ANTI-INFLAMMATORY ACTIVITY OF EXTRACTS AND ISOLATED ALKALOIDAL FRACTION FROM LEAVES OF *BRYOPHYLLUM PINNATUM*

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Summary

The water, petroleum ether, chloroform, acetone and methanolic extracts and the alkaloidal fraction of methanolic extract from the leaves of *Bryophyllum pinnatum* were evaluated for anti-inflammatory activity against carrageenan induced rat paw edema, formalin induced paw edema, changes in lysosomal enzyme (cathepsin-d) levels and DPPH free radical scavenging activity. The methanolic extract of *Bryophyllum pinnatum* leaves and the alkaloidal fraction of methanolic extract showed significant ($P<0.001$) anti-inflammatory activity in all (early, intermediate and late) stages of inflammation against carrageenan induced paw edema, significant ($p<0.01$) reversal of changes in lysosomal enzyme (cathepsin-d) levels during formalin induced paw edema and significant DPPH free radical scavenging activity ($p<0.001$ for methanolic extract and $p<0.05$ for alkaloidal fraction). Thus the alkaloidal fraction may be majorly responsible for the anti-inflammatory activity of the methanol extract and further quantitative separation and activities studies may help isolate an anti-inflammatory alkaloid compound from the leaves.

Keywords*:* *Bryophyllum pinnatum;* Anti-inflammatory activity; Carrageenan; Edema, lysosomal enzymes.

Introduction

Bryophyllum pinnatum (Crassulaceae) is a widely used medicinal plant in traditional system with a wide range of biological activities. It is a succulent glabrous herb 0.3-1.2 m high, stems obtusely 4-angled, older stem light colored, younger stem reddish speckled white, leaves variable, decussate, with the lower leaves usually simple or occasionally compound, the upper usually 3-5 or 7-folliolate, long-petiole, petiole united by a ridge round the stem. Leaflets are ovate or elliptical, crenate or serrate and are rich in alkaloids, triterpenes, glycosides, flavanoids, steroids and lipids (1). The leaves contain a group of chemicals called bufadienolides which are very active showing similarity in structure and activity as two other cardiac glycosides, digoxin and digitoxin (2). The leaves are used as astringent, refrigerant, emollient, mucilaginous, haemostatic, vulnerary, depurative, constipating, anodyne, carminative, disinfectant and tonic**.** They are useful in vitiated conditions of *pitta* and *vata, as perayurvedic system,* for haematemesis, haemorrrhoids, menorrhagia, cuts and wounds, discolourations of the skin, boils, sloughing ulcers, ophthalmia, burns, scalds, corn, diarrhea, dysentery, vomiting (3).
This study was undertaken to evaluate the anti-inflammatory activity of the water, petroleum ether, chloroform, acetone and methanol leaf extracts and alkaloidal fraction of *Brophyllum pinnatum* by various methods.

Material and Methods

Collection of plant material:

The leaves of *Bryophyllum pinnatun* were collected from the herbal garden of B.B.D.N.I.T.M, Lucknow, U.P., India, and were identified and a voucher specimen deposited in the taxonomy division, N.B.R.I, Lucknow, U.P., India (voucher specimen ref. no. NBRI/CIF/Re/08/2008/32**)**.

Animals

Sprague Dawley rats (120-150 g) and Swiss albino mice (40-50 g) were used in these experiments. They were housed in polypropylene cages and were kept in room temperature maintained under controlled condition. All animals were fed with a standard diet ad libitum and had free access to drinking water. Animal study was performed in animal house of B.B.D.N.I.T.M., Lucknow, India (Reg No- 645/02/c/CPCSEA) in accordance with approved norms and protocols.

Extraction and column fractionation:

Preparation of extracts

The dried and powdered leaves were successively extracted with various solvents in Soxhlet apparatus starting with non polar and ending with polar solvents. The solvent extracts were concentrated separately using Buchi rota evaporator and dried under vaccum. The dried extracts were preserved in dessicator until further use.

Column fractionation of methanolic extract

The methanol extract was subjected to column chromatography on a silica gel column (60-80 mesh) with methanol as a stationary phase. After allowing proper time for segregation and stabilization of fractions on the column, first elution was done using chloroform (50 ml) after leaving the solvent for 10 minutes in the column for sufficient partitioning and then collection of 10 ml fractions in 5 test tubes at the rate of 20-25 drops per minute. All the fractions in the test tubes were subjected to chemical test and chromatography (TLC) for identification of components. Further elution was similarly carried out using chloroform:methanol (70:30), chloroform:methanol (50:50), chloroform:methanol (30:70) and pure methanol. Mobile phases used For TLC, were as follows:

- Ethyl acetate: Methanol: Water (100: 13.5: 10)
- Ethyl acetate: Methanol: Water (81:11: 8)
- Ethyl acetate: Formic acid: GAA: Water (100:11: 11: 26)

All fractions showing single spot TLC were analyzed spectroscopically for UV λ_{max} (Double beam UV-VIS Spectrophotometer, 2201, Systronics) to investigate nature of constituents. Fractions showing single spot TLC with similar Rf. values and UV scan were combined and subjected to precipitation of components by using various solvents of appropriate polarity, pH combination and by lyophilisation. All precipitated compounds were subjected to IR (FT-IR, SHIMADZU) to confirm the nature of functional group. After chloroform, second elution was done by adding 50 ml chloroform: methanol (70: 30) and collecting 5×10 ml fractions, subjected to TLC, UV scanning and precipitation as above. Fractionation was continued by using chloroform: methanol (50: 50), chloroform: methanol (30: 70), and fractions similarly obtained and treated as above. Lastly 50 ml methanol was used as eluent and fractions were collected.

Acute toxicity study

Mice were divided into six groups of ten animals each. One group served as a control and received 0.9% NaCl alone (10ml/kg) given intraperitoneally (i.p.), while the remaining five groups were treated with increasing doses of extracts intraperitonealy. The mortality rate within a 72 h period was determined and the LD_{50} was estimated according to K Ghedira et al (4). According to the results of the acute toxicity tests, the doses for administration were chosen for experiment.

Anti-inflammatory activity (5)

Effect of extracts and fractions of Bryophyllum pinnatum on carageenan- induced paw edema in rats:

The anti-iflammatory activity of various extracts and fractions of *Bryophyllum pinnatum* on carageenan-induced paw edema was determined. The animals were divided into groups consisting of six rats each. The control group received 0.2ml of 2.5% DMSO and 2.5% tween-20, the standard group received indomethacin (10 mg/kg) and the test groups received the various leaf extracts or the alkaloidal fraction i.p. Fifteen minutes after intraperitoneal administration of different

substances, 0.1ml of 1 % carageenan suspension was injected to all animals in the right hind paw. The paw volume upto the tibiotarsal articulation was measured using a Ugo Basil Plethysmometer. The measurements were done at 0 hr (before carageenan injection) and 1, 2, 3, 4 and 5 h later. The edema was expressed as an increase in the volume of paw, and the percentage of inhibition for each rat and each group was obtained as follows.

$$\text{Percentage of inhibition} = \frac{(V_t - V_o)\ \text{control} - (V_t - V_o)\ \text{treated}}{(V_t - V_o)\ \text{control}} \times 100$$

Effect of extract/fractions on lysosomal enzyme (cathepsin-d) in formalin induced inflammation:

Anti-inflammatory effects of extract/fractions were investigated in an aseptic arthritis model, which was induced by formaldehyde. Rats were sacrificed on the 7th by decapitating. Plasma was separated from the blood collected with EDTA. Blood drawn was centrifuged for 10 min at 2000 rpm and separated plasma was used for estimation of cathepsin-D which is a lysosomal enzyme which causes degrative changes during inflammation.

Cathepsin–D: It was determined by the method of Etherington (6) in which cathepsin -D liberates TCA soluble poducts (which was estimated for tyrosine content by folins phenol reagent) on incubation with 15% buffered hemoglobin substrate (buffered with Sodium acetate buffer (pH 3.6). The substrate mixture (0.9 ml) was incubated with tissue homogenate (0.5 ml) at 37^{o}C for 2 hrs, reaction arrested with 1ml 10 % TCA, mixture centrifuged and 1 ml supernatant mixed with 1 ml 5% Sod. Hydroxide and 4.5 ml alkaline Copper reagent and 0.5ml Folin's Phenol reagent and absorbance noted at 620 nm after 15 mins along with standard Tyrosine solution (10mg/100ml) in 0.1 N. HCl treated similarly, also performing a blank at the same time. The enzyme activity is expressed as μ mol. of tyrosine liberated /mg protein/min.

Antioxidant activity of extracts/fractions by in-vitro method of DPPH free radical scavenging effect: (7)

Free radical scavenging activity of the crude extracts of leaves of *Bryophyllum pinnatum* and the alkaloidal feaction of methanol extract was established by measuring the decrease in absorbance of 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical. Equal volume of 10^{-4} and 0.01% (w/v) solution of vitamin-E as a natural antioxidant and 3-tert-butyl-4-hydroxyanisol (BHA) as a synthetic antioxidant were mixed and incubated at 30^{O} C° for 15 min in the dark. Final mass ratio of the components in the mixture was 1/25 for DPPH/ *Bryophyllum pinnatum* extract and 1/0.5 for DPPH/reference. Control solution containing equal volume of DPPH and methyl alcohal was used as blank (A_{blank}). Methyl alcohal crude extract mixture was also prepared and its absorbance at 517 nm was recorded against methyl alcohal. Actual absorbance originated from the inhibition of DPPH (A_{517}) was evaluated by substracting the absorbance of methyl alcohal crude extract (or reference compound) mixture, from absorbance of corresponding DPPH-crude extract (or reference compound) mixture at 517 nm. Decrease in absorbance indicated the antioxidant activity. Radical scavenging activity was expressed as percentage inhibition of DPPH and estimated by following formula reported in the Reference.

$$\% \text{ inhibition of DPPH} = 1 - \frac{A_{517}}{A_{blank}} X100$$

Statistical analysis

All data were analyzed statistically by using one way new man-Keulis ANOVA with repeated measures. The level of significance was set at $p<0.05$.

Results and Discussion

Acute toxicity study

Intraperitoneal administration of different extracts and fractions of leaves at 600 mg/kg did not produce any mortality, while 20% animals died at 700 mg/kg and 100% mortality was reported at 900 mg/kg body weight dose. Thus LD_{50} was calculated as 742 mg/kg body weight. The dosing was kept well below this dose.

Anti-inflammatory activity

Effect of extracts and fractions of Bryophyllum pinnatum on carageenan- induced paw edema in rats

The intraperitoneal administration of the various extracts and fractions of leaves of *Bryophyllum pinnatum* reduced significantly the paw edema induced by carageenan by 7.54 % (petroleum ether), 9.43 % (chloroform), 13.20 % (acetone), 18.54 % (aqueous), 71.69 % (alkaloidal fraction), three hours after the injection of a noxious agent. After the intraperitoneal administration of the methanolic extract, significant activity was observed at the third hour after carageenan injection, with 72.64% reduction in paw volume. Standard drug (indomethacin) decreased paw edema by 65.09 % at the third hour.

Table I: Effect of extracts/fraction on paw edema induced by carrageenan in rats

Group (s)	Dose (mg /kg)	Mean paw volume ± SEM(ml) and % Inhibition (P.I)					
		Before carrageenan	Time after Carrageenan injection				
			+1h	+2h	+3h	+4h	+5h
I.	-	0.80 ± 0.03	1.23± 0.07	1.60 ± 0.09	1.86 ± 0.07	1.95 ± 0.05	1.80 ± 0.04
II.	10	0.83 ± 0.01	1.01 ± 0.03*** (58.13)	1.12 ± 0.02*** (63.75)	1.20 ± 0.03*** (65.09)	1.28 ± 0.04*** (60.86)	1.00± 0.06*** (53.01)
III.	500	0.82 ± 0.03	1.16 ± 0.003 (20.93)	1.54 ± 0.002 (10.0)	1.80 ± 0.004 (7.54)	1.92 ± 0.002 (4.34)	1.76 ± 0.004 (6.0)
IV.	500	0.82 ± 0.03	1.14 ± 0.004 (25.58)	1.50 ± 0.003 (15.0)	1.78 ± 0.006 (9.43)	1.90 ± 0.004 (6.08)	1.78 ± 0.003 (4.0)
V.	500	0.84 ± 0.01	1.10 ± 0.003* (39.53)	1.48 ± 0.007 (20.0)	1.76± 0.002 (13.20)	1.88 ± 0.003 (9.56)	1.68 ± 0.007 (16.0)
VI.	500	0.85 ± 0.01	1.04 ± 0.001*** (55.80)	1.02 ± 0.003*** (77.50)	1.14 ± 0.008***(72.64)	1.22 ± 0.006*** (67.82)	1.25 ± 0.002***(60.01)
VII.	500	0.82 ± 0.05	1.08± 0.004 (40.25)	1.30± 0.007 (22.36)	1.50± 0.047 (18.54)	1.72± 0.021 (14.23)	1.68± 0.074 (18.14)
VIII.	500	0.80 ± 0.02	1.02 ± 0.003*** (48.83)	1.00 ± 0.004*** (75.0)	1.10 ± 0.006***(71.69)	1.20 ± 0.002*** (65.21)	1.22 ± 0.004***(58.0)

Group-I Diseased control; Group-II Diseased rat treated with standard (indomethacin) drug; Group-III Diseased rat treated with pet-ether extract, Group-IV Diseased rat treated with chloroform extract; Group-V Diseased rat treated with acetone extract; Group-VI Diseased rat treated with methanol extract; Group-VII Diseased rat treated with aqueous extract; Group-VIII Diseased rat treated with alkaloidal fraction ,*$p<0.05$, **$p<0.01$, ***$p<0.001$, all values in Mean ± SEM

Carrageenan-induced rat paw edema is a suitable test for evaluating anti-inflammatory drugs and has been frequently used to assess the effects of natural products against inflammation (8, 9, 10, 11). It is reported that carrageenan induces inflammation by enhancing PGE_2 release and leukocytes migration. It also enhances the expression of COX-2 in epidermis, skeletal muscle and inflammatory cells in air-pouch models, suggesting that prostaglandin E_2 production is linked through the expression of COX-2 (12). Inflammation induced by carrageenan involves three distinct phases of the release of the mediator, including serotonin and histamine in the first phase (0 - 2 h), kinins in the second phase (3 h), and prostaglandin in the third phase (>4 h) (13).The alkaloidal fraction from the methanolic extract significantly inhibited paw edema induced by carrageenan in the first phase and the effect was prominent up to three hours, suggesting its inhibitory effect on the release of histamine and/or serotonin involved in the first phase of inflammation. It was also effective in all phases showing its inhibition of kinins as well as arachidonic acid. This antiedematous response was also significantly reduced in rats pre-treated with indomethacin, a known cyclooxygenase inhibitor. Methanol extract fraction showed prominent inhibition till the end of five hours showing that their effect was due to inhibition of histamine in the first phase as well as partly due to inhibition of kinins.

We may conclude that these results support the traditional use of this plant in some inflammatory and painful conditions and confirm the presence of active chemical compounds in the alkaloidal fraction from the methanolic extract of the leaves related to these activities.

Effect of extract/fractions on lysosomal enzyme (cathepsin-d) level in formalin induced inflammation in rats:

Out of petroleum ether, chloroform, acetone, methanol and aqueous extracts from *Bryophyllum pinnnatum* leaves, methanol extract was more significant ($p<0.01$) in percentage inhibition of lysosomal enzymes. The alkaloidal fraction from the methanol extract of *Bryophyllum pinnatum* leaves significantly inhibited ($p<0.01$) lysosomal enzymes in animals induced with formaldehyde edema.

Table-II: Effect of extracts/fractions on lysosomal enzyme (cathepsin-D) in formalin induced inflammation in rats

Group(s)	Cathepsin (µ mol. Of tyrosine liberated/mg protein/min.	
	Absorbance	**% Inhibition of absorbance**
I.	0.43 ± 0.03	--
II.	0.98 ± 0.06	--
III.	0.48 ± 0.02***	51.02
IV.	0.82 ± 0,04	16.32
V.	0.68 ± 0.02*	30.61
VI.	0.76 ± 0.08	22.44
VII.	0.50 ± 0.03**	48.90
VIII.	0.64 ± 0.023	34.69
IX.	0.56 ±0.02**	42.85

Group I- Normal control; Group II- Formalin induced diseased control; Group III- Diseased rat treated with standard (Diclofenac) drug; Group IV- Diseased rat treated petroleum ether extract 500mg/kg; Group V- Diseased rat treated chloroform extract 500mg/kg; Group VI- Diseased rat treated with acetone extract 500mg/kg; Group VII- Diseased rat treated methanol extract 500mg/kg; Group VIII- Diseased rat treated aqueous extract 500mg/kg; Group-IX Diseased rat treated with alkaloidal fraction 500mg/kg; *p<0.05, **p<0.01, ***p<0.001, all values in Mean ± SEM.

Alternation in lysosomal integrity and metabolism of connective tissue are the prominent features in adjuvant arthritis which is a systemic disease. Glycoproteins, glycosaminoglycans and collagen are the major macromolecules in synovial tissues of considerable importance. Glycoproteins may be involved in maintaining the structural stability of collagen fibrils and thus stabilizing the tissue and. are primarily responsible for their antigenic property in tissue transplants. The level of glycoproteins increases in arthritic rats due to the increased connective tissue activating factor, as found in inflammatory conditions.

Numerous animal tissues contain a group of cytoplasmic organelles called lysosomes which are characterized by their acid hydrolases content (14) which are extruded out into the extra cellular environment during endocytosis of the immune complexes by the leucocytes (15). Significantly increased activities of lysosomal enzymes are found in arthritic rats than control animals and their level increases in extra cellular fluid by decrease in lysosomal stability (16) and their increased release during arthritic condition alters glycoproteins and glycosaminoglycans metabolism and are also involved in the destruction of structural macromolecules in connective tissue and cartilage during rheumatoid arthritis by destroying proteoglycans and treatment of arthritic rats with alkaloidal fraction, decreased the level of glycoproteins and lysosomal enzymes, which may be due to stabilization of lysosomal membranes. This activity may give synovial damage inhibiting property to these fractions.

The alkaloidal fraction and the methanolic extract may exert their effects either by modifying the lysosomal membrane in such a way that it is capable of fusing with the plasma membranes and thereby preventing the discharge of acid hydrolases or by inhibiting the release of discharge of acid hydrolase or by inhibiting the release of lysosomal enzymes (17). An important mechanism of anti-inflammatory activity has been found to be the membrane stability-modulating effect. As the development of inflammatory process is correlated with release of lysosomal enzymes, the reduction in their release confirms the therapeutic usefulness of the said fraction, and supports further work on quantitative isolation of the components from the alkaloidal fraction and further study on other components.

Antioxidant activity of extracts/fractions by in-vitro method of DPPH free radical scavenging effect:

Methanolic extract produced a dose dependent free radical scavenging activity with the maximum activity (63.97 %) at the highest dose comparable to the standard BHT scavenging capacity. The alkaloidal fraction showed less scavenging capacity.

Table-III: Effect of extracts/fractions for their anti-oxidant activity using in-vitro method of DPPH free radical scavenging activity

Group(s)	Con. (µg /ml)	DPPH radical scavenging activity	
		Absorbance	% Inhibition of Absorbance
I.	---	1.399 ± 0.048	--
II.	1000	0.368 ± 0.0030***	73.69
III.	100	1.270 ± 0.0080	09.22
IV.	100	0.965 ± 0.040*	28.60
V.	100	1.250 ± 0.0250	10.65
VI.	100	0.504 ± 0.0450***	63.97
VII.	100	0.698 ±10.017	50.10
VIII.	100	0.701 ±0.0020*	49.89

Group I-Control blank; Group II-Standard (BHT) treated; Group III-Pet-ether extract treated; Group IV-chloroform extract treated; Group V-acetone extract treated; Group VI-methanol extract treated; Group VII-aqueous extract treated; Group VIII alkaloidal fraction treated; *$p<0.05$, **$p<0.01$, ***$p<0.001$, all values in Mean ± SEM.

Free radicals are a major cause of initiation of cellular injury. Free radicals cause oxidative stress which may lead to disease like cancer, tissue injury, aging, arthritis etc. (7), (18). Antioxidant activity is determined by the capacity of a compound to prevent damage to important cellular components by various ROS (reactive oxygen/oxidant species), for example free radical, reactive Hydroxyl species or even ferric free radicals which are oxidizing agents. An anti-oxidant compound should show antioxidant activity and prevent lipid peroxidation and damage to lipids which form important and integral part of various cellular components. At the same time these compounds should not cause any damage to other structural units of the cell for example, the carbohydrates etc.

The anti-inflammatory activities of some of the above fractions showed against various inflammatory models earlier may thus be due to the antioxidant activity which may prevent damage to the cells and thus prevent progression of inflammatory cellular damage.

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