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Pharmacological evaluation of smooth muscle relaxant and cardiac-modulation potential of *Phyla nodiflora* in *ex-vivo* and *in-vivo* experiments

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ABSTRACT

Objective: To investigate the pharmacological basis of its uses of *Phyla nodiflora* (*P. nodiflora*) for the treatment of anomalies predominantly by smooth muscle containing tissues such as gastrointestinal/vascular/broncho spasm and cardiovascular modulation.
Methods: The crude hydroalcoholic extract of *P. nodiflora* (Pn.Cr) and its fractions were evaluated on isolated rabbit jejunum, rat trachea, aorta and atrium. To access the contractile or relaxant effects of testing materials, the tissues were mounted in isolated organ bath and responses were recorded with transducers coupled with data acquisition system. BALB/c mice were challenged with castor oil for the assessment of *in-vivo* antidiarrheal activity. Normotensive rats were used for *in-vivo* hypotensive study.
Results: Hydroalcoholic extract of Pn.Cr at variable concentrations inhibited the natural spontaneous rhythm and 80 mmol/L K^+ mediated contractions in isolated segment of jejunum with EC_{50} values of 3.18 and 1.91 mg/mL respectively. Verapamil, a Ca^{2+} channel blocker, demonstrated akin pattern in jejunum and therefore possibly suggesting calcium blocking activity. On isolated rat tracheal tissue, Pn.Cr showed relaxation of high-K^+ and carbachol mediated contractions (EC_{50} values: 1.24 and 2.42 mg/mL). Pn.Cr treatment relaxed the rat aortic ring in a cumulative doses with high-K^+ and phenylephrine-induced contractions (EC_{50} values 0.25 and 0.92 mg/mL). Activity based fractionation of Pn.Cr showed that dichloromethane fraction was more potent for relaxing the tissues spasm compared to aqueous fraction. *In-vivo* experiments, significant protection by Pn.Cr ($P < 0.05$) was observed in castor oil-induced diarrhea (50–500 mg/kg) whereas hypotensive effect in anesthetized rats was seen at the dose range of 1–10 mg/kg of Pn.Cr ($P < 0.05$).
Conclusion: This study suggests the blockage of calcium channel in the smooth muscles as a pharmacological application to make usage of *P. nodiflora* in the management of diarrhea, asthma and hypotensive effect.

1. Introduction

Phyla nodiflora (*P. nodiflora*) known as "Bukanbooti" in Urdu, is a wild medicinal herb, an important member of the family Verbenaceae and has been used traditionally for the treatment of diarrhea, micturition and dysuria [1]. In Central America it is used for the treatment of asthmatic disorders and other conditions such as bronchitis and cough [2,3]. In India decoction the plant is used to treat diarrhea in children, also used as delivery tonic [4]. In Pakistan and Bangladesh culture people use *P. nodiflora* for the treatment of number diseases like constipation, eczema, heat stroke, rheumatic and neurological problems, gonorrhea, fever, skin problems, body pain, headache, dizziness and backache [5]. It possesses diuretic, antibacterial, anti-inflammatory, antifungal, anticancer, larvicidal, hepatoprotective, hypolipidemic, antidiabetic, central nervous system inhibitory, hypotensive and skin whitening activities [6–12].

Previous phytochemical analysis reported the existence of variety of chemical compounds in *P. nodiflora* such as

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flavonoids, triterpenoids (lippiacian and halleridone) [3,13,14]. Phenols (lipofolrin A, lipoflorin B, nepetin, batalilfolin), flavones and flavone glycosides like 6-hydroxyluteolin-7-*O*-apioside and luteolin-7-*O*-glucoside nepetin 7-sulfate, demethoxycentaureidin, hispidulin 7-sulfate, hispidulin 7,4′-disulfate, jaceosidin 7,4′-disulfate, nepetin 3′,4′-disulfate, nodifloretin 6,7-disulfate, 6-hydroxyluteolin 6,7-disulfate, nodifloretin 7-sulfate, 6-hydroxyluteolin 6-sulfate, 6-hydroxyluteolin 7-sulfate, jaceosidin 7-sulfate, nepetin 7-sulfate, and hispidulin 4′-sulfate have been isolated from various extracts of *P. nodiflora* [3].

Glycosides such as β-sitosterol glycosides and stigmasterol glycosides have also been identified to be present in *P. nodiflora*. Presence of carbohydrates like glucose, fructose, maltose and lactose has been reported [13].

Smooth muscles play pivotal functions in the regulation of gastrointestinal (GIT) tract, bronchioles, trachea and vascular system [15,16]. Any abnormality in regulation and or function of smooth muscle contractility of GIT tissues eventually results in various disorders ranging from intestinal spasm, cramps, hyper/hypo-motility and diarrhea. Smooth muscles of the vascular beds particularly arteries and arterioles are very susceptible and anomalies with their function can cause serious alterations in blood pressures [17]. Asthma is chronic disease strongly modulated by smooth muscles contractility of the bronchi and bronchioles. In the above mentioned complications, intracellular calcium and associated calcium channels play significant function in the regulation of smooth muscle activity. Moreover, the contractile smooth muscles are highly sensitive to calcium concentration and show versatile responses based on the intracellular and extracellular concentration surrounding the cell.

Because of limited scientific literature pertaining to *P. nodiflora*, the current study was intended to explore the therapeutic potential of the selected plant on the isolated tissues (jejunum, trachea and aorta) of animals to justify the smooth muscle relaxant properties. Additional *in-vivo* experiments were performed for assessment of hypotensive potential. These studies mimic the human situation of gastrointestinal, respiratory and cardiovascular ailments.

2. Material and methods

2.1. Collection of P. nodiflora and extract preparation

Above ground parts of *P. nodiflora* were harvested from the fields of District Lodhran, Punjab Pakistan and authenticated by an expert taxonomist (voucher no: R.R. Stewart 607). After an appropriate period of shade drying (2-weeks) the coarse powder was prepared by means of herbal grinder. One kilogram grounded plant material was macerated in 70% hydroalcoholic solution (30% water and 70% methanol) for the purpose of extraction at room temperature for a period of period of 7–10 d with frequent agitation [15]. The macerated material was filtrated through fine filter paper and obtained filtrate was concentrated on a rotary evaporator at controlled pressure and temperature (37 °C). The percentage yield of crude extract of *P. nodiflora* (Pn.Cr) was 14%.

The methanolic extract of Pn.Cr was further fractioned in dichloromethane (DCM) and aqueous (Aq) fractions. Briefly 50 g of Pn.Cr was mixed with definite proportion of water and DCM in separating funnel and smoothly shaken for 15 min. The lower (DCM) and upper layers (aqueous) were collected three times and subsequently concentrated in to dichloromethane fraction of *Phyla nodiflora* (Pn.DCM) and Pn.Aq fractions by rotary evaporator.

2.2. Drugs and chemicals

All chemicals utilized in current study were of high percentage purity. These were mostly purchased from Sigma Aldrich (merged with Merck Ag, Germany), Merck (Germany), and Alfa Acer (USA). For the isolated tissues and *in-vivo* studies the standard drugs were acetylcholine (Ach), carbachol, verapamil hydrochloride, phenylephrine, norepinephrine, potassium chloride, and atropine sulfate. For the preparation of physiological buffer solutions the used chemicals were NaCl, $CaCl_2$, NaH_2PO_4, KH_2PO_4, $MgCl_2$, $MgSO_4$, KCl, $C_6H_{12}O_6$ and sodium citrate. Before execution of experiments, all solutions were freshly prepared in double deionized water or 0.9% sodium chloride.

2.3. Animals

Sprague Dawley rats (150–300 g) and BALB/c mice (20–35 g) of either gender while the rabbits of local breed (1.2–1.8 kg) were used for the experiments. These animals were accommodated (transparent polycarbonate cages) in the animal facility of Faculty of Pharmacy at B.Z University, Multan with the standardized conditions of the temperature (23–25 °C) humidity (50%–55%) and light (12 h light and dark cycle). Customized self-made standard diet (protein content >21%, carbohydrate >60%) was given to them with free access to water. Food was withdrawn from experimental animals 16–20 h before the execution of experiments but had *ad libitum* water. All protocols and exploratory approach utilized in the investigation were followed the rules set up by the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council (1996). Care was exercised for the minimal number of animal usage defined by the principle of 3Rs, *i.e.*, Replacement, Reduction and Refinement. The departmental ethical committee has further approved the protocols vide No.09/PEC/2015.

2.4. Ex-vivo experiments on isolated tissues

2.4.1. Experiments for spasmolytic activity on jejunum preparation

To study the antispasmodic activity of *P. nodiflora* we used rabbit jejunum. Rabbits were kept on starvation 24 h before the start of experiment. On experiment day rabbit was sacrificed, jejunum was dissected out, cleaned off the mesenteries and divided into small pieces 2–3 cm in length. Each segment of jejunum was hanged in 15 mL tissue bath containing normal Tyrode's solution. The temperature of the bath was kept constant at 37 °C with continuous supply of carbogen. Totally, 1 g tension was applied to the tissue. Tissue was kept free to equilibrate for a period of 30–45 min and during this period no drug was applied but it was repeatedly washed with fresh Tyrode's solution. Then tissue was stabilized by repeated exposure of acetylcholine (0.3 μmol/L) after each remain as 3-minute interval. Spasmolytic action of plant material was investigated by cumulative administration of test drug. This effect was measured as% age of jejunum spontaneous contractions obtained immediately before the application of test materials [15,16]. Percentage relaxation was then calculated from the obtained data. Calcium channel blocker

activity was determined by the inhibitory effect of Pn.Cr on 80 mmol/L K^+-induced contractions in jejunum. Finally the calcium channel blocking activity was assessed by the method used previously [18,19].

2.4.2. Bronchodilator study on tracheal preparations

Isolated rat trachea was used to study the bronchodilator activity of crude extract of *P. nodiflora*. Rats were kept on starvation 24 h before the start of experiment, killed by head blow and trachea was removed. It was cut into rings with a width of 3–4 mm, having minimum of two cartilages. From the ventral side the tracheal ring was cut longitudinally against the smooth muscle. The preparation was hanged in a tissue organ bath filled with Krebs's solution at 37 °C with continuous supply of 5% CO_2 and 95% O_2. Then, 1 g tension was applied to tissue throughout the experiment. The tracheal tissue was equilibrated for 45 min before the addition of any drug. Bronchodilator effect of *P. nodiflora* was observed on K^+-80 mmol/L and carbachol (CCh)-induced contractions via isometric transducers (MLT 0201, Panlab, Spain) connected to Power lab data acquisition system [20].

2.4.3. Study of vasodilation effect on isolated rat aorta

Vasodilation potential of plant extract was evaluated by using isolated rat aorta with adaptation of the reported procedure [18–21]. Rat was sacrificed by head blowing and descending thoracic aorta was dissected out and pieces of 2–3 mm were formed. Each isolated rat aortic tissue segment was suspended in tissue organ bath filled with Kreb's solution. Temperature of tissue organ bath was fixed at 37 °C with continuous supply of carbogen. Then, 2 g preload tension was applied to the tissue and left it to equilibrate for a period of 1 h. Tissue was repeatedly exposed to phenylephrine (1 μmol/L) or high potassium (K^+-80 mmol/L) for the purpose of stabilization. The vasodilator effect of *P. nodiflora* was studied by cumulative addition of plant extract to precontracted aortic rings. Changes in the tension of pre contracted aortic rings were measured via isometric transducer MLT 0201 (Panlab, Spain) which was in connection to Power Lab data acquisition system (ADInstruments, Australia). Precontracted response of the tissue was considered as control and the effect of test material compared to it.

2.4.4. Study of ionotropic and chronotropic effect on isolated rabbit atria

Right atria was isolated carefully from heart of rabbit and hanged in tissue organ bah having Kreb's solution with continuous supply of carbogen. Ionotropic and chronotropic effect of atria were recorded under 1.0 g pressure by isometric transducer associated with PowerLab. Atrial tissues were permitted to equilibrate for a time of 30 min with persistent buffer change after at regular intervals preceding administration of any drug. Effect of drug on the force and rate of contractions was calculated as the percentage of baseline contractions before the addition of drug [22].

2.5. In-vivo experiments

2.5.1. In-vivo antidiarrheal activity

Randomly selected BALB/c mice were arranged in six groups, five mice in each group. These mice were kept in individual cages lined with blotting paper. On the day of experiment diarrhea was induced in all animals by oral ingestion of castor oil (10 mL/kg). After 1 h, 0.9% saline (10 mL/kg) was administered orally to 1st group (the negative control) and loperamide (15 mg/kg) to 2nd group which served as positive control. While groups 3–6 were treated respectively with increasing doses of Pn.Cr, *i.e.*, 50, 100, 300 and 500 mg/kg. After treatment all animals were observed up to 6 h and total number of feces was counted individually for each animal [23].

2.5.2. Antihypertensive activity

Hypotensive activity of Pn.Cr was studied by the previously described method [22,24]. Ketamine (50–80 mg/kg) and diazepam (5 mg/kg) were used intraperitoneally to anesthetize the rats. Anesthetized rat was placed in a supine position on dissecting board. Temperature of the SD rat was kept constant up by putting it on isothermic warming cushion at 37 °C. A small incision was made to uncover trachea, jugular veins and carotid arteries. Spontaneous breathing was maintained by cannulation of trachea with 18 gauge polyethylene tube. Jugular vein was cannulated to administer drug/s. Cannulation was performed in carotid artery with polyethylene 50 tube filled with heparin solution (60 IU/mL) and attached with disposable BP transducer (MLT0699, ADInstruments, Australia), further connected to PowerLab data acquisition system for the measurement of blood pressure. Standard medications were administered in 0.1 mL followed by 0.1 mL of 0.9% saline flush through jugular vein. Transducer was calibrated prior to experiment with the help of mercury sphygmomanometer. Moreover cannulated rat was given 0.1 mL heparinized solution in order to prevent coagulation. To check the animal behavior toward hypertensive and hypotensive drugs acetylcholine (1 μg/kg) and epinephrine (1 μg/kg) were administered intravenously to every animal before the administration of any drug. After attaining the equilibrium, 0.1 mL of extract or drug was administered intravenously followed by normal saline flush [24]. Drug response was calculated as percent difference of basal value.

2.6. Acute toxicity assay

For acute toxicity assay three groups of mice, five mice in each group were selected [22]. The 1st group was given 1 g/kg of Pn.Cr orally while the 2nd and 3rd groups received 2 and 3 g/kg in a volume of 10 mL/kg respectively. The animals were observed for 24 h for behavioral changes and mortality but with full access to water and food.

2.7. Statistical analysis

For isolated tissues, data were represented as mean ± SEM (n = number of individual experiments) and median effective concentrations (EC_{50}) with 95% confidence intervals. Concentration response curves were assessed by statistical software (Graphpad Prism, USA) by means of nonlinear regression. One-way ANOVA followed by Dunnett's multiple comparison test with defined control group as a reference was used for the calculation of *in-vivo* antidiarrheal activity. Student's t-test was used for the analysis of data for invasive blood pressures values. In all cases, P-values < 0.05 were considered to be statistically significant.

3. Results

3.1. Ex-vivo studies

3.1.1. Effect on rabbit jejunum

Significant relaxation of spontaneous and high potassium (K^+-80 mmol/L)-induced contractions of jejunum was observed after the application of Pn.Cr in concentration range from 0.01 to 10.00 mg/mL (Figure 1B and C). Calculated EC_{50} values were 3.18 mg/mL (95% *CI*: 2.25–2.54 mg/mL) for the spontaneous and 1.91 mg/mL (95% *CI*: 1.38–2.66 mg/mL) for high potassium respectively (Table 1). Moreover Pn.Cr at the dose of 0.30 and 1.00 mg/mL inhibited the Ca^{2+} mediated contractile effect of the tissue (Table 2). Comparably a calcium channel blocker drug verapamil also inhibited spontaneous and K^+-80 mmol/L-induced contractions. Respective EC_{50} values of verapamil response were 0.25 mg/mL (95% *CI*: 0.21–0.33 mg/mL) and 0.07 mg/mL (95% *CI*: 0.05–0.10 mg/mL) (Table 1) and shifted the calcium response curves toward rightwards by inhibiting the contractile response of Ca^{2+} (Table 2). Pn.DCM showed both contractile effect at small doses and relaxant effect at high doses (Table 1). Pretreatment of tissue with atropine abolished the contractile response (Data not shown). EC_{50} values of the relaxant effect of Pn.DCM was 1.38 mg/mL (95% *CI*: 0.70–2.83 mg/mL) for spontaneous, 0.12 mg/mL (95% *CI*: 0.07–0.19 mg/mL) for high-K^+-induced contractions respectively (Table 1) while for spontaneous with atropine the observed EC_{50} was 0.61 mg/mL (95% *CI*: 0.29–1.38 mg/mL) (Data not shown). With the application of Pn.Aq, partial relaxation of high-K^+-induced contractions were noticed (Data not shown) but complete relaxation of spontaneous contractions was achieved with EC_{50} value of 1.36 mg/mL (95% *CI*: 1.03–1.83 mg/mL). Moreover, in a situation of low-K^+-induced contractions assessed EC_{50} value of Pn.Aq was 3.74 mg/mL (95% *CI*: 2.13–6.72 mg/mL) akin to a potassium channel opener (Data not shown).

3.1.2. Effect on isolated rat trachea

Pn.Cr in a concentration from 0.01 to 5.00 mg/mL resulted in complete relaxation of precontracted trachea with high-K^+

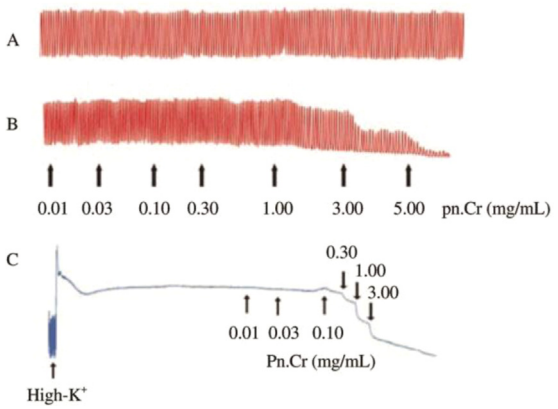

Figure 1. Spasmolytic effect of Pn.Cr on isolated segment of rabbit jejunum.

Tracings showing A) Control response of isolated rabbit jejunum before any drug administration; B) Effect of Pn.Cr on normal rhythmic contractions; C) Effect of Pn.Cr on precontracted isolated rabbit jejunum by 80 mmol/L of potassium. Pn.Cr was administered in the order of increasing final bath concentration (n = 4–6 individual experiments).

Table 1

Relaxant effect of aqueous methanolic extract and its fractions in percentage on spontaneous rhythm and chemically high-K^+-induced contractions in isolated rabbit jejunum.

Group	Treatment	Dose (mg/mL)								EC_{50}
		Control[a]	0.01	0.03	0.10	0.30	1.00	3.00	5.00	
Pn.Cr	Spontaneous	0	0.94 ± 0.73	4.53 ± 1.15	9.88 ± 0.31	19.07 ± 2.24	33.95 ± 3.42	56.44 ± 3.94	100.00 ± 0.00	3.18 ± 0.42
	High-K^+	0	1.50 ± 0.89	4.06 ± 1.53	7.43 ± 2.45	18.36 ± 4.27	43.13 ± 4.24	69.26 ± 5.46	100.00 ± 0.00	1.91 ± 0.53
Pn.DCM	Spontaneous	0	−76.33 ± 5.13[b]	−71.86 ± 5.69	−54.17 ± 4.67	−31.03 ± 4.86	19.24 ± 3.02	100.00 ± 0.00	–	1.38 ± 0.06
	High-K^+	0	4.19 ± 1.61	14.45 ± 2.72	32.59 ± 2.71	100.00 ± 0.00	–	–	–	0.12 ± 0.01
Verapamil[c]	Spontaneous	0	2.33 ± 1.45	15.67 ± 2.33	35.54 ± 1.29	64.57 ± 2.94	100.00 ± 0.00	–	–	0.25 ± 0.33
	High-K+	0	8.67 ± 1.29	26.56 ± 3.29	60.02 ± 3.59	100.00 ± 0.00	–	–	–	0.07 ± 0.01

Values are expressed as mean ± SEM (n = 4–6 individual experiments). Pn.Cr: crude hydroalcoholic extract of *P. nodiflora*. Pn.DCM: Dichloromethane fraction of *P. nodiflora*.

[a] Control response was considered as 100% contracted and zero (0%) relaxed before the administration of any drug/extract. [b] Negative sign (−) indicates the contractile effect. [c] With unit µmol/L.

Table 2

Calcium channel blocking effect of different doses of aqueous methanolic extract of *P. nodiflora* (Pn.Cr) and verapamil as indicated by decrease in contractile effect of control ($CaCl_2$).

Group	Dose log [Ca^{2+}] (mol/L)								
	−4.0	−3.7	−3.4	−3.1	−2.8	−2.5	−2.2	−1.9	−1.6
Control ($CaCl_2$)	22.14 ± 3.52	40.66 ± 2.77	59.99 ± 4.44	65.09 ± 4.01	78.17 ± 2.73	90.61 ± 1.04	96.01 ± 0.77	98.75 ± 0.64	100.00 ± 0.00
Pn.Cr (0.3 mg /mL) + $CaCl_2$	8.99 ± 2.00	16.59 ± 2.14	29.02 ± 3.37	40.15 ± 3.32	49.05 ± 4.68	60.65 ± 3.92	62.97 ± 3.19	67.43 ± 2.61	68.69 ± 2.38
Pn.Cr (1.0 mg /mL) + $CaCl_2$	1.18 ± 1.18	9.72 ± 3.82	11.70 ± 1.84	18.42 ± 3.52	27.81 ± 5.82	34.19 ± 4.56	39.09 ± 4.04	43.13 ± 2.31	45.33 ± 3.07
Verapamil (0.1 µmol /L) + $CaCl_2$	0.50 ± 0.50	4.99 ± 3.21	10.80 ± 2.62	25.47 ± 1.59	40.94 ± 6.40	56.04 ± 3.54	66.93 ± 1.65	75.65 ± 4.42	82.37 ± 2.05
Verapamil (0.3 µmol /L) + $CaCl_2$	0	0.39 ± 0.22	0.64 ± 0.38	2.27 ± 0.89	8.17 ± 4.41	29.13 ± 2.37	42.28 ± 4.89	56.34 ± 5.32	59.94 ± 3.20

Data is shown as mean ± SEM (n = 4–6 individual experiments).

(80 mmol/L) and carbachol (CCh) 1 µmol/L with respective EC_{50} values of 1.24 mg/mL (95% *CI*: 0.90–1.86 mg/mL) and 2.42 mg/mL (95% *CI*: 1.88–3.14 mg/mL) (Table 3) same as verapamil. Moreover more pronounced pattern of relaxation was observed against high-K^+ and CCh-induced contractions by the application of Pn.DCM with EC_{50} values of 0.09 mg/mL (95% *CI*: 0.05–0.15 mg/mL) and 0.24 mg/mL (95% *CI*: 0.16–0.40 mg/mL) (Table 3) but Pn.Aq fraction showed incomplete relaxation of high-K^+ and CCh-induced contractions even at the higher dose of 10.00 mg/mL (Data not shown).

3.1.3. Effect on isolated rat aorta

Vasorelaxant effect was seen when Pn.Cr administered to tissue bath containing rat aortic ring (Table 4). Vasoconstriction caused by high-K^+ was inhibited by Pn.Cr with an EC_{50} value of 0.25 mg/mL (95% *CI*: 0.17–0.36 mg/mL) where as in the case of phenylephrine (PE) Pn.Cr completely relaxed the contracted tissue with EC_{50} values of 0.92 mg/mL (95% *CI*: 0.61–1.42 mg/mL) (Table 4). Pn.DCM caused relaxation of high-K^+ and PE-induced vasoconstrictions with EC_{50} values of 0.11 mg/mL (95% *CI*: 0.06–0.17 mg/mL) and 0.41 mg/mL (95% *CI*: 0.25–0.67 mg/mL) (Table 4). While Pn.Aq when tested on aortic tissue precontracted with high-K^+ and PE showed incomplete relaxation even at highest dose of 10.00 mg/mL (Data not shown).

3.1.4. Effect on isolated rabbit atrium

Carefully isolated right atrium of the rat showed typical spontaneous beating pattern with defined force and rate of contraction (inotropic and chronotropic) in *ex-vivo* environment. When spontaneous beating right atrium was challenged in cumulative increasing concentration of hydroalcoholic extract Pn.Cr (0.10–10.00 mg/mL), it showed the reduction of force of contraction (decrease inotropic effect) with EC_{50} value of 7.11 mg/mL (95% *CI*: 4.11–12.68 mg/mL) but no effect was observed on rate of contraction (Figure 2A and B).

Table 3

Broncho-relaxant effect of aqueous methanolic extract of *P. nodiflora* (Pn.Cr) and its dichloromethane fraction (Pn.DCM) on percentage basis in high-K^+ and 1 µmol/L CCh-induced contractions on isolated rat trachea.

Group	Treatment	Dose (mg/mL)								EC_{50}
		Control[a]	0.01	0.03	0.10	0.30	1.00	3.00	5.00	
Pn.Cr	High-K^+	0	0.00 ± 0.00	4.42 ± 1.37	10.26 ± 2.05	44.15 ± 1.75	100.00 ± 0.00	–	–	1.24 ± 0.15
	CCh	0	0.56 ± 0.37	2.30 ± 1.47	4.76 ± 2.30	11.70 ± 3.17	32.61 ± 3.51	72.72 ± 2.37	100.00 ± 0.00	2.42 ± 0.25
Pn.DCM	High-K^+	0	12.30 ± 2.91	25.44 ± 5.85	51.18 ± 3.34	100.00 ± 0.00	–	–	–	0.09 ± 0.01
	CCh	0	6.31 ± 2.41	16.28 ± 3.29	36.99 ± 7.24	66.09 ± 4.30	100.00 ± 0.00	–	–	0.24 ± 0.09

Values are expressed as mean ± SEM (n = 3–5 individual experiments).
[a] Control response was considered as 100% contracted and zero (0%) relaxed before the administration of any drug.

Table 4

Vaso-relaxant effect of aqueous methanolic extract of *P. nodiflora* (Pn.Cr) and its dichloromethane fraction (Pn.DCM) on percentage basis in high-K^+ and phenylephrine (PE) (1 µmol/L) induced contractions on isolated rat aorta.

Group	Treatment	Dose (mg/mL)								EC_{50}
		Control[a]	0.01	0.03	0.10	0.30	1.00	3.00	5.00	
Pn.Cr	High-K^+	0	7.83 ± 0.80	20.38 ± 1.72	35.90 ± 425	55.06 ± 3.62	95.32 ± 4.67	100.00 ± 0.00	–	0.25 ± 0.08
	PE	0	4.08 ± 0.75	10.74 ± 1.36	19.73 ± 2.42	39.05 ± 4.33	60.86 ± 2.12	100.00 ± 0.00	–	1.03 ± 0.31
Pn.DCM	High-K^+	0	7.39 ± 1.60	22.79 ± 3.51	44.58 ± 3.74	100.00 ± 0.00	–	–	–	0.39 ± 0.16
	PE	0	0.56 ± 0.56	11.33 ± 4.90	25.47 ± 6.25	39.40 ± 2.85	100.00 ± 0.00	–	–	1.39 ± 0.66

Values are expressed as mean ± SEM (n = 3–5 individual experiments).
[a] Control response was considered as 100% contracted and zero (0%) relaxed before the administration of any drug.

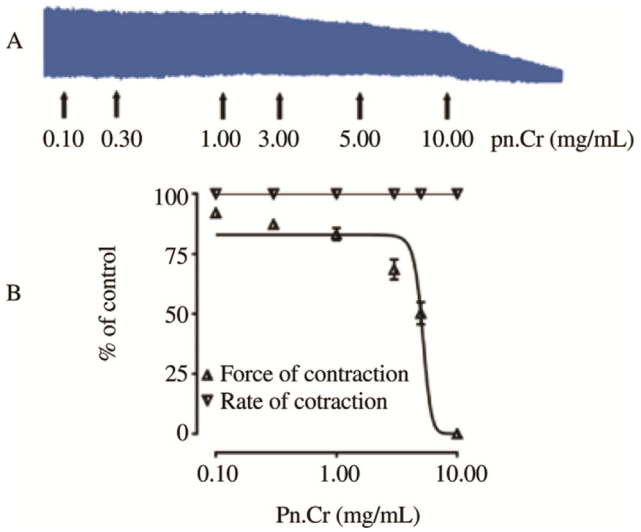

Figure 2. Effect of Pn.Cr on isolated atrium of rabbit and its graphical presentation.
A)Tracings showing the effect of Pn.Cr on rabbit atrium B) Graph showing the effect of Pn.Cr on normal response of isolated rabbit atrium (Values are represented as mean ± SEM, n = 3 individual experiments).

3.2. In-vivo studies

3.2.1. In-vivo antidiarrheal activity

Treatment with Pn.Cr in dose range from 50 to 500 mg/kg showed significant protection ($P < 0.05$) from diarrhea induced by oral administration of castor oil in mice. In a total time span of 6 h, there was significant reduction in total no of feces in 100, 200, 300 and 500 mg/kg treated animals ($P < 0.001$ compared to castor oil treated group). Loperamide (15 mg/kg), which is a potent antidiarrheal drug, showed the prominent effect on reduction in number of feaces ($P < 0.001$) as shown in Figure 3.

3.2.2. Hypotensive activity

In anesthetized normotensive rats, intra-jugular administration of Pn.Cr in a dose-dependent manner demonstrated the decrease in SBP, DBP and MAP. The most significant effect on blood pressure parameters was seen at the dose of 3 and 10 mg/kg ($P < 0.001$ compared to normotensive control). Pn.Cr at the dose of 3 mg/kg decreased SBP by (76.0 ± 2.3) mm Hg (35.00%; $P < 0.001$ *vs* normal SBP), DBP by (63.2 ± 1.7) mm Hg (30.40%; $P < 0.001$ *vs* normal DBP) and MAP by (67.6 ± 1.9 mm) Hg (32.60%; $P < 0.001$ *vs* normal MAP). Whereas Pn.Cr at the dose of 10 mg/kg decreased SBP by (70.5 ± 2.4) mm Hg (40.21%; $P < 0.001$ *vs* normal SBP), DBP by (56.2 ± 2.0) mm Hg (38.14%; $P < 0.001$ *vs* normal DBP) and MAP by (60.1 ± 2.0) mm Hg (40.38%; $P < 0.001$ *vs* normal MAP) (Table 5).

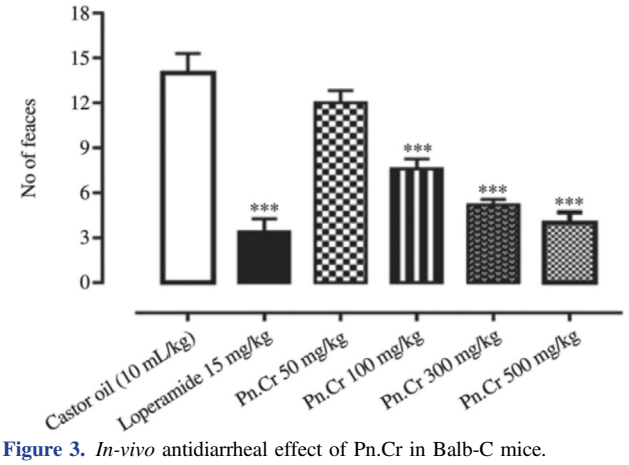

Figure 3. *In-vivo* antidiarrheal effect of Pn.Cr in Balb-C mice.
The number of feces in control and treated animals with Pn.Cr and loperamide were counted for duration of 6 h. Data are mean ± SEM of 5–6 individual experiments. One-way ANOVA followed by Dunnett's multiple comparison test was applied for data analysis. $^{**}P < 0.01$, $^{***}P < 0.001$ in comparison with control.

Table 5
Hypotensive effect of aqueous methanolic extract of aqueous methanolic extract of *P. nodiflora* (Pn.Cr) on various blood pressure parameters in normotensive anesthetized rats (mmHg).

Group	SBP	DBP	MAP
Control	118.0 ± 2.6	90.8 ± 1.2	100.5 ± 2.8
Verapamil 1 mg/kg	71.4 ± 2.7	49.7 ± 2.1	57.3 ± 2.4
Pn.Cr 1 mg/kg	99.6 ± 1.9*	78.0 ± 1.1**	85.4 ± 1.6*
Pn.Cr 3 mg/kg	76.0 ± 2.3***	63.2 ± 1.7***	67.6 ± 1.9***
Pn.Cr 10 mg/kg	70.5 ± 2.4***	56.2 ± 2.0***	60.1 ± 2.0***

Data are expressed as mean ± SEM (n = 5 individual experiments), Student t-test was utilized to analyze data. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

3.2.3. Acute toxicity

For evaluation of acute toxicity studies Pn.Cr was given to three groups (5 mice/group) in increasing order (3, 5 and 10 mg/kg). Mortality of mice was observed for 24 h and no toxicity or mortality was observed.

4. Discussion

P. nodiflora has been used traditionally by different communities throughout the world for the treatment of various ailments. Therefore current research work was planned with the intention to explore its medicinal uses for the treatment of abnormalities predominated by smooth muscle containing tissues such as gastrointestinal/vascular/broncho spasm and cardiovascular modulation on pharmacological basis.

Gastrointestinal smooth muscles show spontaneous contractions with periodic generation of slow waves and spike potentials, which are evoked due to the changes in membrane potential [25–27]. These spontaneous contractions of intestinal smooth muscles with slow waves and spike potential are generated by the interstitial cells of Cajal [27–29], slow waves propagate throughout smooth muscle cells via gap junctions and initiate spontaneous rhythmic contractions by the opening of specific calcium channels (L-type) from which calcium rushed into the cell [30,31]. Any change in gastrointestinal contractility results in various gastrointestinal problems like constipation, intestinal spasm and diarrhea [32]. Because of its use to treat diarrhea, mice were induced diarrhea mediated by castor oil to test antidiarrheal activity by using Pn.Cr. Like loperamide, a standard antidiarrheal drug, Pn.Cr showed prevention as an antidiarrheal agent in a dose-dependent pattern thus providing proof for its antidiarrheal use. In GIT, castor oil gets converted to ricinoleic acid via hydrolysis with help of an enzyme lipase and causes swelling and disturbances with intestinal mucosa which result in hyper-secretions and peristalsis occurs [33]. As a result powerful contractions are produced in transverse and distal colon and diarrhea takes place. Antidiarrheal drugs like loperamide decrease motility and secretions of intestine [15]. Isolated rabbit jejunum in a rhythmic spontaneous fashion is considered to be best preparation to study antispasmodic and anti-motility effects

and hence was used in our study to evaluate Pn.Cr [18,19]. In jejunum preparation, the Pn.Cr exhibited dose-dependent relaxation of isotonic and chemically (high-K^+) induced contractions. Relaxations by Pn.Cr were comparable to that of antispasmodic effect of verapamil possibly indicating calcium antagonistic effect of Pn.Cr. Contraction of smooth muscles is dependent on presence of free calcium in cytoplasm, which causes activation of contractile elements of smooth muscle cells. A raise in free calcium inside the cell happens either by the invasion of calcium through calcium channels (L-Type) or discharge of calcium from stores in sarcoplasmic reticulum inside the cell [27,34]. Blockage of entry of calcium from the calcium channels (L-type) or diminished amount of calcium release from stores inside the cell may be responsible for the inhibition of spontaneous contraction of jejunum. In agreement to many reports, increased extracellular level of potassium opens voltage dependent calcium channels (L-type) and allows entry of calcium to the cell thus facilitating the contraction [35,36]. Calcium channel blocker are said to be those drugs which have the potential to relax contraction induced by high-K^+ [37,38]. Thus the relaxation produced in high-K^+ mediated contractions in secluded rabbit jejunum by Pn.Cr may propose the presence of calcium channel antagonist activity [39]. Proposed calcium antagonistic effect of *P. nodiflora* was further confirmed when it showed the concentration dependent shifting of calcium response curve to rightward with the suppression of maximal effect like verapamil which is being used as a standard calcium channel blocker and standard intestinal spasmolytic drug for *in-vitro* and *ex-vivo* experiments [36,40–42]. Activity based fractionation of crude extract interestingly showed that hydro-fraction (Pn.Aq) inhibited spontaneous contractions plus low-K^+ mediated contractions but incomplete inhibition of high-K^+ mediated contractions might be indicating the occurrence of potassium channel opener activity [39]. While dichloromethane fraction (Pn.DCM) showed contractile response at low doses and relaxant response at high doses, this contractile response was blocked by atropine indicating the presence of some of the cholinergic activity. Calcium channel blockers and potassium channel openers are considered as useful drugs in gut disorders characterized by hyperactivity like diarrhea and abdominal cramps [18,19]. Presence of calcium channel blocker and potassium channel opener activity in *P. nodiflora* may provide the scientific rational of its use in diarrhea/hyperactive gut.

Pn.Cr produces dose-dependent inhibition of CCh and high-K^+ mediated contractions with more potent inhibitory action against high-K^+ mediated contractions. High-K^+ produces induction of contractions in isolated trachea by opening voltage dependent L-type calcium channel while CCh causes contraction of trachea by activating muscarinic receptors (M_1 or M_3) and finally increasing the cytosolic free calcium level [41]. Relaxation of high-K^+ mediated contractions at doses lower than that compared to CCh-induced contractions is evident that use of *P. nodiflora* in the treatment of asthma is due to calcium channel antagonism [15]. Aqueous fraction (Pn.Cr) when applied to high-K^+ and CCh-induced contractions, it showed incomplete relaxation possibly with the non existence of bronchodilator components. Pn.DCM showed complete relaxation of both high-K^+ and CCh-induced contractions with more potent action against high-K^+-induced contractions showing the presence of calcium channel blocker activity.

P. nodiflora was studied on vascular and heart tissues for possible vasorelaxant and cardiac inhibitory effect, because calcium channel blockers have firm standing in treatment of hypertension [43]. When applied to high-K^+ and PE mediated contractions in segregated rat aorta, Pn.Cr relaxes the induced contractions. PE induces contractions in aortic preparations by opening of α-receptors linked with IP_3/DAG pathway while high-K^+ causes the contraction of aorta by opening of voltage gated calcium channels (L-type) [44]. Relaxant effect of Pn.Cr on high-K^+ mediated contractions at lower dose than that of PE-induced contraction is the indication of dominant calcium channel blocker activity. Dominant vasorelaxant effect of *P. nodiflora* in DCM fraction as aqueous fraction showed incomplete relaxation of aortic preparation. This vasorelaxant activity was further confirmed when Pn.Cr was given intravenously to normotensive anesthetized rats and reduction of systolic and diastolic blood pressures was seen. Ionotropic effect on atrium was repressed by Pn.Cr in spontaneously contracting isolated rabbit atrium. Cardiac inhibitory effect of *P. nodiflora* may be the result of blockage of calcium channels which has been observed in intestinal, tracheal and vascular experiments, as calcium channel blockers have negative ionotropic effect on cardiac muscles [45].

Overall, performed *ex-vivo* and *in-vivo* study showed that blockade of calcium channels may be the basis for medicinal uses of *P. nodiflora* in the treatment of GIT spasm, asthma and hypertension.

Pharmacologically, *P. nodiflora* possess antispasmodic, bronchodilator, vasodilator and hypotensive properties as explored in the study. This study provides the preliminary evidences that blockade of calcium channels may be the possible mechanism of its action. It is also concluded that dichloromethane fraction is more potent than aqueous faction and crude extract. Nevertheless, more comprehensive studies are required to elaborate the safety, efficacy and toxicity of *P. nodiflora* and further chemical fingerprinting.

Conflict of interest statement

The authors declare no conflict of interest.

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