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Gastroprotective and Ulcer-Healing Mechanisms of Ellagic Acid in Experimental Rats

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ABSTRACT: Ellagic acid (EA), a plant-derived polyphenol, exhibits antioxidant, anti-inflammatory, and gastroprotective effects. Its gastroprotective mechanisms have not been fully elucidated nor have its effects on chronic ulcer previously been described. Toward these ends, the antiulcer activities of EA were evaluated in acute (ethanol and indomethacin) and chronic (acetic acid) ulcer models in Wistar rats. In this study, oral administration of EA significantly prevented the gastric ulceration caused by ethanol, indomethacin, and acetic acid treatments. Its gastroprotective mechanism in ethanol-induced ulcer were partly due to intensification in the endogenous production of nitric oxide, an antioxidant effect by replenishing depletion of endogenous nonprotein sulfhydryls and attenuation of tumor necrosis factor-α increase, whereas in indomethacin ulcer, it is partly due to a reduction in the plasma level of leukotriene B_4. In acetic acid ulcer, promotion of ulcer-healing effects was partly due to attenuation of the elevated levels of the inflammatory cytokines TNF-α, interferon-γ, and interleukins-4 and -6. These findings suggest that ellagic acid exerts its antiulcer activity by strengthening the defensive factors and attenuating the offensive factors.

KEYWORDS: ellagic acid, antiulcer mechanism, antioxidant, ulcer healing, nitric oxide, cytokines

INTRODUCTION

Gastric ulcer is a recurrent chronic illness that affects approximately 10% of the world population.[1] It is defined as an integrity disturbance of the gastric and/or duodenum mucosa, which causes local defect or excavation due to active inflammation.[2] Gastric ulcer is caused by varieties of both endogenous and exogenous factors, which include, among others, acid, pepsin, stress, and noxious agents such as alcohol, nonsteroidal anti-inflammatory drugs (NSAIDs), *Helicobacter pylori* bacteria, smoking, and alcohol consumption.[2] There are several defensive mechanisms that serve to protect the gastric and duodenal mucosa from the plethora of both exogenous and endogenous offensive factors. These include mucus−bicarbonate barrier, mucosal blood flow, endogenous prostaglandins (E_2 and I_2), nitric oxide (NO), antioxidant enzymes, and nonenzymatic antioxidants.[3,4]

However, when damage has occurred, there exist ulcer-healing mechanisms that serve to reverse the damage caused to any portion of the mucosa. These different processes that are involved in ulcer healing and repair are controlled and regulated by cytokines, growth factors, and some transcription factors that are overexpressed or activated over the injured area or ulcer margins.[5]

Although the precise mechanisms of gastric ulcer formation are still being unraveled,[6] the mechanisms by which some of the obnoxious agents bring about gastric ulcer have been partly revealed. *H. pylori*, known to be the major cause of active chronic gastritis, for example, has been shown to produce various cytokines that are related to neutrophil or mononuclear cell accumulation, including interleukins (IL) IL-1β, IL-6, and IL-8, interferon γ (IFN-γ), and tumor necrosis factor α (TNF-α).[7]

Ethanol is also known to stimulate inflammation through imbalance between pro-inflammatory cytokines such as IL-1β, IFN-γ, and TNF-α and anti-inflammatory cytokines such as IL-10.[8] NO is considered to be one of the most important defensive endogenous agents in the gastric mucosa.[4] Inhibition of mucosal synthesis of NO by NG-nitro-L-arginine methyl ester (L-NAME) renders the stomach more susceptible to the damaging effects of ethanol and other noxious agents, whereas administration of NO increases the resistance of the gastric mucosa to injury induced by ethanol and NSAIDs.[4]

NSAID-induced gastric damage partly depends on their ability to reduce prostaglandin production through inhibition of cyclooxygenase (COX) pathways and partly on COX-independent mechanisms.[4] The combined effects of these two mechanisms leads to marked oxidative tissue injury, which significantly contributes to the NSAID-induced mucosal injury.[9]

The therapeutic approach to peptic ulcer treatments is broadly directed toward reducing the effect of the offensive agents and strengthening of the defensive factors. However, such treatments are not completely effective and produce mild to serious adverse effects, especially for long-term users.[10,11]

Several plant-derived compounds have been investigated for their antiulcer and cytoprotective activities in experimental rodents.[10] Many epidemiological studies have indicated that

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consumption of a plant-based diet high in phenolic content is associated with the prevention of some chronic diseases.[12]

Ellagic acid (EA) is a polyphenol obtained from some plants. It is found in high quantities in nuts and fruits such as strawberries, raspberries, grapes, and blackberries as ellagitannins.[13] EA is used as a food additive due to its antioxidative properties.[14] EA has received attention as an agent that may have potential bioactivities in preventing chronic diseases.[15] It has been credited with several biological activities including anticancer, antimutagenic,[13,16] antioxidant,[17] anti-inflammatory,[18] antimicrobial,[19] and inhibition of UV-induced wrinkling.[15]

Gastroprotective properties of EA has been proven for swim-stress, ethanol, and ischemic/reperfusion-induced ulcers.[20−22] Its antiulcer effect is partially attributed to its inhibitory action on the gastric H^+, K^+-ATPase,[20] in vivo antioxidant property[22] and anti-*H. pylori* activity.[23]

EA, as demonstrated in different studies, is known to inhibit leukocyte recruitment and adherence to the endothelium through inhibition of reactive oxygen species (ROS) generation, cytokine-induced ROS generation, inflammation, and expression of adhesion molecules.[24,25]

However, there is a dearth of information concerning the gastroprotective mechanisms of EA in acute ulcer models, concerning the involvement of nonprotein sulfhydryls (NP-SH), gastric mucus, NO, and TNF-α in ethanol-induced ulcer (EtOH ulcer) and the involvement of prostaglandin E_2 (PGE_2), leukotriene B_4 (LTB_4), and TNF-α in indomethacin (IND)-induced ulcer. Moreover, there is no available literature, to the best of our knowledge, concerning its ulcer-healing activity and its probable mechanism(s) of action in acetic acid induced chronic ulcer (AA ulcer) model.

This work was therefore aimed at exploring further the various mechanisms involved in the gastroprotective effects of EA in rat models of gastric ulcer specifically focusing on the role of NP-SH, PGE_2, gastric mucus production, NO, TNF-α, LTB_4, cytokines (IL-1β, IL-4, IL-6, and IL-10), and vascular endothelial growth factor (VEGF) in both chronic (AA ulcer) and acute (EtOH and IND) ulcer models.

MATERIALS AND METHODS

Animals. Adult male Wistar rats were used in this study (150−200 g body weight, 7 weeks old) with the exception of the NP-SH experiment, for which female rats (180−200 g body weight, 8 weeks old) were used. They were obtained from the Central Animal House of the Federal University of Mato Grosso (UFMT), Brazil. The animals were kept in polypropylene cages at 22 ± 2 °C, with controlled 12 h dark/light cycles, and had free access to standard Purina chow and water ad libitum. They were allowed to acclimatize to the laboratory environment in this condition for 48 h. Groups of five rats were housed in one cage. The cages were lined with wire mesh to prevent coprophagy. The experimental protocols were approved by the Ethics Research Committee, following the International Principles for the Biomedical Research involving the use of animals (CIOMS/OMS, 1985).

Drugs and Reagents. EA, cimetidine, indomethacin, NG-nitro-L-arginine methyl ester (L-NAME), carbenoxolone, Alcian blue, ethylenediaminetetraacetic acid (EDTA), Tris (Trizma), 5,5′-dithiobis(nitrobenzoic acid) (DTNB), *N*-acetylcysteine (NAC), and reduced glutathione (GSH) were obtained from Sigma Chemical Inc. (St. Louis, MO). Ranitidine (Pylorid) was purchased from Glaxo-SmithKline (Brazil). Ethanol, $MgCl_2$, ethyl ether, methanol, and acetic acid were obtained from Synth (Brazil). Evans blue was purchased from Merck (Brazil), and trichloroacetic acid (TCA) was purchased from Vetec (Brazil). Quantikine rat TNF-α/TNFSF1A and Parameter LTB_4 and PGE_2 were obtained from R&D Systems Inc. (Minneapolis, MN). Plex kit for rat cytokines (RCYTO-80K) and fluorescence Luminex device were from Genese (São Paulo, Brazil). All drugs were prepared immediately before use.

Ethanol-Induced Gastric Ulcer (EtOH Ulcer). The experiment was carried out in accordance with a modified method of Robert et al.[26] Briefly, animals fasted for 18 h were orally treated by gavage (with the aid of oral gavage feeding tube) with the vehicle (distilled water, 10 mL kg^{-1}), EA (3, 10, and 30 mg kg^{-1}), or ranitidine (50 mg kg^{-1}). One hour after the treatment, each animal received 75% ethanol (10 mL kg^{-1}, po), and 1 h later, the animals were lightly anesthetized, the blood was collected for TNF-α analysis, and then the animals were sacrificed with an ether overdose. The stomachs were removed and opened along the greater curvature, washed with cold saline solution, and distended between two glass plates for better visualization. The ulcerated area was drawn on transparency paper and expressed in terms of percentage of total area of the gastric body (mm^2), using Image J (Java image processing and analysis software).

Determination of NP-SH in EtOH Ulcer. To evaluate EA's effect on NP-SH levels in the gastric tissue of animals subjected to EtOH ulcer, spectrophotometric analysis was made following the Sedlak and Linsay[27] method with modifications.

Briefly, six groups of eight rats were pretreated with EA (3, 10, and 30 mg kg^{-1}, po), NAC (50 mg kg^{-1}, po), or vehicle (distilled water, 10 mL kg^{-1}, po) 1 h before ethanol treatment. A normal control group ($n = 8$), which received only saline but not ethanol, was also included. All animals were sacrificed by cervical dislocation, and the glandular segment from each stomach was homogenized in 5 mL of ice-cold sodium EDTA (0.02 M) and filtered. The filtered homogenate was mixed and treated as previously described.[27] Absorption was measured at 412 nm within 5 min. The concentration of NP-SH was calculated on the basis of the standard curve of GSH.

Determination of Mucus in the Gastric Mucosa. To determine the effect of the treatments on gastric mucus production in EtOH ulcer, a modified method of Corne et al.[28] was used. The glandular segment of the stomach was weighed and transferred into a test tube containing 5 mL of 2% Alcian blue in a 0.16 M sucrose solution. After two consecutive rinses with 5 mL of 0.25 M sucrose, 5 mL of 0.5 M $MgCl_2$ was added in each test tube for the extraction of mucus content with the dye. The glandular segment remained in this solution for 2 h, with intermittent agitation. After 2 h, 4 mL of the resultant blue solution was agitated vigorously with 4 mL of ethyl ether until the formation of an emulsion and was centrifuged for 10 min at 3500*g*. The absorption of the aqueous phase was measured with a spectrophotometer at 598 nm, and the concentration of Alcian blue was calculated using a calibration curve of Alcian blue; the results were expressed in micrograms of Alcian blue per gram of glandular tissue.

Ethanol-Induced Ulcer in Rats Pretreated with L-NAME. To investigate the involvement of endogenous NO in the gastroprotection of EA, rats were fasted for 18 h and pretreated with L-NAME (70 mg kg^{-1}) or saline ip. Thirty minutes later, animals received an oral dose of vehicle (10 mL kg^{-1}), carbenoxolone (100 mg kg^{-1}), or EA (10 mg kg^{-1}) and L-arginine (200 mg kg^{-1}, ip). After 60 min, gastric ulcer was induced with EtOH, scored, and measured as for the ethanol-induced gastric ulcer above.

Indomethacin-Induced Ulcer (IND Ulcer). To induce gastric lesions by indomethacin, a modified method of Djahanguiri[29] was employed. Rats were fasted for 24 h and treated orally with the vehicle (10 mL kg^{-1}), EA (3, 10, and 30 mg kg^{-1}), or cimetidine (100 mg kg^{-1}). One hour after the treatment, 30 mg kg^{-1} indomethacin (dissolved in 2% sodium bicarbonate) was administered subcutaneously (sc). After 4 h, the animals were sacrificed with an ether overdose, the stomachs were removed, and 1 mL of 5% formol solution was injected for demarcation

of the ulcerated area. After 15 min, the stomachs were opened along the greater curvature, the gastric content was discarded, and the mucous membrane was delicately washed with 0.9% cold saline. For determination of the ulcer index, scores were attributed as previously described.[30]

Determination of the Plasma Levels of LTB_4 in IND Ulcer. To determine the blood plasma level of LTB_4 in IND ulcer, ulcer was induced as described above for indomethacin ulcer induction. For blood plasma collection, animals were anesthetized with ether, and the blood was collected in EDTA vacutainers. The samples were centrifuged at 3500*g* for 10 min, and the plasma was separated and stored in aliquots at −20 °C. A commercial ELISA kit (Parameter, R&D Systems) was utilized for determining the level of LTB_4 following the manufacturer's instructions. Highly hemolyzed samples (one sample of the vehicle and one of the normal control) were excluded. The results are expressed in picograms per milliliter.

Determination of PGE_2 Level in IND Ulcer. To quantify PGE_2 levels, rats were distributed into six groups ($n = 8$). After a 24 h fast, the animals received pretreatment of 0.9% saline (0.1 mL/rat, sc, normal control group, group 1) or indomethacin (dissolved in 2% sodium bicarbonate solution) 30 mg kg^{-1}, sc (groups 2−6). Thirty minutes after pretreatment, vehicle (groups 1 and 2), EA at 3, 10, and 30 mg kg^{-1} (groups 3−5), or carbenoxolone at 100 mg kg^{-1} (group 6) was administered orally. Thirty minutes after treatments, all of the animals were sacrificed and their abdomens opened. A sample of the corpus was excised, weighed, and suspended in 1 mL of 1 mM sodium phosphate buffer (pH 7.4). The tissue was finely minced with scissors and then incubated at 37 °C for 20 min. PGE_2 in the buffer was measured using a commercial ELISA kit (Parameter, R&D Systems) following the manufacturer's instruction. Absorbance was read at 450 nm. The results are expressed in picograms per milliliter.

Acetic Acid-Induced Chronic Ulcer. A modified method of Takagi et al.[31] was used. Rats were subjected to an adaptation period of 3 days before the experiment, receiving chow daily for 2 h with free access to water. In addition, the animals also received orogastric gavage of 1 mL of distilled water twice daily. On the day of the experiment, the animals were anesthetized with ether, the abdominal wall was opened by laparatomy, and gastric ulcer was induced by injection of 20% acetic acid (50 μL) in the submucosal layer of the stomach. The abdominal cavity was washed with saline and the abdominal wall sutured. One day after the surgery, the rats were treated with an oral dose of the vehicle (10 mL kg^{-1}), EA (3, 10, and 100 mg kg^{-1}), or cimetidine (100 mg kg^{-1}), twice daily for 14 days. A control group received only distilled water (10 mL kg^{-1}, po). On the last day of the treatment, the animals were fasted for 14 h and were sacrificed by cervical dislocation. The stomachs were removed, opened along the greater curvature, and washed with saline. The ulcerated area (mm^2) and the thickness (mm) of the injury were determined with a digital pachymeter (Digimess, Brazil).

Determination of the Plasma Level of TNF-α. To determine the blood plasma levels of TNF-α in EtOH and AA ulcer models, a commercial ELISA kit (Quantikine, R&D Systems) was utilized according to the manufacturer's instructions. Blood samples was collected and processed as described in the procedure for PGE_2 determination in IND ulcer above. The results are expressed in picograms per milliliter.

Determination of Cytokines IL1-β, IL-4, IL-6, IL-10, IFN-γ, and VEGF in AA Ulcer. Total blood was collected from the inferior vena cava from animals with AA ulcer, in tubes containing 5% EDTA, and centrifuged at 3000 rpm for 10 min, and the plasma was separated and frozen at −20 °C until the assay. For determination of the plasma levels of IL1-β, IL-4, IL-6, IL-10, IFN-γ, and VEGF, a multiplex kit for rat cytokines (RCYTO-80K) was used according to the manufacturer's instructions, and the fluorescence was determined using a fluorescence (Luminex, Genese, São Paulo-SP, Brazil) device.

Statistical Analysis. The results of the parametric tests were expressed in terms of the mean ± standard error of mean ($\overline{X} \pm$ SEM).

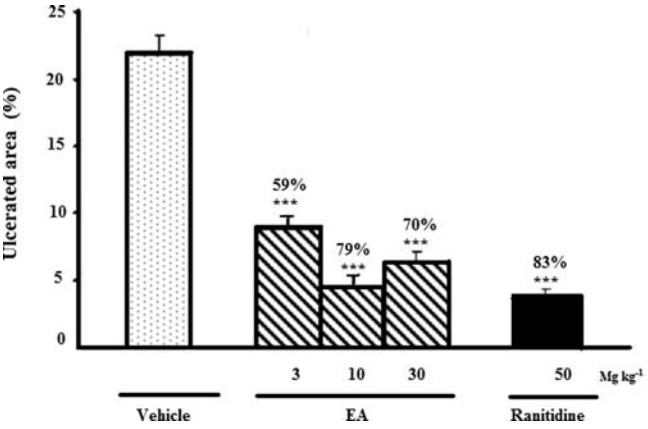

Figure 1. Effect of ellagic acid (EA) (3, 10, and 30 mg kg^{-1}) on gastric lesions induced by 75% ethanol (po) in rats. The ulcerative area (%) was express as the mean ± SEM, $n = 8$. ANOVA was followed by Student−Newman−Keuls's test. ***, $p < 0.001$ compared with vehicle.

Table 1. Effect of Ellagic Acid (EA) on Nonprotein Sulfhydryls (NP-SH) in the Gastric Lesions Induced by Ethanol in Rats

treatment	dose (mg kg^{-1}, po)	NP-SH[a] (μg g^{-1} tissue)
normal control		260.7 ± 10.31
vehicle control		118.8 ± 4.5 †††
EA	3	215.1 ± 13.2 ***
	10	200.2 ± 17.9 **
	30	161.2 ± 17.3
NAC[b]	50	203.8 ± 21.4 **

[a] Results are expressed as the mean ± SEM, $n = 8$. One-way ANOVA was followed by Tukey−Kramer's test. †††, $p < 0.001$ compared with normal control; **, $p < 0.01$, and ***, $p < 0.001$ compared with vehicle control. [b] NAC, *N*-acetylcysteine.

One-way ANOVA was used for comparisons of means followed by Student−Newman−Keuls test or Tukey post-test. For comparison of the medians, a Krustal−Wallis test was carried out followed by Dunn's test. The confidence level $p < 0.05$ was used.

RESULTS

Ethanol-Induced Ulcer. The administration of ethanol (po) to the vehicle group (control) produced extensive ulcerations in the form of hemorrhagic erosions (Figure 1) in the glandular portion of the gastric mucosa (21.8 ± 1.5%). The oral pretreatment with EA (3, 10, and 30 mg kg^{-1}) significantly reduced gastric injury by 59, 79, and 70% ($p < 0.001$), respectively, whereas ranitidine (50 mg kg^{-1}), the standard drug used, also significantly inhibited the injuries by 83% ($p < 0.001$).

Determination of Nonprotein Sulfhydryls (NP-SH). As illustrated in Table 1, pretreatment of the animals with EA significantly augmented the content of NP-SH content only in the 3 mg kg^{-1} (215.1 ± 13.2 μg g^{-1}, $p < 0.001$) and 10 mg kg^{-1} (200.2 ± 17.9 μg g^{-1}, $p < 0.01$) groups compared with the control group (vehicle, 118.8 ± 4.5 μg g^{-1}). It should also be noted that the lower dose was more potent in eliciting a positive response. The NAC-treated group also showed significant increases in the NP-SH content (203.8 ± 21.4 μg g^{-1}, $p < 0.01$).

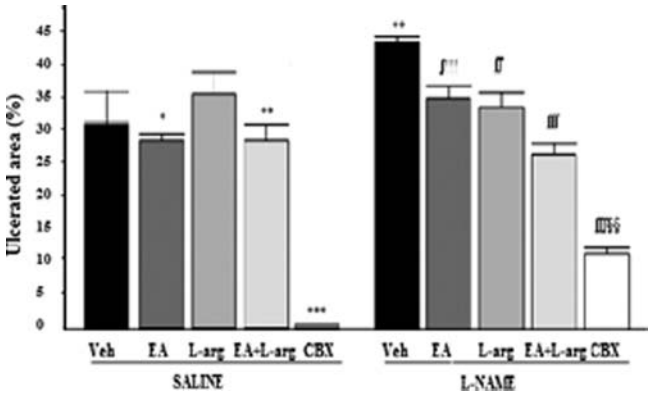

Figure 2. Effect of ellagic acid-EA (10 mg kg^{-1}, po) on gastric lesions induced by 75% ethanol (po) in rats pretreated with saline or L-NAME (inhibitor of NO synthase) ip. The columns represent the mean ± SEM, n = 9−10. ANOVA was followed by Tukey's test. Pretreatment: *, p < 0.05 vs saline/vehicle; **, p < 0.01 vs saline/vehicle; ***, p < 0.001 vs saline/vehicle; ∫, p < 0.05 vs L-NAME/vehicle; ∫∫, p < 0.01 vs L-NAME/vehicle; ∫∫∫, p < 0.001 vs L-NAME/vehicle; †††, p < 0.001 vs saline/AE10; §§p < 0.01 vs saline/carbenoxolone.

Table 2. Effect of Ellagic Acid (EA) on Gastric Ulcer by Indomethacin (30 mg kg^{-1} sc) in Rats[b]

treatment	dose (mg kg^{-1})	ulcer index[a]	% inhibition
vehicle (control)		17 (14; 22)	
EA	3	3 (1; 4) **	82
	10	4.5 (3; 6) **	74
	30	4 (3; 5.5) **	77
cimetidine	100	2 (2; 3) **	88

[a] Results are expressed as medium (Q1; Q3), n = 8. One-way ANOVA, Kruskal Wallis test was followed by Dunn's test. **, p < 0.01 compared with vehicle.

Determination of Mucus in the Gastric Mucosa. Oral pretreatment with EA (3, 10, and 30 mg kg^{-1}) did not modify production of protective mucus in the stomach of the animals subjected to ethanol treatment (76.6 ± 3.8, 90.6 ± 5.3, and 64.9 ± 1.8 μg g^{-1}, respectively) as compared to the vehicle (control) group (78.8 ± 2.3 μg g^{-1}). Moreover, these values were significantly lower than those of the NAC (112.9 ± 6.5 μg g^{-1}, p < 0.001) and normal (162.6 ± 5.1 μg g^{-1}, p < 0.001) groups.

Ethanol-Induced Ulcer in Rats Pretreated with L-NAME. Gastric mucosa has been shown to be capable of synthesizing NO de novo from L-arginine.[32] In addition, pretreatment with inhibitors of NO synthase such as L-NAME has been demonstrated in several studies to worsen EtOH ulcer.[33] In the first part of this experiment, gastric lesions were produced in all animals pretreated with saline before ulcer induction (Figure 2). EA (10 mg kg^{-1}) and CBX (20 mg kg^{-1}) significantly attenuated the gastric lesions by 28% (p < 0.01) and 99% (p < 0.001), respectively. L-Arg (200 mg kg^{-1}) alone had no significant effect on the gastric lesions. When L-Arg was coadministered with AE, there was no enhanced protective effect.

In the second part of the experiment, animal pretreatment with L-NAME increased the severity of the gastric lesions (Figure 2). The gastric lesions in the vehicle group with L-NAME/vehicle were significantly (p < 0.01) increased by about 34% compared to the saline-treated vehicle. This deleterious effect was also observed in EA-treated animals, causing about 50% (p < 0.001) increase in the gastric lesions compared to the EA/saline group. Treatments with L-Arg alone and with EA significantly attenuated this deleterious effect of L-NAME by about 22% (p < 0.01) and 19.6% (p < 0.001), respectively. Co-administration of L-Arg with EA enhanced the gastroprotective effect of EA by about 2-fold (39% inhibition, p < 0.001) as compared to EA alone (19.6%).

Table 3. Effect of Ellagic Acid (EA) on Plasma Levels of LTB_4 in Rats Subjected to Indomethacin-Induced Ulcer

treatment	dose (mg kg^{-1}, po)	LTB_4[a] (pg mL^{-1})
normal control		309.1 ± 9.1
vehicle (ulcerated)		360.1 ± 7.8 †
EA	3	308.7 ± 6.5 **
	10	306.2 ± 11.2 **
	30	341.3 ± 4.2

[a] Results are expressed as the mean ± SEM, n = 7−8 samples (plasma). One-way ANOVA was followed by Tukey's test. ††, p < 0.01 compared with normal control; **, p < 0.01 compared with vehicle (ulcerated).

Indomethacin-Induced Ulcer. The index of ulcer produced by sc administration of indomethacin (Table 2) to the vehicle group (control) was 17 (14; 22). The administration of EA (3, 10, and 30 mg kg^{-1}) significantly decreased this index by 82, 74, and 77%, respectively (p < 0.01). The animals treated with cimetidine (100 mg kg^{-1}) presented an injury index significantly lower than the vehicle group, inhibiting this index by 88% (p < 0.01).

Determination of the Plasma Levels of LTB_4 in IND Ulcer. In indomethacin-induced ulcer experiments, the plasma levels of LTB_4 (Table 3) in the groups treated with EA were significantly lower in the 3 and 10 mg kg^{-1} groups (308.7 ± 6.5 and 306.2 ± 11.2 pg mL^{-1}, respectively, p < 0.01), reaching basal levels as in the normal group (not ulcerated) (309.1 ± 9.1 pg mL^{-1}, p < 0.01) in comparison to the vehicle group (ulcerated) (360.1 ± 7.8 pg mL^{-1}).

Determination of PGE_2 Synthesis. Treatment with EA (3, 10, and 30 mg kg^{-1}) did not modify the production of PGE_2 in the animals treated with indomethacin (2737 ± 166, 3570 ± 392, and 3319 ± 463 pg mL^{-1}, respectively), keeping the PGE_2 content at the same levels as that of the vehicle group (treated only with indomethacin and distilled water) (2881 ± 225 pg mL^{-1}) and were significantly lower than in the normal group (not treated with indomethacin) (4900 ± 196 pg mL^{-1}, p < 0.01). Animals treated with carbenoxolone showed elevated PGE_2 levels in gastric mucosa (4383 ± 713 pg mL^{-1}, p < 0.05) in comparison to the vehicle group.

Acetic Acid-Induced Chronic Ulcer. The area and the thickness of the vehicle group were 11.5 ± 1.65 mm^2 and 3.05 ± 0.19 mm, respectively (Table 4). Postoperative treatment with EA (3, 10, and 30 mg kg^{-1}, po) for 14 days did not reduce the ulcerated area, but showed a significant reduction in the ulcer thickness by 21.3% (2.40 ± 0.10 mm, p < 0.01), 25.6% (2.27 ± 0.20 mm, p < 0.01), and 26.2% (2.25 ± 0.21 mm, p < 0.05), respectively, in comparison to the vehicle group (3.1 ± 0.19 mm). Cimetidine, the reference drug used in this model, significantly reduced the ulcerated area (5.15 ± 1.3 mm^2, p < 0.05) and the thickness of the ulcer by 33.4% (2.03 ± 0.17 mm, p < 0.01).

Determination of the Plasma Level of TNF-α in EtOH and AA Ulcer Models. The plasma levels of TNF-α in normal

animals (not ulcerated) were significantly lower (5.4 ± 1.2 pg mL^{-1}, $p < 0.01$) than in the group subjected to the ethanol-induced ulcer (11.1 ± 1.1 pg mL^{-1}) (Figure 3). Animals subjected to EtOH ulcer but treated with EA had significantly lower levels of TNF-α only in the 10 mg kg^{-1} dose (6.2 ± 1.0 pg mL^{-1}, $p < 0.01$).

Likewise, in the model of AA ulcer (Figure 3), the plasma levels of TNF-α were significantly lower in animals treated with EA at the doses of 3 mg kg^{-1} (8.8 ± 1.2 pg mL^{-1}, $p < 0.01$), 10 mg kg^{-1} (6.9 ± 1.5 pg mL^{-1}, $p < 0.001$), and 30 mg kg^{-1} (8.3 ± 1.4 pg mL^{-1}, $p < 0.01$) compared to the vehicle group (ulcerated) (19.3 ± 3.5 pg mL^{-1}).

Determination of Plasma Levels of Cytokines IL1-β, IL-4, IL-6, IL-10, IFN-γ, and VEGF. Table 5 shows the results of effects of EA on plasma cytokine levels in the AA ulcer model. Compared to the normal value (108.8 ± 33.9 pg mL^{-1}), ulceration drastically increased the plasma levels of IL-4 by 6-fold (661.7 ± 154.3 pg mL^{-1}, $p < 0.05$). EA significantly suppressed the increase by 65, 60.6, and 44.2% at 3 mg kg^{-1} (231.7 ± 45.8 pg mL^{-1}), 10 mg kg^{-1} (260.9 ± 82.5 pg mL^{-1}), and 30 mg kg^{-1} (369.5 ± 158.8 pg mL^{-1}, $p < 0.05$), respectively, as compared to the ulcerated vehicle group.

In a similar fashion, ulceration increased the plasma levels of IL-6 by 12.5-fold (5810.4 ± 1412.1 pg mL^{-1}, $p < 0.01$) as compared to the normal group (465.6 ± 283.6 pg mL^{-1}) (Table 5). EA significantly inhibited this increase by 75, 73, and 48% at 3 mg kg^{-1} (1444.4 ± 337.2 pg mL^{-1}, $p < 0.01$), 10 mg kg^{-1} (1561.2 ± 738.1 pg mL^{-1}, $p < 0.01$), and 30 mg kg^{-1} (3022.8 ± 661.5 pg mL^{-1}, $p < 0.05$), respectively, as compared to the ulcerated vehicle group.

The same trend was observed in the case of IFN-γ. Acetic acid ulceration significantly increased the levels of IFN-γ by 7.2-fold (1724.4 ± 288.6 pg mL^{-1}, $p < 0.01$) as compared to the normal group (240.6 ± 61.3 pg mL^{-1}). Pretreatment with EA significantly inhibited the IFN-γ increase by 83.8, 63, and 54% at 3 mg kg^{-1} (279.4 ± 106.6 pg mL^{-1}, $p < 0.01$), 10 mg kg^{-1} (625.2 ± 289.2 pg mL^{-1}, $p < 0.05$), and 30 mg kg^{-1} (776.7 ± 231.2 pg mL^{-1}, $p < 0.05$), respectively, when compared to the ulcerated vehicle group.

IL1-β, IL-10, and VEGF levels were below detectable levels on the 14th day of ulcer induction in this model.

Table 4. Effect of Ellagic Acid (EA) on Gastric Ulcer by Acetic Acid in Rats

treatment	dose (mg kg^{-1})	thickness[a] (mm)	damage area[a] (mm^2)
sham		0.0 ± 0.0 ***	0.0 ± 0.0 **
vehicle		3.05 ± 0.19	11.50 ± 1.65
EA	3	2.40 ± 0.10 **	11.04 ± 0.95
	10	2.27 ± 0.20 **	10.26 ± 0.37
	30	2.25 ± 0.21 *	9.85 ± 1.71
cimetidine	100	2.03 ± 0.17 **	5.15 ± 1.25 *

[a] Results are expressed as the mean ± SEM, $n = 8$. One-way ANOVA was followed by Tukey's test. ***, $p < 0.001$; **, $p < 0.01$; and *, $p < 0.05$, compared with vehicle.

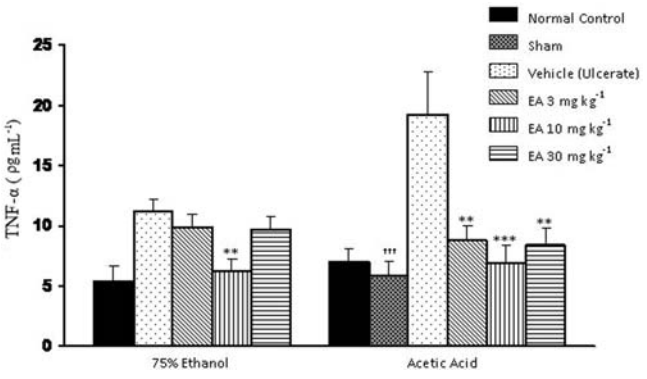

Figure 3. Effect of ellagic acid (EA) (3, 10, and 30 mg kg^{-1}) on plasma levels of TNF-α (pg mL^{-1}) in rats subjected to (75%) ethanol- and (20%) acetic acid-induced ulcers. Results are expressed as the mean ± SEM, $n = 7-8$ samples (plasma). ANOVA was followed by Tukey's test. **, $p < 0.01$, and ***, $p < 0.001$ compared with vehicle (ulcerated); †††, $p < 0.001$ compared with the vehicle (ulcerated).

DISCUSSION

The involvement of oxidative stress in several alcohol-related illnesses such as gastric cancer, ulcer, liver pathology, myopathy, and cerebella atrophy is well documented.[34−36] Oxidative stress in cells or tissues is an enhanced generation of ROS, which eventually leads to an imbalance between pro-oxidants and antioxidants.[37] ROS generation in cells may result in damage to important biomolecules such as cell membrane lipids and DNA.[38]

Drugs with antioxidant properties tend to protect the mucosa from the induced damage of these agents.[39] Oral administration of EA significantly prevented gastric injuries by ethanol in the present study (Figure 1). This further confirms similar previous studies on EA.[20−22]

The modulating effect of EA on mucosal mucus levels was also investigated. EA had no significant effect on mucus production in the stomachs of animals subjected to EtOH ulcer. This indicates that mucus production is not involved in the gastroprotective effect of the EA in the EtOH ulcer model utilized in this study, although there is considerable controversy concerning the protective role of mucus in the gastric mucosa against direct damaging effects from necrotizing agents such as ethanol.[4]

Ethanol-induced gastric injury results in significant reduction in the levels of NP-SH in the gastric mucosa, and the recovery of

Table 5. Effect of Ellagic Acid (EA) on Levels of Inflammatory Cytokines IL-4, IL-6, and IFN-γ from Animals with Chronic Ulcer Model Induced by Acetic Acid in Rats

treatment	dose	IL-4[a] (pg mL^{-1})	IL-6[a] (pg mL^{-1})	IFN-γ[a] (pg mL^{-1})
sham		108.8 ± 33.9	465.6 ± 283.6	240.6 ± 61.3
vehicle (10 mL kg^{-1})		661.7 ± 154.3 †	5810.4 ± 1412.1 ††	1724.4 ± 288.6 ††
EA (mg kg^{-1})	3	231.7 ± 45.8 *	1444.4 ± 337.2 **	279.4 ± 106.6 **
	10	260.9 ± 82.5 *	1561.2 ± 738.1 **	625.2 ± 289.2 *
	30	369.5 ± 58.8 *	3022.8 ± 661.5 *	776.7 ± 231.2 *

[a] Results are expressed as the mean ± SEM, $n = 8$. One-way ANOVA was followed by Student−Newman−Keuls test. **, $p < 0.01$, and *, $p < 0.05$, compared with vehicle. ††, $p < 0.01$, and †, $p < 0.05$, compared with sham.

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the NP-SH levels seems to be important in the gastroprotection exerted for some drugs. NP-SH, mainly GSH, is the antioxidant compound involved in the maintenance of gastric integrity.[40] It controls the cascade of inflammatory cytokines[17] and promotes detoxification and excretion of ROS produced by aggressors, such as ethanol, indomethacin, and stress.[41,42] EA significantly preserved the level of NP-SH in the gastric mucosa of rats subjected to EtOH-ulcer, but to a lesser extent than the basal level. Several studies have demonstrated the antioxidant activitiesof EA. It is capable of attenuating lipid peroxidation in the gastric mucosa,[22] liver,[38] and plasma.[43] It was also able to attenuate production of myeloperoxidase (MPO) in the intestinal mucosa[44] and caused an increase in the endogenous levels of GSH in the liver.[17,38]

NO is considered to be one of the most important defensive endogenous agents in the gastric mucosa.[45] It is synthesized by the enzyme nitric oxide synthase (NOS) from L-arginine.[46] NO, together with the prostaglandins, preserves gastric mucosa integrity.[45,47] The constitutive forms of NOS, neuronal NOS (nNOS) and endothelial NOS (eNOS), play a physiological role in the homeostasis of the gastrointestinal tract, and the inhibition of these enzymes can result in disturbance of gastrointestinal motility, blood flow, and acid secretion.[11] We therefore evaluated the possible involvement of NO in the EA gastroprotective effect.

As can be clearly deduced from our results with L-NAME-pretreated animals (Figure 2), EA gastroprotective effects are brought about by its ability to promote endogenous production of protective NO because L-NAME pretreatment reversed its gastroprotective action. Furthermore, a synergistic effect with L-Arg was only observed in the presence of L-NAME, thus indicating the involvement of the L-arginine−NO pathway.

NSAIDs are considered an established cause of peptic ulcers in humans and rats.[45] LTB_4 contributes to the NSAID gastric injury by promoting intense chemotaxis of the neutrophils and leukocyte adherence to the vascular endothelium, thus stimulating ROS release by neutrophils[11] and indirectly stimulating the formation of other inflammatory mediators, such as TNF-α.[48] Moreover, production of LTB_4 and TNF-α is significantly higher in patients with *H. pylori* infection, indicating that the specific antagonism of its receptors may be an important therapeutic target in the treatment of the *H. pylori*[49] and of associated NSAID gastritis.[11]

In this study, EA significantly reduced the ulcer index and gastric levels of LTB_4 in the indomethacin ulcer model (Tables 2 and 3). This finding is of considerable importance in that LTB_4 has been suggested in several studies to play a significant role in the pathogenesis of NSAID-induced gastric injury.[50] LTB_4 is a potent inflammatory and vasoconstriction mediator. Thus, EA may have an inhibitory effect on 5-lipooxygenase (LOX) enzyme that is responsible for LTB_4 and other leukotrienes, thereby reducing free radicals and gastric damage, because inhibition of biosynthesis of cytoprotective prostaglandins by indomethacin causes overproduction of leukotrienes and other products of the LOX pathway.[51] EA may thus be a promising drug in the cases of peptic ulcer associated with the use of NSAIDs and chronic gastritis caused by infection with *H. pylori*.

Prostaglandins produced by cyclooxygenase-1 (COX-1), mainly PGI_2 and PGE_2, play important roles in the protection of the gastric mucosa.[4] They increase the secretion of mucus and bicarbonate, maintain the blood flow of the mucosa, increase resistance of the epithelial cells, and reduce the leukocytes recruitment to the mucosa.[4] In this study, the EA did not modify the PGE_2 levels in gastric mucosa, indicating that the gastroprotection for the EA occurs independently of the PGE_2. This result partially explains our earlier observation concerning mucus production because PGE_2 is known to stimulate epithelial cells to secrete bicarbonate and mucus production.[4] These observations in all suggest that EA had little or no effect on COX-1. However, Karlsson et al.[52] observed decreased PGE_2 levels and other prostaglandin-synthesizing enzymes in human monocytes due to EA administration, although the study was in human monocytes and inflammation was induced by lipopolysaccaride.

It is also pertinent to note that the antioxidant property of EA may also have contributed to its gastroprotective function in the case of IND ulcer as we have demonstrated for EtOH ulcer.[41,42]

Chronic ulcer induced by acetic acid resembles the human gastric ulcer in terms of pathological characteristic and healing process.[53] Five days after the injection of acetic acid, a round and deep ulcer appeared in the place of the application, which is considered the first day of ulceration. EA had no significant effect on the ulcerated area (mm^2), but it significantly reduced the thickness (mm) of the injury (Table 4).

Cytokines are produced mainly by macrophages and the Th cells in response to inflammatory stimuli.[54] Macrophages produce pro-inflammatory cytokines, which include among others TNF-α and IL-6 and anti-inflammatory cytokines such as IL-10.[55]

TNF-α has a central role in initiating the cascade of other cytokines and factors that make up the immune response to infection. It is involved in many forms of injury to the gastric mucosa associated with infection with *H. pylori*, use of NSAIDs,[56] and ethanol.[57] The inhibition of the TNF-α synthesis results in the reduction of the harmful effect of these ulcerogenic agents.[56] Brzozowska et al.[58] showed that the levels of pro-inflammatory cytokines such as IL-1β and TNF-α in diabetic rats are higher, resulting in persistent inflammatory reaction that delays the healing of the ulcer induced by acetic acid. In the model of ulcerative colitis induced by sodium dextran sulfate, Ogawa et al.[44] suggested the suppression of pro-inflammatory cytokines as the mechanism involved in the anti-inflammatory effect of EA. Our data seem to support this postulation as EA attenuated the gastric injury due to acetic acid by reducing the levels of TNF-α (Figure 3), the cytokine which is known to impair ulcer healing by interacting with mucosal restitution and angiogenesis.[59]

We also noted increases in IL-4 and IL-6 due to AA induction. These increases were substantially inhibited by oral administration of EA at all doses (Table 5). These observations further confirm that EA's ability to resolve acetic acid-induced chronic ulcer is partly dependent on its attenuation of increase in these inflammatory cytokines. Although IL-4 is said to be an anti-inflammatory cytokine in nature, excessive increase in the anti-inflammatory cytokines may exacerbate inflammatory injury. Sprague and Khalil[54] noted that the net inflammatory response of a cytokine is determined by a host of factors such as the timing of cytokine release, the local environment in which they are released, the presence of synergistic or competing factors, cytokine receptor density, and tissue responsiveness to each cytokine. As we noted in our study, all of these cytokines were excessively increased in all animals that received acetic acid as compared to the normal control group.

IFN-γ, a pro-inflammatory cytokine secreted by Th1 cells, was also observed to be increased manyfold by acetic acid treatment, thus implicating it in this ulcer model. EA significantly attenuated the increase in IFN-γ (Table 5). This is quite interesting as IFN-γ activity and *H. pylori* density correlated well in both acute and chronic inflammation in *H. pylori* human infections.[60] EA may thus be useful in the case of ulcer due to *H. pylori* infection.

In general, the antiulcer effect of EA was more effective at the lowest dose of 3 mg/kg bw (Tables 1, 2, and 5), and sometimes maximal positive response is attained at a 10 mg/kg bw dose (Tables 3 and 4; Figures 1 and 3), beyond which it declines. The exact mechanism responsible for this effect is not known. However, various hypotheses may be postulated to explain this phenomenon. First, EA being a pleitropic molecule may act by binding to a yet to be identified receptor(s); thus, desensitization of the receptor(s) may occur at higher drug dose, especially in the chronic ulcer model, thereby resulting in little or no effect as compared to the lower dose. Second, higher doses of EA may have an untoward effect on other systems, thus provoking a negative response toward the antiulcer activity of EA as observed for the lower dose. Third, it is also possible that higher dose of EA may provoke several physiological response mechanisms which may alter the effective concentration of EA or several other factors too numerous to mention.[61]

These postulates are strengthened on the basis of the following premises: phenolic phytochemicals, especially biphenyls such as EA, are structurally similar to many biological signaling molecules, which interact with the receptors on the cell surface that are responsible in activating many biological signaling processes.[13] Phenolic phytochemicals including EA have been documented to also mimic the functions of biological signaling molecules and can trigger the signaling transduction.[13,62] Moreover, plant-derived polyphenols have been demonstrated to be good chelators. Chelation of calcium ion in the extracellular matrix or in the cytosol, for example, may alter net concentration of free calcium.[13] Because many signaling cascades are sensitive to calcium gradient across the cell membrane, an apparent modulation of cellular concentration of calcium either by direct binding and or by modulating the calcium-sensing receptor can activate these cellular signaling cascades, which can result in the changes in many physiological pathways thus affecting the potency of EA.[13]

Finally, phenolic phytochemicals have been shown to possess anticancer properties by activating enzymatic systems (phase II) involved in the detoxification of xenobiotics.[13] On this basis, it is could be said that a higher dose predisposes the physiological system to excrete more EA, thus lowering its effective physiological concentration. Hence, diminished antiulcer effects are observed for the higher dose.

The levels of IL-1β, IL-10, and VEGF were, however, below measurable levels in this AA ulcer model. This might have been due to natural decay of these cytokines and the growth factor.

The present study, to our knowledge, is the first to demonstrate that the gastroprotective effect of EA in experimental rats is mediated in part through modulations of NO, TNF-α, NP-SH, and LTB_4 levels, thus indicating additional mechanisms for the antiulcer effect of EA. On the one hand, these include the increase in endogenous NP-SH and NO and the reduction in the plasma TNF-α and gastric LTB_4 levels, whereas, on the other hand, the gastroprotective effect of EA occurs independently of the production of gastric mucus and PGE_2. Ellagic acid, being a polyphenol widely distributed in dietary foods, may be a good candidate for subsequent development as a multitargeting antiulcer drug.

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ABBREVIATIONS USED

EA, ellagic acid; EtOH, ethanol; IND, indomethacin; AA, acetic acid; NP-SH, nonprotein sulfhydryls; TNF-α, tumor necrosis factor-α; LTB_4, leukotriene B_4; IFN-γ, interferon-γ; IL, interleukin; NSAID, nonsteroidal anti-inflammatory drugs; COX, cyclooxygenase; PGE_2, prostaglandin E_2; NO, nitric oxide; L-NAME, NG-nitro-L-arginine methyl ester; EDTA, ethylenediaminetetraacetic acid; DTNB, 5,5′-dithiobis(nitrobenzoic acid); NAC, *N*-acetylcysteine; GSH, reduced glutathione; VEGF, vascular endothelial growth factor; MPO, myeloperoxidase; NOS, nitric oxide synthase.

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