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Ileal Relaxation Induced by *Mentha longifolia* (L.) Leaf Extract in Rat

Mohammad Kazem Gharib Naseri, Zahra Gharib Naseri,
Maryam Mohammadian and Marzie Omidi Birgani
Physiology Research Center, Department of Physiology, School of Medicine,
Ahwaz Jundishapur University of Medical Sciences, P.O. Box 61335-189, Ahwaz, Iran

Abstract: The effect of *Mentha longifolia* (L.) leaf hydroalcoholic extract (MLE) was examined on rat ileal smooth muscle contractions. Last portion of ileum from male adult Wistar rat was mounted in an organ bath containing Tyrode solution. The tissue was contracted by carbachol (CCh, 10 μM), KCl (60 mM) and $BaCl_2$ (4 mM) and then MLE (0.0625-1 mg mL^{-1}) was added to the bath cumulatively. The effect of MLE on KCl-induced contraction was examined after tissue incubation with propranolol (1 μM), naloxone (1 μM) and N^{ω}-nitro-L-arginine methyl ester (L-NAME, 100 μM). The effect of MLE on $CaCl_2$-induced ileal contraction in Ca^{2+}-free with high potassium Tyrode solution was also evaluated. The role of potassium channels was examined by ileum incubation (5 min) with tetraethylammonium (TEA, 1 mM). The results showed that KCl-, CCh and $BaCl_2$-induced ileal contractions were inhibited ($p<0.001$) by cumulative concentrations of MLE with the same potency. In addition, MLE (0.25-1 mg mL^{-1}) inhibited ($p<0.01$) ileal contractions induced by $CaCl_2$ (0.45-2.7 mM) in a concentration-related manner. The antispasmodic effect of MLE was affected neither by propranolol, L-NAME nor by naloxone. The MLE concentration-response curve was shifted to the right ($p<0.05$) by tissue incubation with TEA. From results it may be suggested that *Mentha longifolia* hydroalcoholic leaf extract induces its spasmolytic activity mainly through disturbance in calcium mobilization and partly by potassium channels activation. Present results show that *Mentha longifolia* leaf extract exerts relaxant effects on intestinal smooth muscle, consistent with the traditional use of the plant to treat gastrointestinal disorders such as diarrhea and colic.

Key words: *Mentha longifolia*, rat, ileum, antispasmodic

INTRODUCTION

The genus Mentha belongs to the family Lamiaceae consisting of about 25-30 species (Shaiq Ali *et al*., 2002); most of them are found in temperate regions of Eurasia, Australia and South Africa. The aromatic Mentha herbs are perennials found in damp or wet places and members of this genus are the most important sources of essential oil production in world (Shaiq Ali *et al*., 2002). In Northern area of Pakistan, *Mentha longifolia* (L.) or horsemint (locally called bonooh) is used for stomach, liver problems and vomiting (Khan and Khatoon, 2008). In Iranian traditional medicine, *M. longifolia*, locally called poneh kohi, is used for gastric disorders. Latif *et al*. (2006) has reported that this herb is used traditionally for treating diarrhea in children and preventing vomiting. The following properties have been demonstrated in *M. longifolia* enhancing bactericide effects of some drugs (Shahverdi *et al*., 2004), inhibitory activity against HIV-1 (Amzazi *et al*., 2003), antimicrobial and scavenging free radical activity (Mimica-Dukic *et al*., 2003), antimycotic (Abou-Jawdah *et al*., 2002), anthelmintic activities (Kozan *et al*., 2006), antiemetic effect in young chickens (Hosseinzadeh *et al*., 2004). Piperienone and piperitone oxide and five flavonoids have been isolated from essential oil of this herb (Ghoulami *et al*., 2001) and isolation of β-sitosterol glycoside (longiside-A and B) and flavanone glycoside (longitin) (Shaiq Ali *et al*., 2002; Shaiq Ali *et al*., 2006) from *M. longifolia* have been reported. Although, this herb has been used to treat various gastrointestinal disorders such as abdominal pain, flatulence and colic, however, its effect on smooth muscle has not been scientifically evaluated yet. Therefore, the aim of the present study was to investigate the effects of *M. longifolia* leaf hydroalcoholic extract (MLE) on rat ileum and to study the involved mechanism(s).

MATERIALS AND METHODS

Plant material and powder preparation: *Mentha longifolia* was collected from Masjed Soleiman Mountains (Northeastern of Khuzestan province) in October 2007 and authenticated by Dr. Sedighi Dehkordi from Ahwaz Shahid Chamran University, Department of

Corresponding Author: Mohammad Kazem Gharib Naseri, Physiology Research Center, Department of Physiology, School of Medicine, Ahwaz Jundishapur University of Medical Sciences, P.O. Box 61335-189, Ahwaz, Iran

Horticultural Science and a voucher was deposited at herbarium of the same department for further references. The leaves were dried under shade and powdered by an electrical grinder. The powder was extracted by macerating method using 70% alcohol for 72 h at room temperature and mixed occasionally daily. The mixture was then filtered (Whatman No. 1), filtrate was concentrated in rotary evaporator and dried at room temperature to obtain a dark green powder (yield: 22.7%). The extract was stored at 4°C until being used and dissolved in bath solution before using in experiments.

Chemicals and reagents: Propranolol, carbachol, N^{ω}-nitro-L-arginine methyl ester (L-NAME), tetra-ethylammonium (TEA) were purchased from Sigma (USA) and naloxone was purchased from Tolidaru (Iran). Other chemicals were purchased from Merck (Germany).

Animals: All rats used in this study were treated in accordance with principals and guidelines on animals care of Ahwaz Jundishapur University of Medical Sciences (AJUMS). Male Wistar adult rats (194.4±5.8 g) were obtained from AJUMS Animal House and kept at 12 h light/dark cycle and at 20-24°C with free access to food and water. Rats were starved of food but not water for 24 h before experiment.

Ileum preparation: On the day of experiment the rats were sacrificed by a sharp blow on the head. A piece (2 cm) was prepared from the terminal ileum (taken within a distance of 2-3 cm from the caecum) and mounted in an organ bath containing Tyrode solution (10 mL) between two stainless steel hooks vertically. The lower hook was fixed at the bottom of the organ bath and upper one was connected to an isotonic transducer (Harvard transducer, UK) connected to a recorder (Harvard Universal Oscillograph, UK). The Tyrode solution (pH 7.4 and 37°C) composition was (in mM): NaCl (136); KCl (5); $CaCl_2$ (2); $NaHCO_3$ (11.9); $MgCl_2$ (0.98) NaH_2PO_4 (0.36) and glucose (5.55) which continuously was bubbled with air (Madeira *et al*., 2002). The initial tension was 1 g throughout the experiment and equilibrium period was 60 min. After equilibrium period, the ileum was contracted either by KCl (60 mM), carbachol (CCh, 10 μM) or $BaCl_2$ (4 mM) and once the plateau was achieved, the extract was added cumulatively (0.0625-1 mg mL^{-1}) to the organ bath. The effect of extract was also studied in separate tissues after either 30 min incubations with 1 μM of propranolol, 30 min with naloxone or 20 min (Izzo *et al*., 1998) with L-NAME (100 μM) as non-selective β-adrenoceptors, opioid receptors antagonists and nitric oxide synthase inhibitor, respectively. To evaluate the MLE effect on $CaCl_2$-induced ileum contraction, in Ca^{2+}-free and rich KCl (60 mM) Tyrode solution, the tissue was depolarized and then $CaCl_2$ was applied cumulatively (0.45-2.7 mM) before and after tissue incubation (3 min) with extract (0.25-1 mg mL^{-1}).

To evaluate the role of potassium channels, tissue preparation was incubated (5 min) with tetra-ethylanımonium (TEA, 1 mM), then contraction was induced by CCh (10 μM) and thereafter MLE was added cumulatively (0.0625-1 mg mL^{-1}). Separate ileum preparations were used for each spasmogens or antagonists.

Statistical analysis: The plateau of ileal contraction induced by KCl or CCh was regarded as 100% and percentage of relaxation was calculated from changes in the contraction. Results were expressed as mean±SEM of n experiments (n indicates the number of tissues and coincides with the number of animals). Comparison between to sets of data was made by Student's t-test. For comparison of one control with several experimental groups, a one-way Analysis of Variance (ANOVA) was used. Analysis of variance (two-way) was used to compare different cumulative concentration-effect curves. A p-value <0.05 was considered significant.

RESULTS

Effect of MLE on ileal contractions induced by applied spasmogens: *Mentha longifolia* leaf hydroalcoholic extract (MLE) reduced the ileum contractions induced either by KCl (60 mM), CCh (10 μM) or $BaCl_2$ (4 mM) significantly (one-way ANOVA, $p<0.001$) and in a concentration dependent manner. As Fig. 1 shows the MLE antispasmodic effects on contractions induced by these spasmogens are identical. Eight animals were used for each spasmogen.

Effect of the MLE on $CaCl_2$-induced ileal contractions: In Ca^{2+}-free with high K^+ (60 mM) Tyrode solution, applying cumulative concentrations of calcium chloride (0.45 to 2.7 mM) induced ileal contractions in a concentration dependent manner ($p<0.01$) as control curve shows in Fig. 2. Three minutes incubation of tissue preparation with MLE (0.25, 0.5 and 1 mg mL^{-1}) reduced the contractions evoked by $CaCl_2$ in a concentration-related manner. The $CaCl_2$-induced contractions in ileum before and after incubation with extract (0.5 mg mL^{-1}) were significantly different (two-way ANOVA, $p<0.01$). Seven animals were used for each MLE concentration.

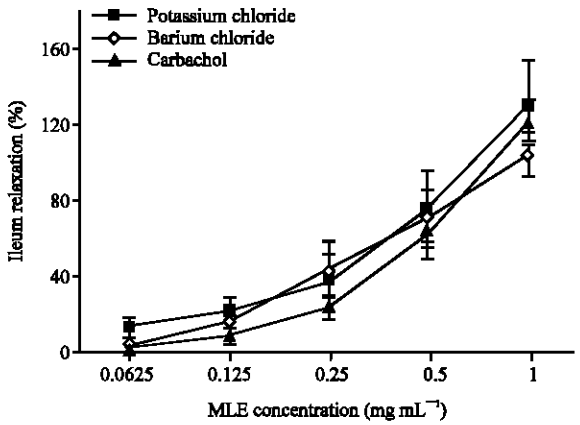

Fig. 1: Effect of *Mentha longifolia* hydroalcoholic extract (MLE) on rat ileal contractions induced by KCl (60 mM), $BaCl_2$ (4 mM) and carbachol (10 μM). Two-way ANOVA indicated that these three concentration-related responses are not significantly different. Each point represents mean±SEM of 8 observations for each extract concentration

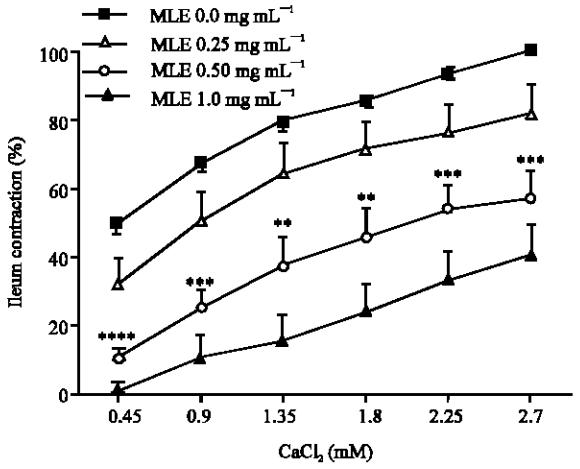

Fig. 2: Spasmogenic effect of $CaCl_2$ on rat ileum before (0.0 mg mL^{-1}) and after 3 min tissue incubation with different concentrations of *Mentha longifolia* hydroalcoholic extract (MLE). The extract antispasmodic effect at 0.5 mg mL^{-1} in compare to 0.0 mg mL^{-1} is significantly different (two-way ANOVA, $p<0.01$). Each point represents mean±SEM of 7 observations for each MLE concentration. (0.0 mg mL^{-1} vs 0.5 mg mL^{-1}, Student's t-test, **p<0.01, ***p<0.001, ****p<0.0001)

Effect of MLE after ileum incubation with propranolol, naloxone or L-NAME: Incubation of tissue preparation either 30 min with propranolol (1 μM, n = 7), naloxone

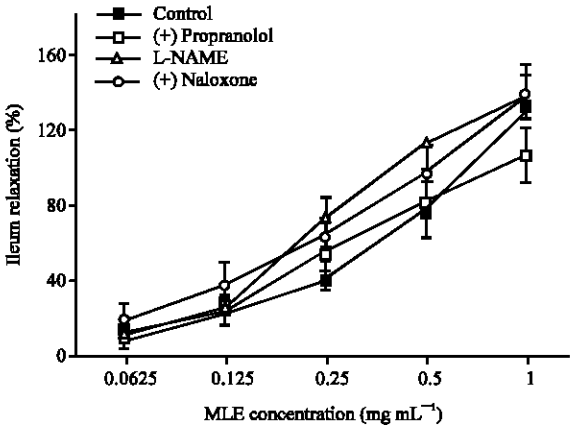

Fig. 3: Antispasmodic effect of *Mentha longifolia* hydroalcoholic extract (MLE) on KCl (60 mM)-induced ileal contraction before (Control, n = 8) and after tissue incubation either with propranolol (1 μM, 30 min, n = 7), L-NAME (100 μM, 20 min, n = 9) or naloxone (1 μM, 30 min, n = 6). The extract spasmolytic activity is not affected by tissue incubation with these antagonist or inhibitor. Each point represents mean±SEM of number of observations (n) mentioned above for each protocol

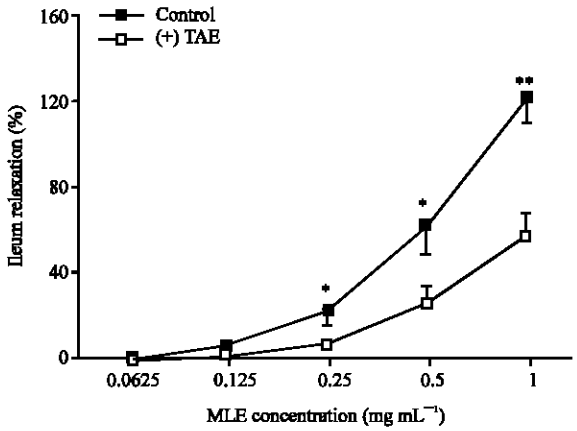

Fig. 4: Spasmolytic effect of *Mentha longifolia* hydroalcoholic extract (MLE) on carbachol 10 μM-induced ileal contraction before (Control) and after tissue incubation with tetraethylammonium (TEA, 1 mM, 5 min) as a non-specific K^+ channels blocker. Incubation of tissue preparation with TEA attenuated (two-way ANOVA, $p<0.05$) the MLE antispasmodic activity. Each point represents mean±SEM of 8 and 7 experiments for control and TEA protocols respectively (Student's t-test, *p<0.05, **p<0.001)

(1 μM, n = 6), or 20 min with L-NAME (100 μM, n = 9), as β-adrenoceptor antagonist, opioid receptor antagonist and nitric oxide synthase inhibitor, respectively, did not alter the spasmolytic effect of MLE on KCl-induced ileum contractions (Fig. 3).

Effect of ileum incubation with TEA on the MLE antispasmodic activity: The antispasmodic effect of MLE (0.0625-1 mg mL^{-1}) on CCh-induced ileal contraction was shifted to right after 5 min tissue incubation with 1 mM of TEA (two-way ANOVA, $p<0.05$, n = 7) which its results are shown in Fig. 4.

DISCUSSION

The distinctive finding in this study is that extract from *Mentha longifolia* leaf hydroalcoholic extract (MLE) has a myorelaxant effects on isolated preparations of rat intestinal ileum. The contraction of gastrointestinal smooth muscle depends on the mediation of intracellular Ca^{2+} and is accomplished by the process of excitation-contraction coupling (Zhang *et al*., 2005). A high-K^+ medium could depolarize the cellular membrane of ileum smooth muscle (Bolton, 1979; Zhang *et al*., 2005). Moreover, it is well known that KCl-induced contraction in smooth muscle is due to an increase in Ca^{2+} influx through voltage-operated Ca^{2+} channels (Kaya *et al*., 2002; Borrelli *et al*., 2006) which the L-type of these channels has been shown to exist in rat ileum (El Bardai *et al*., 2004). In addition, it has been suggested that the substance that inhibits high K^+ contractions is considered as a blocker of Ca^{2+} influx (Gilani *et al*., 2005). Ghoulami *et al*. (2001) has reported that *Mentha longifolia* has high content piperitenone and piperitenone oxide and the relaxant activity of piperitenone oxide on guinea pig ileum has been reported (Sousa *et al*., 1997).

It is accepted that CCh-induced contractile response following receptor activation require an increase in intracellular Ca^{2+} which is provided by both Ca^{2+} influx through L-type Ca^{2+} channels and Ca^{2+} release from intracellular calcium stores (Tanovic *et al*., 2000). On the other hand, $BaCl_2$ may act directly on the smooth muscle (Ozaki *et al*., 2006) or induce smooth muscle contraction by nonspecifically blocking the K^+ channels (Liu *et al*., 2001). Although, the applied spasmogens have different modes of action but identical concentration-response curves effect of MLE on these spasmogens contractile activity indicates that MLE might be acting via a non-specific mechanism and also at Ca^{2+} entry level as a common step in the contraction mechanism elicited by the agonists. Furthermore, this suggestion is supported by the MLE spasmolytic effect on $CaCl_2$-induced contractions since, in the Ca^{2+}-free and high K^+ Tyrode solution, the tissue was depolarized by high K^+ (Fujimoto and Mori, 2004) however, in this study ileal contractions was occurred only after applying $CaCl_2$ in the organ bath as reported by Zhang *et al*. (2005). Therefore, it may be assumed that MLE has inhibited the Ca^{2+} influx. The same suggestion has been made for vasorelaxatory effect of rotundifolone which has been found in *Mentha longifolia* (Guedes *et al*., 2004).

It is very unlikely that the antispasmodic effect of MLE is due to antimuscarinic action, since the extract also inhibited the contractions induced by $BaCl_2$ and KCl which do not act through a receptor-mediated mechanism.

It has been shown that activation of β-adrenoceptor in ileal smooth muscle leads to relaxation (Brown and Summers, 2001). In order to assess if the extract relaxed intestine by binding on β-adrenoceptor, the relaxing effect of the extract was examined in the presence of propranolol. We found that propranolol does not attenuate the activity of the extract, suggesting that MLE does not have any effect on beta adrenergic receptors. Nitric oxide synthase elevates nitric oxide (NO) production which in turn relaxes ileum by promoting cGMP synthesis (Kanada *et al*., 1992). But, the ineffectiveness of L-NAME (as a nitric oxide synthase inhibitor) to reduce the extract spasmolytic effect indicates that NO was not involved in the extract activity. This result is in agreement to a report describing that rotundifolone found in *Mentha longifolia* essential oil exhibits aorta relaxation was not dependent to nitric oxide synthesis (Guedes *et al*., 2004).

Activation of opioid receptors relaxes ileum (Gray *et al*., 2005) but the extract activity was unaffected by naloxone (as a non-selective opioid antagonist) which indicates that the MLE activity was not mediated via these receptors. Another possible mechanism was potassium channels activation by the extract. Present results show that TEA, as a non-specific potassium channel blocker, attenuated the MLE effect on CCh-induced ileal contraction which may indicate that MLE induces its effect, at least in part, through activation of these channels. Considering the demonstration of ceramides such as longifoamide-A and B in *Mentha longifolia* (Shaiq Ali *et al*., 2006) and the spasmolytic activity of these agents (Jang *et al*., 2005) the spasmolytic activity of MLE could be attributed to the ceramides. Furthermore, it has been reported that β-sitosterol which exist in *Mentha longifolia*, exhibits Ca^{2+} channel-blocking action (Gilani *et al*., 2008), therefore, the spasmolytic activity of MLE could be related to activity of this compound as well.

CONCLUSION

It seems that the *Mentha longifolia* leaf extract spasmolytic effect on rat ileum has been occurred mainly through the voltage operated Ca^{2+} channels. This study showed the relaxant effect of MLE on the ileum contractions induced by three different spasmogens. Thus, the *in vitro* antispasmodic activity of MLE supports a rational suggesting basis for folk and traditional use of the *Mentha longifolia* in gastrointestinal cramps, diarrhea and colic. Present results may suggest the beneficial effect of this herb for treatment of diarrhea. However, more detailed phytochemical studies are necessary to identify the active principle(s) and exact mechanism(s) of action.

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