

Original Research Article

Gastroprotective effect of the root extract of *Alpinia officinarum* Hance (Zingiberoside) against acute indomethacin-induced gastric injuries in rats: Involvement of H⁺/K⁺-ATPase and prostaglandin E receptors

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Abstract

Purpose: To investigate the protective effects of *Alpinia officinarum* root ethanol extract (AOE) and galangin against acute indomethacin-induced injury on rat gastric mucosa

Methods: Sprague-Dawley rats were daily treated with bismuth potassium citrate (0.08 g/kg), AOE at doses of 0.09, 0.18 and 0.36 g/kg; and galangin (0.2 g/kg) for 15 days. Then, gastric injury on rats was induced by intragastric administration of indomethacin (30 mg/kg). Blood flow and thickness of gastric mucosa were determined using neutral red clearance test and Alcian blue staining. The activity of H⁺/K⁺-ATPase was assayed using a biochemical kit. Prostaglandin E receptor expressions were assayed by western blotting.

Results: High doses of ethanol extract of *Alpinia officinarum* root significantly inhibited H⁺/K⁺-ATPase activity by 8.12 % ($p < 0.01$), increased gastric mucosal blood flow ($p < 0.001$), enhanced mucus thickness ($p < 0.05$), and elevated the activities of prostaglandin E receptors 1 and 4 ($p < 0.05$). Galangin significantly inhibited H⁺/K⁺-ATPase activity by 4.82 % ($p < 0.05$) and increased gastric mucosal blood flow ($p < 0.01$).

Conclusion: The ethanol extract of *Alpinia officinarum* root attenuates indomethacin-induced gastric injury by reinforcing gastric mucosal barrier and inhibiting excessive gastric acid secretion. Thus, the extract can be potentially developed for management of gastric injuries.

Keywords: Galangin, Gastric mucosal barrier, Gastric acid, Prostaglandin, Indomethacin

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INTRODUCTION

The worldwide use of nonsteroidal anti-inflammatory drugs (NSAIDs) has increased the incidence of gastric injury [1,2]. It is generally

known that reinforcing gastric mucosal barrier and inhibiting excessive gastric acid secretion are crucial strategies in ameliorating gastric ulcer induced by NSAIDs [3]. Prostaglandin E₂ (EP) receptors are closely related to the regulation of

mucosal defense and vascular perfusion [4]. Gastric acid secretion is closely associated with the hydrogen/potassium-adenosine triphosphatase (H^+/K^+ -ATPase) [5]. Thus, maintenance of activities of prostaglandin E_2 receptors while attenuating H^+/K^+ -ATPase activity have become important therapeutic strategies in the treatments of gastrointestinal ulcers or gastroesophageal reflux disease.

In a previous study, it was reported that the *Alpinia officinarum* Hance (Zingiberaceae family) root extract (AOE) markedly reversed gastric injury caused by indomethacin by increasing COX-1 activity while inhibiting COX-2 activity [6]. The ameliorated gastric mucus conditions and markedly increased VEGF levels in AOE treatment group led to a hypothesis that AOE may also reinforce the gastric mucosal barrier by increasing gastric blood flow and maintaining mucosal integrity via prostaglandin E_2 receptors. Furthermore, some flavonoids, like baicalein, myricetin, and flavonoid-rich extracts have been reported to inhibit H^+/K^+ -ATPase activity [7-9]. Moreover, AOE is rich in flavonoids with 11.8% galangin and 2.3% kaempferide. Therefore, another hypothesis was developed that AOE may decrease gastric acid secretion, by inhibiting H^+/K^+ -ATPase activity.

To get a better understanding of the gastroprotective mechanisms of AOE, an indomethacin-induced acute gastric damage rat model was established. In this model, AOE was given to healthy rats for 15 consecutive days before indomethacin treatment. Blood flow and gastric mucus thickness were evaluated, in addition to assay of activity of H^+/K^+ -ATPase and expressions of EP receptors.

EXPERIMENTAL

Agents and drugs

The ethanol extract of roots of *Alpinia officinarum* prepared in the authors' laboratory contained five major constituents: 21.1 % DPHC (cas: 24192-01-6), 20.3% DPHA (cas: 68622-73-1), 11.8% galangin, 5.1 % DPHB (cas: 79559-60-7) and 2.3 % kaempferide [6]. Indomethacin and bismuth potassium citrate group were purchased from Sigma-Aldrich. Galangin was bought from Pufei De Biotech Co. Ltd (Chengdu, China).

Experimental animals

Sprague-Dawley (SD) rats weighting 190 - 210 g were purchased from Tianqing Biotechnology (Changsha, China), and they were acclimatized to the animal house conditions (12 h light/dark

cycle, 25 – 26 °C temperature) for 7 days, prior to commencement of the study. All animal experiments were performed under the guidelines of the Care and Use of Laboratory Animals [10], with the approval of the animal ethics committee of Hainan Medical University (Reg. no. 201812023/HMU).

Drug treatment

Forty-two SD rats of both sexes were randomly assigned to seven groups: high, medium and low AOE groups (AOE-H, 0.36 g/kg; AOE-M, 0.18 g/kg and AOE-L, 0.09 g/kg); galangin group (GAL, 0.2 g/kg); bismuth potassium citrate group (POS, 0.08 g/kg); control group (CON) and indomethacin group (MOD, 30 mg/kg). The drugs were intragastrically administered to the rats once a day for 15 days, while equivalent volumes of vehicle were administered to rats in CON and MOD groups. Gastric injury was induced using indomethacin 12 h after drug administrations on day 15, while the CON group was administered vehicle. The vehicle was 1% (w/v) sodium carboxymethyl cellulose containing 2% (w/v) glycerol, and it was given at a dose of 2.5 mL/kg body weight.

Neutral red clearance test

One hour after indomethacin was given, each rat was anesthetized with urethane and a gastric fistula was made. Then, using a micro infusion pump, neutral red (5 mg/ml) was injected through the right femoral vein at the rate of 3 mg/kg/h for 5 min. Then the concentration of neutral red was changed to 0.5 mg/ml, and the injection through the femoral vein was continued at the same rate (3 mg/kg/h). The gastric juice was washed away with normal saline at the first hour. Then, the pumping was continued for another 3h. Each rat was sacrificed at the end of the third hour, and 1 mL blood was collected through heart puncture. The blood was injected into a test tube containing 0.1 mL of 0.1 M NaOH, followed by addition of 10 mL of ethyl ether. The mixture was vortexed and allowed to stand for 10 min. Then, 2 mL of 0.1 M HCl was added to the supernatant, and the mixture was vortexed and left to stand for 10 min.

Each rat stomach was completely removed into a 50 ml centrifuge tube. The gastric juice was collected via centrifugation at 3000 rpm for 10 min, and then filtered. The gastric juice was treated in the same way as blood as indicated above, except that the volumes of 0.1 M NaOH and 0.1 M HCl were 0.5 and 4 mL, respectively. The absorbances of all samples were read at 540 nm in a spectrophotometer against 0.1 M

HCl which was used as blank.

The amounts of neutral red in the gastric juice and serum was obtained from a neutral red standard curve, and the gastric mucosal blood flow (GMBF) was calculated as in Eq 1.

$$\text{GMBF (mL/kg/h)} = 2G/[(B-b) \times 3W \dots\dots (1)$$

where G stands for neutral red concentration of gastric juice, W stands for rat body weight, (B-b) stands for neutral red concentration in serum, and b is a constant calculated from five different blank rat sera under the same treatment.

Determination of gastric mucus

The opened rat stomach was immersed in 0.1% Alcian blue solution (10 mL/sample) and incubated for 2 h. Then, the segments were washed for 1h with 0.25 M sucrose so as to remove excess dye. Thereafter, the segments were immersed in 0.5 M MgCl₂ for 2 h to extract the mucus–dye complex. The dye extract was mixed with diethyl ether, and the mixture was centrifuged at 3500 rpm for 10 min. The absorbance of the supernate was read at 598 nm in a spectrophotometer. The results were extrapolated from an Alcian blue standard curve, and presented as µg Alcian blue / g tissue.

Assay H⁺/K⁺-ATPase activity

The glandular segments were weighed and a 10 % tissue homogenate solution was prepared in normal saline. The homogenate was centrifuged at 2500 rpm for 10 min, and the supernate was diluted to 2 % with normal saline. The H⁺/K⁺-ATPase activity of each sample was assayed using a biochemical kit (First Division of Nanjing Jiancheng Bioengineering Research Institute, A069). The enzymatic reaction and the phosphorus determination reaction were performed following the manufacturer's instructions. At the end of the reactions, the solutions were cooled to 25°C, and absorbance was measured at 660 nm using a microplate reader.

Western blot analysis of EP receptors

The mucosal tissue proteins were extracted and uniform concentrations of proteins were fractionated with 10% SDS-PAGE, and transferred to PVDF membranes. The membranes were blocked with 5% skim milk for 1.5 h and subsequently probed overnight at 4°C with the primary antibodies against EP-1 (1:1000, ThermoFisher, PA5-76888), EP-2 (1:2000, Abcam, ab167171), EP-3 (1:2000,

ThermoFisher, MA1-12647), EP-4 (1:2000, Santa, sc-55596) and β-actin (1:3000, Abcam, ab8226). Thereafter, the membranes were incubated with secondary antibody at 25°C for 1.5 h. A Bio-Rad ChemiDoc XRS+ chemiluminescence image analysis system was used to analyze the band densities.

Statistical analysis

The data are expressed as mean ± SEM, and they were compared using One-way ANOVA or Kruskal-Wallis *H* test. Values of *p* < 0.05 were considered statistically significant.

RESULTS

AOE and galangin increased gastric mucosal blood flow

As shown in Figure 1, GMBF was increased significantly in rats treated with AOE or bismuth potassium citrate (*p* < 0.001), when compared with the MOD group. Galangin showed less effect on GMBF (*p* < 0.01), when compared with the MOD group. Only GAL (*p* < 0.01) and MOD (*p* < 0.001) groups differed significantly from the CON group, indicating that GMBFs in the other groups were protected by the pretreatments with AOE and bismuth potassium citrate.

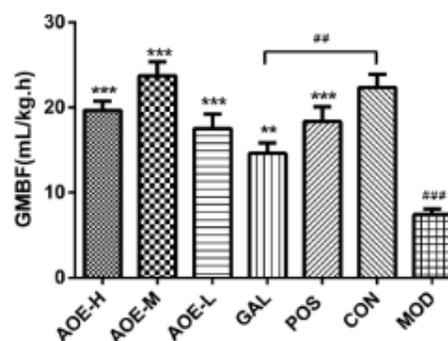


Figure 1: Effect of AOE and galangin on the GMBF in acute indomethacin-induced gastric injury rat model. ##*P* < 0.01, ###*p* < 0.001, vs CON group; ***p* < 0.01, ****p* < 0.001, vs MOD group

AOE, but not galangin, increased gastric mucus thickness

The tissue value of Alcian blue was 0.58 ± 0.05 µg/g in MOD group, 0.88 ± 0.11 µg/g in POS group, 0.88 ± 0.10 µg/g in AOE-H group, and 0.96 ± 0.09 µg/g in AOE-M group (Figure 2). These data reveal that AOE-H (*p* < 0.05), AOE-M (*p* < 0.01) and POS (*p* < 0.05) significantly increased rat gastric mucus thicknesses, when compared with corresponding value in MOD group. However, the gastric mucosal thickness

that resulted from pretreatment with galangin ($0.65 \pm 0.04 \mu\text{g/g}$ Alcian blue/tissue) did not significantly differ from that in the MOD group. When compared with CON group, AOE-H, AOE-M and POS groups showed no statistical differences, indicating that due to pretreatment with AOE and bismuth potassium citrate, the gastric mucus walls in these groups were not significantly damaged by indomethacin.

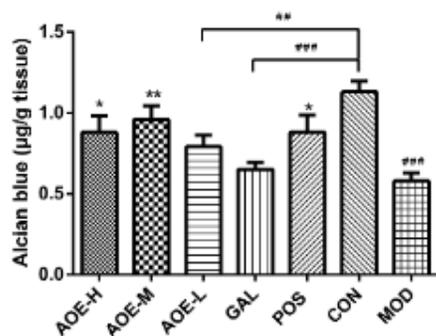


Figure 2: Effects of AOE and galangin on mucosa thicknesses in acute indomethacin-induced gastric injury rat model. ## $p < 0.01$, ### $p < 0.001$, vs the CON group; * $p < 0.05$, ** $p < 0.01$, vs the MOD group

AOE and galangin inhibited H^+/K^+ -ATPase activity

Figure 3 showed the *in vivo* effects of AOE and galangin on H^+/K^+ -ATPase activity in acute indomethacin-induced gastric damage rat model. High and medium dose of AOE significantly decreased H^+/K^+ -ATPase activity by 8.12 % ($p < 0.05$) and 12.19 % ($p < 0.01$), respectively. In contrast, low dose of AOE did not alter H^+/K^+ -ATPase activity. Galangin and bismuth potassium citrate significantly decreased H^+/K^+ -ATPase activity by 4.82 % ($p < 0.05$) and 4.70 % ($p < 0.05$), respectively.

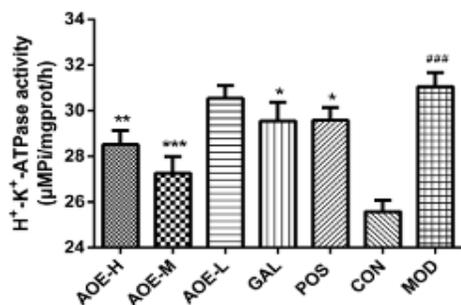


Figure 3: Effects of AOE and galangin on H^+/K^+ -ATPase activity in acute indomethacin-induced gastric injury model. ### $p < 0.001$, vs CON group; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, vs MOD group

AOE, but not galangin, up-regulated EP1 and EP4 protein expressions

The results of Western blot assays for EP1 to EP4 receptors are shown in Figure 4. All EP receptors were suppressed by indomethacin ($p < 0.05$ or $p < 0.01$), except EP2 receptor. Up-regulations of EP1 receptor were observed in AOE-H ($p < 0.05$) and POS ($p < 0.01$) groups. The expression level of EP4 receptor in AOE-H group was significantly increased, when compared with MOD group ($p < 0.05$). There were no statistically significant differences in EP2 and EP3 receptor levels when the drug treatment groups were compared with MOD or CON group. Galangin produced no significant effects on EP receptors.

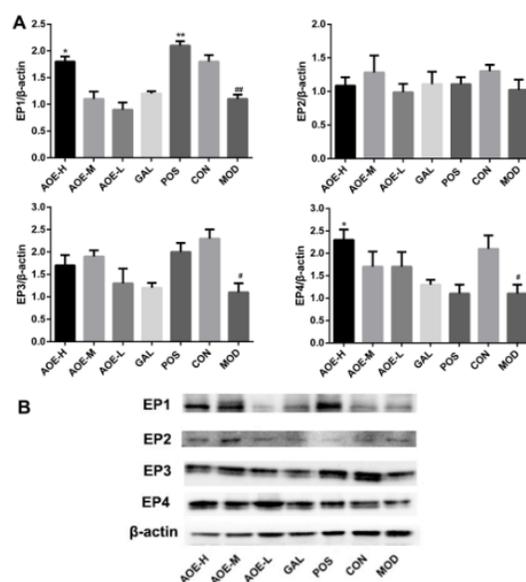


Figure 4: Effects of AOE and galangin on prostaglandin E_2 receptors in acute indomethacin-induced gastric injury model. EP: prostaglandin E_2 receptor. # $p < 0.05$, ## $p < 0.01$, vs CON group; * $p < 0.05$, ** $p < 0.01$, vs MOD group

DISCUSSION

The NSAIDs are widely used as antipyretic, analgesic and anti-inflammatory agents which inhibit cyclooxygenase (COX) activity. However, about 10-25 % of long-term NSAID users develop gastric ulcers, and some may even suffer from gastric bleeding and perforation. Moreover, the pharmacological mechanism associated with NSAIDs is the exact cause of gastric lesions, thereby making it difficult to eliminate the side effects. Cyclooxygenases play important roles in maintenance of gastric mucosal function. Prostaglandins (PGs) derived from COX-1 protect the gastric mucosa from harmful stimuli, while COX-2-derived

prostaglandins accelerate the healing of gastric injuries. Indomethacin, which is widely used in producing experimental gastric injury models, suppresses prostaglandin synthesis by inhibiting both COX-1 and COX-2 [11]. Besides, indomethacin also damages the gastric mucosa directly due to its acidic nature. Therefore, acute indomethacin-induced gastric injury rat model was used in this study to reflect topical and systemic actions of NSAIDs. In previous research, the combined effect of AOE/galangin and indomethacin was similar to that of selective COX-2 inhibitor (COXIB). However, reports have demonstrated that COXIB treatments impaired the healing of ulcers due to absence of endogenous PGs [12]. Therefore, the gastroprotective effects of AOE and galangin may be stronger if they are used before indomethacin treatment. Thus, AOE and galangin were given to rats 15 days before acute indomethacin treatment, based on preliminary experiments.

In the pathogenesis of indomethacin-induced severe gastric injury, increased gastric acid secretion is a vital event [13]. The destruction of gastric mucosal defense is induced by excessive gastric acid, thereby triggering gastric injuries and mucosal barrier collapse [14]. Researchers have found out that H^+/K^+ -ATPase transports K^+ into the cell from the extracellular fluid via self-phosphorylation and dephosphorylation, while pumping intracellular H^+ out of the cell against pH gradient, thereby controlling gastric acid secretion [16]. Besides, the expression of H^+/K^+ -ATPase precisely reflects gastric acid secretion [15]. Therefore, H^+/K^+ -ATPase was used as an index to accurate measurement of the gastric acid secretion of rats in the indomethacin-treated rat model. Drugs which can suppress the H^+/K^+ -ATPase activity are used in clinical treatment of gastric injury so as to reduce excessive gastric acid.

Data from H^+/K^+ -ATPase activity assays showed that AOE and galangin inhibited the enzyme to different degrees, which means that excessive gastric acid secretion was under control. Similar results were obtained in some flavonoid studies [7-9]. Therefore, AOE and galangin may regulate gastric acid secretion in indomethacin-induced acute rat gastric mucosa damage model by inhibiting H^+/K^+ -ATPase. It is worthy of note that the cardiovascular risk associated with NSAIDs and COXIBs has become a concern recently [17]. The US Food and Drug Administration (USFDA) strengthened its warning about the risks of myocardial infarction and stroke due to use of non-aspirin NSAIDs [18]. A meta-analysis has suggested that the combination therapy of COXIB and proton pump inhibitor (PPI) is better

than the combined effect of traditional NSAID and PPI in reducing the risks of recurring gastric bleeding and cardiovascular diseases [19]. The primary function of a PPI is to inhibit H^+/K^+ -ATPase activity. Thus, H^+/K^+ -ATPase inhibition may be an important component in the attenuation of COXIB-induced cardiovascular and gastrointestinal diseases. Therefore, the combined use of AOE/galangin and indomethacin may be similar in effect to combination of COXIB and PPI, which may attenuate both cardiovascular and gastrointestinal risks in high risk NSAIDs users. This hypothesis needs to be confirmed through more experiments.

Mucosal defense and vascular perfusion are two defensive factors in gastric mucosal barrier, and they play early and critical roles against NSAID-induced ulcers. When the basement membrane is exposed due to injury, a "mucoid cap" is formed over the base membrane mucus to protect it. The maintenance of the "mucoid cap" is dependent on uninterrupted mucosal blood flow [20]. The results of neutral red clearance test and gastric wall mucus determination showed that AOE significantly protected the GMBF and mucus thickness from indomethacin-induced injuries. Mucus thickness in AOE-M group was close to normal level. Gastric mucosal blood flow in AOE-M group was even higher than that in CON group. These results demonstrate that AOE exerted a strong gastroprotective effect and strengthened gastric mucosal barrier effectively. The AOE-M group produced the best gastroprotective effect, even when compared with the AOE-H group. A possible explanation may be that the pungent property of DPHC, the main component of AOE, constituted a stimulus to the gastric mucosa at the high dose. This weakened the capacity of gastric mucosa defense after 15 days of exposure, resulting in inability to resist indomethacin-induced gastric damage.

The prostaglandin E_2 receptor is divided into four specific G-protein coupled subtypes i.e. EP 1 - EP 4, and its distribution accounts for the multiple effects of prostaglandin E_2 [4]. Reports have demonstrated that prostaglandin E_2 combines with EP4 receptor to stimulate mucus secretion, and combines with EP1 to stimulate bicarbonate secretion in the stomach [21, 22]. Basal mucosal blood flow is maintained by prostaglandin E_2 through combining with EP2 or EP 4 receptor. [23]. The healing of gastric ulcer is mediated by prostaglandin E_2 through its combination with EP4 receptor [16]. Moreover, EP agonists attenuate gastric injuries by reproducing the functions of reduced PGs, while

EP antagonist significantly eliminate the protective effect of prostaglandin E₂. The EP agonists improve basal mucosal blood flow, with the exception of EP1 agonists [23]. In addition, the potent protective effect of prostaglandin E₂ against indomethacin-induced gastric injury can be reproduced by EP1 agonists, but not by other EP agonists [4]. These reports strongly indicate the important roles of prostaglandin E₂ receptors in suppressing gastric acid secretion and improving thickness of gastric mucus layer and gastric blood flow. Consistent with these findings, AOE improved the EP1 and EP4 receptor levels, suggesting that it may protect the gastric mucosa and improve mucosal blood flow condition by enhancing EP1 and EP4 expressions.

This study has some limitations. The agonists and antagonists of prostaglandin E₂ receptors should be used subsequent studies to verify the results obtained here. The effects of AOE and galangin on COXIB-induced cardiovascular and gastrointestinal diseases need to be further investigated.

CONCLUSION

This study demonstrates that the mechanism underlying the gastroprotective effect of ethanol extract of roots of *Alpinia officinarum* against indomethacin-induced gastric injury involves a combination of multiple targets. The extract inhibited excessive gastric acid secretion by inhibiting H⁺/K⁺-ATPase activity. It increased the GMBF and mucus thickness, and reinforced gastric mucosal barrier by triggering the expressions of prostaglandin E receptor 1 and prostaglandin E receptor 4. Galangin significantly inhibited H⁺/K⁺-ATPase activity and enhanced gastric mucosal blood flow. These findings provide new insights into the mechanisms underlying the gastroprotective effect of *Alpinia officinarum*, and show its promising potential for clinical use in treating gastric mucosal injury induced by NSAIDs.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by Jingwen Gong, Xuguang Zhang, Yingfeng Tan, Hailong Li, Jie Hou and Junqing Zhang, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Junqing Zhang and Jie Hou conceived and designed the study. Jing-wen Gong collected, analyzed the data and wrote the manuscript. Xuguang Zhang and Yingfeng Tan conducted some of the experiments. Hai-long Li participated in the study design and revision of the manuscript.

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