Research Paper

Network Pharmacology Based and Molecular Docking Prediction of the Active Ingredients and Mechanism of Ziziphi spinosae semen-Schisandrae chinensis fructus for Application in Insomnia Treatment

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To investigate the molecular mechanism of *Ziziphi spinosae* semen and *Schisandrae chinensis* fructus in the treatment of insomnia is the objective of the study. First, the compounds and corresponding target genes of *Ziziphi spinosae* semen and *Schisandrae chinensis* fructus were obtained by traditional Chinese medicine systems pharmacology database and analysis platform database and genes associated with insomnia were obtained by GeneCards database. The results were imported into Cytoscape software to construct a network (Herbal-active compound- Target-disease related genes network). Then, gene ontology and Kyoto encyclopedia of genes and genomes enrichment analyses were performed for common genes in disease and compounds (Target-disease related genes). Finally, the key genes were identified by Cytoscape software and the molecular docking of key genes was verified by Autodock software. A total of 21 components and 43 target genes were screened and 41 target-disease related genes were identified. 191 gene ontology functional terms and 26 Kyoto encyclopedia of genes and genomes pathways show that neuroactive ligand-receptor interactions including cholinergic, norepinephrine and dopaminergic signals play an important role in the treatment of insomnia. Solute carrier family 6 member 3, cholinergic receptor nicotinic alpha 7 subunit, nuclear receptor subfamily 3 group C member 1, cholinergic receptor nicotinic alpha 2 subunit, estrogen receptor 1, cholinergic receptor muscarinic 1, acetylcholinesterase and progesterone receptor were identified as key genes, and their molecular docking results showed that all compounds could stably bind to the active pockets. Through these techniques, the multi-component synergistic mechanism of *Ziziphi spinosae* semen and *Schisandrae chinensis* fructus in the treatment of insomnia can be elucidated.

Key words: *Ziziphi spinosae* semen, *Schisandrae chinensis* fructus, molecular docking, network pharmacology, insomnia

Insomnia has become a common phenomenon, accounting for up to 10 % of the total population, insomnias often have frequent difficulty in falling asleep and maintaining sleep even when they have sufficient sleep time[1,2]. Insomnia has a huge impact on patient's quality of life and social economy. In a survey, they used Quality Adjusted Life Years (QALYs) as a measure of health status burden and the results showed that the loss of QALYs associated with insomnia in adults was significantly greater than that of other diseases (including arthritis, hypertension, etc.,). This suggests that insomnia is a major source of QALYs losses[3]. Insomnia is also a major risk factor for increased cardiovascular disease[4]. A meta-analysis of 11 prospective cohort studies involving 58 924 subjects with at least 1 y of interviews found that single or combined symptoms of insomnia were significantly associated with increased incidence of hypertension[5]. In addition, it is not difficult to find evidence that insomnia increases the risk of coronary heart disease and heart failure in several large prospective cohort studies[6,7]. Insomnia puts a lot of strain on social production and the economy, and research shows that insomnia is associated with the productivity loss index

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at work and insomnia is the powerful predictor of productivity[8]. The direct and indirect cost of treating insomnia alone is estimated to be as high as 100 billion United States (US) dollars per year[9], more surprisingly, the 10 billion US dollars economic loss due to insomnia was found in 2019-2020 which is equivalent to 0.73 % of Australia's Gross Domestic Product (GDP)[10].

Insomnia often has a persistent negative psychosocial trigger, of which depression is an important factor[1,11,12]. Insomnia and depression often come together[11]. A meta-analysis of 172 077 participants in 34 prospective cohort studies shows that insomnia increases with the increase of depression risk[13]. In addition, insomnia is also a non-sexual symptom of brain degenerative diseases (including Alzheimer's disease, Parkinson's disease, and *so on*) in the elderly, which is likely to be associated with the damage of the brain area of the disease itself or sleep[14].

Although the current Cognitive Behavioral Therapy for Insomnia (CBT-I) is considered the most promising treatment for insomnia, there is also a problem of resource allocation, compliance, high recurrence rate and high cost[1]. A combination of CBT-I and medication is advocated, but the long-term use of prescription drugs often leads to serious adverse consequences such as dependency, mental nervous system and other accidents[1,15]. However, in China, the history of using traditional Chinese medicine to treat insomnia according to the actual situation has exceeded in 2000 y[16]. There is no doubt that this has a clear and unique advantage in treating insomnia and its research potential is huge. A recent meta-analysis on the treatment of primary insomnia showed that Shenqi Wuweizi tablets, Zao Ren An Shen capsules, Suanzaoren decoction, Bailemian capsules and other composite herb combinations had a significant advantage in different aspects of the effectiveness and safety in terms of sedative hypnosis[17]. Sufficient evidence can also be found in some *in vivo* studies, such as Suanzaoren decoction combined with Lorazepam can significantly improve the symptoms of chronic insomnia patients, its effect is better than conventional treatment[18]. Zao Ren An Shen capsules can significantly improve Pittsburgh Sleep Quality Index (PSQI) and hemorheology indexes of elderly insomnia patients[19]. Noteworthy, all of these classic composite herb combinations have one thing in common that they all use *Ziziphi spinosae* semen (ZSS) and *Schisandrae chinensis* fructus (SCF) as common compatibility, which is definitely no accident. Due to their mechanism of action in the body is hard to explain[20], we choose to use the network pharmacology method to explain their compounds and the relationship of the target gene in order to try to more complete and systematic understanding of ZSS and SCF in insomnia[21], and verify their relationship by simulated molecular docking.

MATERIALS AND METHODS

Data collection and preparation:

Screening of ZSS-SCF drug compounds: Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP) is a computer program for the study of Chinese herbal medicine and pharmacology of traditional Chinese medicine system database and analysis platform[22]. Only need to upload the name of ZSS and SCF, the ideal compound and its corresponding Oral Bioavailability (OB) and the Drug-Likeness (DL) will be easy to get. OB represents the compounds through the blood vessels into the cycle of drug ratio[23] and DL indicates the similarity of compounds to known drugs. OB and DL are important tools for pharmacokinetics[24]. Therefore, the retrieved herbal information is imported into Excel and qualified active compounds were obtained according to OB≥30 % and DL≥0.18 as screening conditions[25].

Acquisition of potential target genes: Similarly, all compounds and target genes can be found in TCMSP. The UniProt database contains extensive protein sequence resources and associated detailed annotations[26]. So only by entering the target gene names obtained in TCMSP into the UniProt database (http://www.uniprot.org/), we can find the target gene of the human species.

Acquisition of disease-related genes and Target-Disease Related Genes (T-DRGs): Genecards (https://www.genecards.org/) combines more than 150 web resources, that is a powerful human gene bank for genetic function analysis[27]. We use insomnia related search words which including "Disorders of initiating and maintaining Sleep", "Early awakening", "Nonorganic insomnia", "Primary insomnia", "Transient insomnia", "Secondary insomnia", "Sleep initiation dysfunction", "Sleeplessness", "Insomnia", "Psychophysiological insomnia" and "DIMS" to search for insomnia related genes in Genecards. In addition, also add search for genetic databases such as PharmGKB (https://www.pharmgkb.org/), OMIM (https://omim.org/), Drugbank (https://db.idrblab.org/).

All target genes of identified compounds and insomnia are put into Bioinformatics and evolutionary genomics system (http://bioinformatics.psb.ugent.be/webtools/Venn/), and a Venn graph is created. The cross part of the Venn graph indicates the common genes in disease and compounds (T-DRGs). T-DRGs are thought to be an effective target for the treatment of insomnia.

Pharmacological network and enrichment analysis:

Establishment of pharmacological network: The integration of T-DRGs, active compounds and Chinese herbs were imported into Cytoscape Version 3.8.2 to construct a complex target network, which visualized the interaction law between active compounds and targets.

Enrichment analysis: Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis is mainly used to explain the connection between basic genes and core functions or pathways, which helps us to understand the key role of genes[28]. Database for Annotation, Visualization and Integrated Discovery (DAVID) (https://david.ncifcrf.gov/summary.jsp) database provides the function that widely collected data translated into the biological significance and contributes to the analysis of genomic data sets[29]. The use of DAVID for GO and KEGG analysis is of great significance to further explore the mechanism of ZSS-SCF in treating insomnia.

Protein-Protein Interaction (PPI) network construction and key gene screening:

PPI network construction: The T-DRGs are entered into the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database to generate a PPI network that helps to analyze protein interaction relationships.

Screening of Key Genes (KGs): The results were imported into Cytoscape (Version 3.8.2) and the interaction network parameters of each gene encoded protein were calculated by the algorithm of the CytoNCA plugin. As a centrality tool for biological network computation and analysis, CytoNCA plug-in can provide 8 centrality measures, including Betweenness Centrality (BC), Closeness Centrality (CC), Degree Centrality (DC), Eigenvector Centrality (EC), Local Average Connectivity-based method (LAC), Network Centrality (NC) scores[30]. The genes were screened according to the median value of the parameters of interest and the results were constructed into a sub-network. CytoHubba plug-in is another network node ordering system based on network characteristics[31]. Another sub-network is built by using cytoHubba plug-in. The gene shared by the two subnetworks is the KG, which is considered to be the key effective target of ZSS-SCF in the treatment of insomnia.

Molecular docking:

Molecular docking techniques were used to verify details of interactions between compounds and target genes[21]. We respectively collected data from Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB) database (https://www.rcsb.org/) to obtain protein two-dimensional structure and from PubChem database (https://pubchem.ncbi.nlm.nih.gov/) to obtain the corresponding compound structure. Since the more stable the docking and the less the binding energy[32], the small molecule of the compound was imported into ChemBio Three-Dimensional (3D) software to calculate the 3D structure of the minimum energy quantization. AutoDock (1.5.6) software was used to dehydrate and hydrogenate the receptor and the location of the center was calculated and an active binding pocket was formed. Ligand-receptor docking was performed by computer and finally PyMOL (2.5) software was used to visualize the docking relationship.

RESULTS AND DISCUSSION

Results of compound screening and target gene prediction were shown below. After collation, it was found that a total of 21 active compounds (13 in ZSS and 8 in SCF) and 112 target genes were collected and relevant information was listed in the table (Table 1). The target genes and 5820 insomnia related genes from GeneCards database were made into Venn diagram (fig. 1a), and then 41 T-DRGs were found. Herbal-active Compound-T-DRGs network (H-C-T network, fig. 1b) shows the complex relationship between the active compound, T-DRG and herbal medicine, which is made up of 62 nodes (21 active compounds and 41 T-DRGs) and 98 edges. We found a compound that matched multiple target genes, and a target gene could also be associated with more than one compound, and the other, dl-nuciferine and (S)-coclaurine were the two most effective compounds.

GO analysis is a system that is widely used to analyze the classification of genetic functions such as Biological Processes (BP), Cell Components (CC) and Molecular Functions (MF)[33]. 191 GO terms were identified from

TABLE 1: THE ACTIVE COMPOUNDS (OB≥30 % AND DL≥0.18) AND CORRESPONDING TARGET GENES WERE SCREENED

Herb	Ingredient number	Ingredient name	OB %	DL	Target gene
ZSS	MOL001522	(S)-Coclaurine	42.35	0.24	CHRM1, SCN5A, PTGS2, RXRA, PDE3A, ADRA1A, ADRA1B, SLC6A3, ADRB2, SLC6A4, HSP90AA1, MAOB, PCP4, DRD1, CHRM3, ADRA2A, CA2, sADRA2C, ADRA1D, OPRM1, PRKACA, NCOA2
ZSS	MOL001525	Daucosterol	36.91	0.75	PGR, NCOA2
ZSS	MOL001531	dl-Nuciferine	29.26	0.4	PTGS1, DRD1, CHRM3, CHRM1, AR, SCN5A, CHRM5, PTGS2, ADRA2A, ADRA2C, CHRM4, RXRA, OPRD1, AChE, ADRA1A, CHRM2, ADRA2B, ADRA1B, SLC6A3, ADRB2, ADRA1D, CHRNA2, SLC6A4, DRD2, OPRM1, CHRNA7
ZSS	MOL001532	Phytosterol	36.91	0.75	PGR
ZSS	MOL001539	Sanjoinenine	67.28	0.79	F10, PTGS2, HSP90AA1
ZSS	MOL001542	Swertisin	31.83	0.75	AR, TOP2A
ZSS	MOL001546	Zizyphusine	41.53	0.55	PTGS1, CHRM3, CHRM1, AR, SCN5A, PTGS2, RXRA, AChE, TOP2A, HSP90AA1, NCOA1, PCP4
ZSS	MOL000211	Mairin	55.38	0.78	PGR
SCF	MOL009199	Interiotherin B	31.76	0.77	TOP2A
SCF	MOL009213	Kadsulignan B	30.63	0.84	ESR1, AR
SCF	MOL009219	Neokadsuranic acid C	35.4	0.85	NR3C1
SCF	MOL009220	Neokadsuranin	33.35	0.88	AR, PTGS2
SCF	MOL009235	Angusifolin B	34.82	0.56	TOP2A
SCF	MOL004624	Longikaurin A	47.72	0.53	CHRM1, CHRM2, PRSS1
SCF	MOL005317	Deoxyharringtonine	39.27	0.81	AR, NR3C2
SCF	MOL008956	Angeloylgomisin O	31.97	0.85	AR, F10, TOP2A
SCF	MOL008957	Schizandrer B	30.7	0.83	PTGS2, TOP2A
SCF	MOL008968	Gomisin-A	30.69	0.78	F2, AChE
SCF	MOL008974	Gomisin G	32.68	0.83	TOP2A
SCF	MOL008978	Gomisin R	34.84	0.86	ESR1, AR, F10, PTGS2, TOP2A PRSS1, NCOA2
SCF	MOL008992	Wuweizisu C	46.27	0.84	AChE, DPP4

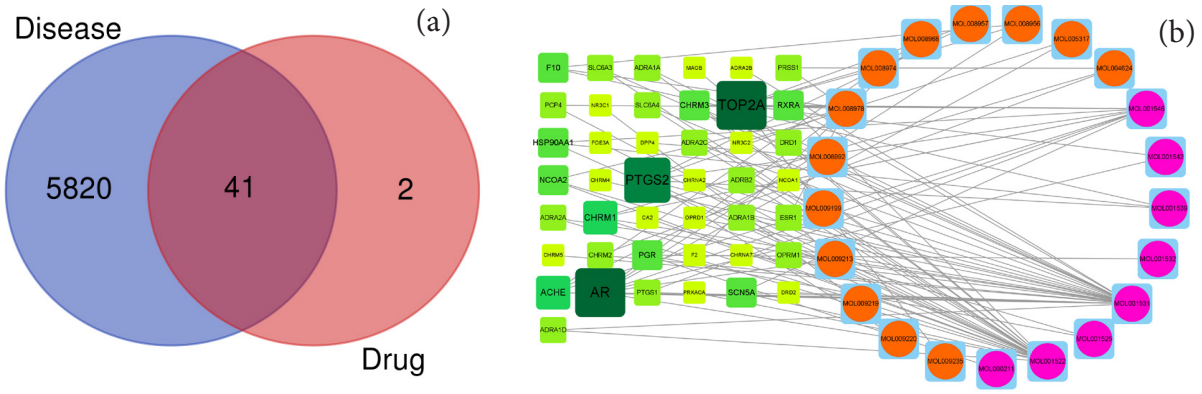

Fig. 1: (a) Venn diagram indicates common targets of ZSS-SCF (drug) therapy and insomnia (disease); (b) H-C-T network of ZSS-SCF: Orange and pink circles indicate ingredients of ZSS and SCF respectively. The darker the color and the larger the size, the larger the target gene degree value represented by the square

the DAVID database and bubble diagrams (fig. 2a-fig. 2c) for the first 10 terms of BP, CC and MF were made respectively by R language and Perl plugins. In these GO terms, adenylate cyclase activation of adrenergic receptors and cholinergic synaptic transmission are the most important processes in 125 BP. 24 CC shows that these active compounds are concentrated in the function of cell membrane, cytoplasmic membrane, nerve axon and postsynaptic membrane. In 42 MF, there are some neural receptors, including G-protein coupling acetylcholine receptor, alpha (α_1/α_2-adrenaline energy receptor, steroid hormone receptor, etc. The GO string diagram (fig. 2d) shows the two most significant terms in the BP, CC and MF respectively and the T-DRGs that participate in these functions. There are about 30 T-DRGs positioned in the cell membrane.

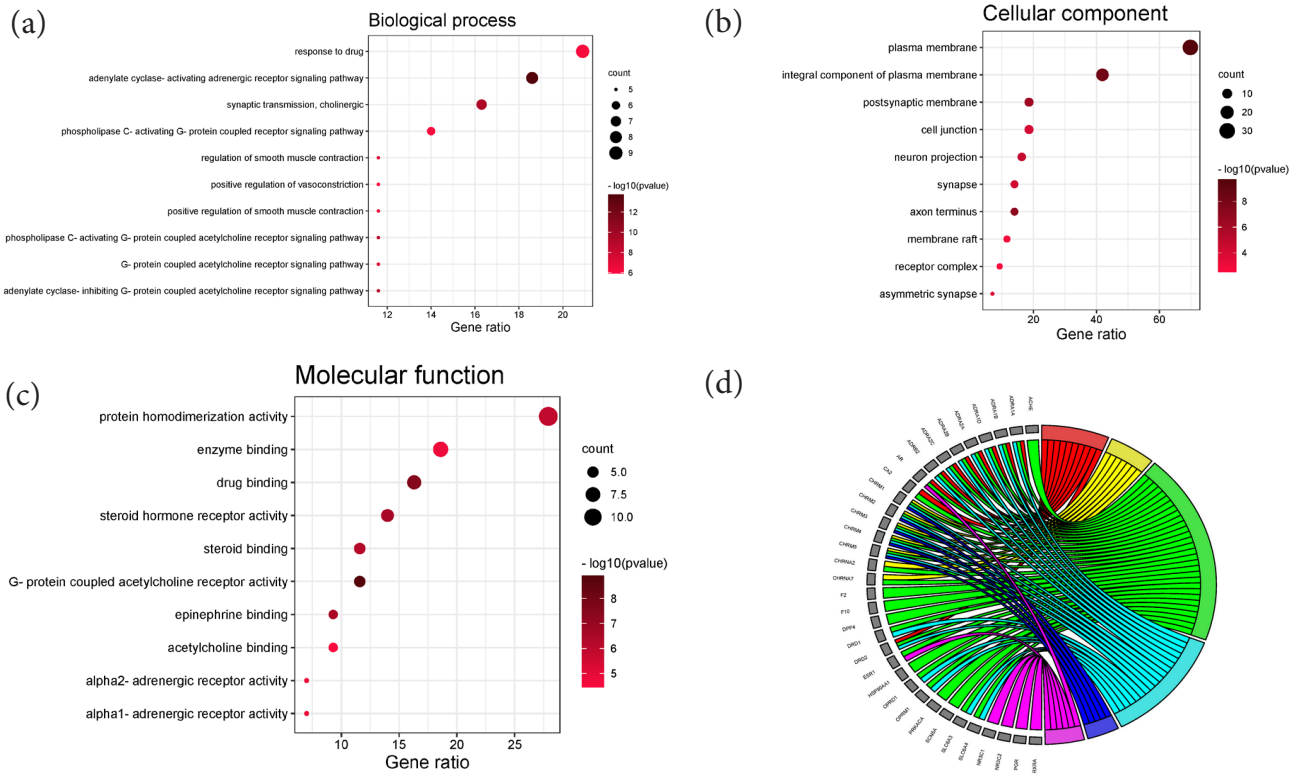

Fig. 2: GO enrichment analysis of T-DRG, (a) The bubble diagram shows biological process; (b) The bubble diagram shows cellular components; (c) The bubble diagram shows molecular function; (d) The GO enrichment analysis string diagram shows the six colors on the right represent six functions and the 34 T-DRGs on the left. The number of lines in a function is proportional to gene enrichment; GO: 0071880-adenylate cyclase-activating adrenergic receptor signaling pathway; GO: 0007271-synaptic transmission, cholinergic; GO: 0005886-plasma membrane; GO: 0005887-integral component of plasma membrane; GO: 0016907-G-protein coupled acetylcholine receptor activity; GO: 0003707-steroid hormone receptor activity, GO Terms (■) GO 0071880; (□) GO 0007271; (■) GO 0005886, (□) GO 0005887, (■) GO 00016907, (■) GO 0003707

The signal pathway of 26 significant enrichment ($p<0.01$) was discovered through the KEGG analysis. It is noteworthy that the neural ligand-receptor interaction signaling pathway is the most enriched and the enrichment of T-DRGs is the largest. Calcium signal, cyclic Guanosine Monophosphate-Protein Kinase G (cGMP-PKG), cyclic Adenosine monophosphate (cAMP) and some synapses including cholinergic, serotonergic and dopaminergic synapses are also significantly enriched. The enrichment of the top 20 paths with small p values has been shown in the bubble graph (fig. 3a) and their names and corresponding term ID are listed in the table (Table 2). The corresponding relationship between significantly enriched signal pathways and T-DRGs are shown in the signal pathway-T-DRGs network (P-T-D network) (fig. 3b), which consists of 63 nodes (26 signaling pathways and 37 genes) and 137 edges. Without doubt, the neural ligand-receptor interaction signaling pathway (fig. 3c) is the object of our most attention and most of these T-DRGs are concentrated on receptors such as choline, adrenaline, Dopamine (DA), opioids and steroid hormones.

The PPI network exported from the STRING database is shown (fig. 4a). A new protein interaction network (fig. 4b) was constructed by Cytoscape software that consists of 38 nodes and 128 edges. Then, parameters (including BC, CC, DC, EC, LAC and NC) of each gene were calculated by CytoNca plug-in. The 9 T-DRGs larger than the median of the six parameters form a subnetwork (fig. 4c). All genes related parameters of the subnetwork are shown in the table (Table 3). In addition, another subnetwork consisting of 10 T-DRG was obtained by cytoHubba plug-in (fig. 4d). Finally, 8 KGs are generated, which included Solute Carrier Family 6 Member 3 (SLC6A3), Cholinergic Receptor Nicotinic Alpha 7 Subunit (CHRNA7), Nuclear Receptor Subfamily 3 Group C Member 1 (NR3C1), Cholinergic Receptor Nicotinic Alpha 2 Subunit (CHRNA2), Estrogen Receptor 1 (ESR1), Cholinergic Receptor Muscarinic 1 (CHRM1), Acetylcholinesterase (AChE) and Progesterone Receptor (PGR) according to sorting by CytoNca plug-in.

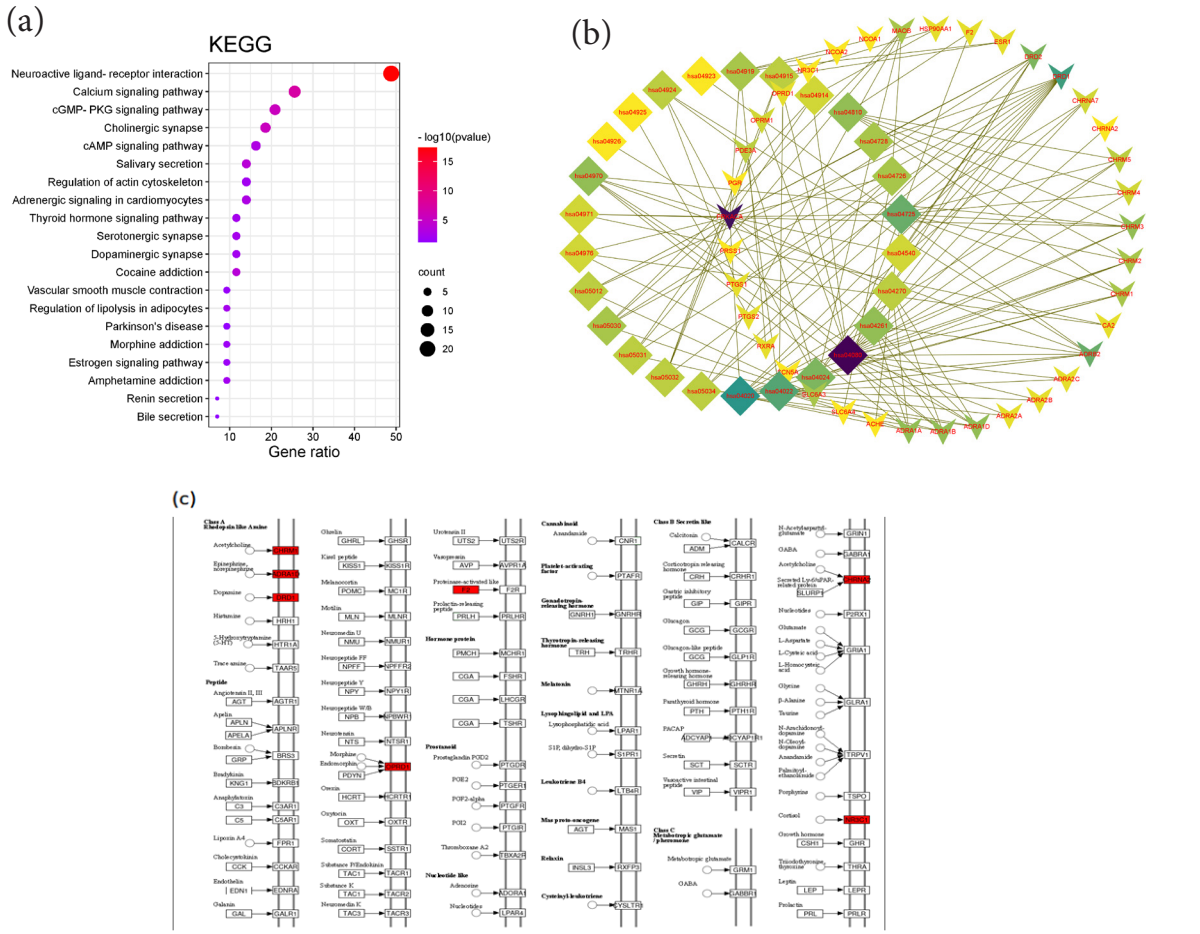

Fig. 3: KEGG enrichment analysis of T-DRG, (a) The bubble diagram shows the top 20 pathways of KEGG enrichment analysis; (b) P-T-D network of KEGG enrichment analysis: Diamonds represent 26 pathways and s-resizes represent 37 T-DRG; (c) Potential targets and mechanisms of ZSS-SCF pair in neural ligand-receptor interaction signaling pathway

TABLE 2: NAMES AND TERM ID OF THE FIRST 20 KEGG ENRICHED PATHWAYS

Term	Path name
hsa04080	Neuroactive ligand-receptor interaction
hsa04020	Calcium signaling pathway
hsa04022	cGMP-PKG signaling pathway
hsa04725	Cholinergic synapse
hsa04970	Salivary secretion
hsa05030	Cocaine addiction
hsa04024	cAMP signaling pathway
hsa04261	Adrenergic signaling in cardiomyocytes
hsa04726	Serotonergic synapse
hsa04923	Regulation of lipolysis in adipocytes
hsa04919	Thyroid hormone signaling pathway
hsa04728	Dopaminergic synapse
hsa05031	Amphetamine addiction
hsa04810	Regulation of actin cytoskeleton
hsa05032	Morphine addiction
hsa04915	Estrogen signaling pathway
hsa04270	Vascular smooth muscle contraction
hsa05012	Parkinson's disease
hsa04924	Renin secretion
hsa04976	Bile secretion
hsa04971	Gastric acid secretion
hsa05034	Alcoholism
hsa04914	Progesterone-mediated oocyte maturation
hsa04540	Gap junction

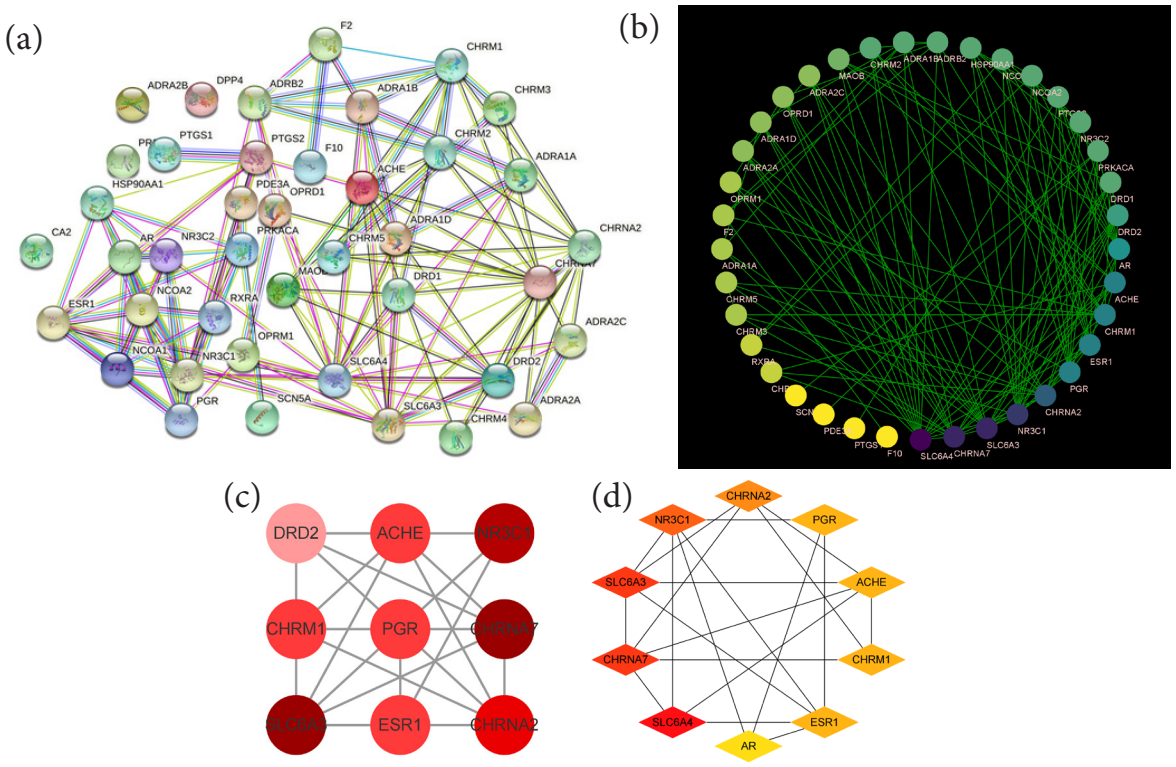

Fig. 4: Screening process for KG, (a) PPI network from the STRING database; (b) PPI network organized by Cytoscape software; (c) The subnetwork is screened by the algorithm stipulated by the CytoNCA plug-in and consists of 9 target genes; (d) A subnetwork of target genes with top 10 degree of value and the algorithm of degree value is specified by cytoHubba plug-in

TABLE 3: PARAMETERS OF 9 TARGET GENES SCREENED BY THE ALGORITHM OF CYTONCA PLUG-IN

Name	BC	CC	DC	EC	LAC	NC
AChE	94.58208568	0.507042254	10	0.24166	4	6.01429
SLC6A3	192.1066686	0.590163934	15	0.30489	3.333333333	8.03343
DRD2	35.54484118	0.514285714	8	0.22775	4.5	5.49048
CHRNA7	114.5898475	0.514285714	15	0.30823	4.933333333	11.8016
CHRM1	71.92516699	0.467532468	10	0.18999	3.4	6.16667
CHRNA2	57.90267713	0.486486486	12	0.24888	4.5	9.11688
NR3C1	304.4970998	0.580645161	14	0.24834	4.857142857	10.1361
PGR	36.65020886	0.444444444	10	0.15285	5.2	7.5
ESR1	109.0580512	0.52173913	10	0.19274	4.6	6.375

The 8 KGs and the corresponding active compounds were simulated to the molecular docking respectively and the important parameters information of these receptors-ligand docking is listed in the table (Table 4), including the ligand and receptor names, receptor protein PDB ID, docking energy, central coordinate and grid size. The best docking mode is determined by the AutoDock software. All of the docking energy is less than -5 kcal/mol, which means that the docking is good[34].

The four ligand-receptor docking models with the lowest binding energy are shown in fig. 5. The first three KGs were selected according to the sequence of parameters calculated by CytoNCA plug-in and their simulated molecular docking models were shown in fig. 6. The ligands of these models are fully wrapped in active pockets and at least one hydrogen bonding ligand configuration.

TABLE 4: ALL KGS OF MOLECULAR DOCKING MODEL RELATED INFORMATION

Targets	PDB-ID	Core active ingredient	Binding energy/ (kcal/mol)	Grid center (xyz)	Grid size (xyz)
SLC6A3	AF-Q01959-F1	dl-Nuciferine	-6.9	-14.239, -23.595, 3.181	106, 110, 72
SLC6A3	AF-Q01959-F1	(S)-Coclaurine	-6.5	-14.239, -23.595, 3.181	106, 110, 72
CHRNA7	5AFN	dl-Nuciferine	-8.5	19.267, 5.881, 28.251	88, 80, 80
NR3C1	AF-P04150-F1	Neokadsuranic acid C	-7.8	-8.21, 0.852, -6.411	126, 122, 126
CHRNA2	AF-Q15822-F1	dl-Nuciferine	-6.2	-25.742, -9.133, 5.379	126, 86, 126
ESR1	1xpc	Kadsulignan B	-5.9	23.553, 4.423, 20.424	46, 56, 54
ESR1	1xpc	Gomisin R	-6.7	23.553¤4.423 ¤20.424	46, 56, 54
CHRM1	6WJC	(S)-Coclaurine	-7.2	-15.171, -18.987, 59.241	60, 74, 90
CHRM1	6WJC	dl-Nuciferine	-8	-15.171, -18.987, 59.241	60, 74, 90
CHRM1	6WJC	Zizyphusin	-8.3	-15.171, -18.987, 59.241	60, 74, 90
CHRM1	6WJC	Longikaurin A	-9.1	-15.171, -18.987, 59.241	60, 74, 90
AChE	6NTO	dl-Nuciferine	-8.7	-33.918, -21.387, 56.538	64, 72, 86
AChE	6NTO	Zizyphusine	-9	-33.918, -21.387, 56.538	64, 72, 86

AChE	6NTO	Gomisin-A	-6.7	-33.918, -21.387, 56.538	64, 72, 86
AChE	6NTO	Wuweizisu C	-7.8	-33.918, -21.387, 56.538	64, 72, 86
PGR	AF-P06401-F1	Daucosterol	-6.6	-4.961, 4.815, 0.984	126, 126, 126
PGR	AF-P06401-F1	Phytosterol	-6.3	-4.961, 4.815, 0.984	126, 126, 126
PGR	AF-P06401-F1	Mairin	-7.6	-4.961, 4.815, 0.984	126, 126,126

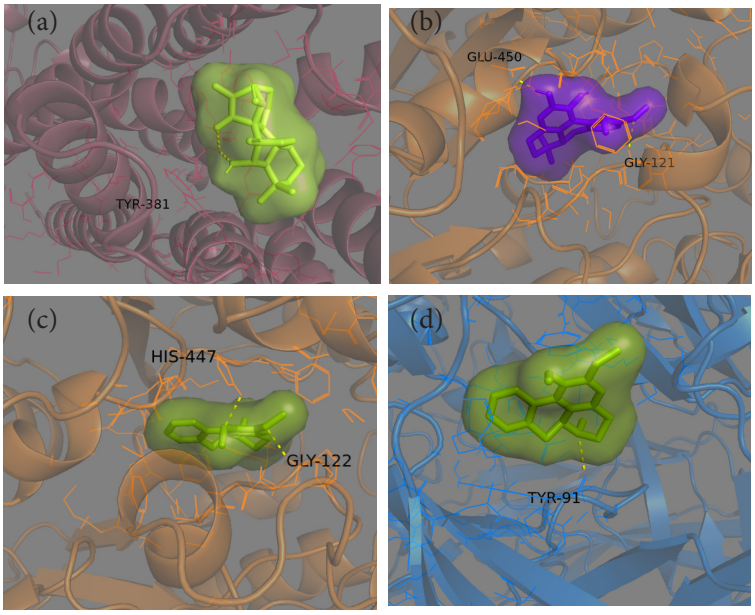

Fig. 5: Four ligand-receptor docking models with the lowest binding energy, (a) CHRM1 and longikaurin A (configuration: 1/20); (b) AChE and zizyphusine (configuration: 1/20); (c) AChE and dl-Nuciferine (configuration: 1/20); (d) CHRNA7 and dl-Nuciferine (configuration: 1/20)

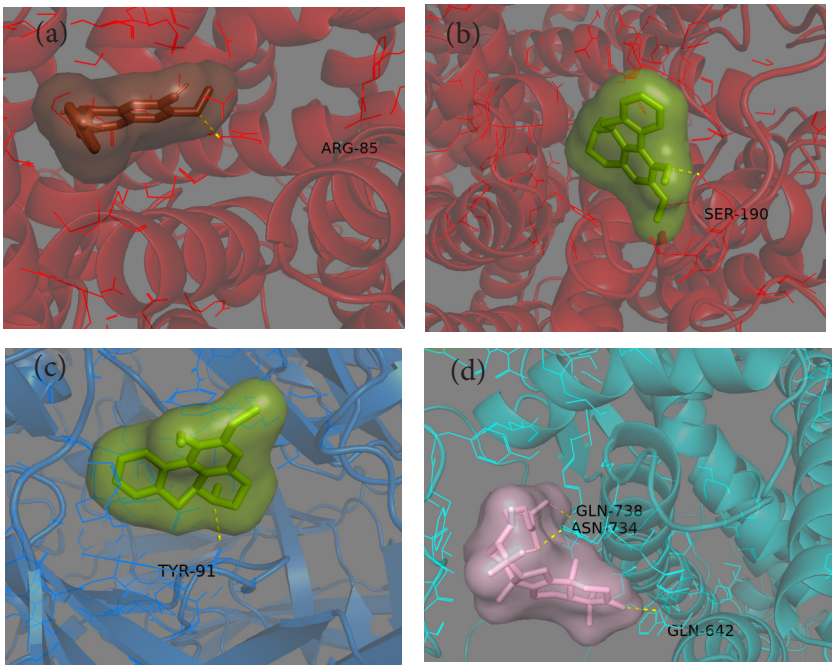

Fig. 6: Four ligand-receptor docking models, whose receptors are encoded by the top three KGs screened by the algorithm of the CytoNCA plug-in respectively, (a) SLC6A3 and (S) Coclaurine (configuration: 3/20); (b) SLC6A3 and dl-Nuciferine (configuration: 2/20); (c) CHRNA7 and dl-Nuciferine (configuration: 1/20); (d) NR3C1 and neokadsuranic acid (configuration: 3/20)

In this study, two herbs were identified with 21 compounds and 112 potential target genes. Then, 41 T-DRGs were predicted and they were rich in 191 GO terms and 26 KEGG pathways. Finally, eight KGs were predicted, these KGs are found to be closely and stably bond to the compound by simulating molecular docking.

The results show that ZSS can improve insomnia by regulation of Serotonin or 5-Hydroxytryptamine (5-HT), glutamate, gamma-Aminobutyric Acid (GABA), Norepinephrine (NE), DA and other monoamine and amino acid neurotransmitters[35]. SCF has the potential to alter the behaviour of the 5-serotonergic and aminobutyric systems and have sedative-hypnotic activity[36]. Dl-Nuciferine and (S)-Coclaurine were found to be the most active compounds by H-C-T network analysis. Studies have shown that smoking lotus leaves rich in dl-Nuciferine can bring feelings of relaxation[37]. The potential of dl-Nuciferine in the treatment of insomnia is enormous and it is a potent inhibitor of AChE and is also excitatory to 5-HT_{2C} receptor. Dl-Nuciferine selectively binds tightly to several key amino acid residues of rat 5-HT_{2A} receptor *via* the H bond[38,39]. The results of simulated molecular docking showed that dl-Nuciferine could bind closely to five KGs encoded proteins, including SLC6A3, CHRNA7, CHRNA2, CHRM1 and AChE (the binding energy <-6 and hydrogen bonding). These results indicate that dl-Nuciferine may play a role in regulating DA system and Acetylcholine (ACH)-associated receptors, which is consistent with previous studies. Coclaurine interacts with melatonin to produce sedative effects[40]. (S)-Coclaurine, as one of the configurations, has antagonistic activity against D1 and D2 DA receptors and is a potential target of psychiatric diseases[41]. In this study, Gomisin A and Wuweizisu C are active components of SCF, and act on ACH-associated receptors, which is what we focus on. Similar to dl-Nuciferine, Gomisin A significantly inhibits AChE activity and modulates ACH levels[42]. It also ameliorates nerve damage by reducing striatum toxicity induced by 3-Nitropropionic Acid (3-NPA) in Huntington's Disease (HD) mouse models due to its anti-inflammatory and antioxidant activity[43]. Wuweizisu C has potential therapeutic effects on a variety of neuropsychiatric diseases through the regulation of NE, DA and 5-HT[44]. It also inhibits oxidative stress, modulates apoptotic signaling and cytotoxicity, and is effective in neurodegenerative diseases[45].

By combining KGs, KEGG and GO analyses, ACH-associated receptors are widely acted by multiple compounds of the two herbs and cholinergic synaptic transmission is the main process in which T-DRGs is involved, suggesting that the cholinergic system may be the most important pathway in the treatment of insomnia by ZSS and SCF. Using a recording method of channelrhodopsin-2-labeled neurons, they found that cholinergic neurons, along with glutamate and albumin-positive GABAergic neurons form circuits in the basal forebrain and participate in Sleep/Wake (S/W) regulation[46]. Cholinergic neurons are significantly active during Rapid Eye Movement (REM) sleep, but activated cholinergic neurons rapidly induce wakefulness[46]. Among the 8 KGs we predicted, ACH-associated receptors include CHRNA7, CHRNA2, CHRM1 and AChE. The simulated molecular docking showed that CHRM1 and AChE could stably and closely bind to a variety of compounds (binding energy <-6, with a large number of hydrogen-bonded configurations), suggesting that CHRM1 and AChE may be the most important targets of ZSS-SCF. Long-term use of AChE inhibitors significantly increases the risk of depression and alters sleep structure through the Hypothalamic-Pituitary-Adrenal-axis (HPA axis), such as increasing REM tension activity and decreasing REM duration[47]. CHRM1 is a key molecule in the regulation of REM sleep and the maintenance time of REM and Non-Rapid Eye Movement sleep (NREM) was moderately or more shortened in mice with this gene knocked out[48]. Nicotinic acetylcholine receptor (CHRNA) is a potential therapeutic target for major depression and CHRNA7 is the most reported. Adolescents who carry CHRNA7 tend to be more likely to have major depressive disorders[49]. Curiously, there is also evidence that CHRNA7 deficient mice are more likely to exhibit depressive symptoms[50] and the use of CHRNA7 agonist can promote the release of 5-HT and improve depressive behavior in depressed mice[51].

In addition to the cholinergic system, the noradrenergic and dopaminergic neuroregulatory systems play an important role in maintaining wakefulness[52]. We also found that Adrenergic Receptor A1 (α_1AR), Adrenergic Receptor A2 (α_2AR), Dopamine Receptor 1 (D12R) and Dopamine Receptor 2 (D22R) are the targets of active compounds in the neuroactive ligand receptor interaction pathway and GO analysis also suggested that adenylate cyclase activation of adrenergic receptors was a significantly enriched biological process, which revealed that noradrenergic and dopaminergic signaling pathways were key to the efficacy of the drug. Both of these signaling systems are important enhancers of cortical activation and behavioral arousal[53] and the

signals transmitted by α1AR and α2AR are mainly from the S/W regulating LC network[53,54]. Using cortical electroencephalography combined with optrode recording, they found that α1AR primarily activating neurons that are active during waking, while sleep-active neurons receive inhibition of α2AR in ventrolateral preoptic nucleus (VLPO)[55]. D12R and D22R are related to the rhythm of sleep activity, recent results show that greater eveningness and physical inactivity are associated with high D12R in caudate nucleus and high D22R in nucleus accumbens, respectively[56]. SLC6A3 is the KG with the highest centrality predicted in this study. It mainly encodes DA transporters and reuptake of DA from synaptic cleft to presynaptic neurons, which is the main mechanism of DA system regulation in the striatum and the key to the pathogenesis of neurological and psychiatric diseases[57,58]. SLC6A3 and D22R genes jointly determine increased drowsiness, attention deficit, and θ/α wave energy ratio changes in healthy subjects after sleep deprivation[59]. In addition, delta (δ) and mu (μ) opioid receptors are also typical neuroactive receptors that are significantly enriched, which are associated with depression. The higher the availability of μ opioid receptors, the lower the depressive mood[60]. Activation of the δ opioid receptor, however, can be a rapid antidepressant[61].

In the combination of ZSS-SCF, although SCF does not seem to be as prominent as ZSS, it is exciting to note that ESR1 in KGs is the main target of SCF. A questionnaire showed a significant positive correlation between ESR1 messenger Ribonucleic acid (mRNA) expression level and the personality characteristics of female depressed patients[62] and a recent study also showed that inhibition of ESR1 mRNA can play an antidepressant role, especially in female population[63]. NR3C1 is one of the KGs of SCF acts alone and plays an important role in depression regulation through the HPA axis[64]. They found that increased promoter methylation of the NR3C1 exon accelerated the encoding of Glucocorticoid Receptor (GR), further influencing depression in children with early adversity[65].

As with other network pharmacology, the selection of the intersection of ZSS-FCS and insomnia targets in this study not only follows the operational process of network pharmacology, but also makes virtual screening results more reliable[66]. However, there are some unavoidable problems. Some of their potential effects may have been overlooked in this classic combination, such as OB and DL not having the desired active ingredient, target genes that may have been missed, whether two herbs cooked together will react to form new active compounds that enhance their efficacy and mitigate their toxic side effects, etc.

As described above, the complex relationship between the active components of ZSS and SCF and their targets has been elucidated by means of network pharmacology and simulated molecular docking. The molecular mechanism explains the use of ZSS and SCF in the treatment of insomnia and similar results have been obtained from previous *in vivo* experiments. The system analysis in this paper can provide a comprehensive idea for further research. The datasets presented in this study can be found in online repositories. (https://data.4tu.nl/account/home).

Conflict of interests:

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

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This article was originally published in a special issue,"New Advancements in Biomedical and Pharmaceutical Sciences" Indian J Pharm Sci 2022:84(2) Spl Issue "29-41"