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Research Article

Muscle Relaxant and Sedative-Hypnotic Activities of Extract of *Viola betonicifolia* in Animal Models Supported by Its Isolated Compound, 4-Hydroxy Coumarin

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The crude methanolic extract of the whole plant of *Viola betonicifolia* (VBME) was investigated for anxiolytic, muscle relaxant, sleep induction, antidepressant, and sedative activities) to ascertain its scientific values. VBME showed a significant ($P < 0.05$) dose dependent anxiolytic action in staircase test. In muscle relaxant paradigms, a dose dependent muscle relaxation was observed. For phenobarbitone sleep induction test, VBME notably ($P < 0.05$) reduced the latency time and increased total sleeping duration. Our tested extract was found free of any antidepressant activity, while the movement was significantly ($P < 0.05$) shortened in locomotor activity. The whole plant of *V. betonicifolia* led to the isolation of 4-hydroxyl coumarin (4HC) which showed substantial safety profile in acute toxicity test. When challenged in Traction and Chimney tests, it showed significant ($P < 0.05$) muscle relaxant effect in both muscle relaxant paradigms at 20 and 30 mg/kg during various assessment times. Nevertheless, 4HC was devoid of sedative and hypnotic potentials. In conclusion, VBME had strong muscle relaxant and sedative-hypnotic properties, while its isolated compound, 4HC, possessed a significant muscle relaxant action with substantial safety profile without sedative-hypnotic effects.

1. Introduction

Viola betonicifolia is found in various countries of the world such as Pakistan, India, Nepal, Sri Lanka, China, Malaysia, and Australia. In Pakistan, this plant is available in Swat, Hazara, and Dir districts. The folk use of this plant is antipyretic, astringent, diaphoretic, anticancer, and purgative [1]. *V. betonicifolia* has been used in the treatment of various neurological disorders, including epilepsy and insomnia [2]. Additionally, *V. betonicifolia* has been used in the treatment of sinusitis, skin and blood disorders and pharyngitis [3]. Roots are used for kidney diseases, pneumonia, and bronchitis. Flowers are recommended for the treatment of asthma, cough, and colds, while leaves are useful for the treatment of boils [4]. Recently we have tested the crude methanolic as well as the subsequent solvent fraction of *V. betonicifolia* for various pharmacological activities [5–9]. The current study was designed to isolate pure compounds and then check their biological potential for rationalization of the neuropharmacological potential of the whole plant of *V. betonicifolia*.

2. Material and Methods

2.1. Plant Material. The whole plant of *V. betonicifolia* was collected from Swat, Khyber Pakhtunkhawa, Pakistan, in April 2010. Plant specimen was identified by Professor Dr. Muhammad Ibrar, Taxonomy Section, Department

of Botany, University of Peshawar, and a specimen was deposited there in the herbarium under voucher number 6410/Bot.

2.2. Extraction, Fractionation, and Isolation. 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 [6]. The methanolic extract was filtered and concentrated under vacuum at low temperature (45°C) resulting in crude methanolic extract (1.98 kg, 22% w/w). The crude methanolic extract (1.60 kg) was dissolved in distilled water and further fractionated into various solvent fractions such as *n*-hexane, chloroform, ethyl acetate, *n*-butanol, and aqueous fractions yielding *n*-hexane (706 g, 44.13% w/w), chloroform (9 g, 0.56% w/w), ethyl acetate (16 g, 1.00% w/w), *n*-butanol (265 g, 16.56% w/w), and aqueous fractions (498 g, 31.13% w/w). The chloroform and ethyl acetate fractions were combined and subjected to column chromatography. The column was eluted with chloroform: *n*-hexane solvent system, starting from pure *n*-hexane and then chloroform was used in various percentages with *n*-hexane which yielded fractions 1–12. The fraction no. 5 was rechromatographed over the silica gel and eluted with ethyl acetate and *n*-hexane. The compound was purified by using silica gel column chromatography (column 32 mm × 40 cm), by using ethyl acetate and hexane as eluting solvent. The compound **1** was purified as 4-hydroxy coumarin (4HC) from this fraction using solvent system 35% ethyl acetate and *n*-hexane.

2.3. Animals. BALB/c mice of either sex were used in all experiments. Animals were purchased from the Pharmacology Section of the Department of Pharmacy, University of Peshawar, Peshawar, Pakistan. The animals were maintained in standard laboratory conditions (25°C and light/dark cycles, i.e., 12/12 h) and were fed with standard food and water *ad libitum*. The experimental protocols were approved by the ethical committee of the Pharmacy Department, University of Peshawar, Peshawar, Pakistan. The experiments were performed with the rulings of the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council [10].

2.4. Acute Toxicity. The acute toxicity test was carried out for 4HC to evaluate any possible toxicity. BALB/c mice (n = 6) of either sex were treated with different doses (50, 100, and 150 mg/kg, p.o.), while the control group received saline (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 [11].

2.5. Muscle Relaxant Activity

2.5.1. Traction Test. In this procedure, a metal wire was coated with rubber and both ends of the wire were rigidly supported with stands about 60 cm above the laboratory bench. The animals were divided into groups (n = 6). Group I was treated with distilled water (10 mL/kg, i.p.), which served as negative control and group II was treated with diazepam 1.0 mg/kg, i.p., which was positive control. The remaining groups were treated with VBME (300, 400, and 500 mg/kg i.p.) or 4HC (10, 20, and 30 mg/kg). After 30 min of the pervious treatments, each animal was hanged by their hind legs from the wire and the time of hanging was recorded for 5 s. The failure to hang less than five seconds reflects the presence of muscle relaxant property and vice versa [12].

2.5.2. Chimney Test. This test was performed according to a well established method [13]. A pyrex glass tube (30 cm long and 3.0 cm 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 VBME (300, 400, and 500 mg/kg i.p.) or 4HC (10, 20, and 30 mg/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; the animal tried to climb the tube with a backward movement. The mouse which failed to reach up to the mark within 30 s was considered with relaxed muscles.

2.6. Sedative-Hypnotic Activites

2.6.1. Sedative Activity. The apparatus used for this activity consisted of an area of white wood (150 cm diameter) enclosed by stainless steel walls and divided into 19 squares by black lines. The open field apparatus was placed inside a light- and sound-attenuated room. BALB/c mice (18–22) of either sex (n = 6) weighing 22 ± 2 g were used. Animals were acclimatized under red light (40 Watt red bulb) one hour before the start of the experiment in the laboratory with food and water *ad libitum*. Animals were divided into groups each of six mice. Group I was treated with distilled water (10 mL, i.p.), which served as negative control, and group II was treated with diazepam 0.5 mg/kg, i.p., which was positive control. Animals of remaining groups were treated with VBME (300, 400, and 500 mg/kg i.p.) or 4HC (10, 20, and 30 mg/kg i.p.). After 30 min each animal was placed in the center of the box and the numbers of lines crossed were counted for each mouse [14, 15].

2.6.2. Hypnotic Activity. Animals were divided into five groups (n = 6); the control group was treated with distilled water (10 mL/kg, i.p.), standard group was treated with diazepam (4 mg/kg, i.p.), and the remaining groups were treated with VBME (300, 400, and 500 mg/kg i.p.) or 4HC (10, 20, and 30 mg/kg, i.p.). After 30 min of treatment all animals were injected with phenobarbitone sodium 35 mg/kg, i.p. Each animal was observed for onset and duration of sleep. The duration of sleep or hypnosis was considered the loss of postural reflux [16].

2.7. Spectroscopic Study of 4-Hydroxy Coumarin. The structure of the isolated compound was elucidated using different spectroscopic techniques including ^{1}H-NMR, ^{13}C-NMR,

Table 1: Effects of VBME (%) on muscle relaxation (Chimney test and Traction test).

Group	Dose/kg	Chimney test (%)			Traction test (%)		
		30 min	60 min	90 min	30 min	60 min	90 min
Control	10 mL	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
Diazepam	1 mg	100 ± 0.00***	100 ± 0.00***	100 ± 0.00***	100 ± 0.00***	100 ± 0.00***	100 ± 0.00***
VBME	0.3 g	10.12 ± 0.88	14.11 ± 0.97	11.09 ± 2.11	12.54 ± 1.10	15.23 ± 2.13	10.34 ± 1.90
	0.4 g	55.13 ± 1.23*	61.56 ± 0.65*	58.23 ± 0.56*	65.02 ± 0.23*	72.12.04 ± 0.56*	68.04 ± 0.34*
	0.5 g	77.08 ± 0.11**	80.76 ± 0.02**	80.55 ± 0.21**	75.05 ± 0.12**	78.03 ± 0.08**	77.67 ± 0.00**

Values represent the percentages of mice ($n = 6$) showing negative effects in Chimney and Traction tests, 30, 60, and 90 min after treatment with distilled water (10 mL/kg), VBME (0.3, 0.4, and 0.5 g/kg), or diazepam (1 mg/kg). Data are presented as mean ± S.E.M ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, all compared with control.

HMBC, HMQC, NOESY, COSY, HREI-MS, and IR. IR spectra were recorded on a Vector 22 (Bruker) Fourier-transform infrared (FT-IR) spectrometer, using KBr windows with CH_2Cl_2 as solvent against an air background. ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker Avance spectrometer. The 2D-NMR spectra were recorded on a Bruker Avance NMR spectrometer. Mass spectra (EI and HR-EI-MS) were measured in an electron impact mode on Finnigan MAT-312 and MAT-95 XP spectrometers, and ions are given in m/z (%). TLC was performed on precoated silica gel F-254 plates (E. Merck); the detection was done at 254 nm and by spraying with ceric-sulphate reagent. Column silica gel (E. Merck, 70–230 mesh) and flash silica gel (E. Merck, 230–400 mesh) were used for column chromatography.

2.8. Statistical Analysis. Results are expressed as mean ± S.E.M. One-way ANOVA was used for comparison test of significant differences among groups followed by Dunnet's multiple comparison posttest. A level of significance ($P < 0.05$ or 0.01) was considered for each test.

3. Results

3.1. Effect of VBME in Muscle Relaxant Activity

3.1.1. Effect of Traction Test. The percent negative effect in Traction test is shown in Table 1. The effect of all posttreatment was observed at 30, 60, and 90 min. The maximum effect was shown after 60 min of drug treatment. The effect was dose dependent as the highest outcome was observed at 0.5 g/kg (80.76%) and the lowest action was 14.11% at 0.3 g/kg. The significant activity was shown by 0.5 and 0.4 g/kg in comparison with control group.

3.1.2. Effect of Chimney Test. The significant ($P < 0.01$) percent negative effect was observed by VBME at the doses of 0.4 and 0.5 g/kg. The muscle relaxant activity was dose dependent as shown in Table 1. The most significant ($P < 0.001$) skeletal muscle relaxation was observed against diazepam which was used as positive control. The effect of diazepam was foremost among the tested doses.

Table 2: Effects of VBME open field test (sedative effect).

Treatment	Dose	No. of lines crossed in 10 min
Distilled water	10 mL/kg	126 ± 1.23
VBME	0.3 mg	90 ± 1.72
	0.4 mg	70 ± 1.67*
	0.5 mg	30 ± 0.89**
Diazepam	0.5 mg	5 ± 0.02***

Values represent the number of lines crossed by animal in open field, 30 min after treatment with distilled water (10 mL/kg), VBME (0.3, 0.4, and 0.5 g/kg), or diazepam (0.5 mg/kg). Data are presented as mean ± S.E.M ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, all compared with control.

3.2. Effect of VBME in Sedative-Hypnotic Tests

3.2.1. Effect of Sedative Test. The plant was sedative at all the applied doses and the locomotion was decreased with increasing the dose as shown in Table 2. The animals were almost immobile and were nonactive at the dose of 0.5 g/kg while the remaining treatments also showed significant ($P < 0.05$) results.

3.2.2. Effect of Hypnotic Test. The effect of phenobarbitone in sleep induction is shown in Table 3. VBME significantly ($P < 0.01$) reduces the latency time and potentiates the duration of sleep in dose dependent manners. The duration of sleep in diazepam treated group was 56.45 min, while duration of sleep in diazepam plus VBME groups was 8.13, 13.98, and 25.23 min for 0.3, 0.4, and 0.5 g/kg, respectively.

3.3. Characterization of 4HC. The isolated compound was confirmed as 4-hydroxy coumarin (Figure 1). 4HC was isolated as a white powder (m.p. 113–114°C) from chloroform and ethyl acetate combined fraction of methanolic extract. Its EIMS showed molecular ion peak at m/z 162 and fragment peaks at m/z 120 and 92. The molecular formula of the compound, $C_9H_6O_3$, was determined from EIMS and ^{13}C-NMR (BB and DEPT). ^{1}H-NMR spectrum (Table 4) exhibited resonances at δ 5.67 (s, H-3), 7.32 (m, H-5), 7.33 (dd J =

Table 3: Effect of VBME in phenobarbitone-induced sleep (hypnotic test).

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***
VBME	0.3 g/kg	23.45 ± 0.87	8.13 ± 0.97
	0.4 g/kg	15.78 ± 0.78*	13.98 ± 1.76*
	0.5 g/kg	10.98 ± 0.91**	25.23 ± 1.46**

Values represent the onset of sleep and duration of sleep (in min) after treatment with distilled water (10 mL/kg), VBME (0.3, 0.4, and 0.5), or diazepam (4 mg/kg) and then all groups were treated with phenobarbitone (35 mg/kg). Data are presented as mean ± S.E.M ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, all with respect to control.

Table 4: ^{1}H- and ^{13}C-NMR chemical shift values of 4-hydroxy coumarin.

C. no.	δ_C	δ_H (J, Hz)
2	162.7	—
3	92.47	5.67 s
4	166.0	—
5	133.4	7.63 dd (7.5, 1.5)
6	124.1	7.85 m
7	124.6	7.32 m
8	117.2	7.33 dd (7.5, 1.5)
9	154.9	—
10	116.7	—

7.5, 1.5 Hz, H-8), 7.63 (dd, $J = 7.5$, 1.5 Hz, H-5), and 7.85 (m, H-6). ^{13}C-NMR spectrum (BB, and DEPT) (Table 4) showed resonances for all nine carbons including five methine and four quaternary carbons. The structure of the compound was further confirmed by using 2D-NMR technique (HSQC, HMBC, and COSY). Key COSY and HMBC interactions in the compound are shown in Figure 2. The spectroscopic data were compared with reported spectroscopy of 4-hydroxy coumarin.

3.4. Acute Toxicity Test. No behavior change or mortality was observed at tested doses (50, 100, and 150 mg/kg) and therefore the compound is considered safe up to the dose of 150 mg/kg.

3.4.1. Effect of 4HC in Traction and Chimney Tests. The percent negative effect in Traction test is shown in Table 5. The effect of all posttreatments was observed after 30, 60, and 90 min of the treatment. The maximum effect was shown after 60 min. The effect was not dose dependent as no activity was observed at the dose of 10 mg/kg, while the maximum activity was observed at 30 mg/kg (27.04%) after 60 min of the treatment. The significant ($P < 0.05$) activity was shown by 20 and 30 mg/kg of 4HC in comparison with negative control group. The significant ($P < 0.05$) percent negative effect was observed by 4HC at the dose of 30 mg/kg. The muscle relaxant activity was not dose dependent as shown in Table 4.

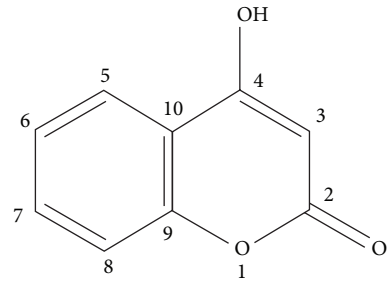


Figure 1: Structure of 4-hydroxy coumarin.

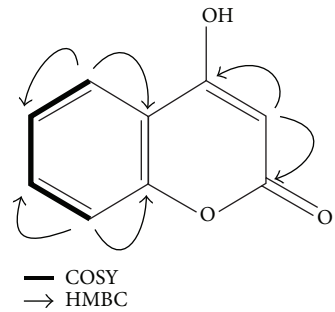


Figure 2: Key COSY and HMBC interactions.

The standard reference drug (diazepam) was the most significant ($P < 0.001$) as compared to the test compound and negative control group. The muscle coordination effect of the tested compound was weaker than that of diazepam (1 mg/kg).

3.4.2. Effect of 4HC in Sedative-Hypnotic Tests. The 4HC was not sedative in all applied doses (10, 20, and 30 mg/kg) and the locomotor behavior remained normal as shown in Table 6. Similarly, no sleep inducing effect was observed as shown in Table 7.

4. Discussion

Therapeutic potentials of Pakistani medicinal plants have been well recognized including neurological disorders [17–19]. The current study describes the muscle relaxant and sedative-hypnotic activities of crude methanolic extract of *V. betonicifolia* (VBME) and its isolated compound, 4HC, in various animal paradigms.

Muscle relaxant like effects of drug substances are usually assessd in Chimney test. In this test, climbing capacity of test animals in a specially designed apparatus is observed during various assessment times, which could diffidently be altered in relaxed muscles. VBME exhibited muscle relaxing activity in a dose dependent manner during various assessment times (30, 60, and 90 min) similar to bromazepam. Similar trend was observed for 4HC, when tested for its muscle relaxant properties in said the paradigms.

Open-field- and phenobarbitone-induced sleeping time assays are frequently employed as prognostic tests for the

Table 5: Effects of 4HC (%) on muscle relaxation (Chimney test and Traction test).

Group	Dose/kg	Chimney test (%)			Traction test (%)		
		30 min	60 min	90 min	30 min	60 min	90 min
Control	10 mL	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
Diazepam	1 mg	100 ± 0.00***	100 ± 0.00***	100 ± 0.00***	100 ± 0.00***	100 ± 0.00***	100 ± 0.00***
4HC	10 mg	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
	20 mg	15.13 ± 1.34*	20.21 ± 1.35*	20.45 ± 0.44*	4.50 ± 1.67*	6.55 ± 1.22*	3.54 ± 1.89*
	30 mg	30.34 ± 1.54**	37.33 ± 1.57**	35.45 ± 1.45**	25.34 ± 0.23**	27.04 ± 1.77**	26.78 ± 2.56**

Values represent the percentages of mice ($n = 6$) showing negative effects in Chimney and Traction tests, 30, 60, and 90 min after treatment with distilled water (10 mL/kg), 4HC (10, 20, and 30 mg/kg), or diazepam (1 mg/kg). Data are presented as mean ± S.E.M, ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{**}P < 0.001$, all compared with control.

Table 6: Sedative effect of 4HC in open field test.

Treatment	Dose	No. of lines crossed in 10 min
Distilled water	10 mL/kg	126 ± 1.23
4HC	10 mg	120.14 ± 1.65
	20 mg	122.37 ± 1.43
	30 mg	120.34 ± 1.12
Diazepam	0.5 mg	5 ± 0.02***

Values represent the number of lines crossed by animal in open field, 30 min after treatment with distilled water (10 mL/kg), 4HC (10, 20, and 30 mg/kg), or diazepam (0.5 mg/kg). Data are presented as mean ± S.E.M ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, all compared with control.

Table 7: Effect of 4HC on phenobarbitone-induced sleep.

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***
4HC	10 mg/kg	26.23 ± 1.34	7.25 ± 1.46
	20 mg/kg	26.54 ± 2.08	7.27 ± 1.76
	30 mg /kg	25.35 ± 1.87	6.24 ± 1.78

Values represent the onset of sleep and duration of sleep (in min) after treatment with distilled water (10 mL/kg), 4HC (10, 20, and 30 mg/kg), or diazepam (4 mg/kg) and then all groups were treated with phenobarbitone (35 mg/kg). Data are presented as mean ± S.E.M ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, all with respect to control.

assessment of sedative-hypnotic properties [17, 20]. Pretreatment of mice with VBME showed dose dependent reduction in locomotive activity in open field test as compared to control. Similarly, it had reduced latency time and potentiated sleeping duration in phenobarbitone-induced sleeping time test. The reduction in the frequency and amplitude of motion could be attributed to the sedative effect of VBME [21]. The resulting sedative-hypnotic effects of VBME were similar to standard drug used (bromazepam). However, when 4HC was studied for sedative-hypnotic effects, it was found inactive.

Researchers believed that the sedative-hypnotic, anxiolytic, and muscle relaxant effects of benzodiazepines like bromazepam are mostly due to interference with the action of gamma aminobutyric acid ($GABA_A$) [9]. Additionally, the anxiolytic action of benzodiazepines may be due to direct activation of glycine synapses in the brain [22]. Studies revealed that benzodiazepines bind to the gamma subunit of the $GABA_A$ receptor, implicating structural modification of the receptor and thus causing an increase in $GABA_A$ receptor activity. Benzodiazepines do not substitute for GABA, which bind at the alpha subunit, but increase the frequency of channel opening events, which leads to an increase in chloride ion conductance and inhibition of the action potential [23]. The overall effects of VBME were similar to standard drug used (bromazepam).

In conclusion, the present study presented convincing evidence that the crude extract of the plant (VBME) exhibited significant muscle relaxant activity augmented by its isolated compound, 4HC, while the spontaneous movements of the animals were not affected by any of the test doses of 4HC. Additionally, the VBME possessed strong sedative-hypnotic properties while 4HC was devoid of sedative-hypnotic actions. Further detailed studies could lead to clinical utility.

Conflict of Interests

The authors have declared that there is no conflict of interests.

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