



International Journal of Pharmacology

ISSN 1811-7775

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The Spasmogenic and Spasmolytic Activities of *Lavandula stoechas* are Mediated Through Muscarinic Receptor Stimulation and Calcium Channel Blockade

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Abstract: The crude extract of *Lavandula stoechas* (Ls.Cr) and its fractions were studied *in vitro* for the possible presence of spasmogenic and spasmolytic constituents to rationalize some of the traditional uses. Ls.Cr (1-10 mg mL⁻¹) caused atropine-sensitive spasmogenic effect in guinea pig ileum. In spontaneously contracting rabbit jejunum, Ls.Cr (0.03-1 mg mL⁻¹) caused a transient spasmogenicity followed by relaxation at higher doses. Ls.Cr also relaxed high K⁺- induced contractions at the similar dose range (0.03-1 mg mL⁻¹), which suggests that the spasmolytic effect is mediated through Calcium Channel Blockade (CCB). The CCB effect was confirmed when pretreatment of the tissue with Ls.Cr produced a dose-dependent shift in the Ca⁺⁺ dose-response curves to the right, similar to that produced by verapamil. Activity-directed fractionation revealed that the spasmolytic effect is concentrated in the petroleum fraction while the spasmogenic effect is more evident in the aqueous fraction. These data indicate the presence of both spasmogenic and spasmolytic components mediated through muscarinic receptor activation and calcium channel blockade, respectively. This study also resolves the controversial results obtained from earlier studies and may explain some of its medicinal uses in gut disorders, like constipation and spasm.

Key words: *Lavandula stoechas*, spasmogenic, spasmolytic, calcium antagonist, muscarinic stimulation

INTRODUCTION

Lavandula stoechas (Fam. Labiatae) is an evergreen plant locally known as Ustu-khuddoos which is indigenous from Arabic and Mediterranean coasts to Asia Minor (Nadkarni, 1976). The flowers and aerial parts of the plant possess medicinal value. Traditionally, *Lavandula stoechas* has been used for the treatment of various diseases such as flatulence, colic, chest affections, nervous headaches, (Usmanghami *et al.*, 1997). *Lavandula stoechas* has been found to possess diaphoretic, expectorant, antispasmodic, antiphlogistic and emmenagogue properties (Nadkarni, 1976). The oil is extensively used in aromatherapy as a holistic relaxant (Evans, 1996). The plant is also being used traditionally for mild constipation.

L. stoechas has been extensively studied phytochemically, with limited work on pharmacological aspects. It contains 0.5-1.2% of volatile oil (Sharma *et al.*, 1983), oleanolic acid, ursolic acid, vergatic acid, longipinene derivatives (Ulubelen *et al.*, 1988), sterols,

β -sitosterol, α -amyrine, α -amyrine acetate, lupeol, erythroidiol and flavonoids luteolin, acacetin and vitexin (Ulubelen and Olcay, 1989). The essential oil also known as lavender oil, is obtained by distillation from the flowers and contains more than 51 compounds, the important of which are borneol, linalol, linalyl acetate, geraniol, cineol, fenchone, α -pinene, pinocarvyl acetate, camphor, eucalyptol, myrthenol (Kokkalou, 1988) and myrthenyl acetate (Evans, 1996). A smooth muscle relaxant principle, 7-methoxycoumarin, has also been isolated (Manzoor-I-Khuda and Khan, 1969).

Lavandula oil has been reported to have antispasmodic activities in guinea pig ileum (Izzo *et al.*, 1996) but the exact mechanism of action was not established. In another study, it was shown that the spasmolytic activity of *Lavandula* oil in gut preparations is often preceded by an initial spasmogenesis (Lis-Balchin *et al.*, 1996). Similarly, the *in vivo* experiments in dogs showed that the oil diluted in water, when infused through the gut, caused an increase in tone and rhythmic contractions as well as peristalsis (Plant, 1920; Plant and

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Miller, 1926). In our preliminary study on gut preparation (Gilani *et al.*, 2000), we observed that the methanolic extract possessed antispasmodic and anticonvulsant activities. However, there is no other study available in the literature explaining the exact nature of spasmogenic and/or spasmolytic activities. In this study we report that the spasmogenic and spasmolytic activities of *Lavandula stoechas* in isolated gut preparations are mediated through stimulation of muscarinic receptors and calcium channel blockade, respectively. The activity-directed fractionation revealed that the spasmogenic activity is separated in the aqueous fraction while the spasmolytic activity is concentrated in the petroleum fraction.

MATERIALS AND METHODS

Plant material and preparation of crude extract: The dried flowers of *Lavandula stoechas* (500 g) were purchased from a local market in Judia Bazar, Karachi and were cleaned off from soil and adulterant materials like flower stalks. A sample of the *Lavandula stoechas* was deposited at the herbarium of the Natural Products Research Unit at the Department of Biological and Biomedical Sciences, Aga Khan University, Karachi, Pakistan.

The plant material was ground to course powder, soaked in 70% aqueous methanol at room temperature for three days with occasional shaking and filtered. The procedure of soaking and filtration was repeated twice. The combined filtrate was dried through evaporation on Rotary Evaporator under reduced pressure to a thick, semi-solid pasty mass of dark brown color; i.e., the crude extract (Ls.Cr) with approximately 19% yield. Ls.Cr was completely solubilized in both saline and distilled water for use in *in vivo* and *in vitro* experiments, respectively.

Bioassay guided fractionation of Ls.Cr: The crude extract of *Lavandula stoechas* (Ls.Cr, 64.45 g) was dissolved in 800 mL of distilled water, and successively extracted with petroleum spirit, chloroform and ethyl acetate using solvent-solvent extraction method (Williamson *et al.*, 1998). The procedure is shown in Fig. 1. In summary, the aqueous solution of crude extract was shaken vigorously with petroleum spirit in a separating funnel. The mixture was allowed to separate into two layers. The Petroleum spirit layer (upper) was removed. The extraction with Petroleum spirit was repeated twice. All the Petroleum spirit fractions were combined and evaporated on Rotary Evaporator i.e., the Petroleum fraction (Ls.Pet).

Similarly the procedure was successively repeated with two other solvents i.e., Chloroform and ethyl acetate.

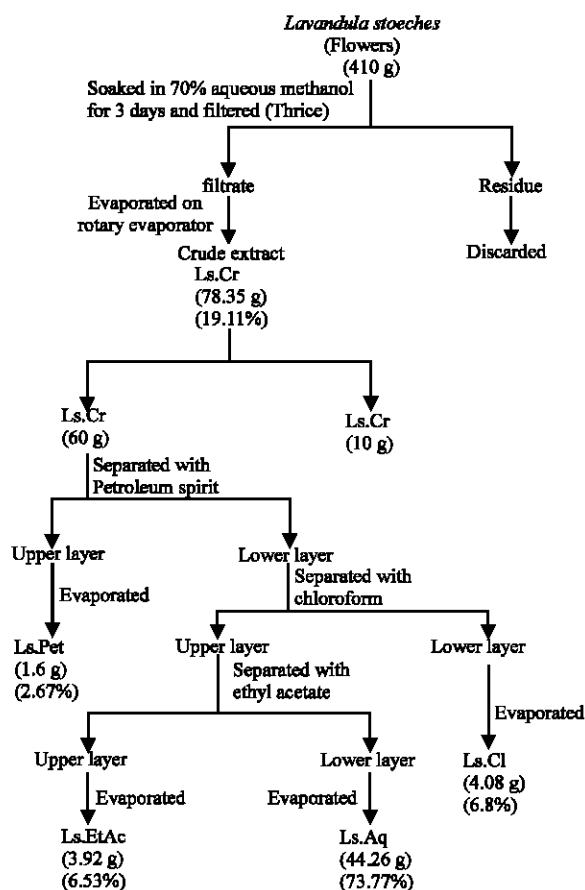


Fig. 1: Schematic diagram for the preparation of 70% aqueous- methanolic crude extract of *Lavandula stoechas* (Ls.Cr) and its fractionation into Petroleum (Ls.Pet), Chloroform (Ls.CHCl₃), Ethyl acetate (Ls. EtAc) and Aqueous (Ls.Aq.) fractions

The resultant fractions were named as Ls. CHCl₃ and Ls. EtAc fractions, respectively. While the residue left over named as Ls.Aq. fraction. The yields of individual fractions are given in the Fig. 1.

Drugs, standards and animals: The following reference chemicals were obtained from the sources specified: acetylcholine chloride, atropine sulphate, histamine dihydrochloride, pyrilamine maleate, verapamil hydrochloride (Sigma Chemical Company, St. Louis, MO, USA).

The following chemicals were used to make the physiological salt solutions: potassium chloride (Sigma Chemical Company, St. Louis, MO, USA), calcium chloride, glucose, magnesium chloride, magnesium sulphate, potassium dihydrogen phosphate, sodium bicarbonate, sodium chloride, sodium dihydrogen

phosphate, (E. Merck, Darmstadt, Germany) and ethylenediaminetetra-acetic acid (BDH Laboratory Supplies, Poole, England). Buffers and other chemicals were of extra pure analytical grade. All chemicals used were of the highest purity grade. Stock solutions of all the chemicals were made in distilled water and the dilutions were made fresh on the day of the experiment.

Isolated tissue experiments: The isolated tissue experiments were carried out according to the procedures laid down earlier (Gilani *et al.*, 2005a).

Guinea-pig Ileum and Rabbit Jejunum: Animal were starved for 24 h with free access to water and were sacrificed by cervical dislocation. The abdomen was cut open and a piece of ileum 10-20 cm long, about 15 cm proximal to the ileo-cecal end removed. The segment of ileum about 2 cm long was hanged in a 10 mL tissue bath containing Tyrode's solution, bubbled with a mixture of 95% oxygen and 5% carbon dioxide (carbogen gas) and maintained at 37°C. The composition of Tyrode's, 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). One end of the segment was attached to the metal tissue hook and the other was attached by a cotton thread to an isotonic transducer and connected to a Harvard Student Oscillograph. Under these conditions, guinea-pig ileum, behaves as a quiescent preparation and is considered more suitable to test spasmogenics (Gilani *et al.*, 2005b). A preload of 1 g was applied to each tissue and kept constant throughout the experiment. The tissue was washed several times within 5 min interval and was allowed to equilibrate for 30 min before isotonic contractions to a sub-maximal concentration (0.3 μM) of Ach were recorded. An agonist contact time of 20 sec, was used together with a 3 min interval between doses. Once the tissue was stabilized with reproducible effects from the doses of the standard, test materials were tested. This same protocol was used for rabbit jejunum. The spontaneously contracting rabbit jejunum was mainly used to assess for spasmolytic activity. The plant materials were added in a cumulative fashion (Van Rossum, 1963) to obtain dose-response curves.

Determination of calcium antagonist activity: The calcium antagonist activity was assessed using isolated rabbit jejunum as described earlier (Taqvi *et al.*, 2006). The tissue was pre-contracted with a high dose of K⁺, 80 mM (Farre *et al.*, 1991). Following the plateau, the test material was introduced in a cumulative manner to obtain dose-dependent inhibitory curve (Van Rossum, 1963). This relaxant effect was expressed as per cent of the control

response exhibited by K⁺. The Ca⁺⁺ antagonist activity of the plant material was confirmed by the protocol described below. After stabilization of a tissue in normal Tyrode's solution, Ca⁺⁺ was removed from the tissue by replacing this solution with a Ca⁺⁺-free Tyrode's solution containing ethylenediaminetetra-acetic acid, EDTA, (0.1 mM, 30 min) with the following composition: NaCl 136.9, KCl 2.68, MgCl₂ 0.49, NaH₂PO₄ 0.32, Glucose 5.05, NaHCO₃ 11.9 and EDTA 0.1. Later, this was also replaced by Ca⁺⁺-free and K⁺-rich Tyrode's solution with composition (mM): KCl 50, NaCl 91.04, MgCl₂ 1.05, NaHCO₃ 11.87, NaH₂PO₄ 0.41, Glucose 5.55 and EDTA 0.1. Following an incubation period of 30 min, control Concentration-Response Curves (CRCs) of Ca⁺⁺ were obtained. When the control CRCs of Ca⁺⁺ were found super-impossible (usually after two cycles), the tissue was pretreated with the plant extract or verapamil for 60 min to test CCB effect. The CRCs of Ca⁺⁺ were constructed in the presence of different concentrations of the test material.

Data analysis: All data expressed are mean±standard error of the Mean. The significant differences between two groups and more than two groups were analyzed using student's t-test and one-way ANOVA, respectively. The p-values<0.05 noted as significantly different.

RESULTS AND DISCUSSION

In isolated guinea pig ileum Ls.Cr caused a concentration-dependent spasmogenic effect at the dose range of 1-10 mg mL⁻¹ (Fig. 2). The maximum response achieved (57.1±3.57, n = 3) was significantly less than that of acetylcholine maximum (p<0.01) (Fig. 2), which indicates that either the plant extract has a partial agonistic activity or the spasmogenic effect is accompanied by a spasmolytic effect.

We previously observed that the spasmogenic effect in the plant is usually mediated through acetylcholine like activity (Bashir *et al.*, 2006; Gilani and Attau-Rehman 2005; Gilani *et al.*, 2000, 2006) To see whether the contractile effect of the crude extract was also mediated through an acetylcholine-like mechanism, the tissue was pretreated with atropine (0.1 μM), an antimuscarinic agent (Arunlakhshana and Schild, 1959). This treatment abolished the spasmogenic effect of the crude extract, similar to that of acetylcholine, while the stimulatory effect of histamine remained unchanged, suggesting that the stimulatory effect is specifically mediated through acetylcholine like mechanism. This cholinergic effect was further confirmed when pretreatment of the tissues with pyrilamine, a histamine (H₁) receptor blocker

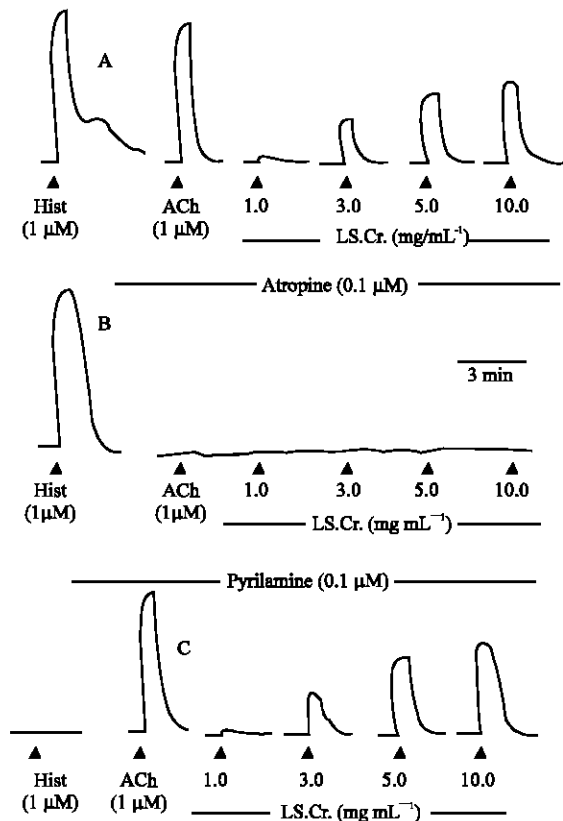


Fig. 2: Effect of Ls. Cr on Guinea pig ileum the absence A and presence of atropine B and Pyrilamine C

(Sharif *et al.*, 1994), did not alter the response of the plant extract or Ach, while completely blocked the effect of histamine as expected (Fig. 2). Acetylcholine is a neurotransmitter released by the parasympathetic nervous system, mediates its action in the gut by stimulation of M₃ receptor subtypes and atropine blocks all muscarinic receptor sites (Brown and Taylor, 2001). Through this mechanism, acetylcholine plays important physiological role to regulate the peristaltic movements of the gut (Brown and Taylor, 1996).

When tested on hyperactive smooth muscle preparation (spontaneously contracting rabbit jejunum), the spasmogenic effect was less marked, followed by a spasmolytic effect at higher concentrations. The spasmogenic component was not studied further in this preparation as the effect was neither dose-dependent, nor reproducible and disappeared after repeated administration of the plant extract. In our previous study we reported that the spasmolytic effect of Ls.Cr was usually mediated through calcium channel blocker(s)-like activity (Gilani *et al.*, 2000). When Ls.Cr was tested for its possible Calcium antagonist effect, it caused dose-

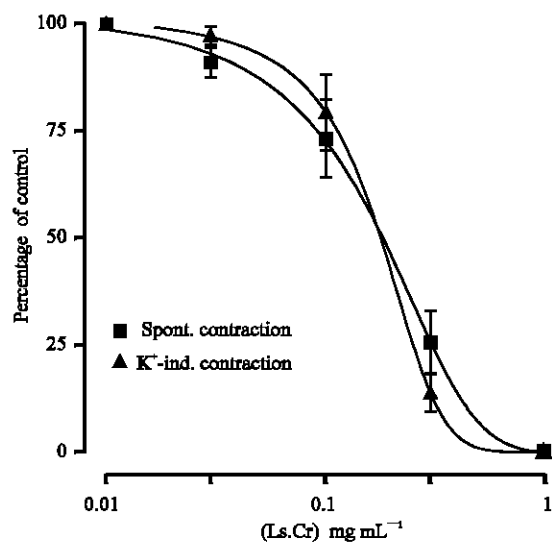


Fig. 3: Dose-dependent inhibitory effect of the crude extract of *Lavandula stoechas* (Ls.Cr) on spontaneous and K⁺- induced contractions in rabbit jejunum. The values shown are mean±SEM of four determinations

dependent relaxation of high K⁺(80 mM)-induced contractions in rabbit jejunum (Fig. 3). Contractions induced by high K⁺ depends upon ingress of Ca⁺⁺ into the cells through voltage-dependent calcium channels (Bolton, 1979; Van Breeman *et al.*, 1979). Sufficient calcium ions enter through these membrane channels to activate intracellular contractile proteins and cause smooth muscle contraction (Adelstein and Eisenberg, 1980) and high K⁺ depolarization-induced contractions only utilize Ca⁺⁺ influx (Loutzenhiser *et al.*, 1985; Chiu *et al.*, 1986). Calcium Channel Blockers (CCBs) such as diltiazem, nifedipine and verapamil interfere with smooth muscle contractions by binding to voltage-sensitive calcium channels in a way that prevents depolarization-induced Ca⁺⁺ influx (Fleckenstein, 1977). Thus the inhibition of high K⁺-induced contraction of smooth muscle by the plant extract may be visualized as an outcome of restricted Ca⁺⁺ entry through voltage-dependent calcium channels. The CCB-like activity was confirmed when pretreatment of the tissue with Ls.Cr caused right-ward shift in the Ca⁺⁺ curves in a dose-dependent manner (Fig. 4). Verapamil, a standard calcium channel blocker (Hamilton *et al.*, 1986) also produced similar shift in the Ca⁺⁺ curves as expected (Fig. 4).

In order to separate constituents responsible for spasmogenic and spasmolytic activities, activity-directed fractionation was carried out using different solvents (Fig. 1). Following fractions were obtained, petroleum

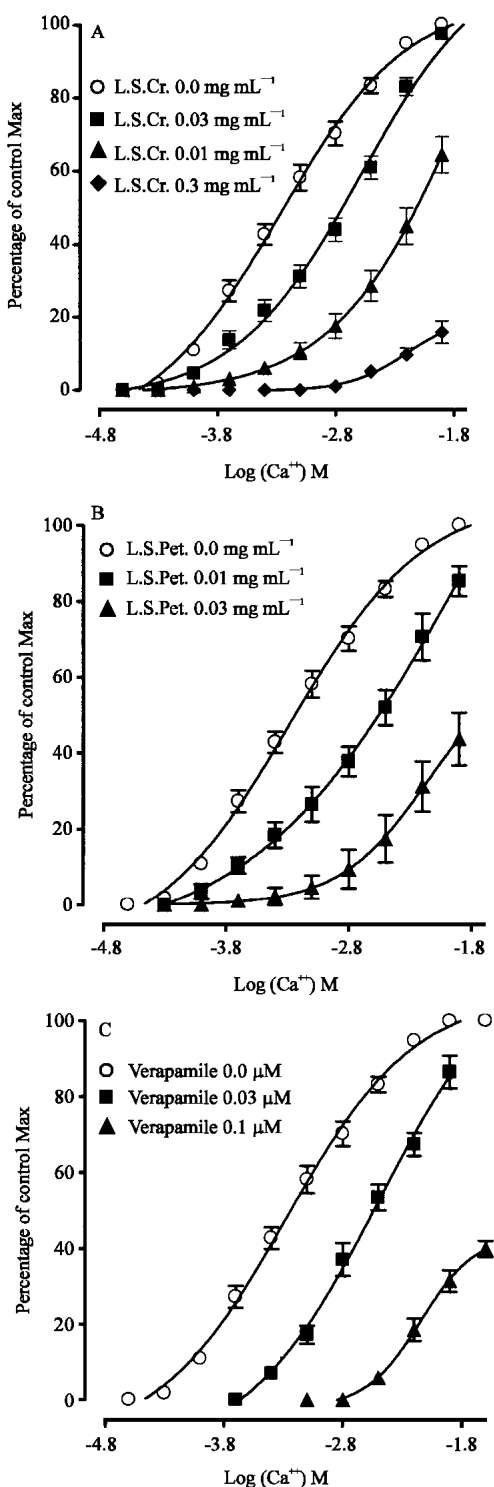


Fig. 4: Effect of increasing doses of (A) *Ls.Cr* (n = 4), (B) *Ls.Pet* (n = 3) or (C) Verapamil (n = 7) on Ca²⁺ dose-response curves constructed in Ca²⁺-free and K⁺-rich (80 mM) medium in isolated rabbit jejunum (values shown are mean±SEM)

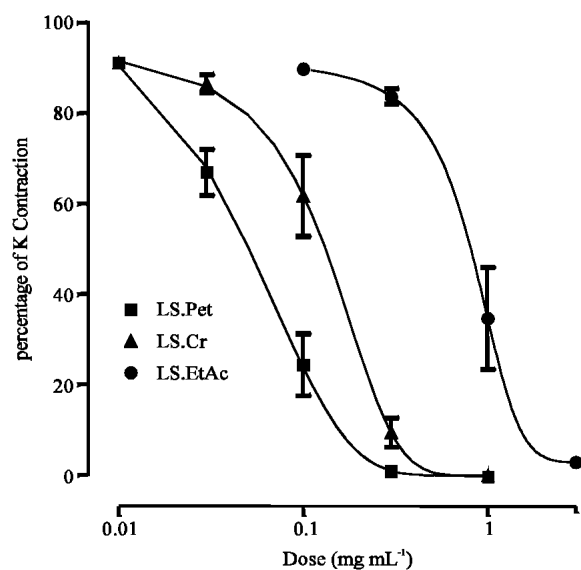


Fig. 5: Comparison of dose-dependent inhibitory effects of *Lavandula stoechas* crude extract (*Ls. Cr.*) and its petroleum (*Ls.Pet*) and ethyl acetate (*Ls.EtAc*) fractions on K⁺- induced contractions in rabbit jejunum. The values shown are mean±SEM of four determinations

fraction (*Ls.Pet*), chloroform fraction (*Ls.CHCl₃*) and ethyl acetate fraction (*Ls.EtAc*) and aqueous fraction (*Ls.Aq.*). The chloroform fraction was insoluble in water and other permitted solvents for assays hence excluded from further studies.

The CCB-like activity was separated in the petroleum and ethyl acetate fractions (*Ls.Pet* and *Ls.EtAc*) as tested against high K⁺-induced contractions in rabbit jejunum. The comparative dose-response curves of different fractions are shown in Fig. 5. Both *Ls.Pet* and *Ls.EtAc* were found to relax spontaneous and K⁺-induced contractions in rabbit jejunum. The comparative IC₅₀ values of the crude plant extract (*Ls.Cr*) and its resultant petroleum ether (*Ls.Pet*) and ethyl acetate (*Ls.EtAc*) fractions were found to be 0.19±0.04; 0.1±0.03; 0.36±0.08 and against high K⁺-induced contractions (0.18±0.02; 0.07±0.01; 0.92±0.14), respectively. *Ls.Pet* was the most potent fraction of the crude extract. *Ls.Pet* caused dose-dependent (0.01-0.03 mg mL⁻¹) relaxation of spontaneous contractions as well as high K⁺-induced contractions. The comparative calcium curves of *Ls.Cr* and *Ls.Pet* are shown in Fig. 3. Furthermore, this fraction was devoid of acetylcholine like activity (data not shown). However, the cholinergic constituents were separated in the aqueous fraction. This fraction was more potent and efficacious in its cholinergic activity than the parent crude extract and was devoid of CCB like activity. The comparative curves are shown in Fig. 6.

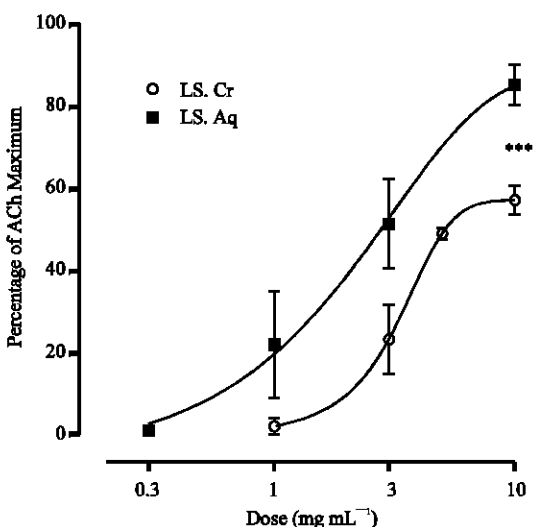


Fig. 6: The comparative stimulatory effects of the crude extract of *Lavandula stoechas* (Ls.Cr) and its aqueous fraction (LS.Aq) in guinea-pig ileum. The values shown are mean±SEM of 3-6 determinations (***) $p < 0.001$

In summary, *Lavandula stoechas* (Ls. Cr) exhibits both spasmogenic and spasmolytic activities, mediated through cholinergic and CCB like mechanisms, respectively. In bioassay-guided fractionation, CCB like activity was concentrated in LS.Pet fraction while cholinergic activity in the aqueous fraction. This study resolves some contradictory results reported earlier and may explain the medicinal use of the plant in the gut disorders like, constipation and spasm.

ACKNOWLEDGMENT

This study was supported in part by Higher Education Commission, Government of Pakistan through Ph.D. scholarship to Ms. Qaiser Jbeen was on study leave from Islania University Bahawalpur.

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