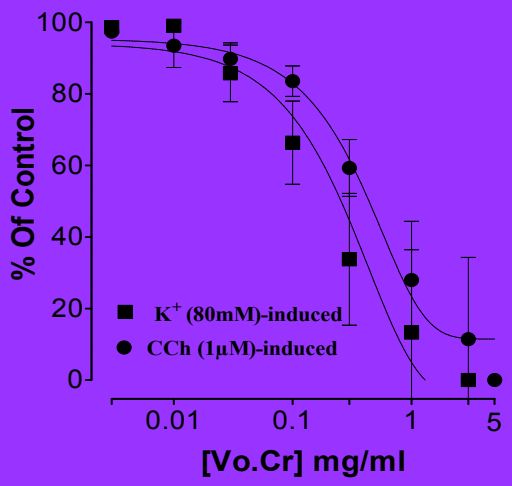

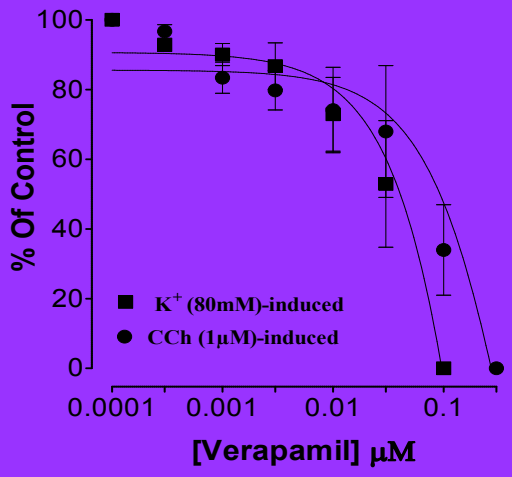

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Pharmacological basis for the medicinal use of *Viola odorata* in diarrhea, bronchial asthma and hypertension

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Abstract

Viola odorata is traditionally used in the management of gastrointestinal, respiratory and vascular disorders. The present study was undertaken to validate its folkloric uses. The application of *V. odorata* to spontaneous contractions in isolated rabbit jejunum preparation exerted relaxant effect through decrease in magnitude and frequency of contractions. Moreover, it also caused relaxation of K^+ (80 mM)-induced contractions and shifted the Ca^{2+} concentration response curves toward right in isolated jejunum similar to verapamil (standard Ca^{2+} channel blocker), confirming Ca^{2+} channel blocking activity. *V. odorata* also caused relaxation of carbachol (1 µM)- and K^+ (80 mM) -induced contractions in isolated rabbit tracheal preparations comparable to verapamil, reflecting that observed relaxant effect may be the outcome of anti-muscarinic and/or Ca^{2+} channel blocking activities. It also exerted relaxant effect on phenylephrine (1 µM)- and K^+ (80 mM)-induced contractions in isolated rabbit aortic preparations thus providing rationale for its folkloric uses to treat diarrhea, asthma and hypertension.

Introduction

Viola odorata, Linn. (Violaceae), locally called banafsha, is widely distributed throughout the world including Pakistan (Said, 1972; Baquar, 1989). The plant has traditionally been used to manage bronchial asthma, cough, bronchitis (Nadkarni, 1976; Pullaiah, 2006), anxiety (Keville, 1991) and hypertension (Duke et al., 2002). It is also used as expectorant and laxative (Ahmad et al., 2009).

The plant is reported to possess antioxidant (Ebrahimzadeh et al., 2010), diuretic, laxative (Vishal et al., 2009), analgesic (Barkatullah et al., 2012), anti-inflammatory (Koochek et al., 2003), antipyretic (Khattak et al., 1985), sedative (Alireza and Ali, 2013), hypotensive and lipid lowering effect (Siddiqi et al., 2012). Moreover, it has been reported to possess antibacterial (Ramezani et al., 2012; Khan et al., 2011), anthelmintic activity (Colgrave et al., 2008), anti-fungal (Pawar and Thaker, 2006) and mosquito repellant activity (Amer and Mehlhorn, 2006).

The qualitative investigation revealed presence of violanthin, flavonoids, glycosides (Khare, 2007), stigmasterol (Mittal, 2013), violaquercetin, saponins, alkaloids, vitamins (Kathi, 1991), phenols (Ebrahimzadeh, 2010), glucosides, violin (Prajapati et al., 2004), violanthin and violanin (Rastogi, 1979), vanillic acid (Evans, 1996), benzofuranone (Akhbari et al., 2012), glucopyranosides, (Karioti et al., 2011), shikimic acid (Anca et al., 2009), cycloviolacin, (Rosengren et al., 2003; Craik et al., 1999), dimethyldodecane (Cu et al., 1992), dimethylheptane (Beierbeck and Saunders, 1980), glucopyranoside (Henrick and Jefferies, 1964), violacin (Ireland et al., 2006) and peptide (Svangård et al., 2003).

V. odorata has the folkoric repute of providing relief in ailments pertaining to gastrointestinal, respiratory and cardiovascular system. The present study was undertaken to validate its folkloric uses in native systems of medicine.

Materials and Methods

Plant material and preparation of extract

The aerial parts of *V. odorata* were collected in May, 2012 from the botanical garden of Pakistan Institute of Forestry, University of Peshawar and were identified by the kind cooperation of an expert taxonomist (Prof. Altaf Ahmad Dasti), at the Institute of Pure and Applied Biology, Bahauddin Zakariya University, Multan.

The plant material was shade dried and rendered free of adulterants by manual picking and was grinded to coarse powder with special herbal grinder. The powdered material (1 kg) was macerated in 70% aqueous-methanol for 2 weeks with occasional shaking. The soaked material was passed through a muslin cloth to remove the vegetative debris and the fluid obtained was subsequently filtered through a Whatman No. 1 filter paper. The filtrate was evaporated on a rotary evaporator (Rotavapor, BUCHI labrotechnik AG, Model 9230, Switzerland) at 40°C under reduced pressure to dark brown thick paste like semisolid material. The percentage yield of crude *V. odorata* was calculated to be 7.3% approximately. The extract obtained was stored in amber colored air tight jars at -40°C.

Chemicals

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

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

Animals and housing conditions

Animals (♂/♀) used in this study were local strain rabbits (1.0-1.8 kg). These were housed under controlled environmental condition (23-25°C) at the animal house of Faculty of Pharmacy, Bahauddin Zakariya University, Multan. Rabbits were provided with refresh green fodder and tap water *ad libitum*. The animals were deprived of food 24 hours prior to the experiments but were given free access to water. Rabbits were sacrificed following a blow on the back of head to use for *in vitro* studies.

Preliminary phytochemical analysis

The crude extract of *V. odorata* was subjected to qualitative phytochemical analysis for the presence of alkaloids, saponins, anthraquinones, coumarins, sterols, terpenes, flavonoids and phenols (Janbaz and Saqib, 2015).

In vitro experiment

Isolated tissues experiments were performed as described previously (Janbaz et al., 2013).

Isolated rabbit jejunum preparations

Plant extract was tested on isolated rabbit jejunum preparations for possible presence of spasmogenic and/or spasmolytic activity. Isolated rabbit jejunum segments of approximately 2 cm in length were suspended in isolated tissue baths containing Tyrode's solution, at 37°C, aerated with carbogen (95%O_2 and 5%CO_2). The composition of the Tyrode's solution (mM) was: KCl (2.68), NaC1 (136.9), $MgC1_2$ (1.05), $NaHCO_3$ (11.9), NaH_2PO_4 (0.42), $CaC1_2$ (1.8) and glucose (5.55). A preload of 1 g was applied and intestinal responses were recorded isotonically through Power Lab data acquisition system (AD Instruments, Sydney, Australia) attached to a computer installed with lab chart software (version 7.1). The tissues were allowed to equilibrate for at least 30 min prior to the addition of any drug. Isolated rabbit jejunum preparations exhibit spontaneous rhythmic contractions and allowed testing of the antispasmodic (relaxant) effect without application of an agonist (Janbaz et al., 2015a; Saqib et al., 2012). The observed response of the test material was quantified by the application of doses in a cumulative fashion. The relaxant effects on the part of test substance were taken as the percent change in spontaneous contractions of the preparation recorded immediately before the addition of test substances.

The possible mechanism of the relaxant activity of the test material was investigated through the relaxation of the observed sustained spasmodic contractions following exposure to high concentration of K^+ (80 mM) (Farre et al, 1991). The 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.

Calcium channel blocking effect of the test substance was confirmed by the method described previously (Janbaz et al., 2015b). The isolated rabbit jejunum preparation was allowed to stabilize in normal Tyrode's solution, which was subsequently replaced for 30 min with Ca^{2+}-free Tyrode's solution to which EDTA (0.1 mM) was added in order to remove calcium from the tissue. This bath solution was further replaced with K^+-rich and Ca^{2+}-free Tyrode's solution, having the

following composition (mM): KC1 (50), NaCl (91.04), $MgCl_2$ (1.05), $NaHCO_3$ (11.9), NaH_2PO_4 (0.42), glucose (5.55) and EDTA (0.1). Subsequent to an incubation period of 30 min, cumulative Ca^{2+} concentrations were applied to the tissue bath to obtain control calcium dose -response curves. On achievement of the super-imposable control calcium dose-response curves (usually after two cycles), the tissues were then washed and allowed to equilibrated with the plant extract for 1 hour and then the concentration response curves of Ca^{2+} were recorded and compared to the control curves. The dose-response curves of Ca^{2+} were recorded in the presence of different concentrations of the plant extract in tissue bath (Janbaz et al., 2015b)

Isolated rabbit tracheal preparations

Rabbit tracheas were dissected out and kept in Kreb's solution having the following composition (mM): NaCl (118.2), $NaHCO_3$ (25), $CaCl_2$ (2.5), KCl (4.7), KH_2PO_4 (1.3) $MgSO_4$ (1.2) and glucose (11.7). The trachea was cleaned free from the surrounding fatty tissues and rings of 2-3 mm width containing 2-3 cartilages were prepared. Each ring was opened by a longitudinal incision on the ventral side opposite to the smooth muscles layer to form a strip with smooth muscles layer in middle and cartilages on both sides. These tracheal preparations were mounted in 20 mL organ bath containing Krebs solution being maintained at 37°C and aerated with carbogen. A preload tension of 1 g was applied and tissue preparations were allowed to be equilibrated for 1 hour prior to any challenge by the drug. Tissue preparations were stabilized by repeated applications of carbachol (1 µM) until constant responses were recorded. The carbachol (1 µM)- and high K^+ (80 mM)-induced sustained contractions were subsequently used for testing of different doses of the test material in a cumulative fashions. The isometric responses were recorded through Power Lab data acquisition system (AD Instruments, Sydney, Australia) attached to a computer installed with lab chart software (Version 7.1). The standard drug with Ca^{2+} channel blocking effect (verapamil) was tested on high K^+ (80 mM)- and carbachol-induced spastic contractions in order to confirm the possible mechanism of action (Janbaz et al., 2014a)

Isolated rabbit aorta preparation

To see the effect of plant extract on systemic vascular resistance, rabbits of either sex were sacrificed by a blow on the back of head and descending thoracic aorta was dissected out and kept in the normal Kreb's solution having composition as described earlier. It was then cut into rings of about 2-3 mm width and each ring was mounted in a tissue bath containing Kreb's solution. Temperature was maintained at 37°C and tissue was continuously aerated with carbogel. A preload of 2 g was applied to each preparation and allowd to equilibrate for a period of 1 hour. After equilibrium, tissue was stabilized by repeated exposure to K^+ (80 mM) or phenylephrine (1 µM) depending upon the protocol of the experiment. The vasorelaxant/ vasoconstrictive effects of the test substances were studied by addition in tissue organ baths containing pre-stabilized tissue in a cumulative manner (Janbaz et al., 2014b). Changes in isometric tension of aortic rings were obtained via force-displacement transducer (Model FORT100, WPI, USA) coupled to Power Lab data acquisition system (AD Instruments, Sydney, Australia) and computer running Lab Chart software (version 7.1).

Statistical analysis

The data was expressed as mean ± standard error of mean (S.E.M., n = 5) and median effective concentration (EC_{50}) with 95% confidence interval (CI). The statistics applied was Student's t-test. The logarithmic, dose/ concentration-response curves of different treatments were analyzed by non-linear regression using computer software (Graph Pad Software, San Diego, CA, USA).

Results

Preliminary phytochemical analysis of *V. odorata* revealed the presence of alkaloids, flavonoids, glycosides, steroids, terpenes, saponins and tannins among the methanol soluble extractable constituents of *V. odorata*.

Effect on isolated rabbit jejunum preparations

The crude extract of *V. odorata* on application to the spontaneously contracting isolated rabbit jejunum preparations exerted relaxant effect in tissue bath concentration dependent manner with EC_{50} value of 0.05 mg/mL (95% CI: 0.03-0.08 mg/mL; n=5). Moreover, it caused complete relaxation of K^+ (80 mM)-induced contraction with EC_{50} value of 0.41 mg/mL (95% CI: 0.14-1.15 mg/mL; n=5). Verapamil (standard Ca^{2+} channel blockers), relaxed the spontaneous and K^+ (80 mM)-induced contractions with respective EC_{50} values of 0.49 µM (95% CI: 0.35- 0.73 µM; n=5) and 0.33 µM (95% CI: 0.16-0.66 µM; n=5) (Figure 1). Furthermore, extract of plant also caused rightward shift of concentration response curves for Ca^{2+} in a manner comparable to verapamil in isolated rabbit jejunum preparations (Figure 2).

Effect on isolated rabbit tracheal preparations

The crude extract of *V. odorata* exerted relaxant effect on application to carbachol (1 µM) and K^+ (80 mM)-induced contractions in isolated rabbit tracheal

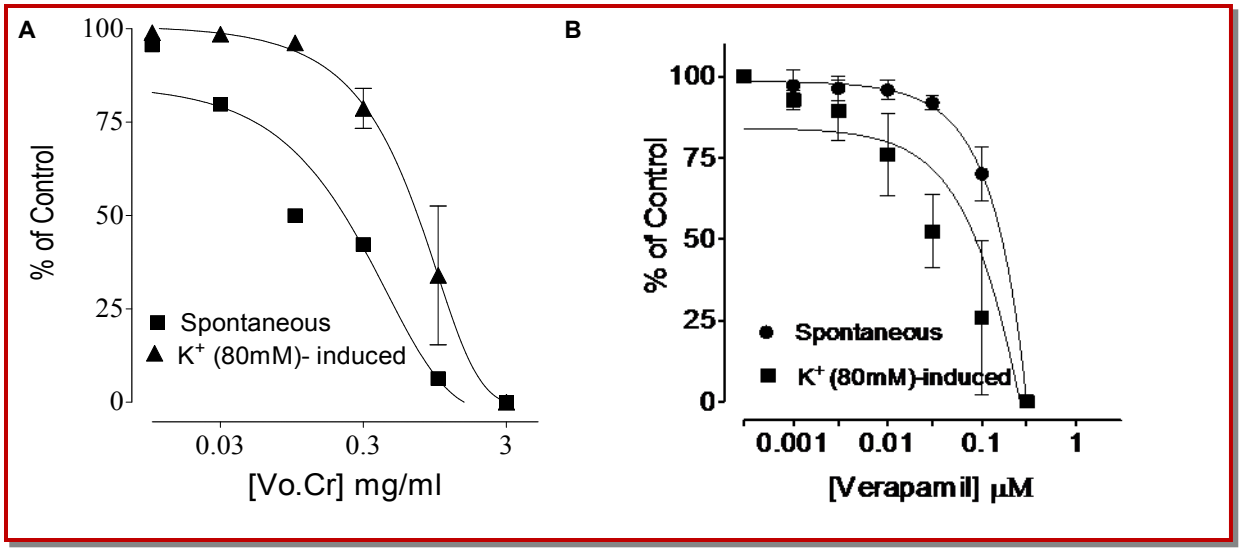

Figure 1: Effect of crude extract of *V. odorata (Vo. Cr)* (A) and verapamil (B) on spontaneous and K^+ (80 mM)-induced contractions in isolated rabbit jejunum preparations (values are expressed as mean ± S.E.M.; n=5)

preparations with respective EC_{50} values of 0.54 mg/mL (95% CI: 0.30-0.96 mg/mL; n=5) and 0.68 mg/mL (95% CI: 0.37-1.24 mg/mL; n=5). Similarly, verapamil also caused relaxation of carbachol (1 µM) and K^+ (80 mM)-induced contractions with respective EC_{50} values of 2.15 µM (95% CI: 0.03-4.26 µM; n=5) and 0.32 µM (95% CI: 0.02-0.62 µM; n=5) (Figure 3).

Effect on isolated rabbit aortic preparations

The *V. odorata* crude extract on application to isolated rabbit aortic preparation, exerted relaxant effect on phenylephrine (1 µM)-induced contractions in isolated rabbit aortic preparations up to the extent of 5 mg/mL tissue bath concentrations with EC_{50} values of 5.37 mg/mL (95% CI: 3.97-6.65 mg/mL; n=5), whereas K^+ (80 mM)-induced contractions in isolated rabbit aorta were relaxed at lower tissue bath concentrations with EC_{50} values of 1.5 mg/mL (95% CI: 0.34-6.66 mg/mL; n=5).

The standard Ca^{2+} channel blocker (verapamil), relaxed the phenylephrine (1 µM) and K^+ (80 mM)-induced contractions with respective EC_{50} of 1.08 mg/mL (95% CI: 0.08-2.52; n=5) and 0.55 mg/mL (95%CI: 0.04-2.10; n=5) (Figure 4).

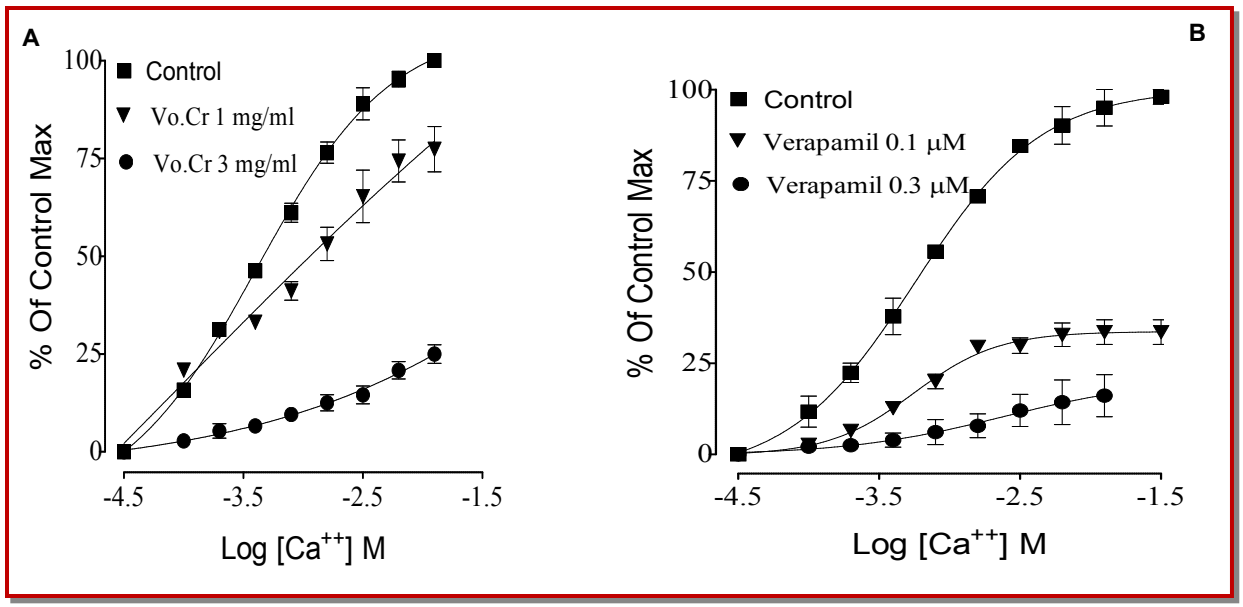

Figure 2: Effect of crude extract of *V. odorata (Vo. Cr)* (A) and verapamil (B) on concentration-response curves for Ca^{2+} in isolated rabbit jejunum preparations (values are expressed as mean ± S.E.M.; n=5)

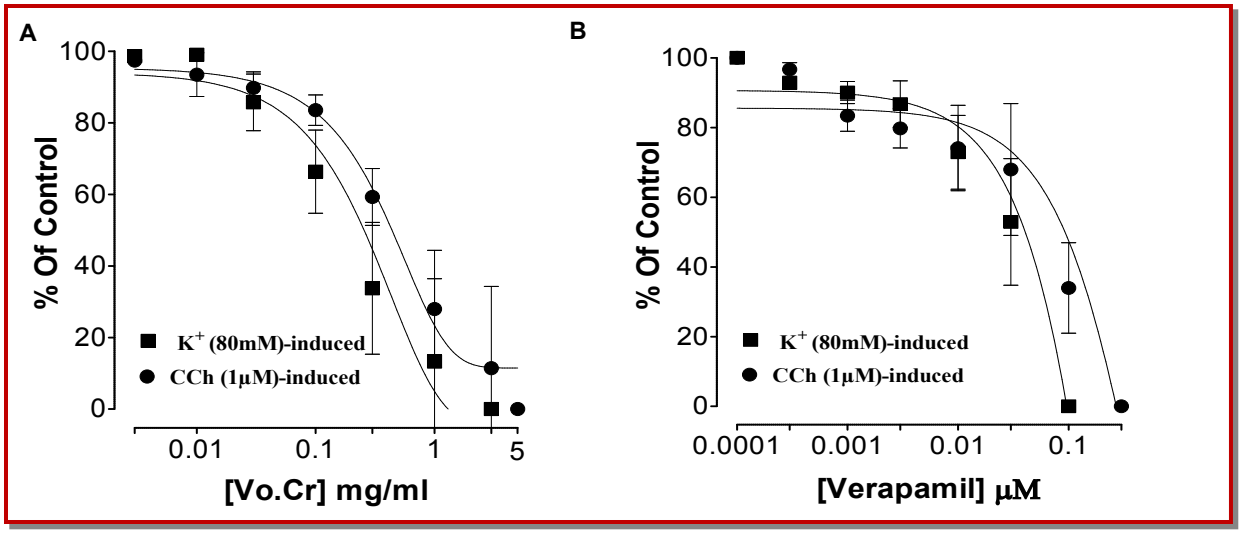

Figure 3: Effect of crude extract of *V. odorata* (Vo. Cr) (A) and verapamil (B) on carbachol (1 µM) and K^+ (80 mM)-induced contractions in isolated rabbit tracheal preparations (values are expressed as mean ± S.E.M.; n=5)

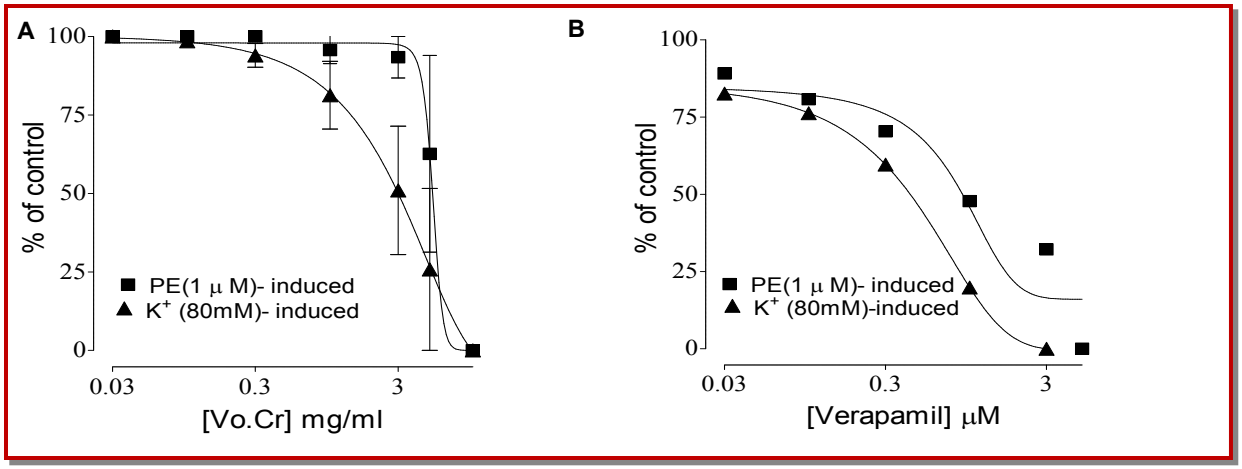

Figure 4: Effect of crude extract of *V. odorata* (Vo. Cr) (A) and verapamil (B) on phenylephrine (PE) (1 µM) and K^+ (80 mM)-induced contractions in isolated rabbit aortic preparations (values are expressed as mean ± S.E.M.; n=5)

Discussion

Preliminary phytochemical analysis of *V. odorata* revealed the presence of alkaloids, flavonoids, glycosides, steroids, terpenes, saponins and tannins among the constituents of *V. odorata*.

The *V. odorata* exerted relaxant effect on spontaneous contractions in isolated rabbit jejunum preparation, i.e., exhibiting antispasmodic activity. Studies reported on plant materials reflected anti-spasmodic activity may possibly be mediated through blockade of Ca^{2+} channels (Janbaz et al., 2015b). The contractile activities of smooth muscle preparations, i.e., isolated rabbit jejunum preparations is mediated through increase/ decrease of cytoplasmic free Ca^{2+} concentration (Karaki et al., 1997). The intracellular Ca^{2+} concen-tration is known to be increased either influx through voltage dependent Ca^{+2} channels or Ca^{2+} released from sarcoplasmic stores (Godfraind et al., 1986). The spontaneous contractions in isolated rabbit jejunum preparations are manifestation of alternative depolarization and repolarization, where tissues at height of depolarization, permit fast influx of Ca^{2+} through voltage dependent Ca^{+2} channels (Brading, 1981). Thus, the spasmolytic effect on the part of *V. odorata* may possibly be mediated through either blockade of voltage dependent Ca^{+2} channels or suppression of Ca^{2+} release from sarcoplasmic reticulum. The isolated smooth muscle preparations on exposure to K^+ (80 mM) exhibit sustained contractile activity due to rapid influx of extracellular Ca^{2+} through opened voltage dependent Ca^{+2} channels (Bolton, 1979; Godfrained et al., 1986) and relaxant effect of extract on K^+ (80 mM)-induced contractions may possibly mediated through Ca^{2+} channel blockade (van Rossum, 1963). The above-mentioned findings were confirmed further as extract treatment in isolated rabbit jejunum preparation caused decreased response to Ca^{2+} and rightward shift of the

concentration response curves for Ca^{2+} in a manner similar to verapamil as standard Ca^{2+} channel blocker (Fleckenstein, 1977). The Ca^{2+} channel blockers is an established class of therapeutic agents and are known to be effective in hyperactive diseases of the gut (Brunton et al., 1996).

The *V. odorata* caused relaxation of carbachol (1 µM)- and K^+ (80 mM)-induced contractions in isolated rabbit tracheal preparations in a manner comparable to verapamil and is possibly mediated through blockade of Ca^{2+} channels. The Ca^{2+} channel blockers are useful bronchodilator in conditions of increased sensitivity of the airway (Ahmed, 1992), hence this study provided a scientific basis to validate traditional uses of *V. odorata*, Linn. in the management of respiratory disorders including asthma, cough and bronchitis.

The *V. odorata* caused complete relaxation of the phenylephrine (1 µM)- and K^+ (80 mM)-induced contractions in isolated rabbit aorta preparations, however, phenylephrine-induced contractions were found to be relaxed at elevated tissue bath concentrations. The isolated rabbit aorta preparations have been used for characterization of Ca^{2+} channel blocking activities (Janbaz et al., 2014b), which on exposed to K^+ (80 mM), resulted in contraction of smooth muscles via opening of voltage dependent Ca^{2+} channels. The increase in intracellular Ca^{2+} due to increased influx of Ca^{2+} can cause further Ca^{2+} release from sarcoplasmic reticulum (Gurney, 1994; Karaki et al., 1997). Similarly, phenylephrine-induced contraction in vascular smooth muscles is known to be mediated through increase in cytsoplasmic Ca^{2+}through two possible means, i.e., Ca^{2+} influx via receptor operated channels and subsequent release of Ca^{2+} from intracellular stores (Graham et al., 1996). The relaxation of phenylephrine-induced contractions on the part of *V. odorata* at elevated tissue bath concentrations can be viewed on focusing the point that *V. odorata* like other Ca^{2+} channel blockers can only block Ca^{2+} influx through voltage dependent Ca^{2+} channels and do nothing with Ca^{2+} influx through receptor operated channels and subsequent increase in intracellular Ca^{2+} due to release of Ca^{2+} from intracellular stores (Graham et al., 1996). The observed relaxant effect of *V. odorata* on aorta may provide a scientific basis for the folkloric use of *V. odorata* in the management of hypertension.

The *V. odorata* exhibited Ca^{2+} channel blocking activity in isolated rabbit tissue preparations (i.e., jejunum, trachea and aorta) which can be attributed to the presence of alkaloids (Khalid et al., 2004; Gilani et al., 2005,), flavonoids (Revuelta et al., 1997; Di-Carlo, 1993) and tannins (Azhar et al., 1997) among the constituents of *V. odorata* detected in the preliminary phytochemical screening.

Ethical Issue

All the experiments performed were complied with the rulings of Institute of Laboratory Animal Resources, Commission on Life Sciences (NRC, 1996), approved by the Ethical Committee of Bahauddin Zakariya University, Multan.

Conflict of Interest

The author(s) declare that there is no conflict of interests regarding the publication of this article.

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