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# Formulation and Pharmacological Evaluation of a Medicine-Food Homology Herb-Based Formula for Sleep Enhancement

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## ABSTRACT

Sleep disorder is a widespread health concern with limited safe therapy options. Medicine-food-homology (MFH) plants offer a promising source for developing a natural sleep-improving dietary supplement because of their nutritional and medicinal qualities. According to traditional medicine principles and the medicinal properties of MFH herbs, we created an MFH-based sleep-modulating formula (SMF), which consists of *Ziziphus spinosa* semen, *Gastrodiae elata* rhizome, *Dendrobium officinale*, *Polygonati rhizome*, *Panax notoginseng* flower and *Radix Glycyrrhizae* preparata. The main active components and quality marker of SMF were analysed. A murine pentobarbital-induced sleep model was constructed to evaluate the sleep-modulating effect of SMF. The results showed that 35 pharmacologically active compounds were identified from SMF. Seven compounds were selected as quality markers to control the quality of SMF. Notably, oral administration of SMF improved the disorder of the neurotransmitter system and exerted a synergistic hypnotic effect with pentobarbital by improving the occurrence rate of sleep and shortening sleep latency. Network pharmacology analysis predicted signalling pathways, such as neuroactive ligand-receptor interactions, serotonergic/dopaminergic synapses and thyroid hormone signalling, to be involved in the sleep modulation of SMF. Molecular docking revealed that SMF exerts therapeutic effects through interactions with key targets, including HTR2A, HTR2C, ADRB2, DRD2, GABRA1, GABBR1 and MAOA. In conclusion, these findings suggest that SMF has a potential sleep-promoting effect by restoring neurotransmitter balance and mediating neurological pathways in mice. This study also elucidates the active constituents and sets quality control criteria for SMF, facilitating its translational application.

**Abbreviations:** 5-HT, serotonin; ACN, acetonitrile; ADRB2, adrenoceptor beta 2; ANOVA, one-way analysis of variance; ARRIVE, Animal Research: Reporting of In Vivo Experiments; BW, body weight; DA, dopamine; DRD2, dopamine receptor D2; GABA<sub>A</sub>,  $\gamma$ -aminobutyric acid sub-type A receptors; GABBR1, gamma-aminobutyric acid type B receptor subunit 1; GABRA1, gamma-aminobutyric acid type A receptor subunit alpha1; GC, *Radix Glycyrrhizae* preparata; HESI, heated-electrospray ionization source; HJ, *Polygonati Rhizoma*; HPLC-DAD, high-performance liquid chromatography with diode array detector; HTR2A, 5-hydroxytryptamine receptor 2A; HTR2C, 5-hydroxytryptamine receptor 2C; ICH, International Council for Harmonisation; ICR, Institute of Cancer Research; LOD, limit of detection; LOQ, limit of quantification; LORR, loss of righting reflex; MAO, monoamine oxidases; MAOA, monoamine oxidase A; MFH, medicine-food homology; NE, noradrenaline; PDB, protein data bank; PPI, protein-protein interaction; QE, Q-Exactive; REM, rapid eye movement; RSD, relative standard deviation; SMF, sleep-modulating formulae; SMILES, Simplified Molecular-Input Line-Entry System; SQH, *Panax notoginseng* flower; STRING, Search Tool for the Retrieval of Interacting Genes/Proteins; SZR, *Z. spinosa* semen; TM, *Gastrodiae elata* Bl. Rhizoma; TPSH, *Dendrobium officinale* Kimura et Migo; UPLC, Ultra High-Performance Liquid Chromatography.

Jasmine U. Ting, Chunxue Zhao, and Jia Xu contributed equally to this work.

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## 1 | Introduction

In modern societies, insomnia is a widespread chronic sleep disorder, affecting about 10%–30% of the global population regardless of age, gender or ethnicity. Insomnia is characterised by difficulties in sleep initiation or maintaining sleep, which leads to interruptions to multiple regulatory systems, including physiological hyperarousal of the nervous system, dysregulation of the hypothalamic–pituitary–adrenal (HPA) axis and hormonal dysfunction (DelRosso et al. 2025; Zhang et al. 2025). Conventional treatments, including cognitive behavioural therapy for insomnia and hypnotic medications, can provide short-term relief but are frequently associated with side effects such as dependence, tolerance and cognitive impairment (Zhang et al. 2025; Madari et al. 2021; Zhong et al. 2024). These limitations have led to the development of other kinds of safer and more effective strategies for sleep disorders to satisfy clinical requirements. Nowadays, many herbal and natural supplements are commonly used as cost-effective, accessible and safer alternatives for managing sleep disorders (Zhong et al. 2024; Arnao and Hernández-Ruiz 2018). Recent surveys indicate that the worldwide sleep supplement market was worth \$6.8 billion in 2024 and is expected to grow at a compound annual growth rate of 5.1% to reach \$11.8 billion in 2033 (Vinayak 2025). With the concept of diet therapy steadily coming into public awareness, medicinal food products have begun to attract attention in the sleep-improving supplement market due to their powerful effects on health, safety and tolerability.

In China, the usage of medicine food homology (MFH) herbal resources to assist or replace drugs in treating sleep disorders has a long history. As a unique type of Chinese botanical drug, the MFH herbs exhibit advantages over usual herbal medicine in high nutritional value, healthcare activities and long-term safety profiles. Many formulas consisting of MFH resources have been summarised, achieving satisfactory results in alleviating sleeping disorders through modulating the function of multiple parts of the body (Ma et al. 2024). Suan-Zao-Ren decoction (SZRD) is one of the most popularly used formulae for the treatment of different types of insomnia, depression, neurasthenia, restlessness and vertigo. This formula is composed of *Ziziphus spinosa* semen (Suan-Zao-Ren), *Rhizoma Chuanxiong* (Chuan-Xiong), *Poria* (Fu-Ling), *Anemarrhenae Rhizoma* (Zhi-Mu) and *Glycyrrhizae Radix Et Rhizoma/Radix Glycyrrhizae* preparata (Gan-Cao), which exerts synergistic action working together to achieve therapeutic effects. Modern pharmacological research has shown that SZRD exerts the sedative and hypnotic effect through regulating the levels of neurotransmitters (L. Chen et al. 2020; Cho et al. 2012; Yang et al. 2023; Dong et al. 2021). SZRD can also improve insomnia via regulating the structure of the bacterial community (Zhang et al. 2025). In addition to *sour jujube semen*, a variety of MFH plants such as *Polygonati Rhizoma* (Huang-Jing), *Gastrodiae elata* Bl. (Tian-Ma) and *Dendrobium officinale* Kimura et Migo (Tie-Pi-Shi-Hu) were also frequently applied sedative-hypnotic drugs in traditional Chinese medicine (TCM) (Kuboyama et al. 2017; H. B. Zhou et al. 2023; H. P. Zhou et al. 2025). Given the importance of MFH herbs in modulating sleep quality, it is expected that developing an MFH-based formula would provide a new opportunity for management and daily intervention of sleep disorders.

According to traditional medicine theories and the medicinal properties of MFH herbs, six MFH herbs, including stir-fried *Z. spinosa* semen, *G. elata* Bl. *rhizome*, *D. officinale*, stewed *Polygonati rhizome*, *Panax notoginseng* flower and *Radix Glycyrrhizae* preparata, were selected to construct a sleep-modulating formula (SMF) used as a sleeping-improving dietary supplement. Ultra-high performance liquid chromatography–quadrupole-electrostatic field Orbitrap mass spectrometry (UPLC–Q-Exactive/MS) analysis was used to qualitatively analyse the chemical constituents of SMF. Twelve of the main constituents were verified using reference standards. Then, these constituents serve as indices to optimise the extraction process, including soaking time, decocting time and reflux conditions. The established extraction process was validated for its reproducibility over 10 consecutive batches. Meanwhile, a high-performance liquid chromatography with diode array detector (HPLC-DAD) analysis method was established for the quality control of SMF, and the method was validated according to International Council for Harmonisation (ICH) guidelines. Subsequently, the potential sleeping-modulating effects of SMF were evaluated using a pentobarbital-induced sleep mouse model following the protocol issued by State Administration for Market Regulation of China for Health Food Function Testing and Evaluation Methods (2023 Edition). Furthermore, network pharmacology was employed to predict the underlying mechanisms of SMF contributing to the sleep-improving effects. The workflow of this study is presented in Figure 1.

## 2 | Methodology

As the formulated sleep-modulating formulae (SMF) are aimed to be marketed as a dietary supplement, the herbs incorporated in the formulae must be included in the food–medicine homology list published by the Ministry of Health, PRC. Furthermore, the often-used herbs for insomnia treatment (H. B. Zhou et al. 2023) and the ancient formula ‘Suan-Zao-Ren decoction’ were analysed to serve as a basis for formulating the SMF. Based on the ‘Jun-Chen-Zuo-Shi’ principle, SMF comprises the following herbs: stir-fried *Ziziphi spinosae* semen (SZR), *G. elata* Rhizoma (TM), *D. officinale* (TPSH), stewed *Polygonati Rhizoma* (HJ), *P. notoginseng* flower (SQH) and *Radix Glycyrrhizae* preparata (GC) at a ratio of 3:3:2:2:1:1. The detailed formulation process was described in the supplementary information, and Waters H-Class ultra high-performance liquid chromatography (UPLC) equipped with a diode array detector (DAD) was utilised to monitor the process. On the other hand, Thermo Vanquish UPLC (Thermo Fisher, USA) in tandem with Q-Exactive (QE) high-resolution mass spectrometer (Thermo Fisher, USA) and heated-electrospray ionization source (HESI) was used to determine the phytoconstituents present in the individual herbs and SMF formulae and identify the quality markers for SMF, which were further verified by standards.

### 2.1 | Qualitative Analysis of the Phytoconstituents Present in the Formulae

#### 2.1.1 | Sample Preparation

All six aforementioned herbs were weighed and mixed at a ratio of 3:3:2:2:1:1, with only sour jujube semen being ground into

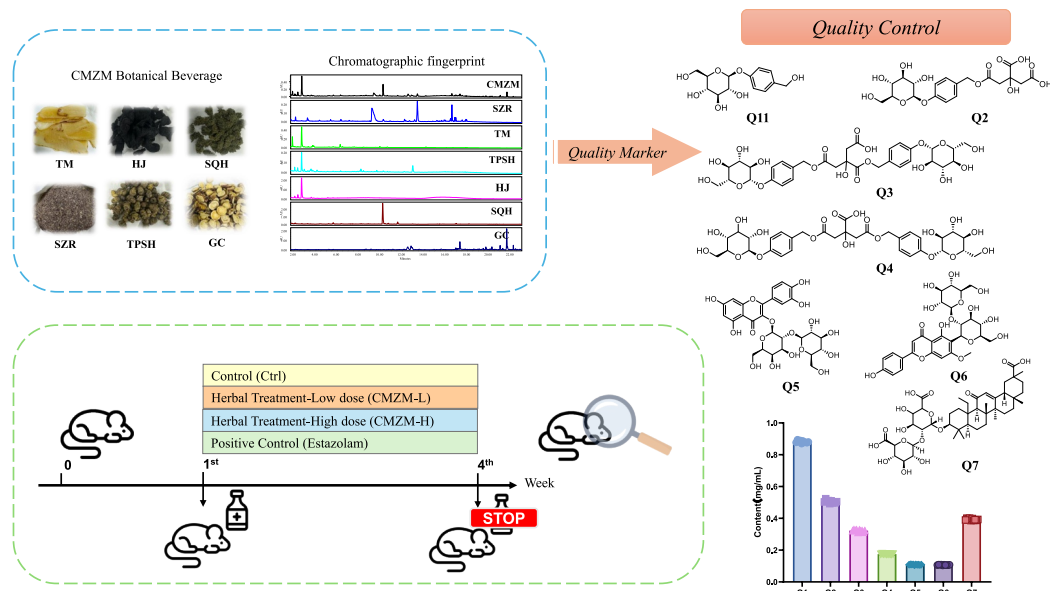


**FIGURE 1** | Schematic overview of the research workflow: (A) Qualitative analysis of the phytoconstituents present in SMF; (B) defining quality markers of SMF and its quantitative analysis; (C) evaluating the sleep-modulating activity of SMF through preclinical evaluation and network pharmacology.

powder, whereas the other herbs retained their ready-to-use form (standard decoction pieces). Thereafter, these six herbs (weighed 12 g in total) were soaked in deionised water in 10-fold volume for 5 h to ensure the full extraction of the water-soluble phytoconstituents present. Followed by, the herbs immersed in deionised water undergo extraction for 2 h by refluxing twice. The extracts obtained were then combined after filtration via a fibre cloth. Similarly, the extracts of the individual herbs (5 g each) were also obtained using the same process. Then, samples (1 mL) were taken from all extracts, and the supernatant was obtained after centrifugation at 15,000 rpm for 15 min. The supernatant yield from the extracts was then filtered with a 0.22  $\mu$ m-membrane and analysed.

### 2.1.2 | Chromatographic Conditions for Qualitative Analysis

The chromatographic fingerprint of the individual herbs and SMF was obtained from Waters H-Class UPLC-DAD equipped with ACQUITY UPLC HSS T3 (2.1 mm  $\times$  100 mm, 1.8  $\mu$ m). For this analysis, mobile phases at a flow rate of 0.3 mL/min consisted of (A) 0.1% (v/v) of formic acid (Sigma-Aldrich, USA) in water and (B) HPLC-grade acetonitrile (ACN, Fischer Scientific, USA) were used. The column temperature was maintained at 50°C, the sample holder was maintained at 15°C, and the injection volume was 5.0  $\mu$ L. After various optimizations, the optimal gradient condition used for analysis as follows: 0–2 min, 5% B; 2–4 min, 8% B; 4–7 min, 13% B; 7–9 min, 15% B; 9–12 min, 16% B; 12–15 min, 25% B; 15–18 min, 37% B; 18–20 min, 45% B; 20–22 min, 60% B and 22–23 min, 90% B.

### 2.1.3 | Identifying the Phytoconstituents Present in the Formulae

For mass spectrometry analysis of the extracts, Q-Exactive mass spectrometer (Thermo Scientific, San Jose, USA) was operated in both positive and negative ion modes (full scan/ddMS<sup>2</sup>) and the MS parameters were set as follows: –3.5 kV spray voltage,

45 arb sheath gas flow rate, 10 arb auxiliary gas flow rate, 350°C capillary temperature, 400°C auxiliary temperature, full MS resolution of 70,000 and MS/MS resolution of 17,500, scan range of  $m/z$  100–1500. The incremental collision energies were 10, 30 and 50 eV. Three of the most abundant ions were selected as precursor ions to obtain the MS/MS spectra in each cycle. The data obtained were then processed in Xcalibur 4.1 software (Thermo Fischer).

### 2.1.4 | Defining the Quality Marker

The SMF extract was centrifuged for 15 min at 4000 rpm, and the supernatant was further concentrated (up to 80 mL) in vacuo. Subsequently, a 200  $\mu$ L sample was taken and further diluted, equating to 0.5 g of herbs per millilitre. After centrifugation at 15,000 rpm for 15 min, the samples prepared were subjected to analysis after syringe filtration (0.22  $\mu$ m membrane). In contrast to the earlier analysis, the injection volume was set to 2  $\mu$ L.

## 2.2 | Quality Control of the Formulae

Based on the known bioactivities, chromatographic fingerprint and the Chinese Pharmacopoeia standard, these seven constituents were selected as the quality marker of the formulae: Gastrodin (Q1), Parishin E (Q2), Parishin B (Q3), Parishin C (Q4), Quercetin 3-O-D-glucosyl-(1  $\rightarrow$  2)-galactoside (Q5), Spinosin (Q6) and Glycyrrhizic acid (Q7). The reference standards (Purity  $\geq$  98%) used were bought from Chengdu DeSiTe Biological Technology Co. Ltd. (Q1, Q2 and Q7), Chengdu Must Bio-Technology Co. Ltd. (Q3 and Q4) and Wuhan ChemFaces Biochemical Co. Ltd. (Q5 and Q6).

### 2.2.1 | HPLC-DAD Condition

To address the limitation, high-performance liquid chromatography (HPLC) Agilent 1290 (Agilent Technologies Inc.) with

DAD detector was used for quantitative analysis, allowing a more compatible method transfer for the quality control process. Considering the specifications in the Chinese Pharmacopoeia general chapter <0512>, the optimal chromatographic condition was listed in Table 1. Whereas the details on the optimization done beforehand were described in the supplementary information.

2.2.2 | Linearity, LOD, and LOQ

The reference standards bought were accurately weighed and dissolved separately in 100% methanol. The final concentration of these stock solutions was 1.45 mg/mL **Q1**, 1.46 mg/mL **Q2**, 1.25 mg/mL **Q3**, 1.39 mg/mL **Q4**, 1.25 mg/mL **Q5**, 1.39 mg/mL **Q6** and 1.00 mg/mL **Q7**. The mixed standard solutions of the calibration curve (linearity) were prepared by taking appropriate volumes of the stock solutions and diluting them with methanol to achieve the following final concentrations (per 1 mL): 145, 120.83, 96.67, 72.5, 48.33 and 24.17 µg for **Q1**; 135, 112.5, 90.0, 67.5, 45.0 and 22.5 µg for **Q2**; 135, 112.5, 90.0, 67.5, 45.0 and 22.5 µg for **Q3**; 138.5, 115.42, 92.33, 69.25, 46.17 and 23.08 µg for **Q4**; 125.0, 104.17, 83.33, 62.5, 41.67 and 20.83 µg for **Q5**; 128.44, 102.75, 77.06, 51.63, 25.82 and 12.91 µg for **Q6**; and 100, 50, 25, 12.5, 6.25 and 3.125 µg for **Q7**. Thus, the mixed standard solution was further diluted until the signal-to-noise (S/N) ratio reached 3 and 10, which corresponded to the limit of detection (LOD) and limit of quantification (LOQ), respectively.

2.2.3 | Precision, Repeatability and Stability

To evaluate the precision and sample stability, a 200 µL sample was taken from the concentrated **SMF** extract and diluted to yield an herb-to-solvent ratio of 0.1323 g herbs per millilitre (mL). The sample solution was further filtered through a 0.22 µm-membrane before analysis. The intra-day ( $n = 6$ ), inter-day ( $n = 3$ ) system precision and sample stability (within 48 h) were evaluated in accordance with ICH guidelines. Six sample solutions were prepared to assess the repeatability of sample

preparation. Variations observed were presented as relative standard deviations (RSD, %).

2.2.4 | Spike Recovery

Accuracy was evaluated by spiking a known amount of standard at three concentration levels (approximately 50%, 100% and 150% of the original concentration,  $n = 3$ ) into the corresponding sample solution (Table 2). The recoveries were computed using the formula: recovery (%) = (found amount – original amount)/spiked amount × 100%.

2.2.5 | Quantitative Analysis of SMF

Each batch of samples (a total of 10 batches) was diluted to 0.1324, 0.0825 and 0.0459 g/mL of the raw herbal material, ensuring that the concentrations of all seven quality markers would fall within the linear range of the calibration curve. Thereby, simultaneous quantification of **Q4**, **Q5** and **Q6** can be done at the sample solution with a concentration of 0.1324 g of herbs/mL, **Q2**, **Q3** and **Q7** at 0.0825 g of herbs/mL and **Q1** at 0.0459 g/mL of herbs.

2.3 | Sleep-Modulating Activity of the Formulae

Prior to the experiment, 604.9 g of raw herbal material yielded 211.59 g of freeze-dried powder **SMF**, establishing the raw herb-to-freeze-dried powder ratio to be 2.86:1. Of note, only a single batch of **SMF** was used across all biological experiments described in this study. The recommended daily dosage is 12 g of raw herb material for adults with 60 kg body weight, which derived from the dosage ranges specified in the Chinese Pharmacopoeia and classical traditional Chinese medicine (TCM) literatures (L. Chen et al. 2020; Cho et al. 2012; Yang et al. 2023; Dong et al. 2021). Thus, the equivalent dosage of freeze-dried powder was calculated to be 69.95 mg/kg body weight. This conversion was used as the basis for subsequent dose extrapolation in preclinical studies. For interspecies conversion to mice, the body surface area normalisation method (Meeh–Rubner formula) was employed, with a conversion factor of 9.1, a value that is standard in Chinese pharmacological research and recommended in the State Administration for Market Regulation of China for Health Food Function Testing and Evaluation Methods (2023 Edition). According to the aforementioned protocol, this conversion yielded a mouse low dose of 636.55 mg/kg and a high dose of 1.91 g/kg (3× low dose) for dose-response assessment.

Suitable sample size determination was based on a priori power calculations performed with G\*Power (v.3.1.9.4). The specific parameters for the power analysis are as follows: Expected effect size (Cohen's  $d = 0.5$ , medium effect, based on the results of our preliminary experiment and relevant literature reports), significance level ( $\alpha = 0.05$ ) and statistical power ( $1 - \beta = 0.8$ ). Based on these parameters, the minimum required sample size per group was calculated to be 8–12 animals.

The in vivo study was conducted using male ICR mice that were randomly allocated into four experimental groups ( $n = 10$ –18) for a 36-day investigation of sleep-modulating effects. The groups consisted of: (1) control group receiving ultrapure water (Control,  $n = 10$ ), (2) low-dose herbal treatment group (**SMF-L**,  $n = 18$ )

TABLE 1 | HPLC-DAD chromatographic conditions.

| Gradient programme   |                                               |           |
|----------------------|-----------------------------------------------|-----------|
| Time (min)           | % A (0.1% formic acid in H <sub>2</sub> O)    | % B (ACN) |
| 0                    | 95                                            | 5         |
| 10                   | 92                                            | 8         |
| 15                   | 87                                            | 13        |
| 25                   | 80                                            | 20        |
| 35                   | 50                                            | 50        |
| 40                   | 35                                            | 65        |
| 41                   | 0                                             | 100       |
| Other specifications |                                               |           |
| Column               | Agilent Zorbax SB-C18 (250 mm × 4.6 mm, 5 µm) |           |
| Column temperature   | 30°C                                          |           |
| Injection volume     | 10 µL                                         |           |

**TABLE 2** | Linearity, detection limits, precision, stability and reproducibility of the method.

| Marker | Regression equation    | $r^2$  | Linearity range ( $\mu\text{g/mL}$ ) | LOD ( $\mu\text{g/mL}$ ) | LOQ ( $\mu\text{g/mL}$ ) | Precision        |                  | Stability in 48 h (RSD%) | Reproducibility (RSD%) |
|--------|------------------------|--------|--------------------------------------|--------------------------|--------------------------|------------------|------------------|--------------------------|------------------------|
|        |                        |        |                                      |                          |                          | Intra-day (RSD%) | Inter-day (RSD%) |                          |                        |
| Q1     | $y = 14.755x - 3.4367$ | 0.9991 | 24.17–145                            | 1.56                     | 5.15                     | 0.87             | 1.07             | 1.15                     | 0.99                   |
| Q2     | $y = 20.28x + 161.71$  | 0.9995 | 22.5–135                             | 3.13                     | 10.31                    | 1.25             | 0.51             | 0.38                     | 1.30                   |
| Q3     | $y = 24.144x + 202.65$ | 0.9993 | 22.5–135                             | 1.56                     | 5.15                     | 0.60             | 0.23             | 0.81                     | 1.32                   |
| Q4     | $y = 24.58x + 352.2$   | 0.9992 | 23.08–138.5                          | 1.56                     | 5.15                     | 1.26             | 0.35             | 1.13                     | 1.61                   |
| Q5     | $y = 57.17x + 166.31$  | 0.9991 | 20.83–125                            | 1.56                     | 5.15                     | 0.86             | 1.51             | 1.20                     | 1.27                   |
| Q6     | $y = 71.465x + 80.562$ | 0.9992 | 12.91–128.44                         | 0.78                     | 2.58                     | 1.29             | 0.30             | 1.76                     | 0.64                   |
| Q7     | $y = 18.317x + 14.582$ | 0.9998 | 3.13–100                             | 1.56                     | 5.15                     | 0.52             | 0.61             | 0.77                     | 0.86                   |

Note: Gastrodin (Q1), parishin E (Q2), parishin B (Q3), parishin C (Q4), quercetin-3-O-glucosyl-(1 → 2)-galactoside (Q5), spinosin, (Q6) and glycyrrhizic acid (Q7).

administered 636.55 mg/kg of SMF, (3) a high-dose herbal treatment group (SMF-H,  $n = 18$ ) given 1.91 g/kg and (4) a positive control group (Estazolam,  $n = 15$ ) treated with 0.2 mg/mL estazolam solution in 0.5% CMC-Na. All treatment was administered daily through oral gavage at a volume of 400  $\mu\text{L}$  for 40 g mice, with the SMF solutions prepared at concentrations of 63.65 mg/mL (low dose) and 190.95 mg/mL (high dose) in ultrapure water to achieve target dosages. The comprehensive evaluation protocol systematically assesses different aspects of sleep modulation through multiple behavioural assessments conducted at specific timepoints: Direct sleep observation on Day 30, followed by a series of pentobarbital-challenge tests—subthreshold hypnosis testing (Day 32), sleep latency measurement (Day 34), and sleep duration evaluation (Day 36). Throughout the process, all mice continued to receive their respective treatments and underwent body weight monitoring every 5 days. All animal procedures complied with ARRIVE guidelines and approved by the Institutional Animal Care and Use Committee (IACUC) under protocol code BCAA0315 on November 11, 2023.

### 2.3.1 | Direct Sleep Test

Through 30 consecutive days of oral administration, the mice were observed for sleep induction between 30 and 60 min after treatment. Sleep was determined by loss of righting reflex (LORR) for 60 s. The duration from LORR was recorded as sleep time. The number of animals exhibiting sleep and their respective sleep durations were documented across all four experimental groups.

### 2.3.2 | Subthreshold Pentobarbital Hypnosis Test

Through preliminary studies, the subthreshold hypnotic dose of pentobarbital sodium was established at 30 mg/kg body weight (BW), which is the maximum dose maintaining righting reflex in 80%–90% of untreated mice. After 32 days of intervention, mice received 30 mg/kg BW of pentobarbital after 20 min of post-administration. Across the groups, sleep induction was quantified by counting mice LORR > 60 s within 30 min of post-injection.

### 2.3.3 | Pentobarbital Sleep Latency Test

On the 34th day, mice were intraperitoneally injected with 42 mg/kg BW pentobarbital sodium (predetermined, can induce

100% sleep incidence) 20 min after treatment. Thus, LORR for  $\geq 60$  s, with the interval between injection and reflex disappearance, was recorded as sleep latency. Comparative analysis of latency differences was conducted across all four experimental groups.

### 2.3.4 | Pentobarbital-Induced Sleep Prolongation Test

After 36 days of continuous treatment, mice received an intraperitoneal injection of pentobarbital sodium (45 mg/kg BW) 30 min after treatment. This sub-hypnotic dose (inducing 100% sleep incidence with limited duration) was used to evaluate sleep-modulating effects. Similarly, sleep onset was confirmed by sustained LORR for 60 s, with total sleep time measured from reflex loss to recovery. The comparative effects of all four treatment groups were systematically analysed.

### 2.3.5 | Organ Index

After 16 h of fasting (access to water is available), mice were euthanised to collect the blood, liver, spleen, kidneys and whole brain (excluding the brainstem). Blood from the organs was removed by rinsing with saline, dried and weighed. Brain tissues were sectioned, rinsed, weighed and flash-frozen in liquid nitrogen within saline-containing tubes for storage at  $-80^\circ\text{C}$ , refraining from freeze–thaw cycles to preserve sample integrity.

### 2.3.6 | Neurotransmitter Profiling

To profile the neurotransmitter level present in the blood serum and the whole brain (excluding the brainstem), the following ELISA kits for mice were bought from Shanghai Enzyme-linked Biotechnology Co. Ltd.: serotonin (5-HT, Catalogue No.: ml001891), noradrenaline (NE, Catalogue No.: ml063805) and dopamine (DA, Catalogue No.: ml002024).

### 2.3.7 | Statistical Analysis

All data are presented as mean  $\pm$  standard deviation (SD) unless specified otherwise. Statistics for body weight, sleep duration and latency, the levels of DA, NE and 5-HT in the serum and brain were calculated using GraphPad Prism (Version 9.2.0) Statistics software, with each dot representing an individual animal. One-way analysis of variance (ANOVA) was conducted

in accordance with standard procedures (a continuous variable). Before ANOVA, homogeneity of variance was assessed using the Brown–Forsythe test. When the variance was homogeneous, the  $F$ -statistic was calculated; if  $F < F_{0.05}$ , we concluded no significant differences among group means; if  $F \geq F_{0.05}$  and  $p \leq 0.05$ , post-hoc pairwise comparisons between multiple experimental groups and the control group were performed using Dunnett's multiple comparisons test. Differences were considered to be significant when  $p$  value was less than 0.05. Fisher's exact test was used while evaluating the number of animals exhibiting sleep onset. Results were considered statistically significant at  $p < 0.05$ .

## 2.4 | Network Pharmacology and Molecular Docking Analysis

The SMILES expression of the phytoconstituents identified from SMF was obtained from the PubChem (<https://pubchem.ncbi.nlm.nih.gov/>) database. Thereafter, the SMILES formulas were input to SwissTarget Prediction (<http://www.swisstargetprediction.ch/>) and NetInfer (<https://lmd.ecust.edu.cn/netinfer/>) to obtain the protein targets of each SMF constituent. To obtain the genes associated with sleep issue, 'insomnia', 'sleep disorder' and 'sleep deprivation' are the keywords used to search on these reliable databases: GeneCards (<https://www.genecards.org/>), DisGeNET (<https://www.disgenet.org/>) and OMIM (<https://www.omim.org/>) based on earlier studies. Through Venn diagram (<https://bioinfo.gp.cnb.csic.es/tools/venny/index.html>) cross genes in common to the components and disease were further analysed to construct the protein–protein interaction (PPI) and component–target network in STRING (<https://cn.string-db.org/>) and Cytoscape 3.10, respectively. Aside from STRING, ShinyGo 0.82 (<https://bioinformatics.sdstate.edu/go/>) was also utilised in the enrichment analysis.

Afterwards, molecular docking was performed to validate the interactions between SMF constituents and core targets identified in the network pharmacology and enrichment analyses. Thus, protein structures of high-priority targets were retrieved from the RCSB protein data bank (PDB, <https://www.rcsb.org/>). The 3D structures of the ligands were obtained from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>). Docking simulations were carried out using AutoDock Vina, and the resulting binding poses and affinities were analysed to elucidate potential mechanisms of action. For visualisation, BIOVIA Discovery Studio 2021 and PyMOL were used.

## 3 | Results and Discussion

Following the principle of TCM formulations, SMF employs *G. elata* (TM) and *Z. spinosae* semen (SZR) as synergistic monarch (*Jun*) herbs to address sleep disorders through meridian-specific actions. *G. elata* targets the liver meridian, suppressing wind and promoting sedation, whereas sour jujube semen nourishes heart yin and liver blood to calm the mind. The formula is further enhanced by minister (*Chen*) herbs such as *Polygonati Rhizoma* (HJ), which harmonises heart-kidney interactions, and *D. officinale* (TPSH), addressing liver-kidney yin deficiency. To enhance the efficacy, *P. notoginseng* flower (SQH) and *Radix Glycyrrhizae* preparata (GC) were both considered as the assistant (*Zuo*) and guide (*Shi*) herbs that harmonise qi, clear heat and reduce fatigue.

Hence, these herbs were selected to formulate the SMF due to their distinct medicinal properties and ability to address insomnia through various pathways.

### 3.1 | Formulation of SMF and Its Qualitative Analysis

To establish the optimal processing conditions, UPLC-DAD was utilised to monitor the formulation process which UPLC fingerprints obtained from the single factor investigation are presented in Supporting Information S1: Figure S1–S3. Comparable peak areas (Supporting Information S1: Figure S1) were found on the primary components of both preparations; however, the powdered form was found to be prone to scorching during the extraction process, which would significantly compromise its suitability for production. As a result, the standard decoction piece was selected as the preferred material for preparation. Notably, the reflux extraction of the decoction pieces for 2 and 5 h of soaking time (Supporting Information S1: Figure S2) was found to be the most optimal condition among all. The repeatability of the process was also validated, with a variance within 5% of the sum in the primary peak areas across batches (Supporting Information S1: Figure S3A). Furthermore, chromatographic data (Supporting Information S1: Figure S3B) revealed complete extraction of the characteristic markers by the second cycle, with the third reflux cycle (Supporting Information S1: Figure S3C) showing only marginal gains in major components. In turn, these findings established a two-stage protocol, comprising 5 h of initial soak followed by two cycles of reflux extract for 2 h. This streamlined approach would ideally balance the compound integrity and extraction efficacy of the process to enhance its reproducibility and scalability.

To identify potential quality markers of the formulae, UPLC/Q-Exactive-MS was utilised to determine the phytoconstituents present in the product (Supporting Information S1: Figure S4), and its source was identified based on the profile of the individual herbs (Figure 2). A total of 35 constituents (Table 3) were identified, and the MS<sup>2</sup> fragmentation pattern (Supporting Information S1: Figure S5) was further verified from the literature. Of which, the identified compounds could mainly be structurally classified into five key categories: (1) Phenolics (e.g., rutin and liquiritin derivatives) that suppress wakefulness via inhibition of neuroinflammatory responses and  $\gamma$ -aminobutyric acid sub-type A receptors (GABA<sub>A</sub>), whereas their antioxidant properties protect sleep-regulating neurons (Ríos et al. 2022). (2) Parishins (Parishin A–E), which are found to enhance sleep quality by influencing pathways such as the GABA pathway (Duan et al. 2025). (3) The alkaloid trigonelline, which could trigger adenosine accumulation, leading to prolonged deep sleep (Membrez et al. 2024). (4) Saponins exhibit subclass-specific effects, such as licorice-derived types (glycyrrhizic acid) influencing signalling pathways of GABA and serotonin (Cho et al. 2012), ginsenosides (Rb1/Rg3) generate neurosteroids for GABAergic modulation (H. Chen et al. 2018; Xu et al. 2018), whereas spinosin directly activates 5-HT receptors (Zhao et al. 2025). (5) Coumarins such as isoglycycoumarin were reported to prolong melatonin activity through monoamine oxidases (MAO) inhibition (Bhawna et al. 2022). Of such, the diverse chemical characteristics found in SMF would likely synergistically promote sleep quality by

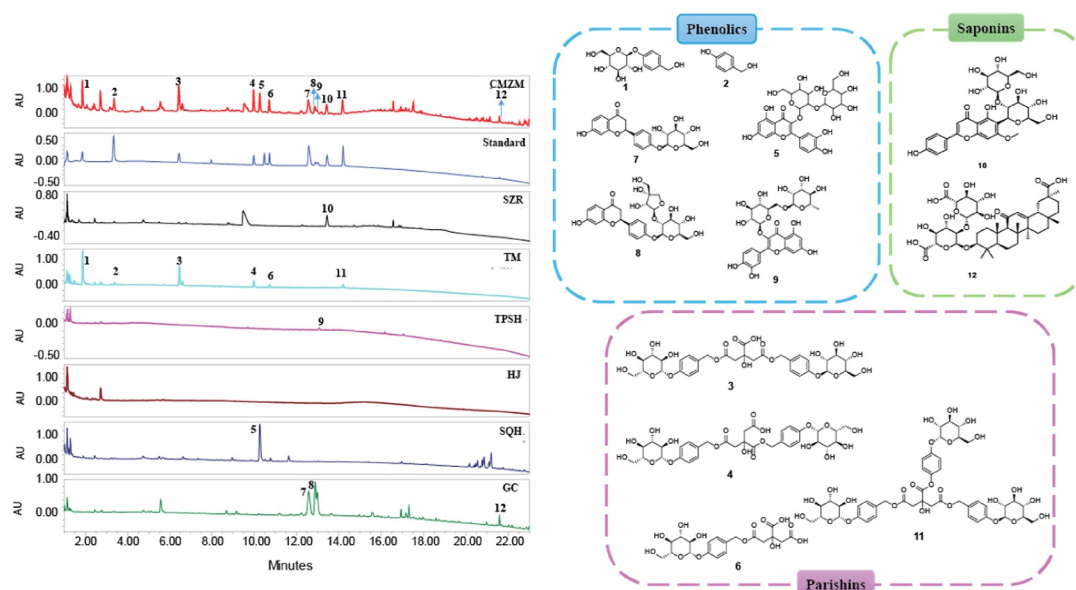


**FIGURE 2** | UPLC fingerprint of SMF, standards, and the individual herbs: *Ziziphi spinosae* semen (SZR), *Gastrodiae elata* Rhizoma (TM), *Dendrobium officinale* (TPSH), stewed *Polygonati Rhizoma* (HJ), *Panax notoginseng* flower (SQH), and *Radix Glycyrrhizae* preparata (GC).

influencing various pathways such as serotonergic and adenosinergic pathways (initiate sleep onset), GABA (enhance REM sleep) and combating inflammation (maintain sleep continuity) rather than a simple hypnotic mechanism. Hence, subsequent assays included in the *in vivo* investigation evaluate the alterations in the neurotransmitter profiles (both serum and brain) of mice after a period of herbal treatment.

Before assessing the reproducibility of the designed formulation, 12 of the identified constituents (Figure 2) were further confirmed by their corresponding standards ( $\geq 98\%$ ), which are usually the quality control indicators of the herbs as specified in the Chinese Pharmacopoeia (2020 edition). Covering the majority of the herbs used in SMF, these 12 compounds are considered the quality control indicators of the process: (1) gastrodin, (2) *p*-hydroxybenzyl alcohol, (3) parishin C, (4) parishin B, (5) quercetin-3-*O*-glucosyl-(1  $\rightarrow$  2)-galactoside, (6) parishin E, (7) liquiritin, (8) liquiritin apioside, (9) rutin, (10) spinosin, (11) parishin and (12) glycyrrhizic acid. Based on the UPLC fingerprint, 10 batches of the SMF (Supporting Information S1: Figure S6) produced seem to be comparable ( $RSD \leq 5\%$ ) among batches. These findings suggest a well-balanced approach to the compound integrity along with the reproducibility and scalability of the designed formulation process.

## 3.2 | Quantitative Quality Control Assessment of SMF

### 3.2.1 | Optimization of Analytical Method

Among all, **Q1–Q7** were selected as the quality markers in the pilot batch. Aside from being the characteristic constituents, these constituents were also reported by earlier studies to induce sleep through direct or indirect biological pathways (Li et al. 2025).

To ensure the efficiency of the method in analysing the targeted compounds, various conditions were assessed and presented in the supplementary information (Supporting Information S1:

Figure S7). Among which, Agilent ZORBAX SB C-18 (250  $\times$  mm  $\times$  4.6 mm, 5  $\mu$ m) showed excellent separation, and was considered for the succeeding experiments. Different column temperature, gradient conditions, and mobile phases were also evaluated, with column temperature at 30°C, 0.1% formic acid (FA) in H<sub>2</sub>O and acetonitrile (ACN) being the best among all. Of which, peaks corresponding to the quality markers were well-separated (Figure 3), enabling efficient quantification. By evaluating the chromatogram of various wavelengths, the result revealed that the optimal response of the analytes under 225 nm was the best, establishing the most promising detection wavelength for quantitative analysis.

### 3.2.2 | Method Validation

**3.2.2.1 | Linearity, LOD and LOQ.** This study considered an external standard calibration method which all seven analytes show good linearity ( $r^2 = 0.9991$ – $0.9998$ ) within their respective concentration ranges (Table 2). Of which, the corresponding LOD and LOQ values for each analyte are also presented in Table 2.

**3.2.2.2 | Precision, Reproducibility, and Stability.** Both intra-day ( $n = 6$ ) and inter-day ( $n = 3$ ) system precision were evaluated (Table 2), where samples are stored in the autosampler at room temperature. A great system precision was observed with all RSD values found to be less than 1.5%. To assess the uniformity in sample preparation, a reproducibility test (Table 2) was done, with an RSD ranging from 0.86% to 1.61%. Lastly, the sample stability under 48 h (Table 2) stored at room temperature was also evaluated, with the RSD ranging from 0.38% to 1.15%. These results indicated the stability of the sample over 48 h and the robustness of the analytical method.

**3.2.2.3 | Accuracy.** The accuracy of the method was assessed by spiking a known amount of standard at three concentration levels (about 50%, 100% and 150% of the original concentration) into the respective sample ( $n = 3$ ). As presented in Table 4, the

TABLE 3 | Phytoconstituents identified from SMF.

| No. | RT/min | [M – H] <sup>–</sup> | [M + H] <sup>+</sup> | MS/MS (m/z)                                       | Identification                              | SZR | TM | TPSH | HJ | SQH | GC |
|-----|--------|----------------------|----------------------|---------------------------------------------------|---------------------------------------------|-----|----|------|----|-----|----|
| 1   | 5.91   | 459.1096             |                      | 111.0074                                          | Parishin E*                                 |     | +  |      |    |     |    |
| 2   | 8.32   | 285.0951             |                      | 123.0437                                          | Gastrodin*                                  |     | +  |      |    |     |    |
| 3   | 9.24   |                      | 342.173              | 297.1146, 265.0882                                | Trigonelline                                | +   |    |      |    |     |    |
| 4   | 9.74   | 727.2017             |                      | 423.0887, 161.0436, 111.0074                      | Parishin B*                                 |     | +  |      |    |     |    |
| 5   | 10.23  | 625.1343             |                      | 300.0242, 271.0219                                | Quercetin-3-O-glucosyl (1 → 2) galactoside* |     |    |      |    | +   |    |
| 6   | 10.45  | 727.2018             |                      | 423.0887, 161.0437, 111.0075                      | Parishin C*                                 | +   |    |      |    |     |    |
| 7   | 11.7   | 609.1399             |                      | 255.0271, 227.0325                                | Kaempferol-3-O-glucosyl (1 → 2) galactoside |     |    |      |    | +   |    |
| 8   | 12.53  | 549.1556             |                      | 429.0995, 255.0636, 119.0487                      | Liquiritin apioside                         |     |    |      |    |     | +  |
| 9   | 12.67  | 417.1146             |                      | 255.0635                                          | Liquiritin                                  |     |    |      |    |     | +  |
| 10  | 12.97  | 549.1557             |                      | 255.0635                                          | Isoliquiritin apioside                      |     |    |      |    |     | +  |
| 11  | 13.19  | 609.1401             |                      | 300.0242                                          | Rutin                                       |     |    | +    |    |     |    |
| 12  | 13.41  | 607.1607             |                      | 427.0988, 307.0578, 292.0347                      | Spinosin*                                   | +   |    |      |    |     |    |
| 13  | 14.04  | 995.2933             |                      | 727.2018, 423.0888, 161.0437, 111.0075            | Parishin*                                   | +   |    |      |    |     |    |
| 14  | 16.38  | 783.2069             |                      | 427.0988                                          | 6"-Feruloylspinosin                         |     |    |      |    |     |    |
| 15  | 19.31  | 983.4403             |                      | 821.3885, 351.0533                                | Licorice saponin A3                         |     |    |      |    |     | +  |
| 16  | 19.73  | 1341.6534            |                      | 1209.614, 1077.5651                               | Notoginsenoside Q                           |     |    |      |    | +   |    |
| 17  | 19.73  | 1239.6245            |                      | 1107.5837, 945.5346                               | Notoginsenoside Fa                          |     |    |      |    | +   |    |
| 18  | 19.73  | 879.3925             |                      | 351.053                                           | Uralsaponin M                               |     |    |      |    |     | +  |
| 19  | 19.88  | 837.3823             |                      | 351.0532                                          | Licorice saponin G2                         |     |    |      |    |     | +  |
| 20  | 20.01  | 1209.6148            |                      | 1077.5733, 945.5317, 783.4826, 621.4320, 459.3795 | Notoginsenoside FP2                         |     |    |      |    | +   |    |
| 21  | 20.08  | 1107.5841            |                      | 945.5343, 783.4787, 621.4293, 459.3791            | Ginsenoside Rb1                             |     |    |      |    | +   |    |
| 22  | 20.3   | 1209.6148            |                      | 1077.5739, 915.5211, 783.4807, 621.4304, 459.3793 | Ginsenoside Ra1                             |     |    |      |    | +   |    |
| 23  | 20.35  | 1077.5740            |                      | 945.5335, 783.4838, 621.4312, 459.3787            | Ginsenoside Rc                              |     |    |      |    | +   |    |
| 24  | 20.42  | 1205.5825            |                      | 1073.5410, 911.4999, 749.4376                     | Jujuboside A                                | +   |    |      |    |     |    |
| 25  | 20.68  | 1077.5737            |                      | 945.5271, 783.4832, 621.4312, 459.3787            | Ginsenoside Rb2                             |     |    |      |    | +   |    |
| 26  | 21.23  | 821.3883             |                      | 351.0532                                          | Glycyrrhizic acid*                          |     |    |      |    |     | +  |
| 27  | 21.75  | 945.5328             |                      | 783.4810, 621.4298, 459.3787                      | Ginsenoside Rd                              |     |    |      |    | +   |    |
| 28  | 21.83  | 821.3879             |                      | 351.053                                           | Licorice saponin H2                         |     |    |      |    |     | +  |
| 29  | 22.08  | 915.5227             |                      | 783.4816, 621.4307, 459.3791                      | Ginsenoside Rd2                             |     |    |      |    | +   |    |
| 30  | 22.33  | 915.5230             |                      | 783.4760, 621.4308, 459.3817                      | Notoginsenoside Ft1                         |     |    |      |    | +   |    |
| 31  | 22.64  | 367.1147             |                      | 309.0372                                          | Isoglycycomarin                             |     |    |      |    |     | +  |
| 32  | 22.93  | 915.5229             |                      | 783.4818, 621.4308, 459.3795                      | Notoginsenoside Fe                          |     |    |      |    | +   |    |
| 33  | 22.99  | 915.5231             |                      | 783.4820, 621.4307, 459.3797                      | Notoginsenoside ST4                         |     |    |      |    | +   |    |
| 34  | 23.38  | 783.4819             |                      | 621.4308, 459.3801                                | Ginsenoside Rg3                             |     |    |      |    | +   |    |
| 35  | 23.46  | 783.4821             |                      | 621.4309, 459.3802                                | Ginsenoside F2                              |     |    |      |    | +   |    |

Note: Detected (+), verified with reference standard (\*). *Ziziphi spinosae* semen (SZR), *Gastrodiae elata* Bl. Rhizoma (TM), *Dendrobium officinale* (TPSH), stewed *Polygonati Rhizoma* (HU), *Panax notoginseng* flower (SQH), and *Radix Glycyrrhizae* preparata (GC).

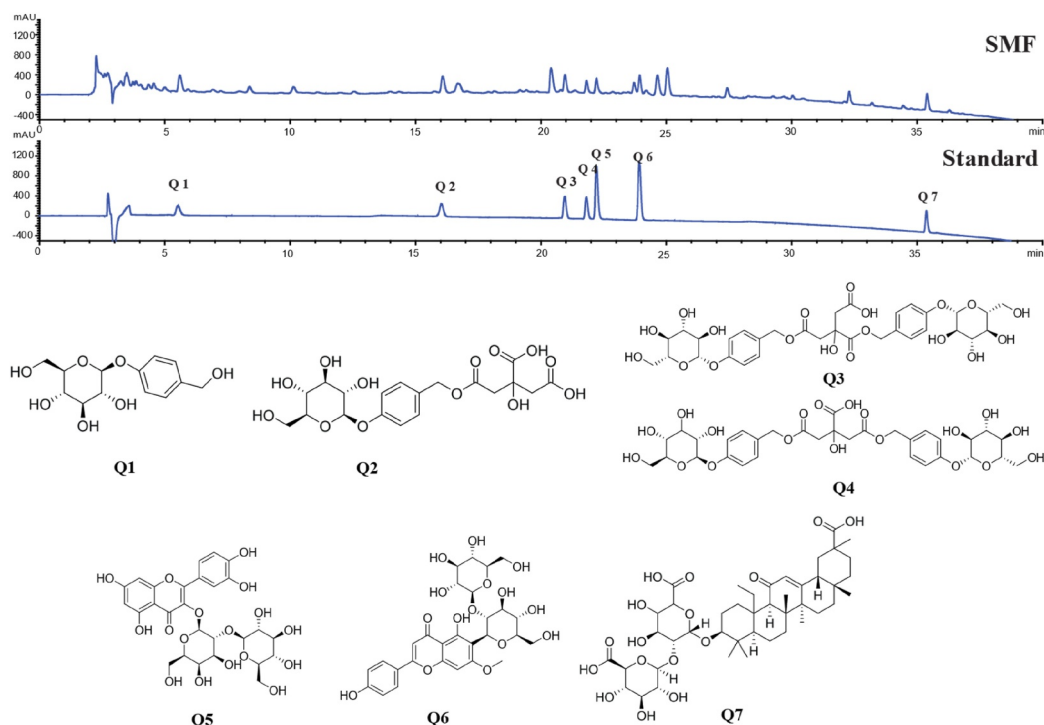


**FIGURE 3** | Quality control HPLC profile of the SMF aligned with the reference standards: gastrodin (Q1), parishin E (Q2), parishin B (Q3), parishin C (Q4), quercetin-3-*O*-glucosyl-(1 → 2)-galactoside (Q5), spinosin (Q6), and glycyrrhizic acid (Q7).

recovery (%) of the analytes varied from 97.55% to 103.16%, indicating acceptable accuracy of this method.

### 3.2.3 | Quantitative Analysis of SMF

For simultaneous quantification of all seven analytes, 10 batches of SMF were prepared in triplicate dilutions (0.1324, 0.0825, and 0.0459 g/mL of crude herbal material) to ensure concentrations of the markers remained within the linear range of the calibration curves (Supporting Information S1: Figure S8). These diluted samples were then analysed using HPLC under optimum chromatographic conditions. The content of the seven compounds (Table 5) added up to 2.03 mg/mL in SMF, with minimal variations observed between batches (RSD not more than 1.77%). Among all, **Q1** was found to be the most prevalent ( $0.88 \pm 0.01$  mg/mL) constituent, followed by **Q2** ( $0.51 \pm 0.01$  mg/mL), **Q7** ( $0.39 \pm 0.01$  mg/mL) and **Q3** ( $0.32 \pm 0.01$  mg/mL), whereas the concentrations of **Q4**, **Q5** and **Q6** in SMF exceeded 0.1 mg/mL.

## 3.3 | In Vivo Investigation of Sleep-Modulating Activity

The experimental results indicated that the SMF exhibited significant sleep-modulating effects while maintaining an excellent safety profile across multiple evaluation criteria. As shown in Figure 4A, no significant differences in body weight were found between herbal treatment groups (SMF-L and SMF-H) as compared to the control. Starting from day 10, the positive control group (Estazolam) exhibited a significant and gradual decrease in body weight compared to the control group. Effect sizes for pairwise comparisons at each time point were quantified using Cohen's *d*. On days 5 and 10, trivial effects and small effect were observed in positive groups, respectively ( $F = 0.187$ ,  $p = 0.905$ ,

$\eta^2 = 0.007$ ,  $|d| < 0.2$  and  $F = 1.872$ ,  $p = 0.152$ ,  $\eta^2 = 0.058$  and  $0.2 \leq |d| < 0.5$ ). A medium effect was detected in day 15 positive group ( $F = 3.267$ ,  $p = 0.031$ ,  $\eta^2 = 0.095$  and  $|d| = 0.526$ ), whereas large effects were observed in day 25 ( $F = 4.892$ ,  $p = 0.005$ ,  $\eta^2 = 0.132$  and  $|d| = 0.861$ ) and day 30 ( $F = 5.128$ ,  $p = 0.004$ ,  $\eta^2 = 0.139$  and  $|d| = 0.809$ ) positive groups, indicating significant biological differences in weight loss between positive group and control group at day 25 and day 30 (Cohen 1988). These findings suggested potential side effects associated with conventional sleep medications on long-term treatment.

Furthermore, comprehensive organ index analyses revealed an excellent safety profile of SMF, with no significant changes observed in liver, kidney, spleen, or brain weights as compared to control groups (Supporting Information S1: Figure S9). Thereby, this observation suggests that SMF shows no evidence of organ toxicity or injuries, which is a significant advantage over some conventional sleep medications known to cause metabolic disturbances and injuries (Van Gastel 2022). Although organ indices alone provide limited safety assessment, the herbs considered in SMF are not only traditional Chinese medicines but also considered as edible for food use, which their safety has been well documented in previous studies (Yang et al. 2023; Dong et al. 2021). Yet, future studies incorporating biochemical and histological assessment may further strengthen the safety profile of this formulation (SMF).

### 3.3.1 | Sleep-Modulating Efficacy of SMF

The direct sleep observation tests (Table 5) confirmed that SMF administration did not induce a direct hypnotic effect, as evidenced by the absence of righting reflex loss in all treatment groups.

**TABLE 4** | Accuracy of the method and average contents of the seven compounds in 10 batches of SMF.

| Marker | Spike<br>(µg) | Accuracy         |                 |            | Average<br>content<br>(mg/mL) |
|--------|---------------|------------------|-----------------|------------|-------------------------------|
|        |               | Recovery<br>(µg) | Recovery<br>(%) | RSD<br>(%) |                               |
| Q1     | 74.35         | 75.59            | 101.67          | 1.23       | 1.44 ± 0.01                   |
|        | 125.87        | 124.65           | 99.03           | 0.43       |                               |
|        | 177.92        | 174.20           | 97.91           | 1.74       |                               |
| Q2     | 35.94         | 37.30            | 103.78          | 1.55       | 2.21 ± 0.03                   |
|        | 54.08         | 54.69            | 101.13          | 0.59       |                               |
|        | 110.19        | 99.56            | 99.37           | 0.51       |                               |
| Q3     | 21.79         | 22.24            | 102.06          | 1.00       | 1.76 ± 0.03                   |
|        | 33.53         | 33.89            | 101.06          | 2.17       |                               |
|        | 59.35         | 60.19            | 101.42          | 1.09       |                               |
| Q4     | 15.41         | 15.79            | 102.46          | 1.69       | 1.73 ± 0.01                   |
|        | 45.95         | 46.84            | 101.94          | 2.93       |                               |
|        | 65.84         | 64.65            | 98.19           | 1.62       |                               |
| Q5     | 13.87         | 13.79            | 99.41           | 2.16       | 2.16 ± 0.03                   |
|        | 29.61         | 30.40            | 102.67          | 1.43       |                               |
|        | 42.42         | 41.54            | 97.93           | 0.71       |                               |
| Q6     | 13.81         | 14.24            | 103.16          | 1.87       | 2.53 ± 0.04                   |
|        | 29.29         | 29.88            | 102.02          | 1.97       |                               |
|        | 53.11         | 53.59            | 100.91          | 1.10       |                               |
| Q7     | 30.17         | 31.32            | 103.82          | 1.81       | 1.46 ± 0.02                   |
|        | 57.06         | 55.66            | 97.55           | 0.75       |                               |
|        | 88.10         | 88.49            | 100.45          | 2.32       |                               |

Note: Gastrodin (Q1), parishin E (Q2), parishin B (Q3), parishin C (Q4), quercetin-3-O-glucosyl-(1 → 2)-galactoside (Q5), spinosin (Q6), and glycyrrhizic acid (Q7).

**TABLE 5** | Direct hypnotic effects of SMF in mice.

| Group     | Number<br>of<br>animals<br>(n) | Number of<br>animals with<br>sleep onset (n) | Incidence of<br>sleep<br>onset (%) |
|-----------|--------------------------------|----------------------------------------------|------------------------------------|
| Control   | 10                             | 0                                            | 0                                  |
| SMF-L     | 18                             | 0                                            | 0                                  |
| SMF-H     | 18                             | 0                                            | 0                                  |
| Estazolam | 15                             | 0                                            | 0                                  |

Further evaluation of pentobarbital-induced sleep (Figure 4B) reveals that SMF does not extend sleep duration relative to control, whereas the positive group shows extremely significant differences ( $F = 4.2876$ ,  $p = 0.0075$  and  $\eta^2 = 0.1924$ ). Cohen's  $d$  showed that only the Estazolam group had a large effect size difference ( $|d| = 0.8769$ ) compared with the control group, whereas the differences between SMF-L/SMF-H groups and the control group were minimal ( $|d| = 0.1897$  and  $|d| = 0.1235$ , respectively). Moreover, SMF administration also significantly reduced sleep latency (Figure 4B), with both dosage groups showing extremely significant differences versus control.  $F = 14.7289$ , which exceeded the critical value ( $F_{0.05}(3,56) = 2.76$ ), confirming significant mean differences among

the four groups ( $p < 0.05$ ). A value of  $\eta^2 = 0.3745$  indicated that grouping was the core source of variation in the results. Cohen's  $d$  revealed that there was an extra-large effect size difference between the Estazolam group and the control group ( $|d| = 2.1583$ ), and a large effect size difference between the SMF-L/SMF-H groups and the control group ( $|d| = 0.8972$  and  $|d| = 0.8215$ ).

The subthreshold pentobarbital hypnosis test (Table 6) confirmed that SMF administration for 32 days led to dose-dependent increases in sleep occurrence rate, with 55.6% in the low-dose and 61.1% in high-dose groups showing significantly higher sleep occurrence rates than the vehicle control (10%) ( $p < 0.05$ ), though less than the positive control (100%,  $p < 0.001$ ). Consequently, these collective findings demonstrate that SMF enhances sleep quality by significantly accelerating sleep onset and extending sleep duration, without exhibiting the direct sedative properties or growth-impairing effects associated with conventional sleep aids.

3.3.2 | Impact of SMF on the Level of Neurotransmitter in Blood and Brain

Neurochemical investigations (Figure 4C) provided valuable mechanistic insights into the sleep-modulating action of SMF by influencing multiple targets to affect the sleep-wake regulation. Thus, the SMF can selectively modulate key neurotransmitter systems involved in sleep physiology. Notably, SMF significantly reduces serum DA levels ( $F = 4.0287$ ,  $p = 0.0218$ ,  $\eta^2 = 0.3241$ ) while maintaining brain DA concentrations ( $F = 1.2879$ ,  $p = 0.2936$ ,  $\eta^2 = 0.1142$ ). Such observation suggested that SMF may influence the peripheral wake-promoting signals without disrupting central dopaminergic function, which can potentially reduce arousal states while avoiding the cognitive side effects often associated with direct CNS dopamine suppression (Bucher et al. 2024; Schelp et al. 2017).

Interestingly, SMF could have a dramatic effect on 5-HT systems, with a highly significant increase in cerebral 5-HT content ( $F = 46.5892$ ,  $p < 0.0001$  and  $\eta^2 = 0.8472$ ) that is crucial for initiating and maintaining slow-wave sleep (Cespuglio 2018; Oikonomou et al. 2019). For NE, SMF shows a more pronounced effect, with high-dose administration, which elevates serum NE ( $F = 4.5892$ ,  $p = 0.0087$  and  $\eta^2 = 0.3876$ ) while leaving brain NE levels unchanged ( $F = 0.4287$ ,  $p = 0.7338$  and  $\eta^2 = 0.0492$ ), indicating primarily peripheral activity that may help in sustaining physiological arousal balance without overstimulating central wake-promoting pathways (Gottesmann 2011; Mitchell and Weinshenker 2009). Together, these changes in the neurotransmitter levels prove that SMF is a sophisticated sleep modulator by simultaneously enhancing sleep-promoting 5-HT activity while tempering wakefulness-associated in central signalling. Such a balanced approach would differ fundamentally from the generalised CNS depression caused by conventional sedative hypnotics (Mitchell and Weinshenker 2009). The targeted neuromodulation ability of SMF would likely improve the sleep quality without causing next-day drowsiness or other common side effects of sleep aids (Mitchell and Weinshenker 2009; Fornaro et al. 2024; Fitzgerald and Vietri 2015). The distinct neurotransmitter profile of the whole-brain homogenates (excluding brainstem) also suggests potential

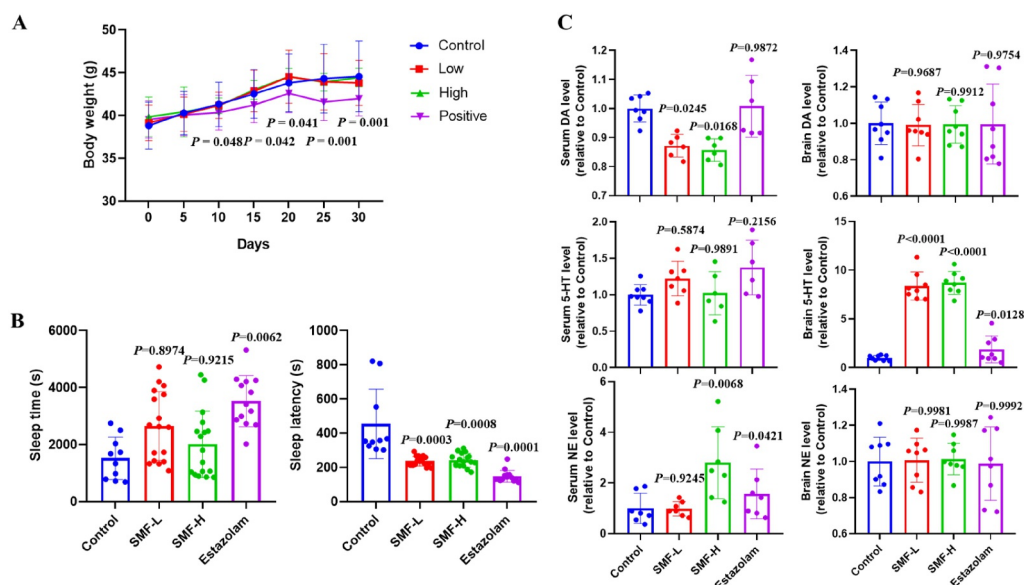


**FIGURE 4** | Effect of SMF on pentobarbital sodium-induced sleep test and neurotransmitter level in mice: (A) body weight; (B) sleep duration and latency induced by pentobarbital sodium. Data are expressed as the mean  $\pm$  SD. (C) The levels of DA, NE, and 5-HT in the serum and brain. Data are expressed as the mean  $\pm$  SD.

**TABLE 6** | Effect of SMF on hypnosis induced by subthreshold doses of pentobarbital sodium.

| Group     | Number of animals (n) | Number of animals with sleep onset (n) | Incidence of sleep onset (%) |
|-----------|-----------------------|----------------------------------------|------------------------------|
| Control   | 10                    | 1                                      | 10                           |
| SMF-L     | 18                    | 10                                     | 55.6*                        |
| SMF-H     | 18                    | 11                                     | 61.1*                        |
| Estazolam | 15                    | 15                                     | 100***                       |

Note: As compared to the control group, \* $p < 0.05$  and \*\*\* $p < 0.001$  for the SMF low-dose, SMF high-dose, and positive control groups.

applications for specific sleep disorders characterised by hyperarousal or sleep maintenance difficulties, though additional research to explore these possibilities. For more precise mechanistic insights, future studies may also examine specific sleep-regulating regions such as the hypothalamus or prefrontal cortex to better localise the neurochemical effects of SMF.

### 3.4 | Predicted Targets and Biological Pathways Influenced by SMF

To further enhance the understanding of the mechanism, network pharmacology analysis was used to link the sleep-modulating activity with the phytoconstituents identified from the formulae. Upon observation, the 35 constituents present in SMF are found to potentially influence 633 known target symbols, and 80 of them (Figure 5A) were found to be target genes related to insomnia, sleep disorder and sleep deprivation gathered from different databases. Through KEGG pathway enrichment analysis, the genes influenced by SMF mainly belong to the following pathways: neuroactive ligand–receptor interactions

(e.g., HTR2A and HTR2C), serotonergic/dopaminergic synapses, Alzheimer's/Parkinson's pathways and thyroid hormone signalling (Figure 5B). Such findings further corroborate the neurotransmitter profile observed from the SMF-treated group, increased in central serotonin via HTR2A/HTR2C activation (Wood et al. 2011) while modulating peripheral DA/NE through respective synapse targets, providing testable hypotheses for future mechanistic studies. In addition, other pathways might seem to be unrelated to sleep modulating, but they suggest the multi-pathway involvement of SMF and its potential to simultaneously influence neurotransmitter systems, neuroprotective mechanisms and cellular regulation pathways (Mitchell and Weinshenker 2009; Monti and Jantos 2008). Hence, highlighting the complex polypharmacology of SMF that exerts its effect through a network of interactions, as presented in Figure 5C. The core targets of SMF (as analysed by CentriScape) are highlighted in light green, which functional clustering (Supporting Information S1: Figure S10) revealed these genes do target five synergistic pathways: nuclear receptor signalling for long-term regulation (Jin et al. 2025), GPCR-cAMP/cGMP cascades for rapid signalling (Parmar et al. 2021), nitric oxide modulation for neurovascular control (Lee et al. 2019), presynaptic machinery for neurotransmitter release (Oh et al. 2018) and GABA receptors for excitatory/inhibitory balance (Zhu et al. 2022). The predicted multi-tiered targeting ability of SMF promotes sleep through complementary mechanisms and comprehensive neuromodulation. Supporting these findings, GO enrichment analysis (Figure 5D) provides a mechanistic foundation for the monoamine neurotransmitter regulation ability of SMF via comprehensive synaptic modulation, including chemical synaptic transmission and neurotransmitter receptor activity. A strong representation would be the serotonin-related terms that support the observed elevated central 5-HT profile while modulating peripheral DA/NE through catecholamine-binding targets such as DRD 2 and ADRB2 (Eisenhofer et al. 2004; Missale et al. 1998).

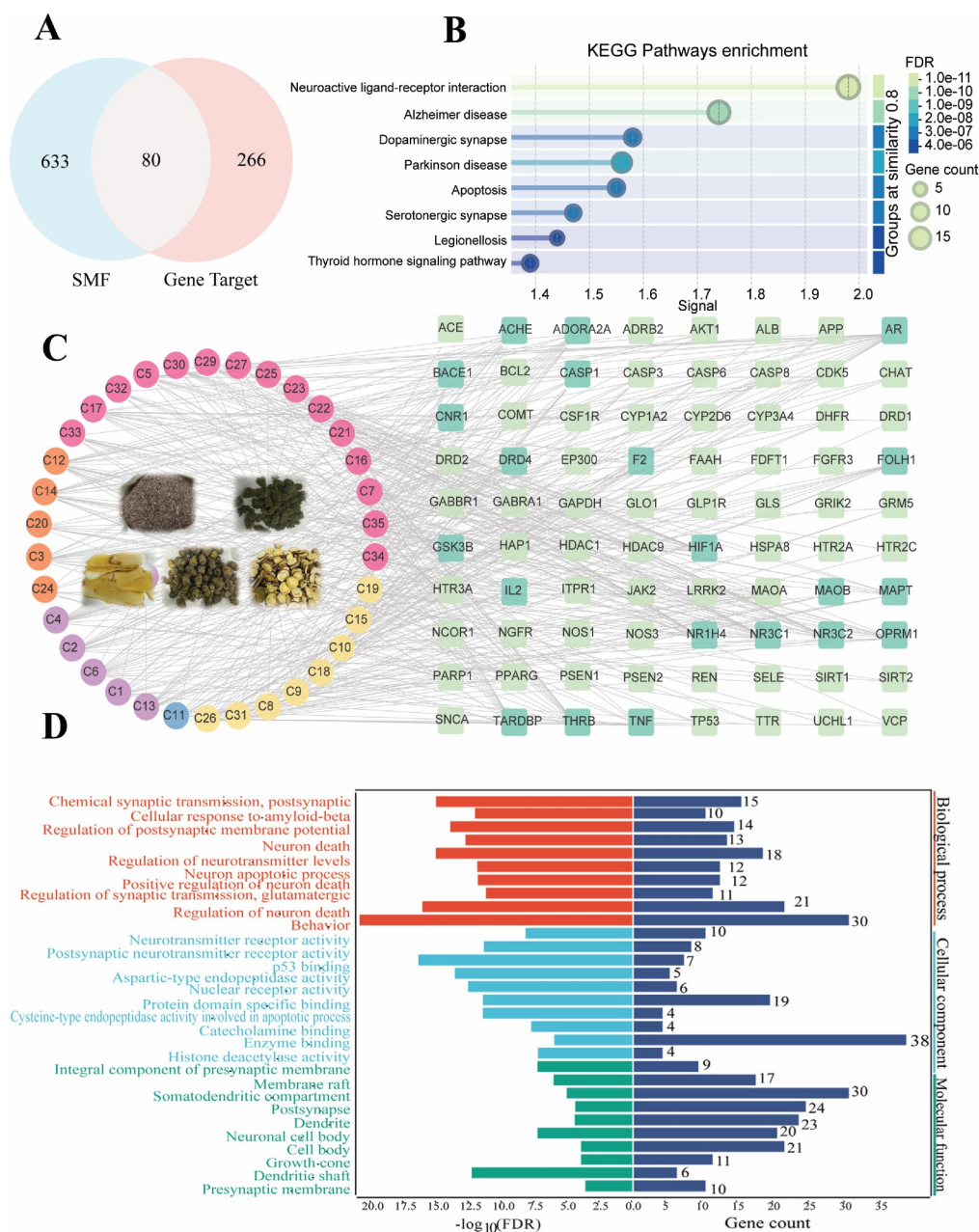


**FIGURE 5** | Network pharmacology analysis of SMF's multi-target mechanisms: (A) Venn diagram of SMF and disease-related targets; (B) KEGG pathway enrichment analysis of 80 overlapping targets; (C) herb-component-gene interaction network, in which the core targets are highlighted with light green; (D) GO enrichment analysis of core targets.

### 3.5 | Molecular Docking Validation of Network Pharmacology Prediction

To refine the numerous constituent-target interactions predicted by network pharmacology, molecular docking analysis was performed, focusing on the biologically relevant targets. The selection was strategically guided by the KEGG pathway enrichment analysis (Figure 5B), which identified neuroactive ligand-receptor interaction as the most enriched pathway where SMF exerts its effects. This finding aligned with GO enrichment (Figure 5D) of the core targets of SMF, which revealed significant terms in biological processes, such as regulation of synaptic transmission and neurotransmitters. Based on consolidated

evidence, seven high-priority receptors were selected, including HTR2A, HTR2C, ADRB2, DRD2, GABRA1, GABBR1 and MAOA, whose combined modulation provides a direct and plausible explanation for the in vivo neurochemical changes (Figure 6A). Thus, the molecular docking analysis revealed that gastrodin, the most abundant constituent in the formulae, stands out as the multi-target ligand of the selected targets. Gastrodin (Figure 6B and Supporting Information S1: Figure S11) demonstrated promising binding affinity across most of the targets, with a binding energy ranging from  $-5.7$  to  $-7.2$  kcal/mol (Supporting Information S1: Table S1). Notably, gastrodin docked into the benzodiazepene (BZP) site of GABA<sub>A</sub> receptor (GABRA1) with a

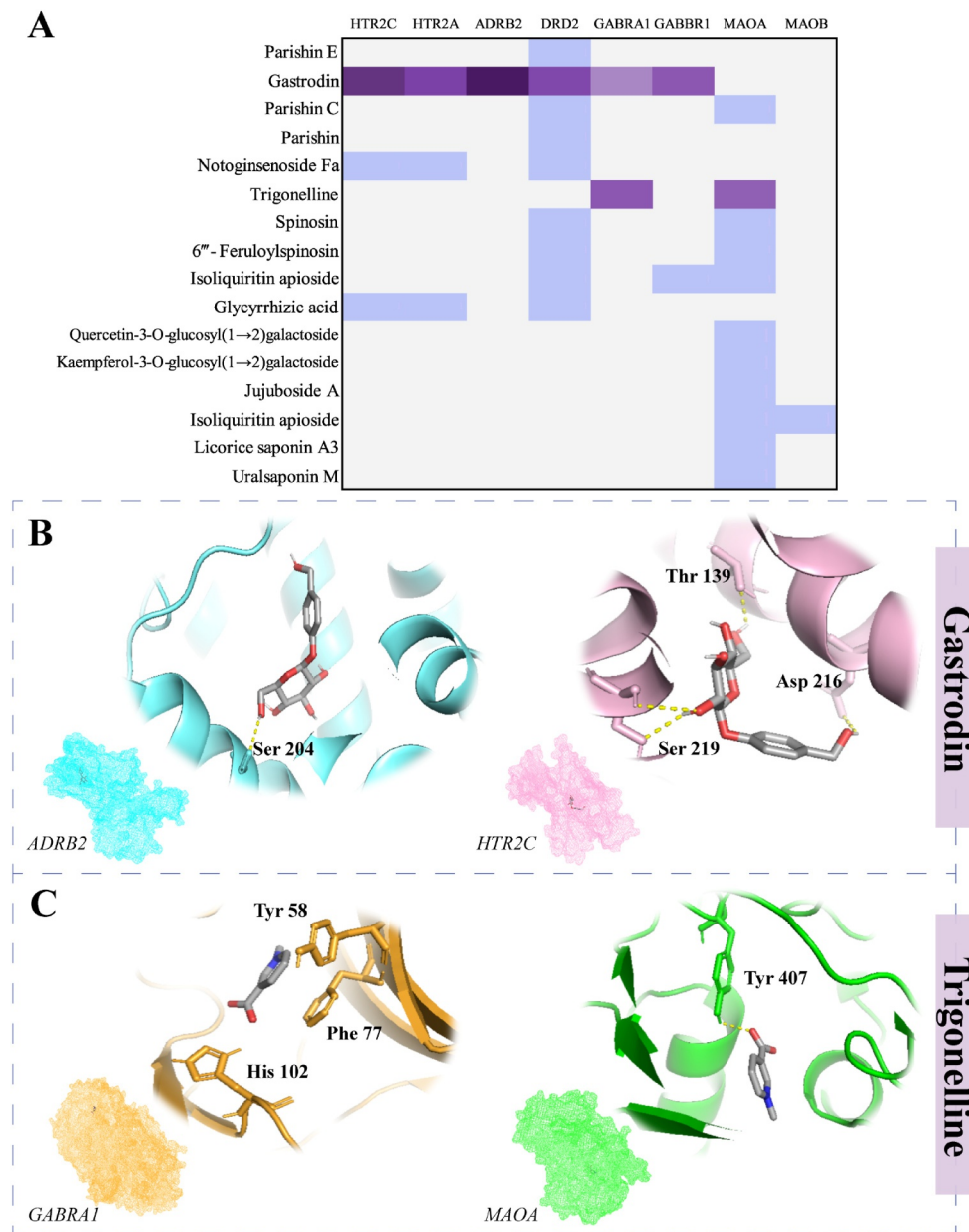


**FIGURE 6** | Molecular docking analysis identifies key SMF constituents and their docking pose: (A) network of SMF constituents predicted to influence the high-priority receptors, with violet shading indicating binding affinity (darker = stronger); (B) binding poses of gastrodin with its highest-affinity targets, ADRB2 (cyan) and HTR2C (pink); (C) binding poses of trigonelline with GABRA1 (yellow) and MAOA (green). Key interacting residues are shown, and hydrogen bonds are indicated by yellow dashed lines.

binding energy of  $-4.0$  kcal/mol. Whereas such a result reflects the weaker binding affinity of gastrodin and the absence of a key bond with Histidine 102 (His 102), an essential interaction for a strong sedative (Zhu et al. 2022). Thereby, this aligns with the in vivo observation that SMF prolonged sleep. Furthermore, the interaction of gastrodin on serotonergic-related receptors (HTR2C and HTR2A) and modulating GABA<sub>A</sub> receptors would likely create a homeostatic counterbalance, resulting in the stable central catecholamine levels observed in vivo (Berg et al. 2008; Szabo and Blier 2001; Bruni et al. 2021). On the other hand, binding of gastrodin to ADRB2 (may function as agonist) and DRD2 (may function as agonist) receptors could influence the release of NE and DA from sympathetic nerve terminals and

adrenal medulla, explaining the change in neurochemical levels in the serum (Wang et al. 2018; Stjärne 2005). Aside from gastrodin, trigonelline (Figure 6C and Supporting Information S1: Figure S12) also exhibits moderate binding affinity ( $-5.7$  kcal/mol) to the BZP site of the GABA<sub>A</sub> receptor, which interacts with His 102, suggesting a more classical modulating effect (Zhu et al. 2022). Other than the GABA receptor, trigonelline was also found to influence monoamine oxidase A (MAOA) with a binding energy of  $-5.4$  kcal/mol. The selectivity of trigonelline for MAOA over MAOB, as visualised in the network pharmacology results (Figure 6A), is particularly noteworthy given the primary role of MAOA in serotonin metabolism. This selective inhibition provides a novel mechanistic explanation for the elevated central

serotonin levels observed in vivo, as reduced MAOA activity would directly decrease 5-HT degradation (Son et al. 2008). Unlike gastradin, trigonelline may contribute to sleep-modulating effects through this specific enzymatic inhibition, working synergistically to elevate monoamine neurotransmitter availability and promote sleep. For the other predicted constituents (Figure 6A), the bulkiness of the structures (MW > 500 Da) does lead to weak or unfavourable binding energies. Parishins are well-known prodrugs that can be hydrolysed in vivo and function as a favourable reservoir of gastradin (Tang et al. 2015). Whereas the other predicted constituents are likely to be metabolised into smaller bioactive metabolites (e.g., aglycone) and may still act through other indirect mechanisms (Feng et al. 2024; Bakr et al. 2022; Wu et al. 2025). As a result, molecular docking analysis predicted that gastradin and trigonelline are the potential multi-target ligands within SMF, which could be responsible for direct modulation of the key receptors in the sleep–wake pathways, providing a direction for further mechanistic studies of SMF activity. Although the current study provides phenomenological evidence of efficacy, the direct validation of these specific molecular targets will be the focus of our future investigations to confirm the proposed mechanisms.

## 4 | Conclusion

This study constructs an MFH-based sleep-modulating formula (SMF), evolved from a traditional Chinese herbal prescription ‘Suan-zao-ren Decoction’, consisting of stir-fried *Z. spinosa* semen, *G. elata* rhizome, *D. officinale*, stewed *Polygonati rhizome*, *P. notoginseng* flower and *Radix Glycyrrhiza* preparata. By UPLC/Q-Exactive-MS, a total of 35 chemical constituents were identified through their MS<sup>2</sup> profiles, and 12 of them were further confirmed by their corresponding standards. Seven constituents, including gastradin, parishin E, parishin B, parishin C, quercetin-3-O-glucosyl-(1 → 2)-galactoside, spinosin and glycyrrhizic acid, were selected as the seven potential quality markers for controlling and evaluating the quality of SMF produced. These compounds are stable at room temperature for over 48 h. Of note, oral administration of SMF substantially improved sleep quality in the pentobarbital-induced sleep murine model via restoring sleep-related neurotransmitter balance by elevating cerebral 5-HT synthesis and serum NE levels while reducing serum DA levels. Importantly, the results of histological analyses revealed no significant abnormalities observed in immune-related organs, suggesting the short-term safety of SMF at the evaluated doses. The mechanistic investigation through network pharmacology predicted that neuroactive ligand–receptor interactions, serotonergic/dopaminergic synapses, and thyroid hormone signalling might have played a role in mediating the sleep-improving effect of SMF. Integrating docking simulation, the analysis not only reveals gastradin (the most abundant constituent in SMF) as the potential primary multi-target ligand modulating sleep–wake pathways but also found trigonelline as a selective MAOA inhibitor, which may serve as a potential explanation for the elevated monoamine level observed in vivo. Although this study has provided promising findings, several limitations deserve careful consideration. First, the proposed mechanisms of SMF for sleep enhancement were derived from network pharmacology and molecular docking analysis, which warrant further experimental validation through in

vitro or in vivo studies. Second, although randomisation was applied in group allocation, blinding was not implemented during data collection and outcome analysis for this study. Key endpoints including sleep latency, loss of righting reflex and recovery time are subjective and prone to observer bias without blinding, which constitutes a potential limitation of this research.

Collectively, this study demonstrates that SMF possesses sleep-promoting properties and may serve as a promising MFH-based sleep supplement to address the growing need for safe and effective sleep management solutions.

## Author Contributions

**Jasmine U. Ting:** investigation, conceptualization, writing – original draft, methodology, formal analysis. **Chunxue Zhao:** formal analysis, methodology, conceptualization, investigation. **Jia Xu:** conceptualization, investigation, methodology, formal analysis. **Chenrui Liu:** investigation. **Yungang Tian:** conceptualization, data curation. **Min Ye:** conceptualization, project administration, supervision. **Tao Cui:** supervision, project administration, conceptualization. **Jian Huang:** conceptualization, project administration, supervision, writing – review and editing.

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## Ethics Statement

The animal study protocol was approved by the Institutional Animal Care and Use Committee (IACUC): Protocol code BCAA0315 and date of approval November 11, 2023.

## Conflicts of Interest

The authors declare no conflicts of interest.

## Data Availability Statement

The data that supports the findings of this study are available in the supplementary material of this article.

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## Supporting Information

Additional supporting information can be found online in the Supporting Information section.

**Supporting Information S1:** pfm270009-sup-0001-suppl-data.pdf.