Original Research

The gastroprotective effect of pogostone from Pogostemonis Herba against indomethacin-induced gastric ulcer in rats

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Abstract

Pogostemonis Herba, known as ''Guang-Huo-Xiang'' in Chinese, has been widely used in the treatment of gastrointestinal dysfunction. Pogostone is one of the major constituents of Pogostemonis Herba. The aim was to scientifically evaluate the possible gastroprotective effect and the underlying mechanisms of pogostone against indomethacin-induced gastric ulcer in rats. Rats were orally treated with vehicle, lansoprazole (30 mg/kg) or pogostone (10, 20 and 40 mg/kg) and subsequently exposed to acute gastric lesions induced by indomethacin. Gross evaluation, histological observation, gastric mucosal superoxide dismutase activity, glutathione content, catalase activity, malonaldehyde level and prostaglandin E2 production were performed. Immunohistochemistry and reverse transcription polymerase chain reaction for cyclooxygenase-1 and cyclooxygenase-2, as well as terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling assay, immunohistochemistry for heat-shock protein 70, B-cell lymphoma-2 and Bax were conducted. Results indicated that rats pretreated with pogostone showed remarkable protection from the gastric mucosa damage compared to vehicle-treated rats based on the ulcer index and inhibition percentage. Histologically, oral administration of pogostone resulted in observable improvement of gastric injury, characterized by reduction of necrotic lesion, flattening of gastric mucosa and alleviation of submucosal edema with hemorrhage. Pogostone pretreatment significantly raised the depressed activities of superoxide dismutase, glutathione and catalase, while reduced the elevated malonaldehyde level compared with indomethacin-induced group. Pogostone-pretreated group induced a significant increase in gastric mucosal prostaglandin E2 level and obvious up-regulation of protein levels and mRNA expressions of cyclooxygenase-1 and cyclooxygenase-2. Furthermore, antiapoptotic effect of pogostone was verified by terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling assay, and the apoptotic process triggered by pogostone involved the up-expression of heat-shock protein70 and B-cell lymphoma-2 protein, and suppression of Bax protein expressions in the ulcerated tissues. It is speculated that the gastroprotective effect of pogostone against indomethacin-induced gastric ulceration might be associated with its stimulation of cyclooxygenase-mediated prostaglandin E2, antioxidant and antiapoptotic effect.

Keywords: Pogostone, gastroprotective, antioxidant, prostaglandin E2, antiapoptosis, indomethacin, rats

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Introduction

Peptic ulcer disease (PUD) is a common disorder of the entire gastrointestinal tract. This disease occurs mainly in the stomach and the proximal duodenum, which affects a large proportion of the world population.[1] Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most widely used drugs in the treatment of arthritis, inflammation, and cardiovascular protection.[2] However, NSAIDs'

application is commonly associated with the occurrence of varied adverse digestive events, including gastric mucosal ulceration, bleeding and perforation, as well as an increased risk of other severe complications. NSAIDs-induced gastric damage is the major side effect of this kind of drug.[3] Previous reports suggested that the pathophysiology of gastric injury evoked by NSAID was predominantly ascribed to their ability to reduce prostaglandin (PG) production through the inhibition of cyclooxygenase (COX).[4] PGs play important roles in modulating the mucosal integrity and various functions of the gastrointestinal tract, such as stimulating the secretion of bicarbonate and mucus, maintaining the blood flow of the mucosa, and regulating mucosal cell renewal.[5] Additionally, tissue oxidation, lipid peroxidation, and apoptosis or programmed cell death are believed to be other main factors that contribute to the pathogenesis of NSAID-induced gastric damage.[6,7]

Although several types of chemical drugs are used in the treatment of gastric ulcer, unfortunately, most of them exhibit potent side effects. Hence, the search for safe and effective gastroprotective agents is essential.[8] Medicinal plants are important sources of new agents with potential therapeutic effects. There has been renewed interest in identifying new antiulcer drugs from natural sources. And exploration of effective antiulcer agents derived from medicinal plants that may present less side effects are becoming more and more important as an alternate therapy for PUD.

Pogostemonis Herba, known as ''Guang-Huo-Xiang'' in Chinese, is the dried aerial part of *Pogostemon cablin* (Blanco) Benth (Labiatae).[9] Clinically, it has been widely used for the treatment of gastrointestinal disorders such as vomiting, diarrhea, nausea, and *H. pylori*-related gastritis in Philippines, Malaysia, India, and China.[10] Pharmacological studies exhibited that the Pogostemonis Herba has a variety of activities including anti-inflammatory,[11] antiemetic,[12] and antimicrobial[13] actions. Pogostone (PO, $C_{12}H_{16}O_4$, the chemical structure is shown in Figure 1) is the major constituent of Pogostemonis Herba. This compound has been demonstrated to exert anti-inflammatory[14] and antibacterial[15] activities. Moreover, our previous studies have also revealed that PO possessed gastroprotective effect against ethanol-induced gastric ulcers, which is attributable to its ability in improving the antioxidant and anti-inflammatory status, stimulating the prostaglandin E2 (PGE_2) content and preserving the non-protein sulfhydryl (NP-SH) level.[16] In an effort to further characterize the gastroprotective effect of PO, the present investigation was initiated to explore the potential involvement of gastric COX-mediated PGE_2 level, antioxidative as well as apoptosis-related parameters in indomethacin-induced gastric ulcerations.

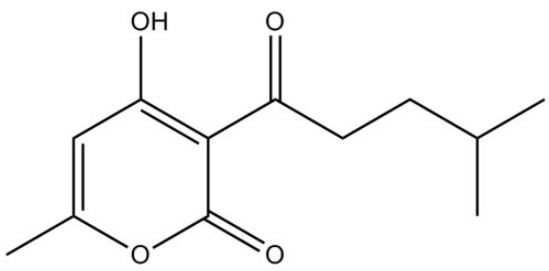

Figure 1 The chemical structure of pogostone (PO)

Materials and methods

Chemicals and reagents

Lansoprazole tablets were purchased from Tuobin Pharmaceutical Factory (Shantou, China) and indomethacin was obtained from Sigma-Aldrich. The ultrapure water was purified using a Milli-Q gradient water purification system (Millipore, Bedford, MA, USA). Superoxide dismutase (SOD), glutathione (GSH), catalase (CAT) and malonaldehyde (MDA) assay kits were purchased from Jiancheng Company (Nanjing, China). All other chemicals and reagents were of analytical grade.

Extraction and isolation of PO

The aerial parts of *P. cablin* were collected from Maoming, Guangdong province (China) in September 2013 and authenticated based on its microscopic and macroscopic characteristics by Prof. Xiaoping Lai at School of Chinese Materia Medica, Guangzhou University of Chinese Medicine, where a voucher specimen (no. 140110) was deposited.

PO was isolated from *P. cablin* as previously described.[17] The dried aerial parts of *P. cablin* (9.0 kg) were exhaustively extracted through water–steam distillation and the white PO crystal (372.8 mg, yield 0.0041%) was obtained by crystallization. Its chemical structure was identified by comparing its spectral data (MS, ^{1}H and ^{13}C-NMR) with those published previously,[18] and its purity was above 98% as determined by HPLC using a previously described method.[19]

Animals

Male Sprague-Dawley rats (6–7 weeks old, 200–250 g, SYXK(YUE)2013-0085) purchased from Laboratory Animal Center of Guangzhou University of Chinese Medicine (Guangzhou, China) were fed a certified diet with free access to tap water under an environmentally controlled condition ($22 \pm 2^{\circ}$C, relative humidity of $50 \pm 5\%$) with a 12 h light/dark cycle. Prior to experimentation, all rats were fasted for 24 h and housed in cages with raised floors of a wide mesh to prevent coprophagy. Animal experiments were performed in accordance with procedures approved by the Animal Experimental Ethics Committee of Guangzhou University of Chinese Medicine (dSPF 2014 021), and the experimental protocols followed the ''*Guide for the Care and Use of Laboratory Animals*''.[20]

Treatment of animals

Prior to the experiments, the rats were acclimatized for one week with standard laboratory diet and water. Male Sprague-Dawley rats were randomly grouped into six groups ($n = 6$) and then the control and vehicle groups received distilled water and vehicle, respectively,

throughout the course of the experiment. The prevention groups received lansoprazole (30 mg/kg) and different doses of PO (10, 20 and 40 mg/kg) dissolved in physiological saline for seven consecutive days before the treatment with indomethacin. The dose of PO was selected based on the previous investigation[16,21] and preliminary experiment.

The rats were fasted for 24 h with free access to drinking water prior to indomethacin administration. The rats ($n = 6$) were pretreated orally with distilled water (control), vehicle, lansoprazole (30 mg/kg), and PO (10, 20 and 40 mg/kg). After 1 h, all rats except the control group received indomethacin (50 mg/kg) to induce gastric ulcer. Six hours after indomethacin treatment, the animals were anesthetized and their stomachs were rapidly removed and opened along the greater curvature. Then the stomachs were rinsed with ice-cold saline to remove the gastric contents and blood clots in order to assess the extent of gastric damage. Thereafter, the stomachs were photographed and the ulcer area (mm^2) was measured using Image J software (developed by the National Institutes of Health, USA). The inhibition percentage was calculated using the following formula: ([Ulcer $Area_{(Vehicle)}$ − Ulcer $Area_{(Treated)}$]/Ulcer $Area_{(Vehicle)}$) × 100%. The stomach samples were scrapped after the scans and rapidly frozen at −80°C until histological slides and biochemical analyses.

Histological examination of gastric tissue

For histological assessment, samples of the stomach of each rat were fixed in 10% buffered formalin and embedded in paraffin. The stomach sections were cut to a thickness of 5 μm and stained with hematoxylin and eosin (H&E); the extent of mucosal ulceration, hyperemia, necrosis, edema, cellular infiltrate and goblet cell hyperplasia were observed microscopically.

Measurement of SOD, GSH, CAT activity, and MDA levels

After the macroscopic analyses, SOD, GSH and the CAT enzymatic activities, and the MDA levels in rat stomach tissues were determined. To prepare the tissue homogenates, stomach tissues were homogenized in homogenization Tris buffer (20 mM, pH 7.5) on ice using Ultra Turraks Homogenizer (IKA, Germany). Homogenates were filtered and centrifuged by a refrigerated centrifuge at 11,940 *g* at 4°C for 10 min. Then, these supernatants were used for the determination of activities of SOD and GSH, and the levels of CAT, MDA. The concentration of protein in the supernatants was obtained by the Bradford method using bovine serum albumin (BSA) as a standard. The activities of SOD and GSH, as well as the levels of CAT and MDA were determined using commercial assay kits according to the manufacturer's instructions (Jiancheng Company, Nanjing, China).

Determination of PGE_2

For measurement of the level of PGE_2 in the stomach tissue homogenate, the supernatant was determined by using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Abingdon, UK). The optical densities were measured at a wavelength of 450 nm and the results were expressed as ng/g protein.

Immunohistochemistry for COX-1 and COX-2

Slides of paraffin-embedded gastric tissues were deparaffinized in xylene and rehydrated in a graded ethanol series and then under running water for 5 min. The inhibition of endogenous peroxidase activity was followed by antigen retrieval using microwave heating. Slides were incubated with specific primary antibodies that recognized either COX-1 (dilutions 1:200) or COX-2 (dilutions 1:500). Overnight, sections were then incubated with biotinylated anti-mouse secondary antibody followed by peroxidase-labeled streptavidin-horse radish protein (HRP) complex and 3,3′-diaminobenzidine tetrahydrochloride (DAB). Specimens were counterstained with hematoxylin and examined under a light microscope. The mean value of integrated optical density (IOD) was calculated using Image-Pro Plus software (IPP, version 6.0, Media Cybernetics, Inc.).

Determination of COX-1 and COX-2 mRNA by RT-qPCR

Expressions of mRNA for COX-1 and COX-2 were determined using reverse transcription polymerase chain reaction (RT-PCR) as described previously.[21] Samples of gastric mucosa were immediately frozen in liquid nitrogen and stored at −80°C until RNA extraction. After the extraction of total RNA using RNAiso Plus Reagent (Takara, Japan), the concentration of RNA was checked at 260 nm, and the purity was measured by the A260/A280 ratio. Complementary DNA was synthesized from 5 μg total RNA using reverse transcription reagent kit with gDNA eraser (Takara BIO, Japan) following manufacturer's instructions. Expressions of COX-1 and COX-2 were performed in Applied Biosystems 7500 Real-Time PCR System (Life Technologies, USA) using SYBR® Premix Ex TaqTM II kit (Takara BIO, Japan) as recommended by the manufacturer. The sequences of the primers are listed in

Table 1 Primer sequences

Gene	Forward primer (5′ to 3′)	Reverse primer (3′ to 5′)
COX-1	CCCACCTTCCGTAGAACAGG	GAGCAACCCAAACACCTCCT
COX-2	CATTGACCAGAGCAGAGAGAT	CTTTCTCTCCTGTAAGTTCTT
GAPDH	CCTCGTCTCATAGACAAGATGGT	GGGTAGAGTCATACTGGAACATG

COX: cyclooxygenase; GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

Table 1. PCR cycling conditions was set at 1 cycle of predenaturation at 95°C for 30 s, 40 cycles at 95°C for 5 s and 58°C for 30 s. At last, melting curve program was added. The PCR data were analyzed by Applied Biosystems 7500 Real-Time PCR System software and the fold change in cDNA (target gene) relative to the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) endogenous control was calculated using $2^{-\Delta\Delta Ct}$ method. All the RT-PCR experiments were conducted strictly according to the MIQE guidelines.[22]

Terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling assay for apoptosis

The terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) assay was performed using an Apoptosis Detection Kit (Roche, Manheim, Germany) according to the manufacturer's instructions. In brief, dewaxed mucosal sections were digested with proteinase K for 30 min at room temperature. After endogenous peroxidase had been washed and inactivated with 3% H_2O_2 in methanol for 15 min at room temperature, the slides were rinsed with PBS and incubated with 50 μl of TUNEL reaction mixture containing TdT and dUTP in the humidified chamber for 60 min at 37°C. The slides were counter-stained lightly with Mayer Heamatoxylin. The numbers of TUNEL-positive cells were counted using IPP software.

Immunohistochemical staining for heat-shock protein 70, B-cell lymphoma-2 and Bax

The protein markers heat-shock protein (HSP) 70, B-cell lymphoma-2 (Bcl-2), and Bax were detected in the gastric tissues by immunohistochemistry staining according to the manufacturer's protocol (Santa Cruz Biotechnology, Inc. American). The tissue sections were heated at 60°C for 1 h in a hot air oven. The tissue sections were deparaffnized in xylene and dried with ethanol; 0.03% H_2O_2 solution was added to the stomach tissue biopsies incubation at 37°C for 10 min. After that, the antigens were recovered in 0.01 mol sodium citrate buffer (pH = 6) after boiling in a microwave. Positive findings of the immunohistochemical staining were observed as a brown staining under a light microscope.

Statistical analysis

All data were presented as mean ± SEM. Statistical analysis of data was performed with an SPSS 17.0 statistical package (IBM, USA). One-way analysis of variance (ANOVA) was used to analyze data differences among groups, followed by Dunnett's significant *post-hoc* test. Differences were considered as statistically significant when $P < 0.05$.

Results

Gross evaluation of gastric lesions

Ulcerative indices and gastroprotection percentage were determined in rats with indomethacin-induced ulcers by measuring ulcerative lesion area. The results are shown in Figures 2 and 3. As expected, there were no macroscopic or microscopic lesions in the control rats. The vehicle-treated group presented severe mucosal injury with ulcer area of $24.22 \pm 1.31\,mm^2$ ($P < 0.01$). A significant decrease ($P < 0.01$) in the ulcer area was demonstrated in the

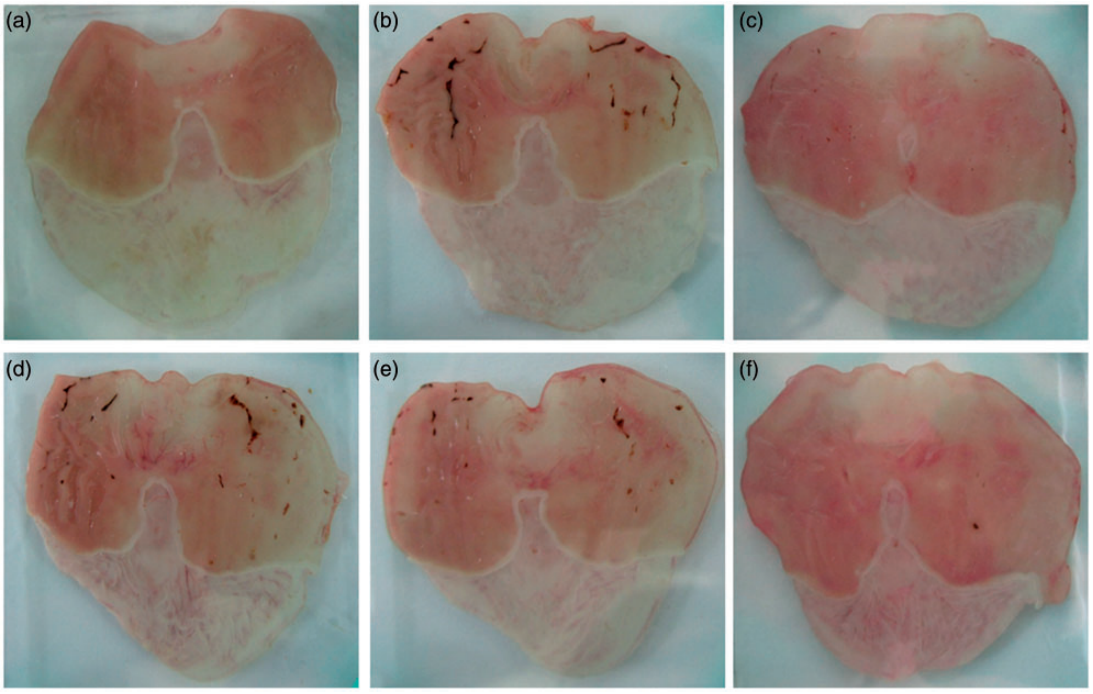

Figure 2 Effect of PO on the macroscopic appearance of the gastric mucosa in indomethacin-induced gastric lesions in rats. (a) control gastric mucosa; (b–f) indomethacin-induced ulcer with different degrees of hemorrhagic bands; (b) vehicle; (c) lansoprazole 30 mg/kg; (d–f) PO of different doses (10, 20, and 40 mg/kg). (A color version of this figure is available in the online journal.)

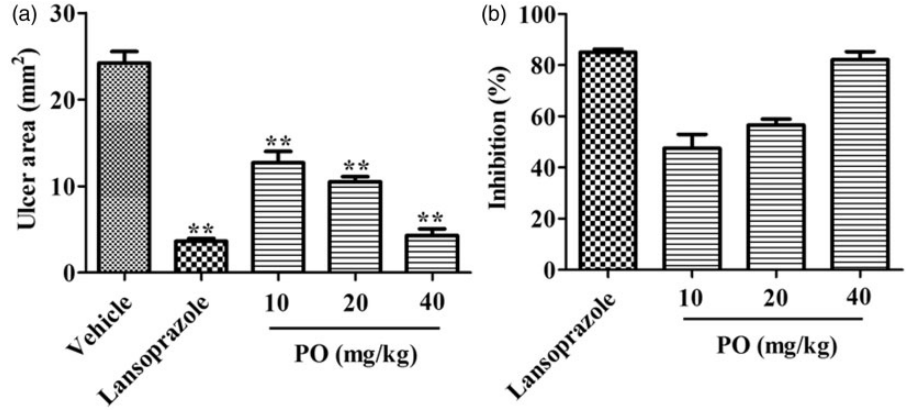

Figure 3 (a) Gastric ulcer area (mm^2) of rat stomachs ($n = 6$) induced by indomethacin after pre-treatment with vehicle, lansoprazole (30 mg/kg) or PO (10, 20, 40 mg/kg). The results were expressed as mean ± SEM and analyzed by ANOVA followed by Dunnett's test. *$P < 0.05$, **$P < 0.01$ versus the vehicle group. (b) Ulcer inhibition (%). PO: pogostone

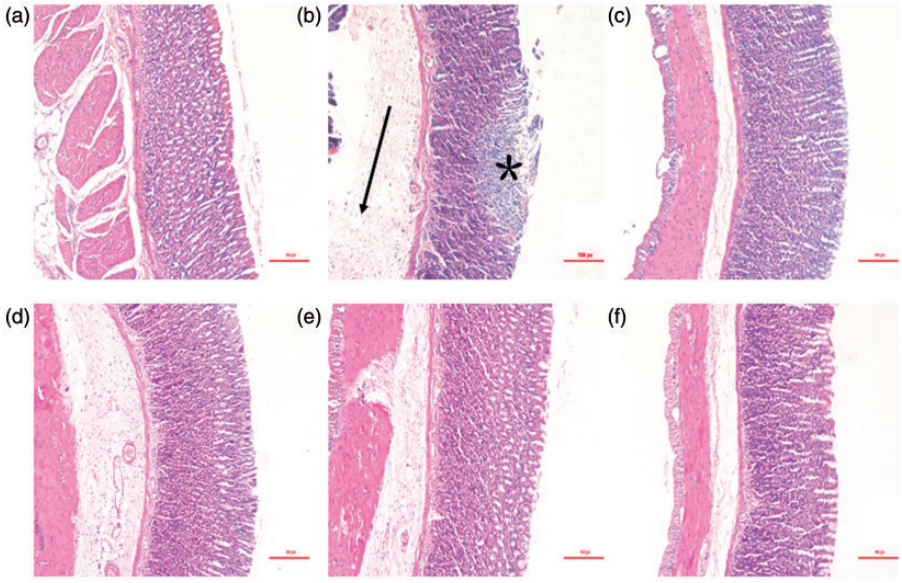

Figure 4 Effect of PO on histological evaluation in indomethacin-induced ulcer (HE staining; magnification 100 ×). (a) control stomach: Control gastric epithelium with organized glandular structure and normal submucosa; (b–f) indomethacin-induced ulcer; (b) Rats pretreated with vehicle: *indicates damaged mucosal epithelium with disrupted glandular structure, and arrow depicts edema of submucosa and inflammatory infiltrate; (c) lansoprazole (30 mg/kg); (d) PO 10 mg/kg; (e) PO 20 mg/kg; (f) PO 40 mg/kg. (c), (d), (e) and (f) show a recovery in mucosal epithelium and reorganized glandular structure as well as improvement of edema under treatment with lansoprazole or PO, respectively. (A color version of this figure is available in the online journal.)

lansoprazole-treated group with an average of $3.63 \pm 0.32\,mm^2$ ($85.01 \pm 1.31\%$ inhibition). For the PO-pretreated groups (10, 20 and 40 mg/kg), the ulcer area was significantly decreased ($P < 0.01$) in a dose-dependent manner, with the minimum ulcer area ($4.31 \pm 0.76\,mm^2$) and the highest inhibition rate ($82.20 \pm 3.13\%$) was observed at the dose of 40 mg/kg. Treatment with 20 and 10 mg/kg PO resulted in an average gastric ulcer area of $10.51 \pm 0.58\,mm^2$ (inhibitory rate $56.61 \pm 2.41\%$) and $12.70 \pm 1.31\,mm^2$ (inhibitory rate $47.56 \pm 5.39\%$), respectively.

Histological evaluations of gastric lesions

Results of histological analyses of the gastric mucosa by H&E staining are depicted in Figure 4. Some necrotic lesions, extensive edema of the submucosal layer, and a severe disruption of the gastric mucosal epithelium lining were displayed in the indomethacin-exposed group (Figure 4(b)). Gastric lesions were erosive and ulcerative. In rats treated with indomethacin and PO, the stomach histopathology improved and showed some mild gastric mucosal injury with less superficial disruption of the gastric epithelial lining, reduced amounts of erosive lesions, minor lesions of hemorrhagic necrosis and edema in the gastric mucosa. Of which, pretreatment with 40 mg/kg PO exhibited remarkable protection via reduction of the gastric ulcer area and submucosal edema, which was comparable to the reference drug lansoprazole. Overall, tissues that received pretreatment with PO had

comparatively better protection of the gastric mucosa in a dose-dependent manner.

Antioxidant activity

Reactive oxygen species (ROS) are implicated in the mechanism of acute and chronic ulceration in gastric mucosa, and scavenging these free radicals is one of the mechanisms implicated in the prevention of gastric ulcers.[23,24] In order to explore the potential involvement of antioxidant defenses in the gastroprotective property of PO, the antioxidant activities (SOD, CAT, and GSH) and lipid peroxidation levels (MDA) were probed. The data are summarized in Table 2. The vehicle group significantly (all $P < 0.01$) decreased the gastric mucosal activities of SOD, CAT, and GSH and increased MDA levels as compared to control rats, thus eliciting the oxidative damage. However, pretreatment with PO (10, 20 and 40 mg/kg) alleviated the oxidant status by significantly augmenting endogenous SOD ($P < 0.05$, $P < 0.01$ and $P < 0.01$), GSH (all $P < 0.01$) and CAT ($P < 0.05$, $P < 0.01$ and $P < 0.01$) activities, and diminishing the constituent MDA level (all $P < 0.01$) in the gastric mucosa in a dose-dependent manner, respectively, as compared with the vehicle group. The maximal effect was observed at the dose of 40 mg/kg.

Effects of PO on PGE_2 levels

The main importance of the systemic effects of NSAIDs, in terms of inducing gastric ulceration, is their ability to suppress PG synthesis. PGs play important roles in modulating the mucosal integrity and various functions of the gastrointestinal tract.[5] Based on this consideration, an experiment was performed to examine the possible effects of PO and lansoprazole on gastric PGE_2 content. As shown in Figure 5, gastric mucosal PGE_2 content was significantly decreased by pretreatment with indomethacin compared with the control group ($P < 0.01$). However, pretreatment with PO at dose of 10, 20, and 40 mg/kg markedly promoted the increment of gastric mucosal PGE_2 levels to 58.65 ± 0.95, 64.90 ± 0.87 and 67.65 ± 1.95 ng/g in a dose-related manner ($P < 0.05$, $P < 0.01$ and $P < 0.01$, respectively), when compared with the vehicle group (49.70 ± 1.71 ng/g).

Expression of COX-1 and COX-2 proteins

The NSAIDs exert both their therapeutic and toxic effects mainly by decreasing levels of circulating PGE at the gastric mucosa via inhibition of COX isozymes.[25] COX-1 and COX-2 are key enzymes in the biosynthesis of PGs and positively maintain gastric mucosal integrity. The immunohistochemical stainings of COX-1 and COX-2 expressions in the gastric mucosa of different groups are shown in Figure 6. The immunohistochemistry of normal gastric tissues showed up-regulated COX-1 expression and a relatively lower expression of COX-2. Indomethacin administration decreased ($P < 0.01$) the expressions of COX-1 and COX-2 by 60% and 62%, respectively, compared to the normal group. However, pretreatment with PO significantly increased both COX-1 (1.36-fold, $P < 0.05$; 1.77-fold, $P < 0.01$ and 2.25-fold, $P < 0.01$) and COX-2 (1.30-fold, $P < 0.05$; 1.35-fold, $P < 0.05$ and 2.19-fold, $P < 0.01$) expressions, compared to those of the vehicle group.

Expression of COX-1 and COX-2 mRNA

Expression of mRNA for COX-1 and COX-2 was determined using RT-PCR as described previously. The assays show that both COX-1 and COX-2 mRNA were suppressed after administration with indomethacin and significant down-regulations of transcript COX-1 and COX-2 expressions (1.69-fold and 3.56-fold, respectively) were observed. However, in rats pretreated with PO, COX-1 and COX-2 mRNA expressions were significantly enhanced (1.24-fold, $P < 0.05$; 1.47-fold, $P < 0.05$; 1.55-fold, $P < 0.05$; and 1.88-fold, $P < 0.01$; 2.43-fold, $P < 0.01$; 2.70-fold, $P < 0.01$, respectively), in parallel to vehicle-treated counterpart, which was even comparable to lansoprazole treatment (Figure 7). These findings collaborated with the cellular expressions of COX-1 and COX-2 proteins as detected by immunohistochemistry.

Apoptosis of stomach tissue cells

Apoptosis plays an important part in the cycle of cellular turnover in the gastrointestinal tract.[26] The detection of gastric epithelial apoptosis was detected by TUNEL assay. As shown in Figure 8, the presence of apoptotic cells in the surface epithelium was observed occasionally in the control group (0.24 ± 0.02). Exposure to indomethacin resulted in a

Table 2 Effect of PO on SOD, GSH, CAT and MDA levels in the stomach tissue of rats ($n = 6$) with or without indomethacin

Group	Dose (mg/kg)	SOD (pg/mg protein)	GSH (pg/mg protein)	CAT (pg/mg protein)	MDA (pg/mg protein)
Control	–	50.84 ± 2.19	47.80 ± 2.69	36.94 ± 3.74	7.13 ± 0.73
Vehicle	–	29.09 ± 0.99^a	21.52 ± 0.83^a	9.49 ± 0.61^a	14.25 ± 0.61^a
Lansoprazole	30	45.75 ± 1.13^b	41.57 ± 2.81^b	24.23 ± 0.87^b	8.74 ± 0.35^b
PO	10	34.58 ± 0.54^c	30.34 ± 1.09^b	14.53 ± 1.06^c	10.79 ± 0.48^b
PO	20	40.81 ± 1.61^b	35.37 ± 2.16^b	18.70 ± 1.37^b	8.69 ± 0.88^b
PO	40	47.77 ± 2.31^b	38.59 ± 2.37^b	29.05 ± 2.23^b	7.70 ± 0.66^b

PO: pogostone; SOD: superoxide dismutase; GSH: glutathione; CAT: catalase; MDA: malonaldehyde.
The results were expressed as mean ± SEM and analyzed by ANOVA followed by Dunnett's test.
[a] $P < 0.01$: significantly different from the control group.
[b] $P < 0.01$: from the vehicle group.
[c] $P < 0.05$.

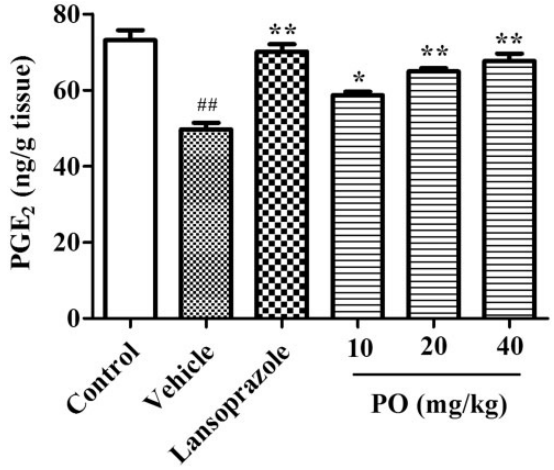

Figure 5 Effect of PO on gastric tissue homogenate content of prostaglandin E2 (PGE_2) level of rat stomachs ($n = 6$) induced by indomethacin after pretreatment with vehicle, lansoprazole (30 mg/kg) or PO (10, 20, 40 mg/kg). The results were expressed as mean ± SEM and analyzed by ANOVA followed by Dunnett's test, $^{\#\#}P < 0.01$: significantly different from the control group and $^{*}P < 0.05$, $^{**}P < 0.01$: from the vehicle group.
PO: pogostone

significant increase of apoptotic ratio (1.00 ± 0.36, $P < 0.01$), while pretreatment with PO markedly suppressed the DNA fragmentation in a dose-related manner, with respective to the vehicle group (0.62 ± 0.08, 0.39 ± 0.05, 0.29 ± 0.03, all $P < 0.01$).

Immunohistochemical staining of HSP70, Bcl-2 and Bax

It was previously reported that apoptosis or programmed cell death is one of the main factors that contributes to the gastric ulcer formation. Blocking of apoptotic cell death is an important mechanism implicated to control gastric lesions.[27] HSP70 is crucial for the maintenance of cell integrity during normal cellular growth, as well as during pathophysiological conditions.[28] In an intention to gain further insight into the mechanism of healing of the gastric ulcer promoted by PO, the behavior of PO in the gastric ulcer was also examined for expression of HSP70, Bcl-2, and Bax proteins by immunohistochemical analysis. The results are shown in Figure 9. Immunohistochemistry showed up-regulation of HSP70 and Bcl-2 proteins when pretreated with lansoprazole or PO, in comparison to vehicle group (all $P < 0.01$), while Bax protein displayed the opposite

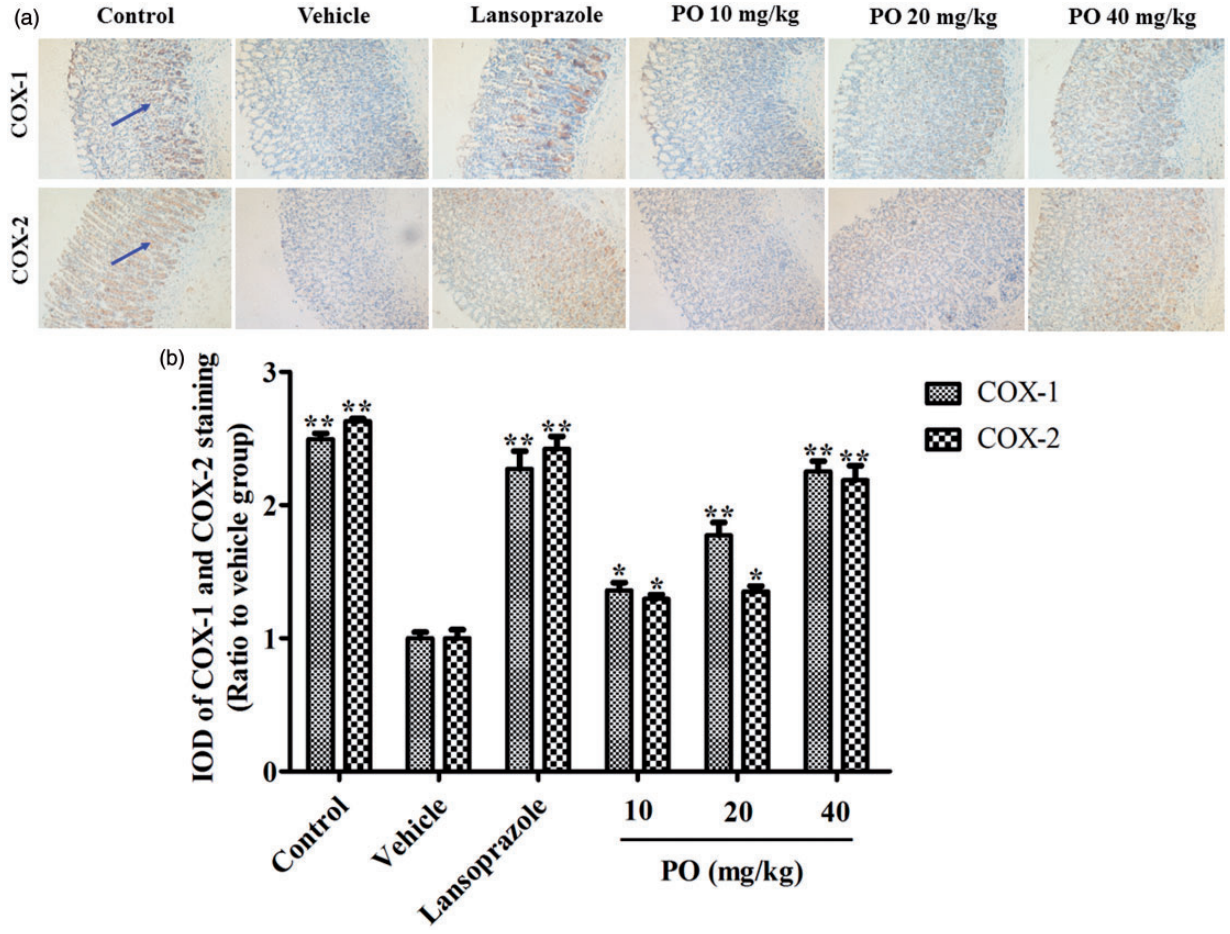

Figure 6 (a) Effect of PO on the immunohistochemistry analysis of the expression of COX-1 and COX-2 enzymes (200 ×) in the stomachs of rats in indomethacin-induced gastric ulcer. First row: immunohistochemistry staining of COX-1 protein; second row: immunohistochemistry staining of COX-2 protein. The blue arrow points to COX protein accumulation. (b) The statistical results were calculated according to the integral optical density (IOD). The results were expressed as mean ± SEM and analyzed by ANOVA followed by Dunnett's test. $^{*}P < 0.05$, $^{**}P < 0.01$ versus the vehicle group. (A color version of this figure is available in the online journal.)
PO: pogostone; COX: cyclooxygenase

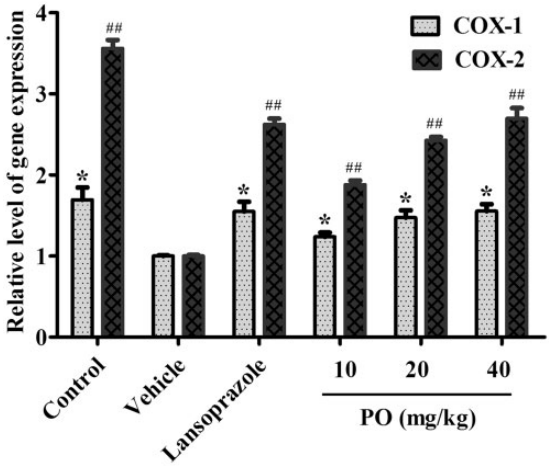

Figure 7 Effects of PO on mRNA expression of COX-1 and COX-2 in gastric mucosa of rats ($n = 6$) submitted to gastric ulcer induced by indomethacin. The results were expressed as mean ± SEM and analyzed by ANOVA followed by Dunnett's test. $^{*}P < 0.05$, $^{\#\#}P < 0.01$ versus the vehicle group.
PO: pogostone; COX: cyclooxygenase

result in those groups, which experienced significant down-regulation compared with the vehicle group ($P < 0.05$, $P < 0.01$ and $P < 0.01$).

Discussion

Gastric ulcer is the most common global disorder of the gastrointestinal tract, with increasing incidence and prevalence. Owing to the undesired effects of modern drugs, the concentration toward medicinal plant products for the treatment of gastrointestinal diseases is gaining increasing importance around the world. Pogostemonis Herba has been widely used for the treatment of functional gastrointestinal disturbance. In our previous study, we have shown that PO exhibited gastroprotective effects against ethanol-induced lesions of rat gastric mucosa, and the gastroprotective effect was associated with its effects on stimulation of PGE_2, improvement of antioxidant and anti-inflammatory status, as well as preservation of NP-SH.[16] Furthermore, oral administration of PO in female mice gave an LD_{50} value of 355 mg/kg,[17] and intravenous administration of an escalating dose of PO in the male mice produced an LD_{50} value of 163 mg/kg,[14] indicating a relatively safety profile. Patchouli alcohol (PA), another major ingredient of Pogostemonis Herba, was reported to exert protection of gastric mucosa against injury against ethanol- and indomethacin-induced gastric ulcers in rats.[21]

In the present study, we aimed to evaluate the possible gastroprotective effect of PO against indomethacin-induced gastric damage in rats. Here, we demonstrated that indomethacin exerted injuring effect on the epithelium, leading to the formation of characteristic necrotic lesions. It was observed that the total gastric lesion areas were significantly reduced ($P < 0.01$) in a dose-dependent manner by repeated administration of PO at 10, 20, and 40 mg/kg in contrast with the indomethacin-treated group. Histopathological staining further confirmed the ability of PO to prevent indomethacin-induced gastric damage in less severe histopathological alterations compared to the control group. The results obtained in histopathological analysis displayed that indomethacin administration caused the development of gastric mucosal injuries characterized by hemorrhage, mucosal edema, and epithelial cell loss, whereas pretreatment with PO was able to inhibit such alterations. Based on these results, macro- and microscopic evaluations indicate that PO possessed gastroprotective potential in NSAID-induced ulcer model.

It has been established that the activation of tissue oxidation is one of the most important detrimental effects evoked by NSAIDs in the digestive system. In particular, indomethacin-induced gastric ulcer is a multifactorial process of which ROS plays a vital role in gastric damage either by its direct oxidative action[29,30] or through apoptotic cell death. Antioxidants have been observed to protect gastric mucosa from ulceration.[31] Preventive antioxidants such as SOD, CAT, and GSH are amongst the first line of defense against ROS. SOD is one of the most effective intracellular enzymatic antioxidants, and it acts by catalyzing the dismutation of superoxide into oxygen and hydrogen peroxide.[32] GSH as an endogenous antioxidant is important for the maintenance of mucosal integrity, and its depletion in the gastric mucosa induces macroscopic mucosal ulceration.[33] CAT is another enzymatic antioxidant, which is principally present in peroxisomes and coverts H_2O_2 to water and non-reactive oxygen species.[34] MDA, a by-product of lipid peroxidation, is regarded as a reliable index of oxidative tissue damage.[24]

Our preceding exploration demonstrated that prior administration of PO could remarkably increase SOD, GSH, and CAT activities, and appreciatively reduce the levels of MDA in the ethanol-induced gastric ulcer and consequently resulting in diminished gastric lesions.[16] In the present study, our finding demonstrated that pretreatment with PO significantly alleviated oxidative stress by reducing levels of MDA and restoring depressed cellular SOD, GSH, and CAT activities, thereby leading to reduced oxidative stress and elevated total antioxidant status in indomethacin-induced gastric ulcer. These results suggest that the activation of antioxidant mechanisms might contribute favorably to the ulcer attenuation of PO. Taken together, these findings support a significant role played by the antioxidant actions of PO in counteracting the gastric damage induced by ethanol or NSAID administration. It is also worthy to mention that, PA also exerted the gastroprotective property partly by inhibiting oxidative gastric damage. Earlier evidence suggested that the effect of Pogostemonis Herba to preserve mitochondrial action during oxidative stress might be a pivotal mechanism obligatory for its protective effect against cell death.[35] Based on these considerations, it was implied that PO and PA might be important contributors responsible for the ROS-scavenging effect of Pogostemonis Herba.

Indomethacin, a non-steroidal anti-inflammatory drug (NSAID), causes gastric ulcers mainly by inhibiting COX-mediated PGs synthesis. PGE_2 plays a vital role in the integrity maintenance of gastric mucosal defense by increasing of mucus secretion and stimulating bicarbonate anions

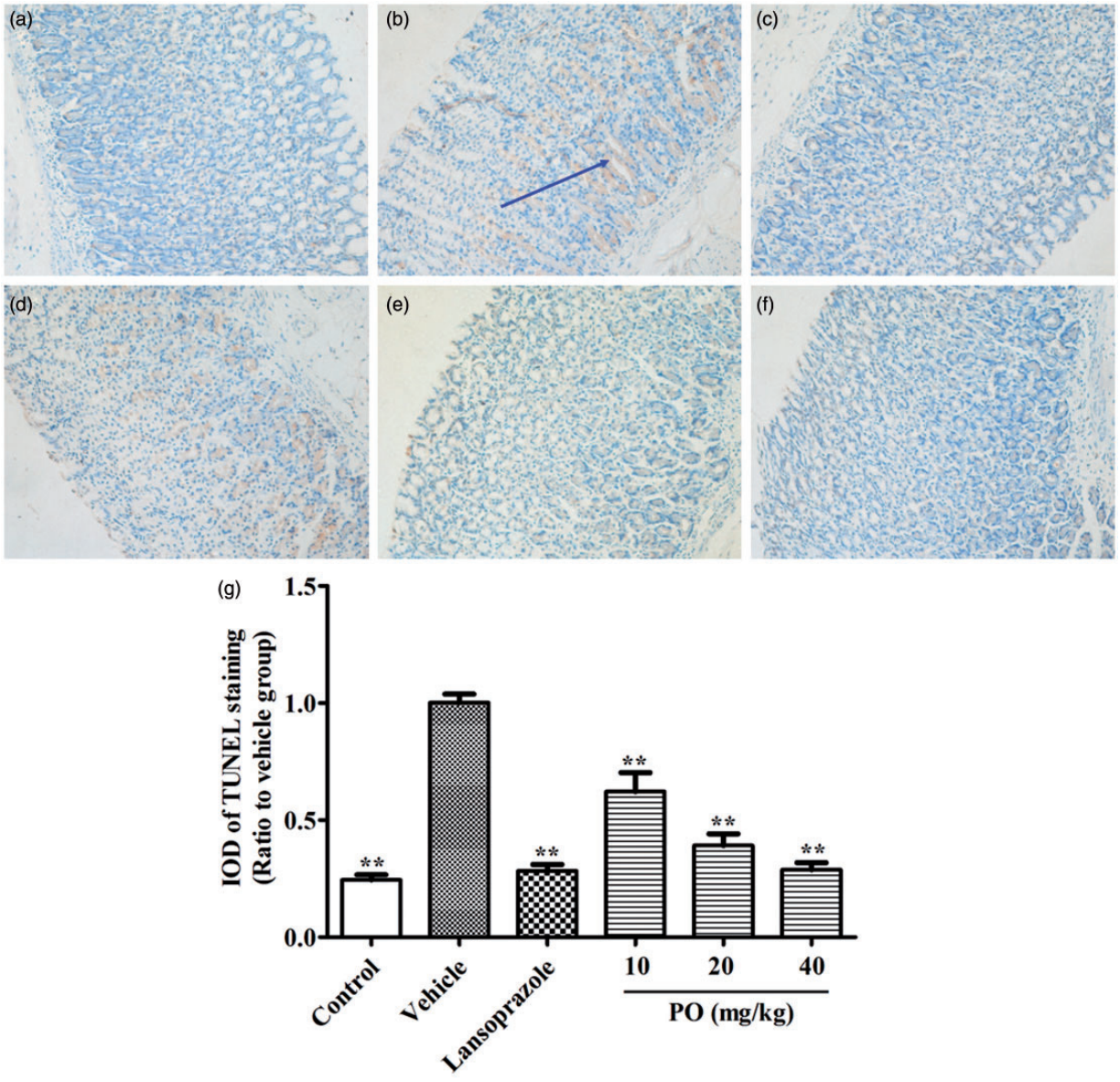

Figure 8 TUNEL staining of apoptotic stomach tissue in the six groups (200 ×). (a) control group; (b–f) indomethacin-induced ulcer: (b) Rats pretreated with vehicle: the blue arrow points to positive TUNEL staining; (c) lansoprazole (30 mg/kg); (d) PO 10 mg/kg; (e) PO 20 mg/kg; (f) PO 40 mg/kg. (g) The statistical results were calculated according to the integral optical density (IOD). The results were expressed as mean ± SEM and analyzed by ANOVA followed by Dunnett's test. *$P < 0.05$, **$P < 0.01$ versus the vehicle group. (A color version of this figure is available in the online journal.)
PO: pogostone

production, which neutralizes acidic gastric content and increases mucosal blood flow.[36] In this study, as expected, mucosal PGE_2 production was markedly suppressed ($P < 0.01$) in indomethacin-induced rats compared with the control group. While repeated administration of PO (40 mg/kg) substantially promoted the mucosal PGE_2 levels ($P < 0.01$). Considering that indomethacin-induced ulcerations are mainly attributed to the inhibition of PGE_2 synthesis, these results suggest the pivotal role of PGE_2 in the gastroprotection offered by PO against the ulcerogenic effect of indomethacin.

COX is the key enzyme that catalyzes PG biosynthesis and exists in two isoforms, namely COX-1 and COX-2 isoforms. COX-1 is the enzyme that provides the dominant source of PG synthesis in the stomach, and COX-2 also conduces to the glandin-mediated mucosal defense. Gastric ulcerogenic effects of NSAIDs are not only mediated by the inhibition of COX-1, but also require the inhibition of COX-2.[37] COX-2 compensates the temporary loss of COX-1 occurring in the mucosa and safeguards gastric mucosal integrity, playing an essential role in the healing of gastric ulcers; therefore, COX-2 inhibition results in delayed ulcer healing.[38] According to our previous work, PA was demonstrated to exert gastroprotective effect against indomethacin-induced gastric ulcers in rats, which might be partly attributed to up-regulation of COX-1 and COX-2 mRNA expression.[21] In the current work, PO pretreatment induced an increment of both mRNA and protein expressions of

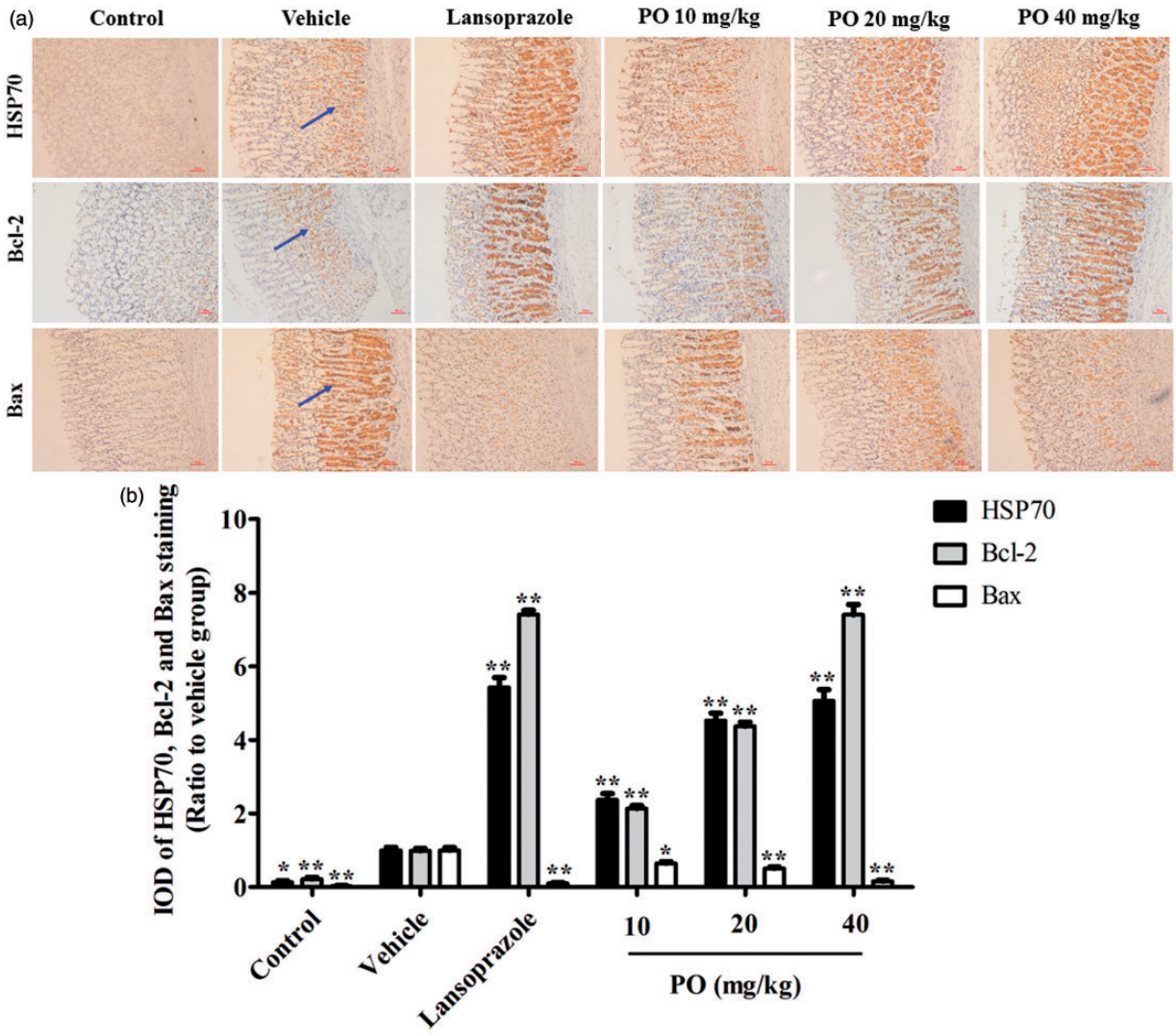

Figure 9 (a) Effect of PO on the immunohistochemistry analysis of the expression of HSP70, Bcl-2 and Bax protein expressions (200 ×) in the stomachs of rats in indomethacin-induced gastric ulcer. First row: immunohistochemistry staining of HSP70 protein; second row: immunohistochemistry staining of Bcl-2 protein; third row: immunohistochemistry staining of Bax protein. The blue arrow points to protein accumulation. (b) IOD of HSP70, Bcl-2 and Bax protein expressions (ratio to the vehicle group) in the stomachs of rats in indomethacin-induced gastric ulcer. The results were expressed as mean ± SEM and analyzed by ANOVA followed by Dunnett's test. $^{*}P < 0.05$, $^{**}P < 0.01$ versus the vehicle group. (A color version of this figure is available in the online journal.)
PO: pogostone; HSP: heat-shock protein; Bcl-2: B-cell lymphoma-2

gastric COX-1 and COX-2 post indomethacin administration, possibly contributing to the maintenance of gastric PGE_2, and therefore resulting in gastric mucosa protection. Herewith, it is conceivable that COX-mediated PGE_2 biosynthesis might take a significant part in the beneficial effect of PO in the healing of ulcerative damage elicited by indomethacin.

Acute and chronic gastric ulcers in rats were observed to be associated with HSP70 induction.[39] HSPs contribute to mucosal protection and ulcer healing by regulating the activity of enzymes such as COX and nitric oxide synthase,[40] as well as by increasing mucosal blood flow.[41] The over-expression of HSPs is supposed as a major indicator of the protective effect of many agents against oxidative damage.[42] In the present investigation, PO and lansoprazole were able to exhibit the up-regulation of HSP70 proteins and counteract the detrimental effects of this NSAID on ulcer healing. This observation led us to hypothesize that the induction of HSP70 protein synthesis might play an essential role in the beneficial effects of PO on mucosal injury, possibly associated with the alleviation of ROS-mediated gastric oxidative stress.

Apoptosis and proliferation are fundamental mechanisms for cell death and survival and differentiation in the gastric mucosa. Gastric mucosa depends on the balance between cell death and cell renewal, and gastric lesion is responsible for the increment of apoptosis and/or inhibition of cell proliferation.[43] The antiapoptotic effect of PO was first investigated by TUNEL assay. As indicated by the results, indomethacin administration led to significant increment of TUNEL-positive cells. However, for rats subjected to PO pretreatment, the presence of apoptotic cells

and nuclear DNA fragmentations in stomach tissue decreased significantly. These findings suggest that inhibition of apoptosis might contribute to the protective effect of PO against indomethacin-induced gastric ulcer in rats.

The Bcl-2 family proteins are the main organizers of apoptotic cell death, which inhibit apoptosis and facilitate cellular survival and differentiation, whereas the Bax protein promotes the apoptosis process and inhibits the function of Bcl-2. In many experimental ulcer models, apoptosis stems from the disturbance in the equilibrium of antiapoptotic Bcl-2 and pro-apoptotic Bax proteins.[27] In order to elaborate the possible role involved in the antiapoptotic effect of PO on indomethacin-induced gastric mucosal damage, as possible mechanism contributing to its healing action, the expressions of biochemical indicators of apoptosis Bcl-2 and Bax proteins were detected by immunohistochemistry. Results indicate that pretreatment with PO resulted in a remarkably enhanced expression of Bcl-2 protein. By contrast, the immunostained localization of the pro-apoptotic Bax protein in PO or lansoprazole pretreated rats was down-regulated substantially when compared with the vehicle group (Figure 9). Based on this observation, it might be suggested that antiapoptotic effect was possibly implicated in the gastroprotective activity of PO against indomethacin-induced gastric tissue injury.

Conclusion

In conclusion, PO was demonstrated to exert gastroprotective effect against indomethacin-induced gastric injury, and the mechanisms of this protective effect were potentially associated with stimulation of COX-mediated PGE_2, enhancement of the cellular antioxidant mechanism by replenishing gastric SOD, CAT and GSH levels along with reducing lipid peroxidation, the depression of mucosal apoptosis as well as activation of HSP70. The findings of the present investigation further provide a scientific support towards the traditional uses of Pogostemonis Herba in treating stomach disease, and broaden our understanding of the potential application of PO as a safe and effective alternative/complementary to conventional medication in treating gastric ulcer.

Authors' contributions: XYC and HMC contributed equally to this work. ZRS, XPL and HMC designed and conceived the study. ZBZ, YFZ, ZQS, YZL and XZ performed the experiments. YHL, LDF and XQH analyzed the data. XYC and JHX wrote the manuscript. All authors have read and approved the final manuscript.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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