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Gastroprotective properties of *Myristica malabarica* against indometacin-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 indometacin-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 (Biswas 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

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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 indometacin-induced gastric ulceration in mice and compare it with the activity of the proton pump inhibitor (PPI) omeprazole. Compared with the H_2-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; Chiverton 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* was 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). Alcian blue, indometacin, bovine serum albumin (used for measurement of protein concentration using a standard assay), haematoxylin and alum (for preparation of the haematoxylin solution), eosin, butylated hydroxytoluene (BHT), guanidine hydrochloride (HCl), trifluoroacetic acid (TFA), omeprazole and Trizma base were purchased from Sigma Chemicals (St Louis, MO, USA). Other reagents used were 35% hydrogen peroxide (Lancaster, Morecambe, UK), 2,4-dinitrophenyl hydrazine (DNPH), disodium hydrogen phosphate and sodium dihydrogen phosphate from BDH (Poole, Dorset, UK), sucrose and 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) from SRL (Mumbai, India) and kits for detection of urea, creatinine and serum glutamic pyruvic transaminase (SGPT) (all from Liqui Bangalore, India), and serum glutamic oxaloacetic transaminase (SGOT, Bayer AST, India).

Instrumentation

Absorbance spectrophotometry was carried out at 25°C using a Jasco (Shachioji, Tokyo, Japan) V-550 UV-Vis spectrophotometer. Wavelength scans and absorbance measurements were made in 1 mL quartz cells with a 1 cm path length.

Preparation of plant extracts

Dry fruit rinds (20 g) of *M. malabarica* were powdered with a grinder and extracted successively with ether, methanol and water (60 mL for 4 days 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 below 40°C in-vacuo to obtain the respective extracts. These were designated as rampatri ether (7.30 g, 36.5%), methanol (RM; 5.77 g, 28.9%) and aqueous extracts (0.42 g, 0.02%), respectively. Extracts were stored in a vacuum desiccator. Since RM had been previously found to possess antioxidant activity, this extract was used for anti-ulcer experiments.

Identification of phytoconstituents in RM

The chemical constituents of RM were analysed by HPLC with a Jasco model PU-2080 plus chromatogram using Hypersil GOLD column (250×4.6 mm, particle size 5 μm; Thermo Electron Corporation, Waltham, MA, USA). The eluent was acetonitrile/water (60:40) at a flow rate of 1.0 mL min^{-1}. Peaks were detected at 345 nm.

Preparation of drugs

RM and omeprazole were prepared as aqueous suspensions in 2% gum acacia as the vehicle, and administered to the mice by oral gavage. The major constituent phenolics – malabaricone B and malabaricone C – were also used as the drugs in some experiments to evaluate the active constituents of RM.

Animals and experimental protocol for ulceration

Male swiss albino mice were bred at the Dr B. C. Roy Post Graduate Institute of Basic Medical Sciences, Kolkata, India and the BARC Laboratory Animal House Facility, Mumbai, India. Mice were procured after obtaining clearance from the respective animal ethics committees of the two centres (Post Graduate Institute of Basic Medical Sciences, Kolkata Animal Ethics Committee 507/CPCSEA, Sanction No. IAEC/SB-2/2004/UCM-16, dated 06.15.04 and BARC Animal Ethics Committee (BAEC), laboratory animal facility. sanction no. BAEC/03/05, dated 11.07.05) and were handled according to International Animal Ethics Committee Guidelines. The mice (25–30 g) were reared on a balanced laboratory diet (NIN, Hyderabad, India) and were given tap water ad libitum. They were kept at 20 ± 2°C, 65–70% humidity and a 12 h day–night cycle.

At the beginning of each experiment, animals were identified by typical notches in the ear and limbs and then randomized. This was done so that all experiments were performed in a blinded fashion.

Ulceration was induced by administering indometacin (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 24 h before ulcer induction but had free access to tap water.

Standardization of drug dose

For the standardization of drug doses, RM (5, 10, 20, 30, 40 and 60 mg kg^{-1} p.o.) and omeprazole (0.2, 0.5, 1, 2, 3, and 5 mg kg^{-1} p.o.) were given as a single dose per day for up to 7 days, 6 h after indometacin administration. Each group consisted of five mice, and each experiment was repeated three times. The mice were killed on the first, third, fifth and seventh days, 4 h after administering the last dose of test drug.

The extent of ulcer healing was assessed from the macroscopic damage scores (MDS) of the untreated and treated ulcerated mice on respective days. The optimal treatment regimen (drug doses and period of treatment) was determined from the MDS obtained in the groups of mice receiving the treatment described above.

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 drug treatment. Stomach ulceration in the untreated mice also peaked on the third day after indometacin 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 indometacin and killed after 10 h (considered as 1 day); Group III = ulcerated with indometacin, 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% formol 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 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 Swarnakar et al (2005). DNPH (4 mL, 10 mM) in 2 M 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 6 M 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 ($\epsilon = 2.2 \times 10^4\,\text{M}^{-1}\,\text{cm}^{-1}$).

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/TBA/HCl (2 mL, 15% TCA, 0.375% TBA, 0.25 M 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 ($\epsilon = 1.56 \times 10^5\,\text{M}^{-1}\,\text{cm}^{-1}$).

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 1200 *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 ($\epsilon = 13.6 \times 10^4\,\text{M}^{-1}\,\text{cm}^{-1}$).

Mucin assay

Levels of free mucin in gastric tissue were estimated using the method of Tariq & Moutaery (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 mM 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 ×20 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 bled from the retro-orbital complex, and the serum of each mouse was analysed for urea, creatinine, SGPT and SGOT using an autoanalyser (Randox daytona, UK) and commercially available kits.

Statistical analysis

Parametric data, which included all the biochemical parameters, were analysed using 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. Data are presented as mean ± s.e.m. Non-parametric data (histology scoring) were analysed using the Kruskal–Wallis test (non-parametric ANOVA) followed by Dunn's multiple comparison post hoc test. These data are presented as medians (ranges). A P value below 0.05 was considered significant. The IC50 values (dose that gave 50% ulcer healing) of the test samples were estimated using Probit analysis, and the significance level was determined using the chi-square test.

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 indometacin (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.

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 was 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 (Biswas et al 2003).

For the untreated mice, peak ulceration (maximum MDS) was observed on the third day of indometacin 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 ($P < 0.01$).

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

Dose (mg kg^{-1})	Macroscopic damage score			
	Day 1	Day 3	Day 5	Day 7
Ulcerated untreated	1.32 ± 0.05	3.46 ± 0.09**	1.24 ± 0.12	0.65 ± 0.09*
RM treated				
5	1.35 ± 0.09	2.85 ± 0.12	1.15 ± 0.08	0.6 ± 0.06
10	1.32 ± 0.09	2.25 ± .12	1.05 ± 0.08	0.55 ± 0.06
20	1.25 ± 0.08	1.92 ± 0.08	0.95 ± 0.05	0.45 ± 0.03
30	1.22 ± 0.06	1.45 ± 0.05	0.85 ± 0.05	0.35 ± 0.03
40	1.1 ± 0.06	0.95 ± 0.05‡	0.38 ± 0.04	0.19 ± 0.06
60	0.95 ± 0.06†	0.83 ± 0.05	0.32 ± 0.02	0.18 ± 0.08
Omeprazole treated				
0.2	1.25 ± 0.06	3.25 ± 0.08	1.15 ± 0.09	0.65 ± 0.06
0.5	1.21 ± 0.08	2.55 ± 0.09	1.1 ± 0.07	0.64 ± 0.06
1	1.15 ± 0.07	2.05 ± 0.06	1.05 ± 0.04	0.62 ± 0.05
2	1.05 ± 0.08†	0.98 ± 0.06	0.9 ± 0.05	0.58 ± 0.07
3	0.95 ± 0.09††	0.82 ± 0.06‡	0.72 ± 0.03	0.55 ± 0.07
5	0.92 ± 0.08††	0.78 ± 0.07	0.68 ± 0.04	0.52 ± 0.05

*$P < 0.01$, **$P < 0.001$ 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.

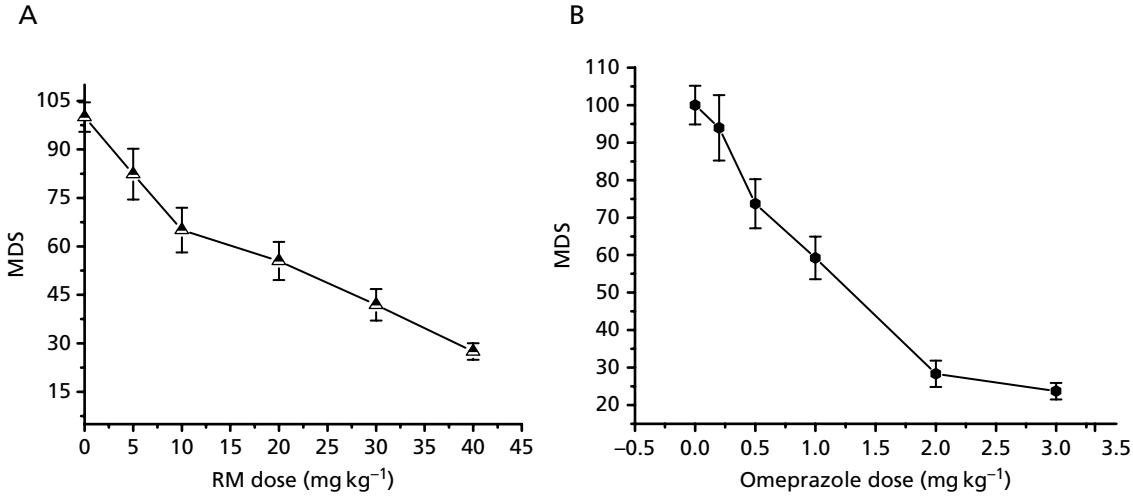

Figure 1 Concentration-dependent healing capacities of methanol rampatri extract (RM) (A) and omeprazole (B) on the third day after indometacin-induced stomach ulceration in mice, as revealed from macroscopic damage scores (MDS). MDS was measured 4 h after the last dose of each drug; the MDS in ulcerated 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% ($P < 0.01$) 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 indometacin-induced stomach ulcers in mice, shown by histological parameters. Histological parameters were assessed in terms of damage and inflammatory scores by analysing 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)

Treatment (group)	Days after ulcer induction	Inflammatory score	Damage score
Untreated (II)	1	1.80 (1.6–2.0)	1.70 (1.2–2.0)
RM (IV)	1	1.0 (0.8–1.4)*	0.60 (0.2–0.8)**
Omeprazole (V)	1	1.0 (0.8–1.2)*	0.4 (0.2–0.6)**
Untreated (III)	3	2.4 (2.0–2.8)‡	3.0 (2.6–3.3)†
RM (VI)	3	0.6 (0.4–0.8)**	0.8 (0.4–1.0)**
Omeprazole (VII)	3	0.8 (0.6–1)**	0.4(0.2–0.6)**

*$P < 0.005$; **$P < 0.001$ compared with untreated mice on same day; $^{\dagger}P < 0.01$, $^{\ddagger}P < 0.005$ compared with group II mice.

Qualitative assessment

The time-dependent indometacin-induced gastropathy and its subsequent healing by RM and omeprazole is shown in Figure 2. Macroscopic and histopathological examinations revealed that indometacin caused marked damage to the glandular portion of the gastric mucosa. Within 6 h after indometacin 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).

Indometacin-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 indometacin intake alone and following administration of RM and omeprazole on the extent of lipid

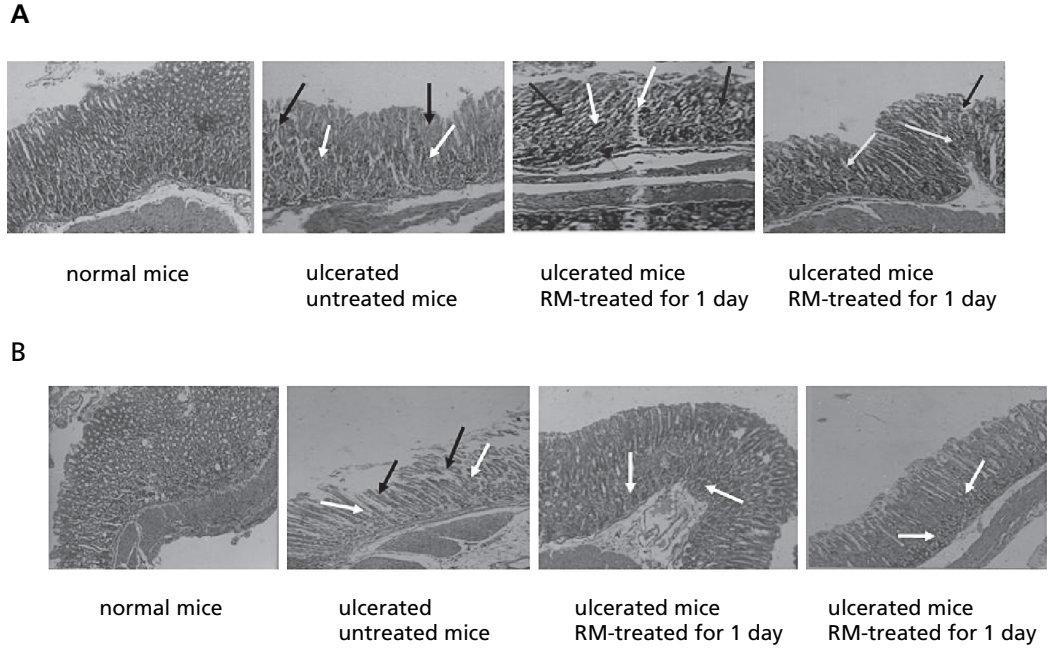

Figure 2 Histological assessment of acute gastric mucosal injury induced by indometacin (18 mg kg^{-1}) in mice and its prevention by methanol rampatri extract (RM) (40 mg kg^{-1}) and omeprazole (3 mg kg^{-1}). Stomach sections were taken 4 h after the last dose of each drug on that day. Images are stomach sections from normal, and ulcerated untreated and treated mice on day 1 (A) and day 3 (B). White and black arrows indicate mucosal damage and inflammatory cells, respectively.

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.

Indometacin 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 43.5% 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 179% ($P < 0.001$) 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.

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 indometacin, 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 ($P < 0.05$).

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 carbonyls, mucin and non-protein thiol in gastric tissue of mice on day 1 after ulcer induction (induced by indometacin 18 mg kg^{-1} p.o.). Values are mean ± s.e.m. (n = 15)

Parameter	Unulcerated controls	Untreated ulcerated controls	RM-treated	Omeprazole-treated
TBARS (nmol mg protein^{-1})	0.84 ± 0.02	1.25 ± 0.07**	1.12 ± 0.07	1.01 ± 0.05‡
Protein carbonyls (nmol mg protein^{-1})	0.96 ± 0.04	1.85 ± 0.07†	1.76 ± 0.07	1.61 ± 0.07
Non-protein thiols (nmol mg tissue^{-1})	1.88 ± 0.049	1.55 ± 0.05*	1.78 ± 0.04‡	1.81 ± 0.03‡
Mucin (μg g tissue^{-1})	300.97 ± 15.89	200.10 ± 20.8*	320.78 ± 18.66‡‡	260.33 ± 19.01‡

*$P < 0.05$; **$P < 0.005$; $^{\dagger}P < 0.001$ vs unulcerated control mice; $^{\ddagger}P < 0.05$; $^{\ddagger\ddagger}P < 0.001$ 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 carbonyls, mucin and non-protein thiol in gastric tissue of mice on day 3 after ulcer induction (induced by indometacin 18 mg kg^{-1} p.o.). Values are mean ± s.e.m. (n = 15)

Parameter	Unulcerated controls	Untreated ulcerated controls	RM-treated	Omeprazole-treated
TBARS (nmol mg protein^{-1})	0.85 ± 0.03	1.91 ± 0.12**	1.08 ± 0.05†	1.10 ± 0.06†
Protein carbonyls (nmol mg protein^{-1})	1.01 ± 0.05	2.82 ± 0.11**	1.90 ± 0.14†	1.87 ± 0.14†
Non-protein thiols (nmol mg tissue^{-1})	1.85 ± 0.04	1.70 ± 0.05	2.10 ± 0.07*	2.35 ± 0.07*
Mucin (μg g tissue^{-1})	313.35 ± 13.3	220.35 ± 17.91*	354.43 ± 13.71‡	301.05 ± 13.67†

*$P < 0.05$; **$P < 0.001$ vs unulcerated controls; $^{\dagger}P < 0.05$; $^{\ddagger}P < 0.001$ vs ulcerated untreated mice.

Effect of RM and omeprazole on mucin contents in stomach tissue

Our studies revealed that administration of indometacin 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 indometacin 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 intake 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 canaliculi. There was no sign of any inflammatory infiltrate or distorted cytoarchitecture, congestion or necrosis. Likewise, normal renal microarchitecture with well-differentiated cortex and medulla was found in the kidneys. Glomeruli, tubules and papilli, 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–Jenning's 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 antioxidative 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 indometacin, 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

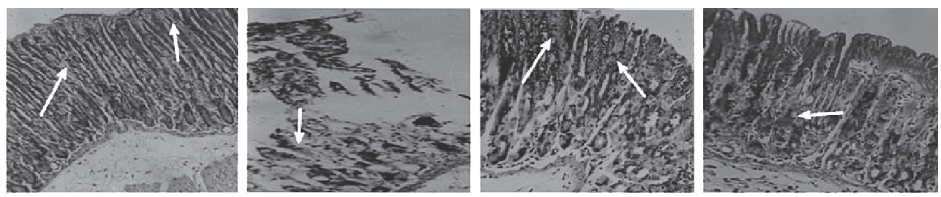

Figure 3 Depletion of mucin associated with indometacin-induced acute gastric ulceration, and its prevention by methanol rampatri extract (RM) (40 mg kg^{-1}) and omeprazole (3 mg kg^{-1}) as revealed by periodic acid–Schiff staining. Stomachs were excised 4 h after the last dose of the drug on the third day. White arrows denote the PAS-positive areas (×20).

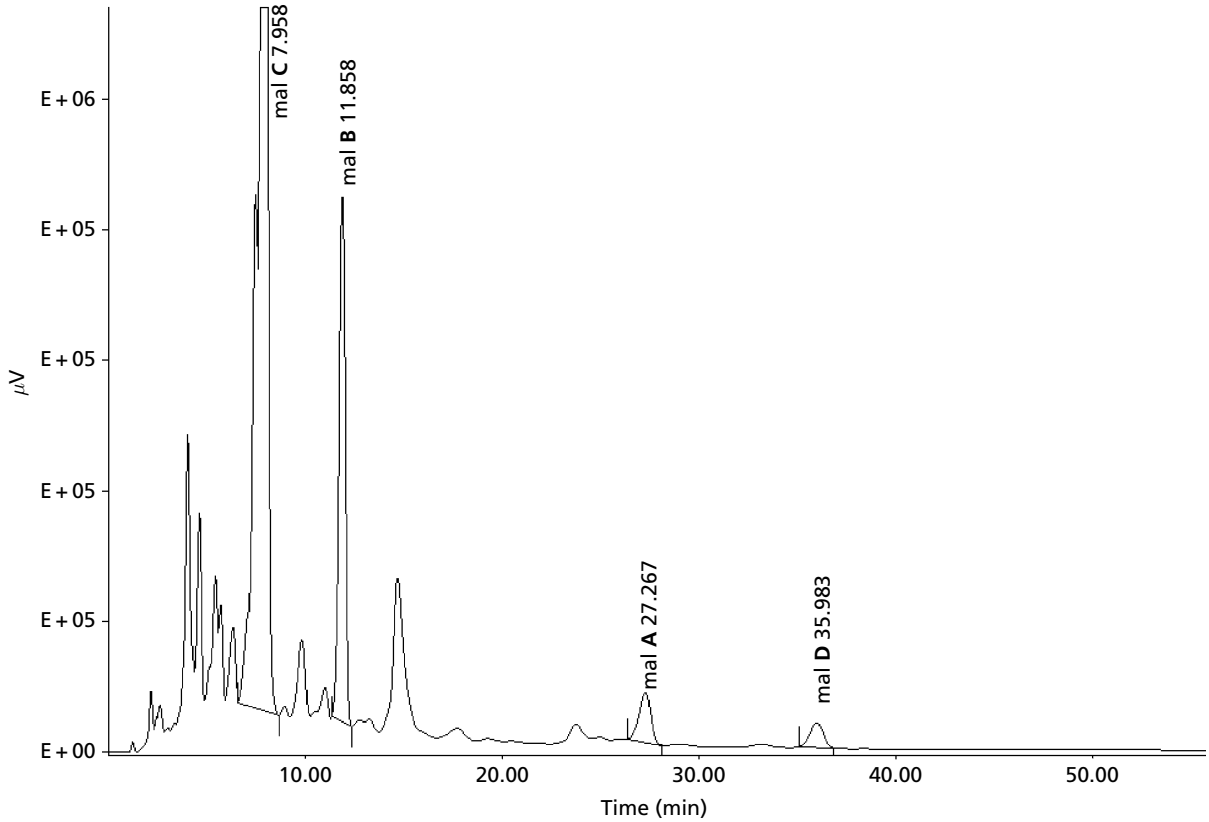

Figure 4 HPLC profile of a methanol extract of fruit rind of *Myristica malabarica* (RM). The chemical constituents of RM were detected at 345 nm. Malabaricones (mal) A–D were identified using standards; retention times were 27.3, 11.9, 8.0, and 36.0 min, respectively.

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)

Parameters	Normal mice	RM-treated mice
SGOT (IU L^{-1})	168.21 ± 10.75	173.5 ± 15.84
SGPT (IU L^{-1})	72.14 ± 6.79	75.84 ± 6.21
Urea (mg dL^{-1})	29.25 ± 1.19	31.4 ± 2.51
Creatinine (mg dL^{-1})	0.29 ± 0.05	0.38 ± 0.02

epithelial recovery after acute injury by forming a mucoid cap beneath which re-epithelization occurs (Wang at 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 indometacin-induced gastric lesions in mice. Administration of indometacin 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 indometacin-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 indometacin-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 disulphide 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 to 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 promotes healing of gastric lesions induced by acute intake of indometacin.

It is known that the free radicals generated by exogenous and endogenous agents can easily produce mucosal damage (Bernier & Florent 1986). Increased mucus production usually assists the healing process by protecting the ulcer crater against irritant stomach secretions (HCl and pepsin) (James 1964), thereby enhancing the local healing process. In this study, mucin secretion was found to be decreased in mice given indometacin, observed by both the Alcian blue assay and PAS staining methods. This indicated reduced ability of the mucosal membrane to protect the mucosa 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 mucous 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 mucus restoration is less evident with omeprazole. The anti-ulcerative 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; Baolin et al 2001). Our results suggest a predominant antioxidative 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 reducing substrates for the oxidized intermediates of prostaglandin H synthase (PGHS), thereby accelerating the peroxidase cycle, and by functioning 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 indometacin-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-toxicty, RM appears to be a promising herbal anti-ulcer preparation that merits further investigation.

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