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Original Article

Anti-inflammatory effect of *Pycnocycla spinosa* extract and its component isoacetovanillone on acetic acid induced colitis in rats

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Abstract

Colitis is an inflammatory disease of the intestine with unknown etiology involving multiple immune, genetic and environmental factors. We were interested to examine the effect of total extract from *Pycnocycla spinosa* Boiss. on the treatment of experimental colitis. Mediators involved in colonic inflammation are prostaglandins, interleukins, leukotriene as well as an increase in myeloperoxidase (MPO) activity. Therefore, MPO activity was also determined in this research. *P. spinosa* hydroalcoholic extract (5, 10 and 20 mg/kg) or isoacetovanillone (2, 5 and 10 mg/kg) were administered orally, started 2 h before induction of colitis by intrarectal administration of acetic acid (3%) in rats. Prednisolone (4 m/kg) was used as the standard drug for comparison. Biochemical evaluation of inflamed colon was done using assay of MPO activity. After 5 days treatments, mucosal ulceration was evaluated. Intrarectal instillation of acetic acid caused significant inflammatory reactions as indicated by macroscopic and microscopic changes. The activity of MPO increased in vehicle treated groups while recovered to normal level by pretreatment of animals with *P. spinosa* extract, isoacetovanillone and prednisolone. *P. spinosa* and isoacetovanillone-treated groups showed significantly lower score values of macroscopic and microscopic characters when compared with the vehicle treated negative control group. The beneficial effect of *P. spinosa* was comparable with that of prednisolone. This research has shown the anti-inflammatory potential of *P. spinosa* extract and isoacetovanillone in experimentally induced colitis.

Keywords: Colitis; *P. spinosa*; Hydroalcoholic extract; Isoacetovanillone

INTRODUCTION

Colitis is inflammation of the inner lining of the colon characterized by motility and secretion disorders (1). It may cause abdominal pain and diarrhea with or without blood. There are numerous causes of colitis including infection, inflammatory bowel disease, ischemic and microscopic colitis (2,3). In inflammatory diseases, increase in the number of intestinal endocrine cells are also associated with chronic mucosal damage (4,5). The enterochromaffin cells that secrete serotonin are the largest population of enteroendocrine cells in the gastrointestinal mucosa (6). In addition to neuronal and mast cells activities, the enterochromaffin cells activation may also play a significant role in the inflammatory process of colitis. In the etiology of colitis most attention has been paid to the role of mast cells and the endocrine cells in regulation of inflammatory and immune responses. Mast cells can release significant amounts of pro-inflammatory mediators (7-9). However, it is well known that various gastrointestinal motility disorders are associated with defects of the enteric nervous system (10,11). Inflammation is associated with changes in both the structure of the enteric nervous system and neurotransmitter contents and receptors (12). Furthermore, increasing evidence suggests that the secretory function of mast cells is also regulated by the nervous system (13). Therefore, drugs which can affect enteric nervous system activity could also be useful in the treatment of colitis.

Pycnocycla spinosa Decne. exBoiss. (Fam. Apiaceae) is a wild plant growing in many parts of Iran (14,15). Hydroalcoholic extract of

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P. spinosa possesses antispasmodic activity on isolated ileum and inhibits contraction induced by acetylcholine, serotonin, KCl or neuronal stimulation (16,17). In addition *P. spinosa* extract exhibited anti-diarrheal activity in animal model (16). *P. spinosa* extract contains alkaloids, flavonoids and saponin-like components, among which the flavonoids had pronounced pharmacological activities (17). Isolation of pure substances resulted in identification of at least four active components (18,19). Isoacetovanillone is an active component that is identified in the hydroalcoholic extract of *P. spinosa* (18,19).

Acetovanillone (apocynin) is a structural isomer of isoacetovanillone (19). Acetovanillone possessed anti-inflammatory activity and can prevent formation of free radicals, oxygen and peroxide ions (20-22). It is believed that these effects are due to inhibition of nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) enzyme (23). Furthermore, in animal model, acetovanillone has reduced colonic damage in colitis and attenuated inflammation related to the activity of myeloperoxidase (MPO) and reduced number of macrophages and mononuclear leucocytes in the colon (23).

As isoacetovanillone is a structural isomer of acetovanillone, it is possible that this compound also possesses anti-inflammatory and anti-colitis activities. Another reason which may indicate that this compound might be useful for the treatment of colitis would be its inhibitory effects on 5-hydroxytryptamine induced spasm (18). So far, there is no scientific report about anti-inflammatory effect of isoacetovanillone or the extract of *P. spinosa*. Therefore, the objective of this research was to evaluate anti-colitis effect of isoacetovanillone and *P. spinosa* extract and to assess their anti-inflammatory effects in animal model of induced colitis.

MATERIALS AND METHODS

Plant extract and isolation procedures

In flowering season (July), the aerial parts of *P. spinosa* were collected from Isfahan University campus located in the base of the Soffeh Mountain in the south of Isfahan-Iran. A voucher specimen of the plant (A24) is deposited in the herbarium of the School of Pharmacy and Pharmaceutical Sciences at the Isfahan University of Medical Sciences (Iran). Plants were dried in shade and powdered using an electrical miller. The total extract was prepared by percolation (24). The solvent was then evaporated and the yield of dried extract was determined.

Animals

Male Wistar rats weighing 200 ± 20 g were used in the present study. They were housed in separate cages (3 per cage) at room temperature (21-23 °C) with free access to food and water. Animal handling was carried out according to the National Research Council, guide for the care and use of laboratory animals as recommended by university authorities (25).

Chemicals and *solutions*

Isoacetovanillone, hexadecyltrimethyl-ammonium bromide, O- dianisidine HCl, and acetic acid were purchased from Sigma Co. (China). Prednisolone powder was obtained from Iran Hormone Pharmaceutical Co. (Tehran, Iran). Other chemicals were of analytical grade and purchased from Merck Chemical Company (Germany).

Dried *P. spinosa* extract was prepared as 5 mg/ml stock solution in ethanol (5%) (Estalak, Iran). Isoacetovanillone (sigma Co.) was also prepared as 2 mg/ml stock solution in ethanol (5%). Prednisolone (Iran Hormone Co.) powder (50 mg) was dissolved in distilled water/tween 80 (0.1%) to prepare 4-mg/ml stock solution.

Induction of colitis

Animals were fasted for 24 h and lightly anesthetized with diethyl ether. Colonic inflammation was induced by rectal administration of 2 ml acetic acid (3%). The rats were then held in an upside-down position for 2-3 min to avoid immediate anal leakage of the instillate. Thereafter, the rats were returned to their cages and had access to food and water *ad libitum*. Sham group was treated in the same way with normal saline (2 ml/rat) (26).

Animal grouping

Animals were randomly allocated to different groups: Sham, negative control, positive control and the tests groups. For each group at least six rats were used. Sham or normal group just received distilled water per oral (p.o.) (2 ml/rat) without induction of colitis. Negative control group was acetic acid-induced colitis rats which treated with vehicle p.o. (5% ethanol, 2 ml/rat). The *P. spinosa* extract groups were treated with doses of 5, 10 and 20 mg/kg (p.o.). The isoacetovanillone was given with doses of 2, 5 and 10 mg/kg (p.o.). The reference group was treated with prednisolone (p.o., 4 mg/kg).

All the treatments were started 2 h before induction of the colitis and continued daily for 5 consecutive days.

Macroscopic studies

Animals were sacrificed using ether inhalation on the sixth day of treatment. The distal colon of animals was removed and cut longitudinally, gently cleaned in physiological saline to remove fecal residues, weighed and processed for the assessment of macroscopic, histological scores and biochemical markers. Following preparing photos of distal colons, ulcer area was determined by Fiji P (Image Analysis Program, version 2). For each specimen, wet distal colon weight (8 cm from the anus) and colonic weight/length ratio (mg/cm) were measured. Treated sections of colon thereafter were collected and immediately frozen in liquid nitrogen for the measurement of MPO activity (27,28). Score were: 0, no ulcer; 1, mucosal erythema only; 2, mucosal edema and slight bleeding or erosions; 3, moderate edema, bleeding ulcers or erosions; 4, severe ulceration, erosions, edema and tissue necrosis and perforation. The ulcer index was determined by summing the mean ulcer score and the mean ulcer area.

Myeloperoxidase activity determination

MPO activity was measured according to the method described by Bradley and coworkers (26). As we described in our previous papers, tissues were weighed and placed in 1 ml of 10 mM potassium phosphate buffer contained 0.5% HTAB and then homogenizied with 1 ml HTAB in buffer solution at 4 °C. The suspensions were centrifuged at 20000 rpm for 15 min. In order to determine MPO activity, O-dianisidine dihydrochloride (1.6 mM) and hydrogen peroxide (0.1 mM) were added on top of medium. The absorbance of the reaction mixture was recorded at 450 nm with a UV-Vis spectrophotometer. The results were expressed as unit/100 g of wet colon weight (29-35).

Histological studies

The colon was scored for microscopically visible damage on a scale of 0 to 10 by 2 observers who were unaware of the treatment, according to the criteria described by Dieleman and colleagues (36) (Table 1), which take into account the extent and the severity of colonic damage.

Table 1. Scoring system for pathological assessment of colitis.

Scoring parameter	Score definition
Inflammation severity	0: None 1: Mild 2: Moderate 3: Severe
Inflammation extent	0: None 1: Mucosa 2: Mucosa and submucosa 3: Transmural
Crypt damage	0: None 1: Basal 1/3 damaged 2: Basal 2/3 damaged 3: Crypts lost, surface epithelium present 4: Crypts lost, surface epithelium lost

To process for histopathological studies, colonic specimens which were fixed in 10% formalin in phosphate buffered saline embedded in paraffin and cut into 4-µm sections. Then paraffin sections were deparaffinized with xylene and then hydrated and stained with hematoxylin and eosin (H&E). The stained sections were assessed for any inflammatory changes including infiltration of cells, necrosis, or damage to tissue structures. Total colitis index was measured by summing three sub-scores (inflammation severity, inflammation extent, and crypt damage).

Statistical analysis

One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post hoc test was used to compare the results of parameteric colonic weight/length ratio changes, MPO activity, and ulcer index values. Nonparametric data of colitis including macroscopic and histological scores were analysed by Mann-Whitney *U* test. The *P* values of 0.05 and less were considered statistically significant.

RESULTS

In the negative control group, acetic acid caused inflammation, sores and swelling in the lining of the injured segment of the rectum. In addition to inflammation, hemorrhage, ulcer, necrosis and thickened colon was visible while in the Sham group treated with normal saline there was no sign of redness or inflammation (Fig. 1).

The MPO activity of negative control group also showed significant increase in comparison to Sham group. Prednisolone treated group showed significantly lower score values of macroscopic and microscopic characters when compared with the negative control group (Figs. 1 and 2).

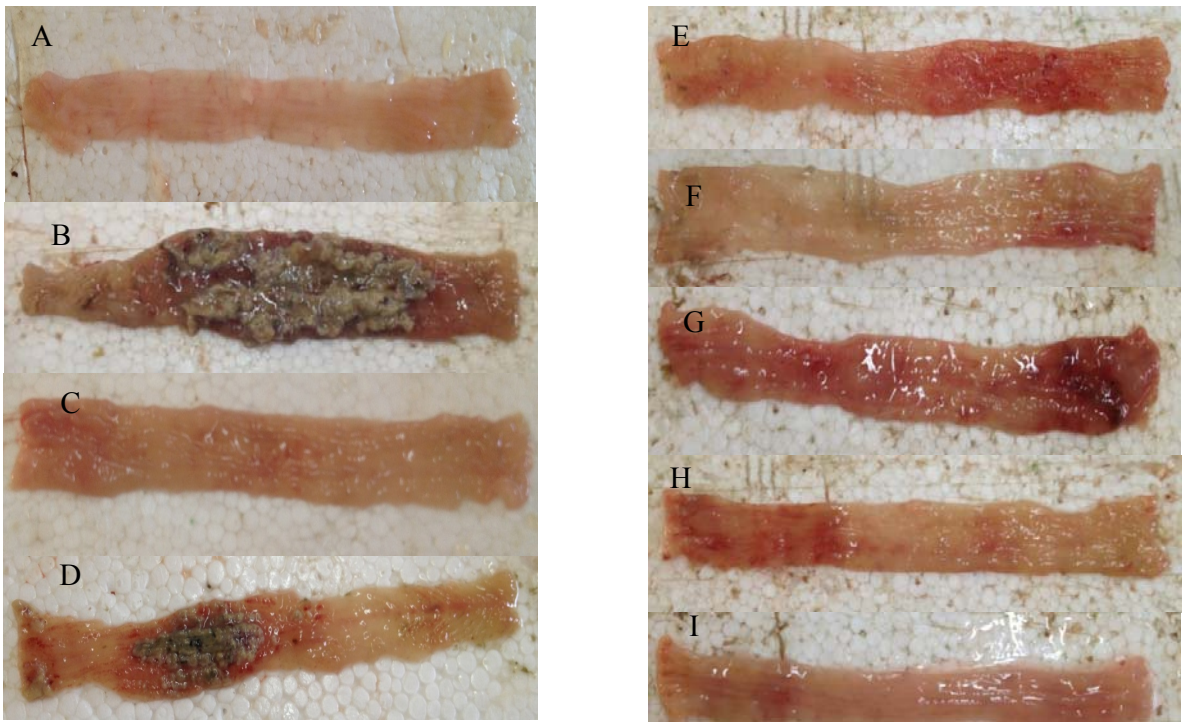

Fig. 1. Macroscopic presentation of acetic acid-induced colitis in rats. A; sham, B; control, C; prednisolone (4 mg/kg), D; isoacetovanillone (2 mg/Kg), E; isoacetovanillone (5 mg/Kg), F; isoacetovanilone (10 mg/Kg), G; *P. spinosa* extract (5 mg/Kg), H; *P. spinosa* extract (10 mg/Kg), I; *P. spinosa* extract (20 mg/Kg).

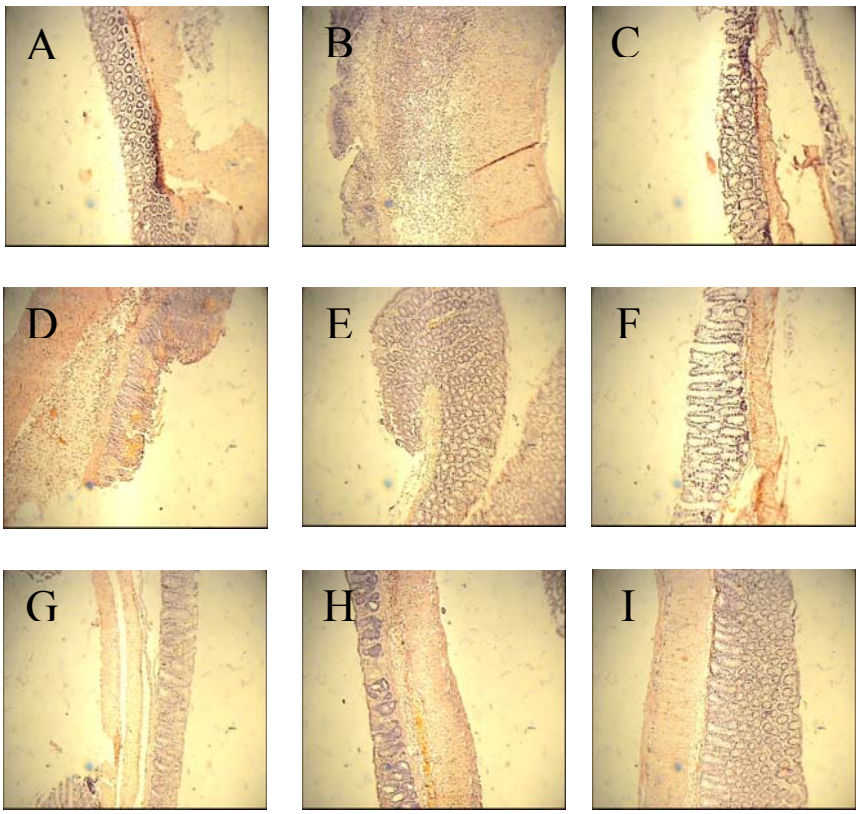

Fig. 2. Microscopic presentation of acetic acid-induced colitis in rats (hematoxylin and eosin staining; original magnification × 10). A; sham, B; control, C; prednisolone (4 mg/kg), D; isoacetovanillone (2 mg/Kg), E; isoacetovanillone (5 mg/Kg), F; isoacetovanilone (10 mg/Kg), G; *P. spinosa* extract (5 mg/Kg), H; *P. spinosa* extract (10 mg/Kg), I; *P. spinosa* extract (20 mg/Kg).

In addition, all the assessed ulcer parameters were relatively reduced in comparison with the negative control group. MPO activity was also decreased in prednisolone treated group close to the normal level. The change in MPO activities in colon segment homogenates of treated animals is shown in Fig. 3.

Both isoacetovanillone and *P. spinosa* extract inhibited acetic acid induced inflammation and MPO activities. The mean percentages of decreases in MPO activity in prednisolone (4 mg/kg), isoacetovanillone (5 mg/kg) and *P. spinosa* extract (5 mg/kg) treated groups were 52%, 40% and 55%, respectively (Fig. 3). In fact, there were no statistically significant differences between inhibitory effects of prednisone and *P. spinosa* extract on inhibiting MPO activity.

Both isoacetovanillone and *P. spinosa* reduced ulcer area in rat colitis induced by acetic acid in a dose-dependent manner. The mean percentage of reduction in ulcer area relative to the negative control group was 70% for isoacetovanillone (10 mg/kg) and 83% for *P. spinosa* extract (20 mg/kg), respectively (Fig. 4). There was no statistically significant difference in improving ulcer between prednisolone and *P. spinosa* extract (20 mg/kg).

Tissue edema which is quantified as colonic weight/length ratio is presented in Fig. 5. There was significant increase in colonic ratio of negative control group compared to the Sham group. Both isoacetovanillone and *P. spinosa* extract significantly reduced the colonic ratio. There were no statistically significant differences between inhibitory effects of isoacetovanillone or *P. spinosa* extract with that of prednisolone.

Scores for tissue sections prepared for microscopic examination are shown in Fig. 6. The microscopic examination was quantified as total colitis index as described in Table 1. The Sham group was given the lowest score. Prednisolone significantly reduced total colitis index in comparison with the negative control

group. Both isoacetovanillone and *P. spinosa* extract reduced total colitis scores (Fig. 6). However, they were less effective than prednisolone. For instance, isoacetovanillone (10 mg/kg) and *P. spinosa* extract (20 mg/kg) reduced the total colitis index by 40% and 55%, respectively while prednisolone reduced the colitis index by 69% (Fig. 6).

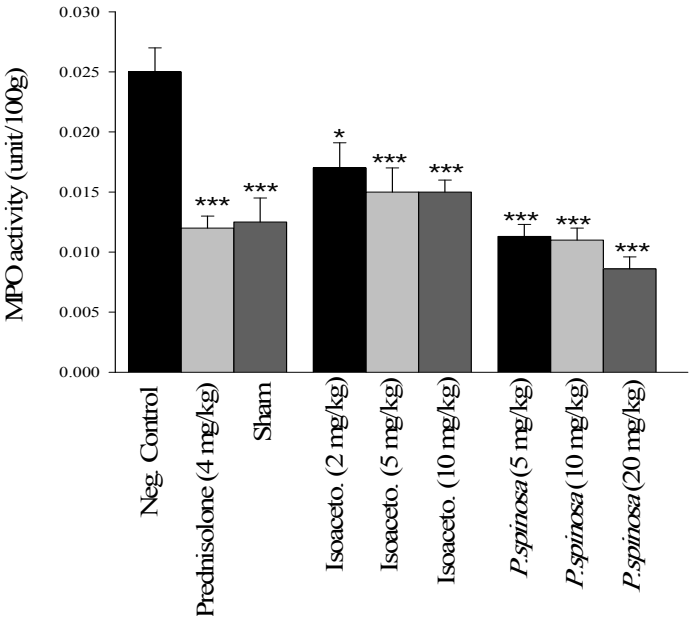

Fig. 3. Effect of oral administration of hydroalcoholic extract of *P. spinosa*, isoacetovanillone and prednisolone on myeloperoxidase activity in the rat colon 5 days after induction of the colitis with acetic acid (%3). MPO activity was measured as unit per 100g wet tissue in treated areas of the colon. Each value represents mean ± SEM (n=6). Stars shows statistically significant difference in comparison with the negative control group (**P*<0.05, ****P*<0.001). One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison *post hoc* test was used for statistical comparison of the data.

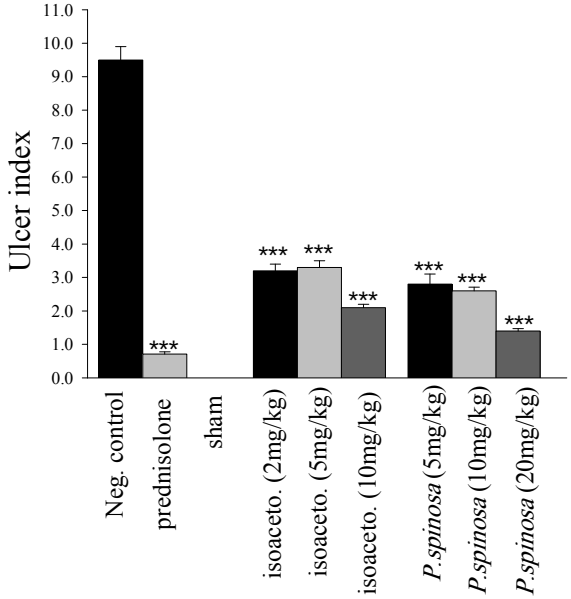

Fig. 4. Effect of oral administration of hydroalcoholic extract of *P. spinosa*, isoacetovanillone and prednisolone on ulcer index in the rat colon 5 days after induction of colitis with acetic acid (%3). Each value represents mean ± SEM (n=6). Stars shows statistically significant difference in comparison with the negative control group (****P*<0.001). One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison *post hoc* test was used for statistical comparison of the data.

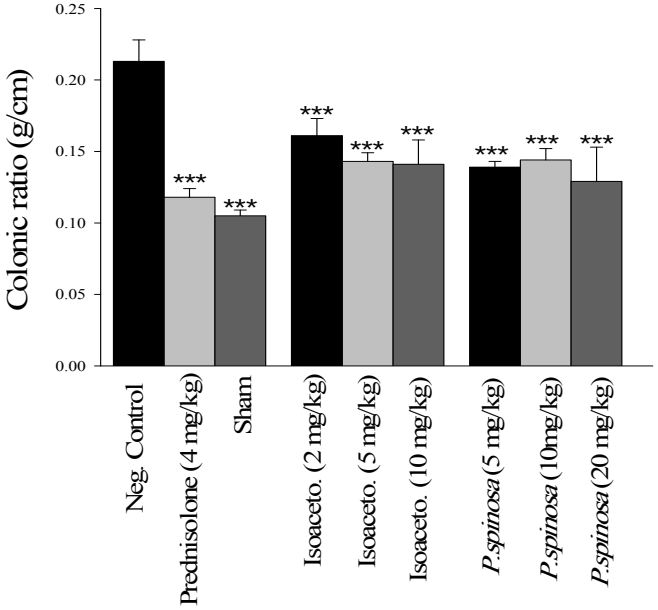

Fig. 5. Effect of oral administration of hydroalcoholic extract of *P. spinosa*, isoacetovanillone and prednisolone on colonic ratio in the rat colon 5 days after induction of colitis with acetic acid (%3)**.** Colonic ratio was calculated as the weight of wet tissue (g) over 8 cm of treated area of colon. Each value represents mean ± SEM (n=6). Stars show statistically significant difference in comparison with the negative control group (****P*<0.001). One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison *post hoc* test was used for statistical comparison of the data.

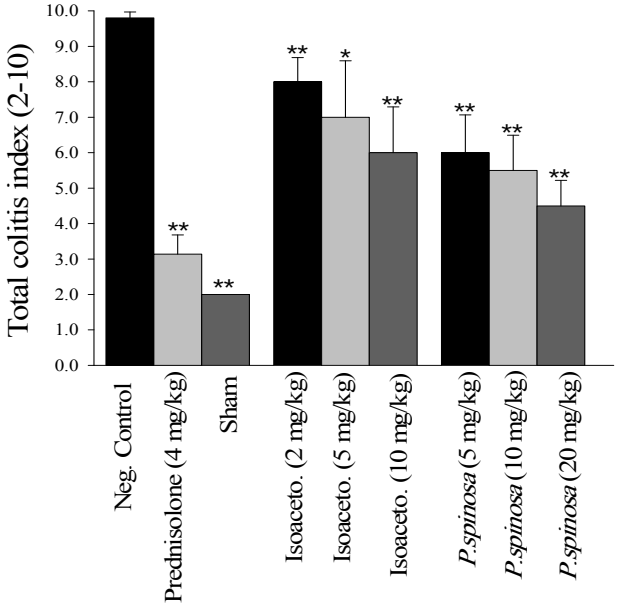

Fig. 6. Effect of oral administration of hydroalcoholic extract of *P. spinosa*, isoacetovanillone and prednisolone on microscopic and histopathological parameters of the rat colon on 5 days after induction of colitis with acetic acid (%3)**.** Each value has been represented as mean ± SEM (n=6). Stars shows statistically significant difference in comparison with the negative control group (**P*<0.05, ***P*<0.01). Comparison of the total colitis index was made by the Mann-Whitney *U* test.

DISCUSSION

The intestinal inflammation is histologically characterized by infiltration of polymorphonuclear leukocytes, monocytes and macrophages. They are activated by various mediators including prostaglandins, leukotrienes, platelet-activating factor and cytokines (37). MPO is an enzyme found in neutrophils and its activity in the colon is linearly related to the infiltration of neutrophils. The assessment of MPO activity is well established for quantification of intestinal inflammation (38). In colitis model, MPO is considered as a major enzyme responsible for tissue damage. Together with the membranous NADPH oxidase, MPO is involved in the formation of reactive oxygen species and oxidation of biological materials (38,39).

Acetic acid induced colitis is an easily inducible model of colitis, and has a good similarity to the inflammatory mediators profile in human intestinal inflammation (40,41). The protonated form of the acid liberates protons within the intracellular space and causes a massive intracellular acidification resulting in massive epithelial damage. It affects the distal colon portion and induces non-transmural inflammation, massive necrosis of mucosal and submucosal layers, mucosal edema, neutrophil infiltration of the mucosa and submucosal ulceration. The inflammatory response initiated by acetic acid includes activation of cyclooxygenase and lipooxygenase pathways (41-43). Prednisolone as an anti-inflammatory agent decreases the recruitment of macrophages in the affected area and suppresses the synthesis of many inflammatory mediators, e.g. production of IL-1 from monocytes, production of IL-2 and tumor necrosis factor from lymphocytes. Prednisolone also inhibits the phospholipase A_2 enzyme and thus decreases availability of prostaglandins and leukotrienes (44).

The results showed that *P. spinosa* extract has a good potential to suppress colitis in rat. Biochemical assays well indicated that administration of *P. spinosa* reduces MPO activity, which is established as an indicator of oxidative stress and marker of colitis (37-39). Interestingly, *P. spinosa* extract at a dose of 20 mg/kg was comparable with prednisolone (4 mg/kg) which showed significant protection against acetic acid-induced colitis.

Hydroalcoholic extract of *P. spinosa* has shown antispasmodic activity on isolated ileum and inhibits contraction induced by acetylcholine, serotonin, KCl or neuronal stimulation (16-18). The extract of *P. spinosa* contains various components including flavonoid substances (17,19). Flavonoids are a class of plant phenolics with significant antioxidant and chelating properties. Their positive effects come from their ability to inhibit lipid peroxidation, chelate redox-active metals and attenuating other procedure involving in production of reactive oxygen species (39,40).

At least four components are identified in *P. spinosa* extract (19). These include isoacetovanillone, isovanilline, 6-(4-hydroxy-3-methoxyphenyl)-hexanonic acid and 3,7,10,14,15-pentaacetyl-5-butanoyl-13,17-epoxy-8-myrsinene (18,19). All these substances possess antispasmodic activity and have reduced incidence of diarrhea induced by castor oil and sulphate magnesium (45). Furthermore, by inhibiting gut motility, these compounds have reduced ileum charcoal meal transit *in vivo* (46-47). As isoacetovanillone is a structural isomer of acetovanillone, we have used animal model of colitis to investigate if this compound possess anti-inflammatory activity.

Isoacetovanillone at doses which have been shown to have anti-diarrheal activity (2 mg/kg and 5 mg/kg) significantly reduced the incidence of colitis in rat. The improvement in the colonic inflammation is accompanied by a significant reduction in MPO activity. It is not clear if the inhibition of MPO activity is a direct affect on the enzyme or is an indirect action of the drug on other inflammatory mediator pathways which consequently reduces MPO activity. *P. spinosa* extract at similar doses reduced both colonic inflammation and MPO activity. There are several reports about anti-colitis activity of other plant extract (27,29,31,33-35). However, inhibition of colonic inflammation were seen with dose ranges of 200-800 mg/kg, while, *P. spinosa* was found to be effective with dose

range of 5-20 mg/kg. *P. spinosa* hydroalcoholic extract at similar doses (2-10 mg/kg) also shown anti-diarrheal activity in mice (46,48). Although this experiment supports the idea that the anti-colitis activity of *P. spinosa* extract could be due to the presence of isoacetovanillone but since the active doses of *P. spinosa* extract and isoacetovanillone are relatively close to each other, it is likely that other components in the extract may also play significant roles. One of these components could be 3,7,10,14,15-pentaacetyl-5-butanoyl-13,17-epoxy-8-myrsinene because it was found to be the most potent identified components in the extract of *P. spinosa* (19). Furthermore, it inhibits ileum spasm induced by KCl, serotonine, acetylcholine and neuronal stimulation like isoacetovanillone (48). Therefore, examination of this compound on rat induced colitis is suggested.

CONCLUSION

In conclusion, we have shown that both *P. spinosa* extract and isoacetovanillone possessed anti-inflammatory activity and prevented injuries due to administration of acetic acid in the colon. Therefore, the first concept that may come to mind is that *P. spinosa* possesses inhibitory effects on the synthesis or release of inflammatory mediators in colitis. Further development of *P. spinosa* extract and its active components for the treatment of colitis is recommended.

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