Innovare Academic Sciences

International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 7, Issue 9, 2015

Original Article

BARLERIA MONTANA WIGHT AND NEES–A PROMISING NATURAL ANTI-INFLAMMATORY AGENT AGAINST FORMALIN INDUCED INFLAMMATION

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Received: 23 May 2015 Revised and Accepted: 06 Jul 2015

ABSTRACT

Objective: To evaluate the anti-inflammatory activity of the ethanolic leaf extract of *Barleria montana* (*B. montana*) against formalin induced inflammation.

Methods: Male albino wistar rats were pretreated with oral doses of 100 mg, 200 mg and 300 mg of the extract for 30 days and the animals received a single dose of sub plantar injection of formalin (0.1 ml/kg body weight (bw.)) Indomethacin (25 mg/kg bw.) was used as the standard drug. The effect of the extract on paw thickness, biochemical and hematological parameters was investigated along with histopathological studies.

Results: The results revealed that the extract exhibited an effective dose dependent activity against formalin induced inflammation with a maximum activity at a dosage of 300 mg/kg bw. which was comparable with the standard drug.

Conclusion: The results signify promising activity of ethanolic leaf extract of *B. montana* against formalin induced inflammation in albino rats. The extract was also found to exhibit appreciable antioxidant activity.

Keywords: *Barleria montana*, Formalin, Inflammation, Indomethacin.

INTRODUCTION

Inflammation, despite being a defense mechanism can induce, maintain or aggravate many diseases such as asthma, arthritis and other autoimmune diseases with its complex events and mediators involved in the inflammatory reaction [1]. Over the years the role of inflammation in the pathogenesis of atherosclerosis [2], insulin resistance [3], tumour progression and that many cancers arise from sites of infection, chronic irritation and inflammation [4] have been well documented. Growing evidence also shows that inflammation not only contributes to cancer development, but also affects chemotherapy efficacy and resistance [5]. In such cases, the defense reaction themselves may cause progressive tissue injury and hence, anti-inflammatory or immunosuppressive drugs may be necessary to modulate the inflammatory process [6].

Medications that work to reduce inflammation come in two major categories: Steroids and Non-steroidal anti-inflammatory drugs (NSAIDs). However, while long-term use of corticosteroids are known to cause several severe side effects like hyperglycemia, insulin resistance, diabetes mellitus and osteoporosis [7], prolonged use of NSAIDs can injure the gastric and duodenal mucosa leading to considerable morbidity and mortality [8]. These negative effects of commercial anti-inflammatory drugs make them untenable for long-term treatment of inflammation and have prompted the need for alternative anti-inflammatory medicines with the highest efficacy and less or no side effects.

Encompassing approximately 6000 medicinal plant species, the medicinal flora of Asia and Pacific comprises a fantastic source of pharmacologically active products and the number of plant species principally used for the treatment of inflammation can be estimated to be more than 380 [9]. Phyto compounds from plants such as polyphenols, flavonoids, terpenes, quinines, catechins, and alkaloids have been screened for their capacity to modulate the expression of pro-inflammatory signals thereby assessing their capacity as anti-inflammatory agents. However, there are many more medicinal plants whose anti-inflammatory potential has not yet been explored.

B. montana (Acanthaceae) is an erect herb, growing on exposed slopes and among rocks in the hills predominantly found in the mountains of Western Ghats and sparsely in Eastern Ghats. Alkaloids, terpenoids, flavonoids, tannins, coumarin, sterol and quinine have been reported in this plant with flavonoids and irudoid glycosides being the predominant phytoconstituents [10]. Even though *B. montana* has been traditionally used for centuries for treating wounds, diabetes, cough, inflammation and is also known to possess hepatoprotective activity [10] its anti-inflammatory activity has not been explored. Hence the present study aims to evaluate the anti-inflammatory activity of *B. montana* against formalin induced inflammation.

MATERIALS AND METHODS

Plant material

Fresh leaves of *B. montana* Wight & Nees were collected from Kolli Hills, Eastern Ghats, identified by Prof. Dr. P. Jayaraman, Director, Institute of Herbal Botany, Plant Anatomy Research Centre, Chennai, Tamilnadu and authenticated with the specimens deposited at RAPINAT Herbarium (RHT), Department of Botany, St. Joseph's college, Tiruchirappalli.

Preparation of plant extract

The plant material (Leaf) was shade dried and coarsely powdered. About 500 g of plant material was soaked in ethanol for 48h. After 48h of soaking the solvent was distilled off under reduced pressure and dried in vacuum condition.

Experimental animals

Wistar strain of albino rats weighing 120g-150g were obtained from Tamilnadu Veterinary and Animal Sciences University, Chennai. They were fed with standard rat chow pellet obtained from Sai Durga Food and Feeds, Bangalore, India and water ad libitum. Animals were maintained in a standard animal house in a controlled environment (temperature 25+2 °C and 12h dark/light cycle). The study was conducted after obtaining the necessary clearance from Institutional Animal Ethical Committee.

Experimental design

Group I	Normal male albino wistar rats
Group II	Received sub-plantar injection of 0.1 ml/kg bw. of formalin
Group III	Pretreated with ethanolic leaf extract of *B. montana* (100 mg/kg bw.) (Single dosage of formalin on 30th day)
Group IV	Pretreated with ethanolic leaf extract of *B. Montana* (200 mg/kg bw.) (Single dosage of formalin on 30th day).
Group V	Pretreated with ethanolic leaf extract of *B. Montana* (300 mg/kg bw.) (Single dosage of formalin on 30th day).
Group VI	Treated with formalin+standard drug indomethacin (25 mg/kg bw.)

Measurement of paw edema

The increase in paw edema was measured by Vernier caliper method [11]. The paw thickness was measured before and 6 days after induction of inflammation by using Vernier caliper. The difference in paw thickness after and before induction of inflammation was calculated and presented as a mean increase in paw thickness (mm). The ability of anti-inflammatory drug to suppress paw inflammation was expressed as a percentage of inhibition of paw edema [12] and this percentage can be calculated according to the following equation:

Percentage of inhibition (%) = 100 × (1-X/Y).

Where, X = mean increase in paw volume, thickness or weight of treated rats

Y = mean increase in paw volume, thickness or weight of control rats.

Biochemical investigation

The levels of enzymatic antioxidants like reduced glutathione (GSH) [13], glutathione peroxidase (GPx) [14], superoxide dismutase (SOD) [15] and non-enzymatic antioxidant lipid peroxide (LPO) [16] were assessed. Serum levels of marker enzymes aspartate transaminase (SGOT) [17], alanine transaminase (SGPT) [17], alkaline phosphatase (ALP) [17] and creatine kinase (CK) [18] were estimated.

Haematological investigation

The level of Hemoglobin (Hb) [19] was determined and enumerations of red blood cells (RBC), white blood cells **(**WBC) [19], neutrophils, eosinophils, lymphocytes and basophils [20] were also done.

Histopathological studies

Histology of the liver tissues was performed by the method of [21].

Statistical analysis

All the results were expressed as mean±Standard Error of Mean±SEM. The data was statistically analyzed by one–way analysis of variance (ANOVA). P values<0.05 were considered as significant.

RESULTS

Effect of ethanolic leaf extract of *B. montana* on paw thickness

In the present study, subcutaneous injection of diluted formalin into a hind paw elicited a biphasic pattern of pain-related behaviors, an early short-lasting neurogenic phase followed by a second and more sustained inflammatory phase when compared with normal control groups. However, pretreatment with ethanol leaf extract of *Barleria montana* showed a significant inhibition ($p<0.05$) in the late phase of formalin induced pain in a dose dependent manner which was well comparable with the standard indomethacin (table 1).

Table 1: Effect of ethanolic leaf extract of *B. montana* on paw thickness

Groups	Paw thickness (cm^2)
Group I	0.93±1.30*
Group II	1.61±1.22^{*,**,a}
Group III	1.25±1.58
Group IV	1.09±1.78
Group V	0.94±1.30^{**,a,b}
Group VI	0.93±0.36 ^{a,b}

*-Significant when compared between Group 1 and Group 2 ($p\leq 0.05$, n=6), **-Significant when compared between Group 2 and Group 5 ($p\leq 0.05$, n=6), a-Significant when compared between Group2, Group 5 and Group 6 ($p\leq 0.05$, n=6) , b-Non significant when compared between Group 5 and Group 6 ($p\leq 0.01$, n=6)

Effect of ethanolic leaf extract of *B. montana* on the antioxidant status of the experimental models

In the present study formalin induced experimental animals showed a decrease in the levels of antioxidant enzymes which had a direct impact on the levels of lipid peroxide (LPO) when compared with normal control groups indicating tissue damage. Pretreatment with ethanolic leaf extract of *B. montana* resulted in a significant increase ($p<0.05$) in the enzymatic antioxidant values and decrease in the LPO levels when compared with the standard drug treated groups, which are an indication of the antioxidant property of *B. montana* (table 2).

Table 2: Effect of ethanolic leaf extract of *B. montana* on antioxidant status and LPO levels

Groups	Superoxide dismutase (mM of epinephrine oxidized/min/g tissue)	Glutathione peroxidase (µmoles of GSH oxidised/min/g tissue)	Reduced glutathione (mg/g tissue)	Lipid peroxide (nmoles of mda/g tissue)
Group I	12.19±0.07*	4.85±0.04*	18.15±0.01*	215.41±2.40*
Group II	4.60±0.08^{*,**,a}	1.16±0.05^{*,**,a}	8.66±0.60^{*,**,a}	636.20±1.58^{*,**,a}
Group III	7.46±0.05	2.40±0.05	12.23±0.07	561.85±1.78
Group IV	9.87±0.07	4.12±0.04	16.35±0.07	425.67±1.51
Group V	11.87±0.05^{**,a,b}	4.71±0.03^{**,a,b}	17.98±0.05^{**,a,b}	241.26±1.09^{**,a,b}
Group VI	12.02±0.06 ^{a,b}	4.93±0.03 ^{a,b}	18.07±0.05 ^{a,b}	217.25±1.78 ^{a,b}

*-Significant when compared between Group 1 and Group 2 ($p\leq 0.05$, n=6), **-Significant when compared between Group 2 and Group 5 ($p\leq 0.05$, n=6), a-Significant when compared between Group2, Group 5 and Group 6 ($p\leq 0.05$, n=6) , b-Non significant when compared between Group 5 and Group 6 ($p\leq 0.01$, n=6)

Effect of ethanolic leaf extract of *B. montana* on Hb, RBC and WBC levels

Decreased levels of Hb and RBC were well observed in formalin induced experimental rats when compared with normal control groups which however on pretreatment with ethanolic leaf extract of *B. montana* in a dose dependent manner showed significant progress ($p<0.05$) in the levels of Hb and RBC which was well comparable with the standard drug treated rats (table 3). Similar results were observed in WBC levels.

Table 3: Effect of ethanolic leaf extract of *B. montana* on Hb, RBC and total WBC levels

Groups	Hb (g %)	RBC millions of cells/mm^3	Total WBC 1000's of cells/mm^3
Group I	13.21±2.20*	5.87±0.12*	4.01±4.56*
Group II	9.16±1.45^{*,**,a}	3.33±0.25^{*,**,a}	8.12±5.63^{*,**,a}
Group III	10.12±0.16	4.51±0.10	7.23±6.16
Group IV	11.97±0.23	4.93±0.09	5.64±2.96
Group V	12.97±0.07^{**,a,b}	5.57±0.12^{**,a,b}	4.31±2.86^{**,a,b}
Group VI	13.30±0.24^{a,b}	5.87±0.05 ^{a,b}	4.17±3.03 ^{a,b}

*-Significant when compared between Group 1 and Group 2 ($p\leq 0.05$, n=6), **-Significant when compared between Group 2 and Group 5 ($p\leq 0.05$, n=6), a-Significant when compared between Group2, Group 5 and Group 6 ($p\leq 0.05$, n=6) , b-Non significant when compared between Group 5 and Group 6 ($p\leq 0.01$, n=6)

Effect of ethanolic leaf extract of *B. montana* on neutrophils, lymphocytes, eosinophils and basophil levels

The present investigation showed elevated levels of neutrophils, lymphocytes, eosinophils and basophils in formalin induced experimental animals when compared with normal control groups. However, there was a significant reduction ($p<0.05$) in the population of neutrophils, eosinophils, lymphocytes and basophils in experimental rats pretreated with ethanolic leaf extract of *B. montana* which was well comparable with the standard drug treated groups (table 4).

Effect of ethanolic leaf extract of *B. montana* on serum marker enzyme and creatine kinase levels

In the present study, increased levels of SGOT, SGPT and ALP were observed in formalin induced animals when compared with normal control groups which subsequently decreased ($p<0.05$) on dose dependent pretreatment with ethanolic leaf extract of *B. montana* which was well comparable with the standard drug treated groups indicating inhibition of inflammation. Creatine kinase, was found to be increased in formalin induced groups but was subsequently decreased on pretreatment with ethanolic leaf extract of *B. montana* at dose levels of 100, 200, 300 mg/kg bw. whose effect was on par with the standard drug treated groups (table 5).

Histopathological studies

Histopathological sections of normal rat paw showed a normal architectural pattern of cartilage and osteoblast. The edematous tissue section of formalin induced rats showed a loss of cartilage, osteoblast hyperplasia and accumulation of abundant mono and poly morpho nuclear cells in the joint and congestion of vessels and loss of marrow and disarray, which were everted back to normalcy on pretreatment with ethanolic leaf extract of *B. montana* (fig. 1).

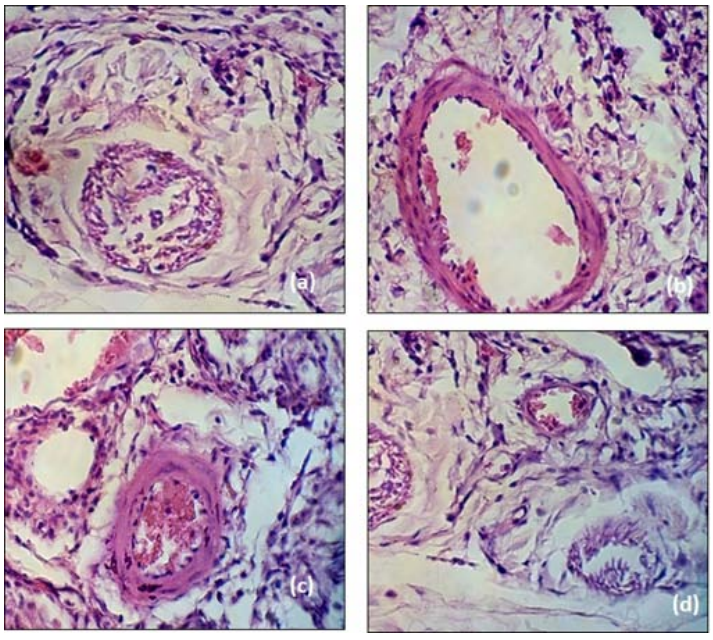

Fig. 1: **Histopathological studies–Inflammation**

(a) Normal hind paw of albino wistar rats showing normal architectural pattern of cartilage and osteoblast. (b) Edematous tissue section of formalin induced rats showing a loss of cartilage, osteoblast hyperplasia, and accumulation of abundant mono and poly morpho nuclear cells in the joint and congestion of vessels and loss of marrow and disarray. (c) Ethanolic leaf extract of *B. montana* (300 mg/kg bw.) pretreated albino wistar rats showing moderate reduction in the cartilage. No osteoblast hyperplasia, moderate accumulation of mono and poly morpho nuclear cells in the joint, healing of vessels and gain of marrow in formalin induced experimental rats (d) Edematous tissue section of formalin induced rats treated with standard drug indomethacin showing normal architectural pattern of cartilage and osteoblast. Magnification-250 X,Stain: Haematoxylin-Eosin stain.

Table 4: Effect of ethanolic leaf extract of *B. montana* on neutrophils, lymphocytes, eosinophil and basophil levels

Groups	Neutrophils (%)	Lymphocytes (%)	Eosinophils (%)	Basophils (%)
Group I	54.36±0.17*	41.13±0.08*	4.16±0.06*	1.16±0.06*
Group II	87.40±1.67*,**,a	63.52±0.17*,**,a	7.79±0.07*,**,a	4.18±0.07*,**,a
Group III	79.36±0.23	56.48±0.27	6.19±0.06	3.19±0.06
Group IV	66.85±0.22	50.80±0.05	5.73±0.08	2.73±0.08
Group V	60.37±0.18**,a,b	44.61±0.17**,a,b	3.98±0.09**,a,b	1.98±0.09**,a,b
Group VI	56.26±0.33 a,b	42.13±0.06 a,b	3.51±0.06 a,b	1.51±0.06 a,b

*-Significant when compared between Group 1 and Group 2 (p≤ 0.05, n=6), **-Significant when compared between Group 2 and Group 5 (p≤ 0.05, n=6), a-Significant when compared between Group2, Group 5 and Group 6 (p≤ 0.05, n=6), b -Non significant when compared between Group 5 and Group 6 (p≤0.01, n=6)

Table 5: Effect of ethanolic leaf extract of *B. montana* on Serum marker enzyme and Creatine kinase levels

Groups	SGOT (IU/l)	SGPT (IU/l)	ALP (IU/l)	CK(IU/l)
Group I	37.97±1.69*	34.16±1.39*	99.77±1.89*	278.56±1.78*
Group II	89.89±1.96*,**,a	78.89±1.77*,**,a	229.97±1.75*,**,a	479.99±1.69*,**,a
Group III	76.79±1.55	73.86±1.57	196.83±1.81	384.76±1.86
Group IV	69.99±1.79	55.69±1.97	186.36±1.87	318.75±1.72
Group V	59.88±1.97**,a,b	48.59±1.87**,a,b	141.47±1.98**,a,b	269.46±1.56**,a,b
Group VI	38.45±1.52 a,b	33.39±1.89 a,b	110.85±1.76 a,b	238.45±1.78a,b

*-Significant when compared between Group 1 and Group 2 (p≤ 0.05, n=6), ** -Significant when compared between Group 2 and Group 5 (p≤ 0.05, n=6), a-Significant when compared between Group2, Group 5 and Group 6 (p≤ 0.05, n=6), b -Non significant when compared between Group 5 and Group 6 (p≤0.01, n=6)

DISCUSSION

The formalin method is an important animal model in the study of acute long-lasting inflammatory pain. A reduction in paw swelling size is a good index in determining the protective action of anti-inflammatory agents [22]. In the present study, sub-cutaneous injection of diluted formalin into a hind paw elicited a biphasic pattern of pain-related behaviors, an early short-lasting neurogenic phase followed by a second and more sustained inflammatory phase. However, pretreatment with ethanolic leaf extract of *B. montana* showed a significant inhibition in the late phase of formalin induced pain in a dose-dependent manner which was well comparable with the standard indomethacin and also corroborated with the findings of [22].

Free radical production that occurs during development of inflammation in the cartilage leads to decreased GSH, GPx, and SOD levels as a result of their consumption during oxidative stress and cellular lysis. Reactive oxygen species (ROS) destroy antioxidant systems leading to oxidative damage and lipid peroxidation because of the reduced endogenous antioxidant defense system [23]. This decrease contributes to increased cellular damage by favoring an attack by free radicals. The present study has shown similar findings with depleted levels of antioxidants and increased level of LPO in formalin induced rats showing loss of cellular integrity, but pretreatment with ethanolic leaf extract of *B. montana* significantly reversed the depleted levels of antioxidants, probably by competing with scavenging of free radicals and as a result helped to maintain the integrity of cellular membranes in the injured cartilage. Flavonoids, which were found to be higher in ethanolic leaf extract of *B. montana* can act as an antioxidant because of its ability to scavenge free radicals.

Cytokines produced by neutrophils are good indicators of progressive inflammation as neutrophils are important sources of free radicals at the site of inflammation [24]. A notable increase in the population of neutrophils in the present study was suggestive of propagation of inflammation, which was well reduced and reverted close to normalcy in groups pretreated with ethanolic leaf extract of *B. montana* with increasing concentrations. Eosinophils are granulated molecules that release lipid derived mediators which stimulate responses in tissues, which are indicative of inflammation. They act as important marker molecules in infectious conditions. The present investigation showed an elevated level in their count indicative of the release of cytokines and interleukins in formalin induced experimental animals accounting to inflammation. However, the pretreated groups showed a good reduction in the population of eosinophils that indicate the restoration of normal tissues. Lymphocyte is the predominant cell in chronic inflammation. It can cause permanent distortion of the tissue. Elevated levels of lymphocytes were observed in formalin induced experimental rats indicative of tissue damage,which was reversed in pretreated groups. Basophils appear in many specific kinds of inflammatory reactions, particularly those that cause allergic symptoms. They can be found in unusually high numbers at sites of inflammation which was well observed in the formalin induced rats [25]. However, the pretreated groups showed a good reduction in the population of basophils. The reduction in the population of neutrophils, eosinophils, lymphocytes and basophils in formalin induced rats pretreated with ethanolic leaf extract of *B. montana* shows its anti-inflammatory property.

Low concentrations of Hb and RBC are important patho-physiological alterations noted in chronic inflammatory diseases such as rheumatoid arthritis, which is commonly associated with conditions of anoxia. This depletion in the levels of Hb and RBC was well observed in formalin induced experimental rats suggestive of the fact that there was a low supply of oxygen to the injured tissues. Such a decline in the levels of Hb and RBC was also reported by Hull *et al.*, 2003 [26]. Pretreatment with ethanolic leaf extract of *B. montana* in a dose-dependent manner showed good progress in the levels of Hb and RBC indicating less necrosis and regeneration of normal tissues, which was well comparable with the standard drug treated rats.

WBC plays an inevitable role in body's defense mechanism. The increase in WBC count during inflammation may be due to the release of interleukins, responsible for the production of both granulocytes and macrophage colony stimulating factor [27]. In the present study, the level of WBC was found to be higher in formalin induced experimental animals. Pretreatment with ethanolic leaf extract of *B. montana* at the dose levels of 100, 200, 300 mg/kg bw. effectively reduced the WBC count that indicated recovery from the inflammatory process.

Most of the anti-inflammatory drugs exert their beneficial effect by inhibiting either release of lysosomal enzymes or by stabilizing lysosomal membrane, which is one of the major events responsible for the inflammatory process. The increased levels of lysosomal enzymes like SGOT, SGPT and ALP in case of inflammation are good indicators of liver and kidney impairment [28]. In the present study, pretreatment with ethanolic leaf extract of *B. montana* at dose levels

of 100, 200 and 300 mg/kg bw. decreased the levels of SGOT, SGPT, ALP that had increased after formalin induction.

Creatine kinase is an important serum marker enzyme that is often used as an important diagnostic tool to ascertain the severity of necrosis or tissue damage. A rapid increase in the concentrations of creatine kinase is directly related to irreversible tissue injury [29]. A two-fold increase in formalin induced experimental animals was observed in the present study, indicative of tissue damage which on pretreatment with ethanolic leaf extract of *B. montana* showed reversion to normalcy in a dose-dependent manner.

Histological studies carried out in rats following pretreatment with ethanolic leaf extract of *B. montana*, especially at a dosage of 300 mg/kg bw. revealed down regulation of the inflammatory infiltration with associated tissue damages showing reduction in the cartilage, no osteoblast hyperplasia, moderate accumulation of abundant mono and poly morpho nuclear cells in the joint, healing of vessels and gain of marrow in formalin induced rats as compared to untreated rats. This change may also be attributed due to restoration of the antioxidant status reflected in the biochemical parameters [25].

CONCLUSION

The results obtained in the present study clearly indicate a promising anti-inflammatory activity of ethanolic leaf extract of *Barleria montana* against formalin induced inflammation in albino rats. The extract was also found to exhibit appreciable antioxidant activity which can be considered as a contributing factor to its biological activity. However, further studies are needed to isolate the phyto compound(s) present in this plant and to evaluate their anti-inflammatory activity.

CONFLICT OF INTERESTS

The authors declare that they have no conflicts of interest concerning this article.

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