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Regulation of arginase/nitric oxide synthesis axis via cytokine balance contributes to the healing action of malabaricone B against indomethacin-induced gastric ulceration in mice

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1. Introduction

Gastric ulcer is a complex pluricausal disease and is known to develop due to loss of balance between aggressive and protective factors [1]. The non-steroidal anti-inflammatory drugs (NSAIDs) are known to cause gastric ulceration and delay ulcer healing, which is generally explained in terms of cyclooxygenase (COX) inhibition, reduced prostaglandin (PG) synthesis, and the impaired PG-mediated angiogenesis. However, the complete mechanism underlying this effect is not completely understood. Various other factors, especially the nitrogen-metabolizing enzymes are also key contributors in host immune defense mechanisms and wound healing [2–4]. In acute inflammatory responses, such as wound healing, heat stroke and glomerulonephritis, arginine has been implicated as an important regulator of diverse pathways including generation of polyamines and the cytosolic free radical molecule, nitric oxide (NO) [5]. The arginine pathway plays a vital role in wound healing since L-arginine becomes an essential amino acid after wounding with almost undetectable levels in the wound milieu [6]. Studies have shown that arginine itself has advantageous effects on cutaneous healing by enhancing cell proliferation and collagen synthesis as well as breaking strength [7]. Further, NO, produced from arginine also plays an important role in inflammatory processes as a mediator of macrophage function [5,7,8]. The temporal switch of arginine, as a substrate for the inducible nitric oxide synthase (iNOS)/NO axis to arginase/polyamine axis is regulated by the inflammatory cytokines. However, little is known on the interplay of cytokines and the NO synthesis pathway during indomethacin-induced gastric ulceration. After trauma, the Th1/Th2 imbalance with Th2 predominance is reflected by an increase of the arginase inducing cytokines such as IL-4, IL-10, and TGFβ [9].

The fruit rind of the plant Myristica Malabarica (Myristicaceae) (popularly known as rampatri, Bombay mace, or false nutmeg) is used as an exotic spice in various Indian cuisines. Although not substantiated adequately, it is credited with hepatoprotective, anticarcinogenic, and antithrombotic properties, and is found as a constituent in some Ayurvedic preparations such as pasupasi. Earlier, we have reported impressive in vitro antioxidant activity [10] of its constituent phenol malabaricone B. Very recently, we have found that treatment with mal B (10 mg kg⁻¹ × 3 days, p. o.) shifted the iNOS/NO axis to the arginase/polyamine axis as revealed from the increased arginase activity (51.6%, P<0.001), eNOS expression, and reduced iNOS expression, total NOS activity (∼75%, P<0.001), and NO level (50.6%, P<0.01). These could be attributed to a favourable anti/pro inflammatory cytokines ratio, generated by mal B. The healing by omeprazole was however, not significantly associated with those parameters.

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Malabaricone B (Mai B) was isolated from the methanol extract of the dry fruit rind of M. malabarica as reported earlier and characterized from its spectral and chemical analysis [9]. Analysis with a Bio-Whitaker QCL1000 kits (Cambridge, MA, USA) revealed it to be practically free of endotoxin, while its chemical purity was confirmed by high performance liquid chromatography (HPLC) analysis with a Jasco model PU-2080 plus chromatogram, and elemental analysis. $\alpha$-arginine, indomethacin, isonicotroperoxiphenone, Bradford reagent, Triton X-100, leupeptin, phenylmethylsulfonyl fluoride (PMSF), glycine, sodium dodecyl sulfate (SDS), acrylamide, bis-acrylamide, Tween 20, ethylenediamine-nitrosonicotinamide (EDTA), 3,3',5,5'-tetramethylbenzidine (TMB), MnCl$_2$, urea, omeprazole, Trizma base, cetrimide, and nitrocellulose membrane were procured from Sigma Chemicals (St. Louis, MO). Other reagents used were disodium hydrogen phosphate and sodium dihydrogen phosphate (BDH, Pool Dorset, U.K.), sulphuric acid, hydrochloric acid, phosphoric acid, sodium chloride (Thomas Becker, Mumbai, India), horseradish peroxidase (HRPO, Sisco Research Laboratory, Mumbai, India), rabbit polyclonal NOS and eNOS antibodies (Santa Cruz Biotechnology, Delaware, USA), peroxidase conjugated anti-rabbit IgG antibody, enhanced chemiluminescence detection kit (Roche, Mannheim, Germany), NOS and NO assay kits (Calbiochem, California, USA), TGF-P1 (Pierce Biotechnology, Rockford, USA).

2. Materials and methods

2.1. Chemicals and reagents

Mal B was isolated from the methanol extract of the dry fruit rind of M. malabarica by liquid chromatography (HPLC) analysis with a Jasco model PU-2080 plus chromatogram, and elemental analysis. $\alpha$-arginine, indomethacin, isonicotroperoxiphenone, Bradford reagent, Triton X-100, leupeptin, phenylmethylsulfonyl fluoride (PMSF), glycine, sodium dodecyl sulfate (SDS), acrylamide, bis-acrylamide, Tween 20, ethylenediamine-nitrosonicotinamide (EDTA), 3,3',5,5'-tetramethylbenzidine (TMB), MnCl$_2$, urea, omeprazole, Trizma base, cetrimide, and nitrocellulose membrane were procured from Sigma Chemicals (St. Louis, MO). Other reagents used were disodium hydrogen phosphate and sodium dihydrogen phosphate (BDH, Pool Dorset, U.K.), sulphuric acid, hydrochloric acid, phosphoric acid, sodium chloride (Thomas Becker, Mumbai, India), horseradish peroxidase (HRPO, Sisco Research Laboratory, Mumbai, India), rabbit polyclonal NOS and eNOS antibodies (Santa Cruz Biotechnology, Delaware, USA), peroxidase conjugated anti-rabbit IgG antibody, enhanced chemiluminescence detection kit (Roche, Mannheim, Germany), NOS and NO assay kits (Calbiochem, California, USA), TGF-P1 (Pierce Biotechnology, Rockford, USA).

2.2. Characterization and purity data of mal B

HPLC (column: Hypersil GOLD 250x4.6 mm, particle size 5 μm; Thermo Electron Corporation, Waltham, MA, USA, eluent: acetonitrile/water (60:40), flow rate: 1.0 ml/min, peaks detection at 345 nm): Rt = 11.86 min (single peak). IR: 3441, 1684 cm$^{-1}$; $^1$H NMR (MeOH-d$_4$): δ 1.31 (6, 8H, 2×H4-17), 1.43-1.58 (8H, 2×H3 and H8), 2.47 (8, H3, H8), 3.09 (8, 2H, ArCH$_2$), 3.08 (8, 2H, ArCH$_2$), 4.78 (8, 1H, -OH), 6.52 (8, 2H, H-3', H-5'), 6.66 (8, 2H, H-2', H-6'), 6.95 (8, 2H, H-3', H-5'), 7.17 (8, 8H, H-1', H-4'), 13C NMR (MeOH-d$_4$): 24.1, 28.8, 29.0, 31.4, 34.7, 44.5, 114.8, 129.2, 134.8, 135.4, 153.1, 160.9, 164.9, 207.8. Anal. Calcd for C$_{21}$H$_{26}$O$_4$ (mol. wt. 342.43): C, 73.66; H, 7.65%. Found: C, 73.71; H, 7.78%.

2.3. Instrumentation

The absorbance spectrophotometry was carried out at 25 °C using an ELISA reader (Biotech Instruments, USA). The bands obtained from the western blots were quantified using the Gelquant software (DNR BioImaging System, version 2.70, Israel).

2.4. Preparation of the drugs

The drugs were prepared from mal B and omeprazole as aqueous suspensions in 2% gum acacia as the vehicle, and administered to the mice orally.

2.5. Protocol for ulceration and biochemical studies

The mice, bred at the BARC Laboratory Animal House Facility, Mumbai, India were procured after obtaining clearance from the Institutional Animal Ethics Committee (BAEC). The animals were handled following Institutional Animal Ethics Committee Guidelines, and the experiments were permitted by BAEC. Male Swiss albino mice (25-30 g) were reared on a balanced laboratory diet as per National Institute of Nutrition, Hyderabad, India and given tap water ad libitum. They were kept at 20±2 °C, 65-75% humidity, and day-night cycle (12 h/12 h). To perform all the experiments in a blinded fashion, the animals were identified by typical notches in the ear and limbs, and randomized, before the experiments. The mice were divided into four groups (each containing five mice), and each experiment was repeated three times. Ulceration was induced in the groups II–IV mice by administering indomethacin (18 mg kg$^{-1}$, p. o.) dissolved in distilled water and suspended in the vehicle, gum acacia (2%) as a single dose. The animals were deprived of food but had free access to tap water, 24 h before ulcer induction. The mice of groups I and II, serving as normal and ulcerated controls respectively were given the daily oral dose of vehicle (gum acacia in distilled water, 0.2 ml) only. The groups III and IV mice were given mal B (10 mg kg$^{-1}$×3 days, p. o.) and omeprazole (3 mg kg$^{-1}$×3 days, p. o.) respectively, starting the first dose 6 h post indomethacin administration. After 6 h of the last dose of the drugs, the mice were sacrificed after an overdose of thiopental, the stomach was opened along the greater curvature, and the wet weights of the tissues were recorded. The stomach glandular portion from five animals were pooled, rinsed with appropriate buffer, homogenized in the same buffer under cold condition and used for assessing the expression of different NOS and assaying arginase and MPO activities. The total NOS activity and the immunological parameters were analyzed both at the tissue and serum levels, while the NO level was assayed using the serum samples.

2.6. Assessment of ulceration and assay of mucosal neutrophil counts

The glandular portions of the stomach were fixed in 10% formal saline solution for 24 h, embedded in a paraffin block, and cut into 5 μm sections, which were placed onto glass slides, and stained with haematoxyline and eosin for histological examination under a light microscope. One centimetre lengths of each histological section was divided into three fields. The histological damage score was assessed by scoring each field on a 0–4 scale as described previously [13]: 0 = normal mucosa; 1 = epithelial cell damage; 2 = glandular disruption, vasoco­ cession or oedema in the upper mucosa; 3 = mucosal disruption, vasoco­ cession or oedema in the mid-lower mucosa; 4 = extensive mucosal disruption involving the full thickness of the mucosa. The overall mean value of the histological damage score for each of the fields was taken as the histological ulcer index for that section. Similarly, inflammatory scores [14] were assigned after reviewing all slides to assess the range of inflammation as follows: 0 = normal mucosa; 1 = minimal inflammatory cells; 2 = moderate number of inflammatory cells; 3 = large number of inflammatory cells.

Following a reported procedure [15], the mucosal neutrophil numbers were counted in the same number of fields around the blood vessel and non-blood vessel areas in the mucosa of each section. The experiments were performed by two investigators blinded to the group and treatment of animals. The sections were coded to eliminate any observer bias. Data for the histological experiments are presented as mean±S.E.M. from a minimum of three sections per
animal and five animals per group. At least 15 fields were evaluated to count an average neutrophil number.

2.7. Assay of myeloperoxidase (MPO) activity

The MPO activity was assayed following a reported method [16] with slight modifications. The glandular portions of the stomach tissues were homogenized for 30 s in a 50 mM phosphate buffer (pH 6.0), containing 0.5% CTAB and 10 nM EDTA, followed by freeze thawing three times. The homogenate was centrifuged at 12,000 ×g for 20 min at 4 °C. The supernatant was collected, and the protein content determined. The supernatant (50 µl) was added to 80 mM phosphate buffer, pH 5.4 (250 µl), 0.03 M TMB (150 µl) and 0.3 M H₂O₂ (50 µl). After incubating the mixture at 25 °C for 25 min, the reaction was terminated by adding 0.5 M H₂SO₄ (2.5 ml). The absorbance of the mixture at 450 nm was recorded using HRPO as the standard. The MPO activity was expressed as mU/mg protein.

2.8. Arginase assay

Following a known method [17] with minor modifications, the assay was carried out using the glandular portions of the stomach tissue homogenate prepared in ice-cold 25 mM Tris-HCl buffer (pH 7.5) followed by centrifugation at 12,000 ×g for 30 min at 4 °C. The reaction mixture (200 µl) containing 0.5 M L-arginine (pH 9.7), 1 mM MnCl₂, and the tissue extract (100 µl) was incubated for 20 min at 37.4 °C. The reaction was stopped by adding an acid mixture (800 µl, H₂SO₄-H₃PO₄-H₂O, 1:3:7) and 3% isonitrosopropiophenone, followed by heating at 100 °C for 45 min, and the absorbance at 540 nm was read. The data were quantified from a calibration curve prepared using urea (1.5–120 µg), and normalized for tissue protein. One unit (U) of enzyme activity is defined as the amount of enzyme that catalyses the formation of 1 µmol of urea/min.

2.9. Total NOS assay

The NOS activity in the serum and tissue was measured using a commercially available colorimetric kit following manufacturer’s protocol.

2.10. Western blot analyses of tissue iNOS and eNOS expressions

The glandular part of the stomach tissue 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 PMSF (0.1 µM). Following centrifugation at 15,000 ×g for 30 min at 4 °C, the supernatant was collected, and the protein concentration measured. The proteins (40 µg) were resolved by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. The membrane was blocked for 2 h in TBST buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.02% Tween 20) containing 5% fat-free milk powder and incubated overnight at 4 °C with rabbit polyclonal iNOS or eNOS antibody. The membrane was washed over a period of 2 h with TBST and incubated with peroxidase conjugated anti-rabbit IgG (1:2500 dilution). The bands were detected using an enhanced chemiluminescence detection kit and quantified.

2.11. Assay of NO

Following manufacturer’s instruction, the serum NO level was measured using a commercially available colorimetric kit that measures the total nitrite concentration of the sample.

2.12. Assay of cytokines

The IL-4, IL-6, IL-1β, IL-10, and TNF-α levels in serum and glandular part of the stomach tissue were estimated using commercially available ELISA kits following manufacturer’s protocols. The method of TGF-β1 estimation [18] in sera was adopted after acidification to include the active and latent forms of the cytokine. Briefly, 96-well high binding ELISA plates were coated with anti-mouse TGF-β1 monoclonal antibody and incubated overnight at 4 °C. After blocking for 30 min at 37 °C, the wells were washed once with TBST buffer, the samples were activated by acid treatment followed by neutralization. The samples along with the standards were seeded to each well at an appropriate dilution, and incubated at room temperature for 30 min. The wells were washed (5 times), diluted polyclonal antibody (100 µl) added, and the mixture incubated further for 2 h at room temperature. The wells were washed, and incubated for 2 h after addition of TGF-β1 HRPO conjugate (100 µl). After the final wash, TMB (100 µl) was added to each well, the mixture incubated for 15 min, the reaction was stopped by 1 N HCl, and the absorbance at 450 nm was read.

2.13. Statistical analysis

The values are expressed as the mean±S.E.M. The data were analyzed by a paired Student’s t test for the paired data, or one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparisons post hoc test.

3. Results

Earlier we have observed peak ulceration in mice on the 3rd day after indomethacin (18 mg kg⁻¹, single dose) administration, and a three-day treatment with mal B (10 mg kg⁻¹ day⁻¹) and omeprazole (3 mg kg⁻¹ day⁻¹) provided optimal ulcer healing [11,12]. Hence, the present experiments were also carried out under the above conditions.

3.1. Ulcer healing by mal B

Compared to untreated mice, those receiving treatment with mal B and omeprazole showed reduction of the histological damage score by 83.2% and 78.7% (P<0.001) respectively. Likewise, the respective inflammatory scores were also reduced (P<0.001) by 67.9% (mal B) and 61.3% (omeprazole) (Fig. 2).
Fig. 3. Effects of Mal B and omeprazole in modulating the mucosal MPO level in the indomethacin-induced ulcerated mice. The supernatant of the gastric tissue homogenate was incubated with TMB and H2O2 in a suitable buffer, and the MPO activity (mean±S.E.M., n=15) was assayed from the absorbance at 450 nm against HPO as the standard. *P<0.01 compared to normal mice; **P<0.001 compared to untreated mice.

3.2. Regulation of the mucosal MPO activity

Compared to the normal mice, ulceration increased the mucosal MPO in the gastric tissues of untreated mice by 36.4% (P<0.01) (Fig. 3). This was brought down both by Mal B (29.2%, P<0.05) and omeprazole (21.5%, P<0.05). The effect of omeprazole was not significantly different from that of Mal B.

3.3. Regulation of the mucosal neutrophil counts

At peak ulceration, the mucosal neutrophil counts increased 3.46 folds, compared to normal mice (P<0.001). However, treatment with Mal B reduced it by ~70% (P<0.001), which was better (P<0.01) than that of omeprazole, the latter reducing the neutrophil counts by 22.7% (P<0.05) only (Fig. 4).

Fig. 4. Effects of administration of Mal B and omeprazole on indomethacin induced neutrophil accumulation in the mucosal compartment. Mucosal neutrophils numbers (mean±S.E.M., n=15) were counted from histological sections of gastric corpus. #P<0.001 compared to normal mice; *P<0.05, **P<0.001 compared to untreated mice; fP<0.05 compared to omeprazole treatment.

3.4. Regulation of the mucosal arginase activity

The indomethacin-mediated stomach ulceration depleted (30.8%, P<0.01) the arginase level considerably, compared to the normal mice (Fig. 5). Three-day treatment with Mal B and omeprazole enhanced it by 51.6% (P<0.001) and 22.9% (P<0.05) respectively, compared to the untreated mice. The results of Mal B and omeprazole were significantly different (P<0.01).

3.5. Regulation of the NOS activity

Compared to the normal mice, ulceration drastically increased the serum and mucosal NOS activity (Fig. 6) by ~5.6 folds and 6.1 folds respectively (P<0.001). Mal B suppressed (P<0.001) both serum and tissue NOS activities by ~75% and 77% respectively, compared to the untreated mice. The NOS activity (mean±S.E.M., n=15) was measured using a colorimetric kit. #P<0.001 compared to normal mice; *P<0.001 compared to untreated mice; fP<0.01 compared to omeprazole treatment.
Serum Tissue
Animat group

Fig. 9. Regulation of the serum and mucosal TNF-α levels by Mal B and omeprazole after indomethacin administration. The TNF-α levels (mean±S.E.M, n=15) were measured by EUSA. *P< 0.001 compared to normal mice; *P<0.05, **P<0.01 compared to untreated mice; fP<0.01 compared to omeprazole treatment.

3.8. Regulation of the serum Th1 (TNF-α, IL-1β, and IL-6) and Th2 (IL-4 and IL-10) cytokines

Compared to the normal value, ulceration drastically increased the serum and mucosal TNF-α levels (Fig. 9) by ~3.83 and 2.89 folds respectively (P<0.001). Mal B suppressed both these parameters by 46.9% and 42.6% (P<0.01), compared to the untreated mice. Omeprazole, however, reduced the serum and mucosal TNF-α levels by 16.5% (P<0.05) and 8.7% respectively, which were much less (P<0.01) than that of Mal B.

The serum and mucosal IL-1β levels were also markedly increased (P<0.001) by ~13 folds and 3.8 folds respectively of the normal values, due to ulceration (Fig. 10). Compared to the untreated mice, treatment with Mal B reversed the changes, although the effect of omeprazole on the IL-1β expression was much less.

3.7. Regulation of the serum NO level

At peak ulceration, there was a significant increase (2.2 folds, P<0.001) in the serum nitrite level compared to the normal mice. Treatment with Mal B and omeprazole reduced it by 50.6% (P<0.01) and 27.5% (P<0.05) respectively, the effect of Mal B being significantly better (P<0.01) than that of omeprazole (Fig. 8).

Fig. 8. Effects of Mal B and omeprazole in regulating serum NO level in the indomethacin-induced ulcerated mice. The NO levels (mean±S.E.M, n=15) were measured using a colorimetric kit. *P<0.001 compared to normal mice; *P<0.05, **P<0.01 compared to untreated mice; fP<0.01 compared to omeprazole treatment.

Omeprazole, however, reduced the serum and tissue NOS activities by 62.1% and 59% respectively which were significantly less than that of Mal B (P<0.01).

3.6. Modulation of the mucosal iNOS and eNOS expressions

The Western blots of the iNOS and eNOS expressions in the gastric mucosa of the normal, ulcerated and drug (Mal B or omeprazole)-treated mice are shown in Fig. 7. The iNOS expression was very high in the ulcerated tissues, but insignificant in normal gastric tissues. Quantification of the bands revealed that stomach ulceration increased the expressions of iNOS (4.4 folds, P<0.001), but reduced the eNOS expression (46%, P<0.01), compared to normal mice. Treatment with Mal B or omeprazole reversed the changes, although the effect of omeprazole on the eNOS expression was much less.

Fig. 7. Western blots of the iNOS and eNOS expressions of normal, ulcerated, and Mal B and omeprazole-treated gastric tissues of mice. Briefly, 60 µg proteins from the tissue extracts were resolved by 10% SDS-polyacrylamide gel electrophoresis and then electrotransferred to nitrocellulose membrane. The bands were detected using an enhanced chemiluminescence detection kit and quantified using Kodak gel quant software.

3.5. Modulation of the mucosal NO synthase activities

Ulceration increased the gastric mucosal NO synthase activities by ~13 folds and 3.8 folds respectively of the normal values, due to ulceration (Fig. 8). Compared to the untreated mice, treatment with Mal B suppressed these parameters by 55.8% and 50.0%, while omeprazole suppressed these by 20.4% and 14.8% only.

The Western blots of the iNOS and eNOS expressions in the gastric mucosa of the normal, ulcerated and drug (Mal B or omeprazole)-treated mice are shown in Fig. 7. The iNOS expression was very high in the ulcerated tissues, but insignificant in normal gastric tissues. Quantification of the bands revealed that stomach ulceration increased the expressions of iNOS (4.4 folds, P<0.001), but reduced the eNOS expression (46%, P<0.01), compared to normal mice. Treatment with Mal B or omeprazole reversed the changes, although the effect of omeprazole on the eNOS expression was much less.

Fig. 8. Regulation of the serum and mucosal TNF-α levels by Mal B and omeprazole after indomethacin administration. The TNF-α levels (mean±S.E.M, n=15) were measured by EUSA. *P<0.001 compared to normal mice; *P<0.05, **P<0.01 compared to untreated mice; fP<0.01 compared to omeprazole treatment.

3.7. Regulation of the serum NO level

At peak ulceration, there was a significant increase (2.2 folds, P<0.001) in the serum nitrite level compared to the normal mice. Treatment with Mal B and omeprazole reduced it by 50.6% (P<0.01) and 27.5% (P<0.05) respectively, the effect of Mal B being significantly better (P<0.01) than that of omeprazole (Fig. 8).

Fig. 9. Regulation of the serum and mucosal TNF-α levels by Mal B and omeprazole after indomethacin administration. The TNF-α levels (mean±S.E.M, n=15) were measured by EUSA. *P<0.001 compared to normal mice; *P<0.05, **P<0.01 compared to untreated mice; fP<0.01 compared to omeprazole treatment.

3.8. Regulation of the serum Th1 (TNF-α, IL-1β, and IL-6) and Th2 (IL-4 and IL-10) cytokines

Compared to the normal value, ulceration drastically increased the serum and mucosal TNF-α levels (Fig. 9) by ~3.83 and 2.89 folds respectively (P<0.001). Mal B suppressed both these parameters by 46.9% and 42.6% (P<0.01), compared to the untreated mice. Omeprazole, however, reduced the serum and mucosal TNF-α levels by 16.5% (P<0.05) and 8.7% respectively, which were much less (P<0.01) than that of Mal B.

The serum and mucosal IL-1β levels were also markedly increased (P<0.001) by ~13 folds and 3.8 folds respectively of the normal values, due to ulceration (Fig. 10). Compared to the untreated mice, treatment with Mal B suppressed these (P<0.001) these by 55.8% and 50.0%, while omeprazole reduced these (P<0.05) these by 20.4% and 14.8% only. The
Fig. 11. Regulation of the serum and mucosal IL-6 levels by mal B and omeprazole after indomethacin administration. The IL-6 levels (mean±SEM, n=15) were measured by ELISA. *P<0.001 compared to normal mice; **P<0.001 compared to untreated mice; †P<0.01 compared to omeprazole treatment.

The effect of mal B was significantly better than that of omeprazole (P<0.01). Compared to the normal value, ulceration also increased the serum and mucosal IL-6 levels (Fig. 11) by 3.2 folds and 2.8 folds respectively (P<0.001). Mal B suppressed these by 57.5% and 53.4% (P<0.001), compared to the untreated mice. Omeprazole reduced (P<0.05) the serum and mucosal IL-6 levels by 15.3% and 17.8%, which were significantly better than that of omeprazole (P<0.01).

The serum and tissue IL-6 levels (Fig. 12) in the ulcerated mice was reduced by 31.2% and 32.6% (P<0.01) respectively, compared to the normal mice. Treatment with mal B improved it appreciably both at the serum and tissue levels (74.4% and 61.9%, P<0.001). The effect of omeprazole (13.7% and 25.6% increase at serum and tissue levels respectively) was significantly less (P<0.01) than that of mal B.

The serum and tissue IL-10 levels (Fig. 13) in the ulcerated mice was reduced by 37.8% (P<0.01) and 41.3% (P<0.01) respectively, compared to the normal mice. Treatment with mal B appreciably (P<0.001) increased the serum and tissue IL-10 levels by ~72% and 81.6% respectively, while the effect of omeprazole (19.4% and 20.6% increase at serum and tissue levels) was significantly less (P<0.01) than that of mal B.

3.9. Modulation of the serum TGF-β1 level

Compared to the normal value, ulceration reduced (P<0.01) the level of serum and mucosal TGF-β1 (Fig. 14) by 36.4% and 47.5% respectively. Treatment with mal B and omeprazole increased it by 48.7% (P<0.01) and 20.5% (P<0.05) respectively at the serum level. At the mucosal compartment, mal B and omeprazole augmented it by...
66% (P<0.001) and 21.1% (P<0.05) respectively. The better potency of mal B over omeprazole was more pronounced at the tissue level (P<0.01), compared to that in serum (P<0.05).

4. Discussion

The NSAIDs including indomethacin are most widely prescribed for the treatment of pain and inflammation. However, they are also known to cause gastrointestinal (GI) damage, characterized by hyperemia, and increased vascular permeability, as well as delayed ulcer healing [19], wherein several factors such as enzymes, cytokines, and soluble mediators, liberated during the inflammatory response play crucial roles. The impressive healing capacity of mal B (IC50=9.39±0.75 mg kg⁻¹) against the indomethacin-induced gastric ulceration in mice [12] encouraged us to investigate its probable modulatory effect on arginase and NO as well as the Th1/Th2 cytokines profiles, since these are some of the established mediators of wound healing.

Earlier, we have established the healing action of mal B by histology [11]. Quantification of the histological slides in terms of damage score and inflammatory score provided a better assessment of the quality of healing. This was also substantiated from our results with the MPO assay and neutrophil counts. MPO, a marker of neutrophil aggregation at the site of inflammation is frequently increased in ulcerated conditions, and reduced during wound healing [20]. We also observed that while indomethacin administration enhanced the gastric mucosal MPO activity, and neutrophil counts, treatment with mal B (10 mg kg⁻¹ *3 days) and omeprazole (3 mg kg⁻¹ *3 days) reduced it almost equally. These results are consistent with our present and previously reported [11] healing data. Given that mal B contains a resorcinol moiety, it might induce an irreversible, hydrogen peroxide-dependent loss of activities of the heme-containing peroxidases such as MPO.

Metabolism of arginine that can be catalyzed by arginase and NOS, plays a vital role in gastric ulceration and its healing. Upregulation of arginase increases the level of polyamines, which play a significant role in wound healing. The regulatory role of arginase in acute intestinal inflammation and tissue repair has been demonstrated [22,23]. On the other hand, catalysis of L-arginine by NOS produces NO, which can play dual roles in gastric mucosal defense and injury. NOSs exist as constitutive (eNOS), and inducible isoforms (iNOS). The low concentration of NO, produced by the endothelial NOS (eNOS), one of the eNOS isoforms helps wound healing by increasing blood flow [24] and angiogenesis [25,26] in the damaged gastric mucosa. However, the enhanced generation of NO by the iNOS may contribute to the pathogenesis of various gastroduodenal disorders including peptic ulcer [20,27]. Thus, the temporal switch between the inflammatory processes [9,28].

Our results showed that ulceration down regulated the mucosal arginase level, while increasing the iNOS expression significantly. This suggested a shift of the arginine metabolism towards the NO/ iNOS pathway during ulceration. The elevated expressions of iNOS accounted for the increased total NOS activity as well as serum nitrite level due to ulceration. The augmented neutrophil counts due to the indomethacin treatment, as observed in this study, would generate more iNOS-derived NO, and superoxide radicals enhancing oxidative mucosal damage.

Treatment with mal B and omeprazole, especially the former restored the arginase activity almost to normalcy. The effects of mal B and omeprazole on the reduction of the iNOS expression, total NOS activity and NO level were almost similar. Earlier, using eNOS-deficient mice, the importance of eNOS and eNOS-derived NO in regulating microvascular structure during acute inflammation has been demonstrated [29]. The improved arginase activity and favourable eNOS/ iNOS ratio, caused by treatment with mal B may be the key contributing factors in its efficient ulcer-healing. Our results suggested that the eNOS-derived NO contributed maximum to the ulcer healing property of mal B, although a role for neuronal NOS-derived NO cannot be excluded. In contrast, despite showing less effect on modulating eNOS/NO expressions and NO production, omeprazole provided excellent healing. This may be due to other operative mechanism in its healing action as observed by us and others [11,30].

The indomethacin-induced gastropathy is attributed to the increased expression of pro-inflammatory cytokines [31,32], which correlates with the extent of ulceration. Even the cross-talk amongst NO and arginase/polyamine is guided by the cytokine profile of the host [2,3]. In view of this, the immune response due to ulceration, and its modulation by mal B and omeprazole was monitored. This enabled us to associate the inflammatory response with a better prognosis.

Indomethacin administration raised the levels of the pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) while reducing the anti-inflammatory cytokines (IL-4, IL-10 and TGF-β), thereby creating a cytokine imbalance. Increased TNF-α is known to increase iNOS activity by promoting binding of NF-κB to the iNOS promoter [28,33].

We selected IL-1β since it modulates ulcer healing via the COX-2 pathway. Blocking the expression of TNF-α, and IL-1β might attenuate the induction of iNOS expression and NO generation [33,34]. Likewise, IL-4 that remains under the influence of NO, controls the expression of growth factors, and production of the proinflammatory cytokines such as TNF-α. Our result of decreased IL-4 level due to ulceration is consistent with an earlier report [35]. The anti-inflammatory cytokine IL-10 also plays a key role in inflammation. After its initial depletion by NSAIDs, its concentration rises slowly to counter-regulate the production of the pro-inflammatory cytokines. Given that IL-10 is stimulated by PGE2, we also observed reduction of IL-10 on indomethacin administration, as reported earlier [36].

Treatment with mal B reversed the imbalance by reducing the Th1 cytokines drastically, and restoring the levels of IL-4, IL-10, and TGF-β to near normalcy, at both tissue and serum levels. Possibly, mal B increased the IL-10 due to its ability to increase PGE2 synthesis [12]. The upregulation of the anti-inflammatory cytokines by mal B is likely to inhibit the stimulatory effect of indomethacin on the level of pro-inflammatory cytokine release in blood and gastric mucosa, as observed by us.

The enhanced IL-4 level by mal B would trigger the TGF-β-SMAD-signaling pathway to stimulate the extracellular remodeling and subsequent tissue repair. The immunosuppressive Th2 cytokine, TGF-β has a direct role in stimulating epithelial restitution [37]. Besides suppressing the IFN-γ-induced iNOS gene expression and thereby generation of excess NO, it also increases arginase activity during inflammatory processes [9,28,38]. The altered arginase activity and NO expressions, observed during ulceration, and mal B treatment are consistent with their respective effects in modulating the mucosal TGF-β status. In contrast, the effect of omeprazole on the cytokines levels was not very much, as reported earlier [37]. This was consistent with its lesser effect in regulating the arginase and NOS activities, compared to mal B.

Overall, mal B modulated the cytokine profile to shift the balance in favour of arginase/polyamine vs-o-vit iNOS/NO pathway, and also improved the expression of beneficial eNOS. A combination of all these might tilt the balance in favour of the repair mechanisms, explaining its ulcer-healing action. The bimodal nature of general immune responses is explained by the Th1/Th2 paradigm [39]. 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 highlighted that the balance of the pro- and anti-inflammatory, as well as regulatory cytokines could play a significant role in the NSAID-induced gastric mucosal injury.
References


A Un-coated PVDF membrane

B PVDF membrane coated with lipid solution

C PVDF membrane coated with lipid/oil/lipid tri-layer
Angiogenic and Cell Proliferating Action of the Natural Diarylnonanoids, Malabaricone B and Malabaricone C during Healing of Indomethacin-induced Gastric Ulceration

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Purpose. To evaluate the plant phenolics, malabaricone B (mal B) and malabaricone C (mal C) in healing stomach ulcer by modulating angiogenesis.

Materials and Methods. Male Swiss albino mice, ulcerated with indomethacin (18 mg/kg, p. o., single dose) were treated up to 7 days with different doses of mal B or mal C. The healing capacities of the drugs and their effects on the angiogenic parameters were assessed.

Results. Maximum ulceration, observed on the 3rd day after indomethacin administration was effectively healed by mal B and mal C (each 10 mg/kg, p. o. *3 days), the latter showing equivalent potency (-18% p <0.001) as that of Omez (3 mg/kg, p. o.*3 days) and misoprostol (10 µg/kg, p. o.x3 days). Compared to the untreated mice, those treated with mal B or mal C respectively for 3 days increased the mucosal EGF level (139 and 178%, p<0.001), the serum VEGF level (56%, p<0.01 and 95%, p<0.001) and microvessels formation (37%, p<0.05 and 62%, p<0.01), while reducing the serum endostatin level (37%, p<0.05 and 61%, p<0.01). The relative healing capacities of mal B and mal C correlated well with their respective abilities to modulate the angiogenic factors. The healing by Omez and misoprostol was not due to improved angiogenesis.

Conclusions. The drugs, mal B and mal C could effectively heal indomethacin-induced stomach ulceration in mice by promoting angiogenesis.

KEY WORDS: angiogenesis; EGF; endostatin; gastrointestinal toxicology; malabaricone; VEGF.

INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are associated with an increased prevalence of gastroduodenal ulceration (1) and their continued use in the presence of ulceration results in delayed healing (2,3). Consequently, prevention of NSAID-mediated gastrointestinal disorder continues to be of concern for both clinical practitioner and researchers. Several mechanisms including mucosal blood flow (4), cellular proliferation and migration at the ulcer edge, and maturation of granulation tissue at the ulcer base (5) contribute to ulcer healing. Angiogenesis, the formation of new blood vessels, is an important component of granulation tissue maturation and plays a crucial part in wound healing (6). Hence, drugs that can modulate the angiogenic response during acute inflammatory insult of the NSAIDs are potential targets for therapeutic research. In spite of their efficacy in managing the NSAID induced gastric ulceration, the currently available synthetic anti-ulcer drugs confer side effects, and are expensive especially for the rural population. Development of suitable formulations from dietary sources might provide antulcer medications with less or no toxicity, and affordability.

The fruit rind of the plant Myristica malabarica (Myristicaceae) (popularly known as rampatri, Bombay mace, or false nutmeg) is used as an exotic spice in various Indian cuisines. Although not substantiated adequately, it is credited with hepatoprotective, anticarcinogenic, and antithrombotic properties, and is found as a constituent in many Ayurvedic preparations such as pasupasi. Very recently, we have found (7) that two of its constituent diarylnonanoids, malabaricone B (mal B) and malabaricone C (mal C), especially the latter possess better in vitro antioxidant activity than curcumin. The chemical structures of the malabaricones are shown in Fig. 1. Given that many anti-ulcer drugs exert their action via antioxidative activity (8), we were interested to study the healing property of mal B and mal C against indomethacin-induced acute gastric ulceration of mice vis-à-vis that of the commercial drugs, omeprazole (Omez) and misoprostol. Another objective of the investigation was to correlate the ulcer healing capacity of the test samples with their ability to correlate the ulcer healing capacity of the test samples with their ability to...
modulate different angiogenic factors and influence the vascularity of granulation tissue in gastric ulcers of mice.

MATERIALS AND METHODS

Chemicals and Reagents

The malabaricones (designated as mal B and mal C respectively) were isolated from the methanol extract of dried fruit rind of *M. malabarica* as reported earlier (7). Indomethacin, bovine serum albumin (BSA), omeprazole (Omez), misoprostol, 3,3'-diaminobenzidine (DAB), rabbit anti-mouse EGF and Trizma base were procured from Sigma, St. Louis, USA. The vascular endothelial growth factor (VEGF) ELISA kit and peroxidase conjugated goat anti-rabbit IgG were from EMD Biosciences, Sandiego, USA while endostatin and von Willebrand Factor (rabbit anti-human) were from Chemicon, Temecula, USA. Other reagents used were hydrogen peroxide (35%, Lancaster, Morecambe, UK), disodium hydrogen phosphate and sodium dihydrogen phosphate from BDH, Poole Dorset, U.K, haematoxylin (mono-hydrate) and eosin yellowish (both from Merck, Mumbai, India), potash alum (S.D. Fine Chem., Mumbai, India), and horse and goat sera (Banglore Genie, Banglore, India).

Preparation of the Drugs

The drugs were prepared from mal B, mal C, Omez and misoprostol as aqueous suspensions in 2% gum acacia as the vehicle, and administered to the mice orally.

Experimental Protocol for Ulceration and Healing

The mice were bred at Dr. B. C. Roy Post Graduate Institute of Basic Medical Sciences, Kolkata, India and BARC Laboratory Animal House Facility, Mumbai, India. They were deprived of food but had free access to tap water 24 h before ulcer induction. The ulcers in the mice was induced by administrating a single dose of indomethacin (18 mg/kg, p. o.) dissolved in distilled water and suspended in 2% gum acacia. Our studies with 5, 10, 15, 18, 20, 25 and 30 mg/kg, p. o. of indomethacin revealed that the lowest doses (5 and 10 mg/kg) provided minor ulceration after 6 h of its administration, while the higher doses (25 and 30 mg/kg) led to mortality. The chosen dose (18 mg/kg) produced optimal ulceration, with inflammation and mucosal insult, without causing any mortality to mice.

Standardization of Doses of Mal B and Mal C

For this, mice were given mal B or mal C (2, 5, 10, 15 and 20 mg/kg) as a single dose per day up to seven days, starting from 6 h of indomethacin administration. Five mice were taken in each treatment regime and each experiment was repeated three times. The mice were sacrificed on the respective days 4 h after the administration of the drugs. The extent of healing was assessed from the macroscopic damage scores (MDS) of the untreated and treated ulcerated mice, measured on first, third, fifth and seventh days and the treatment regime (dose and time) of the drugs was optimized. During these experiments, the normal and ulcerated control mice of the respective days of ulceration were given the vehicle oral dose of gum acacia in distilled water (0.2 ml per mouse) only.

Assessment of Ulceration and Healing from MDS

The mice were sacrificed after an overdose with thiopental. The stomach from the normal and treated groups were removed rapidly, opened along the greater curvature, and thoroughly rinsed with normal saline. The ulcerated gastric mucosal areas were visualized using a transparent sheet and a dissecting microscope. The MDS was assessed (9) 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, 4–mucosa full of lesions. (lesions–hemorrhagic erosions, hyperemia–vascular congestions). The experiments were performed by two investigators blinded to the groups and treatment of animals.

Studies on the Histopathological and Biochemical Parameters

Our MDS results revealed that maximum ulceration in the untreated mice as well as best healing with the drug-treated mice were evident on the third day after indomethacin administration. Hence, we assessed the histopathological and biochemical parameters under the optimized treatment regime [mal B and mal C (each 10 mg/kg), Omez (3 mg/kg) and misoprostol (10 µg/kg)] up to the 3rd day of ulceration only. The dose of Omez, which is also the recommended therapeutic dose for humans, was decided based on the results of our own studies (10). The dose of misoprostol was optimized by carrying out separate experiments with 2–15 µg/kg of the drug (data not shown). For the histopathological and biochemical assays, the following 11 groups of mice were selected from those used for the MDS assay: group I—normal mice; group II—ulcerated mice and sacrificed after 10 h (considered as 1 day); group III—ulcerated mice, and sacrificed after 3 days; groups IV–VII—ulcerated mice, treated with mal B, mal C, Omez and misoprostol respectively for 1 day, and sacrificed 4 h after administration of the drugs; groups VIII–XI—ulcerated mice, treated with mal B, mal C, Omez and misoprostol respectively for three days, and sacrificed 4 h after administration of the last dose of the drugs.
Ulcer Healing by Malabaricones via Angiogenesis

Histopathological Studies of Stomach Tissues

For the histological studies, the ulcerated portions of the stomach were sectioned after fixing in 10% formal saline solution. After 24 h of fixation followed by embedding in a paraffin block, it was cut into sections of 5 micron onto a glass slide, stained with haematoxylin–eosin and examined under a light microscope.

Quantification of Epidermal Growth Factor (EGF) Expression

For immunostaining EGF, the paraffin-embedded sections were processed following a reported procedure (11), with slight modification. Briefly, the stomach specimens were fixed in neutral-buffered formalin within 30 min of harvesting. After deparaffinization in xylene, the sections were treated with a graded series of alcohol and subsequently rehydrated in PBS at pH 7.5. Following blocking of the endogenous peroxidase activity with 3% hydrogen peroxide in PBS, samples were exposed to protein blockers (5% normal horse serum, 1% normal goat serum in PBS) and incubated overnight at 4°C with primary antibody at the appropriate dilution. In control sections, only PBS was added omitting the antibodies. After incubation for 1 h at room temperature with peroxidase conjugated goat anti-rabbit IgG, a positive reaction was detected by exposure to DAB for 2 to 5 min. The slides were counterstained with Meyer’s haematoxylin, and the intensities of the immunolocalized areas were quantified using Biovis MV500 software. Five areas from each section were scanned and the integrated optical density (IOD) in each area was calculated. The IOD of the negative control was subtracted from the IOD of each experimental section for each animal in all the groups.

Assay of VEGF and Endostatin

The serum VEGF and endostatin were measured using the blood samples drawn from the descending aorta, with commercially available ELISA kits following manufacturer’s instruction.

Quantification of Angiogenesis by von Willebrand Factor VIII (vWF VIII)

The number of microvessels in the ulcer was assessed from vWF, following a reported immunohistochemical procedure (12) with slight modifications. Briefly, following digestion of the tissue section with 0.1% trypsin, endogenous peroxidase activity as well as nonspecific protein binding sites were blocked. The sections were incubated with the polyclonal rabbit antihuman Factor VIII-related antigen for 2 h at room temperature and the peroxidase method was used to assay the formation of microvessels. Any positive-staining endothelial cells or endothelial cell clusters that were clearly separated from adjacent microvessels were considered an angiogenic microvessel. The vascular areas immediately adjacent to the normal tissue of the stomach served as internal quality controls. The microvessels (under ×400 magnification) on coded slides in five randomly selected microscopic fields of mucosal erosions were counted, and the data were averaged.

Acute Toxicity Assay of Mal B and Mal C on Mice

The acute toxicity of mal B and mal C on mice was studied by oral gavage of the drugs (each 500 mg/kg) and observing the animals for one month. The experiments were carried out with 15 mice and repeating the experiment twice. At the end of the observation period, the animals were sacrificed and the histology of the liver and kidney was assessed. For renal and liver function tests, animals were bled from the retro-orbital complex, and the serum of each mouse was analyzed for urea, creatinine, SOFT, and SGOT with an autoanalyser (Randox daytona, UK) using the respective kits.

Statistical Analysis

The data are presented as mean±S.E.M. Parametric data which includes all the biochemical parameters were analyzed using a paired ‘t’ test for the paired data or one way analysis of variance (ANOVA) followed by a Dunnett multiple comparisons post test. Nonparametric data (macroscopic scoring) were analyzed using Kruskal–Wallis test (nonparametric ANOVA) followed by a Dunn’s multiple comparisons post test. In addition to all the tests, Bonferroni correction was also carried out for knowing the simultaneous statistical inference among the groups under investigations. The IC50 values of the malabaricones were estimated using the Probit analysis and the significance level of the analyses was also investigated by the chi-square test. A probability value of p<0.05 was considered significant.

RESULTS

Standardization of Treatment Regime with Mal B and Mal C for Gastric Ulcer Healing

The time course of the extent of macroscopic damage due to gastric ulceration and its prevention by different doses of mal B and mal C are shown in Fig. 2a and b. The mice receiving vehicle only showed no lesions in the gastric mucosa. On third day of ulceration, the MDS value reached maximum increasing by −107% compared to that on day one (p<0.001). The autohealing was prominent on the seventh day after ulceration, when the MDS value was reduced by 44.1% compared to that on day 1 (p<0.01).

Both mal B and mal C, at all the chosen doses showed maximum ulcer healing on the third day of ulceration, and the effect was dose-dependent. Compared to the respective ulcerated controls, treatment with mal B (10 and 20 mg/kg) for one and 3 days reduced the MDS by 17.8–24.3% (p<0.05) and 61.9–69.8% (p<0.001) respectively. The effects of mal B (10–20 mg/kg) were significantly better (p<0.05) than that at its lower dose (5 mg/kg).

In contrast, mal C showed much better results than mal B at any given dose and treatment period. For example, compared to the ulcerated untreated mice, the mice receiving mal C (10–20 mg/kg) showed a significant (p<0.01) reduction in the MDS even on the day of ulcer induction. Prolonging the treatment for three days reduced the MDS by 78.1–85.7% (p<0.001), compared to the group III mice. The results of the higher doses (10–20 mg/kg) of mal C were similar, and significantly different (p<0.01) from those receiving mal C...
Days of ulceration

Days of ulceration

Fig. 2. a Comparative dose-dependent healing capacity of mal B against indomethacin-induced stomach ulceration in mice. Stomach ulceration in mice was induced by oral administration of indomethacin (18 mg/kg). Different doses of mal B were used for the experiments. The ulcer indices in terms of the macroscopic damage scores (MDS) were measured on different days after indomethacin administration and the values are mean±SEM (n=15). *p<0.01; **p<0.001 compared to normal mice; *p<0.05, **p<0.01 compared to respective ulcerated mice; *p<0.05 compared to mice treated with 5 mg/kg of mal B.

b Comparative dose-dependent healing capacity of mal C against indomethacin-induced stomach ulceration in mice. Stomach ulceration in mice was induced by oral administration of indomethacin (18 mg/kg). Different doses of mal C were used for the experiments. The ulcer indices in terms of the macroscopic damage scores (MDS) were measured on different days after indomethacin administration and the values are mean±SEM (n=15). *p<0.01; **p<0.001 compared to normal mice; *p<0.05, **p<0.01 compared to respective ulcerated mice; *p<0.05 compared to mice treated with 5 mg/kg of mal C.

In comparison, treatment with Omez (3 mg/kg) and misoprostol (10 μg/kg) for three days reduced the MDS by 76.3 and 78.8% respectively, compared to that in the untreated mice (p<0.001).

For the untreated mice, peak ulceration (maximum MDS) was observed on the third day of indomethacin administration. Hence, this time point was selected to find out the IC50 values of mal B and mal C. Considering the MDS values of the third day ulcerated untreated mice as 100%, the IC50 values of mal B and mal C were found to be 8.61±0.65 and 6.01±0.42 mg/kg respectively, which were significantly different (p<0.05; Fig. 3).

Effect of the Drugs on Biochemical Parameters

Overall, treatment with both mal B and mal C (10 mg/kg) for 3 days after ulcer induction provided optimal ulcer healing. Hence, only those mice receiving mal B, mal C (each 10 mg/kg), Omez (3 mg/kg) or misoprostol (10 μg/kg) up to 3 days were selected for assessing the angiogenic parameters. The comparative results of the untreated and treated groups of mice are summarized in Tables 1 and 2.

Macroscopic and Histological Assessment

At 6 h post indomethacin administration, macroscopic evaluation was performed on the basis of severity of hyperemia (vascular congestions) and hemorrhagic erosions. On the day of ulcer induction itself, hemorrhagic lesions covering the total glandular area of the stomach was evident with the untreated mice. However, mucosal hyperemia along with hemorrhage reached maximum on the third day of ulceration along with the disruption of the gel-like mucin cover in the untreated group. Administration of the drugs had immediate effect, reducing the glandular hemorrhagic lesions substantially. Prolonging the treatment for three days reduced the mucosal congestion and restored the hydrophobic mucous layer to near normalcy.
Table 1. The Effect of Indomethacin-Mediated Stomach Ulceration of Mice on the Levels of Serum VEGF and Endostatin, and Tissue EGF and vWF on the First Day of Ulceration and their Modulation by Mal B, Mal C, Omez and Misoprostol

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group I Normal control</th>
<th>Group II Ulcerated control</th>
<th>Group IV mal B-treated</th>
<th>Group V mal C-treated</th>
<th>Group VI Omez-treated</th>
<th>Group VII Misoprostol-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGF (integrated O.D. × 10[^3])</td>
<td>2.31 ±0.21</td>
<td>6.24±0.55[^a]</td>
<td>12.95±1.09[^**]</td>
<td>14.14±1.35[^<strong>][^</strong>*]</td>
<td>6.32±0.46</td>
<td>7.68±0.62[^**]</td>
</tr>
<tr>
<td>VEGF (ng/ml)</td>
<td>10.09±0.97</td>
<td>9.05±0.52</td>
<td>8.89±0.89</td>
<td>8.66±0.77</td>
<td>8.19±0.69</td>
<td>9.39±0.86[^**]</td>
</tr>
<tr>
<td>Endostatin (ng/ml)</td>
<td>2.42±0.26</td>
<td>15.21±1.84[^a]</td>
<td>15.61±1.58</td>
<td>14.07±1.12</td>
<td>15.95±1.88</td>
<td>14.39±1.54</td>
</tr>
<tr>
<td>vWF (microvessels/field)</td>
<td>36.14±3.58</td>
<td>35.58±3.58</td>
<td>32.54±2.8</td>
<td>34.86±2.65</td>
<td>33.85±2.88</td>
<td>36.01±3.45</td>
</tr>
</tbody>
</table>

*a Stomach ulceration in mice was induced by oral administration of indomethacin (18 mg/kg). Mal B, Mal C (each 10 mg/kg x 1 day), Omez (3 mg/kg x 1 day) and misoprostol (10 µg/kg x 1 day) were used as the drugs. The assays were carried out 4 h after the drug administration. The values are mean±SEM (n=15).

[^a]p<0.001 compared to normal mice
[^**]p<0.05 compared to ulcerated untreated mice
[^***]p<0.01 compared to misoprostol-treated mice
[^****]p<0.001 compared to misoprostol-treated mice

Table 2. The Effect of Indomethacin-Mediated Stomach Ulceration of Mice on the Levels of Serum VEGF and Endostatin, and Tissue EGF and vWF on the Third Day of Ulceration and their Modulation by Mal B, Mal C, Omez and Misoprostol

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group I Normal control</th>
<th>Group III Ulcerated control</th>
<th>Group IV mal B-treated</th>
<th>Group V mal C-treated</th>
<th>Group VI Omez-treated</th>
<th>Group VII Misoprostol-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGF (integrated O.D. × 10[^3])</td>
<td>2.30±0.22</td>
<td>8.20±0.89[^***]</td>
<td>19.59±1.87[^b][^d]</td>
<td>22.81±1.99[^b][[^d]</td>
<td>9.95±0.98[^****]</td>
<td>11.41±1.61[^**]</td>
</tr>
<tr>
<td>VEGF (ng/ml)</td>
<td>11.78±0.75</td>
<td>6.47±0.96[^***]</td>
<td>10.12±1.6[^d][[^c]</td>
<td>12.64±1.28[^d][[^c]</td>
<td>7.15±0.95</td>
<td>7.90±0.84[^***]</td>
</tr>
<tr>
<td>Endostatin (ng/ml)</td>
<td>2.64±0.35</td>
<td>25.15±2.15[^***]</td>
<td>15.87±0.69[^***][[^c]</td>
<td>9.71±0.93[^d][[^c]</td>
<td>21.73±2.55</td>
<td>21.78±1.54</td>
</tr>
<tr>
<td>vWF (microvessels/field)</td>
<td>37.12±2.95</td>
<td>25.28±2.54[^a]</td>
<td>34.54±3.42[^***][[^c]</td>
<td>40.85±3.95[^d][[^c]</td>
<td>26.65±2.54</td>
<td>29.54±2.75[^***]</td>
</tr>
</tbody>
</table>

*a Stomach ulceration in mice was induced by oral administration of indomethacin (18 mg/kg). Mal B, Mal C (each 10 mg/kg x 1 day), Omez (3 mg/kg x 1 day) and misoprostol (10 µg/kg x 1 day) were used as the drugs. The assays were carried out 4 h after the last dose of the drugs. The values are mean±SEM (n=15).
[^**]p<0.05 compared to normal mice
[^***]p<0.01 compared to normal mice
[^****]p<0.001 compared to normal mice
[^b]p<0.05 compared to ulcerated untreated mice
[^c]p<0.01 compared to ulcerated untreated mice
[^d]p<0.001 compared to ulcerated untreated mice
[^e]p<0.05 compared to Omez and misoprostol-treated mice
[^f]p<0.01 compared to Omez and misoprostol-treated mice
[^g]p<0.001 compared to Omez and misoprostol-treated mice
[^h]p<0.001 compared to Omez and misoprostol-treated mice
Consistent with macroscopic observations, the histological examination of the untreated ulcerated mice stomach also revealed immediate (first day) superficial damage to the glandular portion. Subsequently, exfoliation of gastric epithelial cells, disruption of mucosal layer and strong infiltration by inflammatory cells were observed at peak ulceration. Treatment with the drugs showed re-epithelialization and progressive regeneration of the mucosal architecture starting from the first day. Treatment for three days helped in the proliferation of the gastric epithelial cells which migrated over and into the ulcer crater forming a region with maximal repair activity (Fig. 4a–e). Formation of microvessels was also apparent. Among the drugs, mal C, Omez and misoprostol promoted much faster ulcer healing than mal B.

Effect of the Drugs on Macosal EGF of the Ulcerated Mice

Immunohistochemistry of the stomach tissue of mice (Fig. 5) showed that administration of the drugs led to an increased expression of EGF in mucosa of the ulcer margin. EGF was found to be immunolocalized in proliferative zone cells and in some parietal cells in the gastric oxyntic mucosa. Quantification of the immunopositive areas revealed increased EGF expression by 2.7 (p<0.001) and 3.6 fold (p<0.001) respectively on the first and third days of ulceration in the untreated mice compared to that in normal mice. Treatment with mal B and mal C for 1 day increased the EGF expression by 107.6 and 126.6% respectively compared to that of the

A- Day1

B- Day 3

Fig. 4. Histological assessment of acute gastric mucosal injury induced by indomethacin (18 mg/kg) in mice and its prevention by mal B (10 mg/kg), mal C (10 mg/kg) and Omez (3 mg/kg). Section of mice stomachs obtained from a normal control mice on day 3; b untreated control mice on the third day of ulceration; c–e ulcerated mice treated with mal B, mal C and Omez for 3 days. Black and white arrows indicate inflammatory cells and mucosal damage, respectively.

Fig. 5. Immunohistochemical staining of EGF expression in indomethacin administered acute gastric ulcers of mice and its modulation by mal B (10 mg/kg), mal C (10 mg/kg) and Omez (3 mg/kg). A EGF expression on day 1; B EGF expression on day 3. a normal control mice; b ulcerated untreated mice, c–e ulcerated mice treated with mal B, mal C and Omez. EGF immunostaining was carried out using the peroxidase conjugate. Original magnification x400.
Ulcer Healing by Malabaricones via Angiogenesis

untreated mice (p < 0.001). Prolonging the treatment for 3 days with mal B and mal C increased the EGF expression by 138.9 and 178.2% respectively, compared to the group III mice (p < 0.001). Omez increased the EGF expression by 21.3% (p < 0.05), without showing any immediate effect. However, misoprostol augmented it by 23.0% (p < 0.05) and 39.1% (p < 0.01) respectively compared to those of the group II and III mice.

Effect of the Drugs on the Serum VEGF and Endostatin Levels of the Ulcerated Mice

Indomethacin administration reduced the serum VEGF level by 45.1% on the third day of ulceration, compared to that normal mice (p < 0.01). Compared to the untreated mice, treatment with mal B, mal C and misoprostol for 3 days increased the serum VEGF level by 56.4% (p < 0.01), 95.4% (p < 0.001) and 22.1% (p < 0.05) respectively. The effects of mal B (p < 0.01) and mal C (p < 0.001) were significantly better than those of Omez and misoprostol. Omez did not alter it notably. However, our separate experiments carried out with higher doses (5, 10 and 15 mg/kg x 3 days) of Omez revealed that it could increase the VEGF level by ~20.0% compared to that of the group III mice (p < 0.05) only at ≥10 mg/kg.

Indomethacin administration caused a marked upregulation of the serum endostatin level by 6.3 and 9.5 fold on the first and third days ulceration, compared to that of the normal mice (p < 0.001). Treatment with mal B and mal C for three days reduced the level of serum endostatin by 36.9% (p < 0.05), and 61.4% (p < 0.01) respectively compared to the group III mice. In contrast, both Omez (3–10 mg/kg) and misoprostol (10 μg/kg) reduced the serum endostatin level by ~13.5% only, which was significantly less than that of mal B (p < 0.05) and mal C (p < 0.01). Even at higher doses, misoprostol did not alter the parameter significantly (data not shown).

Effect of the Drugs on vWF VIII of the Ulcerated Mice

The number of microvessels in the ulcerated mice was reduced by 31.9% than that in normal mice (p < 0.05), without being affected on the day of ulcer induction. Compared to the group III mice, treatment with mal B and mal C for 3 days increased the mucosal microvessels by 36.6% (p < 0.05) and 61.6% (p < 0.01) respectively. Treatment with misoprostol for 3 days significantly enhanced the parameter (16.9%) compared to the ulcerated untreated animals (p < 0.05). Omez (3 mg/kg x 3 days) was ineffective, but at a dose of 10 mg/kg, it increased the mucosal microvessels by 15.6% (p < 0.05), compared to that of the group III mice.

Studies on Acute Toxicity of Mal B and Mal C in Mice

The possible toxic effect of mal B and mal C (each 500 mg/kg) on mice revealed no observable physical sign change, with normal food and water intake as well as stool during the experimental period. Normal hepatic microarchitecture, laminar arrangement of hepatocyte, central vein, portal triad, and biliary canaliculi without any inflammatory infiltrate or necrosis were observed in the mice livers receiving the drugs. Likewise, normal renal micro-architecture with well-differentiated cortex and medulla and without hemorrhage, or inflammation was found in the kidneys.

DISCUSSION

Clinical and experimental data indicate that, besides inducing gastric ulceration, the NSAIDs also delay the healing of gastroduodenal ulcers by interfering with the action of growth factors, decreasing epithelial cell proliferation in the ulcer margin, decreasing angiogenesis in the ulcer bed, and slowing maturation of the granulation tissue (13). Angiogenesis, requiring the concerted interaction of a variety of cellular systems is a pivotal process in all types of wound healing, including the healing of gastric ulcers (14). It is regulated by proangiogenic factors such as VEGF, fibroblast growth factor, and EGF, as well as antiangiogenic factors (such as endostatin). An imbalance in the production of antiangiogenic versus proangiogenic factors could result in impaired angiogenesis and ulcer healing (15). It is possible that the differential efficacy of some of the ulcer-healing drugs could be attributed to their divergent effects on angiogenesis.

In the present study, we studied the possible healing effects of mal B and mal C against indomethacin-induced acute gastric lesions in mice, and rationalized their potency with their capacity in augmenting the growth of new blood vessels and modulating different angiogenic factors. For this, we focused on EGF, VEGF, and endostatin, and also analyzed the microvessel formation.

Both proton pump inhibitors (PPI) and prostaglandin (PG) preparations are believed to prevent NSAID-induced gastric ulceration and circumvent the delayed healing. But it remains unclear which of these drugs is superior. Hence, we compared the efficacy of mal B and mal C with that of Omez, a PPI, and misoprostol, a PGE1 analogue. We choose Omez as one of the positive controls, since it is reported to have greater efficacy and tolerability in the management of NSAID-associated GI side effects including those in clinical conditions (16,17). On the other hand, the choice of misoprostol was obvious, considering its specific use against the NSAID-induced gastropathy. Apart from counteracting the inhibition of PG production and reducing the secretion of gastric acid, it also maintains the gastric mucosal barrier and mucosal blood flow (1,18).

Our macroscopic and histopathological examinations revealed that administration of indomethacin caused marked damage to the gastric mucosa with elongated hemorrhagic lesions in the glandular portion. The maximum ulcerative damage, observed on the third day after indomethacin administration, was, however, acute in nature as evident from natural recovery of the gastric tissues even without any treatment. However, the healing was only partial (~44%) even after seven days. In comparison, the mice treated with mal B, mal C, Omez and misoprostol showed significantly faster and better healing within three days. The effects of mal C, Omez and misoprostol were comparable, and much better than that of mal B. The healing observed on extending the treatment up to seven days with both mal B and mal C was only marginally better than that observed with the three-day treatment regime. However, a major part of this was due to natural healing.
The growth factor, EGF accelerates gastroduodenal ulcer healing by stimulating cell migration and proliferation in epithelial cell monolayers, tissue repair, increasing release of gastric mucin, and attenuating gastric acid secretion (19). EGF increases the MAZ kinase activity by activating the EGF receptor, and also stimulates PG synthesis that keep the gastroduodenal cell loss/renewal tightly regulated to prevent ulceration and hyperplasia (20,21). A marked reduction in the concentration of EGF has been reported in the gastric juice of both duodenal and gastric ulcer in a larger population of patients in the active stage (22). The role of the endogenous EGF in ulcer healing is also unequivocally confirmed (23).

Our result on the increase in EGF level of the ulcerated mice over that of normal control mice is consistent with the requirement of more EGF for ulcer healing. This was increased further by mal B and mal C, while misoprostol and Omez showed significantly less effect. The present finding with mal B and mal C appears promising given that drugs that can stimulate endogenous EGF level are attractive for treating gastric ulcer. Of the many growth factors, VEGF promotes endothelial proliferation and migration, and accelerate ulcer healing (24). It promotes restoration of the connective tissue and microvessels (angiogenesis) in injured mucosa. Indomethacin inhibits ADP-induced platelet aggregation and release of the α-granule, which stores VEGF (25). Consequently, indomethacin treatment would reduce VEGF release. In contrast, endostatin, the most potent inhibitor of angiogenesis (26) acts via inhibition of endothelial cell growth and migration, apoptosis promotion, and antagonization of VEGF (27).

We also observed significantly reduced serum VEGF, and increased endostatin levels due to the indomethacin-induced ulceration. Three-day treatment with mal B and mal C reversed these changes simultaneously. In contrast, although misoprostol and omeprazole provided excellent healing, their effect in modulating VEGF and endostatin levels was marginal.

Gastric ulcer healing entails several distinct repair mechanisms. The epithelial cell proliferation and migration from the ulcer edge across the ulcer bed is accompanied by maturation of granulation tissue beneath the ulcer base. Within this tissue vascular endothelial cells form new capillaries to restore the microvasculature, while fibroblasts restore the lamina propria. The degree of neoangiogenesis, as assessed by using specific endothelial markers including vWF, CD31, and CD34 in experimental ulcer models correlates well with the extent and speed of ulcer healing. Among the markers, vWF acts as a cofactor for platelet binding to expose extracellular matrix in injured vessel walls. It is implicated in the angiogenic functions of VEGF, and is reported to increase endothelial cell adhesion, helping maintenance of endothelial integrity (28). The endothelial vWF secretion (29), which is also induced by VEGF is crucial for platelet adhesion to subendothelial collagen, and upregulation of tissue factor.

With regard to NSAID's interference with angiogenesis during gastric ulcer healing, our studies revealed that indomethacin administration led to a progressive reduction of the number of microvessels in granulation tissues. Maximum reduction of the number of microvessels was observed on the third day of ulceration, reflecting inhibition of angiogenesis and the associated delay in ulcer healing in mice. Treatment with mal B, mal C and misoprostol, but not Omez for three days increased the number of microvessels significantly compared to that in the untreated ulcerated mice. The superior result with mal C was consistent with its better ability to increase the proangiogenic factors (EGF, VEGF), and reduce the serum endostatin level, compared to mal B.

In contrast to the malabaricones, modulation of angiogenesis was not the major contributing factor in the ulcer healing by the low doses of Omez, as reported earlier (30). Possibly, at these doses, Omez acts via its anti-secretory (31) and antioxidant action (8). Interestingly, the ulcer healing by Omez (10 mg/kg×3 days) was associated with increased angiogenesis as revealed from the increase in VEGF and vWF levels. However, at ≥10 mg/kg, Omez might be promoting cell proliferation and angiogenesis as reported earlier (32).

Overall, besides causing ulceration, indomethacin also delayed ulcer healing. Its significant effect in modulating serum levels of endostatin and VEGF levels was consistent with impairment of ulcer healing. In normal mice, both these factors were detected in serum. Both the test drugs, mal B, and mal C could accelerate the healing of gastric ulcer within three days of treatment, compared to normal healing without any treatment. Between these, mal C was more potent, showing equivalent efficacy as that of the commercial drugs, Omez and misoprostol. However, while mal C enhanced the angiogenesis for the healing, the mode of action of Omez, at the lower doses was different. Both mal B and mal C were found to be non-toxic to mice even at a very high single dose of 500 mg/kg. All these results favored mal C as a most potent ulcer-healing drug for further evaluation.

REFERENCES


Gastroprotective properties of *Myristica malabarica* against indomethacin-induced stomach ulceration: a mechanistic exploration

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**Abstract**

The healing activity of the methanol extract of the spice rampatri/*Myristica malabarica* (RM) and omeprazole against the indomethacin-induced stomach ulceration has been studied in a mouse model. Treatment with RM (40 mg kg\(^{-1}\) per day) and omeprazole (3 mg kg\(^{-1}\) per day) for 3 days could effectively heal the stomach ulceration, as revealed from the ulcer indices and histopathological studies. Compared with the ulcerated group, treatment with RM and omeprazole for 3 days reduced the macroscopic damage score by approximately 72% and 76%, respectively (P<0.001), establishing the efficacy of RM. The extent of ulcer healing offered by 3 days' treatment with RM or omeprazole was better than that observed with natural recovery over 5 and 7 days (P<0.05). The healing capacities of RM and omeprazole could be attributed to their antioxidant activity as well as the ability to enhance the mucin content of the gastric tissues. Both drugs reduced lipid peroxidation (by 42-44%) and protein carbonyl content (by 34%), and augmented non-protein thiol levels beyond normal values. Furthermore, RM improved the mucin level beyond the normal value, while omeprazole restored it to near normalcy.

**Introduction**

Stomach ulcers induced by non-steroidal anti-inflammatory drugs (NSAIDs) are a major problem ranking fourth in terms of causing morbidity and mortality (Wolfe et al 1999). Currently, use of NSAIDs accounts for approximately 25% of gastric ulcer cases, and this number is increasing (Dhikav et al 2003). Consequently, prevention of gastrointestinal disorders continues to be of concern for both clinical practitioners and researchers. In spite of efficacy in managing NSAID-induced gastric ulceration, the currently available synthetic anti-ulcer drugs confer mild to severe side-effects (Akhtar et al 1992), and are expensive, particularly for people living in rural areas. Development of suitable formulations from dietary sources may provide anti-ulcer medications with less toxicity that are more widely affordable (Yesilada & Gurbuz 2003).

It is now well established that many anti-ulcer drugs exert their action via antioxidative properties (Biswa et al 2003). Assessment of dietary antioxidants for their anti-ulcer action might therefore provide inexpensive and non-toxic medications. For this, spices possibly have the best potential, as these are widely consumed and are known to provide domestic remedies for various human disorders (Rastogi & Mehrotra 1991).

The fruit rind of the plant *Myristica malabarica* (Myristicaceae) (popularly known as rampatri, Bombay mace or false nutmeg) is used as an exotic spice in various Indian cuisines. It is credited with hepatoprotective, anticarcinogenic and antithrombotic properties, and is found as a constituent in many Ayurvedic preparations, such as pasupasi. However, most of the medicinal attributes of the spice have not been adequately substantiated.

Recently, the superoxide-scavenging activity and inhibition of prolyl endopeptidase by the methanol extract of *M. malabarica* (RM) have been reported (Khanom et al 2000). The phenolic compounds present in the resin of *M. malabarica* seeds have also been found to prevent the oxidation of various edible oils and fats more efficiently than butylated hydroxytoluene (Duggal & Kartha 1956). We have recently reported that RM shows...
impressive antioxidant activity in vitro (Patro et al. 2005). In view of these observations, it was of interest to study the healing property of RM against acute indomethacin-induced gastric ulceration in mice and compare it with the activity of the proton pump inhibitor (PPI) omeprazole. Compared with the H2-receptor antagonists or prostaglandin analogues, omeprazole has been found to have greater efficacy and tolerability in the management of NSAID-associated gastrointestinal side effects (Jones et al. 1987; CAnything et al. 1989). Its superiority has also been confirmed in clinical studies (Lad & Armstrong, 1999).

Materials and Methods

Materials

The dry fruit rind of *M. malabarica* were purchased from the local market. 2-Thiobarbituric acid (TBA), ethanol, butanol and ethyl acetate were purchased from E. Merck (Mumbai, India); trichloroacetic acid (TCA) was from Thomas Baker (Mumbai, India). Aclain blue, indomethacin, bovine serum albumin (used for measurement of protein concentration using a standard assay), haematoxyline and alun (for preparation of the haematoxyline solution), eosin, butylated hydroxyltiane (BHT), guaandin hydrochloide (HCl), thiourea (TFA), omeprazole and Trizma base were purchased from Sigma Chemicals (St Louis, MO, USA). Morecambe, UK), 2,4-dinitrophenyl hydrazine (DNPH), albumin (used for measurement of protein concentration using a standard assay), haematoxyline and alun (for preparation of the haematoxyline solution), eosin, butylated hydroxyltiane (BHT), guaandin hydrochloide (HCl), thiourea (TFA), omeprazole and Trizma base were purchased from Sigma Chemicals (St Louis, MO, USA). Other reagents used were 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydrogen peroxide (Lancaster, Mo, USA), 35% hydro
Assessment of ulceration and healing

Mice were killed with an overdose of thiopental. The stomach was removed rapidly, opened along the greater curvature, and thoroughly rinsed with normal saline. The ulcerated gastric mucosal areas were visualized using a transparent sheet and a dissecting microscope. The MDS was assessed by grading the gastric injury on a 0–4 scale, based on the severity of hyperaemia and haemorrhagic lesions: 0 = almost normal mucosa; 0.5 = hyperaemia; 1 = one or two lesions; 2 = severe lesions; 3 = very severe lesions; 4 = mucosa full of lesions (lesions = haemorrhagic erosions; hyperaemia = vascular congestion) (Dokmeci et al. 2005). The experiments were performed by two investigators blinded to treatment of animals.

Studies on the histopathological and biochemical parameters

The MDS results revealed maximum ulcer healing after 3 days of treatment. Stomach ulceration in the untreated mice also peaked on the third day after indomethacin administration. Hence, we assessed the histopathological and biochemical parameters under the optimized treatment regimen of RM 40 mg kg⁻¹ p.o. and omeprazole 3 mg kg⁻¹ p.o. up to the third day of ulceration only. For this, the following seven groups, each containing five mice, were selected from those used for the MDS assay and the data shown are derived from three replicates: Group I = normal untreated unulcerated; Group II = ulcerated with indomethacin and killed after 10 h (considered as 1 day); Group III = ulcerated with indomethacin, killed after 3 days; Group IV and V = ulcerated and treated with RM and omeprazole, respectively, and killed 4 h after administration of the test drugs; Groups VI and VII = ulcerated and treated with RM and omeprazole, respectively, and killed on the third day, 4 h after the last dose of the test drugs.

Histopathological studies of stomach tissues

The ulcerated portions of the stomach were fixed in 10% formalin solution for 24 h, embedded in a paraffin block, and cut into 5 μm sections, which were placed onto glass slides, and stained with haematoxylin and eosin for histological examination under a light microscope. One centimetre lengths of each histological section was divided into three fields. The histological damage (HD) score was assessed by scoring each field on a 0–4 scale as described previously (Dokmeci et al. 2005): 0 = normal mucosa; 1 = epithelial cell damage; 2 = glandular disruption, vasocongestion or oedema in the upper mucosa; 3 = mucosal disruption, vasocongestion or oedema in the mid-lower mucosa; 4 = extensive mucosal disruption involving the full thickness of the mucosa. The overall mean value of the HD score for each of the fields was taken as the histological ulcer index for that section.

Similarly, inflammatory scores (Beck & Xavier 2000) were assigned after reviewing all slides to assess the range of inflammation as follows: 0 = normal mucosa; 1 = minimal inflammatory cells; 2 = moderate number of inflammatory cells; 3 = large number of inflammatory cells.

The macroscopic and histological experiments were performed by two investigators blinded to the group and treatment of animals. The sections were coded to eliminate any observer bias. Data for the macroscopic and histological experiments are presented as mean ± s.e.m. and medians (ranges), respectively, from a minimum of three sections per animal and five animals per group.

Quantification of protein and lipid damage during ulceration and healing

The glandular stomach tissues from five animals were pooled, rinsed with appropriate buffer and used for biochemical studies. The wet weight of the tissues was recorded, and experiments were carried out in triplicate. Glandular portions from the control, ulcerated and drug-treated mice taken at different time intervals were homogenized with a glass–Teflon homogenizing tube in 50 mM phosphate buffer (pH 7.4) and centrifuged at 1200 g to obtain the supernatant.

The amount of protein carbonyls in the tissue homogenate was determined using the method reported by Swamakar et al. (2005). DNPH (4 mL, 10 mM) in 2mL HCl was added to the supernatant (1.0 mL), which was incubated for 1 h with intermittent shaking. Ice-cold 20% aqueous TCA solution (5 mL) was added and the mixture incubated for 15 min. The precipitated protein was washed three times with ethanol–ethyl acetate (1:1), then dissolved in 1 mL of a solution containing 64 mM guanidine HCl in 20 mM potassium phosphate (monobasic) adjusted to pH 2.3 with TFA. After centrifuging, the absorbance of the supernatant was read at 362 nm (ε = 2.2 x 10⁵ M⁻¹ cm⁻¹).

For the analysis of lipid peroxidation (measured in terms of thiobarbituric acid reactive species [TBARS]), a 10% homogenate from each sample was prepared in a buffer (320 mM sucrose, 5 mM HEPES, 20 mM EDTA and 0.01% BHT). Samples were centrifuged at 1200 g for 15 min and the supernatant centrifuged at 12000 g for 30 min to obtain the mitochondrial pellet. The pellets were then washed with buffer (150 mM KCl and 20 mM phosphate buffer) and finally suspended in 50 mM phosphate buffer, pH 7.4. The mitochondrial membrane fraction (1 mL) was treated with TCA/THA/HCl (2 mL, 15% TCA, 0.375% TBA, 0.25 mM HCl containing 0.01% BHT), heated in a boiling water bath for 15 min, cooled and centrifuged at 3000 g for 5 min. The absorbance of the supernatant was measured at 535 nm (ε = 1.56 x 10⁶ M⁻¹ cm⁻¹).

Measurement of non-protein thiol (NP-TSH)

Gastric mucosal NP-TSH was measured using the method reported by Sedlak & Lindsay (1968). Briefly, fundic stomach homogenates from control, ulcerated and drug-treated mice were prepared in 0.2 M Tris-HCl buffer, pH 8.2 containing 20 mM EDTA and centrifuged at 12000 g for 15 min. An aliquot of the homogenate (1 mL) was precipitated with ice-cold 20% TCA (1 mL) and centrifuged at 3000 g for 5 min. The supernatant (1 mL) was added to 2 mL 0.8 M Tris-HCl buffer, pH 9, containing 20 mM EDTA, and mixed with 0.1 mL 10 mM DTNB. The absorbance of the yellow chromogen was measured at 412 nm (ε = 13.6 x 10⁶ M⁻¹ cm⁻¹).
Mucin assay

Levels of free mucin in gastric tissue was estimated using the method of Tariq & Montaery (2005). Briefly, the glandular portion of the stomach was separated from the lumen, weighed and transferred immediately to 10 mL 0.1% w/v Alcian blue solution (in 0.16 M sucrose solution buffered with 0.05 M sodium acetate, pH 5.8). After staining for 2 h, excess dye was removed from the tissue by two successive rinses with 10 mL 0.25 M sucrose solution. The dye complexed with the gastric wall mucus was extracted with 10 mL 0.5 M magnesium chloride by intermittent shaking (1 min) at 30 min intervals for 2 h. The blue extract (2 mL) was vigorously shaken with an equal volume of diethyl ether. The resulting emulsion was centrifuged at 1200 g for 10 min, and the absorbance of the aqueous layer was read at 580 nm. The quantity of Alcian blue extracted per g wet glandular tissue was calculated from a standard curve prepared using various concentrations of Alcian blue.

Mucin detection using a periodic acid-Schiff (PAS) method

The adherent mucous layer was detected using the method described by McManus (1946). Briefly, stomach sections were dewaxed, subjected to diastase treatment and then treated with periodic acid for 5 min. The sections were washed with distilled water, covered with Schiff’s reagent for 15 min, washed with water and the nuclei stained with Harris haematoxylin. After another washing with water and rinsing with absolute alcohol, slides were cleaned with xylene and mounted for visualization at x20 under a light microscope.

Acute toxicity of RM in mice

To study the acute toxicity of RM, mice were given a single dose of 500 mg kg\(^{-1}\) by gavage and observed for 1 month. The experiments were performed twice, using 15 mice for each experiment. At the end of the observation period, the animals were killed and the histology of the liver and kidney was assessed. For renal and liver function tests, animals were killed and the serum of each mouse was analysed for urea, creatinine, SGPT and SGOT using typical time-dependent acute lesions in the gastric mucosa, measured in terms of MDS. The MDS value increased by 162.1% on day 3 compared with that on day 1 (P<0.001). Autohealing started on the fifth day and was more pronounced on the seventh day after ulceration, when the MDS value was reduced by 50.8% compared with that on day 1 (P<0.01). The dose-dependent and day-by-day healing capacities of RM and omeprazole are shown in Table 1. On the day of ulcer induction, effective healing was observed only with the higher concentrations of RM and omeprazole. The extent of MDS reduction by RM (60 mg kg\(^{-1}\)) was 28.0% (P<0.05), while that by omeprazole (2, 3 and 5 mg kg\(^{-1}\)) was 20.5% (P<0.05), 28.0% and 30.3% (P<0.01), respectively.

On the third day after ulceration, both drugs, at all doses tested, showed maximum MDS reduction compared with values in the respective ulcerated control groups. A dose-dependent reduction of MDS by RM and omeprazole was noticed up to a dose of 40 mg kg\(^{-1}\) and 3 mg kg\(^{-1}\), respectively, beyond which the extent of MDS reduction was insignificant. The MDS reduction by RM and omeprazole at these doses were 72.5% (P<0.001) and 76.3% (P=0.001), respectively. The effects of the lower doses of the drugs were significantly less than with these doses.

The healing observed on extending the treatment with either RM or omeprazole for up to 7 days was only marginally better than that observed with the 3-day treatment regimen. However, a major part of this was due to autohealing, with less contribution by RM. Notably, omeprazole showed severe adverse effects (data not shown) at a dose of 20 mg kg\(^{-1}\).

Overall, treatment with RM (40 mg kg\(^{-1}\)) and omeprazole (3 mg kg\(^{-1}\)) for 3 days after ulcer induction provided optimal and comparable ulcer healing (72.5% and 76.3%). In view of these observations, all subsequent experiments were carried out with the same treatment regimen. The chosen dose of omeprazole is also the recommended therapeutic dose for humans and was used earlier in a murine model (Biswa et al 2003).

For the untreated mice, peak ulceration (maximum MDS) was observed on the third day of indomethacin administration. Hence, this time point was selected to find out the IC50 values of RM and omeprazole. Taking the MDS values on the third day in ulcerated untreated mice as 100%, the IC50 values of omeprazole and RM were found to be 1.68±0.18 and 23.30±3.50 mg kg\(^{-1}\), respectively (Figure 1), which were significantly different from each other (P<0.01).

Results

Standardization of drug doses for ulcer healing

The doses of drugs for effective ulcer healing were optimized by treating mice with various doses of RM (5, 10, 20, 30, 40 and 60 mg kg\(^{-1}\)) and omeprazole (0.2, 0.5, 1, 2, 3 and 5 mg kg\(^{-1}\)) daily for up to 7 days. The MDS values of the treated and untreated mice were compared each day. The mice receiving vehicle only showed no mucosal lesions. Treatment of mice with indomethacin (18 mg kg\(^{-1}\)) produced typical time-dependent acute lesions in the gastric mucosa, measured in terms of MDS. The MDS value increased by 162.1% on day 3 compared with that on day 1 (P<0.001). Autohealing started on the fifth day and was more pronounced on the seventh day after ulceration, when the MDS value was reduced by 50.8% compared with that on day 1 (P<0.01). The dose-dependent and day-by-day healing capacities of RM and omeprazole are shown in Table 1. On the day of ulcer induction, effective healing was observed only with the higher concentrations of RM and omeprazole. The extent of MDS reduction by RM (60 mg kg\(^{-1}\)) was 28.0% (P<0.05), while that by omeprazole (2, 3 and 5 mg kg\(^{-1}\)) was 20.5% (P<0.05), 28.0% and 30.3% (P<0.01), respectively.

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Gastroprotective property of Myristica malabarica

Table 1 Time- and dose-dependent healing capacities of methanol rampatri extract (RM) and omeprazole against indomethacin-induced stomach ulcers in mice, as shown by the macroscopic damage score, given as mean ± s.e.m. (n = 15)

<table>
<thead>
<tr>
<th>Dose (mg kg⁻¹)</th>
<th>Macroscopic damage score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
</tr>
<tr>
<td>Ulcerated untreated</td>
<td>1.32 ± 0.05</td>
</tr>
<tr>
<td>RM treated 5</td>
<td>1.35 ± 0.09</td>
</tr>
<tr>
<td>10</td>
<td>1.32 ± 0.09</td>
</tr>
<tr>
<td>20</td>
<td>1.22 ± 0.08</td>
</tr>
<tr>
<td>30</td>
<td>1.22 ± 0.06</td>
</tr>
<tr>
<td>40</td>
<td>1.1 ± 0.06</td>
</tr>
<tr>
<td>60</td>
<td>0.95 ± 0.06**</td>
</tr>
<tr>
<td>Omeprazole treated</td>
<td></td>
</tr>
<tr>
<td>0.2</td>
<td>1.25 ± 0.06</td>
</tr>
<tr>
<td>0.5</td>
<td>1.21 ± 0.08</td>
</tr>
<tr>
<td>1</td>
<td>1.15 ± 0.07</td>
</tr>
<tr>
<td>2</td>
<td>1.05 ± 0.08</td>
</tr>
<tr>
<td>3</td>
<td>0.95 ± 0.09**</td>
</tr>
<tr>
<td>5</td>
<td>0.92 ± 0.08**</td>
</tr>
</tbody>
</table>

*P < 0.01, **P < 0.001 compared with the first day ulcerated control. †P < 0.05, ‡P < 0.01 vs ulcerated controls of the same day. \( P < 0.001 \) vs ulcerated controls of the same day and \( P < 0.05 \) vs third day in ulcerated mice that received lower doses of RM and omeprazole.

Figure 1 Concentration-dependent healing capacities of methanol rampatri extract (RM) (A) and omeprazole (B) on the third day after indomethacin-induced stomach ulceration in mice, as revealed from macroscopic damage scores (MDS). MDS was measured 4 h after the last dose of each drug, the MDS in ulcerated untreated mice was taken as 100. The values are mean ± s.e.m (n = 15). The IC50 values (concentration that produces 50% ulcer healing) of RM and omeprazole (determined by Probit analysis) were significantly different (\( P < 0.01 \)).

Histopathological assessment of the gastric ulcer healing capacity of RM

Quantitative assessment

Compared with the ulcerated mice, mice treated with RM or omeprazole for 1 day showed reductions in HD scores by 64.7% and 76.5%, respectively (\( P < 0.001 \)). Likewise, the respective inflammatory scores were also reduced by 44.4% by RM and omeprazole (\( P < 0.005 \)).

Compared with the 1-day ulcerated mice (group II), the HD and Inflammatory scores for the ulcerated untreated mice (group III) increased by 76.5% (\( P < 0.001 \)) and 33.3% (\( P < 0.005 \)), respectively. Treatment with RM and omeprazole for 3 days reduced the HD score by 73.3% and 86.7%, respectively, and the inflammatory score by 75.0% and 66.7%, respectively, compared with group III mice (\( P < 0.001 \)). The results are summarized in Table 2.
Table 2: Time-dependent healing capacities of methanol rampatri extract (RM) (40 mg kg⁻¹) and omeprazole (3.0 mg kg⁻¹) against indomethacin-induced stomach ulcers in mice, shown by histological parameters. Histological parameters were assessed in terms of damage and inflammatory scores by analyzing the data from a minimum of three sections per animal. Significant differences in the histological scores were observed for the ulcerated untreated and ulcerated drug-treated mice. Data are presented as medians (ranges) (n = 5 per group).

<table>
<thead>
<tr>
<th>Treatment (group)</th>
<th>Days after ulcer induction</th>
<th>Inflammatory score</th>
<th>Damage score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated (II)</td>
<td>1</td>
<td>1.80 (1.6-2.0)</td>
<td>1.70 (1.2-2.0)</td>
</tr>
<tr>
<td>RM (IV)</td>
<td>1</td>
<td>1.0 (0.8-1.4)</td>
<td>0.60 (0.2-0.8) **</td>
</tr>
<tr>
<td>Omeprazole (V)</td>
<td>1</td>
<td>1.0 (0.8-1.2)</td>
<td>0.4 (0.2-0.6) **</td>
</tr>
<tr>
<td>Untreated (III)</td>
<td>3</td>
<td>2.4 (2.0-2.8)</td>
<td>3.6 (2.6-3.3) **</td>
</tr>
<tr>
<td>RM (VI)</td>
<td>3</td>
<td>0.5 (0.4-0.8)</td>
<td>0.8 (0.4-1.0) **</td>
</tr>
<tr>
<td>Omeprazole (VII)</td>
<td>3</td>
<td>0.8 (0.6-1)</td>
<td>0.6 (0.2-0.6) **</td>
</tr>
</tbody>
</table>

*P < 0.005; **P < 0.001 compared with ulcerated mice on same day;
¢P < 0.01, aP < 0.05 compared with group II mice.

Qualitative assessment

The time-dependent indomethacin-induced gastropathy and its subsequent healing by RM and omeprazole is shown in Figure 2. Macroscopic and histopathological examinations revealed that indomethacin caused marked damage to the glandular portion of the gastric mucosa. Within 6h after indomethacin administration, superficial erosion and mild inflammation in the stomach were observed, indicating acute ulceration. On the day of ulcer induction, loss of foveolar structure along with cryptic architecture was the prominent feature in most of the untreated mice. Also, mild inflammatory infiltrate containing neutrophils was observed in the lamina propria. Treatment with either RM or omeprazole even for 1 day resulted in observable regenerative changes in the mucosal architecture. This was evident from the localized damage with patchy areas of denuded structural epithelium (Figure 2a).

Indomethacin-induced gastropathy became much pronounced on the third day, showing multiple punched-out areas of ulceration with inflammatory infiltrate containing neutrophils and macrophages in the mucosa, submucosa and muscle coat, along with haemorrhagic serosa. A large number of abnormal cells with altered nucleus-to-cytoplasm ratio were noticed. Treatment with RM or omeprazole for 3 days was associated with a reduction in the number of inflammatory cells and an increase in the number of healthy normal cells in the gastric mucosa, submucosa, serosa and muscle layers, with minimum mucosal congestion. Mucosal hyperplasia along with cryptic proliferation with no frank denudation was a major hallmark following treatment with RM or omeprazole. The omeprazole-treated mice showed better mucosal healing than the RM-treated group. However, there was less mucosal inflammation in the RM-treated mice than in the omeprazole-treated mice (Figure 2).

Effect of RM and omeprazole on lipid peroxidation and protein oxidation in stomach tissue

The effects of indomethacin intake alone and following administration of RM and omeprazole on the extent of lipid peroxidation and protein oxidation were determined. The results are presented in Table 3, which shows a significant decrease in the levels of lipid peroxidation and protein oxidation in the RM and omeprazole-treated groups as compared to the untreated group. This indicates a protective effect of RM and omeprazole against the effects of indomethacin on lipid and protein peroxidation.

Figure 2: Histological assessment of acute gastric mucosal injury induced by indomethacin (18 mg kg⁻¹) in mice and its prevention by methanol rampatri extract (RM) (40 mg kg⁻¹) and omeprazole (3 mg kg⁻¹). Stomach sections were taken 4 h after the last dose of each drug on that day. Images are stomach sections from normal, ulcerated untreated and treated mice on day 1 (A) and day 3 (B). White and black arrows indicate mucosal damage and inflammatory cells, respectively.
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Peroxidation (measured in terms of TBARS), protein oxidation (measured in terms of protein carbonyl content), the thiol-dependent defence system (NP-TSH) and mucin content in the gastric tissues of mice are shown in Table 3 and Table 4.

Indomethacin administration markedly stimulated lipid peroxidation in gastric tissues, and the TBARS content was elevated by approximately 50% in 1 day (*P* < 0.005) compared with that in the control mice. RM reduced it marginally (10.4%) and omeprazole had a more pronounced effect (19.2%, *P* < 0.05). After 3 days of ulceration, the TBARS content in the group III mice increased by ~125% compared with that in normal mice (*P* < 0.001). Treatment with RM and omeprazole for 3 days reduced it by 45.5% and 42.4%, respectively, compared with the untreated ulcerated mice (*P* < 0.05).

Compared with the normal value, the protein carbonyl content of the ulcerated mice was significantly elevated, by 92.7% on the first day (*P* < 0.001) and by 79% (*P* < 0.001) on the third day of ulceration. Treatment with RM or omeprazole for 1 day reduced the protein carbonyl content marginally. However, by day 3 of treatment, both drugs reduced the protein carbonyl content by about 34% compared with that of the untreated ulcerated mice (*P* < 0.05).

We also evaluated the antioxidant activity of RM (single 60 mg kg⁻¹ dose) in the ulcer-healing experiments. Under these conditions, TBARS and protein carbonyl contents were reduced by 20.4% and 14.7%, respectively, compared with values in ulcerated mice. The antioxidant activity of RM was less impressive at a low concentration (40 mg kg⁻¹) and was improved significantly by using RM at a higher dose, or for a longer period.

### Table 3: Effect of methanol rampatri extract (RM) (40 mg kg⁻¹) and omeprazole (3 mg kg⁻¹) on the levels of thiobarbituric acid reactive species (TBARS), protein carbonyl, mucin and non-protein thiol in gastric tissue of mice on day 1 after ulcer induction (induced by indomethacin 18 mg kg⁻¹ p.o.). Values are mean ± s.e.m. (n = 15)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unulcerated controls</th>
<th>Untreated ulcerated controls</th>
<th>RM-treated</th>
<th>Omeprazole-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBARS (nmol mg protein⁻¹)</td>
<td>0.84 ± 0.02</td>
<td>1.25 ± 0.07 **</td>
<td>1.12 ± 0.07</td>
<td>1.01 ± 0.05 **</td>
</tr>
<tr>
<td>Protein carbonyl (nmol mg protein⁻¹)</td>
<td>0.96 ± 0.04</td>
<td>1.85 ± 0.05 **</td>
<td>1.76 ± 0.07</td>
<td>1.61 ± 0.07</td>
</tr>
<tr>
<td>Non-protein thiol (nmol mg tissue⁻¹)</td>
<td>1.88 ± 0.049</td>
<td>1.55 ± 0.02 *</td>
<td>1.78 ± 0.04</td>
<td>1.81 ± 0.03</td>
</tr>
<tr>
<td>Mucin (μg tissue⁻¹)</td>
<td>300.97 ± 15.89</td>
<td>300.10 ± 20.84*</td>
<td>320.78 ± 18.66 **</td>
<td>260.33 ± 19.01</td>
</tr>
</tbody>
</table>

* *P* < 0.05; **P* < 0.005; *P* < 0.001 vs unulcerated control mice; *P* < 0.05; **P* < 0.001 vs untreated ulcerated mice.

### Table 4: Effect of methanol rampatri extract (RM) (40 mg kg⁻¹ per day for 3 days) and omeprazole (3 mg kg⁻¹ per day for 3 days) on the levels of thiobarbituric acid reactive species (TBARS), protein carbonyl, mucin and non-protein thiol in gastric tissue of mice on day 3 after ulcer induction (induced by indomethacin 18 mg kg⁻¹ p.c.). Values are mean ± s.e.m. (n = 15)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unulcerated controls</th>
<th>Untreated ulcerated controls</th>
<th>RM-treated</th>
<th>Omeprazole-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBARS (nmol mg protein⁻¹)</td>
<td>0.85 ± 0.03</td>
<td>1.91 ± 0.12 **</td>
<td>1.08 ± 0.05</td>
<td>1.10 ± 0.06 **</td>
</tr>
<tr>
<td>Protein carbonyl (nmol mg protein⁻¹)</td>
<td>1.01 ± 0.05</td>
<td>2.82 ± 0.11 **</td>
<td>1.90 ± 0.14</td>
<td>1.87 ± 0.14</td>
</tr>
<tr>
<td>Non-protein thiol (nmol mg tissue⁻¹)</td>
<td>1.85 ± 0.04</td>
<td>1.70 ± 0.05</td>
<td>2.10 ± 0.07 *</td>
<td>2.35 ± 0.07 *</td>
</tr>
<tr>
<td>Mucin (μg tissue⁻¹)</td>
<td>313.35 ± 13.3</td>
<td>220.35 ± 17.91 *</td>
<td>354.43 ± 13.71</td>
<td>301.05 ± 13.67</td>
</tr>
</tbody>
</table>

* *P* < 0.05; **P* < 0.001 vs unulcerated controls; *P* < 0.05; **P* < 0.001 vs ulcerated untreated mice.

**Effect of RM and omeprazole on NP-TSH in stomach tissue**

Our results revealed that ulceration decreased NP-TSH significantly (17.6%, *P* < 0.05) in the 1-day untreated ulcerated mice compared with normal mice. Treatment with RM or omeprazole for 1 day increased the NP-TSH content by 15-17% (*P* < 0.05) compared with that of the ulcerated mice. Three days after ulcer induction, the NP-TSH content in the untreated mice was restored to 92% of the normal value. Both drugs increased the NP-TSH level beyond the normal value (*P* < 0.05), omeprazole being more effective.

**Autohealing of gastric ulceration and effect on biochemical parameters**

The results above showed that stomach ulceration started within 6 h of administering indomethacin, and reached its peak after 3 days, when maximum oxidative damage was also noticed. We also followed natural recovery in the absence of any treatment for up to 7 days. Autohealing began 5 days after ulcer induction but was more pronounced after 7 days, when noticeable regeneration atypia in the gastric gland was observed. This also suggested that the ulceration was acute. The rate of healing was significantly slower in untreated mice than in drug-treated animals. Autohealing was accompanied by reductions in TBARS (34%) and protein carbonyl content (30%), together with an increase in mucin content (13%) compared with 3-day untreated mice. These values were significantly less than those observed in mice treated with RM or omeprazole for 3 days (*P* < 0.05).
Effect of RM and Omeprazole on mucin contents in stomach tissue

Our studies revealed that administration of indomethacin led to an immediate reduction in mucin secretion on the first day (33.5%, \(P<0.05\)) compared with levels in normal mice but remained almost the same on the third day after ulcer induction. Treatment with RM and omeprazole for 1 or 3 days augmented the mucin content by about 60% (\(P<0.001\)) and 30–36% (\(P<0.05\)), respectively, compared with the levels in ulcerated untreated mice.

Mucin detection

The photomicrographs shown in Figure 3 represent the PAS-stained stomachs of normal mice and untreated and drug-treated ulcerated mice of the 3-day groups. Administration of indomethacin caused a breach of the continuity of the mucous layer, allowing the secreted gastric juice to cause widespread damage (topical effect). The thickness of the mucous layer was depleted with the progress of ulceration, which was reflected in a reduction in PAS-stained areas.

Administration of RM restored the mucus secretion, which was apparent within 1 day of treatment (data not shown). Continuous administration of RM led to a substantial continuous PAS-positive mucous gel layer covering the surface of the gastric mucosa. A bright-purple stained area covering the mucosa and extending up to the gastric pits was noticed. Administration of omeprazole, however, did not cause much change in the mucus profile, suggesting other mechanisms of action in its ulcer healing action.

Chemical constituents of RM and identification of the active principles

The HPLC chromatograph (Figure 4) of RM showed a number of peaks from which malabaricone B (6.7%), malabaricone C (21.2%) and their glycosides were identified as the major constituents, along with minor amounts of malabaricone A (0.6%) and malabaricone D (1.2%) (Patro et al. 2005). The HPLC data revealed the presence of 2.6 mg malabaricone B and 8 mg malabaricone C in 40 mg RM. Hence, to identify the active principles of RM, the ulcer-healing capacities of malabaricone B (2.6 mg kg\(^{-1}\)) and malabaricone C (8 mg kg\(^{-1}\)) were assessed. Treatment of the ulcerated mice for 3 days with malabaricone B and malabaricone C reduced the MDS by 36.2% and 69.1%, respectively.

Acute toxicity of RM in mice

The possible toxic effect of RM at a dose of 500 mg kg\(^{-1}\) on mice was also evaluated. There were no observable physical signs, and mice had normal food and water as well as stool production during the experimental period. Administration of RM did not have any obvious effect on liver or kidney histology. Mice treated with RM showed normal hepatic microarchitecture, with laminar arrangement of hepatocytes, central vein, portal triad and biliary canaliculii. There was no sign of any inflammatory infiltrate or distorted cyto-architecture, congestion or necrosis. Likewise, normal renal micro-architecture with well-differentiated cortex and medulla was found in the kidneys. Glomeruli, tubules including papillii, were found to be normal, and there was no haemorrhage, congestion, inflammatory infiltrate or necrosis.

Serum levels of urea, creatinine, SGPT and SGOT in the normal and RM-treated mice were analysed to evaluate renal and liver function. Quality control checks were performed with Randox level-1 and level-2 controls and Levi-Jennings’ charts were plotted. The control values were found to be within ±2 s.d. The comparative data (Table 5) of the normal and the RM-treated mice confirmed the non-toxicity of RM even at a high dose.

Discussion

It is well established that oxidative stress plays a key role in the induction and pathogenesis of gastroduodenal injury, and antioxidants offer effective protection/cure against gastric injury (Bilici et al. 2002). The anti-oxidative role of the anti-ulcer drug omeprazole has been confirmed recently (Biswas et al. 2003). It is proposed that the gastrotoxicity of the NSAIDs, including indomethacin, in animals can be attributed to induction of reactive oxygen metabolites.

Ulcer healing is a complex process, involving a combination of wound retraction and re-epithelization (Tabor & Tabor 1984). Release of preformed mucus plays a role in promoting

![Figure 3](https://via.placeholder.com/150)

Figure 3: Depletion of mucin associated with indomethacin-induced acute gastric ulceration, and its prevention by methanol rampatri extract (RM) (40 mg kg\(^{-1}\)) and omeprazole (3 mg kg\(^{-1}\)) as revealed by periodic acid-Schiff staining. Stomachs were excised 4 h after the last dose of the drug on the third day. White arrows denote the PAS-positive areas (×20).
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**Figure 4** HPLC profile of a rampatri methanol extract of fruit rind of *Myristica malabarica* (RM). The chemical constituents of RM were detected at 345 nm. Malabaricones A–D were identified using standards; retention times were 36.0, 11.9, 8.0, and 27.3 min, respectively.

**Table 5** Acute liver and renal toxicity of methanol rampatri extract (RM) 500 mg kg⁻¹ in mice, assessed by serum concentrations of glutamic pyruvic transaminase (SGPT), glutamic oxaloacetic transaminase (SGOT), urea and creatinine. Data are mean ± s.e.m. (n=30 mice)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal mice</th>
<th>RM-treated mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>SGOT (IU L⁻¹)</td>
<td>168.21 ± 10.75</td>
<td>173.5 ± 15.84</td>
</tr>
<tr>
<td>SGPT (IU L⁻¹)</td>
<td>72.14 ± 6.79</td>
<td>75.84 ± 6.21</td>
</tr>
<tr>
<td>Urea (mg dL⁻¹)</td>
<td>29.25 ± 1.39</td>
<td>31.4 ± 2.51</td>
</tr>
<tr>
<td>Creatinine (mg dL⁻¹)</td>
<td>0.29 ± 0.03</td>
<td>0.38 ± 0.02</td>
</tr>
</tbody>
</table>

Table 5 Acute liver and renal toxicity of methanol rampatri extract (RM) 500 mg kg⁻¹ in mice, assessed by serum concentrations of glutamic pyruvic transaminase (SGPT), glutamic oxaloacetic transaminase (SGOT), urea and creatinine. Data are mean ± s.e.m. (n=30 mice)

epithelial recovery after acute injury by forming a mucoid cap beneath which re-epithelization occurs (Wang et al 1997). Besides providing significant buffering capacity for the neutralization of luminal acid, the mucus can protect against endogenous aggressors such as acid, pepsin and oxidants produced in the gastric lumen, as well as against exogenous damaging agents such as NSAIDs. The haemorrhagic mucosal ulcers associated with NSAIDs may result from a decreased production of gastric mucus (Rainsford 1978; Naito et al 1995). Thus, drugs that arrest ulcer progression by antioxidant action and also increase the synthesis and secretion of gastric mucus would be expected to accelerate healing of gastric ulcers.

**RM** has powerful antioxidant activity in vitro, which encouraged us to investigate its possible healing effect against indomethacin-induced gastric lesions in mice. Administration of indomethacin to mice induced marked damage to the gastric mucosa, with marked subjective damage and inflammatory scores, as evidenced by macroscopic and histopathological examinations. Our study shows that RM had potent healing effect on indomethacin-induced gastric lesions in mice, providing impressive healing within 3 days of treatment. In untreated ulcerated mice, ulcer craters receded through the process of autohealing, but this took about 7 days. The extent of autohealing after 7 days was significantly less than the healing observed in mice treated with RM or omeprazole for 3 days.

Tissue damage is associated with generation of free radicals, leading to loss or impairment of protein synthesis (Szabo et al 1985; El-Missiry et al 2001) and damage to key biomolecules. This might lead to aggravated tissue damage during stomach ulceration. Our results show that indomethacin-induced stomach ulceration was accompanied by severe oxidative stress in gastric tissue, causing damage to lipids and proteins. Treatment with RM for 3 days provided marked suppression of oxidative damage, reflecting its excellent radical-scavenging capacity.

The role of endogenous sulphydryl compounds in mucosal protection has been demonstrated previously in ethanol-induced gastric injury, in which the development of damage
was accompanied by a decrease in mucosal sulphydryl compounds (Naito et al. 1995). Sulphydryl compounds scavenge free radicals produced following tissue injury. They may also protect mucus, since mucus subunits are joined by disulfide bridges which, if reduced, render mucus water-soluble (Avila et al. 1996). Both RM and omeprazole produced immediate restoration of the NP-TSH level that was reduced by ulceration. Continued treatment for 3 days with the drugs increased the NP-TSH level beyond the normal value (P<0.05).

Thus, overall RM provided a marked suppression of oxidative damage and brought most of the biochemical parameters near normalcy. The positive effect of RM on all biochemical parameters was significantly better than that observed with natural recovery, even up to 7 days after ulcer induction. Thus, RM can retard ulcer progression and promote healing of gastric lesions induced by acute intake of indomethacin.

It is known that the free radicals generated by exogenous and endogenous agents can easily produce mucosal damage (Bernier & Floret 1986). Increased mucus production usually assists the healing process by protecting the ulcer crater against irritant stomach secretions (HCl and pepsin) (James 1986), thereby enhancing the local healing process. In this study, mucus secretion was found to be decreased in mice given indomethacin, observed by both the Alcian blue assay and PAS staining methods. This indicated reduced ability of the mucosal membrane to protect the mucus from physical damage and back diffusion of hydrogen ions. Treatment with RM significantly accelerated the ulcer healing process, which was associated with an increase in the adherent mucus layer in the gastric mucosa. The free-radical scavenging property of RM may be responsible for protecting the gastric mucosa against oxidative damage.

Our results indicate that enhanced mucus modulation induced by RM plays a significant role in its ulcer-healing effect, whereas mucous restoration is less evident with omeprazole. The anti-inflammatory activity of omeprazole is attributed primarily to its ability to suppress gastric acid secretion via inhibition of H+/K+ ATPase in gastric parietal cells. Long-term use of omeprazole has been reported to inhibit production of gastric mucin (Yoshimura et al. 1996; Basolin et al. 2001). Our results suggest a predominant anti-oxidative mechanism in its mode of action, confirming a recent finding (Biswas et al. 2003).

We used a crude plant extract for these studies. Such use of a crude extract may be beneficial since it may act in a variety of ways, to provide additive or, in some cases, potentiating effects. Our results revealed the presence of four malabaricones (A–D) as well as their glycosides in RM. Of these, the major constituent, malabaricone C alone accounted for most of the ulcer-healing activity of RM. The healing activity of malabaricone B was lower but this may be because it is present in a lower concentration in RM. The marginally better activity of RM compared with malabaricone C might be due to contributions of some of its other constituents, including malabaricone B. Given that some drugs can show mild-to-severe side effects even after short-term intake, we also evaluated the possible toxic effects of RM at a dose of 500 mg kg–1 in mice. The results suggested that RM given at the current dose does not have any side-effects in mice.

Both PPIs and prostaglandin preparations are believed to prevent NSAID-induced gastric ulcers and to promote the delayed healing of gastric ulcers caused by NSAIDs. We did not find any significant anti-secretory activity of RM (data not shown). Ulceration due to NSAIDs is also believed to occur because of non-selective inhibition of cyclooxygenases, which hampers the release of mucus because of reductions in prostaglandin synthesis. Many phenolic compounds stimulate prostaglandin synthesis by acting as electron-donating co-substrates for the peroxidase component of PGHS (Alanko et al. 1999). Thus, it would be of interest to study the effect of RM on the prostaglandin-dependent pathway of gastric ulcer healing. Investigations into this are currently in progress in our laboratory.

Conclusion

Our study establishes that RM possesses significant healing property against indomethacin-induced stomach ulcers in mice, which is marginally less than that of omeprazole. The drug has strong anti-inflammatory properties, as evident from its immediate healing effect within 6 h of administration. However, the effect was more pronounced with treatment for 3 days. The healing action of RM was due to the antioxidant action of its constituent phenolics, malabaricone B and C, especially the latter, which may protect the gastric mucosa and, in turn, the stomach epithelium from oxidative damage. In view of these encouraging results coupled with its non-toxicity, RM appears to be a promising herbal anti-ulcer preparation that merits further investigation.

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