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Original article

Anti-inflammatory activity of rhein isolated from the flowers of *Cassia fistula* L. and possible underlying mechanisms

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ABSTRACT

Objective: Anti-inflammatory activity of rhein in animal models with potential mechanism of actions.
Methods: Rhein was isolated from *Cassia fistula* L. flowers collected in Chennai, Tamil Nadu, India. Its anti-inflammatory activity was then investigated in Wistar rats and mice using carrageenan-induced hind paw oedema, croton oil-induced ear oedema, cotton pellet-induced granuloma and acetic acid-induced vascular permeability models.
Results: Administration of rhein (10, 20, 40 mg/kg) significantly ($p < 0.05$) inhibited carrageenan-induced paw oedema in rats and croton oil-induced ear oedema in mice in dose-dependent manners. Continual administration of rhein to rats using implanted cotton pellets significantly ($p < 0.05$) reduced granuloma formation (20 mg/kg: 17.24%; 40 mg/kg: 36.12%) compared to control group animals. Administration of rhein increased the activities of catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GSH-px) and decreased the levels of nitrite, interleukin-6 (IL-6), interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), malondialdehyde (MDA) and vascular endothelial growth factor (VEGF) compared to control animals. Western blotting results revealed that rhein diminished carrageenan-induced cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS) and increased heme oxygenase (HO)-1, nuclear factor erythroid 2–related factor 2 (Nrf2), peroxisome proliferator-activated receptor gamma (PPAR)-γ and heat shock protein (HSP)-72 expression after 6 h in the paw oedema model.
Conclusion: The anti-inflammatory mechanisms of rhein might be related to decrease in the levels of MDA, iNOS and COX-2 and the stimulation of HO-1, PPAR-γ and Nrf2 expression via increases in the activities of CAT, SOD and GSH-px through the suppression of nitrite, TNF-α, IL-6 and IL-1β.

1. Introduction

Inflammation is a multifaceted biological process involving vascular tissues and nonspecific responses employed by both the innate and acquired immune responses against infection, irritants, injury, and damaged cells. Inflammation is generally classified as acute or chronic and encompasses a cascade of biochemical events in different cell types. Acute inflammation is the early response and is characterized by greater movement of plasma and innate immune cells, such as macrophages and neutrophils, from the blood circulation into the wounded tissues. Chronic inflammation encompasses a constant variation in the variety of cells present at the inflammatory reaction site and is associated with concomitant damage and curing of the injured tissue (Ferrero-Miliani et al., 2007). Medicinal plants are known to play vital roles as sources of active anti-inflammatory agents. According to the World Health Organization (WHO), around three-quarters of the world's

Abbreviations: CAT, catalase; SOD, superoxide dismutase; GSH-px, glutathione peroxidase; IL-6, interleukin-6; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; MDA, malondialdehyde; VEGF, vascular endothelial growth factor; COX-2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase; Nrf2, nuclear factor erythroid 2–related factor 2; PPAR-γ, peroxisome proliferator-activated receptor gamma; HSP-72, heat shock protein; WHO, World Health Organization; CMC, carboxymethylcellulose; AUC, area under the curve; ANOVA, one-way analysis of variance; MPO, myeloperoxidase; *C.fistula*, *Cassiafistula* L.

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population rely on traditional remedies for their healthcare. It is obvious that numerous plants have been used in traditional medicine to treat diverse inflammatory disorders and have been thought to possess wound curing activities (Gacche et al., 2011).

Cassia fistula (*C. fistula*) L., (Leguminosae), a semi-wild Indian Laburnum and a typical vanishing species, is also referred to as golden shower or pudding pipe. *C. fistula* is found in several areas, including Asia, Mexico, South Africa, West Indies, Brazil, and East Africa. The plant possesses attractive bunches of yellow flowers. The entire plant is used for diarrhea treatment, while the flowers, fruits and seeds are used for the treatment of fever, skin diseases, leprosy and abdominal pain by ethnic peoples (Bahorun et al., 2005). The seeds are beneficial in treating jaundice (Asolkar et al., 1992), skin disorders, nausea and swollen throat. *C. fistula* has been reported to have anti-inflammatory activity (Danish et al., 2011) and its leaves have been shown to possess hepatoprotective (Bhakta et al., 1999), wound healing (Bhakta et al., 1997a) and hypoglycemic activity (Bhakta et al., 1997b).

It has been reported that *C. fistula* flowers and leaves possess anthraquinone, oxyanthraquinone, tannin, volatile oils and rhein (Chopra et al., 2006). The current study was initiated to evaluate the anti-inflammatory activity of rhein isolated from *C. fistula* flowers.

2. Material and methods

2.1. Animals

Adult male Wistar albino rats (200–220 g) and mice (24–28 g) were used in the studies. Animals were keep up on a 12 h light/-dark cycle at 25 °C ± 1 °C with moisture of 60–70%; with free access to diet and water ad libitum and were amended for a minimum of fourteen days (2 weeks) prior to the studies. All experiments were carried out with six animals in each group. All the animal studies were performed in accordance with Ethics Committee norms (permit number IAEC-ERI-LC-02) and CPCSEA guidelines.

2.2. Chemicals and drugs

Indomethacin and croton oil were obtained from Sigma-Aldrich (St. Louis, MO, USA). Carrageenan and carboxymethylcellulose (CMC) were obtained from Himedia (Mumbai, Maharashtra, India). ELISA kits for IL-6 and TNF-α were from purchased from BioLegend (San Diego, CA, USA). Antibodies against iNOS and COX-2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). HSP-72 antibody was purchased from Stressgen Bioreagents (Victoria, British Columbia, Canada). Antibodies against HO-1 and Nrf2 were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). All additional chemicals used were of analytical reagent grade.

2.3. Rhein identification and characterization

The isolation and description of rhein has been previously reported by Duraipandiyan et al. (2012). The chemical structure of rhein is shown in Fig. 1.

2.4. Evaluation of rhein acute toxicity

To evaluate the acute toxicity of rhein, different doses (20, 40, 80 and 160 mg/kg) were administered orally with 0.5% CMC as a vehicle, and toxic responses such as loss of weight, respiratory distress, uncoordinated muscle movements, unusual locomotion and mortality were evaluated over a period of 24 h.

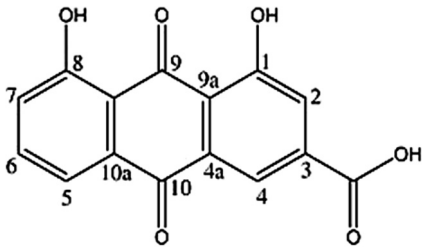

Fig. 1. Rhein (1,8-dihydroxyanthraquinone-3-carboxylic acid) isolated from *C. fistula* flower.

2.5. Croton oil-induced ear oedema in mice

Ear oedema assays were performed as described in a previous report (Antonisamy et al., 2011). Five groups of mice were treated with CMC (0.5%), indomethacin (10 mg/kg) or rhein (10, 20 and 40 mg/kg). Sixty minutes after treatment, ear oedema was induced in mice by the topical application of 10 μl of freshly prepared croton oil (5% in acetone) to the inner surface of the right ear. The inner surface of the left ear received an identical volume of vehicle (acetone). Four hours after the application of the irritant agent, the animals were sacrificed and both ears plugs (6 mm Ø) were removed and their weight was recorded. To evaluate the oedematous response, the weight difference between the left (W_l) and right (W_r) ears was measured. The percentage of oedema inhibition was calculated using the following equation:

$$\text{Inhibition } (\%) = ((W_l - W_r)\ \text{control} - (W_l - W_r)\ \text{test drug}) * 100/(W_l - W_r)\ \text{control}$$

2.6. Carrageenan-induced paw oedema in rats

Male Wistar rats (200–220 g) were divided into six different groups consisting of 6 animals each. Rhein (10, 20 and 40 mg/kg) and indometacin (10 mg/kg) dissolved in 0.5% CMC were administered orally 1 h before carrageenan induction. Subsequently, 0.1 ml of carrageenan (1%) in saline was subcutaneously injected into the plantar side of the rat right hind paw. Paw thickness in all animals was measured using a digital vernier caliper initially (0 h) and then at 1, 2, 3, 4, 5 and 6 h after the carrageenan injection (Antonisamy et al., 2011). The area under the curve (AUC) was evaluated for each dose.

2.7. Cotton pellet-induced granuloma formation in rats

The activity of rhein on the prolonged or proliferative phase of inflammation was evaluated in the cotton pellet-induced granuloma rat model (Winter and Porter, 1957). Animals were divided into 5 groups consisting of 6 animals each. Each rat was anaesthetized with sodium pentobarbitone (60 mg/kg) and sterilized cotton pellets weighing 35 ± 1 mg each were implanted subcutaneously through a slight incision made on the axilla region of the rats. Various groups of animals were treated with rhein (10, 20 or 40 mg/kg) or indometacin (10 mg/kg) once every day for seven sequential days starting on the day of cotton pellet implantation. The control group was treated with vehicle alone (1 ml/kg). Animals were sacrificed on the eighth day and the cotton pellets shielded by the granulomatous tissue were cautiously removed, unnecessary tissues were removed and the pellets were dried up in a hot air oven at 60 °C until recovery of constant weight and cotton pellet dry weight was recorded. Granuloma weight was determined by subtracting the cotton pellet weight on 0 day (before implantation) from the cotton pellet weight on the eighth day.

The inhibition percentage of granuloma formation was calculated using the following equation:

$$\text{Inhibition } (\%) = (W \text{ pellet control} - W \text{ pellet test drug}) * 100/(W \text{ pellet control})$$

2.8. Acetic acid-induced vascular permeability in mice

The activity of rhein on vascular permeability was evaluated using a previously published method (Whittle, 1964). Briefly, sixty minutes after oral treatment with indomethacin (10 mg/kg) or rhein (10, 20 or 40 mg/kg), 0.2 ml of Evans blue dye (0.25% in saline) was intravenously injected through the tail vein. Control animals received an equal volume of vehicle (0.5% CMC). After thirty minutes, animals were treated with an intraperitoneal (i.p.) injection of 1 ml/100 g of acetic acid (0.6%, v/v). Thirty minutes later, all animals were killed and the peritoneal cavity was washed with 3 ml of normal saline and collected in individual heparinized tubes followed by centrifugation. The dye content of the supernatant was measured using a spectrophotometer at 610 nm.

2.9. Statistical investigation

Data were assessed using one-way analysis of variance (ANOVA) with Tukey's multiple comparison post hoc tests. $P < 0.05$ was considered to be statistically significant.

3. Results

There were no behavioral alterations and no mortality was observed within 24 h of oral administration of rhein at different doses. The outcomes revealed that rhein administered up to an oral dose of 160 mg/kg was non-toxic in animals.

Carrageenan-induced acute hind paw oedema in rats was significantly increased in control animals and concurrently reduced in a dose-dependent manner by treatment with rhein. As demonstrated by the area under curve (AUC), rhein exhibited dose dependent inhibitory activity, although indomethacin (10 mg/kg) showed higher inhibitory activity than rhein (40 mg/kg); these values were however not statistically significant (Fig. 2A and B). The levels of pro-inflammatory cytokines such as TNF-α (7.39-fold), IL-1β (3.19-fold), IL-6 (5.71-fold) and nitrite (10.01-fold) were significantly increased in carrageenan-induced animals compared to normal controls. However, rhein dose-dependently reduced the levels of these biomarkers. Rhein used at 40 mg/kg showed higher inhibitory activity against TNF-α (4.86-fold), IL-1β (2.57-fold), IL-6 (3.94-fold) and nitrite (2.13-fold) compared to levels in the carrageenan-induced animals. Indomethacin (10 mg/kg) showed greater inhibitory activity against TNF-α (5.82-fold), IL-1β (2.80-fold), IL-6 (4.50-fold) and nitrite (3.16-fold) levels than did rhein (40 mg/kg). However, these values were not significant except for nitrite levels (Fig. 3A and B).

The levels of MDA (2.52-fold) and VEGF (1.99-fold) increased in carrageenan-induced control animals compared with normal animals. However, rhein treated animals exhibited dose-dependent

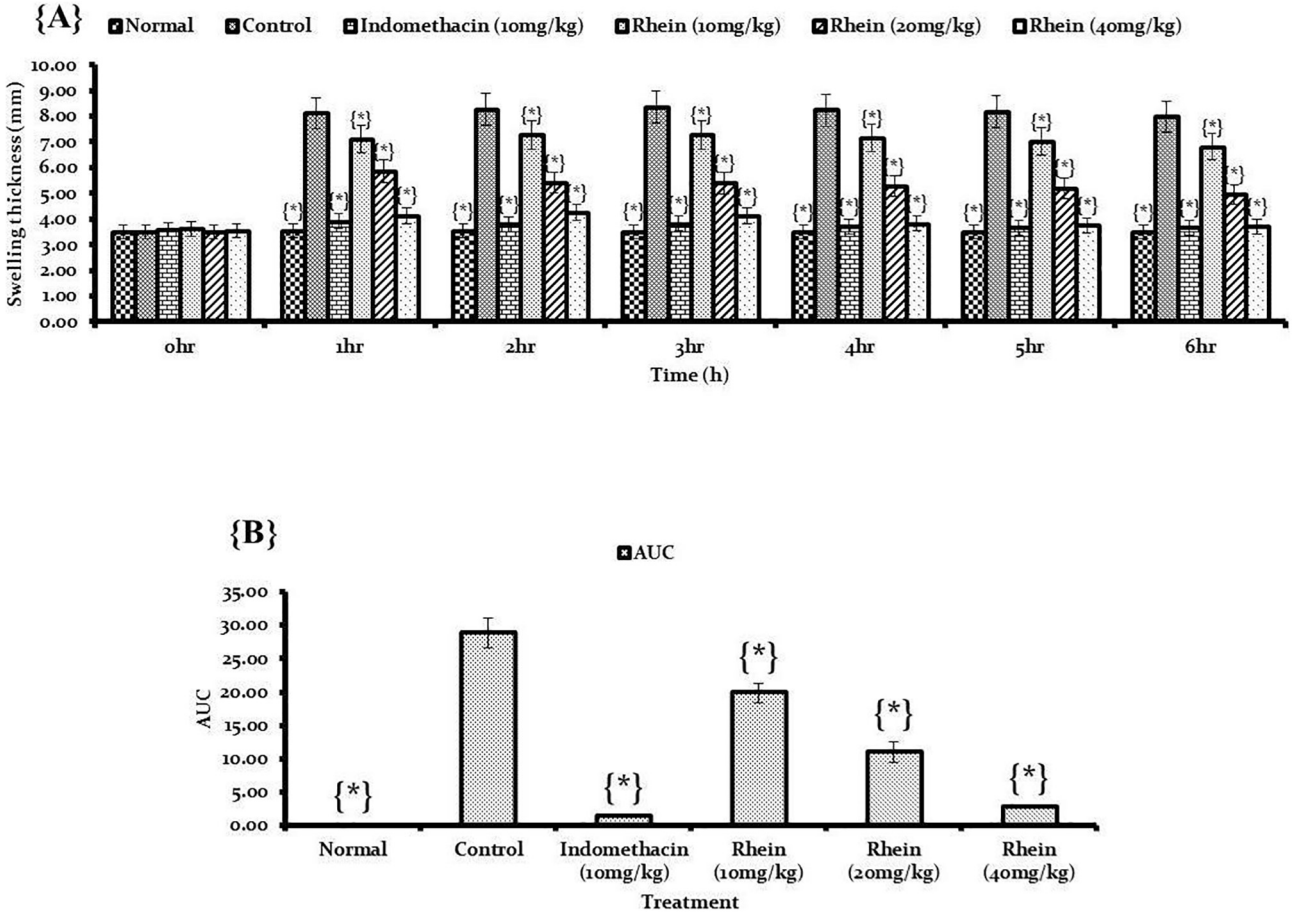

Fig. 2. (A) Effects of rhein (10, 20 and 40 mg/kg) and indomethacin (10 mg/kg) in carrageenan-induced paw oedema in rats. (B) Calculated AUC up to 6 h after induction with carrageenan. Values are expressed as mean ± SD, $n = 6$, $^{*}p < 0.05$ compare control with all the groups.

reductions in the levels of MDA (1.39-fold at 10 mg/kg, 1.53-fold at 20 mg/kg and 1.92-fold at 40 mg/kg respectively) and VEGF (1.14-fold at 10 mg/kg, 1.36-fold at 20 mg/kg and 1.82-fold at 40 mg/kg respectively) compared to the levels in carrageenan-induced control animals (Fig. 4A and B). CAT (2.70-fold), SOD (2.50-fold) and GSH-px (2.04-fold) levels were significantly decreased in carrageenan-induced animals compared to the normal animals. But, the treatment with rhein at 20 mg/kg (CAT (1.74-fold), SOD (1.55-fold) and GSH-px (1.87-fold)) and 40 mg/kg (CAT (2.19-fold), SOD (2.09-fold)) and GSH-px (2.17-fold)) resulted in significant and dose-dependent increases in CAT, SOD and GSH-px levels compared to the carrageenan-induced control animals (Fig. 4 C and D).

Compared with the croton oil-induced control animals, the level of croton oil-induced ear oedema was significantly and dose-dependently reduced by treatment with rhein (13.91-fold at 10 mg/kg, 45.19-fold at 20 mg/kg and 64.09-fold at 40 mg/kg) (Fig. 5A). The myeloperoxidase (MPO) level was significantly increased in croton oil-induced control animals by 4.58-fold compared to the normal animals. However, treatment with rhein significantly and dose-dependently reduced the MPO level by 1.26-fold at 10 mg/kg, 1.49-fold at 20 mg/kg and 3.45-fold at 40 mg/kg compared to the croton oil-induced control animals (Fig. 5B). Rhein significantly inhibited cotton pellet induced granuloma by 17.24% at 20 mg/kg, and 36.12% at 40 mg/kg compared to the control animals. The lower dose of rhein (10 mg/kg) did not result in significant activity in the cotton pellet induced granuloma assay. The reference drug indomethacin (10 mg/kg) showed higher inhibition (40.98%) on granuloma formation compared to the rhein (40 mg/kg), however, the values were not statistically significant (Fig. 5C). Acetic acid-induced vascular permeability was significantly decreased by rhein treatment in a dose-dependent manner (19.67% at 10 mg/kg, 37.70% at 20 mg/kg and 47.54% at 40 mg/kg) compared to the control rats. Indomethacin (10 mg/kg) exhibited greater inhibitory activity (60.65%) against acetic acid-induced vascular permeability than did rhein (40 mg/kg). However, these values were insignificant (Fig. 5D).

The results of immunoblotting revealed that the levels of COX-2 (6.61-fold) and iNOS (3.00-fold) increased significantly in carrageenan-induced control animals compared to normal rats. But, treatment with rhein significantly reduced the expression of COX-2 (2.95-fold at 20 mg/kg, 5.46-fold at 40 mg/kg) and iNOS (1.64-fold at 20 mg/kg, 3.08-fold at 40 mg/kg) in a dose-dependent manner. The levels of PPAR-γ (1.55-fold at 20 mg/kg, 2.59-fold at 40 mg/kg) and HSP-72 (1.56-fold at 20 mg/kg, 2.29-fold at 40 mg/kg) were significantly and dose-dependently increased due to rhein treatment compared to levels in carrageenan-induced control animals (Fig. 6). Cytoplasmic (2.15-fold at 10 mg/kg, 3.54-fold at 20 mg/kg, 5.70-fold at 40 mg/kg) and nuclear Nrf2 (1.84-fold at 10 mg/kg, 2.56-fold at 20 mg/kg, 3.45-fold at 40 mg/kg) levels were significantly increased in the rhein treated groups compared to the indomethacin-induced animals (Fig. 6). The HO-1 protein expression level was significantly increased by rhein (1.90-fold at 10 mg/kg, 2.56-fold at 20 mg/kg, 4.75-fold at 40 mg/kg) compared to indomethacin-induced control animals (Fig. 6).

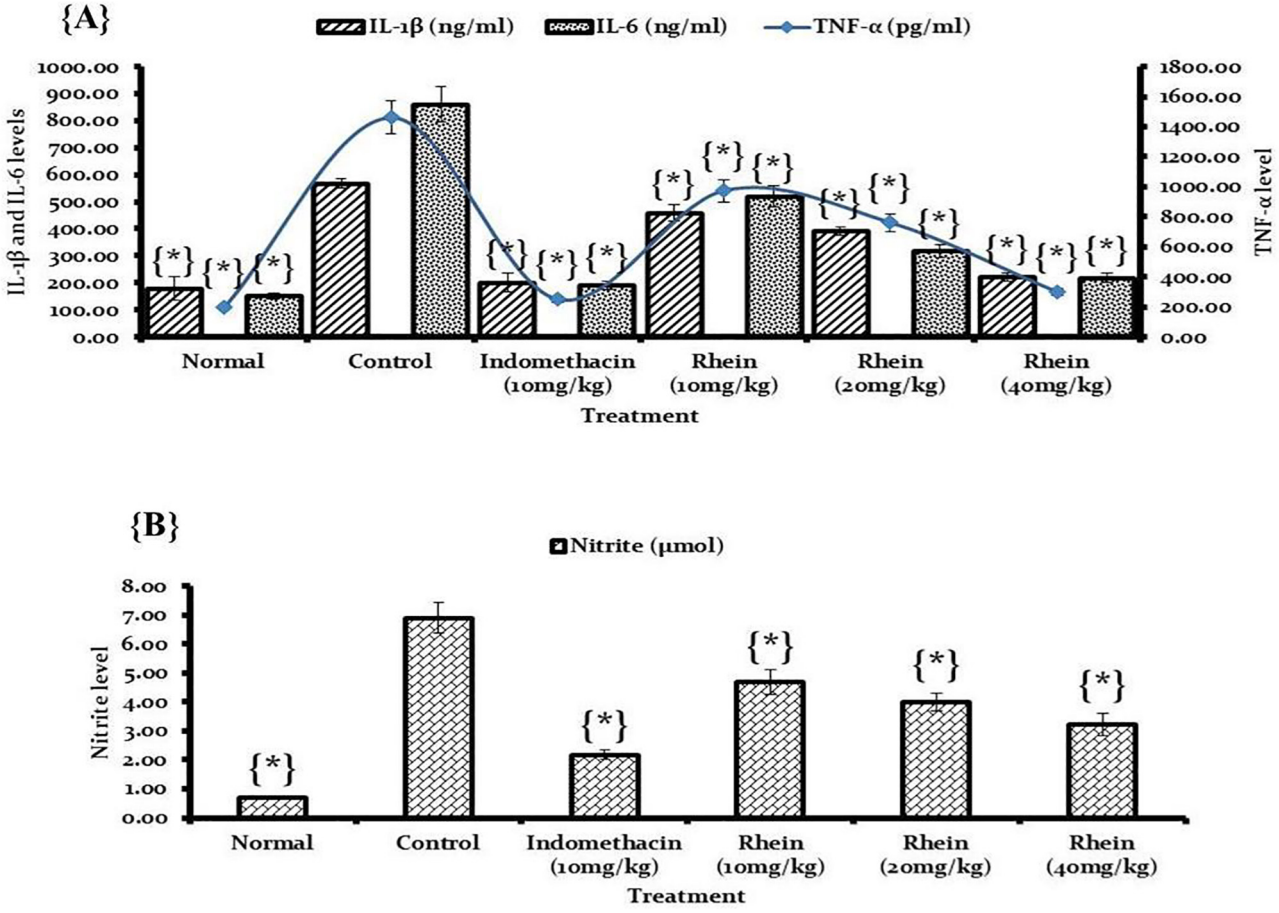

Fig. 3. (A) Effect of rhein (10, 20 and 40 mg/kg) and indomethacin (10 mg/kg) in IL-1β, IL-6 and TF-α level, (B) nitrite level in carrageenan-induced paw oedema in rats. Values are expressed as mean ± SD, $n = 6$, $^{*}p < 0.05$ compare control with all the groups.

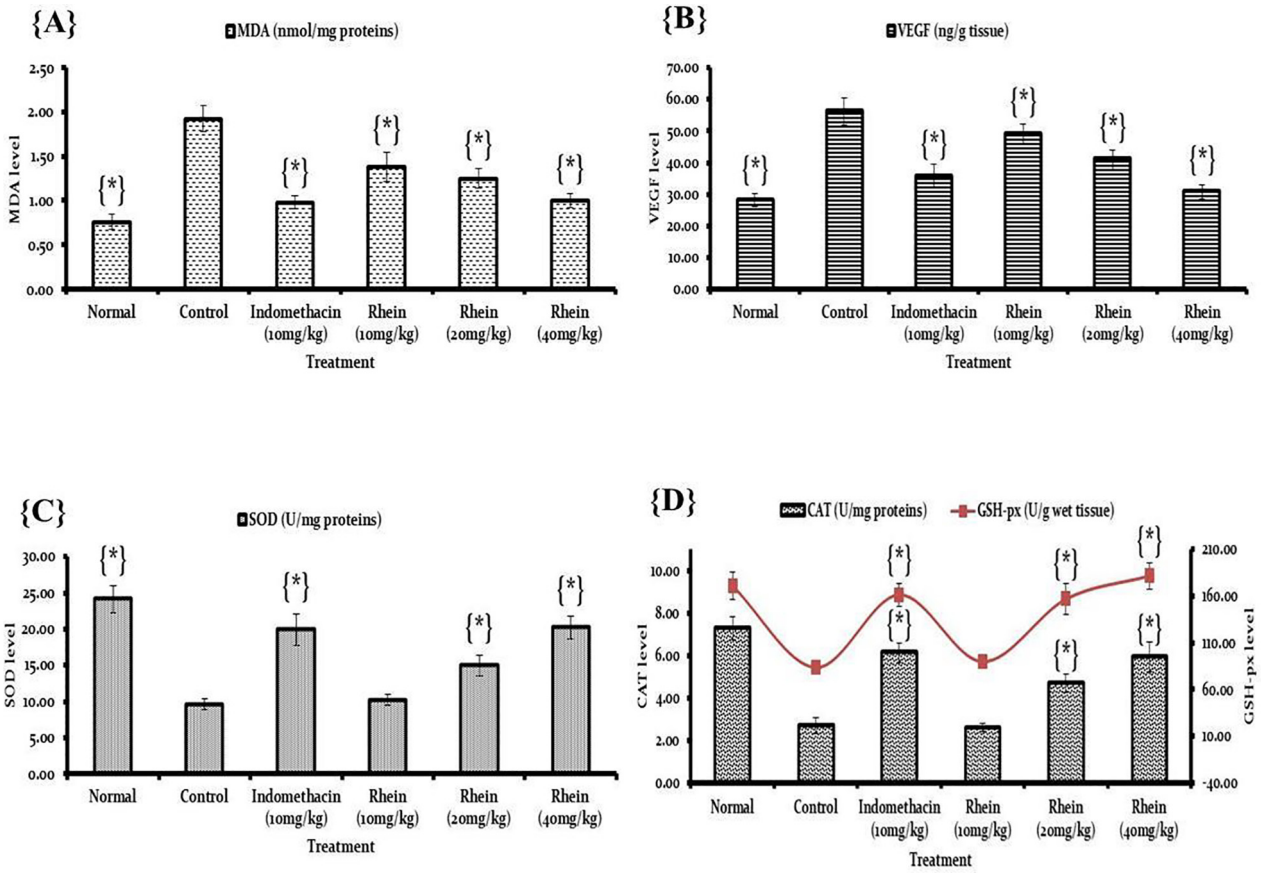

Fig. 4. (A) Effect of rhein (10, 20 and 40 mg/kg) and indomethacin (10 mg/kg) in MDA level, (B) VEGF level, (C) SOD level, (D) CAT and GSH-px level in carrageenan-induced paw oedema in rats. Values are expressed as mean ± SD, $n = 6$, $^{*}p < 0.05$ compare control with all the groups.

4. Discussion

Rhein is a quinone compound isolated from the flowers of *Cassia fistula* L. (Duraipandiyan et al., 2012). To date, the anti-inflammatory activity of this compound has not been described. To the best of our knowledge, this is the initial description on the anti-inflammatory activity of rhein investigated in an in vivo model.

In this experimental study, to assess the anti-inflammatory activity of rhein, we analysed carrageenan-induced hind paw oedema, croton oil-induced ear oedema, acetic acid-induced vascular permeability and cotton pellet induced granuloma models. These animal models are commonly used for the preliminary analysis of the effectiveness of anti-inflammatory drugs (Shu et al., 2006; Zhu et al., 2011; Santos and Rao, 2000; Antonisamy et al., 2015). The overall results demonstrated significant and dose-dependent anti-inflammatory activity associated with rhein (10, 20 and 40 mg/kg).

Inflammation develops through a series of three different levels. The first level involves an increase in vascular permeability that causes exudation of protein rich fluids from the blood stream into the tissue interstitial space; the second level comprises leukocyte infiltration from the blood stream into the interstitial tissue and the third level consists of granuloma formation and tissue repair (Eddouks et al., 2012; Rathi et al., 2015). Hence, it is crucial to assess the effect of any particular test substance on these distinct levels of inflammation when evaluating the anti-inflammatory potential of the substance. Consequently, acetic acid-induced vascular permeability, croton oil-induced ear oedema and carrageenan-induced hind paw oedema assays were conducted to assess the first level of inflammation. The second level of inflammation was evaluated using an MPO accumulation assay, while the third level of inflammation was analysed using the cotton pellet induced granuloma test.

Inflammation induced using the ear oedema model is one of the predominant tests (Jung et al., 2014) broadly recognized as a means of exploring novel anti-inflammatory remedies. Croton oil induction leads to the activation of phospholipase A2, followed by the synthesis of prostaglandins and leukotrienes, which in turn leads to the infiltration of leukocytes and oedema formation (Otuki et al., 2005). Infiltration of polymorphonuclear leukocytes into inflamed tissues with inflammatory reactions can be identified using the marker enzyme MPO present on neutrophils containing intracellular granules (Cabrini et al., 2011). In this study, rhein resulted in dose-dependent reductions in both oedema and MPO levels in the croton oil-induced ear oedema model. Rhein (40 mg/kg) exhibited parallel effects similar to those of indomethacin with respect to the inhibition of MPO and ear oedema.

Another model for the analysis of anti-inflammatory activity of natural products is the carrageenan-induced paw oedema test which is a well-replicated and investigated model (Meira et al., 2014). Carrageenan-induced paw oedema possesses two different phases including an initial phase (up to 2 h) and a late phase (2–6 h) (Winter et al., 1962). The initial phase is associated with the release of pro-inflammatory molecules, such as histamine, serotonin and bradykinin, from the injured tissues (Di Rosa et al., 1971). The late phase is associated with enriched production of PGs, NO, TNF-α, IL-6, and IL-1β, which may perhaps worsen the inflammatory response (Antonisamy et al., 2011).

Polymorphonuclear leukocyte migration in carrageenan-induced inflammation is triggered by the production of

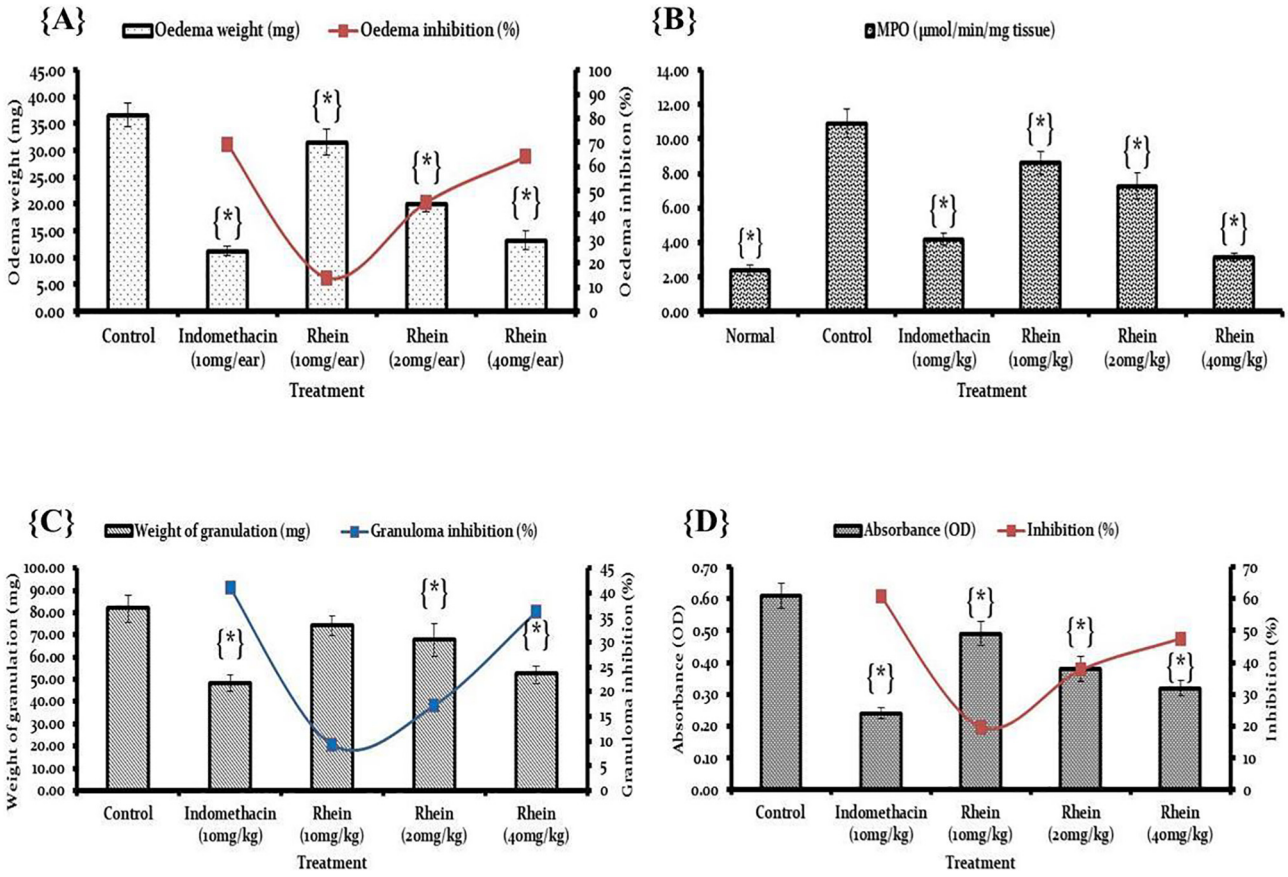

Fig. 5. (A) Effect of rhein (10, 20 and 40 mg/kg) and indomethacin (10 mg/kg) on ear oedema level, (B) MPO level induced by croton oil in mice, (C) cotton pellet induced granuloma level in rats, (D) acetic acid-induced vascular permeability level in rats. Values are expressed as mean ± SD, $n = 6$, $^{*}p < 0.05$ compare control with all the groups.

pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 (Li et al., 2011; Nardi et al., 2007; Nandhini and Stella Bai, 2015). In this study, rhein resulted in a dose-dependent reduction in both phases of hind paw oedema induced by carrageenan, along with reductions in TNF-α, IL-1β, and IL-6 compared to control animals. Hence, the acute anti-inflammatory mechanism of rhein may possibly be associated with inhibition of TNF-α, IL-1β, and IL-6. Pro-inflammatory mediators, including PGE_2 and NO, are produced by induced COX-2 and iNOS respectively (Mitchell et al., 1993). Consequently, suppression of COX-2 and iNOS expression and decreases in PGE_2 and NO production represent a significant strategy to treat inflammatory diseases. The present exploration revealed a significant and dose-dependent downregulation of COX-2 and iNOS protein expression upon rhein treatment; but use of rhein at 10 mg/kg did not result in any significant reduction in COX-2 expression levels. These results verified that the acute anti-inflammatory effect of rhein may possibly be due to the inhibition of PGE_2 and NO synthesis. This suggested mechanism is parallel to that of indomethacin, used in this study as a reference drug, which facilitates an anti-inflammatory effect through the inhibition of PGE_2 and NO production during carrageenan-induced acute inflammation (Fialkow et al., 2007).

Heat shock proteins (Hsp), also designated stress proteins, are highly conserved molecules present in all cellular organisms (eukaryotes and prokaryotes). The main starring roles of Hsp are as chaperones that fold and transport proteins as soon as cells are exposed to stress of different types. Hsp are expressed at low levels during physiological situations. Pathological stressful stimuli can induce noticeable increases in intracellular Hsp synthesis, a process called the cellular stress response (Lindquist and Craig, 1988).

The protective role of Hsp has been demonstrated in a vast variety of animal model inflammation studies (Jaattela et al., 1992; Ianaro et al., 2001; Van Molle et al., 2002; Kalaiselvi et al., 2016). It was reported that HSP-72 significantly reduced carrageenan-induced inflammation in inflamed paw tissues (Ianaro et al., 2003). In the present study, rhein used at 20 and 40 mg/kg resulted in dose-dependent increases in HSP-72, thereby suggesting a defensive role for rhein in acute anti-inflammatory effects.

Furthermore, rhein exerts significant control on the expression of PPAR-γ in swollen paw tissues. PPARs are ligand-activated transcription factors under the nuclear hormone receptor superfamily, which contains the classical steroid, thyroid, and retinoid hormone receptors as well as several orphan receptors. Among PPAR isotypes, PPAR-γ plays a key role as an adipogenesis regulator and adipocyte genes expression responsible for lipid metabolism (Tontonoz et al., 1994). Agonists for PPAR-γ have been shown to modify the inflammatory responses of numerous cell types by inhibiting the expression of pro-inflammatory cytokines (Ricote et al., 1998; Jiang et al., 1998), iNOS (Petrova et al., 1999), and COX-2 (Chawla et al., 2001). However, it has also been described that cytokines, such as TNF-α, considerably antagonize PPAR-γ synthesis (Jiang et al., 1998). In this study, rhein significantly and dose-dependently increased the level of PPAR-γ compared to levels in control animals. It is possible that the rhein-mediated TNF-α inhibition led to augmentation of PPAR-γ, which in turn inhibited the synthesis of pro-inflammatory cytokines, iNOS and COX-2. During inflammation, various roles have been suggested for PPAR-γ, however, it is still unclear how acute inflammation is affected by the PPAR-γ signaling pathway. A previous publication reported that the activity of nuclear factor-κB (NF-κB), activator

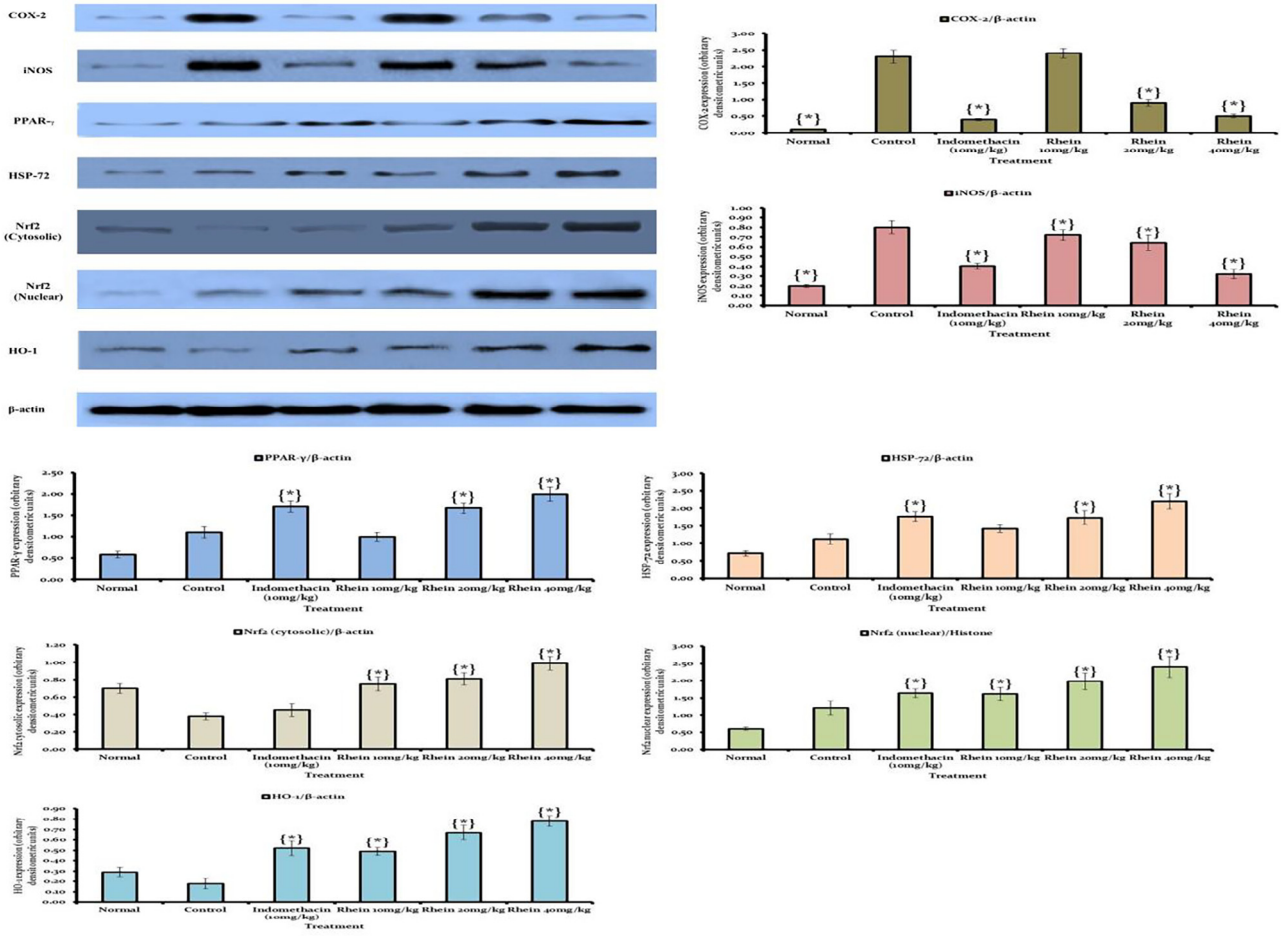

Fig. 6. (A) Effect of rhein (10, 20 and 40 mg/kg) and indomethacin (10 mg/kg) on the protein expression level of COX-2, iNOS, PPAR-γ, HSP-72, Nrf2 (cytosolic), Nrf2 (nuclear), and HO-1 in carrageenan-induced paw oedema in rats. Levels of proteins of interest were normalized to the level of β-actin. $^{*}p < 0.05$ compare control with all the groups. Representative Western blot from three separate experiments is shown.

protein-1 (AP-1), and signal transducer activities were downregulated by activated PPAR-γ (Staels et al., 1998). Among these, NF-κB was revealed to play a more central role in the activation of genes encoding pro-inflammatory cytokines, cell adhesion molecules, COX-2 and iNOS. In the present scientific analysis, COX-2, iNOS, pro-inflammatory cytokines such as TNF-α, IL-6, IL-1β and adhesion molecules including VEGF were significantly inhibited by rhein treatment. Consequently, the anti-inflammatory activity of rhein may be mediated by inhibition of NF-κB, although other possible mechanisms cannot be excluded.

Vascular permeability or microvascular permeability are other vital pathophysiological processes that drive the inflammatory process via augmentation of inflammatory mediators such as prostaglandins, histamine, and serotonin in peritoneal fluids and lead to capillary vessel dilation, which in turn increases vascular permeability (Nardi et al., 2007; Oka et al., 2007). The acetic acid-induced vascular permeability test is a well-known method for analyzing vascular permeability in animal models. Our experimental results demonstrated that rhein (10–40 mg/kg) significantly reduced vascular permeability in a dose-dependent fashion. Hence, based on this outcome, we propose that the anti-inflammatory activity of rhein on the acute inflammation phase might be connected with inhibition of the release of inflammatory mediators and inhibition of vasodilation.

Cotton pellet-induced granuloma is a well-known model for chronic inflammatory conditions, in which granulomatous tissue formation is identified using the dry weight of a cotton pellet (Winter and Porter, 1957; Antonisamy et al., 2011). Inflammatory mediators such as cytokines (Moore et al., 1998), chemokines (Lukacs et al., 1994), and eicosanoids (Kamei et al., 2004) are recognized to be involved in the formation of the granuloma. Granuloma is an immensely vascularised reddish tissue mass formed during the repair process of inflammation involving the proliferation of neutrophils, macrophages, fibroblasts, and the multiplication of small blood vessels (Bhattacharya et al., 1992). During angiogenesis, nutrients and oxygen are supplied by the recruited inflammatory cells to the inflammatory site and promote granuloma tissue formation (Ghosh, 2005). In our studies, rhein (10–40 mg/kg) treatment significantly inhibited granuloma tissue formation by overwhelming dynamic events like angiogenesis and/or other inflammatory mediators.

Oxidative stress activates inflammatory pathways (Reuter et al., 2010). The antioxidant enzymes such as CAT, SOD, and GPx are the main defense system against oxidative injuries. Stimulation of these antioxidant enzymes through the Nrf2 transcription factor represents the best anti-inflammatory approach to attenuate ROS-induced damage. In basal environments, the Nrf2 transcription factor is bound and retained by the Keap1protein in the cytosol. During exposure to ROS, Nrf2 may dissociate from Keap1 and translocate into the nucleus where it activates and regulates antioxidant genes (Hybertson et al., 2011). In the present study, CAT, SOD, and GPx levels and the protein expression levels of Nrf2 and HO-1 were reduced in carrageenan-induced oedema. However, these reductions were significantly increased by rhein

treatment, indicating the possible role of Nrf2 in antioxidant enzymes induction.

In conclusion, *C. fistula* has been used traditionally as an anti-inflammatory remedy. Based on current results we consider that the compound rhein present in *C. fistula* may be in charge for this. Present experimental results revealed that rhein isolated from the flowers of *C. fistula* L. exhibited considerable anti-inflammatory activity in different inflammation experiments in animal models. The levels of anti-oxidant system enzymes (SOD, CAT, GSH-px), HO-1, Nrf2, PPAR-γ and HSP-72 were significantly affected by oedema induction. Treatment with rhein eased the aggressive effects of oedema on these biological markers. The anti-inflammatory properties of rhein may be associated with its positive effects on the anti-oxidant system in rats with hind paw oedema. Moreover, it was confirmed that rhein significantly inhibited acetic acid-induced vascular permeability, croton oil-induced ear edema, cotton pellet-induced granuloma formation and overwhelms inflammatory cells recruitment.

Conflict of interest

The authors declared no competing interests.

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