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

Aurelio Ocaña-Fuentes *, Elena Arranz-Gutiérrez, Francisco Javier Señorans, Guillermo 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

* **Corresponding author**: aurelio.ocanna@uam.es (A. Ocaña-Fuentes).

Tel.: +34 914978128; fax: +34 914978255.

This is the accepted version of the following article: Ocana-Fuentes, A., Arranz-Gutierrez, E., Senorans, F. J., & Reglero, G. (2010). Supercritical fluid extraction of oregano (Origanum vulgare) essentials oils: anti-inflammatory properties based on cytokine response on THP-1 macrophages. Food and Chemical Toxicology, 48(6), 1568-1575, https://doi.org/10.1016/j.fct.2010.03.026 which has been published in final form at: https://www.sciencedirect.com/science/article/pii/S0278691510001754

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.

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

1. INTRODUCCTION

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 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 CO2 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 CO2 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 CO2. 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 CO2 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 × 105 cells/ml at 37 °C in 5% air 95% CO2. 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 × 105 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% CO2. 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% CO2. 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 Cu2 + 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% CO2. 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 × 105) 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 12,000g (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 incubated at room temperature for 10 min. After incubation, samples were centrifuged at 12,000g, 4 °C for 10 min. A gel-like pellet was formed and the isopropyl alcohol removed. The pellet was washed with

200 mL of 75% Ethanol in DEPC treated H2O, and centrifuged at 7600, 4 °C for 5 min. The ethanol was then removed and the pellet let to dry until colorless. Total RNA was then dissolved in 15 μL of DEPC H2O, incubated at 55 °C for 10 min and stored at −80 °C for future use.

2.9. Gene expression quantification

Total RNA isolated from THP-1 cells was quantificated of IL-1β, IL-6, IL-10 and TNF-α gene expression using real-time PCR. Total RNA (10 ng/μL) was used as template for cDNA synthesis using the High Capacity Archive Kit from Applied Biosystems, according to the manufacturer's instructions. Real-time PCR was performed using Taqman Probes (Applied Biosystems) following the manufacturer's recommendations. The Taqman probes used were: Hs99999029_m1 for IL-1β, Hs00174131_m1 for IL-6, Hs999999035_m1 for IL-10, Hs00174128_m1 for TNF-α, Hs01075529_m1 for iNOS, Hs00153133_m1 for Cox-2, Hs00765730_m1 for NFkβ-1, Hs01115512_m1 for PPAR-γ, and Hs99999901_s1 for 18S rRNA. Gene expression levels were then normalized to 18S rRNA expression and compared.

2.10. Statistical analysis

All data were expressed as the mean ± SEM (Standard Error of Mean). For single variable comparisons, Student's t-test was used. For multiple variable comparisons, data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test and Bonferroni test when necessary using the GraphPad Prism statistical software (GraphPad Software Inc. Windows Version 5). P values less than 0.05 were considered significant.

3. RESULTS

3.1. Composition of the supercritical oregano fractions

Two fractions of the *O. vulgare* leaves extract, oregano S1 and oregano S2, were isolated using supercritical fluid extraction with CO2 and their composition was determined by gas chromatography-mass spectrometry (GCMS) (see Table 1). For both fractions, the main compounds present were trans-sabinene hydrate, thymol and carvacrol. Chemical structures of these compounds are shown in Fig. 1.

3.2. Effect of supercritical oregano S1 and S2 fractions on THP-1/Macrophage-like cell viability

Prior to the bioactivity study to evaluate anti-inflammatory properties, citotoxicity of fractions oregano S1 and oregano S2 was determined. For each fraction a MTT essay was performed for several concentrations during 24 and 48 h of incubation. Fig. 2 shows the effects of the oregano extracts in THP-1 derivated macrophages. For both fractions (oregano S1 and oregano S2), and for incubations up to 48 h there was no significant decrease in cell viability for concentrations up to 30 μg/mL. For concentrations higher than 30 μg/mL of oregano S1 and S2 there is a reduction in cell viability in a dose-dependent manner. These results suggest that concentrations above 30 μg/mL are toxic and their use was discarded for the present work.

3.3. Effect of supercritical oregano S1 and S2 fractions in the pro- and anti-inflammatory cytokines release

To activate the THP-1 macrophage, Cu2+ oxLDLs were added to the incubation medium. These oxLDL treated cells showed an increase in total protein secreted (data not shown) which was used as an indication of macrophage activation.

For both incubation periods (24 and 48 h) oxLDL-activated cells showed an increase in the release of all the pro- and anti-inflammatory cytokines analyzed (TNF-α, IL-1β, IL-6, and IL-10, respectively) comparing to non-activated controls (Fig. 3, Fig. 4).

Treatment of activated cells with supercritical S1 and S2 oregano extracts results in an overall reduction of pro-inflammatory cytokines release. Cells incubated for 24 h with oregano S1 or oregano S2, showed a decrease in the release levels of TNF-α in a dose-dependent manner, reaching basal levels of TNF-α release when incubated with 30 μg/mL of extract. When the treatment endured for 48 h, concentrations of only 30 μg/mL of oregano S1 and S2 fractions produced a significant decrease in TNF-α release. Non-activated cells were also treated with oregano S1 and S2 fractions. Although there was a dose dependent decrease in TFN-α, this result was found not significant within the concentration range used for 24 h treatments. On contrary, a significant decrease in TNF-α release was observed for 30 μg/mL of extract when the treatment was of 48 h. In this case, the use of oregano S2 fraction showed a much more significant decrease.

As for IL-1β, treatment with any of the oregano fractions led to a decrease in the cytokine release to basal levels in activated cells. For non-activated cells, there were no significant effects after treatment for both 24 and 48 h incubations.

Activated cells treated with oregano S2 for 24 h showed a decrease in IL-6 secretion in a dose-dependent manner, up to concentrations of 30 μg/mL. In activated cells treated with oregano S1 there was a decrease of IL-6 secretion up to not activated controls. For concentrations of oregano S1 and S2 fractions of 30 μg/mL results showed an evident decrease in IL-6 secretion when compared to non-activated control cells. For 48 h incubation assays, results for both S1 and S2 fractions showed a significant decrease in IL-6 secretion compared to the activated control. In cells treated with 10 and 20 μg/mL of oregano S2 the secretion of IL-6 was significantly lower than the basal secretion in non-activated controls. Regarding the non-activated cells treated with oregano S1 and S2 fractions for 24 and 48 h, results showed a clear reduction in IL-6 secretion in respect to non-activated control, for 10 and 20 μg/mL concentrations, being the treatment with the lowest concentration the most effective in reducing IL-6 secretion. On contrary, for both of the

oregano fractions, 30 μg/mL treatment did not reduce the IL-6 secretion when compared to non-activated controls.

IL-10 secretion increased after treatment with any of the oregano extracts used. For 24 h treatments, there was a similar increased secretion of IL-10 for both extracts and for either activated or non-activated cells. The lower significant increase in IL-10 secretion was achieved when cells were incubated with 30 μg/mL of oregano S2. For 48 h treatments in activated cells, both extracts induced the same increase in IL-10 expression of around 2.5 times the activated control. Non-activated cells treated with any fraction extract had a higher increase in IL-10 secretion than activated cells did. This increase was similar in both extracts except for 30 μg/mL of oregano S2, which was lower.

To test anti-inflammatory effects a group treated with indomethacin (5 μg/ml) was carried out as positive standard control (Kim et al., 2008, Berg et al., 1999). Extracts S1 and S2 in general shown a better anti-inflammatory effect that indomethacin at this concentration, diminishing more the pro-inflammatory cytokines (TNF-α and IL-1β) that indomethacin did and inducing anti-inflammatory release of IL-10 that was not observed with indomethacin.

3.4. Effect of supercritical oregano S1 and S2 fractions in the gene transcription of inflammatory cytokines

Relative quantification (RQ) determinates the change in expression of a nucleic acid sequence relative to a control. Fig. 5, Fig. 6 represent the mRNA expression for each cytokine based on their RQ values.

In agreement with the results present for cytokines release, gene transcription of analyzed cytokines showed an increase in oxLDL-activated cells when compared to non-activated cells in both, 24 and 48 h, periods of incubation.

TNF-α gene expression decrease only in activated cells treated with oregano S2 at 24 h of incubation. Treatment of non-activated cells with either oregano S1 or oregano S2 showed no reduction in the transcription of this gene. In contrast to the results presented for 24 h of incubation, at 48 h, transcription of the TNF-α gene increased significantly for both activated and non-activated cells.

On the other hand, expression of IL-1β gene decreased in activated cells treated with both oregano fractions when compared to activated control cells at 24 h of incubation. However, longer incubation times led to a significant increase in its expression. On contrary, in the case of non-activated cells, treatment with oregano S2 increased the transcription of IL-1β at 24 h of incubation. This increase was found to be three times higher at incubation times of 48 h. Treatment with oregano S1 showed little change transcription at 24 h, although results showed an increase of the IL-1β gene at 48 h.

Regarding IL-6 gene transcription, in the case of activated cells, the treatment with any of the oregano fractions for both 24 and 48 h incubation times, led to gene transcription levels closed to the one attained when working with non-activated control cells. Only oregano S2 caused a significant reduction in IL-6 transcription when compared with levels of not activated cells during 24 h of incubation.

At 24 h IL-10 gene transcription increased twice when cells were treated with either oregano S1 or oregano S2 extract in activated cells of treatment compared to non-treated cells levels. On contrary, at incubation times of 48 h gene expression levels in activated cells treated with oregano were reduced up to non-activated cells levels. Finally, in the case of non-activated cells treated with any of oregano fractions and at both periods of incubation, transcription of IL-10 gene did not change compared to non-activated cells.

Similarly to cytokine protein release, anti-inflammatory gene expression was compared to a group treated with indomethacin (5 μg/ml). After 24 h of treatment, extracts S1 and S2 in general shown a high anti-inflammatory effect that indomethacin. Nevertheless at 48 h opposite effect was observed. Gene expression of anti-inflammatory cytokine was attenuated, improved the pro-inflammatory ones and presented worse anti-inflammatory effects than indomethacin at this period of treatment.

4. DISCUSSION

Results present in this work suggest that supercritical oregano S1 and S2 fractions may act as effective inhibitors of oxLDL induced pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) secretion, and also as enhancers of the anti-inflammatory cytokine IL-10 secretion, in macrophage THP-1 cells. Pro-inflammatory cytokines levels decreased in a dose-dependent manner with any oregano fraction used at incubation times of both 24 and 48 h. These results were in agreement with the ones regarding the transcription of cytokines genes at 24 h of incubation. Other authors have previously reported an increment in cytokine secretion in activated macrophages treated with oxLDL (Osterud and Bjorklid, 2003). Similar results have been described for essential oils extracted from *Cinnamomum osmophloeum*, an herb traditionally used in Asia as food and as a medicine which contains cinnamaldehyde. These essentials oils have been also used in murine macrophages, causing anti-inflammatory effect by decreasing TNF-α, IL-6 and IL-1β secretions (Chao et al., 2005). To our surprise, after 48 h of treatment, TNF-α and IL-1β gene transcriptions were increased. IL-10 is an anti-inflamatory interleukin that inhibits the secretion of pro-inflammatory cytokines, probably by down-regulating NF-kB expression (Bogdan et al., 1991, Schottelius et al., 1999). Translocation of NF-kB into the nucleus it is prevented by IL-10, which blocking IkB kinase activity and also prevents TNF-α induced degradation of IkB kinase (Bhattacharyya et al., 2004, Liu, 2005, Schottelius et al., 1999). NF-kB is overexpressed in inflammation and enhance pro-inflammatory cytokines like TNF-α, IL-1β and IL-6 (Asadullah et

al., 2008, Tak and Firestein, 2001). According to our results, supercritical oregano extracts caused a downregulation of these cytokines and hence an anti-inflammatory effect.

Main compounds present in supercritical oregano extract were sabinene hydrate, thymol and carvacrol. Anti-inflammatory effect of thymol has been demonstrated in human neutrophiles incubated with 10 or 20 μg/ml of this compound (Braga et al., 2006). Mice edema is reported to be reduced with a topical application of 100 μg/cm2 of carvacrol (Sosa et al., 2005). Moreover, antioxidant properties of thymol and carvacrol have been demonstrated in several studies, suggesting their use as nutraceuticals ingredients in the development of novel functional foods. Derivatives of thymol and carvacrol have been described as antioxidant according to the DPPH radical scavenging method (Mastelic et al., 2008). Essential oils from oregano and their components carvacrol and thymol inhibited 3-nitrotyrosine formation, biomarker of the oxidative stress, supporting the nutraceutical value of oregano and the potential of thymol and carvacrol in preventing the formation of toxic products by the action of reactive nitrogen species (Prieto et al., 2007). Also, thymol and carvacrol prevented autoxidation of lipids (Yanishlieva et al., 1999). However, anti-inflammatory or antioxidant effects of sabinene hydrate have not been described in literature so far.

In summary, CO2 oregano supercritical extracts showed anti-inflammatory properties by: (a) reducing the release of pro-inflammatory cytokines, and (b) increasing the anti-inflammatory secretion in activated macrophages. Our extracts, oregano S1 and oregano S2 presented no significant differences on their composition, citotoxicity or effects on the cytokine response. So any of the fractional conditions used on SFE are able to obtain fraction extracts with anti-inflammatory properties. These results suggest that oregano supercritical extracts could be used as novel options for treatment of chronic diseases based on inflammatory processes.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the financial support from Foncasal Trading S.L. (Project CENIT HIGEA CEN-20072003), the Comunidad Autonoma de Madrid (ALIBIRD, project number S-505/AGR-1469) and Consolider Ingenio 2010 (Project CSD/2007/063). E. Arranz would like to thank Ministerio de Educación for the FPU predoctoral fellowship. Thanks to the Servicio de Bioquímica e Investigación, Hospital Ramón y Cajal for the donation of the LDLs and especially to Gema de la Peña Martin.

REFERENCES:

Almeida, P., Ferreira, S., 2007. Crossover pressure for SFE of spearmint (Mentha spicata L.) essential oil with pure CO2 and with CO2 plus ethanol. In: I Iberoamerican Conference on Supercritical Fluids, Prosciba 2007. Book of Abstracts, p. 31.

Asadullah, K., Sterry, W., Volk, H.D., 2008. Interleukin-10 therapy review of a new approach. Pharmacol. Rev. 55, 241–255.

Berg, J., Fellier, H., Christoph, T., Grarup, J., Stimmeder, D., 1999. The analgesic NSAID lornoxicam inhibits cyclooxygenase (COX)-1/-2, inducible nitric oxide synthase (iNOS), and the formation of interleukin (IL)-6 in vitro. Inflamm. Res. 48 (7), 369–379.

Bhattacharyya, S., Sen, P., Wallet, M., Long, B., Baldwin, A.S., Tisch, R., 2004. Immunoregulation of dendritic cells by IL-10 is mediated through suppression of the PI3K/Akt pathway and of IkappaB kinase activity. Blood 104, 1100–1109.

Bogdan, C., Vodovotz, Y., Nathan, C., 1991. Macrophage deactivation by interleukin 10. J. Exp. Med. 174, 1549–1555.

Braga, P.C., Dal Sasso, M., Culici, M., Bianchi, T., Bordoni, L., Marabini, L., 2006. Anti-inflammatory activity of thymol: inhibitory effect on the release of human neutrophil elastase. Pharmacology 77, 130–136.

Bukovska, A., Cikos, S., Juhas, S., Il'kova, G., Rehak, P., Koppel, J., 2007. Effects of a combination of thyme and oregano essential oils on TNBS-induced colitis in mice. Mediat. Inflamm. 2007, 23296.

Cavero, S., Jaime, L., Ibañez, E., Señoráns, F.J., Reglero, G., 2006. Supercritical carbon dioxide extraction of compounds with antimicrobial activity from Origanum vulgare L.: determination of optimal extraction parameters. J. Food Protect. 69 (2), 369–375.

Chao, L.K., Hua, K.-F., Hsu, H.-Y., Cheng, S.-S., Liu, J.-Y., Chang, S.-T., 2005. Study on the antiinflammatory activity of essential oil from leaves of Cinnamomum osmophloeum. J. Agric. Food Chem. 53, 7274–7278.

Chun, S., Vattem, D.A., Lin, Y.T., Shetty, K., 2005. Phenolic antioxidants from clonal oregano (Origanum vulgare) with antimicrobial activity against Helicobacter pylori. Process Biochem. 40, 809–816.

Hansson, G.K., 2005. Inflammation, atherosclerosis, and coronary artery disease. New Engl. J. Med. 352, 1685–1695.

Juhás, S., Cikos, S., Czikková, S., Veselá, J., Il'ková, G., Hájek, T., Domaracká, K., Domaracky´ , M., Bujnáková, D., Rehák, P., Koppel, J., 2008. Effects of borneol and thymoquinone on TNBS-induced colitis in mice. J. Folia Biol. 54, 1–7.

Jung, S.H., Park, H.S., Kim, K.S., Choi, W.H., Ahn, C.W., Kim, B.T., Kim, S.M., Lee, S.Y., Ahn, S.M., Kim, Y.K., 2008. Effect of weight loss on some serum cytokines in human obesity: increase in IL-10 after weight loss. J. Nutr. Biochem. 19, 371–375.

Kim, S.K., Kim, H.J., Choi, S.E., Park, K.H., Choi, H.K., Lee, M.W., 2008. Anti-oxidative and inhibitory activities on nitric oxide (NO) and prostaglandin E2 (COX-2) production of flavonoids from seeds of Prunus tomentosa Thunberg. Arch. Pharm. Res. 31 (4), 424–428.

Libby, P., 2008. Role of inflammation in atherosclerosis associated with rheumatoid arthritis. Am. J. Med. 121, S21–S31.

Liu, Z.G., 2005. Molecular mechanism of TNF signaling and beyond. Cell Res. 15, 24–27.

Loizzo, M.R., Menichini, F., Conforti, F., Tundis, R., Bonesi, M., Saab, A.M., Statti, G.A., Cindio, B.D., Houghton, P.J., 2009. Chemical analysis, antioxidant, anti- inflammatory and anticholinesterase activities of Origanum ehrenbergii Boiss and and Origanum syriacum L. essential oils. Food Chem. 117, 174–180.

Masteli, c, J., Jerkovic, I., Blazevic, I., Poljak-Blazi, M., Borovi´c, S., Ivancic-Bace, I., Smrecki, V., Zarkovic, N., Brci´c-Kostic, K., Vikic-Topic, D.Z., Müller, N., 2008. Comparative study on the antioxidant and biological activities of carvacrol, thymol, and eugenol derivatives. J. Agric. Food Chem. 56, 3989–3996.

Mukhopadhyay, M., 2000. In: Mukhopadyay, M. (Ed.), Natural Extracts Using Supercritical Carbon Dioxide. CRC Press, Boca Ratón, FL, USA, p. 3.

Osterud, B., Bjorklid, E., 2003. Role of monocytes in atherogenesis. Physiol. Rev. 83,1069–1112.

Prieto, J.M., Iacopini, P., Cioni, P., Chericoni, S., 2007. In vitro activity of the essential oils of Origanum vulgare, Satureja montana and their main constituents in peroxynitrite-induced oxidative processes. Food Chem. 104, 889–895.

Rodríguez-Meizoso, I., Marin, F.R., Herrero, M., Señorans, F.J., Reglero, G., Cifuentes, A., Ibáñez, E., 2006. Subcritical water extraction of nutraceuticals with antioxidant activity from oregano. Chemical and functional characterization. J. Pharm. Biomed. Anal. 41, 1560–1565.

Schottelius, A.J., Mayo, M.W., Sartor, R.B., Baldwin, A.S., 1999. Interleukin-10 signaling blocks inhibitor of kappaB kinase activity and nuclear factor kappaB DNA binding. J. Biol. Chem. 274, 31868–31874.

Sosa, S., Altinier, G., Politi, M., Braca, A., Morelli, I., Della Loggia, R., 2005. Extracts and constituents of Lavandula multifida with topical anti-inflammatory activity. Phytomedicine 12, 271–277.

Steinbrecher, U.P., 1987. Oxidation of human low density lipoprotein results in derivatization of lysine residues of apolipoprotein B by lipid peroxide decomposition products. J. Biol. Chem. 262 (8), 3603–3608.

Tak, P.P., Firestein, G.S., 2001. NF-kB: a key role in inflammatory diseases. J. Clin. Invest. 107, 7–11.

Wojdylo, A., Oszmianski, J., Czemerys, R., 2007. Antioxidant activity and phenolic compounds in 32 selected herbs. Food Chem. 105, 940–949.

Wu, S.-J., Ng, L.-T., 2007. Tetrandrine inhibits proinflammatory cytokines, iNOS and COX-2 expression in human monocytic cells. Biol. Pharm. Bull. 30, 59–62.

Yanishlieva, N.V., Marinova, E.M., Gordon, M.H., Raneva, V.G., 1999. Antioxidant activity and mechanism of action of thymol and carvacrol in two lipid systems. Food Chem. 64, 59–66.

Youdim, K.A., Deans, S.G., 2000. Effect of thyme oil and thymol dietary supplementation on the antioxidant status and fatty acid composition of the ageing rat brain. Brit. J. Nutr. 83, 87–93.

Zhao, G., Etherton, T.D., Martin, K.R., Vanden Heuvel, J.P., Gillies, P.J., West, S.G., Kris-Etherton, P.M., 2005. Anti-inflammatory effects of polyunsaturated fatty acids in THP-1 cells. Biochem. Biophys. Res. Commun. 336, 909–91.

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	R.I.	% Area (oregano S1)	% Area (oregano S2)
Sabinene	10.2	971,00	n.d.	1.04
Alpha-terpinene	12.5	1015,00	n.d.	0.74
P-cymene	12.9	1023,00	5.70	1.78
Limonene	13.1	1027,00	n.d.	0.57
Gamma-terpinene	14.9	1057,00	2.48	3.74
Cis-sabinene hydrate	15.39	1065,00	2.76	3.67
Trans-sabinene hydrate	17.17	1096,00	45.21	45.05
Linalool	17.3	1100,00	2.29	1.62
4-terpineol	21.7	1175,00	2.60	5.14
Alpha-terpineol	22.5	1189,00	2.37	2.84
N-I	25.0	1231,00	n.d.	0.73
Thymyl methyl ether	25.6	1240,00	1.33	2.09
Trans-sabinene hydrate acetate	26.17	1250,00	1.55	0.87
Linalyl acetate	26.4	1254,00	1.62	1.51
Thymol	28.6	1291,00	24.10	19.81
Carvacrol	29.2	1300,00	7.99	7.17
Trans-caryophyllene	37.8	1412,00	n.d.	1.63

Fig. 1. Chemical structures of the main compounds present in the supercritical extracts of oregano S1 and oregano S2: (a) trans-sabinene hydrate, (b) thymol and (c) carvacrol.

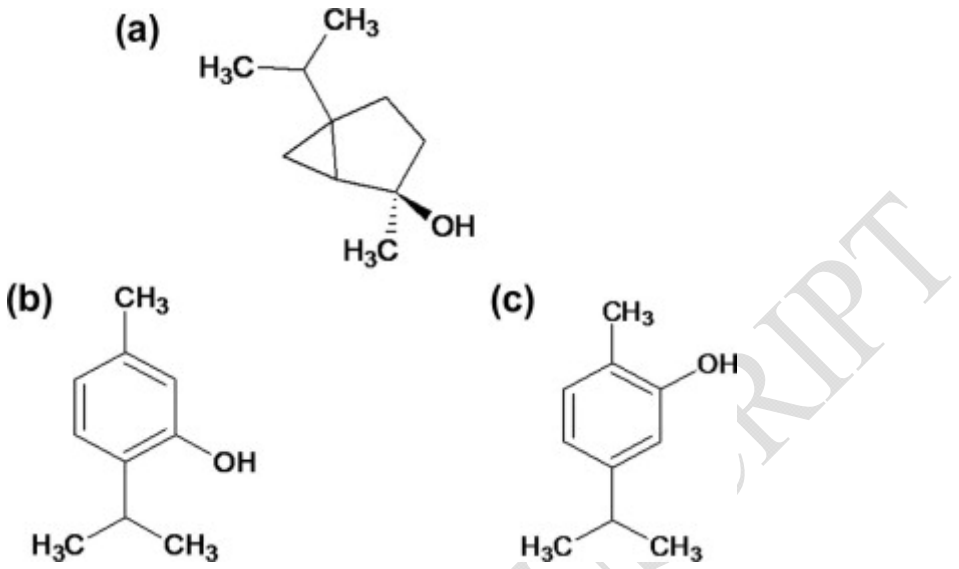

Fig. 2. Effects of S1 and S2 supercritical fractions from oregano on macrophage-THP-1 cell viability. Cells were treated with increasing concentrations of oregano extracts, from 0 to 70 µg/ml, for 24 h (2A) or 48 h (2B). Cell viability was determined by the MTT assay. Values represent the mean ± SEM of six independent experiments and statistic signification is represented by $**P$ values less than 0.01 (very significant), and $***P$ values less than 0.001 (extremely significant).

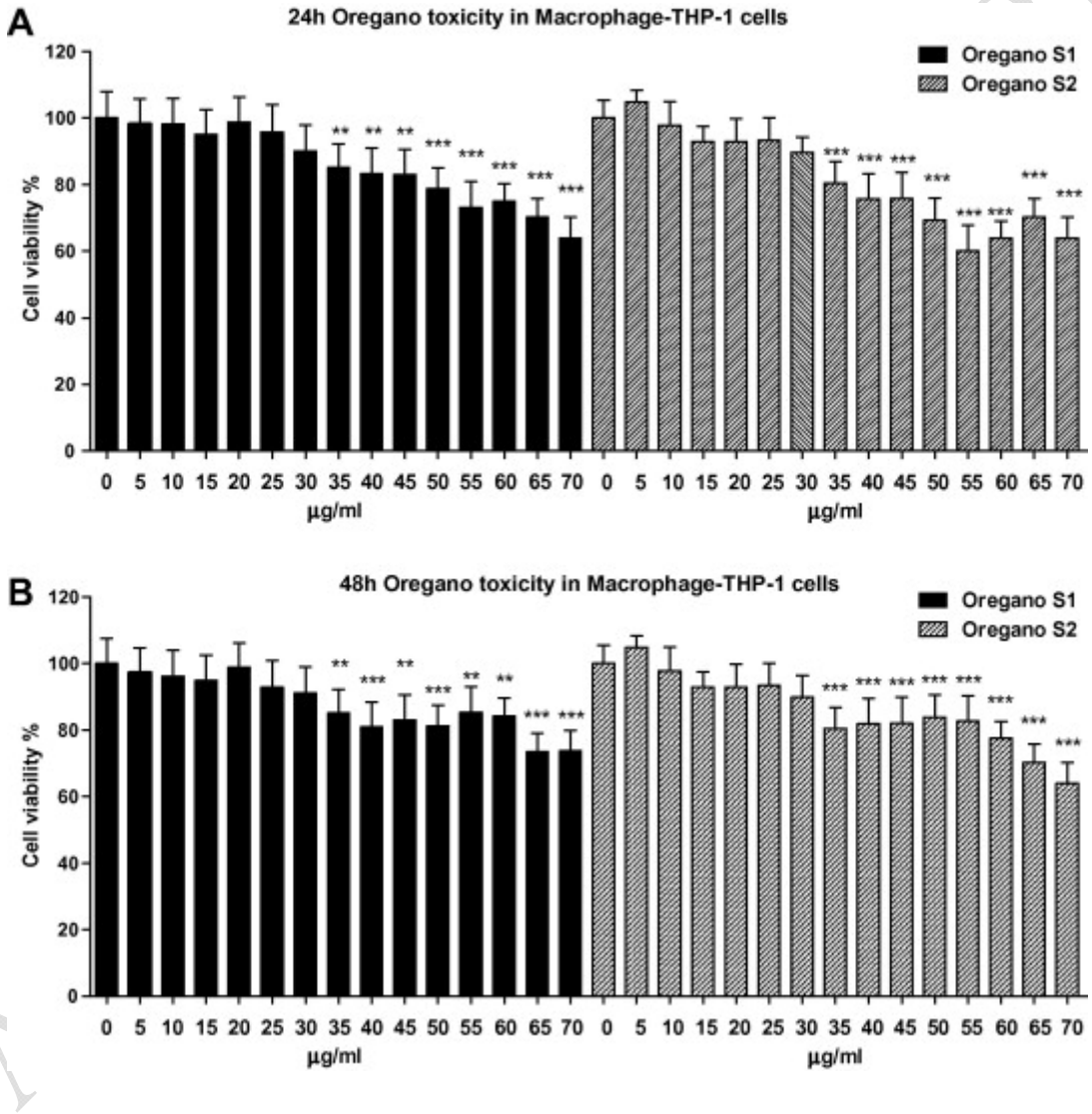

Fig. 3. 24 h dose–effect of 0, 10, 20 and 30 μg/ml of S1 and S2 supercritical oregano fractions on the production and secretion of TNF-α (3A), IL-1β (3B), IL-6 (3C) and IL-10 (3D) on macrophage-THP-1 cells. Cells were differentiated and treated as described in Material and Methods section. Indomethacin is a positive anti-inflammatory control. Data represent means ± SEM calculated from six independent experiments with three replicates for each treatment. Statistic Dunnett's multiple comparison test VS Ctrl + oxLDL signification is represented by ∗*P* values: less than 0.05 (significant), ∗∗*P* values: less than 0.01 (very significant), and ∗∗∗*P* values: less than 0.001 (extremely significant). Statistic Bonferroni multiple comparison test VS Ctrl-oxLDL signification is represented by ♦♦*P* values: less than 0.01 (very significant), and ♦♦♦*P* values: less than 0.001 (extremely significant).

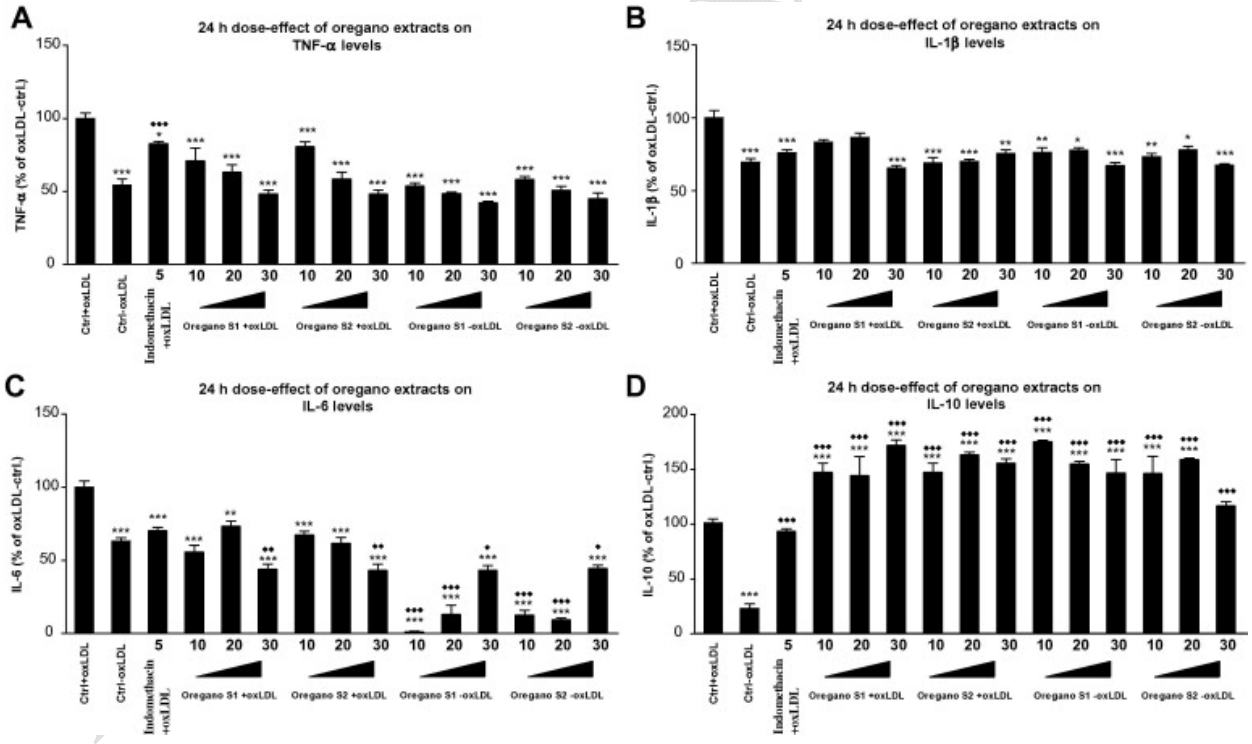

Fig. 4. 48 h dose–effect of 0, 10, 20 and 30 μg/ml of S1 and S2 supercritical oregano fractions on the production and secretion of TNF-α (4A), IL-1β (4B), IL-6 (4C) and IL-10 (4D) on macrophage-THP-1 cells. Cells were differentiated and treated as described in Material and Methods section. Indomethacin is a positive anti-inflammatory control. Data represent means ± SEM calculated from six independent experiments with three replicates for each treatment. Statistic Dunnett's multiple comparison test VS Ctrl + oxLDL signification is represented by ∗*P* values: less than 0.05 (significant), ∗∗*P* values: less than 0.01 (very significant), and ∗∗∗*P* values: less than 0.001 (extremely significant). Statistic Bonferroni multiple comparison test VS Ctrl-oxLDL signification is represented by ♦♦*P* values: less than 0.01 (very significant), and ♦♦♦*P* values: less than 0.001 (extremely significant).

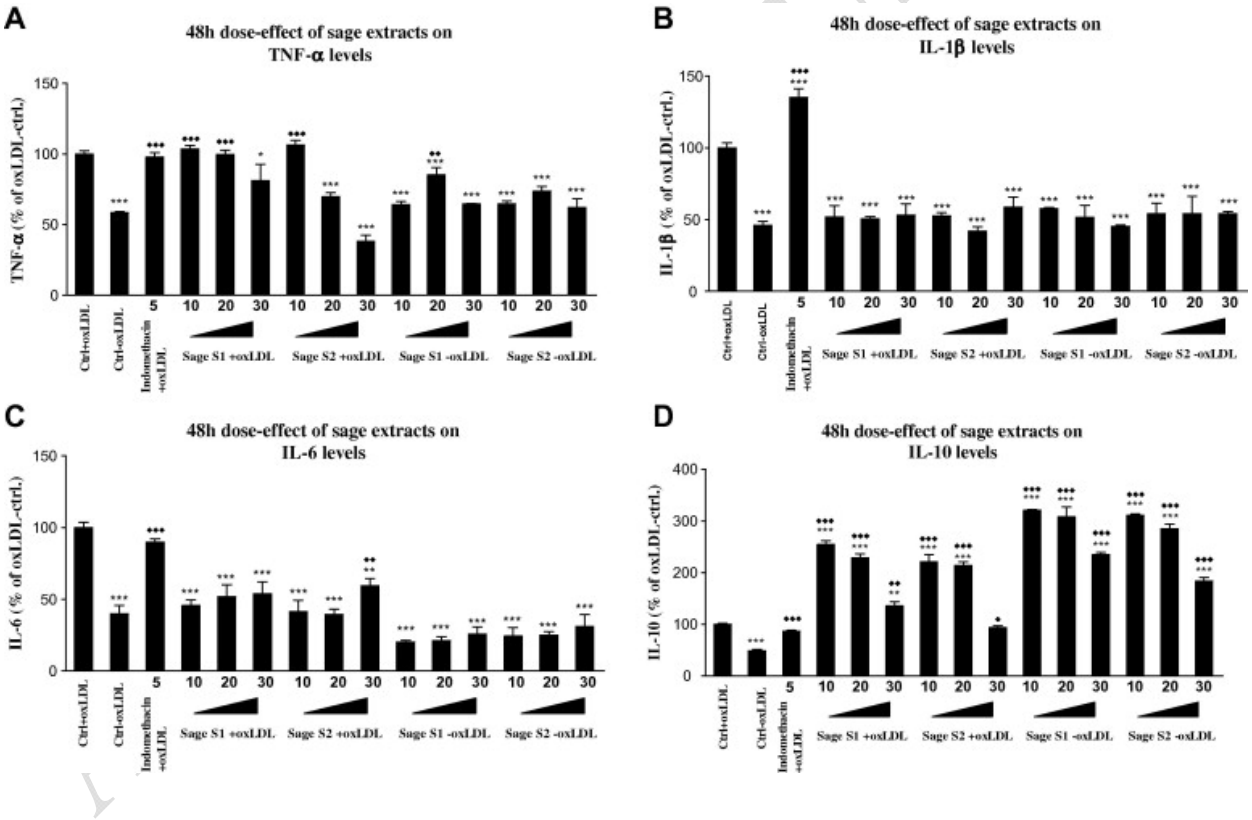

Fig. 5. Effect after 24 h of treatment with 30 µg/ml of S1 and S2 supercritical oregano fractions on the relative transcription gene quantification (RQ) of TNF-α (5A), IL-1β (5B), IL-6 (5C) and IL-10 (5D) on macrophage-THP-1 cells. Cells were differentiated and treated as described in Material and Methods section. Indomethacin is a positive anti-inflammatory control. Data represent means ± SEM calculated from six independent experiments with three replicates for each treatment. Statistic Dunnett's multiple comparison test VS Ctrl + oxLDL signification is represented by ∗*P* values less than 0.05 significant ∗∗*P* values less than 0.01 very significant and ∗∗∗*P* values less than 0.001 extremely significant. Statistic "Bonferroni multiple comparison test VS Ctrl-oxLDL signification is represented by ♦♦*P* values less than 0.01 very significant and ♦♦♦*P* values less than 0.001 extremely significant.

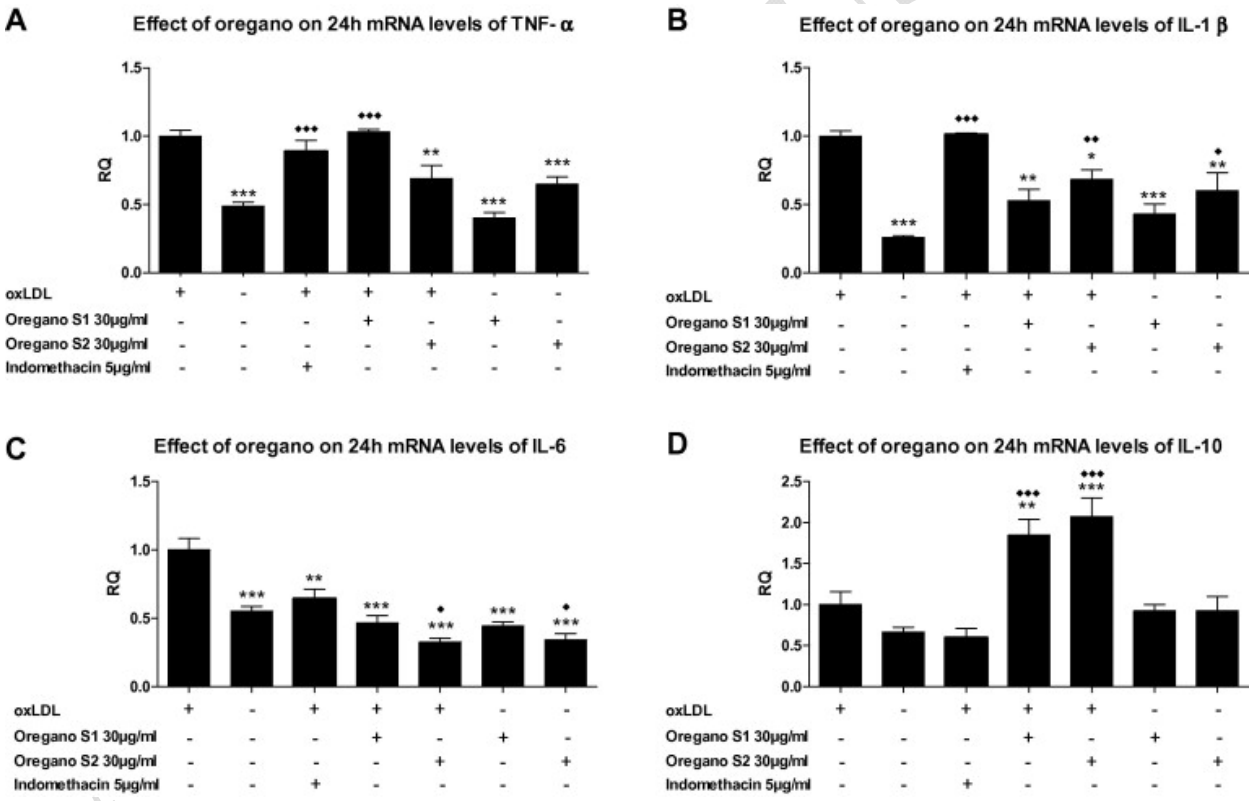

Fig. 6. Effect after 48 h of treatment with 30 µg/ml oregano S1 and S2 supercritical oregano fractions on the relative transcription gene quantification (RQ) of TNF-α (6A), IL-1β (6B), IL-6 (6C) and IL-10 (6D) on macrophage-THP-1 cells. Cells were differentiated and treated as described in Material and Methods section. Indomethacin is a positive anti-inflammatory control. Data represent means ± SEM calculated from six independent experiments with three replicaticates for each treatment. Statistic Dunnett's multiple comparison test VS Ctrl + oxLDL signification is represented by ∗*P* values less than 0.05 significant ∗∗*P* values less than 0.01 very significant and ∗∗∗*P* values less than 0.001 extremely significant. Statistic "Bonferroni multiple comparison test VS Ctrl-oxLDL signification is represented by ♦♦*P* values less than 0.01 very significant and ♦♦♦*P* values less than 0.001 extremely significant.