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Original Article

Antidiarrhoeal assessment of hydroalcoholic and hexane extracts of *Dracocephalum kotschyi* Boiss. and apigenin in mice

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Abstract

Dracocephalum kotschyi Boiss, a member of Labiatae family, is a native plant to Iran, which has been reported to have immunomodulatory, antihyperlipidemic and antispasmodic activities. The objective of this research was to study the antispasmodic and antidiarrhoeal activities of hydroalcoholic and hexane extracts of *D. kotschyi* in mice. Furthermore, the antidiarrhoeal and antispasmodic effect of apigenin, a flavonoid constituent of *D. kotschyi*, was also studied. Hydroalcoholic and hexane extracts were obtained from aerial part of *D. kotschyi* using percolation method. Antispasmodic effect of the test compounds was assessed by measurement of small intestine transit following oral administration of a charcoal meal. Diarrhoea was induced by administration of either castor oil (0.5 ml) or magnesium sulphate ($MgSO_4$) (10% w/v solution). Both the hydroalcoholic and hexane extracts of *D. kotschyi* (10 and 20 mg/kg) reduced the intestinal charcoal meal transit. Loperamide (2 mg/kg) and apigenin (2 and 10 mg/kg) inhibited intestinal movement of the charcoal meal and also inhibited castor oil and $MgSO_4$-induced diarrhoea. The hydroalcoholic and hexane extracts of *D. kotschyi* (10 and 20 mg/kg) also significantly inhibited the castor oil and $MgSO_4$-induced diarrhoea in mice in comparison with the vehicle-treated control groups. This study confirms that both the hydroalcoholic and hexane extracts of *D. kotschyi* has antispasmodic and antidiarrhoeal properties *in vivo* and could be a suitable remedy for treatment of gastrointestinal disorders in which smooth muscle spasm and/or diarrhoea plays a significant roles.

Keywords: *Dracocephalum kotschyi*; Antispasmodic; Antidiarrhoeal; Apigenin; Ileum transit

INTRODUCTION

Dracocephalum kotschy Boiss. (Labiatae family) is a medicinal plant, which grows in many parts of Iran (1,2). Badrandjboie-Dennaie and Zarrin-giah are local Persian names of this plant (3). *D. kotschyi* is an aromatic plant which is enriched in various essential oil including α-pinene, neral, geraniol, α-citral, limonene, cyclononadiene, terpinene-4-ol, linalool, carveol, myrcene, germacrene–D, isopinocarveol and α-terpineol (4). In Iranian traditional medicine, this plant has been used as a remedy for treatment of inflammatory pain, ulcer and fever (5-7). This medicinal plant also is used for many aliments such as muscle spasm, congestion, bloating, and other gastrointestinal disorders. Several pharmacological activities have been attributed to *D. kotschyi*. For instance, the essential oil of *D. kotschyi* has shown to have antinociceptive effects in mice (7). The hydroalcoholic extract of *D. kotschyi* is reported to have antihyperlipidemic effect in animal model (8). The leaf extract of *D. kotschyi* inhibits tumor proliferation and has potential anti-cancer properties in mice (9,10).

D. kotschyi extract has also been used as antispasmodic agent in Iranian traditional medicine (1,7). Both the essential oil and the hydroalcoholic extract of *D. Kotschyi* reported to have spasmolytic activities on isolated ileum (4,11). The *D. kotschyi* extract in concentration dependent manner reduced ileum contractions induced by KCl, acetylcholine and neuronal stimulation with

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IC_{50} values of 36 ± 5.1, 101 ± 9.5 and 96 ± 7.1 µg/ml respectively (11). Therefore, the extract of *D. kotschyi* has a potent antispasmodic activity *in vitro*. It is believed that the antispasmodic effect of plant extract is mainly due to the presence of flavonoids constituents (6). The flavonoid constituents of *D. kotschyi* extract includes, apigenin, calycopterin, xanthomicrol, isokaempferide, luteolin, luteolin 7-O-beta-D-glucopyranoside, luteolin 3'-O-beta-D-glucuronide, apigenin 4'-O-beta-D-glucopyranoside, acacetin 7-O-beta-D-glucopyranoside and rosmarinic acid (6). Flavonoids are widely distributed in the plant kingdom and occur in many medicinal plants (12). Apigenin is one of the common flavonoid present in medicinal plants (12).

So far, there is no report about antispasmodic or antidiarrhoeal effect of *D. kotschyi* extract *in vivo*. Therefore, the aim of current research was to examine the antispasmodic as well as antidiarrhoeal effects of *D. kotschyi* extract in mice. In addition, the effect of apigenin, a flavonoid constituent of *D. kotschyi*, was also examined for comparison with the effect of the extract with similar experimental protocol.

MATERIALS AND METHODS

D. kotschyi aerial parts were purchased from a cultivated farm Fereydun-shahr (in Isfahan province, Iran) and identified at the Botany Department of the Faculty of Sciences, University of Isfahan. A voucher specimen (No. 1519) was deposited at the herbarium of the School of Pharmacy and Pharmaceutical Sciences of Isfahan University of Medical Sciences.

The plant materials were dried in shade and grained to powder using electrical miller (Moulinex, France). The hydroalcoholic and hexane extracts were prepared by percolation method (13). The ratio of plant powder to solvent for hydroalcoholic and hexane extracts were 1:10 and 1:8, respectively. The yields of hydroalcoholic and hexane extracts were 31% and 2%, respectively.

Drugs and solutions

The following drugs and solutions were used in this research: *D. kotschyi* hydroalcoholic and hexane extracts, loperamide and apigenin. The hydroalcoholic extract was made up as 10 mg/ml stock solution in 50% ethanol and diluted in distilled water to obtain concentrations of 1 and 0.5 mg/ml. The hexane extract was made up as 10 mg/ml stock solution in ethanol and further serial dilution was made in distilled water (1 mg/ml and 500 µg/ml). Loperamide was dissolved in ethanol as 1 mg/ml stock solution and was further diluted with distilled water (100 µg/ml). Apigenin was made up as 1 mg/ml stock suspension or solution in either 1% carboxymethyl cellulose (CMC) or ethanol. Further dilution was made in distilled water (500 and 100 µg/ml respectively). Magnesium sulfate was made up as 10% stock solution in distilled water. Charchol (3%) plus tragacanth powder (5%) suspension were prepared in distilled water. All drugs were purchased from Sigma, while the chemicals were from Merck (Germany). Castor oil was purchased from Hannan company (Iran).

Pharmacological studies

Male albino mice (25-30 g), bred in School of Pharmacy and Pharmaceutical Sciences (Isfahan University of Medical Sciences, Iran) animal house were kept at room temperature. The animals were fasted overnight prior to the experiments with free access to water. All animals were handled in accordance with the internationally accepted principles for laboratory animal use and care (14).

In this study effect of hydroalcoholic and hexane extracts of *D. kotschyi*, loperamide (2 mg/kg) and apigenin (2 and 10 mg/kg) were examined on gut motility using charcoal meal transit test. In addition, antidiarrhoeal effects of above substances were examined on castor oil and magnesium sulphate ($MgSO_4$)-induced diarrhoea. All experiments were conducted in parallel with control groups treated with equivalent volume of the vehicle. Each dose of drug was examined on 10 separate mice. Stock solution was adjusted in such way that each mouse was given 0.5 ml of test drugs or extracts.

Charcoal meal transit test

In this test, movement of charcoal meal in the intestine was assessed. For this purpose *D.*

kotschyi hydroalcoholic extract (10 and 20 mg/kg), hexane extract (10 and 20 mg/kg), apigenin (2 and 10 mg/kg) or loperamide (2 mg/kg) was given orally to mice and 30 min later 0.5 ml of charcoal meal containing 3% charcoal plus 5% tragacanth suspension was administered orally. Forty five minutes after charcoal meal administration, each animal was sacrificed and distance of charcoal movement in the small intestine was measured using a ruler.

Castor oil-induced diarrhoea

In this test, preventive effect of *D. kotschyi* hydroalcoholic extract (10 and 20 mg/kg), hexane extract (10 and 20 mg/kg), apigenin (2 and 10 mg/kg) or loperamide (2 mg/kg) were examined on diarrhoea induced by oral administration of 0.5 ml castor oil. Half of the animals were treated with the equivalent volume of the vehicle. Castor oil was administered 30 min after oral administration of drugs or vehicle (15,16). Each animal was placed under a large glass funnel and time of induction of diarrhoea and number of wet defecation on tissue paper was recorded over 3.5 h at fixed interval time. Filter papers were replaced accordingly to eliminate over spotting of wet defecation.

Magnesium sulphate-induced diarrhoea

In this case, each mouse was initially treated orally with 0.5 ml of $MgSO_4$ solution (2 g/kg) and placed under funnel for assessment of diarrhoea. Half an hour later *D. kotschyi* hydroalcoholic extract (10 and 20 mg/kg), hexane extract (10 and 20 mg/kg), apigenin (2 and 10 mg/kg), loperamide (2 mg/kg) or vehicles were given intragastrically to the animals and incidence of diarrhoea was assessed as explained above (15,16).

Measurement and statistical analysis

Number of wet defecation over the course of study was used for the assessment of total diarrhoea index. Ileum transit was expressed as percentage of charcoal moved from pylorus to the caecum relative to the whole length of the ileum.

All results were expressed as mean ± standard error of mean (SEM) and compared with corresponding vehicle-treated control group using unpaired Student's t-test. *P* values less than 0.05 was considered statistically significant. Two ways analysis of variance (ANOVA) was used for comparison of two different experimental groups. SigmaPlot computer program (version 11) was used for statistical analysis and plotting the graphs.

RESULTS

Charcoal meal transit test

In the control group, during 45 min test time, the charcoal meal moved about 92% of the total small intestine. Loperamide significantly reduced the distance moved by charcoal meal. In fact loperamide reduced charcoal meal movement by 65% (Fig. 1). In the control groups treated with 2.5% or 5% ethanol, there was no difference in the charcoal meal movements, although 10% ethanol slightly reduced the charcoal meal intestinal transit. Higher concentration of ethanol (20% ethanol) had profound effect and therefore drug suspension in 1% CMC was used.

The hydroalcoholic extract of *D. kotschyi* (10 and 20 mg/kg) significantly reduced the distance moved by charcoal meal intestinal transit in comparison with the vehicle treated control groups by 28% and 35% respectively (Fig. 1). Similarly the hexane extracts of *D. kotschyi* (10 and 20 mg/kg) reduced the charcoal meal movements by 25% in comparison with vehicle-treated control groups (Fig. 1). Apigenin with doses of 2 mg/kg and 10 mg/kg also inhibited intestinal movement by 35% and 20% respectively in comparison with the its own control group (Fig. 1).

Castor oil-induced diarrhoea

At least half an hour after administration of castor oil, first sign of wet defecation was observed in the animals. The severity of the diarrhoea gradually increased and within 1.5 h reached its peak and thereafter gradually subsided (Fig. 2). In the control group treated with castor oil, all the animals had clear diarrhoea while in the loperamide treated group 4 animals had no sign of diarrhoea at all.

In the rest of the group, loperamide caused a significant delay in induction of diarrhoea (Table 1) and substantially reduced the severity of diarrhoea (Fig. 2).

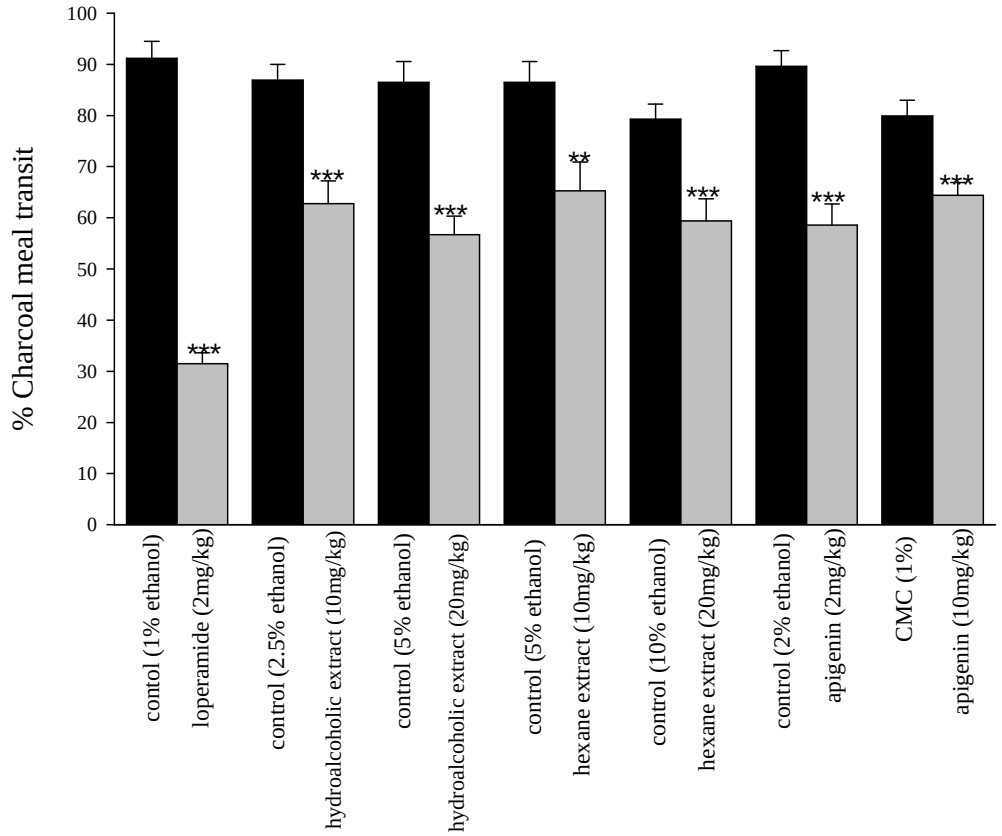

Fig. 1. Effect of hydroalcoholic and hexane extracts of *Dracocephalum kotschyi*, apigenin and loperamide on intestinal distance moved by charcoal meal (0.5 ml; per orum) in comparison with the vehicle treated control groups in mice. Values are expressed as the Mean ± SEM for each group. ***P*<0.01, ****P*<0.001 in comparison with corresponding vehicle-treated control group (Student's t-test). The number of observations was 10 mice in each group. CMC: carboxymethyl cellulose.

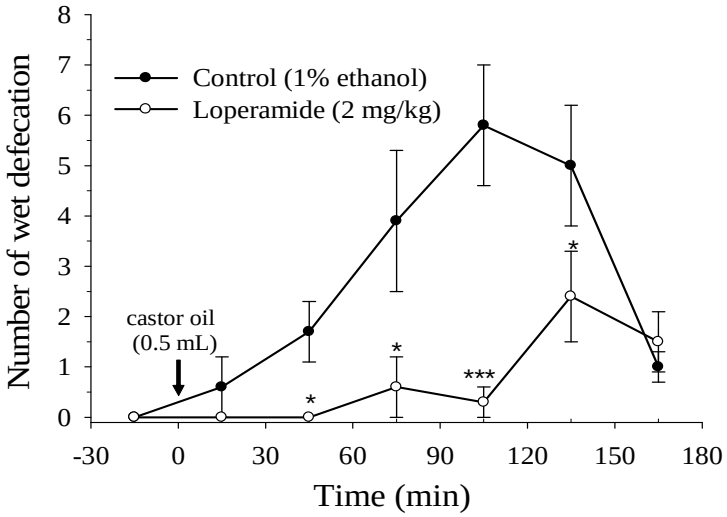

Fig. 2. Effect of loperamide on castor oil induced diarrhoea over a 3-h time course. Loperamide was given orally 30 min before oral administration of 0.5 ml castor oil. Each point presents mean incidence of wet defecation at 30 min interval times. The data are presented as the Mean ± SEM (n = 10). Differences in the mean values among loperamide and control group are statistically significant (*P*<0.01; two way ANOVA). Stars show the statistically significant differences between incidence of diarrhoea and the vehicle treated control at corresponding time intervals. **P*<0.5, ****P*<0.001 (Student's t-test).

The total incidence of diarrhoea was reduced by 73% in loperamide treated group in comparison with the vehicle treated control (Fig. 3).

The hydroalcoholic extract of *D. kotschyi* (10 and 20 mg/kg) significantly reduced the castor oil-induced diarrhoea by 52% and 67% in comparison with the vehicle treated control groups respectively (Fig. 3). One animal in each group had no sign of diarrhoea at all. Similarly, the hexane extract of *D. kotschyi* (10 and 20 mg/kg) inhibited castor oil-induced diarrhoea by 40% and 60% respectively in comparison with the vehicle treated control groups (Fig. 3).

Apigenin, a flavonoid constituent of *D. kotschyi*, with doses of 2 mg/kg and 10 mg/kg also inhibited castor oil-induced diarrhoea by 36% and 63% respectively in comparison with the control group (Fig. 3).

Magnesium sulphate-induced diarrhoea

In the control group, 30 min after administration of $MgSO_4$ (0.5 ml of 10% solution) initial sign of wet defecation was observed. The peak effect of diarrhoea was seen 45 min later and thereafter the incidence of the diarrhoea gradually subsided (Fig. 4). Therefore, 3 h time course of study was a suitable time for studying the antidiarrhoeal effect of drugs in this model (15,16).

Table 1. Effect of hydroalcoholic and hexane extracts of *Dracocephalum kotschyi*, apigenin and loperamide on induction time of diarrhoea in comparison with vehicle-treated control groups following oral administration of castor oil (0.5 ml) in mice.

Drugs	Dose (mg/kg) (vehicle)	Delay in induction of diarrhoea (min)	
		Test group	Control group
Hydroalcoholic extract of *D. kotschyi*	10 (2.5% ethanol)	89 ± 10	85 ± 7
	20 (5% ethanol)	90 ± 10	71 ± 6
Hexane extract of *D. kotschyi*	10 (5% ethanol)	88 ± 6.1	72 ± 6
	20 (10% ethanol)	96 ± 5.5*	81 ± 4
Apigenin	2 (2% ethanol)	93 ± 8.5	79 ± 7
	10 (1% CMC)	94 ± 10	71 ± 8.5
Loperamide	2 (1% ethanol)	125 ± 10***	58 ± 7.5

The number of observations was 10 mice in each group. Values are expressed as mean ± SEM for each group. *P<0.05, ***$P<0.001$ in comparison with corresponding vehicle-treated control group (Student's t-test). CMC; carboxymethyl cellulose.

Table 2. Effect of hydroalcoholic and hexane extracts of *Dracocephalum kotschyi*, apigenin and loperamide on induction time of diarrhoea in comparison with vehicle-treated control groups following oral administration of 10% $MgSO_4$ solution (0.5 ml) in mice.

Drugs	Dose (mg/kg) (vehicle)	Delay in induction of diarrhoea (min)	
		Test group	Control group
Hydroalcoholic extract of *D. kotschyi*	10 (2.5% ethanol)	58 ± 5*	41 ± 4
	20 (5% ethanol)	64 ± 4***	42 ± 3
Hexane extract of *D. kotschyi*	10 (5% ethanol)	50 ± 2.5*	40 ± 3
	20 (10% ethanol)	68 ± 3*	56 ± 2
Apigenin	2 (2% ethanol)	63 ± 3***	43 ± 4
	10 (1% CMC)	55 ± 3	49 ± 4
Loperamide	2 (1% ethanol)	65 ± 3***	39 ± 2

The number of observations was 10 mice in each group. Values are expressed as mean ± SEM for each group. *P<0.05, ***P<0.001 in comparison with corresponding vehicle-treated control group (*Student's t-test*). CMC: carboxymethyl cellulose.

In the control group treated with $MgSO_4$, all the animals had clear diarrhoea while in the loperamide treated group 5 animals had no sign of diarrhoea at all. Loperamide suppressed the induction (Table 2) and the severity of diarrhoea (Fig. 4). The total incidence of diarrhoea was reduced by 85% in loperamide-treated group in comparison with the vehicle-treated control (Fig. 5).

The hydroalcoholic extract of *D. kotschyi* (10 and 20 mg/kg) significantly reduced the $MgSO_4$-induced diarrhoea by 45% and 66% in comparison with the vehicle-treated control groups, respectively (Fig. 5). Similarly, the hexane extract of *D. kotschyi* (10 and 20 mg/kg) reduced $MgSO_4$-induced diarrhoea by 44% and 61% respectively in comparison with the vehicle treated control groups (Fig. 5).

Apigenin (2 and 10 mg/kg) inhibited $MgSO_4$-induced diarrhoea by 70% and 59% respectively in comparison with the vehicle treated control group (Fig. 5). Comparison of apigenin control groups receiving either low concentration of ethanol (2%) or CMC show that CMC also has slight effect on intensity of diarrhoea or time of induction. Administration of higher concentration of ethanol obscured the results and was relatively toxic.

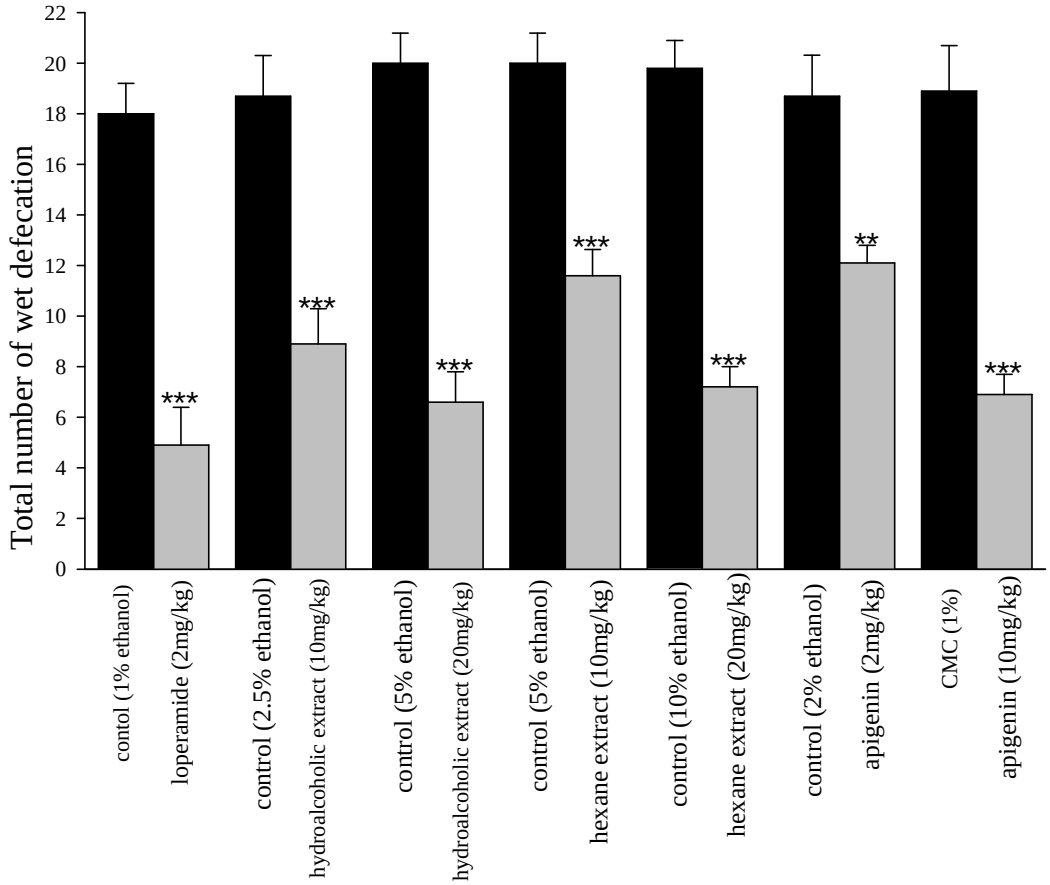

Fig. 3. Effect of hydroalcoholic and hexane extracts of *Dracocephalum kotschyi*, apigenin and loperamide on castor oil diarrhoea (0.5 ml; P.O.) in comparison with the vehicle treated control groups in mice. Values are expressed as the Mean ± SEM for each group. $^{**}P<0.01$, $^{***}P<0.001$ in comparison with corresponding vehicle-treated control group (Student's t-test). The number of observations was 10 mice in each group. CMC; carboxymethyl cellulose.

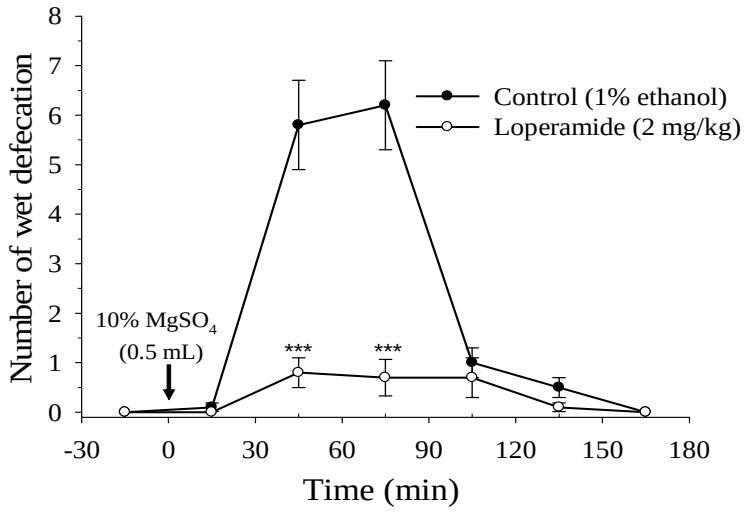

Fig. 4. Effect of loperamide on $MgSO_4$-induced diarrhoea over 3 h time course. Loperamide was given orally 30 min before oral administration of 0.5 ml of 10% $MgSO_4$. Each point presents mean incidence of wet defecation at 30 min interval times. The data are presented as the Mean ± SEM (n = 10). The difference in the mean values among loperamide and control group are statistically significant (P<0.001; two way ANOVA). Stars show the statistically significant differences between incidence of diarrhoea and the vehicle-treated control at corresponding time intervals. ***P<0.001 (Student's t-test).

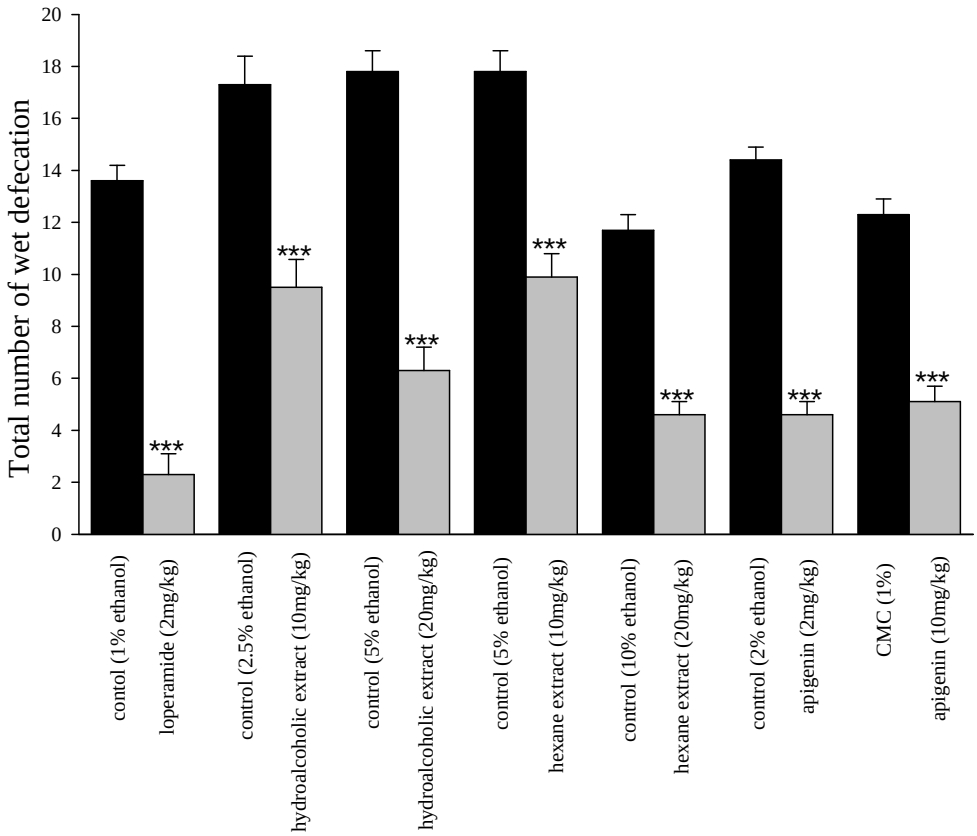

Fig. 5. Effect of hydroalcoholic and hexane extracts of *Dracocephalum kotschyi*, apigenin and loperamide on diarrhoea induced by 0.5 ml of $MgSO_4$ in comparison with the vehicle treated control groups in mice. Values are expressed as mean ± SEM for each group. ***P<0.001 in comparison with corresponding vehicle-treated control group (Student's t-test). The number of observations was 10 mice in each group. CMC; carboxymethyl cellulose.

DISCUSSION

D. kotschyi extracts have been used for their antispasmodic and analgesic effect in Iranian folk medicine (5). *In vitro* studies of *D. kotschyi* extract on isolated ileum has supported the effectiveness of *D. kotschyi* extracts as an antispasmodic agent (4,11). In this research, the antispasmodic action of *D. kotschyi* extracts was further investigated *in vivo* by assessment of intestinal transit of charcoal meal. In addition, the antidiarrhoeal effect of *D. kotschyi* extracts was examined on animal model of diarrhoea induced by castor oil and $MgSO_4$.

Diarrhoea results from an imbalance between the absorptive and secretory mechanism in the intestinal tract, which is accompanied by an excess loss of fluid in the faeces. In some types of diarrhoea, the secretory component is predominant, while other types of diarrhoea are characterized by intestinal hyper motility (17).

Castor oil is regarded as a stimulant and irritant laxative. When given orally, it has a laxative effect (18,19). It is believed that the laxative effect of castor oil is mediated by ricinoleic acid, a hydroxylated fatty acid released from castor oil by intestinal lipases. The released ricinoleic acid induces a strong laxative effect (20-22). Evidence has been provided that ricinoleic acid can directly affect intestinal motility (23,24). In addition to direct effect on intestinal motility, ricinoleic acid has also an effect on intestinal ion transport and water flux (25,26). Whether these effects are mediated by the enteric nervous system (27) or are direct effects on intestinal smooth muscle remained unclear. More recent research suggested that castor oil-induced laxation is mediated via ricinoleic acid activating prostaglandin EP_3 receptors**.** EP_3 prostanoid receptor is specifically activated by ricinoleic acid and that it mediates the pharmacological effects of castor oil (28).

$MgSO_4$ is a non-absorbable osmotic substance that attracts and retains water in the intestinal lumen, increasing intraluminal pressure that mechanically stimulates evacuation of the bowel (29). Magnesium-containing agents also cause the release of cholecystokinin, which increases intestinal motility and fluid secretion (30).

Loperamide which was used as the standard drug in this research decreases circular and longitudinal smooth muscle activity of small intestine (31) and this explains the delay in movements of charcoal meal. Antidiarrhoeal action of loperamide is exerted by slowing intestinal transit and increasing contact time, and perhaps by directly inhibiting fluid and electrolyte secretion and/or stimulating salt and water absorption (32). Loperamide could act by several different mechanisms mediated principally through either μ- or δ-opioid receptors on enteric nerves, epithelial cells, and smooth muscle cells in the gastrointestinal tract. These mechanisms include effects on intestinal motility (μ-receptors), intestinal secretion (δ- receptors), or absorption (μ- and δ- receptors) (30).

In the gastrointestinal transit test, the *D. kotschyi* extracts and apigenin with oral doses of 10 mg/kg retarded the gastrointestinal transit of the charcoal meal in mice indicating a clear antispasmodic activity *in vivo*. In the castor oil and $MgSO_4$-induced diarrhoea experiments, the extracts of *D. kotschyi* and apigenin delayed the induction of diarrhoea and produced a marked antidiarrhoeal effect in mice. Part of antidiarrhoeal activity could be due to the inhibition of gut motility but inhibition of $MgSO_4$-induced diarrhoea indicate that reduction in water absorption may have contributed to the antidiarrhoeal action of *D. kotschyi* extracts.

The components which has been identified in *D. kotschyi* extract are calycopterin, xanthomicrol, isokaempferide, luteolin, apigenin, luteolin 7-O-beta-D-glucopyranoside, luteolin 3'-O-beta-D-glucuronide, apigenin 4'-O-beta-D-glucopyranoside, acacetin 7-O-beta-D-glucopyranoside and rosmarinic acid (6). We have selected apigenin as an active compound of *D. kotschyi* extract in order to compare its antispasmodic and antidiarrhoeal activity. Apigenin is a natural product belonging to the flavenoid class that is the aglycone of several naturally occurring glycosides (12). Apigenin is also found in other fruits and vegetables, including parsley, celery, celeriac, and chamomile (33). A variety of potential

biological activities of apigenin have been reported. For instance, apigenin has been shown to prevent renal damage caused by cyclosporin in rats (34,35). Apigenin may have a chemopreventive role in leukemia (36). The exact mechanism of action of apigenin is not known. However, apigenin is said to be a ligand for central benzodiazepine receptors, exerting anxiolytic and slight sedative effects (37,38). It may also affect the adenosine receptors (39) and blocks N-methyl-D-aspartate receptors (40). Apigenin may act as a monoamine transporter activator (41) and is reported to be a potent inhibitor of CYP2C9 (42). In addition, like various other flavonoids, apigenin has been found to possess nanomolar affinity for the opioid receptors (43). Effect of apeginin on opioid receptors could explain the similarities between antispasmodic and antidiarrhoeal action of loperamide and apigenin. Inhibitory effect of apigenin (2 mg/kg) on $MgSO_4$-induced diarrhoea was relatively close to that loperamide (2 mg/kg) while on castor oil-induced diarrhoea, apigenin was less effective than loperamide with similar doses. Antispasmodic and antidiarrhoeal effect of apigenin was quantitatively very similar to effect of *D. kotschyi* hydroalcoholic extracts. Therefore, it can be concluded that the apigenin in not the sole component responsible for gastrointestinal action of *D. kotschyi* extracts. *D. kotschyi* extract also reported to have antihyperlipidemic effect with doses of 80-120 mg/kg in rats (8). On the other hand, antidiarrhoeal effect of *D. kotschyi* extracts was seen with doses of 10 and 20 mg/kg. Therefore, gastrointestinal effect is seen with lower doses of *D. kotschyi* extract.

CONCLUSION

In this research, we have provided pharmacological evidence for antispasmodic and antidiarrhoeal of *D. kotschyi* extracts. At least part of gastrointestinal of *D. kotschyi* extract is due to presence of apigenin but other constituents may have a contribution which needs to be investigated. As the effective dose of *D. kotschyi* extracts are close to dose of loperamide, a full drug design and development process for this *D. kotschyi* extracts is recommended.

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