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Suppressive effect of pectic polysaccharides extracted from *Rauwolfia verticillata* (Lour.) Baill.var.hainanensis Tsiang on inflammation by regulation of NF- κ B pathway and interleukin-17 in mice with dextran sulphatesodium-induced ulcerative colitis

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ABSTRACT

Objective: To investigate the effects of pectic polysaccharides extracted from *Rauwolfia verticillata* (Lour.) Baill.var.hainanensis Tsiang on an experimental murine colitis model.

Methods: Experimental colitis was induced by dextran sulfate sodium (DSS), and mice were divided into 4 groups: control, DSS alone, DSS plus SASP, DSS plus pectic polysaccharides. The disease activity index (DAI) and histological score were observed. The tumor necrosis factor (TNF)- α and interleukin (IL)-17 levels were measured by enzyme-linked immunosorbent assay. I κ B and NF- κ B p65 expression were assessed by western blot analysis. Myeloperoxidase (MPO) activity was determined by using MPO assay kit. **Results:** Administration of pectic polysaccharides significantly reduced the severity of DSS-induced colitis as assessed by DAI and histological score, and resulted in down regulation of MPO activity and NF- κ B p65 expression and subsequent degradation of I κ B protein, strikingly reduced the production of TNF- α and IL-17. **Conclusions:** Pectic polysaccharides extracted from *Rauwolfia verticillata* (Lour.)Baill.var. hainanensis Tsiang exerts beneficial effects in experimental colitis and may therefore provide a useful therapeutic approach for the treatment of UC.

1. Introduction

Ulcerative colitis (UC) is one of the idiopathic forms of inflammatory bowel disease (IBD). It is a chronic, relapsing inflammatory disease of the colon, characterized by ulcers in the colon and rectum. The etiology is still largely unknown. Environmental and genetic factors in combination with the microbial flora or specific microorganisms trigger an event, leading to the activation of an intestinal immune response[1]. Immune and non-immune cells create a cross talk via the secretion of soluble mediators and expression of cell

adhesion molecules, resulting in further cell activation[2]. Mediators such as cytokines and chemokines play a role in cell recruitment and polarization, intercellular signal amplification or activation and differentiation. Considering these aspects, medical management of inflammatory bowel disease has changed considerably over the past decade[3].

Although much progress has been made in the management of the disease, definitive causal therapies for human UC until now are not available. In fact, 5-aminosalicylic acid and its derivatives are still the drugs of choice for current medical treatment; corticosteroids, azathioprine, mercaptopurines and cyclosporine are used in more severe forms of the disease. However, the therapies not only show limited benefits but also have serious side effects[4]. Plant-sourced formulations purportedly possess anti-inflammatory and radical scavenging properties. Despite the lack of sufficient information on

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the safety of herbal products, their use as alternative and/or complementary medicine is globally popular.

Rauwolfia verticillata (Lour.) Baill. var. *hainanensis* Tsiang is an erect evergreen shrub growing wild in the islands of Hainan. The dried root of *Rauwolfia verticillata* (Lour.) Baill. var. *hainanensis* Tsiang, according to the Pharmacopeia of the People's Republic of China (2010 ed), is a traditional Chinese herbal medicine. It has been earlier shown to possess an anti-inflammatory effect. In China, *Rauwolfia verticillata* (Lour.) Baill. var. *hainanensis* Tsiang has been extensively used for more than 1 000 years in the treatment for lowering blood pressure, treating arrhythmia and tumors. However, *rauwolfia* is rarely used to cure UC. Several researches found the pectin obtained from *Rauwolfia serpentina* L. could inhibit colitis in mice^[5]. However, no systematic studies on its anti-inflammatory activity and mechanisms have been reported.

It is widely accepted that activation of the nuclear factor- κ B (NF- κ B) signaling pathway and overexpression of associated cytokines such as tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), IL-10, and interferon- γ (IFN- γ) play key roles in the development of UC^[6,7]. NF- κ B regulates the expression of multiple pro-inflammation genes and is thus a key player in maintaining immune system homeostasis. Thus, inhibition of NF- κ B and its associated molecules may be a novel therapeutic tool ^[8].

An increasing number of studies have focused upon the role of Th17 cells in the pathogenesis of UC. Th17 cells develop from naive T lymphocytes through distinct pathways from classical Th1 and Th2 cells. Th17 cells secrete IL-17, which promotes the recruitment of inflammatory cells into the intestinal mucosa via its ability to enhance the synthesis of chemoattractants and adhesion molecules on epithelial, endothelial and mesenchymal cells. It has been generally accepted that Th-17 probably plays a key role in the occurrence and development of UC^[9,10].

Therefore, we hypothesized pectic polysaccharides from *Rauwolfia verticillata* (Lour.) Baill. var. *hainanensis* Tsiang could exert its anti-inflammatory effect on UC by inhibiting the activation of NF- κ B signaling pathway. In this study, we extracted pectic polysaccharides from *Rauwolfia verticillata* (Lour.) Baill. var. *hainanensis* Tsiang and elucidated the effects of anti-inflammatory in a murine model of dextran sulfate sodium (DSS)-induced colitis, which resembles human UC, in order to provide experimental evidence that pectic polysaccharides serves as a possible treatment for patients with UC.

2. Material and methods

2.1. Pectic polysaccharide extracted from *Rauwolfia verticillata* (Lour.) Baill. var. *hainanensis* Tsiang

Rauwolfia verticillata (Lour.) Baill. var. *hainanensis*

Tsiang was obtained from the Jianfengling National Forest Park of Ledong li autonomous county, Hainan province, China. The extract method of pectic polysaccharide was in accord with Popov *et al.*^[5]. Briefly, the rootstock (5 g) dried at 40 °C was treated with diluted HCl (up to pH 4.4) at 50 °C for 3 h and the material obtained was extracted with 0.7% aqueous ammonium oxalate. A polysaccharide fraction was precipitated with four volumes of 96% ethanol. After centrifugation at 8 000 g for 10 min, precipitate was collected and dried at 40 °C. Subsequently, the precipitate was dissolved in distilled water and stored at -20 °C.

2.2. Animals

A total of 40 female BALB/c mice (6–8 weeks old, 17–22 g) were purchased from the Huaxi Medical Animal Center of Sichuan University (Sichuan, China). All animals were acclimated under standard conditions and maintained in a 12-h light/12-h dark cycle at a temperature of 23 °C ± 1 °C with 55%±5% humidity. The animals were maintained with free access to a standard diet and tap water. All experimental procedures were approved by the local Animal Care and Use Committee, and were conducted following the guidelines of the animal care policy.

2.3. Drugs and reagents

DSS was purchased from Beijing Bitab Biotechnology Co. Ltd. (Beijing, China). Salicylazosulfa-pyridine (SASP, used as a positive control drug) was purchased from

Fuda Pharmaceutical Co. Ltd (China). The mice TNF- α and IL-17 enzyme-linked immunosorbent assay (ELISA) Kit, a product of R and D Systems (United States), was obtained from Shanghai Chuanxiang Biotechnology Co. Ltd. (Shanghai, China). The antibodies used in this study were anti-NF- κ B p65 (ab16502, Abcam), anti-inhibitor of NF- κ B (I κ B) α (ab32518, Abcam). Goat anti-rabbit IgG were purchased from Boster Biotechnology (Wuhan, Hubei province, China).

2.4. Induction of colitis

BALB/c mice were randomly assigned to the following groups ($n=10$ per group): control, DSS alone (DSS), DSS plus SASP (100 mg/kg), DSS plus pectic polysaccharides (200 μ L). Colitis was induced in BALB/c mice by adding DSS (molecular weight: 36–50 kDa; MP Biomedicals) to drinking water at a level of 4% for 7 days. Control animals were given water only. After 7 d, DSS was then removed from the drinking water and the animals received 200 μ L of normal saline (control group and UC group) or SASP (100 mg/kg, UC plus SASP group) or 200 μ of pectic polysaccharides (UC plus pectic polysaccharides group) orally each day. All of the mice were sacrificed by cervical dislocation after 14 days.

2.5. Evaluation of disease activity index

The mice were checked daily for colitis based on body weight monitoring, gross rectal bleeding, and stool consistency. The disease activity index (DAI) was assessed in accordance with the method described by Murano *et al* [11]. The DAI was assessed by an investigator who was blind to the experimental groups.

2.6. Sample collection and histopathological examination

Mice were then sacrificed, and the colon and rectum were dissociated. After removal of the entire colon and rectum, the total length was measured, and the colon was then opened longitudinally, gently washed with ice-cold saline and blotted dry with filter paper. The colon tissue was then weighed and cut into several segments. A 1-cm colon segment 2 cm from the anus was fully expanded, affixed to filter paper, and then fixed in 10% neutral formalin. The tissues were then embedded in paraffin, stained with hematoxylin and eosin, and assessed under light microscopy. Colonic damage was scored as described previously. The remaining colon tissue was stored at -80°C for biochemical measurements, Western blot analysis and ELISA analysis.

2.7. Determination of IL-17 and TNF- α level

The murine colon tissues were used for cytokines ELISA analysis. In addition, the murine colon homogenates were obtained by handwork, and centrifuged at 3 000 rpm, 10 min. The amounts of cytokines in the supernatants for TNF- α and IL-17 were determined by ELISA according to the manufacturer's instructions.

2.8. Western blot analysis

Nuclear and cytoplasmic extracts were prepared using a nuclear and cytoplasmic protein extraction kit (Beyotime, China). Briefly, colon tissues (approximately 80 mg) were homogenized in cytoplasmic extraction buffer. Tissue homogenates were rapidly lysed by vortexing. The homogenate was centrifuged at 12 000 *g* for 10 min at 4°C and proteins were collected from the supernatant. The pellets were re-suspended in 50 μL of nuclear protein extraction buffer, lysed by ultrasound in an ice water bath, and then centrifuged to yield the nuclear fraction. Protein concentration was measured with a BCA protein assay kit. Protein samples (50–80 μg) were separated using 8%–12% SDS-PAGE gels and transferred onto PVDF membranes. The membranes were blocked with 5% skim milk in Tris-buffered saline with Tween 20 for 2 h at room temperature and then incubated with primary antibodies to anti-NF- κB p65 and anti-I κB α , at a 1:1 000–1:10 000 dilution overnight at 4°C . Then, the membrane was washed 3 times for 10 min each and incubated with a horseradish

peroxidase-conjugated anti-rabbit IgG antibody. Protein bands were detected using enhanced chemiluminescence detection kit (Beyotime Institute of Biotechnology) and visualized by exposure to photographic film. Finally, the densitometry values of each protein analyte normalized to GAPDH were compared.

2.9. Myeloperoxidase activity

Myeloperoxidase (MPO) activity, an index of leukocyte recruitment, was measured with a myeloperoxidase assay kit according to the manufacturer's instructions (CytoStore, Alberta, Canada).

2.10. Statistical analysis

Statistical analyses were performed using SPSS software 11.5 for Windows. All results are expressed as mean \pm SD. Quantitative data were tested for homogeneity of variance. If the variance was homogeneous, one-way ANOVA followed by the Bonferroni post hoc test was used. Comparisons between groups of nonparametric data were made using the Kruskal-Wallis test followed by the Mann-Whitney *U* test. $P < 0.05$ was considered significant.

3. Results

3.1. Effects of pectic polysaccharides on colonic mucosal damage in DSS-induced colitis

After induction of colitis with DSS, the colonic mucosa showed congestion, erosion, and hemorrhagic ulcerations. DAI score were monitored each day. Mice given 4% DSS in their drinking water for 7 d developed symptoms of colitis. Compared with control mice, the DAI value was significantly greater from day 9 to 14 in mice treated with DSS ($P < 0.05$, Table 1). Compared to the UC group, the colitis symptoms were relieved from day 12 to 14 in mice of the UC plus pectic polysaccharides group and UC plus SASP group.

Treatment of mice with pectic polysaccharides and SASP significantly meliorated experimental colitis as assessed by DAI and histological injury scores. As shown in Table 1 and Figure 2. Compared with normal control group, the DAI scores were increased markedly in mice with DSS-induced colitis ($P < 0.05$, Table 1). There are no statistically significant between the two drug treatment groups.

After induction of colitis with DSS, Histological findings demonstrated marked epithelial destruction, inflammatory infiltration, crypt distortion, and submucosal edema (Figure 1). The control group given normal saline showed no histological alterations. Similarly, mice given SASP exhibited virtually the same normal histology with no inflammatory cell infiltration, oedema or crypt abscesses. Severe submucosal oedema, erosion, ulceration, inflammatory

cell infiltration and extensive destruction of mucosal layer were observed in the mucosa of UC group animals. UC plus pectic polysaccharides group revealed attenuation in inflammation, characterized by suppression of erosion, ulceration, reduction in inflammatory cellular infiltrate, and protection against epithelium damage, although oedema was still existed (Table 2).

Table 1

DAI of colitis in mice from four groups.

Group (n=10)	1st day	9th day	12th day	14th day
Control	0	0 ^b	0 ^b	0 ^b
DSS	0	6.53±1.49 ^a	4.13±1.52 ^a	3.46±1.49 ^a
DSS plus SASP	0	6.14±1.23 ^a	2.98±1.16 ^{a,b}	1.14±1.12 ^{a,b}
DSS plus pectic polysaccharides	0 ^a	6.22±1.26 ^a	3.02±1.27 ^{a,b}	1.25±1.27 ^{a,b}

Female BALB/c mouse were given 4% DSS, in their drinking water during the first 7 days, excepted control mouse. Subsequently, DSS was removed, and the animals received normal saline (control group and DSS group) or SASP (UC plus SASP group) or pectic polysaccharides (UC plus pectic polysaccharides group) orally each day. The DAI value was calculated as described under experimental.

^a*P* <0.05 vs. control group; ^b*P* <0.05 vs. DSS group.

3.2. Effects of pectic polysaccharides on expression of NF- κ B p65 and I κ B- α

When mice were treated with 4% DSS, the levels of NF- κ B p65 were significantly increased, otherwise I κ B- α protein was decreased in colonic tissue (Figure 2). In the case of NF- κ B p65, treatments with Pectic polysaccharides and SASP resulted in a decrease of expression. However, I κ B- α protein was increased in UC plus pectic polysaccharides group and UC plus SASP group, when compared with UC group in murine colon tissues by Western blot (*P*<0.05, Table 3).

3.3. Effects of pectic polysaccharides on TNF- α and interleukin-6 serum levels

IL-17 and TNF- α are considered important inflammatory mediators that play a key role in the pathogenesis of UC. In normal stimulation, colon cells release of pro-inflammatory cytokines, including IL-17 and TNF- α is protective in fighting off pathogens like bacteria, for example. To determine the effect of Pectic polysaccharides on major inflammatory cytokines in the colon, we determined the levels of IL-17 and TNF- α (Figure 3). After 7 d of DSS administration, the levels of IL-17 and TNF- α increased significantly. Pectic polysaccharides and SASP group; Likewise, IL-17 and TNF- α were down regulated in colon culture supernatants of UC patients by cultured with pectic polysaccharides and SASP group (Figure 4B). These results indicated that pectic polysaccharides have anti-inflammatory effects in the DSS-induced colitis mouse

model.

3.4. Effects of pectic polysaccharides on myeloperoxidase activity

Evaluation of leukocyte recruitment was assessed by the measurement of myeloperoxidase activity. As shown in Table 2, MPO activity levels were low in the colonic tissues of normal control mice and markedly increased in mice with DSS-induced colitis. However, MPO activity was significantly lower in mice with pectic polysaccharides and SASP than those with DSS-induced colitis (*P*<0.05).

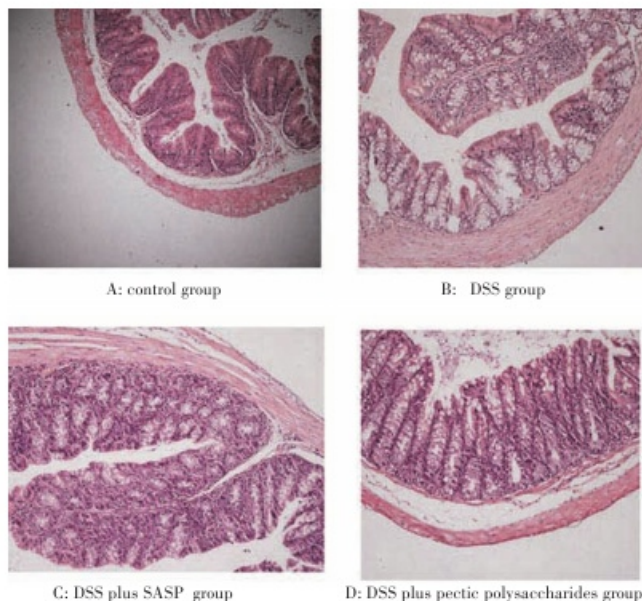


Figure 1. Hematoxylin and eosin staining (magnification $\times 100$) of colonic mucosal tissue section from mice control (A, normal control, n=10), DSS (B, DSS + saline, n=10), DSS plus SASP (C, DSS + SASP, n=10), DSS plus pectic polysaccharides (D, DSS + pectic polysaccharides, n=10).

Compared with that of normal controls, colon of DSS-treated mice showed complete destruction of epithelial architecture with loss of crypts and epithelial integrity, submucosal edema, and intense inflammatory cellular in all layers. Pectic polysaccharides and SASP treatment attenuated morphological damage but showed mild cellular infiltrate.

Table 2

Effect of pectic polysaccharide on clinical indices and histological injury scores.

Group	n	Histological scores
control	10	0 ^b
DSS	10	16.78±2.48 ^a
DSS plus SASP	10	5.78±1.96 ^{a,b}
DSS plus pectic polysaccharides	10	6.42±2.08 ^{a,b}

^a*P* <0.05 vs. control group; ^b*P* <0.05 vs. DSS group.

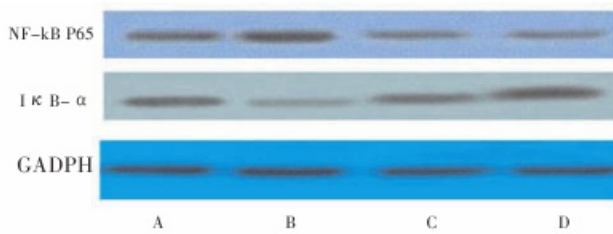


Figure 2. Western blot results display protein expression of NF- κ B P65/I κ B α in the colonic tissues.

A: control group; B: DSS group; C: DSS plus pectic polysaccharides group; D: DSS plus SASP group.

The expression of NF- κ B P65 was increased in DSS-treated mice, which was markedly inhibited in pectic polysaccharides and SASP treated mice. In contrast, the expression of I κ B α protein was decreased in DSS-treated mice compared with normal control group, but increased significantly after treatment of pectic polysaccharides and SASP. $P < 0.05$ versus DSS group and normal control group, each group, $n=10$.

Table 3

Western blot results display protein expression of NF- κ B P65/I κ B α in the colonic tissues.

Group	n	NF- κ B P65	I κ B α
control	10	24.47 \pm 3.83 ^b	43.18 \pm 2.13 ^b
DSS	10	54.62 \pm 11.45 ^a	18.16 \pm 4.19 ^a
DSS plus SASP	10	29.64 \pm 7.64 ^{ab}	37.71 \pm 3.88 ^{ab}
DSS plus pectic polysaccharides	10	31.41 \pm 8.43 ^{ab}	39.28 \pm 3.75 ^{ab}

^a $P < 0.05$ vs. control group; ^b $P < 0.05$ vs. DSS group.

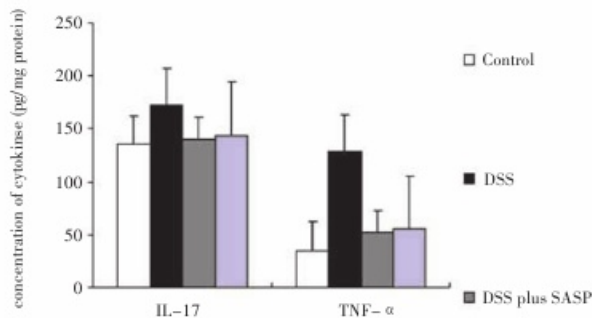


Figure 3. Pectic polysaccharides reduced proinflammatory cytokines production in colitis.

After 14 d, mice were sacrificed and colon tissues were used for evaluating the levels of IL-17 and TNF- α by ELISA analysis.

4. Discussion

UC is a chronic, relapsing disease that causes inflammation and ulcerations of the colonic mucosa with a variable extent and severity. The etiology of UC remains essentially unknown but the results from many studies in humans and animal models suggest that it is related to an abnormal immune response in the gastrointestinal tract, possibly associated with genetic and environmental – mainly microbial – factors[12]. Aminosalicylates, glucocorticoids and immunosuppressive drugs have been mainly used for the treatment and maintenance of remission of UC, but the side effects or toxicity of these drugs represents a major clinical problem[13,14]. For these reasons, natural medicine

has become an alternative therapy in addition to the conventional therapies that are used to treat UC.

In the present study, we demonstrated that pectic polysaccharides extracted from *Rawolfia verticillata* (Lour.) Baill.var.hainanensis Tsiang has an anti-inflammatory effect on colonic injury provoked by oral supplementation with DSS in mice. DSS-induced colitis is a well-established model that is phenotypically similar to UC in humans[15]. Oral administration of DSS for several days, leads to colonic epithelial lesions and acute inflammation characterized by the presence of neutrophils and macrophages within damaged segments. The reason for the deleterious effects of DSS is not well understood, however, epithelial cell permeability and macrophage activation have been proposed as potential mechanisms[16]. We administered pectic polysaccharides to evaluate the role of treatment with this product. The results presented herein clearly indicated that pectic polysaccharides efficiently relieved the symptoms of DSS-induced colitis in mice. In this study, we showed that pectic polysaccharides decreased DAI scores in mice with DSS-induced colitis, mitigated colitis-induced histological damage by suppression of erosion, ulceration, reduction in inflammatory cellular infiltrate.

To further characterize the nature of the inhibitory effect of pectic polysaccharides on pro-inflammatory proteins production, the NF- κ B signal transduction pathway was examined. NF- κ B is the major transcription factor which mediates inflammatory signaling[17,18]. A large number of studies showed that NF- κ B played a broad regulatory role in ulcerative colitis, promoted expression of various pro-inflammatory cytokines including IL-17 and TNF- α . NF- κ B exists mainly as a heterodimer composed of subunits of the Rel family, p50 and p65. In resting stage, NF- κ B normally localizes to the cytoplasm, where it is bound by I κ B α protein. During inflammatory stimulus, I κ B α is phosphorylated by I κ B kinase, subsequently degraded by proteasome, then NF- κ B gets released and translocates into the nucleus, where it triggers the transcription of multiple genes involved in inflammatory cascade[19]. The results of this study suggested that pectic polysaccharides increased the production of I κ B α protein and decreased the expression of NF- κ B p65. These indicated that pectic polysaccharides exhibited anti-inflammatory effects on ulcerative colitis which might be due to the inhibition of NF- κ B signal transduction pathways[20].

In pathological conditions, including oxidative stress, toxicity, colon cells can be over stimulated and produce excess pro-inflammatory cytokines which result in inflammatory bowel disease like UC. IL-17 and TNF- α have been described as a key molecule in UC pathogenesis[21]. These cytokine, recruits leukocytes to inflammatory sites, stimulates monocytes and vascular endothelial cells to express cytokines, induces the cascade effects for other cytokines, and finally results in inflammatory lesions in tissues. Our data showed that pectic polysaccharides

reduced protein expression of IL-17 and TNF- α in mice with DSS-induced colitis. These results suggested that pectics exerted anti-inflammatory effects in UC. The results consisted with previous studies.

The reduction of inflammatory mediators IL-17 and TNF- α expression induced by pectic polysaccharides can be correlated with its antioxidant properties. The effects of antioxidant agents have been ascribed by some authors to inhibition of activation of the NF- κ B, which is activated by ROS with the subsequent induction and expression of various cytokines (such as IL-17) that are involved in the induction and development of UC.

A previous study has demonstrated the ability of pectic polysaccharides to modulate macrophage function through inhibition of chemotaxis and phagocytosis. Macrophages are one of the main sources of cytokines (ie. IL-17 and TNF- α), therefore, a possible modulation of macrophage activity by pectic polysaccharides could influence the decrease in cytokine production. This result suggests an important role for pectic polysaccharides as a modulator of the immune system and should be taken into account for future investigations.

In conclusion, the results showed that pectic polysaccharides extracted from *Rauwolfia verticillata* (Lour.) Baill. var. hainanensis Tsiang is able to prevent body weight loss and colon shortness, as well as increased the production of I κ B- α protein, decreased the expression of NF- κ B p65, inhibited the level of IL-17 and TNF- α . *Rauwolfia verticillata* (Lour.) Baill. var. hainanensis Tsiang might ameliorate ulcerative colitis and exhibit its anti-inflammatory effects via increased expression of I κ B- α proteins and suppressing NF- κ B translocation.

Conflict of interest statement

We declare that we have no conflict of interest.

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