Research Article

Antidiarrheal and antispasmodic activities of *Vincetoxicum stocksii* are mediated through calcium channel blockade

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Abstract

This study was carried out to explore the mechanism underlying antidiarrheal and antispasmodic activities of *Vincetoxicum stocksii.* The crude extract of *V. stocksii* provided 12-24% protection from castor oil-induced diarrhea at the dose of 300-1,000 mg/kg, similar to loperamide. In isolated rabbit jejunum preparations, *V. stocksii* caused inhibition of the spontaneous and high K^+ (80 mM)-induced contractions, with respective EC_{50} values of 2.5 (1.7-3.9) and 1.0 mg/mL (0.6-1.4), similar to that caused by verapamil, suggesting the calcium channel blocking effect. Loperamide caused inhibition of spontaneous and high K^+-induced contraction, with respective EC_{50} values of 8.6 (6.3-10.1) and 9.1 µM (7.3-12.8). The calcium channel blocking activity was further confirmed when pretreatment of the tissues with *V. stocksii* (1-3 mg/mL) caused a rightward displacement of the Ca^{++} concentrations response curves, similar to that produced by verapamil; constructed in Ca^{++} free medium. These data indicate that the crude extract of *V. stocksii* contains calcium channel blocking constituents that may possibly explain its medicinal use in hyperactive states of gut, such as, diarrhea and spasms.

Introduction

Vincetoxicum stcoksii Ali and Khatoon belongs to the Asclepiadaceae (milkweed) family and is a group of perennial climbing leafy vines (Zaidi and Crow, 2005). *Vincetoxicum* is distributed throughout the tropical American continent, Europe and Asia. Phytochemistry of the plant revealed the presence of some glycosides and alkaloids (Zaidi and Crow, 2005). The plant is not well studied biologically or pharmacologically, except for antibacterial and antifungal activities.

This study describes the antidiarrheal and antispasmodic activities of the crude extract of *V. stocksii* with possible mode of action explored.

Materials and Methods

Plant materials

Aerial parts of *V. stocksii* (800 g) were obtained from Quetta Baluchistan and authenticated by Prof. Musaddar A Zaidi. A voucher specimen (VT-LF-09-02-57) was deposited at the herbarium located at the Department of Biological and Biomedical Sciences, Aga Khan University, Karachi, Pakistan.

Preparation of crude extract

Preparation of the crude extract was carried out as described previously (Gilani et al., 2005). The powdered material was soaked in 70% aqueous-methanol for three

days with occasional shaking. It was filtered through a muslin cloth and then through a whatmann qualitative grade 1 filter paper. This procedure was repeated thrice and the combined filtrate was evaporated on a rotary evaporator at 37°C under reduced pressure (-760 mm Hg) to a thick, semi-solid mass of brown color, i.e. the crude extract, yielding approximately 41% (w/w). The crude extract was solubilized in normal saline for use in the *in vivo* and *in vitro* experiments. *V. stocksii* crude extract was screened for the presence of saponins, flavonoids, flavanols, flavones, tannins, phenols, coumarins, sterols, terpenes, alkaloids and anthraquinones by using methods described by Wall et al. (1952).

Chemicals

The following reference chemicals were obtained from the sources specified: Loperamide hydrochloride, acetylcholine chloride, verapamil hydrochloride, potassium chloride (Sigma Chemical Company, St. Louis, MO, U.S.A.) and castor oil (Karachi Chemical Industries, Karachi, Pakistan). All chemicals used were of the highest purity grade available. Stock solutions of all the chemicals were made in distilled water and the dilutions were made fresh in normal saline on the day of the experiment.

Experiments performed complied with the rulings of the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council (National Research Council, 1996) and were approved by the Ethical Committee of Aga Khan University Karachi, Pakistan. BALB-c albino mice (20-25 g) and local rabbits (1.5-2 kg) of either sex used in the study were bred and housed in the animal house of Aga Khan University in a controlled environment (23-25°C). Animals were given tap water *ad libitum* and a standard diet.

Antidiarrheal protocol

The *in vivo* antidiarrheal activity of the extract was investigated following the methods previously described (Awouters et al., 1978; Jebunnessa et al., 2009; Shah et al., 2010). In the present study BALB-c albino mice were fasted for 18 hours. The animals were divided in five groups, housed in five steel cages with five mice in each and the bottom of each cage was covered with a blotting sheet. The first group received saline (10 mL/kg, p.o.) as the vehicle control and so acted as the negative control. The second group received castor oil. Groups third and fourth received two doses (300 and 1,000 mg/kg) of the crude extract of *V. stocksii*, which were selected on a trial basis and administered orally by an intra-gastric feeding needle. Groups fifth received loperamide (10 mg/kg) orally, and served as positive control. One hour after treatment each animal received 10 mL/kg of castor oil orally and was then observed for defecation. Up to 4 hours after the castor oil challenge, the presence of diarrheal droppings was noted on blotting sheets in the individual cages. Percent protection against the castor oil-induced diarrhea was calculated based on the number of dry feces in each cage in comparison to the wet.

The isolated tissue experiments were carried out as described previously (Shah et al., 2010). The animals had free access to water but were fasted for 24 hours before the experiment. The animals were sacrificed by cervical dislocation, the abdomen was cut open and the jejunal portion isolated out. Preparations 2 cm long were mounted in 10 mL tissue baths containing normal Tyrode's solution maintained at 37°C and aerated with a mixture of 5% carbon dioxide in oxygen (carbogen). The composition of Tyrode's solution, in mM, was: KCl 2.7, NaCl 136.9, $MgCl_2$ 1.1, $NaHCO_3$ 11.9, NaH_2PO_4 0.4, Glucose 5.6 and $CaCl_2$ 1.8 (pH 7.4). A preload of 1 g was applied and the tissues kept undisturbed for an equilibrium period of 30 min after which control responses to a sub-maximal dose of acetylcholine (0.3 μM) were obtained and the tissue presumed stable only after the reproducibility of the said responses.

Under these experimental conditions, rabbit jejunum exhibits spontaneous rhythmic contractions, allowing the testing of relaxant (spasmolytic) activity directly without the use of an agonist (Gilani et al., 1994).

Calcium channel blocking activity

To assess whether the spasmolytic activity of the test substances was mediated through calcium channel blockade, high concentration of K^+ (80 mM), as K^+, was used to depolarize the preparations (Farre et al., 1991). K^+ (80 mM) was added to the tissue bath, which produced a sustained contraction. Plant extract and standards were then added in a cumulative fashion to obtain concentration-dependent inhibitory responses (van-Rossum, 1963). The relaxation of intestinal preparations, precontracted with high K^+, was expressed as percent of the control precontraction.

To confirm the calcium antagonist activity of test substances, the tissue was allowed to stabilize in normal Tyrode's solution, which was then replaced with Ca^{++}-free Tyrode's solution containing EDTA (0.1 mM) for 30 min in order to remove Ca^{++} from the tissues. This solution was further replaced with K^+-rich and Ca^{++}-free Tyrode's solution, having the following composition: KCl 50, NaCl 91.0, $MgCl_2$ 1.1, $NaHCO_3$ 11.9, NaH_2PO_4 0.4, glucose 5.6 and EDTA 0.1 mM, with pH of 7.4. Following an incubation period of 30 min, control concentration-response curves of Ca^{++} were obtained. When the control concentration-response curves of Ca^{++} were found super-imposable (usually after two cycles), the tissue was pretreated with the plant extract for 60 min to test the possible calcium channel blocking effect. The concentration-response curves of Ca^{++} were reconstructed in the presence of different concentrations of the test material.

Statistics

All the data expressed are mean ± standard error of the mean (SEM), and the median effective concentrations (EC$_{50}$ values) are given with 95% confidence intervals (CI). The statistical parameter applied is the Student's t-test with $p<0.05$ noted as significantly different (GraphPad Prism).

Results and Discussion

The crude extract of *V. stocksii* was tested for its anti-diarrheal activity. In the castor oil-induced diarrhea in mice, the crude extract of *V. stocksii* , like loperamide, a standard antidiarrheal agent (Reynolds et al., 1984), inhibited significantly ($p<0.05$) the frequency of defecation as well as wetting of feces when compared with the untreated group (i.e. mice receiving neither extract, nor loperamide, but castor oil only). Crude extract of *V. stocksii* and loperamide reduced greatly the wetness of the fecal droppings and provided around 12.7 $\pm$ 3.1 to 24.4 $\pm$ 4.5 and 94.8 $\pm$ 1.4% protection, respectively (Table I). The induction of diarrhea by castor oil results from the action of recinoleic acid formed in the hydrolysis of the oil (Iwao and Terada, 1962), which produces changes in the transport of water and electrolytes and results in a hypersecretory response and generation of giant contraction of the intestine (Croci et al., 1997).

Thus, a potential antidiarrheal agent may exhibit its antidiarrheal effect by inhibiting either gut motility and/or electrolyte out flux (Croci et al., 1997). The protective effect of the crude extract of *V. stocksii* against the castor oil-induced diarrhea in mice, similar to loperamide, suggests that it has either an inhibitory effect on contraction or on electrolyte out flux. To see its possible inhibitory effect on gut motility, the *V. stocksii* was further studied in the *in vitro* experiments.

When tested in isolated rabbit jejunum preparations, cumulative addition of plant extract, verapamil and loperamide caused concentration-dependent inhibition of the spontaneous contractions, with respective EC$_{50}$

values of 2.5 mg/mL (1.7-3.9), 0.2 μM (0.1-0.3) and 8.6 μM (6.3-10.1) (Figure 1), thus showing smooth muscle relaxant (spasmolytic) activity.

Our previous observation revealed that the spasmolytic constituents present in the extracts of different medicinal plants mediate their effect usually through a calcium channel blocking effect (Gilani et al., 2005; Shah et al., 2010). To see whether the spasmolytic effect of the plant extract observed in this study is also mediated through a calcium channel blocking like effect, a high concentration of K$^+$ (80 mM) was produced sustained contractions. The crude extract was then added in a cumulative fashion, where it caused a concentration-dependent relaxation of the induced contractions with an EC$_{50}$ value of 1.0 mg/mL (0.6-1.4), as shown in Figure 1, suggests that the spasmolytic effect is possibly media-ted through a calcium channel blocking-like effect. Similarly, verapamil and loperamide also caused a concentration-related inhibitory effects against high K$^+$-induced contractions with respective EC$_{50}$ values of 0.03 μM (0.02-0.04) and 9.1 μM (7.3-12.8) (Figure 1). Crude extract of *V. stocksii* was more potent against K$^+$-induced contractions, similar to verapamil, which suggests that plant extract mediates its spasmolytic effect possibly through calcium channel blockade .

The contractions induced by high K$^+$ (>30 mM) are dependent on the entry of Ca^{++} into the cells through voltage dependent channels (Bolton, 1979) and a subs-tance which can inhibit high K$^+$-induced contractions is therefore, possibly considered to be a calcium channel blocker (Godfraind et al., 1986). Thus, the inhibition of high K$^+$-induced contractions of rabbit jejunum by Vs.Cr may reflect the restricted Ca^{++} entry via voltage-dependent channels.

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| Table I				
Effect of the crude extract of *Vincetoxicum stocksii* on castor oil-induced diarrhea in mice				
Group	Dose	Total number of feces in 4 hours	Total number of wet feces in 4 hours	Protection (%)
Control	10 mL/kg	12.7 ± 0.9	0.4 ± 0.2	99.0 ± 0.9
Castor oil	10 mL/kg	13.8 ± 0.2	13.0 ± 0.8	1.0 ± 0.2
Castor oil + extract	10 mL/kg + 300 mg/kg	13.6 ± 1.5	87.3 ± 3.1	12.7 ± 3.1[a]
Castor oil + extract	10 mL/kg + 1,000 mg/kg	6.8 ± 0.9	75.6 ± 4.5	24.4 ± 4.5[b]
Castor oil + loperamide	10 mg/kg +10 mg/kg	8.8 ± 1.2	0.4 ± 0.3	94.8 ± 1.4
Results are mean ± SEM; n= 5; [a]Significantly different from control, $p<0.05$; Significantly different from control, $p<0.01$				
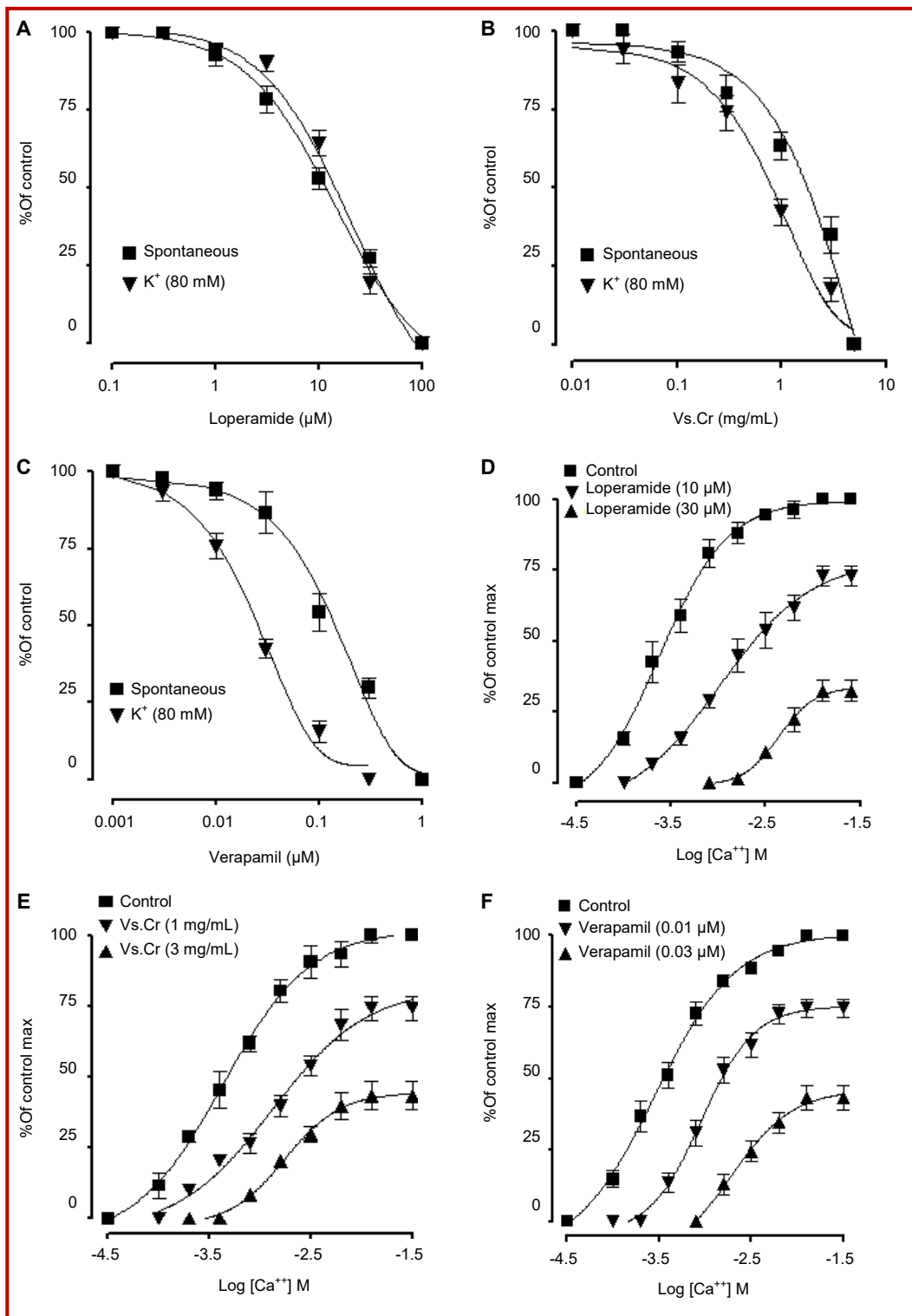
Figure 1: Concentration-response curves of (A) the crude extract of *Vincetoxicum stocksii* (Vs.Cr), (B) verapamil and (C) loperamide on spontaneous and K+ (80 mM)-induced contractions in isolated rabbit jejunum preparations. Figures D, E and F, show respectively, the effect of Vs.Cr verapamil and loperamide on the Ca++ concentration-response curves in isolated rabbit jejunum preparations. Values shown are mean $\pm$ SEM; n= 6-9

extract may reflect the restricted Ca^{++}entry via voltage-dependent channels.

This hypothesis was further strengthened when pre-treatment of the tissues with crude extract of *V. stocksii* (1-3 mg/mL) caused a rightward shift in the Ca^{++} CRCs (Figure 1D), similar to verapamil (Figure 1). Pre-treatment of the tissues with loperamide also caused a rightward shift in the Ca^{++} CRCs (Figure 1), which is in accordance to its known calcium channel blocking effect at antidiarrheal doses (Reynolds et al., 1984). These data indicate that crude extract of *V. stocksii* possesses a calcium channel blocking effect similar to verapamil, which provides pharmacological basis to its anti-diarrheal and antispasmodic effects, as calcium channel blockers are considered useful in diarrhea and gut spasms (Pasricha, 2006).

Preliminary phytochemical analysis of the plant extract revealed the presence of tannins and glycosides while other groups were found absent. The plant derived tannins are known to possess calcium channel blocking and antidiarrheal activities (Zhu et al., 1997) and the presence of tannins in this plant may explain the anti-diarrheal and the calcium channel blocking activities of the plant.

Conclusion

Crude extract of *V. stocksii* possesses antidiarrheal and antispasmodic effects mediated possibly through calcium channel blockade.

Acknowledgement

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