16.24
	30–34	11.2	9.45–12.95
	35–39	27.3	24.83–29.77
	40–44	23.6	21.25–25.95
	>45	13.2	11.33–15.07
Residence	Urban	37	34.33–39.67
	Rural	63	60.33–65.67
Religion	Christian	100	
Level of education	No education	4	2.91–5.09
	Low/primary level	18	15.87–20.13
	Medium level	57	54.26–59.74
	College degree	21	18.74–23.26

Note. The 95% confidence limits (95% CL) are calculated relative to the population of herbal treatment employing women.

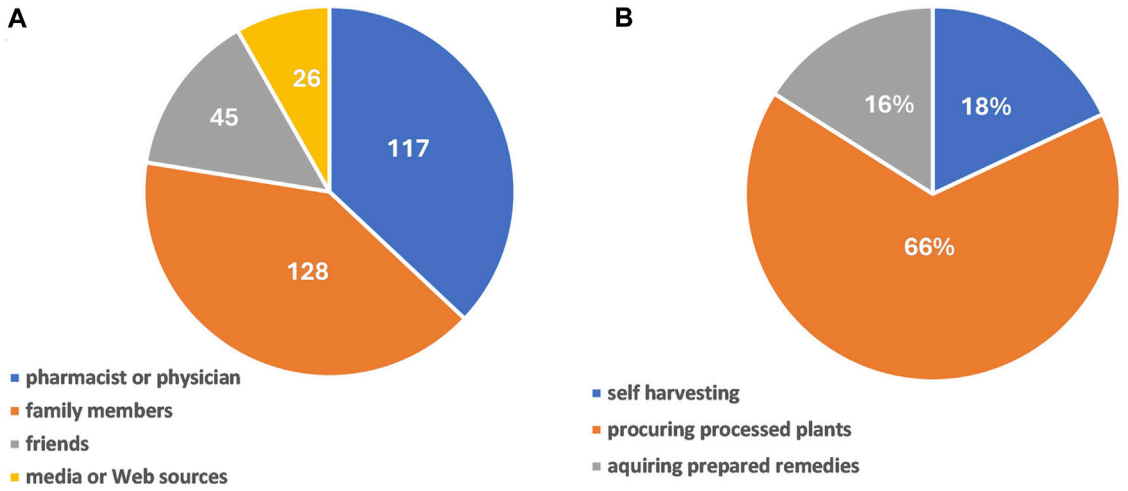

FIGURE 3
Recommendation sources for medicinal plant usage (with each category representing the number of informants) **(A)**, and the sourcing of the botanical remedies documented in our survey (with each category representing the percentage of informants) **(B)**.

with expertise in phytotherapy and ethnomedicine. The questionnaire was strictly confidential, anonymized, and non-compulsory. The purpose, methodology, and objective of the study were explained to all participants, and written informed consent was obtained subsequently. The interviews occurred in an individual manner and took between 20 and 30 min to complete.

The questionnaire comprises structured open-ended and close-ended questions regarding the medicinal plant species used, their indications and ethnopharmacological activities, sources of knowledge, plant part(s) used, type of preparation, routes of administration, concomitant use of conventional drugs, and treatment outcomes (for further details, please refer to the Table 1, Supplementary Material). The demographic data of each participant (age, gender, area of residence, level of education) were recorded, together with the age when the children first received the herbal remedy. Standardized ethnobotanical procedures that confer transparency (e.g., availability of information on the frequency of citations), important to assess the relative cultural rank of a species, were followed (Heinrich et al., 2009). Since the present study was essentially a survey conducted within a hospital unit, the collection of voucher specimens was not feasible. The specific study design resembled

TABLE 5 The medicinal plants recorded in the study, with their corresponding therapeutic use, route of administration, mode or preparation, use value citation index, and fidelity level.

Nr	Latin name	Family name	English name	Romanian name	Origin	Indications and targeted body systems (nr. of mentions and FL)	Ethnopharmacological activities	Parts used	Route of administration	Preparation mode	Body systems	Actions	UVc
1	*Abies alba* Mill	Pinaceae	Silver fir	Brad argintiu	Native	Acute respiratory diseases	Expectorant	Buds	Internal	Syrup	1	1	0.055
						Bronchitis (12, 100)							
2	*Allium cepa* L	Amaryllidaceae	Onion	Ceapa	Exotic	Respiratory	Calming	Bulb, leaves	External	Baked, poultice	1	3	0.074
						Asthma (1, 4.1)	Relaxing						
						Bronchitis (23, 95.9)	Anti-inflammatory						
3	*Allium sativum* L	Amaryllidaceae	Garlic	Usturoi, ai	Exotic	Digestive	Anti-parasitic	Bulb, leaves	Internal	Raw bulb	1	1	0.012
						Intestinal worms (2, 50)			External–intra rectal				
						Intestinal parasites (2, 50)							
4	*Allium ursinum* L	Amaryllidaceae	Wild garlic, ramson	Leurda, usturoiul ursului	Native	General	Tonic	Leaves	Internal	Raw leaves	1	1	0.025
						Weakness (5, 100)	Immunostimulant						
5	*Anethum graveolens* L	Apiaceae	Dill	Marar	Native	Digestive	Relaxing Carminative	Aerial parts	Internal	Infusion	1	2	0.055
						Abdominal cramps (2, 13.3)		Seeds					
						Infantile colic (13, 86.7)							
6	*Arnica montana* L	Asteraceae	Mountain arnica	Arnica	Native	Musculoskeletal: Trauma (87, 100)	Vulnerary	Leaves	External	Ointment	1	2	0.264
							Anti-inflammatory						
7	*Calendula officinalis* L	Asteraceae	Common marigold	Galbenele	Native	Skin	Emollient	Capitulum	External	Ointment	1	3	0.365
						Diaper rash (92, 72.4)							
						Eczema (11, 8.7)	Vulnerary						
						Wounds (3, 2.4)	Anti-infective						
						Burns (3, 2.4)							
						Skin infections (18, 14.2)							

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TABLE 5 (*Continued*) The medicinal plants recorded in the study, with their corresponding therapeutic use, route of administration, mode or preparation, use value citation index, and fidelity level.

Nr	Latin name	Family name	English name	Romanian name	Origin	Indications and targeted body systems (nr. of mentions and FL)	Ethnopharmacological activities	Parts used	Route of administration	Preparation mode	Body systems	Actions	UVc
8	*Carum carvi* L	Apiaceae	Caraway	Chimen	Native	Digestive Cramps (2, 13.3) Infantile colic (13, 86.7)	Carminative	Seeds	Internal	Infusion	1	1	0.034
9	*Chelidonium majus* L	Papaveraceae	Greater celandine	Rostopasca, negelarita	Native	Skin Warts (37, 100)	Anti-infective Immune stimulant	Latex	External	Sap	1	2	0.095
10	*Cucurbita pepo* L	Cucurbitaceae	Pumpkin	Dovleac, bostan	Exotic	Digestive Intestinal worms (18, 100)	Anti- parasitic	Seeds	Internal	Seeds	1	1	0.049
11	*Foeniculum vulgare* Mill	Apiaceae	Fennel	Fenicul	Exotic	Digestive Intestinal cramps (2, 13.3) Infantile colic (13, 86.7)	Carminative	Fruits	Internal	Infusion	1	1	0.055
12	*Helianthus annuus* L	Asteraceae	Common sunflower	Floarea soarelui	Exotic	Ear Ear pain (2, 100)	Analgesic	Seeds oil	External	Seed oil	1	1	0.006
13	*Hippophae rhamnoides* L	Elaeagnaceae	Sea buckthorn	Catina alba, catina de rau	Native	Respiratory Respiratory infections (74, 100)	Tonic Immune stimulant	Fruits	Internal	Syrup, infusion	1	2	0.224
14	*Hypericum perforatum* L	Hypericaceae	Saint John's wort	Sunatoare, pojarnita	Native	Digestive Liver diseases (4, 28.5) Gallbladder dyskinesia (10, 71.5)	Vulnerary	Aerial parts	Internal	Infusion	1	1	0.043
15	*Matricaria spp.*	Asteraceae	Chamomile	Musetel, romanita	Native	1. Internal -Digestive Diarrhea (14, 11) Infantile colic (86, 68) 2.External–Skin and mucosa	Relaxing Carminative Anti-inflammatory	Capitulum	Internal External	Infusion Decoction	2	4	0.301

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TABLE 5 (*Continued*) The medicinal plants recorded in the study, with their corresponding therapeutic use, route of administration, mode or preparation, use value citation index, and fidelity level.

Nr	Latin name	Family name	English name	Romanian name	Origin	Indications and targeted body systems (nr. of mentions and FL)	Ethnopharmacological activities	Parts used	Route of administration	Preparation mode	Body systems	Actions	UVc
						inflammation (19, 15)							
						Eczema (5, 4)	Anti-infective						
						Eye infection (3, 2)							
16	*Mentha spp.*	Lamiaceae	Mint	Menta, izma buna	Native	Digestive	Relaxing	All green parts	Internal	Infusion	1	4	0.509
						Diarrhea (154, 93.3)	Carminative						
						Bloating (11, 6.7)	Anti-infective						
							Anti-flatulent						
17	*Pimpinella anisum* L	Apiaceae	Anise	Anason	Exotic	Digestive	Relaxing Carminative	Fruits	Internal	Infusion	1	2	0.055
						Intestinal cramps (2, 13.3)							
						Infantile colic (13, 86.7)							
18	*Prunus avium (L.) L*	Rosaceae	Cherry	Cires	Native	Renal Urinary tract infections (2, 100)	Diuretic	Stalks	Internal	Infusion	1	1	0.006
19	*Raphanus raphanistrum subsp. sativus (L.) Domin*	Brassicaceae	Black radish	Ridiche neagra	Exotic	Respiratory Bronchitis (2, 100)	Anti-inflammatory	Root	Internal	Sap with honey	1	3	0.006
							Calming						
							Expectorant						
20	*Rosa canina* L	Rosaceae	Dog rose	Maces	Native	Respiratory Acute infective diseases (17, 100)	Tonic	Fruits	Internal	Infusion, syrup	1	3	0.052
							Anti-infective						
							Anti-inflammatory						
21	*Thymus serpyllum* L	Lamiaceae	Wild thyme	Cimbrisor de camp	Native	Respiratory Acute infective diseases (12, 100)	Anti-infective Expectorant	Aerial parts	Internal	Infusion	1	2	0.04
22	*Tilia tomentosa* Moench	Malvaceae	Silver linden	Tei argintiu, tei alb	Native	Psychological: Agitation (3, 5.5)	Relaxing Calming Sedative	Flowers	Internal	Infusion	2	3	0.153
						Anxiety (1, 2)							

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TABLE 5 (*Continued*) The medicinal plants recorded in the study, with their corresponding therapeutic use, route of administration, mode or preparation, use value citation index, and fidelity level.

Nr	Latin name	Family name	English name	Romanian name	Origin	Indications and targeted body systems (nr. of mentions and FL)	Ethnopharmacological activities	Parts used	Route of administration	Preparation mode	Body systems	Actions	UVc
						Respiratory							
						Acute infective diseases with cough (50, 92.5)							
23	*Triticum aestivum* L	Poaceae	Wheat	Grau	Exotic	Skin	Emollient	bran	External	Decoction	1	1	0.018
						Eczema (6, 100)							
24	*Urtica dioica* L	Urticaceae	Common nettle	Urzica	Native	General	Tonic	Young leaves	Internal	Infusion, young leaves cooked	1	1	0.101
						Weakness (13, 38.2)							
						Blood							
						Anemia (21, 61.8)							
25	*Viola tricolor* L	Violaceae	Heartsease	Trei frati patati	Native	General	Anti-allergic	Flowering branches	Internal	Infusion	1	1	0.012
						Allergies (4, 100)							

Legend- FL-fidelity level; UVc -use value citation index.

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TABLE 6 The correlation between categorical parameters as estimated by Fisher's exact test.

		Residency		p-value	Education		p-value
		Rural	Urban		Inferior	Superior	
Used plants	≤2	123	68	0.56	62	129	**E-08**
	>2	82	53		9	126	
Ailments treated	≤2	129	73	0.64	61	141	**E-06**
	>2	76	48		10	114	
Administration route	External	51	29	0.89	18	62	0.88
	Internal	154	92		53	193	
Plant procurement	Purchased	154	109	**0.0008**	68	195	**8E-05**
	Harvested	51	12		3	60	
Preparation method	cooked	10	1	0.14	0	11	**0.0001**
	decoction	5	4		2	7	
	infusion	121	84		59	146	
	ointment	25	15		8	32	
	raw	23	11		2	32	
	syrup	21	6		0	27	
Recommendation source	family	95	46	**0.01**	26	115	**0.0001**
	friends	39	23		9	53	
	medical_prof	65	37		36	66	
	web_media	6	15		0	21	

Legend: As multiple (12) comparisons were performed, the significance level (commonly set at 0.05) was lowered according to Bonferroni correction: the corrected significance level was 0.05 divided by the number of comparisons, namely, $\alpha_{corrected} = 0.05/12 \approx 0.004$. The comparisons that yielded significant results were bold-typed.

that of other studies, such as surveys evaluating the use of herbal remedies by pharmacy customers (Knotek et al., 2012; Raal et al., 2013; Zeni et al., 2017) or by members of various minorities (Bhamra et al., 2017). The assessment of herbal authenticity was not within the scope of this study. The accuracy of the taxonomy and nomenclature of vascular plants was verified by cross-referencing with World Flora Online (WFO) (www.worldfora online.org).

Following the collection and analysis of the questionnaires, we compiled a list of medicinal plants based on informant input. To assess the conformity and/or novelty of our results, we conducted a systematic search of the ethnomedical or bioscientific data on the identified medicinal plants. This comprehensive search was carried out across PubMed and Google Scholar databases. Our strategy involved using specific phrases such as: [(Latin name of the plant) OR (vernacular name of the plant)] AND (children OR toddler OR pediatrics OR adults OR indication OR biological activity). For example, a search query could include (Foeniculum vulgare OR fennel) AND (children OR toddler OR pediatrics OR adults OR indication OR biological activity). This method enabled us to analyze existing literature and evaluate the medicinal properties and potential applications of the identified plants within the age groups of interest across various medical contexts, and in adulthood, specifically in the pathologic spectrum reported by our informants.

2.3 Data analysis

In this article, the terms "variable" and "parameter" will be considered equivalent and used interchangeably. The same holds for the terms "association" and "correlation." The statistical analysis consisted of both descriptive and inferential statistics. Descriptive statistics included the calculation of the median and interquartile range (from the first to the third quartile) to characterize numerical parameters such as mother age, used plants count, treated ailments count, and child age when the first herbal treatment was given.

Based on the number of plants used, the mothers were divided into two groups: one comprising those who utilized one or two plants (n = 191), and the other consisting of those who employed more than two plants (n = 135). Similarly, concerning the number of treated ailments, two groups of informants were defined: one including those who used plants for treating one or two ailments in their children (n = 202), and the other, those who treated more than two diseases (n = 124).

Shapiro-Wilk test was used to assess the normality of data distribution. Inferential statistics encompassed univariate analysis (UVA) and multivariate analysis (MVA). UVA included: 1. regression by Spearman's method, used to estimate the correlation between two numerical parameters 2. Mann-Whitney (Wilcoxon) test with continuity correction for

TABLE 7 The correlation between numerical and dichotomous categorical parameters as estimated by Mann-Whitney (Wilcoxon) test.

Numerical parameters	Categorical parameters	#pts. Yes	#pts. No	Median yes [IQR]	Median no [IQR]	W Statistics	p-value
Treatment duration [days]	Internal administration	**246**	**80**	**5 [4–7]**	**7 [5–10]**	**14243.5**	**8E-10**
Ailments treated count	Internal administration	246	80	2 [1–3]	2 [1–3.25]	10372.5	0.5
Used plant count	Internal administration	246	80	2 [1–4]	2 [1–3]	9347.5	0.5
Mothers' age [years]	Internal administration	246	80	37 [31–43]	37 [31–41]	9406	0.6
Child age at first herbal treatment	Internal administration	246	80	2 [0.5–4]	2.5 [0.33–4]	9843.5	1
Mothers' age [years]	Ailments treated >2	124	202	38 [34–42.25]	36 [28–42]	10688	0.026
Treatment duration [days]	Ailments treated >2	124	202	5 [5–7.25]	5 [4–7]	11735	0.3
Child age at first herbal treatment	Ailments treated >2	124	202	2 [0.33–4]	2 [1–4]	13007	0.6
Used plant count	Higher educational level	**255**	**71**	**2 [1–4]**	**2 [1–2]**	**5461**	**1E-07**
Ailments treated count	Higher educational level	**255**	**71**	**2 [1–3.5]**	**2 [1–2]**	**6122.5**	**2E-05**
Mothers' age [years]	Higher educational level	**255**	**71**	**38 [33–42]**	**32 [23–41]**	**6338.5**	**1E-04**
Child age at first herbal treatment	Higher educational level	**255**	**71**	**3 [0.5–5]**	**2 [0.79–2]**	**6544.5**	**3E-04**
Treatment duration [days]	Higher educational level	255	71	5 [5–7]	5 [4–7]	7993.5	0.1
Ailments treated count	Favorable outcome	274	52	2 [1–3]	3 [1–3.25]	8268	0.06
Used plant count	Favorable outcome	274	52	2 [1–3]	2.5 [1–4]	7884.5	0.2
Mothers' age [years]	Favorable outcome	274	52	37 [31–42]	38 [31.25–43]	7595.5	0.4
Treatment duration [days]	Favorable outcome	274	52	5 [5–7]	5 [4–10]	6678.5	0.5
Child age at first herbal treatment	Favorable outcome	274	52	2 [0.5–4]	2 [0.31–5]	6803	0.6
Child age at first herbal treatment	Purchased	**263**	**63**	**2 [0.33–4]**	**3 [2–5]**	**11007**	**5E-05**
Ailments treated count	Purchased	**263**	**63**	**2 [1–3]**	**3 [2–4]**	**10266**	**0.002**
Used plant count	Purchased	**263**	**63**	**2 [1–3]**	**3 [2–4]**	**10113**	**0.005**
Mothers' age [years]	Purchased	263	63	37 [29–42]	38 [33.5–42.5]	8956.5	0.3
Treatment duration [days]	Purchased	263	63	5 [4.5–7]	5 [5–10]	8836	0.4
Ailments treated count	Urban residency	121	205	2 [1–3]	2 [1–3]	11733.5	0.4
Used plant count	Urban residency	121	205	2 [1–4]	2 [1–3]	11737	0.4
Mothers' age [years]	Urban residency	121	205	37 [31–42]	37 [31–43]	12734.5	0.7
Child age at first herbal treatment	Urban residency	121	205	2 [0.67–4]	2 [0.42–4]	12114	0.7
Treatment duration [days]	Urban residency	121	205	5 [4–7]	5 [5–7]	12220.5	0.8
Mothers' age [years]	Used plant >2	135	191	38 [34–42]	36 [28–42.5]	10867.5	0.016
Treatment duration [days]	Used plant >2	135	191	5 [5–7]	5 [4–7]	12052.5	0.3
Child age at first herbal treatment	Used plant >2	135	191	3 [0.33–4]	2 [0.58–4]	12293.5	0.5

Legend: W statistics and *p*-values were calculated by means of Mann-Whitney test. As multiple (31) comparisons were performed, the significance level (commonly set at 0.05) was lowered according to Bonferroni correction: the corrected significance level was 0.05 divided by the number of comparisons, namely, $\alpha_{corrected} = 0.05/31 \approx 0.0016$. The comparisons that yielded significant results were bold-typed. #pts. = patients count; "yes" and "no" in the 3rd–5th columns refer to categorical parameters in the second column. The median and the interquartile range [IQR] in the 5th and 6th columns are those of the numerical parameter in the 1st column. For example, the numbers in the first row mean that 246 mothers declared having internally administered the herbal remedy, while 80 mothers used it externally; the median duration of internally given treatment was 5 days, while median duration of externally administered treatment was 7 days.

TABLE 8 The correlation between numerical and multivalent categorical parameters as estimated by Kruskal-Wallis test.

Parameter	Values	Mothers' age [years] median (IQR)	Kruskal-Wallis chi-squared	p-value
Preparation method	cooked	36 (33–41)	6.2665	0.2811
	decoction	37 (36–40)		
	infusion	37 (28–42)		
	ointment	37 (30.5–41)		
	raw	39 (36–43.8		
	syrup	38 (31.5–40)		
Recommendation source	medical professionals (MP)	35 (26.2–40.8)	20.224	0.00015 (MP–WM: 0.001; MP–Fr: 0.0025; MP–Fa: 0.03; Fa–WM: 0.02)
	family (Fa)	37 (28–42)		
	friends (Fr)	38.5 (34.2–43)		
	web, media (WM)	42 (38–46)		

Legend: Kruskal-Wallis test was employed to calculate Kruskal-Wallis chi-squared statistics (in the 4th column) and *p*-values (in the 5th column). The parenthesis in the last cell of the table contains the *p*-values obtained by two-by-two comparisons using the pairwise Wilcoxon test, while Benjamini-Hochberg procedure was employed to decrease the false discovery rate. ~ = compared with.

exploring the correlation of various numerical parameters with binary (i.e., with only two possible values) categorical parameters 3. Kruskal-Wallis test, employed to evaluate the correlation of numerical parameters with multivalent (i.e., with more than two possible values) categorical parameters 4. Fisher's exact test is used to explore the correlations between various categorical parameters. In particular, Fisher's exact test was utilized to determine whether there is an association between the social background (rural vs. urban) and the employment of medicinal plants. The strength of the association was estimated by odds ratio. The accompanying 95% confidence intervals for odds ratio are an estimation of the real value in the population: there is a 95% probability that the real value in the population falls within the confidence interval. Of course, it is difficult to decide whether the mothers in this tertiary care pediatric hospital are a representative sample of the entire population of mothers.

When multiple comparisons were performed by Mann-Whitney test, the significance level (commonly set at 0.05) was lowered according to Bonferroni correction: the corrected significance level was determined by dividing 0.05 by the number of comparisons. In cases where Kruskal-Wallis test yielded a significant result, pairwise Wilcoxon tests were subsequently conducted using Benjamini-Hochberg procedure to reduce the false discovery rate.

After having established by UVA the variables significantly associated with used plants count, treated ailments count, and purchase method, MVA was performed to determine the variables independently associated with each of these parameters. The method employed for MVA was backward stepwise regression. For each dependent variable, regression was first performed on all n (supposedly) independent variables pointed out by UVA as being statistically significantly associated with the dependent variable. Among the variables proven to lack a statistically significant ($p > 0.05$) contribution, the one with the smallest contribution (which was also the variable with the smallest t_value) was eliminated. Subsequently, regression was performed again on the remaining n - 1 variables, and once more, the variable with the smallest contribution was eliminated. This process continued iteratively until all remaining variables were statistically significantly associated with the dependent variable. The steps of the MVA for all three dependent variables are shown in Table 1.

All statistical calculations and resulting graphical representations were performed using R language and environment for statistical computing and graphics (version 4.2.3.). The employed packages were dplyr, tidyverse, ggplot2, ggpubr, mosaic, e1071, and mediation.

We divided the data regarding indications into use categories of diseases (Table 2), according to the International Classification of Primary Care (ICPC)(WHO Second edition ICPC-2, 2012), which was adapted to fit the ethnomedical reality (Staub et al., 2015).

The collected data were analyzed using quantitative methods. Use-Value Citation Index, Fidelity Level, and Informant Consensus Factor were calculated (Friedman et al., 1986; Phillips and Gentry, 1993; Hoffman and Gallaher, 2007; Heinrich et al., 2009; Hajdari et al., 2018; Trotter and Logan, 2019).

The Use-value citation index (UV_c) evaluates the relative importance of each species based on its cited uses, and it is calculated for all taxa with the formula:

$$UV_c = \left(\sum U_{is}\right)/N,$$

where U_{is} is the sum of the total number of all individual use citation reports concerning a given taxa, divided by the total number of informants (N) (Phillips and Gentry, 1993; Hoffman and Gallaher, 2007).

The fidelity level (FL) represents the percentage of informants reporting the usage of a certain medicinal plant for the same major indication (Friedman et al., 1986) and was calculated to determine

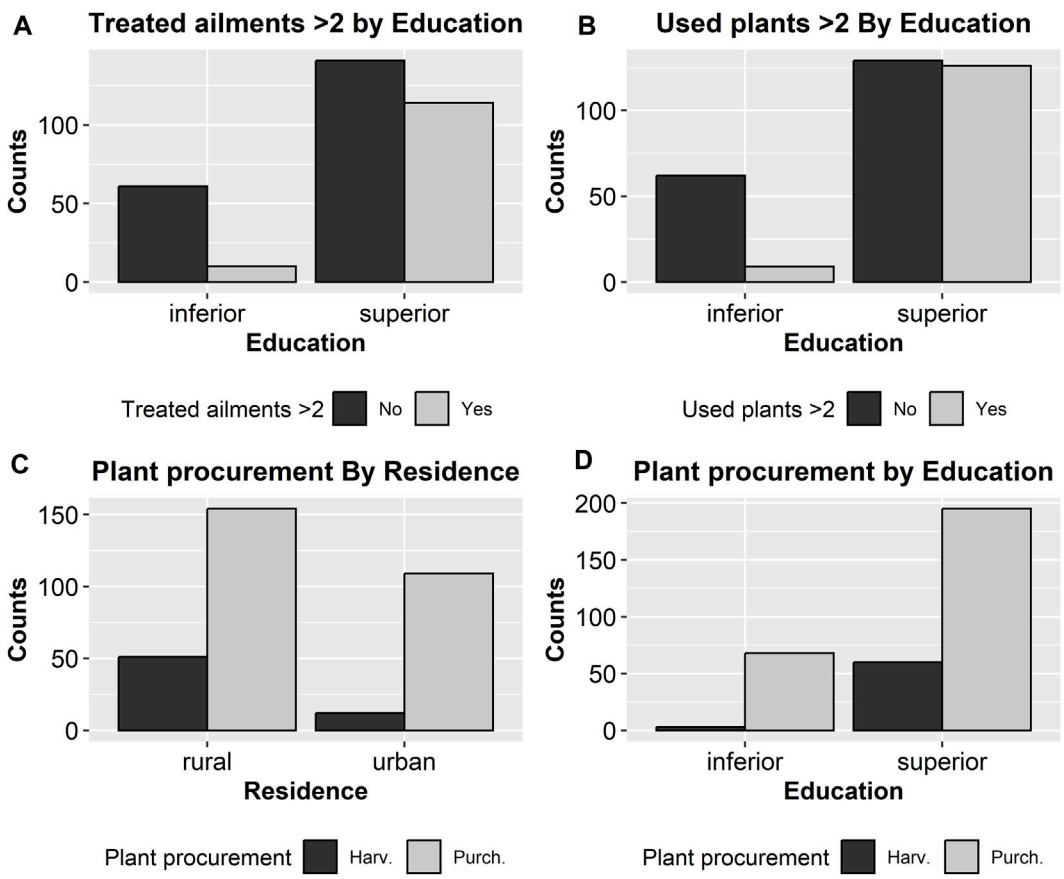

FIGURE 4
Significant correlations between binary categorical parameters as revealed by Fisher's exact test. The corresponding *p*-values are 10^{-6} **(A)**, 10^{-8} **(B)**, 0.0008 **(C)**, 8×10^{-5} **(D)**.

the most important species used to treat the most frequently reported diseases or ailments.

$$\mathrm{FL}\ (\%) = Np/N \times 100,$$

where *Np* is the number of informants reporting the usage of a plant species to treat a particular disease, and *N* denotes the total number of informants utilizing the plant as a treatment for any given disease (Friedman et al., 1986; Mahomoodally and Sreekeesoon, 2014).

The categories selected for the Informant Consensus Factor (F_{ic}) analysis are outlined in Table 3. Each taxon's usage was assigned to the relevant category before analysis, employing the following formula:

$$F_{ic} = (N_{uc}\text{-}N_t)/(N_{uc}\text{-}1),$$

where N_{uc} is the total number of use citations in each category and N_t is the number of taxa used in that category. F_{ic} estimates user consensus regarding the medicinal plants. The value of this factor ranges from 0 to 1. High F_{ic} values (close to 1.0) are obtained when relatively few species are reported to be used by a large proportion of informants for a particular nosological category, whereas lower F_{ic} values indicate that informants disagree upon the taxa to be used in the treatment within a certain category.

Binomial distribution was used to calculate the confidence limits for the various percentages yielded by our study sample.

3 Results

3.1 Demographic characteristics of participants

A total of 550 mothers living in Southern Romania were initially interviewed with a median age of 37 (interquartile range (IQR) of 31–42 and a skewness of −0.2194345). Among them, 224 respondents (40.72%, 95% confidence limits (95% CL): 38.63–42.82) did not utilise herbal remedies based on raw-plant material in treating their children (Figure 2). Reasons for this included: not being aware of this option (187 mothers, among whom 138 were nonetheless interested in using plants to treat their children), having learned from media that plant treatment is not useful or may even be toxic (22 mothers), and considering herbal remedies as inefficient (15 mothers).

Additionally, 74 informants indicated advertising of the pharmaceutic products as one of the factors influencing their decisions. Concerns were raised by 53 informants about plant contamination by pollutants. They declared that due to the lack of time and opportunity to harvest plants of trusted quality, they used pharmaceutical agents based on plant extracts, but mixed with synthetic compounds, vitamins, and minerals that are generally recommended by physicians, pharmacists, friends, and in the media. Due to the mixed nature (herbal and non-herbal/natural and

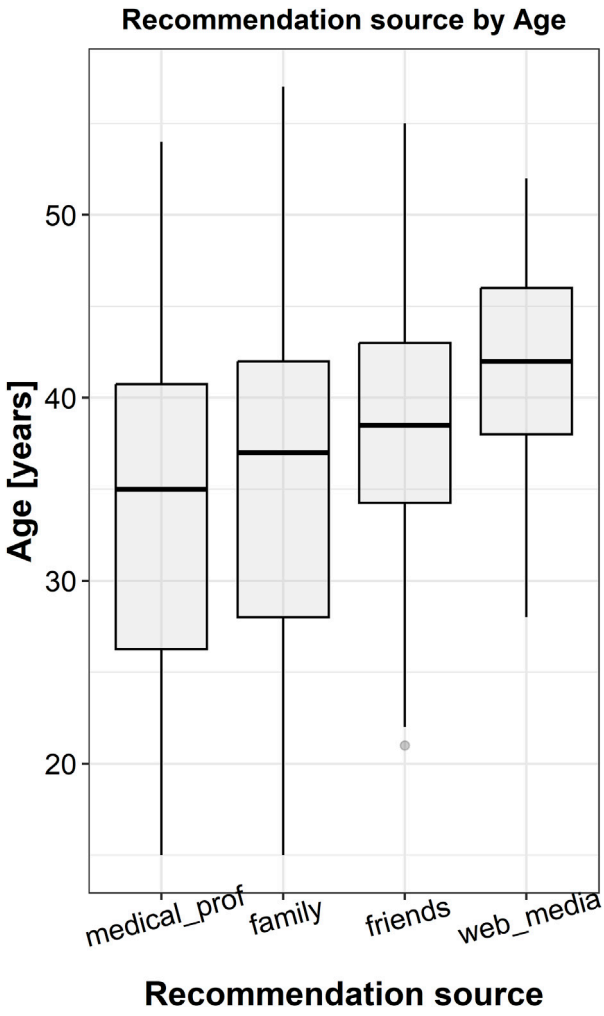

FIGURE 5
Significant correlation between the source of information and mothers' age as revealed by Kruskal-Wallis test. The corresponding *p*-value is 0.00015.

synthetic) of the medicines used, we decided to exclude them from enrollment in the study.

The employment of medicinal plants was declared by 177 and denied by 76 out of 253 mothers living in rural communities. Out of 297 mothers living in urban areas, 149 have confirmed the employment of medicinal plants, while 148 have denied it. Fisher exact test yields a less than 0.00001 *p*-value, indicating a strong association between social background and the utilization of medicinal herbs. Indeed, mothers of rural background have a higher propensity to use medicinal plants compared to mothers of urban background, as reflected by the odds ratio of 2.31 with a 95% confidence interval of 1.63–3.29.

The remaining 326 mothers (59.27%, CL95%: 57.18%–61.37%), who declared the use of medicinal plants, used plant treatment based on raw vegetal material (self-harvested or bought) for their children's illnesses and were included in the study (Figure 2).

These herbal therapy users originated from 14 counties of Southern Romania: Argeș (18), Braila (4), Bucuresti (114), Buzau (6), Calarasi (20), Constanta (6), Dambovita (21), Dolj (7), Giurgiu (21), Gorj (6), Ialomita (19), Ilfov (29), Mehedinti (2), Olt (8), Prahova (19), Teleorman (18), Tulcea (3), Valcea (5) (Figure 1).

Approximately one-third (37%) of the participants resided in rural areas, while the remaining majority (63%) lived in urban areas. In terms of age distribution, 97% of the mothers were above 18 years old, with a significant majority falling into the 35–39 and 40–44 age groups. Regarding educational attainment, 4% of the respondents had no formal education, 18% of them had completed primary education, 57% had attained secondary-level education, and 21% held a college degree (Table 4).

From this point on, the 95% confidence limits were calculated relative to the population of herbal treatment employing women. In 117 cases (35.89%, 95% CL: 33.23%–38.55%) the treatment was recommended by a pharmacist or a physician, in 128 cases (39.26%, 95% CL: 36.56%–41.96%) by family members, in 45 cases (13.80%, 95% CL: 11.89%–15.71%) by friends, and in 36 cases (11.04%, 95% CL: 9.3%–12.78%) the information was found in media or on the Web (Figure 3A). Interestingly, more than one-third of the informants (40.49%, 95% CL: 37.77%–43.21%) stated that herbal remedies were the only treatment used, whereas the others (59.51%, 95% CL: 56.79%–62.23%) associated plants with allopathic drugs. In 196 of the cases, children were given both raw plant-based remedies and pharmaceutical drugs based on plant extracts.

An impressive 88% (95% CL: 86.2%–89.8%) of the informants reported an improvement in the disease course following herbal therapies and consequently declared their intention to use and recommend the use of plant-based remedies in the future.

3.2 Diversity of plant species used for pediatric treatment

The collected data revealed that a total of 25 medicinal plant species from 15 families were used to treat pediatric diseases. Plant indications and activities, parts used, way of administration, UVc, FL, and targeted body systems are provided in Table 5.

Apiaceae and Asteraceae were the dominant families with four species each followed by Amaryllidaceae (three species) and Lamiaceae and Rosaceae (two species). The highest proportion of the medicinal plants used is herbaceous (76%), followed by trees (12%), shrubs (8%), and grasses (4%). The remedies were made from fruits, leaves, roots, seeds, stems, buds, flowers, bulbs, or sap. In some cases, a combination of plants was administered.

Only 18% (95% CL: 15.87%–20.13%) of the probands have harvested themselves the plants cultivated in their gardens or growing in the surroundings, whereas the majority of 82% (95% CL: 79.87%–84.13%) have purchased processed plant parts (66%, 95% CL: 63.38%–68.62%) or already prepared remedies (16%, 95% CL: 13.97%–18.03%) from pharmacies, specialized stores, and local markets (Figure 3B).

Various methods of preparation were used for the 25 medicinal plant species: decoction, infusion, ointment, poultice, and syrup, with infusion as the most preferred method (56%), followed by decoctions and poultice (12%). Conversely, ointments, syrups, and the raw administration of plant parts, sap, or seeds were less frequently utilized, accounting for only 8% of the preparations. Some of the medicinal remedies were administered with the meal, either cooked (e.g., garlic bulbs) or raw (e.g., sea buckthorn fruits). Water was the main solvent employed for the herbal treatments.

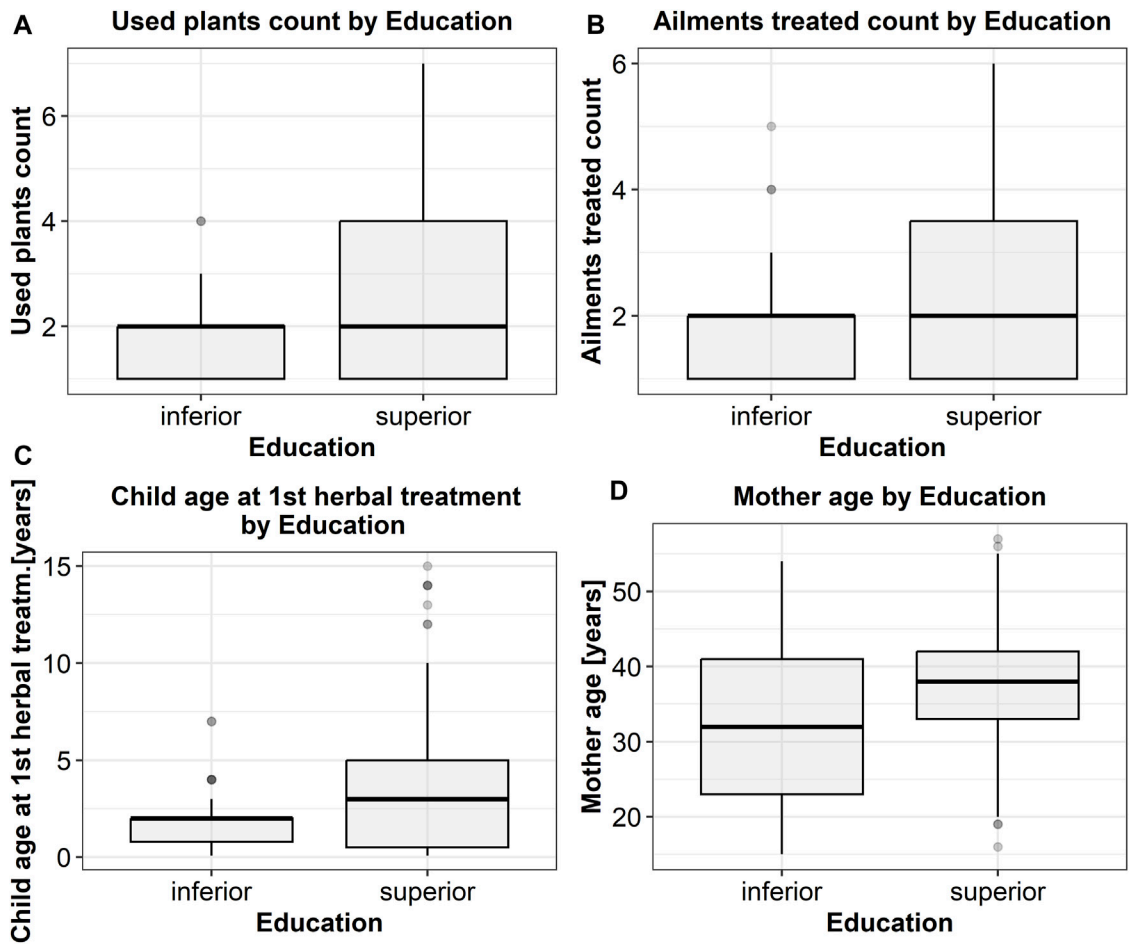

FIGURE 6
Significant correlation between numerical and categorical parameters as revealed by Mann-Whitney test. The corresponding *p*-values are 10^{-7} **(A)**, 2×10^{-5} **(B)**, 3×10^{-4} **(C)**, 10^{-4} **(D)**.

The remedies were administered orally, as baths with cold or warm infusions, or as external applications (crushed or baked plant materials covering the affected body parts). In one case of intestinal worms (oxyurids), a garlic clove was inserted in the anus.

3.3 Pediatric diseases treated using medicinal plants

The diseases were grouped into seven categories, depending on the targeted body systems, among which digestive, skin, and respiratory tract diseases featured predominantly. Informant consensus factor (F_{ic}) for each nosological category is provided in Table 3.

The most versatile species, having the largest area of indications were chamomile (*Matricaria spp*) and silver linden (*Tilia tomentosa* Moench.).

Monotherapy preparations were predominant, although some diseases were treated with polyherbal mixtures. For instance, an infusion of a mixture containing fennel (*F. vulgare* Mill.), caraway (*Carum carvi* L.), dill (*Anethum graveolens* L.) seeds, and chamomile capitulum was used to treat infant colic, beginning at 1.5-month of age. Mint (*Mentha spp*.) leaves combined with chamomile capitulum were employed to treat diarrhea, while Saint John's wort (*Hypericum perforatum* L.) flowers with mint leaves were used to treat gallbladder dyskinesia.

Silver fir (*Abies alba* Mill.), onion (*Allium cepa* L.), black radish (*Raphanus raphanistrum subsp. sativus* (L.) Domin), dog rose (*Rosa canina* L.), wild thyme (*Thymus serpyllum* L.) and silver linden were indicated for the treatment of respiratory diseases. Anise (*Pimpinella anisum* L.), chamomile, fennel, mint, caraway, Saint John's wort, garlic (*Allium sativum* L.), pumpkin (*Cucurbita pepo* L.) and dill were administered to treat digestive ailments, whereas for skin conditions, chamomile, common marigold (*Calendula officinalis* L.), greater celandine (*Chelidonium majus* L.), and wheat (*Triticum aestivum* L.) were prescribed as plant remedies.

3.4 Medicinal plants ordered according to their ethnopediatric relevance

The highest number of mentions was registered for mint. It was used by 165 out of 326 herbal treatment employing women (50.61%, 95% CL: 47.84%–53.38%), with a mean administration time of 4.8 days. Interestingly, FL is 93.3 for diarrhea and only 6.7 for bloating. The youngest patient treated in this manner was a 3-month-old. Chamomile

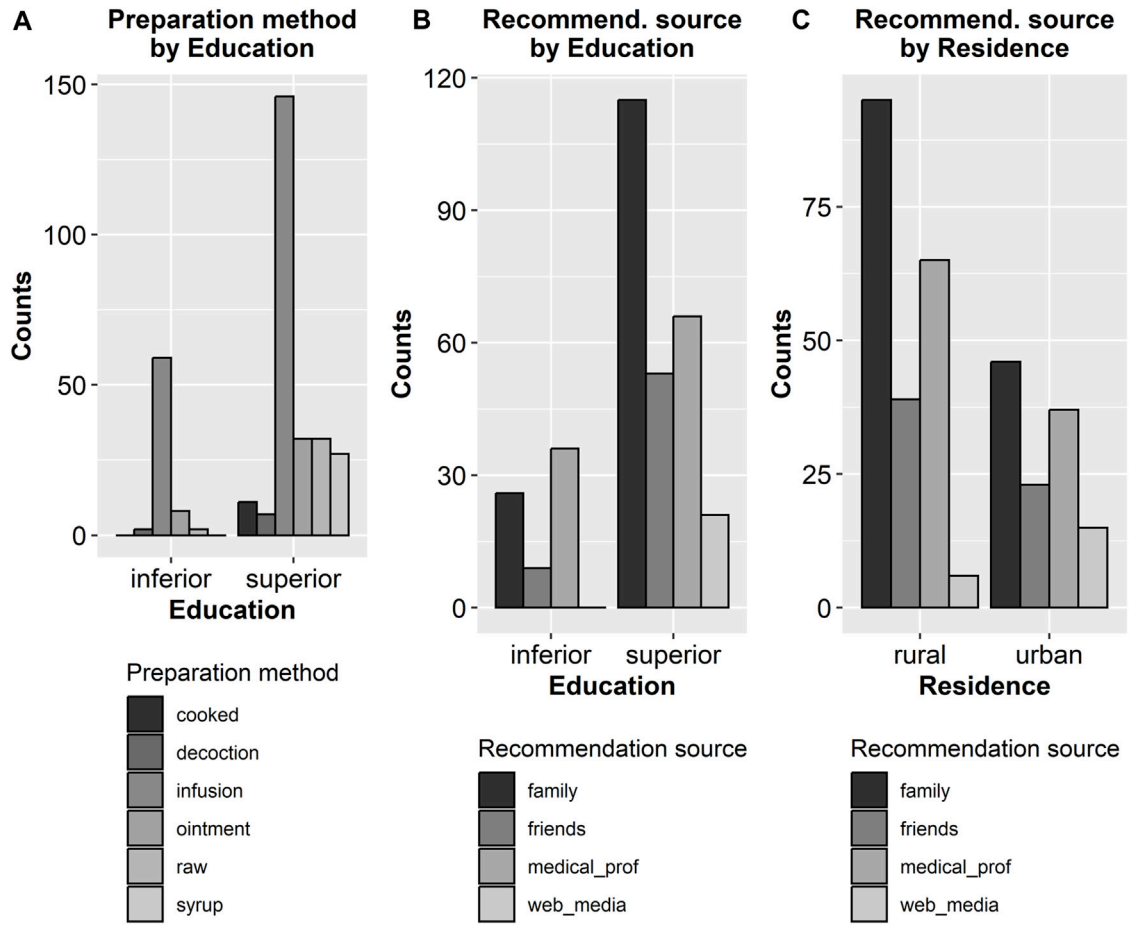

FIGURE 7
Significant correlations between binary and multivalent categorical parameters as revealed by Fisher's exact test. The corresponding *p*-values are 0.0001 **(A)**, 0.0001 **(B)**, 0.01 **(C)**.

the next most frequently referenced plant, was reported 128 times (39.26%, 95% CL: 36.56%–41.97%), with the highest FL (68) recorded for infantile colic.

Common marigold was identified in 127 cases (38.96%, 95% CL: 36.26%–41.66%), being indicated in the treatment of various skin ailments, such as diaper dermatitis (FL 72.4), eczema (FL 8.7), etc. Prepared as an ointment, it was applied to the affected area since the first month of life. Another plant administered in ointments was the mountain arnica (*Arnica montana* L.) with 87 mentions (26.69%, 95% CL: 24.24%–29.14%). The indications were traumatic lesions (FL 100), such as concussions, sprains, and hematoma. The youngest patient that received this preparation was 2 years old.

Sea buckthorn (*Hippophae rhamnoides* L.) fruits, with 74 mentions (22.7%, 95% CL: 20.38%–25.02%), were indicated as a tonic in treatment of weakness and as an immunostimulant agent in acute respiratory diseases (FL 100). A three-year-old child was the youngest recipient. Also, ramson (*Alliumm ursinum* L.) was indicated as an immunostimulant, being eaten raw in salads (FL- 100). Silver linden flowers were referenced 54 times (16.56%, 95% CL: 14.51%–18.62%) and were utilized in the treatment of respiratory disorders, notably cough (with a FL- 92.5), as well as agitation and anxiety. The youngest patient treated was 3 years of age.

Greater celandine latex was mentioned in 37 cases (11.35%, 95% CL: 9.59%–13.11%), for the treatment of skin verruca (Fl- 100), with an anti-infective role. The patient at the lowest end of the age spectrum was 2 years old. Common nettle (*Urtica dioica* L.) young leaves were employed in 34 cases (10.43%, 95% CL: 8.74%–12.12%) to treat anemia (FL- 61.8) and weakness (FL-38.2), and a 1.5 years old was the youngest among the patients. Boiled wheat bran was applied as a poultice on dry dermatitis lesions (FL-100) with very good results.

Garlic bulbs and pumpkin seeds were indicated in intestinal parasites (FL-50 garlic) and worm treatment (FL-50 garlic, FL-100 pumpkin), whereas black radish sap with honey and dog rose was administered in acute respiratory diseases and cough (FL-100), in children aged over 3 years old.

3.5 Alignment of our data with other ethnopediatric sources or with the available bioscientific evidence

Our search in PubMed and Google Scholar for available data on the listed medicinal plants, comprising 25 in total, revealed the

following results (for more details, please refer to the Table 2, Supplementary Material):

1) 22 plants were reported to have ethnopediatric practices in other countries. The plants lacking documented use in this regard are black radish, wheat, and wild thyme.
2) 21 plants were evaluated in clinical studies or mentioned in case reports involving children. The ones that have not undergone such investigations are black radish, silver fir, ramson, and silver linden.
3) 21 plants were assessed in clinical studies or mentioned in case reports in adults for similar purposes as those reported in our present research. Those that have not been subject to such scrutiny are fennel, greater celandine, pumpkin, and ramson.
4) 24 plants were reported to have *in vivo* various biological activities, supporting the ethnopediatric use documented herein. Silver fir was the only one without *in vivo* research backing.
5) 23 plants were found to exhibit various biological activities *in vitro*, consistent with their ethnopediatric use as reported in the present study. Those lacking *in vitro* studies include caraway and sea buckthorn.
6) 14 plants have been extensively studied, with scientific literature covering research for each category among the five mentioned above: anise, arnica, chamomile, cherry (*Prunus avium* (L.) L.), common marigold, common nettle, dill, garlic, heartsease (*Viola tricolor* L.), mint, onion, rosa canina, Saint John's wort, and sunflower.

Based on this data, it appears that although the herbal species mentioned here for ethnopediatric practices are quite common, only part of them have been extensively researched regarding the illnesses reported by the respondents interviewed herein. Furthermore, the majority of the medical uses documented by the mothers enrolled in our survey align with the scientific literature.

3.6 Statistical considerations

The relationships between categorical variables like age groups, urban/rural residence, education levels, and numerical variables (e.g., mother's age, count of used plants, count of treated ailments) were explored by conducting correlation and multivariate analyses, as outlined in the Data analysis section.

Shapiro-Wilk test demonstrated that none of the numerical variables followed a normal distribution. Therefore, non-parametric tests were used to assess the associations between numerical and categorical variables.

The associations between categorical parameters as evaluated by Fisher's exact test are shown in Table 6.

The associations between numerical and binary categorical parameters as appraised by Mann-Whitney test are shown in Table 7, while those between numerical and multivalent categorical parameters as estimated by Kruskal-Wallis test, are displayed in Table 8.

4 Discussions

More than half (59%) of the mothers interviewed reported using herbal remedies based on raw plant material (either self-harvested or purchased) to treat their children's medical conditions. The primary sources of knowledge for these remedies were family and health professionals such as pharmacists and physicians. The significant proportion of informants (40.49%) who indicated that herbal remedies were the only treatment administered to their children could be attributed to several factors. Previous studies have suggested that parents may avoid pharmaceutical agents, including concentrated plant extracts, due to concerns about potential harm to their children, favoring unprocessed plant-based treatments instead (Ventola, 2010; Lucas et al., 2018). Additionally, mothers may consider herbal remedies as providing satisfactory results.

An anticipated outcome was that regarding the rural-urban disparity: the mothers of rural background have a higher propensity to use medicinal plants compared to mothers of urban background. This result may have various explanations. Adherence to urban culture may be responsible for a shift in the perceptions and attitudes toward traditional healthcare practices (Pimentel et al., 2021). Conversely, rural culture is characterized by slower economic development and higher poverty, leading to a preference for low-cost or homemade remedies over synthetic drugs.

The current study identified 25 medicinal plant species belonging to 15 families used in treating children's diseases in Southern Romania. While direct comparisons to historical records, based on a larger overall knowledge of local healers, midwives, and doctors may not be ideal, in the absence of other local contemporary investigations on the same topic, we contrasted our findings with data reported in our previous study on plants historically relevant in Romanian ethnopediatrics. This previous study documented 153 medicinal plants from 52 families, excluding edible varieties like ramson (Petran et al., 2020).

Notably, there has been a significant decrease in the number of medicinal plants used for pediatric purposes, suggesting a reduction in ethnopediatric knowledge transmitted through generations in Romania. This decline, while serving as an indirect indicator, requires further validation. Comparing the therapeutic uses of the various plants reported in the present survey with those documented in our previous investigation, we found that only two plant species, *F. vulgare,* and *P. anisum,* were used for infantile colic in both studies. A clinical trial carried out in Italy confirmed the effectiveness of *F. vulgare* in treating infant colic when administered in combination with *Matricariae recutita* and *Melissa officinalis* (Savino et al., 2005). Some pediatric employments, traditionally acknowledged but previously unreported in Romania, were identified for four plant species: ear pain for *Helianthus annuus*, digestive diseases for *H. perforatum*, and respiratory disorders for *T. serpyllum* and *T. tomentosa*. However, our present study indicated fewer reported utilizations for most plant species compared to our previous research.

Asteraceae, Apiaceae, and Amaryllidaceae were the dominant families used in herbal remedy preparation. The utilization of a particular plant species to treat entirely different conditions could be attributed to the large range of active phytocompounds present in the plant, acting on various body systems. For instance, medicinal plants identified in our survey for treating a certain ailment were mentioned for dissimilar curative properties in other European countries: *A. sativum,* mentioned as an antiparasitic agent in our study, was found to be effective for whooping cough in Italy (Muhe et al., 1994); *C. carvi* used as

carminative for children in Southern Romania, was employed for enuresis in Italy (Leporatti and Ghedira, 2009); *C. majus,* utilized for curing warts in our study, was reported in Poland for treating jaundice and digestive tract parasites (Zielińska et al., 2018); *H. perforatum,* used by Romanian mothers for treating children's liver diseases, was used in Poland for burns treatment (Coppock and Dziwenka, 2021); *U. dioica,* popular for treating pediatric weakness and anemia in the geographic area investigated herein, was noted to be used as an antitussive in Spain (Rigat et al., 2015), whereas *V. tricolor*, utilized as an antialergic in our study, was used for treating seborrhoea of the scalp in nursing infants in Poland (Witkowska-Banaszczak et al., 2005), and as an expectorant in Ukraine (Herasymova et al., 2022).

Conversely, the use of the same plant species for treating a particular illness across various geographical regions may not only indicate their wide habitat range but also the widespread recognition of their efficacy in treating the respective illness. Similar ethnopediatric uses documented in our survey were also acknowledged in other European countries for the same medicinal plant: the treatment of respiratory diseases in Bosnia-Herzegovina with *Abies alba* (Prazina et al., 2011); the antiparasitic effects of *A. sativum* in Italy (Pieroni et al., 2005; Passalacqua et al., 2007; Dutto et al., 2012); *Allium ursinum* L. used as a tonic both in Italy and Poland (Leporatti and Ivancheva, 2003; Luczaj et al., 2012; Sõukand, 2016); recovery after local trauma with *Arnica montana* noted in Ireland (Crowe and Lyons, 2004); vulnerary effects of *Calendula officinalis* reported in Russia (Shaparenko et al., 1979); skin warts treated with *C. majus* in Italy (Leporatti and Corradi, 2001; Muršić, 2020); and *Matricaria chamomilla*, mentioned for treating digestive illnesses and colic in Germany (Nathan and Scholten, 1999).

The majority of the participants in our study have purchased plants and herbal remedies from specialized stores, local producers, and pharmacies. The remainder have harvested them from home gardens and available lands in the vicinity of their homesteads to avoid traveling long distances in their search for wild sources. Corresponding results have been reported in other surveys conducted in Eastern Europe (Pawera et al., 2017).

Most plant species were utilized primarily for managing respiratory, digestive, and dermatological conditions, likely stemming from the high prevalence of these diseases in children living in Southern Romania ("Institutul National De Sanatate Publica. Raportul Național de Sănătate a Copiilor Și Tinerilor Din Romania, 2011," 2011). Infusion stood out as the predominant method for preparing herbal medicine, while water was the preferred solvent utilized. Alcoholic extracts are not commonly recommended in pediatric preparations due to their toxic potential (Gaw and Osterhoudt, 2019). The use as herbal baths in small children may have a rationale: transdermal delivery of pharmacological agents in this age group may be more efficient than their internal administration in adults (Delgado-Charro and Guy, 2014; Proksch, 2014; Visscher et al., 2015).

Regarding the minimal age of administration, we found that 7 out of the 25 medicinal plants were administered to babies younger than 3 months old: *Matricaria spp*, *Calendula officinalis*, *C. carvi*, *A. graveolens*, *F. vulgare*, *P. anisum*, *Mentha spp*. This information may be particularly relevant, considering the specific precautions imposed by the safety concerns associated with the employment of herbal treatment in newborns. Some plants (e.g., *Foeniculum vulgare*, *Matricaria spp*, *Calendula officinalis*) are acknowledged as safe for use in infants (Alexandrovich et al., 2003; Gozum et al., 2007; Martinelli et al., 2017; Sharifi-Heris et al., 2018) or as having minor negative outcomes such as skin irritations and/or reddening due to hypersensitivity reactions to the calendula extract in creams (Guala et al., 2007). However, more severe adverse events have been reported for certain plants, such as toxicity in two breastfed newborns resulting from excessive maternal use of *P. anisum* and other herbs, probably owing to the excretion of anethole in breastmilk (*Anise.*, 2006; Khoda Karami et al., 2008). There is a general scarcity of clinical studies in newborns and small children due to ethical concerns, but the available data on the use of herbs in nursing mothers may partially fill this gap (*Peppermint*, 2006).

4.1 Available scientific data on the most used medicinal plants for children's diseases in Southern Romania

The majority of medicinal plants utilized by the mothers participating in our study to treat their children were employed for similar ailments as reported in ethnobotanical surveys, clinical studies, and case reports conducted worldwide in both children and adults, as revealed by our literature search in PubMed and Google Scholar (summarized in Table 2, Supplementary Material). This indicates their accurate utilization by our informants and the preservation of a solid knowledge base regarding the curative properties of the botanical remedies. Another issue highlighted by our search of the ethnomedical or bioscientific data on the identified medicinal plants was the scarcity of clinical studies on pediatric treatments with botanical remedies.

Mint and chamomile, the plants with the highest number of mentions in our survey were largely employed for ethnopediatric uses in other countries. The efficacy and safety of common marigold as a topical remedy for treating diaper dermatitis, an indication mentioned in the present study, was reported in a randomized comparative trial that included infants less than 3 years old. In topical applications, marigold yielded better outcomes than aloe (Panahi et al., 2012). Additionally, in another study conducted on 171 children aged 5–18 years with otalgia due to acute otitis media (an indication not mentioned in our study), Sarrell et al., 2003 found clinical benefits for naturopathic herbal extract ear drops containing marigold flowers, among other ingredients.

Mountain arnica has been utilized for treating various types of traumatic lesions. Scientific evidence supports its use in combination with echinacea to facilitate the detachment of the umbilical cord (Perrone et al., 2012) and for soft-tissue bruising in children aged 0–8.5 years (Thompson et al., 2010).

Ramson is most commonly consumed as food or spice in many countries (Abbet et al., 2014; Pawera et al., 2017; Mustafa et al., 2020). However, caution should be exercised, particularly in vulnerable groups such as young children, due to reported gastrointestinal toxicity in the literature (Fuchs et al., 2011; Lüde et al., 2016). Notably, a search with the phrase "*Allium ursinum* OR ramson OR wild garlic AND children" in PubMed and ScienceDirect databases provided no clinical studies. Silver linden's flowers were employed to treat respiratory diseases, agitation, and anxiety. Some scientific evidence for anxiolytic activity is available (Gutiérrez et al., 2014), but it is not focused on pediatric use. Furthermore, another search using the phrase "*T. tomentosa* OR silver linden AND children" in PubMed and ScienceDirect databases revealed no clinical study.

Greater celandine fresh latex was reported here to be applied on skin verruca. Interestingly, in our previous historical review on Romanian medicinal plants with ethnopediatric use (1860s–1970s), another plant part and indication were identified: the root of greater celandine was administered as a bath for general strengthening (Petran et al., 2020).

Boiled wheat bran was found to be used mainly externally in dry dermatitis, whereas wheat in spray formulations was reported to be efficient for controlling gingival inflammation in schoolchildren (Bello et al., 2020). *In vitro* studies showed that wheat extract has modulated the expression of inflammation-associated molecules (Funel et al., 2020), and speeded up keratinocyte healing and tissue regeneration (Tito et al., 2020; Morretta et al., 2022). Clinical studies indicated wound healing effects as well, including the curing of venous leg ulcers (Romanelli et al., 2015) and the moisturizing effects of this plant extract (Boisnic et al., 2019).

Black radish, used for the treatment of respiratory disorders and cough in our study, was not yet mentioned in the scientific literature for pediatric use.

4.2 Dynamics of the ethnopediatric use of medicinal plants in Romania

Our research has identified a considerable decline in ethnobotanical knowledge compared to the earlier period examined by our team from 1860 to 1970, with only 25 medicinal plants identified, as opposed to the 153 previously documented (Petran et al., 2020). Several potential explanations for this trend may include the following:

- The information was collected from subjects residing in a relatively narrow geographical area i.e., Southern Romania.
- The respondents included in the study were domestic users of medicinal herbs, and not specialized healers possessing a sound knowledge of the matter.
- The accelerated economic development may have caused a gradual replacement of herbs by conventional drugs during the last decades. It is well-known that the globalization trend is associated with the abandonment of ancestral traditions by younger generations (Lee et al., 2001).
- The health benefits of medicinal plants as well as the results of the ethnopharmacological research are scarcely acknowledged and advertised.
- The health insurance systems provide no financial support for the use of herbals. By contrast, in other countries (e.g., Germany), such facilities are granted for the herbal treatment of children under a certain age or with certain ailments (e.g., developmental disorders), even without the need of a medical prescription (Du et al., 2014).
- The number of traditional healers (who might impart their knowledge to other members of the community) has tremendously decreased in modern times.
- Transmission of ethnobotanical knowledge declined due to the vicissitudes characteristic of the communist period (e.g., informational censorship, marginalization, and oppression of bourgeois experts and monastic communities).
- Deforestation in Romania has become a huge community problem (Petrişor and Petrişor, 2018; 2017; Peptenatu et al., 2020). According to the data available in the literature, deforestation may harm biodiversity and medicinal plant use (Shanley and Luz, 2003).

4.3 Statistical considerations

Statistical analysis revealed intriguing and valuable findings regarding the relationships between categorical and numerical variables documented in this study. Fisher's exact test (Table 6) demonstrated that only the education level (among the categorical parameters) was significantly associated with both the number of employed plants and the number of treated ailments (the higher the educational level, the greater the number of employed plants and the number of treated ailments) (Figure 4).

The manner herbs were procured is also correlated with education level—somehow counterintuitively, mothers with higher educational levels had a greater propensity to harvest the plants themselves. A rural background is also associated with a higher probability of using plants harvested (rather than purchased) by the mothers (Figure 5). Remarkably, education level was not associated with residency status.

Fisher's exact test (Table 6) demonstrated that the preparation method was also influenced by education level: while infusion was by far the most prevalent preparation method in both subgroups (lower and higher educated), higher-educated mothers utilized a more diverse range of preparation methods, in contradistinction to lower educated mothers, which used almost exclusively infusion as a preparation method (Figure 6).

Education level also influenced the recommendation source employed by the mothers (Table 7): for lower educated mothers, medical professionals, and family members (in this order) were the most important sources, while for higher educated mothers, family members (not medical professionals) were by far the most important source, while web and media were also a significant source (absent in lower educated mothers) (Figure 7).

A similar result was found for the association between residency and the source of information: for mothers originating from a rural environment, family members and medical professionals (in this order) being by far the most important sources, while for the mothers dwelling in urban areas the information sources were more uniformly represented, with the web and media having greater importance (Figure 4).

Kruskal-Wallis test too revealed an interesting and rather unexpected result (Table 8): mothers using the web and media as an information source were generally older, while those seeking the advice of medical professionals tended to be younger (Figure 4)

Mann-Whitney test (Table 7) confirmed that a higher educational level was associated with a greater number of both employed herbs and treated diseases, as well as with a later start in treating the children with herbs. In other words, lower-educated mothers had a tendency to initiate herbal treatment for their children at an earlier stage. Harvesting (as opposed to purchasing) medicinal plants was also associated with a higher

count of employed herbs and treated disorders, and a later start in the employment of herbal treatment in children. This association can be attributed, at least partially, to the correlation between a higher education level and a more pronounced inclination to harvest (rather than purchase) the plants.

In the MVA, age, residency, education level, and procurement method were all entered as putative independent parameters in the determinism of both the count of employed plants and the count of treated disorders (dependent parameters). MVA revealed that education level was the only independent determinant for both of these parameters. Interestingly, both residency and education level emerged as independent determinants of procurement method, while age did not show significant independent influence.

4.4 Study limitations

The number of informants included in the study was relatively reduced, mainly due to the specific restrictive conditions during the SARS-COV19 pandemic. Furthermore, the majority of informants resided in urban areas, whereas traditional knowledge regarding the therapeutic use of herbal remedies is typically better preserved in rural zones. Consequently, the conclusions drawn from our survey may not apply to the entire population of Romania, as our informants were exclusively from Southern Romania, and other regions may exhibit variations in medicinal flora and/or folk medical traditions.

Additionally, no herbal authentication was conducted, with the results solely relying on self-reports from our informants. Although botanical authentication would have been ideal, it was deemed impractical for commercial plant products subjected to processing, such as fragmentation or powdering, which eliminates morphological characteristics necessary for accurate macroscopical analyses (Pawar et al., 2017; Ichim et al., 2020).

Furthermore, while all vernacular names of medicinal plants provided by informants were common, there were two instances where these denominations failed to differentiate between several similar taxa in the genus Mentha and Matricaria. As a result, a definitive botanical identification of these two particular taxa cannot be asserted.

5 Conclusion

Medicinal plants continue to hold a relatively important role in treating various children's diseases in Southern Romania, although the number of taxa used nowadays seems to have decreased compared to the past. This survey highlighted that the level of education was associated with the number of employed plants and the range of ailments treated, while residency (rural *versus* urban) was not. Interestingly, both residency and education influenced the method plants were procured: the inclination to harvest (rather than purchase) the plants was associated, as expected, with a rural background and, surprisingly, with a higher educational level.

Whether this traditional knowledge is fading away or dynamically adapting to economic, political, and cultural changes amid the pressures of world globalization is yet to be established (Heinrich, 2010; Pardo-de-Santayana et al., 2022). Ethnopediatric practices in Romania constitute a valuable heritage that urgently requires protection against potential cultural erosion and warrants further exploration to unlock their maximum benefits.

Data availability statement

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

Author contributions

MP: Visualization, Investigation, Conceptualization, Writing–review and editing, Writing–original draft, Methodology, Formal Analysis, Data curation. DD: Validation, Software, Writing–review and editing, Writing–original draft, Methodology, Formal Analysis, Data curation. IS: Writing–review and editing, Writing–original draft, Supervision, Project administration, Methodology, Formal Analysis, Conceptualization. AV: Writing–review and editing, Writing–original draft, Supervision, Methodology, Formal Analysis, Data curation, Conceptualization. MG: Writing–review and editing, Writing–original draft, Visualization, Validation, Supervision, Project administration, Methodology, Formal Analysis, Data curation, 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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2024.1377341/full#supplementary-material

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Modified oxylipins as inhibitors of biofilm formation in *Staphylococcus epidermidis*

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New approaches to combating microbial drug resistance are being sought, with the discovery of biofilm inhibitors considered as alternative arsenal for treating infections. Natural products have been at the forefront of antimicrobial discovery and serve as inspiration for the design of new antibiotics. We probed the potency, selectivity, and mechanism of anti-biofilm activity of modified oxylipins inspired by the marine natural product turneroic acid. Structure-activity relationship (SAR) evaluation revealed the importance of the *trans*-epoxide moiety, regardless of the position, for inhibiting biofilm formation. *trans*-12,13-epoxyoctadecanoic acid (**1**) and *trans*-9,10 epoxyoctadecanoic acid (**4**) selectively target the early stage of biofilm formation, with no effect on planktonic cells. These compounds interrupt the formation of a protective polysaccharide barrier by significantly upregulating the *ica* operon's transcriptional repressor. This was corroborated by docking experiment with SarA and scanning electron micrographs showing reduced biofilm aggregates and the absence of thread-like structures of extrapolymeric substances. *In silico* evaluation revealed that **1** and **4** can interfere with the AgrA-mediated communication language in Staphylococci, typical to the diffusible signal factor (DSF) capacity of lipophilic chains.

KEYWORDS

oxylipins, fatty acids, biofilm, *Staphylococcus epidermidis*, antimicrobial

Introduction

Microorganisms usually occur as aggregates of microcolonies. This clustering can be associated with substratum attachment and matrix encapsulation, which forms biofilm and is ubiquitous in nature. This mode of growth is essential to the survival of some microorganisms thriving in nutrient-depleted conditions and extreme environments (Hall-Stoodley et al., 2004). Because of this protective form, pathogenic bacteria like *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* have become increasingly resistant to antibiotic treatments (Singh et al., 2021). The apparent medical implication of biofilm suggests a need for understanding its complex system to optimize possible mitigation strategies.

Biofilm formation usually starts with the attachment of free-living planktonic cells to a suitable substratum. Initial adherence of bacterial cells can be influenced by environmental factors such as pH, material type, temperature, and hydrophobicity (Dhar and Han, 2020). During this reversible stage, attachment is also mediated by microbial inherent properties like pili, flagellum, surface adhesins, and signaling molecules. This is followed by an irreversible stage of microbial network maturation with distinct Extracellular Polymeric Substance (EPS) formation. EPS is a mixture of

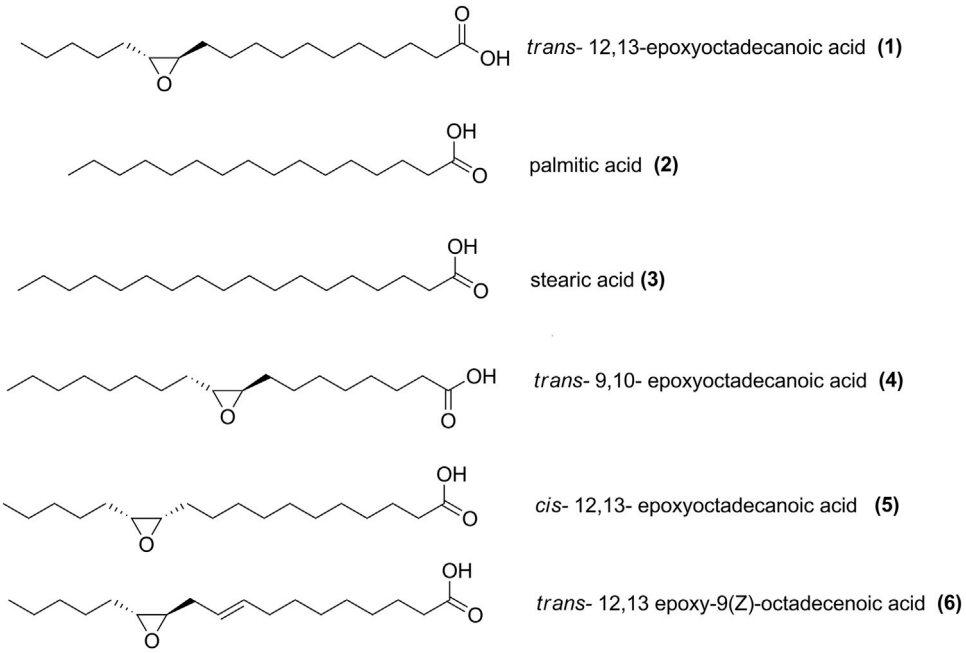

FIGURE 1
Structure of modified oxylipins used in this study.

protein, nucleic acids, and polysaccharides, which may vary per pathogen (Arciola et al., 2015). This encapsulation allows the bacteria to store nutrients, block drug penetration, and facilitate communication via quorum sensing. Ultimately, planktonic cell dispersal can happen on the outer layer of matured biofilms, which can spread and mediate systemic infections in clinical settings (Dhar and Han, 2020).

The marine ecosystem remains a rich repository of bioactive compounds (Jiménez, 2018; Petersen et al., 2020; Deng et al., 2022). Various host organisms and associated microbes were reported as good sources of antibiofilm compounds (Deng et al., 2022). Several marine-derived compounds were characterized to produce anti-biofilm properties with varying target domains, such as quorum sensing (QS)-inhibitor psammaplin A (Oluwabusola et al., 2022), EPS-disruptor cyclic dipeptide (L-Leucyl-L-propyl) (Gowrishankar et al., 2014), pre-formed biofilm suppressor butenolide (Yin et al., 2019), and bacterial aggregation downregulator rodriguesine A (Lima et al., 2014). Three modified fatty acids were previously purified from the shipworm endosymbiont *Teredinibacter turnerae* 991H.S.0a.06 (Lacerna et al., 2020). Turneroic acid, a new modified oxylipin characterized by an epoxide moiety and a hydroxy group, inhibited biofilm formation in *S. epidermidis* RP26A (Lacerna et al., 2020). Preliminary structure-activity relationship (SAR) showed that *trans*-12,13-epoxyoctadecanoic acid (**1**) has a high selectivity index for anti-biofilm activity against *S. epidermidis* with no antiproliferative effects against MDCK NBL-2 cell line (Lacerna et al., 2020). SAR showed the importance of the epoxide moiety in selective biofilm inhibition against *Staphylococci* (Lacerna et al., 2020).

Related compounds to **1** have been reported to exhibit different bioactivities but have not been assessed for antibiofilm properties in representative microbial pathogens. The saturated fatty acids palmitic (**2**) and stearic (**3**) acids are ineffective as antibiotics or biofilm inhibitors (Sanabria-Ríos et al., 2020). The racemic mixture of 9,10-epoxyoctadecanoic acid demonstrated antimicrobial activity against a *Pseudomonas syringae* strain but was ineffective against other subtypes and other plant pathogens including *Xanthomonas* and *Erwinia* (Prost et al., 2005). Meanwhile, 12,13-epoxy-9(*Z*)-octadecenoic acid inhibited the plant fungi *Phytophthora* and *Cladosporium* (Prost et al., 2005).

Herein, we report our efforts to expand the SAR using additional analogues of modified oxylipins and further elucidate its antibiofilm mechanism. We assessed the effects of modified oxylipins on the different stages of biofilm formation using static microdilution assays, microscopy, gene expression, and docking analysis.

Materials and methods

Compounds

Fatty acids palmitic (**2**) and stearic acids (**3**) were sourced from Sigma-Aldrich (St. Louis, MO, United States of America). *Trans*-12,13-epoxyoctadecanoic acid (**1**), *trans*-9,10-epoxyoctadecanoic acid (**4**), *cis*-12,13-epoxyoctadecanoic acid (**5**), *trans*-12,13-epoxy-9(*Z*)-octadecenoic acid (**6**) were purchased from Larodan Fine Chemicals (Solna, Sweden) (Figure 1). Stock solutions were prepared in DMSO. The positive control chloramphenicol was sourced from Sigma-Aldrich (St. Louis, MO, United States of America). Filtered sterilized DMSO (1%) was used as negative control.

Bacterial cultures and culture conditions

Inoculum preparation was based on the methods of Lacerna et al. (2020), as adapted from Sarker et al. (2007). Briefly, Gram-(+) biofilm formers *Staphylococcus aureus* ATCC 6538 and *S. epidermidis* RP262A ATCC 35984 and Gram-(−) *P. aeruginosa* ATCC 41501 were revived on Tryptic Soy Agar TSA (Pronadisa, Spain) plates for biofilm studies and Mueller Hinton Agar (MHA) (Himedia, India) for planktonic setup. Tryptic Soy Broth (TSB) (Himedia, India) with 1% glucose and Mueller Hinton Broth (MHB) (Himedia, India) were used in the assays for biofilm and planktonic cells, respectively, unless otherwise stated. Microbial cell culture was adjusted to match 0.5 McFarland standard and was further diluted, equivalent to 5×10^5 colony forming units/mL (cfu/mL). The 96-well polystyrene flat bottom plates (Costar 3596, United States of America) and 6-well plates (Costar 3506, United States of America) were covered with the corresponding sterile polystyrene lid and sealed with a parafilm to ensure a tight lid.

Biofilm inhibitory concentration (MBIC) and minimum inhibitory concentration (MIC) determination

Assessment of the biofilm inhibitory activity of **1**-**6** was based on the method of Lacerna et al. (2020), as adapted from Skogman et al. (2012). Microbial pathogens were treated with two-fold serial dilution of **1**-**6**, starting with 128 μg/mL to 1 μg/mL. MHB and TSB culture media were used for planktonic and biofilm quantitation, respectively. The 96-well flat-bottom polystyrene plates (Costar 3,596, United States of America) were incubated for 18–20 h at 37 °C without shaking to promote biofilm formation. The formed biofilm was quantified using Alexa Fluor™ 488 (WGA 488, Thermo Fisher, United States of America) probe (0.05 μg/mL). The bound dye was solubilized with 33% acetic acid and quantified spectrophotometrically at 485 nm/420 nm excitation filters and 528 nm/520 nm emission filters. In another set of plates, the planktonic cells were monitored at the end of the incubation and quantified using 0.02% resazurin (Sigma, United States of America). The fluorescence was measured at 530 nm excitation and 590 nm emission using a microplate reader (Biotek Synergy HT, Winooski, United States of America). Percent inhibition was calculated relative to the solvent control. MBIC and MIC are the lowest concentration where ≥98% biofilm and ≥98% planktonic cells inhibition, respectively, were observed.

Pre-formed biofilm disruption assay

S. epidermidis was incubated on TSB culture medium for 18–20 h at 37 °C without shaking to allow biofilm formation in a 96-well flat bottom polystyrene plate (Costar 3596, United States of America). The planktonic cells were removed and replaced with fresh medium and treated with two-fold dilution of **1** and **4**, starting from 128 μg/mL to 4 μg/mL. At the end of the 18 h incubation, the metabolic activity in the biofilm was quantified using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma Aldrich). MTT quantifies viability through the conversion of the yellow tetrazolium MTT dye to a purple formazan product by mitochondrial reductases. The metabolic activity was assessed by reading the absorbance at 570 nm (Biotek Synergy HT, Winooski, United States of America). In a duplicate plate, the microbial density of the biofilm was assessed using WGA 488 probe according to the methods described above. Biofilm disruption was assessed relative to the solvent control (1% DMSO).

Time-kill kinetics

A 24-h monitoring of *S. epidermidis* biofilm and planktonic growth was performed using TSB and MHB, respectively. Each setup in a 96-well polystyrene flat bottom plate (Costar 3596, United States of America) was treated with **1** and **4** (24 μg/mL) or solvent control (1% DMSO). Planktonic cell growth was assessed using MTT (Sigma Aldrich, United States of America) while biofilm was quantified using WGA 488 reagent. Absorbance and fluorescence readings were taken every 2 h using Biotek Synergy HT (Winooski, United States of America).

Microscopy

Sterile glass coverslips (Thickness #1) were placed in 6-well flat bottom polystyrene plate (Costar 3506, United States of America). Briefly, *S. epidermidis* adjusted inoculum was transferred on each well, followed by TSB containing 24 μg/mL of compounds **1** or **4** (1% DMSO). Plates were incubated at 37°C for 24 h without shaking to facilitate biofilm formation.

The microbial content was aspirated at the end of incubation, and fixed with methacarn solution (methanol: chloroform: glacial acetic acid, 6: 3: 1) for 48 h (Dassanayake et al., 2020). Biofilms were rinsed with sterile Phosphate Buffer Saline (PBS), post-fixed with 1% osmium tetroxide (Electron Microscopy Science, Hatfield, United States of America) for 30 min, and washed with distilled water every 5 min (4x). Finally, the coverslips were dehydrated by an increasing series of ethanol: 30% for 5 min, 50% for 10 min, 70% for 15 min (2×) 95% for 20 min (2×), and 100% for 20 min (2×). Further chemical drying was done using increasing concentration of hexamethyldisilazane (HMDS) (Sigma Aldrich, Germany) every 20 min (1:1 EtOH:HMDS, 1:3 EtOH:HMDS, and 100% HMDS). Platinum coating for 40 s and 40 mAmps was conducted using Hitachi MC1000 sputter coater. SEM imaging was performed using a Hitachi SU Field Emission Scanning Electron Microscope.

RNA extraction, quantitative real time-PCR analysis

S. epidermidis was cultured in TSB medium in a flat bottom polystyrene plate (Costar 3,506, United States of America). Compounds **1** and **4** (24 μg/mL), or 1% DMSO were added to plates and incubated at 37°C without shaking. At the end of the 24 h incubation, RNA extraction was performed on the total *S. epidermidis* population, consisting of both planktonic cells and biofilm, according to Juhlin et al. (2017) with some

modifications. Bacterial lysis was performed using acid-washed glass beads (400–600 μm; Sigma Aldrich, United States of America) and TriZol-chloroform phase separation (Juhlin et al., 2017). Total RNA was extracted from the aqueous phase using Qiagen RNeasy® Mini Kit according to the Manufacturer's protocol. The purity and quantity of RNA were assessed using NanoSpec and Qubit™ RNA BR assay. RNA was further reverse transcribed into cDNA using SuperScript IV Reverse Transcriptase (Invitrogen, Lithuania). Gene expression was quantitatively determined by real-time PCR (Applied Biosystems™ ABI 7500) using SYBR Select Master Mix (Applied Biosystems, Thermo Fisher Scientific). The housekeeping gene *gyrB* was used to normalize the relative expression data of the genes of interest using the $2^{-\Delta\Delta CT}$ method. Primer sequence of the target genes are as follows: *icaR* forward 5′-CATTGACGGACTTTACCAGTTTT-3′, and reverse 5′-ATCCAAAGCGATGTGCGTAG-3'; *icaB* forward 5′-GAAACAGGCTTATGGGACTTTG-3′, and reverse 5′-CAAGTGCGCGTTCATTTTT-3'; and *gyrB* forward 5′-TGACGAGGCATTAGCAGGTT-3′, and reverse 5′-GTGAAGACCGCCAGATACTTT-3'.

Docking study

The structure of oxylipin **4** was obtained from PubChem (CID: 15,868) while the crystalline structures of Staphylococcal accessory regulator A (SarA) (PDB ID: 2FNP) and accessory gene regulator (AgrA) (PDB ID: 4XYO) were recovered from Protein Data Bank. Other ligands (**1** and **5**) were created using ChemDraw Ultra 12.0 (Cousins, 2005) and optimized using Avogadro (Hanwell et al., 2012). *In silico* molecular docking was carried out using AutoDockTools version 1.5.7 (Goodsell and Olson, 1990). Prior to docking, the water molecules from the protein were removed. Kollman charges were assigned while polar hydrogen bonds were included in the existing macromolecule. The ligand torsion was made rigid between carbons C8-C11 to maintain the *trans*-configuration. Grid map was optimized to include the surrounding residue with grid point spacing of 0.375 Å for AgrA and 0.558 Å for SarA. Lamarckian genetic algorithm was used to analyze the docking process with 50 runs and 300 population size. The complex with the least binding free energy and high clustering record was selected. Docking results were visualized by PyMOL Molecular Graphics System Version 1.2r3pre Schrödinger, LLC; Protein-Ligand Interaction Profiler (PLIP) (Adasme et al., 2021); and UCSF Chimera (Pettersen et al., 2004).

Staphyloxanthin quantification

The bacterial suspension of *S. aureus* ATCC 6538 was incubated with **1** or **4** (24 μg/mL) for 24 h at 37°C without shaking in a 6-well flat bottom polystyrene plate (Costar 3506, United States of America). Cell pellets were collected by centrifugation at 10,000 rpm for 10 min, washed with sterile PBS and resuspended in 150 μL methanol for 30 min at 55°C. The absorbance of the extracted carotenoid was measured at 465 nm using microplate reader (Biotek Synergy HT, Winooski, United States of America). The production of *S. aureus* pigment was calculated relative to the solvent control.

Results

Structural configurations of the epoxide moiety can influence the biofilm inhibitory activity of modified oxylipins

Oxylipins **1, 4, 5** and **6** inhibited biofilm formation, with MBIC ranging from 24 to 96 μg/mL against *S. epidermidis*, and 64–128 μg/mL against *S. aureus* (Table 1). The most potent oxylipins **1** and **4** both have a *trans*-epoxide but differed on the position of the epoxide moiety. In contrast, the *cis*-epoxide bearing oxylipin **5** showed two-fold lower activity. This suggests that the configuration of the epoxide moiety, but not the position, may be critical for the biofilm inhibitory activity. Compound **6** had close to four-fold higher MIC among the epoxide-bearing oxylipins and may suggest the negative impact of the presence of an unsaturation in the aliphatic chain for bioactivity. The saturated fatty acids **2** and **3** were the least effective in inhibiting biofilm formation, corroborating the importance of the epoxide moiety for bioactivity. The inhibitory activity of the *trans*-epoxide bearing oxylipins showed selectivity to the Gram-(+) *Staphylococcus* species, with no inhibitory activity against the Gram-(−) *P. aeruginosa*. Because of the potent activity of **1** and **4**, these were used to further characterize the mechanism of biofilm inhibitory activity of modified oxylipins.

Further validation was done through a time course monitoring of the metabolic activity and biofilm formation in *S. epidermidis* for 24 h (Figure 2). Reduced metabolic activity was evident starting at 10 h post-incubation with **1** and **4** at 24 μg/mL. Biofilm matrix was absent in oxylipin-treated wells (Figure 2) and suggests selective targeting of **1** and **4** against microbial attachment of *S. epidermidis* during biofilm formation.

Oxylipins can reduce metabolic activity but are unable to disperse mature biofilm

Compounds **1** and **4** did not significantly disrupt mature *S. epidermidis* biofilm even at concentration up to 128 μg/mL (Figure 3). However, a concentration-dependent reduction in metabolic activity was observed in the pre-formed biofilm when treated with the oxylipins. A 36%–60% metabolic inhibition was observed at concentrations >8 μg/mL for both **1** and **4**.

Oxylipins increased *icaR* gene expression

To provide insights to the molecular mechanisms underlying biofilm formation, we assessed the effects of **1** and **4** on the *ica* operon. We prioritized the gene expression analysis for *icaR* and *icaB* given the clear association of these with biofilm formation. These two genes from the *icaADBC* transcription machinery are involved in extracellular matrix production and essential in biofilm attachment and structuring (François et al., 2023). *icaB* is

TABLE 1 Minimum biofilm inhibitory concentration (MBIC) and minimum inhibitory concentration (MIC) of 1–6 against *Staphylococcus epidermidis* RP62A ATCC 35984, *S. aureus* ATCC 6538, and *P. aeruginosa* ATCC 41501 using WGA 488 and resazurin-based microdilution assays.[a]

Compounds	MBIC and MIC (μg/mL)					
	S. epidermidis RP262A ATCC 35984		*S. aureus* ATCC 6538		*P. aeruginosa* ATCC 41501	
	Biofilm	Planktonic	Biofilm	Planktonic	Biofilm	Planktonic
1	24	>128	64	>128	>128	>128
2	>128	>128	>128	>128	>128	>128
3	>128	>128	>128	>128	>128	>128
4	24	>128	64	>128	>128	>128
5	48	>128	128	>128	>128	>128
6	96	>128	128	>128	>128	>128
Chloramphenicol	8	8	16	16	50	50

[a]Data is the mean of two independent biological replicates with three technical replicates each. MBIC, and MIC, defined as the lowest concentration causing ≥98% biofilm and ≥98% planktonic cells inhibition, respectively.

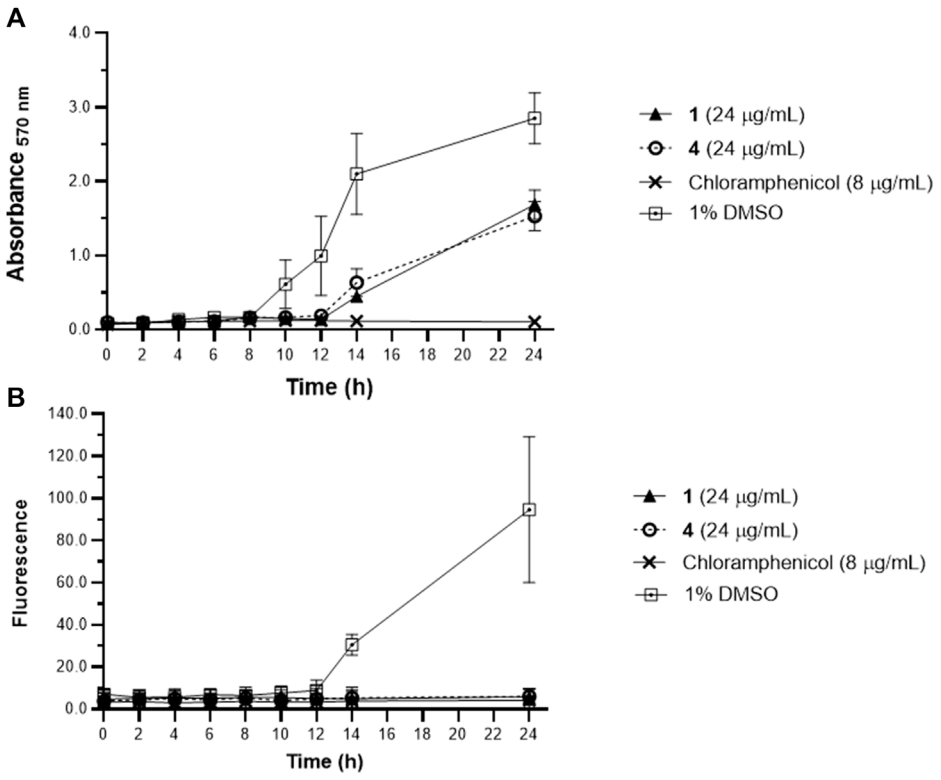

FIGURE 2
Metabolic activity of planktonic *Staphylococcus epidermidis* assessed by MTT **(A)**, and biofilm growth quantified by WGA 488 **(B)** from time-kill assay. *Staphylococcus epidermidis* were treated with **1** and **4** at MBIC = 24 μg/mL and the metabolic activity and biofilm were monitored every 2 h. Reduced metabolic activity was observed at 10 h post-incubation with **1** and **4**. Data are presented as mean ± SD of two independent biological replicates performed in triplicate (n = 6).

responsible for the deacetylation and polymerization of *N*-acetylated β-1,6-linked *N*-acetylglucosamine (PNAG), while *icaR* encodes for the repressor of *ica* operon (François et al., 2023). The effect of **1** and **4** on the gene expression of biofilm-relevant genes *icaB* and *icaR* was determined using qRTPCR. A significant increase in *icaR* transcript level was observed (Figure 4). There was an ~3.58- and 3.71-fold increase in *icaR* expression with 24 μg/mL treatment of **1** and **4**, respectively. Consequently, *icaB* expression was downregulated by **1** and **4**, with 0.57 and 0.56-fold reduction in transcript level, respectively.

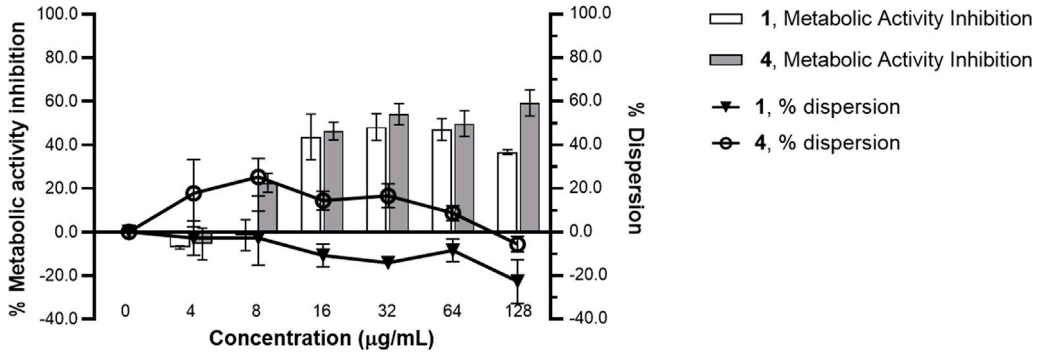

FIGURE 3
Metabolic inhibition and dispersion profile of increasing concentrations of **1** and **4** against *Staphylococcus epidermidis* RP62A ATCC 35984 pre-formed biofilm. A 24 h-old *Staphylococcus epidermidis* biofilm was treated with **1** and **4** from 0 to 128 µg/mL MBIC for 24 h. Using MTT reagent and WGA 488, metabolic activity and dispersive capacity, respectively, were measured. % metabolic activity and % dispersion was calculated relative to the solvent control (1% DMSO). A significant reduction in metabolic activity was observed with treatments of **1** and **4**. Data presented is representative of one biological replicate (mean ± SD) from two independent biological replicates with three technical replicates each.

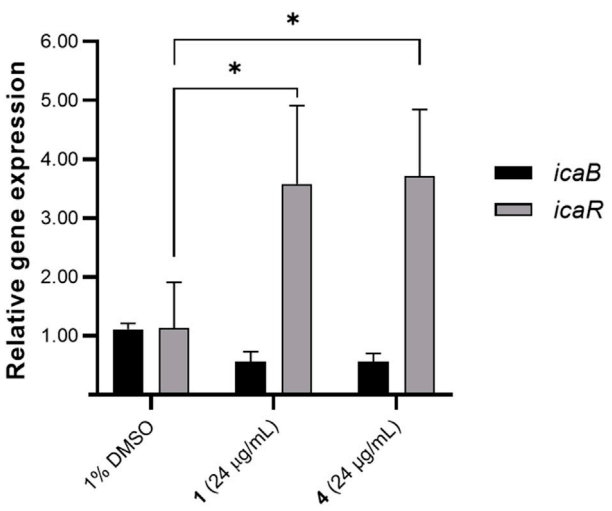

FIGURE 4
Effects of compounds **1** and **4** (24 µg/mL) on *icaB* and *icaR* expression. Data were analyzed using one-way ANOVA and Tukey's *post hoc* test at *p*-value <0.05. Biofilm relevant genes *icaB* and *icaR* were differentially regulated by **1** and **4**. Data presented is representative of one biological replicate (mean + SD) from two independent biological replicates with four technical replicates.

Oxylipins decreased EPS and adherent micro-colonies

SEM was performed to assess the phenotypic effects of the oxylipins on *S. epidermidis* morphology and biofilm. Untreated *S. epidermidis* showed the typical morphology, with circular microcolonies that are clumped together (Figure 5). Attachment of the cells is facilitated by EPS that appeared as thread-like structures in the SEM micrographs (Figure 5). Treatment with oxylipins **1** and **4** showed a significantly reduced number of attached cells and EPS (Figure 5). The observed phenotype with **1** and **4** treatments were significantly different from the negative control.

Oxylipins putatively interact with quorum sensing regulator proteins

To substantiate the observed *ica* regulation and SEM observation, we assessed the interaction of **1, 4,** and **5** on the global regulatory protein SarA, which positively enhances *ica* operon and consequently, PIA/PNAG synthesis. SarA modulates key virulence factors which allows the transition from planktonic growth to biofilm state (Tormo et al., 2005). SarA interplays with the quorum sensing accessory gene regulator (AgrA) (Reyes et al., 2011). Given that SarA influences the *agr* pathway, we included AgrA to the *in silico* assessment. Additionally, oxylipins share structural similarity with known diffusible signal factors (DSFs) which also interferes with quorum sensing through the SarA pathway (Schmidt et al., 2003; Qazi et al., 2006; Biswas and Götz, 2022). We hypothesize that because of the structural similarity of these oxylipins with diffusible signal factors, compounds **1** and **4** are likely to have an analogous mechanism of action in inhibiting biofilm formation. Therefore, we used molecular docking to demonstrate the possible interactions of the modified oxylipins with Agr and SarA.

In silico docking of **1**, **4,** and **5** (Figure 6; Supplementary Table S1) was done using AutoDock (version 1.5.7). The oxylipin analogues were docked in helices α1 - α5 of SarA receptor. Estimated binding energy obtained from SarA ranged from −3.65, −5.03 and −3.79 kcal/mol from oxylipins **1**, **4**, and **5**, respectively (Supplementary Table S1). Hydrogen bonds were noted in the oxylipin-receptor complexes, with critical interactions formed with the epoxide and terminal carboxylic acid moiety (Figure 6; Supplementary Table S1). Hydrophobic interactions were seen in Val116a, Phe134b, Asn161b, Tyr162b.

In the case of AgrA, the binding energy obtained from the molecular docking ranged from −3.28, −4.85 and −2.76 kcal/mol from oxylipins **1**, **4**, and **5**, respectively. The compounds occupied a conserved hydrophobic site formed by helix α1-sheet β1 (Figure 6; Supplementary Table S1). Hydrogen bonding interactions of **1**, **4**, **5** with AgrA were largely similar, with key interactions observed with Asn185, Glu188, and Glu144.

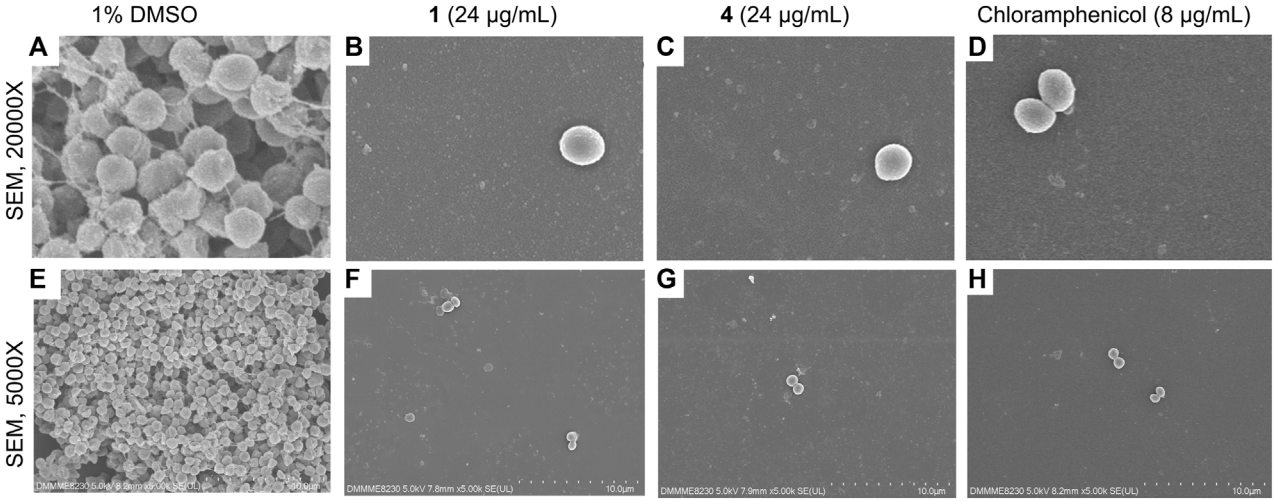

FIGURE 5
SEM micrographs of *Staphylococcus epidermidis* at ×20,000 magnification **(A–D)** and 5,000x **(E–H)** at 24-h post-incubation with **1** and **4** (24 μg/mL), positive control chloramphenicol (8 μg/mL), and solvent control (1% DMSO). Micrographs reveal reduction in attached cells and visible EPS with **1** and **4**.

Oxylipins reduce carotenoid production in *S. aureus*

AgrA expression can influence several virulence factors including pigment formation in *S. aureus* (Tan et al., 2022). Both *S. aureus* and *Staphylococcus lugdunensis* with mutant *agrA* operon were found to have reduced pigmentation as compared to wildtype strains (Aubourg et al., 2022; Tan et al., 2022; Cella et al., 2023). On this basis, we validated the docking analysis of AgrA using staphyloxanthin quantification. Staphyloxanthin is quantified by carotenoid or yellow pigment production in *S. aureus*. Oxylipins **1** and **4** caused a 24% and 29% decrease in staphyloxanthin production, respectively (Figure 7). The decrease in staphyloxanthin production, and decreased EPS and microcolonies observed in the SEM corroborates the cellular permeability action of the oxylipins at the early stages of biofilm formation.

Discussion

We further probe the mechanism of action and identify the structural determinants for the biofilm inhibitory activity of modified oxylipins. Our results corroborate the findings of Lacerna et al. (2020) and highlight the additional structural motifs that are responsible for the biofilm inhibitory activity of modified oxylipins. The addition of an unsaturation to the aliphatic chain negatively impacts the antibiofilm activity of this class of compounds. Positional isomers of modified oxylipins showed comparable bioactivity. This is analogous to the observation in cyclopropyl-bearing fatty acid, where similar inhibitory activities in *Escherichia coli* and *Pseudoroseovarius crassostreae* were obtained despite the modification involving the cyclopropyl unit (Amiri Moghaddam et al., 2018). The *trans*-epoxide **1** and **4** may provide an optimum spatial and electrostatic interaction with the cellular target, compared to its *cis*-isomer. SAR evaluation of cerulenin analogues revealed that *trans*-2,3-epoxydodecanoic acid is 5x more potent HIV protease inhibitor than *cis*-epoxydecenoic (Blumenstein et al., 1989). By disruption of peptidoglycan synthesis and fatty acid synthesis, increased antimicrobial activity was also reported from unsaturated linoleic and arachidonic acid as compared to their saturated counterpart (Zheng et al., 2005; Kenny et al., 2009; Casillas-Vargas et al., 2021).

Interestingly, these oxylipins are structurally related to the *P. aeruginosa* derived fatty acids (10*S*)-hydroxy-(8*E*)-octadecenoic acid (10-HOME) and 7*S*,10*S*-dihydroxy-(8*E*)-octadecenoic acid (7,10-DiHOME). 10-HOME and 7,10-DiHOME are upregulated during biofilm formation and virulence in *P. aeruginosa* (Martínez and Campos-Gómez, 2016; Martínez et al., 2019). In contrast to 10-HOME and 7,10-DiHOME, **1**-**6** did not affect biofilm formation in *P. aeruginosa*, whether inhibition or promotion. The epoxide-bearing oxylipins showed selective bioactivity against the Gram-(+) *S. aureus* and *S. epidermidis* and may suggest the role of the epoxide moiety not just for potent bioactivity but also for selectivity.

To further probe the cellular effects of these modified fatty acids, we undertook a gene to phenotype approach. We focused on *icaB* and *icaR* transcripts that are part of the *ica* operon responsible for the synthesis of polysaccharide intercellular adhesin/polymeric *N*-acetyl-glucosamine (PIA/PNAG) exopolysaccharides. *icaR* serves as a transcriptional repressor and regulates the activation of downstream *icaADBC* (François et al., 2023). *icaB* deacetylates and polymerizes PIA/PNAG in *S. aureus* and *S. epidermidis* resulting in enhanced protective layer and ideal surface adherence of PIA/PNAG (Cerca et al., 2007; Arciola et al., 2015). Significant upregulation of the *icaR* repressor can compromise the synthesis of EPS component polysaccharide intercellular adhesin (PIA) or polymeric *N*-acetyl-glucosamine (PNAG) under an *ica*-dependent pathway. Microbial isolates bearing single nucleotide polymorphisms in *icaR* showed decreased exopolysaccharide production (Morales-Laverde et al., 2022), directly demonstrating the impact of *icaR* on PIA/PNAG. Disruption of PIA/PNAG can impair not just the structural integrity of the biofilm matrix

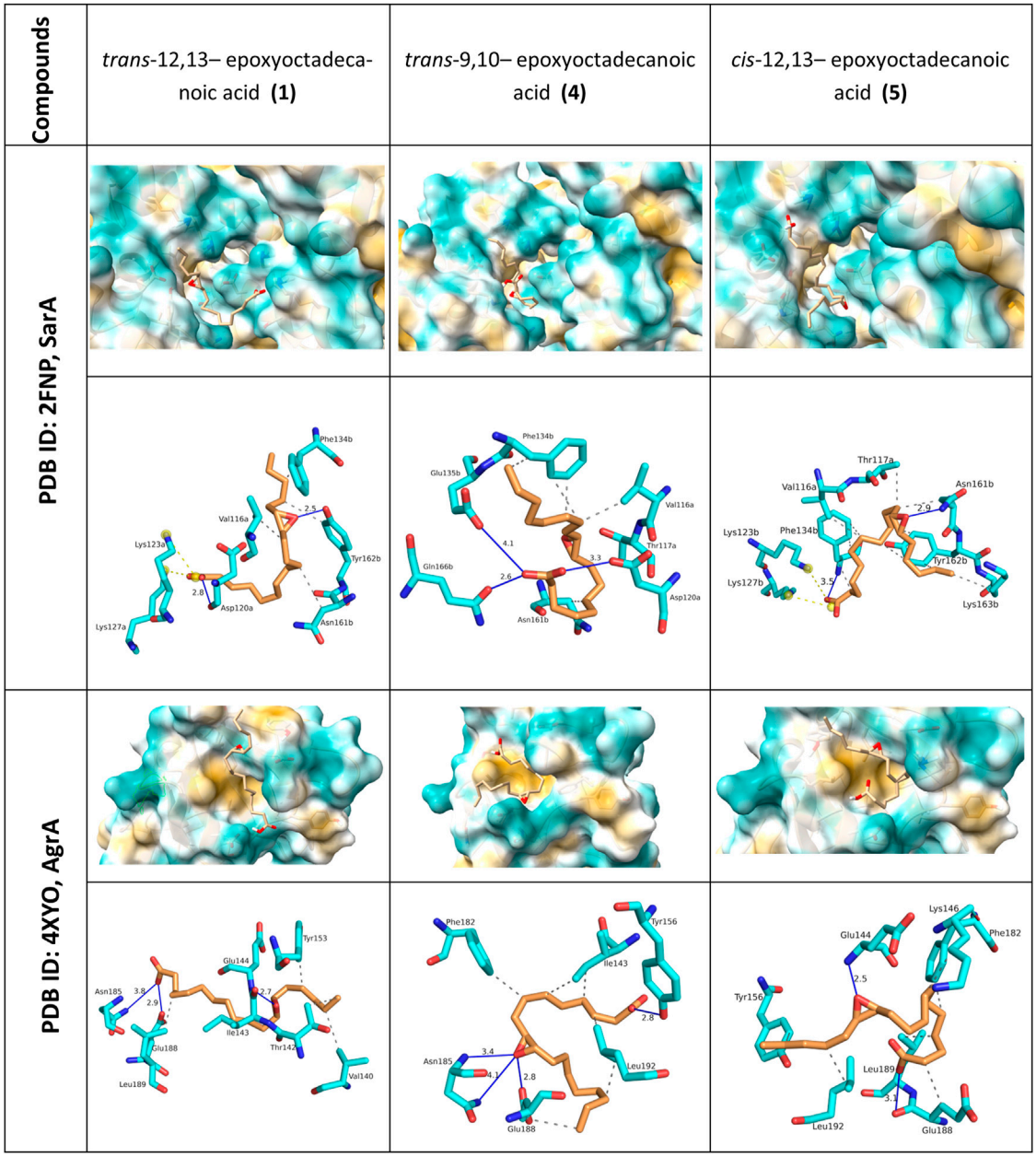

FIGURE 6
Visualization of molecular docking analysis of oxylipin analogues with AgrA and SarA. 3D surface interactions for both protein complexes are shown with closeup view in the protein surfaces. 2D interaction with AgrA and SarA are represented by hydrogen bonds D-A (blue lines), hydrophobic contacts (grey dashed line), and salt bridges (yellow dashed line). Critical interactions were observed between SarA and AgrA with the epoxide moiety of the oxylipins.

but can also decrease the pathogenicity and virulence of *Staphylococcus* (Nguyen et al., 2020; Cheung et al., 2021; Peng et al., 2023). Treatment of **1** and **4** showed an increase in *icaR* transcript level that consequently translated to a modest decrease in *icaB*. SEM analysis confirmed a decline in EPS in oxylipin-treated *S. epidermidis* compared to the solvent control. The observed cellular effects of **1** and **4** are similar to those observed for quercetin, gallic acid and (+)-nootkatone, where suppression of biofilm formation is obtained through modulation of the *ica* operon in Staphylococci (Guo et al., 2021; Mu et al., 2021).

The *ica* operon is regulated by the Staphylococcal accessory regulator (SarA) and consequently, by the quorum sensing accessory gene regulator (AgrA). SarA is a DNA binding protein that belongs to the global modulators of various virulence factors in Staphylococci and increases the production of toxins, fibronectin, and fibrinogen (Cheung et al., 2008). SarA can regulate the *ica* operon and the quorum sensing regulator *agr* operon (Peng et al., 2023). SarA binds to the *ica* operon with high affinity and enhances *ica* expression (Tormo et al., 2005). Interaction of SarA with AgrA leads to enhancement of the conformational bending of promoter during *agr* locus transcription (Reyes et al., 2011).The *ica* operon is, however, not affected directly by *agr* expression.

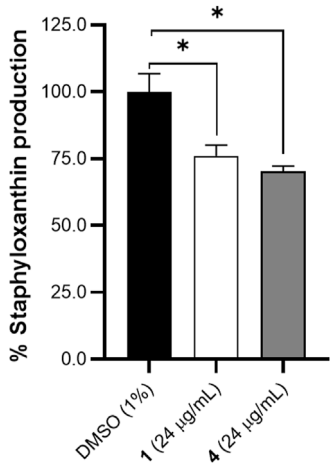

FIGURE 7
Effects of compounds **1** and **4** (24 µg/mL) on staphyloxanthin production in *S. aureus* after 24 h of treatment. A significant decrease in pigment was observed with treatment of **1** and **4**. Data were analyzed using one-way ANOVA with multiple comparisons at $p < 0.05$. Data is representative of one biological replicate (mean +SD (n = 3) from two independent experiments with three technical replicates each.

The *agr* pathway is often associated with virulence, pathogenesis, and toxin production in *S. aureus* (Le and Otto, 2015). The *agr* operon is involved in both biofilm formation and dispersion during the maturation stage (Le and Otto, 2015). A functional *agr* pathway is important in biofilm structural integrity but in some cases can induce biofilm formation (Gray et al., 2013; Le and Otto, 2015). Despite the divergent phenotypic expressions of *agr* QS system, several authors still consider the *agr* system to be prospective targets in biofilm mitigation studies (Park et al., 2007; Gray et al., 2013; Ganesh et al., 2022). Specific interference with AgrA-DNA binding domain is one of the possible ways to decrease or block the RNA III concentration (Gray et al., 2013).

Several diffusible signal factors (DSF) composed of short to medium chain alkyl chains were reported to regulate cell communication in prokaryotes (Kumar et al., 2020). Hydroxy-bearing DSF such as the 3-hydroxyhexadecanoic acid from *Ralstonia solanacearum* (Flavier et al., 1997), is structurally similar to the oxylipins tested. Due to limited protein structures in *S. epidermidis*, we used the crystallized structures of staphylococcal accessory regulator (SarA) and accessory gene regulator A (AgrA) obtained from closely related *S. aureus*. Both AgrA and SarA proteins in *S. aureus* and *S. epidermidis* displayed sequence homology ranging from 68% to 84% (Fluckiger et al., 1998; Otto et al., 1998; Van Wamel et al., 1998).

At the molecular level, our docking assessment showed that oxylipins occupy a hydrophobic pocket in both SarA and AgrA, with structural changes possibly resulting in alteration of specific amino acid binding sites. While the binding pockets occupied by **1** and **4** in SarA and AgrA are not directly involved in target gene binding, this may possibly disrupt the stability of the interaction with the consensus target gene. The observed activity of the compounds may be attributed to the steric hindrance introduced while interacting with multiple amino acids involved in enhancing the stability with the consensus target gene. Leu186 and Lys187 are critical amino acids in AgrA that form hydrogen bonding interactions with the promoter DNA, leading to stabilization of the complex (Rajasree et al., 2016). In comparison with other epoxide bearing QS inhibitors such as cerulenin, fosfomycin, and ambuic acid, **1** and **5** also showed similar hydrogen bonding interactions with Glu144 and Lys146 in the AgrA complex (Refai et al., 2023). The binding of **1**, **4**, and **5** to SarA is reminiscent of the interactions of other natural products carvacrol, eugenol, and hesperidin (Selvaraj et al., 2020; Vijavyakumar et al., 2022). Key interactions were observed in Asn161b, Thr117a, Gln166b (Selvaraj et al., 2020; Vijayakumar et al., 2022).

The molecular docking results were validated by the differential gene regulation of the *icaR* and *icaB* and staphyloxanthin levels. RNA III-independent *agr* pathway is involved in carotenoid production in *S. aureus* (Queck et al., 2008). Mutations on *agr* can lead to variations in virulence factors including loss of pigment production, hemolysin activity, and oxidative stress regulation (Aubourg et al., 2022; Tan et al., 2022). The impact of *agr* gene expression levels on biofilm formation is, however, less definitive compared to other genes such as *icaA* and *icaR* (Arciola et al., 2015). Phenotypic variations in virulence factors have been observed in *agr*-related studies, a case from myriad of factors including staphylococcal strain dependency, compound dosage response, and culture conditions (Yarwood and Schlievert, 2003; Pant et al., 2022; 2023). The molecular interactions and phenotypic observation is congruent with the observations for 3-hydroxybenzoic acid (Ganesh et al., 2022) and ω-hydroxyemodin (Daly et al., 2015) as disruptors of biofilm formation in *S. aureus*.

Staphyloxanthin production in Staphylococci is relevant not just in aggregation during biofilm formation, but also contribute to other virulence factors such as regulating toxic free radicals, and recognition of neutrophil attack (Elmesseri et al., 2022; Cella et al., 2023). A decreased staphyloxanthin production was observed for treatment with compounds **1** and **4**. Staphyloxanthin production is an important adaptation for *S. aureus* by acting as a scavenger of reactive oxygen species (Elmesseri et al., 2022). Inhibition of this phenotype clearly demonstrates the cellular permeability of the modified oxylipins in *S. aureus*. At the same time, the SEM experiment using *S. epidermidis* showed very distinct growth morphology with solvent control, further demonstrating the absorption of compounds **1** and **4**.

Conclusion

Effective mitigation strategies against biofilm formation are crucial for public health. The ability of *S. epidermidis* to colonize biomaterials and to cause persistent clinical infections remains a threat worldwide. The *trans*-epoxide moiety in *trans*-9,10-epoxyoctadecanoic acid (**1**) and *trans*-12,13- epoxyoctadecanoic acid (**4**) imparts selectivity and potent activity against biofilm formation in *S. epidermidis*. The *trans*-epoxide bearing modified oxylipins modulate the transcript levels of key biofilm target genes and consequently, decreasing the phenotypic traits of biofilm formation such as EPS and pigment production.

Data availability statement

The data presented in the study are deposited in FigShare, http://doi.org/10.6084/m9.figshare.25745811.

Ethics statement

Ethical approval was not required for the studies on animals in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used.

Author contributions

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

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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

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Exploring the role of Chinese herbal medicine in the long-term management of postoperative ovarian endometriotic cysts: a systematic review and meta-analysis

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Background: Ovarian endometriotic cysts (OEC) represent the primary manifestation of endometriosis, constituting a hormonally dependent inflammatory disorder in gynecology. It significantly affects the quality of life and reproductive health of women. It is worth noting that traditional Chinese medicine (TCM), especially Chinese herbal medicine (CHM), has been widely applied in mainland China due to its unique therapeutic system and commendable clinical efficacy, bringing new hope for preventing and managing OEC.

Objective: This study aims to evaluate the efficacy and safety of CHM in the management of postoperative OEC. Simultaneously, it seeks to explore the medication laws, therapeutic principles, and specific treatment mechanisms of CHM.

Methods: Eight electronic databases were searched from their inception to 01 November 2023. Randomized controlled trials (RCTs) assessing the therapeutic effects and safety of CHM for postoperative OEC were included. The risk of bias for each trial was assessed using the Cochrane Collaboration's tool. The certainty of the evidence was evaluated using the GRADE profiler 3.2. Additionally, we extracted formulation from the included studies, conducting a thorough analysis.

Results: **(i)** Twenty-two RCTs involving 1938 patients were included. In terms of the primary efficacy outcome, the CHM group demonstrated a potentially lower recurrence rate compared to both control (odds ratio (OR) = 0.25; 95% confidence intervals (CI): 0.10–0.64) and conventional western medicine (CWM) (OR = 0.26; 95% CI: 0.11–0.65) groups. Furthermore, the joint application of CHM and CWM resulted in a significant reduction in the recurrence rate (OR = 0.26; 95% CI: 0.17–0.40). **(ii)** Regarding secondary efficacy outcomes, (a) Total clinical efficacy rate: CHM showcased an augmentation in clinical effectiveness compared to both the control (OR = 4.23; 95% CI: 1.12–15.99) and CWM (OR = 2.94; 95% CI: 1.34–6.43) groups. The combined administration of CHM and CWM substantially enhanced overall

clinical effectiveness (OR = 3.44; 95% CI: 2.37–5.00). (b) VAS Score: CHM exhibited the capacity to diminish the VAS score in comparison to surgery alone (Mean difference (MD) = −0.86; 95% CI: -1.01 to −0.71). Nevertheless, no substantial advantage was observed compared to CWM alone (MD = −0.16; 95% CI: -0.49 to 0.17). The integration of CHM with CWM effectively ameliorated pain symptoms (MD = −0.87; 95% CI: -1.10 to −0.65). (c) Serum Level of Cancer antigen 125 (CA125): the CHM group potentially exhibited lower CA125 levels in comparison to CWM alone (MD = −11.08; 95% CI: -21.75 to −0.42). The combined intervention of CHM and CWM significantly decreased CA125 levels (MD = −5.31; 95% CI: -7.27 to −3.36). (d) Pregnancy Rate: CHM exhibited superiority in enhancing the pregnancy rate compared to surgery (OR = 3.95; 95% CI: 1.60–9.74) or CWM alone (OR = 3.31; 95% CI: 1.40–7.83). The combined utilization of CHM and CWM demonstrated the potential to enhance pregnancy rates compared to CWM (OR = 2.99; 95% CI: 1.28–6.98). Concerning safety outcome indicators, CHM effectively decreased the overall incidence of adverse events and, to a certain extent, alleviated perimenopausal symptoms as well as liver function impairment. **(iii)** Most of CHMs were originated from classical Chinese herbal formulas. *Prunus persica* (L.) Batsch (Taoren), *Angelica sinensis* (Oliv.) Diels (Danggui), *Salvia miltiorrhiza* Bunge (Danshen), *Paeonia lactiflora* Pall. (Chishao), and *Corydalis yanhusuo* W.T.Wang (Yanhusuo) were most frequently used CHM.

Conclusion: CHM may be a viable choice in the long-term management of postoperative OEC, with the potential to enhance clinical efficacy while decreasing recurrence and adverse effects.

KEYWORDS

Chinese herbal medicine, ovarian endometriotic cyst, postoperative treatment, randomized controlled trial, systematic review, meta-analysis

1 Introduction

Ovarian endometriotic cysts (OEC) arise from the growth of ectopic endometrial tissue within the ovarian cortex (Bulun et al., 2019), constituting a major manifestation of endometriosis and contributing to 17%–44% of its incidence (Wang G. et al., 2022). Clinically, OEC is characterized by pelvic pain, infertility, abnormal menstruation, significantly impacting the quality of life and reproductive health of women of childbearing age (Bonavina and Taylor, 2022). Currently, conservative surgery is the principal treatment for reproductive-age patients with OEC (Falcone and Flyckt, 2018). Unfortunately, the recurrence rate within 5 years post-surgery can reach up to 50% (Ceccaroni et al., 2019), rendering OEC a chronic condition. Post-surgery, dienogest and gonadotropin-releasing hormone agonists (GnRH-α) are commonly prescribed to eradicate microscopic lesions and mitigate OEC recurrence. Yet, prolonged use may suppress ovarian function and delay pregnancy (Zhao, 2023). Furthermore, adverse effects including vasoconstriction symptoms, insomnia, irregular vaginal bleeding, and gastrointestinal discomfort, pose significant challenges in the long-term management post-OEC surgery (Sauerbrun-Cutler and Alvero, 2019; Della Corte et al., 2020; Konninckx et al., 2021). According to a report in The Lancet (Taylor et al., 2021), endometriosis has been considered a systemic disease that may affect liver and adipose tissue metabolism, trigger systemic inflammation, alter brain gene expression, and lead to pain sensitization and mood disorders (Taylor et al., 2021). Therefore, seeking more effective and tolerable postoperative treatments for OEC holds significant clinical relevance.

In recent years, Chinese herbal medicine (CHM) has emerged as a promising alternative therapy within the field of gynecology due to its unique treatment system. Contemporary research has further validated the crucial role of CHM in treating a variety of gynecological diseases, including OEC. For instance, studies have shown CHM's ability to induce apoptosis in ovarian cancer cells through multiple signaling pathways (Wang et al., 2024), ameliorate perimenopausal syndrome by regulating hormones secreted by the ovaries (Xue et al., 2022), and exhibit potential therapeutic effects on pregnancy-related diseases, such as recurrent spontaneous abortion, pre-eclampsia, and gestational diabetes (Fang et al., 2023). Furthermore, a meta-analysis demonstrated that CHM significantly alleviates pain associated with endometriosis with fewer side effects compared to conventional therapies (Lin et al., 2023). These findings affirm the applicability of CHM in treating gynecological diseases and provide a scientific basis for its integration into the modern medical system. Therefore, exploring the role of CHM in the long-term management of postoperative OEC is not only a venture into the modern application of traditional medicine but also critically important for the advancement and development of innovative treatment methods in gynecology.

In the postoperative treatment of OEC, CHM adopts a patient-centered approach, adhering to natural law principles and emphasizing holistic care. It aims to bolster healthy qi, dispel pathogen, and facilitate the body's recovery (Xu et al., 2020). The mechanisms through which CHM aids in the postoperative management of OEC primarily include: 1) regulating the immune system to promote immune balance and enhance immunity, thereby facilitating the elimination of ectopic

endometrial tissue (Zhang et al., 2013; Shen et al., 2016; Song et al., 2021); 2) exerting anti-inflammatory effects to significantly reduce postoperative inflammation and alleviate pain (MERESMAN et al., 2021; Chen et al., 2024); 3) modulating the endocrine system to correct endocrine dysregulation, inhibit the proliferation of ectopic tissue, and restore ovarian function (Lai et al., 2021; Ma et al., 2021; Zhao, 2023); (4) promoting blood circulation to repair tissue damage and decrease the recurrence of OEC (Zhou et al., 2019; Zhao et al., 2020); (5) directly targeting ectopic endometrial tissue to inhibit its growth and invasion (Pang et al., 2021; Tan et al., 2021; Huang et al., 2022; Zhang et al., 2023). Collectively, CHM plays a comprehensive and multi-level therapeutic role in the long-term management of postoperative OEC.

A considerable volume of clinical research, encompassing case reports, case series, and randomized controlled trials (RCTs), has demonstrated that CHM could decrease recurrence rates, boost pregnancy rates, and improve the quality of life, bringing new hope for clinical management and the development of novel therapeutic approaches for postoperative OEC (Ma, 2011; Ding and Shi, 2012; Zhang, 2012; Zhou and Liu, 2013; Li, 2014; Chen, 2015; Chen et al., 2015; Dou, 2015; Du, 2015; Han, 2016; Xing, 2016; Zhou, 2016; Chen, 2018; Hu and Y, 2018; Lu et al., 2019; Qiu and Wan, 2019; Song et al., 2019; Wang and Bo, 2019; Liu, 2020; Zhao and Xiao, 2020; Wu and Deng, 2021; Tong et al., 2023). Despite this, debates regarding its efficacy and safety persist. Although 3 systematic reviews regarding CHM treatment for postoperative OEC have been published in advance respectively, some issues were also identified. Two reviews investigated the efficacy of *Salvia miltiorrhiza*-containing CHM (Gao Q. et al., 2022) and *black cohosh* extracts (Peng et al., 2020) in improving the low estrogen status induced by GnRH-α in postoperative endometriosis patients. However, the outcome measures and the scope of exploring CHM were relatively limited. Regarding the review by Dr. Fan et al., (Fan et al., 2022), more databases and RCTs should be updated to reduce potential bias. Therefore, this study has updated the relevant literature, conducted a comprehensive systematic review of RCTs to evaluate the current clinical evidence on CHM for postoperative OEC. Additionally, it provided a summarizing analysis of medication characteristics and treatment principles, aiming to offer assistance for clinical medication.

2 Methods

This study was conducted and reported according to the guidelines of Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) 2020 (Page et al., 2021) (Supplementary Appendix S1).

2.1 Eligibility criteria

2.1.1 Types of studies

RCTs which have evaluated the efficacy of CHM for OEC were included in this study.

2.1.2 Types of participants

Patients received conservative surgical intervention (laparoscopic or laparotomy excision or ablation of lesions while preserving the uterus and ovaries) and had a pathological diagnosis of OEC. In order to ensure including all relevant studies, no restriction on age and nationality was specified.

2.1.3 Types of interventions

Patients in the treatment group should be treated by CHM or combination of CHM and CWM. Patients in the control group should be treated by CWM or CHM placebo or be a blank control. CWM in the treatment and control group must be identical in name, usage, dosage, etc. No restrictions on dosage forms, route of administration, quantity, or treatment course of CHM was specified.

2.1.4 Types of outcome measures

The primary efficacy outcome measure was defined as recurrence rates, while secondary outcomes consist of total clinical efficacy rate, VAS score, serum level of Cancer antigen 125 (CA125), and pregnancy rate. Safety outcome measures encompass the total incidence of adverse events as well as the specific rate of adverse reactions.

2.1.5 Exclusion criteria

RCTs will be excluded if the following conditions are met: (a) clinical experiences, theoretical discussion, reviews, commentaries, editorials, case reports, case series, and experimental studies; (b) Studies limited to surgical exploration, diagnosis, or staging without any intervention on the lesions in patients; (c) Control groups receiving CHM treatment, with either no established control group or inconsistency in baseline treatments between groups; (d) no detailed information regarding clinical efficacy could be extracted; and (e) duplicate publications.

2.2 Literature search

Relevant literature assessing the efficacy and safety of CHM for OEC was searched in 8 electronic databases including PubMed, Web of Science, Cochrane Library, EMBASE, Chinese National Knowledge Infrastructure (CNKI), VIP Information Database (VIP), Chinese Biomedical Literature Database (CBM), and Wanfang Database from inception up to 01 November 2023. The following grouped keywords and Mesh/Emtree thesauri were used as search terms and modified according to each database: “Ovarian endometriotic cyst” “ovarian chocolate cyst” “Postoperative Period” “Surgical Procedures, Operative” “Laparoscopes” “Chinese herbal medicine” “traditional Chinese medicine” “zhong yi yao” “zhong yao” “formula” “decoction” “pill” “capsule” “granules” “powder” “paste” “recipe” “clinical trial” “randomized controlled trial”, etc. Two different authors (DN Ding and SX Liu) independently conducted the literature search and evaluated the results. The search strategies for all the electronic databases can be found in Supplementary Appendix S2. To minimize bias, we also retrieved the ongoing registered clinical trials and unpublished papers on CHM for OEC. No language and status restriction were set in this review.

2.3 Study selection and data extraction

Trials were selected according to the inclusion/exclusion criteria by reading the titles, abstracts and (or) full texts of

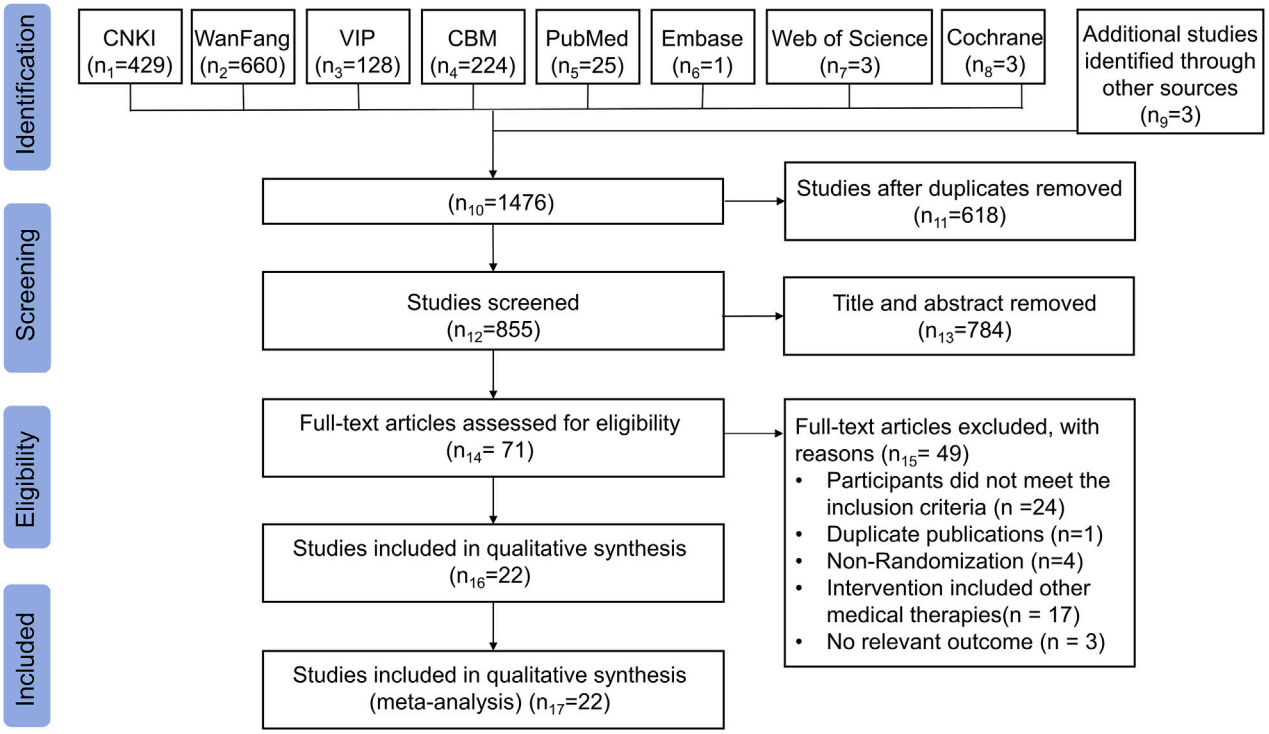

FIGURE 1
Flow diagram of study selection and identification.

the published articles. Two authors (SX Liu and FY Liu) independently selected the studies and extracted data using a pre-designed data extraction sheet, evaluated and cross-checked. Detailed information of enrolled study was listed as follows: (a) basic characteristics of included studies: title of study, authors' name, publication date, sample size, diagnostic criteria, methodological quality, therapeutic schedule in treatment and control groups, components and dosage of CHM, withdrawals, and course of treatment; (b) basic characteristics of included patients: age, gender, duration of disease, size and staging of OEC, previous medical history, and laboratory examination; (c) both primary and secondary outcome measures; and (d) adverse effects. If disagreements on data extraction were identified, a third party (FJ Han) was consulted.

2.4 Assessment of methodological quality

Methodological quality of the included trials was also assessed by 2 authors (SL Hao and Y Shen) independently. According to Cochrane Collaboration's tool (Sterne et al., 2019), 7 fields of risk of bias (ROB) were evaluated as below: random sequence generation, allocation concealment, blinding of participants and personnel, blinding of outcome assessment, incomplete outcome data, selective reporting, and other bias. Each field was assessed to be "yes" (low ROB), "no" (high ROB), or "unclear" (unclear ROB). The inconsistencies were discussed with the third author (FJ Han).

2.5 Data analysis

Review Manager software (RevMan, Version 5.4, Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2020) was utilized to conduct data analysis of dichotomous and continuous outcome measures, which were extracted from the original studies. Mean difference (MD) was utilized for data measurement of continuous outcomes, while odds ratio (OR) for dichotomous outcomes. All of them were expressed with a 95% confidence interval (CI). When no statistical heterogeneity was identified (heterogeneity test, $p \geq 0.10$, or $I^2 \leq 50\%$), fixed-effects model was selected, otherwise random-effects model was applied. Subgroup analysis and/or sensitivity analysis was conducted to identify the sources of heterogeneity. Funnel plot was also used to evaluate the publication bias when over 10 trials were included in the analysis. A significant difference was considered when $p < 0.05$.

Furthermore, in accordance with the GRADE (Grading of Recommendations Assessment, Development and Evaluation criteria) (Guyatt et al., 2008a), the quality of evidence for each outcome was assessed using GRADE profiler 3.2.

3 Results

3.1 Study selection

The flowchart of literature identification and screening is depicted in Figure 1. In total, 1,476 related literature was derived from the above 8 electronic databases, among which three are

ongoing clinical trials (Chictr, 2020; Chictr, 2023). After removing duplicate publications, 855 studies remained. Subsequently, 784 studies were excluded for not being RCTs, specifically including reviews, commentaries, editorials, case reports, experimental studies, data mining articles, and irrelevant to postoperative OEC after scanning titles and abstracts. Furthermore, after reviewing the remaining 71 full texts, an additional 49 studies were excluded for the following reasons: participants did not meet the inclusion criteria (n = 24); duplicate publications (n = 1); non-randomization (n = 4); intervention included other medical therapies (n = 17); no relevant outcome (n = 3). Ultimately, 22 eligible RCTs were included (Ma, 2011; Ding and Shi, 2012; Zhang, 2012; Zhou and Liu, 2013; Li, 2014; Chen, 2015; Chen et al., 2015; Dou, 2015; Du, 2015; Han, 2016; Xing, 2016; Zhou, 2016; Chen, 2018; Hu and Y, 2018; Lu et al., 2019; Qiu and Wan, 2019; Song et al., 2019; Wang and Bo, 2019; Liu, 2020; Zhao and Xiao, 2020; Wu and Deng, 2021; Tong et al., 2023).

3.2 Characteristics of included trials

The basic characteristics of enrolled studies and subjects are presented in Table 1, including sample size, age, intervention, treatment duration, follow-up duration, and outcome measures. All included studies were conducted in China and published in Chinese between 2011 and 2023. There were altogether 1938 patients enrolled in this review, with the sample sizes ranged from 48 to 160. Four studies assessed the efficacy of CHM alone compared to CWM, one study compared the efficacy of CHM alone with a blank control, and the remaining studies evaluated the efficacy of CHM combined with CWM compared to standalone CWM. Treatment duration varied from 3 to 6 months. Mean follow-up durations ranged from 3 to 36 months. Recurrence rate was reported in 17 studies (Ma, 2011; Zhang, 2012; Zhou and Liu, 2013; Li, 2014; Chen, 2015; Chen et al., 2015; Dou, 2015; Han, 2016; Xing, 2016; Zhou, 2016; Hu and Y, 2018; Qiu and Wan, 2019; Song et al., 2019; Wang and Bo, 2019; Liu, 2020; Zhao and Xiao, 2020; Tong et al., 2023). Total clinical efficacy rates were reported in 16 trials (Ma, 2011; Ding and Shi, 2012; Zhou and Liu, 2013; Li, 2014; Chen et al., 2015; Dou, 2015; Du, 2015; Zhou, 2016; Chen, 2018; Hu and Y, 2018; Lu et al., 2019; Song et al., 2019; Wang and Bo, 2019; Zhao and Xiao, 2020; Wu and Deng, 2021; Tong et al., 2023). VAS score was reported in 10 trials (Zhang, 2012; Chen, 2015; Chen et al., 2015; Han, 2016; Xing, 2016; Hu and Y, 2018; Song et al., 2019; Zhao and Xiao, 2020; Wu and Deng, 2021; Tong et al., 2023). Serum level of CA125 was reported in 11 trials (Zhang, 2012; Chen, 2015; Dou, 2015; Du, 2015; Han, 2016; Xing, 2016; Zhou, 2016; Hu and Y, 2018; Lu et al., 2019; Liu, 2020; Zhao and Xiao, 2020). Pregnancy rate was reported in 6 trials (Ding and Shi, 2012; Li, 2014; Qiu and Wan, 2019; Liu, 2020; Zhao and Xiao, 2020; Tong et al., 2023). Fourteen trials reported adverse events (Ding and Shi, 2012; Zhang, 2012; Zhou and Liu, 2013; Chen, 2015; Dou, 2015; Du, 2015; Han, 2016; Xing, 2016; Zhou, 2016; Hu and Y, 2018; Lu et al., 2019; Qiu and Wan, 2019; Wang and Bo, 2019; Liu, 2020).

3.3 Assessment of methodological quality

As shown in Figure 2, the methodological quality of the included studies was evaluated based on the criteria in the Cochrane handbook. Detailed information on the sequence generation of randomization was not reported in 7 trials (Ma, 2011; Ding and Shi, 2012; Zhou and Liu, 2013; Han, 2016; Zhou, 2016; Song et al., 2019; Tong et al., 2023). There were no statistically significant differences in baseline between the intervention and control groups across all enrolled studies. A specific method of allocation concealment was not described in this review. Detailed information regarding blinding of patients and investigators was unclear in all enrolled trials. Outcome data were obtained for nearly all randomized groups of subjects. All studies were free of bias from other sources. Although all studies were unclear on the blinding of outcome assessment, patients with OEC have objective evaluation indexes for recurrence, efficacy, pregnancy and serum CA-125 levels, and it was difficult to affect the outcome's evaluation. No information mentioned that the results were analyzed in accordance with a published pre-specified analysis plan. Consistent outcome measures and data analysis methods were used for all included studies.

3.4 Description of single herb and CHM

Twenty-two CHM were used in this review, including five dosage formulations: decoction (11/22, 50.00%), capsule (7/22, 31.82%), pill (2/22, 9.10%), granule (1/22, 4.55%), and decocted extract (1/22, 4.55%). Decoction was the most commonly used formulation, accounting for the highest percentage. Table 1 illustrates the administration of CHM in each trial, and the specific components of CHM used in the included studies can be found in Supplementary Appendix S3.

The frequency of each Chinese herb in this review was also summarized manually. In total, 97 Chinese herbs were included, with a cumulative frequency of 236. Classified by CHM efficacies, they were divided into 15 categories. The top three categories were invigorate blood and dissolve stasis (81/236, 34.32%), supplement deficiency (61/236, 25.85%), and soothe the liver/rectify qi (32/236, 13.56%), refer to Table 2 for details. Additionally, specific components of CHM are listed in Table 3. The top 5 ranking CHM were *Prunus persica* (L.) Batsch [Rosaceae; Persicae Semen] (Taoren) (10/236, 4.24%), *Angelica sinensis* (Oliv.) Diels [Apiaceae; Angelicae Sinensis Radix] (Danggui) (9/236, 3.81%), *S. miltiorrhiza* Bunge [Lamiaceae; Salviae Miltiorrhizae Radix et Rhizoma] (Danshen) (9/236, 3.81%), *Paeonia lactiflora* Pall. [Paeoniaceae; Paeoniae Radix Rubra] (Chishao) (8/236, 3.39%), and *Corydalis yanhusuo* (Y.H.Chou & Chun C.Hsu) W.T.Wang ex Z.Y.Su and C.Y.Wu [Papaveraceae; Corydalis Rhizoma] (Yanhusuo) (7/236, 2.97%). The top three CHM natures were warm (92/236, 38.98%), cold (79/236, 33.47%), and neutral (63/236, 26.69%), while the top three flavors were bitter (132/389, 33.93%), sweet (111/389, 28.53%), and acrid (103/389, 26.48%). In terms of channel entries, the top four were the foot *jueyin* liver channel (181/598, 30.27%), the hand *shaoyin* heart channel (93/598, 15.55%), the foot *taiyin* spleen channel (88/598, 14.72%), and the foot *shaoyin* kidney channel (80/598, 13.38%). Further details can be found in Table 4.

TABLE 1 Basic characteristics of included trials and subjects.

References	Sample size (T/C)	Age (years)	Intervention	Control	Treatment duration	Follow-up duration	Outcome measures
Tong QL et al., 2023	126 (63/63)	T: 29.14 ± 5.23 C: 29.37 ± 6.02	CHM (*Fufang Xuanju* capsules)	Blank Control	CHM:1.26 g, tid, discontinue during menstruation, 4 weeks/course * 3 courses	12 months	①②③⑤
Wu L et al., 2021	106 (53/53)	T: 34.32 ± 2.35 C: 34.96 ± 2.15	CHM (*Cinnamon Twig and Poria* pills) + Leuprorelin	Leuprorelin	CHM:1.35 g, bid, discontinue during menstruation, 4 weeks/course * 6 courses Leuprorelin: Once every 4 weeks * 6 courses	6 months	②③
Zhao XJ et al., 2020	116 (58/58)	T: 29.41 ± 5.27 C: 29.45 ± 5.21	CHM (*Yishen Shugan* decoction) +Triptorelin	Triptorelin	CHM:1 dose/d (100 mL, tid), discontinue during menstruation, 4 weeks/course * 6 courses Triptorelin: Once every 4 weeks * 6 courses	6 months	①②③④⑤
Liu X 2020	66 (33/33)	T: 31.53 ± 6.09 C: 32.07 ± 4.81	CHM (*Huayu Xiaozheng* decoction) + Goserelin	Goserelin	CHM:1 dose/d (200 mL, bid), discontinue during menstruation, 14 days/course * 3 courses Goserelin: Once every 28 days * 3 courses	7 months	①④⑤⑥
Qiu YF et al., 2019	68 (34/34)	T: 28.25 ± 6.12 C: 28.19 ± 6.04	CHM (*Wenshen Xiaozheng* decoction) + Triptorelin	Triptorelin	CHM:1 dose/d (150 mL, bid), start on Day 5 of Menstrual Cycle, 14 days/course * 3 courses Triptorelin: Once every 28 days * 6 courses	24 months	①⑤⑥
Wang L et al., 2019	116 (58/58)	T: 32.15 ± 3.18 C: 33.21 ± 3.68	CHM (*Xiaojin* capsules) +Leuprorelin	Leuprorelin	CHM: 1.5 g, bid, 4 weeks/course * 6 courses Leuprorelin: Once every 4 weeks * 6 courses	12 months	①②⑥
Song HP et al., 2019	160 (80/80)	T: 37.12 ± 2.59 C: 36.25 ± 3.72	CHM (*Kuntai* capsules) + Leuprorelin	Leuprorelin	CHM: 2 g, tid, 28 days/course * 3 courses Leuprorelin: Once every 28days * 3 courses	4 months	①③
Lu YH et al., 2019	86 (43/43)	T: 29.17 ± 3.28 C: 28.76 ± 3.92	CHM (*Guizhi Fuling* capsules) + Gestrinone	Gestrinone	CHM: 0.93 g, tid, 4 weeks/course * 6 courses Gestrinone: 2.5 mg, qd, twice a week, 4 weeks/course * 6 courses	6 months	②④⑥

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TABLE 1 (*Continued*) Basic characteristics of included trials and subjects.

References	Sample size (T/C)	Age (years)	Intervention	Control	Treatment duration	Follow-up duration	Outcome measures
Hu YY et al., 2018	90 (45/45)	T: 30.9 ± 5.54	CHM (*Dan'e Fukang* decocted extract) + Triptorelin	Triptorelin	CHM: 15 g, bid, start orally on Day 10 prior to menstruation, 14 days/course * 3 courses	6 months	①②③④⑥
		C: 30.20 ± 6.12			Triptorelin: Once every 28days * 3 courses		
Chen M et al., 2018	100 (50/50)	T: 31.6 ± 4.72	CHM (*Neiyi* decoction) + Triptorelin	Triptorelin	CHM: 1 dose/d (100 mL, bid), discontinue during menstruation, 4 weeks/course * 6 courses	9 months	②
		C: 30.90 ± 5.01			Triptorelin: Once every 4 weeks * 6 courses		
Zhou Q et al., 2016	106 (53/53)	T: 31.94 ± 2.80	CHM (*Fuzheng Xiaoyi* decoction) + Goserelin	Goserelin	CHM: 1 dose/d (100 mL, bid), 4 weeks/course * 3 courses	36 months	①②④⑥
		C: 32.64 ± 2.47			Goserelin: Once every 4 weeks * 6 courses		
Xing LM 2016	72 (36/36)	T: 29.64 ± 4.91	CHM (Empirical formula) + Mifepristone	Mifepristone	CHM: 1 dose/d (100 mL, bid), * 3 months	6 months	①③④⑥
		C: 30.56 ± 5.85			Mifepristone: 10 mg, qd, * 3 months		
Han B 2016	60 (30/30)	T: 30.35 ± 5.40	CHM (*Turtle Shell* decocted pills) + Leuprorelin	Leuprorelin	CHM: 3 g, tid, discontinue during menstruation, 28 days/course * 6 courses	6 months	①③④⑥
		C: 28.90 ± 5.49			Leuprorelin: Once every 28days * 6 courses		
Du X 2015	120 (60/60)	T: 31.72 ± 6.57	CHM (*Neiyi* decoction) + Triptorelin	Triptorelin	CHM:1 dose/d (200 mL, bid), start on Day 5 of Menstrual Cycle, 4 weeks/course * 6 courses	6 months	②④
		C: 33.76 ± 5.73			Triptorelin: Once every 4 weeks * 6 courses		
Chen LQ et al., 2015	48 (24/24)	T: 28.44 ± 2.37	CHM (*Xuefu Zhuyu* capsules) + Triptorelin	Triptorelin	CHM: 2.4 g, bid, 30 days/course * 3courses	3 months	①②③
		C: 27.49 ± 1.95			Triptorelin: Once every 28days * 5 courses		
Chen JJ 2015	72 (36/36)	T: 30.17 ± 4.08	CHM (*Bushen Huayu* decoction) + Mifepristone	Mifepristone	CHM: 1 dose/d (100 mL, bid), take for 1 week, then discontinue for 1 week, 4 weeks/course * 3 courses	6 months	①③④⑥
		C: 29.79 ± 4.15			Mifepristone: 10 mg, qd, 4 weeks/course * 3 courses		

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TABLE 1 (*Continued*) Basic characteristics of included trials and subjects.

References	Sample size (T/C)	Age (years)	Intervention	Control	Treatment duration	Follow-up duration	Outcome measures
Dou N 2015	70 (35/35)	T: 26.12 ± 4.21	CHM (*Danbie* capsules)	Gestrinone	CHM: 1.9 g, tid, discontinue during menstruation, 28 days/course * 6courses	12 months	①②④⑥
		C: 28.72 ± 6.14			Gestrinone: 2.5 mg, qd, twice a week, 4 weeks/course * 6 courses		
Li S 2014	60 (30/30)	T: 29.4 ± 5.30	CHM (Empirical formula)	Triptorelin	CHM: 1.5 dose/d (100 mL, bid), 10 days/course * 3 courses	12 months	①②⑤
		C: 28.9 ± 5.41			Triptorelin: Once every 28 days * 3 courses		
Zhou D et al., 2013	120 (60/60)	T: 34.1 ± 1.8	CHM (Empirical formula) + Mifepristone	Mifepristone	CHM: 1 dose/d (100 mL, qd), 4 weeks/course * 6 courses	6 months	①②⑥
		C: 34.5 ± 1.1			Mifepristone: 12.5 mg, qd, 4 weeks/course * 6 courses		
Zhang XN 2012	60 (30/30)	T: 29.70 ± 4.23	CHM (*Muda Tang* granules)	Gestrinone	CHM: 2 sachets, bid, discontinue during menstruation, 4 weeks/course * 3 courses	6 months	①③④⑥
		C: 29.87 ± 4.43			Gestrinone: 2.5 mg, qd, twice a week, 4 weeks/course * 3 courses		
Ding XQ et al., 2012	56 (27/29)	T: 35.84	CHM (Empirical formula)	Gestrinone	CHM: 1 dose/d (100 mL, tid), discontinue during menstruation, 4 weeks/course * 3 courses	36 months	②⑤⑥
		C: 34.54			Gestrinone: 2.5 mg, qd, twice a week, 4 weeks/course * 3 courses		
Ma L et al., 2011	60 (30/30)	T: 31.7 ± 4.79	CHM (*Xiaojie An* capsules) + Gestrinone	Gestrinone	CHM: 0.76 g, tid, 8 weeks/course * 3courses	36 months	1 ②
		C: 31.7 ± 4.79			Gestrinone: 2.5 mg, qd, twice a week, 8 weeks/course * 3 courses		

AbbreviationC: control; CHM: chinese herbal medicine; T: treatment; 'bid' (bis in die) means twice a day; 'qd' (quaque die) means once a day; 'tid' (ter in die) means three times a day. ①: Recurrence rate; ②: Total clinical efficacy rate; ③: Visual analog scale score; ④: Serum level of CA125; ⑤: Pregnancy rate; ⑥: Adverse events.

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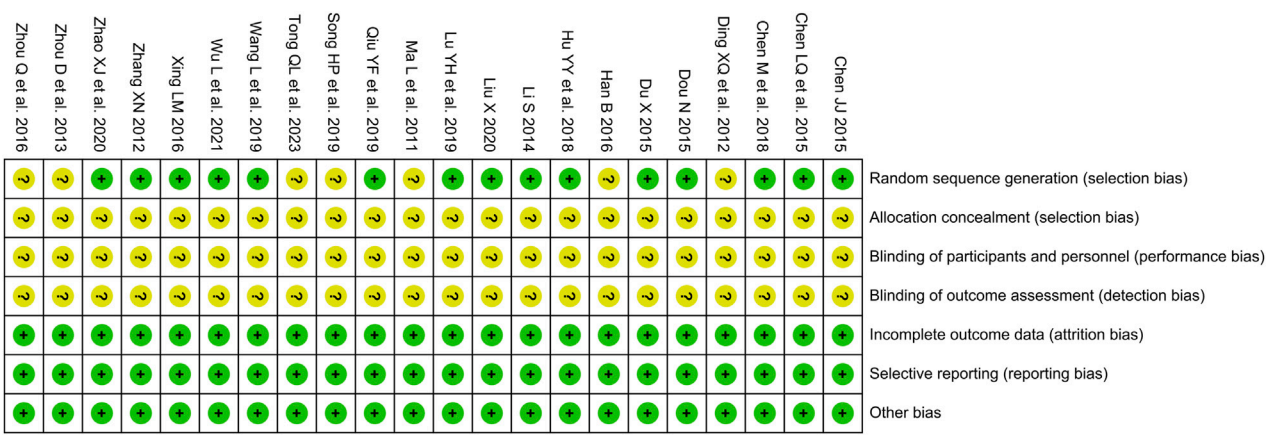

FIGURE 2
Risk of bias summary.

TABLE 2 Categorization of Chinese herbal medicine efficacies in the included studies.

Cat	Qty	Freq	Pct.(%)	Cat	Qty	Freq	Pct.(%)
Invigorate blood and dissolve stasis	21	81	34.32	Resolve toxin and dissipate binds	5	5	2.12
Supplement deficiency	23	61	25.85	Calm the liver and extinguish wind	2	5	2.12
Soothe the liver/Rectify qi	13	32	13.56	Promote digestion and resolve stagnation	2	4	1.69
Heat-clearing	11	14	5.93	Warm the interior	2	2	0.85
Dissolve phlegm	5	9	3.81	Promote astriction	2	2	0.85
Promote urination and percolate dampness	3	7	2.97	Dispel wind and overcome dampness	2	2	0.85
Release the exterior and dissipate cold	1	6	2.54	Open the orifices	1	1	0.42
Stanch bleeding	4	5	2.12				

Abbreviation: Cat.: category; CHM: chinese herbal medicine; Freq: Frequency; Pct.: percentage; Qty.: quantity.

3.5 Efficacy assessment

3.5.1 Recurrence rate

Current guidelines for diagnosing and treating endometriosis suggest that patients with postoperative recurrence of OEC may experience similar or exacerbated clinical symptoms (Gynecology and Obsterics, 2019; Association, 2021). Bimanual gynecological examinations have identified cystic masses in the bilateral adnexal regions and palpable nodules in the posterior vaginal fornix, which are often tender. Additionally, ultrasounds have detected cysts in the adnexal area (Gynecology and Obstetrics, 2019; Association, 2021; Becker et al., 2022). Of the 22 studies reviewed, 17 addressed OEC recurrence rates. Eight of these studies defined recurrence as the reappearance of ovarian endometriotic lesions via ultrasound examination (Zhang, 2012; Han, 2016; Zhou, 2016; Hu and Y, 2018; Wang and Bo, 2019; Liu, 2020; Zhao and Xiao, 2020; Tong et al., 2023). Seven studies defined OEC recurrence as the reappearance of both endometriotic lesions and clinical symptoms (Zhou and Liu, 2013; Li, 2014; Chen, 2015; Chen et al., 2015; Dou, 2015; Xing, 2016; Qiu and Wan, 2019). Meanwhile, two studies characterized OEC recurrence as either the recurrence of endometriotic lesions or clinical symptoms (Ma, 2011; Song et al., 2019). Recurrence rate was quantified as the ratio of recurrent cases to the total patient count (Ma, 2011; Zhang, 2012; Zhou and Liu, 2013; Li, 2014; Chen, 2015; Chen et al., 2015; Dou, 2015; Han, 2016; Xing, 2016; Zhou, 2016; Hu and Y, 2018; Qiu and Wan, 2019; Song et al., 2019; Wang and Bo, 2019; Liu, 2020; Zhao and Xiao, 2020; Tong et al., 2023).

3.5.1.1 CHM alone vs blank control

In a comparative analysis of CHM *versus* a blank control, one study assessed the recurrence rates of OEC in groups of 63 patients each (Tong et al., 2023). Recurrence was identified by the reappearance of endometriotic lesions. The result demonstrated that CHM was superior to blank control in decreasing the recurrence rate (1 trial, n = 126; OR = 0.25; 95% CI: 0.10–0.64; p = 0.004; Figure 3A).

3.5.1.2 CHM alone vs CWM

The comparative impact of CHM *versus* CWM on recurrence rates was assessed across three studies (Zhang, 2012; Li, 2014; Dou, 2015). As shown in Figures 2A,B fixed-effect model was used due to no obvious heterogeneity observed. Meta-analysis showed that the overall recurrence rate was lower with CHM alone than with CWM (3 trials, n = 190; OR = 0.26; 95% CI: 0.11–0.65; I^2 = 0%, p = 0.004; Figure 3B). Furthermore, in two of these studies, recurrence was

TABLE 3 Frequency of Chinese herbal medicine Utilization in the Included Studies.

Full name	Chinese name	Freq	Pct. (%)	Full name	Chinese name	Freq	Pct. (%)	Full name	Chinese name	Freq	Pct. (%)
Prunus persica (L.) Batsch [Rosaceae; Persicae Semen]	Taoren	10	4.24	*Trionyx sinensis* Wiegmann [Trionychidae; Trionycis Carapax]	Biejia	2	0.85	*Liquidambar formosana* Hance [Altingiaceae; Liquidambaris Resina]	Fengxiangzhi	1	0.42
Angelica sinensis (Oliv.) Diels [Apiaceae; Angelicae Sinensis Radix]	Danggui	9	3.81	*Equus asinus* L. [Equidae; Asini Corii Colla]	Ejiao	2	0.85	*Boswellia sacra* Flück. [Burseraceae; Olibanum]	Ruxiang	1	0.42
Salvia miltiorrhiza Bunge [Lamiaceae; Salviae Miltiorrhizae Radix et Rhizoma]	Danshen	9	3.81	*Lycium barbarum* L. [Solanaceae; Lycii Fructus]	Gouqi	2	0.85	*Campsis grandiflora* (Thunb.) K.Schum. [Bignoniaceae; Campsis Flos]	Lingxiaohua	1	0.42
Paeonia lactiflora Pall. [Paeoniaceae; Paeoniae Radix Rubra]	Chishao	8	3.39	*Cervi Cornu Degelatinatum*	Lujiaoshuang	2	0.85	*Achyranthes bidentata* Blume [Amaranthaceae; Achyranthis Bidentatae Radix]	Niuxi	1	0.42
Corydalis yanhusuo (Y.H.Chou & Chun C.Hsu) W.T.Wang ex Z.Y.Su and C.Y.Wu [Papaveraceae; Corydalis Rhizoma]	Yanhusuo	7	2.97	*Panax notoginseng* (Burkill) F.H.Chen [Araliaceae; Notoginseng Radix et Rhizoma]	Sanqi	2	0.85	*Moschus berezovskii* Flerov [Moschidae; Moschus]	Shexiang	1	0.42
Neolitsea cassia (L.) Kosterm. [Lauraceae; Cinnamomi Ramulus]	Guizhi	6	2.54	*Carthamus tinctorius* L. [Asteraceae; Carthami Flos]	Honghua	2	0.85	*Citrus reticulata* Blanco [Rutaceae; Citri Reticulatae Pericarpium]	Chenpi	1	0.42
Sparganium stoloniferum (Buch.-Ham. ex Graebn.) Buch.-Ham. ex Juz. [Typhaceae; Sparganii Rhizoma]	Sanleng	6	2.54	*Calamus draco* Willd. [Arecaceae; Draconis Sanguis]	Xuejie	2	0.85	*Magnolia officinalis* Rehder &E.H.Wilson [Magnoliaceae; Magnoliae Officinalis Cortex]	Houpo	1	0.42
Curcuma longa L. [Zingiberaceae; Curcumae Rhizoma]	Ezhu	6	2.54	*Lindera aggregata* (Sims) Kosterm. [Lauraceae; Linderae Radix]	Wuyao	2	0.85	*Citrus reticulata* Blanco [Rutaceae; Citri Reticulatae Pericarpium Viride]	Qingpi	1	0.42
Paeonia × *suffruticosa* Andrews [Paeoniaceae; Moutan Cortex]	Mudanpi	6	2.54	*Citrus* × *aurantium f. aurantium* [Rutaceae; Aurantii Fructus]	Zhiqiao	2	0.85	*Citrus reticulata* Blanco [Rutaceae; Citri Reticulatae Semen]	Juhe	1	0.42
Cyperus rotundus L. [Cyperaceae; Cyperi Rhizoma]	Xiangfu	6	2.54	*Smilax glabra* Roxb. [Smilacaceae; Smilacis Glabrae Rhizoma]	Tufuling	2	0.85	*Melia azedarach* L. [Meliaceae; Toosendan Fructus]	Chuanlianzi	1	0.42
Cuscuta chinensis Lam. [Convolvulaceae; Cuscutae Semen]	Tusizi	5	2.12	*Scutellaria baicalensis* Georgi [Lamiaceae; Scutellariae Radix]	Huangqin	2	0.85	*Dolomiaea costus* (Falc.) Kasana and A.K.Pandey [Asteraceae; Aucklandiae Radix]	Muxiang	1	0.42
Glycyrrhiza glabra L. [Fabaceae; Glycyrrhizae Radix et Rhizoma]	Gancao	5	2.12	*Rheum officinale* Baill. [Polygonaceae; Rhei Radix et Rhizoma]	Dahuang	2	0.85	*Platycodon grandiflorus* (Jacq.) A.DC. [Campanulaceae; Platycodonis Radix]	Jiegeng	1	0.42
Conioselinum anthriscoides 'Chuanxiong' [Apiaceae; Chuanxiong Rhizoma]	Chuanxiong	5	2.12	*Prunus mume* (Siebold) Siebold & Zucc. [Rosaceae; Mume Fructus]	Wumei	2	0.85	*Pyrrosia lingua* (Thunb.) Farw. [Polypodiaceae; Pyrrosiae Folium]	Shiwei	1	0.42

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TABLE 3 (*Continued*) Frequency of Chinese herbal medicine Utilization in the Included Studies.

Full name	Chinese name	Freq	Pct. (%)	Full name	Chinese name	Freq	Pct. (%)	Full name	Chinese name	Freq	Pct. (%)
Bupleurum chinense DC. [Apiaceae; Bupleuri Radix]	Chaihu	5	2.12	*Gallus gallus domesticus* Brisson [Phasianidae; Galli Gigerii Endothelium Corneum]	Jineijin	2	0.85	*Dianthus chinensis* L. [Caryophyllaceae; Dianthi Herba]	Qumai	1	0.42
Carapichea ipecacuanha (Brot.) L.Andersson [Rubiaceae; Poria]	Fuling	5	2.12	*Crataegus monogyna* Jacq. [Rosaceae; Crataegi Fructus]	Shanzha	2	0.85	Fossilia Ossis Mastodi	Longgu	1	0.42
Dipsacus asper Wall. Ex DC. [Caprifoliaceae; Dipsaci Radix]	Xuduan	4	1.69	*Typha angustifolia* L. [Typhaceae; Typhae Pollen]	Puhuang	2	0.85	*Pheretima aspergillum* (E.Perrier) [Pheretimidae; Pheretima]	Dilong	1	0.42
Paeonia lactiflora Pall. [Paeoniaceae; Paeoniae Radix Alba]	Baishao	4	1.69	*Cibotium barometz* (L.) J.Sm. [Cibotiaceae; Cibotii Rhizoma]	Gouji	1	0.42	*Abutilon indicum* (L.) Sweet [Malvaceae]	Mopancao	1	0.42
Astragalus mongholicus Bunge [Fabaceae; Astragali Radix]	Huangqi	4	1.69	*Rubus chingii* Hu [Rosaceae; Rubi Fructus]	Fupenzi	1	0.42	*Coptis chinensis* Franch. [Ranunculaceae; Coptidis Rhizoma]	Huanglian	1	0.42
Eupolyphaga sinensis Walker [Corydiidae; Eupolyphaga Steleophaga]	Tubiechong	4	1.69	*Cornus officinalis* Siebold & Zucc. [Cornaceae; Corni Fructus]	Shanzhuyu	1	0.42	*Iris domestica* (L.) Goldblatt & Mabb. [Iridaceae; Belamcandae Rhizoma]	Shegan	1	0.42
Trogopterori Faeces	Wulingzhi	4	1.69	*Ganoderma lucidum* (Curtis) P.Karst.[Polyporaceae; Ganoderma]	Lingzhi	1	0.42	*Lobelia chinensis* Lour. [Campanulaceae; Lobeliae Chinensis Herba]	Banbianlian	1	0.42
Gynochthodes officinalis (F.C.How) Razafim. and B.Bremer [Rubiaceae; Morindae Officinalis Radix]	Bajitian	3	1.27	*Rhodiola crenulata* (Hook.f. and Thomson) H.Ohba [Crassulaceae; Rhodiolae Crenulatae Radix et Rhizoma]	Hongjingtian	1	0.42	*Scutellaria barbata* D.Don [Lamiaceae; Scutellariae Barbatae Herba]	Banzhilian	1	0.42
Rehmannia glutinosa (Gaertn.) DC. [Orobanchaceae; Rehmanniae Radix Praeparata]	Shudi	3	1.27	*Atractylodes macrocephala* Koidz. [Asteraceae; Atractylodis Macrocephalae Rhizoma]	Baizhu	1	0.42	*Berberis bealei* Fortune [Berberidaceae; Mahoniae Caulis]	Gonglaomu	1	0.42
Eucommia ulmoides Oliv. [Eucommiaceae; Eucommiae Cortex]	Duzhong	3	1.27	*Cnidium monnieri* (L.) Cusson [Apiaceae; Cnidii Fructus]	Shechuangzi	1	0.42	*Melicope pteleifolia* (Champ. ex Benth.) T.G.Hartley [Rutaceae]	Sanchaku	1	0.42
Epimedium sagittatum (Siebold & Zucc.) Maxim. [Berberidaceae; Epimedii Folium]	Yinyanghuo	3	1.27	*Codonopsis pilosula* (Franch.) Nannf. [Campanulaceae; Codonopsis Radix]	Dangshen	1	0.42	*Forsythia suspensa* (Thunb.) Vahl [Oleaceae; Forsythiae Fructus]	Lianqiao	1	0.42
Sargassum pallidum (Turn.) C.Ag. [Sargassaceae; Sargassum]	Haizao	3	1.27	*Bolbostemma paniculatum* (Maxim.) Franquet [Cucurbitaceae; Bolbostematis Rhizoma]	Tubeimu	1	0.42	*Liquidambar formosana* Hance [Altingiaceae; Liquidambaris Fructus]	Lulutong	1	0.42
Fritillaria thunbergii Miq. [Liliaceae; Fritillariae Thunbergii Bulbus]	Zhebeimu	3	1.27	*Momordica cochinchinensis* (Lour.) Spreng. [Cucurbitaceae; Momordicae Semen]	Mubiezi	1	0.42	*Aconitum kusnezoffii* Rchb.[Ranunculaceae; Aconiti Kusnezoffii Radix Cocta]	Zhicaowu	1	0.42

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TABLE 3 (*Continued*) Frequency of Chinese herbal medicine Utilization in the Included Studies.

Full name	Chinese name	Freq	Pct. (%)	Full name	Chinese name	Freq	Pct. (%)	Full name	Chinese name	Freq	Pct. (%)
Curcuma longa L. [Zingiberaceae; Curcumae Radix]	Yujin	3	1.27	*Polistes olivaceous* (DeGeer) [Eumenidae; Vespae Nidus]	Fengfang	1	0.42	*Zingiber officinale* Roscoe [Zingiberaceae; Zingiberis Rhizoma]	Ganjiang	1	0.42
Leonurus japonicus Houtt. [Lamiaceae; Leonuri Herba]	Yimucao	3	1.27	*Catharsius molossus* Linnaeus [Scarabaeidae]	Qianglang	1	0.42	*Foeniculum vulgare* Mill. [Apiaceae; Foeniculi Fructus]	Xiaohuixiang	1	0.42
Spatholobus suberectus Dunn [Fabaceae; Spatholobi Caulis]	Jixueteng	3	1.27	Nitrum	Xiaoshi	1	0.42	Atramentum	Xiangmo	1	0.42
Commiphora myrrha (T.Nees) Engl. [Burseraceae; Myrrha]	Moyao	3	1.27	*Pinellia ternata* (Thunb.) Makino [Araceae; Pinelliae Rhizoma]	Banxia	1	0.42	*Cirsium japonicum* DC. [Asteraceae; Cirsii Japonici Herba]	Daji	1	0.42
Litchi chinensis Sonn. [Sapindaceae; Litchi Semen]	Lizhihe	3	1.27	*Descurainia sophia* (L.) Webb ex Prantl [Brassicaceae; Descurainiae Semen]	Tinglizi	1	0.42	*Cirsium arvense* var. *arvense* [Asteraceae; Cirsii Herba]	Xiaoji	1	0.42
Ostrea gigas Thunberg [Ostreidae; Ostreae Concha]	Muli	3	1.27	*Gleditsia sinensis* Lam. [Fabaceae; Gleditsiae Spina]	Zaojiaoci	1	0.42				
Taxillus chinensis (DC.) Danser [Loranthaceae; Taxilli Herba]	Sangjisheng	2	0.85	*Euonymus alatus* (Thunb.) Siebold [Celastraceae]	Guijianyu	1	0.42				

Abbreviation: Freq: Frequency; Pct.: percentage.

specifically defined as the reappearance of both endometriotic lesions and clinical symptoms (Li, 2014; Dou, 2015), showing the CHM group to have a significantly lower postoperative recurrence rate than the CWM group (2 trials, n = 130; OR = 0.20; 95% CI: 0.07–0.58; I^2 = 0%, p = 0.003; Figure 3B). Conversely, one study identified recurrence solely based on the return of endometriotic lesions (Zhang, 2012), with the analysis revealing no significant difference in efficacy between CHM and CWM in reducing recurrence rates (1 trial, n = 60; OR = 0.64; 95% CI: 0.10–4.15; p = 0.64; Figure 3B).

3.5.1.3 CHM + CWM vs CWM

Thirteen studies evaluated the effectiveness of combining CHM with CWM in reducing the recurrence rate of OEC (Ma, 2011; Zhou and Liu, 2013; Chen, 2015; Chen et al., 2015; Han, 2016; Xing, 2016; Zhou, 2016; Hu and Y, 2018; Qiu and Wan, 2019; Song et al., 2019; Wang and Bo, 2019; Liu, 2020; Zhao and Xiao, 2020). These trials included a total of 1,132 patients, evenly split between the combination treatment and CWM-only groups. The absence of heterogeneity across the studies supported a uniform analysis. The results indicate that the combination of CHM with CWM significantly reduced the overall postoperative recurrence rate of OEC (13 trials, n = 1,132; OR = 0.26; 95% CI: 0.17–0.40; I^2 = 0%, p < 0.00001; Figure 3C). The analysis further categorized the studies into three groups based on their recurrence definitions, demonstrating the combination therapy's significant advantage in reducing recurrence rates. The benefits were evident in (a) reducing the recurrence of endometriotic lesions alone (Han, 2016; Zhou, 2016; Hu and Y, 2018; Wang and Bo, 2019; Liu, 2020; Zhao and Xiao, 2020) (6 trials, n = 548; OR = 0.24; 95% CI: 0.13–0.44; I^2 = 0%, p < 0.00001; Figure 3C), (b) reducing the recurrence of both endometriotic lesions and clinical symptoms (Zhou and Liu, 2013; Chen, 2015; Chen et al., 2015; Xing, 2016; Qiu and Wan, 2019) (5 trials, n = 364; OR = 0.31; 95% CI: 0.15–0.64; I^2 = 0%, p = 0.002; Figure 3C), and (c) reducing the recurrence of either endometriotic lesions or clinical symptoms (Ma, 2011; Song et al., 2019) (2 trials, n = 220; OR = 0.25; 95% CI: 0.10–0.64; I^2 = 11%, p = 0.004; Figure 3C).

3.5.2 Total clinical efficacy rate

The total clinical efficacy rate was evaluated in sixteen studies (Ma, 2011; Ding and Shi, 2012; Zhou and Liu, 2013; Li, 2014; Chen et al., 2015; Dou, 2015; Du, 2015; Zhou, 2016; Chen, 2018; Hu and Y, 2018; Lu et al., 2019; Song et al., 2019; Wang and Bo, 2019; Zhao and Xiao, 2020; Wu and Deng, 2021; Tong et al., 2023). Eleven of these studies (Ma, 2011; Zhou and Liu, 2013; Chen et al., 2015; Dou, 2015; Zhou, 2016; Chen, 2018; Hu and Y, 2018; Song et al., 2019; Wang and Bo, 2019; Zhao and Xiao, 2020; Wu and Deng, 2021) defined efficacy as follows: (a) significantly effective: complete lesion resolution and symptom relief; (b) effective: lesion size reduction and symptom alleviation; (c) ineffective: no symptom improvement or exacerbation, along with OEC recurrence. Furthermore, five studies (Ding and Shi, 2012; Li, 2014; Du, 2015; Lu et al., 2019; Tong et al., 2023) classified efficacy into four categories: (a) cured: total lesion disappearance and symptom resolution; (b) significantly effective: symptom resolution and lesion size reduction; (c) effective: symptom alleviation without notable lesion size change; (d) ineffective: no improvement or symptom exacerbation, alongside

TABLE 4 Analysis of Chinese herbal medicine natures, flavors, and channel entries in the included studies.

Medicinal nature	Freq	Pct.(%)	Medicinal flavor	Freq	Pct.(%)	Channel entry	Freq	Pct.(%)
Warm	92	38.98	Bitter	132	33.93	the foot *jueyin* liver channel	181	30.27
Cold	79	33.47	Sweet	111	28.53	the hand *shaoyin* heart channel	93	15.55
Neutral	63	26.69	Acrid	103	26.48	the foot *taiyin* spleen channel	88	14.72
Hot	2	0.85	Salty	23	5.91	the foot *shaoyin* kidney channel	80	13.38
			Sour	12	3.08	the hand *taiyin* lung channel	49	8.19
			Astringent	8	2.06	the foot *yangming* stomach channel	36	6.02
						the hand *yangming* large intestine channel	22	3.68
						the foot *taiyang* bladder channel	19	3.18
						the foot *shaoyang* gallbladder channel	17	2.84
						the hand *taiyang* small intestine channel	6	1.00
						the hand *jueyin* pericardium channel	4	0.67
						the hand *shaoyang* san jiao channel	3	0.50

Abbreviation Freq: Frequency; Pct.: percentage.

OEC recurrence. Despite slight variations in the efficacy evaluation criteria across these studies, the definition of "ineffective" remains consistent. Furthermore, all studies employ a uniform method to calculate the total clinical efficacy rate, defined as the proportion of effective cases (calculated by subtracting the number of ineffective cases from the total patient count) to the overall patient population, facilitating meta-analysis.

3.5.2.1 CHM alone vs blank control

A single study evaluated the impact of CHM alone compared to a blank control on the overall clinical efficacy rate (Tong et al., 2023). The result indicated that CHM outperformed the blank control in improving clinical efficacy (1 trial, n = 126; OR = 4.23; 95% CI: 1.12–15.99; p = 0.03; Figure 4A).

3.5.2.2 CHM alone vs CWM

The comparative efficacy of CHM alone and CWM on the overall clinical efficacy rate was assessed in three studies (Ding and SHI, 2012; Li, 2014; Dou, 2015). With no notable heterogeneity detected between the studies, a fixed-effects model was applied. Meta-analysis revealed that CHM achieved a higher clinical efficacy rate than CWM (3 trials, n = 186; OR = 2.94; 95% CI: 1.34–6.43; I^2 = 0%, p = 0.007; Figure 4B).

3.5.2.3 CHM + CWM vs CWM

A meta-analysis of twelve studies (Ma, 2011; Zhou and Liu, 2013; Chen et al., 2015; Du, 2015; Zhou, 2016; Chen, 2018; Hu and Y, 2018; Lu et al., 2019; Wang and Bo, 2019; Zhao and Xiao, 2020; Wu and Deng, 2021) evaluated the combined effect of CHM and CWM *versus* CWM alone on the total clinical efficacy rate. The analysis included 614 patients in each of the combination and CWM-only groups, with no significant heterogeneity detected across the studies. The findings demonstrated that the combination of CHM and CWM significantly enhanced the overall clinical efficacy rate compared to CWM alone (12 trials, n = 1,228; OR = 3.44; 95% CI: 2.37–5.00; I^2 = 0%, p < 0.00001; Figure 4C).

3.5.3 VAS score

3.5.3.1 CHM alone vs blank control

One study reported the effect of CHM alone *versus* a blank control on VAS scores, involving 126 patients (Tong et al., 2023). The result indicated that CHM could ameliorate postoperative pain symptoms in patients with OEC (1 trial, n = 126; MD = −0.86; 95% CI: -1.01 to −0.71; p < 0.00001; Figure 5A).

3.5.3.2 CHM alone vs CWM

The effect of CHM alone *versus* CWM on VAS score was evaluated in 1 trial involving 60 patients (Zhang, 2012). The result showed that there was no significant difference between CHM and CWM on VAS score (1 trial, n = 60; MD = −0.16; 95% CI: -0.49 to 0.17; p = 0.34; Figure 5B).

3.5.3.3 CHM + CWM vs CWM

Eight studies compared the variation in VAS scores between intervention and control groups (Chen, 2015; Chen et al., 2015; Han, 2016; Xing, 2016; Hu and Y, 2018; Song et al., 2019; Zhao and Xiao, 2020; Wu and Deng, 2021). There were 354 patients in the combination group and 354 patients in the CWM group. As depicted in Figure 5C, meta-analysis using a random-model suggested that CHM combined with CWM remarkably reduced the VAS score compared to CWM alone (8 trials, n = 708; MD = −0.87; 95% CI: -1.10 to −0.65; I^2 = 91%, p < 0.00001; Figure 5C).

3.5.4 Serum level of CA125

3.5.4.1 CHM alone vs CWM

The efficacy of CHM alone compared to CWM on serum level of CA125 was assessed in two studies involving 130 patients (Zhang, 2012; Dou, 2015). Meta-analysis revealed that the serum CA125 level was lower with CHM treatment than with CWM (2 trials, n = 130; MD = −11.08; 95% CI: -21.75 to −0.42; I^2 = 88%, p = 0.04; Figure 6A).

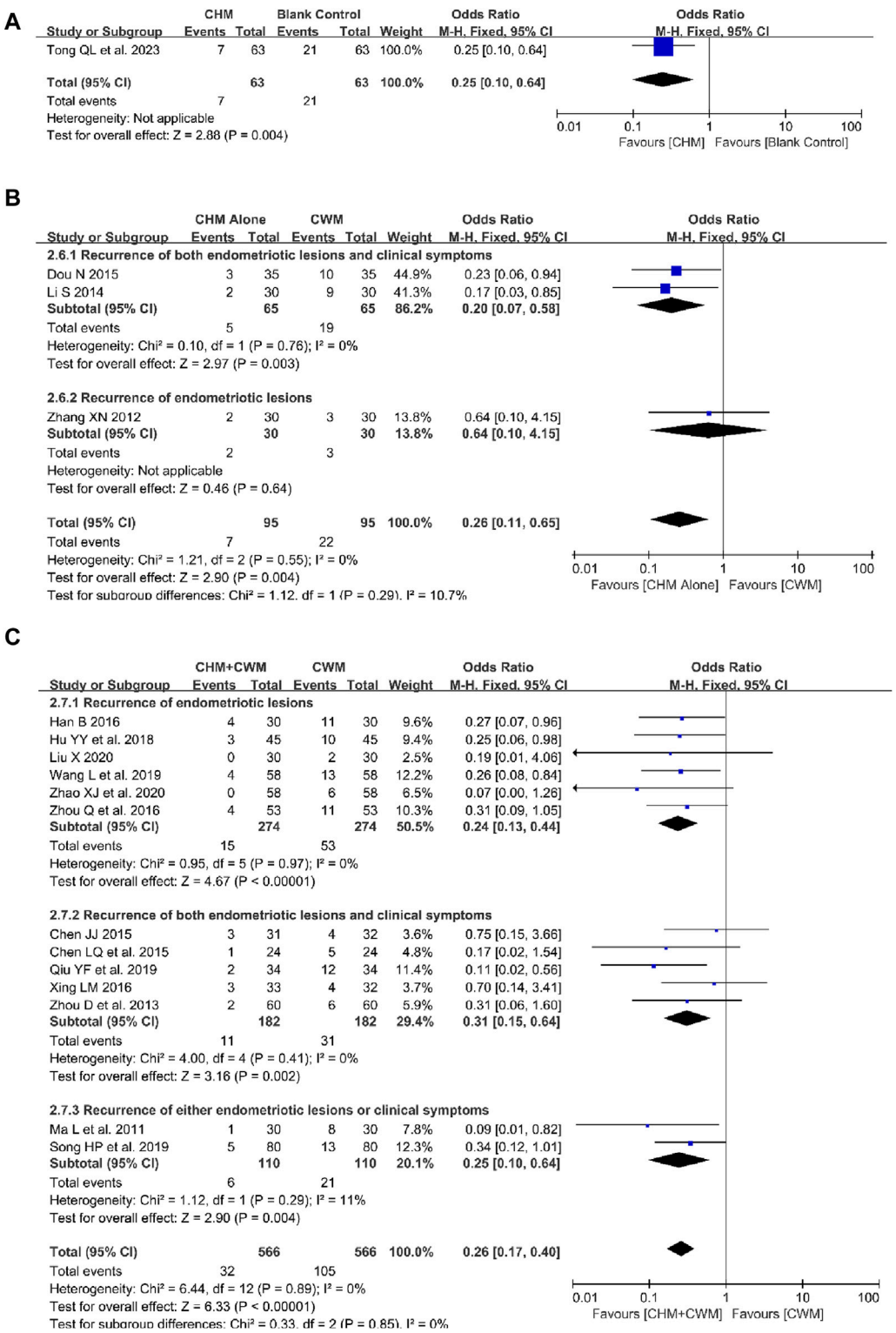

FIGURE 3
Forest plot illustrating the effect on recurrence rate of different intervention modes of CHM: **(A)** CHM Alone vs Blank Control, **(B)** CHM Alone vs CWM, **(C)** CHM + CWM vs CWM.

3.5.4.2 CHM + CWM vs CWM

Nine studies evaluated the effect of CHM combined with CWM on serum level of CA125 (Chen, 2015; Du, 2015; Han, 2016; Xing, 2016; Zhou, 2016; Hu and Y, 2018; Lu et al., 2019; Liu, 2020; Zhao and Xiao, 2020). Each group, the combined treatment and CWM alone, included 383 patients. A meta-

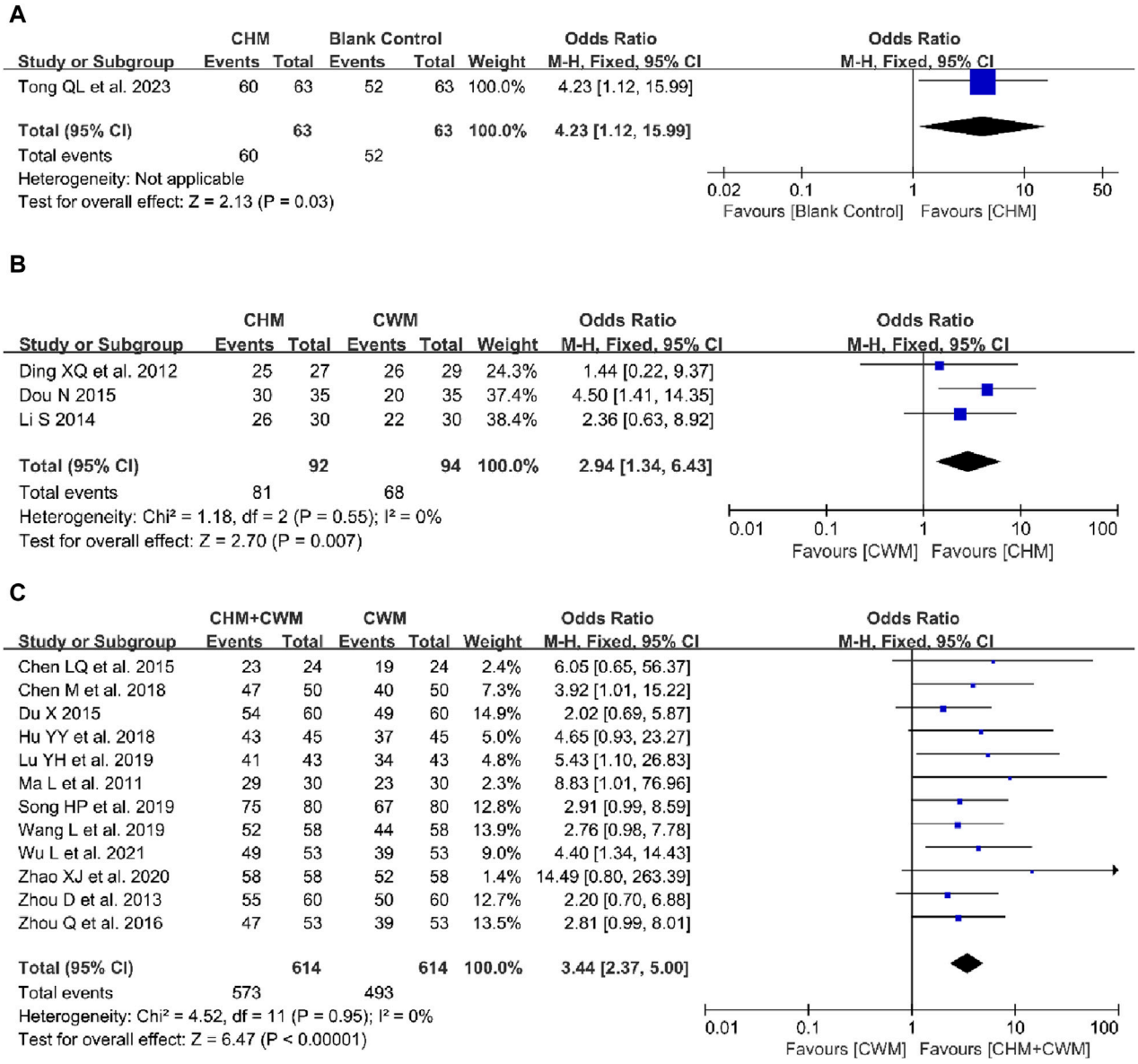

FIGURE 4
Forest plot illustrating the effect on total clinical efficacy rate of different intervention modes of CHM: **(A)** CHM Alone vs Blank Control, **(B)** CHM Alone vs CWM, **(C)** CHM + CWM vs CWM.

analysis employing a random-effects model demonstrated a significant reduction in serum CA125 levels with the CHM and CWM combination (9 trials, n = 766; MD = −5.31; 95% CI: -7.27 to −3.36; I^2 = 86%, p < 0.00001; Figure 6B).

3.5.5 Pregnancy rate

3.5.5.1 CHM alone vs blank control

One study reported the effect of CHM alone compared to a blank control on pregnancy rate, involving 126 patients (Tong et al., 2023). The result showed that CHM could increase the pregnancy rate in postoperative patients with OEC (1 trial, n = 126; OR = 3.95; 95% CI: 1.60–9.74; p = 0.003; Figure 7A).

3.5.5.2 CHM alone vs CWM

As shown in Figure 7B, we applied a fixed-effect model because no obvious heterogeneity was observed. The result suggested that the postoperative pregnancy rate with CHM was higher than with CWM (Ding and Shi, 2012; Li, 2014) (2 trials, n = 116; OR = 3.31; 95% CI: 1.40–7.83; I^2 = 0%, p = 0.007; Figure 7B).

3.5.5.3 CHM + CWM vs CWM

Three studies evaluated the impact of combining CHM with CWM on the pregnancy rate (Qiu and Wan, 2019; Liu, 2020; Zhao and Xiao, 2020). The analysis included 122 patients in each of the combination and CWM-only groups, with no significant heterogeneity across the studies. The combined treatment of CHM and CWM was found to significantly enhance the

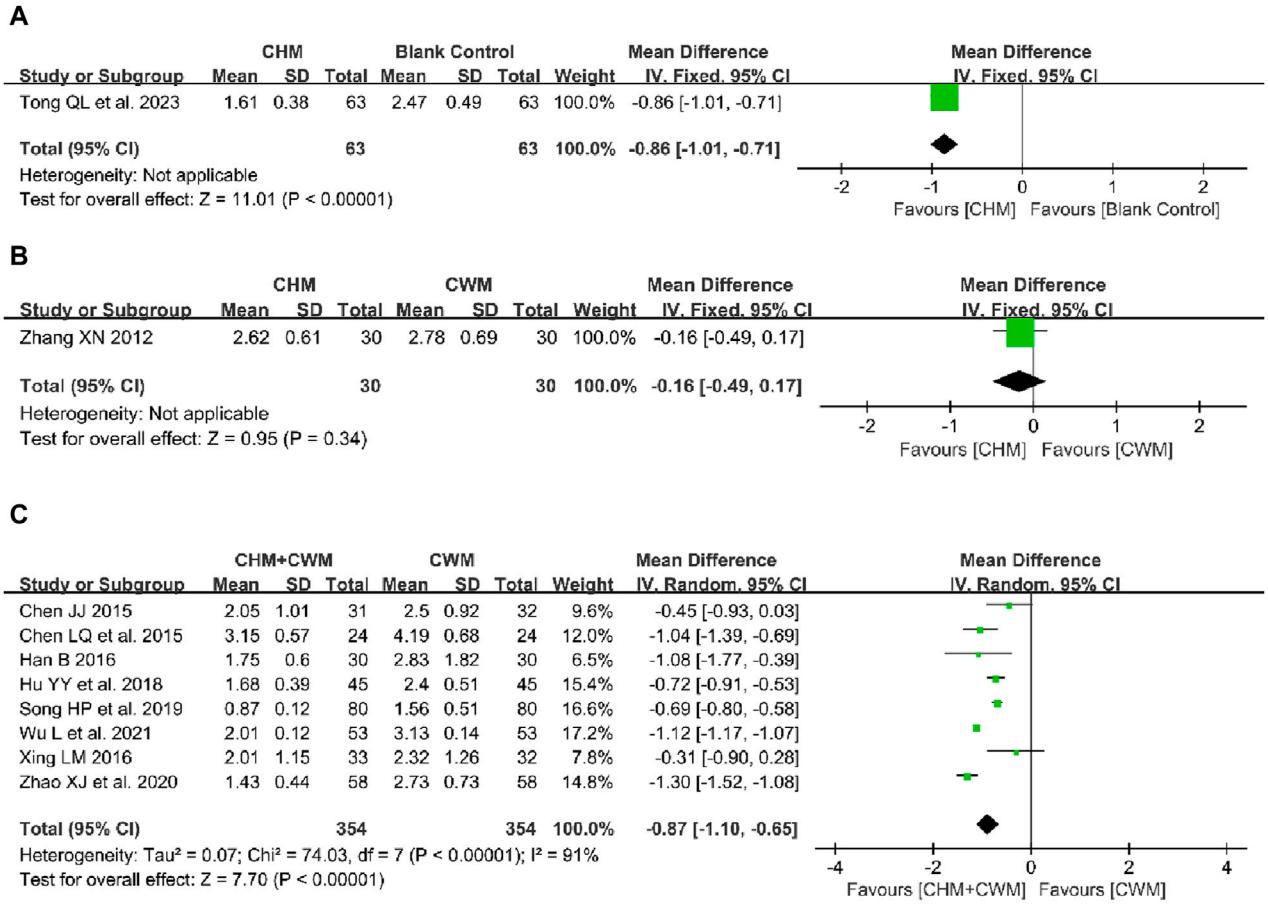

FIGURE 5
Forest plot illustrating the effect on VAS score of different intervention modes of CHM: **(A)** CHM Alone vs Blank Control, **(B)** CHM Alone vs CWM, **(C)** CHM + CWM vs CWM.

pregnancy rate (3 trials, n = 244; OR = 2.99; 95% CI: 1.28–6.98; I^2 = 0%, p = 0.01; Figure 7C).

3.5.6 Adverse events

In this review, adverse events were reported in 14 of 22 studies (63.64%) (Ding and Shi, 2012; Zhang, 2012; Zhou and Liu, 2013; Chen, 2015; Dou, 2015; Du, 2015; Han, 2016; Xing, 2016; Zhou, 2016; Hu and Y, 2018; Lu et al., 2019; Qiu and Wan, 2019; Wang and Bo, 2019; Liu, 2020). Within these, two trials (Lu et al., 2019; Wang and Bo, 2019) found no adverse effects in either the CHM or CWM groups. The remaining 12 studies documented various adverse effects, including perimenopausal symptoms (such as amenorrhea, hot flashes, irritability, decreased libido, insomnia, and irregular vaginal bleeding), androgenic response (acne, weight gain, breast reduction, edema), gastrointestinal discomfort (nausea, vomiting), physical pain (headache, breast pain, limb joint pain, muscle pain), allergic reactions (itching of the skin, urticaria, rash), and hepatic function impairment.

3.5.6.1 CHM alone vs CWM

Three studies (Ding and Shi, 2012; Zhang, 2012; Dou, 2015) encompassing 186 patients reported adverse effects in the CHM alone and the CWM group, displaying no heterogeneity across the studies. The incidence of adverse reactions was significantly lower in the CHM group compared to the CWM group (OR = 0.05; 95% CI: 0.01–0.25; I^2 = 0%, p = 0.0003; Figure 8A). Notably, adverse events such as perimenopausal symptoms (Ding and Shi, 2012; Zhang, 2012; Dou, 2015), androgenic reactions (Ding and Shi, 2012), and hepatic impairment (Ding and Shi, 2012; Zhang, 2012) were documented. Meta-analysis, as illustrated in Table 5, revealed a reduced incidence of perimenopausal symptoms (3 trials, n = 186; OR = 0.09; 95% CI: 0.02–0.50; I^2 = 0%, p = 0.004) and hepatic impairment (2 trials, n = 116; OR = 0.11; 95% CI: 0.01–0.94; I^2 = 0%, p = 0.04) in the CHM group compared to the CWM group. However, the difference in androgenic responses was not statistically significant (1 trial, n = 56; OR = 0.14; 95% CI: 0.01–2.80; p = 0.31).

3.5.6.2 CHM + CWM vs CWM

Eleven studies investigated adverse reactions in groups receiving either a combination of CHM with CWM or CWM alone (Zhou and Liu, 2013; Chen, 2015; Du, 2015; Han, 2016; Xing, 2016; Zhou, 2016; Hu and Y, 2018; Lu et al., 2019; Qiu and Wan, 2019; Wang and Bo, 2019; Liu 2020). A fixed-effects model meta-analysis, depicted in Figure 8B, showed that the combination therapy significantly reduced the overall incidence of adverse events compared to

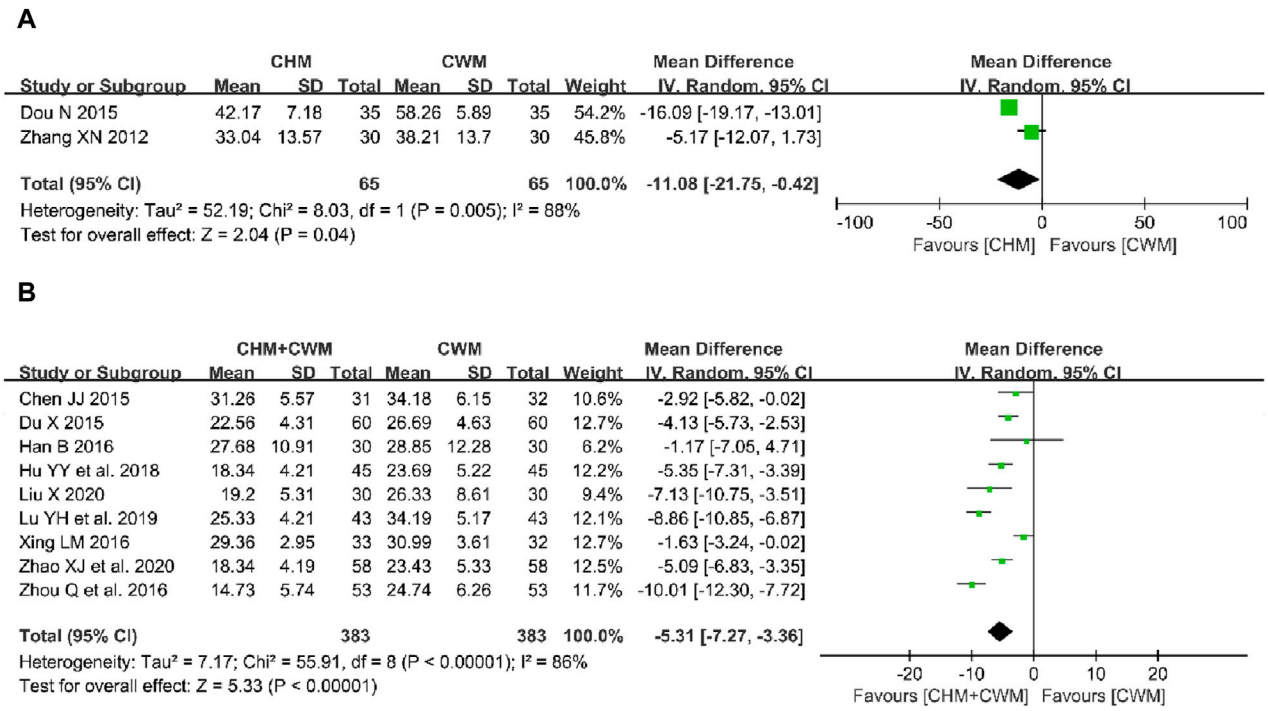

FIGURE 6
Forest plot illustrating the effect on serum level of CA125 of different intervention modes of CHM: **(A)** CHM Alone vs CWM, **(B)** CHM + CWM vs CWM.

CWM alone (OR = 0.37; 95% CI: 0.26–0.55; I^2 = 29%, p < 0.00001). Specific adverse reactions reported included perimenopausal symptoms (Chen, 2015; Du, 2015; Han, 2016; Xing, 2016; Zhou, 2016; Hu and Y, 2018; Qiu and Wan, 2019; Liu, 2020), androgenic response (Zhou and Liu, 2013; Du, 2015; Zhou, 2016), gastrointestinal discomfort (Zhou and Liu, 2013; Du, 2015; Zhou, 2016; Hu and Y, 2018; Qiu and Wan, 2019), physical pain (Du, 2015; Hu and Y, 2018; Qiu and Wan, 2019), allergic reactions (Du, 2015; Hu and Y, 2018), and hepatic function impairment (Zhou and Liu, 2013; Chen, 2015; Xing, 2016; Liu, 2020). Further analysis presented in Table 5 revealed that patients in the combined therapy group experienced fewer perimenopausal symptoms (8 trials, n = 632; OR = 0.36; 95% CI: 0.21–0.61; I^2 = 0%, p = 0.0001) and hepatic function impairment (4 trials, n = 308; OR = 0.39; 95% CI: 0.17–0.92; I^2 = 0%, p = 0.03) than those receiving CWM only. However, no significant differences were observed in androgenic responses (3 trials, n = 346; OR = 0.58; 95% CI: 0.20–1.65; I^2 = 0%, p = 0.31), gastrointestinal discomfort (5 trials, n = 504; OR = 0.75; 95% CI: 0.36–1.58; I^2 = 0%, p = 0.45), physical pain (3 trials, n = 278; OR = 0.63; 95% CI: 0.16–2.45; I^2 = 0%, p = 0.50), and allergic reactions (2 trials, n = 210; OR = 0.37; 95% CI: 0.08–1.63; I^2 = 0%, p = 0.19) between the two groups.

3.6 Subgroup analysis and sensitivity analysis

3.6.1 Recurrence rate

Based on the follow-up duration, we conducted a subgroup analysis on the postoperative recurrence rates in patients with OEC. As illustrated in Figure 9A, the recurrence rate in the group treated with CHM was lower than that in the CWM group at 12 months postoperatively (Li, 2014; Dou, 2015) (2 trials, n = 130; OR = 0.20; 95% CI: 0.07–0.58; I^2 = 0%, p = 0.003; Figure 9A), with no significant difference observed at 6 months postoperatively (Zhang, 2012) (1 trial, n = 60; OR = 0.64; 95% CI: 0.10–4.15; p = 0.64; Figure 9A). Meta-analysis revealed that patients receiving combined CHM and CWM treatment had significantly lower recurrence rates than those treated with CWM alone across several post-operative periods: 3–6 months (Zhou and Liu, 2013; Chen, 2015; Chen et al., 2015; Han, 2016; Xing, 2016; Hu and Y, 2018; Song et al., 2019; Zhao and Xiao, 2020) (8 trials, n = 722; OR = 0.31; 95% CI: 0.19–0.53; I^2 = 0%, p < 0.0001; Figure 9B), 7–12 months (2 trials, n = 176; OR = 0.24; 95% CI: 0.08–0.74; I^2 = 0%, p = 0.01; Figure 9B), 13–24 months (1 trial, n = 68; OR = 0.11; 95% CI: 0.02–0.56; p = 0.008; Figure 9B), and 25–36 months (2 trials, n = 166; OR = 0.22; 95% CI: 0.08–0.61; I^2 = 0%, p = 0.004; Figure 9B).

3.6.2 VAS score

A subgroup analysis of postoperative VAS scores in OEC patients was performed, categorized by treatment duration. Analysis revealed that a 6-month CHM and CWM treatment led to significantly lower VAS scores (Han, 2016; Zhao and Xiao, 2020; Wu and Deng, 2021) (3 trials, n = 282; MD = −1.13; 95% CI: -1.18 to −1.08; p < 0.00001) than a 3-month regimen (Chen, 2015; Chen et al., 2015; Xing, 2016; Hu and Y, 2018; Song et al., 2019) (5 trials, n = 426; MD = −0.70; 95% CI: -0.79 to −0.61; p < 0.00001) (Figure 10). Furthermore, the combination of CHM and CWM notably decreased VAS scores compared to CWM alone, regardless of the treatment's duration (3 or 6 months). The results of this meta-

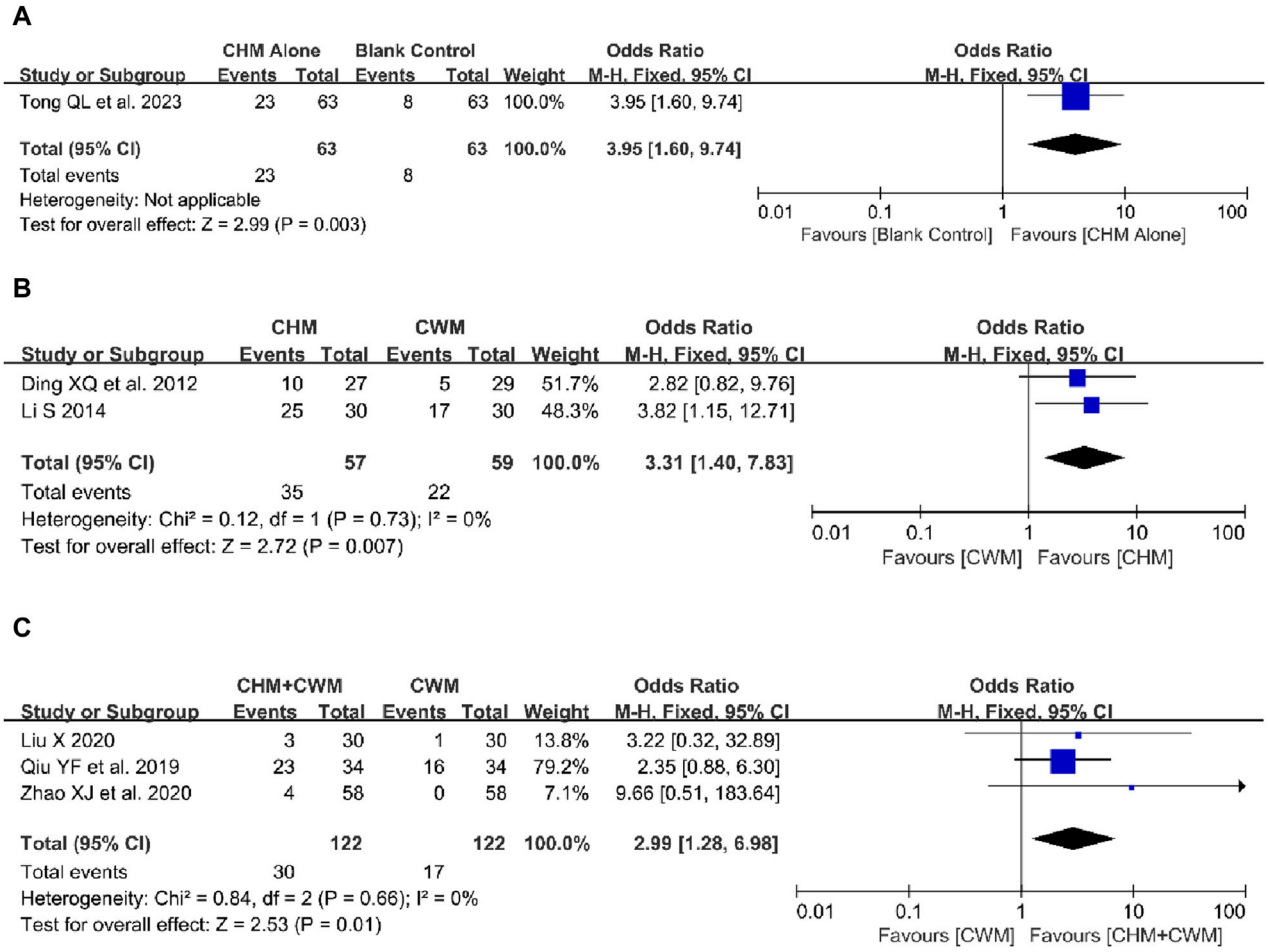

FIGURE 7
Forest plot illustrating the effect on pregnancy rate of different intervention modes of CHM: **(A)** CHM Alone vs Blank Control, **(B)** CHM Alone vs CWM, **(C)** CHM + CWM vs CWM.

analysis can be considered stable since no significant changes were noted in the leave-one-out sensitivity analysis.

3.6.3 Serum level of CA125

A subgroup analysis was conducted on postoperative serum CA125 levels in OEC patients treated with a CHM and CWM combination, targeting specific Western medications. Heterogeneity within subgroups was reduced. As shown in Figure 11, the meta-analysis results indicated a significant decrease in serum CA125 levels, following the combination of CHM with Goserelin (Zhou, 2016; Liu, 2020) (2 trials, n = 166; MD = −8.93; 95% CI: -11.66 to −6.19; I^2 = 42%, p < 0.00001), Triptorelin (Du, 2015; Hu and Y, 2018; Zhao and Xiao, 2020) (3 trials, n = 326; MD = −4.78; 95% CI: -5.79 to −3.77; I^2 = 0%, p < 0.00001), Gestrinone (Lu et al., 2019) (1 trial, n = 86; MD = −8.86; 95% CI: -10.85 to −6.87; p < 0.00001), and Mifepristone (Chen, 2015; Xing, 2016) (2 trials, n = 128; MD = −1.93; 95% CI: -3.34 to −0.53; I^2 = 0%, p = 0.007). However, no significant differences were found in serum CA125 levels between groups combining CHM with Leuprorelin and those using Leuprorelin alone (HAN, 2016) (1 trial, n = 60; MD = −1.17; 95% CI: -7.05 to 4.71; p = 0.70).

Besides, the meta-analysis of CA125 levels in the CHM alone *versus* CWM group exhibited significant heterogeneity (I^2 = 95%). However, due to the limited number of included articles (only 2), it was not possible to conduct subgroup analyses. Hence, based on sensitivity analysis, the original studies were examined. The results revealed that patients who received CHM treatment for a duration of 6 months exhibited significantly reduced serum CA125 levels compared to the control group (Dou, 2015) (MD = −16.09; 95% CI: -19.17 to −13.01; p < 0.00001). Nonetheless, following a 3-month CHM intervention, there was no discernible variation in the serum CA125 levels of the two groups (Zhang, 2012) (MD = −5.17; 95% CI: -12.07 to 1.73; p = 0.14). Differential treatment duration might be the primary source of heterogeneity.

3.7 Publication bias

Publication bias was detected through the funnel plot analysis of recurrence rate, total clinical efficacy rate and adverse event. The asymmetry suggested mild publication bias in the study (Figure 12).

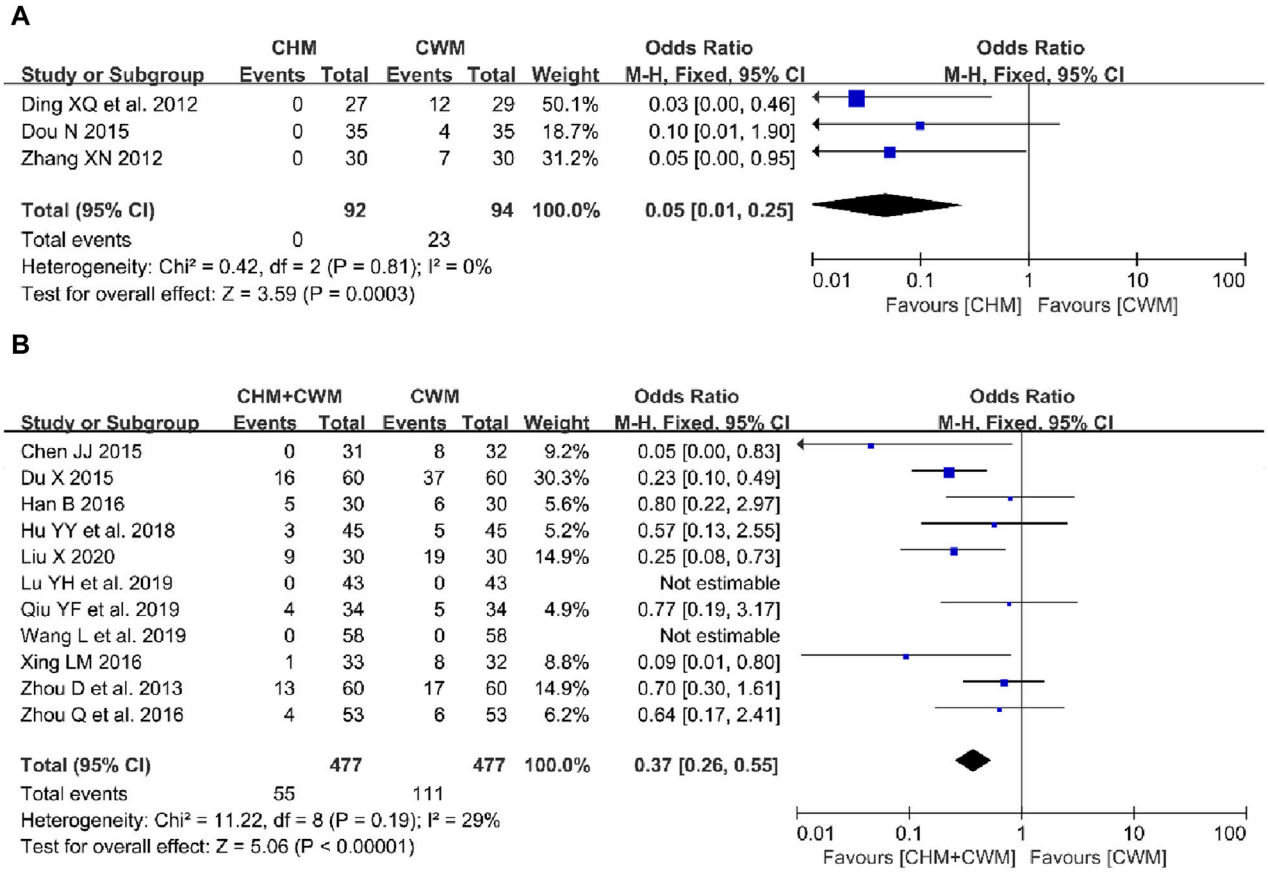

FIGURE 8
Forest plot illustrating the effect on adverse events of different intervention modes of CHM: **(A)** CHM Alone vs CWM, **(B)** CHM + CWM vs CWM.

3.8 GRADE evaluation of evidence quality

According to the GRADE standard (Guyatt et al., 2008b), GRADE profiler 3.2.2 was used to evaluate the quality of evidence for each outcome. Outcome indexes were classified into four grades of high quality, moderate quality, low quality and very low quality according to five aspects of research limitation, inconsistency, indirectness, imprecision, and publication bias. The evidence Profile with quality assessment and summary of findings were reported in Table 6.

4 Discussion

OEC, a prevalent disorder in women of reproductive age, is known for its persistent nature and propensity for recurrence (Ceccaroni et al., 2019). Recently, some academics have incorporated the notion and framework of chronic disease management into the therapy of OEC, aiming to provide patients with comprehensive, uninterrupted, and proactive techniques for managing their condition (Falcone and Flyckt, 2018). Traditional Chinese Medicine (TCM) boasts a history spanning thousands of years. Recent studies have confirmed that CHM exerts therapeutic effects on OEC through multitarget mechanisms, minimizing adverse effects (Flower et al., 2012; Meresma et al., 2021; Chen et al., 2023; Zhao, 2023). To our knowledge, this is the first comprehensive systematic review and meta-analysis that integrates discussions on the principles of TCM treatment and laws of formula composition to assess the efficacy and safety of CHM for postoperative OEC in the English language.

4.1 Summary of evidence

4.1.1 Efficacy

The primary results derived from the meta-analysis are as follows.

(**i**) Conservative surgery combined with CHM treatment may reduce the recurrence of endometriotic lesions, enhance the overall clinical efficacy and pregnancy rates, and alleviate postoperative pain.

(**ii**) In comparison to CWM alone, CHM alone may offer higher clinical efficacy and pregnancy rates, reduce endometriotic lesion recurrence, and lower serum CA125 levels, without a significant improvement in pain symptoms.

(**iii**) The combination of CHM and CWM, *versus* CWM alone, can significantly increase the overall clinical efficacy and pregnancy rates, while also reducing the recurrence of endometriotic lesions and clinical symptoms, lowering CA125 levels, and alleviating postoperative pain.

TABLE 5 The effect of CHM intervention on the incidence of specific adverse events.

Outcome or subgroup	Studies	Patients	Heterogeneity		Effect measure	Or (95% CI)	*p*
			p	I^2/%			
CHM Alone vs CWM							
Perimenopausal Symptoms	3 (Ding and Shi, 2012; Zhang, 2012; Dou, 2015)	186	0.98	0	Odds Ratio	0.09 [0.02, 0.50]	0.004
Androgenic Response	1 (Ding and Shi, 2012)	56	-	-	Odds Ratio	0.14 [0.01, 2.80]	0.31
Hepatic Function Impairment	2 (Ding and Shi, 2012; Zhang, 2012)	116	0.92	0	Odds Ratio	0.11 [0.01, 0.94]	0.04
CHM + CWM vs CWM							
Perimenopausal Symptoms	8 (Chen, 2015; Du, 2015; Han, 2016; Xing, 2016; Zhou, 2016; Hu and Y, 2018; Qiu and Wan, 2019; Liu, 2020)	632	0.73	0	Odds Ratio	0.36 [0.21, 0.61]	0.0001
Androgenic Response	3 (Zhou and Liu, 2013; Du, 2015; Zhou, 2016)	346	0.81	0	Odds Ratio	0.58 [0.20, 1.65]	0.31
Hepatic Function Impairment	4 (Zhou and Liu, 2013; Chen, 2015; Xing, 2016; Liu, 2020)	308	0.41	0	Odds Ratio	0.39 [0.17, 0.92]	0.03
Gastrointestinal Discomfort	5 (Zhou and Liu, 2013; Du, 2015; Zhou, 2016; Hu and Y, 2018; Qiu and Wan, 2019)	504	0.99	0	Odds Ratio	0.75 [0.36, 1.58]	0.45
Physical Pain	3 (Du, 2015; Hu and Y, 2018; Qiu and Wan, 2019)	278	0.87	0	Odds Ratio	0.63 [0.16, 2.45]	0.50
Allergic Reactions	2 (Du, 2015; Hu and Y, 2018)	210	0.93	0	Odds Ratio	0.37 [0.08, 1.63]	0.19

Abbreviation: CHM: chinese herbal medicine; CWM: conventional western medicine.

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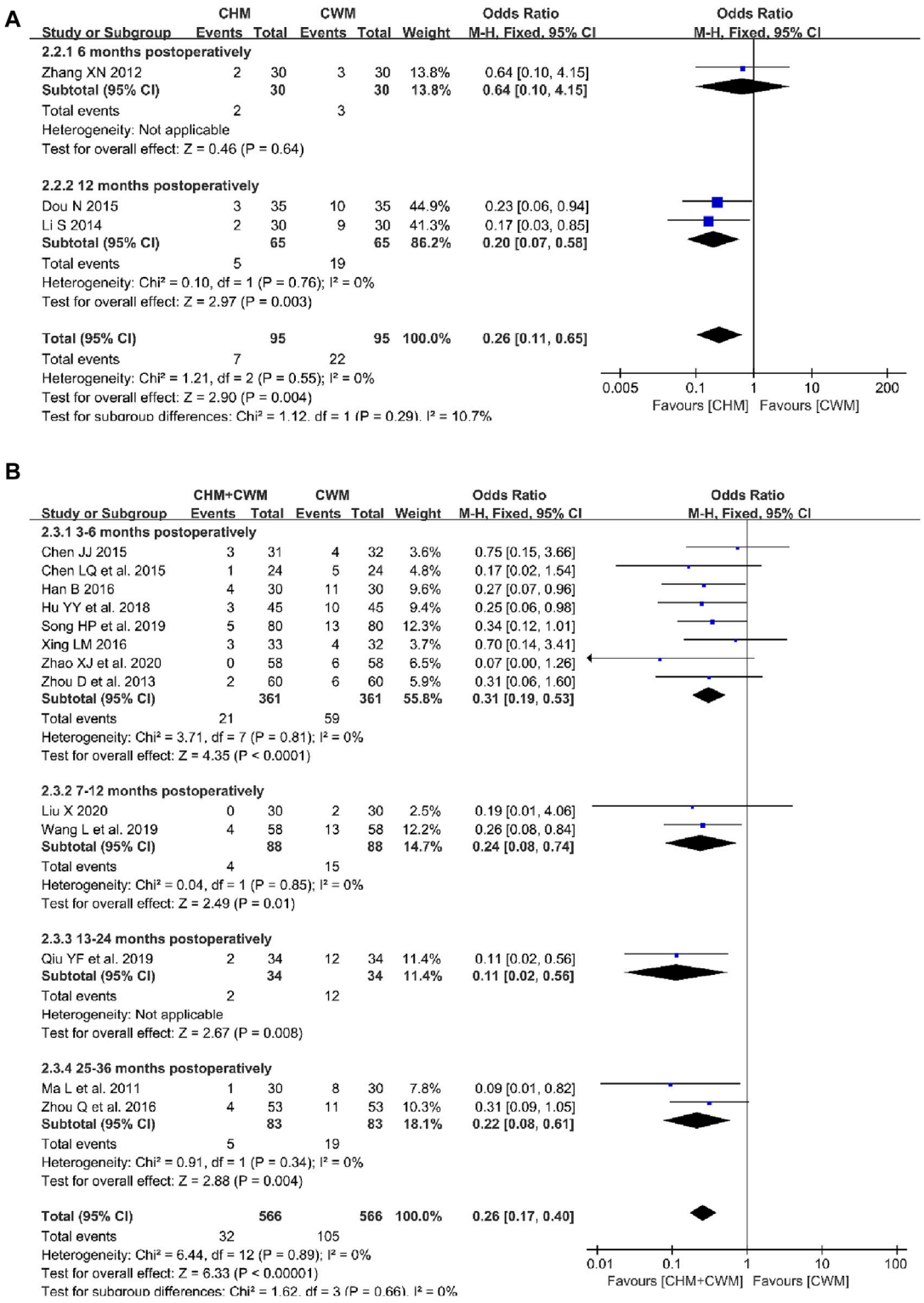

FIGURE 9
Forest plot illustrating the effect of CHM intervention modes on the recurrence rate of OEC during different follow-up periods: **(A)** CHM Alone vs CWM, **(B)** CHM + CWM vs CWM.

(**iv**) Concerning the primary outcome, recurrence rate, CHM intervention has demonstrated efficacy in reducing both endometriotic lesion and clinical symptom recurrence. This might be attributed to its regulatory effects on the body's internal environment, immune enhancement, inflammation suppression, and angiogenesis inhibition (Meresman et al., 2021; Wu et al., 2022). Subgroup analysis revealed that CHM, compared to CWM,

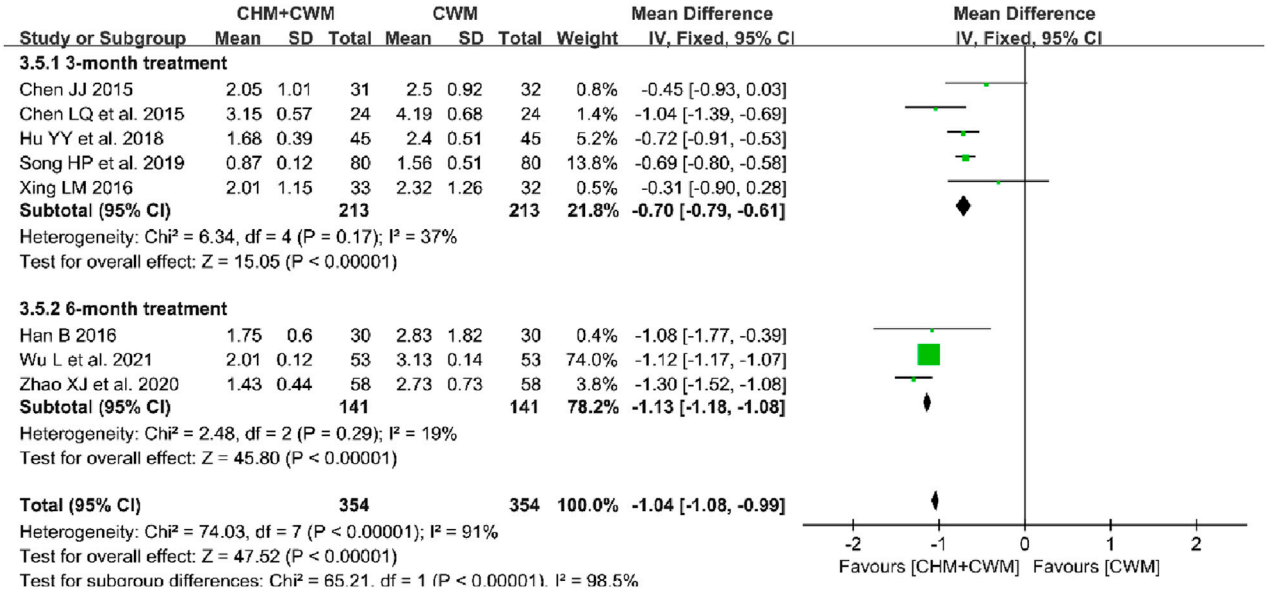

FIGURE 10
Forest plot depicting the effect of different durations of postoperative CHM combined with CWM treatment on reducing the VAS score.

significantly lowered the 12-month postoperative recurrence rate in OEC patients. However, no significant difference was observed at the 6-month follow-up, suggesting the therapeutic effects of CHM may be more pronounced over a longer duration. This observation aligns with the holistic and gradual healing approach traditionally attributed to CHM, which may not only target the symptoms but also the underlying imbalances contributing to the disease's recurrence (Zhao, 2023). Moreover, our analysis demonstrated that combining CHM with CWM could consistently reduce the recurrence rate across all follow-up intervals (3–36 months) compared to using CWM alone. This synergy suggested that CHM might enhance the efficacy of CWM, thereby offering a more sustainable and tolerable long-term management strategy for OEC.

(**v**) The effect of CHM on alleviating postoperative pain in patients with OEC was evaluated using the Visual Analogue Scale. Our research indicated that the use of CHM alone does not significantly outperform CWM in terms of pain improvement. However, it is important to note this conclusion is based on limited evidence, highlighting the need for further rigorous studies to fully explore the potential of CHM as an independent modality for postoperative pain relief. Conversely, our analysis suggested a potentially positive role for CHM as an adjunct to CWM in postoperative OEC management, particularly in offering more effective pain relief in long-term treatment. This not only reflects the comprehensive effects of CHM in regulating pelvic microcirculation and alleviating inflammatory infiltration (Gao Y. et al., 2022; Lin et al., 2022; Yue et al., 2022) but also underscores the significance of an integrated Chinese and Western medical treatment strategy in enhancing postoperative quality of life.

(**vi**) CA125 is recognized as a marker for ovarian epithelial cell tumors. Although it exhibits lower sensitivity and specificity, making it not the most reliable indicator for diagnosing endometriosis (Association, 2021), elevated levels of CA125 are associated with the staging and clinical types of endometriosis (Kovalak et al., 2023). It is commonly used in clinical settings as a monitoring indicator to assess the progression of OEC and the response to treatment (Foster, 2016; Hirsch et al., 2016; Liu, 2020; Zhao and Xiao, 2020). Therefore, in this study, we have included CA125 levels as a secondary outcome, supplementing it with clinical outcomes such as recurrence rate and VAS score, to facilitate a more comprehensive evaluation of the therapeutic effects of CHM in the long-term management of OEC. Our meta-analysis results indicated that CHM interventions could effectively reduce postoperative CA125 levels. Notably, in subgroup analyses, combinations of CHM with Goserelin, Triptorelin, Gestrinone, and Mifepristone treatments have shown significant effects in lowering CA125 levels. However, combining CHM with Leuprorelin did not exhibit a synergistic effect in reducing CA125 levels, necessitating further in-depth research to explore potential influencing factors on its efficacy. These results prompt further considerations for treatment choices, with detailed comparisons between different treatment groups aiding clinicians in formulating more precise treatment plans. Sensitivity analysis revealed that after 6 months of treatment, the CA125 levels in the CHM group were lower than those in CWM group, with no significant difference observed at 3 months of treatment. As OEC is a chronic condition characterized by a long course and a propensity for recurrence, it is advisable in clinical treatment to consider extending the medication duration appropriately based on the patient's condition, to enhance clinical efficacy.

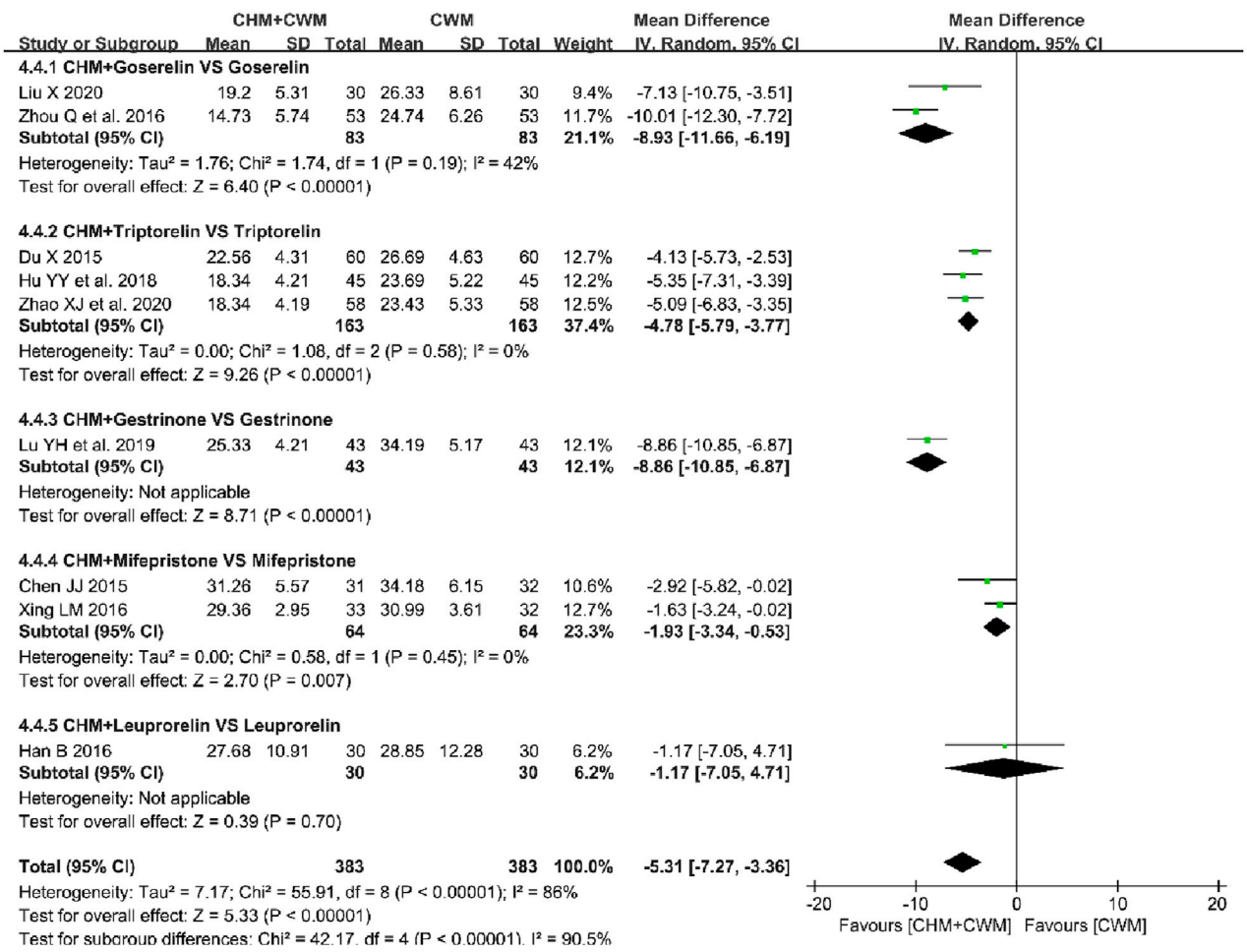

FIGURE 11
Forest plot illustrating the effect of different Western medications combined with CHM on the reduction of serum CA125 levels.

4.1.2 Safety and adverse events

Regarding safety, previous meta-analyses focused on the overall adverse effect incidence rates of CHM treatment in postoperative OEC patients (Fan et al., 2022). In contrast, specific adverse reaction indicators could more accurately highlight TCM's advantages in pattern differentiation and treatment. This study revealed that CHM, alone or combined with CWM, was superior in reducing the overall adverse event incidence and improving perimenopausal symptoms and liver function. CHM mimics sex hormone effects, activating the hypothalamic-pituitary-ovarian axis and enhancing local microcirculation to improve ovarian blood supply (Chen, 2018). Consequently, it improves ovarian function, addresses low estrogen levels, and alleviates symptoms like tidal fever, night sweats, sleep disturbances, and irregular vaginal bleeding in the perimenopausal period. Additionally, according to TCM theory, the liver, viewed as pivotal in women's prenatal basis, plays a crucial role in physiological functioning. If the liver fails to govern the free flow of qi, it could lead to the occurrence of gynecological diseases. Therefore, it is important to emphasize the regulation of viscera and bowels postoperatively. Orderly transformation and qi flow may contribute to alleviating liver function impairment (Zhang et al., 2022).

4.2 Therapeutic principles and medication laws of CHM

4.2.1 Therapeutic principles

This study conducted a summary and synthesis of the included literature, revealing that medical practitioners adhered to the guiding principles of TCM "treat disease before it arises" and "concept of holism" in the treatment of postoperative OEC. To achieve personalized therapy, they clinically based their approach on the patient's constitution, supporting right and dispelling evil, and utilizing pattern identification as the basis for determining treatment. In my opinion, achievement of clinical efficacy is closely related to traditional medical experience and extensive use of a large number of classical Chinese herbal formulas. Although not recorded in TCM, it does not affect the understanding of TCM pathogenesis and the clinical treatment of OEC. As symptoms and signs including tongue coating and pulse are the basis of diagnosis and treatment in TCM, TCM syndrome and formulae syndrome rather than disease were focused accordingly. The following text will describe and discuss specific treatment principles from two perspectives.

(i) prevent disease before it arises, and expel pathogens to prevent recurrence

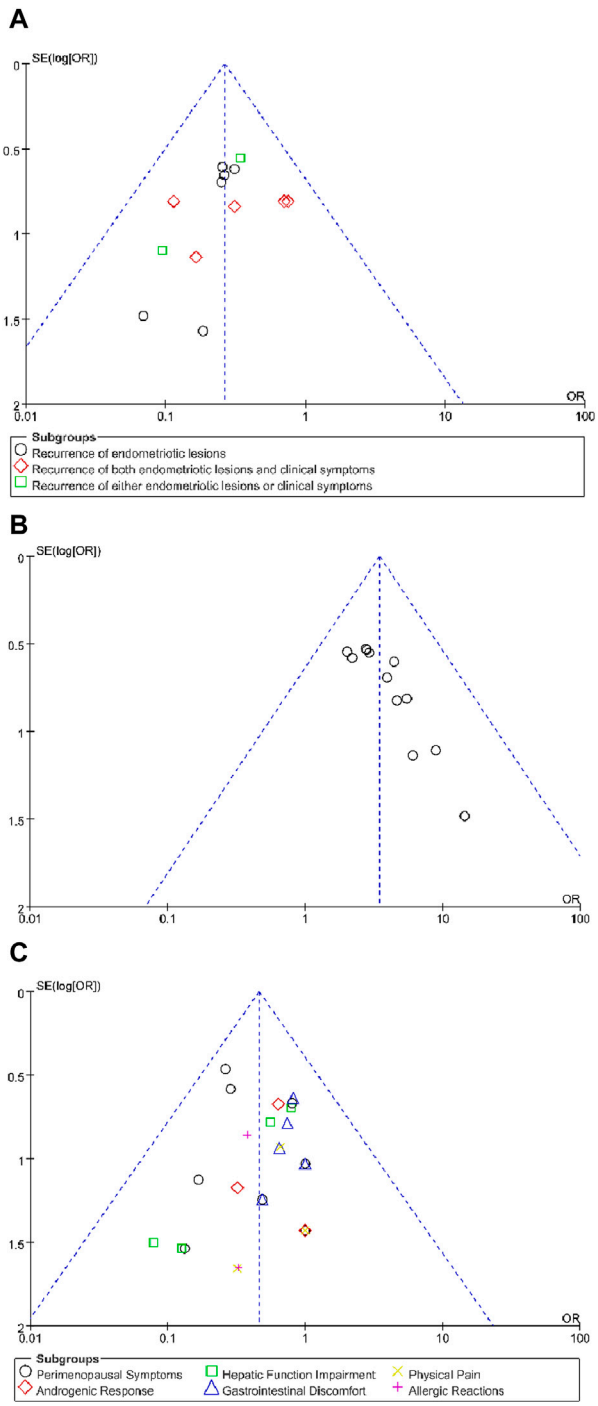

FIGURE 12
Funnel plot of relevant outcomes: **(A)** Recurrence rate, **(B)** Total clinical efficacy rate, **(C)** Adverse event.

According to TCM theory, OEC are classified as "*zhēng jiǎ*" (concretions and conglomerations), with blood stasis being pivotal in their development (Zhao, 2023). While surgical intervention can address the pathological product of "static blood", it does not eradicate the underlying factors leading to postoperative recurrence. Consequently, postoperative treatment in TCM prioritizes the removal of excess pathogens and emphasizes the enhancement of blood circulation and the resolution of stasis, as outlined in Table 2. Clinically, tailored to the individual's constitution, employs flexible application of CHMs known for their efficacy in breaking stasis and invigorating blood, including *P. persica* (L.) Batsch (Taoren), *A. sinensis* (Oliv.) Diels (Danggui), and *S. miltiorrhiza* Bunge (Danshen) to prevent the recurrence of OEC.

(ii) Based on individual conditions, support right and dispel evil, aiming to adjust the body's constitution to a natural balance.

Following OEC surgery, patients may experience diminished immune function, and inadequate postoperative care might lead to a deficiency of healthy qi, disrupting the normal circulation of qi and blood. This disruption fosters the regeneration of static blood, perpetuating a cycle of OEC recurrence. Therefore, the postoperative care should comply with the human body's innate resistance to evil and the physiological characteristics of the internal organs. The treatment aimed to supplement deficiency and support right, and regulate the viscera and bowels (Table 2). Observing the natures, flavors, and channel entry by these herbs (Table 4), their combination demonstrated the following effects: a) harmonizing the warm and cold natures to balance yin and yang; b) acrid opening and bitter downbearing to regulate the movement of qi; c) the combination of acrid and sweet medicinals supports yang, nourishing qi and blood; d) incorporating the expulsion of pathogen within tonifying effects. As shown in Table 3, the tonifying CHMs primarily included *Cuscuta chinensis* Lam. (Tusizi), *Glycyrrhiza glabra* L. (Gancao), *Dipsacus asper* Wall. Ex DC. (Xuduan), *P. lactiflora* Pall. (Baishao), *Astragalus mongholicus* Bunge (Huangqi), focusing on blood supplementation and invigoration while boosting qi and warming yang, simultaneously nourishing the blood, and dispelling stasis. Additionally, postoperative medication primarily targeted the foot *jueyin* liver channel, followed by the hand *shaoyin* heart channel, the foot *taiyin* spleen channel, and the foot *shaoyin* kidney channel (Table 4). Furthermore, recognizing the emotional challenges faced by patients post-surgery, the treatment also aims to soothe the liver, nourish the heart, fortify the spleen, and reinforce the kidney. Achieving equilibrium in *zang-fu* functions ensures a harmonious flow of qi and blood, fostering a balanced constitution and natural elimination of blood stasis.

4.2.2 Description of high-frequency CHM

The most frequently used CHMs were also analyzed in this study. Among the top five, *P. persica* (L.) Batsch (Taoren) possesses a bitter flavor, a neutral nature. It targets the hand *shaoyin* heart, foot *jueyin* liver, and hand *yangming* large intestine channels, exhibiting functions of invigorating blood and dissolving stasis. Its active metabolite, Amygdalin (Figure 13A), exhibits antioxidative, anti-tumor, anti-inflammatory properties, immune modulation, and analgesic effects (Barakat et al., 2022). Research has shown Amygdalin's potential in triggering apoptosis in tumor cells and causing cell cycle arrest (Saleem et al., 2018).

Angelica sinensis (Oliv.) Diels (Danggui) is recognized for its sweet, acrid taste and warm nature, interacting with the foot *jueyin* liver, hand *shaoyin* heart, and the foot *taiyin* spleen channels. It aids in blood supplement and invigoration, menstrual regulation, and pain alleviation. Ferulic acid (Figure 13B), the active metabolite of Danggui, offers vascular endothelial protection through the ERK1/2 and

TABLE 6 GRADE rating of the quality of each outcome.

Group	Outcomes	Anticipated absolute effects* (95% CI)		Relative effect (95% CI)	No of participants (studies)	Certainty of the evidence (GRADE)
		Risk with comparison	Risk with intervention			
Intervention: CHM Alone **Comparison:** Blank Control	Recurrence Rate	333 per 1,000	111 per 1,000 (48–242)	OR 0.25 (0.1–0.64)	126 (1 study)	⊕⊕⊖⊖ low[1,2]
	Total Clinical Efficacy Rate	825 per 1,000	952 per 1,000 (841–987)	OR 4.23 (1.12–15.99)	126 (1 study)	⊕⊕⊖⊖ low[1,2]
	Visual Analog Scale Score	-	MD 0.86 lower (1.01–0.71 lower)	-	126 (1 study)	⊕⊕⊖⊖ low[1,2]
	Pregnancy rate	127 per 1,000	365 per 1,000 (189–586)	OR 3.95 (1.6–9.74)	126 (1 study)	⊕⊕⊖⊖ low[1,2]
Intervention: CHM Alone **Comparison:** CWM	Recurrence Rate	232 per 1,000	73 per 1,000 (32–164)	OR 0.26 (0.11–0.65)	190 (3 studies)	⊕⊕⊖⊖ low[1,2]
	Total Clinical Efficacy Rate	723 per 1,000	885 per 1,000 (778–944)	OR 2.94 (1.34–6.43)	186 (3 studies)	⊕⊕⊖⊖ low[1,2]
	Visual Analog Scale Score	-	MD 0.16 lower (0.49 lower to 0.17 higher)	-	60 (1 study)	⊕⊕⊖⊖ low[1,2]
	CA125	-	MD 14.28 lower (17.09–11.47 lower)	-	130 (2 studies)	⊕⊖⊖⊖ very low[1,2,3]
	Pregnancy rate	373 per 1,000	663 per 1,000 (454–823)	OR 3.31 (1.4–7.83)	116 (2 studies)	⊕⊕⊖⊖ low[1,2]
	Adverse event rate	245 per 1,000	16 per 1,000 (3–75)	OR 0.05 (0.01–0.25)	186 (3 studies)	⊕⊕⊖⊖ low[1,2]
	Recurrence Rate (6 months postoperatively)	100 per 1,000	66 per 1,000 (11–316)	OR 0.64 (0.1–4.15)	60 (1 study)	⊕⊕⊖⊖ low[1,2]
	Recurrence Rate (12 months postoperatively)	292 per 1,000	76 per 1,000 (28–193)	OR 0.2 (0.07–0.58)	130 (2 studies)	⊕⊕⊖⊖ low[1,2]
	Adverse event rate (Perimenopausal Symptoms)	138 per 1,000	14 per 1,000 (3–74)	OR 0.09 (0.02–0.5)	186 (3 studies)	⊕⊕⊖⊖ low[1,2]
	Adverse event rate (Androgenic Response)	103 per 1,000	16 per 1,000 (1–243)	OR 0.14 (0.01–2.8)	56 (1 study)	⊕⊕⊖⊖ low[1,2]
	Adverse event rate (Hepatic Function Impairment)	119 per 1,000	15 per 1,000 (1–113)	OR 0.11 (0.01–0.94)	116 (2 studies)	⊕⊕⊖⊖ low[1,2]
Intervention: CHM + CWM **Comparison:** CWM	Recurrence Rate	186 per 1,000	56 per 1,000 (37–84)	OR 0.26 (0.17–0.4)	1,132 (13 studies)	⊕⊕⊕⊖ moderate[1]
	Total Clinical Efficacy Rate	803 per 1,000	933 per 1,000 (906–953)	OR 3.44 (2.37–5)	1,228 (12 studies)	⊕⊕⊕⊖ moderate[1]
	Visual Analog Scale Score	-	MD 1.04 lower (1.08–0.99 lower)	-	708 (8 studies)	⊕⊕⊖⊖ low[1,3]
	CA125	-	MD 5.1 lower (5.8–4.4 lower)	-	766 (9 studies)	⊕⊕⊖⊖ low[1,3]
	Pregnancy rate	139 per 1,000	326 per 1,000 (171–530)	OR 2.99 (1.28–6.98)	244 (3 studies)	⊕⊕⊖⊖ low[1,2]
	Adverse event rate	233 per 1,000	101 per 1,000 (73–143)	OR 0.37 (0.26–0.55)	954 (11 studies)	⊕⊕⊕⊖moderate[1]

(Continued on following page)

TABLE 6 (*Continued*) GRADE rating of the quality of each outcome.

Group	Outcomes	Anticipated absolute effects* (95% CI)		Relative effect (95% CI)	No of participants (studies)	Certainty of the evidence (GRADE)
		Risk with comparison	Risk with intervention			
	Recurrence Rate (3–6 months postoperatively)	163 per 1,000	57 per 1,000 (36–94)	OR 0.31 (0.19–0.53)	722 (8 studies)	⊕⊕⊕⊖moderate[1]
	Recurrence Rate (7–12 months postoperatively)	170 per 1,000	47 per 1,000 (16–132)	OR 0.24 (0.08–0.74)	176 (2 studies)	⊕⊕⊖⊖ low[1,2]
	Recurrence Rate (13–24 months postoperatively)	353 per 1,000	57 per 1,000 (11–234)	OR 0.11 (0.02–0.56)	68 (1 study)	⊕⊕⊖⊖ low[1,2]
	Recurrence Rate (25–36 months postoperatively)	229 per 1,000	61 per 1,000 (23–153)	OR 0.22 (0.08–0.61)	166 (2 studies)	⊕⊕⊖⊖ low[1,2]
	VAS Score (3-month treatment)	-	MD 0.7 lower (0.79–0.61 lower)	-	426 (5 studies)	⊕⊕⊕⊖moderate[1]
	VAS Score (6-month treatment)	-	MD 1.13 lower (1.18–1.08 lower)	-	282 (3 studies)	⊕⊕⊖⊖ low[1,2]
	CA125 (CHM + Goserelin VS Goserelin)	-	MD 8.93 lower (11.66–6.19 lower)	-	166 (2 studies)	⊕⊕⊖⊖ low[1,2]
	CA125 (CHM + Triptorelin VS Triptorelin)	-	MD 4.78 lower (5.79–3.77 lower)	-	326 (3 studies)	⊕⊕⊖⊖ low[1,2]
	CA125 (CHM + Gestrinone VS Gestrinone)	-	MD 8.86 lower (10.85–6.87 lower)	-	86 (1 study)	⊕⊕⊖⊖ low[1,2]
	CA125 (CHM + Mifepristone VS Mifepristone)	-	MD 1.93 lower (3.34–0.53 lower)	-	128 (2 studies)	⊕⊕⊖⊖ low[1,2]
	CA125 (CHM + Leuprorelin VS Leuprorelin)	-	MD 1.17 lower (7.05–4.71 lower)	-	60 (1 study)	⊕⊕⊖⊖ low[1,2]
	Adverse event rate (Perimenopausal Symptoms)	174 per 1,000	70 per 1,000 (42–114)	OR 0.36 (0.21–0.61)	632 (8 studies)	⊕⊕⊕⊖moderate[1]
	Adverse event rate (Androgenic Response)	58 per 1,000	34 per 1,000 (12–92)	OR 0.58 (0.20–1.65)	346 (3 studies)	⊕⊕⊕⊖moderate[1]
	Adverse event rate (Hepatic Function Impairment)	117 per 1,000	49 per 1,000 (22–109)	OR 0.39 (0.17–0.92)	308 (4 studies)	⊕⊕⊕⊖moderate[1]
	Adverse event rate (Gastrointestinal Discomfort)	67 per 1,000	51 per 1,000 (25–102)	OR 0.75 (0.36–1.58)	504 (5 studies)	⊕⊕⊕⊖moderate[1]
	Adverse event rate (Physical Pain)	36 per 1,000	23 per 1,000 (6–84)	OR 0.63 (0.16–2.45)	278 (3 studies)	⊕⊕⊖⊖ low[1,2]
	Adverse event rate (Allergic Reactions)	57 per 1,000	22 per 1,000 (5–90)	OR 0.37 (0.08–1.63)	210 (2 studies)	⊕⊕⊖⊖ low[1,2]

*The risk in the intervention group (and its 95% confidence interval) is based on the assumed risk in the comparator group and the relative effect of the intervention (and its 95% CI). CI: Confidence interval; MD: Mean difference; OR: Odds ratio. Factors of downgrade: 1 Unclear risk of detection bias, selection bias and performance bias; 2 Sample size is less than the optimal information size; 3 Significant statistical heterogeneity. GRADE Working Group grades of evidence: High quality: Further research is very unlikely to change our confidence in the estimate of effect. Moderate quality: Further research is likely to have an important impact on our confidence in the estimate of effect and may change the estimate. Low quality: Further research is very likely to have an important impact on our confidence in the estimate of effect and is likely to change the estimate. Very low quality: We are very uncertain about the estimate.

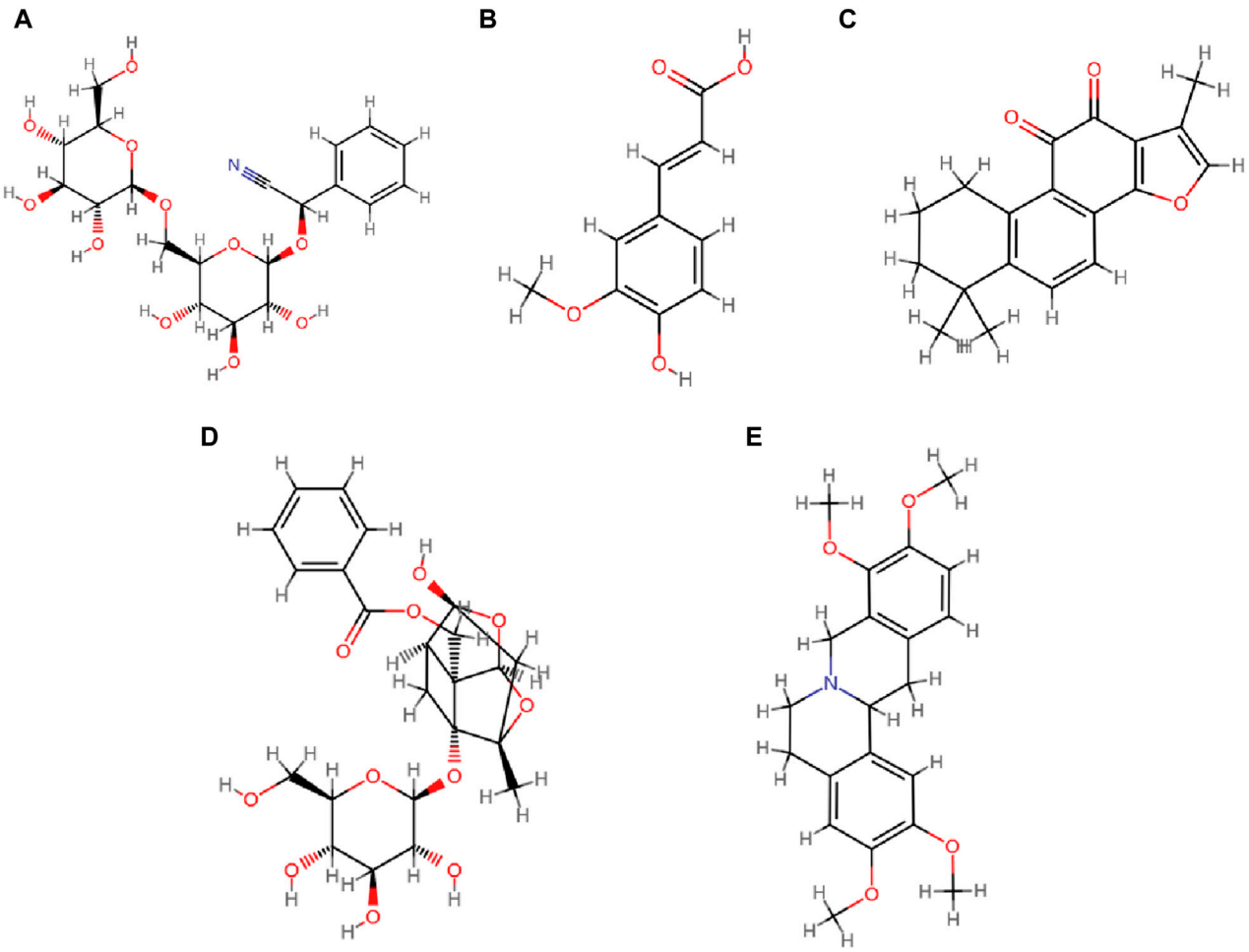

FIGURE 13
Chemical structures of the main active metabolites of most frequently used CHM for postoperative treatment of OEC: **(A)** Amygdalin, **(B)** Ferulic acid, **(C)** Tanshinone IIA, **(D)** Paeoniflorin, **(E)** Tetrahydropalmatine.

NO/ET-1 pathways and exhibits anti-fibrosis properties via the TGF-β/Smad and MMPs/TIMPs systems (Li et al., 2021; Li et al., 2022).

Salvia miltiorrhiza Bunge (Danshen) has a bitter flavor and a slightly cold nature, entering the hand *shaoyin* heart channel and the foot *jueyin* liver channel. Its functions involve invigorating blood, dissolving stasis, regulating menstruation, and calming the mind, making it a crucial botanical drug in gynecology. Its principal active metabolite, tanshinone IIA (Figure 13C), plays a pivotal role in mitigating adhesion, invasion, and angiogenesis, thereby improving the pathological morphology of ectopic endometrium and inhibiting the formation of endometriotic lesions (Chen and Gong, 2020; Zhang et al., 2023).

Paeonia lactiflora Pall. (Chishao) is utilized to alleviate heat, cool the blood, and eliminate blood stasis, addressing conditions such as concretions, conglomerations, and amenorrhea effectively. Its active metabolite, paeoniflorin (Figure 13D), exhibits anti-inflammatory, antioxidant, immunomodulatory, and anti-tumor properties (Zhang and Wei, 2020; Wang Xz. et al., 2022). Furthermore, research suggests that paeoniflorin could regulate the metabolic expression of various pathways in a rat model of endometriosis (Wu et al., 2019).

Corydalis yanhusuo W.T.Wang (Yanhusuo) is widely used to alleviate general body pain. It exhibits significant anti-inflammatory, analgesic, neuroprotective, and antitumor properties, attributed to tetrahydropalmatine as its active metabolite (Figure 13E). A combination of tetrahydropalmatine, ferulic acid, and ligustrazine has been shown to inhibit epithelial-mesenchymal transition through the Wnt/β-catenin pathway (Zhang et al., 2021), exerting an anti-proliferative effect on endometriosis via modulation of the Notch pathway (Dai et al., 2023).

It is noteworthy that, oxidative stress, a negative effect stemming from the imbalance of oxidation system in the body, plays a significant role in apoptosis, aging, and the onset of various diseases (Reuter et al., 2010; Poprac et al., 2017; Hajam et al., 2022). OEC is intricately linked to oxidative damage, both in terms of its initiation and development (Matsuzaki and Schubert, 2010; Hayasti et al., 2020; Koninckx et al., 2021). Plant polyphenols, including flavonoids, tannins, phenolic acids, etc., have the ability to scavenge free radicals within the body, combat lipid oxidation, delay organismal aging (Yahfoufi et al., 2018; Maleki et al., 2019; Nani et al., 2021), and exhibit anti-inflammatory, anti-tumor, and analgesic properties (Arablou and Kolahdouz-Mohammadi,

2018; Liu et al., 2022). Numerous CHMs and natural metabolites, including the five botanical drugs previously mentioned, possess certain antioxidant, anti-tumor, anti-inflammatory, and anti-aging effects. These attributes underline the potential of CHMs in the multifaceted and comprehensive treatment of OEC.

4.3 Limitations and future perspectives

This review acknowledges several limitations that warrant consideration. First, suboptimal methodological design is a common issue in most included trials, demanding a careful explanation of our results. Second, the absence of sample size estimation in many studies undermines the precision of results, particularly in smaller-scale research. Third, the omission of OEC staging information in some studies may influence the aggregated results regarding OEC recurrence. Finally, the GRADE evaluation for various outcomes was of moderate to low quality, indicating constraints in stability and reliability of conclusions.

To address these limitations and improve the quality of future research, the following strategies are recommended. First, significant drawbacks regarding the sequence generation of randomization, concealment of allocation, reporting on blinding, attrition, and pre-estimation of sample size should be considered to minimize potential biases. Second, adopting a multicenter, large-sample research design is advisable to ensure the reliability and generalizability of results. Third, a detailed analysis of variables such as surgical techniques, patient age, severity of condition, and OEC staging could yield deeper insights. Fourth, integrating various types of real-world studies could be considered to enhance the evaluation of the therapeutic effects of CHM. Finally, future studies could further identify the specific components or formulations of CHM responsible for clinical benefits and elucidate the mechanisms of their synergistic effects with CWM. This would lay the groundwork for more personalized and effective treatment paradigms, integrating the best of traditional and modern medicine.

5 Conclusion

In general, this systematic review and meta-analysis suggested that CHM may be beneficial for the treatment of postoperative OEC in reducing recurrence, improving clinical efficacy, and decreasing side effects. CHM could serve as a potential candidate or supplemental therapy for postoperative OEC in the battle for long-term management. However, considering the limitations of existing studies, further large-scale, high-quality, and rigorously designed trials are warranted to substantiate the conclusions.

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

DD: Conceptualization, Data curation, Formal Analysis, Writing–original draft. SL: Writing–review and editing, Data curation, Formal Analysis. FL: Data curation, Writing–review and editing. SH: Writing–review and editing, Methodology. CZ: Methodology, Writing–review and editing. YS: Methodology, Writing–review and editing. WW: Writing–review and editing, Data curation. QC: Data curation, Writing–review and editing. FH: Writing–review and editing, Funding acquisition, Methodology, Supervision.

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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.2024.1376037/full#supplementary-material

SUPPLEMENTARY APPENDIX S1
List of PRISMA 2020 statements.

SUPPLEMENTARY APPENDIX S2
Search strategies for all the electronic databases.

SUPPLEMENTARY APPENDIX S3
Components of CHM in the included studies.

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Acer tegmentosum Maxim and *Bacillus subtilis*-fermented products inhibit TNF-α-induced endothelial inflammation and vascular dysfunction of the retina: the role of tyrosol moiety in active compounds targeting Glu^{230} in SIRT1

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Acer tegmentosum Maxim (AT) is a medicinal plant used to treat hepatic, neurological diseases, and cancer. However, the beneficial effects of AT on endothelial dysfunction have not been reported yet. In this study, we evaluated the effects of AT and the main compounds against TNF-α-mediated inflammatory responses and their possible mechanism of action. The anti-inflammatory effect and its molecular mechanism were analyzed by adhesion assay, immunoblotting, promoter-luciferase assay, ELISA, RT-PCR, immunocytochemistry, immunoprecipitation, siRNA gene knockdown, docking, and molecular dynamics simulation. AT and its compounds salidroside and tyrosol reduced TNF-α-induced adhesion between monocytes and endothelial cells. Fermentation of AT with *Bacillus subtilis* converted salidroside to tyrosol, which is salidroside's aglycone. The fermented AT product (ATF) potently inhibited TNF-α-mediated monocyte adhesion with higher potency than AT. AT or ATF abrogated TNF-α-induced expression of adhesion molecules (VCAM-1 and ICAM-1) and production of MCP-1 with the inhibition of phosphorylated MAP kinases. TNF-α-mediated NF-κB transactivation and RelA/p65 acetylation were suppressed by AT and ATF through the interaction of NF-κB with sirtuin-1 (SIRT1), an NAD^+-dependent histone deacetylase. *Sirt1* gene knockdown diminished the protective effects of AT and ATF against TNF-α-mediated signaling and inflammatory response. Interestingly, SIRT1 protein expression was significantly increased by ATF and tyrosol rather than by AT and salidroside, respectively. Molecular docking showed that the tyrosol moiety is critical for the interaction with Glu^{230} of SIRT1 (PDB ID:

Abbreviations: AT, *Acer tegmentosum* Maxim; ATF, AT fermented product; ICAM-1, intercellular adhesion molecule-1; MD, molecular dynamics; NTD, N-terminal domain; Rg, radius of gyration; RMSD, root-mean-square deviation; RMSF, root-mean-square fluctuation; SAL, salidroside; SASA, solvent-accessible surface area; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SIRT1, sirtuin-1; STAC, sirtuin-activating compounds; STZ, streptozotocin; TNF, tumor necrosis factor; TYR, tyrosol; VCAM-1, vascular cell adhesion molecule-1.

4ZZH and 4ZZJ) for the deacetylase activity. Molecular dynamics revealed that tyrosol can induce the movement of the N-terminal domain toward the catalytic domain of SIRT1. This study demonstrates the potential of AT and ATF to prevent endothelial inflammation and vascular dysfunction of the retina by the MAPK/NF-κB/SIRT1 signaling pathways and targeting of the tyrosol moiety to Glu^{230} in SIRT1.

KEYWORDS

Acer tegmentosum Maxim, anti-inflammation, NF-κB, SIRT1, tyrosol

1 Introduction

Acer tegmentosum Maxim (Aceraceae) has been used as a medicinal plant to treat liver diseases (such as hepatitis, cirrhosis, and hepatocellular carcinoma) in East Asian countries (Hwang et al., 2013; Chang et al., 2017; Bae et al., 2022). Recently, the Ministry of Food and Drug Safety (MFDS) of South Korea approved AT as a food material without acute toxicity (approval #2018-7), and LD_{50} was reported to be over 2,000 mg/kg in rats (Hwang et al., 2013; Park et al., 2019). *Acer tegmentosum* Maxim extracts (AT) abrogated high-fat diet-induced triglyceride and cholesterol accumulation in animal study (Cho et al., 2020). AT protected H_2O_2-induced damage via MAPK signaling pathways in hepatocytes (Park et al., 2019). Salidroside (SAL), the main compound of AT, reduced the hepatic triglyceride content and increased insulin sensitivity in the liver tissues of obese mice or leptin-deficient ob/ob mice (Wang et al., 2016a; Zheng et al., 2018). Tyrosol (TYR), which is SAL's aglycone, alleviated ROS production in palmitic acid-induced oxidative stress in hepatocytes and reversed dysregulated lipid deposition in high-fat diet-fed mice (Sarna et al., 2016). TYR attenuated the inflammatory response and maintained the capillary barrier (Kim et al., 2017). In addition, TYR could penetrate and accumulate in cells and improve the intracellular antioxidant defense system (Di Benedetto et al., 2007).

Flavonoids and phenolic compounds are large-class secondary metabolites found in plants (Huynh et al., 2014). There are several methods to extract phenolic compounds from plants, including physicochemical and chemical processes. On the other hand, fermentation promotes the release and metabolism of polyphenols from plant sources, which improve their functionality (Huynh et al., 2014). Previously, we reported that *Bacillus subtilis*-fermented products were biotransformed into secondary metabolites, thereby enhancing the antioxidant effect (Gum et al., 2017). The content of SAL in AT extracts is approximately 6%–8%, while the content of TYR is very low (Hwang et al., 2013; Park et al., 2019). *Bacillus subtilis* produces β-glucosidase, which can cause deglycosylation of the glycol residue of SAL, generating its aglycone TYR (Nemeth et al., 2003; Huynh et al., 2014; Gum et al., 2017). *Bacillus subtilis* has been used in the fermentation of natto (Japanese fermented soybeans) and other soybean products to enhance their antioxidant effects (Huynh et al., 2014).

The vascular system plays a critical role between tissue and circulation. Endothelial cells, a major component of the innermost layer of blood vessels, act as a barrier to maintain vascular homeostasis and the circulatory system (Piao et al., 2018). Vascular inflammation is characterized by the activated endothelial cells, which is associated with various diseases, including the cardiovascular complication linked to metabolic syndrome (Lenin et al., 2018; Nguyen et al., 2019). Activated endothelial cells upregulate adhesion molecules such as VCAM-1 and ICAM-1, promoting the recruitment of immune cells to the vascular wall, which exacerbates inflammation and contributes to disease progression (Ouyang et al., 2024). Therefore, therapeutic strategies that effectively target activated endothelial cells are required to reduce vascular inflammation.

Tumor necrosis factor (TNF)-α, an inflammatory cytokine, plays a pivotal role in the disruption of vascular circulation, causing endothelial dysfunction in many pathophysiological conditions including diabetes and metabolic syndrome (Ouyang et al., 2024). TNF-α activates three major MAPK cascades and NF-κB (a transcription factor), mediating the transcription of inflammatory genes (Choi et al., 2018). Under TNF-α stimulation, NF-κB translocates to the nucleus, where it undergoes posttranslational modifications, including phosphorylation, methylation, ubiquitination, and acetylation (Yang et al., 2010). The acetylation at Lys^{310} of RelA/p65 is crucial as it prolongs the stability of RelA/p65 and enhances the full transcriptional activity of NF-κB (Huang et al., 2010). Sirtuin-1 (SIRT1, an NAD^+-dependent histone deacetylase) deacetylates histone protein such as NF-κB (Chen et al., 2002). Therefore, SIRT1 activation abrogates TNF-α-stimulated endothelial cell inflammation via NF-κB transactivation (Pan et al., 2016). We also previously reported the beneficial role of SIRT1 as a transcriptional regulator of NF-κB in endothelial cells in the anti-inflammatory response (Nguyen et al., 2019).

Since SIRT1 is linked to multiple biological processes, it has been studied as a therapeutic target for metabolic disorders and inflammation (Dai et al., 2015). The first generation of small-molecule sirtuin-activating compounds (STAC) was a group of related plant polyphenols, such as resveratrol and quercetin (Hubbard and Sinclair, 2014). The Glu^{230} residue of the N-terminal domain (NTD) in hSIRT1 plays a critical role in the direct allosteric activation and stabilization of SIRT1 by STAC (Dai et al., 2015). Therefore, compound targeting the Glu^{230} residue of SIRT1 is a good approach to study when studying SIRT1 activation (Hubbard et al., 2013; Dai et al., 2015).

Our present study is the first to evaluate the protective effect of AT against endothelial inflammatory responses by TNF-α and the enhanced anti-inflammatory effect of *B. subtilis*-fermented ATF. Additionally, the role of NF-κB and SIRT1 as a mechanism in the anti-inflammatory effect was identified, and it was proven that the main compounds of the candidates target the Glu^{230} residue of SIRT1 using molecular docking.

2 Materials and methods

2.1 Reagents

The dried twig of AT was purchased from Omniherb (Gyeongbok, Korea). TNF-α (T6674), anti-β-actin antibody

(A1978), thiazolyl blue tetrazolium bromide (MTT) (M2128), streptozotocin (S0130), poly-L-lysine (P4707), and bovine serum albumin (BSA) (A8806) were purchased from Sigma-Aldrich (St. Louis, MO, United States). Lipofectamine 2000, goat anti-rabbit IgG (#65-6120), and goat anti-mouse IgG (#62-6520) were purchased from Invitrogen (Carlsbad, CA, United States). Anti-intercellular adhesion molecule-1 (ICAM-1) (sc-7891 and sc-8439), anti-NF-κB (sc-8008), anti-SIRT1 (sc-74504), anti-vascular cell adhesion molecule-1 (VCAM-1) (sc-8304) antibodies, anti-IgG-FITC (sc-2010), protein G agarose (sc-2002), DAPI (sc-3598), and Immuno *In situ* Mount (sc-45088) were obtained from Santa Cruz Biotechnology (CA, United States). Calcein-AM (C3100MP), goat anti-mouse Alexa Fluor 488 (A32723), and goat anti-rabbit Alexa Fluor 568 (A11011) were purchased from Thermo Fisher Scientific (Waltham, MA, United States). Anti-phospho-ERK1/2 (#9101), anti-ERK1/2 (#9102), anti-phospho-p38 MAPK (#9211), anti-p38 MAPK (#9212), anti-phospho-SAPK/JNK (#9255), and anti-JNK2 (#9258) antibodies were obtained from Cell Signaling Technology (Beverly, MA, United States). All materials used for SDS-PAGE were purchased from Bio-Rad (Hercules, CA, United States). The *Sirt1* small interfering RNA (siRNA, sc-40986) and non-targeting scrambled RNA (control siRNA, sc-37007) were obtained from Santa Cruz Biotechnology (Sparks, MD, United States).

2.2 AT extraction and fermentation

Extraction of AT was performed as previously reported, with minor modifications (Yu et al., 2010). AT (400 g) was powdered and extracted with 2.5 L of 70% ethanol at 80°C for 2 h. The ethanol extract was filtered through a Whatman #2 filter paper (Whatman International, Maidstone, United Kingdom) and concentrated using a rotary evaporator (model VV2000; Heidolph, Walpersdorfer, Germany) to obtain the final extraction. The samples were frozen in −70°C before being freeze-dried using a FD8505S freeze-dryer (Ilshin Biobase Co., Ltd., Busan, South Korea), and a voucher (AT-0100) specimen was deposited in the pharmacology laboratory at the College of Oriental Medicine, Kyungju, South Korea. The final yield of the ethanol extract was 19.6%. The content of salidroside, the major component of AT, was calculated from the relevant peak area using an external standard method and quantified as 6.41% (w/w) in AT (Bae et al., 2022) (Supplementary Figure 2).

A total of 300 mg AT extract was fermented with 100 mL Luria–Bertani (LB) media containing 0.2–20% *B. subtilis* NCDO 1769T (X60,646) culture for 7 days (ATF) and then frozen at −70°C before use (ATF-0100) (Gum et al., 2017). Negative AT samples were subjected to 100 mL LB media without *B. subtilis* for 7 days (AT). After fermentation, both AT or ATF samples were stored in −70°C for further experiments.

2.3 HPLC analysis

HPLC analyses were performed as previously published with minor modifications (Meng et al., 2006). The HPLC analyses of samples were carried out using an Agilent 1260 HPLC system (1260 Infinity II; Agilent Technologies, Santa Clara, CA, United States) coupled to the Agilent G7115A-1260 DAD WR detector (diode array detector). In brief, 10 μL of AT or ATF samples (3 mg/mL) were injected into the Agilent ZORBAX Eclipse plus C18 column (4.6 mm × 250 mm, 5 μm) at 25°C. The mobile phase was 0.1% formic acid in H_20 (A) and 100% MeOH (B), run at 1 mL/min; peaks were detected with a UV detector at 225/278 nm. The ratios of the A/B solvent were 80/20, 80/20 to 0/100 (linear gradient), and 0/100 at running times of 0–10, 11–30, and 31–35 min, respectively. SAL or TYR at 500 ppm was used as the standard. The SAL and TYR peaks were observed at 7.5 and 9.5 min, respectively.

2.4 Cell culture

EA. Hy926, a human umbilical vein endothelial cell line fused with the human carcinoma cell line (CRL-2922), was purchased from the ATCC (Rockville, MD, United States). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (LM 001-05, Welgene, Gyeongsangbuk-do, Korea) containing 10% FBS, 50 μg/mL streptomycin, and 50 units/mL penicillin at 37°C in a humidified 5% CO_2 atmosphere. THP-1 cells (ATCC® TIB-202™), a monocyte cell line, were purchased from the ATCC (Rockville, MD, United States). The cells were cultured in RPMI 1640 medium (LM 011-01, Welgene, Gyeongsangbuk-do, Korea) containing 10% FBS, 0.05 mM 2-mercaptoethanol, 50 μg/mL streptomycin, and 50 units/mL penicillin. Cells were treated with TNF-α for the indicated time with or without candidates (AT, ATF, SAL, or TYR) pretreated for 1 h.

2.5 Cell viability assay

Cells were seeded in a 96-well culture plate. After reaching 80% of confluence, cells were incubated with candidates at different concentrations for 24 h. The MTT solution (5 mg/mL) was incubated for 2 h. The assays were stopped by adding DMSO, and the cell viability was observed at 520 nm using a Sunrise™ Absorbance microplate reader (Tecan, Bionics, Seoul, Korea).

2.6 Adhesion assay

The monocyte–endothelial interaction was evaluated by the measurement of fluorescent labeling monocytes, as previously described (Nguyen et al., 2019). Endothelial cells were seeded in a 96-well culture black plate and then incubated with candidates for 1 h, following exposure of 10 ng/mL TNF-α for 6 h. Human monocyte cells were labeled with calcein-AM at 37°C for 1 h. Calcein-AM-labeled cells were co-cultured with the monolayer of endothelial cells for 1 h. After the incubation, the recruited monocytes were measured using microplate reader (Bio-Tek Instruments, Winooski, VT, United States) with excitation wavelength 490 nm and emission wavelength 510–570 nm. The average fluorescence intensity represented the number of monocytes adhered to endothelial cells.

2.7 Enzyme-linked immunosorbent assay (ELISA)

MCP-1 cytokine levels in the culture supernatants were detected by ELISA using a commercial Human MCP-1 ELISA kit (Pierce Biotechnology, IL, United States), according to manufacturer's protocol. The OD values were measured using a Sunrise™ Absorbance microplate reader (Tecan, Bionics Co., Seoul, Korea). Concentrations of MCP-1 cytokines were extrapolated from the standard curves provided.

2.8 RT-PCR

RT-PCR was performed as previously reported (Kim et al., 2008). The primers encoding MCP-1 (forward primer 5′ -GAT GCAATCAATGCCCCAGTC -3′ and reverse primer 5′- TTTGCT TGTCCAGGTGGTCCAT -3′) and β-actin (forward primer 5′-GACTACCTCATGAAGATC -3′ and reverse primer 5′-GATCCACATCTGCTGGAA -3′) were synthesized from Bioneer (Cheongwon, Korea).

2.9 Immunoprecipitation and immunoblotting

Immunoprecipitation and immunoblotting were performed, as previously reported (Nguyen et al., 2019). For immunoprecipitation, cell lysates were incubated with antibodies against SIRT1 or NF-κB at 4°C for 2.5 h and protein G plus-agarose beads at 4°C for 1 h. The immunocomplexes were washed with IP buffer and dissolved by boiling. Samples were then run on SDS-PAGE gels. In immunoblotting, primary antibodies were incubated at 4°C overnight, and secondary antibodies were incubated at room temperature for 1 h. The target proteins were developed using an ECL chemiluminescence detection kit (Amersham Biosciences, Bucks, United Kingdom). The protein expressions were normalized with β-actin immunoblot. The densitometry was measured using Gel-Pro Analyzer software (Media Cybernetics, MD, United States).

2.10 Promoter-luciferase assay

The full-length VCAM-1 and ICAM-1 firefly luciferase reporter gene construct (VCAM-1: -1,716 to +119 bp, ICAM-1: -1,350 to +45 bp) were transfected as previously reported (Kim et al., 2008; Nguyen et al., 2019). The luciferase activities in cell lysates were measured using a GloMax 20/20 Luminometer (Tuner BioSystems, Sunnyvale, United States). The relative luciferase signal was normalized by dual transfection with the pSV-β-galactosidase control vector.

2.11 Immunocytochemistry

Cells were seeded in an 8-well chamber slide system (Nunc Lab-Tek II, Seoul, Korea). Immunofluorescence-labeled cells were evaluated as previously reported (Wang et al., 2016b). After drug treatment for the indicated time, cells were fixed with ice-cold MeOH for 15 min. After permeabilizing and blocking, the anti-NF-κB primary antibody was incubated overnight at 4°C. The secondary antibody against anti-mouse-FITC was then incubated. The images were taken in random regions using a BioTek Lionheart FX Automated Microscope (Agilent, VT, United States).

2.12 Transfection of siRNAs

Cells were transfected with siRNAs using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, United States), following the manufacturer's protocol. Human *Sirt1* or scrambled siRNA (10 nM) was transiently transfected for 3 h to knock down *Sirt1*. After an 18 h-recovery period, promoter-luciferase assay, immunoblotting, and adhesion assays were performed, as described above.

2.13 Protein sequence alignment

The full sequence alignment of human SIRT1 (NP_036370.2) was obtained from NCBI by FASTA. The crystal structures of SIRT1 PDB IDs 4ZZH and 4ZZJ were retrieved from the Protein Data Bank online database (PDB). The sequences were imported, and the structure-based sequence alignment was performed using BioEdit software version 7.2 (Pannek et al., 2017).

2.14 Molecular docking

Three-dimensional sequences of SIRT1 with the NTD region were retrieved from PDB: 4ZZH and 4ZZJ (www.rcsb.org). All structures were optimized by the removal of ligands, substrates, ions, and water molecules. The active compounds' (SAL and TYR) structures were downloaded from the NCBI PubChem online database (http://pubchem.ncbi.nlm.nih.gov/) and energy minimized. Molecular docking of PDB and compounds was analyzed by AutoDockZn and PyRx softwares, to predict the binding affinity and ligand conformational change. The proper docking conformations were chosen based on binding affinity scores and the root-mean-square deviation (RMSD) values. The binding pockets and interactions between the ligand and protein were visualized by PyMOL and Biovia Discovery Studio 2021 software applications.

2.15 Molecular dynamics simulation

The docked complexes of the active compounds and SIRT1 were used as the initial coordinates for molecular dynamics (MD) simulations using the GROningen MAchine for Chemical Simulations (GROMACS) (Shamsi et al., 2024). MD simulations were performed with GROMACS version 5.1.2 with the CHARMM36-jul2022 force field. The complexes were immersed in a dodecahedron box and solvated using the CHARMM-modified

TIP3P water model. The topologies of SAL and TYR were generated using the CHARMM General Force Field (CGenFF). The systems were neutralized with counter ions. The energy minimization process was initiated with restraints on both the ligand and protein, followed by an unrestrained minimization step using the steepest descent method. The equilibration phases were conducted by NVT ensemble for 100 ps, followed by NPT ensemble for an additional 100 ps, with the temperature maintained at 300 K. The final MD trajectories were run for 100,000 ps (100 ns) with a 2.0-fs time step. Post-simulation analysis was performed using the following GROMACS utilities: root-mean-square deviation (RMSD), root-mean-square fluctuation (RMSF), radius of gyration (Rg), solvent-accessible surface area (SASA), and hydrogen bond analysis. The conformational changes in the ligand–protein complexes were visualized at the 20-ns interval using PyMOL software.

2.16 Mouse experiments

The animal study was conducted in accordance with the institutional guidelines based on the principles of laboratory animal care (NIH publications No. 85-23, revised 1996) and Korean laws of laboratory animal care (Health & Welfare Committee Registration No. 9025, revised 2010) and was approved by the Institutional Animal Care and Use Committee of the Dongguk University (protocol number IACUC-2023-15). Male C57Bl/6JBomTac mice were purchased from Daehan Bio Link (Chungbuk, South Korea). The 5-week-old mice received three consecutive intraperitoneal injections of streptozotocin (STZ) at a dose of 55 mg/kg in citrate buffer (0.1 M, pH 4.5). One week after STZ-treatment, mice with glucose levels exceeding 250 mg/dL were divided into four groups: control, STZ, STZ + AT, and STZ + ATF. AT or ATF (50 mg/kg) was orally administered daily for 4 weeks. All mice were anesthetized with pentobarbital sodium, and their eyes were collected and stored in 4% paraformaldehyde until further processing.

2.17 Histological evaluation

Eye samples were soaked in 30% sucrose (0.2 M phosphate buffer) at 4°C overnight before fixing in the optimal cutting temperature (OCT) compound for cryosectioning. Retina radial sections (8 μm) were placed on poly-L-lysine-coated slides and heated at 60°C for 2 h. Samples were permeabilized with washing buffer (0.1% Triton X-100) at room temperature for 20 min and then rinsed with PBS twice for 5 min. Retinal samples were blocked with buffer containing 5% fetal bovine serum, 2% BSA, and 0.1% Triton X-100 at room temperature for 30 min. Tissue samples were stained with primary antibodies for VCAM-1 and ICAM-1 (1:400 dilution) overnight at 4°C. The sections were then incubated with secondary antibodies conjugated to goat anti-mouse Alexa Fluor 488 and goat anti-rabbit Alexa Fluor 568 (1:600 dilution) for 30 min at room temperature. For counterstaining, DAPI was incubated with the sections for 5 min. Sections were analyzed using the BioTek Lionheart FX Automated Microscope (Agilent, VT, United States).

2.18 Statistical analysis

Statistical analyses were performed using SPSS 13.0. (IBM SPSS, Chicago, United States), and all data are presented as the mean ± standard error (S.E.). The statistical comparisons between the groups were determined using one-way ANOVA, followed by Dunnett's *post hoc* test.

3 Results

3.1 Effects of AT, SAL, and TYR on TNF-α-induced monocyte–endothelial interactions

In vascular inflammatory conditions, circulating monocytes bind and migrate through the blood vessels' endothelial monolayer, which plays a pivotal role in the initiation of vascular dysfunction (Nguyen et al., 2019). Therefore, we investigated the effects of AT, SAL (the main bioactive compound of AT), and TYR (the aglycone of SAL) on the TNF-α-stimulated adhesion of monocytes to endothelial cells. The concentrations of candidates used in this study did not affect cell viability (Supplementary Figure 1). Endothelial cells were pretreated with AT, SAL, or TYR for 1 h, and following TNF-α exposure for the next 6 h. We then measured the attached calcein-AM-labeled monocytes to the endothelial cells. TNF-α induced monocyte adhesion (~5.5-fold) compared to the control (Figure 1). AT, SAL, and TYR suppressed the elevated monocyte attachment by TNF-α with IC_{50} values of 1.62 μg/mL, 0.94 μM, and 0.14 μM, respectively (Figures 1A, B). TYR exhibited high potency in reducing TNF-α-induced monocyte recruitment on endothelial cells. These results suggest that TYR may exert beneficial effects as an active metabolite in inhibiting monocyte adhesion to endothelial cells.

3.2 Effects of AT and ATF on TNF-α-induced adhesion molecule expression and transactivation

SAL is the major compound of AT, whereas TYR is very minor (Hwang et al., 2013; Lee et al., 2014). We previously demonstrated that fermentation with *B. subtilis* subsp. *subtilis* NCDO 1769T (X60646) showed enhanced beneficial effects through secondary metabolite production (Gum et al., 2017). Therefore, we performed AT fermentation with *B. subtilis* subsp. *subtilis* NCDO 1769T (X60646). The fermented product of AT showed a bioconversion from SAL to TYR (Supplementary Figure 2), analyzed by HPLC. In ATF, SAL and TYR contents were 4.44% (w/w) and 0.75% (w/w), respectively. The elevation of monocyte recruitment under TNF-α exposure was inhibited by ATF with an IC_{50} value of 0.49 μg/mL (Figure 1C). The lower IC_{50} value of ATF indicates that ATF is more effective than AT in inhibiting monocyte attachment to endothelial cells, which may be attributed to the biotransformation of SAL in AT.

TNF-α induces endothelial cell activation, leading to transactivation of adhesion molecules such as VCAM-1 and ICAM-1, which represents the initial step in vascular inflammation (Nguyen et al., 2019). The previous report showed that TNF-α increased VCAM-1 expression, which peaked at 12 h, while ICAM-1 expression showed a sustained induction pattern until 24 h (Nguyen et al., 2019). Therefore, we performed immunoblotting with VCAM-1 and ICAM-1 after the

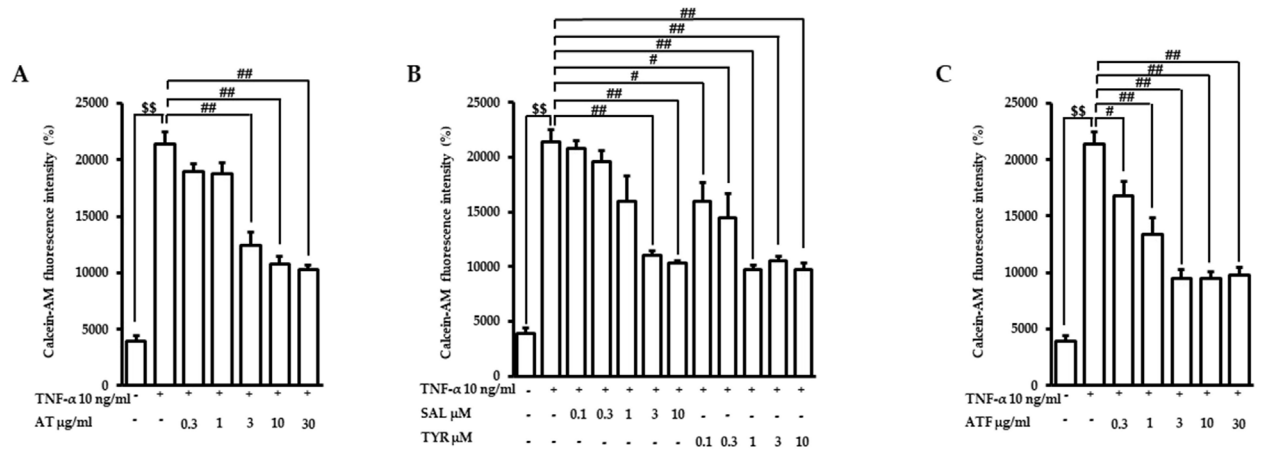

FIGURE 1
Effects of candidates on TNF-α-induced monocyte recruitment to endothelial cells. Cells were pretreated with **(A)** AT, **(B)** SAL or TYR, and **(C)** ATF at the indicated doses for 1 h and followed by TNF-α incubation for 6 h. Calcein-AM-labeled monocytes were co-cultured with endothelial cells for the next 1 h. The monocyte-attached endothelial cells were evaluated based on fluorescence intensities. ($^{\$\$}p < 0.01$ for control vs. TNF-α; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ for TNF-α vs. the candidate-treated group).

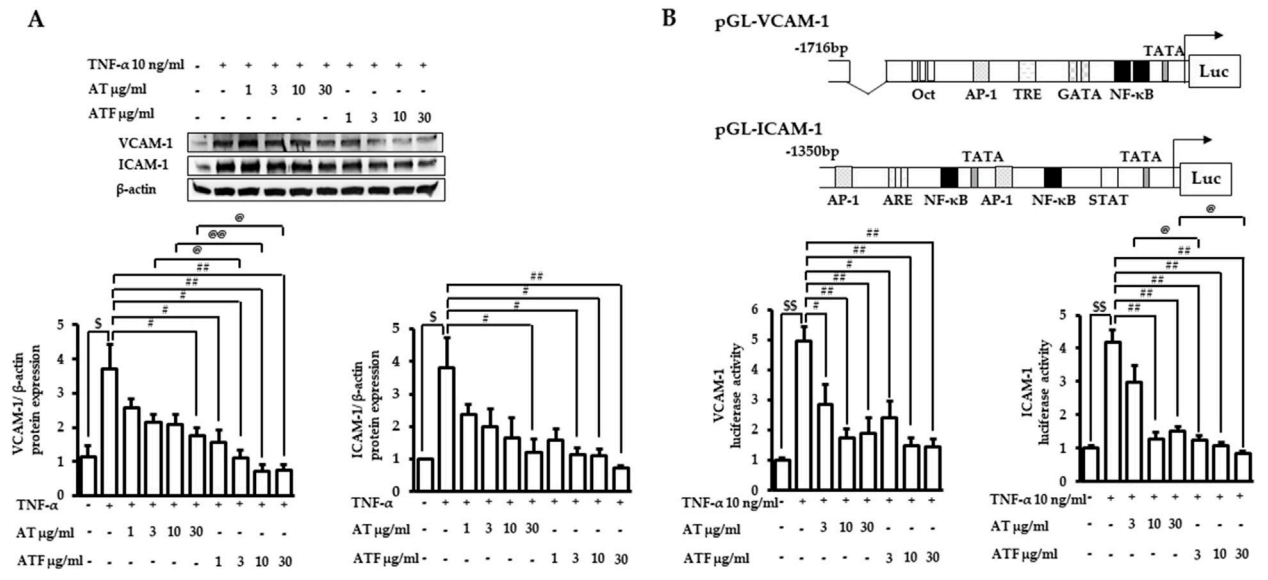

FIGURE 2
Inhibitory effects of AT and ATF on TNF-α-induced adhesion molecules. **(A)** Cells were pretreated with AT or ATF for 1 h, followed by TNF-α incubation. VCAM-1 or ICAM-1 protein expressions were determined after 12 or 24 h of TNF-α incubation, respectively. Cell lysates were analyzed by immunoblotting using specific antibodies against VCAM-1 and ICAM-1. The protein amounts in each lane were normalized to the β-actin control density. The data were obtained from multiple analyses (n = 4). **(B)** Cells were transfected with VCAM-1 or ICAM-1 promoter constructs. After 18 h of recovery, cells were incubated with AT or ATF for 1 h and then TNF-α for the additional 18 h. Cell lysates were used for the reporter gene assay. The data were obtained from multiple analyses (n = 3). ($^{\$}p < 0.05$, $^{\$\$}p < 0.01$ for control vs. TNF-α; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ for TNF-α vs. candidate-treated group; $^{@}p < 0.05$, $^{@@}p < 0.01$ for the same concentration between each candidate).

indicated times of TNF-α incubation with AT or ATF pretreatment. The protein expressions of VCAM-1 and ICAM-1 were elevated ~4-folds under TNF-α exposure, which were significantly inhibited by AT or ATF (Figure 2A).

We performed reporter gene assays in cells transfected with the promoter of VCAM-1 or ICAM-1 to determine transcriptional activity under TNF-α exposure. TNF-α increased the luciferase activity of VCAM-1 by 5-fold and ICAM-1 by 4-fold (Figure 2B). Incubation with AT or ATF strongly attenuated TNF-α-mediated transactivation. AT and ATF inhibited VCAM-1 luciferase activity with a similar effective range, with IC_{50} values of 2.86 μg/mL and 2.09 μg/mL, respectively. The stronger protective effect of ATF was shown in ICAM-1 luciferase activity with an IC_{50} value of 1.27 μg/mL compared to AT with an IC_{50} value of 3.09 μg/

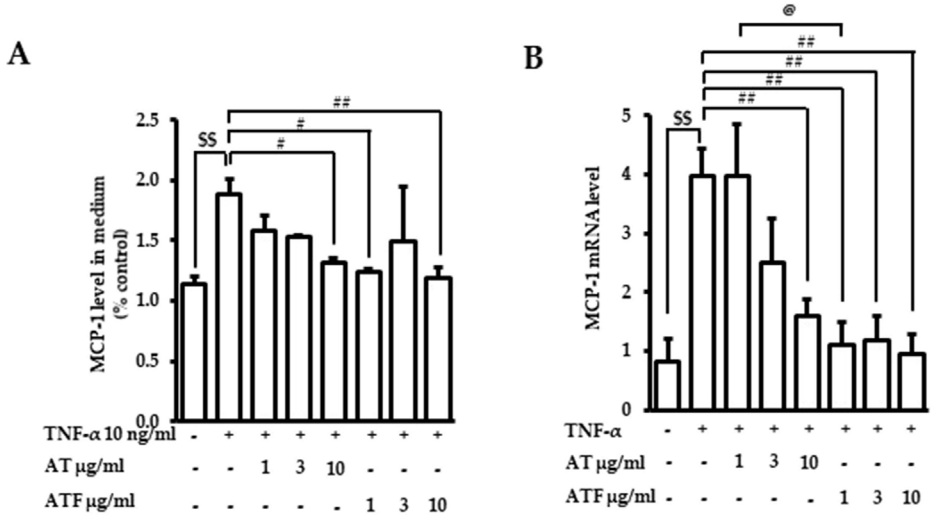

FIGURE 3
Effects of AT and ATF on TNF-α-induced MCP-1 production and mRNA level. Cells were treated with AT or ATF at the indicated doses for 1 h before TNF-α incubation. **(A)** The MCP-1 level was measured after 36 h of TNF-α treatment with or without AT or ATF by ELISA. The data were obtained from multiple analyses (n = 3). **(B)** The mRNA level of MCP-1 was measured after 6 h of TNF-α exposure by RT-PCR. The data were obtained from multiple analyses (n = 5). ($^{SS}p < 0.01$ for control vs. TNF-α; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ for TNF-α vs. candidate-treated group; $^{@}p < 0.05$ for the same concentration between each candidate).

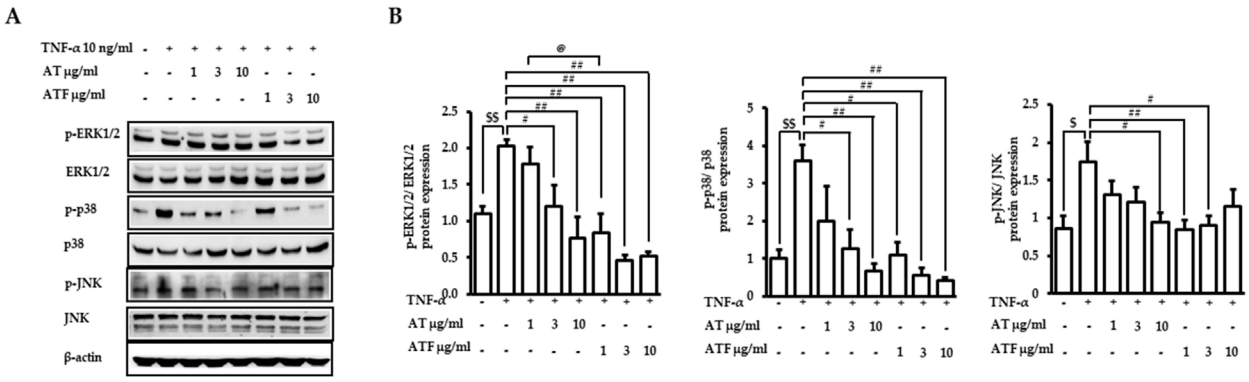

FIGURE 4
Effects of AT and ATF in the TNF-α-induced MAPK signaling pathway. **(A)** Cells were pretreated with AT or ATF for 1 h and incubated with TNF-α for 30 min or 5 h, as described in the *Materials and methods* section. The expressions of phosphorylated and total MAPKs in cell lysates were detected by Western blot. **(B)** Quantitative analyses of the densitometric intensity of immunoblotting were carried out to quantify MAPK levels. The relative expression ratios of each p-ERK, p-p38 MAPK, and p-JNK were normalized to each EKR, p38 MAPK, and JNK. The data were obtained from multiple analyses (n = 4). ($^{S}p < 0.05$, $^{SS}p < 0.01$ for control vs. TNF-α; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ for TNF-α vs. candidate-treated group; $^{@}p < 0.05$ for the same concentration between each candidate).

mL. The significant suppression of ATF in the attenuation of TNF-α-mediated transcriptional activation of adhesion molecules was consistent with the inhibition of those expressions.

3.3 Effects of AT and ATF on TNF-α-induced pro-inflammatory cytokine production

MCP-1 is one of the key cytokines that regulates the migration and infiltration of monocytes/macrophages, which initiates further inflammatory cellular responses (Tedgui and Mallat, 2006). MCP-1 is induced and secreted in endothelial cells stimulated by TNF-α (Meng et al., 2022). Therefore, we tested whether AT or ATF prevented TNF-α-induced MCP-1. We first measured MCP-1 secretion in the culture media after TNF-α incubation for 36 h. AT or ATF pretreatment abrogated TNF-α-induced MCP-1 production (Figure 3A). Next, we evaluated the level of MCP-1 mRNA. After 6 h of TNF-α treatment, MCP-1 mRNA levels increased 4-fold (Figure 3B). AT and ATF significantly suppressed MCP-1 mRNA, and inhibition by ATF was stronger than that by AT. These results support the hypothesis that ATF is potent due to its conversion to the active compound TYR.

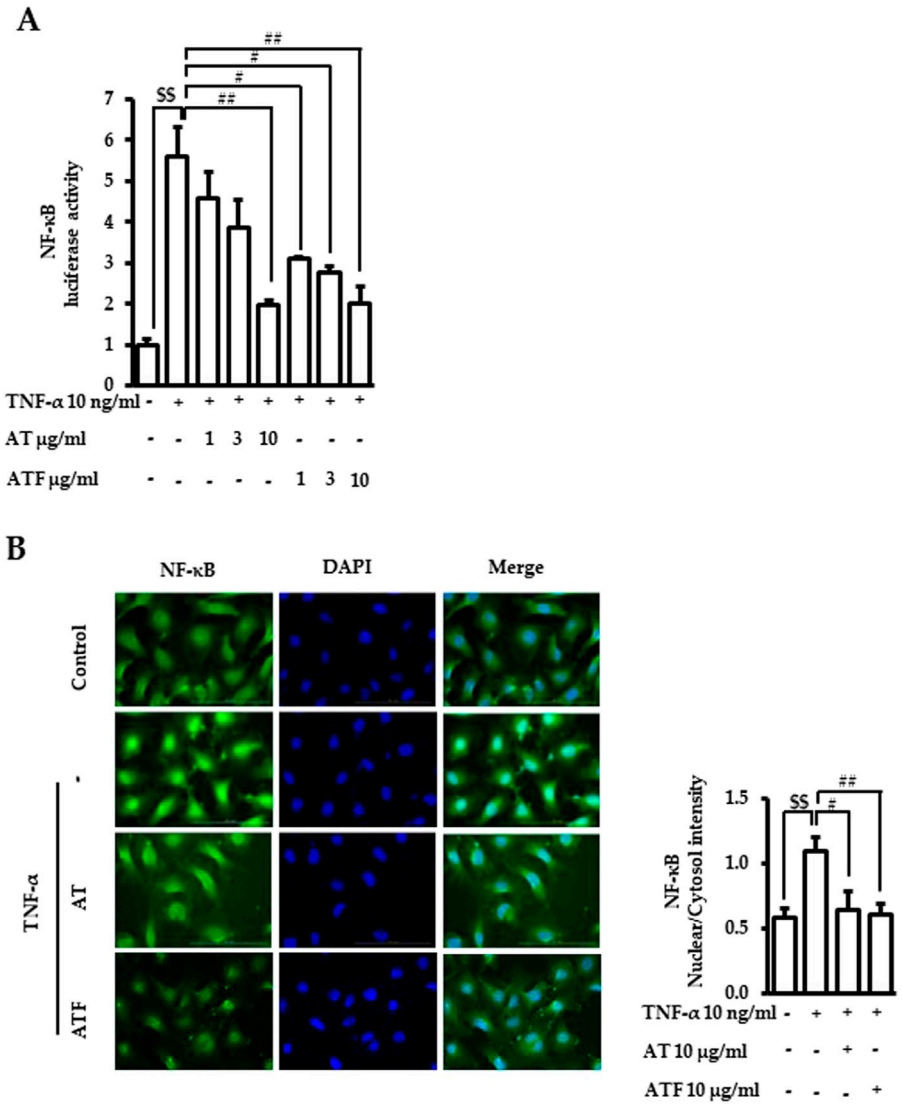

FIGURE 5
Inhibitory effects of AT and ATF on TNF-α-induced NF-κB transactivation and nuclear translocation. **(A)** Cells were transiently transfected with NF-κB promoter gene construct and subsequently treated with TNF-α for 18 h with or without AT or ATF pretreatment for 1 h. Luciferase activity was determined in lysates. The data were obtained from multiple analyses (n = 3). **(B)** Cells were pretreated with AT or ATF for 1 h and TNF-α for the next 1 h. Immunocytochemistry was carried out, as described in *Materials and methods*. NF-κB p65 was stained green, and nuclei were stained blue. The representative image (left panel) and the quantitative analyses of the fluorescence intensities (right panel) are shown. The data were obtained from multiple analyses (n = 5). ($^{SS}p < 0.01$ for control vs. TNF-α; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ for TNF-α vs. candidate-treated group).

3.4 Upstream signaling of the protective effects of AT and ATF against TNF-α exposure

MAPKs play an important role in the regulation of inflammatory signaling cascades (Dong et al., 2020). We performed immunoblotting of phosphorylated forms of ERK1/2, p38, and JNK to analyze the upstream signaling of AT and ATF in response to TNF-α exposure. Cells were treated with AT or ATF at a dose range of 1–10 μg/mL. Then, TNF-α was incubated for the next 30 min to detect phosphorylated forms of p38 and ERK1/2. Phosphorylated JNK was observed after incubation with TNF-α for 5 h. TNF-α significantly increased the phosphorylation of ERK1/2, p38, and JNK, which were ameliorated by AT or ATF pretreatment (Figure 4).

NF-κB is a master transcriptional factor that regulates inflammatory gene expression (Baker et al., 2011). Therefore, the effects of AT and ATF on NF-κB transcriptional activation under TNF-α exposure were determined. Pretreatment of AT or ATF abrogated TNF-α-induced transcriptional activity of NF-κB with IC_{50} values of 3.77 μg/mL and 0.90 μg/mL, respectively (Figure 5A). TNF-α-induced translocation of NF-κB from the cytoplasm into the nucleus was observed, which was inhibited by AT or ATF (Figure 5B). These results suggest that AT and ATF inhibit the transcription of the inflammatory genes by blocking TNF-α-induced nuclear translocation and transcriptional activity of NF-κB.

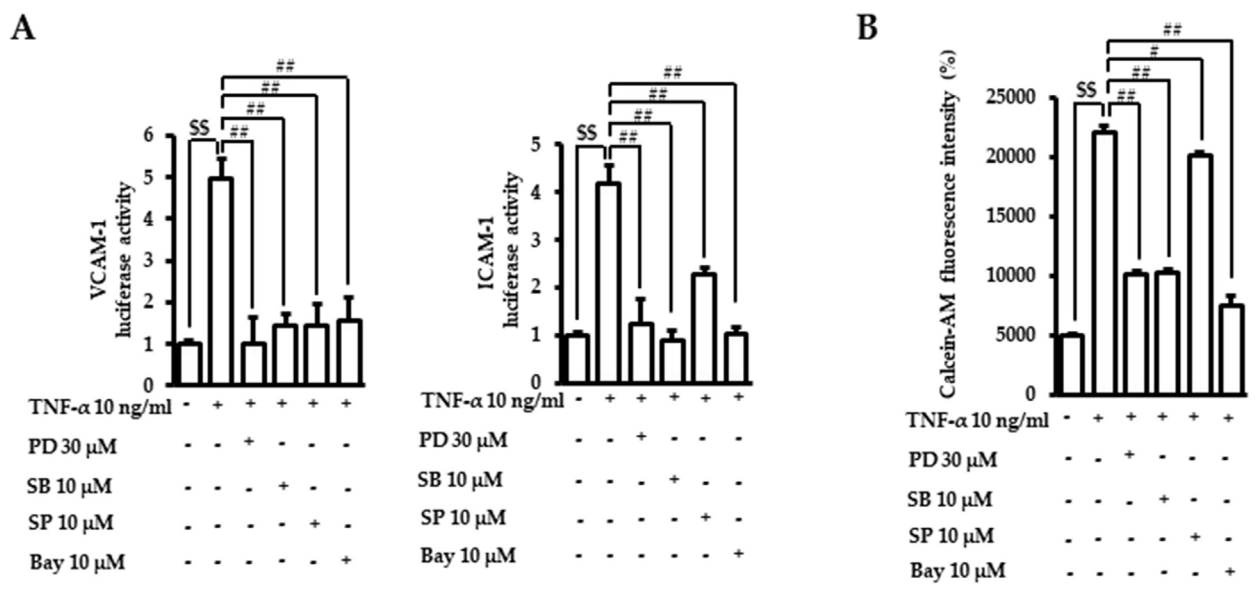

FIGURE 6
Role of MAPK/NF-κB in TNF-α-induced adhesion molecule transactivation and monocyte recruitment. **(A)** VCAM-1 or ICAM-1 promotor gene analyses were performed, as described in Figure 2B. Cells were treated with PD98059 (PD, ERK1/2 inhibitor), SB203580 (SB, p38 inhibitor), SP600125 (SP, JNK inhibitor), or Bay11-7082 (Bay, NF-κB inhibitor) for 1 h. Cells were then exposed to TNF-α for 18 h. The data were obtained from multiple analyses (n = 3). **(B)** Adhesion assays between monocytes and endothelial cells were performed, as described in Figure 1. After incubation with TNF-α with or without chemical inhibitors of MAPK and NF-κB, the intensity of the fluorescent calcein-AM signal was assessed with a fluorometer. The data were obtained from multiple analyses (n = 3). ($^{SS}p < 0.01$ for control vs. TNF-α; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ for TNF-α vs. candidate-treated group).

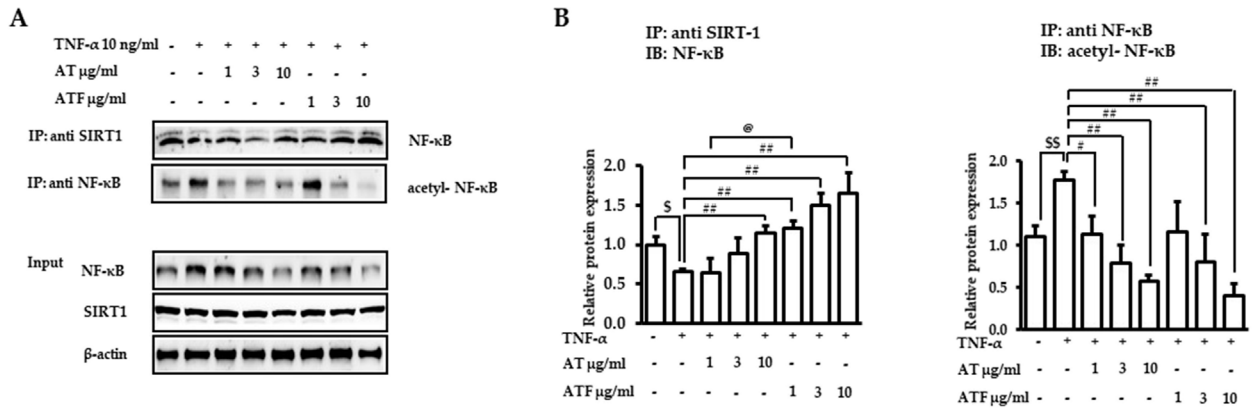

FIGURE 7
Interaction of NF-κB and SIRT1 induced by AT and ATF. **(A)** Cells were pretreated with AT or ATF for 1 h and then exposed to TNF-α for 30 min. The lysates were immunoprecipitated with an anti-SIRT1 antibody or anti-NF-κB antibody and then subjected to immunoblotting using anti-NF-κB and anti-p65 acetyl K310 antibodies, as described in the *Materials and methods* section. The NF-κB and SIRT1 levels in input samples were determined by immunoblotting. **(B)** Graphs represent relative densitometric intensities of NF-κB and acetylated NF-κB obtained from multiple analyses (n = 4). ($^{S}p < 0.05$, $^{SS}p < 0.01$ for control vs. TNF-α; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ for TNF-α vs. candidate-treated group; $^{@}p < 0.05$ for the same concentration between each candidate).

We then confirmed the role of MAPKs and NF-κB in the regulation of adhesion molecule expressions and adhesion between endothelial cells and monocytes using chemical inhibitors. Cells were preincubated with the indicated doses of inhibitors, including PD98059 (ERK1/2 inhibitor), SB203580 (p38 inhibitor), SP600125 (JNK inhibitor), and Bay11-7082 (NF-κB inhibitor), and following TNF-α incubation. The TNF-α-induced luciferase activities of adhesion molecules VCAM-1 and ICAM-1 were mitigated by inhibitors of MAPK and NF-κB (Figure 6A). The attachment of calcein-AM labeled monocytes to endothelial cells induced by TNF-α was also inhibited by the blocking of MAPKs and NF-κB (Figure 6B). These results confirmed that MAPK/NF-κB signaling pathways play an important role in TNF-α-regulated endothelial cell inflammation.

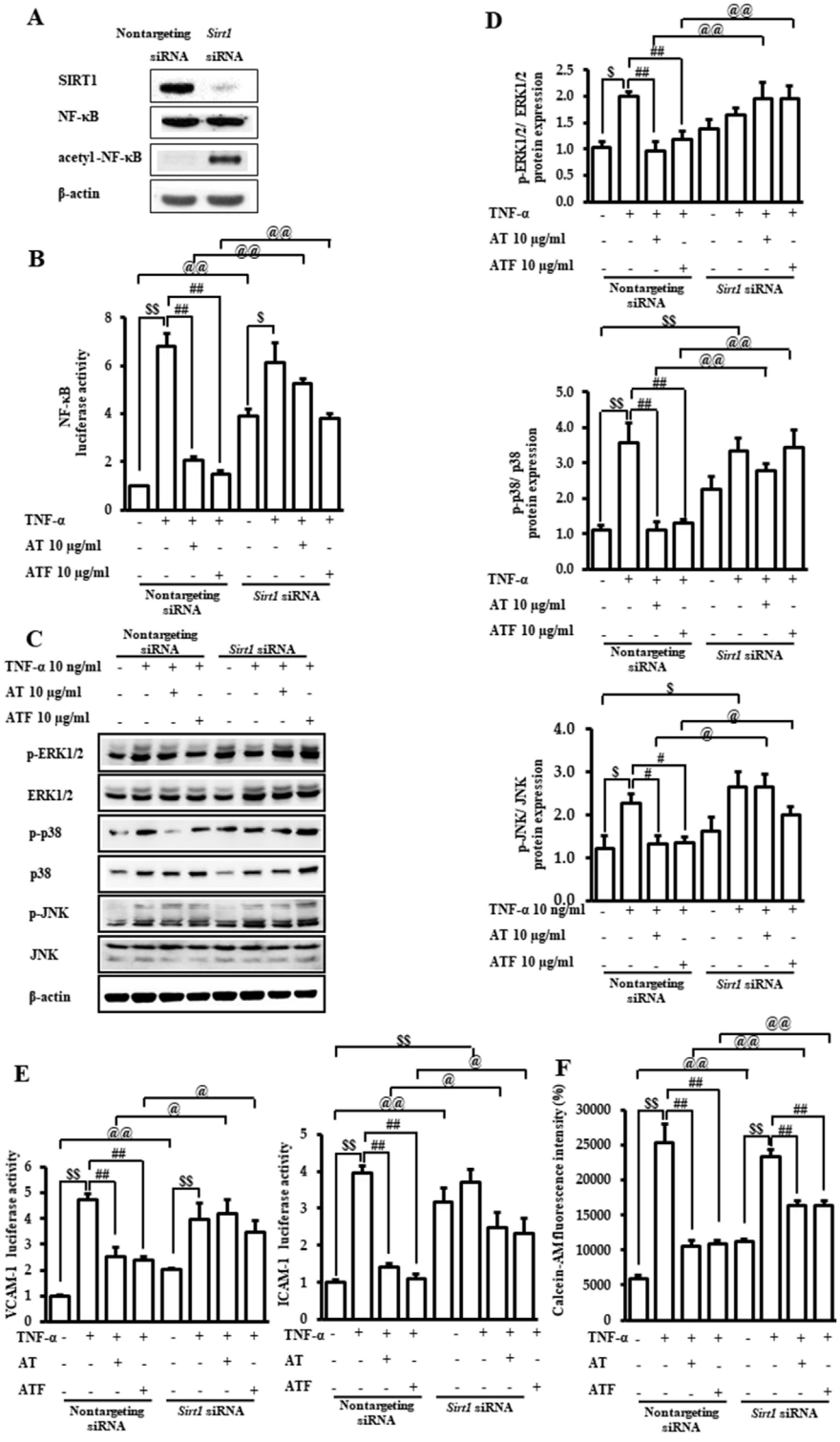

FIGURE 8
Role of SIRT1 in the protective effect of AT and ATF against TNF-α. Cells were transiently transfected with either non-targeting siRNA or *Sirt1* siRNAs. **(A)** Lysates were subjected to immunoblotting using anti-SIRT1, anti-NF-κB, and anti-p65 acetyl K310 antibodies. **(B)** Cells were pretreated with AT or ATF at the indicated concentrations for 1 h and then exposed to TNF-α for 18 h. NF-κB transactivation was analyzed with or without *Sirt1* gene knockdown using a reporter gene assay, as described in Figure 5A. Data were obtained from multiple analyses (n = 3). **(C)** Cells were incubated with AT or ATF for 1 h, followed by treatment of TNF-α for the indicated times, as shown in Figure 4. The representative expression levels of phosphorylated and total MAPKs in cell lysates were detected by Western blotting. **(D)** Graphs representing the relative densitometric ratio of each p-ERK, p-p38 MAPK, and p-JNK were normalized to their respective total EKR, p38 MAPK, and JNK levels (n = 4). Data were obtained from multiple analyses (n = 4). **(E)** VCAM-1 and ICAM-1 luciferase activities were measured, as described in Figure 2B. Data were obtained from multiple analyses (n = 3). **(F)** Attachment of monocytes to
(*Continued*)

FIGURE 8 (Continued)
endothelial cells was evaluated based on fluorescence intensities, as shown in Figure 1. Data were obtained from multiple analyses (n = 3). ($^{\$}p < 0.05$, $^{\$\$}p < 0.01$ for control vs. TNF-α; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ for TNF-α vs. candidate-treated group; $^{@}p < 0.05$, $^{@@}p < 0.01$ for each candidate between non-targeting siRNA group and *Sirt1* siRNA group).

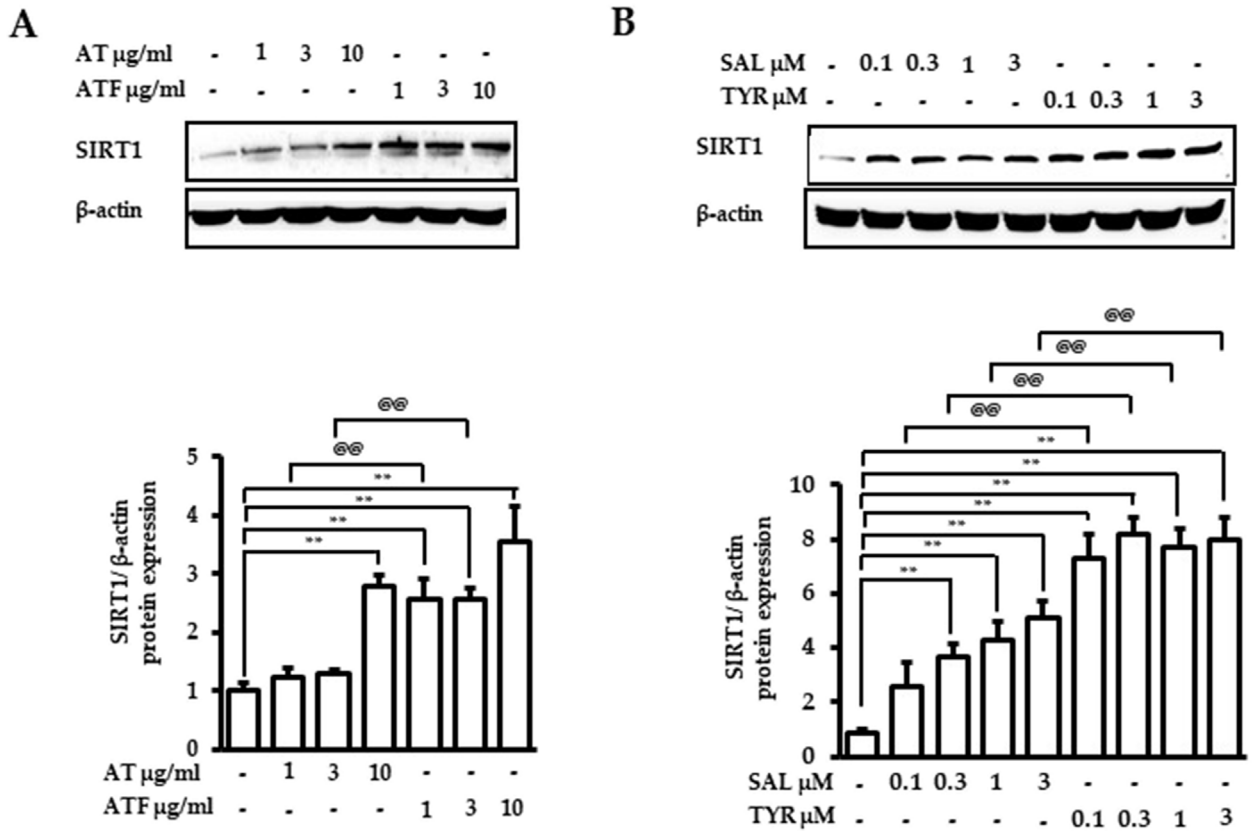

FIGURE 9
Effects of AT, ATF, SAL, and TYR on the SIRT1 expression. Cells were treated with **(A)** AT, ATF, **(B)** SAL, and TYR at the indicated concentration for 24 h. Cells lysates were used for Western blot. The representative blots of SIRT1 are shown in the upper panel, and graphs indicate relative densitometric intensities obtained from multiple analyses (n = 4). ($^{**}p < 0.01$ for control vs. candidate-treated group; $^{@@}p < 0.01$, for the same concentration between each candidate).

3.5 Inhibition of TNF-α-induced NF-κB acetylation by AT and ATF via the NF-κB and SIRT1 interaction

NF-κB posttranslational modification is a critical step in regulating inflammatory gene expression (Chaturvedi et al., 2011). SIRT1, an NAD^+-dependent deacetylase, binds to NF-κB and inhibits transcriptional activity of NF-κB by deacetylating the RelA/p65 subunit at Lys^{310} (Yeung et al., 2004). Therefore, we tested whether SIRT1 was involved in AT or ATF-mediated NF-κB inhibition. NF-κB was bound to SIRT1 in the resting state, which failed to induce NF-κB transcriptional activity. The NF-κB/SIRT1 complex was dissociated in response to TNF-α, which was inhibited by AT or ATF with high potency in ATF (Figures 7A, B). TNF-α exposure resulted in the release of NF-κB from SIRT1 and increased acetylation of NF-κB at Lys^{310}. AT or ATF inhibited the acetylation of Lys^{310} by promoting the interaction between NF-κB and SIRT1, thereby maintaining NF-κB in a deacetylated state. ATF significantly maintained the binding of NF-κB and SIRT1, implying that ATF exhibited a stronger effect in enhancing the NF-κB/SIRT1 interaction than AT did. Thus, the inhibitory effect of AT and ATF on NF-κB transactivation is due to the suppression of acetylated p65, which is attributed to SIRT1-mediated posttranslational modifications of NF-κB.

3.6 The role of SIRT1 in the protective effects of AT and ATF against TNF-α exposure

The role of SIRT1 in the AT and ATF against TNF-α-mediated MAPK/NF-κB signaling pathway and the regulation of adhesive molecules were evaluated by *Sirt1* gene knockdown. Silencing *Sirt1*

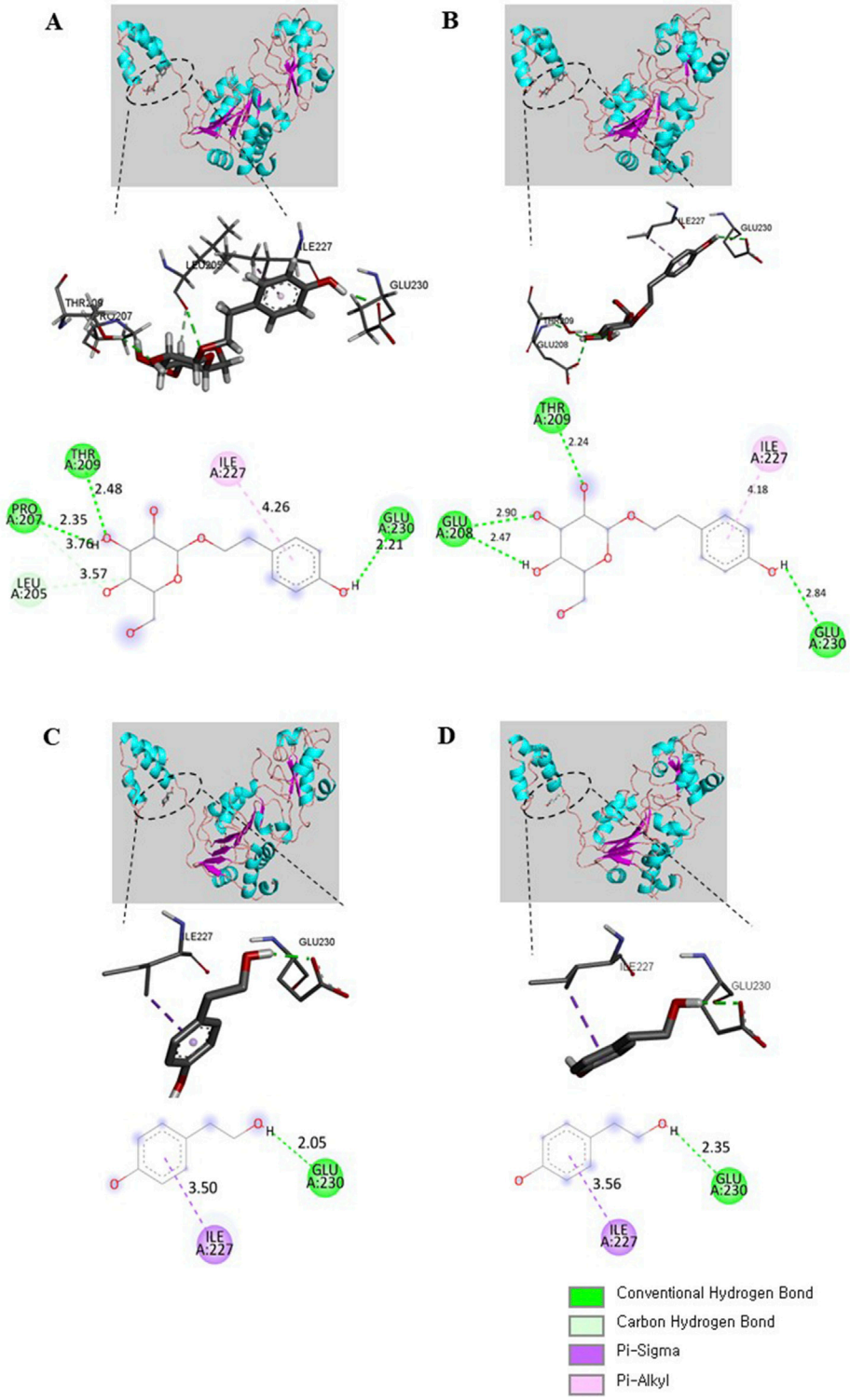

FIGURE 10
SAL and TYR binding to SIRT1 NTD. The binding modes of SAL with **(A)** 4ZZH and **(B)** 4ZZJ or TYR with **(C)** 4ZZH and **(D)** 4ZZJ are illustrated. The interactions of SAL or TYR on the crystal structures are displayed in 3D and 2D diagrams. Deep green, conventional hydrogen bond; light green, carbon hydrogen bond; deep purple, pi–sigma bond; light purple, pi–alkyl bond.

significantly reduced its expression (Figure 8A). SIRT1-deficient cells increased NF-κB transactivation along with K310 acetylation of RelA/p65 in the absence of TNF-α, which is consistent with previous findings (Nguyen et al., 2019). The inhibitory effects of AT and ATF on NF-κB transactivation in response to TNF-α were diminished in SIRT1-deficient cells compared to the control group, indicating that SIRT1 is required for AT and ATF to suppress NF-κB acetylation (Figure 8B). Additionally, the differential regulation of MAPK

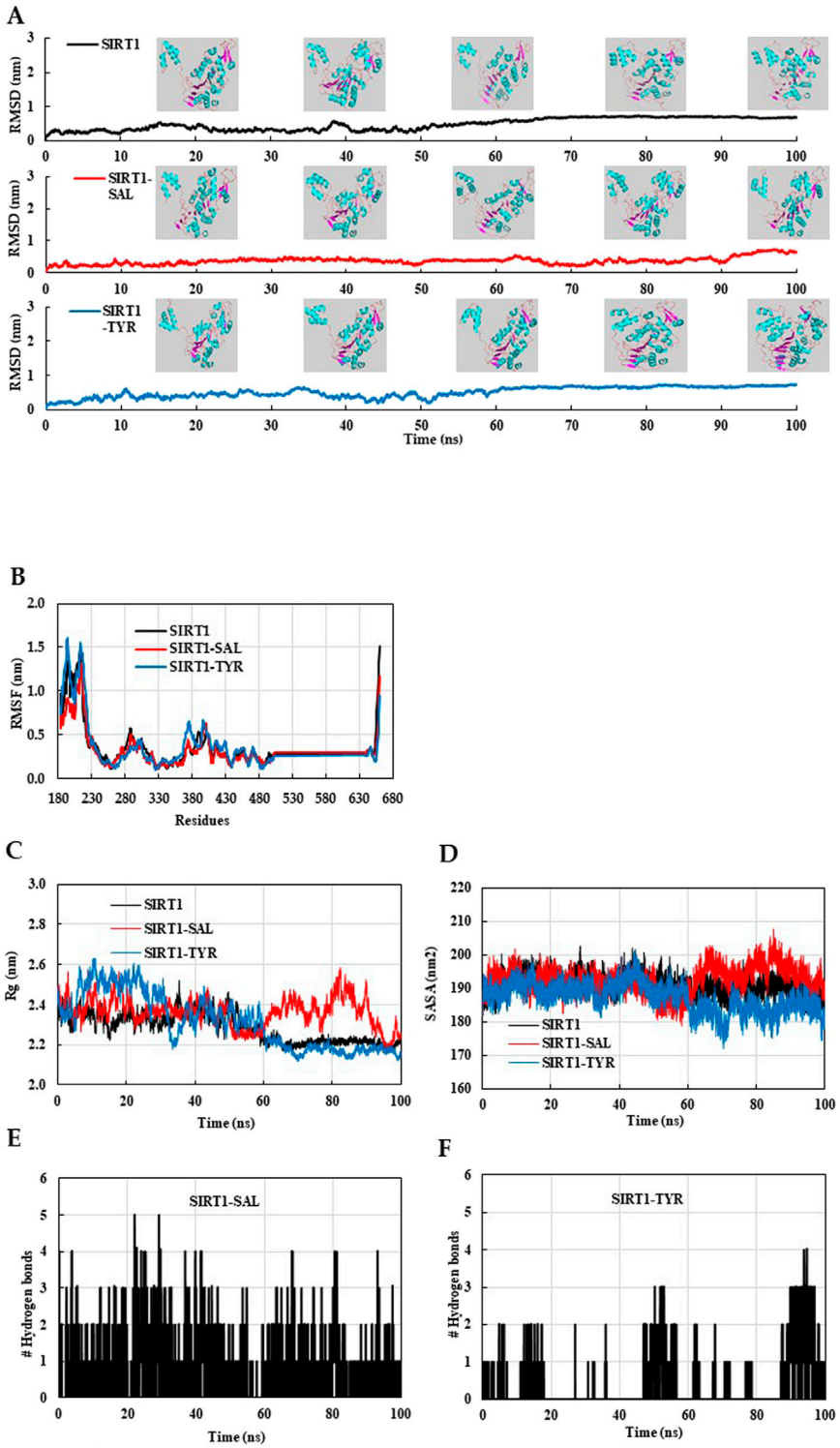

FIGURE 11
MD simulation analysis of native SIRT1, SIRT1–SAL and SIRT1–TYR models. The final MD trajectories of native SIRT1, SAL bound SIRT1, and TYR bound SIRT1 were analyzed over a 100-ns simulation. **(A)** RMSD plot of the SIRT1 in complex with SAL and TYR. **(B)** Residual fluctuation (RMSF) plot of SIRT1 before and after SAL and TYR binding. **(C)** Time evolution of the radius of gyration (Rg), **(D)** solvent-accessible surface area (SASA) plot, **(E)** Hydrogen bonds of the SAL–SIRT1 complex, and **(F)** Hydrogen bonds of the TYR–SIRT1 complex.

phosphorylation by AT and ATF was attenuated in *Sirt1* siRNA-transfected cells (Figures 8C, D). In these cells, AT and ATF also failed to inhibit TNF-α-induced VCAM-1 and ICAM-1 transactivations (Figure 8E). Furthermore, *Sirt1* silencing mitigated the inhibitory effects of AT and ATF on TNF-α-induced adhesion, as measured by the fluorescent calcein-AM

TABLE 1 MD parameters for native SIRT1, SAL–SIRT1, and TYR–SIRT1 systems.

Complex	Average RMSD (nm)	Average RMSF (nm)	Average Rg (nm)	Average SASA (nm^2)
4ZZJ	0.482	0.390	2.285	191.176
SAL–4ZZJ	0.373	0.343	2.364	192.455
TYR–4ZZJ	0.510	0.400	2.315	187.198

signal on endothelial cells, which is consistent with NF-κB regulation (Figure 8F). These findings suggest that SIRT1 plays a critical role in the protective effects of AT and ATF against TNF-α-mediated vascular inflammation.

3.7 SIRT1 targeting of AT, ATF, and their bioactive compounds

We found that SIRT1 is an important target of AT or ATF for regulating the anti-inflammatory responses. We next determined SIRT1 expression by AT, ATF, SAL, or TYR after 24 h. Compared to AT, ATF significantly increased the expression of SIRT1 by 3.5-fold (Figure 9A). SAL and TYR also exhibited 5-fold and 8-fold upregulation of SIRT1 protein expression, respectively, supporting the idea that these candidates increase both SIRT1 activity and SIRT1 expression (Figure 9B).

We next performed molecular docking to analyze how SAL or TYR (the active compounds in AT and ATF) interacts with SIRT1. The multiple alignment results of hSIRT1 and two SIRT1 homologous residues (PDB IDs 4ZZH and 4ZZJ) indicate that all structures show strong homology (>90%) with the NTD and the catalytic domains of hSIRT1 (Supplementary Figure 3). The interaction of bioactive compounds with the allosteric region of the NTD can initiate conformational changes, leading to stabilization and induction of deacetylase activity of SIRT1 (Bonkowski and Sinclair, 2016). Hence, we generated molecular docking of the NTD region of SIRT1 with SAL or TYR, and detailed interactions were illustrated; all four docking results showed the same binding pocket regions (Figure 10). The glycol moiety of SAL interacted with SIRT1 via hydrogen bonds to Leu^{205}, Pro^{207}, Thr^{209} (4ZZH), Glu^{208}, and Thr^{209} (4ZZJ); tyrosol residue of SAL showed interactions with Ile^{227} and Glu^{230} (Figures 10A, B). Interestingly, TYR also interacted with 4ZZH and 4ZZJ at Ile^{227} and Glu^{230}, which were identical to the tyrosol residue of SAL (Figures 10C, D). These docking data indicate that the tyrosol moiety in both SAL and TYR bind to the same binding pocket of SIRT1 and that the binding distance toward SIRT1 is closer in TYR than in SAL, which could potentially regulate SIRT1 activity.

3.8 Molecular dynamics (MD) analysis of the docked SIRT1–SAL and SIRT1–TYR models

We evaluated the structural stability and dynamic behavior of ligand–protein complexes using MD simulation. The RMSD plot showed that the initial fluctuations of native SIRT1 and SIRT1–TYR persisted up to 50 ns, and then, the structures stabilized up to 100 ns (Figure 11A). Interestingly, the SIRT1–TYR complex showed movement of the allosteric region toward the catalytic region of SIRT1 after 80 ns, suggesting that TYR's interaction with SIRT1 NTD may contribute to SIRT1 activation (Figure 11A, inset). The average RMSD and RMSF of SIRT1–SAL were 0.373 nm and 0.343 nm, respectively, which were lower than those of SIRT1–TYR (Table 1). The initial fluctuations in the RMSF plot of combined trajectories may be due to the random interactions in the NTD region. TYR interacted with the SIRT1 NTD, vibrating more strongly than SAL. The slight vibration of the TYR–SIRT1 complex in the catalytic region indicates the role of TYR in the regulation of SIRT1 interaction (Figure 11B). Next, the trajectories of the MD simulation were analyzed by Rg, SASA, and the number of hydrogen bonds. The Rg plot revealed distinct deviation patterns between the SAL–SIRT1 and TYR–SIRT1 complexes (Figure 11C). The Rg values of SIRT1–SAL and SIRT1–TYR were unstable during the initial 40 ns. After 60 ns, the Rg of SIRT1–TYR stabilized and remained below 2.2 nm, which was lower than the Rg of SIRT1–SAL (2.2–2.6 nm). The mean SASA value of SIRT1–TYR was the lowest compared to native SIRT1 and SIRT1–SAL (Table 1). The SASA plot showed that SIRT1–SAL fluctuated after 60 ns, suggesting slight flexibility of SIRT1 upon ligand binding. In contrast, the SASA value of SIRT1–TYR significantly decreased compared to that of SIRT1–SAL at the end of the simulation (after 60 ns) (Figure 11D). SAL exhibited a higher number of hydrogen bonds with SIRT1, with a maximum of five bonds, while TYR showed a more variable number of hydrogen bonds throughout the simulation (Figures 11E, F). These MD results revealed important insights into the interaction of SAL and TYR with SIRT1, suggesting that SAL and TYR have different interaction mechanisms with SIRT1.

3.9 Effects of AT and ATF on the STZ-treated mouse retina

Diabetic retinopathy (DR) is characterized by retinal nerve deformation and microcirculatory disturbances caused by an inflammatory process (Gerhardinger et al., 2005; Yang et al., 2022). Hyperglycemia induces adhesive molecules like VCAM-1 and ICAM-1, which contribute to damage in the retina, the neuro-vascular coupling tissue. Therefore, we evaluated the levels of VCAM-1 and ICAM-1 in STZ-treated retinal sections. STZ induced VCAM-1 in the inner plexiform layer of the retina (Figures 12A, C). AT and ATF attenuated STZ-induced VCAM-1 expression. ICAM-1 expression increased in the retinal nerve fiber layer of the STZ-treated retina sections, and AT and ATF moderately inhibited ICAM-1 distribution in this layer, with ATF showing a more potent effect (Figures 12B, C). These results suggest that AT and ATF reduce STZ-induced diabetic retina damage by inhibiting adhesion molecules.

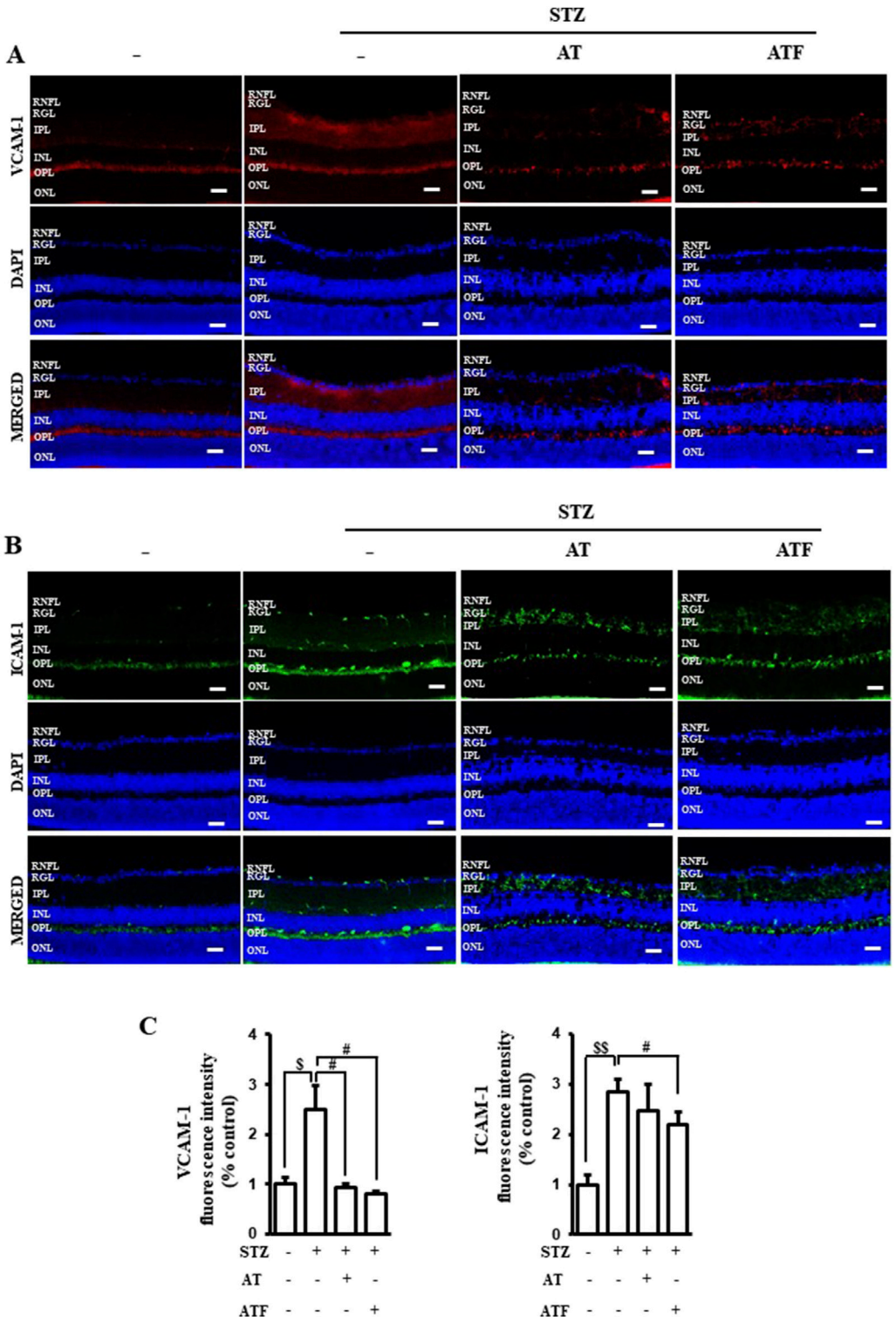

FIGURE 12
Effects of AT and ATF on STZ-treated retinal adhesion molecules. Representative immunostainings of **(A)** VCAM-1 and **(B)** ICAM-1 in retinal sections from STZ-induced or STZ + AT and STZ + ATF (50 mg/kg)-treated groups are shown. **(C)** Quantifications of VCAM-1 and ICAM-1 positive intensity in the retina are shown. Nuclei were identified using 4′,6-diamidino-2-phenylindole (DAPI) (blue). STZ, streptozotocin; RNFL, retinal nerve fiber layer; RGL, retinal ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer. Scale bar 100 μm.

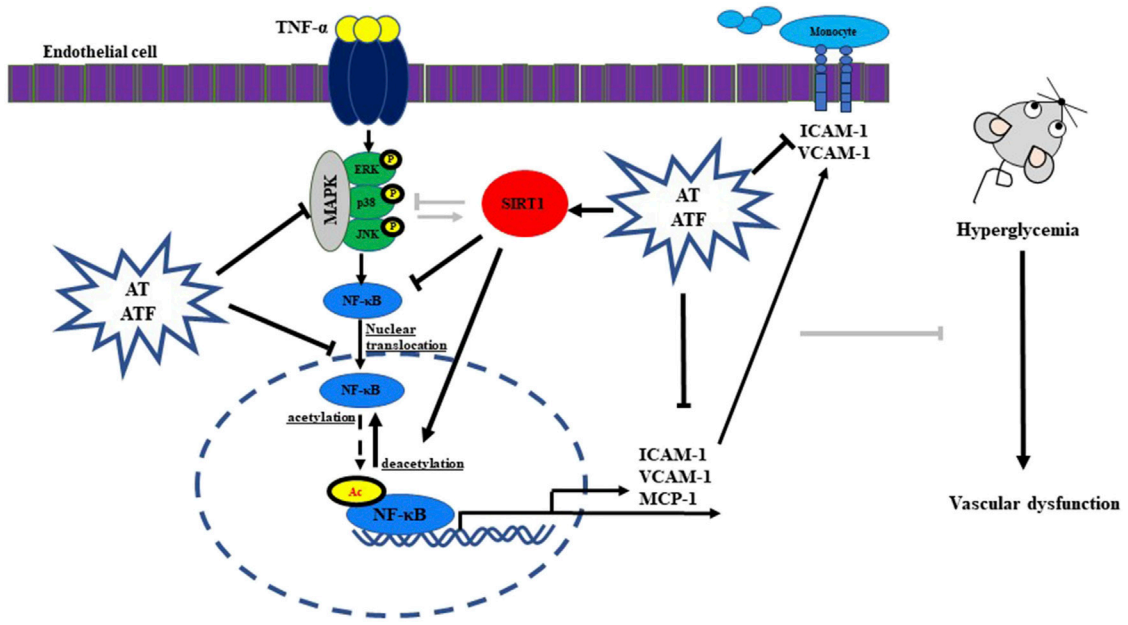

FIGURE 13
Schematic summary of the protective effects of AT and ATF.

4 Discussion

AT is a medicinal herb used in Asian countries to treat various diseases (Yu et al., 2010; Park et al., 2019; Cho et al., 2020; Bae et al., 2022). AT exhibits antioxidant, anti-inflammatory, and anti-lipogenic properties and outstanding protective effects against liver diseases (Yu et al., 2010; Park et al., 2019). Recently, we reported that AT significantly blocked cholestasis-induced liver damage via the inhibition of inflammation and fibrosis (Bae et al., 2022). In hepatic diseases such as hepatitis and cirrhosis, elevated systemic and hepatic TNF-α levels have been linked to vascular dysfunction (Meng et al., 2022). Endothelial cells in a vessel are a major component cell type involved in vascular function since they act as the first barrier against injury (Kitada et al., 2016). Endothelial dysfunction is involved in vasoinflammation, leading to complications such as retinal microvascular damage (Lenin et al., 2018; Ouyang et al., 2024). No studies have been reported on whether AT exerts an inhibitory effect on vascular inflammation. The present study is the first to assess the protective effect of AT against TNF-α-induced endothelial inflammation.

Inflammatory processes, including monocyte adhesion to endothelial cells, are considered early key events and critical steps in response to stimuli (e.g., TNF-α) (Nguyen et al., 2019). AT suppressed TNF-α-induced monocyte adhesion to endothelial cells at the IC_{50} value of 1.62 μg/mL. SAL, the main compound of AT, also suppressed adhesion with the IC_{50} value of 0.94 μM, indicating that SAL acts as a bioactive compound in AT. Surprisingly, TYR showed higher potency than SAL against TNF-α-induced adhesion (IC_{50} = 0.14 μM). SAL constitutes 6%–8% of the extracts obtained from various parts of the AT plant (Hwang et al., 2013; Park et al., 2019). The TYR content in AT is much less compared to SAL (Hwang et al., 2013). Based on the IC_{50} (potency) differences between SAL and TYR, we considered the possibility that the antiadhesive effect of AT could be improved by converting SAL of AT to TYR. Fermented soybeans with *B. subtilis* resulted in the highest concentration of isoflavone aglycones and, thus, a decrease in isoflavone glucosides (Huynh et al., 2014). *Bacillus subtilis* shows potential to release phenolic acids and flavonoids from plant sources, enhancing the yield of polyphenols. Additionally, it can produce β-glucosidase, which facilitates the deglycosylation of SAL. Therefore, we carried out AT fermentation using *B. subtilis*. AT fermentation resulted in an increase in TYR and a decrease in SAL. The adhesion assay indicated that ATF with an IC_{50} value of 0.49 μg/mL is better in inhibiting monocyte adhesion to endothelial cells compared to AT with an IC_{50} value of 1.62 μg/mL. These findings suggest that ATF may be a promising protective product against vasoinflammation.

Most herbal medicinal plants containing polyphenols are administered orally. Plant phenols often exist as glycosides, which have the potential of deglycosylation, producing its aglycone metabolites in the gastrointestinal system *in vivo*. The content of SAL is the active standard component to evaluate the quality of AT (Hwang et al., 2013). In the pharmacokinetic study, oral administration of SAL *in vivo* failed to show deglycosylation of SAL into TYR, whereas TYR was identified after intravenous injection of SAL (Guo et al., 2012). The aglycone metabolite, TYR, has potent pharmacological properties, but it is difficult to be biotransformed from SAL of AT in the body. These findings are consistent with previous reports showing that only 2% SAL exists in plasma as the aglycone metabolite TYR (Guo et al., 2012). In this current study, the fermentation processes with *B. subtilis* resulted in microbial conversion. We focused on the conversion of SAL (the main compound in AT) to TYR, although various metabolites can be produced by microbial fermentation. The stability of SAL and TYR is stable without degradation under all storage conditions, suggesting that those substances are resistant to breakdown in the fermented solution (Guo et al., 2012).

Vascular inflammation could be initiated when monocytes circulating in the bloodstream are recruited to sites of inflammation in the vascular endothelium (Nguyen et al., 2019). AT and ATF significantly suppressed TNF-α-induced transactivation and expression of VCAM-1 and ICAM-1 in human endothelial cells. The production of MCP-1, a proinflammatory chemokine, was also significantly inhibited by AT and ATF. Chemokines are secreted in response to signals such as pro-inflammatory cytokines (including TNF-α), where they play an important role in selectively recruiting monocytes (Nguyen et al., 2019). These results suggest that vascular endothelial cells are a major action site of AT and ATF to prevent the early stages of vascular inflammation. ATF is more effective in blocking TNF-α-induced adhesion molecules and MCP-1 in endothelial cells. This enhanced anti-inflammatory effect of ATF seems to be due to the pharmacological potency of TYR and its longer $T_{1/2}$ than SAL (Guo et al., 2012).

Multiple MAPK signaling cascades operate in a large modular network that regulates inflammation (Schulze-Osthoff et al., 1997). MAPKs, including ERK, p38 MAPK, and JNK, are phosphorylated by cytokines including TNF-α, which is associated with inflammatory gene expression. We found that TNF-α increased phosphorylated ERK and p38 MAPK at early time points and JNK phosphorylation at a late time point in endothelial cells, which is consistent with a previous report (Lo et al., 2014). AT and ATF significantly inhibited TNF-α-mediated ERK and p38 MAPK phosphorylation. However, AT and ATF showed a weak inhibitory effect on TNF-α-mediated JNK activation, suggesting that these candidates differentially regulate the MAPK pathway. AT itself increased MAPK phosphorylation in hepatocytes, whereas AT significantly suppresses ERK and p38 MAPK in endothelial cells, demonstrating the controversy of AT in the regulation of the MAPK pathway (Kim et al., 2015; Zou et al., 2015; Park et al., 2019). These results claim that AT may differentially affect MAPKs depending on the cell type and organ.

NF-κB is a key transcriptional factor that regulates inflammatory genes (Liu et al., 2017). The NF-κB motif in the promoters of ICAM-1, VCAM-1, and MCP-1 is particularly important for the induction of these genes (Rothgiesser et al., 2010; Meng et al., 2022). AT and ATF inhibited the TNF-α-induced nuclear translocation and transcriptional activity of NF-κB. The transcriptional activity of NF-κB is regulated by RelA/p65 posttranslational modifications such as phosphorylation, methylation, ubiquitination, and acetylation (Yang et al., 2010). Among these, the acetylation at Lys^{310} of RelA/p65 is crucial because it is required for the full transcriptional activity of NF-κB (Chen et al., 2002). SIRT1, a deacetylase enzyme, selectively interacts with RelA/p65 to mediate the deacetylation of Lys310 in RelA/p65, thus inhibiting NF-κB transcriptional activity (Yeung et al., 2004). We previously reported that TNF-α-stimulated NF-κB activation was regulated by the interaction between NF-κB and SIRT1 (Nguyen et al., 2019). In this study, AT and ATF reduced the TNF-α-induced dissociation of the NF-κB/SIRT1 complex, resulting in deacetylation of RelA/p65 and a subsequent decrease in NF-κB transcriptional activity. ATF showed stronger effect at lower doses compared to AT. These results suggest that SIRT1, a transcriptional regulator of NF-κB, may serve as a direct target of AT and ATF. Additionally, we confirmed the role of SIRT1 in the inhibition of NF-κB and inflammatory responses by AT and ATF using the *SIRT1* gene knockdown experiment.

Overexpression of SIRT1 and treatment with STAC improves the physiological function of SIRT1 (Bonkowski and Sinclair, 2016; Chen et al., 2024). AT and ATF significantly upregulated SIRT1 expression, with higher levels in ATF. TYR significantly increased SIRT1 expression, with higher potency and efficacy than SAL. The enhanced increase of SIRT1 expression by ATF and TYR supports the idea that the tyrosol moiety may be important for increasing the activity of SIRT1 and stabilizing its deacetylase activity.

The proven protective role of increased SIRT1 activity in various dysfunctions makes this enzyme a promising therapeutic target (Dai et al., 2015). A single point mutation of the Glu^{230} residue of NTD in SIRT1 has been shown to impair activation by direct allosteric activation of SIRT1 or tight substrate binding of SIRT1 and substrate by STAC, demonstrating the importance of the NTD and the Glu^{230} residue for SIRT1 activation by STAC (Dai et al., 2015; Hou et al., 2016; Liu et al., 2023). The SIRT1 NTD can physically modulate the association of SIRT1 and NF-κB, thereby decreasing the acetylation of NF-κB and suppressing inflammation (Ghisays et al., 2015). Medical plants selected as the SIRT1 activator form hydrophobic interactions with Ile^{223} and Ile^{227}, while nicotinamide (as a SIRT1 inhibitor) fails to interact with Ile^{227}, suggesting that Ile^{223} and Ile^{227} could be key residues (Azminah et al., 2019). In molecular docking, the hydroxyl groups of SAL or TYR form hydrogen bonds with the side chain of the Glu^{230} residue in NTD of SIRT1. The distances of TYR toward Ile^{227} and Glu^{230} residues of SIRT1 were closer than those observed for the tyrosol moiety of SAL, suggesting that TYR might have a higher binding affinity for the SIRT1 NTD. MD simulations showed that SAL forms strong interactions with the NTD of SIRT1, stabilized by multiple hydrogen bonds. Interestingly, while TYR forms fewer hydrogen bonds with SIRT1, its secondary structure remains more compact, indicating a potential resistance to unfolding in solvent. The sustained stabilities of the SAL-bound and TYR-bound SIRT1 complexes indicate that both SAL and TYR interact favorably with the allosteric region of SIRT1, inducing conformational changes that enhance its deacetylase activity. The SIRT1–TYR complex exhibited a shift in the allosteric region toward the catalytic region of SIRT1 and displayed high fluctuation in the catalytic region of SIRT1. These results suggest that the interaction with TYR increases the flexibility of this region, leading to conformational changes that promote SIRT1 activity.

Notably, the association of NF-κB/SIRT1 and the deacetylation of p65 by ATF were observed to be higher than AT. This study suggests that TYR may be more potent in regulating SIRT1 activity, based on both structural and biochemical evidences. Further investigation is required to assess whether TYR activates SIRT1 toward the substrate RelA/p65, which may be crucial for the tyrosol moiety-dependent stimulation of RelA/p65 deacetylation. We plan to explore this in our upcoming research.

Diabetic retinopathy is a vascular disorder of the retina (Gerhardinger et al., 2005). STZ-induced hyperglycemia triggers the activation of the vascular endothelial layer of the inner blood–retinal barrier, leading to impairment of retina, a neurovascular coupling tissue (Lenin et al., 2018). In the present study, AT and ATF suppressed STZ-induced retinal adhesion molecules, consistent with the findings of cell studies. These

results suggest a potential protective role on the increased leukocyte adhesion and transmigration across the blood–retinal barrier into the retinal tissue of these candidates against diabetic retinopathy. Expanding this research to clinical trials focused on diabetic retinopathy would be an important next step to confirm the validity of these finding.

5 Conclusion

We demonstrated that AT and ATF exert a protective effect against TNF-α-induced endothelial dysfunction. AT and its compounds, salidroside and tyrosol, attenuate TNF-α-mediated monocyte-endothelial cell adhesion and inhibit key biomarkers of endothelial inflammation, including adhesion molecules (VCAM-1 and ICAM-1) and the pro-inflammatory cytokine MCP-1 and NF-κB transactivation via an interaction with SIRT1. Notably, fermentation of AT with *B. subtilis* converts salidroside into tyrosol, which exhibits greater potential in suppressing endothelial inflammation (Figure 13). These findings have important implications for fermentation strategies aimed at producing high-value extracts from plants, such as ATF, with potential effects in inhibiting vascular inflammation. Additionally, our results reveal that the tyrosol moiety of salidroside and tyrosol targets the Glu^{230} residue of SIRT1 NTD, which inhibits NF-κB acetylation. These insights support the rational design of new candidates targeting the Glu^{230} residue in SIRT1 NTD to inhibit endothelial inflammation and related complications in metabolic syndrome.

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 Dongguk University (protocol number IACUC-2023-15). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

PN: writing–original draft, data curation, methodology, validation, and visualization. JW: data curation, validation, formal analysis, investigation, and writing–review and editing. MC: conceptualization, funding acquisition, supervision, writing–original draft, and writing–review and editing.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2024.1392179/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Cell viabilities of **(A)** AT, ATF, **(B)** SAL, and TYR. Cell viability was measured by MTT assay. The data obtained from multiple analyses (n = 3).

SUPPLEMENTARY FIGURE 2
Representative HPLC chromatograms of AT and ATF. AT, ATF samples, and standard controls (SAL and TYR) were separated using HPLC. Column: C18 (4.6 mm × 250 mm, 5 μm particle size); temperature: 25°C; mobile phase: 0.1% formic acid in H_2O **(A)** and MeOH 100%, flow rate: 1.0 mL/min; detection: λ 225/ 278 nm.

SUPPLEMENTARY FIGURE 3
Homology modeling of SIRT1. **(A)** Schematic diagram showing functional domains in full-length hSirt1. **(B)** Multiple sequence alignment of the hSIRT1 sequence and PDB IDs 4ZZH and 4ZZJ. NTD, N-terminal domain; CTD, C-terminal domain.

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