

Relaxant effect of four fractions separated from alkaloid extract of *Pycnocycla spinosa* on rat isolated ileum

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Abstract

Hydroalcoholic extract of *Pycnocycla spinosa* was shown to have spasmolytic action *in vitro* and antidiarrhoeal effect *in vivo*. The hydroalcoholic extract of *P. spinosa* is composed of alkaloid, flavonoid and saponin components. Alkaloid fraction of *P. spinosa* contains the most active constituents. Therefore, the aim of this research was to separate different fractions of alkaloid extract of *P. spinosa* and screen for their spasmolytic activity on rat isolated ileum. Alkaloid fraction of *P. spinosa* was separated by fractional liberation technique. Four fractions were separated from the alkaloid rich fraction using thin layer chromatography. All 4 separated fractions inhibited the spasmolytic response to 80 mM KCl in a concentration-dependent manner. Two of the separated fractions also inhibited the response to ACh and 5-HT. This study showed the relationship between antispasmodic actions of different components presented in 4 fractions obtained from alkaloid extract of *P. spinosa*.

Keywords: *Pycnocycla spinosa*; Hydroalcoholic extract; Spasmolytic; Alkaloid extract

INTRODUCTION

Pycnocycla spinosa Decne. exBoiss. var. *spinosa* (Fam. Umbelliferae) is an essential oil-containing wild plant growing in Iran (1). Hydroalcoholic extract of *P. spinosa* is a potent relaxant of isolated ileum (2) and its anti-spasmodic action is very similar to that of dicyclomine (3). In addition, *P. spinosa* extract was shown to have antidiarrhoeal action *in vivo* (2). Hydroalcoholic extract of *P. spinosa* at antidiarrhoeal doses had no serious central or cardiovascular adverse effects and it was acting more selectively on ileum smooth muscle (4). In addition, the lethal dose causing 50% mortality (LD₅₀) was above 140 mg/kg indicating a relatively good margin of safety in animal models (5). High extract yield (14% w/w), and selective pharmacological activity are characteristics which make *P. spinosa* extract a suitable candidate to be used as antidiarrhoeal agent. As the main antidiarrhoeal action of *P. spinosa* extract is due to its antispasmodic action, it is necessary to identify

more active component(s). The hydroalcoholic extract is composed of alkaloid, flavonoid and saponin components. The antispasmodic action of *P. spinosa* extract is mainly due to alkaloid and flavonoid rich fractions with the alkaloid fraction being the most potent constituents (6). Therefore, objective of this research was to further separate alkaloid fraction components of *P. spinosa* extract and investigate their spasmolytic action on rat isolated ileum.

MATERIALS AND METHODS

Plant material

Aerial parts of *P. spinosa* were collected in June 2003 from Isfahan University campus and identified in the Biology Department of Isfahan University. A voucher specimen was authenticated and then deposited in the herbarium of the School of Pharmacy and Pharmaceutical Sciences (Isfahan, Iran). The aerial part of the plant was dried in shade. The total hydroalcoholic extract was obtained by

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percolation (7). Alkaloid rich fraction was extracted from the total extract using a general method based on acidifying/basifying and extraction by chloroform (8-10). The alkaloid fraction then was further separated by thin layer chromatography (TLC).

TLC analysis

The concentrated alkaloid extract was spotted on coated silicagel plates and chromatographed in saturated chamber containing $\text{CHCl}_3:\text{MeOH}:\text{NH}_3$ (10:4:1) solvent mixture for 1.5 h. Identified bands then were scraped off and eluted with methanol and centrifuged for 5 min to separate the Silicagel. Visualization of the separated bands was carried out under UV light (365 nm).

Drugs and solutions

Acetylcholine chloride (ACh), 5-hydroxytryptamine (5-HT) were purchased from Sigma (USA). 5-HT was made up as 10 mM stock solution in distilled water. ACh was made up as 100 mM stock solution in distilled water and acidified with a drop of acetic acid, dilution was made in distilled water. KCl was made up as 2 M stock solution in distilled water. The total, alkaloid and all extract fractions were made up as 10 mg/ml stock solution in 70% ethanol, dilution being made in distilled water. Tyrode's solution composed of (mM): 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 was made up in distilled water and bubbled with CO_2 until the pH was adjusted to 7.4; thereafter, gassing continued with O_2 . All chemicals, unless otherwise stated, were purchased from Merck.

Experimental procedure

The contraction procedure design was as described in previous studies (2,6). Male Wistar rats (200-250 g) bred and held in Isfahan School of Pharmacy and Pharmaceutical Sciences animal house at room temperature and feed with standard rat food plates. On day of the 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. Then the

connective tissue was carefully trimmed from the tissue and then suspended in Tyrode's solution at 37 °C and bubbled with oxygen. From a resting tension of 1 g, isotonic contractions, elicited by KCl, ACh or 5-HT were recorded using Harvard isotonic transducer and displayed on a Harvard Universal Oscillograph pen recorder device. Extracts were added directly to the organ bath in volumes usually not exceeding 5% of bath volume (20 ml organ bath). A concentration-response curve was obtained by cumulative addition of the extracts at 15 min intervals after addition of 80 mM KCl. The effects of ACh (500 nM) and 5-HT (1 μM) were studied with a contact time of 30 s and time cycle of 3 min. These concentrations caused 70%-90% of the maximum attainable response. Each concentration of the extract was at least 10 min in contact with the tissue before its effect was evaluated. All experiments were conducted in parallel with time-matched controls using the tissue from same animal and adding an equivalent volume of vehicle in lieu of the extracts.

Measurements and statistical analysis

Ileum contractions were measured as maximum changes in tension from pre-drug baseline within the contact time or as area under the curve produced by tissue contractions at 5 min intervals just before addition of next concentration of the extract (or vehicle) and expressed as percentage of control induced response for each tissue. Concentration of the extract causing 50% inhibition of the maximum response (IC_{50}) was determined for each tissue. Mean and standard error of mean (S.E.M.) values were calculated for each group of results and significance of differences between the means was calculated by analysis of variance (ANOVA). Furthermore, inter-group comparison between corresponding points was made by two-tailed paired Student's t-test. Differences were considered statistically significant when $P < 0.05$.

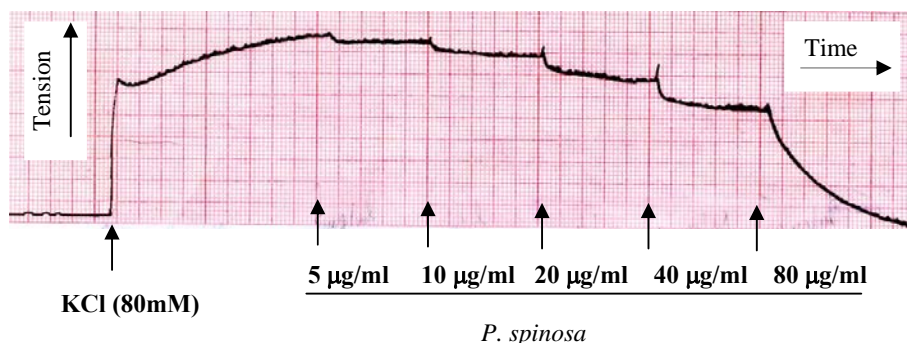


Fig. 1. Original trace shows relaxant effect of cumulative addition of *P. spinosa* hydroalcoholic extract on tonic contraction induced by KCl in isolated ileum of rat.

RESULTS

Extraction of plant material

Alkaloid rich fractions were extracted from dried hydroalcoholic extract. TLC was able to resolve four fractions, namely F1, F2, F3 and F4. These fractions were identified by their Rf value F1 (0-0.4), F2 (0.43-0.47), F3 (0.52-0.70) and F4 (0.80-0.85), respectively.

Antispasmodic study

Rat ileum suspended in Tyrode's solution under 1 g weight had a stable tension. KCl (80 mM) produced a sustained tonic contraction. ACh and 5-HT caused a rapid contraction, reaching their maxima within 30 s of contact. In a single experiment the total hydroalcoholic extract inhibited KCl response with complete relaxation at 80 µg/ml bath concentration (Fig. 1), confirming previous work (2). Alkaloid extract of *P. spinosa* in a concentration-dependent manner inhibited the ileum contraction induced by 80 mM KCl (Fig. 2) with an IC_{50} value of 11 ± 1.1 µg/ml (n=6). With 20 µg/ml bath concentration response to KCl was almost abolished. The inhibitory effect of alkaloid extract was reversible after washing the tissue with fresh Tyrode's solution. In a similar way F1 fraction inhibited the tonic contraction to KCl ($IC_{50} = 27 \pm 6.5$ µg/ml, n=6) (Fig. 3). The F4 fraction also similarly inhibited the tonic contraction induced by KCl ($IC_{50} = 10 \pm 0.8$ µg/ml, n=6) (Fig. 4). The F2 fraction ($IC_{50} = 32 \pm 4$ µg/ml, n=6) and F3 fraction ($IC_{50} = 34 \pm 5$ µg/ml, n=6) at relatively higher concentrations had

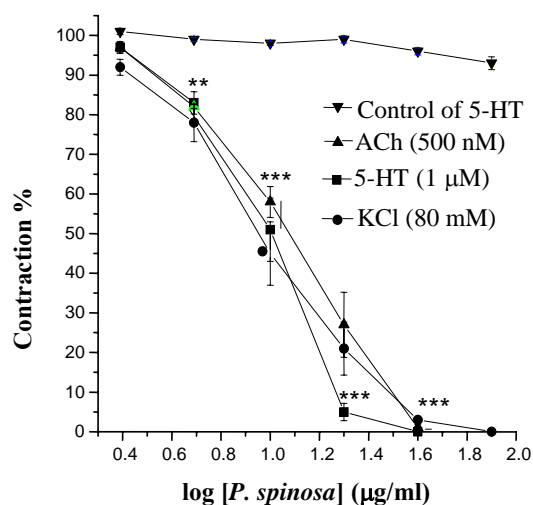


Fig. 2. Effect of alkaloid rich fractions of *P. spinosa* on tension development to KCl, ACh and 5-HT in isolated ileum of rats. Ordinate scale: The extracts effects expressed as % of contraction compare to control. Abscissa scale: \log_{10} concentration of the extracts. The points are mean and the vertical bars show the S.E.M. (n=6). There was no statistically significant change over the course of studies in the control group (ANOVA). ** $P < 0.01$, *** $P < 0.001$ compared with the corresponding point in the control group (Student's t-test).

inhibitory effect on ileum contractions due to KCl (Fig. 4). During the course of experiments there were no significant changes in KCl responses of the tissues treated with equivalent volume of the vehicle.

Relaxant effect of more active fractions (F1 & F4), were further examined on contraction induced by ACh and 5-HT and compared with the alkaloid extract. Alkaloid extract of *P. spinosa* reduced the tissue response to 5-HT ($IC_{50} = 14 \pm 2.0$ µg/ml, n=6) in a concentration dependent manner, inhibiting the

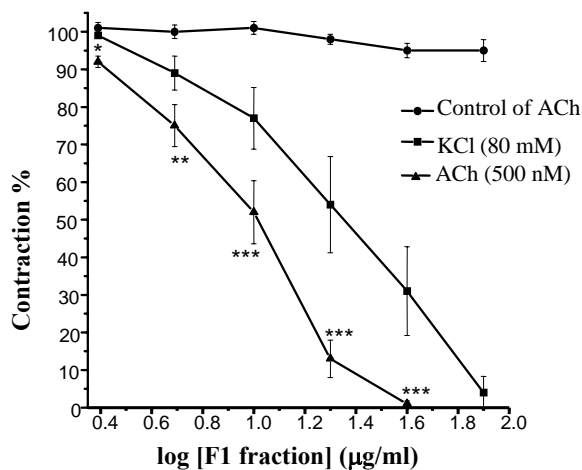


Fig. 3. Effect of fraction F1 separated from alkaloid extract of *P. spinosa* on tension development to KCl and ACh in isolated ileum of rats. Ordinate scale: The extracts effects expressed as % of contraction compare to control. Abscissa scale: \log_{10} concentration of the extracts. The points are mean and the vertical bars show the S.E.M. (n=6). There was no statistically significant change over the course of studies in the control group (ANOVA). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with the corresponding point in the control group (Student's t-test).

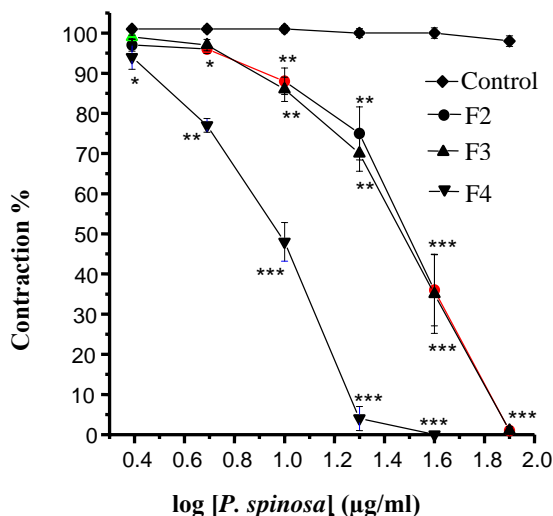


Fig. 4. Effect of three fractions separated from alkaloid extract of *P. spinosa* on tension development to KCl (80 mM) in isolated ileum of rats. Ordinate scale: The extracts effects expressed as % of contraction compare to control. Abscissa scale: \log_{10} concentration of the extracts. The points are mean and the vertical bars show the S.E.M. (n=6). There was no statistically significant change over the course of studies in the control group (ANOVA). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with the corresponding point in the control group (Student's t-test).

response by 97% at 40 µg/ml concentration (Fig. 2). Furthermore, alkaloid fraction at 2.5 µg/ml to 40 µg/ml bath concentrations inhibited the ACh (500 nM) contraction and at the highest used concentration completely removed the response to ACh ($IC_{50} = 11 \pm 1.0$ µg/ml, n=6) (Fig. 2). The inhibitory effect of F4 fraction on ACh response ($IC_{50} = 10 \pm 1.4$ µg/ml, n=6) and 5-HT response ($IC_{50} = 10 \pm 1.2$ µg/ml, n=6) were very similar to that of the KCl response. However, the F1 fraction at lower concentrations inhibited the tissues responses to 5-HT ($IC_{50} = 17 \pm 1.5$ µg/ml) and ACh ($IC_{50} = 11 \pm 1.5$ µg/ml) compared with KCl response (n=6, Fig. 3). Equivalent volume of vehicle (ethanol) had no significant effect to either ACh or 5-HT responses.

DISCUSSION

P. spinosa Decne. exBoiss. var. *spinosa* (Fam. Umbelliferae) is a wild plant growing in Iran (1). Hydroalcoholic extract of *P. spinosa* has spasmolytic effect *in vitro* (2). In addition *P. spinosa* extract at oral dose of 1 mg/kg inhibits castor oil induced diarrhoea in mice (2). Alkaloid fraction of *P. spinosa* has shown to have the most active constituents (6). The antidiarrhoeal effect of *P. spinosa* extract is very likely due to its antispasmodic action. Therefore, objective of this research was to separate different fractions of alkaloid extract of *P. spinosa* and screen for their spasmolytic activity *in vitro* to find out which fraction is mainly responsible for inhibition of ileum contraction induced by three different spasmogen (KCl, ACh and 5-HT). Gastrointestinal smooth muscle contraction is dependent on the intracellular Ca^{2+} concentration. In general, there are two types of excitation-contraction coupling based on the type of mechanism responsible for changes in Ca^{2+} concentration. Electromechanical coupling requires changes in membrane potential, which in turn activate the voltage-dependent Ca^{2+} channel to trigger influx of Ca^{2+} . KCl (80 mM) used extracellularly mainly caused contraction in this way, while ACh and 5-HT act via specific receptors and can produce changes in tension (11), without necessarily first affecting membrane potential. Both ACh

and 5-HT, have functional roles in intrinsic contraction of gastrointestinal tract. ACh is a neurotransmitter at post-ganglionic parasympathetic neurons that innervate the gut. The response to ACh is mediated by activation of two types (M_2 and M_3) of muscarinic receptors (12,13). Activation of these receptors results in an increase in intracellular Ca^{2+} , an effect mediated by inositol triphosphate acting on internal calcium stores (11,14,15). Serotonin (5-HT) is also an important substance in the gastrointestinal tract and is present in both enterochromaffin cells of the mucosa and neurons of the mesenteric plexus; it affects both secretion and motor activity (16,17). Although, atropine can completely abolish the effects of ACh on the gastrointestinal tract, it incompletely inhibits the effects of vagal impulses on motility of the gut. There are evidences, which suggest that 5-HT is partly responsible for the remaining response (18). Atropine has no effect on KCl induced contraction on rat ileum (19) but nifedipine totally blocks the contractile response of KCl (11). However, nifedipine only partially diminishes the phasic response to ACh (11). This effect of nifedipine brought suggestion that ACh response depends on release of internal Ca^{2+} , as well as, entry from extracellular space through voltage-dependent calcium channels (11). 5-HT causes contraction of isolated strip of ileum, mainly by direct effect on the muscle cells. The contractile effect of 5-HT on rat ileum was blocked by methylsergide, mianserin and ketanserin (20). The rank order of potency of 5-HT receptor selective agonist and antagonist suggest involvement of a $5-HT_2$ receptor (20). Neither terodotoxin nor atropine affected contraction of 5-HT, suggesting a smooth muscle localization of these $5-HT_2$ receptor subtypes (20).

By comparing IC_{50} values of different separated fractions, it can be seen that the alkaloid extract of *P. spinosa* has similar inhibitory action on rat ileum contraction induced by KCl, ACh and 5-HT. However it is relatively more potent than the total hydroalcoholic extract. None of the four separated fractions were more active than the alkaloid extract. In fact two of the fractions (F2 & F3) were slightly less active than the

alkaloid extract. These results indicate that after separation procedure some of the activity is being lost. Provided that the fractionation technique hadn't affected the ingredient of the components, it can be concluded that the constituent may have additive or synergism effect. As the concentration of all studied fraction are less than concentration of total hydroalcoholic extract, therefore, it is very likely that all the fractions playing some part in the antidiarrhoeal action of the *P. spinosa* extract. Nevertheless, the inhibitory mechanism of action might be different with different fractions. For instance if inhibitory effect of fraction F1 on contraction induced by KCl, ACh and 5-HT are compared, it can be seen that the order of relaxation is $ACh > 5-HT > KCl$, while fraction F4 has a similar inhibitory action on these three spasmogens. Another point, which needs to be taken into consideration, is molecular weight of the constituents. As we are unaware of their molecular weight we can only make qualitative comparison between the fractions.

From this study it can be concluded that all different fractions separated from alkaloid extract of *P. spinosa* contribute to the spasmolytic activity of this herbal plant. That might be because these constituents are structurally very similar, therefore it is suggested that structure of antispasmodic constituents be phytochemically characterized, in order to identify a suitable lead compound.

ACKNOWLEDGMENT

We would like to acknowledge that this research was supported by Isfahan University of Medical Sciences, Isfahan, Iran. We would also like to thanks Dr. Sajjadi for his help and advise on extract separation, and Mr. Mehregan (botanist) in Biology Department of Isfahan School of Pharmacy and Pharmaceutical Sciences.

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