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The Effect of *Artemisia fragrans* Willd: Essential Oil on Inducible Nitric Oxide Synthase Gene Expression and Nitric Oxide Production in Lipopolysaccharide-stimulated Murine Macrophage Cell Line

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ABSTRACT

The genus *Artemisia* is estimated to comprise over 800 species with anti-cancer, anti-fungal, anti-oxidant and anti-inflammatory properties. *Artemisia fragrans* (*A. fragrans*), a species that belongs to genus *Artemisia*, is rich in monoterpenes and sesquiterpenes derivatives. Due to anti-inflammatory properties of monoterpenes and sesquiterpenes, we aimed to investigate the effect of *A. fragrans* essential oil on mRNA expression of inducible nitric oxide synthase (iNOS) gene and nitric oxide (NO) production in Lipopolysaccharide (LPS)-stimulated RAW264.7 cell line. NO, which is synthesized by iNOS, is the main macrophage-derived inflammatory mediator.

The oil obtained from the *A. fragrans* was prepared from aerial parts of the plant. Chemical composition of essential oil was analyzed by gas chromatography–mass spectrometry (GC/MS).The cytotoxicity of various concentrations of essential oil was evaluated by mitochondrial reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) test assay. The effect of different doses (1.75-7 mg/mL) of *A. fragrans* oil on mRNA expression of iNOS gene and NO production in LPS-stimulated RAW 264.7 cells was assessed by real-time PCR method and Griess reagent, respectively.

In GC/MS analyses of *A. fragrans* oil, 32 compounds were identified. The main components of the oil were camphor and 1, 8-cineole. The results demonstrated that the essential oil of *A. fragrans* (1.75- 7 mg/mL), in a dose-dependent manner, inhibits mRNA expression of iNOS induced by LPS in the RAW264.7 cells without cytotoxic effect even at higher doses. The results of iNOS were consistent with the results of NO production.

Our preliminary results suggest the possible anti-inflammatory effect of *A. fragrans*. Further studies are needed to determine the full pharmacokinetics of *A. fragrans* activity in vivo.

Keywords: *Artemisia fragrans*; Essential oils; Inflammation; Macroghage; Gene expression; Nitric Oxide synthase

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INTRODUCTION

Inflammation is a physiological and protective response to infection or tissue injury. This phenomenon may be acute or chronic. Acute inflammation results in deletion of the damaging factor and accelerates healing process. If an inflammatory reaction prolongs without control, chronic inflammation occurs which results in intensive injuries of cells and tissues. Based on the evidences, chronic inflammation plays a critical role in various pathological conditions, including cardiovascular diseases, cancers, diabetes, arthritis, Alzheimer's disease, pulmonary diseases and autoimmune disorders.[1, 2]

Macrophages are the main immune system cells involved in inflammatory responses. These cells produce inflammatory mediators upon activation. Lipopolysaccharide (LPS), a component of the cell wall of gram-negative bacteria, activates macrophages to release inflammatory mediators including, nitric oxide (NO), prostaglandins (PGs) and inflammatory cytokines such as IL-1β, IL-6 and TNF-α.[3-6]

NO is generated from L-Arginine by nitric oxide synthase (NOS). Three distinct isoforms of nitric oxide synthases exist: two constitutive isoforms including endothelial nitric oxide synthase (eNOS), neuronal nitric oxide synthase (nNOS) and inducible nitric oxide synthases (iNOS).[5, 7-9] The eNOS and nNOS help to sustain homeostasis of cardiovascular and nervous system whilst the iNOS is induced in response to internal and external stimuli and mostly involved in host defense mechanisms in macrophages, smooth muscles and liver. NO production mostly influenced by overexpression of iNOS within inflammatory conditions by activated macrophages.[10] Although several medications are currently available to remedy inflammation, the applications of these drugs are limited due to several side effects and low efficiency. Hence, researches have focused to investigate alternative medications with less side effects and better efficiency.[1]

The genus *Artemisia* is a member of the *Asteraceae (Compositae)* family with more than 800 species which are mostly distributed in Asia, Europe and North America. There are about 34 annual and perennial *Artemisia* species in Iran.[11-14] This genus has numerous applications such as food preservatives and flavoring, preparation of pharmaceuticals and cosmetics.[15]. On the other hand, the pollen of *Artemisia* is one of the most important causes of seasonal allergies. So far, there are several reports that *Artemisia* can cause allergic rhinitis and asthma.[16-18]

The essential oils and extracts isolated from various *Artemisia* species are source of active compounds and secondary metabolites with anti-malarial, anti-tumor, anti-fungal, antiviral, anti-hepatitis, antispasmodic, anti-oxidant, anti-inflammatory properties and others.[9, 15] Investigations on different species of *Artemisia* have shown the presence of polyacetylenic compounds, sesquiterpene lactones, flavonoids and essential oils.[19] The terpenoids are one of the main family members of secondary metabolites that were shown to have medicinal applications for human disease. For instance, artemisinin has been administrated as the sole drug to treat malaria in most parts of the world that have become resistant to synthetic drugs.[20]

According to previous studies, different *Artemisia* species, have anti-inflammatory properties through inhibition of NO production.[7, 21-24] *Artemisia fragrans* (*A. fragrans*) is a perennial herb, which grows in the Azerbaijan, Mazandaran, Qazvin and Tehran provinces of Iran and is known for its strong fragrance.[15, 25] Phytochemical researches on the oil of *A. fragrans*, showed an abundance of monoterpens and sesquiterpens derivatives.[15, 26-28] Some studies suggest the therapeutic potential of these compounds against inflammation.[29-32] There is no report regarding anti-inflammatory activities of *A. fragrans* in the literature. Therefore, this study aimed to investigate the effect of this species' essential oil on mRNA expression of iNOS and NO production in LPS-stimulated RAW264.7 macrophages model.

MATERIALS AND METHODS

Materials

LPS (from *Escherichia coli*, serotype O111:B4), Dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), Fetal bovine serum (FBS) and L-Glutamine were obtained from GIBCO (Grand Island, NY, USA).

Extraction of Essential Oil

A. fragrans oil (IBRCP1000564) was supplied by

Iranian Biological Resource Center (IBRC, Haligerd, Iran). The fresh aerial parts (stem and leaf) of *A. fragrans* sample were air-dried at room temperature (30±5ºC) and then grounded. The essential oil was extracted from 100 grams of sample by hydrodistillation using a clevenger type apparatus for three hours.[25] The collected essential oil was dried over anhydrous sodium sulphate and stored at 4˚C until analyzed.

Gas Chromatography-Mass Spectrometry (GC-MS) **Analysis of Essential oil**

The analysis of the essential oil's composition was carried out using an Agilent 6890 gas chromatography system (Agilent Technologies, CA, USA), with a BPX5 capillary column (30 m×0.25mm×0.25 µm film thickness), equipped with an Agilent 5973 mass selective detector. For GC-MS analysis, an electron ionization system with ionization energy of 70 eV was used. Carrier gas was helium at a flow rate of 0.5 mL/min. The oven temperature was programmed at 50 ºC for 5 min initially and increased at the rate of 3 ºC /min to 240 ºC, and finally raised to 300 °C at 15 °C/min. Injector and detector temperatures were set at 220 and 290 °C, respectively. 1 μ L of diluted samples (in n-hexane) was injected manually in the splitless mode. The components of the oil were identified by comparing their mass spectra with those of Wiley /NBS Registry of Mass Spectral Database libraries and with authentic compounds; and then confirmed by comparison of their retention indices with authentic compounds or with the compounds reported in literature. [33] The retention indices were calculated for all volatile constituents using a homologus series of n-alkanes injected under conditions equal to those of samples.

Cell Culture

RAW264.7 cell line (IBRCC10072), a BALB/c-derived murine macrophage cell line, was purchased from the Iranian Biological Resource Center (IBRC, IRAN, Tehran). The cells were maintained in DMEM supplemented with 10% FBS, 2mM L-Glutamine, without antibiotics, at 37˚C in a humidified atmosphere of 95% air and 5% CO_2. For all experiments, the cells were grown to 80–90% confluency and were subjected to no more than 20 cell passages.[22, 34-36]

Cytotoxicity Assay

The effect of *A. fragrans* essential oil on the viability of RAW 264.7 cells was evaluated by MTT assay. The mitochondrial-dependent reduction of MTT to formazan, a yellow product that is soluble in cell culture medium, was used to measure cell respiration as an indicator of cell viability.[37] Briefly, RAW 264.7 cells were seeded in 96-well plates at the density of 5×10^4 cells/100 µL medium/well at 37˚C to reach 70-80% confluency. After 24 h of incubation, the adhered cells were treated with different concentrations of the essential oil (1.75-7 mg/mL). Twenty-four hours later, after changing the medium, MTT was added to a final concentration of 0.5 mg/mL, and the cells were incubated for 4 h at 37˚C and 5% CO_2. After incubation, the medium was removed and the formazan precipitate was solubilized in DMSO. The absorbance was measured at 570 nm on a microplate reader (Biotek, Winooski, VT, USA).

Measurement of NO Production by LPS-induced Macrophages

The RAW 264.7 cells were seeded at a density of 5×10^5 cells/well in 24 well plates and incubated for 18 h at 37˚C and 5% CO_2.[38] The cells were stimulated with LPS (1 µg/mL) for 20 h. After incubation, the cells were treated with *A. fragrans* essential oil (1.75, 3.5 and 7 mg/mL) for 24h. The quantity of nitrite in the culture medium was measured using commercial NO detection kit (Sigma, St. Louis, MO, USA). Briefly, 100 µL of cell culture medium was mixed with 100 µL of Griess reagent and incubated at room temperature for 10 min. Then the absorbance was measured at 540 nm in a microplate reader. The amount of nitrite as an indicator of NO production was determined on the basis of a sodium nitrite standard curve.

Extraction of RNA and Quantitative Real-time PCR (*q*PCR) Analysis

Total RNA was isolated from fresh cells using the RNeasy Plus Mini kit (Qiagen, Valencia, USA).The purity and concentrations of RNA were determined using the ND-3800 spectrophotometer (Nano-drop Technologies, Hercuvan, Malaysia). For cDNA preparation, total RNA (2 µg) was reverse transcribed using a Prime Script RT reagent kit (Takara Bio Inc, Tokyo, Japan) according to the manufacturer's protocol. Following RNA extraction and cDNA synthesis, *Q*pcr was performed on a Step One real-time PCR instrument (Applied Bio systems, Foster City, USA) using 5×

HOT FIREPol EvaGreen *q*PCR Mix Plus (ROX) kit (Solis BioDyne, Estonia). The *q*PCR steps were as follows: initial denaturation at 95˚C for 15 min; amplification for 40 cycles of denaturation at 95˚C for 15 s, annealing at 61˚C for 20 s, extension at 72˚C for 20 s. Analysis of melting curve confirmed the specificity of amplicons. The specific primers used to determine the expression levels of these mRNAs via *q*PCR are listed in Table 1.

Table 1. Primer sequences used in quantitative real-time PCR for assesing iNOS mRNA expression level

Target	Type	Sequences
GAPDH	Sense	5´-GACTTCAACAGCAACTCCCAC-3´
	Anti-sense	5´-TCCACCACCCTGTTGCTGTA-3´
iNOS	Sense	5´-TCACGCTTGGGTCTTGTTCA-3´
	Anti-sense	5´-TCCAAATCCAACGTTCTCCGT-3´

Statistical Analysis

Statistical analyses were performed in Prism 6 (Graph Pad Software, Inc. San Diego, USA) using one-way analysis of variance (ANOVA).A p value of <0.05 was considered statistically significant.

RESULTS

Chemical Composition of *A. fragrans* Essential Oil

Thirty-two compounds were identified in the essential oil, representing 93.5 % of the total composition (Table 2). The oil contained monoterpenoids (90.52%) and sesquiterpenoids (0.75%) constituents. The main compounds of the essential oil were camphor (31.84%) and 1, 8-Cineole (29.01%), respectively. According to these results, the essential oil of *A. fragrans* is rich in monoterpenoids.

Effect of *A. fragrans* Essential Oil on RAW 264.7 Cell Viability

MTT test analyses indicated that treatment of RAW 264.7cells with low concentrations (up to 7 mg/mL) of *A. fragrans* essential oil had no toxic effect on cells. Therefore, concentrations of 1.75-7 mg/mL of *A. fragrans* essential oil (cell viability> 90%) were chosen for further experiments (Figure 1).

Effect of *A. fragrans* Essential Oil on LPS-induced NO

The effect of *A. fragrans* essential oil on NO production was evaluated in LPS-stimulated RAW 264.7 cells. Treatment of cells with LPS (1 μg/mL) for 20 h, increased the production of nitric oxide. However, treatments of LPS-induced cells with non-toxic concentrations of *A. fragrans* essential oil (7, 3.5 and 1.75mg/mL), significantly reduced NO production in a dose-independent manner (p<0.0001), compared with LPS-treated cells. The greatest decrease was observed with 7 mg/mL *A. fragrans* treatment (Figure 2).

Effect of *A. fragrans* Essential Oil on mRNA Expression Levels of iNOS Gene

The mRNA expression of iNOS in the cells treated with *A.fragrans* oil was determined by real-time PCR method. Unstimulated RAW264.7 cells (as negative control group) did not exhibit mRNA expression of iNOS, whereas LPS-stimulated cells (as positive control group) strongly increased the expression of iNOS. The addition of *A. fragrans* essential oil (7, 3.5

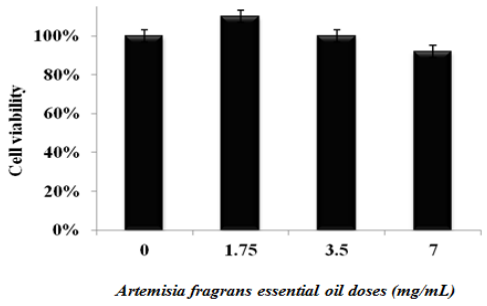

Figure1. Effects of *Artemisia fragrans* essential oil on cell viability. RAW264.7 cells were treated with various concentrations of *A. fragrans* essential oil for 24 h. Cell viability was determined by MTT assay as described in materials and methods. Cell viability in absence of *A. fragrans* treatment was taken as 100%. The results were expressed as mean±SD of three independent experiments.

Table 2. Chemical composition of the essential oil from *Artemisia fragrans*.

Peak.No.	Compounds	KI[a]	Peak area (%)
1	2E-Hexenal	860	0.09
2	α-Thujene	923	0.24
3	α-Pinene	934	0.7
4	Camphene	952	4.85
5	Sabinene	975	0.37
6	β-Pinene	980	0.37
7	Myrcene	992	0.06
8	Mesitylene	1000	0.18
9	α-Phellandrene	1010	0.72
10	α-Terpinene	1020	0.45
11	1,2,4-Trimethyl benzene	1027	0.11
12	o-Cymene	1030	1.19
13	1,8- Cineole	1037	29.01
14	ɣ-Terpinene	1063	0.29
15	Terpinolene	1089	0.06
16	Filifolone	1108	1.85
17	cis-p-Menth-2-en-1-ol	1133	6.24
18	α-Campholenal	1136	0.12
19	trans-p-Menth-2-en-1-ol	1152	4.04
20	Camphor	1160	31.84
21	Pinocarvone	1174	0.46
22	Borneol	1183	2.99
23	Terpinen-4-ol	1190	0.98
24	cis-Piperitol	1219	1.67
25	cis-Carveol	1244	0.11
26	E-Ocimenone	1250	0.15
27	Carvone	1257	0.51
28	Bornyl acetate	1291	0.83
29	Piperitenone	1408	2.27
30	Germacrene D	1489	0.32
31	Bicyclogermacrene	1504	0.34
32	Spathulenol	1590	0.09
	Monoterpene Hydrocarbons		44.65
	Oxygenated Monoterpenes		45.87
	Sesquiterpene Hydrocarbons		0.66
	Oxygenated Sesquiterpenes		0.09
	Other compounds		2.23
	Total identified		93.5

a. KI: Kovats indices

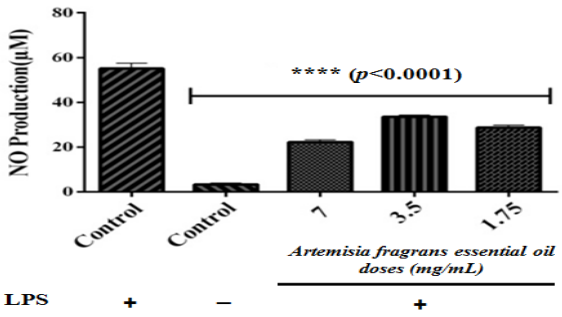

Figure2. Effects of *Artemisia fragrans* essential oil on lipopolysaccharide-induced NO production in RAW 264.7 macrophages.

The cells treated with 1μg/mL of LPS for 20 h and then treated with various concentrations (1.75, 3.5 and 7mg/mL) of *A. fragrans* essential oil for 24 h. At the end of incubation, 100μL of the medium was removed to measure NO production. Negative control values were obtained in the absence of LPS. In the culture medium, NO production was measured by the Griess reaction as described in materials and methods. All values represent mean ± SD of three independent experiments performed in triplicate. **(p<0.0001) indicates statistically significant differences from the LPS-treated group.**

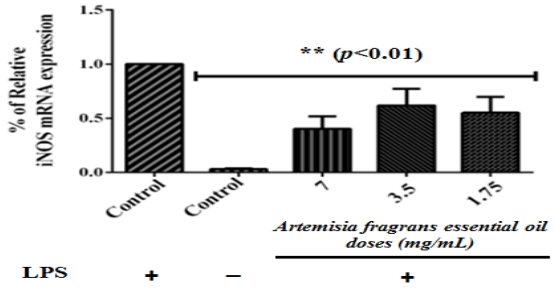

Figure3. Effects of *Artemisia fragrans* essential oil on iNOS mRNA expression in LPS-stimulated RAW 264.7 macrophages. The cells were treated with 1μg/mL of LPS for 20h and then were treated with various concentrations (1.75, 3.5 and 7 mg/mL) of *A. fragrans* essential oil for 24 h. Total RNA was isolated and subjected to real-time PCR to measure iNOS mRNA level. GAPDH was used as an internal control for standardization. All values represent mean±SD of three independent experiments performed in triplicate. ** (p<0.01) indicates statistically significant differences from the LPS-treated group.

and 1.75 mg/mL), in a dose-dependent manner, significantly decreased mRNA expression levels of iNOS gene in LPS-induced cells (p<0.01). The greatest inhibitory effect was observed at the highest concentration (7 mg/mL) (Figure3).

DISCUSSION

Chronic inflammation can lead to degenerative diseases and cancer,[9, 36-41] Hence, efforts have been made to regulate the inflammatory response. Along with current medical treatments for inflammatory diseases, extensive researches have been initiated to assess traditional therapeutic methods based on plant-derived products

The application of these products is in demand due to ease of access, fewer side effects and being natural and non-synthetic, compared to other methods of prevention and treatment of diseases. Among the plant products are essential oils obtained from aromatic plants, which may have anti-inflammatory activities. The major components of the oils are terpenes with monoterpenes and sesquiterpenes dominant.[9, 39] Numerous studies has been done on the application of plant phytochemicals to control and inhibit factors involved in inflammatory pathways. A number of these factors are the molecules involved in inflammatory arachidonic acid-independent pathways such as NO, synthesized by iNOS, the overproduction of which in inflammatory diseases and cancer has been reported.[40, 42-50]

Artemisia extracts or essential oils have been considered as one of the main traditional medicines. A large number of different species of this genus produce essential oils applied in traditional and modern medicine as well as in food, cosmetics and pharmaceuticals.[9] In this study, we have selected the essential oil of *A. fragrans* and investigated its effects on the NO production and mRNA expression of iNOS in LPS-stimulated RAW264.7 murine macrophage. In RAW 264.7 cells, LPS induces iNOS transcription and NO production. Hence, RAW 264.7 cells are considered as a model for evaluation of potential inhibitors of the inflammatory pathway leading to iNOS induction and NO production.[45, 51-53]

In this research, the results of GC-MS analysis indicated the *A. fragrans* essential oil is rich in monoterpens derivatives, main compounds of which included camphor and 1,8-cineole, respectively. This finding was consistent with the previous studies reported by Morteza-Semnani et al. and Delazar et al.[27, 28] Safaei-Ghomi et al. reported that the main components in *A. fragrans* oil were 1,8-cineole and camphor, respectively.[15] Furthermore, in a study by Barazandeh et al., 1,8-cineole and α-thujone were also identified in considerable amounts in the *A. fragrans* oil.[26] Shafaghat et al. investigated the composition of the essential oil extracted from *A. fragrans* leaves. The major components of the oil were chrysanthenone and 1,8-cineole.[54] According to previous studies, camphor (a bornate derivative), and 1,8-cineole comprise the main components of many species of genus *Artemisia*.[15, 55-59] The variations in the profile, quality and amounts of the constituents of essential oil from *Artemisia* species might have resulted from several factors including altitude and time of collection, growth stage, fertilizer and soil pH, climatic and experimental conditions.[9,15,60] MTT assay was performed to determine the viability of the cells in presence of essential oil and to obtain the most appropriate concentrations of oil to treat the cells. The results of MTT assay showed that low concentrations (1-9 mg/mL) of *A. fragrans* essential oil had no cytotoxic effect on RAW 264.7 cell viability. So, to determine the anti-inflammatory effects of the *A. fragrans* on macrophages, concentrations (1.75-7 mg/mL) of the essential oil were selected for the following experiments of this study.

The present study demonstrated that the essential oil of *A. fragrans* significantly (p<0.0001) reduced NO production in LPS-activated RAW 264.7 cells. Moreover, the effect of the oil on NO production was associated with the decline of iNOS mRNA expression.

The results of this study are in accordance with several studies, which have demonstrated that essential oils or extracts of *Artemisia* species inhibited NO production and iNOS expression in LPS-stimulated RAW 264.7 cells. Xi Tan et al. found a decrease in the iNOS gene expression and NO production in LPS-stimulated RAW264.7 cell line treated with ethanolic extract of *A. anomala* S. Moore.[7] Deok Jeong et al. performed a similar study on various concentrations of alcoholic extract of *A. asiatica* and found that LPS-induced RAW264.7 cell line showed a significant decrease (p<0.05) in NO production.[61] In addition, W.J. Yoon et al. reported a significant decrease in iNOS gene expression and NO production in response to *A. fukudo* essential oil.[21] In a study by Xiaoxin X. Zhu et

al. on Arteannuin B, one of the sesquiterpen derivatives obtained from *A. annua* L. it was found that this compound had an inhibitory effect on NO production.[62] Hee-Kyoung Lim et al. showed that ethanol extract of *A. capillaries* reduced NO production by downregulating iNOS transcription.[23] A similar study by Su-Tze Chou et al. demonstrated the inhibitory effects of *Achillea millefolium* L. oil, a member of the *Asteraceae* family, on NO production and iNOS expression in LPS-induced RAW 264.7 macrophages.[63] In current study, we used the essential oil of *A. fragrans* containing different effective compounds mainly camphor and 1,8-cineol which according to the literature have pharmaceutical application.[29, 31-32]. Several studies have confirmed increased levels of NO in the airways of patients with asthma and animal models of asthma.[64] Since *A. fragrans* oil inhibits the generation of NO by activated macrophages, this plant may be helpful to treat asthma.

The results of this study revealed that *A. fragrans* essential oil affect arachidonic acid-independent inflammatory pathway through the reduction in iNOS gene expression.

The reduction in amount of NO, the product of the iNOS enzyme, is associated with iNOS at transcription level. Our preliminary data suggest that *A. fragrans* oil contains potential componentss that exhibit anti-inflammatory effects through downregulating iNOS at mRNA expression level. Thus, further studies on essential-oil-isolated compounds are necessary to accurately determine the possible mechanisms of action and the potential therapeutic targets.

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