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Mentha longifolia lowers blood pressure in anesthetized rats through multiple pathways

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Abstract

This study was aimed to investigate the effect of the extract of *Mentha longifolia* on blood pressure and the possible mechanisms. In anesthetized rats, the crude extract of *M. longifolia* and aqueous and chloroform fractions caused a dose-dependent fall in mean arterial pressure. Atropine pretreatment abolished the effect of extract and aqueous fraction but did not change that of chloroform fraction. In rabbit aortic rings, crude extract relaxed phenylephrine (1 μM) and high K^+ (80 mM) pre-contractions. Chloroform fraction was more potent against high K^+, similar to verapamil and caused a rightward shift in the Ca^{++} concentration-response curves. Aqueous fraction partially relaxed high K^+ pre-contractions. In rat aortic rings, crude extract and aqueous fraction-induced endothelium-dependent atropine-sensitive vasodilator effect. Extract and fractions also relaxed high K^+ precontractions. In guinea pig atrial strips, crude extract and chloroform fraction suppressed force and rate of contractions, similar to verapamil. In conclusion, *M. longifolia* lowers blood pressure through Ca^{++} channel blockade and atropine-sensitive-NO pathway.

Introduction

Mentha longifolia L., (family; Labiatae), is an aromatic perennial herb, commonly known as "wild mint", found in the Northern areas of Pakistan (Baquar, 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 system (Lewis and Elvin-Lewis, 1977) and has been used in heart diseases (Duke, 1997). Additionally, the plant has a reputation for its medical use in other conditions, such as diarrhea, gut spasm (Amini, 1997), indigestion and flatulence (Watt and Breyer-Brandwijk, 1962; Duke, 2002). *M. longifolia* has been reported for diverse biological activities, such as antimicrobial and anti-oxidant (Mimica-Duki et al., 1999), antimycotic (Abou-Jawdah et al., 2001), antiHIV (Amzazi et al., 2003), antiulcer (Gul et al., 2015), anthelmintic (Kozan et al., 2006), antipyretic (Amabeoku et al., 2009), anti-cancer (Sharma et al., 2014) and antidiarrheal (Jalilzadeh-Amin and Maham, 2015). The plant has been traditionally used in the management of heart diseases but current literature lacks scientific evidence for its use in these conditions.

The present investigation was carried out to explore blood pressure lowering effect of the crude extract and the aqueous and chloroform fractions of *M. longifolia* and its possible mechanisms using the *in v*ivo and *in vitro* pharmacological protocols.

Materials and Methods

Plant material

Fresh leaves of *M. longifolia* (3 kg) were collected in the

Swat District, Khyber Pukhtoon Khwa (KPK), Pakistan. It was authenticated by Mr. Mehboob-ur-Rehman, Department of Botany, Government Postgraduate Jehanzeb College, Saidu Sharif, Swat, KPK, Pakistan. A voucher specimen (Ml-L-08-04) has been deposited at the herbarium located at the Department of Biological and Biomedical Sciences, Aga Khan University, Karachi, Pakistan.

Preparation of crude extract and fractionations

Plant material was shade dried and coarsely grounded, soaked in 70% aqueous-methanol for three days and filtered through a filter paper (Shah et al., 2011; Shah and Gilani, 2011; Khan et al., 2013). The procedure was repeated thrice and the combined filtrate was evaporated in a rotary evaporator at 37°C under reduced pressure (-760 mm Hg) to a thick, semi-solid mass of dark color, i.e. the crude extract, yielding approximately 38% (w/w). The crude extract was soluble in normal saline.

Activity-directed fractionation was carried out as described (Williamson and Okpako, 1998). A known quantity (80 g) of the extract was dissolved in distilled water, added an equal volume of chloroform, shaken vigorously and allowed for layer separation. The upper layer, chloroform, was acquired and the same procedure was repeated twice more. Combined filtrate was concentrated in a rotary evaporator for getting the chloroform fraction. The remaining layer was evaporated and the resultant fraction was considered as an aqueous fraction. The yield of the chloroform and aqueous fractions was 28.6% (w/w) and 40% (w/w), respectively.

Drugs and standards

Drugs used in this study were purchased from the source specified: Pentothal sodium (Abbott Laboratories, Pakistan), acetylcholine chloride, norepinephrine bitartrate, atropine sulfate, phenylephrine hydrochloride, DMSO, potassium chloride, isoprenaline hydrochloride, verapamil hydrochloride, and Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME) (Sigma chemicals company, USA). All chemicals used were of the highest purity grade available. Stock solutions of all the chemicals were made in normal saline and distilled water. Dilutions were made fresh on the day of the experiment.

Animals

Sprague-Dawley rats (180-200 g), local breed rabbits (1.5-2 kg) and guinea-pigs (350-550 g) of either sex used in the study were bred and housed in the animal house of Aga Khan University in a controlled environment (23 -25°C). Experiments performed complied with the rulings of the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council (National Research Council, 1996). Animals were given tap water *ad libitum* and a standard diet.

Effect on blood pressure in anesthetized rats

As described previously (Gilani et al., 2007), adult Sprague-Dawley rats were anesthetized with sodium thiopental (90-100 mg/kg, i.p.). The trachea was cannulated with a polyethylene tubing (PE-20) to facilitate spontaneous respiration. The arterial pressure (AP) was recorded from the right common carotid artery via an arterial cannula (PE-50) connected to a pressure transducer and PowerLab (ML845) data acquisition system (ADInstruments, Australia). After 20 min of cannulation, control responses of standards, such as acetylcholine (ACh; 1 mg/kg) and norepinephrine (NE; 1 mg/kg) were obtained by intravenous injection into the left jugular vein before testing the effect of the extract. Each intravenous injection was followed by a flush of 0.1 mL saline.

Some anesthetized rats were pretreated with atropine (2 mg/kg) for 20 min. In the atropine-treated rats, the effect of the extract on mean arterial pressure was determined and compared with untreated. Mean arterial pressure was calculated from the sum of diastolic blood pressure plus one-third pulse width and changes in mean arterial pressure were expressed as percent of control (percent fall in mean arterial pressure).

Determination of vasodilator effects

Rabbit thoracic aorta

Rabbit aortic rings (2-3 mm) were mounted in 10 mL tissue bath filled with normal Kreb's solution (37°C) and continuously bubbled with carbogen (5% CO_2 in O_2) as described earlier (Gilani et al., 2005). The composition of normal Kreb's solution was (mM); NaCl 118.2, KCl 4.7, $NaHCO_3$ 25.0, $CaCl_2$ 2.5, KH_2PO_4 1.3, $MgSO_4$ 1.2, and glucose 11.7. A preload of 2 g was applied to each tissue and a pre-incubation period of 1 hour was allowed before studying the effect of test materials. Changes in isometric tension of the rings were measured through a force transducer (Fort-100, WPI, UK), coupled with a bridge amplifier (Transbridge TBM4) and PowerLab (ML845) data acquisition system (ADInstruments, Australia).

Phenylephrine (1 μM) or high K^+ (80 mM) were used to induce steady contractions and cumulative concentration-response curves (CRCs) of crude extract and fractions were determined for possible vasodilator effect. The inhibitory effect was expressed as percent of the induced contractions.

In a separate set of experiments, an attempt was made to see, if the relaxation induced by the test materials involve Ca^{++} influx via voltage-dependent Ca^{++} channels (VDCs). An indirect approach was followed (Taqvi et al., 2008; Shah and Gilani, 2009); accordingly,

aortic rings were washed few times with Ca^{++}-free solution before the construction of control CRCs of Ca^{++} (as $CaCl_2$). When the control CRCs of Ca++ were found superimposable, then tissue was pretreated with the test material for 30 min to test for possible Ca^{++} channels blocking effect. A parallel control was also run using saline under similar experimental conditions.

Rat aorta

Rat thoracic aorta was dissected out and 2-3 mm rings were suspended in a 10 mL tissue bath as described previously (Furchgott and Zawadaski, 1980; Shah and Gilani, 2009). In some rings, the endothelium was deliberately removed by gentle rubbing of the intimal surface with forceps. Two stainless-steel hooks were passed through the lumen of each ring, one hook was anchored to a steel rod at the bottom and the other was attached to a force transducer (Fort-100, WPI, UK). A preload of 1 g was applied to each preparation. The aortic rings with intact endothelium that produced less than 50 % relaxation in response to acetylcholine (1 µM) were discarded. Individual rings were mounted in 10 mL tissues baths at 37°C, bubbled with carbogen. Changes in isometric tension were recorded through a force transducer (Fort-100, WPI, UK), coupled with bridge amplifier (Transbridge TBM4) and PowerLab (ML845) data acquisition system (ADInstruments, Australia).

Endothelium-dependent vasorelaxation

As described previously (Shah and Gilani, 2009; Karaki and Weiss, 1988) when the phenylephrine (1 µM)-induced tension reached a plateau, the test materials were cumulatively added to the organ baths and CRCs were constructed. The rings with intact and denuded endothelium were always tested in parallel.

The nature of vascular relaxation was further studied; endothelium-intact rings were incubated with L-NAME (10 µM) and atropine (1 µM) for 30 min, extract and fractions were added cumulatively to the PE (1 µM)-induced contractions. CRCs were compared before and after treatment.

For the determination of possible effect on the VDCs, high K^+ (80 mM) was used, which produced sustained contractions (Ajay et al., 2003). Crude extract and fractions were added cumulatively and relaxation was expressed as percent of the induced contraction.

Effect on cardiac contractility

Guinea pig atria

Spontaneously beating paired or right atria were dissected out from guinea-pigs (350-550 g), and mounted in 20 mL tissue baths filled with normal Kreb's solution at 32°C and aerated with carbogen gas as described previously (Gilani et al., 2005; Shah and Gilani, 2009). A preload of 1 g was applied and control responses of ACh (1 µM) and isoproterenol (1 µM) were obtained at least in duplicate. Tension changes in the tissue were recorded through a Grass force-displacement transducer (model FT-03) using Grass model 7 Polygraph.

Statistics

All the data expressed are mean ± standard error of the mean (SEM), and the median effective concentrations (EC50 values) are given with 95% confidence intervals (CI). The statistical analysis was performed by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls multiple comparison tests when a significant difference was present. The $p<0.05$ was noted as significantly different.

Results

Effect on blood pressure in anesthetized rats

In normotensive rats under anesthesia, norepinephrine and acetylcholine were used as standards, which produced rise and fall in arterial pressure, respectively (Figure 1A). The crude extract of *M. longifolia* caused a dose-dependent fall in mean arterial pressure. The percent fall observed at doses (mg/kg) of 3, 10, 30 and 100 was; 9.7 ± 2.6, 18.5 ± 2.1, 32.0 ± 4.2, and 44.0 ± 5.9 mm Hg, respectively (Figure 1B). Rats pretreated with atropine (2 mg/kg), found resistant to the blood pressure lowering effect at lower doses (3 and 10 mg/kg), while the effect at higher doses was partially blocked ($p<0.001$). The percent fall observed at higher doses (30 and 100 mg/kg) was; 13.0 ± 1.5 and 20.3 ± 3.1 mm Hg, respectively (Figure 1B).

The aqueous fraction was found more efficacious in lowering mean arterial pressure than the parent crude extract or the chloroform fraction (Figure 1). Pre-treatment of rats with atropine (2 mg/kg) abolished the effect of the aqueous fraction ($p<0.001$) (Figure 1C). Chloroform fraction was the least potent anti-hypertensive and its effect was not affected by atropine pretreatment (Figure 1D).

Effect on isolated rabbit thoracic aorta

When tested against phenylephrine and high K^+-induced contractions, crude extract caused equipotent relaxation, with respective EC_{50} values of 1.20 (0.5-1.9) and 1.0 mg/mL (0.2-1.6), as shown in Figure 2A. Pre-incubation of aortic rings with crude extract (0.3-3 mg/mL) caused a rightward shift in the Ca^{++} CRCs (Figure 2B), constructed in Ca^{++}-free medium, similar to that of verapamil (Figure 2H). Unlike the parent crude extract, the aqueous fraction had no inhibitory effect on phenylephrine pre-contractions, though produced weak inhibition against high K^+ pre-contractions (Figure 2C).

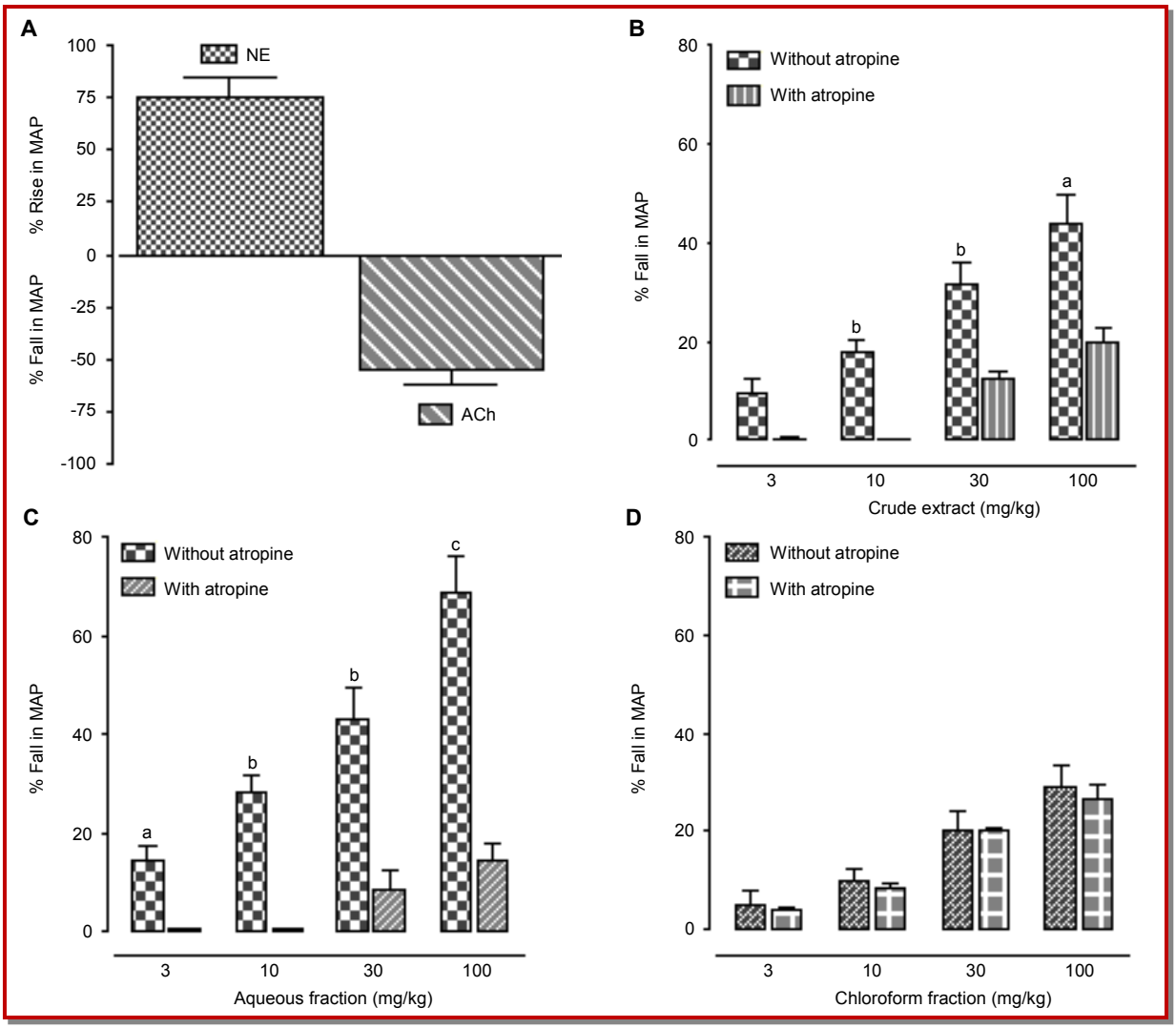

Figure 1: Effect of (A) norepinephrine (NE) and acetylcholine (ACh) on mean arterial pressure (MAP) in normotensive anesthetized rats. B, C and D show effect of the crude extract of *M. longifolia*, its aqueous and chloroform fractions, respectively, on MAP. Values shown are mean ± SEM (n = 5). [a]p<0.01, [b]p<0.001, [c]p<0.0001; compared with values obtained in the presence of atropine

Similarly, aqueous fraction had no significant effect on the Ca^{++} CRCs (Figure 2D).

The chloroform fraction was similar to the parent crude extract in inhibiting high K^+ pre-contractions but was less potent in inhibiting phenylephrine pre-contractions, with respective EC50 values of 1.9 (0.05-0.4) and 7.6 mg/mL (5.6-10.3) (Figure 2E). Pre-incubation of the aortic rings with chloroform fraction (0.3-3 mg/mL) also caused a rightward shift in the Ca^{++} CRCs (Figure 2F).

Effect on isolated rat thoracic aorta

The cumulative addition of crude extract (0.01-10 mg/mL) produced vascular relaxant effect on phenylephrine pre-contractions in aorta rings with intact endothelium. Incubation of the intact aortic rings with L-NAME (10 µM) and atropine (1 µM), significantly ($p<0.01$) inhibited the inhibitory effect of crude extract and shifted the CRCs to the right (Figure 3A).

Endothelium-dependent vasorelaxation

In aortic rings with denuded endothelium, the potency of crude extract for its inhibitory effect was reduced to around 10-fold (Figure 3A). The EC50 values in the intact and denuded endothelium were 0.2 (0.2-0.6) and 6.1 mg/mL (4.5-11.1), respectively. Chloroform fraction also produced inhibitory effect against high K^+ pre-contractions (Figure 3A).

Among the tested fractions, the aqueous fraction exhibited endothelium-dependent L-NAME/atropine-sensitive vasodilator effect (Figure 3B). Unlike the aqueous fraction, the chloroform fraction exhibited endothelium-independent vasodilator effect (Figure 3C), similar to that of verapamil (Figure 3D). The

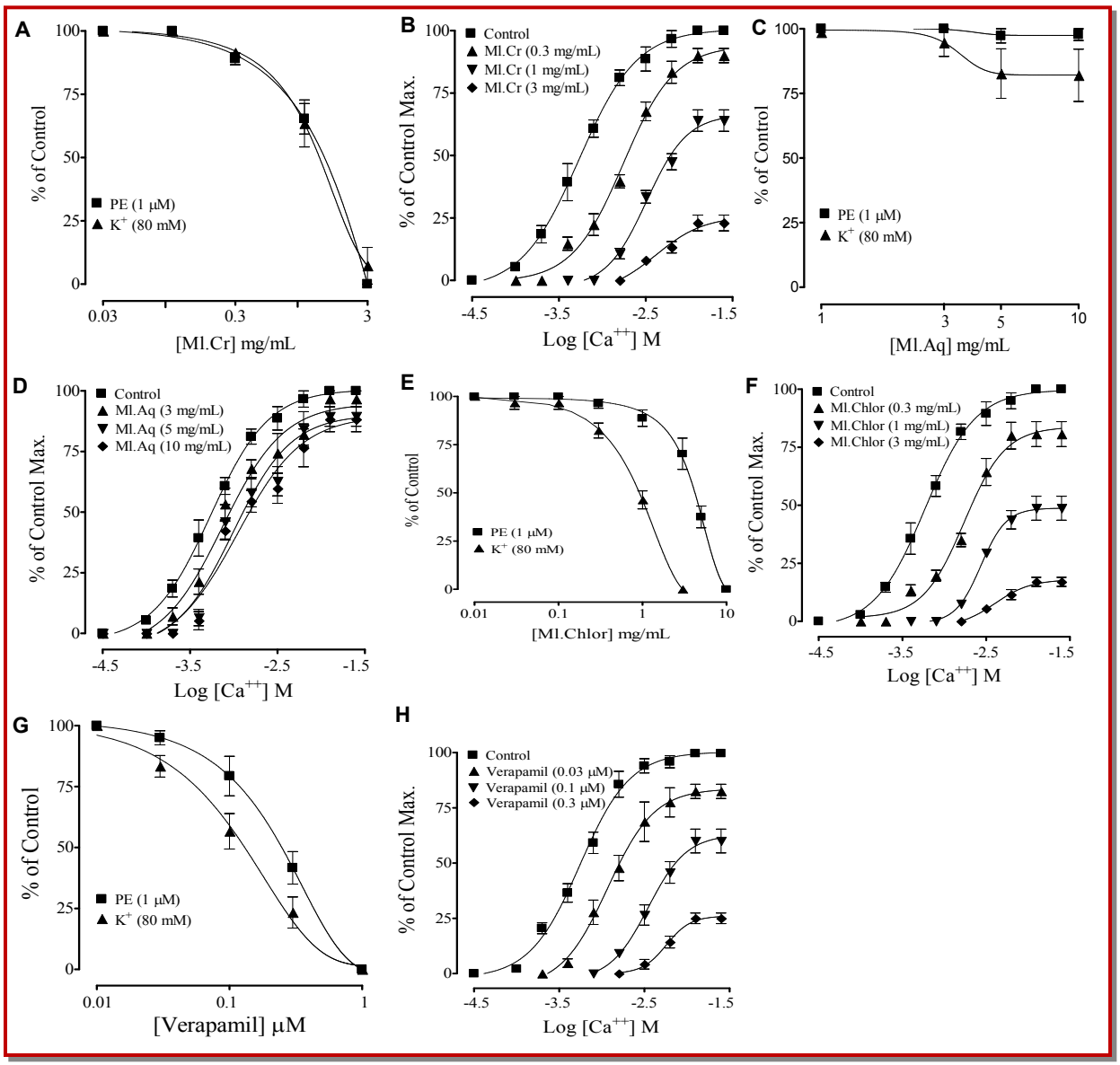

Figure 2: Concentration-dependent inhibitory effect against phenylephrine (PE) and high K^+ (80 mM)-induced vasoconstriction by the (A) crude extract of *M. alongifolia* (Ml.Cr); its (C) aqueous (Ml.Aq) and (E) chloroform (Ml.Chlor) fractions and (G) verapamil, in isolated rabbit aorta preparations. B, D, F and H show, respectively, the effect of Ml.Cr, Ml.Aq, Ml.Chlor and verapamil on the Ca^{++} concentration-response curves, constructed in Ca^{++} -free medium. Values shown are mean ± SEM of 6 determinations

aqueous fraction partially relaxed high K^+-induced contractions (Figure 3B), while the chloroform fraction caused complete relaxation, similar to the parent crude extract and verapamil (Figure 3D).

Effect on isolated guinea pig atria

In isolated guinea pig atrial preparations, crude extract caused inhibition of force and rate of spontaneous contractions at similar concentrations, with respective EC_{50} values of 6.9 (5.3-9.1) and 5.3 mg/mL (3.6-7.7) (Figure 4A).

Among the fractions tested, the aqueous fraction was least effective, which caused partial inhibition of force and rate of atrial contractions (Figure 4B). The chloroform fraction was similar to the parent crude extract with strong inhibition of both force and rate of contractions (Figure 4C), similar to that of verapamil (Figure 4D).

Discussion

M. longifolia has been used traditionally in heart diseases (Duke, 1997), but current literature lacks a scientific base of its use in heart diseases, such as

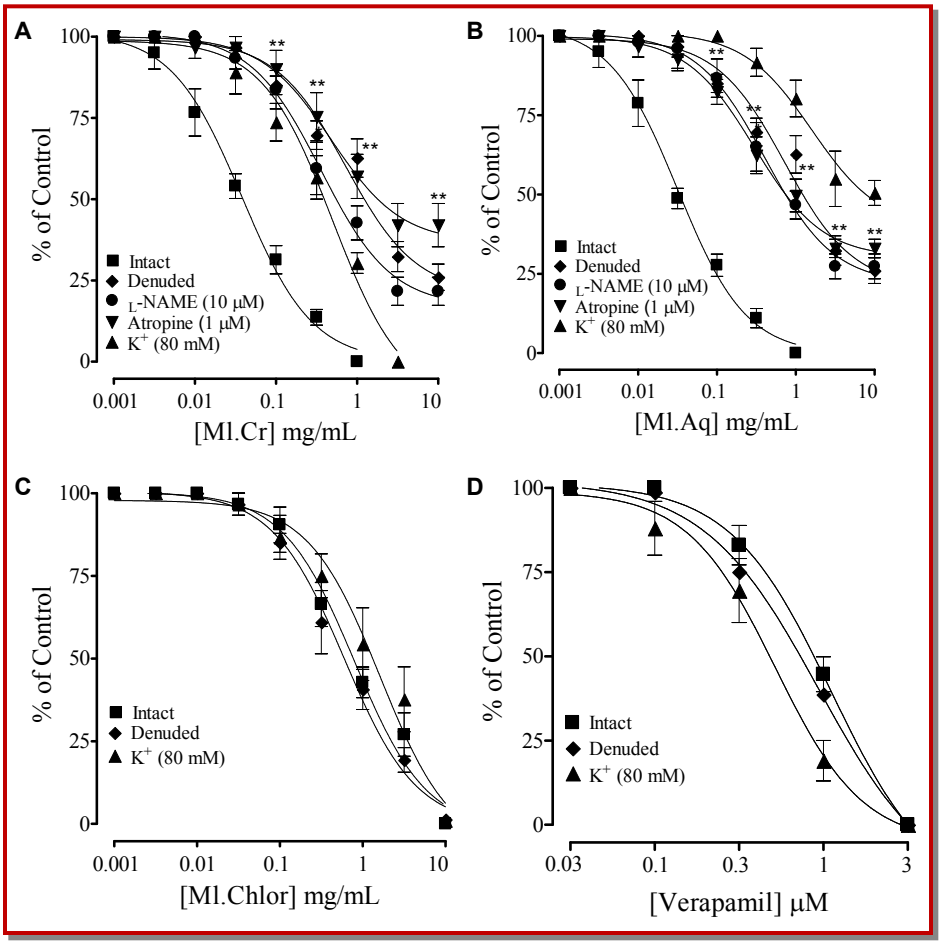

Figure 3. Vasodilator effect of the crude extract of *M. longifolia* (Ml.Cr), its fractions and verapamil in isolated rat aorta preparations. A and B depict endothelium-dependent (L-NAME/atropine-sensitive) and endothelium-independent vasodilator effect of Ml.Cr and itsaqueous (Ml.Aq) fraction on phenylephrine (PE) pre-contractions and also against high K^+ pre-contractions, in the intact aortic rings. C depicts endothelium-independent vasodilator effect of the chloroform fraction (Ml.Chlor) of *M. longifolia* against high K^+ and phenylephrine (PE) pre-contractions in the intact aortic rings. D depicts endothelium-independent vasodilator effect of verapamil against phenylephrine (PE) and high K^+ pre-contractions. Values shown are mean ± SEM of 7 determinations. $^{**}p<0.001$ compared with intact

hypertension. The crude extract of *M. longifolia* and its polar and non-polar fractions when tested for their blood pressure lowering effect, they caused a dose-dependent fall in mean arterial pressure in normotensive anesthetized rats. The aqueous fraction being the most effective, while the chloroform fraction was least effective.

We have previously observed that the blood pressure lowering effect of the crude extracts from different medicinal plants is mediated partly through the cholinergic pathway (Shah and Gilani, 2009; Shah and Gilani, 2011). To see, if the blood pressure lowering effect of the crude extract and fractions was mediated through an acetylcholine-like mechanism, rats were pretreated with atropine, a muscarinic receptor antagonist (Arunlakshana and Schild, 1959). This pretreatment abolished the blood pressure lowering effect of the crude extract and aqueous fraction at lower doses. While at higher doses their effects were partially abolished. The effect on blood pressure of the aqueous fraction was more sensitive to atropine pretreatment. However, this pretreatment did not affect the blood pressure lowering effect of the chloroform fraction. These data indicate that the crude extract contained atropine-sensitive blood pressure lowering constituent (s) concentrated in the aqueous fraction, while atropine insensitive separated into the chloroform fraction. This is in line with the general concept that the natural products, in their crude form contain effect enhancing property, by virtue of acting through multiple pathways (Gilani and Atta-ur-Rahman, 2005). The functional nature of blood pressure lowering effect of the crude extract and fractions was further studied using isolated tissue preparations.

In isolated rabbit aortic rings pre-contracted with phenylephrine and high K^+, cumulative addition of

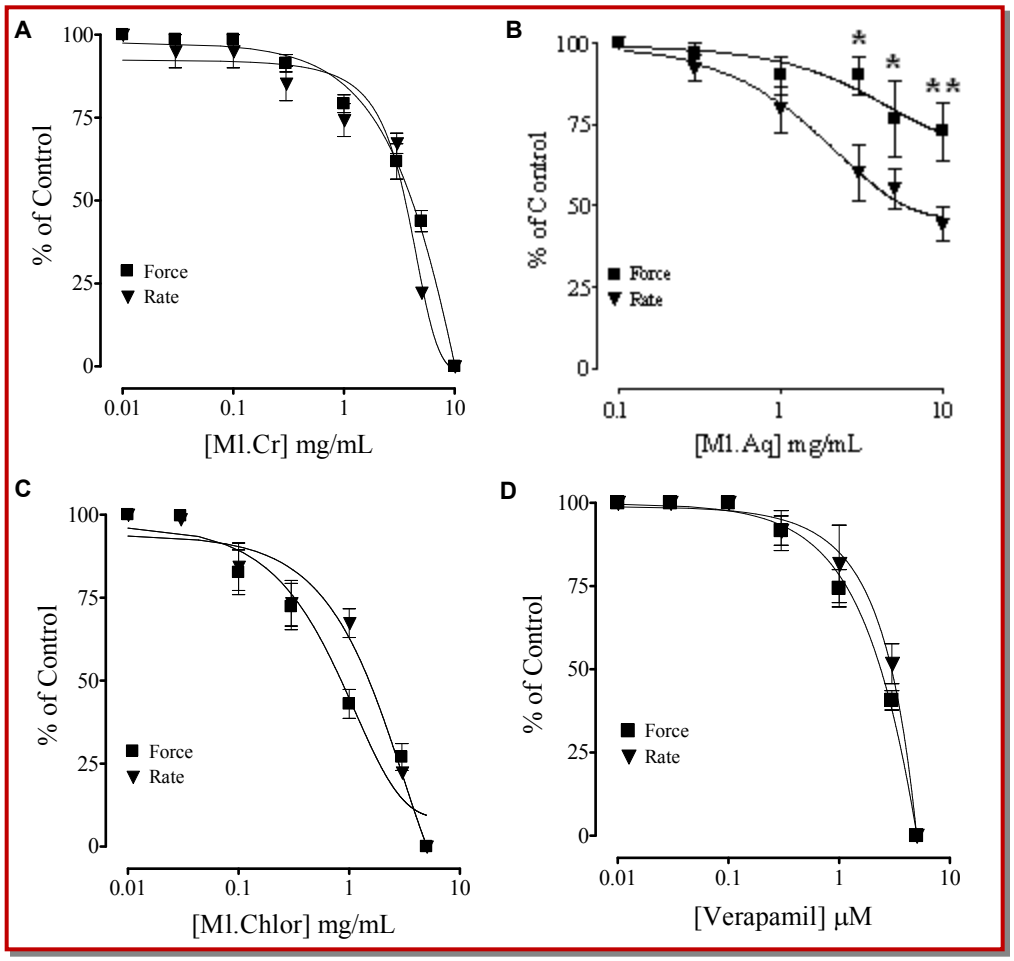

Figure 4: Inhibitory effect of (A) the crude extract of *M. longifolia* (Ml.Cr); (B) aqueous (Ml.Aq) and (C) chloroform (Ml.Chlor) fractions and (D) verapamil on the force and rate of spontaneous atrial contractions in isolated guinea pig atrial preparations. Values shown are mean ± SEM (n = 4-6). $^{*}p<0.01$, $^{**}p<0.001$

crude extract caused non-specific relaxation. Phenylephrine and high K^+ are known to increase cellular Ca^{++} level by allowing Ca^{++} influx through receptor-operated and voltage-dependent Ca^{++} channels (VDCs), respectively (Jiang et al., 2005). We hypothesized that the inhibitory effect of the extract may involve interference with Ca^{++} entry through VDCs. This hypothesis was further strengthened when pretreatment of the tissues with crude extract caused a rightward shift in the Ca^{++} concentration response curves, constructed in Ca^{++} -free medium, similar to verapamil, a Ca^{++} channel blocker (Fleckenstein, 1977). Among the fractions tested, the aqueous fraction failed to inhibit phenylephrine-induced contraction, with no appreciable effect against high K^+-induced contractions. Similarly, the aqueous fraction had no effect on Ca^{++} CRCs, suggesting that the Ca^{++} channel blockade is not the dominant mechanism of vasodilator effect. On the other hand, the chloroform fraction was more potent against high K^+ than phenylephrine-induced contractions, similar to verapamil, and also caused a rightward shift in the Ca^{++} CRCs, indicative of shifting CCB constituent(s) into a non-polar fraction.

The endothelium is an important regulator of vascular tone and arterial pressure, which releases several mediators including nitric oxide (Feletou and Vanhoutte, 1988; Campbell et al., 1996). Therefore, the nature of vasodilator effect was further studied in rat aorta to distinguish the endothelium-dependent and independent effect. In isolated rat aorta preparations precontracted with phenylephrine, the crude extract was about 35 times more potent in its relaxant effect in the endothelium-intact than endothelium-denuded preparations, indicating the endothelium-dependent vasodilator effect of the plant extract.

Pretreatment of aortic rings (endothelium-intact) either with L-NAME, a NO synthase inhibitor (Fantel et al., 1997) or atropine, blocked the vasodilator effect, which indicates that the crude extract contains constituent(s), which either act directly on endothelial cells to release NO or stimulate vascular muscarinic receptors linked NO release (Furchgott and Zawadaski, 1980). This NO of either mechanism has a role in the endothelium-

dependent vasorelaxation of the extract and fraction. The endothelium-dependent and atropine-sensitive vasodilator effect of the crude extract supports the partial atropine-sensitive blood pressure lowering effect observed in anesthetized rats.

Among the tested fractions, the endothelium-dependent vasorelaxant effects were found in the aqueous fraction, while chloroform fraction exhibited endothelium-independent activity. The aqueous fraction partially inhibited high K^+ pre-contractions, while the chloroform fraction caused complete inhibition, similar to that of verapamil. These data indicate that the endothelium-dependent atropine-sensitive vasodilator effect of the aqueous fraction is the dominant mechanism. This accounts for the blood pressure lowering effect of the plant extract, in addition to the Ca^{++} channel blocking activity (endothelium-independent) mainly present in the chloroform fraction.

To see, whether the blood pressure lowering property of the extract and fraction involved an effect on heart rate and contractility, we used isolated spontaneously beating guinea pig paired or right atrial preparations. Crude extract and the chloroform fraction caused marked inhibition of both force and rate of contractions, while the aqueous fraction partially inhibited both force and rate of contractions. The cardiac inhibitory effect of the crude extract and chloroform fraction appear to be mediated through Ca^{++} channel blockade and represent a dominant cardiac depressant mechanism. While the partial cardiac depressant effect of the aqueous fraction indicates that only muscarinic receptor-linked constituents are concentrated in this particular fraction, as such constituents are known having no prominent inhibitory effect on heart particularly on contractility (Crystal et al., 2001).

Conclusion

The crude extract of *M. longifolia* possesses a combination of vasodilator and cardiac depressant constituents responsible for the blood pressure lowering effect. The vasodilatory effect is mediated through a combination of Ca^{++} channel blockade (concentrated in a non-polar fraction) and endothelium-dependent pathway linked to vascular muscarinic receptors (concentrated in a polar fraction).

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