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Analgesic, anti-inflammatory and local anesthetic activity of methanol extract of *Bryophyllum pinnatum leaves*

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Abstract
In order to scientifically evaluate some of the ethnomedical uses of *Bryophyllum pinnatum* leaves, the present study was undertaken to explore analgesic, anti-inflammatory and local anesthetic activity of *Bryophyllum pinnatum* leaves methanol extract (BPME) in experimental animal models.
The analgesic effect of the herb's methanol leaf extract was evaluated by the 'hot-plate' and 'acetic acid' test models of pain in mice. The anti-inflammatory effects of the plant's extract were investigated using Carageenan induced hind paw edema in rats and local anesthetic activity was studied using foot withdrawal reflex in frog.
Methanol extract of the plant showed significant dose depended analgesic activity up to 83.79% against chemically induced pain in mice compared to 97.93% produced by piroxicam and showed mild anti-inflammatory activity up to 43.10% against Carageenan induced hind paw edema in rats compared to 75.66% produced by piroxicam but failed to reveal any analgesic activity against thermal stimuli using hot plate method and did not show any local anesthetic activity.
The antihistaminic activity and different chemical constituents (flavonoids, polyphenols, triterpenoids) of the herb might have attributed to its analgesic and anti-inflammatory activities.

Keywords: Analgesic, anti-inflammatory, carageenan, *Bryophyllum pinnatum*

Introduction
Herbal medicine is an integral component of traditional medicine. About 80% of the world population is dependent wholly or partially on plant based drug [1]. In the present global scenario, the demand for herbal drugs is growing exponentially throughout the world mainly because of their safety, potency and cost effectiveness. So, presently, it is becoming the need of the hour to carry out extensive research on some of the medicinal plants used in traditional medicine. It is essential to blend the traditional knowledge of the folklore with experimental pharmacology for evaluating the efficacy and safety of the herbal drugs.
Plants of the genus Bryophyllum (family Crassulaceae) occur in tropical Africa, America and Asia, Hawaii, India, China, Australia and Madagascar, and have been traditionally used in these regions in multiple pathological situations [2, 3]. Previously it was reported that the leaves of *Bryophyllum pinnatum* possesses antiulcer [4], anti-inflammatory [5], hepatoprotective [2] analgesic [6], antinociceptive, antidiabetic [5], insecticidal [7], antitumorous [7], muscle relaxant and sedative [8], tocolytic [9], antihistaminic [10], antimicrobial [11] and antilithic activity [12, 13].
Different chemical constituents namely bufadienolides [7], flavonoid glycosides [14], phenols [15], polyphenols, triterpenoids of amyrin structure, phytosterols [16, 17] and organic acids [18] etc. have been identified in the preparations of Bryophyllum pinnatum plant, which are likely to have therapeutic potentials.
In order to scientifically evaluate some of the ethnomedical uses of Bryophyllum pinnatum leaves, the present study was undertaken to explore the analgesic, anti-inflammatory and local anesthetic activity of *Bryophyllum pinnatum* leaves methanol extract (BPME) in experimental animal models.

Materials and Methods
Collection of the plant materials and preparation of the methanol extract
The fresh leaves of *Bryophyllum pinnatum* Salisb. (synonym *Kalanchoe pinnata* Pers.) plant were collected from the medicinal garden of CVSc, AAU, Khanapara for the experiment after subsequent identification and authentication by Botanical Survey of India, Shillong, Meghalaya, India.

After chopping, air drying and pulverization of fresh leaves, the crude extract was obtained by soaking 500 g of air dried finely powdered leaves in 1 liter of methanol for 48 hours at room temperature. The extract was filtered through a buckner funnel using whatman no. 1 filter paper. Exactly 500 ml of extract was collected and concentrated up to 10 ml with the help of a rotary evaporator. The concentrated extract was again dried in a hot water bath at 40 ºC. The dried extract was collected, weighed and stored in an air tight vial at 4 ºC in the refrigerator.

Experimental animals

Laboratory animals like adult albino mice (15-30 g), adult albino Rats (150-230 g) and adult frogs (100-200 g) of either sex were used for the present study. All the experimental animals were kept under standard management conditions with *ad libitum* food and water supply.

Acute toxicity study

For acute toxicity study by oral route, mice were divided into 6 groups consisting of 6 mice in each group. Mice were fasted overnight and given water *ad libitum* before experiment. Tween-80 (20%) was administered orally to group-I, which was served as vehicle control whereas BPME @ 0.8, 1.0, 1.2, 1.6 and 1.8 g $Kg^{-}1$with vehicle was administered respectively to group II, III, IV, V and VI as single oral dose.

For acute toxicity study by intra-peritoneal (i.p.) route, mice were divided into 6 groups consisting of 6 mice in each group. Mice were fasted overnight and given water *ad libitum* before experiment. Tween-80 (20%) was administered intraperitoneally to group-I, which was served as vehicle control whereas BPME @ 0.5, 1, 1.5, 2 and 2.5 gKg^{-1} with vehicle was administered respectively to group II, III, IV, V and VI as single intra-peritoneal dose.

The volume administered for each dose was calculated by using the formula: V= (BW× A) / S

Where, V= volume of the extract,

A= amount of extract in $mgKg^{-1}$,

S= strength in $mgml^{-1}$ of the extract solution

BW= body weight of the animal in Kg.

Animals were observed for 48 hours for mortality and 14 days for behavioral changes.

Analgesic activity

For studding analgesic activity of BPME, hot plate method and chemical method (acetic acid writhing test) were carried out in albino mice.

Hot Plate method

The hot plate test was carried out in mice of either sex [19]. The mice were placed on the hot plate maintained at 55 ^{0}C and the time between placement on the hot plate and the occurrence of either licking of the paws or jumping off from the hot plate was recorded as the reaction time (sec). Four groups (n = 5) of mice were taken for this study. Group I served as vehicle control, group II, II and IV received BPME orally @ 100, 300 and 900 $mgKg^{-1}$, respectively. Reaction time was recorded at 0, 30 and 60 minutes after BPME treatment.

The maximum reaction time was fixed at 45 sec to prevent any injury to the tissues of the paws. If the reading exceeds 45 sec, it would be considered as maximum analgesia. The maximum possible analgesia (MPA) was calculated by following formula [20].

$$MPA = \frac{C_t - C_o}{45 - C_o} \times 100$$

Chemical method

The chemical method (acetic acid writhing test) for screening non-narcotic analgesics was followed [21]. Adult non pregnant female albino mice showing at least 40 to 45 stretching episodes in 20 minutes after intra-peritoneal administration of 0.7 % acetic acid solution @ 70 $mgKg^{-1}$ body weight were selected for this experiment. All the mice were fasted overnight before the experiment.

The mice were divided into 5 groups consist of 5 animals in each group. Group I served as vehicle control, group II, III and IV received BPME orally @ 100, 300 and 900 mg/Kg^{-1} respectively. Group V received piroxicam orally @ 10 $mgKg^{-1}$.

After 1 hour of pre treatment with piroxicam or BPME, mice were injected with 0.7 % acetic acid solution intra-peritoneally @ 70 $mgKg^{-1}$ body weight. Total numbers of stretching episodes for 20 minutes were recorded by putting the mouse individually into a 1 liter beaker. The percent protection was calculated using the formula [22]:

$$\text{Percent protection} = \frac{\text{(Control mean – treated mean)}}{\text{Control mean}} \times 100$$

Local anesthetic activity

Local anesthetic activity of plant's methanol extracts (BPME) was studied using foot withdrawal reflex in frog [23]. The frogs (weighing 150- 200 g) were divided into 4 groups (n = 6). Group I served as vehicle control, group II received 2 % lignocaine and group III and IV received BPME @ 20 and 30 $mgml^{-1}$ respectively.

Each frog was pithed and a lateral cut was made high across the abdomen and all the contents were removed to form a sac. The frog was pinned on a board in such a way that its hind legs were hanging free. The foot was immersed in 0.05 (N) HCl which cause a reflex withdrawal immediately. The foot was washed immediately by distilled water and after the muscle gets relaxed the drug was placed in the sac to block the sciatic nerve. After every 1 minute, the withdrawal reflex was tested and foot was washed after each testing. Fresh frogs were used for each concentration of BPME and lignocaine. When response was no longer obtained with 0.05 (N) HCl it was tried with 0.5 (N) and 1 (N) HCl.

Anti-Inflammatory Activity

The anti-inflammatory effect of the plant's methanol extract (BPME) was evaluated by using Carageenan induced hind paw edema in albino rats [24]. Oedema was induced by injecting 0.1 ml of 1% (w/v) carageenan (Duchefa Biochemie) in saline solution, subcutaneously into the sub-planter region of the right hind paw of the rats. The oedema volume was determined using plethysmometer.

A plethysmometer was made for the measurement of rat paw volume [25]. A micropipette of 2 ml capacity was connected by means of plastic tubing to a 5 ml glass syringe. The proximal end of the micropipette was connected by rubber tubing to a glass vessel of 3 cm diameter. About 4 ml of mercury was filled in a syringe and the mercury level adjusted to 0 mark of the micropipette with the help of a 2 ml syringe. The space between the zero mark of the micropipette and a fixed mark

on the glass vessel was filled with water. The normal volume of the right hind paw of each rat was recorded by dipping them in glass vessel up to the level of lateral malleolus. The increased level of water in glass vessel was readjusted to the prefixed mark with the help of a connecting syringe. The reading at the point of water and mercury interface in the micropipette indicates the volume of each foot. The right hind paw was marked just beyond the lateral malleolus so as to fix a constant level up to which the rat's paw must be dipped in water.

Rats were divided into 5 groups of 5 animals each. Group I served as vehicle control. Group II, III and IV received BPME orally @ 100, 300 and 900 mgKg^{-1}, respectively. Group V received piroxicam orally @10 mgKg^{-1}. After 1 hour of pre treatment with BPME or piroxicam, rats were injected sub-cutaneously with 0.1 ml of carageenan under the planter surface of the right hind paw. Hind paw volume for inflammation was recorded after 5 hours of carageenan injection. Volume of inflammation was calculated by subtracting initial volume from the edema volume. The percent reduction was calculated according to the following formula [26]:

$$\text{Percent reduction} = \frac{(C_t - C_0)\ \text{control} - (C_t - C_0)\ \text{treated}}{(C_t - C_0)\ \text{treated}} \times 100$$

Where,

C_t = Edema volume

C_0 = Initial volume

$(C_t - C_0)$ treated = Volume of inflammation in treated group

$(C_t - C_0)$ control = Volume of inflammation in control group

Results and Discussion

There was no mortality in mice receiving single oral doses of BPME. Mice could tolerate up to 1.8 gKg^{-1} and 2.5 gKg^{-1} as single oral and intraperitonial dose respectively without showing any symptoms of toxicity, which might be due to lack of toxic principle in *Bryophyllum pinnatum* leaves [27]. Similarly, no symptoms of toxicity was evident in mice after administration of methanolic extract @ 2.4 gKg^{-1} i.p. [28] and in rat after administration of *B. pinnatum* juice @ 5 g Kg^{-1} i.p. [29].

The BPME showed analgesic activity by chemical method against acetic acid induced writhing syndrome in mice. Percent protections in acetic acid induced writhing in mice after single oral dose of BPME @ 100, 300 and 900 mgKg^{-1} were 43.45%, 70.70% and 83.79%, respectively as compared to 97.93% after single oral dose of piroxicam @ 10 mgKg^{-1} (Table 1). Hot plate method failed to reveal significant analgesic activity even after the single highest oral dose of BPME @ 900mgKg^{-1} (Table-2); which indicates that, BPME does not act like narcotic analgesics. The failure of the extract to inhibit paw licking or jumping behaviors in mice on hot plate method indicates that BPME might not be acting at supraspinal level [30].

Analgesic activity was reported after intraperitoneal dose of BPME @ 100 mg Kg^{-1} against acetic acid induced writhing in mice [31]. Intraperitoneal injection of acetic acid produces pains through activation of chemosensitive nociceptors [32] or irritation of the visceral surface, which lead to the liberation of histamine, bradikynin, prostaglandins and serotonin [33]. The acetic acid writhing test is used to evaluate the analgesic activity of non-narcotic analgesics. The marked analgesic activity by the chemical method indicates that the BPME might act like other non narcotic analgesics [21].

In the present study BPME could not block the foot withdrawal reflex in frog as compared to 2% lignocaine (Table 3). Hence, it reveals that BPME does not possess any local anesthetic activity [23].

Oral administration of BPME at higher dose levels (300 and 900 mgKg^{-1}) showed mild anti-inflammatory activity against carageenan induced hind paw edema in rats (Table 4). Anti-inflammatory activity of Bryophyllum leaves against carageenan induced pedal edema in rat was also reported after intraperitoneal administration of the plants methanolic [34, 31] and aqueous extract [35]. It was evident that certain flavonoids are potent inhibitors of several inflammatory mediators [36] and some isolated plant sterols were reported to have analgesic properties [37]. Thus, non narcotic analgesic and anti-inflammatory activities showed by BPME were might be due to the presence of chemical constituents like flavonoids, phytosterols, polyphenols, triterpenoids etc. [5] and might be due to its antihistaminic activity [10]. Further investigations will be required to identify the active fraction and to elucidate the exact mechanism. Experimental evidence obtained in the present laboratory animal study indicates that methanol leaf extract of the herb possesses non narcotic analgesic and anti-inflammatory properties. These observations lend pharmacological support to the reported folkloric uses of the plant's leaves in the management and control of pain and inflammatory conditions.

Table 1: Analgesic activity against acetic acid induced writhing syndrome in mice

Treatments	Dose (mgkg^{-1})	Writhing numbers in 20 minutes (Mean ± SE)	Per cent Protection
Vehicle Control	Tween80 (20%)	48.33 ± 1.82^{a}	0.00
BPME	100	27.33 ± 1.73^{b}	43.45
	300	14.16 ± 3.10^{c}	70.70
	900	7.833 ±0.60^{c}	83.79
Piroxicam	10	0.50 ± 0.34^{d}	97.93

(Mean having different subscripts differ significantly (P<0.01), SE=Standard Error, N=5)

Table 2: Analgesic activity by Eddy's hot plate method in mice

Treatment	Dose mgKg^{-1}	Reaction time(sec) after treatment		
		0 min	30 min	60 min
Vehicle Control	Tween80 (20%)	3.00±0.3162	3.00±0.4472 (0%)	3.40±0.5099 (0%)
BPME	100	2.6±0.2449	3.6±0.2449 (0.02%)	4.20±0.7348 (0.03%)
	300	2.6±0.2449	3.00±0.5477	3.80±0.3742

			(0%)	(0.02%)
	900	2.6±0.2449	3.60±0.400 (0.02%)	4.40±0.7483 (0.04%)

(Values in the parenthesis indicates maximum possible analgesia (MPA)
The mean values are not significant, SE=Standard Error, N=5)

Table 3: Local anaesthetic activity by foot withdrawal reflex in frog

Treatments	Dose (mgml^{-1})	Time of disappearance of withdrawal reflex (min) (Mean ± SE)
Vehicle Control	Tween80 (20%)	34 ± 0.70
BPME	20	33.60 ± 0.50
BPME	30	33 ±0.31
Lignocaine 2%	20	0.00

(The mean values are not significant, SE=Standard Error, N=6)

Table 4: Anti-inflammatory activity against Carrageenan induced hind paw edema in albino rats

Treatments	Dose (mgkg^{-1})	Volume of inflammation after 5 hours of Carrageenan injection (Mean ± SE)	Per cent Reduction
Vehicle Control	**Tween80 (20%)**	0.664 ± 0.04^{a}	0.00
BPME	100	0.582 ± 0.06^{a}	14.08
	300	0.548 ± 0.04^{ab}	21.16
	900	0.464 ± 0.06^{ab}	43.10
Piroxicam	10	0.378 ± 0.04^{b}	75.66

(Mean having different subscripts differ significantly (P<0.01), SE=Standard Error, N=5)

Conclusion

From the present study it may be concluded that *Bryophyllum pinnatam* leave methanol extract (BPME) is practically nontoxic and posses non narcotic analgesic and mild anti-inflammatory activity but do not possess narcotic analgesic and local anesthetic activity. The antihistaminic activity and different chemical constituents (flavonoids, phytosterols, polyphenols, triterpenoids etc.) of the herb might have attributed to its analgesic and anti-inflammatory activities.

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