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ORIGINAL ARTICLE

The Gastroprotective Effect of *Alpinia officinarum* Extract on Indomethacin-Induced Topical Injuries in RGM-1 Cells: Involvement of H^+/K^+-ATPase- and Mitochondrial-Mediated Apoptosis

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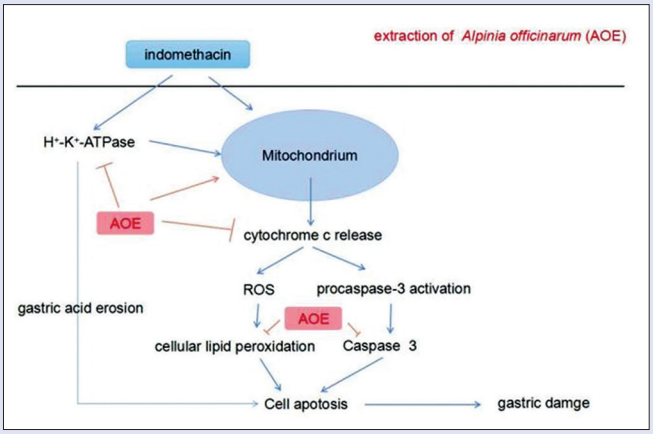
ABSTRACT

Background: Topical effects are essential mechanisms of nonsteroidal anti-inflammatory drugs (NSAIDs)-induced gastric damage. Our previous study showed that the extract of *Alpinia officinarum* (AOE) may have some protective effects against the topical effects of indomethacin (INDO). The aim of this study was to elucidate the protective effects and mechanisms of *A. officinarum* against INDO-induced topical injuries to gastric mucosa. **Materials and Methods:** 0.5 mM INDO was used to cause topical effects to rat gastric epithelial cells (RGM-1). Meanwhile, AOE (2.5 μg/mL) and galangin (GAL) (0.05 mM) were added, respectively, to explore their protective effects. The cell proliferation, mitochondrial viability, and mitochondrial-mediated apoptosis were assessed by flow cytometry, inverted fluorescence microscope, or microplate reader. Pro- and cleaved-caspase-3 were detected by Western blot method. **Results:** AOE and GAL could significantly protect INDO-damaged RGM-1 cells by promoting cell proliferation, upregulating mitochondrial viability, inhibiting mitochondrial cytochrome c release into cytoplasm, inhibiting lipid peroxidation and caspase-3 activity, and suppressing H^+/K^+-ATPase activity. **Conclusion:** The gastroprotective effects of AOE and GAL were closely associated with suppressing the gastric acid secretion and restraining mitochondrial-mediated apoptosis. These data provided new perceptions into interpreting the underlying mechanisms of gastroprotective effects of *A. officinarum* and showed a promising clinical use in treating gastric mucosal injury induced by NSAIDs.

Key words: Caspase-3, cytochrome c, H^+/K^+-ATPase, lipid peroxidation, mitochondrial, topical effect

SUMMARY

- Topical effects are essential mechanisms of nonsteroidal anti-inflammatory drug (NSAID)-induced gastric damage. It initiates the mucosal erosion by disrupting the gastric epithelial cell barrier, and then, excessive gastric acid and other stimuli destroy the gastric mucosa and cause gastrointestinal damages. Our earlier study indicated that the extract of *Alpinia officinarum* (AOE) may have some protective effects against the topical effects of indomethacin (INDO). This study established that AOE and its representative monomer compound galangin significantly protected INDO-damaged RGM-1 cells by promoting its cell proliferation, upregulating mitochondrial viability, mitochondrial cytochrome c concentration, and procaspase-3 protein expression, downregulating cleaved-caspase-3 protein expression and cytosol cytochrome c concentration, and suppressing H^+/K^+-ATPase activity. The results illuminated that the gastroprotective effect of *A. officinarum* was closely connected with suppressing the gastric acid secretion and restraining mitochondrial-mediated apoptosis. These data provided new insights into interpreting the underlying mechanism of *A. officinarum*'s gastroprotective effect and displayed an auspicious clinical use in treating gastric mucosal injury induced by NSAIDs.



Abbreviations used: AOE: The extract of *Alpinia officinarum*; RGM-1: Rat gastric epithelial cells; NSAIDs: Nonsteroidal anti-inflammatory drugs; COXIB: COX-2 inhibitors; ROS: Reactive oxygen species; INDO: Indomethacin; VEGF: Vascular endothelial growth factor; GAL: Galangin; CCK-8: Cell counting kit-8.

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INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most frequently prescribed drugs with noticeably gastrointestinal (GI) side effects.[1] The pathogenesis of NSAID-induced GI injury is complex and contains both topical and systemic effects. Topical injury initiates the initial mucosal erosion by disrupting the gastric epithelial cell barrier.[2] Early studies were focused on the systemic effects, especially the cyclooxygenase-prostaglandin mechanisms.[3-5] However, observed GI damage appeared modest in COX-inhibited models,[6,7] and decreased mucosal prostaglandins were fewer important in the pathogenesis of the small bowel damage.[8] Furthermore, selective COX-2 inhibitors (COXIB) did not eradicate the risks of gastroduodenal ulcers and other complications.[9] These results recommended that treatments aimed at the effects of systemic NSAIDs were not enough to eradicate the GI injury. Therefore, studies on topical effects are increasing.

Topical effects are common to all conventional NSAIDs according to their acidic properties.[1] Accumulated NSAIDs (like indomethacin [INDO]) in gastric epithelial cells uncouple mitochondrial oxidative phosphorylation to disintegrate the mitochondrial membrane potential, which induces mitochondrial permeability transition pore, resulting in release of cytochrome c. Then, reactive oxygen species (ROS) generation is triggered, thereby causing cellular lipid peroxidation and caspase cascade, resulting in cellular apoptosis eventually.[1,10] NSAIDs also increase membrane permeability by inducing changes in membrane hydrophobicity, thickness, and bending stiffness. Gastric acid can lead to cell death by apoptosis and necrosis and develop into gastric ulcers under this broken gastric mucosal barrier condition. Studies have found that topical effects play an important role in initiating GI damage.[11-13] These researches propose that topical effects are also essential mechanisms of NSAID-induced gastric damage.

Alpinia officinarum is a traditional Chinese medicinal plant which has been used for stomach diseases for over thousands of years.[14] In our earlier study, *A. officinarum* extract (AOE, mainly contains flavonoids and diarylheptanoids) remarkably inverted the gastric injury caused by INDO and its mechanisms were related to some systemic targets.[15] However, the improved bending stiffness of gastric mucus and markedly increased vascular endothelial growth factor levels from that study also recommended that AOE may also have some effects on the topical effects of NSAIDs. Some flavonoids like baicalein, myricetin, quercetin, etc. were reported to inhibit H^+/K^+-ATPase activities.[16-19] Therefore, flavonoids in AOE may also possess the H^+/K^+-ATPase inhibition effects. It is known that H^+/K^+-ATPase is the proton pump in the gastric mucosa and is mainly responsible for the acidification of the gastric contents. Depressing the H^+/K^+-ATPase activity and reducing gastric acid secretion is a commonly used clinical intervention for gastric and duodenal ulcers. Furthermore, flavonoids are known for their anti-oxidation effects. Thus, a hypothesis raised that AOE may act against the tropic effects of NSAIDs by decreasing the cellular lipid peroxidation and inhibiting the acid erosion. Thus, the aim of this study was to explore the gastroprotective effects and underlying mechanisms of AOE on the topical effects of INDO. Galangin (GAL), the most abundant flavonoid in AOE, was also examined in this study.[15] We used rat gastric mucosa epithelial cells (RGM-1) to perform the following tests: (1) cell proliferation, (2) cellular apoptosis, (3) cellular lipid peroxidation, (4) caspase activation, and (5) H^+/K^+-ATPase activity.

MATERIALS AND METHODS

Reagents and chemicals

The procedures for preparing of AOE was stated in our previous research.[15] The contents in prepared AOE were identified by Nuclear Magnetic Resonance (NMR) and mass spectrometry, and quantitative analysis was performed by high-performance liquid chromatography (HPLC).[20] The contents of the main substances in AOE are as follows: 13.14% GAL, 3.19% kaempferide, 20.35% DPHA, 4.90% DPHB, and 20.39% DPHC. Ethanol in the extracts was completely removed by heating to 80°C. Purified GAL was separated from AOE by Waters e2695 HPLC, and the purity was 99.99%. INDO was purchased from Sigma Chemical Corp. (St. Louis, USA).

Cell culture

RGM-1 cells (4×10^5 cells/well) were grown in a DMEM/F12 medium containing 10% fetal bovine serum and 1% antibiotic (100 ug/mL streptomycin and 100 units/mL penicillin) at 37°C in a humidified 5% CO_2 atmosphere. Cell growth was observed daily, and the experiments were performed when the monolayer cells were attached to an adherent level of about 80%.

Cytotoxicity assay

Cell injury of INDO was examined by the cell counting kit 8 (CCK8) according to the manufacturer's instructions (KGA312-1, Jiangsu Keygen Biotech Co. Ltd., China). The RGM-1 cells at a concentration of 4×10^5 cells/well were seeded in a 96-well plate for 24 h. After that, RGM-1 cells were cultured with 0-, 0.5-, 1.0-, or 2.0-mM INDO for 0, 2, 4, 12, 18, and 24 h. Afterward, RGM-1 cells were cultured with serum-free media containing 10 μL CCK8 for 2 h. The absorbance of each well at 450 nm was measured by a microplate reader (Tecan Austria GmbH, Austria).

Cell modeling

The RGM-1 cells were seeded for 24 h and then treated with AOE (2.5 μg/mL, final concentration) and INDO (0.5 mM, final concentration) or GAL (0.05 mM, final concentration) and INDO (0.5 mM, final concentration), respectively. Then, the cells were continuously cultivated for 18 h.

Cell viability assay

After continuous cultivation, Methyl thiazolyl tetrazolium (MTT) assay was also used to assess the cell proliferation. The procedures were followed by the manufacturer's instructions.

Annexin V-FITC/PI cytometric assay

Eighteen hours after continuous cultivation, the cells were harvested and stained positively for Annexin V-FITC and PI double-staining (AP101-100-kit, Multi Sciences Co. Ltd., China). The apoptosis was detected using a flow cytometer (NovoCyte 2060R, ACEA Biosciences Inc., USA).

Mitochondrial membrane potential assay

Eighteen hours after continuous cultivation, the cells were harvested. The viability of mitochondria was measured by JC-10-Mitochondrial membrane potential assay kit according to the manufacturer's instructions (40752ES60, Yeasen, China).

Mitochondrial cytochrome c release analysis

Mitochondria in the RGM-1 cells were isolated using a mitochondrial separation kit (SM0020, Solarbio Technology Co. Ltd., China) and lysed with 2% CHAPS, which was dissolved in Tris buffer (25 mM Tris, 150

mM NaCl). Cytochrome c levels of cytoplasm and mitochondria in the RGM-1 cells were detected using the cytochrome c ELISA kit (MCTC0, R&D Co. Ltd., USA).

Lipid peroxidation assay

RGM-1 cells were treated with AOE (2.5 μg/mL) and GAL (0.05 mM) for eighteen hours. Cells was incubated with 200 μM DPPP (110231-30-6, TCI Co. Ltd., China) at 37°C for 1 h following the manufacturer's procedures. The absorbance was measured at 352 nm (excitation wavelength) and 461 nm (emission wavelength).

Western blot analysis

RGM-1 cells were treated with AOE (2.5 μg/mL) and GAL (0.05 mM) for eighteen hours. Cells were lysed for 30 min in ice-cold lysis buffer (Beyotime Biotechnology, Shanghai, China). The cell lysates were cleared by centrifugation for 15 min at 12000 xg. Protein concentration was determined by BCA kit (CW0014S, Cwbio Co. Ltd., China). The extracted proteins were separated using a 10% SDS-PAGE gel and then transferred onto PVDF membranes. The membranes were incubated with 5% skimmed milk at room temperature for 2 h and then incubated with pro-caspase-3 (ab184787, Abcam, US), cleaved caspase-3 (ab49822, Abcam, US), and GAPDH (TA-08, Alfetronic, China) primary antibodies at 1:2000, 1:2000, and 1:2000, respectively, at 4°C overnight, and then, the secondary antibody-labeled HRP was added. The target protein bands were visualized on ChemiDoc™ XRS + system (Shanghai, China) using Super ECL plus. Protein strips were analyzed using Image J software (Bethesda, MD, USA).

H^+/K^+-ATPase activity assay

RGM-1 cells were treated with AOE (2.5 μg/mL) and GAL (0.05 mM) for eighteen hours. Agents from the H^+/K^+-ATPase activity test kit were added according to the manufacturer's instructions. After the enzymatic reaction and the phosphorus determination reaction were completed, the solution was cooled at room temperature and the absorbance was measured by a microplate reader at 660 nm. The activity of H^+/K^+-ATPase was then calculated according to the equation provided by the manufacturer's instructions.

Data analysis

All data were analyzed using IBM SPSS Statistics 19 (Chicago, IL, USA). The quantitative comparison between two groups was performed by an independent sample *t*-test, the quantitative comparison between multiple groups was performed by one-way ANOVA, and the pairwise comparison was performed by LSD and S-N-K. The test level is $\alpha = 0.05$.

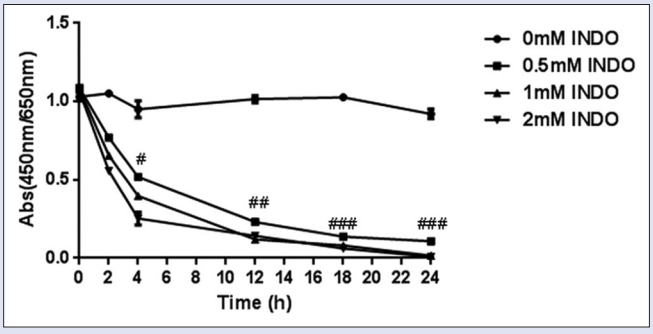

Figure 1: INDO-induced RGM-1 cellular injury. Damage to cells depended on time and dose of INDO. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ when 0.5 mM INDO compared with the control group. The significant differences of other INDO groups compared with the control group were omitted in this figure. RGM-1: Rat gastric epithelial cells; INDO: Indomethacin

RESULTS

The extract of *Alpinia officinarum* and galangin promoted indomethacin-damaged RGM-1 cell proliferation

The cellular injury caused by INDO is presented in Figure 1. All cells treated with INDO showed suggestively reduced absorbance value of cell counting kit-8 (CCK-8) than the control group. Absorbance values decreased in a dose-dependent and time-dependent manner. After 24 h of INDO treatment, cells showed nearly no absorbance. These results indicate that INDO induces cellular injury in a dose-dependent and time-dependent manner, and after 24 h treatment, it induces cellular death. 0.5 mM INDO and 18 h experiment duration was chosen for following experiments according to the severe but not fatal injuries on RGM-1 cells.

The cellular protective effect of AOE and GAL on INDO-induced cellular injury was also investigated. Figure 2 shows the absorbance values of CCK-8 18 h after 0.5 mM INDO treatment with/without AOE or GAL. The results displayed that AOE and GAL accelerated the INDO-damaged RGM-1 cell proliferation in different degrees compared with the model group. ($^{**}P < 0.01$ and $^{***}P < 0.001$, respectively). Based on the present data, 2.5 μg/mL AOE and 0.05 mM GAL were used for further *in vitro* studies.

The extract of *Alpinia officinarum* and galangin attenuated indomethacin-damaged RGM-1 apoptosis

To investigate the underlying mechanisms of AOE and GAL on the INDO-damaged RGM-1 cell, we first measured their effects on cell apoptosis by flow cytometry. As shown in Figure 3, the apoptosis in the INDO group was suggestively higher than those in the control group ($^{\#}P < 0.05$) and the apoptotic cells peaked at 32.53%.

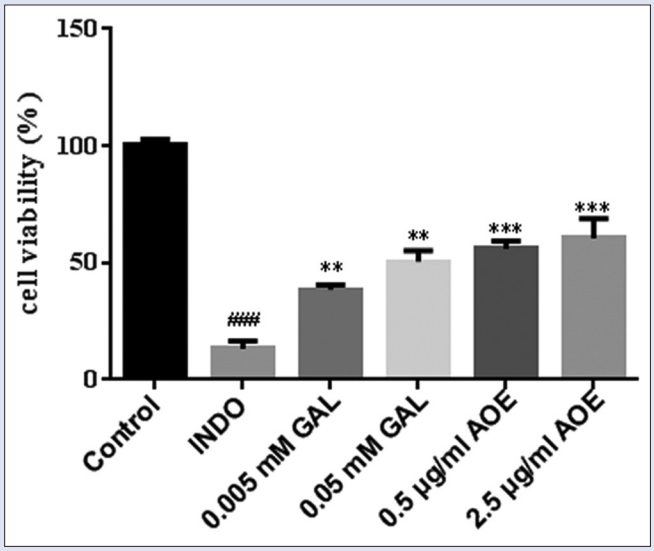

Figure 2: Cellular protective effect of AOE and GAL on INDO-induced RGM-1 cellular injury. After 18 h treatment, AOE and GAL accelerated the INDO-damaged RGM-1 cell proliferation. $^{\#\#\#}P < 0.001$ compared with the control group; $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the model group. AOE: The extract of *Alpinia officinarum*; GAL: Galangin; INDO: Indomethacin; RGM-1: Rat gastric epithelial cells

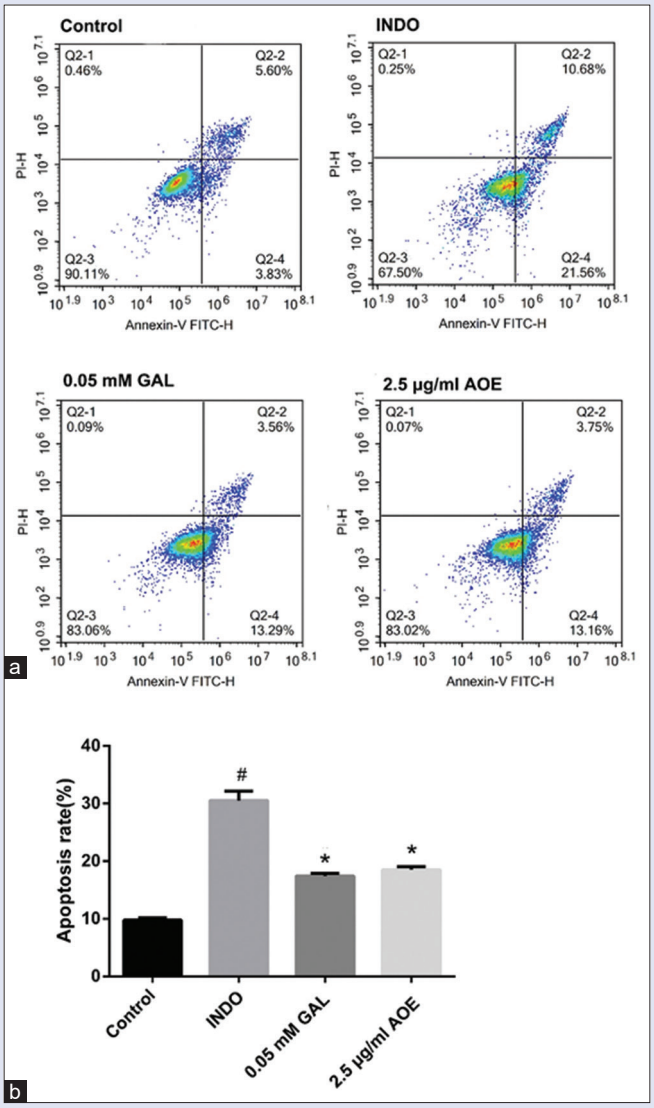

Figure 3: Cellular apoptosis (a) and the apoptosis rate (b) of RGM-1 cells in different groups. After 18 h treatment, AOE and GAL significantly reduced cellular apoptosis induced by INDO. #$P < 0.05$ compared with the Control group; *$P < 0.05$ compared with the model group. AOE: The extract of *Alpinia officinarum*; GAL: Galangin; INDO: Indomethacin; RGM-1: Rat gastric epithelial cells

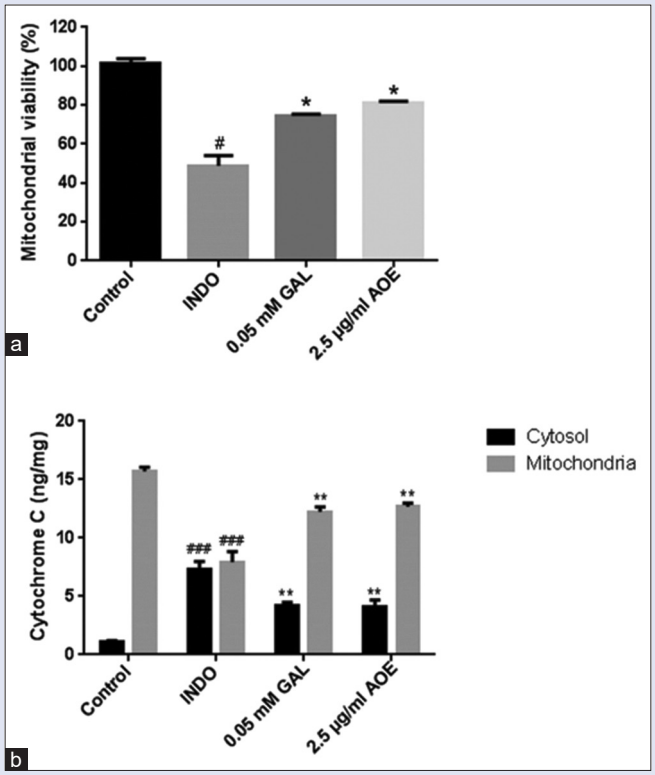

Figure 4: Mitochondrial membrane potentials (a), cytochrome c concentrations in the cytosol and mitochondria (b) of RGM-1 cells in different groups. After 18 h treatment, AOE and GAL significantly decreased mitochondrial damages induced by INDO. #$P < 0.05$ compared with the control group; *$P < 0.05$ compared with the model group. AOE: The extract of *Alpinia officinarum*; GAL: Galangin; INDO: Indomethacin; RGM-1: Rat gastric epithelial cells

With the treatment of AOE or GAL, apoptosis was suggestively suppressed ($P < 0.05$, respectively), which came down to 16.94% and 16.30% in the AOE and GAL groups.

The extract of *Alpinia officinarum* and galangin restrained indomethacin-damaged RGM-1 cell apoptosis by blocking mitochondria-dependent apoptotic pathway

The important role of mitochondria in NSAID-induced apoptosis has been established. Therefore, mitochondrial membrane potential, cytochrome c release, lipid peroxidation, and caspase activation were investigated to determine the influences of AOE and GAL in mitochondria-mediated apoptotic pathway.

As shown in Figure 4, in the INDO group, the mitochondrial viability and concentrations of cytochrome c in the mitochondria were obviously reduced ($^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$, respectively). Concentrations of cytochrome c in the cytosol were suggestively elevated compared with the control group ($^{\#\#}P < 0.01$, respectively). AOE and GAL effectively reversed deteriorative mitochondrial viabilities and cytochrome c distributions compared with the INDO group (*$P < 0.05$ and **$P < 0.01$, respectively).

As shown in Figure 5, the DPPP staining degree in the INDO group was significantly higher than that in the control group ($^{\#}P < 0.05$), suggesting that INDO induced the lipid peroxidation of RGM-1 cells to disturb cell metabolism. DPPP staining degrees of AOE and GAL were improved compared with that in the INDO group (*$P < 0.05$, respectively), indicating that AOE and GAL can decrease the lipid peroxidation induced by INDO.

As shown in Figure 6, the relative expressions of 32 kDa pro-caspase-3 protein and its active 17 kDa form in the CON group were 1.36 ± 0.02 and 0.16 ± 0.02, respectively. The pro-caspase-3 protein was cleaved to its active 17 kDa form at 18 h in the INDO group ($^{\#}P < 0.05$), and their final relative expressions were 0.75 ± 0.08 and 0.95 ± 0.07, respectively. The relative expressions of pro-caspase-3 protein and cleaved caspase-3 protein were 1.04 ± 0.03 and 0.36 ± 0.02 in the GAL group and 1.08 ± 0.02 and 0.28 ± 0.02 in the AOE group, respectively. The densities of cleaved caspase-3 protein in GAL and AOE groups were significantly decreased ($^{\#}P < 0.05$) and showed no significant difference when compared with the control group ($P > 0.05$), suggesting the density of cleaved caspase-3 protein in those groups were back to normal levels.

The extract of *Alpinia officinarum* and galangin inhibited the H^+/K^+-ATPase activities

It is generally known that the activity of H^+/K^+-ATPase is an important indicator for evaluating antigastric acidity.[24,25] As shown in Figure 7, in the INDO group, the H^+/K^+-ATPase activity increased noticeably compared with the control group ($^{\#\#}P < 0.01$). Treatment with AOE or GAL led to an inhibition of H^+/K^+-ATPase activity compared with the INDO group ($^*P < 0.05$).

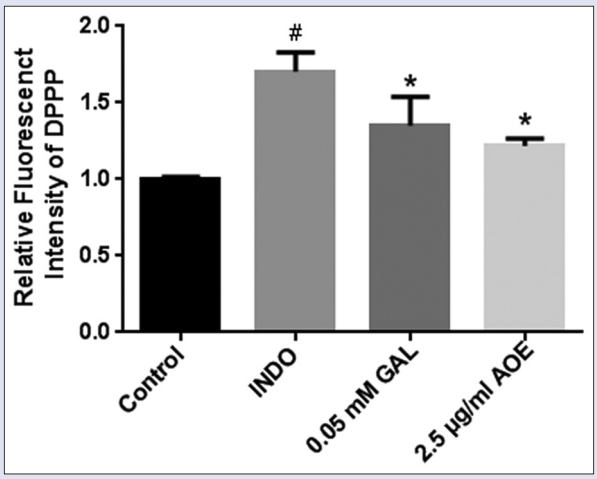

Figure 5: Lipid peroxidation of RGM-1 cells in different groups after 18 h treatment. AOE and GAL significantly decreased the cellular lipid peroxidation induced by INDO. #$P < 0.05$ compared with the Control group; *$P < 0.05$ compared with the Model group. AOE: The extract of *Alpinia officinarum*; GAL: Galangin; INDO: Indomethacin; RGM-1: Rat gastric epithelial cells

DISCUSSION

As there are no referable data to ensure the dose of AOE for experiments in this study, the cytotoxicity of different magnitude concentrations of AOE and GAL was verified in our preliminary experiment. *A. officinarum* ethanol extract showed no significant cytotoxicity compared with healthy RGM-1 cells, and a similar phenomenon was observed in 0.05 mM GAL. The time interval between AOE/GAL and INDO treatment was also verified, and better gastroprotective effects were achieved when added with INDO in the same time. 2.5 μg/mL AOE and 0.05 mM GAL significantly reversed the cytotoxicity caused by INDO, suggesting a protective effect to the RGM-1 cells.

As a result of apoptosis, the loss of epithelial cells in gastric mucosa is visible in gastric mucosa lesion, which is also the inevitable cause of gastric ulcer.[20] NSAIDs were reported to cause epithelial cell apoptosis by their topical effects. The mitochondrial membrane potential of epithelial cell was decreased, when the integrality of the mitochondrial membrane was destroyed by NSAIDs, resulting in the release of cytochrome c from the mitochondria into the cytosol, and then activating ROS and caspase-3, triggering cellular lipid peroxidation and apoptosis.[1,10] In this study, INDO treatment caused the aforementioned phenomenon to RGM-1 cells. The results are consistent with previous reports, suggesting that INDO induces apoptosis to epithelial cells by its topical effects.[10,21,22] Annexin V-FITC and PI dyes are used to detect the cell apoptosis, and results presented that AOE and GAL significantly attenuated the RGM-1 cell apoptosis induced by INDO.

Mitochondrial membrane potential, cytochrome c release, lipid peroxidation, and caspase activation were performed to further investigate the anti-apoptosis mechanisms of AOE and GAL. The upregulated mitochondrial membrane suggested AOE and GAL decreased mitochondrial damages induced by INDO, maintaining the normal function of mitochondrial membrane. After stabilized the mitochondrial membrane, AOE and GAL were also found to reverse

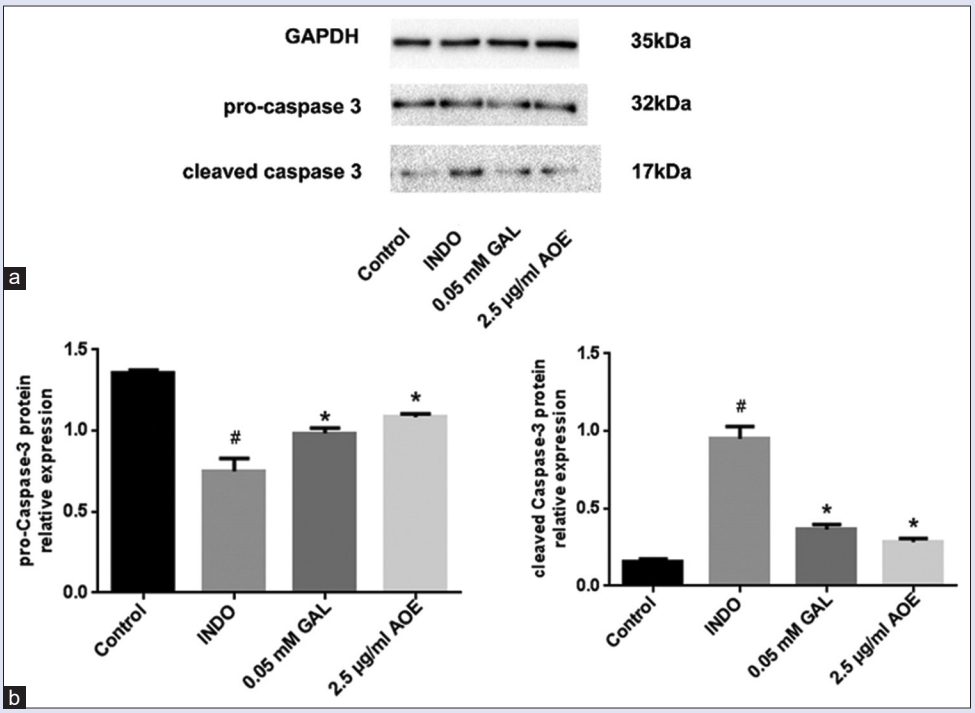

Figure 6: Western blots of pro- and cleaved-caspase-3 proteins (a) and the relative expressions (b) in different groups after 18 h treatment. #$P < 0.05$ compared with the control group; *$P < 0.05$ compared with the model group

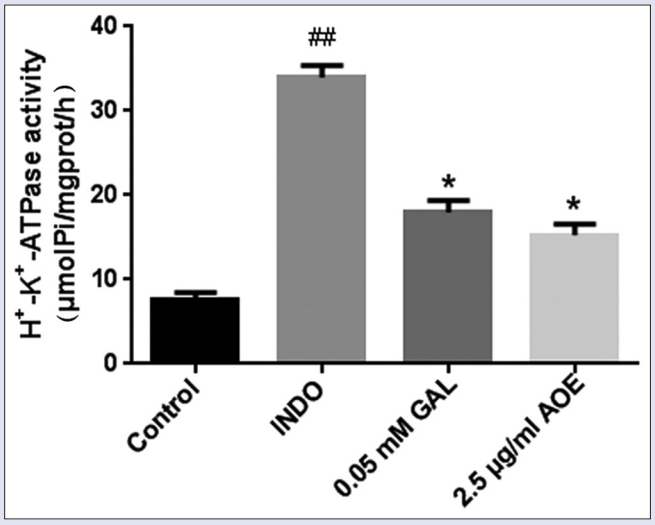

Figure 7: Effects of AOE and galangin on decreasing H^+/K^+-ATPase in RGM-1 cells. #$P < 0.05$ compared with the Control group; *$P < 0.05$ compared with the Model group. AOE: The extract of *Alpinia officinarum*; RGM-1: Rat gastric epithelial cells

the release of cytochrome c, and concentrations of cytochrome c were noticeably decreased in the cytosol and significantly increased in the mitochondria in AOE or GAL treatment group. Increased cytochrome c in the cytosol forms an apoptosome together with Apaf-1 and pro-caspase-9 and then activating pro-caspase-3 into cleaved caspase-3, triggering apoptosis.[23] The increased pro-caspase-3 proteins and decreased cleaved caspase-3 proteins by AOE and GAL further illuminated their anti-apoptosis mechanisms. These results are similar to researches about the inhibition of caspase cascade and apoptosis.[24,25] Caspase-3 is a key enzyme in the caspase cascade, and the activation of caspase-3 makes the apoptosis inevitable.[26] INDO-induced apoptosis is proved to be regulated by the apoptosis-related proteins and downstream effectors including Bcl-2 family proteins. INDO and its derivatives were also proved to be inhibitors on Bcl-2 proteins.[16] Thus, the Bcl-2 family proteins may be intricate in the protective effects of AOE as well. Further experiments are desirable to prove this.

Mitochondrial damage and the release of cytochrome c lead to the production of ROS. ROS may directly oxidize cellular proteins, lipids, or nucleic acids and cause general damage or dysfunction or may initiate the cell death process through affecting various signaling cascades leading to necrosis and apoptosis.[27] AOE and GAL did lessen the lipid peroxidation according to data in this study. It was in accordance with some initial studies, which found that GAL employed a protective effect on the mitochondria with decreased production of ROS, along with the release of cytochrome c reduction and the expression of activated caspase-3.[18] However, GAL was also found to induce apoptosis via accumulation of ROS in some cancer cells.[19,28] The inconsistent results make the effects and underlying mechanisms more difficult to explain. The results may be influenced by doses, cell lines, and many other factors. Cao *et al*. had found that mitochondrial permeability transition pore sealing agents and antioxidants were used to prevent the oxidative stress and mitochondrial dysfunction induced by COXIBs.[29] As AOE and GAL play an antioxidant effect, it is possible that AOE and GAL could attenuate the side effects of COXIBs.

It is generally known that suppressing excessive gastric acid secretion as well as reinforcing the gastric mucosal barrier function is one of the major tactics to treating gastric ulcer. INDO causes gastric injury by increasing the secretion of gastric acid. It has been suggested that the phenomena of the above are closely related to NSAID-induced COX inhibition.[22] Both COX-1 and COX-2 proteins are expressed by gastric glands and gastric mucosa. As approved, prostaglandin derived from COX can inhibit gastric acid secretion via prostaglandin E receptor 3 (EP3) and prostacyclin receptors (IP),[1] and a decrease in prostaglandin synthesis via COX-1 inhibition could potentiate basal and gastric acid secretion.[28] Excessive gastric acid and ROS overproduction give rise to gastric mucosal epithelial cell apoptosis, bringing about gastric mucosal injury and mucosal barrier destruction.[30] Previous reports usually use the pH value of gastric fluid to measure the gastric acid secretion. However, the pH value of the gastric fluid reflects the total acidity formed by the chyme, gastric acid secreted by the animal itself, and other body fluid and is vulnerable to the food intake and drinking water. Therefore, the pH value of gastric fluid does not precisely reflect the ability of the animal's gastric acid secretion. H^+/K^+-ATPase transports ion K^+ into the cell from the extracellular fluid by its own phosphorylation and dephosphorylation, while pumping the intracellular H^+ out of the cell against the pH gradient.[31] Researchers found that the expression and activity of H^+/K^+-ATPase are reliable with the gastric acid secretion, which means that the activity of H^+/K^+-ATPase can be a precise reflection of the gastric acid secretion. Besides, inhibition of H^+/K^+-ATPase is also the main mechanism of proton-pump inhibitor (PPI). A meta-analysis suggested that a selective COX-2 inhibitor plus a PPI is superior to a traditional NSAIDs plus a PPI in the prevention of recurrent upper GI bleeding and showed no observed cardiovascular diseases in aspirin users.[32] These reports suggested that inhibition of H^+/K^+ - ATPase activity may be a good way to alleviate COXIB-induced cardiovascular and gastrointestinal diseases. In the present study, activated H^+/K^+-ATPase was found in INDO-treated RGM-1 cells. Similar results were also obtained in some early studies that INDO induction increases proton-pump expression in parietal cells.[33,34] INDO was found to enhance the α- and β-subunits of H^+/K^+-ATPase, increased proton-pump expression, and activity, with concomitant inhibition of PGE2 synthesis in parietal cells, suggesting that inhibition of COX-1-derived PGE2 synthesis increases gastric acid secretion via improved expression and activation of the proton pump.[33] However, Du *et al.*[35] had found that INDO showed a noncompetitive inhibitory effect on hog gastric H^+/K^+-ATPase vesicles by both a direct effect on the hydrolytic and H^+ transport functions of the enzyme and a disturbing effect on the lipid bilayer of the vesicles. A possible explanation about the contradictory facts may be that INDO did show a direct non-competitive inhibition on H^+/K^+-ATPase *in vitro*, but the results from pure enzymes condition *in vitro* do not necessarily consist with the outcomes of complex conditions *in vivo*. Due to the activation of COX-PGs-H^+/K^+-ATPase signaling pathway and the proliferation of rgm-1 cells, H^+/K^+-ATPase is activated. The inhibitory effect of INDO on H^+/K^+-ATPase may be too small to show. Data from this study showed that AOE and GAL inhibited the H^+/K^+-ATPase activities in different degrees. Similar results have been observed in some flavonoids, and extraction contains plenty of flavonoids.[16-19] Therefore, AOE and GAL were possibly served as H^+/K^+-ATPase inhibitors to regulate the gastric acid secretion in this experiment. As AOE and GAL were proved to increase the COX-1 activity and had no influence on COX-2 inhibition in our last research,[15] the combination use of AOE/GAL with NSAIDs maybe an auspicious therapy for high-risk NSAID users.

The gastroprotective effects of GAL (0.05 mM) was weaker than AOE (2.5 μg/mL), indicating that GAL may not be the primary effective compound in AOE. This finding is consistent with our earlier research.[15] DPHA and DPHC might be responsible for the strong effects of AOE owing to their significant antioxidant and anti-inflammatory potential.

CONCLUSION

This article established that AOE and GAL significantly protected INDO-damaged RGM-1 cells by promoting its cell proliferation, upregulating mitochondrial viability, mitochondrial cytochrome c concentration, and procaspase-3 protein expression, down-regulating cleaved-caspase-3 protein expression and cytosol cytochrome c concentration, and suppressing H^+/K^+-ATPase activity. The results illuminated that the gastroprotective effect of *A. officinarum* was closely connected with suppressing the gastric acid secretion and restraining mitochondrial-mediated apoptosis. These data provided new insights into interpreting the underlying mechanism of *A. officinarum*'s gastroprotective effect and showed a hopeful clinical use in treating gastric mucosal injury induced by NSAIDs.

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Conflicts of interest

There are no conflicts of interest.

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