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Analgesic and Anti-inflammatory Activities of Ethanolic Leaf Extract of *Phyllanthus acidus* L. on Swiss Albino Mice

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Authors' contributions

This work was carried out in collaboration between all authors. Author IJB designed and wrote the research protocol. Authors MSH and SA performed the experiments, managed the literature searches and wrote the manuscript. Overall review of the manuscript has done by author IJB. Author YB performed the statistical analysis. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The aim of the present study was to investigate the analgesic and anti-inflammatory potential of the ethanolic extract of *Phyllanthus acidus* (Family: Phyllanthaceae) leaves on swiss albino mice.
Study Design: The extract was divided into two concentrations (100 mg/kg and 200 mg/kg body weight) and was used for the examination of analgesic and anti-inflammatory activities on swiss albino mice.
Place and Duration of Study: Department of Pharmacy, Southeast University, Dhaka-1213, Bangladesh, from July to December 2015.
Methodology: The analgesic activity was evaluated using acetic acid induced writhing method, formalin induced paw licking method, tail immersion method and eddy's hot plate method. Carrageenan induced hind paw edema was performed to evaluate anti-inflammatory activity.
Results: The extract exhibited significant ($P < .001$) inhibition of writhing at the dose 200 mg/kg body weight compared to control in acetic acid induced writhing method. In formalin induced paw

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licking method, both the early and late phases significantly ($P < .001$) increased analgesia in a dose dependant manner. The extract at the dose of 200 mg/kg body weight was highly significant ($P < .001$) at 1h interval as compared to control in both tail immersion and eddy's hot plate method and inhibited 52.94% and 50% analgesia respectively. In anti-inflammatory activity test, the crude extract was highly significant ($P < .001$) and inhibited inflammation with time in a dose dependant manner.

Conclusion: Our study reveals that *P. acidus* leaves extract possess significant analgesic and anti-inflammatory activity and the dose 200 mg/kg body weight is more significant than 100 mg/kg body weight in all of the methods.

Keywords: Phyllanthus acidus; acetic acid induced writhing; analgesic; anti-inflammatory.

1. INTRODUCTION

From the beginning of human civilization, use of plants and plant products were traced as medicine [1]. Due to the proper weather and fertile soil, Bangladesh is a great source of medicinal plants and for the purpose of traditional medication about 500 species are being used here [2]. Inflammation is a biological process in response to tissue injury. An increase in blood vessel wall permeability followed by migration of immune cells at the injury site can lead edema formation during inflammation. Pain is frequently related with inflammation which is an ill-defined, unpleasant sensation and may be caused by nociceptive and inflammatory agents. However, excessive inflammation contributes to many acute and chronic human diseases [3-5].

Inflammation and pain adheres in virtually all animal and human diseases as a result they have become the focus of global scientific research [6]. Many drugs such as nonsteroidal anti-inflammatory drugs (NSAIDs) and corticosteroids have been developed but they are not safe because of their adverse side effects, for example gastric lesions, ulcers, hypertension and cardiac abnormalities [7,8]. Therefore, drugs lacking those effects are searched all over the world as alternatives to NSAIDs and opiates. In this process, the quests of the aptitude of plant-based drugs used in the traditional medicine have been given great attention because they are cheap and have little side-effects [9,10].

P. acidus commonly named as arbori in Bangladesh and also called gooseberry tree, is one of the trees with edible small yellow berries fruits in the Phyllanthaceae family. In this family, *Phyllanthus* is the largest genus and it has a distinct diversity of growth forms including annual and perennial herbaceous, arborescent, climbing, floating aquatic, pachycaulous, and phyllocladous. It is a curious and ornamental shrub or tree, 2-9 m high with spreading, dense, bushy crown of thickish, rough, main branches. Leaves are simple, oblong, acute or obtuse, slightly oblique to 14 mm long and 6 mm broad and have the inconspicuous flowers in pairs in their axils. It has highly acidic fruit that contains 40 mg/kg ascorbic acid [11-13].

Plants from the genus *Phyllanthus* is rich in many secondary metabolites such as alkaloids, tannins, flavonoids, lignans, phenolics and terpenes. *P. acidus* contains phyllanthusols A and B, aglycon, saccharide, various bioactive compounds such as adenosine, kaempferol and hypogallic acid, 2, 3-dihydroxybenzoic acid (DHBA). The pentacyclic triterpenoids, phyllanthul and olean-12en-3β-ol (β-amyrin) have been isolated from the bark of *P. acidus* skeels [11,14,15].

Previous investigation revealed that various parts of *P. acidus* showed many biological activities including antinociceptive [16], emetic and purgative [17], antihypertensive [15], hepatoprotective [18], antibacterial [19] and antitumor activity [20]. *P. acidus* fruit is liver tonic and a blood purifier which is used in several vitiated conditions of jaundice, bronchitis, constipation, vomiting, biliousness, urinary concretions and piles in ayurvedic system of medicine [21].

The present study was conducted to estimate the analgesic and anti-inflammatory activities of ethanolic extract of *Phyllanthus acidus* (EEPA).

2. MATERIALS AND METHODS

2.1 Drugs and Chemicals

All the chemicals used in this study were of analytical grade. Carrageenan was purchased

from Sigma-Aldrich, USA. Indomethacine and diclofenac sodium was purchased from Laz pharma, Dhaka, Bangladesh.

2.2 Plant Material Collection and Authentication

The fresh mature leaves of *P. acidus* were collected from Ashuganj, Brahmanbaria, Bangladesh in February 2014. The plant was identified and authenticated by an expert botanist of Bangladesh National Herbarium (DACB), Mirpur, Dhaka (Accession No. 39673) and a voucher specimen has been deposited in the herbarium for future reference.

2.3 Preparation of Extract

The collected leaves were shade dried without exposing them to direct sunlight. The dried leaves were ground to coarse powder with a mechanical grinder (Grinding mill) and powdered sample was kept in clean closed glass containers pending extraction. About 250 g of dried sample was subjected to extraction by 99% ethanol with a volume of 1000 ml for 15 days with stirring and agitation for allowing total extraction process. After the extraction process the *P. acidus* leaf extract was filtered with sterilized cotton filter followed by whatman filter paper. The filtrate was collected in a beaker. The extract obtained after filtration was concentrated by using a rotary evaporator at 60℃.

2.4 Experimental Animal

Swiss Albino mice (30–35 g) of either sex were used to evaluate *in vivo* analgesic and anti-inflammatory activity. They were collected from the Animal Resources Branch of ICDDR, B (International Centre for Diarrheal Disease and Research, Bangladesh) and were housed under standard environmental condition (24±1℃) with 12:12 h light-dark cycle. The animals were fed rodent food and water *ad libitum*. According to the institutional animal ethics, Department of Pharmacy, Southeast University, all animals were adapted to the laboratory environment for at least 1 week before the experimental period.

2.5 Analgesic Activity Test

2.5.1 Acetic acid induced writhing method

The ethanolic extract of *P. acidus* leaves were studied for analgesic activity using acetic acid induced writhing method in mice [22]. The animals were divided into four groups including control (Group I), positive control (Group II) and two test groups (Group III-IV). The control group was treated with 1% tween 80 in saline water at the dose of 10 ml/kg p.o and the positive control group received indomethacin (standard drug) at the dose of 10 mg/kg orally. The animals of group III and group IV is treated with the plant extract in two different doses 100 mg/kg and 200 mg/kg body weight orally. 30 minutes after administration of vehicle, standard drug and test sample, 0.7% acetic acid is injected intraperitoneally at a dose of 10 ml/kg body weight and the intensity of analgesic behavior was quantified by counting the total number of writhes over a period of 30 minutes. The percentage analgesic activity was calculated as follows:

$$\text{Percentage analgesic activity} = [(N_c - N_t)/N_c] \times 100\%$$

Where N_c is the average number of writhes of the control group and N_t is the average number of writhes of the test/positive control group.

2.5.2 Formalin induced paw licking method

The formalin-induced paw licking method was performed according to Ghareate et al. [23]. For this method, animals were kept into four groups including 4 mice in each group and were treated in the following manner: group I received vehicle (isotonic saline solution, 0.9%), group II received indomethacin as standard drug at the dose of 10 mg/kg body weight and group III-IV received ethanolic extract of *P. acidus* leaves at a dose of 100 mg/kg and 200 mg/kg. One hour after oral administration of vehicle, standard drug and test sample, mice received 50 µl of 2% formalin in sub plantar region of hind paw and the number of paw licking was measured in each mouse from 0-5 min and 20-30 min. The number of paw licking in first 5 min indicate response to neurogenic pain and the number of paw licking in 20-30 min indicate inflammatory pain.

2.5.3 Tail immersion method

Tail immersion test was performed for evaluating analgesic activity by Mali et al. [24]. In this test, mice were divided into four groups of four mice each were treated orally with vehicle (isotonic saline solution, 0.9%), diclofenac sodium as standard drug (9 mg/kg) and ethanolic extract of *P. acidus* (100 and 200 mg/kg). One hour after administration of vehicle, standard drug and test sample, the tip of tail was immersed up to 5 cm

in hot water maintained at 58℃. Sudden withdrawal of the tail from the hot water was taken as the reaction time. To avoid damage to the tail cut off time of 20 s was maintained. The reaction time was measured at 0, 1, 2, 3, 4 and 5 h.

2.5.4 Eddy's hot plate method

Eddy's hot plate method was performed according to Mali et al. [24]. Pain reflex was measured using a Le7406 hot plate (Panlab S2, Cornella, Barcelona, Spain) in response to the thermal stimulus. Mice were divided into four groups of four mice in each group and were given orally with vehicle (1 ml/kg), diclofenac sodium (9 mg/kg) and ethanol extract of *P. acidus* leaves at the dose of 100 mg/kg and 200 mg/kg body weight. One hour after administration of vehicle, standard drug and test sample mice were placed on the hot plate maintained at 55 ℃ to obtain their response to the plate and the reaction time was noted by the latency to leak the paw or jump from the hot plate. The reaction time was evaluated at 0, 1, 2, 3, 4 and 5 h.

2.6 Anti-inflammatory Activity

2.6.1 Carrageenan-induced hind paw edema in mice

The method described by Mali et al. [24] was used to study acute inflammation. In this experiment, mice were divided into four groups in four mice each and were treated orally with vehicle 1 ml/kg (Group I), indomethacin 10 mg/kg (Group II) and ethanolic extract of *P. acidus* leaves 100 and 200 mg/kg body weight (Group III and IV) respectively. One hour after the administration of vehicle, standard drug and plant extract, 0.1 ml of 1% w/v of carrageenan suspension in 0.9% normal saline was injected into the sub planter region of left hind paw of mice. The paw volume was determined with a micrometer screw gause at 1, 2, 3 and 4h after the administration of the drug and the extract. The percentage inhibition in paw volume after administration of the extract was calculated using following formula:

$$\text{Percentage inhibition in paw volume} = (1-V_t/V_c) \times 100$$

Where, V_t is the mean paw volume in control group and V_c is the mean paw volume in test group.

2.7 Statistical Analysis

Data was expressed as mean ± SEM and analyzed with one-way analysis of variance (ANOVA) followed by Dunnett's test for comparing control and various groups. MS Excel 2010 (Roselle, IL, USA) was used for the statistical and graphical evaluations. A probability of P = .05 was considered as statistical significant compared to control group.

3. RESULTS

3.1 Analgesic Activity

3.1.1 Acetic acid induced writhing method

The results of the ethanolic extract of *P. acidus* leaves on acetic acid induced writhing in mice are shown in Table 1. The percentage inhibition of writhing produced by the extracts at the dose of 200 mg/kg body-weight was 55.48% and that result was statistically significant (P < .001) compared to the standard drug indomethacin, which showed 74.56% writhing inhibition.

Table 1. Effects of ethanolic extract of *P. acidus* leaves on acetic acid induced writhing in mice

Treatment	Avg. no. of writhing	% Inhibition
Control	35.38±3.98	-
standard	9.00±1.29***	74.56
EEPA 100 mg/kg	21.75±1.60**	38.52
EEPA 200 mg/kg	15.75±1.65***	55.48

*Values are reported as mean ± S.E.M. for group of four animals (n = 4). Values are analyzed as compared to control using one way ANOVA followed by Dunnett's test. Asterisks indicated statistically significant values from control, * indicates P = .05, ** indicates P < .01 and *** indicates P < .001*

3.1.2 Formalin induced paw licking method

The effects of the ethanol extract of *P. acidus* leaves on formalin induced paw licking in mice are shown in Table 2. In first phase of formalin induced pain model, ethanol extract at 200 and 100 mg/kg body weight produced 51.47% and 29.41% inhibition of pain response whilst at second phase *P. acidus* leaves extract produced 50.34% and 28.28% inhibition of pain response respectively. We found that *P. acidus* extract showed analgesic effect both in the early and late phase of formalin test which indicates that the extract exerts its analgesic effect through the peripheral and central mechanism.

3.1.3 Tail immersion method

Inhibition of painful sensation was observed against tail immersion method after oral administration of 200 mg/kg and 100 mg/kg body weight of *P. acidus* leaves extract and the result has been summarized at Table 3 and the percent of inhibition at various times are given at Fig. 1. The maximum effect observed at 1 h after administration of the extract at dose 200 mg/kg and then it was gradually decreasing. Standard drug diclofenac sodium produced significant activity up to 5 h. Although the duration of analgesic activity was less than the standard, results represent significant analgesic activity.

Table 2. Effects of ethanolic extract of *P. acidus* leaves on formalin induced paw licking in mice

Treatment	Number of paw licking		% inhibition at different time (minutes)	
	0-5	20-30	0-5	20-30
Control	34.00±1.83	36.25±1.38	-	-
Standard	9.25±0.85***	11.50±0.29***	72.79	68.28
EEPA 100 mg/kg	24.00±1.29***	26.00±2.12***	29.41	28.28
EEPA 200 mg/kg	16.50±1.19***	18.00±0.82***	51.47	50.35

*Values are reported as mean ± S.E.M. for group of four animals (n = 4). Values are analyzed as compared to control using one way ANOVA followed by dunnett's test. Asterisks indicated statistically significant values from control, * indicates P= .05, ** indicates P < .01 and *** indicates P < .001*

Table 3. Effects of ethanolic extract of *P. acidus* leaves on tail immersion method in mice

Treatment	Reaction times					
	0h	1h	2h	3h	4h	5h
Control	8.00±0.41	8.50±0.29	8.25±0.25	7.50±0.29	7.50±0.29	7.75±0.25
Standard	7.50±0.29	2.00±0.41***	2.00±0.41***	2.00±0.41***	2.25±0.25***	2.50±0.29***
EEPA 100 mg/kg	7.75±0.25	5.50±0.29**	5.50±0.29***	5.50±0.96	6.00±1.22	7.00±0.41
EEPA 200 mg/kg	7.75±0.48	4.00±0.71***	4.25±0.25***	4.50±0.96*	5.00±0.71	6.50±0.65

*Values are reported as mean ± S.E.M. for group of four animals (n = 4). Values are analyzed as compared to control using one way ANOVA followed by Dunnett's test. Asterisks indicated statistically significant values from control, * indicates P= .05, ** indicates P < .01 and *** indicates P < .001*

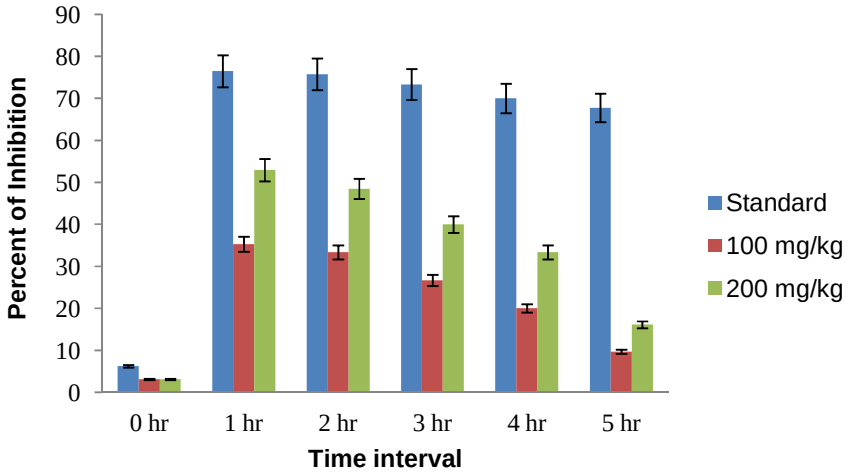

Fig. 1. Percentage of inhibition of pain at various time intervals in tail immersion method

3.1.4 Eddy's hot plate method

P. acidus leaves extract showed significant ($P < .001$) analgesic activity on hot plate method like tail immersion method. The analgesic activity obtained after administration of the extract was dose dependent and the maximum effect observed after 1 h as summarized in Table 4 and the percent of inhibition at various times are given at Fig. 2. At dose 200 mg/kg body weight it showed its maximum inhibition (50%) and the most significant increase in latency period was noticed as compared to the standard drug diclofenac sodium which showed 79.12% inhibition (Fig. 2).

3.2 Anti-inflammatory Activity

3.2.1 Carrageenan-induced hind paw edema in mice

The anti-inflammatory effect of the crude ethanol extract of *P. acidus* using carrageenan induced edema test is represented at Table 5 and the percent of inhibition at various times are given at Fig. 3. *P. acidus* leaves extract showed a significant ($P < .001$) inhibition of increase in paw edema compared to the standard drug indomethacin. The maximum inhibitory effect of the extract was recorded with a dose of 200 mg/kg body weight and was noticed at 4 h.

4. DISCUSSION

In the present study, *P. acidus* leaves extract inhibited writhing response in acetic acid induced writhing test, inhibited licking in formalin induced paw licking method, increased reaction time in tail immersion method, increased reaction time in hot plate method and reduced carrageenan induced paw edema. Acetic acid-induced writhing method is a well recommended method in estimating medicinal agents for their peripheral analgesic property [25]. A painful reaction and acute inflammation occurs in the peritoneal area when animals are intraperitoneally treated with acetic acid. The stimulation of peritoneal nociceptors is indirect and occurs with the release of endogenous substances, which stimulate nervous endings. Induction of pain caused by releasing endogenous substances as well as some other pain mediators such as arachidonic acid via cyclooxygenase, and prostaglandin biosynthesis. Sensation of pain elicited by producing localized inflammatory response in acetic acid induced writhing method due to release of free arachidonic acid from tissue phospholipids via cyclo-oxygenase (COX) and producing prostaglandin specifically PGE2 and PGF2α, the level of lipoxygenase products may also increases in peritoneal fluids [26,27]. These prostaglandin and lipoxygenase products are responsible for pain. There are various peripherally acting analgesic drugs such as ibuprofen, aspirin, diclofenac sodium, indomethacin that have been reported to inhibit acid induced writhing by inhibition of prostaglandin synthesis [3]. The substance inhibiting writhing is considered as analgesic by inhibiting prostaglandin, a peripheral mechanism of pain inhibition [25].

The strong analgesic activity of *P.acidus* leaves extract may be due to the interference of their active principle(s) with the release of pain mediators. Acetic acid induced writhing method was selected to investigate peripheral analgesic effect of extract whereas tail immersion method was selected to investigate central analgesic activity. The drugs acting against tail immersion induced pain attributed their actions through mu (μ) opioid receptors rather than kappa (κ) and delta (δ) receptors [28]. In this study we found our plant is active against tail immersion method which indicates that *P.acidus* leaves extract has central analgesic effect. To get specific result and to avoid misinterpretation of the result formalin test was carried out which comprises of two different phase. The early phase indicates centrally mediated pain, which has a result of direct stimulation of nociceptors, the late phase pain is caused by local inflammation with a release of inflammatory and hyperalgesic mediators. So this model is used to screen the analgesic substances and also for elucidating the mechanism of analgesia [29,30]. Results showed that ethanol extract produced better activity and the effect of extract may medicate through both peripheral and central mechanism. Hot plate method was also selected to investigate the central analgesic effect. In hot plate test, *P.acidus* leaves extract significantly enhanced paw withdrawal latencies to thermal stimulation in mice. The hot plate test involves the transmission of pain from the periphery via C fibers to the spinal cord. The ethanol extracts could therefore be acting by inhibiting the transmission of pain via C fibers to the CNS [31].

The present study was also conducted to evaluate the probable anti-inflammatory activity of the ethanol extract of *P. acidus* leaves on mice. Carrageenan-induced hind paw edema is a suitable animal model to assess the anti-

inflammatory activity of natural products as well as synthetic chemical compounds [23]. The presence of edema is one of the prime signs of inflammation [32]. Edema formation due to carrageenan in paw is a biphasic event [24]. The first phase is due to the release of histamine and serotonin whereas the second or delayed phase is involved with neutrophil infiltration and release of other neutrophil derived mediators, eicosanoid release and production of free radicals [33,34]. Edema production in between early and late phase is involved with the release of kinin like substances (e.g. bradykinin), which later induces the biosynthesis of prostaglandin and other autacoids, which are responsible for formation of the inflammatory exudates [34,35].

Table 4. Effects of ethanolic extract of *P. acidus* leaves on Eddy's hot plate method in mice

Treatment	Reaction time					
	0H	1H	2H	3H	4H	5H
Control	6.25±0.25	6.00±0.41	6.00±0.48	6.25±0.48	6.50±0.29	6.25±0.25
Standard	5.50±0.29	1.25±0.48***	1.50±0.29***	1.75±0.48***	2.00±0***	2.50±0.29**
EEPA 100 mg/kg	6.00±0.58	3.75±0.48*	4.00±0.41**	5.00±0.71	5.25±1.03	5.75±1.25
EEPA 200 mg/kg	5.75±0.25	3.00±0.71**	3.25±0.25***	3.50±0.29**	4.75±0.63	5.50±0.29

*Values are reported as mean ± S.E.M. for group of four animals (n = 4). Values are analyzed as compared to control using one way ANOVA followed by Dunnett's test. Asterisks indicated statistically significant values from control, * indicates P = .05, ** indicates P < .01 and *** indicates P < .001*

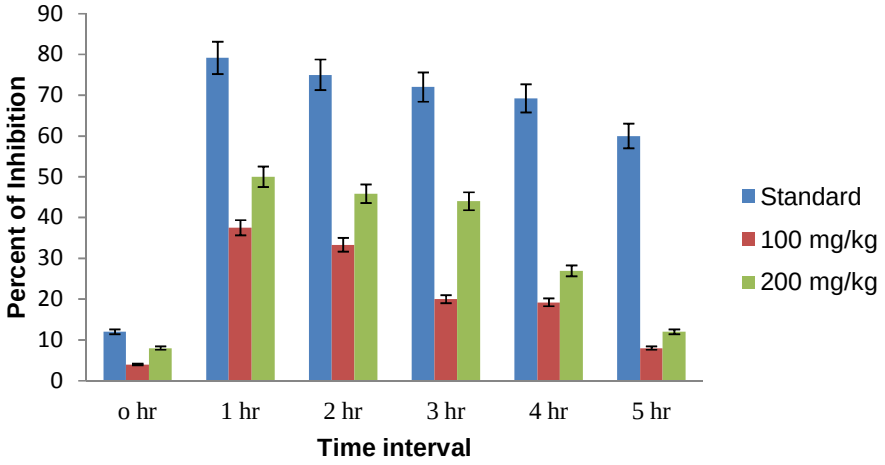

Fig. 2. Percentage of inhibition of pain at various time intervals in eddy's hot plate method

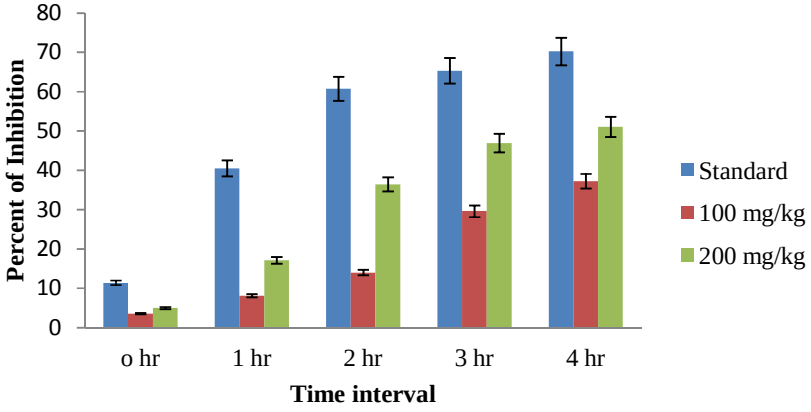

Fig. 3. Percentage of inhibition of inflammation at various time intervals in carrageenan induced hind paw edema

Table 5. Effects of ethanolic extract of *P. acidus* leaves on carrageenan induced paw edema

Treatment	Reaction time				
	0H	1H	2H	3H	4H
Control	17.50±0.61	13.88±0.52	13.38±0.55	12.25±0.43	11.75±0.32
Standard	15.50±0.29	8.25±0.25***	5.25±0.95***	4.25±0.25***	3.50±0.29***
EEPA 100 mg/kg	16.88±0.50	12.75±0.29	11.50±0.33	8.63±0.80***	7.38±0.72***
EEPA 200 mg/kg	16.63±0.24	11.50±0.29**	8.50±0.65***	6.50±0.29***	5.75±0.63***

*Values are reported as mean ± S.E.M. for group of four animals (n = 4). Values are analyzed as compared to control using one way ANOVA followed by Dunnett's test. Asterisks indicated statistically significant values from control, * indicates P= .05, ** indicates P < .01 and *** indicates P < .001*

5. CONCLUSION

From the obtained result, we can reach a conclusion that *P. acidus* possesses significant analgesic and anti-inflammatory activities. Despite remarkable analgesic and anti-inflammatory activity of the plant, it requires further studies to identify various phytochemicals responsible for the above mentioned pharmacological activities.

CONSENT

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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