ANALGESIC AND ANTI-INFLAMMATORY ACTIVITIES OF ETHANOLIC EXTRACT OF LEAVES OF *PUNICA GRANATUM* L. ON EXPERIMENTAL ANIMAL MODELS.

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Summary

The present study was designed to evaluate the analgesic and anti-inflammatory activity of the ethanolic extracts of leaves of Punica granatum *Linn.* (EEPG). The extract was prepared by percolation method and acute oral toxicity test was performed as per OECD guidelines. The central analgesic activity was assessed using tail-flick method. The peripheral analgesic activity was assessed using acetic acid induced writhing method. Anti-inflammatory activity was assessed using carrageenan induced paw edema. It has been shown that EEPG (500 mg/kg s.c) and pethidine (5 mg/kg s.c) significantly increased the pain threshold as assessed by increase in the latency period or basal reaction time. Naloxone (1mg/kg s.c) was used to find the central mechanism of action. EEPG (500 mg/kg s.c) combined with naloxone (1 mg/kg s.c) significantly decreased the latency period indicating some agonistic activity of EEPG for the opioid receptors as the probable mechanism of action. EEPG(500 mg/kg p.o) and aspirin(100 mg/kg p.o) also significantly reduced acetic acid induced writhing response showing peripheral analgesic activity. It has also been shown that EEPG (500 mg/kg orally) and aspirin (100 mg/kg p.o) significantly reduced carrageenan induced paw edema. The result, thus justifies the traditional use of Punica granatum in inflammatory and painful conditions.

Keywords: Punica granatum, Analgesic, Anti-inflammatory, Carrageenan

Introduction

Pain is an unpleasant sensation localized to a part of the body. It is often described in terms of a penetrating or tissue-destructive process and/or of a bodily or emotional reaction. [1]. Inflammation is a protective response intended to eliminate the initial cause of cell injury as well as the necrotic cells and tissues resulting from the original insult. It is intimately interwoven with repair processes whereby damaged tissue is replaced by regeneration of parenchymal cells, and/or filling of any residual defect with fibrous scar tissue.[2]. The pomegranate, *Punica granatum L*., an ancient, mystical, and highly distinctive fruit, is the predominant member of two species comprising the Punicaceae family.The pomegranate is native from the Himalayas in northern India to Iran and has been cultivated and naturalized since Ancient times over the entire Mediterranean region. It is also found in India.In Ayurvedic medicine, the pomegranate is considered "a pharmacy in itself" and is used as anti-parasitic agent, a blood tonic and to heal aphthae, diarrhoea and ulcers. Pomegranate also serves as a remedy for diabetes in the Unani System of Medicine practiced tn the Middle East and India[3]. It is cultivated almost throughout Assam, commonly known as Dalim in Assamese and Bengali. Bark of stem and root is used as astringent, anti-helminthic, specifically in tapeworm. Fruit rind is used in diarrhoea and dysentery. Seeds are used as stomachic. Pulp is used for cardiac conditions and as astringent. Fresh juice is used for cooling and refrigerant action. Juice of flowers mixed with juice of durba in equal parts is used to stop bleeding from nose[4]. Active constituents isolated from the plant are the alkaloids pellentrine, isopellentrine and methyl isopellentrine. B-sitosterol, friedelin, and D-mannitol have also been reported to be present in stem,roots and roots [5]. Current research seems to indicate the most therapeutically beneficial pomegranate constituents are ellagic acid ellagitannins (including

punicalagins), punicic acid,flavonoids,anthocyanidins,anthocyanins,and estrogenic flavonols and flavones[3].Traditionally,decoction of leaves has been used to treat painful and inflammatory conditions. However,no scientific data is available on this. Hence, the present work was undertaken to evaluate the analgesic and anti-inflammatory activities of the ethanolic extract of *Punica granatum* L. on experimental animal models.

Methods

Plant material

Plants of Punica granatum were collected from the campus of Assam Medical College, Dibrugarh, Assam. The plants were authenticated by Prof. M.Islam, Department of Life Sciences, Dibrugarh University, Assam.

Plant Extract

The leaves of the plants were air-dried in shade. These were then powdered and ethanol extracts were prepared using 95% ethanol by percolation method[6]followed by evaporation in a rotary evaporator under controlled temperature, and reduced pressure. A net yield of 90 g (18%) was obtained by percolating 500 g of dry powder of leaves.

Animals

All the animals used in the study were procured from Central Animal House, Assam Medical College & Hospital, Dibrugarh, Assam. The study was conducted in accordance with CPCSEA (Committee for the Purpose of Control and Supervision of Experiment on Animals) guidelines and the study was approved by the Institutional Animal Ethical Committee (Registration no.-634/02/a/CPCSEA). They were fed with standard diet and water *ad libitum* was provided. Experimental animals used were healthy albino rats of the species *Rattus norvegicus* of either sex weighing 150-200 gm and healthy albino mice of the species *Mus Musculus* of either sex weighing 20-30 gm.

Chemicals and Drugs

The following chemicals and drugs were used: 3% gum acacia suspension, normal saline, pethidine, naloxone, aspirin, carrageenan.

Acute toxicity study

Acute toxicity test was done for the ethanolic extract of Punica granatum following OECD 425 guidelines[7]. An arbitrary dose 500 mg/kg was selected for the study, as the extract was found safe even at doses more than 2000 mg/kg without any sign of toxicity or mortality.

Method for central analgesic activity

Healthy albino rats of either sex weighing 150-200 g were divided into five groups with six animals in each group. The groups with their respective treatments were

Group A- Control, vehicle normal saline (NS) 5 ml/kg subcutaneously

Group B- EEPG 500 mg/kg subcutaneously

Group C- Naloxone 1 mg/kg subcutaneously

Group D- EEPG 500 mg/kg subcutaneously + Naloxone 1mg/kg subcutaneously

Group E- Pethidine 5 mg/kg subcutaneously

The central analgesic activity was tested by tail-flick method[8]. The tail flick latencies or the basal reaction time of the animals were assessed using analgesiometer (Elite). Basal reaction time of radiant heat was taken by placing the tip (last 2 cm) of the tail on the radiant heat source. A cut-off period of 10 sec was observed to prevent damage to the tail. Reaction time were recorded at pre-drug, 15, 30, 60, 90, 120,150 and 180 min after administration of vehicle or drugs. Here, pethidine was used as the standard drug and naloxone was used to determine the mechanism of action.

Method for peripheral analgesic activity

Healthy albino mice of either sex weighing 20-30 g were taken and divided into three groups with six animals in each group. The groups with their respective treatments were

Group A- Control, normal saline 5 ml/kg orally.

Group B- EEPG 500 mg/kg orally

Group C- Aspirin 100 mg/kg orally

The peripheral analgesic activity was tested with glacial acetic acid induced writhing response[9]. One hour after administration of drugs, induction of writhing was done in mice by giving intraperitoneal injection of acetic acid at a dose of 5 ml/kg body weight. The number of writhing responses were counted and recorded for 20 min in each group and the percentage protection was noted. Here, aspirin was used as the standard drug at the dose of 100 mg/kg per orally.

Method for anti-inflammatory activity

Healthy albino rats of either sex weighing 150-200 g were taken and divided into three groups of six animals. The groups with their respective treatments were

Group A- Control 3% gum acacia-5 ml/kg

Group B- EEPG 500 mg/kg orally

Group C- Aspirin 100 mg/kg orally

The anti-inflammatory activity of EEPG against acute inflammation was tested by carrageenan-induced rat paw edema method.Carrageenan –induced paw edema is the simplest and the most widely used model for studying the anti-inflammatory activity of new compounds.0.1 ml of 1% carrageenan in normal saline was injected into the subplantar region of the rat hind paw . The animals were treated with 3% gum acacia, EEPG and asprin in the respective groups 1hr before carrageenan injection. The paw volume was measured plethysmometrically just before carrageenan injection at 0 hour, then at 1^{st} , 2^{nd} , 3^{rd} & 4^{th} hour after carrageenan injection[10] Increase in paw volume was measured as the difference between the paw volume at '0' hr and paw volume at the respective hour. The percentage inhibition of rat paw edema was calculated after each hour of carrageenan injection upto 4 hours by the formula described by Sudjarwo Agus[11].

% inhibition= (Control mean – Treated mean) / Control mean x 100

Statistical Analysis

Statistical analysis was done using one way ANOVA followed by Dunnett's Multiple Comparison test. Significance level of < 0.05 was considered as significant[12].

Results

In acute oral toxicity tests, the LD50 of EEPG to be more than 2000mg/kg.

The ethanolic extract of Punica granatum showed significant central analgesic activity as compared to control ($p<0.01$; Table 1) as evidenced by significant increase in the latency time. Significant peripheral analgesic action was also observed with EEPG and aspirin as compared to control ($p<0.01$; Table 2) as evidenced by inhibition of abdominal writhes produced by acetic acid. There was also significant ($p<0.01$;Table 3) reduction in carrageenan induced paw edema by EEPG and aspirin. The maximum inhibition was seen at the end of 1st hour.

TABLE 1: REACTION TIME IN SEC (MEAN +/- SEM)

GROUPS	Drug Dose mg/kg s.c	Pre-drug reaction time(in sec) Mean +/- SEM	15 min	30 min	60 min	90 min	120 min	150 min	180 min
Group A	Normal Saline (5ml/kg)	3.6 +/- 0.09	3.55 +/- 0.05	3.6 +/- 0.13	3.55 +/- 0.15	3.51 +/- 0.15	3.55 +/- 0.11	3.7 +/- 0.16	3.75 +/- 0.12
Group B	EEPG (500mg/ kg)	3.3 +/- 0.09	3.5 +/- 0.07	3.8 +/- 0.07	4.15 +/- 0.07 [a]	4.5 +/- 0.06 [a]	4.2 +/- 0.12 [a]	3.7 +/- 0.12	3.5 +/- 0.05
Group C	Naloxone (1 mg/kg)	3.3 +/- 0.04	3.1 +/- 0.06[a]	3.0 +/- 0.06 [a]	2.9 +/- 0.07 [a]	2.6 +/- 0.13 [a]	2.6 +/- 0.13 [a]	2.7 +/- 0.13 [a]	3.0 +/- 0.05 [a]
Group D	EEPG (500mg/ kg) + Naloxone (1 mg/kg)	3.3 +/- 0.08	3.2 +/- 0.04 [a]	2.8 +/- 0.07 [a]	2.8 +/- 0.07 [a]	2.4 +/- 0.06 [a]	2.4 +/- 0.06 [a]	2.8 +/- 0.08 [a]	3.3 +/- 0.09 [a]
Group E	Pethidine (5 mg/kg)	3.7 +/- 0.15	4.1 +/- 0.15 [a]	5.0 +/- 0.08 [a]	5.05 +/- 0.09 [a]	5.88 +/- 0.11 [a]	5.6 +/- 0.17 [a]	4.75 +/- 0.18 [a]	4.15 +/- 0.09 [a]
One way ANOVA	F df P	3.97 25,4 >0.01	21.75 25,4 <0.0001	97.67 25,4 <0.0001	86.9 25,4 <0.0001	284.6 25,4 <0.0001	112.5 25,4 <0.0001	34.08 25,4 <0.0001	23.23 25,4 <0.0001
One way ANOVA followed by Dunnett's a= $p<0.05$ vs Control									

TABLE 2

GROUPS	DRUG DOSE (mg/kg) PER ORALLY	NUMBER OF WRITHING MOVEMENTS (Mean+/-SEM)	PERCENTAGE OF PROTECTION S.C (%)
GROUP A	Normal saline 10 ml/kg	69.5+/-0.56	___
GROUP B	EEPG 500 mg/kg	48+/-0.88[a]	89.93
GROUP C	Aspirin 100 mg/kg	7+/-0.26[a]	30.94
One Way ANOVA	F df P	2611 15,2 <0.0001	
a= p< 0.05 vs Control; One way ANOVA followed by Dunnett's Multiple Comparison Test			

TABLE 3

Drug	Drug Dose	Mean increase in paw volume(Mean ±SEM)(mL) (% Inhibition within parentheses)			
		1st hr	2nd hr	3rd hr	4th hr
A (Control)	5 mg/kg	0.22 ± 0.02	0.30 ±0.02	0.56 ± 0.01	0.32± 0.02
B (EEPG)	500mg/kg	0.14 ± 0.01[a] (36.54%)	0.16 ±0.01[a] (47.24%)	0.25 ±0.01[a] (55.58%)	0.16 ±0.01[a] (48.48%)
C (Asprin)	100mg/kg	0.12 ±0.01[a] (46.82%)	0.13 ±0.17[a] (55.15%)	0.21 ± 0.02[a] (62.06%)	0.13 ± 0.01[a] (57.00%)
ANOVA	F	10.12	45.84	180.7	60.91
	df	17,2	17,2	17,2	17,2
	P	<0.05	<0.05	<0.05	<0.05
Values are expressed as Mean±SEM a= p<0.05 vs Control One way ANOVA followed by Dunnett's Multiple Comparison Test were done					

Discussion

Our study showed that ethanolic extracts of the leaves of Punica granatum produced significant analgesia, both centrally and peripherally. The extract (500 mg/kg s.c) and pethidine showed significant increase in the reaction time. Pre-treatment with naloxone significantly decreased the reaction time producing hyperalgesia while combined treatment of EEPG (500 mg/kg s.c) and naloxone (1 mg/kg s.c) produced significant decrease in the reaction time as compared to EEPG alone. Naloxone is a competitive antagonist at all types of opioid receptors. It also blocks the actions of endogenous opioid peptides[13].In the face of a variety of physical (pain) or psychological stressors, an increased release of a variety of opioid peptides occurs[14]. This indicates the involovement of endogenous opioid peptides in mediation of analgesic activity of *Punica granatum* which seems to be its probable central mechanism of action. However, since there is almost complete inhibition of analgesic activity of EEPG after naloxone, opioid mechanisms may also be involoved. The extract (500 mg/kg orally) and aspirin (100 mg/kg orally) significantly reduced the number of writhes induced by acetic acid. Acetic acid causes algesia by liberating endogenous substances including serotonin, histamine, prostaglandins, bradykinin and substance P which stimulate pain nerve endings. Local peripheral receptors are postulated to be partly involved in the abdominal constriction (writhing response). The abdominal constriction is related to the sensitization of nociceptive receptors to prostaglandins[15]. Standard NSAIDs like aspirin offer relief from inflammatory pain by suppressing the formation of pain substances in the peripheral tissues, where prostaglandins and bradykinin were suggested to play an important role in the pain process. Prostaglandins elicit pain by direct stimulation of sensory nerve endings to other pain provoking stimuli[16]. Therefore, it is likely that EEPG suppresses the formation of these substances or antagonize the action of these substances which may serve as its peripheral mechanism of analgesic activity. Carrageenan induced paw edema is a suitable test for evaluating anti-inflammatory drugs which has frequently been used to assess the anti-edematous effect of natural products. Development of edema in the paw of the rat after injection of carrageenan is a biphasic event. The initial phase observed during the first hour is attributed to the release of histamine and serotonin. The second phase of edema is due to the release of prostaglandins, protease and lysosome[15]. Our study showed that the EEPG (500 mg/kg) produced significant reduction of the carrageenan induced paw edema suggesting its anti-inflammatory activity. The maximum inhibition was seen at the end of 1st hour. Flavonoids are known to target prostaglandins which are involved in the late phase of acuteinflammation and pain perception[17]. Therefore, flavonoids present in the leaves of Punica granatum may be responsible for its analgesic and anti-inflammatory activities.

The present study demonstrated that the ethanolic extracts of Punica granatum showed significant analgesic and anti-inflammatory activity thereby establishing its traditional use in inflammatory and painful conditions. However further studies and development of more purified product of leaves of *Punica granatum* are required for proper clinical use.

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