Forsythia suspensa (Thunb.) Vahl extract ameliorates ulcerative colitis via inhibiting NLRP3 inflammasome activation through the TLR4/MyD88/NF-κB pathway

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Abstract

Background: Ulcerative colitis (UC) is a chronic inflammatory disease caused by abnormal immune system reactions resulting in inflammation and ulcers in the large intestine. Phillygenin (PHI) is a natural compound found in Forsythia suspensa (Thunb.) Vahl, known for its various bioactivities, including anti-inflammatory, anti-obesity, and antipyretic activities. However, the potential anti-inflammatory effects of PHI on UC and its underlying mechanisms are still poorly understood. Methods: In this study, we investigated the therapeutic effects of PHI on acute UC induced by DSS and TNBS. We evaluated the effects of PHI on disease activity index, body weight, mortality, intestinal mucosal barrier, cytokine secretion, and macrophage infiltration into colon tissue using various techniques such as flow cytometry, immunofluorescence, ELISA, RT-qPCR, and Western blotting. Results: Our findings revealed that PHI has therapeutic properties in UC treatment. PHI was able to maintain body weight, reduce disease activity index and mortality, restore the intestinal mucosal barrier, and inhibit cytokine secretion. Flow cytometry assay and immunofluorescence indicated that PHI reduces macrophage infiltration into colon tissue. Additionally, both in vivo and in vitro results suggested that PHI may exert anti-inflammatory effects by downregulating the TLR4/MyD88/NF-κB pathway, inhibiting NLRP3 inflammasome activation. Conclusion: In conclusion, PHI possesses anti-inflammatory properties and has the potential as a therapeutic agent for the treatment of UC. Our study provides insights into the underlying mechanisms of PHI's therapeutic effects and highlights the potential for further research in developing PHI-based treatments for UC.

Title page

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Keywords : ulcerative colitis, Phillygenin, macrophages, NLRP3 inflammasome

* Background

Ulcerative colitis (UC) is an autoimmune disease that affects the colon and rectum. It is characterized by diffuse, superficial, and localized inflammation of the mucosa[1, 2]. Although the causative factors of ulcerative colitis are not fully understood, multiple causes have been identified, including changes in the intestinal flora,

genetic susceptibility, immune response disorders, and environmental factors. Clinical treatment usually involves 5-aminosalicylic acid, corticosteroids, thiopurines, and biological agents such as anti-tumor necrosis factor (TNF)-α antibodies. However, these treatments can cause specific side effects, including diarrhea, complicated myocarditis, and hemolytic anemia [3].

Macrophages, which is considered as central mediators of intestinal immune homeostasis and inflammation, exert pathological influences in both UC and Crohn's [4]. Toll-like receptors (TLRs) are activated by pathogen-associated molecular patterns (PAMPs), which in turn activate macrophages leading to excessive inflammation and tissue damage, causing colitis [5]. Moreover, innate immune signaling via cytokine receptors and TLRs mediated macrophages activation act as a primary response to promote NOD-like receptor family pyrin domain containing 3 (NLRP3) transcription through NF-kB activation[6].

The NLRP3 inflammasome is a cytosolic protein complex consisting of NLRP3, apoptosis-associated speck-like protein containing CARD (ASC), and pro-Caspase1. This complex is present in various immune cells such as granulocytes, macrophages, and lymphocytes and is crucial for maintaining gut homeostasis[7]. Toll-like receptors (TLRs) activate the NLRP3 inflammasome through the adaptor protein MyD88, which leads to the phosphorylation of the nuclear factor kappa-B (NF-ϰB), ultimately triggering downstream inflammation. The NLRP3 inflammasome identifies a range of signals such as stress, foreign microorganisms, and endogenous danger signals, producing interleukin-1β (IL-1β) and IL-18, thereby promoting inflammation [8, 9]. IL-1β plays an important role in the colon and is mainly derived from macrophages in the lamina propria. It has various functions such as guiding neutrophils to infected or damaged sites, enhancing T cell proliferation, promoting phagocytosis to destroy bacteria, and activating additional pathways to upregulate cytokines, amongst others [8, 10].

Forsythia suspensa (Thunb.) Vahl is an ornamental shrub and its fruits were used as a well-known Chinese herbal medicine with detoxifying and heat-clearing properties. Over the past few decades, several monomeric compounds with antioxidant, anti-inflammatory, anti-viral, neuroprotective, and antibacterial effects have been identified from *Forsythia suspensa* [11]. One of these compounds, phillygenin (PHI), is a lignan known to inhibit inflammation, ameliorate liver fibrosis, inhibit epithelial-mesenchymal transition, reduce nonalcoholic fatty liver disease, and regulate proliferation and apoptosis [12-15]. PHI is involved in several pathways including the SHP-1/JAK2/STAT3, AMPK/ERK/NF-ϰB, and TLR4/MyD88/NF-ϰB pathways [16-18]. However, the impact of PHI on inflammatory bowel disease remains understudied. In the current study, we performed a detailed study on the pharmacological impact of PHI on colitis and its mechanism of action.

* Methods

Drugs and reagents

Preparation of PHI via phillyrin hydrolysis: Naturally occurring phillygenin (from an in-house natural product library, > 97% purity), Synthetic phillygenin (> 98% purity) for*in vivo* study from phillyrin hydrolysis (FigS1 to S5). The extraction of PHI was extracted, identified, and characterized. Briefly, Phillyrin (3.0 g, 5.62 mmol) was dissolved in 120 ml of methanol in a 250 ml of flask, to this solution was added 2 M HCl (24 ml, 12 mmol, 8.6 eq.). The reaction was refluxed at 65°C overnight. The reaction mixture was cooled to room temperature and adjust pH to 5˜6 with sat. $NaHCO_3$, the resulting mixture was evaporated under reduced pressure and then was extracted with EtOAc (100 ml) three times. The organic phase was washed with brine (100 ml) two times, dried with anhydrous MgSO4, and then evaporated. The residue was subjected to a silica gel column eluting with dichloromethane/methanol (20/1) to yield PHI (1.58 g, 4.25 mmol, 76%). White solid, ESI-MS *m/z* (m): 373.3 $[M + H]^+$, 371.2 $[M - H]^-$;^{1}H NMR (500 MHz, $CDCl_3$): δ6.94-6.84 (6H, m, Ar-H), 5.59 (1H, s, 4'-OH), 4.87 (1H, d, J = 6.6 Hz, H-7), 4.43 (1H, d, J = 7.2 Hz, H-7'), 4.13 (1H, dd,J = 1.3, 9.5 Hz, H-9'ax), 3.91 (3H, s, 3-OMe), 3.90 (3H, s, 3'-OMe), 3.89 (3H, s, 4-OMe), 3.86 (2H, m, H-9eq and H-9'eq), 3.33 (2H, m, H-8, H-9ax), 2.91 (1H, m, H-8'); ^{13}C NMR (125 MHz, $CDCl_3$) δ 148.8 (C-3), 148.0 (C-4), 146.7 (C-3'), 145.3 (C-4'), 133.0 (C-1'), 131.0 (C-1), 119.2 (C-6'), 117.7 (C-6), 114.2 (C-5'), 111.0 (C-5), 109.0 (C-2), 108.5 (C-2'), 87.7 (C-7'), 82.0 (C-7), 71.0 (C-9'), 69.7 (C-9), 55.9 (3

× OMe), 54.5 (C-8'), 50.2 (C-8).

Dextran Sulfate Sodium (DSS, MP Biomedicals); Urine fecal occult blood test kit (Nanjing Jiancheng Bioengineering Institute); Goat anti-rabbit Alexa Fluor 488 conjugate (Abcam); DAPI (Sigma-Aldrich); RNA simple total RNA kit (Tiangen Biotech); Hifair II 1st Strand cDNA Synthesis SuperMix for qPCR (gDNA digester plus, Yeasen), Hieff qPCR SYBR Green Master Mix (High Rox, Yeasen); Protease inhibitor cocktail (Roche Life Science); BCA protein assay kit (Thermo Fisher Scientific); 2,4,6-trinitro-Benzenesulfonic acid (TNBS, Sigma-Aldrich); Antibodies against Occludin, TLR4 (Abcam); Antibodies against E-cadherin, NF-κB, p-NF-κB, Cleaved Caspase-1, MyD88, NLRP3, ASC (Cell Signaling Technology); HRP-conjugated monoclonal mouse anti-GAPDH (KangChen); Anti-mouse CD16/32 mAb, BV510-conjugated anti-CD45 mAb, BUV395-conjugated anti-CD11b mAb, APC-conjugated anti-CD11c mAb, PE-conjugated anti-Ly6G mAb, PerCP-Cy5.5-conjugated anti-CD3 mAb (BD Bioscience); FITC-conjugated anti-F4/80 mAb (Thermo Fisher Scientific); FITC-dextran 4KD (Sigma-Aldrich); FBS (Gibco); Colony-stimulating factor (M-CSF, Peprotech); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide (MTT, Sigma-Aldrich); Lipopolysaccharide (LPS, Sigma-Aldrich); Tissue protein extraction reagent (Thermo Fisher Scientific); Mouse TNF-α, IL-6 (BD Biosciences); IL-1β ELISA kit (Thermo Fisher Scientific); Phorbol myristate acetate (PMA, Sigama-Aldrich); Adenosine triphosphate (ATP, Sigma-Aldrich).

Animals

Female BALB/c mice (6-8 weeks), male and female C57BL/6 mice (8 weeks) were purchased from Beijing Huafukang Biotechnology Co., Ltd. The animals were kept in a specific pathogen-free environment with controlled conditions, including a 12-hour light/dark cycle, 22 ± 1 temperature, and 55 +- 5% relative humidity. All experiments were performed according to the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the Bioethics Committee of the Shanghai Institute of Materia Medica (IACUC Protocol #2022-06-ZJP-169; #2022-06-ZJP-177).

Drug treatment and assessment of DSS-induced colitis

For the induction of DSS-induced experimental colitis, C57BL/6 mice were randomly divided into four groups, including a normal control group, a vehicle control group, and two drug treatment groups (60 mg/kg PHI and 20 mg/kg PHI). The normal group received sterile water throughout the experiment, while the other groups were given 2% DSS for 7 days and followed by a 3-day recovery period on regular drinking water. During the experiment, the body-weight loss ratio, stool consistency and rectal bleeding were assessed daily according to the described criterion (see Table 1). The total score of these parameters represents the disease activity index (DAI). On day 10, mice were anesthetized using intraperitoneal injection of 4% chloral hydrate. The peripheral blood, colon, and mesenteric lymph nodes were collected for subsequent analysis.

Table 1. Criteria for DAI: weight loss, stool consistency and fecal blood

Score	Weight loss (%)	Stool consistency	Blood
0	0	Normal	Negative hemoccult
1	1-5	Soft but still formed	Weakly positive hemoccult
2	6-10	Soft	Positive hemoccult
3	11-20	Very soft; wet	Blood traces in stool visible
4	>20	Watery diarrhea	Gross rectal bleeding

Drug treatment and assessment of TNBS-induced colitis

For the induction of TNBS-induced experimental colitis, C57BL/6 mice were randomly divided into three groups, including a normal group, a vehicle control group, and a drug treatment group that received 2.5% 2,4,6-trinitro-Benzenesulfonic acid (TNBS) by clysma on the first day of the experiment. The drug treatment group received 40mg/kg PHI orally. The body-weight loss ratio and survival rate were monitored. At the

end of the experiment, mice were anesthetized using intraperitoneal injection of 4 % chloral hydrate. The peripheral blood, colon, and mesenteric lymph nodes were collected for subsequent analysis.

Histological analysis

The colons tissue specimens were fixed in 10% formalin for 24 h, dehydrated, and paraffin-embedded. Hematoxylin and eosin (H&E) staining was performed to observe the colon tissue pathology under an optical microscope. The scoring criteria consisted of the following: 0, no evidence of inflammation; 1, low-level inflammation with scattered mononuclear cells (1-2 foci); 2, moderate inflammation with multiple foci of mononuclear cells; 3, high-level inflammation with increased vascular density and significant wall thickening; and 4, maximal inflammation with transmural leukocyte infiltration and loss of goblet cells. Additionally, periodic acid-Schiff stain (PAS) and Alcian blue staining techniques were used to examine the epithelial goblet cells, characterizing neutral mucin and acidic mucins, respectively. The Leica DM6B laser microdissection system was utilized to obtain images.

FITC-dextran intestinal permeability assay

Mice were fasted overnight and orally administered FITC-dextran (600 mg/kg). After 4 h, their serum were collected and the fluorescence intensity of FITC-dextran was measured at 480 excitation wavelength and 520 emission wavelength.

Flow cytometry assay

Single suspensions of mouse mesenteric lymph nodes or peripheral blood mononuclear cells (PBMCs) were prepared. Then the single cells were blocked with purified anti-mouse CD16/CD32 mAb for blocking and flow cytometry antibodies: BUV395-conjugated CD11b, FITC-conjugated CD11c, PE-conjugated Ly6G, BV510-conjugated F4/80, APC-conjugated Ly6C, BUV395-conjugated CD4, Percy-cy5.5-conjugated CD3 and PE-conjugated CD69. After incubation at 4 for 20 min, the cells were washed once with phosphate buffer saline and analyzed using a BD LSR Fortessa Flow Cytometer. The data were analyzed with FlowJo software.

Cell culture and treatment

Splenocytes were cultured in triplicate for 48 h in the presence or absence of the compounds and were stimulated with 5 μg/mL concanavalin A (Con A) to induce T cell proliferation and 10 μg/mL lipopolysaccharide (LPS) to induce B cell proliferation. The cells were pulsed with 0.5 μCi/well of [3H] thymidine for 8 h and harvested on glass fiber filters. The incorporated radioactivity was counted using a Beta Scintillation Counter (MicroBeta TriLux, PerkinElmer Life Sciences, Boston, MA, USA).

Bone marrow-derived macrophages (BMDMs) were differentiated as described previously. Briefly, BMDMs were separated from the femur and tibia bones of male C57BL/6J mice and then cultured for 7 days in RPMI-1640 medium containing 10% FBS and 10 ng/mL of M-CSF. Differentiation of THP-1 cells was induced by 100 ng/mL PMA for 48 h. The differentiated cells were treated with 1 μg/mL LPS in the absence or presence of PHI.

Immunofluorescence

For tissue samples, paraffin-embedded intestinal sections were dewaxed in xylene and rehydrated through gradient alcohols. After being blocked with 5% BSA, the tissue sections were stained with anti-F4/80. The signals were detected by goat anti-rabbit Alexa Fluor 647 conjugate and then counterstained with DAPI. For BMDMs, cells are fixed by 4% paraformaldehyde, permeated by 1% Triton X-100, and blocked by immunostaining blocking solution. Anti-NLRP3 is used to incubate cells at 4 overnight, followed by detection with goat anti-rabbit IgG Alexa Fluro 488 conjugate staining. The cell nucleus is then stained by DAPI. Captured images are analyzed using the Leica TCS SPS microscope and the Leica Application Suite X.

RNA extraction and real-time quantitative polymerase chain reaction

RNA lysate RZ is used to homogenize mice colons, followed by RNA extraction using the RNAsimple total RNA kit. Reverse transcription is carried out with HifairTM II 1st strand cDNA synthesis supermix for qPCR, and real-time quantitative PCR is performed with SYBR Green Realtime PCR Master Mix on an Applied Biosystems 7500 Fast Real-Time PCR system. PCR amplification is performed with the primers listed in Table 2.

Table 2. Sequences of primers.

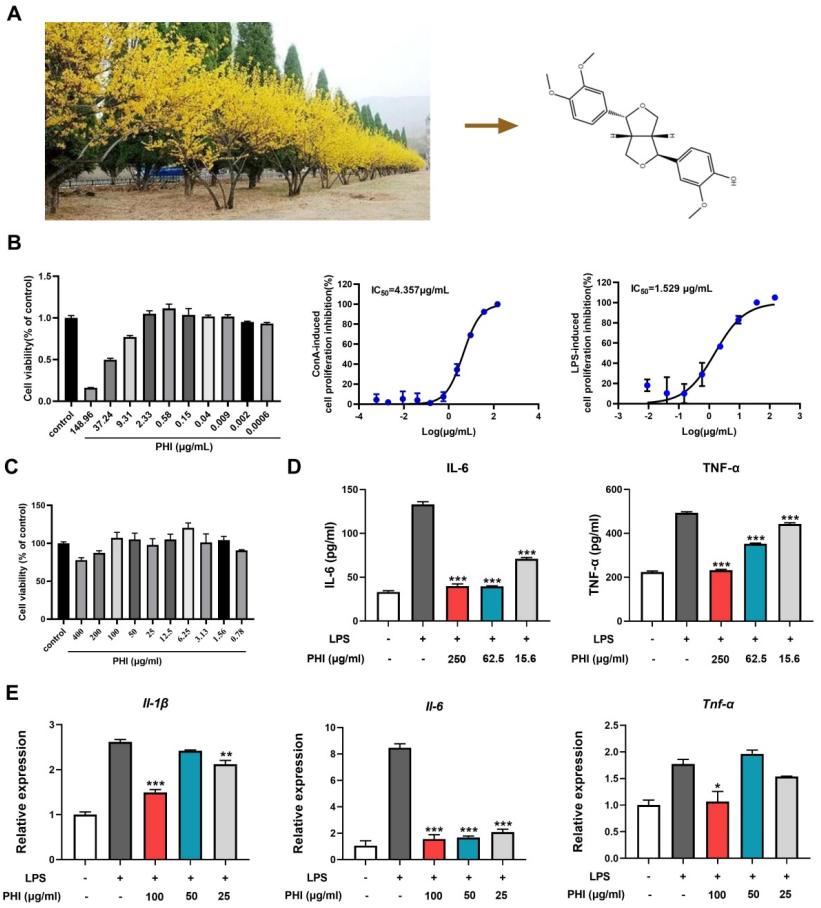
Genes	Forward (5'-3')	Reveres (5'-3')
Mouse β-actin	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT
Mouse IL-1β	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
Mouse IL-6	CCAAGAGGTGAGTGCTTCCC	CTGTTGTTCAGACTCTCTCCCT
Mouse TNF-α	ATGTCTCAGCCTCTTCTCATTC	GCTTGTCACTCGAATTTTGAGA
Mouse TLR4	ATGGCATGGCTTACACCACC	GAGGCCAATTTTGTCTCCACA
Mouse NLRP3	ATTACCCGCCCGAGAAAGG	TCGCAGCAAAGATCCACACAG
Mouse ASC	AGACATGGGCTTACAGGAGCTG	CCACAAAGTGTCCTGTTCTGGC
Mouse Caspase 1	ACAAGGCACGGGACCTATG	TCCCAGTCAGTCCTGGAAATG
Human β-actin	TTGTTACAGGAAGTCCCTTGCC	ATGCTATCACCTCCCCTGTGTG
Human IL-1β	AGCTACGAATCTCCGACCAC	CGTTATCCCATGTGTCGAAGAA
Human IL-6	ACTCACCTCTTCAGAACGAATTG	CCATCTTTGGAAGGTTCAGGTTG
Human TNF-α	CCTCTCTCTAATCAGCCCTCTG	GAGGACCTGGGAGTAGATGAG

Statistical analysis

RNA lysate RZ is used to homogenize mice colons, followed by RNA extraction using the RNAsimple total RNA kit. Statistical differences were detected by GraphPad Prism 8.3 using a two-tailed Student's t-test or one-way ANOVA with Dunnet's multiple comparisons tests. $P< 0.05$ were considered significant.

Results

PHI exhibited immunosuppressive activity *in vitro*



The extraction of PHI was extracted, identified, and characterized (Fig. 1A, FigS1 to S5). In the present study, we found that PHI exhibits immunosuppressive activity *in vitro* . PHI was analyzed to establish appropriate levels for subsequent use that showed minimal signs of cytotoxicity on both murine splenocytes (Fig. 1B) and THP-1cells over a 24h time course growing in cultures (Fig. 1C).

Fig. 1. Phillygenin exhibited favorable immunosuppressive activity *in vitro*. (A) Chemical structure of PHI. (B) The cell viability of splenic lymphocytes with PHI treatment and IC_{50} on ConA or LPS-induced lymphocyte proliferation. (C) The cell viability of THP-1 with PHI treatment. (D) Cytokine secretion levels in LPS-induced THP-1 culture. (E) The gene expression level of cytokine in LPS-induced THP-1.

Specifically, the CC50 of PHI on murine splenocytes was found to be 35.43 μg/mL, and it also exerted ideal immunosuppressive effects on ConA- and LPS-induced lymphocyte proliferation, with IC50 values of 4.357 and 1.539 μg/mL, respectively (Fig. 1B). Furthermore, the anti-inflammatory activity of PHI on PMA-induced human macrophages THP-1 was also confirmed. The ELISA assay indicated that PHI could effectively inhibit the secretion of IL-6 and TNF-α induced by LPS (Fig. 1D). On the genetic level, PHI was found to effectively inhibit expressions of IL-1β, IL-6, and TNF-α. Taken together, these results indicate that PHI is a promising candidate for immunosuppressive therapy *in vitro*(Fig. 1E).

PHI ameliorated DSS-induced murine acute ulcerative colitis

We first investigated the therapeutic effects of PHI in DSS-induced colitis. 2% Dextran Sulfate Sodium

(DSS) in drinking water ad libitum for 7 consecutive days, followed by treatment with different doses of PHI (60 mg/kg and 20 mg/kg) throughout the whole experiment (Fig. 2A)

Using disease activity index (DAI) as a measure of UC severity, the mice in both DSS- and 2,4,6-trinitro-Benzenesulfonic acid (TNBS)-induced colitis experienced significant weight loss, severe diarrhea, and bloody stool after chemical induction. Treatment with PHI resulted in a reversal of these symptoms, as well as a restoration of immune hyperfunction-induced splenomegaly and colon shortening (Fig. 2B and 2C). PHI also exerted positive effects on maintaining the intestinal mucosal barrier by reducing goblet cell loss, mucous thinning, and intestinal permeability (Fig. 2D, E).

In addition, the histologic analysis indicated that PHI treatment restored the colon structure, prevented crypt destruction, and inhibited inflammatory cell infiltration. The expression of tight junction proteins in the colon was also restored, further demonstrating PHI's beneficial effect on intestinal barrier function (Fig. 2F).

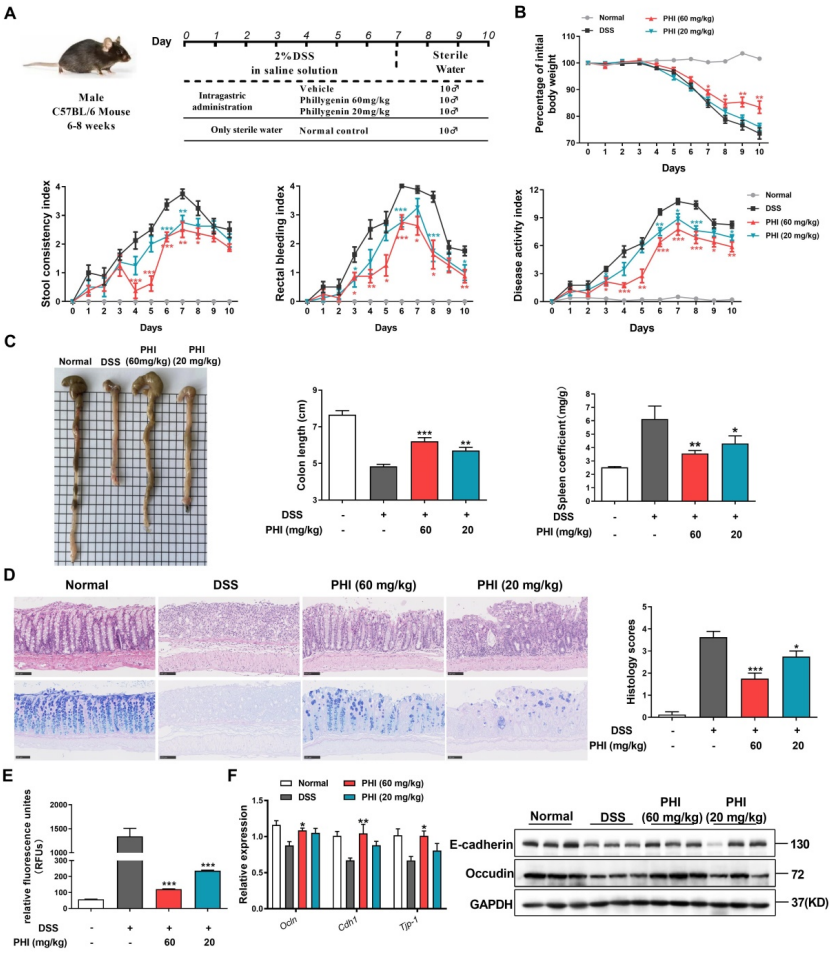


Fig. 2. PHI ameliorated the experimental symptoms in DSS-induced murine acute UC. (A) DSS-induced murine model. (B) Body weight change (% initial body weight), diarrhea, stool bleeding, and disease activity index (DAI). (C) Representative images of colon morphology, quantitative colon length, and the spleen coefficient. (D) Representative images of H&E, PAS-Alcian blue staining of colon sections, and histological scores, bar = 100 μm. (E) Fluorescence intensity of FITC-dextran in serum. (F) The mRNA

and protein levels of tight junction proteins in the intestine. Compared with the model control group *p ¡0.05, **p ¡0.01, ***p ¡0.001, n = 6-8.

PHI ameliorated TNBS-induced murine ulcerative colitis

To better explore the effect of PHI on inflammatory bowel disease, we also used TNBS-induced mouse model. Used 2.5% TNBS to cause gut inflammation. Mice were treated with 40 mg/kg PHI from day 0 to the endpoint (Fig. 3A). Similarly, PHI significantly improved the survival rate, colon length, and spleen coefficient of TNBS-exposed C57BL/6 mice. (Fig. 3B and 3C). Histopathological examination of the colon section revealed evident improvement in inflammatory cell infiltration in PHI (40 mg/kg) treated colitis mice, compared with the TNBS-treated mice (Fig. 3D).

Overall, the study provides evidence that PHI has a favorable pharmaceutical effect in different chemically induced UC models and may have potential as a therapeutic agent for the treatment of UC.

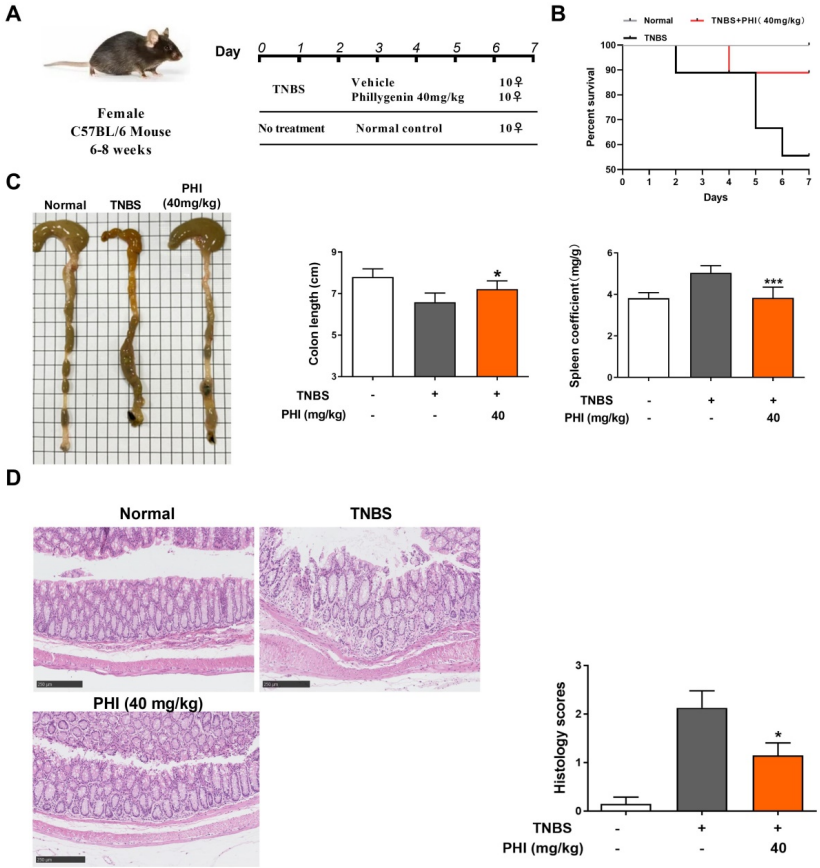


Fig 3. PHI ameliorated the experimental symptoms in TNBS-induced murine acute UC. (A) TNBS-induced murine model. (B) Survival rate. (C) Representative images of colon morphology, quantitative colon length, and the spleen coefficient. (D) Representative images of H&E and histological scores. Compared with the model control group *p ¡0.05, **p ¡0.01, ***p ¡0.001, n = 8-10.

PHI reduced the infiltration of inflammatory cells

Neutrophils and macrophages infiltrations are recognized as evaluative parameters for the severity degree of colitis, and flow cytometry was used to assess changes in the immune cell populations in MLN and

peripheral blood. The population of monocytes ($CD11b^+$), macrophages ($CD11b^+F4/80^+$), neutrophils ($CD11b^+Ly6G^+$), and dendritic cells ($CD11b^+CD11c^+$) were significantly increased in both MLN and peripheral blood of DSS-induced UC mice. However, both high and low doses of PHI were found to reduce the population of macrophages in MLN and peripheral blood monocyte cells, with high doses of PHI having an inhibitory effect on neutrophils of PBMC (Fig. 4A-B). In TNBS-induced mice, the population of macrophages decreased, and PHI reduced the level, which was consistent with the findings in DSS-induced mice(Fig. 4C-D). Immunofluorescent staining with F4/80 showed severe infiltrations of macrophages in DSS-induced mice colon, which were reduced by PHI (Fig. 4E), suggesting that future research may focus on macrophages' role in the mechanism of action of PHI.

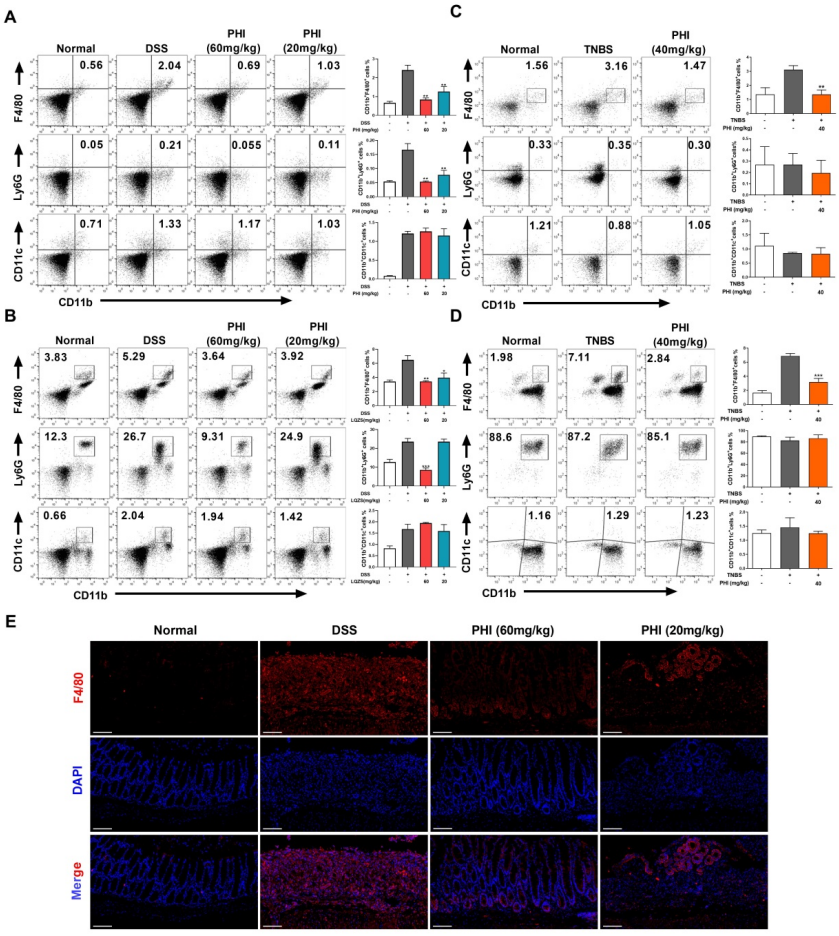


Fig. 4. PHI inhibited inflammatory cells in MLN and PBMC of DSS and TNBS-induced ulcerative colitis. (A and B) Flow cytometry and quantitative analysis of mesenteric lymph nodes (MLNs) and peripheral blood mononuclear cells in the DSS-induced murine model, including macrophages ($CD11b^+F4/80^+$), neutrophils ($CD11b^+Ly6G^+$), and dendritic cells ($CD11b^+CD11c^+$). (C and D) Flow cytometry and quantitative analysis of mesenteric lymph nodes (MLNs) and peripheral blood mononuclear cells in TNBS-induced murine model. (E) Representative images of immunofluorescence staining of F4/80, bar = 100μm. Compared with the model control group $^*p$ ¡0.05, $^{**}p$ ¡0.01, $^{***}p$ ¡0.001, n = 3-4.

ΠΗΙ ιμπροεδ υλχερατιε χολιτις βψ ινηιβιτινγ τηε αχτιατιον οφ ΝΛΡΠ3 ινφλαμμασομε τηρουγη ΤΛΡ4/ΜψΔ88/ΝΦ-κΒ πατηωαψ

Proinflammatory mediators such as cytokines IL-1β, IL-6, and TNF-α play key roles in UC, and the secretion level of these cytokines in serum or colon was found to rise sharply in the presence of UC. After PHI treatment, these inflammatory factors were significantly inhibited at both the gene and protein levels in the colon and serum (Fig. 5A-C).

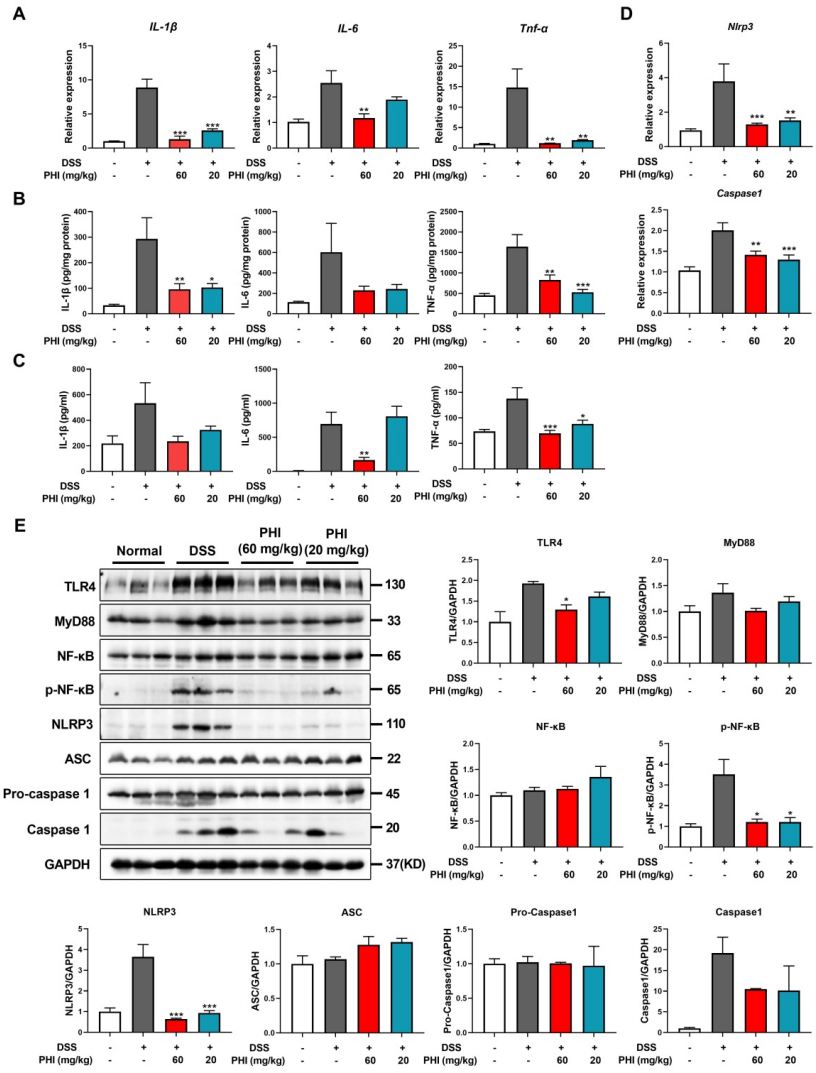


Fig. 5. PHI ameliorated inflammation via the NLRP3 inflammasome pathway. (A) The gene expression level of cytokines in colonic homogenates. (B and C) The protein levels of cytokines in colonic homogenates and serum. (D) The gene expression level of Nlrp3 and Caspase 1. (E) The protein expression of NLRP3 inflammasome and its upstream pathway. Compared with the model control group *p ¡0.05, **p ¡0.01, ***p ¡0.001, n = 3-4.

Given the remarkable effect of PHI on IL-1β, and NLRP3 inflammasome was reported to be an important protein complex for IL-1β production, we detected the expression level of NLRP3 inflammasome relative proteins. As shown in Fig. 5D-E, the expression of both NLRP3 and Caspase1 was increased dramatically with DSS induction, which was restrained in PHI-treated group.

In summary, the present study demonstrated that PHI improved UC by inhibiting the activation of NLRP3 inflammasome through the TLR4/MyD88/NF-κB pathway. These findings have important implications for the development of future UC treatments

ΠΗΙ ιμπροεδ ΛΠΣ ανδ ΑΤΠ-ινδυςεδ ΒΜΔΜ ινφλαμματιον βψ ινηιβιτινγ τηε αςτιατιον οφ ΝΛΡΠ3 ινφλαμμασομε βψ ΤΛΡ4/ΜψΔ88/ΝΦ-κΒ πατηωαψ

Macrophages are known to play a critical role in both the progression of inflammation and the eventual remission of disease during intestinal mucosal repair. To investigate the efficacy of PHI *in vitro* , bone marrow-derived macrophages (BMDM) were utilized in an *in vitro*experiment due to their significant impact on macrophages compared to other myeloid cells. In the initial phase of the experiment, the impact of PHI on cell viability was examined, and three safe concentrations were selected (100, 50, and 25 μg/mL) for subsequent research (Fig. 6A). BMDM was then stimulated with lipopolysaccharide (LPS), a typical TLR4 agonist, to induce inflammatory cytokine production. PHI displayed potent anti-inflammatory properties on BMDM, which was reflected in a reduction in genetic and protein expression levels of IL-1β and IL-6 under LPS induction (Fig. 6B-C).

Based on these findings, we tested the effect of PHI on NLRP3 inflammasome *in vitro* . The NLRP3 inflammasome was activated by LPS and NLRP3 agonist adenosine triphosphate (ATP). As with the results in UC mice colon, PHI inhibited the expression of NLRP3 and Caspase1, especially NLRP3, which was also demonstrated by using cellular immunofluorescence (Fig. 6D-F). The influence mechanism was also proven, as it inhibited the upstream pathway as a depressant. By combining the results in both *in vivo* and *in vitro* experiments, the conclusion was that PHI had a positive effect on ulcerative colitis, and it inhibited inflammation by preventing NLRP3 inflammasome activation through the TLR4/MyD88/NF-κB pathway (Fig. 7).

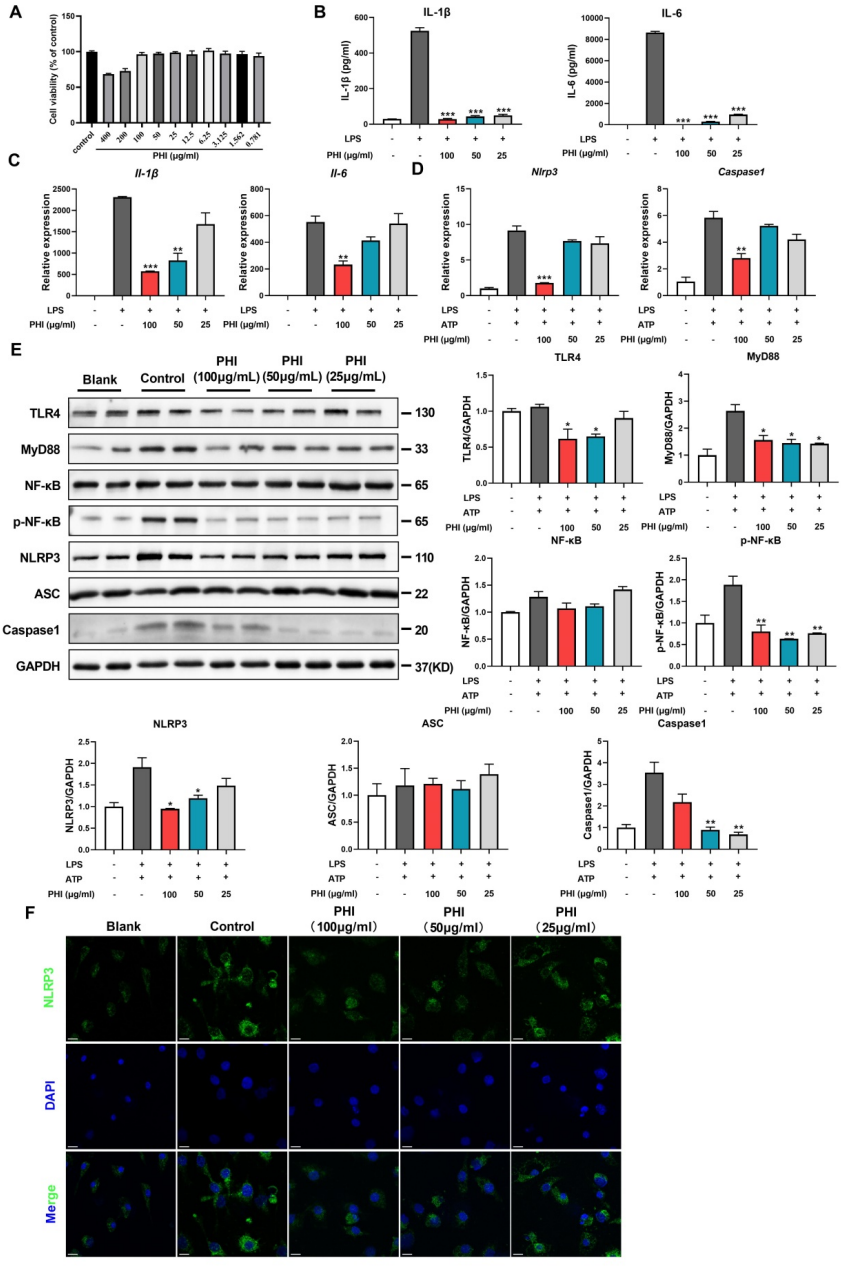
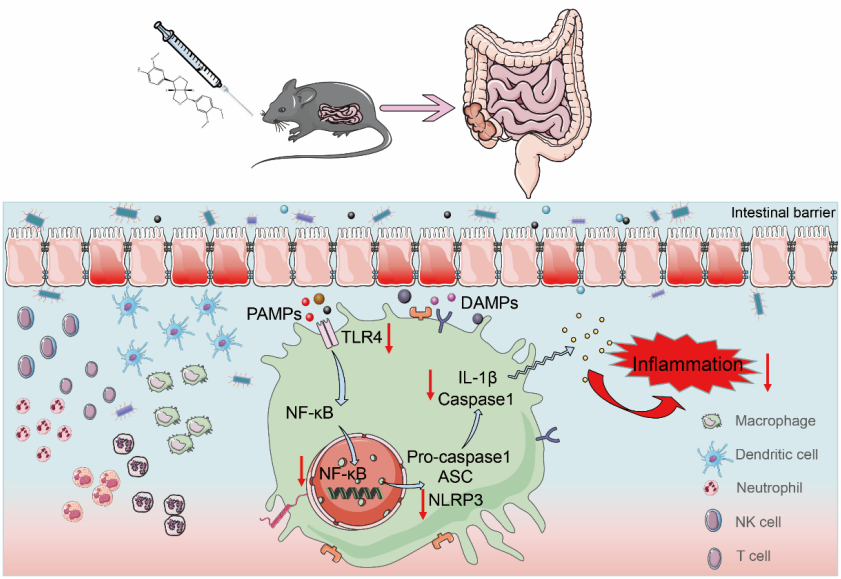


Fig. 6. PHI inhibited the inflammation of BMDMs by inhibiting the NLRP3 inflammasome pathway. (A) The cell viability of BMDM with PHI treatment. (B and C) The protein and gene expression levels in LPS-induced BMDM. (D) The gene expression level of Nlrp3 and Caspase 1. (E) The protein expression of inflammasome and its upstream pathway. (F) Representative images of immunofluorescence staining of NLRP3, bar = 10 μm. Compared with the LPS or LPS&ATP-stimulated group *p ¡0.05, **p ¡0.01, ***p ¡0.001, n = 3-4.



Φιγ. 7. Γραπηιςαλ συμμαρψ οφ αμελιορατιον οφ υλςερατιε ςολιτις βψ ΠΗΙ ια ινηιβιτινγ ΝΛΡΠ3 ινφλαμμασομε αςτιατιον τηρουγη τηε ΤΛΡ4/ΜψΔ88/ΝΦ-κΒ πατηωαψ.

* Discussion

UC is an inflammatory disease characterized by inflammation of the colon mucosa, which affects the rectum and extends proximally[19]. The incidence of UC has been increasing every year due to the industrialization of various countries and the westernization of people's eating habits [20]. Although there have been therapeutic advancements in UC, there is still a treatment gap from people's ideal state[21]. Consequently, an increasing number of studies have explored the potential use of active monomers in functional food or traditional Chinese medicine to treat colitis[22]. *Forsythia suspen* sa, a deciduous shrub with bitter and cold characteristics that align with anti-inflammatory Chinese medicine, has been used historically for fever, inflammation, gonorrhea, and erysipelas. Through exploring the immunosuppressive activity of *Forsythia suspensa's*components in abnormal splenic lymphocyte proliferation induced by ConA and LPS, we identified a beneficial component known as PHI with an IC50 of 4.359 μg/mL and 1.539 μg/mL, respectively. Additionally, PHI inhibited cytokine secretion in LPS-stimulated THP-1 cells, which motivated us to explore its therapeutic effect on clinical inflammatory diseases using mouse models.

Next, significant therapeutic effects of PHI were found in mouse models of UC, as reflected by its ability to maintain body weight, reduce disease activity index and mortality, restore the intestinal mucosal barrier, and inhibit cytokine secretion (Fig. 2 and Fig. 3). To investigate the protective effects of PHI on the gastrointestinal tract, H&E staining, PAS-Alcian blue staining, and FITC-dextran were used to prove the therapeutic effects of PHI on maintain intestinal morphology, reduce goblet cell loss, inflammatory cell infiltration, and decrease intestinal permeability. In inflammatory bowel diseases, barrier function is maintained by the mucus layer and epithelial cells connected by tight junction proteins. In DSS-induced colitis, the expression of tight junction-associated protein E-cadherin and Occludin was decreased, and PHI was able to restore their expression as shown in Fig.2E, which reduced intestinal damage and restored mucosal barrier function. Furthermore, Macrophages play a critical role in the progression of inflammation and the disease's remission during intestinal repair. Flow cytometry analysis of MLN and PBMC showed that PHI could reduce macrophage infiltration in both colitis models. Immunofluorescence performed on colon tissues also demonstrated that PHI significantly inhibited macrophage infiltration in DSS-induced colitis (Fig.4E). Cytokines are also important mediators in UC's enhancement and continuation, and their presence directly causes mucosal and tissue damage, inducing disease-specific immune responses in UC. The levels of

pro-inflammatory cytokines including IL-1β, IL-6, and TNF-α in serum and colon were determined, and we found that PHI could effectively inhibit their secretion, especially the secretion of IL-1β.

The activation of IL-1β in response to infection, mucosal injury, and stress triggers a local mucosal immune response, recruiting neutrophils to the affected site and activating the NF-κB pathway, which leads to the upregulation of pro-inflammatory cytokines and chemokines [5]. Furthermore, IL-1β is the most likely effector molecule downstream of the NLRP3 inflammasome. Given the significant impact of PHI on macrophages and IL-1β, the current study focused on the mechanism of action of the NLRP3 inflammasome. We found that PHI inhibited inflammasome protein levels at both the genetic and protein levels. Starting with its upstream pathway, macrophages exposed to stimuli, such as ligands for TLR4, activate the transcription factor NF-κB, which then upregulates NLRP3 expression [23]. Specifically, TLR4 and MyD88 expression increased, and the phosphorylation of NF-κB increased markedly, but PHI inhibited this pathway and restrained the activation of the NLRP3 inflammasome. Finally, we conducted *in vitro*explorations with BMDM and found that PHI suppressed inflammation by inhibiting the activation of the NLRP3 inflammasome through the TLR4/MyD88/NF-κB pathway.

*** Conclusion**

In conclusion, PHI treatment exhibited significant improvements in UC by inhibiting the activation of the NLRP3 inflammasome via the TLR4/MyD88/NF-κB pathway. These findings provide potential mechanisms underlying the therapeutic effects of PHI on UC.

*** Ethics approval and consent to participate**

All procedures followed the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the Bioethics Committee of the Shanghai Institute of Materia Medica (IACUC Protocol #2021-04-ZJP-145; #2021-07-ZJP-151; #2022-01-ZJP-161).*** Consent for publication**

We declare that the publisher has the author's permission to publish the relevant contribution.

*** Competing interests**

The authors declare no competing interests.*** Funding**

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*** Authors' contributions**

XT, LC performed most of the experiments; SSL, JYY, SHF, XYX, CMW, DL, FHZ, and XQY performed some experiments; SJH, SY, ZML, AND JPZ designed the experiments; SJH, and JPZ interpreted the results; XT and ZML wrote the paper.

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Not applicable.

*** Availability of data and material**

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Appendix A. Supplementary material

Abbreviations:

UC, ulcerative colitis; IBD, inflammatory bowel disease; TNF, anti-tumor necrosis factor; PAMPs, pathogen-associated molecular pattern; TLRs, Toll-like receptors; NLRP3, NOD-like receptor family pyrin domain containing 3; ASC, apoptosis-associated speck-like protein containing CARD; NF-ϰB, nuclear factor kappa-B; PHI, phillygenin; DSS, dextran sulfate sodium; DAI, disease activity index; TNBS, 2,4,6-trinitro-Benzenesulfonic acid; PBS, phosphate-buffered saline; H&E, hematoxylin, and eosin; PAS, periodic acid-Schiff stain; FITC-d, fluorescein isothiocyanate- dextran; PBMC, peripheral blood mononuclear cells; MLN, mesenteric lymph nodes; M-CSF, mouse colony-stimulating factor; BMDM, bone marrow-derived macrophage; ELISA, enzyme-linked immunosorbent assay; LPS, lipopolysaccharide; ATP, adenosine triphosphate; DAPI, 4',6-diamidino-2-phenylindole; ConA, concanavalin A; PMA, phorbol 12-myristate 13-acetate.

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