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The antioxidant activity of Trachyspermum ammi essential oil and thymol in murine macrophages

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ABSTRACT

Essential oils are abundant resources of several bioactive compounds, which possess therapeutic effects and have been at the heart attention of the research as the natural additives for the shelf-life extension of food products. This study aimed to investigate, in vitro radical scavenging and antioxidant capacities of *Ttrachyspermum ammi* (TEO) and its main components. Furthermore, this study sought to determine the antioxidant enzyme responses to TEO at the gene expression levels. The inhibitory effects of TEO and its main components on superoxide and nitric oxide production and NADH oxidase (NOX) and nitric oxide synthase (NOS) expression examined in lipopolysaccharide (LPS)-stimulated macrophages. TEO and thymol displayed a robust antioxidant activity while γ-terpinene and p-cymene have presented a few antioxidant activities. TEO at 10 μg/mL strongly reduced NO but potently increased reactive oxygen species (ROS) in LPS-stimulated macrophages. TEO significantly decreased inducible nitric oxide synthase (iNOS) mRNA expression but upregulated NOX mRNA in LPS-stimulated macrophages at 10 μg/mL. TEO had strong synergism with LPS to enhance ROS, a condition that is suitable against tumors propagation. Thymol at 10 μg/mL reduced NO, and ROS production and iNOS mRNA and NOX mRNA expression in LPS-stimulated macrophages but γ-terpinene and p-cymene did not show such activities. These differential activities between TEO and its pure chemical components strongly recommend that TEO antioxidant activity may be mainly due to the presence of thymol and also a strong synergism between all monoterpenes and monoterpenoids components of essential oils to the presentation a biological action.

1. Introduction

Trachyspermum ammi (Apiaceae family) has a vital role in pharmaceutics and the food industry that is extensively used as a flavoring ingredient in a wide variety of fields and as a traditional folk remedy. The plant has several functional characteristics including anti-spasmodic, carminative and stimulant properties (Amiri and Joharchi, 2016; Johri, 2011). It is used traditionally as a useful remedial agent for diarrhea, atonic dyspepsia, piles, and abdominal tumors, abdominal pains, and loss of appetite, bronchial problems, asthma, galactagogue, and amenorrhea. Traditionally, Persian practitioners applied the eye and ear drop formulated from seeds of *T. ammi* to control the infected conditions and enhance the auditory weakness. In the field of respiratory, *T. ammi* is alleged to be useful for coughing, pleurisy, and dysphonia. Fruits were widely administered for liver, spleen as well as gastrointestinal disorders such as nausea, vomiting, reflux, abdominal cramps and loss of appetite (Bairwa et al., 2012; Dubey and Kashyap, 2015). Modern pharmacological studies proved various biological activities of this plant such as anti-fungal, anti-microbial, antioxidant, cytotoxic, abortifacient, antitussive, anthelmintic, anti-nociceptive, hypolipidemic, anti-hypertensive, anti-spasmodic, broncho-dilating action, anti-lithiasis, diuretic, and anti-filarial (Boskabady et al., 2014; Zarshenas et al., 2013).

It has accepted that, multiple biological actions of medicinal plant and their phytochemical especially essential oils originated from their potential to scavenge biological oxidants/radicals including superoxide anion, hydrogen peroxide, hydroxyl radical, nitric oxide, peroxynitrite and their byproducts such as lipid peroxides and thiobarbituric acid reactive substances (Elshafie and Camele, 2017; Singh et al., 2018). These highly reactive oxidants/radicals can oxidatively modify lipids, sugar, nucleic acids, proteins, enzymes and metabolites leading to oxidative stress and ultimately cell damage. The cell damage caused by

Abbreviations: ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); DPPH, 2,2-diphenyl-1-picrylhydrazyl; LPS, Lipopolysaccharide; NOS, Nitric Oxide Synthase; NOX, NADH Oxidase; ROS, Reactive Oxygen Species; RI, Retention indices; TEO, *Ttrachyspermum ammi* essential oil

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these unstable free radicals and the change in the balance between oxidants and antioxidants appear to be a major contributor to aging and pathological degenerative diseases such as cancer, cardiovascular disease, cataracts, immunodeficiency, brain dysfunction, and depression. Neutralization and deactivation of oxidants/radicals by natural antioxidants are efficient ways to protect the cells against the oxidative damage (Nimse and Pal, 2015; Rodríguez et al., 2016).

In this study, the radical (ABTS and DPPH) scavenging properties along with antioxidant activity (against biological oxidants such as superoxide ion and nitric oxide) of the TEO and its main components were evaluated. The inhibitory effect of TEO and its main components on ROS and NO production, in lipopolysaccharide (LPS)-induced macrophages was examined. The level of potential modulating effects of emulsified TEO and its main components on the expression of NOX subunits (p22phox, p40phox, p47phox, p67phox, p91phox) and iNOS mRNAs in LPS-stimulated macrophages investigated.

2. Materials and methods

2.1. Chemicals and reagents

Polysorbate 20 (CID:443314), sodium nitrite (CID:23668193), Sodium nitroprusside (CID:11963622), Sodium sulphate (CID:24436), Naphthylethylenediamine (CID:15107), Sulfanilamide (CID:5333), phosphoric acid (CID:1004), Ethylenediaminetetraacetic acid (EDTA) (CID:6049), Gallic acid (CID:370), Nitro blue tetrazolium (CID:9853362), Nicotinamide adenine dinucleotide (CID:439153), Phenazine methosulfate (CID:9285), Thymol (CID: 6989), γ-Terpinene (CID:7461), p-Cymene (CID:7463).

2.2. Essential oil preparation

The aerial parts of T. ammi dried in the shade for 72 h. The plants (100 g) were hydro-distilled in distilled water (1000 mL) for 3 h using a Clevenger apparatus. In this method, plant materials were soaked in water. The water boiled to produce steam bearing the most volatile chemicals. The steam was then chilled, and the resulting distillate collected. The essential oil normally float on top of the aromatic water and was separated from aromatic water via a decanting funnel. The obtained essential oil and aromatic water were stored at 4 °C.

2.3. Identification of essential oil chemical components

The essential oil was analyzed using an Agilent 7890A series gas chromatograph (Agilent, Palo Alto, CA, USA) equipped with a flame ionization detector (FID) on a fused silica capillary HP-5 column (30 m × 0.32 mm, i.e., and film thickness 0.25 μm). Retention indices (RI) were defined on n-alkanes (C8–C25) retention times (RT) as standard that was injected after essential oil under the same chromatographic conditions. The compounds of essential oils were identified by software of GC-MS apparatus with comparison of retention times and fragmention patterns of the related peake with those reported in the liberaries of Weily and NIST (NIST05a and Wiley 7n Libraries).

2.4. Preparation of essential oil emulsion

To prepare polysorbate-essential oil-aromatic water emulsion, 1 mL of essential oil added to 100 mL of aromatic water. Polysorbate-20 (100 μg/mL) added to essential oil-aromatic water mixture and the mixture incubated at 30 °C for 24 h and then at 4 °C for 48 h. At this time, a milky emulsion formed. These milky emulsions used for antioxidant analysis.

2.5. Total phenol content

The amount of total phenol content in of essential oil-aromatic water emulsion was determined using Folin-Ciocalteu reagent using gallic acid as a standard. Briefly, 200 μL of antioxidant samples or standard gallic acid solutions (0–500 μg) was added to 1 mL Folin-Ciocalteu reagent (10%) and 0.3 mL Na_2CO_3 (10%). Then the mixture was left at ambient temperature for 10 min. Absorbance was read at 765 nm using spectrophotometer (Ainsworth and Gillespie, 2007). The total phenol content of essential oil-aromatic water obtained 1700 μg/mL of gallic acid equivalents that then adjusted to 1000 μg/mL using deionized water for antioxidant characterization.

2.6. Extracellular antioxidant activity of TEO

2.6.1. ABTS radical scavenging assay

Essential oil (0–200 μg/mL) were added to 1.0 mL of diluted ABTS radical solution. After mixing, the absorbance (A) was read at 734 nm. The percentage of light absorbance reduction used to calculate ABTS radical scavenging. The 50% ABTS inhibition (IC50) calculated using the inhibition percentage/various antioxidant concentrations graph (Floegel et al., 2011).

2.6.2. DPPH radical scavenging assay

Essential oil (0–200 μg/mL) were added to 1.0 mL of DPPH (0.2 mM) in 95% methanol. The mixture was allowed to incubate in the dark for 30 min at room temperature. The decrease of absorbance (A) at 517 nm for each sample was observed. The percentage of light absorbance reduction at 517 nm was taken as DPPH radical scavenging activity. The 50% DPPH inhibition (IC50) was obtained using the inhibition percentage/various antioxidant concentrations graph (Floegel et al., 2011).

2.6.3. Superoxide radical scavenging assay

Essential oil (0–200 μg/mL) incubated with 1.0 mL superoxide radical reaction mixture at room temperature for 15 min. The reaction mixture in phosphate buffer (10 mM, pH 7.4) contains phenazine methosulfate (20 mM), nicotinamide adenine dinucleotide (300 mM), nitro blue tetrazolium (50 mM). After incubation, the absorbance was read at 560 nm. The percentage of light absorbance reduction at 560 nm was taken as superoxide radical scavenging. The graph that plotted the percentage of superoxide radical scavenging against different essential oil concentrations was used to determine IC50 (Tarpey et al., 2004).

2.6.4. Nitric oxide scavenging assay

Essential oil (0–200 μg/mL) were incubated with 0.5 mL of sodium nitroprusside (SNP) (20 μg/mL prepared in 100 mM sodium citrate pH 5) at 37 °C. After 2 h incubation, 0.5 mL of Griess reagent was added, and the absorbance (A) was recorded at 540 nm. The percentage of light absorbance at 540 nm was taken as nitric oxide scavenging potential. The 50% NO inhibition (IC50) calculation was performed using the inhibition percentage/various essential oil concentrations graph (Tarpey et al., 2004).

2.6.5. Low-density lipoprotein (LDL) oxidation inhibition assay

Essential oil (0–200 μg/mL) were incubated with 1.0 mL of LDL solution (500 μg/mL in 10 mM phosphate buffered saline). Immediately, 1 mL of cupric sulphate (10 μM in 10 mM phosphate buffered saline) was added to LDL solution and then incubated at 37 °C for 10 h. After incubation, the light absorbance at 234 was recorded. The percentage light absorbance reduction at 234 nm was taken as LDL oxidation inhibition. The 50% LDL oxidation inhibition (IC50) was calculated from a graph that plotted inhibition percentage against different concentrations of antioxidant (Lopez-Alarcon and Denicola, 2013).

2.7. TEO antioxidant activity on macrophage cells

2.7.1. Macrophage culture

Macrophage cells were harvested from peritoneal fluid of BALB/c mice in 5.0 mL of sterile phosphate-buffered saline (PBS), pH 7.4. The cells were washed twice by centrifugation at 1700*g* for 5 min at 4 °C with PBS. The cells were then re-suspended in 1.0 mL RPMI-1640 culture medium (Gibco) with 10% fetal bovine serum (FBS) (Gibco) and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin), counted and used for the following tests.

2.7.2. Macrophage viability assay

Macrophages (2×10^4 cells per well) were incubated for 24 h (at 37 °C in 5% CO2) with different concentrations of emulsified essential oils (0–100 μg/mL). After that, 10 μL of MTT (5 mg/mL) added to each well and incubated for an additional 4 h at 37 °C followed by treatment with 100 μL of DMSO. The absorbance of each well was determined by spectrophotometer at the wavelength of 570 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 concentration provided a 50% inhibition (IC50) calculated from a graph, plotting the inhibition percent against different essential oil concentrations (Kavoosi and Amirghofran, 2017).

2.7.3. Determination of intracellular reactive oxygen species (ROS)

Intracellular ROS was measured using (2, 7-dichlorofluorescein diacetate) DCFDA cellular ROS Detection Assay Kit (ab113851). Macrophages were added to 96-well plates (25 × 104 cell/well, 200 μL/well) and incubated for 24 h. Culture media were removed, and the cells were washed in 1X PBS. Macrophages were incubated with the diluted DCFDA solution (25 μM) for 45 min at 37 °C in the dark. Then, DCFDA solution was removed, and cells were washed by 100 μL/well of 1X PBS. Afterward, macrophages were incubated in the presence of 1 μg/mL LPS along with the non-cytotoxic concentration of essential oil (10 μg/mL) for 6 h. Finally, the signal was detected at an excitation wavelength of 485 nm and an emission wavelength of 535 nm using fluorescent microplate reader (Bognar et al., 2013).

2.7.4. Determination of intracellular nitric oxide

Macrophages were seeded into a 24 well tissue culture plate (2×10^6 cells/mL, 1 mL/well) and incubated overnight (at 37 °C under humidified air containing 5% CO2) to adhere. Some wells were pre-treated with the non-cytotoxic concentrations of essential oil (1, 5 and 10 μg/mL) 2 h before LPS treatment (1 μg/mL, from *Escherichia coli*). After incubation for 24 h, 100 μL of culture supernatant was mixed with 100 μL of Griess reagent (1% sulfanilamide in 5% phosphoric acid, 0.1% N-1-naphthyl ethylenediamine dihydrochloride in water) in 96 well plates, incubated at room temperature for 10 min and the nitrite content was determined at 540 nm using a microplate reader (BioTek, USA). Sodium nitrite (100 mM sodium nitrite in water) was used for standard curve preparation (Schmolz et al., 2017).

2.7.5. Measurement of thiobarbituric acid reactive substances (TBARS)

Macrophages were incubated with different concentrations of emulsified essential oil (10 μg/mL) and LPS (1 μg/mL) for 24 h. Cells were harvested and lysed then the amount of TBARS content was determined using thiobarbituric acid (TBA) as a chromogen and malondialdehyde standard. Briefly, one volume of supernatants or standard malondialdehyde (50 μM in acetic acid pH 4) were mixed with one volume of thiobarbituric acid (200 μM in acetic acid; pH 4) at 95 °C for 1 h. After cooling at room temperature, the absorbance was read at 532 nm (Bostoglou, 1994).

2.8. Real-time studies

2.8.1. Macrophages culture for real-time PCR

Macrophages were cultured at 37 °C in a DMEM medium containing 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and 10% heat-inactivated FCS. Cultures were allowed to grow until confluence at which point adherent macrophages were scraped from the flask and washed with a warm medium (25 °C). Cells were counted, and their viability was determined by trypan blue dye. Cells were seeded at a concentration of 2 × 106 cells per mL in 24-well tissue culture plates in triplicate (Tissue, Culture Plate, Jet Biofil, Kyoto, Japan). After culturing for 18 h to allow cells to adhere, non-adherent cells were removed by gentle rinsing with the medium. Remaining adherent cells were then cultured in either the presence or absence of medium bearing 1 μg LPS/mL. After 2 h, essential oil was added at a final concentration of 10 μg/mL (the most effective dose). After 24 h of incubation at 37 °C, the culture supernatants in each well were removed and the cells harvested for RNA extraction and quantitative real-time polymerase chain reaction (RT-PCR) analysis.

2.8.2. Quantitative RT-PCR

Total RNA from cells isolated with RNX-plus buffer (Cinagen, Tehran, Iran) in an RNase-free microtube according to the manual instruction. The first strand cDNA synthesis was performed by 1 μg RNA using first strand cDNA kit (Fermentas, Hanover, MD) according to manual instruction. The cDNA was then used as the template for quantitative real-time PCR. Primer design, in form of exon junction was carried out using Allele ID 7 software for the internal control glyceraldehydes -3- phosphate dehydrogenase (GAPDH) (GeneBank NM-008084) and test genes p22phox (cytochrome b-245, alpha polypeptide (Cyba); Gene Bank NM-007806.3), p40phox (neutrophil cytosolic factor 4 (Ncf4); Gene Bank NM-008677.2), p47phox (neutrophil cytosolic factor 1 (Ncf1); Gene Bank NM-001286037.1), p67phox (neutrophil cytosolic factor 2 (Ncf2); Gene Bank NM-010877.4), gp91phox (cytochrome b-245, beta polypeptide (Cybb); Gene Bank NM-007807.5) and inducible nitric oxide synthase (iNOS, Gene Bank NM-010927) genes (Table 1). The gene expression quantification was performed using the relative standard curve method, and all data were normalized to the absolute control group and subsequently normalized to the gene expression of GAPDH. The amplification reactions were carried out in a line-Gene K thermal cycler (Bioer Technology Co., Hangzhou, China). For quantitative RT-PCR data, relative expressions of NOX and iNOS

Table 1
Primer used for real-time analysis.

Genes	Accession No.	Sense sequence	Anti-sense sequence
GAPDH	NM-008084	5′-CGGTGTGAACGGATTTGGC-3′	5′-TGAGTGGAGTCATACTGGAAC-3′
NOX p22	NM-007806.3	5′- ATGGAGCGATGTGGACAG-3′	5′- ACCGACAACAGGAAGTGG-3′
NOX p40	NM-008677.2	5′-CAACAAAGACTGGCTGGAG-3′	5′-CCGCAATGTCCTTGATGG-3′
NOX p47	NM-001286037.1	5′- ATGGCACAAAGGACAATC-3′	5′- ACCTGAGGCTATACACAAG-3′
NOX p67	NM-010877.4	5′- CAGCCACAGTCAGCAGAG-3′	5′-GCACAAAGCCAAACAATACG-3′
NOX p91	NM-007807.5	5′-TGT GGCTGTGATAAGCAGGAGTTC-3′	5′-TTGAGAATGGAGGCAAAGGGCG-3′
iNOS	NM-010927	5′- CTGGAGGTTCTGGATGAG-3′	5′- CTGAGGGCTGACACAAGG -3′

Primer design, in form of exon junction was carried out using Allele ID 7 software for the internal control and test genes from *Mus musculus* sequence.

genes were calculated based on the threshold cycle (CT) method. The CT for each sample was calculated using the Line-gene K software. Accordingly, fold-expression of target mRNAs over the reference values were calculated by equation 2-ΔΔCT, where ΔCT was determined by subtracting the corresponding internal control CT value from the specific CT of targets, and ΔΔCT was obtained by subtracting the ΔCT of each experimental sample from that of the control sample (Larionov et al., 2006).

2.9. Statistical analysis

The data were analyzed with one-way analysis of variance (ANOVA) using the SPSS software (SPSS Inc., Chicago, IL, USA) and significant differences between treatments were analyzed by Tukey test at $P < 0.05$. The data presented here were analyzed as a completely randomized design with three replicate experiments and expressed as the means ± standard deviation.

3. Results and discussion

3.1. The chemical composition of TEO

The basic ingredients of TEO were thymol (40.25%), γ-terpinene (38.68%) and p-cymene (15.84%) (Table 2). Our results have been consistent with previous findings, for instance, Mahboubi and Kazempour (2011) reported that T. ammi oil is composed of thymol (45.9%), γ-terpinene (20.6%), and p-cymene (19%) as the major components. The minor components were also identified ethylene methacrylate (6.9%), β-pinene (1.9%), and hexadecane (1.1%). The essential oil of T. ammi, found in Iran, was analyzed by GC-FID and GC-MS that comprised of thymol (67.4%), p-cymene (17.9%) and γ-terpinene (11.3%) as the major constituents (Vitali et al., 2016). Accordingly, in our study, the primary chemical composition of T. ammi were monoterpenes (58%) and monoterpenoids (42%).

Table 2
Chemical composition (percent area) of *Trachyspermum ammi* essential oil (TEO) analyzed by GC-MS.

No	RT	RI	Formula	Compounds	Percent area
1	5.72	924.773	C10H16	α-Thujene	0.15
2	5.92	931.939	C10H16	α-Pinene	0.40
3	6.32	946.562	C10H16	Camphene	0.03
4	7.00	971.153	C10H17	Sabinene	0.15
5	7.11	975.336	C10H16	β-Pinene	1.71
6	7.49	989.16	C10H16	Myrcene	0.52
7	7.95	1004.26	C10H16	α-Phellandrene	0.10
8	8.14	1009.51	C10H16	δ-3-Carene	0.05
9	8.38	1016.22	C10H16	α-Terpinene	0.16
10	8.64	1023.51	C10H14	**p-Cymene**	**15.85**
11	8.77	1027.13	C10H16	Limonene	0.00
12	8.86	1029.4	C10H18O	1,8-Cineole	0.52
13	9.43	1045.21	C10H16	β-Ocimene	0.01
14	10.03	1061.81	C10H16	**γ-Terpinene**	**38.68**
15	10.93	1086.82	C10H16	Terpinolene	0.00
16	14.02	1163.61	C10H18O	Borneol	0.00
17	14.50	1175.13	C10H18O	Terpinene-4-ol	0.05
18	15.05	1188.58	C10H18O	α-Terpineol	0.06
19	17.28	1241.5	C11H16O	Carvacrol methyl ether	0.00
20	19.41	1291.73	C10H14O	**Thymol**	**40.25**
21	19.77	1300.24	C10H14O	Carvacrol	0.73
22	22.74	1371.38	C12H16O2	Carvacrol acetate	0.02
23	24.63	1417.42	C15H24	(E)-Caryophyllene	0.00
				Monoterpenes	58.00
				Monoterpenoids	42.00

Retention indices (RI) were defined on n-alkanes (C8–C25) retention times (RT) as standard that were injected after essential oil under the same chromatographic conditions. The compounds were identified by comparison of RI with those reported in the literature.

3.2. Extracellular antioxidant activity

Consistent with previous work, in-vitro analysis of data indicated that TEO and thymol displayed an ABTS, DPPH, superoxide anion and nitric oxide scavenging and LDL peroxidation inhibition activities (Table 3). An antiradical agent, gallic acid, was used as a positive control. Results demonstrated that the antioxidant capacity of TEO is comparable to gallic acid. According to the findings, the antioxidant activity of thymol and monoterpenes (cymene and terpinene) was fewer than TEO. Previous studies have shown that the antioxidant capacity of oxygenated monoterpenes is mainly due to their redox properties, which permit them to act as hydrogen donors, reducing agents, singlet oxygen quenchers and metal chelators. The hydrogen atom of hydroxyl groups of phenol compounds can be donated to free radicals, thereby preventing other compounds to be oxidized (Pérez-Rosé;s et al., 2016). With these functions, essential oils scavenge reactive oxygen/nitrogen species and to avoid lipid oxidation in the food and biological samples and thus reduce damage in biological membrane and may protect tissues against oxidative damage. The antioxidant activity of thymol and thymol-rich essential oil in two lipid systems consisting of a purified fraction of triacylglycerols of lard and sunflower oil has been reported (Yanishlieva et al., 1999). According to the results of the investigation of thymol and carvacrol antioxidant activity in roasted sunflower seeds, it has been suggested that these monoterpenes inhibit the formation of oxidative deterioration compounds like peroxides and hexanal and undesirable off-flavors such as oxidized and cardboard flavors and consequently are effective in protection of food products (Quiroga et al., 2015). Altogether, according to the results and previous studies, this high antioxidant capacity of TEO may be linked to the strong synergism between monoterpenes (cymene and terpinene), oxygenated monoterpenes or phenolic monoterpenes (thymol) in the cocktail of essential oil.

3.3. Intracellular antioxidant activity

Intracellular analysis demonstrated that TEO increased the levels of ROS (6037 ± 881) and NO (15.125 ± 0.02) relative to control (4634.33 ± 119.3 and 10.75 ± 0.01 respectively) in macrophages, while, the main TEO components did not stimulate ROS and NO production (data not shown). LPS stimulation of macrophages resulted in an increase in ROS and NO level. Treatment with TEO (10 μg/mL) significantly increased ROS production in LPS-treated cells indicating the stimulatory effect of TEO on ROS generation proposing a synergism between LPS and TEO to augment ROS production in macrophages stimulated with LPS (Table 4). Thymol (10 μg/mL) significantly reduced ROS production in LPS-treated cells suggested a differential capacity between TEO and thymol in ROS production while γ-terpinene and p-cymene did not display significant effects on the ROS production. Treatment with TEO and thymol significantly ($p < 0.05$) decreased NO production in LPS-treated cells, but γ-terpinene and p-cymene had no significant effects on the NO production (Table 4). Results depicted that the naïve macrophages and LPS stimulated macrophages have presented the different response to TEO and thymol. According to our results, many studies have shown that oxygenated monoterpenes or phenolic monoterpenes are mainly responsible for the antioxidant activity of EOs, e.g., apocynin and berberine. Apocynin (acetovanillone) extracted from Picroria kurroa is a phenol-bearing compound with a potent antioxidant activity that potently inhibits superoxide production in neutrophil (Cifuentes-Pagano et al., 2012). Berberine a plant-derived alkaloid with a potent antioxidant activity, at non-cytotoxic level inhibited superoxide production in LPS-stimulated macrophages (Sarna et al., 2010). The inhibitory effects of plumbagin (a medicinal plant-derived naphthoquinone) on the inhibition of ROS production LPS-stimulated macrophages has been reported (Ding et al., 2005). The inhibitory effect of thymol and carvacrol on the nitric oxide production in LPS-stimulated macrophages has been reported (Kavoosi et al., 2012).

Table 3
Antioxidant capacity (50% inhibitory concentration (IC50)) of *Trachyspermum ammi* essential oil (TEO) and its main components thymol, γ-terpinene and *p*-cymene in comparison with gallic acid.

Antioxidants	ABTS	DPPH	Superoxide ion	Nitric oxide	LDL oxide
Gallic acid (μg/mL)	26 ± 1.6^{a}	39 ± 2.2^{a}	90 ± 4.0^{a}	110 ± 6.0^{a}	122 ± 5.0^{a}
TEO (μg/mL)	38 ± 2.0^{b}	54 ± 3.2^{b}	83 ± 4.7^{a}	98 ± 5.0^{a}	130 ± 6.4^{a}
Thymol (μg/mL)	62 ± 4.0^{c}	90 ± 5.4^{c}	134 ± 7.0^{b}	158 ± 9.0^{b}	224 ± 10^{b}
Terpinene (μg/mL)	> 500^{d}	> 500^{d}	> 500^{c}	> 500^{c}	> 500^{c}
Cymene (μg/mL)	> 500^{d}	> 500^{d}	> 500^{c}	> 500^{c}	> 500^{c}

The concentrations TEO that could provide 50% radical or oxidant inhibition (IC_{50}) were calculated from the graph that plotted the radical or the oxidant inhibition percentage against different antioxidant concentrations. The values are expressed as means ± standard deviation for at least three replicate experiments. Mean values with different letters within a column are significantly different by Tukey test at ($p < 0.05$).

Furthermore, the inhibitory effects of several thymol-bearing or carvacrol-bearing essential oil on the creation of ROS and NO have reported. It has suggested that this inhibitory effect strongly related to the antioxidant activity of the oxygenated monoterpenes or phenolic monoterpenes (Bazdar et al., 2018; Karami et al., 2018).

Thymol is a monoterpenoids phenol with anti-bacterial, anti-fungal, anti-viral, antiseptic, anti-tumor, antioxidant, *anti*-inflamatory, anti-mutagenic, cell-protective, antiplatelet, and analgesic properties (Salehi et al., 2018). The γ-terpinene is a monoterpenes present in essential oils of a wide variety of plants, which has been reported to possess anti-inflammatory activity by reducing neutrophil migration as well as the production of interleukin-1β and tumor necrosis factor-α (Koziol et al., 2014; De Oliveira-Ramalho et al., 2015). Recently suggested that p-cymene shows a variety of biological activities, which include anti-oxidant by increasing the activity of antioxidant enzymes and reducing oxidative stress, anti-nociceptive, anti-cancer and anti-tumor, anti-inflammatory activities (Paunescu et al., 2015; Selvaraj et al., 2002).

3.4. Modulation superoxide producing an enzyme

The unstimulated macrophages showed a low level of all NOX mRNAs expressions. LPS stimulation of cells increased the all NOX mRNAs expressions. The addition of TEO at 10 μg/mL did not have significant effects on NOX22 and NOX40 genes but increased NOX47, NOX67 and NOX91 mRNA expression in LPS-treated cells (Table 5). Thymol (10 μg/mL) reduced NOX22, NOX40, NOX47, NOX67 and NOX91 mRNAs expression in LPS-treated cells while γ-terpinene and p-cymene did not display significant effects on the expressions of these genes (Table 5). Noteworthy, the synergism existed between TEO and LPS may pave the way to combat pathogenic parasites or tumor cells.

The inhibitory effects of essential oil on the NOX expression and activity confirmed to be some rare studies. *Ocimum basilicum* essential oil mainly composed of methyl chavicol (47%), geranial (19%) and neral (15%) and significantly reduced NOX mRNA expression in LPS-stimulated murine macrophages at non-cytotoxic level (Kavoosi and Amirghofran, 2017). Carvacrol-rich zataria essential mainly composed of carvacrol (52%), thymol (16%) and p-cymene (10%) and significantly reduced NOX mRNA expression in LPS-stimulated murine macrophages (Kavoosi et al., 2012). *Tagetes minuta* essential oil mainly composed dihydroxyacetone (34%), ocimene (20%) and target one (16%) and significantly reduced NOX mRNA (Karimian et al., 2014).

In macrophages, superoxide anion production is under the control of NOX. This multi-protein enzyme complex consists of several subunits including gp91phox, gp67phox, gp47phox, gp40phox, gp22phox and the small GTPase Rac, which assemble on the cellular membrane to activate the enzyme. Activation of NOX by LPS results in conversion molecular oxygen to superoxide anion through a one-electron transfer (Bedard and Krause, 2007). LPS bind to toll-like receptor 4 (TLR-4) and then activates gp91phox subunit of NOX and superoxide production. Also, superoxide or hydrogen peroxide (derived from superoxide by the action of superoxide dismutase) itself activates mitogen-activated protein kinases (MAPK) like; P38 mitogen-activated protein kinase, extracellular-signal-regulated kinase (ERK) and c-Jun N-terminal protein kinase (JNK). MAPKs finally activate nuclear factor-kB (NF-kB) that itself stimulate the production of other protein like interleukin-6 (IL-6), IL-8, IL-17, IL-22, cyclooxygenase-2 and inducible NOS (More and Pai, 2012; Yousefian et al., 2019).

However, the real mechanism of the TEO in the modulation of superoxide production pathway in stimulated macrophages is unknown. Essential oils are lipophilic compounds and may be moved into the cell through the membrane. Ultra violet, fluorescence, and circular dichroism spectroscopy show essential oil interacts with DNA via intercalation. Alteration in DNA ultraviolet spectra at 260 nm by adding essential oil indicated that essential oil had induced conformational changes in DNA by forming DNA-essential oil complexes, which cause deformation of DNA structure. Fluorescence, and circular dichroism spectra confirmed the intercalation of essential oil in DNA double helix (Salehi et al., 2017). Accordingly, the regulatory effects of essential oil on the expression of some genes may be an epigenetic process. If this correct, the site-specific binding of essential oil to a specific sequence of DNA must be determined.

3.5. Modulation of NO-producing enzyme

LPS stimulation of macrophages resulted in an increase in iNOS mRNA expression in comparison to LPS-untreated cells. The addition of

Table 4
Effects of trachyspermum essential oil (TEO) and thymol, γ-terpinene and *p*-cymene on reactive oxygen specues (ROS), nitric oxide (NO) and thiobarbituric acid reactive substances (TBARS) production in LPS-stimulated macrophages.

Treatment	ROS (fluorescence intensity)	NO (μM)	TBARS (nM)
Control	18330 ± 550^{a}	28 ± 1.5^{ab}	14 ± 1.2^{a}
LPS	36225 ± 1000^{e}	50 ± 3.5^{e}	35 ± 2.5^{d}
LPS + TEO (10 μg/mL)	46665 ± 1300^{f}	25 ± 3.3^{a}	17 ± 2.3^{ab}
LPS + Thymol (10 μg/mL)	23677 ± 800^{b}	32 ± 3.0^{bc}	27 ± 2.0^{b}
LPS + Terpinene (10 μg/mL)	33665 ± 670^{d}	39 ± 3.3^{cd}	28 ± 2.7^{c}
LPS + Cymene (10 μg/mL)	30555 ± 750^{c}	37 ± 3.5^{c}	30 ± 2.6^{cd}

The values are expressed as means ± standard deviation for at least three replicate experiments. Mean values with different letters within a column are significantly different by Tukey test at ($p < 0.05$).

Table 5
Effects of Trachyspermum essential oil (TEO) and thymol, γ-terpinene and *p*-cymene on NADH oxidase (NOX) and inducible nitric oxide synthase (iNOS) mRNA expression in LPS-stimulated macrophages.

Treatment	NOX p22	NOX p40	NOX p47	NOX p67	NOX p91	iNOS
Control	1.0 ± 0.4[a]	1.0 ± 0.3[a]	1.0 ± 0.5[a]	1.0 ± 0.3[a]	1.0 ± 0.5[a]	1.0 ± 0.2[a]
LPS (1 μg/mL)	22 ± 2.2[b]	17 ± 1.9[b]	35 ± 2.4[b]	26 ± 1.7[b]	24 ± 2.0[b]	26 ± 2.6[d]
LPS + TEO (10 μg/mL)	26 ± 1.4[b]	19 ± 1.8[bc]	50 ± 3.8[c]	37 ± 3.2[c]	53 ± 3.8[c]	11 ± 1.4[b]
LPS + Thymol (10 μg/mL)	22 ± 1.5[b]	15 ± 1.3[b]	32 ± 1.7[b]	27 ± 2.0[b]	26 ± 1.8[b]	17 ± 1.5[c]
LPS + Cymene (10 μg/mL)	26 ± 2.2[b]	20 ± 1.7[c]	34 ± 2.0[b]	37 ± 3.3[c]	27 ± 2.5[b]	21 ± 2.5[cd]
LPS + Terpinene (10 μg/mL)	26 ± 2.5[b]	22 ± 2.2[c]	35 ± 2.5[b]	33 ± 2.5[c]	28 ± 2.7[b]	22 ± 2.5[d]

The values are expressed as means ± standard deviation for at least three replicate experiments. Mean values with different letters within a column are significantly different by Tukey test at ($p < 0.05$).

TEO and thymol concentrations of 5 and10 μg/mL significantly ($p < 0.05$) decreased the iNOS mRNA expression in LPS-treated cells while γ-terpinene and p-cymene did not display significant effects on the expressions of iNOS (Table 5).

The inhibitory effects of medicinal plant-derived materials on the NOS expression and activity reported by some studies. The volatile oil of Houttuynia cordata inhibited the production of NO by down-regulation LPS-stimulated iNOS expression (Li et al., 2013). Cinnamomi ramulus inhibits LPS-induced production of NO and expression of iNOS by blocking NF-κB activation (Jeong et al., 2008). Polygoni rhizome reduced NO production and iNOS inhibition by inactivation of NF-kB (Seo et al., 2012). Tagetes minute essential oil mainly composed dihydroxyacetone (34%), ocimene (20%) and target one (16%) significantly reduced iNOS mRNA (Karimian et al., 2014). Furthermore, the modulatory effect of *Semecarpus Anacardium* on the nitric oxide production and NOS expression/activity in animal cells reported (Jaya et al., 2010). However, the real mechanism of these essential oils on the inhibition of NO production pathway in stimulated macrophages is unknown.

NO in macrophages is generated by activation of inducible nitric oxide synthase (iNOS). This enzyme can produce high concentrations of NO after stimulation with bacterial endotoxins (LPS) or a variety of pro-inflammatory cytokines such as TNF-α. In response to inflammatory stimuli such as LPS, macrophages secrete a variety of inflammatory mediators such as TNF-α. The production of TNF-α cytokine is essential for the induction of NO synthesis in LPS-stimulated macrophages (Billack, 2006).

The suppression of NO in macrophages by essential oils may be related to nitric oxide scavenging activity or suppression of NOS expression/activity exerted by essential oil. The NO scavenging activity of TEO observed in our research suggests that these products are valuable in reducing oxidative stress damages to biological membranes and can protect various tissues against cumulative oxidative stress including nitric oxide, nitrite, and peroxynitrite.

4. Conclusion

Considering all these findings, as TEO decreased NO and increased ROS in LPS-induced macrophages, it seems that TEO can lead to protective effects during inflammation responses. On the other hand, thymol acted as a potent antioxidant agent and was able to reduce NO or ROS in LPS-induced macrophages. The reduction of NO production may be related to scavenging of NO and inhibition of nitric oxide synthase expression. Treatment of LPS-induced macrophages with TEO leads to an increase in ROS production over than LPS alone that suggesting synergism between LPS and TEO may exist for enhancement of ROS production. TEO and its main components thymol, γ-terpinene and p-cymene, had different action on the ROS production suggesting the factual biological function of essential oils powerfully depends on the synergism between all components in the essential oils or the ration of monoterpenoids to monoterpenes.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101220.

Conflicts of interest

The authors declare that they have no conflict of interest.

References

Ainsworth, E.A., Gillespie, K.M., 2007. Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin-Ciocalteu reagent. Nat. Protoc. 2, 875–877.
Amiri, M.S., Joharchi, M.R., 2016. Ethnobotanical knowledge of Apiaceae family in Iran: a review. Avicen. J. Phytomed. 6, 621–630.
Bairwa, R., Sodha, R.S., Rajawat, B.S., 2012. Trachyspermum ammi. Phcog. Rev. 6, 56–65.
Bazdar, M., Sadeghi, H., Hosseini, S., 2018. Evaluation of oil profiles, total phenols and phenolic compounds in *Prangos ferulacea* leaves and flowers and their effects on antioxidant activities. Biocatalysis and Agricult Biotechnol. 14, 418–423.
Bedard, K., Krause, K.H., 2007. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. Physiol. Rev. 87, 245–313.
Billack, B., 2006. Macrophage activation: role of toll-like receptors, nitric oxide, and nuclear factor kappa ß. Am. J. Pharmaceut. Educ. 70, 102–110.
Bognar, E., Sarszegi, Z., Szabo, A., Debreceni, B., Kalman, A., Tucsek, Z., Sumegi, B., Gallyas Jr., F., 2013. Antioxidant and anti-Inflammatory effects in RAW264.7 macrophages of malvidin, a major red wine polyphenol. PLoS One 8, E65355.
Boskabady, M.H., Alitaneh, S., Alavinezhad, A., 2014. *Carum copticum* L.: a herbal medicine with various pharmacological effects. BioMed Res. Int. 2014. doi.org/10.1155/2014/569087.
Botsoglou, N.,A., Fletouris, D.J., Papageorgiou, G.E., Vassilopoulos, V.N., Mantis, A.J., Trakatellis, A.G., 1994. Rapid, sensitive, and specific thiobarbituric acid method for measuring lipid peroxidation in animal tissue, food, and feedstuff samples. J. Agric. Food Chem. 42, 1931–1937.
Cifuentes-Pagano, E., Csanyi, G., Pagano, P.J., 2012. NADPH oxidase inhibitors: a decade of discovery from Nox2ds to HTS. Cell. Mol. Life Sci. 69, 2315–2325.
Ding, Y., Chen, Z.J., Liu, S., Che, D., Vetter, M., Chang, C.H., 2005. Inhibition of Nox-4 activity by plumbagin, a plant-derived bioactive naphthoquinone. J. Pharm. Pharmacol. 57, 111–116.
Dubey, S., Kashyap, P., 2015. *Trachyspermum ammi*: a review on its multidimensional uses in Indian folklore medicines. Res. J. Med. Plant 9, 368–374.
Elshafie, H.S., Camele, I., 2017. An overview of the biological effects of some mediterranean essential oils on human health. BioMed Res. Int. 2017, 9268468.
Floegel, A., Kim, D.O., Chung, S.J., Koo, S.I., Chun, O.K., 2011. Comparison of ABTS/DPPH assays to measure antioxidant capacity in popular antioxidant-rich US foods. J. Food Compos. Anal. 24, 1043–1048.
Jaya, A., Shanthi, P., Sachdanandam, P., 2010. Modulation of oxidative/nitrosative stress and mitochondrial protective effect of *Semecarpus anacardium* in diabetic rats. J. Pharm. Pharmacol. 62, 507–513.
Jeong, M.Y., Lee, J.S., Lee, J.D., Kim, N.J., Kim, J.W., Lim, S., 2008. A combined extract of *Cinnamomi ramulus, Anemarrhenae rhizoma* and *Alpiniae officinari* rhizoma suppresses production of nitric oxide by inhibiting NF-κB activation in RAW 264.7 cells. Phytother Res. 22, 772–777.
Johri, R.K., 2011. *Cuminum cyminum* and *Carum carvi*: an update. Phcog. Rev. 5, 63–70.
Karami, A., Kavoosi, G., Maggi, F., 2019. The emulsion made with essential oil and aromatic water from *Oliveria decumbens* protects murine macrophages from LPS-induced oxidation and exerts relevant radical scavenging activities. Biocatalysis and

Agricult Biotechnol. 17, 538–544.
Karimian, P., Kavoosi, G., Amirghofran, Z., 2014. Anti-oxidative and anti-inflammatory effects of *Tagetes minuta* essential oil in activated macrophages. Asian Pacif. J. Trop. Biomed. 4, 219–227.
Kavoosi, G., Amirghofran, Z., 2017. Chemical composition, radical scavenging and anti-oxidant capacity of *Ocimum Basilicum* essential oil. J. Essent. Oil Res. 29, 189–199.
Kavoosi, G., Teixeira da Silva, J.A., Saharkhiz, M.J., 2012. 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. 64, 1491–1500.
Koziol, A., Stryjewska, A., Librowski, T., Salat, K., Gawel, M., Moniczewski, A., Lochynski, S., 2014. An overview of the pharmacological properties and potential applications of natural monoterpenes. Mini Rev. Med. Chem. 14, 1156–1168.
Larionov, A., Krause, A., Miller, W., 2005. A standard curve based method for relative real time PCR data processing. BMC Bioinf. 6, 62–68.
Li, W., Fan, T., Zhang, Y., Fan, T., Zhou, P., Niu, X., He, L., 2013. *Houttuynia cordata* Thunb. volatile oil exhibited anti-inflammatory effects in vivo and inhibited nitric oxide and tumor necrosis factor-α production in LPS-stimulated mouse peritoneal macrophages in vitro. Phytother Res. 27, 1629–1639.
López-Alarcón, C., Denicola, A., 2013. Evaluating the antioxidant capacity of natural products: a review on chemical and cellular-based assays. Anal. Chim. Acta 763, 1–10.
Mahboubi, M., Kazempour, N., 2011. Chemical composition and antimicrobial activity of *Satureja hortensis* and *Trachyspermum copticum* essential oil. Iran. J. Microbiol. 3, 194–200.
More, P., Pai, K., 2012. In vitro NADH-oxidase, NADPH-oxidase and myeloperoxidase activity of macrophages after *Tinospora cordifolia* (guduchi) treatment. Immunopharmacol. Immunotoxicol. 34, 368–372.
Nimse, S.B., Pal, D., 2015. Free radicals, natural antioxidants, and their reaction mechanisms. RSC Adv. 5, 27986–28006.
Paunescu, E., Nowak-Sliwinska, P., Clavel, C.M., Scopelliti, R., Griffioen, A.W., Dyson, P.J., 2015. Anticancer organometallic osmium (ii) p-cymene complexes. ChemMedChem 10, 1539–1547.
Pérez-Rosés, R., Risco, E., Vila, R., Peñalver, P., Cañigueral, S., 2016. Biological and nonbiological antioxidant activity of some essential oils. J. Agric. Food Chem. 64, 4716–4724.
Quiroga, P.R., Asensio, C.M., Nepote, V., 2015. Antioxidant effects of the monoterpenes carvacrol, thymol and sabinene hydrate on chemical and sensory stability of roasted sunflower seeds. J. Sci. Food Agric. 95, 471–479.
Rodríguez, J., Martín, M.J., Ruiz, M.A., Clares, B., 2016. Current encapsulation strategies for bioactive oils: from alimentary to pharmaceutical perspectives. Food Res. Int. 83, 41–59.
Salehi, F., Behboudi, H., Kavoosi, G., Ardestani, S.K., 2017. Monitoring ZEO apoptotic potential in 2D and 3D cell cultures and associated spectroscopic evidence on mode of interaction with DNA. Sci. Rep. 7, 2553–2562.
Salehi, B., Mishra, A.P., Shukla, I., Sharifi-Rad, M., Contreras, M.D.M., Segura-Carretero, A., Fathi, H., Nasrabadi, N.N., Kobarfard, F., Sharifi-Rad, J., 2018. Thymol, thyme, and other plant sources: health and potential uses. Phytother Res. doi.org/10.1002/ptr.6109.
Sarna, L.K., Wu, N., Hwang, S.Y., Siow, Y.L., Karmin, O., 2010. Berberine inhibits NADPH oxidase mediated superoxide anion production in macrophages. Can. J. Physiol. Pharmacol. 88, 369–378.
Schmolz, L., Wallert, M., Lorkowski, S., 2017. Optimized incubation regime for nitric oxide measurements in murine macrophages using the Griess assay. J. Immunol. Methods 17, 30249–30251.
Selvaraj, M., Pandurangan, A., Seshadri, K.S., Sinha, P.K., Krishnasamy, V., Lal, K.B., 2002. Comparison of mesoporous Al-MCM-41 molecular sieves in the production of p-cymene for isopropylation of toluene. J. Mol. Catal. A Chem. 186, 173–186.
Seo, H.J., Huh, J.E., Han, J.H., Jeong, S.J., Jang, J., Lee, E.O., Lee, H.J., Lee, H.J., Ahn, K.S., Kim, S.H., 2012. *Polygoni rhizoma* inhibits inflammatory response through inactivation of nuclear factor-kappa B and mitogen activated protein kinase signaling pathways in RAW264. 7 mouse macrophage cells. Phytother Res. 26, 239–245.
Singh, A., Dwivedy, A.K., Singh, V.K., Upadhyay, N., Chaudhari, A.K., Das, S., Dubey, N.K., 2019. Essential oils based formulations as safe preservatives for stored plant masticatories against fungal and mycotoxin contamination: a review. Biocatal. Agricult. Biotechnol. 17, 313–317.
Tarpey, M.M., Wink, D.A., Grisham, M.B., 2004. Methods for detection of reactive metabolites of oxygen and nitrogen: in vitro and in vivo considerations. Am. J. Physiol. Regul. Integr. Comp. Physiol. 286, R431–R444.
Vitali, L.A., Beghelli, D., Nya, P.C.B., Bistoni, O., Cappellacci, L., Damiano, S., Lupidi, G., Maggi, F., Orsomando, G., Papa, F., Petrelli, D., 2016. Diverse biological effects of the essential oil from Iranian *Trachyspermum ammi*. Arab. J. Chem. 9, 775–786.
Yanishlieva, N.V., Marinova, E.M., Gordon, M.H., Raneva, V.G., 1999. Antioxidant activity and mechanism of action of thymol and carvacrol in two lipid systems. Food Chem. 64, 59–66.
Yousefian, M., Shakour, N., Hosseinzadeh, H., Hayes, A.W., Hadizadeh, F., Karimi, G., 2019. The natural phenolic compounds as modulators of NADPH oxidases in hypertension. Phytomedicine 55, 200–213.
Zarshenas, M.M., Moein, M., Samani, S.M., Petramfar, P., 2013. An overview on ajwain (*Trachyspermum ammi*) pharmacological effects; modern and traditional. J. Nat. Remedies 14, 98–105.