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Evaluation of anti-ulcer potential of *Viola odorata* extract by in-vitro models and ethanol-induced gastric ulcer in rats

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ABSTRACT: *Viola odorata*, or sweet violet, is a plant rich in phytochemicals like flavonoids, tannins, saponins, and cyclotides, known for their antioxidant and antimicrobial properties, making it a promising candidate for anti-ulcer therapy. We aimed to evaluate the anti-ulcer potential of a methanolic extract of *Viola odorata* (MEVO) using in-vitro and in-vivo ethanol-induced gastric ulcer in rats. Fordtran's and Vatier's model were used as an in-vitro model which showed that MEVO at 250 mg/ml and 500 mg/ml effectively neutralized acid. The zones of inhibition of MEVO at 250 mg/ml and 500 mg/ml were found to be 9.33 mm and 11.66 mm respectively, compared to amoxicillin (14.33 mm). In the ethanol-induced ulcers rat model, the ulcer index of MEVO (500 mg/ml) was found to be 3.66, while the standard group had an ulcer index of 2.83. These results suggest that *V. odorata* extract possesses acid-neutralizing and antibacterial properties in both in-vitro and in-vivo settings, indicating its potential for anti-ulcer therapy.

KEYWORDS: *Viola odorata*; Anti-ulcer; Fordtran's model; Vatier's model; Ethanol-induced gastric ulcer

1. INTRODUCTION

A lesion in the innermost lining of the digestive tract brought on by the release of gastric acid or pepsin is known as peptic ulcer disease (PUD). The muscularis mucosae of the stomach epithelial layer is disrupted. Although it is not just confined to these regions, PUD typically affects the stomach as well as the first and second portions of the duodenum. Typically, upper abdominal pain from gastric ulcers appears 30 minutes after eating, whereas pain from duodenal ulcers appears considerably later [1]. The development of peptic ulcer disease may be viewed as a combination scenario involving an imbalance between aggravating factors (mucosal blood flow, prostaglandins, the mucus-bicarbonate layer, and cellular renewal) and defensive factors (hydrochloric acid, pepsin, alcohol, bile juice, medications)[2,3].

There are many causes of PUD, however *Helicobacter pylori* (*H. pylori*) and drug-related PUD make up most of the disease's pathogenesis. A gram-negative bacillus called *Helicobacter pylorus* is present mostly in gastric epithelial cells. It is responsible for the cause of 90% of duodenal ulcers and 70% to 90% of stomach ulcers. Lower socioeconomic status individuals are more likely to have *H. pylori* infection, which is frequently developed during childhood. The bacteria can attach to and inflame the stomach mucosa due to its broad spectrum of virulence factors. This causes achlorhydria or hypochlorhydria, which induces stomach ulcers. Non-steroidal anti-inflammatory drugs (NSAIDs) are also the key players in pathophysiology. After *H. pylori* infection, NSAIDs use is the second most frequent cause of PUD. Prostaglandin is often secreted to protect the stomach mucosa. By inhibiting the cyclooxygenase-1 (COX-1) enzyme, NSAIDs prevent the formation of prostaglandins, which lowers the production of gastrointestinal mucus, bicarbonate, and mucosal blood flow. The other rare cause of PUD includes Zollinger-Ellison syndrome, viral infection, stress, malignancy, radiation therapy, chemotherapy, etc [4,5].

Gastric ulcers are most frequently seen in the lesser curvature, whereas the duodenal bulb is where duodenal ulcers are most frequently found. The ulcer has a smooth foundation and a circular to oval shape. While chronic ulcers have raised borders with inflammation, acute ulcers have regular borders. Depending on the location of the disease and age, the signs and symptoms of peptic ulcer disease can differ. The common signs and symptoms include abdominal pain, bloating, abdominal fullness, nausea and vomiting,

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weight loss. Duodenal ulcers commonly cause nocturnal pain. People who have a gastric outlet obstruction frequently describe having a bloated or full abdomen in the past [4,5].

The perennial herb *Viola odorata Linn* also known as sweet violet, Gul banafsha locally in Hindi, of family Violaceae, has heart-shaped, simple toothed leaves with basal arrangement, flowers are deep violet color, European native, cultivated in Kashmir, and in several hill stations in the north and west Asia, North Africa, and Europe [6,7].

Based on the qualitative investigation the chemical component that are present in the *V. odorata* are flavonoids, cyclotides, tannins, saponins, glycosides, alkaloids, etc [8]. Flavonoids are phenolic compounds that have been identified from a wide variety of vascular plants. They function in plants as light filters, photoreceptors, visual attractants, feeding repellents, antioxidants, and antimicrobials. Numerous studies have revealed that flavonoids have biological effects, such as antiviral, anti-inflammatory, antiallergenic, and vasodilating properties. The antioxidant activity of flavonoids, however, has drawn the more attention because of their capacity to both prevent the creation of free radicals and scavenge existing ones [9]. Another constituent tannins are naturally occurring, water-soluble polyphenols that are mostly found in plant-based products, including food. Tannins are an important source of raw materials for an environmentally friendly, sustainable industry. As a result, they are primarily utilized in a variety of industries, including leather, feed, fisheries, drinks, and others. They can also be used as possible medicines, antioxidants, metal chelators, and regulators of the lipid peroxidation process and damaging pro-oxidative enzymes. Recent studies have shown that tannins provide a number of significant human health benefits, including antimicrobial, anticarcinogenic, and anti-inflammatory qualities, making them good prospects for the pharmaceutical and nutraceutical companies [10]. The production of reactive oxygen species (ROS), particularly the hydroxyl radical, has been demonstrated to be a major mediator of lesions brought on by stress, alcohol intake, *H. pylori*, and usage of NSAIDs, among other causes of stomach ulceration [11]. Hence flavonoids and tannins can potentially be used in the treatment of ulcers.

Cyclotides are small peptide, having macrocyclic backbone and a cystine knot. Their lytic activity on cells is characterized by their binding to and disruption of phospholipid membranes [12]. Cycloviolacin O2 which are present in the *V. odorata* is a cyclotide with strong antibacterial action [13]. Thereby it can act as an antibacterial agent in the ulcer and can possibly cure the *H. pylori* infection.

V. odorata is rich in the mucilage which can act as mucoprotective [14]. Tannins have also shown its effectiveness in gastric ulcer by its anti-inflammatory properties [15]. According to various research studies, it was found that the rutin and anthocyanin showed a potential activity as an antiulcer agent due to its antioxidant properties and by inhibiting the proton pump in the gastric mucosa [16,17]. Also, the cyclotide, cycloviolacin O2, has shown a potent antibacterial activity in the research studies which can potentially counter the gram-negative *H. pylori* bacteria [13]. The plant *V. odorata* contains antioxidants like tannins, flavonoids like rutin[18] and anthocyanin [19], cyclotides like cycloviolacin O2 [20]. So, the current study was undertaken to explore the potential of plant *V. odorata* containing all these constituents in the treatment of gastric ulcer, as ROS is one of the major mediator for the generation of the ulcer lesions and also gram-negative *H. pylori* is the leading cause for the gastric ulcer [11]. Hence, the current research was undertaken to explore the potential of *V. odorata* for the anti-ulcer action.

2. RESULTS

2.1. Drug extraction and identification of phytochemicals

The average percentage yield of methanolic extract of *Viola odorata* (MEVO) was found to be 18.13 % and the color of the extract was blackish green. The identification tests of flavonoids, tannins and saponins were found to be positive and the interpretations are depicted in Table 1.

Table 1. Results of phytochemical Identification tests

Sr. No.	Test for	Test name	Interference	Observation
1	Flavonoids	Shinoda test	Positive	Color change to red
2	Tannins	Ferric chloride test	Positive	Color change to dark green
3	Saponins	Frothing test	Positive	Sustained froth for 30 min

2.2. Determination of pH

The pH of distilled water was found to be 6.7, the artificial gastric juice had a pH of 1.26, the standard solution of $NaHCO_3$ (1% w/v) had a pH of 9.4, the test solution of MEVO 250 mg/ml had a pH of 7.96 and pH of MEVO 500 mg/ml was found to be 8.05.

2.3. Determination of the neutralizing effect of prepared solutions on artificial gastric acid

The Fordtran's model was used to evaluate acid neutralization property. The volume of artificial gastric juice required to bring pH 3 of solution of MEVO 250 mg/ml was found to 11 ± 0.58 mL and that of MEVO 500 mg/ml was found to be 12 ± 0.58 ml. The artificial gastric juice used to bring pH 3 of standard NaHCO3 (1%) solution was found to be 14 ± 0.58 ml. The data are presented in mean ± SEM format. There was a significant difference between standard and MEVO 250 mg/ml whereas no significant difference was observed in MEVO 500 mg/ml and standard (Figure 1(A)).

2.4. Vatier's Artificial Stomach

The Vatier's model was used to evaluate acid neutralization property. The time taken by MEVO 250mg/ml to reach pH 3 was found to be 106 ± 2.6 seconds and that of MEVO 500 mg/ml was found to be 112 ± 2.5 seconds. To compare it with standard, the volume consumed by NaHCO3 (1%) to reach pH 3 was found to be 122 ± 1.8 seconds. The data are presented in mean ± SEM format. The NaHCO3 showed better effect compared to test solutions (Figure 1(B)).

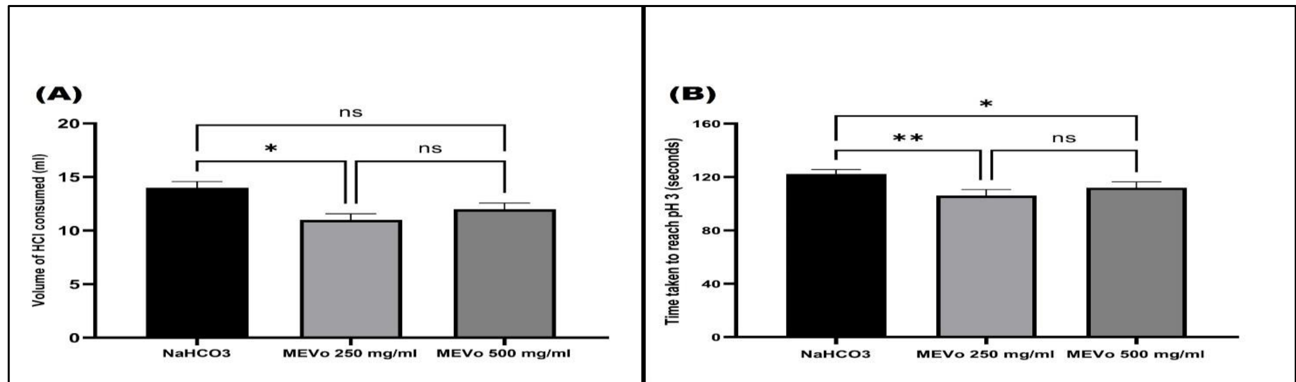

Figure 1. (A) Graphical representation of the results obtained from Fordtran's model. **(B)** Illustrates the outcomes derived from Vatier's model. Data are represented in mean ± SEM format. Here * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, while ns indicates $p > 0.05$.

2.5. Antimicrobial Assay

The zone of inhibition for MEVO 250 mg/ml was found to be 9.33 ± 0.88 mm, the zone of inhibition for MEVO 500 mg/ml was found to be 11.66 ± 0.88 mm, the zone of inhibition for distilled water was found to be 0 mm, the zone of inhibition for the standard amoxicillin 100 mg/ml was found to be 14.33 ± 1.2 mm. The data are presented in mean ± SEM format. The test group (MEVO 500 mg/ml) and standard group showed a significant zone of inhibition but there was no significant difference between standard and test (500 mg/ml) group. These results shows that MEVO (500 mg/ml) and standard amoxicillin (100 mg/ml) were equally effective in anti-bacterial action (Figure 2(A)).

2.6. Ethanol-induced ulcer in rats

In the ethanol induced gastric ulcer model, the ulcer index for the test group (MEVO 500 mg/ml) was found to be 3.66 ± 0.74, the ulcer index for the standard group was found to be 2.83 ± 0.68, the ulcer index for the disease group was found to be 11.83 ± 0.89 and the ulcer index for the normal control was found to be 0. The data are presented in mean ± SEM format. The test group and standard group showed a significant reduction in ulcer index compared to disease group but there was no significant difference between standard and test group (Figure 2(B)).

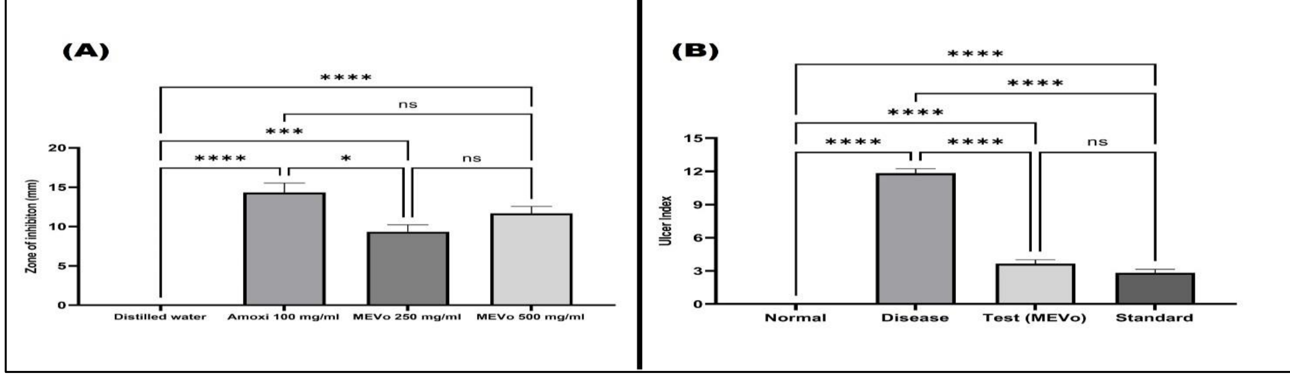

Figure 2. (A) Graphical representation of the antimicrobial assay results. **(B)** Illustrates the outcomes of the ethanol-induced ulcer model. Data are represented in mean ± SEM format. Here * indicates p ≤ 0.05, *** indicates p ≤ 0.001, **** indicates p ≤ 0.0001, while ns indicates p > 0.05.

2.7. Histopathology of stomach

The histopathology of stomach of normal control group (Figure 3), Standard group (Figure 4) and test group (Figure 5) showed no such detrimental damage to the cells and mucosal surface and most of the cells were found to be intact and undamaged. Whereas the disease group showed clear damage of gastric cells and mucosal surface (Figure 6).

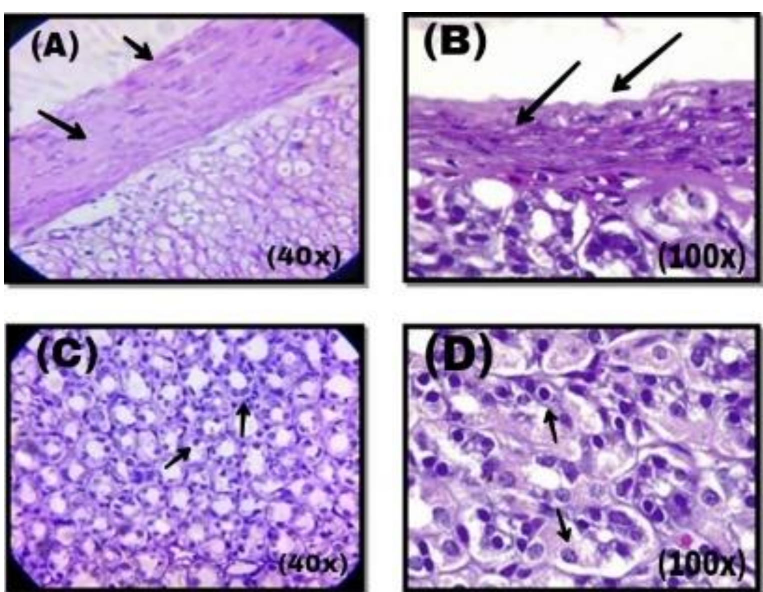

Figure 3. Histopathological changes in stomach of rats treated with only normal saline. (A,C) mucosal surface and gastric cells in 40x magnification; (B,D) mucosal surface and gastric cells in 100x magnification.

3. DISCUSSION

Millions of individuals are impacted by gastrointestinal diseases caused by alcohol, which represent a significant global health issue. The motility and metabolism of the stomach can be directly affected by alcohol use. This results in stomach ulcers and mucosal damage [21]. The increased ROS production is intimately linked to an ethanol-induced stomach ulcer. Damage to stomach cells is caused by an excess of ROS during oxidative stress [22]. Alcohol stimulates the H+/K+-ATP pump in the stomach, which releases pepsin and gastric acid. Additionally, by blocking the K+ and Na+ pumps that cause gastric acid to rise, this action also inhibits blood flow. HCl causes oxidative stress and corrosive damage to the gastric mucus, whereas ethanol worsens gastric injury by attracting immune cells that set off an inflammatory cascade [21].

In our research, we used the perennial herb *V. odorata* which is rich in antioxidants and anti-microbial agents. The herb has shown various pharmacological actions including antidyslipidemic, laxative, antihypertensive, vaginal infection, anti-inflammatory, antioxidant, anticancer, and hepatoprotective.[23]

The in-vitro studies were performed on different concentrations of MEVO (250 mg/ml, 500mg/ml) to identify the optimal and best dose that can show anti-ulcer activity. The in-vitro studies have shown that the plant has acid neutralizing capacity which is one of the major concerns in gastric ulcer disease. Along with that, the MEVO has also shown its anti-bacterial activity against gram-negative bacteria which can potentially counter the gram-negative *H. Pylori* bacteria that causes gastric ulcer.

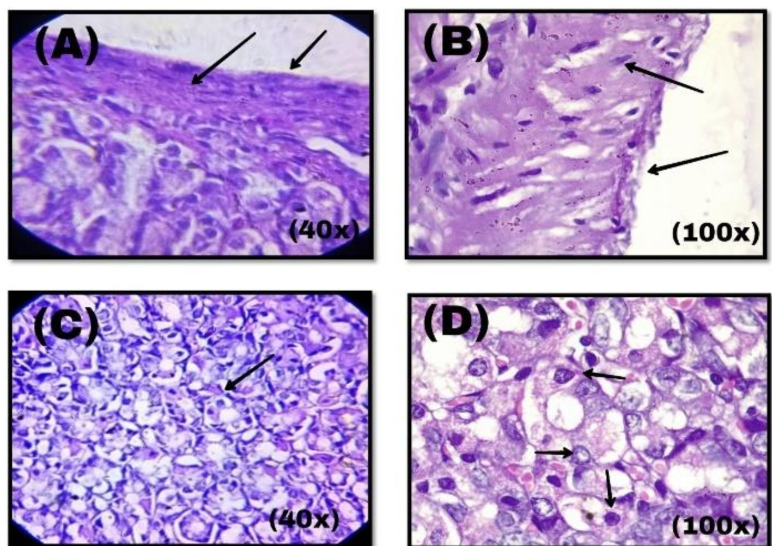

Figure 4. Histopathological changes in stomach of rats treated with standard therapy and ethanol. (A,C) mucosal surface and gastric cells in 40x magnification; (B,D) mucosal surface and gastric cells in 100x magnification.

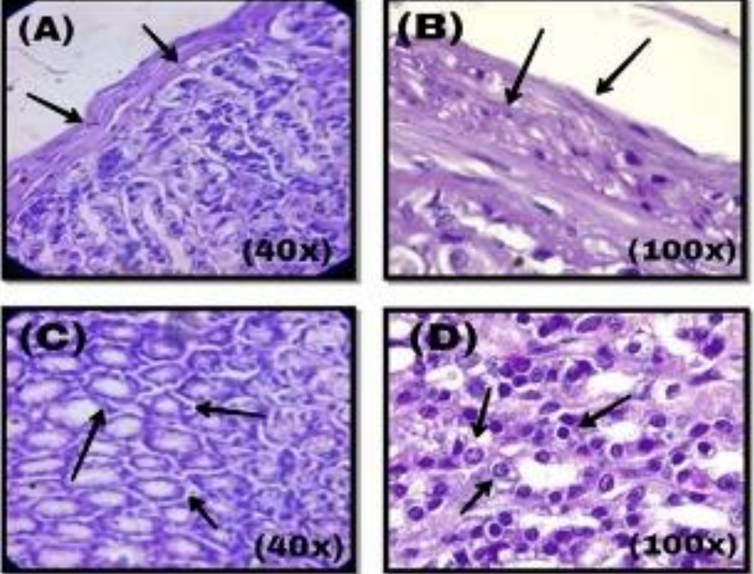

Figure 5. Histopathological changes in stomach of rats treated with MEVO and ethanol. (A,C) mucosal surface and gastric cells in 40x magnification; (B,D) mucosal surface and gastric cells in 100x magnification.

From the result of in-vitro studies, the MEVO 500 mg/ml was selected as an ideal concentration for the further in-vivo study. The ethanol induced ulcer were performed in rats to confirm the in-vitro results showing acid neutralizing property of MEVO. In various epidemiology studies, the prevalence of gastric ulcers were found to be more in female compared to male.[24,25] Also, from the literature search we found that female Wistar rats were used in the studies of ethanol-induced gastric ulcer.[26,27] Hence, we chose female Wistar rats in our study. The results of in-vivo study showed that MEVO at 500 mg/ml concentration and 3 ml/kg dose showed significant gastroprotective effect compared to disease group. The mechanism behind this action were possibly due to the antioxidant actions of flavonoids, tannins and saponins. Also, the pH of MEVO was found to be slightly alkaline which could have act as acid neutralizer in gastric

environment. The result of the current study supports the acid neutralizing property of *V. odorata*, but various other mechanisms, including proton pump inhibition and H-1 receptor inhibition activity, of *V. odorata* can be explored in future research using in-vitro and in-vivo approaches.

The limitations of the study include, firstly, the presence of *H. pylori* was not confirmed in the antimicrobial assay. Furthermore, an antioxidant assay was not conducted to validate whether the gastroprotective effect could be attributed to antioxidants such as flavonoids and tannins.

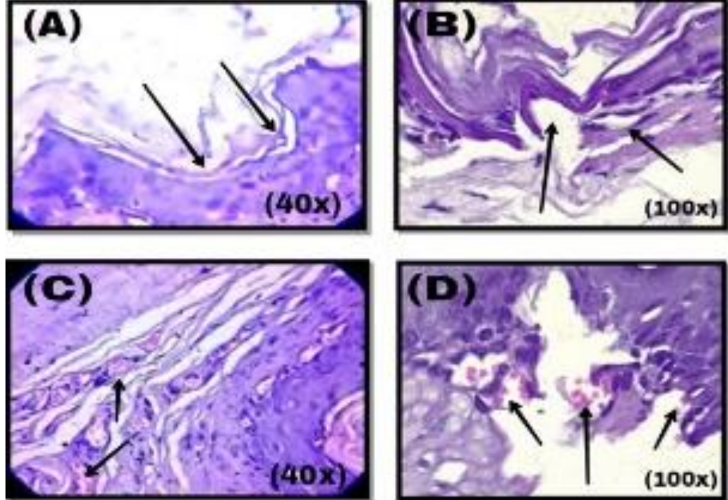

Figure 6. Histopathological changes in stomach of rats treated with normal saline and ethanol. (A,C) mucosal surface and gastric cells in 40x magnification; (B,D) mucosal surface and gastric cells in 100x magnification.

4. CONCLUSION

In conclusion, the in-vitro studies of MEVO have shown supportive results by its acid neutralizing and anti-microbial activity to be used as anti-ulcer agent. Further in-vivo studies have confirmed that the MEVO has potential anti-ulcer activity. However more studies are required to further validate the use of *V. odorata* in management of gastric ulcers.

5. MATERIALS AND METHODS

5.1. Procurement of materials

The whole dried plant of *V. odorata* was ordered from IndianJadiBooti, Delhi; and it was validated at School of Science, RK university (Herbarium no. SOP/RKU/4/2023). The amoxicillin was obtained from Sun Pharmaceutical Industries Ltd. The standard drug containing suspension of aluminium hydroxide, magnesium hydroxide and dimethicone (Gelusil MPS, Pfizer) was obtained from the local pharmacy. Rest of the chemicals used in this study were of analytical grade and obtained from reputed sources like SRL, Mumbai; Rankem Chemicals, Mumbai; Isochem Labs, Coimbatore; Labogens, Ahemdabad.

5.2. Preparation of MEVO using Soxhlet apparatus

The process mentioned by Redfern et al. was followed with slight modification. *V. odorata* plant material was grinded into powder form and placed in a thimble, subjecting it to extraction using methanol at 80 °C for 7-8 hours. The resulting extract (MEVO) in the form of a dark green slurry was obtained after simple distillation and the removal of residual methanol [28].

5.3. Identification of chemical constituents

The various tests were carried out to confirm the presence of compounds of interest like tannins, flavonoids, saponins.

5.3.1. Test for flavonoids

Shinoda test: Each portion of roughly 0.5 g was mixed with about 10 ml of distilled water before being filtered. The filtrate was treated with strong hydrochloric acid and a few pieces of magnesium ribbon. The presence of flavonoids can be known by the color change to red or pink after a short period of time.[29]

5.3.2. Test for tannins

Ferric chloride test: Each portion of roughly 0.5 g was mixed with about 10 ml of distilled water before being filtered. A few drops of 1% ferric chloride solution were added to 2 ml of the filtrate, and a blue-black or dark-green color appearance confirms the presence of tannins.[30]

5.3.3. Test for saponins

Frothing test: In a test tube, 10 mL of distilled water were added to 4 mL of the extract's aqueous solution. The test tube was stopped, agitated forcefully for about five minutes, then let to stand for thirty minutes. The appearance of honeycomb froth indicates the presence of saponins.[31]

5.4. In-vitro studies

5.4.1. Fordtran's model

Preparation of artificial gastric acid

A solution was prepared by dissolving sodium chloride (2 g) and pepsin (3.2 mg) in 500 ml of distilled water. To this mixture, 7 ml of hydrochloric acid (HCl) was added, and subsequently distilled water was added to make total volume 1000 ml. The pH of the solution was then adjusted to 1.2 by the adding either dilute HCl or NaOH.

Preparation of standard and test solutions

Aqueous solution of MEVO of different concentrations (250 mg/ml and 500 mg/ml) was prepared and along with that 1% w/v NaHCO3 aqueous solution was also prepared.

Determination of pH

The prepared solutions of MEVO (250 mg/ml and 500 mg/ml) were used for the pH determination at 25-30 °C. Along with that, the pH of NaHCO3 solution (1% w/v) was also determined. The digital pH meter (IE-702 Insif electronics) was used for this process.

Determination of the neutralizing effect of prepared solutions on artificial gastric acid

Test solutions of MEVO (90 mL each of 250 mg/mL and 500 mg/mL) were warmed to 37 °C and swirled continuously at 30 rpm to mimic the motion of the stomach. With artificial gastric juice, the test solution was titrated until it reached pH 3. For each test solution, the consumed volume (V) of the artificial gastric juice was counted [32]. The experiment was repeated for three times and average value were statistically evaluated.

5.4.2. Vatier's artificial stomach

There are 3 components which formed the modified equipment of Vatier's artificial stomach: a peristaltic pump (P), a pH-recording system (R), and a stomach (S). The model demonstrates three sections in the stomach: S1, S2, and S3.

S1: Served as the mixing vessel for the test solutions and gastric juice.

S2: Inlet for the artificial gastric juice into S1,

S3: Outlet for the artificial gastric juice from S1.

In S1, 100 mL of artificial gastric juice and 90 mL of each test solution were combined. The reaction mixture was heated to 37°C and swirled continuously at 30 RPM using a 2.5-cm magnetic stirring equipment and pH recording meter is attached to it. The artificial gastric juice (S2) was introduced at the rate of 3 ml/min and simultaneously 3 ml was excreted out from the S1. The process was continued till the pH of S1

reached 1.2. The time required to reach the pH 3 was recorded [32,33]. The experiment was repeated for three times and average value were statistically evaluated.

5.4.3. Antimicrobial assay

Collection of bacteria

The bacterial sample was obtained from the foul water through the roadside sewage pit. The bacterial sample was stained using gram staining method and gram-negative strain of bacteria was confirmed.

Procedure

The nutrient agar media was prepared by dissolving the nutrient agar powder (28gm) in warm distilled water (1000mL). Later the media was filled in petri plates and were kept aside to solidify. After that, the bacterial sample was inoculated in the petri dish using a spread plate method. Petri dishes were divided in 4 quadrants and small wells were created in those quadrants to fill the standard and test compound as depicted in Table 2 [34]. The petri dishes were placed in the incubator at 37°C for 24 hours. After 24 hours, the zone of inhibition was measured to evaluate the effect of the test compound [35]. The experiment was repeated for three times and average value were statistically evaluated.

Table 2. Drugs and composition filled in well of petri plate

Well of quadrant	Drug and composition
A	Amoxicillin (100mg/ml)
B	Distilled water
C	MEVO 250mg/ml
D	MEVO 500 mg/ml

5.5. In-vivo model

5.5.1. Ethanol induced ulcer in rats

Animal procurement

The female Wistar rats weighing 200-250 g were procured from the animal house of School of Pharmacy, RK University. A polypropylene cage was used to house the animals and they were acclimatized for 7 days before the initiation of experiment. Standard food and water were provided ad libitum. The temperature was kept at 24 ± 2 °C and relative humidity was maintained at 30-70%.

Ethics approval

The entire animal study was approved by Institutional Animal Ethics Committee (IAEC) of School of Pharmacy, RK University, Rajkot under the prior project proposal number RKCP/COI/Re/22/126.

In-vivo experiment procedure

The rats were fasted for food 18 hours ahead of the experiment but were given unrestricted access to water. The grouping of animal was done as depicted in Table 3, six animals were allocated in each group. The disease control group received normal saline, test group received 3 ml/kg MEVO solution prepared in distilled water (concentration 500 mg/ml), the standard group received 3 ml/kg antacid suspension containing mixture of dimethicone (10 mg/ml), magnesium hydroxide (50 mg/ml) and aluminium hydroxide (50 mg/ml) 30 minutes before giving them 5 ml/kg of ethanol (90 percent). After an hour of given ethanol, the animals were euthanized by excess CO2. The stomachs were removed, cut along the greater curvature, and gently cleaned under running water. The mucosal site was elevated as stomachs were stretched out on a foam pad (Figure 7)[22]. Based on visual observations, the scoring of ulcer was given as depicted in Table 4 [36]. The ulcer index (UI) was calculated by the following formula:

$$\boldsymbol{UI = (Un + Us + Up) \times 10^{-1}}$$

Where, Un = average number of ulcers per animal, Us = average number of severity of scores and Up = percentage of animals with ulcers [37].

Table 3. Grouping of animals

Sr no.	Groups	Intervention
Group 1	Normal control	3ml/kg of normal saline was administered to this group.
Group 2	Disease control	5ml/kg of ethanol (90 percent) was administered to this group.
Group 3	Standard control	3ml/kg of suspension containing mixture of dimethicone (10 mg/ml), magnesium hydroxide (50 mg/ml) and aluminium hydroxide (50 mg/ml) was administered to this group.
Group 4	Test control	3ml/kg of MEVO solution (500 mg/ml concentration) was administered to this group.

Table 4. Scoring of gastric ulcer

Observations	Severity score
Normal colored stomach	0
Red coloration	0.5
Spot ulcer	1
Haemorrhagic streak	1.5
Deep ulcers	2
Perforation	3

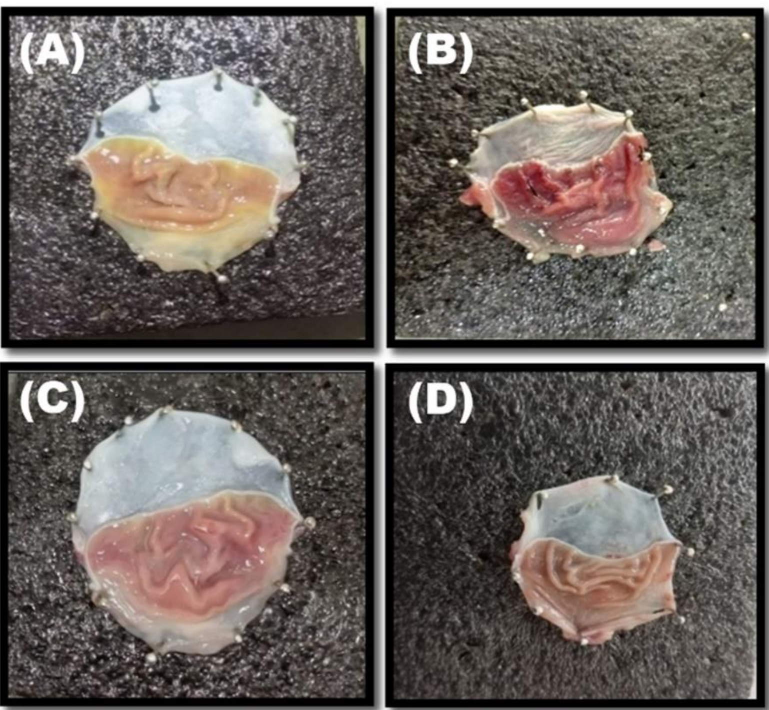

Figure 7. Gross stomach of rats mounted on foam pad. (A) Normal control; (B) Disease control; (C) Test group (MEVO treated); (D) Standard group.

Rationale of dose selection

Ethanol (5 ml/kg) is harmful to the stomach because it easily enters and digests the gastric wall through proteolytic and hydrolytic activities, creates ROS, decreases blood flow, and promotes apoptosis [38]. Based on the prior literature, the ethanol dose (5 ml/kg) for the induction of gastric ulcer in rats was chosen [39]. The dose concentration 500 mg/ml of MEVO was selected based on the results of in-vitro studies. The dose of standard drug was calculated by converting human equivalent dose (HED) to animal equivalent dose (AED) [40]. The animal dose of standard and test were kept similar (3 ml/kg) so that the efficacy at equivalent dose can be compared.

5.6. Histopathological study procedure

A small part from each stomach were fixed in 10% formalin buffer solution for 48 hours. Later the tissues underwent a series of alcohol treatments (50%, 70%, and two rounds of 95% alcohol) to ensure proper dehydration. Afterward, they were kept immersed overnight in a mixture of alcohol and xylene and then cleared with two rounds of xylene.

For sectioning, the tissues were infiltrated with molten paraffin wax, embedded, and cut into 5 µm thick slices using a microtome. These sections were placed on slides and processed further, involving de-waxing with xylene, rehydration with alcohol, staining, and counter-staining. The process mentioned by Ijioma et al. 2018 were followed [41]. After staining, the slides were examined under a phase contrast microscope (Leica Microsystems, Germany) at 40x and 100x magnification.

5.7. Statistical analysis

All the data were presented as mean ± SEM. The data were analysed in the GraphPad prism software (version 9.5.0). The statistical comparison between groups was done using one-way ANOVA followed by Tukey's multiple comparison post-hoc test. P-value < 0.05 was considered as statistically significant.

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Conflict of interest statement: The authors declared no conflict of interest.

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