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ORIGINAL RESEARCH

Mechanisms of Action of *Semen Ziziphi spinosae* in the Treatment of Tourette Syndrome

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Background: *Semen Ziziphi spinosae*, known as Suanzaoren (SZR) in Chinese, is a Chinese herbal medicine widely used in sedatives and tranquilizers. Although SZR is important for the clinical treatment of Tourette syndrome (TS), its mechanism of action remains unclear. Therefore, we investigated the pharmacological mechanisms of SZR in TS treatment using network pharmacology and systems biology approaches.

Methods: The bioactive components and potential targets of SZR were screened using the TCMSP database. UniProt was used to identify targets by mapping the known genes related to SZR. The known genes related to TS were identified by GeneCards and OMIM databases. A protein-protein interaction network was constructed using information from STRING 11.0 database. Cytoscape 3.8.0 software and Bioinformatics online platform were used for plotting this network. Gene ontology and KEGG enrichment analyses were performed using Metascape. Finally, AutoDock was used to verify the molecular docking.

Results: We found that SZR had 10 active compounds. There were 30 overlapping target genes between TS and SZR. These genes were associated with several signaling and metabolic pathways. *AChE, SLC6A4*, and *HTR3A* were the top three hub genes. The active components in SZR had a high binding affinity for the key targets.

Conclusion: SZR therapy for TS could achieve network regulation through the action of various active components of Chinese medicine on different targets and generate a complex regulatory relationship via interaction with potential targets, thereby playing a therapeutic role. Thus, SZR is a potential candidate for treating TS because it regulates nervous system functions.

Keywords: Tourette syndrome, tics, *Semen Ziziphi spinosae*, suanzaoren, network pharmacology, molecular docking

Introduction

Tourette syndrome (TS) is a common childhood-onset neurodevelopmental condition characterized by the presence of multiple sudden, involuntary, rapid, recurrent, and non-rhythmic motor tics and at least one vocal tic that persists for at least a year.[1] The prevalence of TS in school children ranges from 0.3% to 0.9%,[2] while that of other chronic tic disorders ranges from 0.9% to 2.8%.[3] It is widely believed that the occurrence of TS is causally related to the interactions of genetic, neurobiochemical, inflammation-related, immunological, and environmental factors. However, our understanding of the pathophysiological mechanisms of TS remains limited. Current research suggests that abnormalities of neurotransmitters, including dopamine, glutamate, serotonin, and acetylcholine, which participate in the cortico–striato–thalamo–cortical (CSTC) circuit are the primary reason for the development of TS.[4]

Patients with TS may experience subjective discomfort, sustained social problems, and emotional problems.[5,6] The lifetime prevalence of comorbid behavioral disorders is estimated to be 85.7%.[7] Moreover, attention-deficit hyperactivity disorder (ADHD), obsessive-compulsive disorder (OCD), and anxiety disorders are commonly associated with TS.[7,8] About 5% of the patients with TS have life-threatening symptoms, including mild self-injurious behaviors and borderline personality disorder, especially in severe cases.[9] Medications and behavioral treatments are the primary means of treating TS.[10,11] Comprehensive behavioral intervention for tics is the main method used in behavioral treatments. However, this kind of treatment cannot be carried out on a large scale and is suitable only for patients aged 9 years and older with mild

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symptoms. The efficacy of the medications used to treat TS, such as haloperidol, aripiprazole, tiapride, clonidine, and risperidone, is not satisfactory.[10,11] Furthermore, the medications have many side effects, including fatigue, somnolence, and extrapyramidal symptoms.[12,13] Consequently, patients with TS are increasingly using complementary and alternative medicine.[10]

Semen Ziziphi spinosae, also known as spine date seed or suanzaoren (SZR), consists of the dried seeds of *Ziziphus jujuba* and is a widely known Chinese herbal medicine.[14] According to the available literature on traditional Chinese medicine (TCM), SZR is widely used in sedatives and tranquilizers in the treatment of insomnia, anxiety, and other neuropsychiatric diseases.[15,16] Recently, SZR has also shown significant neuroprotective activities in animals with dementia by exerting antioxidant and anti-inflammatory effects.[16] Although previous research has shown that SZR may play an important role in clinical TS treatment, the underlying mechanism is still unclear.[17] Therefore, in this study, systems biology approaches were used to screen the active compounds of SZR, identify its target genes, and explore the potential molecular mechanism of its therapeutic anti-tic effect.

Materials and Methods

Identification of the Active Compounds of SZR

The relevant data for identifying SZR constituents were retrieved from the database of the traditional Chinese medicine systems pharmacology database and analysis platform (TCMSP) database (http://lsp.nwu.edu.cn).[18] The active compounds were screened based on absorption, distribution, metabolism, and excretion (known as ADME screening). Following this, we examined whether the active compounds of SZR met the criteria of oral bioavailability (OB) $\geq$ 25%, drug-likeness (DL) $\geq$ 0.18, and blood-brain barrier (BBB) ≥ -0.3, as recommended in the TCMSP database. OB may represent the percentage of an oral dose of a drug that enters the systemic circulation unchanged.[19] DL is a qualitative term that refers to the optimized pharmacokinetics of a drug and its properties.[20] The BBB restricts proteins and potential diagnostic and therapeutic drugs from entering the brain parenchyma.[21,22] The detailed calculations of these three parameters have been reported by Wang et al.[22]

Identification of Targets Related to Active Compounds of SZR

The targets related to active compounds of SZR were further predicted based on the computer targeting technology developed by the TCMSP. UniProt (http://www.uniprot.org/) is the protein database with the most abundant resources and the most extensive categories.[23] It is composed of data from Swiss-Prot, TrEMBL, and PIR-PSD. Using the UniProt KB search function of UniProt database, SZR target proteins were searched and limited to "human" studies, with the UniProt codes of retrieved active compounds of SZR converted into gene symbols.

Identification of Known Genes Related to TS

Known TS genes were acquired from two existing resources: (1) GeneCards database (https://www.genecards.org/) and (2) Online Mendelian Inheritance in Man (OMIM) database (https://omim.org). The GeneCards database helps in screening correlations between genes and diseases and provides an algorithm to retrieve more relevant targets.[24] The OMIM database includes information on human diseases that describes the categories and names of new diseases as well as the relationship between phenotypes and related etiological genes.[25] The keywords "Tourette syndrome" or "Tic disorder" were entered, and the repeated genes retrieved were removed from the search results and matched with the genes related to the active compounds of SZR to obtain their potential target genes in the treatment of TS.

Protein-Protein Interaction Network of Targets of TS and SZR

Using STRING 11.0, an online software (http://string-db.org),[26] the genes included in the intersection of target genes related to active compounds of SZR and TS were entered, the organism "Homo Sapiens" was selected, the relevant information on the interactions and relationships between proteins were obtained, and a protein-protein interaction (PPI) network was constructed. Cytoscape 3.8.0 software (The Cytoscape Consortium, San Diego, CA, USA) was used for plotting the network between SZR, active compounds, target genes, and TS.

Correlation Pathway and Annotation Analysis

Gene ontology (GO) terms belong to three categories, namely, biological processes, molecular functions, and cellular components.[27] The Kyoto Encyclopaedia of Genes and Genome (KEGG) can consign selected sets of genes to the most relevant signaling pathways.[28] In this study, Metascape (http://metascape.org)[29] was used to conduct GO term and KEGG pathway enrichment analyses of the gene sets to explore the molecular mechanism underlying anti-TS effects of SZR, with the threshold significance value set at the default $P < 0.01$. The enrichment GO term histogram and enrichment KEGG dot bubble were plotted using Bioinformatics (http://www.bioinformatics.com.cn), an online data visualization platform.

Molecular Docking

The crystal structures of the verified components of SZR were obtained from the RCSB Protein Data Bank (PDB, https://www.rcsb.org/).[30] The crystal structure of components were stored as a docking ligand in MOL2 format, and the iGEMDOCK software wa. Finally, AutoDock was used to verify the molecular docking.

Results

Compounds of SZR

Using TCMSP, it was found that SZR includes 33 kinds of traditional ingredients. Among these ingredients, a total of 10 active compounds met the criteria of OB ≥ 25%, DL ≥ 0.18, and BBB ≥ −0.3 (Table 1).

Target Gene Prediction

Target sites of SZR were retrieved from TCMSP, and corresponding targets were selected according to the selected chemical components. Among these components, one candidate did not have any targets, and a total of 42 non-repeating targets were obtained. Moreover, 2624 target genes corresponding to TS were identified from GeneCards and OMIM. There were 30 overlapping target genes between TS and SZR (Figure 1).

Protein-Protein Interaction Network

A PPI network of the 30 overlapping target genes in SZR and TS was constructed using the STRING database (Figure 2). In this study, the genes with high connectivity (≥ 5) were defined as hub genes. Acetylcholinesterase (*AChE*), sodium-dependent serotonin transporter (*SLC6A4*), and 5-hydroxytryptamine receptor 3A (*HTR3A*) were the top three hub genes among the 25 hub genes (Figure 3).

Table 1 Detailed Information on the Active Compounds of SZR

Number	PubChem CID	Molecule Name	OB (%)	DL	BBB
SZR1	64,971	Mairin	55.38	0.78	0.22
SZR2	160,487	(S)-Coclaurine	42.35	0.24	0.06
SZR3	5,742,590	Daucosterol	36.91	0.75	1.15
SZR4	51,346,169	Jujuboside A	34.96	0.62	0.11
SZR5	22,156	dl-Nuciferine	29.26	0.4	1.03
SZR6	222,284	Phytosterol	36.91	0.75	1.16
SZR7	44,566,612	Sanjoinine A	27.32	0.79	−0.01
SZR8	14,729,076	Sanjoinine B	26.86	0.79	−0.2
SZR9	14,729,078	Sanjoinenine	67.28	0.79	−0.24
SZR10	102,063,083	Zizyphusine	41.53	0.55	0.6

Abbreviations: OB, oral bioavailability; DL, drug-likeness; BBB, blood-brain barrier.

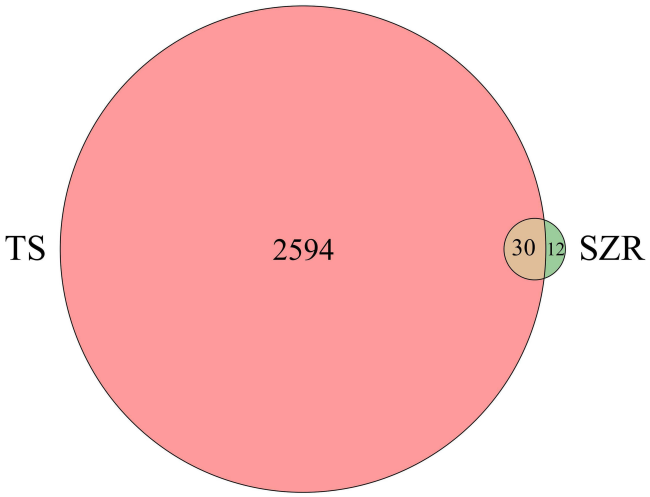

Figure 1 Venn diagram of the target genes for TS and SZR active compounds. TS has 2624 target genes, while SZR has 42 target genes. There are 30 overlapping target genes between the two sets.

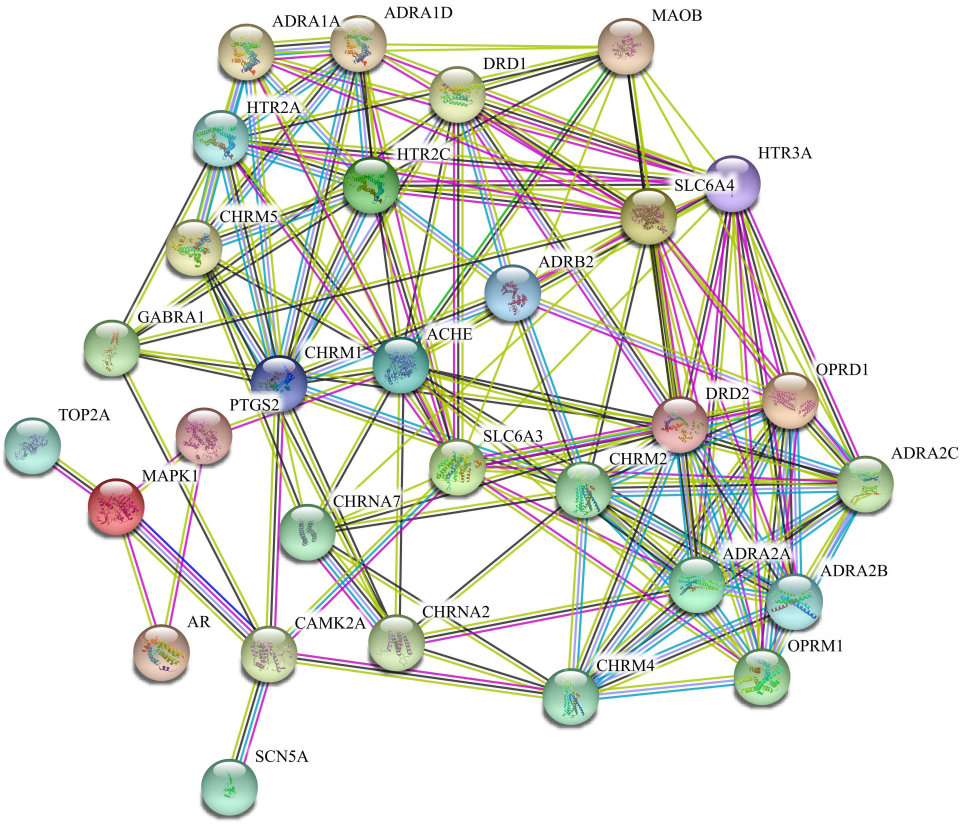

Figure 2 PPI network of the 30 overlapping target genes. Within a PPI network, each protein is represented as a node, and interactions are represented by the lines between the nodes. The number of lines linked to a given node is defined as the connectivity degree.

Network Among SZR, Compounds, Target Genes, and TS

The active compounds of SZR and TS-related overlapping target genes were imported into the Cytoscape 3.8.0 software to construct the network between SZR-compounds-target genes-TS (Figure 4). The results showed that SZR mainly acted on 30 targets through five active compounds, which may affect the occurrence of TS.

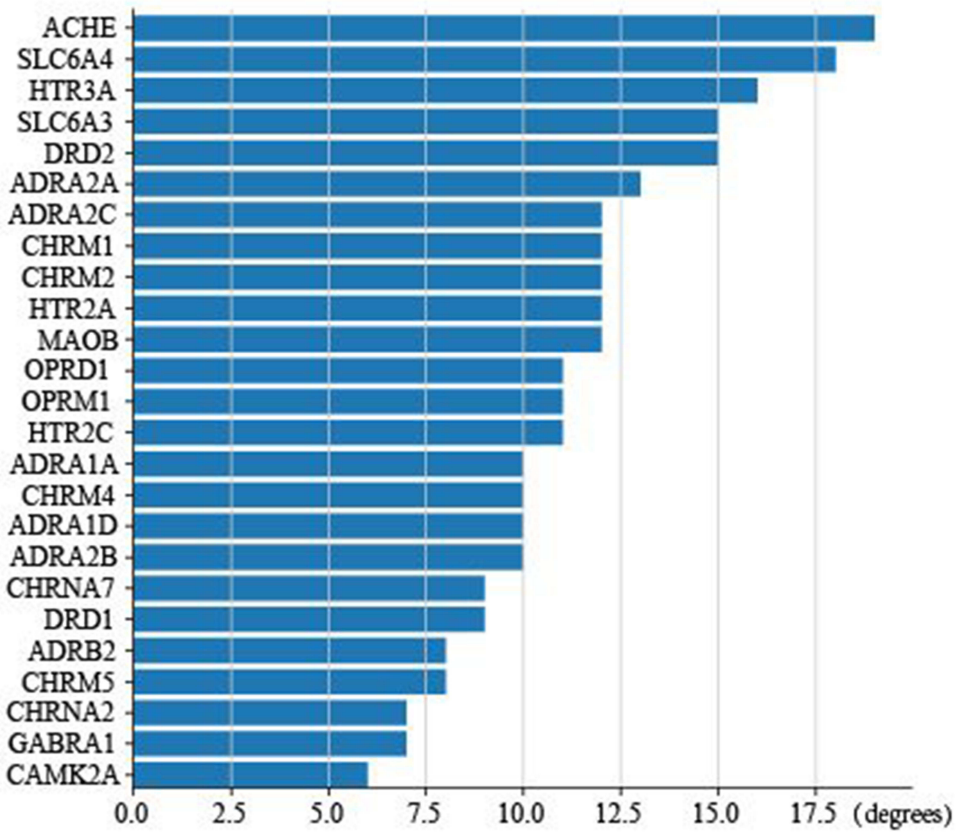

Figure 3 Hub nodes from the PPI network. *AChE* has the highest connectivity degree. Nodes that possess important biological functions usually have a high connectivity degree and are also named hub genes.

GO Term and KEGG Pathway Enrichment Analysis

In each major category of the GO terms (Figure 5), the most significantly enriched terms were "chemical synaptic transmission", "postsynaptic membrane", and "G protein-coupled amine receptor activity." The overlapping target genes were closely related to synapses, receptors, and metabolic signaling pathways (Figures 6 and 7).

Verification of Molecular Docking

When the main active components of a therapeutic agent are docked with a core target, the more stable the binding conformation, the lower the binding energy required. In this study, binding energy < −9.0 kcal/mol was selected as the screening condition, as shown in Table 2. We selected AChE docking with the verified SZR active molecules. (S)-Coclaurine, Sanjoinine B and Zizyphusine had the three lowest energy value, with binding energies of −10.03, −9.76, and −9.7 kcal/mol. Moreover, Pymol 2.1 software was used to create the schematic diagram for the docking result with the lowest binding energy (Figure 8). This shows that (S)-coclaurine has a good binding ability to AChE protein.

Discussion

TS is a neurodevelopmental condition characterized by the presence of tics and associated behavioral problems.[1] Some Chinese herbs may play an important role in clinical TS treatment, *Bombyx Batryticatus* have been used to treat convulsions.[31] *Os Draconis, Concha Ostreae*, and *Radix Bupleuri* have been used to relieve epileptic symptoms.[32] SZR is a Chinese herbal medicine that has been widely used in sedatives and tranquilizers in traditional clinics.[17] Based on the current clinical and experimental research status of SZR treatment for TS, the present study further explored the mechanism of action of SZR by using a systems biology approach.

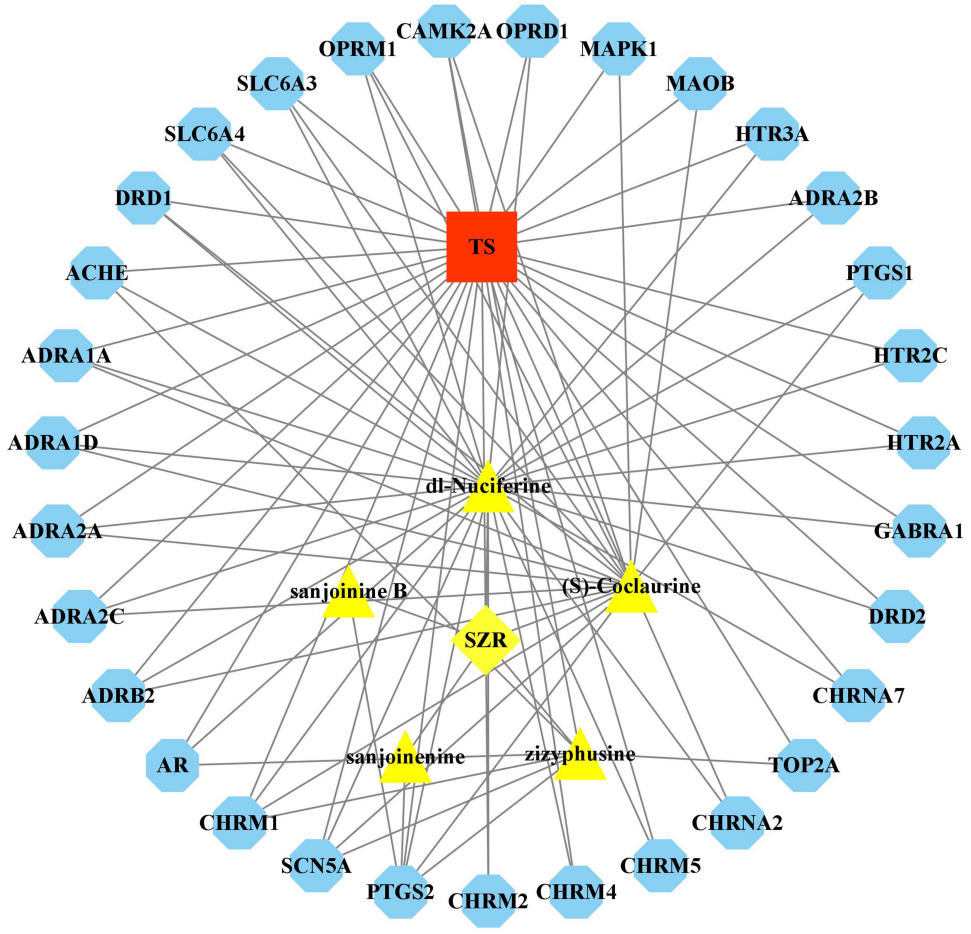

Figure 4 Network of SZR, compounds, target genes, and Tourette syndrome (TS). Red rectangle, TS; yellow diamond, SZR; yellow triangle, compounds; blue octagon, target genes.

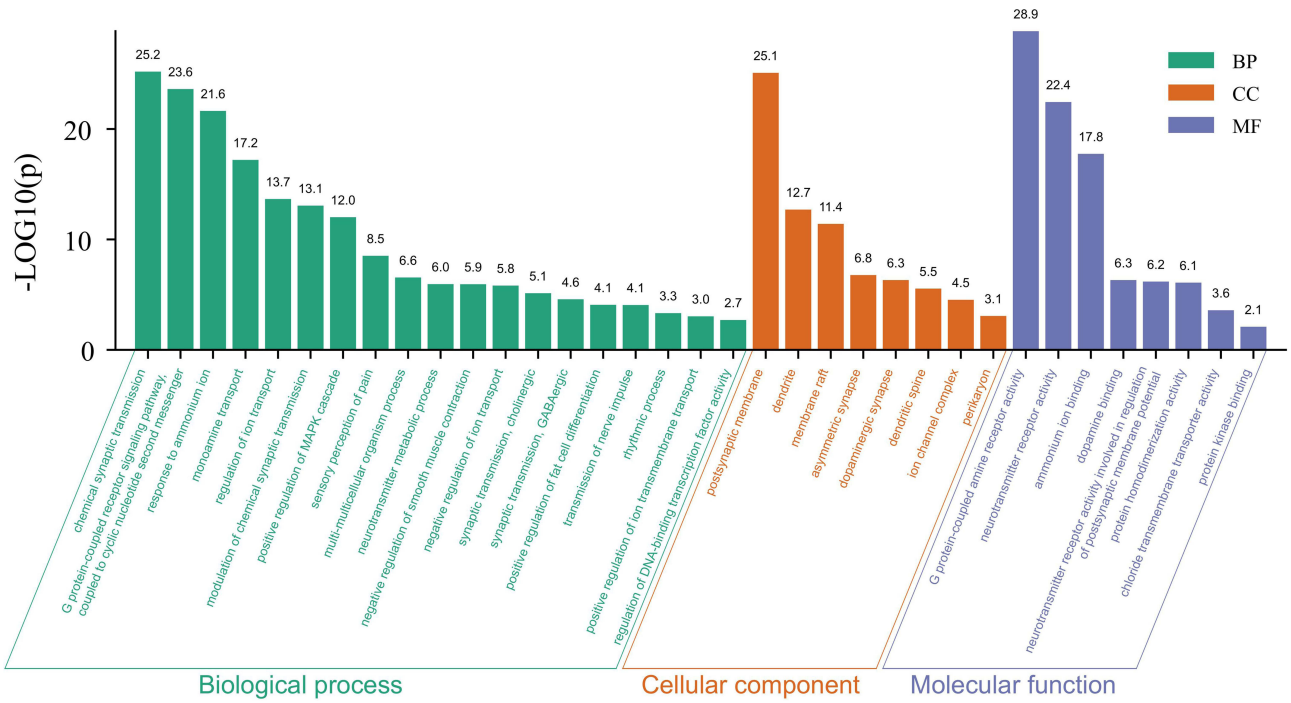

Figure 5 GO term enrichment analysis.

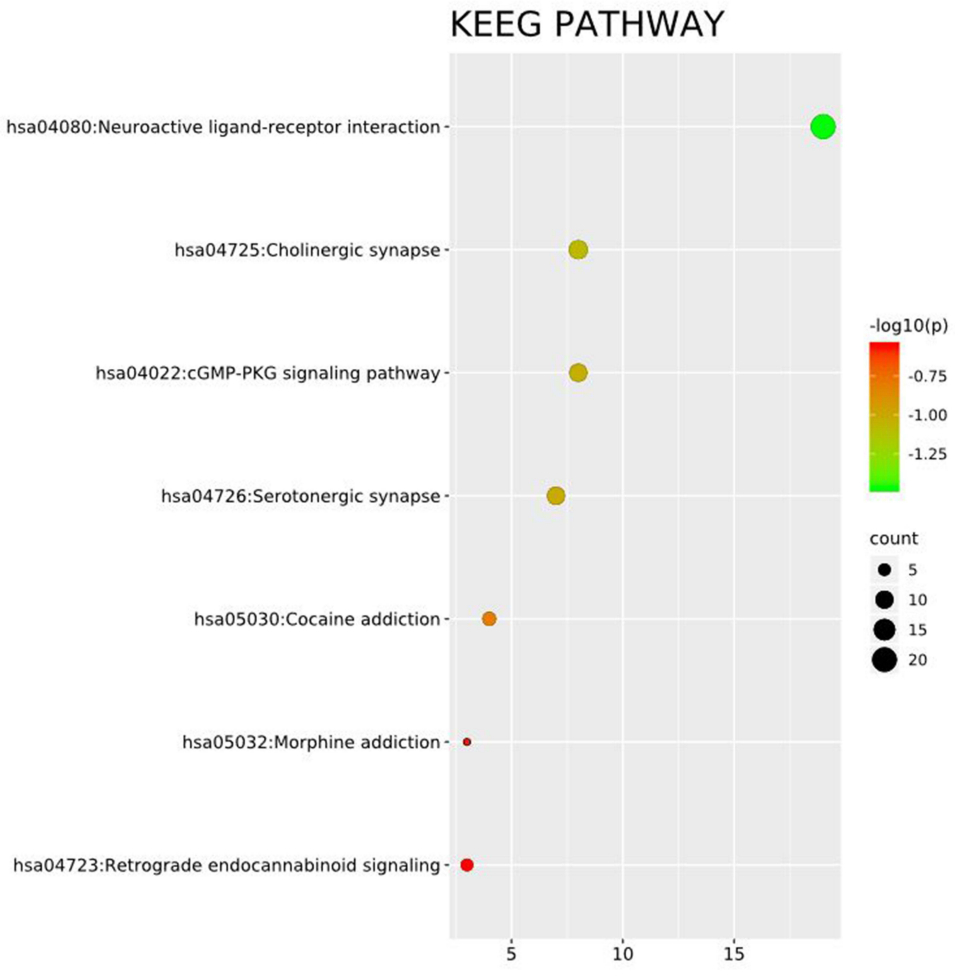

Figure 6 KEGG pathway enrichment analysis.

In this study, SZR was found to have 10 compounds and 42 non-repeating targets. Among the compounds, there were 5 active components: (S)-coclaurine, dl-nuciferine, sanjoinine B, sanjoinenine, and zizyphusine. DL-Nuciferine is an isoquinoline derivative that acts as a D-2-like receptor agonist.[33] Sanjoinine B is a cyclic alkaloid peptide, while zizyphusine, sanjoinenine, and (S)-coclaurine are alkaloids. They all exert diverse effects on nerve cells, neurotransmitters, receptors, and sleep parameters.[34,35] These findings suggest that they may be the key compounds of SZR that play a role in TS treatment. The active amino acid residues bound by (S)-coclaurine and AChE include Glu-199, His-440, Phe-330, Ser-122, and Tyr-334. Hydrogen bonds are formed between the hydroxyl and hydrogen atoms of (S)-coclaurine and the active amino groups of Glu-199, His-440, Ser-122, and Tyr-334. For example, strong hydrogen bonds are formed between hydroxyl protons and amino protons of Glu-199, His-440, Ser-122, and Tyr-334 with distances of 2.1 Å, 2.7 Å, 2.0 Å, and 2.6 Å, respectively. The bond length of a conventional hydrogen bond is markedly less than 3.5 Å. In addition, the benzene ring of (S)-coclaurine can form π-π conjugated interactions with the benzene ring of Phe-330 residue. These hydrogen bonds and π-π conjugate interactions indicate that (S)-coclaurine binds strongly to AChE protein and can match the active pocket of AChE protein suitably.

There were 30 overlapping target genes between TS and SZR. Twenty-five hub genes were identified using PPI network analysis. *AChE, SLC6A4*, and *HTR3A* were the top 3 hub genes among them; they are all closely related to neuropsychiatric diseases. AChE can termination of neurotransmission at cholinergic synapses by rapid hydrolysis of the ACh,[36] and exerts beneficial effects in several neurological disorders such as Alzheimer disease and Creutzfeldt-Jakob disease through its critical role in termination of cholinergic neurotransmission.[37,38] The corpus striatum of the basal

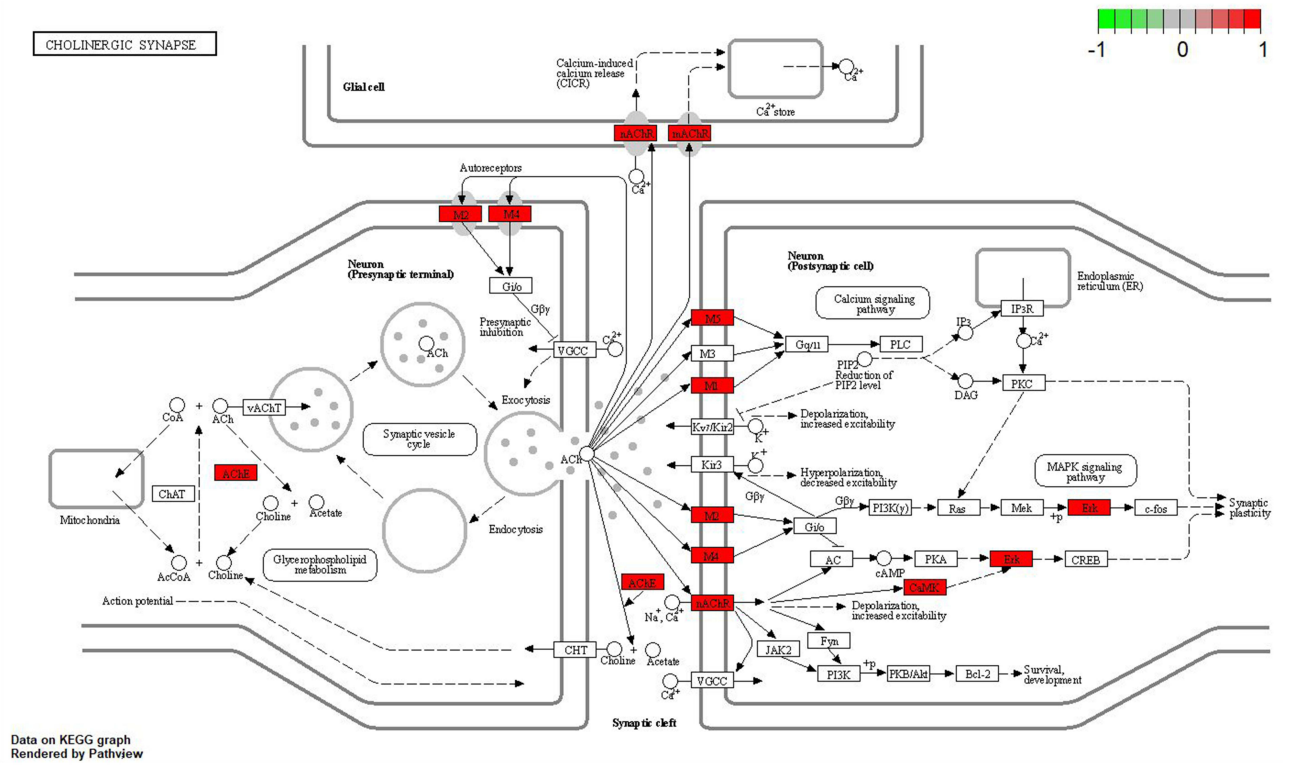

Figure 7 KEGG pathway enrichment analysis demonstrating the interactions among various pathways.

ganglia possesses the highest density of AChE markers, thereby highlighting the importance of AChE in this structure. Accumulating evidence from several animal experiments has indicated that the nicotinic-cholinergic system is involved in physiological motor control. Therefore, the development of AChE drugs can play a pivotal role in treating movement disorders.[39]

Numerous studies have shown that SLC6A4 is associated with the etiology of TS.[40] Increased blood *SLC6A4* mRNA levels in the striatum of TS38 rats has been found to be positively correlated with the severity of the tic.[41,42] The serotonin transporter (SERT)-linked polymorphic region (5-HTTLPR) in the promoter region directly upstream of SLC6A4 has been implicated in the etiology of OCD and TS.[43,44] 5-HTTLPR is a 43-base pair repeat with a long (L) and a short (S) allele. The L allele is associated with increased *SLC6A4* mRNA expression in blood, resulting in reduced serotonergic neurotransmission through the increased serotonin clearance mediated by SERT.[40] SLC6A4 has also been implicated in other neuropsychiatric disorders, such as ADHD and OCD, which are important and common complications of TS.[41] HTR3A is one of the serotonin receptor subtypes. Serotonin has been implicated in various neuropsychiatric disorders; therefore, it may affect the detected variants contributing to the severity of these diseases. Serotonergic dysfunction is thought to be involved in the pathophysiology of TS.[45] These candidate genes offer vast potential benefits for application in gene-targeted therapy of TS.

To further understand the mechanism of action, GO and KEGG enrichment analyses were performed on these target genes. GO enrichment analysis demonstrated that SZR plays a regulatory role in chemical synaptic transmission,

Table 2 AChE Docking Results for the Compounds of SZR

Name	Target	Binding Energy (kcal/mol)	Combination Type
(S)-Coclaurine	AChE	−10.03	Hydrogen bonds, hydrophobic interactions, π-stacking
Sanjoinine B	AChE	−9.76	Hydrogen bonds, hydrophobic interactions, π-stacking
Zizyphusine	AChE	−9.7	Hydrogen bonds, hydrophobic interactions, π-stacking

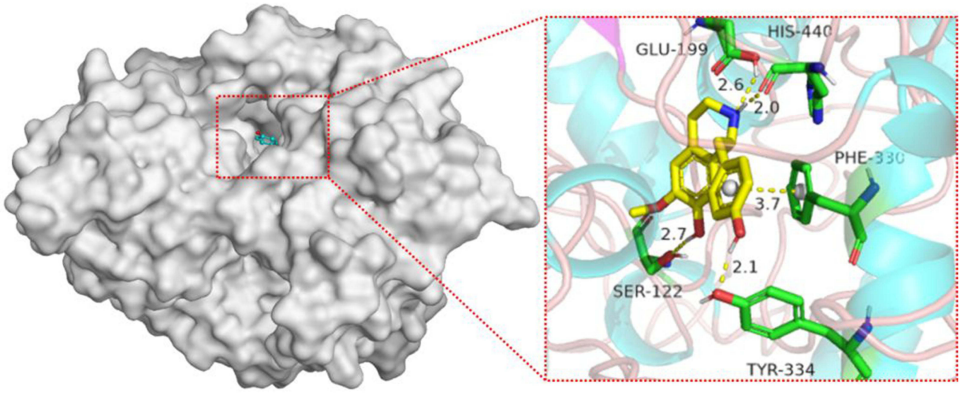

Figure 8 Binding pattern of (S)-coclaurine) and AChE protein.

postsynaptic membrane, G protein-coupled amine receptor activity, and other biological processes. According to the enrichment results of the KEGG pathway, SZR plays a role in treating TS through neuroactive ligand-receptor interaction, cholinergic synapse, cGMP-PKG signaling, serotonergic synapse, cocaine addiction, morphine addiction, and retrograde endocannabinoid signaling. These research results are consistent with the holistic view of TCM and the principle of treatment based on ZHENG differentiation.[46] Therefore, it is speculated that SZR may not only through the action of various active components on different targets, but also through the interaction among potential targets, thereby playing a therapeutic role in TS. Moreover, the present findings can facilitate the isolation of active compounds in SZR to optimize therapy.

Previous studies had shown the therapeutic potential of SZR in treating TS.[15,16] SZR had also been used in a randomized, double blind, double dummy, parallel controlled trial with TS children.[17] Further studies could be designed to subsequent cell and animal experiments to reveal the underlying pharmacological and therapeutic effects of SZR for the treatment of TS.

Limitations

However, the present study had some limitations. First, because we could not validate these results experimentally in this study, the specific mechanism of action is still unclear and needs to be studied further. Second, we chose the criteria of oral bioavailability (OB) $\geq$ 25%, drug-likeness (DL) $\geq$ 0.18, and blood-brain barrier (BBB) ≥ -0.3, as recommended in the TCMSP database, to identified the active compounds of SZR in this study. Thus, the chooses of different criterias may lead to different results.

Conclusions

In general, the present study revealed the active compounds of SZR and possible targets and mechanism of action in the treatment of TS from the perspective of network pharmacology. The key molecules identified in this study are candidate drug targets, and the critical status of these molecules indicates that they may be the key targets of disease control.[43] Network pharmacology is categorized as data mining research, and the research results are mainly based on existing studies and prediction system analysis of active ingredients and targets; therefore, there are some differences between the current results and those of actual experiments and clinical trials, which need to be further verified in the future.

Abbreviations

SZR, Suanzaoren; TS, Tourette syndrome; CSTC, cortico–striato–thalamo–cortical; ADHD, attention-deficit hyperactivity disorder; OCD, obsessive-compulsive disorder; TCM, traditional Chinese medicine; TCMSP, traditional Chinese medicine systems pharmacology database and analysis platform; OB, oral bioavailability; DL, drug-likeness; BBB, blood-brain barrier; OMIM, Online Mendelian Inheritance in Man; PPI, protein-protein interaction; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genome; Ach, acetylcholine.

Data Sharing Statement

The data supporting the findings of the study are obtained from the following databases: TCMSP (http://lsp.nwu.edu.cn), UniProt (http://www.uniprot.org/), GeneCards database (https://www.genecards.org/), Online Mendelian Inheritance in Man (OMIM) database (https://omim.org), STRING 11.0 online software (http://string-db.org), Bioinformatics (http://www.bioinformatics.com.cn), Metascape (http://metascape.org/gp/index.html#/main/step1), and RCSB Protein Data Bank (PDB, https://www.rcsb.org/).

Ethical Approval and Informed Consent

Not applicable – Not required for this study.

Author Contributions

FF was the primary contributor to writing the manuscript. LH and FH participated in research design. All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare no conflicts of interest in relation to this work.

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