Spasmolytic effect of Mentha pulegium L. involves ionic flux regulation in rat ileum strips

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Abstract

Mentha pulegium is commonly known as “poleo” and used for the treatment of diarrhea, headache and cough in Mexican traditional medicine. Organic extracts from aerial parts were evaluated to determine their spasmolytic action on rat isolated ileum test. Hexanic (HEMp), dichloromethanic (DEMp) and methanolic (MEMp) extracts induced a concentration-dependent (0.97 to 1000 μg/mL) antispasmodic effect on spontaneous contractions. DEMp was the most active extract; therefore, spasmolytic mechanism was investigated. This extract (200 μg/mL) induced a significant depression on cumulative concentration-response curve for carbachol and serotonin (P<0.05). Besides, extract decreased and shifted to the right KCl- and CaCl2-induced contraction curves. Moreover, pre-incubation with chlorpromazine (0.001 mM) shifted to the left the relaxant curve. Pre-treatment with L-NAME (1 mM), papaverine (0.01 mM), theophylline (0.01 mM), TEA (1 mM) and glybenclamide (0.1 mM) did not produce any change of the relaxant curves of DEMp. Findings indicate that dichloromethanic extract of M. pulegium induced its spasmolytic effect through Ca2+-influx blockade, which may explain its traditional use against diarrhea.

Key words: diarrhea, Mentha pulegium, traditional medicine, spasmolytic

Introduction

Diarrhea can be caused by an increased osmotic load within the intestine, excessive secretion of electrolytes and water into the intestinal lumen, exudation of protein and fluid from mucosa and, impaired intestinal motility resulting in rapid transit. In most instances, multiple processes are affected simultaneously, leading to a net increase in stool volume and weight accompanied by increases in fractional water content. There are several types of etiologies:
virus, bacteria, parasites, food poisoning, antibiotics, laxative agents, or certain medical conditions that can also lead to diarrhea (Pasricha, 2006). Diarrhea is still one of the major health threats in tropical and subtropical poor countries (Lanata and Mendoza, 2002). The World Health Organization (WHO) has estimated that 3–5 billion cases (1 billion in children <5 years old) and about 5 million deaths are due to diarrhea (2.5 million in children <5 years old) in worldwide each year (Lanata and Mendoza, 2002; Heinrich et al., 2005). In Mexico, infectious intestinal diseases are the 20th most important causes of death (INEGI/Health ministry of Mexico/CONAPO, 2004). In the year 2004, a total of 472,273 deaths were registered and 4,180 (0.9%) were provoked by those diseases. Most affected were children under 4 years old (1,167; 27.92%) and persons >65 years old (1,587; 37.97%). The major impact of these illnesses is morbidity, because it demands primary medical services, hospital-care time and labor days lost. Furthermore, the most highly used drugs for intestinal disease therapies are frequently very expensive, overused and the inadequate use of antibiotics has lead an important increase in the prevalence of multidrug-resistant pathogens. The most important therapy for diarrhea is to ensure that fluid and electrolyte deficits are repaired. These deficits can be repaired with intravenous fluids or oral rehydration solutions. Other important kind of anti diarrheal drugs are opiates, such as loperamide (Imodium) or diphenoxylate with atropine (Lomotil), colloidal intraluminal agents, such as bismuth subsalicylate (Pepto-Bismol) and adsorbants (e.g., kaolin) also can be of use. On the other hand, chemotherapy agents such as antiviral, antibacterial, antiprotozoal and antihelmintic drugs can be used in parasitic-induced diarrhea (Pasricha, 2006).

In Mexico, a large number of plants have been empirically used for the treatment of different diseases, including diarrhea (Aguilar et al., 1994; Monroy-Ortiz and Castillo-España, 2007). One of them is Mentha pulegium L. (Lamiaceae), a shrub native from Europe and West-Africa that grow in deciduous dry forest into Mexican territory. This plant is used in traditional medicine for treat headache, flu and other respiratory illnesses, diarrhea and other gastrointestinal disorders. Furthermore, it is useful to induce the partum contractions in pregnant women as a type of enhancement of the child birth (Monroy-Ortiz and Castillo-España, 2007).

The current study has the aim to evaluate the spasmolytic effects of organic extracts derived from M. pulegium as source of bioactive compounds that act as antidiarrheic agent. And also to determine the mechanism of action of the most active extract through functional in vitro assay using chemical agents that regulate motility and relaxation signaling pathways.

**Material and Methods**

**Chemicals and drugs**

Carbamylcholine HCl (carbachol), serotonin (5-HT), N^ω-nitro-L-arginine methyl ester HCl (L-NAME), atropine, tetraethyl ammonium (TEA), theophylline, chlorpromazine and papaverine were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). All other reagents were analytical grade from local sources.
**Plant material and extraction**

*Mentha pulegium* L. (Lamiaceae) was collected in September 2006. Plant material was taken from its natural habitat, collected and identified by Dr. Patricia Castillo-España. A voucher specimen was deposited at the HUMO-Herbarium of the Centro de Estudios Ambientales e Investigación “Sierra de Huautla” (CEAMISH) of the Morelos State University. The plant material was manually cleaned of solid residues, and then, dried at room temperature (~25°C) during two weeks. Then, dry plant material was pulverized (243 g.) and crude extracts were prepared by successive maceration process using hexane, dichloromethane and methanol (3 times for 72 hours at room temperature). After filtration, extracts were concentrated *in vacuo* at 40°C. Finally, yields of 1.42, 1.92 and 5.26 g. of extracts, respectively, were obtained.

**In vitro rat ileum test**

Healthy male Wistar rats (250–350 g) were used. The animals were maintained under standard laboratory conditions with free access to food and water. All animal procedures were conducted in accordance with our Federal Regulations for Animal Experimentation and Care (Ministry of Agriculture, NOM-062-ZOO-1999, Mexico) and were approved by the Institutional Animal Care and Use Committee.

Rat ileum longitudinal preparations were assayed as described elsewhere. Briefly, animals were sacrificed by cervical dislocation. Tissue segments were dissected out, cleaned and placed in organ baths containing warmed (37°C) and oxygenated (gas mixture O$_2$/CO$_2$, 19:1) Ringer-Krebs-Henseleit solution (14 mL) whose chemical composition (mM) was: NaCl, 118; KCl, 4.7; CaCl$_2$, 2.5; MgSO$_4$, 1.2; KH$_2$PO$_4$, 1.2; NaHCO$_3$, 25.0; EDTA, 0.026 and glucose (dextrose), 11.1 at pH 7.4. One side of the tissue segment (~3 cm) was held to the bottom of organ baths using stainless steel hooks, and the other was attached to a Grass-FT03 force transducer (Astromed, West Warwick, RI, USA) connected to a MP100 analyzer (BIOPAC Instruments, Santa Barbara, CA, USA) under an optimal tension of 1 g. as previously described (Estrada-Soto *et al.*, 2007; Estrada *et al.*, 1999). After stabilization period (30 min), a 10 min-control time range was recorded. Test samples were added to organ bath in cumulative quarter-log concentrations (0.97 to 1000 μg/mL). The pharmacological effect on spontaneous contraction of organic extracts as well as positive controls were determined by comparing muscular tone exerted by tissue contractions before and after sample addition. Muscular tone was calculated from tracings using Acknowledge software (BIOPAC®).

**Determination of mode of action**

In order to establish the spasmolytic mechanism of organic extract, four set of experiments were carried out as described elsewhere (Estrada-Soto *et al.*, 2007).

**Interaction on cholinergic and serotonergic receptors**

The tissues were incubated with extracts (200 μg/mL) during 15 min, and then carbachol (0.006–1.83 μg/mL) was added in half log-units increments.

Tissues were pre-contracted with serotonin (1 μM) during 10 min, and then 200 μg/mL were added to the organ bath.
Regulation of ionic flux (Ca\(^{2+}\) or K\(^{+}\) channels)

The ileum strips were washed with Ca\(^{2+}\)-free Krebs solution (KCl 60 mM) during 10 min, after equilibration, they were incubated with active organic extract (200 \(\mu\)g/mL) about 20 min. Finally, CaCl\(_{2}\) (100 \(\mu\)L) was added in half-log unit increments (0.02–7.35 \(\mu\)g/mL).

Rat tissues were washed with K\(^{+}\)-free Krebs solution for 10 min, after that, they were incubated with active organic extract (200 \(\mu\)g/mL) during 20 min. Finally, KCl (100 \(\mu\)L) was added with half-log unit increments (0.01 to 3.00 \(\mu\)g/mL).

In order to corroborate the participation of selective opening K\(^{+}\) channels in spasmolytic effect of *M. pulegium*, inhibition of this channels were performed through selective inhibitors as glybenclamide (ATP-sensitive K\(^{+}\) channels blocker, 10 mM) and TEA (non-specific K\(^{+}\) channel blocker, 1 mM).

Drugs that affect Ca\(^{2+}\)-mediated signaling pathway in smooth muscle cell

To evaluate the participation of calmodulin-Ca\(^{2+}\) complex (CaM-Ca\(^{2+}\)) inhibition in relaxant effect produced by the extract, rat ileum strips were pre-incubated (0.001 mM) with chlorpromazine 10 minutes before addition of quarter-log concentrations of the extract.

Participation of NO and cyclic nucleotides in extract relaxant effect

L-NAME (1 mM, NOS inhibitor), papaverine or theophylline (100 mM non-specific PDE inhibitors) were previously incubated during 15 min before experiment starting, then, active organic extract (100 \(\mu\)L) was added and cumulative concentration-response curves were performed.

Statistical analysis

All experimental results are expressed as the mean of six experiments ± SEM. Concentration-response curves were plotted using software Microcal™ Origin 6.0 (Microcal Software Inc., USA). Curves were adjusted by non-linear curve fitting sub-program using the same software. Statistical analysis was performed by One-Way Analysis of Variance (ANOVA) and post-hoc Dunnet’s test (sample vs. control). \(P\) values less than 0.05 were considered to be statistically significant.

Results and Discussion

*M. pulegium* L. is used in the Mexican traditional medicine for the treatment of diarrhea and related ailments. In accordance with ethnomedical criteria, this plant is commonly used for treat spasms present in different diseases as gastrointestinal disorders and partum illnesses. Above report suggests that extracts derived from *M. pulegium* would exert relaxation on small intestine motility and in consequence could be used as antidiarrheic agent.

Organic extracts were evaluated using rat ileum strips assay for determine their potential activity as spasmolytic agents for the treatment of diarrhea. Results showed that HEMp (EC\(_{50}\) = 80.79 \(\mu\)g/mL, \(E_{\text{max}}\) = 56.1 ± 2.7\%), DEMp (EC\(_{50}\) = 74.68 \(\mu\)g/mL, \(E_{\text{max}}\) = 57.2 ± 3.9\%) and MEMp (EC\(_{50}\) = 80.79 \(\mu\)g/mL, \(E_{\text{max}}\) = 42.9 ± 4.4\%) have almost same concentration-dependent inhibition
Spasmolytic effect of *Mentha pulegium* on spontaneous contraction of ileum strips (Fig. 1a and 2). Spasmolytic effect could be related with a direct or indirect receptors blockade, ionic channels interaction and/or enhanced second messenger production; as well as other. In order to characterize pharmacological effect of *M. pulegium* spasmolytic action, it was analyzed the effect of carbachol-induced contraction in presence of unique concentration of extracts (200 μg/mL). This experiment allowed identify DEMp as major active extract since it had the major activity to neutralize carbachol-induced contraction (null maximum effect) compared with control ($E_{\text{max}} = 100 \pm 5.36\%$, $EC_{50} = 0.11 \pm 0.01 \mu g/mL$). In contrast, HEMp and MEMp ($E_{\text{max}} = 35.31$ and 51.12%, respectively) showed less...
inhibition activity of carbachol-induced contraction than DEMp (Fig. 3a). Based on these findings, we decided to study DEMp to determine its spasmylytic mechanism of action in rat isolated ileum strips performing functional in vitro experiments through inhibition of receptor-induced contraction (carbachol and serotonin), intracellular mediators or channel blocking involved in smooth muscle contractile and relaxant pathways.

Contraction process mediated by some endogenous neurotransmitters such as acetylcholine and serotonin (5-hydroxitryptamine, 5-HT) induces an activation of G protein-coupled receptor which activate phospholipase C to produce inositol triphosphate (IP₃) and diacylglycerol (DAG), molecules that mediate Ca²⁺ release/entry into smooth muscle cell (Billington et al., 2003). Because of not only acetylcholine can activate this signaling pathway to produce contractile process, we decided to evaluate the effect of DEMp on contractions induced by serotonin (1 μM) to establish or discard selectivity in possible receptor-mediated spasmylytic mechanism. Results showed that stimulation of tissues with 5-HT increased contractile response in comparison with basal spontaneous contraction tissues (Fig. 3b). Moreover, pre-incubation of the ileum strips with DEMp (200 μg/mL) significantly reduce 5-HT-induced contraction (Eₘₐₓ= 1.59 ± 0.2 vs. 0.38 ± 0.3 g) (Fig. 3b). Above findings suggest that spasmylytic effect is not selectively produced mediated by antagonize either acetylcholine or 5-HT receptors since both processes receptor-mediated contractions were inhibited by DEMp. Furthermore, it could be interfering with a common step of the contraction mechanism such as extracellular Ca²⁺-internalization or release from Ca²⁺ depots from sarcoplasmic reticulum (Maciel et al., 2004).

So, in order to test whether DEMp (200 μg/mL) blocks the input of extracellular Ca²⁺, the extract was assayed on the CaCl₂ (Figs. 1b and 4a) and KCl-induced contractions (Fig. 4b). Extract decreased the Eₘₐₓ and shift to the right the KCl (10–1000 μg/mL) and Ca²⁺ (30–100 μg/mL) concentration-response curves (P<0.01). These results suggest that DEMp is blocking the L-type voltage-dependent Ca²⁺ channel and in consequence produce the spasmylytic action

Fig. 2. Relaxing concentration-response curves of extracts obtained from Mentha pulegium on spontaneous contractions of isolated rat ileum strips. Data are expressed as the mean ± S.E.M. of six experiments (*, P<0.01 vs. papaverine).
Spasmolytic effect of *Mentha pulegium* (Gilani *et al.*, 2005; Gilani *et al.*, 2006). The classical signaling pathway through contraction is evoked by the entrance of Ca$^{2+}$ into smooth muscle, Ca$^{2+}$-calmodulin (CaM) complex formation and, finally, myosin light chain (MLC) phosphorylation to induce smooth muscle contraction. Thus, we decided to explore, as alternative mechanism, a possible interaction with Ca$^{2+}$-calmodulin (CaM) complex. Ileum rings were pre-incubated with chlorpromazine (0.001 mM, an inhibitor of Ca$^{2+}$-calmodulin (CaM) complex formation) and relaxant curves of DEMp were shifted to the left ($P<0.05$), which indicates a possible synergic effect between extract and chlorpromazine (Fig. 5). Further experiments are necessary to support this hypothesis.

Second messengers as nitric oxide (NO), cGMP and cAMP are the most important candidates for mediating nonadrenergic-noncholinergic smooth muscle relaxation through the gastrointestinal tract (Estrada *et al.*, 1999; Takahashi, 2003). Therefore, we explored if the antispasmodic activity displayed by DEMp was mediated by NO, cGMP and/or cAMP.
production. Thus, pre-treatment with L-NAME (10 μM, an inhibitor of NOS), papaverine and theophylline (phosphodiesterases inhibitors) did not produce a significant modification of the relaxation curves induced by DEMp (P<0.05) (Fig. 6), suggesting that these second messengers are not related with relaxant effect induced by the extract.

Finally, it was discard the possible participation of a K⁺ channel opening in the spasmolytic action, since relaxant curves was not modified in the presence of glybenclamide (a K_{ATP} channel blocker) or tetraethylammonium (an unspecific potassium channel blocker) (Fig. 7).

In conclusion, DEMp induced its spasmolytic action mainly through blockade of
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extracellular Ca\(^{2+}\) influx, which supports its medicinal uses as antidiarrheic agent.

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**Fig. 7.** Relaxant effect of DEMp on rat ileum strips in the absence (Control, filled circles) and presence of 1 mM TEA (open circles) or 0.1 mM glybenclamide (open triangles). Results are expressed as the mean ± S.E.M. of six experiments (*, P<0.05 vs. relaxant control curves).
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