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Antidiarrheal and antispasmodic activities of *Vitex negundo* are mediated through calcium channel blockade

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Abstract

Vitex negundo has reputation in the traditional medicine in diarrhea and gut spasm. This study was carried out to provide possible pharmacological basis to its medicinal use in hyperactive gut disorders. In castor oil-induced diarrheal model, the crude extract of *V. negundo*, caused a dose-dependent protection (53-71%), similar to loperamide. In isolated rabbit jejunum preparation, *V. negundo* caused inhibition of spontaneous and high K⁺-induced contractions, with EC₅₀ values of 0.36 (0.22-0.61) and 1.30 mg/mL (0.27-5.89; n= 4-5), respectively, suggestive of spasmolytic activity, mediated possibly through calcium channel blockade (CCB). The CCB activity was further confirmed when pre-treatment of the tissue with *V. negundo* (0.03-0.3 mg/mL) caused a rightward shift in the Ca⁺⁺ concentration-response curves (CRCs), similar to diltiazem or loperamide. These data indicate that the antidiarrheal and spasmolytic effects of the crude extract of *V. negundo* are mediated through the presence of CCB-like constituent(s).

Introduction

Vitex negundo Linn., belongs to the family "Verbenaceae" occurs in different parts of Pakistan, such as, Rawalpindi hills, Murree, Muzaffarabad, Mirpur, Lower hazara, Kurram, Swat, Poonch on the bank of streams and water courses (Baquar, 1989). The plant has medicinal reputation in diarrheal and gut spasm, in addition to many other uses, such as, astringent, in rheumatism and joint swelling (Baquar, 1989; Evans, 2006; Shinwari et al., 2003; Usmanghani et al., 1997).

Phytochemical analysis of the plant revealed the presence of casticin, isoorientin, chrysophenol D, luteolin, p-hydroxybenzoic acid and D-fructose. Additionally, alkaloids, glycosides, flavonoids, sterols, resins, tannins, dimethylether of delphinidin and dimethyl ether of leucocyanidin, rhumanoglucosides and flavone glycosides have also been found (Hung et al., 2013).

Pharmacologically the plant has been reported to possess anti-inflammatory (Tandon and Gupta, 2006; Puchpangadan et al., 2006), analgesic, antihistaminic (Dharmasiri et al., 2003), hypnotic (Gupta et al., 1999), antioxidant (Munasinghe et al., 2001), anticancer (Diaz et al., 2003), antiandrogenic (Das et al., 2004; Bhargava, 1989), hepatoprotective (Avadhoot and Rana, 1991) antibacterial (Perumal-Samy et al., 1998) and antifungal (Puchpangadan et al., 2006) activities.

Despite the fact that the plant has been studied extensively phytochemically, research on its pharmacological activities in hyperactive gut disorders is lacking. Therefore, this investigation was carried out to provide possible pharmacological basis to the medicinal use of the plant in hyperactive gut disorders, such as diarrhea and spasms.



Materials and Methods

Aerial parts of *V. negundo* were collected in District Swat, KhyberPakhtunkhwa., Pakistan, and was authenticated by Assistant Professor Mr. Ilyas Iqbal at the Department of Botany, University of Malakand, Chakdara Dir Lower, Pakistan. A voucher specimen (UOM/BGH/149) was deposited at the herbarium of the same Department. The plant materials was shade dried and were clean up of adulterants and approximately 1 kg of the pulverized material was soaked in aqueous-methanol (70%) at room temperature ($25 \pm 2.0^\circ\text{C}$) for three days with occasional shaking. Then it was filtered through a muslin cloth and then through a filter paper. This procedure of soaking and filtration was repeated twice more.

All the filtrates were combined and evaporated to dryness on a Rotary Evaporator under reduced pressure (-760 mmHg) at $35\text{-}40^\circ\text{C}$ to a thick and dark brown material (212.74 g), the crude extract of *V. negundo* (Vn.Cr). The approximate yield was 21.274%. Vn.Cr was solubilized in normal saline (0.9% w/v) and distilled water for *in vivo* and *in vitro* experiments, respectively.

Preliminary phytochemical analysis

Crude extract of *V. negundo* 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).

Drugs and standards

The following reference chemicals were obtained from the sources specified: Loperamide hydrochloride, acetylcholine chloride, verapamil hydrochloride, potassium chloride (Sigma Chemical Company, USA) and castor oil (Karachi Chemical Industries, Pakistan). All chemicals used were of the highest purity grade. 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.

Animals

Experiments performed complied with the rulings of the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council (National Research Council, 1996). 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 under controlled environment ($23\text{-}25^\circ\text{C}$). Animals were given tap water *ad libitum* and a standard diet.

Castor oil-induced diarrhea

The *in vivo* antidiarrheal activity of the extract was conducted following the methods previously described

(Awouters et al., 1978; Jebunnessa et al., 2009; Shah et al., 2011a). In the present study Balb^c albino mice were fasted for 18 h. 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 blotting sheet. The first group received saline (10 mL/kg, p.o.) as the vehicle control and so acted as the negative control. The doses of the crude extract of *Vitex negundo* were selected on a trial basis and administered orally (100, 300 and 1000 mg/kg) by intra-gastric feeding needle to three groups of animals. The fifth group received loperamide (10 mg/kg) orally, 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 later the castor oil challenge, the presence of diarrheal droppings was noted in 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.

Isolated tissue preparations

The isolated tissue experiments were carried out as previously described (Gilani et al., 2005; Shah et al., 2011b). 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 Tyrode's solution maintained at 37°C and aerated with a mixture of 5% carbon dioxide in oxygen (carbogen). The composition of Tyrode's, 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 ($0.3 \mu\text{M}$) 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 testing the relaxant (spasmolytic) activity directly without the use of an agonist (Gilani et al., 2005).

Determination of calcium antagonist activity

To assess whether the spasmolytic activity of the test substances was mediated through calcium channel blockade, high concentration of K^+ (80 mM), as KCl, 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 K^+ , was expressed as percent of the control pre-contraction.

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 91.04, MgCl₂ 1.05, NaHCO₃ 11.90, NaH₂PO₄ 0.42, glucose 5.55 and EDTA 0.1 mM. Following an incubation period of 30 min, control concentration-response curves (CRCs) of CaCl₂ were obtained. When the control CRCs of CaCl₂ 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 CRCs of CaCl₂ were reconstructed in the presence of different concentrations of the test material.

Acute toxicity test

The acute toxicity studies were carried out, as described earlier (Gilani, 1991). Animals were divided in groups of 5 mice each. The test was performed using increasing doses of the plant extracts, given orally, in 10 mL/kg volume to different groups serving as test groups. Another group of mice was administered saline (10 mL/kg, p.o.) as negative control. The mice were allowed food *ad libitum* and kept under regular observation for 6 h and the lethality was recorded after 24 h. Toxic effects as gastrointestinal spasms, anorexia, diarrhea and lethargy were also noticed.

Statistics

All the data expressed are mean \pm standard error of the mean (SEM), and the median effective concentrations (EC₅₀ 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

Based on the medicinal use of *V. negundo* in hyperactive gut disorders, such as diarrhea and spasm (Baquar, 1989; Evans, 2006; Shinwari, et al., 2003; Usmanghani, et al., 1997), its aqueous-methanolic crude extract was tested for the possible antidiarrheal effect in mice. When tested against the castor oil-induced diarrhea in mice, the crude extract of *V. negundo* (Vn.Cr), like loperamide, a standard antidiarrheal agent (Reynolds et al., 1984), significantly ($p < 0.05$) inhibited the frequency of defaecation as well as wetting of faeces when compared with untreated group. The crude extract and loperamide reduced greatly the wetness of the faecal droppings and provided around 53-71% and 93.33% protection, respectively. The induction of diarrhea by castor oil results from the action of ricinoleic acid formed in the hydrolysis of the oil (Iwao and Terada,

1962), which produces changes in the transport of water and electrolytes resulting in a hypersecretory response and generation of giant contraction of the intestine (Crocì et al., 1997). Thus a potential antidiarrheal agent may exhibit its antidiarrheal effect by inhibiting gut motility and/or diarrheal droppings (Crocì et al., 1997). The protective effect of the crude extract of *V. negundo* against the castor oil-induced diarrhea in mice, similar to loperamide, suggests that it has either inhibitory effect on contraction or electrolyte out flux. To see its possible inhibitory effect on gut motility, the Vn.Cr was further studied in the *in-vitro* experiments.

Spontaneously beating isolated rabbit jejunum preparation is used to test possible inhibitory (spasmolytic) effect of test substances without use of a spasmogen (Gilani *et al.*, 2005). When tested in isolated rabbit jejunum preparations, cumulative addition of the Vn.Cr, diltiazem and loperamide, caused concentration-dependent inhibition of the spontaneous contractions (Figure 1), with respective EC₅₀ values of 0.36 mg/mL (0.22- 0.61), 2.62 μ M (0.57-11.95; n=5) and 25.80 μ M (18.80-29.33; n=4) (Figure 2), thus, showing smooth muscle relaxant activity. The contraction of smooth muscle preparations, including rabbit jejunum, is dependent upon an increase in the cytoplasmic free [Ca⁺⁺], which activates the contractile elements (Karaki and Weiss, 1983). The increase in intracellular Ca⁺⁺ occurs either via influx through voltage-dependant Ca⁺⁺ channels (VDCs) or its release from intracellular stores. Periodic depolarization and repolarization regulates the spontaneous movements of the intestine and at the height of depolarization, the action potential appears as a rapid influx of Ca⁺⁺ via VDCs (Brading, 1981). Thus the inhibitory effect of the Vn.Cr on spontaneous movements of rabbit jejunum may appear to be due to CCB effect mediated, possibly, through interference of Ca⁺⁺ influx through VDCs.

To confirm the involvement of Ca⁺⁺ channels in its spasmolytic effect, a high concentration of K⁺ (80 mM) was maintained to depolarize the tissue. The Vn.Cr was then added in a cumulative way, where it caused a concentration-dependent relaxation of the induced contractions with an EC₅₀ value of 1.30 mg/mL (0.27- 5.89; n= 4-5) (Figure 2A), similar to diltiazem (Figure 2B), suggesting that the spasmolytic effect is possibly mediated through CCB. Loperamide also caused concentration-related inhibitory effect against high K⁺-induced contractions with EC₅₀ value of 8.55 μ M (5.80-12.60), as shown in Figure 2C. The crude extract was more potent against K⁺-induced contractions, similar to diltiazem, a typical characteristic of CCB (Godfraind et al., 1986), thus suggesting that Vn.Cr mediates its spasmolytic effect through CCB.

The contractions induced by high K⁺ (>30 mM) are dependent on the entry of Ca⁺⁺ into the cells through VDCs (Bolton, 1979) and a substance which can inhibit

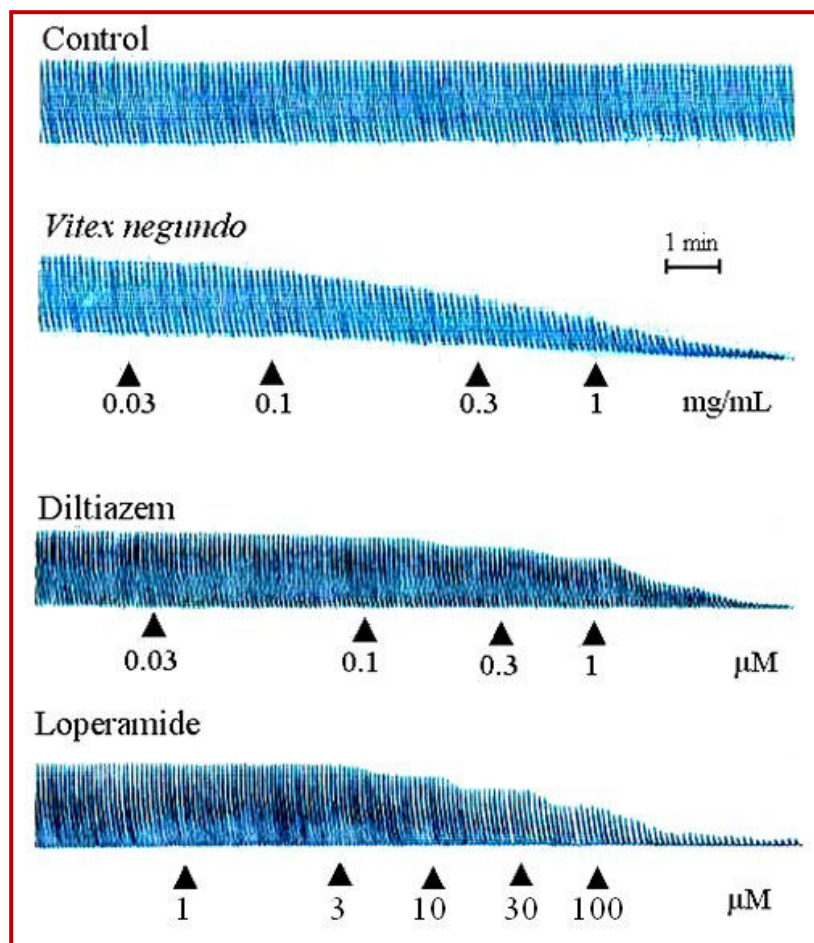


Figure 1: A typical tracing shows the spasmolytic effect of the crude extract of *Vitex negundo*, diltiazem and loperamide on spontaneous contractions in isolated rabbit jejunum preparations

high K^+ -induced contractions is therefore, possibly considered to be a CCB (Godfraind et al., 1986). Thus, the inhibition of high K^+ -induced contractions of rabbit jejunum by Vn.Cr may reflect the restricted Ca^{++} entry via VDCs. This hypothesis was further strengthened when pre-treatment of the tissues with Vn.Cr (0.03-0.3 mg/mL) caused a rightward shift in the $CaCl_2$ CRCs (Figure 2D), similar to diltiazem (Figure 2E). Pretreatment of the tissues with loperamide also caused rightward shift in the $CaCl_2$ CRCs (Figure 2F), which is in accordance to its known CCB effect at antidiarrheal doses (Reynolds et al., 1984). These data indicate that crude extract of *V. negundo* possesses Ca^{++} channel blocking effect similar to diltiazem. These findings provide pharmacological rationale to the antidiarrheal and antispasmodic effects of *V. negundo* because CCBs are considered useful in diarrhea and gut spasms (Brunton, 1996). The presence of flavonoids, and tannins, revealed by preliminary phytochemical analysis, support the CCB effect of the plant extract because plant derived flavonoids (Zhu et al., 1997) and tannins (Zhu et al., 2005) have been found to possess CCB effect,

which might be the active candidate(s) responsible for its medicinal use in diarrhea and gut spasm, though additional mechanisms cannot be ruled out.

In acute toxicity test the crude extract was found safe up to the dose of 10 g/kg, indicates safety of the plant, though safety profile for chronic use needs further studies.

Conclusion

The crude extract of *V. negundo* possesses antidiarrheal and anti-spasmodic effects mediated through calcium channel blockade and provides possible pharmacological base to its medicinal use in hyperactive gut disorders, such as diarrhea and gut spasms.

Acknowledgement

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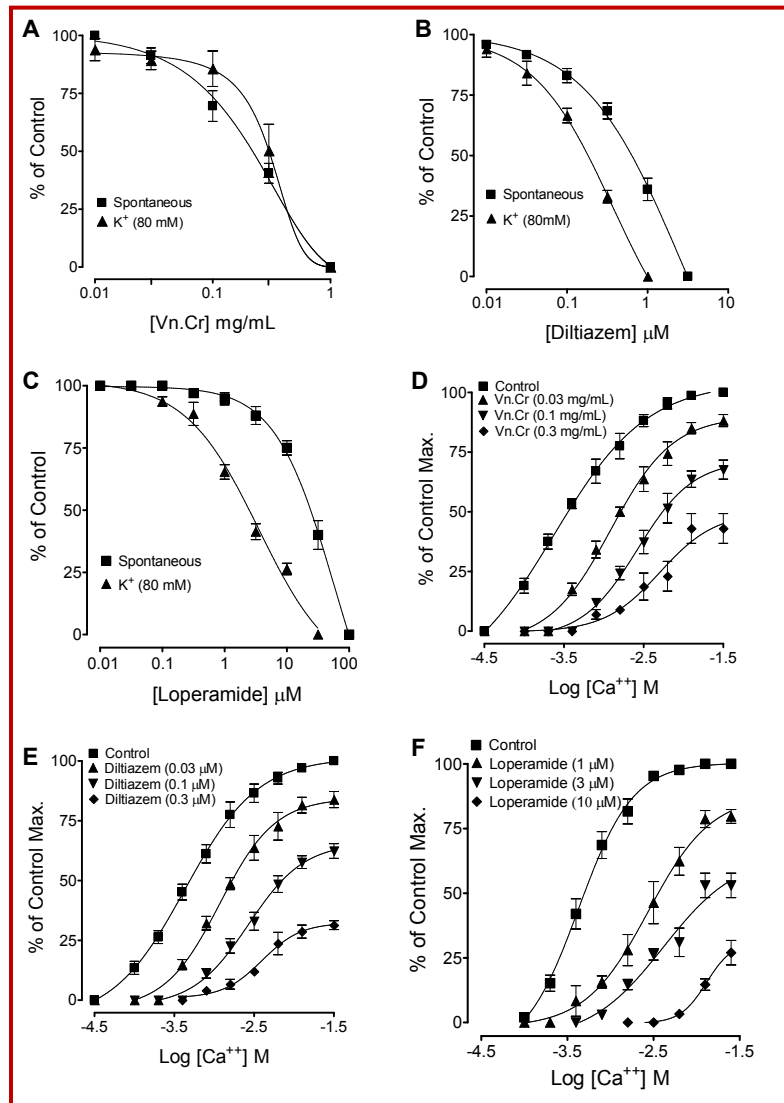


Figure 2: Concentration-response curves of; (A) the crude extract of *Vitex negundo* (Vn.Cr), (B) diltiazem and (C) loperamide on spontaneous and high K⁺ (80 mM)-induced contractions. Figures D, E and F depict the effect on CaCl₂ concentration-response curves in the absence and presence of different concentrations of the Vn.Cr, diltiazem and loperamide, respectively, in isolated rabbit jejunum preparations. Values shown are \pm SEM (n= 4-6)

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