



Antispasmodic effects of hydroalcoholic, aqueous, chloroform, and ethyl acetate extracts of Zaringiah on rabbit ileum smooth muscle contractions

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ABSTRACT

Introduction: Zaringiah (*Dracocephalum kotschyi*) is an Iranian endemic herbal plant naturally growing in the Isfahan and Khorasan provinces. Hydroalcoholic extract of Zaringiah has anti-inflammatory, antispasmodic, and immunomodulatory properties. So far, the effect of Zaringiah extract on contraction induced by histamine and serotonin (5-HT) has not been reported. The objective of this research was to investigate the antispasmodic effect of hydroalcoholic, aqueous, chloroform, and ethyl acetate extracts of Zaringiah on rabbit ileum smooth muscle contractions induced by histamine and 5-HT.

Methods: Khorasani variant of Zaringiah was used in this study. Aqueous extract was prepared by decoction, while hydroalcoholic extract was obtained by the maceration technique. Chloroform and ethyl acetate fractions were obtained using a solvent in solvent extraction technique. Rabbit isolated ileum was set up in an organ bath filled with Tyrode's solution. The effects of the above extracts were examined on contractions induced by histamine or 5-HT and compared with each other.

Results: Hydroalcoholic extract of Zaringiah inhibited the rabbit ileum contractions induced by histamine ($IC_{50} = 76 \pm 7.7 \mu\text{g/mL}$) and 5-HT ($IC_{50} = 60 \pm 6.4 \mu\text{g/mL}$), as well as spontaneous contractions ($IC_{50} = 63 \pm 15 \mu\text{g/mL}$). The aqueous extract, as well as chloroform and ethyl acetate fractions, concentration-dependently inhibited spontaneous, histamine, and 5-HT induced contractions.

Conclusion: There was not a significant difference among the inhibitory actions of hydroalcoholic, aqueous, chloroform, and ethyl acetate extracts of Zaringiah on rabbit ileum, indicating the distribution of active constituents in both polar and non-polar mediums.

Implication for health policy/practice/research/medical education:

This paper supports the traditional idea that boiled extract of Zaringiah (*D. kotschyi*) is effective in alleviating intestinal pain associated with gastrointestinal cramps. Moreover, this paper provides scientific information about the inhibitory effect of Zaringiah extracts on natural contractile activities (i.e., spontaneous contractions) of the intestine, as well as histamine and serotonin-induced contractions.

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Introduction

Dracocephalum, commonly known as dragonhead, is a genus name of flowering plants with great biodiversity (1). At least eight endemic species of *Dracocephalum* have been identified in Iran (2,3). *Dracocephalum kotschyi* Boiss. is a scientific name for Zaringiah, a species of

Dracocephalum, uniquely found in Iran (4). Zaringiah is a special medicinal plant native to temperate regions, which mainly grows in the high altitude in the Zagros and Alborz mountains ranges in Iran (5,6). From the botanical point of view, Zaringiah is an herbaceous perennial plant of the mint family (Labiatae) (4). At least two variants of

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Zaringiah exist. One variant is growing in the Isfahan and Chaharmahal & Bakhtiari regions (Zagros mountains) and the other in Khorasan province (Alborz mountains). Traditionally, local people have used Zaringaih for the treatment of joint edema, gastrointestinal disorders, and respiratory bronchitis (7). In addition, some people believe that it is effective for ailments such as cold, headache, stomachache, toothache, congestion, and the alleviation of kidney and liver diseases (1,4-6). Zaringiah has an essential oil containing fragrant components with the following composition: carveol, α -pinene, geraniol, α -citral, cyclonadiene, linalool, neral, germacrene-D, isopinocarveol, limonene, and α -terpineol (8,9). In recent years, several reports have been published on the biological activities of the Zaringaih essential oil. For example, it had been reported that essential oil has anti-inflammatory and analgesic properties (10,11). There are many reports about the pharmacological activities of hydroalcoholic extract of Zaringaih. For instance, it has been reported that the hydroalcoholic extract of Zaringaih has anti-hyperlipidemia and anti-tumor properties (10,12,13). Furthermore, modern pharmacological investigations have confirmed that Zaringaih has efficient anti-inflammatory and antispasmodic activities (14-20). It is believed that these pharmacological effects are due to the flavonoid constituents of the plant (21). The following flavonoids have been identified in the Zaringaih extract: calycopterin, xanthomicrol, isokaempferide, apigenin, luteolin, luteolin 3'-O-beta-D-glucuronide, apigenin 4'-O-beta-D-glucopyranoside, acacetin 7-O-beta-D-glucopyranoside, luteolin 7-O-beta-D-glucopyranoside, and rosmarinic acid (22,23). Spasmolytic and anti-inflammatory effects of Zaringaih have been attributed to its flavonoid's contents, including apigenin and luteolin (15,16,20). It has been reported that apigenin could alleviate allergic and inflammatory responses in animal model of asthma (24). In addition, apigenin, by inhibiting transforming growth factor- β_1 (TGF- β_1), prevented the proliferation and migration of immune cells in inflammatory respiratory tract conditions (25). Similar effects have been reported for luteolin. For instance, luteolin reduced airway inflammatory response, mucosa production, and other feature of asthma in animal models (26). In addition, apigenin and luteolin prevented respiratory bleomycin-induced fibrosis (27,28). Calycopterin is another flavonoid component of Zaringiah, which is believed to be responsible for its immunomodulatory properties (29). From the above reports, it can be concluded that the hydroalcoholic extract of Zaringiah may have antispasmodic and anti-inflammatory activities both *in vitro* and *in vivo* and could be useful for the treatment of conditions in which tissue inflammation and smooth muscle spasm play a major role. Although in the previous studies, the antispasmodic effect of Zaringiah was suggested to be due to its flavonoid components, however, when the spasmolytic potency of

hydroalcoholic extract of Zaringiah was compared with its flavonoid fractions, it turned out that the flavonoid fraction was less active than the total crud extract (30). Therefore, it is possible that following fractionation of the extract, some active constituents have been dissolved in the chloroform partition. So far, the spasmolytic action of aqueous and chloroform fractions of Zaringiah has not been reported. Therefore, one objective of this research was to study the inhibitory effects of aqueous and chloroform fractions of Zaringiah on ileum contractions. Furthermore, the effects of Zaringiah extracts on histamine- or 5-HT- induced smooth muscle contractions were evaluated. The effects of Zaringiah extracts were also examined on the histamine- and 5-HT- induced contractions in rabbit ileum.

Materials and Methods

Drugs and solutions

Zaringiah extracts and fractions were prepared as explained below. For pharmacological studies, the stock of aqueous extract (100 mg/mL) was made up in 30% dimethylsulphoxide (DMSO) and diluted with distilled water. The other extracts were made up of 100 mg/mL stock solutions in the DMSO and further diluted in 50% DMSO (10 mg/mL). Stocks of histamine (10mM) and 5-HT (25mM) were prepared in distilled water. The physiological Tyrodé's solution was prepared with the following ingredients and gassed with oxygen: NaCl (137mM), KCl (2.7mM), CaCl_2 (1.8mM), NaHCO_3 (11.9mM), MgCl_2 (1.05mM), NaH_2PO_4 (0.42mM) and glucose (5.6mM).

Extraction

The Zaringiah seeds were originally collected from the Khorasan region (Iran) and cultivated on a farm situated in Shahankoh in Fereydunshahr (Isfahan, Iran). The plant was identified as *Dracocephalum kotschyi* variant by an official botanist from Isfahan Natural Resources and Watershed Management Organization. A sample voucher has been deposited in the herbarium room at the School of Pharmacy and Pharmacological Sciences (4030). In the flowering season, the aerial parts of Zaringiah were collected and dried in shadow. The dried materials were reduced to fine powder by grinding in a mill (Keep, Korea). The aqueous extract was prepared using the decoction method and concentrated with rotary, and then freeze dried. The hydroalcoholic extract was prepared by the maceration technique (31). Briefly, plant powder was moisturized for 2 hours before it was macerated using 70% ethanol (8:1 solvent/powder ratio). After 3 days, the elute was emptied and the process was repeated. The extract was concentrated with a semi-automatic rotary vacuum evaporator (Heidolph Instruments, Germany). Further fractionation was carried out using the liquid-liquid extraction procedure (31,32). The concentrated extract was then solubilized in equal volumes of chloroform and water with 20 minutes vigorous agitation and then

separated in a decanter. The water partition was mixed with an equal volume of ethyl-acetate and the process was repeated. The chloroform and ethyl-acetate solvents were evaporated with the rotary at 30°C.

Pharmacological studies

The inhibitory effects of Zaringiah extracts were examined on rabbit ileum contractions. Male rabbits bred locally were anesthetized and asphyxiated by carbon dioxide (33). A portion of the ileum was dissected out, immersed in Tyrode's solution, and oxygenated continuously. The ileum was then cut into short sections in a petri dish filled with Tyrode's solution. Each strip edge was tied with a piece of cotton thread. One end was tied to a tissue holder. The tissue holder was then secured into the organ bath (Palmer, England). The string on the top end of the strip was attached to an isotonic Harvard displacement transducer, and ileum contractions were recorded on the Harvard Universal Oscillograph.

The ileum was then washed several times and allowed to relax. Once a stable baseline was established, a single dose of histamine or 5-HT was added and directed to the organ bath to induce tissue contractions. Following 30 seconds contact time, the tissue was washed with fresh Tyrode's solution thrice. After reproducible responses were obtained, the first concentration of the extract was added to the organ bath, and the effect of histamine or 5-HT was examined in the presence of the extract. The experiment was performed in the presence of the second concentration of the extract and so on, until a full concentration-response curve was constructed. Extract concentrations were selected following a series of pilot experiments. Effects of the extract vehicle (DMSO + water) were also examined on the tissues from the same animal under similar conditions.

The altitude of the recorded tissue contractions was measured from the baseline position and expressed as the percentage of initial contraction prior to the addition of the extract. For spontaneous contraction, the mean of recorded spikes within 5 minutes interval was assessed. A concentration response curve was constructed for each tissue, and the extract concentration causing 50% of maximum inhibitory effect (IC_{50}) was evaluated.

Statistical analysis

Mean and standard error of the mean (SEM) was calculated for each group of data. Intergroup variation was compared using Student's *t* test. Intragroup variation was compared with one-way analysis of variance (ANOVA).

Results

Once setup in the organ bath, the strip of rabbit ileum, suspended under 1 g tension, often exhibited rhythmic spike activities. The spontaneous contractions were relatively uniform, so the quantitative analysis was possible. The hydroalcoholic and aqueous extracts

of Zaringiah, in a concentration-dependent fashion, attenuated the spontaneous contractions. The inhibitory effect of the extract on the spontaneous activities of the tissue was significant in comparison with the time-matched vehicle-treated control groups (Figure 1). Chloroform and ethyl acetate fractions similarly inhibited spontaneous contractions in the rabbit isolated ileum (Figure 1). The extract concentration causing 50% of the maximum response is given in Table 1.

Addition of histamine (10 μ M) or 5-HT (25 μ M) into the organ bath induced clear phasic contractions, reaching the peak within 30 s contact time. Following washing, the tissue quickly relaxed back to its baseline level. Addition of histamine or 5-HT at 10 minutes intervals produced consistent responses. No significant reduction was seen in the contractile response of the vehicle-treated control tissues during the experiment.

The hydroalcoholic extract of Zaringiah also, in a concentration-dependent manner, inhibited the rabbit

Table 1. Comparison of the inhibitory concentrations of Zaringiah extracts on the isolated rabbit ileum contractions

Extract	IC_{50} (μ g/mL)		
	Histamine	5-HT	Spontaneous
Hydroalcoholic	60 \pm 6.4	76 \pm 7.7	63 \pm 15
Chloroform	94 \pm 13	140 \pm 21	90 \pm 13
Ethyl-acetate	86 \pm 16	86 \pm 8.1	66 \pm 7.7
Aqueous	153 \pm 38	256 \pm 65	58 \pm 5

Extract concentration causing 50% of maximum inhibitory response (IC_{50}) was calculated from full concentration response curves. The data has been given as mean \pm SEM (n = 6)

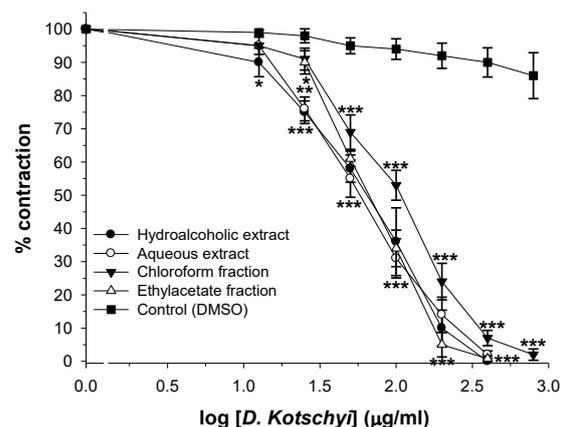


Figure 1. Inhibitory concentration response curve for Zaringiah extracts on the spontaneous contractions in the isolated rabbit ileum. The vertical axis shows the percentage of the recorded contractions relative to the initial control response in the tissue. The horizontal axis shows logarithm of bath concentrations of the extracts. Each point is mean of 6 data and the vertical bars indicate the SEM. The fluctuation in the time-matched vehicle treated control groups was not statistically significant (ANOVA). Stars show the statistical difference between the extract response and its corresponding vehicle (DMSO) treated control groups (Student's *t*-test, * P < 0.05, ** P < 0.01, *** P < 0.001).

ileum contractile responses to histamine and 5-HT (Figures 2 and 3). The inhibitory effect was started with 25 $\mu\text{g}/\text{mL}$ and 200 $\mu\text{g}/\text{mL}$ extract in the bath, and the response to either histamine or 5-HT was diminished. The inhibitory action of Zaringiah extract was observed within 10 minutes contact with the tissue and continued as long as the extract was in the bath. However, following washing the tissue with fresh Tyrode's solution, the normal tissue response to histamine and 5-HT was gradually restored. The IC_{50} values are compared in Table 1. No significant inhibitory effect was seen on the histamine or 5-HT-induced contractions in the tissues treated with the extract vehicle (Figures 2 and 3).

Chloroform fraction elicited from the crude hydroalcoholic extract, concentration-dependently reduced the phasic contractile responses of both histamine and 5-HT in the isolated rabbit ileum (Figures 2 and 3). A significant reduction was seen with 25 $\mu\text{g}/\text{mL}$ extract in the bath, and complete relaxation was achieved with 400 $\mu\text{g}/\text{mL}$ bath concentration. However, the chloroform fraction of Zaringiah at a relatively higher bath concentration inhibited the 5-HT response. The IC_{50} values are presented in Table 1 for comparison.

The ethyl-acetate fraction of Zaringiah also had clear inhibitory effects of the histamine and 5-HT-induced responses (Figures 2 and 3). The reduction in contractile response was concentration-dependent. Significant attenuation in the response was seen with 25 $\mu\text{g}/\text{mL}$ ethyl-acetate extract in the bath and with a bath concentration of 200 $\mu\text{g}/\text{mL}$, total inhibitory effect was accomplished. The ethyl-acetate fraction at similar concentrations prevented the contractile responses of the tissues to the

histamine and 5-HT. Extracts concentrations causing 50% of maximum inhibition are presented in Table 1.

The aqueous extract of Zaringiah also exhibited an inhibitory effect on both histamine and 5-HT-induced contractions in the rabbit intestinal strip (Figures 2 and 3). However, the inhibitory effect was observed with higher bath concentrations in comparison to the other extracts. Total inhibition of histamine and 5-HT responses of the ileum was seen with bath extract concentrations of 800 $\mu\text{g}/\text{mL}$ and 1.6 mg/mL, respectively. The IC_{50} values for comparison are given in Table 1. Following washing the tissue with fresh Tyrode's solution, the contractile responses of histamine, 5-HT, and spontaneous contractile activity were gradually restored.

Discussion

Experimental results of this research indicate that Zaringiah extracts have intense inhibitory action on rabbit ileum smooth muscle contractions. In this work, the spasmolytic potential of aqueous, chloroform, and ethyl-acetate extracts were also demonstrated. Unlike the rat, isolated rabbit ileum has a more stable and regular spontaneous activity in the organ bath. Therefore, in addition to histamine and 5-HT, the effects of the extracts on spontaneous contractions were also evaluated. Zaringiah extracts depressed the ileum spike activities and inhibited spasmogens-induced contractions. Intestinal smooth muscles are innervated by autonomic and enteric nervous systems (34). In addition to direct action on the smooth muscles, spasmogens by stimulating these neurons, can also induce contractions of the ileum. Two layers of longitudinal and circular smooth muscles

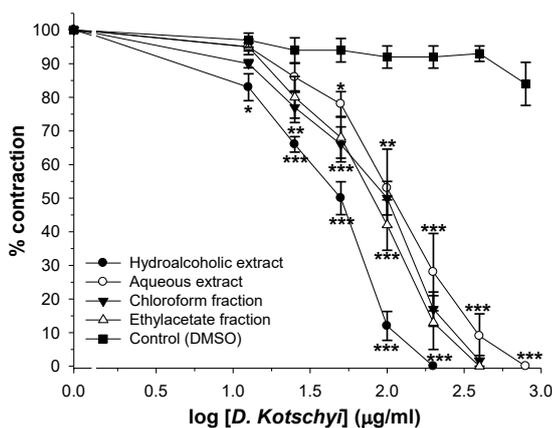


Figure 2. Inhibitory concentration response curve for Zaringiah extracts on the histamine induced contractions in the isolated rabbit ileum. The vertical axis shows the percentage of the recorded contractions relative to the initial control response in the tissue. The horizontal axis shows the logarithm of concentrations of the extracts. Each point is mean of 6 data and the vertical bars indicate the SEM. The fluctuation in the time-matched vehicle treated control was not statistically significant (ANOVA). Stars show the statistical difference between the extract response and its corresponding vehicle (DMSO) treated control groups (Student's *t* test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

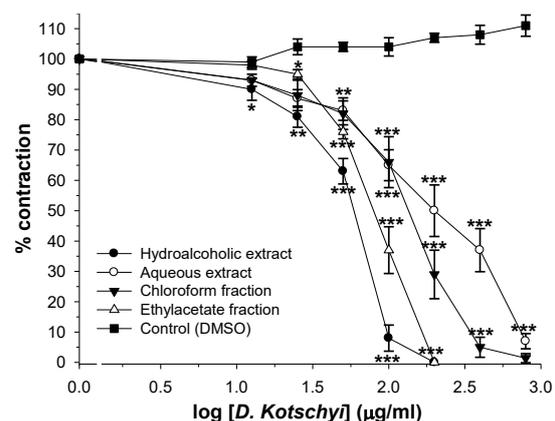


Figure 3. Inhibitory concentration response curve for Zaringiah extracts on the serotonin (5-HT) induced contractions in the isolated rabbit ileum. The vertical axis shows the percentage of the recorded contraction relative to the initial control response in the tissue. The horizontal axis shows the logarithm of concentrations of the extracts. Each point is mean of 6 data and the vertical bar indicate the SEM. The fluctuation in the time-matched vehicle treated control was not statistically significant (ANOVA). Stars show the statistical difference between the extract response and its corresponding vehicle (DMSO) treated control groups (Student's *t* test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

are responsible for the ileum contractions. The inner circular layer is arranged around the lumen, and the outer longitudinal layers are arranged in parallel with the length of the tissue. It was the contractile properties of these muscles that were investigated in this research. The contractile functions of these muscled layers are controlled by the enteric nervous system. The enteric nervous system is comprised of a network of neurons, which receive inputs from the autonomic nerves system. These neurons release various neurotransmitters, including neuropeptides, nitric oxide, histamine, 5-HT, as well as noradrenaline and acetylcholine (34). As soon as the rabbit isolated ileum was set up in the organ bath, spontaneous contractions started. This is because of the activities of pacemaker cells within the ileum wall called the interstitial cell of Cajal (35).

The isolated rabbit ileum rapidly contracted in response to the addition of histamine and 5-HT. The contractile effects of histamine and 5-HT are mediated through specific receptors located on the ileum. At least three types of histaminic receptors have been identified in the intestine (36). Pharmacological investigations have indicated that H₁-histamine receptors located on the longitudinal smooth muscle layers are mainly responsible for histamine contractions. Nevertheless, the stimulation of H₂-histamine receptors on the mesenteric network could also have indirect contributions (36). Abundant amounts of serotonin (5-HT) are found in the gastrointestinal tract (37). Previous studies have shown that 5-HT, both directly and indirectly, could evoke ileum smooth muscle contractions through the stimulation of neurons within mesenteric layers (37).

Hydroalcoholic extract of Zaringiah is enriched in biologically active chemical components, including those with antispasmodic properties. Therefore, it is essential to identify the active substances responsible for the spasmolytic activities on the smooth muscles.

Although some flavonoid constituents in the hydroalcoholic extract of Zaringiah have already been identified, however, it is still not clear which components are regarded as the main antispasmodic substances. Apigenin and luteolin are two flavonoids with spasmolytic activities on the smooth muscles. However, when their inhibitory potencies (i.e., IC₅₀) were compared, the potency of the pure substance was very close to the potency of the crude extract of Zaringiah (15), while the total flavonoids are only accounted for less than 1% of the total extract. Therefore, it is possible that more potent antispasmodic components exist, which have major contribution in the inhibitory actions of the hydroalcoholic extract. Maceration of the plant with 70% ethanol dissolves most of the polar and non-polar substances, while the aqueous extraction mainly takes out water-soluble substances. On the other hand, the chloroform partition mainly solubilizes the non-polar components. Semi-polar constituents such as flavonoids are retained in the ethyl-acetate fraction. The

comparison of the antispasmodic response of aqueous extract with the chloroform and ethyl-acetate fractions indicates that not only flavonoids might be responsible for the antispasmodic action of Zaringiah, but other polar and non-polar components exist in the hydroalcoholic extract of Zaringiah that have substantial contribution in the spasmolytic properties of Zaringiah. Therefore, further separating the ingredients of the aqueous and chloroform extracts with column chromatography and screening for their antispasmodic activities are recommended in order to identify the most active lead compounds.

Conclusion

The hydroalcoholic extract of Zaringiah (in addition to inhibiting the ileum contractions induced by neuronal stimulation, acetylcholine or KCl, at relatively similar concentrations ranges) can suppress histamine and 5-HT-induced contractions, as well as natural spontaneous contractions. Because of the similarity between the inhibitory concentrations of the extracts, it could be concluded that the active substances are distributed in both polar and non-polar mediums. For the separation of active substances, the column chromatography fractionation technique is recommended.

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Authors' contributions

HS was project manager and supervised the pharmacological studies. AY supervised extracts preparation. MD was responsible for the experimental work and analysis of the data. HS was responsible for writing the paper. All authors approved the final manuscript for publication.

Conflict of interests

The authors declare no conflict of interests.

Ethical considerations

Animal care and experiments were performed in accordance with the guidelines for the care and use of laboratory animals of the Isfahan University of Medical Sciences. The project was confirmed by the ethical committee of the university (IR.MUI.RESEARCH.REC.1399.363).

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