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ORIGINAL ARTICLE

Effect of Geraniol and Clarithromycin Combination against Gastric Ulcers Induced by Acetic Acid and *Helicobacter pylori* in Rats

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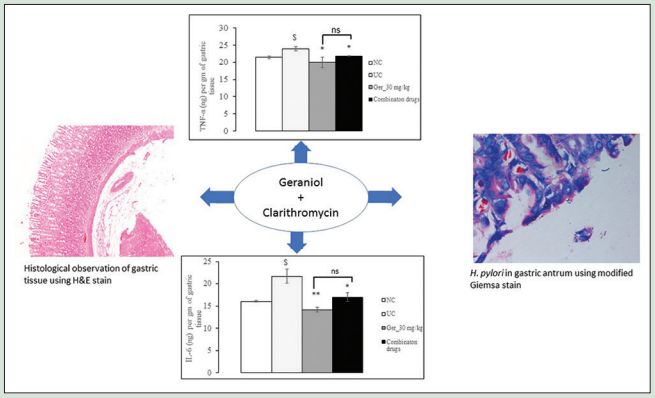
ABSTRACT

Background: Previous work showed geraniol to have anti-*Helicobacter pylori* activity *in vivo* and *in vitro*. Further, it has augmented the anti-*H. pylori* activity of clarithromycin *in vitro*. **Objective:** Geraniol and clarithromycin combination was investigated for anti-ulcer and anti-*H. pylori* activity in rats. **Materials and Methods:** Ulcers were induced by injecting acetic acid into the subserosal layer of the stomach followed by intragastric inoculation of *H. pylori* for 7 days. Geraniol (30 mg/kg) and a drug combination (geraniol, 30 mg/kg and clarithromycin, 25 mg/kg) were administered twice daily for 14 days, and parameters were measured at the end of treatment. **Results:** Geraniol alone or in combination with clarithromycin significantly ($P < 0.05$) lowered the ulcer index and improved the curative ratio. Histopathological examination showed a significant reduction ($P < 0.05$) in the ulcer score in the geraniol and geraniol–clarithromycin treated animals compared with the ulcer controls. Both the treatment groups showed significant ($P < 0.05$) increase in the glutathione level and reduction in the malondialdehyde, interleukin 6, and tumor necrosis factor-α level in the gastric mucosa compared to control group. Both produced a 50% reduction in the rapid urease test, which is further confirmed using modified Giemsa staining. **Conclusion:** Geraniol, alone or in combination with clarithromycin, showed a significant ulcer healing effect which was associated with anti-*H. pylori*, anti-inflammatory, and antioxidant effects in the gastric mucosa. However, the effects of the geraniol and clarithromycin combination were not significantly different from the effects of geraniol alone in this model.

Key words: Anti-inflammatory, antioxidant, antiulcer, clarithromycin, geraniol, *Helicobacter pylori*

SUMMARY

Geraniol and clarithromycin combination was tested against gastric ulcer induced by *H. pylori* and acetic acid in rats. The combination was compared with the geraniol group. Several parameters i.e. ulcer score, total acidity, pH, proinflammatory markers, antioxidant enzymes, *H. pylori* load in gastric mucosa were measured. Geraniol, alone or in combination with clarithromycin, showed a significant anti-ulcer effect by attenuating inflammation, oxidative stress and *H. pylori* infection in the gastric mucosa. However, a significant difference between these two groups was not observed. The present study showed that the addition of clarithromycin to geraniol group conferred no protection against ulceration additional to that seen with geraniol alone.



Abbreviations Used: ANOVA: Analysis of variance, ATCC: American type culture collection, b.i.d: bis in die (twice a day), CLO: Campylobacter-like organism, ELISA: Enzyme-linked immunosorbent assay, FBS: Fetal bovine serum, GSH: Glutathione, H and E: Hematoxylin and eosin, *H. pylori*: *Helicobacter pylori,* IL-1 β: Interleukin-1 β, IL-6: Interleukin-6, IL-8: Interleukin-8, MDA: Malondialdehyde, MHB: Mueller-Hinton broth, MPA: Metaphosphoric acid, RIPA: Radioimmunoprecipitation assay, ROS: Reactive oxygen species, RUT: Rapid urease test, SEM: Standard error mean, SPSS: Statistical packages for Social Science, TBARS: Thiobarbituric acid reactive substances, TNF-α: Tumor necrosis factor-α, UI: Ulcer index, WHO: World Health Organization.

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INTRODUCTION

Peptic ulcer, an upper gastrointestinal disease, affects a large number of people worldwide and is one of the most common diseases of the 21st century.[1] Gastric ulcer and duodenal ulcer, commonly referred to as peptic ulcer disease, are defined as the loss of continuity in part of the gastrointestinal tract wall penetrating the muscularis mucosa with a diameter of at least 0.5 cm.[2] Approximately 50%–75% of the world population is infected with *Helicobacter pylori*. This bacterium plays an important role in the pathogenesis of peptic ulcer diseases and is also classified as a Group 1 carcinogen by the WHO. The prevalence of the infection is higher than 80% in individuals aged above 50 years of age and is greater in developing than the developed countries.[3] In Asia, the prevalence of *H. pylori* infection varies among different countries,

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the reported overall seroprevalence rates ranging from about 31% in Singapore and 36% in Malaysia to 79% in India and 92% in Bangladesh.[4] *H. pylori* are closely associated with the incidence of chronic gastritis, peptic ulcers, gastric mucosa-associated lymphoid tissue lymphoma, and gastric cancer.[5] This spiral-shaped, microaerophilic, Gram-negative bacterium can survive within the extremely harsh environment of the human stomach and establish a chronic infection;[6,7] periplasmic pH value of 4.0–8.5 is favorable for its survival and growth. These periplasmic pH values are attained because the organism produces urease which hydrolyses gastric urea, liberating ammonia, which neutralizes gastric acid.[4] Proton-pump inhibitors along with antibiotics such as amoxicillin plus clarithromycin or metronidazole are currently considered as the first-line treatment for *H. pylori* infection. However, the effectiveness of this therapy is reducing, with eradication cure rates as low as 50%–70%, due to high rates of antibiotic resistance, high rates of antibiotic associated side effects, and low compliance.[8] 10%–20% of *H. pylori* infections persist despite antibiotic treatment,[9] with increasing resistance to clarithromycin,[10] amoxicillin,[11] and especially to metronidazole.[12] Several plant-derived products have been shown to have anti-*H. pylori* actions; these include carvacrol, polyphenolic catechins, tannins, cinnamaldehyde, eugenol, quercetin, licoricidin, licoisoflavone B, berberine, sanguinarine, chelerythrine, protopine, β-hydrastine, mastic, plumbagin, and protocatechuic acid. Combinations of medicinal plants with the present antibiotic regimens have been beneficial in overcoming the antibiotic resistance.[8]

Geraniol (3,7-dimethyl-2,6-octadien-1-ol) is an acyclic monoterpene alcohol found in the essential oils of lemon grass, rose, palmarosa, ginger, orange, lavender, citronella, nutmeg, and other plants.[13,14] It exhibited good antimicrobial activity against a wide spectrum of bacteria and fungi,[15] including *H. pylori*.[16] We found significant antibacterial activity of geraniol against *H. pylori,* and this effect was augmented by combination with clarithromycin or with amoxicillin.[17] We also showed geraniol to have significant antiulcer and anti-*H. pylori* activity in an *in vivo* model of *H. pylori*-induced ulcers.[18] A previous study by de Carvalho *et al*. showed an antiulcer effect of geraniol in acute ulcer models, where gastric ulceration was induced by ethanol or ischemia-reperfusion, and duodenal ulcers were induced by cysteamine.[14] In the present study, we have examined the effects of geraniol alone or in combination with clarithromycin on gastric ulceration induced by acetic acid and anti-*H. pylori* in the rat.

MATERIALS AND METHODS

Drugs and chemicals

Geraniol (98% purity) was procured from Sigma-Aldrich Chemical Co., USA. Clarithromycin (>95% purity) was purchased from PI Chemicals, Shanghai, China. Anaesthetic agents (ketamine, xylazine and zoletil) were obtained from the laboratory animal resource unit, University Kebangsaan Malaysia, Kuala Lumpur, Malaysia. Other chemicals and reagents used in the experiments were of analytical grade.

Micro-organism

H. pylori (ATCC 43504) was purchased from Choice Care, Kuala Lumpur, Malaysia. Microaerophillic conditions were maintained during the subculturing of *H. pylori*. In brief, it was subculture in Mueller-Hinton broth (MHB) supplemented with 10% fetal bovine serum. Incubation was carried out in an anaerobic jar containing microaerophilic gas generator pack (Campy Gen™ 2.5 L, Thermo Scientific, Oxoid Ltd, UK) for 5 days at 37°C to obtain substantial growth of *H. pylori*.

Animals

Adult male Sprague–Dawley rats (200–250 g) were procured and randomly housed in polypropylene cages under standard condition of dark and light cycle (12/12 h), humidity (50%–60%), and temperature (20°C–24°C) maintained at the animal house facility of International Medical University, Bukit Jalil, Kuala Lumpur, Malaysia. All the animals received humane care and had access to food and water *ad libitum*. The experimental protocol obtained prior approval from the animal ethics committee of International Medical University, and experiments were performed according to the criteria outlined in the "Guide for the care and use of Laboratory Animals." The project approval number is BP I-01/13(50)2016.

Rats were grouped into four with six animals in each group. Group 1: normal control (saline, 5 ml/kg, b.i.d); Group 2: ulcer control with *H. pylori* (corn oil, 5 ml/kg, b.i.d); Group 3: geraniol (30 mg/kg, b.i.d), and Group 4: combination drugs (geraniol, 30 mg/kg and clarithromycin, 25 mg/kg).

Ulcer induction and treatment protocol

Ulcer induction procedure was followed according to the method described by Takagi *et al*.[19] In brief, rats were anesthetized intramuscularly by a mixture of xylazine (25 mg/mL), ketamine (25 mg/mL), and zoletil (12.5 mg/mL) at a 1:1:1 volume ratio with 0.1 mL/100 g body weight. The stomach was exposed by laparotomy under anesthesia. Acetic acid (20%, 0.03 mL) was injected using a microsyringe into the subserosal layer of the glandular portion of the stomach. Postsurgical care was provided to all the animals. Rats had free access to food except the periods of 9–10 am and 5–6 pm. This restriction allows the rats in adequate fasting to receive *H. pylori* inoculum and drug.[19] Rats were started inoculated with 1 mL of a confirmed pathogenic strain of *H. pylori* suspended in MHB intragastrically after 24 h of acetic acid-induced ulceration.[20] Inoculation was performed two times in a day for 7 consecutive days. Geraniol, vehicle, or a combination of geraniol and clarithromycin were administered twice daily for 14 consecutive days, starting from the 3rd day after ulcer induction with acetic acid.[20] After treatment, the animals were sacrificed by cervical dislocation. The stomach was removed and evaluated for gastric lesions.

Measurement of gastric ulcer index

The area (mm^2) and diameter of the ulcers were measured using a ruler. The ulcer score was calculated based on the severity of the gastric ulcer lesion as described [Table 1].[21]

The following formula was used to calculate the ulcer index (UI) and percentage of curative ratio.

UI = Total ulcer score/number of animals ulcerated.

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

Measurement of gastric juice volume, pH, and total acidity

Immediately after isolation of the stomach, the pyloric and cardiac end of the stomach was tied with a thread to secure the gastric content. Gastric content was collected into a centrifuge tube by making a small incision

Table 1: Scoring of gastric lesions

Ulcer score	Gastric lesions
0	No lesion
1	Mucosal edema 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

in the greater curvature. 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 0.01N sodium hydroxide using phenolphthalein as an indicator, and the results were expressed as mEq of acid/Liter of gastric content/100 g of body weight (mEq/L/100 g).[14,22]

Estimation of Inflammatory cytokines

Gastric tissues (50 mg) were washed with phosphate buffer (pH 7.4) and cut into appropriate size using scissor. Tissues were homogenized in 500 µL of radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors using small tissue homogenizer. The homogenate was centrifuged at 16,000 × *g* for 10 min at 4°C and supernatants were used for estimation of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) using commercial ELISA kits (BioLegend Inc, USA). The kit protocol was followed for the assay, and the results were expressed as pg of TNF-α or IL-6/g tissue.

Estimation of glutathione

Gastric tissue (100 mg) was washed with phosphate buffer solution (pH 7.4) and homogenized in ice-cold 5% metaphosphoric acid (1 ml/100 mg tissue using a small tissue homogenizer). The homogenate was centrifuged at 10,000 × *g* for 15 min at 4°C. The supernatants were used to determine glutathione (GSH) level. The assay was performed as per the protocol described in the commercial GSH assay kit (Cell Biolabs Inc., San Diego, CA, USA). The value was expressed as µM of GSH/g tissue.

Estimation of malondialdehyde

Gastric tissues (25 mg) were homogenized in 250 µL of RIPA buffer containing protease inhibitors using tissue homogenizer. The homogenate was centrifuged at 16,000 × *g* for 10 min at 4°C. MDA level was measured using TBARS (TCA Method) assay kit (Cayman Chemical Company, Ann Arbor, MI, USA). The values were expressed as µM of malondialdehyde/g tissue.

Rapid urease test

A small area of gastric mucosa (approx. 2 mm^2) from the antral region was used to detect the presence of *H. pylori* using the campylobacter-like organism (CLO) test kits (Kimberly-Clark, USA). The yellow urea gel turned into a bright magenta color due the production of ammonia (alkaline reaction) by *H. pylori*.[23]

Histopathological evaluation

Histopathological examination of the stomach fragments (stored in 10% buffered formalin) was carried out in two staining techniques using hematoxylin and eosin (H and E) and modified Giemsa stains. Modified Giemsa stain was used to detect *H. pylori* and pathogen load in the gastric mucosa. Histopathological changes of the stomach were observed and interpreted under microscope (80i, Nikon, USA) using H and E stained slides. All the observation was conducted by a pathologist who was single blinded to the treatment based on the criteria.[22] Histopathological changes was evaluated by a scoring and they are (1) epithelial cell loss (Score: 0–3); (2) edema in the submucosa (Score: 0–4); (3) hemorrhagic damage (Score: 0–4), and (4) presence of inflammatory cells (Score: 0–3). The maximum total score was estimated up to 14.

Statistical analysis

The data are expressed as mean ± standard error mean (SEM). One-way analysis of variance followed by *post hoc* Tukey–Kramer test was performed to determine the statistical difference between groups. Histopathological data were analyzed using Kruskal–Wallis test followed by *post hoc* Mann–Whitney U-test. Z-test was applied to analyze the CLO test results. Statistical significance is obtained at $P < 0.05$. All the analyses were conducted using IBM SPSS V16.0 software, New York, USA.

RESULTS

Gross evaluation

Ulcer controls showed a large ulcer with hemorrhage and necrosis [Figure 1]. Geraniol alone or combined with clarithromycin significantly reduced the ulcer area as compared to ulcer control group; inflammation and hemorrhagic streaks on the mucosal surface were reduced by both treatments [Figure 1] and the UI was significantly reduced [Table 2], with curative ratios of 36% and 45% for geraniol and the combination, respectively. However, there were no significant differences between the effects of treatment geraniol alone or with the combination.

Measurement of gastric juice volume, pH, and total acidity

Gastric juice volume was significantly ($P < 0.01$) increased in the ulcer control group compared with the normal group [Table 2]. Treatment with geraniol alone produced a significant reduction in the gastric juice volume ($P < 0.05$), although there was no significant reduction in the group treated with geraniol in combination with clarithromycin. The pH of gastric juice in the ulcer control group was significantly ($P < 0.05$) reduced in comparison to the normal control [Table 2]. Treatment with either geraniol alone or with the combination significantly increased the pH compared with ulcer control group. Total acidity in the geraniol-treated and combination-treated rats were significantly ($P < 0.01$ and $P < 0.05$, respectively) reduced when compared with the ulcer control. However, there was no significant difference in pH, gastric juice

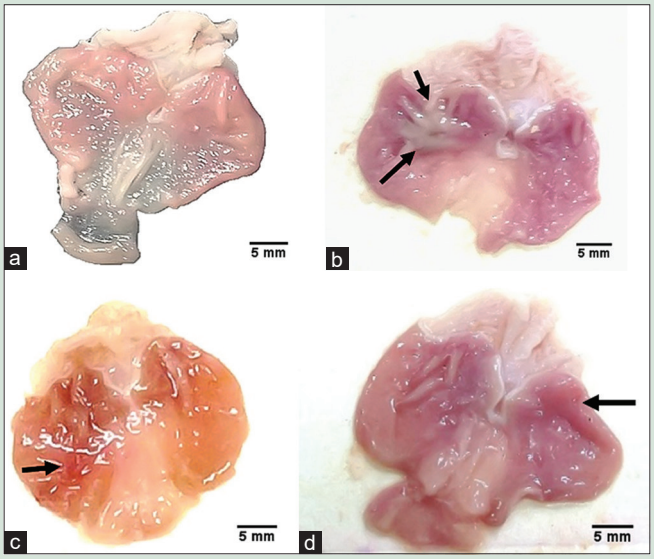

Figure 1: Gross morphological observation of stomach in *Helicobacter pylori* and acetic acid-induced ulcer in rats treated with geraniol alone or in combination with clarithromycin (×1.3). (a) Normal control group having intact stomach with normal morphology of the stomach. (b) Ulcer control group showing extensive damage to gastric mucosa with sever ulceration (black arrow) condition. (c) Geraniol (30 mg/kg, b.i.d.)-treated rats showed mild injuries and ulcer lesions without hemorrhage (black arrow). (d) Combination drugs (geraniol, 30 mg/kg and clarithromycin, 25 mg/kg, b.i.d.) treated rats showed mild injuries without hemorrhage (black arrow)

volume, and total acidity between the geraniol-treated group and the group receiving geraniol in combination with clarithromycin [Table 2].

Estimation of inflammatory cytokines

The content of IL-6 and TNF-α in the gastric mucosa was significantly ($P < 0.05$) elevated in the ulcer control group in comparison to the normal control [Figure 2]. Treatment with geraniol or with the combination significantly reduced the gastric mucosal IL-6 and TNF-α content compared with the ulcer control [Figure 2]. However, there was no significant difference between the geraniol and combination treatment groups.

Estimation of glutathione and malondialdehyde levels

The GSH content in gastric mucosal samples of the ulcer control group was significantly ($P < 0.05$) reduced compared to normal rats. Treatment with either geraniol alone or with the combination significantly ($P < 0.05$) augmented the GSH content of the gastric tissue. However, there was no significant difference in the GSH content between the group treated with geraniol alone and that treated with a combination of geraniol and clarithromycin.

There was a significant elevation in the tissue MDA content in the ulcer control group compared with the normal control [Figure 3]. Treatment with geraniol alone or in combination with clarithromycin significantly ($P < 0.05$) reduced the MDA content, with a significantly more marked reduction in the group receiving the combination [Figure 3].

Rapid urease test

The ulcer control group was uniformly positive for *H. pylori* according to the rapid urease test (RUT) [Table 3]. Treatment with either geraniol or the combination drugs' treatment produced a significant reduction ($P < 0.05$) in *H. pylori*-positive antral samples. However, there was no significant difference between geraniol alone and in combination with clarithromycin.

Table 2: Gastric juice volume, pH and total acidity in acetic acid and *Helicobacter pylori*-induced ulcerated rats treated with geraniol alone or in combination with clarithromycin

Groups	Gastric juice volume (mL)	pH	Total acidity (mEq/L/100 g)	UI
NC	1.63±0.46	4.10±0.33	105±12.17	0.00
UC	5.21±0.75$^{\$\$}$	2.87±0.33$^{\$}$	227.14±25.99$^{\$}$	3.64±0.39$^{\$\$}$
Ger_30 mg/kg	1.89±0.56**	3.52±0.32*	87.86±12.02**	2.33±0.37*
Combination drugs	4.00±0.84	3.48±0.11*	101.17±15.67*	2.00±0.52*

Data are expressed as mean±SEM; n=6 per group; $^{\$}P<0.05$ versus normal control; $^{\$\$}P<0.01$ versus normal control; $^{*}P<0.05$ versus ulcer control; $^{**}P<0.01$ versus ulcer control. NC: Normal control; UC: Ulcer control; Ger_30 mg/kg: Geraniol (30 mg/kg, b.i.d); Combination drugs: Geraniol (30 mg/kg, b.i.d) and clarithromycin (25 mg/kg, b.i.d); UI: Ulcer index; b.i.d: bis in die (twice a day); SEM: Standard error mean

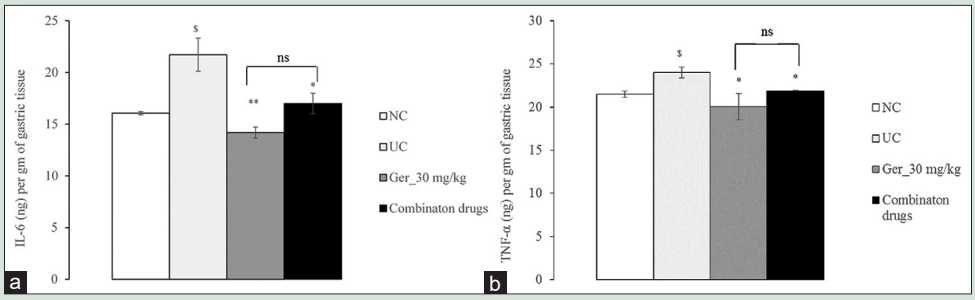

Figure 2: Inflammatory markers (IL-6 and TNF-α) in gastric tissue of *Helicobacter pylori* and acetic acid-induced ulcerated rats treated with geraniol alone or in combination with clarithromycin. (a) IL-6 levels in gastric mucosa. (b) TNF-α levels in gastric mucosa. $^{\$}P < 0.05$ versus normal control; $^{*}P < 0.05$ versus ulcer control; ns: Not significant. Ger_30 mg/kg: Geraniol (30 mg/kg, b.i.d.); Combination drugs: geraniol (30 mg/kg) and clarithromycin (25 mg/kg). NC: Normal control; UC: Ulcer control; IL-6: Interleukin-6; TNF-α: Tumor necrosis factor-α

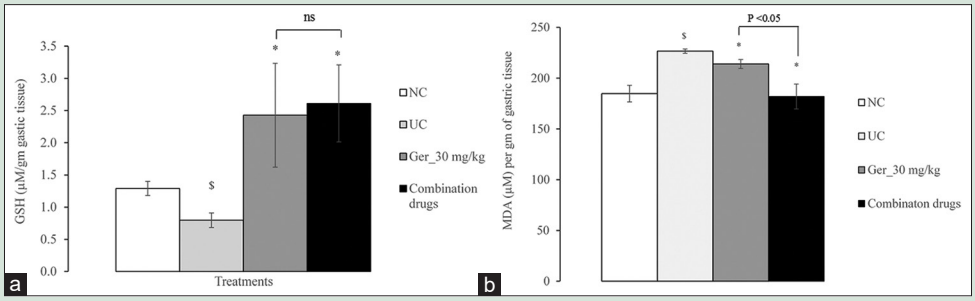

Figure 3: GSH and MDA levels in gastric tissue of *Helicobacter pylori* and acetic acid-ulcerated rats treated with geraniol alone or in combination with clarithromycin. (a) GSH levels in gastric mucosa. (b) MDA levels in gastric mucosa. $^{\$}P < 0.05$ versus normal control; $^{*}P < 0.05$ versus ulcer control; ns: Not significant. Ger_30 mg/kg: Geraniol (30 mg/kg, b.i.d.); Combination Drugs: Geraniol (30 mg/kg) and clarithromycin (25 mg/kg). NC: Normal control; UC: Ulcer control; GSH: Glutathione; MDA: Malondialdehyde

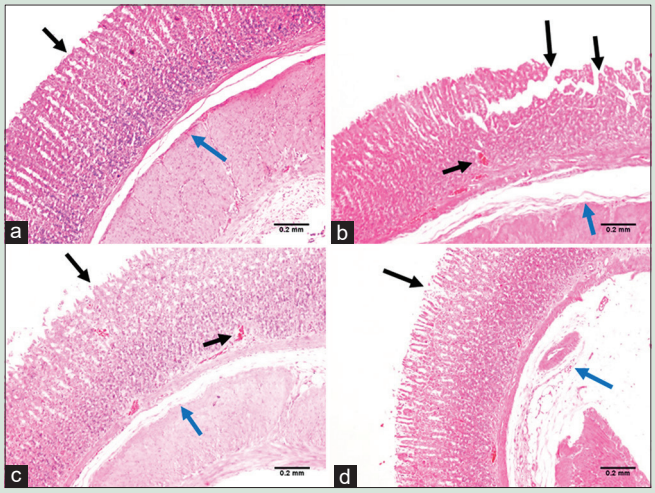

Figure 4: Histological evaluation of gastric tissues using H and E, (×100). (a) Normal control group having intact stomach with normal morphology of the stomach. Black arrow indicates a normal morphology of the stomach with normal mucosal epithelial cells. Blue arrow indicates the normal sub-mucosal and muscular layer of the stomach, (b) ulcer control group showed extensive mucosal damage and ulcerated lesions with hemorrhage; black arrow indicates the extensive damage and loss of mucosal epithelial layer and hemorrhagic lesions. Blue arrow indicates the damage of submucosal layer, (c) Geraniol (30 mg/kg, b.i.d.)-treated rats showed mild injuries with less hemorrhage. Black arrow indicates a mild epithelial cell loss, edema, and neutrophil infiltration in mucosal layer. Blue arrow indicates the normal submucosal layer, (d) Combination drugs: Geraniol (30 mg/kg, b.i.d.)-treated rats and clarithromycin (25 mg/kg, b.i.d)-treated rats showed mild injuries with less hemorrhagic lesions. Black arrow indicates epithelial cell loss. Blue arrow showed the normal submucosal layer

Table 3: Rapid urease test using *Campylobacter*-like organism-test kit

Groups	*H. pylori* positive	*H. pylori* negative	Percentage of inhibition
UC	6 (6)	0 (6)	0
Ger_30 mg/kg	3 (6)	3 (6)	50*
Combination Drugs	3 (6)	3 (6)	50*

Value in parenthesis indicates the total number of rats. Percentage inhibition in CLO test was statistically compared using *Z*-test. *P<0.05 versus UC. UC: Ulcer control; Ger_30 mg/kg: Geraniol (30 mg/kg, b.i.d); Combination drugs: Geraniol (30 mg/kg, b.i.d) and clarithromycin (25 mg/kg, b.i.d); *H. pylori*: *Helicobacter pylori*; b.i.d: bis in die (twice a day); CLO: Campylobacter-like organism

Histopathological evaluation

Histological observation of the ulcer control groups showed extensive lesions in the gastric mucosa, edema in the submucosal layer, and leukocyte infiltration along with hemorrhage [Figure 4]. The scores for both treatment groups were significantly reduced in comparison to the ulcer control [Table 4]. Treatment with either geraniol alone or in combination with clarithromycin produced significant protection, as evident by the presence of less inflammation, hemorrhagic damage, edema, leukocyte infiltration, and epithelial cell loss [Figure 4]. However, there was no difference between the scores for the group receiving geraniol alone and for the group treated with the combination [Table 4].

Modified Giemsa staining confirmed the presence of abundant *H. pylori* cells in the layer between the gastric mucosa and the mucus of the ulcer control rats. Both geraniol and its combination with clarithromycin showed a decrease in the *H. pylori* load in the gastric mucosa in comparison to the ulcer control [Figure 5].

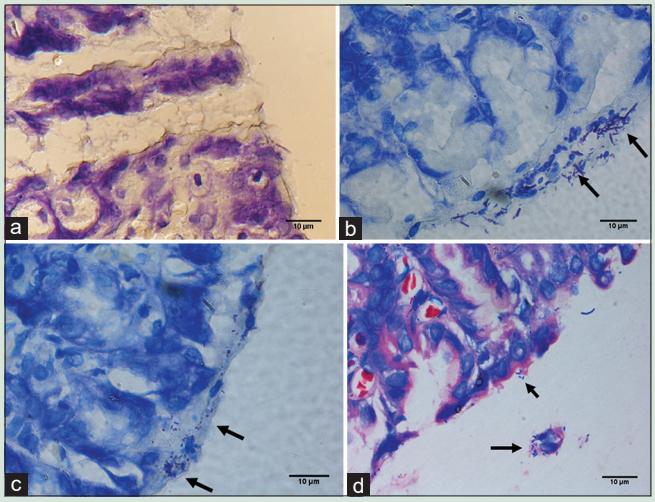

Figure 5: Histological evaluation of gastric tissues for *H. pylori* using Giemsa staining (×1000). (a) Normal control group without *H. pylori* infection. It shows normal architecture of gastric mucosal cells, (b) Ulcer control group with *H. pylori*. Black arrow showed extensive accumulation of *H. pylori* cells in between gastric mucosa and mucus layer, (c) Geraniol (30 mg/kg, b.i.d.)-treated rats. Black arrow showed presence of moderate number of *H. pylori* cells in-between gastric mucosa and mucus layer, (d) Geraniol (30 mg/kg, b.i.d.)-treated and clarithromycin (25 mg/kg, b.i.d)-treated rats. Black arrow showed the presence of mild to moderate number of *H. pylori* cells in-between gastric mucosa and mucus layer. *H. pylori*: *Helicobacter pylori*

DISCUSSION

In the present study, gastric ulcers were induced by administration of acetic acid into the subserosal layer of the stomach followed by introduction of *H. pylori* intragastrically. Acetic acid-induced ulcers heal spontaneously but the introduction of *H. pylori* leads to a significant delay in ulcer healing and development of chronic ulcers.[24] Inflammation, increased apoptosis, overexpression of inflammatory markers, and reduced gastric microcirculation are responsible for delayed ulcer healing.[24] This experimental animal model is well validated,[25] and the basic characteristics of the ulcers are quite similar to the human peptic ulcers in relation to their pathological characteristics and the healing process.[26] We found geraniol to produce a clear and significant reduction in ulceration, as evidenced by the reduction in UI and the improvement in the gross morphology of the mucosa, including reduced leukocyte infiltration and the absence of edema and epithelial cell loss. *H. pylori* infection stimulates gastric acid secretion due to the defect in autoregulation of gastrin release, motor or sensory function of the stomach, and delay in the stomach emptying.[27,28] The results in Table 2 suggest that the pH of geraniol alone is 3.52 and combination is 3.48. The difference is insignificant. However, the pH is significantly increased as compared to ulcer control. Gastric juice volume does not represent completely the gastric acid volume. It also contains mucus which is protective. In geraniol alone group, the gastric juice volume is reduced significantly and the pH is also significantly increased. However, the combination treated group gastric juice volume did not change significantly as compared to ulcer control but pH is increased significantly. This suggests that clarithromycin have effect on mucus secretion, and thus, the pH is not changed but gastric volume is increased due to mucus volume. This is further supported by Gutiérrez-Cabano and Raynald.[29] We showed geraniol to significantly increase the pH and to lower the total acidity and gastric volume. All of these findings support our previous observations.[20]

Table 4: Histological score of rat stomach pretreated with geraniol alone or in combination with clarithromycin in acetic acid and *Helicobacter pylori*-induced ulcer

Groups	Hemorrhagic damage (score 0-4)	Edema (score 0-4)	Epithelial cell loss (score 0-3)	Inflammatory cells (score 0-3)	Total (score 14)
NC	0	0	0	0	0
UC	3$	1$	2.5$	2$	8.5±1.08$
Ger_30 mg/kg	1.5*	0*	1.5*	1*	4.0±0.47*
Combination drugs	1*	0*	1*	1*	3.0±0.67**

Data are expressed as mean±SEM; *n*=6 per group; Histopathological scores were analyzed using nonparametric statistics using Kruskal-Wallis test followed by Mann-Whitney U-test as *post hoc* analysis between two groups. $*P*<0.05 versus normal control; **P*<0.05 versus ulcer control; ***P*<0.01 versus ulcer control. NC: Normal control; UC: Ulcer control; Ger_30 mg/kg: Geraniol (30 mg/kg, b.i.d.); Combination drugs: Geraniol (30 mg/kg, b.i.d) and clarithromycin (25 mg/kg, b.i.d); SEM: Standard error mean; b.i.d: bis in die (twice a day)

RUT is a useful tool for identifying the urease-producing bacteria and shows 100% sensitivity and 89.5% specificity for *H. pylori*, although false positives can arise due to interference of enterobacteriaceae including *Proteus*, *Klebsiella*, and *Yersinia* species.[30] Therefore, it is important to corroborate the RUT using histopathological analysis,[31,32] which was undertaken in the present study. The observation in RUT and histology (Modified Giemsa stain) indicated a significant reduction in the gastric mucosal *H. pylori* burden which could have led to its antiulcer effect. The ability of geraniol to reduce the gastric mucosal *H. pylori* burden supports our previous study.[20] In RUT, both geraniol and its clarithromycin combination showed 50% reduction in *H. pylori* infection. Geraniol and clarithromycin combination did not demonstrate a considerable synergistic effect against *H. pylori* in an *in vivo* model. The failure in synergistic effect of geraniol and clarithromycin could be due to multiple reasons including pharmacokinetic and pharmacodynamic properties of geraniol and clarithromycin.

While the antiulcer effect of geraniol may be in part due to its anti-*H-pylori* effect, involvement of other mechanisms relates to its antibacterial, anti-inflammatory, and antioxidant effect cannot be ruled out. *H. pylori* infection stimulates overexpression of pro-inflammatory cytokines (e.g., IL-6, IL-8, IL-1 β, and TNF-α) in gastric epithelial cells, which act as neutrophil-activating chemokines and lead to leukocyte infiltration.[33] Drugs that attenuate the release of pro-inflammatory cytokines, neutrophil adhesion/infiltration, and inflammation have shown significant effects against *H. pylori*-infected gastric cell damage.[34,35] In the present study, geraniol alone or in combination with clarithromycin significantly reduced gastric mucosal IL-6 and TNF-α levels which could have due to the anti-*H. pylori* action or to an independent anti-inflammatory effect.

Several studies have shown geraniol to have antioxidant properties both *in vitro*[36,37] and *in vivo*.[14,38] Cellular oxidative stress in the stomach results from the excessive accumulation of reactive oxygen species (ROS). These ROS causes gastric tissue injury by damaging membranes and cellular components such as DNA, proteins, and lipids.[39,40] *H. pylori* infection in gastric mucosa encourages leukocyte infiltration, leading to generation of ROS and cellular damage.[41] Thus, drugs having potent antioxidant activities could be beneficial in attenuating the progression of gastric ulcer as well as for its treatment. GSH is an essential endogenous-free radical scavenger that protects cellular damage whereas MDA is the end product of lipid peroxidation and marker of cell membrane damage. Low GSH level in the gastric mucosa facilitates the gastric tissue damage by mediating lipid peroxidation.[42] Thus, GSH and MDA are well correlated in gastric tissue. We showed that the reduced level of GSH and the elevated level of MDA observed in the ulcer control group were essentially normalized by treatment with geraniol alone or in combination with clarithromycin; this may indicate a contribution from the antioxidant effect of geraniol, although these effects may simply reflect the reduced damage produced by other mechanisms. Antioxidative therapy has been shown to stimulate the healing of gastric and duodenal ulcers resistant to existing therapy.[43] Although we did not find any significant difference between geraniol alone and combination group in inflammatory cytokines and GSH level of gastric mucosa, we observed a significant change in gastric mucosal MDA concentration in the combination treated group as compared to geraniol alone. Malondialdehyde has a significant positive relationship with the presence of *H. pylori* in gastric tissues of infected patients.[44] The effect of clarithromycin and geraniol combination on reduction of MDA is significant that implies the reduction of bacterial load in gastric mucosa. We speculate that the combination would have a better effect on reduction in bacterial load in the gastric tissue, which remains a curiosity for further investigation.

CONCLUSION

In the present study, the putative synergistic effect of geraniol and clarithromycin combination was tested by comparing with the geraniol group. However, a significant difference between these two groups was not observed. There is a minimal effect of geraniol on clarithromycin activity or *vice versa* can be anticipated from this observation. Although our previous *in vitro* experiments demonstrated a partial synergistic effect between geraniol and clarithromycin against *H. pylori,* the present study showed that the addition of clarithromycin conferred no protection against ulceration additional to that seen with geraniol alone. The failure to demonstrate synergism between geraniol and clarithromycin in the present study may have a number of explanations. Suppression of acid secretion by geraniol may have shifted *H. pylori* from the antrum to the whole body of the stomach[45] leading to decreased eradication of *H. pylori*, thus preventing any beneficial effect of combination therapy. Further, one of the limitations in this study is that the viable bacterial cell count in gastric tissue was not performed. This test is a better parameter to investigate the viable bacterial load compared to bacterial eradication. In this study, geraniol and clarithromycin combination did not have a significant difference in *H. pylori* eradication, but it may have a better effect in reducing the bacterial load in gastric tissue. Hence, further investigation on the mechanism of antibacterial action against *H. pylori* and its effect with other antibiotics in *H. pylori*-induced gastric ulcers could be of potential interest among researchers.

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Conflicts of interest

There are no conflicts of interest.

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