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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

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ABSTRACT

Oliveria decumbens Vent., is an endemic plant of Iran belonging to the Apiaceae family, where it grows in the arid southeastern regions. In the traditional medicine, it is used in cases of indigestion, diarrhea, abdominal pain and fever. An nanoemulsion including polysorbate (100 μg/mL), *Oliveria decumbens* essential oil (OEO, 1 mL) and *O. decumbens* aromatic water (OAW, 100 mL) was prepared. The radical scavenging and antioxidant capacity of OEO-OAW emulsion was investigated in lipopolysaccharide (LPS)-stimulated murine macrophages. The reduction of nitric oxide (NO), intracellular reactive oxygen species (ROS), and thiobarbituric acid reactive substances (TBARS) at non-cytotoxic concentrations (< 3000 ng/mL) of OEO-OAW was determined. The main components of OEO were thymol (25.5%), carvacrol (23.1%), *p*-cymene (22.1%) and γ-terpinene (17.8%). The emulsion exhibited 1,1-diphenyl-2-picryl-hydrazyl radical (DPPH), 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) radical (ABTS), superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (HO), NO, nitrite (NO_2) and malondialdehyde (MDA) scavenging effects as well as lipid oxidation inhibition at low concentrations (20–200 μg/mL). In addition, at lower doses (1000–3000 ng/mL) it strongly reduced formation of NO, ROS and TBARS in LPS-stimulated macrophages. Taken together, our outcomes corroborate the antioxidant properties of *O. decumbens* formulation and provide new insights into the industrial and pharmacological properties of OEO-OAW.

1. Introduction

Biological oxidants are highly reactive oxygen and nitrogen species, which cause includtion of hydroxyl radical, superoxide anion, hyderogen peroxide, nitric oxide (NO), peroxynitrite, lipid peroxides and thiobarbituric acid reactive substances. They can be formed endogeneously (by the normal cellular metabolism like respiration and stimulation of macrophages) or exogeneously (by ionizing radiation or certain pollutants) (Alzoghaibi, 2013). These highly reactive radicals can oxidatively modify lipids (by production of lipid peroxyl radicals), sugars (by sugar fragmentation and glycolaldehyde formation), nucleic acids (by base modification and strand breaks), protein (by modification of amino acids and peptide cleavage), enzymes and metabolites leading to oxidative stress and ultimately cell damage (Ray et al., 2012). The cell damage resulted from these unstable free radicals and the change in the balance between oxidants and antioxidants seems to be a key supplier to aging and pathological deteriorating illnesses including cardiovascular disease, cancer, cataracts, brain dysfunction, immune system decline, and stress (Carocho and Ferreira, 2013; Lobo et al., 2010). It has been confirmed that the endogenous antioxidant system is not sufficient, and body depends on several dietary antioxidants to keep free radicals at low levels. It has been emphasized that a diet enriched with antioxidants would be necessary for human health. Neutralizaion and deactivation of free radicals by natural antioxidants from plant materials is an efficient way to protect the cells against oxidative damage (Almeida et al., 2011; Gostner et al., 2015).

Essential oils from aromatic and medicinal plants are gaining popularity all over the world. They are composed of mixture of volatile, low molecular weight compounds, including terpenes, phenylpropanoids and aliphatic compounds. Due to the molecular structure and the existence of functional groups and olefinic double bonds, which are relatively lipophilic, soluble in alcohol and nonpolar or weak polar solvents (Brewer, 2011; Rodríguez et al., 2016). Aromatic waters (also known as essential water, hydrolate or hydrosol) are clear and

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saturated aqueous solutions of aromatic volatile substances obtained as a waste of distillation processes. They are composed of water soluble volatile compounds and, to some extent, contain the same components of pure essential oils (Bakkali et al., 2008; Nakatsu et al., 2000). Dispersion of essential oil in aromatic waters using a suitable emulsifier will produce a complex of aromatic water-essential oil mix with a balanced of hydrophilic and lipophilic components that exhibits a broader range of efficacy and functional properties (El Asbahani et al., 2015).

Due to beneficial old diet rich in antioxidants, a lot of interests is focused on the determination of antioxidant capacity of natural products including essential oils. The most popular chemical based approaches for the evaluation of antioxidant capacity are 2, 2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) radical (ABTS) and 1,1-diphenyl-2-picryl-hydrazyl radical (DPPH) scavenging assays (Bakkali et al., 2008; Krishnaiah et al., 2011). The analysis of radical scavenging activity of essential oils from clove, cinnamon, basil, oregano and thyme suggested that terpenoids, especially those endowed with phenolic groups, together with phenylpropanoids, exhibit significant antioxidant effects (Almeida et al., 2011; Tongnuanchan and Benjakul, 2014).

Besides ABTS and DPPH assay that are chemical-based methods, the antioxidant capacity of natural products can be measured against biologically produced radicals likes superoxide ion, peroxyl radicals and hydrogen peroxide (H_2O_2) (Carocho and Ferreira, 2013).

Oliveria decumbens Vent., is an endemic herb of Iran belonging to the Apiaceae family, where it grows in the arid southeastern regions. In the traditional medicine, it is used for indigestion, diarrhea, abdominal pain and fever (Hajimehdipoor et al., 2010). *Oliveria decumbens* essential oil (OEO) and its aromatic water (OAW) have had a vital role in medicine and in food industries, but based to our information there is no published report about the their biological activities.

In this study, the polysorbate-OEO-OAW emulsion was prepared, and its radical scavenging properties, as well as the antioxidant activities against biological oxidants like (H_2O_2), hydroxyl radical, superoxide ion, NO and nitrite, and inhibitory properties against lipid peroxidation were evaluated. Furthermore, to shed light on the OEO-OAW emulsion protective effects in living cells, the inhibitory effects on the synthesis of reactive oxygen species (ROS), NO and thiobarbituric acid reactive substances (TBARS) in lipopolysaccharide (LPS)-stimulated murine macrophages were determined as well.

2. Materials and methods

2.1. Oliveria decumbens essential oil (OEO)

The *O. decumbens* aerial parts were samplelled from its wild habitat in Farashband (52° 12′ E, 28° 49′ N, Altitude 775 m), Fars province, Iran, then were shadedried for 72 h. The plant material (100 g) was immersed in a flask filled with distilled water (1000 mL) and exposed to hydro-distillation using a Clevenger device for 3 h. The essential oil condensed on the top of aromatic water and was isolated from it using a decanting funnel. Afterwards OAW was collected in a different flask. The OEO was dehydrated with anhydrous sodium sulfate and reserved at temperature of 4 °C.

2.2. Chemical analysis of OEO

The essential oil was analyzed with 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.d., 0.25 μm; J & W Scientific, Folsom). The retention indices (RIs) were calculated by a standard mixture of *n*-alkanes (C_8-C_{25}) using the similar chromatographic conditions. The same Agilent gas chromatograph as above coupled with a mass spectrometer detector (Model 5975 C) and equipped with a HP-5 MS fused silica capillary column (30 m × 0.25 mm i.d.; film thickness 0.25 μm; J & W Scientific, Folsom) was used to conduct the GC/MS analysis.

The compounds were recognized through comparing the MS matching and RI value with respect to those stored in the ADAMS library (Adams, 2007).

2.3. Preparation of polysorbate-OEO-OAW emulsion

One mL of OEO was poured into 100 mL of OAW. Polysorbate-20 (100 μg/mL) was poured into the OEO-OAW mixture that was incubated at temperature of 35 °C for 24 h. A milky emulsion was produced at the same time.

2.4. Physical property

The conductivity and pH of the samples were measured using pH-conductometer instrument (Mettler-Toledo, Cleantech, Schaffhausen, Switzerland). The Brookhaven instrument corporation 90 Plus particle size analyzer (New York, 11742, USA) was used to detime the effective hydrodynamic diameter and zeta-potential of particles based on manual instructions. Determination of the effective hydrodynamic diameter of particles was performed according to the principle of Dynamic Light Scattering (DLS). The software of Bi-9000 particle sizing suggested a mean effective hydrodynamic diameter and a measure for polydispersity. The Phase Analysis Light Scattering (PALS) method was applied to determine the particles electrophoretic mobility in the dispersion. The software of Bi-PALS zeta potential analyzer suggested a mean value for electrophoretic mobility and a quantity for zeta-potential with smolouchewsky model.

2.5. Total phenol content

The concentration of total phenol content in OEO-OAW emulsion was measured using the Folin-Ciocalteu technique with gallic acid as a standard. Briefly, 200 μL of samples or standard gallic acid solutions (0–500 μg) were poured into 1 mL of Folin-Ciocalteu reagent (10%) and 0.3 mL Na_2CO_3 (10%), next the resalted solution was shacked at ambient temperature for 10 min. Absorbance was record at 765 nm by means of a spectrophotometer (Ainsworth and Gillespie, 2007). The emulsion was adjusted to 1000 μg/mL of gallic acid equivalent using deionized water for antioxidant characterization.

2.6. ABTS radical scavenging assay

Twenty μL of samples were poured into 1.0 mL of diluted ABTS radical solution and the mixture was stirred. Then, the absorbance (A) of the mixture was recorded at 734 nm. The percentage of ABTS radical scavenging is obtained from the following equation:

ABTS radical scavenging percentage = $[(A_{ABTS} - A_{test}) / A_{ABTS}] \times 100$.

where, A_{ABTS} states to absorbance of ABTS solution and A_{test} represents absorbance of the ABTS solution in the existence of antioxidants. IC_{50} (the concentrations that could cause 50% ABTS inhibition) calculation was performed using the inhibition percentage / various antioxidant concentrations graph (Floegel et al., 2011).

2.7. DPPH radical scavenging assay

Twenty μL of samples were poured into 1.0 mL of DPPH (0.2 mM) in 95% methanolic solution. The solution was incubated at ambient temperature for 30 min. The reduction of absorbance (A) of all samples was observed at 517 nm. The percentage of DPPH radical scavenging performance was measured as follows:

$[(A_{DPPH} - A_{test})/A_{DPPH}] \times 100$;

where A_{DPPH} refers to absorbance of DPPH solution and A_{test} represents absorbance of DPPH solution in the existence of antioxidants. IC_{50} (the concentrations with 50% DPPH inhibition) calculation was performed

using the inhibition percentage / various antioxidant concentrations graph (Floegel et al., 2011).

2.8. H_2O_2 scavenging assay

Incubation of twenty μL of samples was performed using 1.0 mL of H_2O_2 (50 mM prepared in 100 mM phosphate buffer, pH 7.4) at temperature of 37 °C for duration of 60 min. Following incubation, the absorbance (A) was recorded at 230 nm. The H_2O_2 scavenging percentage was computed as follows:

$$[(A_{H2O2} - A_{test})/A_{H2O2}] \times 100;$$

where A_{H2O2} is absorbance of H_2O_2 solution and A_{test} refers to absorbance of H_2O_2 solution in the presence of antioxidants. IC_{50} (the concentrations with 50% H_2O_2 inhibition) calculation was performed using the inhibition percentage / various antioxidant concentrations graph (Tarpey et al., 2004).

2.9. Hydroxyl radical scavenging assay

Incubation of twenty μL of samples was performed with 1.0 mL of Fenton reaction solution at temperature of 37 °C for duration of 60 min. Fenton reaction solution consisted of EDTA (10 mM), $FeSO_4$ (10 mM), 200 μL of H_2O_2 (30%) in sodium phosphate buffer (100 mM), sodium salicylate (2 mM), pH 7.4. Subsequently incubation, the absorbance was measured at 510 nm. The HO scavenging percentage was expressed as follows:

$$[(A_{HO} - A_{test})/A_{HO}] \times 100;$$

where A_{HO} refers to absorbance of Fenton reaction solution and A_{test} represents absorbance of Fenton reaction solution in the existence of antioxidants. IC_{50} (the concentrations that could provide 50% HO inhibition) calculation was performed using the inhibition percentage /various antioxidant concentration graph (Zepp et al., 1992).

2.10. Superoxide radical scavenging assay

Incubation of twenty μL of samples was performed with 1.0 mL of superoxide radical reaction mixture at room temperature for 15 min. The reaction mixture in phosphate buffer (10 mM, pH 7.4) included nicotinamide adenine dinucleotide (300 mM), phenazine methosulfate (20 mM), and nitro blue tetrazolium (50 mM). After incubation, the absorbance was recorded at 560 nm. The percentage of superoxide radical scavenging was expressed as following:

$$[(A_{contol} - A_{test})/A_{control}] \times 100;$$

where $A_{control}$ is absorbance of superoxide radical reaction solution and A_{test} is absorbance of superoxide radical solution in the existence of antioxidants. The graph plotting the percentage of superoxide radical scavenging against different antioxidant concentrations was used to determine IC_{50} (Tarpey et al., 2004).

2.11. Nitric oxide scavenging assay

Incubation of twenty μL of samples was performed with 0.5 mL of sodium nitroprusside (SNP) (20 μg/mL prepared in 100 mM sodium citrate pH 5) at 37 °C. After incubating for 2 h, 0.5 mL of Griess reagent was poured and the absorbance (A) was recorded at 540 nm. The percentage of NO scavenging was determined as following:

$$[(A_{control} - A_{test})/A_{control}] \times 100;$$

where $A_{control}$ = absorbance of Griess reagent in the presence of sodium nitropuroside and A_{test} = absorbance of Griess reagent in the presence of sodium nitropuroside and antioxidants. IC_{50} (the concentrations with 50% NO inhibition) calculation was performed using the inhibition percentage/ various antioxidant concentrations graph (Tarpey et al., 2004).

2.12. Nitrite scavenging assay

Twenty μL of samples were stranded using 0.5 mL of sodium nitrite (10 μg/mL in 100 mM sodium citrate pH 5) at 37 °C. After incubating for 2 h, 0.5 mL of Griess reagent was poured and the absorbance (A) was recorded at 540 nm. The percentage of nitrite scavenging was computed by the subsequent formula:

$$[(A_{control} - A_{test})/A_{control}] \times 100;$$

where $A_{control}$ is absorbance of Griess reagent in the existence of sodium nitrite and A_{test} represents absorbance of Griess reagent in the existence of sodium nitrite and antioxidants. IC_{50} (the concentrations that could provide 50% nitrite inhibition) calculation was performed using the inhibition percentage/ various antioxidant concentrations graph (Tsikas, 2007).

2.13. Linoleic acid oxidation inhibition assay

Twenty μL of samples were incubated with 1.0 mL of linoleic acid emulsion at 37 °C for 24 h. Linoleic acid (40 μL), of polysorbate-20 (20 mg) and 5 mL of phosphate buffer (pH 7) were homogenized to prepare the linoleic acid emulsion. The peroxide formation during linoleic acid oxidation was determined by oxidation of ferrous ion ($FeCl_2$) to ferric ion. Ferric ion forms a complex with ammonium thiocyanate and produces ferric thiocyanate that has a maximum absorbance at 500 nm. The reduction in the absorbance at 500 nm indicates prevention of linoleic acid oxidation. The percentage of linoleic acid oxidation inhibition was expressed by the subsequent equation:

$$[(A_{control} - A_{test})/A_{control}] \times 100;$$

where $A_{control}$ = absorbance of linoleic acid oxidation solution, A_{test} = absorbance of linoleic acid oxidation solution in the presence of antioxidants. IC_{50} (the amount of antioxidant that could provide 50% linoleic acid oxidation inhibition) was computed from a graph plotting the inhibition percentage against different concentrations of antioxidant (Versace et al., 2014).

2.14. Low density lipoprotein (LDL) oxidation inhibition assay

Incubation of twenty μL of samples was performed with 1.0 mL of LDL solution (500 μg/mL in 10 mM phosphate buffered saline). Immediately, 1 mL of cupric sulfate (10 μM in 10 mM phosphate buffered saline) was poured into LDL solution and next incubated at 37 °C for 10 h. After incubation, the light absorbance was recoeded at 234 nm. The percentage of LDL oxidation inhibition was computed as follows:

$$[(A_{control} - A_{test})/A_{control}] \times 100;$$

where $A_{control}$ = absorbance of LDL plus cupric sulfate solution, A_{test} = absorbance of LDL plus cupric solution in the existence of the emulsion. IC_{50} (the effective concentration of antioxidant that could provide 50% LDL oxidation inhibition) was computed from a curve plotting the inhibition percentage against diverse concentrations of antioxidant (López-Alarcón and Denicola, 2013).

2.15. Cell harvest and 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 rinsed two times via centrifuging at 1700 g for duration of 5 min at temperature of 4 °C with PBS. Next, the cells were resuspended in 1.0 mL of RPMI-1640 culture media (Gibco) with 10% FBS (Gibco) and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin), counted and used for the following tests.

2.16. Cell viability assay

Quantification of cell viability was performed via the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium Bromide] assay. Macrophage cells were set to a density of 1.4×10^4 cells/well and planted in 96 well plates and incubated for duration of 24 h. Afterwards, cells were treated with non-cytotoxic concentration of OEO-OAW emulsion (500, 1000, 2000, 3000 ng/mL) and then incubated in 5% CO_2 for 24 h at temperature of 37 °C. Next, supernatant was eliminated and 200 µL/well of MTT solution (0.5 mg/mL in phosphate-buffered saline) was added to each well. After 4 h of incubation at 37 °C in the dark, the solution was removed and dimethyl sulfoxide (DMSO) was poured (100 µL per well). Following incubation in a shaker at 37 °C, formazan crystals were completely dissolved. Finally the absorbance was detected at 492 nm by means of an microplate reader (BioTek, USA) (Kavoosi and da Silva, 2012).

2.17. Determination of intracellular nitric oxide

Macrophage cells were seeded into a 24 well tissue culture plate (2×10^6 cells/mL, 1 mL/well) and incubated overnight (at temperature of 37 °C under humidifed air containing 5% CO_2) to allow adherence. Some wells were pretreated with non-cytotoxic concentration of OEO-OAW emulsion (500, 1000, 2000, 3000 ng/mL) 2 h prior LPS treatment (1 µg/mL, from *Escherichia coli*). After incubation for 24 h, 100 µL of culture supernatant were combined with 100 µL of Griess reagent (1% w/v sulfanylamide, 0.1% w/v naphthylethylenediamine, and 3% H_3PO_4) in 96 well plates, incubated at ambient temperature for duration of 10 min and the nitrite content was measured at 540 nm with an microplate reader (BioTek, USA). Sodium nitrite was utilized for preparing standard curve (Schmölz et al., 2017).

2.18. Determination of intracellular reactive oxygen species (ROS)

Intracellular ROS were determined with 2′, 7′- dichlorodihydrofluorescin diacetate (DCFH2-DA). Cells were added to 96-well plates (2×10^4 cell/well, 200 µL/well) and incubated for 24 h. Culturing media was eliminated and substituted with a new one. Macrophage cells were incubated in the existence or not of 1 µg/mL of LPS together with non-cytotoxic concentration of OEO-OAW emulsion (500, 1000, 2000, 3000 ng/mL) for 20 h. Then DCFH2-DA, at a last concentration of 2 µg/mL, was poured into the medium and incubated for additional 2 h in the dark. Finally, fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 528 nm with fluorescent microplate reader (Bognar et al., 2013).

2.19. Measurement of thiobarbituric acid reactive substances (TBARS)

The macrophage cells were incubated with different concentrations of OEO-OAW emulsion (500, 1000, 2000, 3000 ng/mL) and LPS (1 µg/mL) for 24 h. The cells were harvested and lysed and TBARS content was determined using thiobarbituric acid (TBA) as a choromogen and malondialdehyde as standard. Briefly, one volume of supernatants or standard malondialdehyde (50 µM in acetic acid pH 4) was mixed with one volume of thiobarbituric acid (200 µM in acetic acid; pH 4) at temperature of 95 °C for duration of 1 h. Following cooling at ambient temperature, the absorbance was recorded at 532 nm (Botsoglou et al., 1994).

2.20. Statistical analysis

All data were represented as means ± standard deviations of at least three replication tests. Analysis of variance, Mean comparisons, were performed using SAS 9.4.

Table 1
Chemical composition of *Oliveria decumbens* essential oil (OEO) and aromatic water (OAW) identified by retention index and gas chromatography and gas chromatography-mass spectrometry.

Compounds	Retention index[a]	Literature RI (Adams)	Relative percent in OEO (%)	Relative percent in OAW (%)
α-Thujene	926	924	0.4	
α-Pinene	933	932	0.3	
β-Pinene	976	974	1.8	
Myrcene	990	988	0.6	
n-Decane	1000	1000	traces (< 0.1%)	3.3
α-Phellandrene	1006	1002	0.1	
δ-3-Carene	1010	1008	traces	
α-Terpinene	1016	1014	0.6	
p-Cymene	1026	1020	**22.1**	0.6
Limonene	1027	1024	2.3	
β-Phellandrene	1029	1024	0.6	
1,8-Cineole	1031	1025	0.1	
γ-Terpinene	1059	1054	**17.8**	0.9
Terpinolene	1088	1086	0.1	
Linalool	1098	1095	traces	1.4
Borneol	1165	1165	traces	
Terpinen-4-ol	1177	1174	0.2	0.3
α-Terpineol	1190	1186	traces	0.2
n-Dodecane	1200	1200	traces	0.7
Thymol	1289	1289	**25.5**	**37.6**
Carvacrol	1296	1298	**23.1**	**52.9**
Unknown	1302		0.3	1.2
Thymol acetate	1354	1349	traces	0.1
α-Copaene	1374	1374	traces	
n-Tetradecane	1400	1400	traces	0.3
Myristicin	1520	1517	3.4	traces

[a] Retention indices (RI) were determined using a standard mixture of *n*-alkanes analyzed under the same chromatographic conditions on a HP-5 capillary column.

3. Results and discussion

3.1. Chemical composition

The OEO was characterized by oxygenated monoterpenes (48.9%), mainly phenolics, and monoterpene hydrocarbons (39.87%). The main components of OEO are listed in Table 1. The main components of OEO were thymol (25.54%), carvacrol (23.12%), *p*-cymene (22.07%) and γ-terpinene (17.80%). Notably these components are members of the same biogenetic pathway leading to phenolic monoterpenes (Vitali et al., 2016; Morshedloo et al., 2017).

This composition was quite consistent with that reported in some studies (Amin et al., 2005; Esmaeili et al., 2018; Hajimehdipoor et al., 2010; Sereshti et al., 2011) although slight quali-quantitative differences in these components were observed; they may be related to several factors suh as genetics, environment, harvesting time and essential oil preparation methods. The chemical composition of OAW from dried aerial parts of *O. decumbens* was isolated by the liquid–liquid extraction method and the solvent was removed by nitrogen gas flow. The main components of OAW were carvacrol (52.94%) and thymol (37.63%) (Table 1).

3.2. Physical properties

Conductivity, zeta potential, pH, and hydrodynamic particle size for OEO-OAW emulsion are summarized in Table 2. The product showed low PH, low conductivity and negative surface charge. The hydrodynamic particle size of emulsion was in the nano size. The low pH values could be linked to the phenolic monoterpenes thymol and carvacrol. Indeed, the ionization of the hydroxyl group of the phenol ring can release proton and reduce the pH. The conductivity of a solution

Table 2
Physical and chemical properties of *Oliveria decumbens* essential oil (OEO)-aromatic water (OAW) emulsified in polysorbate-20.

Properties	OEO-OAW
Phenol content in stock solution (μg/mL)	2500 ± 70
Phenol content in working solution (μg/mL)	1000 ± 10
pH	4.05 ± 0.05
Conductivity (μS/cm)	452 ± 8
Mobility (V/cm)	−2.26 ± 0.5
Zeta potential (mV)	−28.95 ± 1.0
Particle size (nm)	320 ± 16
Polydispersity	0.37 ± 0.08

The pH, conductivity, zeta-potential, particle size and total phenol were determined using as reported in materials and methods. The values are expressed as means ± standard deviation of three independent experiments. Mean values with different letters within a row are significantly different from Duncan test at ($p < 0.05$).

depends upon the number of charged groups and hyrophylicity/hydrophobicity index of components in the solution. The conductivity of the OEO-OAW could be due to the combination effects of hydrophobicity/hydrophilicity index and charge groups (Bakkali et al., 2008). Modification of conductivity and pH from neutral states probably can improve physical, chemical and microbial stability of the samples through preventing phase separation. The low zeta-potential (negative surface charge) of OEO-OAW is probably related to its phenol content. The pH of OEE-OAW is acidic, and at this pH some parts of the phenol compound can be ionized (Bilia et al., 2014).

Generally, the highest zeta-potential (surface charge) lead to the lowest particle charge due to an increase in electrostatic repulsion and reduction of particle aggregation. OEE-OAW showed low zeta-potential and particle size, probably related to its high purity (Bilia et al., 2014) (Fig. 1).

3.3. The radical scavenging activity of ABTS and DPPH

The OEO-OAW emulsion exhibited strong ABTS and DPPH radical scavenging acitivties, with IC_{50} values of 28 and 44 μg/mL, respectively (Table 3). This high radical scavenging capacity may be linked to the strong synergism between monoterpene hydrocarbons and oxygenated monoterpenes including phenolic monoterpenes in the mixtures of OEO and OAW. As well recognized, the phenolic compounds antioxidant

Table 3
Antioxidant capacity of *Oliveria decumbens* essential oil (OEO)-aromatic water (OAW) cocktail.

Oxidant/Radical	Antioxidant	IC_{50} (μg/mL)	Gallic acid equivalent (mg/mL)
ABTS	OEO-OAW	28 ± 2.0	175 ± 7.0
DPPH	OEO-OAW	44 ± 4.0	250 ± 10
Hydrogen peroxide	OEO-OAW	226 ± 12	482 ± 14
Hydroxyl radical	OEO-OAW	92 ± 4.0	444 ± 20
Superoxide ion	OEO-OAW	104 ± 5.0	307 ± 16
Nitric oxide	OEO-OAW	116 ± 5.0	500 ± 16
Nitrite	OEO-OAW	75 ± 6.0	425 ± 11
Linoleic acid oxide	OEO-OAW	192 ± 8.0	340 ± 16
LDL oxide	OEO-OAW	130 ± 8.0	250 ± 12
Malondialdehyde	OEO-OAW	122 ± 7.0	434 ± 14

The concentrations OEO-OAW 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. Gallic acid equivalents were calculated by dividing IC_{50} of Gallic acid for radical or oxidant inhibition to IC_{50} of OEO-OAW for inhibition of radical or oxidant.

capacity is mostly because of their redox features, which allow them to play role as reducing agents, hydrogen donors, metal chelators and singlet oxygen quenchers. The hydrogen atom corresponding to phenol compounds hydroxyl groups can be contributed to free radicals, so avoiding oxidization of other compounds (Pérez-Rosés et al., 2016; Prakash et al., 2007; Wojdylo et al., 2007). Essential oils display some modes of antioxidant activity like preventing chain initiation, free radical scavenging, reducing agent, peroxides termination, preventing continuous hydrogen abstraction, quenchers of singlet oxygen production and binding of metal ions transition. Furthermore, essential oils avoid oxidation of lipid in food and biological spicemens, thus protecting membranes and tissues against oxidative damages (Kumar and Rawat, 2013; Lü et al., 2010).

3.4. In vitro ROS scavenging activity and reduction of ROS production in LPS-stimulated macrophages

The OEO-OAW emulsion exhibited strong superoxide, H_2O_2 and hydroxyl radical scavenging activity (Table 3) that may be reflected the synergism between its main constituents as reported in some investigations (Suntres et al., 2015; Vrchovska et al., 2006). *Ex vivo*

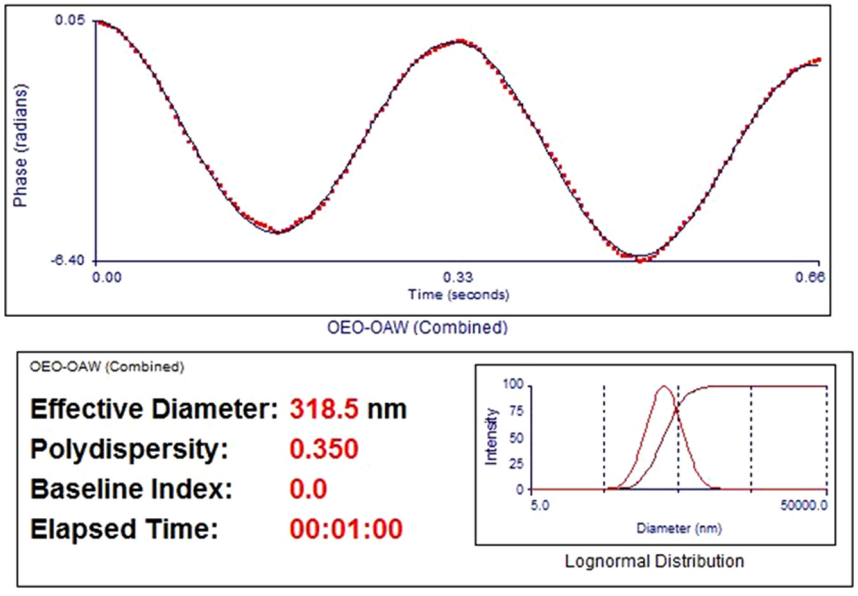

Fig. 1. Zeta-potential and particle size of *Oliveria decumbens* aromatic water emulsified in polysorbate-20.

Table 4
Intracellular antioxidant activity of oleveria essential oil-aromatic water emulsion (OEO-OAW) in lipopolysaccharide (LPS)-stimulated macrophages by reduction of nitric oxide (NO), reactive oxygen species (ROS) and thiobarbituric acid reactive substances (TBARS).

	ROS (fluorescence intensity)	NO (nM)	TBARS (nM)
Control	1409 ± 223	21 ± 2.6	0.33 ± 0.09
LPS	2055 ± 265	33 ± 3.0	0.66 ± 0.10
LPS + OEO (500 ng/mL)	1518 ± 220	21 ± 2.5	0.35 ± 0.03
LPS + OEO (1000 ng/mL)	1099 ± 88	20 ± 2.0	0.27 ± 0.08
LPS + OEO (2000 ng/mL)	1035 ± 170	17 ± 1.6	0.35 ± 0.06
LPS + OEO(3000 ng/mL)	872 ± 230	15 ± 1.7	0.28 ± 0.05

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

analysis demonstrated that LPS stimulation of macrophages increased ROS levels. Pretreatment with OEO-OAW emulsion (2000–3000 ng/mL) significantly decreased ROS production in LPS-treated cells, representing its inhibitory effects against ROS generation (Table 4).

The inhibitory effects of some essential oils such as those from *Mentha longifolia* (Karimian et al., 2013) and *Achillea millefolium* (Chou et al., 2013) on ROS production in LPS-stimulated macrophages were previously observed. Accordingly, the suppression of ROS generation in macrophages by the OEO-OAW emulsion may be related to superoxide/hydroxyl radical scavenging activity and suppression of NOX expression (Xiao et al., 2017). In macrophages superoxide formation is controlled by NADPH oxidase (NOX). This multi-component enzyme contains some cytosolic components comprising p67phox, p91phox, p40phox, p47phox and the small Rho G protein, which collect on the cellular membrane for activating the enzyme. The active NOX enzyme changes molecular oxygen to superoxide anion via a one-electron transfer (Bedard and Krause, 2007). Superoxide anion then transformed to H_2O_2 using superoxide dismutase·H_2O_2 is changed to the hydroxyl radical through the Fenton or Haber-Weiss reactions in the existence of iron. The hydroxyl radical is known to be one of the strongest radicals since it is responsible for generating other dangerous radicals. All major macromolecules comprising lipids, proteins, and especially mitochondrial and nuclear DNA are targets for these toxic radicals (Ray et al., 2012). Therefore, the damages induced by high levels of superoxide and hydroxyl radicals may result in pathogenesis of numerous illnesses like myocardial reperfusion injury and endothelial cell dysfunction (Circu and Aw, 2010). In this research, the crucial role of OEO-OAW emulsion in scavenging superoxide and hydroxyl radicals and reduction of ROS in stimulated macrophages was observed. Therefore this formulation may be employed in treating oxidative stress and associated illnesses.

3.5. *In vitro NO scavenging activity and reduction of NO production in LPS-stimulated macrophages*

The NO and nitrite scavenging capacity of OEO-OAW emulsion was noteworthy and this effect may be ascribed to the synergism between monoterpene hydrocarbons and phenolic monoterpenes occurring in the mixture (Baliga et al., 2003; Suntres et al., 2015). *Ex vivo* analysis indicated that LPS stimulation of macrophages increases NO levels. Pretreatment with emulsion significantly decreased the NO production, indicating the inhibitory effect on NO generation (Table 4). The modulatory effects of *Tagetes minuta* essential oil (Karimian et al., 2014) and *A. millefolium* essential oil (Chou et al., 2013) on NO production in LPS-stimulated macrophages was also reported. NO in macrophages is formed through activating inducible nitric oxide synthase (iNOS). This enzyme can generate high levels of NO following stimulating with bacterial endotoxins (LPS) or different pro-inflammatory cytokines like TNF-α. In response to inflammatory stimuli such as LPS, macrophages secrete a wide range of inflammatory mediators such as TNF-α. The synthesis of TNF-α cytokine is vital for inducing NO production in LPS-stimulated macrophages (Zhu et al., 2018). Although NO is known as an important mediator of macrophage function and is associated with the bactericidal and tumoricidal activity, NO overproduction has been observed in cytokine-induced circulatory shock. In these conditions, excess generation of NO results in systemic vasodilation and fatal decrease in blood pressure and inadequate delivery of blood oxygen supply to vital organs, which consequently leads to multiple organ failure (Fernandes and Assreuy, 2008). NO can also form peroxynitrite, a powerful oxidant and nitrating agent, by reaction with superoxide anion. Peroxynitrite can damage DNA, protein and lipid macromolecules and therefore lead to endothelial dysfunction and associated illnesses like diabetes, hypercholesterolemia and hypertension (Weiming et al., 2002). Unsaturated fatty acids can be nitrated by nitrite. These nitro-fatty acids are known as a group of endogenous anti-inflammatory signaling mediators (Fernandes and Assreuy, 2008). NO and oxygen compete for the same binding site in cytochrome C oxidase. This condition triggers intracellular signaling reactions, including an increase in free oxygen level and over producing mitochondrial ROS, which results in oxidative stress, sereious damages including damage of DNA and consequently the growth of some diseases like cancer (Fernandes and Assreuy, 2008). The suppression of NO in macrophages may be related to NO scavenging activity or suppression of NOS expression/activity or pro-inflammatory cytokines expression by OEO-OAW. Therefore, the effects of OEO-OAW emulsion on the NOS expression /activity or pro-inflammatory cytokines expression should be determined in future studies. The NO scavenging activity of OEO-OAW emulsion observed in our research, suggests that this product is valuable in reducing oxidative stress-induced damages to biological membranes and can defend various tissues against cumulative oxidative stress using NO, nitrite and peroxynitrite.

3.6. *In vitro lipid oxidation inhibition and reduction of lipid peroxidation in LPS-stimulated macrophages*

The OEO-OAW emulsion exhibited a strong inhibition of LDL oxidation, linoleic acid oxidation and malondialdehyde (MDA) production. *Ex vivo* analysis showed that the TBARS (a lipid peroxidation indicator) levels in LPS-stimulated macrophages exposed to emulsion were lower than that in LPS-treated cells (Table 4). Therefore these findings show that OEO-OAW emulsion can strongly suppress the lipid peroxidation in LPS-stimulated macrophages (Xiao et al., 2017). Yanishlieva et al. (1999) found that the antioxidant performance of thymol-rich essential oil and thymol in two lipid systems containing a purified fraction of triacylglycerols of sunflower oil and lard. According to the results of the investigation of thymol and carvacrol antioxidant activity in roasted sunflower seeds, Quiroga et al. (2015) suggested that these monoterpenoids inhibit the creation of oxidative corrosion compounds such as hexanal and peroxides and undesirable off-flavors such as cardboard and oxidized flavors and consequently are effective in protection of food products. Lipid peroxidation is a toxic metabolic process in biological systems, which is implicated in various diseases. The phospholipids of plasma and organelle membrane containing polyunsaturated fatty acids are subject to lipid peroxidation. These lipids are susceptible to oxidation by hydroxyl radical, which can lead to membrane damage (Yanishlieva-Maslarova and Marinova, 2007). The consequences of lipid peroxidation including increased membrane permeability, ultimately lead to an ions influx, mainly calcium, with the cell edema. Subsequent agglomeration of calcium in the mitochondria disrupts mitochondrial membrane potential and may trigger necrotic and apoptotic cell death (Gutteridge and Halliwell, 1990). The linoleic acid and LDL oxidation inhibition and MDA scavenging activity as well as the reduction of TBARS in LPS-stimulated macrophages by the OEO-OAW emulsion confirmed its beneficial role in prevention of membrane damages and lipid peroxidation.

4. Conclusion

The present study supported the use of *O. decumbens* essential oil and aromatic water in the preparation of antioxidant formulations to be used as food preservatives as well as in medicine to prevent oxidative stress and related diseases. The antioxidant capacity exhibited by *O. decumbens* emulsion in both chemical assays and living cells may be attributed to the strong synergism of its constituents, mainly monoterpene hydrocarbons and phenolic monoterpenes. The main effect produced in cells was a decrease in the production of NO and ROS and reduction of TBARS level.

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