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SPASMOLYTIC, BRONCHODILATOR AND VASORELAXANT ACTIVITY OF METHANOLIC EXTRACT OF *TEPHROSIA PURPUREA*

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Abstract: The methanolic extract of the whole plant of *Tephrosia pupurea,* Linn. was subjected to find out its possible therapeutic utility to validate its folkloric use in native systems of medicine. The extract on application to spontaneous contractions in isolated rabbit jejunum preparations exerted a concentration dependent (0.003-3.0 mg/mL) relaxant effect. The extract also caused concentration dependent relaxation of K^+(80 mM)-induced spastic contractions. These findings were further supported by the observations that the extract caused a concentration dependent right ward shift of the Ca^{2+} response curves in manner similar to that of verapamil. The extract exhibited a relaxant effect on carbachol and high K^+(80 mM)-induced contractions of isolated rabbit tracheal preparations in a manner similar to verapamil. The observed non-specific bronchodilator response is possibly mediated through Ca^{2+} channel blockade. Moreover, the extract also exhibited a dose dependent relaxant effect on phenylephrine (1 μM) and K^+(80 mM)-induced contractions in a manner similar to verapamil. On the basis of the above-mentioned findings, it can be concluded that the use of *Tephrosia purpurea,* in gastrointestinal spasm, asthma and hypertension is likely to be mediated through calcium channel blockage.

Keywords: methanolic extract, *Tephrosia pupurea,* bronchodilator, vasorelaxant

Tephrosia purpurea, (Syn: *Galega purpurea*, Linn.; Family: Fabaceae) is locally known as Bansa (Punjabi), Sarphunka (Hindi) and Jangli kulthi (Sindhi). It grows wild throughout Indo-Pak-Bangla Desh subcontinent on hard and stony grounds (1-3). It is branched and sub-erect herbaceous perennial plant, about 30-60 cm in height with spreading branches; leaves imparipinnate, leaflets 11-21, narrow, oblanceloate; flowers red or purple in extra axillary racemes; fruits are slightly curved pods, 3-4 cm long, seeds 5-10 per pod, smooth, shiny grey in coloration. The plants are propagated through seeds (4, 5).

Phytochemical investigations revealed the presence of β-sitosterol, quercetin, lupeol, rutin, delphinidine chloride, cyaniding chloride, isolonchocarpin, lanceolatins A and B, pongamol, karangin, kangone, 5,7-dimethoxy-8-flavanone, 2-methoxy-3,9-dihydroxycoumestone, flevichapparins B and C, methyl karanjic acid and purpurin among the plant constituents (6, 7).

Resistance to the present compounds against management of different diseases has lead to search for the new candidates (8, 9). *Tephrosia purpurea* is reputed to possess diuretic, antipyretic, anti-inflammatory, anti-ulcer, anti-asthmatic, anti-leprosy and anthelmintic properties (10, 11). The aim of this study was to evaluate spasmolytic, bronchodilator and vasorelaxant activity of methanolic extract of *Tephrosia purpurea*.

MATERIALS AND METHODS

Plant material

Whole plant of *Tephrosia purpurea* was collected from the local herbal market of Multan, Pakistan. These plant material was identified/authenticated by the kind cooperation of an expert taxonomist (Prof. Dr. Altaf Ahmad Dasti) at the Institute of Pure and Applied Biology, Bahauddin Zakariya University, Multan and voucher specimen was deposited in the herbarium.

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Preparation of crude extract

The herbal material was shade dried and rendered free of any dust particles or adulterated materials by manual picking. It was subsequently grinded to coarse powder by an electrically driven grinding machine. About 500 g of the coarsely grinded powder materials was soaked in 80% aqueous methanol for eight days with occasional shaking (11). The material was passed through double layered muslin cloth to get rid of organic debris and the fluid portion was filtered through Whatman grade 1 filter paper. The collected filtrate was subsequently concentrated to thick semi solid mass at 37°C on a rotary evaporator (R210, Buchi, Switzerland) under reduced pressure and was dried further through freeze drying and transferred to final containers to kept in refrigerator (-4°C). The approximate yield was 4.0%. Different dilutions of the crude extract were made fresh on the day of experiment.

Chemicals

Acetylcholine chloride, atropine sulfate, carbachol (CCh), histamine, isoprenaline, pyrillamine, potassium chloride, verapamil hydrochloride and phenylephrine (PE), magnesium chloride, ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma Chemicals Co. St Louis, MO, USA. Calcium chloride, glucose, magnesium sulfate, potassium dihydrogen phosphate, sodium bicarbonate, sodium dihydrogen phosphate, and methanol were obtained from Merck, Darmstadt, Germany. Ammonium hydroxide, sodium chloride, and sodium hydroxide were purchased from BDH Laboratory supplies, Poole, England.

The chemicals used in these experiments were of the highest purity and reagent analytical research grade. Stock solutions and subsequent dilutions were made fresh in distilled water on the day of experiment. The drugs were solublized in vehicles which were without any effect on tissue contractility in control experiments.

Animals and housing conditions

Animals (♂/♀) used in this study were rabbits (1.0-1.8 kg), guinea-pigs (500-600 g), Bulb-c mice (20-30 g) and Sprague-Dawley rats (200-300 g). They were housed under controlled environmental condition (23-25°C) at the animal house of The Aga Khan University, Karachi. The animals were given standard diet and tap water *ad libitum*. The animals were deprived of food 24 h prior to the experiments but were given free access to water. Rabbits were sacrificed following a blow on back of head, while rats and guinea-pigs were killed by cervical dislocation to be used for *in vitro* studies, whereas mice were used for the *in vivo* studies. All the experiments performed complied with the rulings of Institute of Laboratory Animal Resources, Commission on Life Sciences (NRC, 1996), approved by the Ethical Committee of The Aga Khan University, Karachi.

Preliminary phytochemical studies

The crude methanolic extract of *Tephrosia purpurea* (Tp.Cr) was subjected to qualitative phytochemical analysis for the presence of alkaloids, saponins, anthraquinones, coumarins, sterols, terpenes, flavonoids and phenols as described by Tona et al. (12).

The crude plant extracts were subjected to preliminary phytochemical screening for qualitative detection of the presence of some constituents of particular phytochemical classes (13). The saponin presence was detected by the froth formation on vigorous shaking of the aqueous extract solution. Development of blue green or dark green coloration on mixing of aqueous $FeCl_3$ with extract solution indicated the presence of phenols and tannins. The coumarins as plant constituents were detected on emission of fluorescence in ultra-violet light from pieces of filter paper which were exposed to the vapors emerging from boiling aqueous solution of plant extract, subsequent to treatment with NaOH. The alkaloid presence was noted by the appearance of yellowish brown coloration on mixing of Dragendorff's reagent (solution of potassium bismuth iodide) with HCl treated aqueous plant extract solution. The appearance of pink, violet or red coloration on exposure to NH_4OH of the mixture of benzene with aqueous solution of plant extract already acidified with 1% HCl was taken as the presence of anthraquinones among the plant constituents.

***In vitro* experiments**

Isolated tissues experiments were performed as described by Gilani et al. (13).

Isolated rabbit jejunum preparations

Plant extract was tested on isolated rabbit jejunum preparations for possible presence of spasmogenic and/or spasmolytic activity. Isolated rabbit jejunum segments of approximately 2 cm in length were suspended in isolated tissue baths containing Tyrode's solution, at 37°C, aerated with carbogen (95% O_2 and 5% CO_2). The composition of the Tyrode's solution (mM) was: KCl (2.68), NaCl (136.9), $MgCl_2$ (1.05), $NaHCO_3$ (11.90), NaH_2PO_4

(0.42), $CaCl_2$ (1.8) and glucose (5.55). A preload of 1 g was applied and intestinal responses were recorded isotonically using Bioscience transducers coupled to Harvard Student Oscillograph. The tissues were allowed to equilibrate for at least 30 min prior to the addition of any drug. Isolated rabbit jejunum preparations exhibit spontaneous rhythmic contractions and allow testing of the antispasmodic (relaxant) effect without application of an agonist. The observed response of the test material was quantified by the application of doses in a cumulative fashion. The relaxant effects on the part of test substance were taken as the percent change in spontaneous contractions of the preparation recorded immediately before the addition of test substances.

The possible mechanism of the relaxant activity of the test material was investigated through the relaxation of the observed sustained spasmodic contractions following exposure to high concentration of K^+ (80 mM) (14). The test material was applied in a cumulative manner to the sustained contractions to achieve concentration-dependent inhibitory responses. The observed relaxant effect of the test material on K^+ (80 mM)-induced contraction was expressed as percent of the control contractile response.

Calcium channel blocking effect of the test substance was confirmed by the method described previously by Gilani et al. (15, 16). The isolated rabbit jejunum preparation was allowed to stabilize in normal Tyrode's solution, which was subsequently replaced for 30 min with Ca^{2+}-free Tyrode's solution to which EDTA (0.1 mM) was added in order to remove calcium from the tissue. This bath solution was further replaced with K^+-rich and Ca^{2+}-free Tyrode's solution, having the following composition (mM): KCl (50), NaCl (91.04), $MgCl_2$ (1.05), $NaHCO_3$ (11.90), NaH_2PO_4 (0.42), glucose (5.55) and EDTA (0.1). Subsequent to an incubation period of 30 min, cumulative Ca^{2+} concentrations were applied to the tissue bath to obtain control calcium dose-response curves (DRCs). On achievement of the super-imposable control calcium dose-response curves (usually after two cycles), the tissues were then washed and allowed to equilibrate with the plant extract for 1 h and then the concentration response curves of Ca^{2+} were recorded and compared to the control curves. The dose-response curves (DRCs) of Ca^{2+} were recorded in the presence of different concentrations of the plant extract in tissue bath.

Isolated guinea-pig ileum preparations

The ileum was dissected and kept in Tyrode's solution having composition as described earlier. Since isolated guinea pig ileum behave as a quiescent preparation, is considered more suitable for spasmogenic activity. The segments, about 2 cm long, were suspended individually in isolated tissue bath, filled with Tyrode's solution, aerated with carbogen and maintained at 37°C. Different concentrations of plant material were applied and tissue was washed every time The isotonic contractions were recorded with Powerlab Data Acquisition System (AD Instruments, Sydney, Australia) attached to a computer installed with labchart software (version 6). Each tissue was allowed to equilibrate for at least 30 min before the addition of any drug. After tissue equilibration, each tissue preparation was repeatedly treated with sub-maximal concentrations (0.3 µM) of Ach at 3 min intervals until constant responses were recorded. Different concentrations of plant material were applied and tissue was washed every time after getting spasmogenic response of each concentration of crude extract and doses were added as bolus dose. Responses were calculated by taking the response of acetylcholine before each bolus dose of plant extract as a control and acetylcholine maximum (Ach max.) response if required, was used as control. Then tissue was washed and procedure was repeated again with sub maximal dose of acetylcholine.

Isolated rabbit and guinea pig tracheal preparations

Rabbit and guinea pig tracheas were dissected out and kept in Krebs 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). The trachea was cleaned free from the adhering fatty tissues and rings of 2-3 mm width containing 2-3 cartilages were prepared. Each ring was opened by a longitudinal incision on the ventral side opposite to the smooth muscles layer to form a strip with smooth muscles layer in middle and cartilages on both sides. These tracheal preparations were mounted in 20 mL organ bath containing Krebs solution being maintained at 37°C and aerated with carbogen. A preload tension of 1 g was applied and tissue preparations were allowed to be equilibrated for 1 h prior to any challenge by the drug. Tissue preparations were stabilized by repeated applications of carbachol (1 µM) until constant responses were recorded. The carbachol (1 µM)- and high K^+ (80 mM)-induced sustained contractions were subsequently used for testing of different doses of the test material in a cumulative fashion. The isometric responses were recorded through Power lab data acquisition sys-

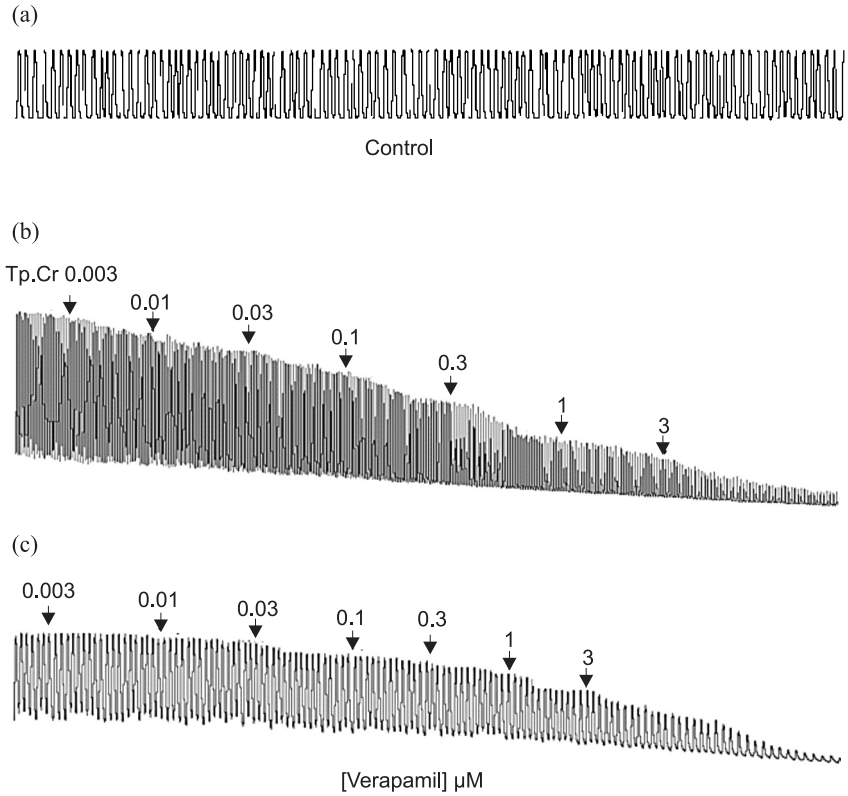

Figure 1. (a) Control, (b) effect of Tp.Cr and (c) effect of verapamil on spontaneous contractions in isolated rabbit jejunum preparations

tem (AD Instruments, Sydney, Australia) attached to a computer installed with lab chart software (Version 6). The standard drugs with Ca^{2+} channel blocking effect (verapamil and papaverine) were tested on high K^+(80 mM)- and carbachol-induced spastic contractions in order to confirm the possible mechanism of action (17).

Isolated rabbit aorta preparations

The effect of Tp.Cr on systemic vascular resistance was assessed on isolated rabbit aorta preparations, rabbits of either sex were sacrificed by a blow on the back of head and descending thoracic aorta was dissected out and kept in the normal Krebs solution having composition as described earlier. It was then cut into rings of about 2-3 mm width and each ring was mounted in a tissue bath containing Krebs solution. Temperature was maintained at 37°C and tissue was continuously aerated with carbogen. A pre-load of 2 g was applied to each preparation and allowed to equilibrate for a period of 1 h. After equilibration, tissue was stabilized by repeated exposure to K^+(80 mM) or phenylephrine (1 µM) depending upon the protocol of the experiment. The vasorelaxant/vasoconstrictive effects of the test substances were studied by addition in tissue organ baths containing pre-stabilized tissue in a cumulative manner. Changes in isometric tension of aortic rings were obtained *via* force-displacement transducer (Model FORT100, WPI, USA) coupled to a transbridge (model TBM4M, WPI, USA) and PowerLab data acquisition system (AD Instruments, Sydney, Australia) and computer running Lab Chart software (version 6).

Statistical analysis

The data were expressed as the mean ± standard error of the mean (SEM, n = number of animals) and the median inhibitory concentrations (IC_{50}) with 95% confidence intervals (CI). The statistics applied Student's *t*-test except in case of castor oil induced diarrhea where χ^2-test was used and $p < 0.05$ was taken as significant difference. Concentration-response curves (CRCs) were analyzed by non-linear regression using Graph Pad program (Graph PAD, San Diego, CA, USA).

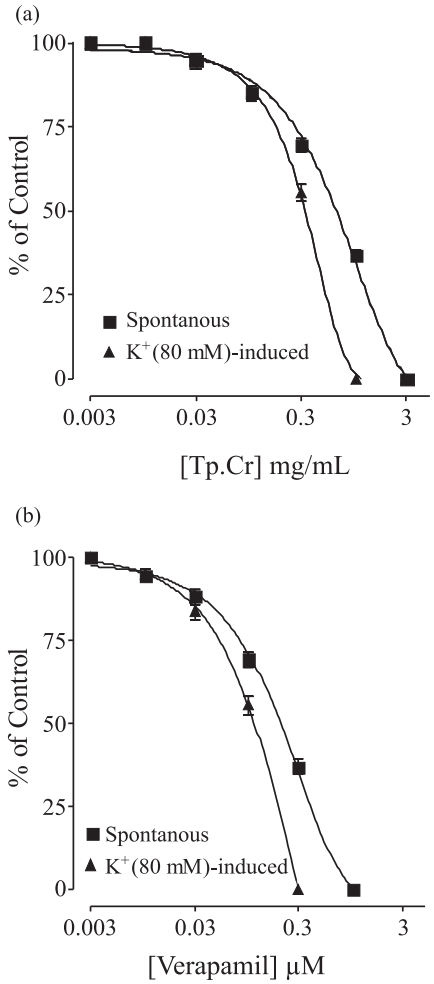

Figure 2. Effects of (a) Tp.Cr and (b) verapamil on spontaneous and K^+ (80 mM)-induced contractions in isolated rabbit jejunum preparations. Values are expressed as the mean ± SEM, n = 5

RESULTS

Phytochemical studies

Preliminary phytochemical studies on *Tephrosia purpurea* were undertaken to reveal the presence of different secondary metabolites of the plant. The outcome of the study indicated the presence of tannins, saponin, flavonoids and phenols among the methanol soluble extractable constituents of *Tephrosia purpurea*.

Effect of crude extract of *Tephrosia purpurea* on rabbit jejunum

The crude extract of *Tephrosia purpurea* exhibited a concentration-dependent inhibitory effect on spontaneous contractions of isolated rabbit jejunum preparations. The inhibitory effect was observed in the dose range of (0.003-3.0 mg/mL) in cumulative manner and EC_{50} value was found to be of 0.658 mg/mL (95% CI; 0.5632-0.7690) (Fig. 1a). The spontaneous contractions were revived following washing the isolated jejunum preparation with Tyrode's solution and addition of atropine (0.1 µM) did not show any effect on the relaxant activity of Tp.Cr. Similarly, verapamil, a standard calcium channel blocker, relaxed the spontaneous contractions of isolated rabbit jejunum preparations in concentration dependent manner in the tissue bath concentration range of (0.003-1.0 µM), with EC_{50} value of 0.208 mg/mL (95% CI; 0.1863-0.2337) (Fig. 1b). Moreover, Tp.Cr also shifted the concentration-response curves (CRC) of Ca^{2+} rightward in a manner similar to verapamil (Fig. 5a,b).

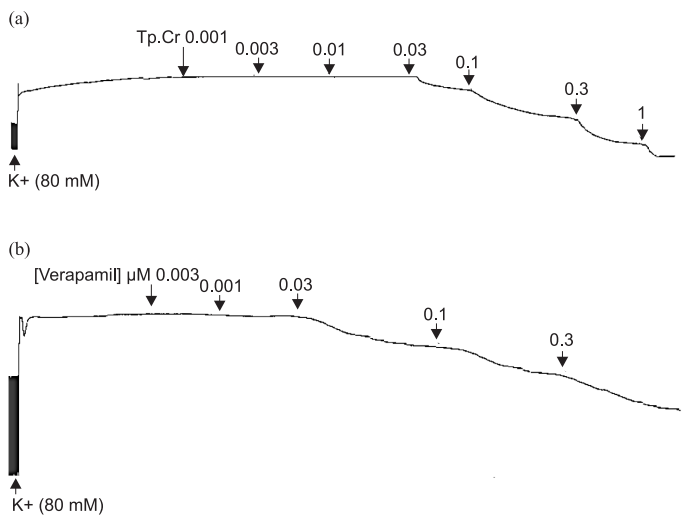

Figure 3. Effects of (a) Tp.Cr and (b) verapamil on K^+ (80 mM)-induced contractions in isolated rabbit jejunum preparations

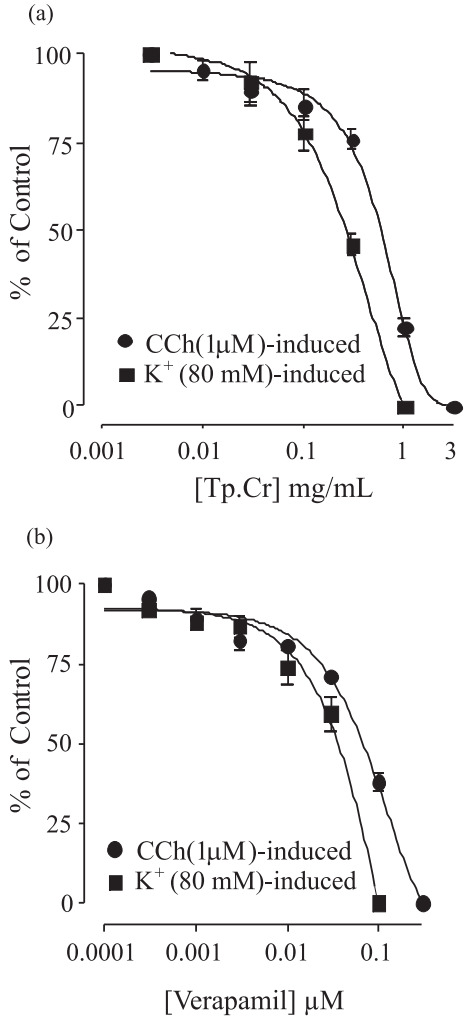

Figure 4. The inhibitory effect of (a) Tp.Cr and (b) verapamil on carbachol (1 μM) and K^+(80 mM)-induced contraction in isolated rabbit tracheal preparations in a concentration dependent manner. Values shown are the mean ± SEM, n = 5

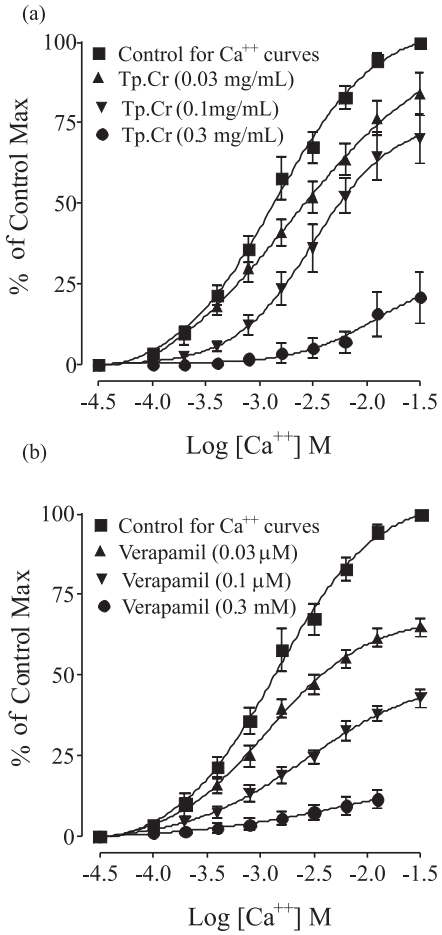

Figure 5. Effect of (a) Tp.Cr and (b) verapamil on concentration response curves of Ca^{2+} in isolated rabbit jejunum preparations. Values shown are the mean ± SEM, n = 5

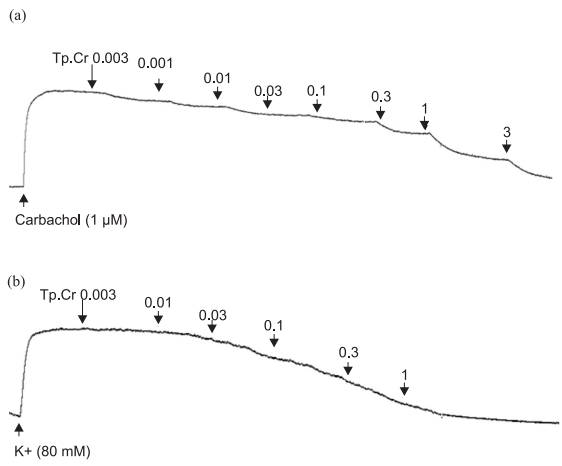

Figure 6. Effect of Tp.Cr on (a) carbachol (1 μM) and (b) K^+(80 mM)-induced contractions in isolated rabbit tracheal preparations

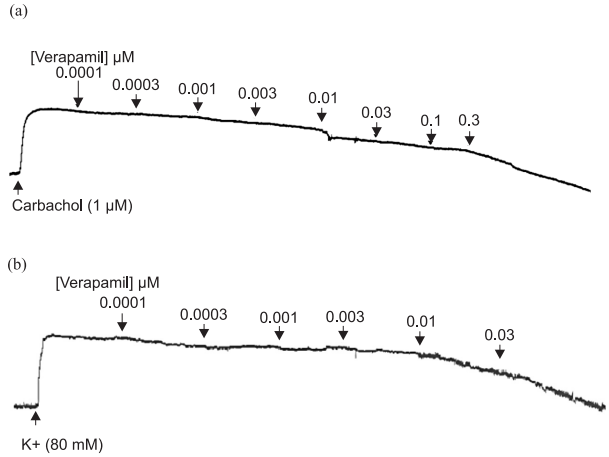

Figure 7. Effect of Tp.Cr on (a) carbachol (1 µM) and (b) K^+ (80 mM)-induced contractions in isolated rabbit tracheal preparations

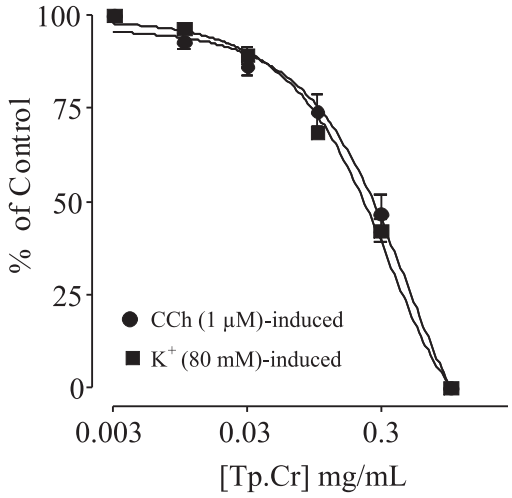

Figure 8. Effect of Tp.Cr on carbachol (1 µM) and K^+ (80 mM)-induced contractions in isolated guinea-pig tracheal preparations. Values are expressed as the mean ± SEM, n = 5

Effect of Tp.Cr on K^+ (80 mM)-induced contractions

Effect of Tp.Cr on K^+ (80 mM)-induced contractions is given in Figures 2-4. The possible mechanism of the observed relaxant activity of the Tp.Cr on the isolated rabbit jejunum preparations was speculated to be mediated through calcium channels blockade and the idea was tested through application of the crude plant extract on high K^+ (80 mM)-induced sustained contractions in the isolated rabbit jejunum preparations. The isolated rabbit jejunum preparations on exposure to high K^+ (80 mM) produced a sustained contraction by depolarization of the tissues. The Tp.Cr on addition to tissue bath in cumulative manner demonstrated a complete relaxation of K^+ (80 mM)-induced contractions in a tissue bath concentration dependent manner at a concentration range of 0.003-1 mg/mL with EC_{50} value of 0.325 mg/mL (95% CI; 0.2877-0.3688), whereas, the standard drug, verapamil, showed complete relaxation of K^+ (80 mM)-induced contractions at a tissue bath concentration range of 0.0003-0.3 µM, with EC_{50} value of 0.114 µM (95% CI; 0.09841-0.1315). Verapamil showed complete relaxation of K^+ (80 mM)-induced contractions at a tissue bath concentration of 0.3 µM.

Effect of Tp.Cr on isolated rabbit tracheal preparations

Tp.Cr on application to the isolated rabbit tracheal preparations in cumulative manner exhibited a concentration dependent relaxant effect in the concentration range of 0.003-3 mg/mL for carbachol-induced contractions and 0.003-1 mg/mL for K^+ (80 mM)-induced contractions. The EC_{50} value for carbachol-induced contraction was 0.6499 mg/mL (95% CI; 0.4719-0.8953) and EC_{50} value for K^+ (80 mM)-induced contractions was 0.2638 mg/mL (95% CI; 0.2199-0.3238) (Fig. 6a, 7a), whereas verapamil showed relaxant effect on carbachol-induced contraction in the tissue bath concentration range of 0.0001-0.3 µM with EC_{50} value of 0.7002 µM (95% CI; 0.6208-0.7899) and K^+ (80 mM)-induced contractions in the concentration range of 0.0001-0.1 µM with EC_{50} value of 0.03689 µM (95% CI; 0.03326-0.04092) (Fig. 6b, 7b).

Effect of Tp.Cr on isolated guinea-pig tracheal preparations

Tp.Cr exhibited the relaxant effect on the carbachol- and K^+ (80 mM)-induced contractions in isolated guinea-pig tracheal preparations with EC_{50}

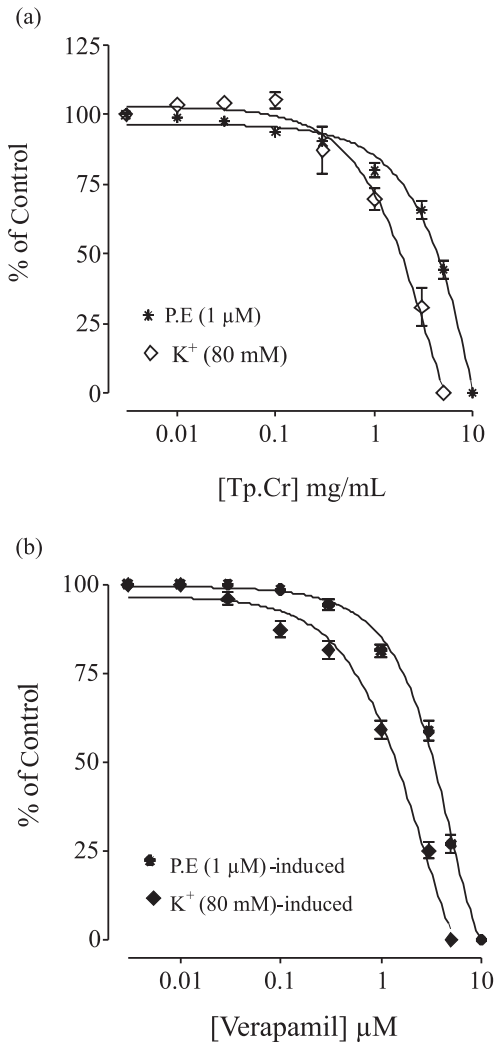

Figure 9. Effect of (a) Tp.Cr and (b) verapamil on phenylephrine and K^+ (80 mM)-induced contractions in isolated rabbit aortic preparations. Values shown are the mean ± SEM, n = 5

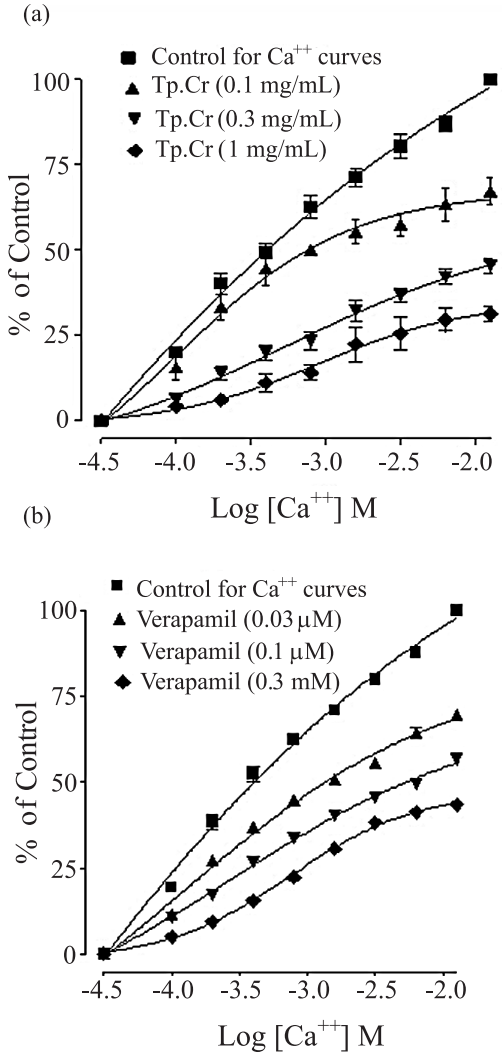

Figure 10. Effect of (a) Tp.Cr and (b) verapamil on concentration response curves of Ca^{2+} in isolated rabbit aorta preparations. Values shown are the mean ± SEM, n = 5

value of 0.2794 mg/mL (95% CI; 0. 2413-0.3236) and 0.2182 mg/mL (95% CI;0. 1900-0.2506), respectively (Fig. 8).

Effect of Tp.Cr on isolated rabbit aorta preparations

Tp.Cr on application to isolated rabbit aorta preparations exhibited a dose dependent relaxant effect on phenylephrine (1 µM) and K^+ (80 mM)-induced contractions and EC_{50} of 4.459 mg/mL (95% CI; 4.208-4.725) and 1.642 mg/mL (95% CI; 1.276-2.114), respectively (Fig. 9a), whereas verapamil on application to the phenylephrine (1 µM) and K^+ (80 mM)-induced contractions in the isolated rabbit aortic preparations showed relaxant effect in the concentration range of 0.003-10 µM with EC_{50} value of 3.509 µM (95% CI; 3.387–3.636) and at concentration range of 0.003-5 µM with EC_{50} value of 1.331 µM (95% CI; 1.113-1.592), respectively (Fig. 9b).

The pretreatment of isolated rabbit aortic preparations with Tp.Cr caused a concentration dependent (0.1-1 mg/mL) rightward shift of Ca^{2+} concentration response curve, similar to verapamil with dose range 0.03-0.3 µM, thus confirming that observed relaxant effect of Tp.Cr was likely to be mediated through calcium channel blocking effect (Fig, 10a, b).

CONCLUSIONS

The methanolic extract of the whole plant of *Tephrosia pupurea,* Linn. on application to spontaneous contractions in isolated rabbit jejunum preparations, exerted a concentration dependent (0.003-3.0 mg/mL) relaxant effect. The extract also caused concentration dependent relaxation of K^+ (80 mM)-induced spastic contractions. These findings were further supported by the observations that the extract caused a concentration dependent rightward shift of the Ca^{2+} response curves in manner similar to that of verapamil. The extract exhibited a relaxant effect on carbachol and high K^+ (80 mM)-induced contractions of isolated rabbit tracheal preparations in a manner similar to verapamil. The observed non-specific bronchodilator response is possibly mediated through Ca^{2+} channel blockade. Moreover, the extract also exhibited a dose dependent relaxant effect on phenylephrine (1 µM) and K^+ (80 mM)-induced contractions in a manner similar to verapamil. On the basis of the above-mentioned findings, it can be concluded that the use of *Tephrosia pupurea,* in gastrointestinal spasm, asthma and hypertension is likely to be mediated through calcium channel blockage.

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