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Action mechanism of the monoterpenes (+)-pulegone and 4-terpinyl acetate in isolated guinea pig ileum

[Mecanismo de accion de los monoterpenos (+)-pulegona y acetato de 4-terpinil en íleon aislado de cobayo]

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Abstract
Recent studies have shown the spasmolytic activity of *p*-menthane monoterpenes (+)-pulegone and 4-terpinyl acetate (4-T) in guinea pig ileum. Since the action mechanism of these monoterpenes in intestinal smooth muscle is unknown, the present study was conducted to characterize their relaxant mechanism in isolated guinea pig ileum. We tested the involvement of voltage-dependent calcium and potassium channels and muscarinic antagonism. Both the monoterpenes caused a shift in the calcium curve to the right with reduction in the maximum effect. Pretreatment with tetraethylammonium chloride partially inhibited relaxation produced by both 4-T and (+)-pulegone. Both compounds caused a shift in the bethanechol curve to the right with reduction in the maximum effect. The results of this study indicate that the mechanisms of action of the smooth muscle relaxant monoterpenes (+)-pulegone and 4-T possibly involve the partial blockade of calcium channels, the activation of potassium channels, and the non-competitive antagonism of muscarinic receptors.

Keywords: (+)-pulegone, 4-terpinyl acetate, monoterpenes, essential oils, spasmolytic activity, ion channels, muscarinic antagonism.

Resumen
Estudios recientes han demostrado la actividad espasmolítica de los monoterpenos p-mentano de (+)-pulegona y acetato de 4-terpinilo (4-T) en el íleon de cobayo. Dado que el mecanismo de acción de estos monoterpenos en el músculo liso intestinal es desconocido, el presente estudio se llevó a cabo para caracterizar su mecanismo relajante en íleon aislado de conejillo de indias. Hemos probado la participación de tanto los canales calcio dependiente de voltaje como los canales de potasio y antagonistas muscarínicos. Ambos monoterpenos causaron un desplazamiento en la curva de calcio a la derecha con la reducción en el efecto máximo. El tratamiento previo con cloruro de tetraetilamonio inhibe parcialmente la relajación producida por tanto 4-T y (+)-pulegona. Ambos compuestos causaron un cambio en la curva de betanecol a la derecha con la reducción en el efecto máximo. Los resultados de este estudio indican que los mecanismos de acción de los monoterpenos relajantes del músculo liso (+)-pulegona y 4-T posiblemente implican el bloqueo parcial de los canales de calcio, la activación de los canales de potasio, y el antagonismo no competitivo de los receptores muscarínicos.

Palabras Clave: (+)-pulegona, acetato de 4-terpinilo, monoterpenos, aceites esenciales, actividad espasmolítica, canales iónicos, el antagonistas muscarínicos.

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INTRODUCTION

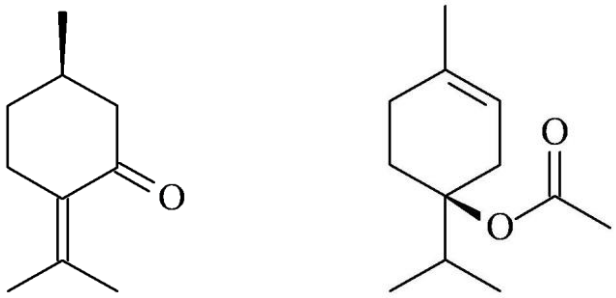
Monoterpenes are chemical constituents present in essential oils commonly found in aromatic plants. Recently, their therapeutic potential has been investigated in several experimental models (Bakkali *et al.,* 2008). Animal studies have shown various biological effects of monoterpenes, such as anticonvulsant (de Almeida *et al.,* 2011), analgesic (De Sousa *et al.,* 2011), anti-ulcer (Siqueira *et al.,* 2012), anti-inflammatory (Brito *et al.,* 2012), hypotensive and vasorelaxant (Santos *et al.,* 2011), and spasmolytic actions (De Sousa *et al.,* 2008; De Sousa *et al.,* 2011).

Modulation of the contraction of smooth muscle forms the therapeutic basis of several drugs, owing to the importance of smooth muscle function in most body organs, including airways, blood vessels, uterus, and gastrointestinal tract (Rasmussen and Barret, 1994). One of the mechanisms regulating muscle contractility is mediated by calcium ions and is dependent on the increase in intracellular calcium concentration (Somlyo and Somlyo, 1994). Smooth muscle relaxation is mediated by physiological processes that involve changes in the mechanism of contraction (Liao *et al.,* 2005).

The control of gastrointestinal tract motility is dependent on classical mediators such as acetylcholine and norepinephrine, and various other neurotransmitters known as non-adrenergic non-cholinergic mediators. Several substances identified in the enteric nervous system of mammals can be released and participate in the control of gastrointestinal motility by acting directly on gastrointestinal tract smooth muscle, causing contraction or relaxation, or indirectly by modulating inhibitory or excitatory mediator release (Furness and Costa, 1987). Examples of substances that produce inhibition of the contractile mechanism include atropine and scopolamine, which are used as antispasmodic drugs for the control of intestinal, uterine, and renal colic (Daniel *et al.,* 2001).

Smooth muscle cells in the walls of many organs are vital to several body functions, and abnormal smooth muscle activity contributes to many pathological processes (Somlyo and Somlyo, 1994; Somlyo *et al.,* 2002). These disorders are thus due to abnormal contraction of smooth muscle cells. Examples of this include angina, which is characterized by spasms of the coronary arteries; asthma, where difficulty in breathing is associated with bronchiospasm; and diarrhea due to increased intestinal motility (Furness and Costa, 1987). Gastrointestinal system-related disorders, such as diarrhea and irritable bowel syndrome, have a high prevalence and significant economic impact, contributing substantially to the total cost of health care (Pittler and Ernst, 1998; Gilani *et al.,* 2007). These disorders are characterized by a wide spectrum of symptoms that are mostly related to changes in intestinal motility without defined histopathological alterations in tissue (Heinle *et al.,* 2006). In this context, compounds with spasmolytic activity are required to treat the abnormal contraction of smooth muscle that underlies hypertension, coronary and cerebral vasospasm, erectile dysfunction, asthma, excessive labor pain, and uterine and intestinal cramping, which are the most common examples of smooth muscle dysfunction in humans (Webb, 2003).

A recent study showed that (+)-pulegone and chemical analogs, such as (-)-carvone and rotundifolone, have spasmolytic effects on guinea pig ileum (De Sousa *et al.,* 2008). The *p*-menthane monoterpenes (+)-pulegone and (-)-carvone are present in several plants of the genus *Mentha* and have in their chemical structure an α,β-unsaturated ketone group. The mechanism of relaxant action of (-)-carvone has been described by Consolini and collaborators (2011), who showed that intestinal muscle relaxation occurs by non-competitive blockade of Ca^{2+} channels. These findings support the relevance of the present study, since the mechanism of the relaxant action of (+)-pulegone has not been elucidated. A comparative study conducted by Andrade and collaborators (2011) has showed a significant spasmolytic activity of 10 structurally related *p*-menthane monoterpenes on isolated guinea pig ileum. The 4-T was the monoterpene that presented higher spasmolytic potency in this study. Due to the potential biological relevance of these monoterpenes, this study aimed to elucidate the mechanism of the muscle relaxant activity of (+)-pulegone and 4-T (**Figure Nº 1**), compounds most potent on isolated guinea pig ileum.



(+)-Pulegone (**1**) 4-Terpinyl acetate (**2**)

Figure Nº 1
Chemical structures of monoterpenes (+)-pulegone and 4-terpinyl acetate.

MATERIALS AND METHODS

Chemicals and solutions

4-Terpineol and (+)-pulegone were purchased from the Aldrich Chemical Company (Jacksonville, FL, USA). The monoterpene 4-T was obtained by acetylation of the alcohol 4-terpineol, using a reaction described by Andrade and collaborators (2011). The isolated compound was identified on the basis of spectral characterization (Infrared, ^{13}C, and ^{1}H nuclear magnetic resonance), TLC behavior and comparison with data from the literature (Andrade *et al.*, 2011). Both monoterpenes were dissolved as emulsions in 10% Tween-80 (VETEC, USA). Nifedipine (Sigma, USA) was solubilized in cremophor, whereas bethanechol (Sigma, USA) and tetraethylammonium chloride (TEA, Sigma, USA) were dissolved in water.

Animals

Male guinea pigs (weighing 300–400 g) were used in these experiments. They were obtained from the Central Animal House of the Federal University of Sergipe, São Cristóvão, Brazil. Two days before conducting the experiments, the animals were housed at 23 ± 2°C with a 12-h light/dark cycle (6 AM to 6 PM in light) in the Department of Physiology. The animals were fasted for 16 h before the experiments, but were allowed free access to water during this time. The experimental protocols were approved by the Ethics Committee on Research Animals of the Federal University of Sergipe, São Cristóvão, Brazil on 05/07/2009 and assigned protocol number 58/11.

The animals were killed by cervical dislocation and exsanguination through the carotid arteries. A 2.0 cm full-thickness segment of the distal portion of the ileum (1 cm proximal to the ileocecal sphincter) was removed and suspended under 1 g of resting tension in a 10 ml organ bath containing Tyrode solution (composition in mmol·L^{-1}: NaCl, 137; KCl, 2.7, $MgCl_2 \cdot 6H_2O$, 0.5; $CaCl_2 \cdot 2H_2O$, 1.8; NaH_2PO_4, 0.4; $NaHCO_3$, 12; glucose, 5.5). Tissues were maintained at 37°C and continuously bubbled with atmospheric air. The ileal strips were allowed to equilibrate for 60 min and the Tyrode solution was replaced every 15 min. The muscle strips were connected to a force transducer coupled to an amplifier-recorder (GOLD, Ohio, USA), and isometric contractions were recorded on a computer.

EXPERIMENTAL PROTOCOL

Spasmolytic activity of 4-T and (+)-pulegone in isolated guinea pig ileum

The concentrations of (+)-pulegone and 4-T used in this study correspond to CE_{50} and CE_{70} values obtained from previous studies (De Sousa *et al.*, 2008; De Sousa *et al.*, 2011).

Investigation of calcium channel blockade

The solutions used included normal Tyrode solution, K60 mM Tyrode solution, and Ca^{2+}-free Tyrode solution. The distal segment (1.5 cm) of the guinea pig ileum was mounted in an isolated organ bath in normal Tyrode nutrient solution for 30 min with successive washes every 10 min. After this period, muscle contraction was induced by 2 consecutive substitutions of normal Tyrode solution for depolarizing Tyrode solution (K60 mM). Afterwards, the preparation was washed using depolarizing Ca^{2+}-free Tyrode solution for a period of 45 min with successive washes every 15 min. Then, 2 cumulative concentration–response curves were obtained for $CaCl_2$. The Ca^{2+} curve was repeated after incubation of the preparation for 15 min with (+)-pulegone at concentrations of 0.4 mM or 1

mM, and 4-T at concentrations of 10 µM or 50 µM. The results were evaluated by comparing the maximum effect (E_{max}) and EC_{50} values for $CaCl_2$ in the absence (control) and presence of (+)-pulegone, and 4-T.

Investigation of potassium channel activation

The ileal segment was mounted in isolated organ bath with Tyrode solution, with washes every 15 min for 60 min. After stabilization, the basal tone was increased by addition of 3 µM bethanechol. After achieving a stable tone, (+)-pulegone (0.4 or 1 mM) or 4-T (10 or 50 µM) were added to obtain a control relaxant response. Then, the preparation was washed every 15 min for 45 min and the above procedure was repeated in segments that were pretreated for 20 min with TEA 1 mM, a nonselective blocker of potassium channels.

Investigation of muscarinic receptor antagonism

The preparation was stabilized in normal Tyrode nutrient solution for 30 min with subsequent washes at every 10 min. After this period, 2 cumulative concentration–response curves for bethanechol were obtained. The bethanechol curve was then repeated after incubation with (+)-pulegone (0.4 or 1 mM), and 4-T (10 or 50 µM) for 15 min. The results were evaluated by comparing the bethanechol maximum effect (E_{max}) and EC_{50} values in the absence and presence of these compounds.

Data presentation and statistical analysis

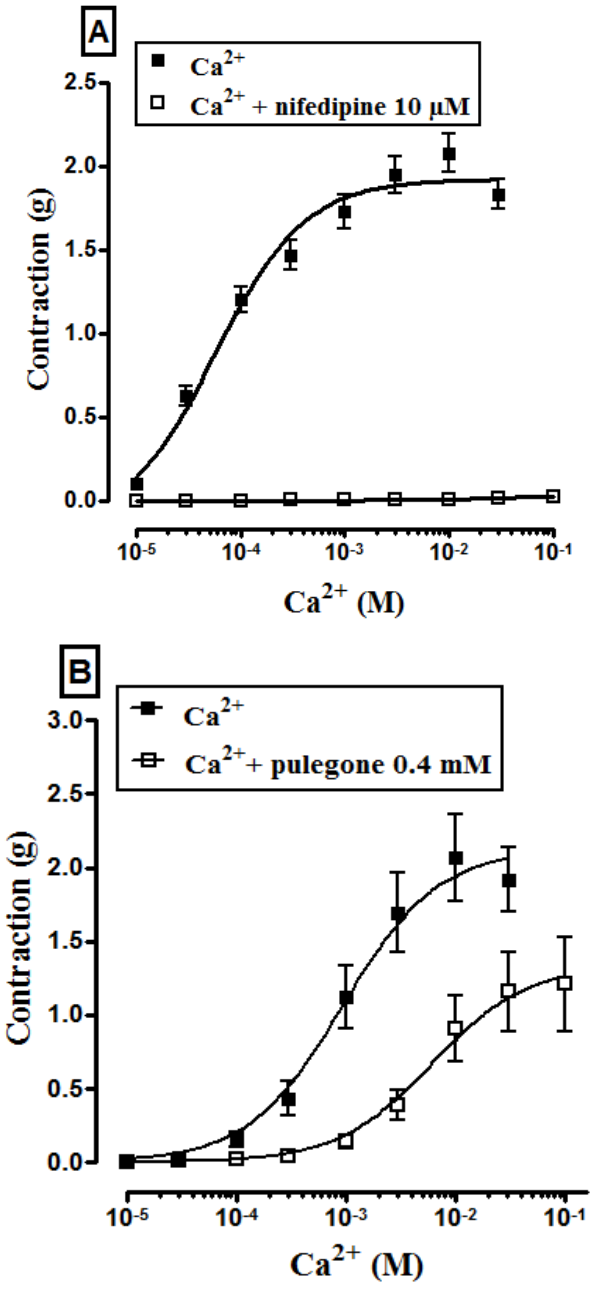
The data are presented as means and 95% confidence intervals, or as mean and standard error of the mean (SEM) of the responses obtained from 6 animals. The data were examined using paired Student's *t* test, and one-way analysis of variance (ANOVA) followed by Tukey's test. An alpha level of 0.05 was considered significant. Statistical analyses were performed using GraphPad Prism© version 5.0 (GraphPad Software Inc., San Diego CA, USA).

RESULTS

Evaluation of the mechanism of action of (+)-pulegone and 4-T. Involvement of Ca^{2+} channels

Incubation with the calcium channel blocker nifedipine (10 µM) completely inhibited the contraction induced by calcium, thus validating the experimental protocol (**Figure Nº 2A**). Treatment with (+)-pulegone at concentrations of 0.4, and 1 mM caused a shift in the calcium curve to the right with reduction in the maximum effect (**Figures Nº 2B and Nº 2C**, respectively). The Ca^{2+} EC_{50} value was increased in the presence of both concentrations of (+)-pulegone (**Table Nº 1**).

Figure Nº 2



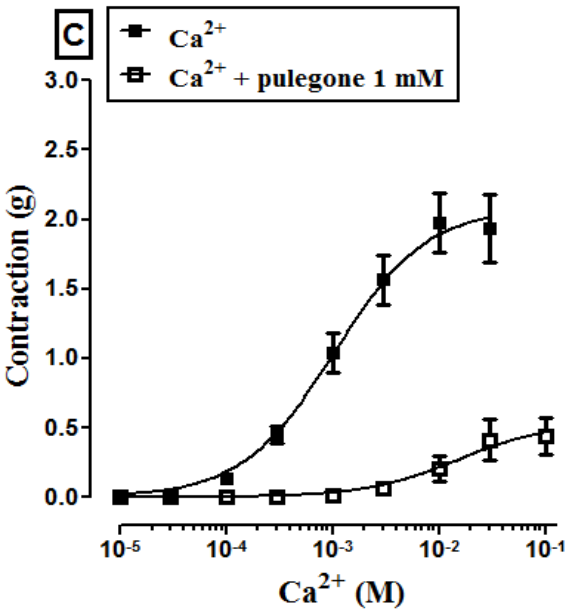


Figure Nº **2**
Cumulative Ca^{2+} concentration–response curves in the absence (negative control) and or presence of nifedipine at 10 µM (**A**) (positive control), (+)-pulegone at 0.4 mM (**B**), and (+)-pulegone at 1 mM (**C**) in isolated guinea pig ileum preparations (n = 6). The symbols and vertical bars represent mean and standard error of the mean (SEM), respectively.

Table Nº 1
Ca^{2+} EC_{50} obtained from Ca^{2+} concentration–response curves in the absence or presence of monoterpenes in isolated guinea pig ileum.

Treatment	Concentration	EC_{50}: Ca^{2+} (control)	EC_{50}: Ca^{2+} + monoterpene
(+)-Pulegone	0.4 mM	0.6 (0.3 - 1.4) mM	5.7 (2.1 - 13.7) mM*
	1 mM	0.7 (0.4 - 1.4) mM	13.6 (7.7 - 23.9) mM*** [aa]
4-T	10 µM	0.2 (0.1 - 0.3) mM	0.4 (0.2 - 0.7) mM
	50 µM	0.4 (0.2 - 0.5) mM	0.9 (0.6 - 1.2) mM** [b]

Data are presented as mean EC_{50} of 6 animals. *p < 0.05; **p < 0.01 ***p < 0.001 versus control, paired Student's t-test. [aa]p < 0.01 versus pulegone 0.4 mM, [b]p < 0.05 versus 4-T 10 µM, one-way ANOVA followed by the Tukey's post.

Addition of 4-T at the concentration of 10 µM did not promote a significant shift in the calcium curve (**Figure Nº 3A**). However, at concentration of 50 µM (**Figure Nº 3B**), 4-T caused a shift in the calcium curve to the right with a reduction in the maximum effect, increasing the Ca^{2+} EC_{50} from 0.4 to 0.9 mM (**Table Nº 1**).

Involvement of K^+ channels

In order to determine if K^+ channels are involved in the effects of (+)-pulegone and 4-T, the relaxation produced by these compounds was evaluated before and after the addition of tetraethylammonium chloride (TEA), a blocker of K^+ channels. Pretreatment with 1 mM TEA reduced the relaxation produced by (+)-pulegone at concentrations of 0.4, and 1 mM by 30.3% and 43.6%, respectively (**Figures Nº 4A and Nº 4B**).

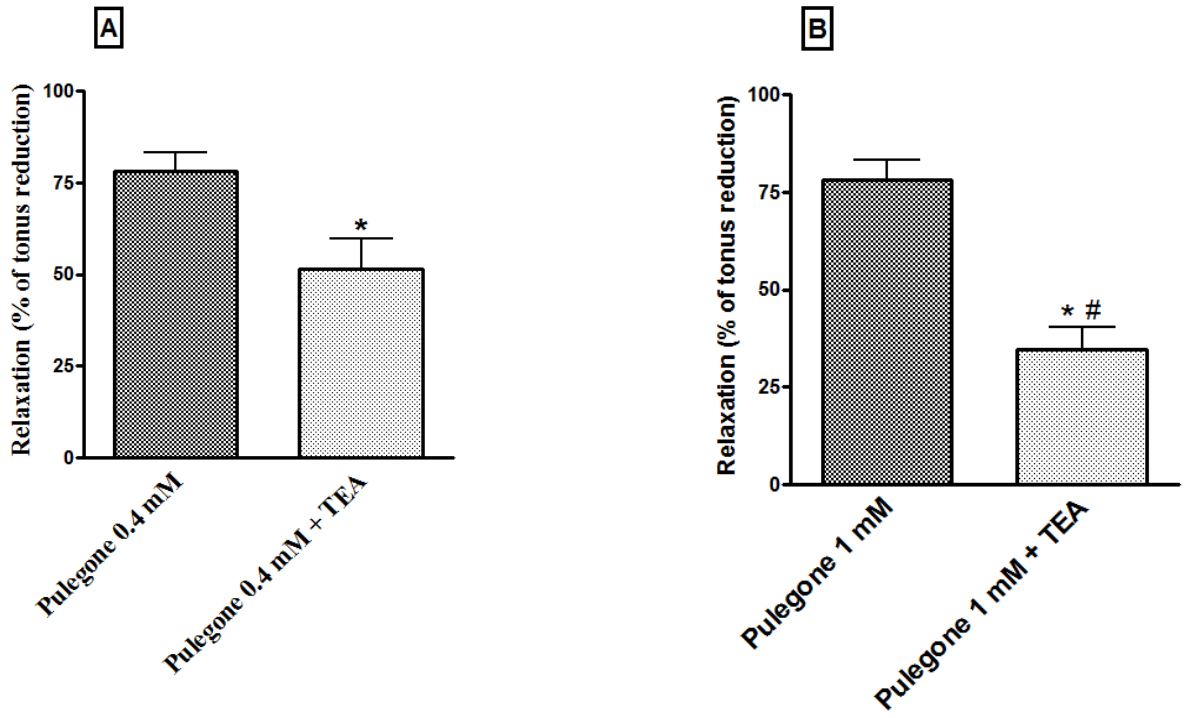


Figure Nº 3

Muscle relaxant effect of (+)-pulegone at concentration 0.4 mM (A) or 1 mM (B) in isolated guinea pig ileum in the absence, and presence of 1 mM tetraethylammonium chloride (TEA). The columns and vertical bars represent the mean ± standard error of the mean (SEM), respectively. *p < 0.05 versus pulegone, Student's *t* test (n = 6). # p < 0.05 versus pulegone 0.4 mM + TEA, one-way ANOVA followed by the Tukey's test.

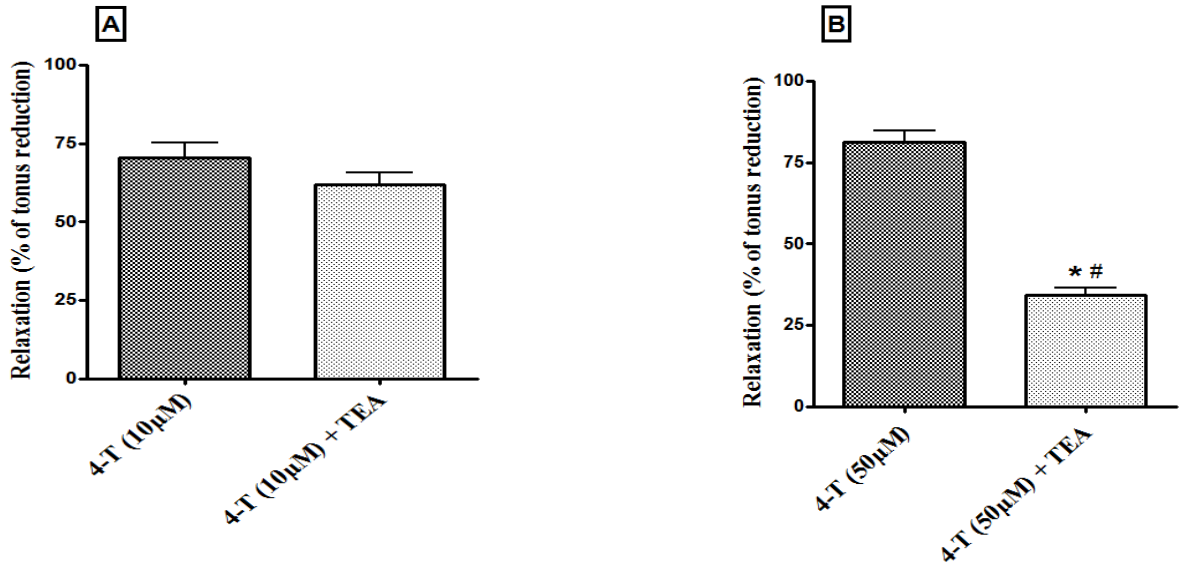


Figure Nº 4

Muscle relaxant effect of 4-T at concentration of 10 µM (A) or 50 µM (B) in isolated guinea pig ileum in the absence and presence of 1 mM tetraethylammonium chloride (TEA). The columns and vertical bars represent the mean ± standard error of the mean (SEM), respectively. *p < 0.05 versus 4-T, Student's *t* test (n = 6). # p < 0.05 versus 4-T (50 µM) + TEA, one-way ANOVA followed by the Tukey's test.

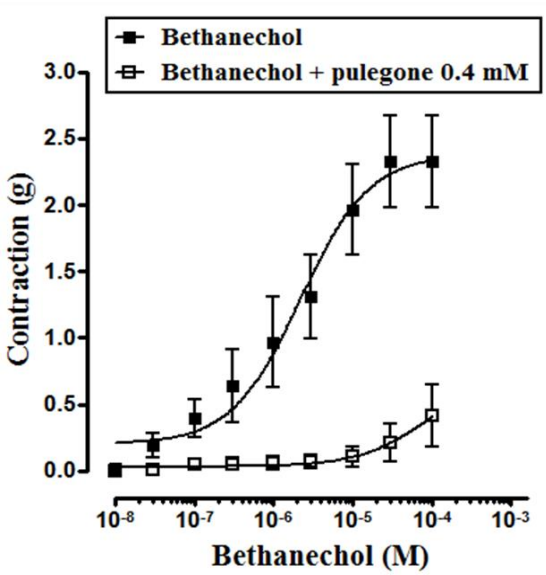


Figure Nº 5
Cumulative bethanechol concentration–response curves in the absence (control) and presence of (+)-pulegone 0.4 mM in isolated guinea pig ileum. The symbols and vertical bars represent mean and standard error of the mean (SEM), respectively.

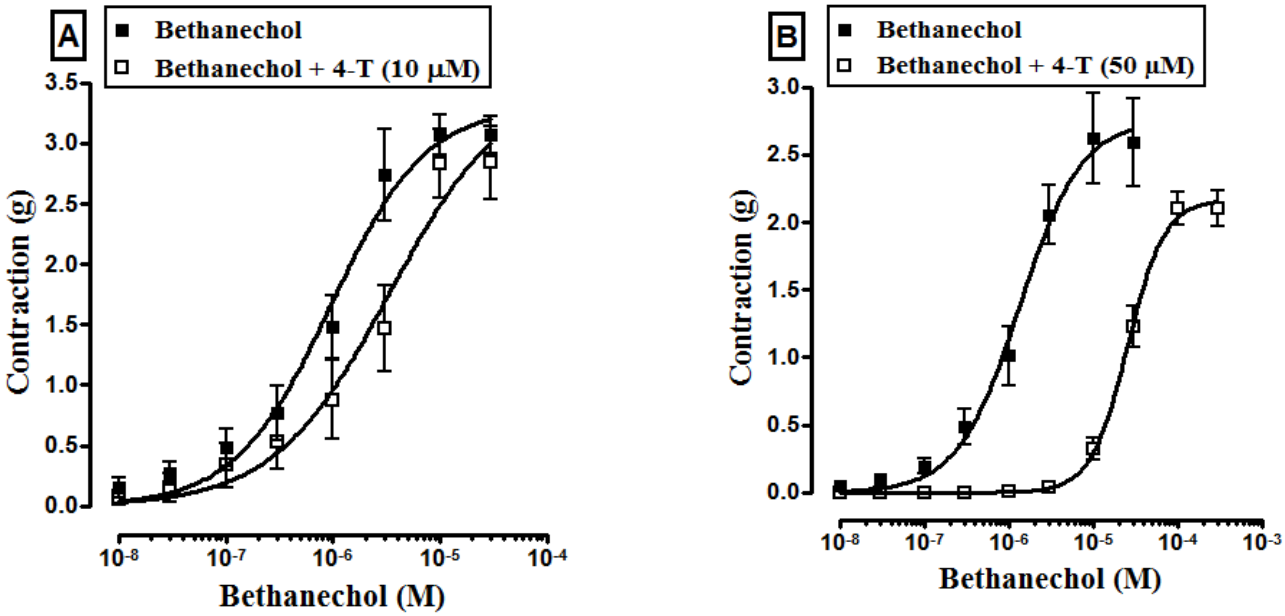


Figure Nº 6
Cumulative bethanechol concentration–response curves in the absence and or presence of 4-T (10 µM) (A) or 4-T (50 µM) (B) in isolated guinea pig ileum. The symbols and vertical bars represent mean and standard error of the mean (SEM), respectively. The shift in the bethanechol curve produced by 4-T (50 µM) was significantly different of the obtained in the presence of 4-T (10 µM), one-way ANOVA followed by the Tukey's test ($p < 0.01$).

The relaxation induced by 4-T at concentration of 10 µM was not reduced by pretreatment with 1 mM TEA (Figure Nº 5A). However, pretreatment with 1mM TEA inhibited the relaxation induced by 50 µM 4-T by 47% (Figure Nº 5B).

Antagonism of muscarinic receptors

Addition of (+)-pulegone at a concentration of 0.4 mM caused a shift in the bethanechol curve to the right with reduction in the maximum effect (Figure 6). Since (+)-pulegone markedly inhibited the maximum effect of bethanechol, it was not necessary to evaluate higher concentrations of this monoterpene. The monoterpene 4-T at concentrations of 10 and 50 µM caused shift in the bethanechol curve to the right with reduction in the maximum effect (Figures Nº 6A and Nº 6B). The EC50 values of bethanechol increased from 0.9 (0.5 – 1.6) mM in the absence of 4-T to 3.3 (0.8 – 13) mM in the presence ($p < 0.002$) of 4-T at 10 µM (Figure Nº 6A). The rightward shift of the bethanechol curve and reduction of the maximum bethanechol effect were more pronounced with 50 µM 4-T. EC50 values of bethanechol were increased from 1.3 (0.8 – 2.2) mM, in the absence of 4-T, to 25 mM (22 – 30) mM in the presence ($p < 0.0001$) of 4-T 50 µM (Figure 6B).

DISCUSSION

The importance of evaluating natural products that have biological activity on smooth muscle lies in the fact that spasmolytic substances are likely to have applications in the treatment of various diseases. This includes conditions such as cerebral vasospasm, asthma, hypertension, and uterine and intestinal spasms, as well as other pathophysiological processes that involve changes in the mechanisms of smooth muscle contraction and relaxation (Magalhães *et al.*, 2008).

Several essential oils affect smooth muscle by decreasing gastrointestinal motility (Magalhães *et al.*, 1998). This has been attributed to their major chemical constituents, such as citral (Sadraei *et al*., 2003), (+)-pulegone (De Sousa *et al.*, 2008), linalol (Mazzanti *et al.*, 1998), (-)-menthol and menthyl acetate (Grigoleit & Grigoleit, 2005), and carvacrol and carvacrol acetate (Baser, 2008; Rivero-Cruz, *et al.*, 2011), which are found in the plants *Melissa officinalis*, *Mentha* × *villosa, Hyssopous officinalis* L., *Mentha piperita*, *Satureja montana* L., and *Origanum compactum*, respectively.

Relaxation of gastrointestinal smooth muscle can occur due to multiple reflexes, such as descending relaxation in the esophagus and in the lower esophageal sphincter during swallowing, the food receptive function in the gastric fundus, in the pylorus during gastric emptying, in the duodenum during the peristaltic reflex, and the internal anal sphincter during defecation (Furness and Costa, 1987). Thus, smooth muscle has become an attractive label for the investigation of substances that promote muscle relaxation (Karaki *et al.*, 1997; Ratz *et al.*, 2004).

According to Rates (2001), studies have historically used a chemical-pharmacological approach to contribute to the research of natural products with therapeutic potential. Therefore, further studies on new muscle relaxant substances are markedly important in pharmacotherapy for the treatment of many conditions, such as hypertension, asthma, diarrhea, and dysmenorrhea, among others. For example, in the study by De Sousa *et al*. (2011), acyclic monoterpene esters, such as citronellyl and linalyl acetates, were shown to have spasmolytic effects in guinea pig ileum. These natural compounds have similar chemical structures, however they differ either in the position of their ester functional groups or their stereochemistry, which confers differences in the specificity of their pharmacological activities. Citronellyl and linalyl acetates are found in the essential oil from *Thymus leptophyllus*, and they have been shown to have significant spasmolytic effects against contractions induced by acetylcholine (Zafra-Polo *et al.*, 1989).

According to Cox (1990), the main mechanisms in which a substance produces smooth muscle relaxation are calcium ion output, increased membrane permeability to potassium, increased levels of the cyclic nucleotides cAMP and cGMP, and direct interference with the action of calcium on contractile proteins. Calcium is an important second messenger that performs essential roles in a wide variety of biological processes, including enzyme regulation, gene expression, protein transport, and mediation of muscle excitation-contraction coupling (Carafoli, 2002). An increase in the intracellular calcium concentration ($[Ca^{2+}]_i$) favors the formation of the calcium-calmodulin complex, which in turn can activate the kinase of the myosin light chain. A reduction in $[Ca^{2+}]_i$ is the primary cause of relaxation in smooth muscle cells (Breemen *et al*., 1979). Considering the importance of calcium ions in smooth muscle contraction, we sought to determine whether muscle relaxation produced by (+)-pulegone and 4-T involves the blockade of extracellular calcium entry.

Treatment with (+)-pulegone at concentrations of 0.4 mM or 1 mM caused a shift in the calcium

curve to the right accompanied by a reduction in the maximum effect (Figures Nº 2C and Nº 2D, respectively). The ester 4-T at concentration of 10 µM did not promote significant shift of the calcium curve (Figure Nº 3A**)**; however, at concentration of 50 µM, 4-T caused shifted the calcium curve to the right with reduction in the maximum effect (Figure Nº 3B). These results suggest that muscle relaxation produced by both concentrations of (+)-pulegone, and by 4-T at 50 µM involves inhibition of calcium influx through voltage-gated calcium channels (Ca_v).

The above results suggest that both 4-T and (+)-pulegone interfere with the activity of Ca_v, but cannot differentiate the channel subtype involved. However, L-type voltage-gated calcium channels (Ca_v-L) are the most highly expressed subtype in guinea pig ileum (Bolton, 1979). Thus the results of the present study are consistent with recent studies that have investigated the smooth muscle relaxant mechanism of 2 *p*-menthane monoterpenes, rotundifolone and (-)-carvone. These previous reports have shown that the vasodilation produced by rotundifolone is mediated by blockade of Ca^{2+} channels and activation of K^+ channels (Silva *et al.*, 2011), and that the intestinal spasmolytic activity produced by (-)-carvone involves non-competitive blockade of calcium ion influx via voltage-gated calcium channels (Consolini *et al.*, 2011).

Another important mechanism that is involved in the relaxation of smooth muscle is the activation of K^+ channels. These channels have a crucial role in controlling membrane potential and are important for the control of vascular tone, since potassium channel activators have been used in the treatment of hypertension (Jackson, 2000). Activation of K^+ channels leads to efflux of potassium ions and hyperpolarization of smooth muscle cells, causing inactivation of voltage-sensitive calcium channels, which results in a decrease in $[Ca^{2+}]_i$ and consequent smooth muscle relaxation (Nelson, 1995). Based on these previous findings, we sought to determine if the monoterpenes (+)-pulegone and 4-T induce ileum relaxation via opening of K^+ channels. Therefore, we compared the muscle relaxing action of (+)-pulegone and 4-T in the absence and presence of 1 mM TEA, a potassium channel blocker. TEA inhibited muscle relaxation produced by both (+)-pulegone concentrations, and the effect was more pronounced against the relaxation produced by (+)-pulegone at 1 mM (43.6%). This result suggests that the mechanism of action of (+)-pulegone involves, at least in part, activation of potassium channels (Figures Nº 4A and Nº 4B, respectively). The relaxant response produced by 4-T was also reduced by pretreatment with TEA; however, this inhibition (47%) was only obtained with 4-T at a concentration of 50 µM. This result also suggests that 4-T also activates K^+ channels (Figure Nº 5B). This activation leads indirectly to blockade of Ca_v, and consequently reduces the influx of calcium ions to the intracellular space. Therefore, the inhibition of the calcium curve shown above can be attributed, in part, to the activation of K^+ channels.

The contractile response of smooth muscle also occurs through the interaction of agonists and receptors, such as acetylcholine and muscarinic receptors. These receptors are coupled to G proteins and are found in effector organs, mainly in the gastrointestinal tract (Brann *et al.*, 1993; Levey, 1993). Thus, one of the mechanisms responsible for muscle relaxation is receptor antagonism, and consequently, decreased coupling to the G_q protein, which then leads to inhibition of the contractile response. This occurs because of inhibition of the intracellular cascade that leads to activation of phospholipase C and the subsequent formation of inositol triphosphate and diacylglycerol. Therefore, there is no release of calcium from the cytoplasmic compartment or activation of protein kinase C.

In the present study, we evaluated the possibility that (+)-pulegone and 4-T produce ileum relaxation via antagonism of muscarinic receptors. Both monoterpenes caused a shift in the bethanechol curve to the right with reductions in the maximal responses (Figures Nº 5 and Nº 6**)**, indicating that the myorelaxant action produced by these two monoterpenes involves noncompetitive antagonism of muscarinic receptors. This mechanism is supported by the other results described in the present study, showing the interaction of these compounds with the calcium and potassium channels, which in turn are related to the reduction in the contractile response to bethanechol. In addition, this noncompetitive antagonism may involve interaction with other muscarinic receptor binding sites or irreversible binding to muscarinic receptors.

CONCLUSION

In conclusion, the results obtained in this study showed that the intestinal muscle relaxation induced by monoterpenes pulegone and 4-terpinyl acetate involves several mechanisms. The relaxation produced by pulegone in isolated guinea-pig ileum occurs via blockade of Ca^{2+} channels, activation of K^+ channels, and noncompetitive antagonism of muscarinic

receptors. In addition, these results also showed that the above mechanisms are responsible by ileum relaxation produced by 4-terpinyl acetate at higher concentrations, and possibly other mechanisms may be responsible by relaxation produced by this monoterpene at lower concentrations. In consequence, future studies are necessary to full characterization of the action mechanism of these monoterpenes.

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