



Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Biomedicine

journal homepage: www.elsevier.com/locate/apjtb



Document heading doi:10.1016/S2221-1691(14)60235-5 © 2014 by the Asian Pacific Journal of Tropical Biomedicine. All rights reserved.

## Anti-oxidative and anti-inflammatory effects of *Tagetes minuta* essential oil in activated macrophages

Parastoo Karimian<sup>1</sup>, Gholamreza Kavooosi<sup>1\*</sup>, Zahra Amirghofran<sup>2</sup><sup>1</sup>Biotechnology Institute, Shiraz University, Shiraz, 71441-65186, Iran<sup>2</sup>Department of Immunology, Autoimmune Disease Research Center and Medicinal and Natural Products Chemistry Research Center, Shiraz University of Medical Sciences, Shiraz, Iran

## PEER REVIEW

**Peer reviewer**

Hasan Salehi, University of Shiraz, Shiraz, Iran.

E-mail: hsalehi@shirazu.ac.ir

**Comments**

TMO displayed an anti-oxidant property by scavenging superoxide, H<sub>2</sub>O<sub>2</sub> and NO radicals, and reduced oxidative stress. The decreased formation of ROS and NOS radicals in macrophages was possibly due to the radical scavenging activity of phenolic groups present in the oil and/or due to an inhibition of iNOS and NOX gene expressions. Furthermore, TMO decreased the expression of the genes for pro-inflammatory cytokine TNF- $\alpha$ . Details on Page 226

## ABSTRACT

**Objective:** To investigate antioxidant and anti-inflammatory effects of *Tagetes minuta* (*T. minuta*) essential oil.

**Methods:** In the present study *T. minuta* essential oil was obtained from leaves of *T. minuta* via hydro-distillation and then was analyzed by gas chromatography-mass spectrometry. The anti-oxidant capacity of *T. minuta* essential oil was examined by measuring reactive oxygen, reactive nitrogen species and hydrogen peroxide scavenging. The anti-inflammatory activity of *T. minuta* essential oil was determined through measuring NADH oxidase, inducible nitric oxide synthase and TNF- $\alpha$  mRNA expression in lipopolysaccharide-stimulated murine macrophages using real-time PCR.

**Results:** Gas chromatography-mass spectrometry analysis indicated that the main components in the *T. minuta* essential oil were dihydrotagetone (33.86%), E-ocimene (19.92%), tagetone (16.15%), cis- $\beta$ -ocimene (7.94%), Z-ocimene (5.27%), limonene (3.1%) and epoxyocimene (2.03%). The *T. minuta* essential oil had the ability to scavenge all reactive oxygen/reactive nitrogen species radicals with IC<sub>50</sub> 12–15  $\mu$ g/mL, which indicated a potent radical scavenging activity. In addition, *T. minuta* essential oil significantly reduced NADH oxidase, inducible nitric oxide synthase and TNF- $\alpha$  mRNA expression in the cells at concentrations of 50  $\mu$ g/mL, indicating a capacity of this product to potentially modulate/diminish immune responses.

**Conclusions:** *T. minuta* essential oil has radical scavenging and anti-inflammatory activities and could potentially be used as a safe effective source of natural anti-oxidants in therapy against oxidative damage and stress associated with some inflammatory conditions.

## KEYWORDS

*Tagetes minuta*, Essential oil, Macrophages, Anti-inflammatory, Antioxidant

### 1. Introduction

*Tagetes minuta* (*T. minuta*) is a tall upright marigold plant in the sunflower (Asteraceae) family. *Tagetes* species originally has been used as a source of essential oil (extracted from leaves, stalks and flowers) for the flavoring in the food industries. The powders and extracts of *Tagetes* are rich in the orange-yellow carotenoid and are used as a food color for foods such as pasta, vegetable oil, margarine, mayonnaises, salad dressing, baked

goods, confectionery, dairy products, ice cream, yogurt, citrus juice, mustard and as colorant in poultry feed[1–3]. *T. minuta* is also extensively used medicinally as a condiment and herbal tea in a wide variety of fields in its native region and as a popular traditional folk remedies and in the complementary and medical therapy. *T. minuta* has several medical benefits such as remedy for colds, respiratory inflammations, stomach problem, anti-spasmodic, anti-parasitic, anti-septic, insecticide and sedative. It is used for chest infections, coughs

\*Corresponding author: Gholamreza Kavooosi, Biotechnology Institute, Shiraz University, P.O.71441-65186, Shiraz, 71441-65186, Iran.

Tel: +98-711-2272805

E-mail: ghkavooosi@shirazu.ac.ir

Foundation Project: Supported by the funding from Shiraz University (Grant no. 88-CR-ACRST-108) and Shiraz University of Medical Science (Grant No. 3937).

Article history:

Received 1 Jan 2014

Received in revised form 10 Jan, 2nd revised form 16 Jan, 3rd revised form 22 Jan 2014

Accepted 26 Feb 2014

Available online 28 Mar 2014

and catarrh, dilating the bronchi, facilitating the flow of mucus and dislodging congestion and can be used in cases of skin infections. It also has a healing effect on wounds, cuts, calluses and bunions<sup>[4–9]</sup>. However, such practices are largely based on folklore and train of traditional medicine rather than evidence-based research.

The most abundant components in *T. minuta* essential oil are dihydrotagetone (unsaturated acyclic monoterpene ketone), ocimene (unsaturated acyclic monoterpene hydrocarbon), tagetone (unsaturated acyclic monoterpene ketone) and limonene (unsaturated monocyclic monoterpene hydrocarbon<sup>[10–14]</sup>). *T. minuta* essential oil has a significant antibacterial activity against both Gram-positive and Gram-negative bacterial<sup>[15–17]</sup>. Several studies have also described antifungal activities of *T. minuta* essential oil against *Candida*, *Penicillium* and *Aspergillus* species<sup>[18–20]</sup>. *T. minuta* essential oil has been shown to possess anti-oxidant activity in 2, 2-diphenyl-1-picrylhydrazyl and 2, 2'-azino-di (3-ethylbenzthiazoline-6- sulphonate) (ABTS) assay<sup>[21,22]</sup>.

Advances in chemical and pharmacological evaluations of *T. minuta* essential oil have occurred in the past recent years; however, several useful features of this plant (*e.g.* the mechanisms underlying its antioxidant and anti-inflammatory effects) have remained unknown. Macrophages play a pivotal role in inflammatory responses. Overproduction of reactive oxygen species (ROS) and nitrogen (RNS) by macrophages is a classic indicator during inflammatory events *in situ*. The production of ROS and RNS radicals are under the control of nicotinamide adenine dinucleotide phosphate oxidase (NOX) and inducible nitric oxide synthase (iNOS), respectively. The aim of the present study was to investigate the level of potential modulating effects of *T. minuta* essential oil on macrophages and their related functions including expression of NOX subunits [p22phox (phagocyte oxidase), p40phox, p47phox and p67phox], NOS and TNF- $\alpha$  mRNAs in lipopolysaccharide (LPS)-stimulated macrophages. In addition, *in vitro* anti-oxidant capacity of *T. minuta* essential oil was examined by assessments of ROS, RNS and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) scavenging ability using ABTS, sodium nitrite, and H<sub>2</sub>O<sub>2</sub> scavenging, respectively. It was expected that these studies would reveal that *T. minuta* essential oil exhibits radical scavenging activity (against superoxide anion, H<sub>2</sub>O<sub>2</sub>, and NO radicals) in macrophages, in part, due to an inhibition of iNOS and NOX gene expression. Furthermore, it was hypothesized for the first time that *T. minuta* essential oil would decrease TNF- $\alpha$  mRNA expression as part of its known anti-inflammatory character and secondarily due to the ongoing quenching of radicals known to trigger formation of these pro-inflammatory cytokines.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Sodium nitrite, sodium sulphate, ABTS, Griess reagent (naphthylethylenediamine, sulfanilamide, phosphoric acid), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), fetal calf serum, Dulbecco's modified eagle medium, L-glutamine, dimethylsulfoxide (DMSO) and LPS (from

*Escherichia coli* 0111; B4) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Fluka (Heidelberg, Germany). RNX-Plus buffer was obtained from Cinagen (Tehran, Iran). All other used chemicals and reagents were of the purest commercially available products.

### 2.2. Plant materials and *T. minuta* essential oil preparation

Seed of *T. minuta* was obtained from Institute of Medicinal Plants, Isfahan, Iran and was grown in green house conditions in Sadra near Shiraz, Iran. Seed of medicinal plant was grown in sterile soil. The Seeds of *T. minuta* were sown in experimental greenhouse, in September 2011. One month later obtained seedlings were transferred to experimental field and distributed homogenously. The aerial parts of plants were harvested at the flowering stage. The leaves of the plants were separated from the stem and were dried in the shade for 72 h. The air-dried leaves (100 g) were hydro-distilled for 3 h using an all-glass Clevenger-type apparatus (Herbal Exir Co., Mashhad, Iran) according to the method outlined by the British Pharmacopeia<sup>[23]</sup>. The yield of *T. minuta* essential oil from leaf material was near 1% (w/w). The obtained essential oil was dehydrated over anhydrous sodium sulphate and stored at 4 °C until analyzed by gas chromatography-mass spectrometry (GC-MS) and was then used.

### 2.3. Identification of the *T. minuta* essential oil components

GC analysis was carried out using Agilent technology chromatograph with HP-5 column (30 m×0.32 mm, internal diameter 0.25  $\mu$ m). Oven temperature was performed as follows: 60 °C to 210 °C at 3 °C/min; 210 °C to 240 °C at 20 °C/min and hold for 8.5 min, injector temperature 280 °C; detector temperature, 290 °C; carrier gas, N<sub>2</sub> (1 mL/min); split ratio of 1: 50. The OBO was analyzed using an Agilent model 7890-A series gas chromatography and Agilent model 5975-C mass spectrometry. The HP-5 MS capillary column (phenyl methyl siloxane, 30 m ×0.25 mm, internal diameter× 25  $\mu$ m) was used with helium at 1 mL/min as the carrier gas. GC oven temperature was programmed from 60 °C to 210 °C at a rate of 3 °C/min and was then increased from 210 °C to 240 °C at rate of 20 °C/min and was kept constant at 240 °C for 8.5 min. The split ratio was adjusted to 1: 50 and the injection volume was 1 mL. The injector temperature was 280 °C. The quadrupole mass spectrometer was scanned over 40–550 amu with an ionizing voltage of 70 eV. Retention indices were determined using retention times of *n*-alkanes (C<sub>8</sub>–C<sub>25</sub>) that were injected after the *T. minuta* essential oil under the same chromatographic conditions. The retention indices for all components were determined according to the method that uses *n*-alkanes as standard. The compounds were identified by comparison of retention indices with those reported in the literature and by comparison of their mass spectra with the Wiley GC-MS Library, Adams Library, Mass Finder 2.1 Library data published mass spectra data<sup>[24]</sup>.

### 2.4. ROS scavenging assay

The ROS scavenging activity of the *T. minuta* essential oil was determined as previously described<sup>[25]</sup>. Briefly,

10  $\mu$ L of the *T. minuta* essential oil (0–500  $\mu$ g/mL in DMSO) was added to 1.0 mL of diluted ABTS radical solution (7 mmol/L ABTS and 2.54 mmol/L potassium persulfate). After mixing, the absorbance (A) was read at 734 nm using an Ultrospec 2000 spectrophotometer (Pharmacia, Uppsala, Sweden). The percentage of ROS scavenging was calculated as  $[(A_{734_{\text{blank}}} - A_{734_{\text{sample}}})/A_{734_{\text{blank}}}] \times 100$ . The concentrations that could provide 50% inhibition ( $IC_{50}$ ) were calculated from the graph that plotted the inhibition percentage against different *T. minuta* essential oil concentrations.

### 2.5. $H_2O_2$ scavenging assay

$H_2O_2$  scavenging activity of the *T. minuta* essential oil was determined as previously described<sup>[26]</sup>. Briefly, 10  $\mu$ L of the *T. minuta* essential oil (0–500  $\mu$ g/mL in DMSO) was incubated with 1.0 mL of  $H_2O_2$  (50 mmol/L in 100 mmol/L phosphate buffer pH 7.4) at 37 °C for 60 min. After incubation, the absorbance (A) was read at 230 nm against a blank solution containing phosphate buffer without  $H_2O_2$  using a spectrophotometer. The percentage of  $H_2O_2$  scavenging was calculated as  $[(A_{230_{\text{blank}}} - A_{230_{\text{test}}})/A_{230_{\text{blank}}}] \times 100$ .  $IC_{50}$  was calculated from the graph that plotted the inhibition percentage against different *T. minuta* essential oil concentrations.

### 2.6. RNS scavenging assay

RNS scavenging activity of the *T. minuta* essential oil was determined as previously described<sup>[26]</sup>. Briefly, 10  $\mu$ L of the *T. minuta* essential oil (0–500  $\mu$ g/mL in DMSO) was incubated with 0.5 mL of sodium nitrite (10  $\mu$ g/mL in 100 mmol/L sodium citrate pH 5) at 37 °C for 2 h. After incubation, 0.5 mL of Griess reagent was added and the absorbance (A) was read at 540 nm using a spectrophotometer. The percentage of RNS scavenging was calculated as follows:  $[(A_{540_{\text{blank}}} - A_{540_{\text{sample}}})/A_{540_{\text{blank}}}] \times 100$ .  $IC_{50}$  was calculated from the graph that plotted the inhibition percentage against different *T. minuta* essential oil concentrations.

### 2.7. Macrophages cell culture

The J774.1A murine macrophage cell line was obtained from the cell bank of the Pasteur Institute of Iran, Tehran. Cells were cultured in Dulbecco's modified eagle medium containing 2 mmol/L L-glutamine, 100 IU/mL penicillin, 100  $\mu$ g/mL streptomycin and 10% heat-inactivated fetal calf serum at 37 °C in a humidified  $CO_2$  incubator. Cultures were allowed to grow until confluence at which point adherent macrophages were scraped from the flask and were washed with warm medium (25 °C). Cells were counted and their viability was determined by trypan blue dye exclusion. The cells were seeded at concentration of  $2 \times 10^6$  cells per millilitre in 24-well tissue culture plates in triplicate (Jet Biofil, Kyoto, Japan). After culturing for 18 h to allow cells to adhere, non-adherent cells were removed by gentle rinsing with medium. Remaining adherent cells were then cultured in the presence or absence of medium bearing LPS (1  $\mu$ g/mL). After 2 h, *T. minuta* essential oil was added at a final concentration of 0–200  $\mu$ g/mL. Two sets of wells without *T. minuta* essential oil but containing LPS and

DMSO solvent (0.1%) were used as negative controls. After 24 h of incubation at 37 °C, the culture supernatants in each well were removed and the cells harvested were used for RNA extraction and real-time PCR analysis.

### 2.8. Cell viability assay

The effect of *T. minuta* essential oil on the viability of J774A.1 cells was determined by MTT assay as described previously<sup>[27]</sup>. Cells ( $2 \times 10^4$  cells per well) were incubated for 24 h (at 37 °C in 5%  $CO_2$ ) with different concentrations (0–200  $\mu$ g/mL) of *T. minuta* essential oil. Thereafter, 10  $\mu$ L of MTT (5 mg/mL) was added to each well and incubated for an additional 4 h at 37 °C followed by treatment with 100  $\mu$ L of lysis buffer (10% SDS in 10 mmol/L HCl). The absorbance of each well was determined by spectrophotometer at dual wavelengths of 570 and 630 nm on a microplate ELISA reader (BioTek Elx 808, Winooski, VT, 05403, USA). Viability percentage was calculated by the following formula (Absorbance of treated cells/Absorbance of corresponding control)  $\times 100$ . The control was *T. minuta* essential oil-untreated cells containing DMSO at the highest concentration used (0.1%). The concentration that provided a 50% inhibition ( $IC_{50}$ ) was calculated from a graph, plotting the inhibition percentage against different *T. minuta* essential oil concentrations.

### 2.9. RNA extraction and cDNA synthesis

Total RNA was extracted using RNX-plus buffer from Cinagen, Tehran, Iran. Briefly, about  $2 \times 10^6$  cells were transferred to 1 mL of RNX-plus buffer in an RNase-free microtube, mixed thoroughly and left at room temperature for 5 min. A volume of 200  $\mu$ L of chloroform was added to the slurry and was mixed gently. The mixture was centrifuged at 13 200 g at 4 °C for 15 min, the supernatant was transferred to a new tube and was precipitated with an equal volume of isopropanol for 15 min on ice. The RNA pellet was washed using 75% ethanol, briefly dried and resuspended in 15  $\mu$ L of RNase free water. The purified total RNA was quantified by NanoDrop ND 1000 spectrophotometer (Wilmington, DE). A sample (0.005 mg) of RNA was used for first strand cDNA synthesis, using 100 pmol oligo-dT (18 mer), 15 pmol dNTPs, 20 U RNase inhibitor, and 200 U M-Mulv reverse transcriptase (all from Fermentas, Hanover, MD) in a 0.02 mL final volume.

### 2.10. Quantitative real-time PCR

Primer design, in the form of exon junction was carried out using AlleleID 7 software (Premier Biosoft Intl., Palo Alto, CA) for the internal controls glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (NM-010927) and  $\beta$ -actin (NM-007393.3) and tested genes NOX p22phox (NM-007806), NOX p40phox (NM-008677), NOX p47phox (NM-010876), NOX p67phox (NM-010877), iNOS (NM-008084) and TNF- $\alpha$  (NM-013693) (Table 1). The GAPDH and  $\beta$ -actin were used as internal control (whose expression proved not to be influenced by LPS) for data normalization<sup>[28]</sup>. Relative real-time PCR was performed in a 20  $\mu$ L volume containing 1  $\mu$ L cDNA, 1 $\times$ Syber Green buffer (Qiagen, Hilden,

Germany) and 4 pmol of each primer. The amplification reactions were carried out in a line Gene k thermal cycler (Bioer Technology Co., Hangzhou, China) with initial denaturing of 94 °C for 2 min, followed by 40 cycles of 94 °C for 10 seconds, annealing temperature of each primer pair was done for 15 seconds and 30 seconds for extension to occur at 72 °C. After 40 cycles, the specificity of the amplifications was checked based on the melting curves resulting from heating the amplicons from 50 °C to 95 °C. All amplification reactions were repeated twice under identical conditions beside a negative control and 5 standard samples. To ensure that the PCR was generated from cDNA and not genomic DNA, proper control reactions were carried out without the reverse transcriptase treatment. For quantitative real time PCR data, relative expression of NOXs, iNOS and TNF- $\alpha$  gene were calculated based on the threshold cycle (CT) method. The CT for each sample was calculated

using the Line–gene K software and the method of Larionov et al.<sup>[29]</sup>. Accordingly, fold–expression of target mRNAs over the reference values were calculated by equation  $2^{-\Delta\Delta CT}$ , where  $\Delta CT$  was determined by subtracting the corresponding internal control CT value from the specific CT of targets, and  $\Delta\Delta CT$  was obtained by subtracting the  $\Delta CT$  of each experimental sample from that of the control sample<sup>[30]</sup>.

### 2.11. Statistical analysis

All data are expressed as means plus standard deviations of at least three independent experiments. The significant differences between treatments were analyzed by One–way analyses of variance (ANOVA) test at  $P < 0.05$  using statistical package for the social sciences (SPSS, Abaus Concepts, Berkeley, CA) and Prism 5 (Graph Phad, San Diego, USA) software.

**Table 1**

Primer used for real–time analysis.

Genes <sup>1</sup>	Accession	Sense sequence	Anti sense sequence
GAPDH	NM–010927	5'–CGGTGTGAACGGATTGGC–3'	5'–TGAGTGGAGTCATACTGGAAC–3'
$\beta$ –actin	NM–007393.3	5'–CCACACCCGCCACCAGTTCC–3'	5'–CTAGGGCGGCCACGATGGA–3'
NOX p22	NM–007806	5'– ATGGAGCGATGTGGACAG–3'	5'– ACCGACAACAGGAAGTGG–3'
NOX p40	NM–008677	5'–CAACAAAGACTGGCTGGAG–3'	5'–CCGCAATGTCCTTGATGG–3'
iNOX p47	NM–010876	5'– ATGGCACAAAGGACAATC–3'	5'– ACCTGAGGCTATACACAAG–3'
NOX p67	NM–010877	5'– CAGCCACAGTCAGCAGAG–3'	5'–GCACAAAGCCAAACAATACC–3'
iNOS	NM–008084	5'– CTGGAGGTTCTGGATGAG–3'	5'– CTGAGGGCTGACACAAGG –3'
TNF– $\alpha$	NM–013693	5'–GTCTCAGCCTCTTCTCATTC–3'	5'– GGAAGTCTCATCCCTTTGG–3'

<sup>1</sup>Primer design, in form of exon junction was carried out using Allele ID 7 software for the internal controls GAPDH and  $\beta$ –actin and test genes NADH oxidase p22 phagocytes oxidase (NOX p22phox), NOX p47phox, NOX p47phox, NOX p67phox, iNOS and TNF– $\alpha$  genes from *Mus musculus* sequence.

## 3. Results

### 3.1. Plant materials

The *T. minuta* essential oil was prepared by water–distillation, and its chemical composition was determined by GC–MS. As shown in Table 2, GC–MS analysis indicated that the main components were dihydrotagetone (33.86%), E–ocimene (19.92%), tagetone (16.15%), cis– $\beta$ –ocimene (7.94%), Z–ocimene (5.27%), limonene (3.1%) and epoxyocimene (2.03%). GC–MS analysis of the essential oil indicated the main components *T. minuta* essential oil were dihydrotagetone, E–ocimene, tagetone, cis– $\beta$ –ocimene, Z–ocimene, limonene and epoxyocimene.

### 3.2. Antioxidant activity of *T. minuta* essential oil

*T. minuta* essential oil displayed a concentration dependent ROS, RNS and H<sub>2</sub>O<sub>2</sub> scavenging activity. IC<sub>50</sub> for ROS, RNS and H<sub>2</sub>O<sub>2</sub> scavenging were (12.0 $\pm$ 3.0), (15.0 $\pm$ 2.5) and (13.0 $\pm$ 4.0)  $\mu$ g/mL of *T. minuta* essential oil, respectively. At concentrations >30  $\mu$ g/mL, the *T. minuta* essential oil significantly scavenges ROS, RNS, and H<sub>2</sub>O<sub>2</sub> by 100%. The *T. minuta* essential oil analyzed here possessed potent *in vitro* ROS, RNS and H<sub>2</sub>O<sub>2</sub> scavenging activity. The *T. minuta* essential oil at >30  $\mu$ g/mL had the ability to scavenge all ROS, RNS and H<sub>2</sub>O<sub>2</sub> radicals, an indicator of its potency as a radical scavenger.

**Table 2**

Chemical composition of *T. minuta* essential oil.

Compounds	Retention index	% of compounds
$\alpha$ –Pinene	933.526	0.32792
Sabinene	973.444	0.39703
cis–3–Hexenyl acetate	1005.680	0.14838
<i>p</i> –Cymene	1025.210	0.93376
Limonene	1029.490	3.10293
cis– $\beta$ –Ocimene	1037.830	7.94067
Dihydrotagetone	1061.290	33.86297
Chrysanthenone	1103.260	0.14646
Allo–cimene	1135.730	0.35852
E, Z–Epoxyocimene	1149.090	2.03534
Tagetone	1160.520	16.15509
cis–Tagetone	1167.400	0.19829
<i>p</i> –Mentha–1,8 dien–3–one	1208.060	0.18894
Z–Ocimene	1238.290	5.27542
E–Ocimene	1250.790	19.9287
Thymol	1282.780	0.47733
Carvacrol	1298.190	0.47014
cis–Isoeugenol	1398.530	0.86560
E–Caryophyllene	1420.970	0.29945
$\alpha$ –Humulene	1455.070	0.14981
Germacrene D	1497.900	0.39897
Spathulenol	1582.190	0.37319

### 3.3. *T. minuta* essential oil reduced cell viability at high concentrations

The MTT assay results indicated that low concentrations (1–50 µg/mL) of *T. minuta* essential oil had no effect on J774A.1 cell viability. However, at higher concentrations (100–200 µg/mL), cell viability was significantly reduced in a concentration-related manner, with the maximum effect (100% cell death) at concentrations >200 µg/mL (Figure 1). Non-cytotoxic concentrations (<50 µg/mL) were thus used for the subsequent studies including expression of genes.

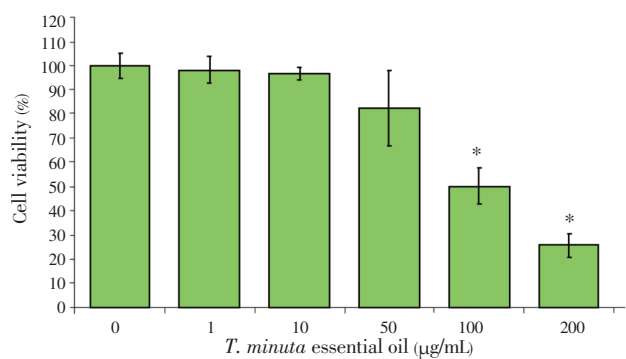
### 3.4. *T. minuta* essential oil reduced NOX p22phox mRNA expression in LPS-stimulated macrophages

The un-stimulated (control) cells showed low level of NOX p22phox mRNA expression. LPS stimulation of macrophages resulted in an increase in NOX p22phox mRNA expression (26.5±1.7) fold of LPS-untreated control cells ( $P<0.001$ ). The addition of *T. minuta* essential oil at 1 to 50 µg/mL

significantly decreased the NOX p22phox mRNA expression in LPS-treated cells from (21.0±2.7) to (4.5±0.8) fold of the control ( $P<0.001$ ) dose-dependently, indicating the inhibitory effect of *T. minuta* essential oil on p22phox mRNA induction/formation (Figure 2).

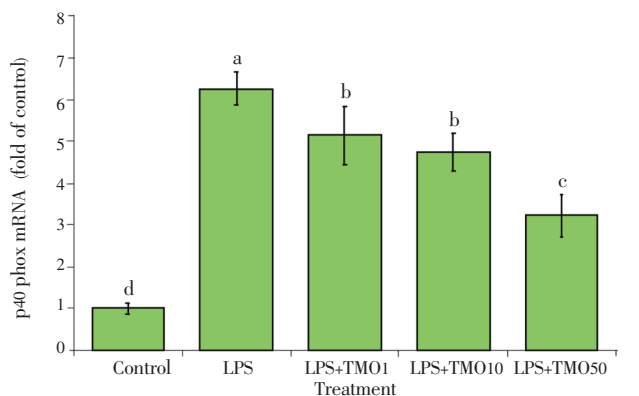
### 3.5. *T. minuta* essential oil reduced NOX p40phox mRNA Expression in LPS-Stimulated Macrophages

The un-stimulated (control) cells showed low level of NOX p40phox mRNA expression while, the expression of NOX p40phox mRNA in LPS-treated cells was (6.3±0.4) fold of the control ( $P<0.001$ ). The addition of *T. minuta* essential oil at 1 to 50 µg/mL significantly decreased the NOX p40phox mRNA expression in LPS-treated cells from (5.1±0.7) to (2.4±0.2) fold of the control, dose-dependently ( $P<0.05$ ), indicating the inhibitory effect of *T. minuta* essential oil on p40phox mRNA induction/formation (Figure 3).



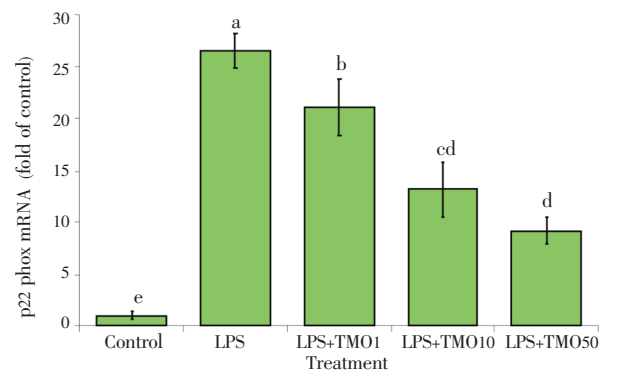
**Figure 1.** Effect of the *T. minuta* essential oil on the viability of J774 cell lines.

TMO: *T. minuta* essential oil. The cells were treated with various concentrations of essential oil (0–200 µg/mL) and incubated for 24 h. Control (0 µg/mL) was cells treated only with the solvent (DMSO) at concentration of 0.1%. Data represent mean±SD from three sets of independent experiments.



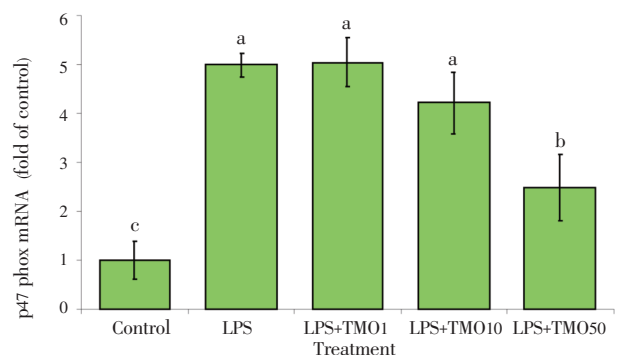
**Figure 3.** Effects of *T. minuta* essential oil on NOX p40phox mRNA expression in LPS-stimulated macrophages.

TMO: *T. minuta* essential oil. The cells were cultured in 24-well plates and treated with and without LPS. Various concentrations of essential oil (1–50 µg/mL) were added. After 24 h, the expression of NOX p47phox mRNA was analyzed by real-time PCR. Cells treated with DMSO as the solvent (control) and cells treated with the solvent and LPS was considered as positive controls.



**Figure 2.** Effects of *T. minuta* essential oil on NOX p22phox mRNA expression in LPS-stimulated macrophages.

TMO: *T. minuta* essential oil. The cells were cultured in 24-well plates and treated with and without LPS. Various concentrations of essential oil (1–50 µg/mL) were added. After 24 h, the expression of NOX p22phox mRNA was analyzed by real-time PCR. Cells treated with DMSO as the solvent (control) and cells treated with the solvent and LPS was considered as positive controls.



**Figure 4.** Effects of *T. minuta* essential oil on NOX p47phox mRNA expression in LPS-stimulated macrophages.

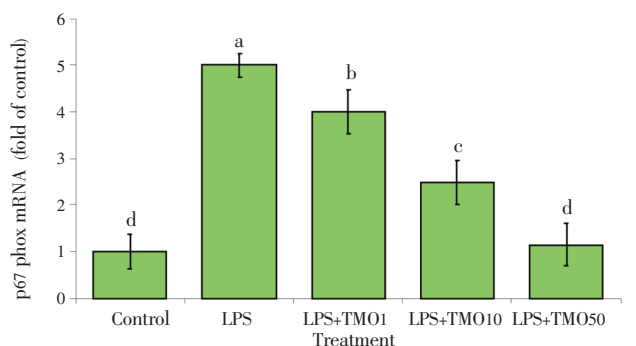
TMO: *T. minuta* essential oil. The cells were cultured in 24-well plates and treated with and without LPS. Various concentrations of TMO (1–50 µg/mL) were added. After 24 h, the expression of NOX p47phox mRNA was analyzed by real-time PCR. Cells treated with DMSO as the solvent (control) and cells treated with the solvent and LPS was considered as positive controls.

### 3.6. *T. minuta* essential oil reduced NOX p47phox mRNA expression in LPS-stimulated macrophages

The un-stimulated (control) cells showed low level of NOX p47phox mRNA expression while, the expression of NOX p47phox mRNA in LPS-treated cells was (5.00±0.25) fold of the control induction/formation ( $P<0.001$ ). The addition of *T. minuta* essential oil at 1 to 50 µg/mL significantly decreased this gene expression in LPS-treated cells from (5.00±0.50) and (1.60±0.28) fold of control, dose-dependently ( $P<0.01$ ) indicating the inhibitory effect of *T. minuta* essential oil on p47phox mRNA induction/formation (Figure 4).

### 3.7. *T. minuta* essential oil reduced NOX p67phox mRNA expression in LPS-stimulated macrophages

With respect to NOX p67phox, a decrease in the gene expression was detected in LPS-stimulated macrophages which were treated with *T. minuta* essential oil. The relative NOX p67phox mRNA expression in cells treated with LPS alone was (5.00±0.25) fold of LPS-untreated the control cells ( $P<0.001$ ). The addition of *T. minuta* essential oil at 1 to 50 µg/mL significantly decreased the NOX p67phox mRNA expression in LPS-treated cells from (4.0±0.5) and (1.0±0.4) fold of the control, dose-dependently ( $P<0.01$ ), indicating the inhibitory effect of *T. minuta* essential oil on p67phox mRNA induction/formation (Figure 5).

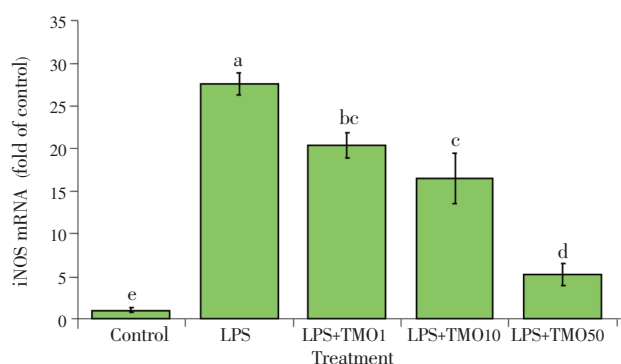


**Figure 5.** Effects of *T. minuta* essential oil on NOX p67phox mRNA expression in LPS-stimulated macrophages.

TMO: *T. minuta* essential oil. The cells were cultured in 24-well plates and treated with and without LPS. Various concentrations of TMO (1–50 µg/mL) were added. After 24 h, the expression of NOX p67phox mRNA was analyzed by real-time PCR. Cells treated with DMSO as the solvent (control) and cells treated with the solvent and LPS was considered as positive controls.

### 3.8. *T. minuta* essential oil reduced iNOS mRNA expression in LPS-stimulated macrophages

LPS stimulation of macrophages resulted in an increase in iNOS mRNA expression (27.5 ±1.4) fold of LPS-untreated cells ( $P<0.001$ ). The addition of *T. minuta* essential oil at concentrations 1 to 50 µg/mL significantly decreased the iNOS mRNA expression in LPS-treated cells from (20.4±1.4) to (3.8±1.0) fold of untreated cells, dose-dependently ( $P>0.05$ ) indicating the inhibitory effect of *T. minuta* essential oil on iNOS mRNA induction/formation (Figure 6).

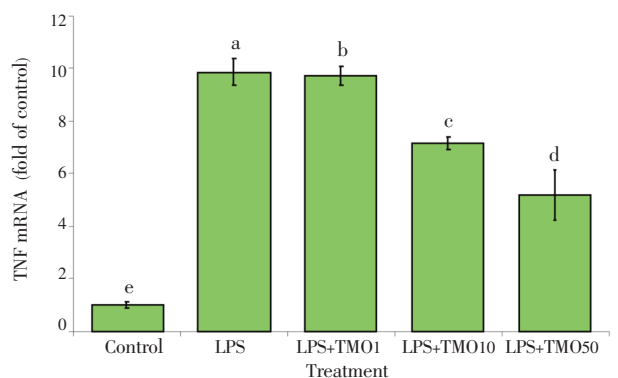


**Figure 6.** Effects of *T. minuta* essential oil on iNOS mRNA expression in LPS-stimulated macrophages.

TMO: *T. minuta* essential oil. LPS-stimulated macrophages. The cells were cultured in 24-well plates and treated with and without LPS. Various concentrations of TMO (1–50 µg/mL) were added. After 24 h, the expression of NOS mRNA was analyzed by real-time PCR. Cells treated with DMSO as the solvent (control) and cells treated with the solvent and LPS was considered as positive controls.

### 3.9. *T. minuta* essential oil reduced TNF-α mRNA expression in LPS-stimulated macrophages

LPS stimulation of macrophages resulted in an increase in TNF-α mRNA expression compared to the conditions in LPS-untreated cells (9.8±0.5) fold ( $P<0.001$ ). The addition of *T. minuta* essential oil at 1 to 50 µg/mL significantly decreased the TNF-α mRNA expression from (9.7±0.4) and (3.3±0.6) fold of the control, dose-dependently (Figure 7). These data indicated the inhibitory effect of *T. minuta* essential oil on TNF-α mRNA induction/formation.



**Figure 7.** Effects of *T. minuta* essential oil on TNF mRNA expression in LPS-stimulated macrophages.

TMO: *T. minuta* essential oil. The cells were cultured in 24-well plates and treated with and without LPS. Various concentrations of TMO (1–50 µg/mL) were added. After 24 h, the expression of TNF mRNA was analyzed by real-time PCR. Cells treated with DMSO as the solvent (control) and cells treated with the solvent and LPS was considered as positive controls.

## 4. Discussion

The antioxidant and anti-inflammatory effects of *T. minuta* essential oil were investigated in the present study. GC-MS analysis of the essential oil indicated the main components

in *T. minuta* essential oil were dihydrotagetone, E-ocimene, tagetone, cis- $\beta$ -ocimene, Z-ocimene, limonene and epoxyocimene. Previous study reported the main components of *T. minuta* essential oil were  $\beta$ -ocimene, dihydrotagetone, tagetone, Z-ocimene and E-ocimene<sup>[11]</sup>. Another study reported thiophenes and polyacetylenic compounds in the *Tagetes* species and *T. minuta* had the highest total thiophene yield<sup>[12]</sup>. Accordingly, the main components of *T. minuta* essential oil could be tagetone (cis/trans, ketone/alcohol, aldehyde/alcohol), ocimene (cis/trans, ketone/alcohol, aldehyde/alcohol) and thiophene derivatives<sup>[11–14]</sup>. For the reasons that, essential oils composition depend on the species, climate, altitude, time of collection and growth stage, thus the plants analyzed in this research had roughly same components with other previously analyzed *T. minuta* essential oil however, showed important differences in their quality and quantity of components.

The *T. minuta* essential oil analyzed here possessed potent *in vitro* ROS, RNS and H<sub>2</sub>O<sub>2</sub> scavenging activity. The *T. minuta* essential oil at >30  $\mu$ g/mL had the ability to scavenge all ROS, RNS and H<sub>2</sub>O<sub>2</sub> radicals, an indicator of its potency as a radical scavenger. ROS are oxygen-derived small molecules, including oxygen radicals such as superoxide, hydroxyl and peroxy and some non-radicals that are easily converted into radicals, such as hydrogen peroxide. ROS, once produced, can interact with various molecules including other small inorganic molecules as well as macromolecules such as proteins and lipids. During these interactions, ROS may destroy or change the function of the target molecule<sup>[31]</sup>. The ROS reducing activity of *T. minuta* essential oil observed in our study imply the beneficial role of this product for reducing damages in biological tissues. The radical scavenging activity of compounds is mainly due to their oxidation-reduction potential, which can play an important role in neutralizing free radicals. This activity is related to phenolic hydroxyl groups<sup>[32]</sup>. *T. minuta* essential oil mainly contains dihydrotagetone, ocimene, tagetone and limonene which all are monoterpenes. This antioxidant activity was confirmed by previous research with IC<sub>50</sub> between 35–344  $\mu$ g/mL<sup>[21,22]</sup>. Thus, *T. minuta* essential oil analyzed in this research showed stronger antioxidant activity rather than previously analyzed one. *In vitro* inhibition of the NO radical is a measure of antioxidant activity of plant extracts. As results of the present study show, the *T. minuta* essential oil used here have the ability to scavenge total RNS at concentration >30  $\mu$ g/mL.

The MTT assay results indicated that low concentrations (1–50  $\mu$ g/mL) of *T. minuta* essential oil had no effect on J774A.1 cell viability (IC<sub>50</sub>=95  $\mu$ g/mL). In order to determine the antioxidant and anti-inflammatory effects of *T. minuta* essential oil on macrophages, the concentrations of >50  $\mu$ g/mL *T. minuta* essential oil which were overtly cytotoxic to the cells were not used. Although the constituents of essential oils can act as antioxidants, they may also act as pro-oxidants and affect inner cell membranes and organelles such as mitochondria in eukaryotic cells. Depending on the type and concentration, this effect may result in cellular cytotoxicity.

In macrophages ROS production is under the control of NOX. This multi-component enzyme consists of several cytosolic components including p91phox, p67phox, p40phox, p47phox

and the small Rho G protein (Rac 1 or Rac 2, Rac: Rho-related C3 botulinum toxin substrate), which assemble on the cellular membrane to activate the enzyme<sup>[33]</sup>. Studies have shown that phosphorylation of p47phox leads to conformational changes, allowing its translocation and interaction with p22phox. Translocation of p47phox brings with it the other subunits, p67phox and p40phox to the membrane<sup>[34]</sup>. Activation of this enzyme complex leads to fusion of the vesicles containing NOX with the plasma membrane or the phagosomal membrane. The active enzyme converts molecular oxygen to superoxide anion through a one-electron transfer<sup>[35]</sup>. As our study showed, *T. minuta* essential oil was able to decrease the expression of key components of NOX. It has been shown that the assembly of p47phox, p67phox and p22phox at the membrane is necessary for oxidase function<sup>[36]</sup>. Thus, it can be assumed that reduced ROS generation by stimulated macrophages in the presence of *T. minuta* essential oil might be, in part, due to the modulation of the expression of NOX subunits.

In addition to ROS, the overproduction of RNS by activated macrophages seems to play an important role in different steps of many inflammatory processes<sup>[37]</sup>. RNS are nitrogen-containing oxidants, mainly NO which is a free radical playing a key role in the pathogenesis of pain and inflammation. NO in macrophages is generated by activation of iNOS. This enzyme has the ability to produce high concentrations of NO after stimulation with bacterial endotoxins or a variety of pro-inflammatory cytokines such as TNF- $\alpha$ , IL-1 and IL-6<sup>[38]</sup>. NO generation involves several steps including the activation of nuclear transcription factor (NF)- $\kappa$ B and subsequent iNOS gene expression<sup>[39]</sup>. NF- $\kappa$ B regulates the expression of various genes involved in inflammatory responses. Its activation can also be regulated by various cytokines, among which TNF- $\alpha$  is the most important. In response to inflammatory stimuli such as LPS, macrophages secrete a variety of inflammatory mediators such as TNF- $\alpha$  and IL-1 $\beta$ . The production of TNF- $\alpha$  cytokine is important for the induction of NO synthesis in LPS-stimulated macrophages<sup>[40]</sup>. As results of this study showed that *T. minuta* essential oil was able to reduce inducible expression of TNF- $\alpha$  gene, which indicated that the reduced NO production seen in the macrophage cultures might be partly related to the suppression of TNF- $\alpha$  expression. TNF- $\alpha$  is known to play a crucial role in inflammatory responses and is involved in the pathogenesis of inflammatory diseases<sup>[41]</sup>. As results of our study showed, *T. minuta* essential oil significantly reduced iNOS mRNA expression in stimulated macrophages. Suppression of TNF- $\alpha$  expression in macrophages as well as reduced iNOS gene expression, due to the *T. minuta* essential oil indicates the ability of this product to diminish immune reactions and provides further evidence that this plant may have potent immuno-modulatory properties.

Considering all these finding, *T. minuta* essential oil displayed an anti-oxidant property by scavenging superoxide, H<sub>2</sub>O<sub>2</sub> and NO radicals, and reduced oxidative stress. This suggested that there was a potential for use of this product in the therapy of oxidative damage, a process that usually accompanies inflammatory conditions. The decreased formation of ROS and NOS radicals in macrophages was possibly due to the

radical scavenging activity of phenolic groups present in the oil and/or due to an inhibition of iNOS and NOX gene expressions. Furthermore, *T. minuta* essential oil decreased the expression of the genes for pro-inflammatory cytokine TNF- $\alpha$ . Therefore, a reduced expression of the above-noted inflammatory enzymes and cytokines could be attributed to a suppression of the NF- $\kappa$ B pathway in the treated cells. These data suggest a potential therapeutic usefulness for *T. minuta* in the modulation of macrophages and provides evidence to support the use of *T. minuta* as a tea/additive/traditional remedy for treatment of inflammatory diseases. Further *in vivo* studies are recommended to more fully understand the therapeutic potential of *T. minuta* essential oil in a multitude of inflammatory disorders.

### Conflict of interest statement

We declare that we have no conflict of interest.

### Acknowledgements

This work was supported by the funding from Shiraz University (Grant no. 88-GR-AGRST-108) and Shiraz University of Medical Science (Grant No. 3937).

### Comments

#### Background

*T. minuta* is an aromatic plant that has several medical benefits such as a remedy for the colds, respiratory inflammations, stomach problems, anti-spasmodic, anti-parasitic, antiseptic and sedative. This work aimed to investigate antioxidant and anti-inflammatory effects of *T. minuta* essential oil.

#### Research frontiers

The aim of the present study was to investigate the level of potential modulating effects of *T. minuta* essential oil on macrophages and their related functions including expression of NOX subunits, NOS and TNF- $\alpha$  mRNAs in LPS-stimulated macrophages. In addition, *in vitro* anti-oxidant capacity of *T. minuta* essential oil was examined by assessments of ROS, RNS and H<sub>2</sub>O<sub>2</sub> scavenging ability.

#### Related reports

Advances in chemical and pharmacological evaluations of *T. minuta* essential oil such as antioxidant and antimicrobial have occurred in the past recent years. However, several useful features of this plant (*e.g.* the mechanisms underlying its antioxidant and anti-inflammatory effects) have remain unknown.

#### Innovations and breakthroughs

These studies would reveal that *T. minuta* essential oil exhibits radical scavenging activity (against superoxide anion, H<sub>2</sub>O<sub>2</sub>, and NO radicals) in macrophages, in part, due to

an inhibition of iNOS and NOX gene expression.

### Applications

These data suggest a potential therapeutic usefulness for *T. minuta* in the modulation of macrophages and provides evidence to support the use of *T. minuta* as a tea/additive/traditional remedy for treatment of inflammatory diseases.

### Peer review

*T. minuta* essential oil displayed an anti-oxidant property by scavenging superoxide, H<sub>2</sub>O<sub>2</sub> and NO radicals, and reduced oxidative stress. The decreased formation of ROS and NOS radicals in macrophages was possibly due to the radical scavenging activity of phenolic groups present in the oil and/or due to an inhibition of iNOS and NOX gene expressions. Furthermore, *T. minuta* essential oil decreased the expression of the genes for pro-inflammatory cytokine TNF- $\alpha$ .

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