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Studies on Blood Pressure Lowering, Vasodilator and Cardiac Suppressant Activities of *Vitex negundo*: Involvement of K^+ Channel Activation and Ca^{++} Channel Blockade

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ABSTRACT
This study was aimed at providing scientific basis for the medicinal use of *Vitex negundo* in hypertension. The *in vivo* study was conducted on normotensive anesthetized rats while *in vitro* studies were conducted on isolated guinea-pig atria and rat aorta preparations by using isometric transducers coupled with Powerlab Data-acquisition system. The crude extract of *Vitex negundo* (Vn.Cr), produced a dose-dependent (10-100 mg kg^{-1}) fall in the arterial pressure of anaesthetized rats. When tested in rat aortic ring preparations, Vn.Cr inhibited the low K^+ (25 mM) with greater potency as compared to high K^+ (80 Mm) and phenylephrine (PE, 1 μM)-induced contractions. Further studies on the inhibitory effect of Vn.Cr against low K^+ revealed that the pretreatment of tissues with tetraethyl ammonium (TEA; 1 mM) shifted the concentration response curves to the right while glibenclamide (Gb; 10 μM) did not show any effect, hence showing the involvement of non-specific type of K^+ channels activation in the vasodilatory effect of Vn.Cr. The plant extract also shifted the Ca^{++} concentration response curves to the right dose-dependently (0.3-1 mg mL^{-1}), like that caused by verapamil. In isolated guinea-pig atria, Vn.Cr (0.1-10 mg mL^{-1}) caused inhibition of atrial force and rate of spontaneous contractions, similar to that exhibited by verapamil. These data indicate that *Vitex negundo* exhibits BP lowering, vasodilator and cardiac suppressant activities, mediated predominantly through K^+ channel activation combined with Ca^{++} channel inhibition.

Key words: *Vitex negundo*, K^+ channel activation, Ca^{++} antagonist, cardio-depressant, vasorelaxant

INTRODUCTION

Vitex negundo (Family: Verbenaceae) commonly known as chaste tree (Tandon, 2005), locally as "Wormandai or Marwandai" (Usmanghani *et al*., 1997; Shinwari *et al*., 2003) is found in different parts of the world including Africa, Europe and different parts of Asia including China, India and Pakistan (Dharmasiri *et al*., 2003). We recently observed that *Vitex negundo* possesses antispasmodic, antidiarrhoeal and bronchodilatory activities (Khan *et al*., 2015). It has been known that the allopathic remedy for hypertension is not always safe, efficacious and is beyond the access and/or affordability of large part of population, who looks for alternative therapy, mostly herbal medicine (Tep-Areenan and Sawasdee, 2011; Khan *et al*., 2014). In the current study, we investigated the effects of *Vitex negundo* on cardiovascular aspects and showed that it exhibits Blood Pressure (BP) lowering, vasodilator and cardio-depressant activities mediated

predominantly by activation of non-specific K^+ channels followed by inhibition of voltage-dependent Ca^{++} channels.

MATERIALS AND METHODS

Plant material and extraction: The plant (aerial parts) was collected from the surrounding locality in District Swat, Khyber Pukhtunkhwa, Pakistan, confirmed by Mr. IlyasIqbal, Assistant Professor, Department of Botany, University of Malakand, Chakdara, Dir Lower, Pakistan. Labelled specimen (UOM/BGH/149), was deposited in the herbarium of University of Malakand. The collected plant materials were treated under shade, cleaned of dirt and a quantity equal to 1 kg being pulverized and soaked in methanol-distilled water mixture (70:30) at 25±2.0°C for three days while stirring occasionally. It was passed through a muslin cloth with subsequent filtration via Whattman paper. Soaking followed by filtration of the solvent mixture was done two-times more. The combined filtrates were concentrated on rotary evaporator under reduced pressure (-760 mm Hg) at 35-40°C to a semisolid, dark brown paste (213.0 g), the crude extract (Vn.Cr). The yield was approximately 21.3%. Vn.Cr was, respectively dissolved in saline (0.9% w/v) and distilled water for *in-vivo* and *in-vitro* procedures.

Drugs and standards: The chemicals used in the study have been listed along with respective source as: Loperamide hydrochloride, acetylcholine chloride, verapamil hydrochloride, potassium chloride (Sigma Chemical Company, St. Louis, MO, USA.) and castor oil (Karachi Chemical Industries, Karachi, Pakistan). The chemicals were of the highest purity grade. Stock solutions of the chemicals were made in distilled water. Fresh dilutions were prepared in saline (0.9% w/v) and distilled water on the day of *in-vivo* or *in-vitro* experiment(s).

Animals: The rodents used, included adult rats (Sprague-Dawley,180-200 g) and guinea-pigs (450-500 g) of local breed and either sex, housed under controlled environment (25±2.0°C) in the animal's lodging of The Aga Khan University. The animals had access to drinking (tap water *ad libitum*) and eating (a balanced diet). The scheduled experiments conformed well to the stated guidelines of the Institute of Laboratory Animal Resources, Commission on Life Sciences, NRC (1996).

Chemicals: The following reference chemicals were obtained from the sources specified: Acetylcholine chloride (ACh), isoprenaline hydrochloride, norepinephrine hydrochloride (NE), phenylephrine hydrochloride (PE) and verapamil hydrochloride, glibenclamide (Gb) and tetraethylammonium (TEA) (Sigma Chemical Company, St. Louis, MO, USA). Pentothal sodium (thiopental) was obtained from Abbot Laboratories, Karachi, Pakistan. The following chemicals were used to make physiological salt solutions: Potassium chloride (Sigma Chemical Company, St. Louis, MO, USA), calcium chloride, glucose, magnesium sulphate, potassium dihydrogen phosphate, sodium bicarbonate and sodium chloride from Merck, Darmstadt, Germany. All chemicals used were of analytical grade.

Measurement of blood pressure in anaesthetized rats: According to the methods described (Consolini *et al*., 1999; Khan *et al*., 2014), thiopentone sodium (80-100 mg kg^{-1}) was injected (i.p.) to anaesthetize the rats. On dissecting table, rats were fixed dorsally. Trachea, left carotid artery and right jugular vein were exposed with a small incision of approximately 1 cm along mid-tracheal line. Polyethylene tube Pe-20 (Clay Adams Division, Becton Dickinson & Company, Parsippany, NJ 07054, USA) was used to cannulate trachea for spontaneous respiration. Polyethylene tube (Pe-50), was used to cannulate the right jugular vein (for administration of drug) and the left carotid artery for connecetion to pressure transducer (MLT 0380/D Reusable BP-Transducer), coupled to ML 224 Quad Bridge Amplifier and Power-Lab ML 4/25 data recording system (AD Instruments, Sydney, Australia) for BP recording (the cannula between artery and transducer was filled with heparinized-saline, 60 IU mL^{-1}). A piece of gauze was soaked in warm saline and used to mask the exposed surface at cannulated site. To prevent coagulation of blood, 0.1 mL heparinized saline (0.9% NaCl) was injected to rats. To maintain rat's body temperature, an overhead lamp was used. *Vitex negundo* (Vn. Cr.) was injected (i.v.) after an equilibrium period of 20 min. Blood pressure was allowed to return to base line between injections. The changes in BP were recognized as difference between the steady state values before and the peak values after administration. Mean Arterial Pressure (MAP) was recorded as the diastolic BP plus one-third of the pulse pressure (systolic BP-diastolic BP). The ACh (1 μg kg^{-1}) and NE (1 μg kg^{-1}) control responses were obtained to ensure the integrity of animals before the injection of any test drug.

Isolated guinea-pig atria: Right atria isolated from the guinea-pigs were mounted in 20 mL tissue baths containing Kreb's solution, at 32°C and aerated with carbogen (95% O_2 in 5% CO_2). Each atria under the resting tension of 1 g, was allowed to beat spontaneously due to pacemaker action (Khan *et al*., 2014). Atria were allowed to equilibrate for a period of 45 min before the application of any drug. Control responses of ACh (1 μM) and isoprenaline (1 μM) were tested at least in duplicate. Changes in atrial force of contraction mediated by drug were taken as the percent change in base-line values, whereas changes in tissue tension were obtained via force-displacement transducer (FT-03) using Grass Model 7 Polygraph.

Rat aorta preparations: Cervical dislocation was performed to scarifice the rats. Thoracic aorta was dissected out from the abdomen, cleaned of adipose tissues, cut into rings (3-5 mm long) and hooked-up separately in 5 mL tissue-bath

with Kreb's solution having the following composition (mM): NaCl 118.2, $NaHCO_3$ 25.0, $CaCl_2$ 2.5, KCl 4.7, KH_2PO_4 1.3, $MgSO_4$ 1.2 and glucose 11.7 (pH 7.4). The solution in the bath-tube was kept at 37°C and aerated (with carbogen) continuously. A load of 1 g was applied to each tissue and a period of 30 min was allowed pass before any test. The tissues were then stabilized with repeated (3-times) exposure to phenylephrine of 1 µM (Gilani *et al*., 2006). The test drug was then checked for its ability to relax the contractions, induced with low K^+ (25 mM), high K^+ (80 mM) and PE, 1 µM. Relaxation of low K^+ (25 mM)-induced contractions by extract would indicate K^+ channel opening effect while inhibition of the contractions induced by K^+ (80 mM) would indicate L-type voltage-operated Calcium Channel Blocking (CCB) mode of vasodilation, whereas, inhibition of the contractions induced by PE, would signify blockade of the Ca^{++} influx through Ca^{++} channels operated by receptors (Godfraind *et al*., 1986). To elucidate further the involvement of the type of K^+ channels, the aortic tissues were pretreated with an ATP-dependent K^+ channel blocker i.e., glibenclamide (Franck *et al*., 1994) and non-specific K^+ channel blocker i.e., TEA (Cook, 1989). The CCB action of drug was confirmed by constructing concentration-response curves (CRC_S) of calcium chloride i.e., Ca^{++} (Jabeen *et al*., 2007). Tissues were first stabilized in Kreb's solution (normal) then replaced with Ca^{++}-free Kreb's solution (with 0.1mM EDTA) to remove the Ca^{++} from tissues. This solution was further replaced with K^+-rich and Ca^{++}-free Kreb's solution, having the following composition (mM): KCl 50, NaCl 50.58, $MgSO_4$ 3.10, $NaHCO_3$ 23.8, KH_2PO_4 1.26, glucose 11.1 and EDTA 0.1. After a period of 1 h, control CRC_S of Ca^{++} were constructed. When the control CRC_S of Ca^{++} were found super-imposable (after two cycles), the tissue was pre-treated with test drug for 45 to 55 min to determine the CCB action. The Ca^{++}-CRC_S were reconstructed in presence of different concentrations of the test material (Rehman *et al*., 2013; Mandukhail *et al*., 2014). Through force transducer (Fort/10, WPI, UK) attached to bridge-amplifier (Transbridge TBM 4M, WPI) and PowerLab ML 845 data acquisition system (AD Instruments, Sydney, Australia), isometric changes in tension were recorded and analyzed.

Statistical analysis: Data obtained are Mean±Standard error of the mean (SEM, n = Number of experiments) and the median effective concentrations (EC_{50}) with 95% Confidence Intervals (CI). Concentration-response curves were analyzed by non-linear regression through GraphPad program (GraphPAD, San Diego, CA, USA).

RESULTS

Effect on blood pressure: The intravenous administration of *Vitex negundo* aqueous-methanol extract caused a dose-dependent fall of arterial pressure in the anaesthetized rats. The percent fall of pressure at 10, 30 and 100 mg kg^{-1}

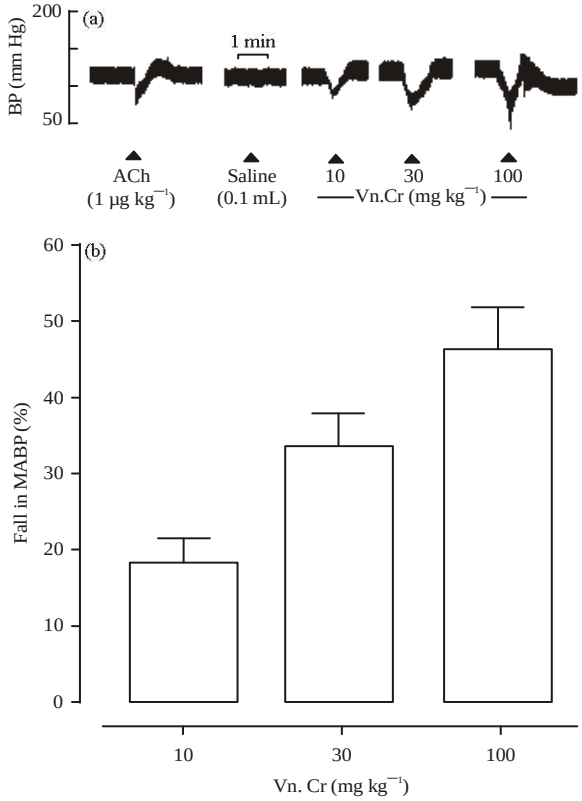

Fig. 1(a-b): (a) Typical tracing of *Vitex negundo* crude extract (Vn.Cr) Blood Pressure (BP)-lowering effect and (b) A bar chart representing hypotensive effect of Vn.Cr on Mean Arterial Blood Pressure (MABP) in anesthetized rats. The dose was administered after the response to the preceding one had returned to normal. Values shown represent Mean±SEM, n = 3

doses was 20.7±2.3, 34.2±3.7 and 49.5±2.4% (n = 3), respectively. Figure 1a shows tracing from a typical experiment, whereas the combined data from different experiments are presented in Fig. 1b.

Effect on isolated rat aorta: When tested against low K^+ (25 mM), high K^+ (80 mM) and PE (1 µM)-induced contractions, the Vn.Cr produced a vasodilator effect with respective EC_{50} values of 0.53 mg mL^{-1} (0.48-0.62, n = 3), 3.26 mg mL^{-1} (2.86-3.78, n = 3) and 3.48 mg mL^{-1} (3.24-4.18, n = 3) thus, showing more potency for low K^+ as compared to high K^+ and PE-induced contractions, as shown in Fig. 2a. When the inhibitory effect of Vn.Cr against low K^+ was reproduced in the presence of glibenclamide (10 µM) or TEA (1 mM), it was found that glibenclamide did not show any inhibitory effect whereas, TEA significantly reversed the inhibitory effect of Vn.Cr against low K^+ (Fig. 2b) evident in terms of rightward shift in the inhibitory concentration response curves of plant extract. Vn.Cr was also tested for its

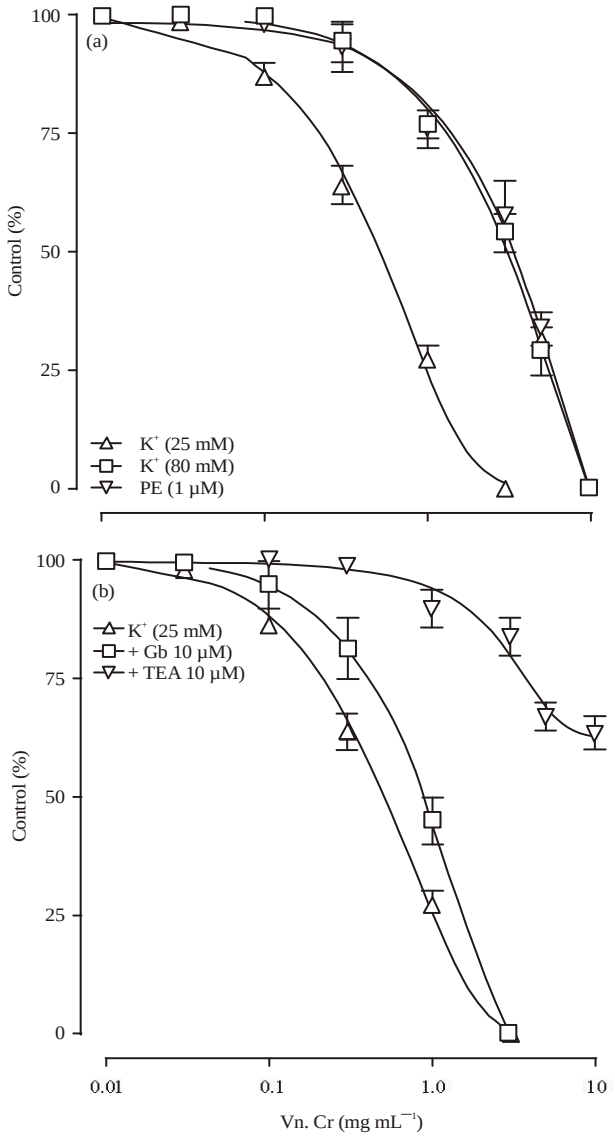

Fig. 2(a-b): Concentration-dependent relaxant effects of (a) *Vitex negundo* crude extract (Vn.Cr) on low K^+, phenylephrine (PE) and high K^+-induced contractions and (b) Vn.Cr on both; low K^+ alone and in the presence of glibenclamide (Gb) and tetraethylammonium (TEA), in isolated rat aortic ring preparations. Values shown are Mean±SEM, n = 3

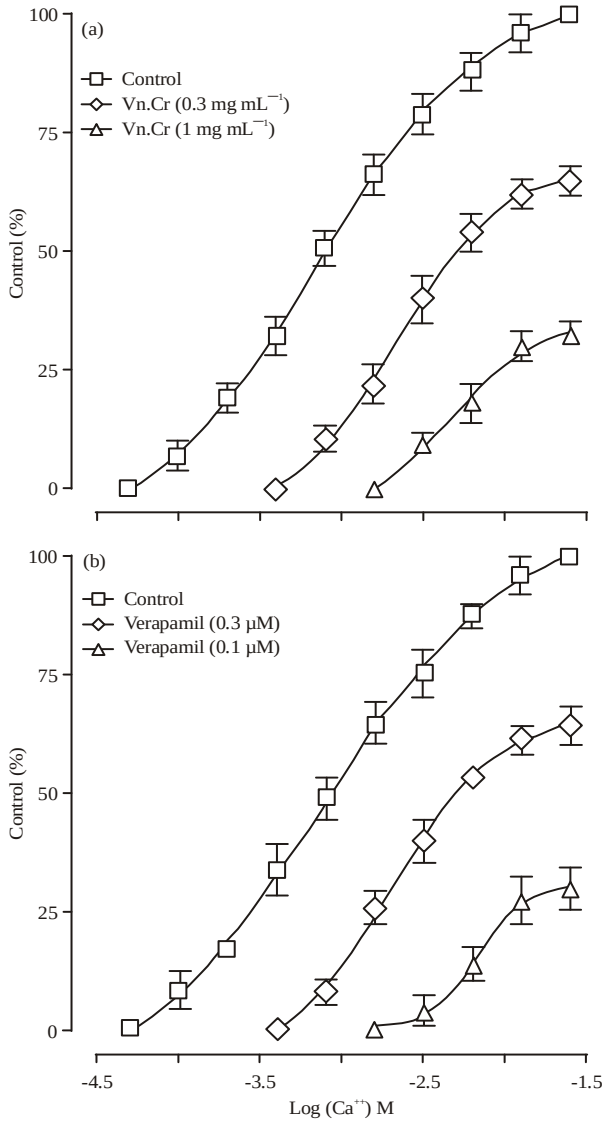

Fig. 3(a-b): Concentration-response curves of Ca^{++} in the absence and presence of different concentrations of (a) *Vitex negundo* crude extract (Vn.Cr) and (b) Verapamil in isolated rat aortic ring preparations. Values shown are Mean±SEM, n = 3

interaction with Ca^{++}, where it shifted the Ca^{++}-CRCs to the right with suppression of the maximum contraction (Fig. 3a). This rightward shift of Ca^{++}-curves was similar to that obtained with verapamil (0.03-0.1 µM, n = 3) as shown in Fig. 3b.

Effect on isolated paired atria: In isolated guinea-pig atria, Vn.Cr exhibited concentration-dependent inhibitory effect on the force and rate of spontaneous contractions at similar concentrations (Fig. 4a) with respective EC_{50} values of 5.56 (5.1-6.0, 95% CI, n = 3) and 5.87 mg mL^{-1} (5.4-6.22, n = 3). Similarly, verapamil caused concentration-dependent inhibitory effect equipotently with respective EC_{50} values of 0.76 (0.6-0.86, n = 3) and 0.98 M (0.84-1.18, n = 3) (Fig. 4b).

DISCUSSION

The crude extract of *Vitex negundo* (Vn Cr.), caused a fall in the arterial BP (dose-dependently), in anesthetized rats. Keeping in view that the BP is constituted of cardiac output and peripheral resistance (Johansen, 1992), the plant's effect

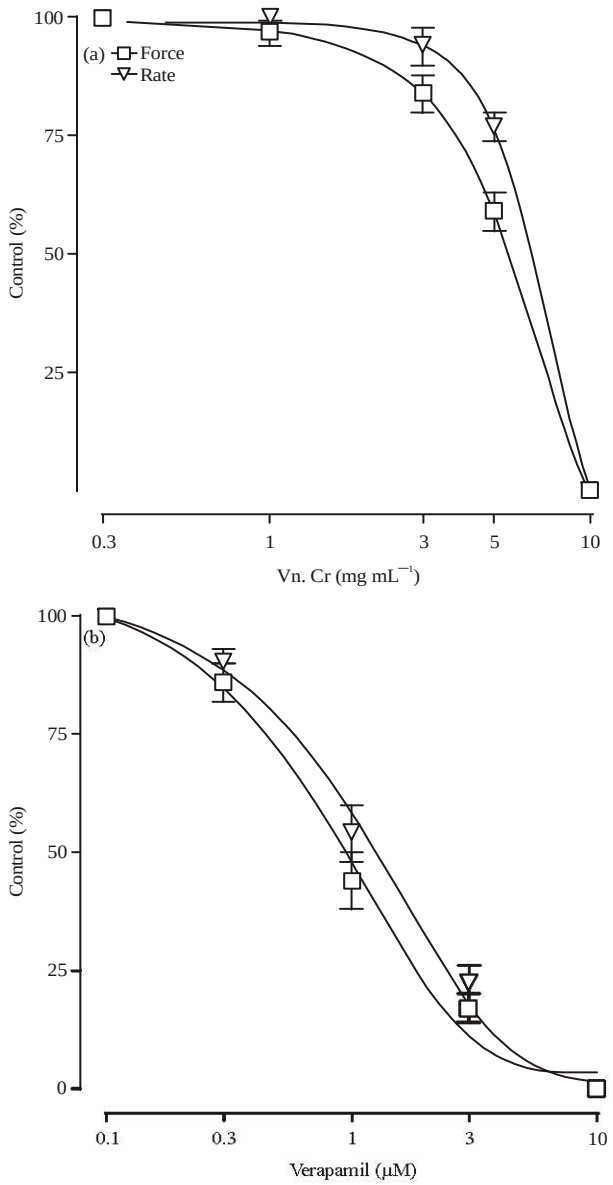

Fig. 4(a-b): Concentration-response curves showing the inhibitory effect of (a) *Vitex negundo* crude extract (Vn.Cr) and (b) Verapamil on force and rate of spontaneous contractions in isolated guinea pig right atria. Values shown are Mean±SEM, n = 3

was studied on heart and vascular tissues to check cardio-depressant and vasodilator actions.

For study on vascular resistance, Vn.Cr was tested in rat thoracic descending aorta to elucidate the underlying mechanism(s) responsible for lowering BP (Khan *et al*., 2014). In isolated rat aorta tissues, *V. negundo* extract was screened against low K^+, high K^+ and PE-induced contractions, thus to distinguish among activities at the activation of K^+ channels, inhibition of voltage-operated and receptor-operated Ca^{++} channels (Shah *et al*., 2014). Vn.Cr relaxed with higher potency the low K^+-induced contractions as compared to high K^+ and PE-induced contractions in aortic rings, indicating that it was acting predominantly through activation of K^+ channels followed by blockade of voltage and receptor-operated Ca^{++} channels (Okumura *et al*., 1993; Musha *et al*., 2005). As the Vn.Cr was found relatively more potent against low K^+-induced contractions (having 10 times lower EC_{50}), further experiments were conducted to know the type of K^+ channels involved in the vasodilatory effect of Vn.Cr. TEA, a non-specific blocker of K^+ channels (Cook, 1989), significantly inhibited the inhibitory effect of Vn.Cr whereas, glibenclamide, an ATP-dependent K^+ channel blocker (Franck *et al*., 1994) remain ineffective, hence indicating the involvement of non-specific K^+ channels. Moreover, Vn.Cr, at higher doses, was also found active against high K^+ and PE-induced contractions, showing the involvement of voltage-dependent Ca^{++} channel blocking effect. The CCB effect of *Vitex negundo* was confirmed, when it shifted the Ca^{++} curves, constructed in Ca^{++} free environment to the right, with the suppression of maximum contractile response, like that caused by verapamil, a standard Ca^{++} antagonist (Fleckenstein, 1977). Vn.Cr was more potent in its inhibitory effect on vascular tissues than cardiac. There is sufficient evidence of heterogeneity of Ca^{++} channels and different Ca^{++} antagonists exhibit selectivity for different organ systems (Farre *et al*., 1991). For example, dihydropyridine antagonists are considered selective for vascular tissues and are more commonly used to decrease blood pressure (Joseph and Barry, 1999).

In guinea-pig atria, Vn.Cr exhibited negative inotropic and chronotropic effects, similar to that caused by verapamil, a standard Ca^{++} channel blocker (Fleckenstein, 1977). The cardiac inhibitory action of the *Vitex negundo* may be due to Ca^{++} antagonist effect, which results in decrease in cardiac output, thus leading to fall in BP.

In conclusion, this study showed that *Vitex negundo* exhibits BP-lowering, cardio-depressant and vasodilatory effects mediated predominantly through activation of non-specific K^+channels followed by inhibitory effect on voltage-dependent Ca^{++} channel. Thus, this study presents the therapeutic potential of *Vitex negundo* to be a useful candidate in hypertension.

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