Integrated Screening of Effective Anti-Insomnia Fractions of Zhi-Zi-Hou-Po Decoction via *Drosophila melanogaster* and Network Pharmacology Analysis of the Underlying Pharmacodynamic Material and Mechanism

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**ABSTRACT:** Insomnia is an anabatic epidemiology, while the mechanism is extremely complicated; it remains one of the major scientific challenges in life sciences. Because of the advantage of having a similar genetic background and circadian rhythm as those of humans, the *Drosophila melanogaster* model organism is hugely popular in sleep-related drug screening studies. Seven-day-old virgin *D. melanogaster* was used to establish the sleep deprivation model by repeated light stimulation at night. Using PySolo activity monitoring system and *Drosophila* activity as indices, the effective fractions of Zhi-Zi-Hou-Po decoction (ZZHPD) for insomnia were screened; the content of monoamine neurotransmitters dopamine (DA), 5-hydroxyindole-3-acetic acid (5-HIAA), Homovanillic acid (HVA), and 5-hydroxytryptamine (5-HT) in the brain of *D. melanogaster* were determined by high-performance liquid chromatography-electro-chemical detection. The herb-compound-target-disease target network were further constructed through network pharmacology to identify the potential targets and pathways of ZZHPD in the intervention of insomnia. Finally, the molecular docking method was used for evaluating the binding characteristics of important compounds from ZZHPD with related targets. The results showed that a certain dose of ZZHPD and its petroleum ether, dichloromethane, ethyl acetate, and *n*-butanol fractions could improve sleep. The dichloromethane fraction from ZZHPD extracts showed the best anti-insomnia effect among all extracts. It can also reduce the content of DA and HVA in the brain of *D. melanogaster* and increase 5-HT and 5-HIAA levels. The network pharmacology showed that the main active ingredients in ZZHPD included magnolol, honokiol, hesperidin, and so forth. According to the screening conditions, there were 71 targets and the result of KEGG enrichment analysis revealed that 73 pathways were associated with insomnia, which were primarily involved in inflammatory response, central neurotransmitter regulation, and apoptosis to relieve insomnia. The molecular docking results clarified that naringenin and apigenin have an intimate relationship with GABA<sub>A</sub> receptor, histamine H1, orexin receptor type 2, and interleukin-6. The mechanism of relieving insomnia is the result of the interaction of multi-components, multi-targets, and multi-pathways, which provides a certain theoretical basis for the treatment of insomnia and related diseases as well as clinical research.

1. **INTRODUCTION**

Being one of the most common sleep disorders in the clinic, insomnia affects 7% adults in the EU and 9−24% adults in the USA.1,2 It has been estimated that the prevalence of at least one insomnia symptom is as high as 33% in the general population.4,5 Stress of society, coupled with negative emotions, including anxiety are largely the causes of insomnia. Insomnia disorder (ID) is defined as a disorder of sleep initiation or maintenance, followed by a feeling of non-restorative sleep and several diurnal consequences ranging from occupational and social difficulties to cognitive impair-

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circadian rhythms,\textsuperscript{2} such as \(\gamma\)-aminobutyric acid (GABA), which is well-known for improving sleep disturbances. In favor of the slow-wave sleep, serotonin [5-hydroxytryptamine (5-HT)] acts as an inhibitory neurotransmitter which is involved in the regulation of circadian system and cognitive function. Dopamine (DA) makes a momentous difference to stimulate the organism to improve wakefulness. 5-Hydroxyindole-3-acetic acid (5-HIAA) and Homovanillic acid (HVA) act separately as their production is parallel to insomnia tendency. Many studies regard insomnia as a disorder of hyperarousal, but the causal relationship between hyperarousal and insomnia remains to be suspicious.

Retrospect to 20th century, \textit{Drosophila melanogaster} was striking in public as a model organism. \textit{D. melanogaster} was screened to investigate human diseases, in which the genetic background of nearly 75\% of human disease genes have corresponding lineal homologues.\textsuperscript{7,8} The use of \textit{D. melanogaster} in sleep assisting marked a milestone in the year 2000, when researchers clarified the concept of its sleep.\textsuperscript{9} Similar to that of humans, the circadian rhythm and effect of age on sleep demand of \textit{Drosophila}, including signal genes that regulate sleep, are highly conserved among mammals.\textsuperscript{10} In addition, the negative effects of impaired learning, memory, and oxidative stress caused by sleep disturbance also occur in human insomnia. Luis published the regulation of homeostasis in neuronal excitability through sleep behavior, in which they researched the effects of Pumilio (Pum) on sleep homeostasis in \textit{D. melanogaster}, and had identified Pum action on neural adaptations.\textsuperscript{11} In the initial stage, our group successfully constructed the \textit{Drosophila} model of sleep deprivation and preceded the pharmacodynamics study of Shuangxia decoction in the treatment of insomnia.\textsuperscript{12}

Traditional Chinese medicine (TCM) has a long history in the therapies of insomnia, which are covered from the perspectives of regulating central neurotransmitters, influencing sleep-related cytokines, and improving the structure of the central nervous system (CNS).\textsuperscript{2} Zhi-Zi-Hou-Po decoction (ZZHPD), comprises of \textit{Gardenia jasminoides} Ellis, \textit{Magnolia officinalis} Rehd. et Wils, and \textit{Citrus aurantium} L, is a traditional Chinese prescription first recorded in TCM bible, “Shang-Han-Lun”, in the Chinese Eastern Han Dynasty (Table 1).\textsuperscript{13}

\begin{table}[h]
\centering
\begin{tabular}{|l|l|}
\hline
ingredients & latin Name  \\
\hline
GAR & \textit{G. jasminoides} Ellis  \\
AUR & \textit{C. aurantium} L  \\
MAG & \textit{M. officinalis} Rehd. et Wils  \\
\hline
\end{tabular}
\caption{Ingredients of ZZHPD}
\end{table}

The clinical application of ZZHPD is primarily to treat depression, and its main ingredient, \textit{M. officinalis} Rehd. et Wils, has been shown to have the effect of antidepressant activity.\textsuperscript{25} Meanwhile, honokiol has an essential effect on NREM sleep. Several longitudinal epidemiological studies have indicated that insomnia is bidirectionally related to anxiety and depression.\textsuperscript{16} However, the mechanism in the treatment of insomnia by ZZHPD is still unclear and requires further exploration.

Network pharmacology is the field in which network biology and multi-pharmacology are combined with the capability of describing complex interactions among biological systems of the human body, drugs, and diseases from a network perspective.\textsuperscript{18} In view of the complexity of multi-components, multi-targets, and synergistic interactions among components of TCM,\textsuperscript{19} the interaction between drugs and diseases is explored through network construction and clarifies the material basis and underlying mechanisms.

In this study, we screened the effective fractions and explored the mechanism of ZZHPD therapy for insomnia based on \textit{D. melanogaster} as the model organism. The concentrations of monoamine neurotransmitters DA, 5-HIAA, HVA, and 5-HT in the brain of \textit{D. melanogaster} were determined. The multi-components, multi-target characteristics, and molecular docking verification of network pharmacology were applied to explore the pharmacological material basis and mechanism.

\section{RESULTS AND DISCUSSION}

\subsection{Effects of Fractions in ZZHPD on Sleep Deprivation of \textit{D. melanogaster}}

Compared with the control group, the nocturnal activity intensity of \textit{D. melanogaster} in the model group significantly increased while the sleep time was decreased \((p < 0.01)\), which demonstrated the sleep deprivation model by repeated light stimulation at night (Figure 1). The PySolo monitoring system was utilized to monitor the activity of \textit{D. melanogaster}. Compared to the model group, the nocturnal activity in the treatment groups was weakened. Furthermore, the sleep duration in the positive group, middle dose of the ZZHPD group, petroleum ether group, dichloromethane group, ethyl acetate group, and low-dose n-butanol group were significantly prolonged \((P < 0.05, P < 0.01)\), especially in the medium dose dichloromethane group. There was no significant difference among the other groups, suggesting that a certain dose of ZZHPD and its different effective fractions can improve the sleep deprivation of \textit{D. melanogaster} (Figure 2).

\subsection{Influence of Neurotransmitters in the Brain of \textit{D. melanogaster}}

According to the above research, the dose of each group was used to determine the influence of DA, 5-HIAA, HVA, and 5-HT in the brain of \textit{D. melanogaster}. Table 2 indicated that they were sacrificed in liquid nitrogen, and their brains were screened and weighed, respectively \((n = 10)\).

As shown in Figure 3, compared with the control group, the DA level in the brain of \textit{D. melanogaster} after sleep deprivation was significantly increased, while those of 5-HT and 5-HIAA significantly decreased \((P < 0.05, P < 0.01)\). The situation reversed in the positive and treatment groups. The effect of the petroleum ether group on neurotransmitters was significant, suggesting that the effective fraction of ZZHPD is more likely to play a role.

\subsection{Network Pharmacology}

\subsubsection{Active Compounds, Drug Targets of ZZHPD, and ID-Related Targets}

The identification of drug–target interactions is crucial to explicitly understand the mechanism of compounds’ interaction at the molecular level and to optimize their therapeutic effect.\textsuperscript{24} It is universal that the higher the content of herbs, the more feasible it is to have a pharmacological effect play a role in therapy. The components that have been reported in literature to have a remarkable effect on insomnia were combined with the quality control compounds of three herbs collected from literature, and thereby, a total of 33 significant compounds were collected in ZZHPD (Table 3).\textsuperscript{25} What is ultimately presented are important compounds, referred to as ID-related targets, such as honokiol, hesperidin, and genipinic acid. Finally, 71 related targets were obtained, which are involved in monoamine
neurotransmitter proteins, inflammatory factors, cell cycle, and apoptotic proteins (Figures 4 and 5).35–38

2.3.2. Construction of Component-Target-Disease Network. The network of protein–protein interaction was established, which possessed the minimum required interaction score (≥0.9) and hid the disconnected nodes in a string (Figure 4b).

To offset the disadvantage of having difficulty in getting the closely connected proteins in protein–protein interaction (PPI) network, we performed topological analysis with degree by Cytoscape (Figure 5). Among the proteins at the top of the topological analysis, inflammatory cytokines and neurotransmitter receptors account for the majority, including IL6 and IL2, as well as receptors mediating the neurotransmitter (HTR1A, DRD2, ADORA1, and DRD3). Furthermore, AKT1, FOS, and TNF were involved in cell proliferation and apoptosis. APP associated with neurite growth and neuronal adhesion was collected with a significant degree. VEGFA, which is a growth factor, played an essential role in angiogenesis and vasculogenesis. We infer that ZZHPD may be closely related to the regulation of inflammation, neurotransmitter receptors, neurite growth, cell proliferation, and apoptosis in the treatment of insomnia. Previous studies have reported that sleep disorder is relevant to the inflammatory response, with higher expression of inflammatory cytokine in insomnia. In particular, the levels of the pro-inflammatory markers, interleukin IL-6, and the acute-phase protein, “C-reactive protein”, increased.26 Dopamine generally exerts its

Figure 1. Locomotor activities of D. melanogaster within 24 h. Abscissa is divided into daytime (white bar, 0–700 min) and nighttime (black bar, 700–1400 min). Ordinate displays the locomotor activities of Drosophila, and the amplitude is positively correlated with the intensity of activity. (a) Control group; (b) model group; and (c) administration group.
actions on the neuronal circuitry via a relatively slow modulation of the fast neurotransmission mediated by glutamate and GABA. HTR1B, DRD2, GABRA1, and other targets associated with the brain CNS are responsible for regulating neuronal activity. Further, the potential herb-compound-target-disease target network was constructed via Cytoscape (Figure 6).

2.3.3. Enrichment Analysis of GO and KEGG. To clearly explore the potential mechanism of ZZHPD on the treatment of insomnia, DAVID database was utilized to perform GO and KEGG pathway enrichment analysis. A total of 387 GO entries were identified, which were mainly enriched in cell signal transduction, cell metabolism, inflammatory response, apoptosis, and angiogenesis (Figure 7). There is exact conformity with the previous expression of the sleep-wake rhythm, neurotransmitters in the CNS, and inflammatory response in insomnia. Molecular functions are mainly reflected in extracellular ligand-gated ion channel activity, receptor and enzyme binding, and cytokine activity. According to \( P < 0.05 \), KEGG enrichment analysis revealed that 73 pathways were

Table 2. Weighting Result of Each Group in Drosophila Brain (\( n = 10 \))

| group   | control | model | PSD | ZZHPD | PE | DT | EA | N-B |
|---------|---------|-------|-----|-------|----|----|----|-----|
| weight/mg | 4.9     | 4.6   | 4.7 | 4.5   | 4.8| 4.5| 4.6| 4.8 |

Figure 2. Effective fractions of ZZHPD on the sleep time of D. melanogaster with sleep deprivation. (a-1–a-3) Control group, model group, and positive group, with different fractions of ZZHPD, respectively. PSD represents pentobarbital sodium; PE represents petroleum ether; DT represents dichloromethane; EA represents ethyl acetate; and N-B represents n-butanol. \# \( p < 0.05 \), \## \( p < 0.01 \) compared with the control group, \* \( p < 0.05 \), \** \( p < 0.01 \) compared with the model group.
associated with insomnia, including neuroactive ligand−receptor interaction, TNF signaling pathway, dopaminergic synapse, GABAergic synapse, and so forth, which were primarily involved in the above biological process to relieve insomnia (Figure 7). We analyzed the top 18 key proteins with degree by topological analysis and top 10 pathways obtained in KEGG (Figure 8). AKT1, FOS, RELA, and ADORA1 were more involved in pathways, which further reflects the activity of ZZHPD in regulating cell apoptosis and anti-inflammatory activity.

2.3.4. Molecular Docking. Based on the reports from earlier studies, the binding energy $\leq -5.0$ kJ·mol$^{-1}$ was used as the paradigm.14 As shown in Table 4 and Figure 9, the analysis results explain that some key compounds of ZZHPD have an intimate relationship with proteins associated with insomnia. Honokiol, naringenin, apigenin, and hesperitin have lower binding energies, suggesting that the index components may play an important role in insomnia, especially naringenin, as well as apigenin, whose stable conformation may act directly on GABA$_A$, histamine H1, and so forth.

2.4. Discussion. Insomnia is commonly regarded as a disturbance in the dynamic balance of molecules in the brain that promote either sleepiness or wakefulness. Based on the reports about the mechanism of insomnia,41 we can learn that insomnia is influenced by various factors which are involved in disorder of hyperarousal; the relationship between stimulants associated with sleep promotion, abnormal regulation of the hypothalamic−pituitary−adrenal axis (HPA), the effects of inflammatory cytokine on slow-wave sleep,29 involvement of multiple neurotransmitters in the CNS,26,30 and circadian rhythm. Prolonged exposure to light decreases the release of melatonin (MT), which is closely related to the regulation of sleep cycle, antioxidant damage, sedative, and hypnotic effects.31 These identifications provide a theoretical basis for the treatment of insomnia. ZZHPD comprised of GAR, AUR, and MAG which are applicable to relieve insomnia caused by dysphoria. In this research, we preliminarily explored the potential active components and the mechanism of ZZHPD acting on insomnia.

Recently, researchers around the world have used multiple methods to establish insomnia models in flies, mainly focusing on repeating light stimulation at night and feeding caffeine.15−17 The method of caffeine is based on the fact that caffeine can act on adenosine and dopamine pathways in the brain to active neurons. It is a common drug-induced pattern with simple preparation and significant effect, which is suitable for behavioral studies of animals with insomnia. But the inhomogeneity for drug-dose, or individual variation between the flies leads to between-group variance. Furthermore, it is a paradox that the signal pathway-confirmed mechanism for drugs to screen sedative hypnotic activity with various substances interact with the organism simultaneously.

Figure 3. Influence of neurotransmitters in the brain of D. melanogaster. DA (a), HVA (b), 5-HT (c), and 5-HIAA (d) were measured after the administration of different fractions of ZZHPD. After sleep deprivation, the DA level significantly increased compared with that of the control group, while those of 5-HIAA and 5-HT decreased. Although the HVA level was not impacted, it is noted that the neurotransmitters had markedly been restored in the group of ZZHPD. #p < 0.05, ##p < 0.01 compared with the control group, and *p < 0.05, **p < 0.01 compared with the model group.
The pattern of repeating light stimulation at night, with no interference from exogenous drugs, is appropriate for research on mechanism of action. Since some of the flies may be insensitive to light condition, the model has possibility of intragroup differences. Hence, we selected the second approach for mechanism exploration. Repeating light stimulation at night can successfully replicate the model of sleep deprivation in D. melanogaster. According to activity monitoring, a certain dose of ZZHPD and its petroleum ether fraction, dichloromethane fraction, ethyl acetate fraction, and n-butanol fraction could improve sleep in sleep-deprived D. melanogaster, which significantly increased the sleep duration in the medium-dose dichloromethane group. Further, experiments indicated that the above effective fractions could reduce the level of DA coupled with its metabolite HVA and increase 5-HT and 5-HIAA levels in the brain of D. melanogaster after sleep deprivation. Based on the neurotransmitters in the CNS, we investigated the effective fractions of ZZHPD on neurotransmitters. It is definite that a certain dose of ZZHPD and its different effective fractions have an effect on relieving sleep to regulate circadian rhythmicity. It may affect the release of monoamine neurotransmitters and amino acid neurotransmitters to cooperate with multiple neurotransmitters to improve insomnia.

Given the exploration of the neurotransmitters in the CNS, from the perspective of network pharmacology, we revealed the material basis and the mechanism of ZZHPD in the treatment of insomnia. Index components and vital components reported in literature were utilized to explore the intervention of insomnia using ZZHPD on the foundation of previous identifications. As acquired in the results of the network, it is identified that 29 active components had common targets with insomnia including magnolol, honokiol, hesperidin, apigenin, genpin, and ferulic acid. Honokiol is one of the substances that act on the GABA_A receptor, which is responsible for NREM sleep. Hesperidin plays an important role in the sedative effect, and genpin, the major bioactive constituent of G. jasminoides Ellis, possesses anti-inflammatory, neuroprotective, and antidepressant activities. The key targets collected by topology analysis covered IL6, APP, AKT1, HTR1A, DRD2, and FOS, which implied that ZZHPD targets collected by topology analysis covered IL6, APP, AKT1, HTR1A, DRD2, and FOS. According to activity monitoring, a certain dose of ZZHPD and its effective fractions could reduce the level of DA and HVA and increase 5-HT and 5-HIAA levels in the brain of D. melanogaster; which significantly increased the sleep duration in the medium-dose dichloromethane group. Further, experiments indicated that the above effective fractions could reduce the level of DA coupled with its metabolite HVA and increase 5-HT and 5-HIAA levels in the brain of D. melanogaster after sleep deprivation. Based on the neurotransmitters in the CNS, we investigated the effective fractions of ZZHPD on neurotransmitters. It is definite that a certain dose of ZZHPD and its different effective fractions have an effect on relieving sleep to regulate circadian rhythmicity. It may affect the release of monoamine neurotransmitters and amino acid neurotransmitters to cooperate with multiple neurotransmitters to improve insomnia.

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Figure 4. Venn and PPI network of ZZHPD. (a) Venn diagram of targets of herb diseases; and (b) PPI network of common targets.

Figure 5. Topology analysis network of common targets in ZZHPD. Size of each label represents its degree; Intensity of the color represents the value of betweenness centrality.

Figure 6. ZZHPD potential herb-compound-target-disease target network. Red nodes represent potential common compound targets in ZZHPD; yellow nodes represent herbs; purple nodes represent compounds; and blue nodes represent disease. GAR: *G. jasminoides* Ellis; AUR: *C. aurantium* L.; and MAG: *M. officinalis* Rehd. et Wils.
validation that ZZHPD can make a difference to the levels of DA and 5-HT in mice. From the literature, it can be noted that researchers have found depression to be associated with the pathogenesis of insomnia. In the antidepressant effects of ZZHPD on rats induced by chronic unpredictable mild stress, the levels of 5-HT and DA in the synaptic cleft can be

Figure 7. GO enrichment analysis and KEGG pathway analysis of potential targets in ZZHPD. Size of the bubbles represents the gene counts of this item, and the colors from cold to warm represent the P values from large to small; top 15 enriched GO and KEGG pathways with P values; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes.

Figure 8. Analysis of the KEGG pathway in ZZHPD.
regulated by the synthesized rate-limiting enzymes and transporters, which are tryptophan hydroxylase 2, tyrosine hydroxylase, serotonin transporter, and dopamine transporter. In addition, honokiol possesses antioxidant, anti-inflammatory, anticancer, antidepressant, and neuroprotective activities. It is reported that honokiol has a high potency in promoting nonrapid eye movement sleep by modulating the benzodiazepine site of the GABA<sub>A</sub> receptor.

GO enrichment analysis results mainly gathered from cell signal transduction, cell metabolism, inflammatory response, and angiogenesis such as GABA signaling pathway, ion transmembrane transport, positive regulation of vasocostriction including IL6 and FOS involved the biological process. Signal pathways enriched by key targets participate in neuroactive ligand−receptor interaction, TNF signaling pathway, cAMP signaling pathway, and GABAergic synapse. It further reflects the regulation of ZZHPD on inflammation and immune response, apoptosis, and neurotransmitters such as DA and 5-HT. As is well known, the permeability of extracellular fluoride ion has a significant effect on the central inhibition of GABA. In the biological process, chloride transmembrane transport, including GABA signaling pathway were enriched, which indicated ZZHPD may regulate the ion channel proteins to promote hypnotic action. In order to verify the pharmacodynamic material basis of ZZHPD for insomnia, molecular docking technology was used to probe the binding activity of the indicative components. We demonstrated that the proteins associated with insomnia had better binding ability, among which honokiol, naringenin, apigenin, and hesperitin had lower binding energies suggesting their greater possibility to play a role.

3. CONCLUSIONS

From the perspective of the effective fractions and network pharmacology, the work preliminarily explored the potential active components and mechanism of ZZHPD on the treatment of insomnia. ZZHPD mainly participates in monoamine neurotransmitters, anti-inflammation, apoptosis, and other pharmacological effects that significantly increase central inhibitory neurotransmitters and reduce the release of excitatory neurotransmitters and inflammatory factors to alleviate insomnia. Further research on how ZZHPD exerts anti-inflammatory activity remains to be carried out. In addition, this work provides theoretic foundation for treatment against insomnia and clinical research of ZZHPD.

4. MATERIALS AND METHODS

4.1. Materials. The processed products of <i>G. jasminoides</i> Ellis (Jiangxi, 160519004), <i>M. officinalis</i> Rehd. et Wils (Sichuan, 160702002), and <i>C. aurantium</i> Ellis (Jiangxi, 160702002) were used as the samples. The following compounds were obtained from the Shanghai Woyong Biological Technology Co., Ltd.: apigenin, hesperidin, hesperitin, caryoptoside, naringenin, etc. The concentration was kept at 10 μg/mL. The molecular docking results of the key active components are listed in Table 4.

Table 4. Docking Results of Quality Control Compounds in ZZHPD

| compound      | molecular formula | binding energy values (GABA<sub>A</sub>) | binding Energy values (D2 dopamine) | binding energy values (orexin receptor type 2) | binding energy values (histamine H1) | binding energy values (interleukin-6) |
|---------------|-------------------|------------------------------------------|-----------------------------------|-----------------------------------------------|-------------------------------------|--------------------------------------|
| magnolol      | C<sub>18</sub>H<sub>18</sub>O<sub>2</sub> | -3.41                                    | -4.19                             | -4.19                                         | -2.31                               | -4.89                                |
| genipin       | C<sub>11</sub>H<sub>14</sub>O<sub>5</sub> | -3.53                                    | -3.17                             | -4.28                                         | -2.56                               | -3.84                                |
| rosmarinic acid| C<sub>11</sub>H<sub>10</sub>O<sub>8</sub> | -2.63                                    | -2.54                             | -4.25                                         | -2.29                               | -4.21                                |
| hesperidin    | C<sub>26</sub>H<sub>34</sub>O<sub>15</sub> | -1.45                                    | -1.96                             | -3.35                                         | -1.41                               | -2.64                                |
| honokiol      | C<sub>18</sub>H<sub>18</sub>O<sub>2</sub> | -4.55                                    | -4.3                              | -4.69                                         | -3.11                               | -4.24                                |
| naringenin    | C<sub>15</sub>H<sub>12</sub>O<sub>5</sub> | -5.01                                    | -4.88                             | -5.23                                         | -6.07                               | -5.55                                |
| magnolol      | C<sub>18</sub>H<sub>18</sub>O<sub>2</sub> | -3.54                                    | -3.29                             | -4.93                                         | -2.98                               | -4.68                                |
| synephrine    | C<sub>6</sub>H<sub>9</sub>N<sub>2</sub>O<sub>2</sub> | -3.12                                    | -2.7                              | -3.17                                         | -2.75                               | -3.26                                |
| apigenin      | C<sub>15</sub>H<sub>10</sub>O<sub>5</sub> | -4.58                                    | -4.66                             | -4.95                                         | -7.08                               | -5.08                                |
| hesperitin    | C<sub>18</sub>H<sub>16</sub>O<sub>6</sub> | -5.15                                    | -4.07                             | -4.82                                         | -3.16                               | -5.57                                |
| ferulic acid  | C<sub>19</sub>H<sub>14</sub>O<sub>4</sub> | -3.87                                    | -2.96                             | -4.97                                         | -3.01                               | -4.25                                |
| caryoptoside  | C<sub>18</sub>H<sub>16</sub>O<sub>11</sub> | -2.49                                    | -2.17                             | -3.17                                         | -1.64                               | -3.49                                |

Figure 9. Molecular docking results in ZZHPD. (a) Magnolol and interleukin-6; (b) apigenin and D2 dopamine receptor; (c) naringenin and orexin receptor type 2; and (d) hesperitin and GABA<sub>A</sub> receptor.
herbs were purchased from Beijing Kangyuan Xiangrui Pharmaceutical Technology Co., Ltd. Pentobarbital sodium was obtained from National Pharmaceutical Group Chemical Reagent Co., Ltd. The other drugs used in this study, 5-HT, 5-HIAA, and HVA standards (purity >98%) were purchased from Shanghai Yuanye Biotechnology Co., Ltd. DA standards (purity >98%) was provided by National institutes for Food and Drug Control. Other chemicals and reagents were of the highest grade available (purity >98%).

4.2. Preparation of Different Extracts of ZZHPD. The proportion of ZZHPD described in Shang Han Lun was converted into G. jasminoides Ellis 9 g, M. officinalis Rehd. et Wils 62.4 g, and C. aurantium L 10 g. With the previous experimental optimization process, its petroleum ether, dichloromethane, ethyl acetate, and n-butanol fractions were separated and dried at 60 °C in a vacuum drying box. The dried powder of extraction part in ZZHPD was obtained and stored in a refrigerator at 4 °C.

4.3. Screening the Effective Fractions of ZZHPD. In this study, Canton-S wild-type D. melanogaster (provided by Institute of Biophysics, Chinese Academy of Sciences) was used to establish a sleep deprivation model by repeated light stimulation at night. The details are as follows: a light-controlled switch was placed in the Drosophila incubator to replicate the sleep-deprivation model between morning and evening with normal light, and after the lights were turned-off, at a fixed time at night. D. melanogaster, cultivated in the culture tube, were transferred every night with reserving ovum and pupa. The newly emerged were diverted in the morning of the next day. Females were collected under CO2 mild anesthesia with a self-made anesthesia device (with a pressure reducing valve to control the flow of CO2 on a pad made of porous material for anaesthetizing) and then fed in a monitoring tube containing basic culture medium.

The Drosophila were randomly divided into control, model, positive drug groups, and low-, medium-, and high-dose extracts of group ZZHPD (n = 32). The control and model groups were transferred into the basic medium monitoring tube without the drug at 7:00 am on the fourth day, while the positive drug group was diverted into the medium monitoring tube containing pentobarbital sodium. The other treatment groups were transferred into the monitoring tube containing 0.5, 2.5, and 5% of drug mass fraction. The activity of Drosophila was monitored from 7:00 a.m. on the 7th day to 7:00 a.m. the next day (habituated 24 h before monitoring).

4.4. Determination of DA, 5-HIAA, HVA, and 5-HT Levels in the Brain of D. melanogaster by High-Performance Liquid Chromatography (HPLC)–Electro-Chemical Detection (ECD). In order to explore the effects of ZZHPD and its petroleum ether, dichloromethane, ethyl acetate, and n-butanol fractions on monoamine neurotransmitters in the brain of D. melanogaster during sleep deprivation, the levels of 5-HT, 5-HIAA, NE, and DA were determined by HPLC with electro-chemical detection (ECD). The HPLC system used was a Waters e2695 Separations Module (Waters Corporation, Milford, MA, USA) with a 2465 electrochemical detector. An Atlantis C18 (2.1 mm × 150 mm, 3 μm) was applied coupled with +0.75 V of detection voltage in ECD. A ratio of 4:96 in methanol-buffer salt solution constituted the mobile phase with isocratic elution with a flow rate of 1.0 mL/min.

4.5. Network Pharmacology Analysis. 4.5.1. Screening of Active Compounds and Targets. The newly developed TCMSP provides up-to-date, quantitative, and system information about TCM ingredients, ADME-related properties, targets, and diseases. The combination of TCMSP and literature was used to identify the active compounds in ZZHPD. The ID-related targets were screened with the key word “insomnia” from GeneCards database.

4.5.2. Network Construction. Venn diagrams are a common visualization chart, which allows spotting shared and unshared identifiers providing an insight on the list’s similarities. For further exploring the relationship between compound targets with ID-related targets, we collected common targets by yven web. The STRING database provides known and predicted protein–protein associations data with confidence scores that quantify their reliability. Based on the STRING database, disconnected nodes were hid in the network, and the minimum required interaction score (≥0.9) was set to obtain the network of PPI. Subsequently, we constructed a potential herb-compound-target-disease target network in ZZHPD using Cytoscape v3.7.2.

4.5.3. Analysis of Network and Molecular Docking. DAVID database was utilized to perform Gene Ontology (GO) and KEGG pathway enrichment analysis so that we can clarify the interaction between related targets and signaling pathway. Molecular docking has the capacity for identification of novel compounds of therapeutic interest or predicting ligand–target interactions at a molecular level. The index components were selected from the active ingredients of ZZHPD, followed by applied AutoDock to fulfill molecular docking with ID-related targets (GABA receptor, D2 dopamine receptor, and orexin receptor type 2, interleukin-6). Finally, Pymol was operated in allusion to the satisfying results of molecular docking.

4.6. Statistical Analysis. The experimental data were analyzed by one-way ANOVA with SPSS software 19.0. Dunnett’s post-test was used for the difference between the groups. The data expressed with the mean ± standard deviation were considered statistically significant at p < 0.05.

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Y.S. and R.Z. participated in literature search, data arrangement, and writing the manuscript. R.L., S.N., and T.L. participated in software operation. H.W., Y.C., T.Y., and Y.Q. participated in manuscript correction. C.Z. and Y.S. designed the experiment.

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Notes
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ABBREVIATIONS
ZHZPD, Zhi-Zi-Hou-Po decoction; GAR, Gardenia jasminoides Ellis; AUR, Citrus aurantium L.; MAG, Magnolia officinalis Rehd. et Wils; TCM, traditional Chinese medicine; PSD, pentobarbital sodium; PE, petroleum ether; DT, dichloromethane; EA, ethyl acetate; N-B, N-butanol; DA, dopamine; 5-HIAA, 5-hydroxyindole-3-acetic acid; HVA, Homovanillic acid; 5-HT, 5-hydroxytryptamine; ADME, absorption, distribution, metabolism, and excretion

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