Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

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Clinical data mining reveals *Gancao-Banxia* as a potential herbal pair against moderate COVID-19 by dual binding to IL-6/STAT3

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ABSTRACT

Background: Coronavirus disease 2019 (COVID-19) keeps spreading globally. Chinese medicine (CM) exerts a critical role for the prevention or therapy of COVID-19 in an integrative and holistic way. However, mining and development of early, efficient, multisite binding CMs that inhibit the cytokine storm are imminent.
Methods: The formulae were extracted retrospectively from clinical records in Hunan Province. Clinical data mining analysis and association rule analysis were employed for mining the high-frequency herbal pairs and groups from formulae. Network pharmacology methods were applied to initially explore the most critical pair's hub targets, active ingredients, and potential mechanisms. The binding power of active ingredients to the hub targets was verified by molecular docking.
Results: Eight hundred sixty-two prescriptions were obtained from 320 moderate COVID-19 through the Hunan Provincial Health Commission. *Glycyrrhizae Radix et Rhizoma* (*Gancao*) and *Pinelliae Rhizoma* (*Banxia*) were used with the highest frequency and support. There were 49 potential genes associated with *Gancao-Banxia* pair against moderate COVID-19 patients. The Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) indicated that *Gancao-Banxia* might act via inflammatory response, viral defense, and immune responses signaling pathways. IL-6 and STAT3 were the two most hub targets in the protein-protein interaction (PPI) network. The binding of five active ingredients originated from *Gancao-Banxia* to IL-6-STAT3 was verified by molecular docking, namely quercetin, coniferin, licochalcone a, Licoagrocarpin and (3S,6S)-3-(benzyl)-6-(4-hydroxybenzyl)piperazine-2,5-quinone, maximizing therapeutic efficacy.
Conclusions: This work provided some potential candidate Chinese medicine formulas for moderate COVID-19. Among them, *Gancao-Banxia* was considered the most potential herbal pair. Bioinformatic data demonstrated that *Gancao-Banxia* pair may achieve dual inhibition of IL-6-STAT3 via directly interacting with IL-6 and STAT3, suppressing the IL-6 amplifier. SARS-CoV-2 models will be needed to validate this possibility in the future.

1. Introduction

Coronavirus disease 2019 (COVID-19), a pandemic caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has spread rapidly worldwide [1]. The ongoing pandemic has already placed a heavy burden on the global public health system, global economy, and social stability. Most COVID-19 patients are moderate [2–5]. Although some drugs and vaccinations have been recommended for use, new effective strategies are still being developed due to side effects, toxicity and allergic reactions [6–10].

Traditional Chinese medicine (TCM) is considered as an indispensable component in Chinese traditional medicine. In the early stage of

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COVID-19 outbreak, TCM schemes have been recommended in the guidelines for COVID-19 [11,12]. TCM mainly exerts the effect of anti-viral infection indirectly by regulating and restoring the equilibrium of the body's immune system [13]. Using of Chinese medicine was confirmed to be associated with regional cure rate in China [14]. A series of clinical trials, case reports and observational studies confirmed the broad prospects of Chinses medicines in preventing or treating COVID-19 in different periods [14–16]. Integrative traditional Chinese and Western medicine has been confirmed to significantly reduce recovery time and decrease the transfer rate from the mild to severe COVID-19 [17]. Many herbal prescriptions showed excellent efficacy, for example, Lianhua Qingwen capsule [18,19], Qingfei Paidu decoction [20,21], Xuebijing injection [22] and Maxing Shigan decoction [23,24]. These formulae play multiple roles through multi-targets and multi-pathways, including mostly antivirus, anti-inflammation and immunoregulation [25,26]. However, the valuable Chinese medicines (CMs) pairs beneficial to COVID-19 and their pharmacological mechanisms remain to be thoroughly investigated.

Chinese medicine formulae records mining is vital for unearthing the concealed current therapies for COVID-19 [27]. For instance, Ren et al. [28] obtained 574 classical prescriptions about treating "Warm diseases", "Pestilence" and "Epidemic diseases" from the *Dictionary of Traditional Chinese Medicine Prescriptions and Pharmacopoeia of the People's Republic of China*. Although this work provided potential CMs pairs or formulae and active ingredients for overcoming COVID-19, some differences may exist between the flexible clinical formulae and invariant ancient prescriptions. Relatively, prescription medication from patients is more conducive to the discovery of effective drugs. Sun et al. [29] screened 179 TCM formulae for COVID-19 from open data sharing platform for Publication and Health and provided candidate CMs groups, such as *Gancao-Huangqin*, *Gancao-Jiegeng*, *Huangqin-Jiegeng-Gancao*. Nevertheless, larger sample sizes are needed to excavate the clinical medication rules of herbs due to the small sample size.

Network pharmacology dedicated to connection of drugs, ingredients, targets and diseases, thereby describing the complex relationship between organisms, potential drugs and specific diseases. Undoubtedly, it is a powerful tool for mining drug combinations, especially prescriptions of TCM [30]. The mechanisms of CMs groups or prescriptions for the potential treatment of COVID-19 have been gradually uncovered by network pharmacology, including *Rhizoma Polygonati* [31], *Pudilan* [32], *Mahuang* and *Xingren* [33], Qingfei Paidu decoction [34] and Lianhua Qingwen capsule [35]. According to the receptor's properties and the manner of interaction between receptors and molecules, molecular docking becomes a novel means for identifying compounds with therapeutic implications [36,37]. Through the above methods, a variety of compositions of TCM have been proven to target the two recognized receptors of COVID-19 successfully, angiotensin-converting enzyme 2 (ACE2) and 3C-like protease (3CL pro) [36,38], including baicalein, quercetin, acetoside and more [28,39,40]. Thus, the combination of network pharmacology with molecular docking is one feasible manner to delve the candidate components and targets of CMs in treating COVID-19.

ACE2 is a well-recognized therapeutic target for COVID-19 [41]. Unfortunately, ACE2 may no longer be an optimal target because of the potent chronic inflammation in the latter stage [42]. During the inflammatory outbreak, the Interleukin-6 (IL-6) family can activate the NF-κB pathway via Signal Transducer and Activator of Transcription 3 (STAT3), which further leads to the activation of IL-6 amplifier (IL-6 Amp), forming a cascade of amplification loops, inducing a series of inflammatory and autoimmune disorders [42–44]. A retrospective study reported that 15.3% (41/227) cases with moderate COVID-19 showed increased serum IL-6 [45]. Remarkably, the proportion with above normal serum IL-6 increased as the diseases progress (severe: 49.25%; critical: 83.33%) [45]. The IL-6 elevation is observed very early after viral infection [46]. Therefore, drugs synergistically binding the IL-6 and STAT3 are crucial to block progress for moderate COVID-19 [42].

This study collected herbal prescriptions used by moderate COVID-19 patients from 14 hospitals in Hunan Province. The association network was used to mine the most critical CMs pair (*Glycyrrhizae Radix et Rhizoma -Pinelliae Rhizoma*, *Gancao-Banxia*). Next, we screened the component targets of *Gancao-Banxia*, targets of interaction with COVID-19 and the potential mechanisms of action though network pharmacology. The core targets (IL-6-STAT3) were obtained by Cytoscape. Finally, the binding abilities of five components derived from *Gancao-Banxia* to IL-6-STAT3 were verified via molecular docking (Fig. 1). In brief, we found that *Gancao-Banxia* may achieve dual inhibition of IL-6-STAT3 via directly interacting with IL-6 and STAT3, which represents a prospective therapeutic strategy for moderate COVID-19 (Fig. 2).

2. Materials and methods

2.1. Data sources and collection

Our research group acquired medical records of 1,014 confirmed COVID-19 cases from the Hunan Provincial Health Commission. There were no restrictions on age and gender in our study. Based on the clinical diagnoses and the Diagnosis and Treatment of COVID-19 by the guidelines on the National Health Commission of China, 755 confirmed moderate patients were screened, inclusive criteria of moderate case were as follows: patients have symptoms including fever and respiratory tract symptoms, etc., and imaging shows pneumonia. Finally, two physicians checked the data, and then we began to gather and collate data on the TCM formulae.

The extracted information included age, gender, hospital length of stay, presenting hospitals and TCM formulae from 320 patients in 14 hospitals (Fig. 3). All prescriptions were administered orally, including decoction and granular formulation. To attain a raw data file, every Chinese medicine in each prescription was entered into the Excel database and standardized the data following the *Pharmacopoeia of the People's Republic of China*. One researcher carried out data input, and two independent staff (RF and TZ) checked and verified the data for accuracy again. The standardized database (Table S1) was used for the subsequent data mining.

2.2. Ethics in publishing

Medical Ethics Committee of The First Hospital of Changsha approved this study (Number: (2021) Ethic [Clinical paper] No.4). The written informed consent was waived.

2.3. Data analyze and association rule analysis

Two methods, frequency analysis and association rule mining, were applied to analyze the collected Chinese medicines [28]. The frequency analysis was utilized for high-frequency CMs. Association rule, a viable approach for revealing the internal structural features of prescriptions [47], was completed with IBM SPSS Modeler 14.1. Under the conditions of established support and confidence, the Apriori algorithm can mine the correlation between different variables, thereby revealing the internal structural features [47]. Specific Apriori algorithm parameters were detailed below: support > 0.2, confidence > 0.8, lift > 1.0 [47]. The combinations of Chinese medicine pairs were extracted and further analyzed. Lastly, the most key Chinese medicine pair was analyzed using network pharmacological approach and molecular docking software.

2.4. Network pharmacology analysis

Components and targets of *Gancao-Banxia*: All chemical compositions and targets of *Gancao-Banxia* were approved by the TCMSP (https://tcmspw.com/tcmsp.php) and Swiss TargetPrediction (http://swisstargetprediction.ch/) databases. In the TCMSP, candidate compounds were screened for the pharmacokinetic parameters with drug-

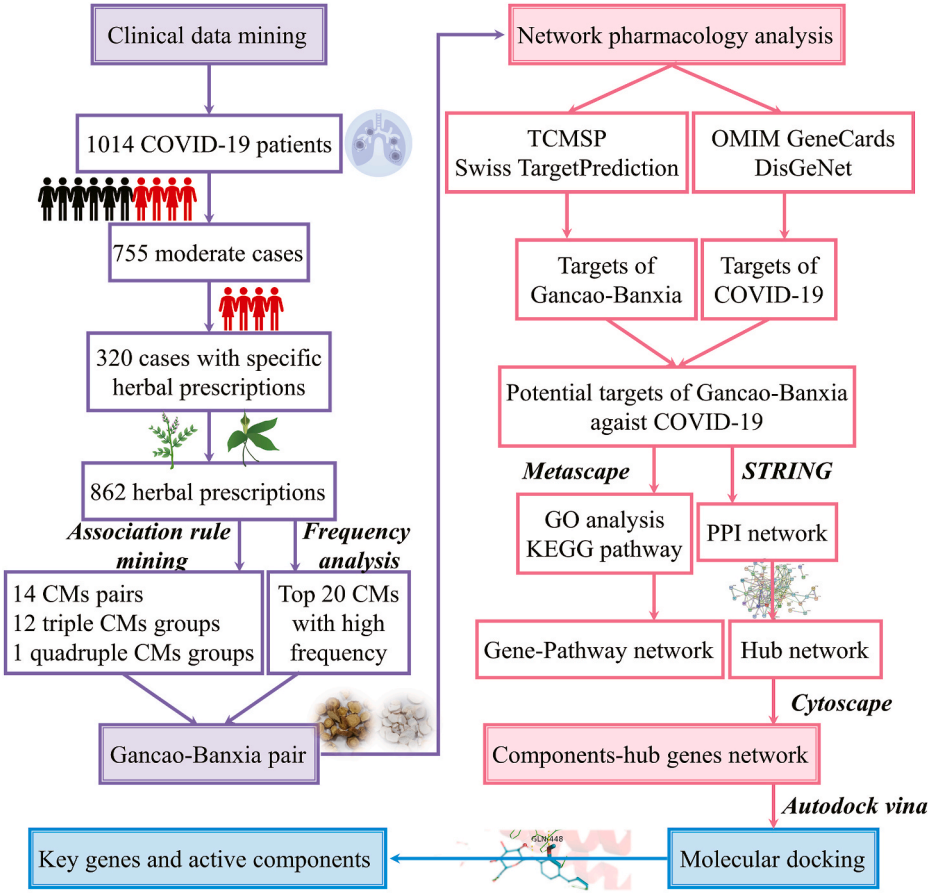

Fig. 1. Study flow diagram. Chinese medicine formulae mining and association rule analysis were utilized for acquiring the CMs-pairs from clinical herbal formulae among moderate COVID-19 cases. The hub network and gene-pathway network of *Gancao-Banxia* against moderate COVID-19 patients were extracted by network pharmacology. The components-core target network was established through Cytoscape. Molecular docking was utilized to provide the docking power of herbal active ingredients to the key genes.

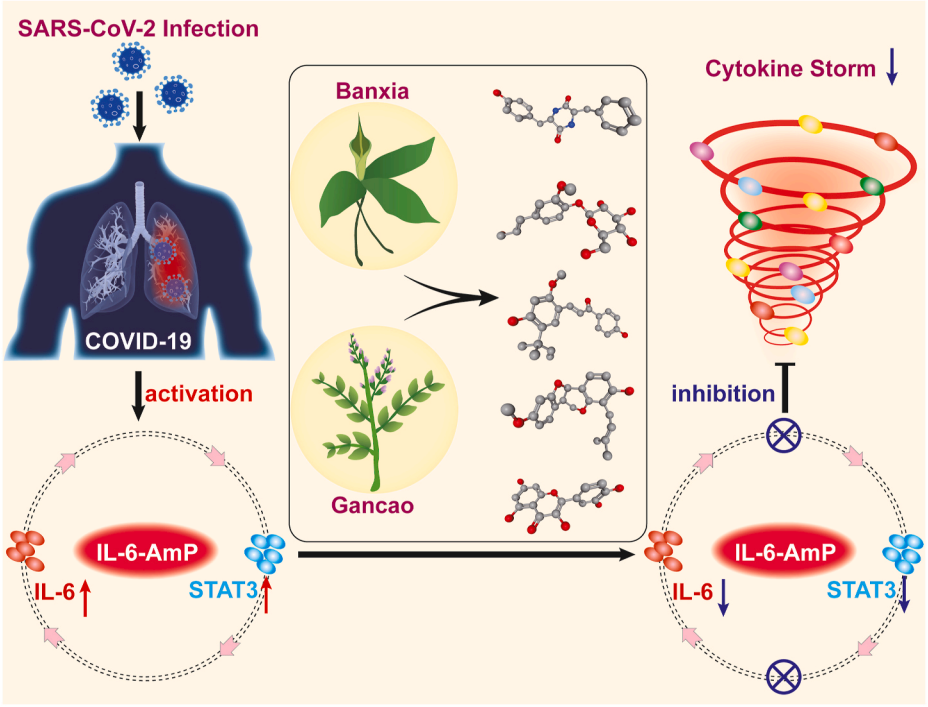

Fig. 2. Schematic of *Gancao-Banxia* pair achieve dual inhibition of IL-6-Amp via directly interacting with IL-6 and STAT3 among moderate COVID-19 patients.

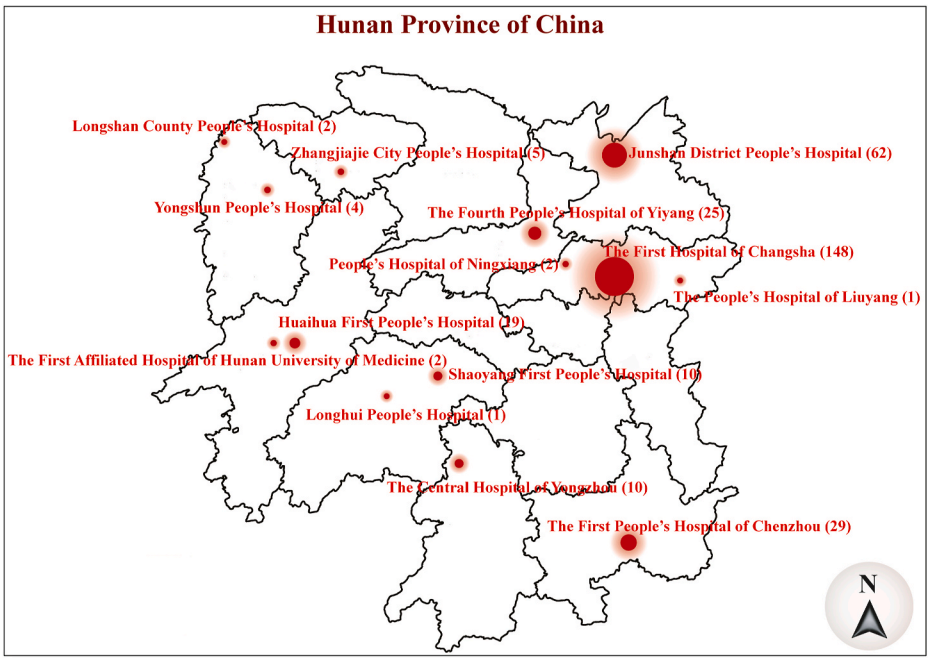

Fig. 3. Location map of the 14 hospitals in Hunan Province. Dot sizes represent patient sample sizes and values in parentheses are for extracted case numbers.

likeness (DL) > 0.18 [48] and oral bioavailability (OB) > 30% [49]. Probability was set to be larger than 0 in the Swiss TargetPrediction database. The intersection portion of two databases was chosen as the target of *Gancao-Banxia*. The ID and gene symbols of candidate targets were standardized by UniProtKB database (http://www.uniprot.org/). All SDF formats of final screened ingredients were downloaded from PubChem (https://pubchem.ncbi.nlm.nih.gov/).

COVID-19 targets: We searched the COVID-19-related targets with the keywords "coronavirus pneumonia," "SARS coronavirus," "novel coronavirus," and "coronavirus" from OMIM (https://www.omim.org/), GeneCards (https://www.genecards.org/) and DisGeNet databases (https://www.disgenet.org/). Our group built gene set related for COVID-19 as the targets of COVID-19. The overlapping genes after integrating COVID-19-related genes and *Gancao-Banxia*-targets were potential targets of *Gancao-Banxia* pair anti-COVID-19.

Protein-protein interaction (PPI): Intersection of targets were utilized for the PPI network construction on the STRING 11.0 platform (https://string-db.org/). "*Homo sapiens*" was then selected. Then, we set the minimum required interaction score to 0.900. After hiding the network's disconnected nodes, results were exported. The topology of PPI network was visualized through the Cytoscape software (3.7.2). Degree Centrality (degree value) is the most direct measure of node centrality in the PPI network. Furthermore, the BisoGenet and CytoNCA apps were utilized to extract the core network from PPI network in Cytoscape with degree cutoff ≥ 14 (twice the median degree). Finally, the core targets were further analyzed.

Enrichment analysis of overlapping genes: Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were performed by the Metascape database (http://www.metascape.org/) ($p < 0.01$). The species was set as "*H. sapiens*." Final GO and KEGG annotation files were downloaded for further analysis.

Network construction: First, the top 20 KEGG pathways were listed according to the p-value. The Cytoscape generated a targets-pathway network and obtained the network of core targets and associated pathways. Second, we set a network about compounds and core targets.

2.5. Molecular docking analysis

The crystal structures of STAT3 (PDB ID: 6tlc) and IL6 (PDB ID: 4cni) were acquired from the RCSB Protein Data Bank (http://www1.rcsb.org/). Ligand files of licochalcone a (PubChem CID: 5318998), Licoagrocarpin (PubChem CID: 15840593), coniferin (PubChem CID: 5280372), (3S,6S)-3-(benzyl)-6-(4-hydroxybenzyl)piperazine-2,5-quinone (PubChem CID: 11438306), and quercetin (PubChem CID: 5280343) were retrieved from PubChem database (https://pubchem.ncbi.nlm.nih.gov/). AutoDockTools 1.5.6, a free tool for the AutoDock Vina program, was employed for preparing the ligands and receptors for ligand docking. Size_x = 40, size_y = 40, size_z = 40 in each target. The grid box center directions of STAT3 are 0.241, 28.864 and 33.563 (center_x, center_y and center_z). The grid box center directions of IL6 are −61.205, 172.516 and 46.418 (center_x, center_y and center_z). Molecular docking was performed with AutoDock Vina, with 8 energy range and 24 exhaustiveness.

3. Results

3.1. Characteristics of 320 moderate COVID-19 patients

Our study enrolled 1014 confirmed patients of COVID-19 from Hunan Provincial Health Commission, including 755 moderate patients (74.46%). In detail, 320 moderate COVID-19 patients had specific TCM prescriptions (42.38%). Of the 320 patients with moderate COVID-19, 152 were male. And the mean age for 320 patients was 44.24 (14.796) years, with a representative age group of 41–60 years

Table 1
Characteristics of 320 patients with moderate COVID-19.

Characteristics	All patients (n = 320)
Mean (SD) age (years)	44.24 (14.796)
Age groups (years)	
≤18	10 (3.13%)
19-40	123 (38.44%)
41-60	141 (44.06%)
61-80	43 (13.44%)
≥81	3 (0.94%)
Sex	
Male	152 (47.50%)
Hospital length of stay (days)	15 (12, 22)

Data are median (IQR), mean (SD) or number (proportion).

(44.06%). The mean duration of hospital stay was 15 days (range 12–22) (Table 1).

3.2. High frequency Chinese medicines from prescriptions

Our study included a total of 862 Chinese medicine formulae (Table S1). We then performed high frequency CMs using frequency analysis, and the top 20 are shown in Table 2. Among them, the highest frequency was *Gancao* (87.12%), followed by *Banxia* (61.83%). Beyond that, the frequency of other medicines was all less than 50% (Table 2). The most frequently used dosage of *Gancao* was 6 g. The commonly prescribed doses of *Banxia* were 10 g, 15 g, and 9 g, of which 10 g took central stage (Fig. S1). The most commonly used compatibility dosage of *Banxia-Gancao* was 10 g–6 g (Fig. S2).

3.3. Analysis of association rules in prescriptions

There were 22 CMs-groups with a high support and confidence, according to the screening conditions (Table 3). Of these, *Gancao-Banxia* pair had the highest support, and *Gancao-Chaihu* with the highest confidence. The vast majority of these CMs-groups contained *Gancao*. Based on the above high frequency medicines, *Gancao* and *Banxia* were considered as the crucial herbal pair for treating moderate COVID-19 patients. This finding prompted us to thoroughly hunt for the possible mechanisms of action of *Gancao-Banxia* group.

3.4. Active ingredients and drug targets screening

All active ingredients were obtained from TCMSP and Swiss TargetPrediction databases. Ultimately, 100 active components of *Gancao-Banxia* pair were screened according to screening criteria (*Gancao*: 88 active components; *Banxia*: 12 active componenets) (Table S2). Additionally, 883 drug targets were determined from all bioactive components. The *Gancao-Banxia* component-targets network was built using Cytoscape software (Fig. 4).

Table 2
High frequency Chinese medicine pairs from 862 formulae (Top 20).

No.	Chinese *Pinyin* name	Latin name	Frequency	Percentage (%)
1	Gancao	*Glycyrrhizae Radix et Rhizoma*	751	87.12
2	Banxia	*Pinelliae Rhizoma*	533	61.83
3	Kuxingren	*Armeniacae Semen Amarum*	383	44.43
4	Fuling	*Poria*	359	41.65
5	Chenpi	*Citri Reticulatae Pericarpium*	352	40.84
6	Yiyiren	*Coicis Semen*	278	32.25
7	Houpo	*Magnoliae Officinalis Cortex*	270	31.32
8	Huangqi	*Astragali Radix*	260	30.16
9	Huangqin	*Scutellariae Radix*	249	28.89
10	Baizhu	*Atractylodis Macrocephalae Rhizoma*	232	26.91
11	Cangzhu	*Atractylodis Rhizoma*	227	26.33
12	Maidong	*Ophiopogonis Radix*	218	25.29
13	Lugen	*Phragmitis Rhizoma*	201	23.32
14	Jiegeng	*Platycodonis Radix*	197	22.85
15	Chaihu	*Bupleuri Radix*	190	22.04
16	Zhebeimu	*Fritillariae Thunbergii Bulbus*	184	21.35
17	Lianqiao	*Forsythiae Fructus*	179	20.77
18	Guanghuoxiang	*Pogostemonis Herba*	174	20.19
19	Sangbaipi	*Mori Cortex*	174	20.19
20	Beishashen	*Glehniae Radix*	173	20.07

Table 3
CMs-groups in 862 prescriptions.

No.	CMs-groups	Support (%)	Confidence (%)
1	Gancao-Banxia	61.83294664	87.80487805
2	Gancao-Kuxingren	44.43155452	90.60052219
3	Gancao-Yiyiren	32.25058005	87.41007194
4	Gancao-Huangqin	28.8863109	90.76305221
5	Gancao-Kuxingren-Banxia	27.95823666	90.04149378
6	Banxia-Chenpi-Fuling	27.37819026	83.89830508
7	Fuling-Baizhu	26.91415313	81.89655172
8	Gancao-Cangzhu	26.33410673	88.54625551
9	Gancao-Maidong	25.2900232	89.44954128
10	Gancao-Yiyiren-Banxia	24.7099768	87.32394366
11	Gancao-Lugen	23.31786543	90.04975124
12	Banxia-Chenpi-Fuling-Gancao	23.08584687	84.42211055
13	Gancao-Yiyiren-Kuxingren	23.08584687	89.44723618
14	Gancao-Jiegeng	22.85382831	90.86294416
15	Gancao-Houpo-Banxia	22.2737819	90.10416667
16	Banxia-Chaihu	22.04176334	81.57894737
17	Gancao-Chaihu	22.04176334	94.73684211
18	Gancao-Huangqin-Banxia	21.6937355	94.11764706
19	Fuling-Baizhu-Gancao	21.57772622	82.79569892
20	Chenpi-Huangqi-Fuling	21.1136891	80.76923077
21	Banxia-Huangqi-Fuling	21.1136891	84.06593407
22	Gancao-Houpo-Kuxingren	21.1136891	89.01098901

Screening conditions: Support >20%, Confidence >80%, Lift >1.0.

3.5. Potential targets of Gancao-Banxia pair against COVID-19

We obtained 162 COVID-19 related genes from OMIM, GeneCards, and DisGeNet databases after removing duplications (Table S3) (Fig. 5A). Then, the 883 drug targets were intersected with 162 COVID-19 targets to acquire the overlapping targets between *Gancao-Banxia* and COVID-19 (Fig. 5B). There were 49 potential genes associated with *Gancao-Banxia* pair against moderate COVID-19 (Table 4). The 49 mapped genes were used to plot a PPI network STRING (Fig. 5C).

3.6. Enrichment analysis of 49 intersecting proteins

The relevant biological functions of *Gancao-Banxia* were revealed by the GO analysis. The top 10 enriched terms of biological process (BP), cellular components (CC), and molecular function (MF) were listed and arranged in Fig. 6A. The main BP included response to bacterium (GO:0009617), regulation of cytokine production (GO:0001817), positive regulation of cytokine production (GO:0001819), regulation of cell adhesion (GO:0030155), and lymphocyte activation (GO:0046649). The clusters in CC were mainly membrane raft (GO:0045121), membrane microdomain (GO:0098857), membrane region (GO:0098589), and side of membrane (GO:0098552). MF analysis demonstrated that the terms primarily involved in cytokine receptor binding (GO:0005126), receptor ligand activity (GO:0048018), receptor regulator activity (GO:0030545), and cytokine activity (GO:0005125).

The possible mechanisms underlying *Gancao-Banxia* against moderate COVID-19 were identified by KEGG pathway. It isn't hard to discover that the kaposi sarcoma-associated herpesvirus infection (hsa05167), human cytomegalovirus infection (hsa05163), Influenza A (hsa05164), and IL-17 signaling pathway (hsa04657) were more important. These results highlighted that *Gancao-Banxia* might regulate three major classes of signaling pathways associated with inflammatory response, viral infections, and immune responses (Fig. 6B).

3.7. Identification of core targets

To identify the core targets, 49 intersection proteins were fed as input data to Cytoscape software. This network included 48 nodes and 182 edges, and the average degree was 7.0 (Fig. 7A). The twice average value of the degree was utilized for identifying the core targets of *Gancao-Banxia* to against moderate COVID-19. Eight pivotal targets

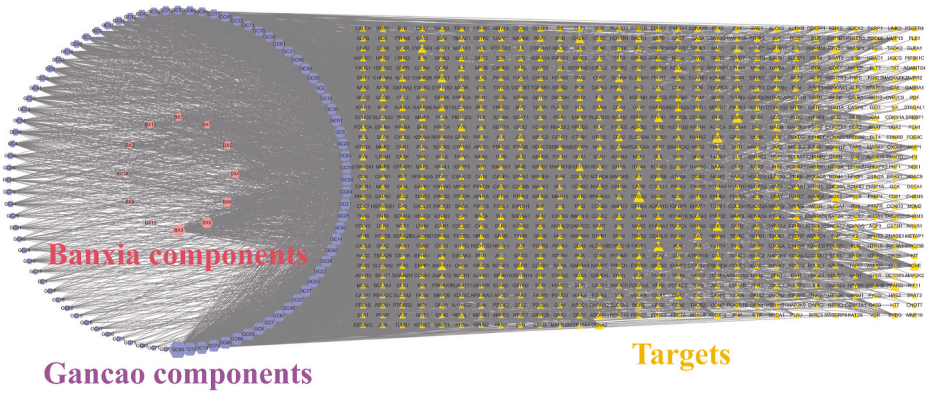

Fig. 4. *Gancao-Banxia* components-targets network. Purple hexagons, orange hexagons, and yellow triangles represent *Gancao* components, *Banxia* components, and putative targets, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

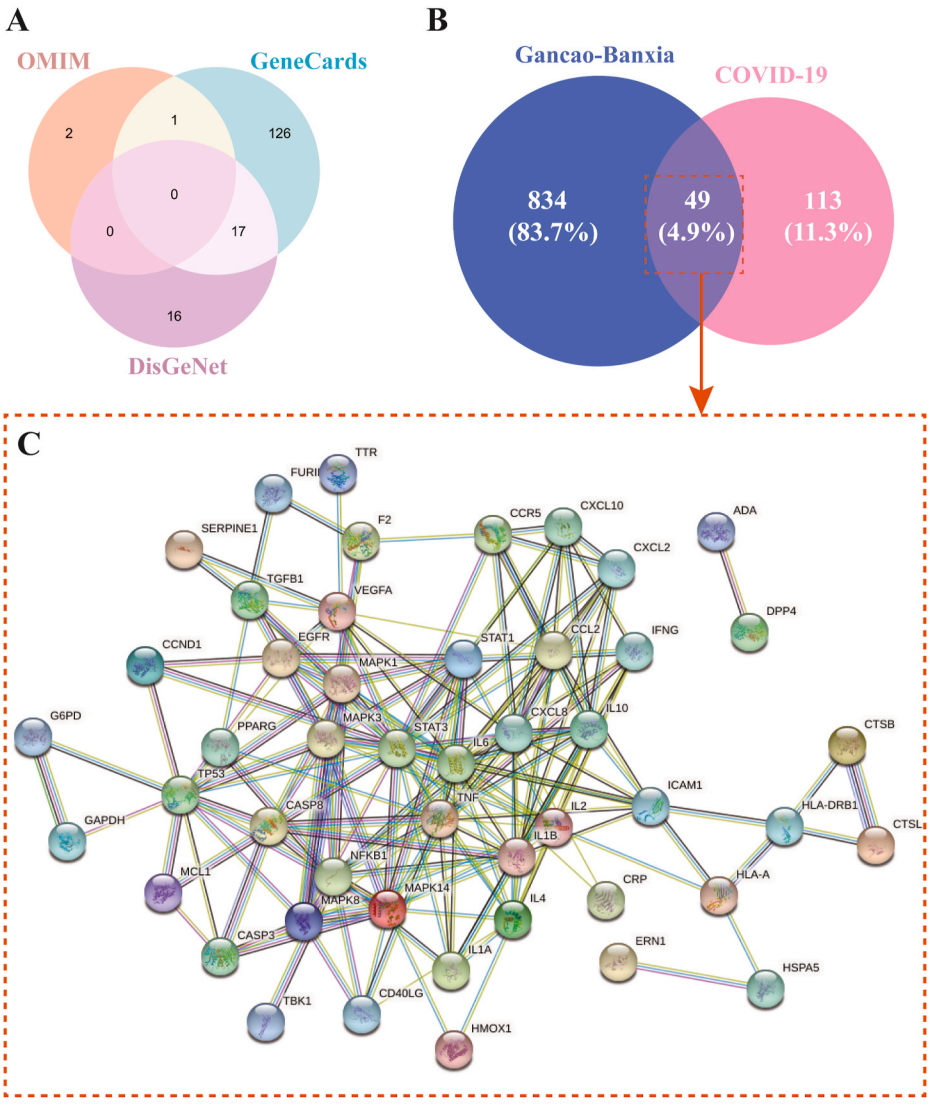

Fig. 5. Candidate targets of *Gancao-Banxia* against moderate COVID-19 patients. **(A)** COVID-19-related gene set. **(B)** The overlapping targets between *Gancao-Banxia* and COVID-19. **(C)** The interaction network of 49 shared proteins (STRING database).

were captured using cytoHubba and indicated in Fig. 7B. Top of the core targets was STAT3, followed by IL-6 and TNF. Further analyzed the betweenness centrality and closeness centrality of IL-6 and TNF, IL-6 was more dominant (Table S4).

We firstly filtered related pathways associated with IL-6 and STAT3 in the top 20 KEGG pathways (Fig. 8A). There were 11 pathways related to STAT3 and 17 connected to IL-6 (Fig. 8B and C). The two targets shared 9 common signaling pathways, including Pathways in cancer,

Table 4
Target information of the intersection of 49 genes.

Uniprot ID	Gene names	Protein names
P37231	PPARG	Peroxisome proliferator-activated receptor gamma
P05121	SERPINE1	Plasminogen activator inhibitor 1
O75460	ERN1	Serine/threonine-protein kinase/endoribonuclease IRE1
P15692	VEGFA	Vascular endothelial growth factor A
Q16539	MAPK14	Mitogen-activated protein kinase 14
P19838	NFKB1	Nuclear factor NF-kappa-B p105 subunit
Q07820	MCL1	Induced myeloid leukemia cell differentiation protein Mcl-1
P00533	EGFR	Epidermal growth factor receptor
P00734	F2	Prothrombin
P27487	DPP4	Dipeptidyl peptidase 4
P42574	CASP3	Caspase-3
P24385	CCND1	G1/S-specific cyclin-D1
P02766	TTR	Transthyretin
P11413	G6PD	Glucose-6-phosphate 1-dehydrogenase
P60568	IL2	Interleukin-2
P05112	IL4	Interleukin-4
P01375	TNF	Tumor necrosis factor
P45983	MAPK8	Mitogen-activated protein kinase 8
P42224	STAT1	Signal transducer and activator of transcription 1-alpha/beta
P09601	HMOX1	Heme oxygenase 1
P05362	ICAM1	Intercellular adhesion molecule 1
P07711	CTSL	Procathepsin L
P27361	MAPK3	Mitogen-activated protein kinase 3
P28482	MAPK1	Mitogen-activated protein kinase 1
Q9UHD2	TBK1	Serine/threonine-protein kinase TBK1
P07858	CTSB	Cathepsin B
P15144	ANPEP	Aminopeptidase N
P11021	HSPA5	Endoplasmic reticulum chaperone BiP
P40763	STAT3	Signal transducer and activator of transcription 3
P01137	TGFB1	Transforming growth factor beta-1 proprotein
P22301	IL10	Interleukin-10
P05231	IL-6	Interleukin-6
P04637	TP53	Cellular tumor antigen p53
Q14790	CASP8	Caspase-8
P01584	IL1B	Interleukin-1 beta
P13500	CCL2	C–C motif chemokine 2
P10145	CXCL8	Interleukin-8
P01579	IFNG	Interferon gamma
P01583	IL1A	Interleukin-1 alpha
P19875	CXCL2	C-X-C motif chemokine 2
P02741	CRP	C-reactive protein
P02778	CXCL10	C-X-C motif chemokine 10
P29965	CD40LG	CD40 ligand
P51681	CCR5	C–C chemokine receptor type 5
P04439	HLA-A	HLA class I histocompatibility antigen, A alpha chain
P09958	FURIN	Furin
P01911	HLA-DRB1	HLA class II histocompatibility antigen, DRB1 beta chain
P00813	ADA	Adenosine deaminase
P04406	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase

AGE-RAGE signaling pathway in diabetic complications, kaposi sarcoma-associated herpesvirus infection, human cytomegalovirus infection, Epstein-Barr virus infection, Hepatitis B, Measles, Th17 cell differentiation and Inflammatory bowel disease.

3.8. Molecular docking of active compounds and IL-6-STAT3 proteins

We obtained active compounds targeting IL-6 and STAT3 from the compounds-targets interaction network (Fig. 9A). As shown in Fig. 9B, four active compounds (licochalcone a, Licoagrocarpin, coniferin, and (3S,6S)-3-(benzyl)-6-(4-hydroxybenzyl)piperazine-2,5-quinone) targeted STAT3. Only one active compound (quercetin) was acquired targeting IL-6 (Fig. 9C). Meanwhile, the results of molecular docking indicated that five active ingredients could readily bind with IL-6 or STAT3 proteins (Fig. 10A and B). Docking scores are listed in Table 5.

In addition to two hydrogen bonds with the key amino acids

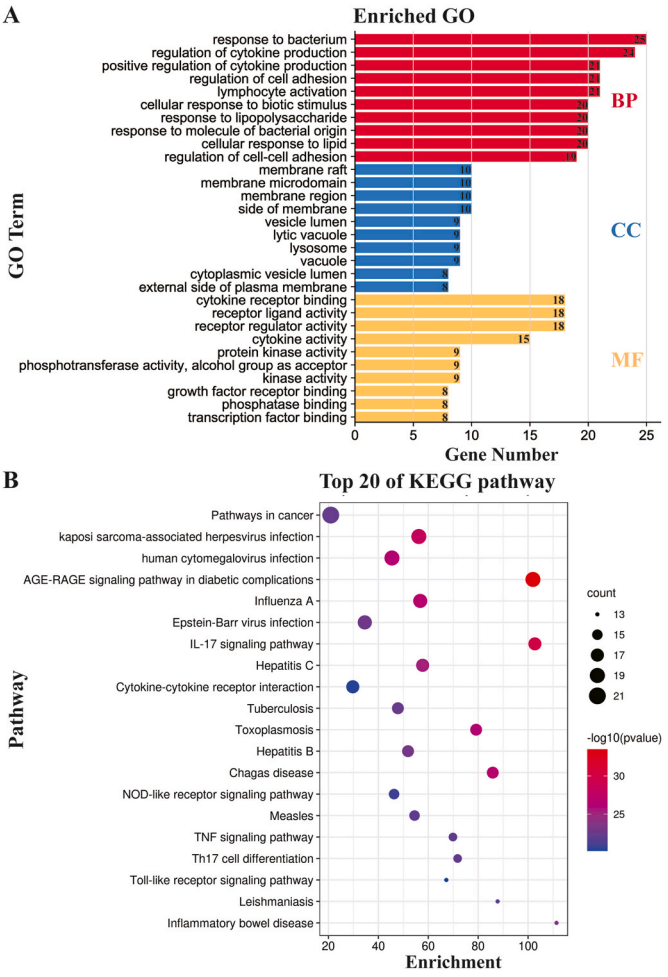

Fig. 6. Functional enrichment analysis of the 49 putative targets of *Gancao-Banxia* for COVID-19 treatment. **(A)** Top ten of biological process, cellular components, and molecular function: numbers in the bar graph represent the number of targets annotated. **(B)** Top 20 KEGG pathways.

including GLN361 and GLU444 Licochalcone a also formed the π-π interaction with TYR447. These bonds ensure that stable complex formation between Licochalcone a and STAT3. Similarly, the binding residues of hydrogen bonds (with TYR446 and LYS282) and π-π bond (with TYR446) were also observed in the three-dimensional picture of Licoagrocarpin. Coniferin and STAT3 formed five hydrogen bonds at GLU357, GLN448, GLU357, GLN361, and TYR446. At the same time, a π-π bond also formed between the Coniferin and HIS447. In the three-dimensional picture of (3S,6S)-3-(benzyl)-6-(4-hydroxybenzyl)piperazine-2,5-quinone), we found that three hydrogen bonds were present at three active sites of STAT3 (GLY449, TYR446, and GLN448) and a π-π bond was located at TYR446. Inconsistently, only three hydrogen bonds were found between the Quercetin and IL-6 at SER193, SER114, and ALA112. Therefore, hydrogen-bonding and π-π interactions exert a major role in determining the inhibitor activities.

4. Discussion

Since the COVID-19 outbreak, many traditional Chinese medicines have played indispensable roles in fighting COVID-19. Regrettably, researching on extracting core Chinese medicines through clinical data mining is still lacking. In the present research, we collected 862 TCM prescriptions from 320 moderate COVID-19 patients. All prescriptions were mined through the association rule mining, and then the *Gancao-Banxia* core pair was screened. We constructed subsequently a *Gancan-*

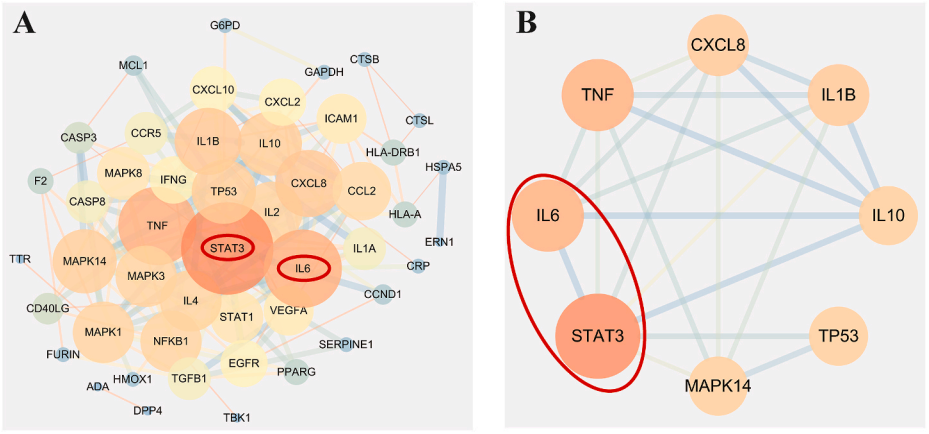

Fig. 7. The core targets of *Gancao-Banxia* against moderate COVID-19. **(A)** Visualized PPI network. **(B)** Eight core targets were screened and identified using BisoGenet and CytoNCA apps with degree cutoff ≥ 14 (twice the median degree). These core targets were connected collectively in a functional map.

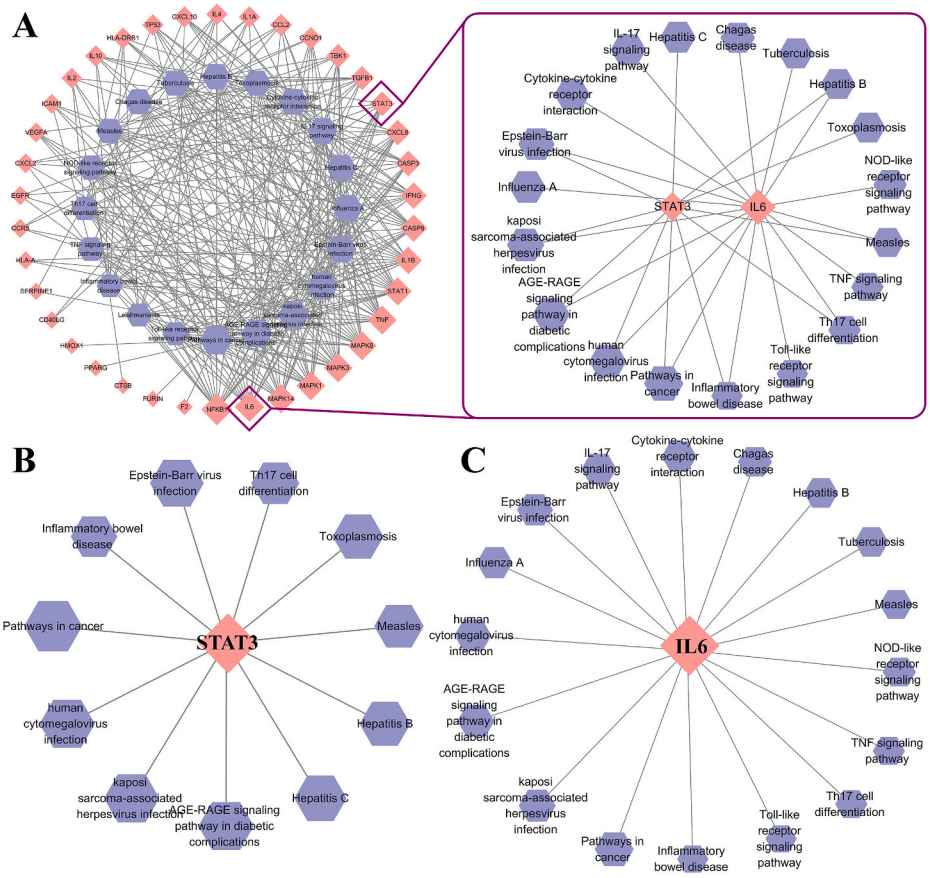

Fig. 8. The related pathways associated with IL-6 and STAT3 in the top 20 KEGG pathways. **(A)** Nineteen shared pathways of IL-6 and STAT3. **(B)** Eleven pathways related to STAT3. **(C)** Seventeen pathways connected to IL-6.

Banxia against moderate COVID-19 related gene set consisting of 49 targets by analyzing the active ingredients of *Gancan-Banxia*. Functional enrichment analysis cleared that *Gancao-Banxia* could regulate inflammatory response pathways, viral defense and immune responses. Core proteins PPI network uncovered eight core targets. We further focused attention on the top two most significant targets (STAT3, IL-6), which can form IL-6-STAT3 axis, facilitating the IL-6 Amp. Eventually, we verified the interaction between active compounds of *Gancao-Banxia* and IL-6-STAT3 by molecular docking. The results of this study discover the potential *Gancao-Banxia* pair and indicate the effectiveness for treating moderate COVID-19 patients from a bioinformatics standpoint. These outcomes may promote target drugs design for IL-6-STAT3 on COVID-19 infection.

In the report, Chinese medicine participation rate was only 42.38%, which is significantly less than the existing literature reports [50]. The main reason is the missing or incomplete detailed prescriptions in many medical records which we did not include in our research. But this did not hinder the use of Chinese medicines at all ages. In addition to young people and middle-aged people, we identified herbal prescriptions were also adopted for the treatment of children and the elderly during their

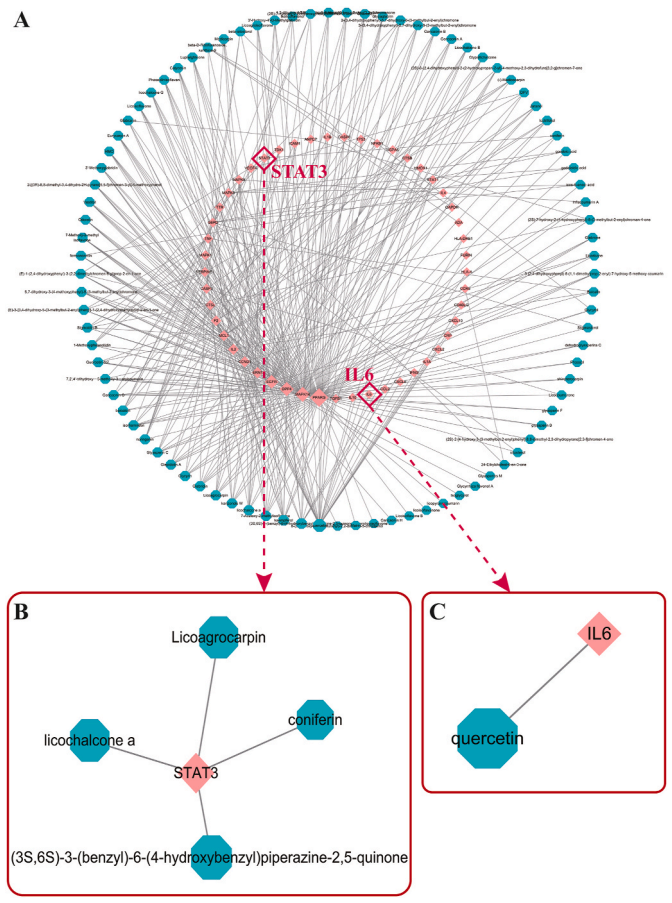

Fig. 9. The compounds-targets interaction network. **(A)** Establish a compounds-targets interaction network through Cytoscape software. **(B)** Four active compounds (licochalcone a, Licoagrocarpin, coniferin, and (3S,6S)-3-(benzyl)-6-(4-hydroxybenzyl)piperazine-2,5-quinone) targeted STAT3. **(C)** One active compound (quercetin) targeted IL-6.

hospitalization.

In most current studies conducted on TCMs therapy for COVID-19 patients using bioinformatics methods, researchers focused on fixed traditional herbal formulae or only used past antiviral prescriptions as data sources to analyze. Despite the antiviral efficacy of these herbs or formulae and potential mechanisms were confirmed, new TCM recipes and CM-pairs may be not easily discernible. Consistent with the results of previous studies, *Gancao* was the most frequently used drug for moderate COVID-19 patients. However, we found that the frequency of *Banxia* ranked second, which is different from the Scutellaria (*Huangqin*) ranked at second place in previous study [51]. This may be the result of discrepancy screening caused by different data sources. We further used association rules to identified *Gancao-Banxia* pair. In addition, some CMs-pairs worth studying have also been discovered, such as *Gancao--Kuxingren*, *Gancao-Yiyiren* and *Gancao-Huangqin*. These groups may better target various symptoms of different patients and provide basis for capturing new potential CMs-pairs for treating moderate COVID-19. Their respective peculiar pharmacological effects need further research to prove. Additionally, the discovery of a combination of three or four herbs may become novel formulae for moderate COVID-19 patients.

Gancao, a medicinal plant in traditional medicine, has the replenish *qi* and invigorate spleen, clear heat and detoxification, and spasmolytic and carminative effects [52]. *Banxia* is traditionally used to eliminate dampness and remove phlegm [51]. A meta-analysis shows that the frequency of *Gancao* and *Banxia* was ranked in the top five during the COVID-19 pandemic [51]. Both herbs have complex pharmacological actions, such as anti-inflammatory, anti-tumor and antibacterial by

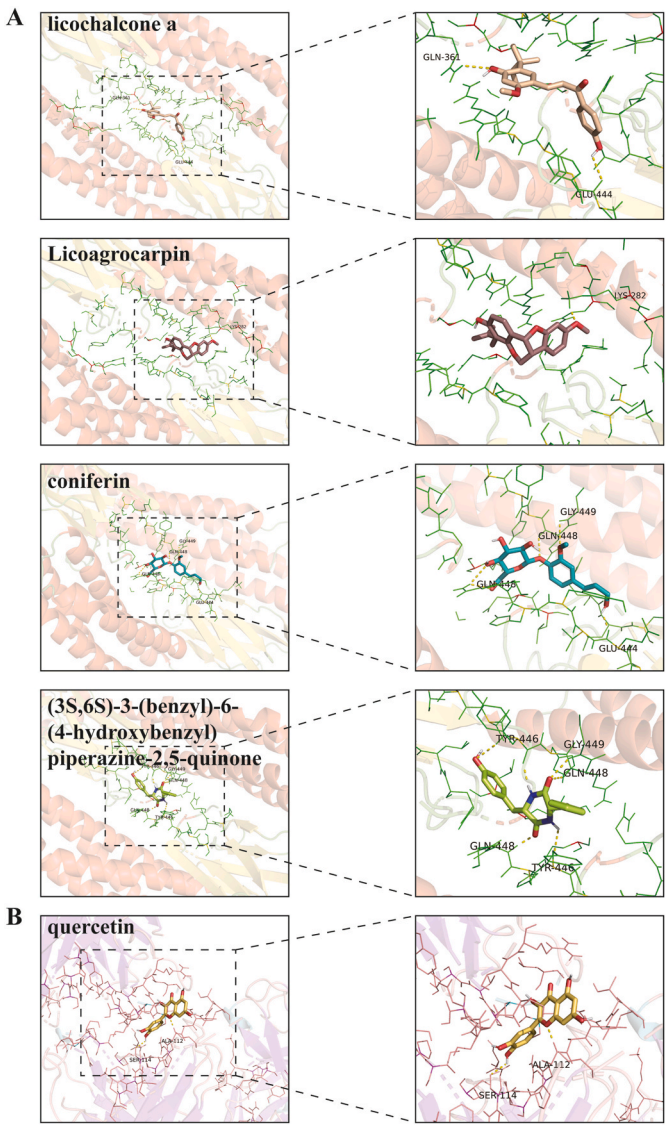

Fig. 10. Molecular docking of *Gancao-Banxia* compounds with IL-6 and STAT3. **(A)** STAT3 with licochalcone a, Licoagrocarpin, coniferin, and (3S,6S)-3-(benzyl)-6-(4-hydroxybenzyl)piperazine-2,5-quinone. **(B)** IL-6 with quercetin.

Table 5
Docking scores of five active compounds.

Target name	ligand name	Pubchem_ID	Docking Score
STAT3	(3S,6S)-3-(benzyl)-6-(4-hydroxybenzyl)piperazine-2,5-quinone	11438306	−8.4
	Licoagrocarpin	15840593	−7.9
	Licochalcone a	5318998	−7.7
	Coniferin	5280372	−7.2
IL-6	Quercetin	5280343	−7.3

accumulated pharmacological studies [53–55]. Our work indicated that the compatibility of *Gancao* and *Banxia* may exert positive effect through the regulation of inflammatory response, viral infection and immune response (Fig. 6). The finding of related pathways provides the possibility for delving into the mechanisms of *Gancao-Banxia* against patients with moderate COVID-19. To define the potential targets of herbal active ingredients, we further screened the most critical proteins and verified the docking capacity between active components and key proteins.

STAT3 and IL-6 are the most critical targets for *Gancao-Banxia* to treat patients with moderate COVID-19 patients. As we all know, IL-6 is the crucial inflammation marker involved in cytokine storm [56]. IL-6 levels increased as the degree of infection worsens during COVID-19 infection [57]. A meta-analysis clearly indicated that elevated IL-6 was significantly associated with adverse outcomes, such as ICU admission, Adult Respiratory Distress Syndrome (ARDS), and high mortality [57,58]. Therefore, for patients with cytokine release syndrome, IL-6 inhibitors may be beneficial [59]. Currently, many herbs, prescriptions or traditional medicine monomers have been proven to reduce IL-6 expression, such as *Glycyrrhizae Radix et Rhizoma* [60], Shufeng Jiedu decoction [61], Lianhua Qingwen [19] and quercetin [62]. Consistent with our research results, we also found that quercetin, the active ingredient of *Gancao*, has a strong docking ability with IL-6, indicating that *Gancao* may primarily reduce the IL-6 levels against the COVID-19 inflammatory outbreak.

More remarkably, STAT3 occupied the core position among key targets (Fig. 7). STAT3 is a cell signal transcription factor in the Signal Transducers and Activators of Transcription (STAT) family and a crucial protein for anti-inflammatory and antiviral [63,64]. IL-6 is the main stimulating factor of STAT3 in the body, especially in the period of inflammatory burst [42]. Conversely, IL-6 signaling mainly acts through the JAK/STAT pathway, predominantly via STAT3 [65]. Both factors can form IL-6 Amp, producing inflammation-related cascade amplification effects. This effect promotes various pro-inflammatory cytokines and chemokines, including IL-6, and recruits macrophages and lymphocytes, thereby strengthening the positive feedback loop formed by IL-6 and STAT3 [42]. Therefore, although ACE2, an important receptor for SARS-CoV-2 to cell entry, provides a key target for early infection, the potential ACE2 dysregulation leads to severe cytokine release syndrome at late time points after infection [42]. An urgent need to develop drugs that dually inhibit IL-6-STAT3 axis to block the amplification of the inflammatory cascade as much as possible. Our research pointed out that STAT3 and IL-6 were the two most critical targets for *Gancao--Banxia*. In addition to the strong docking ability of quercetin with IL-6, the other two active components of *Banxia* (coniferin and (3S, 6S)-3-(benzyl)-6-(4-hydroxybenzyl)piperazine-2,5-quinone) and the two active components of *Gancao* (licochalcone a and Licoagrocarpin) had strong binding power to STAT3. This implies that *Gancao-Banxia* pair can attenuate the cytokine storm by synergistically affecting IL-6 and STAT3 to a greater extent, blocking the amplification effect of IL-6-Amp at multiple sites, resulting in positive impacts on moderate COVID-19.

5. Limitations

Several limitations merit discussion to our study. First, although molecular docking was performed, it can only theoretically prove the relationship between *Gancao-Banxia* and IL-6/STAT3. As our lab is not currently qualified to culture the SARS-CoV-2, so it is difficult for us to finish the experimental verification. Meticulous animal experiments are warranted to verify the key targets in the future. Second, to test the therapeutic efficacy of *Gancao-Banxia* for COVID-19 patients in the following-works, a prospective case-control study also needs to be conducted to exclude a variety of confounders. Third, based on clinical data mining, the results may be affected by the completeness and accuracy of data recording. Therefore, we only entered reliable information, including age, gender, hospital length of stay, and TCM prescriptions. Fourth, the number of enrolled prescriptions was less than the true number of prescriptions due to the incompleteness of medical records. Fifth, we only analyzed two highest-frequency CMs-pair which was obtained from the Hunan province, so the representativeness is not very strong. In addition to exploration of other CMs pairs and groups, future work requires a comparative analysis of various populations in different regions.

6. Conclusion

In short, based on the clinical formulae mining, *Gancao-Banxia* pair might be the key CM-pair and exerted therapeutic effect through the inflammatory response, viral defense and immune responses signaling pathways. Five candidate active ingredients might inhibit IL-6 Amp by dually binding IL-6 and STAT3, exerting an active effect on moderate COVID-19. However, further studies are needed to validate this possibility in the SARS-CoV-2 models.

Ethics approval and consent to participate

Medical Ethics Committee of The First Hospital of Changsha approved this study (Number: (2021) Ethic [Clinical paper] No.4). The written informed consent was waived.

Authors' contributions

YW, RF and WKL designed the project. WKL collected prescription data and carried out data input. RF and TZ checked and verified the data for accuracy again. WKL and RQD analyzed the data. WKL and RQD drafted the manuscript. YW, WKL, RQD, XHG and TT revised the manuscript.

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Consent to publish

All authors agreed on the final version.

Data availability

The data used and/or analyzed in this study are available from corresponding authors upon request.

Declaration of competing interest

The authors declare no potential conflicts of interests.

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List of abbreviations

ACE2	angiotensin-converting enzyme 2
ARDS	Adult Respiratory Distress Syndrome
BP	biological process
COVID-19	Coronavirus disease 2019
CM	Chinese medicine
CC	cellular components
DL	drug-likeness
GO	Gene Ontology
IL-6	Interleukin-6
IL-6 Amp	IL-6 amplifier
KEGG	Kyoto Encyclopedia of Genes and Genomes
MF	molecular function
OB	oral bioavailability
PPI	Protein-protein interaction
STAT	Signal Transducers and Activators of Transcription

SARS-CoV-2 severe acute respiratory syndrome coronavirus 2
STAT3 Signal Transducer and Activator Of Transcription 3
TCM Traditional Chinese medicine;
3CL pro 3C-like protease

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.compbiomed.2022.105457.

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