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Research Article

**ANTI-NOCICEPTIVE AND ANTI-INFLAMMATORY
ACTIVITIES OF ETHANOLIC EXTRACT OF XYLOCARPUS
GRANATUM LEAVES**Ekramul Hasan¹, Rajbongshi Lata¹, Abdur Rouf¹, Howlader Saurav^{2*}¹ Department of Pharmacy, Daffodil International University, Dhanmondi, Dhaka, ^{2*}Howlader Saurav, SouthEast University, Banani, Dhaka, Bangladesh.

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Abstract:

Xylocarpus granatum (Family: Meliaceae) is a mangrove tree species used as herbal remedy for various ailments. But the scientific basis for its medicinal use especially in pain and inflammation remains unknown. Therefore, the present study was aimed to investigate the anti-nociceptive and anti-inflammatory effects of the leaves of *Xylocarpus granatum* in laboratory animals. The anti-nociceptive effect was assessed in mice using acetic acid-induced writhing, formalin-induced paw licking and tail immersion assays. Anti-inflammatory activity was evaluated on carrageenan induced paw oedema in rats. In acetic acid-induced writhing test, the extract at different doses (50 and 100 mg/kg) significantly ($p < 0.001$) and dose-dependently reduced pain by 49.30 and 68.20%, respectively. The extract also significantly inhibited both the early and late phases of formalin-induced paw licking in mice. In the tail immersion test, the extract caused a significant inhibition of pain (68.91% inhibition, after 4 hr.) at a dose of 100 mg/kg. *X. granatum* caused a significant inhibition of paw oedema development in the carrageenan-induced oedema tests. The findings of the study suggested that *X. granatum* leaves have significant anti-nociceptive and anti-inflammatory effects, confirming the traditional use of this plant in the treatment of various diseases associated with pain and inflammation.

Key words: *Xylocarpus granatum*, anti-nociceptive, anti-inflammatory.**Corresponding author:****Howlader Saurav,**

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INTRODUCTION:

Inflammation is a complex physiological and pathological process accompanied by the activation of the immune system, local vascular system, and various cells within the damaged tissue [1]. It is a normal protective response to tissue injury caused by physical trauma (cut, burn or bruise), noxious chemicals, microbiologic agents or even autoimmune disease. Acute inflammation is a part of the defense response by organisms to remove injurious stimuli, such as pathogens, irritants, or physical injury, from tissues and to initiate the healing process. However, epidemiological studies have demonstrated that chronic inflammation is associated with a wide variety of diseases, including cardiovascular diseases, cancers, diabetes, arthritis, Alzheimer's disease, pulmonary diseases, and autoimmune diseases [2]. Although several drugs are currently available for the treatment of chronic inflammation, they also produce various side effects and have relatively low efficacy. Therefore, identification of new molecules with high efficacy and low side effects for the treatment of chronic inflammation related diseases is currently of great interest.

Xylocarpus granatum is a mangrove tree species belonging to the family Meliaceae. It is well distributed in the coastal areas of Australia, South East Asia including Malaysia and Bangladesh, and India [3]. *X. granatum* is a medicinally important mangrove plant the bark of which has been used in traditional medicine for the treatment of fevers, malaria, and cholera [4]. Apart from these traditional uses, scientific studies on *X. granatum* have shown it to possess various biological activities that include antifilarial, antifungal, and anticancer activities [4,5]. The leaves and barks of *X. granatum* have been found to possess significant radical scavenging activity [6,7]. Ethanol extracts of whole fruits of this plant have also exhibited antihyperglycemic and antidyslipidemic activities in streptozotocin (STZ)-induced rats [8]. However, no study has been conducted to evaluate anti-nociceptive and anti-inflammation activity of the leaf extracts from this plant. Therefore, the present study has been carried out to explore the anti-nociceptive and anti-inflammatory activities of ethanolic extracts of *X. granatum* on animal models.

MATERIALS AND METHODS:**Plant Material**

The plant sample of *X. granatum* leaves were collected in September, 2016 from coastal area of Bangladesh. The plant was identified by Bangladesh National Herbarium, Dhaka, where a voucher specimen has been deposited. At first, Leaves were

washed properly to remove dirty materials and air-dried for several days. These were then ground with a hammer grinder for better grinding. The dried leaves were ground into a coarse powder. Then, the dried powder was preserved in an airtight container.

Extract preparation:

The collected plant leaves were washed with water and separated from undesirable materials or plants or plant parts. They were aerated by fan aeration to be partially dried and were next heated in an oven at below 40°C for two days to be fully dried. The fully dried leaves are then grinded to make them powder by the help of a suitable grinder. Then the powders were dissolved in ethanol and kept for a period of 2 days accompanying occasional shaking and stirring. The whole mixture then underwent a coarse filtration by a piece of clean, white cotton material followed by a second filtration through whatman filter paper. The filtrate (ethanol extract) obtained was evaporated by rotary evaporator at 5 to 6 rpm and at 68°C temperature. It rendered a gummy concentrate that was designated as crude extract or ethanolic extract. The crude ethanolic extract was finally dried by freeze drier and preserved.

Experimental Animals:

Swiss Albino mice (25-30 gm) and Wistar male rat (150-200 gm) obtained from the Animal Resource Branch of the International Centre for Diarrhoeal Diseases and Research, Bangladesh (ICDDR, B) were used for the experiment. They were kept in standard environmental condition for one week in the animal house of the Department of Pharmacy, Daffodil International University, Bangladesh for adaptation after their purchase. The animals were provided with standard laboratory food and tap water ad libitum and maintained at natural day night cycle. This study was approved by the Internal Ethics committee for the use and care of laboratory animals, Daffodil International University, Bangladesh.

Drugs and chemicals:

Diclofenac Na (MERCK, Mumbai, India), Indomethacin (BASF Aktiengesellschaft, Germany), Formaldehyde (MERCK, Mumbai, India), Carrageenan (Hi Media Laboratories Pvt. Ltd., Mumbai, India) and 0.9% NaCl saline solution (Square Pharmaceuticals Ltd., Bangladesh) were used. All other reagents were of analytical grade.

Acute oral toxicity test

An acute oral toxicity test was carried out in accordance with the Organisation for Economic Cooperation and Development (OECD) guidelines for testing of chemicals [9]. Swiss albino mice

maintained under standard laboratory conditions being used for the acute toxicity study. A total of three mice from each group received a single oral dose (500, 1000, and 2000 mg/kg bw) of the extract. Animals were fasted overnight prior to administration. After administration of the extracts, food was withheld for a further 3 to 4 h. The animals were then individually observed (with special attention during the first 4 h) for possible behavioural changes, allergic reactions (skin rash, itching), eyes and mucous membrane, and mortality for the next 72 h.

Anti-nociceptive activity

Acetic acid-induced writhing test

For the writhing test, 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 groups III and group IV were treated with plant extracts at two different doses of 50 mg/kg and 100 mg/kg bw orally. Thirty minutes after administration of vehicle, standard drug and test samples, 0.7% acetic acid is injected intraperitoneally (i.p.) at a dose of 10 ml/kg bw and the intensity of analgesic behaviour was quantified by counting the total number of writhes over a period of 30 minutes. The percentage analgesic activity was calculated as follows:

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.

Formalin-induced paw licking test

The formalin-induced paw licking method was performed according to Gharate and Kasture, 2013 [10]. For this method, animals were kept in four groups, with 4 mice in each, 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 bw and group III-IV received ethanolic extract of *X. granatum* (50 and 100 mg/kg of bw). One hour after the oral administration of vehicle, standard drug and test samples, mice received 20 μ l of 2% formalin in sub plantar region of hind paw and the number of paw licking events was measured in each mouse from 0-5 min and 20-30 min. The number of paw licks in the first 5 min indicates response to neurogenic pain and the number of paw licks in 20-30 min indicates inflammatory pain.

Tail immersion method

The tail immersion test was performed to evaluate analgesic activity by Mali et al., [11]. In this test, mice were divided into four groups of 4 mice, each treated orally with vehicle (isotonic saline solution, 0.9%), diclofenac sodium as standard drug (10 mg/kg) and ethanolic extract of *X. granatum* (50 and 100 mg/kg of bw). One hour after administration of the vehicle, standard drug and test samples, the tip of the tail was immersed up to 5 cm in hot water maintained at 55°C. Sudden withdrawal of the tail from the hot water was taken as the reaction time. To avoid damage to the tail, a cut-off time of 20 s was maintained. The reaction time was measured at 0, 1, 2, 3, 4 and 5 h.

Anti-inflammatory Activity

Carrageenan-induced rat paw oedema test

The method described by Mali et al., [11] was used to study acute inflammation. In this experiment, rats were divided into four groups of four mice and were treated orally with vehicle 1 ml/kg (Group I), diclofenac sodium 10 mg/kg (Group II) and ethanolic extract of *X. granatum* (50 and 100 mg/kg of bw for Groups III and IV, respectively). One hour after administration of the 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 the left hind paw of the rat. The paw volume was determined with a micrometre screw gauge at 1, 2, 3, 4 and 5 h after administration of the drug and the extract. The percentage inhibition in paw volume after administration of the extract was calculated using the following formula: Percentage inhibition in rat 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.

Statistical analysis

Statistical analysis was carried out using one-way ANOVA followed by Dunnett's multiple comparisons for analgesic and anti-inflammatory tests. The results obtained were compared with the vehicle control group. P values <0.05, <0.01, <0.001 were considered to be statistically significant.

RESULTS:

Acute oral toxicity test

In the acute toxicity assay, no deaths were observed during the 72 hr. period at the doses tested. At these doses, the animals showed no stereotypical symptoms associated with toxicity, such as convulsion, ataxia, diarrhoea or increased diuresis.

Anti-nociceptive activity**Acetic acid-induced writhing test**

The results of the ethanolic extract of *X. granatum* leaves on acetic acid-induced writhing in mice and percentage inhibition of pain are shown in **Table 1**.

The percentage inhibition of writhing produced by the extracts at the dose of 100 mg/kg bw was 68.20%; that result was statistically significant ($P < 0.001$) compared to the standard drug indomethacin, which showed 75.67% writhing inhibition.

Table 1. Effects of ethanolic extracts of *X. granatum* leaves on acetic acid-induced writhing tests

Group	No of Writhing	% of Inhibition
Control	30.37 ± 3.29
Standard	10.26 ± 2.02***	75.67%
<i>X. granatum</i> (50 mg/kg)	28.15 ± 4.10*	49.3%
<i>X. granatum</i> (100 mg/kg)	16.12 ± 2.23***	68.20%

Values are expressed as mean ± S.E.M. (n=5); significance at *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ as compared to control

Formalin-induced paw licking test

The effects of the ethanol extract of *X. granatum* leaves on formalin induced paw licking in mice and percent of inhibition in both phase are shown in **Table 2**. In the first phase of the formalin-induced pain model, ethanol extract at 100 and 50 mg/kg bw produced 42.14% and 25.84% inhibition of pain

response whilst at the second phase, *X. granatum* leaf extract produced 49.25% and 20.17% inhibition of pain response, respectively. We found that *X. granatum* extract showed an analgesic effect both in the early and late phase of the formalin test which indicates that the extract exerts its analgesic effect through the peripheral and central mechanism.

Table 2. Effects of ethanolic extracts of *X. granatum* leaves on formalin-induced test

Group	Early Phase 0-5 min.	% of inhibition in early phase	Late Phase 20-30 min.	% of inhibition in late phase
Control	34.00 ± 1.05	13.50 ± 2.10
Standard	17.60 ± 1.50	50.15%	2.86 ± 1.30	82.01%
<i>X. granatum</i> (50 mg/kg)	22.50 ± 10.02	25.84%	10.15 ± 5.10	20.17%
<i>X. granatum</i> (100 mg/kg)	20.15 ± 12.39	42.14%	8.12 ± 3.04	49.25%

Values are expressed as mean ± S.E.M. (n=5); significance at *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ as compared to control

Tail immersion test

The effects of the ethanol extract of *X. granatum* leaves on Tail immersion test in mice were significant at the level of $P < 0.001$; these are shown in **Table 3**. In the tail immersion method of analgesic activity, the ethanolic extract of *X. granatum* exhibited potent activity at 100 and 50 mg/kg bw the

response time observed was significantly increased when compared to normal control. However, the standard Diclofenac sodium was found to have better activity than the extract during the 4hr response. Diclofenac sodium showed 80.15% inhibition whereas *X. granatum* showed 68.91% inhibition at tested concentration.

Table 3. Effects of ethanolic extracts of *X. granatum* leaves on tail-immersion method test

Treatment	Retention time in (sec)						% of Inhibition At 4 hr
	Initial	Time after drug administration					
	0 hr	1 hr	2 hr	3 hr	4 hr	5 hr	
Control	12.65 ± 1.5	11 ± 1.16	10.25 ± 0.75	10.55 ± 1.2	14.15 ± 0.78	10.05 ± 0.54
Standard	11.00 ± 0.16*	4.5 ± 1.2**	3.65 ± 1.8*	6.75 ± 1.1**	4 ± 1.15***	5 ± 1.25*	80.15%
<i>X. granatum</i> (50 mg/kg)	12.75 ± 0.6	10.65 ± 0.78**	6.25 ± 0.45	9 ± 0.12	7.75 ± 0.28***	5.56 ± 0.18*	54.63%
<i>X. granatum</i> (100 mg/kg)	15.25 ± 1.1	9.25 ± 0.52*	8.15 ± 1.5*	7.15 ± 0.46**	5 ± 0.81***	6.05 ± 0.28*	68.91%

Values are expressed as mean ± S.E.M. (n=5); significance at *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ as compared to control

Anti-inflammatory activity**Carrageenan-induced rat paw oedema test**

The anti-inflammatory effect of ethanolic extracts of *X. granatum* leaves was observed at the level of $P < 0.001$ when compared with the vehicle distilled water (control group) and Diclofenac sodium (Standard). The percent inhibition of indomethacin in

paw oedema after 4 hrs was recorded as 68.75%. Moreover, 100 mg/kg dose of ethanolic extracts of *X. granatum* leaves exhibited significance percent of inhibition which was recorded as 58.29%. The observations and significance at the level of $P < 0.001$ are shown in Table 4.

Table 4. Effects of ethanolic extracts of *X. granatum* leaves on carrageenan-induced rat paw oedema test

Treatment	Retention time in (sec)					
	Initial	Time after drug administration				% of Inhibition
	0 hr	1 hr	2 hr	3 hr	4 hr	At 4 hr
Control	2.04 ± 0.02	2.15 ± 0.01	2.50 ± 0.02	2.65 ± 0.01*	1.89 ± 0.01
Standard	0.72 ± 0.012*	0.65 ± 0.007*	0.70 ± 0.022	0.55 ± 0.02**	0.42 ± 0.02***	68.75%
<i>X. granatum</i> (50 mg/kg)	0.88 ± 0.007	0.91 ± 0.002**	0.76 ± 0.014*	0.90 ± 0.016*	0.75 ± 0.015*	48.34%
<i>X. granatum</i> (100 mg/kg)	0.53 ± 0.02	0.62 ± 0.03**	0.75 ± 0.016**	0.50 ± 0.009**	0.71 ± 0.017***	58.29%

Values are expressed as mean ± S.E.M. (n=5); significance at *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ as compared to control

DISCUSSION:

Pain and inflammation are associated with pathophysiology of various diseases like arthritis, cancer and vascular diseases. Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used for treatment of pain and inflammation. However, prolonged use of these drugs leads to gastro-intestinal ulcers, bleeding, and renal disorders [12]. Thus, there is a need for discovery of new anti-inflammatory and analgesic drugs without these side effects. A number of natural products are used in various traditional medicinal systems to relief symptoms of pain and inflammation [13]. *Xylocarpus granatum* is reported to contain chemical constituents which may exert analgesic and anti-inflammatory effect; however till now there has been no investigation supporting the anti-nociceptive and anti-inflammatory properties of this plant.

Results of the present study showed that the ethanolic extract of *X. granatum* has marked anti-nociceptive and anti-inflammatory effects with a reasonable safety profile. The anti-nociceptive and anti-inflammatory effect of ethanol leaves extract of *X. granatum* was evaluated for the first time in various experimental test models. Satyanarayana et al., [14] showed that acetic acid induces writhing by stimulating the production of prostaglandins.

Standard analgesic drug Indomethacin has been shown to inhibit prostaglandin synthesis in the brain [15,16]. Since *X. granatum* therapy antagonised acetic acid-induced writhes, it is possible to suggest that the plant extract may be producing anti-nociceptive activity through manipulation of the prostaglandin system. On the other hand, the formalin-induced paw licking model comprises of early phase and late phase. The early phase (immediately after injection) seems to be caused by C-fibre activation due to the peripheral stimulus. The late phase (20 min after formalin injection) appears to depend on the combination of an inflammatory reaction, activation of NMDA and non-NMDA receptors and NO cascade [17] in the peripheral tissue and the functional changes in the dorsal horn of the spinal cord. In our study, the ethanolic extract of *X. granatum* significantly inhibited the late phase of formalin-induced pain. The tail immersion test is used to determine both centrally acting analgesics [9], like Diclofenac Na [18], and peripherally acting analgesics like NSAIDs, which inhibit cyclooxygenase in peripheral tissues, thereby interfering with the mechanism of transduction of primary afferent nociceptors [19]. The results observed for the tail immersion test clearly showed that the ethanolic extract of *X. granatum* possessed a dose dependent anti-nociceptive activity. Carrageenan-induced edema

involves the synthesis or release of mediators at the injured site correlated with early exudative stage of inflammation [20]. These mediators include prostaglandins, especially the E series, histamine, bradykinins, leucotrienes and serotonin all of which also cause pain and fever [21]. The time course of edema development in carrageenan induced paw edema model in rats is generally represented by a biphasic curve. The first phase occurs within an hour of carrageenan injection and is partly due to the trauma of injection and also to histamine and serotonin component. The second phase (over 1h) is mediated by prostaglandins, the cyclooxygenase products, and the continuity between the two phases is provided by kinins [22]. The presence of PGE₂ in the inflammatory exudates from the injected foot can be demonstrated at third hour and period thereafter. Inhibition of these mediators from reaching the injured site or from bringing out their pharmacological effects normally ameliorates the inflammation and other symptoms. Since carrageenan-induced inflammation model is a significant predictive test for anti-inflammatory agents acting by the mediators of acute inflammation [23], the result of our current study is an indication that *X. granatum* can be effective in acute inflammatory disorders. Since the ethanol extract exhibited significant inhibition of edema volume at fourth hour after administration of carrageenan in comparison to control, the possible mechanism of anti-inflammatory activity of the extract may be its ability to inhibit the biosynthesis and/or release of prostaglandin-like substances. However the inhibitory effect on the release of histamine or serotonin like substances cannot be ruled out, because the extract showed significant inhibition of rat paw edema during first hour of carrageenan administration as well. Thus the extract may possess chemical constituents that may cause inhibition of the enzyme 'cyclooxygenase'.

The ethanolic leaf extract of *X. granatum* considerably reduced carrageenan-induced paw oedema in mice. Diclofenac also antagonised carrageenan-induced paw oedema in mice. Carrageenan-induced inflammatory pain is well known to involve inflammatory mediators like cyclooxygenase products (PGE₂), leukotrienes histamine, 5-HT, and cytokines [24], which are released as a result of tissue injury. *X. granatum* reduced carrageenan-induced rat paw oedema, suggesting that the plant may have affected inflammatory mediators to produce its anti-inflammatory activity.

CONCLUSION:

This study revealed the antinociceptive and anti-inflammatory activity of ethanol extract of *Xylocarpus granatum* in a dose-dependent manner. Further investigations are required to isolate the active component of the extract and to confirm the mechanism of action in the development of a potent antinociceptive and anti-inflammatory compound.

Conflict Of Interest

The authors declare no conflicts of interest.

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