Inhibition of Nitric Oxide Production and Proinflammatory Cytokines by Several Medicinal Plants

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ABSTRACT

Background: A number of medicinal plants have been used to treat various immunological diseases. Nitric oxide (NO) has an important regulatory role in the various types of inflammatory processes. **Objective:** To investigate the NO modulatory activity of the extracts of several medicinal plants native to Iran including *Dracocephalum kotschyi*, *Linum persicum*, *Dionysia termeana, Salvia mirzayanii*, *Ferulago angulata* and *Euphorbia cheiradenia*. **Methods:** The methanolic extracts of the plants were prepared and examined for their effects on the NO production by lipopolysaccharide-stimulated mouse macrophages. The level of TNF-α and IL-1β pro-inflammatory cytokines in the macrophage culture were detected using enzyme-linked immunosorbent assay. **Results:** All the extracts at concentration of 50 μg/ml demonstrated a significant decrease in NO production ($p<0.001$) after a 24-hour treatment. This inhibitory effect was also seen after 48 hours. Among the extracts, *L. persicum* was the strongest extract in reducing the NO production at 1 μg/ml after both 24 and 48-hours (nearly 100% inhibition, $p<0.001$). *S. mirzayanii* extract with 66.2 ± 8% inhibition at 50 μg/ml, showed the mildest effects in 48 hour culture. In cytokine release determination, the extract of *L. persicum* significantly inhibited both TNF-α and IL-1β cytokines production by stimulated macrophages ($p<0.001$). *D. kotschyi*, *D. termeana* and *F. angulata* decreased secretion of IL-1β from the cells. **Conclusion:** These results indicate the presence of anti-inflammatory and macrophage inhibitory substances in these plants.

Keywords: Cytokines, Medicinal Plants, Nitric Oxide

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INTRODUCTION

In various investigations, several medicinal plants with immunomodulatory effects have been found and reported to be used in organ transplant rejection and in the treatment of different autoimmune diseases such as rheumatoid arthritis (1-2). The capacity of medicinal herbs particularly on the inhibition of cellular and humoral immune responses could have useful applications in these immune–mediated disorders (3). Both activation of T and B lymphocytes and macrophages play critical roles in pathogenesis of these disorders (4-5). Macrophages are involved in the inflammatory processes by generation of pro-inflammatory cytokines and other inflammatory mediators such as nitric oxide (NO) (6). NO is a highly reactive molecule generated endogenously from L-arginine by a family of NO synthase (NOS) isoenzymes (7). The three main isoenzymes are neuronal, inducible and endothelial NOS. The isoform present in macrophages is an inducible form (iNOS), able to produce high concentrations of NO in many cells after stimulation with bacterial endotoxins or a variety of proinflammatory cytokines including TNF-α, IFN-γ, IL-1, GM-CSF and IL-6 (8). Generation of NO in response to cytokines is part of the inflammatory response and contributes to tissue damage in inflammatory and infectious diseases including septic shock and stroke (9-10).

Increased expression of iNOS and enhanced formation of NO that correlates with disease activity have been shown in peripheral blood mononuclear cells from patients with autoimmune diseases (11). In addition, macrophage NO has been shown to be a key mediator of apoptosis and an important regulator of the Th1/Th2 balance in such diseases (12). It also has an important antimicrobial, anti-tumor and antiviral activity (13).

Due to the diverse climate and geography in Iran, a great number of plants grow of which 1000 species are already recorded as medicinal plants. In our previous studies, the immunomodulatory effects of various medicinal plants in view of their traditional use for various medicinal purposes were studied (14-17). These plants included *Dionysia termeana, Ferulago angulata, Linum persicum, Dracocephalum kotschyi* Boiss, *Salvia mirzayanii* and *Euphorbia cheiradenia*. In the present study the NO modulatory activity of these plants is reported.

MATERIALS AND METHODS

Plant Materials and Preparation of the Extracts. The aerial parts of the plants were collected from Fars province and identified by Mr. Azizolah Jafari from the Department of Botany, Central Research of Natural Resource and Animal Husbandry, Yasuj, Iran. The specimens were deposited in the herbarium of the above mentioned center. Plants were air dried in a shaded area, powdered and defatted with petroleum ether for 4 hours. A methanol extract was obtained by maceration of the plants in 3x1500 ml methanol at room temperature for 48 hours. The methanol extract was filtered and concentrated under reduced pressure. Dried extracts were later dissolved in DMSO followed by RPMI culture medium to obtain 20 mg/ml and mixed at 37°C for 20 minutes. This solution was passed through 0.22 μm filters for sterilization and then diluted with the medium and prepared at different concentrations.

Stimulation of Mouse Peritoneal Macrophages. Male 8-week-old mice were treated with 2 ml of 3% thioglycollate, then after 4 days peritoneal macrophages were obtained

by lavage with RPMI-1640 medium. Cells were centrifuged at 800 x g for 10 minutes and then resuspended in medium supplemented with fetal calf serum (10%) and seeded in 96-well flat-bottom plates at a density of 10^5 cells/well, the plants were then incubated at 37°C in a 95% air and 5% CO2 atmosphere. Two hours later, cells were washed once with medium to remove the non-adherent cells and the remaining cells were incubated with medium containing various concentrations of the extracts and/or previously determined optimal concentration (5 μg/ml) of the bacterial lipopolysaccharide (LPS, Difco laboratories, Mich, USA).

Assay for NO Modulatory Effect. Cells were incubated for 24 and 48 hours and then supernatant was collected. NO production was determined by measuring the optical density (OD) of nitrite accumulated in the culture supernatant at 540 nm using the Griess reagent (1% sulfanilamide, 0.1% N-1-naphthylethylenediamine dihydrochloride in 2.5% H3PO4). The concentration of nitrite was derived from regression analysis using serial dilutions of sodium nitrite as a standard. All experiments were plated in triplicate wells and were performed at least three times. The inhibition percentage was calculated according to the following formula: 100 - [(OD of treated cells/OD of control) ×100]. Control wells included extract-untreated cells stimulating with LPS and containing DMSO at the final concentration equal to test wells.

MTT Assay for Cell Cytotoxicity. For detection of cell cytotoxicity the 3-(4,5-dimethyl-2-thiazyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was performed. In this method mitochondrial respiration, an indicator of cell viability was assessed by the mitochondrial-dependent reduction of MTT to formazan. When supernatant of the wells were removed for NO measurement, cells still adhered to the wells, so cytotoxicity on the cells was determined. 10 μl of MTT (5 mg/ml) was added to the wells and plates were incubated for 4 hours at 37°C. 100 μl DMSO was then added to dissolve the formazan production and then OD was measured on a microplate reader at 570 nm. Control was extract-untreated cells stimulated with LPS and contained DMSO at the final concentration equal to test wells. Viability percentage was calculated according to the following formula: (OD of treated cells/OD of corresponding control) × 100.

Analysis of Pro-Inflammatory Cytokines Secretion by Murine Macrophages. Peritoneal LPS-stimulated macrophages were cultured in the absence or presence of varying concentrations of extracts for 48-hours as mentioned above, and the supernatant was collected. Tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) cytokines were estimated simultaneously using a commercial enzyme-linked immunosorbent assay (ELISA) kit from Biosourse (USA) according to the manufacturer's instructions. The concentration of each cytokine in supernatants was determined by using the standard curve and with a detection limit of 3 pg/ml and 7 pg/ml for TNF-α and IL-1β, respectively. The level of cytokines in each sample was calculated in relation to the extract-untreated control cells containing DMSO and LPS and presented as percent of control.

Statistical Analysis. Statistical significance was determined by SPSS software and by student's t-test. A regression model was used to predict NO concentration based on OD values. The significance level was set at 0.05.

RESULTS

Cytotoxicity Effect of the Extracts. Effects of the methanolic extracts of plants on the growth and viability of activated macrophages was assessed by MTT colorimetric assay. During experimentation in the 48-hour treatment of macrophages, some of the extracts decreased the viability of cells at concentrations of 100 µg/ml and 200 µg/ml (data not shown); However, a viability more than 90% was observed at 50 µg/ml and lower concentrations in all the extracts (except for *D. termeana* with viability of 80.3 at 50 µg/ml) (Table 1), thus these concentrations were used to evaluate their effects on the NO production by activated macrophages.

Table 1. Effects of the extracts at concentration of 50 µg/ ml on the viability and NO production of lipopolysaccharide-activated mouse macrophages after 48-hour treatment.

Extracts	% Viability after 48 hours*	NO concentration (µM)	
		24 hours	48 hours
D. Kotschyi	98.8 ± 4.5	<1.0	<1.0
L. persicum	90 ± 3.1	<1.0	<1.0
S. mirzayanii	91.5 ± 2	15.6 ± 0	12.8 ± 9.5
D. termeana	80.3 ± 9	6.6 ± 8.9	2.5 ± 4.8
F. angulata	94.5 ± 2.3	6.8 ± 2.5	4.1 ± 1.6
E. cheiradenia	98.4 ± 0.9	9.8 ± 6.7	2.7 ± 3.6

*Percent Viability was determined by MTT assay. Data represent mean ± SD of three different experiments.

Effects of the Extracts on NO Production. LPS-stimulated macrophages were studied for NO release in the presence of the extracts. The spontaneously nitrite generation by macrophages treated only with the solvent (no extract, no LPS) after 48 hours was little (<1 µM), whereas stimulation of the cells with LPS resulted in a greatly increased NO generation to the level of 23.6 ± 15.6 and 34.9 ± 7.6 µM after 24 and 48 hours, respectively. Changes in NO production in cell culture supernatants measured after 24 and 48 hours are shown in Figure 1. The level of NO release at 50 µg/ml of the extracts along with the % viability of the cells at this concentration is presented in Table 1. Results obtained for each extract was as follows.

***D. kotschyi*:** This extract at concentrations of 0.1 and 1 µg/ml reduced the production of NO by macrophages after 24 hours (% inhibition= 38.9 ± 14.9 and 51.2 ± 1.4,

respectively) (Figure 1). As the concentration of the extract in the culture medium increased to 50 µg/ml, NO production was nearly 100% inhibited (<1 µM) (Table 1).

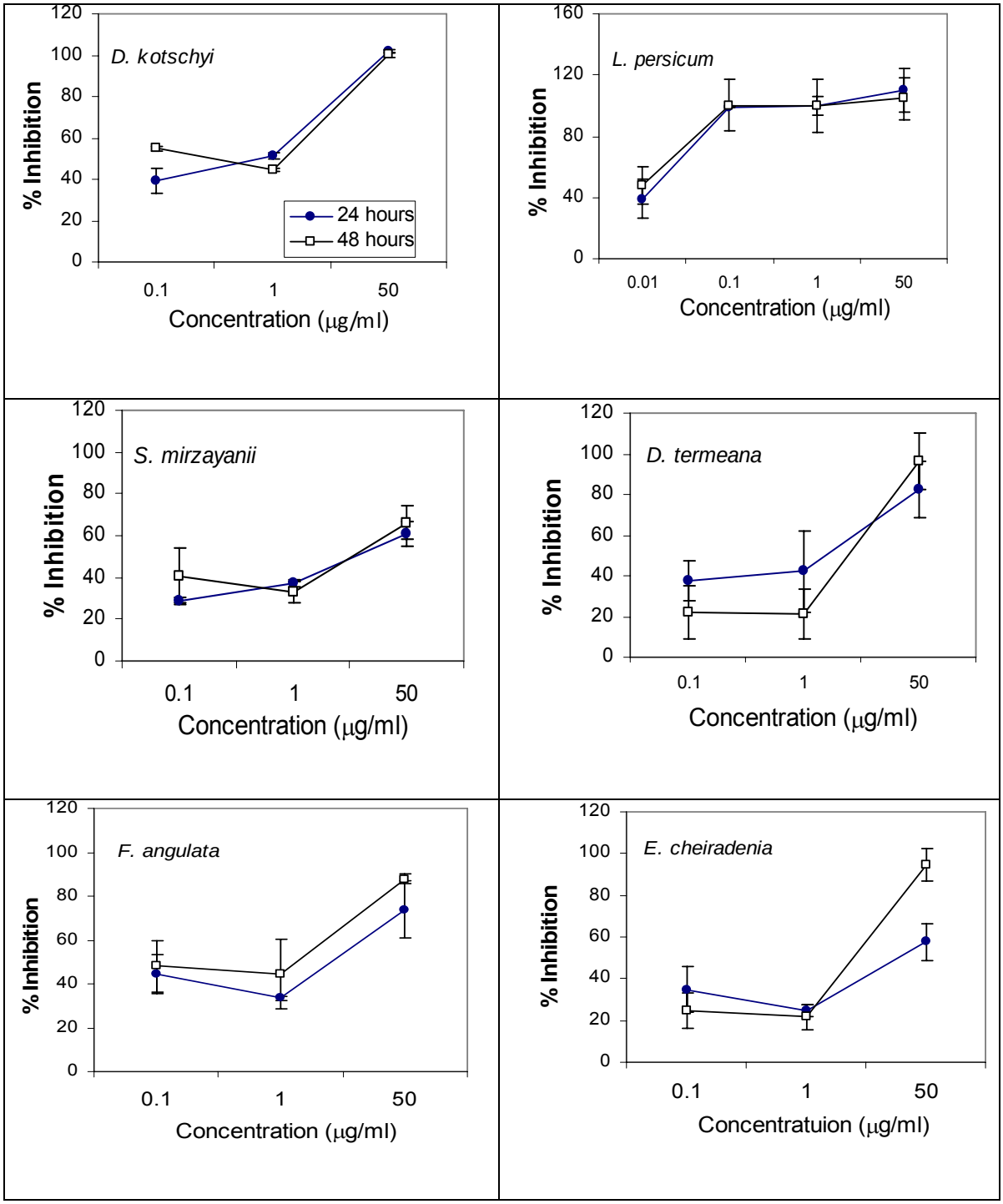

Figure 1. The effect of various concentrations of the plant extracts on the NO production by LPS-stimulated mouse macrophages at 24 and 48 hour incubation. Data represent mean ± SD of three different experiments. Filled circles (●) show % inhibition in NO production after 24 hours and (□) after 48 hours.

***L. persicum*:** The extract of this plant showed a dose dependent inhibitory effect on the NO secretion by the cells. The inhibitory effect observed after 24 hours did not almost change after 48 hours. At 50 µg/ml, the level of NO was <1 µM with nearly100% inhibition.

***S. mirzayanii*:** This extract showed inhibitory effects at all concentrations used, though there was no clear dose-response. No significant difference between NO release after 24 and 48 hours was demonstrated.

***D. termeana*:** This extract decreased NO release at all concentrations used after 24 hours of treatment. The maximum percentage inhibition of NO was 82.3 ± 14.4 at 50 µg/ml.

***F. angulata*:** In terms of this plant, significant changes was detected in NO secretion by macrophages at different concentrations of the extract, the maximum level of NO was 4.1 ± 1.6 µM after 48 hours at 50 µg/ml.

***E. cheiradenia*:** The extract of this plant at concentrations of 0.1 and 1 µg/ml in the 24-hour culture reduced the generation of NO. As the concentration increased to 50 µg/ml, 57.5 ± 9% inhibition in NO secretion was observed. This inhibitory effect was further increased to 94.7 ± 7.6% in the 48-hour culture (Figure 1).

Taken together, all the extracts demonstrated a significant decrease in NO production ($p<0.01$). The percentage inhibition of the extracts at 1 and 50µg/ml after 24 and 48 hours was compared as shown in Figure 2. *L. persicum* was the strongest extract in reducing NO generation at 1 µg/ml in both 24- and 48-hour culture with 104.6 ± 14% and100.2 ± 6.1% inhibition, respectively. Among the extracts *S. mirzayanii* with 66.2 ± 8% inhibition indicated the mildest effects at 50 µg/ml in 48 hour-culture.

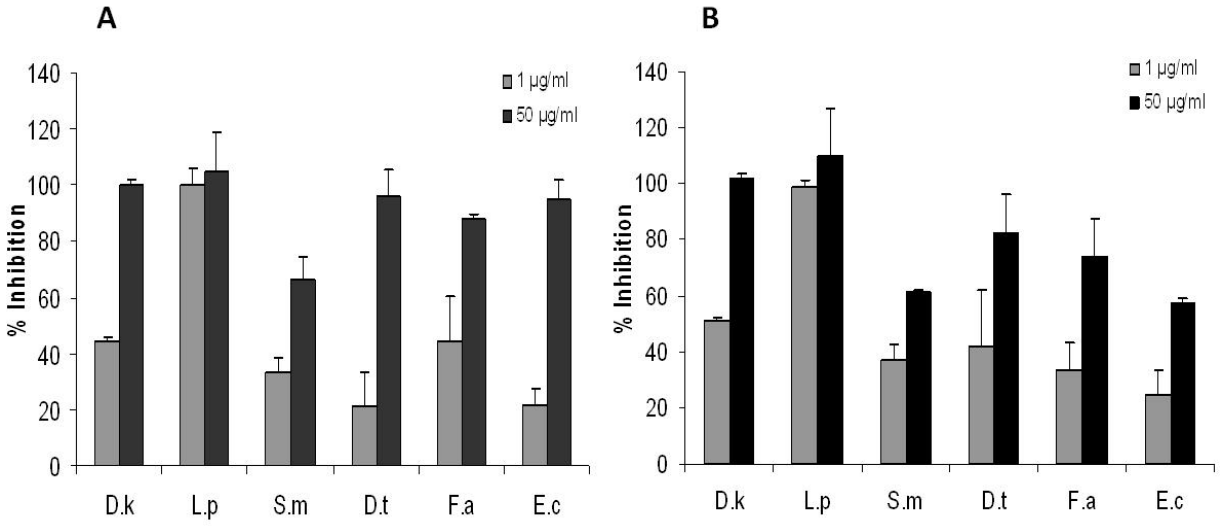

Figure 2. Inhibition of NO production at two concentrations of the extracts on the stimulated macrophages after 24 hour (A) and 48 hour (B) treatment. Data represent mean ± SD of three different experiments. D.k; *Dracocephalum kotschyi*, L.p; *Linum persicum*, D.t; *Dionysia termeana,* S.m; *Salvia mirzayanii*, F.a; *Ferulago angulata* and E.c; *Euphorbia cheiradenia.*

Effects of the Extracts on Production of Pro-Inflammatory Cytokines. The production of TNF-α and IL-1β stimulated by LPS were measured after 48-hour treatment of macrophages with concentrations of 0.1-1 μg/ml of the extracts (Figure 3).

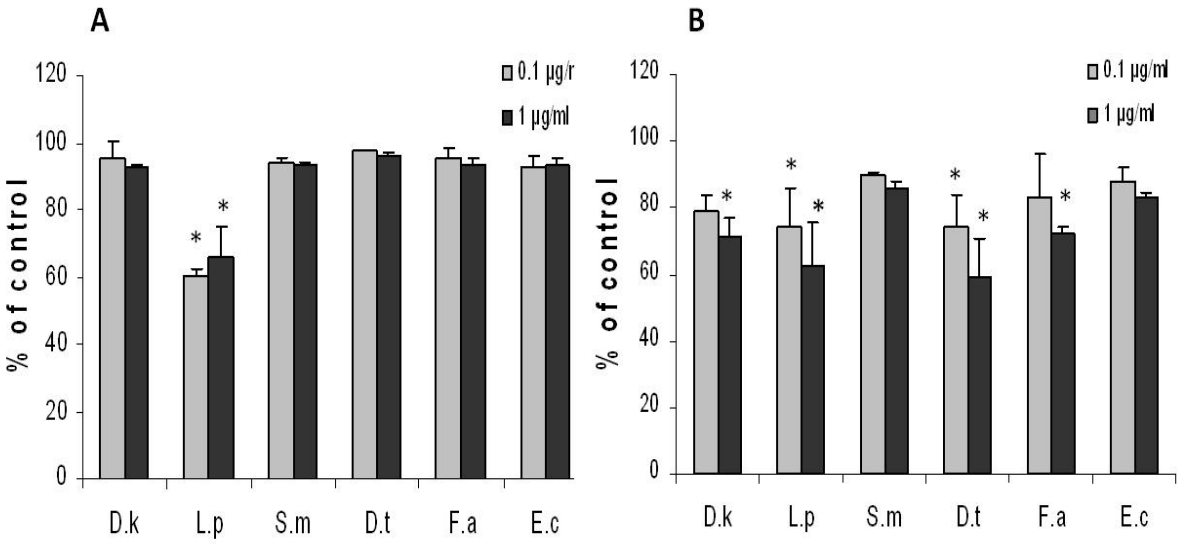

Figure 3. The effect of two concentrations (μg/ml) of the plant extracts on LPS-induced TNF-α (A) and IL-1β (B) production in mouse macrophages. Data represent mean ± SD of two separate experiments. The level of cytokines in each sample measured by enzyme-linked immunosorbent assay was calculated in relation to the control taken to be 100%. The control contains DMSO as the solvent and LPS but not the extract. D.k; *Dracocephalum kotschyi*, L.p; *Linum persicum*, D.t; *Dionysia termeana,* S.m; *Salvia mirzayanii*, F.a; *Ferulago angulata* and E.c; *Euphorbia cheiradenia.* Asterisks represent statistically significance inhibition of cytokines by the extracts compared to the control.

The level of TNF-α and IL-1β in extract-untreated control cells were 1625 ± 63 pg/ml and 1006 ± 158 pg/ml, respectively. Among the extracts, *L. persicum* significantly decreased both TNF-α and IL-1β production. The level of TNF-α and IL-1β secretion in the culture of cells treated with 1 μg/ml of this extract was 66.2 ± 8.9% and 62.3 ± 13.3% of the control, respectively ($p<0.01$). *D. kotschyi,* also slightly decreased the level of TNF-α at 1 μg/ml (1514 ± 87 pg/ml) compared to the untreated control (1625 ± 63 pg/ml) but the result was not significant ($p=0.09$). A significant decrease in the level of IL-1β in the presence of 0.1 and 1 μg/ml of this extract was detected (78.9 ± 4.7% and 71.5 ± 5%) ($p<0.017$). In comparison, the LPS activated production of IL-1β but not TNF-α was inhibited by *D. termeana* in macrophages. The response induced by 0.1 and 1 μg/ml of this extract was 73.9 ± 10% and 58.7 ± 14.7% of the control, respectively ($p<0.01$). *F. angulata* was the other extract with significant inhibitory effect on IL-1β production at 1 μg/ml. Although, other extracts, reduced the level of particularly IL-1β cytokine production in macrophages but the level did not reach to significant.

DISSCUSION

In the present study methanolic extracts obtained from six medicinal plants native to Iran were investigated for their effects on the LPS-induced NO production in activated peritoneal macrophages. For this purpose, first the cytotoxic activity of the extracts against macrophages were studied and then their effects on the NO production was evaluated.

The plants included in our study have previously demonstrated various immunomodulatory effects. One of these plants, *E. cheiradenia* (Euphorbiaceae), is known as *farfion* in Persian and is native to Iran. This plant grows wild on the slopes of Dena Mountain in north of Shiraz in Fars proviance. In Iranian traditional medicine, this plant is used for treatment of infectious diseases such as syphilis. In a previous study this herb showed inhibitory effects on the allogenic and mitogenic lymphocyte proliferation as well as on the growth of several tumor cell lines (18). The other plant, *F. angulata*, is known as *Chovil* (Apiaceae) in Persian, a perennial, herbaceous plant that grows in different areas of Iran including southern parts and is used for infectious diseases in traditional medicine (19). In our study, both of these plants showed significant changes in the level of NO compared to the control at both 24 and 48 hours treatment. It is noted that generally no significant difference between NO release after 24 and 48 hours was demonstrated in the extract-untreated stimulated macrophages, indicating that 24 hours was enough to reach NO release to maximum level. Since both of *E. cheiradenia* and *F. angulata* extracts demonstrated no cytotoxic activity against the macrophages at the concentrations used, the decreased NO production should not be attributed to their cytotoxic effects.

D. termeana, a plant belonging to Primulaceae family, is known as *aros-e-sang* in Persian. This plant has been employed against ulcers, insect bite, as well as diarrhea in folk medicine (20). *D. termeana* showed a decrease in the NO production at all concentrations. Since the viability of macrophages in the presence of 50 μg/ml of this extract was slightly less than the control, the observed inhibitory effect at this concentration could be partly due to its effect on the viability. In our previous study, the inhibitory activity of this plant against proliferated lymphocytes and different tumor cell lines has been reported (21).

Three other plants including *D. kotschyi*, *L. persicum* and *S. mirzayanii* showed also inhibitory activities. *D. kotschyi* belongs to Labiatae family and is known as *badarnjboye* (22) in Persian. The aerial parts of this plant continue to be traditionally used to treat rheumatoid arthritis in Iran. A number of constituents have already been isolated from this plant, and include monoterpene glycosides and trypanocidal terpenoids (23). In our previous study, immunomodulatory effects of this herb have been shown (24). The inhibitory effects of this plant on NO as well as pro-inflammatory cytokines production can justify the therapeutic usefulness of *D. kotschyi* in rheumatoid arthritis which is an inflammatory disorder.

S. mirzayanii known as *maryam-goli* in Persian is native to Iran and widely distributed in this country. This plant is used for the treatment of infectious and inflammatory diseases and as a tonic in folk medicine. The antimicrobial activity of *S. mirzayanii* has been shown to be related to the sesterterpenes isolated from the aerial parts of the plant (25). *S. mirzayanii* has been shown to induce apoptosis in the lymphocytes (26). In our previous study, a sesterterpene known as spathulenol was isolated from this plant.This compound showed the capacity to inhibit proliferation in the lymphocytes and induce

apoptosis in these cells possibly through a caspase-3 independent pathway (27). This plant mildly decreased the NO secretion compared to the others. The traditional use of this plant could be related to its immunomodulatory effects on both lymphocytes (26-28), and macrophages as shown in this study.

In terms of *L. persicum*, previous studies have shown anti-microbial and anti-tumoral affects for this plant and other Linum species (29). *L. persicum*, known as *katan* in Persian is used for its antimicrobial and anti-inflammatory effects in folk medicine. A main constitute of Linum species is lignans derived from podophyllotoxin (29). These compounds are responsible for the cytotoxic activity against various tumor cells reported for these plants (30). As shown in our study, *L. persicum* at concentrations which had no effect on the viability of macrophages inhibited NO release. This allowed us to assume that the decrease in NO secretion was not due to the cytotoxic ability reported for this plant. Among the extracts, *L. persicum* was the strongest one in reducing the NO production in both 24 and 48 hour-culture at 1 μg/ml, indicating the potential activity of this herb on the macrophages.

Under the stimulation of LPS, macrophages produce NO (31). NO generation involves several steps including the activation of nuclear factor (NF)-κB and subsequent iNOS mRNA expression (32). NF-κB activation can be regulated by various cytokines; the most important is TNF-α. Activated monocytes and/or macrophages release a variety of inflammatory cytokines such as TNF-α and IL-1β (33). The production of TNF-α is important for the induction of NO synthesis in LPS-stimulated macrophages (34). Among the extracts, *L. persicum* decreased production of TNF-α, indicating that the reduced level of NO production observed in macrophages culture, might be related to suppression of TNF-α release by this extract. *D. kotschyi* also decreased the level of TNF-α but this effect was not significant. Both of these plants decreased production of IL-1β, which is known to play a crucial role in inflammatory response, a biologic function very similar to TNF-α and involved in the pathogenesis of inflammatory diseases (35). Inhibition of TNF-α and IL-1β secretion from macrophages by these extracts indicate their capacity to diminish immune reactions and provide further evidence that these plants may have potent immunomodulatory properties. In terms of *D. termeana* and *F. angulata*, a decrease in secretion of IL-1β but not TNF-α by stimulated macrophages was detected. It has been shown that regulation of IL-1β but not TNF-α is through MKK3 kinase that in turn activates p38 mitogen-activated protein (MAP) kinase (36). Thus, the differential effect of these extracts on TNF-α versus IL-1β may be related to differential activity of the extracts on the signaling pathways.

In order to understand the mechanism involved in modulation of NO release and cytokine production by these medicinal plants, it is necessary to study the expression of iNOS and NF-κB activation. NF-κB is a key factor regulating the expression of inflammation-associated enzymes and cytokine genes, such as iNOS, COX-2, TNF-α and IL-1β, which contain NF-κB binding motifs within their respective promoters (37).

In conclusion, the present study showed all the extracts down-regulate the release of NO by mouse macrophages. In case of some of the extracts, this effect is likely to be due to inhibition of pro-inflammatory cytokines. The NO down-regulation may reduce pathological harms arise due to an excess in NO production in inflammatory processes. Further study is needed to complete these data and to identify the responsible compounds.

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