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ORIGINAL PAPER

Evaluation of *n*-hexane extract of *Viola betonicifolia* for its neuropharmacological properties

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Abstract *Viola betonicifolia* (whole plant) has been used as a sedative and in various nervous disorders in Pakistani traditional medicines. The *n*-hexane extract of the whole plant of *V. betonicifolia* (HEVB) was investigated for neuropharmacological properties such as anxiolytic, muscle relaxant, sleep induction, antidepressant and sedative to ascertain its folk use. Anxiolytic activity was tested using the staircase test, while the muscle relaxing property of the extract was tested in various muscle relaxant paradigms, i.e. chimney test, traction test, rota rod and inclined plane. In anxiolytic and muscle relaxant tests, HEVB (0.3, 0.4 and 0.5 g/kg, i.p.), diazepam (1 mg/kg, i.p.) or distilled water (10 ml/kg i.p.) were administered 30, 60 and 90 min before performing the tests in mice. HEVB was also screened for a sleep-inducing effect. The antidepressant activity was determined by using the forced swimming test (FST), while line crossing in a special box was used for locomotor activity. HEVB showed a significant ($P < 0.05$) dose-dependent anxiolytic action in the staircase test. In muscle relaxant paradigms, a dose-dependent muscle relaxation was observed. For the phenobarbitone sleep induction test, HEVB notably ($P < 0.05$) reduced the latency time and increased the total sleeping duration. However, HEVB was devoid of any antidepressant activity, while the movements of mice were reduced significantly ($P < 0.05$) in locomotor activity. The results suggest that HEVB has anxiolytic, muscle relaxant, sleep-inducing (sedative) activity and, thus, provides pharmacological justification for the use of this plant as a sedative and for the relief of various nervous disorders.

Keywords *Viola betonicifolia* · Anxiolytic · Muscle relaxant and sleep induction

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Introduction

Viola betonicifolia belongs to the family Violaceace. Locally, it is known as 'banafsha'. It is a perennial herb of 8–20 cm in height. The stem of the plant is absent and its leaves are triangular or obtuse, and the petiole is longer than the lamina. The roots are slender, unbranched and the rhizome is short. *V. betonicifolia* is available in various countries of the world, such as Pakistan, India, Nepal, Sri Lanka, China, Malaysia and Australia [1]. In Pakistan, it is available in the Swat, Hazara and Dir districts. In the Pakistani traditional medicines system, it is used as an antipyretic, sedative, astringent, diaphoretic, anticancer and purgative; it is also used for the treatment of epilepsy and nervous disorders [2]. It is also recommended for the treatment of sinusitis, skin and blood disorders and in pharyngitis [3]. Roots are used to cure kidney diseases, pneumonia and bronchitis. Flowers are used in asthma, cough and cold, while leaves are useful for boils [4]. In

continuation of our research on Pakistani medicinal plants [5–8], we have recently tested various solvent fractions of the whole plant for different in vitro pharmacological activities [9], and, in this paper, we report on the in vivo neuropharmacological profile of *n*-hexane extract of the whole plant of *V. betonicifolia* (HEVB).

Materials and methods

Plant material

Whole plant of *V. betonicifolia* was collected from Swat, Khyber Pakhtunkhwa, Pakistan, in April 2010. The plant specimen was identified by the Taxonomy Section, Department of Botany, University of Peshawar, and a specimen was deposited there in the herbarium under Voucher No. 6410/Bot.

Extract preparation

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 [9]. The methanolic extract was filtered and concentrated under vacuum at low temperature (45°C). The resulting crude methanolic extract (22%) was further fractionated into various solvent fractions, such as *n*-hexane, chloroform, ethyl acetate, butanol and aqueous fractions. The *n*-hexane fraction was used for the investigation of various neuropharmacological activities.

Animals used

BALB/c mice of either sex (20–25 g) were used in all experiments. Animals were purchased from the Pharmacology Section, Department of Pharmacy, University of Peshawar, Peshawar. The animals were maintained in standard laboratory conditions (25°C and light/dark cycles, i.e. 12/12 h) and fed with standard food and water ad libitum. The experimental protocols were approved by the ethical committee of the department.

Acute toxicity test

The acute toxicity test for HEVB was carried out to evaluate any possible toxicity. BALB/c mice ($n = 6$) of either sex were tested by administering different doses (500, 1,000 and 2,000 mg/kg) of HEVB, while the control group received distilled water (10 ml/kg). All the groups were observed for any gross effect for the first 4 h and then mortality was observed after 24 h [10].

Anxiolytic activity

Staircase test

The staircase test was carried out following the available protocol [11], with slight modifications. A stair of five identical steps having dimensions 2.5 × 10 × 7.5 cm were placed on an elevated surface. The animals were divided into control, standard and treated groups, consisting of six mice in each group. The control group was treated with distilled water (10 ml/kg), the standard group was treated with diazepam (1 mg/kg) and the remaining groups were treated with HEVB 0.3, 0.4 and 0.5 g/kg body weight. After 30 min of treatment, the animals were placed on the first step of the elevated stairs and each animal was observed for the number of steps climbed up and the number of rearings over a period of 3 min. A step was considered to be climbed only if the mouse had placed all four paws on the step. The stairs were cleaned from faeces and urine after each mouse performance [11].

Muscle relaxant test

Rota rod

The rota rod used in this test was a metallic rod (3 cm in diameter) coated with rubber and connected to a motor. The rod was rotated at a constant speed, i.e. 9 rpm, and was placed about 60 cm above the table in order to prevent the mice from jumping off the roller. Mice were exposed to the rota rod as a pretest before the experiment and only those mice included in the study were those that remained on the rod for 5 min at a speed of 9 rpm. All the groups ($n = 6$) were treated (i.p.) with diazepam (1 mg/kg), distilled water (10 ml/kg) and HEVB (0.3, 0.4 and 0.5 g/kg) 30, 60 and 90 min before the exposure to the rota rod. Each mouse was exposed for 5 min on the revolving rod and the time spent on the rod was recorded [12].

Traction test

In this procedure, a metal wire coated with rubber was used, both ends of which were rigidly supported with stands about 60 cm above the laboratory bench. Different groups ($n = 6$) were treated with diazepam (1 mg/kg), distilled water (10 ml/kg) and HEVB (0.3, 0.4 and 0.5 g/kg). The animals were exposed to the traction test after 30, 60 and 90 min of treatment. Each animal was hung by their hind legs from the wire and the time of hanging was recorded for 5 s. Failure to hang for less than 5 s was considered as the presence of muscle relaxant activity and vice versa [13].

Chimney test

This test was performed according to a well-established method [14]. A Pyrex glass tube (30 cm long and 3.0 cm in diameter) was used in this test. The tube is marked at 20 cm from the base and all animals were screened after 30, 60 and 90 min of treatment. Different groups ($n = 6$) were treated with diazepam (1 mg/kg), distilled water (10 ml/kg) and HEVB (0.3, 0.4 and 0.5 g/kg). The animal was introduced at one end of the tube and allowed to move up to the mark at 20 cm from the base. When the animal reached the 20 cm mark, the tube was moved immediately to the vertical position and the animal tried to climb the tube with a backward movement. Mice which failed to reach the mark within 30 s were considered to have relaxed muscles.

Inclined plane

The plane used in this procedure consisted of two plywood boards. Both boards were connected with each other in such a way that one board formed the base and the other is fixed with the base at an angle of 65°. Different groups ($n = 6$) were treated with diazepam (1 mg/kg), distilled water (10 ml/kg) and HEVB (0.3, 0.4 and 0.5 g/kg). After 30, 60 and 90 min of treatment, the animals were placed on the upper part of the inclined plane for 30 s to hang or fall [15].

Phenobarbitone-induced sleeping time

Animals were divided into five groups ($n = 6$); the control group was treated with distilled water (10 ml/kg), the standard group was treated with diazepam (4 mg/kg) and the remaining three groups were treated with HEVB (0.3, 0.4 and 0.5 g/kg). After 30 min of treatment, all the animals were injected with phenobarbitone sodium 35 mg/kg (i.p.). Each animal was observed for the onset and duration of sleep. The duration of sleep or hypnosis was considered as the loss of postural reflexes [16].

Antidepressant activity

The antidepressant activity of the extract was evaluated using the forced swimming test (FST). All the mice were trained for swimming in a bath with dimensions (42 × 19 × 19 cm). The bath was filled with water (25 ± 2°C) up to a depth of 15 cm. On the day of experiment, animals were acclimatised with the laboratory environment, i.e. dim red light and soundproofed. Animals were divided into five groups ($n = 6$); control group, standard group (fluoxetine) and the remaining groups were treated with HEVB (0.3, 0.4 and 0.5 g/kg). After the above treatment, the animals were allowed to swim for 6 min, and the durations of immobility were recorded during the last 240 s of the swimming period [17].

Open field test

The apparatus used for this activity consisted of an area of white wood (150 cm in diameter) enclosed by stainless steel walls and divided into 19 squares by black lines. The open field was placed inside a light- and sound-attenuated room. BALB/c mice of either sex ($n = 6$) weighing 22 ± 2 g were used. Animals were acclimatised under red light (40-W red bulb) 1 h before the start of the experiment in the laboratory, with food and water available ad libitum. Animals were administered with distilled water and HEVB (0.3, 0.4 and 0.5 g/kg i.p.). After 30 min, each animal was placed in the centre of the box and the numbers of lines crossed were counted for each mouse [18, 19].

Statistical analysis

The results are expressed as the mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) was used for comparison tests of significant differences among groups, followed by Dunnett's multiple comparison post-test. A level of significance ($P < 0.05$ or 0.01) was considered for each test.

Results

Acute toxicity

No gross behaviour change or mortality was observed at the tested doses and, therefore, the extract is considered to be safe up to the dose of 2,000 mg/kg.

Anxiolytic effect

The number of steps and rearing were significantly ($P < 0.05$) decreased at 0.4 g/kg and 0.5 g/kg ($P < 0.01$). However, at a dose of 0.3 g/kg, the effect was not significant. Diazepam was the most significant ($P < 0.001$) anxiolytic, as shown in Table 1. The extract showed an anxiolytic effect similar to positive controls and was more significant than negative controls.

Muscle relaxant effects

Effect of the rota rod

The rota rod activity of all groups is shown in Fig. 1. The time spent on the revolving rod was significantly ($P < 0.05$) reduced by HEVB (0.4 and 0.5 g/kg) in comparison with

Table 1 Anxiolytic effect of *n*-hexane extract of the whole plant of *Viola betonicifolia* (HEVB) (staircase test)

Treatment	Dose (i.p.)	No. of steps	No. of rearings
Distilled water	10 ml/kg	22.45 ± 1.23	12.22 ± 2.23
Diazepam	1 mg/kg	4.12*** ± 0.60	2.13*** ± 0.30
HEVB	0.3 g/kg	18.21 ± 1.01	10.56 ± 0.54
	0.4 g/kg	10.55* ± 1.08	5.10* ± 1.89
	0.5 g/kg	8.78** ± 1.05	4.94** ± 1.03

Values represent the number of steps taken and number of rearings by animals in the staircase test 30 min after treatment with distilled water (10 ml/kg), HEVB (0.3, 0.4 and 0.5 g/kg) or diazepam (1 mg/kg). Data are presented as mean ± standard error of the mean (SEM) ($n = 6$)

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, all compared with controls

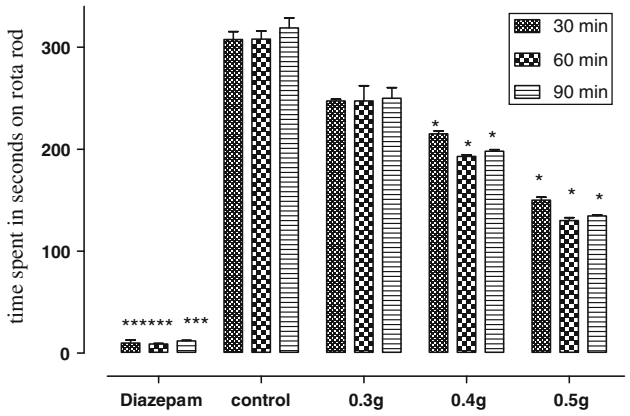

Fig. 1 Effect of *n*-hexane extract of the whole plant of *Viola betonicifolia* (HEVB) on muscle coordination on the rota rod. The *bars* represent the time spent in seconds on the rota rod after 30, 60 and 90 min of treatment with distilled water (10 ml/kg), HEVB (0.3, 0.4 and 0.5 g/kg) or diazepam (1 mg/kg). *$P < 0.05$ and ***$P < 0.001$

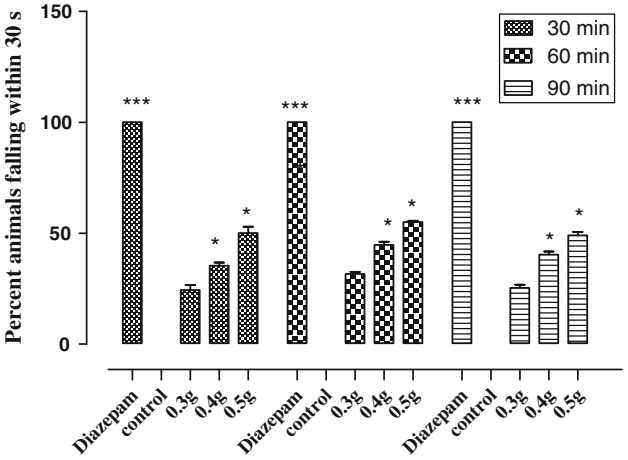

Fig. 2 Effect of HEVB on muscle coordination in the inclined plane. The *bars* represent the percent of time spent in seconds by which the mice slid off the inclined plane 30, 60 and 90 min after treatment with distilled water (10 ml/kg), HEVB (0.3, 0.4 and 0.5 g/kg) or diazepam (4 mg/kg). *$P < 0.05$ and ***$P < 0.001$, both with respect to the control group

the control group. The effect in the reduction of stay on the rota rod was not significant at the dose of 0.3 g/kg. Diazepam, which was the positive control, was more significant ($P < 0.001$) than the tested doses of HEVB.

Effect of the inclined plane

A significant ($P < 0.05$) number of animals fell down from the inclined plane after 30, 60 and 90 min of treatment with distilled water, diazepam or HEVB (0.5 and 0.4 g/kg). The effect of the extract was dose-dependent, as shown in Fig. 2. The maximum action was observed after 60 min. The number of animals which fell down in the case of HEVB were significant compared to the negative control, while the effect of the positive control group was significant compared to the tested extract.

Effect of the traction test

The percent negative effect in the traction test is shown in Table 2. The effect post-treatment was observed at 30, 60 and 90 min. The maximum effect was shown after 60 min. The effect was dose-dependent and the maximum activity was observed at 0.5 g/kg (80.76%), while the lowest effect was 14.11% at the dose of 0.3 g/kg. Significant ($P < 0.05$) activity was shown by 0.5 and 0.4 g/kg of extract in comparison with the control group.

Effect of the chimney test

A significant ($P < 0.05$) percent negative effect was observed by HEVB at the dose of 0.5 g/kg. The muscle relaxant activity was dose-dependent, as shown in Table 2. Effect at the dose of 0.3 g/kg was not significant, while the standard reference drug (diazepam) was the most significant ($P < 0.001$) as compared to the negative control group. The muscle coordination effect of the tested extract was not more than that of diazepam (1 mg/kg).

Table 2 Percent effect of HEVB on muscle relaxation (chimney test and traction test)

Group	Dose	Chimney test (%)			Traction test (%)		
		30 min	60 min	90 min	30 min	60 min	90 min
Distilled water	10 ml/kg	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
Diazepam	1 mg/kg	100** ± 0.00	100** ± 0.00	100** ± 0.00	100** ± 0.00	100** ± 0.00	100** ± 0.00
HEVB	0.3 g/kg	10.12 ± 0.88	14.11 ± 0.97	11.09 ± 2.11	3.54 ± 1.78	4.23 ± 2.23	2.34 ± 1.80
	0.4 g/kg	22.13 ± 1.67	23.56 ± 1.77	25.20 ± 1.06	10.02 ± 0.23	17.12.04 ± 1.76	8.04 ± 1.56
	0.5 g/kg	50.08* ± 1.21	55.16* ± 1.92	49.15* ± 1.01	35.05* ± 1.02	45.03* ± 0.98	37.07* ± 1.09

Values represent the percentages of mice ($n = 6$) showing negative effects in the chimney and traction tests 30, 60 and 90 min after treatment with distilled water (10 ml/kg), HEVB (0.3, 0.4 and 0.5 g/kg) or diazepam (1 mg/kg). Data presented as mean ± SEM ($n = 6$)

*$P < 0.05$ and **$P < 0.01$, both compared with controls

Table 3 Effect of HEVB on phenobarbitone-induced sleep in mice

Treatment	Dose (i.p.)	Onset of sleep (min)	Duration of sleeping (min)
Distilled water	10 ml/kg	25.12 ± 1.25	7.34 ± 2.28
Diazepam	4 mg/kg	5.45*** ± 0.08	56.45*** ± 0.00
HEVB	0.3 g/kg	30.45 ± 1.97	5.13 ± 0.99
	0.4 g/kg	13.79* ± 1.98	18.08* ± 0.76
	0.5 g/kg	9.08** ± 1.01	30.03** ± 1.98

Values represent the onset of sleep and duration of sleep (in min) after treatment with distilled water (10 ml/kg), HEVB (0.3, 0.4 and 0.5 g/kg) or diazepam (4 mg/kg) and then all groups were treated with phenobarbitone (35 mg/kg). Data presented as mean ± SEM ($n = 6$)

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, all compared with controls

Effect of the phenobarbitone-induced sleeping time

The effect of phenobarbitone sleep induction is shown in Table 3. HEVB (0.4 and 0.5 g/kg) significantly ($P < 0.05$) reduced the latency time and potentiated the duration of sleep in dose-dependent manners. The sleep-inducing effect produced in the diazepam-treated group was significant ($P < 0.001$) but the effect produced was less than the action produced by our tested extract.

Antidepressant activity

It is clear from Table 4 that no antidepressant activity was observed at all of the tested doses. The central nervous system (CNS) depressant activity was increased with an increase in the tested dose.

Open field test

The extract was a sedative in all of the applied doses and locomotion was decreased with increasing dose. The animals were almost immobile and were inactive at the dose of 0.5 g/kg. The other treatments also showed significant ($P < 0.05$) results. The number of lines crossed by the negative control group was greater than that of the extract-treated groups, while the number of lines crossed by extract-treated groups was greater than that of the diazepam-treated group, as shown in Table 5.

Table 4 Antidepressant effects of HEVB

Treatment	Dose	Immobility time (s)
Distilled water	10 ml/kg	110 ± 0.09
HEVB	0.3 mg	157 ± 1.07
	0.4 mg	206 ± 1.72
	0.5 mg	215 ± 0.93
Fluoxetine	15 mg	30.34 ± 0.00

Values represent the time of immobility (s) in the forced swimming bath for 6 min after treatment with distilled water (10 ml/kg), HEVB (0.3, 0.4 and 0.5 g/kg) or fluoxetine (15 mg/kg). Data presented as mean ± SEM ($n = 6$)

Table 5 Open field test of HEVB (locomotive activity)

Treatment	Dose	No. of lines crossed in 10 min
Distilled water	10 ml/kg	126 ± 1.23
HEVB	0.3 mg	85 ± 1.72
	0.4 mg	67 ± 2.17*
	0.5 mg	27 ± 1.09**
Diazepam	0.5 mg	5 ± 0.02***

Values represent the number of lines crossed by animals in the box 30 min after treatment with distilled water (10 ml/kg, control), HEVB (0.3, 0.4 and 0.5 g/kg) or diazepam (0.5 mg/kg). Data presented as mean ± SEM ($n = 6$)

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, all compared with controls

Discussion

In the present modern era, anxiety disorders are faced by a high percentage of the world population. The most widely prescribed medications for anxiety disorders are the

benzodiazepines. However, the clinical uses of benzodiazepines are limited due to their adverse effects, such as psychomotor impairment, potentiating other central depressant drugs and dependence liability [20]. Therefore, the search for new and safe medications having anxiolytic properties and being free of complications of benzodiazepines would be of great importance in the treatment of anxiety-related disorders. It is a common perception that natural products are safe. Therefore, *V. betonicifolia* plants were screened with the hope of finding safe and effective natural medicines.

The acute toxicity studies indicate that the plant extract is safe up to 2 g/kg. The staircase test model can be used for the assessment of anxiety (number of rearings) and sedation (number of steps). This test was originally for the investigation of anxiolytic effect in rats [21]. In this study, it was observed that the extract is very anxiolytic, like diazepam, at the dose of 0.4 and 0.5 g/kg, as the number of rearings and the number of steps were significantly decreased by test doses of the extract, therefore, it can be suggested that the extract is anxiolytic and sedative.

The muscle relaxant property of the extract was tested in the inclined plane, traction, climbing and chimney tests and compared to that produced by diazepam. In each case, the muscle relaxant effect was checked after 30, 60 and 90 min following extract administration. The maximum effect was noticed after 60 min following extract administration. In all the experimental models, a significant skeletal muscle relaxation was produced by higher doses of the extract (0.4 and 0.5 g/kg).

The reduction in latency time and the prolongation of sleep duration is an indicator for the centrally acting effect of the extract. The prolongation of barbiturate-induced sleeping time reflects the CNS depression-like activity, as reported in the literature [22]. A significant prolongation of the sleep duration was noticed with increasing dose of the extract, so HEVB is a rich source of sleep-inducing agents. The muscle relaxant, anxiolytic and sedative-hypnotic effects of benzodiazepines like diazepam are mostly attributed to enhance the action of gamma aminobutyric acid ($GABA_A$) [14]. Actually, benzodiazepines bind to the gamma sub-unit of the $GABA_A$ receptor, due to which a structural modification of the receptor results in an increase in $GABA_A$ receptor activity. Benzodiazepines do not substitute for GABA, which bind at the alpha sub-unit, but increase the frequency of channel opening events, which leads to an increase in chloride ion conductance and inhibition of the action potential [23, 24]. According to some researchers, the anxiolytic action of benzodiazepines may be due to the direct activation of glycine synapses in the brain [25]. This may explain the mechanism of action of our tested extract as well, because it is clear from the results that the effect of the extract was similar to diazepam. Alpha 2 receptor involvement has been suggested in anxiety and fear. It has been shown that drugs which increase the level of noradrenaline within the limbic forebrain modulate fear and anxiety [26, 27]. The administration of yohimbine, an alpha 2 antagonist, causes the increased level of noradrenaline within the forebrain that alleviates anxiety [28, 29]. The present study has shown that HEVB alleviates anxiety (Table 1), which may be due to the alpha 2 antagonistic properties of the active ingredients present in plant extract. Diazepam has anxiolytic, sedative-hypnotic and muscle relaxant activity in ascending order of dose and this behaviour is similar with our extract, therefore, HEVB might be involved in the action on both GABA and glycine neurotransmission similarly to diazepam; however, further studies are needed in order to ascertain the exact mechanism of action.

A dose-dependent CNS depression was observed in the FST. The FST is frequently used for the assessment of antidepressant-like activity in animal models. The prolongation of mobility duration indicates antidepressant activity in this model, while shortening mobility duration reflects the CNS depression-like effect [30]. The animals observed to be immobile for longer periods of time as compared to the antidepressant drug reflects that the animal is in a state of tiredness, fatigue and sadness, which are the main symptoms of depressed patients [31]. There was no antidepressant effect shown in comparison with fluoxetine because none of the tested doses of HEVB shortened the duration of immobility. The antidepressant effect of our tested extract was similar to the negative control instead of the positive control, which proves that the extract cannot stimulate the CNS. Furthermore, the extract reduced the locomotor activities in comparison with the control group. The reduction in the frequency and amplitude of motion could be attributed to the sedative effect of the extract [32]. A significant sedative effect was produced by extract (0.4 and 0.5 mg/kg) and diazepam (1 mg/kg). It is very interesting to note that the extract is not antidepressant, as shown in Table 4, and this activity potentiates the CNS depression, as depicted in Table 5. Presently, there is no reported active constituents from this plant; however, a large number of compounds, such as flavonoids, anthocyanins, coumarins, tannins, saponins, carotenoids, phenolic acids [33], cyclotides [34, 35] and triterpenoid saponins [35], have been reported in the literature. The anxiolytic effect of flavonoids [36, 37] and saponins [38] has been previously reported and, therefore, we suggest that these anxiolytic effects may be due to the above constitutes. Our research group is undertaking the project of the isolation of active constitutes from *V. betonicifolia* whole plant.

In conclusion, HEVB possesses anxiolytic, muscle relaxant, hypnotic and sedative effects, and these findings collaborate with the ethno-medicinal uses of this plant. The

isolation of active chemicals from this plant might serve as lead compounds for the synthesis of drugs which could be used in the management of these nervous disorders.

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