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Studies on Cardio-suppressant, Vasodilator and Tracheal Relaxant Effects of *Sarcococca saligna*

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Sarcococca saligna is a shrub that is traditionally used for its medicinal properties in Pakistan. In this study we report the cardio-suppressant, vasodilator and tracheal relaxant activities of the aqueous-methanolic extract (Ss.Cr) of the plant. Ss.Cr, that tested positive for the presence of saponins, flavonoids, tannins, phenols, and alkaloids, exhibited a dose-dependent (0.3-5 mg/mL) negative inotropic and chronotropic effect on the isolated guinea-pig atrium which was resistant to atropine (1 μM) and aminophylline (10 μM) pretreatment. In rabbit thoracic aorta, Ss.Cr dose-dependently (0.1-3 mg/mL) relaxed the high K^+ (80 mM) and phenylephrine (PE, 1 μM)-induced contractions, indicating a possible Ca^{++} channel blocking (CCB) effect. When tested against PE (1 μM) control peaks in normal Ca^{++} and Ca^{++}-free Kreb's solution, Ss.Cr exhibited dose-dependent (0.1-3 mg/mL) inhibition, being more potent in relaxing the PE responses in Ca^{++}-free Kreb's solution, thus indicating specific blockade of Ca^{++} release from the intracellular stores. Ss.Cr also relaxed the agonist-induced contractions in: a) rat aorta irrespective of the presence of endothelium or nitric oxide synthase inhibitor L-NAME and b) rabbit and guinea-pig tracheal strips. The data shows that Ss.Cr possesses possible Ca^{++} channel blocking activity which might be responsible for its observed cardio-suppressant, vasodilator and tracheal relaxant effects though more tests are required to confirm this Ca^{++} channel blocking effect.

Key words: *Sarcococca saligna*, Intracellular Ca^{++} release inhibitor, Cardio-suppressant, Vasodilator, Tracheal relaxant

INTRODUCTION

Sarcococca saligna (D. Don) Muel. Arg. (family, Buxaceae) is a shrub that is widely distributed in the Himalayan region from Afghanistan to the West of Nepal. In the northern areas of Pakistan, it is locally known as 'ban sathra' while is known as 'sweet box' or 'Christmas box' in the west. Different aerial parts, and the whole plant, are traditionally used by the local population for a variety of diseases (Shinwari *et al.*, 2003). The plant has been used in Pakistan for the hyperactive states of the gastrointestinal tract, liver diseases, syphilis, infections, fever, pain, inflammation and rheumatism (Kohli *et al.*, 1967; Rahman *et al.*, 2000, 2003). Of the very few pharmacological studies done on the plant, it is reported to have antibacterial (Rahman *et al.*, 1997, 1998a), antitumor and antiulcer (Qiu *et al.*, 1994) and antidiarrhoeal, antisecretory and acetylcholinesterase inhibitory activities (Gilani *et al.*, 2005). Chemically, the plant is reported to possess steroidal alkaloids, due to the presence of which is responsible for most of the reported activities (Miana and Kiamuddin, 1969; Rahman *et al.*, 1998a, 2000).

The plant is also used in heart diseases (hypertension) and respiratory disorders (Kirtikar and Basu, 1933; Rahman *et al.*, 1998). Thus in this investigation, we report the cardio-suppressant, endothelium-independent vasodilator and tracheal relaxant activities of the aqueous-methanolic crude extract of *S. saligna* using different isolated tissue preparations.

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MATERIALS AND METHODS

Drugs and standards

The following reference chemicals were obtained from the sources specified: acetylcholine chloride (ACh), aminophylline hydrate, atropine sulphate, carbachol chloride (CCh), N_{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME), norepinephrine hydrochloride (NE), phenylephrine hydrochloride (PE) and verapamil hydrochloride (Sigma Chemical Company, St. Louis, MO, U.S.A.). The following chemicals were used to make the physiological salt solutions: potassium chloride (Sigma Chemical Company, St. Louis, MO, U.S.A.), calcium chloride, glucose, magnesium chloride, magnesium sulphate, potassium dihydrogen phosphate, sodium bicarbonate, sodium chloride and sodium dihydrogen phosphate (E. Merck, Darmstadt, Germany) and ethylenediaminetetra-acetic acid (EDTA) (BDH Laboratory Supplies, Poole, England). All chemicals used were of the highest purity grade. Stock solutions of all the chemicals were made in saline and the dilutions were made fresh on the day of the experiment.

Animals

Experiments performed complied with the rules of the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council (NRC, 1996). Balb-C mice (20-25 g), Sprague-Dawley rats (170-200 g), rabbits (1 kg) and guinea-pigs (500-600 g) of either sex (only male rats were used for aorta) used in the study were bred and housed in the animal house of Aga Khan University under a controlled environment (23-25°C). Animals were given tap water *ad libitum* and a standard diet consisting of (g/kg): flour 380, fibre 380, molasses 12, sodium chloride 5.8, nutrivet L 2.5, potassium metabisulphate 1.2, vegetable oil 38, fish meal 170 and powdered milk 150.

Plant material and extraction procedure

The whole plant of *Sarcococca saligna* was acquired fresh from the region of Kashmir in Northern Pakistan in the month of September. After verification, a sample of the plant was deposited at the Herbarium of Department of Biological and Biomedical Sciences, The Aga Khan University, Karachi with the voucher # SS-PL-11-01-35. The plant material was cleaned of any contaminants, soaked in 6 L of 80% aqueous-methanol and kept for a total of 3 days. After three days, it was filtered through a porous cloth and the filtrate collected while the plant material was soaked again in 6 L of the solvent for 3 days. At the end, all of the filtrate was combined, filtered through Whatman qualitative Grade-1 filter paper and then concentrated in a rotary evaporator to obtain a viscous, dark brown coloured crude extract (Ss.Cr) weighing 53.1 g (yield of 21.5%, w/w).

Preliminary phytochemical analysis

The crude extract was screened qualitatively with different organic solvents and reagents for the presence of some phyto-constituents such as saponins, flavonoids, tannins, phenols, coumarins, sterols, terpenes, alkaloids and anthraquinones (Tona *et al.*, 1998). Briefly, saponins were detected by observation of any froth formation following rigorous shaking of the extract dissolved in distilled water. Testing for flavonoids required mixing the extract with $AlCl_3$ and yellow colouration indicated a positive test. Presence of phenols and tannins was determined by appearance of any green or dark green colour after dissolution of extract in aqueous $FeCl_3$. For detecting coumarins, a piece of filter paper was moistened in NaOH and then kept over a test tube with boiling plant extract solution. If the filter paper later showed any yellow fluorescence under UV light, that was taken to indicate a positive test for coumarins. Detection for any sterols and terpenes in the extract involved treatment of the extract with petroleum ether and followed by extraction with $CHCl_3$. The subsequently acquired $CHCl_3$ layer was treated with acetic anhydride and concentrated HCl. The change of pink to purple and green to pink colours was indicative of presence of terpenes or sterols, respectively. Alkaloids were screened by mixing with Dragendorff's reagent. Lastly for detecting anthraquinones, the extract was dissolved in 1% HCl, then in benzene and later if the extract showed pink, violet or red colour with NH_4OH, that indicated a positive test for the presence of anthraquinones.

Isolated guinea-pig atria

The isolated tissue experiments were carried out as previously described (Gilani *et al.*, 1994). Right and left atria from guinea-pigs, killed by cervical dislocation, were mounted separately in 20 mL tissue baths containing Kreb's solution maintained at 32°C and aerated with 5% carbon dioxide in oxygen (carbogen). The composition of Kreb's solution was (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 tissues were allowed to beat spontaneously under the resting tension of 1 g. An equilibrium period of 30 min was given before the application of any drug. Control responses of verapamil (0.3-10 nM) and NE (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.

Isolated rabbit aorta

Rabbits were sacrificed by cervical dislocation. The descending thoracic aorta was removed and cut into 2-3

mm wide rings which were individually mounted in 20 mL tissue baths containing Kreb's solution at 37°C and aerated with carbogen gas. A resting tension of 2 g was applied to each tissue and an equilibrium period of 1 h was allowed before any experimentation. The changes in isometric tensions of the rings were measured via a force-displacement transducer (FT-03) using a Grass Model 7 Polygraph. Following equilibrium period of 1 h, the tissues were stabilized with a fixed dose of PE (1 μM). The tissues were considered stable only when similar responses were obtained from the repeated doses of PE. Effect of the extract was first determined on the resting baseline of the tissue to see if it has any vasoconstrictor effect. The extract was later tested for its ability to relax the contractions induced with PE (1 μM) and high K^+ (80 mM). The ability of the extract to relax K^+ (80 mM)-induced contractions would indicate L-type voltage-operated calcium channel blocking (CCB) mode of vasodilation while inhibition of the PE-induced contractions would signify the blockade of the Ca^{++} influx through the receptor-operated calcium channels (Karaki *et al.*, 1997). In order to study Ca^{++} antagonistic effects of the extract, the tissues were pretreated with hexamethonium for blockade of ganglions. In order to determine if the extract was inhibiting the Ca^{++} release from intracellular stores or Ca^{++} influx across the cell membrane (through the voltage- or receptor-operated channels), the effect of increasing doses of the extract was observed on PE 1 μM peaks (pretreated with each of the increasing extract dose for 1 h) in normal Ca^{++} and in Ca^{++}-free Kreb's solution Ca^{++} omitted and EDTA (0.1 mM) added to ensure total elimination of extracellular Ca^{++} without harmful effects on Ca^{++} inside the cell (Guan *et al.*, 1988). In Ca^{++}-free Kreb's solution, PE acts through stimulation of a_1-adrenergic receptors and then the consequent conversion of phosphatidylinositol to inositol-1,4,5-trisphosphate releases Ca^{++} from the intracellular stores thus bringing about the contraction (Hashimoto *et al.*, 1986). While in normal Ca^{++} Kreb's solution, the PE-stimulated contractions come about possibly through the influx of Ca^{++} *via* a combination of the receptor-operated non-selective cation channels and L-type voltage-operated Ca^{++} channels (Karaki *et al.*, 1997).

Isolated endothelium-intact rat aorta

The procedure of Furchgott and Zawadski (Furchgott and Zawadski, 1980) was followed with some modifications. Thoracic aorta was isolated from male rats. Care was taken in isolating the tissue to avoid any damage to endothelium. Rings, 3 mm wide, were mounted in 5 mL tissue baths with Kreb's solution at 37°C and aerated with carbogen gas. A preload of 1 g was applied to the preparation and the tissue were allowed to incubate for 30 min. Changes in tension were recorded *via* World Precision Instrument's (WPI) Isometric Force transducers (Fort 100) connected to Transbridge 4M and displayed on to a Personal Computer *via* CVMS Data Acquisition System. Following equilibrium period of 30 min, the tissues were stabilized with repeated doses of PE (1 μM). After stabilization, an induced contraction was obtained with PE (1 μM). Once a plateau was achieved, ACh (0.1 μM) was administered upon this PE-induced contraction to confirm endothelium-dependent relaxation. The endothelium lining of the tissues was removed by gentle rubbing, which resulted in the disappearance of this relaxation. To study whether or not the vasodilator effect of the test substance is endothelium-dependent, the PE (1 μM)–induced contraction was preincubated with L-NAME (0.1 mM) for 60 min, to explore for the possibility of an endothelium-dependent vasodilator action (Vanhoutte *et al.*, 1986).

Isolated rabbit and guinea-pig trachea

Trachea was dissected out and kept in Kreb's solution. The tracheal tube was cut into rings, 2-3 mm wide, each containing about 2 cartilages. Each ring was opened by a longitudinal cut on the ventral side opposite to the smooth muscle layer, forming a tracheal strip with a central part of smooth muscle in between the cartilaginous portions on the edges. The preparation was then mounted in a 20 mL tissue bath containing Kreb's solution maintained at 37°C and aerated with carbogen gas. A tension of 1 g was applied to each of the tracheal strip and was kept constant throughout the experiment. The tissue was equilibrated for 1 h after which contractile responses to sub-maximal concentrations of carbachol (CCh) (1 μM) were recorded until constant responses were obtained with a dose interval of 45 min, thus allowing studying the effect of the extract firstly on the resting baseline of the tracheal strip and later against CCh and K^+-induced contractions for a possible tracheal relaxant effect.

Acute toxicity assessment

Animals were divided in groups of 5 mice each and given increasing doses of the extract (1 and 3 g/kg) orally, (in 10 mL/kg volume). Another group of mice was administered saline (10 mL/kg, p.o.) as negative control. The mice were allowed food *ad libitum* and kept under regular observation for mortality up to 24 h after administration of Ss.Cr.

Data analysis

All the data expressed are mean ± standard error of mean (SEM, n=number of experiment) and the median effective concentrations (EC_{50}) with 95% confidence intervals (CI). The statistical parameter applied is analysis of variance (ANOVA) by use of GraphPad program (GraphPAD, San Diego, CA, U.S.A.). A probability less

than 0.05 was considered statistically significant. Concentration-response curves were analysed by non-linear regression (GraphPAD program).

RESULTS

Phytochemical analysis

Ss.Cr showed the presence of saponins, flavonoids, tannins, phenols and alkaloids. None of the other classes of compounds were tested positive in the crude extract.

Effect on guinea-pig atria

Ss.Cr exhibited an inhibitory effect on the force (Fig. 1A) and rate of spontaneous atrial contractions (Fig. 1B). The inhibitory effect was dose-dependent at the dose range of 0.3 to 5.0 mg/mL (Fig. 1) with an EC_{50} value of 2.90 mg/mL (2.42-3.38, 95% CI, n=4) and 3.02 mg/mL (2.83-3.21, n=4) for the force and rate of atrial contraction, respectively. This inhibitory effect of the extract was resistant to the blockade by atropine (1 μM) and aminophylline (10 μM)

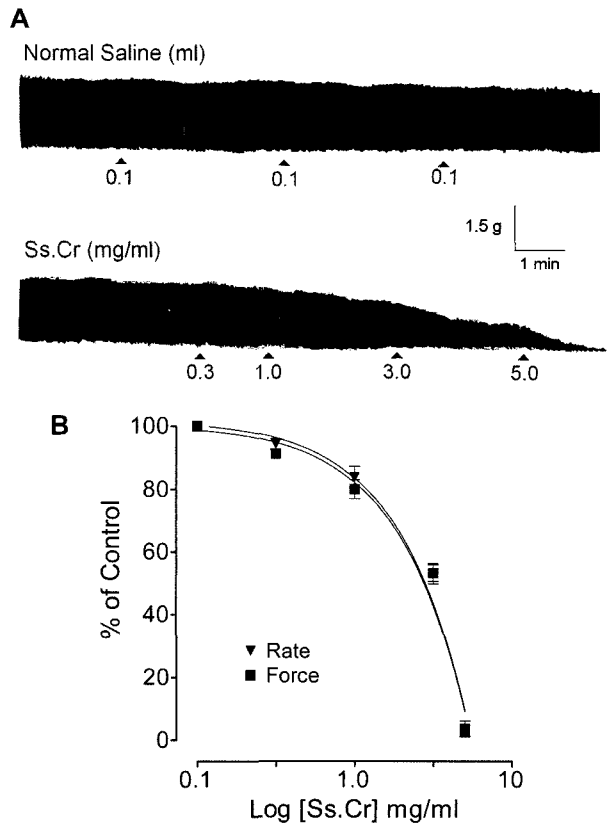

Fig. 1. Typical tracing and dose-response curves showing the inhibitory effect of *Sarcococca saligna* crude extract (Ss.Cr) on **[A]** force and **[B]** force and rate of spontaneous atrial contractions in isolated guinea-pig atria (values shown are mean ± SEM, n=4). In **[B]**, difference was significant between individual doses within the two curves ($P < 0.001$) but the curves were not different from each other ($P > 0.05$); two -way ANOVA.

pretreatment (data not shown).

Effect on rabbit aorta

When tested on the resting base line of the rabbit aorta, Ss.Cr was devoid of any stimulatory effect till the dose of 10 mg/mL. The extract, when tested on high K^+ (80 mM) and PE (1 μM)-induced contractions, produced a dose-dependent vasodilation (Fig. 2A), at a similar dose range (0.1-3.0 mg/mL), with EC_{50} values of 0.41 mg/mL (0.25-0.57, n=4) and 0.55 mg/mL (0.45-0.65, n=4), respectively. When the extract was tested in increasing doses against PE (1 μM) control responses in normal Ca^{++} and Ca^{++}-free Kreb's solution, Ss.Cr (0.1-3 mg/mL) completely relaxed the agonist peaks (Figs. 2B and 2C) with EC_{50} values of 0.95 mg/mL (0.85-1.05, n=4) and 0.40 mg/mL (0.08-0.72, n=4), respectively, being more potent in the latter than the former ($P < 0.001$).

Effect on endothelium-intact rat aorta

Ss.Cr was devoid of any vasoconstrictor activity on the rat aorta baseline till 10 mg/ml. Ss.Cr was then tested on the endothelium-intact rat aorta preparations precontracted with high K^+ (80 mM) and PE (1 μM). The extract inhibited both of the induced contractions (Fig. 3A) at a similar dose range (0.1-3.0 mg/mL) with EC_{50} values of 0.45 mg/mL (0.29-0.61, n=4) and 0.71 mg/mL (0.46-0.96, n=4), respectively. The extract also inhibited the PE (1 mM)-induced contractions, in a similar fashion, in the presence of L-NAME (0.1 mM) pretreatment and in the endothelium-denuded preparations (Fig. 3B) with EC_{50} values of 0.61 mg/mL (0.48-0.74, n=4) and 0.75 mg/mL (0.69-0.81, n=4).

Effect on rabbit and guinea-pig trachea

The extract was devoid of any contractile effect on the tracheal preparations of rabbit and guinea-pig. When tested on agonist-induced contractions, Ss.Cr dose-dependently (0.03-1.0 mg/mL) inhibited the CCh (1 μM) and high K^+ (80 mM)-induced contractions in rabbit (Fig. 4A) with respective EC_{50} values of 0.14 mg/mL (0.11-0.17, n=4) and 0.15 mg/mL (0.12-0.18, n=4), while in the guinea-pig (Fig. 4B) with EC_{50} values of 0.22 mg/ml (0.09-0.35, n=3) and 0.20 mg/ml (0.16-0.24, n=3), respectively.

Acute toxicity assessment

No lethality was observed in mice up to the dose of 3 g/kg.

DISCUSSION

When tested on the isolated guinea-pig atria, the crude extract of *Sarcococca saligna* depressed the force and rate of spontaneous atrial contractions in a dose-depen-

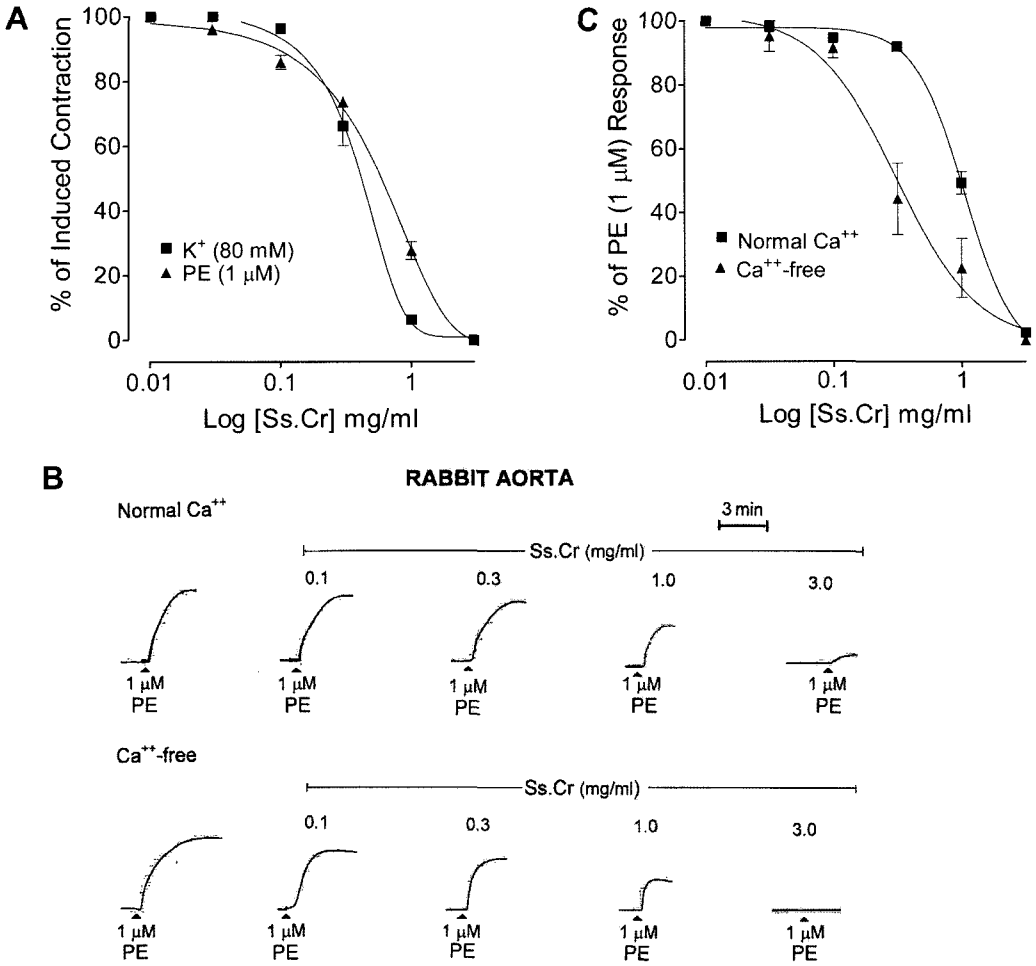

Fig. 2. Figure showing **[A]** dose-response curves for inhibitory effect of *Sarcococca saligna* crude extract (Ss.Cr) on high K^+ and phenylephrine (PE)-induced contractions in rabbit aorta preparations. **[B]** shows a typical tracing while **[C]** shows the corresponding curves for PE control responses (1 μM) in normal Ca^{++} and Ca^{++}-free Kreb's solution in rabbit aorta (values shown are mean ± SEM, n=4). In **[A]** and **[C]**, the difference was significant within individual doses in all the curves ($P < 0.001$). The curves in **[A]** were not different ($P > 0.05$) while the curves in **[C]** were significantly different from each other ($P < 0.001$); two-way ANOVA.

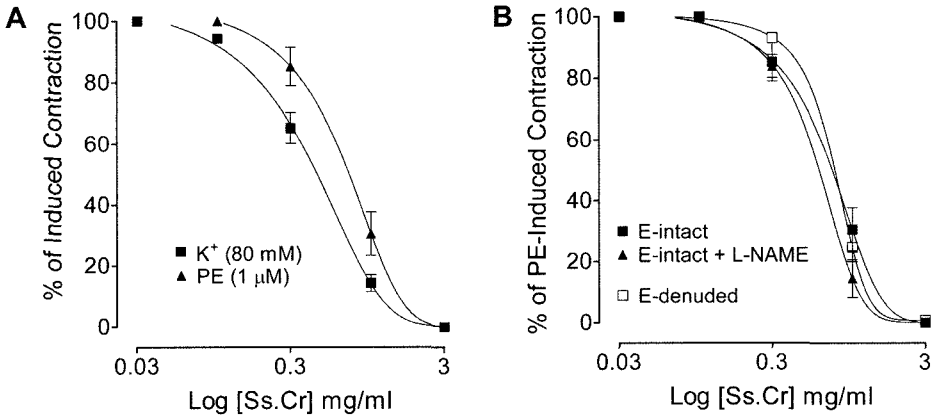

Fig. 3. Dose-response curves showing the inhibitory effect of *Sarcococca saligna* crude extract (Ss.Cr) on: **[A]** high K^+ and phenylephrine (PE)-induced contractions in isolated endothelium-intact rat aorta, and **[B]** on PE-induced contractions in the absence and presence of L-NAME (0.1 mM) in endothelium-intact (E-intact, black symbols) and denuded (E-denuded, hollow symbols) rat aorta preparations (values shown are mean ± SEM, n=4-6). In **[A]** and **[B]**, the difference was significant within individual doses in all the curves ($P < 0.001$) but the curves were not significantly different from each other ($P > 0.05$); two-way ANOVA.

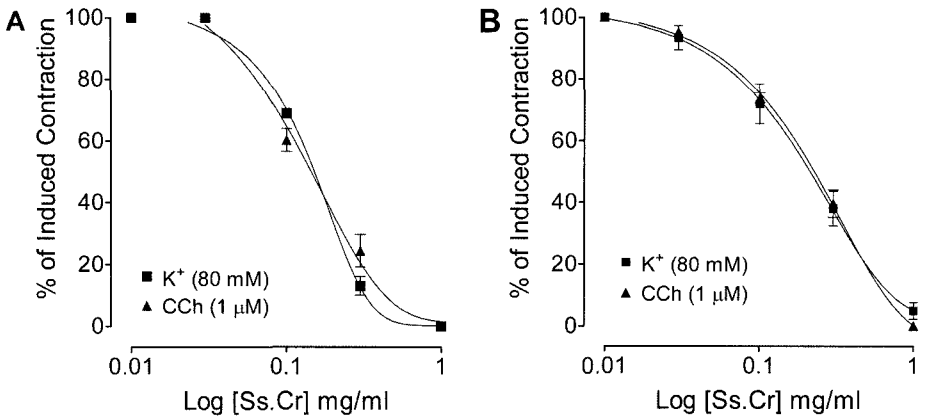

Fig. 4. Dose-response curves showing the inhibitory effect of *Sarcococca saligna* crude extract (Ss.Cr) on high K^+ and carbachol (CCh)-induced contractions in: **[A]** isolated rabbit, and **[B]** guinea-pig tracheal preparations (values shown are mean ± SEM, n=3-4). In **[A]** and **[B]**, there was significant difference within individual doses in all the curves ($P < 0.001$) but the curves were not significantly different from each other ($P > 0.05$); two-way ANOVA.

dent manner. This inhibitory effect on the cardiac tissues was resistant to the blockade by atropine, a cholinergic receptor antagonist (Arunlakhshana and Schild, 1959; Gilani and Cobbin, 1986) and aminophylline, an adenosine receptor blocker (Fredholm and Presson, 1982), indicating that the cardio-suppressant effect of the extract is mediated independent of the cholinergic and adenosine receptor activation.

To see the effect of Ss.Cr on vascular resistance, two different vascular tissues were selected. The rabbit aorta was selected: a) to evaluate the effect of the extract on K^+ and PE-induced contractions and thus to distinguish between activity at the voltage-operated and receptor-operated channels and b) to distinguish the inhibitory effect of the crude extract on membrane bound Ca^{++} channels and those inside the cells. Rabbit aorta is routinely used for the screening of CCBs (Gilani *et al.*, 1994). The second vascular preparation used was rat aorta which is a prototype tissue used for evaluating the endothelium-dependent or independent vasodilatation (Ajay *et al.*, 2003). Rat aorta was selected a) to evaluate the effect of the extract on K^+ and PE-induced contractions and b) to determine if the vasodilator effect of the extract is endothelium-dependent or independent.

Ss.Cr inhibited the PE and high K^+-induced contractions in rabbit aorta, at a similar dose range, indicating that it was acting equipotently through possible blockade of voltage- and receptor-operated Ca^{++} channels (Karaki *et al.*, 1997). The cardio-suppressant and vasodilator activities of the extract are in line with its use in cardiovascular disorders especially in hypertension (Kirtikar and Basu, 1933; Rahman *et al.*, 1998).

Smooth muscle contraction is brought about by the activation of the 1) membrane bound Ca^{++} channels which are the voltage-operated and receptor-operated Ca^{++} channels (Taggart *et al.*, 1997), but this is not the only mechanism for contractility. Ca^{++} influx into the cell can also be guided through 2) Ca^{++} release from the internal stores of inositol triphosphate (IP_3)-sensitive sarcoplasmic reticulum as well (Benham *et al.*, 1986). To assess the activity of the extract on Ca^{++} release from the intracellular stores, PE control responses were taken in the absence and presence of Ss.Cr in rabbit aorta. The crude extract in increasing doses inhibited the agonist peak responses in normal-Ca^{++} and in Ca^{++}-free environment. The inhibitory effect of the extract on PE peaks in a Ca^{++}-free environment was most potent compared to normal-Ca^{++} Kreb's solution, which indicated that the extract was acting *via* specific blockade of the intracellular Ca^{++} release (Hashimoto *et al.*, 1986).

The extract was able to inhibit the high K^+ and PE-induced contractions in endothelium-intact rat aorta. The rat aorta helped in determining that the vasodilator effect of the crude extract was independent of the endothelium as was evident from the fact that the vasodilator effect of Ss.Cr in the endothelium-intact rat aorta was not blocked by L-NAME, a standard nitric oxide synthase inhibitor (Thorin *et al.*, 1998). The vascular endothelium plays a pivotal role in modulating the contractility of the vascular tone through the release of vasodilator and constrictor factors (Vanhoutte *et al.*, 1986). The claim that the vasodilator effect was endothelium-independent was further strengthened when the rat aorta was denuded of endothelium and even then the extract exhibited a relaxant effect on PE-induced contractions at the same concentration as in the intact preparation.

In isolated tracheal chains from rabbit and guinea-pig, the extract dose-dependently inhibited the high K^+ and CCh-induced contractions with a similar potency. The results indicate a tracheal relaxant effect of the extract,

possibly mediated *via* Ca^{++} antagonism. CCBs are known to be useful as tracheal relaxants in disorders characterized by hypermotility of the respiratory tract (Kamei and Kasuya, 1992).

Preliminary phytochemical analysis of the extract showed the presence of saponins, flavonoids, tannins, phenols and alkaloids. Flavonoids have been shown to possess vasodilator activities through endothelium-dependent, -independent and CCB mechanisms (Taggart *et al.*, 1997) while they have also been shown to be protective against cardiovascular diseases (Knekt *et al.*, 1996). Ss.Cr was found to be safe in mice for 24 h when tested up to the dose of 3 g/kg.

The results of the study show that the *S. saligna* crude extract exhibits cardio-suppressant, vasodilator (endothelium-independent) and tracheal relaxant activities possibility mediated through blockade of Ca^{++} channels. From the experiments performed in rabbit aorta with the Ca^{++} levels in the environment of the tissue manipulated, it was observed that the extract was specific in inhibiting the Ca^{++} release from the intracellular Ca^{++} stores. As most of the tests performed only gave an indication into the possible Ca^{++} antagonist effect of the extract, more detailed experiments are required to confirm this Ca^{++} blocking effect possibly *via* use of Ca^{++} fluorescent imaging.

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