Study on the Mechanisms and Experimental Verification of *Cuscuta-Salvia* (Tusizi-Danshen) in the Treatment of Polycystic Ovary Syndrome (PCOS) via Network Pharmacology

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Research

Keywords: Cuscuta-Salvia, PCOS, Network pharmacology, Ovary, Core gene

Posted Date: July 8th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-642886/v1>

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Abstract

Polycystic ovary syndrome (PCOS) is a sort of endocrine disease associated with Reproduction. The formula of *Cuscuta-Salvia* has been widely used in treatment of PCOS in clinic. However, its chemical and pharmacological are still not known in detail. First, the active components of *Cuscuta-Salvia* were identified by UHPLC-ESI-Q-TOF-MS and screened from TCMSP database, and the disease targets were obtained from the DisGeNET and GeneCards databases. Subsequently, common targets between *Cuscuta-Salvia* and PCOS were obtained *via* a Venn diagram. Second, a protein-protein interaction (PPI) network was established. Core genes were selected using Cytoscape software plugin. Third, the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis were performed for common targets using “pathview” package in R. Finally, several core targets were verified *via* determination of qRT-PCR and HE staining. Combined UHPLC-ESI-Q-TOF-MS analysis with network pharmacology study, 14 active components were obtained. Eighty common targets were also obtained. Ten core genes were regulated by *Cuscuta-Salvia* in PCOS, including IL6, AKT1, VEGFA, TP53, TNF, MAPK1, JUN, EGF, CASP3, and EGFR. GO results showed that cellular response to drug, response to oxygen levels, response lipopolysaccharide, and response to molecule of bacterial origin in biological process (BP) category; membrane, transcription regulator complex, nuclear chromatin, postsynaptic membrane, and vesicle lumen in cellular component (CC) category; DNA-binding transcription factor binding, RNA polymerase II-specific DNA-binding transcription factor binding, DNA-binding transcription activator activity, RNA polymerase II-specific, DNA-binding transcription activator activity, and cytokine receptor binding in molecular function (MF) terms. KEGG enrichment pathway mainly involves in PI3K–Akt signaling pathway, MAPK signaling pathway, cellular senescence, TNF signaling pathway, and IL-17signaling pathway. Furthermore, based on an experimental study, *Cuscuta-Salvia* ameliorated the pathology of ovary, live and adipose tissue. Additional, *Cuscuta-Salvia* increased the mRNA expression of VEGFA. *Cuscuta-Salvia* decreased the mRNA expression of IL6, AKT1, TP53, MAPK1, JUN, EGF, AR, LHb, CYP17a1, and CYP19a1. Our results demonstrate that *Cuscuta-Salvia* may provide a novel pharmacology basis in an experimental model of PCOS *via* the regulation of the gene expression. This study lays a basis for subsequent research and clinical application.

1. Instruction

Polycystic ovary syndrome (PCOS) is a common and complex endocrine disorder, with patients suffering from androgen excess, oligomenorrhea or amenorrhea, and polycystic ovaries. These are related to insulin resistance, metabolic abnormality and infertility (Escobar-Morreale et al. 2012), and 18% of women are affected by PCOS in their premenopausal age (Kakoly et al. 2018). At present, PCOS treatment mainly counts on anti-androgen drugs, insulin sensitizers, and ovulation promoting drugs (Zeng et al. 2020).The etiology of PCOS is still unclear and its process is very complex (Liu et al. 2020a). However, traditional Chinese medicine (TCM) possesses multi-targets to treat some diseases.

TCM has been widely applied in the treatment of PCOS in China (Lu et al. 2016), in which *Cuscuta-Salvia* is used to treat PCOS as a kind of common Chinese herb formula in clinic. *Cuscuta* (Tusizi in Chinese)

belongs to convolvulaceae family and is regarded as holoparasitic angiosperms (Kaga et al. 2020). A body of evidences have shown that *Cuscuta chinensis* flavonoids, as main component of *Cuscuta*, have an effect on the endocrine function, which include the improvement of reproductive hormones levels (Han et al. 2020), estradiol (Wei et al. 2020), and glycolipid metabolism (Miao et al. 2019). And according to the theory of TCM, *Cuscuta* possesses the property of nourish liver and kidney, and benefiting Yin. The other herb is *Salvia* (Danshen in Chinese). *Salvia* belongs to the lamiaceae family (Marrelli et al. 2021). Tanshinone is extracted from the roots of *Salvia*. Prior work has reported that tanshinone can improve body weight, ovarian indicators, and other reproductive hormones (testosterone, androstenedione, luteinizing hormone, etc.) (Yu et al. 2014). And according to the theory of TCM, *Salvia* has the property of relieving menstruation and pain. Thus, based on aforementioned properties, we speculated that the combination of *Cuscuta* and *Salvia* could improve PCOS.

In recent time, network pharmacology was used to uncover the active components and potential mechanisms of Chinese herb pair according to the theory of systems biology. The study is designed to screen out these bioactive components and targets of PCOS using network pharmacology. Subsequently, animal experimental verification was carried out to investigate the relationship between candidate genes and potential mechanisms of PCOS, which will enhance the possibility of *Cuscuta* and *Salvia* in the treatment of PCOS. The workflow is presented in Fig. 1.

2. Materials And Methods

2.1 Analysis of *Cuscuta-Salvia* using UHPLC-ESI-Q-TOF-MS

To identify active components of *Cuscuta-Salvia*, an analysis of UHPLC-ESI-Q-TOF-MS was conducted. The conditions were as follows: the chromatographic analysis of *Cuscuta-Salvia* was performed on ACQUITY UPLC BEH C₁₈ column (2.1×100mm, 1.7μm, Waters Corporation, Ireland) at a flow rate of 0.3mL/min and room temperature. The volume of each injection was 3μL. The mobile phase was composed of acetonitrile (A) and 0.1% formic acid (B): 0–2 min, 95% B; 2–22 min, 95%-0% B; 22–23 min, 0%-0% B; 23–23.5 min, 0%-95% B; 23.5–25 min, 95%-95% B. Turboion Spray ion source and ESI positive and negative ion scanning mode were used to perform the Time of Flight Mass Spectrometry (TOF-MS). The optimal TOF-MS conditions were as follows: sample cone was 40 kV, source offset 80 kV, source temperature was 100°C, desolvation temperature: 400°C, cone gas was 50 L/h, desolvation gas was 800 L/h, nebulizer was 6.0 bar, scanning time was 0.2 s/spectra, and scanning m/z range was 50-1200 Da. Under positive ion mode, capillary voltage was 3.0 kV, and capillary voltage was 2.5 kV under negative ion mode. In this study, the active components were identified by SCIEX OS software 1.4 based on the first-order accurate mass number, isotope distribution ratio, and MS/MS of the components.

2.2 Identification for active components of *Cuscuta-Salvia*

To harvest the active components of *Cuscuta-Salvia*, we used databases, including the Traditional Chinese Medicine Systems Pharmacology (TCMSP, <https://tcmspw.com/tcmsp.php>, version 2.3) (Ru et

al. 2014).

Based on drug property of absorption, distribution, metabolism, and excretion (ADME), we screened and identified bioactive components, rather than bad pharmacological compounds (those bad drug ability compounds were removed) (Xu et al. 2020). Thus, to obtain the fully active components, we regarded two conditions as the criteria for screening the candidate components, including oral bioavailability (OB) $\geq$ 30% and drug-likeness (DL) $\geq$ 0.18 (Liu et al. 2018). The results were intersected with the results of UHPLC-ESI-TOF-MS analysis. Finally, the component prediction targets of the two databases were combined to establish the database of chemical constituents of *Cuscuta-Salvia*.

2.3 Collection of candidate genes of polycystic ovary syndrome (PCOS)

We used “Polycystic ovary syndrome” as index keywords and selected species were limited to “Homo sapiens” in search for therapeutic targets for PCOS. To obtain candidate genes of PCOS, databases of DisGeNET (<http://www.disgenet.org/>) and GeneCards (<http://genealacart.genecards.org/>) (relevance scores is 5 or more) were performed (Wang et al. 2020). Duplicates of the results of candidate genes were removed to obtain the target related to PCOS.

2.4 Establishment of herb-compound target-PCOS target network of *Cuscuta-Salvia*

Common targets between herbs and disease were generated *via* establishment of a Venn diagram (<https://bioinfogp.cnb.csic.es/tools/venny/>) of their intersected gene symbols. And then we used Cytoscape v3.7.2 (www.cytoscape.org/) to construct herb-compound target-PCOS target network of *Cuscuta-Salvia* (Yang et al. 2019a). In the network, herbs, bioactive compounds, and their related targets were expressed as nodes, and whereas the interaction between nodes were expressed as edges. The “degree” value of the node is the number of links connected to node. The bigger the degree value is, the more important the target is.

2.5 Establishment of protein-protein interaction (PPI) network

To interpret the interaction between target proteins, the common targets were input into STRING (<http://string-db.org>) online website to obtain PPI network. Protein interactions with a confidence score $>$ 0.4 were analyzed (Wang et al. 2020). Next, the nodes and score information were input into the Cytoscape v3.7.2 software for visual analysis. According to Cytohubba plugin, the top of 10 genes were obtained. And then the potential targets were predicted.

2.6 Gene Ontology (GO) functional enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG)

pathway enrichment analysis

Using “pathview” package in R for *Cuscuta-Salvia* in the treatment of PCOS, GO functional analysis and KEGG pathway enrichment analysis were performed for common targets. GO functional analysis is mainly used to annotate gene functions, including three ontologies: biological processes (BP), cellular components (CC), and molecular functions (MF) (Expansion of the Gene Ontology knowledgebase and resources 2017; Gene Ontology Consortium: going forward 2015). KEGG enrichment analysis is useful for enrichment analysis of common targets of PCOS (Kanehisa et al. 2017). $P < 0.05$ was recognized as statistically significant for both GO and KEGG analysis.

2.7 Preparing for animal models of PCOS in mice

24 female C57BL/6 mice (~ 3 week old) were obtained from the Animal Center of Zhejiang Chinese Medical University (Hangzhou, Zhejiang, China), Certificate NO: SYXK (Zhejiang province) 2021-0012. Mice were housed in a Specific Pathogen Free (SPF)-degree facility (20 ~ 26°C and 12-h light/dark cycle). Standard fodder and tap water were available in the mice cages at the Laboratory Animal Research Center. All the procedures were approved by the Animal Ethics Committee, Zhejiang Chinese Medical University.

All mice were randomly divided into 4 groups: normal control group (NC), model group (Model), *Cuscuta-Salvia* group (CS), and metformin group (Met), 6 mice each group. After adaptive feeding for one week, all mice (except for NC) were intragastrically (i.g.) administrated of letrozole solution (3mg/kg) and NC mice were i.g. administrated of normal saline for one month, respectively. Meanwhile, CS mice were oral given 0.1ml/10g of *Cuscuta-Salvia* extraction, Met mice were oral given 50mg/kg of metformin, and the other mice were oral given the same amount of normal saline for 30 days. Signs of successful modeling: vaginal smears were performed, and estrous cycle stage got disordered.

2.8 PCOS mice weighing

The body weight of the mice was measured every day, ranging from Day 1 to Day 25.

2.9 Oral glucose tolerance test (OGTT) assay

After fasted for 12h, all mice were measured with an ACCU-CHEK Performa at 9 o'clock. All mice were oral given by glucose solution at doses of 2g/kg body weight. One drop of fresh blood was collected from each tail of mice using glucometer to measure glucose concentration. Meanwhile, measurements were performed based on time of 0, 30, 60, 90, and 120 min (Yang et al. 2019b). The total area under the glucose response curve (AUC_{0-2h}) was calculated using GraphPad Prism 6 software.

2.10 Hematoxylin and eosin (HE) staining

Ovary tissues, liver tissues, and adipose tissue from the mice were fixed in 4% PBS-paraformaldehyde solution (pH7.4) at room temperature for 48h and embedded in paraffin. After dehydration and clearing,

these tissues immersed in wax, next cut into 5- μ m-thick slices. The slices were dewaxed and stained with hematoxylin and eosin.

2.11 Determination of quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from ovary tissues in mice using TRIzol reagent (ambion |RNA by life technologies). cDNA was synthesized with FastKing RT kit (With gDNase). Gene expression was determined by qRT-PCR using SYBR Green Master Mix (Bio-Rad) and the primer with the sequences listed in Table 1. The qRT-PCR procedure was performed as follows: UDG activation step at 50°C for 2 min, Dual-Lock™ DNA polymerase step at 95°C for 2 min, denaturation step at 95°C for 15 s, annealing step at 58°C for 15 s, elongation step at 72°C for 1 min, 40 cycles. Relative quantification was calculated using the comparative Ct ($2^{-\Delta\Delta C_t}$) method (Ren et al. 2020).

Table 1
The primers for qRT-PCR

IL6	F: CCAAGAGGTGAGTGCTTCCC	118
IL6	R: CTGTTGTTCACTCTCTCCCT	
ID	Sequence (5'-3')	Product Length (bp)
β -actin	F: TGTGATGGTGGGAATGGGTCAGAA	140
β -actin	R: TGTGGTGCCAGATCTTCTCCATGT	
Akt1	F: ATGAACGACGTAGCCATTGTG	116
Akt1	R: TTGTAGCCAATAAAGGTGCCAT	
TP53	F: GCGTAAACGCTTCGAGATGTT	144
TP53	R: TTTTATGGCGGGAAGTAGACTG	
MAPK1	F: CCCAAGTGATGAGCCCATTG	248
MAPK1	R: TCAATGGAAGGGGACAACTGA	
JUN	F: TCGCTCGGCTAGAGGAAAAA	152
JUN	R: CTGCTGCGTTAGCATGAGTT	
EGF	F: AGGAGGTCCGCTAGAGAAATG	279
EGF	R: CAGGCGATGAACAACCAGTG	
CYP17a1	F: CTCCAGCCTGACAGACATTC	89
CYP17a1	R: CTGAGAACACACTTGGGTCC	
CYP19a1	F: GGGGACAGTATGCTGGCTAA	282
CYP19a1	R: GCCCAGCTTCTCCCTGTAAA	
AR	F: CGAAGTGTGGTATCCTGGTG	132
AR	R: CTGGTACTGTCCAAACGCAT	
FSHb	F: CTGGTGCTGGAGAGCAATCT	174
FSHb	R: ACTTTCTGGGTATTGGGCCG	
LHb	F: AACTCCCAAGCATCAGCCTC	171
LHb	R: AGATGTGGAGGTGGCTAGAG	

2.12 Statistical analysis

All data were showed as mean $\pm$ standard error of mean ($\bar{x}\pm\text{SEM}$). Statistical data were processed using SPSS 25.0 software and GraphPad Prism 6. Analysis of LSD of one-way variance test was used to determine the significance of difference in each group (homogeneity of variance and conformation to normal distribution). Otherwise, analysis of Dunnett was used to determine the significance of difference in groups. A value of $P<0.05$ was considered as statistically significant.

3. Results

3.1 Identification for the active components of *Cuscuta-Salvia*

The total ion flow diagram of *Cuscuta-Salvia* was obtain from UHPLC-ESI-Q-TOF-MS analysis, and the compounds were identified qualitatively by using SCIEXOS software 1.4. As shown in Fig 2 and Table 2, 13 compounds were identified under positive ion mode and 16 compounds were identified under negative ion mode.

Table 2. High resolution mass spectrometry data and elemental composition of *Cuscuta-Salvia*. (No. 1-16 was under negative ion mode and 17-29 was under positive ion mode)

No.	Molecular Name	Molecular Formula	Retention Time (min)	m/z	Measure Molecular Mass (Da)	Molecular Mass (Da)
1	Methyl tanshinate	C ₂₀ H ₁₈ O ₅	1.29	383.1137	338.1155	338.11542
2	Caffeate	C ₉ H ₈ O ₄	2.48	179.0354	180.0427	180.04226
3	Neocuscuscutosside A	C ₃₇ H ₄₆ O ₂₁	4.75	871.2478	826.2496	826.25316
4	Cuscutoside A	C ₃₁ H ₃₆ O ₁₆	5.89	663.1916	664.1989	664.20034
5	Salvianolic acid A	C ₂₆ H ₂₂ O ₁₀	6.2	539.12	494.1218	494.1213
6	Salvianolic acid D	C ₂₀ H ₁₈ O ₁₀	6.54	463.0889	418.0907	418.09
7	Tanshiquinone II	C ₁₉ H ₂₀ O ₄	6.96	357.134	312.1358	312.13616
8	Salvianolic acid C	C ₂₆ H ₂₀ O ₁₀	6.96	537.1043	492.1061	492.10565
9	Danshensu	C ₉ H ₁₀ O ₅	7.24	197.046	198.0533	198.05282
10	Isoimperatorin	C ₁₆ H ₁₄ O ₄	7.51	269.085	270.0923	270.08921
11	Salvianolic acid F	C ₁₇ H ₁₄ O ₆	7.51	313.0742	314.0815	314.07904
12	Tanshinone IIA	C ₁₉ H ₁₈ O ₃	7.87	339.1273	294.1291	294.12559
13	Isotanshinone I	C ₁₈ H ₁₂ O ₃	8.13	311.0488	276.0794	276.07864
14	Salvia miltiorrhiza	C ₂₁ H ₂₀ O ₄	10.2	371.108	336.1386	336.13616
15	Danshinspiroketallactone	C ₁₈ H ₂₂ O ₃	11.21	331.1562	286.158	286.15689
16	Methylenedihydrotanshinquinone	C ₁₈ H ₁₆ O ₃	11.22	325.1086	280.1104	280.10994
17	Protocatechualdehyde	C ₇ H ₆ O ₃	2.5	139.0393	138.0321	138.03169
18	Hydroxytanshinone IIA	C ₁₉ H ₁₈ O ₄	5.89	333.1086	310.1194	310.12051
19	Quercetin-3-O-β-D-glucopyranoside	C ₂₁ H ₂₀ O ₁₂	6.23	465.1032	464.0959	464.09548
20	Quercetin	C ₁₅ H ₁₀ O ₇	6.54	303.0502	302.0429	302.04265
21	Kaempferol-3-o-beta-d-glucoside	C ₂₁ H ₂₀ O ₁₁	7.09	449.1084	448.1011	448.10056
22	Kaempferol	C ₁₅ H ₁₀ O ₆	7.09	287.0555	286.0482	286.04774
23	Lithospermic acid B	C ₃₆ H ₃₀ O ₁₆	7.24	736.1883	718.1545	718.15338

24	Salvianolic acid G	C ₁₈ H ₁₂ O ₇	7.52	341.0662	340.0589	340.0583
25	Lithospermic acid	C ₂₇ H ₂₂ O ₁₂	7.83	539.1203	538.113	538.11113
26	15, 16-dihydrotanshinone I	C ₁₈ H ₁₄ O ₃	12.67	279.1009	278.0936	278.09429
27	Neotanshinone A	C ₁₈ H ₁₆ O ₄	12.67	297.1123	296.105	296.10486
28	Tanshinone IIB	C ₁₉ H ₁₈ O ₄	14.11	297.1488	296.1415	296.14124
29	Cryptotanshinone	C ₁₉ H ₂₀ O ₃	15.13	297.1491	296.1418	296.14124

3.2 Screening of the active components of *Cuscuta-Salvia*

A total of 231 chemical compounds between *Cuscuta* and *Salvia* were collected from TCMSP database, in which included 29 compounds in *Cuscuta* and 202 compounds in *Salvia*. Combined with the parameters of OB $\geq$ 30% and DL $\geq$ 0.18 in TCMSP, the 69 potential active components were screened out *via* removing the duplicate values, in which included 10 compounds related to *Cuscuta* and 59 compounds related to *Salvia*. In addition, the filtered results in TCMSP and the results of UHPLC-ESI-Q-TOF-MS were intersected. Thus, 14 active components were acquired. And the 14 active components were listed in Table 3.

Table 3. *Cuscuta* and *Salvia* active components list.

Herb	MOL ID	Molecule Name	Molecular Formula
<i>Cuscuta</i>	MOL000422	kaempferol	C ₁₅ H ₁₀ O ₆
<i>Cuscuta</i>	MOL000098	quercetin	C ₁₅ H ₁₀ O ₇
<i>Salvia</i>	MOL001942	isoimperatorin	C ₁₆ H ₁₄ O ₄
<i>Salvia</i>	MOL007101	dihydrotanshinone I	C ₁₈ H ₁₄ O ₃
<i>Salvia</i>	MOL007154	tanshinone IIa	C ₁₉ H ₁₈ O ₃
<i>Salvia</i>	MOL007045	3α-hydroxytanshinone IIa	C ₁₉ H ₁₈ O ₄
<i>Salvia</i>	MOL007155	(6S)-6-(hydroxymethyl)-1,6-dimethyl-8,9-dihydro-7H-naphtho[8,7-g]benzofuran-10,11-dione	C ₁₉ H ₁₈ O ₄
<i>Salvia</i>	MOL007088	cryptotanshinone	C ₁₉ H ₂₀ O ₃
<i>Salvia</i>	MOL007108	isocryptotanshinone	C ₁₉ H ₂₀ O ₃
<i>Salvia</i>	MOL007120	miltionone II	C ₁₉ H ₂₀ O ₄
<i>Salvia</i>	MOL007093	dan-shexinkum d	C ₂₀ H ₁₈ O ₄
<i>Salvia</i>	MOL007141	salvianolic acid g	C ₂₆ H ₂₂ O ₁₀
<i>Salvia</i>	MOL007130	prolithospermic acid	C ₂₇ H ₂₂ O ₁₂
<i>Salvia</i>	MOL007111	Isotanshinone II	C ₁₈ H ₁₂ O ₃

3.3 Screening of the candidate genes of the active compounds in *Cuscuta-Salvia*

A total of 404 candidate targets from the 14 active compounds were collected in the TCMSP database. By means of eliminating the overlapping targets, 195 related targets were obtained (Table 4).

Table 4.195 related targets list.

No.	Gene Name	Protein Name
1	NOS2	Nitric oxide synthase 2
2	PTGS1	Cyclooxygenase-1
3	AR	Androgen receptor
4	PPARG	Peroxisome proliferator-activated receptor
5	PTGS2	Prostaglandin G/H synthase 2
6	HSP90AB1	Heat shock protein HSP 90-beta
7	PIK3CG	PI3-kinase subunit gamma
8	PRKACA	cAMP-dependent protein kinase catalytic subunit alpha
9	NCOA2	Nuclear receptor coactivator 2
10	DPP4	Dipeptidyl peptidase 4
11	PRSS1	Protease serine 1
12	PGR	Progesterone receptor
13	F2	Coagulation factor II
14	CHRM1	Muscarinic acetylcholine receptor M1
15	NOS3	Nitric oxide synthase 3
16	GABRA2	Gamma-aminobutyric acid receptor subunit alpha-2
17	ACHE	Acetylcholinesterase
18	SLC6A2	Sodium-dependent noradrenaline transporter
19	CHRM2	Muscarinic acetylcholine receptor M2
20	ADRA1B	Alpha-1B adrenergic receptor
21	GABRA1	Gamma-aminobutyric acid receptor subunit alpha-1
22	TOP2A	DNA topoisomerase 2-alpha
23	F7	Coagulation factor VII
24	RELA	Transcription factor p65
25	IKBKB	Inhibitor of nuclear factor kappa-B kinase subunit beta
26	AKT1	Threonine-protein kinase
27	BCL2	Apoptosis regulator BCL-2
28	BAX	Apoptosis regulator BAX

29	TNF	Tumor necrosis factor
30	JUN	Transcription factor AP-1
31	AHSA1	Activator of 90 kDa heat shock protein ATPase homolog 1
32	CASP3	Caspase-3
33	MAPK8	Mitogen-activated protein kinase 8
34	XDH	Xanthine dehydrogenase
35	MMP1	Matrix metalloproteinase-1
36	STAT1	Signal transducer and activator of transcription 1
37	CDK1	Cyclin-dependent kinase 1
38	HMOX1	Heme oxygenase 1
39	CYP3A4	Cytochrome P450 3A4
40	CYP1A2	Cytochrome P450 1A2
41	CYP1A1	Cytochrome P450 1A1
42	ICAM1	Intercellular adhesion molecule 1
43	SELE	E-selectin
44	VCAM1	Vascular cell adhesion protein 1
45	NR1I2	Nuclear receptor subfamily 1 group I member 2
46	CYP1B1	Cytochrome P450 1B1
47	ALOX5	Polyunsaturated fatty acid 5-lipoxygenase
48	HAS2	Hyaluronan synthase 2
49	GSTP1	Glutathione S-transferase 2
50	AHR	Aryl hydrocarbon receptor
51	PSMD3	26S proteasome non-ATPase regulatory subunit 3
52	SLC2A4	Solute carrier family 2 member 4
53	NR1I3	Nuclear receptor subfamily 1 group I member 3
54	INSR	Insulin receptor
55	DIO1	Death-inducer obliterator 1
56	PPP3CA	Calmodulin-dependent calcineurin A subunit alpha isoform
57	GSTM1	Glutathione S-transferase Mu 1

58	GSTM2	Glutathione S-transferase Mu 2
59	AKP1C3	Aldo-keto reductase family I member C3
60	SLPI	Antileukoproteinase
61	AKR1B1	Aldo-keto reductase family 1 member B1
62	KCMH2	Potassium voltage gated channel subfamily H member 2
63	SCN5A	Sodium channel protein type 5 subunit alpha
64	F10	Coagulation factor X
65	ADRB2	Beta-2 adrenergic receptor
66	MMP3	Matrix metalloproteinase-3
67	RXRA	Retinoic acid receptor RXR-alpha
68	MAOB	Amine oxidase
69	EGFR	Epidermal growth factor receptor
70	VEGFA	Vascular endothelial growth factor A
71	CCND1	G1/S-specific cyclin-D1
72	BCL2L1	Bcl-2-like protein 1
73	FOS	Proto-oncogene c-Fos
74	CDKN1A	Cyclin-dependent kinase inhibitor 1
75	EIF6	Eukaryotic translation initiation factor 6
76	CASP9	Caspase-9
77	PLAU	Urokinase-type plasminogen activator
78	MMP2	Matrix metalloproteinase-2
79	MMP9	Matrix metalloproteinase-9
80	MAPK1	Mitogen-activated protein kinase 1
81	IL10	Interleukin-10 receptor subunit alpha
82	EGF	Pro-epidermal growth factor
83	RB1	Retinoblastoma-associated protein
84	IL6	Interleukin-6 receptor subunit alpha
85	CDKN2A	Cyclin-dependent kinase inhibitor 2A
86	TP53	Cellular tumor antigen p53

87	ELK1	ETS domain-containing protein Elk-1
88	NFKBIA	NF-kappa-B inhibitor alpha
89	POR	NADPH-cytochrome P450 reductase
90	ODC1	Ornithine decarboxylase
91	CASP8	Caspase-8
92	TOP1	DNA topoisomerase 1
93	RAF1	RAF proto-oncogene serine/threonine-protein kinase
94	SOD1	Superoxide dismutase [Cu-Zn]
95	PRKCA	Protein kinase C alpha type
96	HIF1A	Hypoxia-inducible factor 1-alpha
97	RUNX1T1	Protein CBFA2T1
98	CDK1	Cyclin-dependent kinase inhibitor 1 B
99	HSPA5	Endoplasmic reticulum chaperone BiP
100	ERBB2	Receptor tyrosine-protein kinase erbB-2
101	ACACA	Acetyl-CoA carboxylase 1
102	CAV1	Caveolin-1
103	MYC	Myc proto-oncogene protein
104	F3	Tissue factor
105	GJA1	Gap junction alpha-1 protein
106	IL1B	Interleukin-1 beta
107	CCL2	C-C motif chemokine 2
108	PTGER3	Prostaglandin E2 receptor EP3 subtype
109	CXCL8	Interleukin-8
110	PRKCB	Protein kinase C beta type
111	BIRC5	Baculoviral IAP repeat-containing protein 5
112	DUOX2	Dual oxidase 2
113	HSPB1	Heat shock protein beta-1
114	TGFB1	Transforming growth factor beta-1 proprotein
115	SULT1E1	Sulfotransferase 1E1

116	MGAM	Maltase-glucoamylase
117	IL2	Interleukin-2
118	CCNB1	G2/mitotic-specific cyclin-B1
119	PLAT	Tissue-type plasminogen activator
120	THBD	Thrombomodulin
121	SERPINE1	Plasminogen activator inhibitor 1
122	COL1A1	Collagen alpha-1 (I) chain
123	IFNG	Interferon gamma
124	ALOX5AP	Arachidonate 5-lipoxygenase-activating protein
125	PTEN	Phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase
126	IL1A	Interleukin-1 beta
127	MPO	Myeloperoxidase
128	NCF1	Neutrophil cytosol factor 1
129	ABCG2	Broad substrate specificity ATP-binding cassette transporter ABCG2
130	NFE2L2	Nuclear factor erythroid 2-related factor 2
131	NQO1	NAD (P) H dehydrogenase [quinone] 1
132	PARP1	Poly [ADP-ribose] polymerase 1
133	COL3A1	Collagen alpha-1 (III) chain
134	CXCL11	C-X-C motif chemokine 11
135	CXCL2	C-X-C motif chemokine 2
136	DCAF5	DDB1-and CUL4-associated factor 5
137	CHEK2	Serine/threonine-protein kinase Chk2
138	CLDN4	Claudin-4
139	PPARA	Peroxisome proliferator-activated receptor alpha
140	PPARD	Peroxisome proliferator-activated receptor delta
141	HSF1	Heat shock factor protein 1
142	CRP	Cysteine-rich protein 2-binding protein
143	CXCL10	C-X-C motif chemokine 10
144	SHUK	Inhibitor of nuclear factor kappa-B kinase subunit alpha

145	SPP1	Sphingosine-1-phosphate phosphatase 1
146	RUNX2	Runt-related transcription factor 2
147	RASSF1	Ras association domain-containing protein 1
148	E2F1	Transcription factor E2F1
149	E2F2	Transcription factor E2F2
150	ACP3	Prostatic acid phosphatase
151	CTSD	Cathepsin D
152	IGFBP3	insulin-like growth factor-binding protein 3
153	IGF2	Insulin-like growth factor II
154	CD40LG	CD40 ligand
155	IRF1	Interferon regulatory factor 1
156	ERBB3	Receptor tyrosine-protein kinase erbB-3
157	PON1	Serum paraoxonase/arylesterase 1
158	PCOLCE	Procollagen C-endopeptidase enhancer 1
159	NPEPPS	Puromycin-sensitive aminopeptidase
160	HK2	Hexokinase-2
161	NKX3-1	Homeobox protein Nkx-3.1
162	RASA1	Ras GTPase-activating protein 1
163	HTR3A	5-hydroxytryptamine receptor 3A
164	ADRA1A	Alpha-1A adrenergic receptor
165	CHRNA7	Neuronal acetylcholine receptor subunit alpha-7
166	IGHG1	Immunoglobulin heavy constant gamma 1
167	NCOA1	Nuclear receptor coactivator 1
168	DRD1	D (1A) dopamine receptor
169	CHRM3	Muscarinic acetylcholine receptor M3
170	CHRM5	Muscarinic acetylcholine receptor M5
171	CHRM4	Muscarinic acetylcholine receptor M4
172	OPRD1	Delta-type opioid receptor
173	OPRM1	Mu-type opioid receptor

174	FASN	Fatty acid synthase
175	EDNRA	Endothelin-1 receptor
176	EDN1	Endothelin-1
177	NPM1	Nucleophosmin
178	ECE1	Endothelin-converting enzyme 1
179	PARP4	Protein mono-ADP-ribosyltransferase PARP4
180	CALCR	Calcitonin receptor
181	ITGB3	Intergrin beta-3
182	CA2	Intercellular adhesion molecule 2
183	ADRA1D	Alpha-1D adrenergic receptor
184	STAT3	Signal transducer and activator of transcription 3
185	APP	Amyloid-beta precursor protein
186	ESR1	Estrogen receptor
187	DRD2	D (2) dopamine receptor
188	CDK2	Cyclin-dependent kinase 2
189	PIM1	Serine/threonine-protein kinase pim-1
190	NR3C1	Glucocorticoid receptor
191	ESR2	Estrogen receptor beta
192	GSK3B	Glycogen synthase kinase-3 beta
193	CHEK1	Serine/threonine-protein kinase Chk1
194	CCNA2	Cyclin-A2
195	PTPN1	Tyrosine-protein phosphatase non-receptor type 1

3.4 Choosing of the candidate targets of PCOS

To search for candidate targets related to PCOS, we used “Polycystic ovary syndrome” as index keywords to identify 446 potential targets by removing duplicate values from the databases of DisGeNET and Genecard (relevance score $\geq$ 5). 80 common targets between the targets of *Cuscuta-Salvia* (195 potential targets) and the targets of PCOS were collected using a Venn diagram (Fig 3).

3.5 Construction of herb compound-PCOS target network

To determine the interaction between herb compounds and PCOS targets, the active components and common targets were input into Cytoscape software to establish the diagram of herb compound-PCOS target network (Fig 4). According to the value of degree, the top 5 of active components were presented (Table 5).

Table 5. The top 5 of active components of herb compound-PCOS target network.

NO.	Molecular Name	Degree	Closenessc Cntrality
<i>Cuscuta</i> 2	quercetin	69	0.69402985
<i>Cuscuta</i> 1	kaempferol	30	0.44711538
<i>Salvia</i> 3	tanshinone iia	14	0.3907563
<i>Salvia</i> 9	dan-shexinkum d	12	0.38429752
<i>Salvia</i> 12	Isotanshinone II	10	0.37804878

3.6 PPI network and core genes of disease-drug targets

We utilized STRING database to establish a *Cuscuta-Salvia* target network and PCOS targets network, and PPI network was visualized using Cytoscape v 3.7.2 software, which included 80 nodes and 1350 edges (Fig 5). In addition, according to the ranking of degree of nodes in STRING, the top 20 core targets were screened out. At the same time, PPI network was calculated and analyzed using Cytohubba plugin in Cytoscape v 3.7.2. As illustrated in Fig 6 and Table 6, in the PPI network, there was a total of 10 nodes and 45 edges. Ten nodes included IL6, AKT1, VEGFA, TP53, TNF, MAPK1, JUN, EGF, CASP3, and EGFR.

Table 6. Protein target information.

Gene name	Protein name	Degree	Closeness Centrality	Clustering Coefficient
IL6	Interleukin 6	68	0.87777778	0.51448639
AKT1	RAC-alpha serine/threonine-protein kinase	67	0.86813187	0.52464948
VEGFA	Vascular endothelial growth factor A	64	0.83157895	0.56597222
TP53	Cellular tumor antigen p53	63	0.82291667	0.5483871
MAPK1	Mitogen-activated protein kinase 1	62	0.81443299	0.54680063
TNF	Tumor necrosis factor	62	0.82291667	0.57588577
JUN	Transcription factor AP-1	60	0.7979798	0.58248588
EGF	Pro-epidermal growth factor	59	0.7979798	0.58036236
CASP3	Caspase-3	58	0.78217822	0.61766485
MAPK8	Mitogen-activated protein kinase 8	56	0.76699029	0.63181818

3.7 GO functional enrichment analysis and KEGG pathway enrichment analysis

To analyze the biological characteristics of predicted targets of *Cuscuta-Salvia* on PCOS in detail, a GO analysis and a KEGG enrichment analysis were carried out using “pathview” package in *R*. In term of GO terms included biological process (BP), cellular component (CC), and molecular function (MF) terms. It was found that BP category was mainly presented in cellular response to drug, response to oxygen levels, response lipopolysaccharide, and response to molecule of bacterial origin (Fig). CC category was mainly showed in membrane, transcription regulator complex, nuclear chromatin, postsynaptic membrane, and vesicle lumen (Fig). As shown in , MF category mainly included DNA-binding transcription factor binding, RNA polymerase II-specific DNA-binding transcription factor binding, DNA-binding transcription activator activity, RNA polymerase II-specific, DNA-binding transcription activator activity, and cytokine receptor binding. These terms can exert its therapeutic effects on PCOS.

To investigate the underlying pathways of *Cuscuta-Salvia* on PCOS, KEGG enrichment pathway analysis was performed. The filter was also set as an adjusted P -value<0.05 and q-value< 0.05. As showed in KEGG pathways were significantly enriched. The results of KEGG enrichment analysis mainly involved in PI3K–Akt signaling pathway, MAPK signaling pathway, cellular senescence, TNF signaling pathway, and IL-17signaling pathway, etc.

3.8 Effect of *Cuscuta-Salvia* on mice body weight in PCOS mice

To determine whether increased mice body weight in the letrozole-induced PCOS mice model, mice weight was measured. As shown in Fig 8A, compared with the NC group, body weight significantly increased in

Model group after day 15. Compared with Model group, body weight in the CS group and Met group decreased, in which body weight in the CS group was on the greater decrease than in the Met group.

3.9 Detection of OGTT in PCOS mice

To examine glucose tolerance of letrozole-induced PCOS mice model, Oral glucose tolerance test (OGTT) assay was conducted. Compared with NC group, the AUC of model group significantly increased ($P<0.01$). Compared with model group, the AUC of CS group and Met group decreased ($P<0.05$). At the same time, There were no differences of AUC of blood glucose levels between CS group and Met group (Fig 8B-C).

3.10 Histological change in ovary tissues, liver tissues, and adipose tissue

As shown in Fig 9, in mice ovary tissues, NC group mice have many follicles in various stages of development at the site of a peripheral cortex. We observed normal follicular developmental synchronization, stromal tissue, and normal morphology. In term of model group mice, there were a large number of antral follicles. We also observed incompact follicles, increased medullar area, and enlarged vessels network. Also, there were cystic follicles and hemorrhagic cysts. In CS group and Met group, it was found that morphology in ovary was arranged more neatly than model group. Meanwhile, CS group mice were more neatly than Met group mice.

As shown in Fig 10, in mice liver tissues, there were the number of adipocyte in NC group more than model group. Compared with model group, CS group and Met group results showed that the number of adipocyte significant reduced.

As shown in Fig 11, in mice adipose tissue, there was enhanced irregular abdomen adipocyte and inflammatory infiltration in model group. Compared with model group, CS group and Met group results showed that abdomen adipocyte and inflammatory infiltration got improved significantly.

3.11 Effect of *Cuscuta-Salvia* on the ovary tissues mRNA expression of IL6, AKT1, VEGFA, TP53, MAPK1, JUN, EGF, AR, LHb, FSHb, CYP17a1, and CYP19a1 in PCOS mice

qRT-PCR was used to examine the mRNA expression of IL6, AKT, VEGFA, TP53, MAPK1, JUN, and EGF from the mice ovary tissue. As shown in Fig 12, we found the mRNA expressions of IL6, JUN, and EGF in model group significantly increased compared with NC group ($P<0.05$). Compared with model group, the mRNA expressions of IL6 and EGF in CS group decreased, whereas mRNA expression of JUN in CS group significantly decreased ($P<0.05$); the mRNA expressions of IL6, JUN, and EGF significantly decreased ($P<0.01$ or $P<0.05$).

As shown in Fig 12, compared with NC group, the mRNA expressions of AKT1 and TP53 and MAPK1 significantly increased ($P<0.001$ or $P<0.01$ or $P<0.05$). Compared with model group, the mRNA expressions of Akt1, TP53, and MAPK1 markedly decreased in CS group and Met group ($P<0.001$ or $P<0.01$).

As shown in Fig 12, compared with NC group, the mRNA expression of VEGFA significantly decreased ($P<0.001$). Compared with model group, the mRNA expression of VEGFA significantly increased ($P<0.001$ or $P<0.01$).

As shown in Fig 13, compared with NC group, the mRNA expression of AR, LHb, and CYP17a1 significantly increased ($P<0.01$ or $P<0.05$). Compared with model group, the mRNA expression of CYP17a1 significantly decreased in CS group and Met group ($P<0.01$ or $P<0.05$). There were no differences in the mRNA expression of AR and LHb in CS group, though they decreased compared with model group.

As shown in Fig 13, compared with NC group, the mRNA expression of CYP19a1 significantly increased ($P<0.05$). Compared with model group, the mRNA expression of CYP19a1 significantly decreased in CS group and Met group ($P<0.001$ or $P<0.01$).

As shown in Fig 13, compared with NC group, the mRNA expression of FSHb decreased. Compared with model group, the mRNA expressions were no differences in CS group and Met group.

4. Discussion

The global incidence of polycystic ovary syndrome (PCOS) is higher at their productive age. In recent time, more and more TCM formula has been widely used in many diseases, due to their multiple targets. In the present study, network pharmacology was utilized to explore or develop the material basis and underlying molecular mechanism of *Cuscuta-Salvia* in the treatment for PCOS.

First, the components in *Cuscuta-Salvia* was identified using UHPLC-ESI-Q-TOF-MS, the active components and targets of *Cuscuta-Salvia* was selected, and a network of TCM components and targets was established. Based on a network diagram of herb-compound target-PCOS target, we found that quercetin and kaemferol were main active compounds with higher values of degree. Studies on quercetin against PCOS suggested that in clinical trials or animal experiments, quercetin mainly improves lipid abnormalities, enhances sex hormone levels, reduces insulin resistance, and increases anti-inflammation (Khorshidi et al. 2018; Pourteymour Fard Tabrizi et al. 2020; Wang et al. 2017). Prior work have reported that quercetin plays role in insulin resistance, insulin tolerance, and glucose tolerance in type 2 diabetes mellitus as well as other metabolism-related diseases for rodent (Brown et al. 2015). Kaemferol can enhance Akt expression and hexokinase activity in liver, which increase glucose intake and its metabolism, improve mice blood sugar, and enhance insulin sensitivity (Alkhalidy et al. 2018). Additionally, kaemeferol decreases lipid stores induced by palmitic acid, endoplasmic reticulum stress, and pancreatic β cell dysfunction *via* AMPK/ mTOR pathway-mediated lipophagocytosis (Varshney et al. 2018). In our experiments, we found that *Cuscuta-Salvia* could improve mice weight and glucose tolerance (Fig. 8).

Second, eighty common targets were obtained for *Cuscuta-Salvia* and PCOS, which might be targets for *Cuscuta-Salvia* in the treatment for PCOS. Based on the value of degree in Cytoscape v 3.7.2 software, we

found that the 20 core targets were screened out from the 80 common targets. At the same time, on the basis of cytohubba plugin, ten core targets were obtained from the 20 core targets, such as IL6, AKT1, VEGFA, TP53, TNF, MAPK1, JUN, EGF, CASP3, and EGFR.

In PCOS patients, IL6 is recognized as early chronic inflammatory marker (Wu et al. 2019). In addition, IL6 is secret visceral adipose tissue in PCOS group (Ghowasi et al. 2018). In the hyper-androgenic PCOS patients, there is higher AKT1 level (Liu et al. 2018). VEGFA can mediate the formation of PCOS (Butler et al. 2020). TP53 can decrease in PCOS patients (Velazquez et al. 2011). MAPK1 level is higher, leading to insulin resistance (Hou et al. 2019). PCOS-like mice were rescued by regulating JUN pathway (Liu et al. 2020b). The overexpression of EGF is observed in PCOS (Bonetti et al. 2019). CASP3 plays a critical role in the initiation of apoptosis (Daniluk et al. 2019). EGFR can participate in the autophagy activation in PCOS (Li et al. 2018).

Third, we found many pathways, mainly including PI3K-Akt signaling pathway, MAPK signaling pathway, cellular senescence, TNF signaling pathway, and IL-17 signaling pathway. According to literatures, Activated PI3K-Akt signaling pathway can increase insulin sensitivity and regulate androgen level (Qiu et al. 2020; Wu et al. 2018). The suppression of MAPK signaling pathway can reduce PCOS pathology (Zhang et al. 2020). In the meantime, MAPK regulate the induction and progression of EMT, while EMT participates in some biological function, such as uterine development, reproductive disorders, and so on (Hu et al. 2020). Cellular senescence is a normal process due to the formation of oxygen free radical. Cellular senescence is associated with telomere length, and prolonged telomere length can reverse PCOS (Vasilopoulos et al. 2019). TNF is associated with enhanced susceptibility to PCOS (Liu et al. 2018), androgens level is affected by TNF- α (Lang et al. 2019). In PCOS patients, increased IL-17 level can lead to the formation of inflammatory response (Varghese et al. 2019). Prior study have reported that higher IL-17 level in the serum was presented in the etiology of PCOS (Albaghdadi et al. 2019).

Finally, HE staining results showed that in the PCOS model, *Cuscuta-Salvia* administration could improve tissue morphology, and treat PCOS. We selected core targets for qRT-PCR validation and confirmed that this TCM did improve the disease.

Sum up, this study found that 10 core targets and uncovered that *Cuscuta-Salvia* improves the pathology of PCOS.

5. Conclusion

In summary, the present results demonstrated that *Cuscuta-Salvia* exerted its pharmacological effects in PCOS using the combination of network pharmacology and animal experiments verification. Firstly, the main components in *Cuscuta-Salvia* were identified by UHPLC and were screened out by TCMSP database. Next, 80 common targets and 20 pathways were found. Finally, core genes were tested using in vivo experiments.

In conclusion, it was confirmed that the approach was proven to be quick and effective. Also, it can provide some references to similar investigation.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interest.

Funding

This work was supported by the Natural Science Foundation of Zhejiang Province of China (grant number: LQ19H270005), the administration of Traditional Chinese Medicine of Zhejiang Province of China (grant number: 2021ZB212), and Health Science and Technology Project of Hangzhou of China (grant number: No. 002019011).

Authors' contributions

All authors were responsible for the study concept and design: Ying-ying Zhang drafted the paper and participated in the literature search. Yu-tian Zhu and Yi-xuan Wang participated in data preparation. Wang-qiang Chen, Xin Sun, Wei Zhang, and Chen-ye Wang participated in network pharmacology analysis. Ying-ying Zhang and Jian-xiong Ma made some experiments. CD designed this study. All authors approved the final paper.

Acknowledgments

We thank the TCMSP, DisGeNET, GeneCards, STRING, Cytoscape v3.7.2, KEGG, GO databases for providing their platforms and the contributors for their valuable data sets.

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Figures

A.

B.

Figure 2

The total ion flow diagram of *Cuscuta-Salvia* by UHPLC-ESI-Q-TOF-MS. (A) Positive ion mode; (B) negative ion mode.

Figure 3

Venn diagram was applied to present all candidate targets of Cuscuta-Salvia and PCOS.

Figure 4

The network of herb compound-PCOS target in the treatment of PCOS.

Figure 5

PPI network diagram of core targets.

Figure 6

Network diagram of core targets of Cuscuta-Salvia against PCOS.

A.

B.

C.

D.

Figure 7

Representative diagram of GO function analysis and KEGG pathway analysis. (A), (B), and (C): Left, histogram of the biological processes (BP), the cellular component (CC), and the molecular function (MF) of *Cuscuta-Salvia* in the treatment of PCOS, respectively. (A), (B), and (C): Right, bubble chart of the BP, CC, and MF of *Cuscuta-Salvia* in the treatment of PCOS, respectively. (D): Left, histogram of KEEG

pathways of Cuscuta-Salvia in the treatment of PCOS; Right, bubble chart of KEEG pathways of Cuscuta-Salvia in the treatment of PCOS.

A.

B.

C.

Figure 8

Assessment of body weight (A), blood glucose levels (B), and statistical chart of OGTT (C) in PCOS mice. Results are presented as means± SEM, ##P<0.01vs. the NC group; *P<0.05 vs. the model group.

Figure 9

Assessment of pathological change on Cuscuta-Salvia on ovary tissue in PCOS mice on day 30.

Figure 10

Assessment of pathological change on Cuscuta-Salvia on liver tissue in PCOS mice on day 30 (400×).

Figure 11

Assessment of pathological change on Cuscuta-Salvia on adipose tissue in PCOS mice on day 30.

Figure 12

Effects of Cuscuta-Salvia (CS) administration on (A) IL6, (B) AKT1, (C) VEGFA, (D) TP53, (E) MAPK1, (F) JUN, (G) EGF. All data are expression as mean $\pm$ standard error of mean ($\bar{x} \pm \text{SEM}$) (n=3). ###P<0.001, ##P<0.01, and #P<0.05 vs. the normal control group; ***P<0.001, **P<0.01, and *P<0.05 vs. the model group.

Figure 13

Effects of Cuscuta-Salvia (CS) administration on (A) AR, (B) LHb, (C) FSHb, (D) CYP17a1, (E) CYP19a1. All data are expression as mean $\pm$ standard error of mean ($\bar{x} \pm \text{SEM}$) (n=3). ###P<0.001, ##P<0.01, and #P<0.05 vs. the normal control group; ***P<0.001, **P<0.01, and *P<0.05 vs. the model group.