

Pristimerin ameliorates colitis-induced intestinal mucosal injury by inhibiting intestinal epithelial necroptosis

SIQI LIU^{1,2*}, YUANYUAN WANG^{1,2*}, KEYI LU^{1,2}, YIFAN SHI^{1,2}, ZHIBIN WANG³ and ERPING XU^{1,2}

¹Collaborative Innovation Center of Research and Development on The Whole Industry Chain of Yu-Yao, Henan Province, Henan University of Chinese Medicine, Zhengzhou, Henan 450046, P.R. China; ²Academy of Chinese Medical Sciences, Henan University of Chinese Medicine, Zhengzhou, Henan 450046, P.R. China; ³Department of Critical Care Medicine, School of Anesthesiology, Naval Medical University, Shanghai 200433, P.R. China

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Abstract. Ulcerative colitis (UC) is a non-specific inflammatory bowel disease characterized by inflammation of the colonic and rectal mucosa and submucosa and has a globally increasing incidence. A compromised intestinal epithelial barrier function has been established as the primary etiological factor in UC, with necroptosis of intestinal epithelial cells exacerbating barrier disruption. Consequently, the inhibition of necroptosis in these cells has the potential to ameliorate colitis severity and preserve intestinal integrity, thereby offering a promising therapeutic approach for UC management. Pristimerin, a naturally occurring pentacyclic triterpenoid derived from *Tripterygium wilfordii* Hook.f., has been used in the treatment of various diseases. Although pristimerin has been documented to have therapeutic effects on UC, there is a lack of studies exploring its mechanism of

action via necroptosis. The present study aimed to elucidate the role of pristimerin in the treatment of UC by examining its inhibitory effects on necroptosis through both *in vivo* and *in vitro* experimental approaches. Pristimerin was found to markedly enhance body weight, colon length and intestinal barrier function, while concurrently reducing fecal blood loss in murine models of colitis. Furthermore, both *in vivo* and *in vitro*, pristimerin effectively inhibited the phosphorylation of key necroptosis mediators, including receptor-interacting protein kinase 1, receptor-interacting protein kinase 3 and mixed lineage kinase domain-like protein. These findings collectively suggested that the therapeutic effects of pristimerin in UC may be attributed, at least in part, to its ability to suppress necroptosis, thereby improving intestinal barrier integrity.

Introduction

Ulcerative colitis (UC) is a nonspecific inflammatory bowel disease that primarily affects the mucosal and submucosal layers of the colon and rectum. It is clinically characterized by recurrent episodes of abdominal pain, diarrhea and bloody stools. The global incidence of UC is generally increasing (1), particularly in newly industrialized countries, and it contributes to a substantial economic burden (2). Furthermore, patients with UC have a 2.4-fold increased risk of developing colorectal cancer compared with the general population (3). These findings underscore the critical importance of research in inflammatory bowel disease. Owing to its chronic and recurrent nature with idiopathic etiologies (4), UC presents significant challenges in achieving a cure and necessitates prolonged, often lifelong, pharmacological management. The primary therapeutic agents employed in the treatment of UC include sulfasalazine, glucocorticoids, immunomodulators and biologics (5). While these medications offer some clinical efficacy, they are frequently associated with adverse effects and the potential for drug dependence. Consequently, the discovery and development of novel and more effective pharmacological treatments for UC are of paramount importance.

The mechanical barrier, as a critical component of intestinal barrier function, primarily consists of intestinal epithelial cells, intercellular junctions and the mucus layer (6). Intestinal

Correspondence to: Professor Erping Xu, Collaborative Innovation Center of Research and Development on The Whole Industry Chain of Yu-Yao, Henan Province, Henan University of Chinese Medicine, 156 East Jinshui Road, Zhengzhou, Henan 450046, P.R. China
E-mail: Xuerping0371@163.com

Professor Zhibin Wang, Department of Critical Care Medicine, School of Anesthesiology, Naval Medical University, 168 Changhai Road, Shanghai 200433, P.R. China
E-mail: methyl@smmu.edu.cn

*Contributed equally

Abbreviations: UC, ulcerative colitis; RIPK1, receptor-interacting protein kinase 1; RIPK3, receptor-interacting protein kinase 3; MLKL, mixed lineage kinase domain-like protein; TCM, Traditional Chinese Medicine; DSS, dextran sulfate sodium; Nec-1, necrostatin-1; Con, control; P-L, low-dose pristimerin group; DAI, Disease Activity Index; EC50, half-maximal effective concentration; H&E, hematoxylin and eosin; PAS, Periodic acid-Schiff; MUC2, mucoprotein 2

Key words: pristimerin, ulcerative colitis, intestinal barrier, necroptosis

epithelial cells are closely organized and play a pivotal role in nutrient absorption, mucus secretion and antimicrobial production. Intercellular junctions, predominantly tight junctions, ensure the cohesion and integrity of the epithelial cell layer. The mucus layer overlays the surface of the intestinal epithelial cells, providing a protective shield against direct damage from harmful substances.

Cell death serves a crucial role in maintaining homeostasis within organisms. Recently, necroptosis has emerged as a novel mode of cell death, exhibiting morphological similarities to necrosis while possessing distinct programmed characteristics at the molecular level. Although both apoptosis and necroptosis are forms of programmed cell death (7), necroptosis is distinguished as a programmed, inflammatory mode of cell demise. It is characterized by organelle swelling, cell membrane rupture and the subsequent release of cellular contents, which provoke a substantial inflammatory response (8). Necroptosis serves a critical role in the pathogenesis, progression and prognosis of neurodegenerative (9), intestinal (10) and viral infectious diseases (11). In instances where cells are unable to activate the key apoptosis initiator caspase-8, tumor necrosis factor- α (TNF- α) engages with TNF receptor 1, resulting in the activation of receptor-interacting protein kinase 1 (RIPK1). Upon activation, RIPK1 undergoes autophosphorylation at several residues. Within the necrosome, receptor-interacting protein kinase 3 (RIPK3) undergoes phosphorylation, which subsequently facilitates the recruitment and phosphorylation of mixed lineage kinase domain-like protein (MLKL). Following phosphorylation, MLKL oligomerizes and translocates from the cytoplasm to the cell membrane, where it compromises membrane integrity and triggers cell necroptosis (12).

Necroptosis serves a multifaceted role in UC, primarily through the disruption of cellular membrane integrity. This disruption compromises the mechanical barrier function of the intestinal epithelium, facilitating the infiltration of harmful substances into the intestinal tissues, which subsequently triggers further inflammatory responses and tissue damage (13). Consequently, inhibiting necroptosis may alleviate the severity of colitis and protect the intestinal tract (14), thereby offering a therapeutic approach for UC.

Traditional Chinese Medicine (TCM) has demonstrated significant advancements in the treatment of UC, attributed to its multi-component and multi-target properties, which result in effective therapeutic outcomes, as well as low recurrence rates and minimal adverse reactions. Traditional formulations, individual herbal components and active constituents function by modulating inflammatory cytokines, safeguarding the intestinal epithelium and tight junctions and maintaining the integrity of the intestinal barrier (15). In addition, TCM influences the intestinal microecology to modulate immune responses and the composition of the intestinal flora, among other mechanisms, thereby achieving therapeutic efficacy in the management of UC (16). Consequently, there has been increasing scholarly interest in natural compounds derived from TCM. Pristimerin, a naturally occurring pentacyclic triterpenoid compound sourced from the plant *Tripterygium wilfordii* Hook.f., has been documented in the literature to exhibit therapeutic effects against UC (17). Nonetheless, research focusing on the treatment of UC through the inhibition of necroptosis by pristimerin remains unexplored. The present study investigated the effect of the natural active

ingredient pristimerin on UC and further validated, through both *in vivo* and *in vitro* analyses, that the potential mechanism by which pristimerin ameliorates intestinal barrier damage in the treatment of UC involves the inhibition of necroptosis in intestinal epithelial cells.

Materials and methods

Reagents. Dextran sulfate sodium (DSS; molecular weight range: 36,000-50,000; cat. no. CD4421) was obtained from Beijing Coolaber Science & Technology Co., Ltd. Pristimerin (purity: 99.22%, CAS No.: 1258-84-0), sourced from Med Chem Express, was prepared in dimethyl sulfoxide. Necrostatin-1 (Nec-1; cat. no. S8037) was purchased from Selleck Chemicals. Human TNF- α (cat. no. C008), SM-164 Hydrochloride (cat. no. HY-15989A) and z-VAD-fmk (cat. no. T6013) were acquired from Novoprotein, Med Chem Express and TargetMol Chemicals, respectively. Human-derived antibodies included anti-RIPK1 (cat. no. 3493S) and anti-phosphorylated (p-)RIPK1 (cat. no. 65746S) from Cell Signaling Technology, Inc.; anti-RIPK3/p-RIPK3 (cat. no. ab209384), anti-MLKL (cat. no. ab184718) and anti-phospho-MLKL (cat. no. ab187091) from Abcam. Mouse-derived antibodies included: anti-p-RIPK1 (cat. no. BX60008) from Hangzhou Bailing (Biolyx) Biotechnology Co., Ltd.; anti-RIPK1 (cat. no. 3493S) from Cell Signaling Technology, Inc.; anti-p-RIPK3 (cat. no. ab195117) from Abcam; RIPK3 polyclonal antibody (cat. no. 17563-1-A) and anti-MLKL (cat. no. 66675-1-Ig) from Proteintech Group, Inc.; anti-phospho-MLKL (cat. no. bsm-54104R) from Bioss; occludin (cat. no. GB111401) and Zonula Occludens-1 (ZO-1; cat. no. GB111402) from Wuhan Servicebio Technology Co., Ltd.; and anti-GAPDH (cat. no. ab181602) from Abcam. Secondary antibodies included HRP Goat anti-rabbit IgG (cat. no. abs20040) from Absin Bioscience Inc. and HRP-conjugated Goat anti-Mouse IgG (H+L) (cat. no. AS003) from ABClonal Biotech Co., Ltd.

Cell culture. The HT-29 cell line, a widely used model for investigating necroptosis, is derived from human colorectal cancer (18-20). This cell line was obtained from Procell Life Science & Technology Co., Ltd. (cat. no. CL-0118) and authenticated by short tandem repeat DNA profiling.

Cell culture was typically performed in McCoy's 5A medium (cat. no. CM-0118; Procell Life Science & Technology Co., Ltd.) supplemented with 10% fetal bovine serum (cat. no. 164210-50; Procell Life Science & Technology Co., Ltd.) and 1% Penicillin-Streptomycin Solution (cat. no. 15140-122; Gibco; Thermo Fisher Scientific, Inc.). The cells were cultured and grown at a temperature of 37°C and in air with 5% CO₂.

Anti-necroptosis effect of pristimerin. HT-29 cells were seeded in 96-well plates at a density of 1×10⁴ cells/well and cultured for 12 h. Pristimerin was diluted in culture medium supplemented with 10 nM SM-164 and 20 μ M Z-VAD-FMK to achieve final concentrations ranging from 10-0.075 μ M. After 12 h of cell culture, the culture medium was removed and replaced with the prepared pristimerin solutions. After a 30-min pre-treatment with pristimerin, cells were stimulated with 20 ng/ml recombinant human (h-) TNF- α . This point

marked the initiation of TNF- α -SMAC-z-VAD-FMK, necroptosis inducer (TSZ) treatment, which consisted of h-TNF- α in the presence of SM-164 and Z-VAD-FMK. Cell activity was detected after 12 h using CellTiter-Lumi II Luminescent Cell Viability Assay Kit (cat. no. C0056S; Beyotime Institute of Biotechnology).

Double staining of live/dead cells. HT-29 cells were treated with TSZ and pristimerin as aforementioned. Following a 12-h incubation period, 100 μ l of a 1:1,000 dilution of calcein-AM/Propidium Iodide (calcein-AM/PI) working solution was added to each well. The plates were then incubated for 30 min at 37°C in the dark. Cellular viability within each group was subsequently assessed under a fluorescence microscope (TS-2; Nikon Corporation). Red fluorescence indicated dead cells, while green fluorescence denoted live cells.

Western blotting. HT29-cells were seeded at a density of 1×10^6 cells/well in 6-well plates and incubated for 12 h. Subsequently, cells were treated with TSZ and different concentrations of pristimerin (10, 5 and 2.5 μ M) for durations ranging from 0-6 h. Following treatment, cells were lysed with RIPA lysis buffer (Jiangsu Kangwei Biology Co., Ltd.) for 30 min and centrifuged at $12,000 \times g$ for 10 min at 4°C. Protein (20 μ g/lane) was extracted from the supernatant and protein concentration was determined using a BCA protein assay kit (Jiangsu Kangwei Biology Co., Ltd.). After denaturation by boiling, proteins were resolved by 10% SDS-PAGE and transferred to a PVDF membrane (MilliporeSigma). The membrane was blocked with 5% bovine serum albumin (BSA; Wuhan Servicebio Technology Co., Ltd.) for 2 h at room temperature, washed with phosphate-buffered saline and 0.1% Tween-20 (PBST) and then incubated with the primary antibodies at a 1:1,000 dilution overnight at 4°C. The primary antibodies used in this study included the following: Anti-RIPK1 (cat. no. 3493S; 1:1,000) and anti-phosphorylated (p-)RIPK1 (cat. no. 65746S; 1:1,000) from Cell Signaling Technology, Inc., anti-RIPK3/p-RIPK3 (cat. no. ab209384; 1:1,000), anti-MLKL (cat. no. ab184718; 1:1,000), anti-p-MLKL (cat. no. ab187091; 1:1,000) and anti-GAPDH (cat. no. ab181602; 1:50,000) from Abcam. After incubation, the membrane was washed three times with PBST, followed by incubation with horseradish peroxidase (HRP-labeled)-conjugated secondary antibodies for 1 h at room temperature. Secondary antibodies included HRP Goat anti-rabbit IgG (cat. no. abs20040; 1:10,000) from Absin Bioscience Inc. and HRP-conjugated Goat anti-Mouse IgG (H+L) (cat. no. AS003; 1:10,000) from ABClonal Biotech Co., Ltd. After three additional washes with PBST, the membrane was treated with ECL chemiluminescent substrate (Tannen) and imaged using a Bio-Rad imaging system (Bio-Rad Laboratories, Inc.). Band intensities were quantified using ImageJ software (version 1.52; National Institutes of Health).

Animals. A total of 36 C57BL/6J male mice (aged 6-7 weeks; weighing 22-23 g) were obtained from Liaoning Changsheng Biotechnology Co., Ltd. The animals were maintained in a controlled environment with a temperature of 23-25°C, 50-60% relative humidity and a 12-h light/dark cycle. Standard rodent chow and tap water were provided *ad libitum*. All animal procedures were approved by the Animal Care

and Use Committee of Henan University of Chinese Medicine (approval no. DWLLGZR202200023; Henan, China).

Mice model of DSS-induced UC. Following a 1-week acclimation period with standard chow, mice were randomly assigned to a control group (Con) and a dextran sulfate sodium (DSS)-induced colitis model group (DSS). The control group received standard chow and water *ad libitum*. The DSS group received 2.5% DSS in drinking water for 7 days to induce UC. Subsequently, the DSS group was further randomized into five treatment groups: DSS-only, high-dose pristimerin (P-H; 3 mg/kg), low-dose pristimerin (P-L; 1 mg/kg), mesalazine (100 mg/kg) and Nec-1 (5 mg/kg), with six mice per group. The doses of pristimerin were selected based on previous studies and preliminary experiments (21,22). The DSS-only group was maintained on a standard chow diet and water. Treatment groups received their corresponding medications once daily for 5 consecutive days via the following routes: P-H and P-L and mesalazine were administered orally by gavage, while Nec-1 was administered intraperitoneally. On day 12, all animals were sacrificed. The process was performed by certified personnel using an overdose of sodium pentobarbital (150 mg/kg, intraperitoneally). Following the loss of consciousness, cervical dislocation was performed as a secondary method to ensure the cessation of all brain activity. All euthanized animals were confirmed deceased through assessment of the following cessation parameters: Absence of detectable cardiac activity and the loss of corneal reflexes. The predefined humane endpoints used were the inability to access food and water and no animals reached the humane endpoints before the end of the experiment. All animal experiments were conducted at the Animal Experimental Center of Henan University of Chinese Medicine. Blood and colonic tissue samples were collected. Colonic length was measured, and colonic tissue was processed for histological analysis using paraffin-embedded sections. Tissues were fixed in 4% paraformaldehyde fixative (cat. no. G1101; Wuhan Servicebio Technology Co., Ltd.) for 24 h at room temperature and then transferred to a dehydration box for dehydration using a graded alcohol series. After dehydration, the tissues were immersed in wax and embedded using an embedding machine. The wax blocks were cooled at -20°C, trimmed and sectioned to a thickness of 4 μ m.

Disease activity index (DAI). The DAI is a validated scoring system used to assess disease activity in experimental models of UC. It is derived from an understanding of UC pathophysiology gleaned from previous clinical and experimental studies, encompassing a comprehensive evaluation of disease activity (23,24). The DAI is calculated by summing scores from three key indicators: Weight loss, stool consistency and rectal bleeding. Body mass loss of 1-5% was scored as 1, 5-10% as 2, 10-20% as 3 and >20% as 4. Stools were scored according to the nature of the blood in the stools, with occult blood negative scoring 0, weakly positive occult blood scoring 1, moderately positive occult blood scoring 2, strongly positive occult blood scoring 3 and hemorrhage scoring 4. Normal feces was scored as 0, soft scoring 1, soft and thick scoring 2 and soft adherence to the perineal region scoring 3, with diarrhea scoring 4. Daily monitoring of mice included meticulous

observation and measurement of body weight, hair condition, mental status and stool characteristics.

Hematoxylin and eosin (H&E) staining. Mouse colon, heart, lung, liver, spleen and kidney tissues were fixed, dehydrated, embedded in sections and deparaffinized in water. Sections were then stained with hematoxylin for 3–5 min at room temperature, rinsed and blued. Following this, the sections were dehydrated in 95% alcohol for 1 min and counterstained with eosin for 15 sec at room temperature. Finally, the sections were dehydrated, mounted and coverslipped for subsequent imaging and analysis.

Periodic acid-schiff staining. Paraffin sections were dewaxed and rehydrated. Next, these sections were immersed in Periodic acid-Schiff (PAS) staining solution B for 10–15 min at room temperature, followed by thorough washing. Subsequently, sections were immersed in immersion staining solution A for 25–30 min at room temperature, with meticulous attention paid to avoiding exposure to light. Following thorough cleaning, sections were immersed in staining solution C for 30 sec at room temperature to neutralize any residual ammonia and restore the blue color. After rinsing, sections were dehydrated and mounted. An upright light microscope (NIKON ECLIPSE E100; Nikon Corporation) was used for microscopic examination and images were captured for subsequent analysis. The overall morphology was observed, followed by detailed examination at x18 magnification. To ensure representative sampling, ≥ 3 fields of view were analyzed for each sample.

Immunohistochemistry. Paraffin sections of colon tissue were deparaffinized in xylene and subsequently rehydrated through graded alcohols (100, 95 and 70%). Endogenous peroxidase activity was blocked by incubation in 3% H_2O_2 for 10 min, followed by two 5-min washes in phosphate-buffered saline (PBS). The sections were blocked with 3% BSA for 30 min at room temperature. After overnight incubation with the primary antibodies targeting Muc2 (cat. no. GB11344; 1:500; Wuhan Servicebio Technology Co., Ltd.), Occludin (cat. no. GB111401; 1:500; Wuhan Servicebio Technology Co., Ltd.) and ZO-1 (cat. no. GB1114021; 1:2,000; Wuhan Servicebio Technology Co., Ltd.) at 4°C, sections were washed three times in PBS for 5 min each. Following incubation with the appropriate horseradish peroxidase (HRP)-conjugated Goat Anti-Rabbit IgG (H+L) secondary antibodies (cat. no. GB23303; 1:200; Wuhan Servicebio Technology Co., Ltd.) for 50 min at room temperature, protected from light, sections were washed three times in PBS for 5 min each. Color development was achieved using freshly prepared diaminobenzidine (DAB) solution, monitored microscopically and terminated with distilled water rinses. Finally, sections were counterstained with hematoxylin for 3 min at room temperature and dehydrated for mounting. The samples were imaged at x25 magnification using a light microscope (E100; Nikon Corporation).

TUNEL fluorescence assay. Paraffin sections of mouse colon tissue were deparaffinized to water, followed by a 20 min incubation at 37°C with proteinase K working solution for antigen retrieval. Subsequent washes were performed thrice with PBS for 5 min each at room temperature.

Membrane-permeabilization solution was then added to cover the tissue and incubated for 20 min at room temperature, followed by another PBS wash. The sections were subsequently incubated in the TUNEL reaction solution for 1 h at 37°C. The amount of TDT enzyme in the TUNEL kit (cat. no. G1502; Wuhan Servicebio Technology Co., Ltd.) was used according to the number of slides and tissue size, mixed with dUTP and buffer at a 1:5:50 ratio. After thorough washing with PBS, the sections were stained with DAPI for 10 min at room temperature, protected from light. Following a final PBS wash, the sections were mounted with an anti-fluorescence quenching mounting medium. Images were subsequently acquired using a fluorescence microscope (x25 magnification; NIKON ECLIPSE C1; Nikon Corporation).

Immunofluorescence assay. Paraffin-embedded intestinal tissue sections were sectioned into thin slices and subjected to deparaffinization prior to immunofluorescence staining of epithelial monolayers. Following antigen retrieval, sections were blocked with 5% BSA for 30 min at room temperature. Subsequently, primary antibodies against p-RIPK3 (cat. no. ab195117; 1:200; Abcam) and p-MLKL (cat. no. bsm-54104R; 1:300; BIOSs) were applied and incubated overnight at 4°C. After four washes with PBS, sections were incubated with secondary antibodies for 1 h in the dark at room temperature. Nuclear staining was performed using DAPI (cat. no. G1012, Xavier, China) for 10 min at room temperature. Following subsequent washes with PBS, sections were mounted with an antifluorescent mounting medium. Secondary antibodies employed were the Cy3 conjugated Goat Anti-Rabbit IgG (H+L) (cat. no. GB21303; 1:300; Wuhan Servicebio Technology Co., Ltd.) antibodies. Samples were imaged using a fluorescent microscope at x25 magnification (NIKON ECLIPSE C1; Nikon Corporation).

Statistical analysis. Statistical analysis was performed using one-way ANOVA, followed by Tukey's post hoc test for multiple comparisons. $P < 0.05$ was considered to indicate a statistically significant difference. Data were analyzed using SPSS (version 25; IBM Corp.).

Results

Pristimerin exhibits anti-necroptosis activity in HT-29 cells. The chemical structure of Pristimerin is shown in Fig. 1A. An *in vitro* model of TSZ-induced necroptosis was established utilizing HT-29 cells to assess the anti-necroptotic effects of pristimerin. The cells were co-incubated with varying concentrations of pristimerin for a 12-h period. Cell viability was assessed using the CellTiter-Lumi™ II Luminescent Cell Viability Assay Kit. The findings indicated that pristimerin markedly increased cell viability in a dose-dependent manner (Fig. 1B). The half-maximal effective concentration (EC_{50}) was determined to be $8.289 \mu\text{M}$ based on cell viability data (Fig. 1C). Following pristimerin treatment, there was a notable enhancement in cell death, accompanied by a discernible increase in the number of viable cells, as evidenced by staining with the Calcein/PI Cell Viability/Cytotoxicity Assay Kit (Fig. 1D). In this assay, Calcein AM selectively stained viable cells with green fluorescence, while Propidium Iodide marked dead cells with red fluorescence.

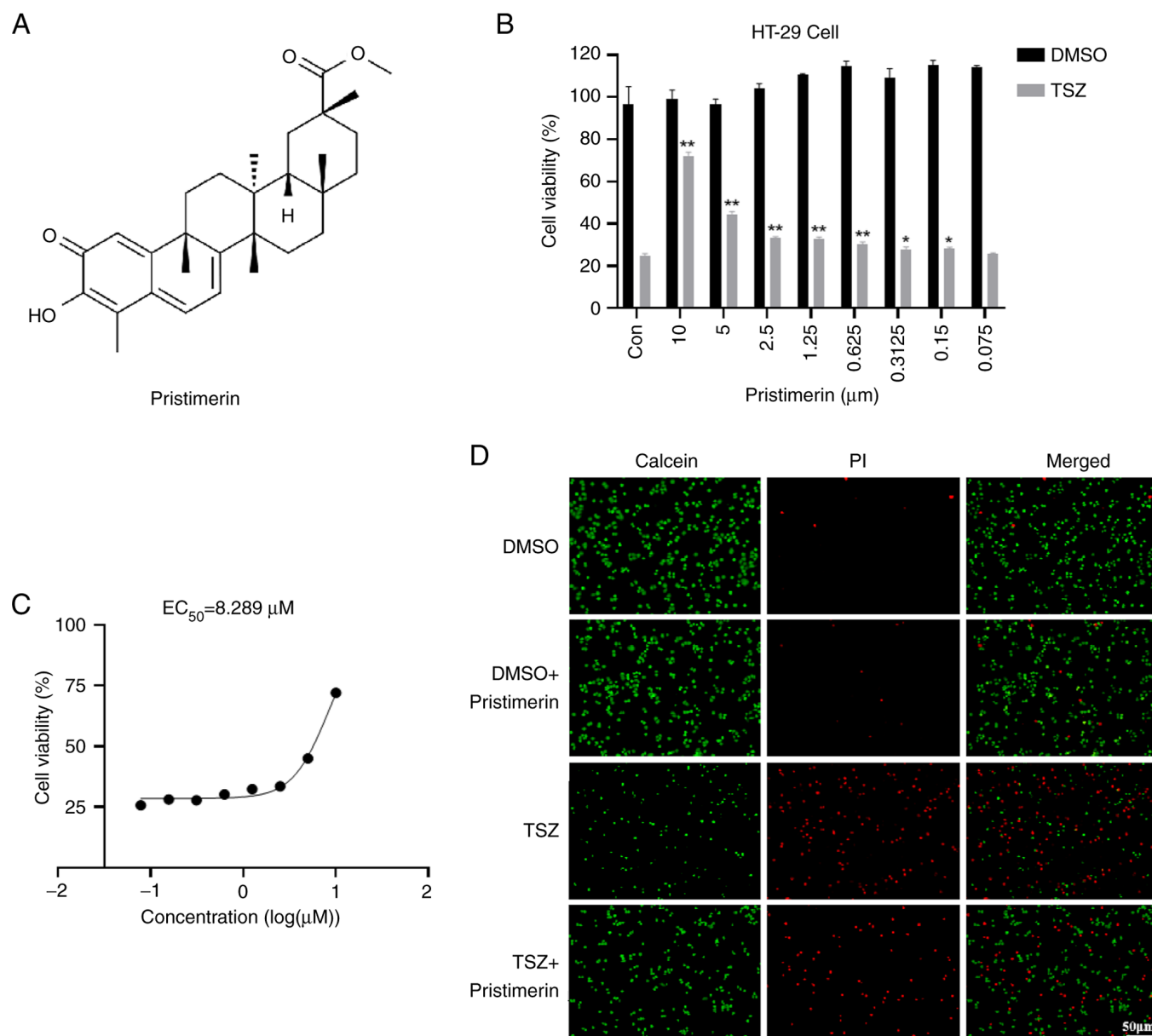


Figure 1. Anti-necroptosis activity of pristimerin *in vitro*. (A) The chemical structure of pristimerin. (B) Different concentrations of pristimerin were used to treat HT-29 cells and then the cells were stimulated with TSZ stimulation for 12 h. The cell viability was detected by chemiluminescence. (C) The EC_{50} value was calculated according to the cell viability. (D) Representative images of calcein-AM/PI staining of pristimerin receiving different treatments for 24 h. ** $P<0.01$, * $P<0.05$ vs. TSZ group. calcein-AM/PI, calcein-AM/Propidium Iodide; TSZ, TNF- α -SMAC-z-VAD-FMK; EC_{50} , half-maximal effective concentration.

Pristimerin inhibits the expression of necroptosis-related proteins. RIPK1 and RIPK3 modulate cell death through processes of polyubiquitination and deubiquitination, thereby playing a crucial role in the mechanism of necroptosis (25). The phosphorylation of MLKL induces its oligomerization, which subsequently leads to the disruption of membrane integrity and the translocation of necroptosomes to cellular or organelle membranes (26). The present study investigated the effect of pristimerin on the phosphorylation status of RIPK1, RIPK3 and MLKL. The experimental findings demonstrated that pristimerin inhibited the phosphorylation of RIPK1, RIPK3 and MLKL in a dose-dependent manner (Fig. 2A). Furthermore, a time-dependent decline in the phosphorylation levels of these proteins upon pristimerin treatment was observed (Fig. 2B).

Pristimerin ameliorates symptoms in DSS-induced UC mice. The DSS-induced UC model was established to evaluate the therapeutic efficacy of pristimerin. Following 7 days of 2.5% DSS administration, all mice exhibited weight loss, hematochezia and decreased stool output, confirming the successful induction of colitis. Subsequently, these mice were randomly assigned into five groups, ensuring no significant baseline differences in body weight or DAI among them. The groups comprised: i) DSS-treated group (DSS), ii) high-dose pristimerin group (P-H; 3 mg/kg), iii) low-dose pristimerin group (P-L, 1 mg/kg), iv) positive control group treated with the standard-of-care UC therapeutic agent mesalazine (100 mg/kg) and v) positive control group treated with the necroptosis inhibitor Nec-1 (5 mg/kg).

Experimental findings demonstrated that pristimerin exerted a significant effect on DSS-induced UC-related

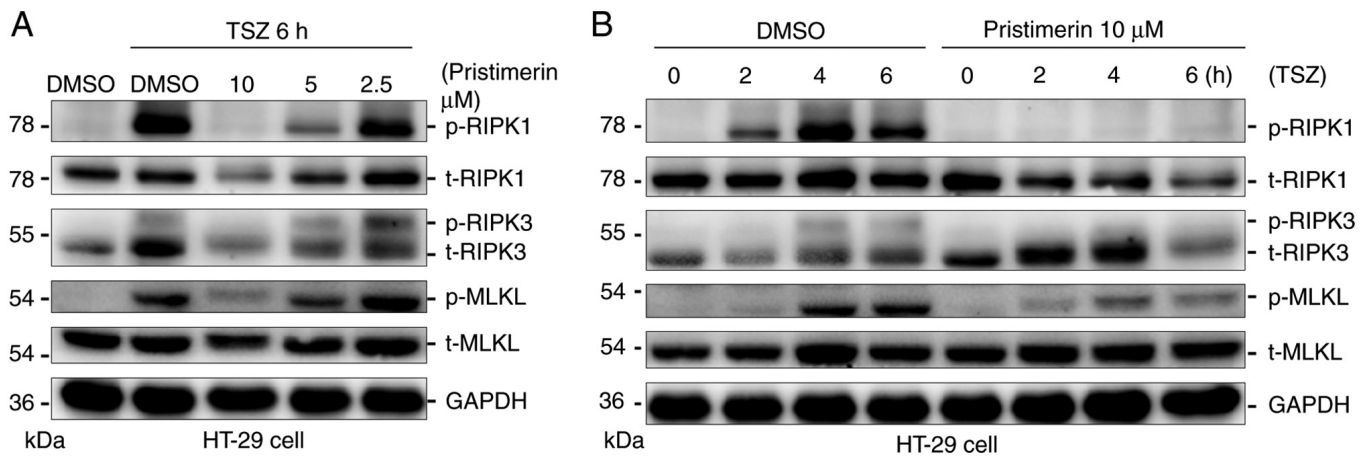


Figure 2. Pristimerin inhibits the activation of the necroptosis signaling pathway. (A) HT-29 cells were treated with TSZ and different concentrations (10, 5 and 2.5 μ M) of pristimerin for 6 h. (B) HT-29 cells were treated with TSZ and pristimerin (10 μ M) for 0, 2, 4 and 6 h. All cells were lysed and subjected to western blotting with the corresponding antibodies. TSZ, TNF- α -SMAC-z-VAD-FMK; p-, phosphorylated; t-, total; RIPK1, receptor-interacting protein kinase 1; RIPK3, receptor-interacting protein kinase 3; MLKL, mixed lineage kinase domain-like protein.

symptoms in mice. A schematic diagram of the DSS-induced UC mouse model and dosing regimen is presented in Fig. 3A. Compared with the model group, the pristimerin high-dose (P-H) group exhibited a slower rate of weight decline on day nine and achieved weight recovery by day 12 (Fig. 3B). This was accompanied by a reduction in DAI scores (Fig. 3C), amelioration of clinical symptoms including blood in stool and stool consistency and an increase in colon length (Fig. 3D and E). The therapeutic efficacy of the P-H group was comparable to that of the positive control groups (Nec-1 and mesalazine) and superior to the P-L group. To evaluate the extent of intestinal injury, histopathological analysis was conducted using H&E staining. The findings indicated that mice in the model group exhibited more severe damage to the colonic mucosal layer, characterized by diffuse infiltration of inflammatory cells within the submucosal layer, along with disruption of crypt architecture and a reduction in crypt numbers. The P-H and P-L groups both demonstrated amelioration of colonic crypt damage and quantity, with the P-H group exhibiting effects compared with the positive control group. This suggested that pristimerin has the potential to repair DSS-induced colonic tissue damage in UC mice (Fig. 3F).

Pristimerin protects the mucus barrier of colonic tissue in DSS-induced UC mice. Goblet cells are integral to the pathogenesis of UC (27). These cells are responsible for the secretion of mucin proteins, particularly mucoprotein 2 (MUC2) (28), which form a substantial component of the intestinal mucus layer, thereby serving as a critical physical barrier, protecting the intestinal epithelium against pathogens and harmful substances. Murine models of DSS-induced UC exhibit a notable impairment of intestinal barrier function, predominantly characterized by the loss of goblet cells and a reduction in glycoprotein synthesis, particularly mucins (29). PAS staining was employed to assess the distribution of polysaccharides and inflammatory damage within colonic tissue. The present study indicated a significant reduction in goblet cell numbers attributable to inflammatory injury, leading to a decrease in PAS-positive

areas in the DSS model. Conversely, treatment with pristimerin demonstrated a notable restoration of goblet cell numbers, suggesting that pristimerin may ameliorate DSS-induced colonic tissue damage in UC mice, as illustrated in Fig. 4A. Immunohistochemical analysis revealed a reduction in MUC2 expression within the model group. In contrast, pristimerin administration markedly enhanced MUC2 expression in a dose-dependent manner, with the P-H group exhibiting effects comparable to those of the positive control drug, Nec-1 (Fig. 4B).

Pristimerin enhances the expression and distribution of tight junction proteins in DSS-induced intestinal epithelial cells of UC mice. Tight junctions between intestinal epithelial cells play a crucial role in preserving the integrity of the intestinal epithelium, with Occludin and ZO-1 being the primary proteins comprising these junctions (30). Immunohistochemical analyses revealed a reduction in Occludin and ZO-1 protein expression in the colonic tissues of mice subjected to the DSS treatment, compared with the control group. This reduction suggested a disruption of the intestinal mucosal barrier in these mice. Notably, the administration of pristimerin, mesalazine and Nec-1 treatments resulted in a significant upregulation of Occludin and ZO-1 proteins (Fig. 5). These findings indicated that pristimerin could promote the repair of the intestinal mucosal barrier and maintain the integrity of the intestinal barrier by upregulating the expression of intestinal tight junction proteins in UC mice.

Pristimerin inhibits decrease of intestinal epithelial cells in UC mice. TUNEL staining was employed to assess apoptosis in intestinal epithelial cells. The model group exhibited a significant increase in the number of TUNEL-positive cells within the intestinal epithelium compared with the control group. However, a reduction in TUNEL-positive cells was observed in both pristimerin-treated groups, with the effect in the P-H group being comparable with that of the positive control groups, mesalazine and Nec-1 (Fig. 6A and D). These findings suggested that pristimerin ameliorated apoptosis

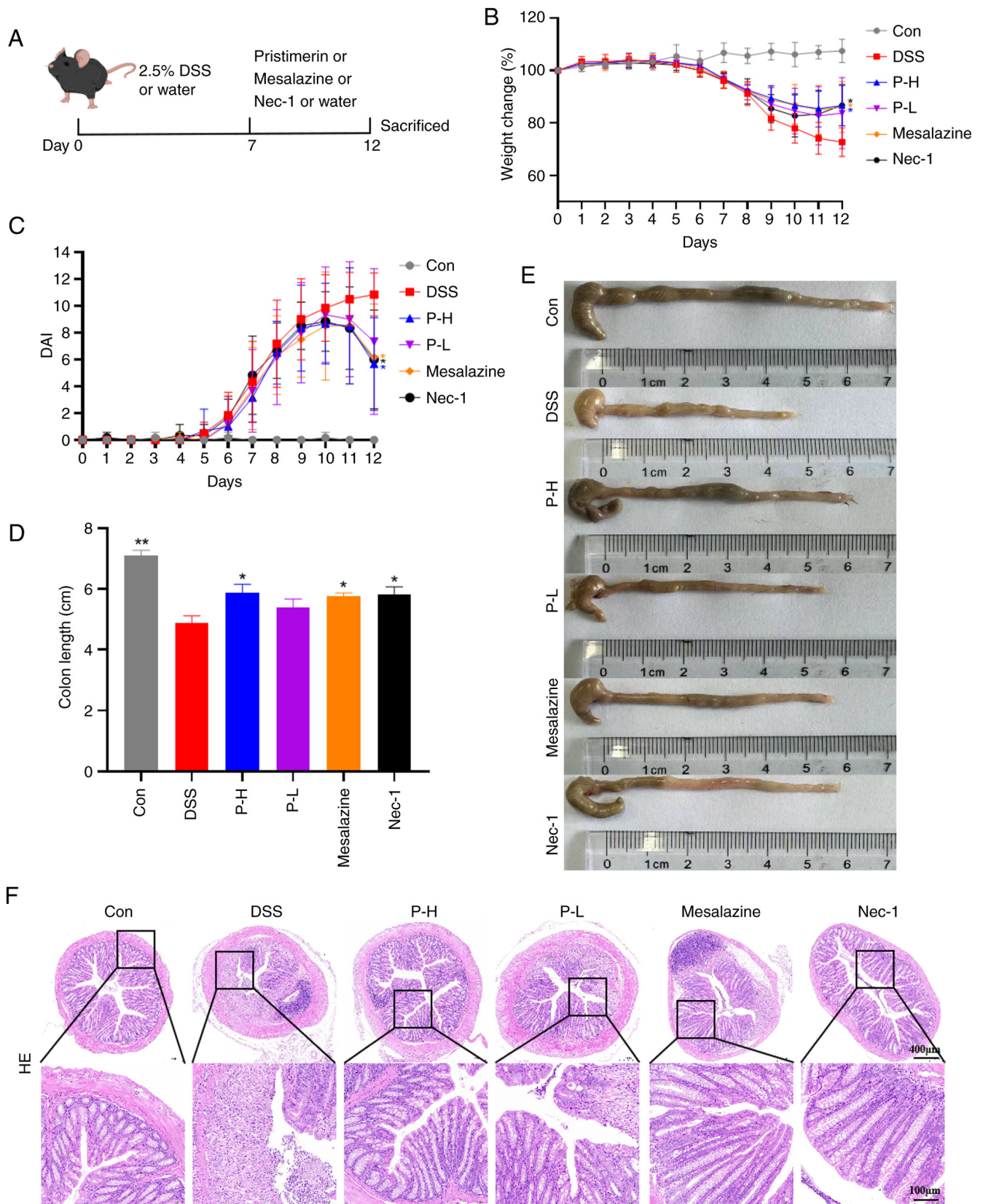


Figure 3. Pristimerin ameliorates symptoms in DSS-induced UC mice. (A) Schematic diagram of the DSS-induced UC mouse model and dosing regimen. (B) Body weight changes of mice throughout the experiment. (C) DAI scores during the experiment. (D) Length of colons on day 12. (E) Representative images of mice colons. (F) Hematoxylin and eosin staining of a representative colonic tissue section. * $P < 0.05$, ** $P < 0.01$ vs. the DSS group. DSS, dextran sulfate sodium; UC, ulcerative colitis; Con, control; Nec-1, necrostatin-1; P-H, high-dose pristimerin group; P-L, low-dose pristimerin group.

in intestinal epithelial cells. Furthermore, increased phosphorylation of RIPK3 and MLKL is recognized as a critical

pathological mechanism in necroptosis (31) with a prevalence exceeding 400 per 100 000 in North America. Individuals

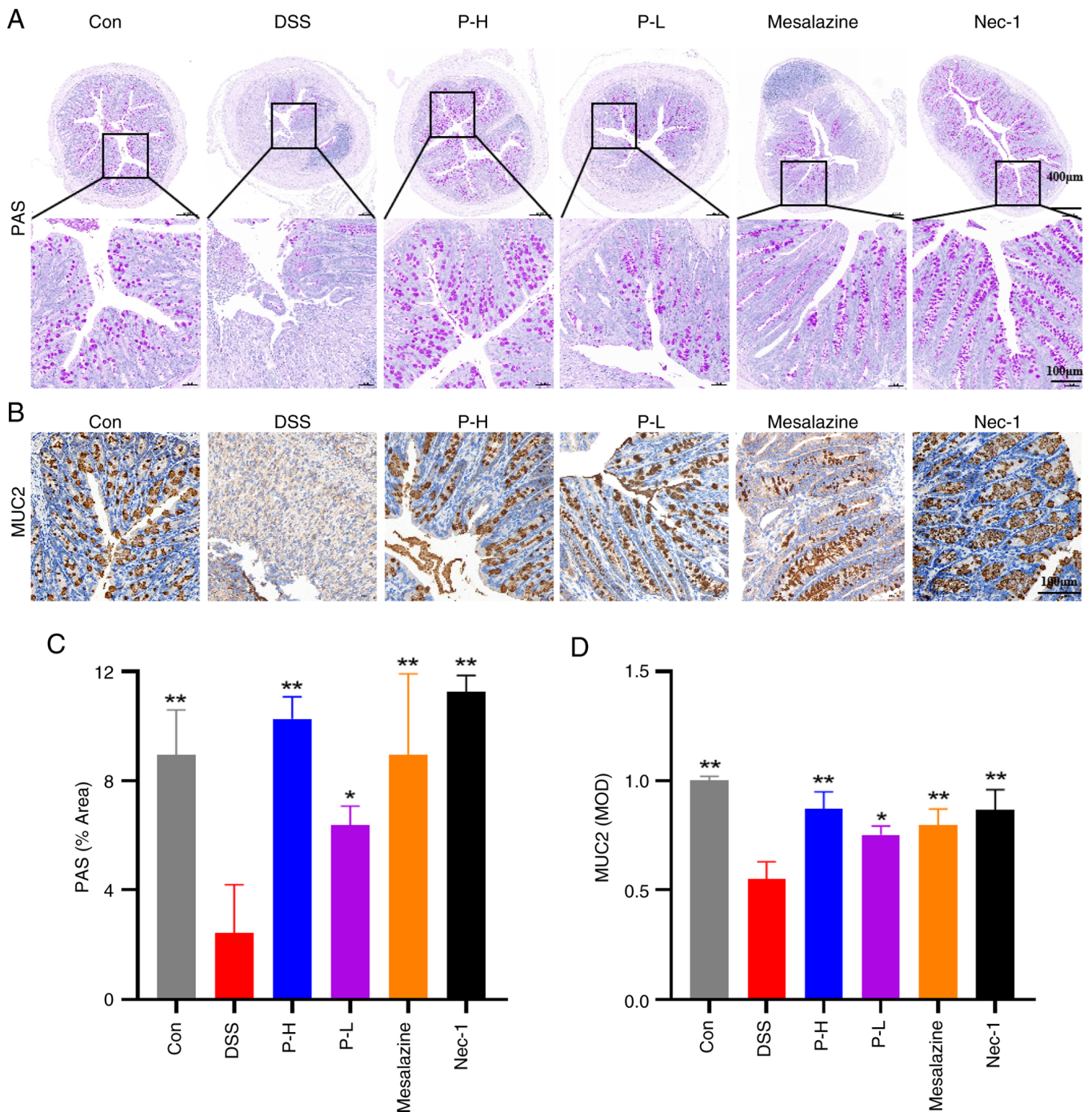


Figure 4. Pristimerin protects the mucus barrier in DSS-induced UC mice. (A) Images of PAS staining of representative colon tissue sections. (B) Images of immunohistochemical MUC2 staining of representative colon tissues. (C) PAS quantitative results. (D) Quantitative results after MUC2 normalization. ** $P < 0.01$, * $P < 0.05$ vs. the DSS group. DSS, dextran sulfate sodium; UC, ulcerative colitis; PAS, Periodic acid-Schiff; MUC2, mucoprotein 2; Con, control; Nec-1, necrostatin-1; P-H, high-dose pristimerin group; P-L, low-dose pristimerin group.

with UC have a lower life expectancy and are at increased risk for colectomy and colorectal cancer. **OBSERVATIONS:** UC impairs quality of life secondary to inflammation of the colon causing chronic diarrhea and rectal bleeding. Extraintestinal manifestations, such as primary sclerosing cholangitis, occur in approximately 27% of patients with UC. People with UC require monitoring of symptoms and biomarkers of inflammation (eg, fecal calprotectin). To evaluate the effect of pristimerin on necroptosis, the distribution of phosphorylated RIPK3 (pRIPK3) and phosphorylated MLKL (pMLKL) in

colonic tissues was analyzed (Fig. 6B and C). These findings indicated that the expression levels of phosphorylated RIPK3 and MLKL were elevated in the colonic tissues of DSS-treated mice. However, administration of pristimerin resulted in a dose-dependent inhibition of these phosphorylation levels. Notably, the effect of P-H was comparable with that observed in the Nec-1 and mesalazine treatment groups (Fig. 6E and F), indicating that pristimerin inhibited necroptosis in the colonic tissue of mice with UC, thereby enhancing intestinal barrier function and exerting therapeutic effects on the condition.

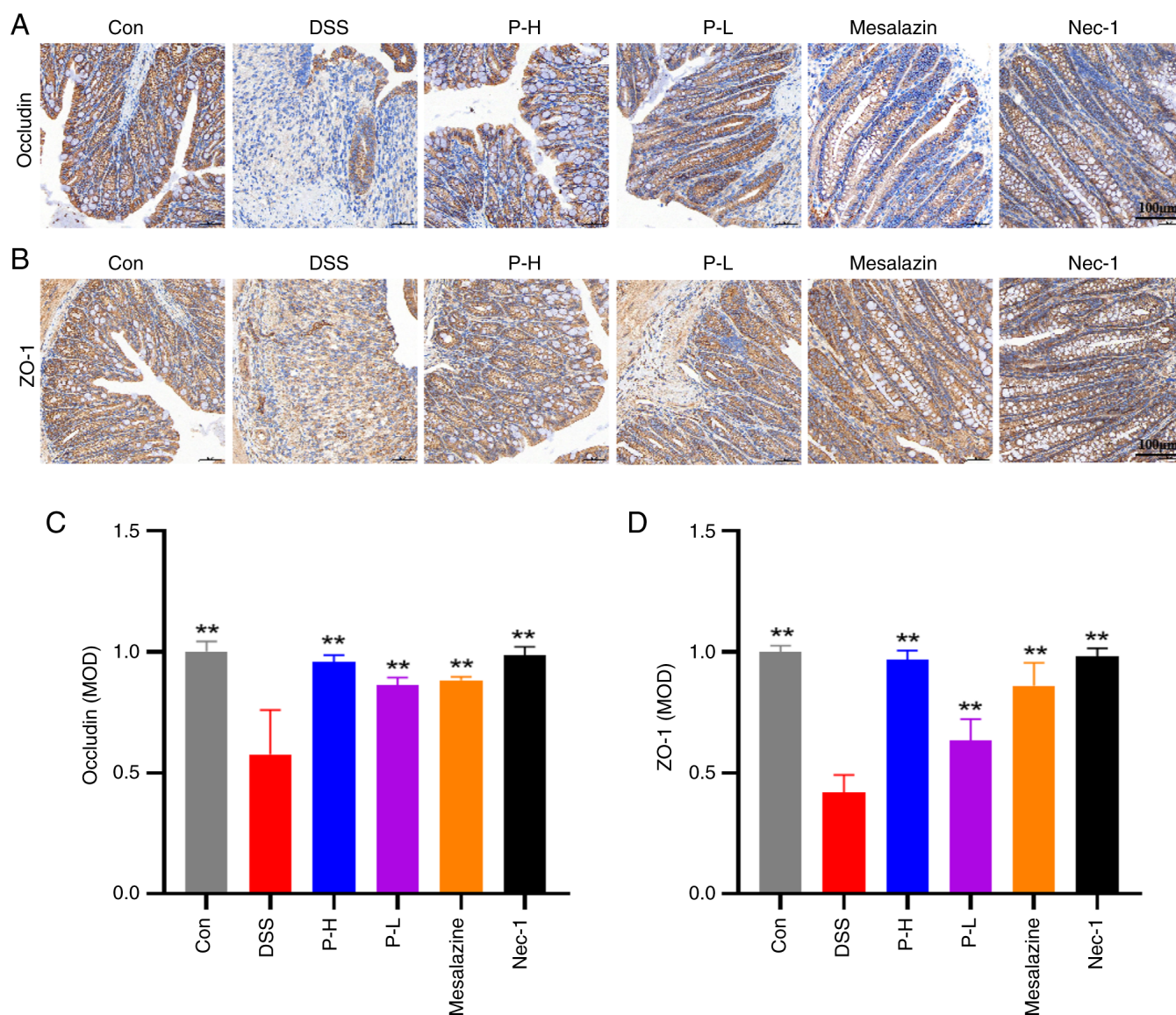


Figure 5. Pristimerin protects mechanical barriers in DSS-induced UC mice. Immunohistochemical detection of the effect of pristimerin on the distribution and expression of (A) Occludin and (B) ZO-1 in DSS-induced UC mice. Quantitative results after normalization of (C) Occludin and (D) ZO-1. ** $P < 0.01$ vs. the DSS group. DSS, dextran sulfate sodium; UC, ulcerative colitis; ZO-1, Zonula Occludens-1; Con, control; Nec-1, necrostatin-1; P-H, high-dose pristimerin group; P-L, low-dose pristimerin group.

Pristimerin yields no toxic effects in UC mice. The present study evaluated the pathology of vital organs, including the heart, liver, spleen, lungs and kidneys, in UC mice administered with a high dose of pristimerin. Histopathological analysis, using H&E staining, revealed no significant differences between the control group and the pristimerin-treated group at the high dose. These findings indicate that pristimerin did not exert toxic effects in mice at the administered dosage (Fig. 7).

Discussion

Despite the availability of certain treatments for UC, their clinical efficacy remains suboptimal. Research indicates that the one-year clinical remission rate with current therapies is ~40%. Moreover, prolonged use of these medications often leads to diminished effectiveness and necessitates alterations in treatment strategies (32). Consequently, to enhance therapeutic

outcomes, improve patient quality of life and effectively prevent and manage complications (33), the investigation of novel pharmacological agents is imperative. TCM has exhibited significant therapeutic efficacy in the management of chronic diseases, attributed to its distinctive advantages. Specifically, monomeric components with well-defined chemical structures and specific activities, extracted from TCM, have demonstrated notable therapeutic effects. Research has identified that herbal monomers such as berberine (34), baicalin (35), triptolide (36) and astragalus polysaccharide (37) mitigate colonic inflammation through various mechanisms. Consequently, further investigation into herbal monomers may offer modern medicine novel insights and therapeutic strategies for the management of UC.

Necroptosis, a regulated form of necrotic cell death, markedly contributes to various pathological conditions, particularly inflammatory responses (38). In UC, necroptosis serves a crucial role by triggering the release of pro-inflammatory

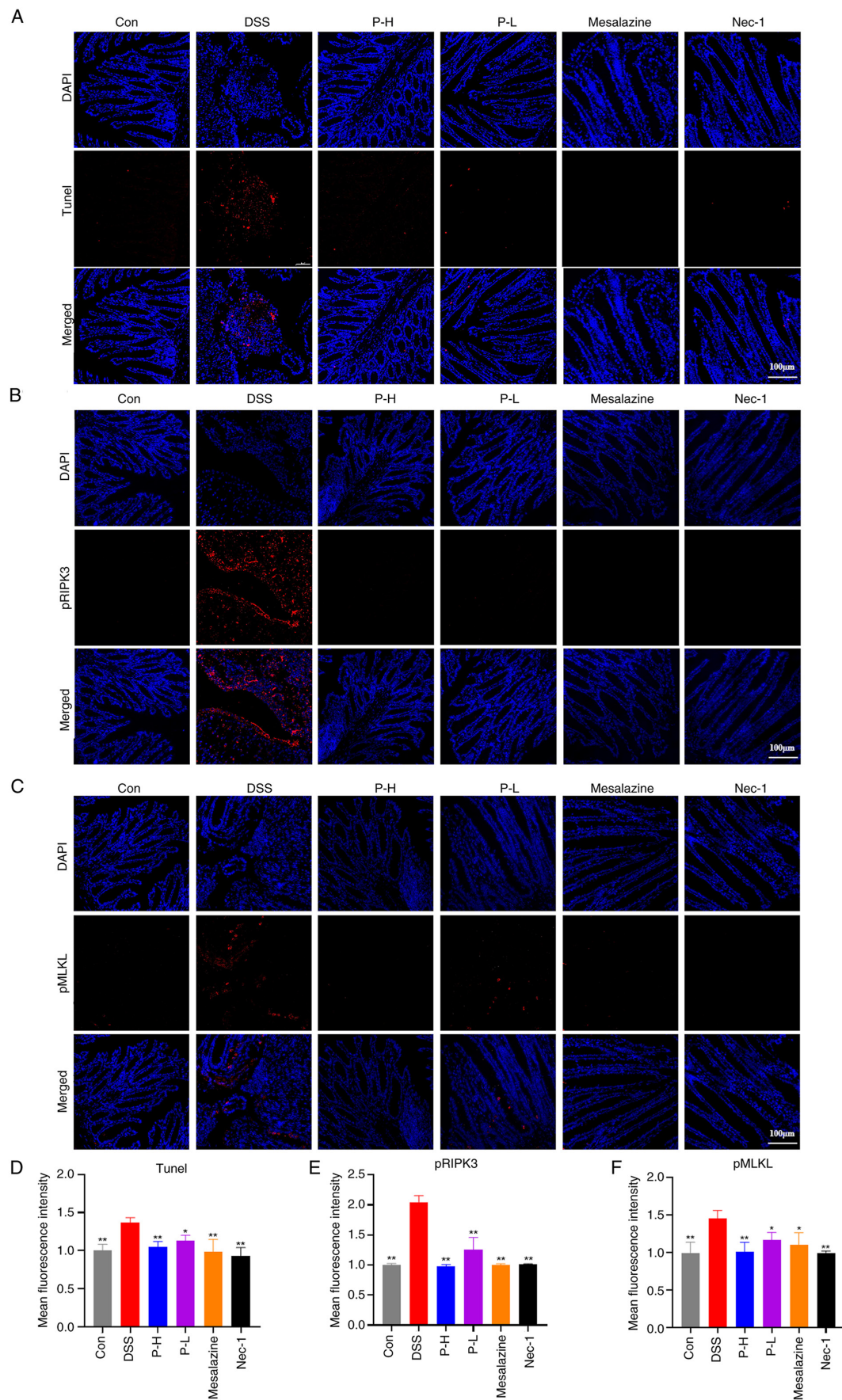


Figure 6. Pristimerin inhibits necroptosis-related protein expression in UC mice. (A) Fluorescence TUNEL detection of apoptosis in intestinal epithelial cells. (B) Immunofluorescence detection of the effects of pRIPK3 and pMLKL. (C) Distribution and expression in intestinal epithelial cells. (D) Quantitative results after normalization of TUNEL, (E) pRIPK3 and (F) pMLKL. ** $P < 0.01$, * $P < 0.05$ vs. the DSS group. UC, ulcerative colitis; p, phosphorylated; RIPK3, receptor-interacting protein kinase 3; MLKL, mixed lineage kinase domain-like protein; DSS, dextran sulfate sodium; UC, ulcerative colitis; ZO-1, Zonula Occludens-1; Con, control; P-H, high-dose pristimerin group; P-L, low-dose pristimerin group; Nec-1, necrostatin-1.

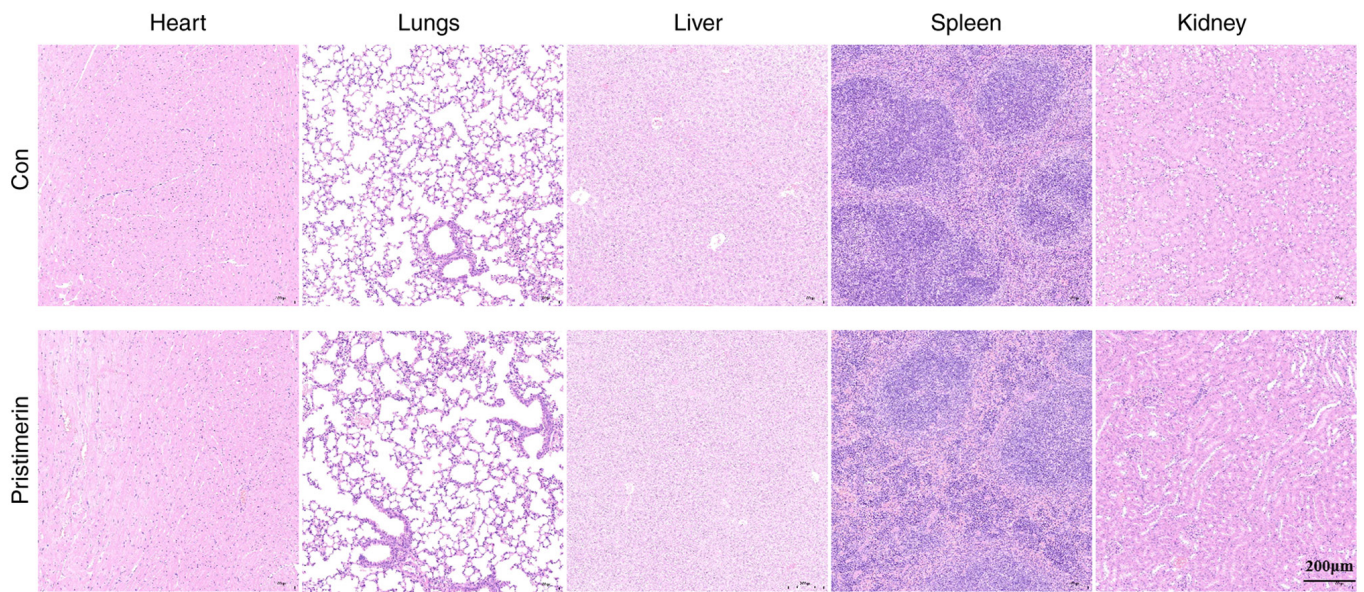


Figure 7. The toxicity of pristimerin on vital organs. Hematoxylin and eosin staining of heart, lung, liver, spleen and kidney tissues of mice. Con, control.

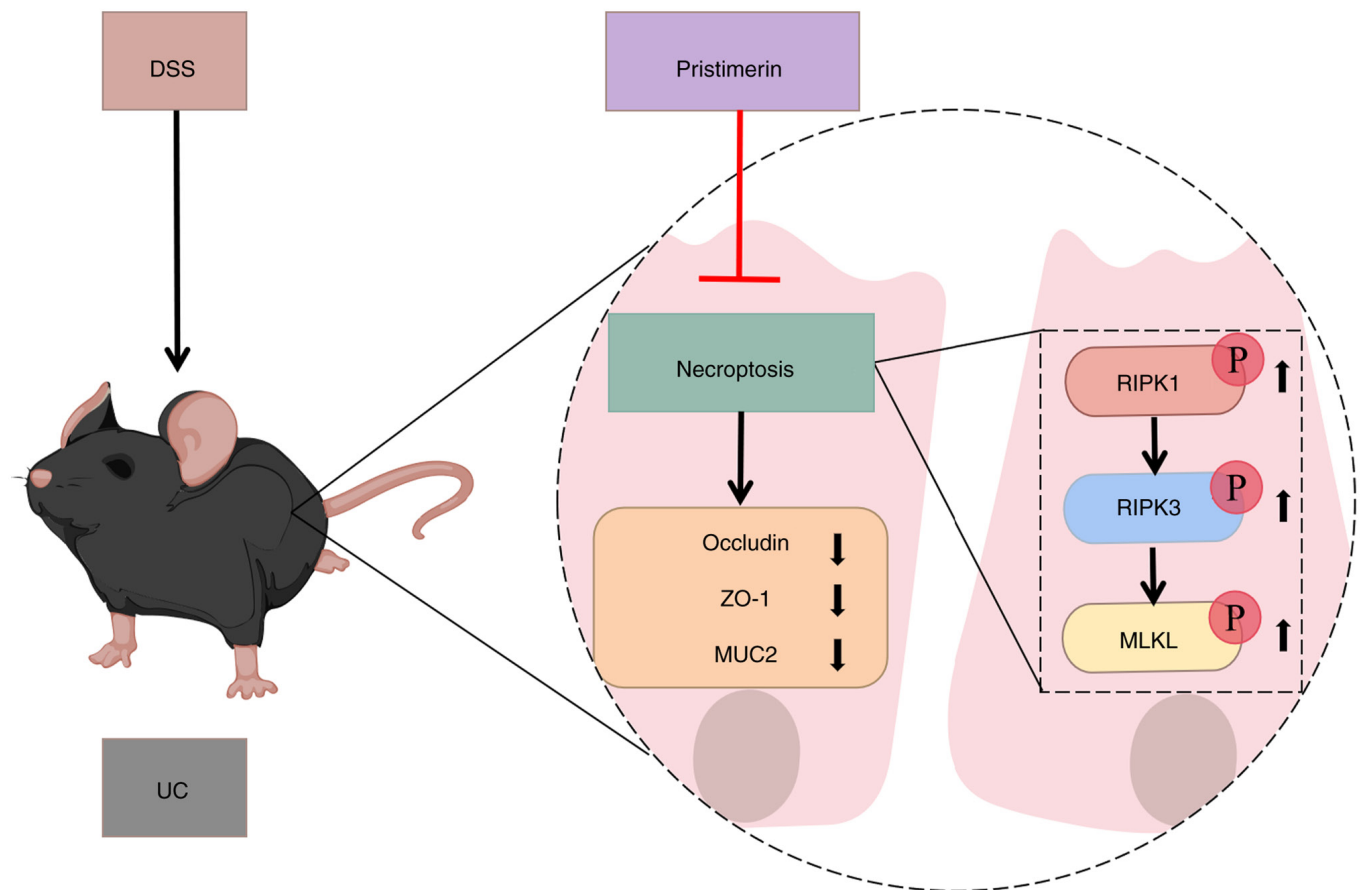


Figure 8. Schematic representation of proposed mechanism by which pristimerin protects against UC. UC, ulcerative colitis; DSS, dextran sulfate sodium; ZO-1, Zonula Occludens-1; MUC2, mucoprotein 2; RIPK1, receptor-interacting protein kinase 1; RIPK3, receptor-interacting protein kinase 3; MLKL, mixed lineage kinase domain-like protein; p-, phosphorylated.

mediators. This activation initiates the RIPK1/RIPK3/MLKL signaling cascade, leading to cellular necroptosis and subsequent exacerbation of the disease (39). Therefore, inhibiting the RIPK1/RIPK3/MLKL signaling pathway presents a

promising therapeutic strategy for mitigating UC severity (40). The discovery of necroptosis inhibitors not only advances our understanding of the necroptosis signaling pathway but also provides a valuable avenue for pharmaceutical research

in diseases associated with necroptosis (41). These inhibitors, particularly those targeting RIPK1, have demonstrated therapeutic potential in models of dermatosis, colitis, arthritis and neurodegenerative diseases (42) by suppressing RIPK1 activity and preventing necroptotic signaling. Inhibitors targeting RIPK3 have been shown to suppress TNF- α -induced necroptosis in human intestinal epithelial cells, thereby enhancing their proliferative capacity (43). Furthermore, the inhibition of MLKL has been observed to mitigate colonic inflammation and reduce colitis-associated tumorigenesis (44). Consequently, the identification and development of novel necroptosis inhibitors hold potential for therapeutic intervention in UC.

In the present study, a murine model of UC was established by administering a 2.5% DSS solution *ad libitum*, a model that more accurately reflects the pathogenesis and clinical manifestations observed in human UC patients. Following treatment with pristimerin, the mice exhibited amelioration in weight loss, reduced DAI scores and a gradual resolution of symptoms, demonstrating the therapeutic efficacy of pristimerin in UC. UC is characterized by dysregulation of epithelial barrier integrity and mucosal damage, accompanied by a marked reduction in mucins and tight junction proteins, as well as increased intestinal permeability (45). In the current study, pristimerin augmented the number of goblet cells and elevated the expression levels of MUC2, Occludin and ZO-1. These findings suggested that pristimerin may mitigate DSS-induced damage to the intestinal mucosal barrier by enhancing the tight junctions between epithelial cells and ameliorating mucosal injury. In HT-29 cells, pristimerin was found to inhibit the phosphorylation of RIPK1, RIPK3 and MLKL in a time- and dose-dependent manner. Furthermore, pristimerin demonstrated a dose-dependent inhibition of p-RIPK3 and p-MLKL expression levels in colonic tissues. Therefore, pristimerin may exert a therapeutic effect on UC by inhibiting the necroptotic signaling pathway. The proposed mechanism is depicted in Fig. 8.

Although pristimerin showed protective effects in UC mouse models, limitations remain. DSS is a synthetic sulfated polysaccharide closely linked to the severity, duration and dosage of colitis. DSS can directly affect colonic epithelial cells, compromising mucosal integrity and animal models of DSS-induced colitis can simulate the symptoms of human UC. Nevertheless, the etiology of human UC is complex, involving immune dysregulation, genetic predispositions and environmental influences, which cannot be entirely replicated in DSS models. The dosage of pristimerin administered in this study was determined to be non-toxic; however, due to the limited range of doses investigated, future research should include more comprehensive dose-response studies. Given the substantial gaps in the existing research on pristimerin, further investigations into its metabolism, distribution and pharmacokinetics *in vivo* are essential. Such studies will facilitate the optimization of clinical dosing regimens and the reduction of potential toxicity. Although pristimerin showed anti-necroptosis effects in *in vitro* and *in vivo* experiments, necroptosis was assessed only by phosphorylation of RIPK1, RIPK3 and MLKL. The specific targets of pristimerin and its effects on upstream and downstream key regulators remain unclear. In future experiments, additional assays are necessary to more comprehensively evaluate the occurrence and inhibitory effects of necroptosis.

In summary, the present study demonstrated that pristimerin can inhibit the phosphorylation of RIPK1, RIPK3 and MLKL, thereby alleviating the symptoms in UC mice and enhancing intestinal barrier function. These findings suggested that pristimerin holds promise as a therapeutic agent for UC. However, the mechanisms underlying cell death are complex and may involve multiple synergistic pathways. Further research is necessary to comprehensively elucidate the precise mechanisms by which pristimerin inhibits cell death in the colonic tissues of UC mice.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

SL and YW conceived the study. EX and ZW determined the design of this study. SL, KL and YS performed experiments and analyzed data. SL and YW wrote and revised the manuscript. EX and ZW contributed to critical revision of this article and supervised the study. SL and YW confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Animal experiments were approved by the Animal Care and Use Committee of Henan University of Traditional Chinese Medicine (approval no. DWLLGZR202200023; Henan, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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