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Reduction of NADH Oxidase, NO Synthase, TNFα, and IL-1β mRNA Expression levels on Lipopolysacharide-Stimulated Murine Macrophages by *Zataria Multiflora*

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A B S T R A C T

Zataria multiflora (ZM) is a thyme-like aromatic plant in the *Lamiaceae* family that grows in central and southern Iran. ZM is extensively used as a flavor ingredient in a wide variety of foods and is used as part of popular traditional folk remedies. In the present study, ZM essential oil (ZMO) was obtained from ZM leaves via hydro-distillation and then analyzed by GC-MS (gas chromatography-mass spectrometry). The anti-inflammatory activity of ZMO was determined via measures of NADH oxidase (*NOX*), inducible nitric oxide synthase (*iNOS*), tumor necrosis factor *(TNF)-α,* and interleukin *(IL)-1β* mRNA expression in lipopolysaccharide-stimulated murine macrophages using real-time polymerase chain reaction (PCR). GC-MS analysis indicated that the main components in the ZMO were carvacrol (29.4%), thymol (25.7%), *p*-cymene (11.2%), linalool (9.3%), and γ-terpinene (8.0%). ZMO significantly reduced *NOX*, *iNOS*, *TNFα,* and *IL-1β* mRNA expression in cells at concentrations of 0.1-1 µg/mL, indicating a capacity for this product to potentially modulate/diminish immune responses. ZMO has anti-oxidant and anti-inflammatory properties and could be potentially used as a safe effective source of natural anti-oxidants in therapy against oxidative damage and a number of inflammatory conditions associated with stress.

Key words: Macrophages, *Zataria Multiflora*, NADH Oxidase, NO Synthase, TNFα, and IL-1β

INTRODUCTION

Zataria multiflora (ZM) is a thyme-like aromatic plant belonging to the Lamiaceae family that grows naturally only in central and southern parts of Iran, Pakistan and Afghanistan. ZM is extensively used as a flavor ingredient in a wide variety of fields in its native region as a popular

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traditional folk remedy [1]. This plant has several properties including the ability to act as a stimulant, diaphoretic, diuretic, anti-septic, anesthetic, anti-spasmodic, anthelminthic, analgesic, and/or anti-tussive agent. Other medicinal uses of ZM include the use in treatment of some gastrointestinal disorders, fever, premature labor pain, bone and joint pain, headache, migraine, diarrhea, vomiting, and the common cold [2]. More recent controlled pharmacological studies show that ZM possesses s wide range of biological properties including anti-microbial, anti-oxidative, anti-nociceptive, spasmolytic, and anti-inflammatory effects [3]. Because of these properties, ZM has played an important role in Iranian traditional medicine and modern pharmacological and clinical investigations. In this context, the ZM essential oil (ZMO) has assumed an important role in both pharmaceutical and food industries.

The most abundant components of ZMO are phenolic monocyclic monoterpenes (thymol and carvacrol), carbure monocyclic monoterpenes (*p*-cymene and γ-terpinene), alcoholic acyclic monoterpenes (linalool), and carbure bicyclic sesquiterpene (caryophyllene) [4, 5]. ZMO is a good source of phenolic monoterpenes (thymol and caracole), with a significant anti-microbial activity against both Gram-positive and -negative bacteria [5-7]. Several studies have also described anti-fungal activities of ZMO against *Candida*, *Aspergillus*, *Malassezia*, *Fusarium*, and *Saprolegnia* species [8-10].

ZMO has been shown to possess strong anti-oxidant activity [11, 12]. Karimian et al. (2012) also showed that ZMO possesses nitric oxide (NO) and malondialdehyde reducing properties and could thus prevent nitrative stress and lipid peroxidation [13]. Recently, ZMO was seen to diminish lipopolysaccharide (LPS)-induced production of NO and hydrogen peroxide (H_2O_2), along with NADH oxidase and NO synthase activities in macrophages [14, 15].

Advances in chemical and pharmacological evaluations of ZMO have been made in recent years; however, several useful features of this plant (e.g., mechanisms underlying its anti-oxidant and -inflammatory effects) remain unknown. The aim of the present study was to investigate the level of potential modulating effects of ZMO on macrophages and their related functions, including the expression of *NOX* subunit (p22phox, p40phox, p47phox, p67phox), *NOS*, *TNFα*, and *IL-1β* mRNAs in LPS-stimulated macrophages. In addition, the *in vitro* anti-oxidant capacity of ZMO was examined by assessments of ROS, RNS, and hydrogen peroxide (H_2O_2) scavenging ability using ABTS, sodium nitrite, and H_2O_2 scavenging assays, respectively. It was expected that these studies would reveal that ZMO 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 that ZMO would decrease *TNFα* and *IL-1β* mRNA expression as both part of its known anti-inflammatory character and due to the ongoing quenching of radicals known to trigger formation of these pro-inflammatory cytokines.

MATERIALS AND METHODS

Chemicals and reagents: Sodium nitrite, sodium sulfate, ABTS, Griess reagent (naphthylethylenediamine, sulfanilamide, phosphoric acid), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), fetal calf serum (FCS), Dulbecco's Modified Eagle Medium (DMEM), L-glutamine, dimethyl sulfoxide (DMSO), lipopolysaccharide (LPS; type 0111:B4 from *Escherichia coli*) and L-nitro-arginine-methyl ester (L-NAME) were all purchased from Sigma (St, Louis, MO) and Fluka (Heidelberg, Germany). RNX-Plus buffer was obtained from

Cinagen (Tehran, Iran). All other used chemicals and reagents were the purest commercially available products.

Plant materials and ZMO preparation: The aerial parts of ZM were obtained from wild plants in the mountains of Arsenjan (Fars Province, Iran). The identification of the plant was confirmed by researchers from the Department of Biology of Shiraz University, Iran and a voucher specimen (24985) deposited at the Herbarium of that department. The leaves of the plants were separated from the stem and were dried in the shade for 72 hr. The dried leaves (100 g) were then hydro-distilled for 3 hr using an all-glass Clevenger-type apparatus (Herbal Exir Co., Mashhad, Iran) following the method outlined by the British Pharmacopeia [16]. The yield of ZMO from leaf material was 2.2% (w/w). The obtained ZMO was dehydrated over anhydrous sodium sulfate and stored at 4°C until analyzed by gas chromatography-mass spectrometry (GC-MS) or used in the *in vitro* studies. The ZMO was dissolved in DMSO and weighed afterwards to obtain approximate density. From these analyses, the measured density of the ZMO stock was found to be 0.986 g/mL for ZMO (or 986 µg ZMO/µL). This stock was, in turn, diluted in a culture medium to yield appropriate concentrations for each given study.

Identification of the ZMO components: GC analysis was carried out using an Agilent technology chromatograph with HP-5 column (30 m × 0.32 mm i.d. × 0.25 µm). The oven temperature was set as follows: 60°C - 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 ZMO 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 i.d × 25 µm) was used with Helium at 1 ml/min as the carrier gas. The GC oven temperature was programmed from 60ºC - 210ºC at a rate of 3ºC/min, then increased from 210ºC to 240ºC at a rate of 20ºC/min, and was then kept constant at 240ºC for 8.5 min. The split ratio was adjusted to 1:50 and the injection volume was 1 mL. The injector temperature was 280ºC. The quadrupole mass spectrometer was scanned over 40-550 amu with an ionizing voltage of 70 eV. Retention indices (RI) were determined using retention times (RT) of n-alkanes (C_8-C_{25}) injected after the ZMO under the same chromatographic conditions. 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 [17, 18].

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 DMEM medium containing 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% heat-inactivated FCS at 37ºC in a humidified CO_2 incubator. Cultures were allowed to grow until confluence at which point adherent macrophages were scraped from the flask and washed with warm medium (25ºC). Cells were then counted and their viability determined by trypan blue dye exclusion. The cells were then seeded at 2×10^6 cells/mL in 24-well tissue culture plates (in triplicate; Jet Biofil, Kyoto, Japan). After culturing for 18 hr to allow cells to adhere, non-adherent cells were removed by the medium with gentle rinsing. Remaining adherent cells were then cultured in the presence or absence of medium bearing 1 µg LPS/mL. After 2 hr, ZMO was

added at a final concentration of 0.1-200 µg/mL. Two sets of wells without ZMO but with LPS and DMSO solvents (0.1%) were used as negative controls. L-NAME (5 µM; inhibitor of NO production) was used as positive control. After 24 hr of incubation at 37ºC, the culture supernatants in each well were removed and the cells harvested for RNA extraction and real-time polymerase chain reaction (PCR) analysis.

Cell viability assay: The effect of ZMO on viability of J774A.1 cells was determined by an MTT assay as described previously [19]. Cells (2×10^4 cells/well) were incubated for 24 hr (at 37 ºC in 5% CO_2) with different concentrations (0 - 200 µg/mL) of ZMO. Thereafter, 10 µl of MTT (5 mg/mL) solution was added to each well and the plate was incubated for an additional 4 hr at 37°C. Each well then received 100 µL lysis buffer (10% SDS in 10 mM HCl) the absorbance in each being determined spectrophotometrically at dual wavelengths of 570 and 630 nm on a microplate ELISA reader (BioTek Elx 808, Winooski, VT). Percentage viability was calculated as: [(absorbance of treated cells/absorbance of control)] × 100. The controls were ZMO-untreated cells containing DMSO at the highest concentration used (0.1%). The concentration that provided the IC_{50} value was calculated from a graph that plotted the inhibition percentage against different ZMO concentrations.

RNA extraction and cDNA synthesis: Total RNA was extracted using RNX-plus buffer from Cinagen (Tehran, Iran). Briefly, 2×10^6 cells were transferred to 1 mL RNX-plus buffer in an RNase-free microtube, mixed thoroughly, and left at room temperature for 5 min. Chloroform (200 µL) was added to the slurry and the sample was mixed gently. The mixture was centrifuged at 13,200 g at 4°C for 15 min, and the resulting supernatant was transferred to a new tube and precipitated with an equal volume of isopropanol for 15 min on ice. The RNA pellet was washed using 75% ethanol, briefly dried, and re-suspended in 15 µL of RNase-free water. The purified total RNA was quantified using a Nano-Drop ND 1000 spectrophotometer (Wilmington, DE). A sample (i.e., 0.005 mg) of isolated 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.

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), 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), *TNFα* (NM-013693), and *IL-1β* (NM-008361) (Table 1). GAPDH and β-actin were used as internal controls (whose expression proved not to be influenced by LPS) for data normalization [20].

Table 1. Primers used for real-time PCR 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	NM-007806	5′- ATGGAGCGATGTGGACAG-3′	5′- ACCGACAACAGGAAGTGG-3′
NOX	NM-008677	5′-CAACAAAGACTGGCTGGAG-3′	5′-CCGCAATGTCCTTGATGG-3′
NOX	NM-010876	5′- ATGGCACAAAGGACAATC-3′	5′- ACCTGAGGCTATACACAAG-3′
NOX	NM-010877	5′- CAGCCACAGTCAGCAGAG-3′	5′-GCACAAAGCCAAACAATACG-3′
NOS	NM-008084	5′- CTGGAGGTTCTGGATGAG-3′	5′- CTGAGGGCTGACACAAGG -3′
TNFα	NM-013693	5′-GTCTCAGCCTCTTCTCATTC-3′	5′- GGAACTTCTCATCCCTTTGG-3′
IL-1β	NM-008361	5′- GAAGAAGAGCCCATCCTC-3′	5′- GTTCATCTCGGAGCCTGTAG-3′

Primer design (in form of exon junction) was carried out using Allele ID 7 software for the internal controls glyceraldehydes-3-phosphate dehydrogenase (GAPDH) and β-actin, and for the test genes NADH oxidase p22 phagocyte oxidase (*NOX p22phox*), *NOX p44phox*, *NOX p47phox*, *NOX p67phox*, inducible nitric oxide synthase (*iNOS*), *TNFα*, and *IL-1β* genes from *Mus musculus* sequences.

Relative real-time PCR was performed in a 20 µL volume containing 1 µL cDNA, 1x Syber Green buffer (Qiagen, Hilden, Germany) and 4 pmoL of each primer. Amplification reactions were carried out in a line Gene k thermal cycler (Bioer Technology Co., Hangzhou, China) with initial denaturing of 94ºC for 2 min, followed by 40 cycles of 94ºC for 10 sec. Annealing temperature (Ta) of each primer pair was done for 15 and 30 sec for extension to occur at 72ºC. After 40 cycles, the specificity of the amplifications was checked based on the melting curves resulting from heating the amplicons from 50°C - 95°C. All amplification reactions were repeated twice under identical conditions beside a negative control and five standard samples. To ensure that the PCR was generated from cDNA rather than genomic DNA, proper control reactions were carried out without the reverse transcriptase treatment. For quantitative real time PCR data, relative expression of *NOX*, *iNOS*, *TNFα*, and *IL-1β* genes 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 put forward by Larionov et al. [21]. Accordingly, fold-expression of target mRNAs over reference values were calculated by equation $2^{-\Delta\Delta CT}$, where ΔCT was determined by subtracting the corresponding internal control CT value from specific CT of targets, and ΔΔCT was obtained by subtracting the ΔCT of each experimental sample from that of the control sample[22].

Statistical analysis: All data are expressed as means plus standard deviations of at least three independent experiments. The differences between treatments were analyzed by one-way analysis of variance (ANOVA) tests at $p < 0.05$, using SPSS (Abaus Concepts, Berkeley, CA) and Prism 5 (Graph Phad, San Diego, CA) softwares.

RESULTS

Plant Materials: The chemical composition of ZMO prepared by water-distillation was determined by GC-MS. GC-MS analysis indicated that the main components were carvacrol (29.4%), thymol (25.7%), *p*-cymene (11.2%), linalool (9.3%) and γ-terpinene (8.0%).

ZMO reduced cell viability at high concentrations: The MTT assay results indicated that low concentrations (0.1-10 µg/mL) of ZMO had no effect on J774A.1 cell viability. However, at higher concentrations (50-200 µg/mL), cell viability was significantly reduced in a concentration-related manner, with the maximum effect (100% cell death) at 200 µg/mL (Fig. 1). Non-cytotoxic concentrations (< 10 µg/mL) were thus used for the subsequent studies including expression of genes.

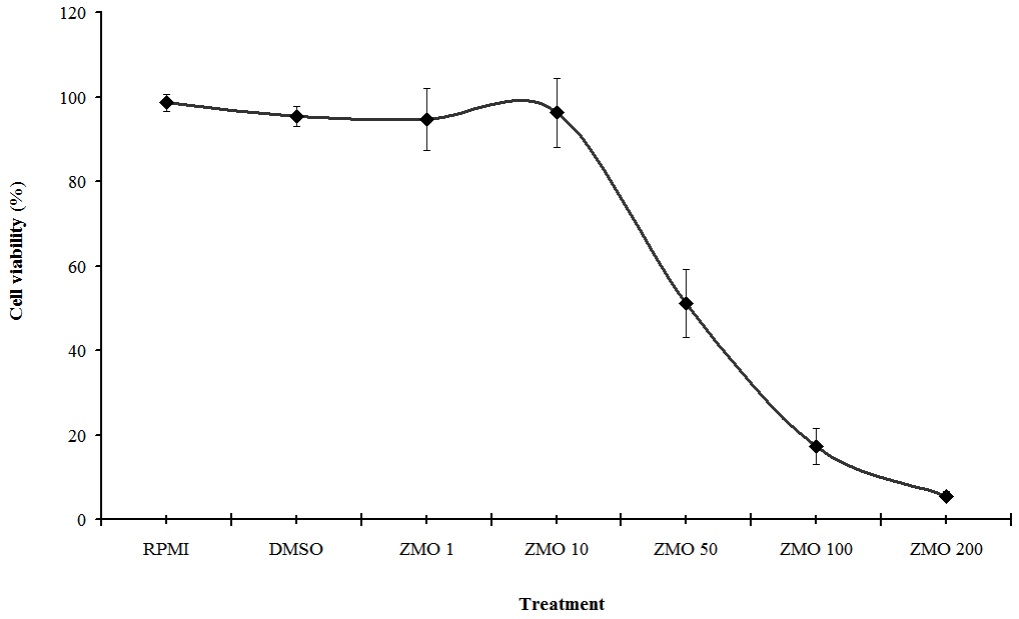

Figure 1. Effect of *Z. multiflora* essential oil (ZMO) on viability of J774 cells. Cells were treated with various concentrations of ZMO (0 - 200 µg/mL) and incubated for 24 hr. Control cells were treated only with solvent (0.1% DMSO). Data shown are the mean ± SD from three sets of independent experiments. Different letters indicate significant differences ($p<0.05$).

ZMO reduced NOX p22phox mRNA expression in LPS-stimulated macrophages: LPS stimulation of macrophages resulted in increased *NOX p22phox* mRNA expression (20.7 [± 1.5]-fold of LPS-untreated control cells, $p<0.001$). The addition of ZMO at 0.001 - 1.0 µg/mL significantly decreased *NOX p22phox* mRNA expression in LPS-treated cells in a concentration-related manner (from 12 [± 1]- to 3.1 [± 0.8]-fold of control; $p<0.05$), indicating an inhibitory effect of ZMO on p22phox mRNA induction/formation (Table 2).

ZMO reduced NOX p40phox mRNA expression in LPS-stimulated macrophages: *NOX p40phox* mRNA expression in LPS-treated cells was 6.00 [± 0.37]-fold of control ($p<0.001$). The addition of ZMO [at all test concentrations] decreased *NOX p40phox* mRNA expression in LPS-treated cells in a dose-related manner (from 3.5 [± 1.0]- to 1.2 [± 0.3]-fold of control ($p<0.05$), indicating an inhibitory effect on *p40phox* mRNA induction/formation (Table 2).

ZMO reduced NOX p47phox mRNA expression in LPS-stimulated macrophages: The stimulation of macrophages with LPS resulted in an increase in *NOX p47phox* mRNA expression (33 [± 4]-fold of control, $p<0.001$). The addition of 0.001 - 1.0 µg ZMO/mL significantly decreased this gene expression in LPS-treated cells in a dose-related manner, from 25 [± 3]- to 11 [± 1]-fold of control ($p<0.05$) (Table 2).

ZMO reduced NOX p67phox mRNA expression in LPS-stimulated macrophages: A decrease in NOX p67phox gene expression was detected in LPS-stimulated macrophages treated with ZMO. The relative *NOX p67phox* mRNA expression in cells treated with LPS alone was 7 [± 1]-fold of LPS-untreated control cells ($p<0.001$). The addition of 0.001 - 1.0 µg ZMO/mL significantly decreased the *NOX p67phox* mRNA expression in LPS-treated cells from 4.8 [± 0.2]- and 2.0 [± 0.5]-fold of the control ($p<0.05$)] (Table 2).

ZMO reduced iNOS mRNA expression in LPS-stimulated macrophages: LPS stimulation of macrophages resulted in an increase in *iNOS* mRNA expression (4.7 [± 0.1]-fold of LPS-untreated cells, $p<0.001$). The addition of ZMO at 0.001 - 1.0 µg/mL significantly decreased *iNOS* mRNA expression in LPS-treated cells almost to the control level [i.e., from 2.8 [± 0.2]- to 0.85 [± 0.15]-fold of untreated cells ($p>0.05$)] (Table 2).

ZMO reduced TNF-α mRNA expression in LPS-stimulated macrophages: LPS stimulation of macrophages resulted in an increase in *TNFα* mRNA expression compared to levels in untreated cells (7.2 [± 0.4]-fold, $p<0.001$). The addition of 0.001 - 1.0 µg ZMO/mL significantly decreased *TNFα* mRNA expression in a concentration-related fashion from 3.6 [± 0.5]- ($p<0.05$) to 1.2 [± 0.2] ($p>0.05$)-fold of control (Table 2). These data indicated the inhibitory effect of ZMO on *TNFα* mRNA induction/formation.

ZMO reduced IL-1β mRNA expression in LPS-stimulated macrophages: *IL-1β* mRNA expression in cells treated with LPS alone was 79 [± 7]-fold of control ($p<0.001$). ZMO treatment significantly decreased this level in a concentration-related manner from 37 [± 8] - to 8.6 [± 4]-fold (each $p<0.05$) of LPS-untreated cells (Table 2). These data indicated the inhibitory effect of ZMO on *IL-1β* mRNA induction/formation.

DISCUSSION

The anti-oxidant and anti-inflammatory effects of ZMO were investigated in the present study. Analysis of the chemical composition of the essential oil by GC-MS indicated that carvacrol, thymol, *p*-cymene, linalool and γ-terpinene were the main constituents. In previous studies, we had analyzed chemical composition of the ZMO from different chemotypes [13]. Our

previous results demonstrated that the main components were carvacrol (0.4-69.3%), thymol (0.1-36%), *p*-cymene (0.2-20.2%), γ-terpinene (0.1-9.7%), linalool (0.2-90.6%), myrcene (0.1-3%), caryophellene (0.6-1.5%), carvacrol methyl ether (0.3-2.8%), and α-pinene (1.6-5.7%) [5, 13]. This indicated that the composition of ZMO is dependent on species, climate, altitude, time of collection, and growth stage of the ZM. The plant analyzed in this research had roughly the same components as other previously-analyzed ZMO.

Table 2. Effects of ZMO on NOX p22phox, NOX p40phox, NOX p47phox, NOX p67phox, NOS, TNFα and IL-1 β mRNA expression in LPS-stimulated macrophages.

Genes	Control	LPS	LPS + ZMO 0.01	LPS + ZMO 0.1	LPS + ZMO 1
NOX p22phox	1 ± 0.13^{d}	21 ± 1.5^{a}	12 ± 0.96^{b}	5 ± 0.4^{c}	4 ± 2^{c}
NOX p40phox	1 ± 0.45^{d}	6 ± 0.5^{a}	$4 \pm .65^{b}$	2.6 ± 0.11^{c}	$1.4 \pm .4^{d}$
NOX p47phox	1 ± 0.25^{d}	33 ± 4^{a}	25 ± 3^{b}	18 ± 1^{c}	15 ± 2^{c}
NOX p67phox	1 ± 0.4^{d}	7 ± 1^{a}	5 ± 0.2^{b}	4.4 ± 0.6^{bc}	3 ± 0.4^{c}
iNOS	1 ± 0.12^{c}	5 ± 0.2^{a}	2.8 ± 0.17^{b}	1.4 ± 0.22^{b}	0.8 ± 0.2^{c}
TNFα	1 ± 0.7^{c}	7 ± 0.4^{a}	3.6 ± 0.6^{b}	2 ± 0.5^{c}	1.4 ± 0.6^{c}
IL-1β	1 ± 0.4^{d}	79 ± 6^{a}	37 ± 5^{b}	12 ± 7^{c}	8 ± 4^{c}

The cells were cultured in 24-well plates and treated with and without LPS. Various concentrations of ZMO (0.01-1 µg/mL) were added. After 24 h, the expression of NADH oxidase p22 phagocyte oxidase (NOX p22phox), NOX p44phox, NOX p47phox, NOX p67phox, inducible nitric oxide synthase (iNOS), TNFα, and IL-1β genes was analyzed by real-time PCR. Cells treated with DMSO as the solvent (Control) and cells treated with the solvent and LPS (LPS) was considered as positive controls. Data represent mean ± SD from three sets of independent experiments. Different letters in each raw show significantly difference ($p < 0.05$).

The ZMO analyzed here possessed a potent *in vitro* antioxidant activity. The ZMO at concentrations > 10 µg/mL had the ability to scavenge all oxidant radicals, an indicator of its potency as a radical scavenger [14, 15]. Reactive oxygen species (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. Once produced, ROS 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 [23]. The ROS reducing activity of ZMO observed in our study implies the beneficial role of this product in 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 [24]. According to GC-MS analysis, ZMO contains high levels of phenolic compounds (thymol and carvacrol). The antioxidant activity of ZMO is thus likely related to these compounds.

In order to determine the anti-oxidant and -inflammatory effects of ZMO on the test macrophages, > 1 µg ZMO/mL oncentrations that were overtly cytotoxic were not used. Though the constituents of essential oils can act as anti-oxidants, 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 previous studies, the hexane extract of ZMO had shown anti-proliferative activity on the peripheral blood lymphocytes at concentrations of > 1 µg/mL, a finding that was similar to our results [25, 26].

ROS production is under the control of NOX. This multi-component enzyme consists of several cytosolic components including; p91phox (phagocyte oxidase), 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 [27]. 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 [28]. 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 a superoxide anion through a one-electron transfer [29]. As our study showed, ZMO 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 [30]. Thus it can be assumed that reduced ROS generation by stimulated macrophages in the presence of ZMO 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 the different steps of many inflammatory processes [31]. RNSs are nitrogen-containing oxidants, mainly NO which is a free radical playing a key role in the pathogenesis of pain and inflammation. *In vitro* inhibition of the NO radical is a measure of antioxidant activity of plant extracts. In our previous study, the effects of various chemotypes of ZMO on NO and hydrogen peroxide scavenging in LPS-stimulated macrophages was shown [14, 15]. As the results of the present study show, the chemotype of the used ZMO had the ability to scavenge total RNS at concentrations of > 10 µg ZMO/mL; this confirmed the previously-reported potential anti-oxidant activity for ZMO [7].

NO in macrophages is generated by the 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 TNFα, IL-1, and IL-6 [32]. NO generation involves several steps including the activation of the nuclear transcription factor (NF)- κB and subsequent iNOS gene expression [33, 34]. NF-κB regulates the expression of various genes involved in inflammatory responses. Its activation can also be regulated by various cytokines, among which TNFα is the most important.

In response to inflammatory stimuli such as LPS, macrophages secrete a variety of inflammatory mediators like TNFα and IL-1β. Production of TNFα cytokine is important for the induction of NO synthesis in LPS-stimulated macrophages [35-37]. As results of this study show ZMO was able to reduce the inducible expression of *TNFα* gene, indicating that the reduced NO production seen in the macrophage cultures might be partly related to the suppression of the TNFα expression. ZMO also decreased expression of *IL-1β*, which like TNFα, is known to play a crucial role in inflammatory responses and is involved in the pathogenesis of inflammatory diseases [38, 39]. As results here showed, ZMO significantly reduced *iNOS* mRNA expression in stimulated macrophages. Suppression of *TNFα* and *IL-1β* expression in macrophages, as well as reduced *iNOS* gene expression due to ZMO indicates the ability of this product to diminish immune reactions and provides further evidence that this plant may have potential immunomodulatory properties.

Considering all these findings, ZMO displayed an anti-oxidant characteristic by scavenging superoxide, H_2O_2, and NO radicals, and also reduced oxidative stress. This suggests a potential use of this product as therapy for 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, ZMO decreased the expression of genes for pro-inflammatory cytokines TNFα and IL-1β as well as NF-κB. Therefore, a reduced expression of the above-noted inflammatory enzymes and cytokines could be attributed to a suppression of the NF-κB pathway in the treated cells. These data suggest a potential therapeutic use for ZM in the modulation of macrophages and provides evidence to support the use of ZM as a tea/additive/traditional remedy for treatment of inflammatory diseases. Further *in vivo* studies are recommended to fully understand the therapeutic potential of ZMO in a multitude of inflammatory disorders.

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