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

Maryam Farghadan1,2, Hossein Ghafoori3, Faezeh Vakhshiteh4, Seyed Abolhassan Shahzadeh Fazeli4, Parvaneh Farzaneh4, and Parviz Kokhaei1

1 Cancer Research Center, Semnan University of Medical Sciences, Semnan, Iran
2 Student Research Committee, Semnan University of Medical Sciences, Semnan, Iran
3 Department of Biology, University of Guilan, Rasht, Iran
4 Human and Animal Cell Bank, Iranian Biological Resource Center (IBRC), ACECR, Tehran, Iran

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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; Macrophage; Gene expression; Nitric Oxide synthase

Corresponding Authors: Parviz Kokhaei, PhD; Department of Immunology, Semnan University of Medical Sciences, Semnan, Iran. Tel: (+98 23) 3365 4362, Fax: (+98 23) 3365 4177. E-mail: parviz.kokhaei@ki.se

Parvaneh Farzaneh, PhD; Human and Animal Cell Bank, Iranian Biological Resource Center (IBRC), ACECR, Tehran, Iran. Tel: (+98 21) 8852 5389, E-mail: pfarzaneh2000@yahoo.com
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 polycytylenic 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.13, 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...
Effect of Artemisia fragrans on iNOS and NO

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±5°C) and then grounded. The essential oil was extracted from 100 grams of sample by hydrodistillation using a clevenger type apparatus for three hours. 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 ºC at 15 ºC/min. Injector and detector temperatures were set at 220 and 290 ºC, respectively. 1 µL 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. The retention indices were calculated for all volatile constituents using a homologous 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% CO₂. For all experiments, the cells were grown to 80% confluence and were subjected to no more than 20 cell passages.

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. Briefly, RAW 264.7 cells were seeded in 96-well plates at the density of 5×10⁴ 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% CO₂. 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 5×10⁵ cells/well in 24 well plates and incubated for 18 h at 37°C and 5% CO₂. 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 µg/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 (qPCR) 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, qPCR was performed on a Step One real-time PCR instrument (Applied Bio systems, Foster City, USA) using 5×
HOT FIREPol EvaGreen qPCR Mix Plus (ROX) kit (Solis BioDyne, Estonia). The qPCR 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 qPCR are listed in Table 1.

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

<table>
<thead>
<tr>
<th>Target</th>
<th>Type</th>
<th>Sequences</th>
</tr>
</thead>
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<tr>
<td>GAPDH</td>
<td>Sense</td>
<td>5'-GACCTCAACAGCAAATCCACAC-3'</td>
</tr>
<tr>
<td></td>
<td>Anti-sense</td>
<td>5'-TCCACACCCCTTGTTGCTGTA-3'</td>
</tr>
<tr>
<td>iNOS</td>
<td>Sense</td>
<td>5'-TCACGCTTGGGTCTTGTTCA-3'</td>
</tr>
<tr>
<td></td>
<td>Anti-sense</td>
<td>5'-TCCAAATCCAACGTCTCCGT-3'</td>
</tr>
</tbody>
</table>

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 264.7 cells 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 1.75 mg/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 and 1.75 mg/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).
Table 2. Chemical composition of the essential oil from *Artemisia fragrans*.

<table>
<thead>
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<th>Peak No.</th>
<th>Compounds</th>
<th>KI*</th>
<th>Peak area (%)</th>
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<td>2E-Hexenal</td>
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<tr>
<td>2</td>
<td>α-Thujene</td>
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<td>5</td>
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<td>β-Pinene</td>
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<tr>
<td>7</td>
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<td></td>
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<tr>
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<tr>
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<td><strong>Total identified</strong></td>
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*KI: Kovats indices*
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 have 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.\(^50\) \(^{42-50}\)

*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.\(^49\) \(^{51-53}\)

In this research, the results of GC-MS analysis indicated the *A. fragrans* essential oil is rich in monoterpenes 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-Ghomí 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.\(^29\) Shafaghat et al. investigated the composition of the essential oil extracted from *A. fragrans* leaves. The major components of the oil were chrysanthene and 1,8-cineole.\(^32\) 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 ethanol extract of *A. anomalá* 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
Effect of Artemisia fragrans on iNOS and NO

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.52 Hee-Kyoung Lim et al. showed that ethanol extract of A. capillaries reduced NO production by downregulating iNOS transcription.53 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.55 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.65 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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