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Anti-inflammatory effect of *Ruta graveolens* L. in murine macrophage cells

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Abstract

Ruta graveolens L. (Rutaceae) is used for several therapeutic purposes worldwide. The present study is designed to investigate the effect of plant extract of *Ruta graveolens* on murine macrophage cells (J-774) challenged with lipopolysaccharide (LPS). LPS induces inflammatory response by stimulating the production of nitric oxide and other mediators. Significant inhibition ($p = 0.01$ to $p < 0.002$) of the LPS-induced nitric oxide production was observed in cells treated with plant extract in a concentration dependent manner. The inhibition observed for the extract was significantly higher than that observed for rutin, a flavonoid constituent of the plant. At 40 μM rutin, a comparable concentration of this flavonoid in the highest concentration (500 μg/ml) of plant extract was used in this study; a 20% inhibition ($p = 0.058$) was observed. Inhibition in inducible nitric oxide synthase (*inos*) gene expression in the cells treated with the plant extract suggests an inhibition at the transcription level. Interestingly, a concomitant decrease in transcription of cyclooxygenase-2 (*COX-2*) gene has also been observed in cells treated with the plant extract and this inhibition is significantly higher than that observed with the highest concentration of rutin (80 μM) used in the study. As an inflammatory response, upregulation of nitric oxide synthase (iNOS) and COX-2 enzymes leads to production of pro-inflammatory mediators, namely nitric oxide and prostaglandins, respectively. Hence, the significant inhibitory effects on both of these inflammatory mediators unravel a novel anti-inflammatory action of this plant.

Keywords: *Ruta graveolens*; Nitric oxide; *inos*; *COX-2*; Anti-inflammatory effect; Rutaceae

1. Introduction

The inflammation is an essential event; this protective response may lead to potentially damaging consequences. Various autoimmune disorders are characterized by marked inflammation and associated failure of repair process. Pro-inflammatory molecules like TNFα, certain interleukins, prostaglandins and even pathogenic concentration of nitric oxide are instrumental in raising such response (Van der Vliet et al., 2000). Many current anti-inflammatory drugs target these mediators at different levels, yet they lack specificity and their untoward effects restrict their long-term use (Dhikav et al., 2002). Hence, there is a constant demand for better therapeutic alternatives. Herbal products are well known for their reputed medicinal properties; however, most of them are empirically used. Several plants of Rutaceae family are used in traditional medicine worldwide. The most common medicinal plant of this family is *Ruta graveolens* L., known as rue and native to Europe. The plant is now available all over the world, though preferably grown in Mediterranean climates. This plant is in medicinal use for various clinical conditions from very ancient time but rationality of its use is still controversial. In homeopathy, rue is an important remedy for deep aching pain and rheumatism besides being used for eyestrain-induced headache (Miguel, 2003). It has also been used as a remedy for gastric disorders, stiff neck, dizziness, headache and so on (Conway and Slocumb, 1979). This hints towards the putative anti-inflammatory property of *Ruta graveolens*.

Rue contains different active compounds, out of them rutin, a flavonoid, is known to have nitric oxide scavenging activity (Van Acker et al., 1995). According to the available literature (CSIR, 1988), *Ruta graveolens* plant contains approximately 2% of rutin. An interesting recent study reported the decrease in lipopolysaccharide- (LPS) induced nitric oxide production by rutin in vivo due to inhibition of nitric oxide synthase (iNOS) protein expression; however, that is mainly claimed to be due to

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hydrolysis of rutin into quercetin (Shen et al., 2002). Rutin is a diglycoside of quercetin that can be hydrolyzed to quercetin in the gastrointestinal tract. In an in vivo model, *Ruta chalepensis* L., a related plant has been found to reduce LPS-induced nitric oxide level (measured nitrite level) without altering the cytokines (Iauk et al., 2004).

High level of different cytokines including TNFα is known to involve in over expression of inducible nitric oxide synthase (*inos*) and cyclooxygenase-2 (*COX-2*) genes, which up-regulate these enzymes (Hughes et al., 1999). Constitutive level of these proteins, particularly iNOS is negligible (Alderton et al., 2001) and is unique to be induced in stress conditions (Madrigal et al., 2001). Similarly, COX-2 undergoes selective upregulation during inflammation (Rohrenbeck et al., 1999). Non-selective and selective (for COX-2) inhibitors of cyclooxygenases are used extensively to combat inflammation (Dhikav et al., 2002). However, their role on nitric oxide production has not been ascertained (Tunctan et al., 2003).

A growing body of evidence in recent years implicated nitric oxide and iNOS activity in different inflammatory pathogenesis (Guzik et al., 2003). Nitrosylation of several functionally important proteins involved in cell proliferation and inflammation have been reported (Foster et al., 2003). More interestingly, nitric oxide has been shown to have the ability to stimulate COX-2 showing a potential synergism (Hughes et al., 1999). Thus, nitric oxide appears to be of crucial importance and hence may be considered as a rewarding target for intervention. Although some plant-derived compounds have shown potential for being therapeutically used (Son et al., 2000), specific inhibitors of nitric oxide production have not been tried therapeutically except L-nitro-arginine methyl ester [L-NAME] (Pechanova et al., 1999).

With this perspective, the present study is designed in murine macrophage cells to investigate, whether whole extract of rue has any effect to combat inflammatory challenge and what is the possible mechanism underlying such effect.

2. Methods

2.1. Preparation of plant extract

The plant *Ruta graveloens* L. was procured from and authenticated by Dr. Prakash Joshi (senior scientific officer) Homeopathic Pharmacopoeia Laboratory (HPL), Ghaziabad, India (this national laboratory is responsible for the validation of all the plants and plant products used in homeopathy). The whole plant was collected during the months of December–January in the year 2002–2003 from HPL herbal garden. The dried plant was extracted three times with 50% methanol at room temperature and the (w/w) extraction yield was 6.5%.

2.2. Percentage analysis of rutin in the test extract by reverse phase HPLC

Twenty micrograms per milliliters of rutin (Sigma) dissolved in methanol was scanned at 200–900 nm (Perkin-Elmer UV–vis spectrophotometer) and the λ_{max} of rutin was determined at 207 nm. The same wavelength was used to analyze the test extract by reverse phase HPLC (Schimadzu LC4A with UV detector and C-18 ODS column, 15 cm long with 4.6 mm diameter) using methanol as eluent. The retention time for rutin at 207 nm was 4.3 min and a peak at the similar retention time was observed in the test extract. Rutin was added to the test extract as an internal standard to confirm that the peak observed in the text extract corresponds to rutin. The percent composition of rutin in the plant extract was calculated by the area covered under the peak of interest by comparing with the area observed for standard rutin of known concentration. Different concentrations of rutin were used to generate a standard curve (5, 10, 15 and 20 μg, $r^2 = 0.9974$). Peaks for the other compounds were also observed but were not analyzed for the present study.

2.3. Cell culture

Murine macrophage cell line (J-774) was obtained from International Center for Genetic Engineering and Biotechnology, India. Cells were cultured in RPMI-1640 medium supplemented with 2 mM glutamine, antibiotics (100 U/ml of penicillin, 100 μg/ml of streptomycin and 0.25 μg/ml of amphotericin-B), 10% heat-inactivated fetal calf serum and maintained at 37 °C in a humidified CO_2 incubator. The culture was allowed to grow to confluence and used for further experiments. The cells were resuspended in RPMI-1640 at a density of 2×10^6 cells/ml. Viability was determined by Trypan blue (0.4% Trypan blue in PBS) exclusion method. The cells were challenged with different concentrations of LPS (Sigma) of *Escherichia coli* (serotype, 055:B5) and LPS concentration was optimized at 1 μg/ml for inducing measurable concentration of nitrite with minimum cytotoxicity. The inhibitory effect of the plant extract on nitric oxide production by murine macrophages was preliminarily studied by simultaneous addition of different concentrations of test extract with LPS (1 μg/ml). Cells were then pretreated with different concentrations of the test extract (100, 200, 300, 400 and 500 μg/ml) and rutin (20, 40 and 80 μM) for 2 h, subsequently washed and then challenged with LPS (1 μg/ml). The cells were then incubated for 4 and 16 h at 37 °C in the humidified CO_2 incubator. Cells after 4 h of incubation were used for RNA isolation and the cell free supernatant after 16 h of incubation was used to detect nitric oxide using Griess nitrite assay (Lee et al., 2003). Similar experiments were performed using rutin at different concentrations along with the plant extract to find the synergistic effect of the rutin. In all the above cases, unstimulated controls were taken under similar culture conditions. The nitric oxide synthesis inhibitor (L-nitro-arginine methyl ester, L-NAME, 200 μM) was used in each assay as positive control.

2.4. Cytotoxicity assay

The cytotoxicity was determined in each experiment using MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide; Sigma) colorimetric assay. Briefly, after 16 h of incubation with or without test extract and or rutin, MTT (100 μl, 5 mg/ml in PBS) solution was added to each well and incubated for 4 h. The medium was then removed from each well and isopropanol containing 0.04 M HCl was added to dissolve