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Anti-inflammatory and analgesic effects of methanolic extract of *Punica granatum* leaves using carrageenan-induced paw edema and acetic acid-induced writhing models

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Abstract
Pain and inflammation are common sensation triggered by various harmful stimuli, leading to release of chemical mediators. *Punica granatum* has traditionally been used for pain relief. To evaluate the *in vivo* anti-inflammatory and analgesic effect of methanolic extract of *Punica granatum* leaves using Swiss albino mice. Anti-inflammatory effect was demonstrated by carrageenan-induced paw edema on experimental model. Acetic acid-induced writhing test was conducted for determining analgesic effect of this extract. The methanolic extract of *Punica granatum* leaves showed dose-dependent effect in the inhibition of paw edema in mice, with maximum effect 34.61% was obtained at 400 mg/kg, and standard diclofenac sodium had 57.69% inhibitory effect on paw edema. In acetic acid acid-induced writhing test, the extract showed 16.66% and 30.55% inhibition at 200 mg/kg and 400mg/kg doses, whereas standard diclofenac sodium showed 77.77% inhibition of writhing. The extract has moderate *in vivo* anti-inflammatory and analgesic effects.

Keywords: *Punica granatum*, analgesic, anti-inflammatory, carrageenan, acetic acid

Introduction
Inflammation is a vital host defense mechanism which is triggered by pathogens, injury, and toxic substances [1, 2]. Healthy wound healing is done in four different phases hemostasis, inflammation, proliferation, and remodeling [3]. Inflammation is vital process to health as the immune system recognizes the injurious substance and removes it from the body through the inflammatory process [4]. While the body removes these harmful elements and start healing for repairing body tissues, secrets different proinflammatory cytokines including IL-6, TNF-α, and C-reactive protein. These proinflammatory cascade of cytokines mediate local inflammation and also activate other signaling pathways and immune cells like neutrophils, macrophages and monocytes [5]. Among the two types of inflammation, during the acute inflammation, the molecular and cellular events lower the chance of tissue injury or infection that are very likely to occur, and this process crucially helps to restore the cellular homeostasis and resolves acute inflammation. However, persistent inflammation may cause damage to different body organs and variety of diseases [6].

Inflammation has five major symptoms: redness, swelling, heat, pain and loss of tissue function. These manifestations emerge as a reflection of increased vascular permeability, allowing serum leakage and immune cells migration. Although the inflammatory process is involved in repairing damage, overproduction of cytokines can be fatal [7, 8]. Inflammation can be triggered by both infectious and non-infectious causes including bacteria, virus and burn, trauma, foreign bodies. When tissues injury occurs, chemical signaling pathways lead leukocytes to the site of injury via chemotaxis, the leukocytes release cytokines and promote healing [8, 9].

Pain is a common phenomenon, and an integral part of inflammation. Inflammatory process triggers pain through releasing pro-inflammatory cytokines. Pain is caused by two-ways communication between the immune system and the nociceptor neurons, while inflammatory mediators modulating sensitivity and immune response. All pain, whether acute or chronic, peripheral or central, arises from inflammation and inflammatory responses [10].

During inflammation, prostaglandin is secreted via the cyclooxygenase pathway and pain is felt. Several Drugs are available to treat pain and inflammation by inhibiting the COX-2

pathways such as NSAIDs and steroidal anti-inflammatory drugs. However, these synthetic drugs have a variety of side effects including gastric lesions, renal failure [11]. Therefore, developing safe, effective and non-toxic anti-inflammatory drugs is an utmost necessity. *Punica granatum* is a granular fruit that is native to Indian subcontinent, Iran and Afghanistan, has also been spread from Persia across the Mediterranean to the regions like Turkey, California and Mexico [12, 13]. Several studies have shown this plant has significant anti-inflammatory effects along with antioxidant, anti-bacterial, analgesic effects [14, 15].

Acute inflammation is a complex process involving excessive production of free radicals, enzyme activation and release of inflammatory kinins. The carrageenan-induced paw edema model remains a gold standard for evaluating acute anti-inflammatory effects of a substance on rats, suppression edema and cytokine modulation [16, 17]. Carrageenan is injected in the subplantar surface of rat paw, inducing edema in a biphasic system. In this study, early-phase edema is mediated by histamine, serotonin, bradykinin, and the late phase is driven by COX-2 or prostaglandins, TNF-α), and interleukin-1 β (IL-1 β) [17, 18].

Materials and Methods

Plant Material Collection

Fresh leaves of *Punica granatum* were collected from Rajshahi, Bangladesh, in January, 2023.

Preparation of Leaves Extract

Extraction was performed by cold extraction process in which the leaves were treated with methanol. The washed, clean, dry leaves were ground into coarse powdered materials. 1 kg powdered leaves were in 2 liter of methanol, in an amber colored bottle, kept for 7 days with occasional stirring [19]. After maceration, the whole mixture was first filtered through cotton and then through Whatman No.1 filter paper. The filtrate was concentrated with a water bath under reduced pressure at 50°C temperature to yield the crude extract.

Drugs and Chemicals

Experimental Animals

The experiments were conducted on Swiss albino mice of both sexes, aged 4-5 weeks, weighting about 25-30 gm. The mice were purchased from the University of Rajshahi. Prior to the experiments, the mice were kept in standard laboratory conditions (temperature:23.0 ± 2.0º, relative humidity: 55 - 65% and 12 h light/12 h dark cycle) and with free access to feed and water. Animals were acclimatized to laboratory condition for one week before the experiments.

Evaluating analgesic activity

Analgesic activity was determined by acetic acid induced writhing method, an assay that demonstrates a nociceptive stimulation in mice [20]. The method was described by [21]. Sharma *et. al*. In this method, acetic acid in injected in the intra-peritoneal route to create pain in mice, therefore, the mice contract their body at regular interval. This contraction is known as writhing. Analgesic activity is determined by the reduction in frequencies of writhing [20, 22].

Acetic Acid-Induced Writhing Test

50 mg of CME were triturated by the addition of small amount of suspending agent (Tween-80). Normal saline (0.9% NaCl) was slowly added to make the final volume up to 2.5 ml. To prepare the standard, (Diclofenac sodium**)** 20 mg was dissolved into 0.9% normal saline and made the volume up to 10 ml. For preparing control sample, tween-80 (1%) was mixed properly in the normal saline to make the volume up to 5 ml.

The experimental animals were randomly divided into four groups consisting of three mice in each group. The groups were denoted from group-I to group-IV. Analgesic activity of CME of *Punica granatum*, two doses 200 mg/kg & 400 mg/kg were carried out with group-III to group-IV, whereas group-I to group-II were employed to evaluate the analgesic activity of the control and standard respectively. Each group of mice received a specific treatment. Prior administering the drugs, each mouse was weighed properly and the doses were adjusted accordingly.

The percent inhibition (% analgesic activity) is calculated by -

$$\% \text{ inhibition} = \left[\frac{A-B}{A}\right] \times 100$$

Where, A= Average number of writhing of control per group
B= Average number of writhing of test per group

Determination of Anti-Inflammatory Activity

Anti-inflammatory activity of test compounds was determined by Carrageenan-induced paw edema in Swiss albino mice [23, 24]. The results obtained were compared with standard NSAID-Diclofenac sodium.

Carrageenan-Induced Paw Edema Method

Experimental animals were randomly selected and divided into four groups consisting of three mice in each group for control, standard and three test samples group respectively. Each group received a particular treatment i.e. control (Saline solution), standard (diclofenac sodium, 20 mg/kg) and the test sample (CME of 200 mg/kg & 400 mg/kg for each sample). Each mouse was weighed properly and the doses of the test samples and standard materials were adjusted according to their body weight. Half an hour after the oral administration of the test materials, 1% carrageenan was injected to the left hind paw of each animal [25]. The volume of paw edema was measured at 0.5, 1, 2 and 2.5 hours using waterslide calipers after administration of carrageenan. The right hind paw served as a reference non-inflamed paw for comparison.

The average percent increase in paw volume with time was calculated and compared against the control group. Percent inhibition was calculated using the formula-

$$\% \text{ Inhibition of paw edema} = \frac{V_c - V_t}{V_c} \times 100$$

Where V_c and V_t represent average paw volume of control and treated animal respectively.

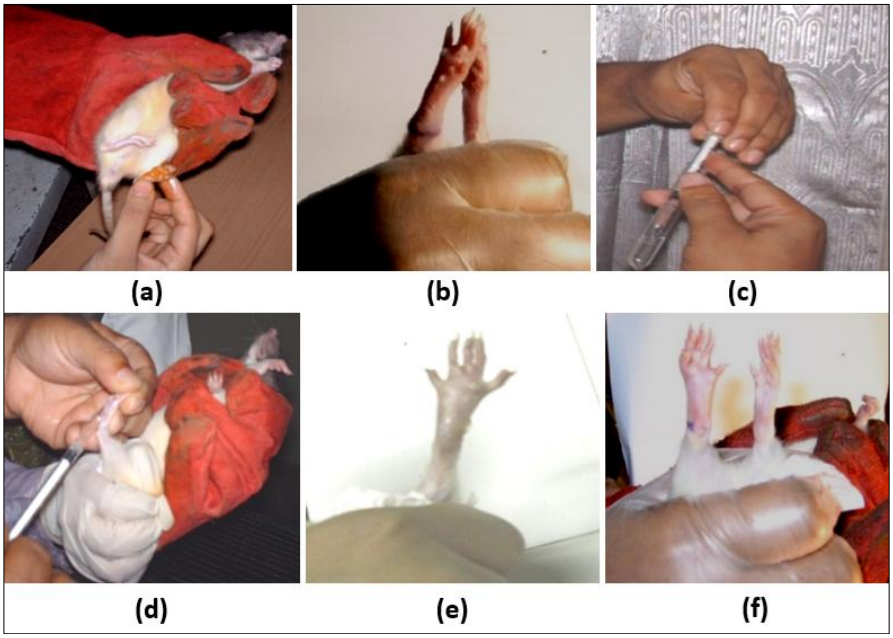

Fig 1: Study design of Carrageenan-induced paw edema method for anti- inflammatory activity. (a) Feeding sample. (b) Marking left paw. (c) Syringe filling with 0.1ml carrageenan. (d) Injected into the left paw of mice. (e) Inflamed paw. (f) Difference between inflamed paw and normal paw.

Statistical Analysis

All data are expressed as Data are presented as Mean ± SD.

Result

Acetic Acid-Induced Writhing Test

In acetic acid induced writhing test, the methanolic extract of *Punica granatum* leaves possessed a significant, dose dependent suppressionin writhing frequency after intraperitoneal administration. At 200 mg/kg and 400 mg/kg doses, crude methanolic extract showed 16.66% and 30.55% writhing inhibition, respectively. In comparison, the standard drug Diclofenac sodium at 20 mg/kg, showed 77.77% writhing inhibition. These findings imply that methanolic extract showed moderate writhing inhibition compared to the standard drug. Analgesic activity of standard and extracts of *Punica granatum* leaves are presented in table- 4.5

Table 1: Analgesic activity of Methanolic extract of *Punica granatum* leaves in writhing method

Sample	Dose (mg/kg)	Writhing number	% Inhibition of Writhing Number
Control	-	36.0±1.0	-
Diclofenac Sodium	20	8.0±1.0	77.77
Methanolic Extract	200	30.0±2.0	16.66
Methanolic Extract	400	25.0±2.0	30.55

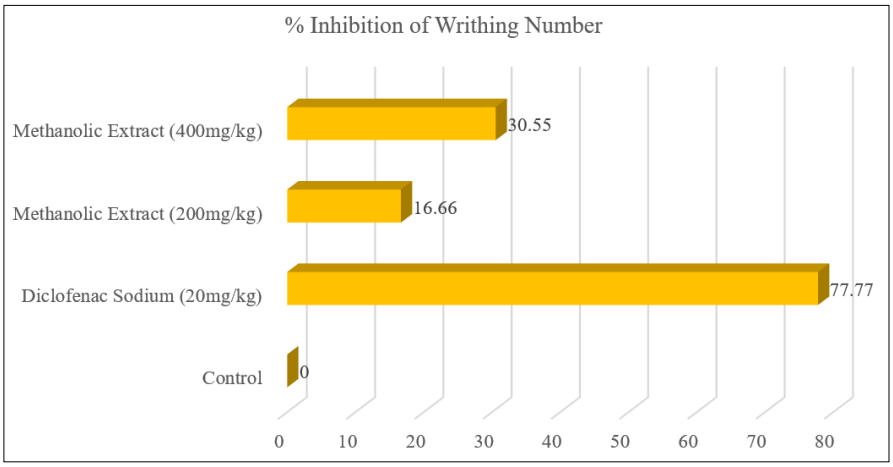

Fig 2: Comparison between Different Samples in Percent Inhibiting of Writhing Number

Study of Anti-Inflammatory Activity

Carrageenan-Induced Paw Eedema Method

Total twelve experimental animals were randomly selected and divided into four groups consisting of three mice in each group for control, standard and two test samples group respectively. Each group received a particular treatment i.e. control (saline solution), standard (diclofenac sodium, 20 mg/kg) and the test sample (CME of 200 mg/kg & 400 mg/kg for each sample). Each mouse was weighed properly and the doses of the test samples and standard materials were adjusted according to their body weight. Half an hour after the intraperitoneal of the test materials, 1% carrageenan was injected to the left hind paw of each animal. The volume of paw edema was measured at 0.5, 2 and 2.5 hours using water slide plethysmometer after administration of carrageen. The right hind paw served as a reference non-inflamed paw for comparison.

Table 2: Anti-inflammatory activity of Methanolic extract of *Punica granatum* leaves in carrageenan-induced paw edema method

Sample	Dose(mg/kg)	Reduction in Paw Volume (cm)				% Inhibition After 2.5 Hours
		0.5h	1h	2h	2.5h	
Control	-	2.2±0.70	2.4±0.42	2.5±0.70	2.6±0.35	-
Diclofenac Sodium	20	1.7±0.12	1.5±0.10	1.2±0.25	1.1±0.30	57.69
Methanolic Extract	200	2.3±0.30	2.0±0.10	2.0±0.35	1.9±0.01	26.92
Methanolic Extract	400	2.2±0.35	2.0±0.35	1.8±0.70	1.7±0.35	34.61

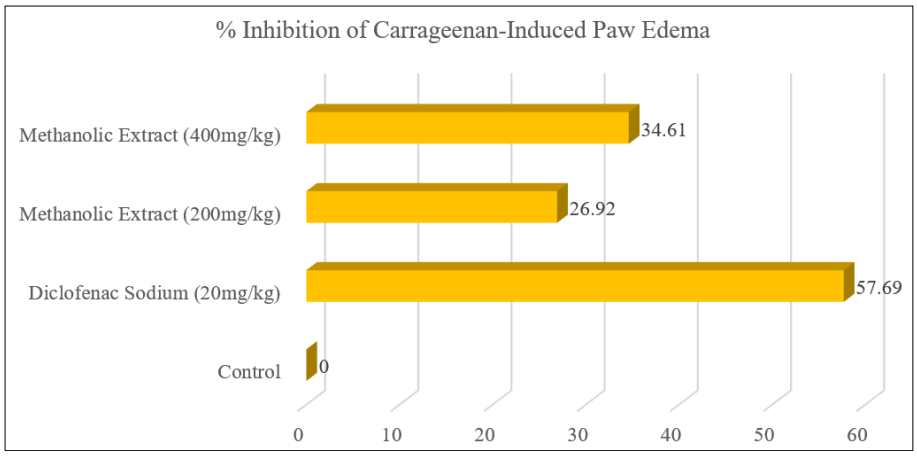

Fig 3: Percentage of Inhibiting the Carrageenan-Induced Paw Edema by Different Samples

Discussion

Studies on inflammation is one of the most principle hubs in recent time [26]. While several anti-inflammatory agents re available in the market, natural remedies are yet appealing in this context due to the adverse effects and toxicities associated with the synthetic drugs. Several phytochemicals are well known for having established anti-inflammatory and analgesic properties, and also they prevent aging and promotes cellular longevity [27]. Excess ROS is considered as the main culprit in inflammation for cellular and organ damage [28]. Tissue damage occurs and inflammatory kinins are released to this response [29]. While studying inflammation and the acute inflammatory effect of both natural and synthetics or even novel compounds, the most popular primary test is done by evaluating the ability to reduce local edema in right paw of mice after administering an irritating agent [30]. In this context, carrageenan-mediated paw edema is widely used experimental model for local and acute inflammation [30, 31]. Carrageenan is injected in the paw of mice, and inflammation results in a biphasic pattern [32]. The release of several chemical mediators triggers edema in tissue via the increase of local blood flow and capillary permeability in the early phase [33]. In the later phase, leukocytes migrates and PG works here [34]. Studies of Mehrzadi *et al*. 2021, Zahra *et al*. 2020 and Hijazy *et al*. 2022 have shown an increase in the mice paw volume after intraplanter injection of carrageenan, compared to the untreated right hind paws [31, 33, 35]. *Punica granatum* have shown potential and dose dependent anti-inflammatory effect with reducing carrageenan-local edema in mice paws. In this study, two doses of CME of *Punica granatum* were used. They had the percentage of inhibition of paw edema were 26.92% and 34.61% for 200mg/kg and 400mg/kg respectively, 2.5 hours after administration via the intraperitoneal route. The paw volume reduced gradually with time, as like the standard diclofenac sodium did. Comparative studies of different *Punica granatum* have been reported in the same experimental model. Waghulde *et al*. 2018 showed the dose- and time-dependent inhibitory effect of oedema of the aril ethanolic extract of *Punica granatum* at 100 mg/kg, 200 mg/kg and 400 mg/kg. At 200 mg/kg, the inhibitory percentage was about 12.40%, 20.23% and 20.53% after 1 hour, 2 hours and 3 hours, respectively. The percentages of inhibition at 400 mg/kg after 1 hour, 2 hours and 3 hours were 7.75%, 28.32% and 21.05%, respectively. The standard indomethacin (5 mg/kg) had the peak effect after 3 hours, which was 45.79% [36]. Another study by Maurya *et al*. (2025) reported the oral administration of fruit peel extract of *Punica granatum* in the reduction of carrageenan-induced paw edema in rats. In this study, three concentrations oedema were taken: 100 mg/kg, 200 mg/kg and 400 mg/kg and observed after 1 hour, 2 hours, 3 hours and 4 hours. At 200 mg/kg, the peak effect was found to be 34.54% after 4 hours. And the inhibitory percentage was maximum at 400 mg/kg after 4 hours, which was 48.72%. Standard ibuprofen had an inhibitory percentage of 51.81% [37].

Pain arises from the effect of different chemical mediators such as PEG2, histamine, bradykinin, serotonin and inflammatory cytokines, including TNF-α, IL-1β, IL-6, and IL-8, in the peritoneal fluid [32]. The acetic acid-induced writhing method is an established method for determining the peripheral anti-nociceptive effect of plant extracts and other substances. After injecting the acetic acid, the COX enzyme is activated and catalyses the conversion of arachidonic acid to PEG2 at the peripheral receptors [38]. The methanolic extract of *Punica granatum* leaves has shown dose-dependent analgesic activity, which had a 16.66% inhibitory effect at 200 mg/kg doses and increased to 35.55% at a dose of 400 mg/kg. Therefore, this extract has a moderate effect in inhibiting acetic acid-induced writhing in mice compared to the standard diclofenac sodium, which had shown 77.77% inhibition of writhing at 20 mg/kg.

Conclusion

The methanolic extract of *Punica granatum* leaves has shown moderate *in vivo* anti-inflammatory and analgesic activities. These effects suggests that it can be promising herbal therapeutic agent in the treatment of pain and inflammation. Further studies for phytochemical screening and compound isolation studies should be done for better understanding to

support its use as a natural remedy for treating pain and inflammation.

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