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

Parastoo Karimian[1], Gholamreza Kavoosi[1*], Zahra Amirghofran[2]

[1]*Biotechnology Institute, Shiraz University, Shiraz, 71441−65186, Iran*

[2]*Department of Immunology, Autoimmune Disease Research Center and Medicinal and Natural Products Chemistry Research Center, Shiraz University of Medical Sciences, Shiraz, Iran*

PEER REVIEW

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−α mRNA expression in lipopolysacharide−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−β−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_{50} 12−15 μg/mL, which indicated a potent radical scavenging activity. In addition, *T. minuta* essential oil significantly reduced NADH oxidase, inducible nitric oxide synthaseand TNF−α mRNA expression in the cells at concentrations of 50 μg/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

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[4–9]. 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)[10–14]. *T. minuta* essential oil has a significant antibacterial activity against both Gram–positive and Gram–negative bacteria[15–17]. Several studies have also described antifungal activities of *T. minuta* essential oil against *Candida*, *Penicillium* and *Aspergilus* species[18–20]. *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[21,22].

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–α mRNAs in lipopolysacharide (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_2O_2) scavenging ability using ABTS, sodium nitrite, and H_2O_2 scavenging, respectively. It was expected that these studies would reveal that *T. minuta* essential oil exhibits radical scavenging activity (against superoxide anion, H_2O_2, 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–α 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, dimethysulfoxide (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[23]. 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 μm). 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_2 (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 μm) 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_8–C_{25}) 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[24].

2.4. ROS scavenging assay

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

10 µL of the *T. minuta* essential oil (0–500 µg/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 [(A734$_{blank}$−A734$_{sample}$)/A734$_{blank}$]×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. H₂O₂ scavenging assay

H$_2$O$_2$ scavenging activity of the *T. minuta* essential oil was determined as previously described[26]. Briefly, 10 µL of the *T. minuta* essential oil (0–500 µg/mL in DMSO) was incubated with 1.0 mL of H$_2$O$_2$ (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$_2$O$_2$ using a spectrophotometer. The percentage of H$_2$O$_2$ scavenging was calculated as [(A230$_{blank}$−A230$_{test}$)/A230$_{blank}$]×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[26]. Briefly, 10 µL of the *T. minuta* essential oil (0–500 µg/mL in DMSO) was incubated with 0.5 mL of sodium nitrite (10 µg/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: [(A540$_{blank}$−A540$_{sample}$)/A540$_{blank}$×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 µg/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 2×10^6 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 µg/mL). After 2 h, *T. minuta* essential oil was added at a final concentration of 0–200 µg/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[27]. Cells (2×10^4 cells per well) were incubated for 24 h (at 37 °C in 5% CO$_2$) with different concentrations (0–200 µg/mL) of *T. minuta* essential oil. Thereafter, 10 µL 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 µL 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)×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 2×10^6 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 µL 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 µL 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 β–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–α (NM–013693) (Table 1). The GAPDH and β–actin were used as internal control (whose expression proved not to be influenced by LPS) for data normalization[28]. Relative real–time PCR was performed in a 20 µL volume containing 1 µL cDNA, 1×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–α 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*[29]. Accordingly, fold–expression of target mRNAs over the reference values were calculated by equation $2^{-\Delta\Delta_{CT}}$, where ΔCT 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 ΔCT of each experimental sample from that of the control sample[30].

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[1]	Accession	Sense sequence	Anti sense sequence
GAPDH	NM–010927	5′–CGGTGTGAACGGATTTGGC–3	5′–TGAGTGGAGTCATACTGGAAC–3′
β–actin	NM–007393.3	5′–CCACACCCGCCACCAGTTCG–3′	5′–CTAGGGCGGCCCACGATGGA–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′–GCACAAAGCCAAACAATACG–3′
iNOS	NM–008084	5′– CTGGAGGTTCTGGATGAG–3′	5′– CTGAGGGCTGACACAAGG –3′
TNF–α	NM–013693	5′–GTCTCAGCCTCTTCTCATTC–3′	5′– GGAACTTCTCATCCCTTTGG–3′

[1]Primer design, in form of exon junction was carried out using Allele ID 7 software for the internal controls GAPDH and β–actin and test genes NADH oxidase p22 phagocytes oxidase (NOX p22phox), NOX p47phox, NOX p47phox, NOX p67phox, iNOS and TNF–α 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–β–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–β–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_2O_2 scavenging activity. IC_{50} for ROS, RNS and H_2O_2 scavenging were (12.0±3.0), (15.0±2.5) and (13.0±4.0) μg/mL of *T. minuta* essential oil, respectively. At concentrations >30 μg/mL, the *T. minuta* essential oil significantly scavenges ROS, RNS, and H_2O_2 by 100%. The *T. minuta* essential oil analyzed here possessed potent *in vitro* ROS, RNS and H_2O_2 scavenging activity. The *T. minuta* essential oil at >30 μg/mL had the ability to scavenge all ROS, RNS and H_2O_2 radicals, an indicator of its potency as a radical scavenger.

Table 2
Chemical composition of *T. minuta* essential oil.

Compounds	Retention index	% of compounds
α–Pinene	933.526	0.327 92
Sabinene	973.444	0.397 03
cis–3–Hexenyl acetate	1 005.680	0.148 38
p–Cymene	1 025.210	0.933 76
Limonene	1 029.490	3.102 93
cis–β–Ocimene	1 037.830	7.940 67
Dihydrotagetone	1 061.290	33.862 97
Chrysanthenone	1 103.260	0.146 46
Allo–cimene	1 135.730	0.358 52
E, Z–Epoxyocimene	1 149.090	2.035 34
Tagetone	1 160.520	16.155 09
cis–Tagetone	1 167.400	0.198 29
p–Mentha–1,8 dien–3–one	1 208.060	0.188 94
Z–Ocimene	1 238.290	5.275 42
E–Ocimene	1 250.790	19.928 7
Thymol	1 282.780	0.477 33
Carvacrol	1 298.190	0.470 14
cis–Isoeugenol	1 398.530	0.865 60
E–Caryophyllene	1 420.970	0.299 45
α–Humulene	1 455.070	0.149 81
Germocrene D	1 497.900	0.398 97
Spathulenol	1 582.190	0.373 19

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).
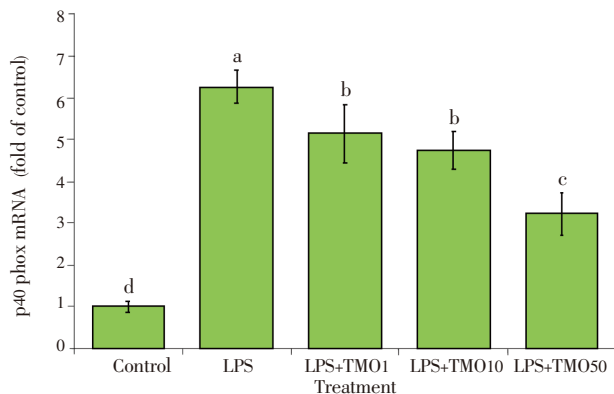
3.5. T. minuta essential oil reduced NOX p40phox mRNA Expression in LPS−Stimulated Macrophages

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).
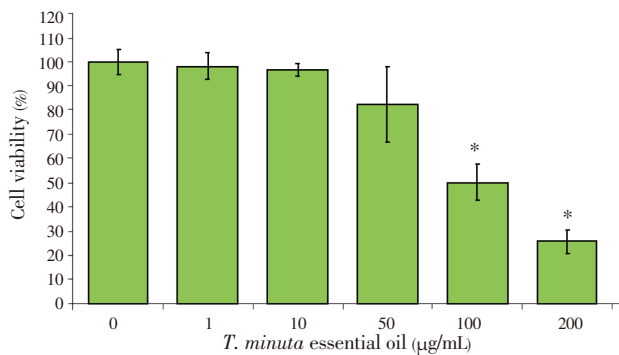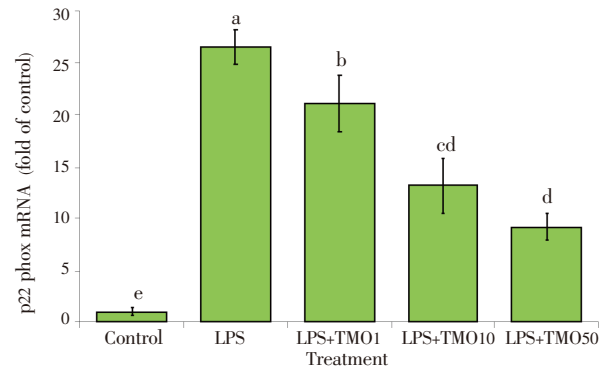

Figure 1. Effect of the *T. minuta* essential oil on the viability of J774 cell lines.

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 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.
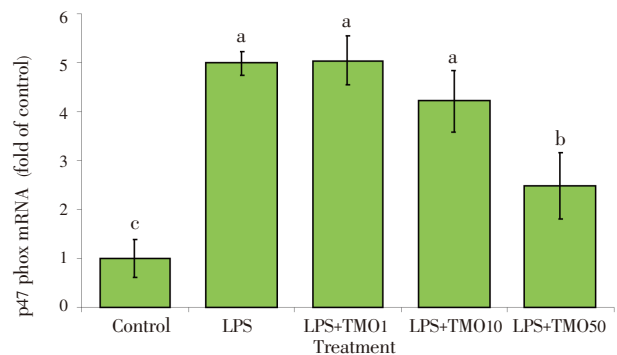

Figure 3. Effects of *T. minuta* essential oil on NOX p40phox mRNA expression in LPS−stimulated macrophages.

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 4. Effects of *T. minuta* essential oil on NOX p47phox mRNA expression in LPS−stimulated macrophages.
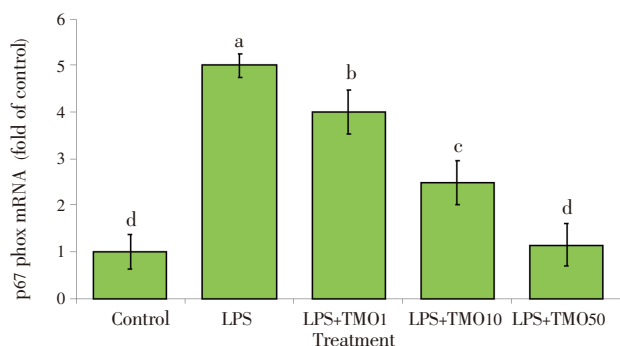
TMO: *T. minuta* essential oil. The cells were cultured in 24−well plates and treated with and without LPS. Various concentrations of TMO (1−50 μg/mL) were added. After 24 h, the expression of NOX p47phox mRNA was analyzed by real−time PCR. Cells treated with DMSO as the solvent (control) and cells treated 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).
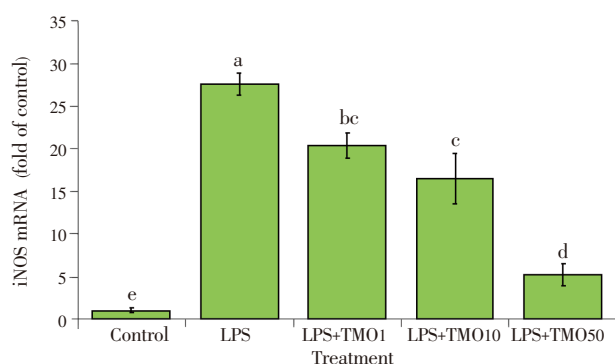
Figure 5. Effects of *T. minuta* essential oil on NOX p67phox mRNA expression in LPS–stimulated macrophages.

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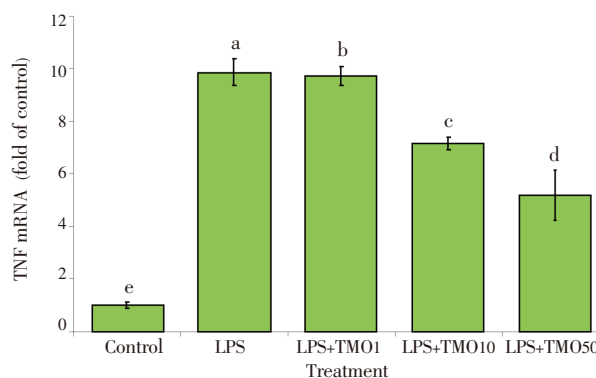
Figure 6. Effects of *T. minuta* essential oil on iNOS mRNA expression in LPS–stimulated macrophages.

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–β–ocimene, Z–ocimene, limonene and epoxyocimene. Previous study reported the main components of *T. minuta* essential oil were β–ocimene, dihydrotagetone, tagetone, Z–ocimene and E–ocimene[11]. Another study reported thiophenes and polyacetylenic compounds in the *Tagetes* species and *T. minuta* had the highest total thiophene yield[12]. 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[11–14]. 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_2O_2 scavenging activity. The *T. minuta* essential oil at >30 μg/mL had the ability to scavenge all ROS, RNS and H_2O_2 radicals, an indicator of its potency as a radical scavenger. ROS are oxygen–derived small molecules, including oxygen radicals such as superoxide, hydroxyl and peroxyl 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[31]. 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[32]. *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_{50} between 35–344 μg/mL[21,22]. 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 μg/mL.

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 (IC_{50}=95 μg/mL). In order to determine the antioxidant and anti–inflammatory effects of *T. minuta* essential oil on macrophages, the concentrations of >50 μg/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[33]. 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[34]. 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[35]. 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[36]. 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[37]. 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–α, IL–1 and IL–6[38]. NO generation involves several steps including the activation of nuclear transcription factor (NF)–κB and subsequent iNOS gene expression[39]. NF–κB regulates the expression of various genes involved in inflammatory responses. Its activation can also be regulated by various cytokines, among which TNF–α is the most important. In response to inflammatory stimuli such as LPS, macrophages secrete a variety of inflammatory mediators such as TNF–α and IL–1β. The production of TNF–α cytokine is important for the induction of NO synthesis in LPS–stimulated macrophages[40]. As results of this study showed that *T. minuta* essential oil was able to reduce inducible expression of TNF–α gene, which indicated that the reduced NO production seen in the macrophage cultures might be partly related to the suppression of TNF–α expression. TNF–α is known to play a crucial role in inflammatory responses and is involved in the pathogenesis of inflammatory diseases[41]. As results of our study showed, *T. minuta* essential oil significantly reduced iNOS mRNA expression in stimulated macrophages. Suppression of TNF–α 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_2O_2 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–α. Therefore, a reduced expression of the above–noted inflammatory enzymes and cytokines could be attributed to a suppression of the NF–kB 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

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–α 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_2O_2 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_2O_2, 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_2O_2 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–α.

References

[1] *Iranian Herbal Pharmacopoeia*. Tehran: Iranian Ministry of Health & Medical Education Publications; 2002.

[2] Nerio LS, Olivero–Verbel J, Stashenko E. Repellent activity of essential oils: a review. *Bioresour Technol* 2010; **101**: 372–378.

[3] Rahimi R, Shams–Ardekani MR, Abdollahi M. A review of the efficacy of traditional Iranian medicine for inflammatory bowel disease. *World J Gastroenterol* 2010; **16**: 4504–4514.

[4] Nikkon F, Habib MR, Saud ZA, Karim MR. *Tagetes erecta* and its mosquitocidal potency against *Culex quinquefasciatus*. *Asian Pac J Trop Biomed* 2011; **1**: 186–188.

[5] Kamaraj C, Rahuman AA, Bagavan A, Elango G, Zahir AA, Santhoshkumar T. Larvicidal and repellent activity of medicinal plant extracts from Eastern Ghats of South India against malaria and filariasis vectors. *Asian Pac J Trop Med* 2011; **4**: 698–705.

[6] Govindarajan M. Larvicidal and repellent properties of some essential oils against *Culex tritaeniorhynchus* Giles and *Anopheles subpictus* Grassi (Diptera: Culicidae). *Asian Pac J Trop Med* 2011; **4**: 106–111.

[7] Aristatile B, Al–Assaf AH, Pugalendi KV. Carvacrol suppresses the expression of inflammatory marker genes in D–galactosamine–hepatotoxic rats. *Asian Pac J Trop Med* 2013; **6**: 205–211.

[8] Maity N, Nema NK, Abedy MK, Sarkar BK, Mukherjee PK. Exploring *Tagetes erecta* Linn flower for the elastase, hyaluronidase and MMP–1 inhibitory activity. *J Ethnopharmacol* 2011; **137**: 1300–1305.

[9] Wang M, Tsao R, Zhang S, Dong Z, Yang R, Gong J, et al. Antioxidant activity, mutagenicity/anti–mutagenicity, and clastogenicity/anti–clastogenicity of lutein from marigold flowers. *Food Chem Toxicol* 2006; **44**: 1522–1529.

[10] Breme K, Tournayre P, Fernandez X, Meierhenrich UJ, Brevard H, Joulain D, et al. Identification of odor impact compounds of *Tagetes minuta* essential oil: comparison of two GC–olfactometry methods. *J Agric Food Chem* 2009; **57**: 8572–8580.

[11] Lopez SB, Lopez ML, Aragon LM, Tereschuk ML, Slanis AC,

Feresin GE, et al. Composition and anti–insect activity of essential oils from *Tagetes* species on *Ceratitis capitata* Wiedemann and *Triatoma infestans* Klug. *J Agric Food Chem* 2011; **59**: 5286–5292.

[12] Marotti I, Marotti M, Piccaglia R, Nastri A, Grandi S, Dinelli G. Thiophene occurrence in different *Tagetes* species: agricultural biomasses as sources of biocidal substances. *J Sci Food Agric* 2010; **90**: 1210–1217.

[13] Mohamed MA, Harris PJ, Henderson J, Senatore F. Effect of drought stress on the yield and composition of volatile oils of drought–tolerant and non–drought–tolerant clones of *Tagetes minuta*. *Planta Med* 2002; **68**: 472–484.

[14] Ranilla LG, Kwon YI, Apostolidis E, Shetty K. Phenolic compounds, antioxidant activity and *in vitro* inhibitory potential against key enzymes relevant for hyperglycemia and hypertension of commonly used medicinal plants, herbs and spices in Latin America. *Bioresour Technol* 2010; **101**: 4676–4689.

[15] Céspedes CL, Avila JG, Martínez A, Serrato B, Calderón–Mugica JC, Salgado–Garciglia R. Antifungal and antibacterial activities of Mexican tarragon (*Tagetes lucida*). *J Agric Food Chem* 2006; **54**: 3521–3527.

[16] Tereschuk ML, Baigori MD, Abdala LR. Antibacterial activity of *Tagetes terniflora*. *Fitoterapia* 2003; **74**: 404–416.

[17] Héthélyi E, Dános B, Tétényi P, Koczka I. GC–MS analysis of the esssential oils of four *Tagetes* species and the anti–microbial activity of *Tagetes minuta*. *Flavor Fragr J* 1986; **1**: 169–173.

[18] Dunkel FV, Jaronski ST, Sedlak CW, Meiler SU, Veo KD. Effects of steam–distilled shoot extract of *Tagetes minuta* and entomopathogenic fungi on larval *Tetanops myopaeformis*. *Environ Entomol* 2010; **39**: 979–988.

[19] Mares D, Tosi B, Poli F, Andreotti E, Romagnoli C. Antifungal activity of *Tagetes patula* extracts on some phytopathogenic fungi: ultrastructural evidence on *Pythium ultimum*. *Microbiol Res* 2004; **159**: 295–304.

[20] Thembo KM, Vismer HF, Nyazema NZ, Gelderblom WC, Katerere DR. Antifungal activity of four weedy plant extracts against selected mycotoxigenic fungi. *J Appl Microbiol* 2010; **109**: 1479–1486.

[21] Gong Y, Liu X, He WH, Xu HG, Yuan F, Gao YX. Investigation into the antioxidant activity and chemical composition of alcoholic extracts from defatted marigold residue. *Fitoterapia* 2012; **83**: 481–489.

[22] Parejo I, Bastida J, Viladomat F, Codina C. Acylated quercetagetin glycosides with antioxidant activity from *Tagetes maxima*. *Phytochemistry* 2005; **66**: 2356–2362.

[23] *British Pharmacopeia*. London: The British Pharmacopoeia Commission; 1998, p. 137–138.

[24] Adams RP. *Identification of essential oil components by gas chromatography/mass spectrometery*. 4th ed. Illinois, USA: Allured Publishing Corporation; 2007, p. 456–494.

[25] Kavoosi G, Rowshan V. Chemical composition, antioxidant and antimicrobial activities of essential oil obtained from *Ferula assa–foetida* oleo–gum–resin: effect of collection time. *Food Chem* 2013; **138**: 2180–2187.

[26] Kavoosi G, Teixeira da Silva JA, Saharkhiz MJ. Inhibitory effects of *Zataria multiflora* essential oil and its main components on nitric oxide and hydrogen peroxide production in lipopolysaccharide–stimulated macrophages. *J Pharm Pharmacol* 2012; **64**: 1492–1500.

[27] Nouri AM, Thompson C, Cannell H, Symes M, Purkiss S, Amirghofran Z. Profile of epidermal growth factor receptor (EGFr) expression in human malignancies: effects of exposure to EGF and its biological influence on established human tumor cell lines. *Inter J Mol Med* 2000; **6**: 495–500.

[28] Barber RD, Harmer DW, Coleman RA, Clark BJ. GAPDH as a housekeeping gene: analysis of GAPDH mRNA expression in a panel of 72 human tissues. *Physiol Genomics* 2005; **21**: 389–395.

[29] Larionov A, Krause A, Miller W. A standard curve based method for relative real time PCR data processing. *BMC Bioinformatics* 2005; **6**: 62.

[30] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real–time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* 2001; **25**: 402–408.

[31] Circu ML, Aw TY. Reactive oxygen species, cellular redox systems, and apoptosis. *Free Radic Biol Med* 2010; **48**: 749–762.

[32] Huang CC, Wang HF, Chen CH, Chen YJ, Yih KH. A study of four antioxidant activities and major chemical component analyses of twenty–five commonly used essential oils. *J Cosmet Sci* 2011; **62**: 393–404.

[33] Groemping Y, Rittinger K. Activation and assembly of the NADPH oxidase: a structural perspective. *Biochem J* 2005; **386**: 401–416.

[34] Minakami R, Sumimotoa H. Phagocytosis–coupled activation of the superoxide–producing phagocyte oxidase, a member of the NADPH oxidase (NOX) family. *Int J Hematol* 2006; **84**: 193–198.

[35] Bedard K, Krause KH. The NOX family of ROS–generating NADPH oxidases: physiology and pathophysiology. *Physiol Rev* 2007; **87**: 245–313.

[36] Kleniewska P, Piechota A, Skibska B, Gorąca A. The NADPH oxidase family and its inhibitors. *Arch Immunol Ther Exp (Warsz)* 2012; **60**: 277–294.

[37] Zielonka J, Zielonka M, Sikora A, Adamus J, Joseph J, Hardy M, et al. Global profiling of reactive oxygen and nitrogen species in biological systems: high–throughput real–time analyses. *J Biol Chem* 2012; **287**: 2984–2995.

[38] Sellers SL, Iwasaki A, Payne GW. Nitric oxide and TNFα are critical regulators of reversible lymph node vascular remodeling and adaptive immune response. *PLoS One* 2013; **8**: 60741.

[39] Lorenzo O, Picatoste B, Ares–Carrasco S, Ramírez E, Egido J, Tuñón J. Potential role of nuclear factor kB in diabetic cardiomyopathy. *Mediators Inflamm* 2011; doi: 10.1155/2011/652097.

[40] Zha LY, Mao LM, Lu XC, Deng H, Ye JF, Chu XW, et al. Anti–inflammatory effect of soyasaponins through suppressing nitric oxide production in LPS–stimulated RAW 264.7 cells by attenuation of NF–κB–mediated nitric oxide synthase expression. *Bioorg Med Chem Lett* 2011; **21**: 2415–2418.

[41] Leonard B, Maes M. Mechanistic explanations how cell–mediated immune activation, inflammation and oxidative and nitrosative stress pathways and their sequels and concomitants play a role in the pathophysiology of unipolar depression. *Neurosci Biobehav Rev* 2012; **36**: 764–785.