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Antispasmodic activity of apigenin and luteolin, two components of *Dracocephalum kotschyi* extract, on rat ileum contractions

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ABSTRACT

Introduction: Aerial parts of *Dracocephalum kotschyi* have been used as antispasmodic agents in Iranian traditional medicine. Recent pharmacological studies confirmed antispasmodic activity of *D. kotschyi* extract. The objective of this research was to investigate antispasmodic activities of apigenin and luteolin to find out if they are responsible for the spasmolytic activity of hydroalcoholic extract of *D. kotschyi*.

Methods: Aerial parts of *D. kotschyi* were extracted with ethanol. Antispasmodic effect of hydroalcoholic extract of *D. kotschyi*, apigenin and luteolin were examined on KCl and/or acetylcholine (ACh)-induced contractions in rat isolated ileum.

Results: Hydroalcoholic extract of *D. kotschyi* concentrations-dependently inhibited KCl and ACh induced contractions with IC_{50} values of 41 ± 10 µg/mL and 133 ± 19 µg/mL, respectively. Apigenin concentrations-dependently inhibited KCl and ACh induced contractions with IC_{50} values of 57 ± 12 µM and 80 ± 18 µM, respectively. Luteolin concentrations-dependently inhibited KCl induced contractions with IC_{50} values of 68 ± 14 µM. Loperamide reduced both KCl and ACh induced contraction with IC_{50} values of 189 ± 44 nM and 82 ± 20 µM, respectively.

Conclusion: In this study apigenin and luteolin were identified as two active ingredients responsible for antispasmodic activities of *D. kotschyi* extract.

Implication for health policy/practice/research/medical education:
Apigenin and luteolin, two active ingredients of *Dracocephalum kotschyi* extract, are responsible for its antispasmodic activity and might be used for this purpose in patients having abdominal spasm.

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Introduction

Dracocephalum kotschyi (Labiatae family) is an endemic herbaceous plant of Iran (1-3). *Dracocephalum* genus plants are used in traditional medicine as carminative and tonic and treatment of ailment such as congestion, headache, stomachache and liver diseases (1-4). Recent pharmacological studies have confirmed some medicinal properties of *D. kotschyi* including antihyperlipidemic (5), immunomodulatory (6), anti-inflammatory (7) and anticancer (8) effects.

In traditional herbal books there are substantial reports about use of *D. Kotschyi* as antispasmodic herbal medicine (9). Recent pharmacological studies have supported the antispasmodic effects of *D. Kotschyi* extract in both isolated ileum and rat uterus smooth muscles (10,11). The *D. kotschyi* extract inhibited the response to KCl, acetylcholine and electrical field stimulations in rat isolated ileum (10). In addition, it has been reported that hydroalcoholic extract of *D. kotschyi* possesses relaxant effect on uterus responses to KCl, acetylcholine and electrical nerve stimulation as well as oxytocin-induced contractions (11). Above findings confirms that *D.*

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kotschyi extract possesses components with antispasmodic activities on smooth muscle of ileum and uterus. Furthermore, hydroalcoholic and hexane extracts of *D. kotschyi* attenuated the intestinal charcoal meal transit, confirming the antispasmodic action *in vivo* (12). The hydroalcoholic and hexane extracts of *D. kotschyi* also significantly inhibited the castor oil and $MgSO_4$-induced diarrhea in mice which could be related to its antispasmodic activity (12). In a separate experiment, the anti-inflammatory effect of hydroalcoholic extract of *D. kotschyi* was examined on acetic acid-induced colitis in rats and it was found to be very effective (13). From these experiments, it can be concluded that *D. kotschyi* extract possesses potent antispasmodic activities both *in vitro* and *in vivo* and could be used as a remedy for the treatment of gastrointestinal disorders especially those associated with intestinal spasm. Therefore, identification of the activity of plants constituents is necessary for drug development. *Dracocephalum kotschyi* is an aromatic medicinal plant enrich in essential oils (5,14), valuable flavonoids (15), monoterpene glycosides and trypanocidal terpenoids (16). Some of the constituents of the *D. kotschyi* extract have already been published (15). These include calycopterin, xanthomicrol, isokaempferide, luteolin, apigenin, luteolin 7-O-beta-D-glucopyranoside, lutcolin 3'-O-beta-D-glucuronide, apigenin 4'-O-beta-D-glucopyranoside, acacetin 7-O-beta-D-glucopyranoside and rosmarinic acid (15). However, so far there is no report about the antispasmodic activity of these components. Therefore, in this research we have investigated antispasmodic effects of apigenin and luteolin and compared with antispasmodic action of hydroalcoholic extract of *D. kotschyi* and loperamide.

Materials and Methods

Plant materials

Aerial parts of *D. kotschyi* Boiss. (Labiatae family) were collected in June 2015 from Chadegan (in Isfahan province-Iran) and identified at the Botany Department of the Faculty of Sciences, University of Isfahan. A voucher specimen of the plant (No. 1519) deposited in the herbarium of the Faculty of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences. The plant materials were dried in shadow and grained to powder using electrical miller (Moulinex, France). The dried plant material was used for extraction by maceration (17). Powdered plant material was thoroughly mixed with 70% ethanol and left for two hours. Wetted plant materials were packed into percolator and soaked in 70% ethanol for three days, with occasional shaking. Plant extract was evacuated from bottom tap passing through cotton cloth placed on the bottom of the percolator. This procedure was repeated three times and the combined filtrate was evaporated using rotary evaporator (Buchi Rotavapor RE) at 50°C to get the final hydroalcoholic extract of *D. kotschyi*. After evaporating the ethanol, amount of dried crude extract was determined (w/w).

Antispasmodic assessment

Experiments were conducted on adult male Wistar rats (180-220 g) bred in School of Pharmacy, animal house. All animals were handled in compliance with the principles of the guide for care and use of laboratory animal care approved by the university committee (18). On the day of experiment, a rat was killed by a blow on the head, followed by exsanguinations. A portion of ileum was removed and placed in oxygenated Tyrode's solution at room temperature. A section of ileum (2-3 cm) was mounted for isotonic contraction under 1 g tension in 20 mL organ bath (Harvard, England) containing Tyrode's solution and continuously gassed with O_2 at 37°C. Ileum contraction was measured using a Harvard isotonic transducer and recorded on a Harvard Universal Oscillograph (England) pen recorder device. Initially the oscillograph was calibrating, and 3 successive washes were given and the tissue was allowed to relax to a stable baseline. When the tissue baseline was established, KCl (80 mM) was added in to the organ bath in order to induce a sustained tonic contraction. The inhibitory effect of *D. kotschyi* extract or drugs were examined on isolated rat ileum contraction induced by KCl. Fifteen minutes after addition of KCl, extract or drugs were added to the bath at 5 minutes intervals in a cumulative manner using two-fold increments in concentration, until maximum inhibition was obtained. In the case of acetylcholine (ACh), non-cumulative manner was used. ACh was added into the tissue bath to give the final bath concentration of 5 µg/mL. ACh was allowed to act for 30 seconds before it was washed with fresh Tyrode's solution. The time-matched control tissues were treated with an equivalent volume of the vehicle.

Drugs and solutions

Solidified extract was weighted and prepared as 20 mg/mL stock solution in dimethyl sulfoxide (DMSO). Further dilutions were made in distilled water. Apigenin and luteolin (Sigma, China) were made up in DMSO as 10mM stock solutions. Loperamide was dissolved in DMSO as 20 mM stock solution. KCl was prepared as 2M stock solution. Acetylcholine (Sigma) was prepared in distilled water as 1mM stock solution. Tyrode's solution (NaCl, 136.9; KCl, 2.68; $CaCl_2$, 1.8; $MgCl_2$, 1.05; $NaHCO_3$, 11.9; NaH_2PO_4, 0.42 and glucose 5.55 mM) was prepared in distilled water. Unless stated, all the chemicals were purchased from Merck (Germany).

Data analysis

The tissue contractile responses were measured as maximum amplitude from pretreatment baseline and expressed as the percentage of the initial contraction

induced by KCl or ACh. All values were quoted as mean ± standard error of the mean (SEM). The IC_{50} value (Drug concentration causing 50% of maximum inhibitory response) was calculated for each tissue and mean and SEM was determined for each group of results. SigmaPlot computer program was used for statistical analysis and plotting the graphs. The IC_{50} values were used as an index for comparison of antispasmodic activities.

Results

Dried hydroalcoholic extract of *D. kotschyi* had dark greenish color. A sample of concentrated extract was thoroughly dried on a hot pate and the yield of dried extract was calculated to be 23% (w/w).

Antispasmodic studies

Rat isolated ileum suspended in Tyrode's solution gradually relaxed to a stable baseline over 15-30 min. KCl (80 mM) caused a tonic contraction which maintained for the duration of the study. Acetylcholine (ACh, 5 µM) caused a rapid phasic contraction in rat ileum during 30 seconds of contact time. KCl and ACh concentrations were chosen to produce maximum contractile responses in the tissue.

Antispasmodic activities of apigenin and luteolin were examined on isolated ileum contraction for comparison with the total extract of hydroalcoholic extract of *D. kotschyi* by constructing full concentration-response curve. Loperamide, an opioid agent with antimotility action, was used as a positive standard drug in this research. As expected, loperamide concentration-dependently inhibited rat ileum contractile responses induced by both KCl and ACh with IC_{50} values of 189±44 nM and 82±20 µM respectively (Figure 1).

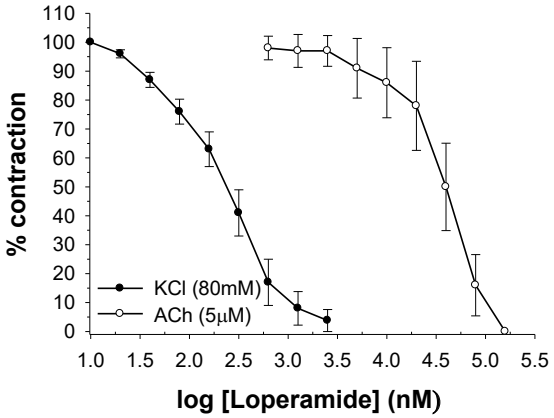

Figure 1. Inhibitory concentration-responses curves for loperamide on contractions induced by KCl and acetylcholine (ACh) in rat isolated ileum.

Ordinate scales: spasm remaining as a % of the contraction prior to drugs addition. Abscissa scales: log_{10} concentration of loperamide. Each point is mean of six experiments and the vertical lines show the SEM (n=6).

Hydroalcoholic extract of *D. kotschyi,* concentration-dependently inhibited both KCl and ACh induced contraction in the rat ileum. The inhibitory effect on KCl response was seen with bath concentration of 8 µg/mL and complete inhibition was achieved with extract concentration of 256 µg/ml (Figure 2). Inhibitory effect of *D. kotschyi* extract on ACh response started with bath concentration of 64 µg/mL and total inhibition was

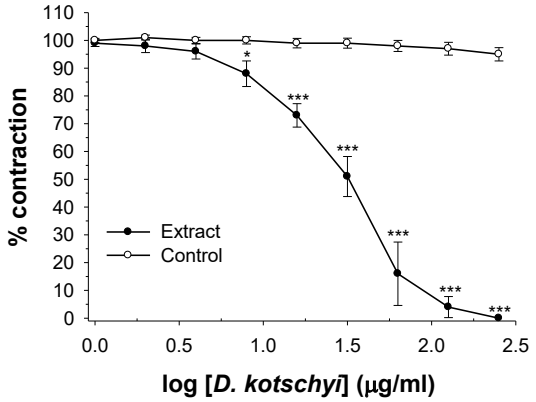

Figure 2. Inhibitory concentration response curves for hydroalcoholic extract obtained from *D. kotschyi* on tonic contractions induced in rat isolated ileum by KCl (80mM).

Ordinate scales: spasm remaining as a % of the contraction prior to drugs addition. Abscissa scales: log10 concentration of extract. Each point is mean of six experiments and the vertical lines show the SEM (n=6). The control groups were treated with equivolume amount of vehicle (DMSO). The stars show the statistically significant difference with corresponding point in the time-matched control groups. *P < 0.05, ***P < 0.001 (Student's t-test).

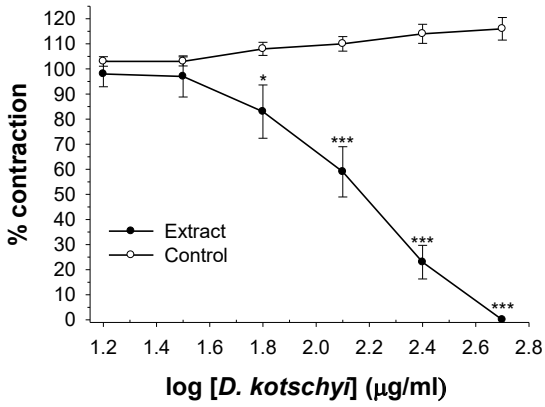

Figure 3. Inhibitory concentration responses curve for hydroalcoholic extract of *D. Kotschyi* on phasic contractions induced in rat isolated ileum by acetylcholine (5 µM). Ordinate scales: spasm remaining as a % of the contraction prior to addition of extract. Abscissa scales: log_{10} concentration of extract. Each point is mean of six experiments and the vertical lines show the SEM (n=6). The control groups were treated with equivolume amount of vehicle (DMSO). Gradual increase in tissue responses in the control group is statistically significant (ANOVA, P < 0.05). The stars show the statistically significant difference with corresponding point in the time-matched control groups. *P < 0.05, ***P < 0.001 (Student's t test).

seen with 512 µg/mL extract in the bath (Figure 3). The inhibitory concentrations causing 50% of maximum response (IC_{50} value) were 41 ± 10 µg/mL and 133 ± 19 µg/mL for KCl and ACh respectively.

Apigenin, a known component of *D. kotschyi*, also in a concentration-dependent manner inhibited KCl, as well as ACh induced contractions with IC_{50} values of 57 ± 12 µM and 80 ± 18 µM respectively. First sign of attenuation of KCl response was seen with 8 µM apigenin in the bath and at bath concentration of 480 µM, apigenin totally removed the KCl response in the rat ileum (Figure 4). Inhibitory effect of apigenin on ACh response was seen with similar concentration ranges (Figure 5). Luteolin, another component of *D. kotschyi* extract, in a concentration-dependent manner inhibited KCl induced contraction in rat ileum with IC_{50} value of 80 ± 18 µM (Figure 5). Initial reduction was observed with bath concentration of 14 µM and at bath concentration of 450 µg/mL, luteolin wiped out the tonic contraction induce by KCl (Figure 6). Potencies of apigenin, luteolin and loperamide are compared in Table 1. In the vehicle treated control groups, equivalent volume of DMSO not only did not reduce ACh response but also was observed a gradual increase in contractile response (Figures 3 and 5). Low concentration of DMSO had no effect on KCl response (Figure 2).

Discussion

Dracocephalum kotschyi hydroalcoholic extract is a relaxant of ileum and uterus smooth muscles and possessed antispasmodic and antidiarrheal activities (10-12). However, *D. kotschyi* extract contains a mixture of several active substances with various pharmacological properties including, anti-inflammatory, anti-cancer, anti-dyslipidimea, etc (5-8). Therefore, it is essential to identify the active substances which could be responsible for antispasmodic activities on smooth muscle. Preparation of standard drug form purified compound is more convenient and administrations of unwanted substances are avoided. Although several constituents of *D. kotschyi* extract are known (15), however, the main problem is that we had no idea which chemical components were

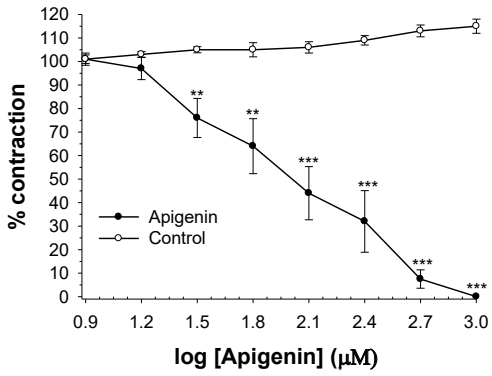

Figure 5. Inhibitory concentration responses curve for apigenin on phasic contraction induced in rat isolated ileum by acetylcholine (5 µM).
Ordinate scales: spasm remaining as a % of the contraction prior to apigenin addition. Abscissa scales: log_{10} concentration of apigenin. Each point is mean of six experiments and the vertical lines show the SEM (n=6). The control groups were treated with equivolume amount of vehicle (DMSO). Gradual increase in tissue response in the control group is statistically significant (ANOVA, $P < 0.05$). The stars show the statistically significant difference with corresponding point in the time-matched control groups. $^{**}P < 0.01$, $^{***}P < 0.001$ (Student's *t* test).

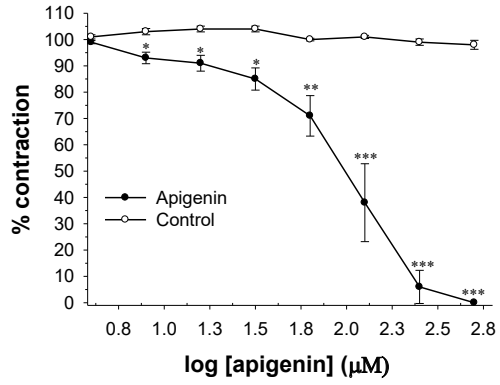

Figure 4. Inhibitory concentration-responses curve of apigenin on the tonic contraction induced by KCl in rat isolated ileum.
Ordinate scales: spasm remaining as a % of the contraction prior to apigenin addition. Abscissa scales: log_{10} concentration of apigenin. Each point is mean of six experiments and the vertical lines show the SEM (n=6). The control groups were treated with equivolume amount of vehicle (DMSO). There are not statistically significant changes in the fluctuation seen in the vehicle treated control group (ANOVA). The stars show the statistically significant differences of the test tissues with the corresponding points in the time-matched control groups. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (Student's *t* test).

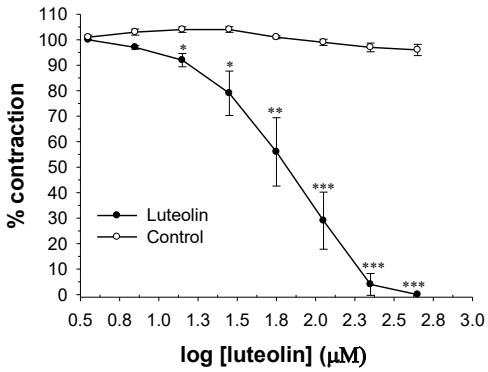

Figure 6. Inhibitory concentration responses curve of luteolin in rat isolated ileum for the tonic contraction induced by KCl (80 mM).
Ordinate scales: spasm remaining as a % of the contraction prior to luteolin addition. Abscissa scales: log_{10} concentration of luteolin. Each point is mean of six experiments and the vertical lines show the SEM (n=6). The control groups were treated with equivolume amount of vehicle (DMSO). Observed fluctuation in the vehicle treated control group response was not statistically significant (ANOVA). The stars show the statistically significant differences of the test tissues with the corresponding points in the time-matched control groups. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (Students *t* test).

Table 1. Comparison of potency of loperamide, apigenin and luteolin for inhibiting KCl (80 mM) and acetylcholine (ACh, 5 μM) responses in rat isolated ileum

	pD_2 values		
	Loperamide	Apigenin	Luteolin
KCl	6.72	4.24	4.10
ACh	4.09	4.10	-

Potency has expressed as pD_2 value (log_{10} potency in molar concentration).

responsible for the antispasmodic activities. Apigenin and luteolin are two flavonoids that have been identified in the hydroalcoholic extract *D. kotschyi* (15). Therefore, antispasmodic activities of apigenin and luteolin were examined on isolated ileum contractions.

In this study loperamide was used as a standard drug and as expected it reduced ileum contraction in a concentration-dependent manner. Loperamide is an opioid agent with antimotility action which inhibits peristalsis and slows gastrointestinal motility by the effect on circular and longitudinal muscle of the intestine (19,20). Loperamide act by several different mechanisms, mediated by various opioid receptors on the enteric nerves and smooth muscles (19,20).

This study revealed that apigenin and luteolin as well as hydroalcoholic extract of *D. kotschyi* have direct relaxant effects on rat ileum smooth contractions by KCl and ACh. ACh is cholinergic neurotransmitter which acts on muscarinic M_3-receptors on smooth muscle of ileum. Muscarinic receptors are G-protein couple receptors (GPCR) and cause the release of Ca^{2+} ions from intracellular stores via increasing phospholipase-C activities and generation of inositol triphosphate (21,22). Addition of high potassium concentration (80 mM KCl) into extracellular solution results in cell membrane depolarization and activation of voltage-gated calcium channels (23). Increase in intracellular Ca^{2+} ion concentrations results in smooth muscle contraction (23). However, other studies have shown that membrane depolarization induced by high concentrations of extracellular KCl can also cause Ca^{2+} sensitization in many smooth muscles (23). In the case of KCl induced tonic contraction it has been proposed that tonic force maintenance in the smooth muscle is dependent on rhoA kinase activity (23). However, some isotype of protein kinase C (PKC) may also play a role in causing KCl-induced sensitization (24). Flavonoids such an apigenin and luteolin are reported to inhibit PKC activities (25-27). Inhibition of PKC by apigenin and luteolin indicates that both apigenin and luteolin are acting intracellulary and this is consistent with inhibition of both ACh and KCl induced contractions. In addition, luteolin is a non-selective competitive inhibitor of phosphodiesterases (28) which could also play a role in its antispasmodic activity. Therefore, apigenin and luteolin are two active ingredients of *D. kotschyi* extract with potent antispasmodic activities.

Comparison of inhibitory concentration at molar concentration showed that potencies of apigenin and luteolin were close to that of loperamide especially for inhibiting acetylcholine response (Table 1). However, *D. kotschyi* extract is a mixture of several substances and comparison of potency in molar concentration was not feasible. Nevertheless, as luteolin and apigenin are found to be relatively potent antispasmodic agents, it can be concluded that they are responsible for antispasmodic activity of *D. kotschyi* extract, although, contribution of other components could not be excluded.

Drugs that reduce spasm in the gut are of great value in gastrointestinal disorders including irritable bowel syndrome and diverticular disease (29). It has been shown that apigenin as well as *D. kotschyi* extract reduce the intestinal transit and has anti-diarrheal activity in mice (12). Therefore, apigenin has been shown to have antispasmodic activities both *in vitro* and *in vivo*. Furthermore, apigenin has anti-inflammatory activities in acetic acid-induced colitis (13). These findings confirm that apigenin is a suitable candidate for the treatment of irritable bowel syndrome in which intestinal spasms and diarrhea are part of the clinical signs.

Conclusion

In this research relaxant effects of apigenin and luteolin were examined on rat ileum and it was found to have direct relaxation actions on gastrointestinal smooth muscle. Apigenin and luteolin not only inhibited contraction induced by ACh but they also were as much potent as loperamide. Therefore, it can be concluded that apigenin and luteolin are two components responsible for antispasmodic activity of *D. kotschyi* extract. However, *D. kotschyi* extract is enriched in bioactive substances. Therefore, further pharmacological researches for identification of other active components with antispasmodic activities are recommended.

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Authors' contributions

MG and GA were responsible for preparation of extract while HS supervised the pharmacological studies. NS was responsible for performing the laboratories work. All contributed to the preparation of the article and confirmed final edition for publication.

Ethical considerations

Ethical issues have been observed by the authors.

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