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## Document heading

# The role of $\alpha$ – and $\beta$ – adrenergic receptors in the spasmolytic effects on rat ileum of *Petroselinum crispum* Latifolium (parsley)

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## ABSTRACT

**Objective:** To investigate *Petroselinum crispum* Latifolium (parsley) for treating stomach and intestinal disorders in Iran. **Methods:** An 80% ethanol extract was prepared from parsley seeds, and its antispasmodic activity assessed by measuring contractions of isolated ilea induced by 60 mM potassium chloride (KCl). A piece of ileum from an adult male Wistar rat was dissected and mounted in an organ bath containing Tyrode's solution, and ileum contractions were recorded by an isotonic transducer under one gram resting tension. The effect of parsley extract was measured in the presence of two antagonists' of  $\alpha$  – and  $\beta$  –adrenoceptors. **Results:** These experiments showed that parsley extract inhibited the response to 60 mM KCl in a concentration-dependent manner ( $P < 0.01$ ,  $n = 7$ ). The spasmolytic effect of parsley extract was unaffected by 1  $\mu$  M phentolamine or 1  $\mu$  M propranolol. **Conclusions:** This study shows that parsley seed extract is a relaxant of isolated rat ileum and the relaxation effect of extract does not involved adrenergic receptors.

## 1. Introduction

About 80% of the world's population relies on the use of traditional medicine, which is predominantly based on plant material[1]. Studies on a number of medicinal plants indicate that promising phytochemicals can be developed for many health problems[2]. Herbal drugs have gained importance in recent years because of their efficacy and cost effectiveness. These drugs are invariably single plant extracts or fractions thereof, or mixtures of fractions and extracts from different plants that have been carefully standardized for safety and efficacy[3]. *Petroselinum crispum* (*P. crispum*) (parsley), which belongs to the Apiaceae family[4] and is presumed to be native to the Mediterranean region, is now cultivated throughout the world. The plant has been used in folk medicine as a diuretic, a stomachic, an emmenagogue and an abortifacient[5]. In previous work, we reported that the inhibitory effect of parsley seed extract on ileum contraction was probably due to blocking of voltage-gated calcium channels[6]. Gastrointestinal motility is an integrated process that includes myoelectrical activity, contractile

activity, tone, compliance and transit[7]. These different phases of motility are generated and modulated by local and circulating neurohumoral substances. The importance of neurohumoral control of motility goes back to 1859, where *C. Bernhard* observed profuse diarrhea (paralytic secretion) and vigorous intestinal motility (hunger contractions) after external asympathetic denervation of the dog gut. The last decade has brought substantial new knowledge about the process, particularly the involvement of the enteric nervous system (ENS)[8]. The enteric nervous system within the gastrointestinal tract contains all necessary elements for autonomic functions. Basic motor events, such as the peristaltic reflex, do not require extrinsic innervation to occur. Nevertheless, the local mechanisms for peristaltic reflex are strongly affected by parasympathetic and sympathetic outflow from the central nervous system and are tuned to the overall needs of the body. The extrinsic nerve supply to the gut is mediated through the mesenteric nerves, including noradrenergic sympathetic nerve fibers, sensory afferent nerve fibers and vagal parasympathetic nerve fibers. One of the main actions of sympathetic nerves is to inhibit motor function in the small intestine[9], which usually causes inhibition of intestinal motility, secretion, and blood flow[10]. However, in the guinea-pig ileum, and indeed in the human small intestine, the muscle layers receive very little direct innervation from sympathetic neurons. It is clear from these experiments that noradrenaline released from sympathetic nerves inhibits

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the action of excitatory motor neurons on this muscle layer, although, the circular muscle is more important than the longitudinal muscle in the intestinal behaviours of peristalsis and segmentation<sup>[11]</sup>. The adrenergic receptors are part of the super-family of G-protein-coupled receptors (GPCRs)<sup>[12]</sup> that bind and mediate the physiologic actions of catecholamines; such as epinephrine and norepinephrine<sup>[13]</sup>. These seven transmembrane-spanning receptors regulate their effector systems via coupling to heterotrimeric ( $\alpha$  –  $\beta$  –,  $\gamma$  – subunit) G-proteins that stimulate their effector systems to mediate physiological effects, such as smooth muscle contraction, neurotransmitter release, and changes in heart rate and contraction. Previous studies suggested that the adrenergic nervous system provides extrinsic tonic inhibitory control of gut motility<sup>[14]</sup>. The localization of adrenergic receptors mediating relaxing action was investigated in innervated and denervated longitudinal muscle strips from guinea pig ileum and rabbit jejunum<sup>[15]</sup>. Sympathetic activity inhibits transmitter release from enteric nerves involved in modulating gut motility and secretion, through the actions of noradrenaline on  $\alpha_2$ -AR as well as  $\beta$ -AR. Presynaptic  $\alpha_2$ -AR terminals inhibit the release of both noradrenaline and acetylcholine<sup>[16]</sup>.  $\beta$ -adrenoceptor proteins are linked via a stimulatory G-protein (GS) to adenylyl cyclase, an enzyme whose activation results in the generation of the second messenger, cAMP. cAMP in turn, activates a number of signaling pathways that result in smooth muscle relaxation<sup>[17]</sup>. Despite a considerable number of reported studies for parsley extract, the mechanism of its effect on ileum smooth muscle has not yet been investigated. The present work was undertaken to evaluate the role of  $\alpha$  – and  $\beta$  – adrenergic receptors on the spasmolytic effect of parsley seed in rat ileum and to evaluate its clinical benefits in gastrointestinal disorders.

## 2. Materials and methods

### 2.1. Plant extract

*P. crispum* Latifolium seeds were collected in April 2006 from Jahad Research Center (Ahwaz) and identified by the Department of Biology, Shahid Chamran University, Ahwaz, Iran. The samples were washed and air-dried under shade and pulverized into a husky powder. One gram of powder was extracted with 10 mL ethanol-distilled water (8:2 w/v), centrifuged for 15 min and the supernatant collected. This process was repeated three times. The ethanol was removed by evaporation<sup>[6, 18, 19]</sup>.

### 2.2. Chemicals and solutions

Potassium chloride (KCl) was from Merck (Germany), propranolol and phentolamine from Sigma (USA). KCl was made as a 60 mM stock solution in Tyrode's solution<sup>[20]</sup>. Propranolol<sup>[9, 21]</sup> and phentolamine<sup>[22]</sup> were made as 1  $\mu$ M stock solutions in Tyrode's solution. The plant extract stock solution was 0.1 g/L Tyrode's. The composition of Tyrode's solution, in (mM was): NaCl(139.9), KCl(2.68), CaCl<sub>2</sub>(1.8), MgCl<sub>2</sub>(1.05), NaHCO<sub>3</sub>(11.9), NaH<sub>2</sub>PO<sub>4</sub>(0.42) and glucose(5.55), made in distilled water<sup>[23, 24]</sup>. All reagents were from Merck.

### 2.3. Animal and ileum preparation

Male Wistar rats (225±25) g from Ahwaz Jundishapur University of Medical Sciences (AJUMS) animal facility were used. Animals were housed in cages at 20–24 °C with a 12 h/12 h light/dark cycle and free access to food and water. They were housed in cages with a mesh bottom to prevent coprophagy and deprived of food but not water 24 h prior to the experiment. Animals were killed by cervical dislocation, and after laparotomy, a strip of ileum dissected and the intraluminal content flushed out with cooled oxygenated Tyrode's solution. The portion of ileum was rapidly isolated, removed and suspended between two stainless steel hooks in a 10 mL jacketed organ chamber containing Tyrode's solution maintained at 37 °C and bubbled with air. The lower hook was fixed at the bottom of the organ bath and the upper one was connected to an isotonic transducer. Tissue were allowed to equilibrate for 60 min before drug additions under a resting tension of 1 g, during which the buffer solution was renewed every 15 min. Isotonic responses elicited by KCl were recorded using a Harvard isotonic transducer and displayed on a Harvard universal oscillograph device. A contraction–response curve was obtained by cumulative addition of extract at 5 min intervals after the addition of 60 mM KCl. Each extract concentration had 5 min of contract with the tissue before effects were evaluated.

### 2.4. Procedure

#### 2.4.1. Effect of extract on KCl-induced ileum contraction

Rat ilea suspended in Tyrode's solution showed irregular spontaneous contractile activity, which was attenuated by changing the bath fluid. 60 mM KCl a non-receptor stimulation for opening Ca<sup>2+</sup> channels produced a sustained tonic contraction, which was maintained during the course of experiments. After administering of 60 mM KCl to the bath solution, various concentrations of extract (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 mg/mL) were added sequentially to the bath.

#### 2.4.2. Effects of an $\alpha$ –receptor antagonist

To investigate the possible mechanisms of the relaxation response induced by extract, the strip of ileum was equilibrated and contracted with 60 mM KCl, then cumulative concentration–effect curve of extract (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 mg/mL) were recorded after 30 min incubation with the  $\alpha$  –antagonist phentolamine at 1  $\mu$ M<sup>[22]</sup>.

#### 2.4.3. Effect of a $\beta$ –receptor antagonist

The non-selective  $\beta$  –adrenoceptor antagonist propranolol was used to characterize the role of  $\beta$  –adrenoceptors in relaxation caused by extract. The preparations were treated with 1 mM propranolol<sup>[9, 21]</sup> for 30 min<sup>[25]</sup>, then contracted with 60 mM KCl, and a cumulative–dose response curve of extract recorded.

### 2.5. Measurements and statistical analysis

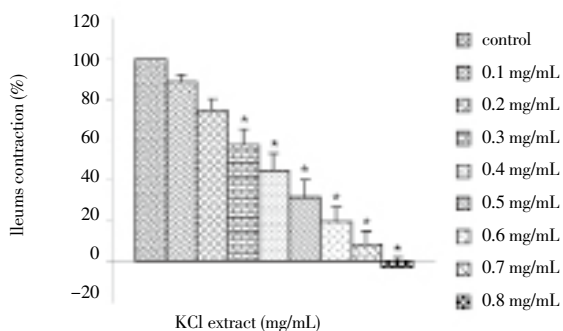
Contractions were measured as a maximum changes in tension within the contract time, from a pre-drug baseline or as the area under the curve produced by tissue contraction at 5 min intervals just before adding the next concentration of the test extract. Data are expressed as a percentage of the

control or maximum induced response for each tissue. Mean and standard error of mean (SEM) values were calculated for each group of results and significance between the means was calculated by one-way analysis of variance (ANOVA) and the post hoc, Tukey test. Values were considered to be significantly different from control when  $P < 0.05$ .

### 3. Results

#### 3.1. Effect of extract on KCl-induced contraction

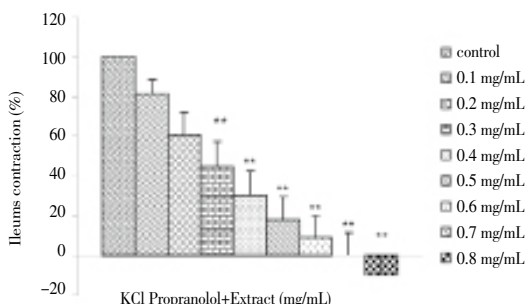
Cumulative additions of extract (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 mg/mL) significantly reduced ileum contraction induced by 60 mM KCl ( $P < 0.001$ ,  $n = 7$ ), in a dose dependent manner (Figure 1).



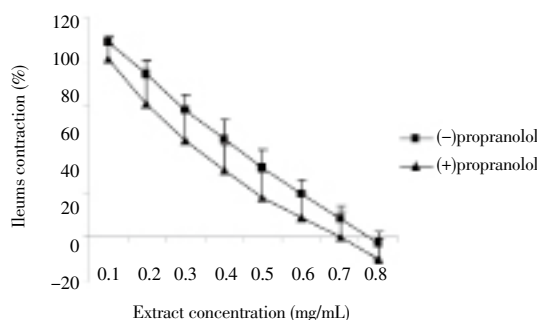
**Figure 1.** Spasmolytic effect of cumulative concentrations of parsley extract on KCl-induced rat ileum contractions ( $n = 7$ ).  $*P < 0.01$ .

#### 3.2. Spasmolytic effect of extract in the presence of phentolamine

Figure 2 shows the effect of pretreatment with phentolamine on the responses of ileum in the presence of extract. The results indicate that the extract inhibited ileum contractions in a concentration-dependent manner. Significant differences between concentrations of extract were observed (Figure 2). Phentolamine, a non-selective  $\alpha$ -adrenoceptor antagonist, not only failed to reduce the antispasmodic activity of the extract, but instead significantly increased the extract activity (Figure 3) ( $P < 0.05$ ).



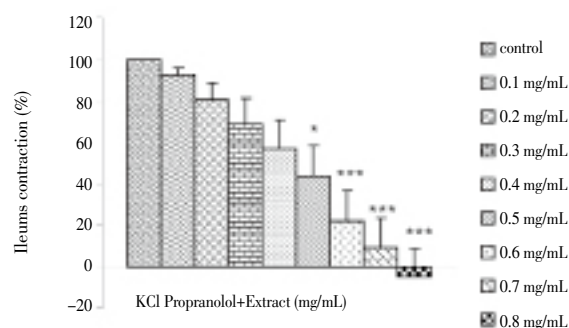
**Figure 2.** Effect of phentolamine on the relaxant activity of parsley extract on ileum contractions induced by KCl (60 mM) ( $n = 7$ ).  $*P < 0.05$ ;  $**P < 0.01$ .



**Figure 3.** Spasmolytic effect of cumulative concentrations of extract on rat ileum in Tyrode's solution. The spasmogenic effect was evaluated in the absence ( $\blacksquare$ ) and presence ( $\blacktriangle$ ) of  $1 \mu\text{M}$  phentolamine ( $n = 7$ ;  $*P < 0.05$ ).

#### 3.3. Spasmolytic effect of extract in the presence of propranolol

Figure 4 shows the effect of pretreatment with propranolol on the response of ileum in the presence of extract. The results indicate that the extract produced a significant relaxation effect on the ileum, with significant differences between concentrations of extract indicated (Figure 4). Propranolol ( $1 \mu\text{M}$ , 30 min,  $n = 7$ ), a non-selective  $\beta$ -adrenoceptor antagonist, did not significantly alter the spasmolytic activity of the extract on KCl-induced ileum contraction (Figure 5).



**Figure 4.** Effect of propranolol on the relaxant activity of parsley extract on KCl-induced ileum contractions ( $n = 7$ ).  $*P < 0.05$ ;  $**P < 0.01$ .

### 4. Discussion

The objective of this work was to study the action of parsley extract on contractile activity of rat ileum induced by a spasmogen, and compare the effects of two adrenergic system antagonists. Ultimately, the goal is to investigate the benefits of this drug for gastrointestinal disorders. Current therapy for some of these disturbances inhibits smooth muscle contractions. This study show that, an alcohol extract of parsley seed is a relaxant of rat isolated ileum *in vitro*.

Studies done in 1984, using intact smooth muscle tissue, showed that a G protein-coupled receptors agonist can produce a greater increase in force for a given increase in  $[\text{Ca}^{2+}]_i$  than KCl. KCl-induced contraction has long been

known to be due to membrane depolarization causing  $\text{Ca}^{2+}$  entry through voltage-operated  $\text{Ca}^{2+}$  channels, activation of  $\text{Ca}^{2+}$ -dependent myosin light chain (MLC) kinase, and an increase in MLC phosphorylation[26]. The phasic contraction is due to direct  $\text{Ca}^{2+}$  influx through L-type voltage dependent channels[27]. Our results showed that parsley seed extract significantly inhibited KCl-induced phasic contractions. This observation suggested that the extract may have an inhibitory effect on L-type voltage-dependent  $\text{Ca}^{2+}$  channels or may reduce the sensitivity of the contractile system to  $\text{Ca}^{2+}$ . The main function of the small intestine is to absorb and secrete. Diarrhea results from an imbalance between the absorptive and secretive mechanisms in the intestinal tract, accompanied by intestinal hurry, which results in fluid excess in the faeces. Reduction of gastrointestinal motility is one of the mechanisms by which many antidiarrheal agents act[28]. The essential parsley oil in this analysis is dominated by five substances: myristicin, apiol,  $\alpha$ -pinene,  $\beta$ -pinene and 1-allyl 1, 2, 3, 4, 5-tetramethoxy-benzene[29]. Some reports indicate that the extracts from the leaves of *Kalanchoe crenata* (*K. crenata*) possess antispasmodic and spasmogenic activities on rat and guinea pig ileum. They also suggest that, the presence of flavonoids in *n*-butanol extracts of *K. crenata* may account for its anti-spasmodic activity[27]. Another report showed that essential oil of *Alpinia speciosa schum* (EOAS) possesses both relaxant and antispasmodic action on the ileum[30]. The inhibitory effect *Teucrium polium* essential oil on smooth muscle is due to an effect on  $\text{Ca}^{2+}$  channels[31]. Researchers have also shown that  $\alpha$ -pinene and  $\beta$ -pinene, two components of the *Ferula gummosa* essential oil, and the hydroalcoholic extract, had clear inhibitory effects on tonic contraction induced by KCl[24]. Flavonoids, secondary plant metabolites occurring widely in vegetables have been shown to display a remarkable array of biochemical and pharmacological actions, including relaxing effects on intestinal smooth muscle[27]. It has been claimed that plants that have essential oils, are generally used traditionally for gastrointestinal disorders. In several studies on the relaxant effects of essential oils, including citral, inhibition of contractile over-activity, or reduction of the ileum inflammatory response is reported to be the basis for the relief of gastrointestinal disorders and diarrheal[28]. Another study suggests that since the inhibitory effects of plant essential oils with different components are qualitatively similar, they may all have a similar mechanism of action[24]. This finding suggests that *P. crispum* extract has the ability to influence the peristaltic movement of intestine, indicating a possible intestinal anti motility activity. However, it seems that the relaxation effect observed for isolated rat ileum contractions, was induced by components present in the essential oil, such as  $\alpha$ -pinene,  $\beta$ -pinene of the *P. crispum* hydroalcoholic extract. In order to test the extract's mechanism of action the role of  $\alpha$ - and  $\beta$ -adrenergic receptors (AR) on extra induced relaxation was investigated. To assess if the extract relaxed intestines by binding to  $\alpha$ - or  $\beta$ -receptors, the relaxing effect of the extract was examined in the presence of phentolamine or propranolol. The ineffectiveness of both on the spasmolytic effect of extract indicates that  $\alpha$ - and  $\beta$ -adrenergic receptors are not involved in this action. Activation of adrenergic receptors in ileal smooth muscle leads to relaxation[27]. Smooth muscle tone is achieved by a balance between the actions of contraction- and relaxation-induced agents and Intracellular signaling pathways for the corresponding processes have been proposed. The pathway from the muscarinic receptors to enhanced contraction clearly involves an increase in cytosolic  $\text{Ca}^{2+}$  levels. The corresponding pathway leads from stimulation of  $\beta$ -adrenergic receptors and an increase in cAMP, to relaxation of phasic smooth muscle[32]. The

localization of adrenergic receptors mediating a relaxing action was investigated in innervated and denervated longitudinal muscle strips from guinea pig ileum and rabbit jejunum[15].  $\alpha_{2a}$ ,  $\beta_1$  and  $\beta_2$ -AR in the ENS of guinea pigs, rats and mice have been demonstrated[16]. Some researchers suggested that in the guinea pig ileum alpha-adrenoceptors mediating relaxation are located only in cholinergic neurons, whereas in rabbit jejunum they are located both in these neurons and in the smooth muscle cells[15]. To date, all three  $\alpha_2$ AR subtypes appear to couple to the same signaling systems, at least in native target cells, which include inhibition of adenylyl cyclase, activation of receptor-operated  $\text{K}^+$  channels, and inhibition of voltage-gated  $\text{Ca}^{2+}$  channels[12]. Doherty *et al* suggested that, the inhibitory effects of  $\alpha_2$ -adrenergic agonists on intestinal motility are due to the presence of the inhibitory  $\alpha_2$  ARs located on the pacemakers neurons of the enteric nervous system, as well as presynaptically on postganglionic neurons innervating intestinal smooth muscle cells[33]. Noradrenaline is an important neurotransmitter in enteric neural reflexes, where it has inhibitory effects on both motility and secretion[11]. In the guinea pig, few noradrenergic fibers are present in the circular or longitudinal muscle, however, implying that sympathetic inhibition of motility is through the myenteric plexus. The effects of noradrenaline on intestinal motility are thought to be through both presynaptic and postsynaptic  $\alpha_2$ -AR as well as through postjunctional  $\beta_1$ -AR[16]. The sympathetic nervous system has a tonic inhibitory effect on intestinal secretion and increase both salt and water absorption. The effects of noradrenaline on secretion are mainly indirect, via the submucosal plexus, although noradrenaline can act directly on epithelial cells. Stimulation of  $\alpha_2$ -AR hyperpolarizes submucosal neurons from the guinea pig ileum by inhibiting voltage dependent  $\text{Ca}^{2+}$  channels and enhancing the  $\text{K}^+$  conductance[16]. In addition, Stebbing *et al* suggested that activation of  $\alpha_2$ -adrenoceptors is necessary for sympathetic inhibition in the small intestine. Radioligand binding studies have confirmed that  $\alpha_2$ -ARs exist peripherally, including in human platelets, submandibular gland, spleen, kidney and ileum[11]. However, our results showed that phentolamine actually enhanced the extract potency. Therefore, the involvement of these receptors in the spasmolytic activity of extract was not supported.

$\beta$ -AR also modulates intestinal motility. Stimulation of  $\beta$ -AR leads to relaxation of both the circular and the longitudinal muscle and several electrophysiological and functional studies have proposed that  $\beta$ -AR are localized postjunctionally on smooth muscle in the guinea pig ileum, the rat ileum and colon, and the dog ileum[16]. There are  $\beta_1$ -,  $\beta_2$ - and  $\beta_3$  adrenoceptors in the rat ileum and the role of  $\beta$ -AR subtypes in mediating smooth muscle relaxation in the rat ileum has been demonstrated[34]. The two principal intracellular pathways for smooth muscle relaxation are believed to be activated by cGMP and cAMP[35]. Various mechanisms have been proposed for  $\beta$ -adrenergic-mediated relaxation of smooth muscle. All theories suggest involvement of cyclic AMP as a second messenger[36]. Many other compounds activate receptors on smooth muscle cells that couple via GS-proteins to adenylyl cyclase. This leads to an increase in intracellular cAMP levels and a subsequent activation of cAMP-dependent protein kinase (PKA). Protein kinase A phosphorylation of MLC kinase prevents the  $\text{Ca}^{2+}$ -calmodulin complex from activating it[35]. The distinctive finding in this study is that propranolol did not significantly effect the activity of the extract, suggesting that extract does not have any effect on  $\beta$ -adrenergic receptors.

We have shown that an alcohol extract of parsley is a relaxant of rat ileum smooth muscle, and that non-selective antagonists of adrenoceptors (phentolamine and propranolol) do not inhibit



relaxation induced by extract. These effects justify its use in folk medicine as remedy for intestinal cramps and diarrhea. We are currently performing further studies to establish the safety of the extract and possibly to isolate the active principle responsible for the observed effects.

### Conflict of interest statement

We declare that we have no conflict of interest.

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