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Rohitukine inhibits NF-κB activation induced by LPS and other inflammatory agents

Amarinder Singh[a,b,*], Pankaj Chibber[a,b], Praveen Kolimi[f], Tanveer Ahmad Malik[d], Nitika Kapoor[c], Amit Kumar[b], Narinder Kumar[e], Sumit G. Gandhi[b,c], Surjeet Singh[a,b], Sheikh Tasduq Abdullah[d], Ram Vishwakarma[a,b], Gurdarshan Singh[a,b,**]

[a] Academy of Scientific & Innovative Research (AcSIR), New Delhi, India
[b] PK-PD-Toxicology and Formulation Division, CSIR-Indian Institute of Integrative Medicine, Canal Road, Jammu 180001, J & K, India
[c] Plant Biotechnology Division, CSIR-Indian Institute of Integrative Medicine, Canal Road, Jammu 180001, J & K, India
[d] Cancer Pharmacology, CSIR-Indian Institute of Integrative Medicine, Canal Road, Jammu 180001, J & K, India
[e] Govt. Science College, Canal Road, Jammu 180001, J & K, India
[f] NIPER, Ahmedabad, India

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ABSTRACT

Rohitukine (referred to as RHK) is a bioactive chromone alkaloid isolated from the leaves of plant *Dysoxylum binectariferum*, which has been reported to possess diverse pharmacological properties for the treatment of inflammatory bowel disease (IBD), diarrhoea and anti-lipidemic. However, the underlying mechanism by which RHK exerts its anti-inflammatory activity has not yet demonstrated. This study aimed to elucidate the anti-inflammatory mechanism of RHK using lipopolysaccharide (LPS) - stimulated J774A.1 macrophage cells and in-vivo inflammatory models. Results demonstrated that RHK treatment could significantly decrease the LPS-induced production of nitric oxide, prostaglandin E2 (PGE2), interleukins (ILs) and tumour necrosis factor (TNF)-α in J774A.1 cells. Molecular studies revealed that RHK inhibited the activation of upstream mediator nuclear factor-κB by suppressing the phosphorylation of IκBα and p65. In in-vivo experiments showed prominent anti-inflammatory activity of RHK. Thus, RHK could be considered as a promising candidate for the treatment of inflammatory diseases.

1. Introduction

Inflammation is a primary complex biological response of the body towards any unwanted stimuli due to invading pathogens, irritants or necrotic cells and so on. It involves the increase in the vascular permeability, blood flow and oedema leading to migration of leukocytes and inflammatory cytokines at the site of injury/stimulus [1]. The purpose of the inflammatory process is to eradicate noxious stimuli, necrotic cells and propagate tissue repair. However, persistent uncontrolled inflammation may precipitate chronic diseases like asthma [2], rheumatoid arthritis [3], ulcerative colitis [4], atherosclerosis [5], pulmonary fibrosis [6], cancer [7], Crohn's disease [8], chronic hepatitis [9] and psoriasis [10]. It is an innate immune response involving immune cells (Macrophages, dendritic cells, Kupffer's cells). Macrophages have a crucial role in the initiation, maintenance and remission of inflammation. Activation of macrophages takes place under the influence of inflammatory cytokines like interferon-gamma, TNF-α, bacterial lipopolysaccharide, various chemical mediators and extracellular matrix proteins, which further initiate the process of phagocytosis and release of other inflammatory mediators like reactive oxygen species, IL-6, TNF-α, nitric oxide, prostaglandin E2 etc. [11].

Gram-negative bacteria cell membrane consists of Lipopolysaccharide (LPS), which is a common endotoxin evading in the environment. LPS binds to TLR4 receptor expressed on epithelial cells, activates nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) downstream signalling pathways via myeloid differentiation factor 88 (MyD88), results in a release of large amounts of pro-inflammatory cytokines by macrophages. In order to mitigate foreign pathogens, these cytokines further recruit neutrophils in the interstitial space, followed by free radicals and reactive oxygen species

* Correspondence to: A. Singh, Discovery Biology, Syngene International Pvt. Ltd., Bangalore, India.
** Correspondence to: G. Singh, PK-PD-Toxicology and Formulation Division, CSIR-Indian Institute of Integrative Medicine, Canal Road, Jammu 180001, J & K, India.
E-mail addresses: amarinder_pharma@yahoo.co.in (A. Singh), singh_gd@iiim.ac.in (G. Singh).

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(ROS). However, this release worsens the condition leading to oedema, pain, necrosis and finally the cell/tissue damage.

Cytokines like IL-1, IL-6, TNF-α, nitric oxide and reactive oxygen species released via LPS stimulation causes prolonged activation NF-κB transcription factor, which further leads to the development of chronic inflammatory diseases. Suppression of aberrant activation of macrophages may have valuable therapeutic potential for the treatment of inflammation-related disorders. Therefore, LPS induced inflammation in macrophage becomes the most widely used model to investigate the anti-inflammatory potential of lead candidates and their mechanism.

The modern scientific trend focused on the use of natural resources for the remission of human ailments. Thus, researchers are more concerned to identify plant-based molecules for their therapeutic potential. Therefore, in this direction, we have investigated the mechanism behind the anti-inflammatory activity of Rohitukine (RHK) which is a plant-based molecule from *Dysoxylum binectariferum* (Meliaceae). *Dysoxylum binectariferum* has been reported to possess various therapeutic activities like contraceptive [12], antidyslipidemic [13], leishmanicidal [14] and inflammatory bowel disorder [15].

RHK is considered to be major active constituent of the plant *Dysoxylum binectariferum*; thus, it possesses various activities like anticancer [16], lipid-lowering [17], antioxidant [18], and gastroprotective [19]. Limited scientific literature is available on the anti-inflammatory effect of RHK, particularly on NF-κB activation, this is the first study to evaluate the effect of RHK in modulating the expression of pro-inflammatory cytokines like IL-1 beta, IL-6, TNF-α, PGE2 and NFκB.

In the present study, we have evaluated the effects of RHK on the modulation of macrophage response to bacterial LPS, which is a well-established, characterised, frequently used model for the mechanistic elucidation of anti-inflammatory effect in vitro.

Therefore, the aim of this study was to investigate whether RHK has an anti-inflammatory ability, through both in-vitro and in-vivo experiments: one using endotoxin-induced macrophage cells, and the other using an endotoxin-induced proinflammatory cytokine production, acetic acid induced pleurisy, carrageenan induce paw oedema, leukocyte migration assay to assess the efficacy of RHK and elucidate the possible anti-inflammatory mechanism.

2. Material and method

Rohitukine (RHK; purity > 98%) was isolated from leaves of *Dysoxylum binectariferum*. J744A.1 cell line was purchased from ATCC. 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide (MTT), 4′,6-diamidino-2-phenylindole (DAPI), Dimethyl sulfoxide (DMSO), and LPS (*Escherichia coli* 055:B5) were procured from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), penicillin and streptomycin, Triton X-100, Mouse Enzyme-linked Immunosorbent Assay (ELISA) were purchased from Invitrogen-Gibco (Grand Island, NY, USA). The goat polyclonal COX-2, goat polyclonal iNOS and rabbit polyclonal NF-κB antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Freund's Complete Adjuvant (FCA) containing 10 mg/mL heat-killed Mycobacterium tuberculosis was procured from Sigma Aldrich Co. USA.

2.1. Plant materials

The dried leaves of *D. binectariferum* (Meliaceae) collected from Forest Research Institute (FRI), Dehradun. A voucher specimen was deposited at the herbarium of CSIR-IIIM Jammu. Isolation of RHK was carried out according to Mahajan V et al. [20]. Briefly, 100 g of shade-dried leaves were taken and powdered. Ultrasonic Extraction was done with 50% ethanol three times (300 mL) at 45 °C with half an hour intervals. The extract was filtered, and the filtrate was evaporated to get the crude suspension, which was further extracted thrice with ethyl acetate (300 mL) in separating funnel by vigorous shaking. The aqueous layer was collected and basified using ammonium hydroxide (29%) to pH 10, followed by extraction with n-butanol. Rohitukine (RHK; 1 g) was obtained by precipitating n-butanol fraction with methanol (percentage yield from leaves is 1%). The structure was confirmed by comparing the of NMR spectroscopic data with previously published spectra [21]. The purity was assessed using HPLC analysis (Fig. 1).

2.2. Preliminary experiment

2.2.1. Evaluation of anti-inflammatory activity of RHK using mouse peritoneal macrophage

3 mL of 3% sodium thioglycollate medium was injected intraperitoneally to harvest Murine macrophages from the peritoneal cavities of BALB/c mice. The cells were transferred to sterile polypropylene conical centrifuge tubes kept on ice and centrifuged at 200g/min for 10 min at 5 °C. Cells were resuspended in DMEM supplemented with 10% fetal bovine serum (FBS; complete medium). Cells were counted in a hemocytometer, and the suspension was adjusted to 1 × 106 cells/mL. Different concentrations of RHK, dexamethasone and methotrexate were tested to verify their influence on the in vitro anti-inflammatory effects on primary mammalian cells. Cells were stimulated with 50 μL of culture medium or a solution of LPS (1 μg/mL). The plates were incubated for an additional 48 h before being used for the proliferation, NO and cytokines assays.

2.3. MTT assay for cell viability

Cell viability was determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction assay. In brief, J774A.1 cells were pre-incubated overnight in 96-well plates at a cell density of 1 × 105 cells per well. After 24 h, cells were treated with various concentrations of RHK (0–100 μM), dexamethasone (10 μM) and methotrexate (0.1 and 1 μM) with or without LPS (1 μg/mL) for another 24 h. After 24 h of incubation, 20 μl of MTT (0.5 mg/mL in PBS) solution was added. The cells were incubated for 4 h at 37 °C, after that the supernatant was removed and 100 μl of dimethyl sulfoxide (DMSO) was added to each well, to dissolve formazan crystals formed after MTT addition. The optical density was measured at 540 nm using Tecan infinite M200 PRO microplate reader. The results were expressed as a percentage of cells viability.

2.4. NO assay

The nitrite concentration as measured in the cell supernatant as an indicator of NO production Using Griess reaction. In order to evaluate the effect of RHK on NO production, J774A.1 cells were treated with or without LPS (1 μg/mL) in the presence of various concentrations of RHK (3, 10, 30 μM, 1 h before LPS treatment). Different concentration of dexamethasone (1, 3, 10 μM) and methotrexate (0.05, 0.1, 1 μM) were used as positive control, respectively to select the effective dose for further experimentation. After 18 h of incubation, the supernatant was collected. 100 μl of supernatant was mixed with 100 μl of Griess reagent (1:1 mixture of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphthyl ethylenediamine-HCL), followed by incubation at room temperature for 10 min in the dark. Resultant absorbance was measured at 550 nm, and NO concentration was calculated with reference to the standard concentration curve of sodium nitrite [22]. Results are expressed as a measure of concentration in NO (μM).

2.5. ROS assay

The generation of intracellular ROS was determined using dichlorofluorescein-diacetate (DCFDA) fluorescent dye. J774A.1 macrophages cells (5 × 105 cells/well) were seeded in 6-well plates for 24 h before treatment. After 24 h, cells were treated with LPS (1 μg/mL) alone and in presences of RHK at 3, 10, 30 μM, dexamethasone (10 μM)

and methotrexate (0.1 μM) for another 18 h. Hydrogen peroxide (125 nM) was used as positive control. Samples were incubated in the dark with 10 nM of DCFDA dye for 30 min. After that, cells were washed with phosphate-buffered saline and fluorescence was analysed using an Olympus X41 microscope at excitation and emission wavelengths of 485 nm and 535 nm, respectively [23].

2.6. Enzyme-Linked Immunosorbent Assay

J774A.1 cells were pre-incubated in 24-well plates (4 × 105) for 24 h. Cells were pretreated with RHK (3, 10, or 30 μM) 1 h before LPS (1 μg/mL) stimulation at 37 °C, 5% CO_2 incubator for 18 h. Dexamethasone (10 μM) and methotrexate (0.1 μM) were used as

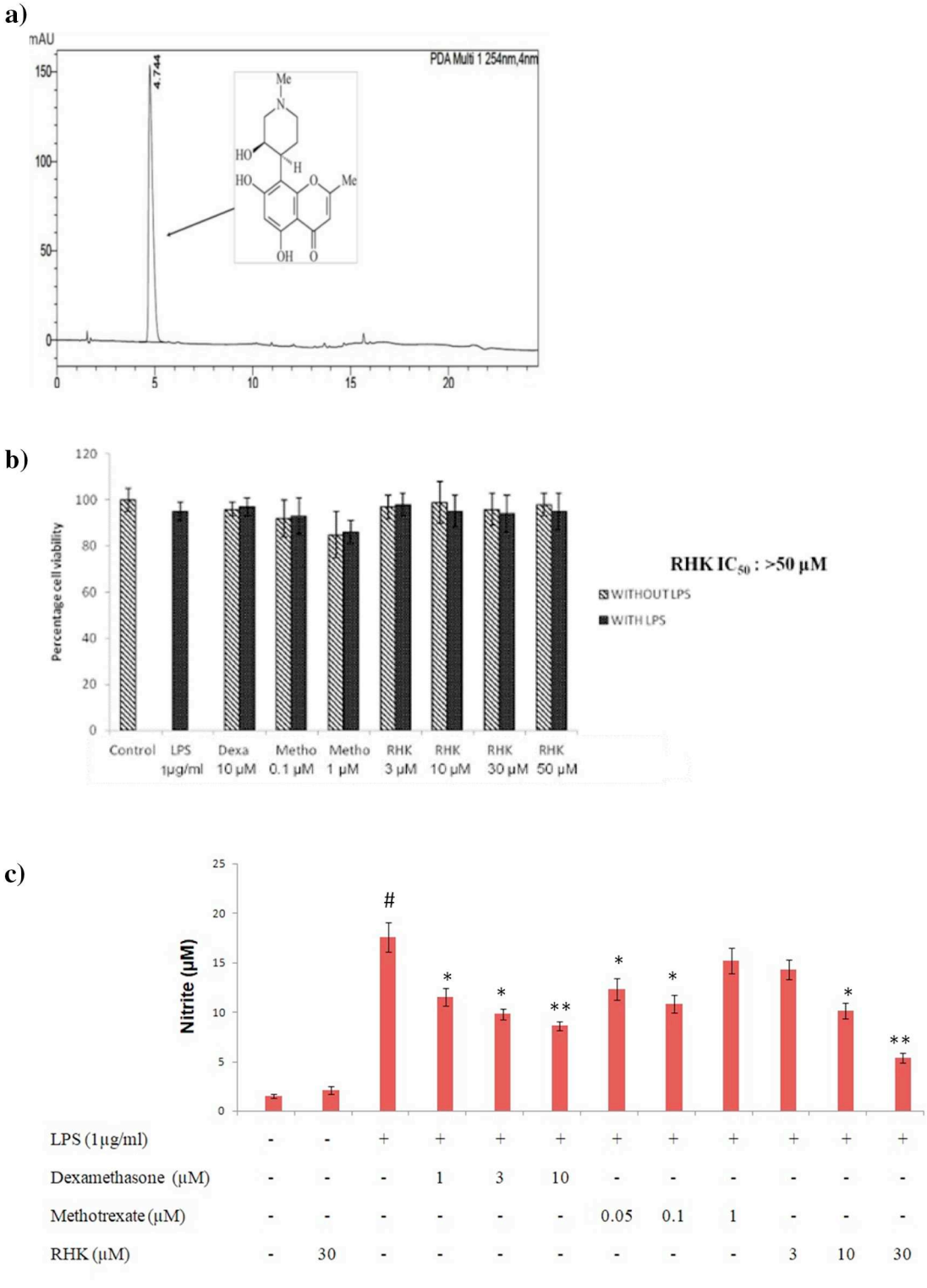

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Fig. 1. a: Structure of RHK and HPLC chromatogram of isolated RHK: A single peak with a retention time of 4.74 min exhibited > 97% of the absorbance eluted and was RHK (97% pure).
b: Effects effect of RHK, dexamethasone (Dexa), methotrexate (Metho) with/without LPS (1 μg/mL) on the viability of J774A.1 cells. J774A.1 cells were incubated with different concentration of RHK (3, 10, 30, 100 μM), dexamethasone (1, 3, and 10 μM) and methotrexate (0.05, 0.1 and 1 μM) in the presence or absence of LPS (1 μg/mL) for 24 h. Cell viability was determined using MTT assay and absorbance was determined at 570 nm. Data are presented as means ± SEM of three independent experiments.
c: RHK significantly attenuated LPS-induced overproduction of NO in J774A.1 macrophage cells. J774A.1 cells were pretreated with various concentrations of RHK, dexamethasone, and methotrexate for 1 h before incubation with LPS (1 μg/mL). The amount of nitrite in the supernatants was measured after 18 h, using Griess reagent. The data represented as mean ± SD for the three independent experiments (n = 3). #p < 0.001 compared with control group. *p < 0.05, and **p < 0.01 were compared with LPS group (one-way ANOVA followed by Dunnett's tests).
d: Effect of RHK on reactive oxygen species (ROS). J774A.1 cells were cultured with RHK (3, 10, 30 μM) for 18 h in the presence or absence of LPS (1 μg/mL). Dexamethasone and methotrexate were used as Positive control. The intracellular ROS was stained with 5-(and-6) chloromethyl-20, 70-dichlorodihydrofluorescein diacetate acetyl ester. Images of J774A.1 cells was captured (n = 3) by confocal laser scanning microscope. The data represented as mean ± SD for the three independent experiments (n = 3). #p < 0.001 compared with control group. *p < 0.05, and **p < 0.01were compared with LPS group (one-way ANOVA followed by Dunnett's tests).

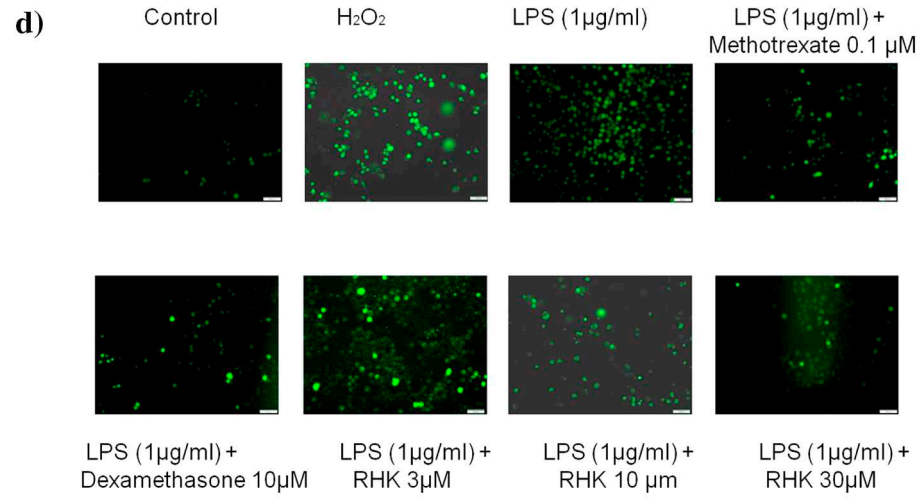
positive control. After incubation, the supernatants were collected and assayed for PGE2, IL-1β, IL-6, and TNF-α using ELISA kits according to the manufacturer's instructions.

2.7. Transient transfection and luciferase assay

J774A.1 cells were transfected with NF-κB-Luc reporter plasmid (Clontech, Shiga, Japan) using Lipofectamine as per the manufacturer protocol. After 48 h of transfection, cells were treated with RHK, 1 h before LPS (1 μg/mL) stimulation for 18 h. After incubation, cells were lysed, and the luciferase activity was evaluated using the Promega luciferase assay system [24].

2.8. Immunocytochemistry for detection of NF-κB

Cultured cells were seeded on coverslips in 35 mm culture dishes at



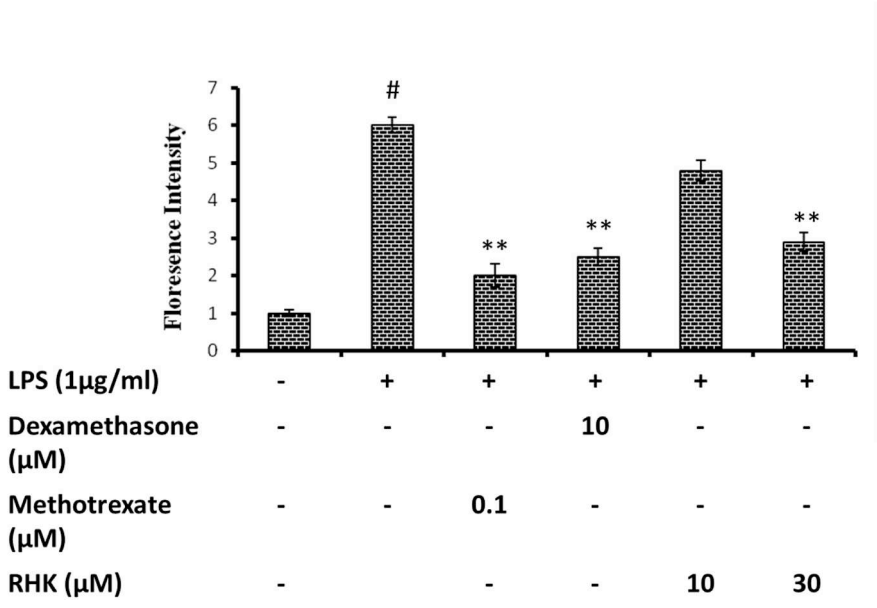


Fig. 1. (*continued*)

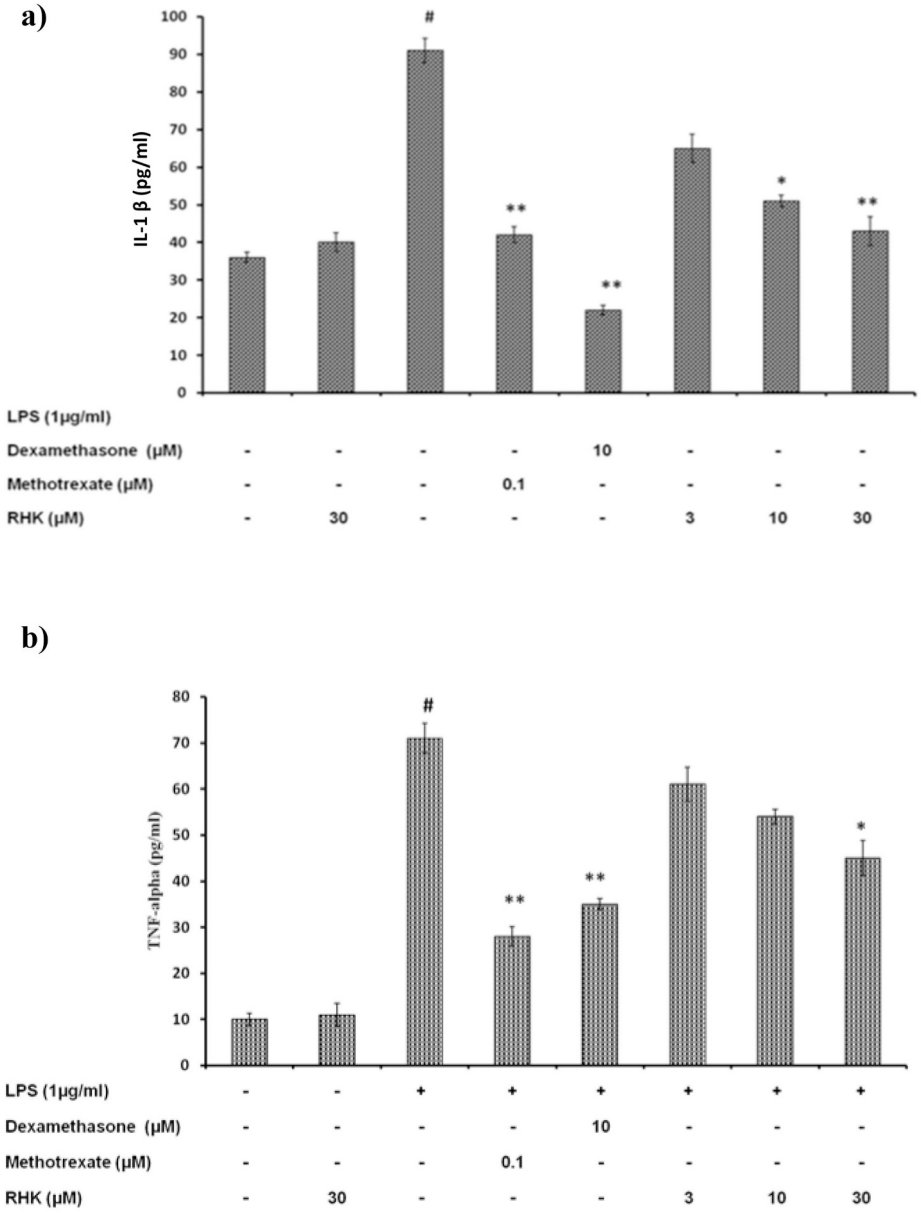


Fig. 2. RHK significantly attenuated LPS-induced overproduction of (a) IL-1 β, (b) TNF-α, (c) IL-6, (d) PGE2 in J774A.1 macrophage cell line: J774A.1 cells were pretreated with various concentrations of rohitukine (RHK), dexamethasone (Dexa), methotrexate (Metho) 1 h before incubation with LPS (1 μg/mL). Level of IL-1β, IL-6, TNF-α and PGE2 in the supernatants were measured after 18 h, using ELISA kits. The data represented as mean ± SD for the three independent experiments (n = 3). #p < 0.001 compared with control group. *p < 0.05, **p < 0.01, when compared with LPS group (one-way ANOVA followed by Dennett's tests).

a concentration of 1 × 105 for 24 h. After 24 h, cells were pretreated with RHK (3, 10, or 30 μM) 1 h before treatment with LPS (1 μg/mL) for 18 h. Dexamethasone (10 μM) and methotrexate (0.1 μM) were used as positive control. Cells were washed twice with PBS and fixed in 4% paraformaldehyde for 15 min at room temperature. Cells were permeabilised in PBS with 0.1% TritonX-100 at room temperature for 10 min. Nonspecific binding sites were blocked by incubating the cells in 10% BSA. Cells were then incubated with NF-kB antibody diluted 1:100 in 0.1% Triton X-100 in PBS for 1 h at room temperature and Alexa Fluor 555 conjugated secondary antibody (Invitrogen) diluted 1:500 in PBS for 1 h at room temperature. Cells were then washed three times with PBS and stained with 4′,6-diamidino-2-phenylindole (DAPI) 1 μg/mL in PBS. The coverslips were mounted on glass slides, and cells were imaged by a laser scanning confocal microscope (Olympus Fluoview FV1000) by using 20× objective lens [25].

2.9. Western blotting analysis

The J774A.1 cells (1 × 107), were cultured in 100 mm Petri dishes for 24 h, after that cells were pretreated with RHK (10, 30 μM) 1 h before treatment with LPS (1 μg/mL) for 2 h in a 37 °C, 5% CO2 incubator. Cells were then harvested on the ice, washed twice using ice-cold PBS, and suspended in 200 μl lysis buffer supplemented with the protease inhibitor. After incubating on ice for 30 min, cell extracts were subjected to centrifugation (12,000 ×*g*) at 4 °C for 15 min to get cell protein, and protein estimation was done using the Bradford method. SDS-PAGE separated proteins and electrotransferred to nitrocellulose membranes (Pierce, USA), then hybridised with the iNOS, COX-2, MAPK pathway (JNK, p38, ERK1/2) IκB-α, p-IκB-α, NFκB p65 specific antibodies. Blots were normalised by use of actin to correct for differences in loading of the proteins. Immunoblot signals were obtained

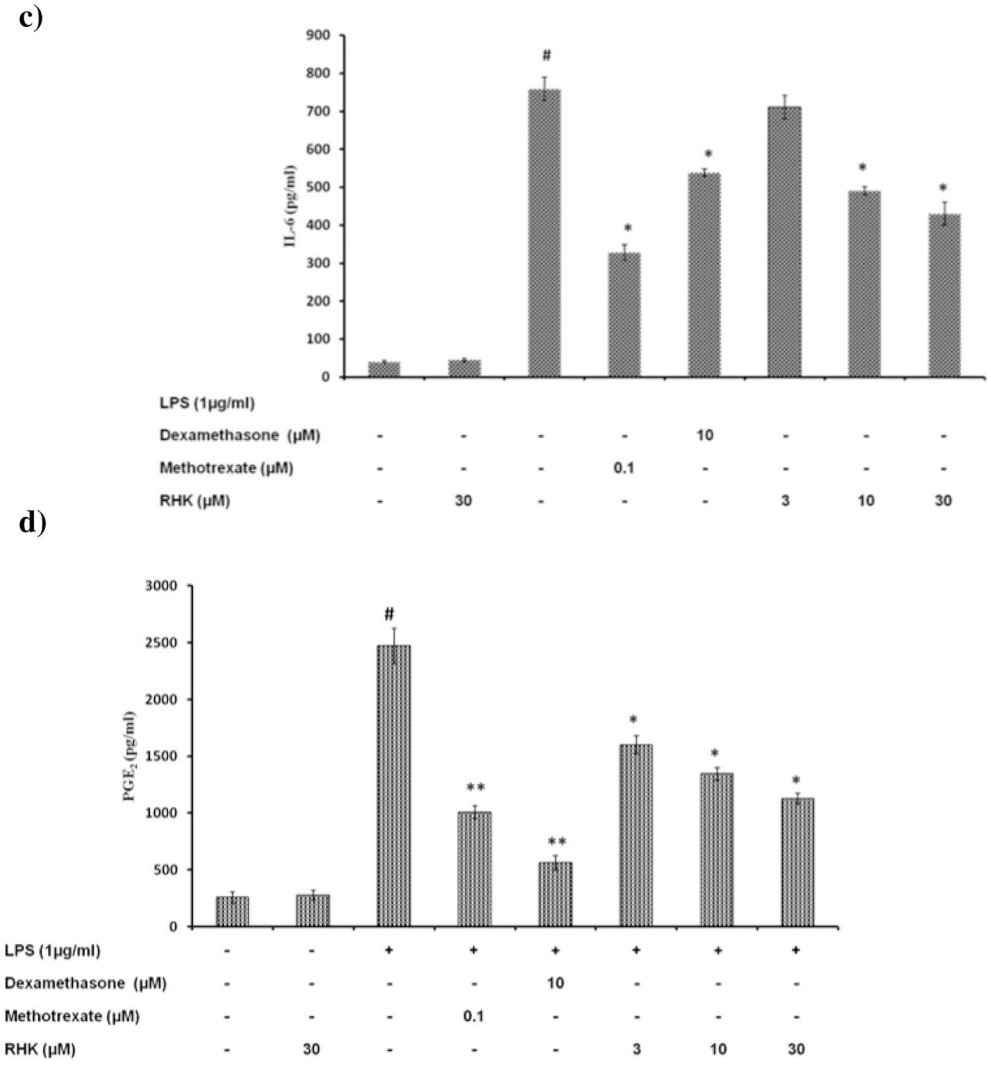


Fig. 2. (*continued*)

using a gel doc from three separate experiments.

2.10. In-vivo anti-inflammatory activity

2.10.1. Animals

Male Wistar rats (120–140 g) and Balb/c mice (22–25 g) procured from the Animal House of Indian Institute of Integrative Medicine, Jammu was used for experimentation. They were housed in standard environmental conditions (a 12:12 h light and dark cycles, temperature 25 ± 1 °C and humidity 55 ± 5%) and fed with standard rodent pellet diet (Ashirwad Feed, Chandigarh, India) and water ad libitum. All the experiments were conducted following the ethical guidelines of the International Association for the Study of Pain [10] and approved by the Institutional Animal Ethics Committee.

2.10.2. LPS induced pro-inflammatory cytokine production in Balb/c mice

Balb/c mice were divided into seven groups (5 animal per group). Group I serves as a negative control (receives only normal saline), group II is control group receives LPS (20 µg/mice i.p., once on the 5th day of the experiment), group III, IV serves as a positive control receives dexamethasone (10 mg/kg p.o. daily for 5 days) and methotrexate (1 mg/kg p.o. on 1, 3 and 5th day). Group V, VI, VII receives RHK at 12.5, 25 and 50 mg/kg p.o. respectively, for 5 days, On the fifth day, 1 h after the last dose of the compound, LPS (20 µg/mice) was injected intraperitoneal. After 4 h of LPS injection, the blood was collected and centrifuged at 5000 rpm for 10 min at 4 °C to separate the serum. Concentrations of IL-1β, IL-6, and TNF-α in the serum samples were determined using commercially available ELISA kits from Invitrogen (USA). Cytokine levels for each time point were measured in two to four independent experiments.

2.10.3. Acetic acid-induced mouse vascular permeability

The acetic acid-induced vascular permeability test with slight modifications was performed according to the method of Whittle (1964). Briefly, 1 h after the oral administration of normal saline (0.9%), RHK (12.5, 25 and 50 mg/kg p.o.), and dexamethasone (10 mg/kg p.o.); 0.1 mL/10 g b.w. of Evans blue (0.5% w/v, in normal saline) was intravenously injected through tail vein followed by intraperitoneal injection of 2% acetic acid at 0.4 mL. After 30 min of acetic acid injection, mice were sacrificed, and the peritoneal cavity was washed three times with saline (10 mL). Washes were collected, and OD measured at 610 nm using UV spectrophotometer (Shimadzu Co. Ltd., Kyoto, Japan). The amount of dye leakage in the supernatant was calculated from the absorbance measurements.

Evans Blue is an alkaline stain that binds albumin. Under physiologic conditions, the endothelium is impermeable to albumin, so Evans blue bound albumin remains restricted within blood vessels. Acetic acid causes increase vascular permeability, leads to leakage of albumin.

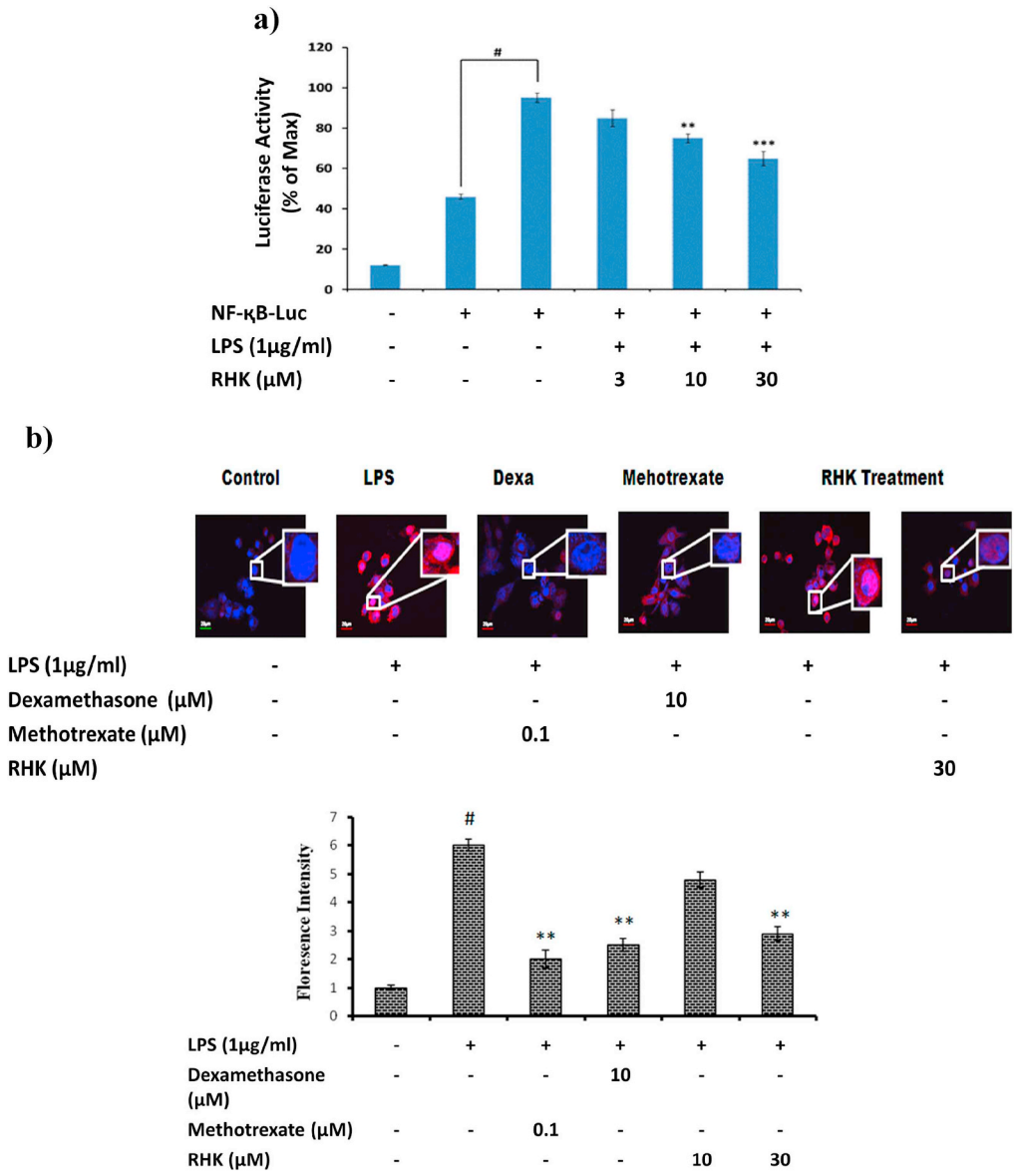


Fig. 3. a: Inhibitory effects of RHK on LPS-induced transcriptional activity of NF-κB in J774A.1 cells. Cells were transiently co-transfected with pNF-κB-luc reporter and pretreated with RHK (3, 10 and 30 μM). After 1 h LPS (1 μg/mL) was added, and cells were further incubated for 3 h. Cells were then harvested, and luciferase activities were determined using a Promega luciferase assay system and a luminometer. Values represent mean ± SD of three independent experiments (n = 3). #$p < 0.001$ compared with control group. *$p < 0.05$, and **$p < 0.01$were compared with LPS group (one-way ANOVA followed by Dunnett's tests).
b: Effect of RHK on translocation of NF-κB using Immunofluorescence microscopic technique: The quantitation of fluorescent intensity was measured using ImageJ software. Red colour signifies translocation of NF-κB in the nucleus. Fluorescent intensity was represented as mean ± SD for the three independent experiments (n = 3). #$p < 0.001$ compared with control group *$p < 0.05$, and **$p < 0.01$ were compared with LPS group (one-way ANOVA followed by Dunnett's tests). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.10.4. Carrageenan-induced paw oedema in Wistar rat

Oedema was induced in groups of five rats by using the method of Winter et al., injecting 100 μl of 1% (w/v) freshly prepared carrageenan solution in normal saline into the sub-plantar region of the left hind paw. Five groups (five rats per group) were taken; group I and V were kept as control and positive standard respectively, whereas group-II, III and IV received different treatment doses of RHK (12.5, 25, 50 mg/kg; p.o.). RHK and ibuprofen were administered orally 45 min before carrageenan injection. The volume of the paw was measured 4 h after carrageenan injection using a volume differential meter (Model 7101, Ugo Basile, Italy) and paw size was determined using micrometer at different interval of time.

2.10.5. Leukocyte migration assay

The assay was carried out by injecting 0.5 mL of 1% of carrageenan solution prepared in sterile normal saline into the pleural cavity of each mouse according to Meacock and Kitchen with little modification [17]. Five groups (five rats per group) were taken; group I and II were kept as control and positive standard respectively, whereas group-III, IV and V received 12.5, 25 and 50 mg/kg o.p. treatment of RHK. The test substances were administered 1 h before the carrageenan injection. Total leukocyte count and the volume of exudates were determined after 24 h of carrageenan injection and compared with the control group.

2.10.6. Chronic inflammation model: Mycobacterium induced arthritis model in Wistar rat

The animals were divided into eight groups consisting of 6 animals in each group (n = 6). Group I-vehicle control rats; Group II-arthritic rats; Group III-arthritic rats orally administered with Ibuprofen (100 mg/kg/day); Group IV-arthritic rats orally administered dexamethasone (10 mg/kg/day); Group V-arthritic rats orally administered methotrexate (1 mg/kg/twice weekly); Group VI, VII, VIII-arthritic rats orally administered RHK (12.5, 25 and 50 mg/kg/day).

2.10.6.1. Induction of arthritis in Wistar rat. Animals were injected with 50 μl of Freund's complete adjuvant (FCA) into the plantar region of the left hind paw. Test substances were administered orally for 14 days from day 0 (one day before injection of Freund's adjuvant). The treatment doses for RHK were fixed based on preliminary results. The changes in the paw volume were measured on alternate days up to 21 days using a plethysmometer (Ugo Basile, Italy) and body weight was recorded on 0, 7th, 14th and 21st day of adjuvant injection. Levels

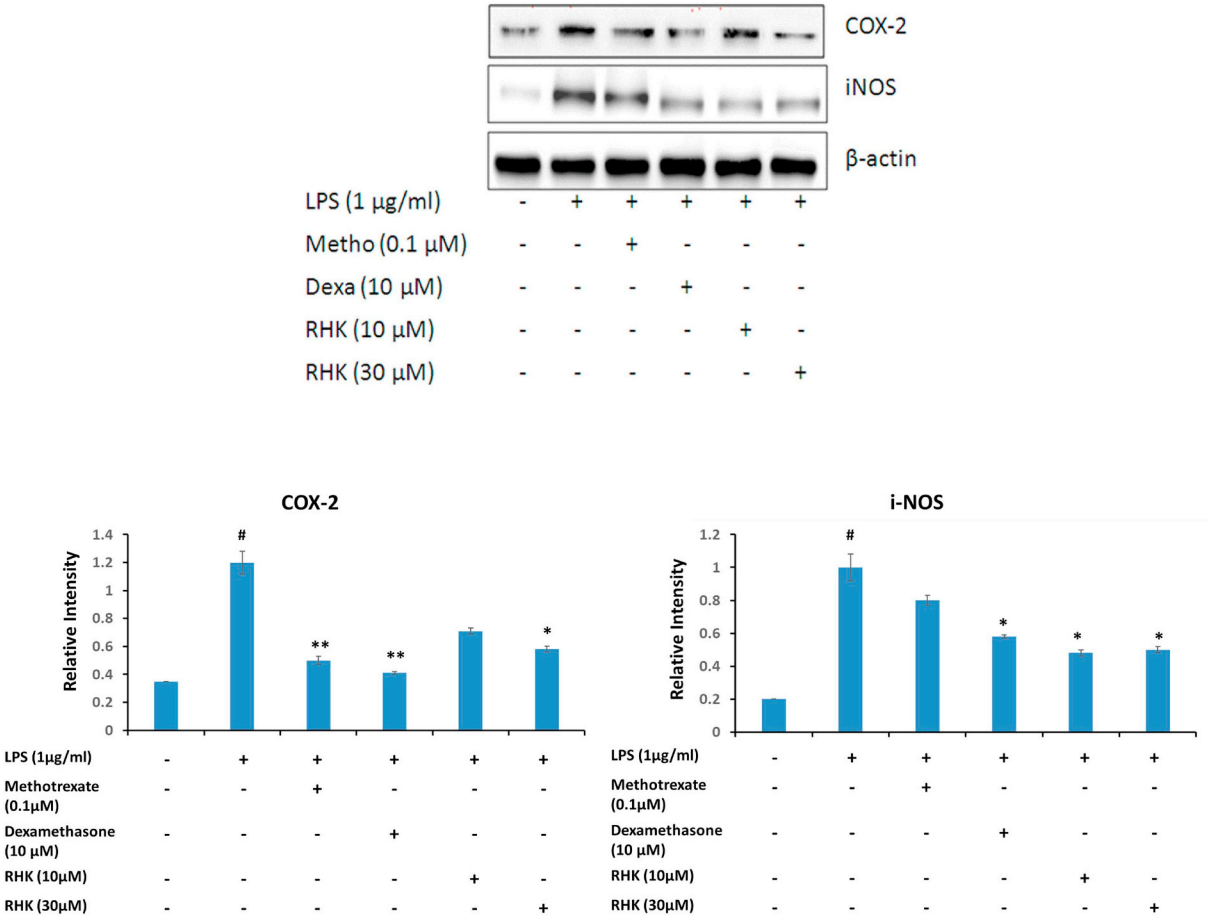


Fig. 4. Effect of RHK treatment on COX-2 and iNOS expression: J774A.1 macrophage cells were pre-treated with different concentrations of rohitukine (RHK), dexamethasone (Dexa), methotrexate (Metho) for 1 h before the addition of LPS (1 μg/mL). Total cellular proteins were analysed after 30 min of LPS addition by western blot with specific antibodies. The data represented as mean ± SD for the three independent experiments (n = 3). #$p < 0.001$ compared with control group *$p < 0.05$, and **$p < 0.01$were compared with LPS group (one-way ANOVA followed by Dunnett's tests). The quantitation of protein bands is expressed as relative intensity.

of pro-inflammatory cytokines and biochemical enzymes like Alkaline Phosphatase (ALP), Serum glutamate pyruvate transaminase (SGPT), Serum glutamate oxaloacetate transaminase (SGOT), locomotor activity, joints radiological assessment, and oxidative stress marker levels were estimated at the end of the study.

2.11. Statistical analysis

The results were expressed as means ± S.E.M. and differences between mean values of normally distributed data were assessed by the one-way analysis of variance (ANOVA) multiple comparisons followed by Dennett's test. The p-value of < 0.05 or less was considered as significant.

3. Results

3.1. Effect of RHK against proliferation, NO and pro-inflammatory cytokines using peritoneal macrophages

The preliminary result showed that RHK did not possess any toxic effect on peritoneal macrophages up to 50 μM concentration. RHK further showed a dose-dependent decrease in the production of NO (12, 46, and 72% at 3, 10 and 30 μM concentration; respectively). However, it has been observed that RHK significantly inhibited the release of proinflammatory cytokines IL-1β (55%), IL-6 (45%) and TNF-α (73%) at the higher dose (30 μM) respectively.

3.2. Effect of RHK on cell viability

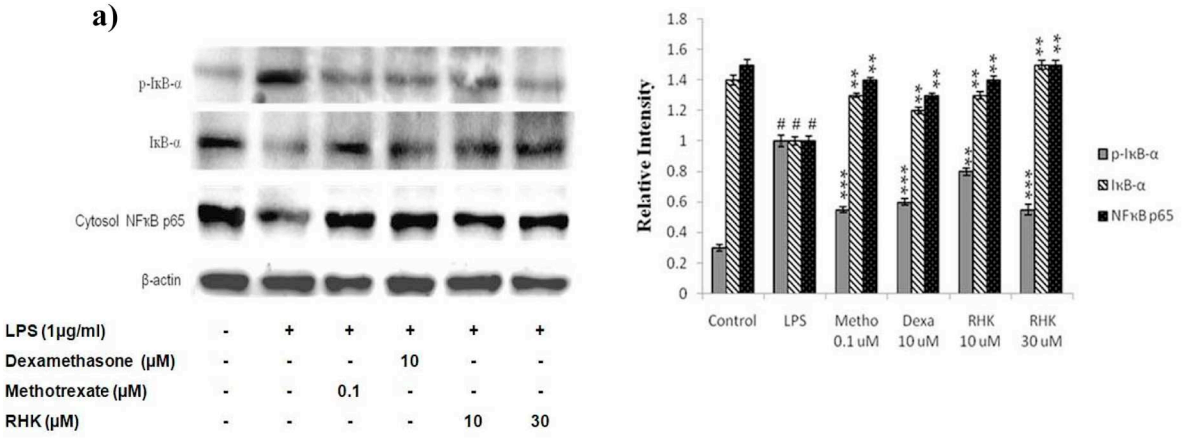
J774A.1 cells were incubated with RHK with and without LPS (1 μg/mL) from 0 to 100 μM and cell viability was measured using MTT assay 18 h later. We found that RKH from 0 to 100 μM had no cytotoxic effects on J774A.1 cells (Fig. 1b). However, dexamethasone and methotrexate were also found to be safe at a dose of 10 and 0.1 μM, respectively. However, methotrexate showed 20% cell growth inhibition at 1 μM concentration.

3.3. Effects of RHK on LPS-induced NO and ROS production

The inhibitory effect of RHK on NO production increased with dose, with the maximal inhibition of about 61% at 30 μM concentration. Dexamethasone, and methotrexate moderately reduced nitrite accumulation with the highest reduction being approximately 55% and 44% respectively (Fig. 1c).

3.4. RHK on LPS-induced pro-inflammatory cytokine production

LPS treatment in J774A.1 macrophages enhanced the release of pro-inflammatory cytokines IL-1β, IL-6, TNF-α compared with those of untreated cells (Fig. 2). In particular, RHK, at a concentration of 30 μM, effectively inhibited LPS-induced release of IL-1β (46%), TNF-α (29%), IL-6 (31%) and PGE2 (48%) compared with those of the LPS-only control (Fig. 2a, b, c and d). These results indicate that RHK inhibited



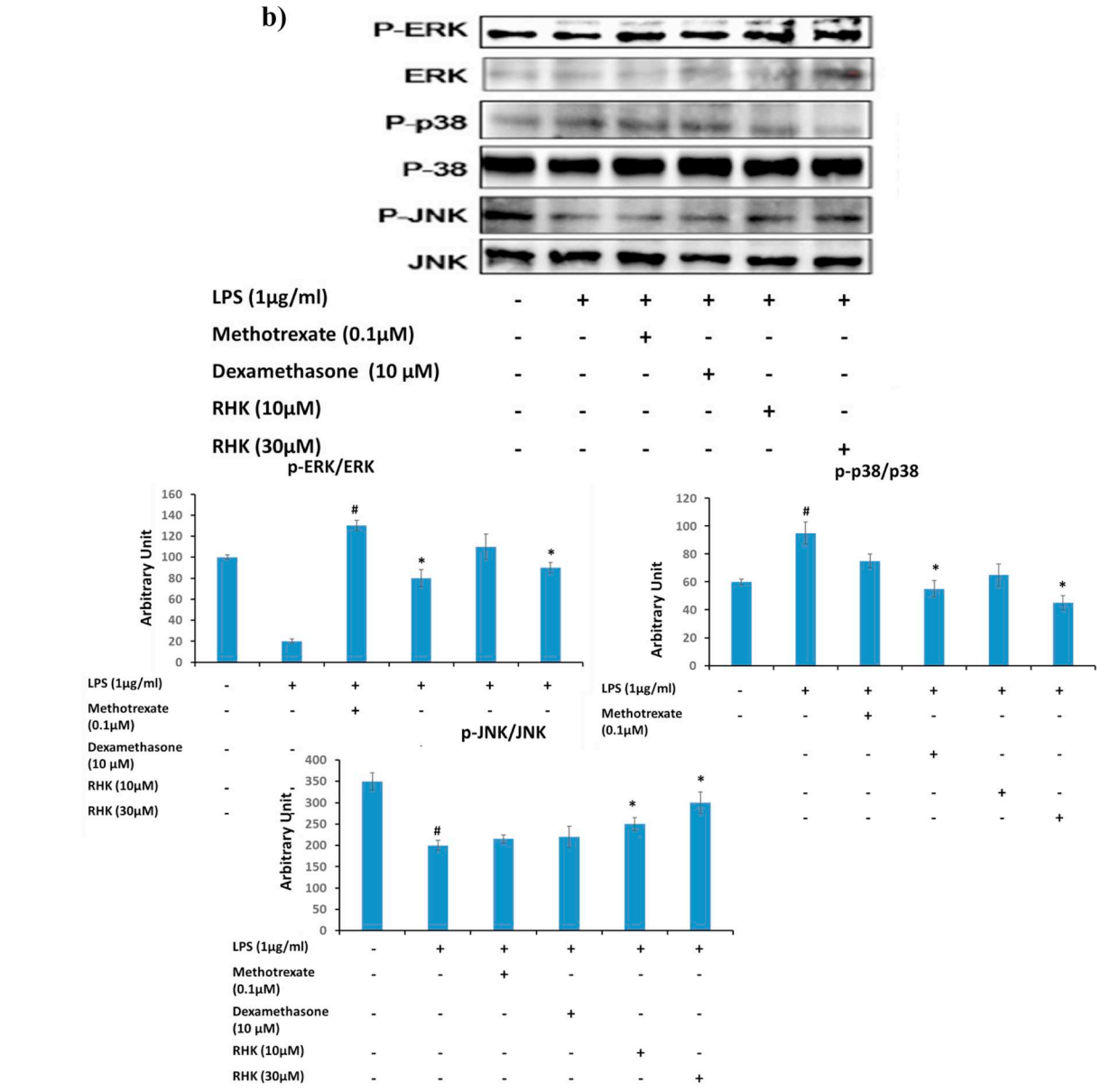


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Fig. 5. Effect of RHK treatment on p-IκB-α, IκB-α, cytosol NfκB p65 expression.
a: Effect of RHK treatment on p-IκB-α, IκB-α, cytosol NF-κB p65 expression: J774A.1 macrophage cells were pre-treated with different concentrations of rohitukine (RHK), dexamethasone (Dexa), methotrexate (Metho) for 1 h and then the cells were incubated with LPS (1 μg/mL) for 30 min. Total cellular proteins were analysed by western blot with specific antibodies. The data represented as mean ± SD for the three independent experiments (n = 3). The quantitation of protein bands is expressed as relative intensity.
b: Effect of RHK treatment on MAPK pathway: J774A.1 macrophage cells were cultured to 80% confluence and treated with RHK, Metho, Dexa or vehicle 1 h before to the stimulation with LPS. Cell lysates were separated by SDS-PAGE electrophoresis and transferred onto 0.2 μm nitrocellulose membranes as described, followed by incubation with Primary antibodies against phosphorylated (active) of p38, ERK and JNK were used to verify the nuclear translocation of the active forms of these signalling intermediates. Beta-actin was served as loading control. Results from representative SDS-PAGE/Western blot analysis Images are representative of three independent experiments. The quantitation of protein bands is expressed as relative intensity.

LPS-induced pro-inflammatory cytokines production in J774A.1 macrophages.

3.5. RHK effect on LPS-induced DNA binding and transcriptional activity of NF-κB

It is well reported that NF-κB is a prominent transcription factor that modulates the levels of pro-inflammatory cytokines (e.g., TNF-α and IL-1β) and inflammatory proteins like iNOS and COX-2 induced by LPS [18], luciferase reporter gene assay using pNF-κB-luc plasmid generated by inserting four spaced NF-κB binding sites into pLuc-promoter vector. This pNF-κB-luc plasmid was transiently transfected into J774A.1 macrophage, which was then pretreated or not with RHK followed by stimulated with LPS. LPS alone showed an almost 3-fold increase in luciferase activity in the transfected cells compared to only vector transfected cells. However, pretreatment with RHK inhibits 35% increase in luciferase activity induced by LPS in a dose-dependent manner (Fig. 3a).

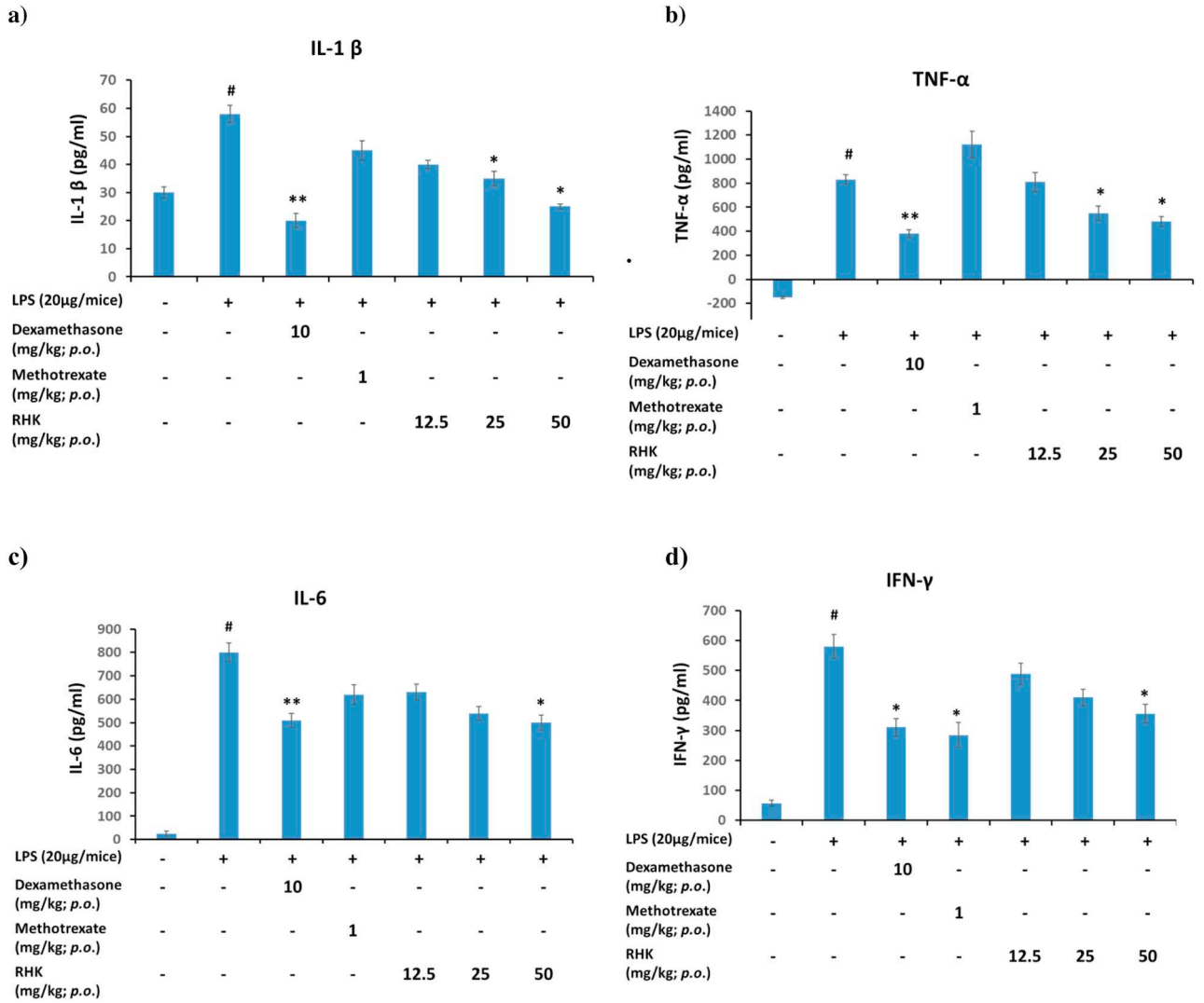

Fig. 6. Effect of RHK against LPS induced iro-inflammatory cytokines production a) IL-1β, b) TNF-α, c) IL-6, and d) IFN-γ: RHK, dexamethasone and methotrexate were administered to BALB/c mice for five consecutive days. On the fifth day of the experiment, LPS (20 μg/mouse; i.p.) was injected, and serum was collected. Levels of pro-inflammatory cytokines were estimated using standard ELISA Kits. The graph represents the mean and SD from three independent experiments (n = 5). #p < 0.001 compared with control group; *p < 0.05, and **p < 0.01 when compared with LPS group (one-way ANOVA followed by Dunnett's tests).

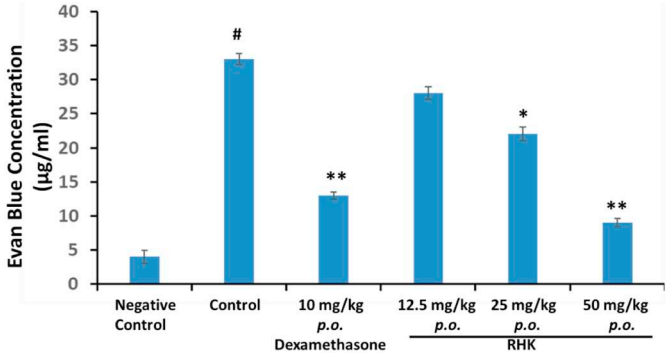

Fig. 7. Effect of RHK against acetic acid induced vascular permeability: The graph represents an inhibitory effect of RHK on vascular permeability. Bar values are the mean ± S.E.M. of five observations (n = 5). Negative control serves as a group of normal mice without any treatment. p * < 0.05 and ** < 0.01 control vs treated group (one-way ANOVA followed by Dunnett's tests).

3.6. RHK inhibits LPS-induced NF-κB translocation using immunocytochemistry

Generally, LPS-stimulated macrophages generate ROS via the activation of an NADPH oxidase, and ROS plays an essential role in NF-κB activation. To determine the effect of RHK to prevent NF-κB nuclear translocation in macrophages stimulated via LPS, immunofluorescence staining was conducted. As shown in Fig. 3b, RHK reversed LPS induced nuclear translocation of NF-κB in macrophages. This effect is imparted due to inhibition of ROS and NO inhibition as shown above.

3.7. RHK inhibits LPS-induced protein expression of iNOS and COX-2

Protein levels of COX-2 and iNOS was reduced by 68% and 54%, respectively, by 30 µM of RHK (Fig. 4). These data indicate that RHK effectively inhibited LPS-induced inflammatory responses by suppressing the expression of iNOS and COX-2 in J774A.1 macrophages cell line.

3.8. Effect of RHK on LPS-induced activation of MAPK pathway and IκB-α, p-IκB-α, NFκB p65

MAPK, NF-κB are LPS-inducible transcription factors that play a central role in the mammalian innate immune response and inflammation. The activation of MAPK and NF-κB are exerted through the regulation of downstream target genes that encode pro-inflammatory cytokines and inducible enzymes, such as TNF-α, IL-6, IL-1β and COX-2. Many studies reported NF-κB activation is induced by LPS, TNF-α or H_2O_2 [20–22].

RHK downregulate the phosphorylation of ERK and JNK was 27% and 54%, respectively, at 30 µM concentration (Fig. 5a), whereas dexamethasone (Dexa) and methotrexate (Metho) showed the similar effect as RHK. Our result indicated that inhibition of ERK, JNK contributes towards the anti-inflammatory effect of RHK against LPS-induced inflammatory response.

3.9. Effect of RHK against LPS induced pro-inflammatory cytokine production in Balb/c mice

We used the acute peritonitis mouse model following LPS (20 µg/mice) injection intraperitoneally. RHK suppressed LPS-induced production of proinflammatory cytokines in macrophages cell line J774A.1. Therefore, we focused on RHK-mediated attenuation of the secretion of these cytokines in vivo. RHK showed maximal inhibition of IL-1β production (58%) at 50 mg/kg p.o.; whereas dexamethasone showed 92% inhibition at 10 mg/kg p.o. However, the effect on TNF-α (45% and 51% respectively) and IL-6 (34% and 38% respectively) inhibition was similar for both RHK (50 mg/kg p.o.) and dexamethasone (10 mg/kg p.o.) (Fig. 6).

3.10. Effect of RHK against acetic acid-induced mouse vascular permeability

The capillary permeability was determined by Evans blue extruded into the peritoneal cavity, measured by the absorbance value of the supernatant. In the present study, the acetic acid-control group showed an increase in Evans blue OD value after administration of acetic acid. However, RHK (12.5, 25 and 50 mg/kg p.o.) significantly ($p < 0.05$) produced a dose-dependent inhibitory effect on the OD by 12.1%, 39.3%, and 75.7%, respectively (Fig. 7).

3.11. Effect of RHK against carrageenan-induced paw oedema in Wistar rat

RHK (12.5, 25 and 50 mg/kg p.o.) significantly ($p < 0.05$) suppressed paw edema dose-dependently 25%, 35% and 58% respectively compared to control group (Fig. 8). Also, paw size reached to the normal state at the end of the study, after treatment with RHK (25 and 50 mg/kg p.o.) and ibuprofen (100 mg/kg p.o.). Results suggest a strong anti-inflammatory activity of RHK.

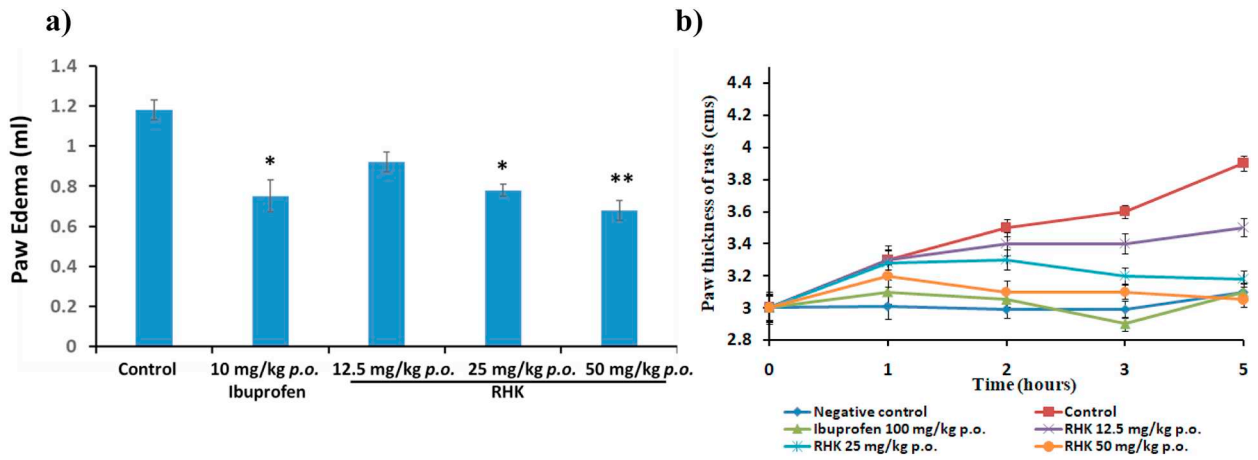

Fig. 8. a: Effect of RHK on paw oedema against carrageenan-induced paw oedema in Wistar rat and b: effect of RHK on paw thickness against carrageenan-induced paw oedema in Wistar rat. Values are the mean ± S.E.M.; of five observations. p * < 0.05 and ** < 0.01 control vs treated group (one-way ANOVA followed by Dunnett's tests).

Table 1
Effect of RHK on pleural exudate volume and total leukocyte count.

Treatment	Dose (mg/kg) p.o.	Volume of exudate (mean ± SEM)	% inhibition	Total leukocyte count × 10^3/μl mean ± SEM	Differential cell count (10^3/uL)					% inhibition
					Neut	Lympho	Mono	Esno	Baso	
Control	–	4.07 ± 0.09	–	49.85 ± 1.7	35.54	4.77	0.39	8.88	0.27	–
Ibuprofen	100	1.53 ± 0.03**	62.41	4.36 ± 0.19***	2.30	0.98	0.50	0.36	0.22	95.95
RHK	12.5	3.83 ± 0.06	5.90	46.88 ± 1.33	33.49	7.30	0.28	5.61	0.20	36.82
RHK	25	1.57 ± 0.09**	61.43	4.91 ± 0.24***	1.18	2.00	0.03	1.56	0.14	82.43
RHK	50	1.63 ± 0.03**	59.95	5.03 ± 0.29***	0.50	2.95	0.02	1.24	0.31	86.04

Pleurisy was induced by injecting 500 μl of 10 mg/mL of carrageenan solution prepared in sterilized normal saline. Values are the mean ± S.E.M. of five observations.
* < 0.05.
** < 0.01.
*** < 0.001.

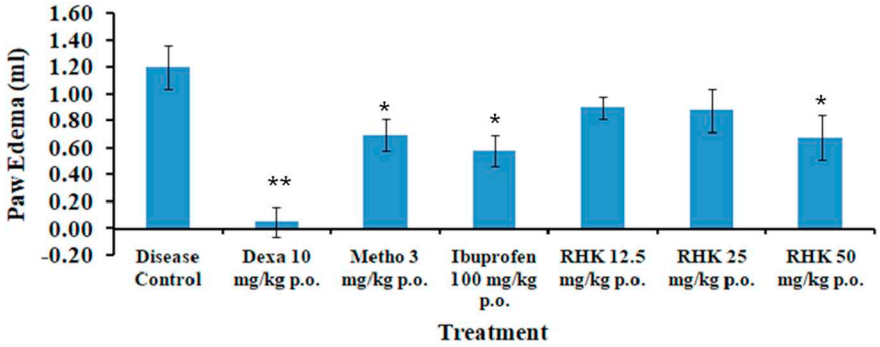

Fig. 9. Paw edema changes during the progression of CIA.

Table 2
Change in biochemcal parameters on RHK treatment.

Parameter	Control	Disease control	Methotrexate 3 mg/kg p.o.	Dexamethasone 10 mg/kg p.o.	Ibuprofen 100 mg/kg p.o.	RHK 25 mg/kg p.o.	RHK 50 mg/kg p.o.
C-reactive protein	0.3 ± 0.0	3.1 ± 0.05**	1.6 ± 0.03*	0.4 ± 0.0	0.38 ± 0.009	0.63 ± 0.02	0.40 ± 0.02
ALP (U/L)	88.4 ± 16.5	116.8 ± 13.0	113.2 ± 25.1	78.0 ± 10.3	72.8 ± 11.6	74.95 ± 17.0	83.17 ± 21.1
SGOT (U/L)	167.5 ± 20.4	156.1 ± 19.4	198.0 ± 25.0	148.8 ± 7.8	160.87 ± 24.81	140.10 ± 18.	161.30 ± 19.2
SGPT (U/L)	55.0 ± 5.5	61.2 ± 6.0	63.7 ± 15.5	43.733 ± 4.4	69.0 ± 18.3	29.35 ± 6.06	27.93 ± 5.65
Urea (mg/dl)	59.8 ± 5.7	50.2 ± 3.6	56.3 ± 2.6	49.8 ± 1.9	42.86 ± 1.57	39.99 ± 10.2	43.23 ± 5.17
Creatinine (mg/dl)	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.308 ± 0.009	0.23 ± 0.02	0.20 ± 0.02
Glucose (mg/dl)	55.9 ± 10.0	74.6 ± 10.7	68.2 ± 14.1	71.8 ± 10.2	57.86 ± 5.50	63.56 ± 14.3	65.83 ± 5.13
Triglyceride (mg/dl)	119.1 ± 7.7	144.7 ± 16.2	163.8 ± 26.8	107.8 ± 24.1	108.68 ± 16.55	113.56 ± 14.3	119.83 ± 15.13
Cholestrol (mg/dl)	107.2 ± 10.2	107.4 ± 7.6	110.6 ± 10.0	108.8 ± 5.7	122.25 ± 10.37	105.94 ± 4.18	106.74 ± 6.78
Uric acid (mg/dl)	3.8 ± 0.7	3.1 ± 0.6	3.2 ± 0.5	2.9 ± 0.5	5.11 ± 0.971	5.87 ± 0.15	6.21 ± 0.28
Total protein (g/dl)	6.4 ± 0.2	7.0 ± 0.3	7.0 ± 0.5	7.1 ± 0.2	6.9 ± 1.626	6.21 ± 0.95	6.68 ± 0.60

Values are the mean ± S.E.M. of five observations.
*p < 0.05, **p < 0.01, ***p < 0.001 when compared with control using one-way ANOVA followed by Dunnett's test.

3.12. Effect of RHK on leukocyte migration assay

Leukocytes migrate to various sites of inflammation/injury is a versatile function of innate and adaptive immunity. However, excess migration of leukocytes leads to worsening further the inflammatory condition leading to severe pain, swelling. Therefore, ideal anti-inflammatory agents should fence this migration. RHK produced significant ($p < 0.01$) inhibition of carrageenan-induced pleuritic exudate volume (59%) only at a dose of 50 mg/kg. However, the leukocyte migration was significantly affected at all the doses applied, reaching the maximum level (86%, $p < 0.001$) at 50 mg/kg. Ibuprofen (100 mg/kg; p.o.), the reference drug used in this study, produced 62% and 95% of inhibitions, respectively, of pleuritic exudate and leukocyte numbers (Table 1). These results indicated that RHK suppressed neutrophils recruitment induced by carrageenan.

3.13. Effect of RHK on chronic model of inflammation/Mycobacterium induced arthritis

3.13.1. RHK restores physical changes induced in arthritic model

The present study involves the evaluation of the in-vivo therapeutic potential of RHK against FCA induced arthritis model. Paw oedema/inflammation is one of the key factors for the determination of the severity of inflammation and the effect of the anti-arthritic regimen. RHK (50 mg/kg; p.o.) showed significant inhibition of paw oedema (54%) compared to Disease control (Fig. 9). There is no change in the body weight of animals when treated with RHK; however, the methotrexate group showed a decline in the body weight, indicating its toxicity.

3.13.2. RHK did not alter biochemical parameters

Biochemical parameters were studied by collecting blood samples at

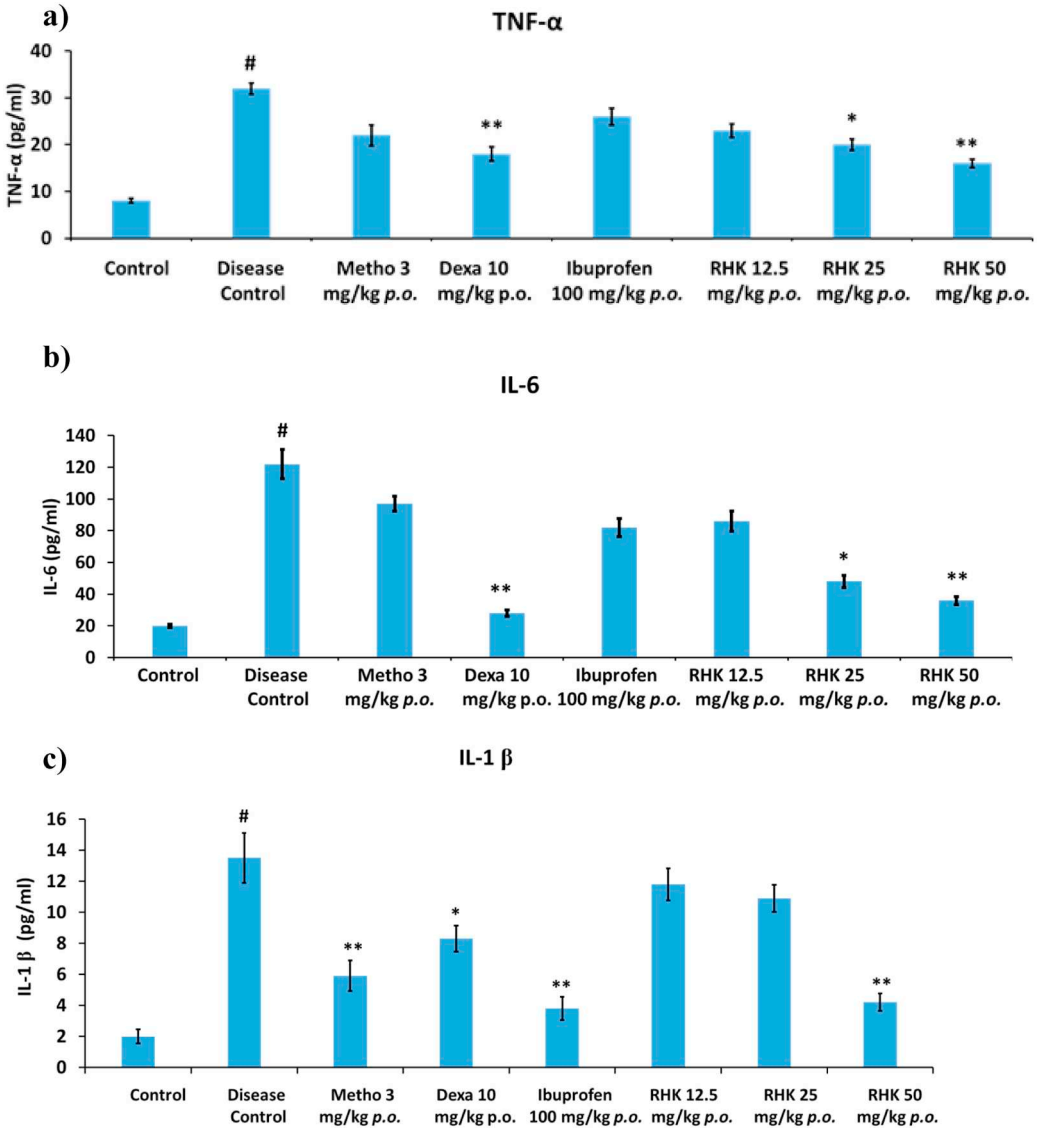

Fig. 10. Effect of RHK on pro-inflammatory cytokines: a) TNF-α, b) IL-6 and c) IL-1β.

the end of the study. Levels of SGPT, SGOT and ALP were increased in all arthritis rats as compared to control rats, C-reactive protein and Haematocrit values give a direct indication about the onset of arthritis. Oral treatment of RHK significantly restored biochemical changes due to arthritis, back to near normal (Table 2).

3.13.3. RHK restores level of pro-inflammatory cytokines

Aetiology of RA involves multiple factors which lead to the involvement of T-cell and B-cells followed by a massive flood of proinflammatory cytokines like TNK-alpha, IL-1beta and IL-6 and many more. These major proinflammatory cytokines are responsible for the destruction of the end organ, despite remission from the cause. Interestingly, we have found that RHK reduced the level of these proinflammatory cytokines in a dose-dependent manner when compared to arthritic rats without any treatment. However, levels of TNF-α, IL-6 and IL-1β were significantly decreased by RHK (52%, 68%, and 71% respectively). Dexamethasone restored the levels of proinflammatory cytokines to normal (Fig. 10).

3.13.4. RHK ameliorate radiographic changes

Radiographs were obtained after completion of the study. Key observations were made based on joint deformities comprise of bone thinning/erosion and decrease in joint space in comparisons to normal control rats. RHK treatment maintains joint architecture and reduces bone erosion in arthritic rats compared to positive controls like dexamethasone, methotrexate and ibuprofen. Results showed marked protection against bone loss and deformities after treatment with RHK (Fig. 11).

3.13.5. RHK abrogates augmented antioxidant enzymes levels

The endogenous antioxidant enzymes remunerate the oxidative damage occurred during arthritic events. There was a profound alteration in the levels of LPO, superoxide dismutase (SOD), catalase (CAT) and glutathione-S-transferase (GST) was observed. The experimental results revealed significant augmentation of serum SOD, CAT and GST of the arthritic group by 74%, 82% and 70% respectively compared to the saline control group. Oral administration of RHK revitalised the change in SOD, CAT and GST levels by 96%, 94% and 98% and ibuprofen fed group exhibited protection only by 40%, 56% and 65% respectively compared to the arthritic group. However, methotrexate (Metho) showed significant high level of MDA production which might be a correlation to its anti-cancer/toxic nature; whereas dexamethasone (Dexa) showed comparative better control on oxidative stress mediators (Fig. 12).

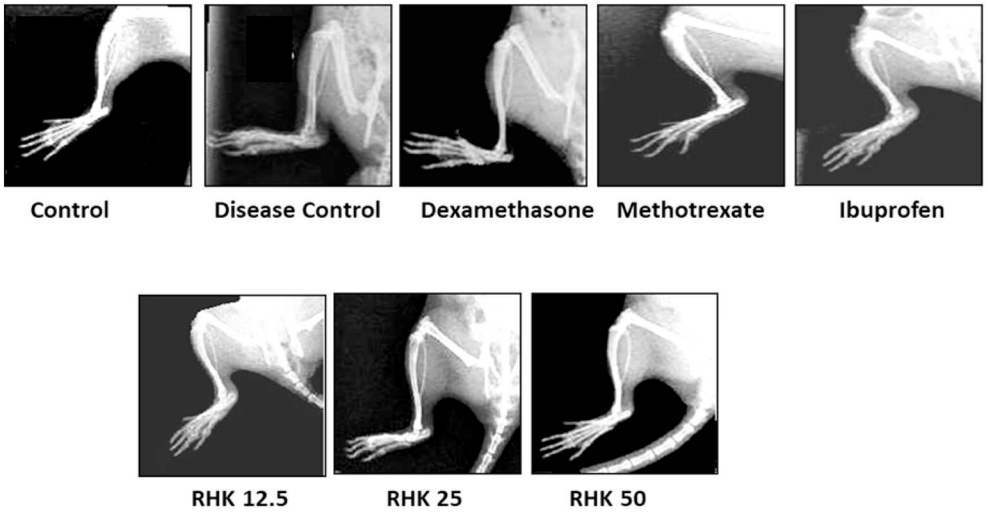

Fig. 11. Radiological assesment for anatomical chages during arthritis progression: (A) representative radiographs of the hind limbs (showing the tibiotarsal and tibiofemoral joints) on day 21.

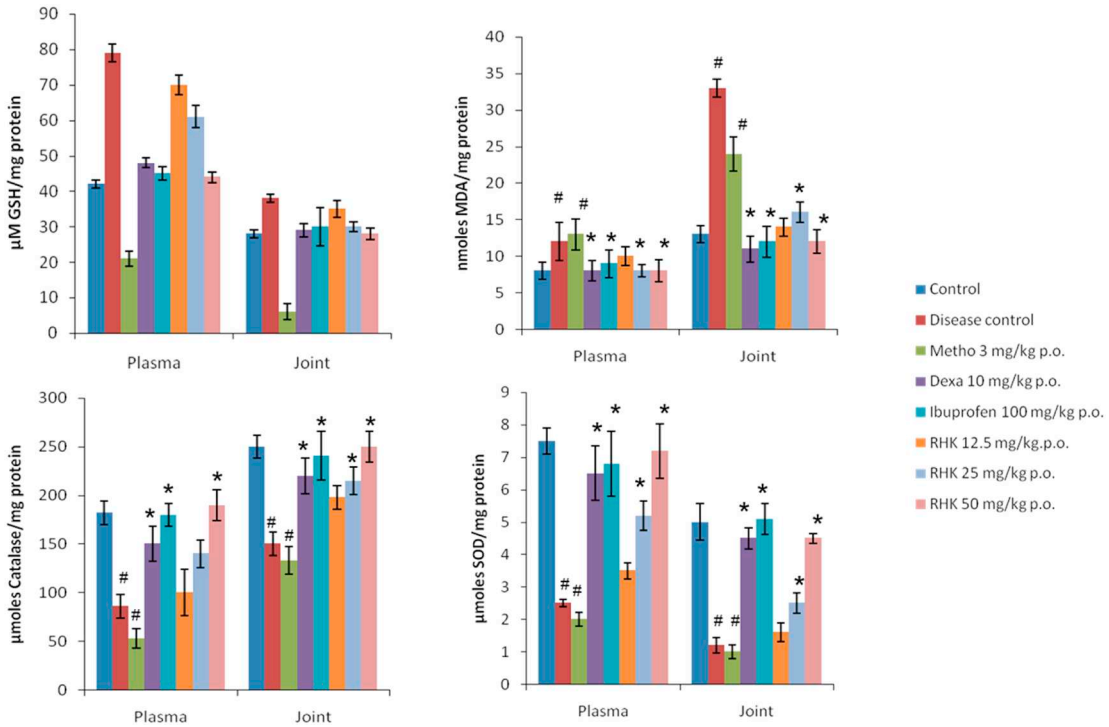

Fig. 12. Effect of RHK on oxidative enzymes.

3.13.6. Spontaneous motor activity

Animals were evaluated for change in the motor activity at the end of the study. The animal group treated with RHK showed improved motor activity in a dose-dependent manner when compared to disease control animals, which further confirms the therapeutic effect of RHK (Table 3).

4. Discussion

The process of inflammation is recognised as a complex series of overlapping cellular and plasma-derived events and differs from species to species, in the same species, from one tissue to another and in the same tissue it varies depending upon the trauma. Despite the diversity of the harmful influences and the tissue involved in inflammation, similar chemical mediators are released in the early event. Different types of the biologically active chemical mediators responsible for the propagation of inflammation are immediately released at the site of inflammation after injury.

In the present study, we demonstrated the influence of RHK protection over the LPS induced inflammatory degeneration in J774A.1 cell line through NFκB deactivation. LPS is a mitogen which activates NFκB via Toll-like receptor 4 (TLR4) and resulted into activation of p38, ERK and JNK via MyD88 stimulates NFκB results in the production of pro-inflammatory cytokine TNF-α, IL-1β, IL-6 and inflammatory mediator iNOS and COX-2 [26]. It has been well documented that NFκB serves as one of the main transcription factors that contribute towards modulation of pro-inflammatory cytokines and inflammatory mediators [27,28].

Thus, researchers are more and more focused on agents, which can regulate NFκB transcription, serves to regulate inflammatory responses

Table 3
Effect of RHK on spontaneous motor activity in mice.

Parameter	Spontaneous motor activity
Control	41.93 ± 4.5
Disease control	14.77 ± 1.0[#]
Methotrexate 3 mg/kg p.o.	36.40 ± 10.4[*]
Dexamethasone 10 mg/kg p.o.	39.77 ± 15.3[*]
Ibuprofen 100 mg/kg p.o.	31.40 ± 10.4[*]
RHK 12.5 mg/kg p.o.	26.15 ± 4.9
RHK 25 mg/kg p.o.	28.43 ± 1.5[*]
RHK 50 mg/kg p.o.	39.90 ± 3.2[*]

Values represent mean ± S.E.M. (n = 5).
[*] Means $p > 0.5$, when compared with disease control group.
[#] Means $p > 0.5$, when compared with control group using one-way ANOVA followed by Dunnett's test.

and promoted to possess chemoprotective therapeutics [29–31].

We found that the suppressive effect of RHK on LPS-induced production of NO was mediated at the transcriptional level. Because of the strong anti-inflammatory properties of RHK, we also assessed its ability to inhibit NO production using LPS-stimulated peritoneal macrophages isolated from mice. Our results demonstrated that RHK inhibited NO production in a concentration-dependent manner using peritoneal macrophages. Additionally, RHK dose-dependently down-regulated LPS-induced iNOS and COX-2 expression.

Inflammation process is associated with the migration of cells within the physiological system [32–34]. The inhibition of leukocytes at the inflammatory site has been suggested as one of the mechanisms of anti-inflammatory drugs. RHK showed a significant decrease in total leukocyte counts and exudate volume at higher dose against carrageenan-induced pleurisy. Moreover, RHK significantly inhibits carrageenan-induced paw oedema in Wistar rat. Thus, it supports potential anti-inflammatory in-vivo.

To further support anti-arthritic activity, the effect of RHK was assessed against Mycobacterium induced arthritis model in Wistar rat. Classic NSAID (ibuprofen), steroid (dexamethasone), immunosuppressant (methotrexate) were taken as positive standards which showed different inhibitory potential. Results depicted that RHK inhibit the paw oedema more pronounced on the un-injected paw, rather than that of the injected paw indicating its role through an immunological pathway.

Radiological assessments revealed that RHK prevented bone degradation/loss and maintained firm joint architecture, whereas ibuprofen has not shown a marked effect. Dexamethasone showed thinning of bone mass compared to control. However, methotrexate also inhibited pannus formation and joint deformities to a great extent.

Biochemical markers are elevated during chronic inflammation [35,36]. The elevated levels of lysosomal enzymes have been reported in chronic inflammation, and reductions of these enzymes occur with anti-inflammatory drugs. Usually, SGOT, SGPT, alkaline phosphatase, protein levels are altered due to multiple factors. Moreover, treatment with conventional available drug lead to increases SGOT, SGPT, and Alkaline Phosphatase and protein levels due to their hepatic and nephritic toxicity [35]. RHK did not alter any biochemical enzymes, thus reveals that it is devoid of any toxic side effects/end-organ toxicity.

Further, RHK at a dose of 50 mg/kg p.o. showed improvement in motor activity compared to control, thus support its effect as a curative agent. RHK also restored homeostasis between the oxidative enzymes like SOD, GSH, LPO, and catalase, which further supports the therapeutic potential of RHK in the control and remission of arthritic symptoms.

In summary, our data indicated that the naturally occurring RHK possesses significant anti-inflammatory activity. RHK suppresses the several pro-inflammatory mediators and cytokines, demonstrate a potential target compound for the treatment of inflammatory diseases.

5. Conclusion

In summary, the anti-inflammatory effect of Rohitukine (RHK) in LPS-activated macrophages is due to down-regulation the secretion of TNF-α, IL-6 and IL-1β. The related mechanism is by preventing the activation of NFκB signalling pathways.

Ethical approval

"This article does not contain any studies with human participants and IAEC approval has been taken before animal's experimentation."

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Conflict of interest

Authors declare that no conflict of interest exists concerning the publication of this work.

References

[1] C.A. Feghali, T.M. Wright, Cytokines in acute and chronic inflammation, Front. Biosci. 2 (1997) d12–d26.
[2] G. Dos Santos, M.A. Kutuzov, K.M. Ridge, The inflammasome in lung diseases, Am. J. Phys. Lung Cell. Mol. Phys. 303 (2012) L627–L633.
[3] J.L. Pablos, J.D. Canete, Immunopathology of rheumatoid arthritis, Curr. Top. Med. Chem. 13 (2013) 705–711.
[4] R. Affendi Raja Ali, L. John Egan, How to manage the risk of colorectal cancer in ulcerative colitis, Curr. Drug Targets 12 (2011) 1424–1432.
[5] P. Libby, History of discovery: inflammation in atherosclerosis, Arterioscler. Thromb. Vasc. Biol. 32 (2012) 2045.
[6] E. Balestro, F. Calabrese, G. Turato, F. Lunardi, E. Bazzan, G. Marulli, et al., Immune inflammation and disease progression in idiopathic pulmonary fibrosis, PLoS One 11 (2016) e0154516.
[7] E. Elinav, R. Nowarski, C.A. Thaiss, B. Hu, C. Jin, R.A. Flavell, Inflammation-induced cancer: crosstalk between tumours, immune cells and microorganisms, Nat. Rev. Cancer 13 (2013) 759–771.
[8] M. Scharl, G. Rogler, Inflammatory bowel disease: dysfunction of autophagy? Dig. Dis. 30 (2013) 12–19.
[9] K.Q. de Andrade, F.A. Moura, J.M. dos Santos, O.R.P. de Araújo, J.C. de Farias Santos, M.O.F. Goulart, Oxidative stress and inflammation in hepatic diseases: therapeutic possibilities of N-acetylcysteine, Int. J. Mol. Sci. 16 (2015) 30269–30308.
[10] K. Reich, The concept of psoriasis as a systemic inflammation: implications for disease management, J. Eur. Acad. Dermatol. Venereol. 26 (2012) 3–11.
[11] N. Fujiwara, K. Kobayashi, Macrophages in inflammation, Curr. Drug Targets Inflamm. Allergy 4 (2005) 281–286.
[12] Govind Keshri, et al., Contraceptive and hormonal properties of the stem bark of *Dysoxylum binectariferum* in rat and docking analysis of rohitukine, the alkaloid isolated from active chloroform soluble fraction, Contraception 76 (5) (2012) 400–407.
[13] Sunil K. Mishra, Shashi Tiwari, Shishir Shrivastava, Ravi Sonkar, Vaibhav Mishra, Sunil K. Nigam, Anil K. Saxena, Gitika Bhatia, Snober S. Mir, Pharmacological evaluation of the efficacy of *Dysoxylum binectariferum* stem bark and its active constituent rohitukine in regulation of dyslipidemia in rats, J. Nat. Med. 72 (4) (2018) 837–845.
[14] V. Lakshmi, K. Pandey, A. Kapil, N. Singh, M. Samant, A. Dube, In vitro and in vivo leishmanicidal activity of *Dysoxylum binectariferum* and its fractions against *Leishmania donovani*, Phytomedicine 14 (1) (2007) 36–42.
[15] Tsuguaki Terashima, Abedul Haque, Yuka Kajita, Akinori Takeuchi, Takashi Nakagawa, Takashi Yokochi, Flavopiridol inhibits interferon-γ-induced nitric oxide production in mouse vascular endothelial cells, Immunol. Lett. 148 (2) (2012) 91–96.
[16] P.M. Kumara, S. Zuehlke, V. Priti, B.T. Ramesha, S. Shweta, G. Ravikanth, et al., *Fusarium proliferatum*, an endophytic fungus from *Dysoxylum binectariferum* Hook.f, produces rohitukine, a chromane alkaloid possessing anti-cancer activity, Antonie Van Leeuwenhoek 101 (2013) 323–329.
[17] P. Mahajan, M. Nikam, A. Asrondkar, A. Bobade, C. Gill, Synthesis, antioxidant, and anti-inflammatory evaluation of novel thiophene-fused quinoline based β-diketones and derivatives, J. Heterocyclic Chem. 54 (2017) 1415–1422.
[18] N. Singh, P. Singh, S. Shrivastva, et al., Naunyn Schmiedeberg's Arch. Pharmacol. 385 (2012) 277.
[19] Salil Varshney, Kripa Shankar, Muheeb Beg, Vishal M. Balaramnavar, Sunil Kumar Mishra, Pankaj Jagdale, Shishir Srivastava, Yashpal S. Chhonker,

Vijai Lakshmi, Bhushan P. Chaudhari, Rabi Shankar Bhatta, Anil Kumar Saxena, Anil Nilkanth Gaikwad, Rohitukine inhibits in vitro adipogenesis arresting mitotic clonal expansion and improves dyslipidemia in vivo, J. Lipid Res. 55 (6) (2014) 1019–1032.

[20] V. Mahajan, N. Sharma, S. Kumar, V. Bhardwaj, A. Ali, R. Khajuria, et al., Production of rohitukine in leaves and seeds of *Dysoxylum binectariferum*: an alternate renewable resource, Pharm. Biol. 53 (2015) 446–450.

[21] D.H. Yang, S.Q. Cai, Y.Y. Zhao, H. Liang, Determination of the absolute configuration of rohitukine, Chin. Chem. Lett. 14 (2003) 720–723.

[22] T. Joo, K. Sowndhararajan, S. Hong, J. Lee, S.-Y. Park, S. Kim, et al., Inhibition of nitric oxide production in LPS-stimulated RAW 264.7 cells by stem bark of *Ulmus pumila* L, Saudi J. Biol. Sci. 21 (2014) 427–435.

[23] H. Raza, A. John, J. Shafarin, NAC attenuates LPS-induced toxicity in aspirin-sensitized mouse macrophages via suppression of oxidative stress and mitochondrial dysfunction, PLoS One 9 (2014) e103379.

[24] J.S. Shin, Y.M. Park, J.H. Choi, H.J. Park, M.C. Shin, Y.S. Lee, et al., Sulfuretin isolated from heartwood of *Rhus verniciflua* inhibits LPS-induced inducible nitric oxide synthase, cyclooxygenase-2, and pro-inflammatory cytokines expression via the down-regulation of NF-kappaB in RAW 264.7 murine macrophage cells, Int. Immunopharmacol. 10 (2010) 943–950.

[25] B.R. Acharya, D. Choudhury, A. Das, G. Chakrabarti, Vitamin K3 disrupts the microtubule networks by binding to tubulin: a novel mechanism of its antiproliferative activity, Biochemistry 48 (2009) 6963–6974.

[26] J.Y. Kim, S.J. Park, K.J. Yun, Y.W. Cho, H.J. Park, K.T. Lee, Isoliquiritigenin isolated from the roots of *Glycyrrhiza uralensis* inhibits LPS-induced iNOS and COX-2 expression via the attenuation of NF-kappaB in RAW 264.7 macrophages, Eur. J. Pharmacol. 584 (2008) 175–184.

[27] J.A. Roman-Blas, S.A. Jimenez, NF-kappaB as a potential therapeutic target in osteoarthritis and rheumatoid arthritis, Osteoarthr. Cartil. 14 (2006) 839–848.

[28] C. Thirunavukkarasu, S.C. Watkins, C.R. Gandhi, Mechanisms of endotoxin-induced NO, IL-6, and TNF-alpha production in activated rat hepatic stellate cells: role of p38 MAPK, Hepatology 44 (2006) 389–398.

[29] M.J. Morgan, Z.G. Liu, Crosstalk of reactive oxygen species and NF-kappaB signaling, Cell Res. 21 (2011) 103–115.

[30] S. Nakajima, M. Kitamura, Bidirectional regulation of NF-kappaB by reactive oxygen species: a role of unfolded protein response, Free Radic. Biol. Med. 65 (2013) 162–174.

[31] J.S. Tilstra, C.L. Clauson, L.J. Niedernhofer, P.D. Robbins, NF-kappaB in aging and disease, Aging Dis. 2 (2011) 449–465.

[32] S.W. Tas, M.J. Vervoordeldonk, N. Hajji, M.J. May, S. Ghosh, P.P. Tak, Local treatment with the selective IkappaB kinase beta inhibitor NEMO-binding domain peptide ameliorates synovial inflammation, Arthritis Res. Ther. 8 (2006) R86.

[33] S.C. Sun, S.C. Ley, New insights into NF-kappaB regulation and function, Trends Immunol. 29 (2008) 469–478.

[34] S. Vallabhapurapu, M. Karin, Regulation and function of NF-kappaB transcription factors in the immune system, Annu. Rev. Immunol. 27 (2009) 693–733.

[35] B. Hoesel, J.A. Schmid, The complexity of NF-kappaB signaling in inflammation and cancer, Mol. Cancer 12 (2013) 86.

[36] G. Madonna, C.D. Ullman, G. Gentilcore, G. Palmieri, P.A. Ascierto, NF-kappaB as potential target in the treatment of melanoma, J. Transl. Med. 10 (2012) 53.