Evaluation of anti-spasmodic effect of *Peucedanum pastinacifolium* extracts on rat's ileum

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Abstract

Genus Peucedanum belongs to the subfamily of Apioideae (Umbelliferae family) and is reported to have many medicinal properties. Several spiecis of Peucedum reported to have antispasmodic activity. *Peucedanum pastinacifolium* species grows in Iran. However, so far there is no report on its antispasmodic activity. The objective of this research was to investigate antispasmodic activities of *P. pastinacifolium* extract using *in vitro* isolated tissue techniques. Hydroalcoholic and hexanoic extracts were prepared by perculation method from aerial part of *P. pastinacifolium*. A portion of rat isolated ileum was suspended under 1g tension in Tyrode's solution at 37 °C and gassed with O_2. Effects of extracts of *P. pastinacifolium* were studied on ileum contractions induced by KCl (80 mM), acetylcholine (ACh, 250 μM) and electrical field stimulation (EFS). The hydroalcoholic extract *P. pastinacifolium* concentration dependently inhibited the response to KCl (IC_{50}=220 ± 30 μg/ml), ACh (IC_{50}=175 ± 15 μg/ml) and EFS (IC_{50}=95 ± 15 μg/ml). The hexanoic extract of *P. pastinacifolium* also had inhibitory effect on ileum contraction induced by KCl (IC_{50}=16 ± 2 μg/ml), ACh (IC_{50}=30 ± 5 μg/ml) or EFS (IC_{50}=11 ± 4 μg/ml). From these experiments it was concluded that both hydroalcoholic and hexanoic extract of *P. pastinacifolium* contain substances which have antispasmodic activities but these substances are mainly concentrated in the hexanoic extract.

Keywords: *Peucedanum pastinacifolium*; Ileum; Antispasmodic

INTRODUCTION

Umbelliferae family is a large family of flowering plants with more than 150 genus and 3000 different species (1). Fruit, leaves, and roots of some of this family plant such as parsley, dill, cumin, and fennel are used in traditional folk medicine (2). Umbelliferae family consists of three main subfamilies including Hydrocotgloideae, Saniculoideae, and Apioideae (2). Genus Peucedanum belongs to subfamily of Apioideae with 120 different species (2). Peucedanum genus is known as Razianeh Kohi in Persian and Hog's Fennel in English with 21 different species grows all over Iran (3). This genus are separated by Flora Iranica into five different species including *Leutea, Demavendia, Cervaria, Zeravschamia,* and *Jahreniopsis* (3). Peucedanum genus is reported to have many medicinal properties. For example they have

antihyperlipidemic and antihypertensive activities (4,5), In addition it is believed that they also have antidiabetic, antimutogenic, antitumor and antioxidant properties (6-9). Other pharmacological activities suggested for this genus include antiplatelet, antimicrobial and antiallergic activities (10-12). Two species of *P. Japonicum* and *P. praeruptorium* are reported to have antispasmodic activities (12). The antispasmodic activity is suggested to be due to the presence of essential oil and furanocoumarins (3,13-15). Additionally, other species of this genus have been used traditionally in the treatment of cold and cough due to pathogenic wind-heat, accumulation of phlegm-heat in the lung; they have also been used as anti-tussive, anti-asthma and a remedy for angina (18). *P. pastinacifolium* Boiss. & Hausskn which is commonly known as "Alafe-Tofangchi" is popular in folk medicine for having antilipidemic and antidiabetic effects

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(4,16,17). It is an endemic species in central parts of Iran and grows on more or less rocky and scree steep slopes. It is used by local inhabitants in central and western Iran as an antihyperlipidemic vegetable which has been verified *in vivo* (4,13). In one study on this plant it is proved that ethanolic extract of the aerial parts of *P. pastinacifolium* can decrease total serum cholesterol, low-density lipoprotein cholesterol, triglyceride levels, and atherogenic indices in hypercholesterolemic rats after eight days (4). In another study, the ethanolic extract of the aerial parts of the same species was injected into streptozotocin-induced diabetic rats and showed the amelioration of their blood lipid profile (13). A phytochemical investigation on acetone extract of *P. pastinacifolium* demonstrated three linear furanocoumarins, bergapten, isopimpinellin, and methoxsalen, and a phenylpropanoid named elemicin (15). Bergapten and isopimpinellin have antifeedant and antimicrobial effects (19,20). Also bergapten is reported to have antimutagenic effects (21). Elemicin which can be found only in essential oil has different effects, among which, antimicrobial and antifungal effects are the most prominent ones (22,23).

The air-dried aerial parts of *P. pastinacifolium* mainly provides about 0.2% of essential oil, whose main components are known to be elemicin (31.1%), limonene (11.6%), myristicin (8.2%), methyl eugenol (6.1%), dillapiole (5.9%), *trans-β*-ocimene (5.9%), and α-pinene (4.9%) (15).

Although two Pecedanum species was reported to have antispasmodic activities, however, so far there is no report on antispasmodic activity of *P. pastinacifilum*. Therefore, the objective of this research was to investigate antispasmodic activity of *P. pastinacifolium* extracts *in vitro* by pharmacological technique.

METHOD AND MATERIALS

Plant materials and extracts preparation

P. pastinacifolium was collected from Golpaygan mountain in flowering season in 2013. A voucher specimen (2831) was deposited in the herbarium of the School of Pharmacy and Pharmaceutical Science at Isfahan University of Medical Sciences. Aerial part of the plant was dried in shade and cut into small pieces and then grounded to powder using electrical miller (Moulinex, France). The hydroalcoholic extract as polar fraction and hexanoic extract as non-polar fraction were obtained by percolation (24) using ethanol (80%) and hexane (100%) respectively. The solvent of the extracts was evaporated to dryness and percentage yield of the dried extract was determined.

Antispasmodic studies

In this research isolated tissue preparation was used for the assessment of antispasmodic activity of *P. pastinacifolium* extracts. Ileum segment were prepared from male Wistar rats (180–220 g), bred in School of Pharmacy animal house (Isfahan), in accordance with the internationally accepted principles for laboratory animal use and care, as recommended by university authority (Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2010) (25).

Longitudinal strips of ileum, 2-3 cm long, were mounted under 1 g resting force in an organ bath (Harvard, England) filled with Tyrode's solution (see drug and solutions section) at 37 °C and gassed with O_2. The tissues were washed several times with fresh Tyrode's solution and allowed to relax to a stable base line. Tissue contractions were recorded on a Harvard Universal Oscillograph (Harvard, England) pen recorder device. Neuronal stimulation was induced using electrical field stimulation technique (EFS, 6 V and 50 Hz for 1s duration) as described before (26,27). Contraction induced by single dose of acetylcholine (ACh, 250 µM, 30 s contact time) was studied in a non-cumulative manner. After reproducible contractions were established, at 12 min intervals, successive concentrations of extracts was added directly into organ bath. In the case of KCl (80 mM), following stabilization of tonic contraction, *P. pastinacifolium* extract was added cumulatively into organ bath using two fold increments in concentration. Initially a number of pilot experiments were carried out for

determination of effective concentration ranges of extract of *P. pastinacifolium*. Then full concentration response curves were obtained for each drug using 8-10 different concentrations of the extracts.

Following completion of the experiment, the tissues were washed with fresh Tyrode's solution and tested for reversibility of the response. All experiments were performed alongside of time-matched vehicle treated controls.

Drugs and solutions

Tyrode's solution composed of: 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 (in mM) were made up in distilled water. Unless stated, all chemicals and drugs were from Merck, Germany.

The following drugs were used in this research: Extracts of *P. pastinacifolium*, acetylcholine hydrochloride (Sigma, Germany). The extracts were made up as 50 mg/ml stock solution in dimethyl sulfoxide (DMSO), dilution being made in 50% DMSO or distilled water (5 mg/ml, 500 µg/ml). KCl (2 M) stock solutions were prepared in distilled water. ACh was made up as 100 mM stock solution and acidified by 1% acetic acid, and further serial dilutions (250 µM) were made in distilled water.

Measurements and statistical analysis

Tissue responses were measured as maximum amplitude of contraction and expressed as the percentage of the initial response in the absence of drugs for each tissue. All the values are reported as mean ± standard error of the mean (SEM). Statistical significance were assessed using a one-way analysis of variance (ANOVA) for repeated measures and when appropriate were compared with the control groups using unpaired *Student's t-test*. Differences were considered statistically significant for *P<0.05*.

Drug concentration causing 50% of maximum response (IC_{50} value) was calculated for each tissue following drawing of full concentration response curves. SigmaPlot computer program (version 11) was used for statistical analysis and plotting the graphs.

RESULTS

Pharmacognosy

The yield of hydroalcoholic and hexanoic extracts of *P. pastinacifolium* were 19% and 0.8%, respectively. Both extracts had dark greenish colour.

Pharmacology

When ileum segment was suspended in the organ bath it started to produce irregular spontaneous contractile activity. Following repeated washing, the ileum gradually relaxed to a stable baseline over 10-20 min. The tissue contracted rapidly to EFS reaching a peak followed by partial relaxation which was then followed by a second peak and then relaxed towards the baseline as described before (28,29). Relaxant effect of *P. pastinacifolium* extracts were examined on these biphasic contractions induced by EFS. Both hydroalcoholic (10 µg/ml-640 µg/ml) and the hexanoic extracts (1.25 µg/ml-160 µg/ml) in a concentration dependent manner inhibited both first and second EFS contractile responses (Fig. 1a, 1b). However the hexanoic extracts was more potent than the hydroalcoholic extract. The IC_{50} value of the hydroalcoholic extract for the initial and the secondary contractile responses were 95 ± 15 µg/ml and 46 ± 10 µg/ml, respectively (n=6). On the other hand, the IC_{50} value of the hexanoic extract for the initial and the secondary contractile response were 11 ± 4 µg/ml and 6 ± 1 µg/ml, respectively (n=6). Over the course of study, an increase in initial contractile response to EFS was observed in the vehicle treated time-matched control tissues (*P<0.05*, ANOVA) while, there was no statistically change in secondary contractile response in the control tissues. The inhibitory effect of both extracts on EFS responses was reversible following washing the tissue with fresh Tyrode's solution.

ACh caused a rapid phasic contraction in rat ileum. After washing the tissue with fresh Tyrode's solution the ileum quickly relaxed to the baseline. The hydroalcoholic extract (2.5 µg/ml-640 µg/ml) and the hexanoic extracts (1.25 µg/ml-160 µg/ml) in a concentration dependent manner inhibited ileum contraction induced by ACh (Fig. 2).
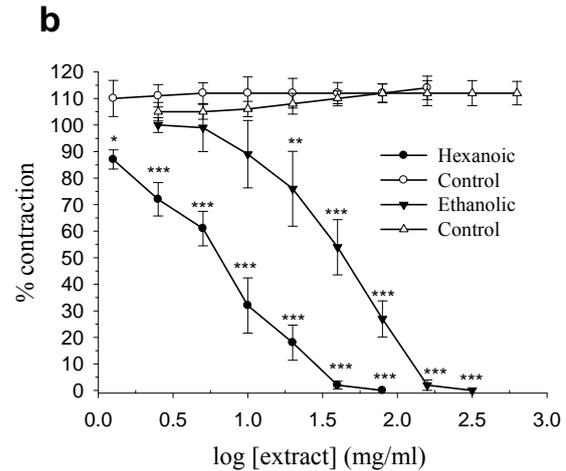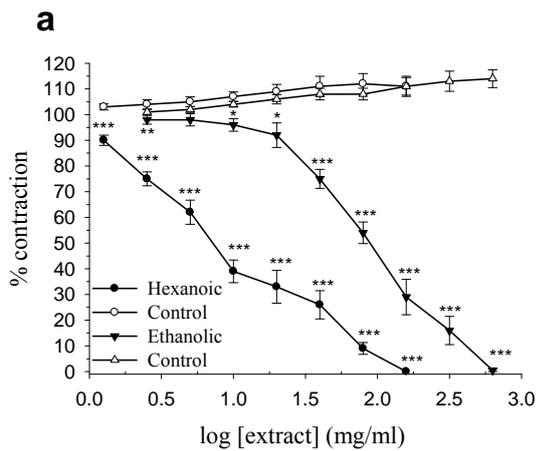
Fig. 1. Effect of *Peucedanum pastinacifolium* hydroalcoholic and hexanoic extracts on tension development to a; first and b; second contractile responses to electrical field stimulation (EFS, 6V, 50Hz, 1 s duration) in isolated ileum of rats. Ordinate scale: ileum contractions expressed as % of initial response to EFS. Abscissa scale: $\log_{10}$ concentration of *Peucedanum pastinacifolium* extracts. Lines drawn through the points, using two fold increments in concentration. The points are mean and the vertical bars show the SEM (n=6). The increase in the response of vehicle treated control tissues only for hydroalcoholic extract in the first EFS response (a) was statistically significant (ANOVA, $P<0.05$). Asterisks show statistical differences between each drug concentration with its corresponding vehicle treated control. Keys: *$P<0.05$, **$P<0.01$, ***$P<0.001$ (*Student's t-test*). Maximum concentration of vehicle (DMSO) in the bath was 1.28%.
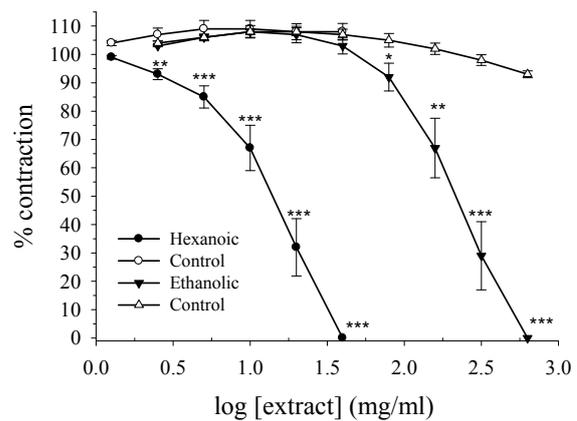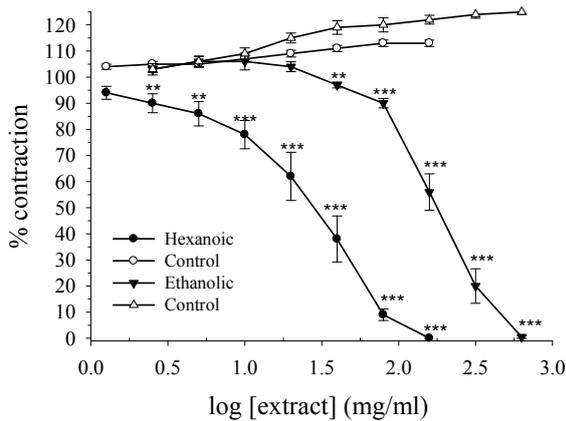

Fig. 2. Effect of *Peucedanum pastinacifolium* hydroalcoholic and hexanoic extracts on tension development to acetylcholine (0.5 µM) in isolated ileum of rats. Ordinate scale: ileum contractions expressed as percent of initial response to acetylcholine. Abscissa scale: $\log_{10}$ concentration of *Peucedanum. pastinacifolium* extracts. Lines drawn through the points, using two fold increments in concentration. The points are mean and the vertical bars show the SEM (n=6). The increase in the response of vehicle treated control tissues is statistically significant (ANOVA, $P<0.001$). Asterisks show statistical differences between each drug concentration with its corresponding vehicle treated control. Keys: **$P<0.01$, ***$P<0.001$ (*Student's t-test*). Maximum concentration of the vehicle (DMSO) in the bath was 1.28% for hydroalcoholic extract and 0.32% for hexanoic extract.

Fig. 3. Cumulative effect of *Peucedanum pastinacifolium* hydroalcoholic and hexanoic extracts on tension development to potassium chloride (KCl, 80 mM), in isolated ileum of rats. Ordinate scale: ileum contraction expressed as percent of initial response to KCl. Abscissa scale: $\log_{10}$ concentration of *Peucedanum pastinacifolium* extract. Lines drawn through the points, using two fold increments in concentration. The points are mean and the vertical bars show the SEM (n=6). The reduction in ileum response only in the control group of hydroalcoholic extract was statistically significant ($P<0.001$, ANOVA). Asterisks show statistical differences between each drug concentration with its corresponding vehicle treated control. Keys: *$P<0.05$, **$P<0.01$, ***$P<0.001$ (*Student's t-test*). Maximum concentration of the vehicle (DMSO) in the bath was 0.4% for hexanoic extract and 1.29% for hydroalcoholic extract.

Although the pattern of inhibitions was similar but the hexanoic extract was more potent. For comparison, the IC_{50} values with the hexanoic extract was 30 ± 5 µg/ml while the IC_{50} values with the hydroalcoholic extract was 175 ± 15 µg/ml. The inhibitory effect of both extract was reversible following removal of extract from organ bath. In the vehicle treated control tissues, corresponding concentration of DMSO gradually increased the ACh responses over the course of studies (Fig. 2) and this increase in ACh response was statistically significant ($P<0.001$, ANOVA).

KCl (80 mM) caused a rapid phasic spasm followed by a stable sustained contraction which was maintained during course of experiment. Both hydroalcoholic and hexanoic extracts of *P. pastinacifolium* concentration dependently inhibited this sustained contraction, but as above, the hexanoic extract was more potent than the hydroalcoholic extract (Fig. 3). At bath concentration of 40 µg/ml the hexanoic extract totally abolished the contractile response to KCl while the hydroalcoholic extracts did not cause any significant inhibition at this concentration (Fig. 3). The inhibitory effect of hydroalcoholic extract started at 80 µg/ml bath concentration and the contractile response to KCl was totally diminished at bath concentration of 640 µg/ml (Fig. 3). The IC_{50} values for hexanoic and hydroalcoholic extracts were 16 ± 2 µg/ml and 220 ± 30 µg/ml, respectively. After washing the tissue with fresh Tyrode's solution the contractile response to KCl was gradually restored. In the vehicle treated time match control there were small but significant reduction in the contractile responses ($P<0.001$, ANOVA) (Fig. 3). The maximum concentration of DMSO for hexanoic and hydroalcoholic extract was 0.4% and 1.29%, respectively. DMSO concentration below 0.4% had no significant inhibitory effect on KCl-induced contractions.

DISCUSSION

The aim of the present study was to investigate antispasmodic effect of *P. pastinacifolium* extracts on isolated ileum contractions indudced by neuronal stimulation (EFS), ACh or KCl. Normal function of gastrointestinal (GI) tract is governed by enteric nervous system (ENS). The ENS consists of a mesh-like system of neurons that is embedded in the lining of the GI tract. It receives inputs from symapthetic and parasympathetic systems, but can act on its own to control the motor as well as secretory functions of the intestine. ENS neurons secrete an intimidating array of neurotransmitters involving many neuropeptides and other neurotransmitters such as ACh, histamine, 5-HT, nitric oxide, and ATP (30,31). The autonomous activity of ENS is responsible for the spontaneous contractile activities seen in the ileum preparation. Abnormal functioning of the ENS recognized as causes of digestive tract disease including small intestinal motility disorders (32).

ACh is a major neurotransmitter that is secreted by neurons during application of EFS. In various GI smooth muscles, ACh produce contractions by activating M_3 muscarinic receptors (33). The M_3 receptor activation, results in the activation of phospholipase C (PLC) and the formation of inositol trisphosphate (IP_3) and diacylglycerol (DAG) (34,35). IP_3 causes Ca^{2+} release from intracellular stores (36,37) and can also mobilize Ca^{2+} secondarily through Ca^{2+}-sensitive or store-dependent mechanisms (38,39). Free intracellular Ca^{2+} ion interacts with calmodulin. Ca^{2+}-calmodulin activates myosin light chain kinase (MLCK) which in turn phosphorylates myosin light chain. The contractile machinery of smooth muscle is activated when the myosin light chain undergoes phosphorylation. Another source of free Ca^{2+} ions is extracellular Ca^{2+} ions. Some channels that occur in the plasma membrane can be activated by membrane depolarization to allow Ca^{2+} entry. High concentration of KCl, causes depolarization of these voltage gated Ca^{2+} channels and in this way increases intracellular Ca^{2+} ion contraction (40).

Our findings show that both polar (hydroalcoholic) and non-polar (hexanoic) extracts of *P. pastinacifolium* had profound inhibitory effect on rat ileum contraction induced by exogenous spasms such ACh and KCl and release of endogenous

neurotransmitters from ENS. However, the hexanoic extract was more potent. For example, when concentration ratio was compared at IC$_{50}$ level, the hexanoic extract was 14 times more potent than the hydroalcoholic extract on inhibiting KCl response. Similarly the hexanoic extract was 6 and 8 times more potent than the hydroalcoholic extract for inhibiting the ACh and EFS responses respectively. As mentioned above, these spasmogens in a different way elevate intracellular free Ca^{2+} ion concentration. Therefore, it is likely that the active component(s) of the extract act at final stage of contraction which is common pathway of contraction for the spasmogens. The antispasmodic is suggested to be due to presence of non-polar components, such as methyl eugenol (41), alpha-pinene (42), and furanocoumarins. Since furanocoumarins, regardless of coumarins, are separated more by non polar phase (43). The considerable inhibitory effect observed with hexanoic extract could be attributed to the presence of higher quantity of lipophilic compounds in the hexanoic extract. Therefore it is likely that the same compounds are responsible for the observed inhibitory effect.

It also should be mentioned that the non polar components which has been indentified in *P. Pastinacifolium* essential oil are elemicin, limonene, myristicin, methyl eugenol, dillapiole, *trans-β*-ocimene, and α-pinene (15).

CONCLUSION

In this research we have shown that the *P. pastinacifolium* extract contains bioactive materials with antispasmodic effect on isolated ileum. The active components probably are furanocoumarins and non-polar component(s); methyl eugenoal and pinene. Identification of other active components for further drug development is recommended.

ACKNOWLEDGEMENTS

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