Immunopharmacological basis of the healing of indomethacin-induced gastric mucosal damage in rats by the constituents of *Phyllanthus emblica*

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Studies were designed to establish the individual active compound present in the ethanolic extract of the plant *Phyllanthus emblica* (EOE), contributing to the healing of indomethacin-induced gastric lesions in rats. The ulcerated rats received EOE, or purified fraction of EOE (which is pure gallic acid, GA). EOE and GA significantly reduced the serum levels of IL-1ß and TNF-α in mice injected with endotoxin, lipopolysaccharide. The experimental groups treated with GA had significantly reduced ulcer index, serum levels of pro-inflammatory cytokines, nitric oxide synthase and interleukin-8 receptor in the gastric epithelial cells, and increased hepatic arginine activity compared to the experimental control group. The studies suggest that GA-induced modification of the inflammatory response contributed significantly in the ulcer-healing process, suggesting the role of GA, a major chemical constituent of *P. emblica*, in the healing of ulcers induced by indomethacin.

Keywords: Gallic acid, indomethacin-induced ulcer, nitric oxide, *Phyllanthus emblica*, pro-inflammatory cytokines.

**GASTROINTESTINAL toxicity associated with nonsteroidal anti-inflammatory drugs (NSAID) may be as high as 4 to 8% per year, despite the recent pharmaceutical advances.** For elderly NSAID users, fatal complications are close to 1 per 1000 person-years of NSAID use, and higher for those with additional risk factors, such as prior history of ulcer disease. NSAID-associated toxicity may be reduced with the use of misoprostol co-therapy to prevent complications. Attempts are being made to find safer and cost-effective therapies to minimize the toxicity of NSAID. An ethanolic extract of *Phyllanthus emblica* (EOE) offered protection against indomethacin-induced gastric lesions. Since net human health effects depend on the concentration of individual molecules present in the EOE, subsequent studies looked at the chemical constituents present in EOE, responsible for the healing of the gastric damage. The aim of the present study was to evaluate the anti-ulcer effect of EOE, and its purified constituent, fraction 3 (E3) containing pure gallic acid (GA), in comparison with a synthetic anti-ulcerogenic drug, misoprostol. Pathogenic mechanisms that have been proposed for NSAIDs-induced gastropathy include the modulation of cyclooxygenase isofoms, COX-1 and COX-2; infiltrating inflammatory cells, and the involvement of inducible nitric oxide synthase (iNOS) and its products, superoxide and peroxynitrite. Since increased production of pro-inflammatory cytokines and increased iNOS activity are involved in gastric mucosal lesions, the present study examined the immunomodulatory properties of EOE and GA on the expression of iNOS, interleukin-8 receptor 1 (CXCR1), and pro-inflammatory cytokines like IL-1ß (interleukin-1ß) and TNF-α (tumor necrosis factor-α), in a rat model with ulcer induced by indomethacin to explain the anti-ulcer effect of the constituents of *P. emblica*. The immunopharmacology of EOE and GA was also studied for its immunomodulatory activity using an in vitro model of concanavalin arginase (Con A)-induced lymphoproliferation and an in vivo model of lipopolysaccharide (LPS)-induced endotoxaemia.

Since polyanimes are known to be involved, the wound healing in the stomach and arginase is known to catalyse the production of ornithine, a key precursor in the production of polyamines, we also looked at the levels of arginase in the liver of ulcerated rats and its modification by the administration of EOE, GA and misoprostol.

**Materials and methods**

**Materials**

MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyl tetrazolium bromide) was obtained from Roche Molecular Biochemicals, Germany. LPS (*Escherichia coli* B55:05) and misoprostol were obtained from Sigma Chemicals, USA.
Extraction of plant materials and further purification: The dried P. emblica fruits (250 g) were chopped into fine pieces, soaked in 95% ethanol for one day with intermittent shaking and the supernatant decanted. The entire process was repeated three times, using a fresh batch of solvent each day. The combined extracts were filtered through a nylon mesh and evaporated in vacuo to obtain a brown, gummy liquid. This was finally lyophilized to obtain an amorphous brown semi-solid (25 g, 10% w/w yield), which was designated as EOE and stored in vacuum desiccators. A part of the EOE (20 g) was subjected to column chromatography over silica gel (350 g), eluting with 5–90% ethyl acetate/petroleum ether to collect twenty-seven fractions (each of 1.0 l). After concentration in vacuo, each of the fractions was tested for their DPPH (1,1-diphenyl-2-picrylhydrazyl) scavenging activity. The four best fractions, designated as E1–E4, were obtained in 0.043, 6.08, 19.31 and 1.6% yields respectively. Fraction E3 showing only a single spot on the thin layer chromatography (TLC) was subjected to preparative TLC (silica gel, 20% methanol/chloroform) to isolate GA (16.61% w/w). GA was characterized by its 1H and 13C NMR spectra as well as X-ray crystallography of its methylated derivative. Both these compounds were identified from their IR and 1H NMR spectra. Considering that GA is the major constituent of EOE, immunopharmacological studies were done with EOE and GA, based on the content of GA in EOE.

Animals: All the experiments were carried out in accordance with the ethical guidelines laid down by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. Wistar rats (100–120 g body wt) and Swiss/Bh inbred male mice (22–24 g body wt) were acclimatized to room temperature at 23.1°C, relative humidity of 50 ± 10% and a 12 h dark/light cycle for one week, before the start of the experiment.

Experimental design: Rats were divided into six groups (A to G). Group A rats were kept as vehicle control, which received gum acacia (2%) at 1400 h, everyday from days 1 to 7. Rats from groups B to F were fasted overnight and indomethacin (30 mg/kg body wt) was given on day 0 at 1400 h. Group B (called 0-day ulcer) rats were sacrificed after 4 h of indomethacin administration, and their serum and tissues were analysed. Group C (experimental control group) rats were fed only the binder gum acacia (2%) at 1400 h, everyday from days 1 to 7. The experimental groups D (called EOE group), E (called GA group) and F (called misoprostol group) were administered the drugs, EOE, GA and misoprostol at a concentration of 100, 19.3 and 1.43 mg/kg respectively, everyday at 1400 h, along with gum acacia, from days 1 to 7. Indomethacin, gum acacia and all the drugs were given by oral intubation using an intubation tube. All the groups were given normal diet and water ad libitum. Group G rats were kept as normal control, which did not receive any treatment. On day 7, the rats were sacrificed at 1800 h by cervical dislocation after overnight fasting and the serum and tissues were analysed.

Quantification of mucosal injury and histology: Mucosal injury was measured by the ulcer index, as reported earlier. The area of injury or spot on the surface on the glandular stomach was expressed as percentage of the total glandular stomach area, and the ulcer index calculated. Histopathological examinations of gastric tissues were done according to the method described earlier. The tissues were fixed in 10% formaldehyde in phosphate buffered saline (PBS) for 24 h. The fixed tissues were sequentially treated with 70, 90% and absolute alcohol for dehydration. Tissues were embedded in paraffin and cut into 3–5 μm sections and stained with haematoxylin and eosin.

Serum cytokine levels: Levels of serum IL-1β and TNF-α were measured using commercial ELISA kits (Opt EIA Elisa kits, BD Biosciences Pharmingen, USA).

Immunostaining for iNOS and CXCR1, and image analysis

Gastric tissue was processed as mentioned earlier for the histology. After deparaffinization and dehydration, the sections were immunostained using specific polyclonal antibodies against iNOS and CXCR1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Briefly, the sections were incubated for 10 min in PBS (pH 7.6), wiped and incubated with blocking solution (normal rabbit serum, 1:5 in PBS) and incubated with primary antibody (1:100) in PBS for 1 h and washed with PBS. In control sections, antibodies were omitted and only PBS was added. The sections were incubated with HRP-labelled secondary antibody in PBS (Santa Cruz Biotechnology) for 1 h. After colour development with diaminobenzidine, the sections were counterstained with haematoxylin, washed, dried, mounted and observed under a microscope (Zeiss, Germany). The intensities of coloured immunolocalized products were evaluated using suitable software (Biovis MV500). Five areas from each section were scanned and the integrated optical density (IOD) in each area was calculated using the software. The IOD of the negative control was subtracted from the IOD of each experimental section for each animal in all groups.

Arginase levels: Liver tissue from the rat was homogenized using a glass teflon homogenizing tube in 50 mM PBS, pH 7.2 under cold conditions. The homogenate was centrifuged at 2000 rpm for 10 min and the supernatant was collected. Arginase activity was measured as reported earlier.
Statistical analyses: Statistical significance was analysed using the one-way ANOVA followed by post-hoc analyses using Scheffe test. Significance was set at $P < 0.05$.

**Results**

**Quantification of mucosal injury**

The stomach from 0-day ulcer had a number of blood clots in the ulcer spots and perforations. The stomach from experimental control group showed lesser spots, but the tissue was hyaline in nature. Rats from EOE, GA and misoprostol groups had no ulcer spots in their stomachs. Ulcer index in the EOE, GA and misoprostol groups was 86, 96 and 87% lower respectively, compared to 0-day ulcer (Figure 1). Ulcer index in the EOE, GA and misoprostol groups was significantly lower ($P < 0.05$) when compared to experimental control group (Figure 1). Ulcer index in the GA group was significantly lower ($P < 0.05$) compared to either EOE or misoprostol group. Histological examination of the gastric mucosal tissues from the 0-day ulcer group revealed detectable ulcerative lesions, with injury in epigastric layer and lamina propria with haemorrhagic lesions (Figure 2b). Normal rat stomach section (Figure 2a) showed integrated and intact layer of gastric mucosa. Stomach from experimental control group (Figure 2c) indicated damage in the mucosal layer with moderate infiltration in the mucous membrane and *Muscularis mucosae*. All the experimental groups exhibited normal intact structure (Figure 2d and e), which is comparable to the vehicle control group. Gastric tissue from misoprostol group (Figure 2f) showed significant healing, but not at par with either EOE or GA group.

**EOE and GA reduce pro-inflammatory cytokines in ulcer**

The serum levels of IL-1$\beta$ and TNF- $\alpha$ were significantly elevated in both the 0-day ulcer group and experimental control group compared to the normal control group (Group G; Table 1). Serum levels of IL-1$\beta$ and TNF- $\alpha$ were significantly decreased in experimental control group compared to the 0-day ulcer group ($P < 0.05$). The serum levels of IL-1$\beta$ and TNF- $\alpha$ decreased further, significantly in rats from EOE and GA groups ($P < 0.05$) compared to those from the experimental control group. EOE and GA groups had undetectable levels of TNF- $\alpha$. Misoprostol group had increased levels of serum TNF- $\alpha$ compared to the experimental control group. Data from group G were not included, as the group had undetectable levels of cytokines.

**EOE and GA reduce pro-inflammatory cytokines in a murine model of septic shock**

Considering that EOE and GA brought down the levels of both IL-1$\beta$ and TNF- $\alpha$ in ulcerated rats, we evaluated EOE and GA in modulating the pro-inflammatory cytokines in an endotoxicemia model in mice. Mice were administered i.p. either EOE (100 mg/kg) or GA (19.3 mg/kg) and immediately LPS (16 mg/kg) was administered. A group of mice were injected only with LPS. Sham group received normal physiological saline, which was the vehicle used for all injections. Serum was collected 4 h after the injection of saline. LPS-injected mice had significantly increased serum levels of both IL-1$\beta$ and TNF- $\alpha$ ($P < 0.05$) compared to the sham group (Figure 3). EOE and GA when administered along with LPS, significantly reduced the levels of both the cytokines, compared to the group of mice injected with LPS ($P < 0.05$).

**Immunostaining and image analysis of CXCR1 and iNOS in gastric tissue**

The expression of CXCR1 and iNOS in gastric epithelial cells was evaluated by immunohistochemistry. Gastric tissues from experimental control group exhibited significantly increased expression of both iNOS and CXCR1 compared to the vehicle control group ($P < 0.05$), indicating a high level of iNOS and CXCR1 activity in the healed ulcer (Figures 4–6). The experimental groups treated with GA and misoprostol had significantly reduced expression of both iNOS and CXCR1 in the gastric tissue compared to the experimental control group ($P < 0.05$).

**Arginase levels**

The experimental group treated with GA had significantly increased hepatic arginase activity ($P < 0.05$) compared to the experimental control group (Figure 7). However,
Figure 2. Histopathology of ulcer. a-f. Histological sections of gastric tissues from vehicle control, 0-day ulcer, experimental control, EOE, GA and misoprostol groups respectively.

Figure 3. Immunomodulatory activity of EOE and GA in a murine model of sepsis. LPS was injected i.p. at a dose of 16 mg/kg. Two groups of LPS-injected mice received either EOE (100 mg/kg) or GA (19.3 mg/kg) i.p. Data are expressed as mean ± SE (n = 5). *P < 0.05 when compared to the sham group, †P < 0.05 when compared to the respective LPS group.

hepatic arginase activity in the experimental group treated with EOE was not statistically different from the experimental control.

Discussion

The results presented here demonstrate the ability of EOE and GA to heal indomethacin-induced gastric ulcer. The ulcer-healing properties of both EOE and GA were comparable to the synthetic drug, misoprostol. The results also suggest that immunomodulatory properties of EOE and GA contribute to ulcer healing.

The profile of cytokine/chemokine persisting at an inflammatory site is important for the development of chronic disease. Production of inflammatory cytokine is stimulated by ulcerogenic factors such as NSAIDs. Inflammatory cytokines such as IL-1β and TNF-α cause recurrence of healed ulcer. Since there is an increased infiltration of neutrophils and expression of pro-inflammatory markers in the healed mucosa during the development of ulcer recurrence, the studies looked at the inflammatory markers of the ulcerated rat, on day 7 of the ulcer, when the ulcer index is at the lowest (Figure 1), with a view to associate the inflammatory response of the host with the ulcer index in rats administered with EOE, GA and misoprostol. Neutrophils infiltrate the superficial portion of the scarred mucosa prior to induction of ulcer recurrence by IL-1β. TNF-α induces apoptosis under chronic inflammatory conditions.
conditions\textsuperscript{14}. TNF-\(\alpha\) also facilitates induction of chemokine expression and is involved in gastric ulcer recurrence\textsuperscript{15}. Based on the above data our results suggest that GA could be contributing to the healing of the ulcer, by bringing down the levels of IL-1\(\beta\) and TNF-\(\alpha\) in the ulcerated rat.

EOE and GA administration just before LPS injection significantly decreased the production of serum pro-inflammatory cytokines. The increased Th\(_1\) (T helper 1) cytokines facilitate the conversion of l-arg by iNOS to produce high-output generation of nitric oxide (NO), which is likely to promote the formation of highly reactive and potentially toxic oxidation products of NO\(^*\), namely ONOO\(^-\), leading to chemical and oxidative stresses\textsuperscript{15}. Blocking the expression of TNF-\(\alpha\) and IL-1\(\beta\) has been demonstrated to attenuate the expression of iNOS\textsuperscript{16}. NO\(^*\) is a crucial mediator of gastrointestinal mucosal defence, but paradoxically, it also contributes to tissue injury in the gastrointestinal tract during inflammatory reactions\textsuperscript{17}. An increase in iNOS activity and a decrease in constitutive NOS activity in the gastric mucosa are closely related to the development of gastric mucosal lesions\textsuperscript{8}. Because the potential high-output source of NO\(^*\) in mammalian cells is iNOS, factors involved in the induction and expression of iNOS activity are key determinants of NO\(^*\)-mediated toxicity. Sustained overproduction of NO\(^*\) by iNOS is detrimental and contributes to inflammation in various gastroduodenal disorders\textsuperscript{18-20}. GA derivative was shown to inhibit iNOS protein in activated macrophages\textsuperscript{21}. This reduction could occur through prevention of the binding of, NF-\(\kappa B\) (nuclear factor-kappa B) to the iNOS promoter, thereby inhibiting the induction of iNOS transcription. The above results also suggest that EOE and GA contribute to the healing of the ulcer, through the down regulation of Th\(_1\) cytokines like TNF-\(\alpha\) and IL-1\(\beta\), resulting in the reduced expression of iNOS.

Extrahepatic arginase activity is known to increase in trauma, which may provide the necessary precursors for cellular proliferation and repair, or play a regulatory role in the production of NO\(^*\) via iNOS\textsuperscript{22}. Arginase is known to protect the gastric epithelial cells against ammonia-induced cell damage.
cell death. Spermine synthesized by the arginase-ornithine decarboxylase pathway was shown to inhibit iNOS translation, NO\(^+\) production, and thus inhibited pro-inflammatory gene expression. Based on the above findings, our data of increased arginase and decreased iNOS, by the administration of GA, may be contributing to the anti-ulcer effect of GA. The hepatic arginase activity in experimental group treated with EOE was not different from the experimental control, which may be due to the effect of GA.

Interleukin-8 (IL-8), which is a CXC chemokine, and iNOS are co-expressed in the gastric mucosa and play an important role in the pathogenesis of \textit{H. pylori}-associated gastroduodenal diseases. IL-8, which is one of the early chemotactic signals released at the site of inflammation, mediates its actions via two cell-surface receptors, CXCR1 and CXCR2, which belong to the seven-transmembrane-domain-type and are coupled to G-proteins. The G-protein coupled receptors are increasingly among today’s number-one category of targets for the pharmaceutical industry. Upregulation of CXCR1, expressed mainly by neutrophils, in inflamed gut and stomach tissues, is involved in the inflammatory process. Studies have demonstrated the mucosal activation of neutrophils and eosinophils precedes pronounced enhancement of mucosal NO\(^+\) production in celiac patients challenged with gluten. Neutrophils are the first recruited effectors of the acute inflammatory response, guided by chemotactic factors. The directed migration of neutrophils to local tissue sites can be either beneficial or harmful. Since CXC chemokine IL-8 is a powerful mediator of this process, its receptor CXCR-1 is a reasonable target for the development of treatments for neutrophil-mediated inflammation.

Pental-oalloyl-beta-D-glucose, a GA-containing molecule and a major constituent of the root cortex of \textit{Paenonia suffruticosa}, is known to inhibit IL-8 gene expression by a mechanism involving its inhibition of NF-\textkappaB activation. Participation of iNOS and neutrophil activation result in the amplification of cell death signalling cascade, and hence contributing to the extent of mucosal injury. Data obtained by immunohistochemistry suggest that pharmacological manipulation of CXCR1 by GA seems to limit the inflammation caused by neutrophil migration and activation.

Therapeutic anti-inflammatory drug molecules are discovered based on their ability to block a host of inflammatory mediators, either at their synthesis level or binding to their designated receptors. The current study
indicated GA to be a major constituent of EOE, responsible for the healing of ulcer. GA decreased the expression of IL-1β and TNF-α, CXCR1 and iNOS and increased the levels of arginase in the host tissues, which was correlated with its ability to heal the ulcer more effectively. The study offers the immunopharmacological basis of the use of GA in the management of peptic ulcer. EOE has low toxicity, as a dose of up to 2 g/kg given orally is non-toxic to mice (data not presented). GA is one of the well-absorbed polyphenols. Since polyphenolics show antioxidant capacity and GA in particular shows the highest antioxidant capacity among the groups, the pharmacological development of GA holds great potential in treating mucosal lesions in the gastrointestinal tract.


Received 27 October 2006; revised accepted 8 March 2007