Food and Chemical Toxicology 48 (2010) 1568–1575

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Supercritical fluid extraction of oregano (*Origanum vulgare*) essentials oils: Anti-inflammatory properties based on cytokine response on THP-1 macrophages

A. Ocaña-Fuentes *, E. Arranz-Gutiérrez, F.J. Señorans, G. Reglero

Departamento de Química- Física Aplicada, Área de Tecnología de los Alimentos, Facultad Ciencias, Universidad Autónoma de Madrid, C/Fco Tomás y Valiente 7, Madrid E-28049, Spain

ARTICLE INFO

Article history:
Received 1 February 2010
Accepted 17 March 2010

Keywords:
Supercritical extraction
Oregano
Anti-inflammatory response
Cytokine
Sabinene hydrate
Thymol and carvacrol

ABSTRACT

Two fractions (S1 and S2) of an oregano (*Origanum vulgare*) extract obtained by supercritical fluid extraction have been used to test anti-inflammatory effects on activated human THP-1 cells. The main compounds present in the supercritical extract fractions of oregano were trans-sabinene hydrate, thymol and carvacrol. Fractions toxicity was assessed using the mitochondrial-respiration-dependent 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) reduction method for several concentrations during 24 and 48 h of incubation. Concentrations higher than 30 μg/mL of both supercritical S1 and S2 oregano fractions caused a reduction in cell viability in a dose-dependent manner. Oxidized-LDLs (oxLDLs) activated THP-1 macrophages were used as cellular model of atherogenesis and the release/secretion of cytokines (TNT-α, IL-1β, IL-6 and IL-10) and their respective mRNA expressions were quantified both in presence or absence of supercritical oregano extracts. The results showed a decrease in pro-inflammatory TNF-α, IL-1β and IL-6 cytokines synthesis, as well as an increase in the production of anti-inflammatory cytokine IL-10. These results may suggest an anti-inflammatory effect of oregano extracts and their compounds in a cellular model of atherosclerosis.

1. Introduction

Atherosclerosis is a progressive disease characterized by the accumulation of lipids and fibrous elements in the large arteries. Chronic inflammation plays an important role in the development of atherosclerosis. This inflammation is the mechanism that the body responses to the interactions between modified lipoproteins, monocytes, macrophages, T-cells and arterial endothelial cells (Hansson, 2005; Libby, 2008; Zhao et al., 2005). Activated leukocytes, endothelial cells and macrophages produce pro-inflammatory cytokines including interleukin (IL)-1β, IL-6, as well as tumor necrosis factor-α (TNF-α) and anti-inflammatory cytokines, like the cytokine IL-10 (Jung et al., 2008; Zhao et al., 2005). These cells also produce pro-inflammatory enzymes, the inducible forms of nitric oxide sintase (iNOS) and cyclooxygenase (COX), which are responsible for increasing the levels of nitric oxide (NO) and prostaglandins (PEG2) and are known to be involved in various chronic diseases including multiple sclerosis or colon cancer (Wu and Ng, 2007).

The use of plants with pharmaceutical properties has received increased interest nowadays from both homeopathic and allopathic branches. Besides, these medicinal plants play an important role in public health, especially in developing countries. Oregano (*Origanum vulgare*) is an aromatic plant of the Mediterranean flora that has been commonly used for medical purposes (Bukovska et al., 2007; Juhás et al., 2008). Some previous studies have reported antioxidant and antimicrobial activities of oregano extracts in the inhibition of *Helicobacter pillori* growth (Chun et al., 2005; Wojdylo et al., 2007). Oregano has also been described as anti-inflammatory when used as treatment for colitis in mice decreasing the levels of the pro-inflammatory cytokines IL-1β, IL-6, GM-CSF and TNFα (Bukovska et al., 2007). Moreover, it has been reported that the use of essential oils from oregano provide a interesting perspective in the prevention of neurodegenerative disorders (Loizzo et al., 2009).

However, the biological activity of these plants strongly depends on their composition. Thymol and carvacrol are two compounds present in oregano with proved antioxidant and antimicrobial properties (Mastelic et al., 2008). Carvacrol has also shown an antiproliferative activity in tumor cells of HeLa (Mastelic et al., 2008). On the other hand, thymol has shown beneficial effects on the antioxidant status of the rat brain, which may in turn have influenced the concentration of docosahexaenoic acid (DHA) (Youdim and Deans, 2000). In this sense, our group have previously

Abbreviations: oxLDLs, oxidized low density lipoproteins; TNF, tumor necrosis factor; IL, interleukin; ELISA, enzyme-linked immunosorbent assay.

* Corresponding author. Tel.: +34 914978128; fax: +34 914978255.
E-mail address: aurelio.ocanna@uam.es (A. Ocaña-Fuentes).

0278-6915/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fct.2010.03.026

demonstrated the antioxidant activity of oregano extracts obtained by subcritical water extraction using *in vitro* assays (Rodríguez-Meizoso et al., 2006).

Supercritical fluid extraction (SFE) with CO_2 is a high-pressure technology, considered an attractive method compared to conventional techniques such as steam distillation or Soxhlet extraction because it avoids solute contamination with solvent residues and the degradation of termolabile compounds (Almeida and Ferreira, 2007). In this sense, supercritical fluid extraction with CO_2 is in increasing demand to produce high-quality extracts from plant material with medicinal properties (Mukhopadhyay, 2000) including oregano (Cavero et al. 2006).

The aim of this study is to describe the anti-inflammatory effects of natural oregano extracts obtained by SFE, in an *in vitro* model of atherosclerosis and other chronic diseases, using human macrophages activated with oxidized low density lipoprotein (oxLDLs). These extracts could be used in the development of new functional foods for the prevention or treatment of inflammation-based chronic diseases.

2. Materials and methods

2.1. Supercritical fluid extraction of plant materials

Dried and cryogenic grinded oregano leaves (*O. vulgare*) were subjected to supercritical fluid extraction (SFE) with CO_2. The supercritical extractions were carried out in a pilot-plant-scale supercritical fluid extractor (Thar Technology, Pittsburgh, PA, USA, model SF2000) of 2 L capacity using pure supercritical CO_2 at a pressure of 30 MPa and a temperature of 40 °C. Oregano extracts were fractionated using a two-cascade depressurized system consisted of two separators (separator 1 and 2). Fractionation conditions were as follows: separator 1 was kept at a constant pressure and temperature of 15 MPa and 40 °C, respectively, whereas separator 2 was maintained at a pressure of 2 MPa, and a temperature of 40 °C. Under these conditions two fractions were obtained, oregano S1 and oregano S2, corresponding to separator 1 and 2, respectively.

2.2. Analysis of the supercritical extract by GC/MS

Characterization of the supercritical oregano fractions oregano S1 and oregano S2 was carried out by a GC-2010 (Shimadzu, Japan), equipped with a split/splitless injector, electronic pressure control, AOC-20i auto injector, GCMS-QP2010 Plus mass spectrometer detector, and a GCMSSolution software. The column used was a ZB-5 (Zebron) capillary column, 30 m × 0.32 mm I.D. and 0.25 μm phase thickness. Helium, 99.996% was used as a carrier gas at a flow of 1 mL/min. Oven temperature programming was 60 °C isothermal for 4 min, increased to 64 °C at 1 °C/min, then increased to 106 °C at 2.5 °C/min. Oven temperature was then increased from 106 to 130 °C at 1 °C/min, and then to 200 °C at 5 °C/min, and then to a final temperature of 250 °C/min at 8 °C/min which was kept constant for 10 min. Sample injections (1 μL) were performed in split mode (1:20). The inlet pressure of the carrier gas was 57.5 kPa. Injector temperature was of 250 °C and MS ion source and interface temperatures were 230 and 280 °C, respectively. The mass spectrometer was used in TIC mode, and samples were scanned from 40 to 500 m/z units. Compounds thymol, carvacrol and linalool were identified by comparison with standard mass spectra obtained in the same conditions and compared with the mass spectra from library Wiley 229. Rests of the compounds were identified by comparison with the mass spectra from Wiley 229 library and by their linear retention index.

2.3. Cell culture

Human THP-1 monocytes cell line (American Type Culture Collection, ATCC) were maintained in suspension in RPMI 1640 culture medium (ATCC) supplemented with 10% FBS (GIBCO), 100 U/ml penicillin (GIBCO), 100 mg/ml streptomycin (GIBCO), 0.05 mM β-mercaptoethanol (Sigma–Aldrich) and 2 mM L-glutamine (GIBCO), at a density of 3–9 × 10^5 cells/ml at 37 °C in 5% air 95% CO_2. Cells were discarded and replaced by frozen stocks every 15 passages.

2.4. Cell differentiation

Cells were pelleted via centrifugation and assessed for viability using the Trypan-blue exclusion method. Viable cells were plated at a density of 5 × 10^5 cells/mL in 96 or 24 wells plates (100 μL and 1 mL respectively) and incubated with 12-myristate, 13-acetate (PMA) 100 ng/ml (Sigma–Aldrich) for 48 h in FBS free medium. Afterwards, the wells were washed with PBS and the treatment initiated.

2.5. Citotoxicity assay

SFE extracts toxicity was assessed using the mitochondrial-respiration-dependent 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) reduction method. THP-1 cells were plated in 96 wells plates, differentiated and incubated with different concentrations of oregano extracts for 24 and 48 h at 37 °C in 5% CO_2. After treatment, the cells were washed with PBS and incubated with MTT 1 mg/mL in PBS for 2 h at 37 °C in 5% CO_2. Afterwards, formazan crystals produced from MTT by the mitochondrial hydrolase, only activates in viable cells were solubilized in lysis buffer (10% SDS in 50% dimetilformamida pH = 7) and the absorbance of each well was then read at 540 nm using a microplate reader (Sunrise Remote, Tecan). The optical density of formazan formed in control cells (without treatment with extract) was taken as 100% viability.

2.6. Bioactivity assay

Fractions S1 and S2 were dissolved in dimethyl sulfoxide (DMSO; Sigma–Aldrich) to stock concentration of 10 mg/mL determined as the maximum doses not cytotoxic by de cell viability assays. THP-1 cells were platted and differentiated in 24 well plates. After differentiation, the cells were washed with PBS and activated with or without Cu_2 + oxidized LDLs (Steinbrecher, 1987) for 24 h and incubated with the extract diluted in FBS free medium, for 24 or 48 h at 37 °C in 5% CO_2. Afterwards, the supernatant was frozen and the cells RNA was isolated. Aliquots were analyzed to determine protein concentration and secreted cytokines.

2.7. Enzyme-linked immuno sorbent assay for quantification of cytokines

Supernatants were centrifuged at 12,000 rpm (Hettich, Universal 320-R, diameter 20 cm) to remove debris, and stored at −80 °C until analysis for cytokine analysis. IL-10, IL-1β, IL-6 and TNF-α were quantified using ELISA kits from BD Biosciences, according to the manufacturer's instructions. 100 μL of 1:10 diluted medium was added to anti-cytokine antibody coated polystyrene wells and incubated for 2 h. After washing, the plates were incubated with biotin-labeled secondary antibody for 1 h. The plates were washed and incubated for 30 min in the dark with substrate solution. Stop solution was added and the absorbance read at 450 nm with λ correction at 570 nm using a microplate reader (Sunrise Remote, Tecan Austria GmbH, Grödig, Austria).

2.8. Total RNA isolation

Total RNA from THP-1 cells was isolated using the Trizol® reagent from invitrogen. Cells (5 × 10^5) were homogenized in 200 μL of Trizol® reagent and, if necessary, stored at −80 °C. Following homogenization, samples were left to rest at room temperature for 5 min. After, 40 μL of chloroform was added, the tubes vigorously shaken for 15 s and let to rest at room temperature for 5 min. Tubes were then centrifuged at 12,000*g* (VWR, Galaxy 4D, diameter 14 cm), 4 °C for 15 min. The aqueous (upper and colorless) phase was transferred to a new tube. Isopropyl alcohol (100 μL) was added to the aqueous phase; the tube was then gently mixed and

Table 1
Composition of the supercritical extracts of oregano (*Origanum vulgare* L.), oregano S1 and oregano S2. Contribution of each compound to the total chromatographic area. N-I: non-identified compound. R.I.: linear retention index. n.d. non-detected.

Compound	Retention time (min)	R.I.	% Area (oregano S1)	% Area (oregano S2)
Sabinene	10.20	971	n.d.	1.04
Alpha-terpinene	12.52	1015	n.d.	0.74
P-cymene	12.94	1023	5.70	1.78
Limonene	13.19	1027	n.d.	0.57
Gamma-terpinene	14.93	1057	2.48	3.74
Cis-sabinene hydrate	15.39	1065	2.76	3.67
Trans-sabinene hydrate	17.17	1096	45.21	45.05
Linalool	17.35	1100	2.29	1.62
4-terpineol	21.74	1175	2.60	5.14
Alpha-terpineol	22.52	1189	2.37	2.84
N-I	25.09	1231	n.d.	0.73
Thymyl methyl ether	25.61	1240	1.33	2.09
Trans-sabinene hydrate acetate	26.17	1250	1.55	0.87
Linalyl acetate	26.40	1254	1.62	1.51
Thymol	28.65	1291	24.10	19.81
Carvacrol	29.23	1300	7.99	7.17
Trans-caryophyllene	37.80	1412	n.d.	1.63