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Chemical composition, radical scavenging and anti-oxidant capacity of *Ocimum Basilicum* essential oil

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ABSTRACT

Ocimum basilicum has several functional characteristics including carminative, stimulant, diuretic, antiseptic, anesthetic, anti-spasmodic, analgesic and anti-tussive properties. *O. basilicum* essential oil (basil oil) was tested for chemical composition and in vitro and ex vivo anti-oxidant activities. The in vitro anti-oxidant capacity of basil oil was examined using 1, 1-diphenyl-2-picryl-hudrazyl radical (DPPH$^{\bullet}$), 2, 2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) radical (ABTS$^{\bullet}$), hydrogen peroxide (H_2O_2), hydroxyl radical (HO$^{\bullet}$), nitric oxide (NO) and nitrite (NO_2) scavenging effects. The *ex vivo* anti-oxidant activity of basil oil was determined through measuring NADH oxidase (NOX) and inducible nitric oxide synthase (iNOS) mRNA expression in lipopolysaccharide-stimulated murine macrophages using real-time polymerase chain reaction (RT-PCR). GC-MS analysis indicated that the main components in the basil oil were methylchavicol (47%), geranial (19%) and neral (15%). Basil oil had effective DPPH$^{\bullet}$, ABTS$^{\bullet}$, H_2O_2, HO$^{\bullet}$, NO and NO_2 scavenging effects. Basil oil significantly reduced NOX and iNOS mRNA expression in lipopolysaccharide-stimulated murine macrophages at concentrations of 1-10 µg/mL. Basil oil had radical scavenging and anti-oxidant activities and could potentially be used as a safe and effective source of natural anti-oxidants in therapy against oxidative damage and stress associated with some inflammatory conditions.

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KEYWORDS
Ocimum basilicum; essential oil; macrophages; NADH oxidase; NOS; anti-oxidant

1. Introduction

An obvious consequence of aerobic metabolism is the production of reactive oxygen species (ROS, including superoxide anion, hydroxyl radical and hydrogen peroxide) and reactive nitrogen species (RNS, including nitric oxide and nitrite) (1). In aerobic organisms, metabolic ROSs are formed by the unavoidable leakage of electrons onto molecular oxygen from the electron transport chain of mitochondria (2), or generated as a metabolic byproduct in peroxisomes (3). Non-metabolic superoxide anion and hydrogen peroxide are actively generated by the action of NADH oxidase (NOX) and superoxide dismutase (SOD), respectively (4). Nitric oxide is generated by the action of nitric oxide synthase (NOS) (5). Animals uses ROS and RNS as second messenger and signal transduction molecules to control a variety of cellular processes including regulation of growth and development and/or responses to biotic an abiotic stimuli (6). However, overproduction of ROS and RNS leads to oxidative stress which causing peroxidation of lipids, oxidation of protein, damage to nucleic acids, inhibition of enzymes, programmed cell death and finally cell death (7). Natural antioxidant obtained from medicinal and aromatic plants can be a simple and accessible way to inhibition of ROS and its worse outcome.

Anti-oxidants have a promising role in food and pharmacological applications as well as in human health. The most popular synthetic anti-oxidants that are commonly used for food and pharmacological applications are phenolic compounds such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ) and propyl gallate (PG) (8). However, due to toxic and carcinogenic effects of synthetic anti-oxidants, their applications have been restricted. There is a growing interest in natural and safer anti-oxidants for food and human applications, and a growing trend in consumers' preferences toward natural anti-oxidants. The beneficial influence of many natural products on human health has been originated from their anti-oxidant capacity. Natural anti-oxidants appear healthier and safer than synthetic anti-oxidants. Thus, natural anti-oxidants have recently appeared as an alternative to synthetic anti-oxidant (9). There has been growing interest in research into the role

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of plant-derived anti-oxidants in food and human health (10). Thus, precise determination and quantification of anti-oxidant capacity in the natural products and food extract lead to apply an appropriate anti-oxidant for a proper use (11).

Ocimum basilicum is among these most popular ingredient herbal teas. *Ocimum* is a culinary plant belonging to the Lamiaceae family. This plant extensively used as a flavor ingredient in a wide variety of fields in its native region as a popular traditional folk remedy or in complementary and alternative medical therapy. It has several characters including the ability to act as a carminative, antiseptic, anesthetic, flatulence, gastritis, anti-spasmodic, anthelmintic, anti-diarrheal, analgesic and anti-tussive agent (12, 13). Because of these properties, *Ocimum* has played an important role in traditional medicine and also in modern pharmacological and clinical investigations. In this perspective, the *Ocimum* essential oil (basil oil) has had a crucial role in both the pharmaceutical and food industries. However, several useful features of this plant including the mechanisms underlying its *ex vivo* anti-oxidant effects have remained unknown.

The aim of this study was to investigate the level of potential modulating effects of basil oil on macrophages and their related functions including expression of NOX subunits (p22phox, p40phox, p47phox, p67phox) and NOS mRNAs in LPS-stimulated macrophages. In addition, *in vitro* anti-oxidant capacity of basil oil was examined by assessments of 1, 1-diphenyl-2-picryl-hudrazyl radical ($DPPH^{\bullet}$), 2, 2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) radical ($ABTS^{\bullet}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\bullet}$), nitric oxide (NO) and nitrite (NO_2) scavenging effects.

2. Materials and methods

2.1. Chemicals and reagents

1-1, diphenyl-2-picryl-hudrazyl radical ($DPPH^{\bullet}$), 2, 2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) radical ($ABTS^{\bullet}$), hydrogen peroxide, sodium nitrite, sodium sulphate, sodium citrate, sodium nitropruside, naphthylethylenediamine, sulfanilamide and phosphoric acid were purchased from Sigma-Aldrich (Saint, Louis, MO, USA). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), fetal calf serum (FCS), Dulbecco's Modified Eagle Medium (DMEM), L-glutamine, dimethysulfoxide (DMSO) and lipopolysacharide (LPS; from *Escherichia coli* 0111; B4) were purchased from Sigma-Aldrich (Saint, Louis, MO, USA). RNX-Plus buffer was obtained from Cinagen (Tehran, Iran). All other chemicals and reagents were the purest commercially available products.

2.2. Plant materials and oil preparation

This study was carried out in the greenhouse and research field station of Faculty of Agriculture, Shiraz University, Shiraz, Iran. The station is located at 1810 m above mean see level, latitude 29° 35′ north and altitude 52° 32′ east with semi-arid climate. The average relative humidity at time of planting was 30%. The averages of minimum and maximum temperatures of the field were 10°C and 38°C, respectively. The soil of experimental field was a clay silt loam with pH of 7.6. Some of the chemical characteristics of the field soil are as follow; conductivity, 1.8 dS/m; organic matter, 0.97%; total nitrogen, 0.54%; phosphor, 20 mg/kg; potassium, 540 mg/kg; copper, 1.1 mg/kg; iron, 4.9 mg/kg; zinc, 1 mg/kg; manganese, 10 mg/kg. Seeds of *O. basilicum* plants were directly seeded in experimental field. During the germination period and during plantlet growth, they were irrigated with tap water. The aerial parts of *O. basilicum* (green basil) were harvested at the flowering stage, and the leaves of the plants were separated from the stem and were dried in the shade for 4–5 days. The air-dried leaves (100 g) were hydro-distilled for 3 hours using an all-glass Clevenger-type apparatus (Herbal Exir Co., Mashhad, Iran). The basil oil was dehydrated over anhydrous sodium sulphate and stored at 4–6°C before Gas chromatography-Mass spectrometry (GC-MS) analysis. The density of basil oil was calculated by digital balance and the average was reported that was 995 mg/mL. Thus, each µL of basil oil is approximately equal to 1 mg. The basil oil was dissolved in DMSO (1% final concentration) and then diluted at appropriate concentrations.

2.3. Identification of the basil oil components

Analysis of essential oil was performed using an Agilent gas chromatograph series 7890-A (Agilent Palo Alto CA) with a flame ionization detector (FID). The analysis was carried out on a fused silica capillary HP-5 column (30 m ×0.32 mm i.d.; film thickness 0.25 µm). The injector and detector temperatures were kept at 250°C and 280°C, respectively. Nitrogen was used as carrier gas at a flow rate of 1 mL/min; oven temperature program was 60–210°C at the rate of 4°C/min and then programmed to 240°C at the rate of 20°C/min and finally held isothermally for 8.5 minutes; split ratio was 1:50. GC-MS analysis was carried out by use of Agilent gas chromatograph equipped with fused silica capillary HP-5MS column (30 m × 0.25 mm i.d.; film thickness 0.25 µm) coupled with 5975-C mass spectrometer. Helium was used as carrier gas with ionization voltage of 70 eV. Ion source and interface temperatures were 230°C and 280°C, respectively. Mass range was from 45 to 550 atomic mass unit (amu). Oven temperature program was the same as that given above for the GC.

Retention indices (RI) were determined using retention times (RT) of n-alkanes (C_8–C_{28}) that were injected after essential oil under the same chromatographic conditions. The retention indices for all components were determined according to the method of using n-alkanes as standard(s). The compounds were identified by comparison of retention indices (RI, HP-5) with those reported in the literature (14).

2.4. DPPH radical scavenging assay

Fifty microliters of basil oil (0–500 µg/mL) were added to 2 mL of 0.2 mM DPPH in 95% methanol. The mixture was mixed and allowed to stand at room temperature in dark for 30 min. The decrease in light absorbance at 517 nm for each sample was determined after 30 minutes using Unico 2100 spectrophotometer (New Jersey, USA). DPPH radical scavenging activity was expressed as percentage of DPPH inhibition using the following formula. DPPH radical scavenging (%) = $[(A_{DPPH} - A_{test}) / A_{DPPH}] \times 100$ (15). Where, A_{DPPH} = absorbance of DPPH solution and A_{test} = absorbance of DPPH solution in the presence of basil oil. The effective concentrations provided 50% DPPH radical scavenging (EC_{50}) were calculated from the graph that plotted the DPPH radical scavenging percent against different basil oil concentrations.

2.5. ABTS radical scavenging assay

Fifty microliters of basil oil (0–500 µg/mL) was added to 1 mL of diluted ABTS radical solution (7 mM ABTS and 2.54 mM potassium persulfate). After mixing, the absorbance was read at 734 nm by using Unico 2100 spectrophotometer (New Jersey). The percentage of ABTS radical scavenging was calculated by using the following formula: ABTS radical scavenging (%) = $[(A_{ABTS} - A_{test}) / A_{ABTS}] \times 100$ (16). Where, A_{ABTS} = absorbance of ABTS solution and A_{test} = absorbance of ABTS solution in the presence of basil oil. The EC_{50} was calculated from the graph that plotted the ABTS radical scavenging percent against different basil oil concentrations.

2.6. Hydrogen peroxide scavenging assay

Fifty microliters of basil oil (0–500 µg/mL) was incubated with 1 mL of H_2O_2 (50 mM in 100 mM phosphate buffer pH 7.4) at 37°C for 60 minutes. After incubation, the absorbance was read at 240 nm using Unico 2100 spectrophotometer (New Jersey). The percentage of H_2O_2 scavenging was calculated by using the following formula: $[(A_{H2O2} - A_{test}) / A_{H2O2} \times 100$ (17). A_{H2O2} = absorbance of H_2O_2 solution and A_{test} = absorbance of H_2O_2 solution in the presence of basil oil. EC_{50} was calculated from the graph that plotted the H_2O_2 scavenging percent against different basil oil concentrations.

2.7. Hydroxyl radical scavenging assay

The hydroxyl radical scavenging activity was investigated using Fenton reaction for hydroxyl radical generation ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + HO^{\bullet}$). Fifty microliters of basil oil (0–500 µg/mL) were incubated with 3 mL of Fenton reaction solution and incubated at 37°C for 60 minutes. Fenton reaction solution was prepared as follow: 10 mM $FeSO_4$, 10 mM EDTA, 2 mM sodium salicylate, 200 µL of H_2O_2 (30%) in 100 mM sodium phosphate buffer pH 7.4. After incubation, the absorbance was read at 510 nm using Unico 2100 spectrophotometer (New Jersey). The percentage of hydroxyl radical scavenging was calculated by using the following formula: $[(A_{HO\bullet} - A_{test}) / A_{HO\bullet} \times 100$ (17). $A_{HO\bullet}$ = absorbance of Fenton reaction solution and A_{test} = absorbance of Fenton reaction solution in the presence of basil oil. EC_{50} was calculated from the graph that plotted the $HO^{\bullet}$ scavenging percent against different basil oil concentrations.

2.8. Nitric oxide scavenging assay

Fifty microliters of basil oil (0–500 µg/mL) was incubated with 0.5 mL of sodium nitropuroside (SNP, 20 µg/mL in 100 mM sodium citrate pH 5) at 37°C for 2 hours. After incubation, 0.5 mL of Griess reagent was added and the absorbance was read at 540 nm using Unico 2100 spectrophotometer (New Jersey). The percentage of nitric oxide scavenging was calculated by using the following formula: $[(A_{control} - A_{test}) / A_{control}] \times 100$ (18). $A_{control}$ = absorbance of Griess reagent in the presence of sodium nitropuroside and A_{test} = absorbance of Griess reagent in the presence of sodium nitropuroside and basil oil. EC_{50} was calculated from the graph that plotted the nitric oxide scavenging percent against different basil oil concentrations.

2.9. Nitrite scavenging assay

Fifty microliters of basil oil (0–500 µg/mL) was incubated with 0.5 mL of sodium nitrite (10 µg/mL in 100 mM sodium citrate pH 5) at 37°C for 2 hours. After incubation, 0.5 mL of Griess reagent was added and the absorbance was read at 540 nm by using Unico 2100 spectrophotometer (New Jersey). The percentage of nitrite scavenging was calculated as follows: $[(A_{control} - A_{test}) / A_{control}] \times 100$ (18). $A_{control}$ = absorbance of Griess reagent in the presence of sodium nitrite and A_{test} = absorbance of Griess reagent in the presence of sodium nitrite and basil oil. EC_{50} was calculated from the graph that plotted the nitrite scavenging percent against different basil oil concentrations.

2.10. 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 at 37°C in DMEM 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 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 mL in 24-well tissue culture plates in triplicate (Tissue, Culture Plate, Jet Biofil, Kyoto, Japan). After culturing for 18 hours 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 1 µg LPS/mL. After 2 hours, basil oil was added at a final concentration of 1 and 10 µg/mL. Two sets of wells without basil oil but containing LPS and DMSO solvent (1% final concentration) were used as negative controls. After 24 hours of incubation at 37°C, the culture supernatants in each well were removed and the cells harvested for RNA extraction and real-time polymerase chain reaction (RT-PCR) analysis.

2.11. Cell viability assay

J774A.1 cells (2×10^4 cells per well) were incubated for 24 hours (at 37°C in 5% CO_2) with different concentrations of basil oil (0–200 µg/mL). Thereafter, 10 µL of MTT (5 mg/mL) was added to each well and incubated for an additional 4 hours at 37°C followed by treatment with 100 µL of lysis buffer (10% SDS in 10 mM HCl). The absorbance of each well was determined by spectrophotometer at dual wavelengths of 570 nm on a microplate ELISA reader (BioTek Elx 808, Winooski, VT, 05403, USA). Viability percent was calculated by the following formula: [(Absorbance of treated cells/Absorbance of corresponding control)] × 100. The control was basil oil-untreated cells containing DMSO at the highest concentration used (1%). The concentration provided a 50% inhibition (IC_{50}) was calculated from a graph, plotting the inhibition percent against different basil oil concentrations (19).

2.13. RNA extraction and cDNA synthesis

About 2×10^6 cells were transferred to 1 mL of RNX-plus buffer (Cinagen, Tehran, Iran) in an RNase-free microtube, mixed thoroughly and left at room temperature for 5 minutes. About 200 µL of chloroform was added to the slurry and was mixed gently. The mixture was centrifuged at 13200 g at 4°C for 15 minutes, the supernatant was transferred to a new tube and was precipitated with an equal volume of isopropanol for 15 minutes on ice. The RNA pellet was washed using 75% ethanol, was 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 (i.e., 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 (Fermentas, Hanover, MD) in a 0.02 mL final volume.

2.14. Quantitative RT-PCR

Primer design, in the form of exon junction was carried out using Allele ID7 software (Premier Biosoft Intl., Palo Alto, CA) for the internal controls glyceraldehydes -3- phosphate dehydrogenase (GAPDH) (NM-010927) and tested genes NOX p22phox (NM-007806), NOX p40phox (NM-008677), NOX p47phox (NM-010876), NOX p67phox (NM-010877) and iNOS (NM-008084) (Table 1). The GAPDH (whose expression proved not to be influenced by LPS) was used as internal control for data normalization (20). Quantitative RT-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. 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 minutes, followed by 40 cycles of 94°C for 10 seconds, annealing temperature (Ta) of each primer pair was done for 15 seconds and 30 seconds for extension to occur at 72°C. After forty 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

Table 1. Primer used for real-time analysis.

Genes[1]	Accession No.	Sense sequence	Anti sense sequence
GAPDH	NM-010927	5′-CGGTGTGAACGGATTTGGC-3′	5′-TGAGTGGAGTCATACTGGAAC-3′
NOX p22	NM-007806	5′- ATGGAGCGATGTGGACAG-3′	5′- ACCGACAACAGGAAGTGG-3′
NOX p40	NM-008677	5′-CAACAAAGACTGGCTGGAG-3′	5′-CCGCAATGTCCTTGATGG-3′
NOX p47	NM-010876	5′- ATGGCACAAAGGACAATC-3′	5′- ACCTGAGGCTATACACAAG-3′
NOX p67	NM-010877	5′- CAGCCACAGTCAGCAGAG-3′	5′-GCACAAAGCCAAACAATACG-3′
NOS	NM-008084	5′- CTGGAGGTTCTGGATGAG-3′	5′- CTGAGGGCTGACACAAGG -3′

Notes: [1]Primer design, in form of exon junction was carried out using Allele ID 7 software for the internal control glyceraldehydes -3- phosphate dehydrogenase (GAPDH) and test genes NADH oxidase p22 phagocytes oxidase (NOX p22phox), NOX p40phox, NOX p47phox, NOX p67phox and inducible nitric oxide synthase (iNOS) genes from *Mus musculus* sequence.

Table 2. Chemical composition of essential oil from *O. basilicum*.

Compounds	RT	RI_{ex}	RI_{let}	% of compounds
α -Pinene	7.699	933	931	0.30
Sabinene	9.003	978	977	0.08
1-Octen-3-ol	9.082	980	982	0.18
5-Hepten-2-one-6-methyl	9.392	991	994	1.10
Unknown	10.200	1009	1010	0.10
p-Cymene	10.571	1025	1024	0.14
Limonene	10.712	1028	1030	0.17
1,8-Cineol	10.990	1036	1035	0.10
trans- β -Ocimene	11.367	1046	1045	0.30
γ-Terpinene	11.810	1058	1057	0.12
cis-Sabinene hydrate	12.378	1073	1069	0.20
2.2-Dimethyl 3,4 octad	13.180	1094	1097	0.09
cis-Verbenol	15.133	1143	1142	0.20
Borneol	15.563	1153	1154	0.51
p-Mentha 1,5-dien-8 ol	16.124	1167	1168	0.42
Methylchavicol	17.971	1212	1210	47.0
n-Octyl acetate	18.141	1216	1216	0.40
Nerol	19.083	1238	1236	3.00
Neural	19.740	1254	1255	15.0
Geraniol	20.212	1265	1266	3.01
Geranial	21.023	1284	1282	19.0
Neryl acetate	24.459	1368	1365	0.30
Unknown	24.830	1377	1374	0.15
Geranyl acetate	25.242	1387	1390	0.32
Methyl eugenol	26.127	1409	1405	0.52
E-Caryophyllene	26.666	1423	1420	2.40
trans -α - Bergamotene	27.205	1436	1440	0.62
α -Humulene	27.986	1456	1458	1.11
Germacrene D	29.061	1483	1480	1.20
trans-α-Bisabolene	31.371	1544	1545	1.61
Caryophyllene oxide	33.075	1589	1586	0.40

Notes: RT = Retention time; RI_{ex} = Retention index calculated on the HP-5 column relative to C8–C28 n-alkanes; RI_{let} = retention index reported in the literature (7).

were repeated twice under identical conditions beside a negative control and 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 RT-PCR data, relative expressions of NOX and iNOS 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 of Larionov et al. (21). 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 ΔΔCT was obtained by subtracting the ΔCT of each experimental sample from that of the control sample (22).

2.15. Statistical analysis

The SPSS statistical package (SPSS, Abaus Concepts, Berkeley, CA) was used for statistical analysis. The data presented here were analyzed as a completely randomized design with three replications and expressed as means ± standard deviation. The significant differences between treatments were analyzed by Duncan tests at $P < 0.05$.

3. Results and discussions

3.1. Basil oil composition

GC-MS analysis (Figure S1 and Table 2) indicated that the main components of basil oil were methylchavicol (47%), geranial (19%), neral (15%), geraniol (3%), nerol (3%) and caryophyllene (2.4%). Accordingly, the most abundant components in basil oil are phenylpropanoids (methychavicol, methyl cinnamate), acyclic alcoholic monoterpenes (linalool, geraniol, nerol), acyclic aldehyde monoterpene (geranial, neral), cyclic ether monoterpenes (1, 8-cineole), and hydrocarbon bicyclic sesquiterpene (caryophyllene). Our experimental results were similar to the results has been reported by Javanmardi et al. (23), Nurzynska (24), Pirmoradi et al (25), Ono et al. (26), Sajjedi (27) and Shirazi et al. (28). However, there are differences in their quality and quantity of the components that is may be attributed to environmental conditions and genetic factors.

3.2. DPPH radical scavenging activity

Basil oil displayed a concentration-dependent DPPH radical scavenging activity (Figure 1) with EC_{50} value of 114 ± 4 μg/mL (Table 3) that is comparable with the results Pirmoradi et al. (25), Gulcin et al. (29) and Salles Trevisan

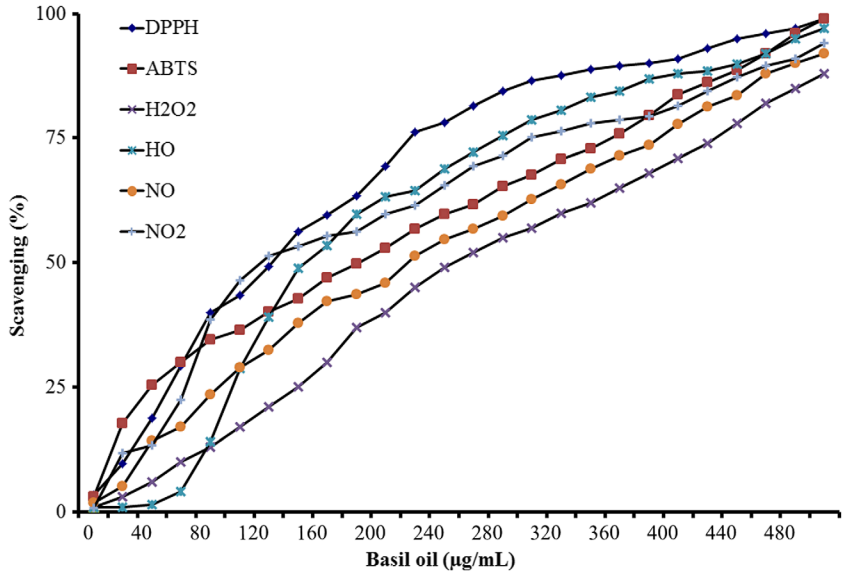

Figure 1. DPPH•, ABTS•, H_2O_2, hydroxyl radical (HO•), nitric oxide (NO) and nitrite (NO_2) scavenging activities of *O. basilicum* essential oil.

Table 3. Effective concentration of O. *basilicum* essential oil required for 50% inhibition (EC_{50}) of DPPH, ABTS, H_2O_2, hydroxyl radical (HO•), nitric oxide (NO) and nitrite (NO_2).

Radicals/oxidants	EC_{50} (µg/mL)
DPPH•	114 ± 4
ABTS•	186 ± 7
H_2O_2	246 ± 3
HO•	138 ± 4
NO	214 ± 5
NO_2	118 ± 4

Data represent mean ± standard deviation from at least three sets of independent experiments. EC_{50} = effective concentration of the test compound which scavenge the radical by 50%.

et al. (30). The principle of DPPH radical assay is based on the reduction of DPPH radical (violet color) to non-radical 1, 1-diphenyl-2-picryl hydrazine (yellow color) in the presence of anti-oxidant. DPPH radical solution is a stable free radical which has a violet color and showed maximum absorbance at 517 nm. In the presence of anti-oxidant, a hydrogen atom and/or electron was transferred to the odd electron in DPPH radical thus the violet color converted to yellow color and the absorbance was decreased. Thus, the decrease in the light absorbance could be reflected to hydrogen atom- and/or electron-donating activity of basil oil (31).

3.3. ABTS radical scavenging activity

Basil oil displayed a concentration-dependent ABTS radical scavenging activity (Figure 1) with EC_{50} value of 186 ± 7 µg/mL (Table 3) that is similar to the results of Javanmardi et al. (23), Shirazi et al. (28) and Bozin et al. (32). The ABTS anti-oxidant assay is based on the generation of a blue/green ABTS radical solution in the presence of an oxidant such as potassium persulfate ($K_2S_2O_8$) which has maximum absorbance at 734 nm. Addition of anti-oxidants to the pre-formed radical cation reduces it to non-radical ABTS, and causes a decrease in the light absorbance at 734 nm. The decrease in the light absorbance at 734 nm directly could be related ABTS radical scavenging and/or electron donating capacity of basil oil (33).

3.4. H_2O_2 and HO• scavenging activity

Basil oil displayed a concentration dependent H_2O_2 and hydroxyl radical scavenging activity (Figure 1) with EC_{50} values of 246 ± 3 and 138 ± 8 µg/mL, respectively (Table 3). The principle of H_2O_2 scavenging is that there is a decrease in the absorbance of H_2O_2 solution in the presence of hydrogen donors (34). In the presence of anti-oxidants (hydrogen donors), H_2O_2 converted to H_2O and molecular oxygen (35). H_2O_2 itself is not a free radical, but is converted by the Fenton or Haber-Weiss reactions to the hydroxyl radical in the presence of ferrous ion. Hydroxyl radical is one of the most dangerous radical as it is involved in generation of other toxic radicals. Hydroxyl radical causes damage to all major classes of macromolecules including; protein, lipids and specially mitochondrial and nuclear DNA (36). Excess generation of H_2O_2 and hydroxyl radical may result in numerous pathological conditions including endothelial cell dysfunction and myocardial reperfusion injury (37). Thus, removing of hydrogen peroxide and hydroxyl radical is very important for protection of pharmaceuticals and food systems against oxidative damages. The H_2O_2 and hydroxyl radical scavenging activity of basil oil observed

in our study imply the beneficial role of basil oil for reducing damages in biological tissues or may protect tissues against oxidative damage by hydroxyl radical derived from H_2O_2.

3.5. Nitric oxide scavenging activity

Basil oil displayed a concentration dependent nitric oxide scavenging activity (Figure 1) with EC_{50} value of 214 ± 5 µg/mL (Table 3). Sodium nitropruside (SNP) acts as a drug by releasing nitric oxide (NO). The principle of this method was that, NO produced by SNP reacts with Griess reagents to generate a pink-red color complex (38). In the presence of NO scavenger (anti-oxidant compounds) the production of this complex is disrupted. Thus, the decrease in the light absorbance at 540 nm could be reflecting NO scavenging activity of the materials (39). Basil oil had the ability to reduced pink-red color complex, thus they have excellent NO scavenging activity. Although NO is essential in tumoricidal and bactericidal functions of macrophages, over production of NO has been implicated in septic/cytokine-induced circulatory shock (40). In septic shock, overproduction of NO leads to massive systematic vasodilation and fatal drop in blood pressure, which causes an insufficient blood and oxygen supply to peripheral organs, leading to multiple organ failure (1). NO can also react with superoxide anion to form peroxynitrite. Peroxynitrite is a powerful oxidant and nitrating agent, which can damage protein, DNA and lipid molecules (5). Damage to biological macromolecules can leads to endothelial dysfunction and many related diseases such as hypertension, hypercholesterolemia and diabetes (37). The NO scavenging activity of basil oil observed in our study imply the beneficial role of these products for reducing damages in biological membrane or may protect tissues against oxidative damage introduced by peroxynitrite.

3.6. Nitrite scavenging effect

Basil oil displayed a concentration dependent nitrite scavenging activity (Figure 1) with EC_{50} value of 118 ± 4 µg/mL (Table 2). Nitrite in the sample is measured using the Griess reagents (sulfanilamide, HCl, N-(1-naphthyl)-ethylenediamine) (41). Nitrite reacts with Griess reagent and produces a chromophore complex with maximum absorbance at 540 nm. Anti-oxidant compounds as nitrite scavenger have the ability to disrupt this red pink-color complex (42). Basil oil could quench the red color complex, thus exhibited nitrite scavenger activity. Inorganic nitrate from dietary sources and drinking water are the major sources of bio-active nitrogen oxides such as nitrite. The bioactivation of the nitrate anion involves initial reduction to nitrite by symbiotic bacteria in the oral cavity (43). A substantial amount of ingested nitrate is actively taken up from the circulation by the salivary glands and then reduced to nitrite by bacteria in the oral cavity. The continuous swallowing of nitrite-containing saliva generates a variety of nitrogen oxides in the acidic location of the gastric lumen (1). The nitrite anion is involved in nitration reactions that are commonly implicated in pathological processes such as inflammation and atherosclerosis. The nitration of unsaturated fatty acids induced by nitrite which in turn mediate adaptive and anti-inflammatory signaling reactions (37). Nitrite binds cytochrome C oxidase (complex IV of electron transport chain), in competition with oxygen. This event elicits intracellular signaling cascades, including the diversion of oxygen to generation of reactive oxygen species with potentially damaging effects and oxidative stress and the development of disease through DNA damaging (43).

3.7. Cytotoxicity of basil oil

The MTT assay results indicated that basil oil inhibited the viability of J774A.1 cells in a concentration-related manner (Figure 2). In order to determine the *ex vivo* anti-oxidant effects of basil oil on macrophages, the concentrations which were overtly cytotoxic to the cells were not used. Lipophilic essential oil components by penetrating through the cytoplasmic and organelles membranes disrupt and permeabilize them and especially damage mitochondrial membranes, these effects may result in cellular cytotoxicity (44). Components of natural products especially phenolic components which show anti-oxidant activity, can be oxidized by reactive oxygen and nitrogen species and thus generate additional radical species like phenoxyl, hydroxyl, superoxide radicals and hydrogen peroxide (45). Indeed, anti-oxidants by interacting with reactive oxygen and nitrogen species are converted into pro-oxidants which are able to oxidize lipids, proteins and DNA (46). Thus, volatile terpenic and phenolic components of essential oils can function as pro-oxidants by affecting the cellular redox status. This event may lead to late apoptosis and/or necrosis including damage to proteins and DNA and overall cytotoxic effects (47). If the anti-oxidant concentrations is too low to permeabilize mitochondrial membranes, their conversion into pro-oxidant may not occur, and the anti-oxidant would keep its activity. Thus, at low concentrations, anti-oxidant was not oxidized and could not damage mitochondria. In contrast, at high concentration anti-oxidants could damage and permeabilize mitochondria, oxidized to pro-oxidant and could react as pro-oxidant, damaging DNA and proteins and finally cell death (48).

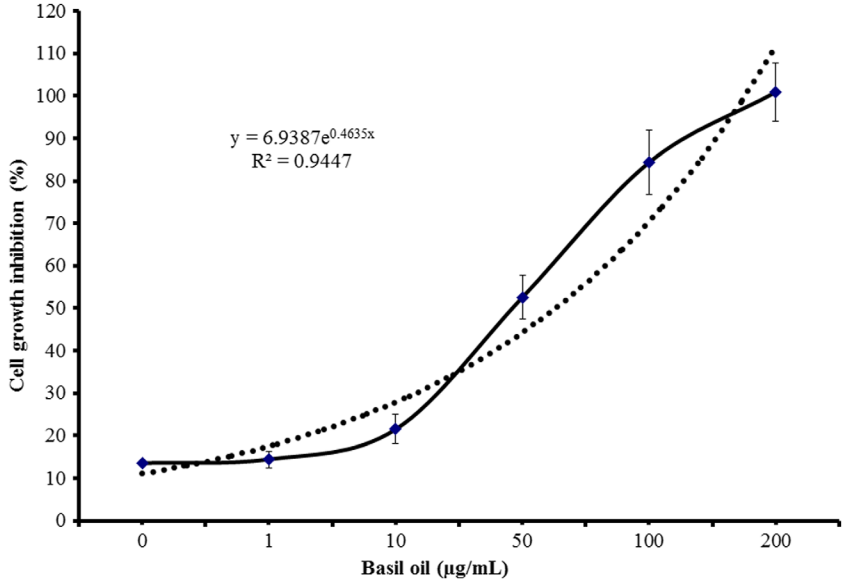

Figure 2. Effect of the *O. basilicum* essential oil on the viability of J774 cell lines. The cells were treated with various concentrations of basil oil and incubated for 24 h. Control (0 µg/mL) was cells treated only with the solvent (DMSO) at concentration of 1%. Data represent mean ± SD from three sets of independent experiments.

Table 4. Effects of *O. basilicum* essential oil (OBO) on NOX p22phox, NOX p40phox, NOX p47phox, NOX p67phox and NOS mRNA expression in LPS-stimulated macrophages.

	NOX p22	NOX p40	NOX p47	NOX p67	NOS
Control	1.0 ± 0.4^{d}	1.0 ± 0.3^{d}	1.0 ± 0.5^{d}	1.0 ± 0.3^{d}	1.0 ± 0.2^{d}
LPS	29 ± 1.8^{a}	16 ± 0.9^{a}	45 ± 2.4^{a}	26 ± 1.7^{a}	28 ± 1.6^{a}
LPS + OBO1	13 ± 1.7^{b}	4.7 ± 0.6^{b}	25 ± 2.2^{b}	6.4 ± 1.2^{b}	9.3 ± 2.2^{b}
LPS + OBO10	9 ± 1.4^{c}	3.0 ± 0.8^{c}	10 ± 0.8^{c}	2.0 ± 0.2^{c}	4.6 ± 0.4^{c}

Notes: The cells were cultured in 24-well plates and treated with and without LPS. Various concentrations of OBO (1-10 µg/mL) were added. After 24 h, the expression of NADH oxidase p22 phagocyte oxidase (NOX p22phox), NOX p44phox, NOX p47phox, NOX p67phox and inducible nitric oxide synthase (iNOS) 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 columns show significantly difference ($p < 0.05$).

3.8. NOX phox mRNA expression

The un-stimulated cells (control) showed low level of NOX p22phox, NOX p40phox, NOX p47phox and NOX p67phox mRNAs expression. LPS stimulation of macrophages resulted in an increase in the all NOX phox mRNA expression in respect to LPS-untreated control cells ($p < 0.01$). The addition of basil oil at 1 and 10 µg/mL significantly decreased all of the NOX phox mRNAs expression in LPS-treated cells ($p < 0.01$), indicating the inhibitory effect of basil oil on NOX phox mRNA induction/formation (Table 4). In macrophages superoxide inion 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 (49). 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 (50). 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 (51). As our study showed, basil 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 NOX function and superoxide anion production (52). The suppression of NOX expression in macrophages, due to the basil oil indicates the ability of this product to diminish superoxide production and oxidative reactions and provides further evidence that this plant may have potent anti-oxidative properties.

3.9. iNOS mRNA expression

LPS stimulation of macrophages resulted in an increase in iNOS mRNA expression in respect to LPS-untreated cells. The addition of basil oil at concentrations of 1

and 10 μg/mL significantly decreased the iNOS mRNA expression in LPS-treated cells, indicating the inhibitory effect of basil oil on iNOS mRNA induction/formation (Table 4). The overproduction of NO by activated macrophages seems to play an important role in different steps of many oxidative stress processes and has a key role in the pathogenesis of oxidative damages (4). 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 (LPS) or a variety of pro-inflammatory cytokines such as TNF-α and IL-1β (53). 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 (54). As the results of our study showed, basil oil significantly reduced iNOS mRNA expression in stimulated macrophages. The suppression of iNOS gene expression, due to the basil oil indicates the ability of this product to diminish oxidative reactions and provides further evidence that this plant may have potent anti-oxidative properties.

4. Conclusions

The main components of basil oil were phenylpropanoids (methychavicol, methyl cinnamate), acyclic alcoholic monoterpenes (linalool, geraniol, nerol), acyclic aldehyde monoterpene (geranial, neral), cyclic ether monoterpenes (1, 8-cineole), and hydrocarbon bicyclic sesquiterpene (caryophyllene). Basil oil was found to be an effective anti-oxidant in several in vitro assays including DPPH radical, ABTS radical, hydrogen peroxide, hydroxyl radical, nitrite and nitric oxide scavenging assay. Basil oil also exhibited *ex vivo* anti-oxidant activity in LPS-stimulated macrophages due to an inhibition of iNOS and NOX gene expressions. Thus, basil oil had radical scavenging activity and could potentially be used as a safe effective source of natural anti-oxidants in therapy against oxidative damages associated with some inflammatory conditions. These data suggest a potential therapeutic usefulness for basil oil in the modulation of macrophages and provides evidence to support the use of basil oil as a tea/additive/traditional remedy for treatment of oxidative stress related diseases. However, further in vivo studies are recommended to more fully understand the therapeutic potential of basil oil in oxidative stress disorders.

Disclosure statement

No potential conflict of interest was reported by the authors.

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