



Study of antispasmodic action of *Lavandula angustifolia* Mill hydroalcoholic extract on rat ileum

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ABSTRACT

Introduction: Lavender (*Lavandula angustifolia* Mill) is a herbal medicine widely used for gastrointestinal (GI) disorders. However, its pharmacological action on isolated ileum has not been studied. In this research effect of hydroalcoholic extract of *L. angustifolia* on isolate ileum contractions was studied and compared with loperamide.

Methods: Hydroalcoholic extract of the plant was prepared by percolation method. The total flavonoid contents were assessed by colorimetric technique. A portion of rat ileum was suspended in an organ bath containing Tyrode's solution. The tissue was kept under 1 g tension at 37°C and continuously gassed with O₂. The tissue was stimulated with KCl (80 mM), acetylcholine (ACh, 2 μM) and electrical field stimulation (EFS). Effect of the *L. angustifolia* extract was studied on ileum contractions and compared with that of loperamide.

Results: The yield of hydroalcoholic extract was 17% with total flavonoid content of 185 μg/mL in the stock solution. Loperamide in concentration dependent manner inhibited ileum contractile response to KCl, ACh and EFS. Hydroalcoholic extract of *L. angustifolia* (8-512 μg/mL) concentration dependently inhibited ileum contraction induced by KCl (IC₅₀ = 88 ± 21 μg/mL), ACh (IC₅₀ = 119 ± 251 μg/mL) and EFS (IC₅₀ = 87 ± 33 μg/mL). The vehicle had no significant effect on ileum contractions.

Conclusion: From this study it was concluded that *L. angustifolia* extract at microgram concentration shows an inhibitory effect on rat ileum smooth muscle. Therefore, isolation and identification of active ingredients are recommended.

Implication for health policy/practice/research/medical education:

Lavender is traditionally used as antispasmodic remedy. This paper provides pharmacological evidence for effectiveness of lavender in inhibiting ileum spasm. The results show that it might be a good source for preparation of new drugs.

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Introduction

Plants ingredients or extracts are widely used for treatment of various ailments (1). The World Health Organization (WHO) estimated that nearly ¾ of people in the world use traditional and folk medicines as a kind of remedy for alleviation of gastrointestinal (GI) diseases (1). One of these plant materials is Lavender which is commonly known in Iran as "Ostokhoddous." Lavender (*Lavandula angustifolia* Mill), belongs to Lamiaceae family and is a widely distributed aromatic herb (2). Most lavender species are indigenous to the mountain regions of the countries bordering the western Mediterranean, the islands of the Atlantic, Turkey, Pakistan and India (3). The

lavender species are evergreen, shrubby plants. They vary from one to three feet high and show a range of leaf and flower shapes. The leaves can be lobed or un-lobed and are sometimes present only at the base of the stems. The color of the flowers can range from blue to violet, and the stem and leaves can range from deep bluish grey to green to discolored brown (4).

Lavender (*L. angustifolia*) contains essential oil, anthocyanins, phytosterols, sugars, minerals, coumaric acid, glycolic acid, valeric acid, ursolic acid, herniarin, coumarin and tannins (5). The composition of lavender essential oil consists primarily of monoterpenoids and sesquiterpenoids. Of these, linalool and linalyl acetate

dominate, with moderate levels of lavandulyl acetate, terpinen-4-ol and lavandulol. 1,8-Cineole, geraniol, β -caryophyllene and camphor are also present in low to moderate qualities. Lavender oil typically contains more than 100 compounds, although a many of these are present at very low concentrations (6). Preparative isolation of lavender extract indicated that Lavender possesses relatively high flavonoid contents (7). Some flavonoids identified in lavender extract are rosmarinic acid, luteolin, apigenin, luteolin 7-O- β -D-glucoside, apigenin 7-O- β -D-glucoside and luteolin 7-O- β -D-glucuronide (8).

Flowers and essential oils of lavender are mostly used in cosmetics and personal care industries (9). Lavender is commonly used, nowadays, in perfumes, soaps, bath and talc powders, candles and perfume. Small amounts are sometimes used to flavor teas and foods (4). Lavender is used in traditional and folk medicines in different parts of the world for the treatment of GI, nervous and rheumatic disorders (10). In Iranian folk and traditional medicines lavender has been used as carminative, diuretic, anti-epileptic, anti-rheumatic and pain reliever especially in headache and migraine. Old famous Iranian practitioners such as Rhazes (11), and Avicenna (12) were well familiar with this plant and mentioned their traditional medicinal books "Continens" and "Canon", respectively (13). In some regions of Iran, the leaves of this plant are claimed to be especially effective against pain and inflammatory diseases including rheumatism and backache (10). In Chinese traditional medicine, lavender is used to treat several conditions including infertility, infection, anxiety, and fever (14,15). In Arabic medicine Lavender is used for the treatment of stomachache and kidney problems (16).

In pharmacological and biological researches, *L. angustifolia* extracts, fractions and essential oils are reported to have relaxant, hypnotic, anti-convulsive, spasmolytic, antioxidant, local anesthetic, anti-bacterial and anti-inflammatory effects (17-21). Recent pharmacological studies confirm the traditional use of lavender for the treatment of painful and inflammatory conditions (10). It has been shown that essential oil of Lavender has spasmolytic effects on smooth muscle on isolated ileum and uterus (22). The antispasmodic actions of lavender were potentiated by application of a nonspecific phosphodiesterase inhibitor and a stereo-selective type 4 phosphodiesterase inhibitor, suggesting that lavender's effects are mediated through cAMP signaling (19,22). So far there is no report of the antispasmodic effect of *L. angustifolia* on isolated ileum, therefore, the purpose of the present study was to investigate the antispasmodic action of *L. angustifolia* extract on rat ileum.

Method and Materials

Plant material and extraction

Top leafy branches of Lavender (Ostokhodous in Persian) were collected from University of Isfahan garden in flowering season (July 2016). The plant was identified

as *L. angustifolia* Mill by botanist Dr Ali Bagheri at Biology Department of Isfahan University. A plant sample was deposited in the herbarium unit at School of Pharmacy (No. 3404).

Plant materials were dried in shadow and any woody branches were separated. The dried materials were then ground to powder using an electric mill (Moulinex). The plant powder was percolated for 24 hours according to pharmacopoeia reference using 70% ethanol (23). The ethanol was then evaporated in the rotary (Buchi Rotavapor RE) at 50°C to give a sticky semisolid product. A sample of the hydroalcoholic extract was completely dried on a heater for determining the percentage of remaining water.

Solvent and solution

The hydroalcoholic extract was made up as 20 mg/mL stock solution in dimethyl sulfoxide (DMSO) and further diluted by distilled water. Acetylcholine (Sigma) was prepared as 1mM stock solution in distilled water. KCl was prepared as 2M stock solution. Loperamide was made up in DMSO as 10mM stock solution. Tyrodé's solution was prepared as follows: NaCl 136mM, KCl 2.7mM, CaCl₂ 1.8mM, NaHCO₃ 11.9mM, MgCl₂ 1.05mM, NaH₂PO₄ 0.42mM and glucose 5.6mM. Aluminum chloride solution was prepared as 20% stock solution in 5% acetic acid in methanol. Quercetin was made up as 1 mg/mL stock solution in methanol. Unless stated all the chemicals were from Merck.

Biochemical standardization of the extract

Total flavonoid content of the hydroalcoholic extract was determined by aluminum chloride colorimetric method (24). Fifty milligrams of quercetin was dissolved in 50 mL methanol and then diluted to 4, 20, 100 and 500 μ g/mL. The diluted standard solutions (0.1 mL) were separately mixed with, 0.1 mL of 20% aluminum chloride, 0.1 mL of glacial acetic acid and 2.7 mL of methanol. After incubation at room temperature for 40 minutes in the dark, the absorbance of the reaction mixture was measured at 415 nm with a Jenway 5105 U.V/Vis spectrophotometer (England). The spectrophotometer was initially calibrated with blank solution. In the blank solution, aluminum chloride was substituted by the same amount of distilled water. The assessment was repeated 6 times (n=6). Similarly, 0.1 mL of hydroalcoholic extracts solution (20 mg/mL) was reacted with aluminum chloride for determination of its total flavonoid content.

Pharmacological studies

All animals were handled in compliance with the principles of the guide for care and use of laboratory animal care approved by university committee (25). On the day of experiment, male Wistar rats (180-220 g) bred in School of Pharmacy animal house were killed by a blow on the head and exsanguination. The abdomen cavity

was then opened up and a portion of ileum was removed and placed in oxygenated Tyrode's solution. Fats and connective tissues were carefully trimmed off and a piece of 2-3 cm long was cut off for contraction studies. For this purpose one end of the tissue was secured into a hook inside organ bath via a cotton thread. The other end of the tissue was connected to Harvard isotonic transducer and contractions were recorded by Harvard Universal Oscillograph apparatus. The organ bath was filled with Tyrode's solution at 37°C and continuously bubbled with oxygen. At the beginning the tissue was washed several times and allowed to reach a stable baseline.

KCl contraction study

After establishment of stable baseline, KCl (80mM) was added into the organ bath in order to produce a tonic contraction in the tissue. Fifteen minutes after addition of KCl, extract (8 to 512 µg/mL) was added into the organ bath in a cumulative manner with 2 folds increment in concentration. Control groups were treated with equivalent volume of vehicle (DMSO). Effect of loperamide was assessed in similar way (5 µg/mL to 1.1 mg/mL) using four fold increment in concentration. Each experiment was repeated on different tissues.

Acetylcholine contraction study

Acetylcholine (ACh) was added in to the organ bath to give final bath concentration of 2µM. After 30S contact time, the tissue was given 3 successive washes with fresh Tyrode's solution. Following interval periods of 5 minutes, ACh was added again as above and the process was repeated until consistent response was produced. After that, first concentration of extract was added and 5 minutes later response of ACh was assessed in the presence of extract. Then next concentration of extract was added and the process repeated until full concentration response curve for the extract was obtained. Effect of loperamide on ACh response was assessed in similar way. Time-matched vehicle treated control group were treated with equivalent volume of DMSO. Each experiment was performed on six different tissues (n=6).

Electrical field stimulation contraction study

Electrical field stimulation (EFS) was delivered via a parallel platinum wire situated on either side of the tissue. The platinum wire was connected to a stimulator and direct square pulses were applied for duration of one second with 6 V voltage output at 50Hz frequency. After establishment of consistent responses to EFS, lavender extract (0.5-256 µg/mL) was added at 15 minutes intervals until maximum response was achieved. Effect of loperamide on EFS response was tested with similar experimental protocol. Time-matched vehicle treated controls were treated with equivalent volume of DMSO.

Measurements and data analysis

For estimation of flavonoid content, average light

absorption for quercetin was plotted against its concentrations. A straight line was carefully fitted to the data and total flavonoid content of the extract was measured by extrapolating the light absorption value of the extract in the X axis of the calibration curve.

Tissue response to spasmogens or EFS were assessed as maximum recorded amplitude relative to the baseline and expressed as percentage of pretreated control response. Mean and standard error of mean (ESM) were calculated for each group of results and full semilogarithm concentration-response curve were plotted. Whenever appropriate, IC₅₀ value (Drug concentration causing 50% of maximum inhibitory effect) was calculated for each tissue and expressed as mean ± SEM. For statistical analysis one way analysis of variance (ANOVA) or Student's t-test was used as appropriate. SigmaPlot computer program (version 11) was used for statistical analysis and plotting the graphs.

Results

Plant phytochemistry

Solidified hydroalcoholic extract of *L. angustifolia* had dark green color. Water content of semisolid concentrated extract was assessed as 42% (W/W). The yield of dried extract was calculated to be 17% W/W. Total flavonoid content in 20mg/ml extract solution was calculated to be 185 µg/mL.

Pharmacological studies

Rat isolated ileum suspended under 1g tension in the organ bath produced small irregular spontaneous contractile activities which was subsided by washing the tissue with fresh Tyrode's solution. Over half and hour period of time the tissue gradually relaxed to a stable baseline.

KCl contraction study

Addition of KCl (80mM) into the organ bath resulted in a fast contraction of ileum followed by a sustained contraction which was maintained during the course of experiment. Cumulative addition of standard drug (loperamide) into the bath reduced the sustained contraction of ileum induced by KCl (Figure 1). The inhibitory effect of loperamide was observed with 30 µg/mL in the bath and total inhibition was achieved with 1.1 mg/mL loperamide in the bath. Loperamide concentration causing 50% of maximum inhibitory response (IC₅₀) was calculated to be 63 ± 27 µg/mL (n=6). In the time-matched vehicle treated control groups which were treated in a similar way with equivalent volume of DMSO, no statistical changes in the sustained contraction induced by KCl were observed (ANOVA, Figure 1).

Cumulative addition of extract into the bath reduced the sustained contraction induced by KCl in a concentration-dependent manner (Figure 2). The inhibitory effect of extract was observed with 16 µg/mL extract concentration in the bath and total inhibition was achieved with concentration of 512 µg/mL. The IC₅₀ value of the extract

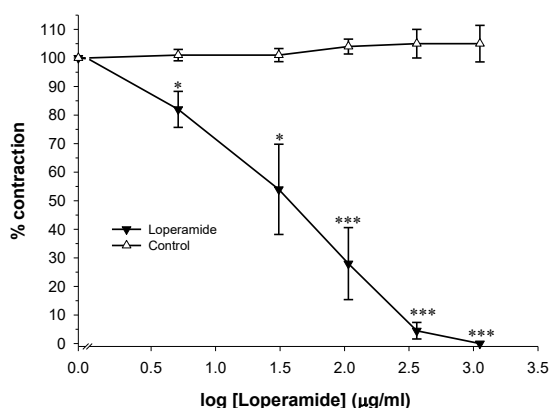


Figure 1. Effect of loperamide on tension development to potassium chloride (KCl, 80 mM) in isolated ileum of rat. Ordinate scale: ileum contractile response expressed a percentage of control before addition of loperamide. Abscissa scale: \log_{10} concentration of loperamide. The points are mean and the vertical bars show the SEM ($n = 6$). There is no statistically significant change in the control group treated with equivalent amount of vehicle (DMSO) over the course of study (ANOVA). Maximum amount of DMSO used was 1.25%. Stars show statistically significant differences in comparison with the corresponding point in the vehicle treated control group. * $P < 0.05$, *** $P < 0.001$ (Student's t test).

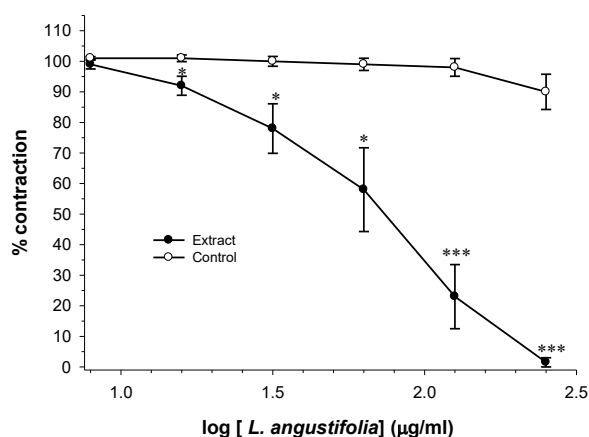


Figure 2. Effect of *Lavandula angustifolia* hydroalcoholic extract on tension development to potassium chloride (KCl, 80mM) in isolated ileum of rat. Ordinate scale: ileum contractile response expressed a percentage of control before addition of extract. Abscissa scale: \log_{10} concentration of extract. The points are mean and the vertical bars show the SEM ($n = 6$). There is no statistically significant difference in the vehicle (DMSO) treated time-matched control tissues (ANOVA). Maximum amount of DMSO used was 2%. Stars show statistically significant differences in comparison with the corresponding point in the vehicle treated control group. * $P < 0.05$, *** $P < 0.001$ (Student's t test).

was calculated to be 89 ± 21 $\mu\text{g}/\text{mL}$ ($n=6$). In the time-matched vehicle treated control groups which were treated in a similar way with equivalent volume of DMSO, no statistical changes in the sustained contraction induced by KCl were observed (ANOVA, Figure 2).

ACh contraction study

Addition of ACh ($2 \mu\text{M}$) into the bath resulted in a fast contraction within 30 second contact time. Following washing the tissue quickly relaxed towards the baseline. Non-cumulative addition of standard drug (loperamide) into the bath in a concentration-dependent manner attenuated the ileum response to ACh (Figure 3). Complete inhibition was observed with $25.6 \text{ mg}/\text{mL}$ loperamide in the bath. The IC_{50} value of the loperamide was calculated to be $4.9 \pm 1.5 \text{ mg}/\text{ml}$ ($n = 6$). In the time-matched vehicle treated control groups no statistical changes in the phasic contraction induced by ACh were observed (ANOVA, Figure 3).

Hydroalcoholic extract of *L. angustifolia* in a concentration-dependent manner inhibited the contractile response to ACh (Figure 4). The inhibitory effect of extract was observed with $16 \mu\text{g}/\text{mL}$ in the bath and total inhibition was achieved with $256 \mu\text{g}/\text{mL}$ extract in the bath. The IC_{50} value of the extract was calculated to be $119 \pm 25.5 \mu\text{g}/\text{mL}$ ($n=6$). Statistically, no significant change was observed in the vehicle treated control groups (ANOVA, Figure 4).

EFS contraction study

Application of EFS resulted in a fast contraction in the isolated rat ileum suspended under 1 g tension in the bath. A second slower contraction was followed before tissue relaxation to the baseline. Regular application of EFS resulted in consistent responses. However, the secondary peak response was less consistent. Loperamide in a concentration-dependent manner reduced EFS responses with IC_{50} value of $178 \pm 73 \mu\text{g}/\text{mL}$ ($n = 5$) (Figure 5). In the

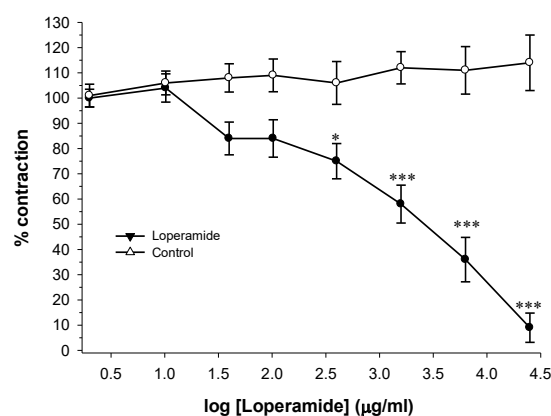


Figure 3. Effect of loperamide on tension development to acetylcholine (ACh, $2 \mu\text{M}$) in isolated ileum of rat. Ordinate scale: ileum contractile response expressed a percentage of control before addition of loperamide. Abscissa scale: \log_{10} concentration of loperamide. The points are mean and the vertical bars show the SEM ($n=6$). There is no statistically significant change in the control group treated with equivalent amount of vehicle (DMSO) over the course of study (ANOVA). Maximum amount of DMSO used was 0.5%. Stars show statistically significant differences in comparison with the corresponding point in the vehicle treated control group. * $P < 0.05$, *** $P < 0.001$ (Student's t test).

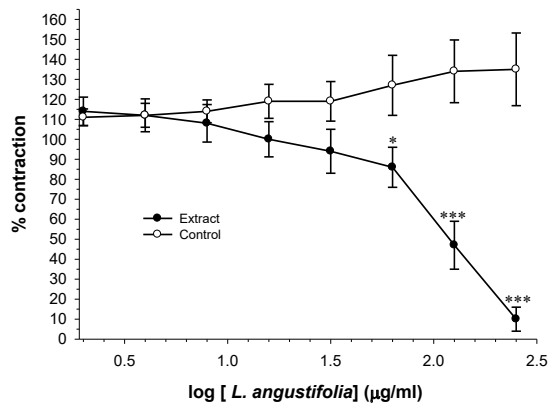


Figure 4. Effect of *Lavandula angustifolia* hydroalcoholic extract on tension development to acetylcholine (ACh, 2µM) in isolated ileum of rat. Ordinate scale: ileum contractile response expressed a percentage of control before addition of extract. Abscissa scale: \log_{10} concentration of extract. The points are mean and the vertical bars show the SEM (n=6). There is no statistically significant changed in the control group treated with equivolume amount of vehicle (DMSO) over the course of study (ANOVA). Maximum amount of DMSO used was 1.25%. Stars show statistically significant differences in comparison with the corresponding point in the vehicle treated control group. * $P < 0.05$, *** $P < 0.001$ (Student's *t* test).

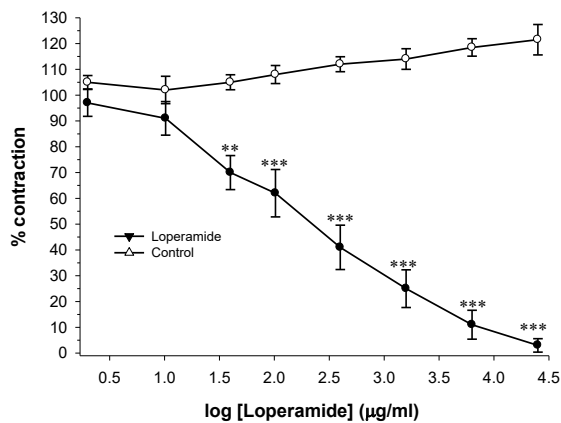


Figure 5. Effect of loperamide on tension development to electrical field stimulation (EFS, 6 V, 50 HZ, 1 s duration) in isolated ileum of rat. Ordinate scale: ileum contractile response expressed a percentage of control before addition of loperamide. Abscissa scale: \log_{10} concentration of loperamide. The points are mean and the vertical bars show the SEM (n=6). There is no statistically significant changed in the control group treated with equivolume amount of vehicle (DMSO) over the course of study (ANOVA). Maximum amount of DMSO used was 0.5%. Stars show statistically significant differences in comparison with the corresponding point in the vehicle treated control group. ** $P < 0.01$, *** $P < 0.001$ (Student's *t* test).

time-matched vehicle treated control groups no statistical changes in the EFS response was observed (ANOVA, Figure 5).

Non-cumulative addition of extract into the bath reduced the contractile response to EFS in a concentration-dependent manner (Figure 6). The inhibitory effect of

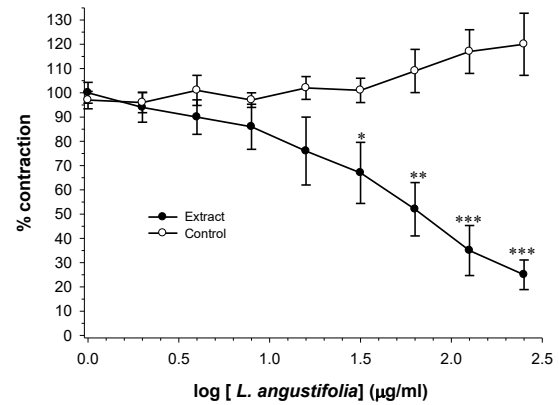


Figure 6. Effect of *Lavandula angustifolia* hydroalcoholic extract on tension development to electrical field stimulation (EFS, 6 V, 50 HZ, 1 s duration) in isolated ileum of rat. Ordinate scale: ileum contractile response expressed a percentage of control before addition of extract. Abscissa scale: \log_{10} concentration of extract. The points are mean and the vertical bars show the SEM (n=6). There is no statistically significant changed in the control group treated with equivolume amount of vehicle (DMSO) over the course of study (ANOVA). Maximum amount of DMSO used was 1.25%. Stars show statistically significant differences in comparison with the corresponding point in the vehicle treated control group. * $P < 0.05$, ** $P < 0.001$, *** $P < 0.001$ (Student's *t* test).

extract was observed with 8 µg/mL in the bath and total inhibition was achieved with 256 µg/mL with IC_{50} value of 87 ± 33 µg/mL (n=6). In the time-matched vehicle treated control groups no significant changes were observed over period of experiments (ANOVA, Figure 6).

Discussion

In Iranian traditional medicine, Lavender is used to alleviate GI disorders including diseases associated with gut motility and spasm (10). Gut motility is controlled by autonomic and enteric nervous system (ENS). ENS is unique in its ability to function independently of the central nervous system (CNS) in the control of the functions of the digestive tract. The ENS controls gut motility and secretion via local reflexes that are triggered by local distension of the intestinal wall, distortion of the mucosa, and chemical contents in the lumen. This neuronal regulation of GI functions is due to the liberation of classical neurotransmitters such as acetylcholine (ACh), noradrenaline, serotonin, GABA and glutamate, but a great number of other neuromediators and hormones also participate in the regulation of functions in the GI tract including vasoactive intestinal polypeptide (VIP), nitric oxide, galanin, motilin, adenosine triphosphate, tachykinins, etc (26).

Intestinal motility can be studied *in vitro* using the organ bath technique, which allows the recording of smooth muscle activity following addition of spasmogen or neurally evoked responses (EFS). Thus, using such an approach we have studied effects of Lavender extract on ileum contraction due to release of various chemical

mediators associated with activity of ENS as well as on exogenous addition of ACh or KCl responses.

KCl has long been used as a convenient stimulus to contract smooth muscle by a highly reproducible and relatively "simple" mechanism involving activation of voltage-operated Ca^{2+} channels that leads to increase in cytosolic free Ca^{2+} ions, calcium-calmodulin-dependent myosin light chain (MLC) kinase activation, MLC phosphorylation and contraction (27).

In ileum smooth muscles, ACh produces contractions by activating muscarinic receptors (28). It is generally assumed that both M_2 and M_3 muscarinic receptors play a key role in mediating this activity (29). The M_3 receptor is coupled preferentially to Gq-type G proteins, resulting in the activation of phospholipase C (PLC) and the formation of inositol trisphosphate (InsP_3) and diacylglycerol (DAG) (30,31). InsP_3 causes Ca^{2+} release from intracellular stores (32,33) and can also mobilize Ca^{2+} secondarily through Ca^{2+} -sensitive or store-dependent mechanisms (34,35). DAG, via activation of protein kinase C, phosphorylates various proteins (36) and can directly activate nonselective cationic channels (37,38). Besides M_3 receptors, smooth muscle tissues also express M_2 receptors. Electrophysiological studies have identified M_2 signaling pathway involving G-mediated activation of smooth muscle cationic channels (39-43). The opening of the cationic channels results in depolarization and the activation of voltage-dependent Ca^{2+} channels (VDCCs), which admit Ca^{2+} into the cell.

Loperamide (used as the standard drug) significantly reduced spasm induced by KCl, ACh and EFS. The inhibitory effect of loperamide is mediated via opioid receptors which exist on both the neurons and the smooth muscles of the gut (44). Loperamide is an opioid that acts on presynaptic μ -receptor located on cholinergic nerve terminal of gut and thereby, inhibits the gut motility and reduces electrolyte and water secretion (45,46). Opioids delay gastric emptying through acting on GI sphincters (45). Stimulation of opioid receptors on the smooth muscle indirectly results in voltage gated Ca^{2+} channels inactivation (46) and that can explain the inhibitory effect of loperamide on KCl induced contraction.

Hydroalcoholic extract of Lavender in a concentration dependent manner inhibited rat ileum contraction induced by neuronal stimulation (EFS), ACh and KCl. Comparison of the pattern of concentration-response curve with loperamide shows a substantial similarity and this may indicate that the inhibitory effect is due to presence of potent ingredient(s) in the extract. Several active constituents have been identified in Lavender extract including rosmarinic acid, luteolin, and apigenin (8). Effect of apigenin on GI disorders such as irritable bowel syndrome, diarrhea and intestinal motility has been reported. Apigenin has antidiarrheal activity and inhibits intestinal peristaltic movement and delays charcoal meal transit (47). Furthermore, apigenin profoundly reduces

the inflammation due to acetic acid induced colitis (48). It has been reported that Lavender extract also possessed anti-inflammatory activities (10) and we have provided scientific evidence for antispasmodic action of Lavender extract. As apigenin has both anti-inflammatory and antispasmodic activities, it should have a significant contribution to antispasmodic action of Lavender extract. Nevertheless, it is unlikely that apigenin is the only active component in the extract. Luteolin has similar structure with that of apigenin and therefore, luteolin as well as other constituents may have contribution in the antispasmodic effect of Lavender extract. As Lavender extract inhibited both ACh and KCl induced contraction, it is possible that there are several active components, some of them may antagonize muscarinic receptors and others act as Ca^{2+} channel blocker which inhibits KCl action. However, as the inhibitory concentration of Lavender extract on ileum contractions induced by ACh, KCl and neuronal stimulation (EFS) are the same. This might indicate that a unique intracellular mechanism is involved which need to be further investigated. Nevertheless, it has been shown that essential oil of Lavender has spasmolytic effects on rat isolated ileum and uterus smooth muscle (19). The antispasmodic actions of lavender were potentiated by a phosphodiesterase inhibitor, suggesting that lavender's effects are mediated via cAMP signaling system (19,22).

Conclusion

We have demonstrated the antispasmodic action of Lavender on isolated rat ileum. The antispasmodic activity of Lavender extract is seen with a concentration similar to that of loperamide. Therefore, isolation and identification of the active ingredients with antispasmodic activity is recommended.

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Authors' contributions

GA was responsible for preparation of extracts while HS supervised the pharmacological studies. MR was responsible for performing the experimental work. All contribute in preparation of the article and confirmed final edition for publication.

Conflict of interests

Authors declare that there is not any conflict of interest.

Ethical considerations

Ethical issues have been observed by the authors.

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