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Article

Network pharmacology strategy to investigate the pharmacological effects of Suanzaoren Decoction on insomnia

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Suanzaoren Decoction (SZRD) is an ancient prescription used in the treatment of insomnia. This study aimed to investigate the components and targets of SZRD in treating insomnia. First, the compounds of five herbs in SZRD were collected from the Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP), and the putative targets for treating insomnia were obtained from DrugBank to construct the herb–compound–target–disease network. A protein–protein interaction (PPI) network was constructed in the STRING database, and then Gene Ontology functional enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were performed to predict the mechanism of action of intersection target. Finally, 30 mice were divided into five groups: control, model, and quercetin groups (100, 50, 25 mg/kg). The sleep latency and duration of pentobarbital-induced sleeping were measured. The production of interleukin-6 (IL-6) and γ-aminobutyric acid (γ-GABA) was detected by using an enzyme-linked immunosorbent assay kit (ELISA), and Gamma-aminobutyric acid type a receptor subunit alpha1 (GABRA1) was tested by Reverse Transcription-Polymerase Chain Reaction (RT-PCR). A total of 152 active ingredients, including 80 putative targets of SZRD, were obtained. The main active compounds included quercetin and kaempferol, and the key targets involved IL-6 and nitric oxide synthase 3 (NOS3). The results of pathway enrichment analysis indicated that the putative targets of SZRD mainly participated in Neuroactive ligand-receptor interaction. The experiment of P-chlorophenylalanine (PCPA)-induced insomnia model showed that quercetin obviously shortened the sleep latency and prolonged the sleep duration of the insomnia model. The production of IL-6, γ-GABA, and GABRA1 mRNA was significantly increased in mice treated with quercetin. This study predicted the active ingredients and potential targets of SZRD on insomnia on the basis of a systematic network pharmacology approach and illustrated that SZRD might exert hypnotic effects via regulating IL-6, γ-GABA, and GABRA1.

Keywords: Suanzaoren Decoction. Insomnia. Network pharmacology.

INTRODUCTION

Insomnia is a sleep disorder charactered by difficulty initiating and maintaining sleep and is associated with medical, emotional, environmental, and behavioral factors (Schutte-Rodin *et al*., 2008). About 25% to 30% of adults are estimated to suffer from insomnia, resulting in poor living quality (Spira *et al*., 2014). At present, medical therapy is the standard treatment approach for insomnia (Sateia *et al*., 2017). The therapeutic strategies for insomnia mainly include benzodiazepine receptor agonists, melatonin receptor agonists, and hypnotic antidepressants (Janto, Prichard, Pusalavidyasagar, 2018). However, the current pharmacotherapy is still unsatisfactory due to contraindications, side effects, high costs, and addictions (Spadoni *et al*., 2011). Traditional Chinese medicine (TCM) has gradually become an alternative option for the treatment of insomnia and has been widely used in

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Asian countries due to its excellent therapeutic effect and safety (Li *et al*., 2020; Liu *et al*., 2016).

Suanzaoren Decoction (SZRD) is an ancient prescription and classic clinical decoction comprising five herbs, including *Ziziphi Spinosae Semen* (ZSS), *Anemarrhenae Rhizoma* (AR), *Poria* (P), *Chuanxiong Rhizoma* (CR) and *Glycyrrhizae Radix Et Rhizoma* (GRR); moreover, it has anticonvulsant, blood-nourishing, cardioprotective, and immunity-enhancing effects (Song *et al*., 2020). In a previous study, SZRD was found to shorten sleep latency and prolong sleep time to improve sleep quality in a sleep-deprivation rat model (Zhan *et al*., 2020). Despite the popular use of SZRD, few studies have explored the potential molecular mechanism for insomnia treatment because of the multiple pathways, target points, and elements of prescription (Lee *et al*., 2018).

Network pharmacology is a new technology that integrates chemoinformatics, bioinformatics, network biology, network analysis, and traditional pharmacology and meets the key ideas of the holistic philosophy of TCM (Berger, Iyengar, 2009; Li, Zhang, 2013). For example, Yu *et al.* (2018) investigated the mechanism of Zuojinwan for the treatment of gastritis by network pharmacology-based strategy.

Consequently, the present study aimed to investigate the components and targets of SZRD to explore its mechanism in the treatment of insomnia. In addition, on the basis of the results of network pharmacology, in vivo experiment was conducted to explore the potential compounds and mechanism of treating insomnia by SZRD.

MATERIAL AND METHODS

Screening chemical compounds and targets of SZRD

The chemical compounds of five herbs (ZSS, P, CR, AR, and GRR) in SZRD were collected from the TCMSP (http://tcmspw.com/) with the limited conditions: "Oral bioavailability (OB)≥30%" and "Drug-likeness (DL)≥0.18" (Ru *et al.* 2014). Then, the potential targets of SZRD were obtained from the DrugBank database (https://go.drugbank.com/), and the gene names were determined by the Uniport database (http://www.uniprot.org/).

Collection of gene targets of insomnia

Human insomnia-related genes were collected from the GeneCards database (https://auth.lifemapsc.com/), OMIM database (https://www.omim.org/), and Therapeutic Target Database (TTD) (http://db.idrblab.net/ttd/) using the keyword "insomnia" on November 12, 2020.

herb-compound-target-disease network constructed

To further determine the molecular mechanism of SZRD on insomnia, the common targets of SZRD and insomnia were determined, and the herb–compound–target–disease network was constructed using Cytoscape 3.6.1, which is a software package for visualizing network analysis. In these graphical networks, the compounds, proteins, or pathways were expressed as nodes, whereas the compound–target or target–pathway interactions were expressed as edges.

PPI network constructed

PPI network data were derived from the STRING database (http://string-db.org/). The common targets of SZRD and insomnia were imported into the STRING database with the limited condition of "*Homo sapiens*" to generate the PPI network. The graphical networks were constructed using Cytoscape 3.6.1.

Pathway enrichment performance

To determine the biological process (BP), molecular function (MF), and cellular component (CC) about the putative targets, Gene ontology (GO) and KEGG analysis was performed in the DAVID database (https://david.ncifcrf.gov/). The top 20 terms were sorted to draw a histogram using GraphPad Prism 8. KEGG database (http://www.genome.jp/kegg/) analysis was visualization by ggplot2 database 2.2.0.

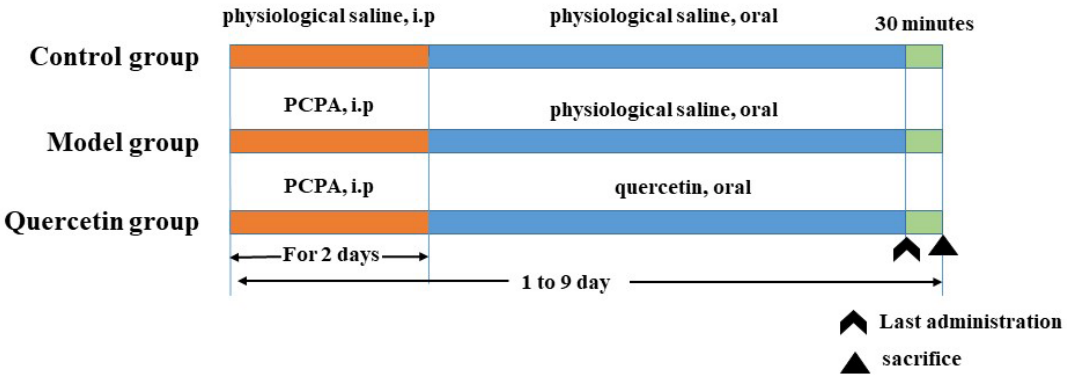
Experiment validation

Animal treated

ICR mice (SPF, weight 20 ± 2 g) were obtained from Zhejiang Experimental Animal Center (NO. SCXK (Zhe) 2019-0001, Zhejiang, China). The animals were maintained in a room with a constant temperature of 23 ± 1 °C, a relative humidity of 30%–40%, light for 12 h from 06:00 to 18:00, and *ad libitum* food and purified water. The animals were randomly divided into five groups as follows: control group (n = 6), model group (n = 6), quercetin high-dose group (Que-H, 100 mg/kg, n = 6), quercetin middle-dose group (Que-M, 50 mg/kg, n = 6), and quercetin low-dose group (Que-L, 25 mg/kg, n = 6).

PCPA-induced insomnia mice model

The insomnia mouse model was established via intraperitoneal injection of PCPA. The PCPA-induced mice were received PCPA in the first and second day and the dose was 300 mg/kg each time. Meanwhile, the control group was intraperitoneally injected with physiological saline. On the third day, the quercetin group mice were orally given different concentrations of quercetin (100, 50, 25 mg/kg), whereas the control and model group mice were orally administered with the same volume of saline for another 7 days. The mice were sacrificed after the final 30 min of administration quercetin or saline (S. Figure.1)



S. FIGURE 1 - The simplified timeline for control, model and quercetin group.

Measurement of sleep latency and duration

Each mouse was intraperitoneally injected with a threshold dose of pentobarbital sodium (45 mg/kg) after the final 30 min of administration quercetin or saline. The sleep latency was recorded from the time of injection to the time when the righting reflex of mice disappeared for 1 min, and the sleeping time was recorded from the time when the righting reflex disappeared until recovery.

Determination of IL-6

The activity of IL-6 in mouse serum, which was collected after mice sacrificed the last administration, was determined using an ELISA (09/2019, Shanghai Enzyme-linked Biotechnology Co., Ltd.) in accordance with the manufacturer's instructions.

Detection of γ-GABA levels in the hypothalamus

After the mice were sacrificed, hypothalamus tissues were taken for the detection of γ-GABA production by ELISA (09/2019, Shanghai Enzyme-linked Biotechnology Co., Ltd.). First, hypothalamus tissues were placed in 1.5 mL eppendorf (EP) tubes and added 9 times the volume of Phosphate Buffered Saline (PBS) buffer solution. Then, the EP tubes were transferred to a high-speed homogenizer for sample homogenization at 65 Hz for

5 min. Finally, the supernatant of hypothalamus tissues (SCIENTZ-48, Zhejiang, China) was collected after centrifugation at a speed of 2500 r/min for 5 min (LX-200, Jiangsu, China). The detection steps were performed in accordance with the manufacturer's protocol.

GABARA1 mRNA expression detected by RT-PCR

The total RNA of mouse hypothalamus was extracted using the TRIzol method. RNA integrity was detected by 1% agarose gel electrophoresis, and the total RNA concentration and OD_{260}/OD_{280} value were measured using a microspectrophotometer. And the satisfactory RNA samples synthesis templates of cDNA. The PCR primers (Table I) were designed on the basis of the NCBI reference sequence database for each gene and using Primer3 software (Rozen, Skaletsky, 2000). β-actin was used as a reference gene, and the expression between groups was compared using the $2^{-\Delta\Delta CT}$ method.

TABLE I - PCR primers

Gene	Sequence
Gabra1	F: 5'-ACCATGCCTAATAAGCTCCTGCGT-3' R: 5'-CAAGTGCATTGGGCATTCAGCTCT-3'
β-actin	F: 5'-TCTTGGGTATGGAATCCTGTGGCA-3' R: 5'-ACAGCACTGTGTTGGCATAGAGGT-3'

DATA AND STATISTICAL ANALYSIS

Statistical analysis was processed with IBM SPSS Statistics 19.0 (SPSS Inc., NY, USA). Data were expressed as the mean ± SD and analyzed using Student's t-test. Differences between groups were considered to be statistically significant if values of $P< 0.05$.

RESULTS

Active ingredient of SZRD screening

The five herbs in SZRD had 152 active ingredients, including 21 compounds of ZSS, 16 compounds of P, 7 compounds of CR, 15 compounds of AR, and 93 compounds of GRR, after screening with the limited condition: "Oral bioavailability (OB)≥30%" and "Drug-likeness (DL)≥0.18" (S.Table I).

The herb-compound-target-disease network analysis

To understand the relationship among herbs, SZRD compounds, putative targets, and insomnia, the herb–compound–target–disease network was constructed. The network had 1128 nodes, including 1 disease, 5 herbs, 80 targets, and 1042 active ingredients. One target could interact with more than one target, and multiple compounds could also act on the same protein targets. MOL000098 (quercetin), MOL00042 (kaempferol), MOL003896 (7-methoxy-2-methyl isoflavone), and MOL004373 (anhydroicaritin) were related to 75, 44, 43, and 37 targets, respectively, and Recombinant Prostaglandin Endoperoxide Synthase 2 (PTGS2), Estrogen Receptor 1 (ESR1), (Recombinant Androgen Receptor) AR, and Calmodulin Dependent Protein Kinase Kinase 2 (CAMKK2) were associated with 95, 81, 71, and 70 compounds (Figure 1).

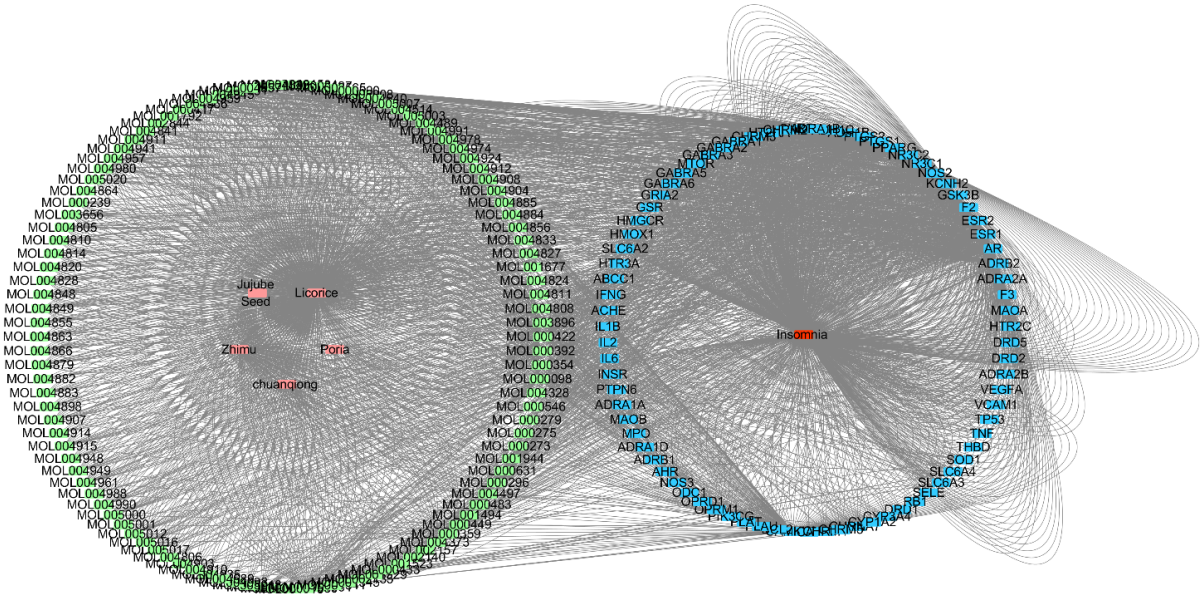


FIGURE 1 - The herb-compound-target-disease network of SZRD and insomnia. The green, orange, blue, red rectangles represented the compounds of SZRD, herbs of SZRD, common targets of SZRD and insomnia, insomnia, respectively. And the lights indicated the association among the compounds, herbs, targets and insomnia. Jujube Seed: *Ziziphi Spinosae Semen*; Poria: *Poria* (P), Chuanqiong: *Chuanxiong Rhizoma* (CR); Zhimu: *Anemarrhenae Rhizoma* (AR), and Licorice: *Glycyrrhizae Radix Et Rhizoma* (GRR).

The PPI network analysis

To explore the mechanism of SZRD in treating insomnia, a PPI network was constructed in the STRING database. As shown in Figure 2, a total of 80 nodes and 653 edges were obtained from the PPI network with confidence level greater than 0.9. IL-6 (degree = 36), NOS3 (degree = 35), Vascular endothelial growth factor A (VEGFA, degree = 35), Tumor necrosis factor (TNF, degree = 33), Catalase (CAT, degree = 32), Prostaglandin G/H synthase 2 (PTGS2, degree = 32), Cellular tumor antigen p53 (TP53, degree = 32), Interleukin-1 beta (IL-1β, degree = 31), and Acetylcholinesterase (ACHE, degree = 30) were obviously higher than other nodes. Especially, IL-6 might act as a bridge to connect other targets.

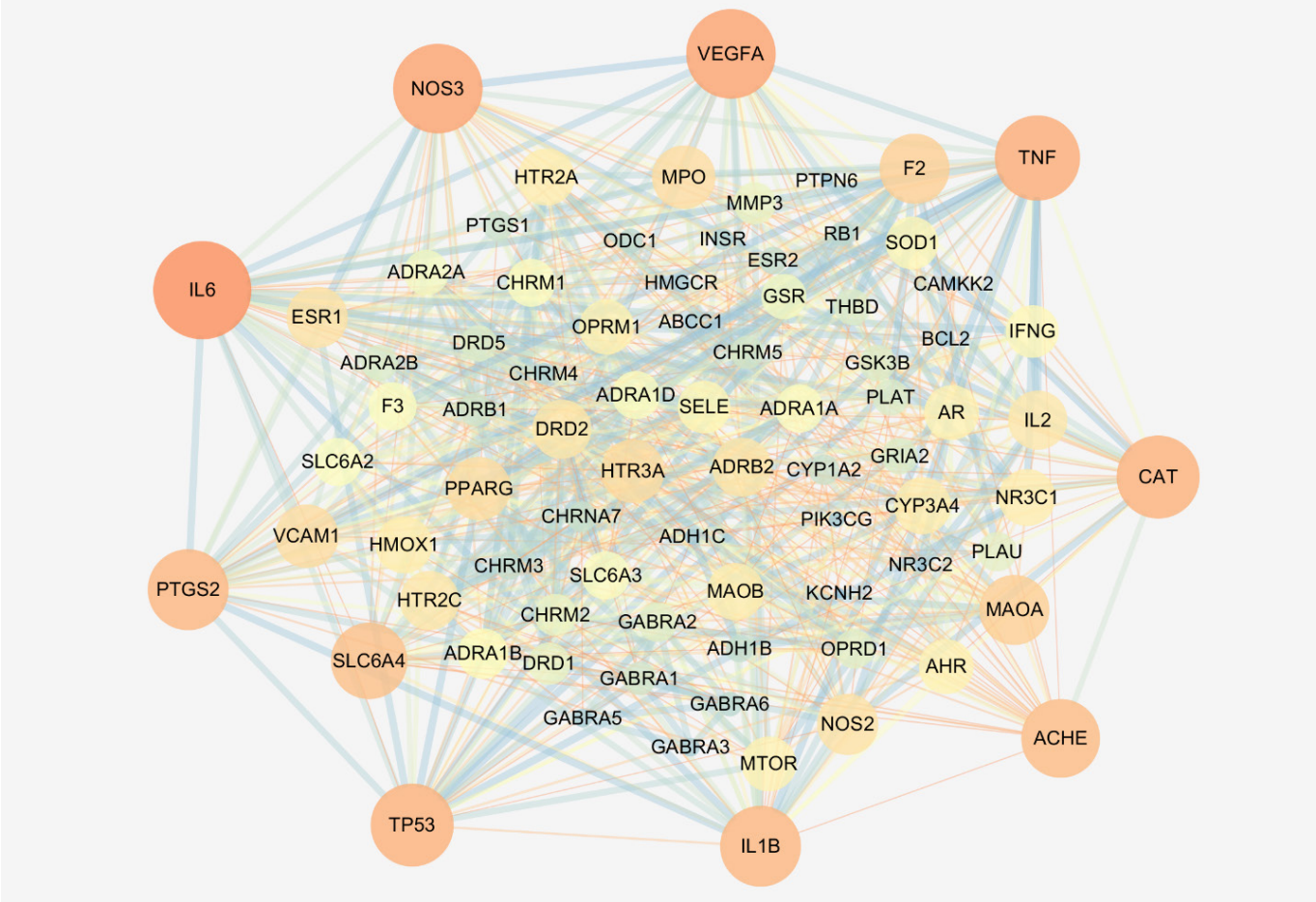


FIGURE 2 - The PPI network of SZRD. The larger the node, the deeper the color, represented the greater the degree of the node.

Gene ontology (GO) functional enrichment analysis

GO functional enrichment analysis were composed with three aspects of BP, CC and MF, and the top 5 items were selected to generate a graph in GraphPad Prism 8 software. The top 3 items of BP were response to drug, positive regulation of nitric oxide biosynthetic process, and adenylate cyclase-activating adrenergic receptor signaling pathway (Figure 3).

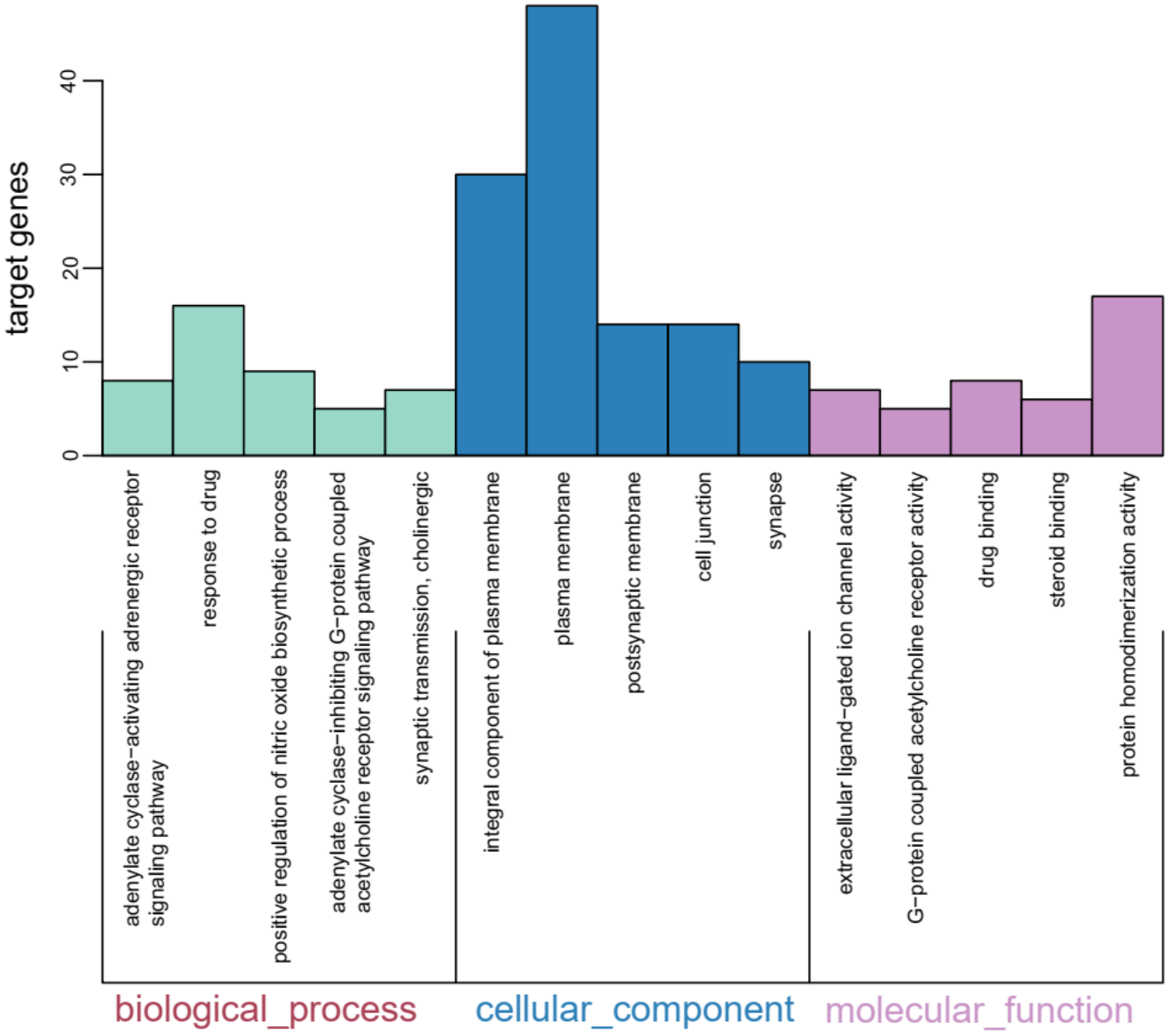


FIGURE 3 - Gene Ontology (GO) enrichment analysis of common targets of compound and insomnia. The analysis items were included with biological process (BP), cellular component (CC) and molecular function (MF).

KEGG pathway enrichment analysis

To determine the underlying mechanism of SZRD in treating insomnia, all of the pathways interacting with the candidate targets were extracted from the KEGG pathway database using DAVID. Then, the "target–pathway" network was constructed, which was composed of the top 10 related pathways, including Neuroactive ligand-receptor interaction, Calcium signaling pathway, Fluid shear stress, and atherosclerosis (Figure 4).

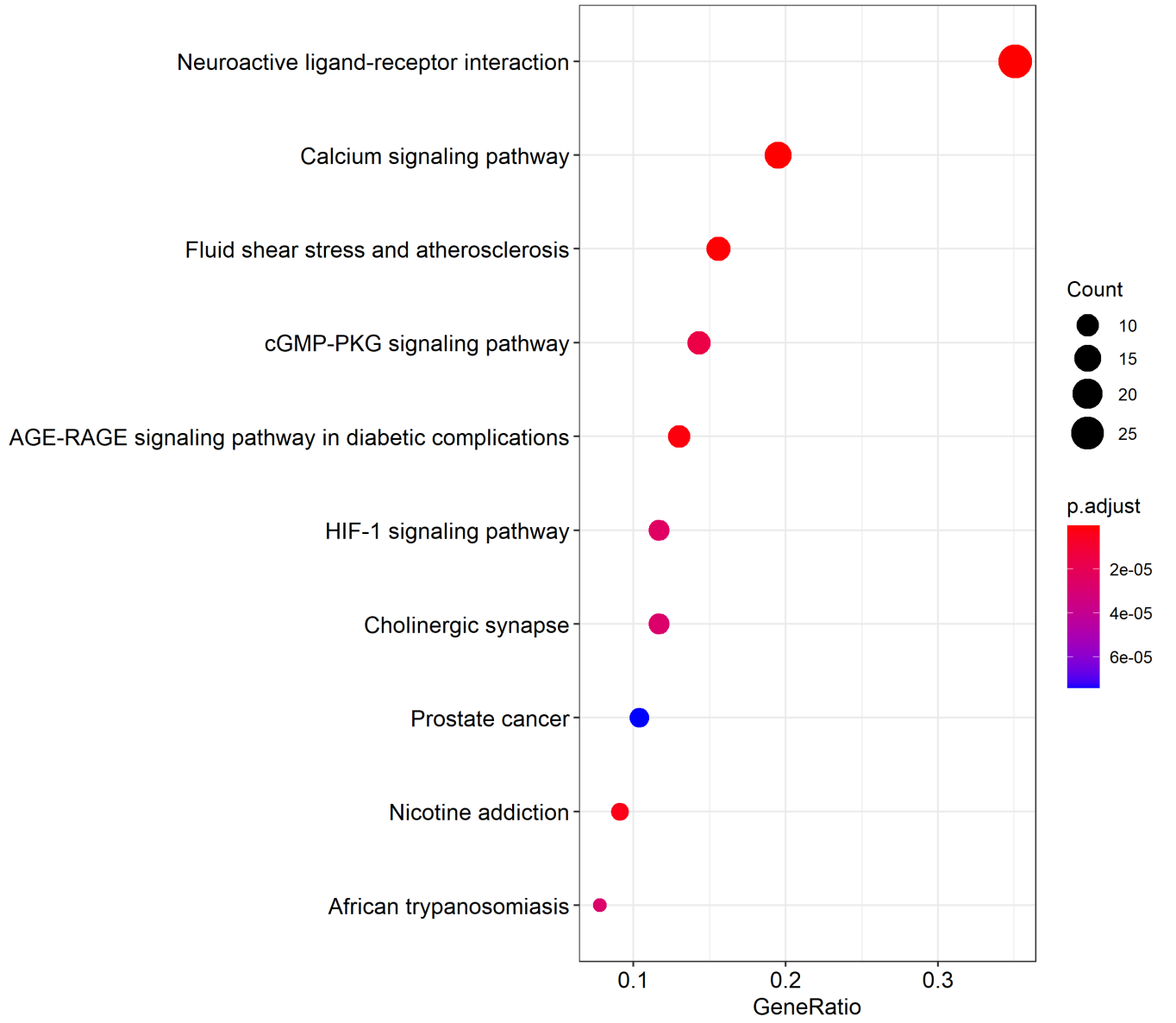


FIGURE 4 - Gene KEGG pathway enrichment. The color of circles represented the Q-value and the size of circles was represented the Count. The vertical axis represents the pathway name; the horizontal axis represents the Gene Ratio.

The effects of quercetin on insomnia mice model

The results of pentobarbital-induced sleeping showed that the sleep latency and sleep duration of model group mice were obviously increased and decreased compared with those of control group mice, respectively ($P < 0.05$, 0.01). The sleep latency and sleep duration of Que-H mice were significantly reduced and increased compared with those of model group mice, respectively ($P < 0.05$, 0.01). Although 50 mg/kg of quercetin (Que-M) had little significant effect on the sleep latency, the sleep duration was remarkably increased compared with the model group (Figure 5A and B).

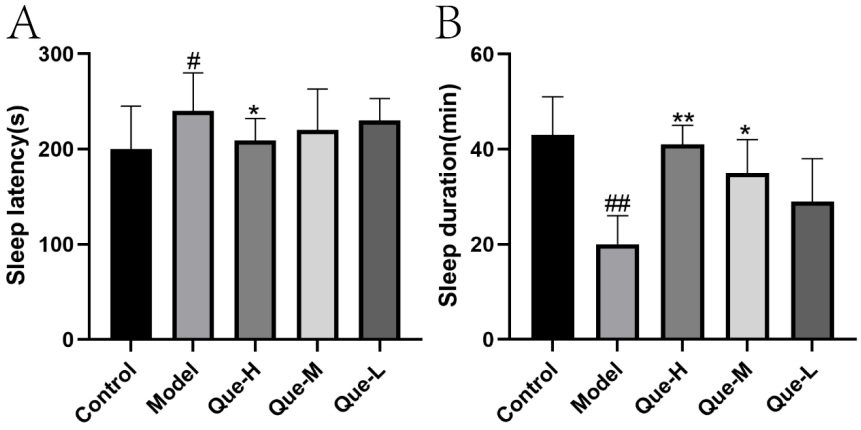


FIGURE 5 - The effects of quercetin (100, 50, 25 mg/kg) on the sleep time and sleep duration by sodium pentobarbital (n=6). The sleep latency (A) and sleep duration (B). Data are expressed as the mean ± SD of three independent experiments. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compare with the control group; $^{*}P < 0.05$ and $^{**}P < 0.01$ compare with the model group.

Quercetin inhibited the production of IL-6

According to the results of the PPI network, IL-6 might be the main target of SZRD in treating insomnia. Therefore, the production of IL-6 in the serum of mice with insomnia was determined to validate the assumption. As shown in Figure 6, PCPA treatment obviously induced the secretion of IL-6 in the model group, whereas treatment with 100, 50, and 25 mg/kg of quercetin in insomnia model mice attenuated the secretion of IL-6, indicating that quercetin could inhibit inflammatory cytokine secretion.

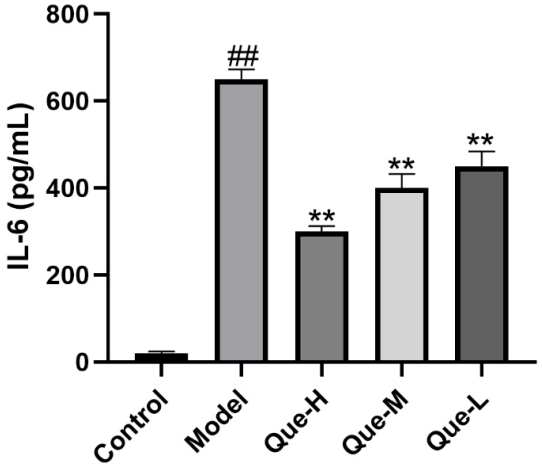


FIGURE 6 - The effects of quercetin on the production of IL-6. The concentration of IL-6 in mice serum were measured by Elisa kit. Data are expressed as the mean ± SD of three independent experiments. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compare with the control group; $^{*}P < 0.05$ and $^{**}P < 0.01$ compare with the model group.

Quercetin increased the levels of γ-GABA and GABRA1 in mice hypothalamus

The KEGG results showed that Neuroactive ligand-receptor interaction was the main pathway in SZRD treatment of insomnia, and it involved 28 genes such as GABRA1. Thus, the expression of GABRA1 mRNA and γ-GABA ligand in the hypothalamus was detected. The results showed that γ-GABA production and relative expression of GABRA1 mRNA were obviously decreased in the model group compared with the control group ($P < 0.01$, 0.05). As expected, high and middle doses of quercetin significantly improved the γ-GABA and GABRA1 mRNA levels of the insomnia model ($P < 0.01$, 0.05) (Figure 7A and B).

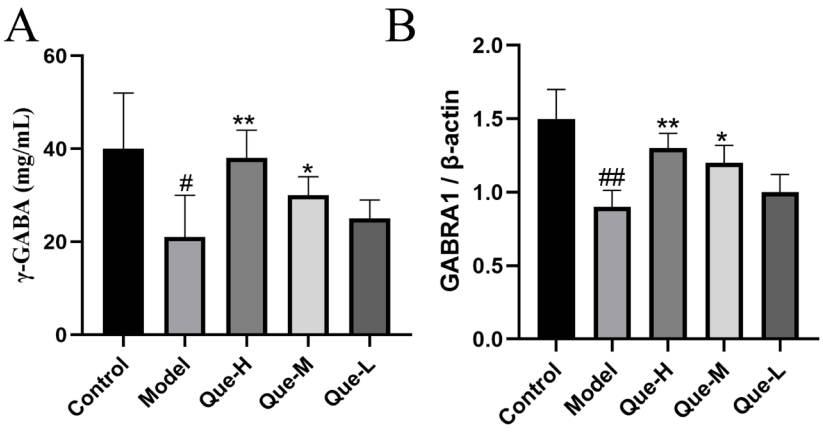


FIGURE 7 - Quercetin improved the γ-GABA production and relative expression of GABRA1 mRNA. γ-GABA production was detected by Elisa kit (A) and GABRA1 mRNA was measured by RT-PCR. Data are expressed as the mean ± SD of three independent experiments. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compare with the control group; $^{*}P < 0.05$ and $^{**}P < 0.01$ compare with the model group.

DISCUSSION

In this study, the potential mechanism of SZRD on insomnia was determined on the basis of network pharmacology, and quercetin was found to be the main active ingredient of SZRD, which might regulate IL-6 and the Neuroactive ligand-receptor interaction pathway for the treatment of insomnia. Then, an insomnia model was established to validate the hypothesis according to the results of TCM network pharmacology.

First, the results of network pharmacology indicated that a total of 152 ingredients, including quercetin and kaempferol, could be the potential compounds of SZRD in treating insomnia. Quercetin, a kind of polyphenol, widely exists in nature and is closely related to neuronal injury and neurodegenerative diseases (Costa *et al.*, 2016; Ossola, Kaariainen, Mannisto, 2009). Several studies had evaluated the antinociception, antidepression, and learning and memory of quercetin and exhibited good treatment for those neurological disorder (Bigelman *et al.*, 2011; Kanazawa *et al.*, 2016). It was found that quercetin of *Flos albiziae* significantly increased the rate of sleep onset, decreased sleeping time, and prolonged sleep latency, which were possibly mediated by the serotonergic system (Ye *et al.*, 2015). Quercetin was also reported to enhance non- rapid eyes movement (REM) sleep and reduce REM sleep, and the GABAergic pathway was found to be partly involved in the latter effect (Kambe *et al.*, 2010). Furthermore, a recent clinically relevant survey indicated that quercetin can be used as complementary treatment for patients with insomnia (Afrasiabian *et al.*, 2019). As the main compound of SZRD, quercetin was presumed to be the active substance that exerts a hypnotic effect. In this study, high dose (100 mg/kg) of quercetin significantly prolonged sleep duration and shortened sleep latency in the insomnia mouse model. Moreover, Qing Cao and Weikai (2009) first separated quercetin from SZRD and identified its structure by spectral analysis. Although the research had been reported that quercetin could be the key active ingredient of SZRD by treating with insomnia, but it also needed to demonstrated quercetin was the main compound of SZRD by chemical analysis in our future research (Zijun *et al.*, 2018). Therefore, quercetin was considered one of the important active ingredients of SZRD in the treatment with insomnia.

The PPI network showed that the degrees of IL-6, NOS3, VEGFA, TNF, CAT, PTGS2, TP53, IL-1β, and ACHE were obviously higher than those of other nodes, and IL-6 played an essential role for its close association with chemical ingredients of SZRD. Accumulating evidence suggested that sleep disturbance is associated with inflammation manifesting in circulating levels of inflammatory markers such as IL-6 (Shearer *et al.*, 2001). IL-6, a pleiotropic cytokine with multiple functions, could be an inexpensive marker to assess sleep loss in humans under various experimental settings (Halota *et al.* 1990).

An animal experiment also found increased plasma IL-6 levels in rats 8 days after chronic total sleep deprivation (Thimgan *et al.*, 2013). In the current study, we also found that the PCPA-induced insomnia mouse model had higher levels of IL-6 compared with the control group, but quercetin could decrease the production of IL-6. The results suggested that quercetin in SZRD improves sleep quality by regulating the secretion of IL-6.

To further explore the potential mechanism of SZRD on insomnia, KEGG pathway enrichment was performed, and the results showed that the Neuroactive ligand-receptor interaction was the most important pathway. Neuroactive ligand-receptor interaction mediated the initiation and progression of insomnia, and it was involved with 28 targets such as GABRA1 in our study (Xu *et al.*, 2020). GABRA1, the receptor of γ-GABA, is the neuronal inhibition in the brain and primary target for benzodiazepines, which are widely used to treat insomnia (Dixon *et al.*, 2015). γ-GABA could bind to GABRA1 causing sedation, muscle relaxion, memory impairment, and anticonvulsant effects (McKernan, Whiting 1996; Mohler, Fritschy, Rudolph, 2002). Enhancing GABRA1 activity inducing the hypnosis was the important treatment for sleep disorders (Krystal *et al.*, 2003). Suanzaoren, Fuling, and Gancao in SZRD were identified as the most frequently used sedative and hypnotic herbs, and their pharmacological actions are related to the stimulation of GABA and GABRA1 (Singh, Zhao, 2017). For example, it was demonstrated that SZRD upregulated the expression of GABRA1 to exert soporific effects (Zhan *et al.*, 2020). Quercetin has also been found to influence the sleep–wake cycle through the activation of GABA(A) receptors (Kambe *et al.*, 2010). On the basis of the results of pharmacology network, GABA and GABRA1 mRNA expressions were detected, which revealed that quercetin increased the levels of GABA and GABRA1 mRNA to improve sleep quality in the insomnia mouse model.

CONCLUSION

The pharmacology network of this study determined the potential targets and novel signaling pathways of SZRD in treating insomnia, which revealed the characteristics of TCM treatment with multi component, multiple target and multiple pathways. The present study also demonstrated that quercetin, the main compound of SZRD, could increase the expression levels of IL-6, GABA, and GABRA1 mRNA to improve sleep quality in the insomnia model. Although this study preliminarily explored the underlying mechanism of SZRD on insomnia, the components and targets of SZRD in the treatment of insomnia should be further investigated.

AVAILABILITY OF DATA AND MATERIALS

All data generated or analyzed during this study are included in this published article.

ABBREVIATIONS

SZRD: Suanzaoren Decoction
GO: Gene Ontology
KEGG: Kyoto Encyclopedia of Genes and Genomes
BP: Biological process
MF: Molecular function
CC: cell component
TCM: Traditional Chinese Medicine

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

FUNDING

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AUTHOR'S CONTRIBUTIONS

Xiaojian Weng, Jimin Jiang conceived and designed the experiments. Hailong Li and Yi Tan contributed significantly to analysis and manuscript preparation.

CONFLICT OF INTEREST

The authors declare no conflicts of interest in association with this manuscript.

ACKNOWLEDGMENTS

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