UNIVERSIDAD DE SANTIAGO DE CHILE

BOLETÍN LATINOAMERICANO Y DEL CARIBE DE PLANTAS MEDICINALES Y AROMÁTICAS
18 (2): 204 - 222 (2019)
© / ISSN 0717 7917 / **www.blacpma.usach.cl**

Blacpma

Artículo Original | Original Article

Evaluation of smooth muscle relaxant potential of *Bismarckia nobilis* (Hildebr. & Wendl.) in diarrhea, hypertension and asthma by *ex-vivo* and *in-vivo* method

[Evaluación del potencial relajante en músculo liso de *Bismarckia nobilis* (Hildebr. & Wendl.) en diarrea, hipertensión y asma por métodos *ex vivo* e *in vivo*]

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Abstract: To explore the mechanistic basis behind smooth muscle relaxant prospective of *Bismarckia nobilis* in gastrointestinal, respiratory and cardiovascular ailments. The methanolic extract of *B. nobilis* and sub-fractions have been evaluated in vitro rabbit isolated tissues, *in vivo* castor oil-induced diarrhea in rats and charcoal meal activity in mice. The *B. nobilis* extract relaxed spontaneous and K^+ (80 mM)-induced contractions in rabbit isolated jejunum preparations, CCh (1 µM) and K^+ (80 mM)-induced contractions in tracheal and bladder preparations, PE (1 µM) and K^+ (80 mM)-induced concentrations in aorta preparations, likewise verapamil. Spasmolytic activity of dichloromethane fraction is stronger as compared to aqueous fraction. *In vivo* castor oil-induced diarrhea in rats and charcoal meal activity in mice further supported spasmolytic activity. *B. nobilis* extract possess anti-spasmodic, anti-diarrheal, airway relaxant and vasodilator activities possible mediated through calcium channel blocking mechanism, justifying therapeutic utility of *B. nobilis* in diarrhea, asthma and hypertension.

Keywords: *Bismarckia nobilis*; Spasmolytic activity; Anti-asthmatic activity; Hypotensive; Diarrhea; Calcium channel blocker

Resumen: El objetivo de trabajo fue explorar el mecanismo de acción relacionado con el efecto relajante del músculo liso inducido por *Bismarckia nobilis* (*B. nobilis*) en enfermedades gastrointestinales, respiratorias y cardiovasculares. El extracto metanólico de *B. nobilis* y subfracciones fue evaluado *in vitro* en tejidos aislados de conejos. Además se evaluó diarrea *in vivo* inducida con aceite de ricino en ratas y la actividad de harina de carbón vegetal en ratones. El extracto de *B. nobilis* relajó tanto las contracciones espontáneas como las inducidas por K^+ (80 mM) en preparaciones de yeyuno aisladas de conejos, las contracciones inducidas por PE (1 µM) y K^+ (80 mM) inducidas en preparaciones de aorta; de manera similar a verapamilo. La actividad espasmolítica de la fracción de diclorometano es más potente en comparación con la fracción acuosa. La diarrea inducida *in vivo* por el aceite de ricino en ratas y la actividad de la harina de carbón vegetal en ratones apoyaron aún más la actividad espasmolítica. El extracto de *B. nobilis* posee actividades antiespasmódicas, antidiarreicas, relajantes de las vías respiratorias y vasodilatadoras, posibles a través del mecanismo de bloqueo de los canales de calcio, lo que justifica la utilidad terapéutica de *B. nobilis* en la diarrea, el asma y la hipertensión.

Palabras clave: *Bismarckia nobilis*; Actividad Espasmolitica; Actividad anti-asmatico; Hipotensivo; Diarrea; Bloqueador de canales de calcio

Recibido | Received: November 29, 2018
Aceptado | Accepted: March 20, 2019
Aceptado en versión corregida | Accepted in revised form: March 24, 2019
Publicado en línea | Published online: March 30, 2019
Este artículo puede ser citado como / This article must be cited as: F Saqib, N Jabeen, M Riaz, G Sechel. 2019. Evaluation of smooth muscle relaxant potential of *Bismarckia nobilis* (Hildebr. & Wendl.) in diarrhea, hypertension and asthma by ex-vivo & in-vivo method. **Bol Latinoam Caribe Plant Med Aromat** 18 (2): 204 – 222.
https://doi.org/10.37360/blacpma.19.18.2.14

ABBREVIATIONS

Acetylcholine (Ach); Aqueous fraction of *Bismarckia nobilis* (Bn.Aq); Calcium channel blocker (CCB); Carbamylcholine HCl (CCh); Concentration response curves (CRCs); Crude extract of *Bismarckia nobilis* (Bn.Cr); Dichloromethane fraction of *Bismarckia nobilis* (Bn.Dcm); Ethylene tetra acetic acid (EDTA); Phenylepherine (PE); Potassium chloride (KCl); Verapamil HCl (VE).

INTRODUCTION

Gastrointestinal, respiratory and cardiovascular ailments are increasing worldwide. Alternative and complementary remedies are obtaining drive for treatment of smooth muscles related ailments like diarrhea, asthma and hypertension. The plants played a pivotal role as silent companion of human being in helping survival through provision of food, clothing, shelter and medicaments for disease control. The remedies for disease management have been obtained from herbal resources because of their efficacy, safety and less side effects than allopathic medicines (Draughon, 2004). In Pakistan, tribal areas and villages are far from cities and local population has trivial belief to use medicinal plants to cure common diseases. The survey of plant related native literature reflected that significant number of human suffering relevant to cardiovascular, respiratory and gastrointestinal tract are likely to be managed by the plant derived drugs (Gilani & Rehman, 2005).

Bismarckia nobilis (*B. nobilis*; Arecaceae), commonly known as Silver Palm Tree, Fan Palm is widely used medicinal plant in Pakistan because of its diversity of folkloric utility by Hakims and traditional healers. Palms are a plant group of 183 Genera and 23,00 species in tropical and subtropical climate regions of earth. Bismarck Palm is 60 feet tall and 20 feet wide (Mitchell, 2012) It is native to Madagascar (Kew, 2013), cultivated all over the world including Florida and Pakistan.

It is an ornamental plant with medicinal value having attractive appearance, intense look and distinguishing silver blue palms (Qureshi *et al*., 2016). It has silver green to blue grey coloured large leaves so termed as frond (Brown, 2011). Its proliferation is from spores. It is entirely dioecious variety having separate male and female plant (Gilman, 1997; Gilman & Watson, 2011).

B. nobilis has folkloric repute to treat stomach abnormalities, diarrhea, fatigue and to heal wounds (Broschat, 2011; Rakotonandrasana *et al.*, 2017). The plant also has role in combating oral infections like tooth deformation (Ranjarisoa *et al.*, 2016). Nobilis species is reported to possess antibacterial activity as well as antifungal effects (Siriken *et al.*, 2018). Moreover, anti-inflammatory (Turk *et al*., 2019), free radical scavenging activity (Dhifi *et al*., 2018) antimicrobial, cytotoxic and immune modulating potential is also present in nobilis species (Siriken *et al.,* 2018).

The plant is scientifically proven to exhibit anti-aging activity (Zheng *et al.*, 2015). It also has anti-oxidant property which is beneficial to treat ischemia, breast cancer and colon cancer (Schauss & Fong, 2013).

B. nobilis contains dietry fibres. Generally, Nobilis species contain essential oils, flavonoids, monoterpenes, sesqueterpenes, alkaloids, tannins, glycosylated flavonoids, megastigmine and tannins. Essential oil of nobilis species contains eucalyptol, α-terpinyl acetate, linalool, methyl eugenol, sabinene and carvacol (Dhifi *et al*., 2018; Siriken *et al.*, 2018). It also possess limonien, 1,8 cineol, linalool, terpinol (Mansour *et al*., 2018; Elkiran *et al*., 2018).

B. nobilis has folkloric reputation of having remedial effects in hypertension, asthma and diarrhea, but scientific evidence for its traditional medicinal usage is lacking so far. As part of our research group continuous studies regarding rationalization of ethnopharmacological prospective of extracts of medicinal plants of Pakistan (Saqib *et al*., 2015; Saqib & Janbaz, 2016; Saqib *et al*., 2018,) effects of crude extract of *B. nobilis* were assessed in isolated tissues of rabbit (aorta, trachea and jejunum) to validate its usage in gastrointestinal, respiratory and cardiovascular diseases.

MATERIAL AND METHODS

Extraction of plant & fractionation

B. nobilis (whole plant) material was collected fresh in March 2017 from botanical Garden of Bahauddin Zakariya University Multan and was verified by an expert taxonomist Dr. Zafar-Ullah-Zafar (Voucher Specimen No. TPL 1.1/record/kew-22257), from Institute of Pure and Applied Biology, Bahauddin Zakariya University, Multan. The plant material was extracted as per reported Method (Saqib *et al*., 2018). Fresh green plant was cut into pieces, dried. It was made free from debris and contaminations and triturated with grinder. About 1000g of *B. nobilis* was

soaked in aqueous methanol (70%) for 3 days with shaking. The saturated liquid was first filtered through Muslin cloth and Whattman-1 filter paper. The filtrate was evaporated on (Rotavapour, BUCHI Labrotechnik AG, Model 9230, Switzerland) at 37°C under reduced pressure connected to a vacuum pump (Buchi VacV-500) and a chiller (B-740) to gain crude extract of *Bismarckia nobilis* (Ba.Cr) honey like thick semisolid mass, stored in amber coloured bottle at room temperature (25°C) with yield (60%). Triple maceration was followed by the same method as described above.

Solvent-solvent extraction (fractionation) was performed following dissolution of 20 g of crude extract (Bn.Cr) in about 100 mL of distilled water to which 100ml of dichloromethane (DCM) was mixed and shaken briskly in a separating funnel to separate DCM layer. The procedure was performed thrice and individually collected DCM fractions were pooled. The DCM portion was vaporized on rotavapor (rotary evaporator) to obtain the dichloromethane fraction (Bn.DCM), while the aqueous portion was lyophilized into aqueous fractions (Bn.Aq) with corresponding yields 15% and 50% (Saqib & Janbaz, 2016).

Chemicals and reagents

Acetylcholine (Ach), Carbamyl choline HCl (CCh), Potassium chloride (KCl), Verapamil HCl and Phenylephrine (PE), Magnesium chloride ($MgCl_2$), Aspirin, Ethylene tetra acetic acid (EDTA) were purchased from Sigma Chemicals Co (St Louis, MO, USA). Whereas Calcium chloride ($CaCl_2$), Glucose, Magnesium sulphate, Potassium dihydrogen phosphate (KH_2PO_4), Sodium bicarbonate ($NaHCO_3$), Sodium dihydrogen phosphate (NaH_2PO_4), Methanol, Sodium chloride (NaCl) were from Merck, Darmstadt, Germany. The fresh dilutions were prepared on each day.

Animals

The white albino rabbits (♂/♀) of weight (1.0–2.0 kg) and 8–9 months in age and Sprague-Dawley albino rats of (♂/♀) 1–2 month in age (250–300g) were kept in cages with sawdust (replaced after every 24h), conserved at ambient temperature of 25°C and exposed to 12h (light/darkcycles) in animal house of Faculty of Pharmacy, BZU, Multan They were given regular diet and tap water ad libitum. The animals were allowed to use H_2O but subjected to overnight fasting prior to experiments. For *In vivo* activity, each random group comprised of 5 animals.

All experiments on animals fulfilled the rules of Commission of Laboratory Animal Resources, of Life Sciences (NRC,2011; Health, 1985) approved by institutional Ethical Committee of B.Z.U, Multan with EC/10/PhL/16 dated 3rd March 2017.

Preliminary phytochemical investigation

Crude extract of *B. nobilis* was initially tested for the existence of alkaloids, coumarins, flavonoids, tannins, phenols, sterols, saponins, terpenes and anthraquinones (Tona *et al*., 1998).

In-vitro experiments

Isolated rabbit jejunum preparations

The rabbits were killed following a blow on neck, abdomens incised and jejunum separated out. The mesenteries were removed and cut into 2–3 cm long segments, The jejunum segments were fixed between 2 stainless steel hooks in10ml tissue bath filled with Tyrode solution (pH 7.4) kept at 37°C by means of circulating thermoregulatory and bubbling with carbogen (95% O_2 and 5% CO_2). One gram tension was applied as pre load on tissue and permitted at equilibrium for forty minutes throughout which the tissue was flushed with fresh solution after 15 min intervals prior to exposure to test substance. The isolated jejunum shows spontaneous rhythmic contractions which were recorded by isotonic transducers connected to a Power Lab Data Acquisition System (AD Instruments, Australia, Sydney) (Saqib *et al*., 2018) plugged with a computer having Lab Chart Software (version7).

Mechanism of calcium channel blockade

To elucidate the possible mechanism of the anti-spasmodic activity, the test material was studied against sustained spasmodic contractions following exposure to high concentration of K^+ (80 mM) (Farre *et al*., 1991). High K^+ (80 mM) induced contractions in isolated smooth muscle preparations is mediated through opening of voltage-dependent Ca^{++} channels, thus allowing influx of extracellular Ca^{++} causing a contractile effect (Bolton, 1979), and the substances capable to decrease high K^+ -induced contractions is considered as blocker of Ca^{++} influx through L-type Ca^{++} channels (Godfrained *et al*., 1986). Once plateau of sustained contractions was obtained, test material

was applied in a cumulative manner to the sustained contractions to achieve concentration dependent inhibitory responses (Van Rossum, 1963). The observed relaxant effect of the test material on K^+ (80 mM)-induced contraction was expressed as percent (%) of the control contractile response.

To confirm the Ca^{++} antagonist action of the test substance, isolated jejunum of rabbit was permitted to stabilize in normal Tyrode solution, later substituted for 45 min with a solution containing normal concentration of K^+ but Ca^{++} was removed and EDTA (0.1 mM) was added to deplete Ca^{++} of the smooth muscles. The tissue was further substituted by K^+ -rich and Ca^{++}-free Tyrode's solution. These CRCs for Ca^{++} were prepared in the presence of different concentrations of test material to observe CCB effect. The CCB action on the part of test drug was confirmed due to shifting of the CRCs (Ca^{++}-free medium) towards right in a concentration dependent manner (Saqib & Janbaz, 2016).

Isolated rabbit tracheal preparation

The tracheal tissue of rabbit was cut apart and divided into 3-4 mm wide ring, each containing around 1-2 cartilages. Every ring was incised longitudinally, the ventral side reverse to the dorsal (smooth muscle) layer, forming a band with smooth muscles sandwiched between cartilaginous edges. The preparation was mounted in a tissue bath (Radnoti) having Krebs's solution (pH 7.4) maintained at 37ºC and bubbled with carbogen (95% O_2+ 5% CO_2). A tension of 1 g was applied to each of the tracheal strip and was kept persistent throughout the experiment. The preparation of trachea was stabilized for 45 minutes and isometric responses were noted via force shift isometric transducers (Model FORT100, USA WPI) connected to a Power Lab Data Acquisition System (AD Instruments, Australia) attached to computer having Lab Chart (version.7). The isolated trachea of rabbit was allowed to equilibrate for one hour before addition of any test drug The relaxant effect of test drug was checked on carbachol (CCh; 1 μM)- and K^+(80 mM)-induced contractions on trachea, where drug was added in a collective pattern compared to control for possible bronchodilator activity (Saqib & Janbaz, 2016).

Isolated rabbit bladder preparation

Rabbit bladder was dissected out and placed in Kreb's solution. The bladder was made clean and cut into transverse directions upto maximum 4mm long piece of tissue. The bladder preparation was placed in 15 ml organ bath filled with Kreb's solution maintained at 37°C and aerated with carbogen. The preload tension of 1 g was applied and tissue preparations were allowed to equilibrate for 30min prior to addition of test material. Tissue preparations were equiliberated by repeated exposure to Carbachol (1 μM) and K^+ (80 mM)-induced sustained contractions were consequently used for testing different doses of plant material in a cumulative manner. The power lab data acquisition system provide help in recording isometric responses through a computer having lab chart software (Santicioli *et al*., 1984).

Isolated rabbit aortic preparation

The rabbit thoracic aorta was dissected out and divided into pieces of 2-3 mm width, placed immediately in 10 ml tissue baths (Radnoti) having Krebs solution, bubbled with carbogen at 37°C A preload tension of 2 g was applied to each preparation of aorta tissue and allowed to stabilize for a period of one hour prior applying test drug. Effect of test drug was first determined on the resting base line of the tissue to see if it has vasoconstrictor effect and later checked by cumulative addition of test drug to tissue bath containing aortic preparation previously constricted by either phenylephrine (1 μM) or K^+ (80 mM) for vasorelaxant activity and responses were measured via a force-shift isometric transducer (Model FORT100, USA WPI,) coupled to Power lab attached to computer having lab chart software (version.7) (Saqib *et al*., 2015).

Isolated rabbit paired atria preparations

Paired atria of rabbits were removed and mounted in a 20 ml tissue bath filled with Krebs-Henseleit (pH.7.4) solution retained at 32°C and aerated with carbogen. The tissue was allowed to equilibrate under 1.0 g basal tension for 20 min before isotonic contractions were recorded. The spontaneous beating of atria were recorded via a force-displacement transducer (FT-03) together with the rate of contractions, which were monitored on a Power Lab. The tissues exhibited spontaneous beating under the resting tension of 1 g due to the presence of pacemaker cells. An equilibrium period of 30 min was provided before the application of any chemical

substance (Saqib & Janbaz, 2016).

In vivo experiments

Castor oil-induced diarrhea in rats

The antidiarrheal activity of test drug was studied on castor oil-induced diarrhea in rats as described previously (Shoba & Thomas, 2001, Uddin, 2006). Rats were fasted for 24 hrs before the experiment. Twenty five rats of equal body weight were randomly divided into five groups (n=5/group), and kept individually in cages lined with blotting paper. The first group served as negative control and was treated with normal saline (10 ml/kg). The second to fourth groups received 100, 200 and 400 mg/kg of test drug orally, respectively. The fifth group was administered with loperamide (10 mg/kg, orally), as positive control. One hour after respective treatment, all the animals received castor oil (10 ml/kg, orally) through a feeding needle. After 5 hour of castor oil administration, the cages were examined for the presence of typical diarrheal droppings; the absence was regarded as a positive result, indicating protection from diarrhea.

% inhibition of wet feces = [(C-T)/C] 100

Where;

C = Castor oil induction wet fecal mass.

T = Treated group wet fecal mass.

Charcoal meal gastrointestinal transit test

Before experiment, 20 mice were starved for 18 hours and divided into four groups. The first group was given saline as negative control and the second group received loperamide (10 mg/kg) as positive control. Different doses of Bn.Cr (200 and 400 mg/kg) were administered to the other groups. After 30 min, each animal was orally given 0.2 ml/15g of charcoal meal (5% deactivated charcoal in 0.5% CMC-Na). Thirty minutes later, animals were killed by the cervical dislocation and abdomens were opened. The distance traveled by charcoal meal from pylorus to the caecum was measured and expressed in centimeters (Khan *et al.*, 2011).

Statistical analysis

The results for *in-vitro* potential are expressed are mean ± standard error of the mean (SEM). The median effective concentrations (EC_{50} value) with 95% confidence interval (CI) were computed using the software Graph Pad Prism (Computer program) version 7.0 (GraphPAD, San Diego, California, USA: http://www.graphpad.com). Concentration-response curves (CRCs) were evaluated by applying a non-linear regression sigmoidal response curve with variable slope.

The statistical parameter applied was one-way analysis of variance (ANOVA) followed by Dunnett's test in the case of *in vivo*, a probability of less than 0.05 ($p<0.05$) was considered statistically significant (Dawson-Saunders & Trapp, 1990).

RESULTS

Chemical analysis of B. nobilis extract

Chemical analysis of *B. nobilis* extract showed existence of alkaloids, tannins, steroids, saponins, anthraquinones and flavonoids and absence of glycosides among aqueous:methanolic extractable constituents.

Isolated rabbit jejunum preparation

Crude methanolic extract of *B. nobilis* (Bn.Cr) showed the relaxant effect on spontaneous contractions of the jejunum concentration at concentration range 0.01-1.0 mg/ml with EC_{50}=0.291 mg/ml (95% CI: 0.271-0.313 mg/ml; n=5).It completely relaxed K^+ (80 mM)-induced contraction at 3.0 mg/ml with EC_{50}=1.914 mg/ml (CI 95%: 1.556-2.355 mg/ml; n=5). Moreover, dichloro-methane fraction (Bn.Dcm) showed relaxation of spontaneous contractions at 0.01-0.3 mg/ml with EC_{50} = 0.747 mg/mL (95% CI: 0.742-0.751 mg/ml; n=5) and K^+ (80 mM)-induced contraction at 0.3 mg/ml with EC_{50} = 0.747 mg/ml (95% CI: 0.743-0.752 mg/ml; n=5) Whereas, aqueous fraction (Bn.Aq) showed slight relaxation of spontaneous contractions and K^+ (80 mM)-induced contraction (Figure No. 1 & No. 2). Verapamil (Standard drug) caused relaxing effect in both spontaneous and K+ (80mM)-induced contractions with EC_{50}=0.712 µM (95% CI: 0.698-0.758) and 0.204 µM (95% CI: 0.198-0.207). Moreover, Bn.Cr showed rightward shift of concentration response curves (CRCs) likewise verapamil (Figure No. 3).

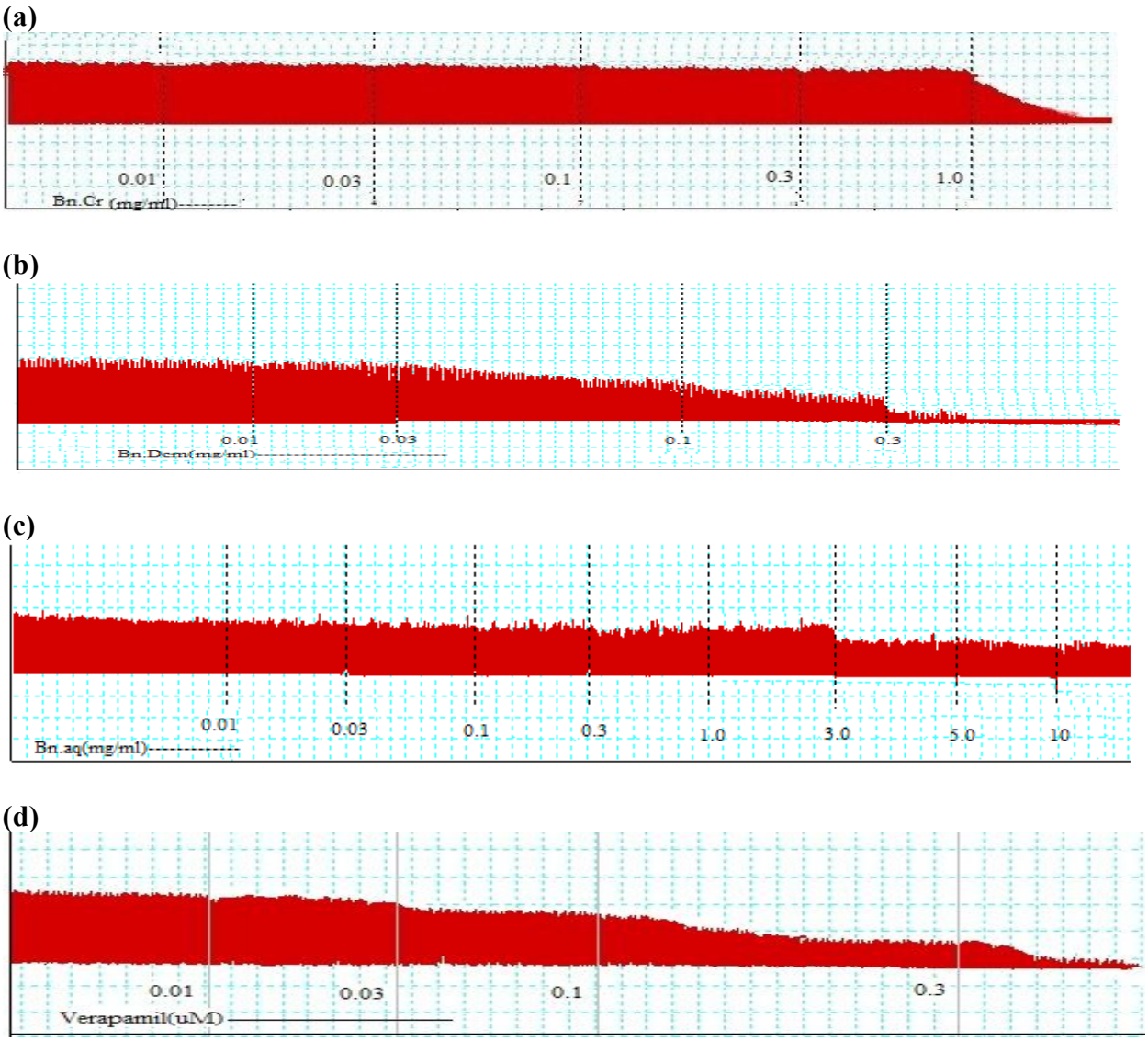

Figure No. 1
Effect of (a) crude extract of *B. nobilis* (Bn.Cr), (b) Dichloromethane fraction (Bn.dcm), (c) Aqueous fraction (Bn.aq) and (d) Verapamil on isolated jejunum preparations.

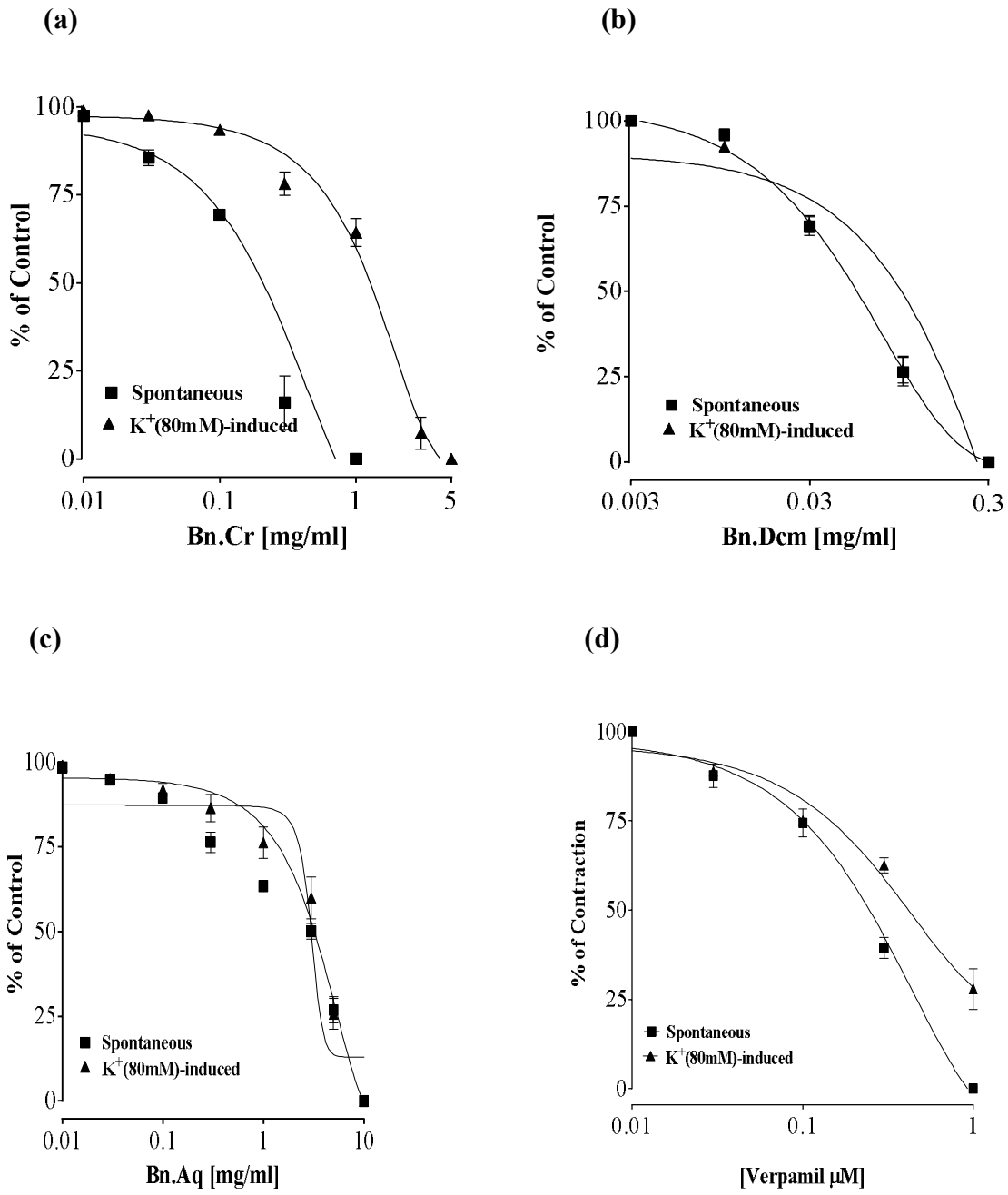

Figure No. 2
Effect of (a) crude extract of *B. nobilis* (Bn.Cr), (b) Dichloromethane fraction (Bn.dcm), (c) Aqueous fraction (Bn.aq) and (d) Verapamil on spontaneous and K^+ (80 mM)-induced contractions on isolated jejunum preparation. (Values are presented as the Mean ± SEM, n=5)

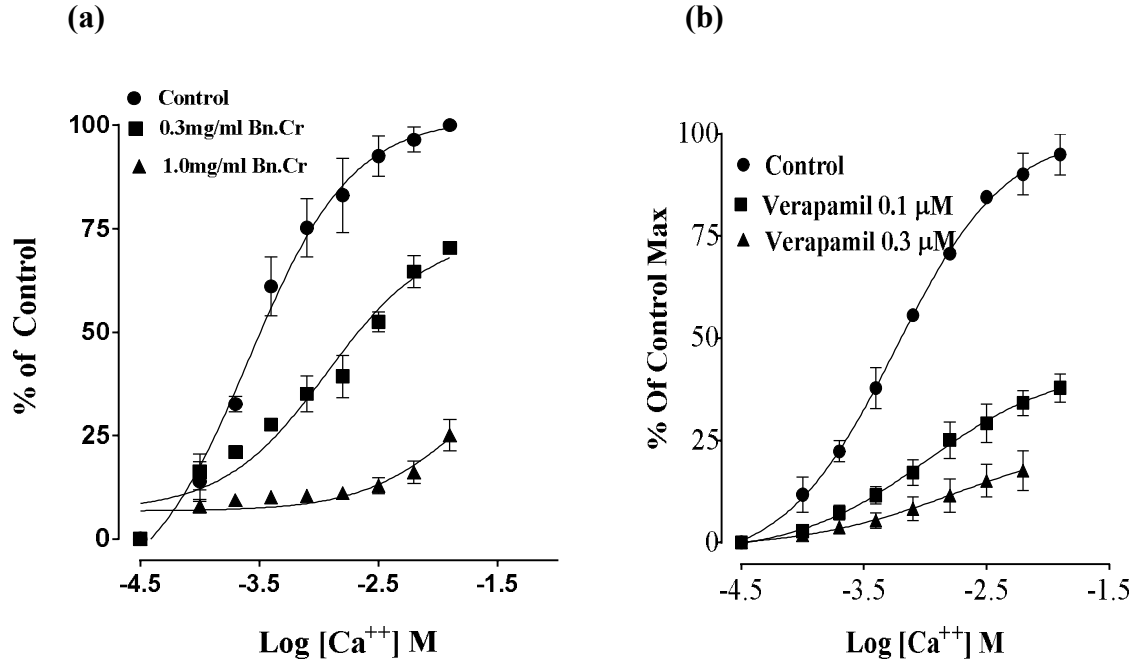

Figure No. 3
Concentration response curves of Calcium in the presence and absence of increasing concentration of crude extract (a) and verapamil (b) in the rabbit isolated jejunum preparations. Values are substituted as Mean ± SEM, n=5

Isolated rabbit tracheal preparations

B. nobilis (Bn.Cr) showed spasmolytic effect on (CCh; 1 µM)-induced and K^+ (80 mM) induced contraction at the concentration of 3.0 mg/ml with EC_{50}=0.517 mg/ml (95% CI: 0.454-0.588 mg/ml; n=5) and 0.457 mg/ml (95% CI: 0.351-0.563; n=5). Moreover, Bn.Dcm relaxed CCh, (1 µM) and K^+ (80 mM) induced contractions at 0.3 mg/ml with EC_{50}=0.747 mg/mL (95% CI: 0.743-0.751 mg/ml; n=5) and 0.746 mg/mL (95% CI: 0.743-0.751 mg/ml; n=5).Whereas, Bn.Aq exerted relaxing effect in CCh (1 µM)-induced contraction 3.0 mg/mL with EC_{50}=0.383 mg/ml (95% CI: 0.353-0.415 mg/ml; n=5) and K^+ (80 mM)-induced contraction 10 mg/ml. Verapamil relaxed CCh (1 µM) and K^+ (80 mM)-induced spasms with EC_{50}=0.310 µM (95% CI: 0.18-0.51 µM) and 0.064 µM (95% CI: 0.037-0.097 µM), respectively (Figure No. 4).

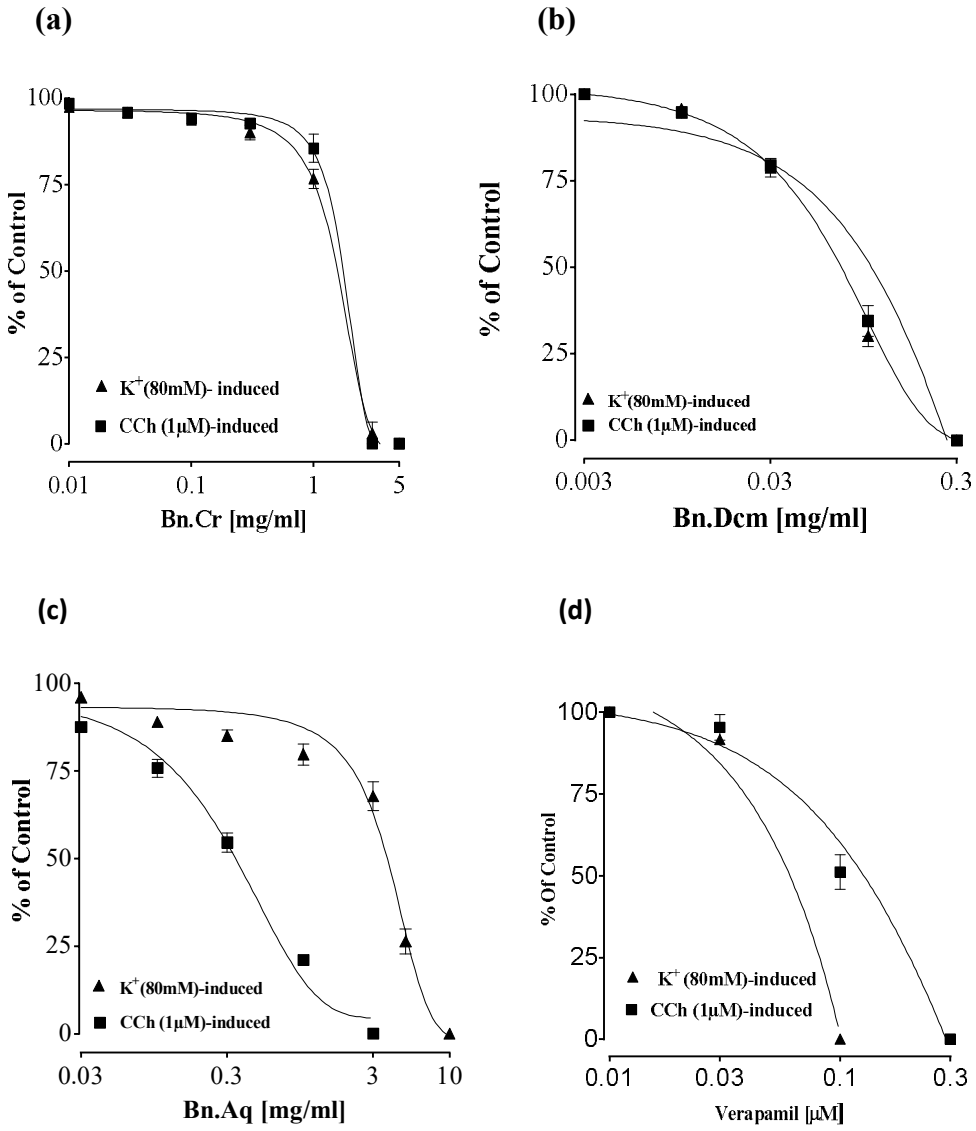

Figure No. 4
Effect of (a) crude extract of *B.nobilis* (Bn.Cr), (b) Dichloromethane fraction (Bn.dcm), (c) Aqueous fraction (Bn.aq) and (d) Verapamil on on Carbachol (1 µM) and K^+ (80 mM) induced contraction on trachea preparations. Values are shown as Mean ± SEM, n=5

Isolated rabbit bladder preparation

B. nobilis (Bn.Cr) exerted relaxant effect on CCh (1 µM) at 0.01 mg/ml with EC_{50}=0.749 (95% CI: 0.745-0.753 mg/ml; n=5) and K^+ (80 mM)-induced contractions at higher dose of 5.0 mg/ml with EC_{50}=0.266 mg/mL (95% CI: 0.240-0.295 mg/ml; n=5). Moreover, Bn.Dcm exerted relaxing effect of CCh (1 µM) and K^+ (80 mM)-induced contractions at concentration of 1 mg/mL and 0.1 mg/mL with EC_{50}=0.515 mg/mL (95% CI: 0.503-0.527 mg/ml;

n=5) and 0.989 mg/mL (CI 95%: 0.987-0.991 mg/mL; n=5).Whereas, Bn.Aq showed mild relaxing effect of CCh and K^+-induced contractions even at the 10 mg mg/ml with EC_{50}=1.839 mg/mL (95% CI: 1.499-2.254) and 10 mg/ml. Verapamil relaxed CCh (1 µM) and K^+ (80 mM)-induced contractions at 0.741 µM (95% CI: 0.740-0.751 µM) and 0.680 µM (95% CI: 0.661-0.690 µM), respectively (Figure No. 5).

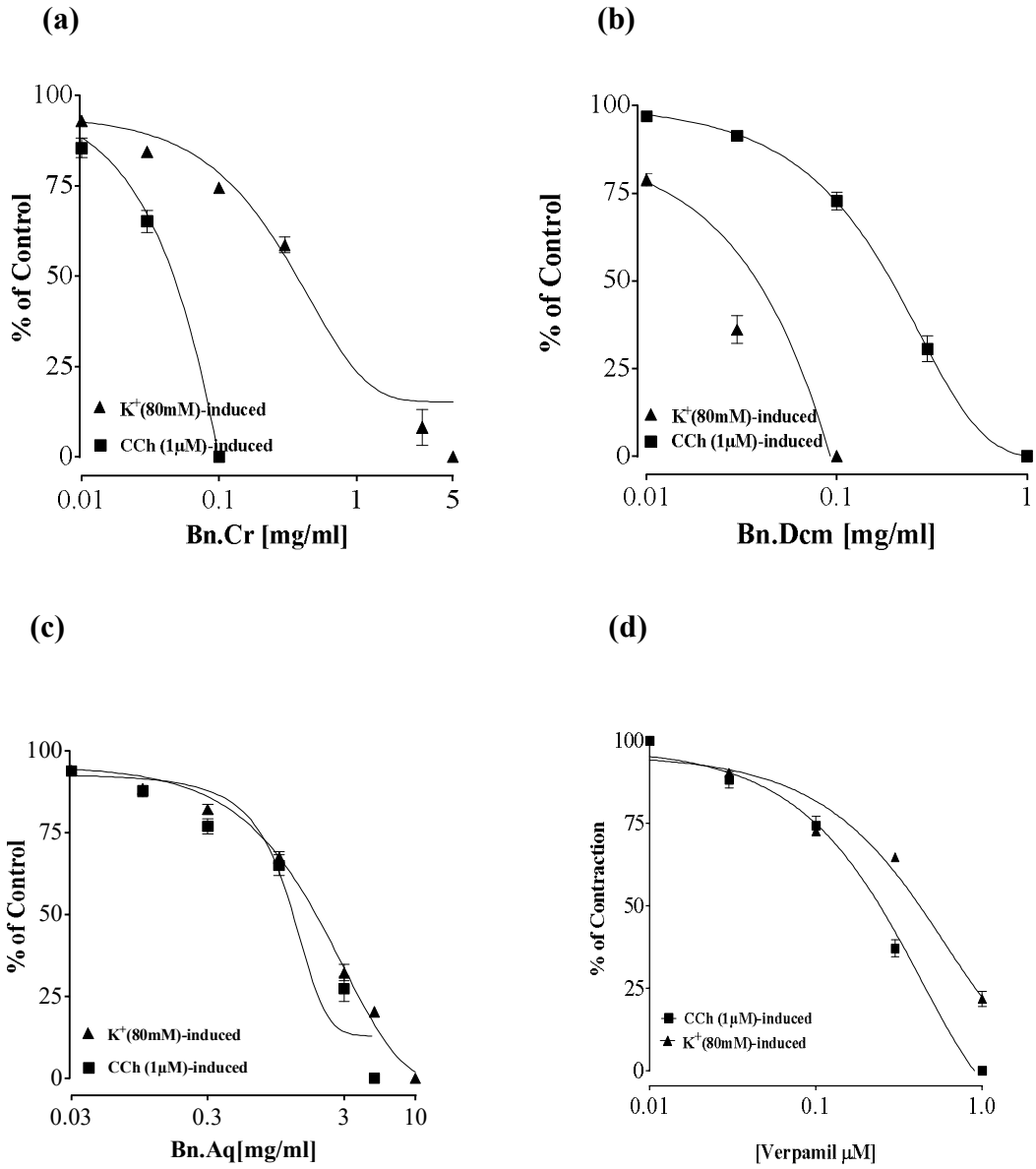

Figure No. 5
Effect of (a) crude extract of *B. nobilis* (Bn.Cr), (b) Dichloromethane fraction (Bn.dcm), (c) Aqueous fraction (Bn.aq) and (d) Verapamil on Carbachol (1 µM) and K^+ (80 mM) induced contractions on isolated bladder. Values are shown as mean ± SEM, n=5.

Isolated rabbit aortic preparation

B.nobilis (Bn.Cr) exerted relaxant effect on PE (1 µM) and K^+ (80 mM)-induced contractions in aorta at concentration of 3.0 mg/ml and 1.0 mg/ml respectively with EC_{50}=0.688 mg/mL (95% CI: 0.623-0.761 mg/lL; n=5) and EC_{50}=0.454 (95% CI: 0.403-0.461 mg/ml; n=5).Moreover, Bn.Dcm exerted relaxant effect at 0.3 mg/mL with EC_{50}=0.750 mg/mL (95% CI: 0.745-0.754 mg/ml; n=5) and 3.0 mg/mL with the EC_{50}=0.909 mg/mL (95% CI: 0.814-1.016 mg/mL; n=5) respectively. Whereas, Bn.Aq exerted relaxant effect on both induced contractions at high concentration of 10 mg/mL with 0.775 mg/mL (95% CI: 0.669-0.899 mg/ml; n=5). Verapamil relaxed the PE 1 µM and K^+ (80 mM)-induced contractions with EC_{50}=0.402 µM (95% CI: 0.251-0.620) and EC_{50}=0.371 µM (95% CI: 0.16-0.86) (Figure No. 6), respectively.

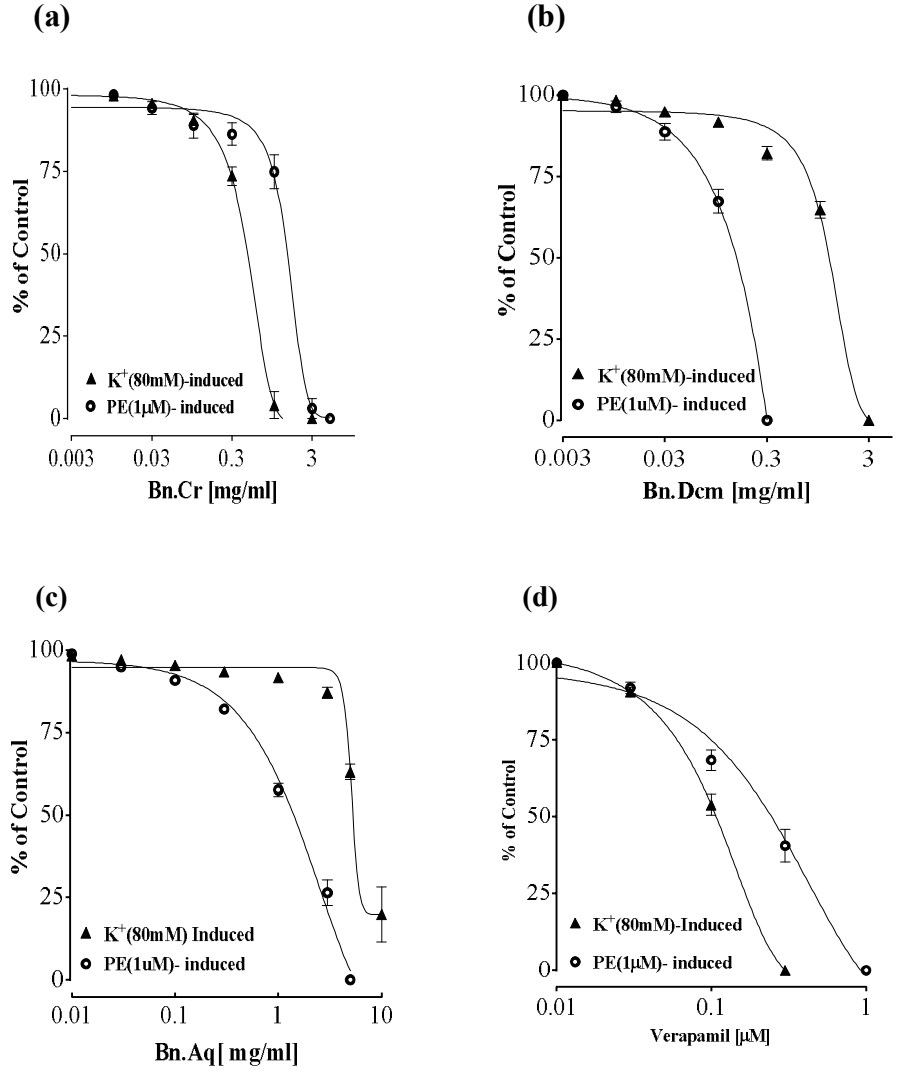

Figure No. 6
Effect of (a) crude extract of *B. nobilis* (Bn.Cr), (b) Dichloromethane fraction (Bn.dcm), (c) Aqueous fraction(Bn.aq) and (d) Verapamil on PE (1 µM) and K^+ (80 mM) induced contractions on isolated aorta. Values are shown as mean ± SEM, n=5.

Isolated rabbit paired atrial preparations

The crude methanolic extract of *B. nobilis* (Bn.Cr) on application to the isolated rabbit paired atria preparations exhibited decrease in the force of contractions (FOC) well as rate of contractions (negative ionotropic and negative choronotropic) effect at tissue bath concentration 3.0 mg/ml with EC_{50}=0.845 mg/ml (CI 95%: 0.486-1.467 mg/ml; n=5) in a manner comparable to verapamil with EC_{50}=0.037 µM (95% CI: 0.025-0.052 µM; n=5) (Figure No. 7 and No. 8).

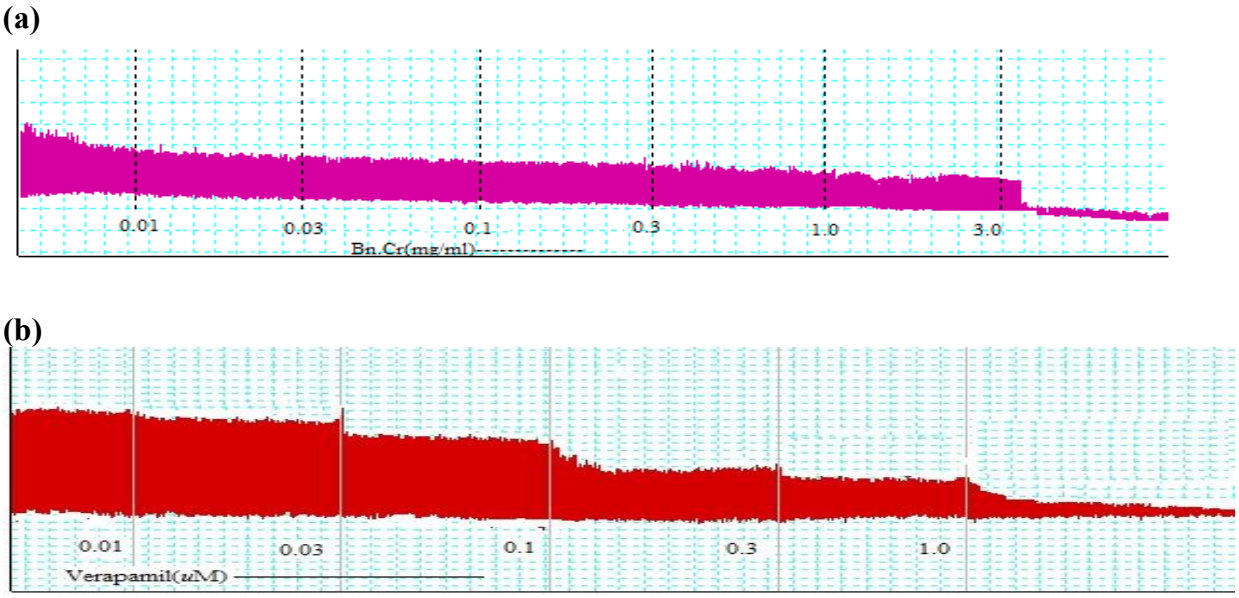

Figure No. 7
Tracing representing effects of (a) crude aqueous ethanolic extract of *B. nobilis* (Bn.Cr) (b) Verapamil on isolated rabbit atrial preparations

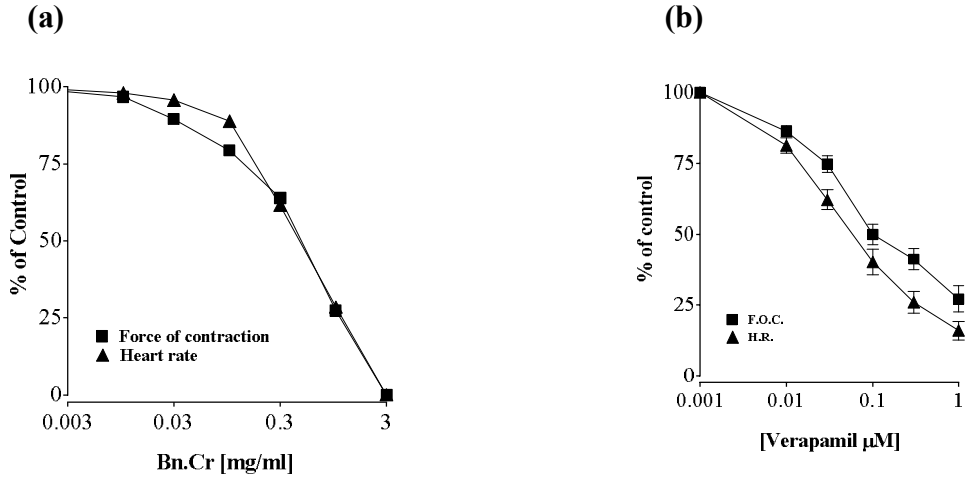

Figure No. 8
Effect of (a) crude aqueous methanolic extract of *B. nobilis* (Bn.Cr) (b) Verapamil on force of contraction and rate of contraction in isolated paired rabbit atria preparations. The values presented are Mean ± S.E.M of 5 preparations obtained from 5 animals

Antidiarrheal activity

The castor oil treatment induced diarrhea in all 5 mice of control group, however, treatment with Bn.Cr at respective oral doses of 100, 200 and 400 mg/kg resulted in 75.32, 90.9 and 82.2% decrease in fecal mass production, whereas Loperamide as control drug caused 97.94% reduction in fecal mass count at a doses of 10 mg/ kg (Figure No. 9).

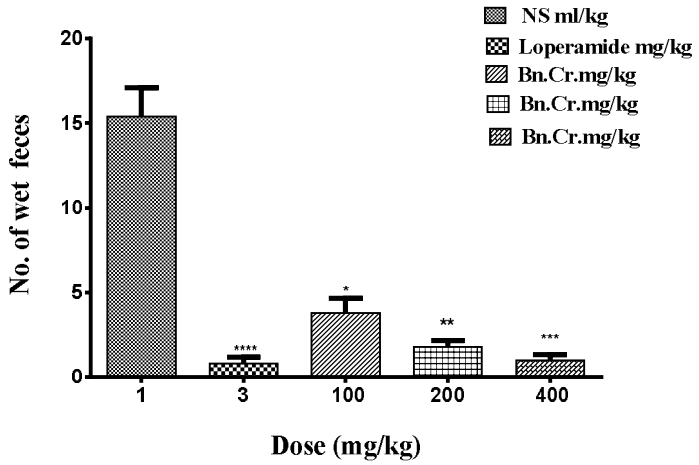

Figure No. 9

Bar graph presenting Effect of crude aqueousm-ethanolic extract of *B. nobilis* (Bn.Cr) and Loperamide on castor oil induce diarrhea in rats. The values are expressed as Mean ± S.E.M. and data was analyzed by one way ANOVA (Dunnett's test) on comparison with control, and p<0.05 was considered significant (*p<0.05, **p<0.005).

Charcoal meal activity

The aqueous-methanolic extract of *B. nobilis* (Bn.Cr) exhibited a dose dependent decrease in the peristaltic propulsive movement of small intestine in mice as reflected by movement of charcoal in intestine of mice in a fixed time following oral administration of charcoal in charcoal meal experiments. The movement of charcoal meal through small intestine in saline treated group of mice was measured to be 34.0 ± 3.9 cm, however, pretreatment of animals with Bn.Cr in doses of 200 and 400 mg/kg resulted in significant (P<0.05) reduction in values to 25.4 ± 0.8 and 15.4 ± 0.6 cm respectively. Similarly, pretreatment with Carbachol (10 mg/kg) to a group of mice also resulted in significant decrease in distance travelled by charcoal meal (09 ± 1.5 cm) as compared to control (**Figure No. 10).**

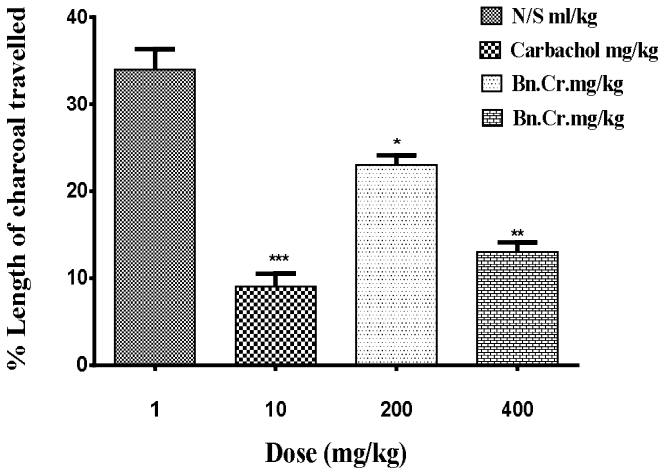

Figure No. 10
Bar graph presenting the effect of crude aqueous-methanolic extract of *B. nobilis* (Bn.Cr) and Loperamide on the peristaltic propulsive movements in mice assessed by the charcoal meal experiments. The values are expressed as Mean ± S.E.M., and data was analyzed by One way ANOVA (Dunnett's test) in comparison with control, and $p<0.05$ was considered significant. (*$p<0.05$, **$p<0.005$)

DISCUSSION

Presently, functional foods and herbal supplements are center of research due to their verified effects on human health. The difference between plants and medicine is disappearing rapidly and it is impossible to draw a line between plants and medicines. Developing countries have folkloric repute of medicines derived from natural sources for use in health care systems. The tribal uses of medicinal plants and herbs in healthcare practices provide guidelines for new domain of research and becoming popular in treatment of ailments. The medicinal and food plants of Pakistan have shown significant benefits to treat the gastrointestinal, respiratory and cardiovascular diseases (Saqib & Janbaz, 2016).

B. nobilis has a traditional repute to provide relief in hyperactive condition of gastrointestinal system such as diarrhea, hence aqueousm-ethanoic extract of *B. nobilis* was tested on spontaneous rhythmic contractions of isolated jejunum preparation to determine possible antispasmodic action.

The plant extract of *B. nobilis* (Bn.Cr) exhibited spasmolytic effect by inhibiting spontaneous contractions of isolated rabbit jejunum. The contraction in smooth muscle is due to increased quantity of free calcium in cytoplasm. This increased concentration of free calcium in the cell is by virtue of either through opening of L-type voltage dependent calcium channels or calcium discharged from intracellular reservoirs in sarcoplasmic reticulum (Saqib *et al*., 2018). The spontaneous rhythmic contractions of the jejunum are synchronized by action potential that is due to continuous depolarization and reploarization (Dimopoulos *et al*., 2007). Depolarization exhibits that the action potential is generated due to inflow of calcium by L-type Ca^{++} channels (Brading, 1981). The subsequent slowdown of the spontaneous contractions in jejunum can be because of interference either with entry of Ca^{++} via voltage dependent Ca^{++} channels (VDCS) or Ca^{++} freed from intracellular supplies of sarcoplasmic reticulum which leads to contraction of smooth muscle (Karaki *et al*., 1997). The resultant inhibition of rhythmic spontaneous contractions in isolated jejunum tissue by Bn.Cr might be due to hindrance either with entry of Ca^{++} through VDCs or obstruction of evoked depolarization (Coburn & Yamaguchi, 1977) due to Ca^{++} discharged from intracellular supplies. As a result, the action potential fall evident by relaxation of jejunum (Saqib & Janbaz, 2016).

It is established fact that K^+ (>30 mM) triggers opening of VDCs (Bolton, 1979), adjusting the Ca^{++} present extracellularly leading to contraction of smooth muscle (Godfraind et al., 1986); therefore the substances capable to reduce high K^+ (80 mM)-induced contractions is anticipated as Calcium channel blocker (CCB). The *B. nobilis* (Bn.Cr) relaxed K^+ (80 mM)-induced contractions in a dose dependent manner, hence showing its relaxant or spasmolytic activity possibly mediated through CCB. The Ca^{++} antagonistic action of *B. nobilis* was further verified due to shifting of the Ca^{++} concentrations response curves rightward on jejunum preparation which is similar to verapamil (a standard calcium channel blocker), and this CCB activity is widely distributed among plant kingdom, proved by sequence of experiments conducted in our research laboratory i.e. *Alternanthera sessilis* (Saqib & Janbaz, 2016), *Michelia champaca* (Saqib *et al*., 2018).

B. nobilis also has traditional uses by local population also in respiratory disorders, i.e. bronchitis, cough and expectoration therefore requires validation. Its aqueous-methanolic extract demonstrated relaxant action on carbachol (1 µM) and high K^+-induced contractions in trachea analogous to verapamil. The relaxation of high K^+-induced contraction shows its Ca^{++} channel blockade effect similar to jejunum tissue. Whereas Carbachol belongs to class of cholinergic agonist drugs and act on Gq- coupled muscarinic M3 receptors which facilitates signaling transduction due to calcium from intracellular storage, and causes contraction by stimulating M3 receptors, which activate Phospholipase pathway (Pang *et al*., 2001). The lipid membrane phosphatidyl inositol 4,5-bisphosphate and phospholipase C produces two potent secondary messengers: 1) inositol 4,5-trisphosphate (IP3) and 2) diacylglycerol (DAG). IP3 binds to receptors on sarcoplasmic reticulum (intracellular Ca^{++} stores), causes the release of stored Calcium ions. This calcium ion along with DAG activates Protein kinase C(PKC) ,i.e. phosphorylate specific target proteins calmodulin and myosin ,which leads to formation of Ca^{++}- calmouldin complex and myosin light chain kinase (MLCK) phosphorylation as a resultant contraction occurs. (Furchgott & Zawadzki, 1980; Kitazawa *et al*., 1989). Hence the relaxant effect by *B. nobilis* extract observed is probably referred as Ca^{++} channel inhibiting mechanism. The Ca^{++} channel inhibitors are very important in airway hyperactivity and indication of this activity can justify the conventional use of the plant in airways diseases (Saqib *et al*., 2018).

Furthermore, the relaxant effect of crude extract of *B. nobilis* as well as its fractions also relaxed CCh, (1 µM) and K^+ (80 Mm)-induced contractions in isolated in bladder preparations possibly mediated through CCB mechanism.

The respiratory diseases are etiologically related to vasospastic problems, so extract of *B. nobilis* was assessed for its potential vasorelaxant capacity. It produced relaxation of phenylephrine and high K^+-induced contractions in rabbit aorta comparable to verapamil. The phenylephrine-induced contraction of aorta is due to enhanced cytosolic calcium via both Ca^{++} entry through receptor operated channels and calcium released from intracellular reservoirs. Hence, it may be opined that relaxation of both high K^+-induced and phenylephrine contractions by crude extract of *B. nobilis* is due to calcium channel blocking mechanism. The *B. nobilis* displayed calcium channel blocker capacity in-vitro on aorta, trachea, bladder and jejunum that may be credited to the existence of alkaloids (Khalid *et al*., 2004), flavonoids (Reuvelta *et al*., 1997) and tannins (DiCarlo *et al*., 1993) amongst the constituents of *B. nobilis* noticed in chemical screening.

The liquid-liquid extraction was performed on Bn.Cr by using Dcm and water. The fractions of Bn.Cr when checked for CCB activity, resulted in manifestation of more dominant activity in Dcm fraction in comparison to aqueous, demonstrating that CCB activity is dominant among the Dcm fraction.

The *B. nobilis* showed negative inotropic and negative chronotropic effects on application to isolated rabbit paired atria preparations, which exhibit spontaneous contractions due to spontaneous depolarization of the pacemaker cells resulting in generation of action potential. The negative ionotropic and negative chronotropic effects in isolated rabbit paired atria preparations on the part of Bn.Cr is likely to be facilitated through reduced availability of cytosolic Ca^{++} ion (Brown & Taylor, 2001) resulting in slowing of the heart rate (chronotropic effect) and force of myocardial contraction (ionotropic effect). The observed decrease in inotropic activity subsequent to increase in tissue bath concentration of Bn.Cr may be due to overwhelming dominance of Ca^{++} channel blocking

activity llikely to be mediated through L-type calcium channels found in the atria (Ooi & Colucci, 2001).

As a therapeutic class, Ca^{++} channel blockers are known to possess potential in the management of gastrointestinal ailments including diarrhea and dysentery (Brunton *et al*., 2008). Additionally, *B. nobilis* also possess folkloric repute for effectiveness in intestinal spasm and diarrhea; hence, crude extract (Bn.Cr) was subjected to series of pharmacological screening tests for possible anti-diarrheal effect on castor oil-induced diarrheal models (i.e., charcoal meal and fecal mass count) in mice. *B. nobilis* slowed the propulsive movement of charcoal meal as well as production of wet fecal masses; thus, provided protection against the castor oil-induced diarrhea. The (Bn.Cr) exhibited anti-diarrheal activity in a manner comparable with loperamide (a standard anti-diarrheal drug) (Reynold *et al*., 1984; Baker, 2006).

The castor oil is known to be hydrolyzed by the intestinal lipases to glycerol and ricinoleic acid in small intestine of mice and ricinoleic acid in turn enhances intestinal secretions, fluid collection and loss of electrolyte (Iwao & Terada, 1962). The substances capable to provide protection against such diarrhea models are not only promising with anti-diarrheal activity but also presumed direct inhibitors of intestinal secretions (Wang *et al*., 2006) and demonstration of such activities by *B. nobilis* may provide a scientific evidence for folkloric use of in diarrhea and intestinal spasm (Pasricha, 2006).

CONCLUSION

The crude extract of *B. nobilis* has shown antispasmodic, bronchodilator, vasodilator, cardiac depressant and hypotensive activities which may be attributed to blockade of voltage dependent Ca^{++} channels. Hence, provided scientific basis for its vernacular use in gastro-intestinal, respiratory and cardiovascular disorders.

ACKNOWLEDGEMENT

This Project was supported by research grant No. 169-26 (2015-2016) approved by Bahauddin Zakariya University.

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