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ORIGINAL RESEARCH

Network Pharmacology and Experimental Validation Reveal Sishen Pill's Efficacy in Treating NSAID-Induced Small Intestinal Ulcers

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Purpose: Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used but often cause small intestinal ulcers (SIUs), for which effective therapies are lacking. Sishen Pill (SSP), a traditional Chinese medicine, shows therapeutic promise, yet its mechanisms remain unclear. This study integrates network pharmacology, molecular docking, and experimental validation to systematically investigate SSP's protective mechanisms against NSAID-induced SIUs.

Patients and Methods: Active SSP ingredients were screened using the Traditional Chinese Medicine Systems Pharmacology (TCMSP) and Encyclopedia of Traditional Chinese Medicine (ETCM) databases. SIU-related targets were retrieved from GeneCards and DisGeNET. Protein-protein interaction (PPI) networks were constructed via STRING and Cytoscape, followed by Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses. Molecular docking (AutoDock Vina, PyMOL) validated ligand-target interactions. In vivo validation employed an indomethacin-induced SIU rat model to assess SSP's effects on ulcer severity, inflammation, oxidative stress, and PI3K/AKT signaling.

Results: We identified 66 bioactive SSP ingredients, 222 drug targets, and 144 SIU-related targets. Molecular docking revealed high binding affinity of SSP components (quercetin, bavachinin, rutaecarpine, evodiamine) to key targets (AKT1, HSP90AA1, IL6, MAPK1, BCL2). KEGG analysis highlighted the PI3K/AKT pathway as central. In vivo, SSP reduced ulcer indices, suppressed pro-inflammatory cytokines (TNF-α, IL-1β, IL-6), and attenuated oxidative stress. SSP also downregulated PI3K and AKT1 mRNA expression, confirming pathway modulation.

Conclusion: This study elucidates SSP's multi-target mechanism against NSAID-induced SIUs, emphasizing its role in suppressing inflammation, oxidative stress, and PI3K/AKT signaling. These findings provide a scientific foundation for SSP's clinical application and highlight its potential as a safe, effective alternative to conventional therapies.

Keywords: nonsteroidal anti-inflammatory drugs, Sishen Pill, small intestine ulcers, network pharmacology, inflammation

Introduction

Medication within the class of Nonsteroidal Anti-inflammatory Drugs (NSAIDs) is extensively acknowledged and employed for its efficacy in analgesic, antipyretic, and mitigating inflammation. [1] However, their prolonged use is associated with various gastrointestinal complications. Mucosal injury induced by NSAID, characterized by inflammation and ulceration, can manifest in both the stomach and proximal duodenum, as well as in the more distal sections of the small intestine. [2,3] The occurrence of NSAIDs induced small intestinal ulcers (SIUs) has seen an upward trend, largely due to the widespread utilization of NSAIDs in chronic pain management, especially among the elderly population. The increase in detection rate is also attributed to the introduction of advanced endoscopic methods, such as capsule endoscopy and double-balloon enteroscopy, which facilitate direct observation of the small intestine. [4,5]

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The precise etiology underlying NSAID-induced SIUs has yet to be fully determined, although numerous theories have been postulated. A widely recognized hypothesis posits that NSAIDs suppress the activity of the cyclooxygenase (COX) enzyme, thereby impeding the synthesis of prostaglandins essential for safeguarding the gastrointestinal mucosa.[6,7] Emerging studies have implicated the gut microbiome in the pathogenesis of SIUs.[8] The existing therapeutic strategies for NSAID-induced SIUs are constrained, primarily consisting of NSAID withdrawal or the administration of proton pump inhibitors (PPIs). However, these treatments do not directly target the small intestine and have been shown to be effective primarily for gastric ulcers.[9] Indeed, these drugs could potentially exacerbate NSAID-induced SIUs by altering the gut microbiota composition.[10] Thus, there exists an urgent requirement to devise alternative treatment approaches for managing NSAID-induced SIUs. Indomethacin (INDO), a type of NSAIDs, is frequently utilized to induce small intestinal ulcers in animal models due to its potent ulcerogenic effect.[11]

The Sishen Pill (SSP), a medicinal formulation from traditional Chinese medicine (TCM), has been extensively employed in the therapeutic management of gastrointestinal disorders, including chronic diarrhea, irritable bowel syndrome (IBS), non-specific colitis, among others.[12] The therapeutic efficacy of SSP is attributed to its multiple components which have diverse biological activities. Recent scientific investigations have validated the clinical benefits of SSP in gastrointestinal health. Studies have demonstrated that SSP exhibits significant protective effects against colitis, mainly through its antioxidant, anti-inflammatory, and cytoprotective activities.[13,14] In addition, SSP has been reported to regulate gut microbiota, enhance intestinal barrier function, and modulate immune responses, thereby improving overall gut health.[15,16] However, the therapeutic potential of SSP in addressing NSAID-induced SIUs has not been extensively researched. The objective of this research is to bridge the existing knowledge gap by examining the possible ameliorative impact of SSP on indomethacin-induced SIUs.

Network pharmacology represents a novel methodology for probing the potency and therapeutic mechanisms underlying TCM.[17] It elucidates the complex web of interactions among various constituents of the medication and their molecular targets, thereby illuminating the cooperative and integrative therapeutic mechanisms characteristic of TCMs.[18] Employing this methodology offers a comprehensive framework for assessing the therapeutic efficacy and intrinsic mechanisms of SSP on indomethacin-induced SIUs.

In this study, we utilized network pharmacology to explore the molecular targets and related signaling pathways of SSP in the treatment of SIUs. Initial validation was accomplished through molecular docking. Subsequently, utilizing a range of experimental methodologies, the influence of SSP in treating NSAID-induced SIUs was confirmed through in vivo experiments. Our findings are expected to provide a theoretical foundation for the therapeutic efficacy of SSP in SIUs.

Materials and Methods

Integrative Network Pharmacology Investigation

Identification of Bioactive Compounds from Sishen Pill (SSP)

Sishen Pill was composed of 6 herbs, including Buguzhi (*Psoralea corylifolia* L.), Wuzhuyu (*Evodiae Fructus*), Roudoukou (*Myristica fragrans Houtt)*., Wuweizi (*Schisandrae Chinensis Fructus*), Shengjiang (*Zingiber officinale Rosc)*., and Dazao (*Jujubae Fructus*). The active compounds of Buguzhi (*Psoralea corylifolia* L.) was retrieved from ETCM Database, accessible at http://www.tcmip.cn/ETCM2/front/ (accessed on 25 July 2024). For the other five TCMs, active constituents were sourced from Traditional Chinese Medicine System Pharmacology Database and Analysis Platform (TCMSP, https://www.tcmsp-e.com/, accessed on 26 July 2024), with selection criteria including oral bioavailability (OB) $\geq$ 30% and drug-likeness (DL) $\geq$ 0.18.

Identification of Target Proteins for Bioactive Compounds

The active components discussed in Section 2.1.1 were employed to explore their prospective protein targets through the TCMSP database. Subsequently, the associated human genes were retrieved from the UniProt database. Complementary data was further supplemented using resources from the PubChem database.

Acquisition of Small Intestinal Ulcers-Related Gene Targets

To identify gene targets associated with small intestinal ulceration (SIU), we utilized the GeneCards database (http://www.genecards.org, accessed on 27 July 2024),[19] employing the keyword "ulceration of small intestine". Targets exhibiting relevance scores exceeding twice the median value were chosen for further analysis. Complementary data were also extracted from the DisGeNET database (https://www.disgenet.com/, accessed on 28 July 2024) to enhance the robustness of our target identification process.

Development and Examination of the Protein-Protein Interaction (PPI) Network

Genes pivotal for the construction of a PPI network were identified by isolating the shared targets between SSP and SIUs, using the Venny online tool. The subsequent construction of the PPI network for these shared targets was facilitated by the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING), available at https://string-db.org/ and accessed on 28 July 2024.[20] This comprehensive database, which encompasses both established and predicted protein interactions, served as a resource for exploring the interrelationships among the identified genes. The study focused on proteins from the species "Homo sapiens", with a minimum confidence threshold established at 0.9. Concurrently, free nodes were excluded to streamline the PPI network visualization. The constructed protein-protein interaction network was then uploaded into Cytoscape software (version 3.10.1) for in-depth analysis. Through the analysis of network topology parameters, the central targets were identified, visualized, and characterized.

Construction of the Bioactive Compounds-Target-Disease Network

For the creation of a comprehensive network visualization that encompasses compounds, their molecular targets, and associated diseases, we utilized Cytoscape version 3.10.1 to import data concerning active constituents and drug-disease intersection targets. Network analysis was conducted utilizing the Network Analyzer plugin, with bioactive constituents and their molecular targets visualized as nodes within the network. Connections representing interactions between these active components and shared targets were illustrated as edges.

Enrichment Analysis of GO Function and KEGG Pathways

Utilizing the DAVID bioinformatics resource (https://david.ncifcrf.gov/home.jsp/, accessed on 29 July 2024), the target genes under investigation were analyzed for GO functional categories and KEGG pathway enrichment. For visualization, we selected GO terms and pathways that were enriched, with a significance threshold of p-values less than 0.05. Bar graphs representing GO enrichment and bubble graphs depicting KEGG pathway enrichment were generated using the website of Microbiotics (https://www.bioinformatics.com.cn, accessed on 1 August 2024).

Molecular Docking

The Network Analyzer plugin was utilized to calculate degree values within the Bioactive Compounds-Target-Disease network, which helped identify the principal bioactive constituents for molecular docking with small ligands. Key molecular targets were determined by analyzing the PPI network. The Protein Data Bank (PDB, https://www.rcsb.org/, accessed on 9 August 2024) was consulted to retrieve relevant protein structures. The preparation of small molecules were conducted using PyMOL, and AutoDock was applied to incorporate hydrogen atoms and to produce output files in the pdbqt format for the ligand. Active ingredient files were sourced from TCMSP. Molecular docking was conducted using AutoDock Vina 1.5.7. The PyMOL was utilized to visualize the docking outcomes for various targets and proteins.

In vivo Experiments

Animals

Male Sprague-Dawley (SD) rats with a weight range of 200–250 g (n = 20) were procured from the Shanghai Laboratory Animal Center (SLACCAS Laboratory Animal Inc., Shanghai, China). The supplier's license number is SCXK (Hu) 2019–0005. The rats were maintained at the Animal Center of the International Institute of Medicine, Zhejiang University, under controlled conditions. The animal housing facility maintained a temperature range of 22°C ± 2°C, complemented by a 12-hour alternating light and darkness cycle facilitated by artificial lighting sources. All animals were allowed to acclimate for one week upon arrival before initiating any experimental procedures. All animal-related

experimental procedures were carried out following the protocols outlined by the Experimental Animal Ethics Committee of Zhejiang University School of Medicine (ZJU20240649).

Drugs

The Sishen Pill (lot: 17080051) was procured from Tong Ren Tang Natural Medicine Co. Ltd., (Beijing, China). The composition included Buguzhi (*Psoralea corylifolia* L.), Wuzhuyu (*Evodiae Fructus*), Roudoukou (*Myristica fragrans Houtt)*., Wuweizi (*Schisandrae Chinensis Fructus*), Shengjiang (*Zingiber officinale Rosc)*., and Dazao (*Jujubae Fructus*). These were formed into pills following the dose ratio of 1:2:4:2:2:2, respectively (100, 200, 400, 200, 200, and 200 g). Although our study primarily relied on network pharmacology to predict the active constituents of SSP, we did not perform our own HPLC analysis. Instead, we refer to the study by Zhang,[21] and Hu,[22] which employed a robust HPLC method to characterize the chemical profile of SSP in its traditional pharmaceutical form. Indomethacin (HY-14397) was purchased from MCE (MedChemExpress, Shanghai, China).

Establishment of Small Intestinal Ulcers

Following a week of acclimatization at the animal center, the laboratory animals were randomly segregated into four distinct groups: Control, Indomethacin (INDO), INDO + SSP (2.5 g/kg), and INDO + SSP (5 g/kg) - each comprising five animals. Indomethacin at 6 mg/kg/day, dissolved in a 5% Na_2CO_3 solution, was administered to the INDO and INDO + SSP groups for seven consecutive days to induce enteropathy, while the Control group received the same volume of the Na_2CO_3 solution alone, as outlined in a previous study.[23] The previously specified dose of SSP was administered intragastrically once daily for five consecutive days once the small intestinal ulceration model had been successfully created. Concurrently, a comparable volume of distilled water was administered orally to rats in the INDO and the Control group. On the 13th day, following an eight-hour fasting period, Carbon dioxide-induced asphyxiation was employed for the humane euthanasia of the animals. Subsequently, blood was executed via cardiac puncture. After careful dissection, the small intestine was soaked in phosphate-buffered saline pH 7.4, rinsed, and finally weighed for analysis. The jejunoileal segment was sectioned into two parts. One section was rapidly frozen using liquid nitrogen, and conserved at a temperature of −80°C for subsequent analysis. Hematoxylin and Eosin (H&E) staining was performed on the remaining segment after it had been fixed in a 10% formalin solution and embedded in paraffin.

Assessment of Ulcer Lesions

Independent evaluators examined small intestine ulcers with magnifying glasses to eliminate bias. The evaluation followed the criteria established by Cantarella et al.[24] Macroscopic lesions of the small intestine were graded from 0 to 5, based on injury severity and hemorrhage formation. The score of 0 indicated normal small intestine mucosa, 1, pinpoint erosions, 2, lesions less than 1 mm, 3, lesions between 1 and 2 mm, 4, lesions between 3 and 4 mm, and 5 lesions exceeding 4 mm. An ulcer index is calculated using the average ulcer score for each animal.

Hematoxylin-Eosin (HE) Staining

After collection, small intestine mucosa samples were stored in paraformaldehyde, then dehydrated with increasing alcohol concentrations. These samples were then rinsed in PBS buffer and then processed for paraffin embedding. Thereafter, sections of the embedded tissue, 4 micrometers in thickness, were prepared and underwent H&E staining. Histopathological alterations in the tissue sections were subsequently evaluated through microscopic observation.

Evaluation of Cytokines Through ELISA Methodology

An ELISA kit was used to measure the activity of IL-6, IL-1β, and TNF-α in rat serum, in accordance with the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Assessment of Myeloperoxidase (MPO) Activity and Oxidative Stress Indexes

The intestinal tissues of rats were analyzed for the activity of myeloperoxidase (MPO), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT), and the level of malondialdehyde (MDA) using specialized assay kits from Nanjing Jiancheng Bioengineering Institute, Nanjing, China.

qPCR Analysis

Isolation of RNA from the intestinal tissues was achieved employing the TRIzol reagent (Vazyme Biotech Co, Nanjing, China). Subsequently, the isolated RNA was subjected to reverse transcription to form complementary DNA (cDNA) utilizing the HiScriptIIQRT SuperMix reagent kit (Vazyme Biotech Co, Nanjing, China). The qPCR SYBR Green Master Mix (Vazyme Biotech Co, Nanjing, China) was used for the quantitative PCR. Table 1 details the primers for quantification of mRNA expression. Expression levels, represented as fold change, were determined applying the $2^{-\Delta\Delta Ct}$ method, employing β-actin as the housekeeping gene.[25] Primers were sourced from Generay Biotech Co., Ltd (Shanghai, China).

Statistical Analysis

We performed all statistical analyses with GraphPad Prism 8 (GraphPad Software, San Diego, California, USA). The multiple comparisons were assessed using a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. The results are presented as the mean values ± standard error of the mean (SEM), with significance determined at $p < 0.05$. To ensure robustness and reliability, each experiment was repeated at least three times.

Results

Outcomes of Pharmacoinformatics Analysis

Screen Active Ingredient of Sishen Pill and Targets of Small Intestinal Ulcers

Active ingredients in Sishen Pill (SSP) were identified through a search using the TCMSP and ETCM databases. Six herbs were identified from SSP: Buguzhi (*Psoralea corylifolia* L.), Wuzhuyu (*Evodiae Fructus*), Roudoukou (*Myristica fragrans Houtt)*., Wuweizi (*Schisandrae Chinensis Fructus*), Shengjiang (*Zingiber officinale Rosc)*., and Dazao (*Jujubae Fructus*). Employing OB ≥ 30% and DL ≥ 0.18 as screening parameters, 72 active compounds were found Supplementary Table 1). After eliminating duplicate compounds and those devoid of associated targets, a cohort of 66 active constituents remained. Notably, Zhang et al employed HPLC-ESI-MS/MS to quantitatively determine nine key bioactive compounds—deoxyschizandrin, γ-schizandrin, schizandrin, schizandrol B, schisantherin A, psoralen, isopsoralen, evodiamine, and rutaecarpine—in SSP of the same pharmaceutical form.[21] Furthermore, Hu[22] and Liu[26] utilized a robust HPLC method to characterize SSP's chemical profile in its traditional formulation. In their study, HPLC analysis successfully separated and identified major bioactive constituents, including bavachin, bavachinin, rutaecarpine, and evodiamine. This externally validated data reinforces our network

Table 1 Primers Utilized in the Current Investigation

Genes	Sequences (5′–3′)
TNF-α	F: CCCTCACACTCAGATCATCTTCT R: GCTACGACGTGGGCTACAG
IL-6	F: TAGTCCTTCCTACCCCAATTTCC R: TTGGTCCTTAGCCACTCCTTC
IL-1β	F: GCAACTGTTCCTGAACTCAACT R: ATCTTTTGGGGTCCGTCAACT
AKT1	F: ATGAACGACGTAGCCATTGTG R: TTGTAGCCAATAAAGGTGCCAT
PI3K	F: ACACCACGGTTTGGACTATGG R: GGCTACAGTAGTGGGCTTGG
β-Actin	F: GTCGTACCACTGGCATTGTG R: GCTGTGGTGGTGAAGCTGTA

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pharmacology predictions and confirms that the key constituents identified via the TCMSP and ETCM databases are indeed present in SSP.

The TCMSP database was engaged to forecast protein targets for the Sishen Pill's constituents. The nomenclature of all target genes was standardized to the official gene symbols from UniProt (UniProKB), with a specification for human genes. After removing duplicate target genes, the final count of unique therapeutic targets was 222.

A search was conducted in the GeneCards database using the keyword "ulceration of small intestine". Candidate targets were shortlisted based on relevance scores that surpassed twice the median value, and additional data were obtained from the DisGeNET database. In total, 2151 target genes related to small intestinal ulcerations (SIUs) were identified.

The selected compounds and target gene were progressively loaded into the Cytoscape to generate a "Drug-Active Ingredient-Target" network as illustrated in Figure 1. Comprising 294 nodes and 1230 edges, this network graphically depicted the interactions among various components and their associated targets.

Identification of Common Targets of SSP and SIUs and Establishment of a PPI Network

Employing a Venn diagram, we mapped the intersecting targets of SSP and SIUs, resulting in the discovery of 144 common targets (Figure 2A). The STRING database was utilized to construct a PPI network, with disconnected nodes were hidden. We applied a filter with a combined score ≥ 0.9 and downloaded the output file in tsv format. This file was uploaded into Cytoscape 3.10.1 for the creation of a pharmacological network and examine significant protein interactions. The final network comprised 130 nodes and 381 edges (Figure 2B). In order to validate the relationships among the bioactive components of SSP and their key targets, a "Bioactive Compounds-Target-Disease" network was developed (Figure 2C).

GO and KEGG Pathway Enrichment Analysis

The DAVID database was used to conduct GO and KEGG analyses on core targets. The GO analysis yielded 487 Biological Process (BP) terms, 68 Cellular Component (CC) terms, and 121 Molecular Function (MF) terms (Figure 3A, Supplementary Table 2). The GO enrichment analysis primarily identified biological processes, with a focus on apoptosis processes,

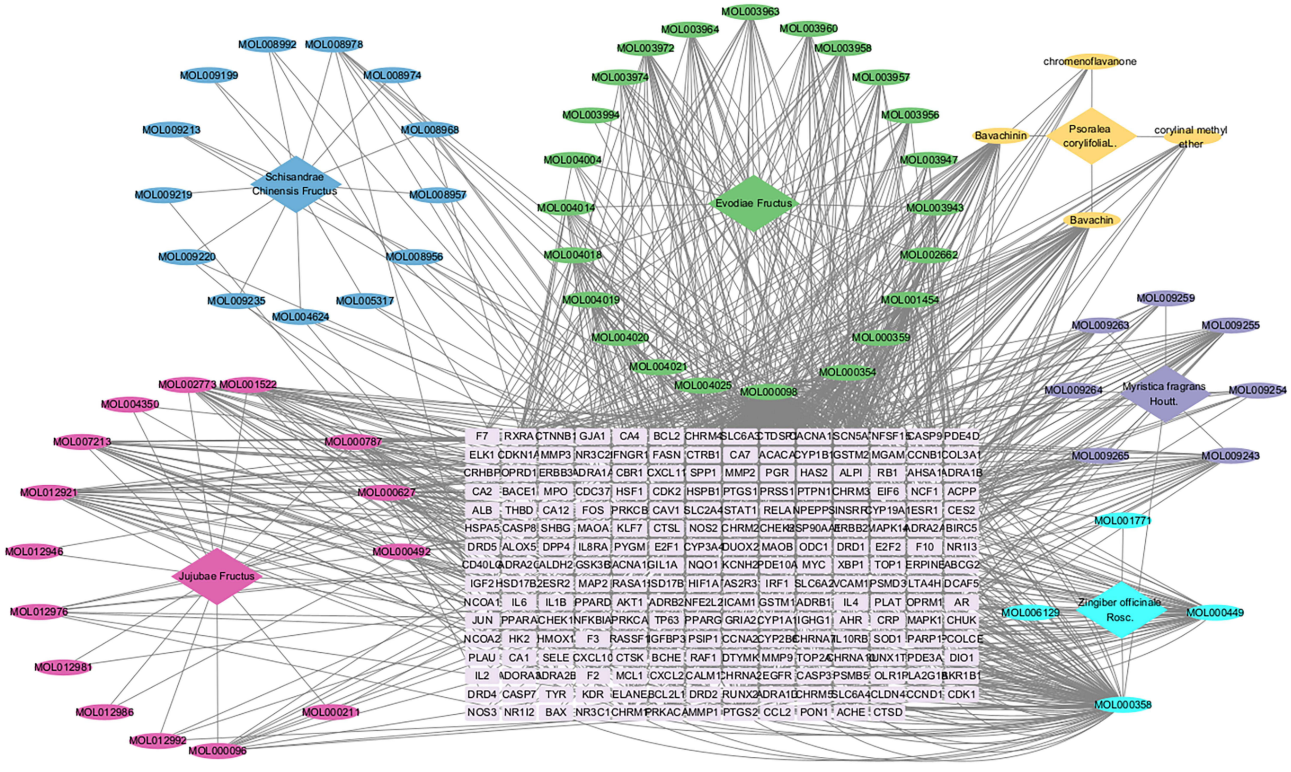

Figure 1 Schematic depiction of the network built with 66 bioactive compounds and 222 prospective target genes from Sishen Pill (SSP). Square-shaped nodes indicate target sites, oval nodes indicate bioactive components, and rhombus nodes indicate pharmaceutical names. Edges indicate interactive relationships between target sites and the bioactive components.

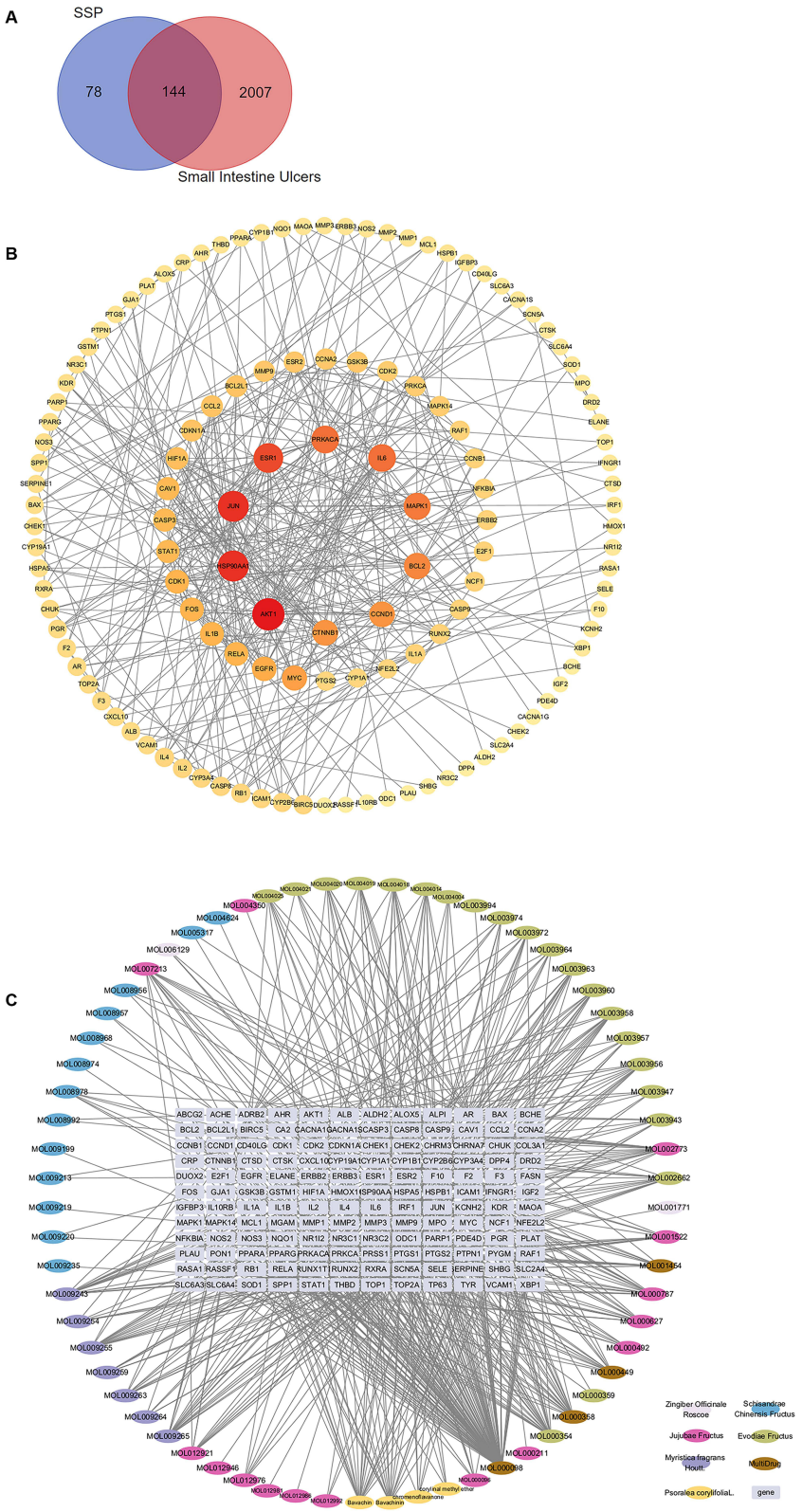

Figure 2 Core Target Identification and Assessment. (**A**) Intersection of genetic overlaps between SSP and SIUs-related protein targets depicted by a Venn diagram. (**B**) A PPI network comprising key target genes, where a higher degree value corresponds to larger nodes and more intense colors. (**C**) A Bioactive Compounds-Target-Disease Network was established, highlighting 66 bioactive constituents and 144 common genes.

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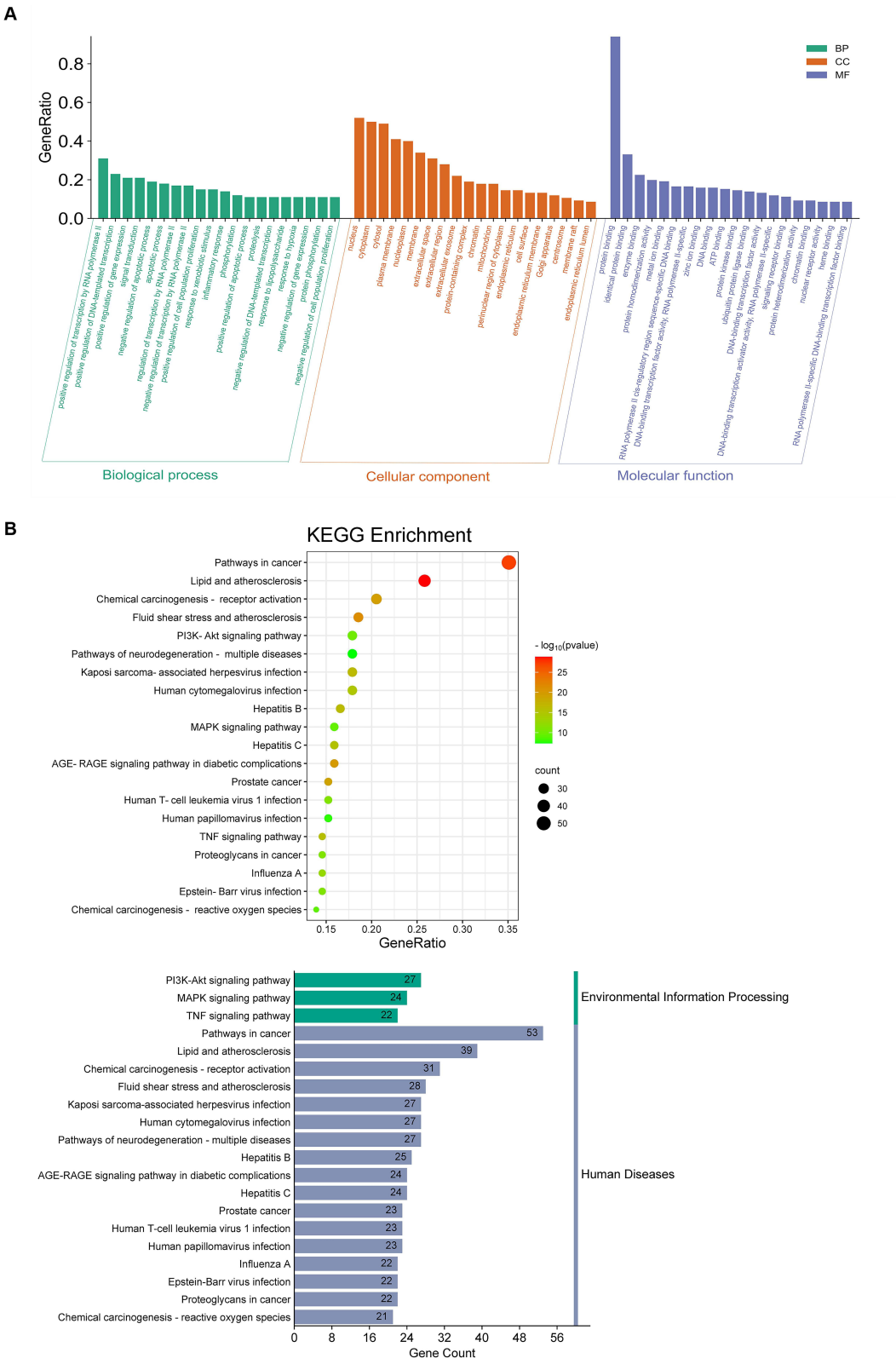

Figure 3 Overview of enriched GO and KEGG pathways for the potential target genes of SSP-treated SIUs. (**A**) Bar plot illustrating the 20 most significantly enriched Biological Process (BP), Cellular Component (CC), and Molecular Function (MF) terms identified through GO enrichment analysis. (**B**) Bubble plot (up) and bar plot (down) of the 20 most significantly enriched KEGG pathways, filtered by a significance threshold of P values < 0.05.

inflammatory response, and cell population proliferation. Cellular Component entries comprised nucleus, cytoplasm, and cytosol. Molecular Function entries encompassed enzyme binding, protein binding, and protein homodimerization activity, among others. This implies that the preventative and therapeutic action of SSP on SIUs involves multiple mechanisms.

A comprehensive analysis identified 152 significant KEGG pathways (Supplementary Table 3), with the top 20 being depicted in Figure 3B. Notable pathways encompassed "Pathways in cancer", "Lipid and atherosclerosis", "Phosphatidylinositol-3-kinase/protein kinase B (PI3K/Akt) signaling pathway", among others. The PI3K/Akt signaling pathway was identified as the most significant and was therefore chosen for further investigation in animal models.

Validation of Molecular Docking

The active components' docking potential was classified according to their degree values, leading to the selection of the top four compounds: Quercetin, Bavachinin, Rutaecarpine, and Evodiamine. The Autodock software was utilized to dock the primary bioactive components with the principal targets and significant pathway proteins. The affinity and stability of the interaction between the bioactive compound and protein targets are reflected by the binding energy, with lower values indicating greater structural stability. Evodiamine with AKT1, Rutaecarpine with HSP90AA1, Rutaecarpine with IL6, Rutaecarpine with MAPK1, and Evodiamine with BCL2 displayed the greatest stability in their bound conformations. Detailed information is depicted in Figure 4A-4E. Figure 4F summarizes the binding energies for these interactions.

Outcomes of in vivo Experimental Verification

SSP Ameliorates Intestinal Damage and Ulceration Caused by Indomethacin (INDO) in Rats

The assessment of the ulcer index was conducted in the small intestine. As shown in Figure 5A, rats in the INDO group exhibited significant mucosal damage in the small intestines. Treatment with SSP at dosages of 2.5 and 5 g/kg resulted in

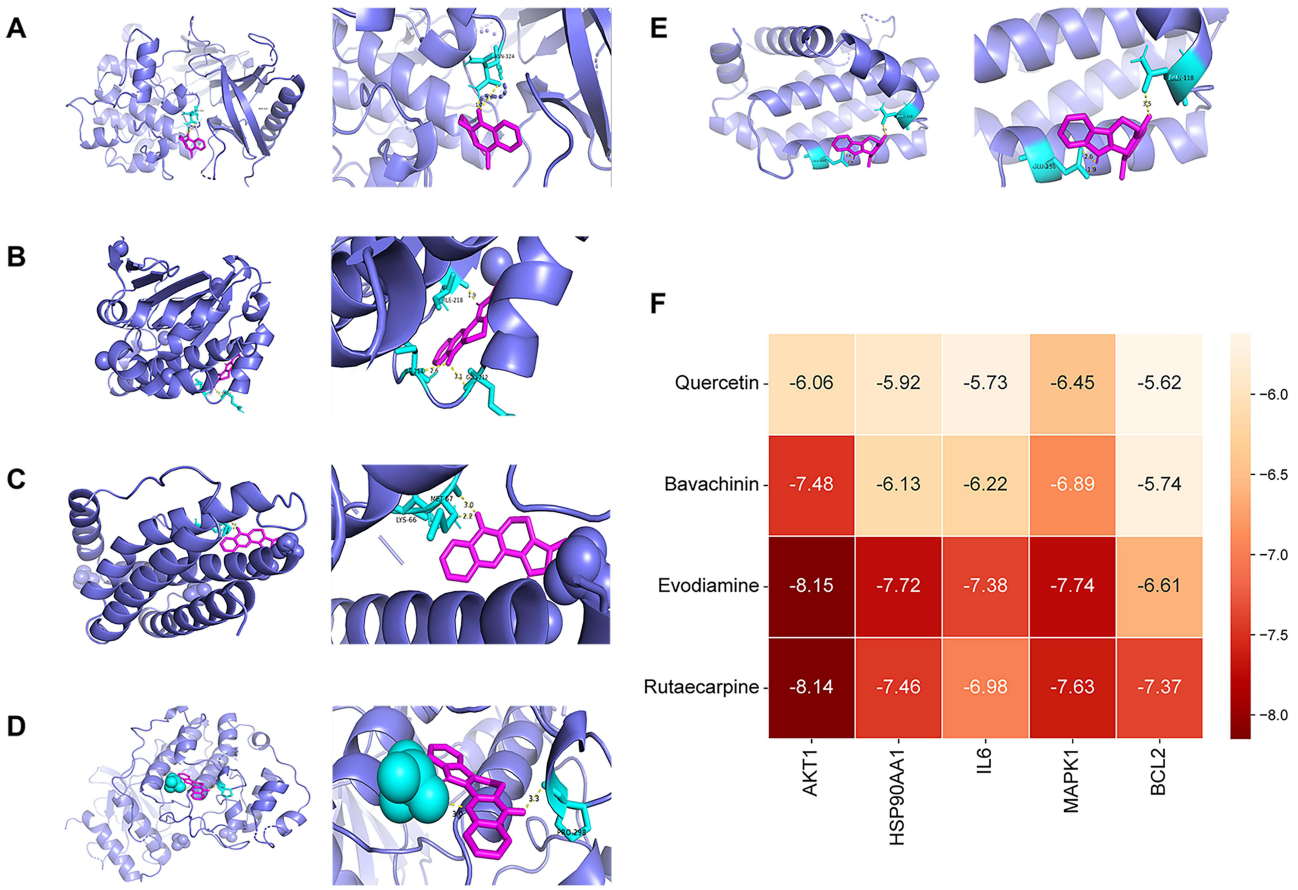

Figure 4 The docking results of four compounds with the central targets. (**A**) Evodiamine with AKT1. (**B**) Rutaecarpine with HSP90AA1. (**C**) Rutaecarpine with IL6. (**D**) Rutaecarpine with MAPK1. (**E**). Evodiamine with BCL2. (**F**) Heat map.

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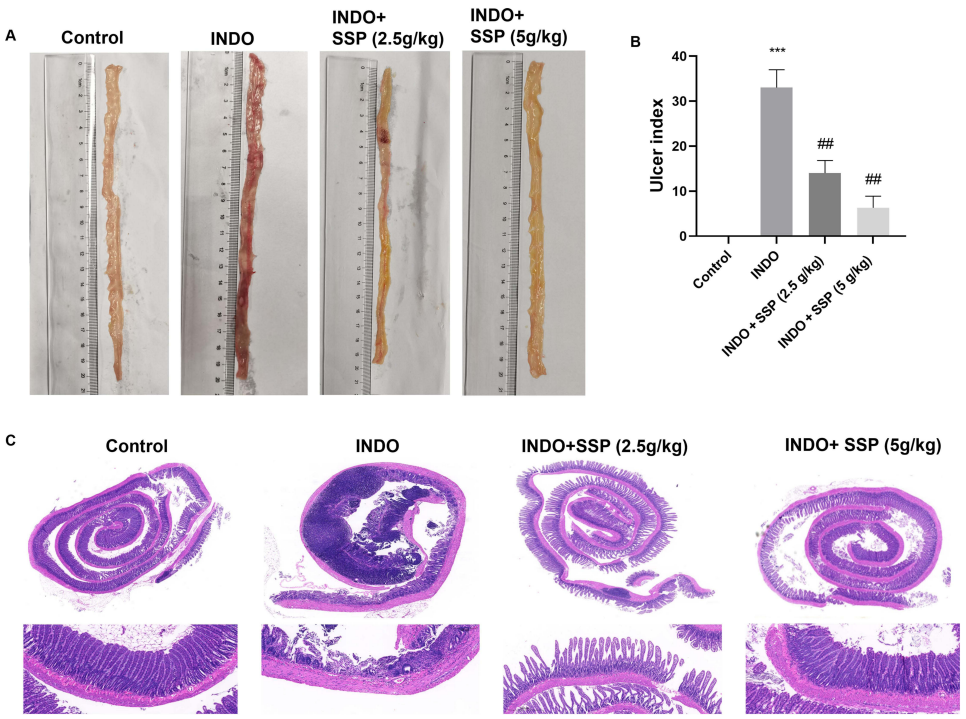

Figure 5 SSP ameliorates intestinal damage and ulcers caused by Indomethacin in rats. (**A**) Image of a rat's small intestine. (**B**) Assessment of ulcer index in the rats. (**C**) Pathological alterations in the small intestinal mucosa. The magnification is at 100x, N= 5. Data are displayed as means ± SEM. *** indicates $P < 0.001$ compared to the Control group; ## indicates $P < 0.01$ compared to the INDO group.

a noticeable reduction in intestinal mucosal ulcers. A significant decrease in the small intestine's ulcer index was particularly evident, with the most substantial effect being observed in the group that received 5 g/kg of SSP (Figure 5A and 5B). Overall, while the INDO group exhibited an elevated ulcer index, SSP treatment dose-dependently inhibited this increase. A histopathological examination of small intestine tissues using HE staining confirmed these observations, which aligned with the ulcer index trend (Figure 5C). The findings suggest that SSP possesses the capacity to mitigate indomethacin-induced mucosal damage in the rat's small intestine.

SSP Protects Against Oxidative Stress and Inflammation Induced by INDO

Serum inflammatory factor levels were assessed via ELISA. As compared to the control group, the INDO group showed a substantial increase in IL-1β, IL-6, and TNF-α activity. Conversely, a notable decrease in cytokine activity was observed in the INDO + SSP (2.5 g/kg) and INDO + SSP (5 g/kg) groups (Figure 6A). Subsequently, the kit detected increased MPO activity in small intestinal tissues post-INDO induction, which significantly decreased following SSP administration (Figure 6B). The results also showed that the levels of antioxidant enzyme activity, specifically SOD, CAT, and GSH-Px, were notably lower in the INDO group than in the control group (Figure 6C–6E), while the MDA activity increased (Figure 6F). Following treatment with SSP, a reversal of the aforementioned indicators in the small intestine was observed.

Effects of SSP on Gene Expression in INDO-Induced Rats

Comparative analysis revealed that the INDO group exhibited higher mRNA levels of TNF-α (Figure 7A), IL-6 (Figure 7B), and IL-1β (Figure 7C). In contrast, the SSP groups exhibited considerable suppression of these inflammatory genes compared to the INDO group. According to the network pharmacology research, the PI3K-AKT signaling pathway is central to the therapy of SIUs. When compared with the control group, INDO showed higher mRNA levels for both AKT1 (Figure 7D) and PI3K (Figure 7E). SSP administration reduced both PI3K and AKT1 mRNA expression in rats with INDO-induced SIUs. These findings imply that SSP may possess anti-inflammatory and immunomodulatory

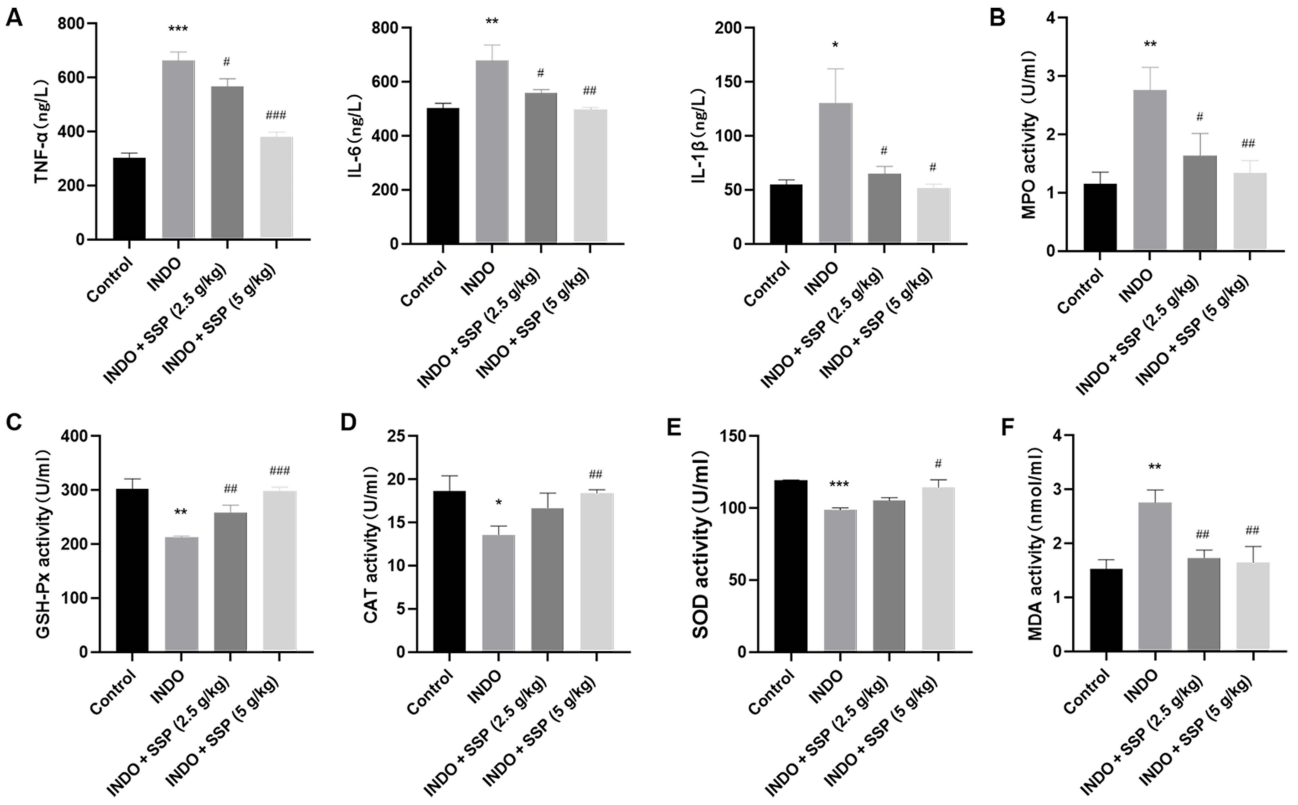

Figure 6 SSP mitigates inflammation and oxidative stress induced by Indomethacin. (**A**) ELISA was employed to quantify serum concentrations of inflammatory factors. (**B**) MPO activity within small intestinal tissues was assessed. (**C-F**) Measurement of oxidative stress-related factors in small intestinal tissues. Sample size (**N**) was 5. Statistical significance is denoted by * for P < 0.05, ** for P < 0.01, and *** for P < 0.001 compared to the Control group; # for P < 0.05, ## for P < 0.01, and ### for P < 0.001 compared to the INDO group.

properties, possibly by modulating the PI3K-AKT signaling pathway, thus affording a protective influence against intestinal damage.

Discussion

Nonsteroidal anti-inflammatory drugs (NSAIDs) have revolutionized pain and inflammation management in clinical practice.[27] Despite this, the utilization of these drugs is linked to a broad spectrum of adverse effects, most notably gastrointestinal complications.[28] A significant proportion of these complications involve the small intestine, leading to the development of small intestinal ulcers (SIUs).[29] Among the prevalent drugs for treating NSAID-induced SIUs, Proton Pump Inhibitors (PPIs), primarily used in NSAID gastropathy treatment, have demonstrated insufficient effectiveness.[10,30] Mucoprotective agents like misoprostol offer an alternative treatment method for NSAID enteropathy, but their high incidence of side effects may limit their application.[10] These challenges highlight the urgent need for novel and effective therapeutic strategies for NSAID-induced SIUs. Traditional Chinese Medicine (TCM) has gained recognition for its safety profile and multi-target therapeutic effects. Sishen Pill (SSP), a well-documented Chinese patent medicine, has been traditionally employed to treat chronic colitis and diarrhea.[31,32] The present research integrated network pharmacology with experimental confirmation to systematically explore the therapeutic potential of SSP in NSAID-induced SIUs.

Our study identified 66 bioactive constituents in SSP, with quercetin, bavachinin, rutaecarpine, and evodiamine emerging as pivotal components. These compounds exhibit potent anti-inflammatory, antioxidant, and barrier-protective properties. Quercetin, a flavonoid with antioxidant and immunomodulatory properties, inhibits proinflammatory cytokines via the cGAS-STING pathway, restoring macrophage polarization and intestinal barrier integrity.[33,34] Bavachinin has been shown to regulate apoptosis through the p53/Bax/Bcl2 axis, reducing crypt cell apoptosis in colitis models.[35]

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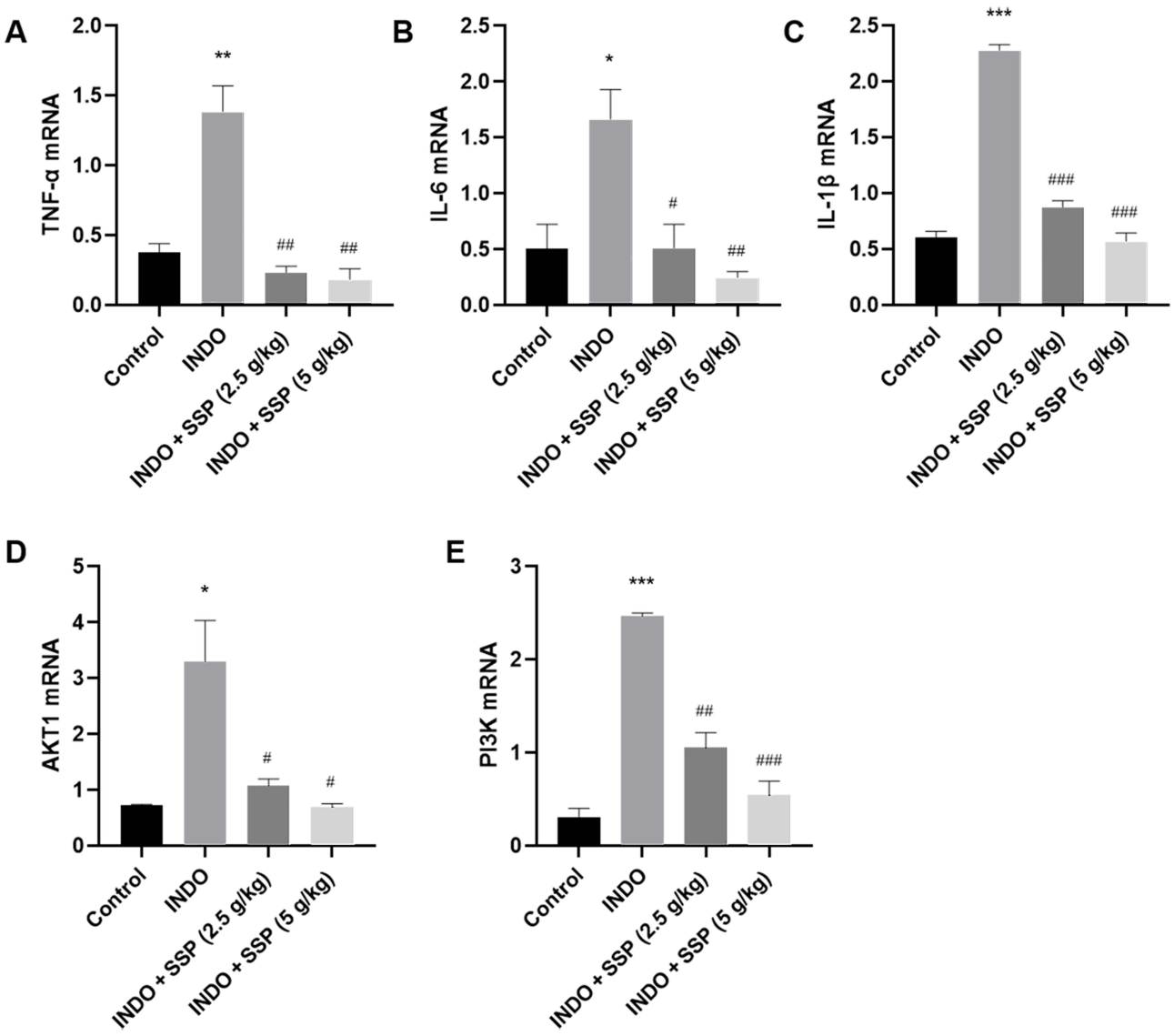

Figure 7 Impacts of SSP on gene expression in rats with INDO-induced SIUs. (**A-C**) SSP effects on the mRNA expression of TNF-α, IL-6, and IL-1β. (**D-E**) SSP effects on the mRNA expression of AKT1 and PI3K. Results are displayed as means ± SEM. N= 5. Statistical significance is denoted by * for P < 0.05, ** for P < 0.01, and *** for P < 0.001 compared to the Control group; # for P < 0.05, ## for P < 0.01, and ### for P < 0.001 compared to the INDO group.

Additionally, rutaecarpine has been reported to attenuate intestinal inflammation via NRF2 activation,[36] while evodiamine modulates gut microbiota composition, enriching short-chain fatty acid (SCFA)-producing species that promote barrier function.[37,38] Analyzing these key constituents, we hypothesize that SSP exerts a synergistic therapeutic effect through its modulation of inflammatory, oxidative, and apoptotic pathways. These findings align with prior studies linking SSP to TLR2/IRAK4/NF-κB, Nrf2/HO-1, and PI3K/AKT pathway.[14,39,40]

Inflammation is a key driver of SIU pathogenesis, primarily mediated by IL-1β, IL-6, and TNF-α, which disrupt epithelial integrity through neutrophil/macrophage-mediated pathways.[41] Our results showed that NSAID exposure significantly elevated these inflammatory markers, whereas SSP administration effectively suppressed them. In addition to its anti-inflammatory properties, SSP also demonstrated strong antioxidant activity, reversing NSAID-induced reductions in GSH-Px, CAT, and SOD, while lowering MPO and MDA levels.[42] These findings suggest that SSP mitigates NSAID-induced SIU progression by modulating inflammatory cytokines and oxidative stress pathways.

The PI3K/Akt pathway was identified as a key regulatory axis in SSP's therapeutic effects. Network pharmacology analysis revealed PI3K/Akt as the most significantly enriched pathway, further validated by molecular docking, which

demonstrated strong binding affinities between SSP components (eg, evodiamine, rutaecarpine) and key PI3K/Akt targets (AKT1, PI3K). In vivo validation revealed that SSP treatment significantly downregulated PI3K and AKT1 mRNA expression in INDO-induced rat models, correlating with reduced ulcer severity and decreased inflammatory cytokine expression. Mechanistically, PI3K/Akt signaling is pivotal in regulating cell survival, inflammation, and tissue regeneration.[43] Upon activation, Akt phosphorylates multiple downstream targets crucial for intestinal homeostasis. Akt phosphorylates BAD (Bcl-2-associated death promoter), preventing its interaction with Bcl2, thereby inhibiting mitochondrial apoptosis.[44] The suppression of Bcl2 mRNA following SSP treatment (Supplementary Figure 1A and B) supports this mechanism, indicating a potential anti-apoptotic effect. Moreover, Akt-mediated activation of mTOR enhances protein synthesis and mucosal repair, while GSK-3β inhibition promotes cell cycle progression through cyclin D1 accumulation.[45] These molecular events likely contribute to the histopathological improvements observed in SSP-treated rats. Additionally, Akt inhibits IκB kinase (IKK), preventing NF-κB nuclear translocation and proinflammatory cytokine release.[46]

SSP promotes mucosal healing through multiple mechanisms. Recent evidence links PI3K/Akt signaling to intestinal barrier integrity and autophagy regulation.[47–49] Dysregulation of this pathway in NSAID-induced injury results in tight junction disruption and increased permeability. Notably, SSP-treated rats exhibited significantly higher occludin and ZO-1 gene expression, suggesting its role in barrier restoration via PI3K/Akt modulation (Supplementary Figure 1C and D). Additionally, rutaecarpine's activation of NRF2 enhances antioxidant defenses, which may further support mucosal healing.[36]

SSP may modulates both innate and adaptive immune responses. By inhibiting the cGAS-STING pathway, quercetin reprograms macrophage polarization from pro-inflammatory M1 to anti-inflammatory M2 phenotypes, reducing IL-6 and TNF-α secretion.[34] Furthermore, SSP downregulates Th17 cell activity while promoting regulatory T-cell (Treg) expansion, rebalancing immune homeostasis in ulcerated tissues.[50] These immunomodulatory effects are critical for resolving chronic inflammation and preventing ulcer recurrence. While our focus was on inflammation and oxidative stress, emerging evidence highlights SSP's potential to modulate gut microbiota.[31] Future studies should explore SSP's impact on microbial diversity and metabolite profiles.

While SSP demonstrated dose-dependent efficacy in rats, its translational feasibility remains a key consideration. Preclinical studies suggest an optimal SSP dosage of 2.5–5 g/kg/day for gastrointestinal disorders, yet human equivalent dosing remains unstandardized. Based on body surface area extrapolation, a tentative human dose of 24.3–48.6 g/day has been proposed, necessitating clinical validation. Additionally, SSP's pharmacokinetic interactions with existing gastroprotective measures require careful evaluation. Co-administration with PPIs (eg, omeprazole, esomeprazole) may influence SSP metabolism via cytochrome P450 2C19 (CYP2C19) inhibition.[51] This could increase plasma concentrations of SSP constituents, potentially enhancing efficacy while raising toxicity risks. Furthermore, chronic PPI use disrupts gut microbiota, whereas SSP promotes the growth of beneficial taxa like Lactobacillus and Bifidobacterium.[31,52] The interplay between PPIs and SSP on microbiota composition warrants further investigation. Clinicians should prioritize weak CYP2C19 inhibitors, advocate fiber- and polyphenol-rich diets, and monitor nutrient levels to optimize therapeutic outcomes. Rigorous clinical trials are imperative to validate these interactions and refine evidence-based guidelines.

Consequently, SSP has demonstrated significant potential in the therapeutic intervention of NSAID-induced SIUs. Whether acting as antioxidants or modulators of cellular signaling, the effects of SSP on oxidative and inflammatory balance are crucial. Despite the promising findings, this study has several limitations. The precise molecular mechanisms by which SSP modulates the PI3K/Akt pathway remain incompletely understood. While network pharmacology and molecular docking identified potential interactions, direct experimental validation at the protein level is lacking. Future studies should employ biochemical assays such as co-immunoprecipitation, or Western blotting to confirm these interactions and delineate the downstream signaling events. Additionally, while this study demonstrated SSP's therapeutic potential in an indomethacin (INDO)-induced rat model of SIUs, interspecies differences in drug metabolism and immune response may limit direct clinical translation. Validation in human-derived intestinal organoid models or well-designed clinical trials is necessary to confirm its efficacy in human populations. Furthermore, this study primarily focused on the PI3K/Akt pathway; however, SIU pathogenesis involves multiple intersecting signaling cascades,

including NF-κB, MAPK, and AMPK pathways. Future transcriptomic and proteomic analyses should be conducted to explore SSP's broader regulatory network and uncover additional therapeutic targets. Finally, a direct comparison between SSP and standard gastroprotective agents, such as proton pump inhibitors (PPIs) or misoprostol, was not performed in this study. Future investigations should include comparative efficacy studies to evaluate whether SSP offers advantages over conventional therapies in NSAID-induced enteropathy. Despite these limitations, the study's findings provide evidence supporting the mechanism of SSP in intervening in NSAID-induced SIUs. These results also provide valuable direction for future research endeavors by the investigative team.

Conclusion

This study underscores the promising therapeutic efficacy of SSP in managing NSAID-induced SIUs, illuminating its complex interactions with various targets associated with the pathogenesis of SIUs. A possible therapeutic mechanism of SSP is its ability to modulate oxidative stress and inflammatory responses, possibly through PI3K/Akt. Our research findings are expected to facilitate the formulation of potent therapeutic approaches aimed at treating NSAID-induced SIUs and to encourage additional studies into the pharmacological properties of traditional Chinese medicine. Future studies should incorporate integrated biochemical assays to confirm the binding interactions between SSP constituents and the PI3K/Akt signaling pathway. Comparative analyses with established gastroprotective agents are essential to rigorously evaluate SSP's efficacy and safety. Moreover, comprehensive metagenomic and metabolomic investigations should be undertaken to delineate SSP's impact on gut microbiota composition and metabolite production.

Abbreviations

NSAIDs, nonsteroidal anti-inflammatory drugs; SSP, Sishen Pill; SIUs, small intestinal ulcers; INDO, indomethacin; TCMSP, Traditional Chinese Medicine Systems Pharmacology; TCM, traditional Chinese medicine; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; MPO, myeloperoxidase; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; CAT, catalase; MDA, malondialdehyde; IL-6, Interleukin-6; TNF-α, Tumor necrosis factor α; IL-1β, Interleukin-1β; PI3K/AKT, Phosphatidylinositol 3 kinase/protein kinase B; Bcl2, B-cell lymphoma-2.

Ethics Approval and Informed Consent

Ethical approval to conduct studies using publicly available databases is exempt under the following legislation: Item 1 and 2 of Article 32 of "the Measures for Ethical Review of Life Science and Medical Research Involving Human Subjects", which was reviewed by the National Science and Technology Ethics Committee, approved by the State Council of China, and jointly promulgated by the National Health Commission, the Ministry of Education, the Ministry of Science and Technology and the State Administration of Traditional Chinese Medicine on Feb. 18, 2023. The animal study protocol was approved by the Ethics Committee of Laboratory Animal Welfare, Zhejiang University (ZJU20240649). All animal experiments were performed by complying with the guidelines for the Protection and Use of Experimental Animals of the Ministry of Science and Technology of China.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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