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Elucidation of the protective effect of schisandrin C originating from *Schisandra chinensis* (Turcz.) Baill. against indomethacin-induced intestinal injury by combining network pharmacology, molecular docking, and experimental validation

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Abstract

Long-term use of nonsteroidal anti-inflammatory drugs (NSAIDs), especially indomethacin, has severe adverse effects on the gastrointestinal system. *Schisandra chinensis* (Turcz.) Baill., an herbal medicine used to treat several gastrointestinal diseases, might effectively reduce the adverse effects of indomethacin. This study aimed to elucidate the main bioactive compound from *S. chinensis* and its molecular mechanism for protection against indomethacin-induced intestinal injury. Network pharmacology, molecular docking, and molecular dynamics were performed to elucidate the key compounds of *S. chinensis*, their relevant targets, and potential molecular mechanisms via various databases, including the TCMS, SwissTargetPrediction, TargetNet, STRING, and DAVID databases. The protective effect of the key compound and its molecular mechanism were validated in intestinal cells and *Caenorhabditis elegans* models. Network pharmacology analysis demonstrated that schisandrin C (SCHC) is the active ingredient of *S. chinensis* and protects against indomethacin-induced intestinal injury. Computational analysis revealed that AKT1, PIK3CA, RELA, EGFR, and GSK3B are potential targets of SCHC. In vitro experiments confirmed that SCHC prevents apoptotic cell death and intestinal permeability dysfunction by modulating the AKT/GSK-3 β pathway and tight junction protein expression. Furthermore, SCHC ameliorated the intestinal atrophy induced by indomethacin and increased the intestinal lysosomal level in *C. elegans*, while its protective effect was abolished in *age-1* and *akt-1,2* mutants, indicating the involvement of the PI3K/AKT pathway. In summary, this study demonstrated that SCHC could be a potential candidate for reversing the side effects of indomethacin in the intestinal tract by combining network pharmacology, molecular docking, and experimental validation.

Keywords Schisandrin C, Indomethacin, Network pharmacology, Intestinal permeability, PI3K/AKT/GSK-3 β

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Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are well-known drugs that are usually used to cure various diseases. Nevertheless, long-term administration of NSAIDs is reported to cause severe damage to the colon or small intestine, such as ulcers, perforations, and strictures [1, 2]. Among NSAIDs, indomethacin is known for its dangerous side effects on the stomach and intestines, such as bleeding, ulcerations, and perforation [3]. Therefore, the dose and duration of treatment are carefully considered to limit severe effects when indomethacin is used in clinical practice.

A previous study has reported that indomethacin can cause intestinal inflammation by inducing the infiltration of the mucosa and elevating the secretion of proinflammatory cytokines by activating NF- κ B [4]. In addition, indomethacin has been reported to induce oxidative stress by impairing mitochondrial function, increasing intracellular reactive oxygen species (ROS) levels, and accelerating intestinal cell death in a human enterocyte model [5, 6]. According to previous studies, the severe side effects of indomethacin are associated with tight junction (TJ) proteins via the MLCK-MLC signaling pathway [7, 8]. TJ proteins are indispensable elements that maintain intestinal barrier integrity, and defects in TJs, which normally occur in inflammatory bowel disease (IBD) patients, result in increased intestinal permeability [9, 10].

Currently, no specific method is available to eliminate the gastrointestinal side effects of indomethacin, and the treatment must be stopped until the side effects are reduced. Revaprazan, a potassium-competitive acid blocker (P-CAB) drug, can protect against indomethacin-induced intestinal damage by altering the expression of various TJ proteins [8]. However, these P-CAB drugs are reported to have some adverse effects, such as diarrhea, dysgeusia, and abdominal pain [11]. Therefore, developing a safe agent from natural sources may be an alternative to protect against the negative impacts of indomethacin on gastrointestinal health [5–7, 12].

Schisandra chinensis (Turcz.) Baill. has been reported to have numerous pharmacological effects, such as anti-fatigue, antioxidant, and anti-inflammatory effects [13]. *S. chinensis* extract has been demonstrated to have a positive effect on the gastrointestinal tract [14, 15]. According to the literature on traditional medicine, *S. chinensis* is normally used to treat persistent diarrhea, dysentery, and reduced body fluids, which are closely linked to intestinal barrier integrity and leaky gut conditions [16]. The Chinese Pharmacopeia 2015 and Vietnamese Pharmacopeia V contain general information on drugs, including the herbal medicine *S. chinensis*, and they indicate that traditional prescriptions of *S. chinensis* are used to treat chronic diarrhea. Therefore, we hypothesize that

S. chinensis could be a potential candidate for preventing severe indomethacin-induced effects on the gastrointestinal system. Lignan compounds are major constituents of *S. chinensis* and have been reported to exhibit various beneficial effects on intestinal health. According to a previous study, schisandrin A protects against inflammation and cell damage induced by deoxynivalenol in Caco-2 cells [17]. Schisandrin B was also demonstrated to prevent ulcerative colitis caused by dextran sodium sulfate (DSS) in a mouse model [18]. Furthermore, gomisin J, gomisin N, and schisandrin C (SCHC) reportedly exhibit anti-inflammatory activity in lipopolysaccharide (LPS)-stimulated macrophages [19]. Our previous study reported that schisandrin C could alleviate IL-1 β -induced intestinal barrier dysfunction by modulating tight junction protein expression [20]. However, the adverse effects of indomethacin on the gastrointestinal system are more complex than those of cytokines, which are related not only to intestinal barrier dysfunction but also to other cellular signaling pathways, including programmed cell death [8, 21]. The protective effects of bioactive compounds from *S. chinensis* against indomethacin-induced intestinal injury and their molecular mechanism remain unknown.

Network pharmacology was first introduced by Hopkins in 2007 and is based on combining different research fields, including pharmacology, systems biology, bioinformatics, and cheminformatics [22]. Recently, network pharmacology has gradually become a widely applied research approach in drug discovery and development, especially in herbal medicine studies, to understand the multiple targets and pathways involved in the molecular mechanism of natural products for treating disease. In addition, molecular docking is a valuable tool for predicting and simulating the interactions and binding affinities between ligands and target proteins. In this study, network pharmacology and molecular docking were integrated to identify the bioactive components of *S. chinensis* that can protect against intestinal injury and leaky gut conditions induced by indomethacin and to elucidate their pharmacological mechanism by identifying potential target proteins and related signaling pathways. In addition, we investigated the severity of intestinal dysfunction induced by indomethacin and evaluated the protective effect of the compound using *in vitro* Caco-2 cells and an *in vivo* *Caenorhabditis elegans* model to validate the prediction from computational analysis.

Materials and methods

Network pharmacology analysis

Screening of bioactive compounds from *S. chinensis* and prediction of their protein targets

The chemical composition of *S. chinensis* was identified by searching the keyword “*Schisandrae chinensis*

fructus” in the Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP: <http://tcmspw.com/tcmsp.php>) [23]. Potential bioactive compounds were selected on the basis of two criteria, namely, drug-likeness (DL) and oral bioavailability (OB), with thresholds of $DL \geq 0.18$ and $OB \geq 30\%$. Afterward, the drug-like properties of the selected compounds were obtained by submitting their Simplified Molecular Input Line Entry System (SMILES) strings to the SwissADME database (<https://www.swissadme.ch/>) [24]. In this database, the DL of a compound is TRUE if its physicochemical properties do not violate any rules, including the rules of Lipinski, Ghose, Veber, Egan, and Muegge. The detailed criteria of each rule are shown in Supplementary Table S2. In addition, the toxicity of each compound was predicted by submitting their SMILES codes to the ProTox 3.0 web server to analyze the LD_{50} values of the compounds, and only compounds categorized in Classes 4–6 were considered safe [25]. Then, the SMILES of the selected compounds were subsequently submitted to the SwissTargetPrediction (<http://www.swisstargetprediction.ch/>) and TargetNet (<http://targetnet.scbdd.com/>) databases to predict the potential protein targets (with probability scores > 0) [26, 27].

Identification of common protein targets related to *S. chinensis* compounds and indomethacin-induced intestinal injury

To identify potential protein targets related to indomethacin-induced intestinal injury, the GeneCards database (<https://www.genecards.org/>) [28] was used to search for the keyword “indomethacin induced intestinal injury”, and only targets with relevant scores ≥ 20 were selected. The OMIM database [29] was also used to obtain the targets by using three keywords, including “intestinal barrier dysfunction”, “intestinal injury”, and “intestinal inflammation”. Subsequently, the common targets were identified by intersecting the potential targets of *S. chinensis* compounds and the disease-related targets obtained from both the GeneCards and OMIM databases via a Venn diagram that was created with the SRplot tool (<https://www.bioinformatics.com.cn/>) [30].

Network construction

To identify the core targets among the intersecting targets, the list of common protein targets was submitted to the STRING database (<https://cn.string-db.org/>) [31] to establish the protein–protein interaction (PPI) network. In this database, the species selected was “*Homo sapiens*”, and only proteins with interaction scores ≥ 0.4 were selected and imported into Cytoscape software (version 3.10.1, Seattle, WA, USA) to construct a PPI network. The network was subsequently analyzed, and the core targets were determined based on the degree value, with the

cutoff value set as the average of the degree value. Next, compound–target–disease (C–T–D) interactions were visualized and analyzed via Cytoscape software to identify the key bioactive compounds of *S. chinensis* involved in protection against indomethacin-induced intestinal injury. Then, the network was analyzed, and compounds with higher degree values were selected as key bioactive compounds. A Sankey diagram showing the associations between compounds and targets was generated via the SRplot tool. Finally, to better understand the molecular mechanisms of the selected compounds against indomethacin-induced intestinal injury, a compound–target–pathway (C–T–P) network was constructed by using the Cytoscape software. This network was used to visualize the complex interactions between the compound, its targets, and the related signaling pathways. The top 10 core targets of the compound with the highest degree values were identified via analysis of the C–T–P network before its bioactivity was validated via in vitro and in vivo experiments. A pie graph was generated via the SRplot tool to visualize the degrees of the top 10 target proteins.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses

The Database for Annotation, Visualization and Integrated Discovery (DAVID; <https://david.ncifcrf.gov/>) [32] was selected to perform GO and KEGG pathway enrichment analyses for the core targets of SCHC, with a p value < 0.05 set as a threshold to detect significant molecular function (MF), cellular component (CC), biological process (BP), and signaling pathways related to the protective effects of SCHC. Then, the SRplot tool was used to visualize the GO and KEGG pathway enrichment analysis results.

Molecular docking

SCHC was virtually docked against target proteins via their atomic coordinates from the Protein Data Bank. The proteins were prepared following standard procedures at Maestro (Schrödinger LLC, NY, 2024-01 release). This involved adding hydrogens, assigning bond orders, removing water molecules, optimizing hydrogen bonds, and minimizing energy via the OPLS4 force field. PROPKA predicted protein ionization states, and ProTAssign optimized hydrogen-bonding networks. Ligand preparation with LigPrep and Epik considered protonation states and tautomeric variations at $pH 7 \pm 2.0$ via the OPLS4 force field. Low-energy stereoisomers were generated, and the most favorable 3D structures with the correct chiralities were selected. These parameterized ligands were then docked onto the target proteins via Glide, a module within the Schrödinger suite [33]. For RELA, putative small-molecule binding pockets were predicted using SiteMap. For the remaining proteins,

binding sites were defined on the basis of cocrystallized ligands. Docking grid boxes were centered on the cocrystallized ligands (or predicted binding pockets) via Glide's grid generation module. The docking simulations were performed in extra precision (XP) mode, and ligands were evaluated based on the Glide XP scoring function, which estimates binding affinity in kcal/mol. The Glide XP scoring function was calculated as described previously [34, 35]. For proteins with promising binding energies (lower than -5 kcal/mol) in Glide XP docking, induced fit docking (IFD) was performed to refine ligand binding poses and account for protein flexibility. The IFD workflow involved three steps, as described previously [36]. The final binding poses obtained from IFD provided a more biologically relevant representation of ligand–protein interactions, capturing induced conformational changes that may occur upon binding. These refined poses were used for further analysis and visualization.

Molecular dynamics (MD) simulation

MD simulations were conducted to evaluate the stability and binding behaviors of the ligand–protein complexes under near-physiological conditions. The final binding poses obtained from IFD were subjected to MD simulations by using Desmond (2024-01 release; Schrödinger LLC, NY, USA). The System Builder tool was employed to prepare the simulation systems. The complexes were solvated by using the TIP3P water model in an orthorhombic box with periodic boundary conditions. Counter ions (Na^+/Cl^-) were added to neutralize the system, and 0.15 M NaCl was included to mimic the physiological ionic strength [37]. The OPLS4 force field was applied to parameterize the system [38]. Before the simulation was initiated, the system was energy minimized and equilibrated. MD simulations were run under the NPT ensemble with a temperature of 300 K and a pressure of 1 bar, which were maintained with a Nose–Hoover thermostat and a Martyna–Tobias–Klein barostat, respectively. A 100-ns simulation was performed for each ligand–protein complex, with trajectories saved every 100 ps for analysis. Postsimulation analyses were conducted by using Desmond's simulation interaction diagram (SID) utility. The root-mean-square deviation (RMSD) and root-mean-square fluctuation (RMSF) were calculated to evaluate the stability of the protein and ligand over time. Additionally, protein–ligand hydrogen bond interactions were monitored throughout the simulation to assess the dynamic behaviors of key interactions. These analyses provided insights into the binding stability and interaction patterns and validated the docking results.

Experimental validation

Chemicals and reagents

Indomethacin, 3,3'-diindolylmethane (DIM), acridine orange (AO) hydrochloride solution, 2,7-dichlorofluorescein diacetate (DCF-DA), fluorescein isothiocyanate (FITC)–dextran, quercetin, and dimethyl sulfoxide (DMSO) were obtained from Sigma (St. Louis, MO, USA). Antibodies against E-cadherin, claudin-1, bcl-2, bax, caspase-3, caspase-9, PARP, Akt, p-Akt, GSK3 β , p-GSK3 β , I κ B α , NF- κ B p65, p-NF- κ B p65, MLC, p-MLC, and PI3K p110 α were obtained from Cell Signaling Technology (Danvers, MA, USA). MLCK antibody was obtained from Sigma. The β -actin antibody, anti-mouse secondary antibody, and anti-rabbit secondary antibody were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). ZO-1, occludin, and claudin-4 antibodies were purchased from Invitrogen (Carlsbad, CA, USA). Transwell plates (12-well; 10.5-mm membrane diameter; 0.4- μ m pores) were obtained from SPL Life Sciences (South Korea). The detailed information on the chemicals and reagents is shown in Supplementary Table S1.

Plant materials and chemical isolation

Schisandra chinensis (Turcz.) Baill. fruits were obtained from the Kyungdong Oriental Herbal Market (Seoul, South Korea). A voucher specimen (accession number KISTNP-0013) was deposited at the KIST Gangneung Institute of Natural Products (Gangneung, South Korea). The plant name *Schisandra chinensis* (Turcz.) Baill. was checked with the World Flora Online website (<https://www.worldfloraonline.org/taxon/wfo-0000495099>). The ethanol extract of *S. chinensis* fruit was prepared by refluxing for 3 h. Then, the high-lignan fraction was partitioned from the ethanol extract using n-hexane as the solvent. As previously described, SCHC (purity: 97.8%) was isolated from the n-hexane fraction [39]. Next, the chemical structure of SCHC was determined by comparing its ^1H and ^{13}C NMR data with those reported in the literature [39]. The ^1H NMR, ^{13}C NMR, and HR-ESI-MS spectra of SCHC are shown in Supplementary Fig. S1.

Cell culture

Caco-2 cells were cultured in the same way as previously reported [40].

Cell viability assay

Caco-2 cells were seeded at a density of 5×10^3 cells/well in a 96-well plate. After 24 h, undifferentiated cells were pretreated with SCHC (2.5–10 μM) or quercetin (10 μM , as a positive control) for 66 h. Then, indomethacin (0.5–2 mM) was added for 6 h. In addition, differentiated Caco-2 cells were seeded in 48-well plates (3×10^4 cells/well) and differentiated for 12–14 days. Similarly, differentiated Caco-2 cells were pretreated with compounds

for 66 h before indomethacin was added. Finally, Caco-2 cell viability was measured with an EZ-Cytox kit (Dogenbio, South Korea) as described previously [41].

Measurement of pro-inflammatory markers in undifferentiated Caco-2 cells

Caco-2 cells were seeded at a density of 5×10^3 cells/well in a 96-well plate. After 24 h, the cells were pretreated with SCHC (2.5–10 μ M) or quercetin (10 μ M) for 66 h. Then, indomethacin (1 mM) was added for 24 h. The cell media were then collected, and the nitric oxide (NO), IL-6, and IL-8 levels were determined using Griess reagents (Promega, USA), human IL-6, and human IL-8 ELISA kits (BioLegend, San Diego, CA, USA), respectively.

Measuring the levels of ROS in undifferentiated Caco-2 cells

The amount of generated ROS was quantified via DCF-DA, as described previously [42]. Caco-2 cells were plated in black 96-well plates (5×10^3 cells/well). After 24 h, the Caco-2 cells were pretreated with SCHC (5–10 μ M) or quercetin (10 μ M, positive control) for 66 h and stimulated with indomethacin (1 mM) for 4 h. Then, the Caco-2 cells were incubated with 10 μ M DCF-DA in phenol red-free DMEM for 20 min. Finally, the Caco-2 cells were washed twice with DPBS, and images were taken using an Operetta high-content imaging system (PerkinElmer, USA). The fluorescence intensity was analyzed by using Harmony software (ver. 3.5).

Apoptosis analysis

Caco-2 cells (3×10^5 cells) were seeded in a 6-well plate and incubated for 24 h. Then, the cells were pre-treated with SCHC (10 μ M) for 66 h and stimulated with indomethacin (1 mM) for 6 h. Subsequently, the cells were harvested and stained with Annexin V-FITC and propidium iodide (PI) for 15 min in the dark, followed by fluorescence cytometric analysis (BD Bioscience FACS Verse, USA).

TEER and FITC-dextran permeability measurements in differentiated Caco-2 cell monolayers

Caco-2 cells were seeded on the apical side of a 12-well transwell plate (3×10^5 cells/well) and differentiated for 12–14 days. To evaluate cell differentiation, the TEER value of each well was measured, and only wells with TEER values between 400 and 500 $\Omega \cdot \text{cm}^2$ were used for the experiment. Then, differentiated Caco-2 cells were pretreated with SCHC (5–10 μ M) or DIM (10 μ M, positive control) for 66 h. Next, the cells were stimulated with indomethacin (1 mM) for 6 h. Afterward, TEER and FITC-dextran permeability were measured as previously reported [40].

Immunoblotting

Caco-2 cells were plated in a 6-well plate at a density of 1.5×10^6 cells/well and differentiated for 12–14 days. Next, the cells were pretreated with SCHC (10 μ M) before being stimulated with indomethacin (1 mM). Proteins were subsequently extracted with RIPA buffer, and the protein concentration was quantified via the Bradford assay. All the other steps of the immunoblotting process were performed in the same way as reported previously [40]. Finally, protein expression was normalized to that of β -actin, and the band intensity was quantified via ImageJ software (NIH, USA).

Immunofluorescence

The influence of SCHC on the ZO-1 protein expression level was evaluated via immunofluorescence microscopy. Caco-2 cells were plated in a 6-well plate (1.5×10^6 cells/well) containing a cover glass in each well and differentiated for 12–14 days. Then, differentiated Caco-2 cells were pretreated with SCHC (10 μ M) for 66 h before being stimulated with indomethacin (1 mM) for 6 h. All other immunofluorescence steps were performed in the same way as described previously [20].

C. elegans and bacterial strain maintenance

All *C. elegans* strains and *Escherichia coli* OP50 were obtained from the Caenorhabditis Genetics Center (University of Minnesota, MN, USA). The following *C. elegans* strains were used in this study: N2 (wild-type), TJ1052 *age-1 (hx546) II*, RB759 *akt-1 (ok525) V*, and VC204 *akt-2 (ok393) X*. The worms were cultured on nematode growth medium (NGM) agar at 20 °C with heat-inactivated (HI) *E. coli* OP50 as a food source. The HI *E. coli* OP50 preparation was performed as previously described [40, 43]. Egg synchronization followed previously described methods [44]. *Pseudomonas aeruginosa* ATCC17503 was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA).

Measurement of intestinal width in C. elegans

The impact of indomethacin on intestinal health was then evaluated in vivo via a *C. elegans* model. To assess the intestinal morphological atrophy induced by indomethacin and the protective effect of SCHC, the width of the *C. elegans* intestine was analyzed as described previously with slight modifications [45, 46]. Synchronized L4-stage *C. elegans* were divided into six different treatment groups, as follows: HI *E. coli* OP50 (control); indomethacin (20 mM); cotreatment with indomethacin (20 mM) and SCHC (10 and 25 μ M, respectively); *P. aeruginosa*; and cotreatment with DIM (100 μ M) and *P. aeruginosa*. *P. aeruginosa* has been reported to induce leaky gut conditions in *C. elegans*, and cotreatment with DIM has been shown to promote recovery [40]. Therefore,

these two groups were used as positive and negative controls, respectively, for this experiment. Following a 2-day chemical treatment, adult worms (2 days post-L4) were mounted on glass slides coated with poly-L-lysine using 10 μ L of 0.25 mM levamisole in M9 buffer. Afterward, the worms were observed under an EVOS[®] microscope (Thermo Fisher, USA). More than ten worms were examined for each condition. The width of the intestine was determined by measuring the diameter of the intestine at a point between the oocyte prior to ovulation and the spermatheca. The diameter of the lumen was subsequently subtracted from the diameter of the intestine. To assess the extent of gut atrophy, the obtained intestinal widths were normalized by dividing them by the corresponding value of the control worms. The intestinal diameters were quantified via ImageJ software.

Intestinal lysosomal staining in *C. elegans*

To assess the impact of indomethacin and SCHC on lysosomal function in the *C. elegans* intestine, intestinal lysosomes were stained with acridine orange (AO), an acidophilic and cell-permeable fluorescent dye, as previously described [47]. Synchronized L4-stage *C. elegans* were divided into six different treatment groups as follows: HI *E. coli* OP50 (control); indomethacin (20 mM); cotreatment with indomethacin (20 mM) and SCHC (10 and 25 μ M, respectively); *P. aeruginosa*; and cotreatment with DIM (100 μ M) and *P. aeruginosa*. Following a 2-day chemical treatment, more than ten worms were incubated in NGM agar coated with 25 μ M AO dissolved in M9 buffer for 1 h at 20 $^{\circ}$ C while avoiding light to prevent bleaching. The worms were subsequently transferred to fresh HI *E. coli* OP50-coated NGM agar for 10 min to remove the excess dye. The stained worms were then observed under an EVOS[®] microscope, and the AO fluorescence intensity was analyzed via ImageJ software.

Statistical analysis

All experiments in this study were performed at least three independent times, and the experimental results are presented as the means $\pm$ standard deviations (SDs). Statistical data were analyzed by one-way ANOVA, and

comparisons between groups were performed via Tukey’s multiple comparison tests with GraphPad Prism 8.0 software (La Jolla, CA, USA). A level of $p < 0.05$ was considered to indicate statistical significance.

Results

Network pharmacology analysis

Screening of bioactive compounds from *S. chinensis* and their drug-like properties

On the basis of the TCMSP database, 130 compounds were found in *S. chinensis*, and following the screening criteria of $DL \geq 0.18$ and $OB \geq 30\%$, eight compounds were satisfied, including gomisin R, angeloylgomisin O, wuweizisu C (SCHC), gomisin G, schizandrer B, deoxyharringtonine, gomisin A, and longikaurin A. Among them, 6/8 compounds were lignan, with the exceptions of diterpenoids and alkaloids, respectively. Next, the SwissADME database was used to calculate the drug-like properties of these bioactive compounds on the basis of their SMILES. The results revealed that only four compounds had DL values as TRUE, namely, longikaurin A, gomisin A, gomisin R, and SCHC (Supplementary Table S2). In addition, the eight bioactive compounds were assessed for potential toxicity through ProTox 3.0, with a focus on potential toxicity and LD₅₀ values. Six compounds with toxicity class 4 were predicted to be non-toxic, whereas deoxyharringtonine (class 2, LD₅₀ = 28 mg/kg) and longikaurin A (class 3, LD₅₀ = 120 mg/kg) were considered toxic for human consumption (Supplementary Table S2). The detailed molecular and pharmacokinetic properties of the eight compounds are shown in Table 1.

Target identification of *S. chinensis* bioactive compounds against indomethacin-induced intestinal injury

Next, the potential targets of eight *S. chinensis* compounds were predicted via the TargetNet and SwissTargetPrediction databases. After removing duplicates, 462 potential targets of eight compounds were obtained from these databases. Next, a total of 532 potential targets related to disease were identified from the GeneCards

Table 1 Molecular and pharmacokinetic properties of eight bioactive compounds originating from *S. chinensis*

Compound	Formula	MW	OB (%)	TPSA	RBN	DL (TCMSP)	DL (SwissADME)
Gomisin R	C ₂₂ H ₂₄ O ₇	400.42	34.84	75.61	2	0.86	TRUE
Angeloylgomisin O	C ₂₈ H ₃₄ O ₈	498.56	31.97	81.68	7	0.85	FALSE
Schisandrin C	C ₂₂ H ₂₄ O ₆	384.42	46.27	55.38	2	0.84	TRUE
Gomisin G	C ₂₉ H ₃₂ O ₈	508.56	32.68	84.84	6	0.83	FALSE
Schizandrer B	C ₂₈ H ₃₄ O ₉	514.56	30.71	101.91	7	0.83	FALSE
Deoxyharringtonine	C ₂₈ H ₃₇ NO ₈	515.60	39.27	103.76	10	0.81	FALSE
Gomisin A	C ₂₃ H ₂₈ O ₇	416.46	30.69	75.61	4	0.78	TRUE
Longikaurin A	C ₂₀ H ₂₈ O ₅	348.43	47.72	86.99	0	0.53	TRUE

*DL, Drug-likeness; MW, Molecular weight; OB, Oral bioavailability; RBN, number of rotatable bonds; TPSA, Topological polar surface area

and OMIM databases, and 91 common targets were identified by intersecting 532 disease-related targets with 462 targets of eight *S. chinensis* components in a Venn diagram (Fig. 1A). These proteins are potential targets related to the protection of *S. chinensis* against indomethacin-induced intestinal injury (Supplementary Table S3).

To understand the interactions between these 91 common targets, a protein–protein interaction (PPI) network was established with the STRING database and Cytoscape software. In this network, nodes represent target proteins, whereas edges signify protein interactions. In this study, the PPI network included 91 nodes and 1261 edges, and the average degree value of the nodes was 27.7 (Supplementary Fig. S2). According to the PPI network analysis results, 37 target proteins with degree centrality values higher than average (>27.7) were selected as core target proteins (Fig. 1B, Supplementary Table S3).

Identification of key bioactive compounds from *S. chinensis* via C–T–D network construction

The C–T–D network was established and analyzed via Cytoscape software to elucidate the key compounds from *S. chinensis* and their potential mechanism of action against indomethacin-induced intestinal injury. In this study, the C–T–D network of *S. chinensis* was constructed with 47 nodes (including 8 potential bioactive compounds and 37 key targets) and 172 edges (Fig. 2A). C–T–D network analysis revealed that the key bioactive compounds from *S. chinensis* were gomisin A, SCHC, schizandrin B, and gomisin G with degree values of 24, 22, 21, and 20, respectively (Fig. 2B and C, Supplementary Table S4). These data indicated that these compounds could interact with most core targets and might be responsible for the protection of *S. chinensis* against indomethacin-induced intestinal injury.

Among these four candidates, two compounds, namely, schizandrin B and gomisin G, violated the DL criteria, according to the SwissADME database (Table 1). Notably, as shown in Table 1, SCHC exhibited good drug-like properties, with the highest oral bioavailability (OB = 46.27%), the lowest number of rotatable bonds (RBN = 2), and the lowest topological polar surface area (TPSA = 55.38 Å). Additionally, the toxicity prediction indicated that SCHC had a low level of toxicity (Class 4) with an LD₅₀ value of 1000 mg/kg (Supplementary Table S2). Our in vitro screening experiments also indicated that among the nine lignan compounds isolated from *S. chinensis* fruit, SCHC could protect against indomethacin-induced death in Caco-2 cells (Supplementary Fig. S6). Therefore, SCHC was selected as a potential candidate for further studies, and we evaluated its effects in various experimental systems that mimicked indomethacin-induced intestinal injury.

GO and KEGG pathway enrichment analysis

The 22 core targets of SCHC were submitted to the DAVID database to analyze GO and KEGG pathway enrichment. The analysis revealed 303 GO terms, comprising 187 BP, 27 CC, and 89 MF terms (Fig. 3A, Supplementary Table S5). Our results indicated that the core targets of SCHC were associated with the top 10 BP terms, including positive regulation of phosphatidylinositol 3-kinase/protein kinase B signal transduction, epidermal growth factor receptor signaling pathway, protein autophosphorylation, positive regulation of cell migration, insulin-like growth factor receptor signaling pathway, negative regulation of gene expression, etc. The analysis revealed that the predicted targets were located in the following CCs: cytosol, nucleus, protein-containing complex, cytoplasm, and plasma membrane. In addition, the core targets were also closely related to various MFs, such as enzyme binding, protein kinase binding, protein kinase activity, protein tyrosine kinase binding, and ATP binding.

KEGG pathway enrichment analysis subsequently revealed 119 signaling pathways (Fig. 3B, Supplementary Table S6). Among these enriched KEGG pathways, the SCHC core genes were closely related to the PI3K–Akt signaling pathway (hsa04151), VEGF signaling pathway (hsa04370), prolactin signaling pathway (hsa04917), ErbB signaling pathway (hsa04012), relaxin signaling pathway (hsa04926), colorectal cancer (hsa05210), thyroid hormone signaling pathway (hsa04919), Ras signaling pathway (hsa04014), C-type lectin receptor signaling pathway (hsa04625), and MAPK signaling pathway (hsa04010). These findings suggest that SCHC may protect against indomethacin-induced intestinal injury through multiple targets and pathways, especially the PI3K/AKT signaling pathway.

Compound–target–pathway (C–T–P) network construction

Next, the C–T–P network was developed, providing a comprehensive view of the molecular mechanism involved in the protective effect of SCHC against indomethacin-induced intestinal injury. The C–T–P network consisted of 43 nodes (including 1 compound, SCHC; 22 key targets; and 20 pathways related to SCHC) and 158 edges (Fig. 4A). According to the C–T–P network analysis results, the top 10 genes associated with the protective effect of SCHC against indomethacin-induced intestinal injury were AKT1, PIK3CA, RELA, MAPK8, EGFR, PRKCA, SRC, GSK3B, KDR, and CASP9 (Fig. 4B). These data were highly consistent with those of the GO and KEGG pathway analyses, suggesting that the protective effect of SCHC could be closely associated with modifications of AKT1, PIK3CA, RELA, GSK3B, and EGFR, which are involved in the PI3K/AKT/GSK-3 β signaling pathway.

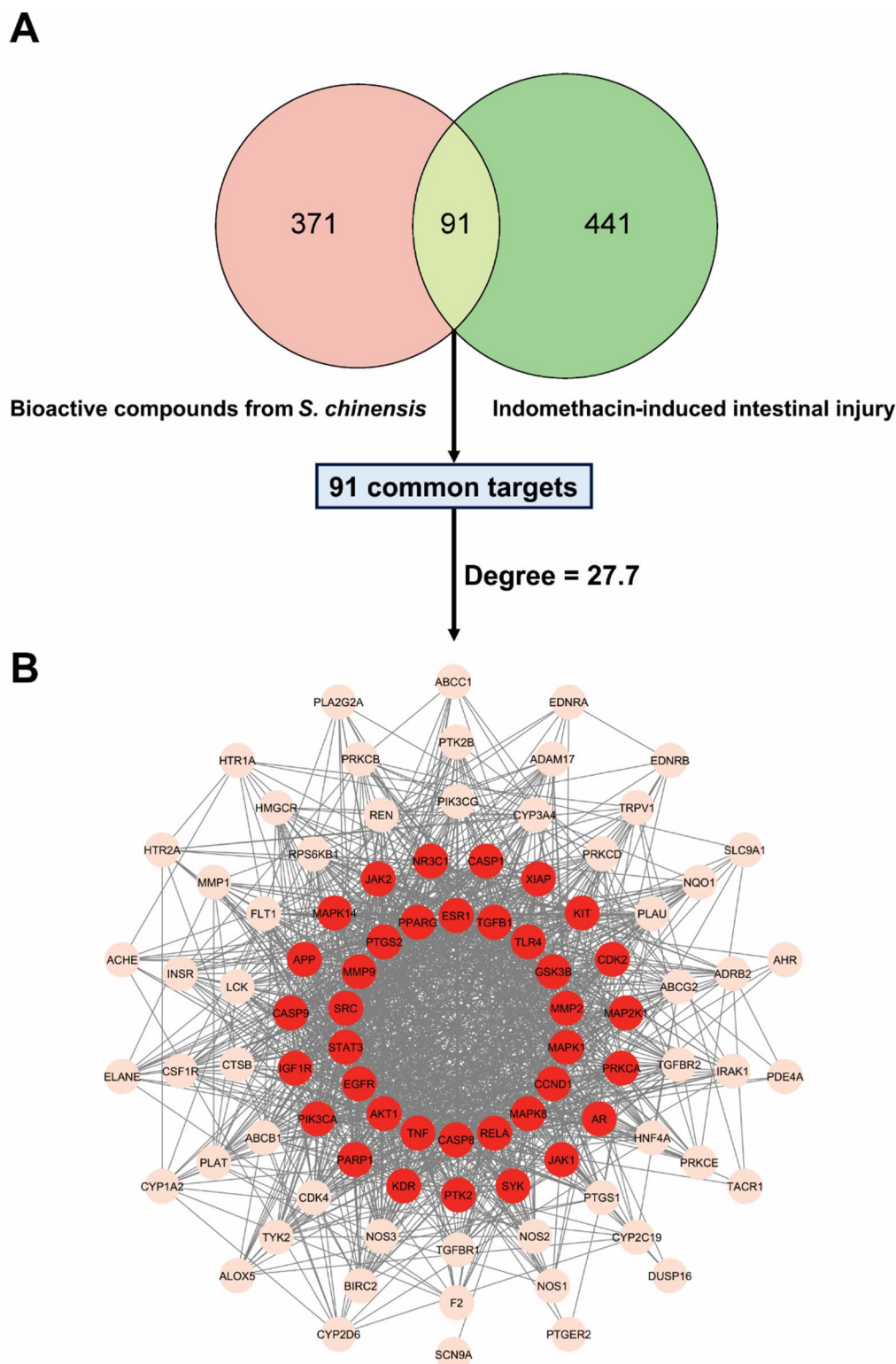


Fig. 1 Identification of the potential targets of *S. chinensis* bioactive compounds against indomethacin-induced intestinal injury. **(A)** Venn diagram of 91 common targets related to *S. chinensis* compounds and indomethacin-induced intestinal injury. **(B)** The PPI network was constructed from 91 common targets and the 37 core targets, with degree values higher than the average (≥ 27.7), indicated by red nodes

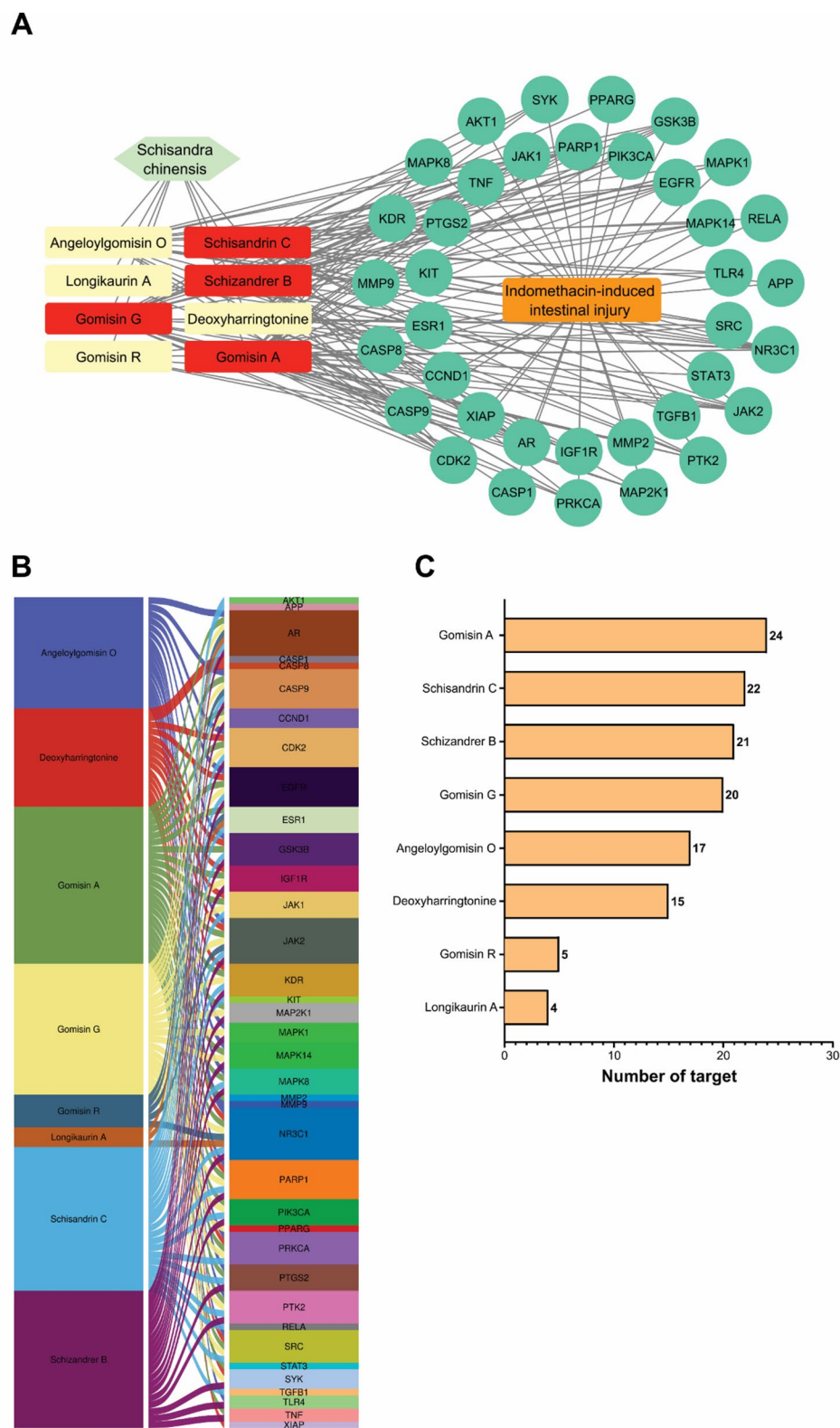


Fig. 2 Identification of key bioactive components from *S. chinensis*. **(A)** Compound–target–disease (C–T–D) network. Red rectangular nodes indicate the top 4 potential compounds with the highest degree in the C–T–D network. **(B)** Sankey diagram of the associations between bioactive compounds from *S. chinensis* and their predicted targets. **(C)** The bar chart displays eight bioactive compounds with their number of targets

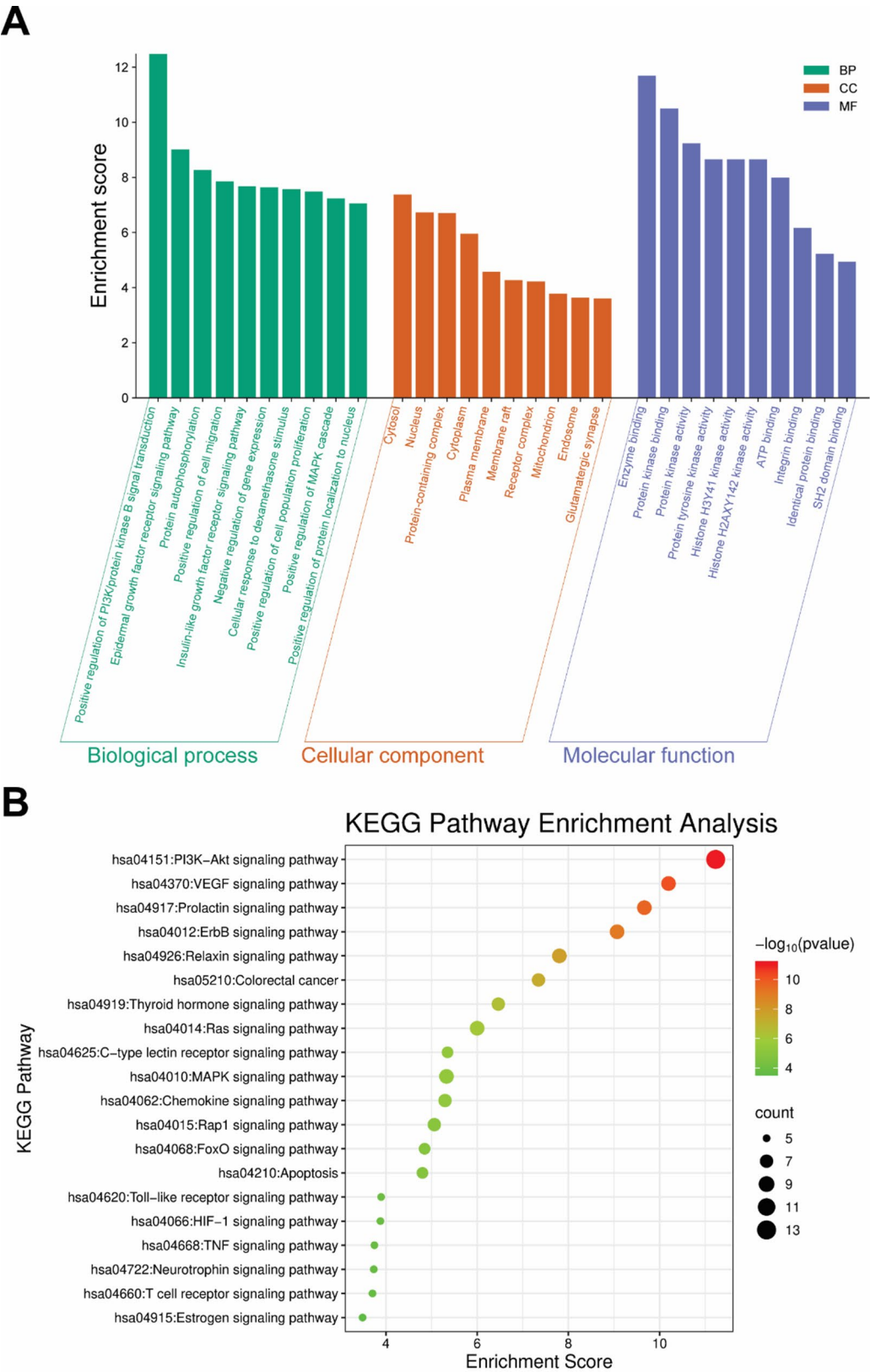


Fig. 3 GO and KEGG pathway enrichment analysis. **(A)** The top 10 biological process (BP), 10 cellular component (CC), and 10 molecular function (MF) terms. **(B)** Bubble plots displaying the top 20 KEGG signaling pathways related to the 22 core targets of SCHC

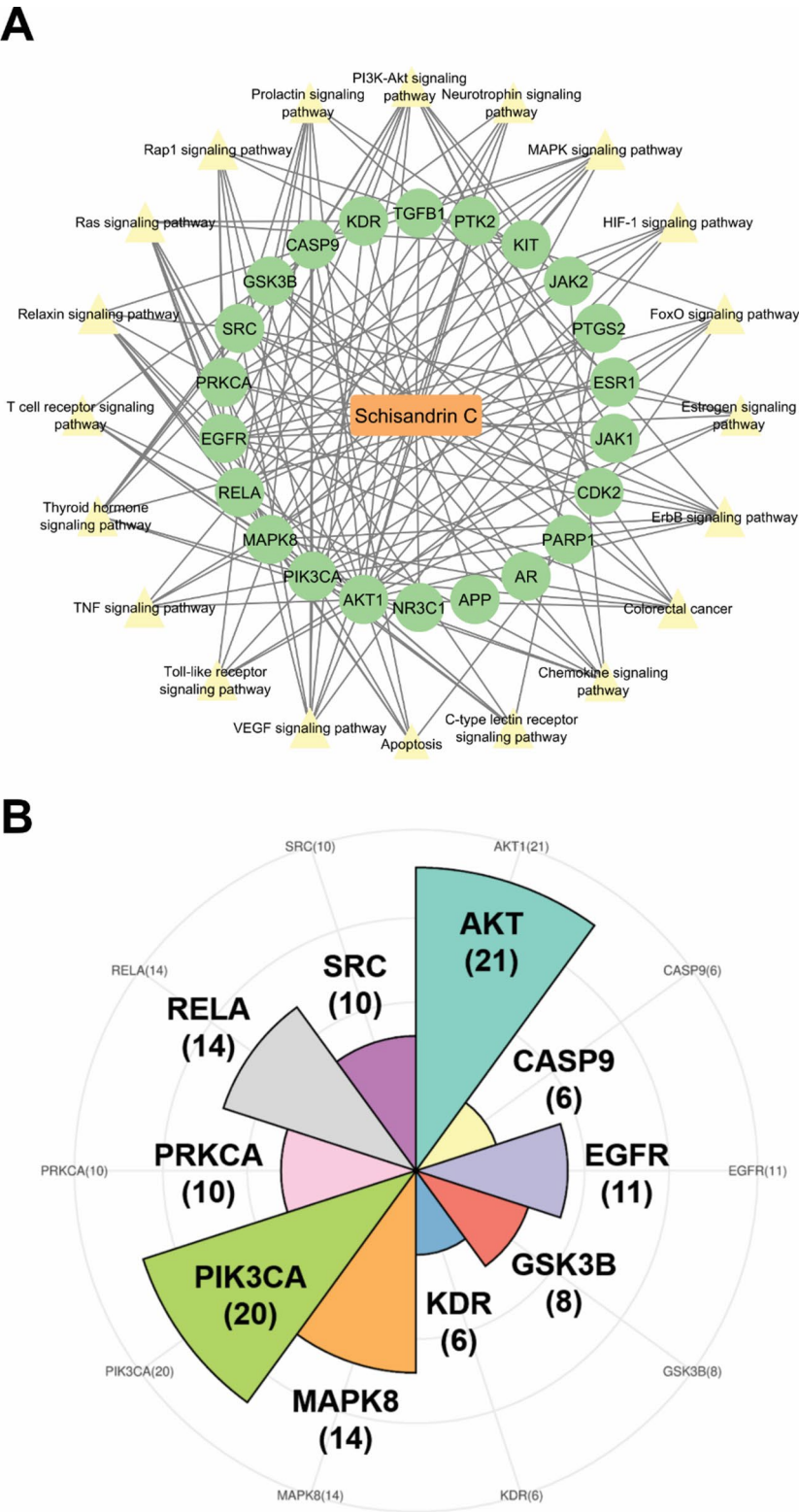


Fig. 4 Construction of a compound–target–pathway (C–T–P) network for SCHC on the basis of its predicted targets and relevant signaling pathways. **(A)** The C–T–P network was constructed with Cytoscape software. **(B)** The pie chart displays the top 10 core targets of SCHC analyzed by the C–T–P network with their degree values

Molecular docking and molecular dynamics simulation

Following the network pharmacology results, the interactions of SCHC with its five core target proteins related to the PI3K/AKT signaling pathway, including AKT1, PIK3CA, RELA, EGFR, and GSK3B, were simulated via molecular docking. The molecular docking data indicated that SCHC has relatively good binding affinity (< -5 kcal/mol) for three targets, namely, GSK3B, EGFR, and PIK3CA. The binding energies between SCHC and these three target proteins were -6.377 (GSK3B), -5.992 (EGFR), and -5.871 kcal/mol (PIK3CA) (Fig. 5, Supplementary Table S7). For these three proteins, IFD was performed to refine the ligand binding poses by incorporating protein flexibility through adjustments of side-chain and backbone conformations. As shown in Fig. 5, the cyclic ether oxygens of SCHC play a crucial role in key interactions within the binding sites. In PIK3CA, all four cyclic ether oxygens form hydrogen or aromatic hydrogen bond interactions with residues S774, W780, K802, and Y836. In EGFR and GSK3B, a single cyclic ether oxygen at position 3 of SCHC forms a hydrogen bond with the amide NH of M793 and V135, respectively. Additionally, the 5-CH group forms aromatic hydrogen bonds with the carbonyl groups of M793 and V135 in EGFR and GSK3B, respectively. These three proteins are reportedly involved in the PI3K/AKT signaling pathway [48]. Therefore, on the basis of molecular docking and network pharmacology analysis, we speculated that the PI3K/AKT signaling pathway might be involved in the protective effect of SCHC.

Based on the molecular docking results, the binding stability of SCHC with PIK3CA, EGFR, and GSK3B was evaluated by MD simulations. First, the RMSD was used as a measure to assess the thermodynamic stability of the ligand-bound systems (Fig. 6A, B, and C). In the PIK3CA and EGFR complexes, the proteins were stabilized between 80 and 100 ns, with RMSD values of approximately 3.5 Å and 4.8 Å, respectively. The GSK3B complex displayed slight RMSD fluctuations up to 40 ns but stabilized thereafter, with RMSD values below 2.8 Å. The ligands remained securely bound within the binding pockets in all cases, indicating stable interactions. These findings demonstrate that the SCHC–target protein complexes maintained thermodynamic stability throughout the simulation.

Additionally, the flexibility within the SCHC–target protein complexes was assessed using RMSF analysis, as shown in Fig. 6D, E, and F. In the PIK3CA complex, most amino acid residues exhibited RMSF values below 5 Å, except for residues in loop regions D209 and I348, which had RMSF values of 5.21 Å and 5.93 Å, respectively. The EGFR complex showed RMSF values below 5 Å for most residues, with the exceptions in loop region A864–K867, which displayed RMSF values ranging from 5.85 Å

to 12.58 Å. These fluctuations may be attributed to the nearby missing loop segment spanning residues 868–875. Similarly, the GSK3B complex had RMSF values below 5 Å, except for residues F293–Q295, which exhibited RMSF values between 5.32 Å and 8.23 Å, likely due to the adjacent missing loop at residues 290–292.

The protein–ligand interactions were monitored throughout the molecular dynamics simulations and categorized into three main types, namely, hydrogen bonds, hydrophobic interactions, and water bridges (Supplementary Fig. S3). For PIK3CA, the results aligned with the initial IFD docking poses, highlighting Y836 and K802 as the key residues involved in interactions with the cyclic ether oxygen at positions 12 and 13, primarily through water bridges. Additional residues, such as D810 and D933, also contributed by forming water bridge interactions with the cyclic ether oxygen at position 12. For EGFR and GSK3B, the primary interactions were mediated by M793 and V135, respectively, forming hydrogen bonds with the cyclic ether oxygen at position 3. Analysis of the MD simulation trajectories revealed that water bridges played a significant role in stabilizing the ligand binding poses, which was not fully apparent from the docking models alone. These observations confirm that the binding poses and interactions identified during IFD were maintained and further stabilized during the simulations. The cyclic ether oxygens, particularly at positions 3 and 12, were identified as critical contributors to binding interactions, consistent with the IFD results.

Experimental validation

Effects of SCHC on indomethacin-induced cell death and inflammation in Caco-2 cells

The network pharmacology results indicated that the effect of SCHC is related to apoptotic cell death (Fig. 3B), and a previous study consistently reported that indomethacin triggered apoptotic cell death in intestinal cells [21]. Therefore, the attenuation of intestinal cell death is helpful in treating leaky gut conditions caused by chronic indomethacin consumption [49]. Our data demonstrated that indomethacin dose-dependently decreased the viability of undifferentiated Caco-2 cells. Compared with no treatment, indomethacin (1 and 2 mM) dramatically reduced the viability of undifferentiated Caco-2 cells (Supplementary Fig. S4).

The protective effect of SCHC was subsequently determined by measuring the viability of indomethacin-stimulated undifferentiated Caco-2 cells. In this study, quercetin, which was reported to protect against indomethacin-induced oxidative stress and inflammation in Caco-2 cells through the NF- κ B and Nrf-2 signaling pathways, was used as a positive control [4]. First, our data revealed that SCHC at concentrations of 20 and 40 μ M had toxic effects on cells by decreasing cell viability

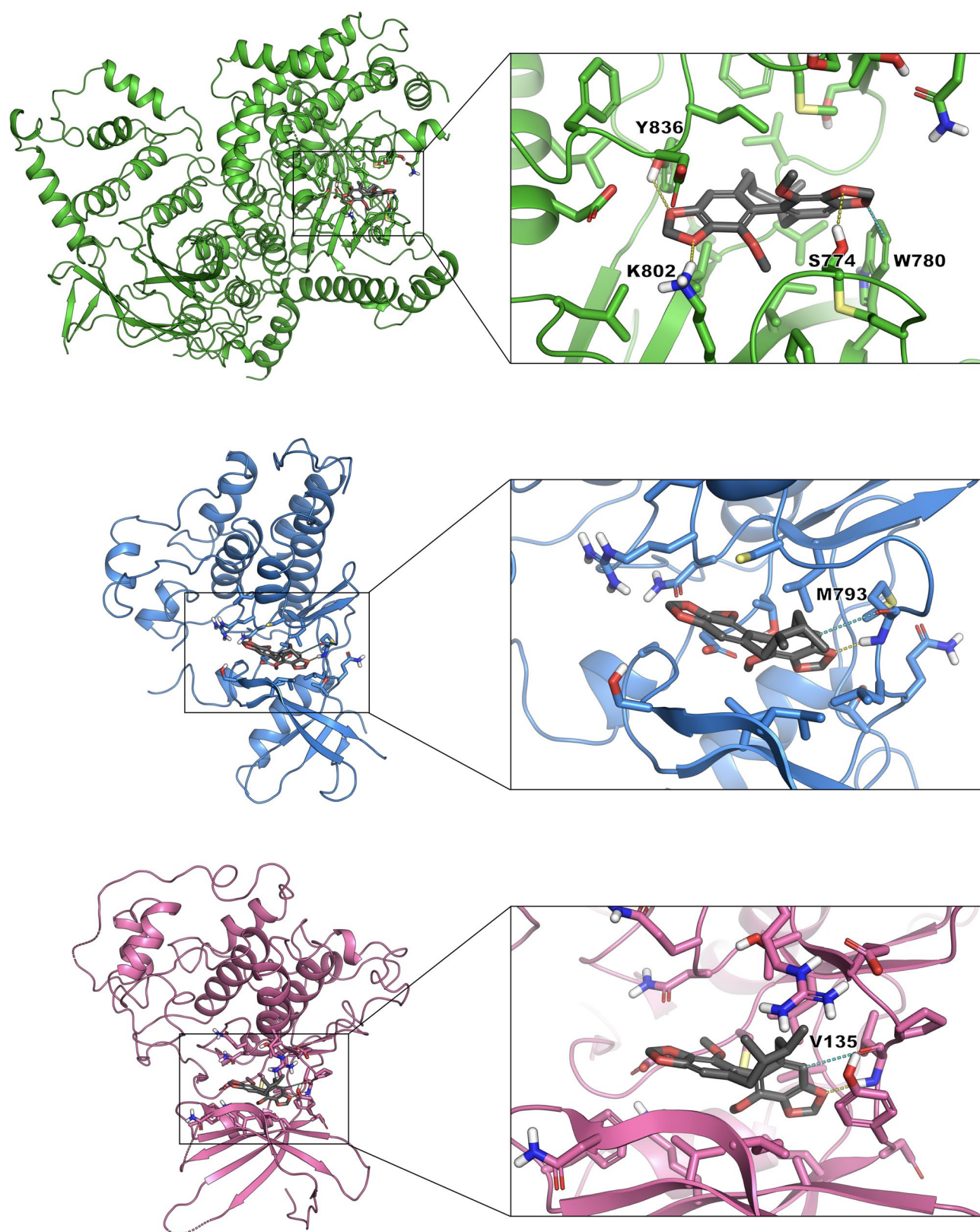


Fig. 5 Induced fit docking (IFD) binding poses of SCHC (dark grey) in PIK3CA (green), EGFR (blue), and GSK3B (pink) with favorable binding energies (< -5 kcal/mol). Sticks represent residues close to the ligand (within 4.5 Å). Key interactions including hydrogen bonds (yellow dashes) and aromatic hydrogen bonds (cyan dashes) are shown, with labels for the involved residues

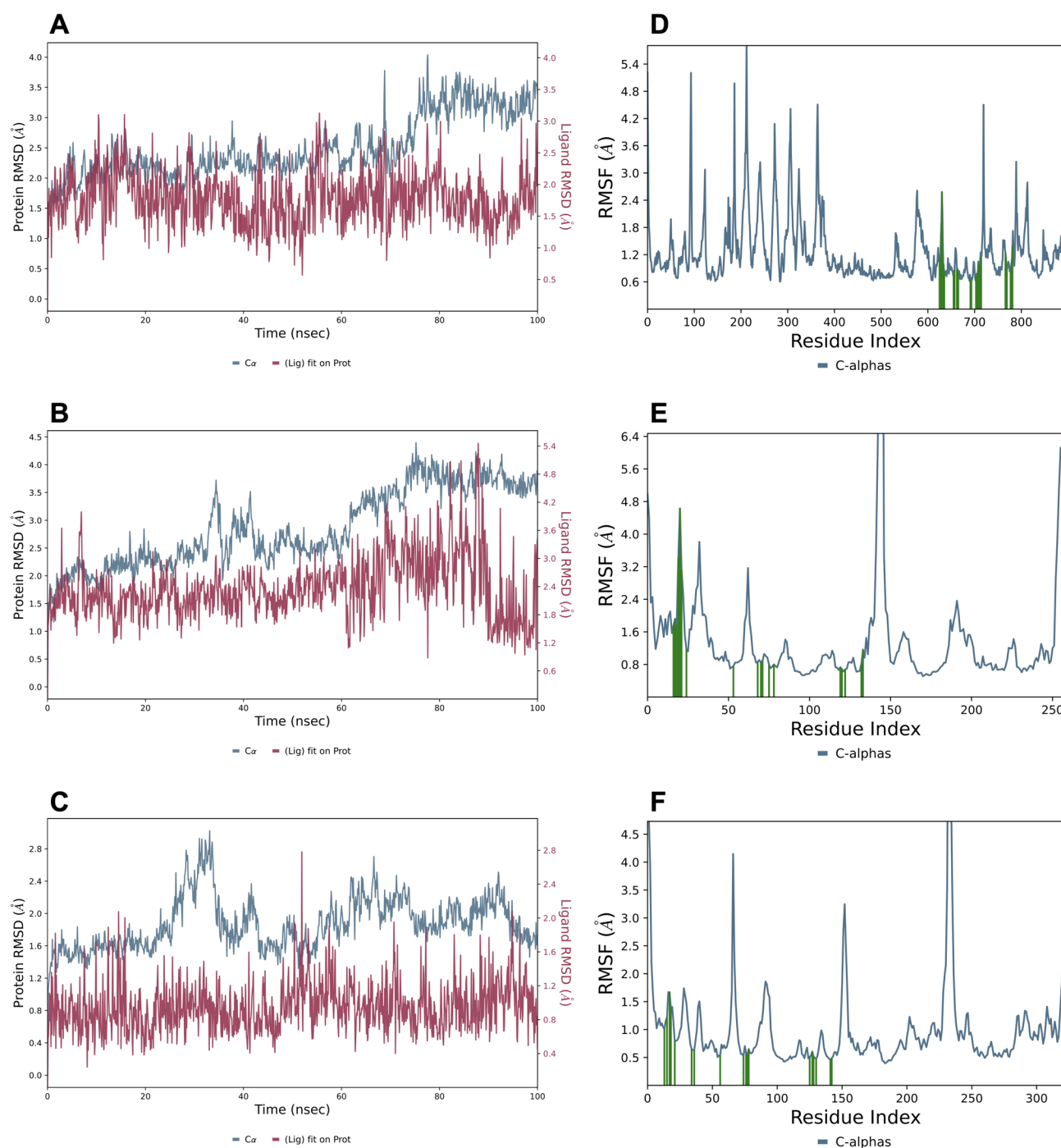


Fig. 6 RMSD and RMSF analyses of selected target protein complexes with SCHC. **(A)** RMSD profile of the PIK3CA complex; **(B)** RMSD profile of the EGFR complex; **(C)** RMSD profile of the GSK3B complex. The protein RMSD of Cα atoms is represented on the left Y-axis, while the ligand RMSD is displayed on the right Y-axis over the simulation time. **(D)** Residue-wise RMSF of Cα atoms in the PIK3CA complex; **(E)** Residue-wise RMSF of Cα atoms in the EGFR complex; **(F)** Residue-wise RMSF of Cα atoms in the GSK3B complex. Ligand contacts are illustrated with green lines

(Fig. 7B). Therefore, SCHC (2.5–10 μM) was tested for its ability to protect against indomethacin-stimulated Caco-2 cells. SCHC (5–10 μM) pretreatment significantly restored cell viability compared with that in the indomethacin single treatment group ($p < 0.05$ and $p < 0.01$, respectively) (Fig. 7C). Additionally, the effect of

SCHC on differentiated Caco-2 cells was also evaluated. Compared with the control, indomethacin (1 mM) dramatically decreased cell viability ($p < 0.001$), whereas pretreatment with SCHC (10 μM) significantly restored cell viability ($p < 0.05$) (Supplementary Fig. S5).

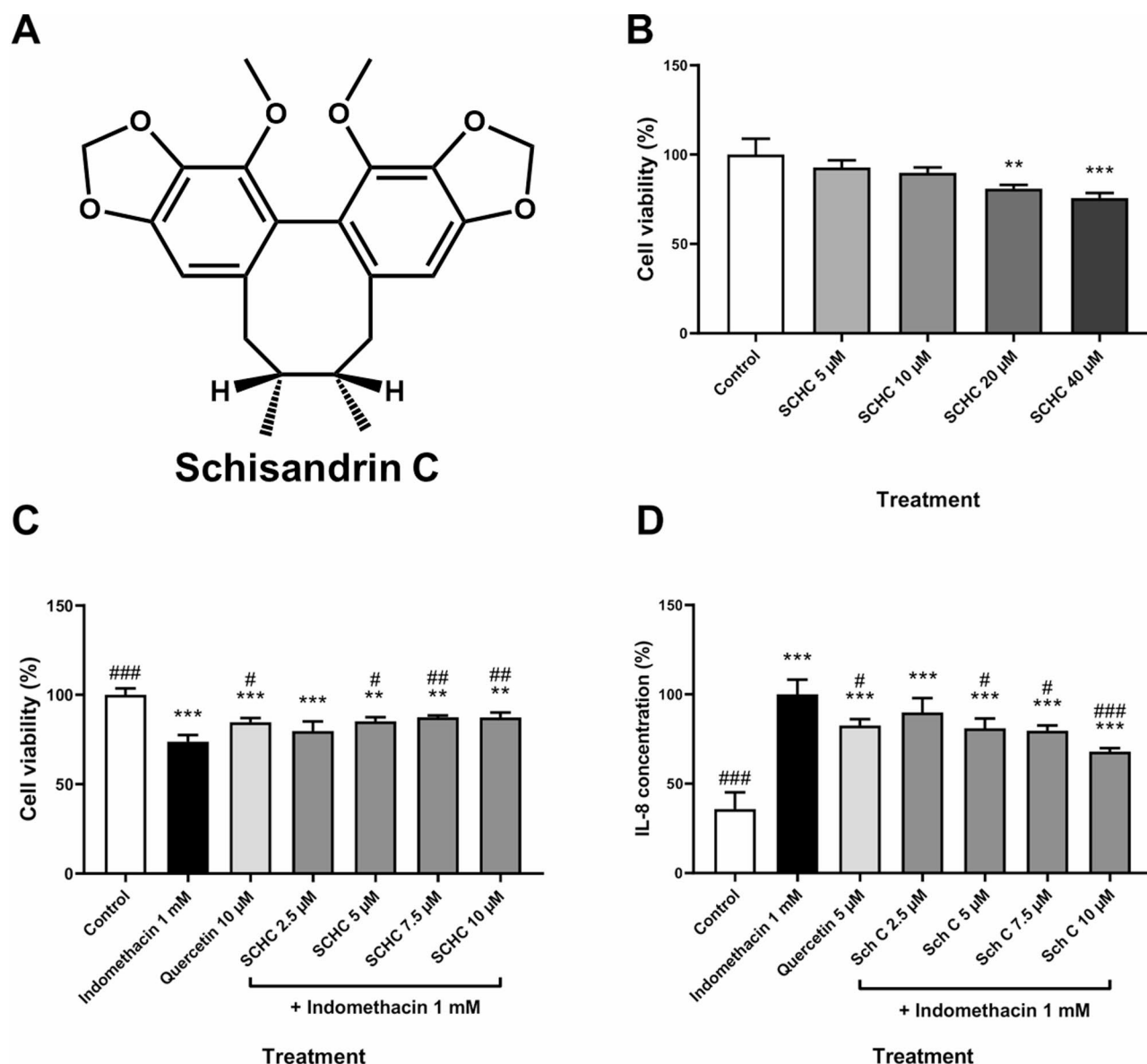


Fig. 7 Chemical structure of SCHC (A). Effect of SCHC on the viability of undifferentiated Caco-2 cells. The cells were treated with SCHC at various concentrations (5–40 μ M) (B). Protective effects of SCHC (2.5–10 μ M) or quercetin (10 μ M, a positive control) against indomethacin (1 mM)-induced death in undifferentiated Caco-2 cells (C). Effects of SCHC (2.5–10 μ M) or quercetin (10 μ M) on indomethacin (1 mM)-induced IL-8 production in undifferentiated Caco-2 cells (D). The data are presented as the means $\pm$ SDs ($n=3$). ** $p < 0.01$, and *** $p < 0.001$, vs. the control. # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$, vs. the indomethacin group. The graphs are representative of three independent experiments

Furthermore, the effect of SCHC on indomethacin-induced intestinal inflammation was also evaluated by measuring the production of NO, IL-6, and IL-8. The results indicated that indomethacin (1 mM) did not induce the secretion of NO and IL-6 in undifferentiated Caco-2 cells (data not shown). Conversely, our data showed that indomethacin (1 mM) dramatically increased IL-8 production in Caco-2 cells ($p < 0.001$; Fig. 7D). Notably, pretreatment with SCHC (5–10 μ M) or quercetin (10 μ M) significantly reduced the IL-8 levels

compared with those in the group treated with indomethacin alone (Fig. 7D).

Effect of SCHC on ROS production in undifferentiated Caco-2 cells

Since ROS are involved in indomethacin-induced intestinal cell death [21], we next evaluated the influence of SCHC on intracellular ROS production in indomethacin-stimulated Caco-2 cells. The results showed that indomethacin (1 mM) treatment dramatically increased the amount of intracellular ROS in differentiated Caco-2 cells

($p < 0.001$). Quercetin, which was used as a positive control, significantly decreased ROS levels ($p < 0.001$). Compared with indomethacin single-treatment, pretreatment with SCHC (7.5 and 10 μM) dramatically inhibited ROS production ($p < 0.001$) (Fig. 8A and B).

Effect of SCHC on TEER and FITC-dextran permeability in intestinal cell monolayers

Because recovery of intestinal barrier function and TJ protein expression are crucial for the treatment of indomethacin-induced intestinal damage [8], we also evaluated the effect of SCHC on indomethacin-induced intestinal barrier dysfunction in Caco-2 cell monolayers. DIM was used as a positive control because it was reported to promote the barrier integrity of the intestinal cell monolayer [40]. Compared with the control treatment, indomethacin (1 mM) treatment for 6 h clearly decreased the TEER value ($p < 0.001$) (Fig. 9A). Pretreatment with DIM (20 μM) increased the TEER, and pretreatment with SCHC (5–10 μM) significantly elevated the TEER (Fig. 9A). Furthermore, FITC-dextran permeability in the Caco-2 cell monolayer was also measured to confirm the protective effect against leaky gut conditions. Indomethacin notably increased intestinal FITC-dextran efflux ($p < 0.001$), and SCHC (5–10 μM) pretreatment potently reduced the efflux of FITC-dextran (Fig. 9B).

Effect of SCHC on indomethacin-induced apoptotic cell death in Caco-2 cells

Indomethacin has been demonstrated to trigger apoptotic intestinal cell death [21], and our computational analysis suggested that the effect of SCHC on indomethacin-induced intestinal injury is associated with the regulation of the apoptosis pathway. Therefore, western blot analysis was conducted to analyze the expression levels of apoptosis-related proteins in indomethacin-treated Caco-2 cells. Our findings revealed that treatment with indomethacin decreased the expression of bcl-2 (an anti-apoptotic protein) and increased the expression of bax (a proapoptotic protein). Conversely, SCHC pretreatment significantly lessened the bax/bcl-2 ratio ($p < 0.01$; Fig. 10A and B). Moreover, our results revealed that cleaved caspase-9, cleaved caspase-3, and cleaved PARP levels were dramatically elevated in the indomethacin-alone treatment group, which demonstrated that indomethacin could induce the activation of apoptotic cell death. SCHC pretreatment effectively decreased the expression levels of apoptosis-related proteins, which indicates that SCHC could suppress the activation of intestinal Caco-2 cell apoptosis (Fig. 10A, C, D, and E). In addition, Annexin V-FITC/PI staining was performed to confirm the effect of SCHC on indomethacin-induced apoptotic cell death. Consistently, indomethacin significantly increased the apoptotic cell population (%) of Caco-2 cells, and pretreatment with SCHC (10 μM)

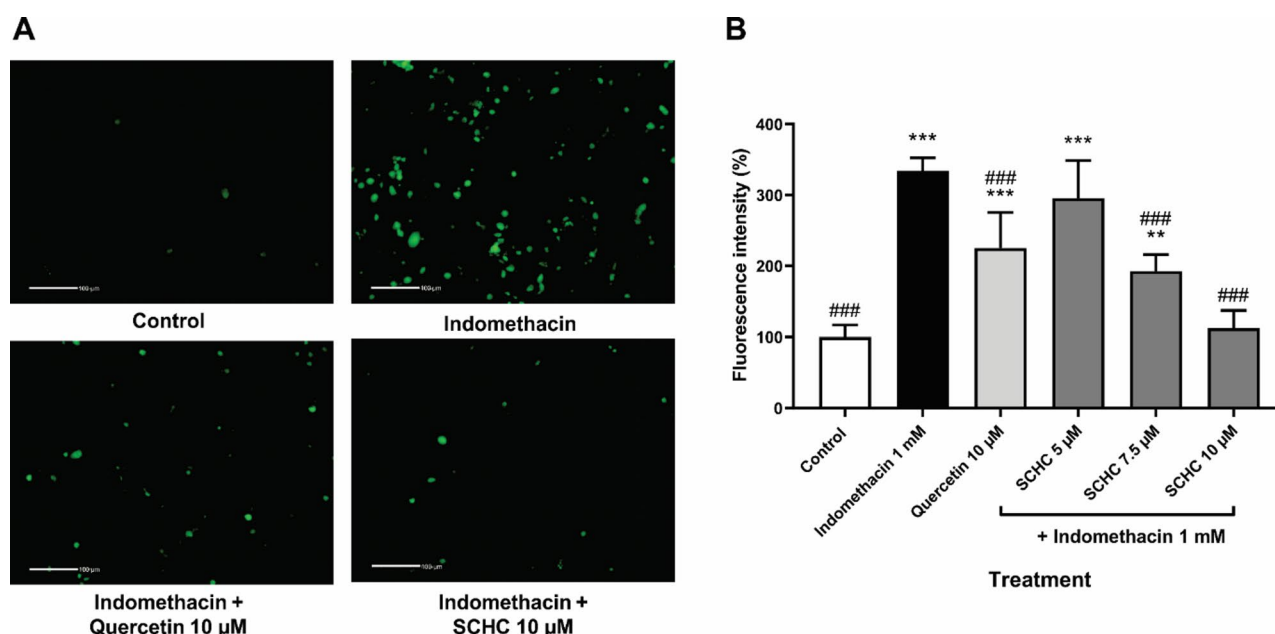


Fig. 8 Effect of SCHC or quercetin (a positive control) on the production of intracellular reactive oxygen species (ROS) induced by indomethacin. Undifferentiated Caco-2 cells were pretreated with SCHC (5–10 μM) or quercetin (10 μM) for 66 h and then stimulated with indomethacin (1 mM) for 4 h. (A) Representative fluorescence images of ROS generation. (B) The quantitative fluorescence intensity reflects the intracellular ROS levels analyzed by Harmony software. The data are shown as the means $\pm$ SDs ($n = 3$) from three independent experiments. ** $p < 0.01$, *** $p < 0.001$ vs. the control group and ### $p < 0.001$ vs. the indomethacin single treatment group. Scale bars = 100 μm

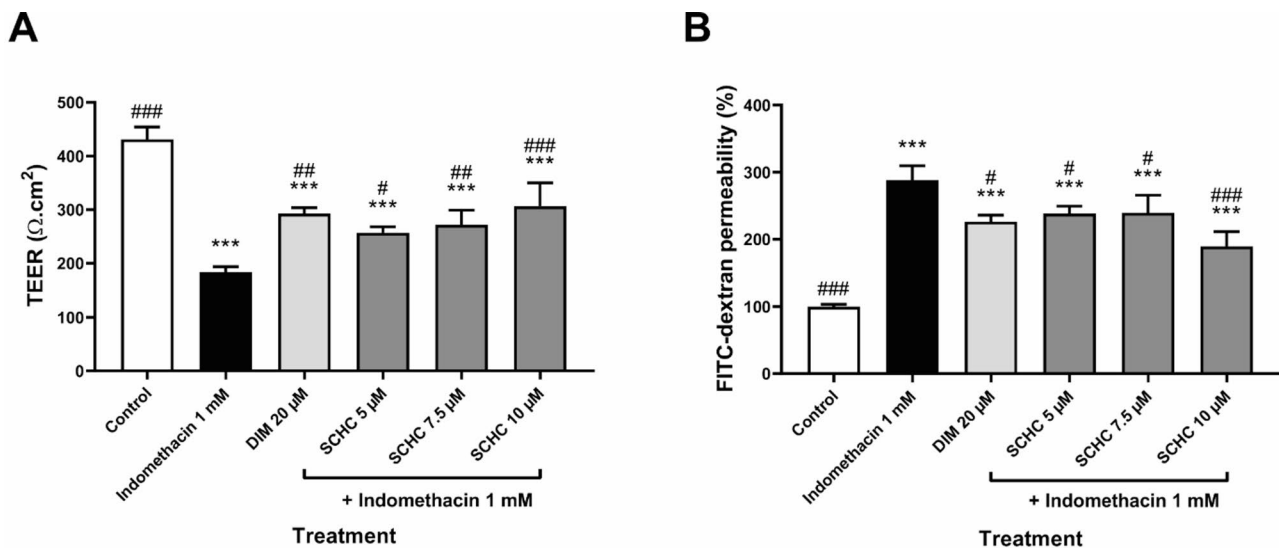


Fig. 9 Effects of SCHC or 3,3'-diindolylmethane (DIM, positive control) on (A) TEER and (B) FITC-dextran permeability in Caco-2 cell monolayers. Differentiated Caco-2 cells were pretreated with DMSO (0.05%), DIM (20 μ M), or SCHC (5–10 μ M) for 66 h. Afterward, indomethacin (1 mM) was added to the apical side for 6 h. *** p < 0.001 vs. control, # p < 0.05, ## p < 0.01, and ### p < 0.001 vs. the indomethacin single treatment group. All the data shown are the means $\pm$ SDs (n = 3), and the graphs are representative of three independent experiments

effectively decreased the apoptotic cell population in indomethacin treated Caco-2 cells (p < 0.001, Fig. 10F).

Effect of SCHC on TJ and AJ protein expression

To clarify the molecular mechanism underlying the protective effect of SCHC against indomethacin-induced intestinal barrier dysfunction, the expression levels of various critical TJ and adherens junction (AJ) proteins, including E-cadherin, claudin-1, claudin-4, occludin, and ZO-1, were analyzed via western blotting, as shown in Fig. 11. As a result, E-cadherin and ZO-1 expression levels were dramatically decreased by treatment with indomethacin alone (p < 0.01 and p < 0.05, respectively) (Fig. 11A and B). Conversely, compared with the indomethacin group, pretreatment with SCHC for 66 h effectively restored the expression levels of ZO-1 and E-cadherin (p < 0.05), which was consistent with the findings of a previous study [8]. Moreover, occludin protein expression was slightly decreased by indomethacin, and SCHC pretreatment increased occludin protein expression, although the difference was not statistically significant. Our data also showed that claudin-1 and claudin-4 expression were not affected by indomethacin (Fig. 11A and B).

Western blot analysis indicated that the expression of ZO-1, a tight junction protein, was most strongly affected by indomethacin. Therefore, the expression level of the ZO-1 protein was also observed via fluorescence microscopy. The expression of ZO-1 in the indomethacin single-treatment group was weaker than that in the untreated group, and SCHC pretreatment significantly restored ZO-1 expression compared with that in the

indomethacin single-treatment group (p < 0.05) (Fig. 11C and D). This microscopic observation was consistent with the western blot results, both of which indicated a decrease in the ZO-1 expression level.

Effect of SCHC on the AKT/GSK-3 β and NF- κ B/MLCK/MLC signaling pathways

Network pharmacology and molecular docking analyses revealed that the protective effect of SCHC against intestinal barrier dysfunction was associated with the PI3K/AKT/GSK-3 β signaling pathway (Figs. 3 and 5). Therefore, p-AKT and p-GSK-3 β expression levels were analyzed. Indomethacin treatment significantly elevated the phosphorylation of AKT and GSK-3 β (p < 0.01 and p < 0.001, respectively) (Fig. 12A, B, and C). Conversely, pretreatment with SCHC significantly decreased the p-AKT/AKT and p-GSK-3 β /GSK-3 β ratios compared with those in the indomethacin single-treatment group (p < 0.01 and p < 0.05, respectively) (Fig. 12A, B, and C).

We also assessed the influence of SCHC on long MLCK expression and MLC phosphorylation because these components regulate the contraction and relaxation of actin fibers and affect intestinal permeability [50]. Indomethacin is known to increase the expression of long MLCK, which is the predominant form in non-muscle cells; as a result, MLC phosphorylation occurs, actin fibers structurally contract, and intestinal integrity decreases [8]. In our experiments, 6 h of indomethacin treatment significantly elevated long MLCK protein expression and MLC phosphorylation (p < 0.05 and p < 0.01, respectively) (Fig. 12A, E, and G). Conversely, pretreatment with SCHC significantly decreased MLCK

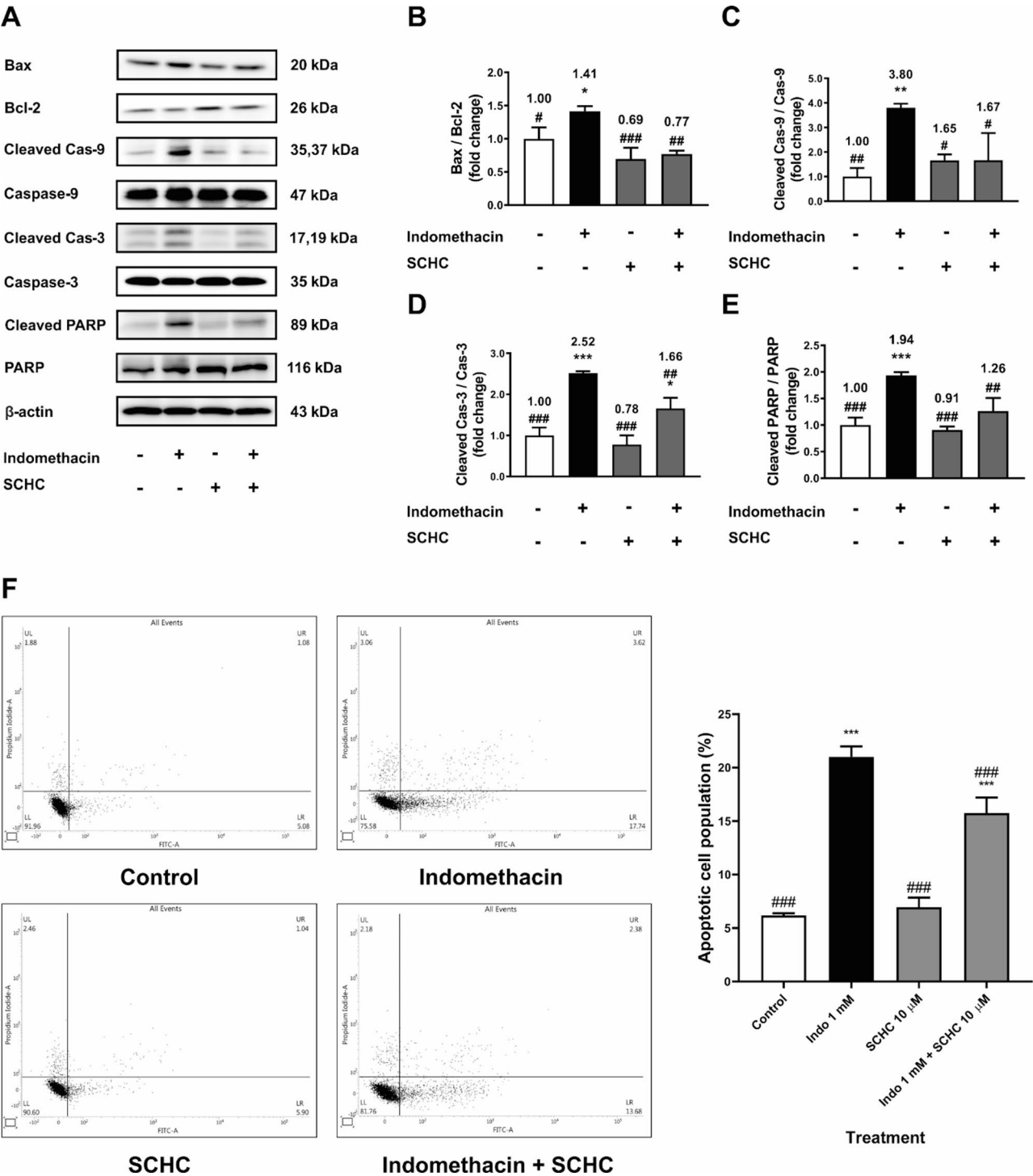


Fig. 10 Effects of SCHC on apoptosis-related protein expression levels were analyzed by immunoblotting. Undifferentiated Caco-2 cells were pretreated with DMSO (0.05%) or SCHC (10 μM) for 66 h. Afterward, the cells were stimulated with indomethacin (1 mM) for 4 h. **(A)** Representative western blot images of bax, bcl-2, cleaved caspase-9, caspase-9 (cas-9), cleaved caspase-3, caspase-3 (cas-3), cleaved PARP, and PARP. **(B)** Bax/bcl-2 ratio, **(C)** cleaved caspase-9/caspase-9 ratio, **(D)** cleaved caspase-3/caspase-3 ratio, and **(E)** cleaved PARP/PARP ratio. **(F)** The effect of SCHC on indomethacin-induced apoptotic cell death in Caco-2 cells was analyzed by flow cytometry with Annexin V-FITC/PI staining. The quantitative data are presented as the means ± SDs from three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control and # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. the indomethacin single treatment group

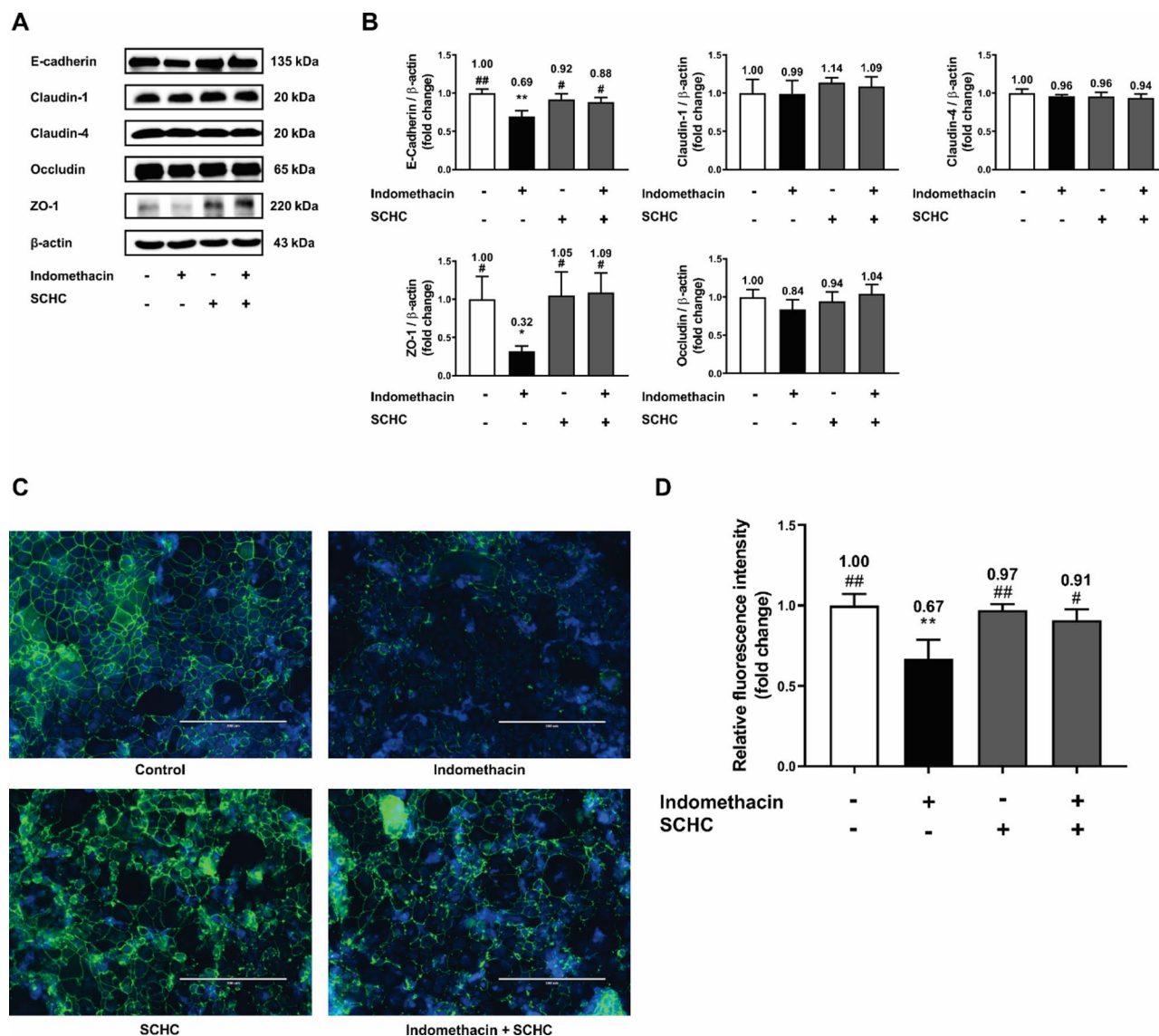


Fig. 11 Effect of SCHC on the expression levels of AJ and TJ proteins (E-cadherin, ZO-1, occludin, claudin-1, and claudin-4). Differentiated Caco-2 cells were pretreated with DMSO (0.05%) or SCHC (10 μ M) for 66 h. Afterward, the cells were stimulated with indomethacin (1 mM) for 6 h. **(A)** Representative western blot images of E-cadherin, claudin-1, claudin-4, occludin, and ZO-1 expression. Relative expression levels of **(B)** E-cadherin, claudin-1, claudin-4, occludin, and ZO-1. **(C)** Representative ZO-1 immunofluorescence images (scale bar = 200 μ m) and **(D)** quantitative ZO-1 expression data were analyzed via ImageJ software. All the data are shown as the means $\pm$ SDs from three independent experiments. * p < 0.05, ** p < 0.01 vs. the control group and # p < 0.05, ## p < 0.01 vs. the indomethacin single treatment group

expression and the p-MLC/MLC ratio compared with those in the indomethacin single-treatment group (p < 0.05) (Fig. 12A, F, and G).

Our network pharmacology analysis revealed that RELA, a subunit of NF- κ B, is a potential protein target of SCHC (Fig. 4). According to a previous study, NF- κ B can translocate to the nucleus, where it binds to the MLCK promoter, thereby increasing MLCK expression levels [51]. Therefore, the expression of I κ B- α and p-NF- κ B was also assessed. Compared with those in the untreated group, indomethacin (1 mM) markedly increased I κ B- α degradation and NF- κ B phosphorylation, thus

demonstrating the activation of the NF- κ B pathway. SCHC pretreatment effectively inhibited the degradation of I κ B- α and decreased the activation or phosphorylation of NF- κ B induced by indomethacin (p < 0.01 and p < 0.05, respectively) (Fig. 12A, D, and E). Taken together, our data suggest that SCHC can regulate the AKT/GSK-3 β and NF- κ B/MLCK/MLC signaling pathways to protect against indomethacin-induced leaky gut conditions.

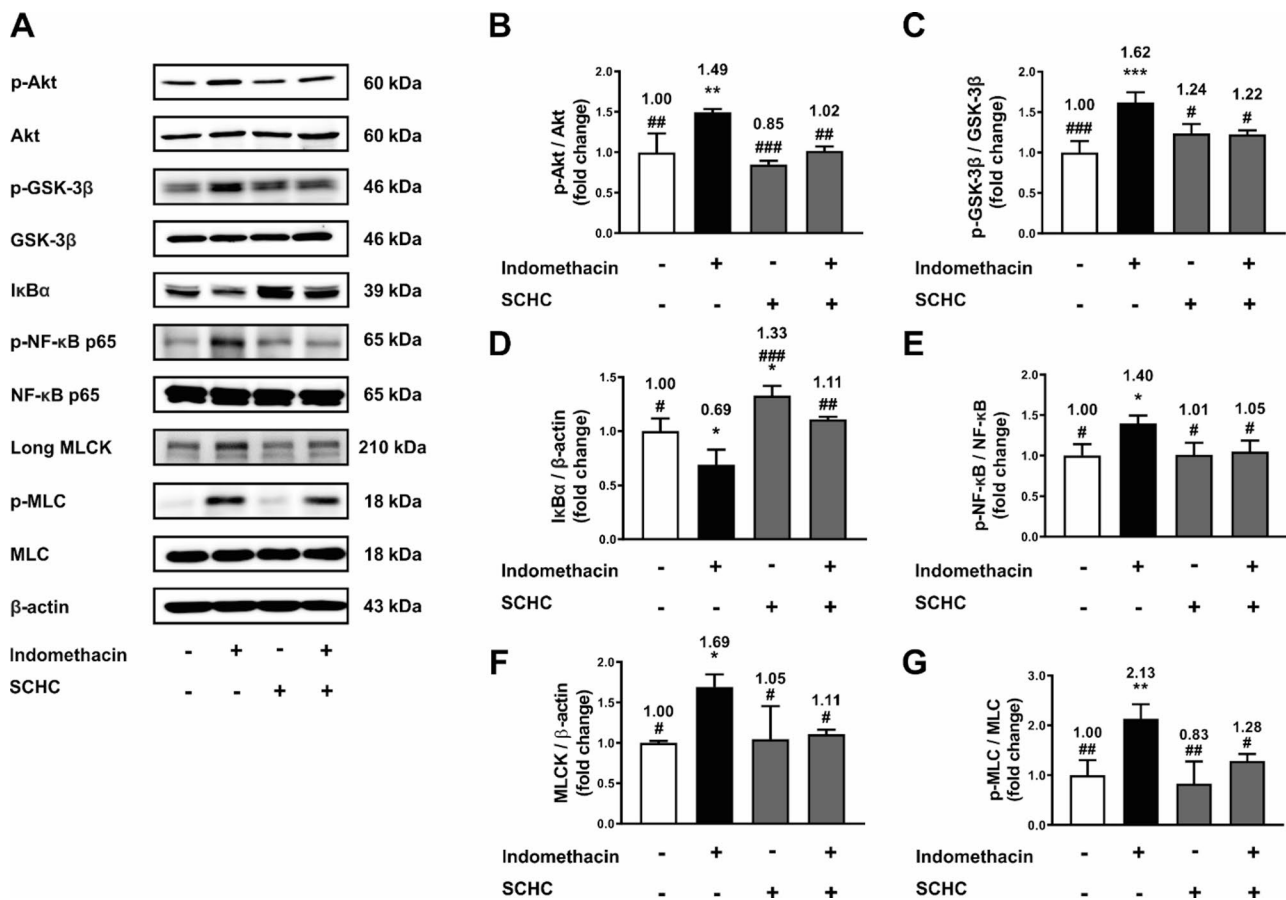


Fig. 12 Effect of SCHC on the AKT/GSK-3 β and NF- κ B/MLCK/MLC signaling pathways. Differentiated Caco-2 cells were pretreated with DMSO (0.05%) or SCHC (10 μ M) for 66 h. Afterward, the cells were stimulated with indomethacin (1 mM) for 6 h. **(A)** Representative western blot images. Relative **(B)** p-AKT/AKT, **(C)** p-GSK-3 β /GSK-3 β , **(D)** I κ B- α / β -actin, **(E)** p-NF- κ B/NF- κ B, **(F)** MLCK/ β -actin, and **(G)** p-MLC (Ser19)/MLC levels were determined by densitometry. The results are shown as the means $\pm$ SDs of three independent measurements. * p < 0.05, ** p < 0.01, *** p < 0.001 vs. control. # p < 0.05, ## p < 0.01, and ### p < 0.001 vs. the indomethacin single treatment group

Effect of SCHC on indomethacin-induced intestinal injury in wild-type *C. elegans* N2

Finally, an *in vivo* *C. elegans* system was used to evaluate the protective ability of SCHC on the intestine because *C. elegans* intestinal epithelium cells are anatomically similar to those of mammals, with the polarized cell structure and apical microvilli [52]. In aged *C. elegans*, the intestinal width tends to decrease with intestinal barrier dysfunction [46, 53]. Therefore, intestinal width can be used as an index to evaluate intestinal morphological atrophy. In this study, we assessed the intestinal width in *C. elegans* after administering indomethacin and SCHC to examine the pathological effect of indomethacin and the protective effect of SCHC in a disease model of indomethacin-induced intestinal atrophy. As a result, *P. aeruginosa* infection, as well as indomethacin (20 mM), significantly decreased the intestinal width of *C. elegans* (p < 0.001, Fig. 13A and B). Conversely, cotreatment with SCHC significantly increased the intestinal width compared with that in the indomethacin single-treatment

group, and SCHC (10, 25 μ M) exhibited dose-dependent activity (p < 0.05 and p < 0.01, respectively) (Fig. 13A and B).

To further demonstrate the protective effects of SCHC, functional lysosome levels in the intestine of *C. elegans* were determined by staining with acridine orange dye. Lysosomes play crucial roles in various cellular processes, including macromolecule digestion and protein degradation, to maintain homeostasis [54]. In aged *C. elegans*, the activity of intestinal lysosome-related organelles is reduced [55]. Treatment with *P. aeruginosa* significantly decreased functional lysosomal levels in the *C. elegans* intestine (Fig. 13C and D). However, DIM did not reverse the intestinal lysosomal dysfunction caused by *P. aeruginosa* (Fig. 13C and D). Indomethacin treatment significantly decreased intestinal lysosomal levels in *C. elegans* (p < 0.001), and SCHC (10 and 25 μ M) treatment reversed the intestinal lysosomal dysfunction induced by indomethacin treatment (p < 0.01) (Fig. 13C and D).

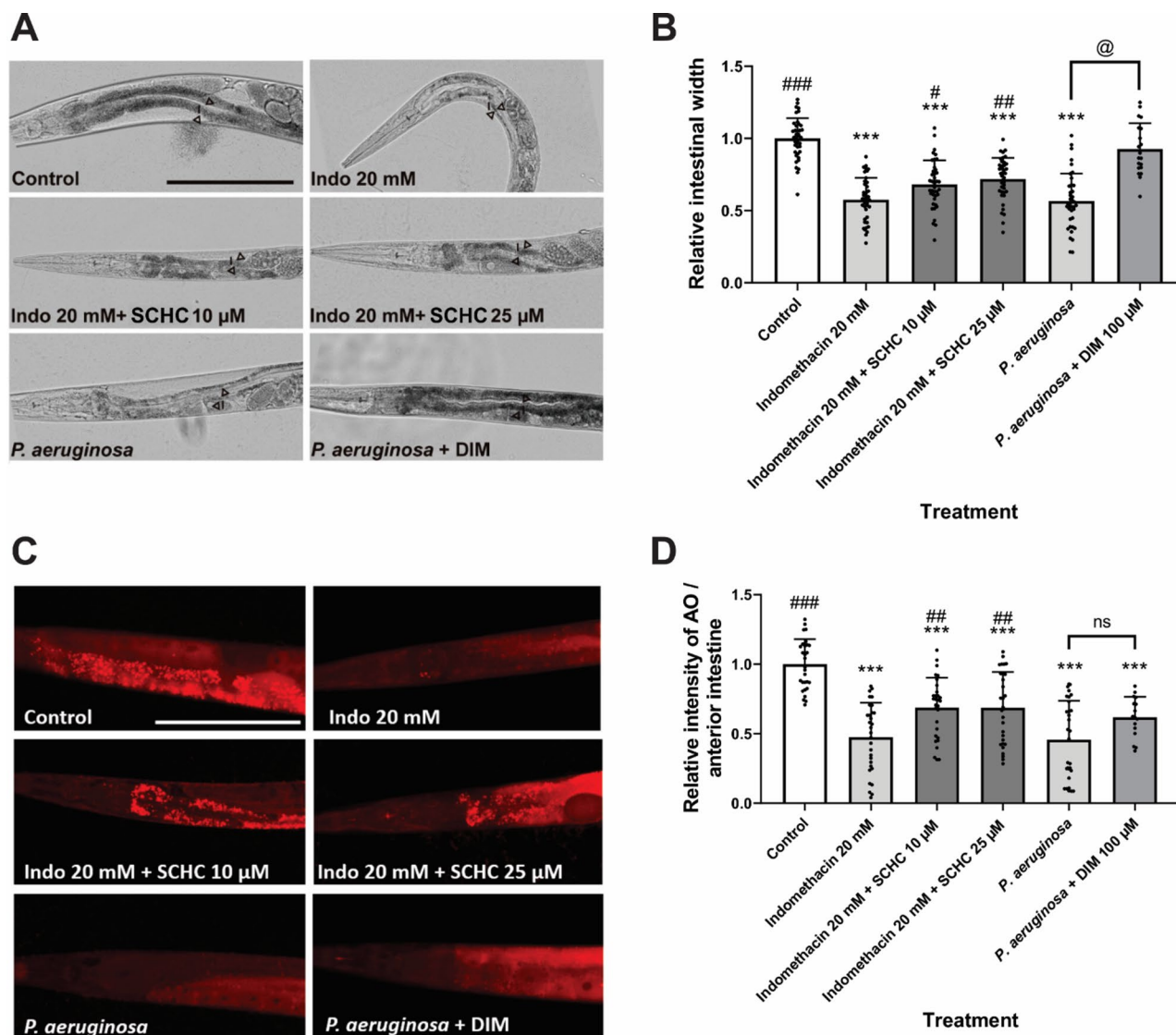


Fig. 13 Effects of SCHC on the indomethacin-induced intestinal width decline and lysosomal dysfunction in a *C. elegans* model. Synchronized L4-stage *C. elegans* N2 worms were fed indomethacin (Indo; 20 mM), indomethacin (20 mM) mixed with SCHC (10 or 25 μ M), *P. aeruginosa*, or *P. aeruginosa* mixed with 3,3'-diindolylmethane (DIM; 100 μ M) for 2 days. **(A)** Representative intestinal images of each treatment group. **(B)** A quantitative graph of the relative intestinal width was generated via ImageJ software. **(C)** Representative images of lysosomes stained with acridine orange (AO) and **(D)** quantification of intestinal lysosome levels were analyzed via ImageJ software. The experimental results are presented as the means $\pm$ SDs from four independent experiments ($n=40$). *** $p < 0.001$ vs. the control group and # $p < 0.05$, ## $p < 0.01$ vs. the indomethacin single-treatment group. @ $p < 0.05$, difference between the *P. aeruginosa* single-treatment group and the *P. aeruginosa* plus DIM group; n.s., not significant. Scale bar = 200 μ m

SCHC did not protect against indomethacin-induced intestinal injury in *age-1*, *akt-1*, and *akt-2* mutant worms

Our network pharmacology analysis and in vitro Caco-2 experiments indicated that SCHC protected against indomethacin side effects by modulating the PI3K/AKT signaling pathway. To further evaluate whether this pathway is involved in the protective effect of SCHC in *C. elegans*, we evaluated the effects of SCHC on *age-1* and *akt-1,2* mutants, which are the orthologs of PI3K and AKT, respectively, in *C. elegans*. The results showed that indomethacin significantly decreased the intestinal

width in all the mutant strains compared with that in the untreated group (Fig. 14A and B). Notably, SCHC (25 μ M) was not able to reverse the intestinal atrophy in these mutant strains ($p > 0.05$). In addition, our data indicated that SCHC (25 μ M) did not reverse the decreases in the lysosome levels induced by indomethacin in the *age-1* (TJ1052), *akt-1* (RB759), and *akt-2* (VC204) mutant strains (Fig. 14C and D). Our findings suggest that SCHC has a potent protective effect against indomethacin-induced intestinal injury in the *C. elegans* model and that the *age-1*, *akt-1*, and *akt-2* genes may play an important

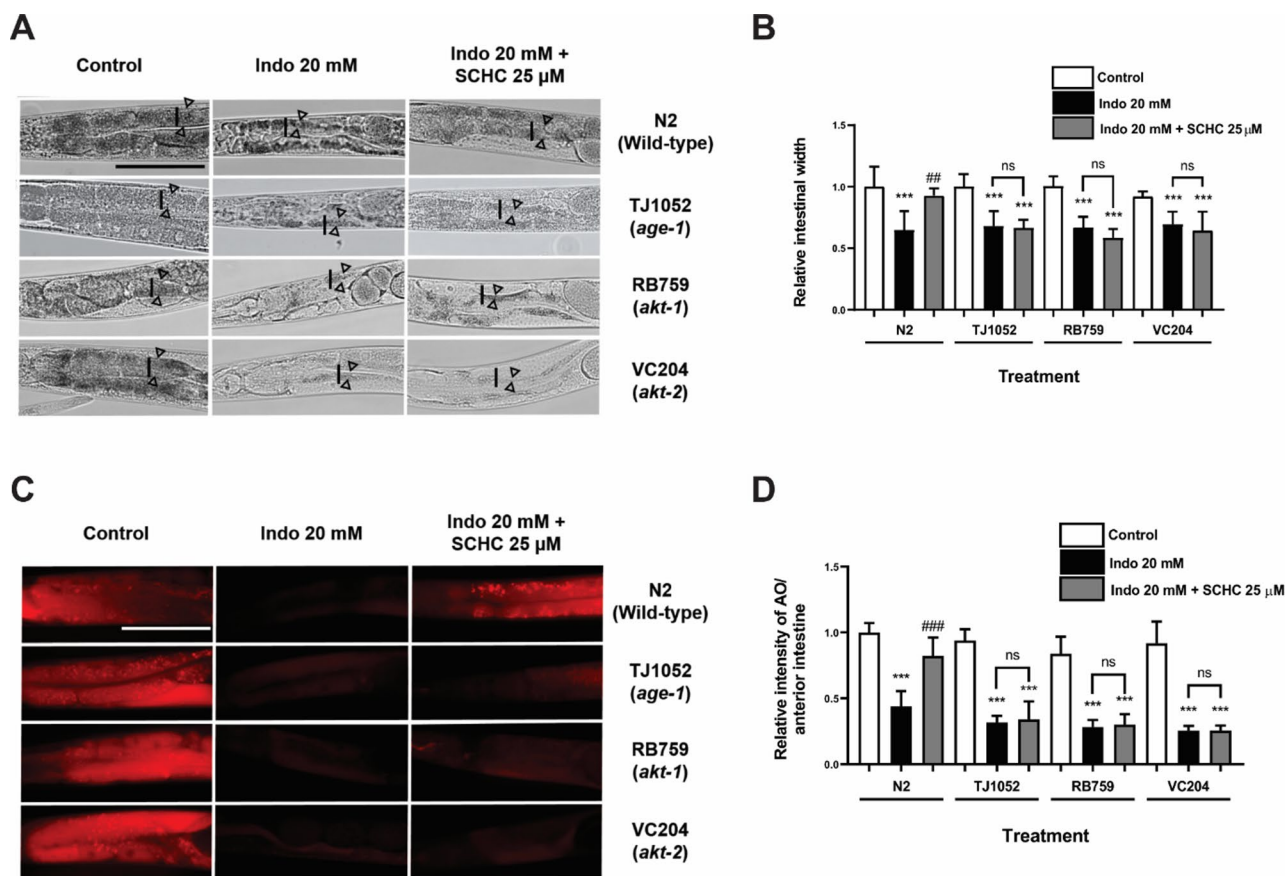


Fig. 14 Effects of SCHC on indomethacin-induced intestinal width decrease and lysosomal dysfunction in a *C. elegans* model. Synchronized L4-stage *C. elegans* TJ1052, RB759, and VC204 worms were fed indomethacin (Indo; 20 mM) or Indo (20 mM) mixed with SCHC (25 μ M). **(A)** Representative intestinal images of each treatment group. **(B)** A quantitative graph of the relative intestinal width was generated via the ImageJ software. **(C)** Representative images of lysosomes stained with acridine orange (AO). **(D)** Intestinal lysosome levels were quantitatively analyzed via the ImageJ software. The experimental results are presented as the means $\pm$ SDs from four independent experiments ($n=40$). *** $p < 0.001$ vs. the control group and ### $p < 0.001$ vs. the indomethacin-alone treatment group. n.s., not significant. Scale bar = 100 μ m

role in the protective effects of SCHC against indomethacin in the *C. elegans* model. Taken together, we confirmed that SCHC modulated intestinal atrophy and lysosome activity in *C. elegans* through the PI3K/AKT signaling pathway.

Discussion

S. chinensis is an herbal medicine used for treating various diseases related to gastrointestinal health. By random screening, our previous study revealed that four lignan compounds of *S. chinensis*, namely, gomisin N, schisan-drin A, schisan-drin B, and SCHC, had anti-inflammatory effects on IL-1 β -stimulated intestinal cells [20]. However, in this study, we speculated that indomethacin exhibits a different mechanism of action in intestinal cells than cytokines. For example, three compounds, gomisin A, gomisin J, and SCHC, showed protective effects against indomethacin (1 mM)-induced intestinal cell death (Supplementary Fig. S6). Hence, we applied a network pharmacology approach to effectively identify the main

bioactive compounds in *S. chinensis* that could protect against indomethacin-induced intestinal injury and elucidate the molecular mechanism underlying the protective effect. Based on network pharmacology and analysis of its drug-like properties, SCHC was selected as the key bioactive component of *S. chinensis* that might play a crucial role in protection against the side effects of indomethacin. The network pharmacology results also indicated that SCHC could exhibit activity through multiple targets and pathways.

In the present study, the potential target proteins of SCHC were predicted via two databases, namely, SwissTargetPrediction and TargetNet. These two databases provide potential targets by comparing the chemical structure of the compound with known ligands collected from all biochemical assays that are based on the direct binding of ligands and proteins [26, 27]. Hence, molecular docking and molecular dynamics were also performed to simulate the interaction between SCHC and its target proteins. The molecular docking and molecular dynamics

simulation results revealed that SCHC strongly interacted with GSK3B, EGFR, and PIK3CA, which suggested that SCHC could affect the PI3K/AKT/GSK-3 β signaling pathway. Our results revealed that SCHC could directly bind to these targets and exert beneficial effects on indomethacin-induced intestinal injury by modulating the structures and activities of these target proteins. However, additional biophysical experiments, such as surface plasmon resonance (SPR) or isothermal titration calorimetry (ITC), are needed in further studies to directly demonstrate the binding of SCHC to these protein targets.

Network pharmacology analysis revealed that the protective effects of SCHC against indomethacin-induced intestinal injury might be associated with apoptotic cell death and the target protein caspase-9. Caspase-9, encoded by the CASP9 gene, is known as the initiator caspase required to activate apoptotic cell death [56]. Our western blot analysis consistently revealed that indomethacin increased cleaved caspase-9 levels, which led to the activation of various downstream pathways, such as the caspase-3 and PARP pathways, which ultimately activated the apoptotic cell death pathway. Our data demonstrated that SCHC significantly inhibited the activation of the apoptotic caspase cascade. In addition, ROS generation induced by oxidative chemical stress is the critical step that causes intestinal cells to undergo apoptotic cell death [21]. SCHC decreased intracellular ROS production in cultured intestinal cells stimulated with indomethacin (Fig. 8). Thus, we speculated that the cytoprotective effect of SCHC is associated with its antioxidant activity. However, SCHC cannot directly scavenge free radicals in 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assays, with IC_{50} values > 1000 μ M (data not shown). A previous study has demonstrated that SCHC can ameliorate angiotensin II-induced endothelial deficits in rat aortic endothelial cells by activating the Nrf-2 pathway [57]. These data suggest that SCHC may also activate the Keap1/Nrf-2/ARE signaling pathway in Caco-2 cells. In this study, Nrf-2 and Keap1 were not predicted to be targets of SCHC by either SwissTargetPrediction or TargetNet, which could be a limitation of target protein prediction. However, our analysis suggests that SCHC could activate Nrf-2 indirectly by targeting proteins that are involved in the upstream signaling pathways of Nrf-2 activation. Otherwise, Nrf-2 could also be activated by SCHC through ROS-related redox signaling originating from direct chemical reactions occurring mainly in mitochondria, rather than through interaction between target proteins and SCHC. Therefore, further in-depth studies are needed to clarify the specific upstream signaling pathways responsible for the protective effect of SCHC on indomethacin-induced apoptotic cell death. In addition to inducing of apoptotic cell death,

indomethacin has also been reported to cause gastrointestinal damage by triggering autophagic cell death [58]. Therefore, further studies are required to elucidate the protective effects of SCHC against indomethacin via other programmed cell death pathways.

In addition to the induction of apoptotic cell death and inflammation in intestinal epithelial cells, a decrease in AJ and TJ protein expression is correlated with intestinal barrier dysfunction. According to a previous pathological molecular mechanism study, indomethacin causes intestinal permeability dysfunction by altering the ERK-MLCK-TJ signaling pathway [8]. Our western blot analysis consistently revealed that indomethacin increased long MLCK expression; we also observed that SCHC could regulate the MLCK-TJ signaling pathway by decreasing MLCK and p-MLC expression levels. However, the upstream molecular signaling pathways by which indomethacin is involved in the upregulation of MLCK have not been identified. According to a previous study, the activation of PI3K/AKT signaling induces the degradation of I κ B- α , leading to the activation of NF- κ B, which can bind to MLCK to induce intestinal barrier dysfunction [59]. Through the combination of computational prediction and western blot experiments in the present study, we demonstrated for the first time that indomethacin induced the AKT/GSK-3 β activation and that SCHC prevented indomethacin-induced intestinal injury by modulating this pathway (Fig. 12). Generally, the AKT activation is mediated by the conversion of PIP2 to PIP3, which is catalyzed by the PIK3CA-encoded PI3K p110 subunit [60, 61]. Network pharmacology and molecular docking analyses indicated that SCHC had high affinity for PI3K p110, which is encoded by PIK3CA. Notably, our western blot analysis results indicated that SCHC treatment reduced the phosphorylation level of AKT but did not alter the expression level of PI3K p110 (data not shown). These data suggested that SCHC could directly bind to PI3K p110 and inhibit its catalytic activity rather than modulate its expression levels.

In addition, our network pharmacology analysis revealed that GSK-3 β was a target protein of SCHC (Fig. 4). GSK-3 β is a downstream target of the PI3K/AKT signaling pathway, where the phosphorylation of AKT prompts GSK-3 β phosphorylation, leading to the inhibition of its normal activity [48]. According to a previous study, the inhibition of GSK-3 β affects epithelial barrier function by decreasing the expression of TJ and AJ proteins, such as claudin-1, occludin, and E-cadherin [62]. In this study, molecular docking data also revealed that SCHC has good binding affinity for GSK-3 β (Fig. 5) and that SCHC decreased the phosphorylation of GSK-3 β induced by indomethacin treatment (Fig. 12). Therefore, SCHC may bind to GSK-3 β and inhibit its phosphorylation, thereby improving intestinal cell permeability. The

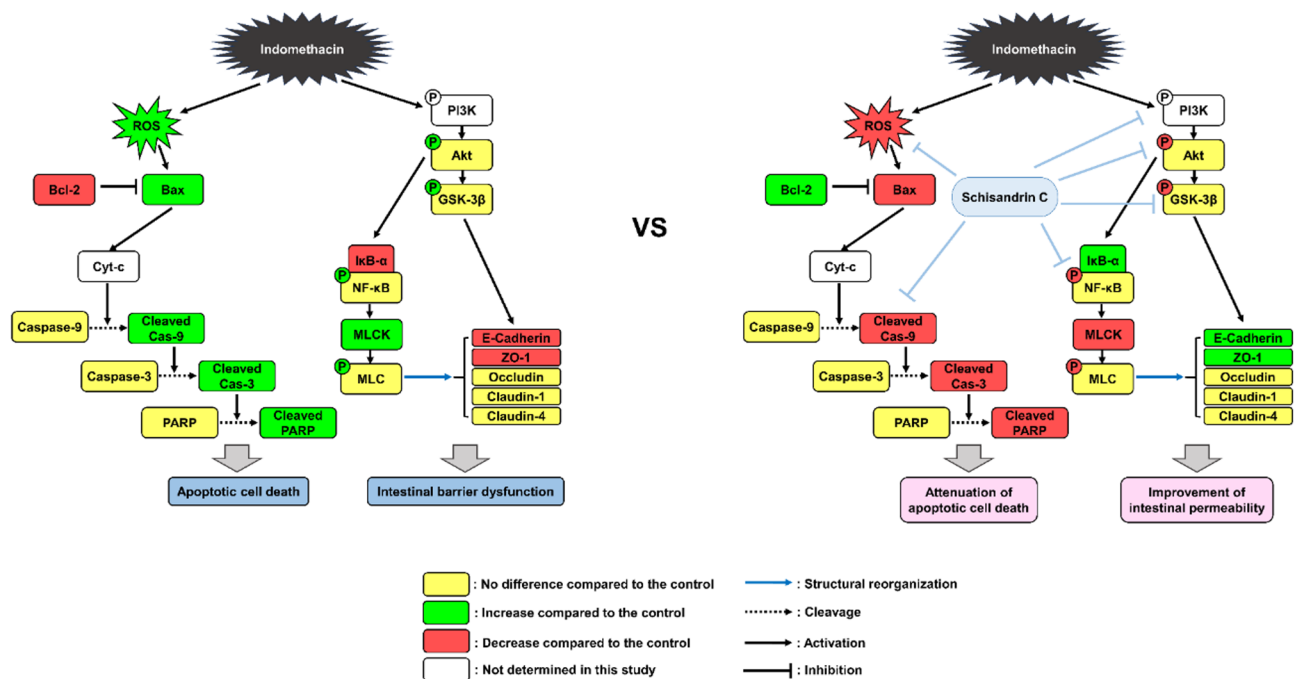


Fig. 15 Diagram illustrating the signaling pathway responsible for the protective effect of SCHC on indomethacin-induced intestinal injury

potential signaling pathways underlying the protective effect of SCHC against indomethacin-induced intestinal injury are summarized in Fig. 15.

C. elegans is a powerful and convenient experimental animal for evaluating the toxicological and pharmacological effects of drugs and candidate chemicals on various intestinal diseases because its intestinal structure is similar to that of the human intestine [40, 43, 63]. In addition, *C. elegans* is also a cost-effective model and can be used for high-throughput screening to discover and elucidate the beneficial effects of natural compounds on intestinal health [63]. For that reason, we selected *C. elegans* as an in vivo model. For the first time, the negative impacts of indomethacin on the intestinal health of *C. elegans* were successfully established. This experimental model will aid in the screening of new bioactive compounds that can protect against indomethacin-induced intestinal dysfunction. Previously, we investigated the effects of two natural compounds, DIM (a functional metabolite found in cruciferous vegetables) and SCHC, on improving intestinal leakage in *C. elegans* induced by pathogenic infection with *P. aeruginosa* [20, 40]. In the present study, we demonstrated that indomethacin could induce dysfunction in lysosome-related granules in the *C. elegans* intestine. Additionally, *P. aeruginosa* treatment also caused lysosomal dysfunction in the intestine of *C. elegans*. Interestingly, while the protective effects of DIM on *P. aeruginosa*-induced intestinal barrier dysfunction did not affect intestinal lysosomal levels, SCHC treatment effectively restored the intestinal structure and intestinal

lysosomal levels, which were disrupted by indomethacin treatment. Our findings suggest that SCHC has a distinct function in regulating both intestinal lysosome levels and intestinal morphology under conditions of indomethacin-induced toxic stress. Lysosomes play a vital role in cellular homeostasis, and the maintenance of lysosomal activity has been demonstrated to be closely related to the regulation of the *daf-16* gene, which is inhibited via phosphorylation upon the activation of *age-1* and *akt-1/2* [55, 64]. Our data showed that the SCHC protective effects were not observed in *age-1*, *akt-1*, or *akt-2* mutant worms, which suggests that SCHC exhibits its beneficial effect through the AGE-1/AKT signaling pathway in the *C. elegans* model. These results are consistent with the network pharmacology predictions and validation in Caco-2 cells in vitro, revealing that SCHC could modulate the PI3K/AKT/GSK-3 β pathway. Thus, our results suggest the potential role for SCHC in protecting against the indomethacin-caused intestinal damage, indicating the importance of this compound in maintaining intestinal health in the contexts of toxic chemical consumption and pathogenic infections.

In summary, we found that SCHC, which originates from *S. chinensis*, is a potential candidate for protection against indomethacin-induced intestinal injury, and the molecular mechanism of SCHC was predicted by network pharmacology analysis. We also demonstrated that treatment with SCHC effectively reversed indomethacin-induced intestinal cell death, inflammation, and barrier dysfunction. Remarkably, treatment with SCHC restored

both the defects in intestinal width and the intestinal lysosomal activity induced by indomethacin in the *C. elegans* model. Taken together, our findings provide strong evidence for the protective effect of SCHC on intestinal injury and suggest that SCHC could be a promising candidate for treating the intestinal side effects of indomethacin. In further studies, preclinical rodent experiments and human clinical trials of SCHC will provide valuable insights into the protective effect of SCHC on indomethacin-induced intestinal dysfunction.

Abbreviations

AKT	Protein kinase B
AJ	Adherens junction
BSA	Bovine serum albumin
DAVID	Database for Annotation, Visualization, and Integrated Discovery
DCF-DA	Diacetyldichlorofluorescein diacetate
DIM	3,3'-Diindolylmethane
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's phosphate-buffered saline
FITC	Fluorescein isothiocyanate
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-regulated kinase
GSK-3 β	Glycogen synthase kinase-3 beta
GO	Gene Ontology
IL-1 β	Interleukin-1 β
IL-6	Interleukin-6
IL-8	Interleukin-8
HR-ESI-MS	High-resolution electrospray ionization mass spectrometry
IBD	Inflammatory bowel disease
IFD	Induced fit docking
KEGG	Kyoto Encyclopedia of Genes and Genomes
MLC	Myosin II regulatory light chain
MLCK	Myosin light chain kinase
NF- κ B	Nuclear factor kappa-B
NMR	Nuclear magnetic resonance
NO	Nitric oxide
OPLS4	Optimized Potentials for Liquid Simulations 4
PARP	Poly (ADP-ribose) polymerase
PI3K	Phosphoinositide 3-kinase
PPI	Protein-protein interaction
RIPA	Radioimmunoprecipitation assay
RMSF	Root-mean-square fluctuation
RMSD	Root-mean-square deviation
ROS	Reactive oxygen species
SCHC	Schisandrin C
STRING	Search Tool for the Retrieval of Interacting Genes/Proteins
TCMSP	Traditional Chinese Medicine Systems Pharmacology
TEER	Trans epithelial electrical resistance
TJ	Tight junction
ZO-1	Zonula occludens-1

Supplementary Information

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Supplementary Material 1.

Supplementary Material 2.

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Author contributions

SHT: Conceptualization, methodology, formal analysis, investigation, writing – original draft. UTTN: Methodology, formal analysis, investigation, writing

– original draft. MRK: Conceptualization, methodology, formal analysis, investigation. HJL: Formal analysis, investigation. EY: Methodology, writing – original draft. WYB: Methodology, formal analysis. SWK: Methodology, formal analysis, writing – original draft. WKK: Methodology, formal analysis. KYC: Methodology. KP: Methodology. KK: Conceptualization, methodology, supervision, writing – review & editing. All authors read and approved the final manuscript.

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Data availability

All data generated or analysed during this study are included in this published article and its supplementary information files.

Declarations

Competing interests

Not applicable.

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