Gastroprotective Effects of Mokko Lactone Against Indomethacin-Induced Gastric Ulcer: Emphasis on its Antioxidant, Anti-inflammatory and Anti-apoptotic Activities

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ABSTRACT

Gastric ulcer is a common gastrointestinal disease with a 5-10% global prevalence. It results from the disturbed balance between protective and destructive factors affecting the gastric lining. Indomethacin was shown to possess great ulcerogenic potential, therefore, it is used to induce gastric ulcers in experimental models. Mokko lactone is a promising guaianolide sesquiterpene lactone with reported antioxidant, anti-inflammatory, and anti-apoptotic properties. Therefore, this study aimed to inspect the defensive effects of mokko lactone against gastric injury induced by indomethacin in rats. The ingestion of a single dose of indomethacin (50 mg/kg) provoked gastric injury manifested as abnormal histological features of the gastric mucosa, which are reflected by the scoring of the gastric injury. Indomethacin-induced gastric injury can be explained through multiple mechanisms including the induction of oxidative stress as proven by increased malondialdehyde (MDA) levels, decreased reduced glutathione concentration (GSH), and decreased the cell defense enzymes, superoxide dismutase (SOD) and catalase, activities. It also increased the gastric tissue expression of the inflammatory proteins, cyclooxygenase-2 (COX-2) and interleukin-6 (IL-6). Moreover, indomethacin decreased the gastroprotective mucin and prostaglandin E2 (PGE2) levels, and increased apoptosis through increased gastric expression of caspase-3. It also induced the degradation of the extracellular matrix by increasing matrix metalloproteinase-9 (MMP-9). Interestingly, the pretreatment with mokko lactone (5 and 10 mg/kg) showed promising gastroprotective activities comparable to those conferred by the potent antiulcerogenic, omeprazole. Pretreatment with mokko lactone restored the normal histological characteristics of the gastric mucosa and decreased the ulcer score; it induced antioxidant effects by reducing the levels of MDA, increased GSH, and induced the SOD and catalase activities; decreased the expression of COX-2 and IL-6; increased gastric mucin and PGE2 content; decreased caspase-3 and MMP-9 tissue expression. In conclusion, mokko lactone induced substantial protective action against gastric damage prompted by indomethacin.

Keywords: Indomethacin; Mokko Lactone; Gastric ulcer, guaianolide sesquiterpene

INTRODUCTION

Gastric ulcer is a widespread gastrointestinal disorder with a 5-10% global prevalence[1]. It results from an imbalance between protective and destructive elements regulating the integrity of the gastric mucosa[2]. The aggressive factors involve smoking, *H. pylori* infection, alcohol, and nonsteroidal anti-inflammatory drugs (NSAIDs). In contrast, the protective factors involve prostaglandin production, mucosal cell renewal, and blood flow[3]. NSAIDs are often utilized for their analgesic, antipyretic, and anti-inflammatory properties[4]. However, NSAID long-term use is one of the most common causes of gastric damage and delayed ulcer healing via several mechanisms, including inhibition of prostaglandin synthesis[5,6].

In this regard, indomethacin was shown to possess greater ulcerogenic potential compared to conventional NSAIDs[5]. Indeed, indomethacin is considered the most frequently used NSAID to experimentally induce gastric ulceration[7]. Indomethacin inhibits prostaglandin synthesis and angiogenesis while inducing free radical generation, pro-inflammatory cytokine synthesis, and cyclooxygenase-2 (COX-2) expression[7,8]. These deleterious effects of indomethacin exposure lead to gastric mucosa injury, which in turn activates cell renewal signaling pathways involving the epidermal growth factor receptor (EGFR) and the extracellular signal-regulated kinases 1/2 (ERK1/2)[9,10].

Natural products are increasingly recognized for their prophylactic and therapeutic properties. In 2018, natural compounds comprised 16% of new drugs approved by the FDA[11]. Sesquiterpene lactones are a class of secondary metabolites found in plants and can be divided based on structure into separate groups, including guaianolides[12]. Guaianolides are known to possess several therapeutic properties,

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such as antioxidant and anti-inflammatory activities[13]. These biological activities of guaianolides might stem from the α-methylene-γ-lactone moiety that potentially interacts with the cysteine sulfhydryl moieties of cellular proteins[14].

Excellent natural sources of guaianolide sesquiterpenes are *Costus speciosus* rhizomes, which are often utilized in India as traditional medicine with anti-inflammatory, hepatoprotective, antidiabetic, antihyperlipidemic, and antimicrobial properties[15]. In this regard, mokko lactone is a promising guaianolide sesquiterpene lactone with reported antioxidant, anti-inflammatory, and anti-apoptotic characteristics[16-18]. It was also shown that mokko lactone treatment confers protection against the toxic effects of doxorubicin exposure in rats[17,18].

Therefore, this study aimed to examine the protective effects of mokko lactone against gastric injury induced by indomethacin in rats.

MATERIALS AND METHODS

Drugs and Reagents: Mokko lactone ML (purity > 98%) was isolated from *Costus speciosus* rhizomes extract as previously described by Sirwi *et al*., 2021[17]. Indomethacin (Catalog number: PHR1247) was procured from Sigma-Aldrich (St. Louis, MO, USA). All the other used reagents were of premium grades.

Animals: Male Wistar rats, weighing about 200 grams, were acquired from the local animal facility, Faculty of Pharmacy, Kind Abdulaziz University. One week prior to the initiation of the study, rats were allowed to familiarize themselves with the workroom environments. During animal residence, they were kept in a normal laboratory setting. The laboratory conditions were adjusted to a room temperature of 22 ˚C ± 2, relative humidity of about 50%, and alternative dark and light cycles of 12/12 hours. Rats were fed with regular chow and supplied with water *ad libitum*. Methods involving animal manipulations and treatments followed the ARRIVE guidelines, and the research protocol was approved by the local Research Ethics Committee (permit # PH-1443-37).

Study Design: The study was performed on thirty rats that were distributed randomly, in groups of six, into five treatment groups as follows:

Group one: The control group received 0.5 % CMC, the vehicle for mokko lactone and indomethacin, at the respective treatment schedule. Group two was treated with 0.5% CMC for seven consecutive days and indomethacin one hour following the last dose.

Group three was treated with mokko lactone 5 mg/kg p.o., suspended in 0.5% CMC, for seven consecutive days, then indomethacin one hour following the last dose.

Group four was treated with mokko lactone 10 mg/kg p.o., suspended in 0.5% CMC, for seven consecutive days, then indomethacin one hour following the last dose.

Group five was treated with omeprazole (30 mg/kg, p.o.) suspended in 0.5% CMC for seven consecutive days, then indomethacin one hour following the last dose.

Gastric ulcer was induced by the oral administration of a single dose of indomethacin (50 mg/kg) on the last day of the experiment, and treatments were given after 24 hours of food deprivation and one hour of water deprivation. Indomethacin, mokko lactone, and omeprazole were suspended in 0.5 % CMC and administered by gastric lavage. Rats were sacrificed by decapitation, six hours next to inducing gastric ulcer using indomethacin. Stomachs were excised, slit at the lengthier arch, and flushed with saline. Part of the tissues were frozen at – 80 ˚C for the biochemical analyses, and the other part was kept in 10 % formalin, then molded into paraffin blocks for the preparation of tissue slides to be used in the histopathological and immunohistochemical assessments. The sample protein concentration was assessed using Bradford Protein Assay Kit (Catalog number, MBS355526), obtained from MyBioSource, Inc., San Diego, CA, USA, and the biochemical parameters results were expressed in terms of mg protein.

Histopathological Examination and Scoring of the Stomach Lesion: Stomach tissue sections, 5 mm thick, were cut using a tissue microtome, then stained with Hematoxylin and Eosin (H and E) and inspected with a light microscope according to Bancroft and Gamble (2008)[19]. A tissue scoring system explained by Shah *et al.,* (1997)[20] was followed to evaluate the histopathological abnormalities matching with specified stomach lesions' degree of severity as follows: epithelial cell sloughing (score: 0–3), bleeding (score: 0–4), inflammatory cell infiltration (score: 0–2) and mucosal attritions (score: 0–4).

Assessment of the Oxidative Stress Biomarkers: The antioxidant potential of mokko lactone against indomethacin-induced oxidative stress was evaluated by detecting the levels of malondialdehyde (MDA), using the MDA colorimetric assay kit, and the levels of reduced glutathione (GSH), using the GSH colorimetric assay kit (Catalog numbers MD2529 and GR2511, respectively). Moreover, catalase (CAT) and superoxide dismutase (SOD), activities were also assessed using the commercially available kits CA2517 and SD252, respectively. Kits were bought from Biodiagnostics (Cairo, Egypt), and all detection procedures were performed by strictly following the kits' directions.

Assessment of the Immunohistochemistry of Stomach Proteins: The protein expressions of cyclo-oxygenase-2 (COX-2), interleukin-6 (IL-6), caspase-3, and matrix metalloproteinase-9 (MMP-9) were assessed using the immunohistochemical technique according to Key 2006[21]. Briefly, stomach tissue sections (5 µm) were deparaffinized before antigen retrieval, then washed for five minutes using 0.1 M PBS (pH 7.4) stomach tissue sections were kept for 18 hours in diluted rabbit primary antibodies: anti-COX-2, anti-IL-6, anti- caspase-3 or anti-MMP-9 (Abcam, Cambridge, UK, Catalog #, ab179800, ab9324, ab184787, and ab76003, respectively). Mouse and Rabbit Specific HRP/DAB Detection IHC kit (Abcam, Cambridge, UK, Catalog # ab64264) was used to stain the target protein. From each sample, five non-overlapping fields were taken and analyzed by ImageJ 1.53t image analysis software (National Institutes of Health, Bethesda, MD, USA). Optical density (OD) was used to express the intensity of protein expression.

Assessment of the Mucin Content in the Stomach: The stomach mucin content was measured using the commercial ELISA kit (Rat-Mucin-1 ELISA Kit, Catalog #: MBS1607935) purchased from MyBiosource (Southern California, San Diego, USA). The assay depends on the ELISA sandwich technique, and all detection procedures were performed by strictly following the kits' directions.

Assessment of the PGE2 Protein levels in the Stomach: The stomach prostaglandin-E2 (PGE2) content was measured using the commercial ELISA kit (Rat-PGE2-ELISA Kit, Catalog number: MBS262150) purchased from MyBiosource (Southern California, San Diego, USA). The assay depends on the ELISA sandwich technique, and all detection procedures were performed by strictly following the kits' directions.

Statistical Analyses: All the data of the study, except the results of the stomach lesion scoring, are considered parametric data and analyzed by one-way ANOVA, followed by the post hoc test, Bonferroni, and the results are presented as means ± SD. Data from the stomach lesion scoring were analyzed using the Kruskal-Wallis test followed by the post hoc test, Dunn's, and presented as medians, 25th and 27th percentiles, maximums, and minimums. *p*-value less than 5% reflects statistically significant differences between treatment groups. GraphPad Prism software 8.0.2 (San Diego, CA, USA) was utilized.

RESULTS

Effect of Mokko Lactone on Indomethacin-Induced Gastric Damage in Rats

Examination of the tissue from the control group showed normal glandular mucosa and submucosa (Fig. 1A). In contrast, intense histopathological perturbations were spotted in the indomethacin-only-treated group. Multifocal ulcerative areas were clear in the glandular mucosa, which are described by desquamation of the epithelial lining together with bleeding and buildup of eosinophilic and karyorrhectic necrotic tissue debris. Tissue sections exhibited increased inflammatory cell infiltration in the mucosa and submucosal layers. Increase in vascular permeability was observed in the submucosal blood vessels with subsequent abundant edema and hemorrhages (Fig. 1B). The examination of the tissue sections from animal pretreated with mokko lactone 5 mg/kg before indomethacin revealed a few changes which are characterized by mild epithelial sloughing with fewer number of inflammatory cells infiltration in the mucosa and submucosal layer. Cystic dilated gastric glands were less commonly detected than in the indomethacin-only-treated rats (Fig. 1C). Pretreatment with mokko lactone 10 mg/kg showed fewer histopathological changes in the examined sections. The mucosa showed increased inflammatory cell infiltration. The submucosa showed edema and congested blood-filled spaces (Fig. 1D). Sections from animals pretreated with omeprazole showed almost normal glandular mucosa. Lower inflammatory cell infiltration with congested blood vessels was also detected (1E). Upon analyzing the scores of the stomach lesions, the intake of indomethacin showed a significantly higher lesion score in comparison to the control group. Contrariwise, pretreatment with mokko lactone (5 or 10 mg/kg) or omeprazole showed a significantly lesser lesion score in comparison to the indomethacin-only-treated group (Fig. 1F).

Effect of Mokko Lactone on the Oxidative Stress Biomarkers in the Gastric Mucosa of Indomethacin-Challenged Rats

As shown in Table 1, indomethacin intake led to a significant increment in the MDA content by 397 %, alongside a significant decrease in GSH content by 70% compared to control rats. Furthermore, the catalytic activities of the antioxidant enzymes SOD and CAT were noticeablyreduced following indomethacin exposure by 59% and 48%, respectively. Both mokko lactone and omeprazole co-treatment at all doses tested substantially ameliorated these pathological changes induced by indomethacin in rat gastric tissues. Interestingly, mokko lactone (10 mg/kg) significantly corrected these indomethacin-induced changes in MDA, GSH, SOD, and CAT by 35%, 77%, 66%, and 65%, respectively.

Effect of Mokko Lactone on Indomethacin-Induced Alterations in Gastric Expression of Inflammatory Markers in Rats.

Indomethacin exposure induced a pro-inflammatory state as indicated by the significantly elevated expression levels of COX-2 and IL-6 in the gastric mucosa by 175% and 156%, respectively **(Fig. 2).** Pretreatment with mokko lactone at 5 mg/kg significantly decreased COX-2 and IL-6 by 33% and 30%, respectively, in comparison to the indomethacin group. Moreover, the expression levels of these pro-inflammatory markers were normalized by mokko lactone at 10 mg/kg and omeprazole co-administration.

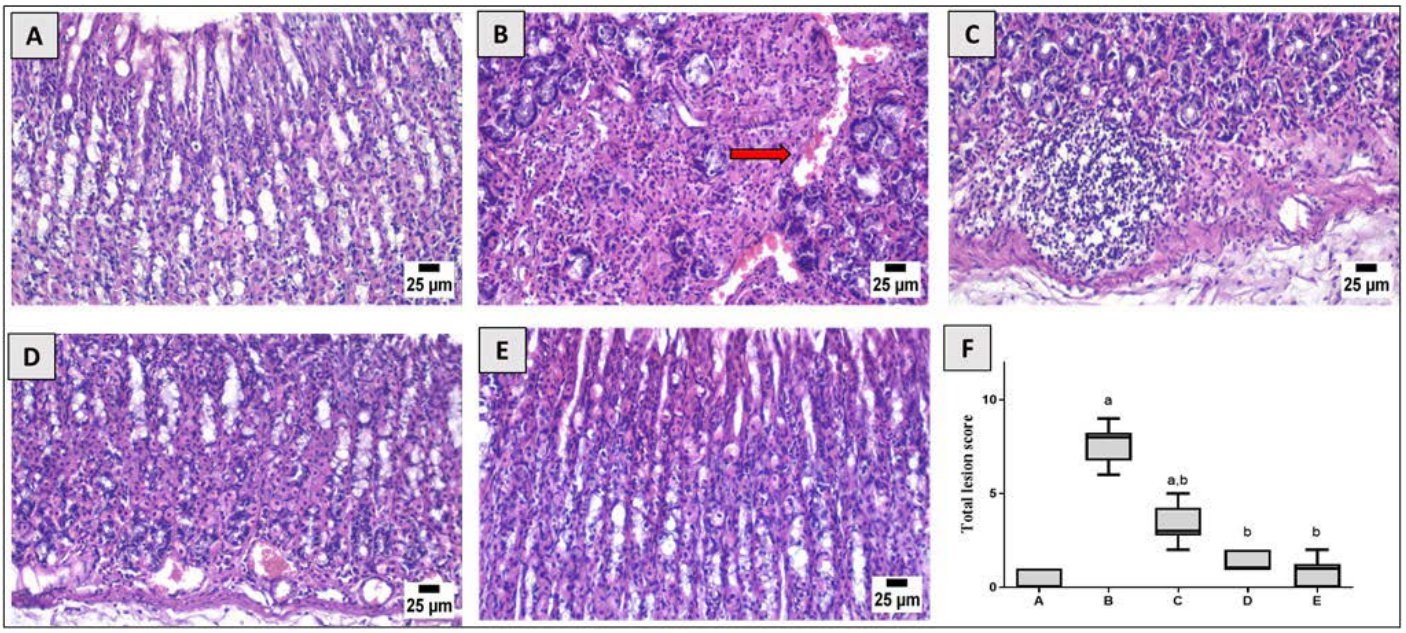

Figure 1: Photomicrographs of the rat stomachs stained with hematoxylin and eosin revealing the histological structure of (A) control; (B) indomethacin-only; (C) indomethacin and mokko lactone low dose-pretreated; (D) indomethacin and mokko lactone high dose-pretreated; (E) indomethacin and omeprazole pretreated groups; (F) ulcer score data tested by Kruskal Wallis followed by Dunn's test. a and b indicate statistically significant referring to the control, and indomethacin-only groups, respectively, at $p < 0.05$.

Table 1: Effect of mokko lactone on indomethacin-induced oxidative stress in rat gastric tissues

	Control	Indo	Indo + ML (5 mg/kg)	Indo + ML (10 mg/kg)	Indo + Omep
MDA (nmol/mg protein)	0.72 ± 0.09	3.58[a] ± 0.62	2.72 [a,b] ± 0.29	2.32 [a,b] ± 0.26	1.74 [a,b,c,d] ± 0.22
GSH (µg/mg protein)	0.44 ± 0.05	0.13 [a] ± .01	0.21 [a,b] ± 0.02	0.23 [a,b] ± 0.02	0.24 [a,b] ± 0.03
SOD (U/mg protein)	43.28 ± 5.6	17.84 [a] ± 2.01	28.50 [a,b] ± 3.2	29.57 [a,b] ± 3.3	38.25 [a,b,c] ± 3.1
CAT (U/mg protein)	0.92 ± .01	0.48 [a] ± 0.5	0.70 [a,b] ± 0.07	0.79 [a,b] ± 0.08	0.77 [a,b] ± 0.07

Data are exhibited as Mean ± SD
Statistical analysis was performed by one-way ANOVA, followed by the Bonferroni
[a,b,c,d] indicate statistical significance referring to the control, Indo, Indo + ML (5 mg/kg), and Indo + ML (10 mg/kg) groups at $p < 0.05$.

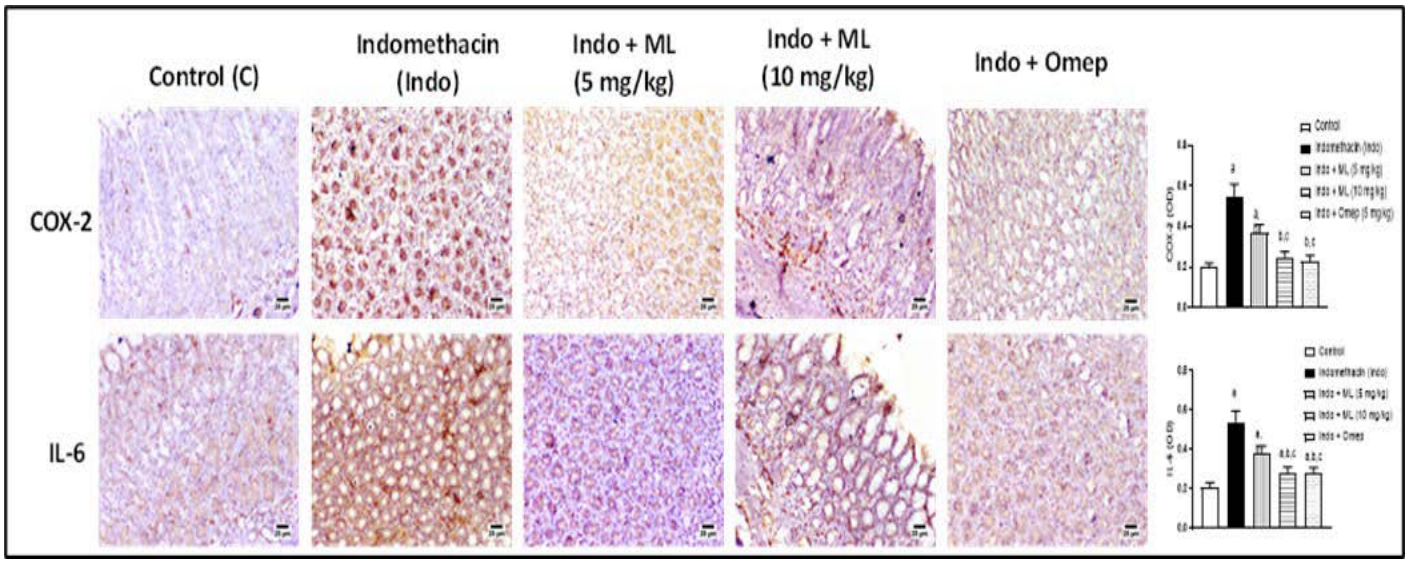

Figure 2. Effect of mokko lactone on gastric expression of COX-2 and IL-6 in rats challenged with indomethacin as evaluated immunohistochemically. Data of the OD were tested by one-way ANOVA, followed by Bonferroni, and presented as means ± SD (n = 6). a, b, or c indicates statistical difference referring to the control, Indo, or Indo + ML (5 mg/kg), respectively at $p < 0.05$.

Effect of Mokko Lactone on Indomethacin-Induced Changes in Mucin and PGE_2 Gastric Expression in Rats

As demonstrated in Figure 3, induction of gastric mucosal injury by indomethacin significantly decreased the content of mucin and PGE_2 by 61% and 68%, respectively. Yet, mokko lactone cotreatment at both doses, 5 and 10 mg/kg, significantly increased the protein content of mucin by 46% and 51%, respectively and PGE_2 by around 65% and 121%, respectively, when compared to the indomethacin group. Omeprazole cotreatment also produced similar findings, where it significantly ameliorated the diminished expression of mucin and PGE_2 combined with indomethacin administration.

Effect of mokko lactone on indomethacin-induced changes in caspase-3 and MMP-9 gastric expression in rats

Immunohistochemical assessment of gastric tissues demonstrated a substantial rise in expression of caspase-3 and MMP-9 following indomethacin administration in rats, as indicated by the higher percentage of immunoreactive cells compared to intact mucosa by 142% for caspase-3 and 100% for MMP-9 (Fig. 4). Cotreatment with mokko lactone at 5 and 10 mg/kg led to significant inhibition of caspase-3 by 25% and 43%, respectively, and MMP-9 by 26% and 29%, respectively, relative to the indomethacin group. Similarly, omeprazole coadministration significantly ameliorated the increased gastric expression of caspase-3 and MMP-9 induced by indomethacin.

DISCUSSION

Gastrointestinal diseases, including gastric ulcers, are driven by various defense factors such as renewal of the gastric mucosa and the mucus-bicarbonate barrier[22,23]. NSAIDs, including indomethacin, are linked to the development of gastric ulcers via multiple mechanisms[24]. For instance, gastric damage induced by indomethacin in rats is known to develop via amplified production of reactive oxygen species, as well as disrupting prostaglandin synthesis[25,26]. On the other hand, mokko lactone is a natural sesquiterpene lactone that acquires significant antioxidant, anti-inflammatory, and cytoprotective activities[17,18]. It was also shown that the structurally related compounds costunolide and dehydrocostus lactone express significant anti-ulcerogenic activities in mice[27]. Hence, the present work aimed to assess the gastroprotective potential of mokko lactone against indomethacin-induced gastric damage in rats.

Mokko lactone in this study showed significant protective activity against mucosal damage as indicated by restoring the normal histological features of the gastric mucosa to a great extent, which was further emphasized by analyzing the gastric lesion scores data. These protective effects against indomethacin-induced mucosal injuries were even statistically comparable to the established gastroprotective effects of omeprazole. Consistently, a previous study showed that costunolide, a structurally related compound, possesses significant protective activity against gastric ulceration in animals, highlighting the potential of sesquiterpene lactones for gastroprotection[27]. In this

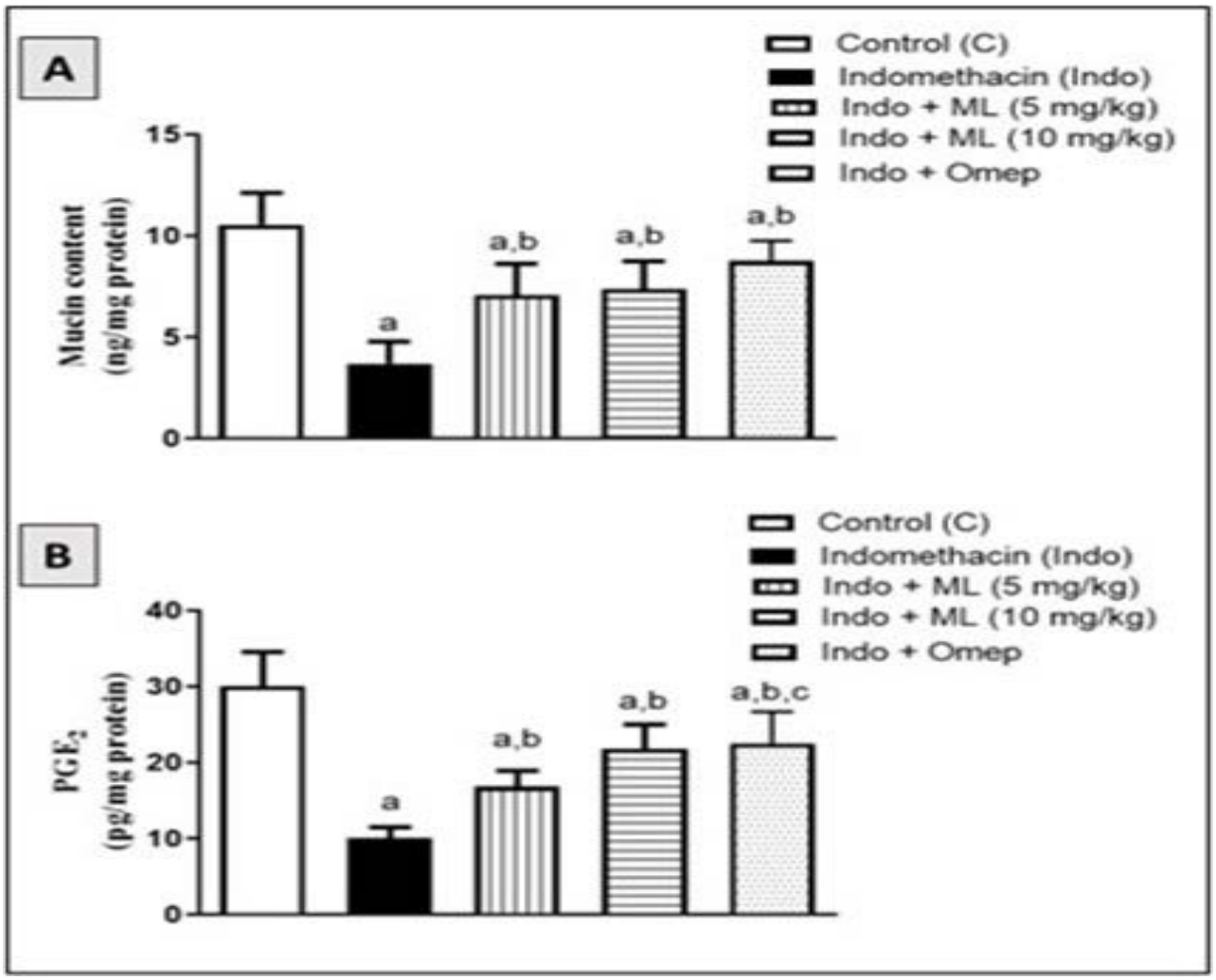

Figure 3. Effect of mokko lactone on gastric content of mucin and PGE_2 in rats challenged with indomethacin. Data were analyzed by one-way ANOVA, followed by Bonferroni, and presented as means ± SD (n = 6). a, b, or c indicates statistical difference referring to the control, Indo, or Indo + ML (5 mg/kg), respectively at $p < 0.05$.

regard, administration of indomethacin resulted in the development of oxidative stress in injured mucosa as showed by the accumulation of the lipid peroxidation product MDA and the reduced antioxidant capacity of GSH, SOD and CAT. This pro-oxidant potential of indomethacin could be derived from its metabolism by peroxidases in the gastric mucosa[28]. Interestingly, mokko lactone significantly reduced gastric oxidative stress associated with indomethacin administration. This antioxidant activity of mokko lactone could be justified by the existence of the α-methylene-γ-lactone ring in its structure. This structural feature enables interaction with many peptides and proteins via their cysteine sulfhydryl structural moieties[14]. This is also in synchronization with the reported antioxidant activity of mokko lactone in different organs[17,18].

Indomethacin exposure also induced a pro-inflammatory state, as evidenced by the increased gastric mucosal expression of COX-2 and IL-6 relative to unchallenged gastric mucosa. According to several reports, increased expression of the inducible COX-2 is indicative of injured gastric mucosa[29]. In contrast, the mokko lactone plus indomethacin group expressed significantly lower levels of mucosal COX-2 and IL-6, underscoring the anti-inflammatory activity of mokko lactone. Comparable anti-inflammatory activity of mokko lactone was also reported in different organs, such as the heart and liver[17,18]. This is also consistent with the anti-inflammatory activity of the structurally related compound dehydrocostus lactone in the context of ethanol-induced gastric ulcer in mice[27]. It should be noted that inflammation is a major cause of oxidative stress and reduced antioxidant capacity, which can further promote the dysregulated oxidative stress status of indomethacin-injured gastric mucosa[30].

Our experimental findings also demonstrated that the indomethacin group expressed lower levels of mucin and PGE2. Other studies also consistently highlighted the inhibitory effect of indomethacin on both mucin and PGE2 levels[31-33]. Compromised mucin content induced by indomethacin challenge is known to promote gastrointestinal damage[33]. On the other hand, the results of the mokko lactone-treated group indicated significant induction of mucin and PGE2 content. Increased expression of mucin and PGE2 is associated with ulcer healing and combating the ulcerative potential of indomethacin on the gastric mucosa[31,33,34].

This study also showed a significant upregulation of mucosal caspase-3, a primary effector during apoptosis, in the indomethacin-administered group. Caspase-3 activation and apoptosis enhancement are critical pathological events in indomethacin-induced gastric ulceration[35]. Mokko lactone significantly ameliorated this increased expression of caspase-3, which is consistent with previous findings[17,18]. Furthermore, MMP-9, an important marker of tissue remodeling in gastric ulcerogenesis[36], was also found in this study to be induced by

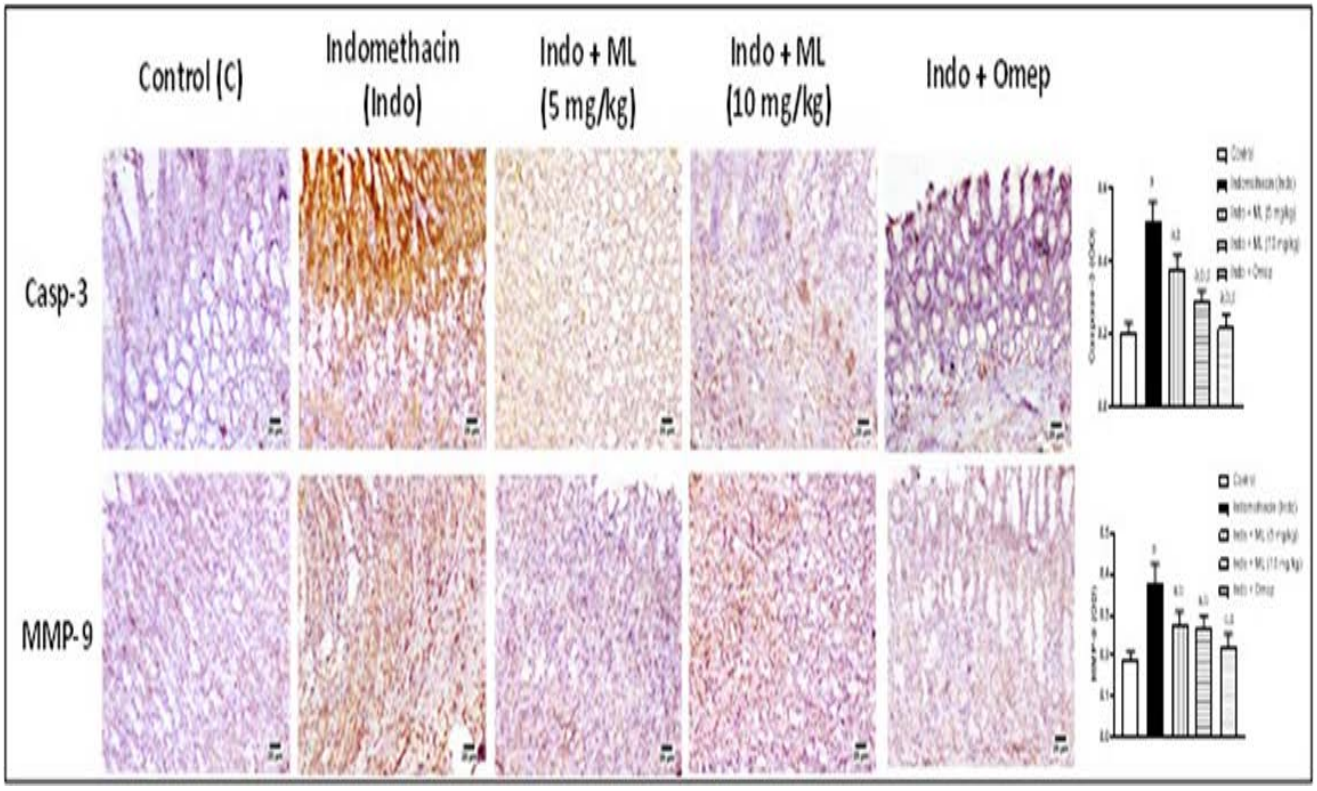

Figure 4. Effect of mokko lactone on gastric expression of Casp-3 and MMP-9 in rats challenged with indomethacin as evaluated immunohistochemically. Data of the OD were tested by one-way ANOVA, followed by Bonferroni, and presented as means ± SD (n = 6). a, b, or c indicates statistical difference from control, Indo, or Indo + ML (5 mg/kg), respectively ($p < 0.05$).

indomethacin in injured gastric mucosa. Increased mucosal expression of MMP-9 can indicate a malfunctioned remodeling process, which can lead to inflammation and ulceration of the gastric connective tissues[36,37]. In contrast, decreased immunohistochemical staining for MMP-9 associated with mokko lactone administration is reflective of reduced proteolysis of the gastric mucosa[38]. These beneficial effects of mokko lactone might stem from its significant antioxidant activity, as oxidative stress was implicated in upregulating MMP production[37].

Acknowledgments

The authors express their gratitude to the Pharmacy students; Omar Sairafi, Sultan Malibari, Faris Deban, Ahmed Alsolami for their technical help in the assessment of gastric ulcer study.

Funding

The Deanship of Scientific Research (DSR) at King Abdulaziz University (KAU), Jeddah, Saudi Arabia has funded this project under grant no (G: 148-249-1443).

CONCLUSION

In conclusion, the findings of this work showed a substantial protective activity of mokko lactone against gastric injury induced by indomethacin in rats. The activity of mokko lactone, particularly at the higher dose, exhibited comparable activities to that of omeprazole. These findings warrant deeper investigations of the specific molecular pathways involved, toxicity profiling, formulation development and escalation to the clinical level.

Authorship Contribution: All authors share equal effort contribution towards (1) substantial contributions to conception and design, acquisition, analysis and interpretation of data; (2) drafting the article and revising it critically for important intellectual content; and (3) final approval of the manuscript version to be published. Yes.

Potential Conflicts of Interest: None

Competing Interest: None

Acceptance Date: 23 June 2025

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