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Research Article

Analgesic and Anti-inflammatory Profile of n-Hexane Fraction of *Viola betonicifolia*

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Abstract

***Purpose**: To evaluate the analgesic and anti-inflammatory activities of n-hexane extract of the whole plant of Viola betonicifolia Sm, family: Violaceace.*
***Methods:** The n-hexane fraction of Viola betonicifolia (VBHF) was tested for its analgesic and anti-inflammatory activities (carrageenan-induced and histamine-induced edema models) in BALB/c mice.*
***Results:** VBHF exhibited significant ($p < 0.01$) analgesic and anti-inflammatory activity at test doses of 100, 200 and 300 mg/kg. The analgesic effect of VBHF was dose-dependent in acetic acid pain model while the extract was a weak analgesic at the dose of 300 mg/kg in hot plate and tail immersion test. Diclofenac sodium and tramadol showed better analgesic properties to the extract. Analgesia was not antagonized by naloxone in the hot plate model. Anti-inflammatory activity against carrageenan-induced edema was 60.8 %; however, histamine-induced inflammation was not antogonised by the extract.*
***Conclusions:** The extract has some analgesic and anti-inflammatory activities. This justifies its use in traditional medicine for pain of management.*

***Keywords:** Viola betonicifolia, Analgesic, Anti-inflammatory.*

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INTRODUCTION

Over the centuries, medicinal plants have been utilized by different communities of the world. Communities in various regions of Pakistan, especially rural areas have been using medicinal plants growing in their areas for centuries for the treatment of diseases [1]. In Pakistan, this traditional mode of therapy is well-established and is known as Hikmat/Tibb. Approximately, 600 - 1000 medicinal plants in the country are reported to be in use for the management of different pathological conditions by more than 40,000 registered and unregistered Hakims or Tabibs [2]. This practice is based on experiences and without any scientific evidence, and therefore, there is need for proper for their proper scientific validation [3].

V. betonicifolia belongs to the family Violaceace. It is known as banafsha, and is a perennial herb of height of 8 - 20 cm. It is stem–less and its leaves are triangular or obtuse, and petiole is longer than the lamina. The roots are short, slender and unbranched, rhizomes. It is found in various countries of the world including Pakistan, India, Nepal, Sri-lanka, China, Malaysia and Australia [4]. In Pakistan, it is found in Swat, Hazara and Dir and used as antipyretic, astringent, diaphoretic, anticancer, febrifuge and purgative; it is also used as anti-epileptic and sedative in nervous disorders [5]. Other uses are the treatment of sinusitis, skin and blood disorders and pharyngitis [6]. Its roots are used for the treatment of kidney diseases, pneumonia and bronchitis while its flowers are used against lung troubles, cough and colds and the leaves are claimed to be effective against boils [7].

The present study was conducted with the aim to prove the analgesic and anti-inflammatory effect of VBHF.

EXPERIMENTAL

Chemicals

Diclofenac sodium (Suzhou Ausun Chemical Co Ltd, China), carrageenan (Sigma Lambda, USA), histamine (Alfa Aesar - A Johnson Matthey Co), naloxone (Acent Scientific Co), Tramadol (Seale Pakistan Ltd) were used in the study. Sterile normal saline was used in all experiments as control while methanolic extract was prepared in normal saline.

Animals

BALB/c mice of either sex (18 – 30 g) were used in all experiments. The animals were obtained from the animal house of the Pharmacology Section of the Department of Pharmacy, University of Peshawar, Peshawar. The animals were maintained in standard laboratory conditions (25 ^{o}C and 12/12 h light/dark cycle) and fed with standard food and water. The experimental protocols for the animal studies were approved by the ethical committee of the Department of Pharmacy, University of Peshawar, Pakistan (approval no. 05/EC/Pharm/2009) and the guidelines of Animals (Scientific Procedures) Act 1986 (A(SP)A 86) were followed [8].

Plant material

The whole plant of *V. betonicifolia* was collected from Swat, Khyber Pakhtunkhawa, Pakistan, in April 2010, and identified by Prof. Dr. Muhammad Ibrar, Department of Botany, University of Peshawar; a specimen was deposited in the department's herbarium (voucher no. 6410/Bot). The whole plant (12 kg) was air-dried, powdered and extracted by maceration with methanol at room temperature for 14 days with occasional shaking. The extract was filtered and concentrated at 45 °C using a rotary evaporator. The methanolic extract was dissolved in distilled water and further fractionated into chloroform, *n*-hexane, ethyl acetate, *n*-butanol and aqueous fractions.

The *n*-hexane fraction was tested for its analgesic and anti-inflammatory activities. This fraction and other standard drugs (diclofenac sodium, tramadol, histamine and carrageenan) were dissolved in normal saline.

Evaluation of analgesic activity

Acetic acid-induced writhing test

BULB/c mice of either sex, weighing 25 − 30 g, were used. The animals were withdrawn from food 2 h before the start of the experiment and divided into five groups. Group I was injected with normal saline intraperitoneally (ip) as control while group II was injected with the standard drug (diclofenac sodium, 10 mg/kg) while the other groups were injected with 100, 200 and 300 mg/kg i.p. of VBHF (extract). The animals were treated i.p. with 1 % acetic acid [9-10] 30 min later. Writhing was assessed 5 min after acetic acid injection and the number of writhes (abdominal constrictions) was counted for 10 min [11].

Acute toxicity study has previously be carried out on VBHF, via i.p. route [12].

Hot plate test

BALB/c mice of either sex, weighing 18 - 22 g, were acclimatized under laboratory conditions 1 h prior to the experiment; food and water were available to the animals *ad libitum*. The animals were pre-tested on a hot plate (Havard) maintained at 55 ± 0.1 °C. Animals having latency time > 15 s on the hot plate were excluded from the subsequent test [13]. The animals were divided in eight groups of six mice each. Group I was treated with saline (control), group II with tramadol (30 mg/kg i.p), [11]and groups III – V with 100, 200 and 300 mg/kg VBHF, i.p.; after 30 min, the animals were placed on a hot plate and latency time (the time the mouse remained on the hot plate (55 ± 0.1 °C) without licking or flicking of the hind limb or jumping) was measured in seconds. In order to prevent the tissue damage, a cut-off time of 30 seconds was set for all animals. To determine the opiodergic mechanism of the analgesic activity of VBHF, groups VI and VII were treated with naloxone (5 mg/kg) s.c.;10 min later, these two groups were treated with VBHF (200 and 300 mg/kg, i.p), while group VIII was treated with Tramadol (30 mg/kg) i.p. after 10 min of naloxone. The latency times for the groups were recorded at 0, 30, 60, 90 and 120 min. Percent analgesia was calculated using Eq 1:

Analgesia (%) = (TL – CL)/(CoT – CL)100 …… (1)

where TL is the latency time of the test extract, CL the latency time of the control group and CoT the cut–off time.

Tail immersion test

BALB/c mice of either sex were divided into five groups of six animals, weighing 18 – 22 g. Normal saline (10 ml/kg), VBHF (100, 200 and 300 mg/kg), and tramadol (30 mg/kg) were administered intraperitoneally to Groups I to V, respectively. Each animal was placed in a vertical position with the tail dipped up to 5 cm in a pot of hot water maintained at 55 ± 0.5 ºC. The time taken to withdraw the tail out of the hot water was taken as the reaction time (Ta). The test was carried out at 0, 30, 60, 90 and 120 min of administration of the drugs [14]. Each animal served as its own control and two readings were obtained for the control at 0 and 10 min. The mean of the two values was taken as the initial reaction time (Tb). The cut-off time, i.e., the time when the test was terminated when there was no response was set at 120 s. Analgesic activity was computed as in Eq 2:

Analgesic activity = (Ta – Tb/Tb)100 ….. (2)

Anti-inflammatory activity

Carrageen-induced paw edema test

Anti-inflammatory activity was determined using mice of either sex (weighing 25 − 30 g).

The animals were randomly divided into five groups each of six animals [15]. Group I was treated with normal saline, group II with standard drug (diclofenac sodium), the other groups with VBHF (100, 200, and 300 mg/kg). Thirty minutes after administration, carrageenan (1 %, 0.05 ml) was injected subcutaneously into the subplantar tissue of the right hind paw. Inflammation was measured using a plethysmometer (LE 7500 Plan Lab S.L, Italy) immediately after injection of carrageenan and then 1, 2, 3, 4 and 5 h later. Mean foot swelling in drug- and standard-treated groups were compared with that of control percent to determine inhibition of edema (anti-inflammatory activity) using Eq 3.

Inhibition (%) = (A - B/A)100 …… (3)

where A is the paw volume of control and B is the paw volume of the other groups.

Histamine-induced paw edema test

The animals were grouped as in the preceding experiment and inflammation was induced by subcutaneous injection of 0.1 ml of freshly prepared solution of histamine (1 mg/ml) into the hind paws of the mice [16]. The inhibition of inflammation and paw volume was as assessed as in the carrageenan-induced edema test.

Statistical analysis

The results obtained were expressed as mean ± SEM (n = 6). Statistical analysis was carried out on the data using ANOVA, followed by post hoc Dunnett's test for multiple comparisons. Activity was considered to be significant at the $p < 0.05$.

RESULTS

Anti-analgesic activity

Analgesic effect was observed at all test doses of the VBHF (100, 200 and 300 mg/kg) and was dose-dependent, as shown in Table 1. Maximum inhibition of pain (85.2 %) was observed at 300 mg/kg dose of extract (VBHF). The other doses of extract also showed significant activity. The data on inhibition of writhing indicate that diclofenac sodium was more effective (96.2 %) than the extract in suppressing pain.

The results of the hot plate test revealed that latency time for the mice increased only at a dose of 300 mg/kg, as shown in Table 2.

Table 1: Effect of intraperitoneal administration of VBHF in acetic acid-induced pain model

Treatment	Dose (mg/kg)	No. of writhings (in 10 min)	Pain reduction (%)
Saline (control)	10 ml/kg	64.80 ± 2.7	-
VBHF	100	48.40 ± 2.6*	59.34
	200	36.6 ± 1.5**	75.67
	300	31.80 ± 1.6**	85.2
Diclofenac	10	10.40 ± 1.4**	96.2

*Data are presented as mean ± S.E.M. group of six animals. *p < 0.05, **p < 0.01*

Table 2: Analgesic activity of VBHF in hot plate pain model

Group	Treatment/kg	0 min	30min	60 min	90 min	120min
Saline	10 ml	9.20 ± 0.02	9.22± 0.08	9.16 ± 0.09	9.20 ± 0.03	9.12 ± 0.11
VBHF	100 mg	9.12 ± 0.21	9.23 ± 0.23	9.26 ± 0.96	9.27 ± 0 .28	9.19 ± 0.29
	200 mg	9.16 ± 0.32	9.26 ± 0.56	9.30 ± 0.27	9.29 ± 0 .28	9.20 ± 0.86
	300 mg	9.25 ± 0.34	10.59± 0.43*	10.88 ± 0.25*	10.59 ± 0.87*	10.19 ± 0.24
Tramadol	30 mg	9.12 ± 0.21	9.23 ±0.23**	9.26 ± 0.96**	9.27 ± 0.28**	9.19 ± 0.2**9

*Values are reported as mean ± S.E.M. (n = 6);.*p < 0.05, **p < 0.01, compared with control*

Table 3: Analgesic effect of tramadol and VBHF antagonized by naloxone, in hot plate pain model

Group	Treatment/ kg	0 min	30min	60 min	90 min	120min
VBHF	200mg	9.24 ±0.45	10.60 ± 0.72	10.80 ± 0.92	10.62 ± 0.34	10.32 ± 0.36
	300 mg	9.22 ±0.76	10.80±0.87	10.75±0.92	10.82±0.72	10.86±0.92
Tramadol	30 mg	9.23 ±0.02	10.22±0.05**	10.02±0.09**	10.24±0. 03**	10.05±0.00**

*Values are reported as mean ± S.E.M. (n = 6);.*p < 0.05, **p < 0.01, compared with control*

Table 4: Analgesic effect ofVBHF, in tail immersion test

Group	Treatment/ kg	0 min	30min	60 min	90 min	120min
Saline		3.22 ± 0.02	3.23 ± 0.04	3.31 ± 0.04	3.28 ± 0.10	3.25 ± 0.12
VBHF	100	3.23 ± 0.62	3.41 ± 0.87	3.50 ± 0.71	3.44 ± 0.74	3.40 ± 0.56
	200	3.20 ± 0.46	3.44 ± 0.02	3.62 ± 0.28	3.57 ± 0.98	3.49 ± 0.34
	300	3.24 ± 0.36	3.55± 0.23*	3.65 ± 0.12*	3.60 ± 0.34*	3.55 ± 0.56
Tramadol	30mg	3.20 ± 0.01	5.60 ±0.03**	5.85± 0.03**	5.79 ± 0.08**	5.71 ± 0.00**

*Values are reported as mean ± S.E.M. (n = 6);.*p < 0.05, **p < 0.01, compared with control*

Overall, analgesic activity was weak and was not antagonised by naloxone as shown in Table 3. The results of tail immersion test are presented in Table 4. A significant analgesic effect was observed at a dose of 300 mg/kg, but no analgesic effect was observed at lower doses.

Anti-inflammatory activity

*A*nti-inflammatory activity of the extract (VBHF) at test doses (100, 200 and 300 mg/kg) is presented in Figure 1 in terms of inhibition (%) of paw volume. Injection of carrageenan caused inflammatory edema in the paw, reaching a maximum at the 4th hour after injection. The anti-inflammatory activity of VBHF (200 and 300 mg/kg) was significant ($p < 0.01$) 2 h after carrageenan administration and was sustained reaching a maximum of 60.8 %. The maximum anti-inflammatory activity of diclofenac sodium (75.2 %) was higher than that of the extract. The activity of VBHF was dose-dependent manner and remained significant for up to 5 h after drug administration.

On the other hand, the inflammation-induced by histamine was not affected by VBHF.

DISCUSSION

The results obtained show that the n-hexane fraction of the whole plant of *Viola betonicifolia* has potent analgesic and anti-inflammatory activities. Acetic acid-induced writhing is a well recommended protocol for evaluating medicinal agents for analgesic property. The pain in this model is induced by the liberation of endogenous substances as well as some other pain mediators such as arachidonic acid via cyclooxygenase, and prostaglandin biosynthesis [11]. This pain paradigm is widely used for the assessment of peripheral analgesic activity due to its sensitivity and response to compounds at a dose that is not effective in other models.

Local peritoneal receptors could be responsible for the abdominal writhing [17]. Pain sensation in acetic acid-induced writhing model is elicited by production of localized inflammatory response due to the release of free arachidonic acid from tissue phospholipids [18] via cyclooxygenase (COX), and also production of prostaglandin specifically, PGE2 and PGF2α; the level of lipoxygenase products in peritoneal fluids may also increases [19]. These prostaglandin and lipoxygenase products cause inflamma-

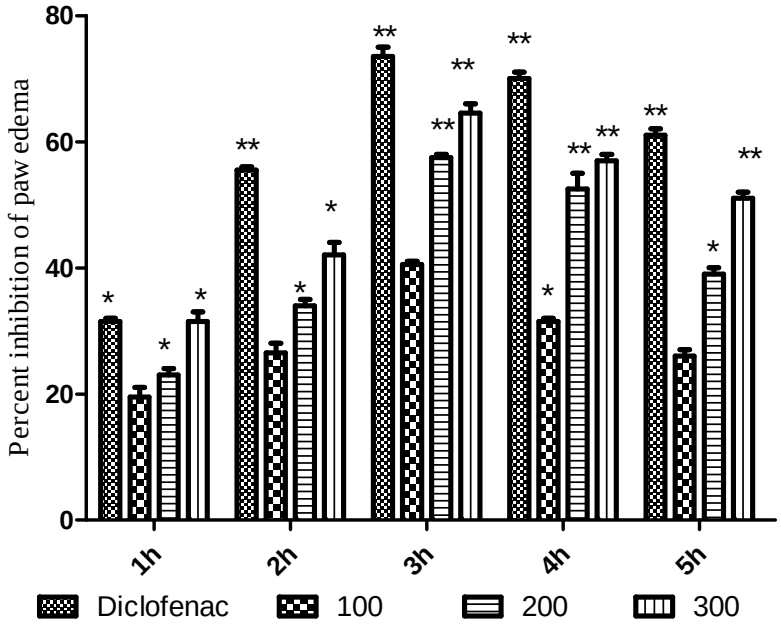

Figure 1: Anti-inflammatory activity of VBHF in carraganeen-induced paw edema in mice.
*Each bar presents the percent inhibition of paw edema after 1, 2, 3, 4 and 5th h of the treatment with diclofenac sodium (10 mg/kg) and VBHF (100, 200 and 300 mg/kg). Values are reported as mean ± S.E.M (n=6). ANOVA followed by Dunnett's test was applied on the data to find the level of significance in comparison with negative control. *P < 0.05, **P < 0.01*

tory pain by increasing capillary permeability. The substance inhibiting writhing has analgesic effect, preferentially by inhibition of prostaglandin synthesis, a peripheral mechanism of pain inhibition [19].

Our findings strongly suggest that VBHF has peripheral analgesic activity and its mechanisms of action may be mediated through inhibition of local peritoneal receptors which may involve cyclooxygenase inhibition. The profound analgesic activity of this extract may be due to interference of its active principle(s) with release of pain mediators.

Thermal nociception models such as hotplate and the tail immersion tests were used to evaluate central analgesic activity. VBHF showed weak activity in thermal pain model, which indicates that VBHF analgesic activity may be peripheral, not central.

Carrageenan-induced paw edema is a well established animal model to assess the anti-oedematous effect of natural activity products. Edema formation due to carrageenan is a biphasic event, with the initial phase occurring at 1 h or 1.5 h and is predominantly a nonphagocytic edema, followed by a second phase with increased edema formation that continues up to 5 h [15]. The initial phase has been ascribed to the action of mediators, such as histamine, serotonin and bradykinin, on vascular permeability [20]. The late or second phase edema has been shown to be a result of the overproduction of prostaglandins [21]. The result of pre-treatment with VBHF demonstrates that the extract is effective in the early phase of inflammation which is due to release of histamine and serotonin primarily. The anti-inflammatory effect of the extract remained significant up to the 5th hour of the experiment. The anti-inflammatory effect of VBHF in the early phase suggests

that the plant may have anti-asthmatic, antitussive and bronchodilator activities. Interestingly, these are some of the well known folk uses of the plant.

CONCLUSION

Findings from this study demonstrate that *Viola betonicifolia* possesses analgesic (peripheral) and anti-inflammatory activities, thus providing justification for folkloric use of the plant as analgesic and anti-inflammatory.

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REFERENCES

1. *Shinwari ZK. Medicinal plants research in Pakistan. J. Med. Plant. Res 2010; 4:161-176.*
2. *Saeed M, Muhammad N, Khan H. Assessment of Heavy Metal Content of Branded Pakistani Herbal Products. Trop J of Pharm Res. 2011; 10: 499-506.*
3. *Saeed M, Khan H, Khan MA, Khan F, Khan SA, Muhammad N. Quantification of various metals and cytotoxic profile of aerial parts of polygonatum verticillatum. Pak J Bot 2010; 42: 3995-4002.*
4. *FOP. Flora of Pakistan. http://www.efloras.org/florataxon.aspx?flora_id=5&taxon_id=200014334.*
5. *Hamayun M. Ethnobotanical profile of Utror and Gabral valleys, district Swat, Pakistan. . Ethnobot Leaflets 2005; (1): 9.*
6. *Bhatt V, Negi G. Ethnomedicinal plant resources of Jaunsari tribe of Garhwal Himalaya, Uttaranchal. Ind. J Trad Knowl. 2006; 5: 331-335.*
7. *Husain SZ, Malik RN, Javaid M, Bibi S. Ethonobotanical properties and uses of medicinal plants of Morgah biodiversity park, Rawalpindi. Pak J Bot. 2008; 40: 1897-1911.*
8. *Guidance on the Operation of the Animals (Scientific Procedures) Act of 1986, The Stationery Office, 15 May 2000, retrieved December 6, 2006.*
9. *Muhammad N, Saeed M, Khan H. Antipyretic, analgesic and anti-inflammatory activity of Viola betonicifolia whole plant. BMC Com AlterMed. 2012; 12:doi:10.1186/1472-6882-12-59.*
10. *Ibrar M, Muhammad N, Barkatullah HK, Jahan F, Ashraf N. Antinociceptive and anticonvulsant activities of essential oils of Zanthoxylum armatum. Phytopharmacol. 2012; 3: 191-198.*
11. *Khan H, Saeed M, Gilani AUH, Khan MA, Dar A, Khan I. The antinociceptive activity of Polygonatum verticillatum rhizomes in pain models. J Ethnopharmacol. 2010; 127: 521-527.*
12. *Muhammad N, Saeed M, Khan H, Haq I. Evaluation of n-hexane extract of Viola betonicifoliafor its neuropharmacological properties. J Nat Med. 2012:DOI 10.1007/s11418-012-0636-0.*
13. *Brochet D, Micó JA, Martin P, Simon P. Antinociceptive activity of beta-adrenoceptor agonists in the hot plate test in mice. Psychopharmacol. 1986; 88: 527-528.*
14. *Aydin S, Demir T, Öztürk Y, Ba er KHC. Analgesic activity of Nepeta italica L. Phytother Res. 1999; 13: 20-23.*
15. *Khan MA, Khan H, Khan S, Mahmood T, Khan PM, Jabar A. Anti-inflammatory, analgesic and antipyretic activities of Physalis minima Linn. J Enz Inhi Med Chem. 2009; 24: 632-637.*
16. *Singh S, Majumdar D, Rehan H. Evaluation of anti-inflammatory potential of fixed oil of Ocimum sanctum (Holybasil) and its possible mechanism of action. J Ethnopharmacol 1996; 54: 19-26.*
17. *Mbiantcha M, Kamanyi A, Teponno R, Tapondjou A, Watcho P, Nguelefack T. Analgesic and Anti-Inflammatory Properties of Extracts from the Bulbils of Dioscorea bulbifera L. var sativa (Dioscoreaceae) in Mice and Rats. Evid Comp Alter Med: eCAM 2011 doi:19.1155/2011/912935*
18. *Zulfiker A, Rahman MM, Hossain MK, Hamid K, Mazumder M, Rana MS. In vivo analgesic activity of ethanolic extracts of two medicinal plants Scoparia dulcis L. and Ficus racemosa Linn. Bio Med. 2010; 2: 42-48.*
19. *Duarte I, Nakamura M, Ferreira S. Participation of the sympathetic system in acetic acid-induced writhing in mice. Braz J Med Bio Res. 1988; 21: 341.*
20. *Maity TK, Mandal SC, Mukherjee PK, Saha K, Das J, Pal M, et al. Studies on antiinflammatory effect of Cassia tora leaf extract (fam. Leguminosae). Phytoth Res. 1998; 12: 221-223.*
21. *Perez-Guerrero C, Herrera MD, Ortiz R, Alvarez de Sotomayor M, Fernandez MA. A pharmacological study of Cecropia obtusifolia Bertol aqueous extract. J Ethnopharmacol. 2001; 76: 279-284.*