Ellagic acid facilitates indomethacin-induced gastric ulcer healing via COX-2 up-regulation

Ananya Chatterjee, Sirshendu Chatterjee, Smita Das, Arpita Saha, Subrata Chattopadhyay, and Sandip K. Bandyopadhyay

1Department of Biochemistry, University College of Medicine, I.P.G.M.E&R, 244B A.J.C. Bose Road, Kolkata -700020, West Bengal, India
2National Tea Research Foundation, Tea Board, 14, B.T.M. Sarani, Kolkata 700001, India
3Bio-Organic Division, Bhabha Atomic Research Center, Mumbai, India

*Correspondence address. Tel: +91-33-30016100; Fax: +91-33-30016170; E-mail: skb.teaboard@gmail.com

The mechanism of indomethacin-induced gastric ulcer healing by ellagic acid (EA) in experimental mice model is described in our study. Ulcer index (UI) and myeloperoxidase (MPO) activity of the stomach tissues showed maximum ulceration on the third day after indomethacin (18 mg/kg, single dose) administration. Preliminary observation of UI and MPO activity suggests that EA possesses ulcer-healing activity. Other anti-ulcer parameters such as the levels of prostaglandin E2, cyclooxygenase (COX) 1 and 2 enzymes, anti-inflammatory cytokines [interleukin (IL)-4 and -5], pro-angiogenic factors, e.g. vascular endothelial growth factor, hepatocyte growth factor (HGF), and endothelial growth factor (EGF) were down-regulated by indomethacin. EA (7 mg/kg/day) treatment for 3 days shifted the indomethacin-induced pro-inflammatory biochemical parameters to the healing side. These activities were correlated with the ability of EA to alter the COX-2-dependent healing pathways. The ulcer-healing activity of EA was, however, compromised by pre-administration of the specific COX-2 inhibitor, celecoxib, and NS-398. Taken together, these results suggested that the EA treatment accelerates ulcer healing by inducing IL-4, EGF/HGF levels and enhances COX-2 expression.

Keywords: celecoxib; COX-2; ellagic acid; ulcer

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Introduction

Gastric ulcer is a common ailment throughout the world, in which the gastric mucosa becomes damaged and perforations lead to bleeding. It affects ~10% of the world population [1]. Varieties of both endogenous and exogenous factors, including acid, pepsin, stress, and noxious agents such as alcohol, non-steroidal anti-inflammatory drugs (NSAIDs), Helicobacter pylori bacteria, smoking, and alcohol consumption are known to cause or aggravate gastric ulcer [2]. NSAID is one of the main causative factors responsible for this gastric ulcer disease. Long-term use of NSAID damages gastric mucosa by reducing prostaglandin (PG) synthesis through inhibition of cyclooxygenase (COX) enzymes and partially on COX-independent mechanisms [3]. NSAIDs are widely prescribed drugs in the world, and are extensively used to alleviate clinical cases especially for pain and inflammation [4]. However, these drugs are well known to induce stomach ulceration, and delay ulcer healing [5]. Among the total gastric ulcer patients, 20%–30% cases have an upward trend due to NSAIDs [6]. Despite recent advances, adequate remedy for the NSAID-induced gastropathy remains elusive. Thus, there is a growing interest in the scientific community to develop drugs from plant origin, which will be cheap, non-toxic, and accessible, particularly to the rural people in the developing countries. Therefore, development of a drug with anti-ulcerogenic property from plant sources without compromising the efficacy and safety would be expected to benefit millions of suffering humanity.

Ellagic acid (EA) (2,3,7,8-tetrahydroxy[1] benzopyrano[5,4,3-cde][1] benzopyran-5,10-dione) is one of the naturally occurring polyphenols (Fig. 1) found in raspberries, strawberries, cranberries, walnuts, Indian gooseberries, pecans, pomegranates, and other plant foods mainly in the form of ellagitannins [7]. Numerous in vitro and animal studies have suggested the anti-proliferative and antioxidant properties of EA [8–11]. Indeed, EA has been shown to exert a potent scavenging action on both superoxide anion and hydroxy anion in vitro [12]. EA can elicit a dose-dependent bactericidal effect to H. pylori, one of the causative factors for severe gastritis and gastric ulcers [13]. EA provides mucosal protective action in the stomach against ethanol or ischemia-reperfusion injury [12,14], and its potency is equivalent to anti-oxidative agent superoxide dismutase. EA can provide significant protection to colonic...
mucosa during the inflammatory response in dextran sulfate sodium-induced ulcerative colitis and reduce the gross mucosal injury, by the inhibition of myeloperoxidase (MPO) activity as well as lipid peroxidation [15]. However, the role of this polyphenol on indomethacin-induced gastric ulcer healing in mice has not yet been reported properly. In this study, we aimed to decipher the plausible mechanistic pathway involved in indomethacin-induced gastric ulcer healing on experimental mice model by EA.

Materials and Methods

Chemicals
EA, indomethacin, omeprazole, 5-bromo-4-chloro-3-indolyl phosphate, nitroblue tetrazolium, Tween-20, Bradford reagent, and hexadecyl trimethyl ammonium bromide (HTAB) were purchased from Sigma (St Louis, USA); ethanol and methanol from E. Merck (Mumbai, India); bovine serum albumin (BSA) from Merck (Darmstadt, Germany); dimethylformamide, and tetramethyl benzidine (TMB) from Acros (Geel, Belgium); polyclonal rabbit anti-goat COX-1 and polyclonal goat anti-rabbit COX-2 antibodies from Santa Cruz Biotechnology (Santa Cruz, USA); PG E2 EIA kit, growth factors such as vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), endothelial growth factor (EGF) enzyme-linked immunoassay (ELISA) kit from Cayman Chemical (Ann Arbor, USA). Cytokines tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-4, IL-6 and IL-10 ELISA kit were purchased from Pierce Biotechnology (Rockford, USA). Other reagents such as 35% hydrogen peroxide (H₂O₂) were purchased from Lancaster (Morecambe, UK); disodium hydrogen phosphate and sodium dihydrogen phosphate from BDH (Poole, Dorset, UK). All the other chemicals used for this study were of the highest purity available from the local suppliers.

Animals
Male Swiss albino mice (aged 6-8 weeks, weight 25 ± 2 g) bred in-house with free access to food and water were used. The mice were kept in 12-h light/dark cycles and housed at 25 ± 1°C. The animal experiments (n = 8) were carried out in accordance with the guidelines of the animal ethics committee of the Postgraduate Institute of Basic Medical Sciences, Kolkata, Animal Ethical Committee, Sanction No IAEC/SB-3/2008/UCM-64 Dated-15/05/08–2011, and were handled following the International Animal Ethics Committee Guidelines, ensuring minimum animal suffering.

Preparation of test samples
The test samples (EA and omeprazole) were prepared as aqueous suspensions using 2% gum acacia as the vehicle and administered to the mice orally. In some experiments, the mice were additionally treated intraperitoneally with COX-2 inhibitors viz. celecoxib (3 mg/kg, once daily) and NS-398 (1 mg/kg, once daily) for 3 days separately.

Experimental protocol for ulceration and assessment of healing
The mice were divided into several groups (eight mice per group) and each experiment was repeated four times. Except for the normal control group, ulceration in the other mice was induced by indomethacin (18 mg/kg, orally, single dose), dissolved in distilled water and suspended in 2% gum acacia as the vehicle [16,17]. The animals were deprived of food but with free access to tap water for 24 h, before ulcer induction. The normal and untreated control groups received the vehicle only throughout the course of the experiments. The treatment groups received different doses of EA (5, 6, 7, 8, and 10 mg/kg once daily) and omeprazole (3 mg/kg, once daily) [18] as positive control for different periods, with the first dose started at 6 h after indomethacin administration. On the first, second, third, fourth, and seventh day, the mice were killed by cervical dislocation under anesthesia (ketamine, 12 mg/kg). Here, only third day data are incorporated. The stomachs from the normal and treated groups were removed rapidly, opened along the greater curvature, and thoroughly rinsed with normal saline.

Histological study of damage score
The fundic portion of stomach was sectioned for histological studies as well as damage score analysis. The tissue samples were fixed in 10% formalin and embedded in paraffin. The sections (5 μm) were cut using microtome, stained with hematoxylin and eosin and assessed under an Olympus microscope (BX41; Tokyo, Japan). From the histological slides, the damage scores were assessed [19] by grading the gastric injury on a 0-4 scale, based on the severity of hyperemia and hemorrhagic erosions: 0, almost normal mucosa; 0.5, hyperemia; 1, one or two lesions; 2, severe lesions; 3, very severe lesions; and 4, mucosa full of lesions (lesions—hemorrhagic erosions, hyperemia—vascular congestions). The sum of the total scores divided by the mean damage score is expressed as the damage score. The
experiments were carried out by two investigators blinded to the groups and the treatment of animals.

Myeloperoxidase assay
MPO activity was determined according to the standard procedure [20] with minor modifications. In brief, whole gastric glandular portions of the stomach taken from all groups (100–150 mg) were homogenized in a 50-mM phosphate buffer (pH 6.0) containing 0.5% HTAB. This was followed by three cycles of freezing and thawing. The homogenate was centrifuged at 12,000 g for 20 min at 4°C. The supernatant (50 μl) was collected for MPO assay and added to 80-mM phosphate buffer, pH 5.4, 30-mM TMB, and 300-mM H2O2, to make a final reaction volume of 500 μl. After the mixture was incubated at 25°C for 25 min, the reaction was terminated by adding 500 mM of H2SO4 and the absorbance was measured at 450 nm.

Western blot analysis of COX enzyme
The glandular part of the gastric mucosa was washed with phosphate-buffered saline (PBS) containing protease inhibitors. Then the tissue was minced and homogenized in a lysis buffer (1 ml) containing 200 μl of 5 × Tris–ethylene-diaminetetraacetic acid buffer, 200 μl of 5 × NaCl, 200 μl of 5 × sodium dodecyl sulfate (SDS), 200 μl of 5 × deoxycholic acid, 200 μl of 5 × Igepal, and 10 μl of protease inhibitor cocktail. The homogenized tissue samples were homogenized and centrifuged at 12,000 g for 10 min, at 4°C and the protein concentration of the supernatant was measured by using commercially available Bradford reagent [21]. The proteins were resolved by 10% non-reducing SDS polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. The membrane was blocked for 2 h at room temperature in 20 mM Tris–HCl, pH 7.4, 150 mM NaCl, and 0.02% Tween 20 (TBST) containing 3% BSA.

Overnight incubation of the membrane at 4°C in primary antibody (1:500) of COX-1, COX-2, and β-actin in TBST buffer containing 3% BSA was undertaken. The membrane was washed three times with TBST buffer and thereafter incubated with alkaline phosphatase-conjugated secondary antibody. Then the membrane was washed again three times with TBST buffer and finally the bands were visualized using 5-bromo-4-chloro-3-indoly1 phosphate/nitro blue tetrazolium substrate solution. Band intensity of COX-1 and COX-2 were measured using ImageJ software and presented in result section as compared with β-actin.

PGE2 assay
Following the harvesting of the stomach, the corpus (full thickness) was excised, weighed (100 mg), and suspended in 10 mM of sodium phosphate buffer, pH 7.4 (1 ml). The tissues were finely minced and incubated at 37°C for 20 min. After centrifugation (9000 g), the PGE2 levels in the supernatant were measured by ELISA, using the PG E2 EIA kit according to the manufacturer’s instructions.

Estimation of tissue cytokine levels
TNF-α, IL-1β, IL-4, IL-6, and IL-10 levels in the tissue homogenate were estimated using commercially available ELISA kits, according to the manufacturer’s protocol.

The glandular part of the gastric mucosa after being washed with PBS containing protease inhibitors, was minced and homogenized in a lysis buffer (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100) containing leupeptin (2 μg/ml) and phenylmethylsulfonyl fluoride (0.4 μM). Then the homogenate was centrifuged at 15,000 g for 30 min at 4°C, and the supernatant was collected and cytokines levels were measured.

The samples along with the standards were seeded to each well at an appropriate dilution and incubated at room temperature for 90 min. The wells were washed (five times), diluted polyclonal antibody (100 μl) was added, and the mixture was incubated further for 2 h at room temperature. The wells were washed again and incubated for 2 h after addition of horseradish peroxidase-conjugated (100 μl) secondary antibody. After the final wash, TMB (100 μl) was added to each well, the mixture was incubated for 15 min, the reaction was stopped by addition of 0.16 M H2SO4, and the absorbance was measured at 450 nm.

Estimation of tissue growth factor levels
Levels of VEGF, EGF, and HGF in the tissue were estimated using commercially available ELISA kits.

Statistical analysis
Data are expressed as the mean ± SD unless mentioned otherwise. Comparisons were made between different treatments using one-way analysis of variance followed by an error protecting multiple comparison procedure, namely Tukey–Kramer post hoc test by Graph Pad InStat (GraphPad Software Inc., San Diego, USA) software for the analysis of significance of all the data.

Results
Histological assessment
Onset of ulceration (i.e. superficial erosion and mild inflammation in the stomach) was observed within 6 h after indomethacin administration, indicating acute ulceration (data not shown). However, on the third day, marked damage to the glandular portion of the gastric mucosa was noticed in the histological photograph of the stomach sections of the third day-ulcerated group of mice. Hemorrhagic serosa was evident on the third day of
ulceration. Treatment with EA and omeprazole for 3 days reduced the number of inflammatory cells and mucosal congestion, and increased the number of healthy normal cells in the gastric mucosa, submucosa, serosa, and muscle layers. The healing effect of EA was slightly better than omeprazole. The histological photographs of stomach sections of the third day groups of normal, ulcerated, and treated mice are shown in Fig. 2.

Assessment of ulcer healing
The mice receiving only the vehicle, showed no lesion in the gastric mucosa. Indomethacin (18 mg/kg, orally, single dose) administration produced acute mucosal lesions in the mice stomach, as evident from damage score analysis. Quantification of the damage scores revealed maximum ulcerative damage on the third day of indomethacin administration. However, the ulcerative damage was reduced on the seventh day (data not shown). Among the chosen doses of EA, best ulcer healing was observed at a dose of 7 mg/kg body weight once daily for 3 days, orally [Fig. 3(A)] at peak ulceration. Compared with the untreated group, the damage scores in the EA and omeprazole-treated groups were reduced by 58.67% ($P < 0.001$) and 33.33% ($P < 0.01$), respectively.

Regulation of mucosal MPO activity
The mucosal MPO activity of the indomethacin-administered mice, increased immediately, reaching the peak value on the third day (Fig. 4). The results were consistent with the damage score data. Treatment with EA (7 mg/kg once daily for 3 days, orally) and omeprazole reduced the MPO activity by 77.98% ($P < 0.001$) and 68.07% ($P < 0.001$), respectively, compared with that of the untreated group [Fig. 3(B)].

Modulation of COX expression
COX-1 and COX-2 expressions in the gastric mucosa of the sham-treated/control, ulcerated untreated and drug (EA or omeprazole)-treated mice were shown in Fig. 4(A). High COX-1 expression and low COX-2 expression were observed in normal mice. Ulceration significantly depleted the expression of gastric COX-1 compared with the normal group. However, COX-2 expression was not altered by indomethacin. Treatment with EA in ulcerated group elevated COX-1 and COX-2 expressions significantly,
compared with the ulcerated-untreated group. In contrast, the effect of omeprazole in ulcerated group was less prominent than EA. Mucosal COX-1 and COX-2 expressions with respect to β-actin for the control, ulcerated, omeprazole, and EA-treated mice were shown in Fig. 4(B,C).

Regulation of PGE2 synthesis

Compared with the normal control, the mucosal PGE2 level was markedly suppressed (2.38 folds) \((P < 0.001)\) in the untreated-ulcerated mice. Treatment with EA and omeprazole for 3 days up-regulated the mucosal PGE2 level by 2.01 \((P < 0.001)\) and 1.61 \((P < 0.01)\) folds, respectively, compared with the untreated group (Fig. 5).

Effect of EA on cytokines and growth factors regulations

Indomethacin administration modulated the balance of pro/anti-inflammatory cytokines and growth factors levels. ELISA study depicted that indomethacin treatment induced TNF-α (1.78 folds, \(P < 0.001\)), IL-1β (1.95 folds, \(P < 0.001\)), IL-6 (1.04 folds, \(P > 0.05\)), and down-regulated IL-4 (2.26 folds, \(P < 0.01\)), IL-10 (1.24 folds, \(P < 0.05\)), VEGF (1.73 folds, \(P < 0.01\)), EGF (1.56 folds, \(P < 0.01\)), and HGF (1.51 folds, \(P < 0.01\)) levels compared with sham-treated mice. However, EA at its dose 7 mg/kg, significantly reduced the pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) levels (1.99 folds, \(P < 0.001\); 1.49 folds, \(P < 0.01\); and 1.64 folds, \(P < 0.01\), respectively) [Fig. 6(A–C)] and induced anti-inflammatory (IL-4 and IL-10) levels (2.57 folds, \(P < 0.001\) and 1.82 folds, \(P < 0.001\), respectively) [Fig. 6(D,E)] and growth factors (VEGF, EGF, and HGF) levels (1.4 folds, \(P < 0.05\); 1.74 folds, \(P < 0.001\); and 1.72 folds, \(P < 0.001\), respectively) [Fig. 7(A–C)] compared with the ulcerated-untreated mice.

Effect of COX inhibitors on the healing property of EA

To confirm the involvement of COX-2 in EA-mediated ulcer-healing pathway, the ulcerated mice were pretreated with COX-2-specific inhibitor celecoxib and NS-398 before EA (7 mg/kg) administration. Ulcer index (UI) [Fig. 8(A)] and MPO activity [Fig. 8(B)] were significantly increased after COX-2 inhibitor pretreatment compared with the EA (7 mg/kg)-treated mice, followed by depletion of IL-4 [Fig. 9(A)], IL-10 [Fig. 9(B)], EGF [Fig. 9(C)], HGF [Fig. 9(D)], and tissue PGE2 [Fig. 8(C)]. However, COX inhibitor pre-treatment drastically reduced the anti-inflammatory cytokine levels, HGF and EGF levels compared with the EA and thereby completely blocked the healing effect of EA.

Discussion

NSAIDs are used generally in rheumatoid arthritis, pyrexia, headache, migraine, acute gout, bone pain etc. for its analgesic, antipyretic and anti-inflammatory effects [22]. Overdose, improper intake or prolonged use sometimes causes severe gastric ulcer and gastrodudonal disorders [22–24]. Gastric ulcer is a common disease and the characteristics of the disease are shared by other inflammatory disorders in the gut, i.e. the migration of activated neutrophils and macrophages are major components of active lesions in ulceration [25–27]. Large numbers of neutrophils and macrophages enter the injured mucosa during acute inflammation, leading to overproduction of oxygen-free radicals [28], ultimately damaging the gastric mucosa and causing perforations that lead to bleeding. Thus, there is a growing interest and needs to find non-toxic, safe, and inexpensive antioxidants as well as anti-ulcer bio-drugs from medicinal plants. For centuries, different herbs have been used in traditional medicine to treat a wide range of ailments, including dyspepsia, gastritis, and peptic ulcer disease [13,29]. Natural antioxidants are usually considered safe by most consumers, and safety tests are not typically required by legislation because natural products are generally recognized as safe [13].
EA is a natural polyphenolic antioxidant of molecular mass 302.197 g/mol and generally found in several fruits and vegetables including blackberries, raspberries, walnuts, grapes, etc. It is the dilactone of hexahydroxydiphenic acid. The antiproliferative and antioxidant properties of EA have stimulated the research into the potential health benefits of EA [30–33].

In this study, we presented the underlying mechanism underlying gastric ulcer-healing property of EA. Although EA is well known for its anti-ulcer activity for long time, its mechanism has not been studied. Hence, evaluation of the anti-ulcerogenic property of EA in indomethacin-induced ulcerated experimental mice model was carried out. EA (7 mg/kg body weight) was administered orally once daily for 3 days. Our macroscopic examinations revealed that administration of indomethacin (18 mg/kg body weight) caused marked mucosal damage in the stomach within 6 h of ulcer induction and maximum ulcerative damage was observed on the third day. And hence EA treatment was started after 6 h of indomethacin treatment and continued for 3 days. During dose standardization of EA, it was observed that among the doses 1, 5, 10, and 50 mg/kg body weight, 10 mg/kg has shown good healing property. A dose less than 5 mg/kg did not show any ulcer-healing effect. Hence, we increased the dose arithmetically from 5 to 10 mg/kg. We found that the dose 7 and 8 mg/kg EA showed best healing properties and hence the dose 7 mg/kg EA was chosen for this study. Treatment with EA reduced the UI 58.67% (P < 0.001) as compared with ulcerated-untreated
mice. Mice receiving only the vehicle showed no lesions in the gastric mucosa.

The MPO activity is known to increase under ulcerated conditions, and reduced during the healing process [34]. It is often used as a risk marker and diagnostic tool for assessing severity of gastric ulcer [35]. In this study, indomethacin administration led to augment the MPO activity in the ulcerated area of the gastric wall. Treatment with EA reduced the MPO activity 77.98% ($P < 0.001$) compared with the indomethacin-treated group. Histology also supported the damage score and MPO data (Fig. 2).

NSAIDs exert their therapeutic action by inhibiting the COXs (COX-1 and COX-2) and decreasing the levels of circulating PGs. However, the reduced level of PGs at the gastric mucosa is known to cause gastric ulceration and also exacerbates pre-existing gastric ulcers in rodents and humans [36,37]. PGs stimulate mucus and bicarbonate secretion as well as mucosal blood flow, and induce angiogenesis [35]. All these factors contribute to accelerated ulcer healing.

In this study, reduced expressions of COX-1 and COX-2 at peak ulceration were evident by western blot analysis (Fig. 4) in the healing model. Treatment with the EA increased the expressions of both the enzymes, the effect being more predominant on COX-2. The western blot analysis of COX supported the colorimetric data of PGE$_2$

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**Figure 6** Effect of EA (7 mg/kg, single dose daily × 3 days, orally) on different cytokines levels in indomethacin (18 mg/kg, single dose, orally) induced ulcerated mice (A) Tissue TNF-α level. (B) Tissue IL-1β level. (C) Tissue IL-6 level. (D) Tissue IL-4 level. (E) Tissue IL-10 level. The values are shown as the mean ± SD. $n = 8$. One-way analysis of variance was followed by Tukey–Kramer post hoc test. **$P < 0.01$** and ***$P < 0.001$** compared with ulcerated-untreated mice.
Colorimetric data showed that indomethacin treatment depleted tissue PGE2 levels. The results are consistent with some earlier reports with indomethacin [38,39]. Decrease in the level of PGE2 has been reported to prevent the PG-mediated angiogenesis [40] that is essential for ulcer healing. Exogenous PGE or its analogs are the drugs of choice in attenuating and healing the indomethacin-induced gastric mucosal damages. In this study, EA enhanced PG synthesis 2.01 folds ($P$, 0.001) compared with ulcerated untreated group. The effect of EA was better than that of omeprazole. However, Beserra et al. [41] showed that EA did not modify the PGE2 levels in their experimental indomethacin-induced ulcerated rat model during ulcer-healing action. The discrepancy in outcome may be due to the difference that lies between the experimental setup and protocol for ulcer induction.

Stimulation of inflammatory cytokines is extremely important in mucosal defense. One of the most prominent modes of mediation of indomethacin-induced gastropathy is the increased expression of the pro-inflammatory cytokines [42,43], which correlates with the extent of ulceration. After trauma, the Th1/Th2 imbalance with Th2 predominance is reflected by an increase anti-inflammatory cytokine [44]. In view of this, the immune response due to ulceration, and its modulation by the EA was monitored. It enabled us to associate the inflammatory response with a better prognosis. Indomethacin administration raised the serum levels of pro-inflammatory Th1 cytokines and reduced the anti-inflammatory cytokine, as reported previously [45]. All these are likely to promote oxidative stress and result in ulceration [46]. The enhancement of the levels of soluble inflammatory modulators by indomethacin reflected a pro-inflammatory trigger.

The bimodal nature of general immune responses is explained by the Th1/Th2 paradigm [47]. The regulatory T cells and Th2 cytokines often collaborate to suppress the Th1 response. Perhaps even more importantly, they strongly promote the mechanism of wound healing. However, the role of cytokine imbalance in gastropathy has not been adequately emphasized.

Our results showed that EA had an impact on immunological parameters. In ulcerated-untreated mice, pro-inflammatory cytokines level increased significantly compared with vehicle-treated group and simultaneously the anti-inflammatory cytokine level was repressed considerably (Fig. 6). However, EA treatment modulated the pro and anti-inflammatory cytokines level at the dose of (7 mg/kg). At this dose it reduced pro-inflammatory cytokines (TNF-$\alpha$, IL-1$\beta$, and IL-6) and concurrently induced the level of tissue IL-4, IL-10 thus exerted its healing effect.

Clinical and experimental data show that traditional NSAIDs delay the healing of gastroduodenal ulcers by interfering with the action of growth factors, decreasing angiogenesis in the ulcer bed, and slowing maturation of the granulation tissues [48]. A large number of factors
including several growth factors regulate wound healing via angiogenesis at its various stages [49–51]. Among these, VEGF triggers endothelial proliferation and migration and accelerates ulcer healing by promoting angiogenesis [50–52]. Likewise, HGF expressed at the ulcer margin to act as trophic factors for the gastric mucosa helps angiogenesis by multiple mechanisms including COX activation and increases EGF expression that ultimately accelerates gastroduodenal ulcer healing by stimulating cell migration and proliferation in epithelial cell monolayers, repairing tissue, increasing release of gastric mucin, and attenuating gastric acid secretion [53]. Our result showed that indomethacin administration decreased mucosal VEGF, EGF, and HGF levels 1.73 ($P < 0.01$), 1.56 ($P < 0.01$), and 1.51 ($P < 0.01$) folds, respectively, compared with the vehicle-treated group. However, EA enhanced growth factor level promisingly (Fig. 7).

Overall, EA enhanced the mucosal PGE2 synthesis by augmenting the COX expression and healed indomethacin-induced gastric ulcer. To substantiate our hypothesis that modulation of COX-2 may primarily account for the excellent ulcer-healing capacity of EA, we studied the effects of celecoxib and NS-398, specific COX-2 inhibitors on the healing capacities of EA (Figs. 8 and 9). It was found that addition of COX-2 inhibitors suppressed COX-2 as well as PGE2 synthesis and thereby abolished the ulcer-healing activity of EA. However, COX inhibitor pre-treatment although did not have any impact on VEGF level (data not shown) but drastically reduced the anti-inflammatory cytokine, HGF and EGF levels and eliminated the healing effect of EA compared with the only EA-treated group. Similarly, Brzozowska et al. [54] showed that classic NSAID such as aspirin and selective COX-2 inhibitor rofecoxib prolonged ulcer healing under diabetic conditions. This is attributed to the suppression of PG in the gastric blood flow at the ulcer margin. In a similar study, Zhao et al. [55] showed that 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid, a potential anion exchanger-2, healed ethanol-induced gastric mucosal injury in experimental rat model by augmenting the COX-2/PGE2 pathway. Above facts suggest that both COX-1 and COX-2 are important sources of PG during ulcer healing in diabetes and thereby strengthen the hypothesis.
that COX-2/PGE2 pathway may be targeted as potential drug design for gastric ulcer healing.

Overall, the EA was able to promote healing of indomethacin-induced gastric ulcers in mice. This result is corroborated by comparison of its effect with that of the positive control, omeprazole. The beneficial effect of the EA was due to its ability to reduce neutrophils infiltration and increase mucosal PGE2 that was down-regulated by indomethacin. EA increased COX expression at the gastric mucosa. All of these factors, especially the modulation of the COX-pathway, helped in up-regulating mucosal growth factors levels and maintaining the balance of pro-/anti inflammatory cytokines levels to accelerate ulcer healing.

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References


Figure 9 Effect of COX inhibitors on the healing activity of EA (7 mg/kg, single dose daily, orally) in indomethacin (18 mg/kg, single dose, orally) induced ulcerated mice. (A,B) Expression levels of tissue anti-inflammatory cytokines (IL-4 and IL-10). (C,D) Expression levels of mucosal growth factors (EGF and HGF). After ulcer induction, treatment was carried out with EA alone or in conjunction with celecoxib (3 mg/kg, once daily) or NS-398 (1 mg/kg, twice daily) for 3 days. The values are shown as the mean ± SD. n = 8. One-way analysis of variance was followed by Tukey–Kramer post hoc test. ***P < 0.001, **P < 0.01 and *P < 0.05, ns P < 0.05.


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