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Integrating pharmacokinetics and network pharmacology to reveal mechanism of Shuangxia decoction in the treatment of insomnia

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ABSTRACT

Objective: Shuangxia Decoction is a traditional Chinese medicine classic formula used clinically to treat insomnia, consisting of *Pinellia ternata* (Banxia) and *Prunella vulgaris* (Xiakucao). The aim of this study is to explore the pharmacokinetic characteristics of the main core components in Shuangxia Decoction based on Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) analysis, as well as to preliminarily investigate its mechanisms of action in treating insomnia using quantitative targeted network pharmacology.

Methods: In this research, an LC-MS/MS method was established to analyze four main core components in Shuangxia Decoction: rosmarinic acid, 4-hydroxy-3-methoxyphenyllactic acid (HMLA), danshensu, and liquiritigenin. The pharmacokinetic characteristics of these phenolic compounds were investigated after oral administration of Shuangxia Decoction in rats. Network pharmacology and molecular docking were used to identify the underlying mechanism of Shuangxia Decoction in treating insomnia.

Results: The results showed that the analysis of the four components was completed within 6 min. The T_{max} for danshensu, HMLA, rosmarinic acid, and liquiritigenin were 0.79 ± 0.09, 0.63 ± 0.12, 0.51 ± 0.21, and 0.38 ± 0.19 h, respectively; their C_{max} were 110.83 ± 10.98, 25.20 ± 4.13, 37.57 ± 7.70, and 22.27 ± 8.75 μg/L, respectively; and their $T_{1/2}$ were 1.20 ± 0.24, 0.79 ± 0.26, 4.93 ± 1.08, and 2.85 ± 0.11 h, respectively. Danshensu exhibited the highest peak concentration (C_{max}: 110.83 ± 10.98 μg/L), while liquiritigenin showed the lowest C_{max} (22.27 ± 8.75 μg/L), likely due to hydrolysis by intestinal carboxylesterases. Network pharmacology results indicated that the main active components of Shuangxia Decoction exert their effects primarily through neuro-signaling pathways such as the dopaminergic synapse and glutamatergic synapse.

Conclusion: This study is the first to explore the pharmacokinetic characteristics of the four core components in Shuangxia Decoction and to provide preliminary predictions of its mechanisms in treating insomnia, laying a foundation for further exploration of its pharmacological mechanisms.

1. Introduction

Shuangxia Decoction (SXD), a two-herb formula containing *Pinellia ternata* (Banxia) and *Prunella vulgaris* (Xiakucao), is a cornerstone in traditional Chinese medicine (TCM) for treating insomnia,[1,2] particularly syndromes associated with liver fire hyperactivity and phlegm-heat disturbance. *Pinellia ternata*[3,4] modulates gastric function and calms the mind, while *Prunella vulgaris*[5,6] clears liver fire and resolves masses, synergistically harmonizing Yin-Yang equilibrium. Phenolic acids, such as rosmarinic acid and danshensu, are key bioactive constituents of SXD, exhibiting anti-inflammatory, antioxidant, and neuroprotective properties.[7] Rosmarinic acid attenuates neuroinflammation via NF-κB

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pathway inhibition,[8,9] whereas danshensu activates the PI3K/Akt signaling pathway to protect neurons.[10] SXD can significantly prolong total sleep duration and reduce sleep latency in rats by downregulating pro-inflammatory cytokines.[11,20]

Despite its clinical efficacy, the pharmacokinetic profile of SXD's phenolic acids remains unclear,[12,13] hindering mechanistic understanding. pharmacokinetic studies are pivotal for elucidating the absorption, distribution, metabolism, and excretion of multi-component herbal formulations, clarifying their "concentration-time-effect" relationships.[14,15] Here, we developed an ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method to simultaneously quantify four phenolic acids in rat plasma and evaluated their pharmacokinetic parameters. This work bridges the gap in SXD's pharmacokinetic research and lays a foundation for rational clinical use.

In recent years, network pharmacology has emerged as a transformative approach in TCM research. This methodology transcends the reductionist paradigm of "single disease-single target-single drug" therapeutics by revealing multi-compound synergism through polypharmacological mechanisms, and enabling precision therapeutic strategies that accelerate drug development pipelines. The inherent advantages of network pharmacology - including its comprehensive analytical framework, systems-level integration capabilities, and emphasis on biological synergy - align particularly well with the multidimensional therapeutic nature of TCM formulations.

Current pharmacokinetic investigations on SXD remain limited, particularly regarding the metabolic profiles of its phenolic acid constituents.[16] To date, no systematic pharmacokinetic study has been reported for phenolic acid components from SXD in rat plasma. Previous studies[17,18] have demonstrated that phenolic acids exhibit significant bioactivities and represent major active constituents in SXD. To bridge this knowledge gap, this study employed a validated UPLC-MS/MS method with network pharmacology method to investigate the pharmacokinetic characteristics and the mechanism of treating insomnia of four representative phenolic acids from SXD: rosmarinic acid, 4-hydroxy-3-methoxyphenyllactic acid (HMLA), danshensu, and liquiritigenin (Fig. 1) in rat models. The findings provide essential pharmacokinetic data and pharmacological mechanism to facilitate its modern research and clinical rational application.

2. Materials and methods

2.1. Chemicals, reagents, animals

Rosmarinic acid (Lot: Y06A9K67402), HMLA (Lot: Z12011 H127185), danshensu (Lot: G24J10L91371), liquiritigenin (Lot: PS0104481BD02B026), and chlorogenic acid (internal standard, IS, Lot: W160108100366) were purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China), The purity of all compounds exceeded 98%. LC-MS grade acetonitrile and formic acid were obtained from Thermo Fisher Scientific (USA). *Pinellia ternata* was supplied by Jiangxi Bairen Chinese Herbal Pieces Co., Ltd. (batch number 190101), while *Prunella vulgaris* was sourced from Hubei Shennong Bencao Chinese Herbal Pieces Co., Ltd. (batch number 20180901). Male Wistar rats (220–240 g) were supplied by SPF Biotechnology Co., Ltd. (Beijing, China; License No. SCXK [Jing] 2019-0010).

2.2. Preparation of lyophilized powder from SXD extract

A mixture of 100 g of *Pinellia ternata* and 100 g of *Prunella vulgaris* was soaked in a 20-fold volume of water for 30 min, followed by reflux extraction for 2 h. The extract was filtered through three layers of gauze. The marc was subjected to a second extraction under identical conditions. The resulting filtrates were combined, concentrated via rotary evaporation, and then lyophilized to obtain 36.7 g of lyophilized powder, yielding an extraction rate of 18.35%. The powder was stored at −80 °C for subsequent use.

2.2.1. Preparation of calibration standards and quality control samples

Stock solutions of rosmarinic acid, danshensu, HMLA, and liquiritigenin were individually prepared at 1 mg/mL using 50% methanol-water (v/v) as the solvent. These stock solutions were subsequently diluted with the same solvent to obtain a mixed standard solution containing rosmarinic acid (5 μg/mL), danshensu (2.5 μg/mL), HMLA (5 μg/mL), and liquiritigenin (2.5 μg/mL).

Different volumes of the mixture of working solutions were transferred into volumetric flasks and then diluted to volume to make the calibration standard samples and quality control (QC) samples. Calibration standard samples of rosmarinic acid and HMLA (1.22, 4.88, 19.53, 78.125, 312.5, 1250, 5000 ng/mL), danshensu and liquiritigenin (0.61, 2.44, 9.77, 39.1, 156.3, 625, 2500 ng/mL) were prepared by spiking blank rat plasma (50 L) with working solutions at different concentrations. The QC samples at three different concentration levels (31.28, 256, and 4000 ng/mL for rosmarinic acid and HMLA, 15.64, 128, and 2000 ng/mL for danshensu and liquiritigenin, representing low, medium and high concentrations, respectively) were prepared by the same fashion as the calibration standard samples. During analysis these QC samples were placed after every five to six unknown samples. Chlorogenic acid was employed as the internal standard (IS) at a constant concentration of 2 μg/mL in all solutions. All solutions were stored at 4 °C and equilibrated to room temperature prior to analysis.

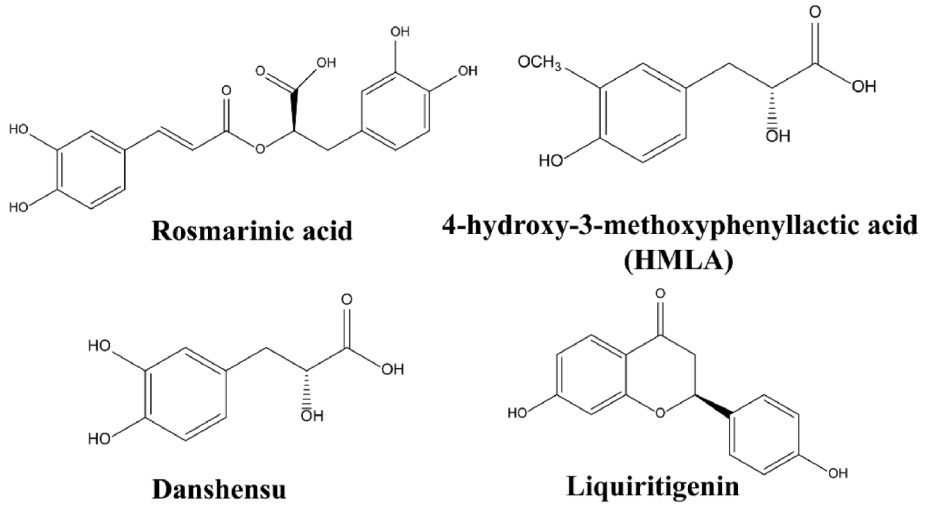

Fig. 1. Chemical structure of four analytes (Rosmarinic acid, 4-hydroxy-3-methoxyphenyllactic acid, Danshensu, and Liquiritigenin) in Shuangxia decoction.

2.3. Plasma sample preparation

Plasma samples (50 μL) were mixed with 20 μL of 50% methanol-water (v/v), 25 μL of 12% hydrochloric acid solution, and 20 μL of chlorogenic acid (internal standard, IS) solution (2 μg/mL). The mixture was vortex-mixed for 3 min, followed by the addition of 250 μL ethyl acetate. After vortexing for another 3 min, the samples were centrifuged at 5000× *g* for 5 min, and the supernatant was collected. The extraction procedure was repeated once, and the combined supernatants were evaporated to dryness under a gentle nitrogen stream at 40 °C. The residue was reconstituted in 100 μL of ultrapure water, vortex-mixed for 3 min, and centrifuged at 12,500× *g* for 15 min at 4 °C. Finally, 2 μL of the supernatant was injected into the UPLC-MS/MS system for analysis.

2.4. Analytical conditions

Chromatographic separation was performed on a Waters ACQUITY UPLC system coupled with a Xevo TQ-S Micro triple quadrupole mass spectrometer (Waters Corporation, Milford, MA, USA). A Waters Acquity UPLC HSS T3 C_{18} column (2.1 mm × 50 mm, 1.8 μm) was used with the column temperature maintained at 40 °C. The mobile phase consisted of 0.1% formic acid in water (A) and acetonitrile (B). A gradient elution program was applied as follows: 0–1 min, 5% B; 1–9 min, 5%–50% B; 9–12 min, 50%–95% B; 12–13 min, 95% B; 13–14 min, 95%–5% B; 14–15 min, 5% B. The flow rate was 0.3 mL/min, the injection volume was 2 μL, and the autosampler temperature was set to 10 °C.

Mass spectrometric detection was conducted using an electrospray ionization (ESI) source in negative ion mode with multiple reaction monitoring (MRM). The monitored ion transitions were as follows: rosmarinic acid (*m/z* 358.98 → 161.02), HMLA (*m/z* 210.97 → 133.8), danshensu (*m/z* 196.95 → 134.97), liquiritigenin (*m/z* 255.04 → 118.95), and the internal standard chlorogenic acid (*m/z* 353.0 → 191.07). Optimized mass parameters included cone voltages of 34 V (rosmarinic acid), 42 V (HMLA), 18 V (danshensu), 34 V (liquiritigenin), and 36 V (chlorogenic acid), and collision energies of 14 eV (rosmarinic acid), 16 eV (HMLA), 16 eV (danshensu), 24 eV (liquiritigenin), and 12 eV (chlorogenic acid). The dwell time was set to 0.028 s. Additional instrument parameters were configured as follows: capillary voltage 3 kV, cone gas flow 50 L/h, desolvation gas flow 1000 L/h, desolvation temperature 300 °C, and source temperature 150 °C. System control and data processing were performed using MassLynx software (Version 4.2, Waters Corporation, USA).

2.5. Method validation

The UPLC-MS/MS method was validated in accordance with the FDA Bioanalytical Method Validation Guidance, including assessments of specificity, linearity, accuracy, precision, lower limit of quantification (LLOQ), matrix effect, recovery, and stability.

2.5.1. Specificity

Specificity was evaluated by analyzing blank plasma samples from six different sources and LLOQ samples to confirm the absence of endogenous interference at the retention times of the analytes and internal standard (IS).

2.5.2. Calibration curve, linearity, and sensitivity

Calibration curves were constructed using weighted ($1/x^2$) least squares regression of the analyte-to-IS peak area ratio (y) versus nominal concentration (x). The correlation coefficient (r) exceeded 0.99 for all analytes. Linear ranges were as follows: rosmarinic acid (0.5–1000 ng/mL), HMLA (0.5–1000 ng/mL), danshensu (0.5–500 ng/mL), and liquiritigenin (0.5–500 ng/mL). The LLOQ, defined as the lowest concentration with a signal-to-noise ratio (S/N) > 10, was 0.5 ng/mL for all analytes.

2.5.3. Precision and accuracy

Precision and accuracy were evaluated using QC samples at LLOQ, low (LQC), medium (MQC), and high (HQC) concentrations. Accuracy, expressed as relative error (RE%), was required to be within ±15% (±20% for LLOQ). Precision, expressed as relative standard deviation (RSD%), was required to be <15% (<20% for LLOQ). Intra-day and inter-day RSD% values were <15%, and RE% values were within ±15%, meeting bioanalytical acceptance criteria.

2.5.4. Extraction recovery and matrix effect

Extraction recovery and matrix effect were assessed at low, medium, and high concentration levels. Recovery was calculated by comparing peak areas of QC samples with those of post-extraction spiked blank plasma. Matrix effect was evaluated by comparing peak areas of post-extraction spiked plasma samples with pure solvent standards. Both recovery and matrix effect ranged from 85% to 115%, demonstrating efficient extraction and minimal matrix interference.

2.5.5. Stability

Stability was evaluated under the following conditions: Room temperature (25 °C) for 12 h, Long-term storage at −80 °C for 30 d, Autosampler storage (10 °C) for 12 h, Three freeze-thaw cycles (−80 °C–25 °C). All analytes remained stable, with measured concentrations within ±15% of nominal values.

2.6. Pharmacokinetic application

To investigate the pharmacokinetic properties of four phenolic acids (rosmarinic acid, HMLA, danshensu, and liquiritigenin) from SXD, a single-dose oral gavage of SXD lyophilized powder (1.92 g/kg) was administered to male Wistar rats (n = 6, body weight 220–240 g; supplied by SpePharm Biotechnology Co., Ltd., Beijing, China). Blood samples were collected via the orbital venous plexus at predefined time points: 0 (pre-dose), 0.083, 0.25, 0.5, 1, 2, 4, 8, 12, 24, and 48 h post-dosing. Plasma was separated by centrifugation (3000× *g*, 10 min) and immediately stored at −80 °C until analysis. All animal procedures were conducted in compliance with a protocol approved by the Institutional Animal Care and Use Committee (IACUC). The study was approved by the Ethics Committee of Xiangyang No. 1 People's Hospital (Ethical Approval Number: XYYYE20250053). The SXD lyophilized powder was prepared in-house by our research group.

Pharmacokinetic parameters, including area under the curve (AUC), maximum plasma concentration (C_{max}), time to C_{max} (T_{max}), total clearance (CL), apparent volume of distribution (V), mean residence time (MRT), and elimination half-life ($t_{1/2}$), were calculated using WinNonlin software (Version 8.0, Certara, Princeton, NJ, USA). Non-compartmental analysis (NCA) was applied to derive pharmacokinetic parameters from plasma concentration-time profiles.

2.7. Targets and mechanisms prediction of SXD based on network pharmacology

The SwissTargetPrediction website (https://SwissTargetPrediction.ch) is used to predict the targets of four components (rosmarinic acid, 4-hydroxy-3-methoxyphenyllactic acid [HMLA], danshensu, and liquiritigenin) with good pharmacokinetic activity in SXD. The OMIM database (https://omim.org/) and GeneCards database (http://www.genecards.org/) are used to predict disease targets using "insomnia" as the search term. The common genes obtained from this analysis represent the key action targets of SXD in the treatment of insomnia. This key action target was then uploaded to the STRING platform (https://string-db.org) with the species set as "*Homo sapiens*". The advanced filter "highconfidence (0.700)" was applied, and the resulting network was downloaded and saved in TSV format. The data was imported into Cytoscape 3.9.1 software, and the NetworkAnalyzer function was used to perform network topology analysis.

2.8. Molecular docking verification

To verify the interaction between the four components with good pharmacokinetic activity in SXD and the targets, molecular docking analysis was performed using the CB-dock2 website (https://cadd.labshare.cn/cb-dock2/php/index.php). First, the protein structures of the target proteins were obtained from the RCSB PDB database (http://www.rcsb.org/). Next, the 3D structures of the active ingredients of NMC were downloaded from the PubChem website (https://pubchem.ncbi.nlm.nih.gov/). Finally, molecular docking simulations were conducted using the CB-dock2 website, and the results were visualized using PyMol.

3. Results

3.1. Method development

This study established an LC-MS/MS analytical method for simultaneous determination of rosmarinic acid, 4-hydroxy-3-methoxyphenyllactic acid (HMLA), danshensu, and liquiritigenin in rat plasma. Through optimization of chromatographic and mass spectrometric conditions, rapid separation and highly sensitive detection of the four compounds were achieved.

For chromatographic optimization, separation was performed on a Waters Acquity UPLC HSS T3 C_{18} column (2.1 mm × 50 mm, 1.8 μm) maintained at 40 °C. The mobile phase consisted of 0.1% formic acid in water (A) and acetonitrile (B), with gradient elution achieving complete separation within 15 min. Mass spectrometric detection was conducted using an electrospray ionization (ESI) source in negative ion mode. All analytes demonstrated optimal responses under negative ionization, with multiple reaction monitoring (MRM) transitions established as follows: rosmarinic acid (*m/z* 358.98 → 161.02), HMLA (*m/z* 210.97 → 133.8), danshensu (*m/z* 196.95 → 134.97), liquiritigenin (*m/z* 255.04 → 118.95), and chlorogenic acid (IS, *m/z* 353.0 → 191.07). Detailed MS/MS parameters including retention times, dwell times, cone voltages, and collision energies are provided in Table 1.

3.2. Method validation

3.2.1. Selectivity

Three types of plasma samples were analyzed by UPLC-MS/MS. As shown in Fig. 2, endogenous substances in blank rat plasma did not exhibit significant interference with the determination of the four target analytes. All compounds displayed well-resolved chromatographic peaks, confirming the high specificity of the method.

3.2.2. Calibration curves

Calibration curves for the four analytes exhibited excellent linearity, with correlation coefficients (rr) exceeding 0.99, indicating a linear relationship between peak areas and concentrations within the selected ranges. The specific linear ranges and regression equations were as follows: For rosmarinic acid, the linear range was 0.5–1000 ng/mL, the regression equation was y = 0.0007x + 0.0061, and the correlation coefficient r = 0.999. For HMLA, the linear range was 0.5–1000 ng/mL, the regression equation was y = 0.0028x + 0.0015, and the correlation coefficient r = 0.999. For Danshensu, the linear range was 0.5–500 ng/mL, the regression equation was y = 0.0059x + 0.0036, and the correlation coefficient r = 0.999. For liquiritigenin, the linear range was 0.5–500 ng/mL, the regression equation was y = 0.0115x + 0.0031, and the correlation coefficient r = 0.999.

3.2.3. Precision and accuracy

Intra-day and inter-day precision and accuracy were evaluated using QC samples at low, medium, and high concentrations. The results demonstrated that the relative standard deviation (RSD%) for both intra-day and inter-day precision was less than 15%, and the relative error (RE%) for accuracy fell within ±15%, meeting the acceptance criteria for bioanalytical method validation. Detailed data are summarized in Table S1.

3.2.4. Extraction recovery and matrix effect

The extraction recovery and matrix effect were evaluated at low, medium, and high concentration levels, respectively. When the extraction recovery was within the range of 85%–115% and the matrix effect was within the range of 85%–115%, it indicated that the established method had good extraction efficiency and matrix tolerance. Detailed data are summarized in Table S2.

3.2.5. Stability

The stability of quality control samples was evaluated under the conditions of being placed at room temperature for 12 h, stored at −80 °C for 30 days, placed in the autosampler (at 10 °C) for 12 h, and under freeze-thaw cycles. The results showed that all the analytes were stable under the above conditions, and the measured concentrations were within ±15% of the theoretical values. Detailed data are summarized in Table S3.

3.3. Pharmacokinetic study

The established UPLC-MS/MS method was successfully applied to investigate the pharmacokinetics of SXD in rats following oral administration. The pharmacokinetic curves of the four compounds are shown in Fig. 3, the detailed pharmacokinetic parameters of four compounds *in vivo* are presented in Table 2. The results demonstrated that the plasma concentrations of rosmarinic acid, HMLA, danshensu, and liquiritigenin varied over time, with all compounds reaching their maximum plasma concentrations (C_{max}) within 1-h post-administration. Among these compounds, danshensu exhibited the highest C_{max} of 110.83 ± 10.98 μg/L, while liquiritigenin showed the longest elimination half-life ($t_{1/2}$) of 2.85 ± 0.11 h. These findings indicate that the phenolic acids in SXD are rapidly absorbed and metabolized *in vivo*, potentially exerting their pharmacological effects through their metabolites.

3.4. Targeted network pharmacology analysis

The rosmarinic acid, 4-hydroxy-3-methoxyphenyllactic acid (HMLA), danshensu, and liquiritigenin from SXD were input into the SwissTargetPrediction website for target prediction, and 313 related targets were obtained. In total, 919 targets related to insomnia were obtained from the OMIM, and GeneCards databases, and 70 intersection targets were identified (Fig. 4A).

The intersection targets were imported into the STRING website to

Table 1
MS/MS transitions and parameters for the detection of the 4 standards and internal standard compound chlorogenic acid.

Analytes	Retention times	Parent (*m/z*)	Daughter (*m/z*)	Dwell (s)	Cone (V)	Collision (V)
Danshensu	1.16	196.95	134.97	0.028	18	16
HMLA	2.43	210.97	133.80	0.028	42	16
Rosmarinic acid	4.68	358.98	161.02	0.028	34	14
Liquiritigenin	5.26	255.04	118.95	0.028	34	24
Chlorogenic acid(IS)	2.49	353.00	191.07	0.028	36	12

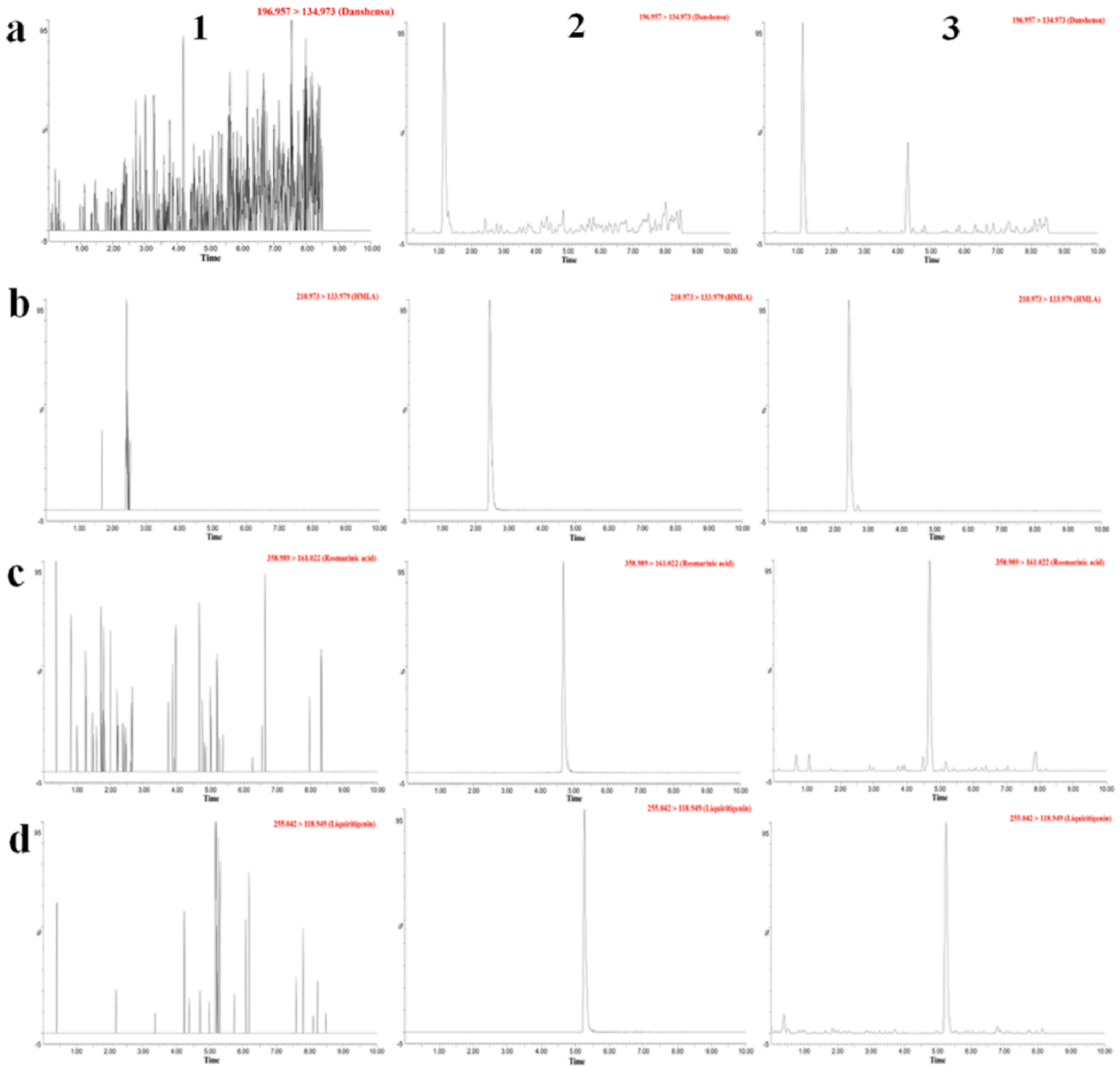

Fig. 2. MRM chromatogram of four analytes (a: rosmarinic acid, b: 4-hydroxy-3-methoxyphenyllactic acid, c: danshensu, d: liquiritigenin) in different samples. (1: Blank plasma; 2: L-QC samples; 3: 1 h plasma sample after administration Shuangxia decoction).

construct the protein–protein interaction network. The topological characteristics of the protein network structure were further analyzed using Cytoscape3.9.1 to screen out the core targets for disease treatment, as shown in Fig. 4B, among which the directly related targets were GABRA1, ADORA2A, DRD1, HTR1A, GABBR1, DRD2, and MAOB from all key targets. GO and KEGG enrichment analyses were performed for the intersection of disease and drug targets using the DAVID database (Fig. 4C). The results showed that the activity of SXD mainly involved glutamatergic synapse, neuronal cell body, cellular response to amyloid-beta, and other biological processes (Fig. 4D). The main pathways enriched by KEGG included pathways of neurodegeneration - multiple diseases, the calcium signaling pathway, and dopaminergic synapse. To elucidate the relationship among components, targets, and pathways, a TCM ingredient–target–pathway network was constructed using Cytoscape 3.10.1 (Fig. 4E). The network visually demonstrated the relationship among the core active ingredients, core targets, and mechanism of action of SXD in the treatment of insomnia.

3.5. *Molecular docking*

In the analysis of the protein–protein interaction networks, potential therapeutic targets associated with insomnia were systematically identified and prioritized for subsequent molecular docking analysis. These proteins were GABRA1 (PDBID: 6 × 3T), ADORA2A (PDBID: 4EJY), DRD1 (PDBID: 7JVP), HTR1A (PDBID: 8FYE), GABBR1 (PDBID: 4MS4), DRD2 (PDBID: 7JVR), and MAOB (PDBID: 7P4H). Molecular docking was performed between these key proteins and the four components with good pharmacokinetic characteristics from the SXD. The results

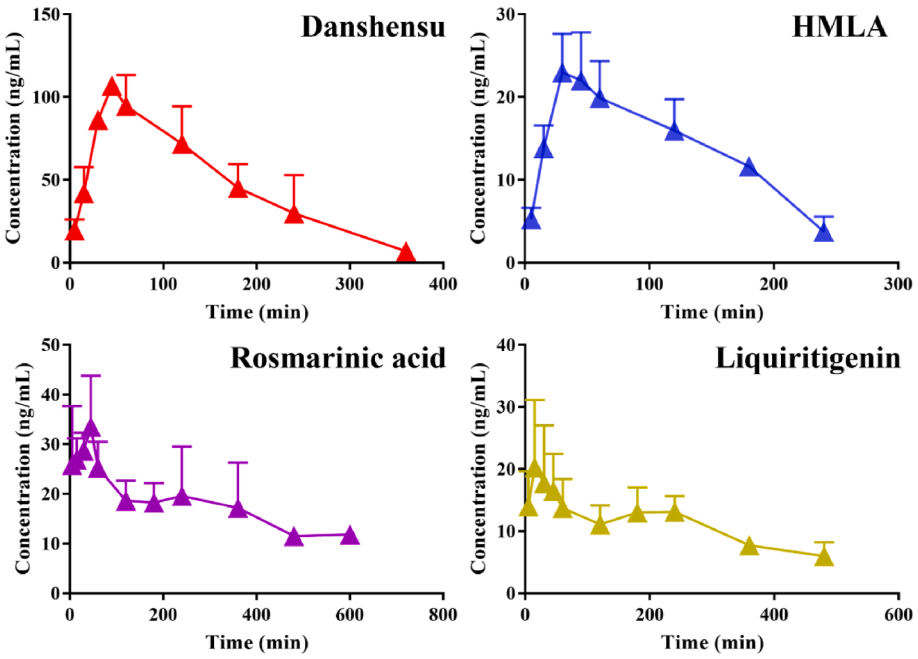

Fig. 3. Average plasma concentration-time curves of four analytes in rats after intragastric administration of Shuangxia decoction.

Table 2
Pharmacokinetic parameters of 4 compounds from Shuangxia decoction.

Compound	Pharmacokinetic parameters					
	T_{max} (h)	C_{max} (μg/L)	AUC_{0-t} (h·μg/L)	$AUC_{0-\infty}$ (h·μg/L)	MRT_{0-t} (h)	$T_{1/2}$ (h)
Danshensu	0.79 ± 0.09	110.83 ± 10.98	276.18 ± 58.11	288.79 ± 58.98	2.05 ± 0.20	1.20 ± 0.24
HMLA	0.63 ± 0.12	25.20 ± 4.13	54.14 ± 7.53	56.71 ± 6.28	1.65 ± 0.05	0.79 ± 0.26
Rosmarinic acid	0.51 ± 0.21	37.57 ± 7.70	174.37 ± 13.76	210.86 ± 18.49	4.76 ± 0.15	4.93 ± 1.08
Liquiritigenin	0.38 ± 0.19	22.27 ± 8.75	83.94 ± 8.74	102.34 ± 10.01	3.36 ± 0.36	2.85 ± 0.11

showed stable docking between the key targets and the active ingredients in SXD (≤5 kJ/mol). Further details are provided in Fig. 5, Table 3, and Table S4.

4. Discussion

This study successfully established a sensitive and reliable UPLC-MS/MS method for the simultaneous quantification of four phenolic acids-rosmarinic acid, HMLA, danshensu, and liquiritigenin in rat plasma. The method demonstrated excellent specificity, with no interference from endogenous substances, and exhibited good linearity, precision, accuracy, extraction recovery (85%–115%), matrix effect (85%–115%), and stability under various conditions. These validated parameters confirm the method's suitability for accurately determining plasma concentrations of the target compounds. Pharmacokinetic analysis following oral administration of SXD revealed rapid absorption of all four compounds, with time to peak (T_{max}) within 1 h. Liquiritigenin showed the fastest absorption ($T_{max} < 0.5$ h), while danshensu achieved the highest peak plasma concentration (C_{max}: 110.83 ± 10.98 μg/L), likely due to its dual role as a constituent of SXD and a hydrolytic metabolite of rosmarinic acid. Despite being a major active component, rosmarinic acid exhibited low systemic exposure (C_{max}: 37.57 ± 7.7 μg/L), attributable to enzymatic hydrolysis of its ester bond by intestinal carboxylesterases. This suggests that its therapeutic effects may arise from bioactive metabolites rather than the parent compound. Notably, HMLA, a metabolite of both rosmarinic acid and danshensu, displayed enhanced lipophilicity, indicating its potential as a pharmacologically active derivative. The analysis of phenolic acids posed challenges due to their structural instability, driven by multiple phenolic hydroxyl and free carboxyl groups. To address this, a sample pretreatment protocol involving ethyl acetate extraction with 12% dilute hydrochloric acid was optimized, effectively reducing matrix interference, improving compound stability, and enhancing sensitivity. Reconstitution in pure water further sharpened chromatographic peaks, ensuring reliable quantification. Despite administering a triple clinical-equivalent dose of SXD, plasma concentrations of the compounds remained low. This paradox-low systemic exposure yet significant efficacy-may be explained by several factors: (1) high intrinsic bioactivity of the compounds at low concentrations, (2) synergistic effects among multiple components in SXD, or (3) extensive metabolism of parent compounds in the gastrointestinal tract and liver, generating undetected active metabolites. Future studies should prioritize profiling gastrointestinal metabolites and evaluating their bioactivity to clarify these mechanisms. The low plasma levels of rosmarinic acid-derived metabolites, which contain polar functional groups (e.g., phenolic hydroxyl and carboxyl groups), suggest limited penetration across biological barriers such as the blood-brain barrier. Instead, SXD or its constituents may exert therapeutic effects via direct interaction with gastrointestinal targets.

Although several studies have reported network pharmacology-based predictions on the mechanisms of SXD in treating insomnia or other diseases,[19] most of them rely on chemical constituents derived from the herbal ingredients or compounds identified in blood via high-resolution mass spectrometry. However, whether these blood-exposed components truly represent the bioactive substances responsible for the therapeutic effects remains debatable. Moreover, it is uncertain whether their concentrations reach pharmacologically effective levels. These issues raise significant concerns, as only compounds with adequate exposure and complete pharmacokinetic profiles can be

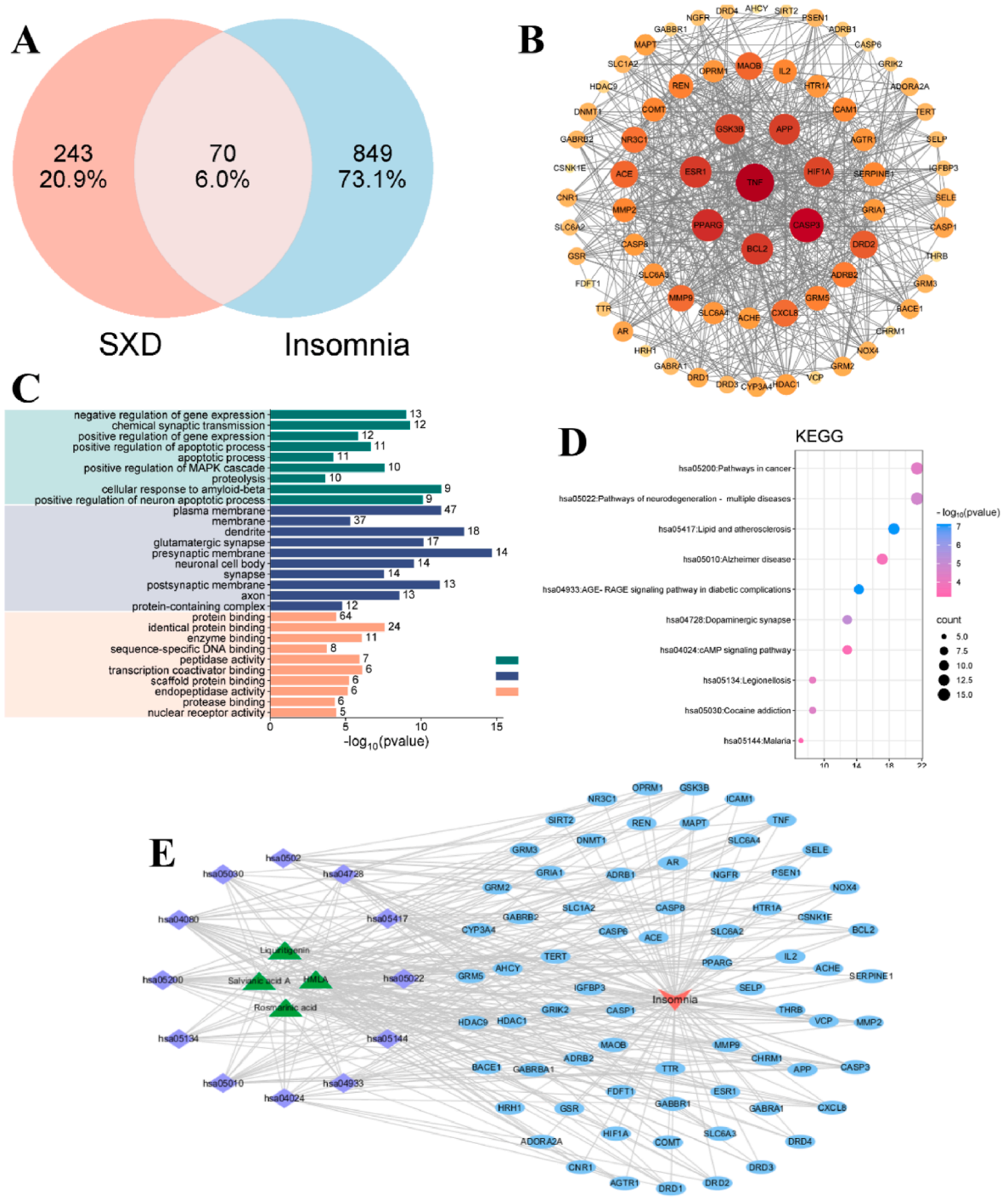

Fig. 4. Network pharmacology analysis of Shuangxia Decoction for the treatment of insomnia. (A: Venn diagram showing the intersection targets between Shuangxia Decoction and insomnia. B: Protein-protein interaction (PPI) network diagram of the intersection targets. C: Bar graph of GO enrichment analysis. D: Bar graph of Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis. E: Network diagram illustrating the relationship between the drug, active ingredients, targets, and pathways for the treatment of insomnia with Shuangxia Decoction).

confidently considered efficacious. Therefore, network pharmacology analyses based on such well-characterized pharmacokinetic compounds would yield more reliable and credible results. Meanwhile, several studies have reported pharmacokinetic investigations of active components in Xiakucao Oral Liquid.[20] However, these studies have primarily focused on the pharmacokinetics of prototype compounds derived from *Prunella vulgaris*, such as hyperoside and salvianic acid A. In contrast, our current research places greater emphasis on rosmarinic acid-a major active constituent of *Prunella vulgaris*—along with its metabolite HMLA, danshensu, and liquiritigenin, a potential active processing-derived residue. Thus, the two lines of inquiry differ in their focal compounds and each possesses distinct characteristics.

In summary, this study represents the first comprehensive pharmacokinetic investigation of four phenolic acids in SXD, providing a robust analytical method and critical insights into their *in vivo* behavior. While rat models cannot fully replicate human physiology, these findings offer valuable guidance for mechanistic research and clinical optimization of SXD. Future work should integrate gut microbiota metabolism, hepatic

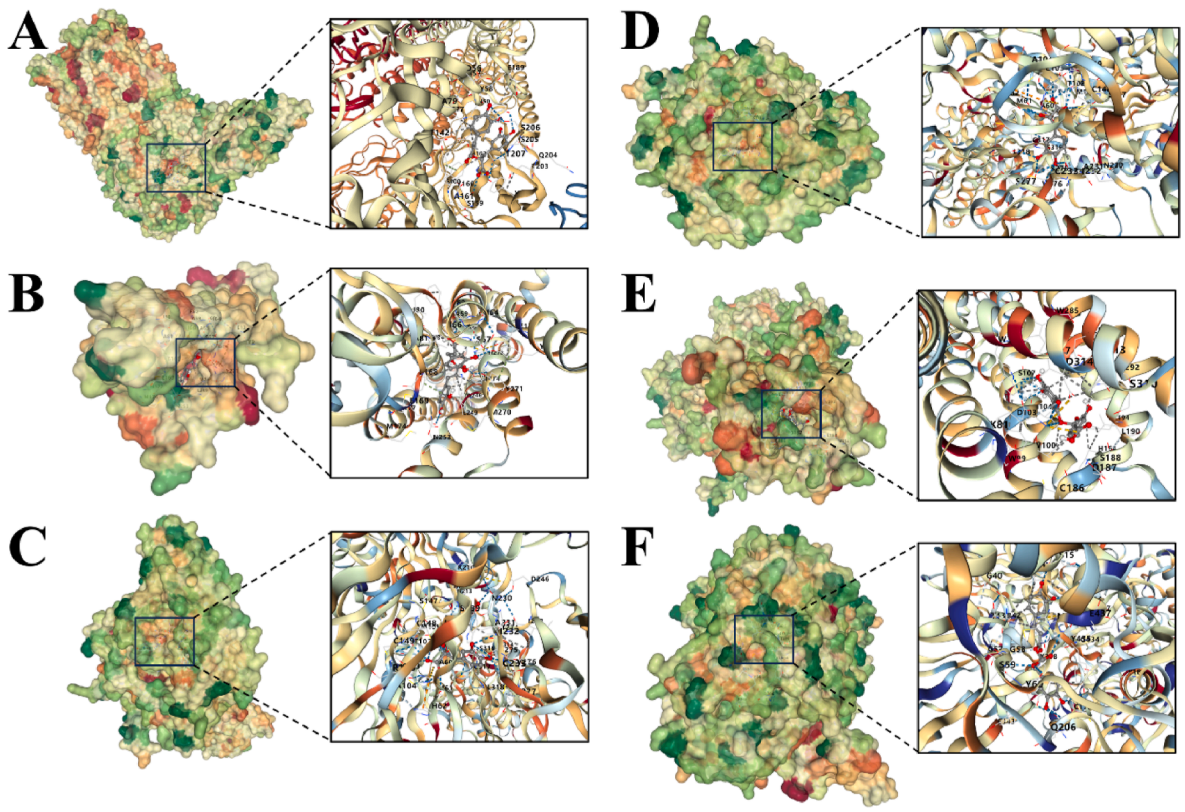

Fig. 5. The molecular docking analysis of four active components of Shuangxia Decoction and key targets. (A) GABRA1 with rosmarinic acid, (B) ADORA2A with rosmarinic acid, (C) HTR1A with rosmarinic acid, (D) HTR1A with danshensu, (E) DRD1 with rosmarinic acid, (F) MAOB with rosmarinic acid.

Table 3
Binding energy of active ingredients and core targets in Shuangxia Decoction (kJ/mol).

Compounds	Rosmarinic acid	HMLA	Danshensu	Liquiritigenin
GABRA1	−8.8	−7.3	−7.3	−9.4
ADORA2A	−10.6	−6.8	−6.7	−10.6
DRD1	−9.0	−7.4	−7.1	−9.1
HTR1A	−9.3	−7.6	−7.2	−9.6
GABBR1	−8.3	−6.4	−8.0	−7.9
DRD2	−9.2	−6.5	−6.4	−8.2
MAOB	−10.1	−7.1	−3.6	−10.0

microsomal incubation, and metabolite profiling to systematically elucidate SXD's metabolic pathways and therapeutic mechanisms, advancing its rational clinical application.

5. Conclusion

In the present study, a rapid analytical method was established using UPLC-MS/MS for simultaneous determination of four primary core components with high-abundance in SXD - rosmarinic acid, HMLA, danshensu, and liquiritigenin. This validated method was successfully applied to plasma pharmacokinetic studies following intragastric administration of SXD in rats. These findings collectively demonstrated that the four aforementioned components exhibited complete pharmacokinetic profiles with significant systemic exposure after oral administration, suggesting their potential as bioactive constituents. Network pharmacological analysis further revealed that these four phenolic acids in SXD primarily exert therapeutic effects through dopaminergic synapse-related pathways and other critical biological routes. Molecular docking simulations indicated favorable binding affinities between these core components and multiple sleep-regulating targets, implying that their pharmacological actions may be mediated through multi-target mechanisms.

CRediT authorship contribution statement

Ke Meng: Writing – original draft, Investigation, Formal analysis. **Miao Xu:** Resources, Formal analysis, Data curation. **Yanping Liu:** Methodology, Formal analysis. **Ying Li:** Methodology, Investigation. **Wei Zhang:** Validation, Investigation. **Yue He:** Writing – review & editing, Investigation, Conceptualization. **Chenning Zhang:** Writing – review & editing, Supervision, Funding acquisition.

Ethical approval

This study was approved by the Xiangyang No. 1 People's Hospital Ethics Committee/Institutional Review Board (Ethical Approval Number: XYYYE20250053).

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhip.2025.11.005.

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