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Anti-inflammatory Effect of Apigenin-7-neohesperidoside (Rhoifolin) in Carrageenin-Induced Rat Oedema Model

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ABSTRACT

Flavonoids are normal constituents of the human diet and are known for a variety of biological activities. They have been reported to bring benefits in lowering inflammation and oxidative stress. The present investigation was performed first, to evaluate the anti-inflammatory activity of rhoifolin and second, to search for the possible contributing mechanisms for this hypothesized effect. Rhoifolin caused a time and reverse dose dependent reduction of carrageenin-induced rat paw oedema. Following 4 hr of treatment, rhoifolin at doses 2.5, 25 & 250 mg/kg caused a significant inhibition of rat paw edema volume by 14, 25 & 45 % respectively in comparison to the control group (74%). In addition to significantly abrogating prostaglandin E2 level, increasing doses of rhoifolin significantly diminished the TNF-α release in the inflammatory exudates. In the same animal model, rhoifolin increased the total antioxidant capacity in a reverse dose order, with the highest capacity obtained with the lowest dose tested. This study demonstrates for the first time the effectiveness of rhoifolin in combating inflammation in carrageenin-induced rat oedema model.

Keywords: TNF-α, prostaglandin E2, Antioxidant capacity.

INTRODUCTION

Inflammation is local response of living mammalian tissues to injury. Inflammation is thus an essential protective process preserving the integrity of organisms against physical, chemical and infective insults (Walport, 1993). However, it is frequent that the inflammatory response to several insults erroneously leads to the damaging of normal tissues (Majno, 1996). In inflammatory processes, the inducible isoform of cyclooxygenase (COX-2) is expressed in many cells and accounts for the release of large quantities of proinflammatory prostaglandins at the site of inflammation (Seibert & Masferrer 1994). Furthermore, the course of inflammation depends on the production of many inflammatory proteins. An important source of these mediators is macrophages. Macrophages activated by lipopolysacharides (LPS) produce many cytokines, e.g. Tumor necrosis factor-α (TNF-α), a cytokine, which plays an important role in inflammation. TNF-α is a pleiotropic pro-inflammatory cytokine that is primarily produced by activated macrophages and many other cell types including lymphocytes, endothelial cells, and mast cells (Rahman & McFadden, 2006; Bradley, 2008).

The upregulated production of TNF- α has been found to be associated with a variety of inflammatory diseases including rheumatoid arthritis, psoriasis, Crohn's disease, and refractory asthma (Palladino *et al*., 2003; Muppidi *et al*., 2004; Bradley, 2008; Kim *et al*., 2008). In this regard, natural products have long gained wide acceptance among the public and scientific community (Bauer, 2000). The anti-inflammatory properties of apigenin are evident in studies that have shown suppression of LPS-induced cyclooxygenase-2 and nitric oxide synthase-2 activity and expression in mouse macrophages (Liang *et al*., 1999). Apigenin treatment resulted in suppression of tumor necrosis factor (TNF) α-induced Nuclear Factor (NF)-κB activation in human umbilical vein endothelial cells (Choi *et al*., 2004). Furthermore, Apigenin from *Chamomilla recutita* were found to significantly inhibit the oedema caused by croton oil. This activity may have been due to a direct inhibition of arachidonic acid metabolism or to other mechanisms such as inhibition of histamine release or promotion of scavenging activity (Della Loggia *et al*., 1986). An important factor determining the efficiency of the absorption of flavonoid glycosides from the intestine is the sugar moiety. Hollman and colleagues showed that quercetin glycosides from onions were absorbed better (52%) than the pure aglycone (Hollman *et al*., 1999). It was reported that rhoifolin has protective effects on the renal cellular membrane and able to ameliorate renal cellular injury. It also exerts anti-diabetic effect through enhanced adiponectin secretion, phosphorylation of insulin receptor-*β*, and GLUT4 translocation (Yokozawa *et al*., 1999). Furthermore, its hepatoprotective effect was reported against carbon tetrachloride induce hepatotoxicity in mice (Perez Gutierrez *et al*., 2011).

Interestingly, the glycoside of apigenin; rhoifolin motivates us to investigate its anti-inflammatory activity which was not previously tested before. Therefore, the present investigation was performed first, to test the hypothesis "Does rhoifolin, the glycoside of apigenin, produce an anti-inflammatory activity in rat paw oedema model? Second, if so then what are the possible contributing mechanisms for this effect?"

MATERIALS AND METHODS

Plant material

Chorisia crispiflora (Bombaceae) leaves were collected from Zoo Garden in Giza, Egypt, 2010 and were authenticated by Prof. Dr Abdel Salam El Noyehy, Prof. of Taxonomy, Faculty of Science, Ain Shams University, Cairo, Egypt. Voucher specimen (voucher specimen number; CCB-73) was deposited at the herbarium of Pharmacognosy Department, Faculty of Pharmacy, Ain Shams University, Cairo, Egypt.

The leaves were dried in shade and reduced to a fine powder. Powder of air dried leaves of *Chorisia crispiflora* (1 kg) was extracted with 70 % ethanol on cold. The extract was completely dried and dissolved in a small amount of water and partioned with *n*-hexane, ethyl acetate, butanol successively. The left water residue was completely dried and extracted with methanol on hot (40ºC). The methanolic extract upon concentration yielded yellow crystals of rhoifolin. Purification to the crystals was achieved by crystallization.

Instruments and materials for phytochemical investigation

Chromatographically pure materials 1 mg each were dissolved in analytically pure methanol then subjected to UV spectroscopic investigation in 4 ml capacity quartz cells 1 cm thick using a Carl Zeiss spectrophotometer PMQ II. $AlCl_3$, $AlCl_3$/HCl, fused NaOAc / H_3BO_3 and NaOMe reagents were separately added to the methanolic solution of investigated material and UV measurements were then carried out. The NMR spectra were recorded on a Varian Mercury VX-500 NMR spectrometer. 1H-spectra run at 300 MHz and 13C- spectra were run at 75.46 MHz in deutrated dimethylsulphoxide (DMSO-d_6). Fig. 1 shows the chemical structure of apigenin 7-neohesperidoside.

Drugs

Indomethacin was purchased from Sigma-Aldrich (St. Louis, MO, USA). Inflammatory-grade carrageenin was purchased from FMC (Rockland, ME, USA). All other chemicals were of analytical grade and of highest purity commercially available.

Kits

PGE2 and TNF-α kits were purchased from Assay Designs Inc. (Ann Arbor, MI, USA). Total antioxidant capacity (TAC) kit was purchased from Biodiagnostics Co. (Cairo, Egypt).

Animals

All animal experimentation procedures followed in the present study were in accordance with the international guidelines governing animal experimentation. Throughout the experiments, adult male Sprague–Dawley rats weighing 150–175 g were used. Animals were housed at a temperature of 23 ± 2 ◦C with free access to water and standard food pellets (El-Nasr Co., Abo-Zaabal, Egypt). Rats were acclimatized in our animal facility for at least 1 week prior to any experiment. Protocol of the present work was approved by Experiments and Advanced Pharmaceutical Research Unit (EAPRU), Faculty of Pharmacy, Ain Shams University, Cairo, Egypt.

Measurement of paw volume in carrageenin-induced rat oedema model

Forty Five rats were equally divided into five groups assigned Latin numbers I–V. Animals were fasted, with free access to water, 16 h before the experiment. Group I was given saline by intragastric tube, while rats in group II were orally treated with indomethacin as standard anti-inflammatory drug (25 mg kg−1). Animals in group III, IV and V received rhoifolinin three oral doses; 2.5 mg kg−1, 25 mg kg−1and 250 ml kg−1, respectively. Dosing volume was kept constant (10 ml kg−1) and was completed with saline when required. The choice of the used doses and time of measurement and sampling was based on pilot studies in our laboratory. Thirty minutes after oral treatment, groups I–V received 0.05 ml carrageenin (1% solution in saline) sc on the

plantar surface of the right hind paw. The right hind paw volume was measured immediately after carrageenin injection by water displacement using UGO-BASILE 7140 plethysmometer (Comerio, Italy) (Winter *et al*., 1962). The percent change in paw volume compared to base line measurement was taken as the criteria of comparison. The volume was measured again 1 and 2 h after carrageenin injection and immediately before decapitation (4 h following injection).

Analysis of PGE2 and TNF-α release in the inflammatory exudate

After decapitation, right hind paws were removed. A volume of 0.1 ml saline containing 10 µM indomethacin was injected to aid removal of the eicosanoid-containing fluid and to stop further production of PGE2. Paws were incised with a scalpel and suspended off the bottom of polypropylene tubes with Ependorff pipette tips to facilitate drainage of the inflammatory exudates. For the purpose of the removal of the inflammatory exudates, paws were centrifuged at 1800 g for 15 min (Mnich *et al*., 1995). PGE2 was quantified in the collected exudates using a quantitative binding PGE2 enzyme immunoassay kit. The kit uses a monoclonal antibody to bind, in a competitive manner, the PGE2 in the sample as well as alkaline phosphatase-labelled PGE2 provided in the kit. The enzyme bound, through PGE2 molecule, to the monoclonal antibodies processes the specific substrate to produce a colour that is measured spectrophotometrically (Virella, 1998).

TNF- α was assayed using rat TNF- α enzyme immunometric assay kit. Rat exudative TNF- α was immobilized on polyclonal antibody bound to microtitre plate. Excess sample was washed. A monoclonal antibody specific to rat TNF- α, coupled to horseradish peroxidase, was added. The monoclonal antibody binds specifically to the immobilized rat TNF- α. Excess monoclonal antibody was washed and the substrate, tetramethyl benzidine, was added. After incubation period, the developed colour was measured spectrophotometrically at 450 nm (Virella, 1998).

Assessment of the total antioxidant capacity

The determination of the antioxidant capacity of the tested samples is performed by the reaction of the antioxidants in the sample with a defined amount of hydrogen peroxide (H_2O_2). The antioxidants in the sample eliminate a certain amount of the provided H_2O_2. The residual H_2O_2 is determined colorimetrically by the enzymatic reaction which involves the conversion of 3,5, dichloro-2-hydroxybenzensulphonate to a colored product (Koracevic *et al*., 2001). Results were expressed as mM residual H2O2.

Statistical analysis

Data are presented as mean ± S.E.M. Multiple comparisons were carried out using one way analysis of variance (ANOVA) followed by Tukey's test for post hoc analysis for the pair wise comparisons. Statistical significance was acceptable to a level of $P<0.05$. Data analysis was achieved using software program Graphpad Instat (version 2).

RESULTS

Effect of rhoifolin on % change of paw volume in carrageenin-induced rat oedema

Intraplantar injection of carrageenin to rats resulted in severe inflammation and significant increase in the mean volume of the challenged paw compared to that of the untreated paws (74% of the untreated paws) (Tab. 1). Pretreatment of rats with rhoifolin significantly inhibited the carrageenin-induced increase in the oedema volume of the paws in a reverse dose manner after 1 and 2 h (Fig. 2). Following 4 hr of carrageenin challenge, doses of 2.5, 25 and 250 mg kg−1 significantly reduced the paw volume to 15, 25 and 45% change, respectively. Similarly, indomethacin-treated group showed significant anti-oedema effect (30 % of the induced paws).

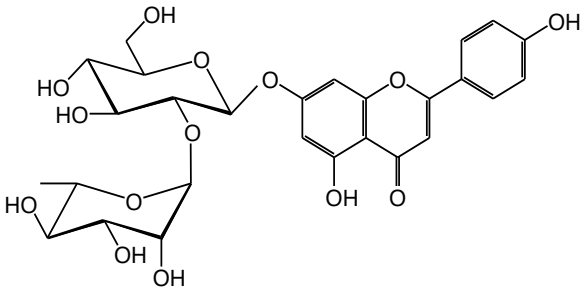

Fig. 1: Chemical Structure of Rhoifolin (apigenin 7-neohesperidoside).

Table. 1: Effect of rhoifolin on carrageenin-induced rat paw oedema model following 4 hrs.

Treatment group	Mean percentage change of paw volume ± SEM
Control	74.3 ± 6.8 [b,c,d,e]
Indomethacin 25 mg/kg	30.9 ± 6.7 [a]
Rhoifolin 2.5 mg/kg	13.6 ± 5.3 [a,e]
Rhoifolin 25 mg/kg	25 ± 4.8 [a]
Rhoifolin 250 mg/kg	44.5 ± 1.3 [a,c]

Each point is the mean ± SEM of 9 rats.
[a]Statistically significant from the control group at $P < 0.05$
[b]Statistically significant from the indomethacin group at $P < 0.05$
[c]Statistically significant from the rhoifolin 2.5 mg/kg group at $P < 0.05$
[d]Statistically significant from the rhoifolin n 25 mg/kg group at $P < 0.05$
[e]Statistically significant from the rhoifolin 250 mg/kg group at $P < 0.05$

Inhibition of PGE2 and TNF-α release by rhoifolinin the inflammatory exudates

In control group, significant increase in PGE2 level was observed 4 h post carrageenan administration. Indomethacin treated animals in group II resulted in mean PGE2 concentration of 310 ± 38 pg/ml in inflammatory exudates in compared to group I of carrageenin challenge that resulted in 810 ± 54 (Fig. 3). Animals receiving rhoifolin showed significant reduction of the PGE2 concentration 2 folds in exudates (to reach 410 ± 37, 410 ± 28 and 410 ± 22 pg/ml) for the doses of 2.5, 25 and 250 mg kg−1, respectively). A significant increase in TNF-α production was observed in control group. Indomethacin treatment after 4 h of injection of carrageenin into the rat paw reduced TNF-α production (7000 pg/ml) compared to that untreated animals in group I (10000 pg/ml rhoifolin injection in doses of 2.5, 25 and 250 mg kg−1

showed significant reduction of TNF-α level by 1.85, 1.7 and 1.3 folds to reach 5400, 5800 and 7900 pg/ml respectively compared to group I (Fig. 4).

Augmentation of the total antioxidant capacity by Rhoifolin in the tested inflammatory model

Rhoifolin showed significant increase of anti-oxidant capacity at dose of 2.5 mg kg−1 by showing 4.7 fold decrease in residual H_2O_2 (0.012 mM), compared to control group (0.056 mM). The middle dose tested of Apigenin 7-neohesperidoside 25 mg kg−1was shown to be equipotent to the standard anti-inflammatory indomethacin tested (0.026 and 0.029 mM residual H_2O_2, respectively). The higher dose tested 250 mg kg−1 was not significantly different from the control group (Fig. 5).

DISCUSSION

Flavonoids are believed to act as health-promoting substances, and some of them act as enzyme inhibitors and antioxidants, and have been reported to have anti-inflammatory properties (Middleton *et al*., 2000; Havsteen, 2002). As an important example, apigenin is a phytopolyphenol widely distributed in the human diet. Like many other flavonoids, apigenin has been reported to exert anti-inflammatory effects such as lowering oxidative stress and preventing the expression of several inflammatory factors, as confirmed by Sawatzky and collaborators (Sawatzky *et al*., 2006). The precise mechanisms by which apigenin and other flavonoids exert their anti-inflammatory properties, however, remain to be elucidated.

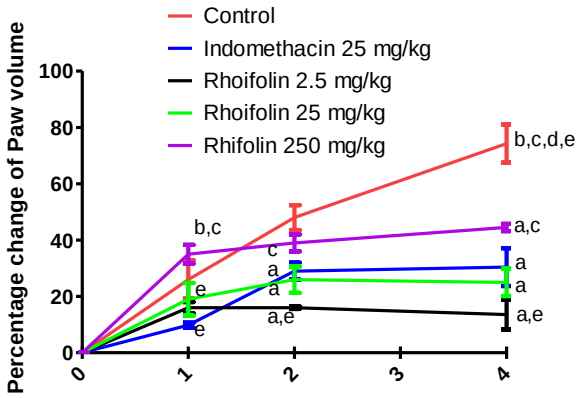

Fig 2. Time and dose dependent effect of rhoifolin on % change of rat paw oedema volume

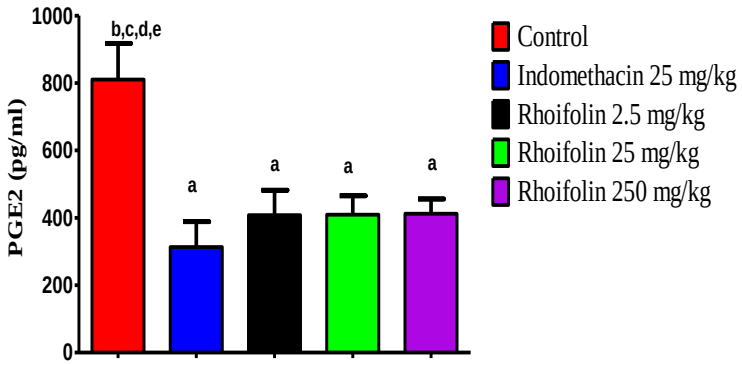

Fig 3. Effect of rhoifolin on PGE2 production in inflammatory exudates.

[a]Statistically significant from the control group at P < 0.05.
[b]Statistically significant from the indomethacin group at P < 0.05.
[c]Statistically significant from the rhoifolin 2.5 mg/kg group at P < 0.05.
[d]Statistically significant from the rhoifolin n 25 mg/kg group at P < 0.05.
[e]Statistically significant from the rhoifolin 250 mg/kg group at P < 0.05.

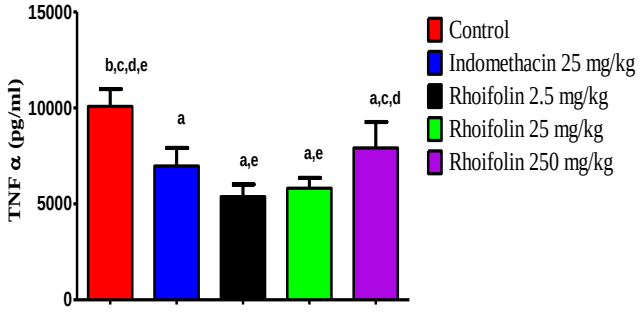

Fig 4. Effect of rhoifolin on TNF-α release in rat paw oedema model.

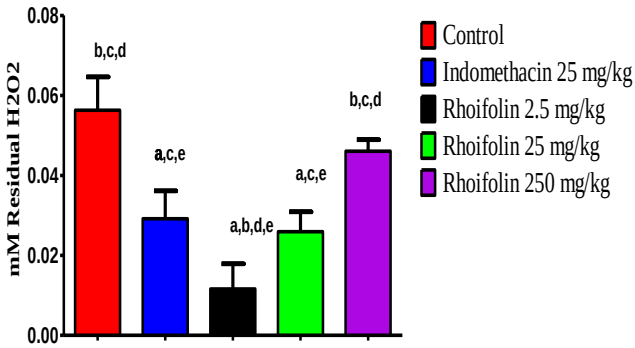

Fig 5. Effect of rhoifolin on total antioxidant capacity in paw oedema experiment in rats.

[a]Statistically significant from the control group at P < 0.05.
[b]Statistically significant from the indomethacin group at P < 0.05.
[c]Statistically significant from the rhoifolin 2.5 mg/kg group at P < 0.05.
[d]Statistically significant from the rhoifolin in 25 mg/kg group at P < 0.05.
[e]Statistically significant from the rhoifolin 250 mg/kg group at P < 0.05.

On the other hand, rhoifolin has never been tested before for its anti-inflamatory activity. Thus our aim in the current study is to evaluate the potential anti-inflammatory activity of rhoifolin in carrageenin-induced rat oedema model. Results showed that rhoifolin caused a time and reverse dose dependent reduction of carrageenin-induced rat paw oedema. Following 4 hr of treatment, rhoifolin at doses 2.5, 25 & 250 mg/kg caused a significant inhibition of rat paw edema volume by 14, 25 & 45 % respectively in comparison to the control group (74%).

Thus, interestingly the higher dose of rhoifolin was observed to be pro-inflammatory as compared to the anti-inflammatory effect of the tested lowest dose of apigenin 7-neohesperidoside. This finding is in accordance with the results reports by (Sawatzky *et al*., 2006), in which the flavonoid apigenin, actually exacerbated inflammation. This finding was further discussed and explained by (Soares & Azevedo, 2006), clarifying that flavonoids are potent phytoestrogens and are capable of inducing estrogen-dependent gene transcription, resulting in activation of several tyrosine kinases (Stroheker *et al*., 2002). Therefore, it is quite possible that flavonoids interact with many signaling pathways as well producing the pro-inflammatroy effect in contrast to the well documented anti-inflammatroy effect.

Such conclusion was further substantiated by assessing PGE2 level in the inflammatory exudates of rat paws in the same model. Prostaglandins have been long recognized as a major mediator of inflammation. They are arachidonic acid metabolites synthesized by COX-1 and COX-2 isozymes (Herschman, 1996). Our results indicate that rhoifolin caused statistically significant reduction of PGE2 content. This finding clearly underlines the anti-inflammatory effect of rhoifolin. In addition to evaluating prostaglandin E2 (PGE2) level in our experiments, the effect of rhoifolin on the (TNF α) release in the inflammatory exudates was also assessed.

TNF- α is a pleiotropic cytokine which plays a critical role in both acute and chronic inflammation (Holtmann *et al*., 2002). More importantly, TNF- α induces the synthesis of PGE2 (Arai *et al*., 1990). In our experimental model, increasing doses of rhoifolin; in particular the lowest dose, strongly and significantly inhibited TNF- α release in the inflammatory exudates. The reduction of TNF-α release by rhoifolin is in agreement with the PGE2 finding obtained in the current study. Thus, an alternative explanation of the resultant reduction of PGE2 level in inflammatory exudates may be through interference with TNF-α release. Furthermore, in the same animal model, rhoifolin also managed to increase the total antioxidant capacity in the inflammatory exudates. This increase in the total antioxidant capacity was also in a reverse dose order, with the highest capacity obtained with the lowest dose tested.

In conclusion, for the first time, rhoifolin was shown to possess a potent anti-inflammatory activity at low doses. This is evidenced by decreased paw oedema, PGE2 content in exudates, and TNF-α formation as well as increase of total antioxidant capacity.

CONCLUSIONS

This study demonstrates the effectiveness of rhoifolin in combating inflammation in carrageenin-induced rat oedema model. These findings suggest that rhoifolin would be useful for the treatment of in combating inflammation via multi-level regulation of inflammatory mediators. These findings consequently merit further exploration of the extract in subsequent *in-vivo* studies and later in controlled clinical trials.

DECLARATION OF INTEREST STATEMENT

Both authors declare that they have no competing financial or personal interest or any kind of conflict of interest relevant to this study.

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