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ORIGINAL RESEARCH

Clinical Analysis and Network Pharmacology in Revealing the Mechanism of Daifu Decoction on the Relapse of UC

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Background: Daifu Decoction (DFD), a patented herbal prescription used to prevent and treat ulcerative colitis (UC). This study aimed to reveal the effect of DFD on the relapse of UC and its mechanism via integrated retrospective clinical analysis, network pharmacology and in vivo and in vitro experimental validation.

Methods: First, the clinical data of UC patients treated with DFD were reviewed from a real-world study (RWS), and the relapse at 24 weeks after drug withdrawal was recorded to evaluate the relapse rate. Next, the chemical components of DFD were identified via ultra performance liquid chromatography–mass spectrometry (UPLC–MS), and the differentially expressed genes (DEGs) between UC patients in the active and remission stages were screened as disease targets related to the relapse of UC from the Gene Expression Omnibus (GEO) database. The core components, targets and key signalling pathways of DFD for preventing the relapse of UC were discussed via network pharmacology. Finally, the above results were verified via molecular docking and in vivo and in vitro experiments.

Results: A total of 475 UC patients were included, and the relapse rate of UC treated with DFD was 23.9%. Additionally, the 221 components identified by UPLC-MS and 398 DEGs related to the relapse of UC enriched the main pathway of the relapse of UC was IL-17 signaling pathway and the inflammatory-related targets, such as IL6, PTGS2, MMP7, MMP3, MMP1. Moreover, molecular docking revealed that the core components of DFD were able to bind to inflammation-related targets, and in vivo and in vitro experiments demonstrated that DFD could inhibit the IL-17 pathway, increase the level of claudin-1, and control inflammation to prevent UC relapse.

Conclusion: DFD can effectively prevent the relapse of UC which may be related to inhibiting the activation of IL-17 signalling pathway.

Keywords: daifu decoction, relapse of UC, real-world study, network pharmacology, differentially expressed genes, IL-17 signalling pathway

Introduction

Ulcerative colitis (UC) is a chronic and nonspecific intestinal inflammatory disease, and its aetiology and pathogenesis have not been fully elucidated. To date, a variety of drugs have been developed and used for treating UC, but some patients still experience recurrent episodes. The cumulative risk of relapse within 10 years has been reported to be 70–80%.[1] Studies have shown that the relapse rate of UC patients treated with aminosalicylic acid, a first-line drug for treating UC, is as high as 49.6%,[2] and the relapse rates of UC patients after receiving biologics and immunosuppressants are 54% and 33%, respectively.[3,4] Therefore, the main dilemma in the current treatment of UC is that the disease is difficult to cure and flare-ups occur frequently, and these dilemmas are due to the lack of clarity surrounding the pathogenesis of UC. At present, there are few available drugs that can prevent the relapse of UC. Therefore, it is imperative to develop novel drugs to prevent the relapse of UC and cure the disease.

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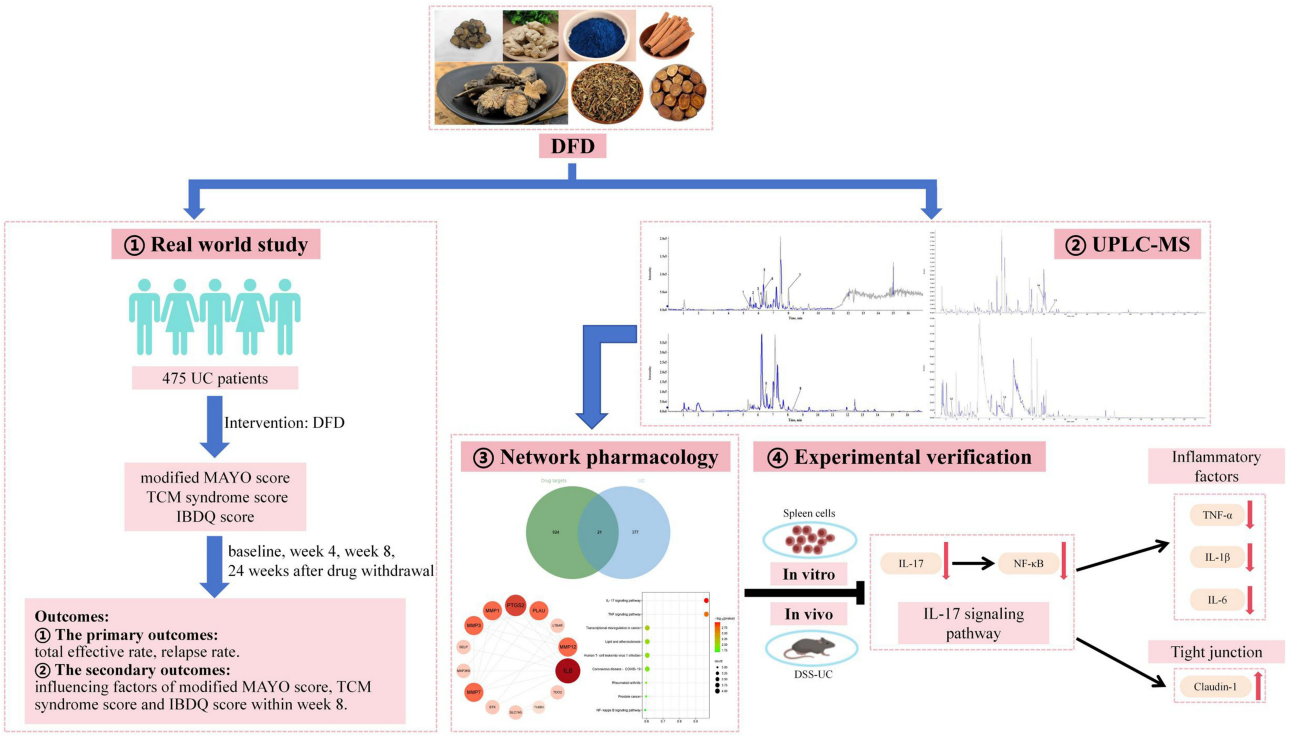
Graphical Abstract



Traditional Chinese Medicine (TCM) has enormous potential in the treatment of UC due to its multi-component, multi-targeted and multi-pathway therapeutic approach, such as *Hericium erinaceus*,[5,6] *Herba Origani*,[7] Shaoyao Decoction,[8] Gegen Qinlian decoction[9] and other herbs and Chinese herbal compounds have been reported to have good therapeutic effects on UC, and TCM has been increasingly applied in the clinical treatment of UC. DFD has been patented by the China National Intellectual Property Administration (Patent name: A traditional Chinese medicine for preventing and treating the recurrence of UC. Patent number: No. 201510162437. X). Our research team applied this formula to treat 1104 UC patients resulted in a total effective rate of more than 90% and a complete remission rate of 21.4%,[10] and to treat refractory UC, the total effective rate was 94.5%,[11] demonstrating that the DFD has a significant effect on UC. Although randomized controlled trials (RCTs) are good sources of reliable evidence reliable, these strictly regulated trials cannot well reflect the real clinical environment.[12] Therefore, we reviewed the clinical data of UC patients in a real-world study (RWS) that were treated with DFD in the past 8 years to investigate the antirelapse effect of DFD. Moreover, the mechanism by which DFD prevents UC relapse is still unclear. Therefore, network pharmacology analysis was performed using the DFD components identified by ultrahigh-performance liquid chromatography–mass spectrometry (UPLC–MS) and the differentially expressed genes (DEGs) between patients in the active and remission stages of UC, which were identified by using data obtained from the Gene Expression Omnibus (GEO) database. Further analyses were performed to identify the pathways involved in the prevention of UC relapse by DFD. Next, the effect of DFD on these pathway was confirmed via in vivo and in vitro experiments to investigate the mechanism by which DFD prevents and treats the relapse of UC.

Materials and Methods

Preparation of DFD

The 42 g DFD formulation was accurately weighed. DFD is composed of seven kinds of traditional Chinese medicine: *Aconitum carmichaelii* Debx (Fuzi) (Lot. No. 2402001), indigo naturalis (Qingdai) (Lot. No. 1706001), *Agrimonia pilosa*

Ledeb (Xianhecao) (Lot. No. 230590701), *Sanguisorba officinalis* L. (Diyu) (Lot. No. 22063002), *Zingiber officinale* Rosc. (Ganjiang) (Lot. No. 2401002), *Cinnamomum cassia* Presl (Rougui) (Lot. No. 99023010) and *Glycyrrhiza uralensis* Fisch (Gancao) (Lot. No. 2212005), and the ratio of these herbs is 5:2:10:10:6:6:3 (Table 1). All the above drugs were purchased from the traditional Chinese Medicine Pharmacy of the General Hospital of Northern Theater Command and were authenticated by Prof. Miao Jiang, Institute of Basic Research In Clinical Medicine, China Academy of Traditional Chinese Medicine, and Yang Gong, Department of Traditional Chinese Medicine, General Hospital of Northern Theater Command. In the Department of Traditional Chinese Medicine of the General Hospital of Northern Theater Command, these herbs were preserved as voucher specimens. Before UPLC–MS, the appropriate amounts of the herbs in DFD were mixed, soaked in 10 times the volume of water, and subjected to reflux extraction for 1 h, after which the supernatant was collected and centrifuged for 15 min (10000 rpm, 4°C). The supernatant was subsequently collected again and filtered through a 0.22 μm filter membrane to obtain the compound sample test solution. Since Qingdai is a powder, 50% ethanol, 75% ethanol and 100% ethanol were used for reflux extraction and supplementary injection.

RWS

Study Design

This was a retrospective single-centre study conducted at the General Hospital of Northern Theater Command, Shenyang, China. On the basis of the medical routine, we collected the diagnosis and treatment information of UC patients who visited the Department of Traditional Chinese Medicine from 2015 to 2023 according to the consensus on the diagnosis and treatment of UC with integrated Traditional Chinese and Western Medicine (2017),[13] and the information was stored in the UC database of the Department of Traditional Chinese Medicine. The information was managed and summarized by professionals, and subsequent analyses were performed after the database was compared twice by two attending physicians. The study was conducted in accordance with the Declaration of Helsinki, and the study protocol was reviewed and approved by the Ethics Committee of the General Hospital of Northern Theater Command (NO.Y(2024) 099). Additionally, the study was registered in the International Traditional Medicine Clinical Trial Registry (ITMCTR2024000560). The patients provided informed consent.

Patients

The patients in the UC database who met the traditional Chinese and Western medicine diagnostic criteria of UC were analysed.[13,14] The inclusion criteria were as follows: regular application of DFD; colonoscopies performed at the initial visit and after 8 weeks of treatment; and no treatment with aminosalicylic acid, glucocorticoids, immunosuppressants, biological agents or small molecule drugs. Patients with incomplete data were excluded.

Sample Size

The sample size calculation was based on the relapse rate, which was the main evaluation index of the study. The expected relapse rate of the study group was (P1)=0.24, the average relapse rate of the other studies was (P0)=0.4, and the superiority margin (SM) = 0.1. The sample size was estimated via a Z test on the basis of the standard deviation of the null hypothesis estimate. At the test level α=0.0500 (one-sided), the experimental group needed at least 345 subjects to draw conclusions of the superiority with 80% power (1-β).[15]

Table 1 Drug Composition of DFD

Drug name	Latin Plant Name	Used Part	Amount (g)
Fuzi	*Aconitum carmichaelii* Debx	Root	5
Qingdai	Indigo Naturalis	Stem and leaf	2
Xianhecao	*Agrimonia pilosa* Ledeb.	Stem and leaf	10
Diyu	*Sanguisorba officinalis* L.	Root	10
Ganjiang	*Zingiber officinale* Rosc.	Root	6
Rougui	*Cinnamomum cassia* Presl	Bark	6
Gancao	*Glycyrrhiza uralensis* Fisch	Root	3

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Intervention

Patients were administered DFD orally twice daily, 50 mL each time, approximately 30 minutes postprandially, until a significant improvement in their condition was observed.

Study Procedures

Data from the time of the first visit (baseline period), at 4 weeks and 8 weeks of treatment (observation period), and at 24 weeks after drug withdrawal (follow-up period) were extracted from the UC database. The information collected during the baseline period included the following: general information of patients (including age, sex, education background and time of first diagnosis); the extent of the colonic lesion, which was determined according to the Montreal classification; extraintestinal manifestations (including joint, skin, mucosal, ocular, hepatobiliary and other extraintestinal symptoms); the presence of abdominal pain, diarrhea, and haematochezia; colonoscopy data; and quality of life, which was determined by the Inflammatory Bowel Disease Questionnaire (IBDQ).[16] The information collected during the observation period included the following: the presence of abdominal pain, diarrhea, and haematochezia; quality of life; adverse reactions; and colonoscopy data, which were collected at 8 weeks of treatment. Information on the relapse of UC was collected during the follow-up period. The primary outcomes were the total effective rate and relapse rate (total effective rate= effective number/total number of people×100%, relapse rate= number of relapses/number of drug withdrawals×100%). Efficacy and relapse were evaluated with the modified MAYO score.[14] The secondary outcomes were the factors influencing the modified MAYO scores, traditional Chinese medicine (TCM) syndrome scores[17] and IBDQ scores of patients within week 8 of treatment, the scoring criteria are shown in Table S1.

UPLC–MS Analysis

The DFD samples were examined via UPLC–MS and separated on an ACQUITY UPLCTM HSS C18 column (10 mm×2.1 mm, i.d. 1.8 μm) (Waters Group Corporation, USA) at 40°C with a flow rate of 0.4 mL/min, and the injection volume was 5 μL. Mobile phases A and B consisted of 0.1% formic acid-water and 0.1% formic acid-acetonitrile, respectively. The gradient elution protocol is displayed in Table 2. Positive and negative ion modes were used for mass spectrometric detection, the retention time and mass spectrometry data were obtained, and the compound structures were identified through data matching with standard reference compounds.

Network Pharmacology

Target Screening of DFD Active Components

All the components identified via UPLC–MS were imported into the Swiss Target Prediction platform (http://www.swisstargetprediction.ch/). The screening conditions were set to *Homo sapiens* and a probability > 0, and DFD targets were obtained after the targets of all chemical components were deduplicated.

Table 2 UPLC Mobile Phase Gradient Elution Protocol

Time (min)	Flow Rate (μL/min)	A%	B%
0.01	400	95	5
6	400	70	30
8	400	70	30
10	400	50	50
12	400	50	50
18	400	0	100
25	400	0	100

Notes: A is 0.1% formic acid-water; B is 0.1% formic acid-acetonitrile.

Screening of Targets Related to the Relapse of UC

We searched the UC dataset in the GEO database (http://www.ncbi.nlms.nih.gov/geo) with the keyword "ulcerative colitis". The GSE53306 dataset, which contains samples from 16 active UC patients and 12 UC patients in remission, was obtained from the GPL14951 [Illumina HumanHT-12 WG-DASL V4.0 R2 expression beadchip] platform and was used as the training dataset for the discovery analysis. All the data used in the study are from the GEO, so there is no need for ethical approval or informed consent. The Limma package in R was used for difference analysis. A log |FC| > 0.585 and adjusted $P < 0.05$ were set as the screening criteria. The DEGs were visualized via heatmaps and volcano maps. Since UC is in the active stage when it recurs, the DEGs between UC patients in the active and remission stages of UC were defined as the targets related to the relapse of UC.

Acquisition of Shared Targets Between Those of DFD and Those Related to the Relapse of UC

Drug targets and disease targets were compared using a bioinformatics platform (https://www.bioinformatics.com.cn/), and a Venn diagram was generated to obtain the common targets. Finally, Cytoscape 3.8.2 was used to construct the drug-ingredient-target network diagram and obtain the core components of DFD.

Construction of a protein–protein Interaction (PPI) Network

The shared drug and disease targets were uploaded to the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database (https://cn.string-db.org/), and a PPI network was subsequently constructed in multiple protein mode, with *Homo sapiens* and medium confidence (0.400) selected. Cytoscape 3.8.2 was used to perform topological analysis and construct the image of the PPI network.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Enrichment Analyses

The shared targets were uploaded to the DAVID database (https://david.ncifcrf.gov/summary.jsp) for GO and KEGG enrichment analysis. GO gene function analysis annotates the role of the target proteins of drug-acting diseases from three aspects: biological process (BP), cellular component (CC) and molecular function (MF). Entries with $p < 0.05$ were taken as the entries with significant enrichment, and the top 3 entries in the GO and KEGG entries were selected for visualization.

Molecular Docking

The 2D structures of the core components were obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). The intersection target protein structures were collected from the PDB database (https://www1.rcsb.org/) and UniProt database (https://www.uniprot.org/). PyMol 2.1.0 software was used to optimize the targets, such as removing water molecules and small-molecule ligands, and AutoDock Tools 1.5.6 was used for hydrogenation and charge processing. Vina-2.0 in PyRx software was used for molecular docking verification and calculation of affinity. Finally, PyMol software and Discovery Studio 2020 client were used to visualize the results.

Animals and Cells

Eighty-six male C57BL/6J mice (18–22 g, 7–8 weeks old) were purchased from Liaoning Changsheng Biotechnology Co., Ltd. (Liaoning, China). All the mice were housed in an air-conditioned room (temperature: 20–26°C, humidity: 50–70%) under a 12 h light/dark cycle with free access to food and water. The study protocol was approved by the ethics committee of the General Hospital of Northern Theater Command (No. 2021JH2/10300109). All experimental procedures followed the guidelines for the Care and Use of Laboratory Animals and were conducted in accordance with the ARRIVE guidelines.

For the extraction and culture of spleen cells, the mice were sacrificed via the cervical dislocation method, after which the spleens were removed, ground and filtered to obtain splenocytes. DMEM (HyClone, SH30243.01) supplemented with 10% foetal bovine serum was used to culture the cells in a 5% CO_2 incubator at 37°C for 24 h.

Drug-Containing Serum Preparation, Cell and Animal Groupings, UC Model Induction and Drug Administration

After 7 days of adaptive rearing, 18 mice were randomly divided into 3 groups (n=6): the drug-free serum group (DFS), DFD serum group (DFDS) and mesalazine sustained-release granule (MES) serum group (MESS). The mice were gavaged twice a day with 0.2 mL of normal saline, DFD (3.185 g/kg) or MES (0.303 g/kg). After 5 days, whole blood samples were collected from the mice, and the serum was obtained after centrifugation and stored at −80°C until further examination. 36 mice were randomly allocated into 4 groups (n=9): the normal control group (NC), UC model group (UC), DFD serum treatment group (DFD), and MES serum treatment group (MES). Except for those in the NC group that drank distilled water freely, the mice drank freshly prepared 3% DSS solution for 10 days. Then, the spleen cells were extracted and cultured as described in section Animals and cells. After 24 h of culture, the medium was discarded, DMEM supplemented with 10% FBS was added to the NC group, DFS serum was added to the UC group, DFDS serum was added to the DFD group, MESS serum was added to the MES group, and the mixture was cultured for 24 h.

For in vivo experiments, 32 mice were randomly divided into 4 groups (n=8): the normal control group (NC), UC model group (UC), DFD treatment group (DFD) and MES treatment group (MES). The mice in the NC group drank distilled water freely, whereas the other mice drank freshly prepared 3% DSS solution for 10 days. Beginning on day 11, the mice given DSS were gavaged with normal saline, DFD (6.37 g/kg/d), or MES (0.61 g/kg/d). On day 21, the eyeballs were removed for blood collection, and the mice were subsequently sacrificed to collect the colon for further experiments.

Calculation of the Disease Activity Index (DAI)

For further analysis of the severity of colitis, the DAI was calculated by measuring body weight and observing stool consistency and rectal bleeding every other day according to previously established scoring criteria.[18]

Calculation of the Colon Mucosa Damage Index (CMDI)

The CMDI was calculated according to a previously proposed method.[19] We observed the general morphology of the colon and measured the length of the colon from the anus to the caecum. The colon was cut longitudinally, rinsed with normal saline, and then observed after drying with filter paper to determine the CMDI.

Haematoxylin and Eosin (H&E) Staining

The colon tissue was fixed in 4% paraformaldehyde, embedded in paraffin and cut into 5-μm-thick sections. The sections were subsequently stained with H&E and observed under a microscope.

Enzyme-Linked Immunosorbent Assay (ELISA)

According to the manufacturer's instructions, ELISA kits were used to measure the levels of IL-17 (Meimian, 231124165M) in the serum or cell culture supernatant and the levels of TNF-α (Thermo Fisher, 88–7324-86), IL-1β (Thermo Fisher, 88–7013A-86), IL-6 (Thermo Fisher, 88–7064-86), IL-17 (Meimian, 231124165M), NF-κB (Meimian, 231124170M), and claudin-1 (Meimian, 231124178M) in the colon tissue.

Immunohistochemical (IHC) Staining

IHC staining was used to measure the levels of TNF-α (Cohesion Biosciences, cpa2174), IL-1β (Santa Cruz, sc52012), IL-6 (Cohesion Biosciences, cpa4914), IL-17 (Abcam, ab79056), NF-κB (Santa Cruz, sc8008) and claudin-1 (CST, #13255) in colon tissue. Paraffin-embedded colon tissue sections were deparaffinized with xylene, hydrated with ethanol, antigen repaired and blocked for IHC analysis. The dilution ratios of the primary antibodies and secondary antibodies are shown in Table S2. Images were collected and analysed via Image-Pro Plus software 6.0.

RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (qPCR)

Total RNA was extracted from splenocytes and colon tissues via TRIzol reagent following the manufacturer's instructions, and cDNA was obtained via reverse transcription. A TB Green Premix Ex Taq kit (TaKaRa, #RR820A) was used for analysis. The primer sequences are described in Table S3.

Statistical Analysis

For the RWS, the Shapiro–Wilk test and Q–Q plot were used to determine the normality of continuous variables. Normally distributed continuous variables are presented as the means ± standard deviations, and comparisons between groups were performed via Student's *t* test. Nonnormally distributed continuous variables are described as medians (lower quartile, upper quartile), and comparisons between groups were based on the Wilcoxon rank-sum test. Categorical variables are described as percentages, and comparisons between groups were performed via the chi-square test. The differences in the modified MAYO score, TCM syndrome score and IBDQ score at different time points were compared via the paired rank sum test. A multilevel model was further used to explore the factors affecting the modified MAYO scores, TCM syndrome scores and IBDQ scores of patients at 8 weeks. SAS 9.4 software was used for statistical analysis, and two-sided $P< 0.05$ were considered statistically significant.

For the animal and cell experiments, SPSS 27.0 and GraphPad Prism 9.0 (GraphPad, USA) were used to analyse the data. The data were presented as the mean ± standard deviation (SD) and all experiments in this study were replicated independently at least three times, and a *t* test was used for comparisons between groups. Heatmaps, Venn diagrams and bubble charts were generated via a bioinformatics platform and R language. $P< 0.05$ was considered statistically significant.

Results

DFD Can Effectively Prevent the Relapse of UC

Participant Characteristics at Baseline

A total of 475 patients were included in the retrospective study (Figure 1). The baseline characteristics of the patients are shown in Table 3.

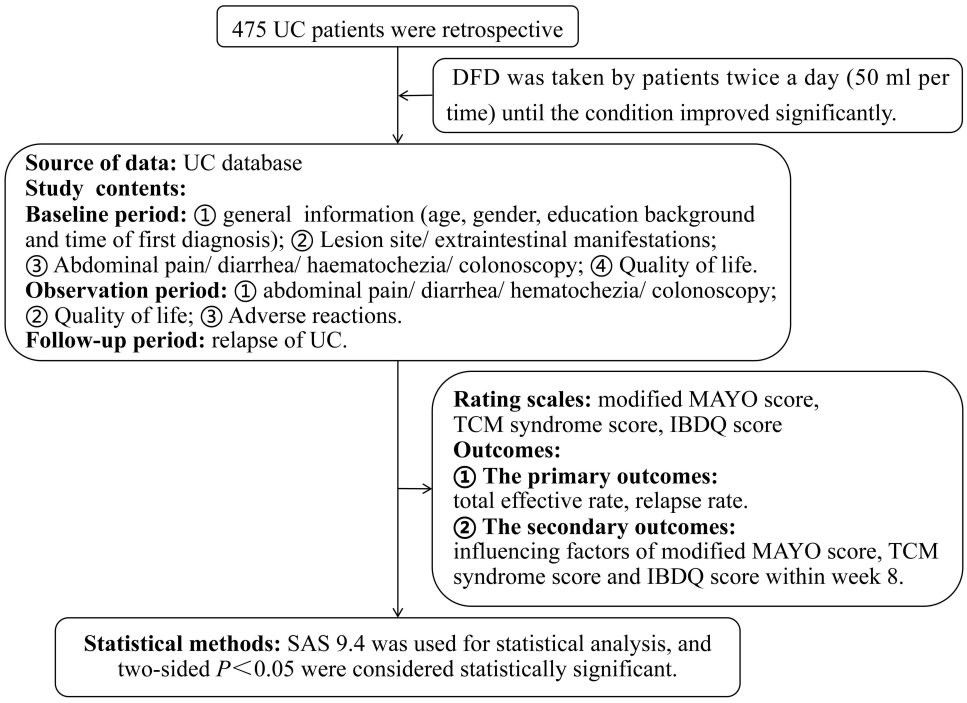

Figure 1 Study flowchart.

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Table 3 Baseline Characteristics

Parameters	Total Population (n=475)	Male (n=262)	Female (n=213)
Age (years)	43.25±13.44	41.91±13.43	44.91±13.29
Level of education n (%)			
High school and below	183 (38.5%)	95 (36.3%)	88 (41.3%)
College and above	292 (61.5%)	167 (63.7%)	125 (58.7%)
Medical History (years)	5 (3, 9)	5.50 (3, 9)	5 (3, 8)
Extent of lesion n (%)			
E1	26 (5.5%)	12 (4.6%)	14 (6.6%)
E2	214 (45.0%)	115 (43.9%)	99 (46.5%)
E3	135 (28.4%)	76 (29.0%)	59 (27.7%)
E1+E3	100 (21.1%)	59 (22.5%)	41 (19.2%)
Extraintestinal manifestations n (%)			
Yes	401 (84.4%)	229 (87.4%)	172 (80.8%)
No	74 (15.6%)	33 (12.6%)	41 (19.2%)

Notes: The extent of the lesion was classified according to the Montreal classification: E1, the lesion was limited to the rectum; E2, the lesion involved the left-sided colon (distal to the splenic flexure); E3, extensive lesions involved the proximal to the splenic flexure and even the entire colon; E1+E3, extensive lesions involved the rectum and entire colon.

Primary Outcomes: DFD Was an Effective Agent for the Prevention of UC Relapse and Treatment of UC

A comparison of the modified MAYO score changes, TCM syndrome score changes, and IBDQ score changes at baseline, week 4 and week 8 via the paired rank sum test revealed that the modified MAYO score and TCM syndrome score gradually decreased and the IBDQ score gradually increased with increasing treatment time (Table 4, Figure 2A-C). After 8 weeks of treatment, the treatment was effective in 446 patients, and the total effective rate was 93.9% (Figure 2D). Among the 475 patients included, a total of 234 patients stopped treatment after their condition improved. However, 56 patients relapsed within 24 weeks after drug withdrawal, and the relapse rate was 23.9% (Figure 2E). These results indicate that DFD was an effective agent for the prevention of UC relapse.

Table 4 Comparison of the Modified MAYO Score, TCM Syndrome Score, and IBDQ Score

Parameter	Total Population (n=475)	Male (n=262)	Female (n=213)
Modified MAYO score			
Baseline	11 (10, 12)	11 (10, 12)	10 (10, 12)
Week 8	2 (0, 4)	2 (0, 4)	2 (0, 3)
TCM syndrome score			
Baseline	18 (15, 21)	18 (15, 21)	18 (15, 21)
Week 4	9 (6, 18)	9 (6, 18)	9 (6, 15)
Week 8	6 (0, 9)	6 (0, 9)	3 (0, 9)
IBDQ score			
Baseline	120 (108, 131)	120 (108, 131)	120 (108, 131)
Week 8	187 (176, 197)	187 (176, 197)	189 (176, 197)

Abbreviations: TCM, traditional Chinese medicine; IBDQ, Inflammatory Bowel Disease Questionnaire.

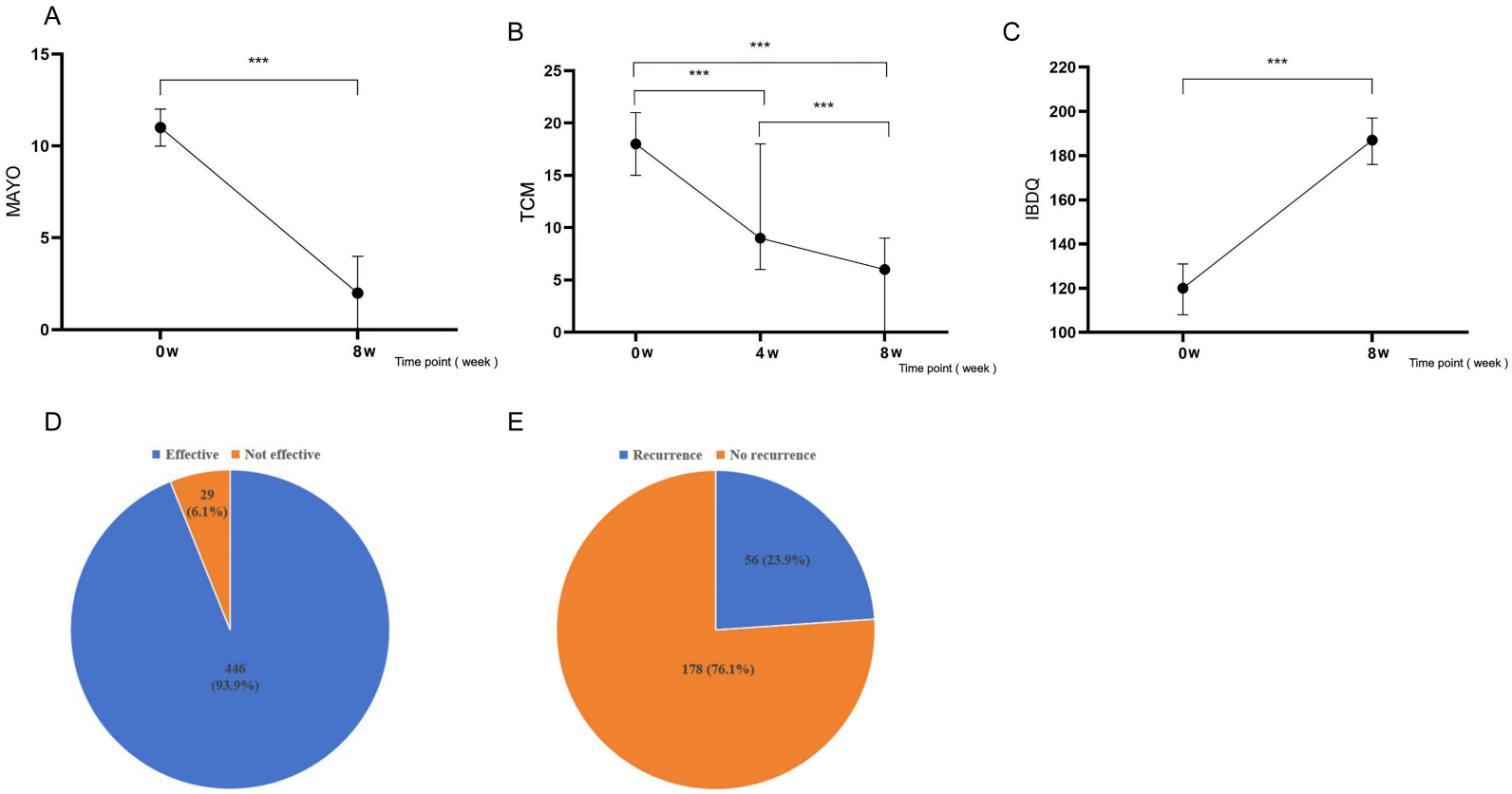

Figure 2 Primary outcomes. Comparison of (**A**) Modified MAYO scores, (**B**) TCM syndrome scores, and (**C**) IBDQ scores at baseline, week 4 and week 8. (**D**) Effective rate. (**E**) Recurrence rate. ***P <0.001.

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Secondary Outcomes: The Factors Influencing Modified MAYO Scores, TCM Syndrome Scores and IBDQ Scores of UC Patients Within week 8

Compared with the modified MAYO score at baseline, the modified MAYO score at week 8 was reduced by 8.35 points (95% CI −8.56, −8.15) on average. Compared to those of patients with only E1 lesions, the modified MAYO scores of patients with E2, E3 and both E1 and E3 lesions increased by 2.01 points (95% CI 1.54, 2.47), 3.20 points (95% CI 2.72, 3.69) and 3.79 points (95% CI 3.30, 4.29) on average at week 8, respectively (Table 5). Compared with that at week 4, the TCM syndrome score was increased by 7.41 points (95% CI 6.67, 8.16) on average at baseline and decreased by 11.71 points (95% CI −12.45, −10.96) on average at week 8. Compared with that of females, the TCM syndrome score of males was increased by 0.94 points (95% CI 0.30, 1.58) on average at week 8. Compared with that of patients with only E1 lesions, the TCM syndrome scores of those with E2, E3 and both E1 and E3 lesions were increased by 3.62 points (95% CI 2.24, 4.99), 4.53 points (95% CI 3.11, 5.95) and 7.11 points (95% CI 5.65, 8.57) on average at week 8, respectively. Compared with that of the college and above groups, the TCM syndrome score of the high school and below patients was increased by 1.11 points (95% CI 0.47, 1.74) on average at week 8 (Table 5). Compared with that at baseline, the IBDQ score increased by 63.01 points (95% CI 60.28, 65.74) on average at week 8. Compared with that of patients with only E1 lesions, the IBDQ scores of those with E2, E3 and both E1 and E3 lesions decreased by 12.37 points (95% CI −18.57, −6.17), 21.17 points (95% CI −28.12, −15.30) and 28.47 points (95% CI −35.06, −21.88) on average within week 8, respectively (Table 5). Overall, the extent of the lesion was an influencing factor of the modified MAYO score and IBDQ score. Moreover, sex, the extent of the lesion, and the level of education were factors influencing the TCM syndrome score.

Adverse Events

In terms of adverse events, no serious adverse events occurred, but 21 patients with adverse reactions were reported during the treatment, including 11 patients with stomach discomfort, 5 patients with rash, 3 patients with liver dysfunction and 2 patients with other adverse events.

Table 5 Factors Influencing the Modified MAYO Score, TCM Syndrome Score, and IBDQ Score Within week 8

Parameter	Modified MAYO Score		TCM Syndrome Score		IBDQ Score	
	OR (95% CI)	*P* value	OR (95% CI)	*P* value	OR (95% CI)	*P* value
Baseline vs week 4			7.41 (6.67, 8.16)	<0.001		
Week 8 vs week 4			−11.71 (−12.45, −10.96)	<0.001		
Week 8 vs baseline	−8.35 (−8.56, −8.15)	<0.001			63.01 (60.28, 65.74)	<0.001
Sex						
Male vs female	0.17 (−0.05, 0.38)	0.133	0.94 (0.30, 1.58)	0.004	0.62 (−2.25, 3.49)	0.672
Extent of lesion						
E1 (Reference group)		–		–		–
E2	2.01 (1.54, 2.47)	<0.001	3.62 (2.24, 4.99)	<0.001	−12.37 (−18.57, −6.17)	<0.001
E3	3.20 (2.72, 3.69)	<0.001	4.53 (3.11, 5.95)	<0.001	−21.17 (−28.12, −15.30)	<0.001
E1+E3	3.79 (3.30, 4.29)	<0.001	7.11 (5.65, 8.57)	<0.001	−28.47 (−35.06, −21.88)	<0.001
Extraintestinal manifestations						
Yes vs None	−0.16 (−0.45, 0.12)	0.267	−0.45 (−1.29, 0.40)	0.300	−2.04 (−5.84, 1.76)	0.293
Level of education						
High school and below vs College and above	0.11 (−0.10, 0.33)	0.294	1.11 (0.47, 1.74)	0.001	1.11 (−1.74, 3.96)	0.446
Medical History						
For every additional year	0.02 (0.00, 0.04)	0.062	0.04 (−0.02, 0.10)	0.157	−0.10 (−0.18, 0.37)	0.491

Notes: E1, the lesion was limited to the rectum; E2, the lesion involved the left-sided colon (distal to the splenic flexure); E3, extensive lesions involved the proximal to the splenic flexure and even the entire colon; E1+E3, extensive lesions involved the rectum and entire colon.
Abbreviations: TCM, traditional Chinese medicine; IBDQ, Inflammatory Bowel Disease Questionnaire.

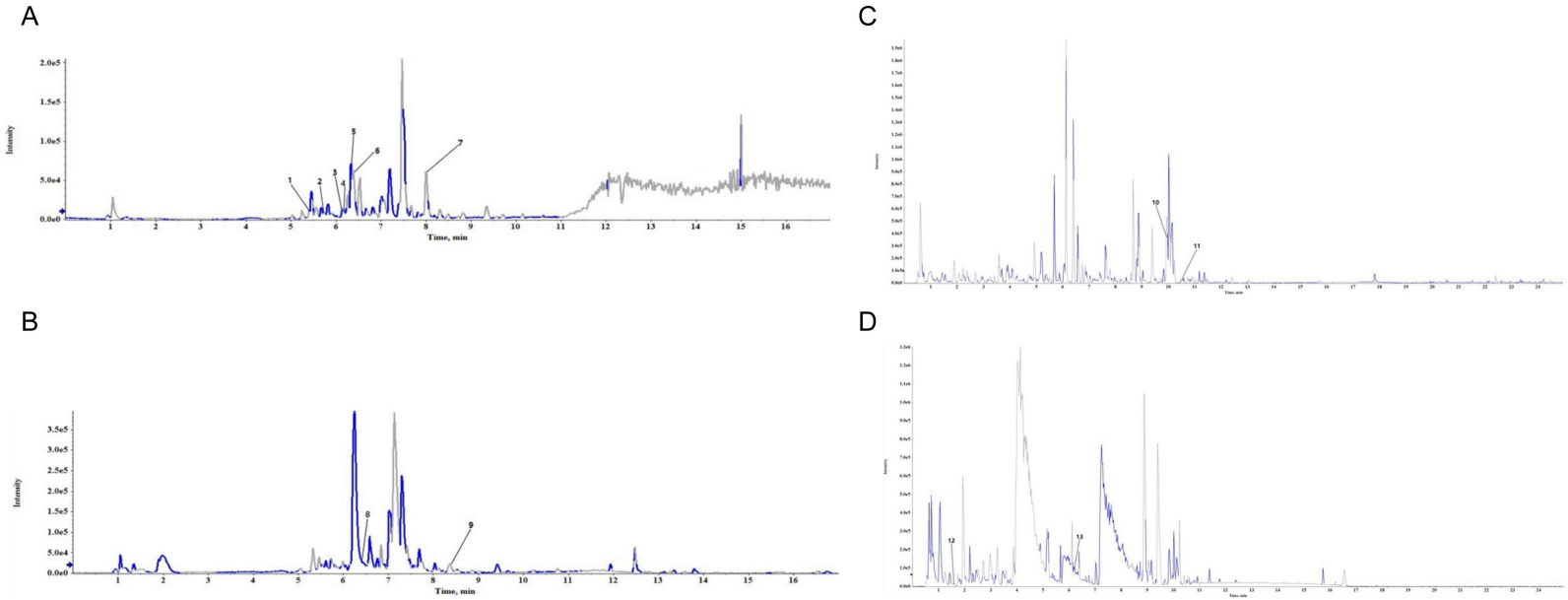

Figure 3 ULPC-MS detection of DFD chemical components. Water-extracted total ion current map of (**A**) positive ions and (**B**) negative ions. Alcohol-extracted total ion current map of (**C**) positive ions and (**D**) negative ions. 1. chlorogenic acid 2. liquiritin 3. isovitexin 4. naringenin 5. kaempferol 6. quercetin 7. 6-gingerol 8. quercitrin 9. ziyuglycoside II 10. indigo 11. indirubin 12. esculetin 13. azelaic acid.

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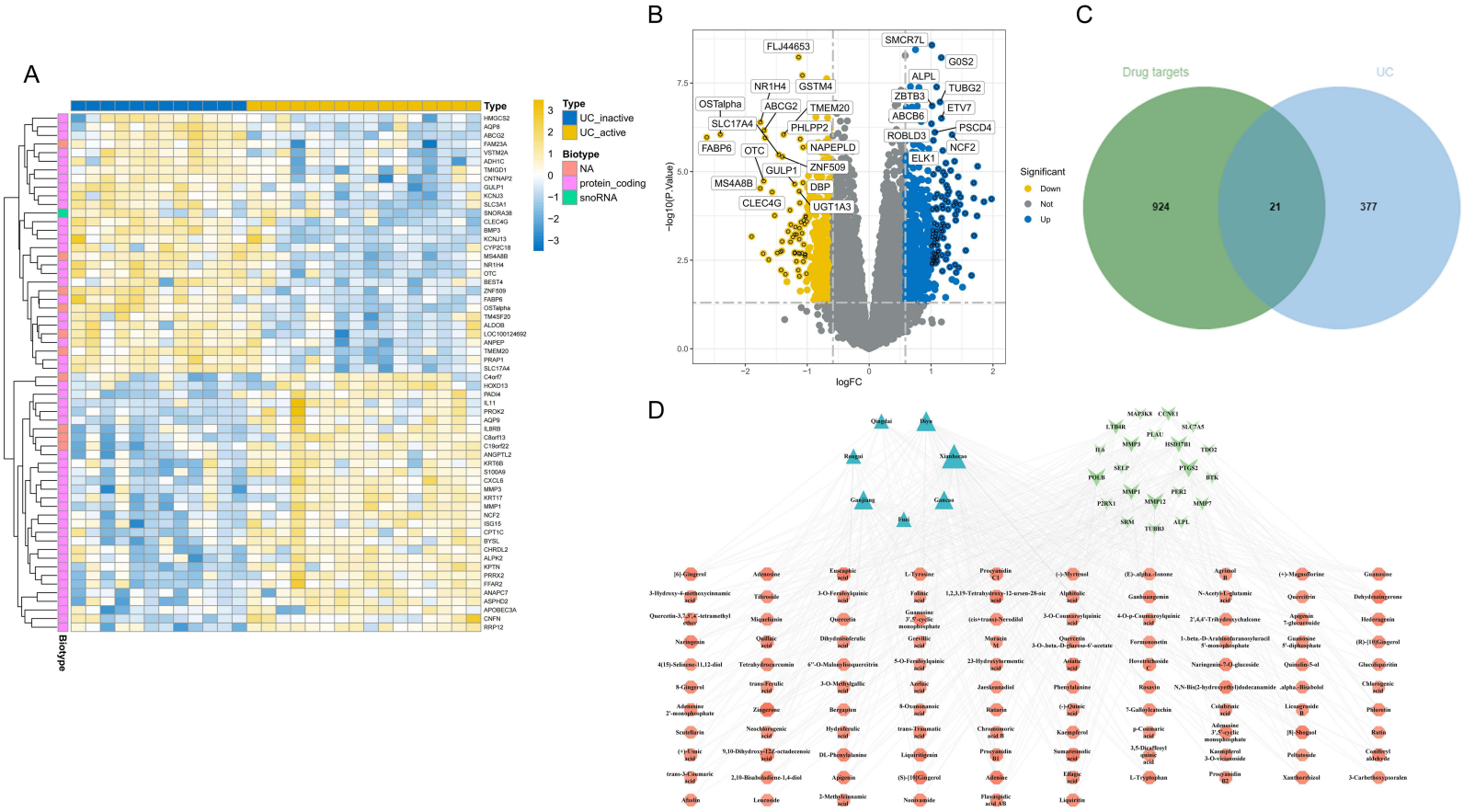

Figure 4 The active components of DFD and targets for DFD in the prevention of UC relapse. (**A**) Heatmap and (**B**) volcano plot of DEGs between UC patients the active and remission stages. (**C**) Venn diagram of shared targets between the drug components and UC. (**D**) Herb-component-target network of DFD.

Identification of the Chemical Components of DFD

In the UPLC-MS analysis, water reflux extraction yielded 219 chemical components, which were mainly flavonoids, isoprene lipids, organic oxygen compounds, amino acids and organic acids; ethanol reflux extraction yielded 181 chemical components. Finally, a total of 221 chemical components, including quercetin, kaempferol, naringenin, indirubin, indigo, isovitexin, liquiritin, chlorogenic acid, 6-gingerol, quercitrin, ziyuglycoside II, esculetin and azelaic acid, were identified after the removal of duplicate components (Figure 3A-D, Tables S4 and S5).

The Common Targets Between Those of DFD Components and Those Related to the Relapse of UC

Using the Swiss Target Prediction platform, 966 DFD active ingredient targets were obtained. To identify targets related to UC relapse, we obtained genomic data from UC patients in the active stage and remission stage from the GEO database and analysed it in R. A total of 680 DEGs were obtained between UC patients in the active stage and remission stage, including 398 DEGs in the active stage and 282 DEGs in the remission stage. The DEGs were visualized via a heatmap and volcano map (Figure 4A and B). Because the disease is in the active stage when UC recurs, the DEGs in the active stage were used as potential targets related to the relapse of UC. Finally, 21 shared drug and disease targets were obtained (Figure 4C). The herb–component–target network diagram was constructed in Cytoscape 3.8.2 (Figure 4D), and the 10 components with the highest degree values, namely, zingerone, dehydrozingerone, sumaresinolic acid, 9,10-dihydroxy-12Z-octadecenoic acid, [8]-shogaol, euscaphic acid, asiatic acid, adenine, 1,2,3,19-tetrahydroxy-12-ursen-28-oic acid, and (S)-[10] gingerol, were identified as the core components of DFD (Table 6).

Construction of the PPI Network and GO and KEGG Pathway Enrichment Analyses

The above 21 shared targets were imported into the STRING database, and after 7 isolated targets were removed, the remaining shared targets were subjected to topological analysis and sorted according to the degree value to obtain a PPI network diagram with 14 nodes and 31 edges (Figure 5A). The darker the colour and the larger the area of the nodes are, the greater the degree value, indicating that the target plays a more critical role in the drug treatment of diseases. These targets include a variety of inflammatory-related targets, such as IL6, PTGS2, MMP7, MMP3 and MMP1, indicating that the mechanism by which DFD prevents the relapse of UC is closely related to inflammation.

The significantly enriched terms identified via the GO enrichment analysis are shown in Figure 5B. Among them, the shared targets were involved mainly in BPs such as extracellular matrix disassembly and the collagen catabolic process. The shared targets were mainly associated with CCs such as the extracellular matrix and extracellular space. The shared targets were mainly involved in MFs such as serine-type endopeptidase activity and endopeptidase activity.

Table 6 Core Compounds of DFD

Name	Degree	Betweenness	Closeness	Source
Zingerone	11	370.66843	0.48188406	Ganjiang, Xianhecao
Dehydrozingerone	9	242.21083	0.4586207	Ganjiang, Rougui
Sumaresinolic acid	9	204.2251	0.475	Diyu, Xianhecao
9,10-dihydroxy-12Z-octadecenoic acid	9	185.13379	0.46830985	Diyu, Ganjiang, Xianhecao, Rougui, Gancao, Qingdai
[8]-shogaol	8	155.92761	0.42903227	Ganjiang
Euscaphic acid	8	153.51178	0.46503496	Diyu, Xianhecao
Asiatic acid	8	268.68314	0.46830985	Diyu, Xianhecao
Adenine	8	185.1227	0.47841728	Diyu, Fuzi, Ganjiang, Xianhecao, Rougui, Gancao, Qingdai
1,2,3,19-tetrahydroxy-12-ursen-28-oic acid	8	145.8012	0.46503496	Diyu, Xianhecao
(S)-[10] gingerol	8	274.11163	0.475	Diyu, Fuzi, Ganjiang, Xianhecao, Rougui, Gancao, Qingdai

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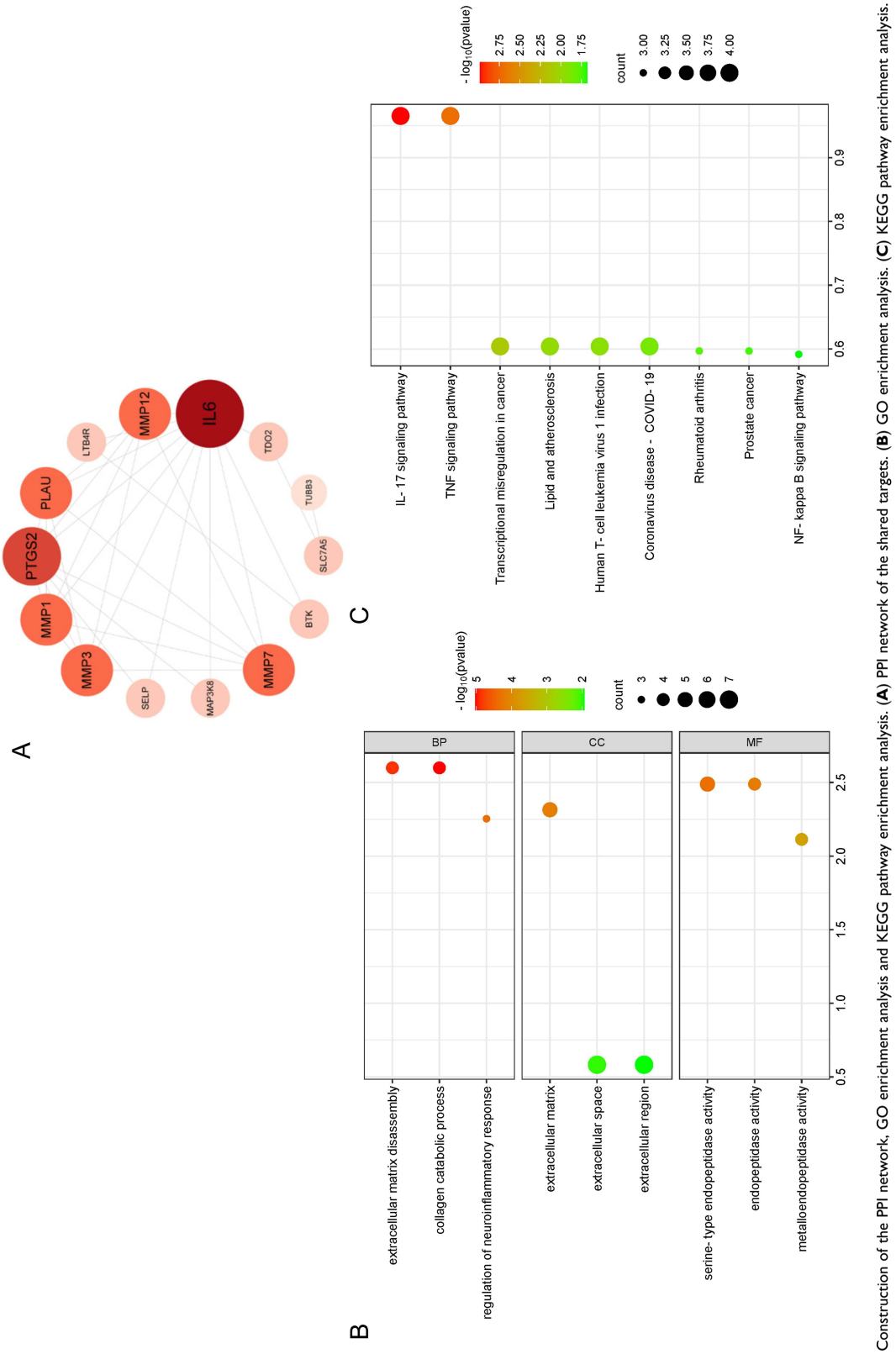

Figure 5 Construction of the PPI network, GO enrichment analysis and KEGG pathway enrichment analysis. (**A**) PPI network of the shared targets. (**B**) GO enrichment analysis. (**C**) KEGG pathway enrichment analysis.

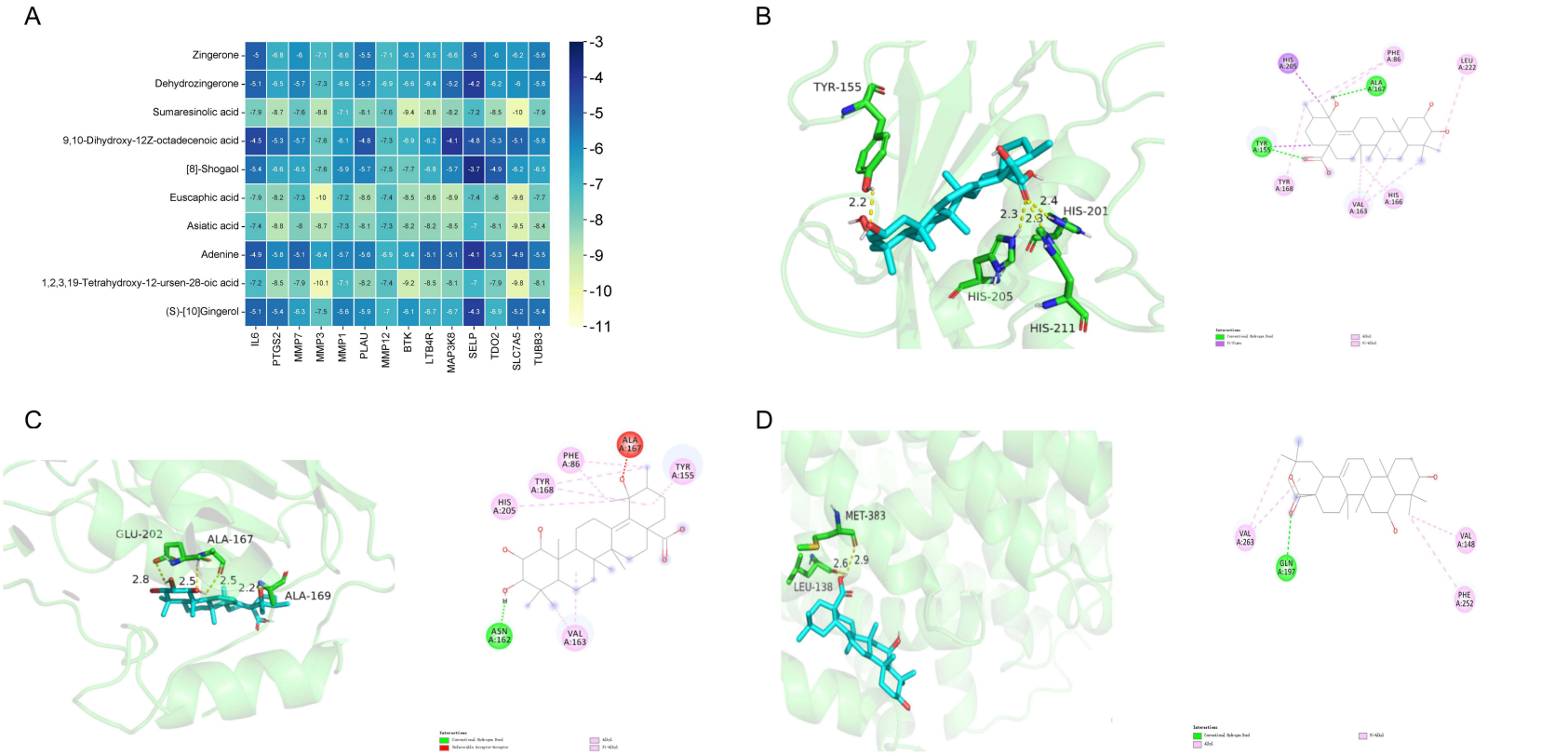

Figure 6 Molecular docking results. (**A**) Heatmap of the binding affinities. (**B**) Molecular docking of MMP3 and Euscaphic acid (affinity: −10.0 kcal/mol). (**C**) Molecular docking of MMP3 and 1,2,3,19-tetrahydroxy-12-ursen-28-oic acid (affinity: −10.1 kcal/mol). (**D**) Molecular docking of SLC7A5 and sumaresinolic acid (affinity: −10.0 kcal/mol).

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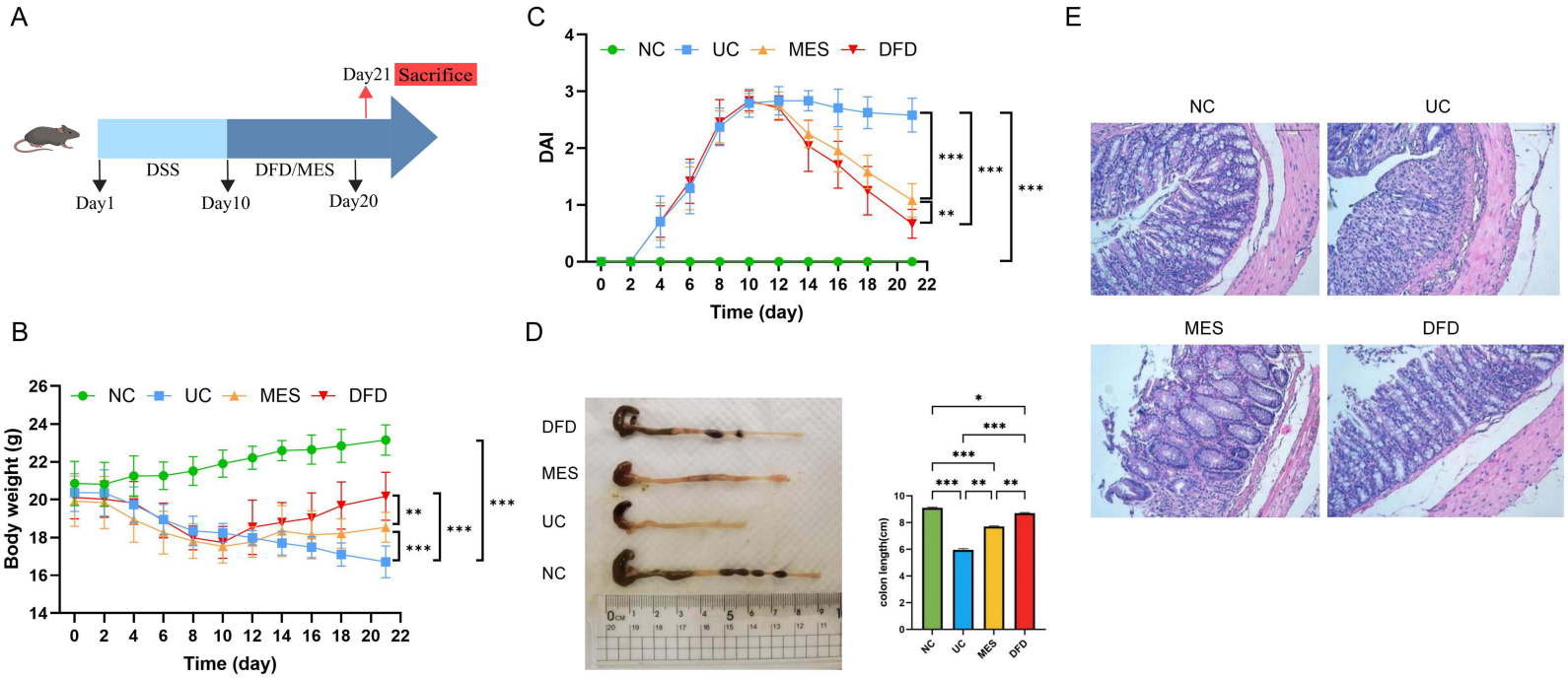

Figure 7 DFD alleviates DSS-induced UC in mice. (**A**) Procedure of mouse intervention. (**B**) Body weight. (**C**) DAI. (**D**) Colon length. (**E**) HE staining (200×). All data are presented as the mean ± SD (n=8). **P* <0.05, ***P* <0.01, ****P* <0.001.

KEGG pathway enrichment analysis revealed 9 enriched pathways, including the IL-17 signalling pathway, the TNF signalling pathway, transcriptional misregulation in cancer, lipid and atherosclerosis, human T-cell leukaemia virus 1 infection, coronavirus disease-COVID-19, rheumatoid arthritis, prostate cancer, and the NF-kappa B signalling pathway (Figure 5C). Among them, the IL-17 signalling pathway was the most enriched pathway. Our research team's previous study revealed that the level of IL-17 in UC patients was significantly greater than that in healthy people and that the level of IL-17 in patients with moderate and severe UC was significantly greater than that in patients with mild UC.[20] The IL-17 signalling pathway is abnormally activated during the recurrence of UC. Therefore, we explored this topic further.

Molecular Docking Verification

To further investigate the potential interactions between DFD active ingredients and the shared drug and disease targets, we performed molecular docking analyses of the 10 core components of DFD, namely, zingerone dehydrozingerone, sumarsinolic acid, 9.10-dihydroxy-12Z-octadecenoic acid, [8]-shogaol, euscaphic acid, asiatic acid, adenine, 1,2,3,19-tetrahydroxy-12-ursen-28-oic acid, and (S)-[10] gingerol and the 14 shared targets, namely, IL6, PTGS2, MMP7, MMP3, MMP1, PLAU, MMP12, BTK, LTB4R, MAP3K8, SELP, TDO2, SLC7A5 and TUBB3, and calculated the affinity between the core components and shared targets. The results revealed that the 10 core components in DFD had suitable binding affinity to the 14 shared targets (Figure 6A). The lower the binding affinity is, the stronger the binding between component and the target, and the more stable the binding. An affinity <-7.0 kcal/mol indicates strong binding activity.[21] The top 3 pairs of ingredient-targets with the best binding affinity (affinity ≤-10.0 kcal/mol) in this study were MMP3- Euscaphic acid, MMP3- 1,2,3,19-Tetrahydroxy-12-ursen-28-oic acid, SLC7A5- Sumaresinolic acid (Figure 6B-D). These results indicate that DFD active components have the potential to bind to the shared targets and thus exert an anti-UC relapse effect.

DFD Alleviates DSS-Induced UC in Mice

The mouse intervention procedure is shown in Figure 7A. The results indicated that DFD significantly reduced the weight loss and DAI of the DSS-treated mice (Figure 7B and C). In addition, DFD significantly alleviated the shortening of colon length induced by DSS and reduced the CMDI (Figure 7D and Table 7). H&E-stained sections can directly reflect the pathological state of colon tissue. As shown in Figure 7E, in the NC group, the intestinal epithelial structure was intact, the morphological structure of the epithelial cells was normal and arranged neatly, and the structure of the gland was clear in the lamina propria. In the UC group, the intestinal epithelial structure was disrupted, the epithelial cells were shed, the glands were arranged irregularly in the lamina propria, and numerous inflammatory cells infiltrated the mucosa and submucosa. In the DFD group, the intestinal mucosal damage basically returned to normal, with an intact intestinal epithelial structure, normal epithelial cell morphology and structure, and abundant glands in the lamina propria. These findings suggest that DFD has good potential for alleviating intestinal injury induced by DSS in mice with UC.

Table 7 Colon Mucosa Damage Index (CMDI) ($\bar{x}$±s, n=8)

Group	N	CMDI
NC	8	0.00±0.00
UC	8	3.25±1.04*
MES	8	1.38±0.52*#
DFD	8	0.75±0.46*#&

Notes: *P<0.001, vs NC group; #P<0.001, vs UC group; &P<0.05, vs MES group.

Abbreviations: NC, normal control group; UC, UC model group; MES, MES treatment group; DFD, DFD treatment group.

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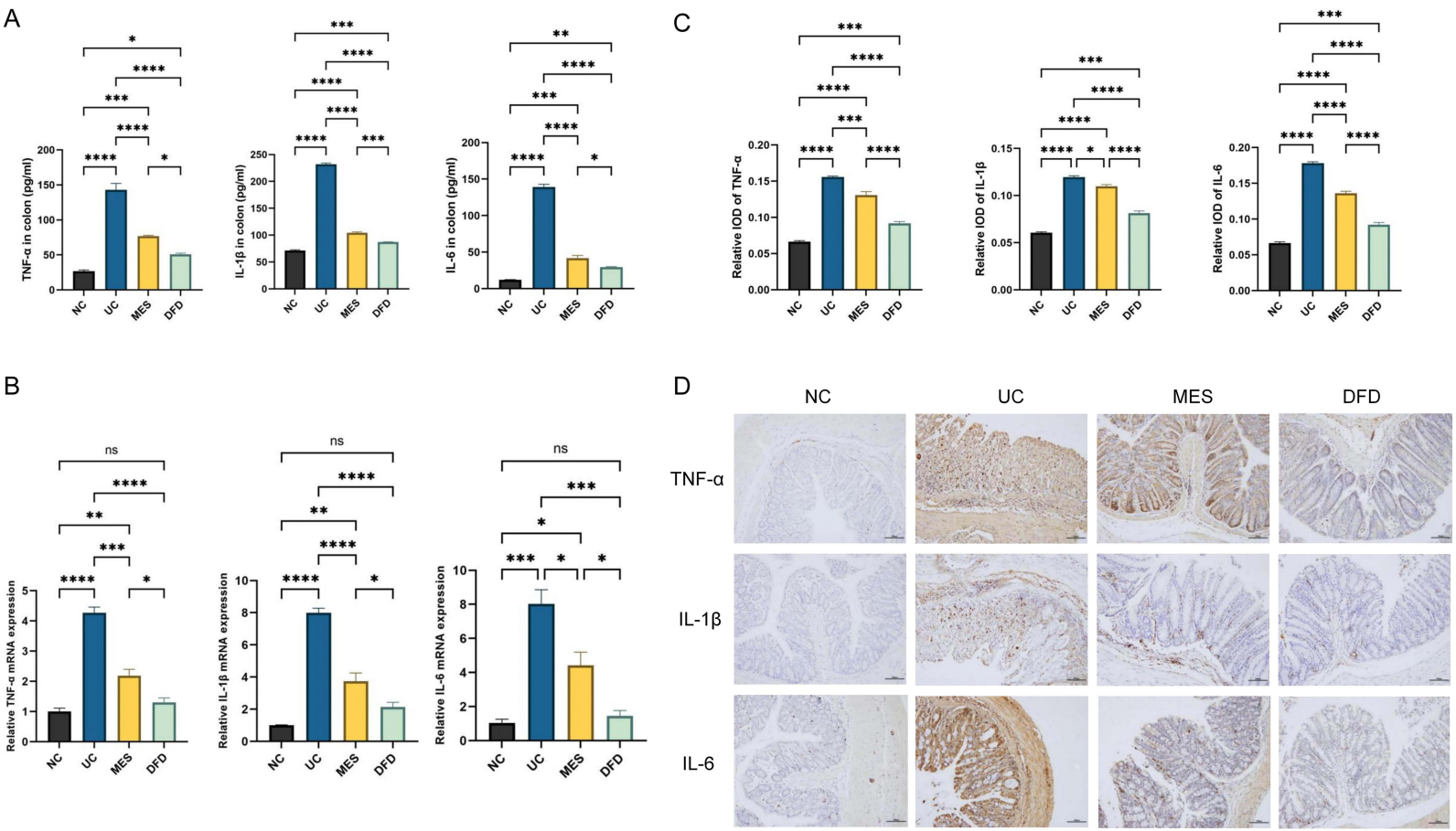

Figure 8 DFD reduces the levels of proinflammatory cytokines in mice with UC. (**A**) ELISA. (**B**) qPCR. (**C**) and (**D**) IHC (200×). All data are presented as the mean ± SD (n=8). **P* <0.05, ***P* <0.01, ****P* <0.001, *****P* <0.0001; ns, not significant.

DFD Reduces the Levels of Proinflammatory Cytokines in Mice with UC

Colon tissues were collected, and ELISAs were performed to measure the levels of proinflammatory cytokines. The results revealed that the levels of proinflammatory cytokines (TNF-α, IL-1β, and IL-6) in the UC group were significantly greater than those in the NC group, indicating a notable inflammatory response in the model mice. DFD effectively reduced the levels of proinflammatory cytokines (Figure 8A). We subsequently investigated whether DFD could affect the levels of proinflammatory cytokines via qPCR and IHC analyses, and the findings were consistent with the ELISA results (Figure 8B–D). These results suggest that DFD can reduce the levels of proinflammatory cytokines in mice with UC.

DFD Ameliorates Intestinal Mucosal Barrier Injury in Mice with UC

Tight junctions (TJs) play an important role in protecting colonic mucosal barrier function. We analysed whether DFD affects the levels of claudin-1, a TJ protein, via ELISA, qPCR and IHC analysis. The ELISA results indicated that the claudin-1 level in the UC group was significantly lower than that in the NC group (P <0.0001) (Figure 9A), which demonstrated that the intestinal mucosal barrier was damaged in the mice with UC. However, treatment with DFD abrogated the decrease in claudin-1 levels in colon tissue (Figure 9A). Similar results were obtained by qPCR and IHC analyses (Figure 9B–D). These findings suggest that DFD has an obvious protective effect on the intestinal mucosal barrier.

DFD Prevents the Relapse of UC via Inhibition of the IL-17 Signalling Pathway

The prevention of UC relapse by DFD is related to the IL-17 signalling pathway, which was confirmed by network pharmacology. NF-κB, a crucial transcription factor downstream of IL-17, plays a pivotal role in the inflammatory process. To clarify the effect of DFD on the IL-17 signalling pathway in mice with UC, we analysed the expression of genes related to the IL-17 signalling pathway via ELISA, qPCR and IHC analysis. The ELISA results revealed that the

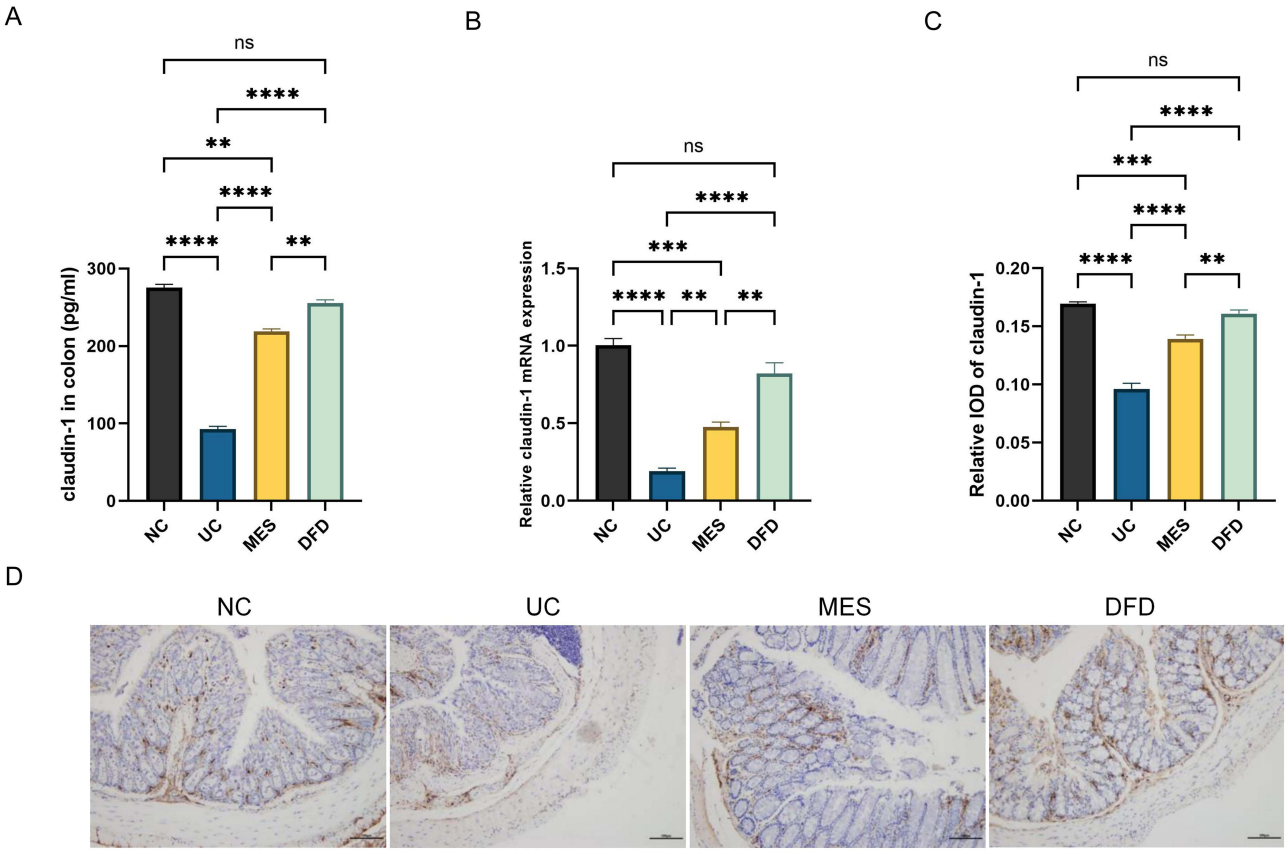

Figure 9 DFD increased claudin-1 expression in the colon tissue of the mice with DSS-induced UC. (**A**) ELISA. (**B**) qPCR. (**C**) and (**D**) IHC analysis (200×). All data are presented as the mean ± SD (n=8). **P <0.01, ***P <0.001, ****P <0.0001; ns, not significant.

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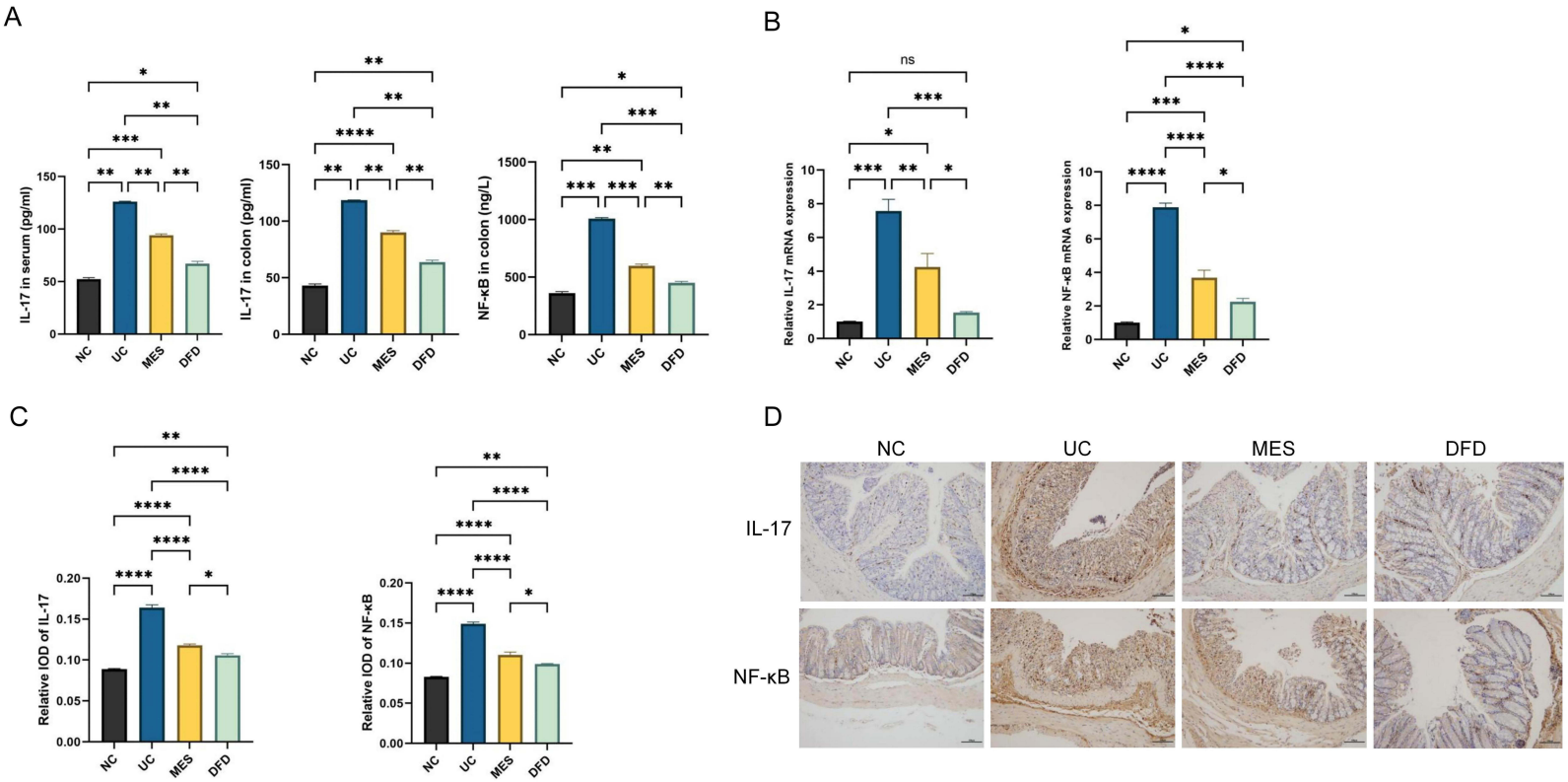

Figure 10 DFD reduced the levels of IL-17 in the serum and the levels of IL-17 and NF-κB in the colon tissue of the mice with DSS-induced UC. All data are presented as the mean ± SD (n=8). (**A**) ELISA. (**B**) qPCR. (**C**) and (**D**) IHC analysis (200×). **P* <0.05, ***P* <0.01, ****P* <0.001, *****P* <0.0001; ns, not significant.

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IL-17 signalling pathway was activated in the context of UC, whereas DFD significantly reduced the levels of IL-17 and NF-κB (Figure 10A). The changes in IL-17 and NF-κB mRNA levels were consistent with the changes detected by ELISA (Figure 10B), and the same results were also shown by IHC analysis (Figure 10C and D). In vitro experiments also revealed that DFD reduced the level of IL-17 in spleen cells (Figure 11A and B). These results suggest that DFD can inhibit the activation of the IL-17 signalling pathway.

Discussion

UC is a recurrent disease in the digestive system and difficult to cure. DFD has been used in the prevention UC relapse for more than 20 years, has been proven to be effective in prior clinical trials,[10,11,22,23] and has been patented by the China National Intellectual Property Administration (No. 201510162437. X). On the basis of the previous findings of our research group, we conducted a retrospective study using data from a RWS. The results showed that UC patients treated with DFD had a lower relapse rate (23.9%), which was more advantageous than that reported in previous studies on Western medical therapy.[2–4] Our findings revealed that there were few patients with adverse reactions (4.4%) to DFD, and the percentage of patients with adverse reactions to DFD was less than those with adverse reactions to 5-aminosalicylic acid (15%) and sulfasalazine (29%),[24] and no serious adverse events occurred. Analysis of the modified MAYO score revealed that the score decreased from 11 (10,12) to 2 (0,4) at week 8 after treatment with DFD, demonstrating that the DFD had a significant anti-inflammatory effect. In conclusion, DFD effectively prevents UC relapse. In this study, a retrospective clinical analysis was conducted using a RWS to explore the anti-UC relapse effect of DFD. Compared with RCTs, RWSs mimic real conditions more closely, which can compensate for the shortcomings of RCTs, and the conclusions obtained also have more practical clinical significance and value.[25]

To study the anti-relapse mechanism of DFD, we used the GEO database to obtain genomic data from UC patients in the active and remission stages,[26] identified DEGs in the active and remission stages as disease targets related to UC relapse, and then conducted network pharmacological analysis together with DFD components detected by UPLC-MS. We concluded that the IL-17 signalling pathway was the most significantly enriched pathway in UC relapse. Because the relapse of UC involves the transformation of the disease from remission to the active phase, comparing the difference between active-phase UC and remission-phase UC can more accurately reflect the mechanism of UC relapse.

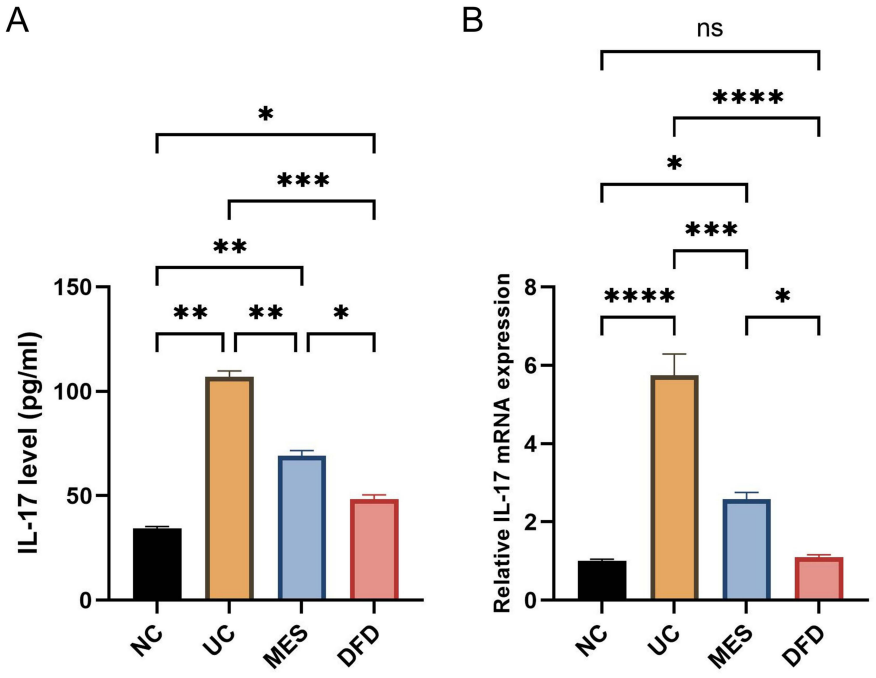

Figure 11 DFD reduced IL-17 levels in spleen cells. (**A**) ELISA. (**B**) qPCR. All data are presented as the mean ± SD (n=8). *P <0.05, **P <0.01, ***P <0.001, ****P <0.0001; ns, not significant.

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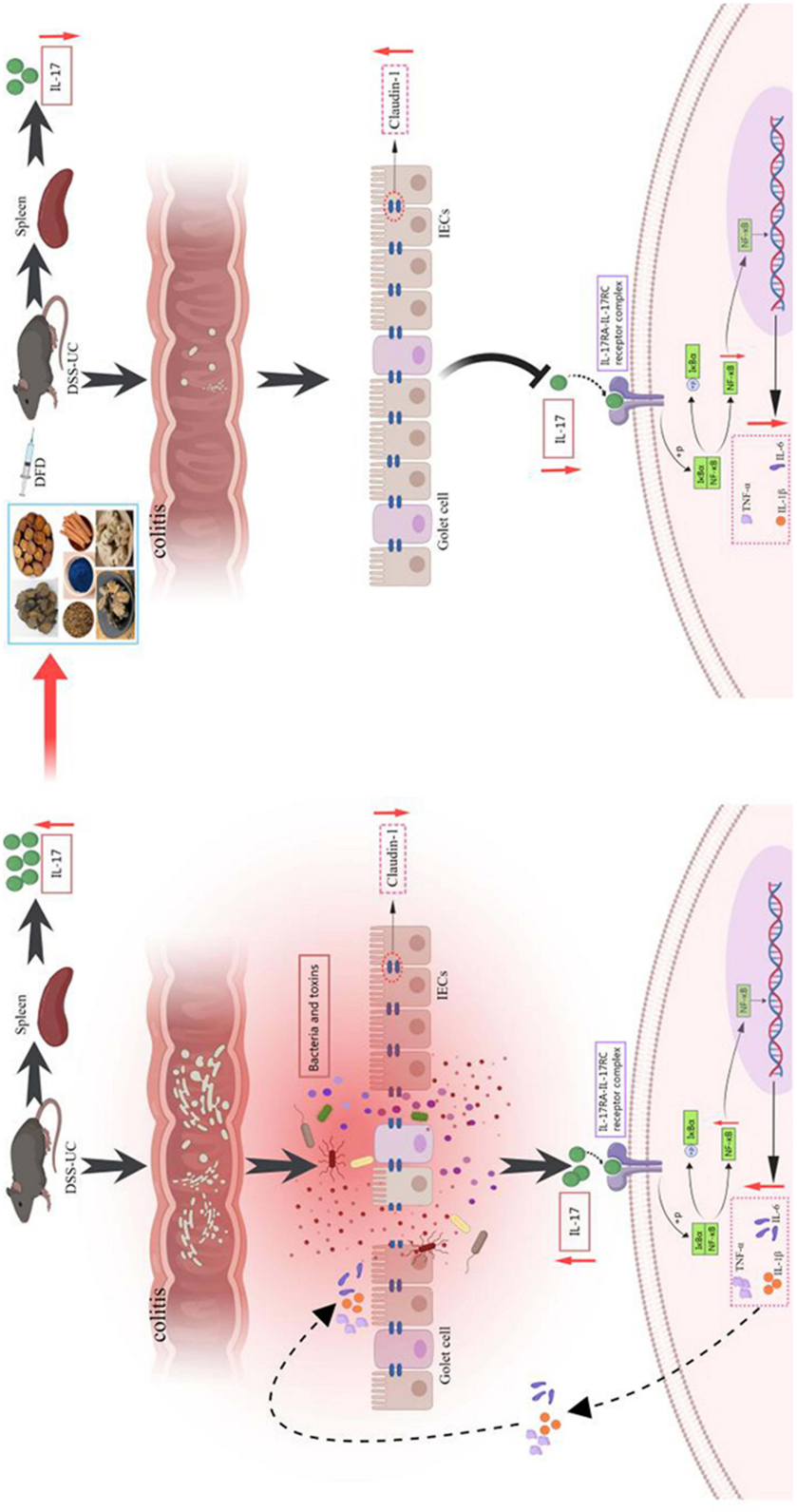

Figure 12 Mechanism by which DFD alleviates DSS-induced UC.

With respect to the enriched IL-17 signalling pathway, other studies have shown that interleukin-17 (IL-17) can activate nuclear factor kappa B (NF-κB), resulting in the production of many proinflammatory cytokines, such as tumour necrosis factor-α (TNF-a), interleukin-6 (IL-6), and interleukin-1β (IL-1β), leading to TJ dysfunction and subsequent destruction of intestinal mucosal barrier function.[27–29] This study also used a DSS-induced UC mouse model to investigate the relevant molecules in the IL-17 signalling pathway. The results demonstrated that the levels of IL-17, NF-κB, TNF-α, IL-1β and IL-6 were significantly elevated and that the level of claudin-1 was increased, suggesting that the IL-17 signalling pathway was activated during the active phase of UC. Treatment of UC model mice with DFD reversed these changes, indicating that DFD can effectively inhibit the activation of the IL-17 signalling pathway and protect the intestinal mucosal barrier of UC model mice, thereby reducing the inflammatory response and preventing the transition of UC to the active phase. Moreover, the in vitro results also revealed the inhibitory effect of DFD on the IL-17 signalling pathway (Figure 12). Therefore, we hypothesized that DFD could prevent the relapse of UC by inhibiting the IL-17 signalling pathway.

We further validated the effect of DFD on the relapse of UC from the perspective of molecular docking. The core components of DFD selected from the database were used as ligands, and the shared drug and disease targets were used as receptors for molecular docking verification. The results revealed that the binding of ligands and receptors was relatively stable, especially for key components in DFD and inflammation-related targets (such as IL6, PTGS2, MMP7, MMP3, and MMP1). These results further confirmed that DFD had a regulatory effect on UC-induced inflammation.

The above results showed that DFD decreased inflammation in UC model mice via multiple components, multiple targets and multiple pathways; thus, DFD is superior to Western medicine, which is composed of only a single compound, for preventing the relapse of UC. At present, there is a lack of effective clinical treatments for the prevention of UC relapse. DFD has great potential for the prevention of UC relapse, providing a new direction for UC treatment.

However, this study has certain limitations. This study focused only on DFD preventing the relapse of UC by regulating the IL-17 signalling pathway, and other signalling pathways may also be closely related to the recurrence of UC, which needs further experimental verification. Therefore, in the future, we will conduct in-depth research on other mechanisms of DFD in the prevention of UC relapse, study the role of the different chemical components of DFD in the prevention of UC relapse, and develop new drugs for UC treatment.

Conclusion

In summary, the present study highlights the potential of DFD in preventing and treating the relapse of UC, and its mechanism of action is related to inhibiting the activation of the IL-17 pathway. This study provides beneficial evidence for the use of DFD in preventing the recurrence of UC, reveals its potential mechanism, and lays a solid foundation for the clinical application and development of DFD.

Abbreviations

CI, confidence interval; CMDI, Colon Mucosa Damage Index; DAI, Disease Activity Index; DFD, Daifu Decoction; DSS, dextran sulfate sodium; ELISA, Enzyme-linked immunosorbent Assay; GO, Gene Ontology; H&E, Hematoxylin and Eosin; IBDQ, Inflammatory Bowel Disease Questionnaire; IHC, Immunohistochemical analysis; IL-17, Interleukin-17; IL-1β, Interleukin-1β; IL-6, Interleukin-6; KEGG, Kyoto Encyclopedia of Genes and Genome; MES, mesalazine sustained-release granules; NF-κB, nuclear factor kappa B; OR, Odds Ratio; PPI, protein-protein interaction; qPCR, quantitative real-time Polymerase Chain Reaction; RWS, real world study; RCTs, randomized controlled trials; TCM, traditional Chinese medicine; TJs, Tight junctions; TNF-α, Tumor necrosis factor-α; UC, ulcerative colitis; UPLC-MS, ultra-high performance liquid chromatography-mass spectrometry.

Data Sharing Statement

Data used to support the findings of this study are available on request from the corresponding author. The authors intend to share individual deidentified participant data, including deidentified individual basic data in the reporting results section of the article, such as the *main text*, tables, figures, and supplementary documents. In addition, authors can also share research documents related to this study, such as research protocols, statistical analysis plans, and statistical codes.

The shared data can be obtained immediately after publication without end date by contacting the corresponding author (Yang Gong, Email gongyang126@126.com).

Ethics Approval and Informed Consent

The protocols and procedures of this study were approved by the ethics committee of General Hospital of Northern Theater Command (NO.2021JH2/10300109, NO.Y(2024)099). This retrospective clinical trial was conducted in accordance with the principles of the Declaration of Helsinki. All patients who participated in the real-world study signed informed consent and all data obtained remained anonymous.

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Disclosure

The authors declare no conflicts of interest in this work.

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