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European Journal of Pharmacology 560 (2007) 69–80
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# Inhibition of lipopolysaccharide-inducible nitric oxide synthase and IL-1β through suppression of NF-κB activation by 3-(1′-1′-dimethyl-allyl)-6-hydroxy-7-methoxy-coumarin isolated from *Ruta graveolens* L.

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Received 16 November 2006; received in revised form 6 January 2007; accepted 10 January 2007
Available online 19 January 2007

**Abstract**

The *Ruta graveolens* L. plant is used in traditional medicine to treat a large number of diseases. The methanol (50%) extract of the whole plant was observed to inhibit the expression of inducible nitric oxide synthase (iNOS) and the cycloxygenase-2 (COX-2) gene in lipopolysaccharide (LPS)-induced macrophage cells (J774A.1, [Raghav, S.K., Gupta, B., Agrawal, C., Goswami, K., Das, H.R., 2006b. Anti-inflammatory effect of *Ruta graveolens* L. in murine macrophage cells. J. Ethnopharmacol. 104, 234–239]). The effect of whole plant extract on the expression of other pro-inflammatory genes such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-12, interferon-γ (IFN-γ) and the activation of nuclear factor-kB (NF-κB) were investigated in LPS stimulated macrophage cells. An active compound was isolated from this methanol extract by further solvent fractionation and reverse phase high performance liquid chromatography (RP-HPLC). The purified compound was identified as 3-(1′-1′-dimethyl-allyl)-6-hydroxy-7-methoxy-coumarin having IUPAC nomenclature of 6-hydroxy-7-methoxy-3-(2-methyl but-3-en-2yl)-2*H*-chromen-2-one by ESI-MS, MALDI, FT-IR and NMR. Effect of this purified compound was assessed on iNOS, COX-2 and various pro-inflammatory cytokine genes and was observed to inhibit both the protein and mRNA expression of iNOS and IL-1β in LPS challenged macrophages. Electrophoretic mobility shift assay (EMSA) and Western blot analyses indicated that the plant extract and the isolated active compound blocked the LPS-induced activation of NF-κB through the prevention of inhibitor-kB (IkB) degradation. The purified compound also showed the anti-oxidant activity. The active compound at a dose of 40 mg/kg body weight was observed to inhibit the iNOS and IL-1β gene expression significantly in endotoxin-induced inflammatory model of BALB/c mice. The low level of nitric oxide production was also observed in the sera of compound treated mice. The normal behavioral condition in LPS challenged BALB/c mice was noticed when these were treated with active compound.


*Keywords:* *Ruta graveolens*; iNOS; IL-1β; NF-κB; IkB; Anti-inflammatory effect

## 1. Introduction

Inflammation is an essential response, but when uncontrolled it may lead to potentially damaging consequences as seen in several inflammatory diseases. Lipopolysaccharide as an endotoxin, induces septic shock and stimulates the production of inflammatory mediators such as nitric oxide (NO), TNF-α, interleukins, prostanoids and leukotrienes (Lee et al., 2003b). NO, prostaglandins and inflammatory cytokines are important pro-inflammatory mediators and are the major targets for the treatment of inflammatory disorders (Hobbs et al., 1999; Turini and DuBois, 2002). Free radical NO is produced by nitric oxide synthase (NOS) enzyme as a by-product during conversion of L-arginine to L-citrulline (Lee et al., 2003b). The inducible NOS (iNOS) is induced by stress, pro-inflammatory cytokines like IL-1β, IFN-γ, TNF-α and bacterial lipopolysaccharide (Nathan and Xie, 1994). Nitric oxide synthase (NOS) plays a major role in regulating vascular tone, neurotransmission, killing of micro-organisms and tumor cells and other homeostatic mechanisms (Mayer and Hemmens, 1997). High levels of NO have been reported in circulatory shock (Szabo, 1995), inflammation (MacMicking et al., 1997) and carcinogenesis (Ohshima and Bartsch, 1994). It has been proposed that iNOS mediated high

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doi:10.1016/j.ejphar.2007.01.002

output production of NO causes cell injury through generation of reactive radicals such as peroxynitrite. It also results in nitrosylation of a number of proteins, some of which are involved in cell signaling (Kuncewicz et al., 2003).

*R. graveolens* L. (Rutaceae) commonly known as rue is known as medicinal plant since ancient times and currently used for treatment of various disorders such as aching pain, eye problems, rheumatism, and dermatitis (Conway and Slocumb, 1979; Miguel, 2003) etc. Rue is a native of the Mediterranean region but cultivated throughout Europe and many Asian countries, including China, India, and Japan (Raghav et al., 2006b). The plant contains more than 120 compounds of different classes of natural products such as acridone alkaloids, coumarins, essential oils, flavonoids and furoquinolines (Kuzovkina et al., 2004). The components of *Ruta* species are of great interest in medicinal chemistry, as these compounds show a broad range of biological activity and a number of them are used in medicine (Ulubelen et al., 1986).

During the inflammatory process, TNF-α and interleukins like IL-1β, IL-12 are produced from macrophages to combat the injury (Weinstein et al., 1991). The uncontrolled feedback inhibition of some of these cytokines results in diseased condition. In some of the inflammatory conditions like rheumatoid arthritis, the natural homeostasis leading to controlled apoptosis/proliferation becomes altered due to imbalance in the cytokine levels (Mountz et al., 2001). TNF-α plays an important role in apoptosis as well as proliferation of cells. TNF-α transduces its signal through its membrane receptors tumor necrosis factor receptor-1 (TNFR-I) and TNFR-II. The cytoplasmic domain of TNFR-I contains a death domain, TNF receptor associated death domain (TRADD) which interacts with Fas associated death domain (FADD) leading to activation of caspases and hence apoptosis. TNFR-II lacks the death domain but the intracellular domain of this receptor contains a consensus motif that allows binding to TNF receptor associated factor 2 (TRAF-2) (Rothe et al., 1995). TRAF-2 activates both NF-κB and JNK (c-*Jun* N-terminal kinase) and mediates its anti-apoptotic effect (Reinhard et al., 1997). Our very recent findings (Raghav et al., 2006a) in peripheral blood mononuclear cells of rheumatoid arthritis patients indicated that an enhanced TNF-α signaling through TNFR-1-TRADD-RIP-TRAF-2, while a suppressed signaling was observed by TNFR-1-TRADD-FADD pathway. Expression of TNF-α and its signaling intermediates (treated with or without the plant extract and the pure compound) involved in the apoptosis/proliferation were also considered as a part of the study.

In inflammatory disorders like rheumatoid arthritis, the joint cartilage undergoes erosion as the inflammation prolongs. In chondrocytes, the IL-1β-induced NO inhibits its proliferation via prostaglandin synthesis (Blanco and Lotz, 1995). The pro-inflammatory cytokine IL-1β also plays an important role in recruitment of adhesion molecules like intracellular adhesion molecule-1, vascular cell adhesion molecule-1 and E-selectin (Ebisawa et al., 1992). These molecules increase the migration of macrophages and other cells at the inflammatory tissue locus. The accumulated inflammatory cells secrete NO, chemokines and other pro-inflammatory molecules. The NO act synergistically along with IL-1β in the process of osteoclast cell development and suppression of cartilage proteoglycan synthesis (Taskiran et al., 1994) leading to bone erosion, and hence joint impairment. Therefore both IL-1β and nitric oxide are important targets to prevent this type of detrimental effect.

NF-κB proteins are a small group of closely related transcription factors and NF-κB is activated by the inflammatory responses during viral and bacterial infections in the cytosol (Grilli and Memo, 1999). It is especially involved in the expression of iNOS, COX-2, TNF-α and IL-1β genes (Kim et al., 2000; Ahn et al., 2005). Recently we reported that the plant extract of rue significantly inhibits the generation of nitric oxide *via* inhibiting the iNOS gene in murine macrophages challenged with bacterial LPS (Raghav et al., 2006b). It also inhibits the expression of COX-2 gene. The present study is designed to enunciate the mechanism of action of the plant extract leading to potential anti-inflammatory effects, and to identify the lead compound/s resulting in the anti-inflammatory effects of the plant.

## 2. Materials and methods

The plant was procured from Homeopathic Pharmacopeia Laboratory (HPL) herbal garden and authenticated by Dr. Prakash Joshi, Senior Scientific Officer, HPL, Ghaziabad, India. (Homeopathic Pharmacopeia Laboratory is a central government organization responsible for validation of all the plants and plant products used in homeopathic medicine).

Anti-NF-κB p65 subunit, anti-IkBα antibodies, anti-rabbit-IgG-horseradish peroxidase conjugate were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibiotic–antimycotic solution (100×), 4-chloronaphthol, Dulbecco's modified eagle's medium (DMEM), MTT [3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide], DCF-DA (2′,7′-dichlorofluorescein-diacetate), 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), phenylmethylsulfonylfloride (PMSF) and protease inhibitor cocktail were purchased from Sigma Chemicals (St. Louis, MO). Gel shift assay kit was purchased from Promega (Madison, WI). Fetal calf serum and total RNA isolation kit was purchased from Biological Industries (Israel).

### 2.1. Preparation of crude plant extract

The whole plant extract was prepared as published earlier (Raghav et al., 2006b). In brief, the plant was grinded and soaked in 50% methanol made in de-ionized water with intermittent shaking and extracted three times at room temperature. The extract was then filtered and lyophilized using freeze drier (Vertis, USA). The dried extract was then weighed and dissolved in 50% methanol for *in vitro* screening.

### 2.2. Fractionation of methanol extract

Dried methanol extract of plant was resuspended in de-ionized water and stirred till a uniform suspension was obtained. The suspension was then exhaustively fractionated using

organic solvents (diethyl ether < chloroform < ethyl acetate) according to their increasing polarity. The solvents were evaporated using rotary evaporator and the samples were lyophilized using freeze drier. The dried powder recovered from each fraction was weighed and dissolved in dimethylsulfoxide (DMSO) at a concentration of 50 mg/ml. Each of the four fractions obtained was then scanned for its effect on nitric oxide inhibition *in vitro*.

### 2.3. Isolation of active compound from diethyl ether fraction

The active diethyl ether fraction (showing significant NO inhibition) was passed through 0.22 μm filter and then loaded on to pre-equilibrated analytical Sunfire™ $C_{18}$ column (5 μm; 4.6 × 150 mm, photodiode array detector, waters system, pump 600e) at room temperature. The isocratic mobile phase containing 30% acetonitrile and 70% MilliQ water with 0.05% TFA was used. The flow rate was maintained at 1 ml/min. Thirteen well separated peaks were obtained (Fig. 1).

Semi-preparative Sunfire™ $C_{18}$ (10 μm; 10 × 250 mm) column was used for collecting the individual peak material in sufficient quantity using the same isocratic mobile phase. After evaporating the solvent the resultant residue from each fraction was stored at 4 °C till use. From each purified sample 2.5 mg/ml of stock was prepared in DMSO for *in vitro* screening of nitric oxide inhibition. The compound showing significant inhibitory effect was subjected to rerun RP-HPLC column using gradient mobile phase to confirm its purity.

### 2.4. Analysis for presence of rutin and quercetin in diethyl ether fraction by RP-HPLC

The presence of rutin and quercetin in the diethyl ether fraction was checked using semi-preparative RP-HPLC as described above. Two hundred micrograms (200 μl) of diethyl ether fraction was injected with or without 40 μg of purified rutin or quercetin (Fig. 2). The retention time of rutin/quercetin thus obtained was compared with that of the active compound.

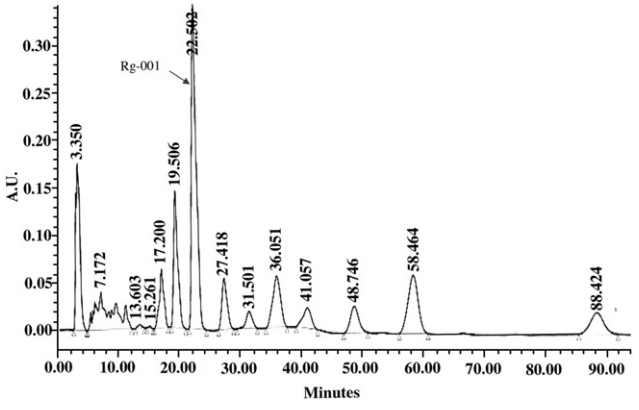


Fig. 1. RP-HPLC profile of diethyl ether fraction showing thirteen different peaks (fractions) including that of the active compound Rg-001. The retention time (min) is mentioned for each peak.

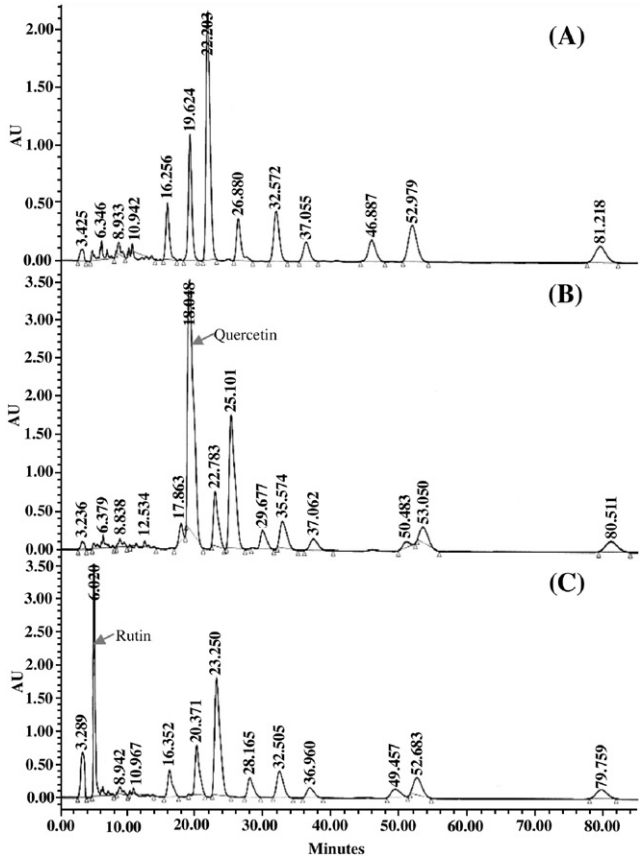


Fig. 2. RP-HPLC of diethyl ether fraction after addition of rutin and quercetin. (A) HPLC profile of diethyl ether fraction (200 μg), (B) HPLC profile of diethyl ether fraction (200 μg) with purified quercetin (40 μg), (C) HPLC profile of diethyl ether fraction (200 μg) with purified rutin (40 μg).

### 2.5. Cells and cell culture

Murine macrophage cells (J774) were grown in DMEM media supplemented with 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. Viability was determined by trypan blue (0.4% Trypan blue in PBS) exclusion method. To test the activity of the compounds, $2 \times 10^6$ cells were plated in 24 well plates and pre-incubated with the plant extract (300, 500 μg/ml), subfractions (50, 100, 150 μg/ml) and the isolated compounds (5, 10, 20 μg/ml) for 2 h and then challenged with LPS (1 μg/ml). The LPS concentration was optimized at 1 μg/ml for inducing pro-inflammatory mediators with minimum cytotoxicity as described earlier (Raghav et al., 2006b). Concentration of crude extract (300, 500 μg/ml) was also considered from our previous observations as $IC_{50}$ was found to be 345 μg/ml however, the subfractions and the active compounds were checked at different concentrations.

After LPS treatment the cells were incubated further for 4, 8 and 16 h. The cell free supernatant was used for nitric oxide and IL-1β measurement while the cell pellets were used for gene expression analysis using reverse transcription-polymerase chain reaction (RT-PCR).

### 2.6. Griess nitrite assay

The nitric oxide production was measured as described by Lee et al. (2003a). Briefly 100 μl of supernatant samples were incubated for 10 min at room temperature with an equal volume of Griess reagent (0.1% naphthalene diamine dihydrochloride, 1% sulfanilamide in 5% $H_2SO_4$) in microtitre plate. The absorbance at 550 nm was measured. Sodium nitrite was used as the standard.

### 2.7. Cell viability assay

The cytotoxicity was determined in each experiment using MTT colorimetric assay. Briefly, after 16 h of incubation with or without test extract/the isolated active compound, 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 the formazan produced in the cells. The optical density of the formazan product in solution was measured with a microplate reader at 570 nm.

### 2.8. Total RNA isolation and RT-PCR

Total RNA was isolated from macrophage cells harvested after 4 h and 8 h of incubation according to the vendor recommended protocol (total RNA isolation kit, Biological Industries, Israel). The RNA was quantified spectrophotometrically and 2 μg of total RNA from each sample was used for cDNA synthesis (single strand cDNA synthesis kit, Clontech, USA). Gene specific PCR for different cytokines and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) as house keeping gene was performed. Primer sequences (Table 1) were designed from cDNA sequences of the specific gene (www.ncbi.nlm.nih.gov) using DNASTAR software. The PCR mixture consisted of 25 mM Taq buffer containing 15 mM $Mg^{2+}$, 5 mM dNTPs, 10 pM each of the forward and the reverse primers, 2 units Taq DNA polymerase enzyme and 2 μl of 1:5 diluted cDNA for a 25 μl reaction. The PCR conditions for the amplification of transcripts were as follows: denaturation at 94 °C for 4 min and 35 cycles at 94 °C for 30 s, primer annealing for 30 s (Table 1), 72 °C for 1 min and then a final extension for 3 min at 72 °C. For the amplification of the G3PDH gene the PCR conditions followed, an initial denaturation at 94 °C for 4 min, 29 cycles at 94 °C for 45 s, 60 °C for 45 s, 72 °C for 2 min and then a final extension for 7 min at 72 °C. The PCR products were analyzed in 1.2% agarose gel electrophoresis and the densitometric analysis of the gene specific PCR products with respect to G3PDH gene was carried out using Image Scan and Analysis System (Alpha-Innotech Corporation, USA) with Digidoc 1201 software.

### 2.9. Preparation of nuclear extracts

Nuclear extract was prepared according to the previously reported methods (Schreiber et al., 1990). Briefly, the murine macrophage cells were pre-incubated with or without whole plant extract (300 and 500 μg/ml), diethyl ether fraction (100 μg/ml) and the active compound, designated as Rg-001, (5, 10 and 20 μg/ml) showing significant nitric oxide inhibition for 2 h followed by induction with LPS (1 μg/ml) for 30 min. The cells were washed with PBS, dislodged using a cell scraper, and pelleted by centrifugation. The cells were resuspended in cell lysis buffer [10 mM HEPES; pH 7.5, 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 0.5% Nonidet-40 and 0.5 mM PMSF along with the protease inhibitor cocktail] and allowed to swell on ice for 10 min. Tubes were vortexed to disrupt cell membranes and then centrifuged at 12,000 *g* at 4 °C for 10 min. The supernatant was stored as cytoplasmic extract and the nuclei pellets were washed thrice with the cell lysis

Table 1
Primer sequences used for the amplification of cDNA prepared from total RNA isolated from murine macrophages

| Gene | Primer sequence | Annealing temperature (°C) | Amplicon length (bp) |
|---|---|---|---|
| TNF-α | 5′ CAGGGGCCACCACGCTCTTC 3′<br>5′ CTTGGGGCAGGGGCTCTTGAC 3′ | 60 | 419 |
| TNFR-I | 5’ GGTGACCGGGAGAAGAGGGAT AGC 3’<br>5′ ACCAGGGGCAACAGCACCGCAGTA 3′ | 62 | 551 |
| TNFR-II | 5′ AGGGACGTTCTCTGACACCAC A 3′<br>5′ GCCTCCGCCATGACTCTT G 3′ | 60 | 570 |
| TRADD | 5′ ACTGATGAAGAGCGCTGTTTGAAT 3′<br>5′ GCTGGAAGGCCTGCTCGTAT 3′ | 58 | 409 |
| FADD | 5′ GCTGCTGCACTCGCTGTCC 3′<br>5′ ACTTCGGGGGTACTTCTCCTCAAT 3′ | 60 | 391 |
| TRAF-2 | 5′ CTACTCCAGCGGTGCCAGATTTT 3′<br>5′ CAGTGCCGTCGCCATTCAAGTAG 3′ | 60 | 403 |
| IL-1β | 5′ CAGGCTCCGAGATGAACAACAAAA 3′<br>5′ TGGGGAACTCTGCAGACTCAAACT 3′ | 60 | 332 |
| IL-12 | 5′ GTGACACGCCTGAAGAAGATGACA 3′<br>5′ CGGCAGTTGGGCAGGTGAC 3′ | 60 | 453 |
| IFN-γ | 5′ GCTCTTCCTCATGGCTGTTTCTG 3′<br>5′ CATCCTTTTTCGCCTTGCTGTT 3′ | 58 | 302 |
| iNOS | 5′ TCACTGGGACAGCACAGAAT 3′<br>5′ TGTGTCTGCAGATGTGCTGA 3′ | 58 | 510 |

buffer and resuspended in the nuclear extraction buffer containing 20 mM HEPES (pH 7.5), 400 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF with protease inhibitor cocktail and incubated in ice for 30 min. The extracted nuclei were pelleted at 12,000 *g* for 15 min at 4 °C and the supernatant was collected as the nuclear extract. The protein concentration was estimated using Bradford's reagent. The nuclear and cytoplasmic extracts were stored at −70 °C.

### 2.10. Western blot analysis

Nuclear and cytoplasmic extracts from macrophage cells were electrophoresed on 12% sodium dodecyl sulfate (SDS) polyacrylamide gels and transferred to nitrocellulose membrane (Pall Life Sciences, USA) in 25 mM Tris, 192 mM glycine, 20% methanol at 15 V for 45 min using semi dry transfer apparatus (Biorad, USA). The protein transferred was checked using 0.01% solution of ponceau-S stain in 1% acetic acid. Non-specific binding sites were blocked by incubating the membrane in 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) at room temperature for 1 h. After washing twice with PBST (PBS with 0.05% Tween-20) buffer the membrane was incubated with primary antibody [polyclonal anti-NF-κB p65 subunit raised in rabbit (diluted 1:500 in PBS containing 0.25% BSA) or polyclonal anti-IkBα antibody raised in rabbit (diluted 1:1000 in PBS containing 0.25% BSA)] for 3 h at room temperature. The membrane was then washed with PBST buffer thrice and incubated with anti-rabbit IgG horseradish peroxidase conjugated (1:1000 diluted in PBS containing 0.25% BSA) at room temperature for 1 h. After extensive washing with PBST buffer, the blots were exposed to peroxidase substrate (15 mg 4-chloronaphthol in 5 ml methanol and 20 ml PBS buffer containing 50 μl of 3% hydrogen peroxide).

### 2.11. Enzyme linked immunosorbent assay (ELISA)

The cell free supernatants with or without treatment with the plant extract (300, 500 μg/ml) or the active compound, Rg-001, (10 and 20 μg/ml) were used for measuring the IL-1β protein using IL-1β ELISA kit (BD Biosciences, USA).

### 2.12. Gel retardation assay

Consensus sequence of NF-κB (Gel shift assay system, Promega) was used for gel shift analysis after end labeling of the oligonucleotide with [γ-$^{32}$P] ATP. EMSA was performed with modifications of a previously published procedure (Schreiber et al., 1990). Briefly, 20 μg of nuclear extract was incubated with 80 fmol of $^{32}$P-end labeled double stranded NF-κB oligonucleotide in 2 μl of 5× binding buffer containing 20% glycerol, 5 mM $MgCl_2$, 250 mM NaCl, 2.5 mM EDTA, 2.5 mM DTT, 0.25 mg/ml poly dI–dC and 50 mM Tris–Cl (pH 7.5), and sterile water in a total 10 μl volume. The specificity of protein binding to the DNA was confirmed by competition reactions, in which a 20-fold molar excess of unlabelled oligonucleotides was added to each reaction mixture before the addition of radiolabelled probe. DNA–protein complexes were analyzed by electrophoresis on a 6% native polyacrylamide gel using Tris–glycine buffer, pH 8.5, followed by autoradiography using Fuji FLA-2000 Phosphorimager.

### 2.13. Effect of the active compound on reactive oxygen species

The effect of the isolated active compound Rg-001 on the reactive oxygen species production by the macrophage cells on stimulation with LPS was evaluated using DCF-DA dye. The cell-permeant dye DCF-DA is oxidized by hydrogen peroxide, peroxynitrite ($ONOO^-$), and hydroxyl radicals (OH.) to yield the fluorescent molecule 2′-7′-dichlorofluorescein. The dye oxidation is an indirect measure of the presence of these reactive oxygen intermediates. The cells ($1 \times 10^6$) were plated in 24 well plate and pre-incubated with the diethyl ether fraction (100 μg/ml) and Rg-001 (20 μg/ml) for 2 h. The cells were then washed and challenged with LPS (1 μg/ml) for 16 h. Following LPS stimulation, cells were incubated with 10 μM DCF-DA (Sigma, USA) for 15 min at 37 °C in $CO_2$ incubator. The cells were then washed with warm PBS and analyzed using florescent microscope (FITC filter) and the images were captured.

### 2.14. In vivo effect of the isolated active compound on BALB/c mice

The BALB/c mice were used to check the *in vivo* effect of the active compound Rg-001 on endotoxin-induced inflammation by LPS. BALB/c mice, 6 to 8 weeks of age, weighing 23–25 g, were housed in micro-barrier cages on sterile bedding and fed ad libitum water and food. Different concentrations of LPS were injected to produce septic shock in mice with measurable concentration of NO in sera, without mortality and the dose of 150 μg/25 g body weight was observed to give optimum results.

The dose of active compound was extrapolated according to the ratio of LPS concentration and the compound used in *in vitro* experiments. Accordingly, 20 μg/ml used *in vitro* corresponds to 3 mg/25 g body weight. However, 1 mg/mice dose gave significant effect against endotoxemia produced in mice.

The animals were divided into three groups containing 6 mice in each group. The active compound (1 mg/25 g body weight) was preinjected intra-peritonially (i.p.) into the mice for 2 h. Then LPS (150 μg/mice) was injected i.p. and the mice were kept for 8 h. After 8 h the normal behavior of the treated and untreated mice were observed blindly by a volunteer. The blood was then drawn from each mice using retro-orbital puncture and collected in acetate-citrate-dextrose buffer. The plasma from the blood samples were then collected by centrifuging at 500 *g* for 10 min and stored at −80 °C till nitrite was analyzed using Griess nitrite assay. The total nitrite level in the plasma samples were detected using total nitrite ($NO_2^-/NO_3^-$) estimation kit (R&D systems, USA). Peritoneal macrophages were also collected by peritoneal lavage to study the gene expression of iNOS and IL-1β. The protocols used for the *in vivo* experiments were approved by the Institutional Animal Ethics Committee.

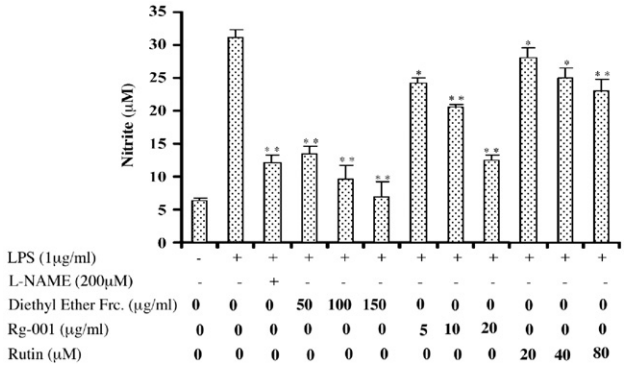


Fig. 3. Nitric oxide level measured as nitrite concentration using Griess nitrite assay in culture supernatant of macrophage cells (J774) pre-incubated for 2 h with diethyl ether fraction (50, 100 and 150 μg/ml) and the active compound Rg-001 (5, 10 and 20 μg/ml) and then challenged with LPS for 16 h. The inhibitory effect of the compounds was compared with only LPS treated cells. The effect of purified rutin (20, 40 and 80 μM) on nitric oxide inhibition was used to compare the efficacy of the isolated compound. (Number of observation, $n=5$). *$P<0.05$, **$P<0.01$.

### 2.15. Scanning densitometry and statistical analysis

Scanning densitometric analysis was performed with Image Scan and Analysis System (Alpha-Innotech Corporation, USA) with Digidoc 1201 software. The values of three separate sets of experiments are expressed as mean ± S.D. The significance of differences from the respective controls was tested using student *T*-test for each paired experiment. $P \leq 0.05$ was considered as significant.

### 2.16. Characterization of the isolated active compound

The purified active compound Rg-001 isolated from the diethyl ether fraction was then characterized using EI-MS, MALDI-TOF, FT-IR and NMR. The sample (Rg-001) was dissolved in MS grade methanol at a concentration of 100 μg/ml for mass estimation. For NMR, the sample (20 mg) was dissolved in deuterated methanol (500 μl) and the analysis was performed using Avance 300 MHz.

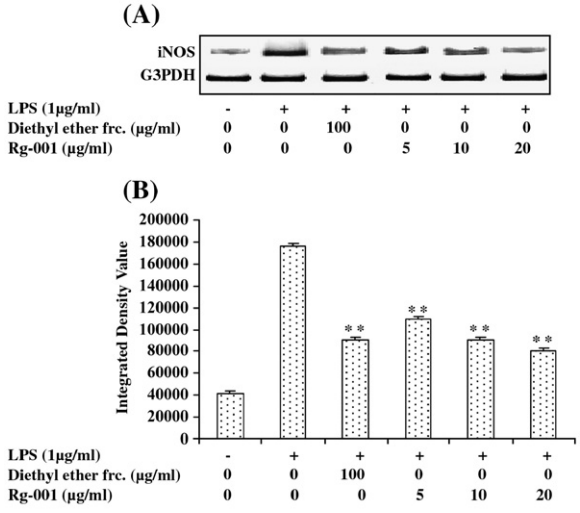


Fig. 4. Effect of diethyl ether fraction (100 μg/ml) and active compound (5, 10 and 20 μg/ml) on LPS-induced iNOS mRNA levels in J774 cells. (A) Gene specific PCR products were analyzed in agarose gel (1.2%). (B) Densitometric analysis with respect to G3PDH gene product was carried out as described in Material and methods. (Number of observations, $n=3$.) The significance was calculated with respect to the only LPS treated cells. **$P<0.01$.

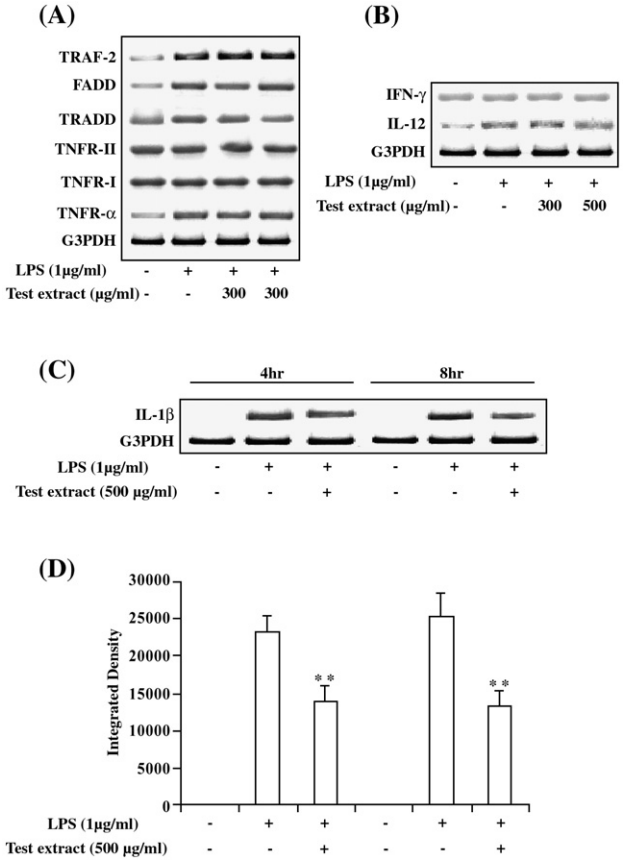


Fig. 5. Gene expression profile of pro-inflammatory genes in murine macrophages treated with or without whole plant extract and challenged with 1 μg/ml LPS for 4 h. (A) Gene expression profile of TNF-α and its associated signaling intermediates leading to apoptosis/proliferation, (B) expression profile of IL-12 and IFN-γ in murine macrophages. G3PDH was used as housekeeping gene. (C) Effect of plant extract on IL-1β gene expression in murine J774 macrophages. (D) Densitometric analysis of the IL-1β PCR products observed in agarose gel. (Number of observations, $n=3$.) The effect was compared with that of only LPS treated control. **$P<0.01$.

### 2.17. Statistics

The values of three separate sets of experiments are expressed as mean ± S.D. The significance of differences from the respective controls was tested using student *T*-test for each paired experiment. $P<0.05$ was considered as significant. *$P<0.05$, **$P<0.01$.

## 3. Results

### 3.1. Fractionation of 50% methanol extract of R. graveolens L.

Methanol extract was subfractionated using different solvents and four fractions were obtained. The yield obtained for

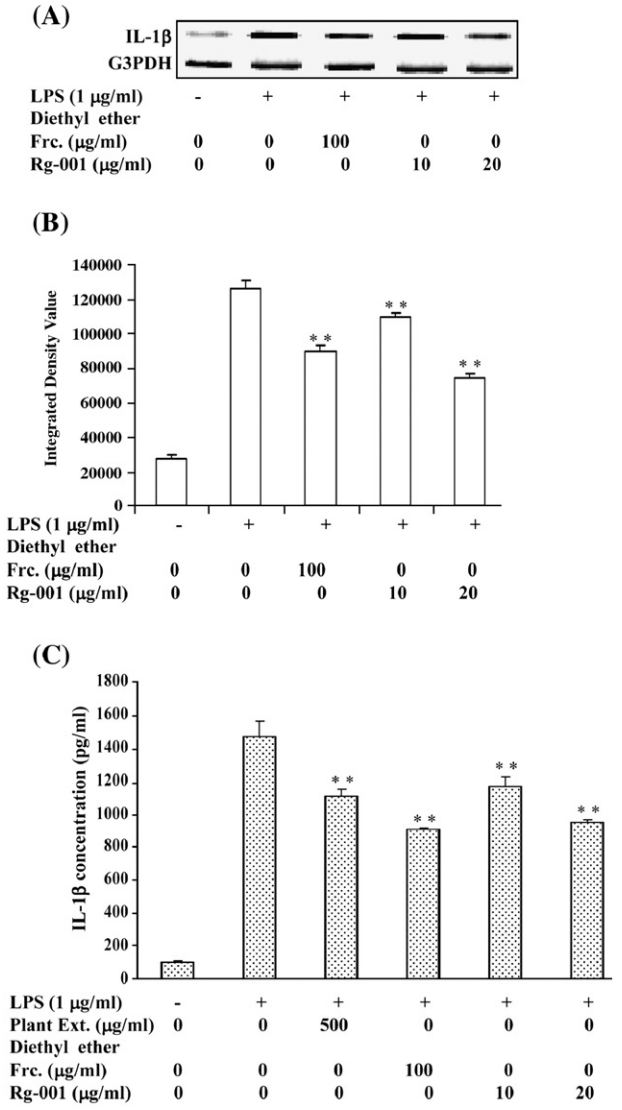


Fig. 6. Effect of diethyl ether fraction (100 μg/ml) and active compound (10 and 20 μg/ml) on LPS-induced inhibition of IL-1β gene expression in murine J774 macrophages. (A) Gene specific PCR products were analyzed in agarose gel (1.2%). (B) Densitometric analysis with respect to G3PDH gene product was carried. (C) Concentration (pg/ml) of IL-1β cytokine in the culture supernatant of macrophage cells treated with or without the whole plant test extract (500 μg/ml), diethyl ether fraction (100 μg/ml) or the active compound (10 and 20 μg/ml) and then challenged with LPS (1 μg/ml) as measured by ELISA. (Number of observations, $n=3$.) Effect was compared with that of only LPS treated control. $^{**}P<0.01$.

diethyl ether, chloroform and ethyl-acetate fractions were 0.31%, 0.11% and 0.26% respectively. Only diethyl ether fraction was observed to inhibit the nitric oxide production by LPS stimulated macrophage cells significantly (70% at 100 μg/ml concentration, Fig. 3). The diethyl fraction was further fractionated by RP-HPLC and thirteen peaks were observed as shown in Fig. 1.

### 3.2. Effect of isolated fractions on nitric oxide production

Thirteen fractions obtained after RP-HPLC were screened for their effect on nitric oxide production on LPS activated macrophage cells and fraction VI (Rg-001, Fig. 1) showed significant inhibition. The retention time (22.582 min) of Rg-001 was distinct from that of quercetin (18.848) or rutin (5.020) as shown in Fig. 2.

Rg-001 was found to inhibit the nitric oxide production significantly (23, 34 and 62%) when the cells were pre-incubated with the purified compound (5, 10 and 20 μg/ml respectively) for 2 h and then challenged with LPS (1 μg/ml, Fig. 3). L-NAME was used as positive control in each experiment. Rutin, present in the plant (known to inhibit the nitric oxide production) was used at 20, 40 and 80 μM concentrations for comparison with the isolated active compound. Both Rg-001 as well as the diethyl ether fraction were observed to inhibit the iNOS gene significantly ($P<0.05$, Fig. 4A and B).

Although the whole plant extract showed inhibitory effect on COX-2 gene expression, but no significant effect was observed with either diethyl ether fraction or the isolated active compound (data not shown).

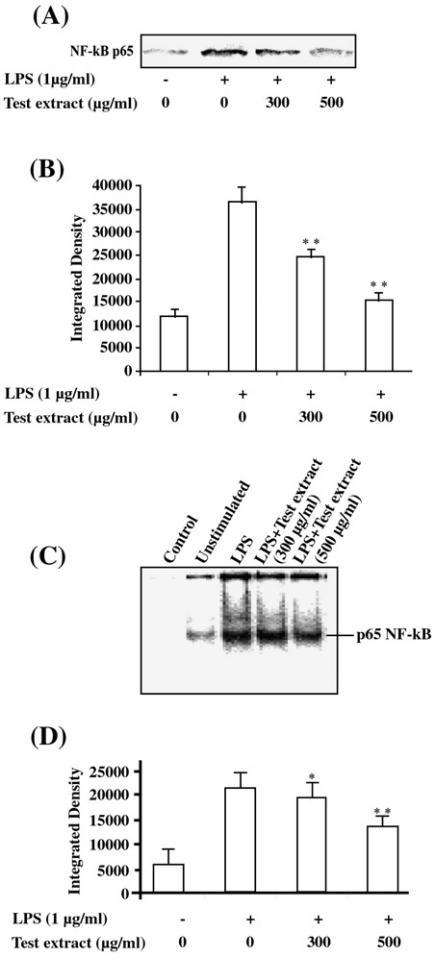


Fig. 7. Effect of the plant extract on LPS inducible NF-κB activation. (A) Western blot analysis of NF-κB using polyclonal antibodies against p65 subunit. Nuclear extract was isolated from cells treated with or without the plant extract and then challenged with LPS (1 μg/ml) for 30 min. (B) Densitometric analysis of the bands obtained after Western blot. (C) Gel shift analysis of nuclear extracts was performed using the consensus sequence of NF-κB as described in Materials and methods. Each lane contained 20 μg of nuclear extracts. (D) Densitometric analysis of the gel shift bands. The effect was compared with that of only LPS treated control. (Number of observations, $n=3$.) $^{*}P<0.05$, $^{**}P<0.01$.

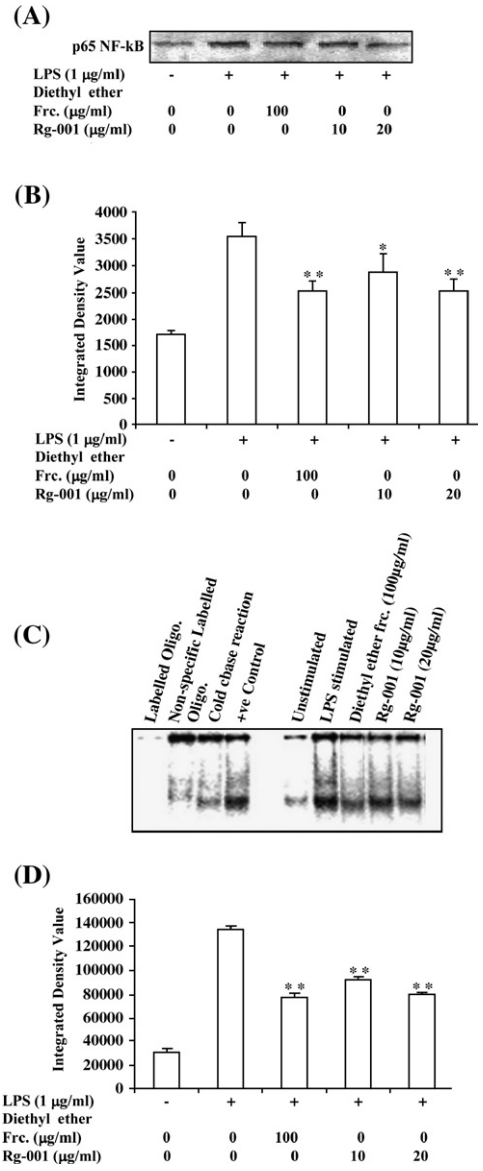


Fig. 8. Effect of diethyl ether fraction and the active compound Rg-001 on LPS-inducible NF-κB activation. (A) Western blot of p65 subunit of NF-κB in J774 macrophages pre-incubated with the diethyl ether fraction (100 μg/ml) and Rg-001 (10 and 20 μg/ml) for 2 h and then challenged with LPS (1 μg/ml) for 30 min. (B) Densitometric analysis of the bands observed in the Western blot, (C) gel mobility shift assay of nuclear extract from diethyl ether fraction and the compound treated or untreated macrophages challenged with LPS for 30 min, (D) densitometric analysis of the bands observed in the gel shift assay. The effect was compared with that of only LPS treated control. (Number of observations, $n=3$.) $^{*}P<0.05$, $^{**}P<0.01$.

### 3.3. Effect on LPS inducible IL-12, IFN-γ, IL-1β, TNF-α and its signaling pathway intermediate genes (TNFR-I, TNFR-II, TRADD, FADD, TRAF-2)

The gene expression of TNF-α gene and those involved in its signaling (leading to apoptosis or proliferation of cells) was also performed for the murine macrophage cells with or without treatment with the whole plant extract (300 and 500 μg/ml). G3PDH gene was kept as housekeeping control. No significant difference ($P>0.05$) was observed for TNF-α or any of the TNF-α signaling genes when compared with the untreated control (Fig. 5A).

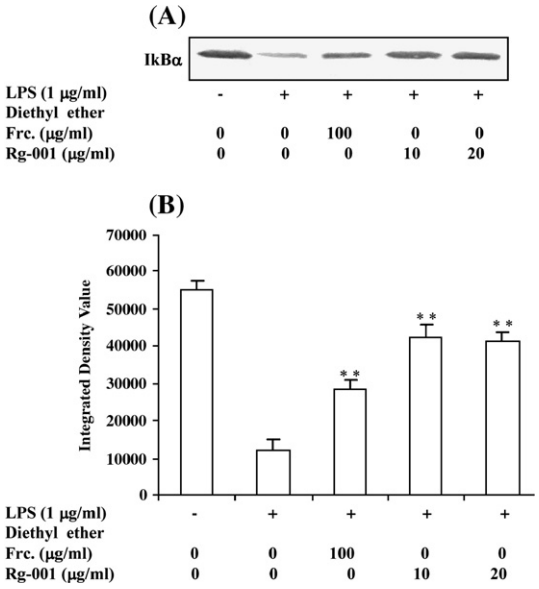


Fig. 9. Effect of the diethyl ether fraction and Rg-001 on activation of IkBα. (A) Western blot analysis of the IkBα in J774 macrophages with or without treatment with the diethyl ether fraction (100 μg/ml) or the active compound (10 and 20 μg/ml) for 2 h and then challenged with LPS (1 μg/ml) for 30 min, (B) densitometric analysis of the bands observed in the blot. ($n=3$.) The comparison was performed with that of only LPS treated control. $^{**}P<0.01$.

The expression of IL-12 and IFN-γ genes was analyzed but no significant effect on their expression was observed after treatment of the cells with the whole plant extract Fig. 5B).

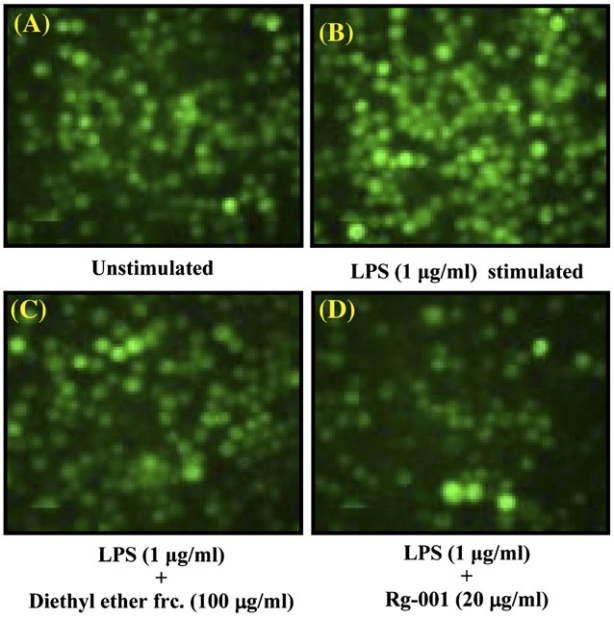


Fig. 10. Effect of the diethyl ether fraction and the active compound Rg-001 on the reactive oxygen species using DCF-DA dye. (A) Unstimulated J774 macrophages, (B) macrophages challenged with LPS (1 μg/ml) for 16 h, (C) macrophages pre-incubated with the diethyl ether fraction (100 μg/ml) and then challenged with LPS, (D) macrophages pre-incubated with the active compound Rg-001 (20 μg/ml) for 2 h following LPS stimulation. The decrease in the green florescence inside the macrophages in comparison to the only LPS treated cells is proportional to decrease in reactive oxygen species production.

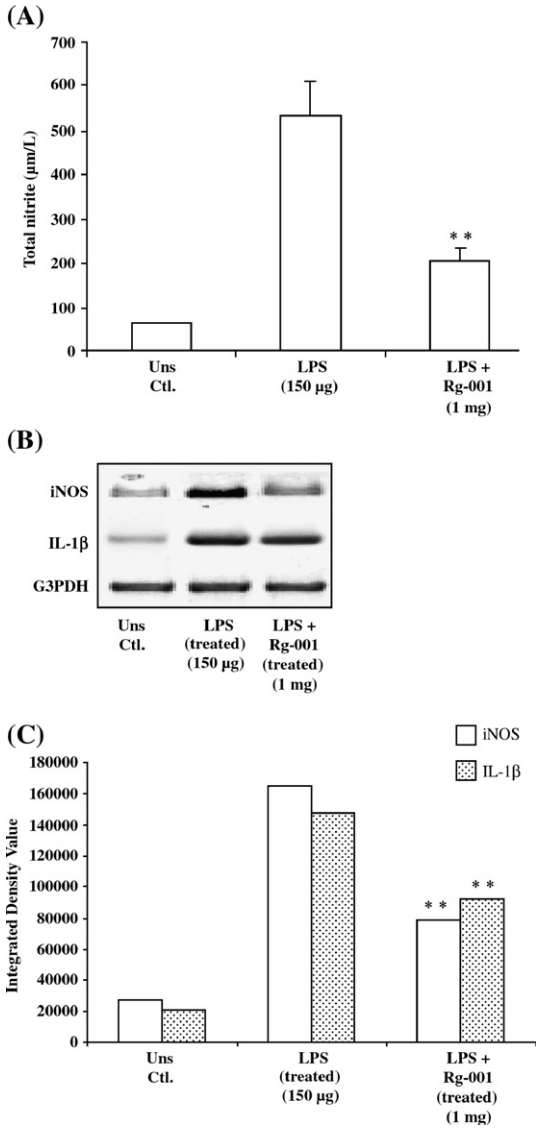


Fig. 11. Effect of the active compound Rg-001 on the total nitrite production and gene expression of iNOS and IL-1β in BALB/c mice. The six mice (23–25 g) were taken in each group. The mice in the treated group were preinjected (i.p.) with the compound (1 mg/25 g body weight) in phosphate buffered saline (PBS) for 2 h and then challenged with LPS (150 μg/mice) for 8 h. (A) The levels of total nitrite concentration in the plasma of compound treated mice were compared with that of LPS challenged mice (positive control). Negative control group of mice was injected with PBS only. (B) Agarose gel (1.2%) showing the iNOS and IL-1β gene expression in peritoneal macrophages. (C) Densitometric analysis of the bands observed for iNOS and IL-1β gene expression, G3PDH was used as housekeeping control. $^{**}P<0.01$.

However, the expression of IL-1β gene was found to be significantly ($P<0.01$) inhibited by the whole plant extract (500 μg/ml) (Fig. 5C and D). The inhibition was observed to be 40% and 48% after 4 h and 8 h of LPS treatment respectively. As the whole plant extract was observed to inhibit the IL-1β gene, the effect of diethyl ether fraction and the purified compound on IL-1β gene expression was also checked. This important pro-inflammatory gene was found to be significantly inhibited ($P<0.05$) by the diethyl ether fraction (100 μg/ml) and the isolated active compound (10 and 20 μg/ml) (Fig. 6A). The inhibition was clearly evident from the densitometric analysis of the PCR products obtained (Fig. 6B). The decrease in the level of IL-1β protein in the culture supernatant was confirmed by ELISA (Fig. 6C).

### 3.4. Effect of whole plant extract, diethyl ether fraction and the active compound (Rg-001) on LPS inducible NF-κB

Activation of NF-κB is critical for induction of both iNOS and IL-1β genes. We determined whether the plant extract suppresses NF-κB activation in LPS-stimulated macrophages (Fig. 7A and B) by immunoblotting using antibody against p65 subunit. Densitometric analysis showed that the whole plant extract inhibited translocation of NF-κB by 33% and 58% with 300 μg/ml and 500 μg/ml concentration respectively.

Incubation of J774 cells with 1 μg/ml LPS for 30 min increased NF-κB binding activity ~4 fold as determined by EMSA. Induction of NF-κB binding activity by LPS was markedly inhibited (18% and 38%) by whole plant extract in a dose dependent manner (Fig. 7C and D).

The NF-κB activation/translocation was also significantly ($P<0.05$) suppressed by the diethyl ether fraction and the isolated active compound, Rg-001 (Fig. 8A and B) as observed by immunoblotting. This was also confirmed by the gel mobility shift assay (Fig. 8C and D). The addition of excess unlabeled consensus oligonucleotide completely prevented the band shifts demonstrating the specificity of the protein–DNA interaction.

The activation/translocation of NF-κB in the nuclear extract correlates with proteolytic degradation of IkB. Presence of significantly ($P<0.01$) increased amount of IkB in the cytosol of the compound treated cells as compared to the only LPS stimulated cells indicated the prevention of IkB degradation by the active compound as shown in Fig. 9A and B.

### 3.5. Anti-oxidant property of diethyl ether fraction and Rg-001

The cells produce large amounts of reactive oxygen species on stimulation with LPS and play an important role in the regulation of cell survival and formation of peroxynitrite by reacting with nitric oxide. It is clearly visible from the Fig. 10 that the diethyl ether fraction and the active compound significantly inhibited the

Table 2
Chemical shift values (ppm) observed in the $^{1}H$ and $^{13}C$ spectrum of the compound Rg-001

| Proton | Shift value (ppm) | Carbon | Shift value (ppm) |
|---|---|---|---|
| H-4 | 7.97 (d) | C-3 | 117.81 |
| H-5 | 7.36 (d) | C-4 | 121.26 |
| H-8 | 7.81 (d) | C-5 | 111.50 |
| H-2′ | 6.01 (s) | C-6 | 122.08 |
| H-3′ | 4.49 (s) | C-7 | 142.45 |
| $2CH_3$ | 1.28 (t) | C-8 | 94.19 |
| | | C-9 | 152.23 |
| | | C-10 | 113.67 |
| | | C-1′ | 33.85 |
| | | C-2′ | 130.69 |
| | | C-3′ | 104.54 |
| | | $2CH_3$ | 27.45 |
| | | $OCH_3$ | 55.05 |
| | | CO | 162.83 |

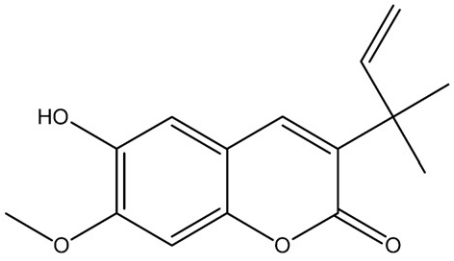


Fig. 12. The deduced structural formula of the active compound Rg-001 and its IUPAC name.

generation of reactive oxygen species by the LPS. The green colored florescence emitted by the cells (oxidized DCF-DA dye) is directly proportional to the reactive oxygen species present in the cells.

### 3.6. Cytotoxicity assay

The macrophage cell viability was observed to be greater then 85% at the highest concentration of the diethyl ether fraction (200 μg/ml) and the active compound Rg-001 (50 μg/ml).

### 3.7. In vivo effect of the active compound on endotoxemia-induced BALB/c mice

The compound was observed to inhibit the iNOS and IL-1β gene *in vitro* through NF-κB suppression that makes it important to be analyzed *in vivo* on an animal model of endotoxin-induced inflammation/septic shock. The compound treated mice were observed to show normal behavioral condition and locomoter activity. The total nitrite concentration was significantly decreased (75%) in the plasma samples of compound treated mice in comparison to LPS challenged group (Fig. 11A).

The RT-PCR analysis for the iNOS and IL-1β gene using the peritoneal macrophages isolated from compound treated mice group showed a significant ($P<0.05$) decrease in the expression of the respective genes as compared to controls (Fig. 11B and C).

### 3.8. Characterization of the purified active compound

IR spectrum of Rg-001 shows –C═O stretching frequency at 1658.67 $cm^{-1}$ and –OH at 3434.95 $cm^{-1}$. The EI mass spectrum of the compound showed the $M^+$ at m/z value of 260.0049 corresponding to the formula $C_{15}H_{17}O_4$. The MALDI-TOF of the compound shows the base peak at 259.477 confirming the mass of the compound by EI-MS. The chemical shift values (ppm) for $^1H$ and $^{13}C$ spectrum of the compound are shown in Table 2. The active compound was identified as 3-(1′,1′-dimethyl-allyl)-6-hydroxy-7-methoxy-chromen-2-one (IUPAC name) having structural formula as shown in Fig. 12.

## 4. Discussion

Different herbs have been described in texts that are being used as anti-microbial, anti-inflammatory and anti-viral medicine to cure allergies, rheumatoid arthritis, infections, wound healing, fever and so on (Borchers et al., 2000). Rue (*R. graveolens* L.) extract has a long history of medicinal usage in homeopathy and traditional medicine worldwide. However, there is a considerable controversy about such use owing to the reported toxicity like uterine bleeding, photo-dermatitis etc. (el Agraa et al., 2002; Gawkrodger and Savin, 1983). We described here an anti-inflammatory property of a purified compound 3-(1′-1′-dimethyl-allyl)-6-hydroxy-7-methoxy-coumarin isolated from the extract of rue.

Rue contains various active compounds like flavonoids, coumarine derivatives, furoquinolines, volatile oils, undecanone and others (Pathak et al., 2003). Rutin, one of the flavonoid constituents of rue, is well known for its nitric oxide scavenging activity. Recently it has been reported that rutin decreases nitric oxide (measured nitrite level) along with the reduction in the iNOS protein in BALB/c mice pretreated with lipopolysaccharide (Shen et al., 2002), but no effect on *in vitro* LPS stimulated murine macrophages (RAW 264.7). However, a related flavonoid quercetin (a derivative of rutin devoid of a glycan called rutinose) has been found to bring forth such decrease in both *in vivo* and *in vitro* conditions. The RP-HPLC analysis of the diethyl ether fraction clearly shows the absence or the negligible presence of rutin or quercetin in the diethyl ether fraction from which the active compound was isolated. Hence, it is clear that the inhibition of nitric oxide production is due to isolated active compound in the diethyl ether fraction and the active compound distinctly different from rutin.

In our earlier study (Raghav et al., 2006b), whole plant extract of rue was observed to inhibit the nitrite level in LPS challenged murine macrophage cells (J774) and the inhibition was much more significant than that with pure rutin. The diethyl ether fraction isolated from the methanol extract and the active compound isolated from the said fraction also significantly inhibited the nitric oxide production. Diethyl ether fraction and the active compound Rg-001, showed a reduction in COX-2 gene expression but the inhibition was not significant as observed with the whole plant extract. Inhibitory effect of plant extract was not found for IL-12, IFN-γ, TNF-α or its signaling pathway intermediate genes. However, expression of IL-1β gene was significantly inhibited by the whole plant extract as well as its diethyl ether fraction and the purified compound.

To the end of the signaling generated by LPS in macrophages, the activation of transcription factors occurs resulting in the production of both pro- and anti-inflammatory mediators. The binding of LPS to (toll like receptor-4) TLR-4 leads to activation of transcription factor NF-κB, through myeloid differentiation factor 88 (Myd 88), TNF receptor associated factor-6 (TRAF-6) and NF-κB inducing kinase (NIK) as signaling intermediates, which widely regulates innate immune responses (Zhang and Ghosh, 2001). Activation of NF-κB has been found to induce the expression of several inflammatory mediators such as iNOS, IL-1β and COX-2 along with many other genes (Lee et al., 2003b). IL-1β has been shown to induce chondrocytes to produce several types of reactive oxygen species, including $H_2O_2$ (Rathakrishnan et al., 1992) and hydroxyl (Tiku et al., 1998) and superoxide (Tawara et al., 1991) radicals. Furthermore, reactive oxygen species have

been shown to be required for IL-1β-induced NF-κB activation and iNOS expression (Mendes et al., 2001). It is also reported that extracellular stimuli such as reactive oxygen species signal the degradation and release of inhibitory unit IkBα through a rapid but complex cascade of events resulting in a rapid translocation of NF-κB to the nucleus (Baldwin, 1996).

Several plant derived coumarins such as 5, 7-dihydroxy-4-methylcoumarin and 7, 8-Dihydroxy-4-methylcoumarin were known to inhibit the activation of NF-κB (Pande and Ramos, 2005). Therefore, the isolated active compound from *R. graveolens* plant, adds a new compound to the list that inhibits the pro-inflammatory mediators like NO and IL-1β through suppression of NF-κB activation.

Increased expression of iNOS is associated with inflammatory responses and also with serious disorders such as septic shock and rheumatoid arthritis (Salerno et al., 2002). In view of the involvement of iNOS in inflammatory processes, we have already checked and reported the inhibition of iNOS gene in macrophages by the *R. graveolens* L extract (Raghav et al., 2006b). Suppression of iNOS was in parallel with the inhibition of NO production.

NF-κB binding site is present in the promoter region of murine iNOS gene and play a pivotal role in LPS mediated induction of iNOS in J774 cells. The present study demonstrated that crude extract of *R. graveolens* L. plant, the diethyl ether fraction and the isolated active compound repress activation of p65/NF-κB by LPS in macrophage cells by inhibiting the activation of IkBα and effectively suppress nuclear translocation of NF-κB.

Macrophages secrete inflammatory mediators including lipid metabolites (e.g. prostaglandins) and other cytokines. COX-2 catalyzes the inducible production of prostaglandins, which clearly represents an important step in the inflammatory process (Wadleigh et al., 2000). The *cis*-acting elements identified on the promoter region of murine COX-2 include NF-κB, C/EBP and CREB (Caivano et al., 2001). The percent inhibition of COX-2 gene indicates that other transcription factors also play an important role in the transcription of this gene. It is also reported that putative NF-κB is not required for the induction of COX-2 by LPS in murine macrophages (Wadleigh et al., 2000) and the C/EBP element is believed to play a critical role in the induction of COX-2 in macrophages (Thomas et al., 2000). A significant inhibition of NF-κB with no significant inhibition of COX-2 gene may therefore indicate a critical role of other transcription factor in regulation of the COX-2 gene.

IL-1β is an important cytokine, which is involved in inflammation and other pathological processes such as rheumatoid arthritis. IL-1β is an important target as it leads to complex cascades along with other mediators such as TNF-α and NO leading to severity in inflammatory disease. NF-κB and C/EBP transcription regulator consensus sites are present in the promoter region of IL-1β gene (Basak et al., 2005). NF-κB activation requires the classical TLR-4 initiated signaling cascade leading to IkB phosphorylation and its degradation (Basak et al., 2005). Since, NF-κB is important for the regulation of IL1-1β gene transcription, the significant inhibition of this cytokine *via* NF-κB inhibition may result in blockade of several inflammatory cascades stimulated by IL-1β. It was clear from the study that the diethyl ether fraction and the active compound Rg-001 present in the plant extract resulted in the inhibition of IL-1β gene through the suppression of NF-κB activation.

The inflammatory disorders are very much associated with the oxidative stress generated by the cells present in the milieu (Haskard, 2004), which can further complicate the pathology. Nitric oxide in excess, produced by the up-regulated iNOS is notoriously involved in such interaction leading to formation of more potent peroxynitrites. This can target multiple proteins to alter their functions to induce cell proliferation and can intervene with normal repair process (Guzik et al., 2003). It was observed that the diethyl ether fraction and the active compound reduced the generation of reactive oxygen species that might result in decreased superoxide radicals thereby resulting in decreased production of peroxynitrite. The isolated active compounds therefore, might result in decreased cytotoxicity induced by the peroxynitrite. This anti-oxidant property may also contribute to inhibition of LPS-induced phosphorylation and degradation of IkB. Therefore, it can be speculated that the IkB remained bound to NF-κB and prevented NF-κB translocation to the nucleus.

Regulation of iNOS and IL-1β is known to occur predominantly on the transcription level, whereby the transcription factor NF-κB plays a crucial role (Heiss et al., 2001). NF-κB is activated in cells challenged with LPS and other inflammatory stimuli and involved in the transcriptional activation of responsive genes (Baldwin, 1996). Previous studies have indicated that NF-κB was activated at 30 min to 1 h after LPS treatment (Kim et al., 2000). In the present study NF-κB translocation was also measured after 30 min induction and was found to show effective translocation by EMSA.

The *in vivo* inhibitory effects on IL-1β gene and nitric oxide production through iNOS gene in the LPS-induced endotoxemia in BALB/c mice using the active compound isolated from the diethyl fraction of the plant clearly demonstrates the anti-inflammatory potential of the plant and the isolated compound.

Hence, the present study with *R. graveolens* L. unravels a novel molecular mechanism working behind the anti-inflammatory effects of the plant.

## Acknowledgements

Authors like to thank Dr. Mahak Singh, Delhi University for his help in elucidating the structure of active compound and Mr. Rajeev Ranjan, M. Sc. for helping in performing the *in vivo* experiments. The IR and EI-MS/MS studies were performed at Delhi University and the NMR and MALDI were performed at National Institute of Pharmaceutical Education and Research (NIPER), Chandigarh.

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