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Research Article
Siegesbeckia pubescens Attenuates Iodoacetamide-induced Colitis in Rats

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Abstract

Background and Objective: The prevalence of Ulcerative Colitis (UC) in most countries has increased and it is necessary to develop new therapeutic options for UC. *Siegesbeckia pubescens* is a traditional Chinese medicine widely used for the treatment of inflammatory and autoimmune diseases in clinic. The purpose of this study is to evaluate its therapeutic effect against UC. **Materials and Methods:** Experimental UC in rats was induced by iodoacetamide (IA) through instilling into the lumen of the colon. The aqueous extract of *S. pubescens* (SPA) was orally administered 3 days before IA instillation and continued upto 4 days. Throughout the experiment, rats were monitored for body weight loss, stool consistency and fecal occult blood which were quantified as Disease Activity Index (DAI). At the end of the experiment, rats were sacrificed and colonic length, weight, macroscopic and histopathological damage were examined. Furthermore, the mRNA expression levels of pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β) were analyzed in the colonic tissues by real-time PCR. The data were examined for their statistical significance of difference with ANOVA and the standard's t-test. **Results:** The results showed that SPA significantly ameliorated typical symptoms of IA-induced rat colitis including weight loss, mucus stool and bloody diarrhea. Moreover, macroscopic and histopathologic scoring showed that SPA suppressed IA-induced colonic edema, hyperemia, necrotic destruction of epithelium and inflammatory cellular infiltration. In addition, SPA inhibited IA-induced pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β) mRNA expression in colon. **Conclusion:** These evidences indicated that SPA has therapeutic effects against IA-induced colitis in rats which suggested the potential of *S. pubescens* as an agent for use in the treatment of UC for the first time.

Key words: *Siegesbeckia pubescens*, traditional Chinese medicine, ulcerative colitis, inflammatory bowel disease, iodoacetamide, autoimmune diseases, anti-inflammatory activity, therapeutic effect

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Ulcerative colitis (UC) and Crohn's disease (CD) represent the two major types of Inflammatory Bowel Disease (IBD). The UC is a chronic idiopathic inflammatory disorder of the rectum and colon. Its typical symptoms include bloody diarrhea, abdominal pain and mucus stool[1]. Anti-inflammatory agents (such as corticosteroids and 5-aminosalicylic acid), immunosuppressants and biologicals have been employed as therapeutic options for UC for decades. However, these treatments are not completely effective in eliminating the disease, there are still patients that experience frequent flares of inflammation or develop a treatment resistant disease with chronic inflammatory activity. Moreover, these treatments are associated with severe adverse events including diarrhea, cramps, abdominal pain accompanied by fever and high blood pressure[2]. Accordingly, the incidence and prevalence of UC in most of developed and developing countries has increased[3]. Thus, there is a need to develop new therapeutic options with minimal side effects.

Treatment of ulcerative colitis by Traditional Chinese Medicine (TCM) has received great interest in recent years. *Siegesbeckia pubescens* makino is a well-known traditional Chinese medicine clinically used in China for the treatment of inflammatory and autoimmune diseases such as rheumatic arthritis and osteoarthritis[4]. It has also been reported that *S. pubescens* ameliorates acute enteritis in clinic[5]. The anti-inflammatory activities of *S. pubescens* have been verified in several animal models such as osteoarthritis rabbits, carrageenan-induced edema rats and hypersensitivity reaction in rats[6-8]. However, in spite of its wide use over a long period of time, to date, there is no further pharmacological evidence of *S. pubescens* on UC treatment. Thus, to evaluate the therapeutic effect of *S. pubescens* on UC, the present investigated the effect of aqueous extract of *S. pubescens* (SPA) on iodoacetamide (IA) induced colitis in rats.

MATERIALS AND METHODS

Preparation and quality control of SPA: The aerial parts of *S. pubescens* makino were obtained from East China Pharmaceutical Group Limited Company and identified by Prof. Xiaoyu Li (Zhejiang Academy of Medical Sciences, Hangzhou, China). A voucher specimen (No. 20121024) was deposited in Laboratory of Natural Products, Zhejiang Academy of Medical Sciences, Hangzhou, China. The plant material (1 kg) was extracted three times with water under boiling. The aqueous extract was filtered and evaporated on a rotary evaporator under reduced pressure to small volume and then lyophilized to yield a brown solid (named SPA, 170.0 g) which was then stored at 4°C until required.

To guarantee the quality of SPA, the amounts of presumed anti-inflammatory active constituents in SPA were determined. The content of total flavonoids was detected by aluminium colourimetric method and expressed as rutin equivalents (mg RE g^{-1} sample)[9]. Three diterpenoids (Kirenol, Pubeside D and Darutoside) in SPA were analyzed by HPLC. In brief, SPA (163.67 mg) was dissolved in methanol and diluted to 25 mL. About 20 mL of the sample solution was injected to an Agilent 1260 HPLC apparatus (Agilent, USA) with a ZORBAX SB C18 (4.6×150 mm, 5 μm) column (column temperature 40°C) and Acetonitrile-water (0-10 min: Acetonitrile from 10-30%; 10-20 min: Acetonitrile 30%) as mobile phase. The flow rate was 1.0 mL min^{-1} and the detection wavelength at 220 nm. The amounts of total flavonoids in SPA were 22.96±0.05 mg RE g^{-1}. The contents of Kirenol, Pubeside D and Darutoside in SPA were 4.50±0.10, 0.80±0.02 and 0.82±0.02 mg g^{-1}, respectively.

Chemicals and reagents: Iodoacetamide (IA) was purchased from Sigma-Aldrich Company (St. Louis, USA). Sulfasalazine (SASP) was supplied by Shanghai Zhongxi Sunve Pharmaceutical Company Limited (Shanghai, China). Occult Blood (OB) reagent (aminopyrine semi-quantitative test) was purchased from Zhuhai Baso Diagnostics Inc (Guangdong China). Trizol was purchased from Invitrogen (Carlsbad, CA, USA); PCR primers and other PCR reagents were purchased from Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai, China). All Other chemicals were of highest purity and analytical grade.

Experimental animals: Sprague-Dawley rats (male, 180-220 g) were purchased from Zhejiang Experimental Animal Center (Hangzhou, China) and acclimatized for a week before use. Rodent laboratory chow and tap water were provided *ad libitum* and maintained under controlled conditions: Temperature 24±1°C, humidity 50±10%, 12 h light per 12 h dark cycle. All the procedures were in strict accordance with the P.R. China legislation on the use and care of laboratory animals and with the guidelines established by the Experimental Animals Center of Zhejiang province and were approved by the Animal Care and use Committee of Zhejiang Academy of Medical Sciences, China.

Induction of experimental colitis in rats and treatment protocols: The experimental rats were randomly assigned to 6 groups: Normal control group, model control group,

positive control group which was received SASP (400 mg kg^{-1}) treatment and SPA (12.5, 50 and 200 mg kg^{-1}) treated group. The drugs were dissolved in distilled water and administered once daily by oral gavages. The treatment protocol was performed as following: Administration of each drug was started 3 days before iodoacetamide instillation and continued upto the day before of the sacrifice of the rats, which took place 4 days after the colitis induction. The same solvent (distilled water) was given to both normal group and colitis group.

Colitis was induced in model control and drug treated groups according to the methodology described previously[10]. Briefly, after being deprived of food for 24 h with free access to distilled water, rats were anesthetized with 10% chloral hydrate (0.30 mL 100 g^{-1}, i.p.) and a flexible catheter was then carefully inserted into the colon and the tip was 8 cm proximal to the anus. To induce colitis, a solution of 0.1 mL of iodoacetamide (6%, v/v) in 1 mL of 1% sodium carboxymethylcellulose (CMC-Na) was instilled into the lumen of the colon and rats were carefully held upside down in a vertical position for 5 min to prevent the leakage of the intra colonic instillation. In normal control experiments, rats received 1% CMC-Na alone using the same technique. The animals were then given free access to food and water.

Throughout the experiment, rats were monitored for body weight loss, stool consistency and fecal occult blood which were assayed by aminopyrine semi-quantitative test method using OB reagent kits. At the end of the experiment, rats were sacrificed and the colons were separated from the proximal rectum, close to its passage under the pelvisternum. The colon length was measured between the ileocecal junction and the proximal rectum. The colon was cut longitudinally and washed with cool saline and then their weight was measured. At last, the colon was used for macroscopic scoring, histopathological examination and biochemical studies.

Evaluation of Disease Activity Index (DAI): Body weight, stool consistency and gross bleeding were recorded daily after iodoacetamide instillation. The DAI was quantified with a clinical score assessing weight loss, stool consistency and bleeding of the colon as described by Cooper *et al.*[11] which was divided by grade 3. Each score was determined as follows: Change in body weight loss (0: None, 1: 1-5%, 2: 5-10%, 3: 10-20% and 4: >20%), stool blood (0: Negative, 1: +, 2: ++, 3: +++ and 4: ++++) and stool consistency (0: Normal, 1 and 2: Loose stool and 3 and 4: Diarrhea). Body weight loss was calculated as the percent difference between the body weight on the day of iodoacetamide instillation and the body weight on any particular day after iodoacetamide instillation.

Evaluation of colon damage by macroscopic scoring: Macroscopic damage was assessed blindly by the scoring system of Wallace *et al.*[12]. The criteria for assessing macroscopic damage and the numerical rating score on a 0-10 scale were as follows: Grade 0: No macroscopic changes, grade 1: Focal hyperemia, no ulcers, grade 2: Ulcer without significant inflammation (hyperemia and bowel wall thickening), grade 3: Ulceration with inflammation at one site, grade 4: Two or more sites of ulceration/inflammation, grade 5: Major sites of damage extending $\geq$1 cm along colon length and grade 6-8: When the area of damage exceeds 2 cm along the colon, the score was increased by one for each additional 1 cm.

Evaluation of colon damage by histopathologic scoring: The colonic tissues were fixed in 4% neutral formalin, dehydrated with increasing concentrations of ethanol, embedded in paraf n and sectioned. Sections (5 μm thick) were mounted on slides, cleared, hydrated and stained with hematoxylin and eosin. Histopathological grading system was used in a blinded manner[13]. Grade 0: Histological findings identical to normal mice, grade 1: Mild mucosal and/or submucosal inflammatory infiltrate and edema, punctuate mucosal erosions often associated with capillary proliferation, muscularis mucosae intact, grade 2: 50% of the specimen display grade 1 changes, grade 3: Prominent inflammatory infiltrate and edema frequently with deeper areas of ulceration extending through the muscularis mucosa into the submucosa, rare inflammatory cells invading the muscularis propria but without muscle necrosis, grade 4: 50% of the specimen display grade 3 changes, grade 5: Extensive ulceration with coagulative necrosis bordered underneath by numerous neutrophils and lesser numbers of mononuclear cells, necrosis extends deeply into the muscularis mucosae and grade 6: 50% of the specimen display grade 5 changes.

Measurement of inflammatory cytokines mRNA expressions by real-time PCR: Colon tissues were lysed with trizol reagent and the total RNA was isolated according to the manufacturer's protocol. The total RNA was reverse-transcribed into cDNA by using oligo (dT) primers. Real-time PCR was performed using the SYBR green PCR master mix. The primer sequences were as follows: TNF-α forward: 5'-CAGGTTCCGTCCCTCTCATA-3' and reverse: 5'-TGCCAGTTCCACATCTCG-3', IL-1β forward: 5'-GCCAACAAGTGGTATTCTCCA-3' and reverse: 5'-CCGTCTTT

CATCACACAGGA-3', IL-6 forwad: 5'-AGTTGCCTTCTTGGGACTGA-3' and reverse: 5'-ACTGGTCTGTTGTGGGTGGT-3', GAPDH forward: 5'-GACATGCCGCCTGGAG AAAC-3' and reverse: 5'-AGCCCAGGATGCCCTTTAGT-3'. The GAPDH was used as an endogenous control. Primer amplification efficiency and specificity were verified for each set of primers. The mRNA expression levels of the tested genes relative to GAPDH were determined using the $2^{\Delta\Delta Ct}$ method and expressed as fold induction[14].

Statistical analysis: The data were expressed as Mean±Standard Deviation (SD) and examined for their statistical significance of difference with ANOVA and the standard's t-test. p-values of less than 0.05 were considered to be statistically significant.

RESULTS

SPA improved pathological symptoms and reduced DAI scores in IA-induced colitis of rats: Based on the clinical usage of traditional Chinese medicine, *S. pubescens* was used by decoction with boiling water in clinic. Therefore, the aqueous extract of *S. pubescens* (SPA) was used to evaluate its therapeutic effect on IA-induced experimental UC in rats. In this study, the rats in the IA-treated groups developed severe colitis rapidly and the typical signs including mucus stool, bloody diarrhea and dramatic body weight loss. After IA instillation, DAI scores according to body weight loss, stool consistency and gross bleeding were recorded daily (Table 1). The DAI in model control group at 1st, 2nd and 3rd day after iodoacetamide instillation were found to be 2.40±0.75, 2.12±0.94 and 1.79±1.03, respectively which were prominently higher than that in normal control rats ($p<0.001$). However, treatment with SPA (50 and 200 mg kg^{-1}) or positive control SASP (400 mg kg^{-1}) significantly reduced DAI scores at 2nd or 3rd day compared to IA-treated model group ($p<0.05$).

Table 1: SPA reduced DAI scores in IA-induced colitis of rats

	After iodoacetamide instillation		
DAI score	1st day	2nd day	3rd day
Normal	0.33±0.70	0.20±0.17	0.00±0.00
IA	2.40±0.75###	2.12±0.94###	1.79±1.03###
IA+SPA (12.5 mg kg^{-1})	2.15±0.76	2.38±0.82	2.02±1.08
IA+SPA (50 mg kg^{-1})	1.82±1.18	1.35±0.84*	0.44±0.75**
IA+SPA (200 mg kg^{-1})	1.60±1.09	1.05±1.09	0.74±1.19*
SASP (400 mg kg^{-1})	2.49±0.46	1.81±0.80	0.82±0.69*

Data are expressed as the Mean±SD (n = 10), ###$p<0.001$ vs. normal control group, *$p<0.05$ and **$p<0.01$ vs. The IA treated only model group, IA: Iodoacetamide, SPA: *S. pubescens* and SASP: Sulfasalazine

SPA attenuated IA-induced macroscopic damage of colon in rats: The colon tissues were collected on 4th day after iodoacetamide instillation when colitis severity reaches the peak. As an indicator of inflammation, the ratio of colonic wet weight to length was firstly measured. The results were shown in Fig. 1a and b, a significant increase of colonic wet weight and decrease of colonic length were found in IA-treated model rats and the ratio of colonic wet weight to length was significantly elevated compared to normal control group ($p<0.001$). However, this elevation was significantly reduced by treatment with SPA (50 and 200 mg kg^{-1}) or SASP (400 mg kg^{-1}) ($p<0.01$). These results were further confirmed by macroscopic scoring. As shown in Fig. 1a and c compared to normal control rats, IA caused colonic edema, hyperemia, inflammation and anabrosis and a significant increase of macroscopic injury severity score in model group rats ($p<0.001$). Treatment with SPA (50 and 200 mg kg^{-1}) or SASP (400 mg kg^{-1}) significantly improved both hyperemia and inflammation in the colons and reduced the macroscopic score compared to IA treated model rats ($p<0.05$, $p<0.01$ and $p<0.001$).

SPA attenuated IA-induced histopathologic damage of colon: The histological observations also supported above results. As shown in Fig. 2 compared to normal control rats, IA-treated model rats showed massive necrotic destruction of epithelium, submucosal edema and inflammatory cellular infiltration through the muscularis, mucosa and submucosa. Globet cells were entirely not present at the surface epithelium compared to normal control rats. However, treatment with SPA (12.5-200 mg kg^{-1}) or SASP (400 mg kg^{-1}) significantly attenuated the extent and severity of the histological signs of cell damage. The colonic mucosa showed ulcers in the process of healing, evolving to a more chronic inflammatory infiltrate with mononuclear predominance and initiation of a repair process. The elevation of microscopic pathological score induced by IA was significantly decreased by SPA or SASP treatment ($p<0.05$ and $p<0.001$).

SPA inhibited IA-induced mRNA expression of pro-inflammatory cytokines in colon: The TNF-α, IL-6 and IL-1β have been well characterized as pivotal pro-inflammatory cytokines during colonic inflammation. In this study, the steady-state mRNA levels of TNF-α, IL-6 and IL-1β in the colonic tissue were measured by using real-time PCR. As shown in Fig. 3, IA significantly increased the mRNA expression levels of these three pro-inflammatory cytokines

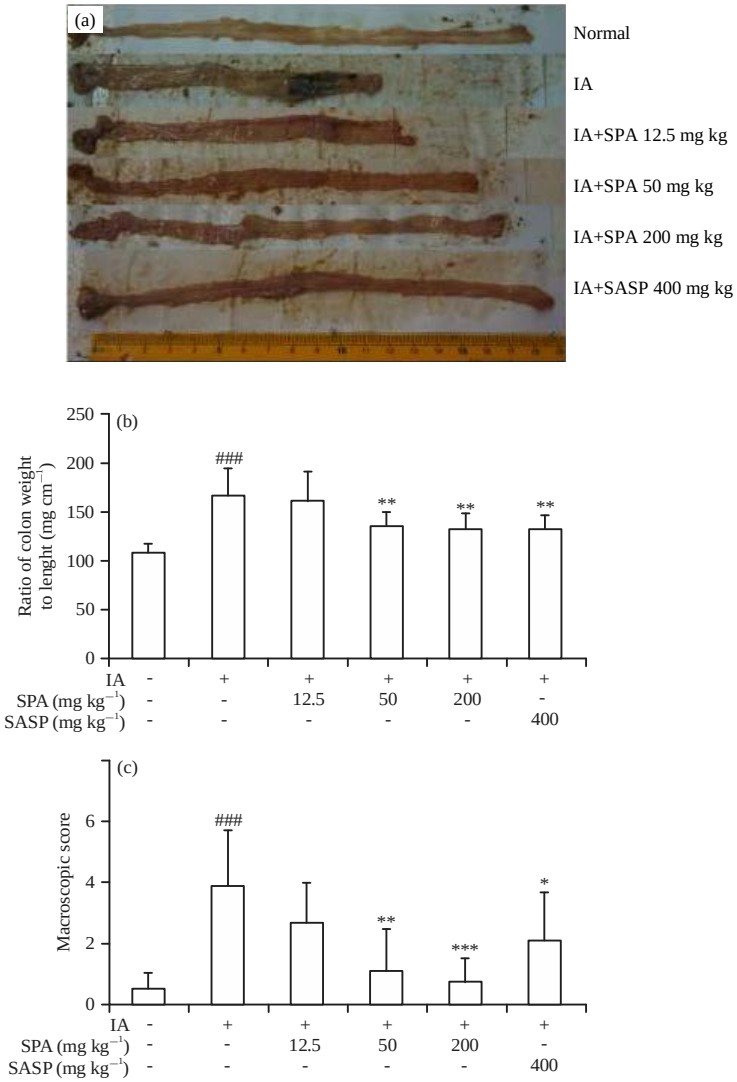

Fig. 1(a-c): *Siegesbeckia pubescens* attenuates attenuated Iodoacetamide-induced macroscopic damage of colon in rats. Experimental UC in rats was induced by IA through instilling into the lumen of the colon. The SPA (12.5, 50 and 200 mg kg^{-1}) and SASP (400 mg kg^{-1}) were orally administered 3 days before IA instillation and continued up to 4 days. At the end of the experiment, rats were sacrificed and the portion of colon was excised for morphological examination. (a) Representative photograph of colon morphology of each group, (b) The ratio of colonic wet weight to length and (C) Macroscopic injury severity score of colon. Data are expressed as the Mean±SD (n = 10). $^{###}$p<0.001 vs. normal control group, *p<0.05, **p<0.01 and ***p<0.001 vs. The IA treated only model group

in the colonic tissues (p<0.01) and these increases were obviously inhibited by SPA (12.5-200 mg kg^{-1}) or SASP (400 mg kg^{-1}) treatment (p<0.05, p<0.01 and p<0.001).

DISCUSSION

The UC is characterized by repeated cycles of colonic mucosal damage, ulceration and regeneration. Although, the etiology and pathogenesis of UC are still poorly understood, its pathogenesis process involves the impairment of epithelial barrier integrity, the penetration of enteric bacteria and other antigens into mucosa as well as the continuous inflammatory reaction[15]. Sulfhydryl compounds play an important role in maintaining mucosal integrity in the gastrointestinal tract by protecting against oxidative stress. The IA is a sulfhydryl blocker, induces colitis through a biochemical mechanism of injury mediated by oxidative free radicals and inflammatory mediators. Intrarectal administration of IA in rats causes maximal injury to the intestine after 3-7 days[16]. This experimental colitis model is widely used to investigate the

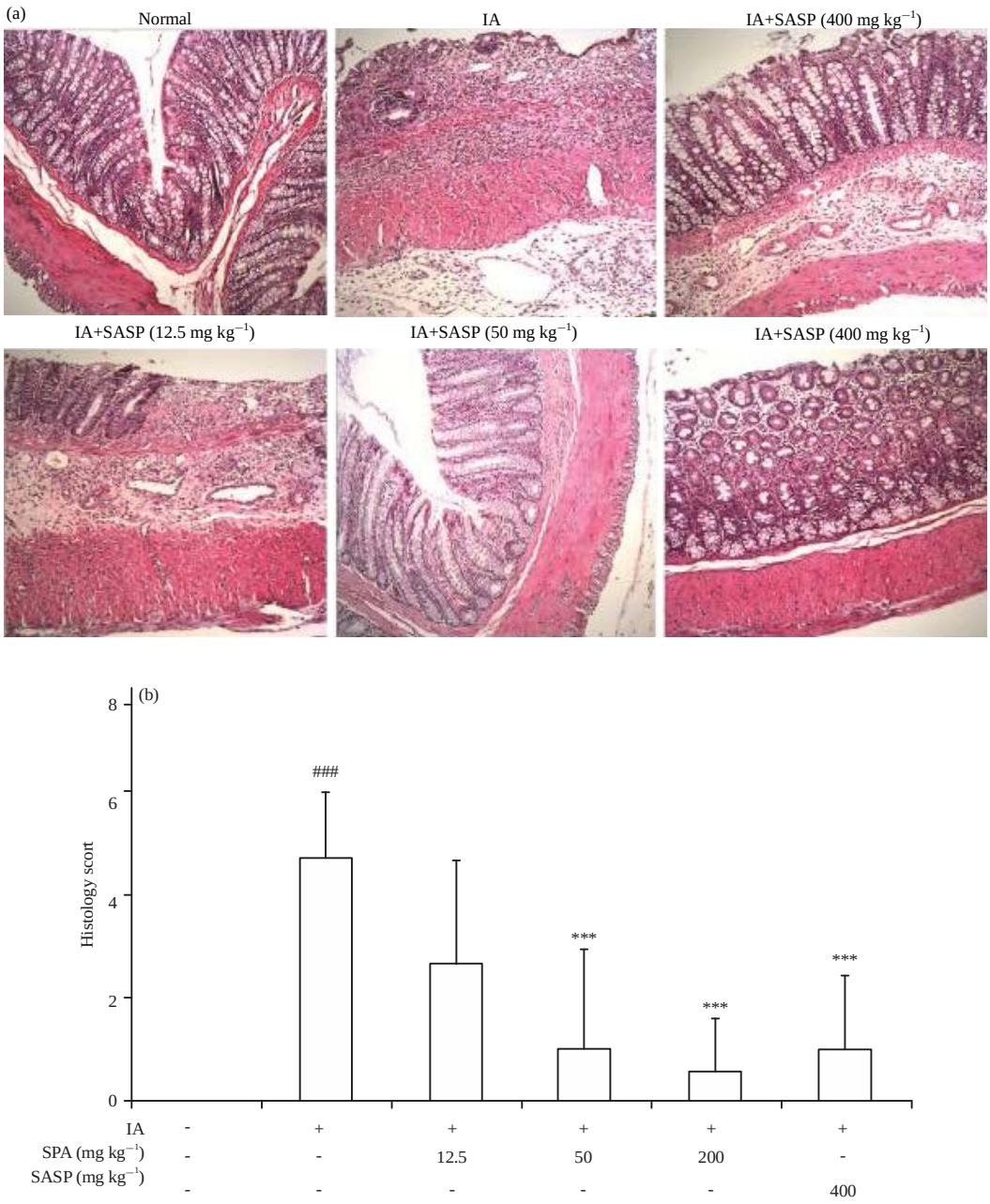

Fig. 2(a-b): *Siegesbeckia pubescens* attenuates attenuated Iodoacetamide-induced histopathologic damage of colon. Sections of the colon were stained with H and E for histopathological analysis. (a) Representative histological photo (100×magnification) and (b) histological score of each group were presented. Data are expressed as the Mean±SD (n = 10). ###p<0.001 vs. normal control group, *p<0.05 and ***p<0.001 vs. The IA treated only model group

mechanism of UC and to evaluate the efficacy of new strategies for UC[17,18]. In this study, 4 days later after being instilled into the colon, IA caused typical symptoms of experimental colitis such as mucosal damage with inflammation, diarrhea with bleeding and a decrease of body weight which was accompanied by enhanced generation of pro-inflammatory mediators (IL-1β, TNF-α and IL-6). These results were consistent with previous studies[16,18].

In previous studies, oral administration of the aqueous extract of *S. pubescens* significantly inhibited immunoglobulin E (Ig E) mediated immediate hypersensitivity reaction in rats and inhibited histamine release from rat peritoneal mast cells[8]. Topical application of the methanolic extract of *S. pubescens* displayed anti-inflammatory activity in carrageenan-induced edema rats[7]. The extract of *S. pubescens* by 50% (v/v) ethanol-water was reported to

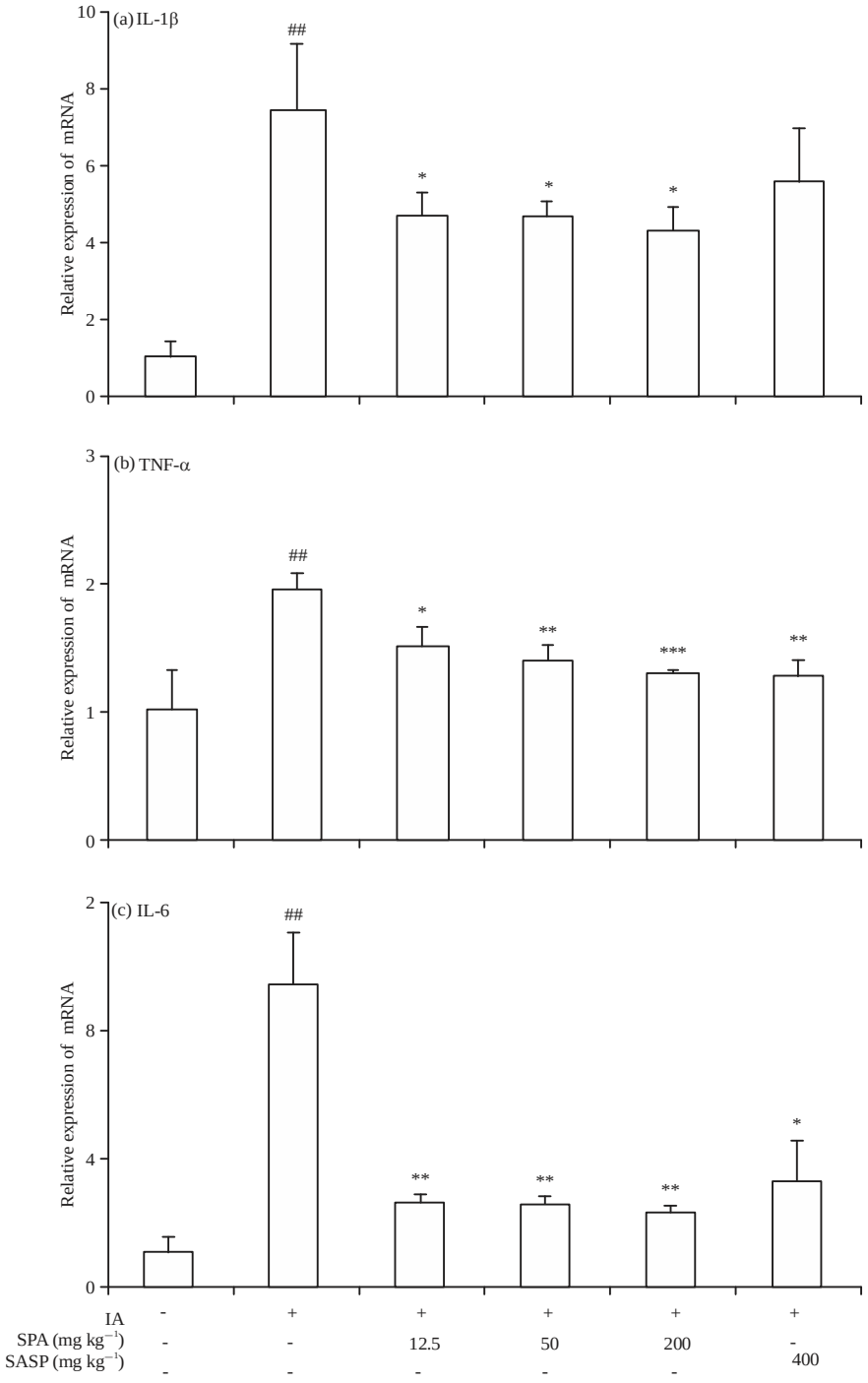

Fig. 3(a-c): *Siegesbeckia pubescens* attenuates inhibited Iodoacetamide-induced mRNA expression of pro-inflammatory cytokines in colon. The total RNA was isolated from colon tissues and reverse-transcribed into cDNA. The mRNA expressions of pro-inflammatory cytokines relative to GAPDH were examined by real-time PCR method and expressed as fold induction. Data are expressed as the Mean±SD (n = 10). $^{\#\#}p<0.01$ vs. normal control group, *p<0.05, **p<0.01 and ***p<0.001 vs. The IA treated only model group

have therapeutic effects on an osteoarthritis rabbit model by prominent cartilage protection through controlling aggrecanase, MMPs/TIMP-1 levels and inflammatory mediators (IL-1β, PGE2 and NO)[6]. In clinic of traditional

Chinese medicine, *S. pubescens* was of ten used by decoction with boiling water for the treatment of inflammatory and autoimmune diseases[4]. Thus, in the present study, *S. pubescens* was extracted with boiling water and oral administration of this aqueous extract (SPA) resulted in a significantly amelioration of typical symptoms of IA-induced rat colitis including weight loss, mucus stool and bloody diarrhea which were quantified as DAI score (Fig. 1). Moreover, macroscopic and microscopic pathologic scoring showed that SPA suppressed IA-induced colonic edema, hyperemia, necrotic destruction of epithelium and inflammatory cellular infiltration (Fig. 1, 2). In addition, IA-induced mRNA expression of pro-inflammatory mediators (IL-1β, TNF-α and IL-6) in the colonic tissues were also obviously inhibited by SPA (Fig. 3). All these results supported that SPA attenuated IA-induced colitis in rats. Hitherto, this is the first time to provide experimental evidences to demonstrate the therapeutic effect of *S. pubescens* on UC.

In the past few years, phytochemical investigations revealed that diterpenoids and flavonoids are the main bioactive constituents in *S. pubescens*[19,20]. These chemical constituents have been reported to have anti-inflammatory activities. It was reported that kirenol attenuated synovial inflammation of collagen-induced arthritis in rats. Siegeskaurolic acid inhibited iNOS and COX-2 expression in RAW 264.7 macrophages. The anti-inflammatory mechanisms of these two main constituents in *S. pubescens* are through interacting with NF-κB[21,22]. Lee *et al*.[23] reported that ent-kaurane and ent-pimarane diterpenes inhibited lipopolysaccharide-induced NO production and the expression of iNOS and COX-2 in BV2 microglia. Wang *et al*.[22] also reported that several sesquiterpenoids and diterpenoids from this plant inhibited LPS plus IFN-γ induced NO production in RAW 264.7 macrophages and FMLP/CB induced superoxide anion generation and elastase release in human neutrophils[24]. Thus, it was conjectured that these bioactive constituents may responsible the therapeutic effect of *S. pubescens* on UC in present study. However, it is needed to be confirmed in future experiments.

CONCLUSION

In summary, it was concluded that *S. pubescens* has therapeutic effects against IA-induced colitis in rats. These findings highlight the potential of this traditional Chinese medicine as an agent for use in the treatment of UC.

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