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Plant metabolites in drug discovery: the prism perspective between plant phylogeny, chemical composition, and medicinal efficacy, volume III

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Plant metabolites in drug discovery: the prism perspective between plant phylogeny, chemical composition, and medicinal efficacy, volume III

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Editorial: Plant metabolites in drug discovery: the prism perspective between plant phylogeny, chemical composition, and medicinal efficacy, volume III

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Editorial on the Research Topic
Plant metabolites in drug discovery: the prism perspective between plant phylogeny, chemical composition, and medicinal efficacy, volume III

There is a Chinese saying that goes, "The most profound takes the simplest form." The phylogenetically close taxa usually have similar phytometabolite profiles, and taxa that are closely related in chemotaxonomy often have analogous ethnopharmacological uses and similar bioactivities. This is the simple core idea of "Pharmacophylogeny," and the proposed term "Pharmacophylomics" aims to disentangle the intricate relationships and connectivity between medicinal plant phylogeny, phytochemical constituents and bioactivities/therapeutic utilities based on emerging omics data (Figure 1) (Hao and Xiao, 2023); the genomic, transcriptomic and metabolomic data are very useful to promote pharmaceutical resource discovery and plant-based drug R&D. In recent years, more phytomedicine researchers become familiar with the theory and methods of pharmacophylogeny (Moutouama and Gaoue, 2024), but the research on the simultaneous examination of phylogeny/phylogenomics, chemical constituents, and bioactivity is still limited. Based on the 21 papers published in Volume I and II of this Research Topic (Hao et al., 2023a), volume III further contributed eight enlightening papers on the phylogenomics, metabolomics, network pharmacology, ethnopharmacology and bioactivity of various medicinal species, covering algae (Su et al.), monocot (Bencheikh et al.; Luo et al.), basal eudicot (Bencheikh et al.; Pan et al.), core eudicot (Ding et al.), Lamiids (Liu et al.) and Campanulids (Luo et al.). These works provide rich phytometabolite and bioactivity information for deeper exploration on pharmacophylogeny, facilitating the analysis of distribution patterns of various medicinal compounds and pharmacological activities on the phylogenetic tree (Hao et al., 2024a), the inference of biosynthetic pathways and

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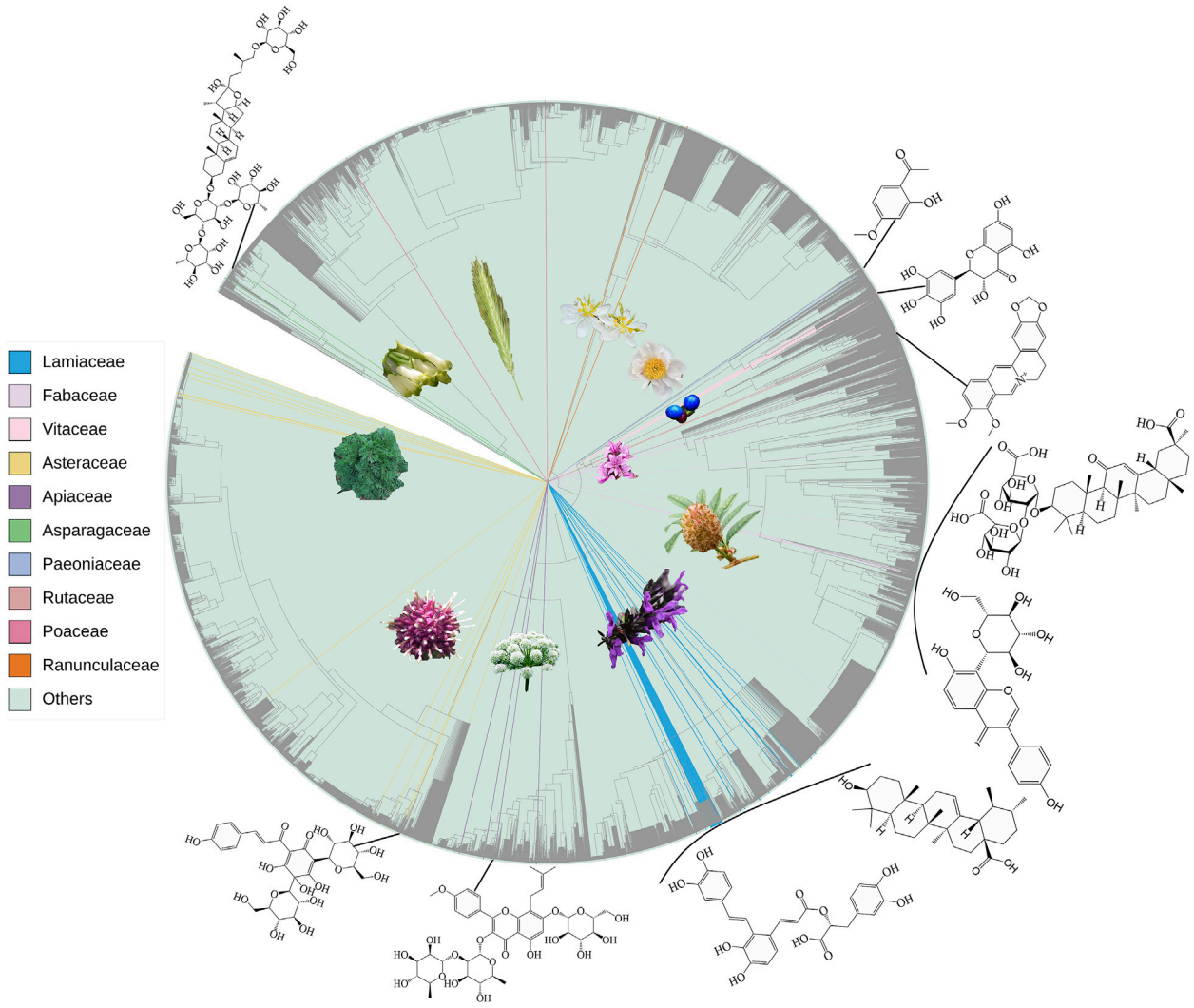

FIGURE 1
Phylogenetic distribution of botanical taxa, reported in Vol III of this Research Topic, as well as representatives of medicinal phytometabolites therein. Among totally 155 species, 85 genera of 44 families, only 118 species, 49 genera of top ten families are shown.

therapeutic mechanisms of phytometabolites, and the search for alternative/complementary medicine sources.

Fermentation is an important processing technique for phytomedicine in transforming and enhancing the active ingredients of herbal medicine via specific microbial processes (Luo et al.). Thus, fermentation adds another dimension to pharmacophylogeny. Similar fermentation strains when used in species with similar phytometabolites—for generating microbial transformations—expands the use of fermented botanical drugs in the areas anti-cancer, hypolipidemic, antioxidant, antimicrobial, cosmetics, and gut microflora regulation. The fermentation technology also reduces toxic effects of some crude drugs, while enhancing drug efficacy (Pan et al.). The puzzling interaction between microbial strains and crude drugs is another intriguing Research Topic. Managing factors affecting the microbial activities and fermentation process is vital for the successful transformation and efficacy improvement of herbal drugs. For example, by assuming that phylogenetically related bacteria and fungi similarly interact with specific plant compounds, one may predict the similarities and differences in the spectrum of transformation products based on the composition of the microbial community. There is much more work to be done in this area, which is not limited to TCM species. Fermentation research can be conducted on all ethnomedicinal species around the world (Bencheikh et al.; Liu et al.) at the genus level. Molecular phylogeny and metabolomic information are essential to understand medicinal value of each microbial/plant genus in developing alternative medicine. This is also applicable within the food medicine continuum (FMC) in ethnomedicinal plants worldwide. Pharmacophylogeny can aid in expand FMC and medicinal plant resources (Hao and Xiao, 2023), authenticate quality control of herbal medicines, predict the chemicals or bioactive constituents of herbs and identify/quantify chemicals. Reports on phytometabolites and pharmacological properties of algae (Su et al.) are relatively

fewer as might be expected by the fewer species number; however, their association with fungi and bacteria in the lichen biome has scarcely been investiaged (Rakotondraibe et al. 2024). In the coming years, pharmacophylogeny and pharmacophylomics can be expected to improve mining of natural products from taxa of various evolutionary levels (Lu and Tang, 2020), refining ethnopharmacology understandings, and therefore advance the sustainable conservation and consumption of natural pharmaceutical resources.

The global medicinal plant diversity is threated by the increasingly intense anthropogenic activities, while phrmacophylogeny and pharmacophylomics help sustainable conservation and utilization of precious phytomedicine resources. Based on the harvest of first two volumes, the volume III (https://www.frontiersin.org/research-topics/62190/plant-metabolites-in-drug-discovery-the-prism-perspective-between-plant-phylogeny-chemical-composition-and-medicinal-efficacy-volume-iii) strives to gain a deeper understanding of phylogeny/evolution, phytometabolites and poly-pharmacology of genera/families of interest. We always advocate to conduct such investigations within the context of pharmacophylogeny and pharmacophylomics, and we are happy to publish such comprehensive work in this volume. The Lamiaceae genus *Dracocephalum*, with more than 30 species, possesses diverse medicinal activities and is traditionally used in Eurasian ethnomedicine (Liu et al.). The geographical distribution, metabolite identification, and bioactivity of *Dracocephalum* species were extensively investigated, but there are debates on the taxonomy of *Dracocephalum* and closely related genera *Hyssopus* and *Lallemantia*, which presents an opportunity for pharmacophylogenetic studies of these medicinal taxa. Liu et al. present a multidimensional view of the geographical distribution, phylogenetics, phytometabolites and chemodiversity, ethnopharmacological uses, and pharmacology of *Dracocephalum*, *Hyssopus*, and *Lallemantia*. The species in the latter two genera are concentrated in southwest Asia and those in *Dracocephalum* are distributed across temperate northern hemisphere. Although all three genera are closely related phylogenetically, the species of *Hyssopus* were intertwined with those of *Dracocephalum* on the phylogenetic tree. Among more than 900 reported phytometabolites of three genera, terpenoids and flavonoids are the most abundant. The newly identified novel metabolites of *Dracocephalum* expand chemical space to be bioprospected. Ethnopharmacologically, these three genera are especially useful in treating respiratory, liver and gall bladder diseases. Phytometabolites of these genera have various bioactivities such as hepatoprotective, anti-inflammation, antimicrobial action, anti-hyperlipidemia, and anti-tumor properties. Integrating phylogenetics and network pharmacology enabled exploring the intricate links between metabolite profiles, traditional efficacy, and modern pharmacology of *Dracocephalum* and its related genera. This study illustrates how to discover potential medicinal value from closely related ethnomedicinal taxonomic groups.

Another endeavor in Vol III is reconstructing the phylogenetic tree of *Glycyrrhiza* and related subfamilies of Fabaceae based on the whole chloroplast (cp) genome sequences (Wu et al.). China has eight species of *Glycyrrhiza* (Chen et al., 2020), which can be classified into two sections based on the presence of glycyrrhizic acid: section *Glycyrrhiza* (*G. uralensis* Fisch., *G. glabra* Linn., *G. inflata* Batal., *G. aspera* Pall., *G. eglandulosa* X. Y. Li) and section *Pseudoglycyrrhiza* (*G. pallidiflora* Maxim., *G. squamulosa* Franch., *G. yunnanensis* Cheng f. et L. K. Dai ex P. C. Li). The cp genome-based phylogeny confirmed this classification, and suggested that *G. gobica* Grankina and *G. uralensis* clustered together, *G. laxissima* Vassilcz. and *G. aspera* clustered together, and other taxa, which are treated as synonyms of *G. uralensis*, *G. glabra* or *G. aspera* in Flora of China, should be considered as independent species. The North American species *G. lepidota* had a lower content of glycyrrhizic acid and was in another group, indicating that the groups containing glycyrrhizic acid were not monophyletic, i.e., the incongruence between phylogenomics and chemotaxonomy. The cp genome-based phylogeny also revealed distinct intraspecific divergence in 38 *Artemisia annua* strains (Ding et al.). These results are very beneficial for us to fully understand the complexity of medicinal kinship at various taxonomic levels.

It is expected that the metabolomic analyses and phytometabolite content determination could reveal the overall similarity of phytometabolite profiles between medicinally important species, such as *Astragalus membranaceus* (Li et al.), *Dioscorea opposita*, and *Rehmannia glutinosa*, and phylogenetically related species, and both molecular authentication and chemotaxonomy could be used to discriminate them from common adulterants. On the other hand, the R&D of innovative herbal medicine formulas can also benefit from detailed pharmacophylogenetic studies, as closely related species can be added to the medicine formulas, and various combinations of them can be tried to observe changes in therapeutic effects, which may improve the efficacy of existing herbal formulas or expand novel therapeutic approaches. The cp genome is an useful genetic resource for phylogeny and evolution studies at both species and subspecies/population levels, while the interspecific/intraspecific chemodiversity could lead to development of novel clinical utility.

In summary, the greatest truths are the simplest; taxa in sister phylogenetic groups have relatively similar requirements for ecological environment conditions (Hao et al., 2024b), they have closely related genetic features, and are more likely to evolve analogous biosynthetic pathways, therefore their chemical ammunition depot could be more similar, resulting in the global resemblance of bioactivity or therapeutic efficacy (Hao et al., 2024a). However, if we want to condense scientific hypotheses and practical solutions from the complex phenomena and vast amounts of data, hard work is essential. Integrating ecological and evolutionary factors helps to gain a more inclusive understanding of phytochemical changes in changing environments (Hao et al., 2023b). With any luck the papers in Volumes I-III of Research Topic could serve as valuable references and enhance researchers' cognizance of pharmacophylogeny, and we wish more scholars intentionally leverage pharmacophylomic methods to the conservation, exploration, and utilization of medicinal species.

Author contributions

D-CH: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Project administration, Supervision, Visualization, Writing–original draft, Writing–review and editing. Y-XW: Methodology, Visualization, Writing–original draft. RS:

Resources, Supervision, Writing–review and editing. C-NH: Resources, Writing–review and editing.

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Conflict of interest

Author RWS is employed by World Botanical Associates Inc. as Principal Investigator.

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

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Comparative genomics and phylogenomics of the genus *Glycyrrhiza* (Fabaceae) based on chloroplast genomes

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Glycyrrhiza (Fabaceae) species are rich in metabolites and widely used in medicine. Research on the chloroplast genome of *Glycyrrhiza* is important for understanding its phylogenetics, biogeography, genetic diversity, species identification, and medicinal properties. In this study, comparative genomics and phylogenomics of *Glycyrrhiza* were analyzed based on the chloroplast genome. The chloroplast genomes of six *Glycyrrhiza* species were obtained using various assembly and annotation tools. The final assembled chloroplast genome sizes for the six *Glycyrrhiza* species ranged from 126,380 bp to 129,115 bp, with a total of 109–110 genes annotated. Comparative genomics results showed that the chloroplast genomes of *Glycyrrhiza* showed typically lacking inverted repeat regions, and the genome length, structure, GC content, codon usage, and gene distribution were highly similar. Bioinformatics analysis revealed the presence of 69–96 simple sequence repeats and 61–138 long repeats in the chloroplast genomes. Combining the results of mVISTA and nucleotide diversity, four highly variable regions were screened for species identification and relationship studies. Selection pressure analysis indicated overall purifying selection in the chloroplast genomes of *Glycyrrhiza*, with a few positively selected genes potentially linked to environmental adaptation. Phylogenetic analyses involving all tribes of Fabaceae with published chloroplast genomes elucidated the evolutionary relationships, and divergence time estimation estimated the chronological order of species differentiations within the Fabaceae family. The results of phylogenetic analysis indicated that species from the six subfamilies formed distinct clusters, consistent with the classification scheme of the six subfamilies. In addition, the inverted repeat-lacking clade in the subfamily Papilionoideae clustered together, and it was the last to differentiate. Co-linear analysis confirmed the conserved nature of *Glycyrrhiza* chloroplast genomes, and instances of gene rearrangements and inversions were observed in the subfamily Papilionoideae.

KEYWORDS

***Glycyrrhiza*, Fabaceae, chloroplast genome, comparative genomics, phylogenomics**

1 Introduction

With the fervent progress of genome projects worldwide in the early 1990s, DNA sequences for phylogenetic studies accumulated rapidly. The integration of phylogenetics and genomics gave rise to phylogenomics and comparative genomics (Eisen, 1998). On the one hand, phylogenomics and comparative genomics utilize large-scale molecular data at the genomic level to determine the phylogenetic relationships between species. On the other hand, they integrate these phylogenetic relationships to investigate patterns and mechanisms of genome evolution (Eisen, 1998). The cell organelle genome in eukaryotes mainly includes all DNA molecules contained in mitochondria and plastids (including chloroplast, chromoplast, and leucoplast), serving as the primary carriers of cytoplasmic inheritance. With the emergence and development of DNA sequencing technologies, the organelle genome has become a crucial tool in research areas such as phylogenetics, biogeography, hybridization, and species identification in eukaryotes (Fujii et al., 2010; Smith, 2015). In plants, the chloroplast genome, characterized by a high copy number and moderate molecular substitution rate, has been utilized in systematic studies across various taxonomic ranks. It has achieved notable success in resolving phylogenetic relationships, particularly in addressing challenging taxonomic groups (Wolfe et al., 1987; Drouin et al., 2008).

The chloroplast genome is predominantly a closed circular DNA, typically ranging in size from 115 kb to 165 kb. In most angiosperms, it exhibits a relatively conserved quadripartite structure, consisting of two inverted repeat regions (IRs), a large single-copy region (LSC), and a small single-copy region (SSC), among which the two single-copy

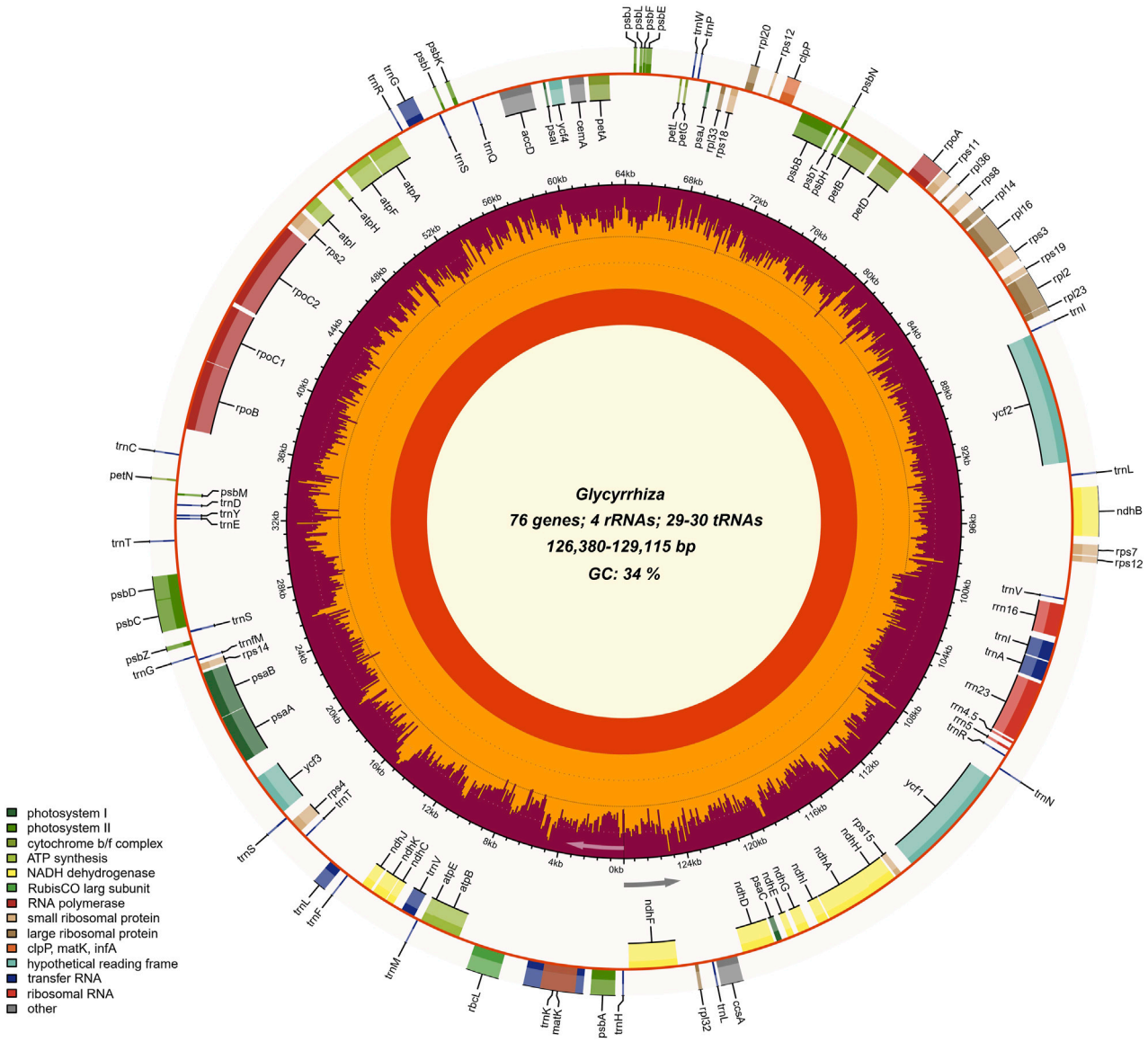

FIGURE 1
Chloroplast genome map of *Glycyrrhiza* species. The gradient GC content of the genome was plotted in the second circle with zero level based on the outer circle. The gene names were labeled on the outermost layer. The gene GC content was depicted with the proportion of shaded areas. Represented with arrows, the transcription directions for the inner and outer genes were listed clockwise and anticlockwise, respectively.

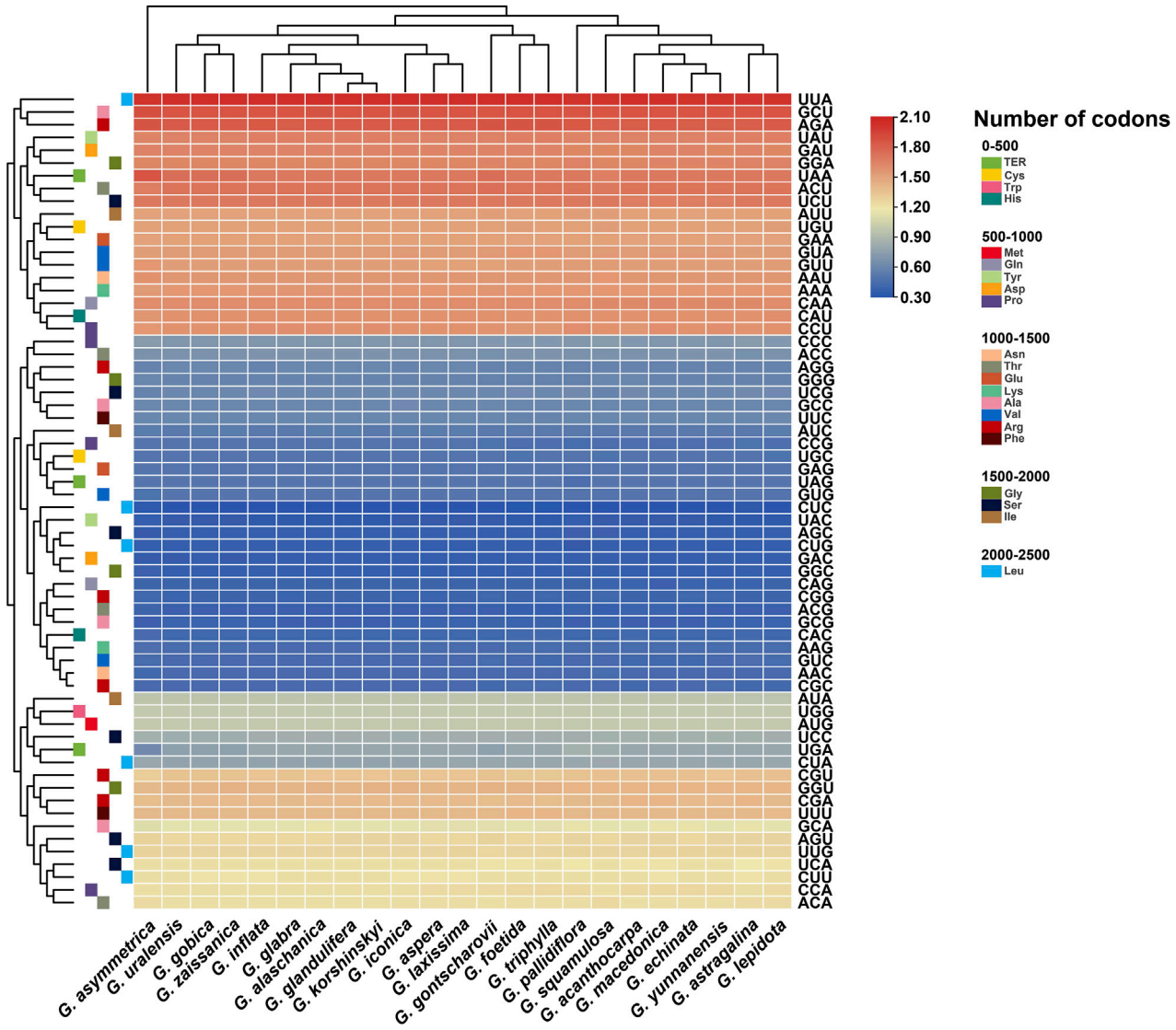

FIGURE 2
Heat map of RSCU values among *Glycyrrhiza* species.

regions are separated by IRs (Jansen and Ruhlman, 2012). However, with the increasing number of reported chloroplast genomes, researchers have found varying degrees and types of size and structural variations in the chloroplast genome in some angiosperm families. The expansion, contraction, and even loss of the IRs are important factors influencing the size of the chloroplast genome (Jansen and Ruhlman, 2012). Research on the chloroplast genome of the Fabaceae family has mainly focused on the Papilionoideae subfamily. Compared with most angiosperms, the chloroplast genome structure in the Papilionoideae subfamily exhibits significant variation. One branch of this subfamily, encompassing all reported species, has experienced the loss of the IR regions in the chloroplast genome. Consequently, this branch is termed the inverted repeat-lacking clade (IRLC) (Wojciechowski et al., 2000). In the IRLC, chloroplast genomes have undergone extensive rearrangements, involving processes such as gene duplication, loss, and sequence inversion (Cai et al., 2008; Sveinsson and Cronk, 2014). For a long period, scholars believed that, except for the Papilionoideae subfamily, chloroplast genomes in other Fabaceae plants were highly conserved (Schwarz et al., 2015). However, subsequent research revealed a roughly 13 kb expansion of the IR into the SSC in the *Acacia* and *Inga* genera (Caesalpinioideae subfamily). Additionally, in the *Acacia* genus, IR underwent an approximately 2 kb contraction at the junction with the LSC (Dugas et al., 2015; Williams et al., 2015).

The genus *Glycyrrhiza*, belonging to the subfamily Papilionoideae of the family Fabaceae, is a perennial herbaceous plant. It is distributed worldwide, with main production areas in Asia and Europe, and a small number can be found in the tropical and subtropical regions of America and Africa. China is located in the central zone of *Glycyrrhiza* resources, and it is also the country with the largest consumption and export volume of *Glycyrrhiza* in the world (Zheng et al., 2015). According to the Flora of China, eight species of *Glycyrrhiza* exist in China, which can be classified into two sections based on the presence of glycyrrhizic acid: section *Glycyrrhiza* (*Glycyrrhiza uralensis* Fisch. ex DC., *Glycyrrhiza*

TABLE 1 Codon usage of the *Glycyrrhiza* species.

Species	GC3s	GC	CAI	ENc	Fop	Gravy	Aromo	L_sym	L_aa
G. astragalina	0.248	0.369	0.167	48.24	0.347	−0.047394	0.113196	21226	22121
G. zaissanica	0.247	0.368	0.167	48.16	0.347	−0.048145	0.113707	21239	22127
G. foetida	0.246	0.368	0.167	48.1	0.347	−0.048522	0.113764	21225	22116
G. alaschanica	0.247	0.368	0.167	48.17	0.347	−0.04951	0.11352	21249	22137
G. korshinskyi	0.247	0.368	0.167	48.17	0.347	−0.049031	0.113526	21239	22127
G. squamulosa	0.247	0.368	0.166	48.19	0.346	−0.046467	0.113602	21231	22121
G. gontscharovii	0.246	0.368	0.167	48.1	0.347	−0.048869	0.113367	21223	22114
G. iconica	0.247	0.368	0.167	48.16	0.347	−0.048692	0.113566	21249	22137
G. acanthocarpa	0.248	0.368	0.167	48.2	0.346	−0.049096	0.113727	21244	22132
G. yunnanensis	0.248	0.368	0.166	48.19	0.346	−0.047484	0.113626	21243	22134
G. glandulifera	0.247	0.368	0.167	48.17	0.347	−0.048778	0.113506	21243	22131
G. asymmetrica	0.248	0.368	0.166	48.25	0.346	−0.045656	0.113857	21285	22177
G. uralensis	0.247	0.368	0.167	48.17	0.347	−0.048963	0.113693	21232	22121
G. echinata	0.247	0.368	0.167	48.15	0.347	−0.046748	0.113728	21232	22123
G. pallidiflora	0.247	0.368	0.166	48.12	0.347	−0.051691	0.113367	21228	22114
G. lepidota	0.247	0.368	0.166	48.1	0.347	−0.047214	0.113471	21236	22129
G. glabra	0.247	0.368	0.167	48.17	0.347	−0.048563	0.113532	21238	22126
G. triphylla	0.247	0.368	0.167	48.12	0.348	−0.047907	0.113844	21228	22118
G. aspera	0.247	0.368	0.167	48.16	0.347	−0.049067	0.113561	21241	22129
G. inflata	0.247	0.368	0.167	48.14	0.347	−0.049559	0.113537	21237	22125
G. macedonica	0.247	0.368	0.167	48.11	0.346	−0.047017	0.113792	21237	22128
G. laxissima	0.247	0.368	0.167	48.17	0.348	−0.048728	0.113561	21241	22129
G. gobica	0.247	0.368	0.167	48.16	0.348	−0.048836	0.113627	21237	22125

GC3s, GC content at the synonymous third codon position; GC, total GC content; CAI, codon adaptation index; ENc, effective number of codons; Fop, frequency of optimal codons; Gravy, influence of protein hydrophobicity on codon usage bias; Aromo, influence of aromatic protein on codon usage bias; L_sym, number of synonymous codons; L_aa, total number of synonymous and non-synonymous codons.

glabra L., *Glycyrrhiza inflata* Batalin, *Glycyrrhiza aspera* Pall., and *Glycyrrhiza eglandulosa* X.Y.Li) and section *Pseudoglycyrrhiza* (*Glycyrrhiza pallidiflora* Maxim., *Glycyrrhiza squamulosa* Franch., and *Glycyrrhiza yunnanensis* S.H.Cheng & L.K.Tai ex P.C.Li) (Li et al., 2010). Among them, the species in section *Glycyrrhiza* contain glycyrrhizic acid, and they are often used as medicine. By contrast, the species in section *Pseudoglycyrrhiza* do not contain glycyrrhizic acid. *Glycyrrhiza* species are widely used in medicine, and the Chinese pharmacopoeia specifies that *G. uralensis*, *G. glabra*, and *G. inflata* are the primary source plants for medicinal materials, with their medicinal parts being the roots and rhizomes. It is known for their effects in tonifying the spleen and invigorating qi, clearing heat and detoxifying the body, relieving coughs, reducing phlegm, alleviating pain, and harmonizing the actions of various other medicinal herbs (Commission, 2020). The species in *Glycyrrhiza* are rich in metabolites. Among them, triterpenoid saponins and flavonoids are two important metabolites that determine the medicinal effects. Modern pharmacological research has discovered that species in *Glycyrrhiza* have various effects, including anti-inflammatory, analgesic, anticancer, antiviral, antioxidant, and hepatoprotective properties (Shin et al., 2007; Hawthorne and Gallagher, 2008; Shin et al., 2008; Ming and Yin, 2013). Besides their medicinal uses, *Glycyrrhiza* species are also applied as an additive and flavoring agent in industries such as food, tobacco, daily chemicals, and animal husbandry (Li et al., 2004). However, there has been ongoing controversy regarding the classification within the *Glycyrrhiza* genus, which has implications for the medicinal, industrial, and scientific applications of *Glycyrrhiza* species, as well as the collection and breeding of their germplasm resources. Non-medicinal species are sometimes sold in the market as medicinal species, and even some scientific studies on pharmacology or chemistry have used misidentified *Glycyrrhiza* materials (Duan et al., 2023).

To date, 23 species in *Glycyrrhiza* with chloroplast genomes have been published. However, comparative genomics studies of the chloroplast genome in *Glycyrrhiza* are scarce. Additionally, research on phylogenomics of the Fabaceae family using chloroplast genome data is limited, and no study has utilized chloroplast genomes to investigate the divergence times of Fabaceae species. This study performed the assembly and annotation of chloroplast genomes for

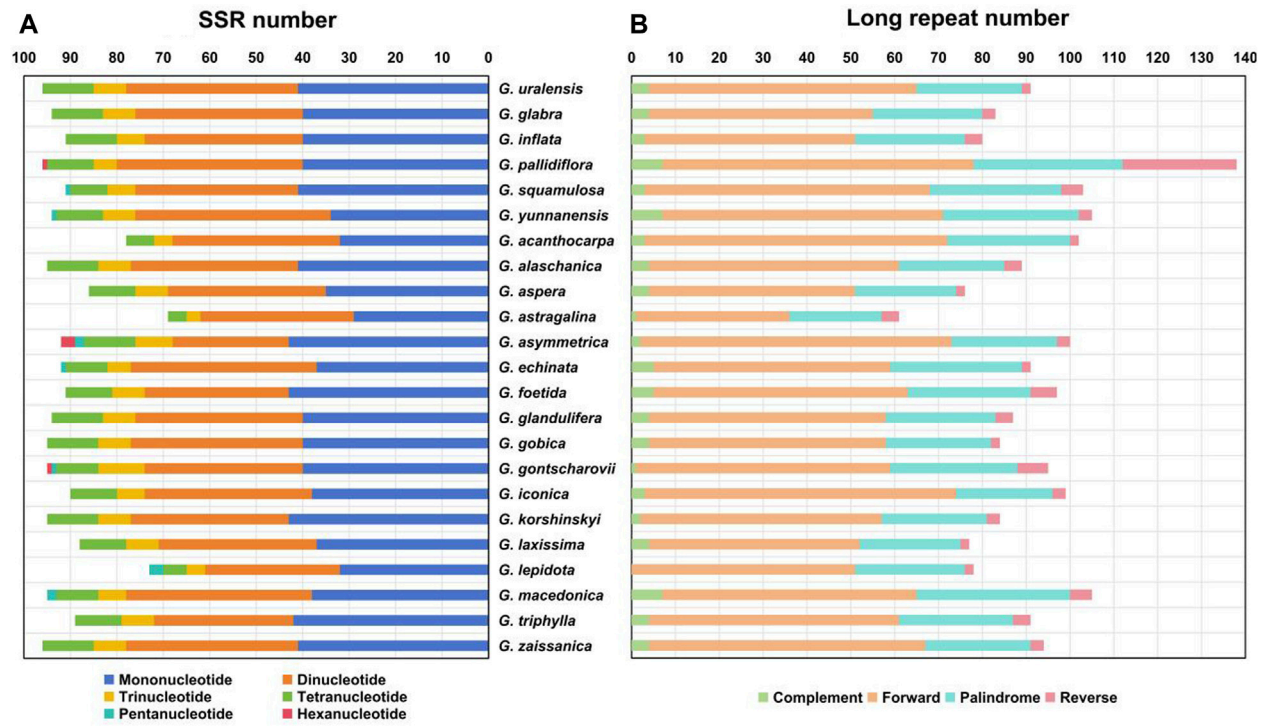

FIGURE 3
Repeat analysis of chloroplast genomes of *Glycyrrhiza* species. **(A)** SSR statistics of *Glycyrrhiza* species. **(B)** Long repeat statistics of *Glycyrrhiza* species. Different types of repeats are indicated by different colors.

six *Glycyrrhiza* species, namely, three from section *Glycyrrhiza* (*G. uralensis*, *G. glabra*, and *G. inflata*) and three from section *Pseudoglycyrrhiza* (*G. pallidiflora*, *G. squamulose*, and *G. yunnanensis*). The research explored the efficiency of different assembly and annotation tools on chloroplast genomes of *Glycyrrhiza* species and analyzed the codon usage, repeat sequences, gene selection pressure, and sequence variations of all species with published chloroplast genomes within the *Glycyrrhiza* genus. It aims to contribute to the understanding of the genetic background and phylogenetic evolution related to the phenotypes in the *Glycyrrhiza* genus. Additionally, an evolutionary tree was reconstructed for all tribes with published chloroplast genomes in the Fabaceae family, providing a systematic evolutionary analysis of Fabaceae species. The study also included analyses of differentiation times and sequence collinearity among Fabaceae species.

2 Materials and methods

2.1 Plant and DNA sources

Fresh leaves of *Glycyrrhiza uralensis* Fisch. ex DC., *Glycyrrhiza glabra* L., *Glycyrrhiza inflata* Batalin, and *Glycyrrhiza pallidiflora* Maxim. were obtained from the Beijing Medicinal Plant Garden, and *Glycyrrhiza squamulosa* Franch. and *Glycyrrhiza yunnanensis* S.H.Cheng & L.K.Tai ex P.C.Li were collected from Hengshui City in Hebei Province and Lijiang City in Yunnan Province, respectively. All samples were identified by Prof. Yulin Lin from the Institute of Medicinal Plant Development (IMPLAD). Voucher specimens were deposited in the herbarium at IMPLAD. Total DNA of the six species was extracted by a DNeasy Plant Mini Kit (Qiagen Co., Germany), and the DNA concentration and quality were assessed through Nanodrop 2000C spectrophotometry and electrophoresis in 1% (w/v) agarose gel, respectively.

2.2 DNA sequencing, assembly, and annotation

The DNA of six *Glycyrrhiza* species was used to generate libraries with average insert size of 500 bp and sequenced using Illumina Hiseq X in accordance with the standard protocol. Low-quality reads resulting from all samples were trimmed by Trimmomatic software (Bolger et al., 2014). GetOrganelle (Jin et al., 2020) and NOVOPlasty (Dierckxsens et al., 2017) were used to assemble the chloroplast genomes. GeSeq (Tillich et al., 2017), CPGAVAS2 (Shi et al., 2019), and PGA (Qu et al., 2019) software were used to annotate the sequences initially and correct them manually. The final complete chloroplast genomes of *G. uralensis*, *G. glabra*, *G. inflata*, *G. pallidiflora*, *G. squamulose*, and *G. yunnanensis* were submitted to GenBank and obtained the accession numbers of PP119344, PP119342, PP119340, PP119341, PP119343, and PP119345, respectively.

2.3 Structural analyses

Chloroplast genome maps were generated using Chloroplot (Zheng et al., 2020) and then manually corrected. CodonW

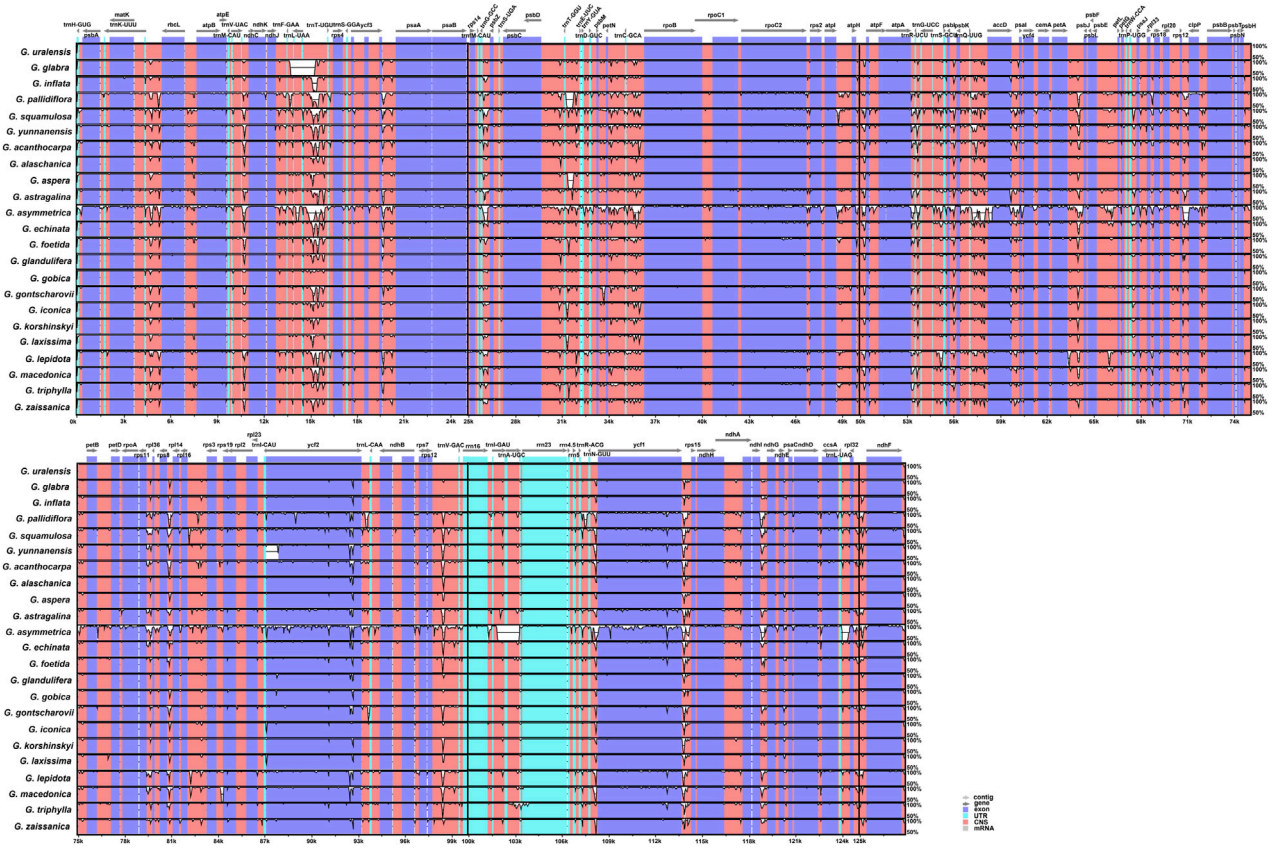

FIGURE 4
Global alignment of chloroplast genomes of 23 *Glycyrrhiza* species. The *x*-axis represents the coordinates in the chloroplast genome. The *y*-axis indicates the average percent identity of sequence similarity in the aligned regions, ranging between 50% and 100%.

software (Sharp and Li, 1987) was adopted to analyze the usage of codon. Simple sequence repeats (SSRs) were detected by MISA (Beier et al., 2017) with the definition of ≥10 repeat units for mononucleotide; ≥5 repeat units for dinucleotide; and ≥4 repeat units for trinucleotide; and ≥3 repeat units for tetranucleotide, pentanucleotide, and hexanucleotide SSRs. The long repeated sequences were detected by REPuter (Kurtz et al., 2001) with length of ≥30 bp and over 90% similarity between two copies.

2.4 Comparative and phylogenetic analyses

The chloroplast genomes of all published *Glycyrrhiza* species (Supplementary Table S1) were compared using the online genome comparison tool mVISTA software (Frazer et al., 2004). The nucleotide diversity values (Pi) of the chloroplast genomes were computed using DnaSP (Librado and Rozas, 2009). KaKs_Calculator v. 2.0 was used to determine the ratio of non-synonymous substitutions (Ka) and synonymous substitutions (Ks) (Wang et al., 2010). Chloroplast genome sequence homology and collinearity were analyzed using Mauve software (Darling et al., 2004). A total of 87 chloroplast genomes of Fabaceae species, including all tribes of Fabaceae with published chloroplast genomes (Supplementary Table S1), were used to construct a phylogenetic tree. MAFFT software (Katoh and Standley, 2013) was used to compare the common protein-coding genes shared by the chloroplast genomes of these Fabaceae species. Maximum likelihood (ML) analysis was carried out by IQ-TREE (Nguyen et al., 2015) based on these common protein-coding genes with a bootstrap of 1000 repetitions. ML analysis was conducted based on the GTR + F + R5 model. The MCMCTREE program in the PAML package (Yang, 2007) was used to estimate the differentiation time of Fabaceae. The topology of the legume ML tree was used to demarcate the differentiation time nodes, and the results were viewed and edited by FigTree software (http://tree.bio.ed.ac.uk/software/figtree/).

3 Results and discussion

3.1 Assembly and annotation of *Glycyrrhiza* chloroplast genomes

Correctly assembling complete chloroplast genomes ensures the reliability and reproducibility of research on the evolution of eukaryotic chloroplast genomes, as well as downstream studies based on chloroplast genomes. Currently, the most widely used tools for assembling organelle genomes are GetOrganelle and NOVOPlasty. This study employed GetOrganelle and NOVOPlasty for the assembly of six *Glycyrrhiza* species. The results revealed that the species successfully assembled using GetOrganelle were *G. uralensis*, *G. squamulosa*, and *G. yunnanensis*, whereas those successfully assembled using NOVOPlasty

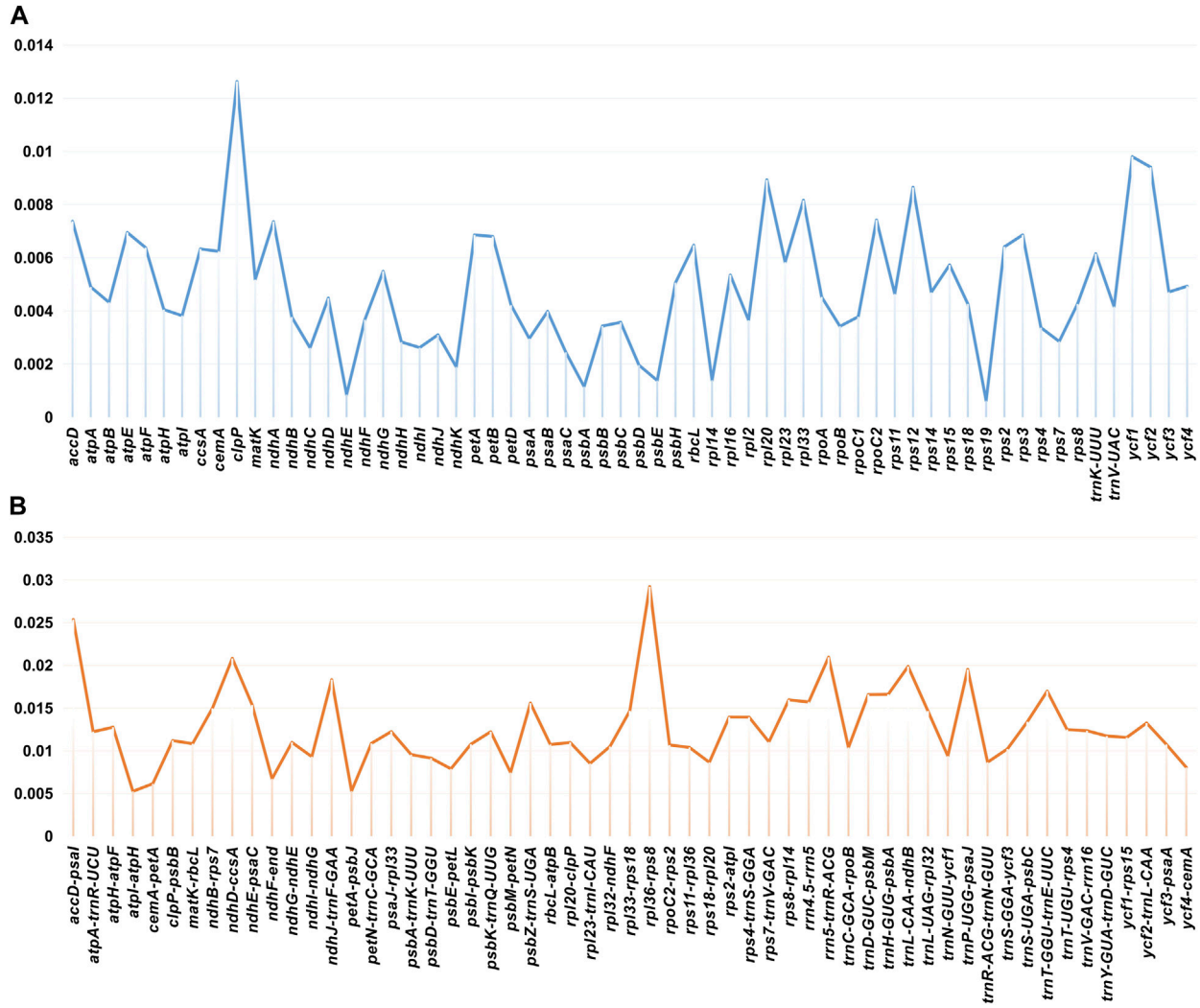

FIGURE 5
Nucleotide diversity of shared various regions with Pi > 0 in chloroplast genomes of 23 *Glycyrrhiza* species. **(A)** Pi values in the genes. **(B)** Pi values in the intergenic spacers.

were *G. glabra*, *G. inflata*, *G. pallidiflora*, and *G. squamulosa*. The final assembled chloroplast genome sizes for the six *Glycyrrhiza* species ranged from 126,380 bp (*G. glabra*) to 129,115 bp (*G. yunnanensis*), with an overall GC content of approximately 34%. The coding sequences (CDS) accounted for the largest proportion, comprising around 52% of the total genome. The chloroplast genomes of the six *Glycyrrhiza* species showed a circular structure (Figure 1). Similar to the published chloroplast genomes of *Glycyrrhiza* species, these six chloroplast genomes also lacked the IRs. The success rates of chloroplast genome assembly for these six *Glycyrrhiza* species were similar between GetOrganelle and NOVOPlasty. However, a study based on testing with a publicly available dataset of reads from 50 plant species showed that, under slightly high computational resource consumption, the default parameter of GetOrganelle achieved a much higher complete circularity rate compared with the best parameter result of NOVOPlasty (Jin et al., 2020). In the benchmark study evaluating mainstream chloroplast genome assembly tools, GetOrganelle achieved significantly higher circularity and accuracy rates compared with other tools; thus, it was recommended as the optimal assembly tool (Freudenthal et al., 2020). Additionally, the output results from GetOrganelle included excellent visualization, allowing for an intuitive assessment of the assembly success without the need for additional verification. This feature significantly reduces the occurrence of false positives.

For chloroplast genome annotation, we employed the widely used organelle genome annotation tools, namely, GeSeq, CPGAVAS2, and PGA. The results indicated that the annotation effectiveness varied among different software tools for the six *Glycyrrhiza* species (Supplementary Table S2). GeSeq provides a comprehensive annotation of genes, and its real-time updated organelle genome database facilitates the selection of closely related species as references, which is crucial for the accuracy of chloroplast genome annotation. Combining these three annotation tools with manual correction, a total of 109–110 genes were annotated, including 76 protein-coding genes, 29–30 tRNAs, and 4 rRNAs, similar to the published *Glycyrrhiza* chloroplast genomes (Supplementary Table S3). Introns can accumulate more mutations than exons and play an important role in gene expression regulation (Kelchner, 2002). Previous studies have shown that introns can increase the expression

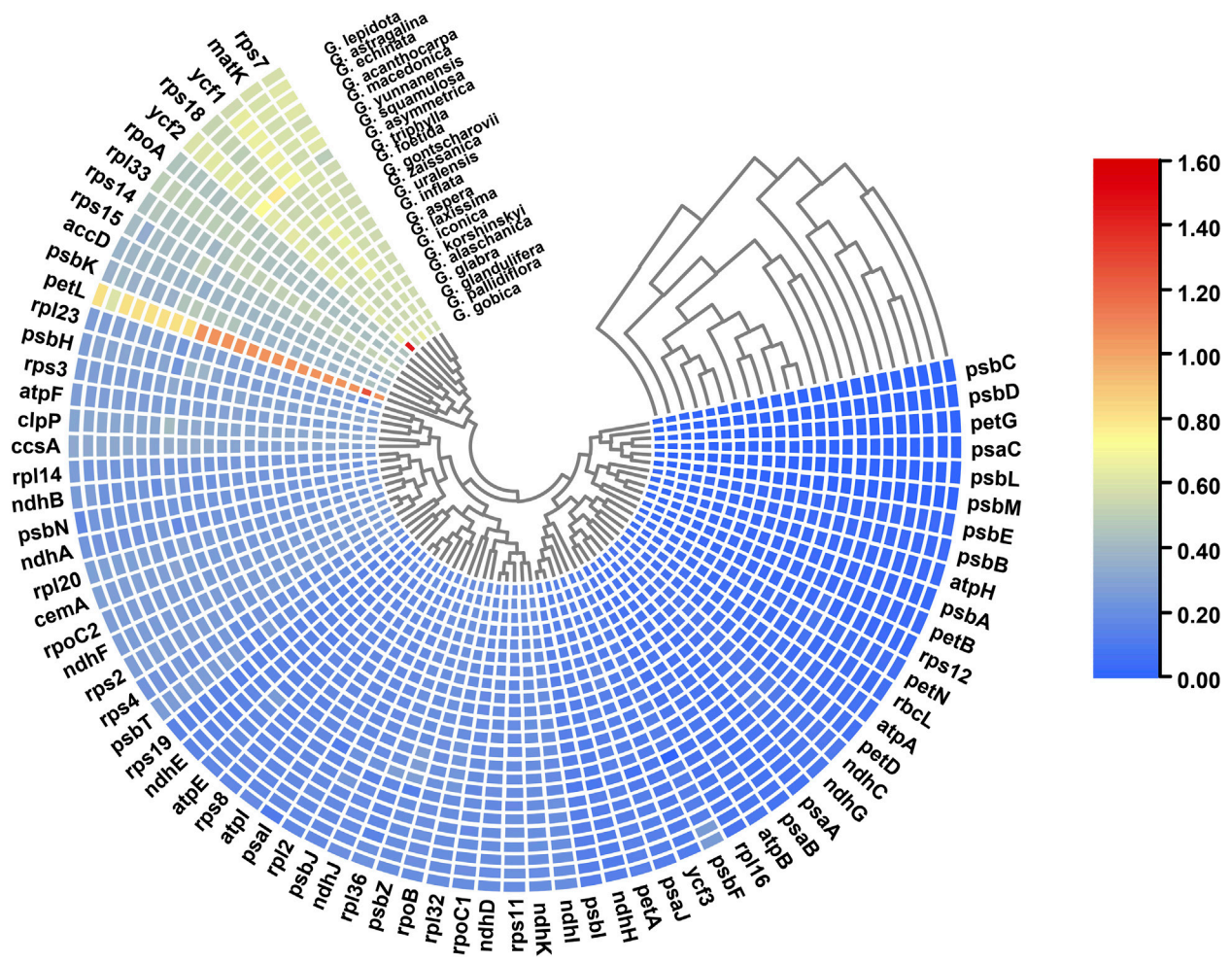

FIGURE 6
Selective pressure of genes in *Glycyrrhiza* chloroplast genomes.

level of foreign genes in eukaryotic genomes (Xu et al., 2003). In the six *Glycyrrhiza* species, there are a total of 16 genes with introns, with one gene (*ycf3*) having 2 introns, and the remaining 15 genes (*atpF*, *ndhA*, *ndhB*, *petB*, *petD*, *rpl16*, *rpl2*, *rpoC1*, *rps12*, *trnK-UUU*, *trnV-UAC*, *trnL-UAA*, *trnG-UCC*, *trnI-GAU*, and *trnA-UGC*) each having 1 intron (Supplementary Table S4). There were two special genes: *rps12* gene was a trans-splicing gene; *trnK-UUU* gene sequence contained the *matK* gene, similar to that of many other plants (Wu et al., 2021).

In summary, various chloroplast genome assembly and annotation tools yield varied results for different species or datasets, even within the same genus. Therefore, it is recommended to use multiple tools for chloroplast genome assembly and annotation, and the results from different tools should be combined to ensure the accuracy of chloroplast genome assembly and annotation.

3.2 Codon usage of *Glycyrrhiza* chloroplast genomes

Codon usage bias refers to the phenomenon that all synonymous codons in protein transcripts are not uniformly used to encode all amino acids in the protein except methionine and tryptophan (Jia and Xue, 2009). Given the difference in the degree of variation and selection pressure, the variation in compositional constraints between different genomes is a key factor in the formation of codon usage bias (Sharp and Li, 1987; Hershberg and Petrov, 2008; Paul et al., 2018). Codon usage bias can be used to investigate the evolutionary history of organisms, predict the expression level, and understand the evolutionary processes acting on genomes at the molecular level (Jia and Xue, 2009; Leffler et al., 2012).

The relative synonymous codon usage (RSCU) of the chloroplast genomes of all published *Glycyrrhiza* species was calculated based on all protein-coding genes (Figure 2). The results showed that the chloroplast genomes of *Glycyrrhiza* species contained 64 types of codons encoding 20 amino acids. Of all amino acid codons, leucine had the highest number of codons, whereas cysteine had the lowest number of codons. Thirty codons were found with an RSCU of >1, of which 29 were A/U-ending codons; 34 codons were found with an RSCU of ≤1, of which 31 were G/C-ending codons. Thus, compared with the G/C-ending codons, the chloroplast genome exhibited a greater bias toward the A/U-ending codons. The highest RSCU value was recorded for UUA and the lowest was recorded for CUC, which both encode leucine. In addition, the codon usage of the chloroplast genomes in section *Glycyrrhiza* was relatively close, whereas that of section *Pseudoglycyrrhiza* was close.

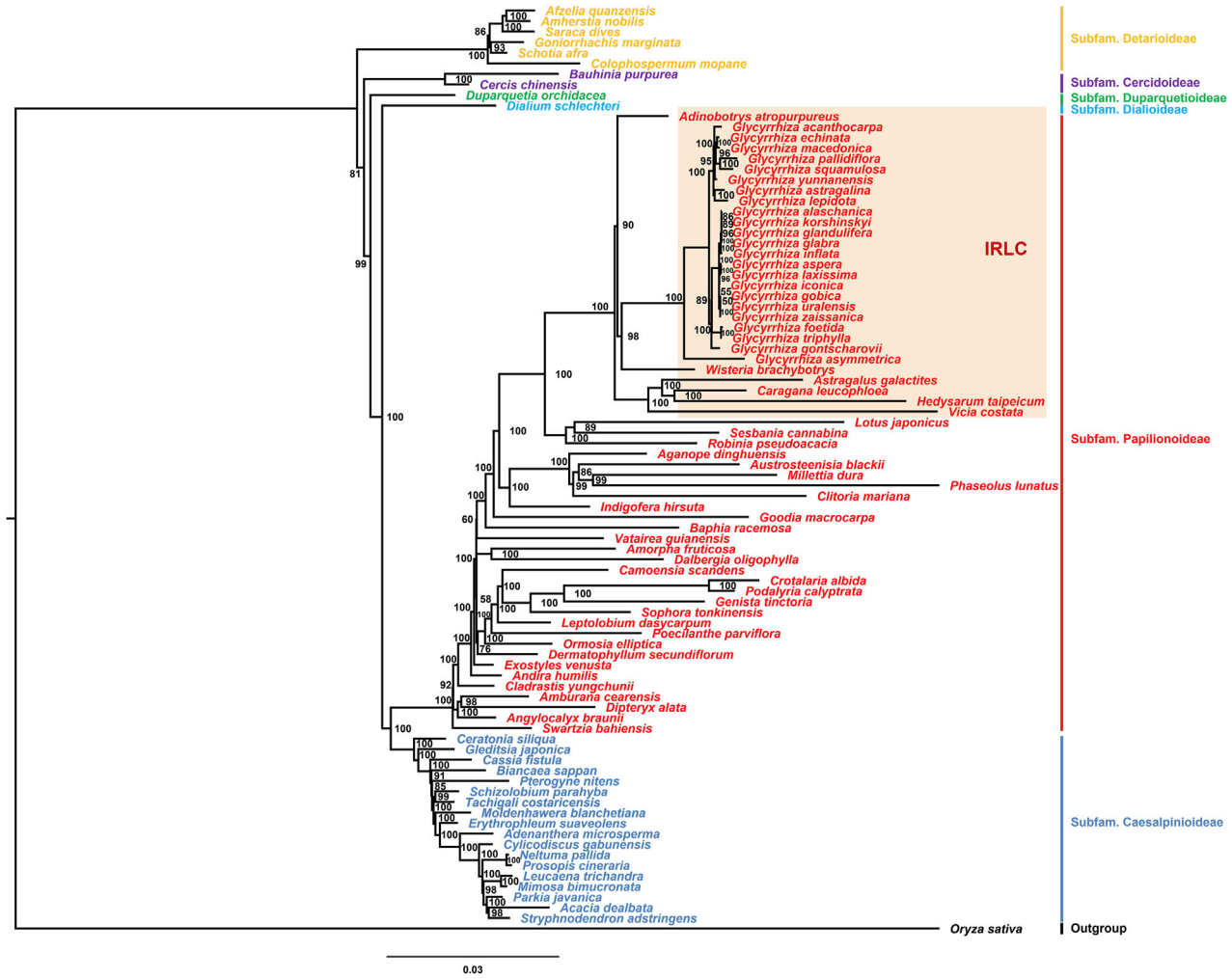

FIGURE 7
Phylogenetic tree constructed using the maximum likelihood (ML) method with a bootstrap of 1000 repetitions based on the chloroplast genomes of Fabaceae species. Numbers at nodes are values for bootstrap support.

GC reflects the strength of directional mutation pressure, whereas GC3s is closely related to codon bias and used as an important basis for analyzing codon usage pattern (Shang et al., 2011; Zhao et al., 2016). GC and GC3s in the codons of these 23 chloroplast genomes were all less than 0.5, indicating that the chloroplast genomes of *Glycyrrhiza* species tended to use A/U bases and A/U-ending codons. Codon adaptation index values and effective number of codon values indicated a slight bias in codon usage in the *Glycyrrhiza* species. The frequency of optimal codons was relatively low. In addition, the hydrophobicity of the protein and the aromatic protein had little effect on codon usage bias (Table 1).

3.3 SSRs and long repeat sequences

SSRs are widely distributed in the chloroplast genome and usually used as important molecular markers for species identification (Yang et al., 2011; Jiao et al., 2012). A total of 69–96 SSRs were identified in the chloroplast genomes of the *Glycyrrhiza* species (Figure 3A). In addition, the base composition of the repeating motifs from mononucleotide repeats to tetranucleotide repeats, which existed in all *Glycyrrhiza* species, had a certain A-T base preference. In these SSRs, mononucleotide repeats were the largest in number, and they were found 31–43 times in these chloroplast genomes. A/T repeats were the most common mononucleotide repeats, whereas the majority of dinucleotide repeats, trinucleotide repeats, and tetranucleotide repeats comprised AT/AT, AAT/ATT, and AAAT/ATTT, respectively. These results were consistent with A-T enrichment in complete chloroplast genomes (Qian et al., 2013). Pentanucleotide repeats were found in *G. squamulosa*, *G. yunnanensis*, *Glycyrrhiza asymmetrica* Hub.-Mor., *Glycyrrhiza echinata* L., *Glycyrrhiza gontscharovii* Maslenn., *Glycyrrhiza lepidota* Pursh, and *Glycyrrhiza macedonica* Boiss. & Orph., and hexanucleotide repeats only existed in *G. pallidiflora*, *G. asymmetrica*, and *G. gontscharovii*.

Except for SSRs, some repetitive sequences with length ≥30 bp and sequence similarity ≥90% are called long repetitive sequences, including complement (C), forward (F), palindrome (P), and reverse (R). In *Glycyrrhiza* species, our results revealed 61 (*Glycyrrhiza*

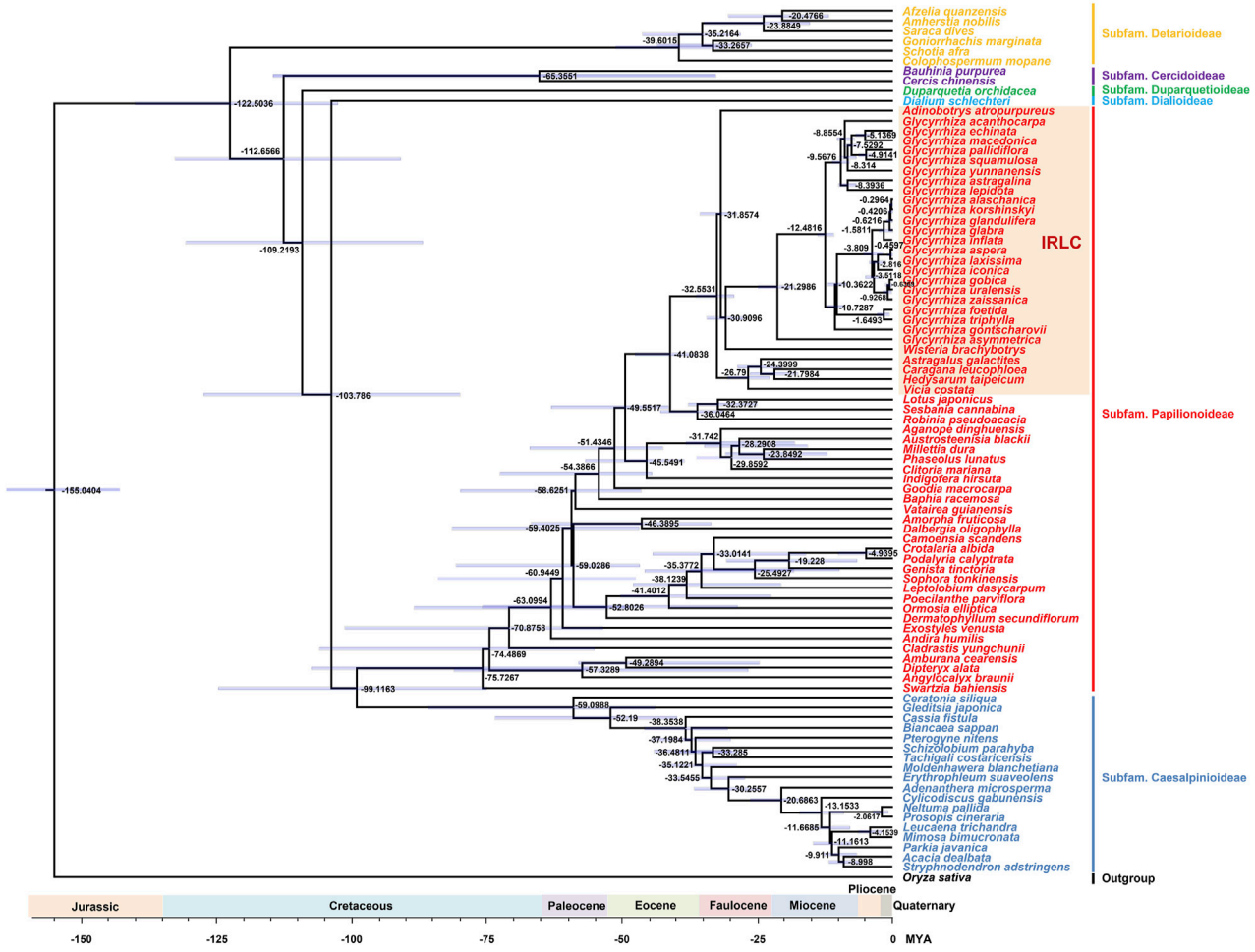

FIGURE 8
Divergence time estimation of Fabaceae species based on chloroplast genomes. The blue bars correspond to the 95% highest posterior density credibility intervals of age estimates. Numbers at nodes are values for divergence time (MYA).

astragalina Gillies ex Hook. & Arn.)–138 (*G. pallidiflora*) long repeats, most of which were F (35–71) and P (21–35) repeats. No C repeat was found in the chloroplast genome of *G. lepidota*. The length of C and R repeats was mainly within the range of 30–39 bp (Figure 3B).

3.4 Variations in chloroplast genomes of *Glycyrrhiza* species

In this study, the complete chloroplast genomes of all published *Glycyrrhiza* species were compared with the *G. uralensis* genome as the reference genome (Figure 4). Overall, the comparative genomic analysis revealed that the 23 *Glycyrrhiza* chloroplast genomes were relatively conserved. Intergenic spacers and intron regions showed more variations than protein-coding regions. Most protein-coding regions had a very high degree of conservation, and rRNA genes were highly conserved with almost no variation.

Nucleotide diversity (Pi) of shared genes and intergenic spacers of the chloroplast genomes of the 23 *Glycyrrhiza* species were calculated. Figure 5 shows the intergenic spacers and genes with Pi > 0. The intergenic spacers had more polymorphisms (average Pi = 0.0127) than the gene regions (average Pi = 0.0049), consistent with mVISTA analysis. Combining mVISTA and Pi, four highly variable regions were screened out, namely, *accD–psaI*, *ndhD–ccsA*, *rpl36–rps8*, and *rrn5–trnR-ACG*, for species identification and relationship analysis.

3.5 Selective pressure analyses

Selective pressure refers to the external forces exerted on a species during the evolutionary process, driving the species to adapt to its natural environment. In genetics, ω = Ka/Ks represents the ratio between non-synonymous mutations (Ka) and synonymous mutations (Ks). In general, synonymous mutations are considered not subject to natural selection, whereas non-synonymous mutations are influenced by natural selection. $\omega > 1$ indicates positive selection; $\omega = 1$ implies neutral evolution, where no selection is acting; $0<\omega < 1$ is considered negative or purifying selection, with small ω values indicating high negative selection pressure (Wang et al., 2010).

The results of selective pressure analyses on *Glycyrrhiza* chloroplast genomes showed that almost all chloroplast genes in *Glycyrrhiza* species

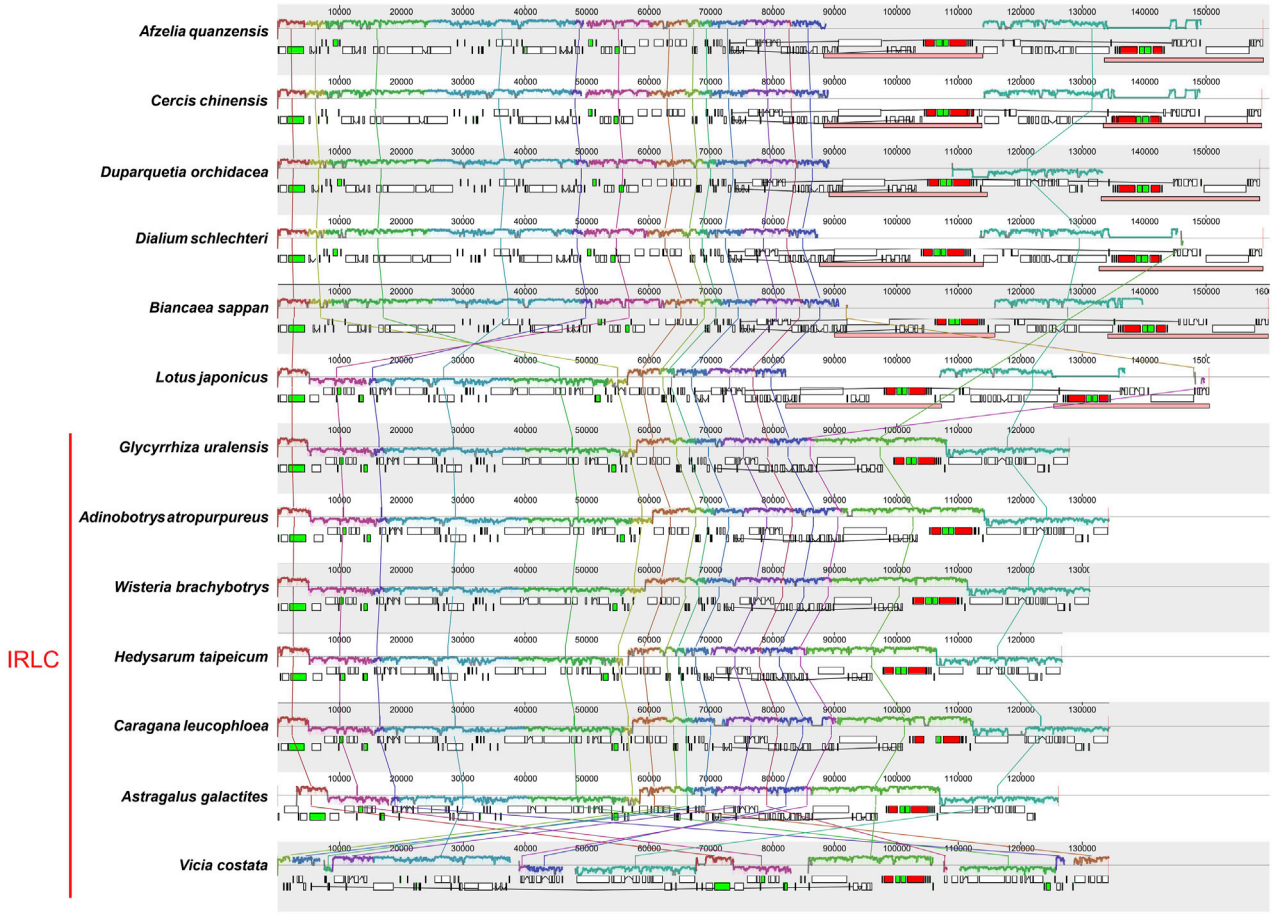

FIGURE 9
Co-linear analysis of Fabaceae chloroplast genomes. Local collinear blocks are represented by blocks of the same color connected by lines. The DNA fragments above the line correspond to the clockwise direction and those below the line are in the counterclockwise direction.

underwent negative or purifying selection, which indicated these genes were relatively conserved. Only *petL* in *G. glabra*, *G. pallidiflora*, *G. uralensis*, *G. inflata*, *Glycyrrhiza foetida* Desf., *Glycyrrhiza glandulifera* Waldst. & Kit., *Glycyrrhiza iconica* Hub.-Mor., *Glycyrrhiza laxissima* Vassilcz., *Glycyrrhiza triphylla* Fisch. & C.A.Mey., *G. aspera*, *Glycyrrhiza alaschanica* Grankina, *Glycyrrhiza gobica* Grankina, *G. gontscharovii*, *Glycyrrhiza zaissanica* Serg., and *Glycyrrhiza korshinskyi* Grig. and *ycf2* in *G. gobica* were positively selected genes, suggesting that some advantageous mutations were positively favored by selection (Figure 6). The positively selected genes may be associated with the adaptation of plants to low-light and low-temperature environments (Yang et al., 2023).

3.6 Phylogenetic analysis

Traditionally, the Fabaceae family is classified into three subfamilies based on the different floral morphologies: Mimosoideae, Caesalpinioideae, and Papilionoideae. Some traditional classification systems, such as the Cronquist system, elevate these three subfamilies to the rank of family (Taubert, 1894). However, molecular studies indicate that Mimosoideae and Papilionoideae are generally monophyletic, but Caesalpinioideae is a highly basal and paraphyletic group. Therefore, the practice of dividing Fabaceae into three subfamilies or three families does not adhere to the principle of monophyly (Taubert, 1894). Given the keen interest of the legume taxonomic community in the subordinal classification system, the Legume Phylogeny Working Group, composed of legume experts worldwide, published a consensual phylogenetic tree of the legume family in 2013, and three schemes for subfamily division were proposed (Group, 2013). Among these schemes, the 15-subfamily scheme retains the traditional concept of the Mimosoideae subfamily, whereas the Caesalpinioideae subfamily is split into several subfamilies (with over half of them distributed in China). The 6-subfamily scheme merges the Mimosoideae subfamily into a newly defined Caesalpinioideae, and it can avoid excessive splitting of the Caesalpinioideae subfamily. Considering that the species diversity of the traditional Mimosoideae and Caesalpinioideae in the Chinese region is not high, the 6-subfamily scheme is more convenient for Chinese scholars compared with the 15-subfamily scheme. In 2017, they officially adopted the 6-subfamily scheme, publishing the comprehensive Linnaean system for the classification of the legume family. They refined the hierarchical system below the subfamily level and above the genus level based on the research

results accumulated in the academic community over the years (Group, 2017).

The chloroplast genome is an important tool to explore the phylogeny of species (Hu et al., 2016). The Fabaceae family comprises 6 subfamilies and 79 tribes. Previous studies have focused on the phylogenetic relationship within the *Glycyrrhiza* genus (Jo et al., 2018; Jia et al., 2019; Duan et al., 2020; Jiang et al., 2020; Duan et al., 2023). Research on phylogenomics of the Fabaceae family using chloroplast genome data is limited. To date, chloroplast genomes of species from 65 tribes have been published. In this study, chloroplast genomes from species of these 65 tribes were used to construct a phylogenetic tree, including all published species of the *Glycyrrhiza* genus (Figure 7). The results indicated that species from the six subfamilies formed distinct clusters, consistent with the classification scheme of the six subfamilies. In the chloroplast genomes of Fabaceae plants, the absence of the IR regions was not unique to the *Glycyrrhiza* genus. Species lacking the IR regions were primarily distributed in various tribes, i.e., Glycyrrhizeae tribe, Adinobotryeae tribe, Wisterieae tribe, Hedysareae tribe, Caraganeae tribe, Astragaleae tribe, and Fabeae tribe in the Papilionoideae subfamily. The results of phylogenetic analysis showed that these tribes lacking the IR regions clustered together. Among them, the Glycyrrhizeae tribe clustered with the Wisterieae tribe and then with the Adinobotryeae tribe. The Hedysareae tribe, Caraganeae tribe, Astragaleae tribe, and Fabeae tribe formed a distinct cluster. In *Glycyrrhiza* species, the species of the section *Glycyrrhiza* and section *Pseudoglycyrrhiza*, distributed in China, respectively clustered together. The North American species *G. lepidota*, although had a lower content of glycyrrhizic acid (Hayashi et al., 2005) and was in another group, indicating that the groups containing glycyrrhizic acid were not monophyletic, consistent with previous studies (Duan et al., 2020). The Flora of China treats *G. alaschanica*, *G. korshinskyi*, and *G. gobica* as synonyms of *G. uralensis*; *G. glandulifera* as a synonym of *G. glabra*; and *G. laxissima* and *G. zaissanica* as synonyms of *G. aspera* (Li et al., 2010). However, according to the results of this study, only *G. gobica* and *G. uralensis* clustered together, *G. laxissima* and *G. aspera* clustered together, and others should be considered as independent species.

3.7 Divergence time estimation

In addition to being frequently used for phylogenetic analysis and species identification, the chloroplast genome is employed for studying species divergence times (Mo et al., 2022). This study explored the divergence times of Fabaceae species using the chloroplast genome (Figure 8). The results indicated that the subfamily Detarioideae was the first to diverge, followed by the subfamily Cercidoideae, subfamily Duparquetioideae, subfamily Dialioideae, subfamily Papilionoideae, and subfamily Caesalpinioideae. In the subfamily Papilionoideae, the IRLC population was the last to differentiate. In *Glycyrrhiza* species, the ones without glycyrrhizic acid differentiated earlier than those with glycyrrhizic acid. This result suggested that the common ancestor of *Glycyrrhiza* lacked glycyrrhizic acid, consistent with previous studies (Duan et al., 2023). Medicinal groups in the Eurasian continent share a common ancestor, and their descendants result from two rapid differentiation events within the last million years. This phenomenon has led to significant morphological over-diversification and taxonomic confusion within this group (Duan et al., 2023).

3.8 Co-linear analysis

A co-linear analysis of the 23 *Glycyrrhiza* chloroplast genomes was conducted, and the *G. uralensis* genome was used as the reference genome. Results showed that the entire genome sequence was a homologous region with no big indels. The 23 chloroplast genomes connected with a line, indicating that the chloroplast genomes of *Glycyrrhiza* species were relatively conserved, and no rearrangement and inversion occurred in gene organization (Supplementary Figure S1). Additionally, a co-linear analysis was conducted on the chloroplast genomes of other species within the Fabaceae family (Figure 9). These species were from the six subfamilies: *Afzelia quanzensis* Welw. (subfamily Detarioideae), *Cercis chinensis* Bunge (subfamily Cercidoideae), *Duparquetia orchidacea* Baill. (subfamily Duparquetioideae), *Dialium schlechteri* Harms (subfamily Dialioideae), *Biancaea sappan* (L.) Tod. (subfamily Caesalpinioideae), and *Lotus japonicus* (Regel) K.Larsen (subfamily Papilionoideae), as well as all the tribes within the subfamily Papilionoideae that lack IR regions. Thus, the chloroplast genomes of *A. quanzensis*, *C. chinensis*, *D. orchidacea*, *D. schlechteri*, and *B. sappan* were relatively conservative, with minimal gene rearrangements and inversions. However, when compared with species in the subfamily Papilionoideae, especially *Vicia costata* Ledeb., the relative positions of some homologous blocks changed, which indicated rearrangements in the sequence. Additionally, homologous blocks below the line indicated reverse complementary alignments, also known as inversion blocks, suggesting possible inversion events. Species with gene rearrangements and inversions may be prone to the structural mutations in DNA strands and accumulate constantly due to the low GC content in their chloroplast genomes under the long evolutionary history (Ding et al., 2021).

4 Conclusion

This study focused on the comparative genomics and phylogenomics of *Glycyrrhiza* based on the chloroplast genome. Comparative genomics results showed that the chloroplast genomes of *Glycyrrhiza* had typically lacking IR regions, and the genome length, structure, GC content, codon usage, and gene distribution were highly similar. Selection pressure analysis indicated overall purifying selection in the chloroplast genomes of *Glycyrrhiza*, and some positively selected genes were potentially linked to environmental adaptation. The results of phylogenetic analysis and divergence time estimation showed that species from the six subfamilies formed distinct clusters, consistent with the classification scheme of the six subfamilies. The subfamily Detarioideae was the first to diverge, followed by the subfamily Cercidoideae, subfamily Duparquetioideae, subfamily Dialioideae, subfamily Papilionoideae, and subfamily Caesalpinioideae. In addition, the IRLC population in the subfamily Papilionoideae clustered together, and it was the last to differentiate. The groups containing glycyrrhizic acid in *Glycyrrhiza* were not monophyletic, and the common ancestor of *Glycyrrhiza* lacked glycyrrhizic acid. Co-linear analysis confirmed the conserved nature of *Glycyrrhiza* chloroplast genomes, as well as instances of gene rearrangements and inversions in the subfamily Papilionoideae.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material. The data presented in the study are deposited in the GenBank repository (www.ncbi.nlm.nih.gov/genbank), accession number PP119344, PP119342, PP119340, PP119341, PP119343, and PP119345.

Author contributions

LW: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Software, Visualization, Writing–original draft. PF: Investigation, Writing–original draft, Formal Analysis. JC: Investigation, Writing–original draft, Formal Analysis. CZ: Investigation, Writing–original draft, Data curation. YL: Resources, Writing–review and editing. ZX: Writing–review and editing, Software. ZW: Resources, Writing–original draft. WG: Resources, Writing–original draft. JS: Methodology, Writing–review and editing. HY: Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Validation, Writing–review and editing.

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Conflict of interest

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Supplementary material

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Diabetes cardiomyopathy: targeted regulation of mitochondrial dysfunction and therapeutic potential of plant secondary metabolites

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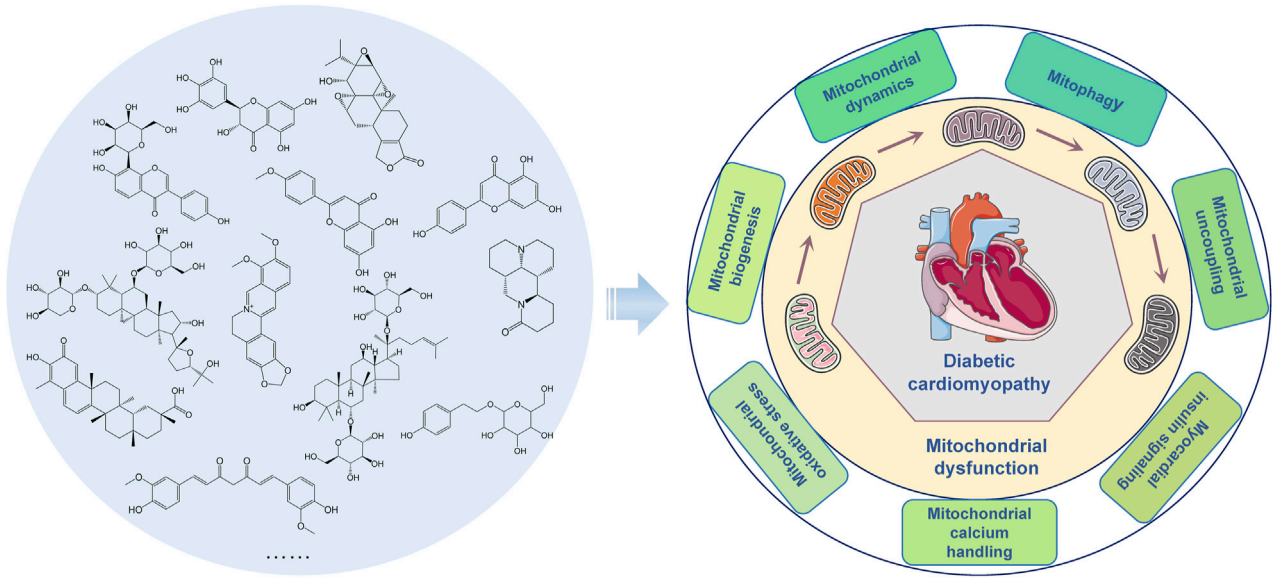
Diabetic cardiomyopathy (DCM) is a specific heart condition in diabetic patients, which is a major cause of heart failure and significantly affects quality of life. DCM is manifested as abnormal cardiac structure and function in the absence of ischaemic or hypertensive heart disease in individuals with diabetes. Although the development of DCM involves multiple pathological mechanisms, mitochondrial dysfunction is considered to play a crucial role. The regulatory mechanisms of mitochondrial dysfunction mainly include mitochondrial dynamics, oxidative stress, calcium handling, uncoupling, biogenesis, mitophagy, and insulin signaling. Targeting mitochondrial function in the treatment of DCM has attracted increasing attention. Studies have shown that plant secondary metabolites contribute to improving mitochondrial function and alleviating the development of DCM. This review outlines the role of mitochondrial dysfunction in the pathogenesis of DCM and discusses the regulatory mechanism for mitochondrial dysfunction. In addition, it also summarizes treatment strategies based on plant secondary metabolites. These strategies targeting the treatment of mitochondrial dysfunction may help prevent and treat DCM.

KEYWORDS

diabetic cardiomyopathy, mitochondrial dynamics, oxidative stress, mitophagy, insulin resistance, plant secondary metabolites

1 Introduction

Diabetes mellitus (DM) is a worldwide public health problem. According to the latest data of the International Diabetes Federation (IDF) in 2021, diabetes has become a health burden affecting 537 million people worldwide. It is estimated that this number will increase to 783 million by 2045 (Saeedi et al., 2019; Sun et al., 2022). The DM can be divided into type 1 (T1DM) and type 2 (T2DM), of which T2DM accounts for more than 90% of human diabetes population. Patients with T1DM or T2DM have a high risk of developing diabetes



GRAPHICAL ABSTRACT

cardiomyopathy (DCM) and even heart failure (Lam, 2015). DCM is a myocardial-specific microvascular complication that results in structural and functional abnormalities of the heart muscle in diabetic patients without other cardiac risk factors such as coronary artery disease, hypertension, and severe valve disease (Heather et al., 2022). It has been estimated that the prevalence of DCM in the general population is ~1.1%, and that it is ~16.9% in diabetics (Rajbhandari et al., 2021). Studies have shown that hyperinsulinemia, insulin resistance, and hyperglycemia are the starting points of the cascade reaction of cardiac dysfunction in DCM (El Hayek et al., 2021; Avagimyan et al., 2022). In the state of high glucose, multiple metabolic pathways are activated and interact with each other, leading to myocardial fibrosis and hypertrophy, cardiomyocyte apoptosis, reduced coronary microcirculation perfusion, and then evolving into diastolic and systolic dysfunction and eventually diabetic heart failure (Schilling, 2015). Furthermore, there are some pathophysiological differences in the triggering of DCM between T1DM and T2DM. Patients with T1DM experience severe insulin deficiency due to autoimmune disease, leading to hyperglycemia and abnormal metabolism and function of myocardial cells. The delayed absorption of calcium in the sarcoplasmic reticulum is associated with hyperglycemia, resulting in impaired left ventricular contractile function, making contractile dysfunction symptoms more typical in T1DM patients. In contrast, T2DM is mainly caused by hyperinsulinemia and insulin resistance. Its clinical manifestations are related to myocardial fibrosis and left ventricular remodeling, leading to increased wall hardness, reduced compliance, and early induction of diastolic myocardial dysfunction in the disease (Nathan, 2015; Waddingham et al., 2015; Prandi et al., 2023).

Mitochondria are important organelles in cells, mainly responsible for the generation of cellular energy. Through the process of oxidative phosphorylation, mitochondria produce adenosine triphosphate (ATP), which provides energy for the normal physiological activities of the cell (Wang et al., 2020). In DCM, mitochondrial dysfunction has a significant impact on heart function. Due to hyperglycemia and insulin resistance, the mitochondrial function in the myocardial cells of diabetic patients may be impaired. Mitochondrial dysfunction leads to reduced ATP production, increased oxidative stress, disrupted calcium ion balance, and subsequently affects the normal function of myocardial cells. Further damage may lead to decreased myocardial contractile force, abnormal cardiac structure and function, ultimately resulting in serious consequences such as heart failure (Zhu et al., 2021). The reasons for hyperglycemia levels leading to mitochondrial dysfunction may be related to the generation of glycation end products, oxidative stress, and abnormal lipid metabolism. Hyperglycemia levels may increase the generation of glycation end products, which can bind to proteins and DNA inside the mitochondria, forming highly pathogenic crosslinks, damaging the structure and function of the mitochondria. Hyperglycemia levels may increase the level of oxidative stress inside the mitochondria, leading to an imbalance in the mitochondrial redox balance, thereby damaging the integrity of the mitochondrial membrane and the function of the electron transport chain, affecting the production of energy in the mitochondria. Additionally, hyperglycemia levels may also cause abnormal lipid metabolism, increasing the burden of lipid oxidation inside the mitochondria, leading to an increase in lipid peroxidation reactions inside the mitochondria, damaging the membrane structure and function of the mitochondria. The treatment strategy for mitochondrial dysfunction in DCM is one of the hot spots of current research (Wang et al., 2020; Wang et al., 2020). Some studies have shown that plant secondary metabolites have the potential to improve mitochondrial function and alleviate the development of DCM (Gao et al., 2022; Sodeinde et al., 2023). The plant secondary metabolites may improve mitochondrial function by regulating mitochondrial dynamics, reducing

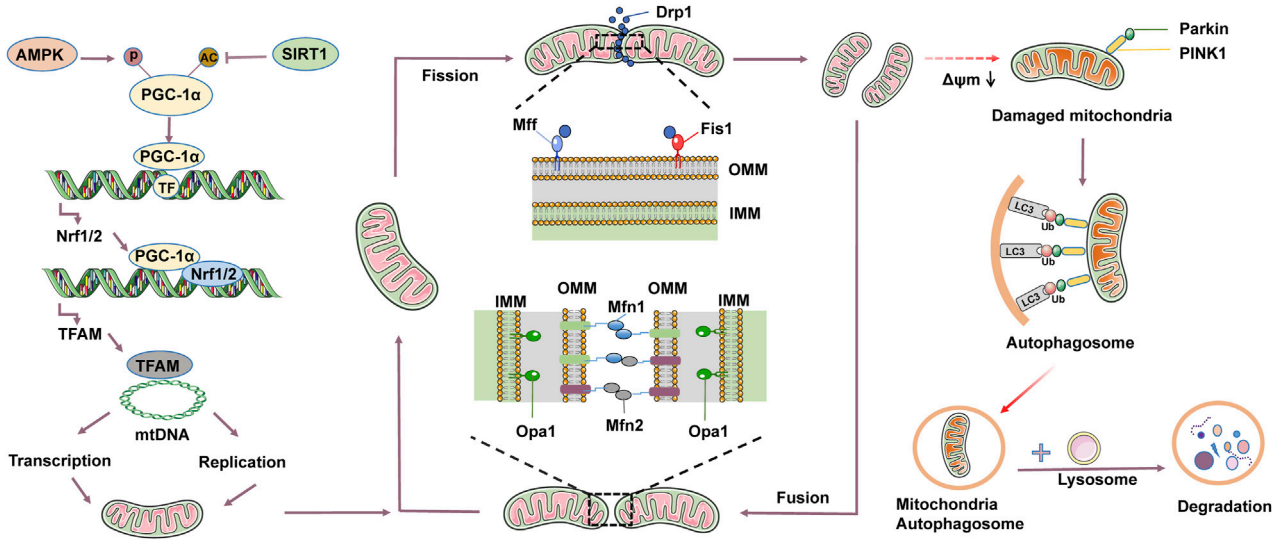


FIGURE 1
Mitochondrial quality control mainly includes mitochondrial dynamics, mitophagy, and mitochondrial biogenesis. Mitochondrial dynamics involve fusion and fission. Mitochondrial fusion is induced by homotypic and heterotypic interactions between mitochondrial fusion proteins 1 and 2 (Mfn1/2) of the outer mitochondrial membrane (OMM) and optic atrophy 1 (Opa1) of the inner mitochondrial membrane (IMM). Mitochondrial fission mainly involves dynamin-related protein 1 (Drp1), mitochondrial fission factor (Mff), and mitochondrial fission 1 (Fis1). Drp1 is recruited to OMM through interactions with Fis1 and Mff. Under physiological conditions, mitochondrial fusion and fission constraint each other to reach mitochondrial dynamic equilibrium. Mitochondrial fission contributes to the clearance of damaged or aging mitochondria, leading to a decrease in mitochondrial membrane potential (Δψm), thereby activating mitophagy. Mitophagy mainly consists of 4 key steps: 1) Depolarization of damaged mitochondria, leading to the loss of membrane potential. 2) Enclosure of mitochondria by autophagosome to form mitochondria autophagosome. 3) Fusion of mitochondria autophagosome with lysosomes. 4) Degradation of mitochondrial contents by lysosomes. Mitochondrial biogenesis is a tightly regulated process. Adenosine monophosphate-activated protein kinase (AMPK) can phosphorylate peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), while sirtuin 1 (SIRT1) can acetylate PGC-1α. PGC-1α activates signaling molecules such as nuclear respiratory factor 1/2 (Nrf1/2) and transcription factor A mitochondrial (TFAM), driving the replication and transcription of mtDNA, and translating into proteins, assembling to form new mitochondria.

oxidative stress, maintaining calcium ion balance, promoting mitochondrial biogenesis, inducing mitophagy, inhibiting mitochondrial uncoupling, and regulating myocardial insulin signaling. Therefore, in-depth research on the mechanisms of mitochondrial dysfunction in DCM and the development of treatment strategies targeting mitochondrial function is of great significance for improving the prognosis of DCM.

2 The role of mitochondrial dysfunction in the pathogenesis of diabetic cardiomyopathy

The pathogenesis of DCM is complex, with early manifestations of myocardial fibrosis, functional remodeling, and associated diastolic dysfunction, progressing to systolic dysfunction, and ultimately leading to heart failure with reduced ejection fraction (EF) values. The diagnostic criteria for DCM include left ventricular diastolic dysfunction and/or reduced left ventricular ejection fraction (LVEF), left ventricular hypertrophy, and interstitial fibrosis, which can be classified as early, late, and end stages (Joubert et al., 2019). Current research indicates that the development of DCM is associated with mitochondrial dysfunction, abnormal glucose and lipid metabolism, oxidative stress, inflammation, and myocardial fibrosis. Among them, mitochondrial dysfunction plays a crucial role in the pathogenesis of DCM (Sangwung et al., 2020). Myocardial mitochondrial dysfunction refers to the accumulation of reactive oxygen species (ROS) in cells induced by factors such as ischemia or hypoxia, resulting in abnormal mitochondrial structure and function (Bozi et al., 2020). In addition, the consequence of mitochondrial dysfunction is the excessive production of ROS in the respiratory chain. When the accumulation of ROS exceeds its limit and is beyond the clearance capacity of the antioxidant system, it promotes cellular oxidative stress and induces tissue damage. Specific factors leading to excessive ROS production include hyperglycemia, hyperlipidemia, inflammatory responses, and more. These factors disrupt the electron transport chain within the mitochondria, leading to increased ROS generation. Excessive ROS negatively impacts cell structure and function, such as oxidizing lipids, proteins, and DNA, damaging cell membranes, mitochondrial membranes, and organelle structures, resulting in cell apoptosis and inflammatory reactions (Dubois et al., 2020). Additionally, mitochondrial dysfunction is often observed in the rat model of streptozotocin (STZ) induced diabetes, which is manifested in the imbalance of mitochondrial structure, the reduction of mitochondrial DNA and the reduction of the level of biologically related messenger RNA, leading to the damage of mitochondrial biology (Marciniak et al., 2014).

Mitochondria are double-membrane organelles that maintain a highly dynamic and multifunctional network (Iannetti et al., 2015). Maintaining the integrity and function of mitochondria is crucial for

cellular physiology, especially in the energy-demanding heart. Within cardiac muscle cells, glucose is oxidized to ATP, which is the main source of cellular energy. The oxidation process mainly occurs within mitochondria, and through the catalytic action of enzymes such as adenosine triphosphate synthase, glucose is gradually broken down and energy is released. The ATP produced provides contraction and relaxation to myocardial cells, thereby maintaining normal heart function (Cree et al., 2017). However, in patients with diabetes, due to insufficient insulin secretion or insulin resistance, glucose cannot be effectively used, and the energy source of the heart turns to fatty acid oxidation (FAO). Over time, long-term dependence on FAO can lead to the accumulation of lipid metabolites in myocardial cells, ultimately leading to mitochondrial dysfunction and cardiac dysfunction (Croston et al., 2014). In summary, mitochondria are not only the main metabolic organelles in cells, but also important regulatory factors for improving insulin resistance. Their dysfunction plays a particularly important role in the development of DCM.

3 Regulatory mechanism of mitochondrial dysfunction in diabetic cardiomyopathy

3.1 Mitochondrial dynamics

Mammalian mitochondria are dynamic organelles with two membranes, constantly changing in length, size, quantity, and shape within the cell (Chang et al., 2022). Mitochondrial dynamics consists of mitochondrial fusion and fission, where fusion is the integration of substances from different mitochondria, while fission is the separation of mitochondria from intact parents (Sygitowicz et al., 2022). The main regulatory factors responsible for mitochondrial fusion in mammalian cells are mitochondrial fusion proteins 1 and 2 (Mfn1/2), located on the outer mitochondrial membrane (OMM), and optic atrophy 1 (Opa1), a protein located on the inner mitochondrial membrane (IMM) (Parra et al., 2011). Mitochondrial fusion is primarily divided into two steps: OMM and IMM fusion. OMM fusion is mainly mediated by Mfn1 and Mfn2, which can form bridges on the outer membrane of mitochondria and promote the outer membrane fusion of two mitochondria. The function of Mfn1 and Mfn2 is to guide the outer membranes of two mitochondria into contact with each other, and then promote the fusion of the outer membranes (Yu et al., 2020; Casellas et al., 2021). IMM fusion is mainly mediated by Opa1 protein. The Opa1 protein forms complexes on the mitochondrial inner membrane, promoting the fusion of the inner membranes of two mitochondria into one. The function of Opa1 is to facilitate membrane fusion, thereby forming a continuous inner membrane structure (Gilkerson et al., 2021; Tokuyama et al., 2023). Mitochondrial fission is the process of dividing intact mitochondrial progenitors into two or more mitochondria, leading to the redistribution of mitochondrial genetic material, structure, and quantity within the cell (Fröhlich et al., 2013). Mitochondrial fission mainly involves dynamin-related protein 1 (Drp1), fission protein 1 (Fis1), and mitochondrial fission factor (Mff) (Yang et al., 2022). Drp1 is a cytoplasmic GTP-dependent driving protein involved in the division process. Drp1 functions by localizing to the OMM, forming a ring around the mitochondria, and then hydrolyzing GTP to cause the ring to contract, resulting in mitochondrial fission. In addition, various post-translational modifications, including phosphorylation, ubiquitination, S-nitrosylation, and acetylation, control the transport of Drp1 from the cytoplasm to the mitochondria. These translation modifications promote mitochondrial fission by enhancing Drp1 oligomerization and its receptor attachment (Jin et al., 2021). Mff and Fis1 can also affect Drp1-induced mitochondrial fission through post-translational phosphorylation (Wang et al., 2022). In DCM, the loss of fusion and fission-related proteins can lead to DCM. Studies have shown that during the development of DCM, Drp1, Mff, and Fis1 are significantly upregulated in myocardial cells, while Opa1 and Mfn/2 are significantly downregulated (Ikeda et al., 2015).

Based on the essential role of mitochondrial dynamics in regulating DCM, some targeted drugs that normalize mitochondrial dynamics are used to treat hyperglycemia induced myocardial injury. Melatonin is an anti-diabetic drug. *In vivo*, it can prevent the occurrence of heart dysfunction in diabetes by inhibiting the mitochondrial fission induced by Drp1. Specifically, melatonin intervention reduces the expression level of Drp1, inhibits mitochondrial ragmentation, suppress oxidative stress, and reduces cardiomyocyte apoptosis by inhibiting SIRT1/PGC-1α dependent mitochondrial fission, thereby improving the mitochondrial function and cardiac function (Ding et al., 2018). Moreover, Mdivi-1 intervention inhibited the metastasis and translocation of Drp1, thus reducing the myocardial infarction area of STZ induced diabetes mice after ischemia reperfusion injury surgery. Mdivi-1 is considered as a Drp1 inhibitor (Ding et al., 2017). In diabetic hearts, mitochondrial fusion promoter M1 significantly increases the expression of mitochondrial fusion and Opa1, while reducing myocardial oxidative stress and improving myocardial fibrosis (Ding et al., 2020; Feng et al., 2021). Equally, dapagliflozin can promote mitochondrial fusion and inhibit fission, accompanied by prolonged cardiac action potential and stable $\Delta\psi m$, which may be due to upregulation of Mfn2 expression (Durak et al., 2018). Nicotinamide riboside (NR) activates SIRT1/PGC-1α/PPARα signaling transduction increases Mfn2 expression and promotes mitochondrial fusion in diabetic db/db mouse, reduces cell apoptosis, and improves heart function (Hu et al., 2022).

3.2 Mitochondrial biogenesis

Mitochondrial biogenesis is a process that maintains the quantity of mitochondria through regeneration, aiming to produce new and healthy mitochondria. The process of mitochondrial biogenesis is complex, mainly regulated by mitochondrial genes (mtDNA) and nuclear genes (nDNA). The mtDNA encode some of the proteins and RNA in the mitochondrial inner membrane, including important protein subunits and tRNA, while most mitochondrial proteins and other components are encoded by nDNA (Cameron et al., 2016; Tao et al., 2022). Under normal conditions, mitochondrial biogenesis enhances mitochondrial oxidative phosphorylation capacity, reduces

pathological oxidative stress, maintains normal mitochondrial physiological function, and meets the energy metabolism needs of the cell. However, when the process of mitochondrial biogenesis is disrupted by exogenous or endogenous factors, it can promote mitochondrial dysfunction, leading to excessive ROS production, causing mitochondrial oxidative stress and calcium overload, thereby triggering cell apoptosis or disrupting cellular homeostasis (Bruggisser et al., 2017). PGC-1α is considered a key central mediator regulating mitochondrial biogenesis, and its expression is regulated by various upstream stimuli and post-translational modifications (Novakova et al., 2022). The expression of PGC-1α is regulated by the activation of transcription factors that act on mtDNA, consisting of sirtuin 1 (SIRT1), myocyte enhancer factor 2 (MEF2), forehead box class-O1 (FoxO1), as well as other signal inducers such as AMPK, AKT-eNOs, and calmodulin dependent protein kinase IV (CaMK IV) (Gleyzer and Scarpulla, 2016; Wang et al., 2022). PGC-1α expression is regulated by post-translational modifications, consist of methylation, acetylation, ubiquitination, and phosphorylation. In addition, PGC-1α collaborating with a series of nDNA transcription factors to regulate downstream signaling pathways, consisting of nuclear respiratory factor 1/2 (Nrf1/2), transcription factor A mitochondrial (TFAM), estrogen related receptors α (ERR-α) and PPARs (Ploumi et al., 2017). PGC-1α promotes mtDNA replication and enhances mitochondrial biogenesis by interacting with its upstream and downstream factors, which is crucial for maintaining the normal physiological function of tissues with high energy metabolism demands. AMPK is a key pathway in energy metabolism, and it is activated when the intracellular ATP level decreases or the intracellular AMP/ATP ratio increases. Activated AMPK can directly phosphorylate PGC-1α, thereby increasing its transcriptional activity. Meanwhile, AMPK can phosphorylate the threonine site 177 and serine site 538 of the PGC-1α promoter, promoting the expression of PGC-1α, a series of mitochondrial target genes, and oxidative metabolism related genes (Fernandez et al., 2011). SIRT1 is a deacetylase that regulates gene expression and metabolic processes within cells, particularly playing a crucial role in energy metabolism and oxidative stress. SIRT1 can increase its expression by deacetylating PGC-1α, thereby promoting mitochondrial biogenesis (Wu et al., 2023). Some evidence has suggested an association between mitochondrial biogenesis and DCM. Diao et al. found reduced mtDNA replication and transcription, damaged mitochondrial ultrastructure, downregulation of PGC-1α, leading to impaired mitochondrial biogenesis and cardiac injury in a DCM rat model (Diao et al., 2021). Research by Tao et al. has shown that MiR-144 is downregulated in HG-induced myocardial cells and STZ-induced DCM rats. Overexpression of MiR-144 enhances mitochondrial biogenesis and inhibits cell apoptosis, while inhibiting MiR-144 shows the opposite results. In addition, Rac-1 has been identified as a regulatory gene of MiR-144. Reduced expression of Rac-1 activates AMPK phosphorylation and PGC-1α deacetylation, leading to increased mitochondrial biogenesis and reduced cell apoptosis (Tao et al., 2020). Adiponectin (APN), as an upstream activator of AMPK, showed a significant decrease in plasma levels of APN in ob/ob mouse. The researchers further validated the hypothesis that there is a causal relationship between APN reduction and mitochondrial biogenic damage. After 1 week of APN treatment in ob/ob mice, activating AMPK and reducing PGC-1α acetylation can increase mitochondrial biogenesis and alleviate mitochondrial diseases. On the contrary, knocking out APN inhibits AMPK/PGC-1α signaling and impairs mitochondrial biogenesis (Yan et al., 2013). Tetrahydrobiopterin (BH4) is a novel endogenous activator of CaMKK2 that can participate in regulating vascular and cardiac function. Research has shown that in the db/db mouse model lacking BH4, ROS production increases and induces mitochondrial dysfunction. Supplementing BH4 can improve cardiac function, correct myocardial morphological abnormalities, and increase mitochondrial biogenesis by activating the CaMKK2/PGC-1α signaling pathway (Kim et al., 2020). Antioxidant pterostilbene in blueberries regulates AMPK/NRF2/HO-1/PGC-1α signal transduction, which can reduce oxidative stress and inflammation and improve mitochondrial biogenesis in a high glucose rat model (Kosuru et al., 2018). In conclusion, an increasing amount of evidence suggests that abnormal mitochondrial biogenesis is a major factor leading to DCM, and regulating the process of mitochondrial biogenesis has become a potential strategy for treating DCM.

3.3 Mitophagy

Mitophagy contributes to maintaining the health and function of mitochondria within cells, which is crucial for cellular metabolism and survival. The process of mitophagy involves the selective targeting of autophagosomes to phagocytize dysfunctional or damaged mitochondria, which are then transferred to lysosomes for degradation (Li et al., 2021; Saito et al., 2021). There are currently three pathways that induce mitophagy, consist of the phosphatase and tensin homologue-induced putative kinase 1 (PINK1)/Parkin pathway, the FUN14 domain-containing (FUNDC1) pathway, and the BCL2/adenovirus E1B 19kDa interacting protein 3 (BNIP3)/NIX pathway (Tong et al., 2021). Among them, the most studied mitophagy pathway is PINK1/Parkin (Andres et al., 2017). PINK1, a serine/threonine kinase, functions as a messenger to relay the collapse of $\Delta\psi m$ to Parkin. Usually, PINK1 is swiftly transported to the mitochondrial matrix and cleaved by mitochondrial proteases (Jin et al., 2010). Therefore, under normal circumstances, the content of PINK1 in mitochondria is relatively low. However, when mitochondria are damaged, the decrease in $\Delta\psi m$ is directly related to the increase in PINK1 on OMM. PINK1 and Parkin jointly control the removal of damaged mitochondria (Zhang et al., 2020). Similarly, Parkin acts as an E3 ubiquitin ligase and remains cytosolic in normal conditions. However, upon depolarization of the mitochondrial membrane, it rapidly translocates to the OMM and ubiquitinates proteins located in the outer membrane, thereby marking them for elimination (Cai et al., 2022). Currently, it has been found that many Parkin substrates accumulate on OMM, such as Mfn1/2, OMM transporters, and voltage dependent anion channels (Poole et al., 2010; Morciano et al., 2020). In DCM, mitophagy enhances the regeneration of cardiomyocyte mitochondria and stimulates biogenesis, which can normalize the morphology and bioenergy of cardiac mitochondria (Wang et al., 2018; Wang et al., 2019). In addition, mitophagy also reduced lipid accumulation, improved mitochondrial homeostasis, and restored the diastolic and systolic

functions of diabetes heart (Zhou et al., 2019). Tong et al. used the mito-Keima method to evaluate mitophagy in the GFP-LC3 mouse myocardial cell model induced by a high-fat diet (HFD). Knocking out Parkin inhibits mitophagy, increases lipid accumulation, and exacerbates diastolic dysfunction. However, injection of Tat-Beclin1 (TB1) can activate mitophagy, reduce lipid accumulation, and prevent diastolic dysfunction in the heart. The study suggests that inhibiting mitophagy leads to mitochondrial dysfunction and lipid accumulation, thereby exacerbating diabetic cardiomyopathy. In contrast, the activation of mitophagy can prevent HFD-induced diabetic cardiomyopathy (Tong et al., 2019). Alisporivir is a non immunosuppressive cyclosporin derivative and a selective inhibitor of mitochondrial permeability transition pore (mPTP). Belosludtseva et al. treated HFD combined with STZ-induced diabetic mice with Alisporivir (2.5 mg/kg/d) for 20 days. Alisporivir improved mitochondrial swelling and ultrastructural changes in the myocardial cells of diabetic mice, increased the mRNA expression levels of Pink1 and Parkin in the heart tissue, and reduced the accumulation of lipid peroxides. The study suggests that Alisporivir can exert a protective effect on the heart by inducing mitophagy (Belosludtseva et al., 2021). Therefore, pharmacological methods of targeting mitophagy may be effective treatment methods to slow down the progression of DCM and improve prognosis (Figure 1).

3.4 Mitochondrial oxidative stress

Diabetic cardiomyopathy is a disease characterized by structural and functional abnormalities of the myocardium caused by hyperglycemia, and its pathogenesis is closely related to mitochondrial oxidative stress. During the process of mitochondrial oxidative phosphorylation (OXPHOS), NADH and FADH2 serve as electron donors, releasing electrons at electron transport chain (ETC) complexes I and II respectively. These electrons travel from complexes I and II through ubiquinone to complex III, and then in complex IV, O_2 combines with the electrons transferred from complex III by cytochrome C, generating H_2O. Protons are sequentially transferred to the intermembrane space through complexes I, III, and IV. Ultimately, the protons in the intermembrane space are transported back to the mitochondrial matrix through complex V, generating ATP. It is worth noting that electrons can easily leak from complexes I and III, leading to the generation of ROS (Teshima et al., 2014; Jia et al., 2016). In the environment of insulin resistance and hyperglycemia, the mitochondrial OXPHOS process is impaired, leading to reduced ATP synthesis and the production of a large amount of ROS. ROS is a highly active molecule that can cause oxidative damage by interacting with proteins, lipids, DNA, and other molecules inside the mitochondria, leading to oxidative stress. Oxidative stress affects the structure and function of mitochondria, thereby influencing cellular energy metabolism and signal transduction. In addition, oxidative stress also activates some proteins on the OMM, such as Bax and Bcl-2, which are involved in regulating cell apoptosis (Quan et al., 2020; Dewanjee et al., 2021).

Link between mitochondria oxidative stress and lipotoxicity. The augmented uptake of fatty acids (FA) by mitochondria and subsequent oxidation in diabetic cardiac tissues may surpass the respiratory capacity of mitochondria, leading to the buildup of harmful lipid metabolites. This accumulation can result in cardiac lipotoxicity and impairment of mitochondrial function (Jia et al., 2018). Adenosine monophosphate-activated protein kinase (AMPK) typically enhances the generation of new mitochondria by activating peroxisome proliferator-activated receptor-γ (PPAR-γ) coactivator-1α (PGC-1α), a key metabolic regulator of mitochondrial biogenesis and respiratory performance (Crisafulli et al., 2020). The impairment of the AMPK/PGC-1α signalling pathways associated with FAO occurs during the advanced stage of DCM, thereby exacerbating mitochondrial dysfunction (Nakamura et al., 2022). Additionally, an increase in FAO can promote the production of ROS and induce cardiac oxidative stress and inflammation. The elevated ROS levels further contribute to mitochondrial dysfunction, leading to lipid accumulation, fibrosis, diastolic dysfunction, and ultimately exacerbating heart failure (Murtaza et al., 2019). Similarly, an increase free fatty acids (FFAs) in the blood can lead to an increase of FA in cardiomyocytes. Excessive FA accumulate in cells in the form of lipid droplets and triglycerides, while diacylglycerol and ceramide also increase (Nakamura et al., 2020). Diacylglycerol triggers the exacerbation of insulin resistance and oxidative stress by activating protein kinase C (PKC). Research has demonstrated that diacylglycerol serves as a toxic lipid intermediate in cardiac tissue (Chokshi et al., 2012). The accumulation of ceramide leads to a substantial production of mitochondrial ROS, which induces mitochondrial dysfunction and oxidative stress within myocardial mitochondria (Law et al., 2018; Kim et al., 2020). Moreover, the anti-diabetic drug empagliflozin (SGLT2 inhibitor) can lead to a decrease in plasma volume and cardiac preload, regulate superoxide dismutase (SOD) levels and lipid metabolism, reduce oxidative stress, improve mitochondrial function, and thus play a protective effect on the heart (Kaludercic et al., 2020).

Recent research has highlighted the close relationship between ferroptosis and mitochondrial oxidative damage. Research shows that abnormal mitochondrial ferroptosis occurs in the heart of diabetes mice, which is mainly manifested by the decrease of Δψm, the downregulation of the expression of SOD and glutathione peroxidase 1 (GPX 1) in mitochondria, and the significant increase in mitochondrial ROS levels (Fang et al., 2020). Furthermore, another study has demonstrated that feeding mice a high-iron diet leads to severe myocardial damage, manifested as iron overload, increased lipid peroxidation, and decreased glutathione levels (Sampaio et al., 2014). Du et al. treated STZ-induced diabetic C57BL/6J mice with canagliflozin for 6 weeks, as well as H_9C_2 cardiomyocytes induced with high glucose (HG) for 24 h. Their *in vivo* and *in vitro* studies showed that canagliflozin inhibits the deposition of total iron and Fe^{2+}, downregulates the expression of ferritin heavy chain (FTN-H), upregulates the cystine-glutamate antiporter (xCT), increases the level of Δψm in the myocardium, reduces ROS levels, and inhibits mitochondrial oxidative damage. This exerts a cardioprotective effect by inhibiting ferroptosis (Du et al., 2022). Ferroptosis is a novel form of programmed cell death, and more clinical research is needed to support its role in the prevention and treatment of diabetic cardiomyopathy. In conclusion, targeting ferroptosis may provide a new strategy for the prevention and treatment of DCM.

3.5 Mitochondrial uncoupling

Mitochondrial uncoupling is an important physiological mechanism that can regulate energy metabolism and heat generation within cells. Under normal circumstances, there is a proton gradient inside the mitochondria, meaning there is a difference in proton concentration between the inner and outer membranes. During the process of uncoupling, the proton gradient is released to maintain the balance of proton concentration. The released protons will bind to the uncoupling proteins (UCPs) protein on the IMM, forming a channel that allows protons to pass through the mitochondrial inner membrane instead of through ATP synthase. In other words, the proton gradient will not be used to produce ATP, but will interact with UCPs to generate heat energy (Azzu et al., 2010). UCPs located on the IMM are considered the main mediators of mitochondrial uncoupling. UCPs family consists mainly of UCP1-UCP5. UCP1 is highly expressed in the mitochondria of adipose tissue and is mainly responsible for temperature regulation. UCP2 is widely expressed in most tissues, such as the myocardium, and is involved in the body's energy metabolism. UCP3 is predominantly expressed in skeletal muscle, while UCP4 and UCP5 are present in brain tissue. The expression of different UCPs in different tissues reflects different physiological functions. Currently, research on DCM mainly focuses on UCP2 and UCP3, especially UCP2 (Mailloux and Harper, 2011; Akhmedov et al., 2015).

In DCM, UCP2 may be involved in the development of the disease through various physiological mechanisms. UCP2 can regulate the permeability of the IMM, increasing the proton permeability within the mitochondria, thereby reducing the electrochemical load of the mitochondria, decreasing the proton gradient within the mitochondria, inhibiting the coupling of the tricarboxylic acid cycle and oxidative phosphorylation, ultimately leading to mitochondrial uncoupling. This uncoupling may result in reduced ATP synthesis within the cells, while increasing the production of ROS within the mitochondria, thereby triggering oxidative stress and cell damage (Cadenas, 2018; Nirengi et al., 2020; Ho et al., 2022). In addition, UCP2 may also be involved in the occurrence of DCM by regulating lipid metabolism. Studies have shown that UCP2 can affect lipid metabolism pathways, including FAO and synthesis, thereby influencing intracellular lipid content and oxidative stress levels. The dysregulation of lipid metabolism in relation to UCP2 regulation may lead to increased lipid accumulation in myocardial cells, thereby affecting myocardial cell function (Diano et al., 2012). Due to the elevated levels of FFA in DCM, the enhanced expression of UCPs is directly induced by PPARα, thereby affecting the permeability and proton leak of IMM, inhibiting ATP production, which is typically observed in failing hearts (Wang et al., 2021). PPARα can promote the oxidative metabolism of FA, including FA uptake, transport, β-oxidation, and the permeability of the IMM. These processes directly impact the electrochemical gradient of the IMM, thus influencing the degree of mitochondrial uncoupling (Lee et al., 2017; Crescenzo et al., 2019; Liu et al., 2022). Mitochondrial uncoupling is mainly mediated by the activation of UCPs. In a report by Dludla et al., it is suggested that guanosine diphosphate (GDP) can inhibit the activation of UCPs, preventing mitochondrial proton leak in diabetic db/db mice (Dludla et al., 2018). Additionally, some studies have suggested that overexpression of UCP2 may lead to mitochondrial dysfunction and exacerbate the development of diabetic cardiomyopathy. The application of PPAR agonists (such as pioglitazone) can regulate the expression of UCP2, significantly reducing the levels of free fatty acids in the plasma of type 2 diabetic patients, increasing Δψm, and restoring normal mitochondrial function (Wassef et al., 2018). Interestingly, in another study, it was found that UCP2 was downregulated in a STZ-induced diabetic mouse model, leading to a decrease in Δψm and an increase in cell death. However, overexpression of mitochondrial aldehyde dehydrogenase (ALDH2) can reverse this situation, resulting in beneficial effects on cardiac structure and function, mitochondrial function, and cell survival (Zhang et al., 2023). In summary, targeted regulation of UCP2 will enhance our understanding of DCM.

3.6 Mitochondrial calcium handling

The mitochondrial calcium handling plays a crucial role in maintaining normal cellular function. Mitochondrial calcium homeostasis is the balance of intracellular calcium concentration maintained by mitochondria by regulating the uptake and release of calcium ions (Diaz et al., 2021). Within mitochondria, Ca^{2+} enhances oxidative phosphorylation activity (including mitochondrial complexes I, III, IV, and complex V), as well as activates pyruvate dehydrogenase complex (PDC), alpha-ketoglutarate dehydrogenase, and isocitrate dehydrogenase to enhance ATP regeneration (Ketenci et al., 2022). Various calcium channel proteins exist on the inner mitochondrial membrane, with the most important ones being mitochondrial calcium uniporter (MCU) and voltage-dependent anion channel (VDAC). MCU is a key protein that regulates the uptake of calcium ions on the IMM, while VDAC is involved in regulating the opening of calcium channels on the OMM. The functional abnormalities or changes in expression levels of these channel proteins may lead to excessive or insufficient uptake of calcium ions in mitochondria, thereby affecting mitochondrial function (Hamilton et al., 2021). Mitochondrial calcium uptake protein 1 (MICU1), located on the inner mitochondrial membrane, interacts with MCU to regulate the uptake of calcium ions by the mitochondria. When the intracellular calcium ion concentration increases, MICU1 binds to the MCU channel and inhibits its activity, thereby reducing the uptake of calcium ions within the mitochondria. This regulatory effect helps maintain the balance of calcium ions within the mitochondria, preventing excessive uptake of calcium ions from damaging mitochondrial function (Ji et al., 2017). In DCM, abnormal mitochondrial calcium handling may lead to mitochondrial dysfunction and damage to myocardial cells. Studies have shown that the expression level of MICU1 is downregulated in 12 week old db/db mouse cardiomyocytes, accompanied by mitochondrial dependent intrinsic apoptosis. In this mouse model, the reconstruction of MICU1 can reduce myocardial hypertrophy and fibrosis, inhibit cell apoptosis, and normalize cardiac function. Furthermore, studies have shown that upregulation of MICU1 increases mitochondrial Ca^{2+} uptake, weakens mitochondrial ROS production and cell apoptosis (Dillmann, 2019). This dysfunctional calcium handling can be rescued by restoring calcium to mitochondria, thereby enhancing mitochondrial activity and energy production. Similarly, in the STZ induced diabetes mouse model, Suarez et al. found that the heart of diabetes mice showed changes in the expression of MCU and MCU members, which led to the decrease of

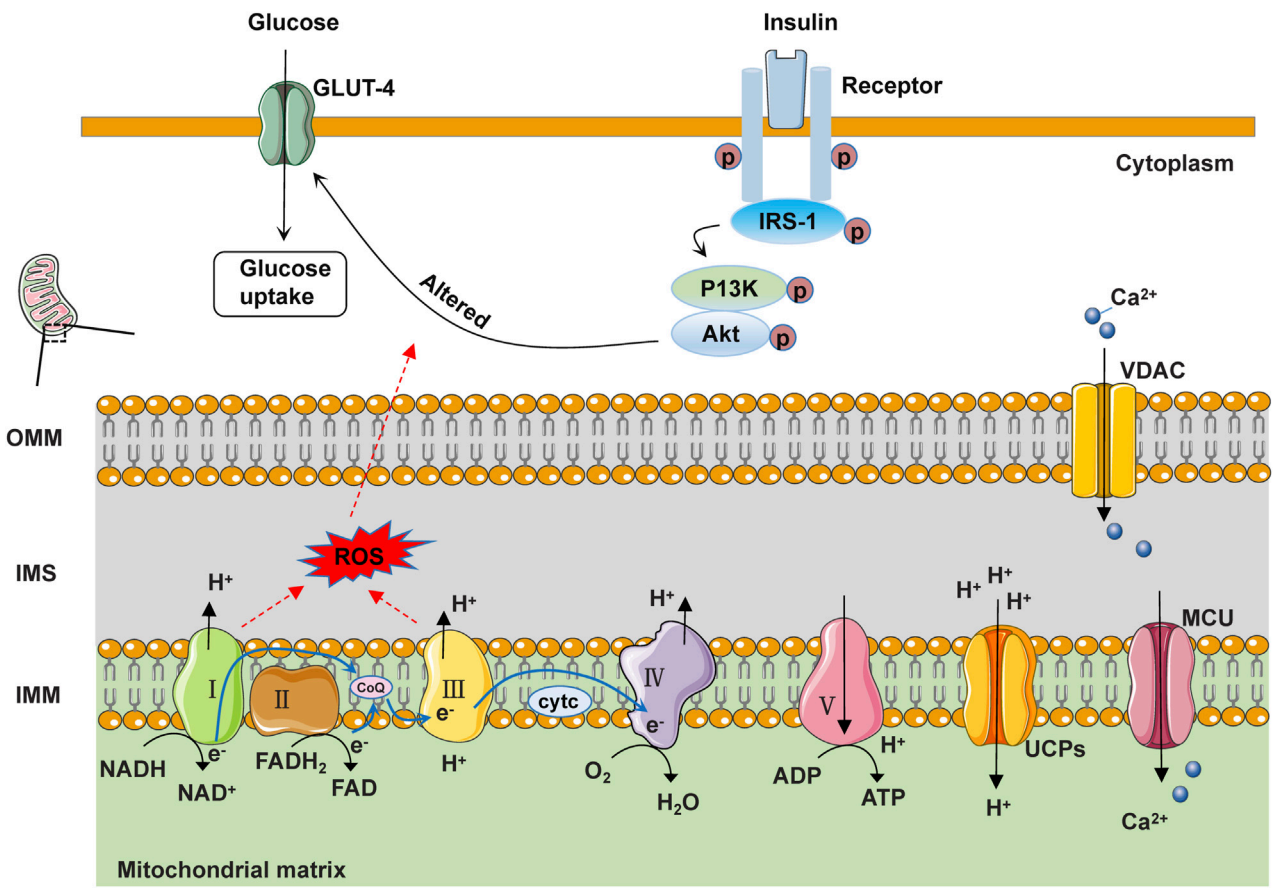


FIGURE 2
Schematic diagram of the structure and function of mitochondria under physiological conditions. In IMM, NADH and FADH2 serve as electron donors, releasing electrons at complexes I and II, respectively. These electrons pass from complexes I and II through ubiquinone to reach complex III. Subsequently, at complex IV, O2 in the matrix accepts electrons transferred from cyt c by complex III, generating H2O. Protons are sequentially transferred to the intermembrane space (IMS) through complexes I, III, and IV. Ultimately, the protons in the IMS are transported back to the matrix through complex V to generate ATP. Additionally, electrons can easily leak from complexes I and III, leading to the formation of reactive oxygen species (ROS). Some protons return to the mitochondrial matrix through uncoupling proteins (UCPs), generating heat. Free Ca^{2+} in the cytoplasm can enter the mitochondrial matrix through the voltage-dependent anion channel protein (VDAC) on the OMM and the mitochondrial calcium uniporter (MCU) on the IMM. When insulin binds to the insulin receptor, the activated receptor phosphorylates the IRS-1 protein. IRS-1 further activates the phosphorylation activity of PI3K and Akt, thereby promoting the transport of GLUT4 and glucose uptake.

mitochondrial Ca^{2+} uptake, mitochondrial energy function and cardiac function. On the contrary, normalization of MCU levels based on adeno-associated viruses in these hearts restored mitochondrial Ca^{2+} homeostasis, reduced PDC phosphorylation levels, improved cardiac energy metabolism and cardiac function (Suarez et al., 2018). In addition, research has shown that GrpE-like 2 (Grpel2) can reduce myocardial ischemia/reperfusion injury by inhibiting mitochondrial calcium overload mediated by MCU. Grpel2 levels are decreased in STZ-induced DCM, and overexpression of Grpel2 can mitigate mitochondrial dysfunction and apoptosis in DCM by maintaining dihydrolipoyl succinyltransferase (DLST) input to mitochondria (Yang et al., 2023).

3.7 Myocardial insulin signalling

Diabetic cardiomyopathy is a heart disease caused by hyperglycemia and insulin resistance. Diabetic patients often experience insulin resistance (reduced sensitivity of cells to insulin), leading to abnormal insulin signal transduction, including reduced expression and function of insulin receptor substrate-1 (IRS-1) and blocked PI3K/Akt signaling pathway. These abnormalities can affect cellular glucose uptake and utilization, leading to cellular metabolic disorders (Salvatore et al., 2021). IRS-1 is one of the key molecules in insulin signal transduction. Under normal circumstances, insulin binds to its receptor, activates IRS-1, and then activates the PI3K/Akt signaling pathway. Activated Akt can promote the translocation and transposition of GLUT4, thereby increasing glucose uptake. However, in DCM, insulin signal transduction is inhibited, leading to a decrease in the phosphorylation level of IRS-1, which affects the function of IRS-1, making it unable to effectively recruit and activate the PI3K/Akt pathway (Chen et al., 2022). This abnormal insulin signal transduction may directly affect mitochondrial function. Mitochondria are the energy production centers within cells, responsible for producing most of the intracellular ATP (Qi et al., 2013). Insulin signaling abnormalities can affect the structure and

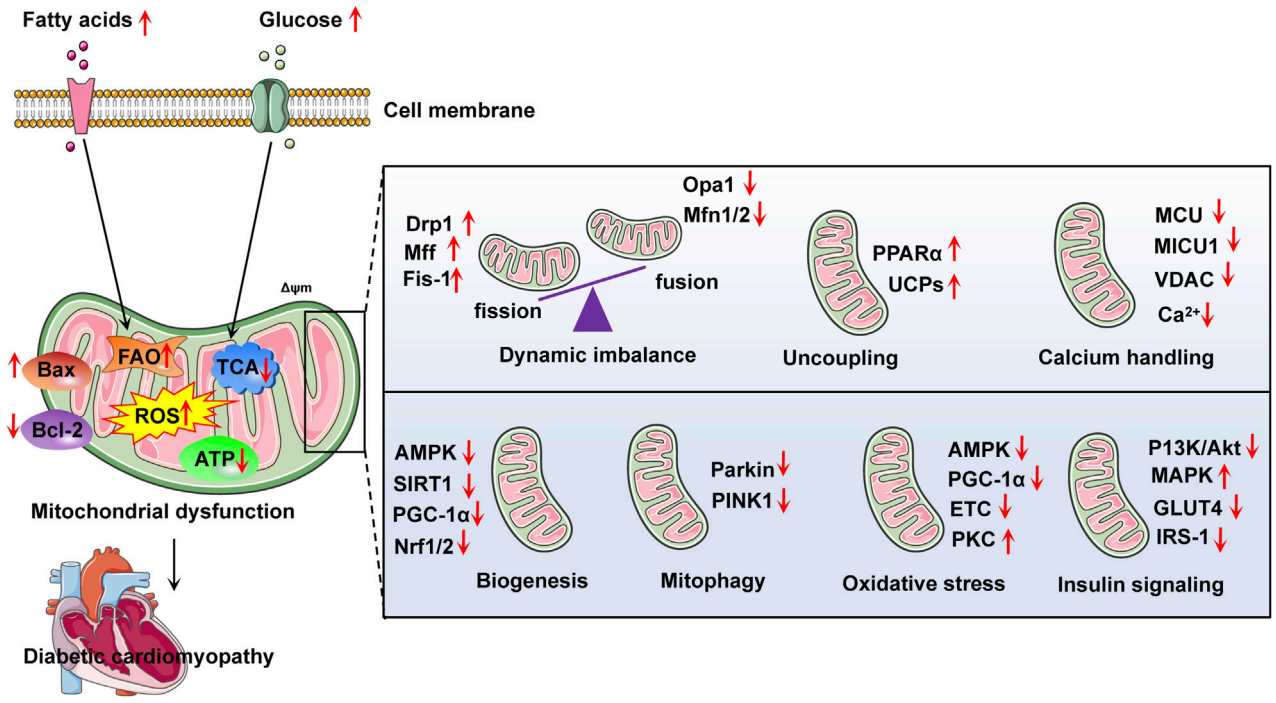


FIGURE 3
Summarizes some relevant targets in the development of diabetic cardiomyopathy with mitochondrial dysfunction. Abnormal metabolism of fatty acid and glucose may lead to mitochondrial dysfunction, thus aggravating the development of diabetes cardiomyopathy. FAO, fatty acid oxidation; ROS, reactive oxygen species; ATP, adenosine triphosphate; Bcl-2, B-cell lymphoma-2; Bax, BCL2-associated X protein; PPARα, peroxisome proliferator-activated receptor-α; MCU, mitochondrial calcium uniporter; VDAC, voltage-dependent anion channel; AMPK, adenosine monophosphate-activated protein kinase; PINK1, phosphatase and tensin homolog induced putative kinase 1; Opa1, optic atrophy 1; Mfn1/2, mitofusion 1/2; Drp1, dynamin related protein 1; UCPs, uncoupling proteins; PKC, protein kinase C; ETC., electron transport chain; IRS-1, insulin receptor substrate 1; Nrf1/2, nuclear respiratory factor 1/2; GLUT4, glucose transporter type 4; Δψm, mitochondrial membrane potential; MAPK, mitogen-activated protein kinase.

function of mitochondria, leading to changes in mitochondrial membrane permeability, disruption of oxidative phosphorylation, and increased oxidative stress. These changes may result in mitochondrial dysfunction and subsequently affect the energy metabolism of myocardial cells (Chen et al., 2022). Research has shown that knocking out the insulin receptor in the heart leads to reduced glucose uptake and increased mitochondrial ROS production in the heart (Zhou et al., 2018; Gargiulo et al., 2020). Knockout of IRS-1 reduces ATP content in myocardial cells, impairs cardiac metabolism and function, increases fibrosis, and exacerbates heart failure (Battiprolu et al., 2012; Bugger et al., 2012). The results of ventricular muscle biopsy obtained from T2DM patients have shown reduced PI3K/Akt signaling, as well as decreased GLUT4 expression and translocation (Hou et al., 2019). The E3 ubiquitin ligase mitsugumin 53 may play a crucial regulatory role in maintaining insulin signaling. Elevated levels of mitsugumin 53 in a T2DM mouse model are associated with increased degradation of insulin receptor and IRS-1 proteins. Overexpression of mitsugumin 53 inhibits insulin signaling transduction and promotes cardiac fibrosis (Song et al., 2013; Liu et al., 2015). Conversely, downregulation of mitsugumin-53 may be a potential therapeutic approach to prevent diabetic cardiomyopathy from progressing to heart failure. In addition, abnormal insulin signaling may also lead to increased apoptosis, while the activation of the mitogen-activated protein kinase (MAPK) signaling pathway may exacerbate this process. Under normal circumstances, insulin promotes cell proliferation and growth, maintains the structure and function of cardiomyocytes, further promotes glucose uptake and utilization, and provides the energy substrates needed for mitochondria by regulating the MAPK signaling pathway. However, hyperglycemia and insulin resistance can lead to increased levels of inflammatory factors and oxidative stress within cells, thereby activating the MAPK signaling pathway. The activated MAPK signaling pathway can promote cardiomyocyte apoptosis, fibrosis, and inflammatory reactions, accelerating the progression of myocardial disease (Jia et al., 2018). In general, insulin signaling transduction regulates various signaling molecules and pathways, affecting the energy metabolism and function of cells. When these signaling pathways become disrupted or imbalanced, it can lead to insulin resistance and the development of diseases such as diabetes. Therefore, a thorough understanding of the molecular mechanisms of insulin signaling transduction is helpful in revealing the pathogenesis of diabetes and providing new targets and strategies for the treatment and prevention of related diseases (Figure 2).

4 Potential targets and therapeutic strategies of mitochondrial dysfunction in diabetes cardiomyopathy

Currently, there is no effective treatment regimen to reverse or control the progression of DCM. Strict blood sugar control is the most accepted initial methods (Thai et al., 2021). After the diagnosis of DCM,

cardiac function changes rapidly and the risk is high of further development into heart failure. Because mitochondrial dysfunction is the most common driving factor of diabetes cardiomyopathy, mitochondrial dynamics imbalance, excessive oxidative stress, damaged mitophagy, impaired mitochondrial biosynthesis, and impaired mitochondrial calcium processing constitute potential therapeutic targets (Varkuti et al., 2020) (Figure 3).

Some antidiabetic medications currently in use may directly or indirectly interfere mitochondrial abnormalities associated with DCM, such as metformin, dapagliflozin, and empagliflozin. Metformin is a widely used diabetes drug in clinic. Metformin promotes mitochondrial autophagy and improves myocardial cell dysfunction through AMPK-dependent or -independent mechanisms in diabetic hearts. Additionally, metformin can stimulate mitochondrial biogenesis in high glucose-induced cardiomyocytes by upregulating transcription factors related to mitochondrial biogenesis (such as PGC-1α and TFAM) (Abdel et al., 2018; Liu et al., 2020). However, the detailed mechanism by which metformin regulates DCM mitochondrial function remains unclear (Packer, 2020). In addition, for diabetic patients at risk of cardiovascular disease, cautious consideration of the use of metformin and close monitoring of cardiovascular events may be necessary. As new hypoglycemic drugs, Dapagliflozin and empagliflozin are sodium-glucose cotransporter-2 inhibitors (SGLT2is) that exhibit protective effects on reducing cardiovascular mortality and heart failure in patients with T2DM. Recent evidence suggests that SGLT2is may play a protective role in the heart by regulating the mitochondrial function in diabetes models. In obese insulin-resistant rats induced by a HFD, administration of dapagliflozin 4 weeks before myocardial ischemia/reperfusion injury can effectively reduce mitochondrial ROS production, swelling, as well as depolarization. Studies have also shown that dapagliflozin enhances mitochondrial ultrastructure by decreasing fragmentation and ridge loss within the mitochondria (Wiviott et al., 2019; Li and Zhou, 2020). Additionally, in STZ-induced diabetic rats fed with a HFD, empagliflozin has been shown to improve atrial structural and electrical remodeling by enhancing mitochondrial respiratory function and biogenesis. It plays a crucial role in mitochondrial biogenesis by activating the PGC-1α-NRF1-TFAM signaling pathway to prevent the induction of atrial fibrillation (Shao et al., 2019). However, the exact role of mitochondrial biogenesis in the occurrence and progression of ischemic cardiomyopathy or atrial fibrillation in diabetic patients remains unclear.

Besides positive effect of antidiabetes drugs on DCM mitochondria, other strategies may also be promising treatments for mitochondrial dysfunction (Ketenci et al., 2022). For example, targeting mitochondrial ROS clearance is a positive potential therapeutic strategy in DCM. The mitochondrial targeted drugs Mn (III) tetrakis (4-benzoic acid) porphyrin (MnTBAP) and mitoquinone (MitoQ) have been shown to have the ability to alleviate oxidative stress in preclinical studies. MnTBAP intervention can reverse myocardial oxidative stress and improve mitochondrial bioenergy in a mouse model of metabolic syndrome. MitoQ treatment can reduce ROS accumulation and demonstrate anti-inflammatory and anti-oxidantion effects in T2DM patients (Ilkun et al., 2015; Escribano et al., 2016). In addition, targeting mitochondrial regulators may also have beneficial effects on DCM. AMPK is the main regulator of mitochondrial energy homeostasis. Activation of AMPK enhances the expression and translocation of GLUT4, enhances insulin stimulated glucose uptake, and promotes mitochondrial biogenesis. Mechanically, activated AMPK in myocardial cells increases glucose uptake and utilization, while also negatively regulating mTOR signaling and gluconeogenesis, lipid as well as protein synthesis (Abdel et al., 2017). The activation of AMPK is essential to prevent the progression of diabetes cardiomyopathy. Therefore, AMPK is considered as an effective target for drug discovery and development to prevent and reverse DCM. Moreover, PPARα plays a crucial regulatory role in mitochondrial oxidative stress and myocardial glucose and lipid metabolism. The role of PPARα in diabetes cardiomyopathy is mainly reflected in regulating lipid metabolism and maintaining energy balance of myocardial cells. It was found that the activity of PPARα was affected by the state of diabetes, and its expression and function might be inhibited, leading to lipid metabolism disorder and myocardial cell function damage (Lee et al., 2013). In addition, the activity of PPARα can also affect myocardial mitochondrial function, including mitochondrial morphology, quantity, respiratory chain complexes, $\Delta\psi m$, and oxidative stress response (Yin et al., 2019). Therefore, in-depth study on the expression and regulation mechanism of PPARα in diabetes cardiomyopathy and the relationship between PPARα and mitochondrial function will help reveal the molecular mechanism of the development of diabetes cardiomyopathy and provide new targets and strategies for disease treatment. Table 1 Summarizes the intervention measures and targets targeting mitochondrial dysfunction in the progress of DCM.

5 Plant secondary metabolites-based diabetes cardiomyopathy targeting mitochondrial dysfunction

Secondary metabolites derived from plants have the properties of being safe, effective, and low in toxicity. Research on the prevention and treatment of diabetes and its complications using these metabolites has attracted increasing attention (Sukhikh et al., 2023). Previous studies have reported that the research on plant secondary metabolites for diabetes mainly focuses on regulating lipid and protein metabolism pathways, insulin signaling pathways, anti-inflammatory responses, and anti-oxidant stress (Shehadeh et al., 2021). In recent years, targeting mitochondrial function has become a promising treatment strategy for various diseases. Therefore, influencing mitochondrial function may have beneficial effects on DCM. This section reviews some biologically active plant secondary metabolites targeting mitochondrial dysfunction for the treatment of DCM (Figure 4).

5.1 Flavonoids

Flavonoids are secondary metabolites of plants, characterized by compounds with a 2-phenylchromen-4-one structure, widely present in plants. They exhibit various pharmacological activities, including antioxidant, anti-inflammatory, and anti-tumor effects, and have been used to treat various diseases (including diabetes) (Shen et al., 2022). Flavonoids are considered promising anti-diabetic drugs, but

TABLE 1 The intervention measures and targets targeting mitochondrial dysfunction in the progress of diabetes cardiomyopathy.

Intervention	Model	Target spots or pathways	Effect	References
Melatonin	STZ injection mice	SIRT1/PGC-1α, Drp1	Inhibit mitochondrial fission, reduce oxidative stress	Ding et al. (2018)
Nicotinamide riboside	db/db mice	SIRT1/PGC-1α/PPARα, Mfn2	Promotes mitochondrial fusion	Hu et al. (2022)
MiR-144	STZ injection mice	AMPK /PGC-1α	Increases mitochondrial biogenesis, reduced cell apoptosis	Tao et al. (2020)
Adiponectin	db/ob mouse	AMPK/PGC-1α	Increases mitochondrial biogenesis	Yan et al. (2013)
Tetrahydrobiopterin	ob/ob mouse	CaMKK2/PGC-1α	Increase mitochondrial biogenesis	Kim et al. (2020)
Tat-Beclin1	High-fat diet -induced GFP-LC3 mouse	Parkin	Induce mitophagy	Tong et al. (2019)
Alisporivir	High-fat diet and STZ injection rats	PINK1, Parkin	Induce mitophagy	Belosludtseva et al. (2021)
Guanosine diphosphate	db/db mice	UCPs	Inhibit mitochondrial uncoupling	Dludla et al. (2018)
Grpel2	STZ injection mice	MCU, DLST	Inhibit mitochondrial calcium	Yang et al. (2023)
Mitsugumin 53	High-fat diet -induced obese mice	IRS-1	Improve insulin resistance	Liu et al. (2015)
Metformin	STZ injection mice	AMPK/PGC-1α, TFAM	Induce mitophagy, improve mitochondrial biogenesis	Liu et al. (2020), Abdel et al. (2018)
SGLT2is	High-fat diet and STZ injection rats	AMPK, PGC-1α, NRF, TFAM	Reduce oxidative stress,improve mitochondrial biogenesis	Li and Zhou (2020), Wiviott et al. (2019)

their poor bioavailability is also recognized. The use of drug delivery technologies such as microencapsulation, nano delivery systems, microemulsions, and enzyme-promoted methylation can enhance the therapeutic effects and bioavailability of flavonoids (Hussain et al., 2020). Flavonoids have a significant hypoglycemic effect by regulating the activity of mitochondrial respiratory chain complexes, affecting oxidative phosphorylation in mitochondria, reducing oxidative stress, improving mitochondrial energy metabolism and ATP synthesis, and helping to reduce the risk of diabetes and its complications (Sapian et al., 2021).

Puerarin is a flavonoid compound isolated from *Pueraria lobata* (Willd.) Ohwi, which has pharmacological activities such as reducing insulin resistance, alleviating inflammatory reactions, improving microcirculation, and inhibiting platelet aggregation (Huang et al., 2020). Cheng et al. found that after 4 weeks of treatment with puerarin (100 mg/kg/d), the expression and translocation of GLUT4 increased, while the expression and translocation of CD36 decreased in STZ and nicotinamide (NA)-induced diabetic mice. Puerarin also enhances Akt phosphorylation, reduces PPARα expression, and improves heart function after myocardial infarction in diabetes mice by regulating mitochondrial energy metabolism (Cheng et al., 2015). In a controlled trial involving 50 patients undergoing heart valve replacement, puerarin appears to enhance the safety and effectiveness of valve replacement surgery. Pretreatment with puerarin reduces the activation of neutrophil NF-κB and the overexpression of IL-6, IL-8, inhibits the release of cardiac enzymes troponin I (cTnI), and creatine kinase isoenzyme MB (CK-MB), indicating a protective effect on the myocardium (Zhou et al., 2019). Additionally, Sun et al.'s research has shown that Puerarin-V (a new crystal form of puerarin) can significantly reduce mitochondrial ROS production, decrease MDA levels, increase the activity of SOD and GSH in the myocardium, improve the activity of the mitochondrial electron transport chain, and enhance the mitochondrial respiratory function related to complexes I/II in DCM mice. These results indicate that puerarin-V plays an antioxidant role in DCM and may be involved in improving mitochondrial dysfunction. Furthermore, the therapeutic effect of puerarin-V in DCM is superior to that of puerarin injection (a marketed drug for myocardial ischemia), indicating that puerarin-V may be an attractive compound for developing anti-DCM drugs (Sun et al., 2022).

Apigenin is one of flavonoids widely found in plants, fruits, and vegetables, with anti-oxidant, anti-inflammatory, and anti-tumor effects (Salehi et al., 2019). Liu et al. research has confirmed that treatment with apigenin (100 mg/kg/d) for 7 months can significantly improve myocardial remodeling and cardiac function in STZ-induced diabetic C57BM/6J mice models. It also inhibits myocardial cell apoptosis, improves myocardial mitochondrial oxidative stress and inflammatory response, and normalizes myocardial mitochondrial energy. These pathological changes are achieved by inhibiting the excessive accumulation of 4-hydroxynonenal through apigenin, upregulating the expression of Bcl-2 and GPx, increasing SOD activity, reducing malondialdehyde (MDA) activity, downregulating the expression of Bax and Cleaved caspase3, and inhibiting the translocation of NF-κB (Liu et al.,

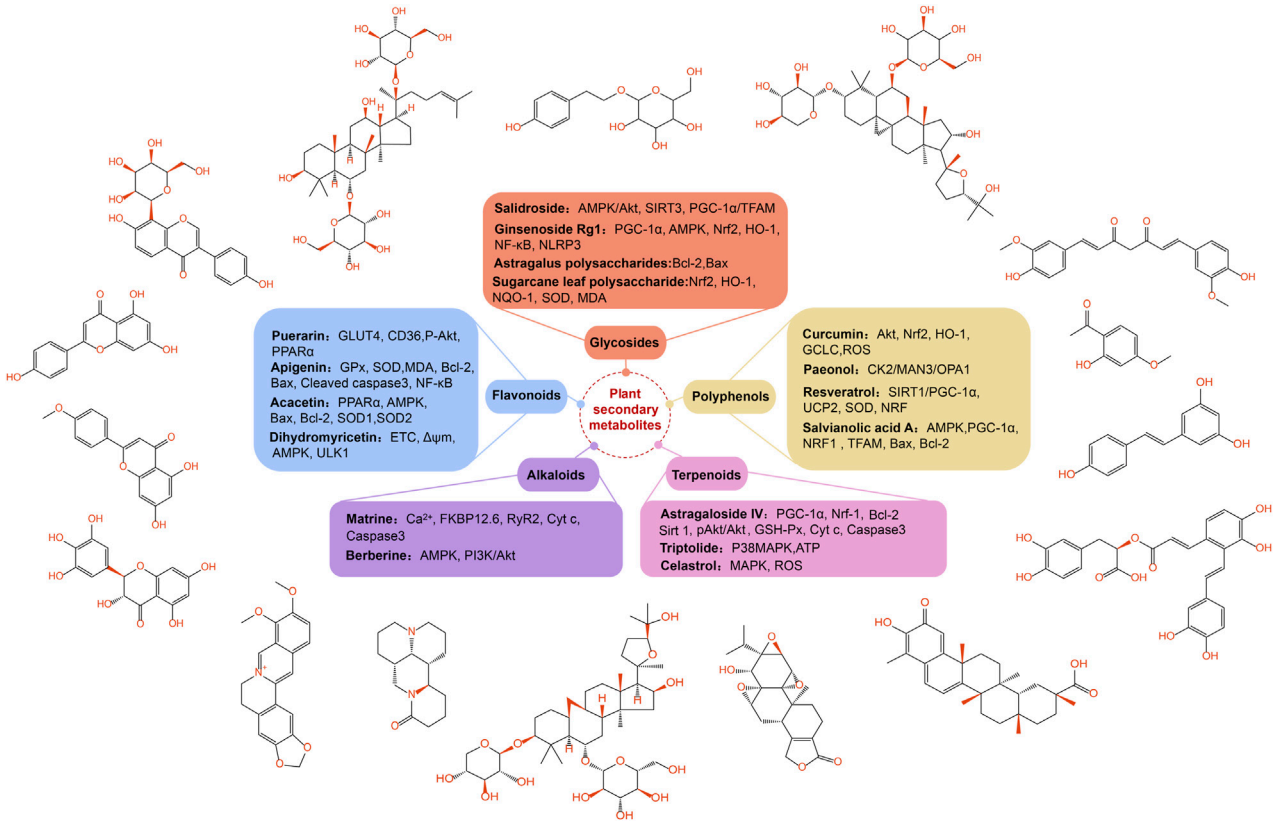


FIGURE 4
The classification of plant secondary metabolites and their main targets of regulating diabetes cardiomyopathy. Some plant secondary metabolites have been found to alleviate the pathological changes of diabetes cardiomyopathy, including flavonoids, polyphenols, terpenes, alkaloids and glycosides. These plant secondary metabolites are mainly regulated through targets related to mitochondrial function. Opa1, optic atrophy 1; UCP2, uncoupling protein 2; Nrf2, nuclear factor E2-related factor 2; HO-1, heme oxygenase-1; GCIC, glutamate-cysteine ligase catalyst; MDA, malondialdehyde; NF-κB, nuclear factor kappa-B; ULK1, unc-51 like autophagy activating kinase 1; GSH, glutathione; GSH-Px, glutathione peroxidase; Nrf-1, nuclear respiratory factor-1; CK2α, casein kinase 2α; Stat3, signal transducer and activator of transcription 3; RyR2, Ryanodine receptor 2.

2017). Additionally, apigenin has been found to reverse mitochondrial dysfunction induced by lipopolysaccharide (LPS), maintaining mitochondrial homeostasis and function by promoting the expression of mitochondrial SIRT3, inducing mitochondrial biogenesis (PGC-1α, TFAM) and fusion proteins (Mfn2, Opa1), and activating mitochondrial autophagy (PINK1, parkin) (Ahmedy et al., 2022). In conclusion, the results suggest that apigenin may be a promising compound for treating diabetic cardiac damage and neurological diseases by targeting mitochondrial function.

Acacetin is a common natural flavonoid compound that can be extracted and isolated from *Carthamus tinctorius* L. Pharmacological studies have shown that it has antioxidant, anti-tumor, anti-inflammatory, and cardiovascular protective effects (Han et al., 2021). The study by Song et al. demonstrated that in the STZ-induced Sprague-Dawley (SD) diabetic rat model, treatment with acacetin (10 mg/kg/d) for 16 weeks activated AMPK protein phosphorylation and regulated the expression levels of PPARα. *In vitro* experiments showed that acacetin (0.3, 1, 3 μM) downregulated the expression of Bax protein in H_9C_2 cells, while up-regulating the expression of Bcl-2, SOD1 (located in the intermembrane space of mitochondria), and SOD2 (mainly located in the mitochondrial matrix). The study suggests that acacetin can reduce oxidative stress, inhibit mitochondria-dependent cell apoptosis, improve mitochondrial function, and alleviate diabetic myocardial damage (Song et al., 2022). In addition, Han et al. found that acacetin can reduce ROS production and levels of MDA, inhibit depolarization of Δψm, upregulate the expression and activity of SOD, Bcl-2, PGC-1α, pAMPK, Sirt1, and Sirt3, and exert its cardioprotective effect. It is worth noting that when Sirt3 is knocked out, the cardioprotective effect of acacetin is eliminated (Han et al., 2020). In conclusion, research shows that acacetin can prevent mitochondrial dysfunction, reduce oxidative stress, and reduce the incidence of cardiovascular disease in diabetes.

Dihydromyricetin is a dihydroflavonol compound widely present in plants of the ampelopsis family, with pharmacological effects including scavenging free radicals, antioxidant, and anti-fibrotic properties (Zhang et al., 2022). Wu et al. found that treatment with dihydromyricetin (100 mg/kg/d) for 14 weeks improved mitochondrial function in STZ-induced diabetic C57BL/6J mice, increasing ATP content in, ETC., and the activity of complex I/II/III/IV/V, restoring Δψm, reducing oxidative stress, and improving mitochondrial energy metabolism. Furthermore, the study also indicated that dihydromyricetin can activate AMPK and the phosphorylation level of unc-51 like autophagy activating kinase 1 (ULK1), enhancing autophagic function in diabetic mice and

preventing the occurrence of cardiac dysfunction (Wu et al., 2017). Hua et al. believe that dihydromyricetin may lower fasting blood glucose and glycated hemoglobin levels in diabetic mice, inhibit the production of ROS in mitochondria, upregulate SIRT3, SOD2 protein expression, and increase mtDNA copy number to suppress oxidative stress in diabetic mice and improve diabetic vascular endothelial dysfunction. This may be achieved through mediating SIRT3-dependent pathways (Hua et al., 2020). These research findings suggest that dihydromyricetin may have significant potential in regulating mitochondrial biosynthesis, stimulating mitochondrial autophagy, and combating oxidative stress.

5.2 Terpenoids

Terpenoids are polymers of isoprene and its derivatives, and they are very important secondary metabolites in plants. Terpenoids have shown anti diabetes properties *in vivo* and *in vitro* studies, which can increase insulin secretion in body tissues, promote the translocation of GLUT4 to increase glucose uptake, protect pancreatic cells and improve the expression of inflammatory factors (Putta et al., 2016). Recent reports indicate that terpenoids may also improve the development of diabetes cardiomyopathy by regulating mitochondrial function (Zhang et al., 2024).

Triptolide is a diterpenoid compound isolated from *Tripterygium wilfordii* Hook, possessing pharmacological effects such as anti-inflammatory, immune regulation, and anti-cancer properties (Gao et al., 2021). Liang et al. treated SD diabetic rats induced by STZ with triptolide (100, 200, or 400 μg/kg/d) for 6 weeks and evaluated cardiac energy metabolism using P-31 nuclear magnetic resonance spectroscopy. The study results indicated that the optimal therapeutic effect was achieved with a dose of 200 μg/kg/d of triptolide, which could enhance cardiac energy metabolism by promoting mitochondrial ATP generation and upregulating the expression of P38 MAPK protein, improving cardiac function in diabetic cardiomyopathy rats through the regulation of MAPK signaling pathways (Liang et al., 2015). Additionally, Pan et al.'s research indicates that during the process of cardiac remodeling, the expression of FoxP3 is downregulated in cardiomyocytes, leading to sustained activation of Parkin-mediated mitochondrial autophagy. However, Triptolide can regulate mitophagy, restoring the activity of FoxP3 in cardiomyocytes. Mechanistically, FoxP3 interacts with a sequence downstream of the binding site for Activating Transcription Factor 4 (ATF4), which involves the promoter of Parkin and sequestered free nuclear ATF4, to reduce the expression of Parkin mRNA during the process of cardiac remodeling. In conclusion, studies suggest that triptolide may be an effective cardioprotective agent (Pan et al., 2022).

Celastrol is one of terpenoids isolated from *T. wilfordii* Hook, which has various biological activities such as anti rheumatoid, anti-tumor, and antioxidant properties (Wang et al., 2020). Wu et al. used network pharmacology to predict the key regulatory targets of celastrol on DCM, and analyzed its biological processes and signaling pathways through animal experiments. The results showed that celastrol (50 μg/kg/d) treatment for 4 weeks could downregulate the expression of P38 protein in the MAPK pathway, reverse the energy remodeling, mitochondrial dysfunction, and oxidative stress induced by STZ-induced SD diabetic rats, thereby delaying the deterioration of heart function and myocardial interstitial fibrosis. The study suggests that the MAPK signaling pathway may be an effective intervention target for DCM (Wu et al., 2022). In another study, celastrol can also alleviate diabetes-induced cardiac damage, inhibit mitochondrial ROS production, and suppress the release of inflammatory factors. The research results indicate that celastrol shows great potential as an effective cardiac protective drug for treating DCM (Zhao et al., 2023).

Astragaloside IV is one of the active ingredients extracted from *Astragalus membranaceus* (Fisch.) Bunge, which has pharmacological effects such as anti-inflammatory, antioxidant, immunomodulatory, and anti-tumor effects (Gao et al., 2022). In a SD rat model of DCM induced by STZ, Zhang et al. confirmed that astragaloside IV (10, 20, and 40 mg/kg/d) treatment for 16 weeks can improve mitochondrial biogenesis by upregulating the expression of PGC-1α and Nrf-1 in myocardial tissue, as well as PGC-1α and Nrf-1 mRNA expression in H_9C_2 cells. This regulation enhances ATP and ADP levels to improve mitochondrial energy metabolism, reduces the expression of cytochrome c (Cyt c) and caspase-3 to inhibit cell apoptosis and myocardial hypertrophy, thereby reducing diabetic myocardial damage (Zhang et al., 2019). Additionally, Zhu et al. have shown that astragaloside IV can downregulate the expression of miR-34a and upregulate the expression of Bcl-2, Sirt1, and pAkt/Akt proteins to protect myocardial cells from high glucose-induced damage (Zhu et al., 2019). These findings suggest that astragaloside IV may exert a protective effect on DCM by promoting mitochondrial biogenesis and inducing mitophagy.

5.3 Polyphenols

Polyphenols refer to secondary metabolites in plants, named for their multiple phenolic groups. They are widely present in traditional herbal medicines and some natural foods. In the treatment of DCM, polyphenols demonstrate significant antioxidant activity, capable of scavenging free radicals, reducing oxidative stress, and thus protecting mitochondria from oxidative damage (Raina et al., 2024).

Curcumin is a polyphenolic compound isolated from the root of *Curcuma longa* L and extensive research has confirmed that it is a highly effective antioxidant (Zheng et al., 2018). In a rat model of SD diabetes established by HFD and intraperitoneal injection of STZ, curcumin (200 mg/kg/d) treatment for 8 months promoted the transfer of Nrf2 to the nucleus through the AKT pathway, increased the expression of the antioxidant factors HO-1 and GCLC, reduced the accumulation of mitochondrial ROS, and mitigated mitochondrial oxidative damage. The study suggests that curcumin inhibits cell apoptosis by activating the AKT/Nrf2/ARE pathway and eliminates the accumulation of superoxide in myocardial cells (Wei et al., 2023). Additionally, Yao et al. research has shown that curcumin can upregulate the expression of AMPK and JNK1 to stimulate mitophagy, as well as upregulate

the expression of Bcl-2 and Bim to reduce cardiomyocyte apoptosis. Further mechanistic studies have indicated that curcumin prevents DCM through the cross-talk between mitophagy and apoptosis mechanisms via the AMPK/mTORC1 pathway (Yao et al., 2018).

Paeonol is a polyphenolic compound isolated from the root bark of *Paeonia suffruticosa* Andr, and it is also the active ingredient of paeonol injection (a marketed antipyretic and analgesic drug), which has pharmacological effects such as anti-inflammatory, neuroprotective, and anti-cardiovascular diseases (Zhang et al., 2019). The research by Liu et al. found that intervention with paeonol (75, 150, or 300 mg/kg/d) for 12 weeks in STZ-induced diabetic rats promoted Opa1-mediated mitochondrial fusion, inhibited mitochondrial oxidative stress, and maintained mitochondrial respiratory capacity and cardiac function in DCM. It is noteworthy that knocking out Opa1 attenuated the protective effect of paeonol in diabetic hearts and high-glucose-treated cardiomyocytes. The study indicates that paeonol is a novel promoter of mitochondrial fusion, providing protection against DCM through the CK2-MAN3-OPA1 signaling pathway (Liu et al., 2021). Additionally, in another study, Ding et al. demonstrated that paeonol can activate the transcription factor Stat3 to promote Mfn2-mediated mitochondrial fusion, not only reducing doxorubicin (Dox)-induced cardiac toxicity, but also preserving Dox's anticancer activity (Ding et al., 2023). In conclusion, these research findings suggest that paeonol may have significant value in preventing or treating diabetes and its complications by regulating mitochondrial dynamics.

Resveratrol is a polyphenolic compound widely present in plants, fruits, and vegetables, with biological activities such as antioxidant, anti-inflammatory, anticancer, and anti-aging (Galiniak et al., 2019). Fang et al. believe that resveratrol (50 mg/kg/d) treatment for 16 weeks significantly alleviated the cardiac dysfunction induced by HFD combined with STZ in SD rats. This was manifested by a significant increase in the activity of manganese SOD, ATP content, mitochondrial DNA copy number, $\Delta\psi m$, and nuclear respiratory factor (NRF), and a significant decrease in MDA and mitochondrial uncoupling protein UCP2 levels. The results indicate that resveratrol alleviates cardiac dysfunction in diabetic rats by improving mitochondrial function through SIRT1-mediated PGC-1α deacetylation (Fang et al., 2018). Similarly, in another study, Diao et al. demonstrated that resveratrol treatment improved mitochondrial function in diabetic rats, inhibited mitochondrial ROS generation, MPTP opening, and Cyto c release. It also suppressed the expression of UCP2 protein, thereby improving cardiac function in diabetic rats (Diao et al., 2019). In conclusion, resveratrol may have a positive impact on the prevention and treatment of diabetic cardiomyopathy by regulating mitochondrial uncoupling.

Salvianolic acid A is a polyphenolic compound isolated from *Salvia miltiorrhiza* Bunge, which has been proven to have various biological activities, including anti-oxidant, anti-inflammatory, anti-fibrotic and neuroprotective, etc (Wang et al., 2019). The research by Gong et al. indicates that Salvianolic acid A (3 mg/kg/d) treatment for 6 weeks in STZ-induced diabetic SD rats significantly enhances the respiratory activity and mitochondrial respiratory function related to complex I/II in diabetic rats, improves the abnormal electrocardiogram in diabetic rats, and inhibits cardiomyocyte apoptosis by down-regulating the expression of Bax and up-regulating the expression of Bcl-2, Caspase3, and Caspase9, thus exerting a protective effect on the heart (Gong et al., 2023). Furthermore, Wang et al.'s research indicates that salvianolic acid A can promote mitochondrial biogenesis in endothelial cells by regulating the expression of AMPK, PGC-1α, NRF1, and TFAM. Mechanistically, salvianolic acid A may activate the AMPK-mediated PGC-1α/TFAM signaling pathway, thereby improving the occurrence of diabetic cardiovascular diseases caused by mitochondrial dysfunction (Wang et al., 2022). In conclusion, Salvianolic acid A can prevent and treat diabetic cardiomyopathy by enhancing mitochondrial respiratory function and promoting mitochondrial biogenesis.

5.4 Alkaloids

Alkaloids are plant secondary metabolites composed of polycyclic aromatic frameworks containing one or more nitrogen atoms. Alkaloids have significant hypoglycemic effects, as they can stimulate glucose uptake and regulate insulin secretion, and are considered allosteric activators of AMPK (Seksaria et al., 2023). It is reported that alkaloids can affect the permeability of mitochondrial membrane, regulate the level of calcium ions in mitochondria, and regulate the imbalance of mitochondrial dynamics, thus reducing the occurrence of mitochondrial dysfunction, which is beneficial to the treatment of DCM (Patalas et al., 2021).

Berberine is an isoquinoline alkaloid that can be extracted and isolated from various plants such as *Coptis chinensis* Franch and *Phellodendron chinense* Schneid. It has biological activities such as lowering blood sugar and regulating lipid metabolism (Pang et al., 2015). In a study by Hang et al., it was shown that in a high glucose-induced H_9C_2 cardiomyocyte hypertrophy model, berberine intervention for 24 h at a concentration of 100 nM can regulate the imbalance of mitochondrial dynamics, promote mitochondrial biogenesis, and activate mitophagy to eliminate damaged mitochondria. These beneficial effects of berberine may be related to the activation of the AMPK signaling pathway (Hang et al., 2018). Additionally, research by Chen et al. demonstrated that berberine can upregulate the Bcl-2/Bax ratio, reduce the expression of Caspase3 protein, and simultaneously activate the PI3K/Akt and AMPK signaling pathways to improve cardiac contractile and diastolic dysfunction in diabetic rat myocardial I/R and inhibit myocardial cell apoptosis (Chen et al., 2014). Therefore, berberine may treat DCM by targeting the AMPK and PI3K/Akt signaling pathways through the activation of mitochondrial biogenesis.

Matrine is an alkaloid extracted and isolated from the dried roots of *Sophora flavescens* Aiton. It exhibits a wide range of biological activities, such as antioxidant, anti-tumor, anti-inflammatory, anti-fibrotic, anti-arrhythmic, and immunomodulatory effects (Wang et al., 2023). In an AGEs-induced SD rat model, matrine (50, 100, and 200 mg/kg/d) treatment for 20 days was found to inhibit the dissociation of FKBP12.6 and RyR2, reduce RyR2 activity and Ca^{2+} levels, decrease the expression levels of cytochrome c and active Caspase3, suppress cell apoptosis, and restore $\Delta\psi m$ (Wang et al.,

2019). Additionally, studies by Liu et al. suggest that matrine significantly reduces mitochondrial ROS production in primary cardiomyocytes of DCM rats, downregulates the expression of Cleaved caspase8 and Cleaved caspase3 proteins to inhibit cardiomyocyte apoptosis. Further research indicates that matrine improves diabetic cardiomyopathy by inhibiting the ROS/TLR-4 signaling pathway (Liu et al., 2015). In conclusion, matrine can effectively improve the occurrence of diabetic cardiac dysfunction and may potentially be developed as a cardioprotective agent.

5.5 Glycosides

Glycosides are an important type of plant secondary metabolites, widely found in plants, fruits, vegetables, and nuts. They include saponin glycosides, flavonoid glycosides, alcoholic glycosides. Phenolic glycosides, coumarin glycosides, and more. Glycosides hold great potential in the prevention and treatment of diabetes and various vascular complications (Yeram et al., 2022).

Ginsenoside Rg1 is a saponin glycoside isolated from *Panax ginseng* C. A. Mey, which has various biological activities such as anti-inflammatory, anti-oxidant, anti-platelet aggregation, anti-cancer, hypoglycemic, and neuroprotective effects (Zhang et al., 2022). Qin et al. showed that ginsenoside Rg1 (20 mg/kg/d) can promote mitochondrial biogenesis by increasing the expression of PGC-1α, AMPK, Nrf2 and HO-1 proteins, and reduce the expression of NF-κB and NLRP3 proteins to reduce oxidative stress after 8 weeks of treatment in STZ induced diabetes Wistar rats. Furthermore, ginsenoside Rg1 has been found to play a cardioprotective role by mediating the mitochondrial-related AMPK/Nrf2/HO-1 signaling pathway (Qin et al., 2019). In another study, ginsenoside Rg1 significantly reduced MDA and caspase-3 levels in the myocardium of diabetic rats, while increasing levels of SOD, catalase, glutathione peroxidase (GSH-Px), and B-cell lymphoma-extra-large (Bcl-xL). This indicates that ginsenoside Rg1's treatment of diabetic rats is associated with inhibiting oxidative stress and alleviating myocardial cell apoptosis (Yu et al., 2015). In conclusion, these findings suggest that ginsenoside Rg1 may have potential preventive and therapeutic effects on cardiovascular damage in diabetic patients by regulating mitochondrial biogenesis, inhibiting oxidative stress, and the mitochondrial-dependent apoptotic pathway.

Salidroside is an alcohol glycoside isolated from *Rhodiola rosea* L, which has a wide range of biological activities, including antioxidant, anti-tumor, antiviral, and hypoglycemic effects (Rong et al., 2020). The research by Li et al. indicates that in a C57BLKS/J mice model induced by HFD and STZ injection, treatment with salidroside (50 or 100 mg/kg/d) for 16 weeks can improve insulin resistance, mitochondrial ultrastructure damage, and restore normal cardiac contractile function in diabetic mice. Further mechanistic studies have shown that salidroside upregulates the expression of SIRT3 protein, promotes the translocation of SIRT3 from the cytoplasm to the mitochondria, increases the deacetylation of mitochondrial protein MnSOD, and upregulates the expression of AMPK, PGC-1α, and TFAM to induce mitochondrial biogenesis (Li et al., 2021). Additionally, salidroside can improve DCM by activating the Akt signaling pathway, upregulating the expression of Nrf2 and the antioxidant factor HO-1 (Ni et al., 2021). In conclusion, salidroside may play an important role in diabetes and its cardiovascular complications by promoting mitochondrial biogenesis and exerting antioxidant stress.

Astragalus polysaccharides are water-soluble polysaccharides extracted from the dried roots of *Astragalus membranaceus* (Fisch.) Bunge, which have anti-oxidant, anti-inflammatory, anti diabetes, immune regulation and other biological activities (Dong et al., 2023). Studies by Sun et al. have shown that astragalus polysaccharides (0.1–3.2 mg/mL, 24 h) can inhibit HG-induced H_9C_2 cell apoptosis by up-regulating the expression of Bcl-2, down-regulating the expression of Bax, and increasing the ratio of Bcl-2/Bax in the mitochondria (Sun et al., 2017). In another study, Chen et al. demonstrated that astragalus polysaccharides can protect the ultrastructure of cell mitochondria, reduce cell apoptosis, increase SOD activity, and thereby reduce oxidative stress induced by HG in H_9C_2 cells (Chen et al., 2018). In conclusion, these results prove that astragalus polysaccharides can prevent and treat DCM through the mitochondria-mediated apoptotic pathway.

Sugarcane leaf polysaccharide is an amorphous polysaccharides isolated from *Saccharum sinensis* Roxb leaves, which has various biological activities such as antioxidant, hypoglycemic, lipid-lowering, antibacterial, and immune regulation (Tang et al., 2019). Studies by Sun et al. have shown that sugarcane leaf polysaccharide (10 and 20 mg/kg/d) can effectively reverse myocardial ischemia-reperfusion injury in diabetic rats, prevent myocardial fibrosis and neutrophil infiltration, increase myocardial tissue SOD activity, reduce MDA and MPO activity, and significantly inhibit the expression levels of TNF-α and IL-6. *In vitro*, it promotes the translocation of Nrf2 from the cytoplasm to the nucleus by activating the Nrf2/HO-1 signaling pathway, upregulates the expression of Nrf2, HO-1, and NQO-1 proteins, reduces ROS production, and restores Δψm to affect myocardial mitochondrial biogenesis (Sun et al., 2023). In addition, Hao et al. suggested that sugarcane leaf polysaccharides can promote the expression of vascular endothelial growth factor (VEGF), enhance the activity of SOD, reduce the levels of MDA, NO, and GSH-Px, strengthen the antioxidant capacity in NOD mice, facilitate the body in clearing oxidative free radicals, and thereby improve the oxidative stress status of pancreatic β-cells (Hao et al., 2018). These research results indicate that sugarcane leaf polysaccharide may prevent DCM through targeting mitochondrial biogenesis and enhancing antioxidant capacity. Table 2 Plant secondary metabolites targeting mitochondrial dysfunction in diabetes cardiomyopathy models.

6 Traditional plants with hypoglycemic and antioxidant properties can regulate mitochondrial function in diabetic cardiomyopathy

Astragali radix (leguminous), huangqi in Chinese, also known as astragalus, the dried root of *Astragalus membranaceus* (Fisch.) Bge. var. mongholicus (Bge.) Hsiao or *Astragalus membranaceus* (Fisch.)Bge., is used as a traditional

TABLE 2 Plant secondary metabolites targeting mitochondrial dysfunction in diabetes cardiomyopathy models.

Category	Metabolites name	Molecular formula	In vivo model	In vitro model	Effect	Possible mechanisms/target	References
Flavonoids	Puerarin	$C_{21}H_{20}O_9$	STZ-NA induced C57BL/6J male mice (100 mg/kg/d, 4 weeks)	—	Improve insulin resistance, regulate mitochondrial respiratory function	GLUT4, CD36,P-Akt, PPARα	Huang et al. (2020)
	Apigenin	$C_{15}H_{10}O_5$	STZ induced C57BL/6J male mice(100 mg/kg/d, 7 months)	High glucose induced H_9C_2 cell (25 μM, 12, 24, 72 h)	Induce mitochondrial biogenesis, promote mitochondrial fusion	GPx, SOD,MDA, Cleaved caspase3, Bcl-2, Bax, NF-κB	Liu et al. (2017)
	Acacetin	$C_{16}H_{12}O_5$	STZ induced SD rats (10 mg/kg/d, 16 weeks)	High glucose induced H_9C_2 cell(0.3, 1, 3 μM, 48 h)	Reduce oxidative stress, inhibit mitochondrial dependent cell apoptosis	PPARα, AMPK, Bax, Bcl-2, SOD1, SOD2	Song et al. (2022)
	Dihydromyricetin	$C_{15}H_{12}O_8$	STZ induced C57BL/6J male mice(100 mg/kg/d, 14 weeks)	—	Reduce oxidative stress, regulate mitochondrial energy metabolism	ETC, Δψm, AMPK, ULK1	Wu et al. (2017)
Terpenoids	Triptolide	$C_{20}H_{24}O_6$	STZ induced SD rats (100, 200, and 400 μg/kg/d, 6 weeks)	—	Improve insulin resistance, regulate mitochondrial energy metabolism	P38MAPK, ATP	Liang et al. (201)
	Celastrol	$C_{29}H_{38}O_4$	STZ induced SD rats(50 μg/kg/d, 4 weeks)	—	Improve insulin resistance, reduce oxidative stress	MAPK, ROS	Wu et al. (2022)
	Astragaloside IV	$C_{41}H_{68}O_{14}$	STZ induced SD rats (10, 20 and 40 mg/kg/d, 16 weeks)	High glucose induced H_9C_2 cell (20, 40 and 80 μmol/L, 48 h)	Promote mitochondrial biogenesis, induce mitophagy	PGC-1α, Nrf-1, MDA, GSH, PI3K/Akt, GSH-Px, Cyt c, Caspase3	Zhang et al. (2019)
Polyphenols	Curcumin	$C_{21}H_{20}O_6$	High-fat diet and STZ induced SD rats (200 mg/kg/d, 8 months)	High glucose induced primary rat cardiomyocytes (14 μm/L, 24 h)	Reduce oxidative stress, induce mitophagy	Akt, Nrf2, HO-1, GCLC, ROS	Wei et al. (2023)
	Paeonol	$C_9H_{10}O_3$	STZ induced SD rats (75, 150, and 300 mg/kg/d, 12 weeks)	High glucose induced primary rat cardiomyocytes (25, 50, 100 and 200 μmol/L, 48 h)	Promote mitochondrial fusion,reduce oxidative stress	CK2/MAN3/OPA1	Liu et al. (2021)
	Resveratrol	$C_{14}H_{12}O_3$	High-fat diet and STZ induced SD rats (50 mg/kg/d, 16 weeks)	—	Inhibite mitochondrial uncoupling	SIRT1/PGC-1α, UCP2, SOD, NRF	Fang et al. (2018)
	Salvianolic acid A	$C_{26}H_{22}O_{10}$	STZ induced SD rats (3 mg/kg/d, 6 weeks)	—	Promote mitochondrial biogenesis, inhibit mitochondrial dependent cell apoptosis	AMPK, PGC-1α, NRF1, TFAM, Bax, Bcl-2	Gong et al. (2023)
Alkaloids	Berberine	$C_{20}H_{18}NO_4$	—	High glucose induced H_9C_2 cell line (100 nM, 24 h)	Promote mitochondrial biogenesis,induce mitophagy	AMPK, PI3K/Akt	Hang et al. (2018)
	Matrine	$C_{15}H_{24}N_2O$	AGEs induced SD rats (50, 100, and 200 mg/kg/d, 20 days)	High glucose induced primary rat cardiomyocytes (0.5, 1.0 and 2.0 mmol/L, 24 h)	Regulate mitochondrial calcium handling	Ca^{2+}, FKBP12.6, RyR2, Cyt c, Caspase3	Wang et al. (2019)

(Continued on following page)

TABLE 2 (*Continued*) Plant secondary metabolites targeting mitochondrial dysfunction in diabetes cardiomyopathy models.

Category	Metabolites name	Molecular formula	In vivo model	In vitro model	Effect	Possible mechanisms/ target	References
Glycosides	Ginsenoside Rg1	$C_{42}H_{72}O_{14}$	STZ induced Wistar rats (20 mg/kg/d, 8 weeks)	—	Promote mitochondrial biogenesis	PGC-1α, AMPK, Nrf2, HO-1, NF-κB, NLRP3	Qin et al. (2019)
	Salidroside	$C_{14}H_{20}O_7$	High fat diet and STZ induced C57BLKS/J mice (50 or 100 mg/kg/d, 16 weeks)	Primary culture of neonatal ratcardiomyocytes(10, 30 μM, 48 h)	Promote mitochondrial biogenesis	AMPK/Akt,SIRT3, PGC-1α/ TFAM,MnSOD	Li et al. (2021)
	Astragalus polysaccharides	$C_{41}H_{68}O_{14}$	—	High glucose induced H_9C_2 cell line(0.1, 0.2, 0.4, 0.8, 1.6 and 3.2 mg/mL, 24 h)	Inhibit mitochondrial dependent cell apoptosis	Bcl-2, Bax	Sun et al. (2017)
	Sugarcane leaf polysaccharide	—	Myocardial ischemia-reperfusion(MI/R) SD rat model (10 and 20 mg/kg/d, 1 weeks)	TBHP induced H_9C_2 cells (25, 50, and 100 μg/mL, 3 h)	Promote mitochondrial biogenesis	Nrf2, HO-1, NQO-1, ROS	Sun et al. (2023)

medicinal plants in China, Iran, Russia, and some other European countries. Astragali radix was first recorded in the Shennong's Classic of Materia Medica and is listed as a qi-supplementing formula. In the theoretical system of traditional Chinese medicine (TCM), astragali radix is sweet in flavor, warm in nature, and acts on the lung and spleen. It has the traditional effects of invigorating qi for ascending, consolidating superficies for arresting sweating and inducing diuresis for removing edema (Liu et al., 2023). Currently, a variety of secondary metabolites have been isolated from the dried roots of astragali radix, including polysaccharides, flavonoids, saponins, amino acids, trace elements, etc. Among them, polysaccharides, flavonoids, and saponins have biological activities such as hypoglycemic, antioxidation, and immune regulation, and are important components of the pharmacological effects of astragali radix. In addition, pharmacological studies have shown that the biological activities of astragali radix, such as antioxidation, hypoglycemic, immune regulation, and antiinflammatory effects, are widely used in the treatment of respiratory, digestive, urinary, and blood system diseases, as well as diabetes and its complications, and have achieved good therapeutic effects (Chen et al., 2020). In previous reports, it has been indicated that astragaloside IV can significantly delay the excessive generation of mitochondrial ROS. It can also exert a protective effect on diabetic cardiomyopathy by upregulating the activities of antioxidant enzymes SOD2, catalase, GSH-Px, and downregulating the expression of c-Jun N-terminal kinase and p38 MAPK (Chen et al., 2018). Astragalus polysaccharides can prevent the occurrence of diabetic cardiomyopathy through the mitochondrial-mediated cell apoptosis pathway (Sun et al., 2017).

Kudzu root (*P. lobata* (Willd.)Ohwi) belongs to the leguminosae and is a homologous medicinal and edible plant. It is mainly distributed in southern China and Southeast Asia. Kudzu root is sweet in flavor, mild in nature, and acts on the spleen, stomach, and lung. It has traditional effects such as expelling pathogenic factors from muscles for clearing heat, relieving rigidity of muscles and activating collaterals. In clinical applications of TCM, kudzu root is often used to treat diabetes, cardiovascular and cerebrovascular diseases, tumors, and other ailments. Kudzu root has various pharmacological effects such as antioxidant, hypoglycemic, anti-inflammatory, anti-tumor, blood pressure lowering, lipid-lowering, heart protection, and memory improvement (Wang et al., 2022). The extract of kudzu root contains abundant flavonoids such as puerarin, daidzein, tectoridin, and luteolin-6-C-glucoside, which have free radical scavenging and antioxidant activities. Its mechanism of action may be related to the regulation of oxidative stress-related factors such as COX-2, SOD, MDA, ET-1, NO, and GSH (Gao et al., 2016; Dong et al., 2024). In DCM, puerarin has been found to modulate mitochondrial function. Studies have shown that puerarin can regulate mitochondrial energy metabolism, reduce oxidative stress and apoptosis of myocardial cells, and improve the symptoms of DCM (Sun et al., 2022). In general, kudzu root is a medicinal plant with wide-ranging biological activities that can improve diabetic heart function by modulating mitochondrial function and other pathways.

Carthami flos (asteraceae), the dried floret of *C. tinctorius* L., is a perennial herbaceous plant with rich medicinal value, mainly distributed in Iran, North Korea, China, Mongolia, Russia and other regions. In clinical applications of TCM, carthami flos is pungent in flavor, warm in nature, and acts on the heart and liver. It is traditionally believed to promote blood circulation for removing blood stasis, regulate qi-flowing for relieving pain, and is mainly used for angina, irregular menstruation, diabetes, and hypertension (Tu et al., 2015). Carthami flos contains compounds such as flavonoids, volatile oils, and polysaccharides, which have biological activities such as

antioxidant and anti-inflammatory effects. Hydroxysafflor yellow A isolated from carthami flos is considered a potential antioxidant, providing protective effects against myocardial damage. Hydroxysafflor yellow A can increase the levels of SOD and GPX 1 in the serum of DCM mice, reduce MDA content, scavenge free radicals, and reduce oxidative stress damage to cardiac mitochondria (Yao et al., 2021). In addition, the essential oil of carthami flos extracted using different solvents was analyzed by gas chromatography-mass spectrometry (GC-MS) technology. The content of n-hexane extract was 97.65%, petroleum ether extract was 98.05%, dichloromethane extract was 98.93%, and the content of steam-distilled extract was 99.68%. *In vitro* pharmacology studies have shown that the n-hexane extract of carthami flos has the best *in vitro* anti-diabetic activity against protein tyrosine phosphatase 1B (PTP1B), demonstrating potential for the treatment of diabetes and obesity (Li et al., 2012).

Gynostemma pentaphyllum (Thunb.) Makino is a kind of cucurbitaceae, mainly distributed in India, Nepal, Bangladesh, China, Myanmar, Laos, Vietnam, Malaysia and other regions. Gynostemma pentaphyllum is spicy and slightly bitter in flavor, warm in nature, and acts on the lung, spleen, and stomach. In the theory of TCM, it has the traditional effects of warming spleen and stomach for dispelling cold, ventilating lung qi for dissipating phlegm and regulating qi-flowing for harmonizing stomach. Gynostemma pentaphyllum is a commonly used plant in TCM for treating diabetes. It has various pharmacological effects such as antioxidant, hypoglycemic, anti-inflammatory, antibacterial, antiallergic, and antitumor properties (Nguyen et al., 2021). Active ingredients in gymnema pentaphyllum, such as saponins and polysaccharides, have a certain hypoglycemic effect, significantly reducing insulin resistance index and improving diabetes and its complications. Gypenoside can lower fasting blood sugar and blood lipids in mice with type 2 diabetes induced by HFD and STZ, and significantly improve glucose tolerance and insulin resistance. Its hypoglycemic effect may be related to the downregulation of key proteins in the AMPK signaling pathway, including phosphoinositide 3-kinase and glucose-6-phosphatase (Song et al., 2022). Similarly, in another study, the extract of gynostemma pentaphyllum significantly reduced the levels of MDA, hydrogen peroxide, peroxynitrite, and ROS in DCM rats, while increasing the levels of GSH, SOD, CAT, and GPx. It also significantly reduced the expression of cytokines and inflammatory parameters (TNF-α, IL-6, IL-1β, COX-2, NLRP3, NF-κB). Furthermore, the extract of gynostemma pentaphyllum also promoted mitochondrial biogenesis in cardiac tissues by enhancing the expression of PGC-1, HO-1, and Nrf2. These results indicate that the gynostemma pentaphyllum has a cardioprotective effect on STZ-induced diabetic cardiac dysfunction by regulating the AMPK/Nrf2/HO-1 pathway (Chen et al., 2022).

7 Conclusion

Diabetic cardiomyopathy is manifested as abnormal cardiac structure and function in the absence of ischaemic or hypertensive heart disease in individuals with diabetes. However, its pathogenesis remains unclear. Mitochondrial dysfunction is an important pathological mechanism leading to the development of the disease. Targeted regulation of mitochondrial function can effectively improve the symptoms of DCM. Targeting mitochondria with plant secondary metabolites may be an effective approach for preventing and treating DCM. This review provides evidence supporting mitochondrial dysfunction in DCM, briefly describes the pathophysiological mechanisms leading to mitochondrial dysfunction, and discusses potential targets and treatment strategies.

Currently, extensive screening research on anti-diabetic drugs has identified plants as the main potential source for drug discovery. Biologically active secondary metabolites in plants, such as flavonoids, terpenes, polyphenols, alkaloids, and glycosides, have been proven to have hypoglycemic effects *in vivo* and *in vitro*. Previous reports indicate that plant secondary metabolites improve hyperglycemia and insulin resistance mainly by regulating lipid and protein metabolism pathways, insulin signaling pathways, anti-inflammatory responses, and antioxidant stress. The key regulatory targets involved include α-glucosidase, α-amylase, dipeptidyl peptidase 4 (DPP-4), protein tyrosine phosphatase 1B (PTP1B), PPARα, GLUT4, and the AMPK signaling pathway (Shehadeh et al., 2021; Sukhikh et al., 2023).

In this review, we have gathered plant secondary metabolites that affect mitochondrial function in the treatment of DCM. Some plant secondary metabolites have biological activities such as hypoglycemic, anti-oxidation, and anti-inflammation, which can protect myocardial cells by improving mitochondrial dysfunction. For example, apigenin can promote mitochondrial biogenesis, induce mitochondrial autophagy to maintain the normal myocardial mitochondrial quality and quantity homeostasis; triptolide can improve insulin resistance, regulate mitochondrial energy metabolism; paeonol can promote mitochondrial fusion, inhibit mitochondrial oxidative stress; resveratrol can regulate the opening of mitochondrial inner membrane channels, regulate the process of mitochondrial uncoupling, and help reduce oxidative stress reactions inside mitochondria; matrine can regulate the opening of mitochondrial calcium ion channels, affecting the balance of calcium ions inside mitochondria; sugarcane leaf polysaccharide can improve insulin resistance and promote mitochondrial biogenesis. However, there are still some challenges in the use of plant secondary metabolites in the treatment of DCM. For example, the pharmacological effects and dosages of plant secondary metabolites are not fully understood, and a more detailed quality evaluation system is needed to verify their efficacy and safety. It is also important to address how to improve the bioavailability and stability of plant secondary metabolites, which can be achieved through nanocarrier delivery technology, chemical modification, and other biotechnological methods. In conclusion, plant secondary metabolites targeted at mitochondria are expected to become an important drug resource for the treatment of DCM, and more clinical experiments are needed in the future to elucidate their mechanisms of action.

Author contributions

XP: Writing–original draft, Methodology. EH: Writing–review and editing, Visualization. FZ: Methodology, Writing–review and editing. WW: Data curation, Writing–review and editing, ZD: Investigation, Writing–review and editing, GY: Formal Analysis, Writing–review and editing. XW: Supervision, Writing–review and editing. JD: Investigation, Writing–review and editing, XH: Conceptualization, Funding acquisition, Writing–review and editing.

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Conflict of interest

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

Publisher's note

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Sargassum pallidum reduces inflammation to exert antidepressant effect by regulating intestinal microbiome and ERK1/2/P38 signaling pathway

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Immune inflammation is one of the main factors in the pathogenesis of depression. It is an effective and active way to find more safe and effective anti-inflammatory depressant drugs from plant drugs. The purpose of this study is to explore the potential of marine plant *Sargassum pallidum (Turn).C.Ag.* (Haihaozi, HHZ) in the prevention and treatment of depression and to explain the related mechanism. Phytochemical analysis showed that alkaloids, terpenes, and organic acids are the main constituents. *In vitro* and *in vivo* activity studies showed the anti-neuroinflammatory and antidepressant effect of *Sargassum pallidum*, furthermore, confirmed that 7-Hydroxycoumarin, Scoparone, and Kaurenoic Acid are important plant metabolites in Sargasum pallidum for anti-neuroinflammation. Mechanism exploration showed that inhibition of ERK1/2/p38 inflammatory signaling pathway contributing to the antidepressant effect of *Sargassum pallidum* in reducing intestinal inflammatory levels. This study confirmed the value of *Sargassum pallidum* and its rich plant metabolites in anti-inflammatory depression, providing a new choice for the follow-up research and development of antidepressant drugs.

KEYWORDS

sargassum pallidum, network pharmacology, inflammation, antidepressant, gut microbiota

Abbreviation: BH, Scoparone; CUR, Curtain Gas; DAPI, 4′,6-Diamidino-2-Phenylindole; DV, Declustering Voltage; EDTA, Ethylene Diamine Tetraacetic Acid; EPM, Elevated Plus Maze Test; GS1, Atomizing Gas; GS2, Auxiliary Heating Gas; HHZ, Sargassum pallidum; IL-6, Interleukin-6; IL-1β, Interleukin-1β; OFT, Open Field Test; 7-OH, 7-Hydroxycoumarin; PBS, Phosphate Buffered Saline; PCR, Polymerase Chain Reaction; SPT, Sucrose Preference Test; TNF-α, Tumor Necrosis Factor-α; TST, Tail Suspension Test; YBK, Kaurenoic Acid.

1 Introduction

Depression is a serious and complex mental disorder, which has become a major killer of human beings. The pathogenesis of depression is very complex, including monoamine depletion, neuroinflammation, genetic and epigenetic abnormalities, neuroendocrine hypotheses, *etc.* Among them, the monoamine depletion hypothesis has always dominated the pathophysiology of depression (Uchida et al., 2018).

At present, the first-line drugs for the treatment of depression: SSRI drugs, As classic monoamine drug, which can cause dry mouth, constipation, orthostatic dizziness, in clinical practice, their long-term use can easily lead to blurred vision and anticholinergic adverse reactions (Uher et al., 2009). With the continuous accumulation of clinical immunological evidence, the research and development of depression prevention and treatment drugs have extended from the excitation of monoamine neurotransmitter neurons to the regulation of central inflammatory immunity (Beurel et al., 2020). Studies have shown that nonsteroidal anti-inflammatory drugs (NSAIDs) can significantly improve depressive symptom (Zuzarte et al., 2018), yet Long term use of non steroidal anti-inflammatory drugs may cause a series of adverse reactions such as nausea, vomiting, gastric ulcers, hypertension, and acute kidney injury (Theken et al., 2020). Due to depression is a chronic disease requiring long-term medication, the safety of long-term medication is the focus of clinical treatment selection and application. Therefore, there is an urgent need to find more safety and less side effect depression prevention and treatment drugs.

Immune inflammation has been proved to be one of the main factors in the pathogenesis of depression, and the development of inflammation is closely related to depression (Rajkowska and Stockmeier, 2013). The imbalance of microorganisms in the body will lead to the release of inflammatory factors increases, and influenced depressive symptoms occur (Beurel et al., 2020). Therefore, regulating the microbiota for the treatment of depression has been received increasing attention (Chudzik et al., 2021). Modern research analysis showed that plant metabolites can improve intestinal microbiota imbalance and immune disorders (Hu et al., 2021; Li et al., 2021). Thus, exploration more phytomedicines in regulating intestinal flora and preventing inflammatory is a promising approach for depression treatment. Among a large number of plant medicines, marine plants are an important component that cannot be ignored. Marine plant drugs contain a large amount of active metabolites, which are widely distributed and have high yields, and are also receiving increasing attention.

The traditional Chinese medicine "haizao (*Sargassum pallidum*)" was first recorded in the "Shennong Materia Medica Classic" and is classified as a medium grade. It has the effects of reducing phlegm, softening hardness, dispersing nodules, promoting diuresis, and reducing swelling. Sargasum is a wild plant resource with high development, utilization, and medicinal value. It has the advantages of rich active ingredients, wide distribution, abundant resources (Premarathna et al., 2020). Modern pharmacology indicates that Sargassum is rich in plant metabolites such as alginate, fucoidan sulfate, and taurine, and has various physiological activities such as antioxidant, anti-tumor, and anti gastric ulcer properties (Chen et al., 2022). The medicinal plants of the genus Sargassum have a wide range of anti-inflammatory activities, some of which also have anti neuroinflammatory effects (Han et al., 2021; Liyanage et al., 2022). Considering the high correlation between neuroinflammation and depression, it is necessary to explored whether Sargasum pallidum has the potential to treat depression, as well as the specific mechanisms of anti-inflammatory depression.

In this article, we used UHPLC-QTOF-MS/MS technology to complete the analysis of plant metabolites contained in the *Sargassum pallidum*, obtaining its chemical profile. The animal model of inflammatory depression induced by LPS and the neuroinflammation model of BV2 cells were selected to carry out pharmacodynamic studies *in vivo* and *in vitro*, based on them, the antidepressant effects of seaweed and its plant metabolites were explored. At the same time, it is found that the gut microbiota and its inhibitory ERK1/2/p38 inflammatory signaling pathway play a crucial role in improving depression. This study confirmed the potential of *Sargasum pallidum* and its rich plant metabolites in anti-inflammatory depression. It also emphasized the important value of phytomedicines in regulating intestinal microbiome and reducing inflammation to play an antidepressant role.

2 Materials and methods

2.1 Consumables and chemicals

Sargassum pallidum(Turn).C.Ag. (Haihaozi, HHZ) (batch number: 19061309) was purchased from Anguo Yifang Pharmaceutical Co., Ltd. (Anguo, China) and was identified as HHZ dried whole herb by Wu Shuyao who is a Chinese pharmacist of Qihuang National Medical College, Jiangxi University of Chinese Medicine. Chemicals and their suppliers are summarized in Supplementary Table S1 of Supplementary Methods.

2.2 Preparation of seaweed

Accurately weigh 500 g HHZ of dried algal bodies, crush them and put them in a 10000 mL round bottom flask. Add 10 times the amount of 80% ethanol (5000 mL) as the solvent, condensing reflux extraction for 8 h, 5 times the amount of solvent was added to the residue for reflux extraction 1 h. The twice obtained extracts mixed was rotary evaporated to remove ethanol, and the remaining concentrated solution was freeze-dried for 24 h. The HHZ extract is dried by a freeze-drying machine and ground into freeze-dried powder, which is stored in a dryer away from light for better storage.

2.3 UHPLC-QTOF-MS/MS analysis

Accurately weigh 1 g of medicinal material powder was weighed and mixed with ten times the amount of 80% methanol (10 mL), followed by ultrasonic extraction for 60 min. The liquid phase and mass spectrometry analysis parameters are shown in Supplementary Table S2.

2.4 Cell lines and culture

The BV2 microglial cell line was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). BV2 cells were maintained in complete DMEM medium containing 10% fetal bovine serum. All cells were incubated at 37°C in a humidified atmosphere of 5% CO_2.

2.4.1 Cell viability assay

BV2 cells were seeded into 96-well plates at an initial density of 6×10^3 cells/well. The cultured cells were divided into Control group, Model group, Positive drug (PAR) group, and different dosage groups. The Control group was not treated, while the remaining groups were added with LPS at a final concentration of 1 μg/mL. HHZ extracts at final concentrations of 20 μg/mL, 40 μg/mL, 60 μg/mL, 80 μg/mL, and 100 μg/mL were added. The final concentrations of 7-hydroxycoumarin (7-OH), Scoparone (BH), and Kaurenoic Acid (YBK) were all 10 μM, and the PAR group was added with paroxetine at a final concentration of 5 μM. Add 10 μL of CCK-8 solution to each well. After incubation for 1.5 h, read the OD value at 450 nm using a microplate reader.

2.4.2 Preparation of medicated serum

54 healthy SPF C57BL/6J were randomly divided into control group and treatment groups. Administer paroxetine group and HHZ plant metabolites group 10 mg/kg, HHZ low, medium, and high doses of HHZ 100 mg/kg, 200 mg/kg, and 300 mg/kg, respectively by gavage, the control group was given an equal volume of physiological saline for five consecutive days. After intraperitoneal injection of pentobarbital anesthesia, blood was collected and centrifuged at 4°C at 3,000 r/min for 15 min. The upper serum was separated, inactivated in a water bath, and packaged with a 0.22 μm filter membrane. It was stored at −80°C for later use.

2.4.3 ELISA

Used Elisa reagent to detect the levels of inflammatory cytokines interleukin-6 (IL-6), NO, IL-4, interleukin-1 β (IL-1 β), and tumor necrosis factor alpha (TNF-α) in BV2 cells.

2.4.4 RT-qPCR

Collect total RNA from BV2 cells and colon tissue using Trizol reagent. Use a transcription kit to reverse RNA into cDNA. The iNOS qPCR primer sequence is upstream:GGCTGCCCCTGGAAGT, downstream:TGCAAGT-GAAATCCGATTGG, and the ZO-1 primer sequence is upstream:CCATCTTGGACGATTGTG, downstream: TAATGCCCGAGCTCCGATG.

2.5 Animal experiments

2.5.1 Animals and drug administration

Male C57BL/6J mice (6°weeks old) were purchased from Hunan Slack Jingda Co., Ltd. Before the experiment, all the mice were exposed to an SPF room at 20°C–22°C, at humidity of 45%–65%, with 12 h light/dark cycle, free access to water and food. The animal experiment protocol was approved by the Animal Ethics Committee of Jiangxi University of Chinese Medicine (Nanchang, China) on 9 November 2021 (Approval Number: JZLLSC20210061). After 1 week of adaptive feeding, the mice were randomly divided into control group, model group, paroxetine positive group 10 mg/kg, HHZ high-dose group 300 mg/kg, HHZ medium-dose group 200 mg/kg, HHZ low-dose group 100 mg/kg, 10 mg/kg of 7-OH group, 10 mg/kg of YBK group, and 10 mg/kg of BH group, eight mice in each group.

2.5.2 LPS injection combined with orphanage method to establish inflammatory depression model and behavioral evaluation

Except for the control group, the other groups were injected with LPS continuously for 5°days, 1 mg/kg once a day. In addition, the model group and treatment group mice were raised in a quiet environment with a single cage, and the padding was changed once a week. The control group mice were raised in the same environment with four mice per cage. The plant metabolites group was administered by intraperitoneal injection, the positive drug group and the HHZ extract were administered by gavage. Depression-like behavior was evaluated by sucrose preference test (SPT), open field test (OFT), elevated plus maze test (EPM) and tail suspension test (TST). All ethology procedures refer to literature and previous work (Song et al., 2021; Pei et al., 2024).

2.5.3 16S r RNA gene sequencing and data analysis

Fresh stool samples were collected in sterile cryovials on the same day and stored in a −80°C freezer until use. The DNA of the fecal samples was extracted and then subjected to Polymerase Chain Reaction (PCR) amplification and product purification. Shanghai Meiji Biomedical Technology Co., Ltd. was authorized to use the MiseqPE300 platform for microbiome analysis.

2.5.4 HE staining

Intestinal tissues were rinsed three times with Phosphate Buffered Saline (PBS), fixed in 4% paraformaldehyde, embedded in paraffin, and made into 5 μM sections. Then sections were deparaffinized with xylene and varying concentrations of ethanol. After HE staining, the pathological changes of intestinal tissue were observed under light microscope.

2.5.5 Immunofluorescence

BV2 cells were placed in confocal dishes, colon paraffin sections (5 μM), and deparaffinized with different concentrations of xylene and ethanol. Antigen retrieval was performed in warmed citrate buffer (10 mM, pH 6.0). Block with goat serum for 1 h at room temperature, use the following primary antibodies: rabbit anti-IL-6 (1:500), rabbit anti-TNF-α (1:500), IL-1β (1:250) rabbit anti-phospho-ERK1/2 (1:25), rabbit anti-phospho-p38-MAPK (1:50). Then store overnight in a 4°C refrigerator. Secondary antibody was added after washing: goat anti-rabbit IgG (H + l) (1 h at room temperature), after addition of 4′,6-Diamidino-2-Phenylindole (DAPI) for nuclear visualization, anti-fluorescent quencher was also dropped on the slide. Images were analyzed by Leica Software and Fiji (ImageJ).

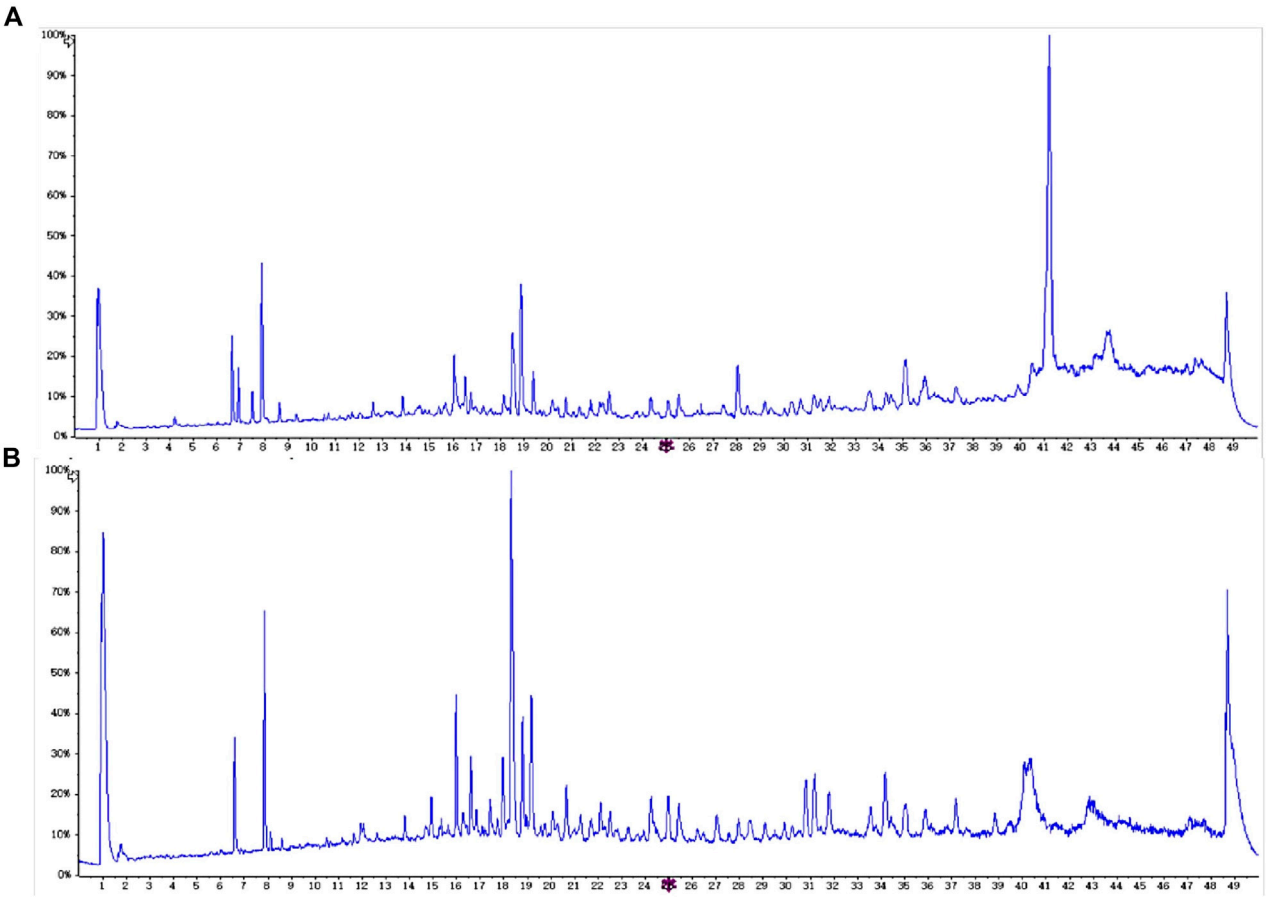

FIGURE 1
HHZ chemical composition analysis, **(A)** positive ion mode, **(B)** negative ion mode.

2.6 Statistical analysis

All data analysis was performed using the GraphPad Prism 8.0 data analysis software. All data are presented as mean ± SD. One-way analysis of variance was used for comparisons involving more than three groups, *t*-test were used for comparisons involving two groups. *p*-value <0.05 was considered statistically significant.

3 Results

3.1 Phytochemical composition

The samples were detected by UHPLC-QTOF-MS/MS, and the obtained data were analyzed using Analyst TF 1.6 and peakview1.2 data processing system (Sciex Corporation). Through mass spectrum fragment ion analysis, mass spectrum database matching and comparison of relevant literature reports, a total of 45 chemical components were identified (Figure 1; Supplementary Table S3). Compared with the composition analysis results of *Sargassum fusiforme (Harv.)Setch.* (YXC) in our research group before, it was found that the amount of alkaloids, glycosides, and lignans in HHZ was higher than that in YXC (Supplementary Figure S4). Comparing the relative content of various metabolites in two types of seaweed, we found that the content of phenylpropanoid and saponins in HHZ was higher. Coumarins rich in HHZ are the main characteristic compounds. (Supplementary Figure S5). These data indicate that HHZ has more advantages in composition and development value than YXC.

3.2 Protective effect of HHZ on BV2 cell inflammation induced by LPS

The results of CCK-8 showed that in the positive drug group, paroxetine could effectively inhibit the activity changes induced by LPS. The HHZ extract inhibited the activation of microglial cells in a dose-dependent manner, and the other 7-OH, BH, and YBK groups decreased the cell activation index after administration, while the inhibitory effect of the BH group was weak. The cell viability of each of the above groups was insignificantly reduced, which also suggested that the administration groups did not produce cytotoxic effects (Figures 2A, B). The difference in cell morphology was observed under the microscope. Most of the normal BV2 cells in the control group were relatively round, with a small cell body diameter, and synaptic growth of cells with longer morphology was not seen. The number of activated cells in the model group treated with LPS increased notably, and the cell body became hypertrophic and the cell synapses grew. The number of activated cells in the administration groups of

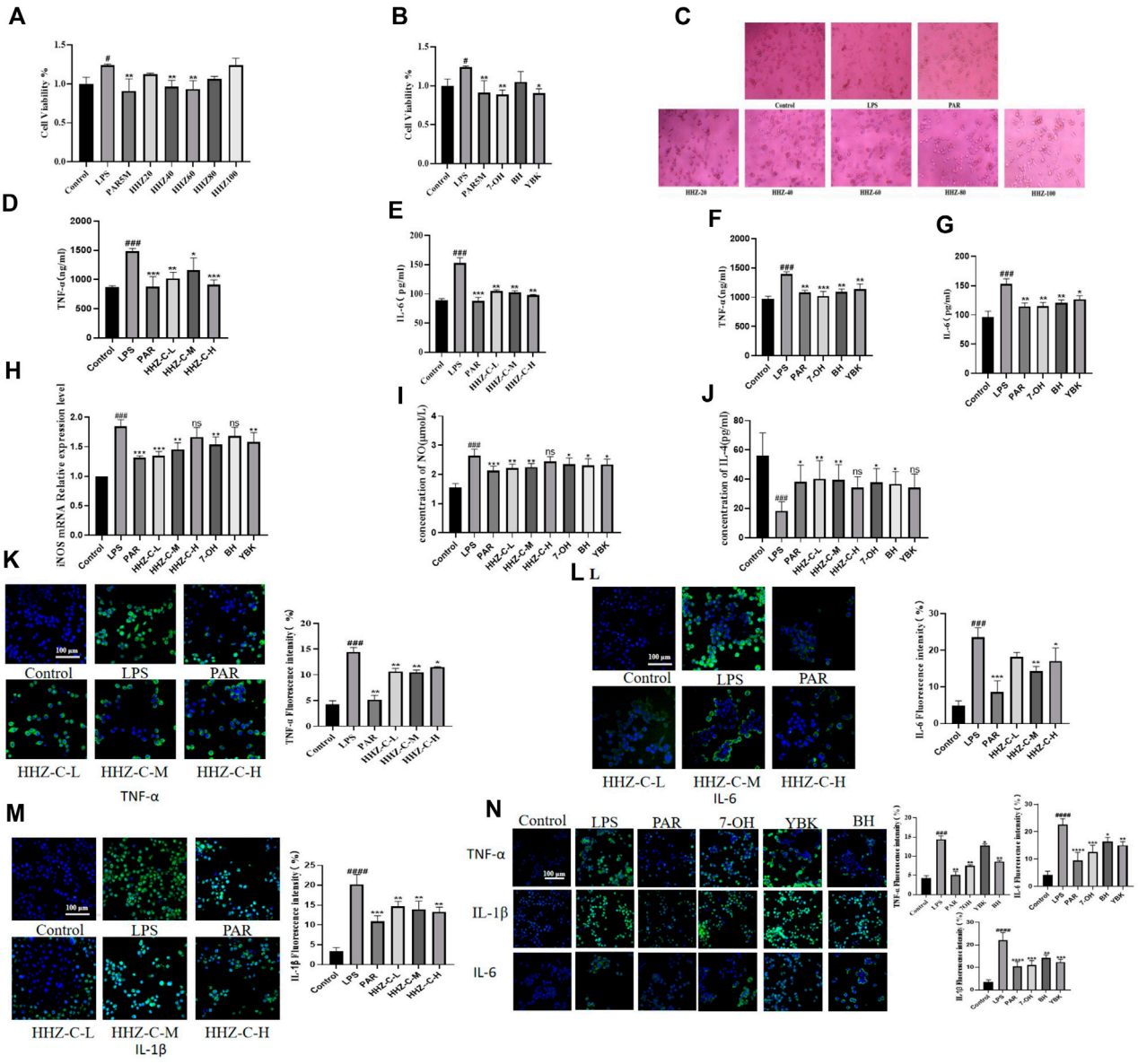

FIGURE 2
Protective effect of HHZ on LPS-induced inflammation in BV2 cells. **(A, B)** CCK-8 cell viability detection of HHZ extract and its active ingredient. **(C)** The effect of HHZ extract and LPS on the morphology of BV2 microglial cells (100X). **(D–G)** ELISA detection of TNF-α and IL-6 levels in BV2 cells. **(H–J)** After treatment with medicated serum, the expression levels of iNOS, NO, and IL-4 in BV2 cells. **(K–N)** Immunofluorescence analysis of TNF-α, IL-6, IL-1β. Values represent the mean ± SD. #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$, ####$p < 0.0001$ means significant difference compared with the control group; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ means significant difference compared with LPS group.

20 μg/mL and 100 μg/mL extracts of seaweed was more, and the number of activated cells increased remarkably, while the number of activated cells in other groups was relatively small (Figure 2C). In summary, 40 μg/mL, 60 μg/mL, and 80 μg/mL seaweed extract can be selected as the subsequent experimental concentration.

In order to further verify the anti-neuroinflammatory effect of HHZ extract and its plant metabolites, we detected the contents of TNF-α and IL-6 of microglial inflammatory factors in each group (Pei et al., 2023). It was found that the contents of TNF-α and IL-6 in the supernatant of BV2 cells in the Model group increased considerably, and different doses of administration could reduce the concentrations of these two inflammatory factors to varying degrees (Figures 2D–G).

Additionally, to determine whether HHZ and its plant metabolites inhibit the inflammatory response of BV2 cells and whether it is related to phenotype polarization, we detected the expression levels of M1 phenotype markers iNOS mRNA and NO in BV2 cells and the content of M2 phenotype marker IL-4 in drug-containing serum. The results showed that compared with the control group, the expression of iNOS mRNA and NO was significantly increased after LPS stimulation, while the content of IL-4 was significantly reduced. After treatment with drug-containing serum, these indicators all showed varying degrees of improvement (Figures 2H–J). Then we measured the inflammatory factors TNF-α, IL-1β, and IL-6 by cell immunofluorescence experiments, and it showed that administration

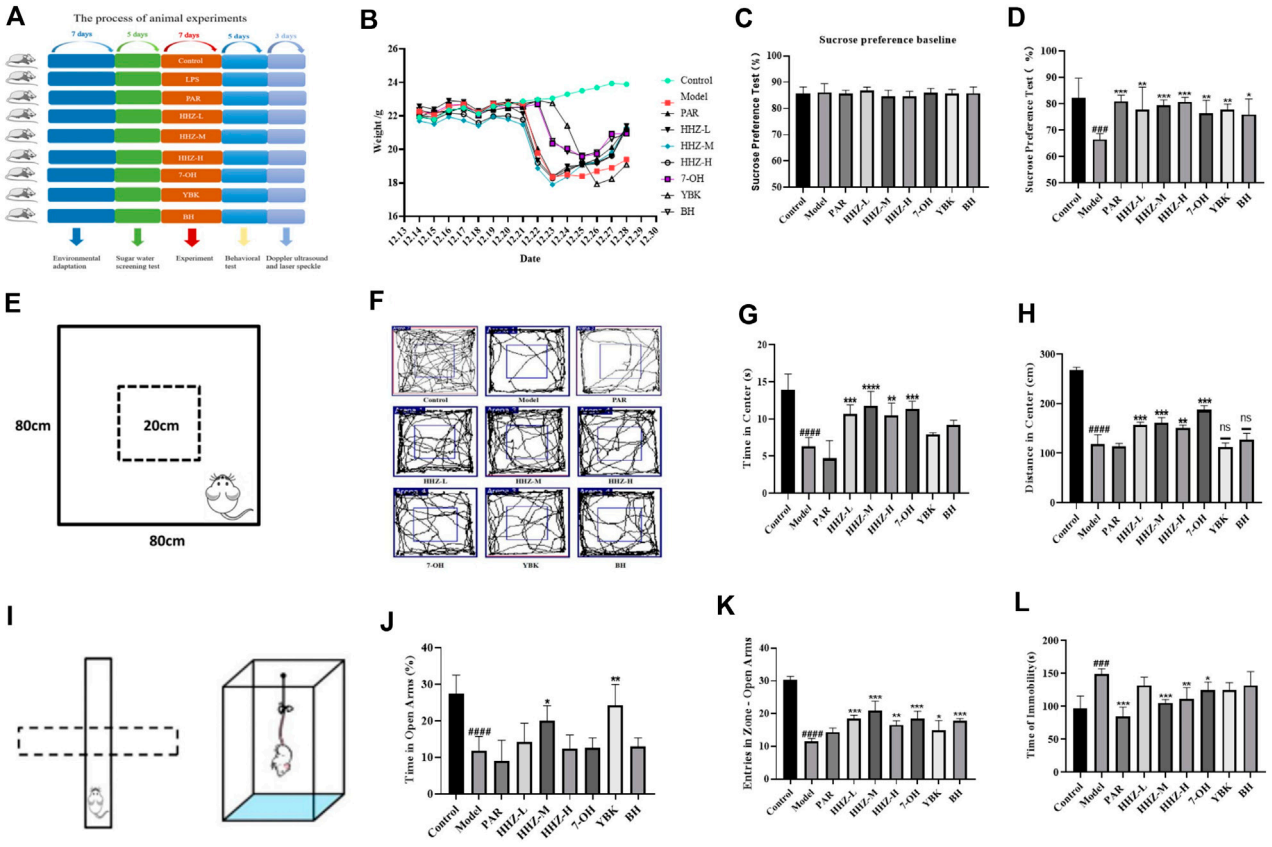

FIGURE 3
Improvement effect of HHZ and its active ingredients on LPS-induced depression-like mice. **(A)** Experimental design. **(B)** The trend of body weight of mice in each group. **(C–D)** Effects of HHZ and its active components on anhedonia in depressed mice in SPT. **(E–H)** Effects of HHZ and its active components on spontaneous locomotion in depressed mice in OFT. **(I–K)** Effects of HHZ and its active components on exploratory behavior in depressed mice in EPM. **(L)** Effects of HHZ and its active components on immobility time in depressed mice in TST. Values represent the mean ± SD. #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ ####$p < 0.0001$ means significant difference compared with Control group; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ means significant difference compared with Model group.

of low, medium and high doses of HHZ extracts could all make the levels of LPS-induced cellular inflammatory factor TNF -α, IL-1β, IL-6 decreased. In the experiment, the level of IL-6 in the low-dose HHZ group showed a certain degree of decrease. Among the three groups, the middle-dose one (60 μg/mL) had best effect (Figures 2K–M). The three monomers of 7-OH, BH and YBK also showed good anti-neuroinflammatory effects, among which 7-OH had the best anti-inflammatory activity (Figure 2N). These data indicate that HHZ and its active ingredients exhibit excellent anti-neuroinflammatory activity and neuroprotective effect in cells *in vitro*. For the purpose of further validating the anti-inflammatory activity and neuroprotective effect of HHZ and its plant metabolites *in vivo*, we used the mouse model of LPS-induced inflammation leading to depression to explore the mechanism of action of HHZ against inflammatory depression.

3.3 Improvement effect of HHZ and its plant metabolites on LPS-induced depression-like mice

Firstly, the body weight changes of the mice in each group were evaluated, and it was found that the body weight of the mice in the Model group was obviously lower than that of the mice in the control group. However, after administration, none of the HHZ dose groups and their active components showed significant changes in mouse body weight (Figure 3B). Mice were evaluated using four behavioral tests: SPT, OFT, EPM, and TST. Compared with the control group, the mice in the Model group had an apparently lower preference for sucrose, and this trend was reversed after treatment with HHZ and its active ingredient (Figures 3C, D). In the OFT, the Model mice showed overt decrease in locomotor activity, the state recovered after treatment with HHZ and its active ingredients. We noticed that moderate doses of HHZ produced a pretty stable effect (Figures 3E–H). In the EPM, compared with the Control group, the percentage of time spent in the open arm and the number of open arm entries were reduced massively in the model group mice, all doses of HHZ and plant metabolites administration groups were able to significantly increase the number of times they entered the open arm, and HHZ-M and YBK groups were also able to significantly increase the stay time of the open arm (Figures 3I–K). And in the TST, the immobilization time of model mice was longer than that of control mice, and the immobility time after treatment with HHZ and its plant metabolites was clearly shorter than that of model group. Among them, the medium dose of HHZ showed the best effect (Figure 3L).

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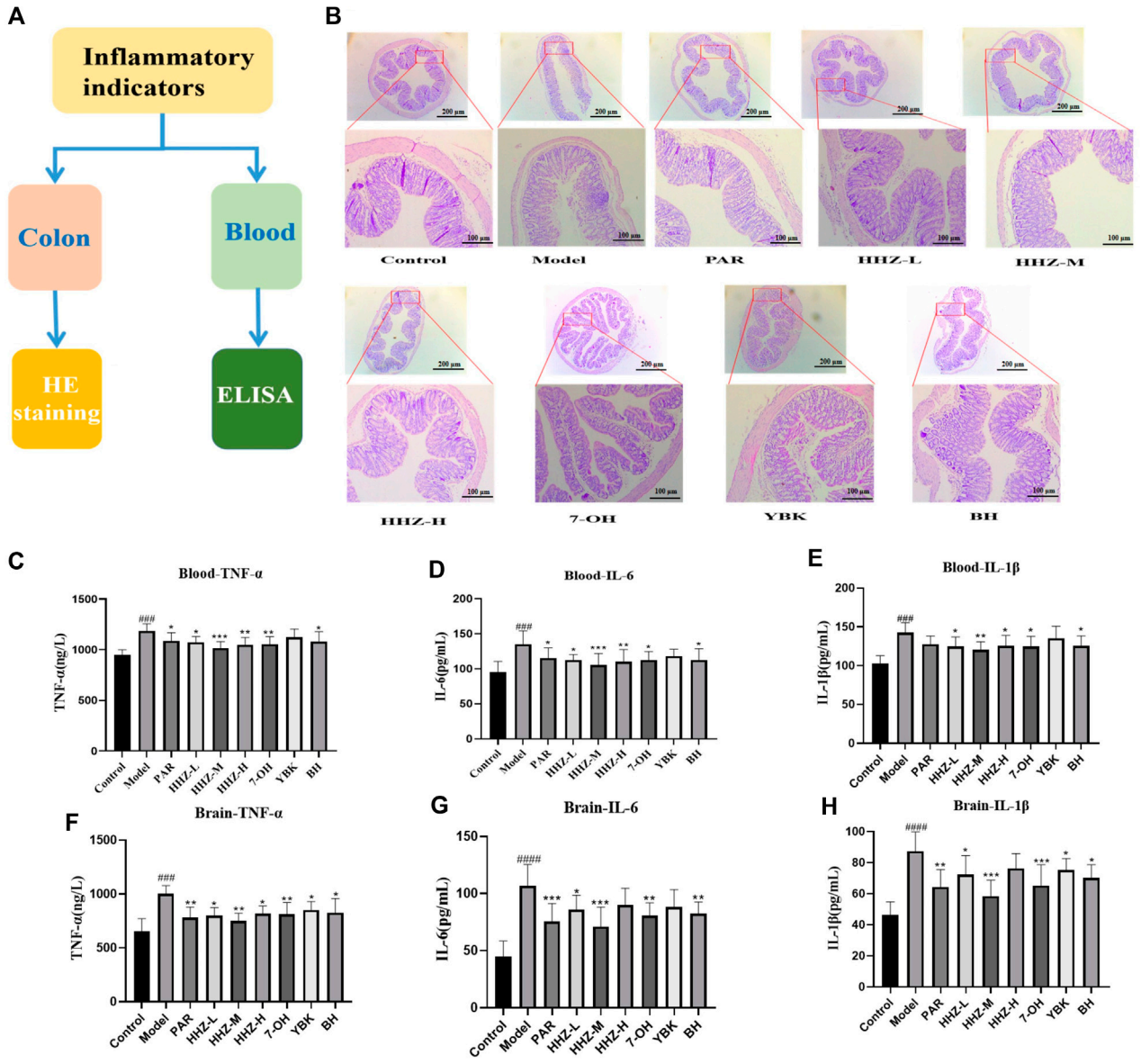

FIGURE 4
The improvement effect of HHZ on the intestinal environment of inflammatory depression mice. **(A)** Experimental design. **(B)** Pathological examination of mouse colons in each group by HE staining. **(C–E)** Elisa detection of serum TNF-α, IL-6, IL-1β levels of mice in each group. **(F–H)** Elisa detection of Brain TNF-α, IL-6, IL-1β levels of mice in each group. Values represent the mean ± SD. #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ ####$p < 0.0001$ means significant difference compared with Control group; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ means significant difference compared with Model group.

3.4 Intestinal inflammation improvement of HHZ in depression mice

The intestinal environment can affect human psychology and behavior by regulating brain development and behavioral patterns through the brain-gut axis. Therefore, we first used HE staining to perform pathological examination on the colon tissues of mice in each group. The pictures showed that the goblet cells in the control group were in complete shape, the crypts were arranged neatly, and there was no conspicuous inflammatory infiltration. After LPS modeling, goblet cell morphocytes and crypts were severely lost in the model group, and obvious inflammatory infiltration appeared at the same time. By administering HHZ and its active ingredients in each group, except for the control group and the model group, the intestinal inflammation of the mice in each group showed varying degrees of recovery, among which HHZ-L, HHZ-M, 7-OH, and BH very well improved the inflammatory state of mice in the model group, and the cell morphology in the other groups was mostly intact (Figure 4B). Then we used ELISA to measure the content of serum and brain inflammatory factors TNF-α, IL-1β, and IL-6 in inflammatory depression mice, and found that HHZ and its three active ingredients can greatly reduce the content of these three inflammatory factors (Figures 4C–H), which show that HHZ and its plant metabolites may exert antidepressant effects by improving intestinal inflammation.

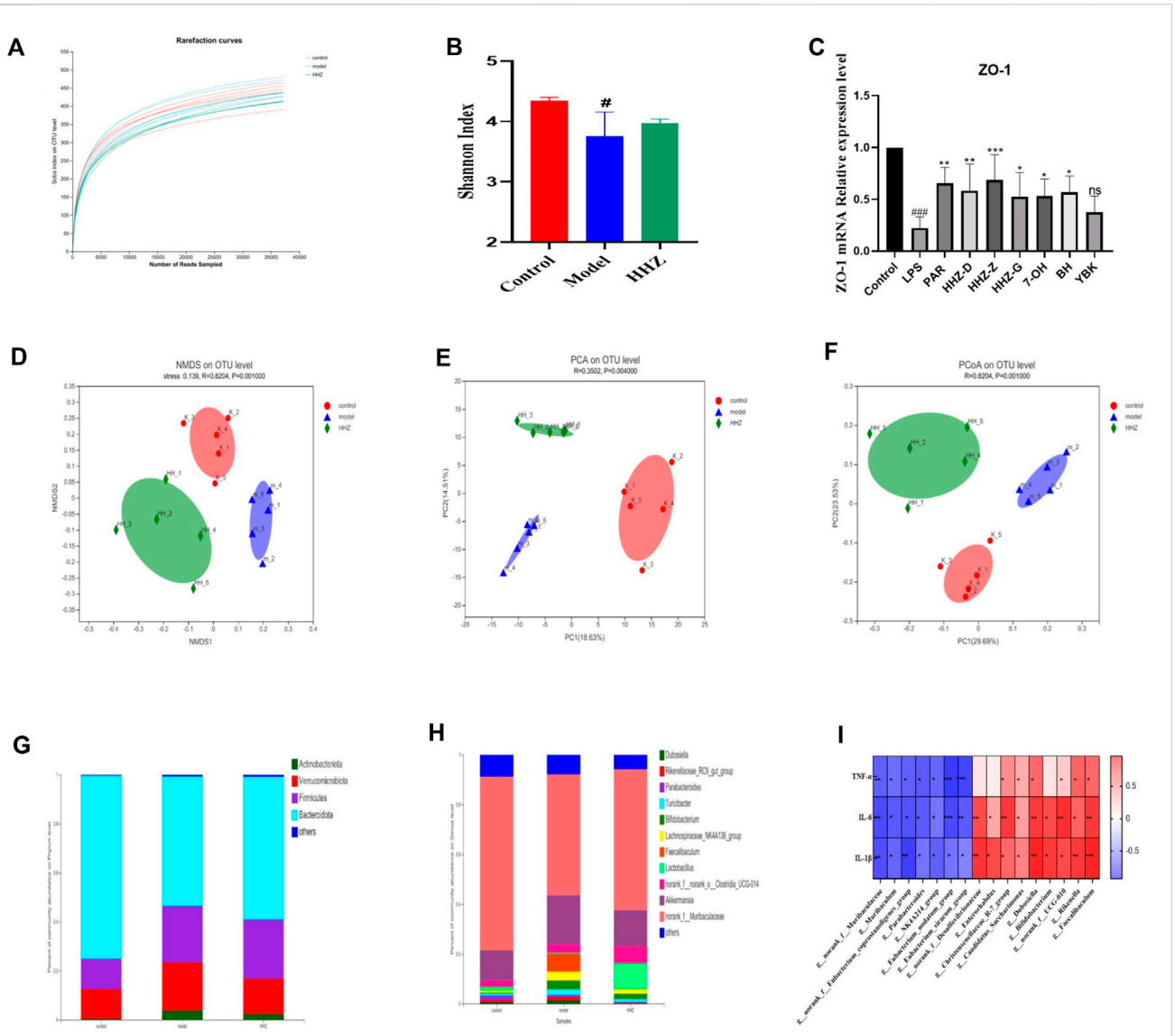

FIGURE 5
The improvement effect of HHZ on the gut microbiota of inflammatory depression mice. **(A–B)** Effects of HHZ on microbial alpha diversity sobs and shannon indices. **(C)** Relative expression of ZO-1 gene in intestinal tissue. **(D)** NMDS method to analyze species differences in microbial communities. **(E)** PCA analysis of gut microbiota β-diversity. **(F)** Differences in gut microbiota between different groups in the PCoA study. **(G)** Differences in microbial communities in feces at the phylum level. **(H)** Distribution of microbial communities in feces at the genus level. **(I)** Correlation between different levels of Spearman's gut bacteria and identification of depression-related inflammatory indices. Values are presented as mean ± SD. #$p < 0.05$ means a significant difference compared with the control group, and the significance of the relevant results is represented by *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

We collected fecal samples from mice in the control group, model group, and HHZ gavage group for 16s RNA sequencing analysis to further explore the impact of HHZ on the intestinal environment, and the obtained Sobs index and Shannon index had no difference between the groups (Figures 5A, B). PCA analysis showed that the β-diversity of the model group was mighty different from that of the control group, while HHZ partially reversed this change (Figures 5D–F), suggesting that HHZ can regulate the diversity of gut microbiota. At the level of phylum classification, compared with the Control group, the richness of Bacteroidota went down, and the richness of Actinobacteriota, Verrucomicrobiota, and Firmicutes rose in the Model group, while the HHZ group reduced the increase of these three bacterial phyla and alleviated the decrease in the richness of Bacteroidota (Figure 5G). At the level of genus classification, compared with the control group, the proportion of norank_ f_ _Muribaculaceae richness in the Model group was distinctly reduced, and the richness of Akkermansia, Bifdobacterium, Lachnospiraceae and NK4A136 group increased. It is worth noting that the HHZ group can recover the richness of norank_ f_ _Muribaculaceae, reduce the proportion of Akkermansia, Bifdobacterium, Lachnospiraceae and NK4A136 group, and greatly increase the richness of Lactobaillus (Figure 5H). The correlation between intestinal flora and serum inflammatory factors was verified by spearman correlation analysis, and the results showed that there was a close relationship between intestinal bacteria and inflammatory factors. Among these bacterial

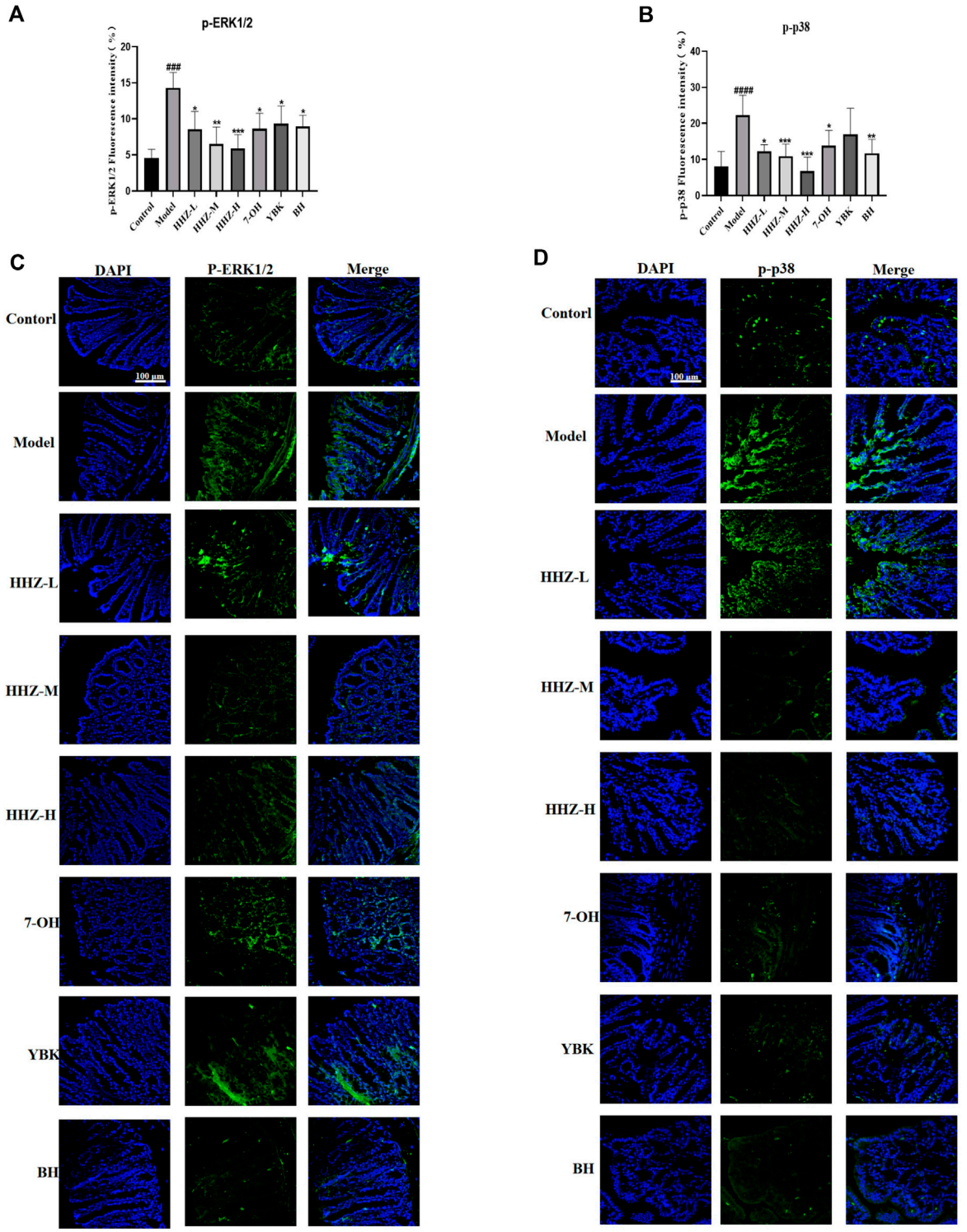

FIGURE 6
Gut microbiota-based antidepressant mechanism of HHZ involves expression inhibition of ERK1/2/P38 signaling pathway. **(A, C)** Immunofluorescent staining of p-ERK1/2 in the colon. **(B, D)** Immunofluorescent staining of p-p38 in the colon. Values represent the mean ± SD. #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ ####$p < 0.0001$ means significant difference compared with Control group; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ means significant difference compared with Model group.

genera, beneficial bacterial genera such as g-norank, f-Muribaculaceae, and g-Muribaculum had a strong negative correlation with the levels of IL-1β, IL-6, and TNF-α, while g-Faecalibaculum, g-Dubosiella, and IL-1β, IL-6, and TNF-α levels showed a strong positive correlation (Figure 5I). In addition, we also used RT qPCR to detect the mRNA level of

intestinal barrier protein ZO-1, and the results showed that compared with the control group, the mRNA expression level of ZO-1 in the Model group was significantly reduced. After administration, the expression of ZO-1 mRNA showed varying degrees of regression (Figure 5C). These data suggested that HHZ exerts antidepressant effects by improving the gut microbiota structure in inflammatory depressed mice.

3.5 Inflammatory inhibition of HHZ on ERK1/2/P38 signaling pathway

Since HHZ can regulate lipopolysaccharide (LPS)-producing intestinal bacteria and inflammatory factors, it is suggesting that the anti-inflammatory depressive effect of HHZ may be related to the ERK1/2/P38 signaling pathway, which is a key and usually stimulated by pro-inflammatory factors (Chahwan et al., 2019). The expressions of p-ERK1/2 and p-p38 were detected in the colon tissue of mice in each group by immunofluorescence method. The results showed that the expression level of p-ERK1/2/p-p38 in model group mice was markedly higher than that in control group mice, while HHZ and its plant metabolites could reverse this trend (Figures 6A–D). It can be seen that HHZ can inhibit the activation of ERK1/2/P38 signaling pathway, and the potential anti-inflammatory depression mechanism of HHZ and its plant metabolites is related to the transmission of ERK1/2/P38 signaling pathway mediated by intestinal flora.

4 Discussion

Many different dosing strategies have been adopted in the past decade to increase the efficacy of conventional antidepressants (Chenu et al., 2006), but clinical responses remain unsatisfactory (Lam et al., 2002), and as many as One-third of depressed patients still are resistant to treatment with these conventional antidepressants (Wohleb et al., 2016). With the further deepening of the research on marine drugs, more and more algae have been developed from simple consumption to medical services. A large number of studies have proved that marine algae have anti-inflammatory, anti-tumor, antibacterial and other effects. In this study, *Sargassum pallidum* and its plant metabolites showed good anti-inflammatory depressive activity, which indicated that the comprehensive utilization of marine algae had a bright prospect.

We established a UHPLC-QTOF-MS/MS analysis method, and identified main plant metabolites of seaweed, mainly alkaloid, terpenoids and coumarins, and also rich in vitamins and amino acids. LPS-induced microglial activation can lead to an inflammatory response and applied in cellular models (Shen et al., 2022; Zhai et al., 2023). Therefore, in this study, LPS (lipopolysaccharide) was used to stimulate BV2 microglia to establish an *in vitro* neuronal inflammation model, and to verify the anti-neuroinflammatory effect of *Sargassum pallidum* and its three plant metabolites. Using the CCK-8 kit to measure the viability of microglial cells, it was found that LPS increased the activity of microglial cells, indicating that the model was successful. The generation of neuroinflammation is inseparable from high levels of inflammatory factors (Han et al., 2024). The levels of IL-1β, TNF-α, and IL-6 were measured by enzyme-linked immunosorbent assay and immunofluorescence assay, among them, the medium dose HHZ group (60 μg/mL) can ensure the highest cell activity while reduced the concentration of the three inflammatory factors mentioned above and avoided the induction of neuroinflammation and neurotoxicity by abnormally activated microglia. This experiment proved that *Sargassum pallidum* and its plant metabolites can inhibit the increase of the secretion of pro-inflammatory factors. It showed that *Sargassum pallidum* and its plant metabolites have anti-inflammatory activity, may have antidepressant activity, and may also have the potential to treat other central diseases caused by inflammation.

In vitro, *Sargassum pallidum* extract can effectively reduce the production of inflammatory factors and protect nerve cells. *In vivo*, *Sargassum pallidum* improve the depression-like behavior of chronic inflammation-induced depression mice. Specifically, after administration, the immobility time in the FST and the TST reduced, while sugar water preference increased in the SPT. The results of *in vitro* and *in vivo* pharmacological effects show that 7-OH, BH, and YBK are important metabolites of the anti-inflammatory and depressive effects of *Sargassum pallidum*.

In the LPS induced depression model, the intestine showed a significant inflammatory response. HE staining of the intestinal tissue verified its pathological changes. The inflammatory factors in the blood have a direct impact on the level of inflammation in the brain, meanwhile also regulated by the level of intestinal inflammation (Wang et al., 2022; Liu et al., 2023). Therefore, the expression levels of inflammatory factors in serum and brain tissue of each group were further detected. The results showed that the expression levels of inflammatory factors in the serum and brain tissue of the model group were significantly increased, at the same time, *Sargassum pallidum* and its active ingredients could improve inflammation to varying degrees The gut microbiota plays an important role in the occurrence and development of depression, and the imbalance of the microbiota will directly affect the rate of neuroinflammation. In the analysis and comparison of the taxonomic levels of the microbiota in each group of mice, we found that the model group had a significant decrease in the abundance of beneficial bacterial genera norank_ f_ Mauribaulaceae and Lactobacaillus, and a significant increase in the abundance of harmful bacterial genera Akkermansia, Bifdobacterium, Lachnospiraceae and NK4A136. *Sargassum pallidum* could significantly increase the number of norank_ f_ Mauribaulaceae and reduce the abundance of Bifdobacterium, La4A136. The quantity of chnospiraceae and NK4A136 is worth noting that HHZ can greatly increase the richness of *Lactobacillus*, *Lactobacillus* is the largest genus of lactic acid bacteria, which not only enhances the intestinal mucosa and the systemic immune system to exert anti-inflammatory effects, but also improves the structure of the intestinal microbiota community by regulating intestinal microbiota metabolism, and exerts antidepressant effects through the brain gut microbiota axis. This may be an important pathway for *Sargassum pallidum* to produce good antidepressant effects.

In order to explore the mechanism of *Sargassum pallidum* reducing intestinal inflammation while regulating the flora, we explored potential ERK1/2/P38 pathway using immunofluorescence. It was found that *Sargassum pallidum* can regulate the ERK1/2/p38 signaling pathway, inhibit the phosphorylation of ERK1/2/p38 protein, and reduce the release of

intestinal inflammatory factors. Meanwhile, 7-OH, BH and YBK can also control the inflammatory factors of BV2 microglial cells *in vitro* by regulating the ERK1/2/p38 signaling pathway. Studies have shown that ERK1/2 and p38 signals participate in the regulation of the secretion and release of inflammatory factors in the inflammatory stress of cells, and regulate the differentiation and apoptosis of cells in stress (Sun et al., 2015). This indicates that HHZ affects the balance of the intestinal microenvironment, improves intestinal immunity, and transmits signals through the central nervous system along the brain gut axis, thereby reducing neuroinflammation which can alleviate the development of depression.

5 Conclusion

This study used LC-MS to analyze the material composition of *Sargassum pallidum*, and found that coumarins are one of the main characteristic plant metabolites. At the same time, combining the *in vivo* and *in vitro* activity studies of LPS inflammatory depression model and BV2 cells, not only confirmed the antidepressant effect of *Sargassum pallidum*, but also exhibited that 7-OH, BH, and YBK having anti neuroinflammation effect, which can significantly reduce and alleviate the inflammatory activation of microglia, and significantly improve the depressive behavior of inflammatory depression mice. Finally, based on 16S rDNA microbiota technology, combined with immunofluorescence staining, it was confirmed that the ERK 1/2/p38 inflammatory signaling pathway is the key pathway for alleviating inflammatory depression in *Sargassum pallidum*. This study may provide valuable insights into the discovery of new therapeutic uses for traditional medicinal plants, and also suggest that *Sargassum* may be a valuable plant for inflammatory or psychiatric diseases.

Data availability statement

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

Ethics statement

The animal study was approved by the Animal Ethics Committee of Jiangxi University of Chinese Medicine (Approval Number: JZLLSC20210061). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

DS: Funding acquisition, Resources, Validation, Writing–review and editing. QL: Data curation, Formal Analysis, Writing–original draft. XL: Methodology, Software, Writing–review and editing. YS: Writing–review and editing. HL: Methodology, Supervision, Writing–review and editing. ZA: Resources, Writing–review and editing. QZ: Visualization, Writing–review and editing. WS: Project administration, Writing–review and editing. MY: Resources, Validation, Writing–review and editing. GZ: Conceptualization, Resources, Writing–original draft.

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Conflict of interest

Author MY was employed by Jiangxi Guxiang Jinyun Comprehensive Health Industry Co Ltd.

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2024.1424834/full#supplementary-material

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A comparative analysis of chloroplast genomes revealed the chloroplast heteroplasmy of *Artemisia annua*

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Artemisia annua L. is the main source of artemisinin, an antimalarial drug. High diversity of morphological characteristics and artemisinin contents of *A. annua* has affected the stable production of artemisinin while efficient discrimination method of *A. annua* strains is not available. The complete chloroplast (cp) genomes of 38 *A. annua* strains were assembled and analyzed in this study. Phylogenetic analysis of *Artemisia* species showed that distinct intraspecific divergence occurred in *A. annua* strains. A total of 38 *A. annua* strains were divided into two distinct lineages, one lineage containing widely-distributed strains and the other lineage only containing strains from northern China. The *A. annua* cp genomes ranged from 150, 953 to 150, 974 bp and contained 131 genes, and no presence or absence variation of genes was observed. The IRs and SC junctions were located in *rps*19 and *ycf*1, respectively, without IR contraction observed. Rich sequence polymorphisms were observed among *A. annua* strains, and a total of 60 polymorphic sites representing 14 haplotypes were identified which unfolding the cpDNA heteroplasmy of *A. annua*. In conclusion, this study provided valuable resource for *A. annua* strains identification and provided new insights into the evolutionary characteristics of *A. annua*.

KEYWORDS

***Artemisia annua*, chloroplast genome, genetic diversity, strains identification, comparative analysis**

Abbreviations: cp, chloroplast; rDNA, ribosomal DNA; WGS, whole genome sequencing; tRNA, transport RNA; ML, maximum likelihood; NJ, neighbor-joining; IR, inverted repeat region; Pi, nucleotide diversity; HTS, high-throughput sequencing; IGV, Integrative Genomics Viewer; LSC, large single-copy region; SSC, small single-copy region; rRNA, ribosomal RNA; CDS, coding sequence; SNPs, single nucleotide polymorphisms; pSNP, partial single nucleotide polymorphism.

1 Introduction

Artemisia annua L. is the only natural source for artemisinin which is used for the treatment of malaria (Klayman, 1985; Cheong et al., 2020; Septembre-Malaterre et al., 2020; Al-Khayri et al., 2022). The selection and identification of elite germplasm of *A. annua* are critical for the high-quality, stable and low-cost production of artemisinin (Ma et al., 2014; Chen et al., 2017; Li et al., 2017; Shen et al., 2017; Ding et al., 2023). The wide range of natural distribution and high genetic heterozygosity due to self-incompatibility of pollination brought great challenges to the breeding of *A. annua* (Ma et al., 2014; Li et al., 2017; Amiryousefi et al., 2018; Ma et al., 2018). Although the genetic background of *A. annua* is relatively complex, the traits, especially artemisinin content, is correlated with their geographical distribution (Li et al., 2017). Moreover, strains of *A. annua* from southern China still had higher artemisinin content than those of northern strains under the same cultivation environment (He et al., 2022), which indicated a relative stable correlation between genetic background and geographical distribution.

Previously, a number of identification and taxonomy studies were conducted at species level of *A. annua* and its closely related species (Li et al., 2017; Shi et al., 2018; Jiao et al., 2023). *A. annua* can be efficiently identified from other *Artemisia* L. species by ITS regions (Li et al., 2017). The nuclear single nucleotide polymorphisms of 205 *Artemisia* species were used to reconstruct the phylogenetic relationships of *Artemisia* (Jiao et al., 2023). Besides, different types of molecular markers were also applied to identify different *A. annua* germplasms. He et al. (2022) applied SSR molecular markers to distinguish *A. annua* strains from different habitats. Ding et al. (2023) found that the ITS2 haplotype analysis is an ideal tool for *A. annua* strains identification based on the polymorphism of ribosomal DNA (rDNA). The cp genome is also an ideal tool for variants/strains identification and the intra-species genetic variations of cp genomes have been reported in many species (Sabir et al., 2014; Lei et al., 2016; Sun et al., 2019; Kim et al., 2020; Song et al., 2020; Xu et al., 2022). Cells of flowering plants possess high copies of their cp genome, and the copy numbers were estimated to range from 1, 900 to 50, 000 copies per cell (Bendich, 1987; Johnson and Palmer, 1989; Morley and Nielsen, 2016). Notably, heteroplasmy of organellar genome that more than one types among multiple copies of organellar genomes, was observed in individuals or even within single cells (Johnson and Palmer, 1989; Sabir et al., 2014; Lei et al., 2016; Ramsey and Mandel, 2019). Compared to DNA fragments, the whole cp genome, with relative long size, may contain sufficient variation and exhibit uniparental unisexual inheritance (Jansen and Ruhlman, 2012; Jensen and Leister, 2014; Ruhlman and Jansen, 2014), which makes it an ideal source for germplasm discrimination and genetic characteristic analysis.

In this study, a total of 38 complete cp genomes of *A. annua* were assembled and annotated. The structures, sequence polymorphisms and phylogenetic relationships of cp genomes were first in-depth analyzed and compared among *A. annua* strains. The cp genome heteroplasmy of *A. annua* was first reported in this study. Intra- and inter-individual genetic diversity of cp genomes of *A. annua* revealed in this study provided important information for the identification and evolution of *A. annua* strains.

2 Materials and methods

2.1 Materials collection, DNA extraction and PCR amplification

Thirty-five individuals of *A. annua* were cultivated in Huairou District, Beijing, from seeds collected from four countries to test and verify the polymorphic loci (Supplementary Table 1). Fresh leaves were snap-frozen with liquid nitrogen and stored at −80°C (Eppendorf, Hamburg, Germany). The total DNA of *A. annua* individuals were extracted by the modified cetyltrimethylammonium bromide (3 × CTAB) method (Allen et al., 2006), and the DNA quality and concentration were measured by electrophoresis in 1.0% agarose gel and the NanoDrop2000 ultra-micro ultraviolet spectrophotometer (Thermo Scientific, MIT, United States). Primers were designed by Primer Premier 5 (Supplementary Table 2). PCR amplification was performed on 25 μL reaction mixtures containing 2X Pro Taq Master Mix (dye plus) 12.5 μL, nuclease-free water 8.5 μL, 1.0 μL each of 10 μM forward and reverse primers, and genomic DNA 1.0 μL. The PCR reaction conditions were listed in Supplementary Table 1. The amplified products were detected by electrophoresis in 1.0% agarose gel. The synthesis of primers, and sequencing of amplification products were conducted by Sangon Biotech Guangzhou branch office.

2.2 WGS data collection, cp genome assembly, and annotation

WGS (whole genome sequencing) datasets of LQ-9 and HAN1 strains, and whole genome resequencing datasets of 36 individuals were obtained from previous study (Liao et al., 2022a) (Supplementary Table 3). The quality of raw sequencing data was evaluated by FastQC 0.11.5 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc) and low quality bases and reads were trimmed and removed by Skewer (Jiang et al., 2014). The LQ-9 cp genome was assembled and as the reference genome for later analysis. WGS reads of LQ-9 were mapped to *A. annua* cp genome (GenBank Accession Number: MF623173) and mapped reads were extracted as cp-like reads. The extracted reads were then assembled into contigs by ABySS 2.0.0 (https://github.com/bcgsc/abyss) (Jackman et al., 2017). Finally, the cp sequence contigs were ordered and concatenated based on the collinearity with reference cp genome sequences. The initial gene annotation was conducted with plann 1.1.2 (Huang and Cronk, 2015) and then validated by BLAST and manually correction. The transport RNA (tRNA) genes were identified with tRNAscan-SE software (Lowe and Chan, 2016). Circular gene maps of the *A. annua* cp genomes were generated using Chloroplot software (Zheng et al., 2020). The cp genomes assembled in this study have been deposited in the Global Pharmacopoeia Genome Database (Liao et al., 2022b) at http://www.gpgenome.com/species/92 under the "SuperBarcodes" section.

2.3 Phylogenetic analysis

A total of 117 complete cp genome sequences from 38 *Artemisia* species, and one complete cp genome from

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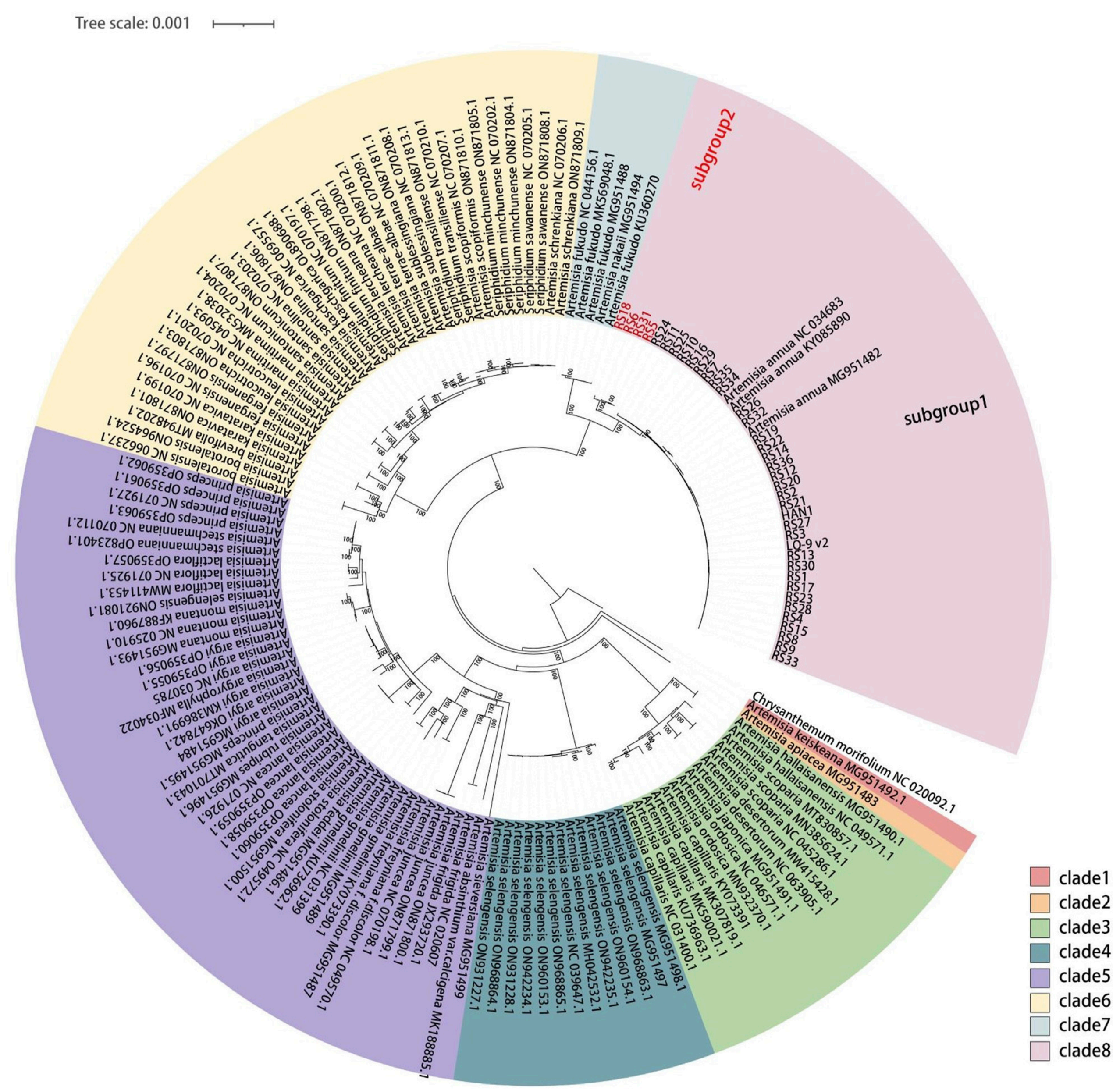

FIGURE 1
Phylogenetic tree obtained using the ML method for *Artemisia* species based on 156 complete cp genomes. Numbers above the branches indicate the ML bootstrap values.

Chrysanthemum were downloaded from the NCBI GenBank (Supplementary Table 4) for phylogenetic analysis. Among which, 115 cp genomes were reassembled since the direction of SSC regions were opposite to *A. annua* cp genome in this study. Multiple sequence alignments were performed with 156 cp genomes (Supplementary Tables 3, 4) using MAFFT 7.313 (Katoh and Standley, 2013). Maximum likelihood (ML) phylogenetic tree was performed by RAxML 8.2.11 (Stamatakis, 2014) with 1,000 bootstrap replicates under the GTR + G model. Neighbor-joining (NJ) tree was constructed using MEGA 7 (Kumar et al., 2016) under the Kimura two-parameter model with 1,000 bootstrap replicates.

2.4 Sequence comparison and nucleotide variation analyses

Whole cp genomes of 38 *A. annua* individuals were aligned using MAFFT 7.313 (Katoh and Standley, 2013). For rearrangement analysis, all aligned sequences were constructed with Geneious Prime program (Kearse et al., 2012). The IRscope program was used to evaluate the expansion and contraction of inverted repeat region (IR) (Amiryousefi et al., 2018). Comparative analysis of cp genomes was performed among *Artemisia* species and visualized by R. Furthermore, nucleotide diversity (Pi) was estimated by sliding window analysis using DnaSP 6.0 with a 600 bp window length and 200 bp step size (Rozas et al., 2017). High

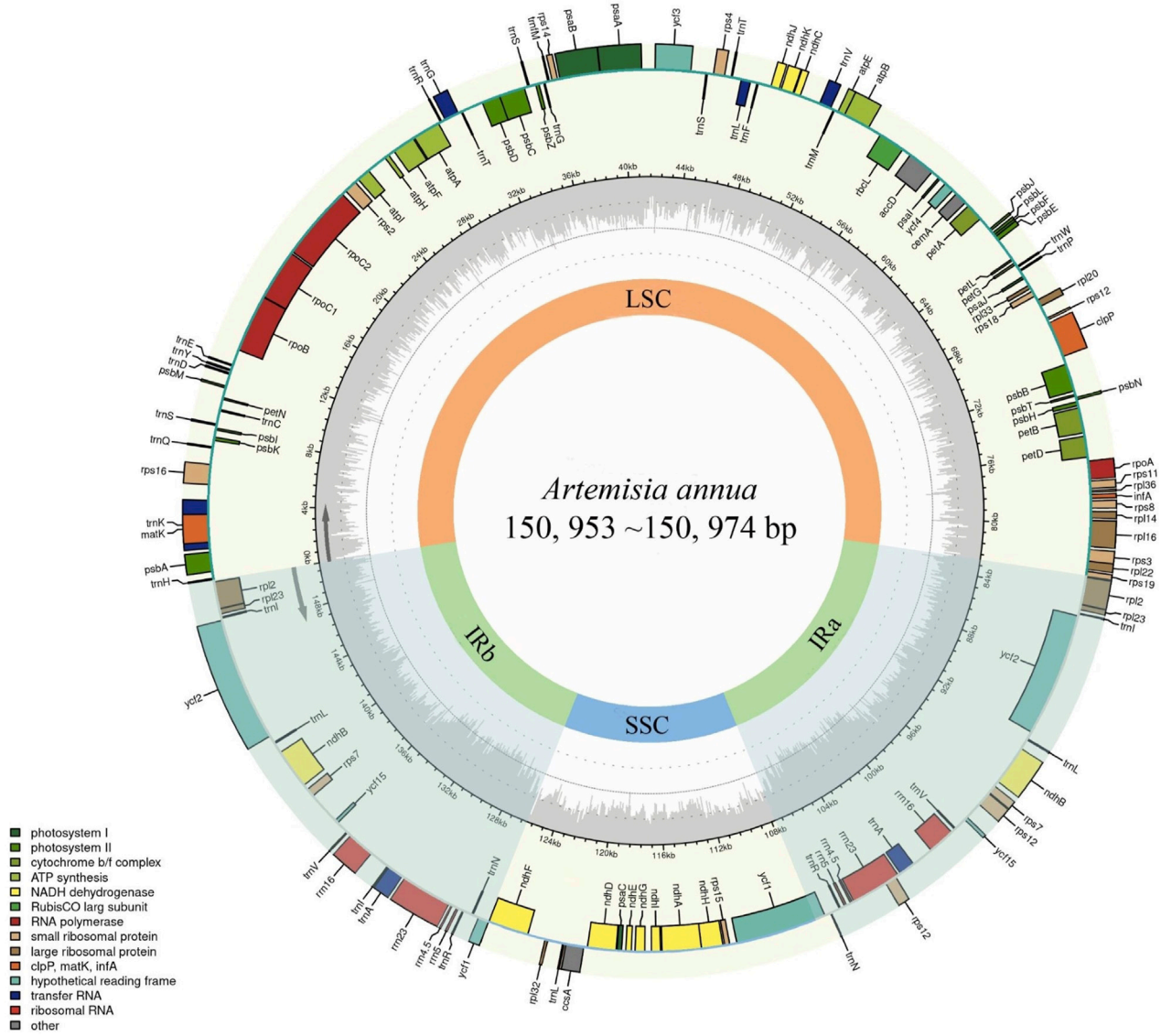

FIGURE 2
Circos plot of the *A. annua* cp genomes. Genes drawn inside the circle are transcribed in the clockwise direction, and those on the outside are transcribed in the counterclockwise direction. The genes are color-coded based on their function. The dark gray plot in the inner circle corresponds to GC content.

throughput sequencing (HTS) data of 38 *A. annua* individuals were aligned to the LQ-9 reference cp genome with Bowtie2 2.4.4 (Langmead and Salzberg, 2012). The variants were called and filtered using BCFtools (http://samtools.github.io/bcftools/) with a minor allele count higher than 1. False positives caused by sequencing errors were excluded by visualizing the sequencing data of mutation sites by Integrative Genomics Viewer (IGV) (Robinson et al., 2011).

3 Results

3.1 Interspecific divergence in *Artemisia* species based on cp genomes

A total of 38 complete *A. annua* cp genomes were assembled with WGS data of 38 distinct individuals released by previous study (Supplementary Table 3) (Liao et al., 2022a). To explore the phylogenetic and evolutionary relationships of *A. annua* populations, a phylogenetic tree was constructed using ML and NJ methods based on 156 complete cp genomes of 38 *Artemisia* species, and *C. morifolium* was included as the outgroup (Supplementary Table 4). The topological congruence between the ML and NJ phylogenetic trees was observed, with the majority of nodes exhibiting robust support values (>99%) (Figure 1; Supplementary Figure 1). Thirty-eight species were divided into two main branches, the first branch only comprising *A. keiskeana* (Figure 1, clade 1), while the second branch was further divided into seven well-supported clades (Figure 1, clades 2–8). *Seriphidium* was considered to be an independent genus (Lin et al., 2011; Haghighi et al., 2014), while this was not supported by the phylogenetic relationship of cp genomes as *Seriphidium* species were close related with *A. schrenkiana* and *A. scopiformis*. The phylogenetic tree also showed that all cp genomes from the same species clustered

TABLE 1 Features of 38 *A. annua* cp genomes.

Sample	Genome size (bp)	LSC length (bp)	SSC length (bp)	IR length (bp)	CDS length (bp)	Number of genes	Number of protein-coding genes	Number of tRNA genes	Number of rRNA genes	Overall GC (%)
LQ-9	150, 953	82, 774	18, 267	24, 956	79, 236	131	87	36	8	37.48
HAN1	150, 956	82, 777	18, 267	24, 956	79, 236	131	87	36	8	37.48
RS1	150, 955	82, 776	18, 267	24, 956	79, 236	131	87	36	8	37.48
RS2	150, 956	82, 777	18, 267	24, 956	79, 236	131	87	36	8	37.48
RS3	150, 957	82, 778	18, 267	24, 956	79, 236	131	87	36	8	37.48
RS4	150, 955	82, 776	18, 267	24, 956	79, 236	131	87	36	8	37.48
RS5	150, 959	82, 706	18, 341	24, 956	79, 236	131	87	36	8	37.47
RS6	150, 957	82, 704	18, 341	24, 956	79, 237	131	87	36	8	37.48
RS7	150, 963	82, 766	18, 285	24, 956	79, 236	131	87	36	8	37.48
RS8	150, 955	82, 776	18, 267	24, 956	79, 236	131	87	36	8	37.48
RS9	150, 955	82, 776	18, 267	24, 956	79, 236	131	87	36	8	37.48
RS10	150, 972	82, 776	18, 284	24, 956	79, 236	131	87	36	8	37.48
RS11	150, 960	82, 763	18, 285	24, 956	79, 236	131	87	36	8	37.48
RS12	150, 963	82, 784	18, 267	24, 956	79, 236	131	87	36	8	37.48
RS13	150, 955	82, 776	18, 267	24, 956	79, 236	131	87	36	8	37.48
RS14	150, 958	82, 779	18, 267	24, 956	79, 236	131	87	36	8	37.48
RS15	150, 955	82, 776	18, 267	24, 956	79, 236	131	87	36	8	37.48
RS16	150, 974	82, 778	18, 284	24, 956	79, 236	131	87	36	8	37.48
RS17	150, 955	82, 776	18, 267	24, 956	79, 236	131	87	36	8	37.48
RS18	150, 962	82, 701	18, 349	24, 956	79, 236	131	87	36	8	37.48
RS19	150, 957	82, 778	18, 267	24, 956	79, 236	131	87	36	8	37.48
RS20	150, 959	82, 780	18, 267	24, 956	79, 236	131	87	36	8	37.48
RS21	150, 957	82, 778	18, 267	24, 956	79, 236	131	87	36	8	37.48
RS22	150, 958	82, 779	18, 267	24, 956	79, 236	131	87	36	8	37.48
RS23	150, 955	82, 776	18, 267	24, 956	79, 236	131	87	36	8	37.48
RS24	150, 964	82, 785	18, 267	24, 956	79, 248	131	87	36	8	37.48
RS25	150, 972	82, 776	18, 284	24, 956	79, 236	131	87	36	8	37.48
RS26	150, 955	82, 776	18, 267	24, 956	79, 236	131	87	36	8	37.48
RS27	150, 956	82, 777	18, 267	24, 956	79, 236	131	87	36	8	37.48
RS28	150, 955	82, 776	18, 267	24, 956	79, 236	131	87	36	8	37.48
RS29	150, 963	82, 766	18, 285	24, 956	79, 236	131	87	36	8	37.48
RS30	150, 955	82, 776	18, 267	24, 956	79, 236	131	87	36	8	37.48
RS31	150, 959	82, 706	18, 341	24, 956	79, 236	131	87	36	8	37.47
RS32	150, 955	82, 776	18, 267	24, 956	79, 236	131	87	36	8	37.48
RS33	150, 955	82, 776	18, 267	24, 956	79, 236	131	87	36	8	37.48
RS34	150, 969	82, 772	18, 285	24, 956	79, 236	131	87	36	8	37.48

(Continued on following page)

TABLE 1 (*Continued*) Features of 38 *A. annua* cp genomes.

Sample	Genome size (bp)	LSC length (bp)	SSC length (bp)	IR length (bp)	CDS length (bp)	Number of genes	Number of protein-coding genes	Number of tRNA genes	Number of rRNA genes	Overall GC (%)
RS35	150, 968	82, 771	18, 285	24, 956	79, 236	131	87	36	8	37.48
RS36	150, 958	82, 779	18, 267	24, 956	79, 236	131	87	36	8	37.48

together, except *A. argyi* and *A. selengensis*. All *A. annua* cp genomes clustered into a same clade and shared the most recent common ancestor with *A. fukudo* and *A. nakaii*, which was consistent with the previous results of Shen et al. (2017) and Jin et al. (2023). Intriguingly, all *A. annua* strains were divided into two subgroups, forming two distinct lineages. Geographically, the first lineage (subgroup 1) containing a mixture of southern and northern strains was defined as a broad lineage, and the second (subgroup 2) was defined as a northern lineage containing only northern strains.

3.2 Conserved cp genome structure in *A. annua* strains

Thirty-eight assembled *A. annua* cp genomes were further annotated and analyzed. All cp genomes possessed the typical quadripartite structure which consisted of a large single-copy (LSC) region, a small single-copy (SSC) region, and two copies of IR regions (Figure 2). All cp genomes had lengths ranging from 150, 953 bp (LQ-9) to 150, 974 bp (RS16) with GC contents ranging from 37.47% to 37.48%. The LSC regions had lengths ranging from 82, 701 bp (RS18) to 82, 785 bp (RS24), and the SSC regions ranged from 18, 267 bp (RS24 *et al.*) to 18, 349 bp (RS18), while the lengths of IR regions were 24, 956 bp in all *A. annua* strains (Table 1), similar to the previously reported *Artemisia* cp genomes (Shen et al., 2017; Kim et al., 2020; Jin et al., 2023). Gene annotation showed that all cp genomes contained 131 genes, including 87 protein-coding genes, 36 tRNA genes and eight ribosomal RNA (rRNA) genes (Table 1). Of these, 17 genes (seven protein-coding genes, six tRNA genes and four rRNA genes) were duplicated in the IR regions in all *A. annua* strains (Supplementary Table 5). In addition, 17 intron-containing genes were identified, of which 15 contained one intron and two contained two introns (Supplementary Table 5).

The IR border structure was conserved in *A. annua* that no IR contraction was observed. In each *A. annua* individual, the junctions between IRs and LSC and SSC were flanked by *rps*19 and *ycf*1, respectively (Supplementary Figure 2), in concordance with other *Artemisia* species (Kim et al., 2020; Jin et al., 2023). Comparison of genome structure showed that *A. annua* cp genomes were highly conserved, and no significant gene rearrangements were observed (Supplementary Figure 3). On the whole, the *A. annua* cp genomes were structural conserved.

3.3 Sequence polymorphisms in *A. annua* cp genomes

In this study, the intraspecific polymorphisms of *A. annua* cp genomes were assessed in inter-individual levels. Complete cp genomes of 38 *A. annua* individuals were aligned using the LQ-9 as the reference genome. In total, 60 polymorphic sites were identified after excluding false positives caused by sequencing errors, including 19 singleton variable sites and 41 parsimony informative sites, among which, 48 SNPs were identified in LSC, 11 in SSC and one in IR regions, respectively (Figure 3A; Table 2). The SSC regions were found to have the highest polymorphisms with a SNP site density of 8.8/10 kb, followed by LSC regions (5.3/10 kb), and the SNP density of IRs regions was only 0.4/10 kb (Table 2), revealing that the IR regions were more conserved than the single-copy regions, which is consistent with the comparative analysis of the whole cp genome of *Artemisia* species (Figure 4). Furthermore, the Pi values of 38 *A. annua* cp genomes were calculated using DnaSP v6.0, and the Pi values ranged from 0 to 0.0013 with an average of 0.00007. Based on DNA polymorphisms, seven highly diverged regions (Pi > 0.0007) were identified, including *rps*16-*trn*Q-UUG, *pet*N-*psb*M, *psb*M-*trn*D-GUC, *rpl*20-*clp*P, *clp*P, *trn*L-UAG-*rpl*32 and *rpl*32 (Figure 3B), similar results have been observed in other *Artemisia* species (Shen et al., 2017; Kim et al., 2020; Jin et al., 2023). Fifteen protein-coding genes were found to containing variations, of which the *ycf*4 gene had the highest Pi value (0.0007) and the *ndh*F gene had the lowest Pi value (0.000002) (Figure 3C; Supplementary Table 6).

In addition, the WGS data of 38 individuals were aligned to LQ-9 reference genome with Bowtie2 for polymorphic sites validation. All 60 polymorphic sites were supported by mapped reads (Figures 3A, D). Notably, most of the polymorphic sites were single nucleotide polymorphisms (SNPs) between *A. annua* individuals, but partial single nucleotide polymorphism (pSNP) which contained more than one alternative bases in one site defined by James et al. (2009) were also found within individuals (Figure 3D). The polymorphic loci were confirmed by Sanger sequencing using the total DNA of 35 *A. annua* individuals (Figure 3F; Supplementary Table 1). In the Sanger sequencing results, the pSNP site generally contained multiple nested-peak which were identified as degenerate bases, and the SNP site showed clean single-peak representing complete base mutations (Figure 3E).

A total of 14 haplotypes were found in 38 *A. annua* individuals, which can be further divided into two distinct lineages as shown above (Figure 1; Figure 3G). Germplasm resources collected from the same location clustered together excluding Gansu, Hubei, and Xizang populations, but strains from different populations were also observed to have the same haplotype. Besides, polymorphism analysis showed that the subgroup 2 (the northern lineage) exhibited more unique polymorphic sites than the subgroup 1 (the broad lineage) and they shared only two polymorphic sites (Table 3). Other *Artemisia* species shared fifteen and four

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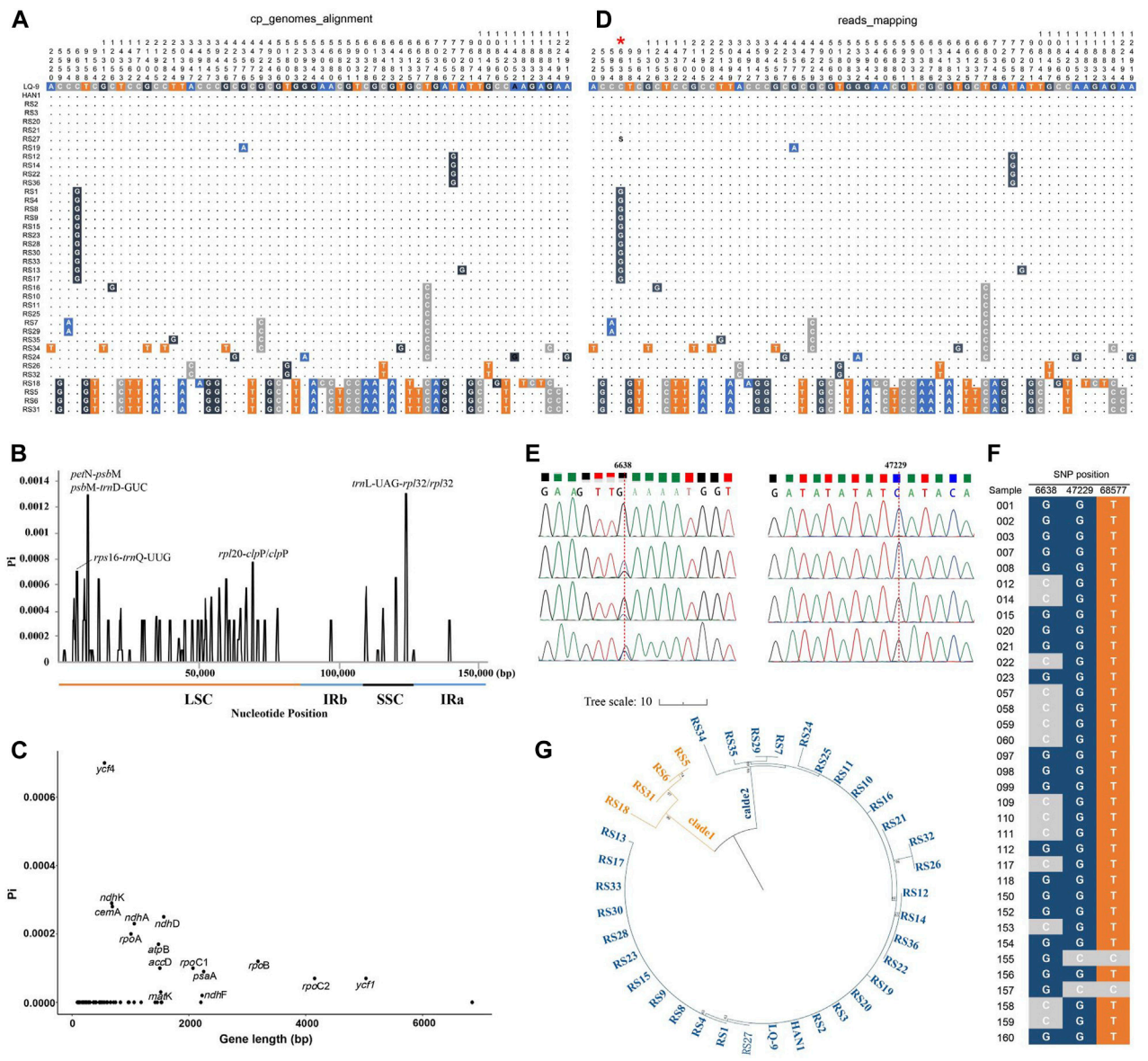

FIGURE 3
Variation of *A. annua* cp genomes. **(A)** Polymorphism analysis based on 38 *A. annua* global sequence alignment; **(B)** Nucleotide diversity of 38 *A. annua* cp genomes; **(C)** Nucleotide diversity of protein-coding genes; **(D)** Polymorphism analysis based on WGS reads mapping, red asterisk represents the pSNP site; **(E)** Partial peak diagram of Sanger sequencing of polymorphic sites 6,638 and 47,229, respectively; **(F)** Polymorphic sites confirmed by Sanger sequencing; **(G)** NJ tree constructed based on 60 polymorphic sites.

TABLE 2 Number of SNPs in each region of cp genomes combing 38 *A. annua* individuals.

Region	Length	SNP count	Transversion	Transition	SNP density/10 kb
LSC	82, 774	48	31	17	5.3
SSC	18, 267	11	7	4	8.8
IR	49, 912	1	1	0	0.4

polymorphic sites with the northern lineage and the broad lineage, respectively (Table 3; Supplementary Figure 4). We proposed that the broad lineage may be the stable and specific evolutionary lineage during the speciation of *A. annua*, and the northern lineage may be the post-forming lineage. The above results showed that *A. annua* cp genomes were highly polymorphic, and the cp variants dataset can be used as the reference dataset for the identification and evolutionary analysis of *A. annua* strains.

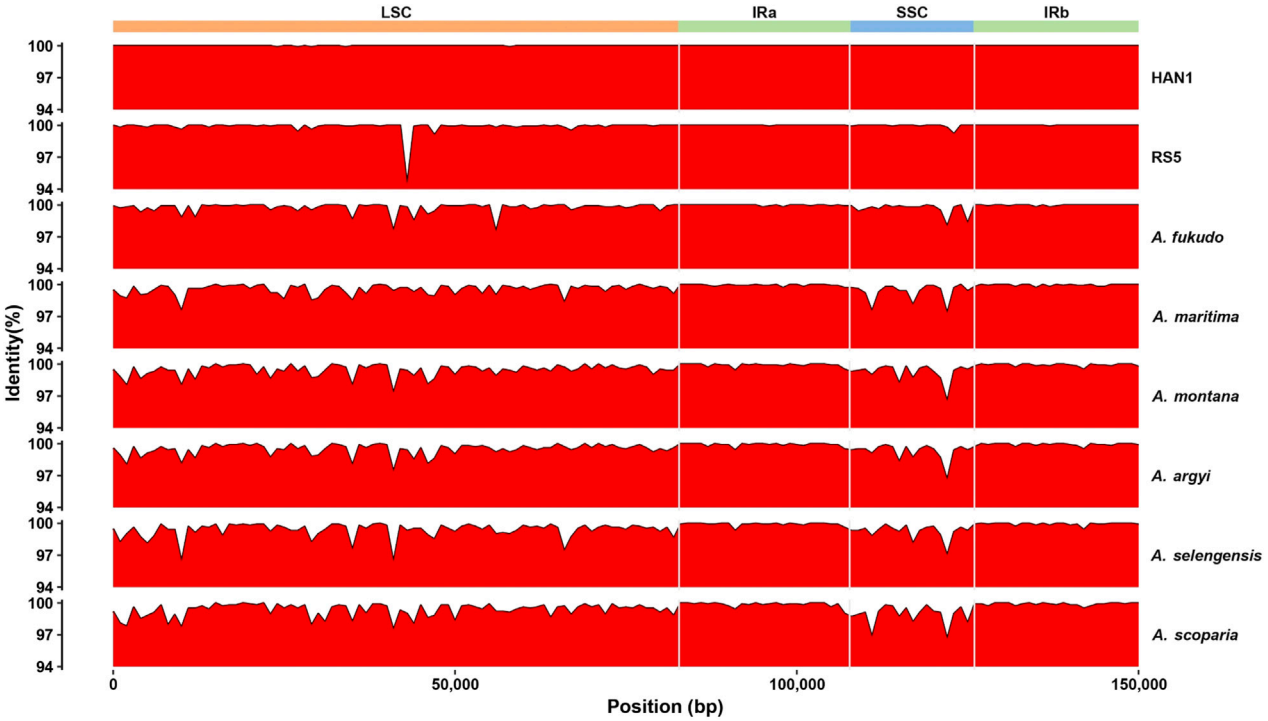

FIGURE 4
Identity plot based on the whole cp genome comparison of *Artemisia* species.

TABLE 3 Comparison of variations between 38 *A. annua* strains and other *Artemisia* species.

	Variation types	Number of polymorphic sites
Subgroup 1 vs. Subgroup 2	Unique (Subgroup 1)	22
	Unique (Subgroup 2)	36
	Common	2
Subgroup 1 vs. Other *Artemisia*	Unique (Subgroup 1)	20
	Common	4
Subgroup 2 vs. Other *Artemisia*	Unique (Subgroup 2)	23
	Common	15

4 Discussion

In this study, the phylogenetic relationships, structures, sequence polymorphisms of *A. annua* cp genomes were analyzed. All *A. annua* cp genomes showed highly conserved in structures, gene numbers and gene order. Minor differences were observed in genome sizes, but the lengths of IR regions were identical in all strains without contraction, similar to the observation in other *Artemisia* species (Shen et al., 2017; Kim et al., 2020; Jin et al., 2023). CpDNA is multicopy and abundant in plant (Bendich, 1987; McPherson et al., 2013; Malé et al., 2014; Morley and Nielsen, 2016), and were thought to be very conserved, as each species is typically characterized by a single cpDNA type. Recently, some genome-based studies have focused on population-level resolution and a single individual per species, and intraspecific genetic variations were detected in many species (Cui et al., 2020; Chen et al., 2022; Huang et al., 2022). Similarly, high sequence polymorphisms were found among *A. annua* populations. Distinct intraspecific divergence was observed, in which a more complex population structure was assumed in broad lineage due to their multiple maternal lineages and relatively higher number of haplotypes.

Although multiple copies and heteroplasmy of organelle genomes had been discovered over a century, the phenomenon of heteroplasmy was often overlooked in previous studies (Bendich, 1987; Ramsey and Mandel, 2019). The mitochondrial heteroplasmy in humans has been studied extensively (Li et al., 2010; Jiang et al., 2023) as its occurrence is strongly related with mitochondrial diseases (Wallace, 1992; Wallace, 1994; Chinnery and Turnbull, 1999; Chinnery et al., 2002). Besides, the heteroplasmy characteristic of mitochondria have also been used as genetic markers in molecular

identification (Salas et al., 2001; Salas et al., 2005; Jiang et al., 2023). Plants possess high copy numbers of cp genomes, and polymorphisms of cp genomes are common at the level of population or species (Johnson and Palmer, 1989; Kim et al., 2020; Song et al., 2020; Xu et al., 2022). However, intra and inter-individual heteroplasmy of cp genomes are rarely reported (Fitter et al., 1996; García et al., 2004; Frey et al., 2005). In this study, the heteroplasmy of *A. annua* cp genomes were first discovered and verified, which may provide an important genetic basis for the accurate identification and evolutionary analysis of *A. annua* strains in the future.

This study represents the first in-depth understanding of *A. annua* cp genomes, and the rich maternal haplotypes composition can be used as the reference dataset for strains identification and breeding of *A. annua*. As cp genome is inherited matrilineally and relatively conserved, the combination of cp genome haplotypes and trait-related loci from nuclear genome should be further applied to *A. annua* strains screening in the future.

5 Conclusion

In the present study, the phylogenetic relationships, structures, sequence polymorphisms of *A. annua* cp genomes were characterized. Phylogenetic analysis of *Artemisia* species showed that intraspecific divergence occurred during the speciation of *A. annua*, forming two distinct lineages, the broad lineage and the northern lineage. The structures of *A. annua* cp genomes were extremely conserved without IR contraction and gene rearrangements observed. Rich sequence polymorphisms were observed among *A. annua* strains, and cpDNA heteroplasmy has been first reported and verified in *A. annua*. A total of 60 polymorphic sites were identified by global sequence alignment and WGS reads mapping, which can be further divided into 14 haplotypes, representing the cp variants dataset of *A. annua*. Our work provides important information for the identification and evolution of *A. annua* strains.

Data availability statement

The WGS data and assembled cp genome data were deposited in the Global Pharmacopoeia Genome Database at http://www.gpgenome.com/species/92 under the "SuperBarcodes" section. Data supporting the findings of this work are available within the paper and its supplemental information files. The datasets generated and analyzed during the study are available from the corresponding author upon reasonable request.

Author contributions

XD: Writing–review and editing, Writing–original draft, Visualization, Validation, Methodology, Data curation, Conceptualization. HP: Writing–original draft, Validation, Software, Investigation, Data curation, Conceptualization. PS: Writing–original draft, Formal Analysis, Data curation, Conceptualization. SiZ: Writing–original draft, Validation, Investigation, Data curation. SB: Writing–original draft, Validation, Methodology, Data curation, Conceptualization. ShZ: Writing–original draft, Validation, Software. CD: Writing–original draft, Validation, Investigation. JC: Writing–original draft, Visualization, Software. LG: Writing–review and editing, Formal Analysis, Data curation. DZ: Writing–review and editing, Methodology. XQ: Writing–review and editing, Writing–original draft, Supervision, Resources, Methodology, Investigation. BL: Writing–review and editing, Writing–original draft, Supervision, Software, Methodology, Funding acquisition, Conceptualization. ZH: Writing–review and editing, Writing–original draft, Supervision, Resources, Methodology, Investigation, Formal Analysis, 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.2024.1466578/full#supplementary-material

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Fermentation: improvement of pharmacological effects and applications of botanical drugs

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Fermentation is an important concoction technique for botanical drugs. Fermentation transforms and enhances the active ingredients of botanical drugs through specific microbiological processes, ultimately affecting their pharmacological effects. This review explores the use of fermented botanical drugs in areas such as anti-tumor, hypolipidemic, antioxidant, antimicrobial, cosmetology, and intestinal flora regulation. It elucidates the potential pharmacological mechanisms and discusses the benefits of fermentation technology for botanical drugs, including reducing toxic side effects, enhancing drug efficacy, and creating new active ingredients. This article also discussesdelves into the common strains and factors influencing the fermentation process, which are crucial for the successful transformation and enhancement of these drugs. Taken together, this study aimed to provide a reference point for further research and wider applications of botanical drug fermentation technology.

KEYWORDS

fermentation, botanical drug, reduced toxicity, pharmacological effect, strains abstract

1 Introduction

Botanical drugs play a significant role in mainstream medicine because of their clinical effectiveness (Lu et al., 2020). They are abundant in active compounds with medicinal value and have been proven to be effective at treating various diseases, although they may have certain toxic side effects. Fermentation is a biochemical process that usually refers to the conversion of raw materials into desired metabolites by microorganisms through their vital activities. It is a simple, natural, and valuable technology (Ai et al., 2019).

In recent years, the application of fermentation technology in the production of botanical drugs has improved the extraction rate of active ingredients, reduced the dosage of botanical drugs, produced new active ingredients, decomposed toxic ingredients, reduced adverse reactions, enhanced drug efficacy, and broadened the use of botanical drugs in disease treatment. Early fermentation methods relied on natural microorganisms, and the microbial fermentation of botanical drugs, as documented in "The Essentials of the Golden Chamber," is exemplified by Qu (Yang et al., 2023a). However, these methods have problems, such as impure strains and unclear definitions of medicinal ingredients.

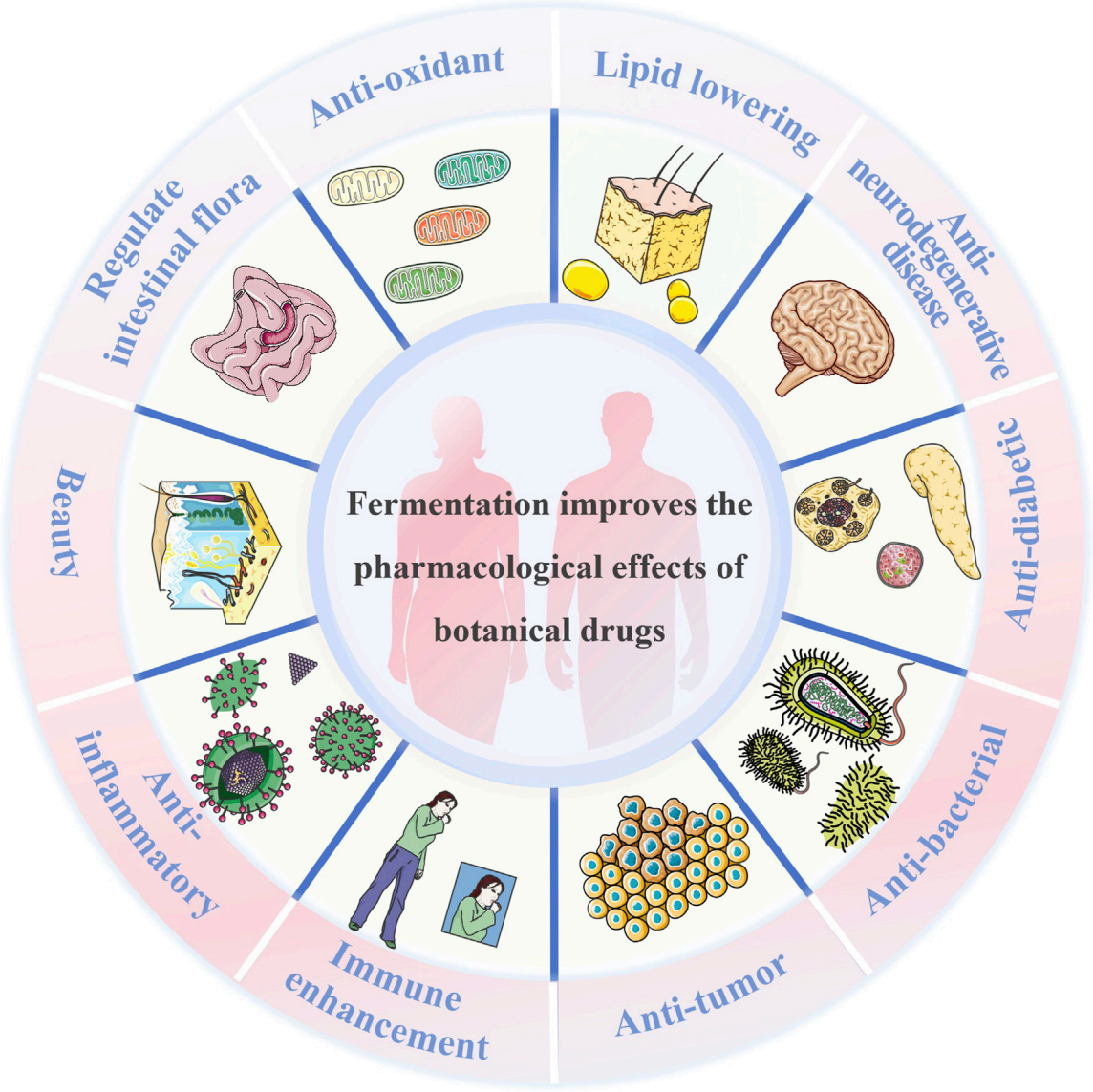

FIGURE 1
Fermentation improves the pharmacological effects of botanical drugs.

Modern fermentation is an advanced pharmaceutical technology, with the advancement of analytical methods to promote a further understanding of the fermentation strains as well as the composition of botanical drugs, to provide a more stable and safe production process for the fermentation, but also for the elaboration of the mechanism of action of fermented botanical drugs, which breaks through the limitations of natural conditions, the method can select certain strains of bacteria, a clear understanding of the products of fermentation, an in-depth understanding of the mechanism of fermentation, and the pharmacological effects of the drug can be evaluated through compositional analysis and biological assessment, thus improving the efficacy of the drug. The pharmacological effect of a drug can be evaluated by component analysis and biological assessment to improve the efficacy of the drug in a targeted manner, making fermentation an important emerging field with great potential for development. For example, fermentation of *Coix lacryma-jobi* using *Lactobacillus plantarum* NCU137 increases the nutrient content of free amino acids, free fatty acids, soluble dietary fiber, and organic acids; reduces the content of the hazardous substance 2-pentylfuran; improves its safety; and produces a natural preservative, acetic acid, which improves the stability of *C. lacryma-jobi* (Yin et al., 2020). Previous reviews have mostly focused on fermentation technology and its strain application; however, there are few comprehensive analyses of how fermentation enhances the pharmacological efficacy of botanical drugs, the specific advantages of the application of this technology to botanical drugs, and the key factors regulating the fermentation process. This study aimed to fill this gap by systematically reviewing the pharmacological enhancement mechanism of fermented botanical drugs, the advantages of the technology, and the key factors influencing the fermentation process, with the aim of providing a scientific and theoretical

basis for further research and wide application of the fermentation technology of botanical drugs.

2 Fermentation improves the pharmacological effects of in botanical drugs

Fermentation of botanical drugs involves a complex series of biochemical reactions and physiological processes. During fermentation, microorganisms utilize polysaccharides, fibers, proteins, and other medicinal components as nutrient sources. This process transforms low-activity ingredients into highly active ones, thereby boosting the efficacy of traditional botanical drugs. Additionally, this leads to the production of new secondary metabolites that play novel roles in the body, as shown in Figure 1.

2.1 Metabolic and endocrine regulation

2.1.1 Lipid lowering

Therefore, fermented botanical drugs may exhibit better lipid-lowering effects. Kim et al. (2015) fermented *Panax ginseng* with red koji from *Monascus* spp. to enhance its anti-obesity effects. In female ICR high-fat diet (HFD) -fed rats, fermented *P. ginseng* treatment significantly reduced the diameter of adipocytes per ovary ($P < 0.01$), abdominal fat pads ($P < 0.05$), and abdominal fat thickness. Additionally, fermented *P. ginseng* partially mitigated the HFD-induced weight gain in a dose-dependent manner. Biochemical and histomorphometric analyses confirmed that fermented *P. ginseng* effectively inhibited HFD-induced metabolic disorders, such as hyperglycemia, hyperlipidemia (shown by decreases in serum low-density lipoprotein (LDL), total cholesterol (TC), and triglycerides (TG), and an increase in high-density lipoprotein (HDL), hepatopathy, and nephropathy in a dose-dependent manner. Furthermore, one study indicated that fermented *P. ginseng* has more favorable pharmacological effects on HFD-induced metabolic issues than an equal dose of *P. ginseng* (Kim et al., 2013). This study showed a significant increase in the concentrations of propionic acid, butyric acid, acetic acid, and total short-chain fatty acids (SCFAs) in the colon of type 2 diabetic rats treated with *L. plantarum* plantarum-fermented *Momordica charantia* juice. This increase may be attributed to the regulation of glucose and lipid metabolism, particularly insulin sensitivity and glucose homeostasis, by SCFAs. It also increased the abundance of gut flora compared to untreated diabetic rats. These results suggest that *M. charantia* juice fermented with *L. plantarum* modulates the gut flora and improves glucose and lipid metabolism (Gao et al., 2019). *Momordica charantia* polysaccharide fermented by *L. plantarum* increased the total amount of SCFAs in the colonic contents of a rat model of type 2 diabetes mellitus and significantly improved blood glucose, lipid, insulin levels, and oxidative stress in diabetic rats compared to unfermented *M. charantia* polysaccharide, thus enhancing the anti-diabetic effect of *M. charantia* polysaccharides in rats (Gao et al., 2018). In a separate study, it was found that exposing rats from the HFD + lipopolysaccharide (LPS) group to *L. plantarum*-fermented *Atractylodes macrocephala*, rather than unfermented, led to a significant decrease in the relative weight of abdominal fat, total fat mass, and relative weight of total fat. Additionally, there was a notable reduction in serum TG levels and aspartate transaminase activity, along with a significant increase in serum HDL levels. The study proposed that The anti-adipogenic and anti-obesity effects of fermented *A. macrocephala* may be attributed, in part, to the inhibition of adipogenesis through various mechanisms such as enhancing glucose uptake, modulating the composition of the intestinal microflora to improve gut permeability, and preventing endotoxemia and associated inflammation (Wang et al., 2015). Ji et al. prepared *Rosa roxburghii* Tratt (RRT) juice into temperature-controlled fermentation vials, inoculated with 200 mg/L of Angel active yeast BV818 for 3 months, and obtained fermented RRT juice (FRRT) by sterilization and filtration, which was fed to subgroups of hyperlipidemic rat models. Metabolomic analysis showed that bile acid metabolism in the fermented prickly pear juice-fed group was significantly different from that in the RRT juice-fed group. It was concluded that FRRT helps to reduce the increase in fecal bile acids (e.g., deoxycholic acid and lithocholic acid) induced by a high-fat diet. This was achieved by affecting bacteria, such as *Lactobacillus* and *Staphylococcus*, which in turn inhibited farnesoid X receptor signaling in the liver. This process maintains the balance of bile acids in the enterohepatic circulation and ultimately improves dyslipidemia (Ji et al., 2022). Red yeast rice (RYR) is produced by fermenting ordinary rice using *M. spp*. Recent studies have shown that RYR can prevent weight gain and reduce fat pad weight, while also improving blood lipid parameters, liver enzymes, and leptin levels in HFD-fed rats. These results suggest that RYR has therapeutic potential in the treatment of obesity and hyperlipidemia (Lee et al., 2015).

2.1.2 Anti-diabetic

Accumulating evidence suggests that fermentation has beneficial effects on the anti-diabetic properties of botanical drugs. Fermented *P. ginseng* has been shown to lower fasting blood glucose and HbA1c in a mouse model of type 2 diabetes by increasing serum insulin and lipocalin levels, reducing TNF-α expression, and enhancing the expression of hepatic PPAR-γ and GLUT-2 genes (Jeon et al., 2013). In a separate study involving STZ-treated rats, *P. ginseng* fermented by *Lactobacillus* reduced blood glucose levels during an oral glucose tolerance test. This is accompanied by increased insulin secretion, ultimately resulting in decreased blood glucose levels. A previous study suggested that higher levels of Rb1, Rb2, Rc, Rd, Rg3 (known for their anti-diabetic properties), and ginsenoside Rh2 (which enhances insulin secretion) in fermented *P. ginseng* than in *P. ginseng* may enhance the anti-diabetic effects (Kim H.-J. et al., 2011). Liu et al. fermented *Gynoacemma pentaphllum* using *Lactobacillus* spp. Y5 and found that the content of anthraquinones, polysaccharides, and cyclic allyl glycosides was higher in fermented *Gynoacemma pentaphllum* than in unfermented *Gynoacemma pentaphllum* ($P < 0.05$); the use of fermented *Gynoacemma pentaphllum* in the treatment of diabetic rats for 8 weeks found that the rats showed a decrease in the level of blood glucose and an increase in body weight ($P < 0.05$) (Liu et al., 2023). Yan et al. investigated the hypoglycemic effects of GeGen QinLian Tangand Fermented GeGen QinLian Tang on high-fat diet and Streptozotocin (STZ)-and streptozotocin-induced diabetic rats

using a combination of non-targeted metabolomics and targeting analysis. The results of the study showed that fermented GeGen QinLian Tang modulated TC, TG, LDL-C, HDL-C, and fasting insulin levels more than unfermented GeGen QinLian Tang, and fermented GeGen QinLian Tang showed a trend towards better recovery in diabetic rats, suggesting that the fermentation technique enhanced the anti-diabetic properties of GeGen QinLian Tang (Yan et al., 2018). *Lactobacillus plantarum*-fermented DangGui BuXue Tang exhibited improved anti-diabetic effects,,such as inhibiting α-glucosidase, demonstrating antioxidant properties through the 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging and T-AOC, and showing anti-glycation capacity in various models (Guo et al., 2020). Li et al. evaluated the hypoglycemic effect of *Lactobacillus shortus* YM 1301-fermented *Polygonatum sibiricum* by analyzing glucolipid metabolism in streptozotocin-induced T2DM rats and a high-fat diet. These findings indicate that fermented *P. sibiricum* demonstrated superior effects on insulin resistance and glycated hemoglobin compared to unfermented *P. sibiricum*. Furthermore, fermented *P. sibiricum* not only boosts AMPK activation, but also increases the ratio of phosphorylated AKT/AKT to mitigate issues with glucose tolerance and insulin resistance (Li C. et al., 2021). Yang et al. investigated the effects of solid-state fermentation products of *Astragalus membranaceus* and *Paecilomyces cicadidae* in diabetic nephropathy (DN) and found that fermented *A. membranaceus* had a significant mitigating effect on mice with diabetic nephropathy (DN), and that fermented *A. membranaceus* significantly reduced urinary proteins, serum creatinine, and blood urea nitrogen in mice with DN. Compared to unfermented *A. membranaceus*, fermented *A. membranaceus* had a better effect on improving renal structure in DN. The results of the *in vitro* experiments indicated that fermented *A. membranaceus* enhanced the autophagy of podocytes, which may delay the onset of DN by inhibiting the PI3K/AKT/mTOR signaling pathway (Yang et al., 2020).

2.2 Immunity and anti-infection

2.2.1 Immune enhancement

Clinical and experimental studies have shown that fermented botanical drugs can modulate immunity through various pathways. Wang et al. fermented *Platycodon grandiflorum* using *Lactobacillus rhamnosus* 217-1 and found a significant increase in polyphenol and flavonoid content in the fermented group compared to the unfermented *P. grandiflorum* ($P < 0.05$), and a significant difference in the scavenging of DPPH free radicals in the fermented group compared with the control group ($P < 0.01$). Treatment of the UC mouse model revealed that fermented *P. grandiflorum* liquid significantly restored dextran sulfate sodium-induced colonic shortening ($P < 0.01$) and significantly increased the mRNA expression of OCLN and TJP1 ($P < 0.01$, $P < 0.05$ vs. Model), suggesting that fermented *P. grandiflorum* may have improved autoimmunity in mice (Wang et al., 2022b). Li et al. examined the impact of fermentation broth (GLFB) on dexamethasone (DEX)-induced immunosuppression in rats using *Lactobacillus acidophilus* and *Bifidobacterium bifidum* for *in vitro* fermentation of aqueous extracts from *Ganoderma lucidum* substrates. These findings indicate that the extract formed through probiotic fermentation modified the composition of the main ganoderic acid components. Furthermore, GLFB notably enhanced immunity and intestinal integrity and rectified intestinal flora dysbiosis in DEX-treated rats (Li Y. et al., 2021). Sun et al. compared the immunomodulatory activity of unfermented Yupingfeng polysaccharides and trans-oligosporic rhizobacteria in weaning Rex rabbits and found that fermented Yupingfeng polysaccharides significantly increased intestinal *Lactobacillus* and Bifidobacterium populations, while reducing the presence of opportunistic pathogens, such as Enterobacteriaceae bacteria and *Streptococcus*. This effect may be attributed to the fact that the fermentation products increased the number of probiotic bacteria involved in colonization antagonism, competition for nutrients, and ultimately enhanced biobarrier function. Thus, inhibition of harmful bacterial proliferation and enhancement of intestinal immunity were effectively achieved (Sun et al., 2016)

2.2.2 Anti-inflammatory

Inflammation is the adaptive response of an organism to infection and tissue damage that restores homeostasis. A successful acute inflammatory response involves the elimination of infectious agents and dissolution and repair. There is emerging evidence that the fermentation of botanical drugs can enhance their anti-inflammatory effects. Yong et al. found that fermentation of *Curcuma longa* by *Lactobacillus* fermentum significantly increased curcumin content by 9.76% and effectively decreased the expression of pro-apoptotic tumor necrosis factor-α and Toll-like receptor-4 in RAW 246.7 cells compared with unfermented *C. longa*. Western blotting analysis further showed that the anti-inflammatory activity of fermented *C. longa* was achieved by inhibiting the c-Jun N-terminal kinase (JNK) signaling pathway, whereas unfermented *C. longa* was not. (JNK) signaling pathway, whereas unfermented *C. longa* did not (Yong et al., 2019). Similarly, another study showed that *A. membranaceus* fermented by *L. plantarum* enhanced its anti-inflammatory properties. Fermentation of *Astragalus membranaceus*resulted in inhibition of lipopolysaccharide (LPS)-induced NO production and downregulation of TNF-α, iNOS, COX-2 and nuclear factor-κB (NF-κB) expression in RAW 264.7 cells (Park et al., 2021). The anti-inflammatory effects of *P. ginseng* fermented with *Lacto. plantarum* KP-4 was more effective than untreated *P. ginseng*. LC–MS/MS analysis showed that the major ginsenosides decreased and the minor ginsenosides Rg3, F2, and Rh1 increased significantly in *P. ginseng* after fermentation, whereas new minor ginsenoside compounds, CK and Rh3, were generated, suggesting that the anti-inflammatory effect of fermented *P. ginseng* was closely related to changes in ginsenosides (Fan et al., 2021). Compared with unfermented *Paeonia lactiflora* extracts, *P. lactiflora* extracts fermented by *L. shortus* 174A significantly increased total phenolic content, decreased intracellular ROS levels, and inhibited NO release, as well as reduced gene expression of the inflammatory cytokines IL-6, TNF-α, and IL-1 (Shakya et al., 2021). Liu et al. used a mouse model of dextran sodium sulfate-induced ulcerative colitis to study the anti-ulcerative effects of fermented and unfermented *Lycium barbarum* juice. The results of this study showed that *L. plantarum*, *Lactobacillus royale* and *S . Streptococcus* spp. Both fermented and unfermented *L. barbarum* juices exhibited positive anti-inflammatory effects. In addition, fermented *L. barbarum* juice

exhibited more significant modulatory effects on serum and colonic inflammatory cytokines and related enzymes, including T-SOD, NO, IL-1β, IL-4 and IL-10, compared to unfermented *L. barbarum* juice (Liu et al., 2021). Oh et al. investigated the effects of a *Morus alba* extract fermented by *L. acidophilus* A4 on intestinal mucositis in a rat model induced by 5-fluorouracil. Results demonstrated that these interventions led to improvements in inflammation by upregulating MUC2 and MUC5AC gene expression, enhancing mucin production, and reducing IL-1β expression and myeloperoxidase levels. Notably, fermented *M. alba* extract exhibited the most significant protective effect compared to the control group (Oh et al., 2017). The production of endotoxins results in increased intestinal permeability, allowing toxins and bacteria to breach the mucosal barrier, triggering the release of inflammatory mediators and causing further harm to the intestine (Di Michele, 2022). Bose et al. evaluated the *in vitro* and *in vivo* protective effects of fermented and unfermented *Coptis chinensis* on LPS-induced rats. The results of the study showed that endotoxin levels were significantly reduced in mice treated with fermented *C. chinensis* compared to the unfermented group ($P < 0.05$), whereas the combination of fermented *C. chinensis* extract and probiotics showed stronger anti-inflammatory activity *in vitro* and was more effective in reducing LPS-induced intestinal permeability (Bose et al., 2012).

2.2.3 Anti-bacterial

Pathogenic bacteria, particularly those related to foodborne illnesses, pose a significant threat to human health owing to their secretion of harmful toxins. The World Health Organization reports that approximately 1.55 billion cases of diarrhea occur worldwide annually, leading to three million deaths in children under 5 years of age, with approximately 70% of these cases attributed to foodborne pathogens (Ju et al., 2019). Studies have shown that Fermented botanical drugs exhibit strong antibacterial properties (Kanklai et al., 2020). A previous study demonstrated that the fermentation of *Portulaca oleracea* exhibited significant antibacterial activity against *Campylobacter* jejuni, a major microorganism that causes diarrheal disease. Among the various strains, *Leuconostoc mesenteroides* KACC 12312 and *lactic acid bacteria* isolated from *P. oleracea* show the highest activity (Bae, 2012). Another study investigated the antibacterial activity of fermented *Hippophae rhamnoide* juice against 10 foodborne pathogens and found that its effectiveness increased after fermentation with *L. plantarum* RM1. An increase in the phenolic content and acidity during fermentation appears to enhance the antimicrobial potential of fermented *H. rhamnoide* juice (El-Sohaimy et al., 2022). *Glycine max* fermented with Rhizopus oligosporus exhibit significant antibacterial activity against Rhizopus oligosporus aureus and *Bacillus subtilis*. This effect may be linked to the synthesis of long-chain polyunsaturated acids and fatty acid antibacterial compounds during fermentation process (Kusumah et al., 2020). *Magnolia officinalis* extracts fermented with Aspergillus niger exhibited enhanced antibacterial activity against a variety of tested strains (*Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Staphylococcus* epidermidis, Propionibacterium acnes, Epidermophyton floccosum, and methicillin-resistant *S. aureus*), with a significant 8-to 20-fold increase compared with unfermented extracts (Wu et al., 2018a).

2.3 Digestive system and microecology

2.3.1 Regulate intestinal flora

Recent studies have demonstrated that fermented botanical drugs can influence the intestine and play a significant role in regulating intestinal flora. Zhao C. et al.(2021) found that *Monascus*-fermented *P. ginseng* could reverse the decrease in species abundance and diversity of the intestinal flora in rats fed a high-fat diet. It has been theorized that this process may elevate the relative abundance of Prevotella while decreasing the relative abundance of Muri, thus enhancing the hydrolysis of ginsenosides and regulating cholesterol levels to improve lipid metabolism disorders. In a murine model of alcoholic liver injury, *P. ginseng* fermented with *Lactobacillus fermentum* was found to enhance the growth of beneficial probiotics such as *Lactobacillus* and *Bifidobacterium*, leading to significant improvements in alcohol-induced intestinal permeability and short-chain fatty acid (SCFA) levels. The study also demonstrated that fermented *P. ginseng* positively affected other SCFA-producing bacterial strains, such as Allobacterium, Ruminococcus, and *Streptococcus*, while reducing the abundance of bacteria linked to inflammatory conditions, ultimately ameliorating intestinal disorders and alleviating inflammation (Fan et al., 2019). Total flavonoids, total triterpenes, and related short-chain fatty acids were significantly higher in *H. rhamnoide* fermentation liquid. The study indicated that *H. rhamnoide* fermentation liquid effectively improved alcohol-induced liver injury.By measuring gut microbiota in mice feces samples, we found that the high-dose group of SFL reversed the declining trend of the gut microbiota Firmicutes/Bacteroidetes (F/B) ratio caused by alcohol, reducing the number of gram-negative bacteroidetes. These findings suggest that SFL has the potential to prevent alcoholic liver disease and modulate intestinal flora composition (Ran et al., 2021). Zhang et al. examined the metabolic functions of the gut microbiota using PICRUSt analysis of 16S rRNA gene sequences and found that *Laminaria japonica* fermented by *Lactobacillus shortcombicus* FZU0713 affected primary and secondary bile acid biosynthesis in a rat model of hyperlipidaemia. It also significantly affected the expression of certain mRNAs associated with lipid metabolism and bile acid homeostasis, including BSEP, CYP7A1, LDLR, HMGCR, CD36, and SREBP1-C. These findings suggest that the microbial fermentation of *L. japonica* has the potential to modulate body health and ameliorate metabolic disorders by regulating the gut flora (Zhang et al., 2021). Duan et al. (2024) found that *L. barbarum* juice fermented with *Lactobacillus paracasei* E10, *L. plantarum* M, and *L. rhamnosus* LGG enhanced intestinal integrity, remodelled the intestinal microbiota by increasing the presence of *Bacteroides* and *Lactobacillus* spp., and altered intestinal microbial metabolites, such as Kyotoferrin, indolactic acid, and N-methyl-serotonin, compared to unfermented *L. barbarum* juice. Correlation analyses showed that E10F, MF, and LGGF increased *Lactobacillus*, indolactic acid, and N-methylserotonin levels, which were positively correlated with reduced inflammation and enhanced hepatic and intestinal functions. This study used ultra-performance liquid chromatography-Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometry (UPLC-Q-Exactive-MS) to identify differential metabolites in *A. membranaceus* fermented by *L. plantarum*, and the results showed that 11 different metabolites, such as raffinose, progesterone, and uridine, might contribute to the fermented *A. membranaceus* ability to alleviate colitis. In addition, fermented *A. membranaceus* alters the structure of the gut microbiota and enriches

Akkermansia and Alistipes, which are positively correlated with short-chain fatty acid production. Compared to mice treated with unfermented *A. membranaceus*, mice treated with fermented *A. membranaceus* showed more pronounced expression of intestinal tight junction and mucus secretory proteins ZO-1, occludin, and MUC2, as well as modulation of apoptosis of intestinal epithelial cells (IECs), which verified the reparative effect of fermented *A. membranaceus* on the intestinal mucosal barrier. Mice supplemented with fermented *A. membranaceus* showed a more pronounced expression of intestinal tight junction proteins and mucus secretory proteins ZO-1, occludin, and MUC2, while apoptosis of intestinal epithelial cells (IECs) was also regulated, which verified the reparative effect of fermented *A. membranaceus* on the intestinal mucosal barrier (Li et al., 2022). Lee et al.(2017) incorporated *Lactobacillus bulgaricus*, *Streptococcus thermophilus*, and *L. acidophilus* into the fermentation process of *M. alba* extracts. These findings indicate that fermented *M. alba* extract leads to a decrease in intestinal transit time and fecal particles in the colon, while also increasing the presence of *lactobacilli* in the stool, thus potentially aiding in the prevention of constipation. Guo et al. (2022) found that a Sijunzi decoction fermented by *L. plantarum* significantly increased the expression levels of AQP1, ZO-1, and occludin mRNA in aquaporin (AQP), and enhanced the level of AQP4 mRNA expression in aquaporin (AQP) deficiency induced by sodium sporatricepsin. This restoration of the intestinal epithelium structure and function, repair of membrane permeability and intestinal barrier, and regulation of water inlet and outlet ability effectively treated diarrhea in rats.

2.4 Neuroprotection and anti-tumour therapy

2.4.1 Anti-neurodegenerative disease

Recent studies have shown that fermented botanical drugs exert neuroprotective effects. Kim et al. investigated the potential anti-neurodegenerative properties of yeast-fermented *Ziziphus jujuba* using a rat disease rat induced by amyloid beta25–35. These results demonstrate that fermented *Z. jujuba* has a positive impact on cognitive function and memory by reducing amyloid beta25–35-induced oxidative stress. Notably, fermented *Z. jujuba* exhibited stronger antioxidant effects on malondialdehyde and nitric oxide in tissues and serum than unfermented *Z. jujuba*, which could be attributed to the enhanced presence of bioactive compounds resulting from fermentation (Kim et al., 2021). Fermentation of *P. ginseng* with *L. paracasei* has been demonstrated to improve spatial memory deficits caused by cerebral ischemia and β-amyloid injection, as well as protect hippocampal neurons from apoptotic death in rats (Nagao et al., 2019). Mei et al. found that Shuan-Tong-Ling fermented by *Lactobacillus*, *Bacillus acetate*, and *Saccharomyces* inhibited neuronal inflammation and apoptosis through the activation of the SIRT1 signaling pathway, which is beneficial for the treatment of cerebral ischemia/reperfusion injury (Mei et al., 2017). A recent study found that fermented *Zingiber officinale* extracts exhibited greater neuroprotective properties than unfermented *Z. officinale* extracts. This was attributed to the metabolic transformation of *Z. officinale* extracts by Aspergillus niger, resulting in the conversion of side chain α,β-unsaturated ketones to 6-paradol and other related metabolites. The increased bioavailability of 6-paradol was identified as a key factor in the enhanced neuroprotective effects (Park et al., 2016).

2.4.2 Anti-tumor

With the increasing research on fermented botanical drugs, it has been found that Botanical components treated with specific microorganisms have also shown significant effects in anti-tumor. Yim et al. found that fermentation of So-Cheong-Ryong-Tang (CY) by *L. fermentum* altered the composition of CY and enhanced the anticancer properties of the fermented CY preparation (FCY). In a xenograft assay, FCY significantly inhibited the tumor growth of subcutaneously injected cancer cells compared to unfermented CY. These findings suggest that the anticancer efficacy of FCY can be enhanced by modifying its active ingredients via *Lactobacillus* fermentation (Yim et al., 2015). Melanoma is one of the most serious malignant epidermal cancers worldwide. *Glycine max* freeze-dried extract and *G. max* water extract were obtained from soybeans fermented with Natto and *B. subtilis* and were evaluated as potential antimelanoma agents. Cytotoxicity experiments demonstrated that *G. max* freeze-dried extrac and *G. max* water extract exhibit notable antimelanoma properties by inhibiting the AMPK signaling pathway, thereby inducing oxidative stress in cancer cells and ultimately triggering apoptosis (Chou et al., 2021).

2.5 Anti-aging and beauty

2.5.1 Anti-oxidant

Research shows that Microbial fermentation plays a crucial role in enhancing the antioxidant capacities of botanical drugs. Furthermore, fermentation can boost the antioxidant activity of plant extracts by increasing the levels of phytochemicals, particularly polyphenols, antioxidant polysaccharides, and antioxidant peptides generated through microbial hydrolysis or biotransformation (Zhao Y.-S. et al., 2021). Li et al. fermented *L. barbarum* juice using food-derived bacterial strains, including *B. subtilis*, *Bacillus licheniformis*, *Lactobacillus reuteri*, and a mixed strain of *L. rhamnosus* and *L. plantarum*. The results showed that the fermentation of *L. barbarum* juice affected the conversion of free and bound forms of phenolic acids and flavonoids and increased their antioxidant capacity (Liu et al., 2019). Another study demonstrated that fermenting pumpkin juice with five species of *Lactobacillus spp*. resulted in abundant organic acids from *Lactobacillus casei paracasei*, whereas *L. plantarum*, *L. acidophilus*, and *Lactobacillus swissii* exhibited strong DPPH and hydroxyl radical scavenging abilities. These abilities are positively correlated with the content of vanillic acid and erucic acid (Sun et al., 2022). Khan et al. *Dimocarpus longan* underwent fermentation with selected strains of lactic acid bacteria (*L. plantarum subsp. Plantarum and L. mesenteroides*). The fermentation process was shown to increase the ferric reducing antioxidant power. Furthermore, fermentation leads to a decrease in the levels of free amino acids responsible for the bitter taste, while increasing the presence of amino acids with antioxidant properties (Khan et al., 2018). It was found that *H. rhamnoide* juice fermented by *L. plantarum* increased 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'azobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) scavenging activity, which was positively correlated with the increase in phenolic compounds (El-Sohaimy et al., 2022). This aligns with the results of Tkacz et al. (2020), who showed that fermentation

leads to the biotransformation of flavonols in *H. rhamnoide* juice, resulting in enhanced antioxidant activity (Tkacz et al., 2020). In addition, studies have indicated that *L. spp*. enhance the antioxidant properties of fermented *Perilla frutescens* seeds. During fermentation, *lactic acid bacteria* release antioxidant phenolics from the cellular matrix of *P. frutescens* seeds through secondary metabolic pathways or extracellular enzymatic action. These phenolics can neutralize free radicals either through hydrogen atoms or direct electron transfer (Kawee-Ai and Seesuriyachan, 2019). Fang et al. examined the impact of *C. lacryma-jobi* fermented by *L. reuteri* on H2O2-induced oxidative stress. The results showed that the fermented group exhibited significant activation of the PI3K/AKT signaling pathway, reduction in intracellular ROS levels, upregulation of the COL-I gene, and a notable decrease in MMP-1 expression, indicating an antioxidant effect (Fang et al., 2022). Furthermore, *A. membranaceus* fermented by *Lactobacillus* MG5125, *B. bifidum* MG731, and *Lactobacillus* MG741 reduced hepatic aspartate aminotransferase, alanine aminotransferase, and lipid peroxidation in the tBHP-injected mouse model. The fermentation of *A. membranaceus* leads to enhanced isoflavone sapogenins through glycoside hydrolysis, thereby boosting the antioxidant activity of *A. membranaceus* (Lee and Kang, 2022).

2.5.2 Beauty

Cosmetics and pharmaceutical industries are seeking new products or enhancements to existing products that contain innovative active ingredients. Botanical products and their active constituents remain consistently popular with consumers, with fermented botanical drug products being particularly popular in Asian countries and gaining more attention in global markets. Lee et al. (2022) mixed root extracts of *Taraxacum mogolicum*, *Arctium lappa*, *Anemarrhena asphodeloides*, *Pueraria lobata*, and *Nelumbo nucifera* fermented with *Saccharomyces cerevisiae* to promote the proliferation and migration of human keratinocytes (HEKa) and fibroblasts (HDF), exhibiting anti-aging properties and potential as active cosmetic ingredients. Fermented *Angelica sinensis* has been shown to enhance extracellular matrix damage induced by UVB irradiation by increasing the production and release of procollagen type-1, while reducing the expression of MMP-1 and elastase in HaCaT (human keratinocyte) or Hs68 (human foreskin fibroblast) skin cells (Kim et al., 2010). Ho et al. performed *in vitro* and *in vivo* experiments on FB-ChiBai (consisting of extracts of *A. macrocephala*, *P. lactiflora*, *Bletilla striata*, *Poria cocos*, *Dictamnus dasycarpus*, *Ampelopsis japonica* and *Tribulus terrestris*), a mixture fermented by *L. rhamnosus*. It was found that FB-ChiBai inhibited melanogenesis in α-melanocyte-stimulating hormone-induced B16F0 mouse melanoma cells without cytotoxicity in the *in vitro* assay. *In vivo*, FB-ChiBai inhibits melanogenesis by inhibiting the CREB/MITF/tyrosinase signaling pathway. These results suggested that FB-ChiBai has the potential to protect against UV-B radiation (Ho et al., 2021). The anti-tyrosinase and anti-wrinkle activities of *Citrus aurantium* flowers fermented by *Lactobacillus brevis* were found to be 5.2-fold and 4.29-fold higher, respectively, than those of the unfermented group. Furthermore, the fermented extract of *L. brevis* exhibits significant inhibitory activity against wrinkle-related enzymes activities (Chen et al., 2022a). Cui et al. (2022) found that fermented *L. japonica* offers skin-protective benefits. The use of *Bacillus siamensis* in the fermentation process of *L. japonica* has been shown to improve the release of its bioactive compounds and exhibit positive effects such as tyrosinase inhibition, skin repair, and anti-wrinkle properties. In a separate study, *B. subtilis* natto-fermented R. *astragali* was found to have skincare benefits. This fermented product notably increased hyaluronic acid production in primary human epidermal keratinocytes and human dermal fibroblasts. It also increased the expression of hyaluronate synthase 3 and hyaluronate synthase 2 mRNA in HaCaT cells and human fibroblasts compared to that in the non-fermented group. These positive effects on the skin can be attributed to isoflavone glycosides or other metabolites formed from the major isoflavones during fermentation (Hsu and Chiang, 2009). A recent study demonstrated that fermentation by *L. brevis* enhanced the anti-wrinkle and whitening effects of *P. ginseng* and reduced its toxicological potential. Higher concentrations of uronic acid, polyphenols, flavonoids, and antioxidant properties were observed in fermented *P. ginseng* compared to non-fermented *P. ginseng*. In addition, fermented *P. ginseng* exhibited stronger tyrosinase and elastase inhibitory effects than *P. ginseng*. Furthermore, fermentation increases the levels of ginsenoside metabolites including Rg3, Rg5, Rk1, compound K, Rh1, F2, and Rg2 (Lee et al., 2012). Cha et al. demonstrated that inhibiting tyrosinase activity and melanin synthesis using a 0.3% (w/v) optimal dose of fermented *Aloe barbadensis* extract was more effective than arbutin and aloesin, common commercial skin-lightening ingredients. Furthermore, fermented *A. barbadensis* extract significantly reduced the expression of microphthalmia-associated transcription factor (MITF), tyrosinase-related protein-1 (TYRP-1), TYRP-2, and tyrosinase (TYR) genes, suggesting a mechanism for inhibiting melanogenesis in the MITF/TYRP-1/TYRP2/TYR pathway. Additionally, a combination of fermented *Glycyrrhiza glabra*, *Broussonetia papyrifera*, *A. sinensis*, *A. macrocephala*, *P. cocos*, *M. alba*, and *Paeonia labiflora* (2% each) with Phellinus linteus exhibited antimelanogenic activity against B16F0 mouse melanoma cells tested in culture (Cha et al., 2012). The study used *L. plantarum*, *L. rhamnosus*, *L. casei*, *Lactobacillus gattii*, and other *Lactobacillus* strains to ferment the *Lepidium meyenii* extract, and found that the secretion of the inflammatory mediator nitric oxide was considerably lower in the fermented than in the unfermented extracts in the RAW264.7 cells, suggesting an effective anti-inflammatory action of the fermented *L. meyenii* and the inhibition of tyrosinase activity, melanin synthesis and melanogenesis (Yang et al., 2023a).

3 Application advantages of fermented botanical drugs

Fermentation has become one of the most important methods for processing botanical drugs (Li et al., 2020). Fermentation improves the solubility and bioavailability of botanical drugs, reduces their toxicity of botanical drugs, and improves their safety and efficacy of botanical drugs. The interaction between microorganisms and botanical drugs in the fermentation process can also produce new active ingredients, and the fermentation method applied to dregs of botanical drugs can also realize the multi-level utilization of traditional botanical drug resources. The advantages of applying fermentation technology to traditional botanical drugs are shown in Figure 2.

3.1 Reduced toxicity

Several studies have found that fermentation alters or breaks down the toxic components of botanical drugs and reduces their

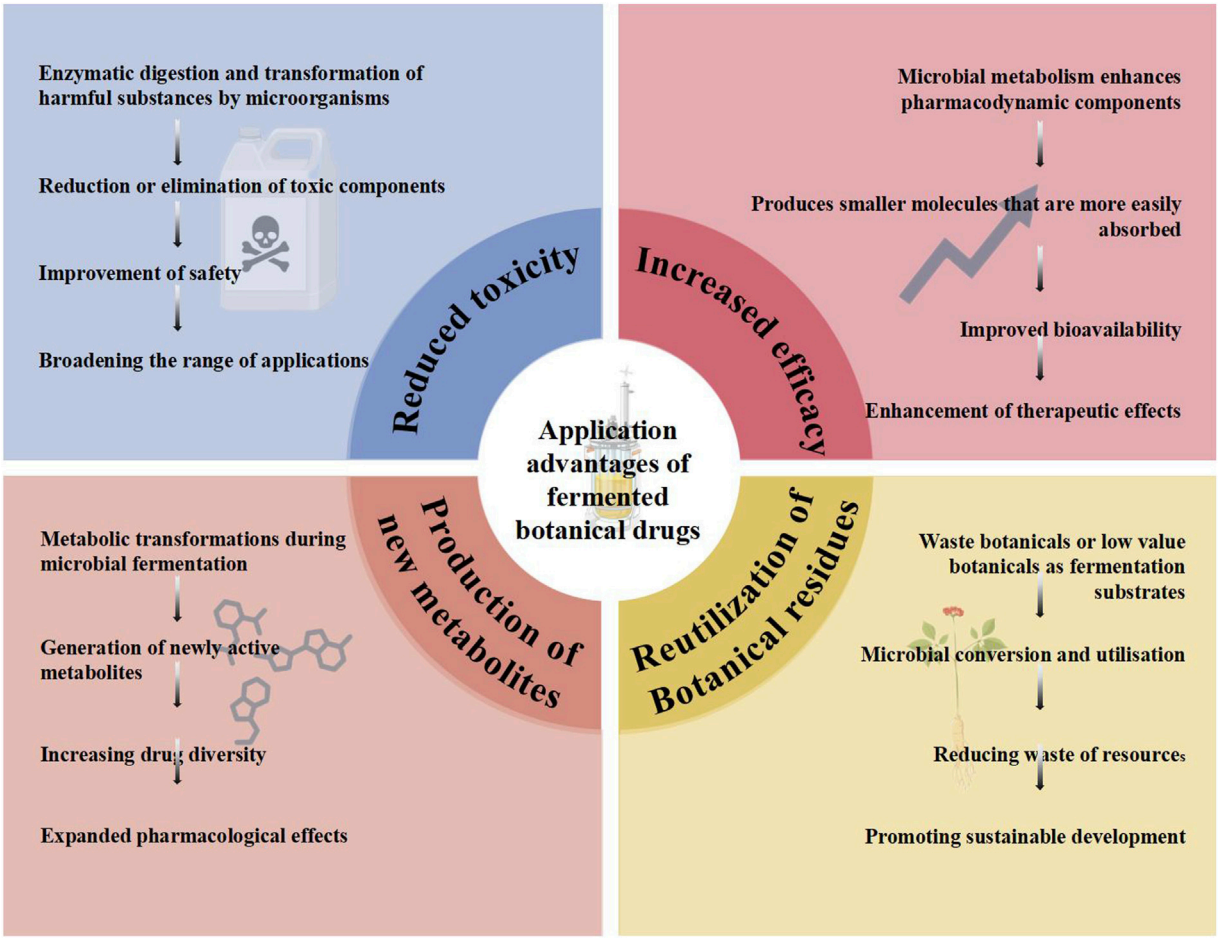

FIGURE 2
Application advantages of fermented botanical drugs.

cytotoxicity compared to non-fermented botanical drugs. Botanical drugs often contain toxic macromolecular substances. In addition, when they enter an organism, they produce irritants that can cause toxicity or other side effects. Fermentation can reduce the adverse effects and toxicity of botanical drugs containing cytotoxic compounds such as heavy metals, toxic glycosides, and toxic proteins (Li J. et al., 2021; Hussain et al., 2016; Li et al., 2022). Aconitine is a potential preventive and therapeutic agent for various diseases; however, it carries the risk of ventricular tachyarrhythmias and cardiac arrest, which can be fatal (Zhou et al., 2021). Therefore, fermentation with probiotics can effectively alleviate this problem. Aconitine is the main ingredient in Huafeng Dan Yaomu and is used to treat hemiplegia, epilepsy, and facial paralysis. The production of Huafeng Dan Yaomu involves fermentation. Cao et al. (2020) investigated the changes in toxic alkaloids during the fermentation process using high performance liquid chromatography (HPLC) and analyzed the changes in the microbial community during the fermentation process using the Illumina MiSeq platform and found that the contents of the toxic alkaloids aconite, neoaconitine, and hypoaconitine decreased during the fermentation process; at the same time, the lower toxicity of benzoylneaconitine and benzoyl hypaconitine, which are less toxic. *Aristolochia debilis*, a traditional Chinese herb used for blood pressure reduction and pain relief, is known for its anti-diarrheic properties. However, due to the nephropathy associated with *aristolochic* acids, botanical drugs have been prohibited for clinical use in China by the State Food and Drug Administration (Yang et al., 2012). Nevertheless, a study on fermenting *A. debilis* with six different medicinal fungi and analyzing the products of aristolochic acids using HPLC-ESI-TOF-MS demonstrated a significant decrease in the content of aristolochic acids after fermentation (Liu et al., 2018). Guo et al. found that ginkgolic acid was significantly reduced to safe levels using *Ginkgo biloba* fermented with *Bacillus subtilis* natto (No. 1A752). The study suggested that the reduction in ginkgolic acid may be due to enzymes secreted by the probiotic bacteria degrading ginkgolic acid or metabolites produced by the probiotic bacteria (e.g., proteins or amino acids), altering the structure of ginkgolic acid (Guo et al., 2018). The removal of toxic ingredients from botanical drugs may lead to a loss of efficacy or even survival due to toxicity, ultimately failing to achieve the intended therapeutic outcome. Fermentation plays a crucial role in efficiently transforming the toxic components of these medicines, generating new chemical compounds that are highly effective and have low toxicity. This process enables the dosage of toxic botanical drugs to be controlled within safe limits, without compromising their therapeutic benefits.

Such fermentation-based approach distinguishes itself from traditional preparation methods and forms the theoretical foundation for the concept of 'removing the poison and preserving the effect' in toxic botanical drugs.

3.2 Increased efficacy

Most active ingredients of botanical drugs are contained within the cell walls. These dense and hard cell walls act as barriers, preventing the active ingredients from easily leaching out and being absorbed by the body. Microorganisms play a crucial role in the extraction of active ingredients. They target different cell wall components, secrete various extracellular enzymes, and break down the tight structure of the cell. This process enlarges the gaps between the cells, allowing for better diffusion of substances in and out of the cells. Ultimately, this not only enhances the extraction rate of active ingredients but also improves their absorption and utilization. Traditional Chinese botanical drugs are usually administered orally, which results in low bioavailability of the active ingredients. However, during microbial fermentation, extracellular enzymes such as cellulase and pectinase produced by microorganisms enter the culture medium, causing the botanical cells to rupture and exposing the active ingredients. Additionally, fermentation is known to improve the absorption and bioavailability of botanical extracts by aiding in the production or conversion of active components into metabolites, or by producing low-molecular-weight substances such as aglycones from glycosides (Joo et al., 2009). Previous studies have shown that microorganism-mediated fermentation of botanical drugs can synthesize important microbial and vegetative secondary metabolites that degrade macromolecular organic substances into small active compounds and increase their therapeutic effects (Stanton et al., 2005; Hussain et al., 2016; Su et al., 2022). Studies have shown that fermented medicinal botanical drugs have stronger anti-ovarian cancer, antioxidant, and neuroprotective effects than unfermented botanical drugs.(Huang et al., 2017; Fu et al., 2014; Tan et al., 2016). Oxalic acid has physiological benefits for human health; however, high concentrations of oxalic acid, an anti-nutritional factor, can bind to essential minerals in the body to form insoluble oxalates, leading to limited mineral absorption and hyperoxaluria (Gomathi et al., 2014; Shi et al., 2018). Filannino et al. used different 8 strains of *Lactobacillus* isolated from fruits, vegetables, and the honeybee intestinal tract as single fermenters, and each strain was selected based on specific technical and functional traits. The determination of organic acids by high performance liquid chromatography (HPLC) revealed that the oxalic acid content in *P. oleracea* fermented by *Lactobacillus kunkeii B7* and *L. plantarum POM1* was reduced (approximately 30%), which promoted the absorption of nutrients in *P. oleracea* (Filannino et al., 2017). Recent research has shown a direct correlation between the oral administration of probiotics and reduced urinary oxalate excretion in both rats and humans. This indicates that probiotic fermentation could be a promising approach to reduce the antinutritional factors of botanical drugs, making them more suitable for human consumption (Abratt and Reid, 2010; Mehra et al., 2022). Nan et al. explored the biotransformation of ginsenosides by *L. fermentum* KP-3 in a high-fat diet-fed mouse model. The results showed that The ginsenoside content increased from 0.746 mg g-1 to 0.939 mg g-1 after fermentation, and the fermentation of *P. ginseng* by *L. fermentum* KP-3 significantly reduced serum TC and LDL levels and inhibited the sharp increase in hepatic alanine aminotransferase (ALT) and alanine oxaloacetate aminotransferase (AST) levels compared to the unfermented group. The significant decrease in the ginsenoside Rb1 content during fermentation was attributed to the conversion of Rb1 to the minor ginsenosides Rg3 and F2 via fermentation, as confirmed by TLC analysis. Ginsenoside Rg3 can be produced directly by eliminating two glucose units at the C-20 position of ginsenoside Rb1, whereas ginsenoside F2 can be derived from ginsenoside Rb1 by removing one glucose unit at the C-3 and C-20 positions of ginsenoside Rb1 (Nan et al., 2018). Major ginsenosides can be converted into deglycosylated and minor ginsenosides, with the general belief that these converted ginsenosides exhibit enhanced bioavailability and more potent pharmacological activity (Bae et al., 2011; Kim S. H. et al., 2011). Solid-state fermentation of *Salvia miltiorrhiza*, *P. grandiflorum*, *Huperzia serrata*, and *G. glabra* using *Aspergillus oryzae* NCH 42, an extracellular ellagitannase-producing fungus, for 5 days at 30°C revealed that extraction of phenolic substances from all four phytomedicines was significantly increased by fermentation, and that the antioxidant and bacteriostatic activities of the extracts were enhanced (Wen et al., 2013). During fermentation of botanical drugs, specific glycosides are converted into smaller hydrophobic molecules. This transformation enhances the effectiveness of original botanical drugs by improving the absorption and bioavailability of their active components in the body. As discussed above, botanical drugs are rich in chemical components, and microbial fermentation resulting in the biotransformation of botanical drugs can generate new metabolites, which involve an interaction between microorganisms and botanical metabolites that ultimately boost the activity and enhance the efficacy of the medicines.

3.3 Production of new metabolites

Fermentation of botanical drugs, a method of changing their composition of botanical drugs, can increase the active ingredients in MFH or produce new active metabolites through the metabolic activity of microorganisms. Probiotic bacteria can synthesize precursor compounds from the active ingredients of botanical drugs, interact with secondary metabolites of microorganisms to form new metabolites, and affect the metabolism of probiotic bacteria to produce novel metabolites. Some active ingredients are found in small amounts in plants, and the need for large quantities of valuable botanical drugs is extremely inconvenient. Direct extraction and isolation of ingredients from medicinal plants is limited by many factors, such as slow plant growth and climate change. Studies have reported that fermented botanical drugs can increase the active components of single botanical drugs, an effect that has great economic potential. Fermentation improves the pharmacological properties of botanical drugs mainly through the modification of naturally occurring molecules such as isoflavones, saponins, phytosterols, and phenols that exert beneficial health-promoting and disease-

preventing effects, in keeping with the "theory of the oriental medicine." In recent years, with the rapid progress in microbial fermentation technologies and in-depth research on the modernization of botanical drugs, microbial fermentation and the transformation of botanical drugs have gained considerable interest and have emerged as new approaches to produce novel active metabolites with potent medicinal value (Wu et al., 2013). The fermentation of medicinal botanical drugs is a decomposition process carried out using microorganisms such as bacteria and fungi. This process is regarded as a valuable biocatalytic technique for producing novel, active, and less toxic bioactive products that may be difficult to obtain using biological systems or chemical synthesis. Shakya et al. fermented *P. lactiflora* extract from *Lactobacillus shortcombii* 174A significantly increases gallic acid content. Additionally, a newly generated bioactive metabolite from fermentation was identified as pyrogallol, demonstrating its ability to inhibit inflammatory responses (Shakya et al., 2021). Many bacterial and yeast strains are used in the fermentation of botanical drugs. *Bacillus subtilis* and *S. cerevisiae* are the two most commonly used strains. The fermentation of *Panax notoginseng* with *Bacillus subtilis* produces a new active substance (ginsenoside RH4), and the fermentation of *A. membranaceus* with *Bacillus subtilis* results in a much higher polysaccharide content and an increased immune-enhancing effect. It has been proposed that bacterial fermentation not only generates bioactive metabolites of flavonoids but also alters their structure. This process includes the deglycosylation, sulfation, or methylation of flavonoids, ultimately influencing their absorption rate and metabolism in the liver (Scalbert and Williamson, 2000). Bacterial fermentation-induced alterations in flavonoid structures can enhance absorption rates and overall absorption levels. This may lead to an increased bioactivity and bioavailability of the active ingredients (Hendrich, 2002), potentially resulting in beneficial effects on bone health (Scholz-Ahrens et al., 2007). Oh et al. found that compared to unfermented WuYao ShunQi San (a traditional Botanical formula consisting of 12 botanical drugs), WuYao ShunQi San fermented by *Lactobacillus* was effective in inhibiting the production of pro-inflammatory mediators such as NO, prostaglandin E2, TNF-a and IL-6, as well as their respective synthase-inducible nitric oxide synthase and cyclooxygenase-2, thus exhibiting potent anti-inflammatory effects. This study showed that fermentation leads to the production of five pro-inflammatory mediators: Nitric Oxide Synthase and Cyclooxygenase-2. The study showed that fermentation led to an increase in the concentration of five unknown compounds, whereas the glycyrrhizin content in the fermented WuYao ShunQi San decreased dramatically to undetectable levels (Oh et al., 2012). Sheih et al. found that fermentation of *A. membranaceus* using *Aspergillus* spp. resulted in a significant increase in the phenolic content of *A. membranaceus*. Fermented *A. membranaceus* showed stronger antioxidant activity against various free radicals than unfermented *A. membranaceus*. In addition, a potent novel phenolic antioxidant, 3,4-bis(4′-hydroxyphenyl) isobutyric acid, with a molecular weight of 272, was isolated from the methanolic extract of fermented *A. membranaceus* (Sheih et al., 2011). Additionally, a notable increase in the concentration of saponin-D, flavonoids, and polyphenols was observed during the fermentation of *Platycodon grandiflorus* root using *L. rhamnosus* 217-1 (Wang et al., 2022b).

3.4 Reutilization of botanical residues

After the extraction of botanical drugs, leftover dregs are often disposed of by dumping, leading to environmental pollution and underutilization of the active ingredients. The increasing production of botanical drugs owing to their growing prevalence of botanical drugs globally poses a significant challenge. Improper handling not only wastes resources, but also contributes to environmental pollution. Botanical drug fermentation has emerged as a promising solution for converting these dregs into valuable resource materials, offering great potential for further development and utilization. Kong et al. selected two endophytic actinomycetes and three endophytic fungi to determine their potential ability to reuse Huazhenghuisheng oral-liquid (HOL) residues. HPLC analyses showed that all endophytic bacteria could produce metabolites from HOL residues, with the most abundant metabolites produced by *Aspergillus cristatus* CB10002. Further studies showed that *A. cristatus* CB10002 could reuse the composite HOL residue. Nine anthraquinone compounds with medicinal value were produced (Kong et al., 2019). As by-products of extraction with water or ethanol, botanical drug residues still contain approximately 30%–50% of the medicinally active substances (Meng et al., 2017). The fermentation of botanical drugs not only enhances potency and detoxification but also utilizes leftover medicinal dregs as a culture substrate. This approach not only addresses potential environmental pollution issues caused by dregs, but also maximizes the proteins and sugars present in them, thereby reducing production costs and optimizing herb resource utilization. Natural environmental factors and human intervention have decreased the production and quality of several commonly used botanical drugs. To maximize the utilization of traditional botanical drug resources, it is important to minimize resource loss. In the case of precious and endangered botanical drugs, identifying and utilizing suitable alternatives can help conserve herbal resources and protect the ecological balance, promoting the sustainable utilization of botanical drug resources.

4 Common strains of botanical drugs fermentation

Microbial fermentation of botanical drugs has a rich historical background and has recently gained significant attention in modernization research on fermented botanical drugs. The key to successful fermentation technology for botanical drugs lies in the careful selection and cultivation of high-quality strains. The types and quantities of microbial secondary metabolites are closely linked to the strains used. Additionally, enzyme systems or secondary metabolites produced by exotic bacterial strains can interact with the intestinal flora of the human body and other

related targets, thereby regulating various body functions and providing therapeutic effects. Therefore, the selection of strains with high yields, efficient conversion, and minimal adverse reactions is crucial for the fermentation of botanical drugs. When selecting microorganisms for the fermentation of botanical drugs, the characteristics of botanical drugs, purpose of fermentation, genetic background and growth characteristics of microorganisms, and actual production conditions should first be considered comprehensively to ensure that the fermentation effect and efficacy of botanical drugs are maximized.

Currently, the microorganisms predominantly utilized in the fermentation of botanical drugs fall into two main categories: fungi and bacteria. Fungi exhibit a notable capacity to break down the active compounds present in botanical drugs under straightforward culture conditions and minimal environmental requirements. Compared with fungal fermentation, bacterial fermentation is characterized by its diversity, rich metabolite content, simple structure, environmental sensitivity, and ease of enhancement. At present, the common microorganisms are *Bacillus* species, such as *Bacillus subtilis*, which have strong resistance and vitality, are able to survive in extreme environments, and produce a variety of enzymes and active substances, which can help the conversion and extraction of botanical drug ingredients; *Lactobacillus*: lactobacilli are often used in botanical drug fermentation to improve the taste and efficacy of botanical drugs, and at the same time, can regulate the balance of the intestinal flora to improve the body's yeasts, such as brewer's yeast, which have a faster growth rate and higher metabolic activity, which can promote the release and transformation of active ingredients in botanical drugs. Moulds: *A. niger*, Aspergillus oryzae, and others Moulds can produce a variety of enzymes and secondary metabolites during the fermentation of botanical drugs, which can facilitate the decomposition and transformation of botanical drug ingredients and improve their efficacy.

5 Influencing factors in the fermentation process of botanical drugs

Botanical drugs is a complex and delicate process, and its effect is jointly influenced by many factors. Firstly, temperature is one of the key parameters in the fermentation process, most microorganisms grow best at around 37°C. However, in the actual fermentation process, the optimal temperature may vary depending on the strain of bacteria and the raw materials of the botanical drugs. Second, the fermentation time should be determined according to the specific growth characteristics of the selected strains; some strains may be more sensitive to specific fermentation times, and a short fermentation time may lead to the failure of full growth and metabolism of the strains, while an excessively long fermentation time may cause aging of the strains or the production of undesirable metabolites. Table 1 lists the effects of temperature and time on the fermentation effect during the fermentation of botanical drugs. In practice, it is necessary to optimize fermentation temperature and time through experimental exploration to obtain the best fermentation effect and product quality.

6 Limitations and risks of fermented botanical drugs

While exploring the promising applications of fermented botanical drugs, it is important not to overlook their inherent limitations and potential risks, as this is crucial to ensure the efficiency of the fermentation process and the stability of the product quality and safety.

6.1 Limitations of fermented botanical drugs

The fermentation of botanical drugs may be limited by the limited resources of strains for use in the fermentation process. Moreover, the stability and activity of the microorganisms are susceptible to environmental parameters, such as temperature, humidity, and pH value, which directly lead to uncertainty about the fermentation effect and the fluctuation of the quality of the product. Although modern fermentation technology has been able to realize a certain degree of parameter monitoring and regulation, it is still a difficult task to completely eliminate the uncertainty in the fermentation process, because it is difficult to accurately control the parameters in the fermentation process, which may affect the quality and stability of the final fermentation products. In addition, the complexity of the composition of botanical drugs seriously affects the quality of the fermented products. Not all components can be effectively utilized or transformed during the fermentation process, and some of the active ingredients may be lost due to inappropriate fermentation conditions. Meanwhile, the newly produced components may have unknown pharmacological effects or safety issues. Currently, the fermented botanical drug industry also faces problems of low process standardization and the lack of unified quality standards and testing methods, which directly increases the difficulty with product quality control. The fermentation process also requires a long period of time and incurs a high cost, due to strain cultivation and fermentation equipment and raw material procurement. In addition, energy consumption during the fermentation process and waste disposal are issues that need to be considered.

6.2 Risks of fermented botanicals

The fermentation of botanical drugs carries risks that should not be overlooked. Some botanical drugs themselves contain toxic components, and the fermentation process may produce new toxic components or increase the levels of the original toxic components, which may pose a potential threat to human health and require rigorous safety assessments. In addition, new components produced during the fermentation process may trigger allergic reactions in humans; therefore, adequate allergy tests and risk assessments are required before their use. Meanwhile, fermented botanical drugs may interact with other drugs, affecting their efficacy or increasing the risk of adverse reactions. Therefore, special attention needs to be paid to drug compounding contraindications when combining drugs. Owing to differences between individual patients and different disease states, there may be uncertainty in the efficacy of fermented botanical

TABLE 1 Fermentation conditions of selected botanicals.

Botanical drug	Fermentation strains	Temperature and time	Chemical component	Pharmacological effects	References
Coix lacryma-jobi	*Lactobacillus plantarum* NCU137	37°C for 36 h	Free amino acids, free fatty acids ↑ 2-pentylfuran ↓	Reduced toxicity	Yin et al. (2020)
Gynoacemma pentaphllum	*Lactobacillus* sp. Y5	35°C for 6 days	Anthraquinones, polysaccharides, cyclic allyl glycosides ↑	Anti-diabetic	Liu et al. (2023)
Platycodon grandiflorum	*Lactobacillus rhamnosus* 217-1	37°C for 24 h	Polyphenols ↑ flavonoids ↑	Immune enhancement, anti-inflammatory	Wang et al. (2022b)
Momordica charantia	*Lactobacillus plantarum*	37°C for 48 h	Propionic acid, butyric acid, acetic acid, short-chain fatty acids ↑	Anti-diabetic	Gao et al. (2019)
Astragalus membranaceus	*Lactobacillus plantarum*	36°C for 36 h	Raffinose, progesterone, uridine ↑	Regulation of the intestinal flora	Li et al. (2022)
Panax ginseng	*Lactobacillus brevis*	37°C for 2 days	Uronic acids, polyphenols, flavonoids ↑	Anti-oxidant	Lee et al. (2012)
Astragalus membranaceus	*Lactobacillus plantarum*	37°C for 15 h	Nitric oxide, hydrogen peroxide ↓	Anti-inflammatory	Park et al. (2021)
Panax ginseng	*Lacto. plantarum* KP-4	37°C for 18 days	Ginsenosides Rg3, F2, Rh1 ↑	Anti-inflammatory	Fan et al. (2021)
Paeonia lactiflora	*Lactobacillus shortus* 174A	37°C for 48 h	Total phenolic content, gallic acid content ↑	Anti-inflammatory	Shakya et al. (2021)
Morus alba	*Lactobacillus acidophilus* A4	37°C for 18 h	Mucin production ↑ myeloperoxidase levels ↓	Anti-inflammatory	Oh et al. (2017)
Coptis chinensis	*Lactobacillus mesenteroides*	35.4°C for 24 h	Endotoxin levels ↓	Anti-inflammatory	Bose et al. (2012)
Panax ginseng	*Lactobacillus*	40°C for 12 h	Ginsenoside Rh2 content ↑	Anti-diabetic	Kim et al. (2011a)
GeGen QinLian Tang	*Saccharomyces cerevisiae*	28°C for 48 h	Flavone aglycones, puerarin ↑	Anti-diabetic	Yan et al. (2018)
DangGui BuXue Tang	*Lactobacillus plantarum*	37°C for 24 h	A-glucosidase ↓	Anti-diabetic	Guo et al. (2020)
WuYao ShunQi San	*Lactobacillus*	37°C for 48 h	—	Anti-inflammatory	Oh et al. (2012)

drugs. Therefore, adequate efficacy assessments and monitoring are required for clinical applications.

7 Conclusion and prospects

With continuous and in-depth study of botanical drug fermentation, this technology is expected to add value to botanical drugs as a novel approach. Through fermentation, the active ingredients of botanical drugs can be transformed and concentrated, the content of active substances increases, and new active ingredients can be produced, thus broadening the clinical application of botanical drugs. For example, polysaccharides and saponins in certain botanical drugs that have undergone fermentation, such as *A. membranaceus* and *A. sinensis*, which are fermented using specific strains of microorganisms, are converted into new substances with greater anti-tumor activity. These new substances can inhibit tumor cell growth. The components of botanical drugs obtained through fermentation techniques, such as certain flavonoids, have shown powerful effects in the inhibition of inflammatory responses.

The potential toxicity of medicines can be reduced, making botanical drugs safer and more effective, and the amount of medicinal materials can be reduced, enabling the multi-level use of botanical drugs. Fermentation technology can transform and modify complex chemical components in botanical drugs through the metabolic activities of microorganisms and act on the toxic components of botanical drugs to change their structure, thus reducing their toxicity. Simultaneously, the interactions between microorganisms and certain components of traditional botanical drugs may produce new substances with improved pharmacological effects and lower toxicity. Fermentation technology can also improve the taste and stability of botanical drugs to increase their safety and allow them to be easily accepted by patients and used for a long time.

In addition, the fermentation of traditional botanical drugs can also expand the scope of application of traditional botanical drugs, so that they can play a greater role in the fields of beauty, healthcare, etc., Botanical drugs are rich in active ingredients that are beneficial to the skin such as polyphenols and flavonoids, which are often found in plant forms and are unfavorable for direct skin absorption. Through fermentation, microbial metabolism can convert these active ingredients into forms that are more easily absorbed by the skin. For example, the antioxidant capacity of certain botanical drugs is significantly enhanced after fermentation, which can effectively resist damage caused by free radicals to the

TABLE 2 Botanical names and medicinal parts.

Scientific name of the drug	Family	Medicinal part	References
Aloe barbadensis Miller	Asphodelaceae	Herba	Chinese Pharmacopoeia Commission. (2020)
Ampelopsis japonica (Thunb.) Makino	Vitaceae	Radix	Chinese Pharmacopoeia Commission. (2020)
Anemarrhena asphodeloides Bge	Asparagaceae	Rhizoma	Chinese Pharmacopoeia Commission. (2020)
Angelica sinensis (Oliv.) Diels	Apiaceae	Radix	Chinese Pharmacopoeia Commission. (2020)
Arctium lappa L	Asteraceae	Fructus	Chinese Pharmacopoeia Commission. (2020)
Aristolochia debilis Sieb.et Zucc	Aristolochiaceae	Fructus	Chinese Pharmacopoeia Commission. (2020)
Astragalus membranaceus (Fisch.) Bge	Fabaceae	Radix	Chinese Pharmacopoeia Commission. (2020)
Atractylodes macrocephala Koidz	Asteraceae	Rhizoma	Chinese Pharmacopoeia Commission. (2020)
Bletilla striata (Thunb.) Reichb.f	Orchidaceae	Rhizoma	Chinese Pharmacopoeia Commission. (2020)
Broussonetia papyrifera (L.) Vent	Moraceae	Fructus	Chinese Pharmacopoeia Commission. (2020)
Citrus aurantium L	Rutaceae	Fructus	Chinese Pharmacopoeia Commission. (2020)
Coix lacryma-jobi L.var.ma-yuen (Roman.) Stapf	Poaceae	Semen	Chinese Pharmacopoeia Commission. (2020)
Coptis chinensis Franch	Ranunculaceae	Rhizoma	Chinese Pharmacopoeia Commission. (2020)
Curcuma longa L	Zingiberaceae	Rhizoma	Chinese Pharmacopoeia Commission. (2020)
Dictamnus dasycarpus Turcz	Rutaceae	Cortex	Chinese Pharmacopoeia Commission. (2020)
Dimocarpus longan Lour	Sapindaceae	Fructus	Chinese Pharmacopoeia Commission. (2020)
Ganoderma lucidum (Leyss.ex Fr.) Karst	—	Fructus	Chinese Pharmacopoeia Commission. (2020)
Ginkgo biloba L	Ginkgoaceae	Folium	Chinese Pharmacopoeia Commission. (2020)
Glycine max (L.) Merr	Fabaceae	Semen	Chinese Pharmacopoeia Commission. (2020)
Glycyrrhiza glabra L	Fabaceae	Radix et rhizoma	Chinese Pharmacopoeia Commission. (2020)
Gynoacemma pentaphllum (Thunb) Mak	Rubiaceae	Herba	Chinese Pharmacopoeia Commission. (2020)
Hippophae rhamnoides L	Elaeagnaceae	Fructus	Chinese Pharmacopoeia Commission. (2020)
Huperzia serrata (Thunb.) Trevis	Lycopodiaceae	Cortex	Kozikowski and Tückmantel. (1999)
Laminaria japonica Aresch	—	Thallus	Chinese Pharmacopoeia Commission. (2020)
Lycium barbarum L	Solanaceae	Fructus	Chinese Pharmacopoeia Commission. (2020)
Lepidium meyenii Walp	Brassicaceae	Rhizoma	Toledo (1998)
Magnolia officinalis Rehd.et Wils	Magnoliaceae	Cortex	Chinese Pharmacopoeia Commission. (2020)
Momordica charantia L	Cucurbitaceae	Fructus	Raman and Lau. (1996)
Morus alba L	Moraceae	Ramulus	Chinese Pharmacopoeia Commission. (2020)
Nelumbo nucifera Gaertn	Nelumbonaceae	Semen	Chinese Pharmacopoeia Commission. (2020)
Paeonia lactiflora Pall	Paeoniaceae	Radix	Chinese Pharmacopoeia Commission. (2020)
Panax ginseng C.A.Mey	Araliaceae	Radix et rhizoma	Chinese Pharmacopoeia Commission. (2020)
Panax notoginseng (Burk.) F.H.Chen	Araliaceae	Radix et rhizoma	Chinese Pharmacopoeia Commission. (2020)
Perilla frutescens (L.) Britt	Lamiaceae	Fructus	Chinese Pharmacopoeia Commission. (2020)
Platycodon grandiflorum (Jacq.) ADC.	Campanulaceae	Radix	Chinese Pharmacopoeia Commission. (2020)
Polygonatum sibiricum Red	Asparagaceae	Rhizoma	Chinese Pharmacopoeia Commission. (2020)
Poria cocos (Schw.) Wolf	—	Sclerotium	Chinese Pharmacopoeia Commission. (2020)
Portulaca oleracea L	Portulacaceae	Herba	Chinese Pharmacopoeia Commission. (2020)

(Continued on following page)

TABLE 2 (*Continued*) Botanical names and medicinal parts.

Scientific name of the drug	Family	Medicinal part	References
Pueraria lobata (Willd.) Ohwi	Fabaceae	Radix	Chinese Pharmacopoeia Commission. (2020)
Rosa roxburghii Tratt	Rosaceae	Flos	Wang et al. (2021)
Salvia miltiorrhiza Bge	Lamiaceae	Rhizome	Chinese Pharmacopoeia Commission. (2020)
Taraxacum mogolicum Hand.-Mazz	Asteraceae	Herba	Chinese Pharmacopoeia Commission. (2020)
Tribulus terrestris L	Zygophyllaceae	Fructus	Chinese Pharmacopoeia Commission. (2020)
Zingiber officinale Rosc	Zingiberaceae	Rhizome	Chinese Pharmacopoeia Commission. (2020)
Ziziphus jujuba Mill.var.spinosa (Bunge) Hu ex H.F.Chou	Rhamnaceae	Semen	Chinese Pharmacopoeia Commission. (2020)

skin and slow skin aging. Fermented botanical drugs can also improve the intestinal microecological environment, promote the value-added of beneficial bacteria, and improve intestinal levels, thereby enhancing overall immunity.

It is important to note that most of the current research on fermented botanical drugs remains focused on simple pharmacological function demonstration, and the exact mechanism of the health benefits of these fermentation products has not yet been elucidated; therefore, there is still a lack of systematic mechanism research and clinical trials. Most studies on fermented botanical drugs have limitations, such as insufficient standardization of experimental design, backwardness of fermentation process detection, control, and technology, unknown mechanism of interaction between microorganisms and botanical drugs, imperfect evaluation system of pharmacological efficacy, and uneven quality of the literature. To promote in-depth research on the fermentation of botanical drugs, it is necessary to improve and innovate against these limitations in the future.

To summarize, the research progress of botanical drug fermentation technology not only enriches botanical drug preparation but also provides strong support for the improvement of the pharmacological effects of botanical drugs and the expansion of their applications. In the future, as botanical drug fermentation technology will continue to improve and innovate, its role in the modernization and internationalization of botanical drugs will be more prominent. For example, genomics, transcriptomics, metabolomics, proteomics, and other multi-omics analysis techniques can be used to study the biological mechanisms involved in the fermentation process of traditional botanical drugs and provide a scientific basis for optimizing the fermentation technology. The rapid development of intelligent and automated technology has brought new prospects for research on botanical drug fermentation. Through the integration of artificial intelligence, machine learning, and other technologies, the fermentation process of botanical drugs can be accurately regulated, fermentation conditions improved, and fermentation efficiency enhanced. Fermentation technology can also be used to produce novel botanical drugs with unique medicinal and biological properties. Fermentation technology can be used to develop functional foods and botanical drug health products by combining yeast and probiotics, which are beneficial to health. By improving the efficacy and reducing the toxicity of botanical drugs through fermentation technology, botanical drugs can become more competitive in the global pharmaceutical market and provide safer and more effective treatment options for patients worldwide, thereby making a greater contribution to human health. All botanical drugs name and family were validated using http://www.plantsoftheworldonline.org and Chinese Pharmacopoeia as shown in Table 2.

Author contributions

XL: Writing–original draft, Writing–review and editing. MD: Writing–review and editing. JL: Writing–review and editing. NG: Supervision, Writing–review and editing. JL: Supervision, Writing–review and editing. YS: Funding acquisition, Resources, Supervision, Writing–review and editing. YY: 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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Ethnobotanical survey and scientific validation of liver-healing plants in northeastern Morocco

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Introduction: Liver diseases represent a significant global health challenge, with primary causes including excessive alcohol consumption, infections, chemotherapy, and autoimmune disorders. Medicinal plants, due to their natural bioactive compounds, hold promise for developing effective treatments and preventive measures against liver ailments. This study aimed to document the use of herbal remedies in northeastern Morocco for liver diseases and correlate these uses with scientific evidence through a bibliometric analysis.

Methods: An ethnobotanical survey was conducted in remote communities of northeastern Morocco from October 2020 to January 2022. A total of 189 informants were interviewed using semi-structured questionnaires to gather information on local medicinal plants used for liver ailments. The data were analyzed using four ethnobotanical quantitative indices: use value (UV), familial use value (FUV), informant consensus factor (ICF), and fidelity level (FL). Additionally, a bibliometric analysis was performed to evaluate the scientific support for the ethnopharmacological uses documented.

Results: The survey identified 45 plant species from 26 different families used in the treatment of liver diseases. The most frequently utilized species were *Cuminum cyminum* L. (UV = 0.1065), *Allium sativum* L. (UV = 0.1015), *Salvia officinalis* L. (UV = 0.0761), *Asparagus officinalis* L. (UV = 0.0558), and *Ziziphus lotus* (L.) Lam. (UV = 0.0457). The Apiaceae family showed the highest familial use value (FUV = 0.1066), followed by Alliaceae (FUV = 0.1015). Liver congestion had the highest informant consensus factor (ICF = 0.83), followed by hepatic colic (ICF = 0.80). Bibliometric analysis revealed that 61% of the plants identified had documented pharmacological effects related to liver health.

Discussion: The study demonstrates that traditional knowledge in northeastern Morocco encompasses a rich diversity of medicinal plants used to treat liver diseases. The high ICF values indicate a strong consensus among informants on the efficacy of these remedies. The correlation between ethnopharmacological use and scientific validation for a significant portion of these plants suggests their potential as reliable

therapeutic agents for liver conditions. However, further scientific investigations are necessary to confirm their efficacy and safety in clinical settings. This research contributes valuable information for future studies on the therapeutic potential of these plants.

Conclusion: This ethnobotanical survey provides a comprehensive database of medicinal plants used in northeastern Morocco for liver diseases. The findings highlight the potential of these plants in developing novel treatments for hepatic conditions, although further research is essential to substantiate their therapeutic claims.

KEYWORDS

ethnobotany, ethnopharmacology, traditional medicine, medicinal plants, liver diseases

1 Introduction

The liver is one of the most critical organs in the human body, playing a pivotal role in several physiological functions, including the regulation of metabolic processes, maintenance of blood sugar levels, bile production, and detoxification of foods, water, drugs, and xenobiotics (Marcellin and Kutala, 2018). These functions are vital for sustaining overall health, as the liver processes everything that enters the body, ensuring that nutrients are metabolized correctly and harmful substances are neutralized. Given its extensive involvement in maintaining homeostasis, the liver is susceptible to various diseases, which can manifest as serious clinical syndromes such as jaundice, hepatitis, hepatocarcinoma, and cirrhosis. Because of its essential functions, the liver is often considered a reflection of an individual's overall health (Rinder et al., 2011).

Liver dysfunction is a significant global health problem, with a variety of causes that contribute to its widespread prevalence. These causes include excessive alcohol consumption, infections (notably viral hepatitis), the use of chemotherapeutic agents, exposure to toxic chemicals, and autoimmune disorders (Wei et al., 2022). The impact of liver diseases is profound, with global mortality rates reaching approximately 2 million deaths annually. Of these, 1 million deaths are attributed to complications arising from viral hepatitis and hepatocellular carcinoma, while another 1 million result from cirrhosis (Asrani et al., 2019). The growing burden of liver diseases has underscored the urgent need for effective therapeutic strategies, particularly in regions where access to conventional medical treatments is limited.

Medicinal plants have long been recognized as a valuable source of therapeutic agents, offering potential remedies for a wide array of health conditions, including liver diseases (Bhagawan et al., 2023a). The use of plants in traditional medicine is deeply rooted in human history, with ethnobotanical practices providing insights into natural remedies that have been utilized for centuries (Bhagawan et al., 2022; 2023b; 2024). In Morocco, traditional herbal medicine remains a cornerstone of healthcare, especially in rural and underserved areas. Recent ethnobotanical research indicates that a significant proportion of the Moroccan population—ranging from 60% to 80%—relies on medicinal plants to meet their healthcare needs (Jamila and Mostafa, 2014; Labiad et al., 2020; Alami Merrouni et al., 2021; Fakchich and Elachouri, 2021; Bencheikh et al., 2022; 2023). This reliance is driven by several factors, including the high cost of conventional medications, limited access to adequate healthcare facilities, and socio-economic challenges, particularly in remote and underdeveloped regions (Bencheikh et al., 2021e; Fakchich and Elachouri, 2021).

The cultural heritage of North-Eastern Morocco, like that of other regions in the country, is steeped in a rich tradition of herbal medicine that dates back to the Arab influence in the 7th century. Over centuries, the indigenous population has developed and maintained extensive knowledge of medicinal plants, which forms the foundation of the region's traditional medical system. This knowledge is passed down orally from one generation to the next, ensuring the continuity of these traditional practices. However, this oral transmission is also a source of vulnerability. The absence of formal documentation and the lack of ethnobotanical archives pose significant threats to the preservation of this cultural heritage. As modern influences encroach and younger generations turn to contemporary medicine, there is a real risk that this indigenous medicinal knowledge, along with the phytogenetic resources it depends on, could be lost (Eddouks et al., 2017).

In Morocco, despite the widespread use of traditional medicine, there is a notable gap in ethnobotanical documentation, particularly concerning medicinal plants used for treating liver diseases. This lack of documented evidence limits the potential for scientific validation and integration of these traditional practices into modern healthcare systems. To address this gap, we propose a study aimed at documenting and analyzing the traditional knowledge related to medicinal plants used in rural areas of North-East Morocco for the treatment of liver diseases. The study will also seek to correlate these traditional uses with scientific evidence through a bibliometric review, thereby providing a comprehensive understanding of the therapeutic potential of these plants and contributing to the preservation of Morocco's ethnobotanical heritage.

2 Materials and methods

2.1 Study area

The Eastern region of Morocco, covers 90,130 km^2, or 12% of the country's total size (Figure 1). This region is limited to the West by the provinces of Al Hoceima, Taza, Boulmane, and Errachidia, to the North by the Mediterranean, to the East, and to the South by the Morocco-Algerian border. The population of this region reached 2,314,346 people (6.8% of the total population), with a density of 26 people per square kilometer, according to the national census report issued in 2014 (RGPH, 2014). According to the High Commission for Planning's survey, the dialect of Arabic was spoken here the most frequently, followed by Berber or Tamazight, which is split into two tiny dialects: Tarifit in the north and tachelhit in the south. The territory's southern zone is characterized by the vast Highlands and Sahara, while the mountainous areas of Beni Snassen, Rif, and Horst reach 1800 m, 1,500 m, and 1,100 m, respectively, elevations. The region also has 200 km of Mediterranean coastline. With hot, dry summers and cooler,

FIGURE 1
Geographic location of the study area.

humid winters, the region has a Mediterranean climate zone, with average annual rainfall ranging from 100 mm in the south to 400 mm in the north. Additionally, the area has a number of protected areas and sites of biological and ecological interest, including Al Hoceima National Park, Benisnassen, Jbel Gorougou, Cap des Trois Fourches, Chekhar, Lalla Chafia, and Lalla Mimouna. In fact, these places had already been chosen because of their biological and ecological characteristics as well as their indigenous flora (Fennane, 2004; Fakchich and Elachouri, 2021).

2.2 Ethnobotanical data collection

The collection of ethnobotanical data on liver diseases was conducted between October 2020 and January 2022 across twelve rural communes located in five provinces of northeastern Morocco. Traditional knowledge was randomly selected from twelve stations studied through structured and semi-structured interviews using a questionnaire sheet with 189 local residents and 8 traditional herbalists participated. Verbal informed consent was gained from informants following verbal explanation of the study aims. The established best practice for ethnobotanical investigations, the International Society of Ethnobiology's Code of Ethics, was followed when conducting interviews (International Society of Ethnobiology, 2006). The questionnaire sheet utilized in this study has two sections: the first lists the respondents' demographic information, and the second lists their floristic and ethnic backgrounds.

2.3 Identification of specimens

We were able to transform the common names of plants identified during our ethnobotanical survey into their botanical names using some relevant references (Bellakhdar et al., 1991; Jamila and Mostafa, 2014). Subsequently, plant samples were collected from various vegetation sites across the northeastern region of Morocco. After the harvest, the botanical identification of the samples was carried out in the Laboratory of Bioresources, Biotechnologies, Ethnopharmacology and Health of the Faculty of Sciences of Mohammed first University, Oujda, Morocco, with the help of available herbaria and a number of essential references such as the catalogue of Moroccan plants and the practical flora of Morocco (Jahandiez and Maire, 1931; 1932; 1934; Fennane et al., 1999; 2007; 2014). After the samples were identified, specimens were placed in the Mohammed First University Herbarium in Oujda, Morocco. Using the World Flora Online (WFO) Plants database

(https://wfoplantlist.org/), all scientific names were reviewed once more. Additionally, a group of flowering plants (angiosperms) known as Angiosperm Phylogeny Group III - 2009 has been given credit for naming all plant families (A.P.G III, 2009).

2.4 Quantitative data analysis

To quantify the ethnobotanical information, we adopted a quantitative analysis using ethnobotanical indices such as the Medicinal Use Value (UV), the Family Use Value (FUV), Informant Consensus Factor (ICF), and the Fidelity Level (FL).

- Medicinal use value (UV)

We analyzed the medicinal use value of each plant species to identify the relative relevance of each plant species that is locally recognized to be utilized in herbal treatments. This index is calculated using the formula below (Tabuti et al., 2003):

$$\mathbf{UV} = \frac{\sum \mathbf{U}}{\mathbf{N}}$$

Where;

UV: medicinal use value, **U:** number of citations per species, **N:** number of informants. The UV value will be larger if a plant has a high utilization ratio, indicating that the plant is significant, however if there are few utilization ratios, it will be near to zero.

- Botanical Family Use Value (FUV)

We used the family use value index to analyze the association between botanical families and users of taxa that correspond to these families. This index is equal to the mean total use value of each species in the family (Hoffman and Gallaher, 2007).

$$\mathbf{FUV} = \frac{\sum \mathbf{UV}}{\mathbf{N}}$$

Where;

FUV is the family use value, UV is the utility value of the family's species, and N is the number 173 of species in the family.

- Informant Consensus Factor (ICF).

The ICF demonstrates the uniformity of traditional knowledge exchange amongst informants regarding the usage of plants to cure different types of diseases. The following formula was used to determine ICF (Bencheikh et al., 2021e).

$$\boldsymbol{ICF} = \frac{\mathbf{Nur} - \mathbf{Nt}}{\mathbf{Nur} - \mathbf{1}}$$

Where;

Nur denotes the number of use-reports for an ailment category and Nt denotes the total number of plants used by all informants for that illnesses category. The ICF values range between 0 and 1, with values close to 0 indicating that the herbs were picked at random or that there was no exchange of information about plant usage within the population. Furthermore, ICF values close to 1 indicate a clear selection of medical species and information sharing about their use in the population.

TABLE 1 Number of informants for each locality.

Provinces	Stations	Number of informants	
		Local residents	Herbalist
Guercif	Ras Laksar	10	0
	Saka	11	1
	Jal	16	0
Jerada	Ain Benimathar	18	0
	Guenfouda	10	2
Berkane	Tafoughalt	11	2
	Ahfir	25	0
Nador	Tiztoutine	10	0
	Bouarg	12	1
Oujda-Angad	Bni Drar	31	2
	Naima	19	0
	Sidi Moussa Lemhaya	16	0
Total	12 stations	189	8

- Fidelity Level (FL).

The level of fidelity (FL) identifies a plant species' ability to effectively combat a certain disease. FL was determined using the formula below (Sreekeesoon and Mahomoodally, 2014).

$$\boldsymbol{FL} = \frac{\mathrm{Ip}}{\mathrm{Lu}} * \mathbf{100}$$

Where;

Lu denotes the total number of interviewers who cited all uses of the particular species for the therapies of all liver pathologies, and **Ip** represents the number of individuals who used a particular species for a specific type of liver disease.

2.5 Pharmacological validation

A bibliographic search was conducted to identify the biological activities of identified plants against liver disease, by the mean of the following databases: PubMed, Science Direct, Google Scholar, Scopus and Web of Science with keywords like "liver disease," "liver disease," "Liver failure," "hepatitis," "Jaundice," and "Hepatoprotective" combined with the scientific name of each plant.

3 Results and discussions

3.1 Informants' sociodemographic profile

A total of 197 informants, including 189 non-specialists and 8 herbalists interviewed for this study. These interviewees are spread over twelve rural stations in five provinces of North-East Morocco (Table 1). The socio-demographic profile of the participants in this

TABLE 2 Socio-demographic characteristics of informants.

Distribution	Categories	Informants number	Percentage of informants
			%
By sex (197)	Men	80	40.6
	Women	117	59.4
By age range	Less than 25 years	3	1.52
	25–45	50	25.38
	46–65	116	58.88
	More than 65 years	28	14.21
By education level	Illitirate	75	38.07
	Primary education	69	35.03
	Secondary education	38	19.29
	University education	15	7.61
By income/month	Unemployed	81	41.11
	500–2000 DH	89	45.17
	2000–6000 DH	12	6.1
	>6000 DH	15	7.61
By choice of medicine	Herbal medicine	101	51.27
	Both conventional and herbal medicine	76	38.58
	Modern medicine	20	10.15

study (The variable comprising age, sex, education level, income and attitude towards drugs) were grouped in the Table 2. Analysis of the data presented in Table 2 shows that Women had the highest share of participants (59%), followed by men (40.6%). The use of medicinal plants for the treatment of liver disease in the study areas is widespread in all age groups. The 46–65 age group is the most represented in this study with a frequency of 58.88%, followed by the 25–45 age group with a percentage of 25.38%, the over-65 age group with 14.21%, and the under-25 age group with a percentage of 1.52%. The results of numerous research have consistently shown that older people had more traditional knowledge on how to use medicinal herbs than did younger people (Alami Merrouni et al., 2021; Bencheikh et al., 2021e; Hachlafi et al., 2022). The discomfort of the younger generation, which tends not to accept popular medicine due to the effect of exotic culture, and the influence of lifestyle modernization can be used to explain the gradual loss of traditional knowledge about medicinal plants (Sargin et al., 2015). The fact that there were fewer informants over the age of 65 (14.21%) is a reflection of the depth of traditional knowledge being lost as rural elders pass away.

In terms of educational attainment, the findings revealed that 38.07% of the informants are illiterate, followed by the categories of secondary and primary education, with percentages, respectively 35.03% and 19.29%, and lastly the university level, with a percentage of 7.61%. These findings are consistent with those of other ethnobotanical studies conducted in various regions of Morocco (Khouchlaa et al., 2017b; Bencheikh et al., 2021e; Hachlafi et al., 2022). The study area's rising illiteracy rate may be caused by the fact that poverty is still pervasive in the rural areas examined. This is indicated in our results, where the majority of respondents had a low socio-economic level (41.11% unemployed, and 45.17% between 500 and 2000 DH/month).

There are differences in how the people in this area feel about treating liver illness. The results shown in Table 2 demonstrate the extreme variety of usage patterns. In fact, the majority of interviews indicated that traditional medicine was their first choice of treatment when they were ill, with a percentage of 51.27%, followed by the use of conventional and herbal medicine in second place, with a percentage of 38.58%, and exclusively modern medicine in third place, with a percentage of 10.15%. Access to modern medication is hampered by a lack of health facilities and trained medical personnel, a lack of infrastructure, particularly paved roads, a lack of transportation options, a lack of logistical support, and the high expense of treating liver disease with modern medicine (El Hassani et al., 2013; Eddouks et al., 2017). The aforementioned factors all strongly encourage rural populations to switch to traditional healthcare, especially the usage of medicinal herbs.

3.2 Diversity of plant species used to treat liver diseases

This study recorded the use of 45 medicinal plants, spread across 26 families and 43 genera, for the treatment of liver disease in the study area. Traditional information on the applications of these plants has been developed (Table 3), including the use value,

TABLE 3 Medicinal plants used in the study area for the treatment of liver ailments.

Botanical family Scientific name (voucher number)	Local name	Therapeutic uses	Part used	Mode of preparation	Mode of administration	UV
ASTERACEAE *Artemisia absinthum* L. (HUMPOM903)	Chiba - lahyat cheikh	Hepatic colic, hepatitis, detoxification, jaundice	Leaves	Decoction	Oral	0.0355
Artemisia herba-alba Asso. (HUMPOM923)	Izri - halfa	Detoxification	Leaves	Decoction	Oral	0.0051
Cynara scolymus L. (HUMPOM924)	Khorchef	Liver diseases, hepatic colic	Stems, flowers	Infusion	Oral	0.0203
Anacyclus pyrethrum (L.) Lag. (HUMPOM925)	Oud alatass	Liver diseases	Stems	Decoction	Oral	0.0051
Reichardia intermedia (Sch.Bip.) Samp. (HUMPOM926)	Nokad	Hepatic colic, liver congestion	Leaves	Decoction	Oral	0.0152
ALLIACEAE *Allium sativum* L. (HUMPOM927)	Thouma	Liver cancer, hepatoprotective effect, liver diseases, hepatitis	Bulb, leaves	Decoction, infusion, in food, maceration	Oral	**0.1015**
ANACARDIACEAE *Pistacia lentiscus* L. (HUMPOM895)	Dro - btam	Liver cancer, liver diseases, hepatic colic	Fruit, leaves	Decoction	Oral	0.0305
APIACEAE *Cuminum cyminum* L. (HUMPOM909)	Kamoun	Detoxification, liver diseases, jaundice, hepatitis	Seed	Decoction, infusion, powder	Oral	**0.1065**
Apium nodiflorum (L.) Lag. (HUMPOM928)	Zyata	Liver diseases, hepatic colic, bile problems	Leaves	Decoction	Oral	0.0254
Pimpinella anisum L. (HUMPOM902)	Habat hlawa - yanssoun	Hepatitis, hepatitis	Leaves, fruit	Decoction, powder, infusion	Oral	0.0152
Coriandrum sativum L. (HUMPOM910)	Kossber	Liver diseases	Leaves	Decoction	Oral	0.0051
APOCYNACEAE *Nerium oleander* L. (HUMPOM901)	Alili, defla	Liver diseases, liver cancer	Leaves	Decoction	Oral	0.0102
ARECACEAE *Cocos nucifera* L. (HUMPOM911)	Noix de coco	Jaundice	Fruit	-	Oral	0.0051
ASPARAGACEAE *Asparagus officinalis* L. (HUMPOM898)	Sekoum	Bile problems, liver stones, hepatitis, jaundice	Stems, leaves	Decoction, infusion, in food	Oral	**0.0558**
COMBRETACEAE *Terminalia arjuna* (Roxb. ex DC.) Wight and Arn. (HUMPOM915)	Aarjouna	Hepatic colic	Leaves	Decoction	Oral	0.0051
FABACEAE *Lupinus albus* L. (HUMPOM929)	Termass	Hepatitis, liver diseases	Fruit, leaves	Decoction, infusion	Oral	0.0254
Ceratonia siliqua L. (HUMPOM931)	Kharoub	Liver diseases, jaundice	Fruit	Maceration, infusion	Oral	0.0102
Glycyrrhiza glabra L. (HUMPOM930)	Arq souss	Liver diseases	Stems	Infusion	Oral	0.0051
PLANTAGINACEAE *Globularia alypum* L. (HUMPOM894)	Tasselgha	Liver diseases, hepatitis	Fruit, leaves, whole plant	Decoction	Oral	0.0203
IRIDACEAE *Crocus sativus* L. (HUMPOM912)	Zaafran lhor	Liver diseases	Flowers	Decoction	Oral	0.0051
LAMIACEAE *Salvia officinalis* L. (HUMPOM904)	Salmiya	Jaundice, liver diseases, hepatitis, liver cancer, detoxification	Leaves, stems	Decoction, infusion	Oral	**0.0761**

(Continued on following page)

TABLE 3 (*Continued*) Medicinal plants used in the study area for the treatment of liver ailments.

Botanical family Scientific name (voucher number)	Local name	Therapeutic uses	Part used	Mode of preparation	Mode of administration	UV
Thymus vulgaris L. (HUMPOM932)	Zaatar	Hepatoprotective effect, liver diseases, hepatic colic	Leaves	Decoction	Oral	0.0355
Ocimum basilicum L. (HUMPOM916)	Rihane - hbek	Liver diseases, jaundice, hepatitis	Leaves	Decoction, infusion	Oral	0.0355
Rosmarinus officinalis L. (HUMPOM919)	Azir - yazir	Hepatitis, detoxification, liver diseases	Leaves	Decoction	Oral	0.0254
Lavandula dentata L. (HUMPOM920)	Khzama	Liver diseases, hepatitis	Flowers, leaves	Decoction, infusion	Oral	0.0203
Mentha pulegium L. (HUMPOM921)	Fliyo	Hepatitis, jaundice	Leaves	Decoction	Oral	0.0152
Mentha spicata L. (HUMPOM913)	Naanaa	Liver diseases	Leaves	Infusion	Oral	0.0051
LAURACEAE *Laurus nobilis* L. (HUMPOM922)	Wrak sidna moussa	Hepatitis	Leaves	Infusion	Oral	0.0051
MALVACEAE *Malva parviflora* L. (HUMPOM905)	Khoubiza	Liver diseases	Leaves	Decoction	Oral	0.0051
MYRTACEAE *Syzygium aromaticum* (L.) Merr. and L.M.Perry (HUMPOM896)	Kronfol	Detoxification, liver diseases	Leaves	Decoction, infusion	Oral	0.0254
Eucalyptus globulus Labill. (HUMPOM933)	Eucalyptus	Liver diseases	Leaves	Oil	Oral	0.0051
OLEACEAE *Olea europaea* L. (HUMPOM908)	Zitoune	Liver diseases	Leaves, fruit	Oil, decoction	Oral	0.0152
PIPERACEAE *Piper nigrum* L. (HUMPOM914)	Flfla kehla	Hepatic colic, liver diseases, liver cancer	Fruit	Decoction, poudre infusion	Oral	0.0355
PLUMBAGINACEAE *Armeria alliacea* (Cav.) Hoffmanns. and Link (HUMPOM899)	Arq wedmi	Liver diseases, jaundice	Fruit, stems	Infusion, decoction	Oral	0.0152
POACEAE *Zea mays* L. (HUMPOM934)	Dorra, kbal	Liver diseases	Cones, fruit	Decoction, infusion	Oral	0.0102
Hordeum vulgare L. (HUMPOM906)	Chaair	Liver diseases	Leaves	Decoction	Oral	0.0051
POLYGONACEAE *Rumex vesicarius* L. (HUMPOM935)	Zriaat lhemida	Detoxification, liver diseases	Seeds, leaves	Infusion, decoction	Oral	0.0203
RANUNCULACEAE *Nigella sativa* L. (HUMPOM917)	Haba kahla	Liver cancer, liver diseases	Seeds	Decoction	Oral	0.0102
RHAMNACEAE *Ziziphus lotus* (L.) Lam. (HUMPOM918)	Nbeg	Jaundice, liver diseases, hepatic colic, hepatitis	Fruit, leaves	In food, maceration, infusion, decoction	Oral	**0.0457**
ROSACEAE *Agrimonia repens* L. (HUMPOM887)	Makerman	Jaundice, liver diseases	Leaves	Decoction	Oral	0.0102
Crataegus monogyna Jacq. (HUMPOM936)	Zaarour	Liver diseases	Leaves	Infusion	Oral	0.0051
RUTACEAE *Citrus × aurantium* L. (HUMPOM937)	Ranj	Liver diseases, liver congestion	Fruit	Decoction, maceration	Oral	0.0254

(Continued on following page)

TABLE 3 (*Continued*) Medicinal plants used in the study area for the treatment of liver ailments.

Botanical family Scientific name (voucher number)	Local name	Therapeutic uses	Part used	Mode of preparation	Mode of administration	UV
THEACEAE *Camellia sinensis* (L.) Kuntze (HUMPOM907)	Atay	Liver diseases	Whole plant	Infusion	Oral	0.0051
ZINGIBERACEAE *Zingiber officinale* Roscoe (HUMPOM938)	Skinjbir	Liver diseases, hepatic colic, jaundice	Whole plant, rhizomes	Decoction, infusion, powder	Oral	0.0254
Curcuma longa L. (HUMPOM939)	Kharkoum	Liver diseases, hepatitis	Whole plant	Powder	Oral	0.0102

Bold indicates the best values.

TABLE 4 Distribution of botanical medicinal families according to species and genera. FUV: Family Use Value.

Family	Number of species	Number of genera	FUV	Family	Number of species	Number of genera	FUV
Lamiaceae	7	6	0.0305	Combretaceae	1	1	0.0051
Asteraceae	5	4	0.0162	Plantaginaceae	1	1	0.0203
Apiaceae	4	4	**0.1066**	Iridaceae	1	1	0.0051
Fabaceae	3	3	0.0135	Lauraceae	1	1	0.0051
Myrtaceae	2	2	0.0152	Malvaceae	1	1	0.0051
Poaceae	2	2	0.0076	Oleaceae	1	1	0.0152
Rosaceae	2	2	0.0076	Piperaceae	1	1	**0.0355**
Zingiberaceae	2	2	0.0178	Plumbaginaceae	1	1	0.0152
Alliaceae	1	1	**0.1015**	Polygonaceae	1	1	0.0203
Anacardiaceae	1	1	**0.0305**	Ranunculaceae	1	1	0.0102
Apocynaceae	1	1	0.0102	Rhamnaceae	1	1	0.0457
Arecaceae	1	1	0.0051	Rutaceae	1	1	0.0254
Asparagaceae	1	1	**0.0558**	Theaceae	1	1	0.0051

Bold indicates the best values.

scientific name, botanical family, popular names, traditional uses, parts utilized, preparation procedure, and mode of administration for each medicinal species.

3.2.1 Frequency of families and their use value

As indicated in Table 4, a total of 26 botanical families were used in rural areas of North-East Morocco for the treatment of liver pathologies. However, the families most used are Lamiaceae (7 species; 6 genera) in the first position, followed by Asteraceae (5 species; 4 genera), Apiaceae (4 species; 4 genera), Fabaceae (3 species; 3 genera), Myrtaceae, Poaceae, Rosaceae and Zingiberaceae with (3 species; 3 genera) for each. There are only one species and one genus for the other families. Similarly, the Lamiaceae, Asteraceae, and Apiaceae botanical families are the ones that are most prevalent in Mediterranean countries (Benítez et al., 2010; Savo et al., 2011). The predominance of the Asteraceae family in the traditional treatment of liver disease has already been confirmed by an ethnobotanical study carried out in the Maritime region of Togo (Kpodar et al., 2016).

Families with high FUV are Apiaceae (0.1066), Alliaceae (0.1015), Asparagaceae (0.0558), and Piperaceae (0.0355) (Table 4). However, there aren't many species in these groups to represent them. It appears that the value of using ethnobotanical families is not dependent on their particular wealth but rather on the significance and value of the use of the individual species (Najem et al., 2019). Additionally, these families' significant FUV would be mostly dependent on their abundance of bioactive compounds, which would confer multiple benefits such as antimicrobial, anti-allergic, anti-oxidant, and anti-inflammatory properties (Bencheikh et al., 2022).

3.2.2 Most used plants species to treat liver diseases according to use value index

In this work, we inventoried 45 different medicinal plants that are utilized to treat liver ailments in rural areas of North Eastern Morocco. Nevertheless, the most widely used plants for the treatment of liver diseases are *Cuminum cyminum* L. (UV = 0.1065), followed by *Allium sativum* L. (UV = 0.1015), *Salvia officinalis* L. (UV = 0.0761), *Asparagus officinalis* L. (UV = 0.0558), and *Ziziphus lotus* (L.)

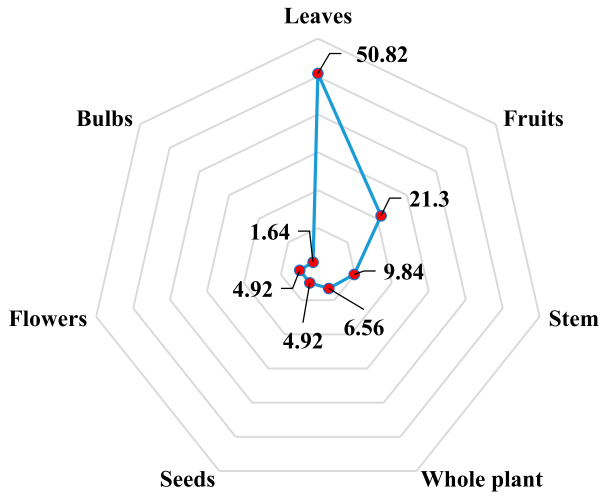

FIGURE 2
Distribution of plant parts by the percentage of use (%).

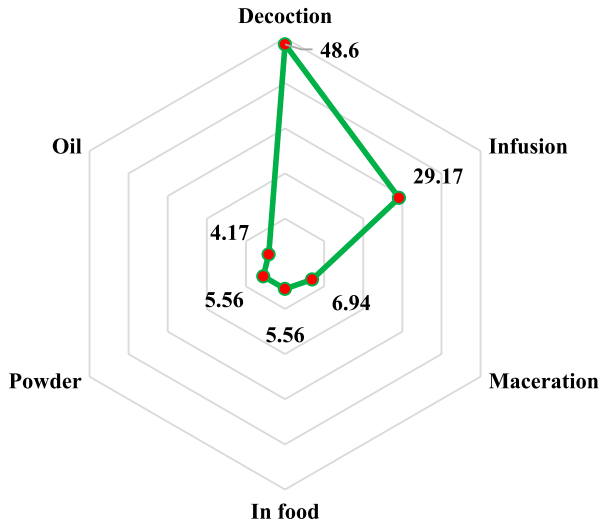

FIGURE 3
Distribution of preparation methods according to percentages of use.

Lam. (UV = 0.0457) (Table 3). These five species made up 27.84% of all use ratios, while the other 40 species only made up 72.16% of all use ratios. Similar studies conducted in other nations have shown that high utilization values have been attained for plants other than those in the current study (Kotoky and Das, 2008; Kpodar et al., 2016). This difference in species similarity could be explained by the difference in bioclimate between countries, which will favor the difference in the abundance of certain plant species from one country to another. In addition, geographic distance between countries has a direct impact on the traditional cultures of indigenous peoples, as evidenced by Alami Merrouni et al. (2021), in which they demonstrated that the increase in distance between countries is accompanied by the increase in the difference in the cultures of these countries and *vice versa*. Thus, all these factors can lead to differences between countries in the use of plant species to treat a particular health condition.

These five medicinal plants were frequently utilized in traditional Moroccan medicine to cure a wide range of illnesses:

Cuminum cyminum L.: This Apiaceae family medicinal plant was one of the first plants grown in Asia, Africa, and Europe (Al-snafi, 2017). Since antiquity, *C. cyminum* seeds have persisted in popularity as culinary seasonings and are widely utilized in folk therapy across a variety of geographic regions. This plant, called in Morocco as "Kammun", is frequently used conventionally to treat digestive system issues, including diarrhea (Jamila and Mostafa, 2014). According to the analysis of the data collected during our investigation, *C. cyminum* is the most widely used to treat liver pathologies in the North-Eastern Moroccan population with a usage value of 0.1065. Indeed, the seeds of *C. cyminum*, in decoction or infusion, are used by the study population as treatment of jaundice, and hepatitis, and thus for liver detoxification. In Ayurveda (former Indian therapeutic system), seeds of *C. cyminum* are traditionally used against jaundice and to improve liver function (Andallu and Ramya, 2007; Johri, 2011).

Allium sativum L.: This plant, called locally as "Thouma" in Morocco, is one of the earliest known to have been cultivated (Thomson and Ali, 2003). Traditional Moroccan medicine makes extensive use of garlic to cure and prevent a wide range of illnesses, including cancer, lung disease, hypertension, diabetes, microbiological infections, infertility, and problems with the kidneys (Fakchich and Elachouri, 2021). According to the results of our investigation, this plant is classified according to its use value in the second position as the plant most used to treat liver diseases in the northeast of Morocco. Indeed, the leaves and bulb of this plant in decoction or infusion are widely recommended by the inhabitants of the study area to fight against liver cancer and hepatitis, and thus declared that it has hepatoprotective effects. Furthermore, it has been reported that portions of this plant are commonly used to heal liver problems in Togo's Maritime region (Kpodar et al., 2016). The bulb of plant is often used to treat jaundice in the southern region of Algeria (Bendaif et al., 2021).

Salvia officinalis L.: This round perennial shrub belongs to the Lamiaceae family and is called to as "Salmiya" in the Oriental area of Morocco. It is indigenous to the Middle East and the Mediterranean, although it has since become naturalized everywhere (Ghorbani and Esmaeilizadeh, 2017). In Morocco, the aerial part of *S. officinalis* is used to handle gastrointestinal problems, metabolic disorders, and renal ailment (Bencheikh et al., 2021e; Fakchich and Elachouri, 2021). Based on the findings of the current investigation, this plant is classified among the three most used medicinal species in the study area for the treatment of liver diseases. Indeed, the leaves and stems of *S. officinalis*, in decoction or infusion are widely used in rural areas of north-eastern Morocco to prevent and treat jaundice, hepatitis, and liver cancer, and thus to detoxify the liver. In the middle Oum Rbia region of Morocco, leaves and whole plant decocted were used for liver problems (Ben Akka et al., 2019). In addition to these local uses, in traditional South-West Algerian medicine, the flowers of this plant were also used to treat liver symptoms (Benarba, 2016).

Asparagus officinalis L.: Since ancient times, asparagus, a perennial herbaceous plant of the Asparagaceae family, has been

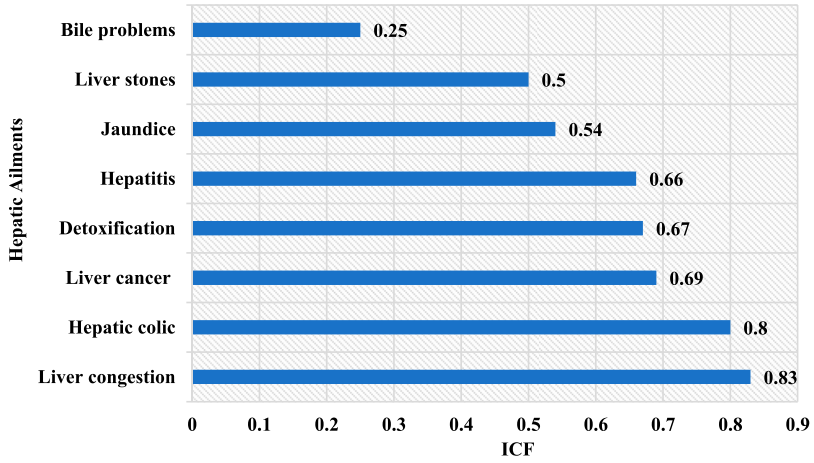

FIGURE 4
Category of hepatic illness and their informant consensus factor (ICF).

utilized extensively in food and medicinal. This plant is called « Sekoum» in Morocco, is used to treat various ailments such as respiratory diseases, digestive problems, kidney diseases, liver diseases and diabetes (Alami Merrouni and Elachouri, 2021; Fakchich and Elachouri, 2021; Bencheikh et al., 2022). In our study, asparagus is ranked fourth among the most cited plants for the treatment of liver patients. In fact, this plant's leaves and stems are frequently used to treat biliary issues, liver stones, hepatitis, and jaundice.

Ziziphus lotus (L.) Lam.: The majority of Africa, numerous Asian nations, including China, Iran, and South Korea, as well as several European nations, including Cyprus, Spain, and Greece, are all home to this medicinal plant (Adeli and Samavati, 2014; Bencheikh et al., 2021d). In Morocco, *Z. lotus* is locally known as "*Sedra*," and "*Nbeg*" for its fruits, and is widely found in arid and semi-arid areas (Bencheikh et al., 2019). Plant parts were traditionally used to combat various health problems such as sedation, anxiety, urinary problems, diabetes, skin infections, scarring, and bronchitis (Khouchlaa et al., 2017a; Bencheikh et al., 2021e; Fakchich and Elachouri, 2021). As per the findings of our survey, *Z. lotus* in rural parts of North-East Morocco, is one of the top five plants used to treat liver disorders such as jaundice, hepatic colic, and hepatitis. Furthermore, the fruits of this medicinal plant are traditionally used to treat lung diseases, jaundice, and as an emollient in El Hammadia, Algeria (Bendaif et al., 2021).

3.3 Ethnic medicinal characteristics

In this study, different parts of plants are used as medicines to treat liver problems in rural areas of North-East Morocco (Figure 2). Thus, on the basis of calculating the percentage of use of each part (%), the leaves (50.82) appear to be the most commonly utilized for the treatment of liver illnesses in the study area, followed by fruits (21.3), stems (9.84), whole plants (6.56), the seeds, and the flowers (4.92) for each, and finally the bulbs (1.64). The leaves are both a source of photochemical reactions and a repository of organic stuff created from them, which explains why they are used so frequently (Bencheikh et al., 2021e). In addition, it is important to avoid pulling out the entire plant or picking up the roots of the plants, as this will promote deforestation and put the species at risk (Kadir et al., 2013). On the contrary, the use of leaves contributes to the conservation and sustainable use of the plant.

As seen in Figure 3, various techniques are used in rural North-East Morocco to make alternative therapies for treating liver disease. Nevertheless, with a percentage of 48.61%, decoction remains the most commonly employed method of preparation, followed by infusion (29.17%), maceration (6.94%), powder and preparation in the diet (5.56% for each), and finally oils with 4.17%. The preparation technique is frequently correlated with the type of use (external or internal); typically, external usage involves the use of a mask, massage, or suppositories, while internal use involves the use of decoction, infusion, maceration, and other techniques (Eddouks et al., 2017). Decoction's supremacy may thus be explained by the fact that it allows for the capture of the greatest amount of bioactive molecules and reduces or eliminates the toxic effects of some recipes (Noureddine et al., 2022).

3.4 Hepatic ailments categories and their informant consensus factor (ICF) values

In this study, we identified eight liver pathologies that were treated with medicinal plants in rural areas of North-East Morocco (Figure 4). The ICF values of the plant species cataloged in this investigation extended from a minimum of 0.25 to a maximum of 0.83. (Figure 5). This index has the highest value for liver congestion (ICF = 0.83), followed by hepatic colic (ICF = 0.80), liver cancer (ICF = 0.69), liver detoxification (ICF = 0.67), hepatitis (ICF = 0.66), jaundice (ICF = 0.54), liver stone (ICF = 0.50), and bile problems with ICF = 0.25. High values (around 1) of this index for liver congestion, hepatic colic, and liver cancer suggest that a small number of species were employed by many informants, reflecting a high level of consensus on the use of plants in the management of these illnesses. The low accord between both interviews was

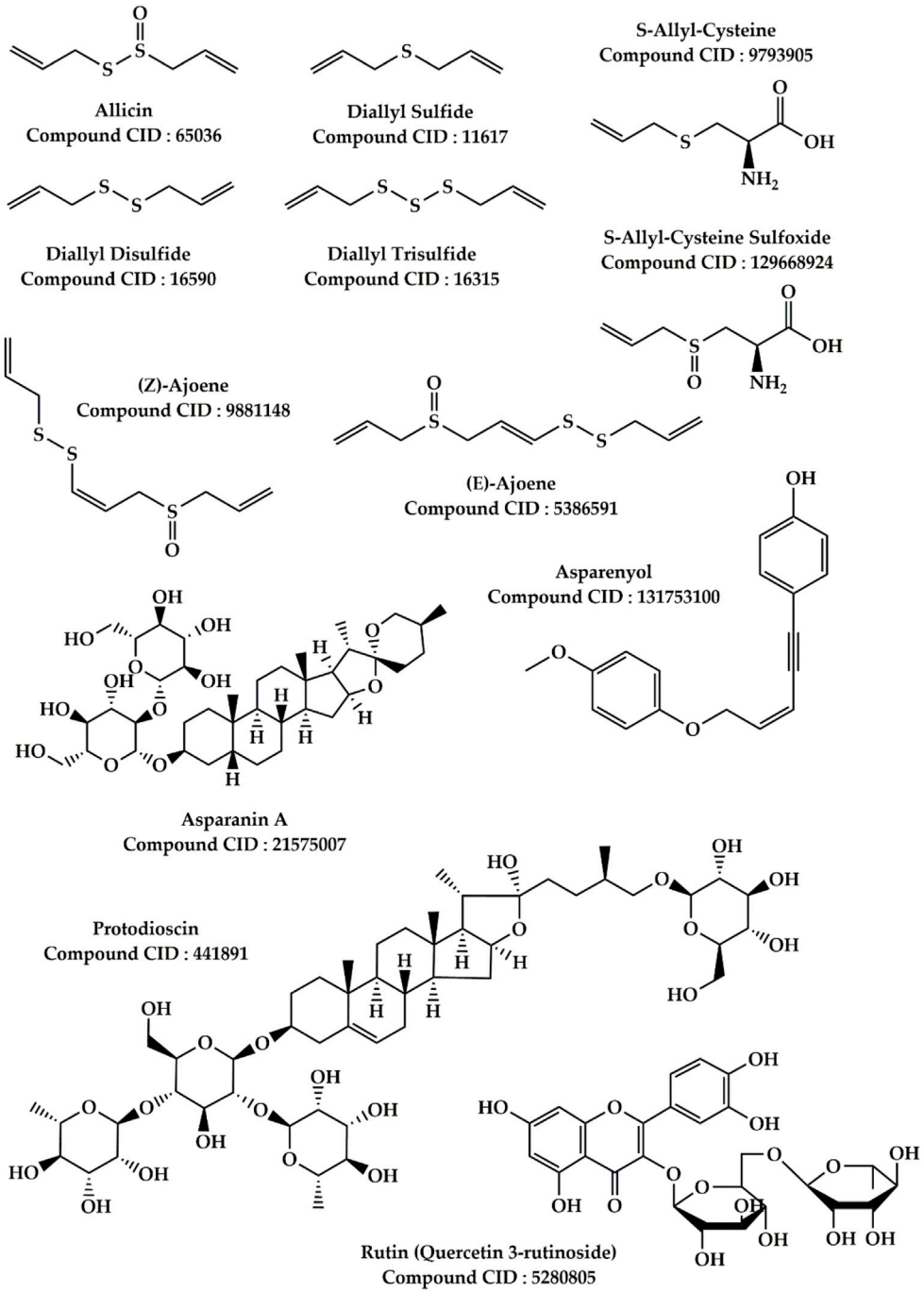

FIGURE 5
Main components found in *Allium sativum* L. and *Asparagus officinalis* L.

witnessed for biliary problems. This could be attributed to a lack of interaction and knowledge exchange among individuals (Al-Qura'n, 2005).

3.5 Fidelity level (FL)

According to the corresponding level of fidelity, we categorized the medicinal plants used to treat liver illness in Table 5. According to our findings, the level of fidelity of plant species for a particular liver condition ranged between 9.09% and 100%. Concerning hepatitis problems, the most important species according to the level of fidelity were *Pimpinella anisum* L. (FL = 100%), *Laurus nobilis* L. (FL = 100%), *Lavandula dentata* L. (FL = 75%), and *Lupinus albus* L. (FL = 66.67%). For the jaundice, *Cocos nucifera* L. (FL = 100%), *Mentha pulegium* L. (FL = 100%), *Z. lotus* (L.) Lam. (FL = 66.67%), and *Agrimonia repens* L. (FL = 66.67%) were the most important. The most widely known species in the hepatic colic

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TABLE 5 Fidelity level values of medicinal plants for each category of liver illness.

Category of illness	Name of species	Fidelity level (FL %)
Hepatitis	*Pimpinella anisum* L	**100.0**
	Laurus nobilis L	**100.0**
	Lavandula dentata L	75.00
	Lupinus albus L	66.67
	Artemisia absinthium L	50.00
	Rosmarinus officinalis L	50.00
	Globularia alypum L	40.00
	Mentha pulegium L	33.33
	Curcuma longa L	33.33
	Cuminum cyminum L	25.00
	Salvia officinalis L	25.00
	Ocimum basilicum L	25.00
	Asparagus officinalis L	16.67
	Allium sativum L	12.50
Jaundice	*Cocos nucifera* L	**100.0**
	Mentha pulegium L	**100.0**
	Ziziphus lotus (L.) Lam	66.67
	Agrimonia repens L	66.67
	Ocimum basilicum L	50.00
	Cuminum cyminum L	37.50
	Armeria alliacea (Cav.) Hoffmanns. and Link	33.33
	Salvia officinalis L	25.00
	Zingiber officinale Roscoe	25.00
	Ceratonia siliqua L	20
	Asparagus officinalis L	16.67
	Artemisia absinthium L	13.64
Hepatic colic	*Terminalia arjuna* (Roxb. ex DC.) Wight and Arn	**100.0**
	Piper nigrum L	60.00
	Reichardia intermedia (Sch.Bip.) Samp	50.00
	Thymus vulgaris L	50.00
	Zingiber officinale Roscoe	50.00
	Cynara scolymus L	37.50
	Pistacia lentiscus L	37.50
	Apium nodiflorum (L.) Lag	37.50
	Artemisia absinthium L	27.27
Detoxification	*Syzygium aromaticum* (L.) Merr. and L.M.Perry	**33.33**
	Rumex vesicarius L	**33.33**
	Artemisia herba-alba Asso	28.57

(Continued on following page)

TABLE 5 (*Continued*) Fidelity level values of medicinal plants for each category of liver illness.

Category of illness	Name of species	Fidelity level (FL %)
	Cuminum cyminum L	25.00
	Rosmarinus officinalis L	25.00
	Artemisia absinthium L	9.09
Liver cancer	*Nerium oleander* L	**66.67**
	Allium sativum L	50.00
	Pistacia lentiscus L	37.50
	Nigella sativa L	33.33
	Salvia officinalis L	25.00
	Piper nigrum L	20.00
Liver congestion	*Citrus* × *aurantium* L	**66.67**
	Reichardia intermedia (Sch.Bip.) Samp	50.00
Liver stones	*Asparagus officinalis* L	**16.67**
Bile problems	*Asparagus officinalis* L	**50.00**

Bold indicates the best values.

group were *Terminalia arjuna* (Roxb. ex DC.) Wight & Arn. (FL = 100%), and *Piper nigrum* L. (FL = 60%). For liver detoxification, plants with the highest FL were *Syzygium aromaticum* (L.) Merr. & L.M. Perry (FL = 33.33%), *Rumex vesicarius* L. (FL = 33.33%), and *Artemisia herba-alba* Asso (FL = 28.57%). *Nerium oleander* L. (FL = 66.67%), and *A. sativum* L. (FL = 50%) were species with the highest fidelity level. For liver congestion, *Citrus* × *aurantium* L. (Fl = 66.67%) was the most important. In the end, *Asparagus officinalis* L. is the most important for liver stone and bile problems. The importance of these plants for the treatment of liver diseases in the study area could be due to their wide use in traditional Moroccan medicine to treat various diseases (Fakchich and Elachouri, 2021).

3.6 Pharmacological confirmation data of the medicinal plants

The current ethnobotanical fieldwork confirmed that the inhabitants of northeastern Morocco has extensive ethnobotanical information concerning the use of herbal remedies in the treatment of liver conditions. These conventional data, which detailed a wide variety of quantitative factors, were particularly intriguing for the goal of bioprospecting to identify novel drugs to cure liver pathological conditions. It could be worthwhile to look up these plants' pharmacological properties in the literature. According to the results of our bibliographic survey, of the 46 plant species registered for the treatment of liver diseases in the study area, 28 plant species from 20 botanical families have already been pharmacologically validated for liver diseases (Table 6). It can be concluded that the majority of them significantly reduce the risk of liver disorders. These findings demonstrated the potential of ethnobotanical knowledge as a preferable traditional database for plant species with beneficial therapeutic effects connected to liver illnesses. The pharmacological data collected for the plants selected during our survey were grouped in the Table 6.

According to the results of our ethnobotanical survey, *C. cyminum* L. (UV = 0.1065), *A. sativum* L. (UV = 0.1015), *S. officinalis* L. (UV = 0.0761), *Asparagus officinalis* L. (UV = 0.0558), and *Z. lotus* (L.) Lam. (UV = 0.0457) are the medical species commonly used in Northeastern Morocco for the treatment or prevention of liver problems. To support the use of these plants in conventional medicine, it may be interesting to further explore and discuss their pharmacological properties related to liver problems. To this goal, we shall explore the pharmacological potential of these herbs in the following paragraphs to validate their benefits against liver diseases:

Allium sativum L. is ranked as the second most used species (UV = 0.1015) for the treatment of liver diseases. According to ethnobotanical findings, this plant is widely used in rural areas of northeastern Morocco for its hepatoprotective effect, against liver cancer, and viral infections (hepatitis). The leaves and bulb of *A. sativum* have demonstrated antioxidant and hepatoprotective effects against ethanol-induced hepatotoxicity in rats (Nencini et al., 2010). Indeed, the administration of an extract of the leaves or bulbs of *A. sativum* At a dose of 250 mg/kg, the Glutathion reductase (GR), catalase (CAT), and superoxide dismutase (SOD) activities were restored, and the levels of malondialdehyde, ascorbic acid, and glutathion were reduced and oxidized in the liver tissue of rats exposed to ethanol (Nencini et al., 2010). A study also discovered that *A. sativum* has a cytoprotective impact in HepG2 cells submitted to mycotoxines, specifically Beauvericin, α-Zearalenol and β-Zearalenol (Juan-García et al., 2021b). The presence of antioxidant compounds, according to the authors, is responsible for this cytoprotective effect, which involves the activation of defensive pathways as an enzymatic defence mechanism from within cells, the control of the cell cycle, and cell death, all of which can be provoked by these mycotoxines (Juan-García et al., 2021b). Another research revealed that an aqueous extract of garlic

TABLE 6 Pharmacological outcomes of medicinal plants cited by locals to cure liver ailments.

Scientifique name	Used parts	Used extracts	Experimental model	Pharmacological uses	Therapeutic doses	Ref.
Allium sativum L	Leaves or bulbs	Aqueous extract	Rats	Hepatoprotective effect against acute ethanol-induced oxidative stress in rat liver	250 mg/kg of b.w (for 5 days)	Nencini et al. (2010)
	Garlic cloves	Ethanol extract	HepG2 cells	Cytoprotection against mycotoxins on HepG2 cells	1% during 24 and 48 h	Juan-García et al. (2021a)
	Bulbs	Aqueous extract	Rats	Protective effect on alloxan-induced elevations of plasma biochemical factors of hepatic functions	100 or 200 mg/kg of b.w/day (for 21 days)	Aprioku and Amah-Tariah (2017a)
Artemisia absinthium L	Aerial parts	Aqueous extract	Mice	Protective effect against CCl_4 and endotoxin-caused liver damage	50–200 mg/kg of b.w (for 7 days)	Amat et al. (2010)
	Aerial parts	Hydroalcoholic extract	Rats	Reduce serum levels of ALT, AST, and oxidative damage in rats to alleviate liver toxicity	100 mg/kg of b.w (for 24 h)	Mohammadian et al. (2016)
	Aerial parts	Ethanol extract and its fractions, petroleum ether, and ethyl acetate	Human hepatoma BEL-7404 cells, and mouse hepatoma H22 cells	Induce apoptosis in Hepatocellular carcinoma cells via the endoplasmic reticulum stress and mitochondrial-dependent pathways to inhibit cell growth	25–150 μg/mL (for 24, 48, and 72 h)	Wei et al. (2019)
	Leaves	Methanol and ethyl acetate extract	Rats	Hepatoprotective effect against diclofenac-induced liver toxicity in rats	50–200 mg/kg of b.w/day (for 5 days)	Antonio et al. (2020)
Asparagus officinalis L	Asparagus spears	Ethanolic and aqueous extracts	Mice	Prevent liver against high-fat diet	200 mg/kg of b.w (for 10 weeks)	Zhu et al. (2010)
	Roots	Hydroalcoholic extract	Rats	Prevent the liver from oxidative stress enhanced by cadmium chloride	100–400 mg/kg of b.w (for 28 days)	Abedi et al. (2018)
Ceratonia siliqua L	Leaves	Aqueous extract	Mouse hepatocellular carcinoma cell line (T1)	Anti-cancer effect against hepatocellular carcinoma cell line	0.2–0.4 mg/mL (for 24 h)	Corsi et al. (2002)
	Leaves	Hydroethanolic and ethyl acetate extracts	Rats	Protective effect against hepatotoxicity caused by CCl_4 in rats	250 mg/kg of b.w (for 8 days)	Hsouna et al. (2011)
	Pods	Aqueous extract	Mice	Improves liver fibrosis caused by *Schistosoma mansoni*	300–600 mg/kg of b.w (daily for 10 days)	Al-Olayan et al. (2016)
	Pods	Aqueous extract	Rats	Hepatoprotective effect against dextran sulfate sodium in rats	50–100 mg/kg of b.w (for 14 days)	Rtibi et al. (2016)
	Seeds	Powder	Rats	Protective effects on ethanol-iduiced hepatotoxicity in rats	15% in diet supplementation	Temiz et al. (2015)
Cocos nucifera L	Inflorescences	Acetone extract	Rats	Protective effect on acetaminophen-caused hepatotoxicity in rats	100–400 mg/kg of b.w (for 14 days)	Chithra et al. (2020)
	Unspecified	Essential oils	Rats	Improved hypothyroidism by the reduction of liver functions	10% in diet (for 6 weeks)	Mohammed et al. (2020)

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TABLE 6 (*Continued*) Pharmacological outcomes of medicinal plants cited by locals to cure liver ailments.

Scientifique name	Used parts	Used extracts	Experimental model	Pharmacological uses	Therapeutic doses	Ref.
Coriandrum sativum L	Fruits	Essential oils	Mice	Hepatoprotective effect against CCl_4-induced liver damage	0.13 g/kg of b.w (during 5 consecutive days)	Samojlik et al. (2010)
	Fruits	Essential oils	Rats	Hepatoprotective effect against liver toxicity-induced by ibuprofen in rats	40 mg/kg of b.w (for fourteen consecutive days)	Baghdadi et al. (2016)
	Leaves and seeds	Coriander sauces	Rabbits	Hepatoprotective effect against CCl_4-induced toxicity in rabbits	15 mL/kg of b.w	Iqbal et al. (2018)
	Areal parts and seeds	Aqueous extract	Rats	Hepatoprotective effect against hepatic injury provoked by lambda-cyhalothrin insecticide	1% (w/w) in died (for 90 days)	Boutlelis et al. (2020)
Crataegus monogyna Jacq	Fruits	Aqueous extract	Rats	Exhibits a protective effect on doxorubicin-induced the liver toxicity	20 mg/kg of b.w (for 28 days)	Shalizar-jalali et al. (2013)
Crocus sativus L	Stigmas	Aqueous extract	Rats	Protective effects against chronic-stress induced oxidative damage liver in rats	30 mg/kg of b.w (daily for 21 days)	Bandegi et al. (2014)
	Flowers	Aqueous extract	Rats	Alleviate methotrexate-induced liver toxicity in rats	80 mg/kg of b.w (for 10 days)	Hoshyar et al. (2019)
	Stigmas	Ethanolic extract	Rats	Protective effect on oxidative damages in aged male rat liver	5–20 mg/kg of b.w (daily for 4-week)	Samarghandian et al. (2016)
	Petals and stigmas	Aqueous and ethanolic extracts	Rabbits	Hepatoprotective effect on amiodarone-provoked liver toxicity in rabbits	100 mg/kg of b.w (for 3 days)	Riaz et al. (2016)
	Stigmas	Ethanolic extract	Rats	Beneficial effect for the liver	0.35 g/kg of b.w (daily for 2 weeks)	Mohajeri et al. (2007)
	Petal	Hydroalcoholic extract	Rats	Anti-inflammatory effect in liver toxicity caused by alcohol consumption	167.5–335 mg/kg of b.w (for 8 weeks)	Azizi et al. (2019)
	Petals	Hydroalcoholic extract	Rats	Hepatoprotective effect against acetaminophen provoked liver toxicity in male rats	10–20 mg/kg of b.w (daily for 6 days)	Omidi et al. (2014)
	Tepals, stigmas and leaves	Hydroethanolic extract	Rats	Protective effects on CCl_4-provoked acute liver toxicity in rats	50 mg/kg of b.w (daily for 14 days)	Ouahhoud et al. (2021a)
Curcuma longa L	Rhizomes	Ethanolic extract	Rats	Prevent on thioacetamide-provoked liver cirrhosis in rats	250–500 mg/kg of b.w (for 8 weeks)	Salama et al. (2013)
	Rhizomes	Ethanolic extract	Chicken	Hepatoprotective effect against aflatoxine-causing liver toxicity	5 mg/kg of b.w (for 28 days)	Gholami-ahangaran et al. (2016)
	Rhizomes	n-Hexane extract	Rats	Protective effect on hepatotoxicity induced by ethanol	200 mg/kg of b.w (for 28 days)	Nwozo et al. (2014)
	Rhizomes	Aqueous extract	Mice	Inhibiting hepatic oxidative stress and inflammatory cytokine secretion in ethanol-induced liver injury	20 mg/kg of b.w (unspecified)	Uchio et al. (2017)
	Rhizomes	Hydro-alcoholic extract	Rats	Inhibiting hepatic oxidative stress in adriamycin-provoked liver toxicity	100 mg/kg of b.w (for 4 weeks)	Article et al. (2024)

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TABLE 6 (*Continued*) Pharmacological outcomes of medicinal plants cited by locals to cure liver ailments.

Scientifique name	Used parts	Used extracts	Experimental model	Pharmacological uses	Therapeutic doses	Ref.
	Unspecified Unspecified		Rats	Protective effect on the liver toxicity provoked by $AlCl_3$	40 mg/kg of b.w (for 8 weeks)	Khouja (2017)
	Rhizomes	Ethyl acetate extract	Rats	Prevent the liver on alcohol-caused hepatotoxicity in rats	100–350 mg/kg of b.w (for 14 days)	Antiya et al. (2021)
Cynara scolymus L	Leaves	Aqueous extract	Rats	Inhibits cholesterol production in hepatocytes	0.1–4.0 mg/mL	Gebhardt (1998)
	Leaves	Ethanol extract	Rats	Prevent liver on CCL_4-caused oxidative stress and liver damage	1.5 g/kg of b.w (for 2 weeks)	Colak et al. (2016)
	Buds	Methanolic extracts	Rat Hepatocytes and on Human Hepatoma Cells	*In vitro* protection against oxidative stress in hepatocytes	400–1,200 µM (for 24, 48, and 72 h)	Miccadei et al. (2008)
	Leaves	Ethanol extract	Rats	Hepatoprotective effect against obesity	200–400 mg/kg of b.w (during 2 months daily)	Salem et al. (2019)
	Receptacle and bracts	Petroleum ether and ethyl acetate extracts	Rats	Reduced liver tissue lesions when damaged by thioacetamide	1.5 g/kg of b.w (daily for 2 months)	El-Mesallamy et al. (2020)
	Roots	Hydro-alcoholic extract	Rats	Prevent liver on CCL_4-provoked hepatotoxicity	300–900 mg/kg of b.w (for 3 days)	Huseini et al. (2011)
	Leaves	Hydroethanolic extract	Rats	Inhibit hepatic oxidative stress in hepatotoxicity induced by diazinon	1,500 mg/kg of b.w (for 15 days)	Ahmadi et al. (2019)
	Leaves	Aliphatic alcohols extract	HepG2 liver cells	Increased the mitochondrial dehydrogenase activities of the human liver HepG2	100 mg/mL for 48 h	Löhr et al. (2009)
	Leaves	Aqueous extract	HepG2 cells	Against genotoxicity of HepG2 cells, and modulate hydrogen peroxide DNA damage	0.62–5.0 mg/mL (for 1 h)	Pereira et al. (2017)
	Leaves	Unspecified	Mice	Prevent the liver against obesity	5% in fied (for 1 month)	Azeem et al. (2016)
Glycyrrhiza glabra L	Roots	Aqueous extract	Rabbits	Protective effect on CCL_4-caused acute liver toxicity	2 g/kg of b.w (for 7 days)	Al-Razzuqi et al. (2012)
	Roots	Aqueous and ethanol extracts	Rats	Protective effect on CCl_4-caused acute liver toxicity	250–500 mg/kg of b.w	Laylani (2016)
	Roots	Ethanolic extract	Rats	Prevent the liver against paracetamol provoked liver acute toxicity	200 mg/kg of b.w (once a day for 7 days)	Tajua et al. (2011)
	Roots	Aqueous extract	Rabbits	Protective effect on CCl_4 induced hepatotoxicity	2 g/kg of b.w (daily for 7 days)	Omar and Omar (2014)
	Roots	Methanolic extract	HepG2	Prevent the HepG2 cell line against H_2O_2	10–100 µg/mL	Shinde et al. (2016)
	Unspecified	Unspecified	Fish hepatocytes (*Cyprinus carpio*)	Hepatoprotective effect against CCl_4-induced hepatocyte damage in common carp	2.5–10 µg/mL (for 4 h)	Yin et al. (2011)
	Roots	Hydromethanolic extract	Mice	Hepatoprotective effect against CCl_4 induced oxidative-stress mediated hepatotoxicity	300 mg/kg of b.w (once a day up to 7days)	Sharma and Agrawal (2014)

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TABLE 6 (*Continued*) Pharmacological outcomes of medicinal plants cited by locals to cure liver ailments.

Scientifique name	Used parts	Used extracts	Experimental model	Pharmacological uses	Therapeutic doses	Ref.
Hordeum vulgare L	Seeds	Unspecified	Rats	Lowering hyperlipidemia and improving liver enzymes and nearly restoring tissues of the liver to their normal structure	10% in died (for 8 weeks)	Abulnaja and Rabey (2015)
	Seeds	Methanolic extract	Rats	Prevent the liver on acetaminophen caused liver toxicity	300–500 mg/kg of b.w (for 3 days)	Pa et al. (2010)
	Seeds	Methanolic extract	Rats	Hepatoprotective effect on ethanol-provoked hepatotoxicity	300–500 mg/kg of b.w (for 18 days)	Shah et al. (2009)
Laurus nobilis L	Leaves	Etahanol extarct	Rats	Protective effect on CCl_4 provoked liver toxicity	0.2 mL per 100 g rat mass	Gasparyan et al. (2015)
	Leaves	Etahanol extarct	Rats	Hepatoprotective on sodium valproate-caused liver damage	150 mg/kg of b.w (for 30 days)	Mahdi et al. (2022)
	Leaves	Methanol extarct	Rats	Prevent liver against paracetamol caused hepatotoxicity	200–400 mg/kg of b.w (for 7 days)	Ravindran et al. (2013)
Lavandula dentata L	Leaves	Aqueous extract	Mice	Hepatoprotective effect against thioacetamide provoked hepatic fibrosis in mice	200 mg/kg of b.w (for 8 weeks)	Almalki (2022)
Malva parviflora L	Whole plant	Methanol extarct	Mice	Hepatoprotective effect against paracetamol-provoked hepatotoxicity in mice	250 mg/kg of b.w (for 7 days)	Mallhi et al. (2014)
Mentha spicata L	Aerial parts	Aqueous extract	Rats	Protective effects against nicotine-induced toxicity in liver of rat	100 mg/kg of b.w (for 2 months)	Ben Saad et al. (2018)
Nigella sativa L	Seeds	Essential oils	Mice	Protective effect on the liver injury provoked by *Schistosoma mansoni*	2.5 and 5 mL/kg (for 2 weeks)	Mahmoud et al. (2002)
	Seeds	Aqueous extract	Rats	Protective effect on CCl_4-caused liver toxicity	250–500 mg/kg of b.w (for 5 days)	Al-ghamdi (2015)
	Seeds	Essential oils	Rats	Hepatoprotective effects in CCl_4-treated rats	0.2 mL/kg (for 60 days)	Kanter et al. (2005)
	Seeds	Unspecified	Patients with non-alcoholic fatty liver disease	Improves biochemical and fatty liver changes in non-alcoholic fatty liver disease patients	1g twice a day for 3 months	Hussain et al. (2017)
	Seeds	Aqueous extract	Mice	Protective effect against N-acetyl-p-aminophenol-induced injury in male mice	0.25 g/kg of b.w (for 30 days)	Hamza and Al-harbi (2015)
	Unspecified	Essential oils	Rats	Protective effects on carboplatin-provoked hepatotoxicity	4 mL/kg	Erisgin et al. (2019)
	Unspecified	Essential oils	Rats	Prevent the liver lesions induced by irradiation	2 mg/kg for 4 weeks	Radwan and Mohamed (2018)
	Seeds	Hydroethanolic extract	Rats	Prevent liver injury on lipopolysaccharide-provoked hepatotoxicity	100–400 mg/kg of b.w (for 2 weeks)	Rats et al. (2018)
	Seeds	Essential oils	Rats	Prevent liver against ethanol provoked oxidative stress and hepatotoxicity	2.5–5.0 mL/kg of b.w (for 3 weeks)	Develi et al. (2014)
	Unspecified	Essential oils	Rats	Prevent against aluminium chloride-provoked liver damage	2 mL/kg of b.w (once per day for 5 weeks)	Bouasla et al. (2014)

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TABLE 6 (*Continued*) Pharmacological outcomes of medicinal plants cited by locals to cure liver ailments.

Scientifique name	Used parts	Used extracts	Experimental model	Pharmacological uses	Therapeutic doses	Ref.
	Unspecified	Essential oils	Rats	Prevent the liver lesions induced by irradiation	1 g/kg of b.w (for 10 days)	Cikman et al. (2014)
	Seeds	Aqueous extract	Rats	Protective and restorative impact on cholestatic liver failure in bile duct ligated rats, possibly via reduced neutrophil infiltration and oxidative stress in hepatocytes	0.2 mL/kg of b.w (for 14 days)	Coban et al. (2010)
	Unspecified	Essential oils	Rats	Improves cisplatin's effect on membrane enzymes, carbohydrate metabolism, and reactive oxygen species in liver	2 mL/kg of b.w (for 14 days)	Farooqui et al. (2016a)
	Unspecified	Essential oils	Rats	Hepatoprotective Effect on CCl_4 caused liver toxicity in adult rats	2–4 mL/kg of b.w (for 2 weeks)	Danladi et al. (2013)
	Seeds	Unspecified	Mice	Protective effect against Dimethylaminoazobenzene provoked liver carcinogenesis in mice	5% (for 32 weeks)	Mohamed et al. (2011)
	Unspecified	Essential oils	Rats	Protective effect in thioacetamide- provoked liver cirrhosis in albino rat	5 mL/kg of b.w (for 8 weeks)	Nehar and Kumari (2013)
	Seeds	Hydroalcoholic extracts	Rats	Protective effect on CCl_4-caused hepatotoxicity	400–800 mg/kg of b.w (for 3 days)	Kingsley (2020)
	Unspecified	Essential oils	Rabbits	Protective effect on CCl4-caused hepatotoxicity	0.2 mL/kg of b.w (for 7 days)	Al-razzuqi et al. (2011)
	Seeds	Aqueous extract	Rats	Protective effect on rifampicin caused hepatotoxicity	2 g/kg of b.w (for 28 days)	Al-azzawi and Baraaj (2016)
	Seeds	Aqueous extract	Rats	Protective effect on thioacetamide-provoked liver fibrosis	50 mg/kg of b.w (for 7 weeks)	Salem et al. (2017)
	Unspecified	Essential oils	Mice	Protective effect on diclofenac sodium and ibuprofen provoked hepatotoxicity	2.5 mL/kg of b.w	Husna and Sajjad (2017)
	Seeds	Unspecified	Chicken	Hepatoprotective effect on aflatoxin-caused liver toxicity	1%	Abosaleh et al. (2019)
	Unspecified	Essential oils	Rats	Reduced the hepatotoxicity provoked by ochratoxin A	0.3 mL/kg of b.w (for 4 weeks)	Alhussaini (2015a)
	Unspecified	Essential oils	Rats	Protective effect on thioacetamide-provoked liver toxicity	10 mL/kg of b.w (for 6 days)	Tanbek et al. (2017)
	Seeds	Unspecified	Rabbits	Protective effect in isoniazid-induced liver toxicity in rabbits	500–1,000 mg/kg of b.w (for 20 days)	Panezai et al. (2022)
	Seeds	Hydro-alcoholic extract	Mice	Protective effect against diethyl phthalate induced changes in mitochondrial enzymatic activities in liver of mice	150–300 mg/kg of b.w (for 30 days)	Prajapati and Verma (2022)
	Seeds	Unspecified	Mice	Protective effect against CCl_4 provoked liver injury in mice	4 mL/kg of b.w (for 3 weeks)	Aleem et al. (2020)
Ocimum basilicum L	Leaves	Aqueous and ethanol extracts	Rats	Hepatoprotective effect on CCl_4-induced liver fibrosis in rats	200 mg/kg of b.w (for 6 weeks)	Yacout et al. (2012)

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TABLE 6 (*Continued*) Pharmacological outcomes of medicinal plants cited by locals to cure liver ailments.

Scientifique name	Used parts	Used extracts	Experimental model	Pharmacological uses	Therapeutic doses	Ref.
	Leaves	Petroleum ether, chloroform, alcohol and Aqueous extracts	Goat liver	Hepatoprotective effect against H_2O_2 and CCl_4 induced hepatoxicity in goat liver	100 mg/kg of b.w (for 5 days)	Meera et al. (2009)
	Unspecified	Essential oils	Rats	Modulates hematotoxicity, reactive oxygen species (ros, DNA damage, and cell cycle arrest caused by β-cyfluthrin in rat liver	3 mL/kg of b.w (every day for a month)	Jebur et al. (2022)
	Leaves	Aqueous extract	Rats	Prevent liver on methotrexate-caused hepatotoxicity	1% (for 42 days)	El Shahat et al. (2017)
	Leaves	Aqueous extract	Rats	Prevent hepatic damage caused by arsenic	400 mg/kg of b.w (once a day for 5 weeks)	Osman et al. (2020)
	Leaves	Aqueous extract	Rats	Hepatoprotective effect on adriamycin-caused liver toxicity	20 mg/kg of b.w (for 8 weeks)	Bayomy et al. (2016)
	Whole plant	Chloroform, diethylether, ethylacetate and methanol extracts	Rats	Hepatoprotective effect on acetaminophen-provoked liver injury	1,200 mg/kg of b.w (for 7 days)	Asala et al. (2021)
Olea europaea L	Leaves	Aqueous extract	Rats	Protective effect on CCl_4-provoked liver toxicity	80 mg/kg of b.w (for 10 days)	Ustuner et al. (2018)
	Leaves	Unspecified	Rats	Protective effect in CCl_4-caused hepatotoxicity	80 mg/kg of b.w (for 3 days)	Vidičević et al. (2020)
	Fruit pulp	Ethanol, n-hexane, ethyl acetate (EA) extracts	Rats	Hepatoprotective effect against high-fat diet-fed	100–300 mg/kg of b.w (for 28 days)	Kim et al. (2014)
Piper nigrum L	Fruits	Methanol extract	Human liver microsomes	Prevent liver	5%	Usia et al. (2005)
	Seeds	hydroalcoholic extract	Mice	Hepatoprotective effects on concanavalin A-provoked liver toxicity	400 mg/kg of b.w	Mushtaq et al. (2021)
	Fruits	Ethanol extract	Rats	Inhibited liver fibrosis induced by thioacetamide	100 mg/kg of b.w (for 28 days)	Dinakar et al. (2010)
	Fruits	Essentiel oils	Mice	Hepatoprotective effect on CCl_4-induced toxicity in mice	2 g/kg of b.w (for 14 days)	Zhang et al. (2021)
Pistacia lentiscus L	Unspecified	Essential oils	Rats	Hepatoprotective effect in rats intoxicated by CCl_4	2–5 mL/kg of b.w (every 3 days for 15 days)	Maameri et al. (2015)
Rosmarinus officinalis L	Leaves	Methanol extract	Rats	Against CCl_4-provoked liver cirrhosis	200 mg/kg of b.w (for 12 weeks)	Alhussaini (2015b)
	Leaves	Etahanol extarct	Mice	Limits weight gain and liver steatosis in mice fed a high-fat diet	200 mg/kg of b.w (for 50 days)	Harach et al. (2010)
	Leaves	Etahanol extarct	*Oncorhynchus mykiss*	Reducing the rate of steatosis in the liver of rainbow trout	0.4–3 g/kg of b.w (for 40 days)	Farooqui et al. (2016b)
	Leaves	Hydroalcoholic extract	Rats	Hepatoprotective effect of on acetaminophen-caused liver damage	100–500 mg/kg of b.w (for 7 days)	Lucarini et al. (2014)
	Unspecified Unspecified		Rats	Prevent liver against etoposide chemotherapy-caused hepatotoxicity	220 mg/kg of b.w (for 6 weeks)	Almakhatreh et al. (2019)

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TABLE 6 (*Continued*) Pharmacological outcomes of medicinal plants cited by locals to cure liver ailments.

Scientifique name	Used parts	Used extracts	Experimental model	Pharmacological uses	Therapeutic doses	Ref.
	Leaves	Hydroalcoholic extract	Rats	Hepatoprotective effect on bile-duct ligation provoked toxicity	500 mg/kg of b.w (for 14 days)	Sadeghi et al. (2020)
	Leaves	Ethanolic Extract	Rats	Prevent liver on alcohol-provoked hepatocytes damage	200 mg/kg of b.w (for 90 days)	Aouad et al. (2021)
	Leaves	Aqueous extract	Rats	Protective effect on trichloroacetate-caused hepatotoxicity	200 mg/kg of b.w (for 2 months)	Abozid and Farid (2018)
	Whole plant	Dichloromethan and methanol extract	HepG2	Regulates metabolism in HepG2 Cells	0 50 µg/mL (for 24 h)	Tu et al. (2013)
Rumex vesicarius L	Whole plants	Methanolic extract	Rats	Hepatoprotective effect on CCl_4-caused toxicity	100–200 mg/kg of b.w (for 7 days)	Ganaie et al. (2015)
	Whole plants	Methanolic extract	Rats	Protective effect in malathion hepatotoxicity	200 mg/kg of b.w (for 28 days)	Mostafa et al. (2018)
Salvia officinalis L	Unspecified	Essential oils	Mice	Hepatoprotective effect against high-fat diet exposition	4 mg/kg of b.w (during 8 weeks)	Koubaa-Ghorbel et al. (2020a)
	Unspecified	Essential oils	Rats	Hepatoprotective effects against vanadium-induced oxidative stress and histological changes in the rat liver	15 mg/kg of b.w	Koubaa et al. (2021a)
	Flowers	Aqueous extract	Rats	Hepatoprotective effect against ethanol induced oxidative stress in rats	50–200 mg/kg of b.w (for 15 days)	Jedidi et al. (2022a)
	Leaves	Hydromethanolic extract	Rats	Hepatoprotective effect against *Aspergillus parasiticus* Aflatoxin-caused liver damage in rats	25–150 mg/kg of b.w	Parsai et al. (2015)
	Aerial parts	Hydroalcoholic extract	Rats	Hepatoprotective effect against isoniazid provoked hepatic damage in rats	100–400 mg/kg of b.w (for 28 days)	Shahrzad et al. (2014a)
	Leaves	Ethanolic extract	HepG2	Prevent HepG2 cells against oxidative stress	0.01–100 mg/mL (for 24 h)	Kozics et al. (2013a)
Syzygium aromaticum (L.)	Flower buds	Etahanol extarct	Rats	Protective effect on hepatotoxicity caused by thioacetamide	800 mg/kg of b.w (for 3 days)	Prasad et al. (2010)
Terminalia arjuna (Roxb. ex DC.) Wight and Arn	Bark	Aqueous extract	Mice	Protect the liver tissues against CCl_4-caused hepatotoxicity	50 mg/kg of b.w (for 1 week)	Manna et al. (2006)
	Bark	Ethanolic extract	HepG2	Thherapeutic effects on human hepatoma cell line, HepG2, and exhibits its cytotoxicity to these cells, and the cell death is mediated by apoptosis	20–100 mg/L (for 48 h)	Sivalokanathan et al. (2006)
	Bark	Aqueous extract	Rats	Hepatoprotective effect against isoniazid provoked toxicity in rats	200 mg/kg of b.w (for 10 days)	Doorika and Ananthi (2002)
	Fruits	Aqueous and ethanol extracts	Mice	Protective effect on cadmium-caused hepatotoxicity	100 mg/kg of b.w (for 7 days)	Ghosh et al. (2010a)
	Bark	Alcoholic and aqueous extracts	Human liver microsomes	Modulatory impacts on the enzyme activity of CYP3A4, CYP2D6, and CYP2C9 in hepatocyte microsomes	2.5–75 µg/mL	Varghese et al. (2015)

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TABLE 6 (*Continued*) Pharmacological outcomes of medicinal plants cited by locals to cure liver ailments.

Scientifique name	Used parts	Used extracts	Experimental model	Pharmacological uses	Therapeutic doses	Ref.
	Bark	Aqueous extract	HepG2	Attenuates toxicity provoked by tert-butyl hydroperoxide in HepG2 cell	25–100 mg/mL	Shivananjappa et al. (2013)
	Bark	Aqueous extract	Rats	Prevent liver against acetaminophen	250–500 mg/kg of b.w (for 14 days)	Kannappan et al. (2020)
	Bark	Aqueous extract	Rats	Protective effect against alcohol caused hepatoxicity in rats	250–500 mg/kg of b.w	Ch et al. (2015)
	Bark	Aqueous extract	HepG2	Reduce basal oxidative stress in HepG2 cells	25–100 µg/mL	Shivananjappa and Joshi (2012)
	Leaves	Aqueous extract	Rats	Attenuated the physiological and histopathological alterations in liver provoked by cisplatin	400 mg/kg of b.w (for 14 days)	Sneha et al. (2021)
	Bark	Aqueous extract	Rats	Protective effects against arsenic-caused aggravation of high fat diet-induced oxidative stress-mediated damages in liver	20–60 mg/kg of b.w (for 8 days)	Dutta et al. (2014)
	Bark	Aqueous extract	Rats	Protective effect against adrenaline-induced hepatic damage in rats through an antioxidant mechanism	10–40 mg/kg of b.w (for 5 days)	Ghosh et al. (2010b)
	Fruits	Ethanolic extaract	Mice	Hepatoprotective effect in acetaminophen intoxicated mice	400 mg/kg of b.w	Saira et al. (2020)
	Bark	Aqueous extract	Rats	Protective effect on acetaminophen-provoked liver damage	250–500 mg/kg of b.w (for 14 days)	Chester et al. (2017)
	Bark	Ethanolic extaract	Rats	Protective effect on paracetamol provoked liver toxicity	200 mg/kg of b.w (once daily for 7 days)	Sangamithira et al. (2011)
	Stem bark	Alcohol extract	Rats	Hepatoprotective effect on CCl_4-caused toxicity	250–500 mg/kg of b.w (for 5 days)	Anbalagan et al. (2007)
Thymus vulgaris L	Unspecified	Alcoholic extract	Broiler chickens	Regulates lipid metabolism in the liver	0.2%–0.6% (for 42 days)	Abdulkarimi et al. (2011)
	Unspecified	Essential oil	Rats	Enhances the total antioxidant potential of hepatocytes	42.5 mg/kg of b.w	Youdim and Deans (1999)
	Leaves	Unspecified	Mice	Boost the activity of xenobiotic-metabolizing enzymes in the liver	0.5%–2.0% (for 7 days)	Sasaki et al. (2005)
	Unspecified	Essential oils	Japanese quails (*Coturnix coturnix* japonica)	Ameliore *in vivo* antioxidant activity in the liver	150–450 mg/kg of b.w	Nutrition et al. (2017)
	Leaves, flowers and stems	Aqueous extract	Rats	Hepatoprotective effect against toxicity in rats exposed to aluminum	150 mg/kg of b.w (daily for 90 days)	Mokrane et al. (2020)
	Unspecified	Aqueous extract	Rats	Improved liver injury induced by paclitaxel in rats	4.5–18 mg/kg of b.w (for 2 weeks)	Salahshoor et al. (2019)
	Aerial parts	Aqueous extract	Rats	Prevent liver against dexamethasone-provoked liver damage	500 mg/kg of b.w (for 8 weeks)	Abou-Seif et al. (2019)
	Leaves	Ethanolic extaract	HepG2	Protective the HepG2 cells oxidative stress	100 mg/mL	Kozics et al. (2013b)
Ziziphus lotus (L.) Lam	Fruits	Aqueous extract	Rats	Protective effect on CCl_4 provoked liver toxicity	200 and 400 mg/kg for 14 days	Bencheikh et al. (2019)

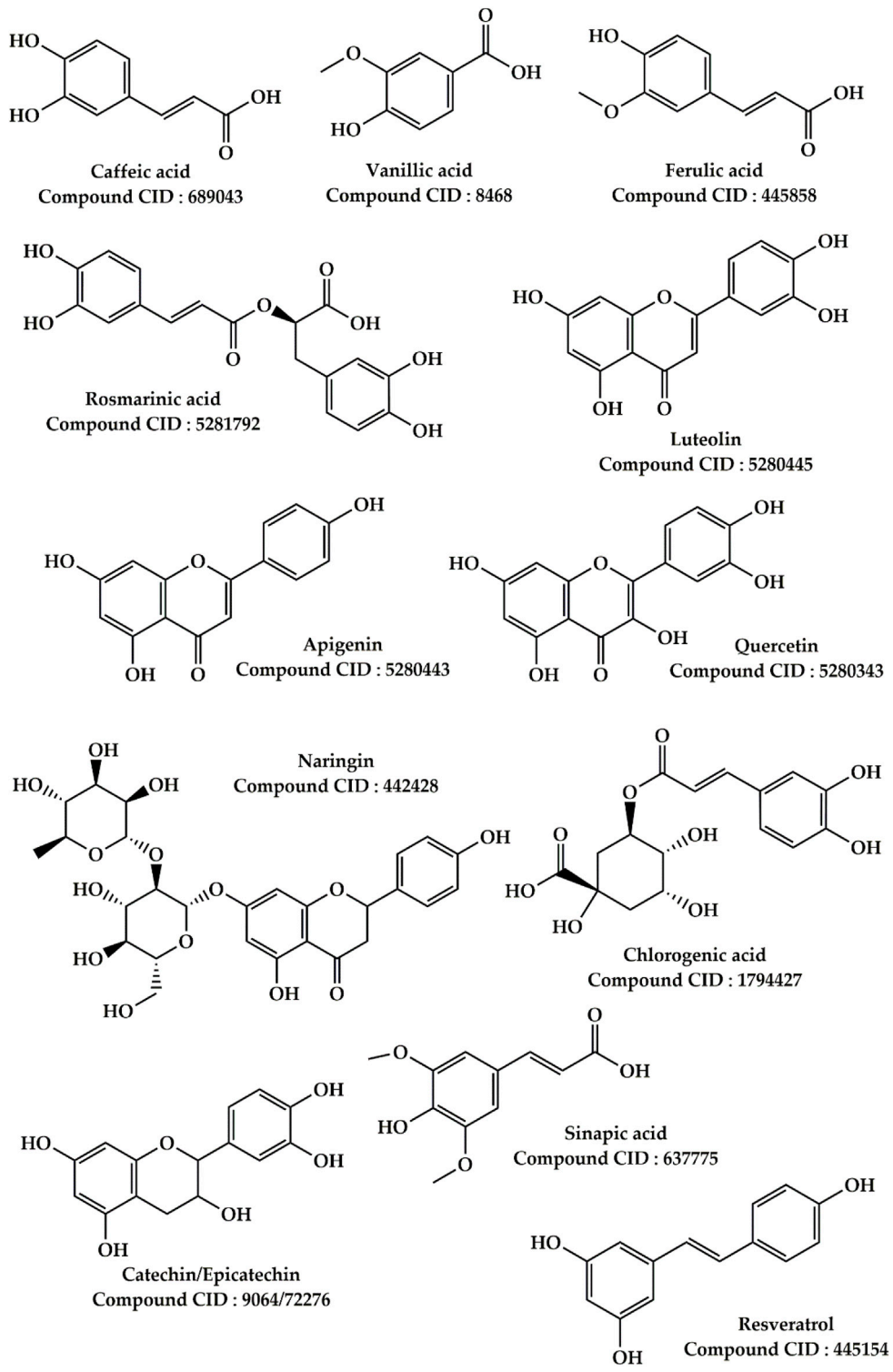

FIGURE 6
Main phytoconstituents found in *Salvia officinalis* L. and *Ziziphus lotus* (L.) Lam.

bulbs reduces alloxane elevation of biological parameters of liver and kidney functions in rats (Aprioku and Amah-Tariah, 2017b). According to prior study, garlic includes a number of bioactive components such as organosulfur compounds, saponins, and phenolic compounds (Bradley et al., 2016; Diretto et al., 2017). Organosulfur compounds such as diallyl thiosulfonate (allicin), diallyl sulfide, diallyl disulfide, diallyl trisulfide, E/Z-ajoene, S-allyl-cysteine, and S-allyl-cysteine sulfoxide have been claimed to be the principal active phytochemicals found in garlic (Figure 5) (Yoo D. Y. et al., 2014; Yoo et al., 2014 M.; Kodera et al., 2017;

Mansingh et al., 2018). These compounds were discovered to be related to the plant's powerful antioxidant and antitumor ability (Bagul et al., 2015).

Asparagus officinalis L. this plant is ranked third among the plants most commonly used for the treatment of liver diseases by the Moroccan population. It is frequently used to treat biliary problems, hepatic stones, hepatitis, and jaundice, as shown in Table 3. It has been reported that the aqueous and ethanolic extract of *A. officinalis* have hypolipidemic and hepatoprotective effects in mice fed a high-fat diet (Zhu et al., 2010). According to the findings of this study, daily treatment of 200 mg/kg of either ethanolic or aqueous extract for 8 weeks enhanced lipid parameters, transaminase (Alanine and Aspartate) activity, superoxide dismutase (SOD) and antioxidant capacity, and hepatic malondialdehyde levels. In addition, an *in vivo* research indicate that the aqueous extract of *A. officinalis* roots has protective properties on cadmium chloride-induced liver injury in rats (Abedi et al., 2018). This investigation found that treatment by aqueous extract of *A. officinalis* roots at 200 and 400 mg/kg for 28 days significantly restored liver biomarkers in cadmium chloride poisoned rats. Several phytochemical investigations have revealed that the main bioactive compounds found in asparagus include phenolic compounds, sterols, and saponins (Jang et al., 2004; Fuentes-Alventosa et al., 2013). Asparanin A, Asparoffin C, Asparoffin D, Asparenyol, Gobicusin B, Protodioscin and 1-methoxy-2-hydroxy-4-[5-(4-hydroxyphenoxy)-3-penten1-ynyl] phenol are the main phytochemicals found in asparagus, with Rutin (Quercetin 3-rutinoside) as the major compound (Figure 5) (Fan et al., 2015). These compounds' antioxidant action is well-known (Sun et al., 2007; Solana et al., 2015; Slatnar et al., 2018), that may contribute in the hepatoprotective effects of the plant.

Salvia officinalis L. This plant is widely used in folk medicine in North East Morocco to treat liver failure. It is ranked fourth of the most commonly used plants, with a use value of 0.0761. According to our findings, various parts of this plant have traditionally been used to treat jaundice, hepatitis, and liver cancer in the study site. Several preclinical investigations on plant parts were carried out to examine its medicinal qualities for liver failure. In fact, 8 weeks of daily administration of *S. officinalis* essential oil at 4 mg/kg enhanced hyperlipidemia, hepatic, and renal lesions in mice fed a high-fat diet (Koubaa-Ghorbel et al., 2020b). This effect of *S. officinalis* essential oil was more effective than that of simvastatin (standard drug for this purpose). In addition, daily intake of 15 mg/kg of the essential oil of *S. officinalis* showed a protective effect against vanadium-induced hepatotoxicity in Wistar rats (Koubaa et al., 2021b). The treatment of rats with 200 mg/kg of *S. officinalis* aqueous extract for 15 days showed protective effects against ethanol-induced hepatotoxicity (Jedidi et al., 2022b). According to the same authors, this effect is reflected in the improvement of plasma transaminase activity and the restoration of hepatocyte structure in rats poisoned with ethanol. Besides, it was shown that administering a hydroalcoholic extract of *S. officinalis* at a dose of 250 mg/kg protected rats from isoniazid-induced hepatotoxicity (Shahrzad et al., 2014b). Furthermore, a previous investigation showed that an ethanolic extract of *S. officinalis* leaves protects human HepG2 cells from hydrogen peroxide and 2,3-dimethoxy-1,4-naphthoquinone-induced DNA damage (Kozics et al., 2013b). As shown in Figure 6, common sage contains a variety of biologically active compounds, primarily two types of relatively abundant phenolic components: phenolic acids (caffeic, vanillic, ferulic, and rosmarinic acids) and flavonoids (luteolin, apigenin, and quercetin) (Lu and Foo, 2002; Roby et al., 2013). These phenolic components are well-known as hepatoprotective agents (Kiokias and Oreopoulou, 2021; Venmathi Maran et al., 2022).

Ziziphus lotus (L.) Lam. According to our ethnobotanical study conducted in several parts of the Moroccan North-East, this plant ranks sixth among the most commonly utilized herbs to cure liver disorders. Indeed, the leaves and fruits of this plant were utilized to treat jaundice, hepatic colic, and hepatitis in the research region. Previous pharmacological work has demonstrated that *Z. lotus* extracts exert hepatoprotective effects at the preclinical stage. In a rat investigation, an aqueous extract of *Z. lotus* fruits was found to have hepatoprotective properties against CCl_4-induced liver damage (Bencheikh et al., 2019). The findings of this study indicate that administration of aqueous extract of *Z. lotus* fruits at doses of 200 and 400 mg/kg restored the biochemical parameters (liver biomarkers) altered during hepatotoxicity induced by CCl_4 injections in rats. Similarly, it has been reported that treatment of rats with the aqueous extract of *Z. lotus* fruit at doses of 200 and 400 mg/kg protects the liver and kidney from gentamicin poisoning (Bencheikh et al., 2021b). In the literature, it has been well demonstrated that the hepatotoxicity caused by the agent CCl_4 and gentamicin is related to the oxidative stress caused by these chemical compounds (Lin and Huang, 2000; Achuthan et al., 2003). In this context, several authors confirm that the use of natural antioxidants to fight against the oxidative stress caused by CCl_4 and gentamicin is the best strategy to prevent hepatotoxicity produced by these hepatotoxic substances (Bencheikh et al., 2021a; Bouhrim et al., 2021; Ouahhoud et al., 2021b). Extracts of *Z. lotus* fruits are high in phenolic compounds such as Rutin, Naringin, Chlorogenic acid, Rosmarinic acid, Quercetin, Catechin, Epicatechin, Sinapic acid, Resveratrol, and Caffeic acid, according to phytochemical research (Figure 6) (Marmouzi et al., 2019; Bencheikh et al., 2021c; 2021d). These photochemical compounds thanks to their antioxidant powers could be responsible for the hypatoprotective effects.

4 Conclusion

This ethnobotanical study reveals that locals in remote areas of northern Morocco possess extensive traditional knowledge about using medicinal plants to treat liver diseases, reflecting the region's floristic richness. The findings demonstrate the potential of these herbs in addressing liver-related health issues within these communities. However, caution is necessary when using these remedies. The study is limited by its small sample size and lack of a control group, which may affect the robustness of the conclusions.

Further research is essential to evaluate the pharmacological benefits and phytochemical components of these plants, identify active ingredients, and confirm their clinical efficacy. Additionally, safety data are needed to standardize dosages and ensure safe use. Addressing these limitations will help in the development of

effective medications derived from these medicinal plants for liver disease treatment.

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

NB: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing–original draft, Writing–review and editing. AE: Formal Analysis, Investigation, Software, Validation, Writing–original draft, Writing–review and editing. AB: Formal Analysis, Investigation, Software, Writing–original draft, Writing–review and editing. MB: Data curation, Investigation, Methodology, Software, Writing–review and editing. AA: Formal Analysis, Validation, Writing–review and editing. MA: Formal Analysis, Resources, Validation, Visualization, Writing–review and editing. RM: Funding acquisition, Resources, Validation, Writing–review and editing. HA-Y: Funding acquisition, Resources, Writing–review and editing. BE: Investigation, Validation, Writing–review and editing. ME: Formal Analysis, Resources, Validation, Visualization, Writing–review and editing.

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Conflict of interest

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

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Corrigendum: Ethnobotanical survey and scientific validation of liver-healing plants in northeastern Morocco

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In the published article, there was an error regarding the **Affiliation** of Mohamed Bouhrim. Instead of having both "Laboratory of Biological Engineering, Team of Functional and Pathological Biology, University Sultan Moulay Slimane Faculty of Sciences and Technology Beni Mellal, Meknes, Morocco" and "Department of Pharmacognosy, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia," they should only be affiliated with **Affiliation 4**, "Laboratory of Biological Engineering, Team of Functional and Pathological Biology, University Sultan Moulay Slimane Faculty of Sciences and Technology Beni Mellal, Meknes, Morocco."

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

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Pharmacophylogenetic relationships of genus *Dracocephalum* and its related genera based on multifaceted analysis

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The Lamiaceae genus *Dracocephalum*, with over 30 species, is believed to have considerable medicinal properties and is widely used in Eurasian ethnomedicine. Numerous studies have researched on the geographical distribution, metabolite identification, and bioactivity of *Dracocephalum* species, especially amidst debates concerning the taxonomy of its closely related genera *Hyssopus* and *Lallemantia*. These discussions present an opportunity for pharmacophylogenetic studies of these medicinal plants. In this review, we collated extensive literature and data to present a multifaceted view of the geographical distribution, phylogenetics, phytometabolites and chemodiversity, ethnopharmacological uses, and pharmacological activities of *Dracocephalum*, *Hyssopus*, and *Lallemantia*. We found that these genera were concentrated in Europe, with species adapted to various climatic zones. These genera shared close phylogenetic relationships, with *Dracocephalum* and *Hyssopus* displaying intertwined patterns in the phylogenetic tree. Our review assessed more than 900 metabolites from these three genera, with terpenoids and flavonoids being the most abundant. Researchers have recently identified novel metabolites within *Dracocephalum*, expanding our understanding of its chemical constituents. Ethnopharmacologically, these genera have been traditionally used for treating respiratory, liver and gall bladder diseases. Extracts and metabolites from these genera exhibit a range of pharmacological activities such as hepatoprotective, anti-inflammation, antimicrobial action, anti-hyperlipidaemia, and anti-tumour properties. By integrating phylogenetic analyses with network pharmacology, we explored the intrinsic links between metabolite profiles, traditional efficacy, and modern pharmacology of *Dracocephalum* and its related genera. This study contributes to the discovery of potential medicinal value from closely related species of *Dracocephalum* and aids in the development and sustainable use of medicinal plant resources.

KEYWORDS

***Dracocephalum*, pharmacophylogeny, geographical distribution, plant metabolites, pharmacological activities**

1 Introduction

The Lamiaceae family is the sixth most diverse in terms of species and the tenth most diverse in terms of genera among angiosperms (Christenhusz and Byng, 2016). With its cosmopolitan distribution, this botanical group is used extensively in various fields. For example, lavender [Lamiaceae; *Lavandula angustifolia* Mill.], which is known for its ornamental cultivation and aromatic qualities, has attracted considerable scholarly attention (Gu et al., 2021). Another member of this family, fresh mint [Lamiaceae; *Mentha piperita* L.], is highly valued for its culinary use (Hsu et al., 2010). Historical records from various regions and ethnic groups have confirmed the traditional medicinal uses of Lamiaceae species. In Traditional Chinese Medicine, the dried roots and rhizomes of *Salvia miltiorrhiza* Bunge [Lamiaceae; Salviae miltiorrhizae radix et rhizoma] is lauded for its therapeutic properties, which encompass the promotion of blood circulation, the alleviation of menstrual discomfort, heat-clearing, and the reduction of swelling (Deng et al., 2016). In the southern regions of India, *Leucas ciliata* Benth [Lamiaceae] have been documented for their efficacy in wound healing and as a snakebites remedy (Akshatha et al., 2021). Additionally, the native American plant *Callicarpa americana* L [Lamiaceae] has been traditionally used by several indigenous tribes in the southeastern United States for its febrifuge, stomachic, and anti-dysenteric properties (Dettweiler et al., 2020).

Globally, the importance of Lamiaceae plants is undeniably prominent. The genera *Dracocephalum*, *Hyssopus*, and *Lallemantia* belong to the Lamiaceae family. In recent years, the phylogenetic relationships among these three genera have attracted the attention of researchers and their taxonomy has been reassessed from multiple perspectives, including bioinformatics, geography, and plant morphology. Among them, *Dracocephalum* has the greatest number of species, characterized by flowers that are typically blue-purple with occasional whites, and bracts, that are often obovate and frequently feature acute teeth or spines, rarely being entire. The floral structure is described as tubular or campanulate-tubular, either straight or slightly curved, and adorned with 15 veins and five teeth (Figure 1). As close relatives of the *Dracocephalum* genus, the flowers of *Hyssopus* and *Lallemantia* genera also typically have a tubular calyx, 15 veins, five teeth, and a bilabiate corolla, with four stamens and a stigma that is bifid at the apex. However, significant differences between the stems and leaves were observed. Plants of these three genera contain several valuable metabolites. Notably, *Dracocephalum* is known for its rich content of essential oils, flavonoids, glycosides, triterpenoids, organic acids, and esters (Lv et al., 2022). The 'A Quick-Consultative Dictionary of World Medicinal Plants' documents the nomenclature, medicinal parts, habitats, and therapeutic properties of the medicinal plants within the *Dracocephalum* genus. A substantial proportion of these records are from diverse provinces across China, where they have garnered attention for their roles in traditional Chinese medicine, particularly for their heat-clearing, blood-cooling, and cough-relieving effects (Jiang, 2015; Yao et al., 2020; Yan et al., 2020).

As living standards progressively increase, societal emphasis on health and wellbeing increases. This trend is mirrored by an increasing interest in herbal resources. However, the growing demand for herbal resources could result in the overexploitation of wild resources and a shortage of medicinal plants that are on the verge of extinction. Thus, sustainable use of medicinal plants has become a pivotal research domain in contemporary society. This field demands a robust foundation of precise knowledge and strategic application of theoretical frameworks, as exemplified by the discipline of pharmacophylogeny. The concept of pharmacophylogeny was proposed by Xiao Peigen in the 1980s and is an emerging discipline that studies the correlation between the molecular phylogeny of medicinal plants, chemical metabolites, pharmacological activities, and traditional applications. Pharmacophylogeny is advantageous owing to its interdisciplinarity and interpenetration feature its research objects, which involve multidisciplinary fields (Chen et al., 2005; Hao and Xiao, 2020). Establishing this subject as an essential guide for the conservation and development of medicinal plant resources is crucial, and justified through long-term research (Gong et al., 2022; Hao et al., 2022). Pharmacophylogeny proposes that phylogenetically closer taxonomic groups, such as families, tribes, or genera, are more likely to possess similar chemical profiles, which contribute to more analogous bioactivities. Pharmacophylogeny principles have been applied to various aspects of imported drug resource substitution, the search for new drugs, and production practices to expand the development and utilization of medicinal resources (Li, 2021; Hao et al., 2022).

In recent years, many studies have been conducted on the genus *Dracocephalum*, covering various aspects such as the geographical distribution of its species, their phylogenetic relationships with other Lamiaceae genera, its metabolites, and its pharmacological activities. Our review meticulously compiled extensive information on *Dracocephalum* species and their closely related genera to clarify the current status of *Dracocephalum* as a medicinal plant through the lens of pharmacophylogenetic principles. This compendium is intended not only to deepen our understanding of *Dracocephalum* and its relatives but also to encourage the sustainable management and application of these precious botanical resources.

For this review, we have primarily referenced 'A Quick-Consultative Dictionary of World Medicinal Plants' and conducted independent searches for the species names across PubMed (https://pubmed.ncbi.nlm.nih.gov/), Google Scholar (https://scholar.google.com), and the China National Knowledge Infrastructure (https://kns.cnki.net/) database, focusing on the most current and authoritative literature. All plant names mentioned in this article have been confirmed on the WFO Plant List (https://wfoplantlist.org/), ensuring that they are accepted species names. The geographical coordinates were sourced from the Global Biodiversity Information Facility (https://www.gbif.org/zh/). Species distribution data were integrated from the WFO, WORLD PLANTS (https://www.worldplants.de/), and iPlant (http://www.iplant.cn/) databases, facilitating the delineation of climatic conditions. Phylogenetic tree sequences are derived from the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/) and the scholarly work of Chen G. W. et al. (2022).

2 Geographical distribution and phylogenetics

2.1 Geographical distribution

The genus *Dracocephalum*, comprising over 60 species, is distributed across the Eurasian and North American tectonic

FIGURE 1
Species within the *Dracocephalum* genus, recognized for their medicinal properties, display certain distinctive features in the field of plant morphology. *D. rigidulum* **(A)**, *D. rupestre* **(B)**, *D. argunense* **(C)**, *D. heterophyllum* **(D)**, *D. moldavica* **(E)**, **(D)** *nutans* **(F)**, *D. forrestii* **(G)**, *D. taliense* **(H)**. The picture comes from shooting.

plates. The speciation of *Dracocephalum* exhibits regional differentiation, featuring four primary distribution areas on the Eurasian plate and one main area on the Indian and American plates. The genus *Dracocephalum* exhibits an endemic distribution, with robust its adaptability observed predominantly within the climatic zones of the Northern Hemisphere, specifically in temperate continental climate areas.

In the Asian continent, the genus *Dracocephalum* shows a rich diversity of species that exhibit distinct climatic differentiation and a geographical distribution gradient from east to west. *Dracocephalum's* distribution transitions from temperate zones to more demanding alpine mountainous regions, with this genus predominantly thriving in temperate continental climates. These climates are marked by significant seasonal variations, pronounced temperature fluctuations, and moderate, seasonally concentrated rainfall, which, along with the region's topography, shape the ecological spread of *Dracocephalum* across alpine, temperate monsoon, and Mediterranean climates.

The aridification of the Asian interior and the uplift of the Qinghai-Tibet Plateau (QTP) are substantial geological and climatic events that have influenced the speciation and distribution of *Dracocephalum*. Interior aridification likely drove the rapid radiation of the genus, whereas the uplift of the QTP initiated its dispersal and diversification in the plateau and surrounding areas (Chen Y. P. et al., 2022). The flora of Central and West Asia are closely related to the QTP, with some species hypothesized to have originated from the plateau and later migrated to other regions (Wen et al., 2014; Zhang et al., 2017). The alpine climate of the QTP, influenced by its distance from the ocean, high terrain, and low temperatures, has fostered a conducive environment for the growth and diversification of this genus. Interestingly, despite the southern part of the American Plate fallings within the temperate continental climate zone, there are no records of *Dracocephalum* in this region, as the genus's adaptive radiation of this genus may be unable to cross the tropical climate zones of the American Plate.

The spatial distribution pattern (Figures 2A, C) shows that *Dracocephalum* has a broader distribution than the *Lallemantia* and *Hyssopus*; however, the latter three genera share overlapping ranges within Europe. *Dracocephalum* is found across Asia and Europe, whereas *Lallemantia* and *Hyssopus* are predominantly present in Southern European countries such as Greece and Italy, in addition to other regions across Europe. The distribution of *Dracocephalum* also extends to the Americas, particularlynorthern Mexico. From the perspective of suitable ecological zones, the genera *Dracocephalum*, *Lallemantia*, and *Hyssopus* are well adapted across much of Europe. *Dracocephalum*, in particular, is amenable to cultivation and thrives along the Mediterranean coast, Central Asia, West Asia, and the western regions of North America. However, most areas south of the equator were not conducive to the cultivation and growth of these three genera (Figures 2B, D).

2.2 Phylogenetics

The genus *Dracocephalum* encompasses a diverse array of over 60 species, approximately 30 of which are recognized for their medicinal properties. The majority of these medicinal species are meticulously documented in the authoritative publication 'A Quick-Consultative Dictionary of World Medicinal Plants', as well as in the traditional medical literature of numerous ethnic groups and within a variety of scholarly texts. Currently, phylogenetic analysis is widely applied in pharmacophylogeny, especially in the exploration and organization of traditional Chinese medicine (Hao et al., 2017; Gong et al., 2022). The medicinal plants of *Dracocephalum* are underpinned by a rich and extensive genomic sequence foundation, which renders them invaluable subjects for extensive and scholarly research.

In 2022, Chen et al. conducted an exhaustive study employing a range of genetic sequences, including chloroplast sequences (rpl32-trnL, trnL-trnF, ycf1, ycf1-rps15), nuclear genes (ITS and ETS), and low-copy nuclear gene sequences (AT3G09060, AT1G09680) (Chen Y. P. et al., 2022). Despite the topological structures of the resultant phylogenetic trees demonstrating variability, these findings suggest that the taxonomic classification of *Dracocephalum* species is a

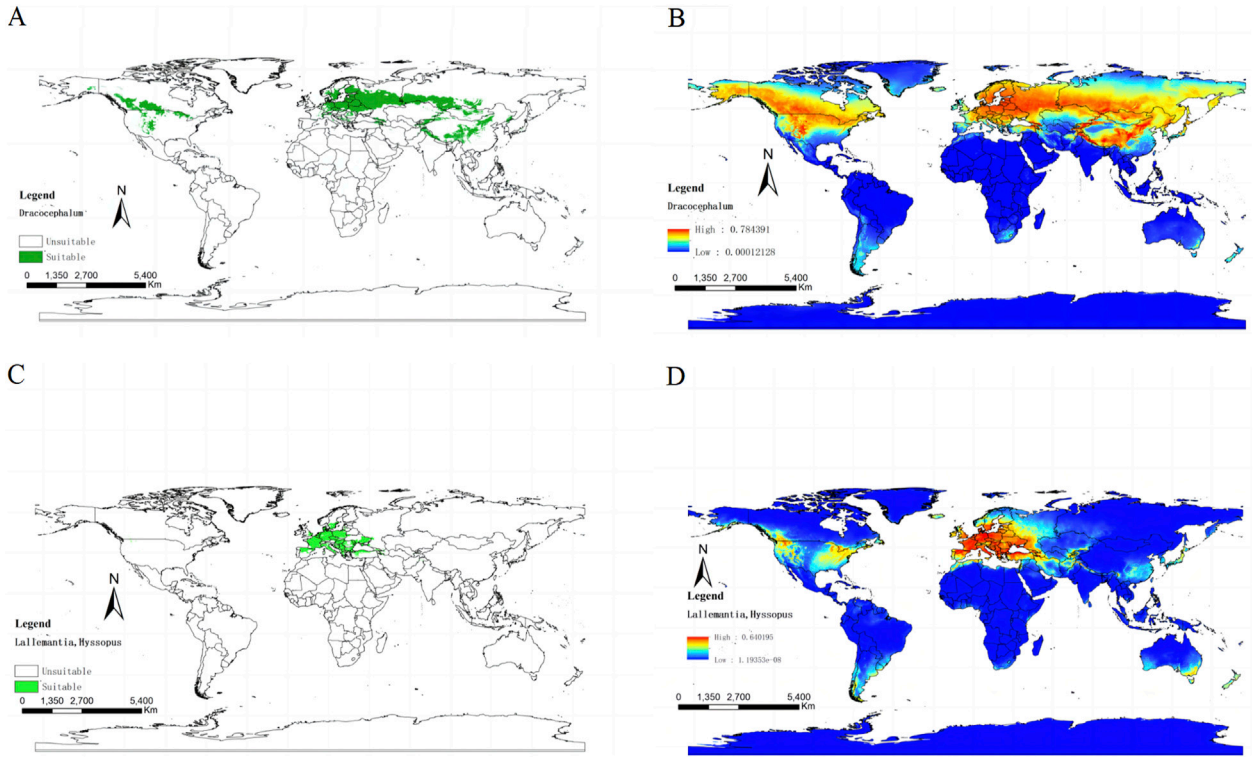

FIGURE 2
Ecologically suitable regions of genus *Dracocephalum* (Green distribution is suitable for the growth area of *Dracocephalum*). **(A)**. Suitability distribution of *Dracocephalum*. From blue to red, the suitability of *Dracocephalum* gradually increases, and yellow is the transition area **(B)**. Ecologically suitable regions of genus *Lallemantia* and *Hyssopus* (Green distribution is suitable for the growth area of *Lallemantia* and *Hyssopus*). **(C)**. Suitability distribution of *Lallemantia* and *Hyssopus*. From blue to red, the suitability of *Lallemantia* and *Hyssopus* gradually increases, and yellow is the transition area **(D)**.

matter of ongoing debate. This is particularly true when considering the phylogenetic interrelationships among *Hyssopus*, *Lallemantia*, and *Dracocephalum*, which present both morphological and taxonomic challenges.

The ITS sequence, which is a standard in pharmacophylogeny analysis, was pivotal in our study (Hao et al., 2017; Wang et al., 2019). We used the Neighbor-Joining (NJ) model to construct phylogenetic trees (Supplementary Figure S1) from these sequences (Supplementary Table S1). These result indicates that within the medicinal flora of *Dracocephalum* and its closest botanical allies, *Hyssopus* is consistently intermingled with the *Dracocephalum* lineage, whereas *Lallemantia* may be more appropriately classified taxonomically as a distinct entity. However, it is important to note that the phylogenetic branches derived from this model had relatively low bootstrap values. In a stark comparison, Chen G. W. et al. (2022) leveraged the Pentapeptide Repeat (PPR) motif and applied the Bayesian Inference (BI) algorithm to uncover a tree with significantly robust branch support. By extracting the topological structure of the medicinal plant branches from their analysis, we delineated a novel phylogenetic tree for the genus *Dracocephalum* and its related medicinal species (Figure 3). Within the *Dracocephalum* genus, species such as *Dracocephalum argunense* Fisch. ex Rchb. and *Dracocephalum bipinnatum* Rupr., demonstrated adaptability to two diverse climatic conditions. It is worth noting that, *Dracocephalum moldavica* L. and *Dracocephalum nutans* L. have the ability to inhabit three separate climatic zones. Furthermore, *Dracocephalum ruyschiana* L. had an even broader distribution across the five climatic regions. In contrast to medicinal plants in the genera *Hyssopus* and *Lallemantia*, which share a close phylogenetic affinity with *Dracocephalum*, only *Hyssopus officinalis* L. and *Lallemantia royleana* (Benth.) Benth. are documented to have distributions spanning multiple climatic zones. This adaptability highlights the ecological plasticity of these species and underscores their potential for medicinal use in various environmental contexts.

3 Phytometabolites and chemodiversity

Our survey systematically catalogued the chemical metabolites from a total of seventeen *Dracocephalum* species, three *Hyssopus* species, and two *Lallemantia* species, resulting in the identification of more than 900 metabolites (Supplementary Tables S3, S4). The predominant metabolites are terpenoids, flavonoids, phenylpropanoids, and phenolic acids. Fatty acids, alkaloids, and steroids, being less prevalent, were categorized under the 'Others' grouping (as depicted in Figure 4A). Our findings, which only provide a consolidated summary, consider the variability in extraction methods reported in the literature. Notably, within the genus *Dracocephalum*, *D. moldavica* exhibited the greatest metabolite diversity, while in *Hyssopus*, *Hyssopus cuspidatus*

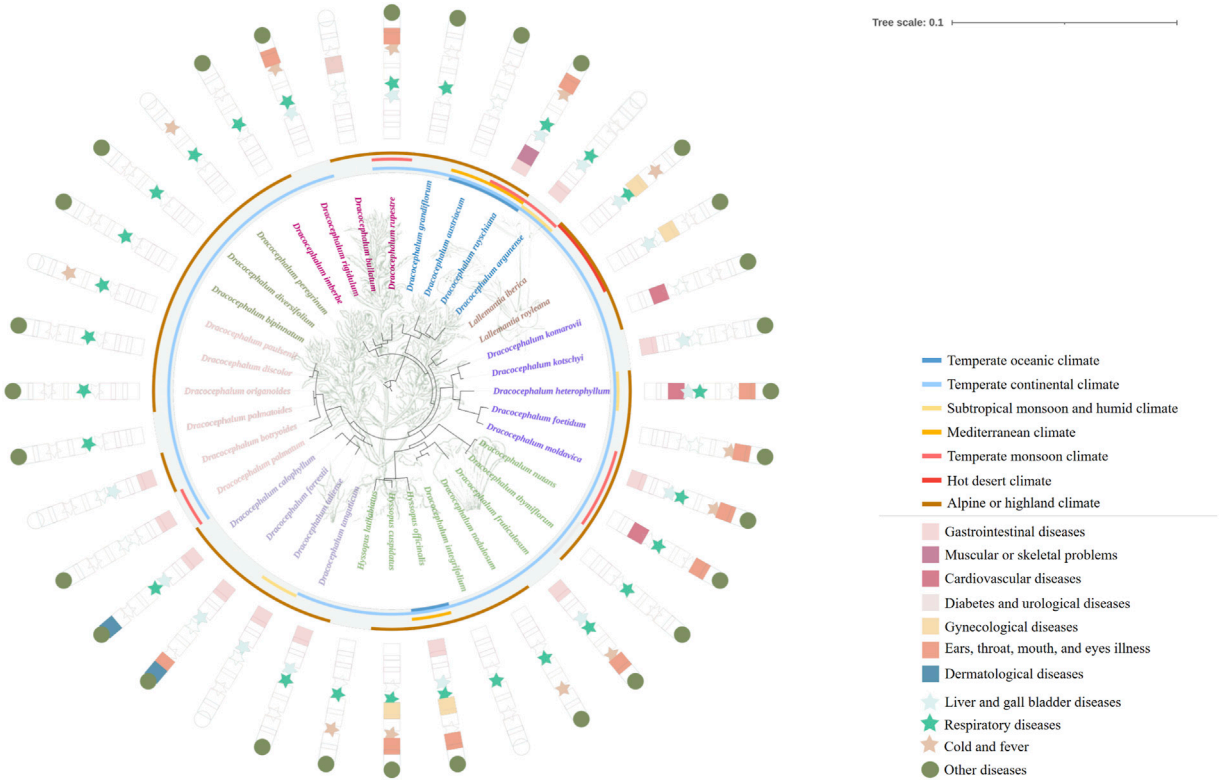

FIGURE 3
This refined phylogenetic tree expands upon the original framework constructed by Chen et al., with selective pruning to emphasize species of Dracocephalum and its closely related genera, recognized for their medicinal properties and historical use in healing practices. The outermost ring of the visualisation encapsulates the climatic distribution characteristics of these plants, collated from the World Plants database [https://www.worldplants.de/world-plants-complete-list/complete-plant-list] and the iPlant database [http://www.iplant.cn/]. The annotations on the outer layer provide insights into the medicinal efficacy attributed to these species, offering a visual representation of their therapeutic potential. The traditional classification of efficacy is shown in Supplementary Table S2, and the climate information of each region is shown in Supplementary Table S5.

Boriss. had the most characterized metabolites. In the genus *Lallemantia*, the number of reported metabolites for *L. royleana* significantly surpassed that for *Lallemantia iberica* (M.Bieb.) Fisch. & C.A.Mey., with a focus on the aerial parts of the plants rather than on the seeds (Figure 4B). Most studies have focused on identifying chemical metabolites in naturally dried whole plants of *Dracocephalum* species, with *D. moldavica*, *Dracocephalum heterophyllum* Benth., and *D. nutans* being the most studied examples (Jiang, 2015). Some studies have specifically analyzed whole plants during the flowering stage, such as *Dracocephalum kotschyi* Boiss., *Dracocephalum palmatum* Stephan ex Willd., *Dracocephalum austriacum* L. and *Dracocephalum botryoides* Steven, which may correspond to the peak times of traditional medicinal activity (Olennikov et al., 2017; Kashchenko et al., 2022). Additionally, some investigations have concentrated on particular plant organs, such as leaves or roots (Poursalavati et al., 2021; Kashchenko et al., 2022). Concurrently, many novel metabolites are continually being discovered within this flora, highlighting the dynamic chemical landscape of this genus. This disparity may be attributed to variations in metabolic pathways, ecological preferences, or evolutionary factors that have shaped the chemical diversity of these plants. It is important to acknowledge that current research on the metabolites of *Dracocephalum* and its closely related genera are not exhaustive, indicating the need for further comprehensive studies. However, the extensive geographical distribution of *Dracocephalum* species, along with the significant altitudinal gradients they inhabit, make them an excellent model for studying the relationship between medicinal plant metabolites and environmental factors such as latitude, longitude, and altitude. This research is crucial for understanding the influence of environmental factors on the chemical profiles of medicinal plants and has implications for biodiversity conservation and the discovery of new pharmaceutical metabolites.

In this review, we focused on terpenoids and flavonoids, which constitute the two most diverse classes of metabolites in the aforementioned genera. Moreover, during our literature review, we found that researchers were often able to successfully isolate and characterize new metabolites from the genus *Dracocephalum*. Among these, some have already made initial contributions to the advancement of human health, whereas others are yet to be investigated because of their unknown activities.

3.1 Terpenoids

Terpenoids are ubiquitous throughout the plant kingdom and play integral roles in both primary and secondary metabolic processes. They are crucial for a myriad of biological functions,

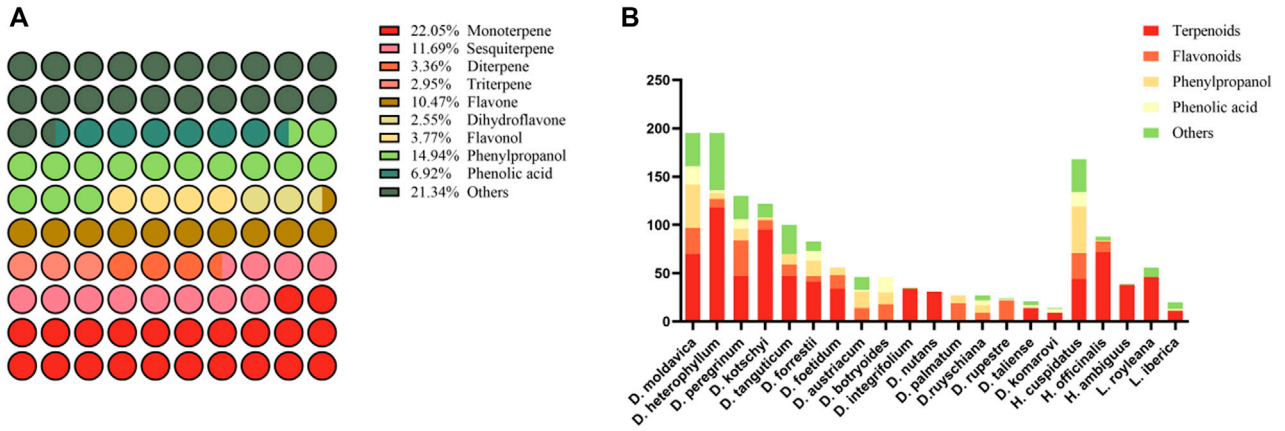

FIGURE 4
A proportional diagram of all metabolites collected from the medicinal plants of the *Dracocephalum* genus and its closely related genera **(A)**, and information on each collected medicinal plant metabolite **(B)**.

such as signal transduction, reproduction, communication, and adaptation of plants to environmental changes, including climate change, as well as their defense mechanisms (Tholl, 2015; Atriya et al., 2023). Terpenoid metabolites within plants can attract pollinating insects and deter or resist predators and other organisms that may harm the plant foliage. Plants use the color and/or aroma of these volatile metabolites to entice pollinators and animals, thereby facilitating seed dispersal (Boncan et al., 2020; Tetali, 2019). Certain terpenoids not only regulate plant growth and development and respond to environmental pressures but also play a central role in the physiological activities of plants (Chen et al., 2020). Additionally, the majority of plant terpenoids possess antimicrobial and insecticidal properties (Yamaguchi, 2022).

In this study, we compiled a collection of 283 terpenoids from the *Dracocephalum* genus, including 216 monoterpenes, 112 sesquiterpenes, 24 diterpenes, and 29 triterpenes. This collection highlights the rich chemical diversity of this class of metabolites and their potential significance in biological and ecological contexts.

3.1.1 Monoterpenes and sesquiterpenes

Monoterpenes and sesquiterpenes are prevalent in the essential oils of plants and represent the most abundant and varied classes within the genera *Dracocephalum*, *Hyssopus*, and *Lallemantia*. In this literature review, with the exception of essential oils from *Dracocephalum tanguticum* Maxim. extracted via supercritical carbon dioxide, which had a combined GC/MS-determined proportion of monoterpenes, oxygenated monoterpenes, sesquiterpenes, and oxygenated sesquiterpenes of less than 70% (Zhang et al., 2007), species such as *D. heterophyllum*, *D. moldavica*, and *D. nutans* all surpassed this percentage threshold (Zhang et al., 2008; Fattahi et al., 2021; Ashrafi et al., 2017). GC-MS analysis of *Dracocephalum integrifolium* Bunge from various regions revealed that these four metabolite classes comprised an average of 95.86% of the total essential oil content (Zhou et al., 2019). Some of these metabolites exhibited toxicity, suggesting their potential for weed management and resistance to pests and pathogens, thereby warranting consideration for the development of novel natural pesticides.

Specific metabolites, such as citral and nerolidol, which are the most abundant in the essential oil of *D. kotschyi*, may also account for its notable *in vitro* antibacterial activity against gram-negative bacteria (Chahardoli et al., 2019). Among *Hyssopus*, *Lallemantia*, and *Dracocephalum*, there was certain overlap in the presence of monoterpene and sesquiterpene metabolites. Furthermore, some metabolites have been identified that are distinguished solely by their optical isomers within these genera. However, the diversity of these shared metabolites is generally less pronounced in *Hyssopus* and *Lallemantia* than in *Dracocephalum*. This distinction underscores the rich chemical landscape of *Dracocephalum*, which may provide a more extensive reservoir of bioactive metabolites for future research and medicinal exploitation.

3.1.2 Diterpenes and triterpenes

The variety of diterpenes and triterpenes detected in *Dracocephalum* plants was far less than that of the monoterpenes and sesquiterpenes. However, it is noteworthy that komaroviquinone, isolated by Uchiyama et al. (2003) from *Dracocephalum komarovii* Lipsky in the early 21st century, has gained particular prominence. In the context of Chagas disease treatment, the natural product komaroviquinone and its derivatives have emerged as promising candidates, offering a novel therapeutic strategy. This study highlights the efficacy of komaroviquinone, which has shown significant anti-trypanosomal activity against Trypanosoma cruzi, the causative agent of Chagas. The potency of this metabolite is especially remarkable, as it demonstrates higher antiprotozoal activity than the standard drug benznidazole, without concurrent toxicity to host cells (Suto et al., 2015). *Dracocephalum forrestii* W.W.Sm. is currently the plant with the most abundant triterpene metabolites, whereas betulinic acid and ursolic acid have been detected in three or more *Dracocephalum* species. During plant desiccation, there may be a general accumulation phenomenon of these antioxidant-rich triterpenoid metabolites (Abbasi et al., 2019). This antioxidant effect originates from the plants themselves, indicating that throughout evolution, plants have developed the capability to synthesize these compounds to protect themselves from oxidative stress.

3.2 Flavonoids

In *Dracocephalum*, 160 flavonoid metabolites were collected, including 94 flavone, 44 flavonol and 22 dihydroflavonoid metabolites. The pioneering documentation of flavonoids within the *Dracocephalum*, as found in current literature, originates from the work of Shamyrina et al., who characterized the presence of apigenin and its glucoside derivative, apigenin 7-*β*-D-glucopyranoside from *D. nutans* (Shamyrina et al., 1975). Subsequently, metabolites such as cosmosiin, pedalitin, and pedaliin were successively isolated from *D. tanguticum* by Zhang et al. (1994). Flavonoids were most widely distributed in vanilla, including isorhamnetin, kaempferol, tilianin, xanthomicrol, 8-hydroxy-salvigenin, chrysoeriol, etc (Sharafi et al., 2014; Zhang et al., 2019). The most widely distributed metabolite are luteolin and its derivatives, which have been identified in *Dracocephalum* (Sharafi et al., 2014). Cosmosiin has also been identified in *D. moldavica*, *D. tanguticum*, and *D. palmatum* (Zhang et al., 2019). Most flavonoids are distributed throughout the plant, but some tend to accumulate in the tissue of specific species. For example, Apigenin-7-*O*-glucoside is predominantly accumulated in the leaves of *Dracocephalum rupestre* Hance (Ferreira et al., 2016); (*2S*)-Isosakuranetin 7-*O*-*β*-D-(6″-o-malonyl) glucopyranoside and (*2S*) -Poncirin can only be detected in the aboveground part of *Dracocephalum fruticulosum* Stephan ex Willd. (Sabrin et al., 2021); chrysoeriol *O*-*β*-D-glucopyranoside was specifically distributed in the aboveground part of *D. nutans* (Conrad et al., 2009); acacetin-7-*O*-(3-*O*-malonyl)-*β*-D-glucopyranoside and acacetin-7-*O*-(2-*O*-malonyl)-*β*-D-glucuronopyranoside are distributed in the aboveground portion of *Dracocephalum foetidum* Bunge (Selenge et al., 2014). In addition, several flavanone glucosides deserve attention. For example, (*2S*) -isosakuranetin 7-*O*-*β*-D-(6″-o-malonyl) glucopyranoside, (*2S*) -poncirin, and isosakuranin, present in *D. fruticulosum*, naringenin-7-*O*-*β*-D-glucuronopyranoside, present in *D. palmatum* (Sabrin et al., 2021; Olennikov et al., 2013). Naringenin-7-*O*-*β*-D-glucuronopyranoside is one of the main substances controlling the bitter taste of plants, which may be a factor affecting the flavor of medicinal materials (Matoušek et al., 2012).

3.3 Chemical metabolites of the first classification

Over the past 20 years, investigators of the *Dracocephalum* genus have made significant strides in successfully isolating and characterizing a plethora of new metabolites. In addition to the aforementioned komaroviquinone, Saeidnia et al. reported the isolation of two novel monoterpene glycosides from *D. kotschyi*: limonen-10-ol 10-*O*-*β*-D-glucopyranoside and limonen-10-ol 10-*O*-*β*-D-glucopyranosyl-(1→2)-*β*-D-glucopyranoside. Regrettably, these metabolites did not exhibit the potent trypanocidal activity observed for limonene-10-aL (Saeidnia et al., 2004). In 2008, Dai et al. extracted 1′-methyl-2′-hydroxyethyl ferulate (a ferulic acid ester) from *Dracocephalum peregrinum* L., which demonstrated a modest inhibitory effect on nitric oxide (NO) production. Cellular viability assays using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] indicated that this metabolite did not significantly affect RAW264.7, at efficacious concentrations (Dai et al., 2008).

In 2009, Fu et al. identified three flavonoid glycosides (Peregrinumin A, B, and C) and one cyanogenic glycoside (Peregrinumcin A) from *D. peregrinum*. These metabolites displayed commendable anti-inflammatory activity *in vitro*, effectively suppressing the production of nitric oxide (NO) in RAW 264.7 and 293 cells activated by lipopolysaccharide (LPS). Notably, Peregrinumin A and Peregrinumcin A exhibit robust anti-inflammatory effects at a dose of 100 mg/mL (Fu et al., 2009). In the same year, Wang et al. isolated four novel spermidine glycosides, dracotanosides A-D, from *D. tanguticum*, yet their biological activities and functions were not elucidated (Wang et al., 2009). In 2009, Li et al. discovered four new metabolites from *D. forrestii*, among which only 3,4,5-trimethoxyphenylethanol *β*-D-glucopyranoside demonstrated modest anti-inflammatory effects (Li et al., 2009).

In 2011, Zeng et al. reported that among the four glycosides found in *D. tanguticum*, benzyl-6-[(*2E*)-2-butenoate]-*β*-D-glucopyranoside exhibited moderate inhibitory activity against NO with an IC_{50} value of 64.33 μM (Zeng et al., 2011). In 2017, Deng et al. identified two metabolites from *Dracocephalum taliense* Forrest ex W.W.Sm., one being the abietane diterpenoid 12-methoxy-18-hydroxy-sugiol and the other a highly oxygenated ursane triterpenoid named 2*α*,3*α*-dihydroxy-11*α*,12*α*-epoxy-urs-28,13*β*-olide. While these did not show significant anti-inflammatory activity, the latter metabolite displayed potential antitumor activity, exhibiting marked cytotoxic effects on the HepG2 (human liver cancer cell line) and NCI-H1975 (human lung adenocarcinoma cell line) with IC_{50} values of 6.58 ± 0.14 μM and 7.17 ± 0.26 μM, respectively (Deng et al., 2017). In 2019, Ma et al. discovered three new phenylacetamide glycosides, dratanguticumides A-C, from *D. tanguticum*. These metabolites showed moderate antihyperglycemic activity, as determined by the glucose consumption rate in 3T3-L1 adipocytes, with rates of 20.80% ± 1.47%, 21.48% ± 2.44%, and 21.57% ± 1.35%, respectively (Ma et al., 2020). In 2021, Zhang et al. identified eight lignans from *D. moldavica* whose biological activities remain to be elucidated (Zhang et al., 2021).

4 Ethnopharmacological uses

The genus *Dracocephalum*, along with its closely related genera *Hyssopus* and *Lallemantia*, presents a rich tapestry of ethnomedical uses across various plant parts such as the roots, stems, leaves, flowers, and seeds. Our comprehensive compilation verified 32 medicinal species within *Dracocephalum*, complemented by four within *Hyssopus* and two within *Lallemantia*, all taxonomically confirmed in the field of botanical science. The application of the whole herb is notably dominant in *Dracocephalum* and *Hyssopus*, representing a substantial 71.9% and 75.0% of the medicinal species from these genera, respectively. Conversely, the medicinal accounts for *Lallemantia* are solely attributed to its seeds (Table 2). To delineate the traditional medicinal attributes of these genera in more detail, we draw on the previous classification (Li et al., 2012a). The traditional medicinal properties have been categorized into 11 therapeutic applications, which encompass a spectrum of conditions, including gastrointestinal diseases; musculoskeletal issues;

cardiovascular diseases; diabetes and urological disorders; gynaecological conditions; ailments of the ears, throat, mouth, and eyes; dermatological conditions; liver and gall bladder diseases; respiratory diseases; colds and fevers; and others (see Supplementary Table S2). We have consolidated this information and presented it visually in Figure 3, which clearly demonstrates that the traditional efficacy of these three genera is predominantly concentrated in addressing respiratory diseases, liver and gall bladder diseases.

4.1 Tibetan plateau and adjacent regions ethnomedicines

Some *Dracocephalum* species are distributed across the QTP and its surrounding regions, establishing themselves as an indispensable element of local and regional ethnopharmacopeia. These plants have become an integral part of traditional medicine for neighboring ethnic groups and countries, underscoring their importance in the ethnomedical landscape.

In Tibetan pharmacopeias dating back to the 17th century, a record exists of a Tibetan medicinal material known as "Priyangu," characterized by its flowers that resemble fluttering blue flags and its whole plant possessing effects in alleviating liver heat and hemostasis. In contemporary Tibetan medicine, *D. tanguticum* isconsidered the mainstream variety of "Priyangu" (Tashi et al., 2022). However, some scholars from China's ethnic minorities suggest that because of ambiguous botanical descriptions in ancient Tibetan medical literature, *Dracocephalum calophyllum* Hand.-Mazz. and *Dracocephalum isabellae* Forrest ex W.W.Sm. may also be used as "Priyangu" to alleviate liver heat (He et al., 2015). Unfortunately, literature regarding the chemical metabolites and pharmacological properties *D. calophyllum* and *D. isabellae* is lacking.

D. forrestii is a Tibetan medicinal plant that is indigenous to the mountainous regions of Yunnan Province, China. The aerial parts of *D. forrestii* are used as diuretic, astringent, and antipyretic agents (Weremczuk-Jeżyna et al., 2020). Given its use as a substitute for *D. tanguticum*, *D. forrestii* has emerged as a medicinal plant that warrants in-depth investigation of its pharmacological activities and clinical applications. *D. taliense* is a species endemic to Yunnan Province. Local communities utilize the entire plant of *D. taliense* for the treatment of hepatic disorders. The plant has demonstrated significant efficacy particularly in cases of hepatitis and jaundice, as well as for the regulation of gastrointestinal health (Deng et al., 2017).

D. heterophyllum is recognized as one of the traditional Tibetan medicinal plants within the *Dracocephalum* genus. In the traditional Tibetan medical system, *D. heterophyllum* is also known as "Ao-Ga" or "Ji-Mei-Qing-Bao," and has been utilized by Tibetan physicians to treat various conditions, including jaundice, liver diseases, coughs, lymphangitis, oral ulcers, and dental disorders (Li et al., 2022). Additionally, the Uyghur people employ *D. heterophyllum* to treat certain cardiovascular and respiratory diseases (Jiang et al., 2018).

D. integrifolium is predominantly found in Central Asia and is referred to as "Marzan Juxi" in traditional Uyghur herbal medicine, where it is used for the treatment of coughs and asthma (Zhou et al., 2019). *D. peregrinum* is widely distributed across Russia, Mongolia, and Northern China. In the traditional medicine of the Kazakh people in Xinjiang, *D. peregrinum* is known as "Tekanbasjelanbas," and the entire plant is commonly used for treating colds and liver diseases (Yan et al., 2020; Dai et al., 2008).

4.2 Ethnopharmacological heritage beyond the Tibetan plateau

Beyond the QTP and its surrounding regions, we provide a comprehensive overview of the utilization of *Dracocephalum* medicinal plants in other countries and regions.

For example, *D. rupestre* is widely distributed across various provinces in China, including Hebei, Shanxi, Qinghai, Inner Mongolia, and Liaoning. According to records in the "Chinese Herbal Medicine Dictionary," the entire plant of *D. rupestre* is used to treat conditions such as externally contracted wind-heat, headache with chills fever, cough, jaundice, and hepatitis. Additionally, the tender stems and leaves of *D. rupestre* form a traditional Chinese medicinal food, known as "Maojian" tea (Wang et al., 2022).

D. austriacum, and *D. botryoides* are characteristic *Dracocephalum* species found in the Caucasus region. In Azerbaijan, these two species are considered medicinal. Local populations add the aerial parts of these plants into noodle soups to treat respiratory and gastrointestinal disorders. In local folk medicine, herbal decoctions of *D. botryoides* are used to treat liver diseases, gastritis, and ulcers, whereas those of *D. austriacum* possess anti-inflammatory properties and aid in wound healing (Kashchenko et al., 2022).

D. komarovii predominantly grows in the high-altitude regions of the Western Tian Shan Mountain range. In Uzbekistan, *D. komarovii* is known as "buzbosh," and the local population utilizes the aerial parts of the plant to prepare a tea that is traditionally consumed to address various ailments, including inflammatory conditions and hypertension (Uchiyama et al., 2006).

D. kotschyi is a traditionally used medicinal plant in Iran, referred to as "Zarrin-giah" in Persian. In the traditional Iranian medical system, this plant is used for its antispasmodic, antihyperlipidemic, and analgesic properties. It is also used to treat symptoms such as headaches, fever, inflammation, bruising, rheumatism, gastric disorders, and liver diseases, and serves as an analgesic for complications related to kidney function. In Iran, it is boiled to alleviate rheumatic pain and to facilitate wound healing. Furthermore, it is recognized for its capacity to strengthen the immune system (Ghavam et al., 2021; Heydari et al., 2019).

From the Caucasus to Siberia and China, the whole herb or seedling of *D. calophyllum* has been reported to have effects on clearing the heat of the liver, stomach, and lungs, stopping bleeding, healing sores, eliminating dampness, relieving itching, and treating dizziness, visceral pus, prurigo rheumatism, hematochezia, hematuria, sore mouth incompatibility, edema, and ascites (Jiang, 2015).

D. moldavica is recognized as a *Dracocephalum* species with the most extensive folk medicinal applications, for which we collected data. In the northern Iranian Boulze Mountains, *D. moldavica* is commonly known by the name 'Badarshoo.' It is used as a food

additive in yogurt or as a processed herb. It can effectively treat stomach and liver diseases, headaches, and congestion. In regions spanning Eastern Europe, Siberia, Mongolia, and China, *D. moldavica* has been used extensively for its traditional therapeutic properties. These include its applications in clearing heat and detoxifying, cooling the blood and purging internal 'fire,' alleviating pain, arresting bleeding, and providing relief from cough and asthma. Additionally, it is recognized for its expectorant effects and capacity to protect liver health (Morshedloo et al., 2020; Zhang et al., 2018). In China, *D. moldavica* is the main composition in certain clinical preparations, such as Qinggan Qiwei powder, Niuhuang Shisanwei pill, and Liganhewei pill (Xing et al., 2021).

In addition, *H. officinalis* essential oils have therapeutic effects on coughing, loss of appetite, fungal infections, spasmodic diseases, and antibacterial activity. The biological activity and aroma of essential oils indicate that they can serve as potential antioxidant food ingredients (Sharifi-Rad et al., 2022). Various compositional metabolites of *L. iberica* and *L. royleana* seeds such as proteins, oils, fatty acids, and carbohydrates play a significant role in their commercial value of the seeds. The oils, composition of fatty acids (linolenic acid, linoleic acid, and oleic acid), and mucilage are the main resources in *Lallemantia* seeds that are used by the food and pharmaceutical industries (Paravar et al., 2023). *L. royleana* is used as a diuretic, nourishing, aphrodisiac, and cough suppressant in traditional and folk medicine in Iran, and is used to treat various neurological, liver, and kidney diseases (Mohammed et al., 2022). The traditional application of the plant indicates that the medicinal value of *Dracocephalum* mainly focuses on clearing away heat and protecting the liver, which echoes the anti-inflammatory and hepatoprotective effects of *Dracocephalum* in modern pharmacological research, possibly through phenols, flavonoids, and terpenoids in *Dracocephalum*. Therefore, combining the anti-inflammatory and hepatoprotective effects of *Dracocephalum* in clinical practice, and effectively improving its clinical utilization rate will be the focus of *Dracocephalum* plant research.

5 Pharmacological activities

The pharmacological properties of *Dracocephalum* have attracted considerable attention in recent years. The main pharmacological activities of *Dracocephalum* include hepatoprotective, anti-inflammatory, antimicrobial, anti-hyperlipidemic, and antitumor. In addition, recent research has reported novel pharmacological effects. Table 3 lists some *in vitro* and *in vivo* pharmacological models and related dosage information used to clarify the pharmacological activities of *Dracocephalum*. The main pharmacological activities of *Dracocephalum* are as follows.

5.1 Hepatoprotective

Elevated levels of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are important markers of LPS and CCl4-induced liver injury (Weber et al., 2003; Xiong et al., 2017). Zhu et al. (2018) established a Kunming mice liver injury model induced by acute CCl_4 and confirmed that the extract of phenols from *D. rupestre* reversed the increase in serum ALT and AST levels induced by CCl_4 in a dose-dependent manner, and lowered them to near-normal levels (Zhu et al., 2018). *D. heterophyllum* can reduce plasma ALT, AST, IFN-γ, and TNF-α concentration in experimental autoimmune uveitis (EAU) mouse models at a dose of 20 mg/kg, indicating that *D. heterophyllum* has certain advantages in protecting against liver damage (Zheng et al., 2016). The MTT cell viability experiment showed that the extract of *H. officinalis*, at a concentration of 500 μg/mL, did not exhibit hepatotoxicity in mouse FL83B hepatocytes. Furthermore, this concentration of the *H. officinalis* extract displayed a protective effect against both palmitic acid-induced and acetaminophen-induced hepatotoxicity. This suggests that it may contribute to the mitigation or prevention of hepatocellular damage induced by these chemical agents to a certain extent.

5.2 Anti-inflammatory activity

Anti-inflammatory drugs are the second largest class of drugs after anti-infective drugs. Therefore, exploring the anti-inflammatory effects of natural products has become a new trend in the research and development of novel anti-inflammatory drugs (Li et al., 2012b). The anti-inflammatory effects of *Dracocephalum* have also been extensively studied, including the anti-inflammatory mechanism underlying its traditional use for treating typhoid fever, common cold, cardiovascular diseases, gastritis, sore throat, rheumatism, pulse disease, and scabies (Sadraei et al., 2017; Nie et al., 2021).

Current research has shown that at the third and fourth hours after administration of carrageenan, the highest dose of 200 mg/kg of methanol extract from *H. officinalis* has a significant inhibitory effect on rat foot edema (Mićović et al., 2022). *In vitro* inflammatory models are often established using LPS-stimulated macrophages (RAW 264.7 cells). Toshmatov et al. isolated new Monoterpene glucosides—komarovin B and komarovin C—from *D. komarovii*, and found that they inhibited LPS-induced NO production in macrophages to alleviate inflammation at concentrations of 1, 10, 50, and 100 μM (Toshmatov et al., 2019). The anti-inflammatory experiment using RAW 264.7 cells showed that dracocephalumoid A, uncinatone, trichotomone F, and caryopterisoid C from *D. moldavica* can inhibit LPS-induced TNF-α, IL-1β or the production of NO has a significant inhibitory effect, with IC_{50} values ranging from 1.12 to 5.84 μM (Nie et al., 2021).

5.3 Antimicrobial

Studies have shown that *D. moldavica* contains geranyl acetate, geranial, and neral, and can effectively inhibit *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhimurium*, and *Listeria monocytogenes* (Aćimović et al., 2022). Essential oil of *H. officinalis* effectively shows antifungal activity at concentrations of 500 and 1,000 μg/mL, with the main active metabolites being isopiperitenone, pinocampheol, and α-pinene (Harčárová et al., 2021). Rosmarinic acid, methyl rosmarinate, butyl rosmarinate, and salvigenin significantly inhibit *E. coli*, *Candida albicans*, and *S. aureus* growth in *H. cuspidatus* (Shomirzoeva et al., 2020). The

antibacterial activity of *D. kotschyi* is mainly indicated by its inhibition of *S. aureus* and *E. coli* (Moridi Farimani et al., 2017). *L. iberica* showed antibacterial activity against *E. coli*, *Pseudomonas aeruginosa*, *S. aureus* and *Enterobacter aerogenes*, and the largest inhibitory activity was observed against *P. aeruginosa* and *E. aerogenes* (Yilmaz Kardas et al., 2023).

5.4 Antihyperlipidemic

Through a rat model, Aslian et al. validated that the *D. kotschyi* can upregulate the expression of p-FOXO1, p-AKT, and PPARγ and downregulate the expression of FOXO1, p-JNK, and SREBP-1 at concentrations of 0.25 and 0.5 mL/rat to exert antihyperlipidemic activity. In the pathogenesis and treatment of hyperlipidemia, multiple key signaling factors play significant roles. PPARγ, as a primary regulator of adipocyte differentiation and lipid metabolism, can promote the storage of fatty acids and the breakdown of lipids when activated, contributing to the reduction of blood lipid levels. FOXO1, acting as a transcription factor, affects lipid metabolism by inhibiting the activity of PPARγ. Phosphorylated FOXO1 (p-FOXO1) is usually associated with the suppression of FOXO1 activity, and its increase may help to reduce the negative impact of FOXO1 on lipid metabolism. Additionally, SREBP-1 influences blood lipid levels by regulating the synthesis of cholesterol and fatty acids. The activation of the AKT signaling pathway, particularly in the form of p-AKT, is crucial for improving insulin resistance and regulating lipid metabolism. The activation of p-JNK is often related to cellular stress responses and inflammatory reactions, and it may exacerbate insulin resistance by promoting the activation of FOXO1 (Aslian and Yazdanparast, 2018). The total flavonoid extract of *D. moldavica* can significantly improve hyperlipidemia in rats by regulating TG, LDLC, HDLC, ICAM-1, VCAM-1, PCNA, and other indicators at doses of 21, 42, and 84 mg/kg and exhibited a dose-dependent effect (Quan et al., 2017). In addition, the study on the protective effects of *L. royleana* seed polysaccharides against liver and kidney injury in rats induced by a high-cholesterol diet indicates their potential to safeguard hepatic and renal functions and tissue integrity, thereby providing a novel avenue for natural products in the management of hyperlipidemia (Mohammed et al., 2022).

5.5 Antitumor

Generally, when evaluating the antitumor activity of natural products, we mainly investigate their ability to inhibit tumor cell proliferation and promote immune cells to secrete cytokines acting on tumor cells (Zhang et al., 2019). Studies have shown that the methanolic extract of *H. officinalis* can increase apoptosis, reduce cell division, reduce tumor volume, and prolong survival in C6 glioma cells, mainly due to the upregulation of p53 and p21 mRNA expression (Khaksar et al., 2022). The *D. taliense* was tested for its antitumor activity against HepG2 cells using flavonoids at concentrations of 10, 20, 40, and 80 μM (Deng et al., 2017).

5.6 Other pharmacological activities

The hypoglycemic effects of *D. tanguticum* were confirmed in 3T3-L1 cells. It exerted moderate effects at a final concentration of 25 μM (Ma et al., 2020). The total flavonoids of *D. moldavica* (25, 50, 100 mg/kg/d) can upregulate MDA, SOD, and GSH-Px expression and downregulate IL-6, IL-8, and TNF-α expression in the brain tissue of model rats, effectively improving myocardial ischemia (Su et al., 2010). Frosmaric acid and oleanolic acid in *D. moldavica* also improve memory impairment (Deepa et al., 2020), whereas tilianin, luteolin, and apigenin improve vascular dementia (Liu M. et al., 2021). The antiviral effects of isosakuranetin glycosides and phenylpropanoid oligomers from *D. foetidum, D. nutans,* and *D. fruticulosum* have been confirmed through *in vitro* experiments (Sabrin et al., 2021). In animal models of systemic and local allergic reactions, Kim et al. found that the water extract of *D. argunense* regulates TNF-α and IL-6 in a dose-dependent manner, indicating its anti-allergic effect (Kim and Shin, 2006).

6 Discussion

Dracocephalum is a large and complex group of plants, and the intricate relationships between it and closely related genera can be discerned from the classification methods applied by scholars throughout history (Chen Y. P. et al., 2022). Preliminary pharmacophylogeny studies suggest that a certain regularity exists among medicinal plants of the genus *Dracocephalum*; species that are phylogenetically closer tend to exhibit some aggregation in terms of geographical distribution, chemical metabolites (the distribution pattern of these metabolites), and therapeutic effects (as reflected in ethnopharmacology and pharmacological activity) (Abbasi et al., 2022). We used Microsoft Excel 2010 software for statistical analysis and Cytoscape 3.7.2 software for visual presentation, establishing a network diagram (Figure 5). This diagram integrated data on plant species, metabolite profiles, pharmacological activities, traditional uses, and medicinal components of 21 medicinal plants from genus *Dracocephalum* and its related genera for a visual analysis. This approach facilitated the exploration of their untapped potential. *D. moldavica*, *D. heterophyllum*, *D. forrestii*, *H. cuspidatus*, and *H. officinalis* all show higher connectivity. Extensive research has been conducted on the metabolites and pharmacological effects of these species, which have significant traditional applications and are known for their terpenoids, flavonoids, and phenylpropanoids with high chemical connectivity. These metabolites have been found to possess hepatoprotective properties, as well as anti-inflammatory, antitumor, and antimicrobial effects (Figure 5). *D. moldavica* and *H. cuspidatus* had the most reported types of metabolites, with relatively balanced proportions. *D. heterophyllum*, *D. kotschyi*, and *D. forrestii* have several reported metabolites, particularly terpenoids in the studied types. In contrast, reports on *D. austriacum*, *D. rupestre*, *D. botryoides*, and *D. palmatum* are predominantly related to their flavonoid and phenylpropanoid contents. This phenomenon indirectly reflects the medicinal tendencies of these species, illustrating a potential dialectical relationship between the traditional efficacy and metabolite diversity.

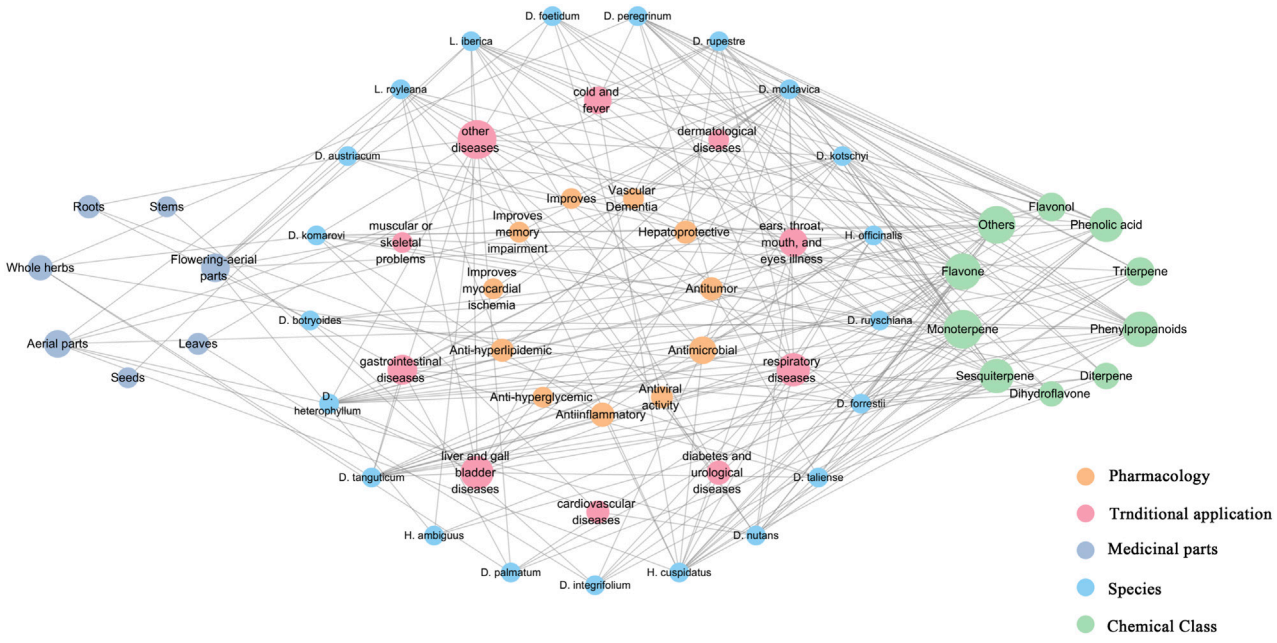

FIGURE 5
Network analysis of genus *Dracocephalum*-chemical metabolites-traditional application and pharmacological activities. (We used Microsoft Excel 2010 software for statistical analysis and Cytoscape 3.7.2 software for visual presentation and established a network diagram. Nodes represent medicinal plants, chemical metabolites, traditional applications, and medicinal parts of *Dracocephalum*, and edges are connected to represent their mutual relationship; Connectivity indicates the number of edges directly associated with a node in the network diagram. The greater the connectivity, the wider the network of the node.

The phylogenetic tree established by Chen G. W. et al. (2022) featured nine clades, eight of which contained species of medicinal varieties. However, the functions of many of these medicinal plants are only documented in traditional applications, without clear pharmacological mechanisms. *D. moldavica* is recognized as the *Dracocephalum* species with the richest known diversity of compounds and the most extensive pharmacological activities. However, some of its traditional medicinal claims remain unverified. Phylogenetic analysis has demonstrated that *D. moldavica*, *D. heterophyllum*, and *D. kotschyi* are closely related. In traditional ethnomedical practices, these species are utilized for the treatment of liver and gall bladder diseases. Specifically, *D. moldavica*, *D. heterophyllum*, and *D. kotschyi* are reputed to possess therapeutic effects against hepatitis, jaundice, and liver pain, respectively. However, in our survey, we identified only studies concerning the ethanol extract of *D. heterophyllum* and its impact on ConA-Induced Acute Hepatitis. Therefore, many extended discussions on the efficacy of medicinal plants are based on traditional applications. Medicinally related species include *D. foetidum* and *D. komarovii*, as well as some closely related plants that have not yet been documented, including *Dracocephalum stamineum* Kar. & Kir., *Dracocephalum renati* Emb., and *Dracocephalum spinulosum* Popov. The lack of documentation for these species may be attributed to their perceived lack of significant medicinal properties or because their potential remains undeveloped or has not been fully investigated. Medicinal plants, such as *D. nutans*, *D. integrifolium*, and *H. cuspidatus* are closely related and form a single branch in the phylogenetic tree. They are all effective in treating respiratory diseases; therefore, we speculate that closely related species such as *Dracocephalum psammophilum* C.Y.Wu & W.T.Wang and *Hyssopus seravschanicus* (Dubj.) Pazij may have a similar efficacy. Similarly, the clade comprising *D. calophyllum*, *D. forrestii*, *D. taliense*, and *D. tanguticum* is known to exhibit therapeutic effects on gastrointestinal conditions, raising the question of whether *Dracocephalum propinquum* W.W.Sm. and *Dracocephalum microphyton* Y.P.Chen, Y.S.Chen & C.L.Xiang share similar beneficial properties, meriting further investigation. Furthermore, although neurological disorders are grouped within the category of "Other diseases," the shared efficacy of *L. royleana* and *L. iberica* in addressing neurological as well as hepatic and renal diseases is particularly noteworthy. Consequently, the closely related species *Lallemantia peltata* (L.) Fisch. & C.A.Mey. and *Dracocephalum scrobiculatum* Regel should also garner the interest of researchers because of their potential applications in this area. In addition, *D. rupestre*, which has the potential for use in medicine and food, can be used as a tea. According to the theory of consanguinity, we speculate that *D. bipinnatum* and *Dracocephalum adylovii* I.I.Malzev are likely homologous plants used in medicine and food. Therefore, there is an urgent need to provide a suitable theoretical and technical system for medicinal and edible homologous items, enhance the scientific connotations of medicinal and edible homologous items, and allow people to use medicinal and edible homologous items to meet the needs of contemporary health and disease (Hao et al., 2022; He et al., 2022; Zhang et al., 2022; Pan et al., 2022; Selvaraj and Gurumurthy, 2022; Yao et al., 2022).

TABLE 1 Distribution of specialized metabolites in *Dracocephalum, Hyssopus*, and *Lallemantia*.

Section/Series	Species	Terpenoids				Flavonoid			Phenylpropanoids	Phenolic acid	Others
		Monoterpene	Sesquiterpene	Diterpene	Triterpene	Flavone	Flavonol	Dihydroflavone			
Sect. Dracocephalum	*D. moldavica*	※**	※	***	*	****	**	*	※****	****	※**
	D. heterophyllum	※※***	※※		**	**	*		**	*	※※**
	D.peregrinum	※	***		**	※	***	*	***	**	※
Sect. Sinodracon	*D. forrestii*	**	***	*	****	*		*	****	**	**
	D. taliense			***	*					*	*
	D. tanguticum	※*	****	*		***		*	***		※*
Sect. Calodracon	*D. rupestre*				*	**	*	**		*	*
Sect.Idiodracon	*D.integrifolium*	****	***								*
	D. nutans	※*	*								
Sect. Ruyschiana	*D.ruyschiana*					**	*		**	*	*
Ser. Angustifoli	*H. cuspidatus*	※***	*		*	***	***		※※	***	※**
Ser. Officinales	*H. officinalis*	※※**	****			**	*		*		*
Others	*D. palmatum*				*	***		**	**		
	D. foetidum	※*	*			***			**		
	D. kotschyi	※※****	※	*	*	**	*		*		***
	D. austriacum					***			****	***	
	D. botryoides					***		**	***	****	
	D. komarovi	*		*	*					*	*
	L. iberica	**	*						*	*	**
	L. royleana	※**	***								**

Note: *, 1–5 metabolites; **, 6–10 metabolites; ***, 11–15 metabolites; ****, 16–20 metabolites; ※, 21–25 metabolites; ※*, 26–30 metabolites; ※**, 31–35 metabolites; ※***, 36–40 metabolites; ※****, 41–45 metabolites; ※※, 46–50 metabolites; ※※*, 51–55 metabolites; ※※**, 56–60 metabolites; ※※***, 61–65 metabolites; ※※****, 66–70 metabolites.

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TABLE 2 Geographical distribution and traditional applications of *Dracocephalum*.

Medicinal plant	Region (From the world plants)	Medicinal parts	Traditional therapeutic properties	References
D. argunense	Siberia (Chita); Russian Far East (Amur, Khabarovsk, Primorye); China (Hebei, Heilongjiang, Jilin, Liaoning, Nei Mongol); Mongolia; North Korea South Korea; Japan (Hokkaido, Honshu)	Aerial part, Whole herb	Gastritis, hepatitis, pulmonary phthisis	Saren and Saren (2002) Kim and Shin (2006)
D. austriacum	Switzerland; Austria; Czech Republic; Slovakia; Hungary; NE-Spain; France; Italy; Romania; C-European Russia; SW-Ukraine; Northern Caucasus; Georgia [Caucasus]; Armenia; Turkey (E-Anatolia, NE-Anatolia)	Whole herb, root	Stop bleeding, granulation promoting (wound healingremedy), decrease inflammation	Sokolov (1991) Kashchenko et al. (2022)
D. bipinnatum	Kazakhstan; Kyrgyzstan; Tajikistan; Afghanistan (Badakshan); China (Xinjiang); Tibet; Pakistan (Chitral, Astor, Gilgit, Baltistan, Hunza); NW-India (Jammu and Kashmir)	Stems, leaves, flowers	Relieve cough and asthma, clear heat	Jiang (2015)
D. botryoides	Armenia; Azerbaijan	Whole herb, root	Liver diseases, gastritis, and ulcers	Sokolov (1991) Kashchenko et al. (2022)
Dracocephalum bullatum Forrest ex Diels	China (Yunnan: Lijiang)	Whole herb	Secure the foetus	Jiang (2015)
D. calophyllum	China (Sichuan: Daocheng, Yunnan: Lijiang)	Whole herb, seedling	Liver heat; stomach heat; lung heat; prurigo rheumatism; stop bleeding, heal sore, eliminate dampness and relieve itching, dizziness, visceral pus, hematochezia, hematuria, edema and ascites	Jiang (2015)
Dracocephalum discolor Bunge	Siberia (Altai, Krasnoyarsk, Tuva); Kazakhstan; Kyrgyzstan; Mongolia	Stems, leaves, flowers	Relieve cough and asthma, clear heat	Jiang (2015)
Dracocephalum diversifolium Rupr	Kazakhstan; Uzbekistan; Kyrgyzstan; Tajikistan	Stems, leaves, flowers	Relieve cough and asthma, clear heat	Jiang (2015)
D. foetidum	Siberia (Altai, Krasnoyarsk, Tuva); Mongolia	Leaves, flowers	Fever; oral cavity diseases; rheumatic edema, and wounds, suppurative diseases, prevent bacterial and fungal infections	Lee et al. (2007) Selenge et al. (2014)
D. forrestii	China (Yunnan: Lijiang)	Whole herb, seedling	Liver heat; stomach heat; lung heat; prurigo rheumatism; stop bleeding, heal sore, eliminate dampness and relieve itching, dizziness, visceral pus, hematochezia, hematuria, edema and ascites	Jiang (2015)
D. fruticulosum	Siberia (Buryatia, Tuva); China (Ningxia); Mongolia	Aerial part	Sore throat; jaundice, liver heat, hepatitis; cold; cough; stomach heat, stomach spasm, stomach bleeding, dysentery; food poisoning, green leg disease, clearing heat and drying dampness, cool blood and stopping bleeding, wound healing, headache	Zhu (1993)
D. grandiflorum	Siberia (Altai, Buryatia, Chita, Irkutsk, Krasnoyarsk, Tuva, W-Siberia); Kazakhstan; Kyrgyzstan; Tajikistan; China (Nei Mongol, Xinjiang); Mongolia	Whole herb	Expelling phlegm and relieving asthma; clearing heat and detoxification	Jiang (2015)
D. heterophyllum	Kazakhstan; Kyrgyzstan; Tajikistan; Afghanistan (Wakhan); China (Gansu, Nei Mongol, Ningxia, Qinghai, Shanxi, Sichuan, Xinjiang); Tibet; Mongolia; NE-Pakistan (Deosai); Nepal; N-India (Himachal Pradesh, Jammu and Kashmir, Uttarakhand, Rupshu, Ladakh, Sikkim, Chumb)	Whole herb, stem, leaves, flowers	Cough, chronic bronchitis; goiter and tumor, mouth ulcers, web-eye; liver heat, jaundice; hypertension; clear heat and headache	Jiang (2015)
D. imberbe	Siberia (Altai, Krasnoyarsk, Tuva); Kazakhstan; Turkmenistan; Uzbekistan; Kyrgyzstan; Tajikistan; China (Xinjiang); Mongolia	Whole herb	Expelling phlegm and relieving asthma; clearing heat and detoxification	Jiang (2015)

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TABLE 2 (*Continued*) Geographical distribution and traditional applications of *Dracocephalum*.

Medicinal plant	Region (From the world plants)	Medicinal parts	Traditional therapeutic properties	References
D. integrifolium	Siberia (Altai); Kazakhstan; Uzbekistan; Kyrgyzstan; Tajikistan; China (Xinjiang); Mongolia	Whole herb, aerial parts	Expectorant, antitussive, antiasthmatic, senile chronic bronchitis	Jiang (2015)
D. isabellae	China (Yunnan: Zhongdian Shan)	Whole herb, seedling	Liver heat; stomach heat; lung heat; prurigo rheumatism; stop bleeding, heal sore, eliminate dampness and relieve itching, dizziness, visceral pus, hematochezia, hematuria, edema and ascites	Jiang (2015)
D. komarovii	Kazakhstan; Uzbekistan; Kyrgyzstan; Tajikistan	Aerial parts	Inflammatory diseases and hypertony	Uchiyama et al. (2003)
D. kotschyi	Iran (EC-Iran, N-Iran, S-Iran, W-Iran)	Whole herb, leaves	Stomachache; liver pain; various cancers, headache, relieve pain	Jiang (2015) Poursalavati et al., 2021 Khamesipour et al. (2021) Cham et al. (2022)
D. moldavica	Siberia (Chita, Krasnoyarsk, Tuva, W-Siberia); Russian Far East (Amur, Primorye); Turkmenistan; Tajikistan; Georgia [Caucasus]; Iran (EC-Iran, N-Iran, Iranian Aserbaijan); China (Gansu, Hebei, Heilongjiang, Henan, Jilin, Liaoning, Nei Mongol, Qinghai, Shaanxi, Shanxi); Mongolia; NW-India (Ladakh)	Whole herb, aerial parts	Sore throat; jaundice, liver heat, hepatitis; fever and cold; cough; stomach heat, stomach spasm, stomach bleeding, dysentery; food poisoning, green leg disease, clearing heat and drying dampness, cool blood and stopping bleeding, wound healing, headache, hematemesis	Zhu (1993) Jiang (2015) Morshedloo et al. (2020) Zhang et al. (2018)
Dracocephalum nodulosum Rupr	Kazakhstan; Uzbekistan; Kyrgyzstan; Tajikistan; China (Xinjiang); Mongolia	Whole herb	Expelling phlegm and relieving asthma; clearing heat and detoxification	Jiang (2015)
D. nutans	Siberia (Altai, Buryatia, Chita, Irkutsk, Krasnoyarsk, Tuva, W-Siberia, Yakutia); Russian Far East (Amur, Khabarovsk, Primorye); Kazakhstan; Kyrgyzstan; Tajikistan; Afghanistan (Kunar/Nuristan); China (Heilongjiang, Nei Mongol, Xinjiang); Mongolia; Pakistan (Chitral, Swat, Gilgit, Astor, Deosai, Baltistan); NW-India (Himachal Pradesh, Jammu & Kashmir, Dras, Zanskar)	Whole herb	Cough sputum panting, chronic bronchitis; eye swelling pain, hypertension; dizziness, tinnitus	Jiang (2015)
D. origanoides	Siberia (Altai, Tuva); Kazakhstan; Kyrgyzstan; Tajikistan; China (Xinjiang); Mongolia	Whole herb	Expelling phlegm and relieving asthma; clearing heat and detoxification	Jiang (2015)
Dracocephalum palmatoides C.Y.Wu and W.T.Wang	China (Xinjiang: Toli Shan)	Whole herb	Expelling phlegm and relieving asthma; clearing heat and detoxification	Jiang (2015)
D. palmatum	Siberia (Yakutia); Russian Far East (Kamchatka, Khabarovsk, Magadan)	Young shoots and flowers	Gastro-intestinal tract disorders and alcoholism	Olennikov et al. (2013) Olennikov et al. (2017)
D. paulsenii	Siberia (Tuva); Kazakhstan; Uzbekistan; Tajikistan; Afghanistan (Wakhan); China (Xinjiang); Mongolia; Pakistan (Chitral, Gilgit); NW-India (Jammu and Kashmir)	Stems, leaves, flowers	Relieving cough, relieving asthma and clearing heat	Jiang (2015)
D. peregrinum	Siberia (Altai, Irkutsk, Krasnoyarsk, Tuva, W-Siberia); Kazakhstan; China (Xinjiang); Mongolia	Whole herb	Clear heat and detoxicate; cooling blood and purging fire; relieving cough and asthma; clear phlegm	Yan (2020)
D. rigidulum	China (Nei Mongol)	Aerial part	Jaundice; sore throat; cold; cough; clearing heat and detoxification, stop bleeding, hematemesis, headache	Huang (2022)
D. rupestre	China (Hebei, Liaoning, Nei Mongol, Qinghai, Shanxi); North Korea	Whole herb, aerial part	Jaundice; sore throat; cold; cough; clearing heat and detoxification, stop bleeding, hematemesis, headache	Jiang (2015) Zhu (1993)

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TABLE 2 (*Continued*) Geographical distribution and traditional applications of *Dracocephalum*.

Medicinal plant	Region (From the world plants)	Medicinal parts	Traditional therapeutic properties	References
D. ruyschiana	Norway; Sweden; Germany (+Bayern, +Sachsen-Anhalt); Switzerland; Liechtenstein; Austria; Poland; Slovakia; Hungary; France; Italy; Croatia; Romania; Estonia; Latvia; Lithuania; Belarus; C-European Russia; E-European Russia; N-European Russia; NW-European Russia; Ukraine; Siberia (Altai, Buryatia, Chita, Irkutsk, Krasnoyarsk, Tuva, W-Siberia, Yakutia); Russian Far East (Amur); Kazakhstan; Turkmenistan; Uzbekistan; Kyrgyzstan; Northern Caucasus; Georgia [Caucasus]; Armenia; Turkey (NE-Anatolia); China (Heilongjiang, Nei Mongol, Xinjiang); Mongolia	Whole herb	Sore throat, laryngitis; jaundice, liver heat, hepatitis; cold; acute respiratory infection, cough; stomach heat, stomach spasm, stomach bleeding, gastric ulcers, dysentery, diarrhea; rheumatoid arthritis; food poisoning, green leg disease, clearing heat and drying dampness, cool blood and stopping bleeding, wound healing, headache	Jiang (2015) Zhu (1993)
D. taliense	China (Yunnan: Heqing, Dali)	Whole herb	Jaundice, hepatitis; adjusting the stomach	Jiang (2015) Deng et al. (2017)
D. tanguticum	China (Gansu, Qinghai, Sichuan: Maoxian, Songpan, Ma'erkang, Rangtang, Aba, Hongyuan, Ganzi, Kangding, Daofu, Luhuo, Ganzi, Dege, Shiqu, Seda, Xiangcheng, Daocheng, Derong, Muli); Tibet; Nepal; N-India (Chumbi)	Whole herb, aerial part, seedling	Chronic gastritis, gastric ulcer, epigastric pain; hepatitis, hepatomegaly; cough, sputum; ascites, edema	Jiang (2015) Guo et al. (2022)
Dracocephalum thymiflorum L	Slovakia; Romania; Bulgaria; S-European Russia; Belarus; C-European Russia; E-European Russia; N-European Russia; NW-European Russia; Ukraine; Siberia (Altai, Buryatia, Irkutsk, Krasnoyarsk, Tuva, W-Siberia); Kazakhstan; Northern Caucasus; Georgia [Caucasus]; Iran (EC-Iran, N-Iran)	Whole herb	Expelling phlegm and relieving asthma; clearing heat and detoxification	Jiang (2015)
Hyssopus ambiguus (Trautv.) Iljin ex Prochorov. and Lebel	Siberia (Altai, W-Siberia); Kazakhstan; Mongolia	Aerial part	Reducing swelling and relieving pain, clearing heat and detoxification	Jiang (2015)
H. cuspidatus	Siberia (Altai); Kazakhstan; China (Xinjiang); Mongolia	Whole herb	Relieve cough and asthma; clear phlegm; clear away the lung-heat, tracheitis, cough; cold and fever; tonsillitis; bladder and kidney stones; night sweats, relieve pain	Jiang (2015) Aihaiti et al. (2022) Jia et al. (2022)
H. latilabiatus	China (Xinjiang)	Whole herb	Heat-clearing, detoxification and anti-inflammation. For colds, fever, cough	Jiang (2015)
H. officinalis	Switzerland; Austria; Slovakia; Hungary; Spain; France; Italy; Montenegro; Serbia; Kosovo; North Macedonia; Albania; Bulgaria; C-European Russia; E-European Russia; Ukraine; Crimea; Siberia (Buryatia); Northern Caucasus; Georgia [Caucasus]; Armenia; Turkey (E-Anatolia, NE-Anatolia, SSW-Anatolia); Iran (EC-Iran, N-Iran, Iranian Aserbaijan); Pakistan (Baluchistan, Chitral, Swat); India (Himachal Pradesh, Jammu and Kashmir, Uttarakhand); Myanmar (Kachin, Sagaing)	Whole herb	Carminative and antispasmodic stomachic; gallstones; kidney stones; chronic bronchitis, asthma; tonsillitis; night sweats; rheumatic pains, bruises, wounds, anxiety, relaxation of muscles	Jiang (2015) Tahir et al. (2018) Semerdjieva et al. (2019)
L. iberica	Turkmenistan; Georgia [Caucasus]; Armenia; Turkey (E-Anatolia, Inner Anatolia, N-Anatolia, NE-Anatolia, S-Anatolia, SE-Anatolia, SE-Anatolia: Mesopotamian Anatolia, SSW-Anatolia, W-Anatolia); Iraq (NE-Iraq, NW-Iraq, SE-Iraq: Mesopotamia); Iran (EC-Iran, N-Iran, Iranian Aserbaijan, S-Iran, W-Iran); Lebanon (Antilebanon, C-Lebanon, coastal W-Lebanon); Syria (Jazira, NW-Syria, Jbel Druze, W-Syrian Mountains); Israel (N-Israel, Judean Desert); Jordania (S-Jordania, W-Jordania)	Seeds	Treating stress, fever, cough, nerve, liver, and kidney diseases	Al-Snafi (2019)

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TABLE 2 (*Continued*) Geographical distribution and traditional applications of *Dracocephalum*.

Medicinal plant	Region (From the world plants)	Medicinal parts	Traditional therapeutic properties	References
L. royleana	Siberia (W-Siberia); Kazakhstan; Turkmenistan; Uzbekistan; Kyrgyzstan; Tajikistan; Armenia; Iraq (S-Iraq, W-Iraq: Desert); Iran (EC-Iran, E-Iran, NE-Iran: Mts., N-Iran, Iranian Aserbaijan, S-Iran, W-Iran); Afghanistan (Baghlan, Balkh, Bamyan, Farah, Faryab, Herat, Kabul, Kandahar, Kunar/Nuristan, Laghman, Logar, Nangarhar, Paktia/Khost, Parwan, Zabul); Syria (C-Syrian Desert); Sinai peninsula (S-Sinai); Saudi Arabia (C-Saudi Arabia, N-Saudi Arabia, Midyan, Asir); Kuwait; China (Xinjiang); Pakistan (Chitral, Baluchistan, Kurram, Khyber, Hazara, Kohat, Pakistani Punjab, Rawalpindi, Swat, Baltistan, Skardu); India (Himachal Pradesh, Uttarakhand)	Seeds	Hepatic; renal diseases; sedation, treatment of various nervous	Ghannadi and Zolfaghari (2003) Jiang (2015)

The genus *Dracocephalum* originated from the steppe-desert biomes of Central and West Asia, and the alpine region of the QTP. It has a long history of medicinal uses. Previous studies showed that this genus spread from East and West Asia to QTP alpine region during the Pliocene. Drought on the Eurasian continent has promoted rapid radiation in the region, and the uplift of QTP has promoted the distribution and diversity of species in this genus. *D. forrestii* occurs only in the northwestern mountains of Yunnan Province, China. *D. taliense* is distributed across Dali, Yunnan. *D. integrifolium* is found primarily in Xinjiang, China. *D. nutans* is found throughout Siberia, Eastern Europe, and Kashmir. It is also seen in Heilongjiang, Inner Mongolia, and northern Xinjiang. *D. moldavica*, and *D. heterophyllum* are found in Inner Mongolia, Gansu, Qinghai, and in Eastern Europe. *Dracocephalum paulsenii* Briq., and *Dracocephalum origanoides* Stephan ex Willd. are distributed in Xinjiang and Russia. *Hyssopus latilabiatus* C.Y.Wu & H.W.Li, *H. cuspidatus*, *D. integrifolium*, and *D. nutans* are distributed in Xinjiang. *H. officinalis* is distributed in Europe, Algeria, Morocco, Iran and other places, whereas *D. moldavica*, *D. rupestre*, *D. heterophyllum*, and *H. officinalis* overlap in Inner Mongolia, Shanxi, Gansu, and Qinghai. *H. cuspidatus* has been found in North Africa, India, Mongolia, Russia, and China (Deepa et al., 2020). *L. iberica* originated in the Caucasus and the Middle East and is widely distributed in some western parts of Europe and Asia, while *L. royleana* grows in Iran and almost all parts of Middle East. The genus *Hyssopus* is widely distributed in Central and West Asia, Siberia, and Europe, whereas the genus *Lallemantia* is distributed in West Asia and *L. royleana* is further extended to Central Asia, which is consistent with our research. We preliminary verified the rationality of combining *Hyssopus* and *Lallemantia* with *Dracocephalum* through the spatial distribution pattern map and the suitable Ecotope (Figure 1). From the perspective of spatial distribution patterns, the distribution of genus *Dracoephalum* is wider than that of *Hyssopus* and *Lallemantia*, but the two have overlapping distributions in the European region. From the perspective of suitable Ecotope, *Dracocephalum*, *Lallemantia* and *Hyssopus* were suitable for growing in western North America and western Asia. Consistent with the results of our previous phylogenetic studies, *Sect. Dracocephalum*, *Sect. Sinodracon*, and *Sect. Keimodracon* are phylogenetically close to each other and can be used to verify the phylogenetic relationships between the *Hyssopus*, *Lallemantia*, and *Dracocephalum* genera at the geographical distribution level (Chen Y. P. et al., 2022).

Dracocephalum grandiflorum L., *D. integrifolium*, *D. nutans*, *D. origanoides*, *D. paulsenii*, *D. forrestii*, *D. heterophyllum*, *D. moldavica*, *D. grandiflorum*, *D. bipinnatum*, *D. peregrinum*, *D. rupestre*, *Dracocephalum rigidulum* Hand.-Mazz., *L. iberica* and *Hyssopus* species share similarities in clearing heat, detoxifying, cooling blood, relieving bleeding, treating bronchitis, and relieving cough and asthma. *D. forrestii* has traditional efficacy in the treatment of cardiovascular diseases and rheumatoid arthritis, which has not yet been proven in pharmacological studies. Both modern pharmacological research and traditional application have shown that *D. rupestre*, *D. nutans*, *D. taliense*, *D. tanguticum*, *D. peregrinum*, *D. heterophyllum*, *H. officinalis*, *L. iberica*, and *L. royleana* possess hepatoprotective activities. The Encyclopedia of Chinese Medicine records that the aboveground parts of *D. moldavica* are used to treat all liver disorders (Editorial Board of Chinese Medical Encyclopedia, 1992). Terpenoids and flavonoids are abundant in closely related species, among which, monoterpenes are the most common, and the dominant flavonoids are flavones and their glycosides (Table 1). We speculate that the traditional therapeutic properties of clearing heat and detoxifying, treating bronchitis, relieving cough, and relieving asthma may be related to the content of monoterpenes and flavonoids in *D. heterophyllum*, *D. rupestre*, *D. forrestii*, *D. integrifolium*, *D. nutans*, *D. origanoides*, *L. iberica*, *H. latilabiatus*, *H. cuspidatus*, which supported the anti-inflammatory activity of *H. cuspidatus* to a certain extent. According to the theory of pharmacosystematics, *D. integrifolium*, *D. nutans*, *D. rigidulum*, *D. origanoides*, *D. forrestii*, and *L. royleana* may be a new potential resource for the development of anti-inflammatory drugs. Both *L. royleana* and *D. moldavica* exhibit antibacterial, and anti-hyperlipidemic activities, possibly owing to the presence of soluble fiber and polysaccharides. Moreover, triterpenoids were concentrated in *H. cuspidatus*, *D. taliense* and *D. forrestii* of *Sect. Sinodracon*, but *Ser. Angustifolii* and *Sect. Sinodracon* and *Sect. Idiodracon* exhibited antibacterial, anti-tussive, and anti-asthmatic effects, implying that known triterpenoids might not be the only

TABLE 3 Pharmacological activities of the medicinal plants of genus *Dracocephalum* and its related genera.

NO	Activities	Species	Extract(s) or main metabolites	Types of study (*In vivo*/*In vitro*)	Dose	Key findings/Mode of action or biochemical and histopathological parameters studied	Reference
1	Hepatoprotective	*D. rupestre*	Phenylpropanoids: rosmarinic acid Flavonoids: eriodictyol	*In vivo* (mice) SOD, MDH, LDH, ALT, AST	50, 100, or 200 mg/kg	↓ALT ↓AST ↓MDH ↓LDH (Excl. 50 mg/kg)	Zhu et al. (2018)
		D. heterophyllum	-	*In vivo* (mice) *In vitro* (Kupffer cells)	20 mg/kg	↓Plasma ALT, AST, ↓IFN-γ, TNF-α	Zheng et al. (2016)
		H. officinalis	-	*In vitro* (FL83B mouse hepatocytes)	500 μg/mL	↑MTT cell viability	Chen et al. (2022a)
2	Anti-inflammatory	*D. komarovii*	Terpenoids: komarovin B, komarovin C, limonen-10-ol 10-*O*-*β*-D-glucopyranosyl-(1→2)-*β*-D-glucopyranoside	*In vitro* (RAW 264.7 cells)	1, 10, 50, 100 μM	↓NO	Toshmatov et al. (2019)
		D. moldavica	Terpenoids: Dracocephalumoid A-E Uncinatone Trichotomone F Caryopterisoid C	*In vitro* (RAW 264.7 cells)	1.12–5.84 μM	↓TNF-α, IL-1β ↓NO	Nie et al. (2021)
		H. officinalis	-	*In vivo* (Rats) *In vitro* (COX-1, COX-2)	50, 100, 200 mg/kg 5, 10, 20 μg/mL	↓COX-1 and COX-2 enzymes	Mićović et al. (2022)
		H. cuspidatus	Phenylpropanoid: Hyssopuside	*In vitro* (RAW 264.7 cells and mouse peritoneal macrophages)	10, 20, 40, 80 μM	↓NF-κB ↓TNF-α, IL-6, IL-1β ↓NO	Liu et al. (2021b)
3	Antimicrobial	*D. moldavica*	Essential oil Terpenoids: Geranyl acetate Geranial, Neral	*In vitro* (*Bacillus cereus*, *Escherichia coli*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, and *Staphylococcus aureus*)	6.5 g/kg successive dilutions (100%–0.39%)	↓*E. coli* ↓*L. monocytogenes* ↓*S. typhimurium* ↓*S. aureus*	Aćimović et al. (2022)
		H. officinalis	Essential oil Terpenoids: isopiperitenone, pinocampheol, α-pinene	*In vitro* (*Fusarium graminearum* CCM F-683 and CCM 8244)	100, 500, 1,000 μg/mL	↓*F. graminearum* CCM F-683 and CCM 8244 growth (Excl. 100 μg/mL)	Harčárová et al. (2021)
		H. cuspidatus	Phenylpropanoids: caffeic acid, rosmarinic acid, oresbiusin A Flavonoids: salvigenin Others: daucosterol	*In vitro* (*Escherichia coli*, *Candida albicans* and *Staphylococcus aureus*)	-	↓*E. coli* ↓*C. albicans* ↓*S. aureus*	Shomirzoeva et al. (2020)
		D. integrifolium	Essential Oils Terpenoids: sabinene, eucalyptol	*In vitro* (*Bacillus subtilis*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Saccharomyces cerevisiae*, and *Candida albicans*)	-	↓*B. subtilis* ↓*P. aeruginosa* ↓*E. coli* ↓*S. cerevisiae* ↓*C. albicans*	Zhou et al. (2019)
		D. kotschyi	Terpenoids: limonene, perilla aldehyde	*In vitro* (*Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922)	-	↓*S. aureus* ↓*E. coli*	Moridi Farimani et al. (2017)
		L. iberica	Flavonoids: Rutin hydrate Phenylpropanoids:p-coumaric acid	*In vitro* (*Escherichia coli* ATCC-8739, *Staphylococcus aureus* ATCC-6538,*Pseudomonas aeruginosa* ATCC-9027 and *Enterobacter aerogenes* ATCC-13048)	0–100 mg/L	↓*P. aeruginosa* ↓*E. coli* ↓*S. aureus* ↓*E. aerogenes*	Yilmaz Kardas et al. (2023)

(Continued on following page)

TABLE 3 (*Continued*) Pharmacological activities of the medicinal plants of genus *Dracocephalum* and its related genera.

NO	Activities	Species	Extract(s) or main metabolites	Types of study (*In vivo*/*In vitro*)	Dose	Key findings/Mode of action or biochemical and histopathological parameters studied	Reference
		L. royleana	-	*Staphylococcus aureus Enterobacter cloacae, Pseudomonas aeruginosa* and *Escherichia coli*	10,50,100 mg/mL	↓↓*S. aureus* ↓*E. cloacae* *P. aeruginosa* ↓*E. coli*	Mahmood et al. (2015)
4	Antihyperlipidemic	*D. kotschyii*	-	*In vivo* (Rats) *In vitro* (3T3-L1 cells)	0.25, 0.5 mL/rat 4 μL/mL	↓fasting blood glucose level, TC, TG, LDL; ↑HDL ↑p-FOXO1, p-AKT, PPARγ; ↓p-JNK, FOXO1, SREBP-1	Aslian and Yazdanparast (2018)
		D. moldavica	Total flavonoids	*In vivo* (Rats)	21 mg/kg, 42 mg/kg, 84 mg/kg	↓TG, LDLC ↑HDLC ↓ICAM-1, VCAM-1, PCNA (Excl. 21 mg/kg)	Quan et al. (2017)
		L. royleana	polysaccharide	*In vivo* (Rats)	200 mg/kg	↑GSH, Vitamin C, GPx, SOD ↓MDA, AOPP ↓CT, TG, LDL ↓AST, ALT, CK, Gamma GT, ALP, Urea, Creatinine, Uric acid	Mohammed et al. (2022)
5	Antitumor	*D. kotschyi*	Flavonoids: Calycopterin, Xanthomicrol	*In vivo* (mice)	20 mg/kg	↓Cell proliferation, VEGF activity	Zamani et al. (2016)
		H. officinalis	Total flavonoids, phenolic	*In vivo* (Rats) *In vitro* (C6 glioma cell)	100 mg/kg 50, 100, 200, 400, and 600 μg/mL	↑p53 and p21 mRNA; ↓SOD, CAT in tumor tissue ↓MTT cell viability (Excl. ≤100 mg/kg)	Khaksar et al. (2022)
		D. taliense	Flavonoid: 12-methoxy-18-hydroxy-sugiol, 2α,3α-dihydroxy-11α,12α-epoxy-urs-28,13β-olide	*In vitro* (HepG2 Cells, NCI-H1975)	0, 0.128, 0.256, 0.512, 1, 2, 5, 10, 20, 40, and 80 μM	-	Deng et al. (2017)
6	Anti-hyperglycemic	*D. tanguticum*	Phenylpropanoids: dratanguticumide B, dratanguticumide C Phenolic acids: dratanguticumide A	*In vitro* (3T3-L1 cells)	25 μM	↓glucose consumption rate	Ma et al. (2020)
	Improves myocardial ischemia	*D. moldavica*	Total flavonoids	*In vivo* (Rats)	2, 5, 12.5 μg/mL	↑LVDP, ±dp/dtmax, CF, HR, SOD, GSH/GSSG ↓CK, LDH, MDA	Jiang (2015)
7	Improve cerebral ischemia	*D. moldavica*	Total flavonoids	*In vivo* (Rats)	25, 50, 100 mg/kg	↓Brain tissue IL-6, IL-8, TNF-α ↑Brain tissue MDA, SOD, GSH-Px	Jia et al. (2017)
8	Improves memory impairment	*D. moldavica*	Phenylpropanoids: rosmarinic acid, oleanolic acid	*In vivo* (mice)	25, 50 and 100 mg/kg, p.o	↑ERK-CREB signaling cascade	Deepa et al. (2020)
9	Improves vascular dementia	*D. moldavica*	Total flavonoids Flavonoid: tilianin, luteolin, apigenin	*In vitro* (SH-SY5Y cells)	25, 50, 100 μg/mL	↑miR-3184–3p ↓miR-6875–5p	Liu et al. (2021a)
10	Antiviral activity	*D. foetidum D. nutans, D. fruticulosum*	Flavonoid: isosakuranetin glycosides; Phenylpropanoid oligomers	*In vitro* (Cells)	3.25–5.75 log10 TCID 50/mL	-	Sabrin et al. (2021)
11	Anti-allergic activity	*Dracocephalum argunense*	-	*In vivo* (mice) *In vitro* (HMC-1)	0.001–1 g/kg BW 0.001–1 mg/mL	↓TNF-α, ↓IL-6	Kim and Shin (2006)

active substances, and other effective metabolites are yet to be identified (Tables 2, 3). Both *Sect. Calodracon* and *Sect. Dracocephalum* contains essential oils, seven-substituted flavonoid glycosides and three-substituted flavanol glycosides (Table 1). *Ser. Officinales* Boris., *Sect. Calodracon*, and *Sect. Dracocephalum* have the common effects of protecting the liver, clearing heat and toxic materials, relieving cough and asthma, and eliminating phlegm. Additionally, *Sect. Calodracon* and *Ser. Officinales* Boris. have the

common effects of cooling the blood, and purging fire. Based on these effects, *Sect. Calodracons* can also prevent bleeding and promote granulation. Therefore, these phylogenetically close species may have similar pharmacodynamic bases, such as chemical composition and therapeutic effects, which are relevant to their clinical efficacy. Phytochemistry research has identified 19 species of *Dracocephalum*, *Lallemantia*, and *Hyssopus*, and found that most of them contain terpenoids, flavonoids, and phenylpropanoids. These metabolites are key substances for medicinal plants to exert pharmacological effects, with good pharmacological activity and potential anti-inflammatory, and hepatoprotective effects. In addition, plant metabolites not only have pharmacological effects but also determine their traditional applications. The genus *Dracocephalum* is widely used for the treatment of cough, fever, and liver and gall bladder diseases. Owing to the similar pharmacological activities of the three genera, *Lallemantia* and *Hyssopus* have similar traditional therapeutic effects. Based on the latest phylogenetic and biogeography studies of *Dracocephalum*, we provide further evidence for the merger of *Lallemani*a and *Hyssopus* into *Dracocephalum* and lay a foundation for finding plants with the medicinal value of this kind in the future.

7 Conclusion and prospects

In conclusion, we have shown that *Hyssopus*, *Lallemantia*, and *Dracocephalum* are closely related in many aspects, including molecular phylogeny, species distribution model, chemical composition, pharmacological activity, and traditional application. The division of these two genera into *Dracocephalum* was reasonable. Most flavonoids have flavonoid glycosides or flavonoid glycosides as their parent nuclear structure; therefore they have anti-inflammatory properties, but their pharmacological effects differ owing are different due to the different positions of the substituents. Modern pharmacological studies have justified some traditional medicinal uses, which may be partly attributed to the bioactivities of terpenoids, flavonoids, phenylpropanoids and phenolic acid, however, pharmacological mechanisms should be revealed to provide a scientific explanation for the traditional curative effect of *Dracocephalum* medicinal plants. In contrast, there are relatively few studies on the chemical metabolites of *Dracocephalum*, drug safety, effectiveness, and corresponding toxicological mechanisms, and the rationale for disease treatment remains uncertain. Although some species are used in traditional therapeutics, they lack verification of modern pharmacological research. Many metabolites have been identified using LC-MS, including indole alkaloids, flavonoids, terpenoids, and phenolic acids; however, some species of the genus *Dracocephalum* remain understudied with respect to their chemical metabolites. This review focused on *Sect. Sinodracon*, *Sect. Idiodracon*, *Sect. Calodracon*, and *Sect. Dracocephalum*. The commonly recorded efficacies of these sections are clearing away heat and toxic materials, relieving cough and asthma, eliminating phlegm, and protecting the liver. While studying the phylogenetic relationships of the genus *Dracocephalum*, we found that species in the *Hyssopus* and *Lallemantia* genera share a similar chemical composition, pharmacological activity, and traditional therapeutic effects. Network pharmacology has made some contributions to clarify the distance of plant genetic relationship from the perspective of mechanism.

In this review, the phamacophylogenetic relationship among *Hyssopus*, *Lallemantia*, and *Dracocephalum* is discussed from many standpoints, and on this basis, the related target pathways are predicted through network pharmacology, which helps to re-examine traditional usage, design/optimize experiments, to further unearth the associations among evolutionary relationship, chemical composition, and pharmacological activity. Based on previous studies, ecological adaptability evaluation was carried out to provide a research basis for the future cultivation of *Dracocephalum*, and clinical substitution of drugs among *Hyssopus*, *Lallemantia*, and *Dracocephalum*. Beyond this, there is considerable scope for future investigation into the conservation and utilization of plant resources, as well as the relationships of medicinal plants among these genera. For example, by employing species distribution prediction models in conjunction with various databases, it was possible to forecast potential suitable growth areas for endangered plants in these genera, followed by targeted conservation and cultivation efforts (Zhang et al., 2023). Furthermore, the application of genomics, transcriptomics, and metabolomics were crucial for exploring the origins of plant medicinal metabolism. Yang et al. (2023) sequenced the genomes of three Solanaceae species that produce hyoscyamine and scopolamine (HS) and one species that does not, revealing a shared biosynthetic pathway for HS across distantly related lineages, contributing to our understanding of HS biosynthesis. Correspondingly, the biosynthetic pathway of komaroviquinone, a distinctive pharmacological effect present from *D. kotschyi*, merits thorough investigation. This study not only enriches the theory of pharmacy but also provides a reference for the medicinal application of *Dracocephalum* medicinal plants. Unfortunately, for a long time, the medicinal value of the 60 species of *Dracocephalum* has not aroused enough attention or in-depth research; only a few species of chemical and pharmacological activities have been given due attention. For this reason, it is particularly urgent to find suitable substitutes and appropriate cultivation techniques. In this study, the genetic relationship between species of *Dracocephalum* was studied under the guidance of the genetic theory of medicinal plants, and it was skillfully transformed into the genetic relationship of modern scientific drugs, which was more helpful to understand and explore the medicinal value of *Dracocephalum*, and also provided the reference for the development of other medicinal plants. In addition, the internal relationships among the medicinal plants of *Dracocephalum* are still unclear, and these areas deserve further study in the future.

Author contributions

HL: Visualization, Writing–original draft, Writing–review and editing. XF: Writing–original draft. YZ: Writing–original draft. GL: Data curation, Investigation, Writing–original draft. CZ: Resources, Supervision, Writing–original draft. Aruhan: Investigation, Visualization, Writing–original draft. T-AD.: Investigation,

Visualization, Writing–original draft. NZ: Resources, Supervision, Writing–original draft. DH: Conceptualization, Methodology, Resources, Supervision, Writing–original draft. ML: Conceptualization, Methodology, Resources, Supervision, 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.

Publisher's note

Supplementary material

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Huyang Yangkun formula regulates the mitochondria pathway of ovarian granulosa cell apoptosis through FTO/m6A-P53 pathway

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Background: Premature ovarian insufficiency (POI) presents a significant challenge to female reproductive health. The Huyang Yangkun Formula (HYF), a traditional Chinese medicinal formulation, has been utilized in clinical settings for the treatment of POI for over a decade. Nevertheless, the therapeutic application of HYF is considerably constrained by the lack of clarity regarding its underlying mechanism of action.

Methods: The experimental procedures entailed administering VCD to female Sprague-Dawley rats at a dosage of 160 mg/kg/day over a period of 15 days, succeeded by a 100-day treatment with HYF. Blood serum samples were collected and analyzed using ELISA to quantify the concentrations of Anti-Müllerian Hormone (AMH), Follicle-Stimulating Hormone (FSH), and Estradiol (E2). The levels of N6-methyladenosine (m6A) were assessed through Dot blot analysis and liquid chromatography-tandem mass spectrometry (LC-MS/MS). Western blotting was employed to validate the differential expression of m6A-related catalytic enzymes and apoptosis-related regulators, including BCL-2, BCL-XL, and MCL-1, which may be implicated in the effects of HYF. Certain shRNA-COV434 cell line was constructed for the exploration of molecular mechanism, and then the potential targets were finally verified by MeRIP-qPCR.

Results: HYF has been identified as having a significant influence on the development of residual ovarian follicles in rats with POI, especially during the initial stages. It was observed that HYF facilitates the progression of escaping antral follicles to full maturation. Additionally, HYF exhibited the capacity to

Abbreviations: POI, premature ovarian insufficiency; VCD, 4-vinylcyclonhexene diepoxide; AMH anti-Müllerian hormone; FSH, follicle-stimulating hormone; E2, estradiol; m6A, N6-methyladenosine; METTL3, methyltransferase 3; METTL14, methyltransferase like 14; ALKBH5 alkB homolog 5; FTO, fat mass and obesity associated; JNK, c-jun N-terminal kinase; P38, mitogen-activated protein kinase14; HO1, heme oxygenase 1; NRF2, nuclearfactor (erythroid-derived2)-like2 protein; NQO1 NADPH quinine oxidoreductase1; P53 (Tp53), tumor protein p53; BCL2, B cell lymphoma 2; BCLXL, B cell lymphoma-extra large; MCL1, myeloid cell leukemia-1; BAX (Bax), BCL2-Associated X; BAK (Bak), BCL2 antagonist/killer; pBIM (phosphorylated), Bcl2 interacting mediator of cell death; PUMA (Puma), p53 upregulated modulator of apoptosis; Caspase, cysteinyl aspartate specific proteinase; MAPK, mitogen-activated protein kinase.

enhance the proliferation of COV434, a human ovarian granulosa cell line, while concurrently inhibiting apoptosis within these cells. Notably, HYF treatment resulted in the downregulation of apoptotic proteins, including BCL-XL, cleaved-caspase 9, cleaved-caspase 3, and Bcl-2. Concurrently, m6A modification is implicated in the regulation of HYF. Both *in vitro* and *in vivo* studies indicate that FTO may play a role in the anti-apoptotic mechanisms mediated by m6A in ovarian granulosa cells influenced by HYF. Moreover, employing qPCR and MeRIP-qPCR techniques, P53 has been identified as the target gene for m6A modification mediated by FTO.

Conclusion: These findings suggest that HYF holds promise as a potential treatment for POI and provide a more comprehensive understanding of the mechanism by which HYF operates, specifically its ability to prevent the BCL-2 mitochondrial apoptosis pathway mediated by P53 in ovarian granulosa cells of POI rats by regulating FTO/m6A-Tp53.

KEYWORDS

m6Amethylation modification, premature ovarian insufficiency, traditional Chinese medicine, apoptosis, P53

The results of this study indicate that HYF shows potential as a therapeutic intervention for primary ovarian insufficiency (POI) and contribute to a more thorough understanding of its underlying mechanisms. Specifically, HYF appears to inhibit the BCL-2 mitochondrial apoptosis pathway mediated by P53 in the ovarian granulosa cells of POI rats, through the regulation of the FTO/m6A-Tp53 axis.

1 Introduction

Premature ovarian insufficiency (POI) is a reproductive endocrine disorder, characterized by a cessation of function, which affects 3.7% of pregnant women under 40 years old (Welt, 2008; Golezar et al., 2019; Li et al., 2023). In addition to infertility, POI is associated with heightened risks of osteoporosis, cardiovascular disease, and premature mortality. This condition exhibits heterogeneity, with potential causes including autoimmune diseases, infections, or iatrogenic factors (Jiao et al., 2021; Ke et al., 2023). The etiology of approximately 80% of cases classified as 'idiopathic POI' remains elusive, indicating a lack of understanding regarding other biologically significant genetic factors contributing to POI (Liu et al., 2023).

In recent years, there has been a growing interest in exploring the involvement of N6-methyladenosine (m6A) regulation in diverse biological processes. Studies employing genetic loss-of-function approaches to investigate m6A methyltransferases, m6A-binding proteins, and m6A demethylases have underscored the crucial role of m6A modifications in governing gene expression during the onset and progression of reproductive development disorders. These disorders encompass various aspects, including sex differentiation, embryonic stem cell differentiation, and oocyte maturation (Lence et al., 2016; Sun et al., 2022). According to recent reports, an upregulation of m6A-modified transcripts has been observed during follicular recruitment or activation, which is regarded as a contributing factor to the dynamic expression of numerous genes associated with folliculogenesis (Hu et al., 2020; Yao et al., 2021). The role of m6A-methyltransferase, specifically methyltransferase-like 3 (METTL3), in facilitating the proliferation of ovarian granulosa cells has been well-documented (Hua et al., 2018). In a study conducted by Boxian Huang et al., the m6A RNA levels in granulosa cells from patients with premature ovarian failure (POF) infertility were examined, revealing an increase in m6A levels in these cells (Ding et al., 2018). A recent study by Liu et al. investigating transcriptome-wide N6-methyladenine (m6A) methylation in granulosa cells of women with diminished ovarian reserve has identified an increased number of m6A-methylated genes in the older age cohort (Liu et al., 2022). These findings indicate that the role of RNA m6A modification in ovarian aging remains inadequately understood and necessitates prompt and comprehensive further investigation.

Traditional Chinese medicine offers an effective treatment for premature ovarian insufficiency, especially for patients who cannot use hormone supplements or are hesitant due to concerns (Fu et al., 2022; Liu and Sun, 2023; Li et al., 2022). The Huyang Yangkun formula (HYF), derived from the traditional Chinese medicine recipe Danggui Buxue Tang, addresses ovarian aging symptoms in perimenopausal women. HYF consists of Astragali Radix, Herba Epimedii, Dioscoreae Rhizoma, Semen Cuscutae, Rehmanniae Radix, Angelicae Sinensis Radix, Glehniae Radix, at a ratio of 5:1:1:1:1:1:1. Based on the findings of our previous study, it has been observed that HYF treatment has demonstrated precise clinical efficacy in the management of POIs. Patients with declining ovarian function can potentially benefit from HYF therapy as it has the potential to enhance serum AMH and E2 levels, as well as regulate menstrual disorders (Li et al., 2020; Yafang, 2018). The depletion of follicles and subsequent ovarian aging can be attributed to abnormal apoptosis of oocytes or granulosa cells. Our previous research has indicated that the MAPK/P53/Bcl-2 family signaling pathways play a role in activating the apoptosis of ovarian granulosa cells (Yang et al., 2017). Building upon these findings, our research group has previously presented preliminary evidence suggesting that HYF treatment may facilitate follicular development through regulating Bcl-2 family-related mitochondrial apoptosis (Wang et al., 2021). Furthermore, it is postulated that the p53-mediated endogenous granulosa cell death pathway serves as the fundamental

TABLE 1 HYF composition.

English names (Chinese names)	Latin names	Botanical plant names	Dose
Milkvetch Root (huangqi)	Astragali Radix	*Astragalus didymophysus* Bunge	50 g
Epimedium (yinyanghuo)	Herba Epimedii	Epimedium brevicornu Maxim	10 g
Common Yam Rhizome (huaishanyao)	Dioscoreae Rhizoma	Dioscorea oppositifolia L	10 g
Dodder Seed (tusizi)	Semen Cuscutae	Cuscuta chinensis Lam	10 g
Rehmannia Glutinosa (shudihuang)	Rehmanniae Radix	ehmannia glutinosa (Gaertn.)	10 g
Chinese Angelica (danggui)	Angelicae Sinensis Radix	Angelica sinensis (Oliv.) Diels	10 g
Coastal Glehnia Root (beishashen)	Glehniae Radix	Glehnia littoralis F Schmidt ex Miq	10 g

mechanism in the induction of follicular atresia. However, due to limited circumstances, the upstream mechanisms by which HYF regulates apoptosis have not been fully elucidated, warranting further investigation.

In this study, we developed an experimental methodology integrating both *in vivo* and *in vitro* approaches with two primary objectives: first, to elucidate the impact of m6A RNA modification on apoptosis in ovarian cells associated with primary ovarian insufficiency (POI); and second, to test our hypothesis that HYF modulates mitochondrial apoptosis in ovarian granulosa cells via the m6A/P53/BCL-2 signaling pathway. Furthermore, the study sought to identify specific targets through gene interference, thereby providing both experimental and theoretical foundations for the effective treatment of POI using HYF.

2 Materials and methods

2.1 Herbal materials and HYF extract preparation

Decoction of HYF, a Chinese herbal medicine prescribed at the clinic, obtained from Guangdong Kangmei pharmaceutical Company, Ltd (Guangdong, China), has been used to treat POI in Guangdong Provincial Hospital of Chinese Medicine for more than 10 years. HYF consists of seven botanical drug(s), including Astragali Radix, Herba Epimedii, Dioscoreae Rhizoma, Semen Cuscutae, Rehmanniae Radix, Angelicae Sinensis Radix, Glehniae Radix, at a ratio of 5:1:1:1:1:1:1 (Table1). After soaking for half an hour, HYF had been boiled for 1.5 h using a reflux extraction device. Then the extracted liquid was collected together, the liquid was concentrated using a rotary evaporator to a final concentration of 1.1 g·mL-1. This concentration of liquid is administered directly to animals by intragastric administration. The drug used *in vitro* cell assay is Freeze-dried, the freeze-drying conditions are: 40°C refrigeration, −20°C freezing for 2 h, −10°C freezing for 16 h, 20°C drying for 36 h, 35°C secondary drying for 36 h. Freeze-dried HYF were weighed and dissolved in F12 medium to a final concentration of 100 mg/mL and centrifuged at 14,000 rpm for 10 min, the supernatant was filtered with 0.22 μL filter before use.

2.2 Analysis of HYF by UPLC-MS/MS

Batch-to-batch consistency was monitored by quantifying marker compounds via UPLC-MS/MS analysis have been reported previously (Wang et al., 2021). Briefly, the chromatographic column was Thermo Hypersil GLOD C18 (2.1 × 100 mm, 1.9 μm). The mobile phase is 0.1% formic acid water (A) and 0.1% formic acid-acetonitrile mixture (D). Gradient elution: 0–2 min, 6% D; 2–25 min, 6%–95% D; 25–30 min, 95% D; 30–32 min, 95%–5% D. Flow rate 0.3 mL/min; Column temperature 40°C; Sample tray temperature 15°C; The sample size was 5 μL.

Mass spectrum conditions: HESI electrospray ion source; Scanning mode Full MS/dd MS2; Positive ion electrospray voltage 3.5 kV, negative ion electrospray voltage 3.2 kV; The sheath gas flow rate was 35 arb and the auxiliary gas flow rate was 10 arb. Capillary temperature 320°C; Probe heater temperature 350°C; Maximum spray current 100 A; S-Lens resolution 60; Scanning range m/z 100–1,500 Da; Quality resolution 70,000 (full width at half maxima, FWHM); Secondary resolution 17,500 FWHM.

2.3 Animal and experimental design

Animal studies were conducted following the guidelines approved by the Guangdong Provincial Hospital of Chinese Medicine Institutional Animal Care and Use Committee (Registration number: 2019032; Approval date: 15 July 2019). The animals used in this study were SPF-grade SD female rats provided by Guangdong Medical Laboratory Animal Center, License No. SCXK (Guangdong) 2019–0,035.

The 28 days old SD rats were randomly divided into 3 groups using random numbers generated by computer (n = 7/group):

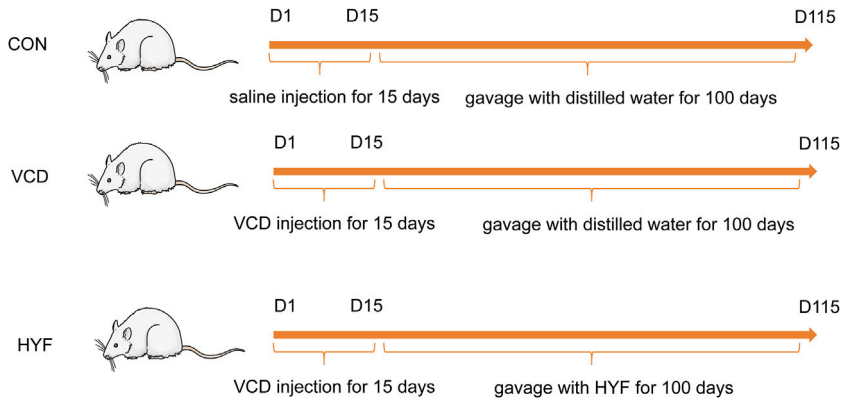

FIGURE 1
Overall animal experimental design.

normal control group (CON), VCD group (MOD) and Huyangyangkun formula group (HYF). Rats in VCD and HYF groups received intraperitoneal injection of VCD (Sigma Aldrich Korea, lot1302946) every day for 15 consecutive days (0.146 mL/kg) (Li et al., 2023). Then, the HYF group was gavaged with HYF (0.297 g/kg) for 100 days. Rats were finally euthanized using overdosed pentobarbital sodium and serum and ovaries were collected. Smears of rat vaginal exfoliated cells were taken between 9:00–10:00 a.m. daily (Figure 1).

2.4 HE staining and follicular count

To determine follicle numbers and observe follicular pathological alterations continuously, ovaries were fixed, embedded and sectioned as described before (Li et al., 2023; Nam et al., 2016). The section thickness was 5 μm, and the slices were successively sliced, and one slice was taken every 9 slices for HE staining. The sections of ovarian tissue stained by HE were observed under a microscope, and follicles were graded and counted according to a unified standard (reported previously), two pathologists performed blind numbers without knowing the grouping and took the mean of their readings for registration. The follicles were divided into 5 stages: primordial follicle, primary follicle, secondary follicle, antral follicle and mature follicle. To avoid the loss and duplication of counting, only the follicles in which oocytes can be observed are counted when counting small sinus follicles, sinus follicles, and mature follicles with larger diameters, and this is not necessary when counting small primary, primary, and secondary follicles.

2.5 Hormonal measurements

Blood was collected from orbital veins of rats at 30, 60, and 100 days after VCD injection for hormonal measurements. All rats were finally euthanized during the interestrous period, and blood was collected from abdominal aorta. Anti-Müllerian hormone (AMH), and estradiol (E2) were detected referring to the instructions of the ELISA kits (CSB-E11162r, CSB-E05110r, C0100090186, CUSABIO BIOTECH, Wuhan), follicle-stimulating hormone (FSH) were provided by (Elabscience, E-EL-R0391c, China).

2.6 Cell culture and plasmid construction

The COV434 line of immortalized granulosa cells were obtained from Sigma. Cells were grown at 37°C in a humidified 5% CO2 and 95% air and cultured in F12 medium (C11330500BT, Gibco, United States) containing 10% FBS (16000044, Gibco, United States) and 0.5% penicillin streptomycin sulfate (15140122, Gibco, United States). The granulosa cell apoptosis model was induced by VCD (Nam et al., 2016). Lentivirus construction was performed by Obio Technology (Shanghai, China). ShRNA interference fragment for Human FTO was designed and constructed into lentiviral vector by molecular biological means (pSLenti-U6-shRNA-CMV-EGFP-F2A-Puro-WPRE). The vector can be used to transfect cells to interfere with the expression of FTO gene in cells. pSLenti-CMV-MCS-3xFLAG-PGK-Puro-WPRE GL119 as a negative control (NC). Then, the COV434 cells were infected according to the optimal MOI value (MOI = 10), followed by screening for high purity infected COV434 cells.

2.7 RNA isolation and m6A quantification

Total RNA in ovaries and COV434 cells was extracted by Trizol reagent (Invitrogen, 15596026, United States) following the protocol. The mRNA was purified by Dynabeads® mRNA Purification Kit (Thermo Fisher).

The purity of mRNA is measured by a NanoDrop method and diluted into different concentrations. For the rigor of the experiment, three experimental methods (ELISA, Dot blot and LC-MS/MS) were choosing to test the change in globa.

m6A levels in mRNA. For enzyme-labeled assay use m6A RNA Methylation Quantification Kit (Cat#ab185912, Abcam,

United Kingdom), the RNA was incubated with m6A antibody in wells, and m6A levels were measured at 450 nm using a microplate reader. For Dot blot analysis and LC-MS/MS, the experimental process has been reported in detail in the previous article (Li et al., 2023), so we will not repeat it here.

2.8 MeRIP-qPCR

The methods for total RNA extraction, mRNA purification were described above, the amount and purity of total RNA were then controlled by NanoDrop ND-1000 (NanoDrop, Wilmington, DE, United States). The integrity of RNA was then detected by Bioanalyzer 2,100 (Agilent, CA, United States), and verified by agarose electrophoresis. The mRNA with PolyA was captured using the oligo (dT) magnetic beads (Dynabeads Oligo (dT) (No. 25–61005, Thermo Fisher, United States). Fragmentation was performed under high temperature conditions using the NEBNext® Magnesium RNA Fragmentation Module (Cat#E6150S, United States) at 86°C for 7 min. Dynabeads Antibody Coupling Kit (Thermo Fisher, CA, United States) and m6A antibody (No. 202003, Synaptic Systems, Germany) was premixed in IP buffer (50 mM Tris-HCl, 750 mM NaCl and 0.5% Igepal CA-630). The IP product was synthesized into cDNA by Invitrogen SuperScript™ II Reverse Transcriptase (Cat#1896649, CA, United States). The resulting cDNA (including IP samples and Input samples) can be directly used for the next MeRIP-qPCR and conventional RT-qPCR.The sequences of the primers are shown in the (Supplementary Table 1).

The enrichment m6A of yap was expressed as the enrichment percentage relative to the input sample (%Input) = $2^{-\Delta\Delta CT}$ (Input)-Ct (MeRIP)× Fd× 100%, where Fd is the input dilution factor (1/8).

2.9 Western blot

Cells and ovaries were harvested and lysed with 1x RIPA buffer (Beyotime, P0013B, China). Protein concentration was detected by Thermo Bicinchoninic acid (BCA, 23,227, United States) kit. Equal amounts of protein were dissolved in 5×loading buffer and separated in 10%–15% SDS polyacrylamide separation gels. The protein was transferred onto PVDF membranes (Beyotime, FFN10, China), then incubated with TBST solution containing 5% BSA for 1.5 h at room temperature and incubated with the primary antibodies at 4°C overnight. The next day, the membranes were washed and incubated for 1 hour with the secondary antibody. After the membranes were covered enhanced chemiluminescence (ECL), the images were shown by ChemiDoc XRS + chemiluminescence imaging system (Bio-rad). The results were analyzed with ImageJ software. Primary antibodies (all diluted at 1:1,000):ALKBH5, ab195377, Abcam; FTO, ab92821, Abcam; pJNK,4,668, CST; P38,8690, CST; PP38,4632, CST NRF2,16396-1-AP, Proteintech; BCL2,2876, CST; BCLXL, ab32370, Abcam; MCL1, ab32087, Abcam; BAX, 2,772, CST; BAK,12,105, CST; pBIM, ab17935, Abcam; PUMA,4,976, CST; Pro-caspase9/Cleaved-caspase9,10380-1-AP, Proteintech; Caspase3,9662, CST; Cleaved-caspase3, 9,664, CST; IgG,7,074, CST.

2.10 Statistical analysis

Data analysis was processed by SPSS 21.0 software. Mean ± Standard or Median (P25~P75) was chosen to describe the data, depending on whether the data meet the normal distribution. *t*-test or Mann-Whitney U test was used to compare CON group with MOD group. One-way ANOVA or Kruskal–Wallis test was used for comparison of groups. $p < 0.05$ was considered statistically significant. And graphs were drawn by Graphpad Prism 8 software.

3 Results

3.1 Characterization of compounds in HYF

Batch-to-batch consistency was monitored by quantifying marker compounds via UPLC-MS/MS analysis. Figure 2A and b display the quantification in negative and positive ion mode. UPLC-MS/MS was used to identify the active constituents of HYF. Nine major active components have been identified through the comparison of standard compounds (Table 2).

3.2 Protective effect of Huyang Yangkun formula on ovarian function in premature ovarian insufficiency (POI) rats

Ovarian function can be predicted by assessing the estrous cycle, follicular development, and hormonal fluctuations. Rats were periodically weighed throughout the experimental period. It was observed that the body weight of rats in the VCD and HYF groups increased at a slower rate than that of the CON group, with this difference becoming evident by the first month of VCD modeling (Day 28). The body weight of rats in the VCD group was found to be lower than that of the CON group ($p < 0.01$), while the HYF group exhibited higher body weight than the VCD group ($p < 0.05$) (Figures 3A, B). The ovarian structures of the rats are depicted in Figure 3C. Bilateral ovaries from each rat were subjected to weight measurement, and their dimensions were recorded, specifically the long diameter (L, in millimeters) and the short diameter (S, mm). The ovarian volume was subsequently calculated using formula V = L *S^2/2, mm3). When compared to the control (CON) group, the VCD group demonstrated a significant reduction in ovarian volume, amounting to 46.89%. In contrast, the group not exposed to VCD exhibited a significant increase in ovarian volume by 39.28% relative to the VCD group (Figure 3D). To evaluate alterations in the estrous cycle, we performed an analysis of vaginal cell shedding at 30, 60, and 100 days following HYF administration, in addition to daily sampling over a period of 10 consecutive days. The VCD model rats exhibited significant disruptions in their estrous cycle, which intensified with prolonged VCD exposure. Similarly, the HYF group experienced disturbances in their estrous cycle; however, some individuals in the HYF group maintained regular estrous cycles by the conclusion of the experiment (Figure 3E; Supplementary Table 2). Figure 3F illustrates the maximum section of pathological sections of the ovary in three groups of rats. The VCD group exhibited significantly lower numbers of follicles at all stages compared to the CON group ($p < 0.01$, $p <$

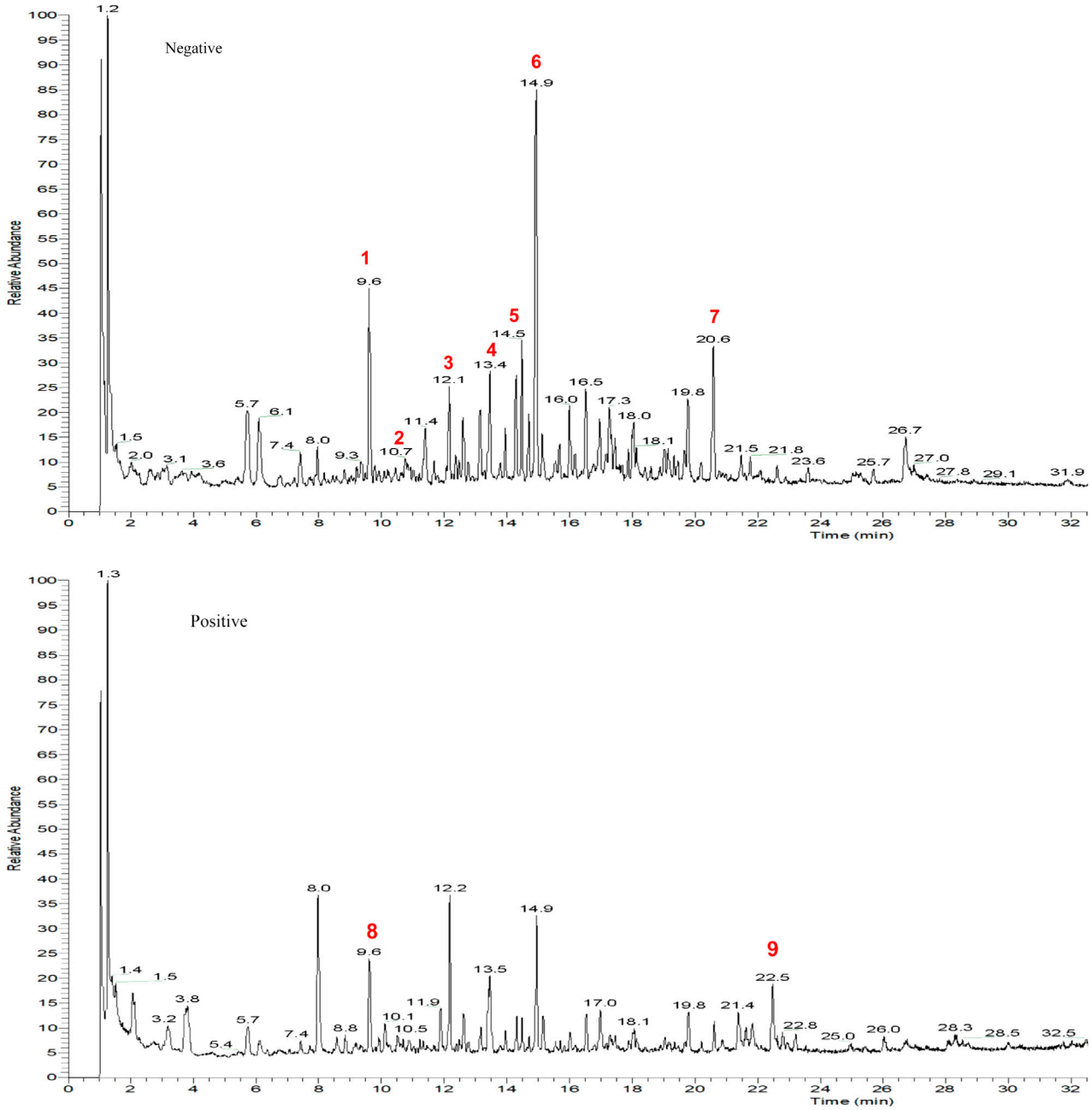

FIGURE 2
TIC (total ion chromatography) of HYF in negative and positive ion modes.1. Hyperoside, 2. Quercitrin, 3. Ononin, 4. calycosin, 5. epimedin B, 6, epimedin C, 7. baohuoside I, 8. ferulic acid, 9. Senkyunolide A.

0.01, $p < 0.01$, and $p < 0.05$, respectively). Following HYF intervention, there was a significant increase in the number of original and mature follicles compared to the VCD group ($p < 0.05$, $p < 0.01$, respectively, Figure 3G).

Serum anti-Müllerian hormone (AMH) levels were employed as a sensitive biomarker for assessing ovarian reserve, with serum samples collected on days 30, 60, and 100 following VCD injection. No significant differences in AMH levels were observed among the three groups at 30 and 60 days post-injection. However, at 100 days post-injection, the serum AMH level in the VCD group was significantly reduced compared to the control (CON) group ($p < 0.01$, Figure 3H). Furthermore, the HYF group demonstrated a significant upward trend in AMH levels 100 days post-VCD injection compared to the VCD group ($p < 0.05$, Figure 3I). Blood samples for follicle-stimulating hormone (FSH) and estradiol (E2) were collected in accordance with the estrous cycle of rats (4–5 days), and a sex hormone cycle line chart was developed to observe their respective trends. The FSH line chart indicates that the CON group is consistent with previous literature (Olvera-Juárez et al., 2020), whereas the line chart for the VCD group exhibits a completely irregular pattern (Figure 3J and Supplementary Figure 1). The E2 line chart indicates that the rats in the normal

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TABLE 2 The imformation of nine compounds identified in HYF by UPLC-MS/MS.

No.	Compound	t_R (min)	Formula	Observed Mass (m/z)	Detection mode (±)
1	Hyperoside	9.66	$C_{21}H_{20}O_{12}$	463.08841	-
2	quercitrin	10.88	$C_{21}H_{20}O_{11}$	447.09348	-
3	ononin	12.11	$C_{22}H_{22}O_9$	475.12457	-
4	calycosin	13.44	$C_{16}H_{12}O_5$	283.06122	-
5	epimedium B	14.50	$C_{38}H_{48}O_{19}$	853.27679	-
6	epimedium C	14.79	$C_{39}H_{50}O_{19}$	867.29297	-
7	baohuoside I	20.60	$C_{27}H_{30}O_{10}$	513.17688	-
8	ferulic acid	9.16	$C_{10}H_{10}O_4$	193.04996	+
9	senkyunolide A	20.12	C19H21ClN2O3S	193.12166	+

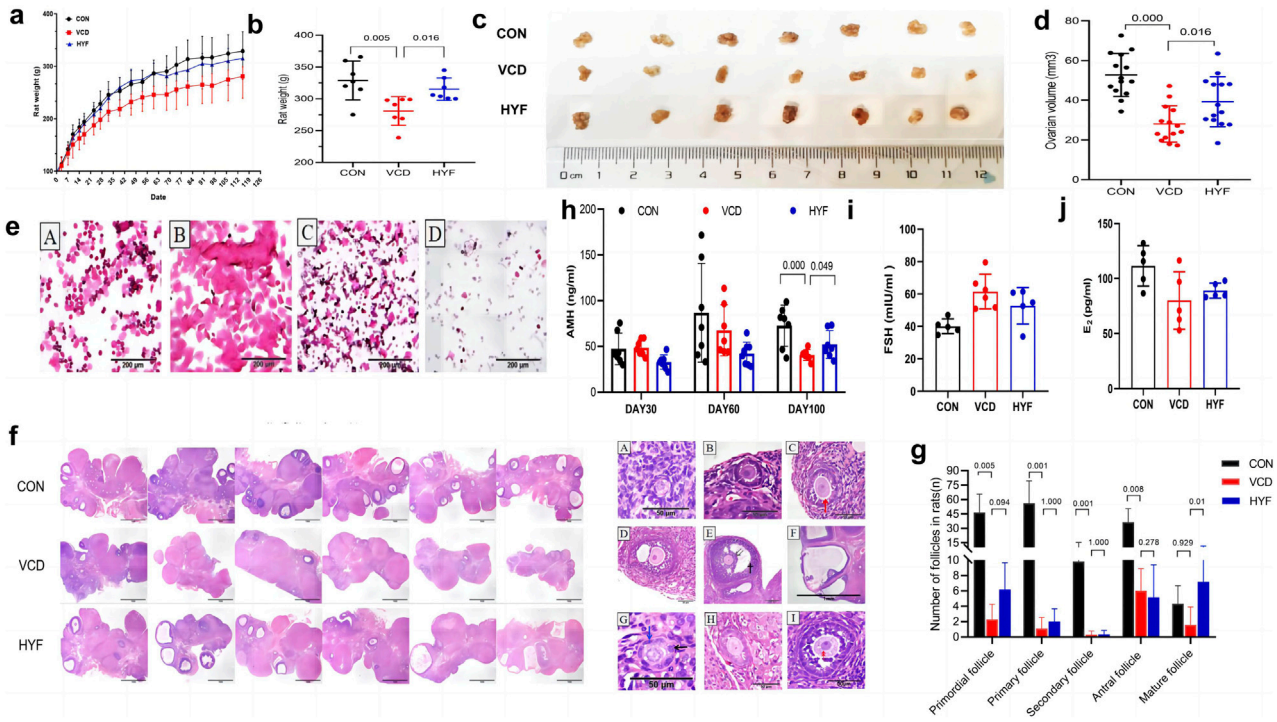

FIGURE 3
Protective effect of HYF on ovarian function in POI rats **(A, B)** Body weight changes in rats (a. Body weight record, b. Final body weight; n = 7; Compared with CON group, $^{\#\#}p < 0.01$; Compared with VCD group, $^{*}p < 0.05$) **(C, D)** Comparison of ovary weight and volume in each group (c. Images of ovaries of rat. d. Ovarian volume; n = 7). **(E)** Representative images of estrous cycle of rats (A. Proestrus, B. Estrus, C. Metestrus, D. Diestrus). **(F)** Morphological changes of ovarian tissue in rats (n = 6, maximum transverse section, HE, ×40, scale bar = 1 mm). **(G)** Number of primordial follicles, primary follicles, secondary follicles, antral follicles and Mature follicles in each group. Data are shown as the mean ± SD, n = 6 **(H, J)** Detection of sex hormone in rats (AMH, FSH and E2, n = 6).

control group display periodic fluctuations, aligning with previous findings by Olvera-Juárez et al. (2020), in contrast, the E2 line chart for the VCD group exhibits a disordered pattern, characterized by the absence of wave crests or the emergence of multiple peaks. Meanwhile, the HYF group shows a slight degree of regularity, evidenced by the presence of wave crests. Furthermore, a comparison of baseline E2 levels revealed no statistically significant differences among the three groups (Figure 3J and Supplementary Figure 1).

3.3 The anti-apoptotic effect of Huyang Yangkun formula is related to jnk/P53/mitochondrial apoptotic pathway

TUNEL staining was first used to detect the apoptosis of ovarian granulosa cells in the CON, VCD and HYF groups. As shown in Figure 4A, CON showed less apoptosis signals to the naked eye, while the ovaries in the VCD-induced POI group showed a lot of apoptosis signals. On the contrary, granulosa cell apoptosis in POI

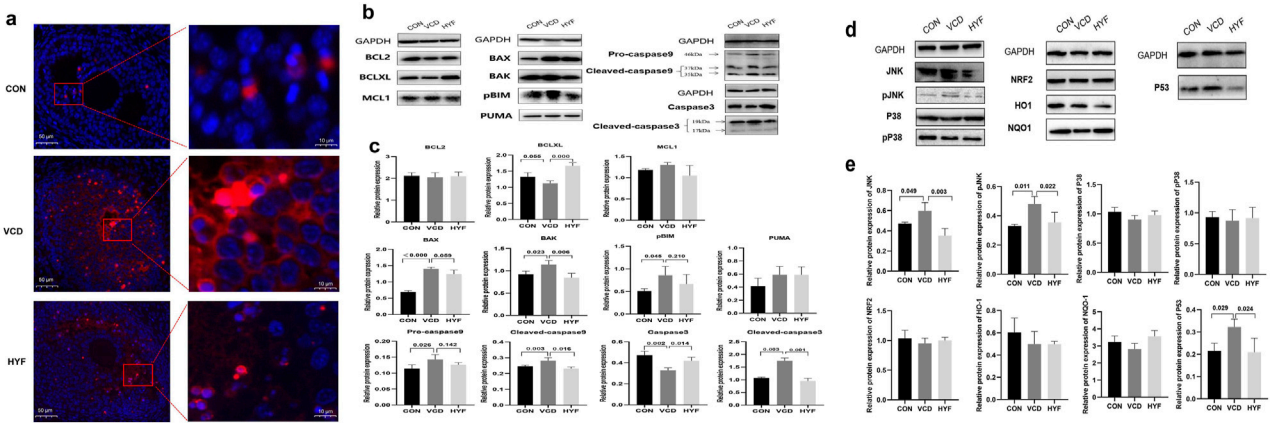

FIGURE 4
HYF exhibited protective effects against VCD-induced apoptosis. **(A)** The apoptosis of ovarian tissue was detected by immunofluorescence. **(B, C)** Western blot analysis of the expression of BCL-2 family and Caspases proteins in rat ovarian tissues (n = 3). **(D, E)** Western blot analysis of protein expression of potential target in rat ovarian tissue (n = 3).

rats decreased after HYF intervention. Meanwhile, according to this result and previous research basis, we detected the expression levels of various anti-apoptotic proteins associated with both extrinsic and intrinsic by WB experiment.

TUNEL staining was initially employed to assess apoptosis in ovarian granulosa cells across the CON, VCD, and HYF groups. In Figure 4A, the CON group exhibited minimal apoptotic signals visible to the naked eye, whereas the VCD-induced POI group demonstrated a substantial increase in apoptotic signals. Conversely, granulosa cell apoptosis in POI rats was reduced following HYF intervention. Based on these findings and previous research, we conducted Western blot (WB) experiments to evaluate the expression levels of various anti-apoptotic proteins associated with both extrinsic and intrinsic pathways.

The results of the rat ovarian tissue test revealed that in the VCD group, there was an increase in the expressions of pro-apoptotic proteins BAX, BAK, and pBIM ($p < 0.01$, $p < 0.05$ and $p < 0.05$, respectively), as well as an increase in the expression of Caspase9 protein in both the precursor Pro-caspase9 and Cleaved caspase9 forms ($p < 0.05$). Additionally, the expression of Cleaved caspase3 was also found to be upregulated ($p < 0.01$). In contrast, the HYF group exhibited an increase in the anti-apoptotic protein BCLXL in the BCL-2 family ($p < 0.01$), a decrease in the pro-apoptotic protein BAK ($p < 0.01$), and a decrease in both Cleaved-caspase9 and Cleaved-caspase3, when compared to the VCD group ($p < 0.01$, Figures 4B, C).

To explore the potential upstream molecular targets of HYF anti-mitochondrial apoptosis, based on the analysis of relevant literature and preliminary research, we established a potential pool of upstream signals consisting of eight potential targets, namely, JNK, pJNK, P38, pP38, NRF2, HO1, NQO1, and P53. These signals were investigated as potential regulators of the mitochondrial apoptosis pathway.

The results indicated that compared to the control group, the expressions of JNK, pJNK, and P53 were significantly increased in the VCD group ($p < 0.05$), while no statistically significant differences were observed for P38, pP38, NRF2, HO1, and NQO1. In comparison to the VCD group, the expression levels of JNK, pJNK, and P53 were significantly downregulated ($p < 0.01$, $p < 0.05$, and $p < 0.05$, respectively), while no statistically significant differences were observed for P38, pP38, NRF2, and HO1 proteins (Figures 4D, E). Our research findings indicate that HYF has a significant inhibitory effect on the expression of P53 protein under VCD stimulation. This suggests that the P53 signal may have a crucial role in the molecular mechanism of HYF. Furthermore, the anti-apoptotic effect of HYF is partially mediated through the JNK in the MAPK kinase system.

3.4 mRNA m6A methylation plays a role in the mechanisms underlying VCD-induced POI and HYF

Evidence suggests that m6A modifications play a significant role in the reduction of ovarian follicular reserve. This study also explores this aspect by extracting RNA from rat ovarian tissues. The quality of the mRNA extraction was evaluated using RNA electrophoresis, as illustrated in Figure 5A. The levels of m6A-RNA modification in rat ovarian tissues were assessed utilizing ELISA, dot blot, and LC-MS/MS methodologies. The results from all three detection techniques demonstrated a consistent trend, revealing that the m6A modification in the VCD group was significantly reduced compared to the CON group ($p < 0.01$). Furthermore, the global m6A level in the HYF group was found to be elevated relative to the VCD group ($p < 0.01$, Figures 5B–D). It is noteworthy that the m6A modification of mRNA in the VCD group demonstrated a reduction of 15.36% relative to the CON group, whereas the HYF group exhibited an increase of 20.71% in m6A modification. Furthermore, to investigate the protective role of m6A involvement in HYF on ovarian granulosa cells, human ovarian granulosa cells (COV434) were employed for relevant analyses. The m6A-RNA modification level in the *in vitro* experiment was evaluated using Dot Blot, which revealed a significant reduction in the m6A modification level in the VCD group compared to the CON group (Figures 5E, F).

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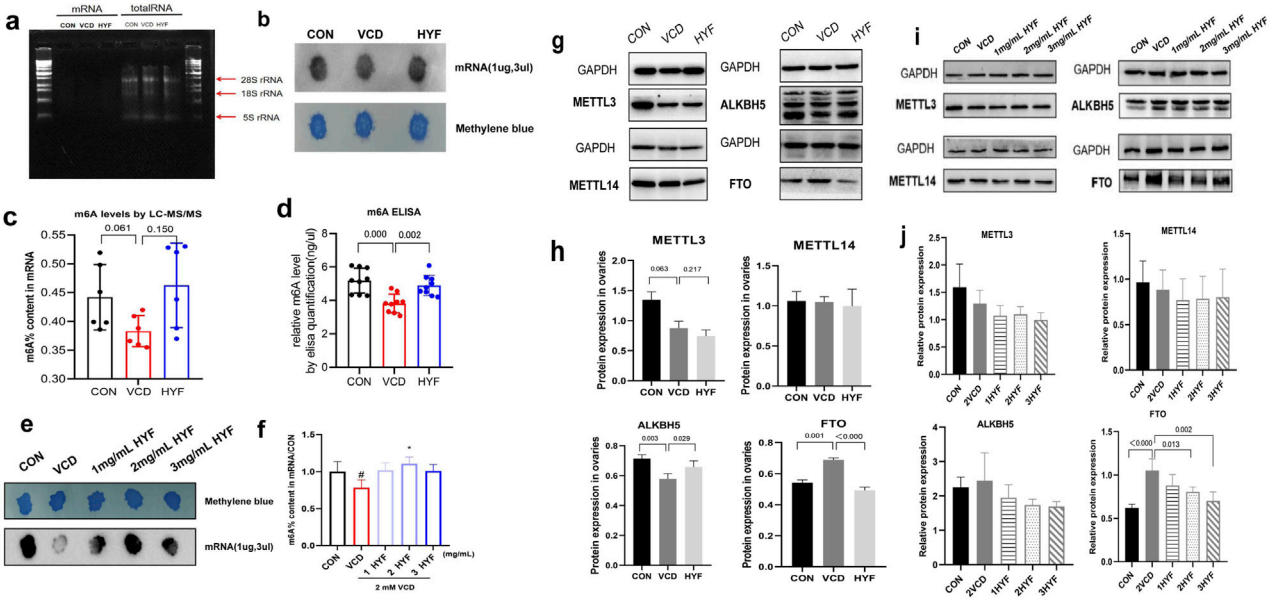

FIGURE 5
Effect of HYF on m6A levels and the expression of methylase *in vivo* and *in vitro*. **(A)** The quality of mRNA extraction was assessed through RNA electrophoresis. **(B)** Rat ovary m6A levels were determined by Dot Blot **(C, D)** Rat ovary m6A levels were determined by m6A ELISA and LC-MS/MS **(E, F)** M6A levels in cov434 cells were determined by Dot Blot and LC-MS/MS **(G, H)** Comparison of the expression levels of m6A related catalytic enzymes in ovary of rats (n = 3) **(I, J)** Comparison of m6A methylase expression levels *in vitro* COV434 cells (n = 3).

In this study, the protein expression levels of key m6A catalytic enzymes, specifically METTL3, METTL14, ALKBH5, and FTO, were analyzed to assess potential alterations in these enzymes within the ovaries of rats across three distinct groups. The expression levels of METTL3 and METTL14 exhibited no significant differences among the groups. Conversely, the expression of the demethylase ALKBH5 was significantly reduced in the VCD group ($p < 0.01$) and significantly elevated in the HYF group ($p < 0.05$). Additionally, the expression of FTO, another demethylase, was significantly increased in the VCD group and significantly decreased in the HYF group ($p < 0.01$, Figures 5G, H). The results indicate possible modifications in m6A-related catalytic enzymes in the ovaries of rats across various experimental groups. Western blot analysis of *in vitro* cells demonstrated a statistically significant difference in FTO expression between the groups, with an F-value of 8.239 and a *p*-value of 0.003 (Figures 5I, J). In contrast, no significant differences were observed in the expression levels of METTL14, ALKBH5, and METTL3 among the groups. This section of the study provides additional evidence that modifications in m6A are intricately linked to the fate of ovarian cells. Furthermore, it clarifies the role of the FTO/m6A axis in the regulatory effects of HYF on ovarian granulosa cells.

3.5 The FTO/m6A-Tp53 pathway is involved in the anti-apoptotic effect of HYF: In vivo POI rat model or *in vitro* VCD-cov434 cell apoptosis model

Previous findings suggested a significant link between FTO/m6A RNA methylation and targets like pJNK, P53, PUMA, and BAX at the protein level, both *in vivo* and *in vitro*. To explore if FTO/m6A, influenced by HYF, is related to the mitochondrial apoptosis pathway triggered by these targets, we conducted two experiments: verifying functional proteins after FTO knockdown *in vitro*, and examining m6A modified transcripts in ovarian samples.

3.6 (1) FTO mediate m6A modification to participate in the anti-apoptosis molecular mechanism of HYF

As shown in Figure 6A, the expression of the FTO protein was significantly reduced in the FTO gene knockdown group compared to the blank control group. Consistent with previous reports, FTO knockdown resulted in a significant increase in the level of m6A (Figure 6B). The proliferation and apoptosis of COV434 cells were not affected by FTO knockdown (Figure 6C). Although FTO knockdown did not ultimately lead to significant changes in apoptosis phenotype, some changes occurred in certain proteins inside the cell, it was observed that the expression of JNK, pJNK, and P53 significantly decreased following FTO knockdown ($p < 0.05$, Figures 6D, E). Likewise, the protein levels of BAX, PUMA, and Cleaved-caspase9 were also diminished upon FTO knockdown ($p < 0.01$, $p < 0.05$, $p < 0.05$, respectively. Figures 6F, G).

3.7 m6A-Tp53may be the pathway through which m6A modification participates in the anti-apoptotic effect of HYF

The potential target proteins were screened and identified through both *in vivo* and *in vitro* experiments, with the results

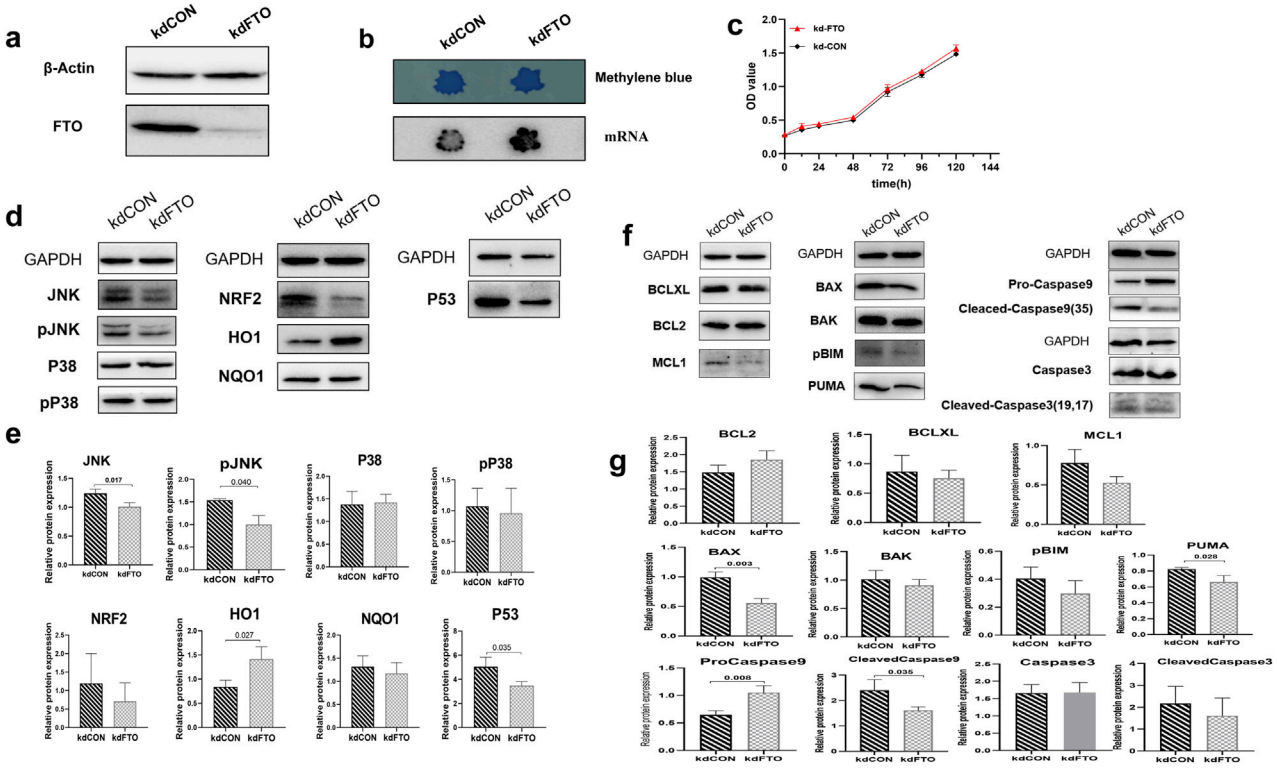

FIGURE 6
FTO mediate m6A modification to participate in the anti-apoptosis molecular mechanism of HYF **(A, B)** Effect of FTO gene knock-down on FTO protein expression and m6A level in ovarian granulosa cells. **(C)** Effect of FTO gene knockdown on ovarian granulosa cell proliferation **(D, E)** Protein changes of ovarian granulosa cell candidate target pool after FTO knockdown, n = 3; Compared with kdCON group **(F, G)** Expression changes of BCL-2 family and Caspases protein in FTO knockdown granulosa cells (n = 3).

being descriptively summarized (Figure 7A). Our findings led to the identification of five potential targets: m6A-TP53, m6A-JNK, m6A-PUMA, m6A-Bax, and m6A-Bak, which are hypothesized to exert significant influence. To validate this hypothesis, we performed preliminary validation experiments on these five core candidate targets using rat ovarian tissue. These experiments specifically examined the differences between groups of total transcripts and m6A-modified transcripts.

As illustrated in Figure 7B, the mRNA expression of Tp53 demonstrated a decreasing trend in the VCD group and an increasing trend in the HYF group, which was contrary to the expression pattern observed for the P53 protein. This result is of considerable interest. Notably, the expression of m6A-Tp53 significantly increased in the VCD group and significantly decreased in the HYF group (Figure 7C, $p < 0.05$). This observation provides a potential explanation for the elevated levels of P53 protein in the HYF group. Moreover, Jnk expression was observed to be upregulated in the VCD group, while no statistically significant difference was detected between the HYF and VCD groups. In contrast, the expression of m6A-Jnk was downregulated in the VCD group, with no significant difference between the HYF and VCD groups (Figures 7D, E). As for Puma, Bax, and BAK, the results demonstrated minimal variation across the three groups, with inconsistent expression levels noted at both the protein and gene expression levels. Moreover, the abundance of m6A-modified transcripts did not correspond to the previously discussed proteins (Figures 7F–K). In conclusion, m6A modifications on Jnk and Tp53 (m6A-JNK, m6A-TP53) may play a contributory role in the pathogenesis of POI, with the regulation of m6A-TP53 potentially having a more significant impact on the anti-apoptotic effects of HYF.

The current study offers preliminary evidence indicating the involvement of m6A-Tp53 in the pharmacodynamic mechanism of HYF *in vivo*. To further investigate this, we performed immunofluorescent staining to assess P53 expression and localization in rat ovaries (Figure 7L). Our results demonstrated a reduced fluorescence intensity in the CON and HYF groups, while a significant increase was noted in the VCD group. Additionally, the fluorescence signals were primarily localized within the follicular granulosa cells, suggesting that the observed alterations in P53 and m6A-Tp53 within the ovary likely originate from these cells.

In addition, the SRAMP (http://www.cuilab.cn/sramp) and RMBase v2.0 databases (http://rna.sysu.edu.cn/rmbase) were employed to forecast the methylation locations within the mRNA sequence. The m6A modification sites pertaining to p53 were primarily located near the initiation sites of gene transcription. A total of eight methylation sites were identified in association with P53, with one discovery exhibiting a very high level of confidence, three discoveries demonstrating high confidence, four discoveries indicating moderate confidence, and five discoveries suggesting low confidence (Figure 7M; Supplementary Table 3; Supplementary Figure 2).

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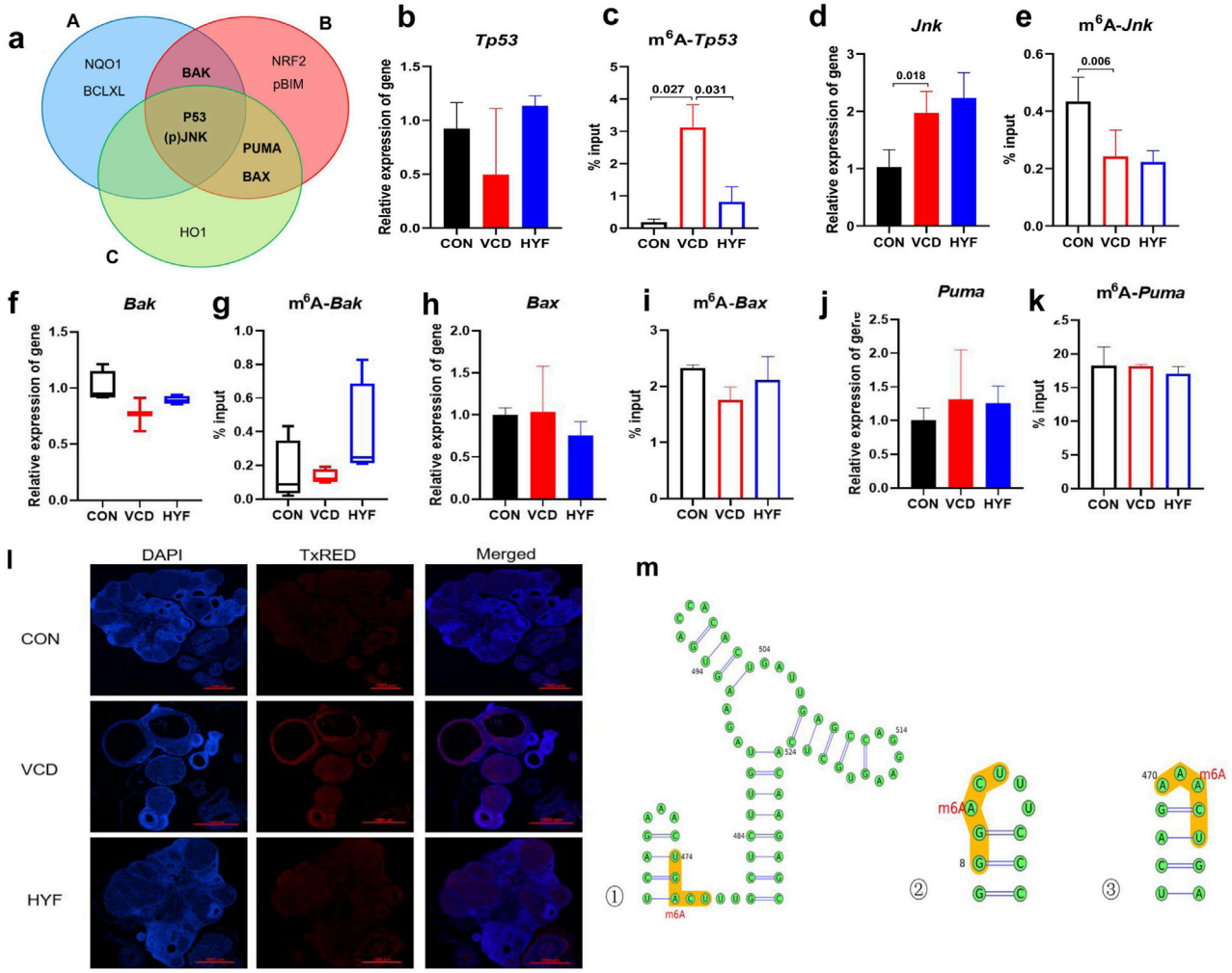

FIGURE 7
The pathways of m6A-Tp53 involved in the anti-apoptotic effect of HYF **(A)** Venn diagram shows the screening results of major differential proteins (A. Differential proteins in animal experiments, B. VCD model cell experiments, C. FTO knockout cell differential proteins **(B–K)** Enrichment of m6A-modified mRNA in ovarian tissue, analyzed by MeRIP-qPCR, the percentage of the input is shown (n = 3–4; b-c. Tp53 and m6A-Tp53; d-e.Jnk and m6A-Jnk; f-g.Bak and m6A-Bak; h-i. Bax and m6A-Bax; j-k. Puma and m6A-Puma). **(I)** Immunofluorescence detection of P53 in rat ovarian tissue **(M)** Prediction of m6A modification sites on Tp53 transcripts (top three confidence levels).

4 Discussion

Premature ovarian insufficiency (POI) is a key cause of female infertility due to early ovarian decline. Currently, no drug can restore POI-related reproductive function, and diminished ovarian function is considered irreversible. However, traditional Chinese medicine shows promise in treating menstrual issues and aiding conception (Li et al., 2022). According to the principles of traditional Chinese medicine, HYF exhibits significant potential in the treatment of POI (Yafang, 2018). VCD is an effective method for creating POI mouse models through continuous intraperitoneal injection of 160 mg/(kgd) VCD for 15 days. Rodent ovaries exhibit POI-like clinical characteristics approximately 70 days after stopping the injections. We utilize these POI models to study HYF's efficacy and mechanism, having previously explored different Chinese medicine intervention times (25, 50, 90, and 105 days). By evaluating follicle development, estrus cycle, and serum sex hormones, we found that ovarian function improved more at 90 and 105 days after HYF treatment compared to 50 and 25 days. Our study indicates that VCD-induced POI causes irreversible ovarian damage without natural repair, but early HYF treatment can partially preserve ovarian function. Given these findings, our aim was to elucidate the underlying mechanisms and examine the potential therapeutic implications for POI.

From a clinical standpoint, anti-Müllerian hormone (AMH) is extensively acknowledged as a dependable and sensitive indicator for evaluating ovarian reserve, owing to its minimal variability across the menstrual cycle (Anderson et al., 2022; Nelson et al., 2023). In our study, it was observed that the administration of HYF significantly increased serum AMH levels ($p < 0.05$, Figure 3H), suggesting a potential protective effect on ovarian reserve function. Furthermore, the follicle count results demonstrated a trend towards an increased number of primary follicles and pre-antral follicles under the influence of HYF, while a slight decrease was observed in the number of antral follicles compared to the VCD-POI group (Figure 3G). This finding seems to contradict the observed

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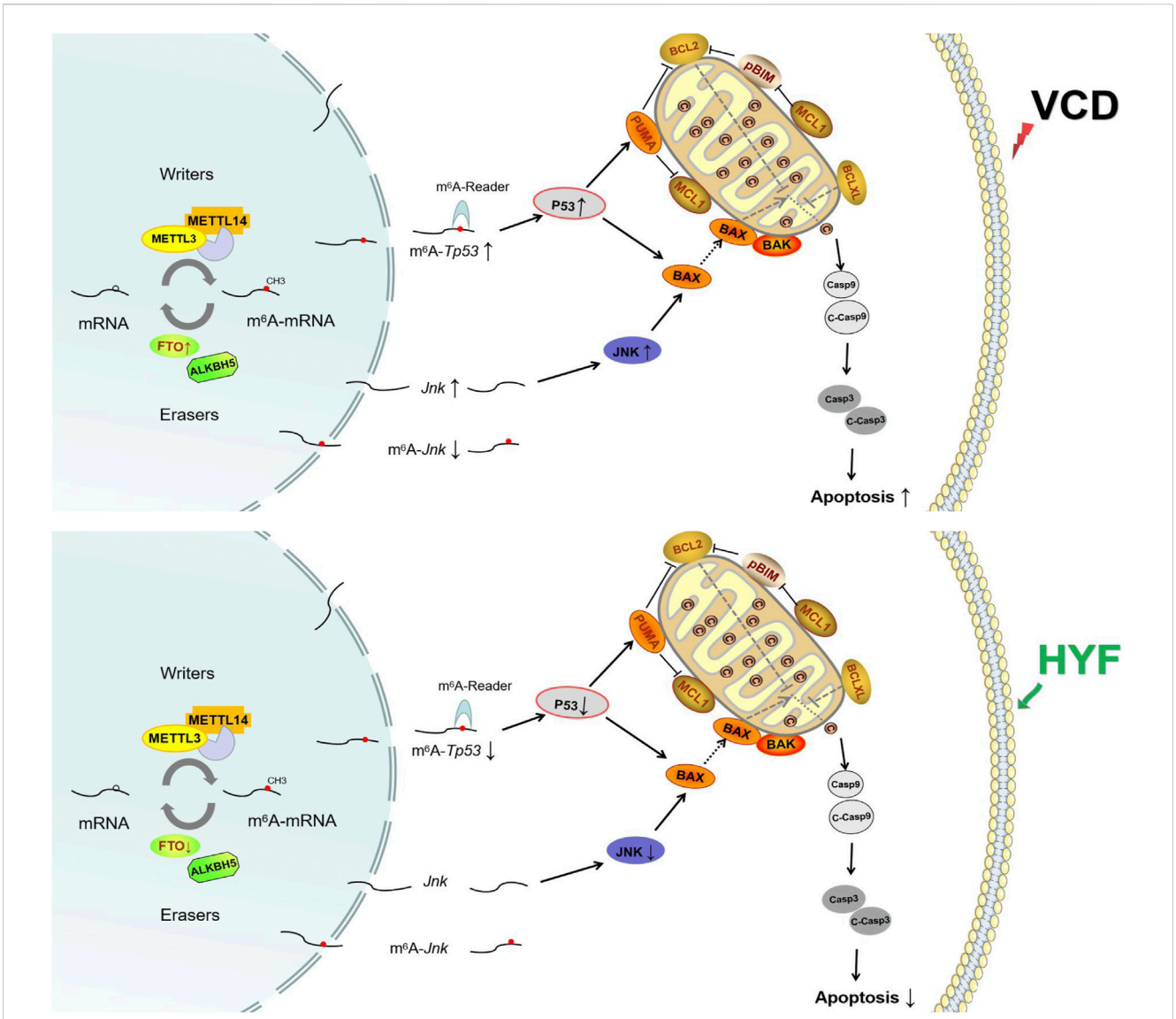

FIGURE 8
The anti-apoptotic mechanism of Huyang Yangkun formula regulates the apoptotic signaling pathway of mitochondrial P53/BCL-2 family in ovarian granulosa cells through FTO/m6A-RNA.

elevation of AMH levels in the HYF group, a phenomenon of significant interest. It is well-established that AMH is synthesized by the granulosa cells of primary, preantral, and small antral follicles. Thus, it is puzzling why AMH levels are elevated despite the lack of a substantial increase in the number of antral follicles. This suggests that two potential factors may influence AMH secretion: the number of granulosa cells and their capacity to produce AMH (Alvaro Mercadal et al., 2015; Desongnis et al., 2021). It is reasonable to hypothesize that in individuals with primary ovarian insufficiency (POI), the basal antral follicles, which contain competent granulosa cells, represent the genuinely viable and effective follicles capable of maturing. In the current study, the administration of HYF led to a significant increase in mature follicles in rat models. Therefore, it can be inferred that HYF may facilitate follicular development by enhancing the competence of granulosa cells within the follicles (Supplementary Figure 3).

Prior research on the role of m6A in ovarian aging disorders has been relatively limited. In a pertinent study by Jiang et al. (2021), a comparative analysis was performed on cumulus granulosa cells from older women with diminished ovarian response (≥37 years old) and younger women with normal ovarian response (<37 years old). The study revealed that increased m6A modification levels were exclusively associated with a decrease in FTO protein, whereas other m6A-related catalytic enzymes did not show significant changes. The m6A RNA levels in granulosa cells from patients with primary ovarian insufficiency (POI) were investigated by Boxian Huang and colleagues (Ding et al., 2018; Huang et al., 2019), who reported an elevation in m6A levels within these cells. Similarly, increased m6A RNA modification levels were observed in CTX-induced POI ICR mice compared to normal ICR mice. However, this finding is inconsistent with our results, which indicate that the decrease in ovarian m6A levels in POI rats induced by VCD may be attributed to the downregulation of the methyltransferase METTL3, coupled with the upregulation of the demethylase FTO. In our earlier study, we analyzed m6A

modification levels in C57 mouse ovaries at different developmental stages: 4 weeks (puberty), 7 weeks (sexual maturity), 9 weeks (body maturity), and 24 weeks (middle and old age). We found that m6A levels increased from puberty to sexual maturity but gradually decreased from sexual maturity to middle and old age (Supplementary Figure 4). The results indicate a pattern of m6A modification in the mouse ovary, with levels rising during reproductive development and decreasing with age. Various experiments suggest that these modifications are influenced by factors like cellular origins, model inductions, and detection methods. In summary, the effects of RNA m6A modification and its catalytic enzymes on ovarian aging are still emerging, requiring further study due to varied outcomes.

The apoptotic function of P53 is stringently regulated, and in quiescent, non-stimulated cells, P53 exists as a minimally stable protein with a relatively short half-life of approximately 30 min. However, when stress-induced damage exceeds the cell's repair capacity, P53 adopts a lethal role by initiating cellular apoptosis (Meulmeester and Jochemsen, 2008; Wu and Deng, 2002). This study explores the key role of the mitochondrial apoptosis signaling pathway and its link to upstream protein signals. We identified and detected candidate proteins *in vitro* and *in vivo*, focusing on core differential proteins, VCD exposure raised ovarian P53 protein levels, activating apoptotic proteins like BAX and BAK and increasing follicle apoptosis. However, TP53 mRNA levels were downregulated. We proposed that P53 undergoes post-transcriptional modifications, the discovered that VCD-induced apoptosis is caused by the upregulation of demethylase FTO, which results in the upregulation of m6A-Tp53 transcripts, which in turn leads to elevated levels of Tp53-induced apoptosis. This observation implies that both FTO and P53 play crucial roles in the regulatory mechanisms governing granulosa cells within follicles. Furthermore, HYF was found to reverse this outcome. This aroused our strong interest, given FTO is a demethylase of N6-methyladenosine (m6A) in RNA, elevated levels of FTO in VCD-treated cells would result in decreased m6A-Tp53 transcripts and reduced P53 levels. Subsequently, we investigated whether the knockdown of FTO could lead to the derepression of P53 expression. The results obtained align with those observed *in vivo*. Unfortunately, we do not have quantitative data or methylation site detection for m6A modifications to TP53 in FTO knockdown cell lines, thus we cannot determine the regulatory role of FTO in tp53 m6A modification, but we suggest that RNA-binding proteins, including m6A reader proteins, may play a role.

Consequently, it is essential to undertake further research to examine the potential modulation of P53 gene and protein expression in granulosa cells, specifically through the investigation of FTO or m6A methylation.

Our study presents several limitations, notably the inadequate detection of the m6A methylase system. Furthermore, the investigation into the m6A reader protein, which is essential for post-modification translation, remains unaddressed. This area warrants further exploration. Future research should include experiments incorporating inhibitors and co-immunoprecipitation (Co-IP) to substantiate the correlation between proteins. Within the limitations of time and resources, the role of m6A-Tp53 in the regulation of the molecular mechanisms of HYF was initially identified and validated under current experimental conditions. However, further research is required to determine whether HYF modulates the transcription of m6A-Tp53 via FTO and to elucidate how it influences downstream signaling through modified translation. This necessitates a more comprehensive experimental design to strengthen the existing evidence.

5 Conclusion

In conclusion, the results of this study indicate that HYF is a promising therapeutic candidate for the treatment of POI. The research utilized both *in vivo* and *in vitro* experiments to conduct an initial exploration of the dynamic regulation of m6A RNA modification influenced by HYF. These findings enhance our understanding of the molecular mechanisms through which HYF preserves ovarian function (Figure 8). Moreover, this study offers a novel examination of the molecular mechanisms by which Chinese herbal compounds protect ovarian function, specifically through the perspective of m6A RNA modification. The findings of this research hold significant implications for future studies conducted in the field of female reproduction.

Data availability statement

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

Ethics statement

The animal study was approved by Guangdong Provincial Hospital of Chinese Medicine Institutional Animal Care and Use Committee (Registration number: 2019032; Approval date: 15 July 2019). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

YL: Methodology, Writing–original draft, Writing–review and editing. LW: Data curation, Writing–review and editing. JL: Writing–review and editing. GN: Project administration, Writing–review and editing. HY: Funding acquisition, Project administration, 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.2024.1491546/full#supplementary-material

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