

A Combination of UPLC-LTQ-Orbitrap-MSⁿ Research, Network Analysis and Experimental Verification Used to Explore the Mechanism of Yangyin Yiqi Huoxue Recipe in Treating Sjogren's Syndrome

Wen-Wen Lu

The First Affiliated Hospital of Shantou University Medical College

Tian-Xiao Fu

The First Affiliated Hospital, College of Medicine, Zhejiang University

Fang-Ping Wu

Zhejiang University of Traditional Chinese Medicine: Zhejiang Chinese Medical University

Fu-lin Xiong

The First Affiliated Hospital, College of Medicine, Zhejiang University

Tian-Yi Li

Zhejiang University of Traditional Chinese Medicine: Zhejiang Chinese Medical University

Guo-lin Wu (✉ wuguolin28@163.com)

Department of Traditional Chinese Medicine, The First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou (310003), China <https://orcid.org/0000-0002-9324-9424>

Research

Keywords: UPLC-LTQ-Orbitrap-MS research, network analysis, Sjogren's syndrome, Chinese medicine, Yangyin Yiqi Huoxue Recipe

Posted Date: September 24th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-80950/v1>

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Abstract

Purpose: To explore the main active ingredients and mechanism of Yangyin Yiqi Huoxue (YYYQH) Recipe in treating Sjogren's syndrome.

Methods: The main active ingredients of Yangyin Yiqi Huoxue(YYYQH) Recipe were obtained through UPLC-LTQ-Orbitrap-MSⁿ research technical analysis. Subsequently, the main active ingredients were analyzed by network pharmacology to explore their important targets and biological processes. Finally, the important target M3 muscarinic acetylcholine receptor (CHRM3) gene was verified by cytological experiments.

Results: We identified 41 active ingredients in the YYYQH Recipe, and identified 26 potential targets through network pharmacology methods. According to the network analysis and literature research, CHRM3 is one of the main targets of YYYQH in the treatment of Sjogren's syndrome. We observed the effect of YYYQH on the CHRM3 and aquaporin 5(AQP5) genes using cytological experiments. The results showed that YYYQH significantly increased the expression of CHRM3 and AQP5 in submandibular gland cells. This may be the mechanism of YYYQH Recipe in improving symptoms such as dry mouth in Sjogren's syndrome.

Conclusions: This study provides a strategy for understanding the mechanism of action of YYYQH in treating Sjogren's syndrome by combining UPLC-LTQ-Orbitrap-MS research, network pharmacology, and experimental validation.

Background

Sjogren's syndrome (SS) is a chronic systemic autoimmune disease. The main organs involved are the salivary glands and lacrimal glands. The symptoms are dry mouth and eyes, which seriously affects the quality of life of patients^[1]. In the clinical practice of treating SS, it has been found that traditional Chinese medicine (TCM) can not only effectively improve patients' symptoms such as dry mouth and eyes and improve their quality of life, but it also has the advantages of a significant curative effect and few side effects^[2, 3].

Yangyin Yiqi Huoxue Recipe (YYYQH Recipe) is a TCM formula used in clinical practice, combining nine kinds of Chinese herbal medicine (*Pseudostellariae Radix*, *Paeoniae Radix Alba*, *Polygonati Rhizoma*, *Fructus Ligustri Lucidi*, *Rhizoma Dioscoreae*, *Schisandrae Chinensis Fructus*, *Mume Fructus*, *Rhodiola rosea L.*, and *Herba Sarcandrae*). In clinical practice, we have found that treatment with YYYQH is effective in treating SS. It not only improves the condition and relieves symptoms such as dry mouth, but also regulates the patient's Th1/Th2 immune balance and reproductive hormone–endocrine–immune function, and improves the quality of life^[4, 5]. Non-human animal experiments also showed that YYYQH Recipe can reduce the amount of water drunk by NOD (Non-obese Diabetic) mice, increase saliva secretion, and reduce the pathological damage of submandibular gland tissue^[6, 7].

In this study, UPLC-LTQ-Orbitrap-MSⁿ research, network pharmacology and experimental verification were applied to investigate the therapeutic mechanism of YYYQH Recipe in the treatment of SS. Specifically, UPLC-LTQ-Orbitrap-MS research technology was used to analyze the main active ingredients in YYYQH Recipe. Subsequently, the main active ingredients were analyzed by network pharmacology to explore their important targets and biological processes. In addition, the important targets were verified by cytological experiments to verify the pharmacological effects of YYYQH Recipe on SS(Fig. 1). These results should clarify the therapeutic mechanism of TCM at the molecular level and improve the effectiveness and specificity of clinical treatment.

Materials And Methods

PART1 UPLC-LTQ-Orbitrap-MSⁿ research and network analysis

1.1 Equipment

UPLC-Triple-TOF/MS system: AcquityTM ultra high performance liquid chromatograph (Waters Corporation, Milford, Massachusetts, USA), Triple TOF 5600 + time-of-flight mass spectrometer, equipped with electrospray ion source (AB SCIEX company, USA); Eppendorf minispan centrifuge (Eppendorf AG, Hamburg, Germany)

1.2 Preparation of test solution

Yangyin Yiqi Huoxue Recipe: *Pseudostellariae Radix* 24 g, *Paeoniae Radix Alba* 18 g, *Polygonati Rhizoma* 18 g, *Fructus Ligustri Lucidi* 15 g, *Rhizoma Dioscoreae* 30 g, *Schisandrae Chinensis Fructus* 10 g, *Mume Fructus* 15 g, *Rhodiola rosea L.* 15 g, *Herba Sarcandrae* 12 g. The above-mentioned medicinal materials were crushed into a fine powder and stirred evenly. To 1 g of the crushed sample was added 15 ml of 80% aqueous methanol solution; the mixture was ultrasonicated for 30 min, centrifuged at 10000 rpm for 20 min, and the supernatant used for testing.

1.3 Chromatographic conditions and mass spectrometry conditions

Chromatographic conditions: The column was a Waters ACQUITY UPLC HSS T3 (150 mm × 3.0 mm id, 1.7 μm); 0.1% formic acid solution was used as mobile phase A, 0.1% formic acid acetonitrile was used as mobile phase B, linear gradient elution, 0 min 5%B; 32 min 40%B; 36 min 95%B; the flow rate was 0.3 ml/min; column temperature 50 °C; detection wavelength 254 nm; injection volume: 2 μl.

Mass spectrometry conditions: UPLC-Triple-TOF 5600 + Time-of-Flight LC/MS: negative ion scanning mode; scanning range: m/z 100–1500; atomizing gas (GS1): 55 psi; atomizing gas (GS2): 55 psi; curtain air (CUR): 35 psi; ion source temperature (TEM): 550 °C (negative); ion source voltage (IS): –4500 V (negative); primary scan: declustering voltage (DP): 100 V; focusing voltage (CE): 10 V; secondary scan: TOF MS² Product Ion²IDA mode was used to collect mass spectrum data; the collision-induced dissociation (CID) energy was –20, –40 and –60 V; before sample injection, a calibrant delivery system (CDS) pump was used for mass axis calibration to ensure that the mass axis error was less than 2 ppm.

1.4 Identification of compounds

Databases (Scifinder, TCMID, TCM@ Taiwan, TCMSP, CNKI, etc.) were searched for terms related to the chemical composition of the nine traditional Chinese medicines of YYYQH X Recipe, to establish the chemical composition database of Yangyin Yiqi Huoxue Recipe, and to calculate the related compounds: [M + H]⁺, [M + Na]⁺ and M⁺ in the positive ion mode, M[–], [M-H][–] and [M + HCOO][–] in the negative ion mode. The exact mass-to-charge ratio (m/z) was used to provide a reference for analysis and identification. During the identification process, the primary mass spectrum data comparison (ΔPPM < 10) was performed to identify possible compounds. On this basis, the compounds were further confirmed through secondary mass spectrometry analysis and literature search and comparison.

1.5 Targets of action of active compounds of Yangyin Yiqi Huoxue Recipe and disease targets of SS

The active compounds obtained above were used to obtain prediction targets through the SwissTargetPrediction database (<http://www.swisstargetprediction.ch/>) and the SEA database (<http://sea.bkslab.org/>). In the Therapeutic Target Database (<http://db.idrblab.org/ttd/>), Drugbank Database (<http://www.drugbank.ca>), and Disgenet Database (<http://www.disgenet.org>), "Sjogren syndrome" was used as the search term to retrieve the disease target of SS; only one duplicate target was retained, and that target was taken to be the target related to SS.

1.6 Network construction and analysis

Mapping the target of the selected active compound with the disease target of SS and taking the intersection, the resulting target was the target of the YYYQH X Recipe activating a blood compound acting on the disease SS. The cytoscape software was used to construct the active compound of Yangyin Yiqi Huoxue Recipe and its target network for SS disease.

1.7 Gene ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) signal pathway analysis

Based on the targets obtained in the above process, the human genome annotation database DAVID 6.8 (<http://david.ncifcrf.gov/>) was used to conduct GO enrichment analysis and KEGG signal pathway analysis, screening with P < 0.05.

PART 2 Experimental verification

2.1 Experimental materials

Cell culture plate (Corning USA, 430167), RPMI-1640 (Solarbio China, 31800022), penicillin and streptomycin mixture (Gibco USA, MP 1670249-M), 0.25% EDTA-trypsin (Gibco USA, T1300), fetal bovine serum (Gibco USA, 10099-141), L-glutamine (Solarbio China, G0200), CCK-8 detection kit (Beyotime China, C0037).

2.2 Experimental instrumentation

Multifunctional microplate reader (Thermo VARIOSKAN LUX), biological safety cabinet (ESCO AC2-4S1), cell incubator (ESCO CCL-170B-8), pipette (ThermoFisher Finn pipette™), electric pipetting aid (RININ PX-100), centrifuge (Beckman Microfuge), ultra-low temperature refrigerator (Thermo Scientific – 80 °C), refrigerated centrifuge (Sigma 3-18K), real-time quantitative PCR instrument (Roche LightCycler®96).

2.3 Cell culture

HSG cells (human submandibular gland cells) were cultured in a 37 °C, 5% CO₂ incubator. The medium used was DMEM/F12 containing 10% (V/V) fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin.

2.4 CCK8 detects cell proliferation

The HSG cell lines were randomly divided into a blank group, phosphate buffered saline (PBS) group, Yangyin Yiqi Huoxue Recipe (YYYQH) group, and hydroxychloroquine (HCQ) group. After the above-mentioned drugs were used for 12, 24, and 48 h, a CCK-8 kit was used to calculate the cell survival rate.

2.5 Quantitative PCR(q-PCR) detection

The HSG cell lines were randomly divided into a blank group, PBS group, YYYQH group, and hydroxychloroquine HCQ group. After 12, 24, and 48 h of drug action, q-PCR was performed to detect the levels of expression of the CHRM3 and AQP5 genes.

2.6 Data processing

Data were presented as mean \pm SD and analyzed using GraphPad Prism 8 (GraphPad Software, Inc., La Jolla, CA, USA). One-way analysis of variance (ANOVA) was used, and a P-value < 0.05 was considered statistically significant.

Results

Part 1

3.1 Identification of active compounds of Yangyin Yiqi Huoxue Recipe based on the UPLC-LTQ-Orbitrap-MS platform

Based on the above sample processing methods and analysis conditions, the Yangyin Yiqi Huoxue Recipe was analyzed. In the positive and negative ion mode, 41 active compounds were screened from Yangyin Yiqi Huoxue Recipe, as shown in Fig. 2 and Table 1.

Table 1
Main active compounds of Yangyin Yiqi Huoxue Recipe

Number	Molecular formula	Compound name	Medicinal materials	Number	Molecular formula	Compound name	Medicinal materials
AI1	C12H18O12	hexose 1-citrate	<i>Polygonati Rhizoma</i>	AI22	C21H34O10	sacranoside A	<i>Rhodiola rosea L.</i>
AI2	C9H11NO2	L-tyrosine	<i>Polygonati Rhizoma</i>	AI23	C48H64O27	Nuezhenoside G13	<i>Fructus Ligustri Lucidi</i>
AI3	C10H13N5O4	adenosine	<i>Rhizoma Dioscoreae</i>	AI24	C21H20O10	kaempferol 3-rhamnoside	<i>Herba Sarcandrae</i>
AI4	C7H6O5	Gallic acid	<i>Paeoniae Radix Alba</i>	AI25	C48H64O27	oleonuezhenide	<i>Fructus Ligustri Lucidi</i>
AI5	C6H8O4	2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	<i>Polygonati Rhizoma</i>	AI26	C19H36O10	Rhodiooctanoside	<i>Rhodiola rosea L.</i>
AI6	C9H11NO2	Phenylalanine	<i>Rhizoma Dioscoreae</i> <i>Polygonati Rhizoma</i>	AI27	C21H36O10	Kenposide A	<i>Herba Sarcandrae</i>
AI7	C6H6O3	5-hydroxymethylfurfural	<i>Polygonati Rhizoma</i>	AI28	C30H32O12	benzoylpaeoniflorin	<i>Paeoniae Radix Alba</i>
AI8	C16H18O9	neochlorogenic acid	<i>Mume Fructus</i>	AI29	C15H10O6	Kaempferol	<i>Herba Sarcandrae</i>
AI9	C14H20O7	Salidroside	<i>Rhodiola rosea L.</i>	AI30	C40H58N8O8	Heterophyllin B	<i>Pseudostellariae Radix</i>
AI10	C15H14O6	Cianidanol	<i>Mume Fructus</i>	AI31	C37H57N7O8	Heterophyllin A	<i>Pseudostellariae Radix</i>
AI11	C16H22O11	secologanoside	<i>Herba Sarcandrae</i>	AI32	C42H56N8O9	Heterophyllin G	<i>Pseudostellariae Radix</i>
AI12	C23H28O12	oxypaeoniflorin	<i>Paeoniae Radix Alba</i>	AI33	C45H67N9O9	Heterophyllin E	<i>Pseudostellariae Radix</i>
AI13	C30H26O12	procyanidin B1	<i>Mume Fructus</i>	AI34	C35H48N6O7	Heterophyllin D	<i>Pseudostellariae Radix</i>
AI14	C15H14O6	L-Epicatechin	<i>Herba Sarcandrae</i>	AI35	C24H32O7	schisandrin	<i>Schisandrae Chinensis Fructus</i>
AI15	C23H28O11	albiflorin	<i>Paeoniae Radix Alba</i>	AI36	C28H34O10	Gomisin D	<i>Schisandrae Chinensis Fructus</i>
AI16	C23H28O11	paeoniflorin	<i>Paeoniae Radix Alba</i>	AI37	C23H28O7	Schisandrol B	<i>Schisandrae Chinensis Fructus</i>
AI17	C41H32O26	penta-O-galloyl glucopyranose	<i>Paeoniae Radix Alba</i>	AI38	C28H36O8	Angeloylgomisin H	<i>Schisandrae Chinensis Fructus</i>
AI18	C30H32O15	6'-O-galloyl paeoniflorin	<i>Paeoniae Radix Alba</i>	AI39	C24H32O6	deoxyschizandrin	<i>Schisandrae Chinensis Fructus</i>
AI19	C31H42O17	Nuezhenide	<i>Fructus Ligustri Lucidi</i>	AI40	C23H28O6	γ-schizandrin	<i>Schisandrae Chinensis Fructus</i>
AI20	C31H42O17	Specnuezhenide	<i>Fructus Ligustri Lucidi</i>	AI41	C23H28O6	Schisandrin B	<i>Schisandrae Chinensis Fructus</i>
AI21	C27H30O16	Herbacetin-7-O-glucorhamnoside	<i>Rhodiola rosea L.</i>				

3.2 The disease targets of SS and the target of action of active compounds in Yangyin Yiqi Huoxue Recipe

From Therapeutic Target Database, Drugbank Database, Disgenet Database, using "Sjogren syndrome" as the search term to search for the relevant targets of SS, only one duplicate target was retained, and a total of 123 disease targets were obtained.

In the SwissTargetPrediction database and the SEA database, the above 41 active compounds were obtained as predicted targets;

Finally, the disease targets of SS and the predicted targets of the active compounds of Yangyin Yiqi Huoxue Recipe were mapped to each other (only one repeated target remained), and 26 effect targets of Yangyin Yiqi Huoxue Recipe in the treatment of SS were obtained, as shown in Fig. 3 and Table 2.

Table 2 Network Statistics of 26 effect targets

shared name	AverageShorte	ClosenessCent	Stress	Degree	BetweennessC	Neighborhood	NumberOf	Radiality
<i>MMP9</i>	2.38709677	0.41891892	2146	15	0.05875355	5.13333333	15	0.65322581
<i>ABCB1</i>	2.4516129	0.40789474	1584	14	0.04358246	5.21428571	14	0.63709677
<i>CYP19A1</i>	2.48387097	0.4025974	1056	11	0.03488776	5.09090909	11	0.62903226
<i>RORC</i>	2.5483871	0.39240506	1208	11	0.02853011	5.72727273	11	0.61290323
<i>CASP3</i>	2.64516129	0.37804878	808	12	0.02309025	4.75	12	0.58870968
<i>HSD11B1</i>	2.74193548	0.36470588	320	7	0.01974163	4.28571429	7	0.56451613
<i>CHRM1</i>	2.74193548	0.36470588	678	8	0.01496092	6	8	0.56451613
<i>CHRM3</i>	2.77419355	0.36046512	434	7	0.01083324	6.14285714	7	0.55645161
<i>IL2</i>	2.80645161	0.35632184	362	8	0.00982137	5.125	8	0.5483871
<i>CYP2D6</i>	2.83870968	0.35227273	326	7	0.00693289	5.71428571	7	0.54032258
<i>BCL2</i>	2.87096774	0.34831461	176	5	0.00748209	4.8	5	0.53225806
<i>TLR9</i>	2.93548387	0.34065934	100	4	0.0027163	6.5	4	0.51612903
<i>CHRM2</i>	3	0.33333333	92	5	0.00225939	6.4	5	0.5
<i>TNF</i>	3.03225806	0.32978723	74	4	0.0018716	5.25	4	0.49193548
<i>TP53</i>	3.03225806	0.32978723	80	5	0.00205515	5.8	5	0.49193548
<i>HLA-DRB</i>	3.12903226	0.31958763	26	3	8.47E-04	5.66666667	3	0.46774194
<i>NR3C1</i>	3.16129032	0.31632653	0	1	0	8	1	0.45967742
<i>SERPINA1</i>	3.16129032	0.31632653	0	1	0	8	1	0.45967742
<i>CHRM4</i>	3.16129032	0.31632653	2	2	7.56E-05	7	2	0.45967742
<i>PSMB9</i>	3.16129032	0.31632653	2	2	7.56E-05	7	2	0.45967742
<i>ST14</i>	3.19354839	0.31313131	2	2	8.81E-05	6	2	0.4516129
<i>IL1B</i>	3.25806452	0.30693069	0	1	0	5	1	0.43548387
<i>ALB</i>	3.29032258	0.30392157	0	1	0	4	1	0.42741935
<i>BLK</i>	3.29032258	0.30392157	0	1	0	4	1	0.42741935
<i>CYP2A6</i>	3.32258065	0.30097087	0	1	0	3	1	0.41935484
<i>CYP2C19</i>	3.32258065	0.30097087	0	1	0	3	1	0.41935484

3.3 Network construction and analysis

The drug-active ingredient-target point network was constructed using Cytoscape software. As shown in Fig. 2, there were 63 nodes and 175 edges, of which yellow indicates Yangyin Yiqi Huoxue Recipe, orange indicates the 41 active compounds, and green indicates the 26 action targets. The edges represent the interaction of active ingredients with SS disease targets.

Each active ingredient could act on multiple targets, and each target was also related to multiple active compounds. This showed that Yangyin Yiqi Huoxue Recipe has a common effect on different compounds when exerting pharmacological effects.

3.4 GO enrichment analysis and KEGG signal pathway analysis

The human genome annotation database DAVID 6.8 was used to carry out GO enrichment analysis and KEGG signal pathway analysis on the 26 target points obtained above, using $P < 0.05$ as the screening condition to screen out biological processes and pathways with a large number of enriched genes.

Table 3
Top eight biological functions from the GO analysis of target genes

Groups	Function	Group Genes
Group00	phospholipase C-activating G-protein coupled acetylcholine receptor	<i>CHRM4, CHRM3, CHRM2, CHRM1</i>
Group01	adenylate cyclase-inhibiting G-protein coupled acetylcholine receptor	<i>CHRM4, CHRM3, CHRM2, CHRM1</i>
Group02	synaptic transmission, cholinergic	<i>CHRM4, CHRM3, CHRM2, CHRM1</i>
Group03	G-protein coupled acetylcholine receptor signaling pathway	<i>CHRM3, CHRM2, CHRM1</i>
Group04	negative regulation of apoptotic process	<i>CASP3, ALB, BLK, BCL2, TP53</i>
Group05	regulation of vascular smooth muscle contraction	<i>CHRM3, CHRM1</i>
Group06	positive regulation of transcription from RNA polymerase II promoter 5	<i>TNF, TP53, NR3C1, IL2, TLR9</i>
Group07	saliva secretion	<i>CHRM3, CHRM1</i>

PART 2

4.1 CCK-8 detects cell proliferation

The test results are shown in the Fig. 6. Compared with the normal control (NC) group, the cell viability of the YYYQHX Recipe group at concentrations of 60 and 80 mg/ml was significantly lower ($P < 0.01$), and with the increase of the drug action time, low-dose YYYQHX Recipe also began to show cytotoxicity. When the drug had acted for 72 h, the cell survival rates in the YYYQHX Recipe group at concentrations of 20, 40, 60, and 80 mg/ml were all $< 90\%$, and the difference between NC group and YYYQHX Recipe groups was statistically significant.

Compared with the NC group, when HCQ was used to treat the cells for 24 h, each dose group showed no cytotoxicity. When the drug action time was prolonged, the cell survival rate started to be lower than 90% at the concentration of 0.28 mg/ml at 48 h, and under the condition of HCQ action for 72 h, the cell viability in the five HCQ dose groups was significantly reduced ($P < 0.01$): the higher the dose, the lower the cell survival rate.

4.2 q-PCR detection

The test results are shown in the Fig. 7. Compared with the control group, YYYQHX Recipe (10 mg/ml) up-regulated the levels of expression of *CHRM3* ($P < 0.05$) and *AQP5* ($P < 0.01$). The HCQ group (3 mg/ml) also showed up-regulation of the expression of *AQP5* ($P < 0.01$), but had no significant effect on *CHRM3*.

Discussion

SS is a chronic autoimmune disease. As with most autoimmune diseases, the etiology of Sjögren's syndrome is not yet fully understood. There is currently no specific medicine in the treatment of SS^[8]. Hydroxychloroquine is a highly safe immunosuppressant with few side effects. It is the drug of choice when SS is mild to moderate. It can relieve clinical symptoms such as dry mouth, dry eyes, joint pain, arthritis, skin lesions and fatigue to a certain extent^[9]. When the patient has severe organ manifestations, the treatment requires the use of large doses of methylprednisolone and cyclophosphamide, which have been proven to be effective^[1].

Traditional Chinese medicine has a long history and good clinical effects in treating diseases such as SS. Traditional Chinese medicine with the characteristics of holistic treatment has attracted increasing attention worldwide because of its satisfactory clinical effects^[10, 11]. As the main body and main form of Chinese medical practice, Chinese herbal medicine involves a variety of herbal ingredients and hundreds of chemical components^[12]. Therefore, it is difficult to understand fully its effective ingredients and mechanisms of action, which depend on the overall interaction between all the ingredients.

In recent years, mass spectrometry technology has been widely used in the study of chemical components of traditional Chinese medicine, activity screening, and the study of small molecular compounds in metabolomics, and has played a huge role in elucidating the material basis and mechanisms of action of traditional Chinese medicine^[13, 14].

In this study, we used UPLC-LTQ-Orbitrap-MSⁿ technology to analyze a total of 41 active ingredients in Yangyin Yiqi Huoxue Recipe, combined with network pharmacological analysis. There appear to be 26 targets of Yangyin Yiqi Huoxue Recipe for treating SS. Among them, the target

gene CHRM3 was most prominent. Through literature research, it was found that CHRM3 is closely related to the pathogenesis of SS. Therefore, CHRM3 was determined to be an important target for cell experiment verification.

Decreased saliva secretion is one of the important clinical manifestations of SS, and CHRM3 is closely related to saliva secretion^[15,16]. Studies have shown that M3 receptors are the most abundantly expressed subtype in the submandibular glands of different species such as humans, rats, mice, and rabbits, and are mainly distributed on acinar cells and vascular endothelial cells^[15].

The development of CHRM3 knockout mice is delayed after weaning, and intraperitoneal injection of the mAChR agonist pilocarpine caused little stimulation of salivation^[17]. Studies have demonstrated that the saliva secretion of CHRM3 knockout mice is about 90% less than that of wild-type mice, and the knockout mice need to drink continuously to supplement the lack of saliva when eating, while M1 receptor gene knockout mice have no such phenotype^[18,19]. This suggests that CHRM3 is the main gene involved in regulating saliva secretion.

AQP5 is an important protein that mediates the rapid transcellular transport of water in salivary gland cells^[20]. AQP5 contributes to salivary secretion in patients with SS^[21]. Abnormal distribution of AQP5 in labial salivary glands is associated with poor saliva secretion in patients with SS, including patients with the complication neuromyelitis optica. In the submandibular glands (SMG) of patients and mice with diabetes, the expression of AQP5 is reduced, which leads to the dysfunction of diabetic SMG, and saliva production decreases^[21].

There are also theories that the abnormal subcellular distribution and transport of AQP5 may be one of the important mechanisms of the pathogenesis of SS^[22]. In the submandibular acinars of the non-obese diabetic(NOD) mice with inflammatory infiltration, the expression of AQP5 in the apical plasma membrane is decreased and the expression in the basement membrane is increased. Konttinen et al.^[23] found that the expression of AQP5 in the salivary gland epithelium of mice with experimental SS was decreased or absent, and the expression of AQP5 increased in the myoepithelial cells. These results indicate that the distribution and content of AQP5 are important factors that determine the transcellular transport of water.

We observed the levels of expression of the CHRM3 and AQP5 genes in submandibular gland cells under the action of Yangyin Yiqi Huoxue Recipe and hydroxychloroquine in experimental cell studies. From the experimental results, we found that the levels of expression of the CHRM3 and AQP5 genes in submandibular gland cells were significantly increased under the action of Yangyin Yiqi Huoxue Recipe. The hydroxychloroquine group showed up-regulation of the expression of the AQP5 gene, but no obvious up-regulatory effect on CHRM3. This experimental result confirmed that CHRM3, obtained from the previous network pharmacological analysis, is one of the important targets of the effect of Yangyin Yiqi Huoxue Recipe. The CHRM3 and AQP5 genes are closely involved in the secretion of saliva. This may be the mechanism by which Yangyin Yiqi Huoxue Recipe can improve symptoms such as dry mouth in SS.

Conclusions

In this study, using a combination of UPLC-LTQ-Orbitrap-MSⁿ technology, network pharmacological analysis and experimental verification, a comprehensive strategy was adopted to illustrate the active ingredients and molecular mechanisms of Yangyin Yiqi Huoxue Recipe in the treatment of SS. Using UPLC-LTQ-Orbitrap-MSⁿ technology, 41 kinds of active ingredient were determined in the herbal database and used for network pharmacological analysis to allow interpretation of the active ingredients in the whole prescription more objectively. Based on the results of network pharmacological analysis and literature research, this study identified CHRM3 as the research target. Through experimental verification of the target CHRM3, the mechanism of Yangyin Yiqi Huoxue Recipe in treating SS was elucidated from the perspective of "drug-component-target". However, because of the small number of targets, research on signal pathways could not be presented here. This study reveals the complex components and pharmacological mechanism of action of Yangyin Yiqi Huoxue Recipe, and identifies potential therapeutic targets, which could provide a theoretical basis for the research and development of new drugs for the treatment of SS.

Abbreviations

CHRM3
M3 muscarinic acetylcholine receptor
AQP5
aquaporin 5
TCM
traditional Chinese medicine
YYYQH X Recipe
Yangyin Yiqi Huoxue
SS

Sjogren's syndrome
HCQ
hydroxychloroquine
q-PCR
Quantitative PCR
SMG
submandibular glands
NOD
non-obese diabetic

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data used to support the findings of this study are available from the corresponding author upon request.

Competing interests

The authors declare that they have no competing interests.

Funding

The study was supported by National Natural Science Foundation of China (No. 81473604) and Natural Science Foundation of Zhejiang Province, China (No. LY19H270013)

Authors' contributions

Ww Lu contributed to analysis and manuscript preparation, was a major contributor in writing the manuscript; Tx FU Fp Wu and Fl Xiong helped perform the analysis with constructive discussions; Ty LI contributed to the conception of the study; Gl WU performed the data analyses and wrote the manuscript; All authors read and approved the final manuscript.

ACKNOWLEDGMENTS

The authors would like to acknowledge the technical support of Ge Zhiwei from the School of Pharmacy, Zhejiang University.

We thank International Science Editing (<http://www.internationalscienceediting.com>) for editing this manuscript.

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Figures

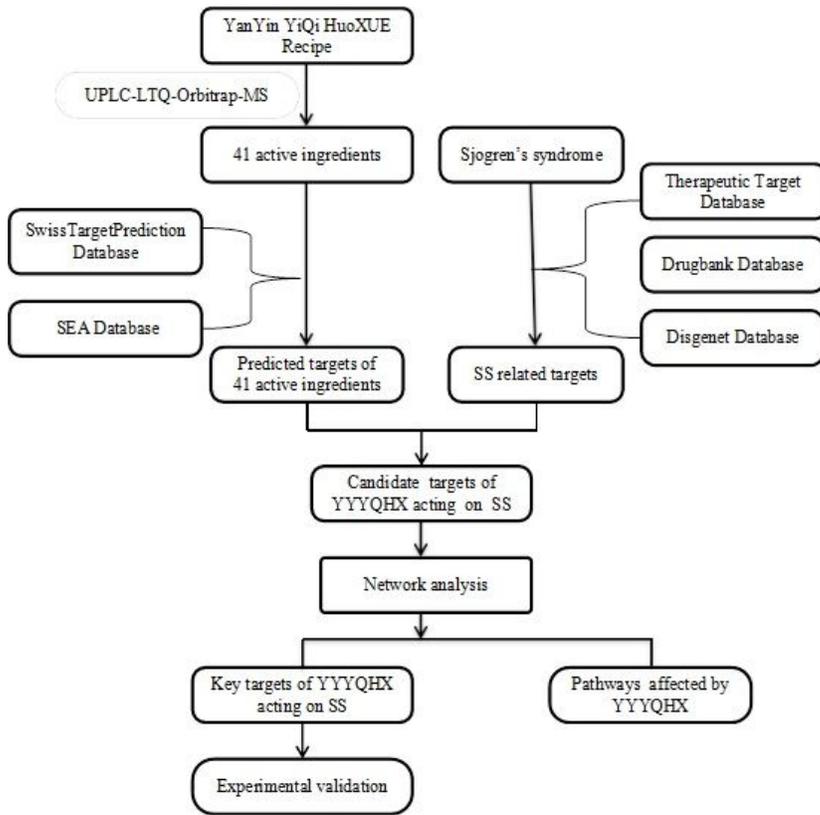


Figure 1

A schematic diagram of UPLC-LTQ-Orbitrap-MS analysis of components, network pharmacology and experimental validation for investigation of the mechanisms of Yangyin Yiqi Huoxue Recipe (YYYQH) against SS.

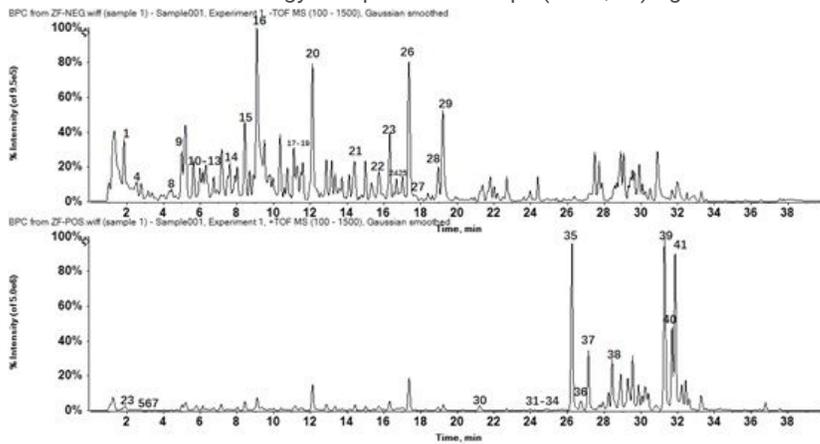


Figure 2

Diagram of the positive and negative ions of Yangyin Yiqi Huoxue Recipe

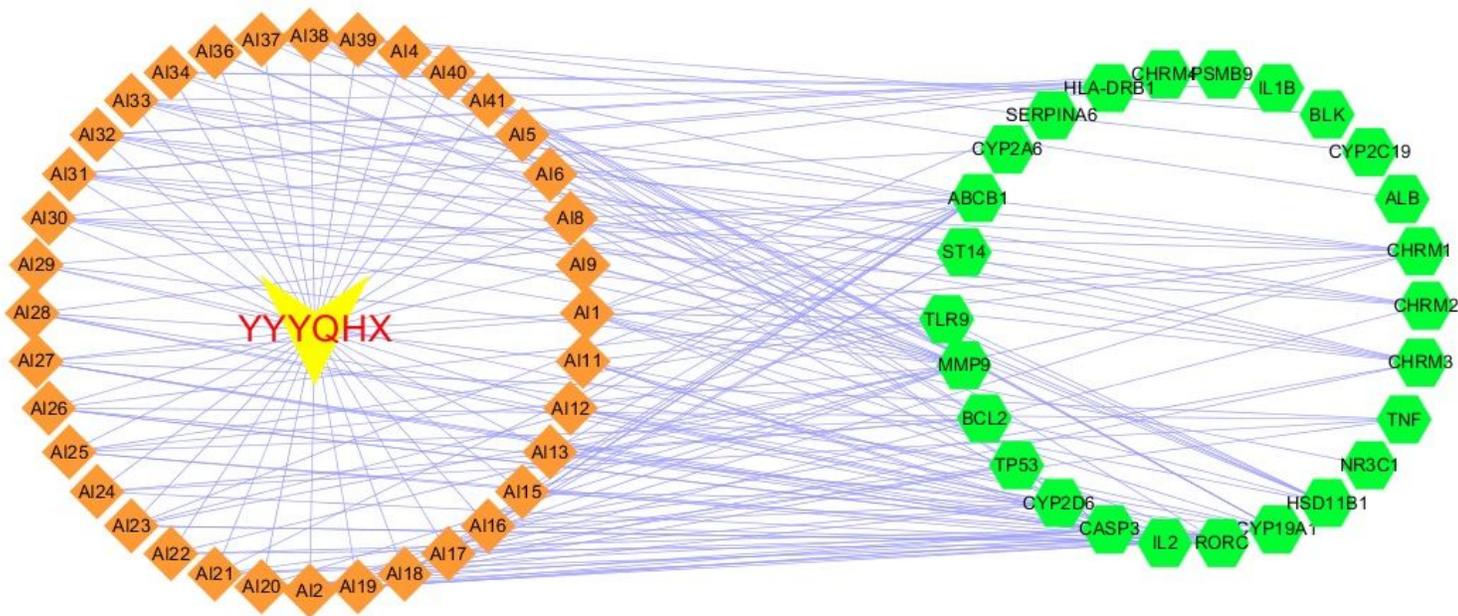


Figure 3

Drug-active compound-target relationship diagram. Yellow indicates YYYQHIX Recipe, orange indicates active compound, green indicates the target of these active compounds for treating SS.

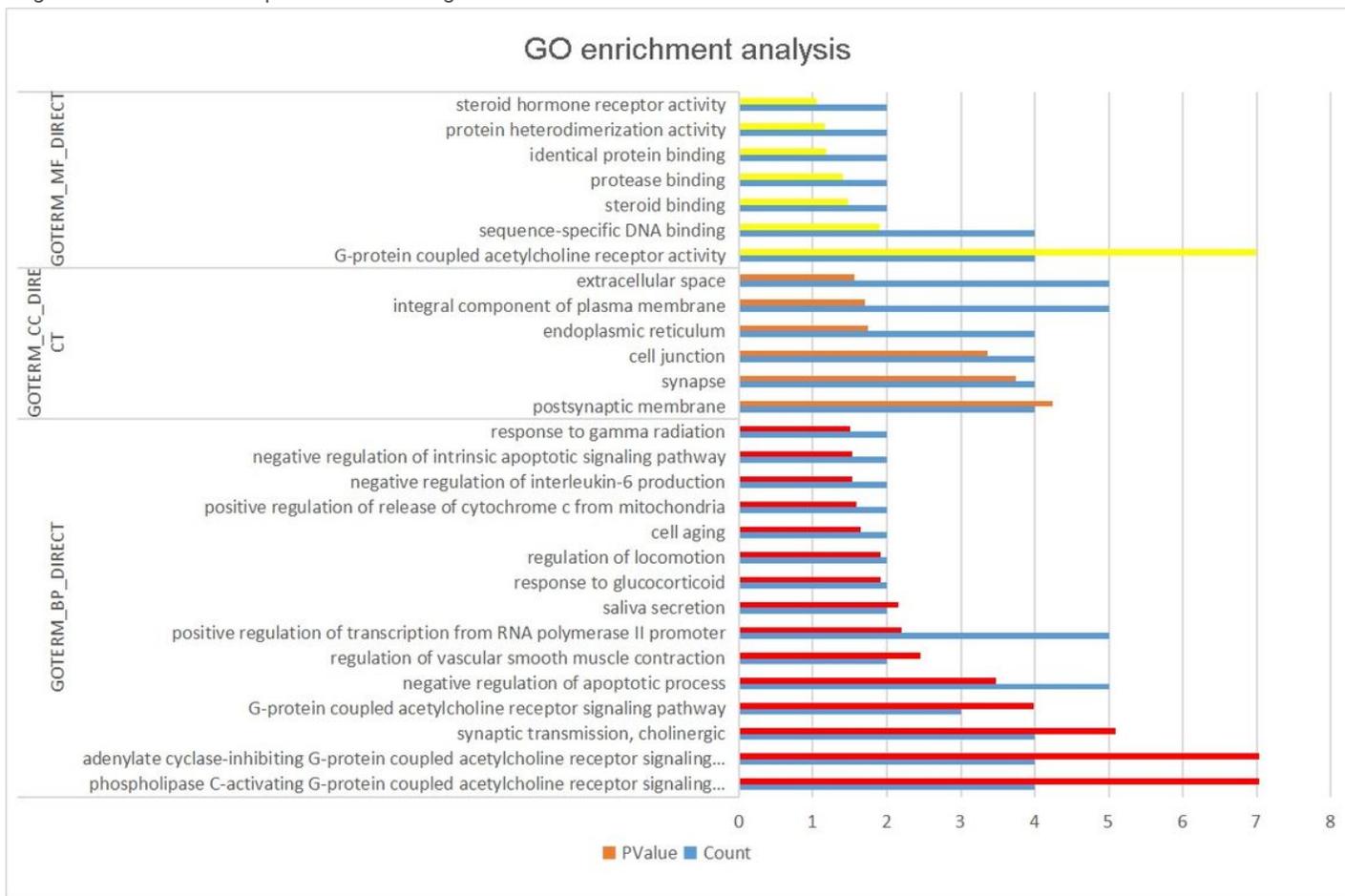


Figure 4

GO enrichment analysis on the 26 target points

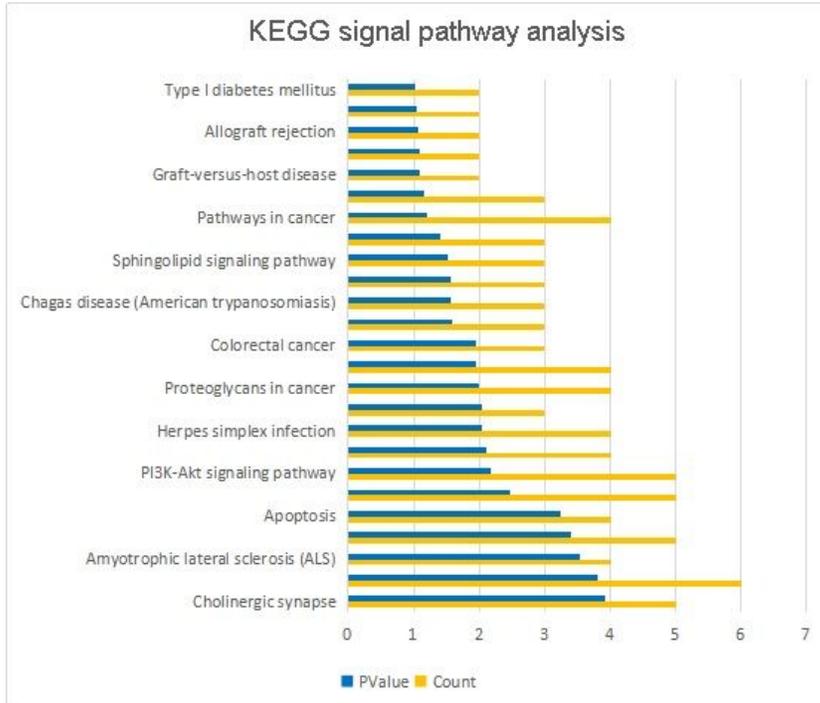


Figure 5

KEGG signal pathway analysis on the 26 target points

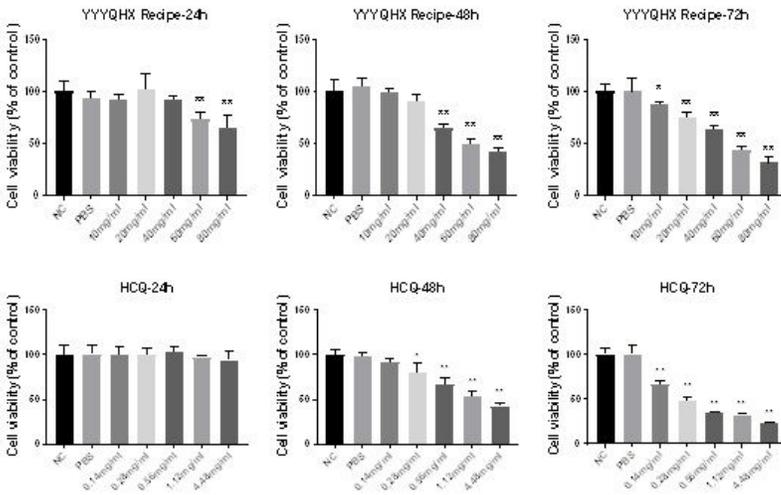


Figure 6

CCK-8 detects cell proliferation in YYYQHX Recipe groups and HCQ Groups

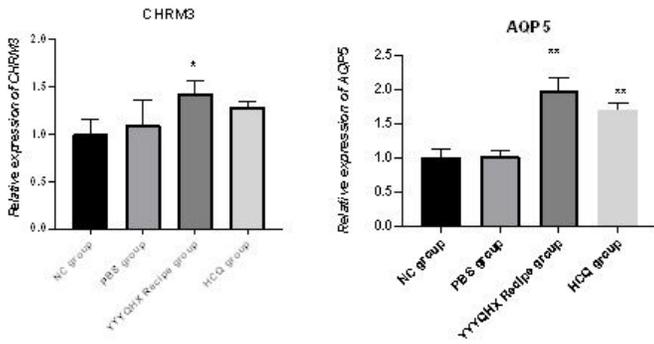


Figure 7

YYYQHX Recipe (10 mg/ml) up-regulated the levels of expression of CHR3 ($P < 0.05$) and AQP5 ($P < 0.01$). The HCQ group (3 mg/ml) also showed up-regulation of the expression of AQP5 ($P < 0.01$).