Original Article

Regulation of the nitric oxide synthesis pathway and cytokine balance contributes to the healing action of *Myristica malabarica* against indomethacin-induced gastric ulceration in mice

Biswanath Maity[1], Debashish Banerjee[1], Sandip K. Bandyopadhyay[1], Subrata Chattopadhyay[2,*]

[1] *Department of Biochemistry, Dr. B.C. Roy Post Graduate Institute of Basic medical Sciences & IPGME&R, 244B, Acharya Jagadish Chandra Bose Road, Kolkata, India;*
[2] *Bio-Organic Division, Bhabha Atomic Research Centre, Mumbai, India.*

ABSTRACT: The role of the ariginine-metabolism in the healing action of the methanol extract of *Myristica malabarica* (rampatri) (RM) and omeprazole (Omez) against indomethacin-induced stomach ulceration in mouse was investigated. Indomethacin (18 mg/kg) was found to induce maximum stomach ulceration in Swiss albino mice on the 3rd day of its administration, which was associated with reduced arginase activity (38.5%, $p < 0.05$), eNOS expression, along with increased iNOS expression, total NOS activity (5.37 fold, $p < 0.001$), NO generation (55.1%, $p < 0.01$), and ratio of pro-/anti-inflammatory cytokines. Besides providing comparable healing as Omez (3 mg/kg × 3 d), RM (40 mg/kg × 3 d, p.o.) shifted the iNOS/NO axis to the arginase/polyamine axis as revealed from the increased arginase activity (59.5%, $p < 0.01$), eNOS expression, and reduced iNOS expression, total NOS activity (73%, $p < 0.001$), and NO level (49.8%, $p < 0.01$). These could be attributed to a favourable anti/pro inflammatory cytokines ratio, generated by RM. The healing by Omez was however, not significantly associated with those parameters.

Keywords: Arginase, Cytokine balance, Gastric ulcer healing, Indomethacin, NOS

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 defence mechanisms and wound healing (*2,3*). In acute inflammatory responses, such as wound healing, heat stroke and glomerulonephritis, arginase has been implicated as an important regulator of diverse pathways including generation of polyamines and the cytostatic free radical molecule, nitric oxide (NO) (*4*). 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 (*5*). Studies have shown that arginine itself has advantageous effects on cutaneous healing by enhancing cell proliferation and collagen synthesis as well as breaking strength (*6*). Further, nitric oxide (NO), produced from arginine also plays an important role in inflammatory processes, being a mediator of macrophage function (*4,6,7*). The temporal switch of arginine as a substrate for the inducible nitric oxide synthase (iNOS)/NO axis to arginase/polyamine axis is subject to regulation by 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 a predominance of the arginase inducing cytokines such as IL-4, IL-10, and TGFβ (*8*).

Very recently, we have documented impressive healing activity of the fruit rinds of the plant, *Myristica malabarica* (Myristicaceae), popularly known as rampatri (*9*). It was found that oral administration of the methanol extract of rampatri (designated as RM) at a dose of 40 mg/kg for three days could effectively heal the indomethacin (18 mg/kg, p.o., single dose)-induced stomach ulceration in mice, reducing the

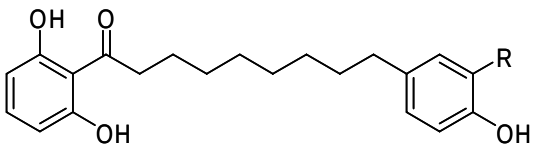
**Correspondence to:* Dr. Subrata Chattopadhyay, Bio-Organic Division, Bhabha Atomic Research Centre, Mumbai 400 085, India;
e-mail: schatt@barc.gov.in

ulcer index from 3.0 to 0.8 (~74%). The healing activity of RM could be partly attributed to its ability to prevent oxidative damages to lipids, thiols and antioxidant enzymes, as well as augmenting mucin status. Subsequently, malabaricones B and C (the chemical structures shown in Figure 1) were found as its active constituents (*10*). However, the extract was more potent than the individual malabaricones at their respective concentrations in the extract (*9*). Hence for the present study, we have used RM to understand the mechanisms of its healing action in terms of its capacity to regulate the arginine metabolism by modulating the balance of cytokines in the process. To this end, we have investigated the effect of RM in elevating arginase activity, and regulating NO production through modulation of NOS expression. In addition, the important role of the pro- and anti-inflammatory, as well as regulatory cytokines *vis-a-vis* their putative role during wound healing was also investigated.

2. Materials and Methods

2.1. *Chemicals and reagents*

The dry fruit rinds of *M. malabarica* were purchased from the local market. L-arginine, indomethacin, isonitrosopropiophenone, Bradford reagent, Triton X-100, leupeptin, phenylmethylsulfonyl fluoride (PMSF), glycine, sodium dodecyl sulfate (SDS), acrylamide, bis-acrylamide, Tween 20, ethylene diamine tetraacetic acid (EDTA), 3,3',5,5'-tetramethyl benzidine (TMB), $MnCl_2$, urea, omeprazole (Omez), Trizma base, cetyl trimethylammonium bromide (CTAB), and nitrocellulose membrane were procured from Sigma Chemicals (St. Louis, MO, USA). Other reagents used were disodium hydrogen phosphate and sodium dihydrogen phosphate (BDH, UK), sulphuric acid, hydrochloric acid, phosphoric acid, sodium chloride (Thomas Becker, Mumbai, India), horseradish peroxidase (HRPO, Sisco Research Laboratory, Mumbai, India), rabbit polyclonal iNOS and eNOS antibodies (Santacruz Biotechnology, Delaware, USA), peroxidase conjugated anti-rabbit IgG antibody, enhanced chemiluminescence detection kit (Roche, Mannheim, Germany), NOS and NO assay kits (Calbiochem, CA, USA), TGF-β1 set (Promega Corporation, Madison, USA) and cytokine ELISA kits (Pierce Biotechnology, Rockford, IL, USA).



Malabaricone B: R = H
Malabaricone C: R = OH

Figure 1. The chemical structures of malabaricone B and malabaricone C present in *M. malabarica*.

2.2. *Instrumentation*

The absorbance spectrophotometry was carried out at 25°C using an ELISA reader (Biotech Instruments, USA). The bands obtained from western blots were quantified using the Gelquant software (DNR Bioimaging System, version 2.7.0, Israel).

2.3. *Preparation of plant extract*

The dry fruit rinds (20 g) of *M. malabarica* were powdered with a grinder and extracted successively with ether, methanol, and water (60 mL × 4 d with each solvent) at room temperature. The supernatants in each case were decanted. The entire process was repeated three times, each of the combined supernatants was filtered through a nylon mesh and evaporated at < 40° C *in vacuo* to obtain the respective extracts, which were stored in a vacuum desiccator. The methanol extract (designated as RM, 28.9%) was used for the present work.

2.4. *Preparation of the drugs*

The drugs were prepared from RM and Omez 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 BARC Animal Ethics Committee, and were handled following International Animal Ethics Committee Guidelines. Male swiss albino mice (25-30 g) were reared on a balanced laboratory diet as per NIN, Hyderabad, India and given tap water ad libitum. They were kept at 20 ± 2°C, 65-70% 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, 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 RM (40 mg/kg × 3 d, p.o.) and Omez (3 mg/kg

× 3 d, 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 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 NOSs and assaying arginase and MPO activities. Other biochemical (total NOS and NO) and immunological parameters were assayed using the serum samples.

2.6. *Assessment of ulcer healing*

The ulcerated portions of the stomach were fixed in 10% formol saline solution for 24 h, embeded in a paraffin block, and cut into 5 µm sections, which were placed onto glass slides, and stained with haematoxylene 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 (DS) was assessed as described previously (*9*).

2.7. *Determination of myeloperoxidase (MPO) activity*

The MPO activity was determined following a reported method (*11*) with slight modifications. Gastric ulcer tissues were homogenized for 30 sec in a 50 mM phosphate buffer (pH 6.0) containing 0.5% CTAB and 10 mM 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 H_2O_2 (50 µL). After incubating the mixture at 25°C for 25 min, the reaction was terminated by adding 0.5 M H_2SO_4 (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 (*12*) with minor modifications, the assay was carried out using the 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 $MnCl_2$, 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, H_2SO_4-H_3PO_4-H_2O, 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 serum NOS activity was measured using the commercially available colorimetric kit following manufacturer's protocol.

2.10. *Western blot analyses of tissue iNOS and eNOS expressions*

The glandular part of the gastric mucosa after being washed with PBS containing protease inhibitors was minced and homogenized in a lysis buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100) containing leupeptin (0.005 µg/mL) and PMSF (0.4 µM/mL). 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 99% 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 (1:2,500 dilution). The bands were detected using an enhanced chemiluminescence detection kit and quantified using the Gelquant software.

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 serum IL-4, IL-6 and TNF-α levels were estimated using commercially available ELISA kits following manufacturer's protocols. The method of TGF-β1 estimation (*13*) 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 90 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-β 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 Dunnet'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, single dose) administration, and a three-day treatment with RM (40 mg/kg/day) and Omez (3 mg/kg/day) provided optimal ulcer healing (*9*). The extent of stomach ulceration and healing by the drugs were quantified from the same histological slide. The optimized healing data, assessed in terms of histological damage scores are provided in Table 1. Hence, the present experiments were also carried out under the above conditions. We preferred using the crude extract (RM) since it was more potent than the individual active components on equivalent basis. This would also be economically beneficial.

3.1. *Regulation of the mucosal MPO activity*

Compared to the normal mice, the mucosal MPO status in the gastric tissues of ulcerated untreated mice increased by 34.3% ($p < 0.05$) (Figure 2). This was brought down significantly by both RM (22.4%, $p < 0.05$) and Omez (20.3%, $p < 0.05$). The effect of Omez was not significantly different from that of RM.

3.2. *Regulation of the mucosal arginase activity*

The indomethacin-mediated stomach ulceration depleted (38.5%, $p < 0.05$) the arginase activity considerably, compared to the normal mice (Figure 3). Three-day treatment with RM and Omez enhanced it by 59.5% ($p < 0.01$) and 19.6% respectively compared to the untreated mice. The results of RM and Omez were significantly different ($p < 0.05$).

Table 1. The optimized healing capacities of RM and Omez against indomethacin-induced stomach ulceration in mice as revealed from the histological damage scores[a]

Group	Damage score
Untreated	3.0 ± 0.07
Extract-treated	0.8 ± 0.03 (73.3)[b]
Omez-treated	0.4 ± 0.02 (86.7)[b]

[a] Stomach ulceration in mice was induced by indomethacin (18 mg/kg, p.o.). RM (40 mg/kg × 3 d) and Omez (3.0 mg/kg × 3 d) were used for the experiments. The histologic damage (DS) were measured on the 3rd day after indomethacin administration by analyzing the data from the review of a minimum of three sections per animal. The values are mean ± S.E.M. ($n = 15$). Figures in parenthesis show % reduction from the experimental control.
[b] $p < 0.001$, compared to the 3rd day ulcerated control.

3.3. *Regulation of the NOS activity*

Compared to the normal mice, a significant increase (5.37 fold, $p < 0.001$) in the total NOS activity was noticed in the ulcerated mice. Both RM and and Omez reduced it by ~73% ($p < 0.001$) (Figure 4).

3.4. *Modulation of the mucosal eNOS and iNOS expressions*

The Western blots of the eNOS and iNOS expressions

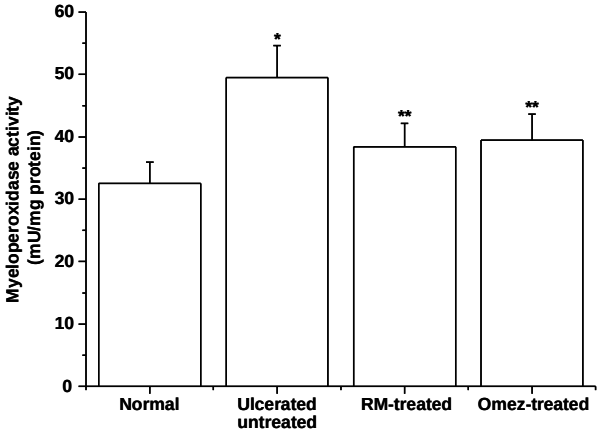


Figure 2. Effects of RM and Omez in modulating the mucosal MPO level in the indomethacin-induced ulcerated mice. The supernatant of the gastric tissue homogenate was incubated with TMB in a suitable buffer and the MPO activity (mean ± S.E.M., $n = 15$) was assayed from the absorbance at 450 nm. $^{*}p < 0.05$ compared to normal mice; $^{**}p < 0.05$ compared to untreated mice.

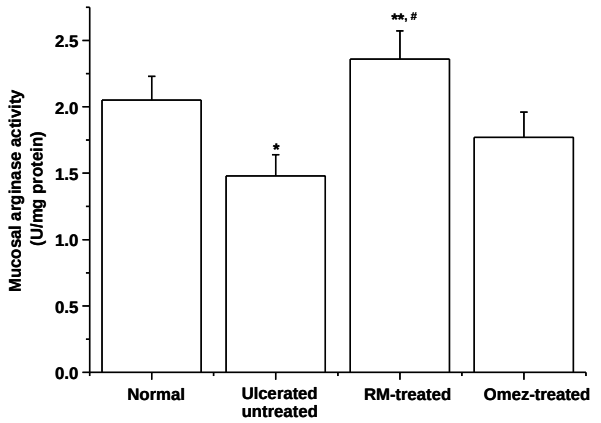


Figure 3. Effects of RM and Omez in modulating the mucosal arginase activity in the indomethacin-induced ulcerated mice. The supernatant of the gastric tissue homogenate was incubated with L-arginine and $MnCl_2$ in a suitable buffer and the arginase activity (mean ± S.E.M., $n = 15$) was assayed from the absorbance at 540 nm. $^{*}p < 0.05$ compared to normal mice; $^{**}p < 0.01$ compared to untreated mice; $^{\#}p < 0.01$ compared to Omez-treated mice.

in the gastric mucosa of the normal, ulcerated and drug (RM or Omez)-treated mice are shown in Figure 5. The eNOS expression was detected in both normal and ulcerated gastric tissues. In contrast, 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 (62%, $p < 0.001$) and eNOS (44%, $p < 0.01$), compared to normal mice. Treatment with RM reduced the iNOS expression (21.6%, $p < 0.05$), along with dramatic increase in the eNOS expression (56.3%, $p < 0.001$), compared to the untreated mice. In contrast, Omez reduced the eNOS expression (23.6%, $p < 0.05$) significantly, but showed insignificant effect on the iNOS expression.

3.5. *Regulation of the serum NO level*

At peak ulceration, there was a significant increase (55.1%, $p < 0.01$) in the serum nitrite level compared to the normal mice. Treatment with RM and Omez reduced it by 49.8% ($p < 0.01$) and 29.3% ($p < 0.01$) respectively, the effect of RM being significantly better ($p < 0.05$) than that of Omez (Figure 6).

3.6. *Modulation of the serum TGF-β1 level*

Compared to the normal value, ulceration reduced the level of serum TGF-β1 (Figure 7) by 48% ($p < 0.001$). Treatment with RM and Omez, however, increased it by 79.6% ($p < 0.001$) and 26.3% ($p < 0.05$)

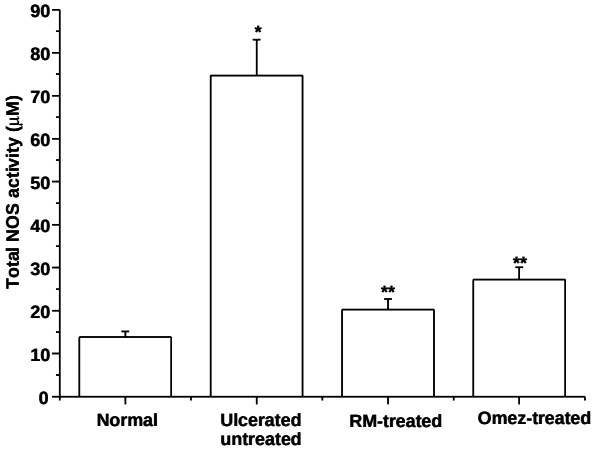


Figure 4. Effects of RM and Omez in regulating the serum total NOS activity in the indomethacin-induced ulcerated mice. The NOS level (mean ± S.E.M., $n = 15$) was measured by ELISA. $^{*}p < 0.001$ compared to normal mice; $^{**}p < 0.001$ compared to untreated mice.

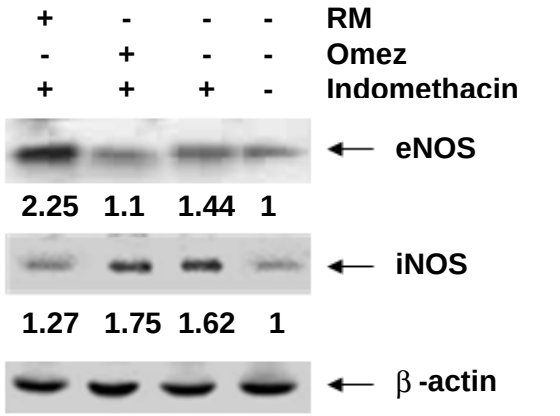


Figure 5. Western blots of eNOS and iNOS expressions of normal, ulcerated and drug-treated gastric tissues of mice. The numerical figures (arbitary unit) reveals the density scanning results, considering that of normal mice as 1.

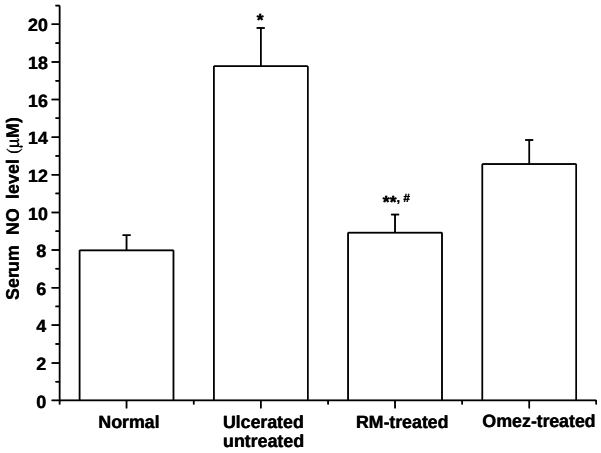


Figure 6. Effects of RM and Omez in regulating serum NO level in acute gastric ulcerated mice. The NO level (mean ± S.E.M., $n = 15$) was measured by ELISA. $^{*}p < 0.01$ compared to normal mice; $^{**}p < 0.01$ compared to the untreated mice; $^{\#}p < 0.05$ compared to Omez-treated mice.

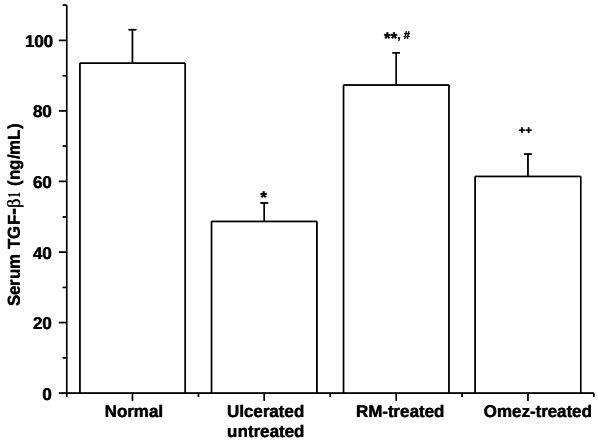


Figure 7. Effects of RM and Omez in regulating serum TGF β1 level in acute gastric ulcerated mice. The serum TGF β1 level (mean ± S.E.M., $n = 15$) was measured by ELISA. $^{*}p < 0.001$ compared to normal mice; $^{**}p < 0.001$, $^{++}p < 0.05$ compared to untreated mice; $^{\#}p < 0.05$ compared to Omez-treated mice.

respectively, compared to the untreated mice. RM was significantly ($p < 0.05$) more potent than Omez.

3.7. *Regulation of the serum Th1 (TNF-α and IL-6) and Th2 (IL-4) cytokines*

Compared to the normal value, ulceration drastically increased the serum TNF-α and IL-6 ~4.4 and ~3.5 folds, respectively ($p < 0.001$). RM suppressed the levels of both TNF-α (66.9%, $p < 0.001$) and IL-6 56% ($p < 0.001$) significantly, compared to the untreated mice. Omez, however, reduced both the cytokines by ~13.3% ($p < 0.05$), which were much less than that by RM.

In contrast, the serum IL-4 level in the ulcerated mice was reduced by 28.3% ($p < 0.05$), compared to the normal mice. Treatment with RM improved it appreciably (95.4%, $p < 0.001$), while the effect of Omez (14.3% increase) was significantly less ($p < 0.001$) than that of RM. The results on the cytokine modulation are summarized in Figure 8.

4. Discussion

The non-steroidal anti-inflammatory drugs (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 (*14*). Ulcer-healing is a complex process involving a combination of wound retraction and re-epithelization wherein several factors such as enzymes, cytokines, and soluble mediators, liberated during the inflammatory response play crucial roles. The impressive healing capacity of RM (IC_{50} = 23.30 ± 3.50 mg/kg) against the indomethacin-induced gastric ulceration in mice (*9*) encouraged us to investigate its probable modulatory effect on arginase and NOS 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 (DS) provided a better assessment of the quality of healing. This was also substantiated from our results with the MPO assay. The MPO activity, a marker of neutrophil aggregation at the site of inflammation is frequently increased in ulcerated conditions, and reduced during wound healing (*15*). Our studies depicted that while indomethacin administration enhanced the gastric mucosal MPO activity, treatment with RM (40 mg/kg × 3 d) and Omez (3 mg/kg × 3 d) reduced it almost equally. These results are consistent with our previous

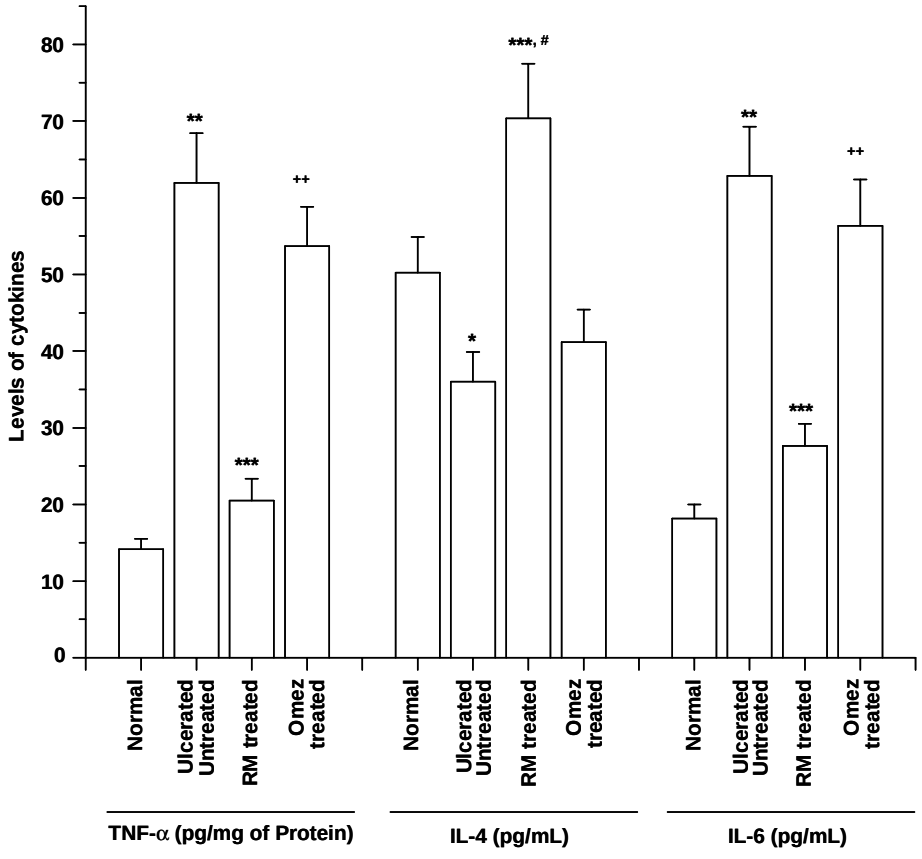


Figure 8. Effects of RM and Omez in regulating serum TNF-α, IL-4 and IL-6 levels in acute gastric ulcerated mice. The cytokine levels (mean ± S.E.M., $n = 15$) were measured by ELISA. $^{*}p < 0.05$, $^{**}p < 0.001$ compared to normal mice; $^{++}p < 0.05$, $^{***}p < 0.001$ compared to untreated mice; $^{\#}p < 0.001$ compared to Omez-treated mice.

histological results, where both RM and Omez were found to produce similar ulcer healing at the designated doses (*9*). It is worth mentioning that extending the period of treatment led to slightly better healing, but a large part of this was due to natural healing. Since that would not provide much information on the drug action, this was not pursued. Likewise, the effect of increasing daily dose of RM was only marginal. The phytochemical analysis of RM revealed the presence of four diarylnonanoids, malabaricones A-D as its major constituents. All these compounds contain a resorcinol moiety, which is known to induce irreversible, hydrogen peroxide-dependent loss of activities of the heme-containing peroxidases such as MPO (*16*).

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 (*17,18*). On the other hand, NOS catabolizes L-arginine by a different pathway to produce L-citrulline and NO. The signaling molecule, NO has long been the subject of extensive research with respect to its role in GI mucosal defense and the pathogenesis of mucosal injury. NOSs exist as constitutive (cNOS), and inducible isoforms (iNOS). Of these, the endothelial NOS (eNOS), belonging to the cNOS isoforms generates only nanomolar concentrations of NO that dilates the blood vessels and increases blood flow in the gastric mucosa (*19*) and also contributes to angiogenesis (*20,21*). The eNOS expression plays a major role in wound healing that gets inhibited if the eNOS expression is depleted (*20,22*). On the other hand, iNOS that can be largely induced under certain pathological conditions (*23*) acts as a killer molecule, and is involved in inflammation. The enhanced generation of NO in the inflamed gastric mucosa may be toxic in the digestive systems, and contribute to the pathogenesis of peptic ulcer disease (*24*). An increase in iNOS activity, and a decrease in eNOS activity in the gastric mucosa are closely related to the development of gastric mucosal lesions. Because the potential high-output source of NO in mammalian cells is iNOS, factors involved in the induction and expression of iNOS activity are key determinants of the NO-mediated toxicity. Sustained overproduction of NO by iNOS is detrimental and contributes to inflammation in various gastroduodenal disorders (*15*).

The intense reciprocal regulation of NOS and arginase activities *in vivo*, and the temporal switch between them decides ulceration and healing (*8,25*). Our results showed considerable down regulation of the mucosal arginase level along with an increased expression of the iNOS due to ulceration. This suggested a shift of the arginine metabolism towards the NO/iNOS pathway during ulceration. The elevated expressions of both iNOS and eNOS accounted for the increased total NOS activity as well as serum nitrite level due to ulceration. The iNOS is probably derived from inflammatory neutrophils and macrophages that would contribute to stimulate inflammatory situation, explaining the ulcerogenic property of indomethacin. Simultaneous generation of superoxide and NO by the macrophages would produce peroxynitrite creating oxidative stress. Its stimulated generation, as observed in this study, may also delay proximal duodenal contractions, by exposing mucus to gastric acidity.

Treatment with RM and Omez, especially the former restored the arginase activity almost to normalcy. RM also raised the eNOS/iNOS ratio to a level favorable for efficient ulcer-healing. This would amount to generation of more polyamine at the expense of the iNOS-derived NO that may be a key contributing factor in the anti-ulcer effect of RM. The reduction of the total NOS activity and NO level by RM was primarily due to suppression of the iNOS expression. Even a moderate suppression of iNOS expression would reduce NO release substantially, since the enzyme is capable of generating NO many folds.

In contrast, despite showing less effect on modulating eNOS/iNOS expressions and NO production, Omez provided excellent healing. This may be due to other operative mechanism in its healing action as observed by us and others (*10,26*). The total NOS reduction by Omez was due to less eNOS expression. This was also evident from the fact that its effect on NO reduction was much less.

Although iNOS is very strongly induced in ulcerated tissue in the stomach, the NO-derived from it does not appear to play a role in modulating healing. Most of the previous studies of NO and inflammation focused on the role of iNOS, whereas the role of eNOS that can also be markedly enhanced by various stimuli or tissue injury has been underestimated. Using eNOS deficient mice, the importance of eNOS and eNOS-derived NO in regulating microvascular structure during acute inflammation has been demonstrated (*27*). Our results suggested that the eNOS-derived NO contributed maximum to the ulcer healing property of RM, although a role for neuronal NOS-derived NO cannot be excluded.

Stimulation of inflammatory cytokines is extremely important in mucosal defense. One of the most prominent modes of mediation of indomethacin-induced gastropathy is the increased expression of the pro-inflammatory cytokines (*28,29*), which also correlates with the extent of ulceration. Even the cross-talk amongst NOS/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 RM and Omez was monitored. This enabled us to associate the inflammatory response with a better prognosis.

Indomethacin administration raised the levels of pro-inflammatory cytokines (TNF-α and IL-6) while reducing the anti-inflammatory cytokines (IL-4 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 (*25,30*). The increased levels of Th1 cytokines due to ulceration would augment the iNOS/NO pathway to produce excess NO, which is likely to promote oxidative stress and result in ulceration (*31,32*). Our result on the decreased IL-4 level due to ulceration was in tune with a previous report (*33*).

Treatment with RM, however, restored the imbalance by increasing the levels of IL-4 and TGF-β beyond the normal values. The upregulation of the anti-inflammatory cytokines by RM is likely to inhibit the stimulatory effect of indomethacin on the level of pro-inflammatory cytokine release in blood and gastric mucosa. The immunosuppressive Th2 cytokine, TGF-β has a direct role in stimulating epithelial restitution (*34*). Besides suppressing the IFN-γ-induced iNOS gene expression and thereby generation of excess NO, it also increases arginase activity during inflammatory processes (*8,25,35*). The altered arginase activity and iNOS expressions observed by us during ulceration, and RM treatment are consistent with their respective effects in modulating the mucosal TGF-β status. The enhanced IL-4 level by RM would trigger the TGF-β-SMAD-signaling pathway to stimulate the extracellular remodeling and subsequent tissue repair. In contrast, except for the TGF-β, the other cytokines were not affected significantly by Omez, as reported earlier (*33*). This was also reflected in its marginal effect in regulating the enzymes, arginase and NOS.

Overall, RM modulated the cytokine profile to shift the balance in favour of arginase/polyamine *vis-a-vis* iNOS/NO pathway, besides improving the eNOS expression. A combination of all these events 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 (*36*). 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.

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