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Prokinetic and laxative effects of the crude methanolic extract of *Viola betonicifolia* whole plant

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Abstract

Background: The present study was aimed to provide ethnopharmacological basis for the medicinal use of *Viola betonicifolia* whole plant in indigestion and constipation.

Methods: Mice were used in the *in-vivo* prokinetic and laxative studies while *in-vitro* experiments were conducted on isolated tissues of rabbit and guinea-pig gut preparations suspended in tissue bath to measure isotonic contractions.

Results: The crude methanolic extract of *Viola betonicifolia* (VBME) showed partially atropine-sensitive prokinetic (50 and 100 mg/kg) and laxative (30 and 100 mg/kg) activities in mice. When tested in isolated rabbit jejunum and guinea-pig ileum, VBME caused dose-dependent contractions at 0.01-0.3 mg/mL and 0.03-5 mg/mL, respectively. The spasmogenic effect was partially sensitive to atropine, while the presence of pyrilamine, SB203186 or hexamethonium had no effect in both gut preparations. The spasmodic effect of VBME was more efficacious in guinea-pig ileum than rabbit jejunum preparation. The phytochemical analysis of the crude methanolic extract for total alkaloids and saponins revealed that the VBME is a rich source of alkaloids and saponins.

Conclusions: This study showed the prokinetic and laxative effects of *Viola betonicifolia* in mice, partially mediated through muscarinic receptor activation. The *in-vitro* spasmodic effect of the plant extract was also partially sensitive to atropine indicating more than one mechanism in the gut stimulant effect. This study provides a rationale for the medicinal use of *Viola betonicifolia* in indigestion and constipation.

Keywords: *Viola betonicifolia*; prokinetic; laxative; cholinergic; jejunum; ileum
Background

*Viola betonicifolia* belongs to family Violaceae locally known as banafsha. It is found naturally in various countries of the world like Pakistan, India, Nepal, Srilanka, China, Malaysia and Australia. In Pakistan, it is available in Swat, Hazara and district Dir. The folk use of this plant is purgative, antipyretic, astringent, diaphoretic and anticancer. It has been used in the treatment of various neurological disorders including epilepsy and insomnia [1]. Additionally, it has been used in the treatment of sinusitis, skin and blood disorders and pharyngitis [2]. Roots are used for kidney diseases, pneumonia and bronchitis. Flowers are recommended for the treatment of cough and colds while leaves are useful for the treatment of boils [3].

Recently we have tested the crude methanolic extract as well as the subsequent solvent fractions of *V. betonicifolia* for various pharmacological activities [4-6]. Regarding the phytochemical study, the VBME contained saponins, flavonoids, tannins, proteins, and phenolic compounds [4, 6]. The current study was designed to provide scientific evidence to the ethnobotanical uses of the plant in the treatment of constipation and indigestion using various pharmacological models.

Method

**Preparation of the crude extract**

Whole plant of *V. betonicifolia* was collected from Swat, Khyber Pakhtunkhwa, Pakistan, in April 2010. Plant specimen was identified by Taxonomist, Department of Botany, University of Peshawar and a specimen was deposited there in the herbarium with voucher number 6410/Bot. The collected whole plant (12 kg) was air dried and powdered. The powder was extracted by maceration with methanol at room temperature for 14 days with occasional shaking. The
methanolic extract was filtered and concentrated under vacuum using rotary evaporator at low
temperature (45 °C) to a thick and dark brown crude extract (VBME). The approximate yield
was 22 % w/w. The methanolic extract was completely soluble in normal saline (0.9% w/v) and
distilled water for the in-vivo and in-vitro experiments, respectively.

**Chemical and reagents**

Acetylcholine perchlorate (ACh), atropine sulphate, carbamylcholine (CCh), histamine
hydrochloride, 5-hydroxytryptamine (5-HT), pyrilamine maleate and hexamethonium chloride
were purchased from Sigma-Aldrich Chemicals Company (St Louis, MO, USA). SB203186 (1-
piperidinylethyl-1H-indole-3-carboxylate) was purchased from Tocris (Ballwin, MO, USA).
Chemicals used for making physiological salt solutions including potassium chloride, calcium
chloride, glucose, magnesium chloride, magnesium sulfate, potassium dihydrogen phosphate,
sodium bicarbonate, sodium dihydrogen phosphate and sodium chloride were obtained from
Merck (Darmstadt, Germany). All chemicals used were of the analytical grade available and
solubilized in distilled water.

**Animals**

BALB/c mice (weighing 20–25 g), guinea-pigs (weighing 400–600 g) and local bred rabbits
(weighing 1-1.5 kg) of either sex, were housed at the animal house of the Aga Khan University
under a controlled environment (23–25 °C). The animals were kept in their respective cages with
sawdust (changed after every 48 h) and were fasted according to the protocols of the study. In
routine, they were given tap water ad libitum and a standard diet consisting of (g/kg): flour 380,
fiber 380, molasses 12, NaCl 5.8, nutrivet L 2.5, potassium metabisulfate 1.2, vegetable oil
38, fish meal 170 and powdered milk 150. The experimental protocols were approved by the
ethical committee of the Department of Pharmacy, University of Peshawar, Peshawar, Pakistan and all the experiments were performed in compliance with the rulings of the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council [7].

**Phytochemical screening**

**Total saponins content**

Saponin contents were determined for the crude extract according to documented method [8]. Briefly, 2 g of test samples were taken in a beaker and 50 mL of petroleum ether was added and heated gently on water-bath to 40°C for 5 min with regular shaking. The petroleum ether was filtered and repeated the operation twice with further 50 mL of pet ether. The marc obtained was extracted with 4 × 60 mL of methanol on gentle heating. The methanol layer was concentrated to approximately 25 mL on water-bath and 150 mL of dry acetone was added to precipitate the saponins, which was followed by filtration and drying in oven at 100°C for constant weight.

**Total alkaloid contents**

The total alkaloid contents of the crude extract of *Viola betonicifolia* was estimated by using method developed previously [8]. Briefly, 2 g of each was defatted by extraction with petroleum ether, heated gently on water- bath to 40°C for 5 min with regular shaking. The marc obtained was acidified with 100 mL of 20% acetic acid in ethanol and allowed to extract for 4 h. The resulting solution was filtered, concentrated and then basified with concentrated ammonium hydroxide to pH 9 followed by precipitation. The final weight of precipitated mass was designated as the total alkaloid contents.
**In-vivo experiments**

**Charcoal meal GI transit test**

The mice were divided into various groups (n=6). The group treated with saline (10 ml/kg) was served as negative control, while the group treated with CCh (1 mg/kg) serves as positive control. The remaining groups were treated with VBME (50 and 100 mg/kg, orally, p.o.). After 15 min of treatment, each animal received 0.3 mL of charcoal meal in the form of suspension in distilled water containing 10% gum acacia and 10% vegetable charcoal. After 30 min of the above treatments animals were killed through cervical dislocation and the whole small intestine was removed. The distance travelled by charcoal was measured and the percent movement was calculated. In order to assess the involvement of acetylcholine like prokinetic effect of the extract and CCh, some groups were treated with atropine (10 mg/kg, i.p.) 15 min prior to the administration of the VBME or CCh [9].

**Laxative activity test**

In accordance with the previous method [10], mice fasted for 6 h before the experiment were placed individually in cages lined with clean filter paper. The animals were divided into 7 groups (n=6); the first group acting as the negative control and administered saline (10 mL/kg, p.o.), while the next group received CCh (1 mg/kg, p.o), which served as the positive control. The third and fourth groups received orally, 30 and 100 mg/kg of VBME, respectively. To determine the mechanism underlying its laxative effect, separate sets of mice (group # 5, 6 and 7) were pretreated with atropine (10 mg/kg, i.p.) 1 h before administration of the extract or CCh. After 18 h, the feces production (total number of feces and total number of wet feces per group) in all
animals was counted, and the percentage increase in wet feces relative to that of total fecal output was recorded, which was considered as the laxative effect [11].

**In-vitro experiments**

By following the previously described methods in earlier studies [12], isolated gut preparations of rabbit jejunum and guinea-pig ileum were obtained subsequent to cervical dislocation of the animals; the abdomen was cut opened, required tissues were isolated out. Tissue preparations jejunum or ileum of 2-3 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 and 95% oxygen (carbogen). The composition of Tyrode’s solution (mM) was KCl 2.68, NaCl 136.9, MgCl₂ 1.05, NaHCO₃ 11.90, NaH₂PO₄ 0.42, CaCl₂ 1.8, and glucose 5.55 (pH 7.4). A preload of 1 g was applied to each tissue, and the contractile responses were recorded using isotonic transducer 50-6360 (Harvard Apparatus, Holliston, MA, USA) coupled with either a student oscillograph (Harvard Apparatus) or PowerLab (ML-845) data acquisition system (AD Instruments; Sydney, Australia) and a computer using chart software (version 5.3). The tissues were allowed to equilibrate for a period of 30 min, and then stabilized with sub-maximal concentration of acetylcholine (ACh, 0.3 µM). The tissues were presumed stable only after the reproducibility of the said responses. The VBME was examined later for any spasmodic activity on jejunum and ileum preparations at concentrations ranged from 0.01 to 5.0 mg/mL.

**Statistical analysis**

All the data expressed are mean ± standard error of mean (S.E.M., n = number of experiments) and the median effective concentrations (EC₅₀ values) with 95% confidence intervals (CI). One way analysis of variance (ANOVA) followed by Dunnett’s test or unpaired t-test was used to assess the laxative activity, while one-way ANOVA followed by Tukey’s test was employed for
the effect of plant extract in charcoal meal transit. The concentration-response curves (CRCs) were analyzed by non-linear regression and two-way ANOVA followed by Bonferroni’s post-test correction or unpaired t-test was used for multiple comparisons of CRCs with the respective control. All the graphing, calculations and statistical analysis were performed using GraphPad Prism 4 for windows (GraphPad Software, San Diego, California, USA).

**Results**

**Phytochemical screening**

**Total saponins**

The crude methanolic extract was found a rich source of alkaloid contents (7.45 mg/g).

**Total alkaloids**

Profound total saponins contents (7.23 mg/g) were found in crude methanolic extract.

**In-vivo findings**

**Effect of VBME on charcoal meal**

VBME exhibited a dose-dependent increase in the propulsive movement of charcoal meal as shown in Figure 1. The percent movement of charcoal meal through the small intestine after treatment with VBME was 58.33 and 79.66 at the test doses of 50 and 100 mg/kg respectively, while in saline and CCh treated groups the charcoal meal travelled 51.66 and 92.67 % respectively. When the effects of VBME and CCh were restudied for their influence on transit of charcoal meal in mice pretreated with atropine, all the excitatory effects were markedly reduced.

**Laxative activity**
VBME treatment produced 61.16 ± 2.18 % and 86.83 ± 4.8 % (n=6) wet feces in mice at 30 and 100 mg/kg, respectively. The positive control, CCh (1 mg/kg) produced 88.16 ± 3.07% wet feces, while the saline treated group did not form any wet feces. When VBME (30 and 100 mg/kg) was studied for its positive influence on wet feces in mice pretreated with atropine, the effect declined to 36.6 ± 5.2 % and 46.3 ± 11.7 %, respectively; further details are shown in Table 1.

**In-vitro findings**

**Effect of VBME on rabbit and guinea-pig gut preparations**

In rabbit jejunum, VBME caused a concentration-dependent stimulant effect at 0.01-0.3 mg/mL, reaching to the maximum effect of 67.33± 2.67% (mean ± S.E.M; n=5) of ACh maximum. Pretreatment of the tissue with atropine (0.1 µM) significantly (p < 0.001) blocked the spasmogenic effect of VBME with remaining maximum spasmodic effect of 23.33 ± 2.40% (mean ± S.E.M; n=4), while the presence of hexamethonium, pyrilamine or SB203186 did not alter (P > 0.05) its effect (Figure 2 A).

When tested in guinea-pig ileum, VBME (0.03-5 mg/mL) exhibited a strong spasmodic effect reaching its highest 96.66 ± 3.33% (mean ± S.E.M; n=5) of ACh maximum. The efficacy of the contractile effect of VBME in guinea-pig ileum was found higher than observed in rabbit jejunum preparations. When the spasmogenic effect of VBME was redetermined in the presence of different antagonists, it was partially blocked in the presence of atropine with remaining maximum spasmodic effect of 28.66 ± 2.90% (mean ± S.E.M; n=5), while remained unchanged (P > 0.05) in the presence of hexamethonium, pyrilamine or SB203186 (Figure 2 B) like that observed in rabbit jejunum.

**Discussion**
Keeping in view the medicinal use of *Viola betonicifolia* in gut disorders, such as indigestion and constipation, its crude extract (VBME) was tested in mice, where it propelled charcoal meal through the small intestine and increased the production of wet feces, hence showing prokinetic and laxative activities, similar to the effect of carbachol, a standard cholinergic agonist and accelerator of intestinal contents (Brown and Taylor, 2006). These gut stimulatory actions of the extract were found partially sensitive to atropine, a muscarinic receptor blocker [13], indicating the presence of some ACh-like component(s) in addition to other gut stimulant constituent(s). ACh is a neurotransmitter of the parasympathetic nervous system and is known to cause gastrointestinal stimulation through the activation of muscarinic receptors [14], hence, the presence of ACh-like constituents explains its medicinal use in constipation and as digestive aid.

To further study the possible mode of the observed prokinetic and laxative properties of the extract, VBME was tested in isolated jejunum and ileum preparations from rabbit and guinea-pig respectively. VBME showed dose-dependent gut stimulant effect, partially sensitive to atropine in both preparations, thus, showing a common mechanism of muscarinic receptor activation along with some additional spasmodic components. However, the efficacy of gut stimulant effect was more in guinea-pig ileum than rabbit jejunum. When further experiments were conducted to know the unknown additional spasmodic components other than cholinergic, gut preparations were restudied in the presence of pyrilamine, a histaminic type-1 (H₁) receptor blocker [15], hexamethonium, a ganglion blocker [16] or SB203186, a serotonergic (5-HT) receptor antagonist [17]. VBME showed complete resistance and clearly suggesting some additional mechanism(s), independent of histamine, nicotine or 5-Hydroxytryptamine (5-HT, serotonin) receptors activation. Other mechanisms known for their gut stimulant property, which have not been ruled out in this study include certain prostaglandins [18], platelet activating factor [19], nitric-oxide-
donating or releasing compounds [20], dopaminergic antagonists [21], cholecystokinin [21] and tachykinins[22].

Recently we have reported the anthelmintic and nematicidal properties of the VBME and its subsequent solvent fractions [4, 23]. It is very interesting that VBME is anthelmintic and laxative at the same time because the laxative and prokinetic property of the test extract will be helpful in the expulsion of worms or nematodes from gut. Regarding the preliminary phytochemical study, the VBME is a rich source of alkaloids, flavonoids, phenolic compounds and saponins [6], while in case of quantitative phytochemical profile of the plant, it is clear from results that VBME is a rich source of alkaloids and saponins. The presence of alkaloids and saponins as the plant constituents, which are known to possess gut stimulatory properties [24-25], may explain the gut stimulant actions of the plant extract, though further studies are required to know the specific chemical(s) responsible for the tested biological activities.

**Conclusion**

This study shows that the prokinetic, laxative and spasmodic activities of *Viola betonicifolia* are partially mediated through muscarinic receptors along with some unknown additional mechanism(s) which are further confirmed by *in-vitro* experiments using rabbit jejunum and guinea-pig ileum preparations. Thus, this study provides sound mechanistic basis for the medicinal use of *Viola betonicifolia* in GI disorders, such as indigestion and constipation.

**List of abbreviation**

VBME: *Viola betonicifolia* methanolic extract

ACh: Acetylcholine

CCh: Carbamylcholine
5-HT: 5-hydroxytryptamine

i.p.: Intraperitoneal

p.o. per oral

GI: Gastrointestinal

CRCs: Concentration-response curves

S.E.M: Standard error of means

**Conflict of Interest**

The authors declare that they have no conflicts of interest.

**Author's contributions**

NM and NR: Performed experimental work

HK: Manuscript writing

MS: Project supervisor

AHG: Project supervisor and technical expertise

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References


Legend to Table and figures

**Table 1.** Effect of atropine on the laxative activity of crude extract of *Viola betonicifolia* (VBME) in mice.

**Figure 1.** Bar diagram showing the dose-dependent effect of crude methanolic extract of *Viola betonicifolia* (VBME) on the travel of charcoal meal through small intestine of mice, in the absence and presence of atropine. One-way ANOVA followed by Tukey’s test. *P* < 0.05, **P** < 0.01 and ***P** < 0.001.

**Figure 2.** The stimulatory effects of the crude methanolic extract of *Viola betonicifolia* (VBME) without and with atropine (0.1 µM), pyrilamine (1 µM), hexamethonium (0.3 mM) and SB203186 (1 µM) in (A) isolated jejunum and (B) guinea-pig ileum preparations. The values shown are mean ± S.E.M of 4-7 individual experiments. *P* < 0.05, **P** < 0.01 and ***P** < 0.001 (Two-way ANOVA, followed by bonferroni post-test correction or unpaired t-test).
Table 1. Effect of atropine on the laxative activity of the crude methanolic extract of Viola betonicifolia (VBME) in mice.

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>defecation/group</th>
<th>number of wet feces/group</th>
<th>% of wet feces</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Saline (p.o., mL/kg)</td>
<td>10</td>
<td>3 ± 0.36</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>Carbachol (p.o.)</td>
<td>1</td>
<td>11.5 ± 0.5**</td>
<td>10.1 ± 0.54*** ±</td>
<td>88.16 ± 3.07</td>
</tr>
<tr>
<td>3</td>
<td>VBME (p.o.)</td>
<td>30</td>
<td>8.3 ± 0.88**</td>
<td>5.1 ± 0.9*</td>
<td>61.16 ± 2.18</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>100</td>
<td>11.1 ± 1.3**</td>
<td>9.5 ± 71**</td>
<td>86.83 ± 4.8</td>
</tr>
<tr>
<td>5</td>
<td>Carbachol + Atropine (i.p.)</td>
<td>1 + 10</td>
<td>4 ± 0.5**</td>
<td>0.83 ± 0.3***</td>
<td>22.5 ± 10.1</td>
</tr>
<tr>
<td>6</td>
<td>VBME (p.o.) + Atropine</td>
<td>30 + 10</td>
<td>5.3 ± 0.9*</td>
<td>1.83 ± 0.3***</td>
<td>36.6 ± 5.2</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>100 + 10</td>
<td>4.6 ± 0.6**</td>
<td>2 ± 0.36***</td>
<td>46.3 ± 11.7</td>
</tr>
</tbody>
</table>

Values shown are mean ± S.E.M, n=6. *P< 0.05, **P< 0.01 and ***P< 0.001 show a comparison of group # 2,3 and 4 vs. group # 1 (One-way ANOVA followed by Dunnett’s test), group # 5 vs. group # 2, group # 6 vs. group # 3 and group # 7 vs. group # 4 (unpaired t- test).