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Original Research Article

Pharmacological basis for medicinal use of Ziziphyus nummularia (Rhamnaceae) leaves in gastrointestinal disorders

Sayed Muzahir Hussain[1], Aslam Khan[2]*, Arif-ullah Khan[1], Neelum Gul Qazi[1] and Fawad Ali[1,3]

[1]Department of Pharmacology, Riphah Institute of Pharmaceutical Sciences, Riphah International University, Islamabad, Pakistan, [2]Basic Sciences Department, College of Science and Health Professions, King Saud Bin Abdulaziz University for Health Sciences, Jeddah, Saudi Arabia, [3]Department of Pharmacy, Kohat University of Science and Technology, Kohat, Pakistan

For correspondence:* ***Email:* *khanasl@ksau-hs.edu.sa, khanas03@ngha.med.sa, aslamkhan_mkd@yahoo.co.uk*

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Abstract

Purpose: *To explore the pharmacological basis for the folkloric use of Ziziphus nummularia for treating diarrhea and gastrointestinal spasm.*
Methods: *Ziziphus nummularia crude extract (Zn.Cr) was investigated for antidiarrheal activity (50, 100 and 300 mg/kg) in terms of reduction diarrhea droppings as well as for antisecretory activity (300 and 1000 mg/kg) in castor-oil induced model in mice. The effect of the extract on potassium chloride (KCl, 80 mM)-induced contractions in isolated rabbit jejunum tissues were was also examined. Furthermore, the antiulcer properties of the extract was assessed in an ethanol-induced gastrointestinal ulcer model.*
Results: *Zn.Cr (50 – 1000 mg/kg) exhibited protective effect against castor oil-induced diarrhea ($p < 0.05$, $p < 0.01$ vs saline group) and intestinal fluid accumulation ($p < 0.001$ vs. castor oil group) in mice. In isolated rabbit jejunum model, Zn.Cr concentration-dependently (0.01, 0.1, 0.3, 0.5, 1 and 3 mg/mL) caused relaxation of spontaneous and KCl-induced contractions, similar to verapamil. Calcium antagonistic effect was indicated, as pretreatment of intestinal tissues with Zn.Cr (0.3, 0.5 and 1.0 mg/mL) produced a rightward shift in Ca^{2+} concentration-response curves, with suppression of maximum contraction. In ethanol-induced gastric ulceration assay, Zn.Cr (300 and 1000 mg/kg) caused 52.5 and 93.6 % inhibition, respectively ($p < 0.001$ vs. saline group).*
Conclusion: *These results reveal that Ziziphus nummularia possesses anti-diarrheal, anti-secretory, anti-spasmodic and anti-ulcer actions, mediated possibly through voltage-gated Ca2+channel blockade.*

Keywords: *Ziziphus nummularia, Anti-diarrheal, Anti-secretory, Anti-spasmodic, Anti-ulcer*

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INTRODUCTION

Ziziphus nummularia (Burm. f.) Wight & Arn. (Rhamnaceae), commonly known as "Jharber", and also as Bairi or Karkanrha in Pakistan, is a branched thorny shrub with a height of 1 - 2 m found in Pakistan, India and Iran. *Ziziphus nummularia* leaves and fruits are used for cold, diarrhea, dysentery, indigestion, inflammation of gums and tonic [1]. The unripe fruits of the plant are prescribed in the management of vomiting, burning sensations and as tonic, while dried fruits are useful as an anticancer, anodyne, refrigerant, sedative, stomachic and in treatment of anemia, bronchitis,

burns, chronic fatigue, diarrhea, hysteria, loss of appetite and pharyngitis [2].

Phytochemical analysis of *Ziziphus nummularia* leaves and fruits confirm the presence of phytoconstituents such as flavonoids, alkaloids, glycosides, pectin, polysaccharides, peptide alkaloids, saponins, sterols, tannins, sterols, triterpenoic acids, fatty acids, ziziphin N, O, P, Q and dodecaacetylprodelphinidin B3 [3]. *Ziziphus nummularia* is reported to possess antitumor [4], anthelmintic [3], antibacterial [5], analgesic and anti-inflammatory [6] properties. In this study, we investigated the anti-diarrheal, anti-secretory, anti-spasmodic and anti-ulcer activities of *Ziziphus nummularia*, to determine whether there is any scientific justification for its folkloric use in hyperactive gut disorders, viz, diarrhea, gastrointestinal spasm and ulcer.

EXPERIMENTAL

Materials

The leaves of *Ziziphus nummularia* were collected from the Sihala, Islamabad in September 2015. The plant was authenticated by Dr. Mushtaq Ahmad, a taxonomist at Department of Plant Sciences, Quaid-a-Azam University, Islamabad and voucher specimen (ISB-814) was submitted to the same Department. The plant material (2 kg) was air dried, powdered and extracted at room temperature with aqueous-methanol (8:2, thrice), to obtain *Ziziphus nummularia* crude extract (Zn.Cr).

Chemicals and drugs

The following standard chemicals were purchased from the sources verified: Acetylcholine chloride (ACh), atropine sulphate, ethanol, loperamide, methanol, omeprazole, papaverine and verapamil hydrochloride (Sigma Chemicals Co, St Louis, MO, USA) and Castor oil from KCL Pharma, Karachi, Pakistan.

Animals

Rabbits (1 - 1.2 kg), Sprague-Dawley rats (180 - 220 g) and Balb/C mice (25 - 30 g) of local breed and either sex were obtained from animal house of the Riphah Institute of Pharmaceutical Sciences, Islamabad, maintained at standard temperature (23 - 25 °C). Plastic cages with sawdust were used for animals, they were fasted before each experiment for 24 h. Animals were provided with tap water *ad libitum.* Animal experiments were performed in accordance with Institute of Laboratory Animal Resources [7] and ethical approval by Ethics Committee of Riphah Institute of Pharmaceutical Sciences (ref no. REC/RIPS/2016/003).

Castor oil-induced diarrhea

Balb/C mice were fasted before the experiment for 24 h, housed in individual cages and were divided in five groups (n = 5). The first group received saline (10 mL/kg, p.o.), served as a negative control. The second, third and fourth group received extract (50, 100 and 300 mg/kg) respectively and loperamide (10 mg/kg, served as a positive control) was given to fifth group. After 1 h of treatment all groups received castor oil (10 mL/kg, p.o.). 4 h post treatment observation was carried out in order to check the presence of diarrheal droppings, absence of diarrheal droppings was recorded as a positive result [8].

Intestinal fluid accumulation

Enteropooling assay was used to study the intestinal fluid accumulation. Overnight fasted mice were placed in five cages with five mice each. Normal saline (10 mL/kg) and castor oil (10 mL/kg, p.o.) were administered to groups I and II respectively. Groups III and IV were treated at dose of 300 and 1000 mg/kg (ip), respectively. Group V was given a standard drug (atropine) at a dose of 10 mg/kg, 1 h prior to diarrhea-induction with castor oil (10 mL/kg, po). The mice were sacrificed 30 min later, and the entire intestines removed and weighed. The results are expressed as (Pi/Pm) x 1000 where Pi is the weight (g) of the intestine and Pm is the weight of the animal [9].

Isolated tissue experiments

The rabbits had free access to water but were fasted for 24 h before experiment. After cervical dislocation of the animal, jejunal portion was isolated and cleaned with the help of Tyrode's solution. 2 cm of jejunal segment was suspended in tissue bath, containing Tyrode's solution (pH 7.4), maintained at standard temperature (37 °C) and aerated with 95 % O_2 and 5 % CO_2 (carbogen). The tissue was allowed to equilibrate for 30 min and an initial loading dose of 1 g of acetylcholine was applied. After achieving normal contractions, At 3 min interval each preparation was subjected to sub-maximal dose of ACh (0.3 µM) until constant responses were recorded via power Lab 4/25 data acquisition system (AD Instrument, Sydney Australia). In the spontaneous contractions of jejunum the inhibitory effects of Zn.Cr was (0.01 - 3 mg/mL) recorded as the % change [8].

Ca^{2+} antagonist assay

To determine the Ca^{2+} channel blockade (CCB) mode of action, method was used, as described by [10]. Once plateau of the induced contraction was achieved (usually within 7-10 min.), the test substances (0.3, 0.5 and 1 mg/mL) was then added in a cumulative fashion to obtain concentration-dependent inhibitory responses. In order to confirm the Ca^{2+} antagonist effect, Tyrode's solution was used to stabilize the tissue and then the solution was replaced by Ca^{2+}-free Tyrode's solution, and further replaced with K^{+}-rich and Ca^{2+}-free Tyrode's solution.

Following the incubation period of 30 min, control concentration-response curves (CRCs) of Ca^{2+} were obtained. When the control Ca^{2+} CRCs were found super-imposable (usually after two cycles) for 60 min, then the tissue was pretreated with the 0.3, 0.5 and 1 mg/mL concentration of plant extract, to test the possible CCB effect [13]. The CRCs of Ca^{2+} were constructed in the presence of different concentrations (0.3, 0.5 and 1 mg/mL) of test extract.

Ethanol-induced ulceration

Rats fasted for 24 h before study was randomly divided into four groups of five animals each. Group I (negative control) received only saline 10 mL/kg body weight p.o. Group II and III pretreated with Zn.Cr at doses of 300 and 1000 mg/Kg, (p.o.) respectively and group IV received omeprazole p.o (30 mg/Kg) used as the standard drug. One hour after drug treatment, absolute ethanol (1.0 mL/100 g, p.o.) was administered to each rat, which was sacrificed one hour later, stomach removed and observed for ulcers in glandular region. Surface area of each lesion was measured and scored by the method as described previously [11]. Ulcer index for each rat was taken as mean ulcer score (0: no ulcer, 1: US ≤ 0.5 mm^2, 2: 0.5 < US ≤ 2.5 mm^2, 3: 2.5 mm^2 < US ≤ 5 mm^2, 4: 5 mm^2 < US ≤ 10 mm^2, 5: 10 mm^2 < US ≤ 15 mm^2, 6: 15 mm^2 <US ≤ 20 mm^2, 7: 20 mm^2 < US ≤ 25 mm^2, 8: 25 mm^2 < US ≤ 30 mm^2, 9: 30 mm^2 < US ≤ 35 mm^2 and 10: US > 35 mm^2). Sum of the length (mm) of all lesions for each stomach was used as the ulcer index (UI). The percentage of inhibition (% I) was calculated using Eq 1.

$$\% I = (USc - USt)100/USc \quad \text{.....} \quad (1)$$

where USc = ulcer surface area of control and USt = ulcer surface area of test drug group animal [11].

Acute toxicity test

Mice were divided in three groups of five mice each. The test was performed using increasing doses of the plant extract (3 and 5 g/kg) given in 10 mL/kg volume. Saline (10 mL/kg, p.o, negative control) was administered to one group. 24 h post study the mice were observed for mortality [12].

Statistical analysis

The data are expressed as mean ± SEM (n = 5) and median effective concentrations (EC_{50}) with 95 % confidence intervals. The data were subjected to one-way analysis of variance (ANOVA) followed by *post-hoc* Tukey test were applied to the data, except in the case of the antidiarrheal data, where Chi square test was used. $P < 0.05$ was considered statistically significant. Concentration-response curves were analyzed by non-linear regression using Graph Pad program (GraphPAD, SanDiego, CA-USA).

RESULTS

Effect of Zn.Cr on castor-oil induced diarrhea

Zn.Cr produced protection against the castor-oil induced diarrhea in mice. Saline treated group shows no protection. Mice pretreated with Zn.Cr (50, 100, and 300 mg/kg) exhibited 20, 40 and 80 % protection respectively ($p < 0.05$ versus saline group). The positive control group, Loperamide (10 mg/kg) showed 100 % protection from diarrhea ($p < 0.01$ versus saline group, Table 1).

Table 1: Protective effect of *Ziziphus nummularia* crude extract (Zn.Cr) and loperamide against castor oil (C.oil)-induced diarrhea in mice

Treatment	No. of mice with diarrhea	Protection (%)
Saline (10 mL/kg) + (C.oil 10 mL/kg)	5	0
Zn.Cr (50 mg/kg) + (C.oil 10 mL/kg)	4	20
Zn.Cr (100 mg/kg) + (C.oil 10 mL/kg)	3	40
Zn.Cr (300 mg/kg) + (C.oil 10 mL/kg)	1*	80
Loperamide (10 mg/kg) + (C.oil 10 mL/kg)	0**	100

* $p < 0.05$, ** $p < 0.01$ compared to saline group, Chi-squared test; n = 5 per treatment group

Effect of Zn.Cr on intestinal fluid accumulation

Zn.Cr produced antisecretory effect. In saline treated group intestinal fluid accumulation was 94.36 ± 2.197 (mean ± SEM, n = 5), castor oil-treated group reduced it to 124.48 ± 0.48 (p < 0.001 vs. saline group). The fluid accumulation was significantly decreased by Zn.Cr to 101.61 ± 3.54 (p < 0.001 vs. castor - oil group) and 88 ± 0.47 (p < 0.001 vs. castor - oil group) at 300 and 1000 mg/kg respectively (Figure 1). The intestinal fluid accumulation was reduced by Atropine (10 mg/kg) to 80.52 ± 1.16 (p < 0.001 vs. castor - oil group).

Effect of Zn.Cr on jejunum contractions

Figure 2 shows the inhibitory effect of Zn.Cr and verapamil against spontaneous and K^+ (80 mM)-induced contractions. Zn.Cr inhibited spontaneous and K^+ (80 mM)-induced contractions with EC_{50} values of 0.30 (0.24 - 0.37, n = 4) and 0.05 mg/mL (0.04 - 0.07, n = 4) respectively, as presented in Figure 2A. Verapamil caused relaxation of spontaneous and K^+ (80 mM)-induced contractions with EC_{50} value of 0.09 (0.07 - 0.11, n = 4) and 0.015 µM (0.012 - 0.02, n = 4) respectively (Figure 2B). Zn.Cr (0.3 - 1.0 mg/mL) shifted Ca^{2+} CRCs to the right with suppression of the maximum contractile response (Figure 2C), like that caused by verapamil at 0.03 - 0.1 µM (Figure 2D).

Effect of Zn.Cr on ethanol-induced gastric ulcers

Zn.Cr, at doses of 300 and 1000 mg/kg, caused 52.5 and 93.6 % inhibition, respectively (p < 0.001 vs. saline group) of ethanol-induced gastric ulcer lesions. Omeprazole (30 mg/kg) showed 97.7 % inhibitory effect (Table 2).

Acute toxicity

The extract did not show any mortality up to the dose of 10 g/kg.

DISCUSSION

Based on ethnopharmacological use of *Ziziphus nummularia* in hyperactive gut disorder, such as diarrhea, spasm and ulcer, Zn.Cr was evaluated for possible anti-diarrheal, anti-secretory and anti-ulcer actions. Isolated intestinal tissue was used for the elucidation of possible underlying mechanism(s) to rationalize aforementioned ethnomedicinal uses of the plant. Zn.Cr showed dose-dependent protective effect against the castor oil-induced diarrhea in mice, similar to loperamide, a standard anti-diarrheal drug [13]. The alteration in transportation of electrolytes and water causing diarrhea, is due to the ricinoleic acid formed as a result of hydrolysis of castor oil, responsible for generation of contractions in the transverse and distal colon [8].

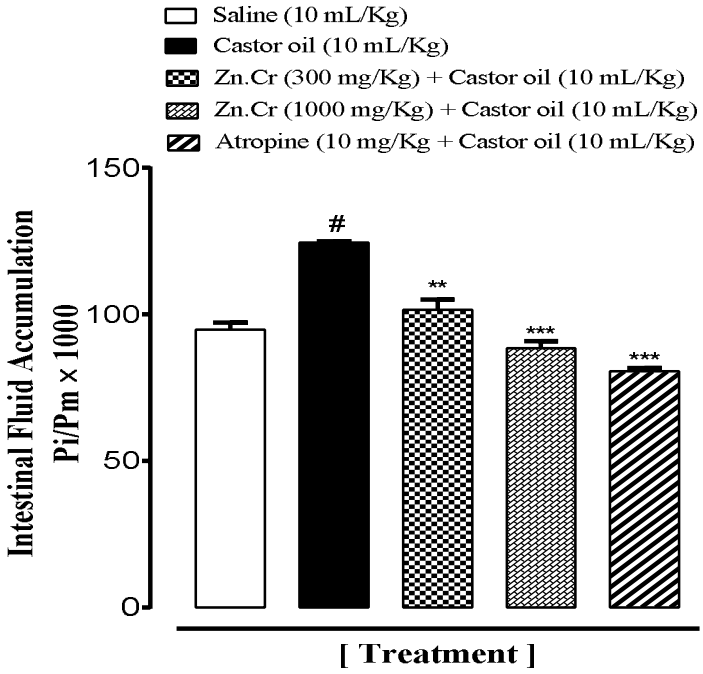

Figure 1: Inhibitory effect of *Ziziphus nummularia* crude extract (Zn.Cr) and atropine on castor oil-stimulated fluid accumulation in mice. Data are mean ± SEM of 5 animals for each experimental group; $^{\#}p$ < 0.001 vs. saline group, $^{**}p$ < 0.01, $^{***}p$ < 0.001 vs. castor oil group, one-way analysis of variance with post-hoc Tukey test

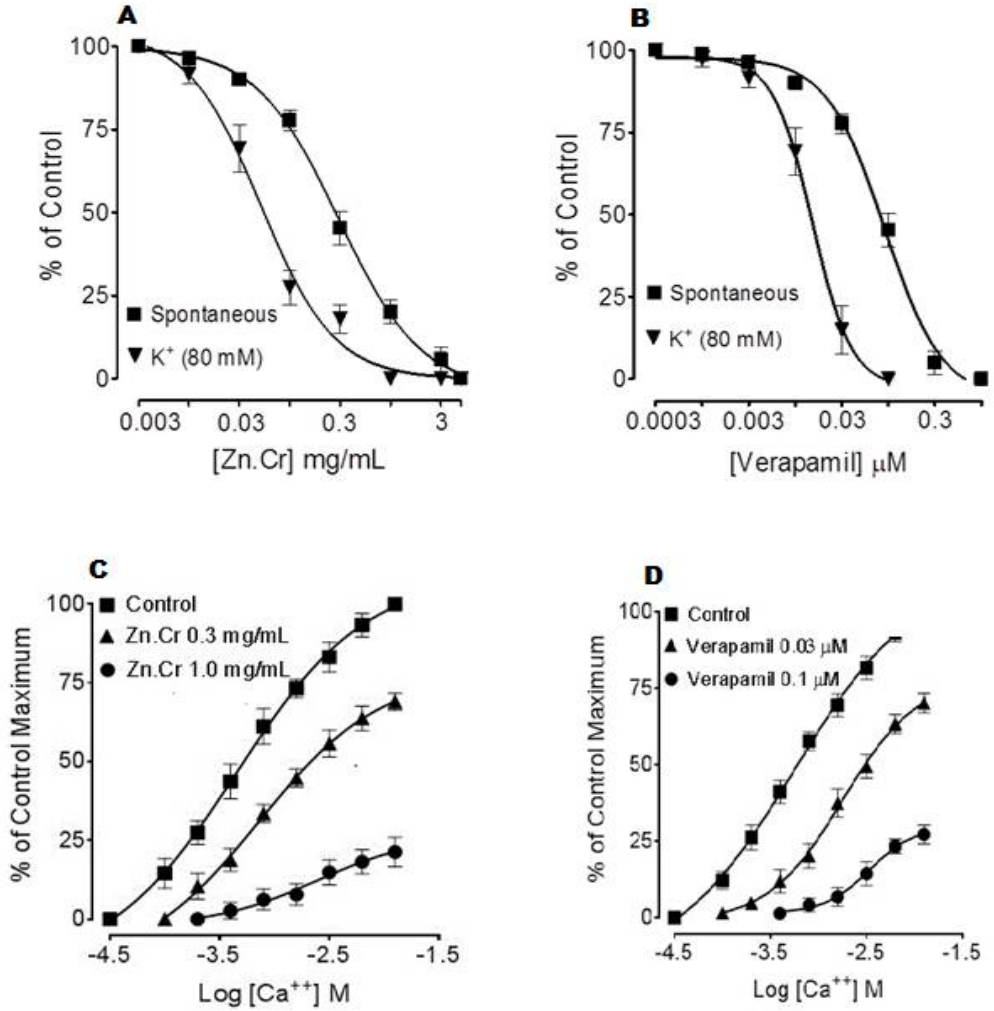

Figure 2: Concentration-dependent inhibitory effect on spontaneous and K^+ (80 mM)-induced contractions of *Ziziphus nummularia* crude extract (Zn.Cr) **(A)** and Verapamil **(B)** on isolated rabbit jejunum preparations. Concentration-response curves of Ca^{2+} in absence and presence of increasing concentrations of *Ziziphus nummularia* crude extract (Zn.Cr) **(C)** and verapamil **(D)** in isolated rabbit jejunum preparations. Values shown are mean ± SEM (n = 4)

Table 2: Protective effect of *Ziziphus nummularia* crude extract (Zn.Cr) and omeprazole against ethanol-induced gastric ulcers in rats

Treatment	Ulcer index	Inhibition (%)
Saline 10 mL/kg + ethanol (1 mL/100 g)	4.42 ± 0.10	-
Zn.Cr (300 mg/kg) + ethanol (1 mL/100 g)	2.32 ± 0.080***	52.5
Zn.Cr (1000 mg/kg) + ethanol (1 mL/100 g)	0.28 ± 0.037***	93.6
Omeprazole (30 mg/kg) + ethanol (1 mL/100 g)	0.1 ± 0.044***	97.7

***$P < 0.001$ compared to control saline group, one-way analysis of variance with post-hoc Tukey test (n = 5)

Thus, an agent having the potential to protect against diarrhea may manifest its effect by inhibiting the contractions and/or decreasing the hypersecretory state in the bowel of the experimental animal. This indicates that either the extract (Zn.Cr) has an inhibitory effect on gut motility or hyper-intestinal secretions. The secretory functions in the gastrointestinal organs are dependent to certain degree on intracellular Ca^{2+} levels, subsequently consequences for gastric acids and intestinal fluid discharge may be affected by medication that hinder Ca^{2+} influx [9]. *Ziziphus nummularia* showed protection against castor oil-induced intestinal fluid accumulation in mice. The antidiarrheal and antisecretory activities of Zn.Cr may be due to gastrointestinal relaxant component(s) present in *Ziziphus nummularia.*

Spontaneous contracting rabbit jejunum preparation is routinely used to determine the spasmolytic effect, without the use of spasmogen

(agonist). Action potentials and slow waves are responsible for the production of spontaneous phasic contractions in intestinal smooth muscles [14]. The antispasmodic effect of Zn.Cr is caused by the inhibition of spontaneous contractions in isolated rabbit jejunum preparations. To determine the spasmolytic action of Zn.Cr, the jejunal preparations was depolarized using high K^+ (80 mM). Through opening of voltage-gated L-Type Ca^{2+} channels thus creating a contractile impact [15]. Any agent that produced effect through the inhibition of high K^+-induced contraction is considered a blocker of Ca^{2+} influx. High K^+ (80 mM)-induced contractions is relaxed by Zn.Cr, similar to that caused by verapamil (a standard Ca^{2+} antagonist) [16]. The plant extract caused a rightward shift in Ca^{2+} CRCs (constructed in the applied K^+-rich and Ca^{2+}-free medium) and suppressed the maximal response, strengthened the presence of Ca^{2+} antagonist constituent(s), as exhibited by verapamil.

The observed anti-diarrheal, anti-secretory, anti-ulcer and anti-spasmodic effects of *Ziziphus nummularia* supports its folk medicinal use in diarrhea, spasm and ulcers. This is expected as Ca^{2+} antagonists are known to possess such activities [8].

Ethanol induced gastric ulcer was employed to study cytoprotective effect of Zn.Cr. Ethanol is one of the most widely recognized used exploratory models for assessment of anti-ulcer activity. Ethanol incites ulcers through systems including mucus depletion, mucosal abrasion, release of superoxide anion, hydro-peroxide free radicals, thus expanded oxidative stress in the tissues and release of inflammatory mediators [11]. Zn.Cr protected the gastric mucosa against ethanol challenge as seen 37 ^oC by the decreased estimation of lesion index when compared with the control group, similar to that caused by omeprazole, used clinically as a drug of choice to treat stomach and duodenal ulcers [17]. The anti-ulcer property of *Ziziphus nummularia* might be due to its CCB effect, as Ca^{2+} antagonist are known to exhibit such action [8].

Oxidative stress also plays an important role in pathophysiology of gastric ulcers [11]. *Ziziphus nummularia* has been reported to possess antioxidant components [18], which may account for its effectiveness as an anti-ulcer agent. The presence of flavonoids and tannins in *Ziziphus nummularia* [3] might partly be responsible for its observed pharmacological effects, as phytochemicals of such classes are well known for anti-diarrheal, anti-secretory, anti-spasmodic, anti-ulcer and Ca^{2+} antagonist activities [19]. It is proposed that these active compounds, would have capacity to invigorate mucus bicarbonate and the prostaglandin secretions and counteract with the weakening effects of reactive oxidants in the gastrointestinal lumen [11] and the existence of these in *Ziziphus nummularia* shows gastrointestinal effects. The presence of other constituents present in the plant cannot be ignored.

In the acute toxicity testing, *Ziziphus nummularia* was safe up to a dose of 10 g/kg, which shows the wide therapeutic range of *Ziziphus nummularia*.

CONCLUSION

The findings of this study indicate that *Ziziphus nummularia* possesses anti-diarrheal, anti-secretory, anti-spasmodic and anti-ulcer effects, mediated possibly via voltage-gated Ca^{2+}channel blockade.

DECLARATIONS

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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