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Original Article

Calycosin attenuates TNBS-induced colitis through inibiting inflammatory cytokine and oxidative stress

Chao Liu[1,2], Pengyuan Zheng[1], Xia Liu[2], Yong Yu[1], Gaofeng Lu[2], Fuai Tang[2], Zhiguo Zhao[2]

[1]Department of Gastroenterology, The Fifth Affiliated Hospital of Zhengzhou University, Zhengzhou 455052, China; [2]Department of Gastroenterology, The Second Affiliated Hospital of Zhengzhou University, Zhengzhou 450014, China

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Abstract: Objective: To investigate the underlying protective effects of Calycosin on experimental colitis. Methods: The effect of Calycosin on pro-inflammatory genes mRNA expression and production were examined using real-time PCR and ELISA. Flow cytometry was used to determine the effect of Calycosin on LPS-induced ROS production in Raw264.7 cells. Western blot assay was used to determine the effect of Calycosin on NF-κB and MAPK pathway activation. For in vivo models, 2.5% TNBS was utilized through the catheter to induce experimental colitis. Body weight and DAI were evaluated every day. H&E staining were used to evaluate the effect on Calycosin on TNBS-induced colon damage. Moreover, MPO levels, MDA, GSH and SOD in TNBS-treated colon were determined by assay kits. Results: Calycosin significantly inhibited LPS-induced pro-inflammatory cytokines mRNA expression and production in Raw264.7 cells. 50 mg/kg Calycosin protect against TNBS-induced experimental colitis in mice, decreased oxidative stress and inhibited TNBS-induced inflammatory cytokine production, which is connected to the inhibition of NF-κB pathway. Conclusion: These findings revealed that Calycosin successfully ameliorated the effect of TNBS-induced colitis in mice.

Keywords: Calycosin, oxidative stress, experimental colitis, inflammation, NF-κB pathway

Introduction

Intestinal inflammation is related to multiple factors including inflammatory bowel disease (IBD). The IBD comprises a set of related diseases such as Ulcerative Colitis (UC) and Crohn's Disease (CD), which has been attributed to the breakdown of tolerance to self-antigens in the intestinal mucosa. UC affects the colon and/or the large intestine, whereas CD causes inflammation that extends through the entire bowel wall. The development of IBD is associated with epithelial cell necrosis and ulceration and local infiltrated immune cells. The increase in incidence of IBD has attracted more attention in recent times [1-4].

At present, the clinical treatment of inflammatory bowel disease is based on immunosuppressants, salicylic acid and glucocorticoids. However, most of these medicines only temporarily alleviate the symptoms, and are associated with mild to serious side effects, leading to their limited clinical applications [5]. Thus traditional Chinese Medicine Formulas are gaining more considerations in the treatment of IBD, largely because of their effectiveness and safety [6]. Some clinical and experimental studies have shown that Chinese formulas for treatment of IBD were effective, and most of their mechanisms were related with anti-inflammatory, anti-oxidant and restoration of the functions of intestinal barrier [7-9].

Calycosin (**Figure 1**), the main component of isoflavones, is widely used as a natural active compound against oxidative stress. It can attenuate mice hepatic fibrosis via activating farnesoid X receptor [10]. Recently, several studies have shown that Calycosin can inhibit growth in a variety of cancers [11-13].

However, whether Calycosin can effectively ameliorate intestinal inflammation remains unknown. The present study was designed to investigate the effects of Calycosin on TNBS-induced colitis in mice of BALB/c. Parameters as body weight, histology of target tissues, cell proliferation, and cytokine production were evaluated in this investigation. Our data suggested that Calycosin modulates the RAW264.7 against LPS in vitro while regulating immune

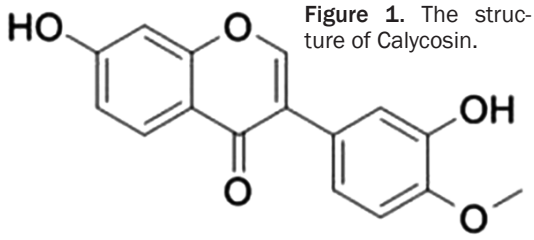

Figure 1. The structure of Calycosin.

responses against TNBS in BALB/c mice through antioxidation and suppression of NF-κB pathway.

Materials and methods

Reagents

Dimethyl sulfoxide (DMSO), isopropanol, ethanol and chloroform were purchased from China national medicine group chemical reagent co., LTD. Calycosin, Lipopolysaccharides (LPS, Escherichia coli, L2630, Serotype 0111:B4), 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT), ammonium persulfate, sodiumdodecyl sulphate, TritonX-100, and Tris-base were procured from Sigma-Aldrich (St. Louis, Missouri, USA). DMEM, RPMI-1640 and fetal bovine serum (FBS), TRIzol were purchased from Life Technologies (Grand Island, New York, USA). The TNF-α ELISA, IL-6 ELISA, MCP-1 ELISA, IL-1β, Nitrite ELISA were from R&D corporation, DCFH-DA Dye was perchased from Beyotime (NanTong, China). Reduced glutathione (GSH) assay kit (A006-2), Superoxide Dismutase (SOD) assay kit (WST-1 method) (A001-3), Malondialdehyde (MDA) assay kit (TBA method) (A003-1) and myeloperoxidase (MPO) assay kit (A044) were all obtained from the Institute of Biological Engineering of Nanjing Jiancheng (Nanjing, China). Anti-IKKα (#11930), anti-IKKβ (#8943), anti-phosphate-IKKα/β (#2697), anti-IκBα (#4814), anti-phosphate-IκBα (#2859), anti-p65 (#8242), anti-phosphate-p65 (#3033), anti-SAPK/JNK (#9-295), anti-phosphate-SAPK/JNK (#4668), anti-Erk1/2 (#4695), anti-phosphate-Erk1/2 (#4-370), anti-p38 (#8690), anti-phosphate-p38 (#4511), β-actin (#5174), HRP conjugated Goat anti-Rabbit IgG (#7074) and RIPA cell lysate (#9806) were purchased from Cell Signaling Technology corporation. Other chemical reagents were ordered from Sangon Biotech (Shanghai, China).

Cell culture

Mice macrophage Raw264.7 cell line was purchased from Chinese academy of sciences and incubated in a humidified CO_2 incubator (5% CO_2/95% air) at 37°C in DMEM medium containing 100 U/ml penicillin, 100 U/ml streptomycin and 10% fetal bovine serum.

Hemolysis test

Whole blood collected from Balb/c mice was mixed with cold PBS in 1:2 ratio, and cells were centrifuged at 500 g for 10 min afterwards the supernatant was discarded. Precipitation was washed with cold PBS 3 times to get purified red blood cells. Red cells suspension treated with different concentrations of Calycosin were incubated for 3 hours under the condition of 37°C, 5% CO_2. Supernatant was collected at 10000 g for 3 min and the release of Heme was measured at 540 nm using microplate reader. A 1% TritonX-100 was set as positive control and the hemolysis rate was 100%.

MTT method

Raw264.7 cells were planted into 96-well culture plate with the density of 5×10^4 per well and was treated with Calycosin for 72 h. A 100 ml/well MTT (1 mg/ml, dissolved in PBS solution) was added into the 96-well plates. After 4 h incubation at 37°C, 100 ml DMSO was also added. One hour later, the absorbance was determined using a multiplate reader at a wavelength of 570 nm.

Animal studies

Animal research was approved by the Animal Ethical and Welfare Committee (AEWC) of the Affiliated Second Hospital of Zhengzhou university (20160912). Male Balb/c mice (6-8 weeks old, 18-20 g, n=35) were obtained from Laboratory Animal Center of Henan Province (Henan, China), and housed under constant conditions (12-h light/dark cycle, room temperature 21 ± 1°C) with clean water and food at all times. Mice were randomly divided into 5 groups according to the weight. Calycosin and 5-ASA were suspened in 0.5% CMC-Na. Normal group and TNBS group administrated with the same volume of 0.5% CMC-Na were used as a negative control. The mice in the model group were gavaged daily with 0.5% CMC-Na containing different doses of Calycosin (25 mg/kg or 50 mg/kg per mouse) or 5-ASA (50 mg/kg per mouse) until day 3.

TNBS induced experimental colitis

A 2.5% TNBS dissolved into 50% ethanol was applied to induce experimental colitis. The mice

were fasted overnight and were then anesthetized with pentobarbital sodium (50 mg/kg, ip) to allow the rectal administration of TNBS. Briefly, the tip of a soft catheter was advanced transanally 3 cm into the distal colon and 100 μl TNBS was instilled intraluminally to induce colitis. The animals were maintained in a head-down position for approximately 60 seconds to prevent leakage of the infusate. Normal group was subjected to the same procedure, infusing 100 μl 50% ethanol instead of TNBS. The survival rate, weight loss and disease activity index (DAI) were observed for three consecutive days. DAI is the sum of the faeces and hematochezia. The score of faeces is followed as: 0= moderate, 1= soft but not stick to the anus, 2= soft and stick to the anus. The score of hematochezia is followed as: 0= no, 1= slightness, 2= a large number.

Myeloperoxidase (MPO) activity

Colon tissues from the same site were weighed and homogenized in ice-cold PBS. The homogenate was centrifuged for 10 min at 8000 g and the supernatant collected for the measurement of myeloperoxidase (MPO) activity in intestinal mucosa using microplate reader according to the manufacturer's instructions on the kit.

Colon oxidative stress

The levels of GSH, SOD and MDA in the supernatant from the homogenate were respectively measured according to the manufacturer's instructions.

Histology

A histological examination was performed on three samples of the colon from the same position of each mouse. The samples were fixed in 10% formalin and embedded in paraffin. 4-μm-thick section was cut and stained with hematoxylin and eosin for analysis.

Real-time PCR analysis

RNA was prepared using 1 ml of TRIzol and the cDNA generated with reverse transcriptase. The qPCR was carried out using the DyNAmo SYBR Green 2-step qRT-PCR kit (Finnzymes, F430L). The sequences of primers are as follows:

TNF-α-forward CTTCTGTCTACTGAACTTCGGG, TNF-α-reverse CAGGCTTGTCACTCGAATTTTG; IL-1β-forward ACGGACCCCAAAAGATGAAG, IL-1β-reverse TTCTCCACAGCCACAATGAG; IL-6-forward CAAAGCCAGAGTCCTTCAGAG, IL-6-reverse GTCCTTAGCCACTCCTTCTG; IFN-γ-forward CCTAGCTCTGAGACAATGAACG, IFN-γ-reverse TTCCACATCTATGCCACTTGAG; MCP-1-forward GTCCCTGTCATGCTTCTGG, MCP-1-reverse GCTCTCCAGCCTACTCATTG; iNOS-forward GCAAACATCACATTCAGATCCC, iNOS-reverse TCAGCCTCATGGTAAACACG; β-actin-forward ACCTTCTACAATGAGCTGCG, β-actin -reverse CTGGATGGCTACGTACATGG.

Data collection was performed using ABI PRISM 7000 Sequence Detection System with the SYBR Green PCR Master Mix (Applied Biosystems).

Enzyme-linked immunosorbent assay (ELISA)

Colon tissues were collected from the same position and 500 μl of homogenization buffer containing protease inhibitors and phosphatase inhibitors added to homogenize for 30 s. The mixture was incubated on ice for 30 min and centrifuged at 12000 g for 5 min. The supernatant was obtained and used to determine the cytokines according to the manufacturer's instructions on the ELISA kit.

Preparation of cellular lysates and Western blot analysis

Raw264.7 were collected after undergoing different treatments. Colon tissues were cut into small pieces and put in eppendorf tubes. The samples were then homogenized using a lysis buffer containing protease cocktail inhibitors (Roche). The homogenate was centrifuged at 14000 g for 15 min at 4°C and the protein concentration determined using BCA kit. Whole tissue lysate (20 μg) was loaded onto 12.5% SDS-PAGE. Electrophoresis was performed using a stacking gel at 80 V for 20 min and a separating gel at 110 V for 70 min. The proteins were transferred to PVDF membranes (Millipore, MA, USA) using an electro-blotting apparatus (Bio-Rad, CA, USA) at 300 mA for 90 min. The membranes were blocked for 1 h in TBST containing 0.1% Tween-20 and 5% dry milk and then incubated overnight with primary antibodies. After washing for 3 times in TBST, membrane was incubated for 2 h with horseradish peroxidase-conjugated secondary antibodies. The optical densities of the antibody-specific bands were

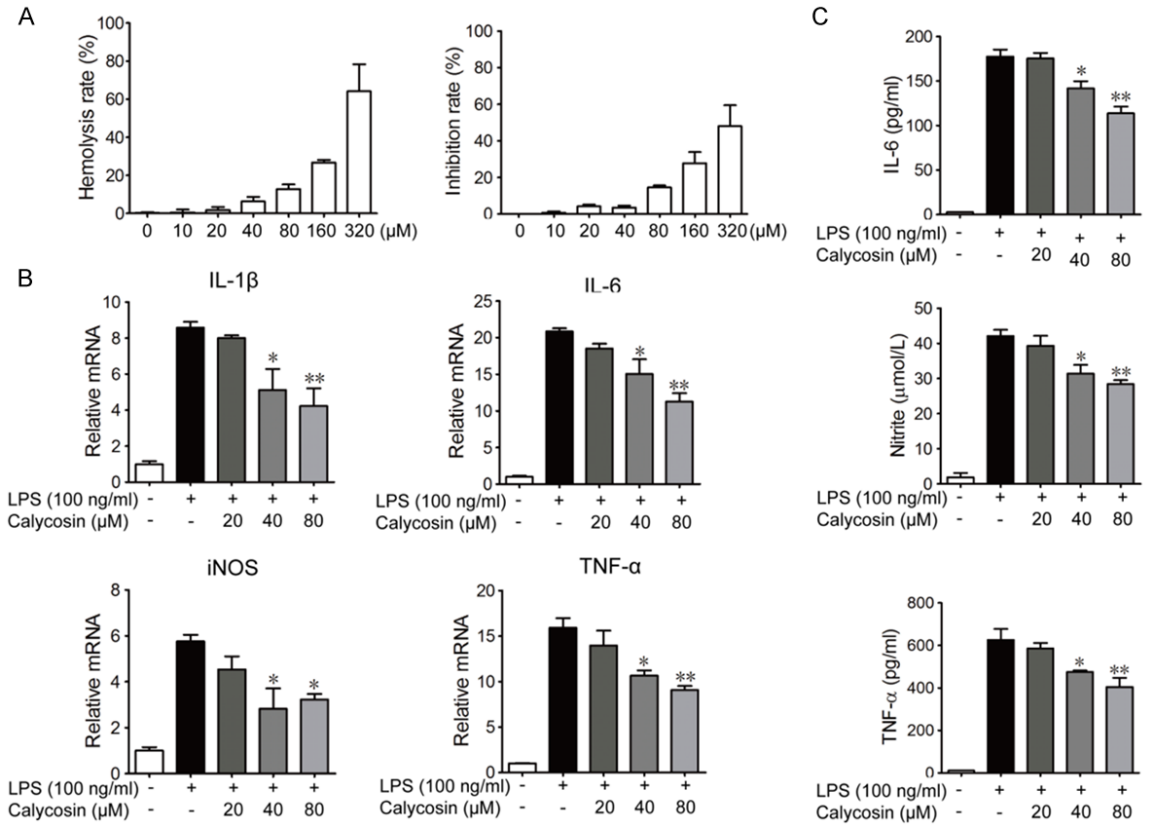

Figure 2. Calycosin decrease the LPS induced inflammatory factors in RAW264.7 cells Hemolysis and inhibition rate of Calycosin were detected to assay the cytotoxicity of Calycosin (A). RNA was isolated using TRIzol and reversed to cDNA for real-time PCR analysis of indicated cytokine mRNA levels such as IL-1β, IL-6, iNOS and TNF-α in each group (n=3) (B). The cytokine mRNA expression level in normal group was set as 100%, and mRNA expression levels in other groups were compared with normal group. RAW264.7 cells suffering LPS were lysated to detect cytokine protein levels including IL-6, Nitrite and TNF-α using ELISA kits (C). The data represent means ± SD (n=3 mice/group). *P < 0.05 compared with LPS vehicle group.

analyzed using a Luminescent Image Analyzer (Alpha, USA). The relative quantity of protein expression was normalized with actin using an optical density ratio of targeted protein/β-actin.

Statistical analysis

The experimental data were expressed as mean ± S.D. of at least three independent experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA) with the SPSS18.0 statistical software. *P* values less than 0.05 were considered statistically significant.

Results

Calycosin protects against LPS-induced inflammation in Raw264.7

In order to discover the toxicity of Calycosin, different concentrations of Calycosin were cultured with red blood cells derived from mice and macrophage Raw264.7 cell line to measure hemolysis rate and inhibition rate. **Figure 2A** shows hemolysis rate is 0.5%, 1.7%, 6.2% and 12.8% respectively after exposing to 10, 20, 40 and 80 μM Calycosin alone for 3 h. However, the hemolysis rates were 26.6% and 64.1% after red blood cells have been exposed to 160 and 320 μM concentrations of Calycosin. After 72 h culturing with Calycosin, macrophage Raw264.7 cells viability was measured using MTT. The inhibition rate was 0.7%, 4.1%, 3.5% and 14.4% respectively in the presence of 10, 20, 40 and 80 μM Calycosin. Nevertheless, the inhibition rate were 27.8% and 48.0% at 160 and 320 μM treatment accordingly. According to the above, 20, 40 and 80 μM Calycosin were applied to assess its anti-inflammation effects in vitro.

Lipopolysaccharides (LPS), the main composition of gram-negative bacteria, can induce

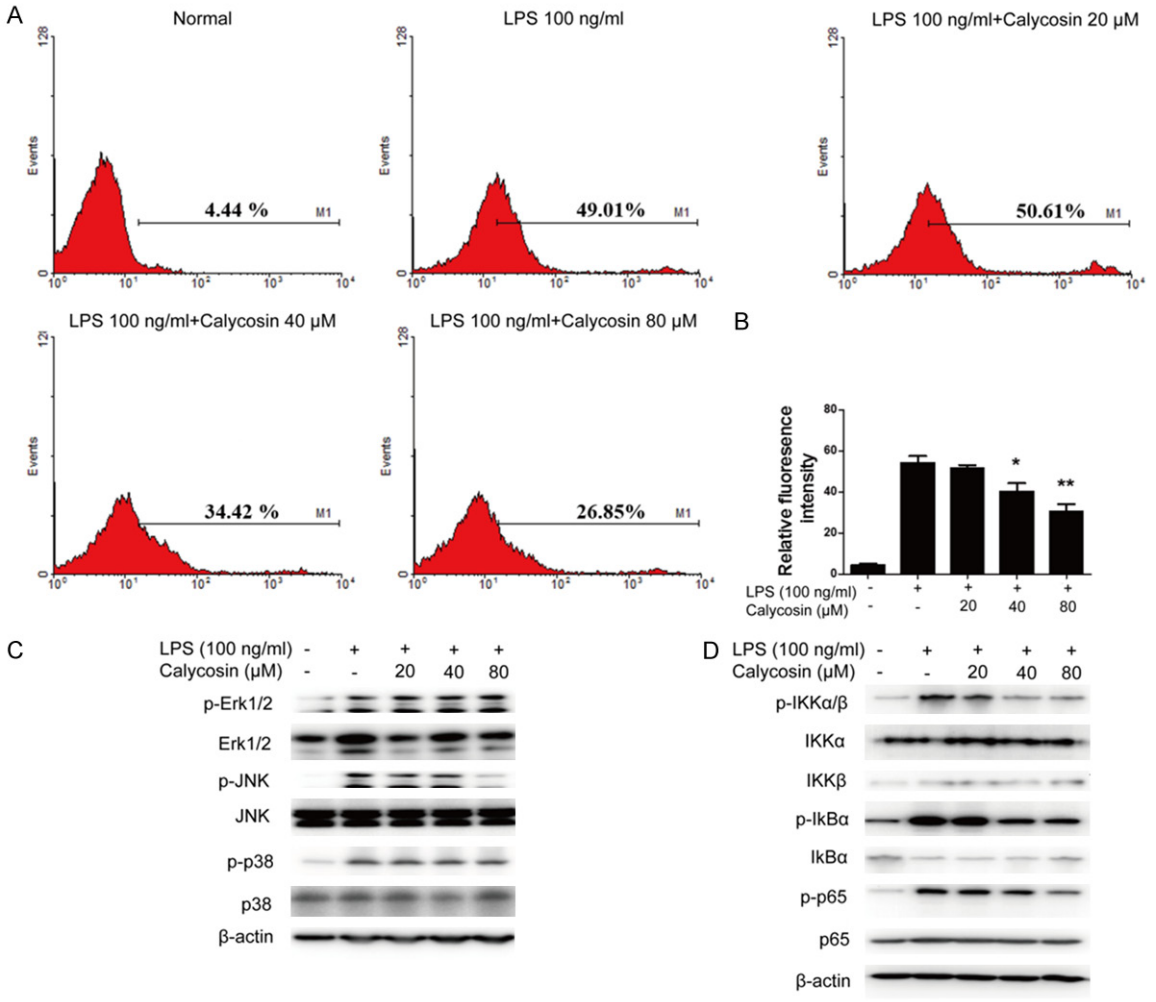

Figure 3. Calycosin alleviates ROS, JNK and NF-κB pathway ROS levels staining DCFH-DA dye were measured using flow cytometry (A). The columns of fluorescent intensity represent 3 independent experiments (B). Total colon tissue lysates were prepared for detecting the activation states of MAPK signal pathway including JNK, ERK and p38 by Western blot (C). NF-κB pathway proteins such as IκBα, p65 and IKKα/β were measured by Western blot (D). *P < 0.05 compared with LPS group.

inflammatory reaction. Raw264.7 cells with a 6 h pretreatment with 20, 40 and 80 μM Calycosin were treated for 24 h with 100 ng/ml LPS. The RNAs were collected and the mRNA of inflammatory cytokines was detected using real-time PCR. As depicted in **Figure 2B**, Calycosin suppressed the proinflammatory factors including IL-1β, IL-6, TNF-α and iNOS in Raw264.7 cells caused by LPS in a dose-dependent manner. The proinflammatory factors released in the medium were measured using ELISA (**Figure 2C**). The concentration of IL-6, Nitrite and TNF-α were 177.3 pg/ml, 626.2 pg/ml and 42.1 μM respectively after LPS alone treatment. A 80 μM Calycosin significantly inhibited the IL-6, NO and TNF-α production. The above results illustrates Calycosin has certain anti-inflammatory effects.

Calycosin attenuates oxidative stress, JNK and NF-κB pathway

LPS, the activator of TLR4, can induce significantly reactive oxygen species (ROS) production, which is involved in inflammatory response and cellular damage in macrophage cells. Calycosin, a kind of Flavonoid, has stronger antioxidant effect. The results from Flow cytometry shows that LPS can bring about a large number of ROS generation in Raw264.7 cells and the relative fluorescence intensity increases from 4.44% to 49.01%. Calycosin inhibits

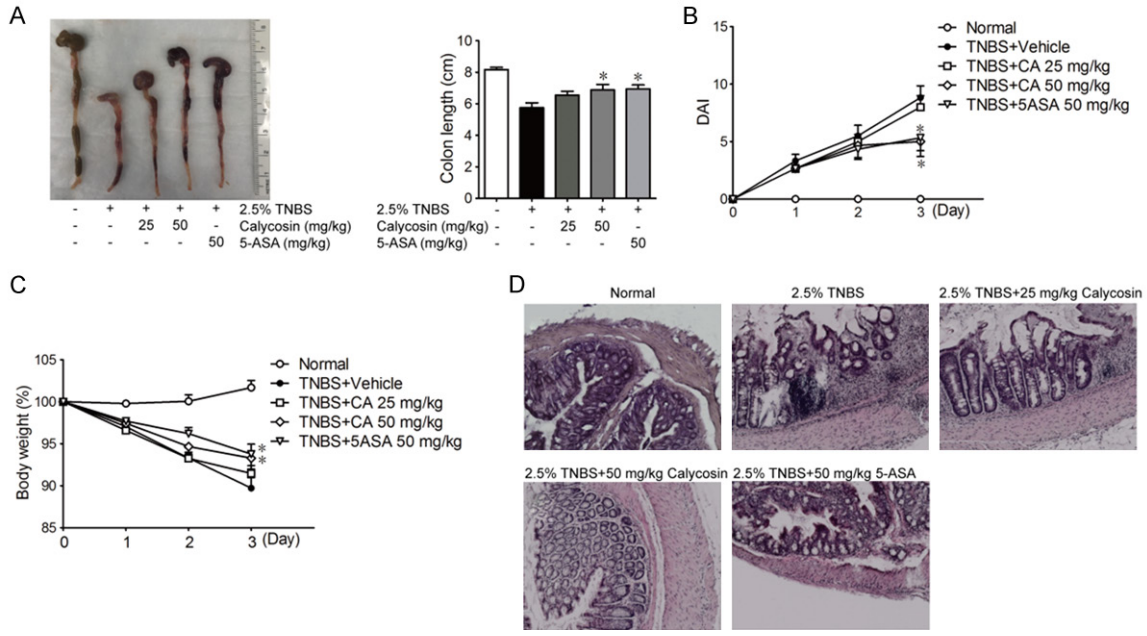

Figure 4. Calycosin attenuates TNBS-induced intestinal injury in mice. The morphology of colon and its length on day 3 in each group (A). The changes of Disease Activity Index during DSS-induced colitis (B). The weight of each mouse was measured daily (C). The histologic findings in TNBS-induced colitis. Colons were excised after 3 days and stained with hematoxylin and eosin (HE). The data represent means ± SD (n=7 mice/group). *P < 0.05 compared with TNBS+vehicle group.

LPS-triggered ROS generation in a dose-dependent manner. The inhibition rate of 40 and 80 μM Calycosin are 34.42% and 26.85%, respectively (**Figure 3A** and **3B**). The MAPK pathway composed of Erk1/2, p38 and JNK is associated with ROS and inflammation. As shown in **Figure 3C**, we observed that LPS increases the phosphorylated Erk1/2, p38 and JNK in 30 minutes. Calycosin decreases the JNK phosphorylation but has no effect on the phosphorylated Erk1/2 and p38. LPS activates NF-κB signaling pathway through TLR4. As depicted in **Figure 3D**, LPS stimulates Raw264.7 cell line for 30 minutes, while LPS-induced IKKα/β phosphorylation is followed by its downstream proteins, the phosphorylated IκBα and p65 using western blot. Calycosin can attenuate IKKα, IKKβ, IκBα and p65 phosphorylation in a dose-dependent manner. These results demonstrate that Calycosin inhibition of LPS-mediated inflammation is involved in anti-oxidation and the suppression of MAPK alongside NF-κB signaling transduction pathway.

Calycosin ameliorates TNBS-induced colitis in mice

A 100 μl 2.5% TNBS was injected into anus duct per day and the colon length was decreased after 3 days induction. As shown in **Figure 4A**, the morphology of large intestine is shorter in TNBS than normal group. The average length was 8.16 ± 0.15 cm, no bloody and intestinal content was good. TNBS induced mice intestine length reduced to 5.75 ± 0.31 cm accompanied with intestinal blood and intestinal content appeared more viscous. The intestine length was 6.55 ± 0.24 cm with some blood, intestinal content was sticky in 25 mg/kg Calycosin group. Compared with TNBS group, administration of 50 mg/kg Calycosin significantly improved the intestine ($p < 0.05$). The length was 6.88 ± 0.34 cm with no obvious blood, and contents hardness is moderate. The disease activity index (DAI) evaluation results shows that mice that were given 2.5% TNBS began to exhibit bloody phenomenon in the second day, and the most serious disease occurring at the third day with DAI score up to 8.83 ± 1.01. The DAI of 25 mg/kg Calycosin scored 8 ± 1.1. However, giving 50 mg/kg Calycosin and 50 mg/kg 5-ASA can significantly inhibit the pathogenesis of inflammatory bowel disease (**Figure 4B**). Moreover, the body weight is decreased significantly during TNBS administration (**Figure 4C**). The weights of 25 mg/kg, 50 mg/kg Calycosin and 50 mg/kg

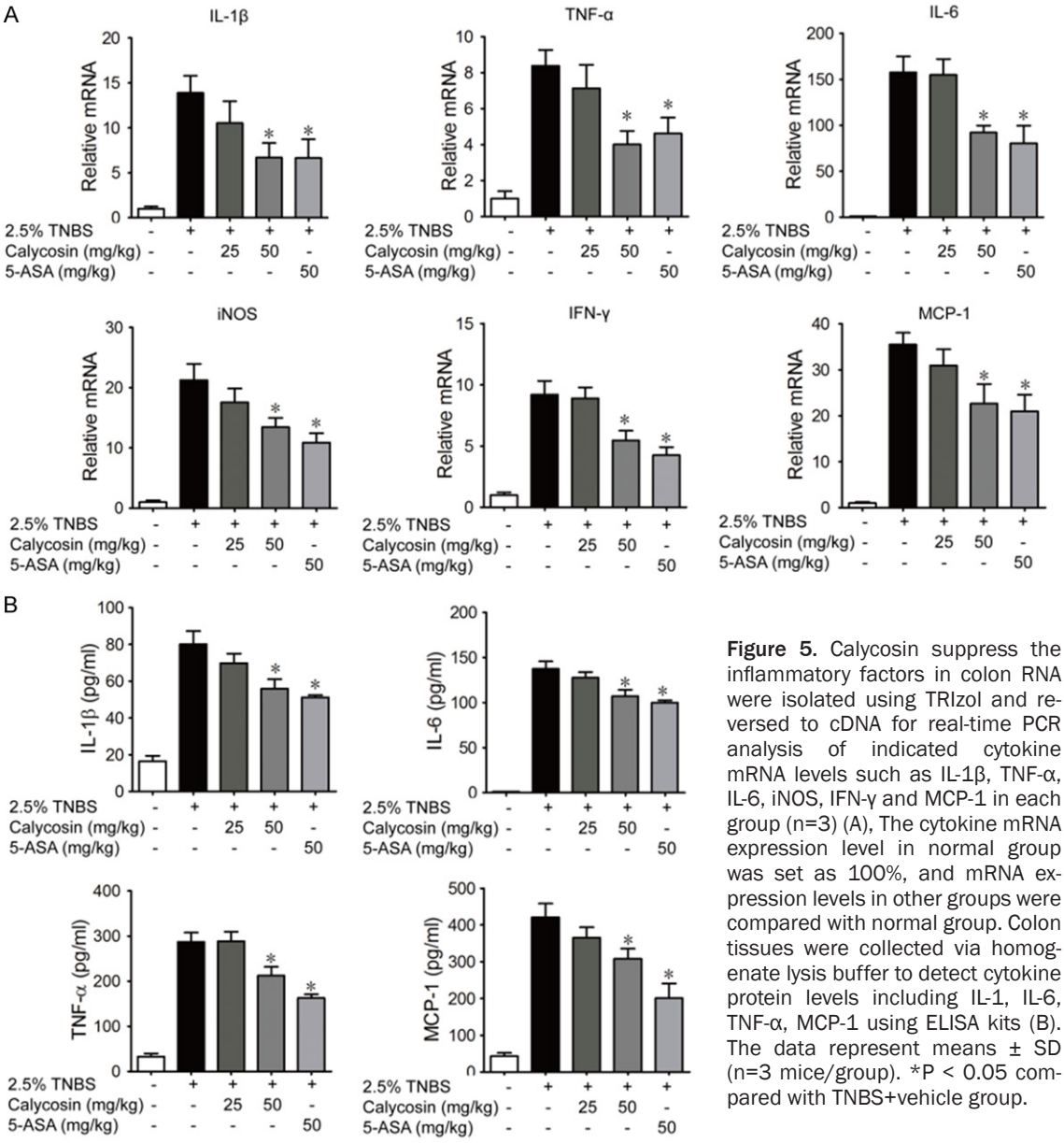

Figure 5. Calycosin suppress the inflammatory factors in colon RNA were isolated using TRIzol and reversed to cDNA for real-time PCR analysis of indicated cytokine mRNA levels such as IL-1β, TNF-α, IL-6, iNOS, IFN-γ and MCP-1 in each group (n=3) (A), The cytokine mRNA expression level in normal group was set as 100%, and mRNA expression levels in other groups were compared with normal group. Colon tissues were collected via homogenate lysis buffer to detect cytokine protein levels including IL-1, IL-6, TNF-α, MCP-1 using ELISA kits (B). The data represent means ± SD (n=3 mice/group). *P < 0.05 compared with TNBS+vehicle group.

5-ASA groups were 91.5%, 93.9% and 93.8% respectively of the original weight on day three. The H&E staining detection is applied to check the pathological morphology. As shown normal group in **Figure 4D**, the intestinal structure integrity is intact, fossae is clearly visible, mucosa tissue is normal and maintenance of goblet cells integrity, as well as little infiltrated inflammatory cell. The model group administered with 2.5% TNBS depicted that goblet cells and fossae are lost, the mucosa severely damaged coupled with infiltrated inflammatory cells. In 50 mg/kg Calycosin and 5-ASA groups, the morphology of intestine tissue is improved, fossae structure is relatively intact, mucosal damage and inflammatory cells infiltration are reduced. The results illustrate Calycosin have protective effect on TNBS induced intestine damage.

Calycosin down-regulates TNBS-induced inflammation

A large number of literature reports that TNBS can significantly induce proinflammatory factors generation in mice colon. After administration, the same parts of the intestine in mice were taken to extract total RNA, while inflammatory factor mRNA levels were also detected. As shown in **Figure 5A**, the intestinal tissue of

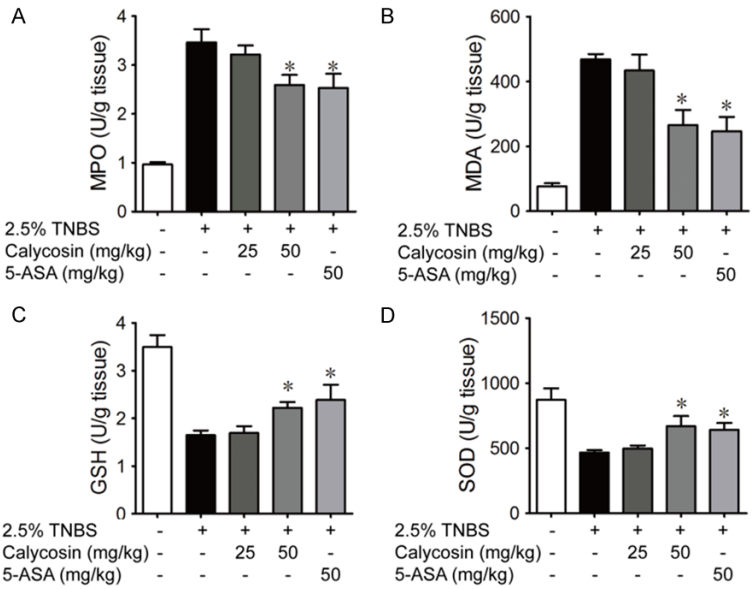

Figure 6. Calycosin suppress oxidative stress in colon tissue in TNBS-treated mice Histogram shows MPO activities (A), MDA levels (B), GSH levels (C) and SOD activities (D) in different treatment groups in colon tissue. The data represent means ± SD (n=7 mice/group). *P < 0.05 compared with TNBS+vehicle group.

mice that were treated with 2.5% TNBS produced higher proinflammatory factor mRNA level than the normal group. Compared with solvent control group, oral 25 mg/kg of calycosin has no improve effect on the inflammation factors, while oral 50 mg/kg significantly suppress the IL-1β, TNF-α, IL-6, iNOS and IFN-γ mRNA levels. Oral 50 mg/kg 5-aminosalicylic acid (5-ASA) as positive drug significantly suppressed the inflammatory cytokines mRNA induced by TNBS. The production of inflammatory cytokines were further measured using ELISA, the results were consistent with mRNA levels (**Figure 5B**). A 50 mg/kg Calycosin significantly suppress the production of IL-1β, IL-6, TNF-α and MCP-1 in TNBS-damaged colon tissue. Experimental results show that Calycosin suppress inflammatory factors to reduce the pathogenesis of inflammatory bowel disease in mice

Calycosin decreases TNBS-triggered oxidative stress

As an indicator of oxidative stress, the activities of myeloperoxidase (MPO) can reflect the infiltration degree of inflammatory cell. **Figure 6A** shows that MPO level of normal group is 0.96 ± 0.05 U/g while MPO level in TNBS induced intestine tissue is 3.46 ± 0.27 U/g. Compared with model group, the MPO in 25 mg/kg Calycosin has no improved effect. However, oral 50 mg/kg Calycosin and 50 mg/kg 5-ASA could significantly inhibit TNBS induced MPO levels ($p < 0.05$), which were determined to be 2.59 ± 0.2 and 2.53 ± 0.29 U/g, respectively. These results show that Calycosin suppress the infiltrated inflammatory cells. As the by-product of membrane lipid peroxidation, MDA is highly detected in oxidative stress. In **Figure 6B**, TNBS induced colitis exhibited high MDA content, while oral 50 mg/kg Calycosin and 5-ASA could inhibit MDA generation significantly. Furthermore, reduced glutathione exert its antioxidant properties via detoxification and maintenance of the normal function of the immune system. SOD is one of vital antioxidant enzymes in the body. TNBS downregulated GSH alongside SOD and destroyed the REDOX balance of intestinal tissue, which increased colon injuries. Compared with TNBS model, oral administration of 50 mg/kg Calycosin and 5-ASA could enhance GSH content and SOD activities (**Figure 6C** and **6D**). The anti oxidative stress capability of Calycosin can improve damaged colon.

Calycosin inhibits NF-κB pathway in TNBS induced colitis in mice

TNBS can significantly induce the phosphorylation of IKKα and IKKβ protein, which is followed by activated IκBα to dissociate p65/p50 dimer. Then, p65/p50 dimer shuttled into the nucleus to launch inflammation gene transcription. Oral 25 mg/kg Calycosin had no effect on the phosphorylation of IκBα, IKKα/β and p65 protein. On the other hand, oral 50 mg/kg Calycosin and 50 mg/kg 5-ASA had no effect on the phosphorylation of IKKα/β, but rather inhibited the IκBα and p65 phosphorylation to reduce the transcription of inflammation factors (**Figure 7A** and **7B**).

The experimental results show that Calycosin protect against TNBS induced intestinal dam-

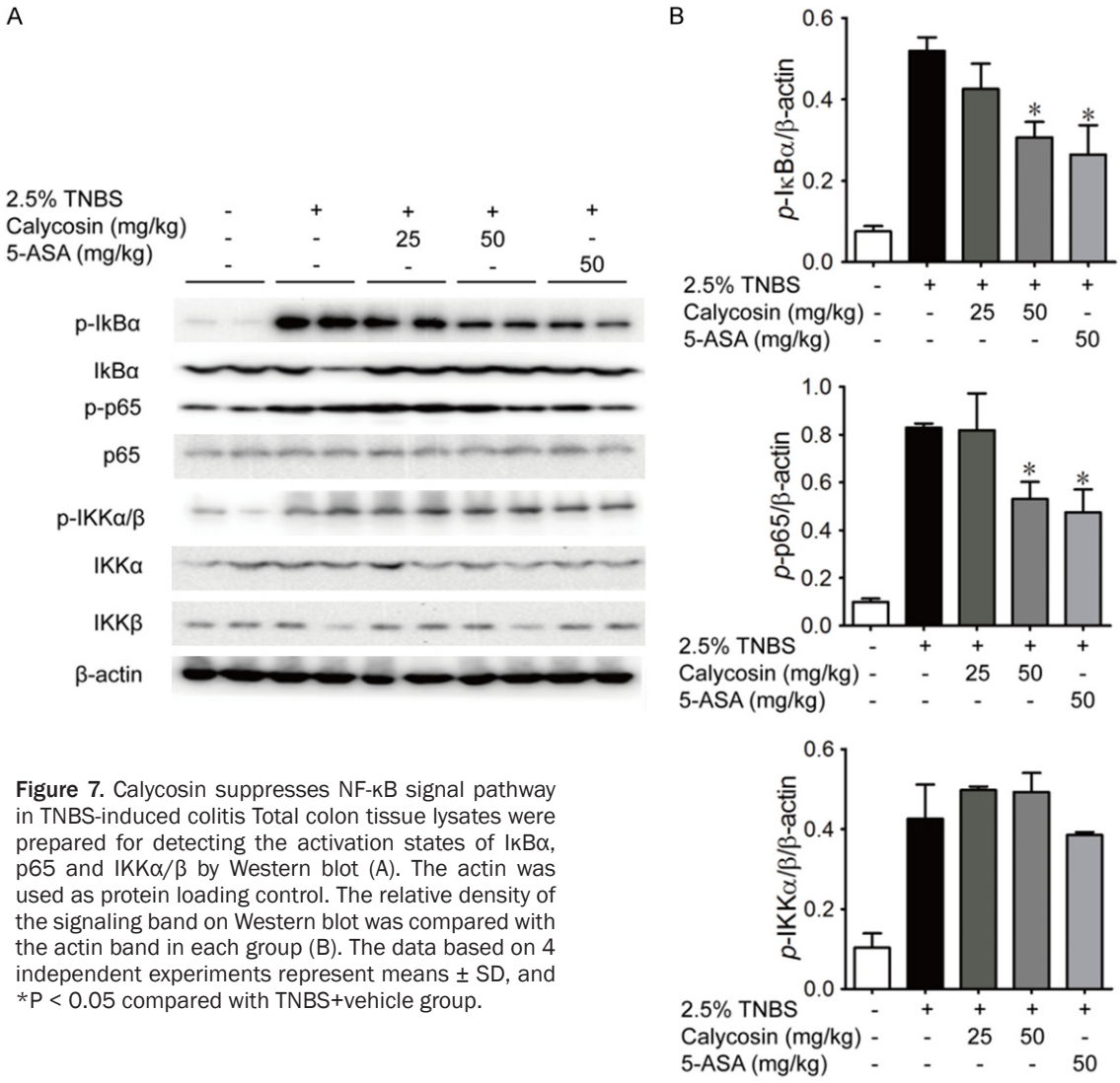

Figure 7. Calycosin suppresses NF-κB signal pathway in TNBS-induced colitis Total colon tissue lysates were prepared for detecting the activation states of IκBα, p65 and IKKα/β by Western blot (A). The actin was used as protein loading control. The relative density of the signaling band on Western blot was compared with the actin band in each group (B). The data based on 4 independent experiments represent means ± SD, and *P < 0.05 compared with TNBS+vehicle group.

age, which might be through the inhibition of NF-κB pathway.

Discussion

Inflammatory bowel disease is a chronic intestinal disorder, including ulcerative bowel disease and crohn's disease. Clinical manifestations are characterized by diarrhea, abdominal pain and hematochezia. A large number of literature report that the number and the role of macrophage is involved in the injured intestinal mucosa [14]. Macrophages express functional T cells stimulate molecules such as CD40, CD80 and CD86 in colitis [15]. In addition, sub population of macrophages in colon tissue also express TLR2, TLR4, CD89 and TREM-1. Compared with macrophages from normal colon tissue, the infiltrated macrophages from IBD patients express higher CD14 levels, which produce a large number of IL-12, IL-23 and TNF-α [16]. These macrophages expressing CD14 generate IFN-γ to induce abnormal macrophage differentiation. Numerous studies have shown that TNF-α released from macrophage is the main source in the pathogenesis of IBD. Monoclonal antibody therapy targeted on TNF-α has been widespread concerned [17, 18]. Interestingly, macrophages affects the balance among Th1/Th2/Th17 to maintain Helper T cells associated immune state and acquired immune response in colon [19]. However, the low activity of immune system is broken in bowel disease, while inflammatory signals promote infiltrated and migrated cells to the intestinal mucosa to expand further damage. Macrophages can drive acquired immune response in body. Different function of macrophages in colon has

different pathogenetic roles of autoimmune bowel disease, which can provide strategies for the prevention and treatment of chronic inflammation.

Flavonoids, with complex structure and multi-functions in pharmacology, are widely distributed in nature. The high chemical reactivity of flavonoids removes biological free radicals and thus has obvious antioxidant effect. Additionally, flavonoids have many pharmacological activities including anti-tumor [20], anti-inflammation [21], neuroprotection [22] and low toxicity properties. We explored the anti-inflammatory activity of Calycosin in vitro and found Calycosin has low toxicity using hemolysis experiment, which is similar to other flavonoids.

Lipopolysaccharide, a component of gram-negative bacteria, trigger pro-inflammation effect mainly through combining the toll like receptor (TLR) in cellular surface. LPS released from gram-negative bacteria cause a fever, microcirculation dysfunction, endotoxin shock and disseminated intravascular coagulation. When TLR4 is combined with LPS, the conformational changes of TLR4 recruits Myeloid Differentiation Primary Response 88 (MyD88), then MyD88 form dimerization with Toll/interleukin 1 receptor domain-containing adapter protein (TIRAP). The MyD88/TIRAP dimer recruits receptor interleukin-1-associated kinase 4 (IRAK4), IRAK4 phosphorylates IRAK1; phosphorylated IRAK1 is released from TLR-MyD complex and is combined with TNF receptor-associated factor 6 (TRAF6). As an E3 ubiquitin ligase, TRAF6 form complex with UBC13 and UEV1A, which activates TAK1 [23]. TAK1 activates the downstream inhibitory kappa B kinase (IKK) and mitogen-activated protein kinases (MAPK) [24]. The IKKα, IKKβ and IKKγ then form a complex to catalyze IκB phosphorylation, which is in turn degraded via ubiquitylation resulting in the nuclear translocation of NF-κB [25]. In affirming the vital role of macrophage in colitis, we found the effective anti-inflammatory dose of Calycosin to be lower than hemolysis dose while concomitantly inhibiting LPS induced inflammation in RAW264.7 cells.

Inflammations are closely related to ROS. It has been reported that LPS can induce higher oxygen free radicals in cells, which could cause cellular damage. LPS mediated ROS signal comes from the mitochondria, which normally boost MAPK activation. LPS induce the phosphorylation of c-Jun and p38, which downregulates UCP2 and activates MAPK. Moreover, macrophages with UCP2 deficiency are characterized by enhanced NO production, increased cellular migration and pro-inflammatory factors secretion [26]. Neutrophil with Nrf2 deficiency produce ROS to promote the generation of TNF-α, IL-6, MIP2 and MCP-1 [27]. Calycosin could significantly inhibit LPS induced ROS generation in macrophages. Using western blot, we found that Calycosin could also suppress the phosphorylation of IKKα/β in a dose-dependent manner in vivo and in vitro. Whether Calycosin can inhibit upstream proteins such as TRAF6, IRAK1, IRAK4 or MyD88 need to be further confirmed. Nevertheless, whether the antioxidative effect occurs earlier than anti-inflammation or not is still uncertain.

In conclusion, it has been shown that Calycosin exerted significant improved effect on LPS-induced inflammation in vitro and TNBS-induced experimental colitis in vivo. The potential mechanism of Calycosin could be the modulation of inflammation signaling pathway and anti-oxidative stress. As a flavonoid without any significant side effect, Calycosin can be developed into a promising drug for intestinal inflammatory diseases.

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Disclosure of conflict of interest

None.

Address correspondence to: Pengyuan Zheng, Department of Gastroenterology, The Fifth Affiliated Hospital of Zhengzhou University, Zhengzhou 455-0052, China. Tel: 86-371-66916883; Fax: 86-371-66916883; E-mail: zhengpengyuandr@126.com

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