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Antidiarrheal action of *Zataria multiflora* hydroalcoholic and hexane extracts in mice

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ABSTRACT

Introduction: *Zataria multiflora* Boiss. is an indigenous herbal plant found in many parts of Iran. This herb is traditionally used as a remedy for treating gastrointestinal disorders including diarrhea. Despite the existence of few pharmacological evidences which support the antispasmodic action of *Z. multiflora in vitro*, there is no scientific report about therapeutic efficacy of *Z. multiflora* in animal models. The objective of this research was to investigate the antispasmodic activity of hydroalcoholic and hexane extracts of *Z. multiflora* on intestinal peristaltic movement as well as assessment of its antidiarrheal action in mice.

Methods: Dried leafy branches of *Z. multiflora* were coarsely powdered and subjected to extraction by ethanol or hexane in a percolator apparatus. Antispasmodic activity of *Z. multiflora in vivo* was assessed by investigating effect of the extracts on intestinal charcoal meal transit. The antidiarrheal activity of *Z. multiflora* extracts was evaluated by castor oil and magnesium sulfate-induced diarrhea. The inhibitory effects of the extracts were compared with the standard drug loperamide.

Results: The antispasmodic activity of *Z. multiflora* (20 & 40 mg/kg) hydroalcoholic and hexane extracts was confirmed by a reduction in the distance traveled by charcoal meal alongside the small intestine. *Z. multiflora* extracts (20 & 40 mg/kg) also significantly attenuated the castor oil and magnesium sulfate-induced diarrhea. Loperamide was more efficacious in reducing number of total stools in both models of diarrhea.

Conclusion: The obtained results have established a pharmacological evidence for the folkloric use of the *Z. multiflora* as an antidiarrhoeal and spasmodic agent.

Implication for health policy/practice/research/medical education:
Zataria multiflora hydroalcoholic and hexane extracts significantly inhibited small intestinal transit and reduced the severity of induced diarrhea and delayed the induction of diarrhea in animal models. These results are in consistent with traditional use of *Z. multiflora* for treatment of gastrointestinal disorders and show promising hope for preparation of a new drug.
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Introduction

Zataria multiflora Boiss. (Family Labiatae) is a medicinal plant which grows in Iran, Pakistan and Afghanistan (1). In Iran this plant is known as Avishan Shirazy and mainly used as food flavoring agent (1). This plant has several traditional medicinal uses including treatment of fever, labor pain, bone and joint pain, headache, migraine, common cold, bloating, nausea, airways obstruction and diarrhea (2). As *Z. multiflora* is a popular medicinal plant among people, its pharmacological properties have been reviewed in a number of scientific reports. There is substantial evidence for the efficacy of this medicinal plant for treatment of ailments such as pain, infection, inflammation, intestinal spasm, diabetes, and protecting liver function (3-8). Research studies revealed that both essential oil and hydroalcoholic extract of *Z. multiflora* contain pharmacologically active substances. Main constituents of the essential oil are thymol, *p*-cymene, carvacrol, linalool, γ-terpinene, *trans*-caryophyllene, α-pinene, β-pinene, carvacrol methyl ether, borneol, *cis*-sabinene hydrate, 1,8-cineole, α-terpinene, α-terpinolene, geraniol, eugenol, terpinene-4-ol, spathulenol, and

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camphor (9). Thymol is the most abundant compound among all constituents. It has been shown that essential oil of *Z. multiflora* subsides inflammatory signs of irritable bowel syndrome (1). The hydroalcoholic extract compositions are aliphatic phenols, alcohols, flavonoid, saponins and tanens. Also, apigenin, dihydroxy aromadendrene, α-tocopherolquinone, luteolin, and 6-hydroxyluteolin glycosides, as well as di-, tri-, and tetramethoxylated are compounds which have been identified in the *Z. multiflora* extracts (10).

Zataria multiflora has been traditionally used for the treatment of a number of gastrointestinal disorders including, stomachache, colitis and diarrhea (1). The anti-colitis action of the *Z. multiflora* has already been shown in animal models of colitis (11). Antispasmodic effect of *Z. multiflora* extract on smooth muscle spasm of rat ileum and uterus has been reported (3,12). However, so far the inhibitory effect of *Z. multiflora* extract on intestinal spasm *in vivo* has not been reported. Therefore, in this research we investigated the gastrointestinal effect of *Z. multiflora* hexane and hydroalcoholic extracts on intestinal movement as well as their antidiarrheal potentials in animal models.

Materials and Methods

Plant material and extraction

Aerial parts of *Z. multiflora* were collected from Ardestan region in spring 2016 (Isfahan, Iran). The plant was dried in shade at room temperature. The plant was identified as *Z. multiflora* and a specimen was deposited at botany herbarium of School of Pharmacy and Pharmaceutical Sciences (reference No. 1483). The stiff branches were separated and the rest was coarsely powdered by an electronic grinder (Moulinex, France) and then subjected to extraction in a percolator.

For the preparation of the hydroalcoholic extract, 200 g of dried and powdered *Z. multiflora* was percolated with 70% ethanol. To obtain the non-polar fractions, a powdered sample of *Z. multiflora* was extracted with n-hexane. The plant powder was percolated for 24 hours according to pharmacopoeia reference (13,14). The solvents were eliminated from the extracts by evaporation under vacuum in a rotary evaporator (Buchi Rotavapor RE) at 50°C. The crude extracts were stored in a refrigerator. A sample of the hydroalcoholic extract was totally dried on a heater for determining the percentage of remaining water.

Animals and solutions

Albino male mice (22-28 g) were purchased from School of Pharmacy animal house and handled according to university guidelines for animal care and handling (15). All animals were fasted overnight before starting the experiment with free excess to water *ad libitum*.

The extracts were prepared as described above. Loperamide and extracts were made up in 70% ethanol as 20 mg/mL stock solution. Further dilutions were made in distilled water as appropriate. Sulfate magnesium solution was prepared as 10% stock solution. For the preparation of charcoal meal tragacanth suspension (5%) was mixed with charcoal suspension (3%) solution. Unless stated, all chemicals were from Merck (Germany).

Gastrointestinal transit test

Animals were fasted for overnight and divided into seven groups. Mice in groups I and II received oral administration of loperamide solution (2 mg/kg) or vehicle (0.35% ethanol). Groups III and IV received the hydroalcoholic extract (20 mg/kg and 40 mg/kg p.o.). Groups V and VI received the hexane extract (20 and 40 mg/kg p.o.). The animals in control group (VII) were treated with equivolume amount of the vehicle (7% ethanol). 0.5 mL of charcoal meal was administered orally using steel feeding tube after 30 minutes and the animals were left in the cage. Forty-five minutes after oral administration of charcoal meal, each animal was anesthetized with diethylether and sacrificed. The entire intestine was carefully dissected out and lined up on the table. The total length of the small intestine and the distance that charcoal meal had traveled was measured. The volume of administrated solution was adjusted as such that all animals were received 0.5 mL of drug or vehicle.

Castor oil-induced diarrhea

Animals were fasted overnight and divided into seven groups as above. On the day of experiment 0.5 mL of hydroalcoholic extract, hexane extract or loperamide were given orally to the animals (n = 10). The animals in control group were treated with equivolume amount of vehicle as above. Half an hour later, diarrhea was induced by oral administration of castor oil (0.5 mL). Each animal was placed on a tissue paper under a glass funnel and observed for any wet defecation for a period of 3 hours.

Magnesium sulfate-induced diarrhea

A similar protocol as for castor oil-induced diarrhea was followed. Diarrhea was induced by oral administration of $MgSo_4$ (2 g/kg). Half an hour later, the effect of *Z. multiflora* hydroalcoholic and hexane extracts were examined on diarrhea induced by $MgSO_4$ and compared with the standard drug loperamide. The animals in control group were treated with vehicle.

Measurement and statistics analysis

Gastrointestinal transit was expressed as the percentage of distance that charcoal moved relative to the whole length of small intestine. Diarrhea was scored as the number of watery defecation at appropriate time. The absorbent paper was changed regularly and number of wet defecations was recorded. Number of diarrheic feces excreted at 30min intervals as well as total wet fecal outputs was calculated. Delay in the induction of diarrhea was the time when first sing of diarrhea was observed following administration of

laxatives. Mean and standard error of mean (SEM) were calculated for each group of results and compared with the control groups using unpaired Student's *t* test. SigmaPlot software (version 11) was used for statistical analysis and plotting the graphs.

Results

Plant extraction

Concentrated hydroalcoholic extract had a brown-greenish color. The water content was calculated to be 20%. The yield of hydroalcoholic extract was 12% (W/W). The hexane extract has brownish color and its yield was 1% (W/W).

Gastrointestinal transit test

In the control group treated with vehicle, the charcoal meal covered up to about three quarters of the entire small intestine (Figure 1). Loperamide (2 mg/kg) significantly reduced the charcoal meal transit by 67% (Figure 1). The hydroalcoholic extract with oral doses of 20 mg/kg and 40 mg/kg reduced gastrointestinal transit by 14% and 40% respectively (Figure 1). Similarly the hexane extract attenuated the charcoal meal transit by 33% and 49% with oral doses of 20 mg/kg and 40 mg/kg, respectively (Figure 1).

Castor oil-induced diarrhea

Mice in the control group produced copious diarrhea following oral administration of castor oil. First sign of diarrhea was observed within first half an hour after castor oil administration and diarrhea reached its peaks about 75 minutes and thereafter gradually subsided down (Figure 2a). On the other hand, animals pretreated with hydroalcoholic extract of *Z. multiflora* and hexane extract of *Z. multiflora* showed a significant delay in onset of diarrhea (Table 1), decrease in the frequency of wet defecation (Figure 3) and the total wet stool recorded over 3 hours time (Figure 4). Few mice showed no sign of diarrhea after treatment with loperamide or extracts. Loperamide reduced the incidence of total wet defecation by 80% while hydroalcoholic extract of *Z. multiflora* with doses of 20 mg/kg and 40 mg/kg reduced the incidence of total wet discharge by 49% and 63%, respectively in comparison to their corresponding control groups. Similarly hexane extract (20 & 40 mg/Kg) reduced the total number of wet defecation by 56% and 73%, respectively over the course of the study (Figure 4).

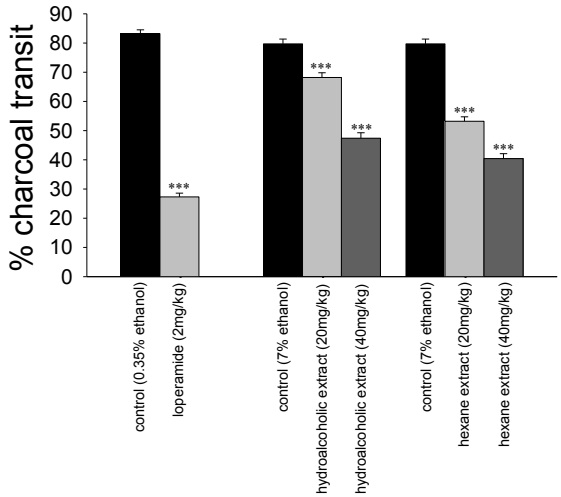

Figure 1. Effect of loperamide, *Zataria multiflora* hydroalcoholic and hexane extracts on small intestinal transit (0.5 mL of charcoal meal). Data are presented as mean ± SEM (n=10). Stars show degree of statistically significant difference in comparison with its appropriate control group (***$P < 0.001$, Student's *t* test).

Magnesium sulfate-induced diarrhea

In the control group treated with the vehicle, oral administration of $MgSO_4$ induced watery stool discharge in all the animals. Time of induction and the peak of diarrhea followed a similar pattern as it was seen with the castor oil (Figure 5). However, the severity of observed watery defecation was less copious than those seem with the castor oil. Loperamide significantly delayed the onset of diarrhea (Table 1) and two mice had no sign of diarrhea

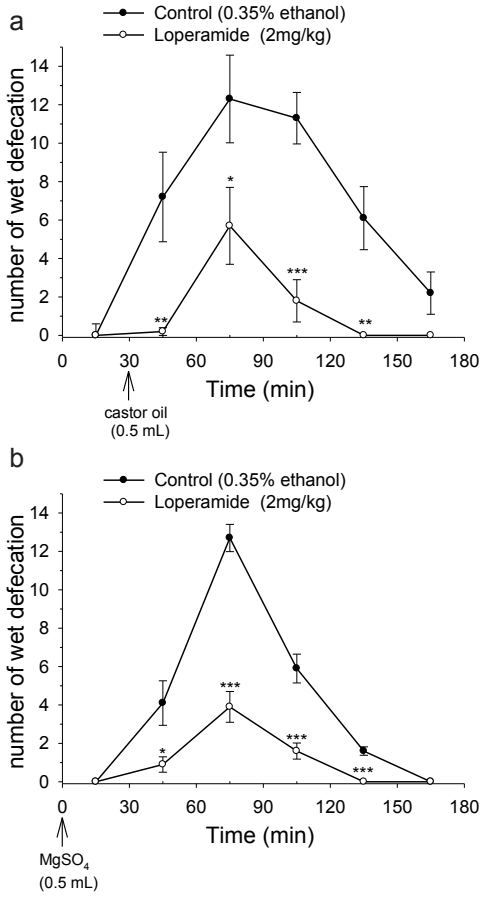

Figure 2. Antidiarrheal activity of loperamide with castor oil (0.5 mL p.o) and $MgSO_4$ (0.5 mL, 10% solution) induced diarrhea. Incident of diarrhea were assessed as number of wet defecation at 30min intervals. Data are shown as mean ± SEM, n=10 for each group. Key: **$P < 0.01$, ***$P < 0.001$ in comparison with corresponding vehicle treated control group (Student's *t* test).

Table 1. Effect of *Zataria multiflora* extracts on induction of diarrhea following oral administration of castor oil and magnesium sulfate in mice

Treatment	Dose	Delay in induction of diarrhea (min)	
		Castor oil	$MgSO_4$
Control group	7% ethanol	50±4.6	54±3.2
Hydroalcoholic extract	20 mg/kg	70±9.5	62±3.6
Hydroalcoholic extract	40 mg/kg	79±11*	61±3.4
Hexane extract	20 mg/kg	63±3.8*	56±3
Hexane extract	40 mg/kg	70±5**	674.5*
Control group	0.35% ethanol	54±6.5	54±3.2
Loperamide	2 mg/kg	75±4.8*	66±5.2*

All data are expressed as mean ± SEM. for each group (n = 10). *P < 0.05, **P < 0.01 in comparison with corresponding control group (Student's *t* test).

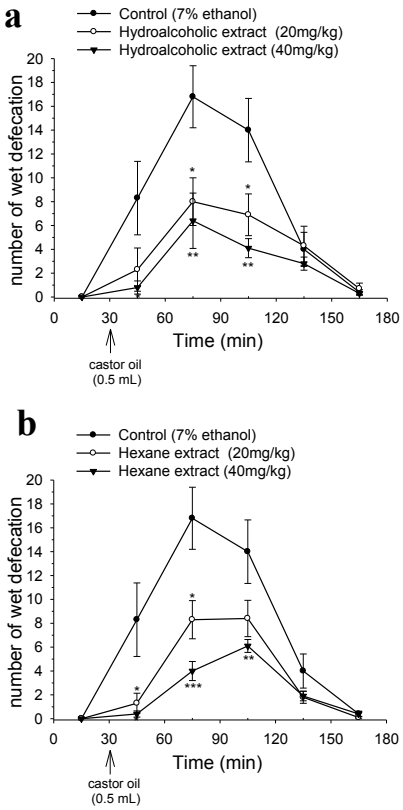

Figure 3. Antidiarrheal activity of *Zataria multiflora* hydroalcoholic (a) and hexane (b) extracts on induced diarrhea by castor oil (0.5 mL p.o). The incidence of diarrhea was assessed as number of wet defecation at 30 min intervals. Data are shown as mean ± SEM (n = 10). Key:*P<0.05, **P < 0.01, ***P < 0.001 in comparison with vehicle treated control group (Student's *t* test).

at all. Loperamide significantly reduced number of wet defecation by 73% in comparison to the vehicle treated control group (Figure 6). Hydroalcoholic and hexane extracts of *Z. multiflora* (20 & 40 mg/kg) significantly reduced the frequency of wet defecation (Figure 5). The incidence of total wet discharge was reduced by 33% and 48% in the test group treated with a dose of 20 mg/kg of hydroalcoholic or hexane extracts, respectively. The

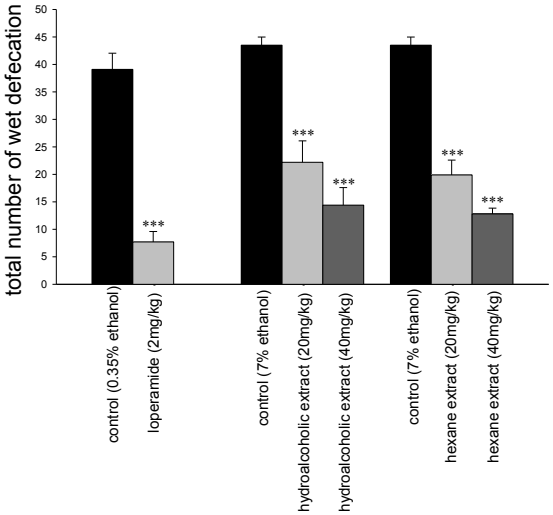

Figure 4. Antidiarrheal activity of loperamide, *Zataria multiflora* hydroalcoholic and hexane extracts on induced diarrhea by castor oil (0.5 mL p.o). Each bar shows the total number of wet defecation following oral administration of castor oil. Data are presented as mean ± SEM (n = 10). Key: ***P < 0.001 in comparison with the corresponding vehicle treated control group (Student's *t* test).

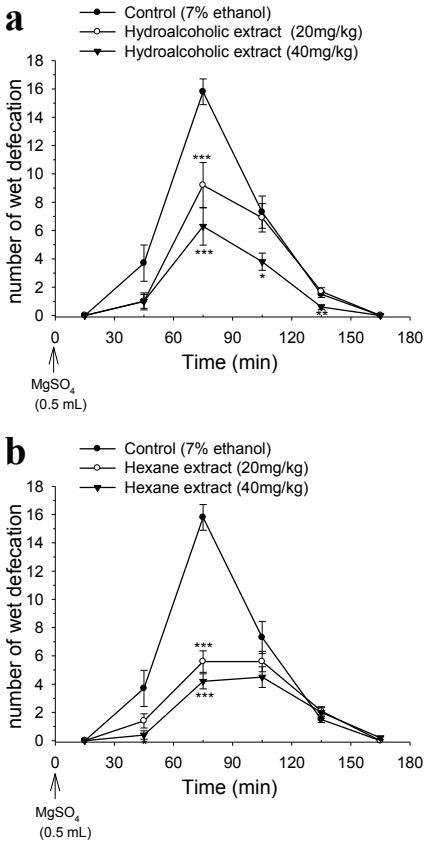

Figure 5. Antidiarrheal activity of *Zataria multiflora* hydroalcoholic (a) and hexane (b) extracts on induced diarrhea by $MgSO_4$ (0.5 mL,10% solution). Incidences of diarrhea were assessed as number of wet defecation at 30 min intervals. Data are as mean ± SEM (n = 10). Key: *P<0.05, **P<0.01, ***P<0.001 in comparison with vehicle treated control group (*Student's t-test*).

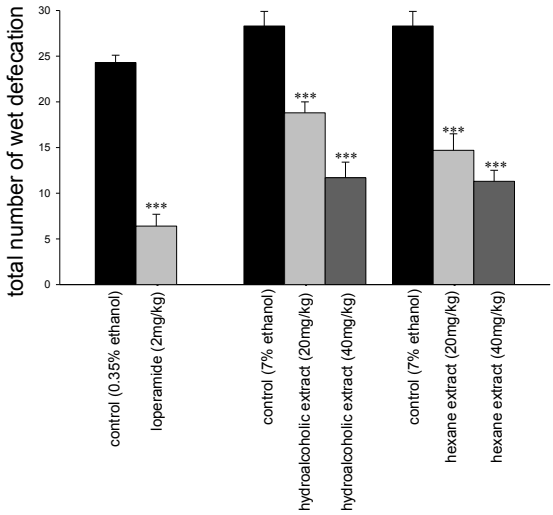

Figure 6. Antidiarrheal activity of loperamide, *Zataria multiflora* hydroalcoholic and hexane extracts on induced diarrhea by $MgSO_4$ (0.5 mL, 10% solution). Each bar shows the total number of wet defecation following oral administration of $MgSO_4$. Data are presented as mean ± SEM (n = 10). *** $P < 0.001$ in comparison with the corresponding vehicle treated control group (Student's *t* test).

severity of the diarrhea was further reduced by increasing the oral dose of the extracts to 40 mg/kg (Figure 5) and the total number of wet defecation over the course of the study was reduced by 58% and 60%, respectively (Figure 4). Loperamide (2 mg/kg), *Z. multiflora* hydroalcoholic and hexane extracts (20 & 40 mg/kg) significantly inhibited small intestinal transit and reduced the severity of induced diarrhea and delayed the induction time of diarrhea.

Discussion

Diarrhea has long been recognized as a common disease of tropical areas and has been responsible for morbidity and mortality of children and infants, especially in developing countries (16). The World Health Organization on its Diarrhoeal Diseases Control Program has emphasized on the use of traditional medicine in order to reduce diarrhea morbidity and mortality (16). Diarrhea is mainly associated with impairment of fluid and electrolyte absorption or intense increase in intestinal motility (17). Herbal medicines have long been used as a remedy for the treatment of gastrointestinal disorders including diarrhea (2). *Z. multiflora* is a medicinal plant which has been used for gastrointestinal discomfort in Asia (2). The main goal of this research was to investigate the antidiarrheal effect of *Z. multiflora* extract in animal models. *In vivo* antidiarrhoeal activity of extracts was assessed using castor oil and $MgSo_4$-induced diarrhea (18). Castor oil is known as a stimulant laxative. It works by increasing the movement of the intestines. Castor oil is non-irritant, but in small intestine it is hydrolyzed to ricinoleic acid which has a strong irritant effect on the gut mucosa (19). Ricinoleic acid induces diarrhea by releasing nitric oxide (NO), stimulating prostaglandin synthesis and increasing peristalsis (20). On the other hand, $MgSo_4$ is an osmotic laxative (18). $MgSO_4$ induces diarrhea by promoting cholecystokinin release from the duodenal mucosa preventing the reabsorption of sodium chloride and water from the lumen (18). Loperamide had profound antidiarrheal activities on both castor oil and $MgSo_4$-induced diarrhea. Loperamide is opioid receptor agonist and acts on μ-receptor on both intestinal smooth muscle and on the nerve terminal innervated gut. This inhibits intestinal peristaltic activity and reduces water and electrolyte secretion (21). Reduction in intestinal motility is associated with inactivating Ca^{2+} ion influx through voltage gated Ca^{2+} channels (22). Reduction in neurotransmitters release, especially acetylcholine, is due to activation of presynaptic opioid receptors which act as autoreceptor on cholinergic nerve terminals (21,22). As intestinal peristaltic movement is mostly controlled by parasympathetic activities, slowing of intestinal movement by loperamide could also be due to reduction in acetylcholine release. Attenuation of parasympathetic activities allows more time for water absorption in gastrointestinal tract. Suppression of $MgSo_4$-induced diarrhea by loperamide could also be explained by inhibition of acetylcholine release and reduction of electrolyte secretions.

Pretreatment of mice with both hydroalcoholic and hexane extracts of *Z. multiflora* provided significant protection against castor oil induced diarrhea. Comparison of antidiarrhoeal action of hydroalcoholic and hexane extract shows that they have a relatively similar effect and this may indicate that the antidiarrhoeal compounds exist in both types of extracts. Hexane extract mainly contain the non-polar constituents while both polar and non-polar constituents exist in hydroalcoholic extract. This may indicate that the active components mainly have non-polar properties. Apigenin has been identified as one the active compounds of *Z. multiflora* extract (10). In another research we have reported the antidiarrheal action of apigenin (23). However, it is likely that other active ingredients exist which need to be identified.

In the case of $MgSo_4$, the extracts were given after induction of diarrhea. Both hydroalcoholic and hexane extracts of *Z. multiflora* reduce severity and number of diarrhea incidence. Within 45 minutes following oral administration of *Z. multiflora* extract, the antidiarrheal action of the extract was visible. This shows that the active ingredient has relatively fast action. When the antidiarrheal doses of the extracts are compared with that of loperamide, it looks that loperamide is more potent the extract. However, the extract is composed of unknown numbers of active and inactive constituents. With disregard of inactive substance it is likely that antidiarrheal activity of *Z. multiflora* extract will not be less than loperamide. Therefore, isolation and identification of all active ingredients of *Z. multiflora* extract are recommended.

Measurement of charcoal meal transit is used as an indication of intestinal peristaltic movement. Hydroalcoholic extract of *Z. multiflora* has potent inhibitory effect on isolated ileum contraction induced by acetylcholine, high potassium concentration and neuronal stimulation (3). Therefore, reduction in charcoal meal transit by hydroalcoholic extract of *Z. multiflora* was expected. Reduction in intestinal motility could also explain the antidiarrheal action of *Z. multiflora* extracts. As the hexane extract shows similar anti-motility activity, it is possible that similar components which exist in both types of extract are responsible for reduction in intestinal motility. Apigenin which is a known constituent of *Z. multiflora* also reduced intestinal motility under similar condition (23). Although apigenin could have a substantial participation in reduction of intestinal movement, however, the role of other substances such those exist in the essential oil should not be ignored. For instance it has been shown that essential oil components such as α-pinene, β-pinene, α-terpineol, geraniol have antispasmodic activities on isolated ileum (24-26). These components are also found in the essential oil of *Z. multiflora*.

Conclusion

In this research pharmacological evidence was presented for the effectiveness of *Z. multiflora* extracts as antispasmodic and antidiarrheal remedies. As the extract is a combination of active and inactive substances, separation and identification of the active ingredients for further investigations and development of lead compounds are recommended.

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Authors' contributions

GA was responsible for preparation of extracts while HS supervised the pharmacological studies. HJ was responsible for performing the experimental work.

Conflict of interests

The authors declare no conflict of interest.

Ethical considerations

Ethical issues have been observed by the authors.

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