Protective activity of geraniol against acetic acid and *Helicobacter pylori*-induced gastric ulcers in rats

Subrat Kumar Bhattamisra, Vivian Lee Yean Yan, Chin Koh Lee, Chew Hui Kuean, Mayuren Candasamy, Yun Khoon Liew, Priyadarshi Soumyaranjan Sahu

Department of Life Sciences, School of Pharmacy, International Medical University, Bukit Jalil, 57000, Kuala Lumpur, Malaysia.

School of Medicine, International Medical University, Bukit Jalil, 57000, Kuala Lumpur, Malaysia.

**A B S T R A C T**

Geraniol, an active constituent of rose and palmarosa essential oils, possesses several pharmacological properties, including antioxidant, antibacterial and antiulcer activity. Geraniol was therefore investigated for its antiulcer and anti-*Helicobacter pylori* activity in rats. Ulcers were induced by injecting acetic acid into the sub-serosal layer of the stomach followed by orogastric inoculation of *H. pylori* for 7 days. Geraniol (15 and 30 mg/kg), vehicle and a standard drug combination (amoxicillin, 50 mg/kg; clarithromycin, 25 mg/kg and omeprazole, 20 mg/kg) were administered twice daily for 14 days. All the parameters were measured at the end of treatment. The ulcer index was significantly (*P* < 0.05) lowered in geraniol and standard drug-treated rats as compared to the *H. pylori* control group (4.13 ± 0.43). Treatment with geraniol (30 mg/kg) significantly (*P* < 0.01) increased the gastric pH along with a reduction in total acidity and gastric juice volume. Geraniol significantly (*P* < 0.05) attenuated the myeloperoxidase activity and augmented the total glutathione level in gastric mucosa. The extent of damage in the stomach was measured using a histopathological score. The score in *H. pylori* control, geraniol (30 mg/kg) and standard drugs was 9, 3.5 and 2.0 respectively. In the rapid urease test, treatment with geraniol (30 mg/kg) and the standard drugs produced a 33% and 67% cure respectively from *H. pylori* infection. Further, the reduction in bacterial load in the gastric mucosa was confirmed using modified Giemsa staining. Geraniol was observed to exhibit significant antiulcer and anti-*H. pylori* activity in a rodent model.

**1. Introduction**

Peptic ulcer is a common gastrointestinal disease and is considered as a global health problem. The etiology of the disease is still not completely understood but it is generally accepted that an imbalance between aggressive (stress, pepsin, acid) and protective (such as bicarbonate, mucin, mucosal blood flow, prostaglandin) factors, leads to the development of gastric ulcer. NSAIDs, *Helicobacter pylori* (*H. pylori*) infection, and hereditary predisposition are some of the risk factors that increase the incidence of gastric ulcer. *H. pylori* infection, which is spread through faecal-oral and oral-oral routes, is one of the prevalent factors causing peptic ulcer disease (PUD). More than 90% of duodenal and 60% of gastric ulcer patients were diagnosed with *H. pylori* infection. Currently, drugs, including antacids, anticholinergic, proton pump inhibitors and H2-receptor antagonists are available for the treatment of gastric ulcers. However, they possess several adverse effects and are non-curative. Current standard first-line therapy for *H. pylori*-positive PUD consists of proton pump inhibitors (PPIs) combined with at least two antibiotics, these being clarithromycin and amoxicillin or metronidazole/tinidazole. Unfortunately, antibiotic resistance in *H. pylori* is increasing and approximately 10–20% of *H. pylori* infections persist despite antibiotic treatment. Meanwhile, *H. pylori* resistance rate for clarithromycin has increased significantly from 10% during 1996–1999 to 17.9% during
2005–2007, whereas *H. pylori* resistant towards amoxicillin was found to be 8.5% in 2016. Metronidazole shows the highest resistance rate, with around 40% in developed countries and >90% in developing countries. It has been challenging to find an anti-ulcer agent that could both address the multifactorial etiologies of gastric ulcers and cure the disease, rather than simply producing an anti-secretory action. Hence, the search for an effective anti-ulcer agent having multiple mechanisms of gastroprotective and anti-bacterial activity is essential to combat the disease.

Plant-derived essential oils are widely used as medicines, insecticides, and perfumes. Geraniol (3,7-dimethyl-2,6-octadien-1-ol) is an acyclic monoterpene alcohol found in the essential oils of lemongrass, rose, palmarosa, ginger, orange, lavender, citronella, nutmeg and other plants. It is commercially used for its fragrance and as a flavour in food industry. Geraniol exhibited good antimicrobial activity against a wide spectrum of bacteria and fungi and inhibited the growth of *H. pylori* with a MIC of 0.53 mg/L. We recently found a significant antibacterial activity of geraniol against *S. aureus*, *E. coli* and *H. pylori* with MIC values of 11.2 mg/ml, 5.6 mg/ml and 7.33 mg/ml respectively. (Unpublished data).

Besides its antibacterial activity, geraniol also exhibited anti-oxidant, anti-ulcer, anti-inflammatory and anti-apoptotic effects. Recent studies showed oral administration of geraniol to protect against gastric ulcers induced by either ethanol or by ischemia-reperfusion, and duodenal ulcers induced by cysteamine in rats. However, the effect of geraniol against *H. pylori*-induced ulceration has not been studied. The present study investigates geraniol for its putative antiulcer and anti-*H. pylori* activity in a rodent model.

### 2. Materials and methods

#### 2.1. Drugs and chemicals

Geraniol (98%) was purchased from Sigma-Aldrich Chemical Co., USA. The anaesthetic agents (ketamine, xylazine and zoletil) were obtained from the animal house of University Kebangsaan Malaysia, Kuala Lumpur, Malaysia. Amoxicillin, clarithromycin, and omeprazole were procured from PI Chemicals, Shanghai, China. All the other chemicals and reagents used in the experiments were of analytical grade.

#### 2.2. Microorganisms

*H. pylori* (ATCC 43504) was purchased from Choice Care, Kuala Lumpur, Malaysia. It was subcultured in Mueller-Hinton broth (MHB) supplemented with 10% fetal bovine serum (FBS) and incubated at 37 °C in an anaerobic jar containing microaerophilic gas generator pack (CampyGen™ 2.5 L, Thermo Scientific, Oxoid Ltd, UK) for five days to obtain substantial growth of *H. pylori*.

#### 2.3. Animal grouping

Adult male Sprague-Dawley rats (200–250 gm) were procured and maintained in the animal house facility of International Medical University, Bukit Jalil, Kuala Lumpur, Malaysia. The rats were housed randomly in polypropylene cages under standard condition of dark and light cycle (12/12 h), humidity (50–60%) and temperature (20–24 °C). During the acclimatisation and experimental period, animals had access to food and water ad libitum. The experimental protocol was presented to Institutional ethics committee of International Medical University, Kuala Lumpur, Malaysia. The research protocol was duly approved (BP I-01/12 (46) 2015) before initiation of the study. All the animals used in this study were received humane care and the experiments were performed according to the criteria outlined in the "Guide for the care and use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institute of Health, USA.

The rats were divided into six groups with six animals in each group. The grouping was as follows. Group 1: normal control (saline, 5 ml/kg, b. i.d); Group 2: ulcer control (acetic acid) (corn oil, 5 ml/kg, b. i.d); Group 3: ulcer control with *H. pylori* (acetic acid and *H. pylori*) (corn oil, 5 ml/kg, b. i.d); Group 4: geraniol (15 mg/kg, b. i.d); Group 5: geraniol (30 mg/kg, b. i.d) and Group 6: standard drugs (amoxicillin, 50 mg/kg; clarithromycin, 25 mg/kg; omeprazole, 20 mg/kg).

#### 2.4. Ulcer induction and treatment protocol

Rats were ulcerated with acetic acid according to the method described by Takagi et al. (1969). Under anaesthesia, laparotomy was performed through a midline epigastric incision; the stomach was exposed and 20% acetic acid (0.03 mL) was injected into the sub-serosal layer of the glandular portion, using a micro syringe (0.05 mL). After suturing the abdominal incision, the animals were returned to their home cages with daily access to food, restricted to the time periods of 9–10 a.m. and 5–6 p.m. This allows adequate fasting for the administration of *H. pylori* inoculum, geraniol and standard drugs.

Twenty 4 h after acetic acid-induced ulceration, the animals were inoculated intragastrically by gavage with 1 mL of a confirmed pathogenic strain of *H. pylori* suspended in MHB; this was undertaken twice daily for 7 days. Control rats and those receiving acetic acid without *H. pylori* infection received MHB orally for the same time period. Geraniol, vehicle and standard drugs were administered twice daily for 14 consecutive days, starting from the third day after ulcer induction with acetic acid. After treatment, the animals were sacrificed by cervical dislocation. The stomach was removed and evaluated for gastric lesions.

#### 2.5. Measurement of gastric ulcer index

The ulcerated area (mm²) and the healing rate (%) were measured...
determined according to the method of Takagi and Okabe (1968). The ulcerated area and diameter (mm²) was measured with a ruler and the ulcer score was calculated based on the severity of gastric ulcer lesion scoring system.²⁴

| Ulcer score | Gastric Lesions                      |
|-------------|-------------------------------------|
| 0           | No lesion                           |
| 1           | Mucosal oedema and petechiae        |
| 2           | One to five small lesions (1–2 mm), |
| 3           | More than five small lesions or one intermediate lesion (3–4 mm) |
| 4           | Two to more intermediate lesions or one gross lesion (≥4 mm) |
| 5           | Perforated ulcers                   |

The ulcer index (UI) and percentage of curative ratio was calculated using the formula below:

\[ UI = \frac{\text{total ulcer score/number of animals ulcerated}}{100} \]

Curative ratio (%) = (UI control – UI treated)/UI control × 100

2.6. Measurement of gastric juice volume, pH and total acidity

Gastric content was secured by tying the pyloric and cardiac end of the stomach with a thread. A small incision was made in the greater curvature and the gastric content was collected into a centrifuge tube. Distilled water (5 mL) was added to the tube and centrifuged at 2000 × g for 10 min. The pH of the supernatant was measured using a pH meter. Total acidity was determined by titrating with 0.01 N sodium hydroxide using phenolphthalein as an indicator and the results were expressed as mEq/L/100 g.²⁴

2.7. Determination of myeloperoxidase (MPO)

The gastric tissue (100 mg) was homogenized using a small tissue homogeniser in 40 mM phosphate buffer at pH 7.4. The homogenates were centrifuged at 13,000 × g for 10 min at 4 °C. MPO activity in the tissue homogenate was estimated as per the protocol described in the commercial MPO kit (BioVision Incorporated, USA). In brief, supernatants were allowed to react with 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB) at 25°C for 120 min and the reaction was terminated by adding the stop mixture. The change in absorbance was measured at 412 nm using a spectrophotometer.

2.8. Determination of total glutathione (GSH)

Gastric tissue (100 mg) was washed with phosphate buffer solution (pH 7.4) and the size was reduced appropriately by chopping with scissors. The tissue was homogenized in ice-cold 5% metaphosphoric acid (MPA) (~1 ml/100 mg tissue using a small tissue homogeniser). The homogenate was centrifuged at 10,000 × g for 15 min at 4°C. GSH level was measured as per the protocol described in the commercial GSH assay kit (Cell Biolabs Inc., San Diego, CA, USA).

2.9. Rapid urease test (RUT)

Gastric mucosa (approximately 2 mm²) from the antral area was removed and the presence of H. pylori was detected using the Campylobacter-like organism (CLO) test kits (Kimberly-Clark, USA). The yellow urea gel turned into a bright magenta colour in the presence of H. pylori, with the production of ammonia (alkaline reaction).²⁵

2.10. Histopathological evaluation

The collected stomach fragments were preserved in 10% buffered formalin. All the specimens were subjected to haematoxylin and eosin (H&E) and modified Giemsa staining. Giemsa-stained slides were observed by a pathologist for H. pylori detection and pathogen load in the gastric mucosa. H&E stained slides were observed and interpreted under a microscope (80i, Nikon, USA) for histopathological changes and graded according to the lesion scoring system by a pathologist who was blind to the treatment based on the criteria.²⁵ The scorings were allotted as 1. Epithelial cell loss (Score: 0–3); 2. Oedema in the submucosa (Score: 0–4); 3. Haemorrhagic damage (Score: 0–4) and 4. Presence of inflammatory cells (Score: 0–3). The maximum total score was thus 14.

2.11. Statistical analysis

The results are expressed as mean ± standard error mean (S.E.M). Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Tukey-Kramer post-test. Histopathological data were analysed using non-parametric statistics using Kruskal-Wallis test followed by Mann-Whitney U test as post-hoc analysis between two groups. Percentage inhibition in CLO test was statistically compared using z-test. The value of P < 0.05 is considered statistically significant. All statistical analyses were done using the SPSS (Statistical packages for Social Science) version 16.0 Software.

3. Results

3.1. Gross evaluation

Treatment with geraniol or standard drugs considerably reduced the ulcer area and also the number of ulcer patches when compared to both ulcer control groups. The ulcer control without H. pylori showed large ulcers with haemorrhage and necrosis, whereas several ulcerogenic lesion areas (3–6 mm) with hyperemia and haemorrhagic streaks were observed in the ulcer control with H. pylori (Fig. 1). Treatment with geraniol and standard drugs resulted in fewer dilated blood vessels and haemorrhagic streaks on the mucosal surface (Fig. 1). Geraniol in both doses and the standard drugs produced a significant reduction in the UI compared with the ulcer control with H. pylori group (Table 1). Geraniol at doses of 15 and 30 mg/kg exhibited a percentage curative ratio of 42% and 52% respectively when compared to ulcer control with H. pylori, whereas the standard drugs treatment produced a maximum curative ratio of 64%.

3.2. Gastric juice volume, pH and total acidity

Gastric juice volume was significantly (P < 0.01) increased in the ulcer control group with H. pylori as compared to the normal group. Treatment with geraniol (15 mg/kg, 30 mg/kg) but not with standard drugs, produced a significant reduction in gastric juice volume (P < 0.05) (Table 1). The pH of gastric juice in the ulcer control groups was significantly (P < 0.05) reduced in comparison to the normal control. Treatment with geraniol (15 mg/kg and 30 mg/kg) or the standard drugs produced a significant (P < 0.05) increase in pH compared with the ulcer with H. pylori control group. There was a marked increase in total acidity in the ulcer control group with H. pylori; this was significantly decreased by treatment with geraniol at both doses or with the standard drugs (P < 0.05) (Table 1).
Fig. 1. Gross morphological observation of stomach in H. pylori and acetic acid-induced ulcer in rats (magnification = 1.3×).

A: Normal control group having intact stomach with normal morphology of the stomach; B: Ulcer control group without H. pylori showing extensive damage to gastric mucosa with severe ulceration and haemorrhage (red arrow) condition; C: Ulcer control group with H. pylori showed extensive mucosal damage and many ulcerated lesions with haemorrhage; D: Geraniol (15 mg/kg, b. i.d.) treated rats showed moderate ulcer lesions with less haemorrhage; E: Geraniol (30 mg/kg, b. i.d.) treated rats showed mild injuries and ulcer lesions without haemorrhage; F: Standard drug (amoxicillin, 50 mg/kg; clarithromycin, 25 mg/kg and omeprazole, 20 mg/kg, b. i.d.) treated rats showed mild injuries and ulcer lesions without haemorrhage.

Table 1
Effect of geraniol on gastric juice volume, pH and total acidity in H. pylori and acetic acid - induced ulcer model.

| Groups              | Gastric juice volume (mL) | pH       | Total acidity (mEq/L/100 g) | Gastric ulcer index (UI) |
|---------------------|---------------------------|----------|-----------------------------|--------------------------|
| NC                  | 1.63 ± 0.41               | 4.1 ± 0.3| 105 ± 19.83                 | 0.00                     |
| UC                  | 2.43 ± 1.20               | 2.6 ± 0.28$ | 140 ± 15.06                 | 2.80 ± 0.68$            |
| UC with H. pylori   | 6.38 ± 0.83$              | 2.2 ± 0.06$ | 315 ± 35.24$               | 4.13 ± 0.43$           |
| Ger_15 mg/kg        | 3.12 ± 1.03*              | 3.4 ± 0.26** | 134 ± 25.42$               | 2.20 ± 0.34*            |
| Ger_30 mg/kg        | 1.79 ± 0.91**             | 4.1 ± 0.26** | 124 ± 17.78**              | 2.00 ± 0.53*            |
| Standard Drugs      | 3.50 ± 1.46               | 4.0 ± 0.44** | 130 ± 26.77**              | 1.50 ± 0.29**           |

Data are expressed as mean ± S.E.M; n = 6 per group; $ P < 0.05 vs normal control; $^5 P < 0.01 vs normal control; * P < 0.05 vs ulcer control with H. pylori; ** P < 0.01 vs ulcer control with H. pylori.

NC: normal control; UC: ulcer control without H. pylori; UC with H. pylori: Ulcer control with H. pylori; Ger_15 mg/kg: Geraniol (15 mg/kg); Ger_30 mg/kg: Geraniol (30 mg/kg); Standard Drugs: amoxicillin (50 mg/kg), clarithromycin (25 mg/kg) and omeprazole (20 mg/kg).
3.3. Rapid urease test (RUT)

Geraniol treatment at 15 mg/kg and 30 mg/kg showed 17% and 33% reduction in H. pylori positive antral samples respectively, whereas treatment with the standard drugs showed a 67% (P < 0.05) reduction (Table 2).

3.4. Determination of myeloperoxidase (MPO)

MPO activity was significantly (P < 0.05) increased in both ulcer control groups in comparison to normal controls. Geraniol (15 mg/kg and 30 mg/kg) significantly (P < 0.05 and P < 0.01 vs. ulcer control with H. pylori respectively) reduced the MPO levels in the stomach. Treatment with the standard drugs also reduced MPO levels (P < 0.05 vs. ulcer control with H. pylori (Fig. 2)).

3.5. Determination of total glutathione (GSH)

The GSH content in stomach samples of both ulcer control groups was significantly (P < 0.05) reduced as compared to normal controls. Treatment with geraniol (30 mg/kg) or with the standard drugs significantly (P < 0.05) augmented the GSH content in the gastric tissue (Fig. 3).

3.6. Histopathological evaluation

H&E staining of the gastric mucosa showed extensive lesions in both ulcer control, groups, with oedema in the sub-mucosal layer and leucocyte infiltration, along with haemorrhage and epithelial cell loss (Fig. 4; Table 3). Treatment with geraniol (30 mg/kg) or with the standard drugs significantly reduced inflammation, oedema, leucocyte infiltration and haemorrhagic damage in comparison to the ulcer control with H. pylori, with a significant reduction in lesion scores (Table 3).

The presence of H. pylori in the gastric mucosa was verified using the modified Giemsa staining technique. Geraniol (15 and 30 mg/kg)-treated animals showed decreased H. pylori load in the gastric mucosa in comparison to ulcer control with H. pylori group. Standard drug therapy significantly reduced the pathogen load, with an absence of detectable H. pylori in the gastric mucosa (Fig. 5).

4. Discussion

H. pylori is considered as the major causative factors in the pathogenesis of peptic ulcer disease in association with predisposing factors. While the anti-ulcerogenic action of geraniol has been demonstrated previously in a rat model in which peptic ulceration was induced by ethanol, ischemia-reperfusion or cysteamine, the present study is the first investigation of the protective action of this agent using a model in which gastric ulceration was induced by a combination of acetic acid and H. pylori infection. Although it is not easy to completely mimic the basic characteristics of human H. pylori infections (an intense active chronic gastritis, either antral or diffuse), and the associated complications (mucosal atrophy and intestinal metaplasia) and pathologies (peptic ulcer, gastric adenocarcinoma and lymphoma), this is a well-validated model. Ulceration using acetic acid spontaneously healed. However, the introduction of H. pylori, as used in the present study, leads to significant delay in ulcer healing and results in the establishment of chronic ulceration. Inflammation, increased apoptosis, over-expression of inflammatory markers and reduced gastric micro-circulation are responsible for delayed ulcer healing. Further,
MPO activity in the ulcerated tissue are much higher than normal gastric tissue. The characteristics of these ulcers are quite similar to the human peptic ulcers in regard with its pathological characteristics and healing process. Previous results showed that rats...
ulcer. Total GSH is an essential component of the endogenous stress remain a major cause for the induction and aggravation of an antioxidant levels are insufficient to counter the excess ROS, resulting in lipid peroxidation, cellular death and tissue damage. The role of ROS in the etiology and pathophysiology of human gastric ulcer development is well established. ROS production and oxidative stress remain a major cause for the induction and aggravation of an ulcer. Total GSH is an essential component of the endogenous defence system; it scavenges toxic oxygen free radicals and peroxides and low GSH levels in the gastric mucosa facilitate gastric tissue damage. A significant reduction in total GSH was seen in both ulcer controls, and the higher dose of geraniol, as well as the standard drugs, significantly increased total GSH levels. The increased level of GSH after geraniol treatment may be a factor in its gastro-protective effects. Our observations concur with the previous findings which showed that the antiulcer effect of geraniol in an acute ulcer model was associated with increased GSH and decreased MPO activity, as well as stimulation of the nitric oxide pathway, increased prostaglandin levels, and increased mucus production. In the present study, the biochemical changes in gastric mucosa due to geraniol were further corroborated by the histopathological evaluation of gastric tissues. The observations from gross morphology, histopathology and biochemical estimations strongly suggest the anti-ulcer potential of geraniol in a chronic ulcer model. Oxygenated monoterpenes like geraniol, carvacrol, α-terpineol, isopulegol have shown potent anti-ulcer activity against acute ulcer models induced by many agents.

H. pylori infection leads to an alteration of the motor or sensory function of the stomach, causing a delayed stomach emptying and increased gastric juice volume. We found geraniol to significantly reduce the volume of gastric juice, as well as to lower the total acidity in the stomach; these effects may indicate a primary anti-secretory effect or that the secretion was reduced secondary to ulcer healing. de Carvalho et al. suggested that the antiulcer activity of geraniol in their acute ulcer model was not related to its anti-secretory action. The reduced gastric acid secretion and gastric juice volume produced by geraniol in the present study may be secondary to ulcer healing, an anti-H. pylori effect or an effect of geraniol on autoregulation of gastrin release.

H. pylori colonise and move chemotactically towards the gastric mucosa layer, even in the acidic gastric environment, by using urease to convert urea into ammonia. The ammonia neutralises...
the acid around the bacteria, thus allowing their survival in an acidic environment.\textsuperscript{56} Ammonia also damages the gastric epithelial cell, as well as reducing the thickness of the mucus gel layer, resulting in chronic colonisation of \textit{H. pylori} and ulcer formation.\textsuperscript{46,49} Detection of urease using the RUT is a minimally invasive test with 100% sensitivity and 89.5% specificity for \textit{H. pylori}.\textsuperscript{50}

However, false positive results can be obtained due to interference by other bacterial species like \textit{Klebsiella pneumonia}, \textit{Enterobacter cloacae}, \textit{Citrobacter freundii}, and \textit{Proteus mirabilis} that also produce urease.\textsuperscript{21} We found both geraniol and the standard drugs to reduce urease production, suggesting a reduction in \textit{H. pylori}, although the standard drug treatment was the most effective, statistical significance being achieved only with this treatment. However, the urease data were supported by the histopathological findings, with histopathology having 97% specificity and 98% sensitivity.\textsuperscript{52,54} The ulcer control rats showed a significant pathogen load and colonies in between gastric mucosa and mucus layer of the stomach. The pathogen load in the gastric mucosal epithelium was reduced by geraniol as well as by the standard drugs.

The combination therapy including two antibiotics and an acid suppressant is effective against the eradication of \textit{H. pylori} and healing of gastritis, gastric/duodenal ulcers. Amoxicillin inhibits the synthesis of glycopeptides of the bacterial cell wall. Whereas, Clarithromycin inhibits the bacterial protein synthesis. The MIC value of amoxicillin and clarithromycin is decreases in higher gastric pH. Proton pump inhibitors like pantoprazole which increase the gastric pH co-administered to augment the antibacterial effects of amoxicillin and clarithromycin as well as improve the ulcer condition.\textsuperscript{53} Geraniol has attenuated the gastric acid secretion and increase the gastric pH in this study. In another study, we have observed anti-\textit{H. pylori} effect of geraniol.\textsuperscript{54} Thus, geraniol possesses both anti-ulcer and anti-\textit{H. pylori} effect which is complementing each other for delivering a better efficacy. Thus our findings suggest a considerable in vivo anti-\textit{H. pylori} activity, supporting the in vitro findings by our group and other researchers.\textsuperscript{19}

5. Conclusion

The antiulcer effect and anti-\textit{H. pylori} action of geraniol in the present study was associated with increased GSH levels and decreased MPO activity, along with reduced gastric secretion. Thus, geraniol has the potential to be developed as a treatment for \textit{H. pylori} associated PUD. The mechanism(s) underlying the antiulcer effect remains to be established but may involve direct gastro-protective effects due to free radical scavenging, as well as an antibacterial action against \textit{H. pylori}.

Conflicts of interest

All authors declare no conflict of interest to disclose.

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