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Neem (*Azadirachta indica*) Lowers Blood Pressure through a Combination of Ca^{++} Channel Blocking and Endothelium-Dependent Muscarinic Receptors Activation

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Abstract: This study was aimed to investigate mechanisms underlying the blood pressure lowering effect of the crude extract of *Azadirachta indica* (Ai.Cr) and its aqueous (Ai.Aq) and ethylacetate (Ai.EtAc) fractions. In normotensive anesthetized rats, Ai.Cr (1-30 mg kg^{-1}) caused a dose-dependent fall in arterial pressure, Ai.Aq being more effective. In isolated rabbit aorta ring preparations, Ai.Cr inhibited phenylephrine (1 μM) and high K^+ (80 mM) pre-contractions, with slightly higher potency against phenylephrine while Ai.EtAc was more potent against K^+, similar to verapamil. The aqueous fraction was equipotent against both pre-contractions. Pre-treatment of aortic rings with Ai.Cr and both of its fractions shifted the Ca^{++} concentration-response curves to the right, similar to verapamil. In isolated rat aorta preparations, Ai.Cr and Ai.Aq exhibited endothelium-dependent $_L$-NAME and atropine-sensitive and Ai.EtAc endothelium-independent vasorelaxation, similar to verapamil. Against high K^+-pre-contractions, crude extract and Ai.EtAc were comparable in potency while, Ai.Aq was less potent. In isolated guinea-pig atrial preparations, crude extract and Ai.Aq were equipotent against both force and rate of contractions while Ai.EtAc was more potent against the rate. These data show that the crude extract of *A. indica* possesses vasodilator effect, mediated through Ca^{++} channel blockade and NO-dependent atropine-sensitive pathways along with cardiac depressant activity which possibly explain its blood pressure lowering effect.

Key words: *Azadirachta indica* (neem), antihypertensive, vasodilator, cardiac depression, Ca^{++} antagonist, NO-dependent cholinergic pathway

INTRODUCTION

The global view is changing towards the development and therapeutic use of safer preparations from medicinal plants for controlling various diseases (Bandyopadhyay *et al*., 2004). *Azadirachta indica* A., Juss, locally famous as "Neem", is a popular herb for its medicinal value in a wide range of diseases including cardiovascular disorders, such as, hypertension and cardiac arrhythmia (Chattopadhyay, 1997; Obiefuna and Young, 2005).

Neem is cultivated in various parts of the Asia (subcontinent) for more than 2000 years (Biswas *et al*., 2002). Its various parts, such as fruit, leaves, seeds and bark are known for medicinal use since time immemorial (Thakur *et al*., 1981). Neem is a household remedy against various human diseases and is also extensively used in Ayurveda, Greco-Arab (Unani) and Homoeopathic medicine. The importance of the Neem tree has been recognized by the US National Academy of Sciences and published a report in 1992 entitled "Neem, a tree for solving global problems" (Biswas *et al*., 2002). A large number of clinical studies are available on the extracts as well as some of its preparations, such as antifertility (Sinha *et al*., 1984), hepatoprotective (Chattopadhyay *et al*., 1992), antiulcer (Chattopadhyay *et al*., 2004), antihypertensive (Obiefuna and Young, 2005), hypoglycemic (Waheed *et al*., 2006),

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antimalarial (Udeinya *et al*., 2008), anti-inflammatory (Akihisa *et al*., 2009), antioxidant (Manikandan *et al*., 2009) and cytotoxic (Kikuchi *et al*., 2011).

The study of Obiefuna and Young (2005) has shown that extract from the leaves of Neem lowers blood pressure in salt-induced hypertension model in rats; however, the underlying mechanism is not yet clear. Therefore, this investigation was aimed to explore the underlying mechanisms responsible for the blood pressure lowering effect of Neem and we showed for the first time that the crude extract prepared from fruits of Neem possesses vasodilator effect, mediated through a combination of Ca^{++} channel blockade and NO-dependent atropine sensitive pathways along with cardiac depressant activity that provides sound mechanistic basis for its blood pressure lowering effect.

MATERIALS AND METHODS

Preparation of crude extract and fractionation: Fruits of *A. indica* (4.5 kg) were collected from the campus of the Aga Khan University, Karachi, Pakistan and soaked in the aqueous-methanol (30:70) for three days with occasional shaking. The material was first filtered through a muslin cloth and then through a Whatman qualitative grade 1 filter paper. This procedure was repeated twice. The combined filtrates were collected and concentrated on rotary evaporator at 35-40°C under reduced pressure (-760 mmHg) to a thick semi-solid mass of yellowish color; the crude extract (Ai.Cr) yielding approximately 10%.

Activity-directed fractionation of the crude extract was carried out by following a standard phytochemical procedure (Williamson *et al*., 1998). A known quantity of the crude extract (60 g) was dissolved in 100 mL of distilled water in a separating funnel. Ethyl acetate (100 mL) was then added and the mixture was shaken vigorously. It was kept for about 30-60 min to let the two layers separate. The upper layer of ethyl acetate was acquired and the same procedure was repeated twice more. All the ethyl acetate layers were collected and concentrated in a rotary evaporator to obtain the ethyl acetate fraction (Ai.EtAc), with approximate yield of 25.2%. The lower layer was also collected and concentrated on rotary evaporator and was considered as aqueous fraction (Ai.Aq), with approximate yield of 70%. The crude extract and aqueous fraction were found soluble in distilled water while the ethyl acetate fraction was dissolved in 10% dimethyl sulfoxide (DMSO). Dilutions of the crude extract and fractions were made fresh on the day of experiment in normal saline for the *in vivo* and *in vitro* experiments, respectively.

Drugs and standards: Drugs used in these experiments were purchased from the source specified: phentothal sodium (Abbott Laboratories, Pakistan). Acetylcholine chloride, norepinephrine bitartrate, atropine sulphate, phenylephrine hydrochloride, DMSO, potassium chloride, isoprenaline hydrochloride, verapamil hydrochloride and Nù-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 experiment.

Animals: Experiments performed complied with the rulings of the Institute of Laboratory Animals Resources, Commission on Life Sciences, National Research Council (NRC., 1996). Sprague-Dawley rats (200-250 g) and local rabbits (1.5-2 kg) of either sex used in the study were breed and housed at the Animal House of the Aga Khan University, Karachi, maintained at 23-25°C and were given tap water *ad libitum* and a standard diet.

Measurement of blood pressure in anesthetized rats: As described previously (Gilani *et al*., 2006; Taqvi *et al*., 2006), adult Sprague-Dawly normotensive rats were anesthetized with sodium thiopental (Pentothal, 90-100 mg kg^{-1}, i.p.). The trachea was cannulated with a polyethylene tubing (PE-20) to facilitate spontaneous respiration. The arterial pressure 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, Sydney, Australia). The animals were allowed undisturbed for at least 30 min before the start of experiment. After 30 min, control responses of standards, such as, acetylcholine (Ach; 1 μg kg^{-1}) and norepinephrine (NE; 1 μg kg^{-1}) were obtained by intravenous injection into the left jugular vein. Each dose was followed by a saline (0.1 mL) flush. Each rat was used as a single experiment.

Some anesthetized rats were pre-treated with atropine (2 mg kg^{-1}) to see, if the blood pressure lowering effect was sensitive to atropine. After 30 min of treatment, the effect of the crude extract and fractions of *A. indica* was determined in the presence of atropine. Mean Arterial Pressure (MAP) in each case was determined from the sum of diastolic blood pressure plus one-third pulse width and changes in MAP were expressed as percent of control (percent fall in MAP).

Rabbit thoracic aorta: Vascular reactivity studies were performed as described previously (Khan *et al*., 2014).

Aortic rings were suspended in isolated tissue baths filled with 10 mL normal Kreb's solution, continuously bubbled with carbogen (5% CO_2 in O_2) at 37°C. 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 resting tension of 2 g was applied to each tissue and an equilibrium period of 1 h in unstretched condition. 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, Sydney, Australia).

Determination of vasodilator and calcium channel blocking activity: As described previously (Khan *et al*., 2014; Gilani *et al*., 2006), phenylephrine (PE; 1 μM) or high K^+ (80 mM) was used to induce steady contractions and cumulative Concentration-Response Curves (CRCs) of extract and its fractions were determined for possible vasodilator effect. The inhibitory effect was expressed as percent of the agonist-induced contractions.

In a separate set of experiments, an attempt was made to see, if the relaxation induced by the crude extract of Neem and its fractions involve Ca^{++} influx via voltage dependent Ca^{++} channels (VDCs). An indirect approach was followed (Taqvi *et al*., 2006; Shah and Gilani, 2009), accordingly, aortic rings were washed 4-5 times with Ca^{++}-free solution before the construction of control CRCs of Ca^{++} (as $CaCl_2$). When the control CRCs of Ca^{++} were found super-imposable, tissues were then pre-treated with the test material at least 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: As described previously (Furchgott and Zawadaski, 1980; Shah and Gilani, 2009), aortic rings with intact and denuded endothelium were suspended in organ baths filled with 5 mL of normal Kreb's solution bubbled with carbogen at 37°C. 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). The aortic rings with intact endothelium that produced less than 40% relaxation in response to acetylcholine (1 μM) were discarded. Individual rings were mounted in 5 mL tissues baths at 37°C, bubbled with carbogen. A preload of 1 g was applied to each preparation and the tissues were equilibrated for 30 min. 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, Sydney, Australia).

Study on vascular tone: A series of experiments were conducted to assess endothelium-dependent and independent effects as described previously (Colussi *et al*., 2011; Shah and Gilani, 2009). When the PE (1 μM)-induced tension reached a plateau, the crude extract of *A. indica* and its fractions were cumulatively added into the tissue baths and CRCs were constructed. The rings with intact or denuded endothelium were always tested in parallel.

To determine the underlying mechanisms of vasodilator effect, endothelium-intact rings were pre-treated with $_L$-NAME (10 μM) and atropine (1 μM) for 30 min before the addition of PE (1 μM). The crude extract of *A. indica* and its fractions were added cumulatively to the sustained PE (1 μM) pre-contractions and the CRCs were constructed again and compared.

High K^+ (80 mM) was also used to produce sustained contractions which allowed studying possible effect of the test material on the VDCs (Karaki *et al*., 1997). Test material was then added cumulatively and relaxation was expressed as percent of the induced contractions.

Guinea-pig atria: As described previously (Gilani *et al*., 2006), guinea-pig right atria were made into strips and suspended in tissue baths filled with 20 mL of Kreb's solution, aerated with carbogen gas at 32°C. The tissues were then allowed to beat spontaneously under the resting tension of 1 g. An equilibrium period of 30 min was allowed before the application of any drug. Control responses of ACh (1 μM) and isoproterenol (1 μM) were obtained at least in duplicate. Tension changes in the tissue were recorded via a Grass force-displacement transducer (model FT-03) using Grass model 7 Polygraph.

Statistics: All data expressed are Mean±Standard error mean (SEM, n = number of experiments) and median effective concentration (EC_{50} values) with 95% Confidence Intervals (CI). The statistical parameter applied is the Student's t-test (paired or unpaired) with $p<0.05$ as significantly different using GraphPAD program (GraphPAD, San Diego, Ca, USA). The concentration response curves were analyzed by non-linear regression.

RESULTS

Effect on blood pressure: Following an equilibrium period of around 20 min, when the baseline of blood pressure was in a steady state, norepinephrine and acetylcholine were tested which produced rise and fall in mean arterial pressure respectively in rats under anesthesia (Fig. 1a).

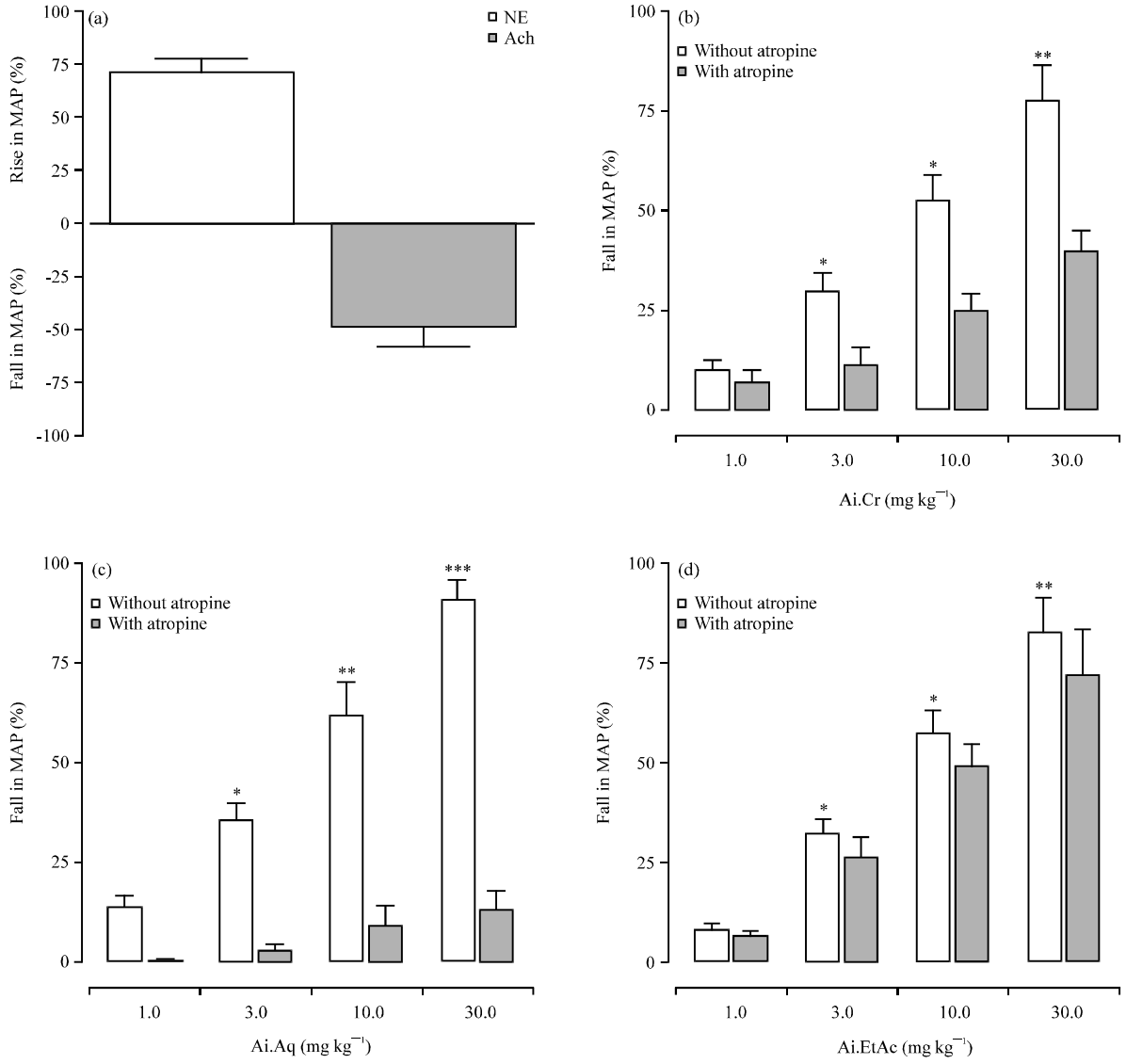

Fig. 1(a-d): (a) Effect of norepinephrine (NE) and acetylcholine (Ach) on Mean Arterial Pressure (MAP) in normotensive anesthetized rats and (b-d) Effect of the crude extract of *Azadirachta indica* (Ai.Cr) and its aqueous (Ai.Aq) and ethyl acetate (Ai.EtAc) fractions on MAP in normotensive anesthetized rats, Values shown are Mean±SEM (n = 6), *p<0.01, **p<0.001, ***p<0.0001, compared with without atropine

The crude extract of *A. indica* (Ai.Cr) was then administered intravenously, where it caused a dose-dependent fall in arterial pressure in normotensive rats. The percent fall observed at doses of 1, 3, 10 and 30 mg kg^{-1} was; 10.0±2.55, 29.75±4.59, 52.0±6.97 and 77.50±8.54 mmHg, respectively (Fig. 1b). Rats pre-treated with atropine (2 mg kg^{-1}), showed diminished response ($p<0.001$) to Ai.Cr on MAP, with respective percent fall of 6.67±3.18, 11.67±3.75, 24.67±4.26 and 39.67±5.24 mmHg, at same doses (Fig. 1b).

Among the fractions studied, the aqueous fraction was found more effective than the parent crude extract or ethyl acetate fraction in lowering BP at the same doses, with respective percent fall of 13.75±2.83, 35.75±4.25, 62.0±8.28 and 91.25±5.15 mmHg (Fig. 1c). Pretreatment of rats with atropine (2 mg kg^{-1}), partially blocked the BP lowering effect of the crude extract and the aqueous fraction (more sensitive to atropine) while effect of the ethyl acetate fraction remained unaltered (Fig. 1d).

Effect on isolated rabbit aorta: When tested against the agonist-induced contractions, Ai.Cr inhibited the phenylephrine pre-contractions more potently than high K^+, with respective EC_{50} values of 0.55 (0.41-0.73) and 1.34 mg mL^{-1} (1.02-1.74) as shown in Fig. 2a. Pre-treatment of aortic rings with Ai.Cr (0.3-3 mg mL^{-1}) caused a rightward shift in the Ca^{++} CRCs (Fig. 2e), constructed in Ca^{++}-free medium, similar to verapamil (Fig. 2h).

Unlike the parent crude extract, Ai.EtAc was relatively more potent in inhibiting high K^+ pre-contractions than phenylephrine, with respective EC_{50} values of 0.33 (0.25-0.43) and 1.25 mg mL^{-1} (0.84-1.86) (Fig. 2b), similar to verapamil (Fig. 2d). Pre-treatment of the aortic rings with Ai.EtAc (0.1-1 mg mL^{-1}) also caused a rightward shift in the Ca^{++} CRCs (Fig. 2f), constructed in Ca^{++}-free medium.

The Ai.Aq was found least potent against both high K^+ and phenylephrine pre-contractions, with respective EC_{50} values of 9.01 (6.76-12.02) and 4.20 mg mL^{-1} (3.08-5.75) (Fig. 2C). Pre-treatment of the aortic rings with Ai.Aq (1-5 mg mL^{-1}) also caused a rightward shift in the Ca^{++} CRCs (Fig. 2g), constructed in Ca^{++}-free medium.

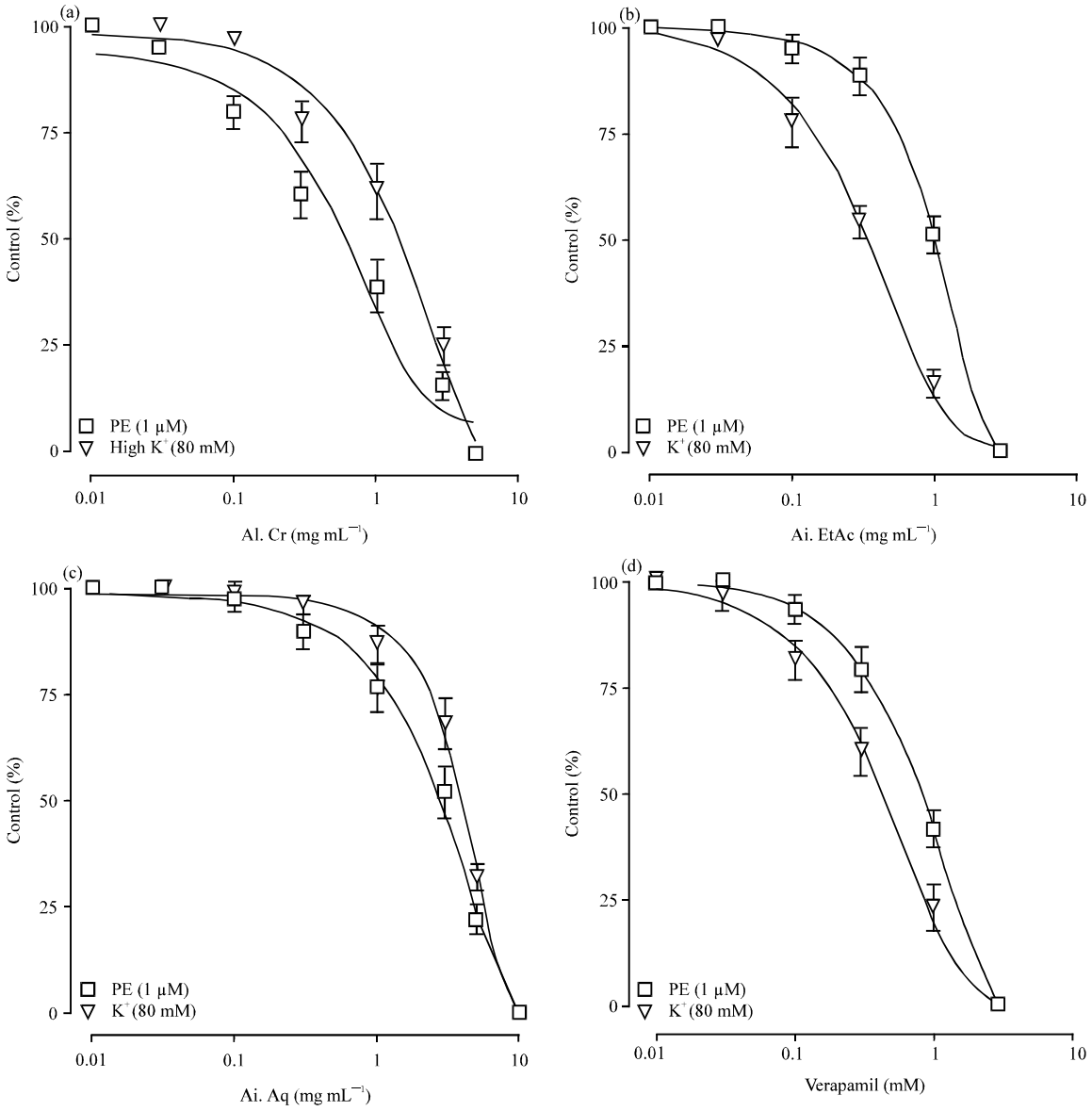

Fig. 2(a-h): Continue

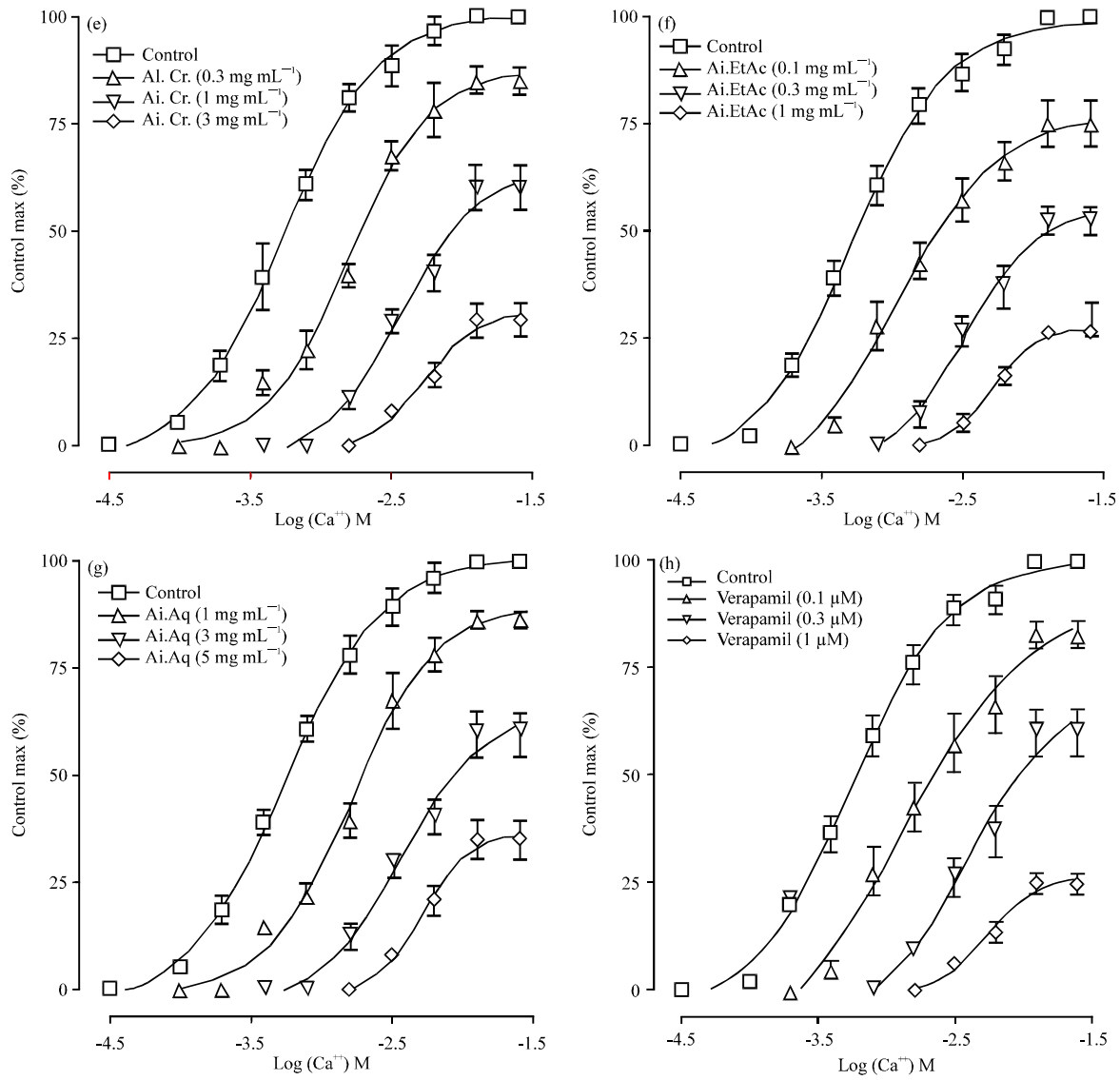

Fig. 2(a-h): Concentration-dependent vasorelaxation against phenylephrine (PE) and high K^+ (80 mM)-induced by the (a) Crude extract of *Azadirachta indica* (Ai.Cr); its (b) Aqueous (Ai.Aq) and (c) Ethylacetate (Ai.EtAc) fractions, (d) Verapamil, in isolated rabbit aorta preparations, (e-h) Effect of Ai.Cr, Ai.Aq, Ai.EtAc and verapamil on the Ca^{++} concentration-response curves, constructed in Ca^{++} -free medium, values shown are Mean±SEM (n = 6-8)

Effect on rat aorta: The cumulative addition of Ai.Cr (0.01-10 mg mL^{-1}) caused inhibitory effect in aorta rings with intact endothelium when pre-contracted with PE (1 µM). Incubation of the intact aortic rings with $_L$-NAME (10 µM) and atropine (1 µM), inhibited significantly ($p<0.01$) the inhibitory effect of Ai.Cr and shifted the CRCs to the right (Fig. 3a). In aortic ring with denuded endothelium, the inhibitory effect of Ai.Cr was significantly ($p<0.001$) blocked (Fig. 3a). Ai.Cr was also found around 16 times more potent in inhibiting high K^+-induced contractions in rat than in rabbit aorta, with EC_{50} value of 0.08 mg mL^{-1} (0.04-0.27) (Fig. 3a).

Among the fractions tested, the aqueous fraction exhibited endothelium-dependent $_L$-NAME/atropine-sensitive vasodilator effect (Fig. 3b). Unlike the crude extract and aqueous fraction, the ethyl acetate fraction exhibited endothelium-independent vasodilator effect

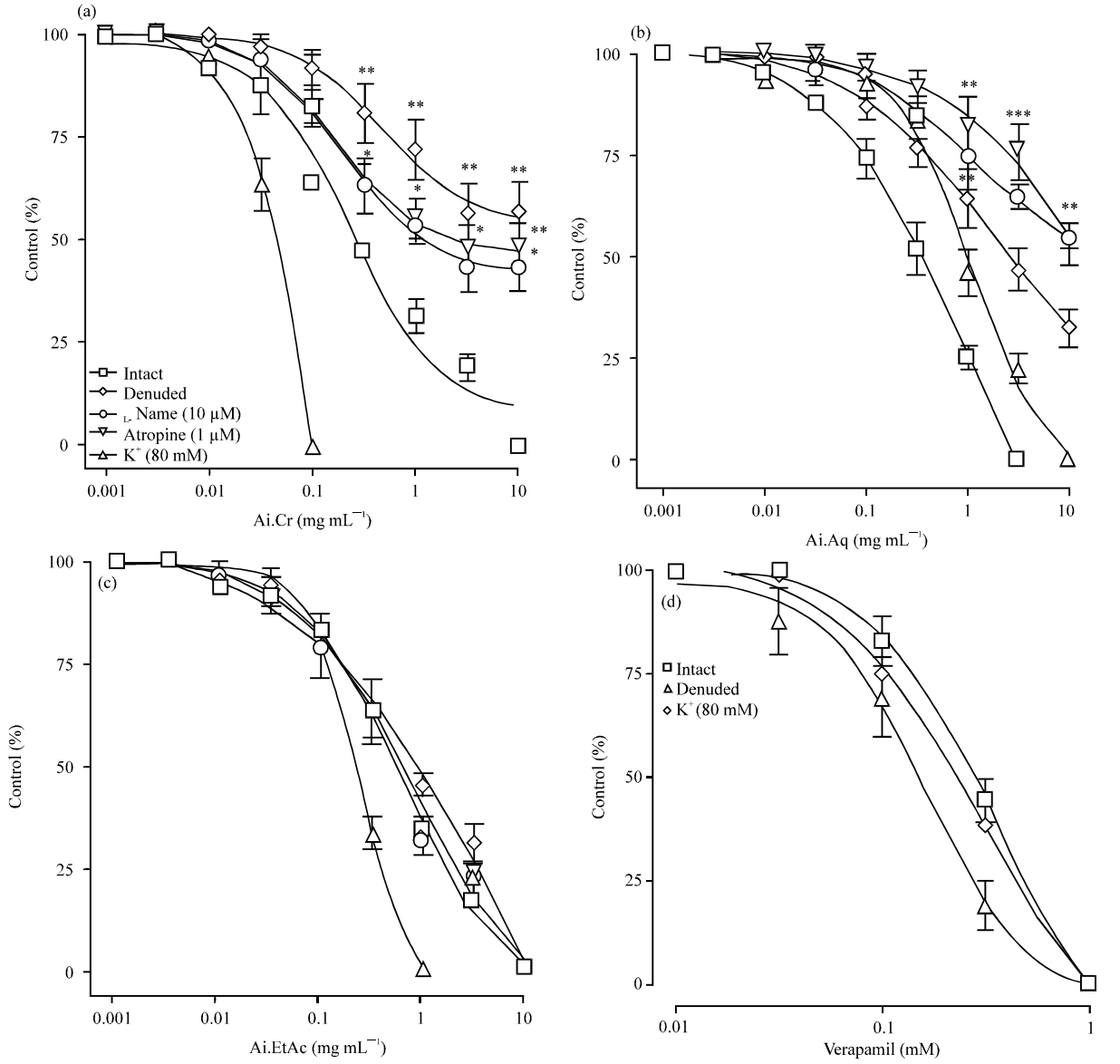

Fig. 3(a-d): Effect of the crude extract of *Azadirachta indica* (Ai.Cr), its fractions and verapamil in isolated rat aorta preparations, (a-b) Endothelium-dependent vasorelaxation induced by Ai.Cr and its aqueous (Ai.Aq) fraction against high K^+ and phenylephrine (PE) pre-contractions in the intact aortic rings, pretreated with $_L$-NAME and atropine and in aortic rings with denuded endothelium, (c) Endothelium-independent vasorelaxation induced by the ethyl acetate (Ai.EtAc) fraction of *Azadirachta indica* against high K^+ and phenylephrine (PE) pre-contractions in the intact aortic rings, pretreated with $_L$-NAME and atropine and in aortic rings with denuded endothelium and (d) Endothelium-independent vasorelaxation induced by verapamil against phenylephrine (PE) and high K^+ pre-contractions, values shown are Mean±SEM (n = 6), $^*p<0.01$, $^{**}p<0.001$, $^{***}p<0.0001$; compared with intact

(Fig. 3c), similar to that of verapamil (Fig. 3d). Both the aqueous and ethyl acetate fractions caused inhibition of high K^+-induced contractions, later being more potent with respective EC_{50} values of 6.88 (5.72-7.98) and 0.76 mg mL^{-1} (0.13-1.88).

Effect on guinea-pig atria: In isolated guinea-pig right atrial preparations, Ai.Cr caused inhibition of atrial force and rate of spontaneous contractions at similar concentrations, with respective EC_{50} values of 1.61 (0.81-3.12) and 1.93 mg mL^{-1} (1.0-3.73) (Fig. 4a).

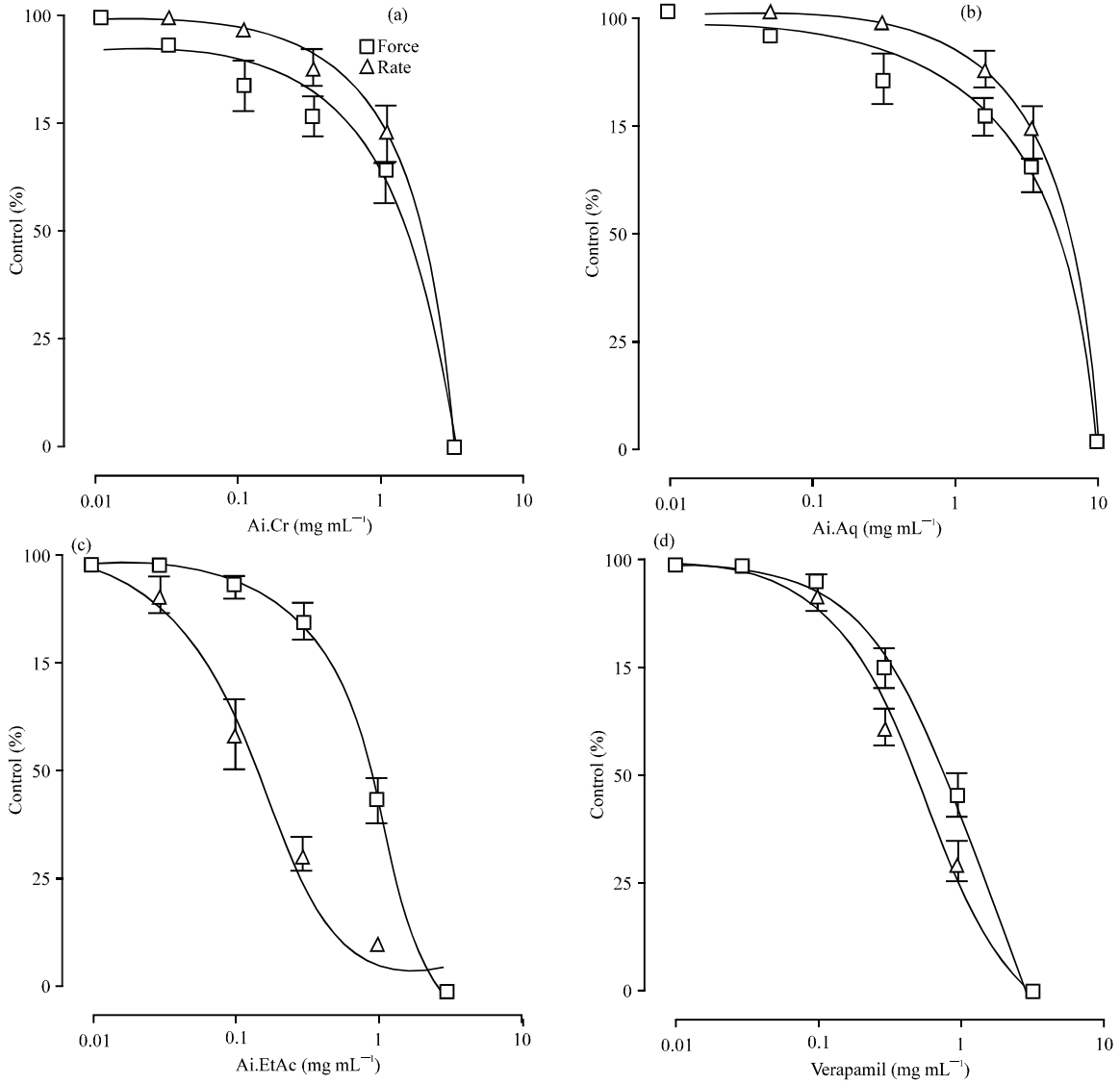

Fig. 4(a-d): Inhibitory effect of (a) Crude extract of *Azadirachta indica* (Ai.Cr); its (b) Aqueous (Ai.Aq), (c) Ethyl acetate (Ai.EtAc) fractions and (d) Verapamil on the force and rate of spontaneous atrial contractions in isolated guinea-pig atrial preparations, values shown are Mean±SEM (n = 5)

Among the fractions tested, the aqueous fraction was least potent in its cardiac suppressant activity and had similar effect on force and rate of spontaneous contractions (Fig. 4b) with respective EC_{50} values of 6.04 (3.44-9.41) and 7.10 mg mL^{-1} (5.23-11.65) (1.702-3.148) (Fig. 4b). The ethyl acetate fraction was not only most potent in its cardiac inhibitory effect but also exhibited selectivity in suppressing rate of contractions with respective EC_{50} values of 0.14 mg mL^{-1} (0.10-0.20) and 1.10 (0.70-1.69) as shown in Fig. 4c. Verapamil suppressed force and rate of atrial contractions at similar doses (Fig. 4d).

DISCUSSION

Based on the medicinal reputation of Neem in cardiovascular disorders (Chattopadhyay, 1997; Obiefuna and Young, 2005) and limited studies in this area, its crude extract and fractions were tested for blood pressure lowering effect in normotensive rats under anesthesia. The crude extract and its aqueous and ethyl acetate fractions caused a dose-dependent fall in arterial pressure, aqueous fraction being the most effective. We previously observed that the BP lowering effect of different medicinal plants was mediated, partially, through

cholinergic pathway (Shah and Gilani, 2011). To see, if the PB lowering effect of the plant extract was mediated through acetylcholine-like mechanism, the rats were pre-treated with atropine, a muscarinic acetylcholine receptor antagonist (Gilani *et al*., 1997). This pre-treatment partially blocked the blood pressure lowering effect of the crude extract indicating the involvement of more than one mechanism. In the atropinized rats, the BP lowering effect of the aqueous fraction was blocked ($p>0.05$) while that of the ethyl acetate fraction remained unchanged ($p>0.05$) which indicates that atropine-sensitive blood pressure lowering constituent(s) is/are, separated into the aqueous fraction while atropine insensitive BP lowering constituent(s) concentrated in the ethyl acetate fraction; interestingly, none of the fractions showed any clear supremacy over the parent crude extract in terms of potency or efficacy; hence, the use of fractionation process may serve the academic purpose only without impact on the clinical application. The crude extract mediates its effect through multiple pathways is likely to have more value. This is in line with 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; Saeed *et al*., 2005; Tep-Areenan and Sawasdee, 2011). Further, the functional nature of blood pressure lowering effect of the crude extract and its fractions was studied in isolated cardiovascular preparations (Thompson and Anderson, 1978; Chattopadhyay, 1997; Obiefuna and Young, 2005).

When tested in isolated rabbit aorta preparation, Ai.Cr exhibited a non-specific vascular inhibitory effect against PE and high K^+. The increase in intracellular Ca^{++} and subsequent vascular contraction in response to high K^+ is a result of Ca^{++} entry through voltage-dependent Ca^{++} channels (Jiang *et al*., 2005). The inhibitory effect of the Ai.Cr against high K^+ pre-contractions may be due to interference with Ca^{++} entry through VDCs. This hypothesis was further strengthened when pre-treatment of the tissues with Ai.Cr caused a right ward 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 ethyl acetate fraction was more potent against high K^+ than PE while the aqueous fraction was almost equipotent against both pre-contractions with less potency than the crude extract and ethyl acetate fraction. However, pre-treatment of the aortic rings with Ai.EtAc and Ai.Aq caused a right ward shift in the Ca^{++} CRCs like the parent crude extract. These data indicate that the Ca^{++} channel blocking constituents present in the parent crude extract was mainly separated into the ethyl acetate fraction which can partly explain the fall in MAP.

The endothelium has emerged as an important regulator of vascular tone and blood pressure which releases several mediators including nitric oxide (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, cumulative addition of Ai.Cr caused endothelium-dependent inhibition of the PE pre-contractions. Similarly, in aortic rings without functional endothelium (denuded), Ai.Cr failed to cause complete inhibition. In intact aortic rings pre-treatment either with $_L$-NAME, a NO synthase inhibitor (Fantel *et al*., 1997) or atropine, blocked the relaxation to Ai.Cr. These data indicate that the crude extract also contains constituents which either act directly on endothelial cells to release NO or stimulate the muscarinic receptors located on vascular endothelial cells (Furchgott and Zawadaski, 1980) to cause endothelium-dependent vascular relaxation. The endothelium-dependent and atropine-sensitive vasodilator effect of the Ai.Cr supports the partial atropine-sensitive blood pressure lowering effect observed in anesthetized rats.

When tested against high K^+ pre-contractions in rat aorta preparations, Ai.Cr caused inhibition of the induced contractions with about 16 times more potency than in rabbit aorta which may account partly for the endothelium-independent vasodilator effect. This difference in the Ca^{++} channel blocking activity in different vascular preparations may be due to the heterogeneity of Ca^{++} channels (Koike *et al*., 1992), though contribution of species differences cannot be ruled out. Among the fractions tested, the endothelium-dependent activity was separated in the aqueous fraction while the endothelium-independent activity was concentrated in the ethyl acetate fraction. The aqueous and ethyl acetate fractions produced relaxation against high K^+ pre-contractions, the aqueous being less potent in rat while the ethyl acetate fraction possessed similar potency as in rabbit aorta. These data indicate that the endothelium-dependent atropine-sensitive vasodilator effect of the aqueous fraction is the dominant mechanism accounting for the blood pressure lowering effect, in addition to the Ca^{++} channel blocking activity (endothelium-independent) retained mainly in the ethyl acetate fraction.

When studied for the cardio-suppressant activity using isolated spontaneously beating guinea-pig paired atrial preparations, Ai.Cr and its aqueous fraction caused inhibition of both force and rate of contractions equally, the aqueous fraction being less potent than the parent extract. Interesting, the ethyl acetate fraction was not only more potent than the parent extract or the aqueous

fraction but also inhibited the rate of spontaneous contractions at concentrations around 8 times less than required to inhibit force of contraction, whereas, verapamil was non-selective in its inhibitory effect on rate and force of atrial contractions. This novel selective inhibitory effect of the ethyl acetate fraction on the rate has more clinical relevance as the tachycardia is more commonly occurring disorder (Buxton *et al*., 2000). The cardiac inhibitory effect of the crude extract and its fractions appear to be mediated through Ca^{++} channel blockade as muscarinic receptor-linked NO effect is not known to produce prominent inhibitory effect on heart (Crystal *et al*., 2001). In a previous study the negative inotropic and chronotropic effects of *A. indica* extract remained unchanged in the presence of atropine (Khosla *et al*., 2011), ruling out the possibility of muscarinic receptor stimulation and/or NO linked pathway. Moreover, the relative potency of the crude extract and its fractions for its cardiac inhibitory effect observed is directly proportional to the potency for Ca^{++} antagonist effect studied in vascular preparations.

CONCLUSION

In summary, these data show that the Neem extract possesses combination of vasodilator and cardiac depressant constituents. The vasodilatory effect is mediated through Ca^{++} channel blockade and NO pathway linked to muscarinic vascular receptors while the cardiac depressant effect is probably the outcome of Ca^{++} antagonist effect. Thus this study provides sound mechanistic base to the blood pressure lowering effect of the plant while the selective inhibitory effect of the ethyl acetate fraction on the cardiac rate is a novel finding which needs further in-depth study along with insight into the chemical constituent(s) and molecular aspects of these findings.

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