

Network Pharmacology-based Strategy for Studying the Mechanism of Gegen Qinlian Decoction for the Treatment of Acute Colitis

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Research

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Abstract

Background: Gegen Qinlian Decoction(GQD) has been used to treat acute colitis (AC) for several years in China and it has shown good efficacy. However, the active components and target proteins of its anti-AC effects remains to be deciphered.

Methods: In this study, serum pharmacochemistry and network pharmacology strategy were integrated to identify the constituents in blood and the mechanism of GQD for the treatment of AC. Ultra-performance liquid chromatography and LTQ-Orbitrap mass spectrometry(UPLC-LTQ-Orbitrap-MS) was used to identify the absorbed components of GQD in rat serum; molecular docking and compound-target network analysis were used to predict candidate targets and critical components in GQD responsible for efficacy; In addition, the Kyoto Encyclopedia of Genes and Genomes(KEGG) pathway analysis and Gene Ontology(GO) enrichment analysis were used to predict the related pathways and biological process respectively; Finally, the model rats with acute colitis were induced by DSS(Dextran Sulfate Sodium) in order to verify the effects and potential mechanism of baicalein, which is an important component of GQD.

Results: Based on our comprehensive systematic approach, 23 components were successfully identified in rat serum after oral administration of GQD. The predicted results of molecular docking indicated that these 23 active components closely interacted with 41 protein targets associated with inflammation, immunity and enteric mucos. Among the 23 compounds identified, baicalin, baicalein, wogonoside, liquiritin and daidzin may be the most important components of GQD. Furthermore, according to GO enrichment analysis, the 41 candidate targets identified were mainly involved in two biological process, immune system process and inflammatory response. The KEGG pathway analysis revealed that 41 candidate targets were associated with 62 biological pathways, including HIF-1 signaling pathway and PI3K/Akt signaling pathway. Animal experiments found that baicalein could inhibit the activation of PI3K/Akt/HIF-1 signaling pathway and significantly reduce pro-inflammatory cytokines, such as IL-1 β , IL-6, IL-8 and TNF- α to alleviate intestinal mucosal damage and achieve a therapeutic effect on AC.

Conclusion: This research not only provides a novel and scientific strategy to better understand the complex mechanism of GQD, but also offers a new perspective to identify and/or discover novel active ingredients of TCM drugs.

Background

Acute colitis (AC) can be induced by bacteria, viruses or parasites. The incidence rates of AC are increasing worldwide. The general symptoms include diarrhea, abdominal pain and vomiting, and seriously affects the quality of life in some patients [1-5]. The current treatment strategy for AC are antibiotic administration. However, long-term use of antibiotics could induce bacterial resistance and dysbacteriosis and is not conducive to patient recovery [6]. Traditional Chinese Medicine (TCM) has been used widely in China for thousands of years and it has unique advantages for treating complex diseases. Clinical studies have demonstrated that several TCM drugs are effective in treating AC[4, 7-10].

GQD is a famous prescription of Zhang Zhongjing. It consists of Puerariae Lobatae radix, Scutellariae radix, Coptidis rhizoma and Glycyrrhizae Radix et Rhizoma. It has been demonstrated to be an effective treatment for AC [11, 12]. However, GQD consists of a mixture of hundreds of ingredients, the active ingredient or ingredients have not been identified, and the “multi-component and multi-target” therapeutic mechanism of GQD is also still unclear, which affected its clinical use to a certain extent. Hence, a promising analysis method is needed for a comprehensive understanding of the mechanism of action of GQD.

In order to decipher the molecular mechanisms involved in GQD efficacy for AC, the components of GQD in the serum were first identified by an UPLC-LTQ-Orbitrap-MS analysis system. And then, the protein targets and related biological processes as well as pathways were recognized by a network pharmacology strategy. UPLC-LTQ-Orbitrap-MS is an analytical technique based on LTQ multi-stage mass spectrometry with Orbitrap high resolution capability. It is a powerful method to rapidly separate and identify blood constituents. Recently, with the rapid development of systems biology and bioinformatics, the emerging network pharmacology has been considered as a promising tool for analyzing TCM formulas [13, 14]. It provides new sights to understand the interactions between drugs and protein targets, and the compound-target model is helpful for understanding the multi-component and multi-targeted therapeutics of TCM formulas [15]. Wei et al [16] applied network pharmacology to reveal that 11 compounds in San-Cao Granule related to 16 targets for treatment of liver fibrosis. Tao et al [17] found that 58 bioactive ingredients from the Chinese herbal Radix Curcumae formula closely associated with 32 potential targets related to the Cardiovascular and cerebrovascular diseases. In addition, they also found that some components could regulate the same protein target simultaneously through network pharmacology analysis. Therefore, network pharmacology has become a promising method in explaining synergistic mechanisms of TCMs.

DSS induced colitis model is widely used to elucidate the the molecular and cellular pathways involved in pathogenesis of ulcerative colitis (UC) and AC [18-21]. It is generally believed that UC is a more serious state of further development of AC [22]. Studies showed that the same concentration of DSS can cause AC rat model in a short time, while for a long time DSS can cause a UC rat model [23]. Here, we refer to the method of literature [24], using a short time to create AC model.

In this study, UPLC-LTQ-Orbitrap-MS was used in combination with a network pharmacology approach to predict crucial components and potential targets of GQD for treating AC. We then determine the effects and mechanisms of these key ingredients to treat AC using rat disease models. This approach will be valuable in understanding the chemical and pharmacological basis of TCM drugs. Figure 1.

Methods

Reagents and materials

Puerariae Lobatae radix (Lot number: 190225), Scutellariae radix (Lot number: 190222), Coptidis rhizome (Lot number: 190124) and Glycyrrhizae Radix et Rhizoma (Lot number: 190307) were purchased from Kangqiao Pharmacy (Shanghai, China) and was authenticated by Yanjun Chen, the chief pharmacist from Hongqiao Pharmacy, Shanghai, China. DSS was purchased from Yeasen Biotechnology (MW: 36000-50000, Shanghai, China) and baicalein (purity $\geq 98\%$) was purchased from Yuanye Biotechnology (Shanghai, China). Salazosulfapyridine (SASP) was purchased from Shanghai Fuda Pharmaceutical Co., Ltd. Mouse IL-6, IL-8, TNF- α and IL-1 β enzyme linked immunosorbent assay (ELISA) kits were purchased from Youxuan Biotechnology (Shanghai, China). Rabbit anti-PI3K, phosphorylated-PI3K, anti-AKT, phosphorylated-AKT, anti-HIF-1 α were purchased from Affinity Biosciences (United States), anti- β -actin were purchased from Servicebio (Wuhan, China).

Blood components analysis of GQD

GQD sample preparation

GQD is composed of Puerariae Lobatae radix, Scutellariae radix, Coptidis rhizoma and Glycyrrhizae Radix et Rhizoma at a ratio of 15:9:9:6. Appropriate amounts of each component were weighed and then processed individually before mixing. Puerariae Lobatae radix was boiled for 20 min, afterwards the other components were added and decocted in boiling water twice for 30 min each time. The mixture was filtered and then evaporated to a final density of 1 g/mL. The final product, GQD, was stored at 4 °C until needed.

Animals and blood collection

Male Sprague-Dawley rats weighing 180-200g were purchased from the Experimental Animal Center of Shanghai University of Traditional Chinese Medicine, license number: SCXK (2018-0006). Animals were maintained in a controlled environment (temperature: 20~25 °C, relative humidity 38~41%) and housed on a 12-hour light/dark cycle. The animal experiment was carried out in accordance with the Institutional Animal Committee of Shanghai University of Traditional Chinese Medicine (Permit No. PZSHUTCM190301004.).

Animals were fed food and water ad libitum. After environmental adaptation, twelve rats were randomly divided into 2 groups, the GQD group (orally administered GQD at a dose of 17 ml/kg) and the blank group (orally administered equal volume of saline). Blood samples were collected from the jugular vein 1 hour after the dose and then centrifuged at 4000 \times g for 10 min at 4 °C. Serum samples were stored at -80 °C until needed.

Serum sample preparation

800 μ L acetonitrile was added to 200 μ L of serum and vortexed well. The mixture was then centrifuged at 12000 \times g for 10 min at 4 °C. Supernatant was transferred to a new tube and dried under a gentle nitrogen stream. Dried residues were then reconstituted in 100 μ L acetonitrile and centrifuged at 12000 \times g for 10 min at 4 °C. 5 μ L of the supernatant was used for UPLC-LTQ-Orbitrap-MS analysis.

UPLC-LTQ-Orbitrap-MS analysis

Thermo Accela UPLC system (Thermo Fisher Scientific, Germany) was used in this study to analyze serum samples. Dikma Endeavorsil C18 (2.1 \times 100mm \times 1.8 μ m) was used for chromatographic separation and the mobile phase consisted of solvent A (0.1% formic acid in water) and solvent B (acetonitrile). The flow rate was kept at 0.3 mL/min, and gradient elution was performed as follows: 0 min~0.5 min, 5%B; 0.5 min~1.5 min, 5%~10%B; 1.5 min~3 min, 10%~14%B; 3 min~4.5 min, 14%B; 4.5 min~8 min, 14%~16.5%B; 8 min~10 min, 16.5%~18.5%B; 10 min~13 min, 18.5~24%B; 13 min~15.5 min, 24~50%B; 15.5 min~17.5 min, 50%~100%B; 17.5 min~20 min, 100%B; 20 min~22.5 min, 100%~5%B; 22.5 min~24 min, 5%B. MS/MS spectrometer analysis was performed on an LTQ-Orbitrap Elite mass spectrometer (Thermo Fisher Scientific, Germany) using the full-scan mode. The mass range was m/z 50-1000 in the positive ion mode. The parameters for electrospray ionization interface were as follows: capillary voltage, 3.8kV; capillary temperature, 350 °C; ion source temperature, 300 °C; sheath gas (N₂) flow rate, 35 arb; auxiliary gas (N₂) flow rate, 15 arb.

Network pharmacology analysis of GQD

Protein targets database building

Firstly, candidate proteins associated with AC were collected from the following resources (1) literature [5-25-30](2) DrugBank (<http://www.drugbank.ca/>) and (3) Therapeutic Targets Database (<http://bidd.nus.edu.sg/group/ttd/>). Secondly, the corresponding protein PDB IDs with x-ray structures were identified from the uniprot database (<https://www.uniprot.org/#>). Finally, proteins with ligand-receptor schematics were downloaded using (RCSB) Protein Data Bank (<https://www.rcsb.org/>).

Molecular docking

The interaction between compounds and protein targets were preliminarily determined using discovery Studio 2016 for molecular docking. All docking studies were performed using a protein-ligand complex with a crystal structure present, similar to the method described previously in Chen et al [31].

Based on the target prediction results, candidate targets were selected based on the following three criteria: i) low binding energy between compounds and proteins by virtual screening (better match degree), ii) the higher docking scores for target proteins (more likely to be potential targets), iii) proteins that closely associated with colitis.

Compound-target network construction

A compound-target network to investigate the anti-AC mechanism of GQD was constructed using Cytoscape 3.6.1 (<http://www.cytoscape.org/>). Cytoscape is widely used in network pharmacology for visualizing molecular and component interaction networks [32]. In the graphical network, compounds and proteins were represented as nodes and intermolecular interactions between molecules (compound-target) were denoted with links[33]. The analysis of network parameters were performed by Network Analyzer (a Cytoscape plug-in).

PPI network construction

The candidate proteins were submitted to the Search Tool, STRING, <http://string.embl.de/> database, to explore the functional interactions between the proteins. The interactions with combined score > 0.4 were selected as significant. The mapped proteins were imported into Cytoscape software to construct a protein-protein interaction (PPI) network. The Network Analyzer of Cytoscape was used to further analyze network parameters, such as the average degree of freedom and degree of freedom etc. Then, set the size and color of the nodes according to the degree of freedom, the bigger the node size and the brighter the color with the greater degree value.

GO enrichment analysis and KEGG pathway analysis

The GO enrichment analysis and KEGG pathway analysis were performed using DAVID online tool. $p < 0.01$ was considered statistically significant. The GraphPad Prism software and Omicshare cloud platform were used to achieve visualization.

Experimental validation

DSS-induced AC models and drug treatments

Animal information and breeding environment are the same as above. The AC rat model was established by administrating DSS in their drinking water. After environmental adaption, forty rats were randomly divided into the following five groups: Normal group, DSS group, baicalein (15 mg/kg) +DSS group, baicalein (20 mg/kg) +DSS group and SASP (400 mg/kg) +DSS group.

Rats in the normal group received distilled water, while rats in the other groups received 7% (w/v) DSS in their drinking water. Seven days later, the AC model was fully established. In the model therapy groups, SASP (40 mg/ml), the reference drug, and baicalein (1mg/ml) were suspended in CMC-Na. Drugs were administered to rats in their respective groups once a day for 7 days. The remaining groups were administered an equal volume of CMC-Na.

Disease activity index(DAI) assessment and histological analysis of colonic

Stool consistency, gross bleeding and body weight of rats were recorded, and were used to assess DAI. The scoring criteria were based on the method previously described [34]. Rats were euthanized at the end of the study and the entire colon was harvested. The colon was opened longitudinally and quickly washed in saline buffer and the morphology of the colon was examined using a microscope (Motic, China). Colonic sections were fixed in 4% polyformaldehyde and embedded in paraffin for histopathological analysis by Hematoxylin & eosin (H&E) staining. Photomicrographs were captured using a digital research photographic microscope (Thermo Corporation, America).

Enzyme-Linked immunosorbent Assay (ELISA) analysis

Blood was collected from abdominal aorta of rats after intraperitoneal anesthesia with 2% pentobarbital sodium, then the whole blood was placed at 4 °C for 2 hours and centrifuged at 3 500 × g for 10 minutes to obtain serum. The enzyme linked immunosorbent assay was used to determine the levels of TNF- α , IL-1 β , IL-6 and IL-8 in rat serum.

Western blot analysis

The proteins samples were prepared from the colon tissue by RIPA lysis buffer, and its concentrations were detected by BCA protein concentration assay kit. The proteins were exposed to electrophoresis on 8% to 12% SDS-PAGE gel and transferred to a PVDF membrane. After blocking with 5 % BSA at room temperature for 1 h, the membrane were incubated with different primary antibodies at 4 °C overnight. Then the PVDF membrane were washed with TBST three times and incubated with HRP conjugated secondary antibodies at room temperature for 1 h. Finally, after washing with TBST three times, the membrane were added with ECL solution and were placed in a gel imager for photographic analysis.

Data analyses

Statistical analysis was performed using GraphPad Prism 6.0 statistical software. Experimental data were expressed as mean \pm standard deviation (mean \pm SD). Statistical significance was determined using One-way ANOVA test, and $p < 0.05$ was considered statistically significant.

Results

UPLC-LTQ-Orbitrap-MS analysis of rat serums after oral administration of GQD

In a previous study, we performed chemical analysis of GQD and 67 constituents were identified. In this study, UPLC-LTQ-Orbitrap-MS was used to detect and identify differential peaks in serum samples from rats administered saline versus GQD in the positive mode. Based on the retention time, MS/MS results and precise molecular mass, 23 components were identified. Among the 23 compounds, seven compounds were from *Coptidis rhizome*, six compounds were from *Puerariae Lobatae radix*, five compounds each were from *Scutellariae radix* and *Glycyrrhizae Radix et Rhizoma*. 13 of them were flavonoids, 7 of them were alkaloids, 2 of them were triterpenoids and triterpenoid saponins, and 1 was coumarin. Detailed information of the 23 blood constituents in GQD administered rat serum are shown in Table1. Structural data was obtained from the NCBI PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>). Total ion chromatographs of UPLC-LTQ-Orbitrap-MS using positive mode are shown in Fig.2.

Network pharmacology analysis of GQD

Putative protein targets collection

44 protein targets (Table2) were obtained, and their x-ray crystallographic structures were downloaded from the (RCSB) Protein Data Bank (<https://www.rcsb.org/>) for high-throughput molecular docking studies. These proteins are associated with inflammation, immunity and enteric mucosa. The pathogenesis of AC is not yet clear, but studies showed that it may be related to immune dysfunction, infectious factors, environmental factors, and other factors [35].

Table1 Analysis of blood constituents in rat serum after Gegen Qilian decoction administration

Peak No.	Retention time(min)	Molecular formula	Error ppm	Theoretical mass [M] ⁺ /[M+H] ⁺ /[M+Na] ⁺ /[M-H] ⁻ m/z	Experimental mass [M] ⁺ /[M+H] ⁺ /[M+Na] ⁺ /[M-H] ⁻ m/z	MS ²	Identified components
1	5.18	C ₂₀ H ₂₄ NO ₄	-2.936	342.16998	342.16898[M] ⁺	297.11[M-C ₂ H ₇ N] ⁺ 265.09[M-C ₂ H ₇ N-CH ₃ OH] ⁺	Magnoflorine
2	5.52	C ₂₁ H ₂₀ O ₉	-3.497	417.11801	417.11655[M+H] ⁺	399.11[M+H-H ₂ O] ⁺ 381.10[M+H-H ₂ O×2] ⁺ 351.09[M+H-H ₂ O×2-CH ₂ O] ⁺	Puerarin*
3	5.99	C ₂₂ H ₂₂ O ₁₀	-3.004	447.12857	447.12723[M+H] ⁺	429.12[M+H-H ₂ O] ⁺ 411.11[M+H-H ₂ O×2] ⁺ 393.10[M+H-H ₂ O×3] ⁺	3'-MethoxyPuerarin
4	7.06	C ₂₁ H ₂₀ O ₉	-3.497	417.11801	417.11655[M+H] ⁺	255.06[M+H-Glucose] ⁺	Daidzin*
5	8.54	C ₁₉ H ₁₆ NO ₄	-3.212	322.10738	322.10635[M] ⁺	294.10[M-C ₂ H ₄] ⁺	Berberrubine
6	9.22	C ₁₅ H ₁₂ O ₄	-3.522	257.08083	257.07993[M+H] ⁺	239.07[M+H-H ₂ O] ⁺ 137.02[M+H-C ₈ H ₈ O] ⁺	Liquiritigenin*
7	9.25	C ₂₁ H ₂₂ O ₉	-3.68	441.1156	441.11398[M+Na] ⁺	321.04[M+Na-C ₄ H ₈ O ₄] ⁺	Liquiritin*
8	11.07	C ₁₉ H ₁₄ NO ₄	-5.512	320.09173	320.08997[M] ⁺	292.10[M-CO] ⁺ 290.08[M-CH ₂ O] ⁺	Coptisine*
9	11.48	C ₂₀ H ₁₈ NO ₄	-5.785	336.12303	336.12109[M] ⁺	320.09[M-H-CH ₃] ⁺ 308.13[M-C ₂ H ₄] ⁺	Epiberberine*
10	11.88	C ₂₀ H ₂₀ NO ₄	-4.923	338.13868	338.13702[M] ⁺	323.12[M-CH ₃] ⁺ 294.11[M-CH ₃ -H-CO] ⁺	Jatrorrhizine*
11	13.88	C ₂₀ H ₁₈ NO ₄	-8.939	336.12303	336.12003[M] ⁺	321.10[M-CH ₃] ⁺ 320.09[M-CH ₃ -H] ⁺ 292.10[M-CH ₃ -HCO] ⁺	Berberine*
12	14.18	C ₂₁ H ₂₂ NO ₄	-6.772	352.15433	352.15195[M] ⁺	337.13[M-CH ₃] ⁺ 336.12[M-	Palmatine*

						CH ₃ -H] ⁺ 308.13[M- CH ₃ -HCO] ⁺	
13	15.05	C ₂₁ H ₂₂ O ₉	-3.48	419.13366	419.13220[M+H] ⁺	257.08[M+H- Glucose] ⁺	Isoliquiritin*
14	15.32	C ₁₅ H ₁₀ O ₄	-2.883	255.06518	255.06445[M+H] ⁺	237.05[M+H- H ₂ O] ⁺ 227.07[M+H- CO] ⁺ 199.08[M+H- CO×2] ⁺	Daidzein
15	15.49	C ₂₁ H ₁₈ O ₁₁	-4.893	447.09218	447.09000[M+H] ⁺	271.06[M+H- Glucuronide acid] ⁺	Baicalin*
16	15.5	C ₁₅ H ₈ O ₅	-4.608	269.04444	269.04321[M+H] ⁺	241.05[M+H- CO] ⁺	Coumestrol
17	16.42	C ₁₆ H ₁₂ O ₅	2.332	285.07575	285.07492[M+H] ⁺	270.05[M+H- CH ₃] ⁺ 257.04[M+H- CO] ⁺ 253.04[M+H- CH ₄ O] ⁺	3'- methoxydaidzein
18	16.54	C ₂₂ H ₂₀ O ₁₁	-3.162	461.10783	461.10638[M+H] ⁺	285.08[M+H- Glucuronide acid] ⁺	wogonoside*
19	17.28	C ₁₅ H ₁₀ O ₅	-2.287	271.06009	271.05948[M+H] ⁺	253.05[M+H- H ₂ O] ⁺ 243.05[M+H- CO] ⁺	Baicalein*
20	17.55	C ₃₀ H ₄₆ O ₄	-6.463	471.34688	471.34384[M+H] ⁺	425.34[M+H- H ₂ O-CO] ⁺ 407.33[M+H- H ₂ O×2-CO] ⁺ 317.12[M+H- C ₉ H ₁₄ O ₂] ⁺	Enoxolone
21	17.56	C ₄₂ H ₆₂ O ₁₆	-3.622	823.41106	823.40808[M+H] ⁺	647.38[M+H- Glucuronide acid] ⁺ 471.35[M+H- Glucuronide acid×2] ⁺ 453.33[M+H- Glucuronide acid×2-H ₂ O] ⁺	Glycyrrhizic acid*
22	17.99	C ₁₆ H ₁₂ O ₅	-8.279	285.07575	285.07339[M+H] ⁺	270.05[M+H- CH ₃] ⁺	Wogonin*
23	18.19	C ₁₆ H ₁₂ O ₅	-6.349	285.07575	285.07394[M+H] ⁺	270.05[M+H- CH ₃] ⁺	Oroxylin-A*

*This compound was identified using reference standards

Table2 Potential protein targets of GQD

ID	Full name of protein	Short name of protein	Classification
P1	Antithrombin-III	SERPINC1	Inflammation
P2	Integrin beta-7	ITGB7	Inflammation
P3	Serine/threonine-protein kinase mTOR	MTOR	Inflammation
P4	Interleukin-13	IL13	Inflammation
P5	Vitamin D-binding protein	GC	Inflammation
P6	P-selectin	SELP	Inflammation
P7	Interleukin-23 subunit alpha	IL23A	Inflammation
P8	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	Inflammation
P9	Bcl-2-like protein 1	BCL2L1	Inflammation
P10	Calcium/calmodulin-dependent protein kinase type IV	CAMK4	Inflammation
P11	Toll-like receptor 4	TLR4	Inflammation
P12	Protein kinase C alpha type	PRKCA	Inflammation
P13	Prothrombin	F2	Inflammation
P14	Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit delta isoform	PIK3CD	Inflammation
P15	Transcription factor p65	RELA	Inflammation
P16	Protein kinase C gamma type	PRKCG	Inflammation
P17	Glucocorticoid receptor	NR3C1	Inflammation
P18	Peroxisome proliferator activated receptor gamma	PPARG	Inflammation
P19	Intercellular adhesion molecule 1	ICAM1	Inflammation
P20	Triggering receptor expressed on myeloid cells 1	TREM1	Inflammation
P21	Proto-oncogene tyrosine-protein kinase receptor Ret	RET	Enteric mucosa
P22	Toll-like receptor 8	TLR8	Inflammation
P23	cAMP-specific 3',5'-cyclic phosphodiesterase 4B	PDE4B	Inflammation
P24	C-type lectin domain family 4 member E	CLEC4E	Inflammation
P25	Bifunctional heparan sulfate N-deacetylase/N-sulfotransferase 1	NDST1	Inflammation
P26	Rho-associated protein kinase 1	ROCK1	Inflammation
P27	Nuclear receptor subfamily 1 group D member 2	NR1D2	Inflammation
P28	Mitogen-activated protein kinase 14	MAPK14	Inflammation
P29	MAP kinase-activated protein kinase 3	MAPKAPK3	Inflammation
P30	Galectin-3	LGALS3	Inflammation
P31	Heat shock cognate 71 kDa protein	HSPA8	Inflammation
P32	Mitogen-activated protein kinase 11	MAPK11	Inflammation
P33	Angiopoietin-1 receptor	TEK	Inflammation
P34	RAC-alpha serine/threonine-protein kinase	AKT1	Inflammation

P35	Hypoxia-inducible factor 1-alpha	HIF1A	Inflammation
P36	Macrophage colony-stimulating factor 1 receptor	CSF1	Immunity
P37	Tyrosine-protein kinase SYK	SYK	Immunity
P38	Interleukin-6	IL6	Inflammation
P39	Ficolin-3	FCN3	Immunity
P40	Tryptase beta-2	TPSB2	Immunity
P41	Neutrophil gelatinase-associated lipocalin	LCN2	Immunity
P42	Methionine-R-sulfoxide reductase B1	MSRB1	Immunity
P43	Tyrosine-protein kinase BTK	BTK	Immunity
P44	Tyrosine-protein kinase JAK2	JAK2	Immunity

Target identification

Molecular docking is a computational technique that is commonly used in structure-based drug design [36]. Molecular docking can predict the binding sites of candidate drugs to disease proteins [37]. Using the molecular docking method, 41 targets were mapped out from 44 targets. Glyceraldehyde-3-phosphate dehydrogenase, MAP kinase-activated protein kinase 3, C-type lectin domain family 4 member E were removed due to its low binding affinity for all candidate compounds. The most promising candidate targets of GQD were Intercellular adhesion molecule 1(ICAM1), Tyrosine-protein kinase JAK2 (JAK2), RAC-alpha serine/threonine-protein kinase (AKT1), Serine/threonine-protein kinase mTOR(MTOR), Peroxisome proliferator activated receptor gamma(PPARG), Transcription factor p65(RELA) and Mitogen-activated protein kinase 14(MAPK14).

Compound-target network construction and analysis

A "compound-target" network was constructed to determine the molecular mechanism of GQD using Cyscape3.6.1. The network consisted of 64 nodes and 667 edges (Fig.3). Nodes denote drug molecules and target proteins, while an edge represents the interaction between a specific molecule and a protein [38]. The results of network analysis are shown in Table3. The "degree" indicates the number of protein targets that a compound can bind to [39]. In the network of GQD, the top 5 components with the highest degree were baicalin, baicalein, wogonoside, liquiritin and daidzin, baicalin can interacted with 37 potential proteins, baicalein, wogonoside, liquiritin and daidzin can interacted with 36 potential proteins.

Table3 Degree of blood components in GQD

Chinese herb	ID	Compound name	Degree
Coptidisrhizoma	C1	Magnoflorine	24
PuerariaeLobataeradix	C2	Puerarin	32
PuerariaeLobatae radix	C3	3'-MethoxyPuerarin	25
PuerariaeLobatae radix	C4	Daidzin	36
Coptidisrhizoma	C5	Berberubine	26
Glycyrrhizae Radix et Rhizoma	C6	Liquiritigenin	29
Glycyrrhizae Radix et Rhizoma	C7	Liquiritin	36
Coptidisrhizoma	C8	Coptisine	29
Coptidisrhizoma	C9	Epiberberine	28
Coptidisrhizoma	C10	Jatrorrhizine	26
Coptidisrhizoma	C11	Berberine	26
Coptidisrhizoma	C12	Palmatine	25
Glycyrrhizae Radix et Rhizoma	C13	Isoliquiritin	31
PuerariaeLobataeradix	C14	Daidzein	26
Scutellariae radix	C15	Baicalin	37
PuerariaeLobatae radix	C16	Coumestrol	23
PuerariaeLobatae radix	C17	3'-methoxydaidzein	26
Scutellariae radix	C18	wogonoside	36
Scutellariae radix	C19	Baicalein	36
Glycyrrhizae Radix et Rhizoma	C20	Enoxolone	22
Glycyrrhizae Radix et Rhizoma	C21	Glycyrrhizic acid	35
Scutellariae radix	C22	Wogonin	27
Scutellariae radix	C23	Oroxylin-A	26

Components–targets interactions analysis

After docking the compounds in Discovery studio 2016, the pymol software is used to provide details about the interplay between proteins and active compound. Here, daidzin and ICAM-1, wogonoside and JAK2 were selected to study the action modes between compounds and proteins, which had low binding energy and high docking scores.

The interplay between daidzin and ICAM-1 is shown in Fig.4-A. The carbonyl can form a hydrogen bond with Lys142, and glycosyl group can form a hydrogen bond with Asp85, Ala84, and Gln166, respectively.

The interaction between wogonoside and JAK2 is shown in Fig.4-B. The carbonyl group on the parent nucleus can form a hydrogen bond with Pro1114, and the glycosyl group can form a hydrogen bond with Phe1116, Arg1117, Gly968, respectively. Lin's research confirmed the interaction between wogonoside and protein JAK2. He discovered that wogonoside could limit the phosphorylation process of JAK2 and reduce the production of inflammatory factor IL-8 [40].

PPI network of GQD anti-AC construction and analysis

The construction and the most significant module analysis of PPI network were performed by STRING and Cytoscape software (Fig.5.). The interactions between the proteins with combined score > 0.4 were selected in this study. The PPI network consist of 36 nodes (genes), 186 edges (interactions) and the average degree of freedom is 9.07. The greater the degree value, the more the biological function of the node in the PPI network. thus, the top 15 proteins with degree greater than 11 were selected as the core targets of GQD against AC (Table 4.). The results were basically consistent with the molecular docking analysis.

Table 4 The top 15 hub genes of GQD anti-AC in the PPI network

No	gene name	Degree	No	gene name	Degree
1	IL6	28	9	TLR8	14
2	TLR4	23	10	IL13	13
3	AKT1	20	11	BCL2L1	13
4	RELA	19	12	MAPK14	13
5	MTOR	18	13	JAK2	12
6	ICAM1	18	14	SELP	11
7	SYK	17	15	LGALS3	11
8	PPARG	15			

GO enrichment analysis

The results of GO enrichment analysis showed that the 41 target proteins were involved in 137 Biological Process, 16 Molecular Function and 15 Cellular Component. Top ten terms of Biological Process, Molecular Function and Cellular Component were selected and revealed in Figure 6 according to *P* value. We found that the therapeutic effect of GQD on AC may involve in Biological Processes such as inflammatory response and innate immune response, Molecular Function such as protein kinase activity and ATP binding, Cellular Component such as cytosol and blood microparticle.

Pathway analysis

KEGG pathway enrichment analysis was performed by using the DAVID online tool. 41 target proteins were involved in 62 signaling pathways. Information on the top 20 pathways are shown in table 5 and Fig 7 .

Table 5 Potential pathways modulated by GQD

Term	Description	Count in gene set	PValue
hsa04066	HIF-1 signaling pathway	10	2.95E-10
hsa05164	Influenza A	11	3.30E-09
hsa04380	Osteoclast differentiation	10	4.84E-09
hsa04664	Fc epsilon RI signaling pathway	8	1.86E-08
hsa04151	PI3K-Akt signaling pathway	13	1.91E-08
hsa05205	Proteoglycans in cancer	10	1.95E-07
hsa04620	Toll-like receptor signaling pathway	8	4.14E-07
hsa04668	TNF signaling pathway	8	4.42E-07
hsa05145	Toxoplasmosis	8	5.34E-07
hsa05200	Pathways in cancer	12	7.99E-07
hsa04071	Sphingolipid signaling pathway	8	9.67E-07
hsa05152	Tuberculosis	9	1.02E-06
hsa05162	Measles	8	1.94E-06
hsa05142	Chagas disease (American trypanosomiasis)	7	6.80E-06
hsa04370	VEGF signaling pathway	6	7.78E-06
hsa04670	Leukocyte transendothelial migration	7	1.22E-05
hsa04917	Prolactin signaling pathway	6	1.65E-05
hsa05169	Epstein-Barr virus infection	7	1.71E-05
hsa05133	Pertussis	6	2.16E-05
hsa04611	Platelet activation	7	2.46E-05

Baicalein alleviated the DSS-induced AC

Changes of DAI scores

The body weights of DSS-induced rats decreased during the research, while treatment with baicalein (20 mg/kg) or SASP could significantly improve DSS-induced weight loss (Fig.8A). In addition to changes in body weight, diarrhea and blood stool of rats were measured daily. On the third day of modeling, presence of soft stool and perianal infection were observed. On the fourth day, blood was present in the stool. The DAI scores of DSS-induced rats significantly increased. Administration of baicalein (20 mg/kg) and SASP had a significant therapeutic effect and reduced DAI scores (Fig.8B).

Changes of pathological

Histopathological analysis indicated that rats in the normal group, the colon glands were arranged neatly, the mucosa was intact, and no inflammatory cells infiltrated into the submucosa. However, the majority of the colon glands in rats induced by DSS were destroyed, the colonic epithelial cells were detached, and numerous inflammatory cells were observed in the submucosal tissues. The pathological condition of rats treated with baicalein (20 mg/kg) or SASP showed significant improvement (Fig.8C).

Changes of pro-inflammatory cytokines

Pro-inflammatory cytokines play a significant role in the process of DSS-induced acute enteritis rat [4]. To investigate whether baicalein is associated with proinflammatory cytokines in the repair of DSS-induced acute enteritis, the levels of TNF- α , IL-1 β , IL-6 and IL-8 in the serum were tested by enzyme linked immunosorbent assay. Baicalein (15, 20 mg/kg) and SASP could remarkably reduce the levels of IL-1 β in the serum which significantly increased after DSS challenge. Baicalein (20 mg/kg) and SASP could significantly reduce the TNF- α , IL-6 and IL-8 in the serum after DSS challenge (Fig.9.). The results indicated that the protective effect of baicalein on DSS-induced colitis is closely related to the down-regulation of the expression of these pro-inflammatory cytokines.

Changes of the PI3K/Akt/HIF-1 signaling pathway

According to the previous pathway analysis studies, we proposed that baicalein may exert its anti-AC effects by regulating the PI3K/Akt/HIF-1 signaling pathway. Therefore, western blot analysis were used to study the expressions of some key proteins in the pathway. As shown in Fig.10, Compared to the DSS group, the protein expressions of phospho-PI3K (p-PI3K) and HIF-1 α were significantly decreased in Baicalein (20 mg/kg) group and SASP group. Similarly, the protein expressions of phospho-AKT(p-AKT) were significantly decreased in Baicalein (15, 20 mg/kg) group and SASP group, and the p-AKT expressions were down-regulated by baicalein dose-dependently. In addition,compared to the DSS group, the expression of total-PI3K, total-AKT were hardly affected by baicalein and SASP.

Discussion

In this study, UPLC-LTQ-Orbitrap-MS was used to analyze and identify the active compounds in rat serum after oral administration GQD, so as to systematically clarify the effective substance of GQD; Then, network pharmacology strategy was used to study the mechanism of GQD for the treatment of acute colitis, including revealing its key components, targets, and regulated pathways; Finally, the therapeutic effects and mechanism of baicalein, which is an important component of GQD, were investigated in dextran sulfate sodium (DSS)-induced AC rats. This article hopes to lay a foundation for the study of quality control and mechanism of GQD, and provide evidence for the investigative modernization of TCM.

There are 23 components were identified from rat serum after oral administration GQD by UPLC-LTQ-Orbitrap-MS analysis system, and the results indicated that flavonoids and alkaloids were the main active ingredients of GQD. The results of molecular docking showed that the constituent may target protein such as ICAM1, JAK2, AKT1, MTOR and PPARG. Here, three representative proteins(ICAM-1, AKT1 and JAK2) were selected to futher discuss their function.

The main cause of acute exacerbation of chronic colitis is the influx of neutrophils in the lining of the epithelium, which leads to the formation of crypt abscesses. After the crypts rupture, the pus flows to the intestine or neutrophils spread into the mucosa or connective tissue. Therefore, the focus of controlling this disease seems to be to limit the damage of neutrophils. The study found that ICAM-1 is key to the flow of neutrophils into the colon [41]. From the link between AC and ICAM-1 genes, ICAM-1 was found to be a clear target for the treatment of AC.

Protein kinase B (AKT) consisted of three isoforms AKT1, AKT2 and AKT3, regulates many processes including cell survival and apoptosis [42]. Studies confirm that the activation of AKT would regulate the entry of nuclear transcription factor (RELA, NF- κ B) into the nucleus, and promote the release of various inflammatory cytokines and pro-apoptotic proteins, initiate inflammatory responses and cell apoptosis [26-43] , So AKT and NF- κ B play key roles in the pathogenesis and treatment of AC.

At present, it is believed that the pathogenesis of colitis is closely related to the disorder of immune regulation function. JAK2 is an essential signaling events in both innate and adaptive immunity, Yang et al. analyzed the genomic differences between colitis patients and normal people, and found that JAK2 gene was significantly associated with colitis in Korea [30].

Subsequently, compound-target network analysis suggested that baicalin, baicalein, wogonoside , liquiritin and daidzin which with the highest degree were the core components of GQD. The therapeutic effect and mechanisms of baicalin and wogonoside on AC rats were confirmed by many consulting published literatures. For example, Sun et al [44] reported that wogonoside could exert repair and therapeutic effects on AC rats by inhibition of NF- κ B and NLRP3 inflammasome pathway. Baicalin could effectively treat DSS-induced ulcerative colitis by inhibiting IL-33 expression and its subsequent activation of NF- κ B [45]...In addition, Hong et al [46] demonstrated the therapeutic effect of baicalein on AC, but its mechanism of action was not deciphered.

KEGG pathway enrichment analysis predicted that GQD could suppress AC by regulating the HIF-1 signaling pathway and PI3K-Akt signaling pathway and Toll-like receptor signaling pathway.

Studiesshowed that in the pathogenesis of AC, intestinal mucosal microcirculation disorder would make the colonic tissue in the environment of hypoxia , and then the colonic tissue will turn on oxidative stress and cause the expression of HIF-1 α , so as to realize the hypoxia tolerance and inflammatory repair of colon tissue. Therefore, HIF-1 signaling pathway plays a key role in maintaining intestinal barrier function and treatment of AC [47-49]. The HIF-1 signaling pathway is derived from KEGG database, as shown in Figure 11, It is found that the expression of HIF-1 α is regulated by PI3K-Akt signaling pathway, which is consistent with the literatures [50-51]. PI3K-AKT signaling pathway can regulate a wide range of cellular, is involved in the regulation and release of multiple pro-inflammatory cytokines, and these pro-inflammatory cytokines play an important role in the development of AC [43]. So we speculated that the active components of GQD may achieve the therapeutic effect on AC by regulating HIF-1 signaling pathway and PI3K-Akt signaling pathway. Toll-like receptor signaling pathway is involved in the activation of innate and adaptive immune responses [52]. Although the pathogenesis of AC is not clear, it is thought to be closely related to the immune

function barrier, intestinal barrier function and environmental factors [53]. Therefore, according to the research results, GQD may improve intestinal inflammation and restore intestinal function through immune regulation to achieve the treatment of AC.

Finally, animal experiments were performed to determine the efficacy of baicalein on AC rats and its potential mechanism. The results showed that after the intervention of baicalein, the weight of rats increased, the DAI scores decreased, and the injury of colon were improved compared with the model group. In addition, the levels of TNF- α , IL-1 β , IL-6 and IL-8 were decreased compared with model rats, the protein expressions of phospho-PI3K(p-PI3K), phospho-Akt(p-Akt) and HIF-1 α were down-regulated compared with model rats, which demonstrated that baicalein may affect the phosphorylation process of PI3K and AKT, thereby inhibiting the PI3K/Akt/HIF-1 signaling pathway and significantly reduce pro-inflammatory cytokines, such as IL-1 β , IL-6, IL-8 and TNF- α , to reduce intestinal mucosal damage and achieve a therapeutic effect on acute colitis.

According to the researches [19, 54-56], on one side, DSS-induced colitis has been considered to be driven by activated intestinal macrophages, which release pro-inflammatory cytokines and chemokines to up-regulate the expression of HIF-1 α , cause tissue damage. In other hand, oxidative stress can cause intestinal epithelial barrier dysfunction and up-regulate the expression of HIF-1 α . The mechanism of action of HIF-1 α need to be further studied in the following work.

Taken together, our study identified 23 active ingredients in rat serum after oral administration of GQD, and the 23 components closely interacted with 41 protein targets associated with inflammation, immunity and enteric mucos, the core components of GQD were baicalin, baicalein and wogonoside etc, the most promising candidate targets of GQD were ICAM1, MAPK14, AKT1 and PPARG etc. The KEGG pathway enrichment analysis predicted that GQD could suppress AC by regulating the HIF-1 signaling pathway, PI3K-Akt signaling pathway and Toll-like receptor signaling pathway. And the animal experiments confirmed that baicalein could ameliorate intestinal mucosal damage by regulate PI3K/Akt/HIF-1 signaling pathway. These results demonstrate that multi-component synergistic system are the advantage of TCM when play a therapeutic role in the treatment of AC, which deserves further exploration.

Conclusion

In this study, we used a UPLC-LTQ-Orbitrap-MS method combined with an integrated network pharmacology strategy were used to identify differential blood components, predict potential targets and identify critical compounds of GQD that were efficacious in the treatment of AC. In addition, animal experiments were performed to determine the efficacy of the predicted components on AC. The network pharmacology method was used to establish a biological network of chemical components interacting with target proteins at the molecular and system-wide level. This approach helped in identifying the molecular mechanisms of GQD action in reducing AC, and should be valuable for understanding the molecular mechanisms of other traditional Chinese medicines.

Abbreviations

GQD:Gegen Qinlian Decoction; AC:acute colitis;UPLC-LTQ-Orbitrap-MS:Ultra-pe-

rformance liquid chromatography and LTQ-Orbitrap mass spectrometry;KEGG:Kyoto Encyclopedia of Genes and Genomes;GO:Gene Ontology;DSS:Dextran Sulfact Sodium;TCM:Traditional Chinese Medicine;UC:ulcerative colitis;SASP: Salazosulfapyridine;ELISA:enzyme linked immunosorbent assay; PPI:protein-protein interaction ;DAI :Disease activity index

;ICAM1:Intercellular adhesion molecule 1; JAK2 :Tyrosine-protein kinase JAK2; AKT1:RAC-alpha serine/threonine-protein kinase;; MTOR:Serine/threonine-protein kinase mTOR; PPARG:Peroxisome proliferator activated receptor gamma; RELA :Transcription factor p65; MAPK14:Mitogen-activated protein kinase 14;AKT:Protein kinase B.

Declarations

Acknowledgements

Not applicable.

Author's contributions

TW, YX and WJ conducted experiments and analyzed the data; TW and RA wrote the manuscript. RA designed the study and guided the experiment. All authors read and approved the final manuscript.

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Availability of data and materials

The data used to support the results of this study can be obtained from the first author upon reasonable request.

Ethics approval and consent to participate

All animal protocols in the study were approved by the Institutional Animal Committee of Shanghai University of Traditional Chinese Medicine. All procedures for the animal study were conducted in line with the relevant specifications of experimental animal welfare and ethics.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflicts of interest.

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Figures

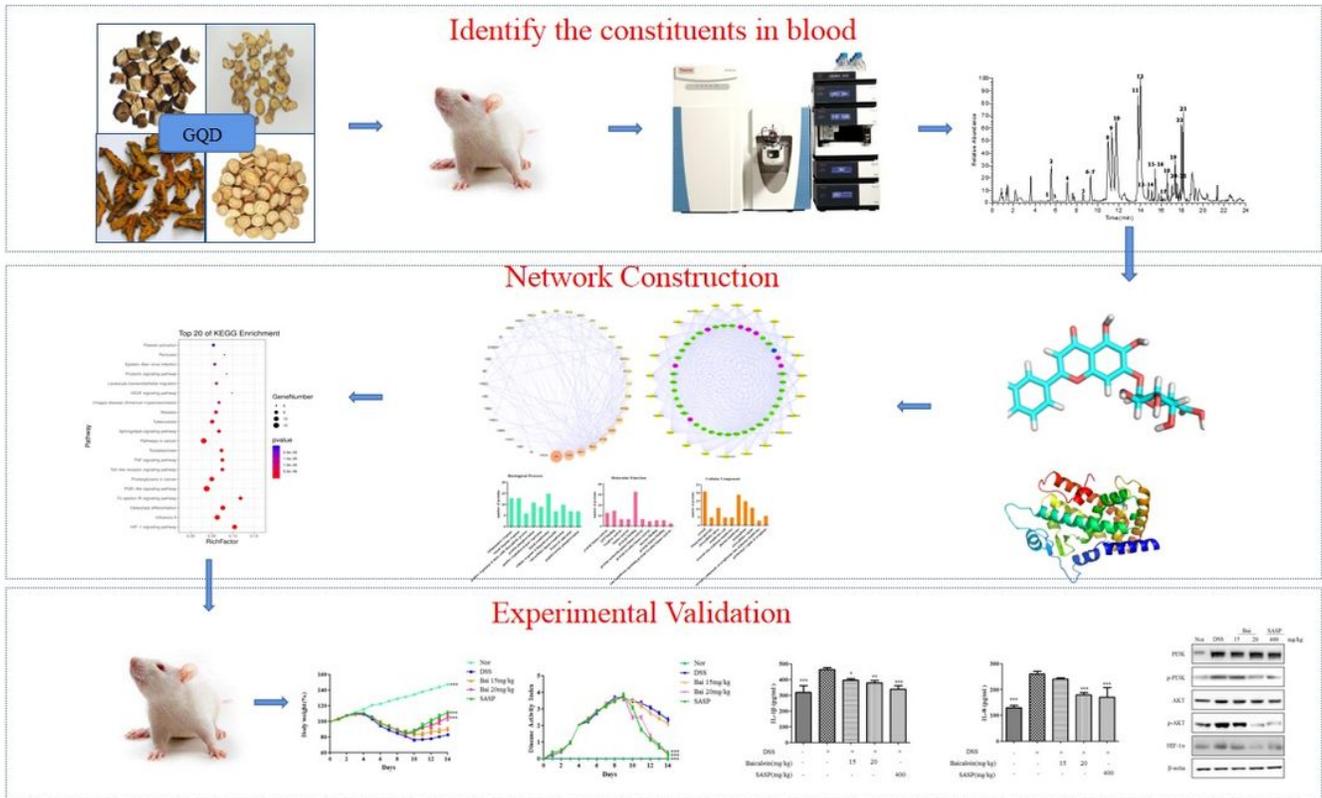


Figure 1

Scheme of the study

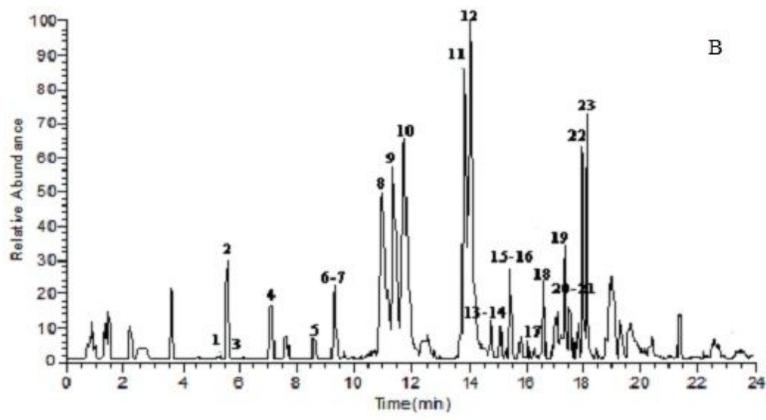
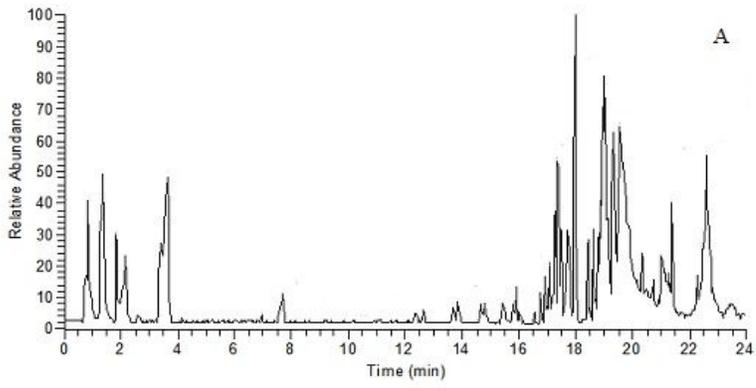


Figure 2

Total ion chromatograms of blank serums samples(A) and GQD-dosed serum samples(B) by UPLC-LTQ-Orbitrap-MS in positive mode.

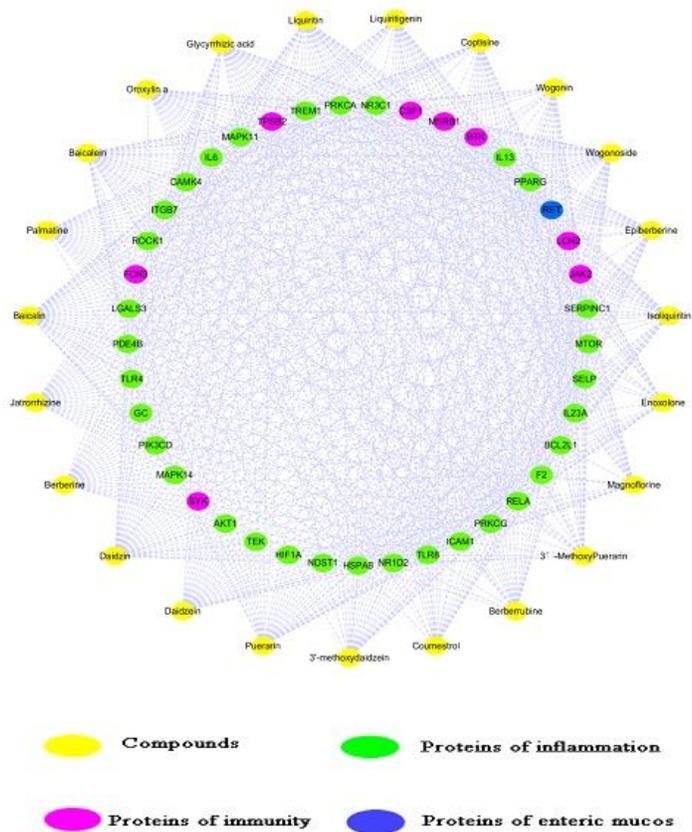


Figure 3

Network of compound-protein interaction induced by GQD. The 23 representative compounds were associated with 41 potential protein targets determined by high-throughput molecular docking analysis. The yellow ellipse nodes represent compounds, while the 41 protein targets identified were associated with inflammation (green ellipse nodes), enteric mucosa (blue ellipse nodes), immunity (purple ellipse nodes).

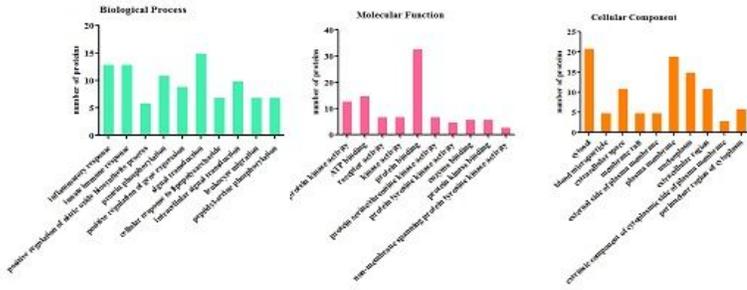


Figure 6

Top 10 terms of Biological Process, Molecular Function and Cellular Component by major hubs from the DAVID database

Top 20 of KEGG Enrichment

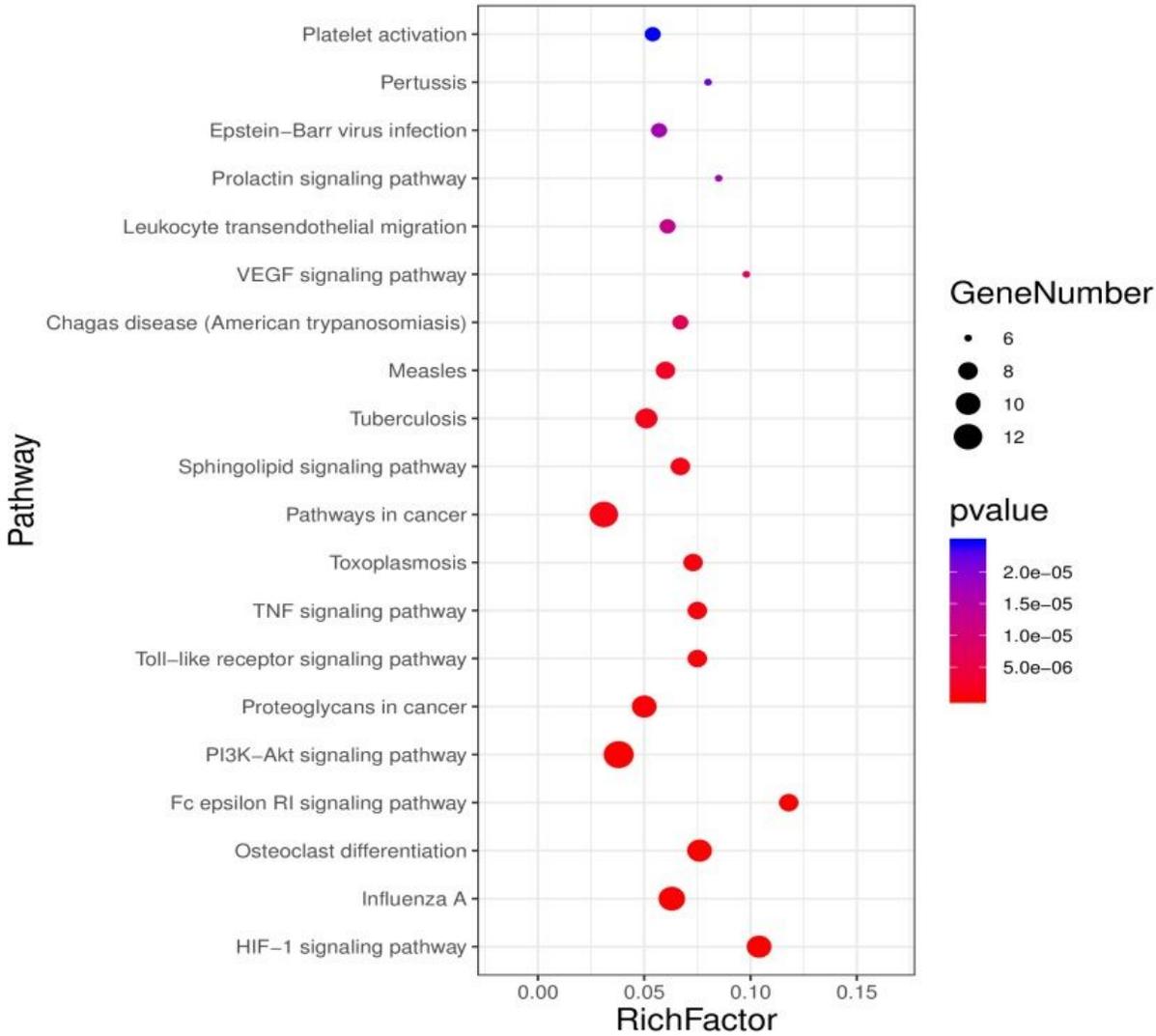
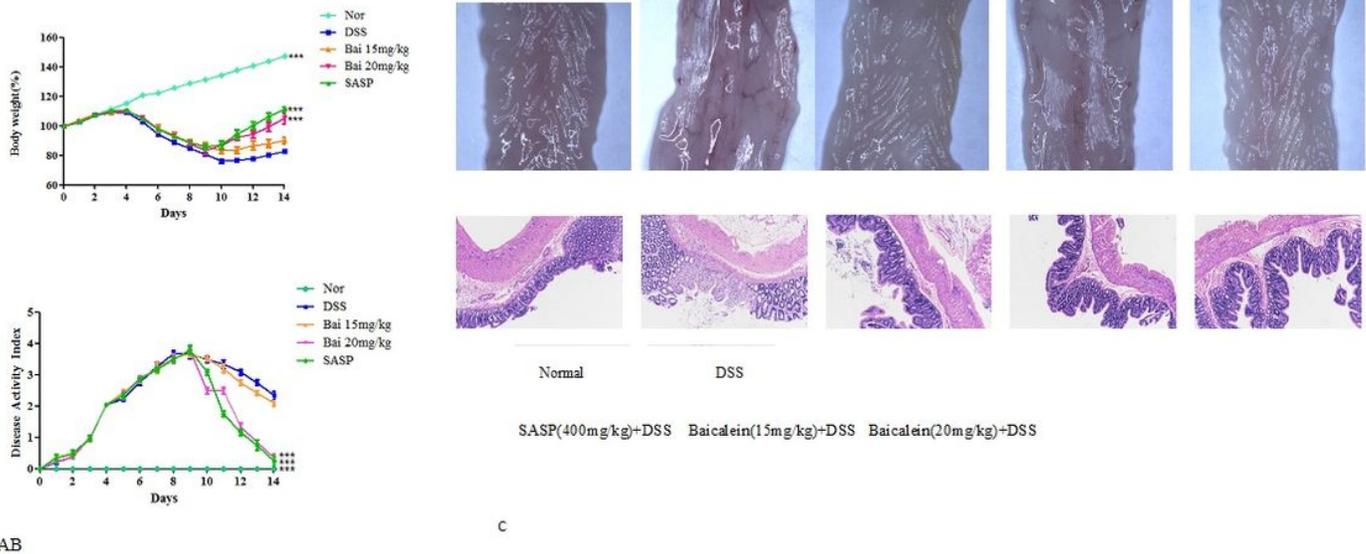


Figure 7

Top 20 pathways enriched by major hubs from the DAVID database



AB

C

Figure 8

(A): Changes in body weight in rats for each group.(B): Disease activity index (DAI) was evaluated daily (C): Baicalein attenuates DSS-induced colon damage in rats. (n=8 rats per group).*P<0.05 compared to DSS-induce AC rats.**P<0.01 compared to DSS-induce AC rats.***P<0.001 compared to DSS-induce AC rats.

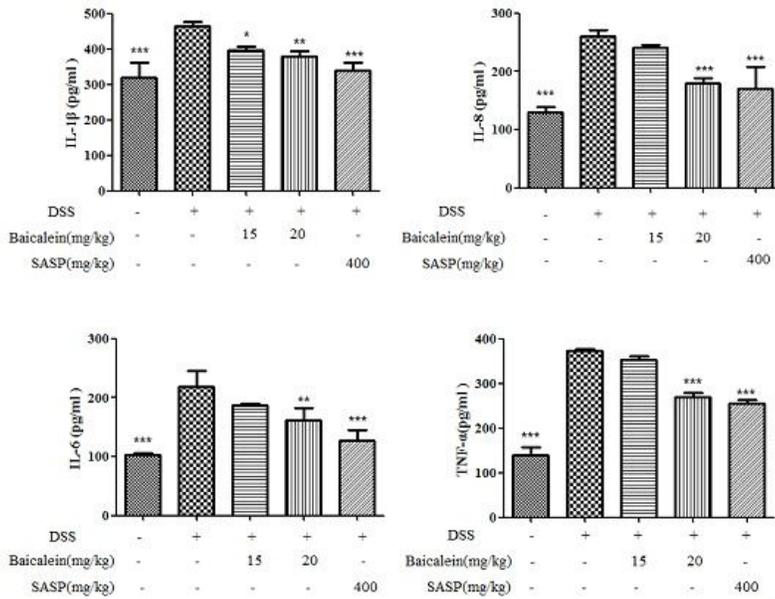


Figure 9

Expression levels of TNF-α, IL-1β, IL-6 and IL-8 in the serum were determined by Elisa. Experimental data were expressed as mean ± SD.*P<0.05 compared to DSS-induce AC rats.**P<0.01 compared to DSS-induce AC rats.***P<0.001 compared to DSS-induce AC rats.

