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Network pharmacology and experimental validation of Compound Kushen Powder for the treatment of diarrhea in vivo

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ABSTRACT

To explore the mechanism of sophora flavescens, cortex fraxini, and pomegranate peel complex powder (Compound Kushen Powder) in the treatment of animal diarrhea, a network pharmacology approach leveraging databases like TCMSP and SwissTarget was applied in this study. Molecular docking was executed between the primary constituents and pivotal targets, enabling an additional refinement of main targets and key medications. Subsequently, a rat diarrhea model induced by folium sennae leaves was established for in vivo validation. The rats were divided into four groups: negative control group, positive control group, positive drug treatment group, and Compound Kushen Powder treatment group. Key protein targets, such as Caspase-3, IL-1β, IL-10, MMP9, STAT3, TNF, TP53, and VEGFA, essential for mitigating diarrhea in response to the composite medication were found through network pharmacology. Additionally, the results of molecular docking analysis unveiled fundamental constituents of Compound Kushen Powder, namely beta-sitosterol, ursolic acid, formononetin, and matrine, which demonstrated significant binding affinities with those identified key protein targets. The results of mRNA and protein expression analyses of rat colonic tissue validated the in vivo alterations of core genes identified through network screening. Except for IL-10 and STAT3, the expression of all targets exhibited noteworthy reductions when compared to the positive control group ($P < 0.05$). These results demonstrated that Compound Kushen Powder can inhibit inflammation and regulate cell apoptosis by modulating signaling pathways such as IL-17, TNF-α, MAPK, and NF-κB. Collectively, this study sheds light on the traditional application of complex powder for the prevention and treatment of diarrhea.

Introduction

Piglet white diarrhea, caused by pathogenic *Escherichia coli*, is a bacterial disease that primarily affects weaned piglets (White et al., 1972). Its notable symptom is the discharge of white, loose feces (Martins et al., 2013). The disease is complicated by the presence of multiple serotypes of pathogenic *E. coli*, which limits immune protection provided by vaccines (Fairbrother et al., 2005). Approximately 55.3 % of *E. coli* isolates collected from piglets suffering from post-weaning diarrhea in Shandong Province, China, were found to harbor at least one virulence gene. Among these, the enterotoxin genes EAST1 (34.9 %) and LT-I (27.1 %) were the most prevalent (Li et al., 2020). Another study isolated 455 *E. coli* strains from 56 swine farms in 15 provinces in China, finding heat-labile enterotoxin (LT) was the most common enterotoxin gene (61.76 %), followed by heat-stable enterotoxin (STb) (33.19 %) and stx2e (21.54 %) (Yang et al., 2019).

Currently, antibiotics and chemical antibacterial agents are the main approaches for prevention and treatment. However, the extensive and improper use of these drugs has led to a significant increase in antibiotic resistance among *E. coli* strains (Arbab et al., 2022; Poirel et al., 2018). The highest levels of antimicrobial resistance in animals have been found in India and China (Van Boeckel et al., 2019). In an epidemiological investigation, of 1871 *E. coli* isolates collected from pigs in China, 91 % exhibited multidrug resistance to last-resort drugs including colistin, carbapenems, and tigecycline (Peng et al., 2022). As the world's largest pig producer, China was also one of the top consumers of colistin

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in veterinary medicine. This widespread usage of colistin led to substantial selective pressure in the veterinary environment, facilitating the emergence of colistin-resistant *E. coli* carrying the mcr-1 gene. Notably, analysis of bacterial isolates demonstrated that approximately 20 % of *E. coli* samples obtained from swine slaughterhouses and meat products tested positive for mcr-1 (Liu et al., 2016b). These surveillance data revealed that China had the highest prevalence of mcr-1-positive Enterobacteriaceae (Van Boeckel et al., 2019), which prompted China to ban the use of colistin in animal feed from May 1, 2017 (Shen et al., 2020). Therefore, immediate actions to preserve antimicrobials and alternative treatments in animal husbandry are in urgent need.

With the development of natural products and herbs in pharmacology, the application of traditional Chinese veterinary medicine formulations on treating white scour of piglets has raised growing interest. Among these treatments, Compound Kushen Powder has demonstrated promising antidiarrheal effects in castor oil-induced diarrhea in mice (Yu et al., 2019). This formulation, composed of sophora flavescens, pomegranate peel, and fraxini cortex in a 3:3:2 wt ratio, effectively increased gastric residue rates and reduced intestinal propulsion in mice. It also significantly inhibited the hyperactivity of intestinal peristalsis caused by neostigmine, with good acute anti-inflammatory activity. An acute toxicity test in mice and the 30-day subchronic toxicity test in rats confirmed that the formulation of Compound Kushen powder is a low-toxic and safe veterinary preparation suitable for clinical application (Yu et al., 2018). Furthermore, in clinical trials for treating E. coli-induced diarrhea in piglets, the high (1.0 g/body weight (kg)/day) and medium (0.5 g/body weight (kg)/day) doses of Compound Kushen Powder achieved cure rates of 85.0 % and 80.0 %, respectively, after five days of administration (Yu et al., 2024). However, the precise mechanisms underlying its actions remain incompletely understood. Traditional Chinese medicine often comprises multiple components and targets various points, making it increasingly challenging to elucidate their mechanisms of action. Network pharmacology, a novel approach to studying and elucidating drug mechanisms, integrates cheminformatics, bioinformatics, network biology, and pharmacology (Duan et al., 2019). It enables the construction of multi-level networks representing "multiple drug components—multiple targets—diseases," allowing for the analysis of interactions between drugs and potential targets (Li et al., 2014). This approach provides new research insights and methods for investigating the mechanisms of action of traditional Chinese medicine.

The present research utilized a combination of network pharmacology, molecular docking, and in vivo experiments to investigate the potential key targets and pharmacological mechanisms underlying the effects of cortex fraxini, sophora flavescens, and pomegranate peel against diarrhea. Quantitative PCR (qPCR) and western blot (WB) were employed to analyze the expression levels of eight distinct proteins (MMP9, TP53, CASP3, VEGF, IL-1β, IL-10, TNFα, STAT3) in the colon tissue of a rat diarrhea model, providing a comprehensive evaluation of their role in the disease process. Focusing on these selected proteins, the present study aimed to uncover potential therapeutic mechanisms of action for traditional Chinese medicine compounds. Our findings shed light on the intricate molecular changes associated with diarrhea and the response to therapeutic interventions.

Materials and methods

Screening of active ingredients in Compound Kushen Powder and target identification for its anti-diarrheal mechanism

By utilizing the Traditional Chinese Medicine Systems Pharmacology Analysis Platform database (TCMSP, http://tcmspw.com/), the chemical constituents of Compound Kushen Powder, including sophora flavescens, cortex fraxini, and pomegranate peel, were identified. Compounds with an oral bioavailability (OB) $\geq$ 30 % and drug-likeness (DL) $\geq$ 0.18 were selected as effective components (Bao-jin, 2022). Simultaneously, compounds obtained from reference literature were included in this study for analysis.

Target proteins for these chemical constituents were extracted from the TCMSP database. In the UniProt KB database (http://www.uniprot.org/), the species "rat" was chosen to search for the corresponding targets of the aforementioned effective components (Huang et al., 2012). To enhance the accuracy of target data, the chemical constituents were also imported into the Swiss Target and DrugBank databases to obtain specific target information. Subsequently, all collected target data were subjected to further analysis after removing duplicates.

Investigation of the intersection target-interaction network between active ingredients in Compound Kushen Powder and diarrheal mechanisms (Lim & Kang, 2018)

GeneCards and Ensembl databases were employed to retrieve target genes associated with the term "Diarrhea" as a key keyword. Subsequently, a Venn diagram was constructed to illustrate the overlap between the target genes corresponding to the active ingredients extracted from the Compound Kushen Powder and those associated with diarrhea. Furthermore, we separately imported information pertaining to the active ingredients and the intersecting target genes into Cytoscape 3.9.1 software in order to construct a network diagram representing the interactions between drugs, active ingredients, and target genes associated with diarrhea.

Protein-Protein interaction (PPI) and core target screening (Liu et al., 2016a)

Imported the intersecting targets into the STRING database (http://cn.stringdb.org/), selected "Multiple proteins," chosen the species "Rat," conducted a relationship analysis between the active ingredients of Compound Kushen Granules and diarrhea targets, and constructed a Protein-Protein Interaction (PPI) network using Cytoscape 3.8.2 software to analyze the important targets of Compound Kushen Powder in treating sennae leaf-induced diarrhea in rats.

GO and KEGG enrichment analysis (Xu et al., 2020)

In the DAVID database (https://david.ncifcrf.gov/), inputted the intersection targets, selected the species as 'rat' for GO biological process and KEGG pathway enrichment analysis, set the threshold at $p < 0.05$, and excluded the presence of false positives. Utilized the Microbioinformatics online website (http://www.bioinformatics.com.cn/) to generate analysis plots and analyzed the regulatory pathways of Compound Kushen Powder in the treatment of castor oil-induced diarrhea in rats.

Active ingredient molecular docking (Meng et al., 2011)

The 3D structures of the core component targets and ligands were retrieved from the chemical database (PubChem) and protein database (RCSB-Pdb). These structures were then processed in AutodockTools, which included hydrogenation and charge removal, before calculating the binding affinity between the target and ligand molecules (Martucci et al., 2004).

Experimental animals

A total of 40 Sprague-Dawley male rats (180–220 g), were purchased from Beijing HFK Bio-Technology.co., LTD. The rats were housed in a specific-pathogen-free (SPF) conditions controlled temperature (25 $\pm$ 1 °C) and humidity (50 $\pm$ 5 %) environment with a 12-h light/dark cycle and allowed free access to sterilized food and water.

Animal experiments

Senna leaves were soaked in distilled water for 30 min, then decocted for 10 min. The solution was reduced under vacuum to a crude drug concentration of 0.3 g/ml and stored at 4 °C for further use.

A combination of 375 g sophora, 375 g pomegranate peel, and 250 g cortex fraxini were boiled twice, each time for two hours, with 15-fold water. The decoctions were combined, filtered, and concentrated to about 0.5 g/ml of the original crude drug. It was then spray-dried, sieved, and mixed with an appropriate amount of lactose to make 500 g of complex powder.

A diarrhea model in rats was established with reference to the literature (Lu et al., 2022). A total of 40 rats were randomly divided into four groups: the negative control group, the positive control group, the positive drug treated group with Rapid Anti-Diarrheal Capsule (1.25 g/kg, Zhengzhou Ruilong Pharmaceutical Co., Ltd), and the Compound Kushen Powder treated group (2.3 g/kg), with 10 rats in each group. Rapid Anti-Diarrheal Capsule is also known as SuXiaoZhiXieJiaoNang, which has Berberine Hydrochloride and Bistort Rhizome as the main components. Except for the negative control group, which received intragastric administration of physiological saline, the other experimental groups were orally administered a solution of Fructus Cannabis (10 mg/kg) in the morning and afternoon, respectively, for a duration of 14 days. On the 15th day, the corresponding drugs were orally administered once a day for seven consecutive days to the dose groups, while the negative control group and positive control group continued to receive intragastric administration of an equivalent volume of physiological saline. Four hours after the final dose of treatment, the rats in each group were anesthetized with 2 % thiopental sodium (Shanghai New Asiatic Pharmaceuticals Co., Ltd., China) and then humanely euthanized, and the colons were collected.

Sample collection and processing

The contents of the intestinal cavity were washed out with physiological saline. Then, tissues from the most severe lesion sites were quickly fixed in a 10 % neutral formalin solution. The remaining colon tissues were stored at −80 °C for future use. Paraffin-embedded tissues were routinely stained with hematoxylin and eosin (HE). The tissue sections were scanned with SQS-600P Slide Scanning system (Shengqiang Technology Co., Ltd., China).

qPCR

Approximately 50 mg of colon tissue was used to extract total RNA using the method provided by the manufacturer (SPAKeasy Tissue/Cell RNA Rapid Extraction Kit, Sicongjie, Batch number: DUKKX). The purity and concentration of RNA were assessed using Nanodrop. The mRNA expression of eight proteins was reversed transcribed to cDNA. This cDNA was used as a template and amplified according to the real-time PCR instructions. The 20 μL PCR reaction system included 10 μL SYBR Green Mix, 1 μL each for upstream and downstream primer sequences, 2 μL template cDNA, and 2 μL DNase and RNase-free water. The cycles included pre-denaturation at 94 °C for 5 min, denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s, for a total of 40 cycles. In accordance with the specific gene sequences of rat TNF-α, IL-1β, IL-10, TP53, CASP3, STAT3, MMP9, and VEGFA available in the GenBank database, primers were designed, and their sequences are provided in detail in Table 1. GAPDH was employed as the internal reference gene, and the $2^{-\Delta\Delta Ct}$ method was utilized to calculate the relative expression of mRNA for each target gene.

Western blot

Approximately 100 mg of colon tissue was used to extract total protein using the manufacturer's instructions (Product number: BC3790, Solabio), and protein content was determined. After boiling and denaturing the protein, it was separated by SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked with 5 % skim milk, incubated with primary antibodies overnight at 4 °C, and washed three times with PBST to remove unbound primary antibodies. The membrane was then incubated in secondary antibody solution at room temperature for 1.5 h, and washed three times with PBST to remove unbound secondary antibodies. The membrane was exposed in a dark room, and the gray values were analyzed using ImageJ software.

Data analysis

For statistical analysis of qPCR and western blot, one-way analysis of variance (ANOVA) was performed followed by Duncan's Multiple Range Test for multiple comparisons. Statistical significance was defined as $P < 0.05$. The analysis was carried out by using SPSS 25.0 software.

Results

Screening of active ingredients in Compound Kushen Powder and identification of their targets in the treatment of diarrhea

By utilizing the TCMSP database, compounds were selected from the herbal ingredients (sophorae radix, pomegranate rind, and cortex fraxini) of Compound Kushen Powder based on criteria of oral bioavailability (OB) ≥30 % and drug-likeness (DL) ≥0.18. Additionally, pertinent literature was consulted to identify the effective constituents of the three traditional Chinese medicines, resulting in a total of 9, 45, and 15 active ingredients, respectively, as outlined in Table 2. These 69 effective constituents were then mapped to potential target genes using the TCMSP database, which were further converted and cross-referenced with Uniprot, Swiss Target, and Drugbank databases. Following the removal of duplicates, a cumulative total of 617 potential target genes for the drug's effective constituents were obtained.

A keyword search for 'diarrhea' was conducted in the GeneCards and Ensembl databases, yielding 1109 potential target genes associated with diarrhea. The intersection of the potential target genes of Compound Kushen Powder's effective constituents and those related to diarrhea resulted in 45 potential target genes, as depicted in Fig. 1.

Table 1
Primer sequence.

Gene name	Forward primer	Reverse primer
TNF-α	5′-GACCCTCACACTCAGATCATCTT-3′	5′-CCTTGAAGAGAACCTGGGAGTAG-3′
IL-1β	5′-CAACTGTCCCTGAACTCAACTGT-3′	5′-GAGATGCTGCTGTGAGATTTGAA-3′
IL-10	5′-TGGGTTGCCAAGCCTTATCG-3′	5′-TTCAGCTTCTCACCCAGGGA-3′
TP53	5′-TGGAGGATTCACAGTCGGATATG-3′	5′-GCCTTCTAACAACTCTGCAACAT-3′
CASP3	5′-GATGCAGCTAACCTCAGAGAGAC-3′	5′-AGTCGCCTCTGAAGAAACTAGTT-3′
STAT3	5′-TGTCAGATCACATGGGCTAAGTT-3′	5′-TGAAACCCATGATGTACCCTTCA-3′
MMP9	5′-TCGAAGGCGACCTCAAGTG-3′	5′-TTCGGTGTAGCTTTGGATCCA-3′
VEGFA	5′-GGCCTCTGAAACCATGAACTTTC-3′	5′-ACACAGGACGGCTTGAAGATATA-3′
GAPDH	5′-GCACCGTCAAGGCTGAGAA-3′	5′-AGCATCGCCCCACTTGATT-3′

Table 2
The effective ingredients of the Compound Kushen Powder.

Drug	Molecular number	Ingredients	Drug	Molecular number	Ingredients
Sophora flavescens	MOL001040	(*R*)-Naringenin	Sophora flavescens	MOL006568	isosophocarpine
	MOL001484	Inermine		MOL006569	(-)−14beta-hydroxymatrine
	MOL003542	8-Isopentenyl-kaempferol		MOL006571	anagyrine
	MOL003673	Wighteone		MOL006590	Thermopsine
	MOL003676	Sophoramine		MOL006591	Thc-9-cooh
	MOL004580	cis-Dihydroquercetin		MOL003627	sophocarpine
	MOL004941	DL-Liquiritigenin		MOL003680	sophoridine
	MOL005100	Rac-Hesperetin		MOL000392	formononetin
	MOL000006	luteolin		MOL005944	matrine
	MOL006561	(+)−14alpha-hydroxymatrine		MOL006562	7,11-Dehydromatrine
	MOL006563	(+)−9alpha-hydroxymatrine		MOL006564	(+)-allomatrine
	MOL006565	AIDS211310		MOL006623	kushenol,t
	MOL006566	(+)-lehmannine		MOL006626	leachianone,g
	MOL006567	(+)-sophoranol		MOL006622	kushenol O
	MOL006570	(-)−9alpha-hydroxysophoramine		MOL006627	Lehmanine
	MOL006582	5α,9α-dihydroxymatrine		MOL006628	(+)-Lupanine
	MOL006583	7,11-dehydromatrine		MOL006630	Norartocarpetin
	MOL006596	Glyceollin		MOL000456	Phaseolin
	MOL003347	hyperforin		MOL006650	(-)-Maackiain-3-O-glucosyl-6′-O-malonate
	MOL006604	Isoxanthohumol		MOL006652	trifolrhizin
	MOL006612	kurarinone		MOL000008	apigenin
	MOL006613	kushenin		MOL000098	quercetin
	MOL006587	N-allomatrine			
Cortex fraxini	MOL001778	Sinapaldehyde	Pomegranate peel	MOL001002	ellagic acid
	MOL000223	caffeic acid		MOL001906	Methylgallate
	MOL003837	escμLetin		MOL002077	(+)-Pelletierine
	MOL004456	EscμLin		MOL000358	beta-sitosterol
	MOL004667	fraxetin		MOL000422	kaempferol
	MOL000511	ursolic acid		MOL000492	(+)-catechin
	MOL000570	Nonox D		MOL000513	3,4,5-trihydroxybenzoic acid
	MOL006712	sinapaldehyde glucoside		MOL009146	punicalagin
	MOL003177	Syringaldehyde		MOL009274	Fritillaziebinol
				MOL000098	quercetin
				MOL000006	luteolin
				MOL009275	Punicalin
				MOL009276	casuarinin
				MOL009271	granatin B
				MOL009272	09,762_FLUKA

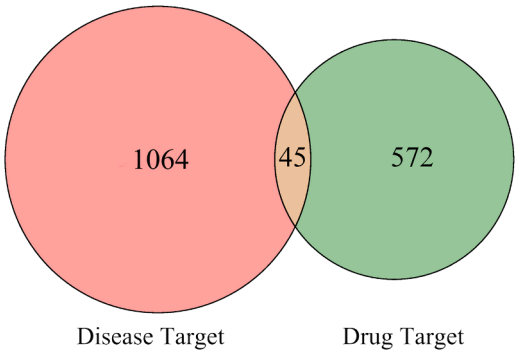

Fig. 1. The intersection of potential targets for the active ingredients of Compound Kushen Powder with those for diarrhea, represented as a line plot in this Venn diagram.

Network diagram of active ingredients and target genes for Compound Kushen Powder

45 potential target genes were considered as the therapeutic targets of Compound Kushen Powder for the treatment of diarrhea. Using Cytoscape 3.9.1 software, a visual analysis was conducted to construct a drug-effective ingredient-target gene network diagram. Key effective ingredients included matrine, ellagic acid, quercetin-3-O-alpha-L-arabinopyranoside, quercetin-3-O-beta-D-glucopyranoside, kaempferol, punicalagin, quercetin, and epicatechin gallate, among others, as detailed in Fig. 2.

PPI network analysis (protein interaction network diagram)

In the STRING database, the 45 intersecting target genes were entered to construct a protein-protein interaction (PPI) network, as illustrated in Fig. 3. A total of 296 edges and 43 nodes were obtained. This network was subsequently imported into Cytoscape 3.9.1, where Network Analysis and topological analysis were applied to construct the PPI network diagram. Eight target genes with the highest connectivity, topological coefficient and neighborhood connectivity were identified as core targets, namely TNF-α, IL-1β, IL-10, TP53, CASP3, STAT3, MMP9, and VEGFA (Table 3).

Go and KEGG analysis

45 core target proteins of Compound Kushen Powder in the treatment of rat diarrhea was analyzed by inputting them into the DAVID database for Gene Ontology (GO) functional enrichment analysis. A total of 454 GO terms were identified, comprising 304 biological process terms, 26 cellular component terms, and 32 molecular function terms. The top 10 statistically significant GO terms from each category were selected to create a bubble chart, as shown in Fig. 4. In the biological process category, the main target proteins were associated with responses to ethanol, lipopolysaccharides, positive regulation of angiogenesis, response to exogenous stimuli, drug responses, and positive regulation of cell proliferation. The cellular component category primarily involved vesicles, membrane rafts, outer mitochondrial membrane, cell surface, and cytoplasm. The molecular function category mainly included peroxidase activity, endopeptidase activity, and peptidase activity. Furthermore, KEGG pathway enrichment analysis

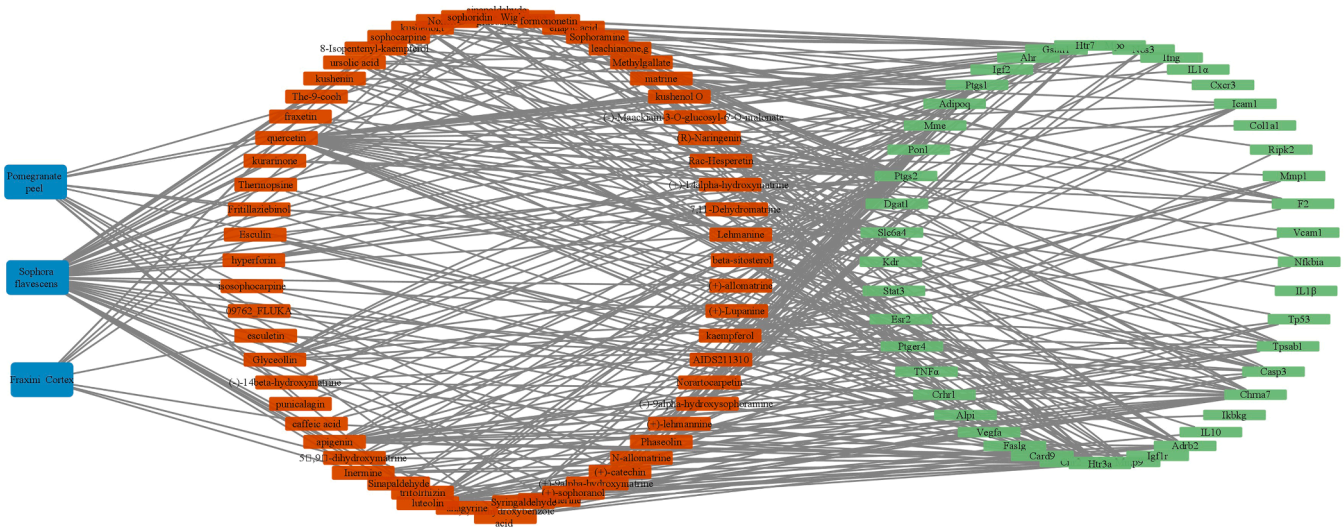

Fig. 2. The network diagram of effective components-target of Compound Kushen Powder.

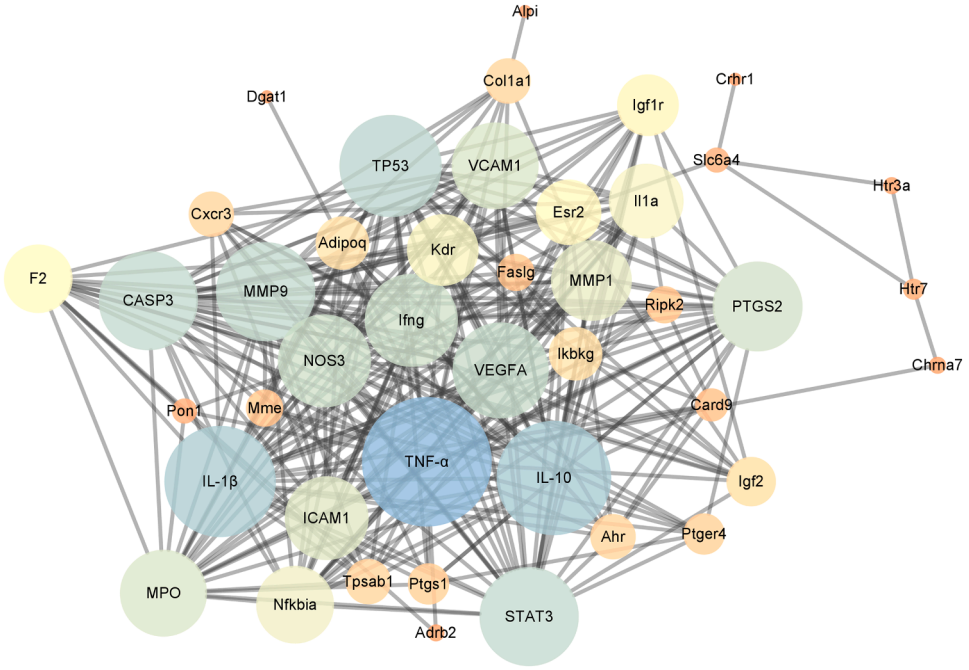

Fig. 3. . The PPI network diagram depicting the targets of intersection between the Compound Kushen Powder and diarrhea in rats.

Table 3
Key nodes of protein information.

Uniprot ID	Gene	Connectivity	Topological coefficient	Neighborhood connectivity
P16599	TNF-α	35	0.22	0.8235
P29456	IL-10	30	0.06	0.7241
Q63264	IL-1β	29	0.05	0.6885
P10361	TP53	26	0.04	0.6774
P55213	CASP3	25	0.03	0.6667
P52631	STAT3	25	0.03	0.6667
P50282	MMP9	25	0.03	0.6667
P16612	VEGFA	24	0.02	0.6667

revealed a total of 90 related pathways. The top 20 pathways, ranked by p-value, are detailed in Fig. 5, primarily involving cancer signaling pathways, the IL-17 signaling pathway, TNF-α signaling pathway, MAPK signaling pathway, and NF-κB signaling pathway.

Molecular docking

The PyMOL Software was utilized to visualize the results of the "drug compound intersection target" interaction network, which included the top 10 components and 21 main active components reported in the literature, linked with the PPI core target gene. The results with the lowest binding energy in each target were selected (Table 4).

A binding energy less than −5.0 kcal/mol indicates a strong correlation between the components of the drugs and the target for treating diarrhea. The 3D and 2D diagrams of the receptor-ligand molecular docking results with a binding energy less than −6.50 kcal/mol are shown in Fig. 5. The optimal combination was observed with ursolic acid and Caspase_3, followed by β-Sitosterol and Caspase_3, and matrine and its derivatives with Caspase_3, IL-1β, and MMP9. The strongest

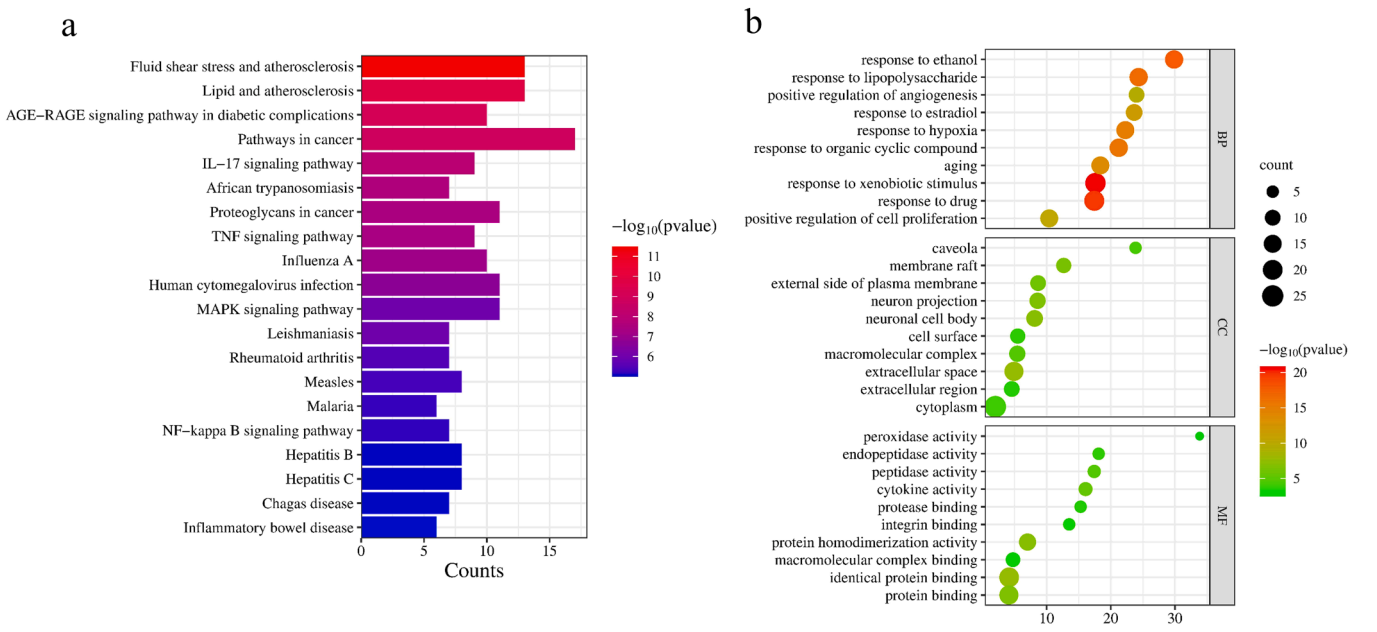

Fig. 4. . KEGG **(a)** and GO **(b)** enriched pathway analysis.

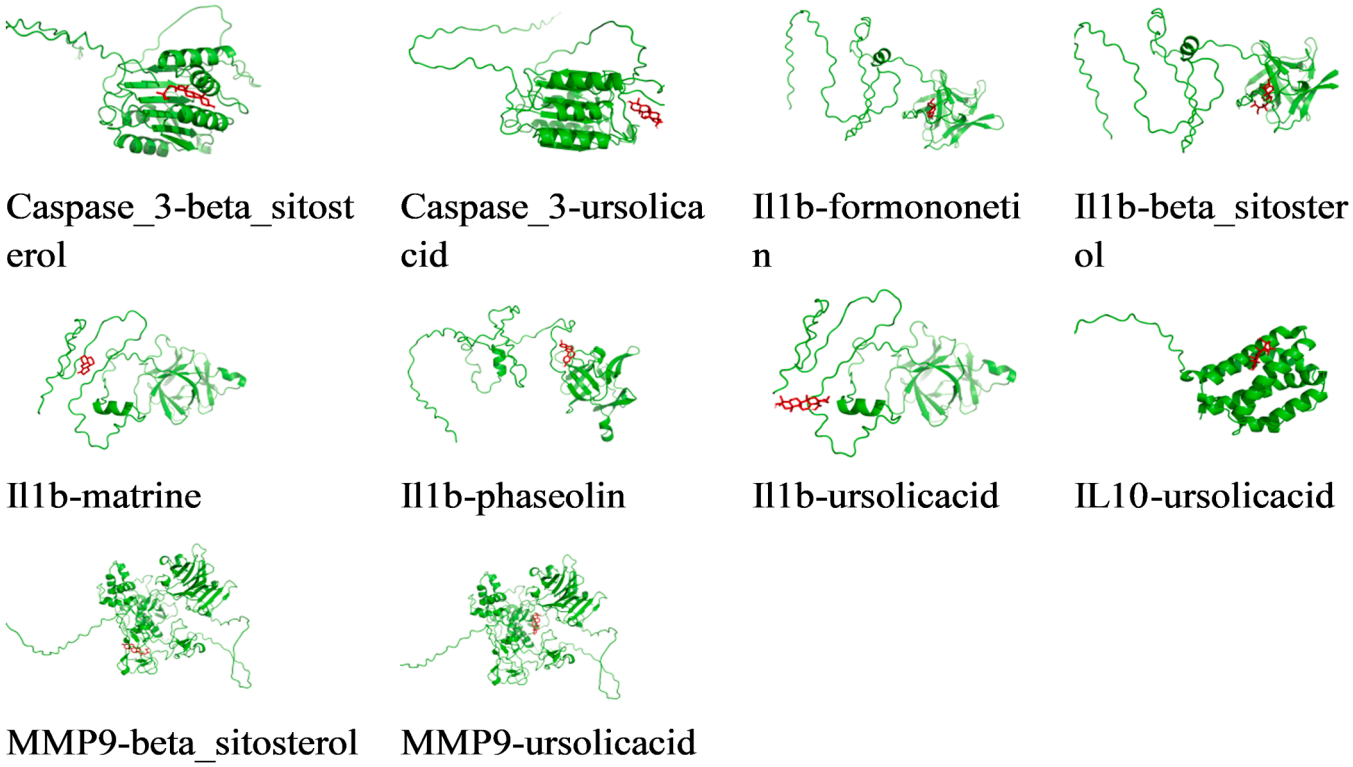

Fig. 5. . 3D pattern of docking between key targets and key component molecules. The color red signifies drug molecules, while green indicates protein molecules.

combination was observed with IL-1β, IL-10, MMP9, STAT3, and TP53.

The 2D analysis of the top 5 combinations with the lowest binding energy reveals specific interactions between the ligands and the target proteins was illustrated in Fig. 6. For example, in the case of beta-Sitosterol, the hydroxyl group forms a hydrogen bond with Ser106A of IL-1β, and hydrophobic bonds with Lys210A, Leu113A, Kys209A, and Ile119A. Ursolic acid forms hydrogen bonds with Ser205A of Caspase_3, and hydrophobic bonds with Phe256A, Trp206A, Ser251A, and Phe250A. It also forms a hydrogen bond with Leu73A of IL-1β and hydrophobic bonds with Trp74A, Phe23A, and Glu71A. In addition, Ursolic acid forms a hydrogen bond with Gln122 of IL-10 and hydrophobic bonds with Arg125 and His32. It also forms a hydrogen bond with Thr183 and Tyr193 of TNF, and hydrophobic bonds with Cys148, Trp192, and Lys190.

Histopathological analysis of the colon tissues

To validate the effects of Compound Kushen Powder on the potential

Table 4
Docking results between potential targets and key ingredients.

No.	Ingredients	Proteins							
		Caspase_3	IL-1β	IL-10	MMP9	STAT3	TNF	TP53	VEGFA
1	Apigenin	−5.13	−5.75	−4.06	−4.35	−4.12	−5.16	−3.65	−3.55
2	Beta_sitosterol	−6.57	−9.28	−5.92	−6.65	−7.14	−6.06	−6.09	−5.65
3	Ellagicacid	−4.51	−5.36	−4.3	−3.95	−3.82	−4.27	−3.28	−3.65
4	Formononetin	−5.13	−6.54	−5.06	−5	−4.51	−5.14	−4.36	−4.83
5	Fraxetin	−4.34	−4.84	−3.88	−4.69	−3.15	−4.17	−3.41	−3.16
6	Glyceollin	−5.49	−6.49	−5.41	−5.2	−5.43	−5.4	−5.31	−4.76
7	Kushenol	−3.53	−5.19	−3.29	−2.18	−2.34	−3.33	−2.33	−3.73
8	Methylgallate	−3.32	−3.61	−3.17	−2.18	−2.67	−3.08	−2.83	−2.57
9	Phaseolin	−5.69	−6.59	−5.87	−5.64	−4.89	−5.46	−5.96	−5.32
10	Ursolicacid	−7.52	−8.03	−8.24	−7.14	−7.39	−7.94	−6.96	−5.99
11	Punicalagin	−5.78	−5.66	−3.2	−1.13	−0.7	−1.8	−1.62	−3.71
12	Oxymatrine	−5.98	−7.45	−5.49	−6.75	−6.3	−5.19	−6.26	−4.29
13	Esculin	−2.85	−4.9	−3.16	−2.56	−2.48	−1.13	−1.55	−1.98
14	Esculetin	−4.34	−5.16	−4.55	−4.5	−4.3	−4.23	−4.38	−3.77
15	Dihydroxymatrine	−5.72	−6.46	−5.5	−5.82	−5.27	−5.27	−5.13	−5.07
16	Alpha-hydroxymatrine	−5.42	−7.1	−5.96	−5.51	−6.13	−5.5	−4.8	−4.75
17	Matrine	−6.11	−6.76	−6.18	−6.06	−6.13	−5.67	−5.84	−5.3
18	Kaempferol	−4.24	−4.96	−3.95	−5.25	−3.88	−3.68	−3.73	−3.34
19	Luteolin	−5.31	−5.83	−3.99	−3.57	−4	−3.88	−3.61	−3.76
20	Quercetin	−3.77	−4.68	−2.92	−2.64	−3.47	−3.29	−2.92	−2.81
21	Caffeicacid	−3.12	−3.92	−2.92	−3.89	−3.78	−3.16	−4.4	−3.08

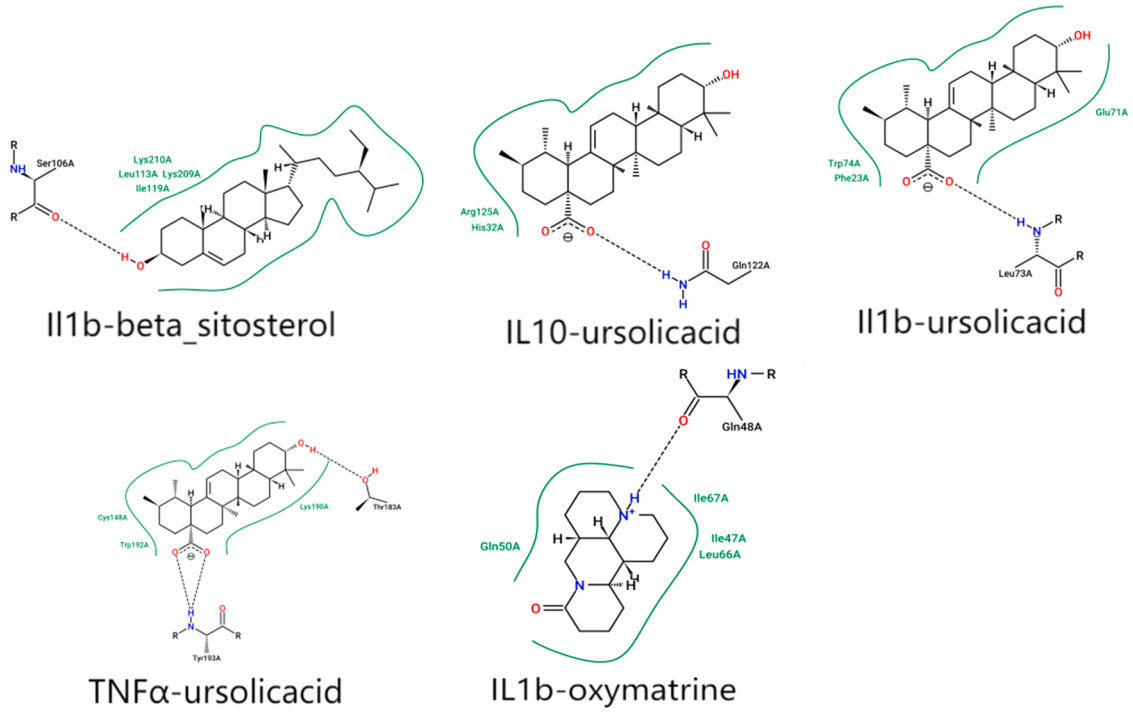

Fig. 6. . 2D pattern of top 5 docking results between key targets and selected component molecules.

key targets identified through network analysis, a rat diarrhea model induced by folium sennae leaves was established. As shown by the HE staining results, the colon tissues of rats in the negative control group were well-organized with intact structures (Fig. 7a), and no lesions were observed. In the diarrhea-induced rats (positive control group), local mucosal epithelial cells of the colon tissues were shed, the number of lymphocytes increased, the intestinal gland were necrotic and loosely arranged, and the fibrous tissues proliferated and hypertrophied (Fig. 7b). The pathological conditions of the colon tissues were significantly improved with no characteristic lesions found in the positive drug group (Fig. 7c) and the Compound Kushen Powder group (Fig. 7d), indicating favorable therapeutic effects.

qPCR

In comparison to the positive control group, the mRNA expression levels of TNF-α, IL-1β, TP53, CASP3, MMP9, and VEGFA genes in the colon tissues of the Compound Kushen Powder group, positive drug treated group, and negative control group were significantly reduced ($p < 0.01$), as shown in Fig. 8 (a, b, d, e, g, h). The mRNA expression level of IL-10 was significantly increased ($p < 0.01$), as shown in Fig. 8(c). In comparison to the positive control group, there was no significant difference in the mRNA expression level of STAT3 gene in the Compound Kushen Powder group and the positive drug control group ($p > 0.05$), while the mRNA expression level of STAT3 in the negative control group was significantly lower than that in the positive control group ($p <$

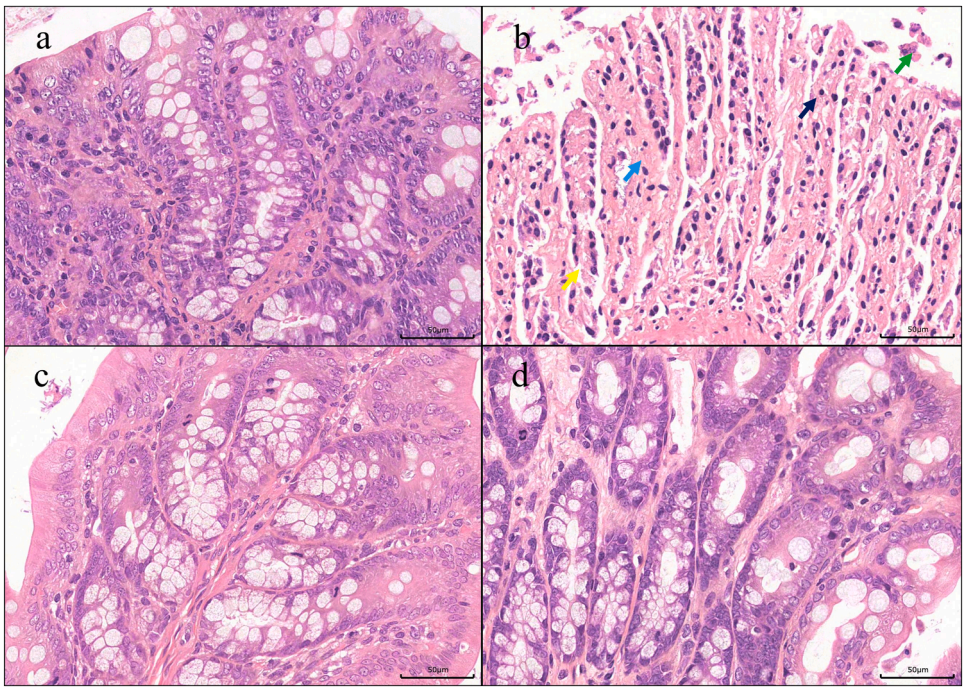

Fig. 7. Representative Hematoxylin and eosin (H&E) stains of rat colon tissues. **a**: Negative control group; **b**: Positive control group; **c**: Positive drug treated group; **d**: Compound Kushen Powder treated group. Black arrow: increased number of lymphocytes; yellow arrow: intestinal gland necrosis; green arrow: mucosal epithelium shedding; blue arrow: Fibrous tissue hyperplasia. Scale bars: 50 μm.

0.01), as shown in Fig. 8(f).

When compared to the negative control group, the mRNA expression levels of TP53, CASP3, MMP9, and VEGFA genes in the Compound Kushen Powder group and the positive drug treatment group showed no significant difference ($p > 0.05$), as shown in Fig. 8 (d, e, g, h). The mRNA expression level of STAT3 was significantly increased ($p < 0.01$), as shown in Fig. 8(f). In comparison to the negative control group, the TNF-α gene mRNA expression level was significantly increased ($p < 0.05$) in the positive drug treatment group, as shown in Fig. 8(a), while the IL-10 gene mRNA expression level was significantly decreased ($p < 0.05$), as shown in Fig. 8(c). When compared to the negative control group, the IL-1β gene mRNA expression level was significantly increased ($p < 0.05$) in the Compound Kushen Powder group, as shown in Fig. 8 (b).

In comparison to the positive drug treatment group, the mRNA expression level of MMP9 in the Compound Kushen Powder group was significantly decreased ($p < 0.05$), as shown in Fig. 8. There were no significant differences in the mRNA expression levels of TNF-α, IL-1β, IL-10, TP53, CASP3, STAT3, and VEGFA genes ($p > 0.05$).

Western blot

The results of protein expression blots are presented in Fig. 9 and Fig. 10. When compared to the positive control group, the levels of TNF-α, IL-1β, TP53, CASP3, MMP9 and VEGFA protein expression in the colon tissues of the Compound Kushen Powder group, positive drug control group, and negative control group were significantly reduced ($p < 0.01$) or significantly decreased ($p < 0.05$), as depicted in Fig. 10 (a, b, d, e, g, h). However, for STAT3 in the colon tissues of the Compound Kushen Powder group, positive drug control group, there was no difference as compared to that in the positive control group (Fig. 10f). When compared to the positive control group, the levels of IL-10 protein expression in the Compound Kushen Powder group, positive drug control group, and negative control group were significantly elevated ($p < 0.05$), as shown in Fig. 10(c).

Discussion

Sophora flavescens, cortex fraxini, and pomegranate peel have been traditionally used for the prevention and treatment of animal diarrhea, but most studies have been limited to their extracts on antimicrobial and immunology research (Guo, 2017). According to the results of network pharmacology, we identified eight indices closely associated with diarrhea in this compound, such as TNF-α, IL-10, IL-1β, TP53, CASP3, STAT3, MMP9, and VEGFA, all of which are intricately linked to immunity. The results of molecular docking revealed that Ursolic acid and beta_sitosterol exhibit the strongest binding capabilities with these indices, indicating that these primary components are involved in the inhibition of diarrhea. The top five docking combinations include Ursolic acid with Caspase_3 (docking score −7.52), IL-1β (docking score −8.03), IL-10 (docking score −8.24), and TNF (docking score −7.94), and beta_sitosterol with IL-1β (docking score −9.28). Ursolic acid has proven efficacious in the treatment of diarrhea by affecting changes in the intestinal flora (Navabharath et al., 2023). These changes correlate with a substantial reduction in the expression of proinflammatory cytokines, including IL-1β, TNF, IL-10, and MMP9 (Cho et al., 2015; Chun et al., 2014; Xu et al., 2022).

These findings suggest that while Compound Kushen Powder influences the expression of several key genes and proteins associated with inflammation and immune response in diarrhea, it does not significantly affect the expression level of the STAT3 protein. This is particularly noteworthy as STAT3 plays a critical role in maintaining the intestinal mucosal barrier and its activation can enhance the production of inflammatory cytokines. The predictive results of the key target STAT3 in your study were inconsistent with experimental validation. This discrepancy may be due to network pharmacology's theoretical model-based predictions, which can lead to false positives. Additionally, the regulation of gene and protein expression by various factors could also contribute to this inconsistency. Chen et al.'s (Chen et al., 2022) study on sophocarpine's effects on DSS-induced intestinal epithelial cell damage found that sophocarpine could reduce inflammatory cytokine release and inhibit p-STAT3 protein elevation, but did not affect STAT3

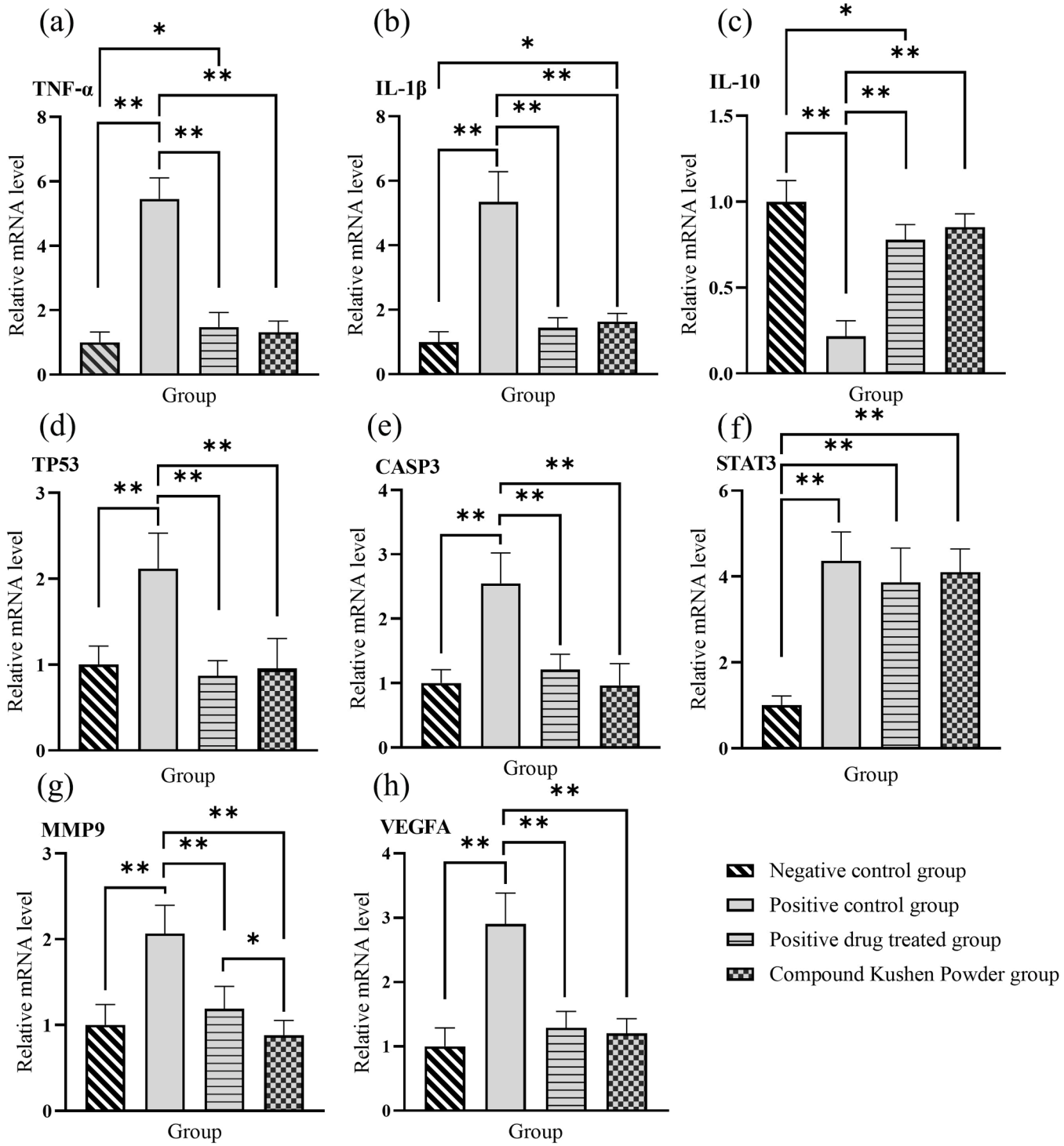

Fig. 8. . Effects of the Compound Kushen Powder on the mRNA expression of **(a)** TNF-α, **(b)** IL-1β, **(c)** IL-10, **(d)** TP53, **(e)** CASP3, **(f)** STAT3, **(g)** MMP-9, and **(h)** VEGFA in the colon of diarrhea induced rats (mean ± SD). Note: **, $P < 0.01$; *, $P < 0.05$.

protein expression. This suggests the importance of further research on Compound Kushen Powder's influence on p-STAT3 protein expression in colon tissue following STAT3 protein activation. Wang et al.'s (Wang et al., 2019) research on grape seed polyphenols demonstrated that high-dose treatment significantly downregulated the gene mRNA expression of inflammatory cytokines and the phosphorylation level of STAT3. This indicates that Compound Kushen Powder's effect on STAT3 gene mRNA and protein expression in colon tissue might be dose-dependent. Therefore, the results suggest the potential effectiveness of Compound Kushen Powder in controlling intestinal inflammation in diarrhea, specifically through its impact on various inflammatory cytokines and proteins, but not significantly on STAT3 protein expression.

Elevated levels of MMP9 have been linked to increased intestinal permeability, a critical factor in the development of diarrhea in various animal models (Bai et al., 2020; Derkacz et al., 2021). MMP9 also plays a significant role in the migration of inflammatory cells to the intestinal mucosa, thereby exacerbating the inflammatory response associated

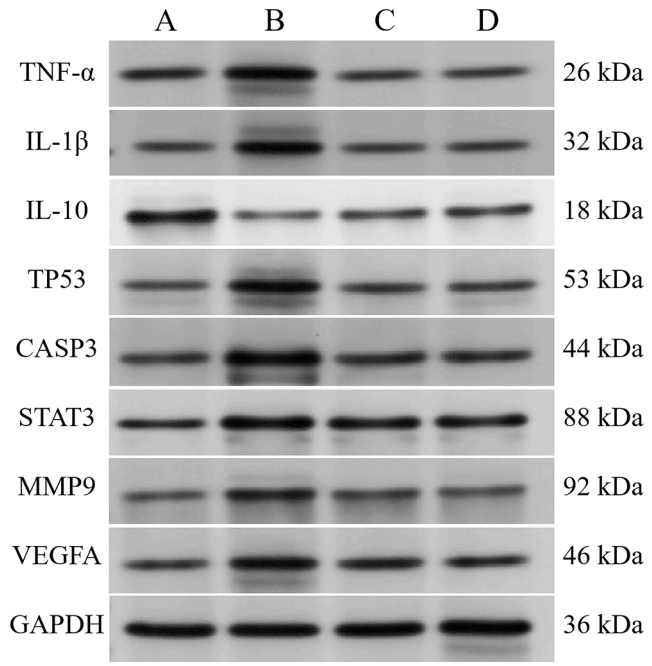

Fig. 9. . Effect of the Compound Kushen Powder on the expression of TNF-α, IL10, IL1β, TP53, CASP3, STAT3, MMP9, and VEGFA proteins in the colon of rats with Folium sennae induced diarrhea (Western blot). **A**: Negative control group; **B**: Positive control group; **C**: Positive drug treated group; **D**: Compound Kushen Powder treated group.

with diarrhea (Derkacz et al., 2021). The degradation and remodeling of the extracellular matrix are primarily facilitated by MMP9, making it a key player in the pathogenesis of diarrhea. In the context of Compound Kushen Powder, the study's findings reveal a significant impact on MMP9 expression. The qPCR results indicated a significant decrease in MMP9 mRNA gene expression in the colon tissues treated with Compound Kushen Powder compared to the positive control group. Furthermore, Western blot analysis showed a notable reduction in MMP9 protein expression in the Compound Kushen Powder treatment group, suggesting that the powder can effectively downregulate MMP9 protein expression. This downregulation is crucial as it indicates the potential of Compound Kushen Powder to inhibit the development of inflammation and tissue damage commonly seen in diarrheal conditions. Previous research has highlighted the anti-inflammatory effects of certain compounds on MMP9 expression (Kou et al., 2020). For instance, luteolin and kaempferol have been found to inhibit MMP9 expression, thus suppressing inflammation and tissue damage. Luteolin achieves this by inhibiting the NF-κB signaling pathway, while kaempferol decreases MMP9 expression and its associated biological effects (Cheng et al., 2019; Mahdiani et al., 2022). These findings align with the results of the current study, suggesting that the active ingredients in Compound Kushen Powder, may exert similar inhibitory effects on MMP9 expression. Furthermore, the study also reported on the IL-1β-induced increase in the permeability of intestinal epithelial tight junctions during diarrhea, leading to cell apoptosis and tissue damage. In this regard, caspase3, a protein involved in apoptosis, showed a significant increase in expression in the Compound Kushen Powder treatment group. Quercetin, a key component of Compound Kushen Powder, has been shown to regulate the expression of proteins such as CASP3 and MMP9, modulating inflammation and apoptosis in colon tissue.

VEGFA, or vascular endothelial growth factor A, is a pivotal angiogenic factor known to promote the proliferation and differentiation of vascular endothelial cells, thereby enhancing vascular permeability and exacerbating the occurrence of inflammation (Melincovici et al., 2018). VEGFA plays a critical role in the regulation of angiogenesis and vascular permeability (Góra-Tybor et al., 2015). Studies by Luo et al (Luo et al., 2020) have indicated that inhibiting the expression of VEGFA protein can ameliorate the inflammatory response in porcine intestinal epithelial cells induced by Clostridium perfringens beta-2 toxin (CPB2). Currently, the active components of Compound Kushen Powder, primarily represented by matrine and oxymatrine, are focused on cancer cell research due to their ability to downregulate the expression of VEGFA protein. Chen et al. (Chen et al., 2013) showed that oxymatrine as the main alkaloid component in Sophora flavescens Ait can suppress the proliferation of human pancreatic cancer and pancreatic cancer xenograft tumors in nude mice by downregulating the expression of the NF-κB-mediated VEGF signaling pathway. Our findings are in line with prior research demonstrating that suppressing VEGFA expression can mitigate inflammation in intestinal cells. This suggests that the key constituents of Compound Kushen Powder, particularly matrine and oxymatrine, may regulate VEGFA expression. Our study provides consistent experimental evidence that Compound Kushen Powder can reduce the mRNA and protein levels of VEGFA in the colon tissues of rats. This reduction may lead to decreased microvascular damage and vascular permeability, thus alleviating inflammation in intestinal cells induced by the diarrhea model. These outcomes validate and substantiate previous research on the regulatory impact of Compound Kushen Powder on VEGFA expression.

TNFα is a potent pro-inflammatory cytokine involved in the initiation and amplification of the inflammatory response (Silva et al., 2022). Increased TNFα and MMP9 expression has been observed in various experimental models of diarrhea, and it can disrupt the intestinal barrier and induce apoptosis in intestinal epithelial cells, contributing to the pathogenesis of diarrhea (Moore et al., 2011; Yamane et al., 2005). IL-10 is an anti-inflammatory cytokine that acts to suppress inflammatory responses, and IL-10 plays a dual role in the context of diarrhea (Zhu et al., 2017). On one hand, IL-10 can protect against excessive inflammation and tissue damage. On the other hand, elevated IL-10 levels may also contribute to the persistence of infection and delay in pathogen clearance (Saraiva & O'Garra, 2010). The balance between the inflammatory factors TNF and IL-10 contributes to the modulation of immune system activity, preventing excessive inflammatory response, and fostering the well-being of intestinal epithelial cells (Waehre et al., 2002). Additionally, the elevated IL-1β levels in this diarrhea model may be attributed to pathogen-specific diarrhea (Villafuerte Gálvez et al., 2022). It is reported that matrine, esculin, luteolin, quercetin, and punicalagin have been found to possess robust anti-inflammatory properties. Their primary mechanism of action involves suppressing the secretion of TNF-α and IL-1β, thereby effectively reducing the inflammatory response. This reduction is achieved through an increase in the level of IL-10 when compared to the positive control group after treatment with either the positive drug or the Compound Kushen Powder, as demonstrated by the results of this study ($p < 0.05$, Fig. 7 and 9). As a tumor suppressor gene p53 in regulating cell cycle arrest, apoptosis, and DNA repair, TP53 mutations have been linked to various gastrointestinal disorders, including inflammatory bowel disease (IBD) and diarrhea (Du et al., 2017; Wang et al., 2021), while CASP3 is a protease that promotes apoptosis by inducing DNA fragmentation. Both TP53 and CASP3 are apoptotic markers closely associated with diarrhea, as excessive apoptosis of colonic epithelial cells can lead to damage to the intestinal mucosa (Bowen et al., 2014). In addition, in vitro experiments demonstrated that CASP3, identified as a key molecular target of Compound Kushen Injection, whose main active compounds were matrine, oxymatrine, sophoridine, and N-methylcytisine, exhibited anti-cancer effects on human hepatocellular carcinoma (Gao et al., 2018).

Conclusion

This study focused on the traditional use of Sophora flavescens, cortex fraxini, and pomegranate peel for the prevention and treatment of animal diarrhea, primarily through their extracts. Our research

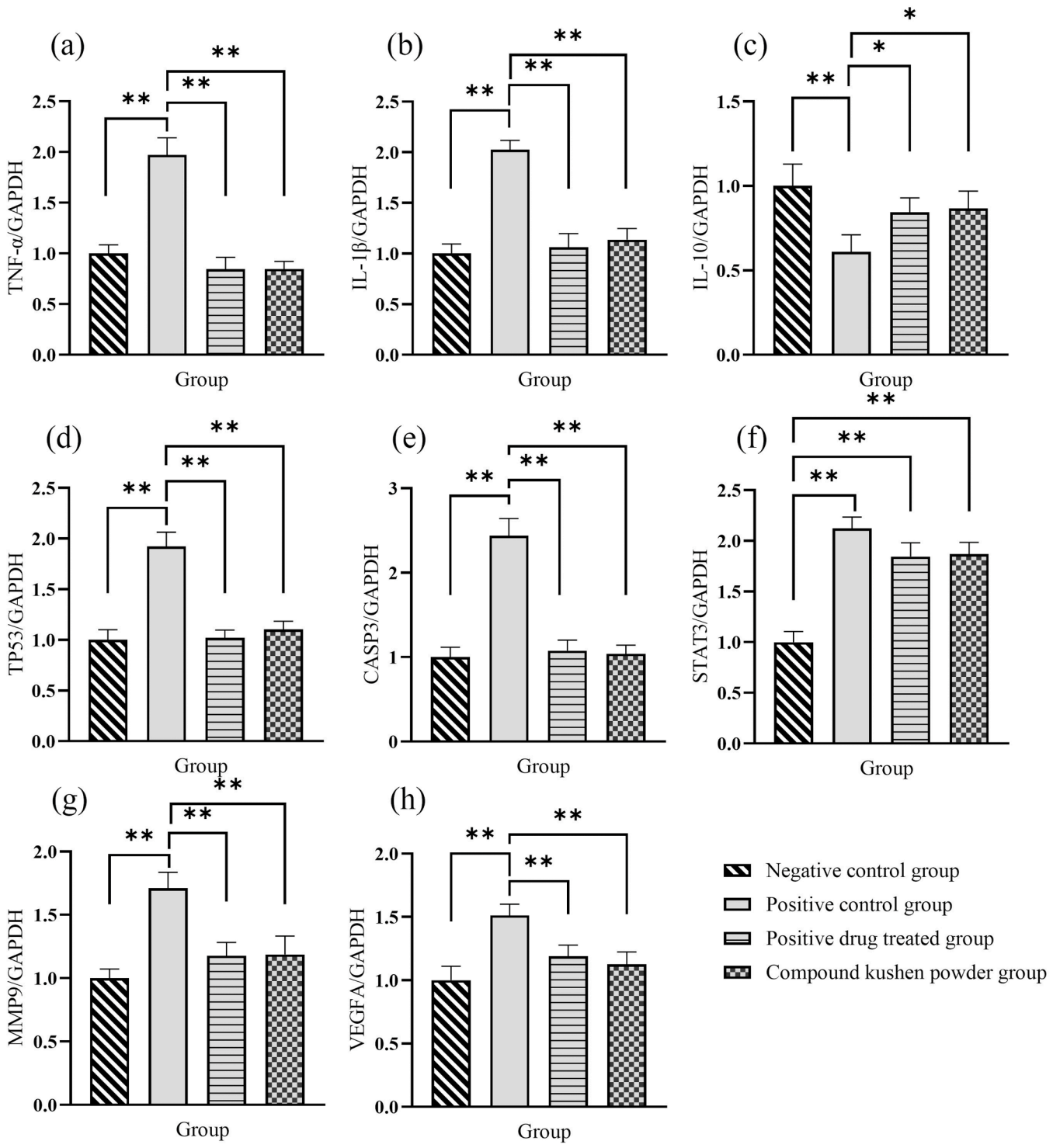

Fig. 10. . Effect of the Compound Kushen Powder on the expression of **(a)** TNF-α, **(b)** IL-1β, **(c)** IL-10, **(d)** TP53, **(e)** CASP3, **(f)** STAT3, **(g)** MMP-9, and **(h)** VEGFA proteins in the colon of rats with Folium sennae induced diarrhea (mean ± SD). Note: **, $P < 0.01$; *, $P < 0.05$.

identified key targets such as TNF, IL-10, IL-1β, TP53, STAT3, CASP3, MMP9, and VEGFA, involved in anti-inflammatory responses. Molecular docking scores highlighted core active ingredients like beta-sitosterol, ursolic acid, and matrine derivatives, each with distinct mechanisms targeting various pathways. However, these findings will require in vitro assays to further confirm target binding and pathway regulation. Overall, this study underscores the potential of these traditional Chinese medicine compounds to regulate diarrheic inflammation. Further validation through functional assays, pharmacokinetic studies, and clinical trials in pigs will be essential to advance these findings toward practical therapeutic alternatives for diarrhea-related conditions.

Ethical statement

All animal procedures were in compliance with the Chinese Animal Welfare Law and were approved by the Experimental Animal Ethics Committee of Yangzhou University (Approval number: SYXK 2022–0011).

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Availability of data and materials

The data generated for this study are available within the article or on request to the corresponding author.

CRediT authorship contribution statement

Bo Yu: Writing – review & editing, Writing – original draft, Conceptualization. **Yuanfeng Zhao:** Writing – review & editing, Writing – original draft, Visualization, Data curation. **Lingling Jiang:** Investigation. **Jingrui Zhou:** Investigation. **Haoxiang Xu:** Methodology. **Lu Lei:** Methodology. **Longxin Xu:** Resources. **Xin Wang:** Data curation. **Shijin Bu:** Writing – review & editing, Supervision, Resources, Conceptualization.

Declaration of competing interest

The authors declare that they have no competing interests.

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Not applicable.

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