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Panax notoginseng attenuates experimental colitis in AOM/DSS mouse model

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Abstract

Patients suffering from inflammatory bowel disease are at a high risk of developing colorectal cancer. To assess the anti-cancer potential of botanicals, in this study, we evaluated the effects of *Panax notoginseng* on azoxymethane (AOM)/dextran sulfate sodium (DSS)-induced colitis. One week after A/J mice received AOM, the animals received DSS for 8 days, or were supplemented with *P. notoginseng* extract, at 30 or 90 mg/kg. DSS-induced colitis was scored with the disease activity index (DAI). The severity of the inflammatory lesions was evaluated by a colon tissue histological assessment. The expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) were also explored. We observed that the effects of *P. notoginseng* on the reduction of colon inflammation, expressed in DAI score, were in a dose-related manner ($P < 0.01$). *P. notoginseng* inhibited the reduction of the colon length and the loss of bodyweight in dose-related manner (all $P < 0.05$). The histological assessment of the colitis and inflammatory related immunohistochemical data also supported the pharmacological observations. Our data suggest that *P. notoginseng* is a promising candidate in preventing and treating colitis and inflammation-associated colon carcinogenesis.

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Conflict of Interest

The authors have no conflict of interest to report.

Keywords

Panax notoginseng; notoginseng; inflammatory bowel disease; colitis; AOM/DSS model; colorectal carcinogenesis

INTRODUCTION

Ulcerative colitis and Crohn's disease are two major forms of inflammatory bowel disease (IBD). The IBD is associated with colonic mucosal ulceration, shortening of the colon, reduced gastric motility and/or diarrhea, stool with blood and/or mucus, and body weight loss (Fiocchi, 1998; Kohn and Meddi, 2012). Patients suffering from this widespread and distressing medical problem are also at a high risk of developing colorectal cancer (Tanaka, 2009; Andersen *et al.*, 2012), a very common form of cancer worldwide (Siegel *et al.*, 2013).

Panax notoginseng is a Chinese herbal medicine that has a long history of use in China and other Asian countries for different medical conditions (Wang *et al.*, 2006). This perennial botanical is distributed throughout southwest China, Burma, and Nepal. The portion of the plant commonly used in remedies is the root, which is dug up after the fruit has ripened. Previous studies have shown that *P. notoginseng* possessed potential anti-tumor activities (Chen *et al.*, 2001; Wang *et al.*, 2007b; Jin *et al.*, 2010). Our group has previously reported that *P. notoginseng* extract increased the effects of 5-FU's cancer chemotherapy, and this synergistic effect between *P. notoginseng* and 5-FU makes it possible to reduce the dose of 5-FU in combination with the herb and thereby decrease 5-FU dose-related toxicity (Wang *et al.*, 2007a). We also demonstrated significant growth inhibitory effects of *P. notoginseng* and its constituents on human colorectal cancer cell lines (Wang *et al.*, 2009b; Sun *et al.*, 2011), although these observations were based only on *in vitro* preparations.

Chronic inflammation in the colon can lead to cancer. Experimentally, azoxymethane (AOM; a mutagenic agent) and/or dextran sodium sulfate (DSS; a pro-inflammatory reagent) have often been used in colorectal cancer chemoprevention animal studies (Tanaka *et al.*, 2003; Neufert *et al.*, 2007; Poudyal *et al.*, 2012; Sliva *et al.*, 2012). In this project, using an AOM/DSS mouse model, the colonic inflammation was established to mimic the inflamed colon and immunopathological disorders observed in humans (Murthy, 2006; Neufert *et al.*, 2007). To evaluate *P. notoginseng*'s anti-colorectal cancer potential, the chemically induced experimental colitis was characterized by a change in stool consistency, blood in stool, reduction of colon length, and loss of body weight. Histological assessment of murine colitis and colon tissue immunohistochemical staining were also conducted to support the pharmacological observations. Our data suggest that as a natural product, *P. notoginseng* is a promising candidate in preventing and treating colitis.

MATERIALS AND METHODS

Chemicals and reagents

HPLC grade ethanol, *n*-butanol, acetonitrile, and DMSO were obtained from Fisher Scientific (Pittsburgh, PA). Milli Q water was supplied by a water purification system (US

Filter, Palm Desert, CA). *P. notoginseng* saponin standards for ginsenosides Rb1, Rc, Rd, Re, and Rg1 were obtained from Indofine Chemical Company (Somerville, NJ); ginsenosides Rg2, Rg3, and notoginsenoside R1 were obtained from the Delta Information Center for Natural Organic Compounds (Xuancheng, Anhui, China). All saponin standards were of biochemical-reagent grade and at least 95% pure as confirmed by HPLC. Azoxymethane (AOM) was obtained from the NCI Chemical Carcinogen Reference Standard Repository, Midwest Research (Kansas City, MO). Dextran sodium sulfate (DSS, molecular weight of 36,000–50,000 Da) was obtained from MP Biomedicals (Solon, OH). Anti–inducible nitric oxide synthase (polyclonal, 1:500) was obtained from EMD Millipore (Billerica, MA). Anti–cyclooxygenase-2 (polyclonal, 1:500) was obtained from Cayman Chemical (Ann Arbor, MI). Avidin-biotin complex (ABC) kit and DAB peroxidase substrate kit were obtained from Vector Laboratories (Burlingame, CA). Hemoccult Sensa test strips were obtained from Beckman Coulter (Brea, CA).

Botanical material, preparation, and phytochemical analysis

The root of *P. notoginseng* (*Panax notoginseng* (Burk.) F.H. Chen), cultivated for 4 years, was obtained from Wenshan (Yunnan, China). Dr. Chong-Zhi Wang authenticated the plant materials and voucher specimen was deposited at the Tang Center for Herbal Medicine Research at University of Chicago (Chicago, IL). The air-dried root was extracted and lyophilized (Wang *et al.*, 2008). Briefly, dried *P. notoginseng* roots were pulverized into fine powder with a pulverizer and passed through a 40-mesh screen. A 100 g of powdered *P. notoginseng* sample was extracted with 70% ethanol, and the solvent of the extract solution was evaporated under vacuum. The dried extract was dissolved in water, and then extracted with water-saturated *n*-butanol. The *n*-butanol phase was evaporated under vacuum and then lyophilized.

Before pharmacological evaluation, the *P. notoginseng* extract was analyzed using HPLC (Wang *et al.*, 2009a; Mao *et al.*, 2012). The HPLC system was a Waters 2960 instrument (Milford, MA) with a quaternary pump, an automatic injector, a photodiode array detector (Model 996), and Waters Millennium 32 software for peak identification and integration. The separation was carried out on an Alltech Ultrasphere C18 column (5 μ, 250 × 3.2 mm I.D.) (Deerfield, IL) with a guard column (7.5 × 3.2 mm I.D.). For HPLC analysis, a 20-μL sample was injected into the column and eluted at room temperature with a constant flow rate of 1.0 mL/min. For the mobile phase, acetonitrile (solvent A) and water (solvent B) were used. Gradient elution started with 17.5% solvent A and 82.5% solvent B. Elution solvents were then changed to 21% A for 20 min, then to 26% A for 3 min and held for 19 min, at 36% A for 13 min, at 50% A for 9 min, at 95% A for 2 min and held for 3 min. Lastly, eluting solvents were changed to 17.5% A for 3 min and held for 8 min. The detection wavelength was set at 202 nm.

Animal treatment

The experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Chicago. All experiments were carried out in male A/J mice, 6–8 weeks old, weighing between 18 and 22 g, obtained from Jackson Laboratories (Bar Harbor, ME). Mice were caged under controlled room temperature, humidity and light

(12/12 h light/dark cycle) and allowed unrestricted access to standard mouse chow and tap water. The mice were allowed to acclimate to these conditions for at least 7 days before inclusion in the experiments.

The experimental protocol is shown in Figure 2(A). There were 4 experimental groups (n = 10 per group). Group 1 was the control. Animals in Group 2 (model group), Group 3, and Group 4 initially received a single intraperitoneal injection of AOM (7.5 mg/kg). One week after the AOM injection (set as Day 1), these animals began to receive 2.5% DSS in drinking water for 8 consecutive days. The animals in Group 3 (low-dose group) and Group 4 (high-dose group) also received *P. notoginseng* in doses of 0.15 mg/ml and 0.45 mg/ml, respectively, in drinking water for 15 consecutive days. We calculated that the *P. notoginseng* daily dose was approximately 30 mg/kg and 90 mg/kg for low-dose and high-dose groups, respectively. The animals were sacrificed on Day 15, and tissue samples were collected for additional observations.

Disease activity index

DSS induced colitis was scored as the disease activity index (DAI) as described previously (Ghia *et al.*, 2006). In brief, the DAI was the combined scores of weight loss (0, none; 1, 0–5%; 2, 5–10%; 3, 10–20%; 4, >20%), stool consistency change (0, none; 2, loose stool; 4, diarrhea), and bleeding (0, none; 1, trace; 2, mild hemoccult; 3, obvious hemoccult; 4, gross bleeding), and then divided by 3. The animals were scored for the DAI at the same time of each day, blind to the treatment. The minimal score was 0 and the maximal score was 4.

Histological assessment

Paraffin-embedded tissue samples were serially sectioned, and some sections were stained with hematoxylin and eosin (H&E). The stained sections were subsequently examined for histopathological changes by a gastrointestinal pathologist.

The histology score was determined by multiplying the percent involvement of each of the three following histologic features by the percent area of involvement (Konoshima *et al.*, 1999): inflammation severity (0, none; 1, minimal; 2, moderate; 3, severe), inflammation extent (0, none; 1, mucosa; 2, mucosa and submucosa; 3, transmural), crypt damage (0, none; 1, one third of crypt damaged; 2, two thirds of crypt damaged; 3, crypts lost, surface epithelium intact; 4, crypts lost, surface epithelium lost), percent area involvement (0, 0%; 1, 1–25%; 2, 26–50%; 3, 51–75%; 4, 76–100%).

Immunohistochemical staining

Some sections of mouse colon tissue were incubated with antibodies against inducible nitric oxide synthase (iNOS; rabbit polyclonal; diluted 1:500; EMD Millipore, Billerica, MA), cyclooxygenase-2 (COX-2; rabbit polyclonal; diluted 1:100; Cayman Chemical, Ann Arbor, MI).

The avidin-biotin indirect immunoperoxidase method was used for immunohistochemistry of paraffin-embedded sections. The Vectastain Elite ABC kit was used according to the manufacturer's instruction (Vector Laboratories, Burlingame, CA). The samples were

incubated with biotin/avidin horseradish peroxidase conjugates and chromogen DAB (3, 3'-diaminobenzidine) (Vector Laboratories, Burlingame, CA). Sections were counterstained with hematoxylin. One set of sections was incubated in phosphate-buffered saline (PBS) as a primary antibody to serve as a negative control. The staining was accomplished by rocking slides using the Antibody Amplifier to ensure consistent, sensitive, and reproducible staining.

Statistical analysis

Data were presented as mean ± standard error (S.E.). Data were analyzed using analysis of variance (ANOVA) for repeated measures and Student's *t*-test. The level of statistical significance was set at $P < 0.05$.

RESULTS

HPLC analysis of *P. notoginseng* saponin profile

The linearity of the analytical method was assayed by analyzing standard solutions in the ranges of 2–400 μg/mL for ginsenoside Rb1 and Rg1, 1–200 μg/mL for notoginsenoside R1, ginsenosides Rc, Rd, Re, Rg2 and Rg3. Calibration curves were constructed from the measured peak areas and the related amount of ginsenosides. For all 8 saponins (Figure 1A), the calibration curves showed good linearity ($R^2 > 0.9990$). *P. notoginseng* saponins in the extract were identified by comparison of their retention times with those obtained from the chromatograms of mixed ginsenoside standards. From the chromatogram of the *P. notoginseng* extract (Figure 1B), four main peaks of notoginsenoside R1, ginsenosides Rg1, Rb1 and Rd were observed. The content of saponins in the extract was calculated using standard curves of *P. notoginseng* saponins. As shown in Figure 1C, the contents of two ginsenosides Rg1 and Rb1, reached over 20% (24.4% and 28.4%, respectively). The contents of other six saponins are: R1, 5.3%; Re, 2.6%; Rg2; 0.4%; Rc, 0.3%; Rd, 7.8%; Rg3, 0.1%. Compared to the total saponin content in the extract (69.3%), the proportion of Rg1 and Rb1 accounted for 76.2% of the total saponin.

P. notoginseng suppressed experimental colitis

After DSS treatments, animals in the model group showed apparent diarrhea and rectal bleeding, starting from Day 4. As the treatment continued, the presence and development of inflammation manifested clearly. The disease severity, scored by the disease activity index (DAI), reached its highest level on Day 9 (Figure 2). Figure 2(B) shows the effects of *P. notoginseng* on the reduction of the DAI score in a dose-related manner ($P < 0.01$). This suppression of the experimental colitis was not only evident during DSS treatment, but also very obvious after the cessation of its administration, suggesting that *P. notoginseng* significantly promoted recovery from the colitis.

P. notoginseng attenuated colitis-induced colon length reduction

As an objective measure of the severity of inflammation, colon length in different groups was measured (Figure 3). The control group animals had an average colon length of 9.8 ± 0.5 cm. The DSS treatment lead to a very significant reduction of colon length, and the average length in the model group animals was reduced to 5.5 ± 0.4 cm ($P < 0.01$ compared

to control). Treatment with low-dose and high-dose *P. notoginseng* inhibited the reduction of the colon in as dose-related manner (6.8 ± 0.6 and 7.5 ± 0.7 cm, $P < 0.05$ and $P < 0.01$) compared to the model group, respectively.

Body weight changes

Figure 4 shows the body weight changes in different experimental groups. Compared to the control group, which had a slow weight gain, the model group had significant weight reduction beginning on Day 7. This reduced weight remained for over 7 days after cessation of the DSS. However, both low-dose and high-dose *P. notoginseng* groups significantly reduced the body weight reduction ($P < 0.05$ and $P < 0.01$ compared to the model group, respectively).

Histological characterization

From H&E staining histological sections, control mouse colon sections showed intact epithelium, well-defined crypt length, no neutrophil infiltration in mucosa and submucosa, and no ulcers or erosions. In contrast, colon tissue from model animals showed severe inflammatory lesions, characterized by complete loss of crypts, surface erosion and ulcerations, lamina propria fibrosis and acute and chronic transmural inflammatory infiltrate. For mice treated with *P. notoginseng*, there was a significant reduction in inflammatory injury. Colon tissue from the low-dose group showed moderate inflammation, patchy architectural distortion, and mild lamina propria fibrosis. From the high-dose group, colon mucosa had tightly packed glands with a normal amount of goblet cells. The lamina propria contains mild patchy neutrophilic infiltrate. Figure 5 shows representative H&E staining histological sections (A), and overall histology scores are provided (B).

Immunohistochemical staining

To explore the impact of *P. notoginseng* on inflammatory markers *in vivo*, we examined iNOS and COX-2 expression using colon tissue immunohistochemical staining. Figure 6(A) shows representative sections of the expression of iNOS, and Figure 6(B) shows expression of COX-2. The immunohistochemical images show that iNOS and COX-2 levels were elevated in DSS treated mice, with most of the staining appearing in the inflammatory cells. Compared to the model group, the expression of iNOS and COX-2 in colon tissue was markedly decreased after *P. notoginseng* treatment, supporting the DAI data and histological evaluation.

DISCUSSION

Colorectal cancer remains a leading cause of morbidity and mortality worldwide. It is a significant contributor to the burden of disease for the American public. In 2013 alone, there is an estimated 142,820 new colorectal cancer cases and 50,830 the cancer-related deaths in the U.S. (Siegel *et al.*, 2013). In fact, 1 in 21 American men and women are at risk for developing invasive colorectal cancer in their lifetime.

Targeting inflammatory pathways has been shown to be effective in preventing the formation of colonic tumors and their malignant progression in both animal and human

studies (Madka and Rao, 2013). Non-steroidal anti-inflammatory drugs (NSAIDS) can reduce colorectal cancer tumorigenesis, but concerns and long-term risks of NSAIDs make this form of chemoprevention unsuitable as a general recommendation (Garcia Rodriguez *et al.*, 2013; Zhou *et al.*, 2013). Given the limitations of today's standards of practice, there is strong motivation for exploring alternative strategies for colorectal cancer and its associated inflammation. In this study, the effects of *P. notoginseng* were evaluated using an AOM/DSS mouse model.

Previously, the *in vitro* anti-proliferative effects of major saponins in *P. notoginseng* were evaluated using human colorectal cancer cell lines. We observed that *P. notoginseng* could arrest colorectal cancer cells in S and G2/M phases. In addition, the remarkable apoptosis induction activities of *P. notoginseng* were shown (Wang *et al.*, 2009b; Sun *et al.*, 2011). From our encouraging *in vitro* data to *in vivo* studies, human colorectal cancer cells can be implanted in immunodeficient nude mice for tumor growth and observation. However, this xenograft mouse model is not specifically designed to investigate colorectal cancer.

Several animal models have been used for colorectal cancer studies, especially mouse models for inflammation related colon cancer. IBD is a group of chronic dysregulated inflammatory conditions in the large and small intestines in humans, and it is well known that chronic inflammation in the colon can lead to cancer (Hanauer, 1996; Geier *et al.*, 2007). In the traditional medical system, anti-inflammatory botanicals had protective effects against DSS-induced colitis (Kim *et al.*, 2011). DSS was used alone to induce colitis in early studies, however, recently published studies often used AOM plus DSS together, since AOM enhanced DSS induced colitis (Tanaka *et al.*, 2003; Long *et al.*, 2013). AOM is a genotoxic, colonic carcinogen routinely used to induce colon tumors in mice (Poole *et al.*, 2004; Neufert *et al.*, 2007). In this study, the mouse colitis was chemically induced by AOM/DSS (Tanaka *et al.*, 2003; Neufert *et al.*, 2007; Poudyal *et al.*, 2012; Sliva *et al.*, 2012). Our inflammation-related mouse model of colorectal carcinogenesis combines AOM and DSS to induce colon lesions with positive inducible nitric oxide synthase and COX-2 (Maltzman *et al.*, 1997; Kohno *et al.*, 2006).

In this study, we showed that, *P. notoginseng* inhibited experimental colitis, in a dose-related manner, resulting in overall attenuation of inflammation DAI, including colon length and body weight changes. The effects of *P. notoginseng* were further confirmed by the H&E staining histological characterization in mouse acute colitis and colon tissue, and supported by our preliminary immunohistochemical staining data, such as expression of iNOS and COX-2.

In our previous studies using a xenograft nude mouse model, the botanical test compound was intraperitoneally injected for accurate dose control (Jin *et al.*, 2012). However, like other herbal medicines, *P. notoginseng* is almost always taken orally (Ushiroyama *et al.*, 2012). In the present study, *P. notoginseng* was administered orally, which is a more safe and practical way to deliver the treatment, and we carefully calculated the ingested dosage. For extended, long-term observation, it would be better to mix the botanical extract with mouse chow, which could be standard chow or chow formulated with high fat to mimic a western diet in humans (Huang *et al.*, 2012; Ohnishi *et al.*, 2012).

The bioactive constituents in *P. notoginseng* are believed as a group of triterpenoids or dammarane saponins. The antioxidant potential of *P. notoginseng* saponins has been reported (He *et al.*, 2012). It is known that oxidant stress plays a role in chemical induced colitis, and the pathological severity can be reduced by antioxidant botanicals (Yasui *et al.*, 2011; Oz *et al.*, 2013). Whether the beneficial effect of *P. notoginseng* on AOM/DSS-induced colitis is linked to the antioxidant activities of saponins in the herb will be conducted in future studies.

Using HPLC analysis, we identified the major constituents of *P. notoginseng* in our study. However, after oral administration, *P. notoginseng* saponins could be metabolized extensively by intestinal microbiota (Wang *et al.*, 2011; Wang *et al.*, 2012a). Previous animal pharmacokinetic evaluations of ginseng compounds have focused on their parent constituents, but their metabolites, especially those transformed by intestinal microbiota, have largely not been carefully evaluated. In addition, the liver plays a major role in drug metabolism. Compounds, including those constituents from *P. notoginseng*, and their metabolites are excreted into the bile duct. Hepatic disposition, biliary excretion, and enterohepatic circulation may affect the pharmacokinetic profile of administered compounds. To obtain a complete pharmacokinetic profile of *P. notoginseng*, we recently developed a dynamic microdialysis sampling method to sensitively analyze the *P. notoginseng* metabolite profile in rat bile, and identified both parent compounds (notoginsenoside R1, ginsenosides Rg1, Rb1, and Rd) and metabolites (ginsenosides Rg2, Rh2, and compound K) (Wen *et al.*, 2012). Our *P. notoginseng* anti-colitis observation should extend to gut microbiome and pharmacokinetic studies.

In this study, we observed that *P. notoginseng* attenuated experimental colitis in a mouse model. In future studies, we should extend the observation time for direct assessment of AOM/DSS-induced, colitis-associated colorectal carcinogenesis. Our study should also include other colorectal cancer animal models, especially the APC mutant *Min* (multiple intestinal neoplasia) mice with detailed mechanisms of actions (Yamada and Mori, 2007; Velmurugan *et al.*, 2010).

Traditional medicine has been practiced for thousands of years based on clinical experience. It is necessary to integrate existing traditional knowledge of diseases with modern biomedical technologies (Wang *et al.*, 2012b). The results from this study provided evidence-based information on complementary and alternative medicine intervention with an important clinical significance. *P. notoginseng*, as a candidate of botanical-based colorectal cancer chemoprevention, should be further tested for the unmet medical needs.

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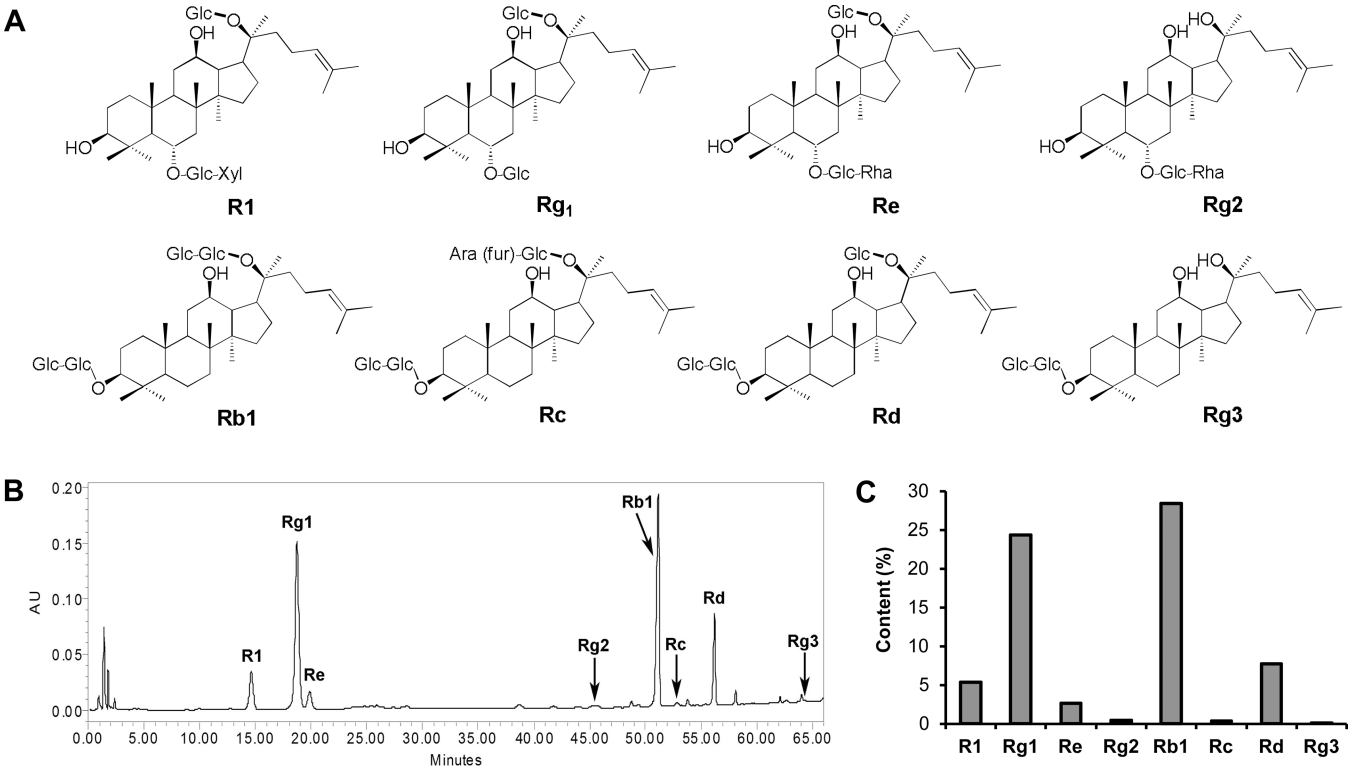

Figure 1.

Chemical structures and HPLC analysis of saponins in *P. notoginseng* extract. The structures of determined saponins are shown in (A). HPLC chromatogram of *P. notoginseng* extract recorded at 202 nm is shown in (B). HPLC conditions are described in the Materials and Methods. Saponin content of determined compounds is shown in (C). Eight *P. notoginseng* saponins were determined: notoginsenoside R1 (R1), and ginsenosides Rg1, Re, Rg2, Rb1, Rc, Rd and Rg3.

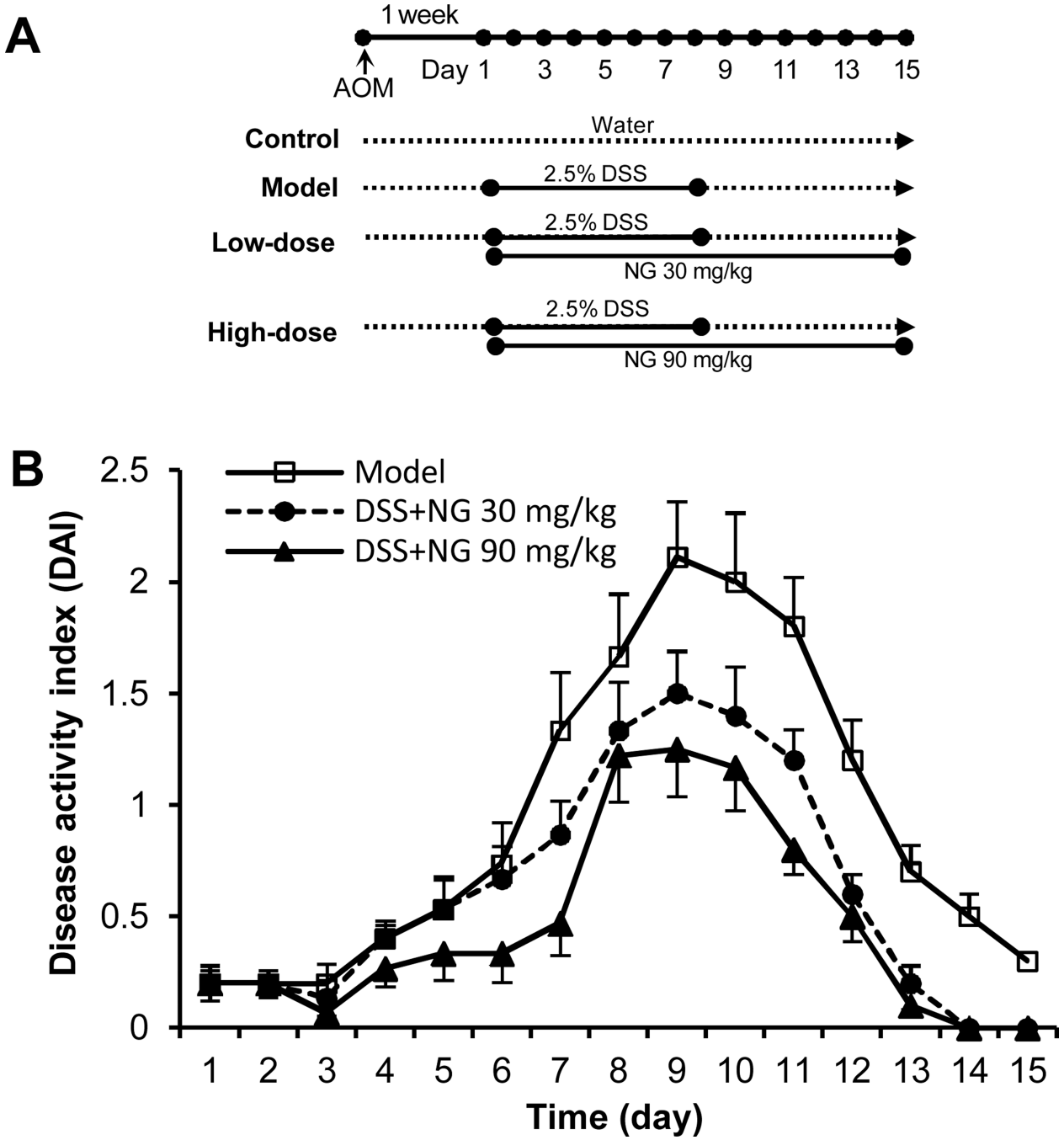

Figure 2.

Effects of *P. notoginseng* on acute experimental colitis in A/J mice. (A) Experimental protocol. (B) *P. notoginseng* attenuated the DSS-induced colitis, expressed as disease activity index (DAI). Data from the control group are all zeros from Day 1 to Day 15 (not shown). The dose-related effects of *P. notoginseng* is observed ($P < 0.01$). NG, *P. notoginseng*.

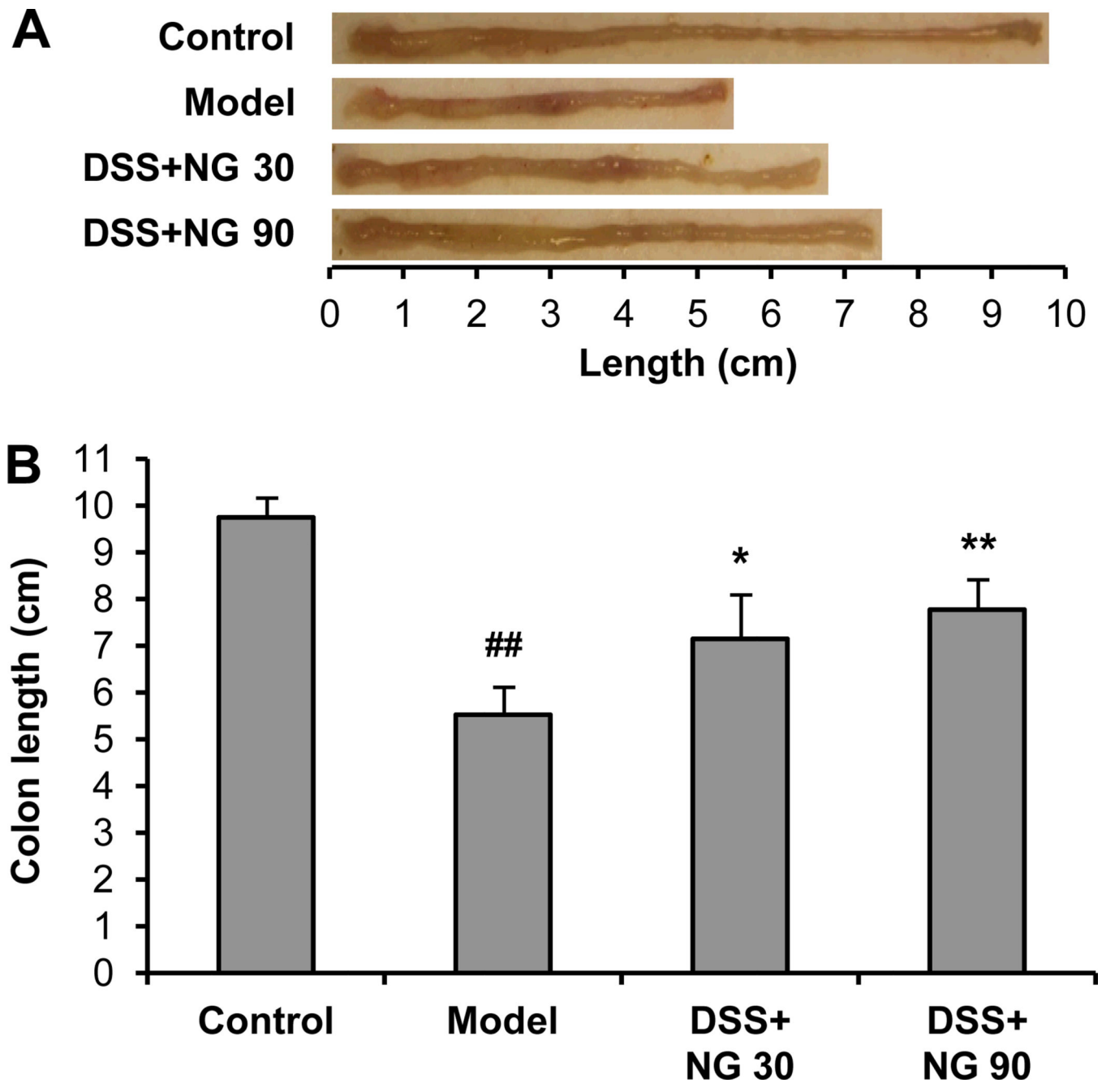

Figure 3.

Mouse colon length changes in different groups. (A) Representative photographic images of the colon. (B) For the model group, DSS treatment very significantly reduced the colon length in comparison with the control group (#, $P < 0.01$). *P. notoginseng* low-dose and high-dose treatment significantly decreased the colon length reduction compared to the model group, respectively (*, $P < 0.05$ and **, $P < 0.01$). NG 30, *P. notoginseng* 30 mg/kg; NG 90, *P. notoginseng* 90 mg/kg.

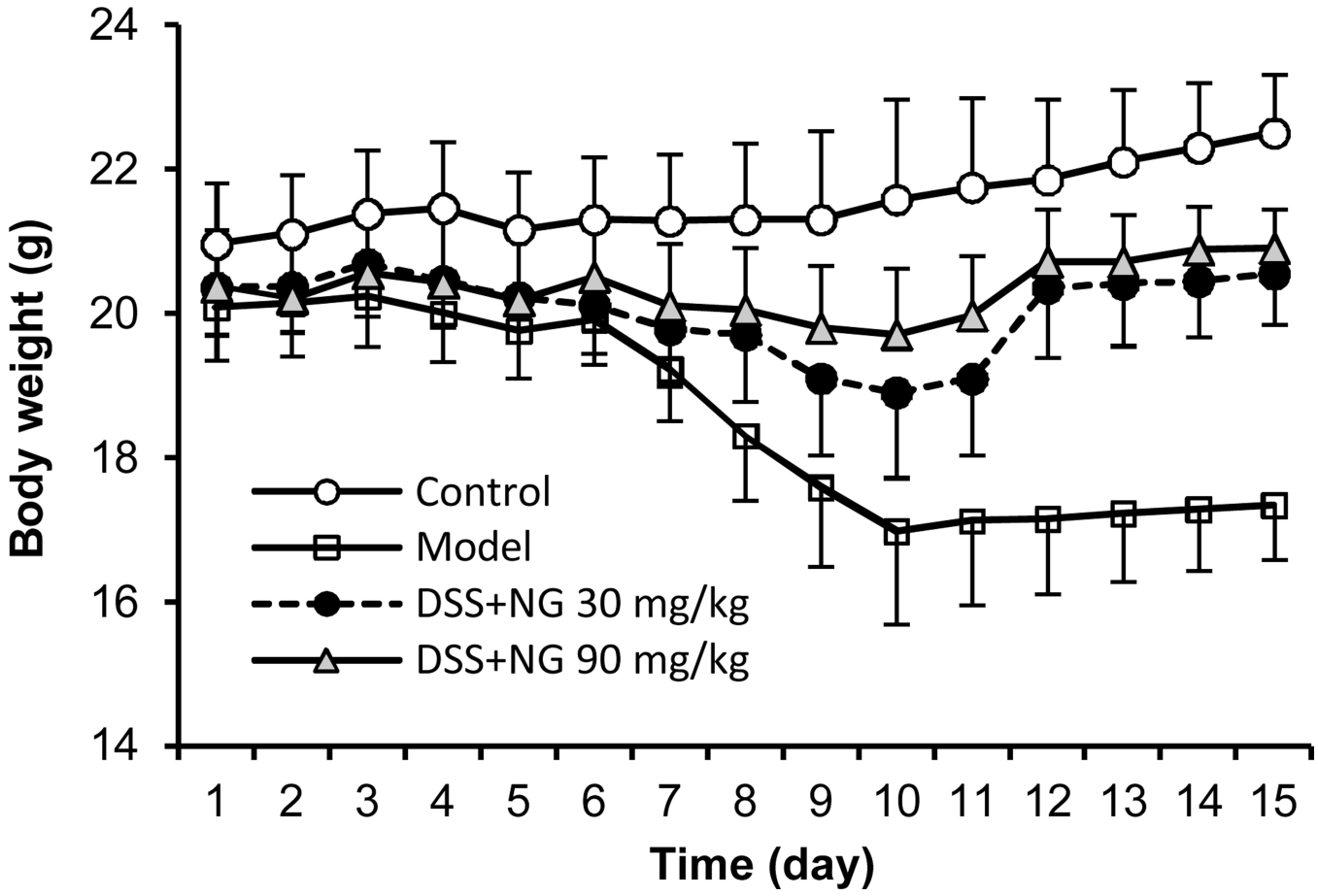

Figure 4.

Effects of *P. notoginseng* on mouse body weight changes. While the model group showed significant weight reduction from Day 7, *P. notoginseng* low-dose and high-dose treatment significantly reduced the DSS-induced weight reduction. ($P < 0.05$ and $P < 0.01$ compared to the model group, respectively).

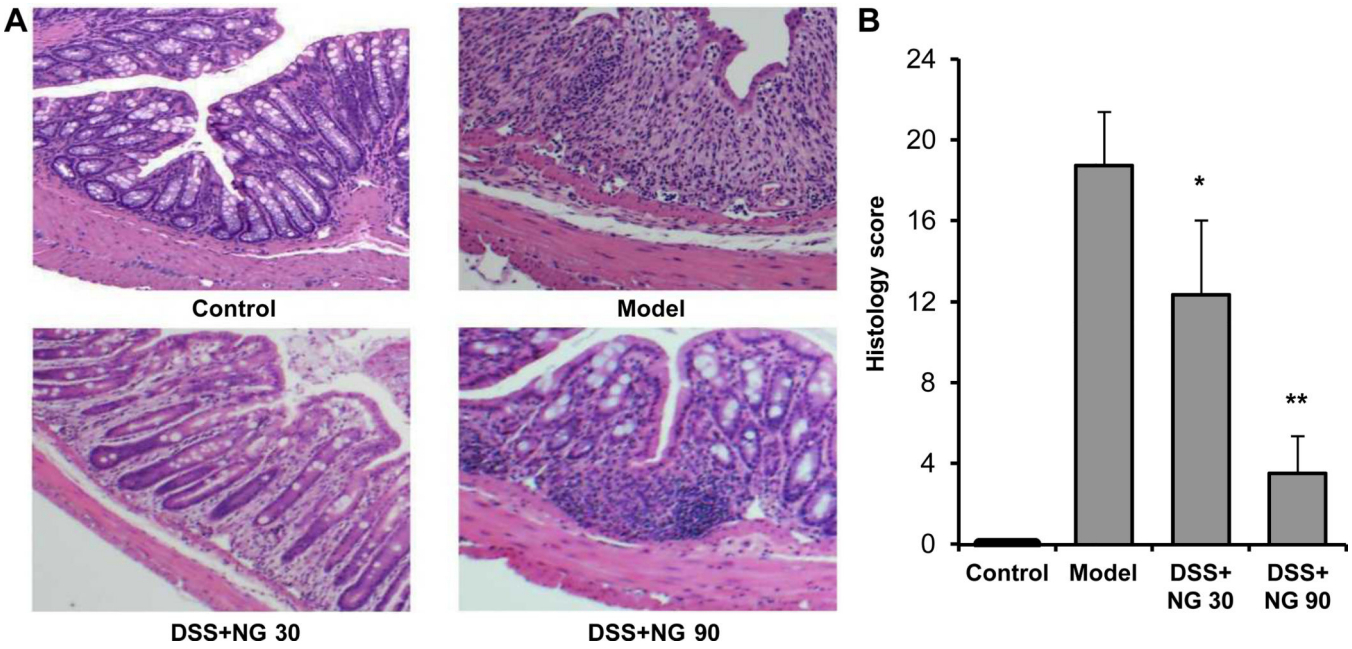

Figure 5.

Effects of *P. notoginseng* on the histological characterization in DSS-induced mouse colitis. (A) Representative H&E staining histological sections of control, DSS only, DSS plus low-dose *P. notoginseng* (NG 30), and DSS plus high-dose *P. notoginseng* (NG 90). (B) Overall histology scores in these different groups. *, $P < 0.05$; **, $P < 0.01$ compared to the model group.

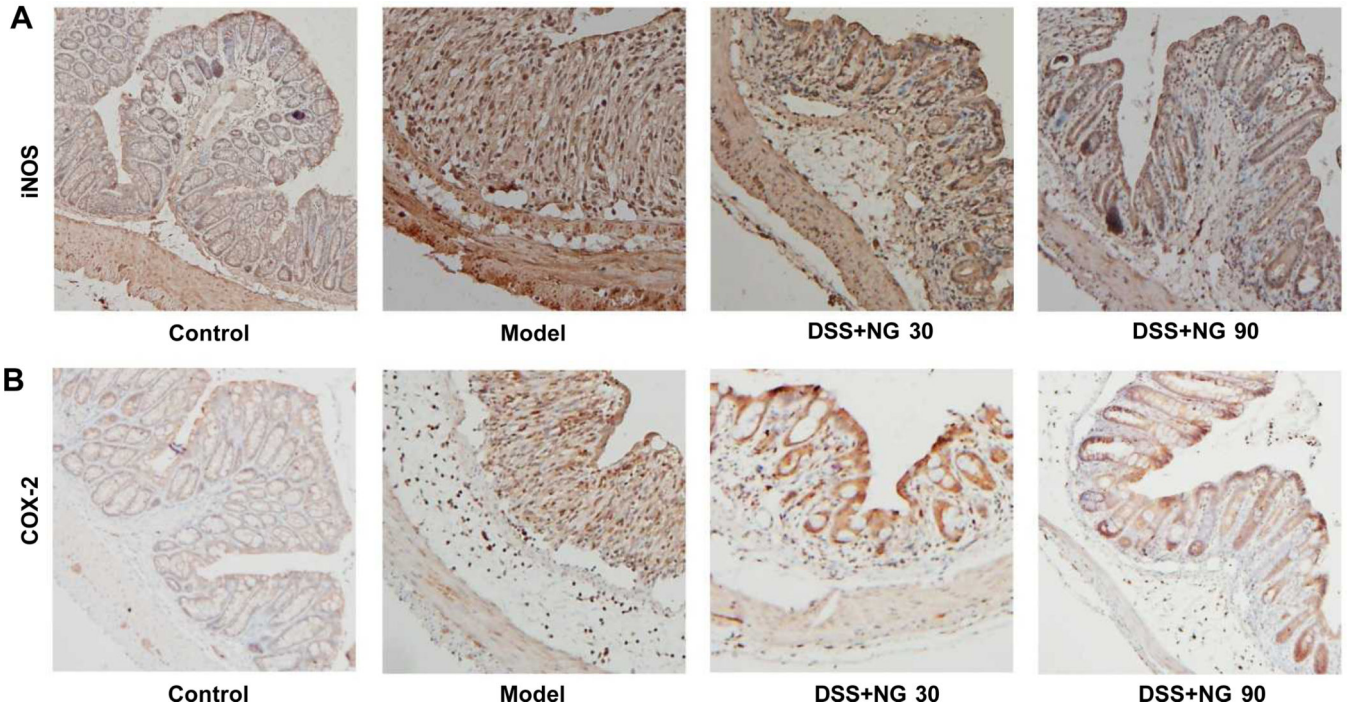

Figure 6.

Mouse colon tissue immunohistochemical staining with expression of (A) iNOS and (B) COX-2. Representative sections of control, DSS only, DSS plus low-dose *P. notoginseng* (NG 30), and DSS plus high-dose *P. notoginseng* (NG 90). DSS treatment induces iNOS and COX-2 expressions. Positive staining is brown colored. Magnification ×200.