

ORIGINAL ARTICLE

Chemical composition and protective effect of oregano (*Origanum heracleoticum* L.) ethanolic extract on oxidative damage and on inhibition of NO in LPS-stimulated RAW 264.7 macrophages

Filomena Conforti¹, Mariangela Marrelli¹, Federica Menichini¹, Rosa Tundis¹, Giancarlo A. Statti¹, Umberto Solimene², and Francesco Menichini

¹Department of Pharmaceutical Sciences, Faculty of Pharmacy, Nutrition and Health Sciences, University of Calabria, Italy, and ²Department of Human Morphology and Biomedical Sciences, University of Milano, Italy

Abstract

The present study shows the chemical profile and the *in vitro* properties (antioxidant and inhibition of nitric oxide production) of the *Origanum heracleoticum* L. (Lamiaceae). The ethanolic extract of the aerial parts is characterized by terpenes and fatty acids. The extract, with high total phenol and flavonoid content, showed a significant radical-scavenging activity (IC₅₀ value of 12.8 µg/mL) using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) test and an interesting antioxidant activity with the β-carotene bleaching test (IC₅₀ values of 12.9 and 14.1 µg/mL at 30 and 60 min of incubation, respectively). The test for the inhibition of NO production, performed using the murine monocytic macrophage RAW 264.7 cell line, showed that the extract had significant activity with an IC₅₀ value of 108.5 µg/mL. The cytotoxic effect of *O. heracleoticum* extract in presence of lipopolysaccharide (LPS) (1 µg/mL) was evaluated but found to be negligible.

Keywords: Antioxidant activity, Lamiaceae, aromatic plant, inhibition of NO production, *Origanum heracleoticum*, radical-scavenging activity, terpenes

Introduction

In traditional societies, nutrition and healthcare are strongly interconnected and many plants have been consumed both as food and for medicinal purposes.^{1,2} The consumption of noncultivated botanicals plays a central role in the diet, but very few ethnopharmacological and phytopharmacological studies have dealt exhaustively with the potential health benefits of such diets. The number of studies on the antioxidant properties of specific plant foods and their phenolic constituents has become very impressive.^{3–5} Oxidative stress is intricately linked with aging-related diseases (ARD), and longevity has been related with variants of the Mediterranean diet and specifically with a high consumption of olive oil, fruits, and vegetables.⁶

It is commonly accepted that in a situation of oxidative stress, reactive oxygen species (ROS) such as superoxide (O₂⁻, OOH⁻), hydroxyl (OH[·]), and peroxy (ROO[·]) radicals are generated. ROS play an important role in the pathogenesis of various serious diseases, such as neurodegenerative disorders, cancer, cardiovascular diseases, atherosclerosis, cataracts, and inflammation.^{7,8} Several anti-inflammatory drugs have recently been shown to have an antioxidant and/or radical-scavenging mechanism as part of their activity.⁹ Thus the neutralization of free radicals by antioxidants and radical scavengers can attenuate inflammation.¹⁰

Nitric oxide (NO) is a diatomic free radical produced from L-arginine by constitutive and inducible nitric oxide synthase (cNOS and iNOS) in numerous mammalian

Address for Correspondence: Dr. Filomena Conforti, Department of Pharmaceutical Sciences, Faculty of Pharmacy, Nutritional and Health Sciences, University of Calabria, I-87036 Rende (CS), Italy. E-mail: filomena.conforti@unical.it

(Received 12 May 2010; revised 19 August 2010; accepted 25 August 2010)

cells and tissues. NO, superoxide (O_2^-), and their reaction product peroxynitrite ($ONOO^-$) may be generated in excess during the host response against viral and anti-bacterial infections and contribute to some pathogenesis by promoting oxidative stress, tissue injury, and even cancer.¹¹

The vernacular name "oregano" is attributed to a vast number of species in over a dozen genera of different families. Today, it is generally accepted that oregano as a matter of fact is a characteristic flavour produced by a number of plant species that yield carvacrol-rich essential oils.¹²

Oregano plays a primary role among temperate culinary herbs in world trade. *Origanum vulgare* L. is the most variable species of the genus and the only one commonly known as oregano in most countries. *Origanum heracleoticum* L. is endemic to the Mediterranean area.¹³ Oregano is found in many areas and is a perennial shrub native to the dry, rocky calcareous soils in the mountainous area of southern Europe and Southwest Asia. It is also cultivated because of its uses as an herb and its therapeutic properties, which have been known since ancient times.¹⁴

The aim of this work was to evaluate the chemical composition and the antioxidant activities of *O. heracleoticum* extract using 2,2-diphenyl-1-picrylhydrazyl (DPPH) test and β -carotene bleaching test. Moreover, the inhibition of NO production in the murine monocytic macrophage cell line RAW 264.7 of *O. heracleoticum* extract was performed to evaluate the anti-inflammatory activity.

Materials and methods

Chemicals

Ethanol, dimethyl sulphoxide (DMSO), sodium carbonate, sodium nitrite, and NaOH were obtained from VWR International s.r.l. (Milan, Italy). Ascorbic acid, β -carotene, propyl gallate, linoleic acid, Tween-20, DPPH, Griess reagent (1% sulphonamide and 0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride in 2.5% H_3PO_4), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Dulbecco's modified Eagle's medium (DMEM), L-glutamine, fetal bovine serum (FBS), antibiotic/antimicotic solution (penicillin/streptomycin), lipopolysaccharide (LPS), indomethacin, Folin-Ciocalteu reagent, $AlCl_3$, quercetin, and chlorogenic acid were obtained from Sigma-Aldrich S.p.A. (Milan, Italy). All other reagents, of analytical grade, were Carlo Erba products (Milan, Italy).

Plant material and sample preparation

The aerial parts of *O. heracleoticum* L. used in this study were collected in August 2007 in Calabria (Southern Italy) and authenticated by Dr. Dimitar Uzunov, Natural History Museum of Calabria and Botanic Garden, University of Calabria (CLU; Italy). A voucher specimen is deposited in the Botany Department Herbarium at the CLU.

The aerial parts (251 g) were exhaustively extracted with ethanol ($3 \times 2.50L$) through maceration ($144h \times 3$

times). The resultant total extract was filtered, dried under reduced pressure (37 g), and analyzed by gas chromatography-mass spectrometry (GC-MS).

GC-MS analyses

To determine the nonpolar composition of the *O. heracleoticum* ethanolic extract, analytical GC was carried out on a Hewlett-Packard 6890 gas chromatograph equipped with a SE30 capillary column (30 m length, 0.25 mm i.d., 0.25 μm film thickness) and interfaced with a Hewlett-Packard 5973 Mass Selective. Ionization of the sample components was performed in Electron Impact (EI, 70 eV) mode, with helium as the carrier gas. Column temperature was initially kept at 50°C for 5 min, then gradually increased to 250°C at 5°C/min rate and finally held for 10 min at 250°C. Injector and detector were maintained at 250°C and 280°C, respectively. Constituents of *O. heracleoticum* extract were identified by comparison of their mass spectra with those stored in Wiley 138 and NIST 98 libraries or with mass spectra from literature and by comparison of their GC retention times with those of standards available in our laboratories.¹⁵

Determination of total phenol content

The amount of total phenolics was estimated spectrophotometrically in the *O. heracleoticum* ethanolic extract using Folin-Ciocalteu reagent.¹⁶ A total of 100 μL of the extract were diluted with 2 mL of distilled water, 0.2 mL of Folin-Ciocalteu phenol reagent was added, and the flasks were shaken vigorously. Subsequently, 1.0 mL of 15% sodium carbonate solution was added and the mixtures were mixed thoroughly again. The mixtures were allowed to stand for 2 h protected from light. The absorbance of the blue colour produced was measured with a spectrophotometer (UV-Vis Jenway 6003) at 765 nm. The levels of total phenolics content were calculated in triplicate on the basis of a standard curve obtained using chlorogenic acid as standard and were expressed in mg/g of extract.

Determination of total flavonoid content

The flavonoid content was determined spectrophotometrically using a method based on the formation of a flavonoid-aluminium complex.¹⁷ One millilitre of the extract was added to a 10-mL volumetric flask. Distilled water was added to make a volume of 5 mL. At time 0, 0.3 mL of 5% (w/v) sodium nitrite was added to the flask. After 5 min, 0.6 mL of 10% (w/v) $AlCl_3$ was added and then at 6 min 2 mL of 1 M NaOH were also added to the mixture, followed by the addition of 2.1 mL distilled water. Absorbance at 510 nm was measured immediately. Quercetin was chosen as a standard and the levels of total flavonoid content were determined in triplicate and expressed in mg/g of extract.

DPPH assay

Free radical-scavenging activity was determined using a method based on the reduction of a methanolic

solution of the coloured free radical DPPH. This experimental procedure was adapted from Wang et al.¹⁸ In an ethanol solution of DPPH radical (final concentration was 1.0×10^{-4} M), the extract at different concentrations (5–100 $\mu\text{g/mL}$) was added. The reaction mixtures were shaken vigorously and then kept in the dark for 30 min. The absorbance of the resulting solutions was measured in 1 cm cuvettes, using a PerkinElmer Lambda 40 UV/VIS spectrophotometer at 517 nm, against blank without DPPH. A decrease in the absorbance of the DPPH solution indicates an increase of DPPH radical-scavenging activity. This activity is given as % DPPH radical scavenging, which is calculated in the equation:

$$\% \text{DPPH radical scavenging} = \left[1 - \left(\frac{\text{sample absorbance with DPPH} - \text{sample absorbance without DPPH}}{\text{control absorbance}} \right) \right] \times 100$$

The DPPH solution without sample solution was used as control. All tests were run in triplicate and the mean values were calculated. Ascorbic acid was used as positive control.

β -Carotene bleaching–linoleic acid assay

Antioxidant activity was determined using the β -carotene bleaching test¹⁹ with some modifications. In brief, 1 mL of β -carotene solution (0.2 mg/mL in chloroform) was added to 0.02 mL of linoleic acid and 0.2 mL of 100% Tween-20. Chloroform was evaporated and the mixture was diluted with 100 mL of water, 5 mL of the emulsion were transferred into different test tubes containing 0.2 mL of sample in 70% ethanol at different concentrations. Propyl gallate at the same concentration was used as positive control. The tubes were then gently shaken and placed at 45°C in a water bath for 60 min. The absorbances of the samples, standard and control, were measured at 470 nm using a PerkinElmer Lambda 40 UV/Vis spectrophotometer against a blank, consisting of an emulsion without β -carotene. The measurement was carried out at initial time ($t=0$) and successively at 30 and 60 min. All samples were assayed in triplicate and the mean value calculated. The antioxidant activity was measured in terms of successful preventing of β -carotene bleaching by using the following equation:

$$\text{Antioxidant activity} = \left[1 - \frac{(A_0 - A_t)}{(A_0 - A_t \cdot)} \right] \times 100$$

where A_0 and $A_0 \cdot$ are the absorbance values measured at the initial incubation time for samples/standard and control, respectively, whereas A_t and $A_t \cdot$ are the absorbance values measure in the samples/standard and control, respectively, at $t=30$ min and $t=60$ min.

Cell culture

The murine monocytic macrophage cell line RAW 264.7 (European Collection of Cell Cultures, London, UK) was grown in plastic culture flask in DMEM with

L-glutamine supplemented with 10% FBS and 1% antibiotic/antimycotic solution (penicillin/streptomycin) under 5% CO_2 at 37°C. After 4–5 days, cells were removed from culture flask by scraping and centrifugating for 10 min under 1500 rpm. The medium was then removed and the cells were resuspended with fresh DMEM. Cell counts and viability were performed using a standard trypan blue cell counting technique. The cell concentration was adjusted to 1×10^6 cells/mL in the same medium. One hundred microlitres of the above concentration were cultured in 96-well plate for 1 day to become nearly confluent. Concentrations ranging from 10 to 100 $\mu\text{g/mL}$ of the samples were prepared from the stock solutions by serial dilution in DMEM to give a volume of 100 μL in each well of a microtiter plate (96-well). Then cells were cultured with vehicle, *O. heracleoticum* EtOH extract in the presence of 1 $\mu\text{g/mL}$ LPS for 24 h.

Assay for cytotoxic activity

Cytotoxicity was determined using the MTT assay reported by Tubaro et al.²⁰ with some modifications. The assay for each sample analyzed was performed in triplicate and the culture plates were kept at 37°C with 5% (v/v) CO_2 for 1 day. After 24 h of incubation, 100 μL of medium was removed from each well. Subsequently, 100 μL of 0.5% w/v MTT (Sigma, Italy), dissolved in phosphate-buffered saline, was added to each well and allowed to incubate for a further 4 h. After 4 h of incubation, 100 μL of DMSO was added to each well to dissolve the formazan crystals. Absorbance values at 550 nm were measured with a microplate reader (GDV DV 990 B/V, Roma, Italy). Cytotoxicity was expressed as IC_{50} , which is the concentration to reduce the absorbance of treated cells by 50% with reference to the control (untreated cells).

Inhibition of NO production in LPS-stimulated RAW 264.7 cells

The presence of nitrite, a stable oxidized product of NO, was determined in cell culture media by Griess reagent (1% sulphonamide and 0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride in 2.5% H_3PO_4).²¹ One hundred microlitres of cell culture supernatant were removed and combined with 100 μL of Griess reagent in a 96-well plate followed by spectrophotometric measurement at 550 nm using a microplate reader (GDV DV 990 B/V, Rome, Italy). Nitrite concentration in the supernatants was determined by comparison with a sodium nitrite standard curve.

Statistical analysis

All experiments were carried out in triplicate. Data were expressed as means \pm SD. The concentration giving 50% inhibition (IC_{50}) was calculated by nonlinear regression with the use of Prism Graphpad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA, USA). The

dose–response curve was obtained by plotting the percentage inhibition versus concentration.

Results and discussion

After maceration with ethanol (99.8%), the total phenol and flavonoid content of *O. heracleoticum* aerial parts extract (yield of 15% relative to dry weight material) was evaluated. The *O. heracleoticum* extract showed a total phenol content, determined by Folin-Ciocolteu assay, of 38.7 mg chlorogenic acid equivalent/g of extract. The flavonoid content, determined using a method based on the formation of a flavonoid–aluminium complex, was 14.4 mg quercetin equivalent/g of extract.

Previously, Tsimogiannis et al.²² examined *O. heracleoticum* as potential source of phenolic antioxidants. The components of the plant were extracted in a Soxhlet apparatus sequentially with solvents of increasing polarity, and in particular with petroleum ether, diethyl ether, and ethanol.²² The *O. heracleoticum* ethanol extract was found to be characterized by rosmarinic acid, apigenin, apigenin glycoside, and carvacrol as major components. According to Vekiari et al.,²³ flavonoid constituents of oregano (*O. vulgare*) are apigenin, eriodictyol, taxifolin, and dihydrokaempferol. Apigenin and luteolin were detected in acetone extracts of Greek oregano (*O. vulgare* ssp. *hirtum*) by Exarchou et al.²⁴ Justesen²⁵ and Justesen and Knuthsen²⁶ identified rosmarinic acid, luteolin, luteolin glycoside, apigenin, apigeninacetyl-diglycoside, and diosmetin-acetyl-glucuronide.

In order to identify putative active nonpolar compounds present within the *O. heracleoticum* extract, the GC-MS analyses were realized. The GC-MS analysis revealed that the *O. heracleoticum* extract is characterized by the presence of four terpenes, namely *p*-cymene, thymol, carvacrol, and neophytadiene, and nine fatty acids and methyl esters, namely methyl palmitoleate, methyl palmitate, myristic acid, methyl heptadecanoate, methyl linoleate, methyl linolenate, methyl stearate, methyl arachidate, and methyl behenate, as major nonpolar constituents (Figure 1).

In addition to the above-referred studies on the polyphenols constituents, oregano was previously analyzed especially for its essential oil. The chemical analysis has shown its constituents to be principally carvacrol and thymol.^{27–29} Vokou et al.,³⁰ examining essential oils of *O. vulgare* ssp. *hirtum* from 23 localities scattered all over Greece, found that in spite of the high variability of individual compounds in the essential oil, the sum of carvacrol, thymol, *p*-cymene, and γ -terpinene was stable amounting to >80%. Carvacrol and thymol appear to be the main components of all *O. vulgare* ssp.,^{31–33} although their content may vary considerably among different populations.^{30,33}

The model of scavenging stable DPPH free radicals can be used to evaluate antioxidant activity in a relatively short time. The absorbance decreases as a result of a colour change from purple to yellow as the radical is

scavenged by antioxidants through donation of hydrogen to form the stable DPPH-H molecule,³⁴ although a recent work suggests that, on the basis of kinetic analysis of the reaction between phenols and DPPH, the reaction in fact behaves like a single electron-transfer reaction.³⁵ It was found that the rate-determining step for this reaction consists of a fast electron-transfer process from the phenoxide anions to DPPH. The hydrogen atom abstraction from the neutral ArOH by DPPH becomes a marginal reaction path, because it occurs very slowly in strong hydrogen-bond-accepting solvents, such as methanol and ethanol. The scavenging effects of extract on DPPH were examined at different concentrations (range between 5 and 1000 $\mu\text{g/mL}$). The extract was able to reduce the stable free radical DPPH to the yellow-coloured DPPH with an IC_{50} value of 12.8 $\mu\text{g/mL}$ (Table 1 and Figure 2).

A second test, oriented towards lipophilic antioxidants, was selected. In the β -carotene/linoleic acid model system, β -carotene undergoes rapid discoloration in the absence of an antioxidant. Ethanol extract of *O. heracleoticum* was able to inhibit the discoloration of β -carotene with IC_{50} values of 12.9 and 14.1 at 30 min and 60 min of incubation, respectively (Table 1 and Figure 3). As reference, IC_{50} of propyl gallate was 1 $\mu\text{g/mL}$, both after 30 and 60 min incubation. Membrane lipids are rich in unsaturated fatty acids that are most susceptible to oxidative processes. Especially, linoleic acid and arachidonic acid are major targets of lipid peroxidation. Radical chain reactions are widely accepted as a common mechanism of lipid peroxidation and it is generally thought that the inhibition of lipid peroxidation by antioxidants may be due to their free radical-scavenging activities. Lipophilic radical scavengers may directly react and quench peroxide radicals to reduce the peroxidation chain reaction and improve the quality and stability of biological membranes and food products.

Several studies evaluated the relationships between antioxidant activity of plant products and their phenolic content. Some authors found a correlation between the phenolic content and the antioxidant activity, whereas others found no relationship. Velioglu et al.³⁶ reported a high correspondence between total phenolic content and antioxidant activity (using β -carotene bleaching method) in selected fruits, vegetables, and grain products. On the other hand, no correlation between antioxidant activity (using methyl linoleate oxidation assay) and phenolic content was observed by Kähkönen et al.³⁷ on some plant extracts containing phenolic compounds.

NO, which is derived from the oxidation of L-arginine through three isoforms of NOS, namely neuronal (nNOS), endothelial (eNOS), and iNOS, is recognized as a mediator and regulator in pathological reactions, especially in acute inflammatory responses.³⁸ High levels of NO cause a variety of pathophysiological processes including inflammation³⁹ and carcinogenesis.⁴⁰ iNOS mainly exists in macrophages, it is expressed by stimulation with endotoxins, tumor necrosis factors, or LPS.

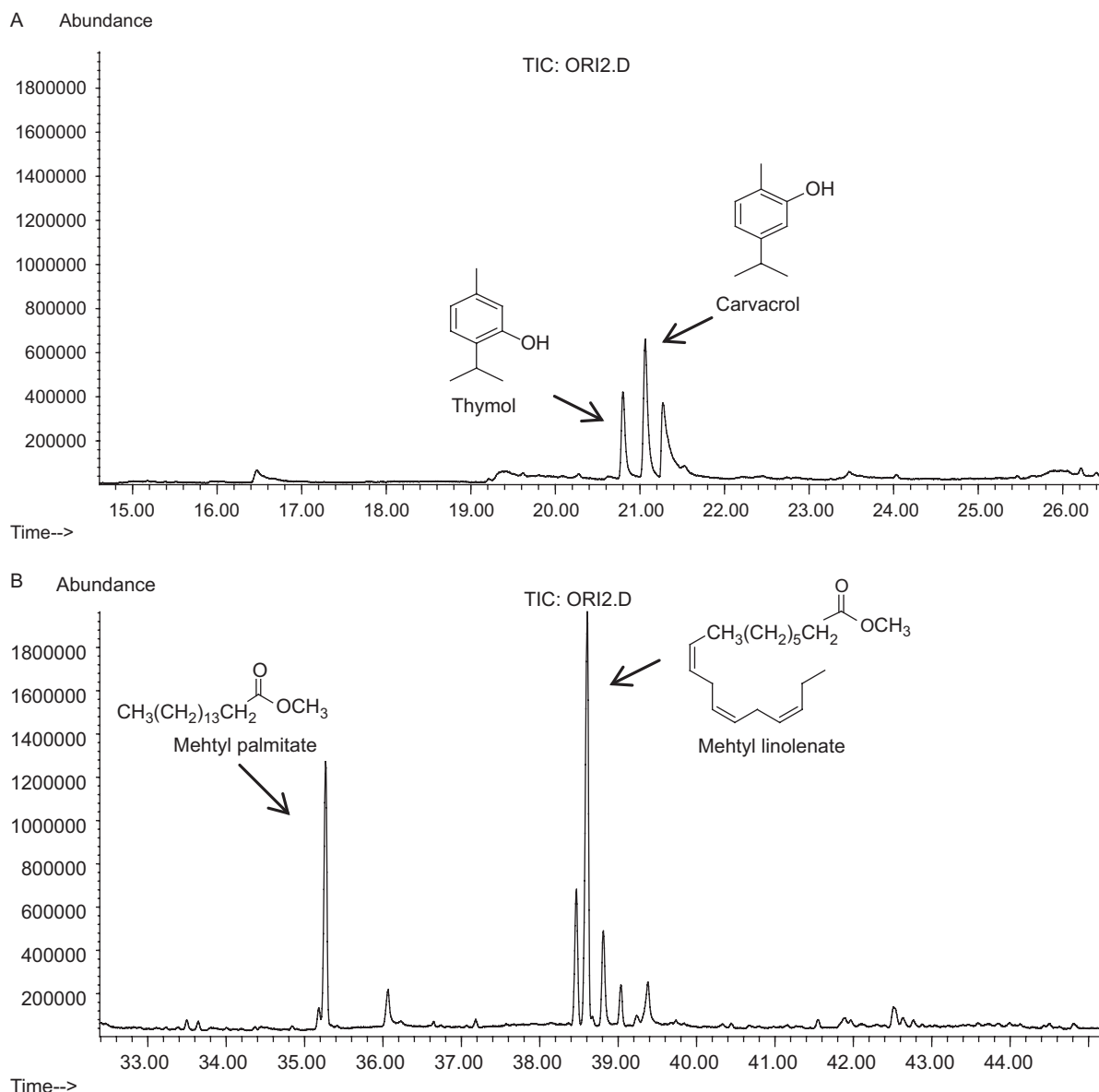


Figure 1. Chromatograms of *O. heracleoticum* extract and structures of the major components: (A) terpenes and (B) fatty acids.

Table 1. IC₅₀ values of antioxidant and NO production inhibition activities of EtOH extract from oregano.

	IC ₅₀ (μg/mL) ^a
<i>DPPH assay</i>	12.8 ± 0.9
Ascorbic acid ^b	2.0 ± 0.01
<i>β-Carotene bleaching test</i>	
30 min of incubation	12.9 ± 0.8
60 min of incubation	14.1 ± 0.8
Propyl gallate ^b	1 ± 0.01
<i>Inhibition of NO production</i>	108.5 ± 1.6
Indomethacin ^b	52.8 ± 1.2

^aData are mean ± SD (*n* = 3).

^bPositive control.

Proinflammatory agents, such as LPS, can significantly increase NO production in macrophages through activation of iNOS.^{41,42} In this study, the NO inhibitory activity

of ethanolic extract of *O. heracleoticum* was evaluated by using a LPS-stimulated RAW 264.7 cell assay. The treatment of RAW 264.7 macrophages with LPS (1 μg/mL) for 24 h induces NO production, which can be quantified by utilizing the chromogenic Griess reaction, which measures the accumulation of nitrite, a stable metabolite of NO. The beneficial effect of *O. heracleoticum* extract on the inhibition of production of inflammatory mediators in macrophages can be mediated through oxidative degradation of products of phagocytes, such as O²⁻ and HOCl. As shown in Figure 4, incubation of RAW 264.7 cells with *O. heracleoticum* extract induced a significant inhibitory effect on the LPS-induced nitrite production. The EtOH extract of *O. heracleoticum* showed significant inhibition of LPS-induced NO production in RAW 264.7 cells in a dose-dependent manner, with an IC₅₀ value of 108.5 μg/mL (Table 1 and Figure 4). Any cytotoxic

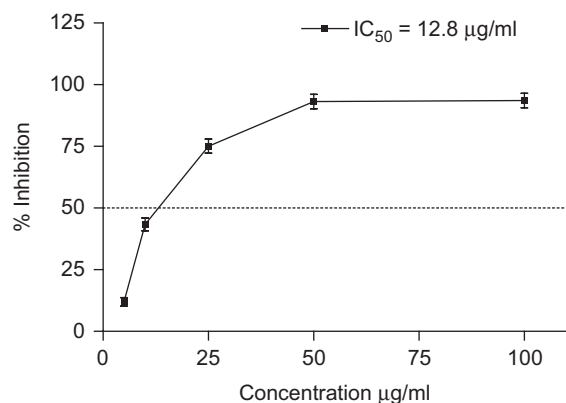


Figure 2. Free radical-scavenging activity of *O. heracleoticum* extract. Data are mean \pm SD ($n=3$). Ascorbic acid (IC_{50} value of 2 $\mu\text{g}/\text{mL}$) was used as positive control.

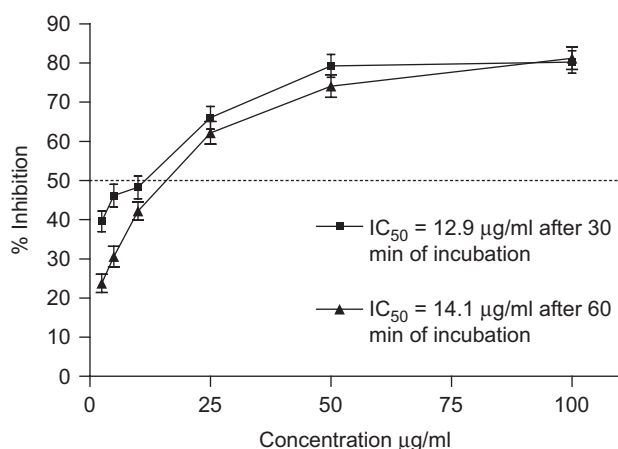


Figure 3. Lipid peroxidation inhibition using β -carotene-linoleic acid system after 30 and 60 min of incubation of ethanolic extract of *O. heracleoticum*. Data are mean \pm SD ($n=3$). Propyl gallate ($IC_{50} = 1 \mu\text{g}/\text{mL}$) was used as positive control.

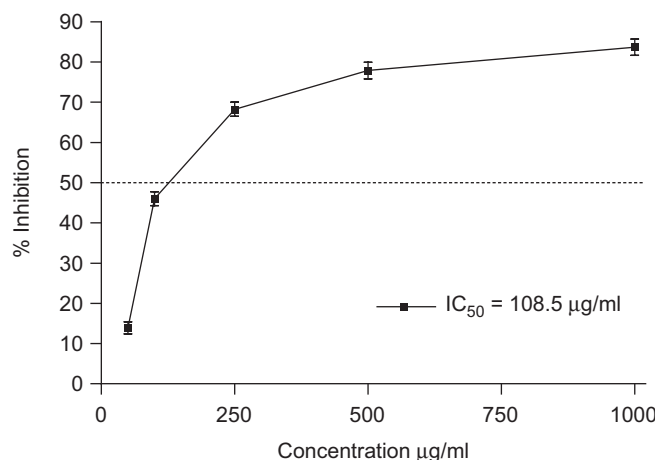


Figure 4. Inhibition of NO production in the murine monocytic macrophage cell line RAW 264.7 of *O. heracleoticum* extract. Data are mean \pm SD ($n=3$). Indomethacin (IC_{50} value of 52.8 $\mu\text{g}/\text{mL}$) was used as positive control.

effect of *O. heracleoticum* EtOH extract in presence of LPS (1 $\mu\text{g}/\text{mL}$) was also evaluated. *O. heracleoticum* extract did not show any cytotoxicity up to 250 $\mu\text{g}/\text{mL}$ concentration.

Previous studies showed that thymol and carvacrol inhibit the peroxidation of phospholipids liposomes and these compounds possess useful antioxidant properties.^{43,44} Carvacrol also showed inhibitory activity on NO production by human leucocytes, whereas thymol, an isomer of carvacrol, showed no activity.⁴⁵ Among polyphenol compounds, flavonoid such as apigenin was also markedly active inhibitors of free radicals and of transcriptional activation of iNOS.^{46,47}

Conclusions

Oxidative damage may initiate and promote the progression of a number of chronic diseases, including inflammation. The present work showed for the first time the *in vitro* activity of *O. heracleoticum* ethanol extract. Further, *in vivo* investigations are needed for a possible usefulness of this extract in the treatment of inflammation. In this study, we have demonstrated that the EtOH extract of *O. heracleoticum* exhibited significant antioxidant activity and an inhibitory effect on NO production (an inflammatory mediator) in macrophages. The observed *in vitro* activities suggest that the investigated plant extract might also exert *in vivo* protective effects against oxidative and free radical injuries occurring in different pathological conditions. Therefore, we propose here the potential benefits of *O. heracleoticum* extract on the basis of the phytochemical characteristics and the observed bioactive properties. The antioxidant and anti-inflammatory properties of naturally occurring compounds appear to contribute to their chemopreventive or chemoprotective activity.

The anti-inflammatory activities of *O. heracleoticum* EtOH extract was evaluated to obtain an insight into

the beneficial effects of this plant species in conditions related to inflammation, reduced risk for cardiovascular diseases, and cancer prevention by acting as anti-inflammatory agents. Further studies of the plant extracts and/or the identified compounds from *O. heracleoticum* on the pharmacokinetics or mode of action on mechanisms of chemopreventive properties are warranted. Also, the extraction technique should be investigated more widely, particularly in view of the application of supercritical fluids. Another point that should be strongly evaluated is the use of emulsions instead of solution in real applications, with the aim to prevent degradation of the extract activity due to oxygen exposure. In conclusion, this work reveals that *O. heracleoticum* can be an interesting source of anti-inflammatory and antioxidant principles with a potential use in different fields (food, cosmetics, pharmaceutical).

Declaration of interest

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