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Pharmacological investigation of activities pertaining to modulation of gastrointestinal, respiratory and cardiovascular parameters by *Indigofera argentea* in experimental models

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Abstract: *Indigofera argentea* is widely used for the management of gastrointestinal, respiratory and cardiac disorders. This study was done to explore scientific basis of its uses. Aqueous methanolic extract of *Indigofera argentea* and its fractions were studied on isolated tissues of rabbit's jejunum, trachea, aorta and atrium. Castor oil induced diarrheal model was used for the study of the antidiarrheal effect and pre-anesthetized rats were used for hypotensive study. Concentration dependent spasmolytic effect of the extract upon isolated jejunum, trachea and aorta was observed. Concentration response curves constructed upon isolated rabbit jejunum, revealed the presence of calcium channel blocker in the plant extract. Moreover, significant reduction ($P<0.05$) in atrial force of contraction but non-significant reduction in rate of contraction was seen by the application of plant extract. Protection ($P<0.05$) against diarrhea was observed by the administration of crude extract to rats which were pretreated with castor oil. When given to rats intravenously, the extract showed hypotensive effect. Experimental findings justified the traditional uses of *Indigofera argentea* on pharmacological basis for the management of disorders pertaining to gut, airway and hypertensive situation.

Keywords: Spasmolytic. hypotensive, tracheorelaxant, antidiarrheal, concentration response curve.

INTRODUCTION

Spasm of gastrointestinal tract (GIT) is a common complication affecting the people of every gender, age and badly affects the quality of life (Janbaz *et al.,* 2015a). Hyperactivity of Calcium (Ca^{++}) in the circular and longitudinal smooth muscles of GIT is believed to be a key player in the spastic activity and therefore antagonism the input of Ca^{++} is a useful pharmacological manipulator (Rahman *et al.,* 2019). Furthermore, bronchial tissues and vascular tissues are enriched with the smooth muscles and any anomaly with the functionality of smooth muscles results in bronchoconstriction and vasoconstriction (Janbaz *et al.,* 2015b). Moreover, diarrhea is another life-threatening situation in which hyper-activity of gut due to excess of Ca^{++} is a critical determinant. Plants and natural medicine are the enormous source of drugs which have ameliorated the spasticity of GIT and other tissues (Aleem *et al.,* 2020). The best-known example extracted from the plant is Hyoscine, a strong regulator of GIT, acts by its antimuscarinic potential. Similarly, population of under developed countries are still looking for complementary and alternative medicines system to treat their respiratory and cardiac problems. These herbal remedies may be thought superior over standard remedial agents by local communities to treat these ailments. Since these plants have various phytoconstituents that have synergistic effects and/or neutralizing of adverse effect potential, therefore established to be comparatively safe for long use (Mehmood *et al.,* 2014).

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In Pakistan, *Indigofera argentea* is *locally* known as Seehan and widely distributed in different geographic areas of Pakistan, Iran, Saudi Arabia, Egypt, Sudan and Somalia. It is an annual herb, erect, much branched up to 30 cm or more tall. Stem glaucous-silvery and with very short glandular hairs. Leaflets 5-7, opposite, obovate, 6 mm long, both sides silvery. Racemes 3-12 flowered, lax, exceeding the subtending leaves. Calyx densely pubescent. Standard pubescent outside. Stamens 3 mm long. Pods straight, spreading 1x 0.1 cm, mucronate, silvery and with very short glandular hairs, 2-5 seeded (Alfarhan *et al.,* 2005). Traditionally it has been used for the treatment of gastrointestinal disorders, jaundice, malaria and headache vertigo, inflammation and body pain (Ahmad *et al.,* 2014). It is also used as analgesic, anti- inflammatory and antipyretic (Javed *et al.,* 2020). The leaves and roots of *Indigofera argentea* are bitter in taste but their oil is used to heal traumatic wounds and ulcers. Pertaining to this plant very minimal scientific evidences are present related to its claimed traditional used in many of the developing countries therefore the current study was designed to explore its usage on the pharmacological background to investigate underlying mechanisms.

MATERIALS AND METHODS

Collection, identification and extraction of plant

Whole plant of *Indigofera argentea* Burm.f was collected from rural area of Bijnot, Cholistan, Bahawalpur, Punjab, Pakistan. The plant was identified by taxonomist at the

Institute of Pure and Applied Biology, Bahauddin Zakariya University, Multan, Punjab, Pakistan with a voucher # www.theplantlist.org/tpl1.1/record/ild-3686. The plant was carefully picked to avoid adulteration. After collection the plant material were shade dried for 12 – 15 days. After adequate time the dried plant material was converted into coarse powder by using a purpose specified herbal grinder (Rahman *et al.,* 2019). Obtained coarse powder was macerated in the 80% hydro-alcoholic mixture (CH_3OH and H_2O) in amber color air tight container. The macerated plant was daily shaken and kept at 25^oC + 5 for a period of 15 days. After completion of 15 days period, soaked plant was strained through strainer to remove insoluble powder material. The filtrate was run through filter paper. The remaining part was resoaked for one week and process repeated twice.

Obtained filtrates were mixed and then dried at low pressure rotavap at 37^oC + 2. A concentrated crude extract of *Indigofera argentea* (Ia.Cr) was obtained. Sufficient amount of preformed extract was mixed in water to obtained saturated solution of extract. This mixture was poured in separating funnel and added to dichloromethane then shake agitativelly. The above procedure repeated thricely for liquid- liquid fractionation. Separated aliquots layer of DCM was combined and then dried at low pressure rotavap at 37^oC + 2 solvent was removed by means of rotary evaporator. The remaining aqueous layer in funnel was collected and dried. The %age yield was 0.4, 0.6 and10.4% for DCM, aqueous and hydro-alcoholic fractions. Extract and its fractions were stored at 4°C for further use. Every day of experimentation fresh solution of crude extract and fraction was prepared.

Animals

Locally breed male rabbits with body weight of 1.6-2 kg (5-8 months), Sprague dawley (SD) rats (250-300 grams) and Swiss albino mice (25-35 grams) both sexes were used in this study. All animals were kept in special animal boxes and gave special animal feed and tap water *ad libitum.* Standard temperature (25^oC) + 5^oC and humidity (50%) + 5% was maintained, with a cycle of 12 hours' day and night. The standard feed was given to animals with protein content was up to 20-25% and carbohydrates 60-65%. Experimental animals had *ad libitum* water but feed was withdrawn 15-20 hours before experiment. Animals were brought to experimental laboratory few hours before the experiment in order to acclimatize the conditions of that room. The rabbits were sacrificed by cervical dislocation (Janbaz *et al.,* 2015a). Then animal was anatomized to obtained jejunum, trachea, aorta and atria for ex-vivo isolated tissue study, whereas rats and mice were used for in-vivo studies.

In each case of animal handling care was exercised to minimize the pain and stress on animal. Animals were used in this study following the animal protocols of Institute of Laboratory Animal Resources (ILAR), Commission on Life Science, National Research Council (NRC, 1996) Washington DC, USA.

These animal protocols were passed by the Ethical Committee vide # 09/PEC/2015 of B.Z.U, Multan, Punjab, Pakistan.

Materials

The experimental work was conducted with highly pure and scientific research grade chemicals and drugs. All was purchased by the Merck (Germany), and Alfa Acer (USA). Loperamide, verapamil hydrochloride and potassium chloride were used as standard drugs for *in-vivo* and *ex-vivo* studies. NaCl, $CaCl_2$, NaH_2PO_4, KH_2PO_4, $MgCl_2$, $MgSO_4$, KCl, glucose and sodium citrate are taken for the preparation of physiological salt solution. All solutions were freshly prepared in distilled water, prior execution of experimental work.

Preliminary phytochemical screenings

For the screening of phytochemicals in Ia.Cr different tests were performed as described by (Janbaz *et al.,* 2012; Rahman *et al.,* 2019).

Ex -vivo experimentations

Spasmolytic study of plant extracts on isolated tissue of rabbit jejunum

Spasmolytic effect of plant extracts was studied on isolated rabbit jejunum preparation (Janbaz *et al.,* 2015a; Khan *et al.,* 2016). Rabbits were starved for 15-20 hours but water *ad libitum* before to conduct study. Animal was sacrificed by cervical dislocation and jejunum was anatomized. Jejunum was cut into 2-3cm segments (Janbaz *et al.,* 2015b). These pieces were cleaned off gently from the feces and fatty material. Then it was fixed with glass rod and hanged in 20ml chamber filled with Tyrode's solution (pH 7.4). The temperature was kept at 37^oC+ 1^oC with uninterrupted carbogen supply.

A preload of 1gram was applied to the suspended tissue. Prior to the application of any drug, the tissue was permitted to stabilize for 30 – 45 minutes. For tissue stabilization, acetylcholine (0.3 µM) was applied for 30 second at 3-5-minute intervals. After tissue stabilization, plant extract was applied in a cumulative order and the response was recorded by isotonic transducer (MLT 0015, Panlab, Spain) coupled with PowerLab data acquisition system. The spasmolytic response was calculated as percentage of the control response.

To assess the calcium channel activity of the plant extract, Ia. Cr was applied to the high-K^+ induced contractions. For confirmation of the calcium channel blocker activity of the plant extract, calcium response curves were constructed as described by the method as discuss by (Rahman *et al.,* 2017a).

Tracheorelaxant activity

To study the tracheorelaxant activity of plant extract, isolated trachea of rabbit was used (Rahman *et al.*, 2017a). After scarification of rabbit, trachea was anatomized and cleaned off adhering tissue dexterously. Isolated trachea was divided into small pieces of 2-3 cartilages length. These small pieces were opened by cutting the cartilage in front of smooth muscle. Then it was hanged in 10 ml tissue organ bath, filled with Kreb's medium (pH 7.4). Temperature was maintained at $37^{o}C$ + $1^{o}C$ with continuous carbogen perfusion. A preload tension of 1.0 gram was applied. Tracheorelaxant activity of the plant extract was determined by the application of Ia.Cr to High-K^{+} and carbachol 3µM (CCh) induced tracheal contractions and the response was recorded with the help of isometric transducer (MLT 0201, Panlab, Spain), linked with Powerlab data acquisition system (Rahman *et al.,* 2019; Aleem *et al.,* 2020).

Vasorelaxant activity

For determination of vasorelaxant effect of plant extract, isolated thoracic aorta of rabbit was used. After scarification of the rabbit, aorta was anatomized, cleaned off the adhering tissues and placed in Kreb's solution. Aorta was divided in to small segments of 0.3-0.4 cm in length. These tissues were hanged in a 10mL tissue organ bath filled with Kreb's solution. A preload of 2 g weight was applied and this force kept maintained during whole study. Tissues were repeatedly exposed to High-K^{+} or phenylephrine (1µM) for tissue stabilization. Vasorelaxant effect of *Indigofera argentea* was studied by the application of plant extract to precontracted tissues and the response was recorded with the help of isometric transducer MLT 0201 attached with Power Lab data acquisition system (Rahman *et al.,* 2017b & 2019).

Atrial contractions

To study the effect of *Indigofera argentea* on rate and force of contractions of atrium, isolated right atrium of rabbit was used. Rabbit was sacrificed and right atrium was removed carefully. Then dissected atrium was hanged in tissue organ bath filled with Kreb's solution. Temperature was kept constant at $37^{o}C$ + $1^{o}C$ with continuous supply of carbogen. After stabilization of the atrial tissue, Ia.Cr was applied to it in a cumulative fashion and the response was recorded using isometric transducer. Negative or positive inotropic and chronotropic effects of the plant extracts were expressed as %age of the baseline response of isolated atrium (Khan *et al.,* 2017; Rahman *et al.,* 2017; Rahman *et al.,* 2019).

In-vivo experiments

Anti-diarrheal activity

For anti-diarrheal study of plant extract five groups of SD rats containing n=6-8 animals in each group were used (shifah *et al.,* 2020). The animals were healthy almost of equal age i.e. 45-60 days, weight range 250-300 grams of either gender were selected for diarrheal protection activity. Food was removed 20-24hours earlier the study but water presents all time (Shahed-Al-Mahmud *et al.,* 2020).. Animals were placed in their respective cages lined with absorbent paper. Castor oil was given orally by oral gavage to all animals 1hour before the dosing of the plant extract for inducing diarrhea. After 1hour 1^{st} group were treated with (10ml/kg) normal saline (as negative control), 2^{nd} group was given (10mg/kg) loperamide (positive control) while the third, fourth and fifth groups were administered different plant extract doses 100mg/kg, 200mg/kg, and 300mg/kg respectively. After 6 hours of dosing, wet, dry and total number of stool (dry and wet) of all animals of all groups (de Souza Monteiro *et al.,* 2018; Li *et al.,* 2019; William *et al.,* 2019).

Hypotensive activity

Previously reported procedure was adopted for the investigation of hypotensive potential of test compound (Rahman *et al.,* 2019). Healthy adult Albino rats of either sex almost of equal age (45-60 days), weight range 250-300 grams of were used. Animals were anesthetized by the administration (i.p.) of diazepam (0.01g/kg) and ketamine (0.075g/kg). As required anesthesia was attained rat was kept on dissecting board in horizontal lying position. To fix the movement of animal, its paws and tail were fixed with sticking tape. Jaw was fixed with rubber band to fix the movement of head and neck. Animal was placed on isothermic rodent pad at temperature $37^{o}C$. For tracheal intubation; linear low-density polyethylene tube (LLDPE 20mm) was administered into trachea.

Jugular vein and carotid artery were cannulated by (LLDPE 20mm) for dosing of test drug. For measuring blood pressure disposable BP transducer (MLT 0699) was connected with carotid artery on one end and its second end was coupled with power lab data acquisition system. Cannulated surface was covered by tissue paper which is moistened with normal saline so that animal tissue remains hydrated. Transducer was calibrated before the execution of experiment. Heparin sulphate (0.1ml) was occasionally injected to inhibit blood clotting during experiment and 0.1mL sodium chloride solution 0.9% was given after each dose. As the animal blood pressure maintained, 0.1ml of plant extract was delivered into jugular vein followed by 0.1mL sodium chloride solution. Verapamil hydrochloride was injected as a standard hypotensive drug. Any decrease in BP was calculated and correlate with effects of verapamil. Different parameters like systolic, diastolic and mean arterial pressure were determined (Aslam *et al.,* 2016; Bilanda *et al.,* 2020).

Acute toxicity study

Acute toxicity study of *Indigofera argentea* was conducted on adult mice (25-35gram) of Swiss albino

strain without gender discrimination. Control group animals were administered normal saline (1omL/kg) orally, while the test group animals were given different doses of Ia.Cr (0.5, 2, 5 and 10g/kg). These animals were kept in controlled environment. The temperature was kept at 25^{o}C + 5^{o}C, humidity 50% + 5% was maintained with a cycle of 12 hours' day and night. These animals were kept under observation for 14 days. For first 12 hours they were examined after every one hour. After that they were examined on daily basis for any physical, behavioral change. Different parameters were like tremors, writhing, convulsions, lacrimation, salivation and mortality rate were monitored (Javed *et al.*, 2020).

STATISTICAL ANALYSIS

Results were presented by mean ± SEM (n=5-10 animals per group) with confidence intervals of 95%. The conc. response curves were calculated by nonlinear regression. Two-Way ANOVA followed by Tukey's test (multiple comparison) was used to evaluate the significant difference among different concentration. One-way ANOVA followed by Dunnett's multiple comparison test was used to analyze antidiarrheal results. While hypotensive results were evaluated by using Student's t-test. Statistical analysis was done using GraphPad Prism version 8.

RESULTS

Preliminary phytochemical analysis

Initial phyto-chemistry of Ia.Cr disclosed that *Indigofera argentea* has alkaloids, flavonoids, glycoside, terpenoids, tannins, and saponins.

Ex-vivo activities

Spasmolytic effect on isolated rabbit jejunum

Spasmolytic effect of crude extract of *Indigofera argentea* (Ia.Cr) was studied by using isolated rabbit jejunum preparation. Application of Ia.Cr to spontaneously contracting isolated jejunum and potassium-80mmole (high-K^+) mediated contractions in isolated jejunal tissue showed inhibition of both contractions. Resulted EC_{50} values were 4.09 ± 0.14 (2.0 to 11.65 mg/ml; by 95% *CI*) and 1.93 ± 0.09 (0.12 ± 0.06 mg/m; by 95% *CI*) respectively. Verapamil the standard calcium channel blocker also used similar results with EC_{50} values of 0.28 ± 0.09 (0.17 to 0.48mg/ml; by 95% *CI*) and 0.08 ±0.06 (0.06 to 0.11 mg/ml; by 95% *CI*) against spontaneous and high-K^+ induced contractions.

Moreover, dichloromethane fraction (Ia.DCM) also resulted the relaxation of spontaneous contractions as well as High-K^+ induced contraction of jejunal tissue with EC_{50} value of 0.20 ± 0.06 (0.15 to 0.27 mg/ml; by 95% *CI*) and 0.12 ± 0.06 (0.09 to 0.17 mg/ml; by 95% *CI*) respectively. However; aqueous fraction (Ia.Aq) was found less potent than hydro-methanolic and DCM fraction. Thus we can conclude that tissue relaxing effect of Ia.Cr and Ia.DCM was significant and equivalent to verapamil effect. Calcium concentration response curves (CRCs) were constructed with and without Ia.Cr and similarly with verapamil for confirmatory result. The fluid that were provided for calcium concentration response curves (CRCs) construction was Ca^{++} free but K^+ enriched. It was observed that pre-exposure with Ia.Cr caused the relaxation of maximally contracted jejunum tissue and shifted the CRCs in the rightward direction.

Consequently, confirmed the Ca^{2+} antagonistic potential of plant extract. To evaluate the calcium channel blocker activity of Ia.Cr, calcium response curves were constructed in the absence and presence of Ia.Cr (0.3-3.0 mg/mL). Ia.Cr caused rightward shifting of the calcium curves with the inhibition of maximum response like verapamil. (fig. 1)

Tracheorelaxant activity

Application of Ia.Cr exhibited inhibition of high-K^+ and carbachol (CCh; 1µM) stimulated tracheal contractions. EC_{50} values of Ia.Cr versus high-K^+ was 0.25± 0.11 (0.007 to 3.01 mg/ml; by 95% *CI*) and versus carbachol (CCh) 1µmol are 0.72± 0.15 (0.32 to 1.51mg/ml; by 95% *CI*) like verapamil. Similarly, Ia.DCM also caused inhibition of high-K^+ and CCh induced contractions but at low concentration with respective EC_{50} values of 0.12 ± 0.09 (0.08 to 0.18 mg/ml; by 95% *CI*) and 0.58 ± 0.08 (0.38 to 0.91 mg/mL by 95% *CI*) in that order. Hence Ia.Aq exhibited partial inhibition of high-K^+ and CCh stimulated contractions (fig. 2).

Vasorelaxant activity

Ia.Cr when applied to isolated aorta of rabbit, resulted in relaxation of high-K^+ and phenylephrine (PE) induced vasoconstriction like verapamil, with EC_{50} values of 1.16 ± 0.07 (0.83 to 1.66 mg/ml; by 95% CI) and 3.85 ± 0.12 (2.19 to 6.95 mg/ml; by 95% CI) respectively. Similarly, Ia.DCM also showed vasorelaxant effect against both high-K^+ and PE induced contractions with respective EC_{50} values of 0.38±0.10 (0.256 to 0.57 mg/ml; by 95% CI) and 3.65±0.18 (1.82to 9.49 mg/ml; by 95% CI). Whereas, Ia.Aq showed prominent effect against PE induced contractions as compared to high-K^+ induced contractions (fig. 3).

Effect on atrial contractions

Application of Ia.Cr to isolated atrium of rabbit decreased the force and rate of contraction with respective EC_{50} values of 0.227 ± 0.197 (2.274 to 12.435 mg/ml by 95% CI) and 1.676 ± 0.22 (0.593 to 9.218 mg/ml by 95% CI). However significant effects were displayed upon force of contraction in contrast to rate of heart (fig. 4).

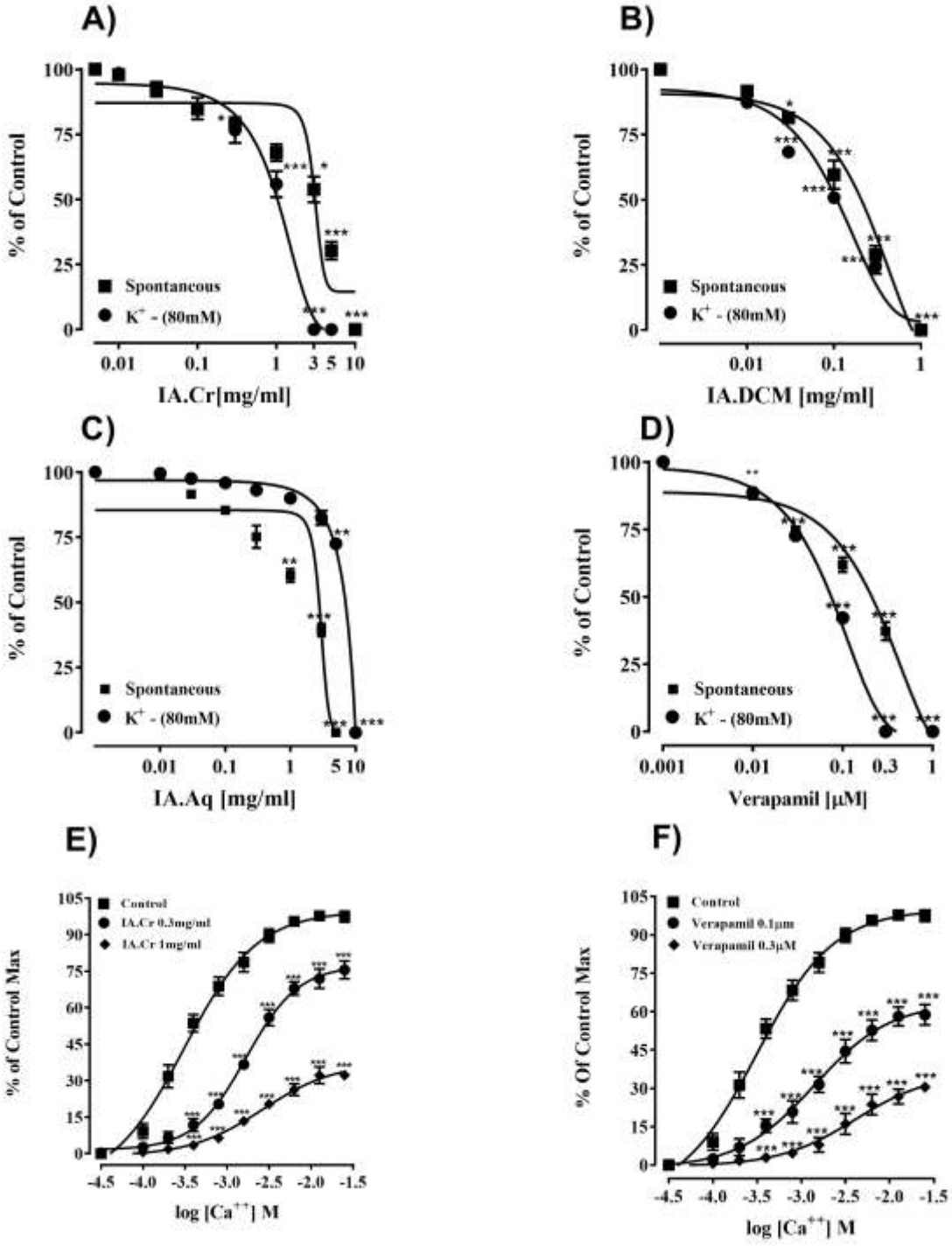

Fig. 1: Spasmolytic effect of (A) Ia.cr, (B) Ia.DCM, (C) Ia.Aq and (D) verapamil upon spontaneous and high-K^+ induced contractions in jejunum. Data are represented as mean ± SEM, n = 5-7 experiments. Two-Way ANOVA pursued by Tukey's post-test was used to analyze the data. E) Ca^{++} response curve (CRC_s) without and with of Ia.cr (F) without and with verapamil upon jejunum tissue and the data of CRCs is represented as mean ± SEM n = 5-7 individual experiment and measured statistically by Two-Way ANOVA pursued by Dunnet's test compared with control. **P*<0.05, ***P*<0.01, ****P*<0.001were regarded statistically significant.

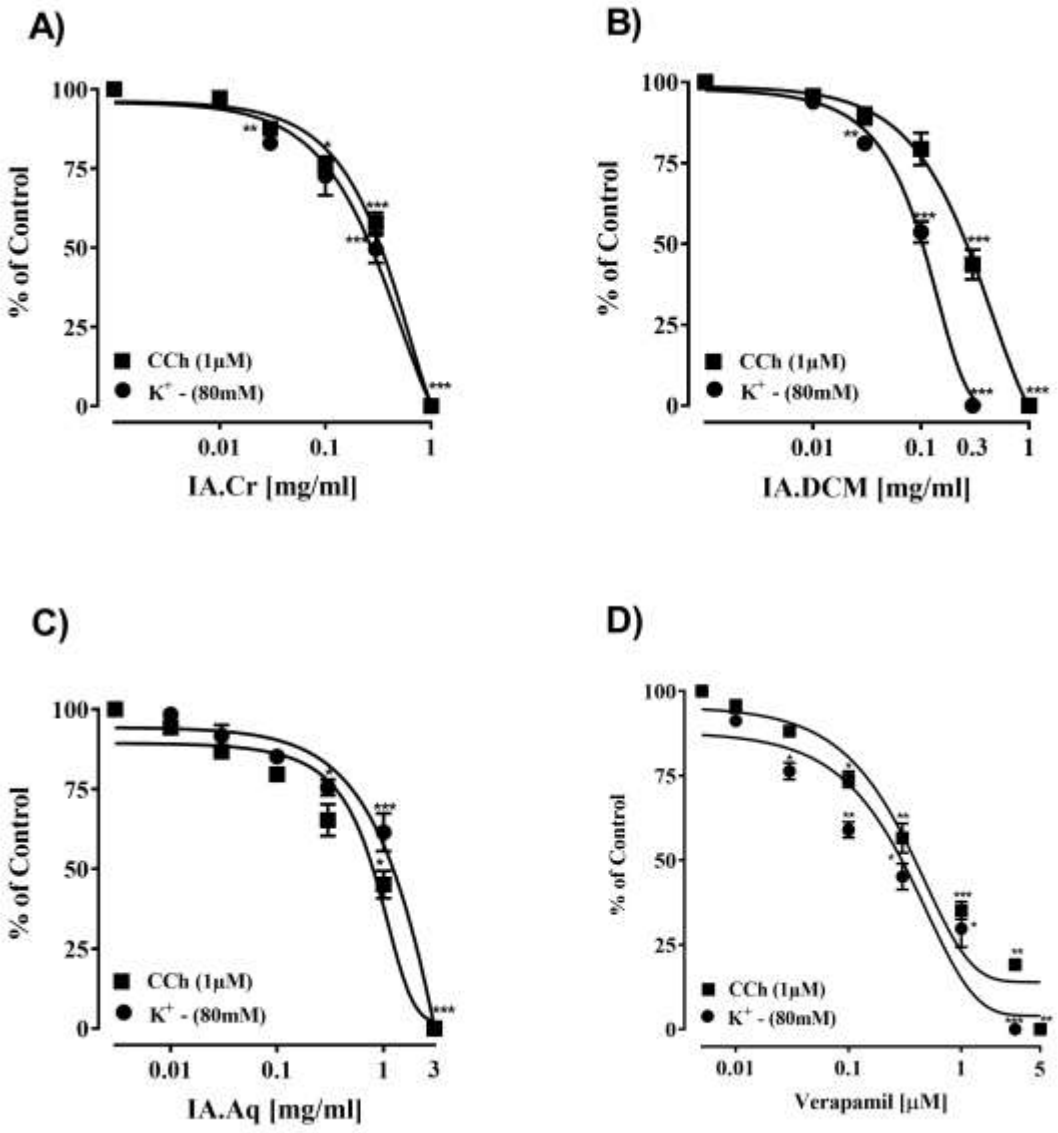

Fig. 2: Concentration dependent relaxing effect of A) Ia.cr, B) Ia.DCM, C) Ia.Aq and D) verapamil on High-K^+ and carbachol (CCh) stimulated contractions of isolated tracheal tissue. Data presented as mean ± SEM, n = 5-7 individual experiments. Two-Way ANOVA followed by Tukey's post-test was used to evaluate the significant changes among different conc. of extract. **P*<0.05, ***P*<0.01, ****P*<0.001were regarded statistically significant.

Table 1: Dose dependent hypotensive effect of *Indigofera argentea*

Group	SBP	DBP	MABP
Control	120.762 ± 2.37	92.876 ± 1.54	108.458 ± 4.85
Verapamil (1mg/kg)	65.658 ± 3.12***	49 ± 2.59***	54.552 ± 1.02***
Ia.Cr (3mg/kg)	104.846 ± 3.67	92.542 ± 5.67	96.644 ± 4.95
Ia.Cr (10mg/kg)	91.186 ± 4.10**	77.378 ± 4.85*	81.982 ± 4.42**
Ia.Cr (30mg/kg)	76.794 ± 5.73***	59.314 ± 4.18***	65.14 ± 4.24***

Results are displayed by way of mean ± SEM; *n* = 5, and analyzed by student *t*-test. Probability values (**P* < 0.05, ***P* < 0.01, ****P* < 0.001) were regarded statistically significant with respect to control.

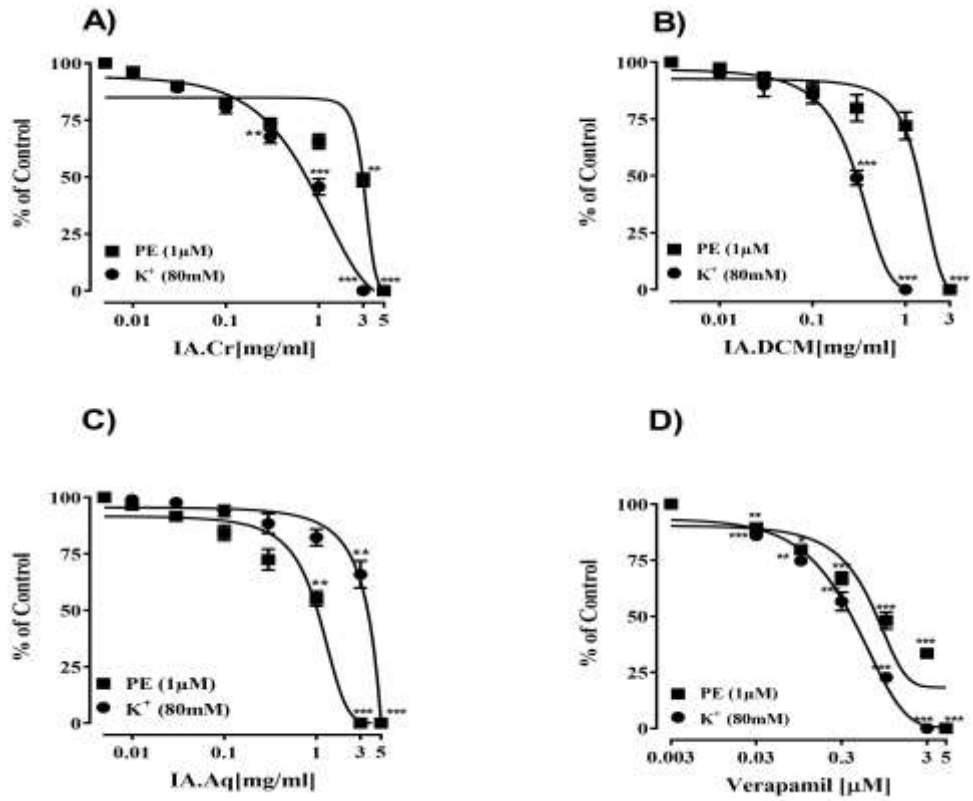

Fig. 3: Concentration dependent vasorelaxant effect of Ia.Cr on A) High-K^+ and B) PE induced contractions of isolated aorta of rabbit. Data presented as mean ± SEM, n = 5-7. Two-Way ANOVA pursued by Tukey's post-test was used to analyzed the data. **P*<0.01, ***P*<0.01, ****P*<0.001were regarded statistically significant.

In-vivo studies

Anti-diarrheal activity

Protection from diarrhea was observed by the application of Is.cr to rats as seen by the reduction in fecal output. Respective no of dry, wet and total feces in castor oil treated animals was 0.83±0.30, 7.34±1.53 and 8.16± 1.32. Loperamide treated animals showed reduction in dry, wet and total number of feces to 3.16±1.01, 1.00±0.36 (*P* <0.001) and 4.16±0.94 (P<0.005) respectively. Administration of Ia.cr 0.1gm/kg exhibited decrease in wet stools count to 4.83±0.65, dry stools to 1.16±0.40 and total stools were 6.00±1.00. Further treatment with Ia.cr 0.2gm/kg and Ia.cr 0.3gm/kg was also exhibited considerable decline in wet stools count that were 1.83 ± 0.47 (*P*<0.001) and 1.16 ± 0.54 (*P*<0.001). Furthur more there is also reduction in total stools count that were 4.33 ± 0.71 (*P* < 0.05) and 3.66 ± 0.80 (P<0.005) with respect to castor oil class respectively (fig. 5).

Hypotensive activity

Indigofera argentea (Ia.Cr) at various doses 3, 10 and 30 mg per kg produced dose dependent hypotension when intravenously administered to pre-anesthetized, normotensive rats (n=5). Ia.Cr 3mg/kg exhibited reduction in systolic blood pressure (SBP) to 104.846 ± 3.67 mmHg, diastolic blood pressure (DBP) to 92.542 ± 5.67 mmHg and mean arterial blood pressure (MABP) to 96.644 ± 4.95mmHg vs normal control. Whereas, Ia.Cr 10mg/kg exhibited decrement in SBP to 91.186±4.10 mmHg (P< 0.01), DBP to 77.378 ± 4.85mmHg (*P* < 0.05) and MABP to 81.982±4.42mmHg (*P*<0.01) vs normal control. Similarly, 30 mg/kg of Ia.Cr produced prominent reduction in SBP to 76.794±5.73 (P<0.001), DBP to 59.314 ± 4.18 mmHg (*P*<0.001) and MABP to 65.14 ± 4.24 mmHg (*P*<0.001) vs normal control (table 1).

Acute toxicity studies

Administration of Ia.Cr to mic e up to the dose of 10g/kg showed no toxic effects in 48 hours and 2 weeks duration. Animals followed their regular sleep and awake pattern. Behaviorally the animals were calm and did not show any signs of behavioral manifestation such as existence of anxiety, despairness and aggression. No tremors, salivation, diarrhea bradykinesia and mortality were observed for the entire observed time.

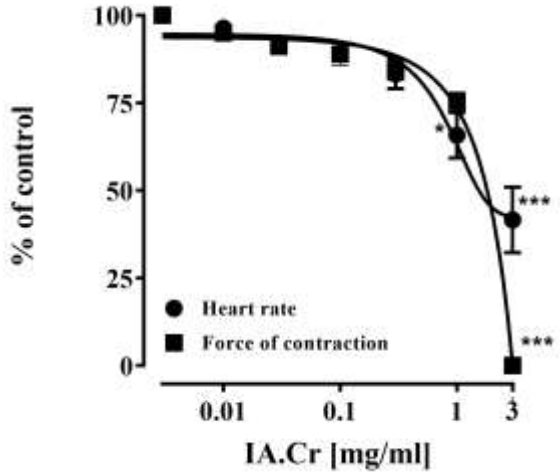

Fig. 4: Inhibitory effect of *Indigofera argentea* on rate and force of contractions of isolated atrium of rabbit. Results were expressed by mean ± SEM, n=6-7. Two-Way ANOVA pursued by Tukey's post-test was used. P values **P*<0.05, ***P*<0.01, ****P*<0.001 were regarded statistically significant.

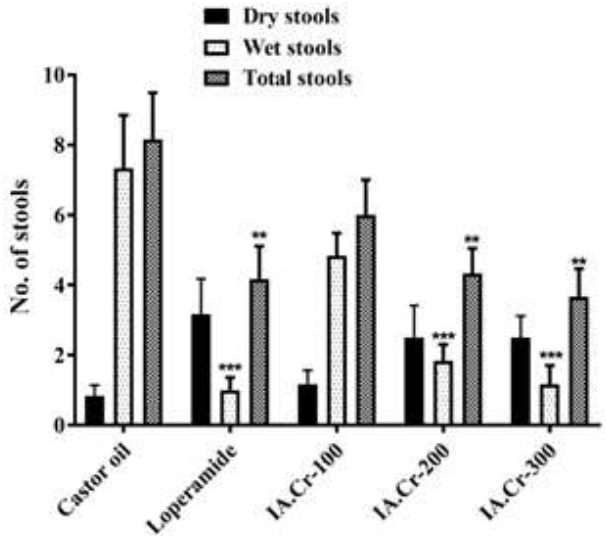

Fig. 5: Bar graph was demonstrating diarrheal protection activity of Ia.cr. Data values are represented by mean ± SEM, n = 6-8. One-Way ANOVA pursued by Dunnett's test was used for analyzing the data with respect to control. ***P*<0.005, ****P*<0.001were regarded statistically significant.

DISCUSSION

Indigofera argentea is used to treat various ailments like jaundice, hepatitis, urticarial and blood purifier (Ahmad *et al.,* 2014) and also as analgesic, anti- inflammatory and antipyretic (Javed *et al.,* 2020). This study was conducted for the evaluation of spasmolytic effect of crude extract of this plant.

Calcium has important role in muscle contraction and maintenance of muscle tone (Sanders *et al.,* 2011). The level of free calcium in sarcoplasm is low as compared to sarcoplasmic reticulum. When action potential initiated due to high k^+ in cell membrane, its open voltage sensitive Ca^{2+}-channels present in cell membrane (sarcolemma) of smooth muscles cells. Cytosolic level of free calcium increases, which opens calcium channels present on sarcoplasmic reticulum membrane by stimulation of ryanodine receptors. As a result, calcium rushed into the cytosol. The released free calcium binds with cytosolic proteinaceous structure known as calmodulin and Ca^{2+}-calmodulin complex was formed. That Ca^{2+}-calmodulin triggers the enzymes (myosin light chain kinases MLCK). MLCK enzymes cascade resulted the phosphorylates of cross bridges heads of myosin filaments (Sweeney & Hammers, 2018). Activated cross bridges bind with actin filaments and as a result contraction of smooth muscles take place (Al-Shboul, 2018; Touyz *et al.,* 2018). Due to increase in contractility of smooth muscles in gastrointestinal system, diarrhea may occur. Similarly, contraction of smooth muscles of respiratory tract results in asthma (Worgall & Worgall, 2018) whereas contraction in smooth muscles of vascular system leads to increase in peripheral vascular resistance (Touyz *et al.,* 2018). In the same way increased gut motility develop diarrhea (Ciobanu & Dumitrascu, 2011; Li *et al.,* 2019). Calcium channels antagonist block the inward entry of calcium in cytosol, ultimately low level of calcium in cytoplasm leads to the relaxation of smooth muscles of gastrointestinal tract, respiratory tract and vascular system. This inhibitory effect on voltage sensitive calcium channels in smooth muscle was useful in ailments like diarrhea, asthma and hypertension. (Aslam *et al.,* 2016; Rahman *et al.,* 2019; Sanders *et al.,* 2012). When we used high-K^+ to smooth muscle tissues direct to the opening of voltage sensitive Ca^{+2}-channels which results in contraction of smooth muscle (Aslam *et al.,* 2016; Iqbal *et al.,* 2019; Aleem *et al.,* 2020)). Ca^{+2}-channels antagonist blocks the smooth muscles that were pre-exposed to high-K^+ and spontaneous contracting smooth muscles. However major effect was observed in opposition to high-K^+ produced contraction with respect to spontaneously induced contractions (Rahman *et al.,* 2017a & 2017b).

Ia.Cr when applied to spontaneously and high-K^+ induced contraction on rabbit's jejunal segment; reduction in contraction was seen in both tissues. Although inhibiting effects were significant with opposition to the contractions mediated by high-K^+ as observed by verapamil against high-K^+ mediated contractions. Similar effects were seen with the administration of Ia.DCM to the jejunal piece of rabbit; although at a reduced dose with respect to Ia.Cr effects. For the confirmation of

calcium channel blocker activity of Indigofera argentea, calcium response curves were constructed. Incubation of tissue with Ia.Cr prior to administration of calcium, resulted in rightward shift of calcium curves with inhibition of maximum contractile effect like verapamil, a calcium channel blocker. The above mentioned *ex-vivo* result was authenticated by protection against diarrhea that was induced by castor oil (shifah *et al.,* 2020). Calcium channel antagonists may be used for the management of gastrointestinal diseases like dysentery and diarrhea (de Souza Monteiro *et al.,* 2018; Khan *et al.,* 2016; Li *et al.,* 2019; Shareef *et al.,* 2014; William *et al.,* 2019). Due to calcium channel blocking potential, *Indigofera argentea*, it can be used in the management of diarrhea (Shahed-Al-Mahmud *et al.,* 2020).

Indigofera argentea was also studied for its possible tracheorelaxant effect. Concentration based inhibitory effects of Ia.Cr, Ia.DCM and Ia.Aq were observed against high-K^+ and CCh induced isolated tracheal contractions like verapamil (Aleem *et al.,* 2020). High-K^+ is well-known to mediate contraction by opening of Ca^{+2}-channels (voltage operated) L-type in smooth muscles (Shareef *et al.,* 2014; William *et al.,* 2019) whereas carbachol (CCh) mediate contraction in tracheal tissue by stimulating M_3 receptors. The ligand attached with receptor and further activate G_q protein. This activated complex then stimulate phospholipase-C (PLC) (Yang *et al.,* 2012). PLC present on the cytosolic face of plasma membrane that splits phosphoinositol 4,5-bisphosphate to diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). Then this IP_3 bind with IP_3 receptor that are present on endoplasmic reticulum and directs to opening of ligand gated Ca^{+2}-channels. Calcium released into cytosol and then activate protein kinase C. DAG also convert inactive protein kinase C (PKC) to active PKC (Katzung & Chatterjee, 2012; Widmaier, Raff, & Strang, 2011). This stimulated PKC ultimately results in bronchoconstriction (Townsend *et al.,* 2013). Ia.Cr produced tracheal relaxant effect against high-K^+ and CCh 1µmol mediated tracheal contractions with major effect was showed against high-K^+ mediated tracheal contraction like verapamil. Similar effects were observed by the administration of Ia.DCM to tracheal tissue. Ia.Cr also showed tracheorelaxant effect but the effect was more pronounced against CCh induced contraction than high-K^+ induced contractions like dicyclomine , an antimuscarinic (Data not shown), indicating the presence of dual mechanism of tracheal dilation, calcium antagonism and muscarinic receptor antagonism (Rahman *et al.,* 2017).

Indigofera argentea was assessed for possible vasorelaxant and hypotensive effect. When Ia.Cr was administered to phenylephrine (PE) and high-k^+ mediated vasoconstriction; it exhibited a concentration dependent vasorelaxation. Significant vasorelaxation was observed versus high-k^+ mediated vasoconstrictions, demonstrating the presence of Ca^{+2}-channel blocker activity (Rahman *et al.,* 2019) Alike Ia.DCM was exhibited relaxation of pre-constricted aorta segment. Ia.Aq fraction also produced relaxation of high-k^+ 80mmol mediated vasoconstriction and in-significant relaxation versus PE 1µmol mediated vasoconstrictions. The vasodilatation responses of Ia.DCM are more prominent with respect to Ia.Cr and Ia.Aq. That results exhibited that dichloromethane fraction is rich in smooth muscle relaxant components such as flavonoids (Vrolijk *et al.,* 2020). Peripheral vascular resistance was reduced due to vasorelaxation of vascular bed. As we administer different doses of Ia.Cr to isolated rabbit atrium, dose dependent reduction in heart rate and force of contraction was observed. Blood pressure is product of (CO) cardiac-output and (PVR) peripheral-vascular-resistance (Rang *et al.,* 2014; Siddiqui *et al.,* 2011). When PVR was increased blood pressure was also increased and similarly when cardiac output (CO) was increased blood pressure was also increased and vice versa (Katzung & Chatterjee, 2012). Ia.Cr exhibited reduction of both CO and PVR that provide solid evidence for the management of hypertension. The hypotensive results were authenticated by *in-vivo* experiment on pre-anesthetized SD rats. When Ia.Cr was given parentally to animals dose dependently reduction in blood pressure was exhibited. From the observations of all above mentioned results it was assumed that all activities of *Indigofera argentea* like tracheorelaxant, antispasmodic, vasorelaxant and hypotensive activities may be due to the blockade of voltage gated Ca^{+2}-channels and muscarinic antagonism.

Phytochemical screening *Indigofera argentea* revealed the presence of components such as flavonoids, alkaloids (Moloudizargari *et al.,* 2013), phenols, saponins and tannins. Whereas HPLC analysis showed the presence of gallic acid, quercetin, caffeic acid, chlorogenic acid, benzoic acid, ferulic acid and p-coumaric acid that are associated with many pharmacological activities (Javed *et al.,* 2020). It has been documented that these phytochemical compounds contain smooth muscle relaxant activities (Vrolijk *et al.,* 2020). Hence it is deduced that these activities could be due to these phytochemical compounds.

CONCLUSION

In the light of above phytochemical screening, *Indigofera argentea* crude extract revealed the presence alkaloids, flavonoids, glycoside, terpenoids, tannins, and saponins. These phytoconstituents are associated with number of pharmacological properties. *Ex-vivo* and *in vivo* experimentation of Indigofera argentea exhibited significant antispasmodic, tracheorelaxant and vasorelaxant activities. Hence present work can be concluded that antagonism of calcium channels is the

major cause of spasmolytic, tracheorelaxant, vasorelaxant, diarrheal protection and hypotensive effects but muscarinic antagonism also involved in tracheal relaxation. But the involvement of any other mechanism cannot be ignored.

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REFERENCES

Ahmad S, Alam K, Wariss H, Anjum, S and Mukhtar M (2014). Ethnobotanical studies of plant resources of Cholistan desert, Pakistan. *Int. J. Sci. Res.*, **3**(6): 1782-1788.

Al-Shboul O (2018). Contraction and relaxation signaling in gastrointestinal smooth muscle. *ECGDS.*, **5**(5): 315-321.

Aleem A, Janbaz KH, Imran I, Wahid M, Bibi S, Afzal K and Asad MHHB (2020). Antiplatelet aggregation, cardiotonic, anti-inflammatory, antioxidant and calcium channel antagonistic potentials of *Nepeta ruderalis* Buch. *Biomed Res. Int.,* **2020**: 2096947.

Alfarhan AH, Al-Turki TA and Basahy AY (2005). Flora of Jizan Region. *Final Report Supported by KACST.*, **1**(2): 545.

Aslam N, Janbaz KH and Jabeen Q (2016). Hypotensive and diuretic activities of aqueous-ethanol extract of Asphodelus tenuifolius. *Bangladesh J. Pharmacol.*, **11**(4): 830-837.

Bilanda DC, Dzeufiet PDD, Fouda YB, Ngapout RF, Tcheutchoua Y, Owona PE and Kamtchouing P (2020). Antihypertensive and antidiabetic activities of *Erythrina senegalensis* DC (Fabaceae) stem bark aqueous extract on diabetic hypertensive rats. *J Ethnopharmacol.*, **246**: 112200.

Ciobanu L and Dumitrascu DL (2011). Gastrointestinal motility disorders in endocrine diseases. *Pol. Arch. Med. Wewn.,* **121**(4): 129-136.

de Souza Monteiro F, Carvalho AFS, de Castro Marques E, Ribeiro RM, Borges ACR and da Rocha Borges MO (2018). Antidiarrhoeal and antispasmodic activity of leaves of *Syzygium cumini* L. (Myrtaceae) mediated through calcium channel blockage. *African J Pharm Pharmacol.*, **12**(1): 11-18.

Iqbal Z, Bello I, Asmawi MZ, Al-Mansoub MA, Ahmad A, Jabeen Q and Fei YM (2019). Vasorelaxant activities and the underlying pharmacological mechanisms of *Gynura procumbens* Merr. leaf extracts on rat thoracic aorta. *Infl. Ammopharmacology,* **27**(2): 421-431.

Janbaz KH, Ahsan MZ, Saqib F, Imran I, Zia-Ul-Haq M, Rashid MA, Jaafar HZ and Moga M (2015a). Scientific basis for use of Pyrus pashia Buch.-Ham. ex D. Don. fruit in gastrointestinal, respiratory and cardiovascular ailments. *PloS One.*, **10**(3): e0118605.

Janbaz KH, Akhtar T, Saqib F, Imran I, Zia-Ul-Haq M, Jansakul C, De Feo V and Moga M (2015b). Pharmacological justification of use of Solena heterophylla Lour. in gastrointestinal, respiratory and vascular disorders. *J. Transl. Med.*, **13**(1): 134.

Janbaz KH, Haider S, Imran I, Zia-Ul-Haq M, De Martino L and De Feo V (2012). Pharmacological evaluation of *Prosopis cineraria* (L.) Druce in gastrointestinal, respiratory, and vascular disorders. *Evid. Based Complementary Altern. Med.*, **2012**: 735653.

Javed F, Jabeen Q, Aslam N and Awan AM (2020). Pharmacological evaluation of analgesic, anti-inflammatory and antipyretic activities of ethanolic extract of *Indigofera argentea* burm. F. *J Ethnopharmacol.*, **259**: 112966.

Katzung BG and Chatterjee K (2012). Vasodilators and the treatment of angina pectoris. *Basic Clin. Pharmacol. Toxicol*, 10th ed., McGraw-Hill New York, **7**: pp.20-25.

Khan AU, Ali S, Gilani AH, Ahmed M and Choudhary MI (2017). Antispasmodic, bronchodilator, vasorelaxant and cardiosuppressant effects of *Buxus papillosa. BMC Complem. Altern. M.*, **17**(1): 54.

Khan H, Saeed M, Gilani AH, Muhammad N, ur Rehman N, Mehmood MH and Ashraf N (2016). Antispasmodic and antidiarrheal activities of rhizomes of *Polygonatum verticillatum* maneuvered predominately through activation of K^+ channels: Components identification through TLC. *Toxicol Ind Health.,* **32**(4): 677-685.

Li Y, Li J, Liu X, Zhang J, Mei X, Zheng R and Zhong S (2019). Antidiarrheal activity of methanol extract of *Sophora tonkinensis* in mice and spasmolytic effect on smooth muscle contraction of isolated jejunum in rabbits. *Pharm. Biol.*, **57**(1): 477-484.

Mehmood MH, Anila N, Begum S, Syed SA, Siddiqui BS and Gilani AH (2014). Pharmacological basis for the medicinal use of Carissa carandas in constipation and diarrhea. *J. Ethnopharmacol.*, **153**(2): 359-367.

Moloudizargari M, Mikaili P, Aghajanshakeri S, Asghari MH and Shayegh J (2013). Pharmacological and therapeutic effects of *Peganum harmala* and its main alkaloids. *Pharmacogn. Rev.,* **7**(14): 199-212.

Rahman HMA, Ahmed K, Rasool MF and Imran I (2017a). Pharmacological evaluation of smooth muscle relaxant and cardiac-modulation potential of *Phyla nodiflora* in *ex-vivo* and *in-vivo* experiments. *Asian Pac. J. Trop. Med.*, **10**(12): 1146-1153.

Rahman HMA, Bashir S, Mandukhail SR, Huda S and Gilani AH (2017b). Pharmacological evaluation of gut modulatory and bronchodilator activities of *Achyranthes aspera* Linn. *Phytotherapy Research*., **31**(11): 1776-1785.

Rahman HMA, Rasool MF and Imran I (2019). Pharmacological studies pertaining to smooth muscle

relaxant, platelet aggregation inhibitory and hypotensive effects of *Ailanthus altissima. Evid. Based Complementary Altern Med.,* **2019**: 1871696.

Rang HP, Ritter JM, Flower RJ and Henderson G (2014). Rang & Dale's Pharmacology E-Book: with Student Consult Online Access: Elsevier Health Sciences, 8th ed., p.776.

Sanders KM (2001). Invited review: mechanisms of calcium handling in smooth muscles. *J. Appl. Physiol.*, **91**(3): 1438-1449.

Sanders KM, Koh SD, Ro S and Ward SM (2012). Regulation of gastrointestinal motility-insights from smooth muscle biology. *Nature Reviews J. Gastroenterol. Hepatol.,* **9**(11): 633.

Shahed-Al-Mahmud M, Shawon MJA, Islam T, Rahman MM and Rahman MR (2020). *In vivo* anti-diarrheal activity of methanolic extract of streblus asper leaves stimulating the Na^+/K^+-ATPase in swiss albino rats. *Indian J. Clin. Biochem*., **35**(1): 72-79.

Shareef H, Rizwani GH, Mandukhail SR, Watanabe N and Gilani AH (2014). Studies on antidiarrhoeal, antispasmodic and bronchodilator activities of *Operculina turpethum* Linn. *BMC Complem Altern M.*, **14**(1): 479.

Shifah F, Tareq AM, Sayeed MA, Islam MN, Emran TB, Ullah MA and Ullah A (2020). Antidiarrheal, cytotoxic and thrombolytic activities of methanolic extract of *Hedychium coccineum* leaves. *Measurement*, **11**: 12.

Siddiqui A (2011). Effects of vasodilation and arterial resistance on cardiac output. *J. Clin. Exp. Cardiolog.*, **2**: 170.

Sweeney HL and Hammers DW (2018). Muscle contraction. *Cold Spring Harb Perspect Biol.,* **10**(2): a023200.

Touyz RM, Alves-Lopes R, Rios FJ, Camargo LL, Anagnostopoulou A, Arner A and Montezano AC (2018). Vascular smooth muscle contraction in hypertension. *Cardiovasc. Res.,* **114**(4): 529-539.

Townsend EA, Siviski ME, Zhang Y, Xu C, Hoonjan B and Emala CW (2013). Effects of ginger and its constituents on airway smooth muscle relaxation and calcium regulation. *Am. J. Respir. Cell Mol. Biol.*, **48**(2): 157-163.

Vrolijk M F, van Essen H, Opperhuizen A, Bast A and Janssen BJ (2020). Haemodynamic effects of the flavonoid quercetin in rats revisited. *Br. J. Pharmacol.,* **177**(8): 1841-1852.

Widmaier EP, Raff H and Strang KT (2011). Vander's human physiology. The Mechanisms of Body Function. **12**: 632-669.

William YN, Gilbert A, Shah AJ, Marius M, Mireille TTL, Vanessa MMM and Khan T (2019). Extracts from the trunk bark of *Distemonanthus benthamianus* Baillon (Caesalpiniaceae) developed antidiarrhoeal activities in rats and mice. *Orient Pharm. Exp. Med.*, **19**(4): 421-433.

Worgall S and Worgall TS (2018). Methods to decrease susceptibility to asthmatic bronchoconstriction. *In*: Google Patents. **10**: 159-678.

Yang Z, Balenga N, Cooper PR, Damera G, Edwards R, Brightling CE and Druey KM (2012). Regulator of G-protein signaling-5 inhibits bronchial smooth muscle contraction in severe asthma. *Am. J. Respir. Cell Mol. Biol.,* **46**(6): 823-832.