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Calcium Channel Blocking Activity of Mentha longifolia L. Explains its Medicinal Use in Diarrhoea and Gut Spasm

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Mentha longifolia has a reputation in traditional medicine in the indications of diarrhoea and gut spasm. This study was carried out to provide a possible pharmacological basis for its medicinal use in hyperactive gut disorders. In a castor oil induced diarrhoeal model, the crude extract of Mentha longifolia (Ml.Cr), at doses of 100–1000 mg/kg, provided 31–80% protection, similar to loperamide. In isolated rabbit jejunum preparations, Ml.Cr caused inhibition of spontaneous and high K+-induced contractions, with respective EC50 values of 1.80 (1.34–2.24; n = 6–8) and 0.60 mg/mL (0.37–0.85; n = 6–8), which suggests spasmodic activity, mediated possibly through calcium channel blockade (CCB). The CCB activity was further confirmed when pretreatment of the tissue with Ml.Cr (0.3–1 mg/mL) caused a rightward shift in the Ca++ concentration–response curves (CRCs), similar to verapamil. Loperamide also inhibited spontaneous and high K+-induced contractions and shifted the Ca++ CRCs to the right. Activity-directed fractionation revealed that the petroleum spirit fraction was more potent than the parent crude extract and aqueous fraction. These data indicate that the antidiarrhoeal and spasmodic effects of the crude extract of Mentha longifolia are mediated through the presence of CCB-like constituent(s), concentrated in the petroleum spirit fraction and this study provides indirect evidence for its medicinal use in diarrhoea and spasm. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: Mentha longifolia; antidiarrhoeal; antispasmodic; calcium channel blocker.

INTRODUCTION

Mentha longifolia L. (family: Labiatae) is an aromatic perennial herb, commonly known as ‘wild mint’, found in the Northern areas of Pakistan (Baquer, 1989). The family Labiatae is one of the major sources of culinary, vegetable and medicinal plants all over the world (Farzaneh et al., 2005). The plant is well known in traditional medicine (Lewis and Elvin-Lewis, 1977), as a cooling medicine and has been used in diarrhoea and gut spasm (Amini, 1997), in addition to many other uses, such as choleretic, carminative (Chopra and Chopra, 1992), in indigestion and flatulence (Watt and Breyer-Brandwijk, 1962; Duke, 2002).

The leaves of Mentha longifolia contain an abundance of volatile oils, the main constituents of which are piperitone (Kokkini and Papageorgiou, 1988) and piperitene (Ghoulami et al., 2001). Other constituents present in the leaves are thymol (Al-Ankari et al., 2004), tannins, saponins and flavonoids (Bourweig and Pohl, 1973).

The plant has been studied for a few biological activities, such as antimicrobial and antioxidant (Mimicasaparins and flavonoids (Bourweig and Pohl, 1973)). The 1962; Duke, 2002). anti-HIV (Amzazi et al., 2003) and anthelmintic (Kozan et al., 2006). In the past, the plant has not been evaluated pharmacologically for its use in hyperactive gut disorders. Therefore, the present investigation was carried out to study the antidiarrhoeal and antispasmodic activities of the plant with the intention of providing a pharmacological base to its medicinal use in these hyperactive gut disorders.

MATERIALS AND METHODS

Plant materials. Fresh leaves of Mentha longifolia (3 kg) were collected in the Swat District, N.W.F.P., Pakistan in the month of August 2004 and authenticated by Mr Mehboob-ur-Rehman, a botanist, at the Department of Botany, Govt. PG Jehanzeb College, Saidu Sharif Swat, N.W.F.P., Pakistan. A voucher specimen (Ml-L-08-04) was deposited at the herbarium located at the Department of Biological and Biomedical Sciences, Aga Khan University, Karachi, Pakistan.

Preparation of crude extract and fractionation. The plant materials were cleaned of adulterant and were coarsely ground. The powdered material (2 kg) was soaked in 70% aqueous methanol for 3 days with occasional shaking. It was filtered through a muslin cloth and then through a filter paper. This procedure was repeated...
thrice and the combined filtrate was evaporated on a rotary evaporator at 37°C under reduced pressure (~760 mm Hg) to a thick, semi-solid mass of dark brown colour, i.e., the crude extract (ML.Cr), yielding approximately 38% (w/w). ML.Cr was solubilized in normal saline for use in the in vivo and in vitro experiments.

Activity-directed fractionation of the crude extract was carried out by standard phytochemical procedures using different organic solvents (Williamson and Okpako, 1998). A known quantity of the extract (80 g) was dissolved in distilled water. This was then introduced in a separating funnel and petroleum spirit (90–100 mL) was then added. This mixture was shaken vigorously, regularly allowing the air to escape out. It was kept for about 30 min to let the two layers separate. The upper layer of petroleum spirit was acquired and the same procedure was repeated twice and all the petroleum spirit layers were collected and concentrated in a rotary evaporator to obtain the petroleum spirit fraction (ML.Pet). The remaining layer was evaporated and the resultant fraction was considered as the aqueous fraction (ML.Aq). The yield of both fractions was 28.6% (w/w) and 40% (w/w), respectively.

**Preliminary phytochemical analysis.** *Mentha longifolia* crude extract was screened for the presence of saponins, flavonoids, flavanols, flavones, tannins, phenols, coumarins, sterols, terpenes, alkaloids and anthraquinones using the methods described by Wall et al. (1952).

**Drugs and standards.** The following reference chemicals were obtained from the sources specified: loperamide hydrochloride, acetylcholine chloride, verapamil hydrochloride, potassium chloride (Sigma Chemical Company, St Louis, MO, USA) and castor oil (Karachi Chemical Industries, Karachi, Pakistan). All chemicals used were of the highest purity grade available. Stock solutions of all the chemicals were made in distilled water and the dilutions were made fresh in normal saline on the day of the experiment.

**Animals.** Experiments performed complied with the ruling of the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council (National Research Council, 1996) and were approved by the Ethical Committee of Aga Khan University Karachi, Pakistan. Balb/c albino mice (20–25 g) and local rabbits (1.5–2 kg) of either sex used were killed by cervical dislocation, the abdomen was cut open and the jejunal portion isolated out. Preparations 2 cm long were mounted in 10 mL tissue baths containing Tyrode's solution maintained at 37°C and aerated with a mixture of 5% carbon dioxide in oxygen (carbogen). The composition of Tyrode's solution, in mm, was: KCl 2.7, NaCl 136.9, MgCl2 1.1, NaHCO3 11.9, NaH2PO4 0.4, glucose 5.6 and CaCl2 1.8 (pH 7.4). A preload of 1 g was applied and the tissues kept undisturbed for an equilibrium period of 30 min after which control responses to a submaximal dose of acetylcholine (0.3 μM) were obtained and the tissue presumed stable only after the reproducibility of the said responses. Under these experimental conditions, rabbit jejunum exhibits spontaneous rhythmic contractions, allowing the testing of the relaxant (spasmolytic) activity directly without the use of an agonist (Gilani et al., 1994).

**Determination of calcium antagonist activity.** To assess whether the spasmolytic activity of the test substances was mediated through calcium channel blockade, a high concentration of K+ (80 mm), as KCl, was used to depolarize the preparations (Farre et al., 1991). K+ (80 mm) was added to the tissue bath, which produced a sustained contraction. Plant extract and standards were then added in a cumulative fashion to obtain concentration-dependent inhibitory responses (Van-Rossum, 1963). The relaxation of intestinal preparations, precontracted with K+, was expressed as the percent of the control precontraction.

To confirm the calcium antagonist activity of test substances, the tissue was allowed to stabilize in normal Tyrode's solution, which was then replaced with Ca²⁺+-free Tyrode's solution containing EDTA (0.1 mm) for 30 min in order to remove Ca²⁺ from the tissues. This solution was further replaced with K⁺-rich and Ca²⁺+-free Tyrode's solution, having the following composition: KCl 50, NaCl 91.04, MgCl2 1.05, NaHCO3 11.90, NaH2PO4 0.42, glucose 5.55 and EDTA 0.1 mm. Following an incubation period of 30 min, control concentration–response curves (CRCs) of CaCl2 were obtained. When the control CRCs of CaCl2 were found superimposable (usually after two cycles), the tissue was pretreated with the plant extract for 60 min to test the possible calcium channel blocking effect. The CRCs of CaCl2 were reconstructed in the presence of different concentrations of the test material.

**Statistics.** All the data expressed are mean ± standard error of the mean (SEM), and the median effective
RESULTS AND DISCUSSION

Based on the medicinal use of *Mentha longifolia* in hyperactive gut disorders, such as diarrhoea and spasm (Amini, 1997), its aqueous methanol crude extract was tested for the possible antidiarrhoeal effect in mice. When tested against the castor oil-induced diarrhoea in mice, the crude extract of the leaves of *Mentha longifolia* (Ml.Cr), like loperamide, a standard antidiarrhoeal agent (Reynolds et al., 1984), inhibited significantly (*p* < 0.05) the frequency of defaecation as well as wetting of faeces compared with the untreated group (i.e. mice receiving neither Ml.Cr, nor loperamide, but castor oil only). Ml.Cr and loperamide reduced greatly the wetness of the faecal droppings and provided around 31.73–80.05% and 97.30% protection, respectively (Table 1). Verapamil, a standard calcium channel blocker (Godfraind et al., 1986) also provided protection from castor oil induced diarrhoea (Table 1). The induction of diarrhoea by castor oil results from the hypersecretory response and generation of giant contraction of the intestine (Croci et al., 1997). Thus, a potential antidiarrhoeal agent may exhibit its antidiarrhoeal effect by inhibiting gut motility and/or electrolyte outflux (diarrhoeal droppings) (Croci et al., 1997).

The protective effect of the crude extract of *Mentha longifolia* against the castor oil-induced diarrhoea in mice, similar to loperamide and verapamil, suggests that it has either an inhibitory effect on contraction or on electrolyte outflux. To see its possible inhibitory effect on gut motility, the Ml.Cr was further studied in *in vitro* experiments.

Spontaneously beating isolated rabbit jejunum preparations were used to test a possible inhibitory (spasmolytic) effect of test substances without the use of a spasmogen (Gilani et al., 1994). When tested in isolated rabbit jejunum preparations, cumulative addition of Ml.Cr, loperamide and verapamil caused concentration-dependent inhibition of the spontaneous contractions (Fig. 1), with respective EC\(_{50}\) value of 1.80 mg/mL (1.34–2.24; *n* = 6–8), 79.73 μm (57.39–110.70) and 0.28 μm (0.13–0.35) (Figs 2 and 3), thus showing smooth muscle relaxant (spasmolytic) activity. The contraction of smooth muscle preparations, including rabbit jejunum, is dependent upon an increase in the cytoplasmic free \([\text{Ca}^{++}]\), which activates the contractile elements (Karaki and Weiss, 1983). The increase in intracellular \([\text{Ca}^{++}]\) occurs either via influx through voltage-dependent \([\text{Ca}^{++}]\) channels (VDCs) or its release from intracellular stores.

### Table 1. Effect of the aqueous methanol crude extract of the leaves of *Mentha longifolia* (Ml.Cr) and verapamil on castor oil-induced diarrhoea in mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose</th>
<th>Total number of faeces in 4 h</th>
<th>Total number of wet faeces in 4 h</th>
<th>Protection (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (saline)</td>
<td>10 mL/kg</td>
<td>14.80 ± 1.10</td>
<td>0.20 ± 0.31</td>
<td>98.46 ± 0.88</td>
</tr>
<tr>
<td>Castor oil</td>
<td>10 mL/kg</td>
<td>11.80 ± 0.80</td>
<td>11.6 ± 0.77</td>
<td>1.33 ± 0.82</td>
</tr>
<tr>
<td>+ Ml.Cr</td>
<td>100 mg/kg</td>
<td>19.80 ± 1.03</td>
<td>14.20 ± 0.96</td>
<td>31.73 ± 1.02*</td>
</tr>
<tr>
<td>+ Ml.Cr</td>
<td>300 mg/kg</td>
<td>20.80 ± 1.14</td>
<td>9.20 ± 0.58</td>
<td>52.85 ± 1.88*</td>
</tr>
<tr>
<td>+ Ml.Cr</td>
<td>1000 mg/kg</td>
<td>13.80 ± 1.15</td>
<td>2.40 ± 0.87</td>
<td>80.05 ± 5.68*</td>
</tr>
<tr>
<td>+ Verapamil</td>
<td>1 mg/kg</td>
<td>9.2 ± ± 2.55</td>
<td>8.89 ± 1.72</td>
<td>23.31 ± 10.2*</td>
</tr>
<tr>
<td>+ Verapamil</td>
<td>3 mg/kg</td>
<td>10.06 ± 1.72</td>
<td>8.50 ± 1.11</td>
<td>46.30 ± 10.2b</td>
</tr>
<tr>
<td>+ Verapamil</td>
<td>10 mg/kg</td>
<td>11.2 ± 1.32</td>
<td>4.0 ± 0.71</td>
<td>62.38 ± 3.87b</td>
</tr>
<tr>
<td>+ Loperamide</td>
<td>10 mg/kg</td>
<td>8.80 ± 1.18</td>
<td>0.4 ± 0.34</td>
<td>97.30 ± 1.67</td>
</tr>
</tbody>
</table>

Ml.Cr, crude extract of the leaves of *Mentha longifolia*.

\(n = 5\) in each case. Mean ± SE.

\(^a\) *p < 0.05*, \(^b\) *p < 0.01* and \(^c\) *p < 0.001* vs control, Student’s *t*-test.
in the sarcoplasmic reticulum. Periodic depolarization and repolarization regulates the spontaneous movements of the intestine and at the height of depolarization, the action potential appears as a rapid influx of Ca\(^{++}\) via VDCs (Brading, 1981). Thus, the inhibitory effect of the Ml.Cr on spontaneous movements of rabbit jejunum may appear to be due to a CCB effect mediated possibly due to interference of Ca\(^{++}\) influx through VDCs.

It was previously observed that the spasmolytic constituents present in different medicinal plants mediate their effect usually through a CCB effect (Gilani et al., 2009).
1999, 2005a, 2005b, 2006). To see whether the spasmolytic effect of the plant extract observed in this study is also mediated through a CCB-like effect, a high concentration of K\(^+\) (80 mM) was introduced to depolarize the tissue. The crude extract was then added in a cumulative fashion, where it caused a concentration-dependent relaxation of the induced contractions with an EC\(_{50}\) value of 0.60 mg/mL (0.37–0.85; \(n=6–8\)), as shown in Fig. 2A, suggesting that the spasmolytic effect is possibly mediated through a CCB-like effect. Similarly, verapamil and loperamide also caused a concentration-related inhibitory effect against high K\(^+\)-induced contractions with respective EC\(_{50}\) values of 0.08 (0.05 to 0.12) and 8.55 \(\mu\)M (5.80–12.60), as shown in Fig. 3. Ml.Cr was more potent against K\(^+\)-induced contractions, similar to verapamil. These data suggest that Ml.Cr mediates its spasmolytic effect possibly through calcium channel blockade.

The contractions induced by high K\(^+\) (>30 mM) are dependent on the entry of Ca\(^{2+}\) into the cells through VDCs (Bolton, 1979) and a substance which can inhibit high K\(^-\)induced contractions with respective EC\(_{50}\) values of 0.08 (0.05 to 0.12) and 8.55 \(\mu\)M (5.80–12.60), as shown in Fig. 3. Ml.Cr was more potent against K\(^-\)-induced contractions, similar to verapamil. These data suggest that Ml.Cr mediates its spasmolytic effect possibly through calcium channel blockade.

Activity-directed fractionation revealed that the petroleum spirit fraction was more potent than the parent crude extract and its aqueous fraction, which suggests that the antispasmodic activity is shifted to the petroleum spirit fraction. The presence of flavonoids, saponins and tannins, revealed by preliminary phytochemical analysis, support the CCB effect of the plant extract because plant derived flavonoids (Zhu et al., 1997), saponins (Kai et al., 1998) and tannins (Zhu et al., 2005) have been found to possess CCB effects, which might be the active candidate(s) responsible for its medicinal use in diarrhoea and gut spasm, though additional mechanisms cannot be ruled out.

This study thus showed that the crude extract of Mentha longifolia possesses antidiarrhoeal and
antispasmodic effects mediated through calcium channel blockade and provides a possible pharmacological base to its medicinal use in diarrhoea and gut spasms. Additionally, this study also provides important information, showing how traditional knowledge is important for the discovery of health products of natural origin.

Acknowledgement
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Conflict of Interest
The authors have declared that there is no conflict of interest.

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