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Cyclooxygenase-2 Inhibitory Effect and Anti-Inflammatory Activity of Pomegranate (*Punica granatum* L.) Rind Extract

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ABSTRACT: ***Inflammation is a normal protective response to tissue damage mediated by Cyclooxygenase-2 (COX-2) enzyme. Cyclooxygenase enzymes are responsible for the production of prostaglandins. Increasing incidence and impact of inflammatory diseases have encouraged the search for new pharmacological strategies to face them. Pomegranate (Punica granatum L.) has been traditionally used as an anti-inflammatory and antioxidant, thus giving scope for anti-inflammatory studies. The aim of the present investigation to determine the anti-inflammatory activity and inhibitory effect on COX-2 enzyme of the ethanol extract of pomegranate rind (EEPR). The animals used in this study were male white rats which were divided into six groups, dose 20 mg/kg BW, dose 40 mg/kg BW, dose 80 mg/kg BW, positive control, negative control, and normal control. Determining of anti-inflammatory activity was carried out by inducing the soles of the rats with carrageenan and then measuring the edema volume using a plethysmometer and COX-2 inhibition is determined spectrophotometrically in a Microplate Reader. The results showed that EEPR (20 mg/kg BW and 40 mg/kg BW, and 80 mg/kg BW) had an inhibitory effect against prostaglandin synthesis by COX-2 and anti-inflammatory activity. These findings suggest that EEPR possesses promising anti-inflammatory activity, which is possibly mediated through inhibition COX-2 enzymes.***
Keywords: Anti-Inflammatory, Punica granatum L, Cyclooxygenase-2 enzyme, Inflammation

1. Introduction

Inflammation is a protective reaction of the microcirculation, initiated after infection and/or injury. Local and systemic inflammatory responses aim to eliminate the inciting stimulus, promote tissue repair and healing and, in the case of infection, establish immune memory such that the host mounts a faster and more specific response on a future encounter [1]. Arachidonic acid, liberated from membrane phospholipids by the action of phospholipase A2, is a central component of inflammatory cascade reactions and is metabolized by two enzymatic pathways into numerous biologically active mediators: prostaglandins and leukotrienes, which induce tissue edema and pain. Traditional treatment of the inflammatory process and the associated pain focused on reducing the production of inflammatory prostaglandins [2].

Pro-inflammatory mediators are the key regulators of physiological process, but uncontrolled production of pro-inflammatory mediators can maintain or amplify the inflammatory response leading to chronic inflammation. Cyclooxygenase is present in two isoforms; COX-1 and inducible form COX-2 [3]–[5]. Down regulation of these mediators can provide anti-inflammatory activity. For these purposes, nonsteroidal anti-inflammatory drugs (NSAIDs) or SAIDS are used to inhibit the pathway of inflammatory mediators. Use of NSAIDS usually results in ulceration of lumen and bleeding of the intestinal mucosa [6]. On the other hand prolonged use of NSAIDs is also associated with severe side effects such as gastrointestinal hemorrhage due to COX-1 inhibition. The new COX-2 selective drugs do not seem to be free of risk either since several COX-2 inhibitors are linked with cardiovascular problems [7]. Consequently, there is a strong need for natural products with minimum side effects. Data indicated that plants possess diverse therapeutic activities including antiinflammatory activities [8].

The fruit of *Punica granatum* L. (pomegranate) has been used as a health-promoting and healing fruit for many years. Fruit of the Punica granatum L. tree has been used since ancient times to treat a wide range of ailments. It has long been used in Middle Eastern and Chinese medicine for its antioxidant, anti-inflammatory [9],

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bactericidal (Millo *et al*., 2017), antiarthritic [10] properties. The pomegranate seed oil has phytoestrogenic compounds and the fruit is rich in phenolic compounds with strong antioxidant activity [11].

Pomegranate rind extract is obtained from the pericarp (rind), which contains the highest concentration of phytochemicals, principally polyphenolic flavonoids, and ellagitannins, including ellagic acid and punicalagin. It has recently been shown that topically applied of *Punica granatum* L. rind extracts, exert a significant anti-inflammatory effect on COX-2 expression in ex vivo skin. Indirectly, this demonstrates that the anti-inflammatory principles, in particular punicalagin, penetrate the skin and modulate COX-2 expression [12]. In previous studies has been shown that topical formulations of standardized pomegranate rind extracts and ellagic acid are promising therapies for contact dermatitis and can be applied as an alternative treatment for cutaneous disorders [13]. The positive effect of the consumption of pomegranate extracts and juice has been demonstrated in relation to inflammation in the gastrointestinal tract, where ellagic acid was stated as the responsible agent [14], [15]. Recent data from in vitro studies have added new insights into the multiple variables that different subclasses of flavonoids can modulate in several stages of inflammation [16]. The flavanol quercetin was found to suppress the expression of COX 2 mRNA in the pouch exudates cells of a rat paw, indicating that the anti-inflammatory action of quercetin may partly due to suppressing the up-regulation of COX-2 [17]; pomegranate juice consumption by patients was found to reduce inflammatory processes in patients with type-2 diabetes [18].

However, the actions of Pomegranate rind extracts on COX-2 in vivo have not been described previously. Thus, in the present study, we explored the effects of Pomegranate rind extracts on Anti-inflammation activity in Carrageenan-induced Rat Paw Edema and the COX-2 inhibitory effect in vivo.

2. Materials and Methods

2.1 Chemical and Reagents

Prostaglandin Endopheroxide Synthase-2 ELISA kit (Elabscience), Carrageenan (Sigma Aldrich), Celecoxib (Pfizer), PELLET HI-PRO-VITE 511 (Charoen Pokphand), Ethanol 70 % (Brataco), Normal Saline 0,9% (PT Otsuka), Na. CMC (Brataco).

2.2. Plant Material

The Rind of *Punica granatum* L. were collected from Koto Tangah, Padang, Indonesia in April 2019. The Rind of Punica granatum L were identified by Dr. Nurainas, a botanist at Herbarium of Andalas University, West Sumatera, Indonesia.

2.3 Preparation of The Ethanol Extract of Pomegranate Rind (EEPR)

The pomegranate rind was sun-dried. The dried rind (350 g) was powdered using a conventional grinder powdered materials were then soaked in Ethanol (70%) by stirring at room temperature for 24 hours. After 24 hours, the materials were filtered. The process was repeated thrice. The filtrates were combined and concentrated under vacuum using a rotary till a brownish semisolid extract, free of solvent (50 g), was obtained. The extract was refrigerated for further pharmacological screenings.

2.4 Experimental Animal

18 adult male Wistar rats with body weights of 180–220 g and aged 2-3 months were obtained from West Sumatera animal houses were used for this study. Animals were housed and cared for under standard conditions with 12 h light/dark circle and were fed with standard pellet diet and water ad libitum. All the animals were acclimatized for a minimum period of 1 week prior to the experiment. After 1 week, animals were randomly selected for different experimental groups (3 animal/ group) and used for the in vivo determination of anti-inflammatory activity. The rats were deprived of food, but not water, for 18–20 hours before an experiment.

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2.5 Evaluation of Anti-Inflammatory activity

The anti-inflammatory activity was further examined by the carrageenan-induced rat paw edema method according to the method of [19], modified by [20]. The experimental groups consisted of 18 rats divided into six groups— Group I: Normal control (received Na. CMC 0,5% p.o only), Group II: Negative control (Carrageenan 1% s.c), Group III: Carrageenan 1% s.c + EEPR (20 mg/kg B.W p.o), Group IV: Carrageenan 1% s.c + EEPR (40 mg/kg B.W p.o), Group V: Carrageenan 1% s.c + EEPR (80 mg/kg B.W p.o), Group VI: Positive control (Carrageenan 1% s.c + Celecoxib 9 mg/kg p.o) were given 1 h before the injection of carrageenan. Edema was induced on rat right hind paw by injection of 0.1 ml of 1% carrageenan in 0.9% saline. After 1 h, 0.1 ml 1% carrageenan was injected subcutaneously into the subplantar area of the right hind paw of each rat except those in Group I. Edema volume was measured every 1 h up to 6 h after the administration of carrageenan. The paw volumes were measured by a plethysmometer. The results were obtained by measuring the volume difference before and after the injection of the right paw. The swelling degree of paw and inhibition rate of edema was calculated as follows :

% edema inhibition = (Vc −Vt) × 100/ Vc;

Vc and Vt are average edema volume of control and test, respectively. At the end of the third hour, the animal blood was collected in heparin-coated tubes. Groups I–VI was used to determining the activities of COX-2.

2.6 Evaluation of COX – 2 Inhibitory Activity

Rat serum was prepared at the 3rd hour after induction of Carrageenan 1%. EEPR (20 mg/kg BW and 40 mg/kg BW, and 80 mg/kg BW) was used for inhibition studies.The ability of the test compound to inhibit COX-2 was determined by using the Enzyme-Linked Immunosorbent Assay (ELISA) kit (Elabscience) according to the manufacturer's instructions. The product of this enzymatic reaction produced a distinct yellow color, determined by spectrophotometrically (Microplate reader) at 450 nm.

Statistical Analysis

The results were analyzed using a statistical program SPSS version 25 (SPSS Inc., Chicago, IL, USA). Data was analyzed using one-way ANOVA followed by Duncan's multiple range test. $p < 0.05$ was considered significant.

3. Results and Discussion

The carrageenan-induced rat paw edema assay has frequently been used to evaluate the anti-inflammatory effect of natural products. The induction of edema by using carrageenan is believed to be biphasic in nature. The first phase involved within 1 h of carrageenan administration is associated with the release of histamine and serotonin from mast cells [21], [22]. The second phase starts after 1 h and is characterized by an increased release of Prostaglandin (PG) in the inflammatory area. During the second phase, the macrophages are known to release the large amounts of interleukin-1 (IL-1) which led to the increased accumulation of polymorphic nuclear cells (PMNs) to the site of inflammation. The activated PMNs then release the lysosomal enzymes and active oxygen species to destroy connective tissue and induce paw swelling [23]. In the present study, the edema was measured after 3 h of carrageenan injection, i.e., during the later stages of the second phase, where the effect of PGs and the released cytokines is prominent. EEPR given at dose (80 mg/kg B.W), were effective in inhibiting the induced paw edema (Table 1).

Tabel 1. ***Effect of EEPR, celecoxib as compared to carrageenan control group at different hours in carrageenan-induced paw edema model***

	Groups	Dose	Percentage (%) of paw edema inhibition[a]
I	Negative control (Na. CMC 0,5%)	2 ml /200g B.W	-
II	Positive control (Carrageenan 1%)	0,1 ml/200g B.W	-
III	EEPR	20 mg/kg B.W	41,58*
IV	EEPR	40 mg/kg B.W	49,69*

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V	EEPR	80 mg/kg B.W	68,68*
VI	Celecoxib[b]	9 mg/kg B.W	27*

[a]Data are expressed as the mean of Three observations (n = 3), [b]Used as positive control
* Significant difference compared to the positive control (P < 0.05)

Table 1 shows the effect of EEPR and standard drug as compared to carrageenan control at different hours in the carrageenan-induced paw edema model. From Table 1, a significant anti-inflammatory activity of EEPR could be confirmed through edema inhibition by 41,58%, 49,69% and 68,68% after 6 h, at doses of 20, 40, and 80 mg/kg, respectively, while celecoxib reduced paw edema by 27% at the same time (p<0,005). Comparison of the effects prolonged for 6 h, it was obvious that 80 mg/kg B.W was the most potent anti-inflammatory dose. Moreover, treatment with EEPR at the same dose (80 mg/kg B.W) significantly suppressed Cox-2, P < 0,005 (Table 2). These results further corroborate previous studies based on Topical application of Pomegranate rind extracts had significant anti-inflammatory effects in ex vivo skin [12]
Moreover, in previous studies, ethanolic extract of *Punica garanatum* L. rind has been shown the positive effect of the consumption of pomegranate extracts and juice has been demonstrated in relation to inflammation in the gastrointestinal tract, where ellagic acid was stated as the responsible agent [14], [15], and topical formulations of standardized pomegranate rind extracts and ellagic acid are promising therapies for contact dermatitis and can be applied as an alternative treatment for cutaneous disorders [13].
From Table 2, a significant COX 2 Inhibitory effect of EEPR could be confirmed through COX-2 inhibition by 49,75 %, 52,17 % and 54,92 % after 3 h, at doses of 20, 40, and 80 mg/kg, respectively, while celecoxib reduced paw edema by 45,12 % at the same time (p<0,005). Comparison of the effects prolonged for 3 h, it was obvious that 80 mg/kg B.W ware the most potent COX 2 Inhibitory effect dose.

Table 2. ***COX 2 Inhibitory effect of EEPR***

	Groups	Dose	Percentage (%) of Cox-2 inhibition[a]
I	Negative control (Na. CMC 0,5%)	2 ml /200g B.W	-
II	Positive control (Carrageenan 1%)	0,1 ml /200g B.W	-
III	EEPR	20 mg/kg B.W	49,75 *
IV	EEPR	40 mg/kg B.W	52,17 *
V	EEPR	80 mg/kg B.W	54,92 *
VI	Celecoxib[b]	9 mg/kg B.W	45,12 *

[a]Data are expressed as the mean of Three observations (n = 3), [b]Used as positive control
* Significant difference compared to the positive control (P < 0.05)

COX and 5-LOX are two important enzymes that catalyze the formation of mediators involved in the inflammatory process. Inhibitors of Coxs are the main strays of current therapy aimed to modulate pain, inflammation, and to control fever [24]. Many COX-2 or 5-LOX inhibitors have been developed as drugs to treat inflammation; however, some have been withdrawn from the market, indicating a need for inhibitors free of side effects [25]. Cyclooxygenase group of enzymes (COXs, prostaglandin-endoperoxide synthases) catalyze two reactions, the first being a cyclooxygenase function consisting of the addition of molecular oxygen to arachidonic acid to form PGG2. The second is the conversion of PGG2 to PGH2 by a peroxidase function. Hence, COX performs the critical initial reaction in the arachidonic acid metabolic cascade, leading to the formation of pro-inflammatory prostaglandins, thromboxane, and prostacyclin. Prostaglandins regulate smooth muscle contractility, blood pressure, and platelet aggregation and induce pain and fever. Inhibition of cyclooxygenase activity is the mechanism by which non-steroidal anti-inflammatory drugs (NSAIDs) exert their analgesic, antipyretic, anti-inflammatory, and antithrombotic effects [26]. COX activity was significantly inhibited by EEPR in rat at the 3rd hour in vivo as compared to control.
These results further corroborate previous studies, different preparations of pomegranate, including extracts from peels, flowers, seeds, and juice, show a significant anti-inflammatory activity in the gut [15], and

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ethanolic extract of *Punica garanatum* L. rind has been shown that topically applied of *Punica granatum* L. rind extracts, exert a significant anti-inflammatory effect on COX-2 expression in ex vivo skin. Indirectly, this demonstrates that the anti-inflammatory principles, in particular punicalagin, penetrate the skin and modulate COX-2 expression [12].

The anti-inflammatory potential of EEPR may be due to the presence of active phytoconstituents such as polyphenolic flavonoids, and ellagitannins, including ellagic acid and punicalagin. The previous report describes that different subclasses of flavonoids can modulate in several stages of inflammation [16]. The flavanol quercetin was found to suppress the expression of COX 2 mRNA in the pouch exudates cells of a rat paw, indicating that the anti-inflammatory action of quercetin may partly due to suppressing the up-regulation of COX-2 [17]. Moreover, topical formulations ellagic acid are promising therapies for contact dermatitis and can be applied as an alternative treatment for cutaneous disorders [13].

This study is the first report on the potent COX-2 inhibitory effect in vivo of the ethanol extract of *Punica garanatum* L. rind (EEPR). Obtained results indicate that the inhibition of prostaglandin production mediated via the arachidonic acid pathway could be responsible for the biological effects of this natural compound.

4. Conclusion

From the results of the present investigation, it can be concluded that the ethanol extract of Punica garanatum L. rind (EEPR) possesses significant and promising anti-inflammatory activity. The mechanism of anti-inflammatory action is assumed to be mediated through the inhibition of COX-2. These findings support the use of the extract in the traditional system of medicine for the management of inflammatory conditions and the results presented also suggest the need for further research on the COX-1 inhibitory effect.

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