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Evaluation of inhibitory activities of plant extracts on production of LPS-stimulated pro-inflammatory mediators in J774 murine macrophages

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Abstract Whole plant methanolic extracts of 14 traditionally used medicinal herbs were evaluated for their anti-inflammatory activity. Extracts of *Grindelia robusta*, *Salix nigra*, *Arnica montana*, and *Quassia amara* showed up to 4.5-fold inhibition of nitric oxide (NO) production in the J774 murine macrophage cells challenged with LPS without cytotoxicity. These four selected extracts significantly reduced the protein levels of inducible NO synthase (iNOS) and the cyclooxygenase-2 (COX-2) as observed by Western blot analysis. Culture supernatants from cells treated with these extracts indicated 3–5-fold reduction of tumor necrosis factor-α (TNF-α). However, only *G. robusta* and *Q. amara* extracts significantly inhibited (by 50%) IL-1β and IL-12 secretions. Furthermore, all these plant extracts were shown to prevent the LPS-mediated nuclear translocation of nuclear factor-κB (NF-κB). All the above observations indicate the anti-inflammatory potential of these plant extracts.

Keywords Anti-inflammatory activity · Plant extract · *Grindelia robusta* · *Salix nigra* · *Arnica montana* · *Quassia amara*

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Introduction

Many plant products from medicinal herbs are historically important for the treatment and prevention of diseases. Natural plant compounds are now gaining more pharmacological attention as many unexplored plant products are showing a wide range of activities like anti-cancer, anti-inflammatory, and anti-aging [1–3]. Nearly 25% of all prescribed drugs are derived from plants with or without further modification [4, 5] and still several pharmacologically active plant-derived compounds remain unexplored [6, 7].

Inflammation acts as a central executor in the pathogenesis of many diseases such as rheumatoid arthritis, arteriosclerosis, myocarditis, infections, cancer, metabolic disorders, and many more [8–10]. Monocytes and macrophages are the key players in inflammatory responses and are also the major sources of pro-inflammatory cytokines and enzymes including tumor necrosis factor-α (TNF-α), interleukins (ILs), cyclooxygenase (COX), and nitric oxide synthase (NOS) [11–13]. These genes of pro-inflammatory mediators are strongly induced during inflammation and are responsible for its initiation and persistence. TNF-α and IL-1β are the cytokines that act as signaling molecules for immune cells and coordinate the inflammatory responses [10]. Cyclooxygenase-2 (COX-2) is an enzyme which is necessary for the production of pro-inflammatory prostaglandins and thus has been a target for many present anti-inflammatory and cancer-preventive drugs [14, 15]. Nitric oxide (NO) is a free radical that mediates many physiological and pathophysiological processes, including neurotransmission and inflammation [16]. Its formation is catalysed by three different isoforms of NOS by the conversion of L-arginine to NO and L-citrulline. Expression of the inducible isoform of NOS (iNOS) in activated

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macrophages is mainly responsible for production of pathological concentration of NO during inflammation. It is well known that nuclear factor-κB (NF-κB) plays the most important role in the immune system [17]. NF-κB is reported to regulate the expression of nearly all inflammatory mediators involved in inflammation [11]. Nuclear translocation of NF-κB in response to various pro-inflammatory stimuli is associated with the activation of inflammatory cascade and therefore, this transcriptional factor is a primary target of many anti-inflammatory therapeutic strategies [16, 18].

Natural plant compounds which are able to suppress the production of inflammatory mediators from activated macrophages can act as potential anti-inflammatory agents. Therefore, this study is aimed to explore and evaluate the anti-inflammatory potential of extracts from some traditionally used medicinal herbs with unidentified anti-inflammatory activities and their mode of action. For the screening of such active components, pro-inflammatory TNF-α, IL-1β, IL-12 and enzyme-encoding genes, iNOS, and COX-2 are chosen as pro-inflammatory markers. The biological activities of the methanolic extracts against inflammation and their mode of action were examined in vitro using LPS-stimulated J774 murine macrophage cells as model.

Materials and methods

Materials

Growth media (RPMI 1640 containing 2 mM L-glutamine) and antibiotic–antimycotic solution were purchased from HiMedia Laboratories (India). Fetal bovine serum (FBS), trypan blue and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), cell culture grade dimethyl sulphoxide (DMSO), L-nitro-arginine methyl ester (L-NAME), phenylmethyl sulphonylfluoride (PMSF) were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Antibodies of iNOS, COX-2, NF-κB, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and β-actin were purchased from Santa Cruz Biotechnology (USA). Enzyme-linked immunosorbent assay (ELISA) kits were purchased from BD Biosciences (USA). Other chemicals and solvents were of reagent grade.

Preparation of plant extract

The plants were procured from, and authenticated by Homeopathic Pharmacopoeia Laboratory (HPL), Ghaziabad, India which is responsible for the validation of all the plants and plant products used in homeopathy. All the dried plants were ground using grinder and the dried plant powders were extracted thrice with methanol (1:10 w/v) at room temperature and evaporated under reduced pressure. The extract powders were dissolved in DMSO to make a stock solution of 50 mg/ml and further dilutions were made with phosphate-buffered saline (PBS; pH 7.4) before use and were filter satirized for cell culture assays. The final concentration of DMSO in the culture medium was kept below 0.2% and diluted extracts.

Cell culture

Murine macrophage cell line (J774) was maintained in the laboratory in RPMI-1640 medium supplemented with 2 mM glutamine, antibiotics (100 U/ml of penicillin, 100 µg/ml of streptomycin and 0.25 µg/ml of amphotericin-B), 10% heat-inactivated fetal calf serum at 37°C in a humidified CO_2 incubator. The culture was allowed to grow to confluence and used for further experiments. The cells were resuspended in RPMI-1640 at a density of 2×10^6 cells/ml. Viability of the cells was determined by Trypan blue (0.4% Trypan blue in PBS) exclusion method. The cells were challenged with different concentrations of lipopolysaccharide (LPS; Sigma) of *Escherichia coli* (serotype, 055:B5) and LPS concentration was optimized (1 µg/ml) for inducing measurable concentration of nitrite with minimum cytotoxicity. The inhibitory effect of the plant extracts on NO production by macrophage was preliminarily studied by the addition of different concentrations of extract (50, 100, 200, 300, 400, and 500 µg/ml) with LPS (1 µg/ml) for 24 h (Supplementary Fig. 1). Cells were then treated with a fixed (100 µg/ml) concentration of the extracts with LPS for all subsequent studies. The cell free culture supernatant after 24 h of incubation was used to estimate NO using Griess nitrite assay [19]. In all the above cases, unstimulated J774 cells receiving an amount of DMSO equal to that present in the plant extract under the same culture conditions were treated as negative control. The nitric oxide synthesis inhibitor L-nitro-arginine methyl ester (L-NAME, 200 µM) was used in each assay as positive control.

Cytotoxicity assay

The cytotoxicity of all the plant extracts on macrophage was determined in each experiment using MTT colorimetric assay. Briefly, the cells were grown in 96-well plates to a density of 5×10^3 cells/well. After 24 h, cells were washed with fresh medium and treated with extracts to the final concentration of 500 µg/ml. After 48 h of incubation, cells were washed and 20 µl of MTT (5 mg/ml in PBS) solution was added to each well and further 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 (Molecular Devices, USA) at 570 nm with background subtraction at 650 nm.

Griess nitrite assay

The NO production was measured as described by Lee et al. [19]. Briefly, 5×10^3 cells/well were seeded in 96-well plates. After 24 h, cells were washed with fresh medium and treated with LPS (1 μg/ml) and plant extracts (100 μg/ml). The nitric oxide synthesis inhibitor L-NAME (200 μM) was used for each assay as positive control. After 24 h of incubation, 100 μl of the culture supernatant was mixed with an equal volume of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine in 5% phosphoric acid) and incubated at room temperature for 10 min. The absorbance at 540 nm was recorded using microplate reader. The nitrite concentration was determined by extrapolation from the sodium nitrite standard curve.

Preparation of nuclear extract

Nuclear extract was prepared according to the previously reported method [20]. Briefly, 1×10^6 murine macrophage cells were pre-incubated with or without the plant extract (100 μg/ml) and LPS for 1 h. The cells were washed with PBS, dislodged and pelleted by centrifugation and resuspended in the 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 (Sigma)] 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 at −70°C till further use as cytoplasmic extract. The pelleted nuclei were washed thrice with the cell lysis 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. Nuclear extract was collected by centrifugation at 12,000*g* for 15 min at 4°C. Protein concentration of the nuclear extract was estimated using Bradford's reagent (BioRad, USA). The extract was either immediately used or stored at −70°C till further use.

Western blot analysis

Equal protein amount (40 μg) of nuclear/cytoplasmic extracts from macrophage were electrophoresed on 12% sodium dodecyl sulfate (SDS) polyacrylamide gel and transferred to nitrocellulose membrane (Pall Life Sciences, USA) in 25 mM Tris, 192 mM glycine, 20% methanol at 20 V for 20 min using semi dry transfer apparatus (Biorad, USA). The transfer of protein to the membrane 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 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), polyclonal iNOS or COX-2 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 conjugated with horseradish peroxidase (1:1000 diluted in PBS containing 0.25% BSA) at room temperature for 1 h. After extensive washing with PBST, the blot was exposed to peroxidase substrate [15 mg 4-chloronaphthol (Sigma) in 5 ml methanol and 20 ml PBS buffer containing 50 μl of 3% hydrogen peroxide]. Nuclear and cytoplasmic proteins of LPS challenged cells were taken as control for integrated density values (IDV) to determine relative expressions of the above mentioned proteins.

Enzyme-linked immunosorbent assay (ELISA)

The cell free culture supernatants were collected after 24 h of incubation of the cells with LPS (1 μg/ml) and plant extracts (100 μg/ml). These supernatants were used for measuring the levels of TNF-α, IL-1β, and IL-12 proteins using ELISA kit (BD Biosciences, USA) according to the manufacturer's instructions. ELISA results were recorded using a microplate spectrophotometer (Molecular devices, USA) at 450 nm and the corrected absorbance was noted as per manufacturer's instruction. Results were expressed in pg/ml. Each sample was measured in triplicate and values were derived from the standard curve.

Nuclear translocation assays of p65

Inhibition of the LPS-induced NF-κB activation in macrophage in response to plant extract was analysed by immunocytochemistry to visualize the nuclear translocation of the transcription factor p65. Cells were seeded into 4-well chamber slides at 5×10^3 cells/well and allowed to adhere for 24 h in RPMI containing 10% of FBS. The cells were then washed thrice with 1 ml of RPMI and were treated with LPS with or without the plant extracts (100 μg/ml) for 60 min. Cells were then washed thrice with 1 ml of cold PBS and fixed with 3.7% formalin in PBS for 10 min, followed by permeabilization with 1% Triton X-100 containing PBS for 30 min. The immunostaining for NF-κB p65 subunit (Santa Cruz, USA) was

carried out following the antibody supplier's (Santa Cruz, USA) protocol. The cells were subsequently incubated with FITC-conjugated secondary anti-rabbit IgG (diluted 1:200 in PBS containing 1% BSA) for 1 h. After washing the cells thrice with PBS, the slides were scanned under the inverted fluorescence microscope (Nikon, Japan) at ×40 magnification using digital camera DXM 20 1200F and ACT-1 software.

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's *t*-test for each paired experiments. $P \leq 0.05$ was considered as significant.

Results

Effect of plant extracts on LPS-induced NO production

Methanolic extracts of 14 different medicinal plants were screened for their anti-inflammatory activities and out of them four extracts (*Grindelia robusta*, *Salix nigra*, *Arnica montana*, and *Quassia amara*) were selected for further studies. These selected plant extracts did not exhibit any significant cytotoxic effect even at a high concentration (500 μg/ml) for 48 h of incubation, and in all cases the viability was found above 92% by MTT assay (Data not shown). Nitric oxide production by LPS-activated cells was found to be significantly inhibited ($P < 0.05$) by all four plant extracts under study in a dose dependant manner (Supplementary Fig. 1). In the present study selected plant extracts (100 μg/ml) were used to evaluate their anti-inflammatory activity. As shown in the Fig. 1 cells treated with whole plant extracts of *G. robusta*, *S. nigra*, *and A. montana* resulted in 2.27, 3.96, and 4.9-fold reduction, respectively in the LPS-induced NO production, while *Q. amara* treatment was able to reduce the same to 5.69-fold ($P < 0.01$). L-NAME (200 μM) was used as positive control in each experiment.

Effect of plant extracts on LPS-induced iNOS and COX-2 protein expressions

The protein expression levels of the two major pro-inflammatory enzymes iNOS and COX-2 in LPS-challenged cells with or without the treatment of plant extract were evaluated by densitometric analysis of the Western blots (Fig. 2a, b). Treatment of LPS increased the protein levels of iNOS and COX-2 in macrophage cells by 5.34- and 4.66-fold, respectively (Fig. 2). A significant reduction ($P < 0.05$) in iNOS protein level was observed with all the four plant extracts and the maximum (4.4-fold, $P < 0.01$) inhibitory effect was observed in cells treated with *Q. amara* extract. Similarly COX-2 protein level also showed a significant decrease in response to plant extract treatment, however, *S. nigra* extract showed a highly significant (2.14-fold, $P < 0.01$) inhibitory effect in the LPS-induced COX-2 protein expression.

Effect of plant extracts on pro-inflammatory cytokines

Macrophage cells treated with LPS exhibited an appreciable increase in the levels of TNF-α, IL-1β, and IL-12 in the culture supernatants (Fig. 3). All the four plant extracts were able to reduce the levels of TNF-α significantly in the culture supernatant and *Q. amara* extract exhibited highest (3.98-fold, $P < 0.01$) inhibitory activity (Fig. 3a). Extracts from *Q. amara* and *G. robusta* showed highly significant (up to 2.25-fold, $P < 0.01$) suppressive effects on the production of IL-1β by the LPS-stimulated macrophages on the contrary no significant effect was observed with *A. montana* extract (Fig. 3b). As seen in Fig. 3c, only *A. montana* and *G. robusta* extracts were able to inhibit the production of IL-12 in response to LPS to significant levels (2.8-fold, $P < 0.01$ and 1.3-fold, $P < 0.05$, respectively).

Effect of plant extracts on NF-κB activation

Activation of NF-κB is the key event for induction of all major inflammatory mediators. Both iNOS and COX-2 are the NF-κB inducible enzymes. Production of pro-inflammatory cytokines is also regulated by NF-κB. It was also observed that the plant extracts were able to inhibit NF-κB activation in LPS-stimulated macrophages as indicated by immunoblots (Fig. 4a). Densitometric analysis of the blots (Fig. 4b) showed that LPS stimulation radically increased NF-κB activation through the nuclear translocation of the p65 subunit of NF-κB. This phenomenon was significantly inhibited ($P < 0.05$) in cells treated with the extracts of *G. robusta*, *S. nigra*, *A. Montana*, or *Q. amara*. Among these, *Q. amara* showed the highest inhibition (2.08-fold, $P < 0.01$) nuclear translocation of NF-κB. These results were also confirmed by the immunostaining of treated cells with antibody against NF-κB p65 subunit as shown in the Fig. 5.

Discussion

Plant kingdom is a rich source of active components that lead to the discovery and development of numerous agents that can be used as medicine against several diseases [4]. Despite the recognized effects of several traditionally used

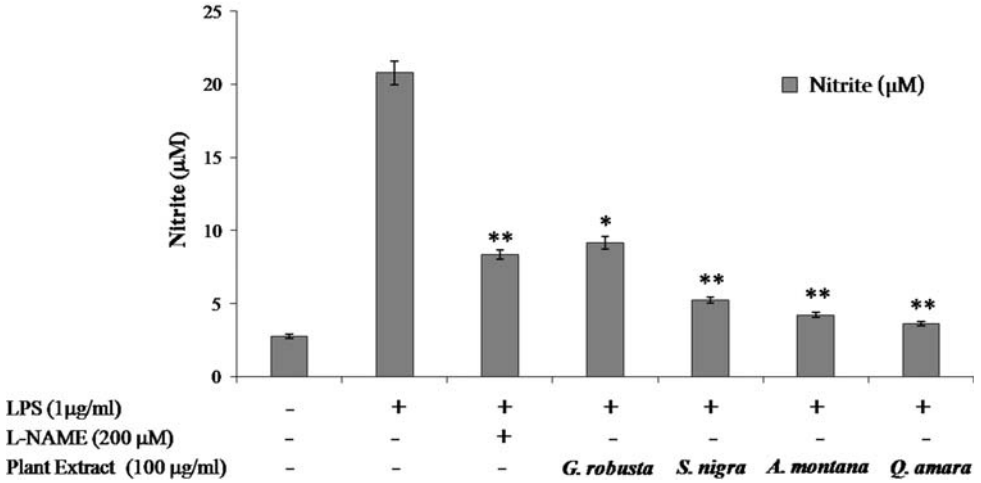

Fig. 1 Inhibition of nitric oxide production measured as nitrite in the cell free culture supernatants of LPS (1 μg/ml) challenged J774 murine macrophages. Experimental details are as given under "Material and methods" and NO concentration in the culture supernatant was determined by Griess assay using sodium nitrite standard curve. Data are presented as means ± SD from three sets of independent experiments. * $P < 0.05$, ** $P < 0.01$ represent significant difference compared with cells treated with LPS alone

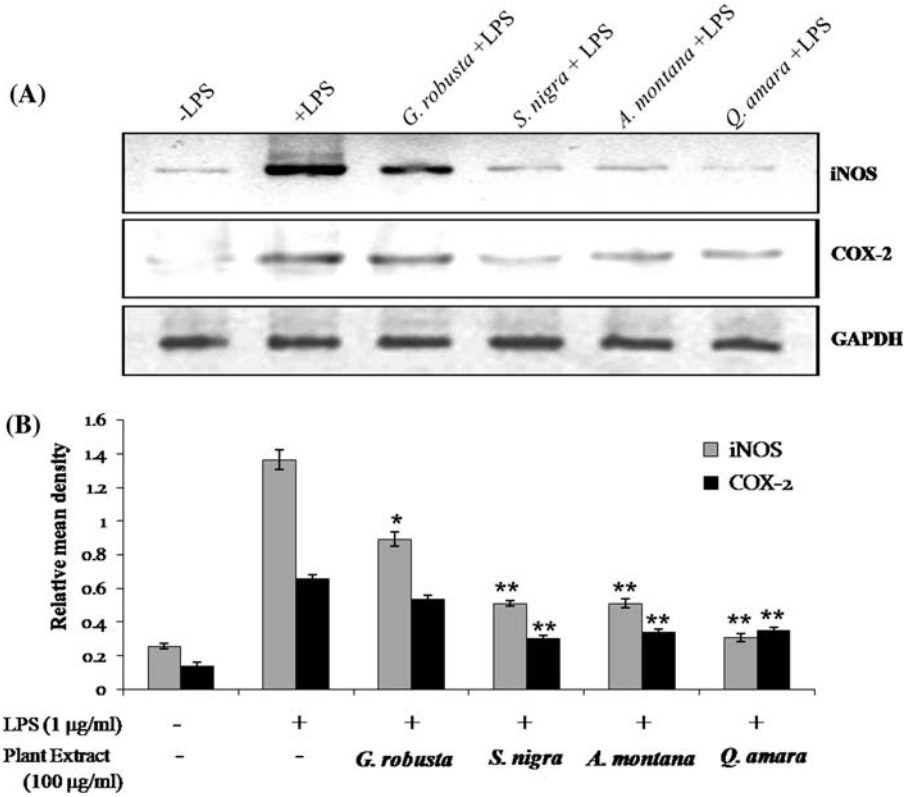

Fig. 2 Inhibition of iNOS and COX-2 enzyme levels in the J774 cells. Cells were treated with indicated concentration of extracts for 24 h. **a** Protein levels of iNOS and COX-2 in the cell lysates were determined using Western blot analysis and GAPDH was used as control. **b** Densitometric analysis of protein bands; data are presented as means ± SD from three sets of independent experiments. Experimental details are as given under "Material and methods". * $P < 0.05$, ** $P < 0.01$ represent significant difference compared with cells treated with LPS alone

medicinal herbs, their pharmacological activities have not been thoroughly investigated regarding their immunological effects. In this study, we have screened and selected whole plant methanolic extracts of some medicinal plants and examined their effects on the production of LPS-induced inflammatory mediators in J774 murine macrophage cell line. Initial screening of plant extracts was performed on the basis of their ability to inhibit the LPS-induced NO production by the macrophage. The cytotoxicity of all the plant extracts was determined on macrophage cells by MTT assay, and four active extracts that did not affect the cell viability up to a concentration of 500 μg/ml were selected for further studies (results not shown). A variety of stimuli, such as with LPS, TNF-α, and IFN-γ can result in the production of massive amount of NO by the activated macrophages which can participate in

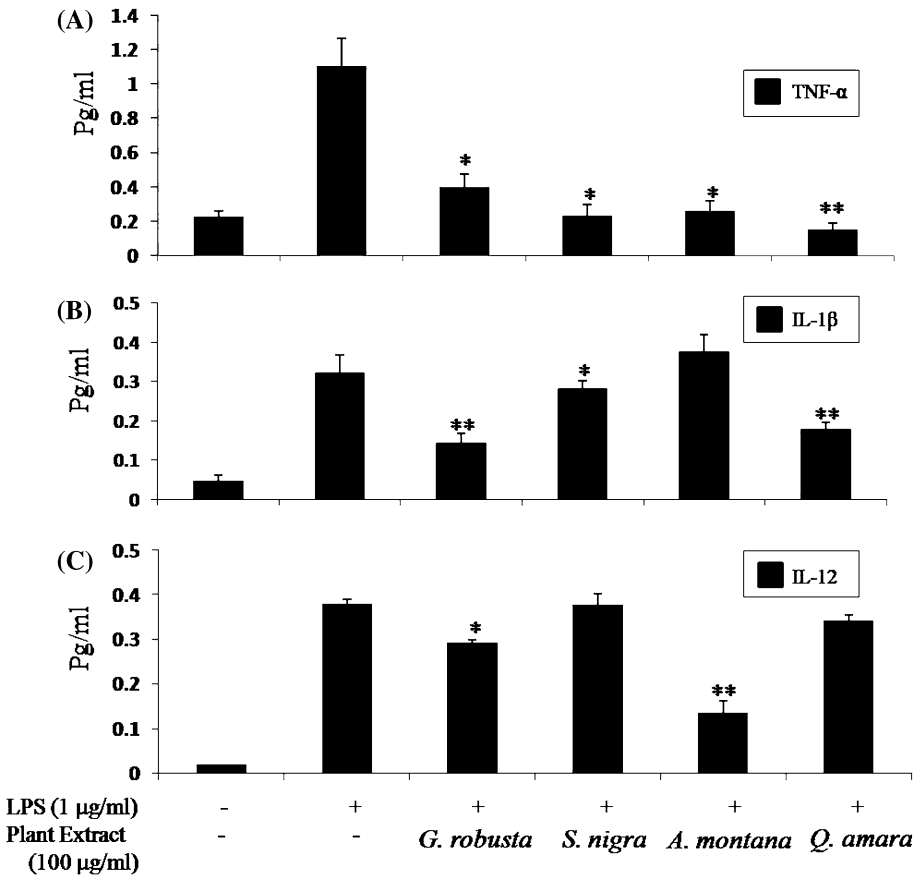

Fig. 3 Inhibition of pro-inflammatory cytokines production by J774 cells. Culture supernatants from treated cells were immunoassayed for TNF-α (panel **a**), IL-1β (panel **b**), and IL-12 (panel **c**) production by ELISA. Data are given as means of means ± SD from three sets of independent experiments. * $P < 0.05$, ** $P < 0.01$ represent significant difference compared with cells treated with LPS alone

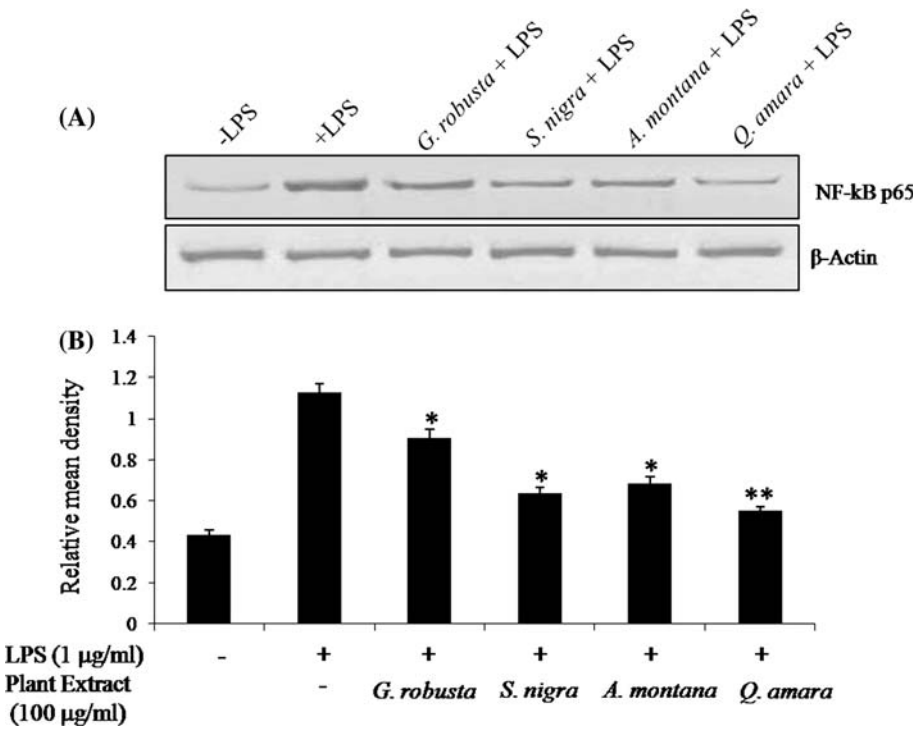

Fig. 4 Inhibition of LPS-induced nuclear translocation of NF-κB in J774 cells. Cells were treated with extracts in the absence or presence of LPS (1 µg/ml) for 30 min as described under "Material and methods". **a** The levels of NF-κB, protein in the nucleus were analyzed by Western blot. This is a representative of three independent experimental set of blots; **b** relative densities of specific genes with the housekeeping gene β-actin. Data represent the means ± S.D.; * $P < 0.05$, ** $P < 0.01$ compared with LPS alone

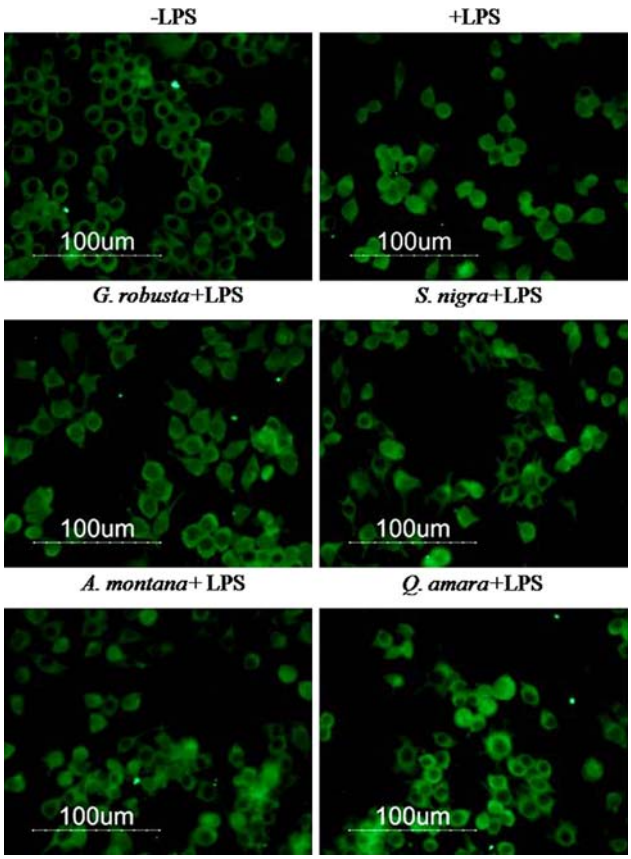

Fig. 5 Inhibition of LPS-induced nuclear translocation of NF-κB in J774 cells as visualized by immunostaining of NF-κB using FITC-conjugated antibodies. The *green fluorescence* indicates the location of p65 protein (magnification $\times$40). A representative figure from three independent sets of experiments is shown

the pathological processes in several acute and chronic inflammatory disorders [21–23]. Therefore, drugs that decrease NO production by transcriptional down-regulation of iNOS gene expression, inhibiting the iNOS gene, major receptors for signaling initiated by LPS, depletion of arginine substrate by arginase, and/or enzyme activity, have appreciable therapeutic effect in the treatment of all major inflammatory and infectious diseases including some neurological diseases [24–26]. In this study, we demonstrate that the plant extracts under study significantly inhibited NO production (Fig. 1) in LPS-stimulated macrophages. Moreover, the inhibitory activity of these extract on LPS-induced NO production was found to be associated with the suppression of iNOS protein expression (Fig. 2). Similarly, COX-2 is an inducible enzyme that catalyzes the production of prostaglandins, which contribute to the inflammatory process and tissue damage. The induction of prostaglandin production by LPS in macrophages is primarily due to the transcriptional activation of the COX-2 gene [27, 28]. It is reported that COX-2 can also be activated by high concentrations of nitric oxide, contributing towards more intense inflammatory responses as seen in many chronic inflammatory disorders [29]. Several natural products of plant origin have been shown to transmit their anti-inflammatory activities through suppression of COX-2 [30], however, for that suppression of nitric oxide production is critical. It was observed that all the plant extracts were able to suppress the COX-2 protein expression in addition to iNOS in LPS-stimulated J774 macrophage cells (Fig. 2). The production of TNF-α and pro-inflammatory interleukins such as IL-1β and IL-12 is a crucial part of the immune response to many inflammatory stimuli. For instance, overproduction of these mediators could be detected in both acute (septic and hemorrhagic shock) [30], as well as chronic (rheumatoid arthritis, atherosclerosis) inflammatory disorders [8–10]. Recently, new approaches on the use of herbal products for the treatment of inflammatory diseases by inhibiting inflammatory cytokines such as TNF- α and interleukins [31] has become an essential area of investigation because of their associated complications. Our results revealed that each of the plant extract could remarkably suppress the production of more than one inflammatory cytokine from LPS-activated macrophages (Fig. 3).

In monocytes/macrophages engagement of toll like receptor-4 (TLR-4) with LPS or other microbial products is known to trigger several intracellular signal transduction cascades. Among the most prominent and best characterized of these is the activation of pro-inflammatory IκB kinase (IKK)-NF-κB pathway leading to NF-κB activation [17, 32]. TLR-induced NF-κB activation represents a critical component of innate host defense system, which is phylogenetically conserved [33]. This signaling pathway induces many genes that encode inflammatory mediators through the activation of transcription factor NF-κB [34]. Therefore, these signaling molecules may represent novel targets for the treatment of patients with inflammatory diseases [35]. The most probable mechanism by which these plant extracts inhibit the expression of these pro-inflammatory mediators seems to involve the inhibition of NF-κB activation. It is evident from our results (Figs. 4, 5) and it was verified that the anti-inflammatory effects caused by these plant extracts are through blockade of NF-κB nuclear translocation and thus, the inhibition of LPS-stimulated expressions of the iNOS and COX-2 genes and production of cytokines. In addition to NF-κB activation, TLRs can also initiate mitogen-activated protein kinase (MAPK) signaling cascades that induce phosphorylation of p38, ERK1/2, and c-Jun NH2-terminal kinase (JNK) and thus activation of several other transcription factors, including activator protein 1 (AP-1) and Elk-1 [32, 36, 37]. However, the direct role of these transcription factors is not well established in transcriptional activation of pro-inflammatory genes in response to LPS stimulation but the

possibility of inhibition of transcription factor(s) other than NF-κB by these plant extracts cannot be ruled out.

In conclusion, this study demonstrated that the methanolic extracts of *G. robusta*, *S. nigra*, *A. montana*, and *Q. amara* inhibit the production of NO, TNF-α, IL-1β, and IL-12 in LPS-stimulated macrophages. The anti-inflammatory effect was also found to achieve by the suppression of the production of iNOS and COX-2 inflammatory mediators. One of the possible mechanisms principally involved in the anti-inflammatory effects of these extracts seems to be the blocking of LPS mediated NF-κB activation. Therefore, these crude extracts from traditionally used medicinal plants are valuable source of some new potential anti-inflammatory compounds that has not been yet explored. These extracts may have therapeutic potential for the modulation and regulation of macrophage activation, and may provide safe and effective treatment options for a variety of inflammation-mediated diseases. However, therapeutic potential of these plant extracts will be further clear when the experiment will be carried out with isolated active principles from the extracts.

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