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Anti-inflammatory effect of dikaempferol rhamnopyranoside, a diflavonoid from *Eugenia jambolana* Lam. Leaves

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Traditionally, the Indian Blackberry or locally called Jamun, *Eugenia jambolana* Lam. (Syn.: *Syzygium cumini*), is well known for its pharmacological potential, particularly anti-inflammatory. Here, we studied kaempferol-7-O-α-L-rhamnopyranoside]-4'-O-4'- [kaempferol-7-O-α-L-rhamnopyranoside (EJ-01) isolated from the *E. jambolana* leaves for possible anti-inflammatory activity. EJ-01 (3, 10 and 30 mg/kg, p.o.) was assessed for anti-inflammatory activity using carrageenan-induced paw edema model in mice by determining edema volume, myeloperoxidase (MPO), nitrite plus nitrate (NOx) and cytokine levels in paw edema tissue. EJ-01 significantly attenuated the edema, MPO levels, tumor necrosis factor-alpha (TNF-α) and interleukin-1beta (IL-1β) levels in the edema of paw at the 5th hour after carrageenan injection at all doses. EJ-01 (30 mg/kg) decreased the nitric oxide (NO) levels of the edema of paw at the 5th hour after carrageenan injection. The anti-inflammatory mechanisms of EJ-01 might be related to the decrease in the level of edema paw by reduced activities of NO and MPO. It probably exerts anti-inflammatory effects through the suppression of TNF-*α* and IL-1β. Therefore, we conclude that EJ-01 could be positively exploited for itspotential benefits against inflammatory diseases and support the pharmacological basis of *E. jambolana* as traditional herbal medicine for the treatment of inflammatory diseases.

Keywords: Diflavonoid, Indian Blackberry, Jamun, Java Plum, *Syzygium cumini*

Research interest in using plants as safe source of new therapeutic agents is ever-growing since decades[1]. Kaempferol is a natural flavonol, a type of flavonoid, that has been isolated from tea[2], cabbage, beans, tomato, grapes, apples and other plant sources[3,4] including aquatic pteridophyte *Azolla microphylla*[5]. Numerous preclinical studies have shown that kaempferol and some glycosides of kaempferol possess a wide range of pharmacological activities *viz*., antioxidant[5,6], antimicrobial[5,6], antiviral[7], anti-inflammatory[8-10], antinociceptive/analgesic[10,11], neuro-protective[12], antidiabetic[13], anticancer[14], antiallergic[15], and others like cardioprotective, anxiolytic and anti-osteoarthritic activities[3].

Eugenia jambolana Lam. (Syn.: *Syzygium cumini*), the Indian Blackberry (Java plum or Jamun), is an evergreen tropical tree belongs to flowering plant of family Myrtaceae, native to Bangladesh, India, Nepal, Pakistan and Indonesia. Crude extracts of leaves and bark of *E. jambolana* have been shown to possess potential antiviral activity against highly pathogenic avian influenza virus (H5N1)[7]. *E. jambolana* seeds have been reported to have anti-inflammatory effects[16]. Significant anti-inflammatory activity was observed with *E. jambolana* bark extract in acute, subacute, and chronic inflammation in rats[17]. The maximum concentration of phenolic compounds in the leaves was found in summer period when the leaves reach maturity. There is direct correlation between the total phenolic and flavonoid compounds in the leaf extract of *E jambolana* and the antiedematogenic and anti-inflammatory activity[18]. Anti-inflammatory activity of *E. jambolana* leaf extracts has been demonstrated in albino rats[19]. We have earlier isolated a pure compound from *E. jambolana* leaves and characterized as kaempferol-7-O-α-L-rhamnopyranoside]-4'-O-4'- [kaempferol-7-O-α-L-rhamnopyranoside (Fig. 1) on the basis of chemical reactions and spectral analysis (Hetero Multiple Bond Coherence NMR) and found to possess analgesic activity[11].

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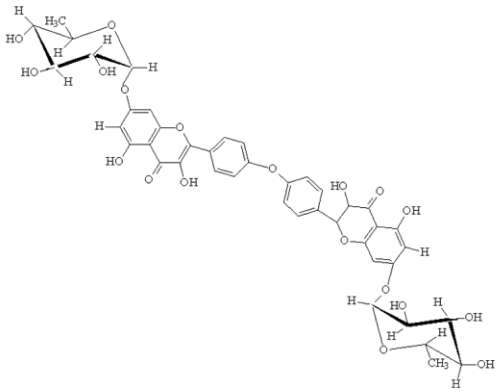

[Kaempferol-7-*O*-α-L-rhamnopyranoside]-4'-*O*-4' [Kaempferol-7-*O*-α-L-rhamnopyranoside]

Fig. 1—Structural formula of EJ-01

In this study, we evaluated its anti-inflammatory properties and focused on determining whether this compound may act as a putative modulator of different inflammatory mediators by testing the mechanisms by which this compound reduces inflammation in carrageenan-induced inflammation in mice.

Materials and Methods

Etoricoxib was obtained from Ranbaxy Research Lab (Gurgaon, India). Mouse tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β) ELISA kits were purchased from eBiosciences (San Diego, Canada). Dipotassium hydrogen phosphate, potassium dihydrogen orthophosphate were purchased from Himedia, Mumbai, India. Carrageenan, *N*-naphthyl-ethylenediamine, sulfanilamide, hexadecyltrimethyl-ammonium bromide (HTAB), o-dianisidine, hydrogen peroxide and all other chemicals were purchased from Sigma-Aldrich (St Louis, USA).

Procurement, grouping and treatment

In the present study, a total of 60 male Swiss albino mice (18-25 g) were used. Healthy mice were procured from Laboratory Animal Resource Section of Indian Veterinary Research Institute. Animals were kept in polypropylene cages at ambient environment (room temperature 24±2°C; relative humidity 60-70%; 12-h light-dark cycle) and maintained on a balanced ration obtained from the Feed Technology Unit of the Institute, offered fresh drinking water *ad libitum.* The housing conditions and experimental protocols were duly approved by the Animal Ethics Committee of the Indian Veterinary Research Institute (IAEC/2009/011). The mice acclimatized for one week were divided into five groups of six animals each as follows: Group I, administered with vehicle and served as vehicle control; Groups II-IV, EJ-01 at 3, 10 and 30 mg/kg p.o., respectively; and Group V, etoricoxib (10 mg/kg) p.o. One hour after vehicle or drug administration, each mouse was injected subplantarly with single dose of 50 μL carrageenan 1% (w/v) in normal saline.

Experiment I

Carrageenan-induced paw edema test

Anti-inflammatory activity of flavonoid, EJ-01 was assessed by the carrageenan-induced hind paw edema[20]. Paw edema volume was measured at 0 hour (before carrageenan injection) and 5th hour (after carrageenan injection). The volume of the mice paw was measured with a plethysmometer (Ugo Basil, Italy) before (V_0) the intraplantar stimulus with carrageenan and 5 h after (V_T) the stimulus. The amount of paw swelling was determined for each mouse and the difference between V_T and V_0 was taken as edema volume (edema mm^3/paw). The edema volume of treated groups was compared with vehicle control.

Myeloperoxidase (*MPO*) *measurement in carrageenan-injected mice paw*

MPO is an enzyme present in the neutrophils and MPO activity is directly correlated to the neutrophil concentration in the inflamed tissue. Tissue damage in inflammation is also brought about by hypochlorous acid generated by MPO activity. After 5th hour of carrageenan injection mice of different groups were anaesthetized with intraperitoneal injection of a mixture of 10 mg/kg xylazine and 100 mg/kg ketamine hydrochloride, skin and adjacent muscle tissues were removed from the paw and homogenized in 1ml of buffer (containing 5 g HTAB/L 50 mM potassium phosphate buffer; pH 6)[21] using a Polytron homogenizer (two cycles of 10 s at maximum speed). After centrifugation at 10000 rpm for 10 min at 4°C, supernatant fraction was assayed for MPO activity, as an index of cellular migration, using the method described by Koike and coworkers[22]. Supernatant (0.1 mL) was added to 2.9 mL of 50 mmol/L potassium phosphate buffer, pH 6.0, containing 0.167 mg/mL o-dianisidine and 0.0005% hydrogen peroxide. The sample absorbance was measured at 460 nm visible light (A460) for 2 min. MPO activity per gram paw tissue (gpt) was calculated by the following formula: MPO activity (units/gpt) = ($\Delta A460$) × (13.5)/paw tissue weight (g), where $\Delta A460$ was the changes in absorbance at 460 nm from 30 to 90 s (of 2 min absorbance measurement) after the initiation of the reaction. The coefficient 13.5 was empirically determined such that 1 unit MPO activity was the amount of enzyme that would reduce 1 mMol peroxide/min.

Experiment II

Paw tissue homogenate preparation for nitrite plus nitrate (*NOx*) *and cytokine assay*

Like experiment I, 30 mice were divided into five groups of six animals each and similar protocol was followed for drug administration and carrageenan injection. At 5th hour of carrageenan injection, mice were anaesthetized with intraperitoneal injection of a mixture of 10 mg/kg xylazine and 100 mg/kg ketamine hydrochloride, skin and adjacent muscle tissues were removed from the paw. Samples were homogenized in 500 µL of the phosphate buffer containing protease inhibitors, using a Polytron homogenizer (two cycles of 10 s at maximum speed). After centrifugation at 10000 rpm for 10 min at 4°C, the supernatants were collected and kept at −80°C until further analysis.

NOx assay in carrageenan-injected mice paw

Anti-inflammatory activity of flavonoid, EJ-01 was assessed by the effect on NOx level in carrageenan injected mice paw at 5th hour after carrageenan injection. Paw NOx level was measured from supernatant by Copper-Cadmium alloy method as described by Sastry and coworkers[23].

Pro-inflammatory cytokine assay in carrageenan-injected mice paw

Anti-inflammatory activity of flavonoid, EJ-01 was assessed by the effect on cytokine level i.e. TNF-α and IL-1β in carrageenan injected mice paw[24] at 5th hour after carrageenan injection. TNF-α and IL-1β levels were determined by ELISA (as per manufacturer's instruction). Results were expressed as picogram (pg) of each cytokine per mL of paw exudates.

Statistical analysis

Data were expressed as Mean ± SEM. The level of statistical significance was determined by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test employing the Graph Pad Prism-4 software. Statistical differences were considered significant at '*P*' value less than 0.05.

Results

Effect of EJ-01 on carrageenan-induced hind paw edema in mice

The results of oral administration of flavonoid, EJ-01 after 5 hour stimuli on carrageenan-induced hind paw edema (mm^3) are summarized in Fig. 2. EJ-01 produced significant inhibition in edema volume at all the doses i.e. 3, 10 and 30 mg/kg (38.30±3.07, 21.60±3.07 and 18.30±3.07) as compared to the vehicle control (60.00±4.47) at 5 h of its administration. The reference drug etoricoxib significantly inhibited the edema at 10 mg/kg (18.30±3.07) following of its administration as compared to the vehicle control.

Effect of EJ-01 on MPO measurement in carrageenan injected mice hind paw

Results of oral administration of EJ-01 on MPO activity (units/g paw tissue) measurement in carrageenan-injected mice hind paw are summarized in Fig. 3A. EJ-01 significantly decreased MPO activity in carrageenan-injected mice hind paw at all

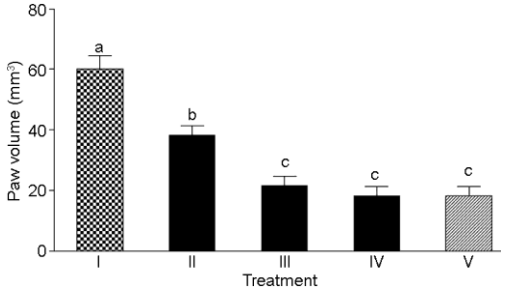

Fig. 2—Effect of EJ-01 on carrageenan-induced hind paw edema in mice at 5th hour. [Group I, vehicle vehicle control; Groups II-IV, EJ-01 at 3, 10 and 30 mg/kg p.o., respectively; and Group V, etoricoxib (10 mg/kg) p.o. Alphabets above the bars represent superscript and have been used to show the significance. Any bar with any other bar shares same superscript then they do not differ significantly. Any bar with any other bar shares different superscript then they differ significantly; n=6]

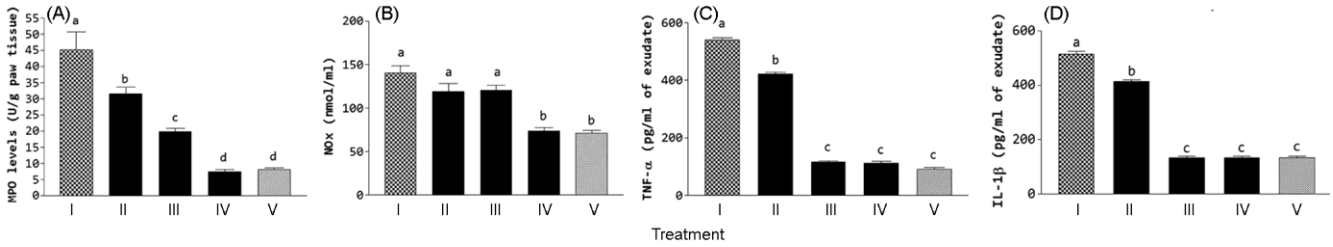

Fig. 3—Effect of EJ-01 on (A) MPO; (B) NOx; (C) TNF-α and (D) IL-1β levels at 5th hour in exudates in carrageenan-injected hind paw of mice. [Group I, vehicle vehicle control; Groups II-IV, EJ-01 at 3, 10 and 30 mg/kg p.o., respectively; and Group V, etoricoxib (10 mg/kg) p.o. Alphabets above the bars represent superscript and have been used to show the significance. Any bar with any other bar shares same superscript then they do not differ significantly. Any bar with any other bar shares different superscript then they differ significantly; n=6]

the doses i.e. 3, 10 and 30 mg/kg (31.58±2.0, 19.90±1.02 and 7.38±0.72) as compared to the vehicle control (45.14±5.56) and the reference drug etoricoxib at 10 mg/kg (8.13±3.07) significantly decreased the MPO activity in carrageenan-injected mice paw as compared to the vehicle control.

Effect of EJ-01 on NOx levels in carrageenan-injected mice hind paw

Results of oral administration of EJ-01 on NOx levels (nmol/ mL of tissue exudates) in carrageenan-injected mice hind paw at 5th hour after carrageenan injection are summarized in Fig. 3B. EJ-01 did not significantly decreased NOx levels in carrageenan-injected mice hind paw at 3 and 10 mg/kg doses as compared to the vehicle control; however, it significantly decreased NOx levels in carrageenan-injected mice hind paw at the higher dose (30 mg/kg; 73.69±3.88) as compared to the vehicle control (139.98±8.67). The reference drug etoricoxib at 10 mg/kg (70.85±3.50) significantly decreased the NOx levels in carrageenan-injected mice hind paw as compared to the vehicle control.

Effect of EJ-01 on pro-inflammatory cytokines: TNF-α and IL-1β levels in carrageenan-injected mice hind paw

The results of oral administration of EJ-01 on pro-inflammatory cytokine TNF-α levels (pg/mL of tissue homogenates) in tissue exudates of carrageenan-injected mice hind paw are summarized in Fig. 3C. EJ-01 significantly decreased TNF-α level in carrageenan-injected mice hind paw at all the doses i.e. 3, 10 and 30 mg/kg (422.91±5.52, 115.70±5.40 and 112.07±5.90) as compared to the vehicle control (540.87±7.54). The reference drug etoricoxib at 10 mg/kg (91.70±4.80) also significantly decreased the TNF-α level in carrageenan-injected mice hind paw as compared to the vehicle control.

The results of oral administration of EJ-01 on pro-inflammatory cytokine IL-1β levels (pg/mL of tissue exudates) in carrageenan-injected mice hind paw are summarized in Fig. 3D. EJ-01 significantly decreased IL-1β levels in carrageenan-injected mice hind paw at all the doses i.e. 3, 10 and 30 mg/kg (413.51±5.82, 133.39±5.76 and 134.15±4.84) as compared to the vehicle control (513.38±10.89). The reference drug etoricoxib at 10 mg/kg (133.41±6.73) significantly decreased the IL-1β levels in carrageenan-injected mice hind paw as compared to the vehicle control.

Discussion

Herbal medicines derived from plant extracts are being increasingly utilized to treat a wide variety of diseases, although relatively only modest acquaintance about their mode of action is available. There is an emergent interest in the pharmacological evaluation of various plants used in Indian traditional systems of medicine. In the present investigation, an attempt was made to evaluate the anti-inflammatory potential of kaempferol-7-O-α-L-rhamnopyranoside]-4'-O-4'- [kaempferol-7-O-α-L-rhamnopyranoside in mice model of carrageenan-induced hind paw edema as an *in vivo* model of inflammation. The isolated compound from *E. jambolana* leaves is chemically kaempferol-7-O-α-L-rhamnopyranoside]-4'-O-4'-[kaempferol-7-O-α-L-rhamnopyranoside, a flavonone and abbreviated as EJ-01. The kaemferols have been subjected to investigation in recent days because of their other beneficial effects along with major anti-inflammatory activity which may have advantage over other investigated drugs[3]. The present investigation was therefore, undertaken with the objective of finding the therapeutic potential of the flavonoid EJ-01 as anti-inflammatory agent.

Carrageenan-induced paw edema, a classical model of acute inflammation, has been widely used in the study of anti-inflammatory agents[25]. The edema, or swelling, one of the cardinal signs of acute inflammation is an important parameter to be considered when evaluating compounds with potential anti-inflammatory activity[26]. The result obtained from the carrageenan-induced paw edema test showed that edema formation attained its maximum 5 h after carrageenan injection and the treatment with EJ-01 inhibited the paw swelling induced by carrageenan (Fig. 2). These findings demonstrate that EJ-01 has a potent in vivo anti-inflammatory activity.

During tissue inflammation, there is normally vasodilation and recruitment of capillaries and at least transient increases in capillary permeability. This leads to the extravasations of plasma proteins and to tissue edema. The application of proinflammatory substances may also change the interstitial structure, contributing to this edema[27]. The present study showed that treatment with EJ-01 can decrease paw edema in mice suggesting an important effect on plasma extravasations. Since inflammation is as a result of a complex biological response to tissue damage and the edema induced in the rodent paw by injection of 1% carrageenan is brought about by autacoids, histamine and 5-HT during the first 1 h, after which kinins act to increase the vascular permeability up to 2.30 h. After that, prostaglandins act from 2.30 to 6 h, which results in migration of leucocytes into the inflammed site[28].

A local increase in concentration of TNF-α will cause the cardinal signs of inflammation to occur[29]. TNF-α and leukotriene B_4 mediates the neutrophil migration in inflammation[30]. TNF-α level were reduced by all the doses of the investigational compound in carrageenan-induced inflammatory exudates and may possibly explain reduced neutrophil migration and ultimately reduction in MPO activity due to carrageenan.

Presence of significant MPO activity is considered a hallmark of cell infiltration (mainly neutrophils) in inflammation. MPO is an enzyme present in neutrophils and at a much lower concentration in monocytes and macrophages[31]. It is well known that the MPO activity is directly proportional to the neutrophil concentration in the inflamed tissue including skin, by which measurement of the enzyme activity has been considered a quantitative and sensitive marker of chemotaxis and neutrophil infiltration in the inflammatory process[21]. Indeed an ability to estimate the quantity of neutrophils in inflamed tissue might prove useful to judge the intensity of inflammation or the effects of experimental or therapeutic maneuvers to alter the inflammatory response. It has been reasoned that MPO, a constituent of neutrophils, would be a marker for tissue neutrophils, since the enzyme is abundant in neutrophils comprising as much as 5% of their mass[32]. In this study EJ-01 produced significant dose-dependent inhibition of MPO levels in carrageenan-injected mice hind paw at all the doses (3-30 mg/kg) as compared to the vehicle control which could decrease tissue damage caused by hydrolytic enzymes and besides by some oxidant species such as hypochlorous acid generated by MPO activity. Experiments with carrageenan clearly showed that EJ-01inhibits neutrophil mobilization to the carrageenan in paw tissue. The decrease in MPO activity corroborates this inhibition in EJ-01-treated mice, since this enzyme is absent from other types of leukocytes[33].

Nitric oxide is a free radical that plays a pivotal role in cell survival and death, and plays various pro-inflammatory effects on many cell types[34]. High levels of NO generated by iNOS in inflamed tissue have been shown to be cytotoxic in studies of many types of inflammatory diseases, including asthma[35], arthritis[36] and cardiovascular diseases[37] leading to cell death[38]. NO is a reactive nitrogen species like molecular oxygen, but in combination with superoxide (O_2^-) it can yield a highly reactive species called peroxynitrite ($ONOO^-$). This can attack critical cellular targets and cause oxidative damage even before the depletion of cells antioxidant defenses[39]. Therefore, NO inhibition in inflammation has the potential therapeutic implications. It has been demonstrated that the NO produced by cNOS is involved in the development of inflammation at early time points following carrageenan administration and that NO produced by iNOS is involved in the maintenance of the inflammatory response at later time points[40]. In this study, EJ-01 produced significant inhibition on NOx levels in carrageenan-injected mice hind paw at the dose 30 mg/kg as compared to the vehicle control suggesting that EJ-01 is at least effective in reducing NO production in late phase of inflammation. Further, previous study in our laboratory with EJ-01 showed its inhibitory action on NOx production in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells[11]. This indicates that the anti-inflammatory mechanism of EJ-01 may be through the L-arginine-NO pathway.

Cytokines are critical to the pathogenesis of inflammatory disorders. The inhibition of their production and action can provide therapeutic benefits. Previous studies have shown significant correlations among cytokine production, COX-2 protein expression and PG synthesis in the paw in which edema was induced by intraplantar injection of carrageenan[41,42].

TNF-α is a major mediator in inflammatory responses, inducing innate immune responses by activating T cells and macrophages and stimulating the secretion of other inflammatory cytokines[43]. The production of multiple proinflammatory cytokines like TNF-α in the edematous paw was also reduced by EJ-01. These results indicate that EJ-01 play a role in the anti-inflammatory activities in carrageenan-induced paw edema through the inhibition of TNF-α activity. IL-1β is a pleiotropic mediator of the host response to infections and injurious insults. It co-ordinates the activities of other cells and cytokines, acts as a costimulant of early innate inflammatory and later specific immune responses[44]. It acts on monocytes and neutrophils, inducing secretion of several cytokines including IL-1β it-self[45]. Findings of the earlier study show positive correlations between significant increase in each of the mediators like IL-1β, nitrite and percentage of peripheral neutrophils in inflammed paw. All the aforementioned changes were consistent with the incidence of edema induced by different used dose levels of carrageenan in a dose-dependent manner[46]. The present results demonstrate

that EJ-01 attenuates the production of TNF-α and IL-1β in paw of carrageenan-injected mice, which might contribute to the mechanism underlying the anti-inflammatory effectiveness of EJ-01 in carrageenan-induced inflammation. Supporting the findings of this study, EJ-01 in the previous study was found to inhibit TNF-α and IL-1β levels in LPS-treated murine macrophage cells *in vitro*[11]. Alternatively, the suppression of TNF-α and IL-1β formation by EJ-01 might reflect its inhibitory effects on neutrophil infiltration. Reduced levels of TNF-α and inhibited expression of IL-1β and TNF- α genes were observed with kaempferol in LPS-stimulated macrophage model[47].

Conclusion

The results of the present study demonstrated that EJ-01 reduced edema formation suggesting its anti-inflammatory effect which is further supported by potent inhibitory action on carrageenan-induced NO, TNF-α and IL-1β productions in inflammed paw. Furthermore, the current results proposed that the possible mechanism by which EJ-01 exerts its anti-inflammatory effect also involves the inhibition of neutrophil infiltration. Therefore, it is concluded that EJ-01 possibly has potential benefits in inflammatory conditions and support the pharmacological basis of *E. jambolana* plant as traditional herbal medicine for the treatment of inflammatory diseases.

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