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Anti-ulcer activity of aqueous ethanol extract of *Rheum spiciforme* and its fractions in animal model

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Abstract

Helicobacter pylori infection, pepsin, ischemia, hypoxia, smoking, alcohol intake, hydrochloric acid, and nonsteroidal anti-inflammatory drugs are the causal factors of ulceration. In third-world countries, approximately one in two individuals has signs of gastric ulceration. Extensive literature suggested that plant-derived drugs hold the potential in the treatment of peptic ulcer, and *Rheum spiciforme* (family: *Polygonaceae*) has been known in the folk medicine for possessing medicinal effect on ulceration. To explore further along this line, the current study was designed to investigate the effects of aqueous ethanol extract of *R. spiciforme* roots and its fractions on peptic ulcer and to identify bioactive compounds responsible for mitigating this pathological condition. Animal model of ethanol-induced gastric ulcer was utilized in this study. The animals were pre-treated with crude plant extract, given at doses 125, 250, and 500 mg/kg, and then orally administered with butanol, aqueous, and dichloromethane fractions at doses of 250 mg/kg for 7 days. Following the treatments, a significant decrease in ulcerative lesions, ulcer index, ulcer severity score, volume, free acidity, and total acidity of gastric juice as well as a marked increase of gastric pH in the groups treated with plant extract at doses of 250 and 500 mg/kg were noted. Moreover, butanol fraction has been shown to produce effects equipotent to that of the reference drug omeprazole. Liquid chromatography-mass spectrometry analyses show that the plant extract contains emodin, aloe-emodin, and quercetin. In conclusion, this study demonstrated that the aqueous ethanol extract of *R. spiciforme* and its butanol fraction exhibited gastro-protective effect.

Keywords: *Rheum spiciforme*; Protective role; Ulcer; Ethanol; Fractions

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1. Introduction

A peptic ulcer is a common pathological condition of the gastrointestinal tract with more a high prevalence in Asian countries[1]. It is defined as a lesion of mucosa that infiltrates into the muscularis mucosae layer of the stomach or duodenal bulb, leaving behind a cavity accompanied by acute and chronic inflammation[2]. Peptic ulcer develops when the equilibrium between the "offensive" and "defensive" factors that govern the integrity of the luminal surface of the epithelial cells is disrupted. The offensive factors include *Helicobacter pylori* infection, pepsin, bile acids, ischemia, smoking, hypoxia, alcohol intake, hydrochloric acid, and nonsteroidal anti-inflammatory drugs, while the mucus

layer, growth factors, bicarbonate, mucosal blood flow, and prostaglandins constitute the line of defense against the ulceration[3].

Peptic ulcer commonly develops along the gastrointestinal tract, especially the stomach and duodenum, where gastric acid and pepsin are secreted. Commonly observed symptoms of peptic ulcer include bloating, burning, pressure or feeling of fullness, occasional bloody or dark stools, pain in the chest, fatigue, vomiting, changes in appetite, indigestion, and weight loss[1]. Numerous drugs, including proton pump inhibitors and H2 receptor antagonists, are included in the treatment regimen of peptic ulcer[4]. Nevertheless, these drugs lead to the occurrence of adverse effects and relapses and interact with other drugs. As an effort to reduce adverse effects stemming from these drugs and prevent relapses, the relentless search for potent herbal medication is still relevant and crucial[5].

Rheum spiciforme, mostly found in the Himalayan region of Kashmir, belongs to family Polygonaceae[6], which comprises 60 species, among which seven species are found in the Indian subcontinent and the Himalayan region of Kashmir. It is a wild plant capable of surviving in harsh environments [7]. The petioles, stems, and leaves of *R. spiciforme* are traditionally included in the meals of a few Tibetan communities[8], while the roots are applied in the treatments of bone fractures, backache, joint pain, joint swelling, rheumatic pain, stomachache, intestinal infections, and dysentery[7]. In light of the potential medicinal values mentioned above, the current study aimed to investigate the anti-ulcer activity of *R. spiciforme*.

2. Materials and methods

2.1. Chemicals and drugs

The list of chemicals and drugs used in his study is as follows: diethyl ether (Riedel-de Haen), sodium hydroxide (Merck®, Germany), 10% formalin, Topfer Reagent, paraffin wax, phenolphthalein, hemotoxylin, eosin, 0.1 N sodium hydroxide, analytical-grade absolute ethanol (Merck®, Germany), n-butanol, dichloromethane (Riedel-de Haen), normal saline, distilled water, hydrochloric acid, Mayer's reagent, Wagner's reagent, sodium chloride, ethanol, and omeprazole (Sigma, Germany).

2.2. Animals

Healthy adult albino rats (6–8 weeks old) of either sex were obtained from the Animal House, College of Pharmacy, University of Sargodha, Sargodha. The rats weighed between 150 and 180 g. Standard rat pellet diet and tap water were given to the animals, which were kept on a standard light-dark cycle (12 h with light on and 12 h in the dark) at room temperature[9]. All animals were treated in accordance with the standard ethical guidelines set forth by the National Research Council[10].

2.3. Collection and authentication of medicinal plant

The identification of *R. spiciforme* based on the ethnobotanical literature[11]. The roots of *R. spiciforme* were acquired from a local market during the full bloom season of the plant, *i.e.*, in July. The identification and authentication of the plant was performed by assistant professor Dr. Shair Wali Khan from Karakoram International University, Gilgit-Baltistan. After cleaning, the dried roots of *R. spiciforme* were powdered using an electric blender.

2.4. Preparation of crude extract and its fractions

Aqueous ethanol extract of *R. spiciforme* was prepared through maceration. For the extraction purpose, the dry powder was immersed in aqueous ethanol (30:70; analytical grade, Merck®, Germany) for 48 h with regular shaking[12]. The mixture obtained was filtered after 3 days using a muslin cloth and grade 1 Whatman filter paper. The filtrate was then passed through a rotary evaporator and afterward, it was dried at room temperature.

Activity-guided fractionation of *R. spiciforme* was performed using three different organic solvents in increasing polarity so that the relative active fraction could be obtained[13]. Next, 100 g of the active fraction was mixed in 250 mL of distilled water to produce aqueous (48%) fraction. Separately, two solvents – dichloromethane and butanol – were added in increasing polarity, eventually generating concentrated dichloromethane (20%) and butanol (25%) fractions, respectively. After drying, these fractions were then applied in the anti-ulcer activity evaluation.

2.5. Evaluation of gastro-protective effect of crude extract on rats with ethanol-induced ulcer

Rats were divided into five groups and each group consisted of six animals (n = 6). Group I was the control group that received a vehicle once per day for 7 days and then was induced for ulcer development on day 7. Group II was the reference group given 20 mg/kg omeprazole (p.o). Groups III, IV, and V were given aqueous ethanol extract of *R. spiciforme* at doses of 125, 250, and 500 mg/kg body weight once per day for 7 days, respectively[14]. Gastric ulcer was induced by administering ethanol (5 mL/kg) to rats after 1-h pre-treatment with crude extract and omeprazole on day 7[15].

2.6. Evaluation of gastro-protective effect of plant extract fractions on rats with ethanol-induced ulcer

In this experiment, the animals were divided randomly into five groups, with each group containing six rats (n = 6).

Group I was the control group that was given a vehicle once per day for 7 days. Group II was the reference group administered with 20 mg/kg omeprazole once per day for 7 days. Groups III, IV, and V were treated with butanol, aqueous, and dichloromethane fractions of plant extract at 250 mg/kg once per day for7 days. Then, gastric ulcer was induced in adherence to protocols briefly mentioned in section 2.5[15].

2.7. Macroscopic evaluation of ulcer

2.7.1. Estimation of ulcer severity

Stomach of each rat was cut open with an incision on the greater curvature. After the gastric contents were removed, gastric lesion on the stomach was examined with the naked eyes. The length of the ulcer was measured using Vernier Calliper, and the ulcer severity was estimated by means of scoring[16].

2.7.2. Estimation of ulcer index

The ulcer index is defined as mean ulcer severity score for each animal. The ulcer index was calculated using the following formula[17]:

$$UI = UN+US+UP\times10-1 \quad (I)$$

where UI = ulcer index, UN = average number of ulcer per animal, US= average severity score, and UP = percentage of animal with ulcer

2.7.3. Percentage inhibition of ulcer

Percentage inhibition of ulcer is also known as percentage protection. Percentage inhibition of ulceration was calculated using the following formula[18]:

$$\text{Inhibition of ulceration} = \frac{(UI_{Ctrl} - UI_{Test})\times100}{UI_{Ctrl}} \quad (II)$$

where UI_{Ctrl} = Ulcer index of control group, and UI_{Test} = ulcer index of test group.

2.7.4. Evaluation of pH

Before pH measurement, 1 mL gastric juice was diluted with 1 mL of distilled water, and then, pH meter was used to measure the pH of the solution[19].

2.7.5. Determination of free and total acidity

One milliliter of gastric juice solution, diluted in a 1:1 ratio with distilled water, was poured into a conical flask. A few drops of a phenolphthalein indicator were added to the solution. Titration was performed with 0.01 N NaOH until a sustained pink coloration appeared. The volume of 0.01 N NaOH used was noted. Topfer reagent was used to determine the free acidity. Titration of gastric juice with 0.01 N NaOH was performed until a yellow coloration appeared. The volume of 0.01N NaOH used was noted. The total and free acidity was calculated using the following formula[17].

$$\text{Acidity} = \frac{V_{NaOH}\times N\times100 \text{ mEq/L}}{0.1} \quad (III)$$

where V_{NaOH} = volume of NaOH solution used, and N = normality.

2.7.6. Determination of gastric juice volume

Gastric juice was centrifuged at 500 rpm for 5 min, and then, the supernatant was separated and its volume was measured using a graduated cylinder[20].

2.8. Liquid chromatography-mass spectrometry (LC-MS) analysis of bioactive fractions

In the present study, active constituents in most active fractions (butanol fractions) were identified by means of LC-MS using methanol (100%) as the mobile phase. A thermo electron (LTQ-Orbitrap XL mass spectrometer) equipped with a nanoelectrospray ion source (Thermo Fisher Scientific, Bremen, Germany) was utilized in positive and negative ionization modes as per requirements.

The separated target compounds were detected in direct injection mode. The collision-induced dissociation (CID) was based on the parent ion fragmentation efficiency. The capillary temperature was set at 280°C and the sample flow at 8 μL/min[21].

2.9. Statistical analysis

Data were analyzed using GraphPad Prism, and descriptive statistics were used to describe the results. The results are expressed as mean ± standard error of mean. One-way analysis of variance (ANOVA) followed by Dunnett and Bonferroni's *post hoc* test was carried out. A P-value <0.05 was considered statistically significant.

3. Results

3.1. Gastro-protective effect of *R. spiciforme* on gastric mucosal injury in ethanol-induced ulcer rat model

The extract at a dose of 125 mg/kg resulted in a non-significant decrease (6.50 ± 0.95) in number of lesions in comparison with the control group (7.83 ± 0.47), whereas a remarkable reduction in the number of lesions in mucosa was observed when the 250 and 500 mg/kg doses were used (2.50 ± 0.4 and 1.66 ± 0.4, respectively). The 125, 250, and 500 mg/kg doses of the plant extract considerably reduced the ulcer severity score (22.67 ± 7.3, 7.16 ± 1.70, and 6.83 ± 1.77) compared to the control group (32.33 ± 2.81).

In addition, the ulcer index was decreased (12.88, 10.97, and 9.18) at doses of 125, 250, and 500 mg/kg; notably, the effect of the 500 mg/kg dose on ulcer index was more pronounced with respect to the other two doses. The gastroprotective effect of the crude plant extract was also measured in terms of percentage inhibition of ulcer; it was found that the extract at the dose of presented 125 mg/kg dose engendered a gastric damage inhibition of 8.13%, while higher doses at 250 mg/kg and 500 mg/kg inhibited the damage by 21.75% and 34.52% (Table 1).

3.2. Gastro-protective effect of crude plant extract on pH, volume, and acidity of gastric juice

R. spiciforme crude extract at 250 and 500 mg/kg doses markedly increased the pH of gastric juice ($P < 0.05$ and $P < 0.001$, respectively) in comparison with control group, while increase in pH was observed following treatment with 125 mg/kg of the extract was non-significant as compared to the control group (Figure 1). The 250 and 500 mg/kg doses of the crude extract reduced the volume of gastric juice significantly ($P < 0.01$), whereas the 125 mg/kg dose only mildly reduced the gastric juice volume (4.46 ± 0.264 mL) in comparison with the control group (4.61 ± 0.47 mL). The 250 and 500 mg/kg doses of aqueous ethanol extract significantly reduced the free acidity ($P < 0.05$ and $P < 0.001$, respectively) as compared to the control group, but the 125 mg/kg dose led to only non-significant decrease in the acidity of gastric juice. On the other hand, total acidity was significantly reduced by 250 and 500 mg/kg doses ($P < 0.05$ and $P < 0.001$, respectively), as shown in Figure 1.

3.3. Effect of *R. spiciforme* root extract's fractions on gastric mucosal injury in rats with ethanol-induced ulcer

The 250 mg/kg dose was used to prepare the butanol, aqueous, and dichloromethane fractions of plant extract. The butanol fraction caused a marked reduction in lesion formation (1.66 ± 0.42) in comparison to the control group (7.16 ± 0.30), as presented in Table 2. Both aqueous and dichloromethane fractions were found to mildly reduce number of lesions (5.50 ± 2.46 and 5.33 ± 0.88, respectively) in comparison with the control group. Butanol fraction of the extract also decreased the ulcer severity score markedly down to 5.66 ± 1.49, while aqueous and dichloromethane fractions reduced the ulcer severity score non-significantly (25.33 ± 7.49 and 23.83 ± 3.21, respectively) as compared with control group (31.50 ± 3.56). The 250 mg/kg dose of butanol fraction reduced the ulcer index to 9.063 ± 0.18, producing an effect resembling that of the reference drug (9.32 ± 0.31). Aqueous fraction significantly lowered the ulcer index (11.41 ± 0.9492) while dichloromethane fraction non-significantly reduced the ulcer index (12.88 ± 0.71) as compared with the control group.

3.4. Effect of fractions of *R. spiciforme* on gastric juice parameters in rats with ethanol-induced ulcer

Both butanol and aqueous fractions significantly elevated the pH of gastric juice ($P < 0.001$ and $P < 0.05$, respectively) as compared to ulcer control, dwarfing the minute, non-significant pH elevation achieved by dichloromethane fraction (Figure 2). Regarding other gastric juice parameters, butanol fraction was singled out for its significant lowering effects on these parameters ($P < 0.001$), namely the volume of gastric juice, free, and total acidity, in comparison with control group.

3.5. LC-MS results

Figure 3 shows the LC-MS chromatogram of butanol fraction of *R. spiciforme* extract detected at negative and positive modes. The peaks of emodin and aloe emodin were obtained at 3.42 retention time with molecular weight 270.00 g/moL. The compounds – emodin and aloe-emodin – were identified based on the molecular weight in the butanol fraction of plant extract, with the aid of a standard reference graph.

4. Discussion

The ulcer usually manifests as an open sore of the skin or the mucosal membrane, characterized by sloughing

Table 1. Gastro-protective effect of *R. spiciforme* crude extract on gastric mucosal injury in rats with ethanol-induced ulcer

Treatment (mg/kg)	Number of lesions	Severity score	Ulcer index	Percentage inhibition of ulcer (%)
Control	7.833 ±0.4773	32.33±2.813	14.02±0.2522	NA
Omeprazole	2.167±0.6009***	8.083±2.758***	9.356±0.3295***	33.26
RS-CE (125 mg/kg)	6.500±0.9574^{ns}	22.67±7.311^{ns}	12.88±0.7148^{ns}	8.13
RS-CE (250 mg/kg)	2.500±0.4282***	7.167±1.701***	10.97±0.1909***	21.75
RS-CE (500 mg/kg)	1.667±0.4216***	6.833±1.778***	9.180±0.2045***	34.52

Notes: Values are expressed as means±standard error of mean (n=6); ***P<0.001, ns: Non-significant versus control group, analyzed by one-way analysis of variance (with Dunnett's test).
Abbreviation: RS-CE: *Rheum spiciforme* crude extract.

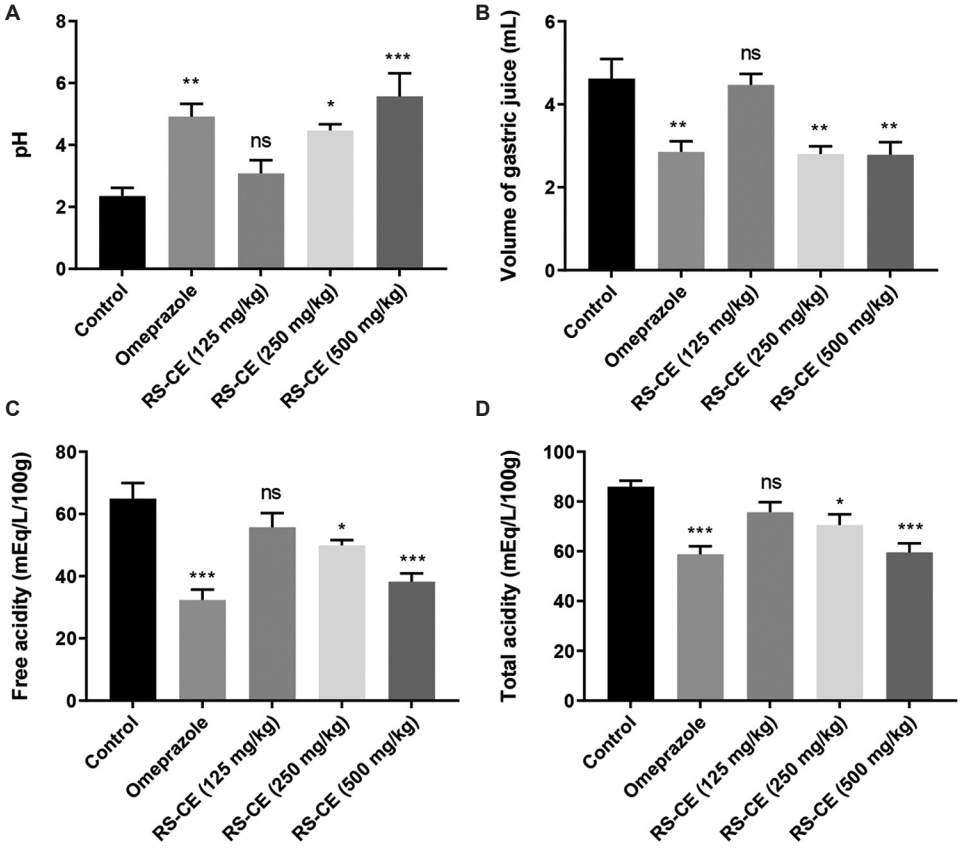


Figure 1. Effect of *Rheum spiciforme* crude root extract on gastric juice parameters: (A) pH, (B) volume of gastric juice, (C) free acidity, and (D) total acidity in rats with ethanol-induced ulcer. Values are expressed as means ± standard error of mean ($n = 6$); *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ns: Non-significant versus control group, analyzed by one-way analysis of variance (with Dunnett's test).
Abbreviations: UC: Ulcer control; RS-CE: *R. spiciforme* crude extract.

Table 2. Effect of plant root extract fractions on gastric mucosal damage in rats with ethanol-induced ulcer

Treatments (mg/kg)	Number of lesions	Ulcer severity score	Ulcer index	Percentage inhibition of ulcer (%)
Control	7.167±0.3073	31.50±3.566	13.87±0.3836	NA
Omeprazole	2.167±0.6009*	7.750±2.620**	9.322±0.3132***	32.79
RS-BF	1.667±0.4216*	5.667±1.498***	9.063±0.1856***	34.66
RS-AF	5.500±2.460^{ns}	25.33±7.491^{ns}	11.41±0.9492*	17.74
RS-DCMF	6.500±0.9574^{ns}	22.67±7.311^{ns}	12.88±0.7148^{ns}	7.14

Notes: Values are expressed as means±standard error of mean (n=6); *P<0.05, **P<0.01, ***P<0.001, ns: Non-significant versus control group analyzed by one-way analysis of variance (with Dunnett's test). Abbreviations: RS-BF: *Rheum spiciforme* butanol fraction, RS-AF: *R. spiciforme* aqueous fraction, RS-DCMF: *R. spiciforme* dichloromethane fraction.

of inflamed dead tissue. The prevalence of peptic ulcer is disproportionately higher in developing countries where it is estimated that about 70% of their population suffers from peptic or gastric ulcers; in contrast, the highest prevalence rate ever recorded from the developed countries is 40%[3].

The present study evaluated the anti-ulcer potential of *R. spiciforme* root extract and its fractions in rats with ethanol-induced ulcer. Several parameters concerning ulcer, such as number of ulcerative lesions, ulcer severity score, percentage inhibition of ulcer, ulcer index, volume, pH, free, and total acidity of gastric juice, were approximated to characterize the gastro-protective effects of *R. spiciforme*. The pH of gastric juice normally ranges between 2.9 and 3.3 but could be reduced to 2.3 following ethanol intake, which triggers ulceration due to a significant decrease in pH. Due to the enhanced production of histamine, the release of gastric juice increases, thereby causing an increase in the hydrogen ions concentration (more acidic pH), which is

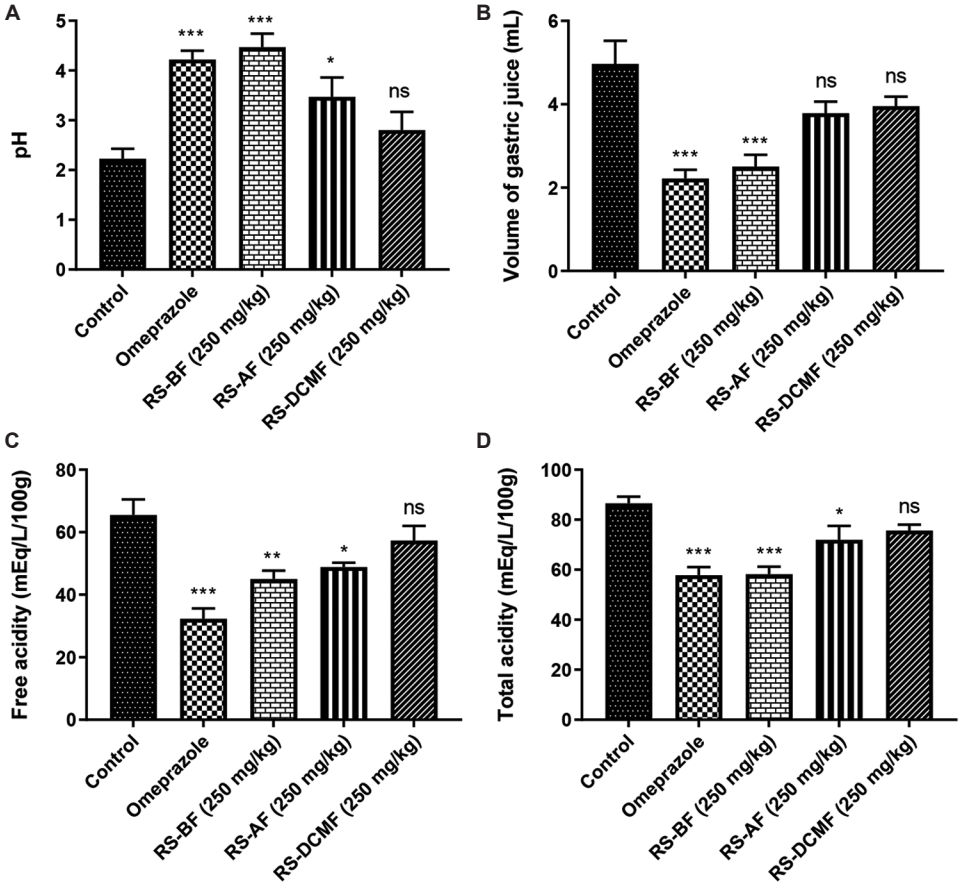


Figure 2. Effect of *Rheum spiciforme* root extract's fractions on gastric juice parameters: (A) pH, (B) volume of gastric juice, (C) free acidity, and (D) total acidity in rats with ethanol-induced ulcer. Values are expressed as means ± standard error of mean (n=6); *P<0.05, **P<0.01, ***P<0.001, ns: Non-significant versus control group analyzed by one-way analysis of variance (Dunnett's test).
Abbreviations: RS-BF: *R. spiciforme* butanol fraction, RS-AF: *R. spiciforme* aqueous fraction, RS-DCMF: *R. spiciforme* dichloromethane fraction.

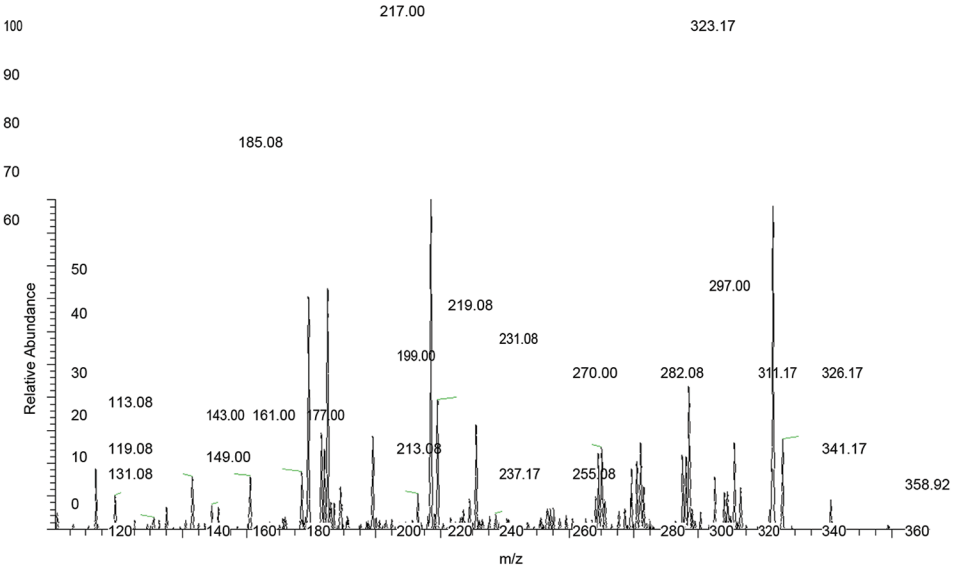


Figure 3. Liquid chromatography-mass spectrometry chromatogram at the retention time 3.42.

claimed to be an offensive factor triggering ulceration. Moreover, parameters such as the number of ulcerative lesions, ulcer severity scores, ulcer index, volume, and acidity of gastric juice were markedly increased[3]. The oral administration of aqueous ethanol extract of *R. spiciforme* at higher doses reduced the number of lesions, ulcer index, and severity score, which were otherwise aggravated by ethanol. Our results were congruent with other published work, in which Ranunculus millefoliatus ethanol extract at 250 mg/kg dose was demonstrated to exhibit anti-ulcer activity[22]. In the clinical setting, omeprazole is used as a standard anti-ulcerative medication. As a proton pump inhibitor, omeprazole prevents ulceration by inhibiting the H-K-ATPase in the stomach wall irreversibly, thereby blocking gastric acid release. This study revealed that the anti-ulcerative efficacy of *R. spiciforme* extract was comparable to that of omeprazole, suggesting that the mechanism underlying the *R. spiciforme*-mediated anti-ulceration might be similar to that of proton pump inhibitor.

As compared to control group, the extracts at 250 and 500 mg/kg doses markedly increased the pH and intuitively reduced the acidity (both free and total) of gastric juice. Likewise, the volume of gastric juice was also significantly decreased in groups of animals treated with the plant extract as compared to the control group (Figure 1). The effects produced by *R. spiciforme* mirrors the anti-secretory activity of *Aloe vera* demonstrated by Borra *et al.*[23].

In the present study, crude extract of *R. spiciforme* was further utilized for the preparation of different fractions (butanol, aqueous, and dichloromethane), which were investigated for their anti-ulcer activity in ethanol-induced ulcer model. Compared with the control group, the butanol fraction stands out to be the only specially prepared fraction capable of producing significant gastro-protective effects, evident by the significant reductions in the severity score and ulcer index ($P < 0.001$) (Table 2). These findings indicated that the butanol fraction was as effective as the reference drug, omeprazole (20 mg/kg), in the treatment of ulcer. The results of the present study, such as the increase in the pH as well as the decrease in the volume, free acidity, and total acidity of gastric juice, were consistent with the findings by Lee *et al.*[24] and Ahmed *et al.*[25]

The ethanol-induced ulcer model treated with aqueous fraction also exhibited fewer lesions and reduced severity score and ulcer index; however, these results were not statistically significant probably due to the presence of less bioactive constituents responsible for the anti-ulcer effect. This justification is inspired by the presence of less bioactive constituents present in the aqueous fraction of root bark of *Aralia elata*[24]. Similarly, the non-significant results observed with the dichloromethane fraction might be due to the absence of bioactive constituents responsible for anti-ulcer activity. A previous study has in fact shown that the *Markhamia tomentosa* leave extract in dichloromethane fraction has non-significant anti-ulcer activity[9].

The anti-ulcer effect of flavonoids, which are cytoprotective constituents, has been confirmed in previous studies[7]. The flavonoids provide defense to the gastric mucosa against damage by enhancing the prostaglandins level in the mucosal lining of the stomach[26]. The increased prostaglandin level in the mucosa of stomach and reduced histamine secretion from mast cells achieved through the blocking of histidine decarboxylase and the inhibition of *H. pylori* growth are the mechanisms behind the gastro-protective effect of flavonoids. In addition, flavonoids act as scavengers of free radicals, which play an important role in the formation of gastrointestinal tract lesions. Hence, coupled with a less toxic profile and properties, flavonoids have high medicinal value in the treatment of ulcers. Apart from that, flavonoids are therapeutically useful in the treatment of gastrointestinal diseases, especially those associated with *H. pylori* infection[27]. Thus, we postulate that the presence of flavonoids is accountable for the anti-ulcer potential of the crude extract of *R. spiciforme*. Furthermore, the LC-MS analysis of the butanol fraction, which is the most active fraction, of the crude extract revealed that the anthraquinones, flavonoids, alkaloids, and carbohydrates are the active constituents of the fraction. We also found that quercetin is the predominant type of flavonoid in the fraction. Quercetin activates the cyclooxygenase, which activates the production of local prostaglandin[28]. Earlier studies have demonstrated that emodin and aloe emodin possess anti-ulcer properties, and the flavonoids, particularly quercetin, possess antioxidant, anti-inflammatory, and anti-ulcer activities[21]. Hence, these active constituents present in the aqueous ethanol extract of *R. spiciforme* and its butanol fraction might play vital roles in suppressing the formation of ulcer.

5. Conclusions

Taken together, the aqueous ethanol extract of *R. spiciforme* possesses gastro-protective activity, on the basis of its capacity in suppressing ulcer formation in animal models and lowering gastric acid secretion. Furthermore, owing to the presence of emodin and aloe emodin, butanol fraction of the extract is more effective than other fractions in improving the ulcer-related parameters.

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Conflict of interest

The authors have no conflicts of interest.

Author contributions

Conceptualization: Hafiz Muhammad Irfan
Investigation: Hafiz Muhammad Irfan, Maham Idrees
Writing – original draft: Hafiz Muhammad Irfan
Writing – review & editing: Kainat Jabeen

Ethics approval and consent to participate

The experiments on animals were performed in accordance with the animal ethics guidelines (Approval No. AEA-PMY-22).

Consent for publication

Not applicable.

Availability of data

The raw data used in this work can be obtained from the corresponding author on reasonable request.

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