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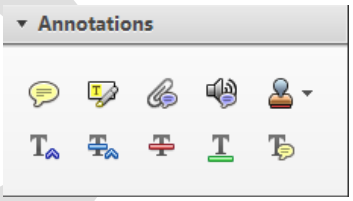

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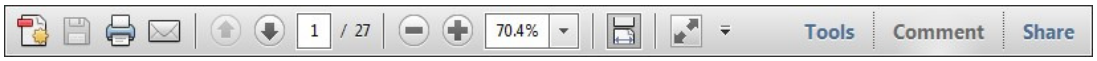

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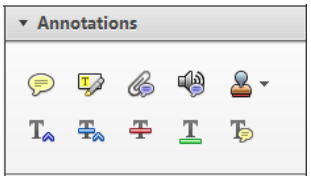

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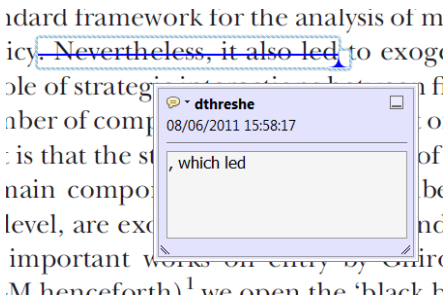

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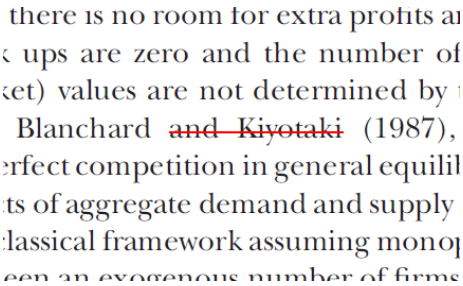

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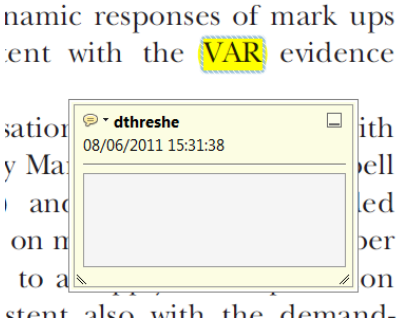

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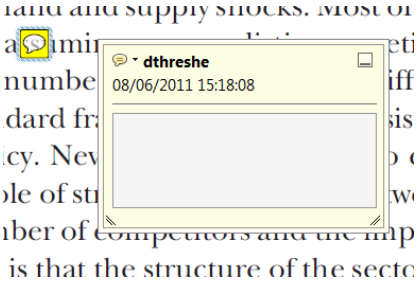

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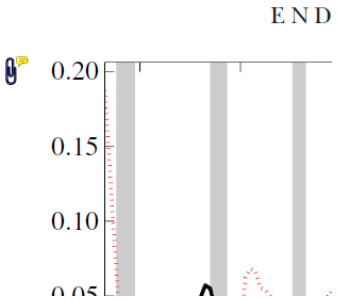

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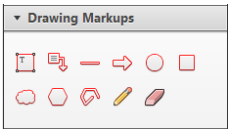

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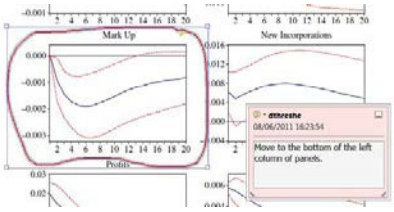

Original article

Anti-inflammatory activity of rosemary extracts obtained by supercritical carbon dioxide enriched in carnosic acid and carnosol

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Summary The *in vitro* anti-inflammatory activity of supercritical rosemary (*Rosmarinus officinalis* L.) extracts (rosemary A and B) is been reported in this study. To achieve that, THP-1 macrophages were activated using lipopolysaccharide or human ox-LDL and secretion and gene expression of TNF-α, IL-1β, IL-6 and IL-10 were evaluated, as well as COX-2 gene expression. Results indicated that both rosemary extracts (A & B) exhibit high anti-inflammatory activity although at a higher extent in case of rosemary B extract (5 μg mL^{-1}), representing a higher quantity of carnosic acid and carnosol than rosemary A. When comparing the activity of the extract to the standard itself, the anti-inflammatory activity of standards of carnosic acid and carnosol was not as intense as that obtained with rosemary B. These data indicated that although carnosic acid content in the extracts is considered as the main anti-inflammatory compound, a synergistic interaction with other compounds may play a significant role in enhancing its activity. Results provided the grounds for possible increase in the application of supercritical rosemary extracts in food formulations for mitigation or prevention of inflammatory diseases.

Keywords Anti-atherogenic activity, anti-inflammatory activity, carnosic acid, rosemary, supercritical extracts.

Introduction

Inflammation is a complex response of the immune system induced by a microbial infection or tissue injury: ischaemic, toxic or autoimmune. In this process, are involved a whole assembly of interactions between soluble factors and cells. Activated macrophages secrete several mediators such as pro-inflammatory cytokines, tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6 and anti-inflammatory, as IL-10. During the inflammatory response, these cells also induce gene expression of pro-inflammatory enzymes as cyclooxygenase-2 (COX-2), responsible of prostaglandin E-2 synthesis (Barton, 2008; Zhang, 2008). Several diseases risen from chronic inflammatory process, like cardiovascular diseases, atherosclerosis, obesity, diabetes, cancer, rheumatoid arthritis or neurodegenerative diseases, as Alzheimer (Nathan, 2002; Medzhitov, 2008).

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Rosemary (*Rosmarinus officinalis* L.) is a common household plant grown in many parts of the world that it is used as a food flavouring agent in cosmetics and in traditional medicine known for its choreretics, hepatoprotective and antitumorigenic activity (Slamenová *et al*., 2002). Besides, a number of studies have claimed that rosemary extracts possess a variety of biological activities such as antioxidant, antitumor, antimicrobial and anti-inflammatory activities (Santoyo *et al*., 2005; Viuda-Martos *et al*., 2008; ilas *et al*., 2012; Yu *et al*., 2013). These activities have been attributed to the presence of a high percentage of phenolic diterpenes in rosemary leaves (Takaki *et al*., 2008). Thus, the main compounds carnosic acid and carnosol of rosemary have been shown to present antioxidant, anti-inflammatory and antiproliferative activities (Rau *et al*., 2006).

Associated with anti-inflammatory effects, Mengoni *et al*. (2011) using two *in vivo* inflammation models indicated that carnosic acid and carnosol significantly decreased expression of IL-1β and TNF-α and completely inhibited COX-2 expression. The inhibitory

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effects of carnosic acid on LPS-induced NO and TNF-α production have been related to the suppression of iNOS and COX-2 expression due to the effect of inhibition in induced NF-κB signalling (Kuo *et al.*, 2011). Carnosol decreases LPS-induced iNOS mRNA and downregulates the inhibitor NF-κB kinase activity in mouse macrophage RAW 264.7 cell line (Lo *et al.*, 2002). However, the commercial application of carnosic acid and carnosol is limited due to the cost of purification procedures. Besides, several authors have reported that some rosemary extracts or fractions presented a similar or superior anti-inflammatory activity that carnosic acid or carnosol alone (Kuo *et al.*, 2011; Yu *et al.*, 2013).

The use of supercritical carbon dioxide to obtain extracts from plants is an attractive separation technique for the recovery of valued compounds as provides a high speed and efficiency of extraction, eliminates concentration steps and avoids the use of organic solvents with the subsequent benefit to the environment; moreover, CO_2 is a food grade nontoxic solvent, nonexplosive and easy to remove from obtained extracts. Thus, the extraction of rosemary leaves using supercritical CO_2 has been intensively investigated, assuring high percentage of phenolic diterpenes, mainly carnosic acid and carnosol (Carvalho *et al.*, 2005; Chang *et al.*, 2008). Parameters like extraction temperature, pressure, type and amount of modifier determine the solubility of these substances in the supercritical CO_2 and thus have a direct effect on their percentage in the extract composition. The advantage of using CO_2 supercritical rosemary extracts over traditional solvent extraction (ethanol, acetone and hexane) and hydrodistillation has been proved by many authors (Carvalho *et al.*, 2005). Despite of these data, only a few studies have evaluated the anti-inflammatory activity of supercritical rosemary extracts (Peng *et al.*, 2007; Kuo *et al.*, 2011).

The objective of this work was to compare the anti-inflammatory activity of two rosemary extracts obtained by supercritical extraction with neat CO_2 or ethanol as cosolvent highly enriched in carnosic acid and carnosol. Furthermore, we compared the anti-inflammatory capacity of rosemary extracts with that of pure carnosic acid and carnosol solutions. The anti-inflammatory activity was evaluated using two *in vitro* models of inflammation with human macrophages. In the first model, THP-1 macrophages were activated using lipopolysaccharide (LPS), which produced a general inflammatory response. In the other model, THP-1 macrophages were activated with human oxidised low-density lipoproteins (ox-LDL), a model that allow us to determine the anti-inflammatory effect of the extracts in an atherosclerotic environment and could be useful to determine the potential activity of the extracts in the prevention of atherosclerosis. This study will prove the urge for increase the application of supercritical rosemary extracts in food formulations for possible prevention of inflammatory diseases.

Material and methods

Chemicals

Carnosol (>95%), camphor (>97%), α-terpineol (>97%) and linalool (>97%) were purchased from Sigma-Aldrich (Spain) while carnosic acid (≥97%), 1,8 cineole (98%) and borneol (>99%) were supplied from Fluka (Spain). Ethanol and phosphoric acid (85%) in HPLC grade were purchased from Panreac (Spain) and acetonitrile was obtained from Lab Scan (Ireland). CO_2 (N38) was supplied from Carburos Metálicos (Spain). Purified water was obtained from a Milli-Q purification system (Millipore, Spain).

Rosemary samples

Rosemary (*R. officinalis* L.) samples consisted in dried leaves obtained from an herbalist' shop (Murcia, Spain). Cryogenic grinding of the samples was performed under liquid nitrogen. The ground material was sieving to the appropriate size (between 200 and 600 μm), and all samples were stored at −20 °C until use.

Extraction methods

Supercritical rosemary extractions were carried out using a pilot-plant supercritical fluid extractor (model SF2000; Thar Technology, Pittsburgh, PA, USA), comprising a 2-L cylinder extraction cell and two different separators (S1 and S2), each of 0.5 L capacity, with independent control of temperature and pressure. For each experiment, the extraction vessel was packed with 0.5 kg of the cryogenically milled and sieved plant particles. Two different extraction conditions were performed to obtain rosemary A, extractor pressure was 30 MPa and temperature 313 K, with a CO_2 flow rate of 60 g min^{-1} during a extraction time of 360 min. In this assay, fractionation of the extracted material during 120 min was carried out then extraction continued for 240 min without fractionation. The fractionation was accomplished by setting the pressure of the first separator (S1) to 10 MPa, while the second separator (S2) was maintained at recirculation system pressure (5 MPa). In this case, two different samples were collected, one per separator; however, only S1 was used and named as rosemary A. Rosemary B was obtained setting an extraction pressure of 15 MPa, temperature of 313 K and 5% w/w ethanol was employed as cosolvent during 180 min of extraction to obtain one sample from S1 without fractionation.

Chemical characterisation of supercritical rosemary extracts

Analysis was carried out to determine the carnosic acid and carnosol content in the samples, using a HPLC (Varian Pro-star) equipped with a Microsorb-100 C18 column (Varian). Mobile phase consisted of acetonitrile (solvent A) and 0.1% of phosphoric acid in water (solvent B) applying the following gradient: from 0 to 8 min, 23% A; increasing from 8 to 25 min up to 75% A; kept constant during 15 min and from 40 to 45 min, initial conditions were gained (23% A). The flow rate was continuous at 0.7 mL min^{-1}, and the detection was accomplished using a diode array detection system (Varian) storing the signal at a wavelength of 230, 280 and 350 nm.

The essential oil compounds present in supercritical rosemary extracts were determined by a GC-2010 (Shimadzu, Japan). 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^{-1}. Compounds 1,8-cineole, α-terpineol, linalool and camphor were identified by comparison with standard mass spectra obtained in the same conditions and compared with the mass spectra from library Wiley 229. The rest of the compounds were identified by comparison with the mass spectra from Wiley 229 library.

Isolation and oxidation of low-density lipoproteins

Low-density lipoproteins (LDLs) were kindly donated by Servicio de Bioquímica e Investigación, Hospital Ramón y Cajal (Madrid, Spain) and isolated from human plasma as described before (Havel *et al.*, 1955). Oxidation of LDLs was done by incubating LDLs with 5 μM $CuSO_4$ for 3 h at 37 °C. Oxidation degree was measured as the amount of thiobarbituric acid reactive substances (TBARS) produced as previously reported (Yancey & Jerome, 1998).

Cell culture and treatment

Human THP-1 monocytes (American Type Culture Collection, ATCC) were cultured in RPMI 1640 culture medium supplemented with 10% FBS, 100 U mL^{-1} penicillin, 100 mg mL^{-1} streptomycin, 2 mM L-glutamine (Invitrogen, Spain) and 0.05 mM β-mercaptoethanol (Sigma-Aldrich) at 37 °C. Cells were plated at a density of 5×10^5 cells mL^{-1} in 24-well plates. Differentiation of monocytes to macrophages (THP-1/M cells) was induced by maintaining the cells with 100 ng mL^{-1} of phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) for 48 h. After differentiation, cells were washed with PBS and incubated with 75 μg mL^{-1} ox-LDLs or 0.05 μg mL^{-1} of LPS (Sigma-Aldrich) in the presence of different concentrations of supercritical rosemary extracts or pure standards for 24 h in a medium without FBS. Then, the supernatant was frozen at −20 °C and cells RNA isolated. Indomethacin (Sigma-Aldrich), an anti-inflammatory drug, was used as a reference.

Cytotoxicity assays

The cytotoxic effect of the extracts and pure standards on THP-1/M cells was tested using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Sigma-Aldrich). THP-1/M cells in 24-well plates were incubated with RPMI containing different concentrations of the extracts for 24 h at 37 °C. Cells were then washed with PBS and 0.5 mg mL^{-1} of MTT were added to each well and incubated for 3 h at 37 °C. Supernatants were discarded and formazan crystals dissolved in an extraction solution [dimethyl sulfoxide (DMSO) and ethanol (Panreac) in a concentration of 1:1 w/w]. Quantification was performed by measuring the optical density at 570 nm using a multiscanner autoreader (Sunrise; Tecan, Spain), using extraction solution as a blank.

Quantification of cytokines by ELISA

The release of TNF-α, IL-1β, IL-6 and IL-10 was measured in the medium of THP-1/M cells treated with ox-LDL or LPS, in the presence of different concentrations of supercritical rosemary extracts or pure standards using ELISA kits (BD Biosciences, Spain), according to manufacturer's instructions. The colour generated was quantified by measuring the optical density at 450 nm with substrate correction at 570 nm using a multiscanner autoreader.

RNA isolation and RT-PCR

Total RNA was isolated from THP-1/M cells using Trizol® (Invitrogen) according to manufacturer's instructions. Reverse transcription of the RNA was performed using High Capacity Archive Kit and GeneAmp PCR System 9700 (Applied Biosystems, Spain) according to the manufacturer's instructions to obtain 20 ng μL^{-1} of cDNA. PCR amplification was conducted in a 10 μL reaction mixture with cDNA, Taqman Gene Expression Master Mix and TaqMan probes (Applied Biosystems), according to the manufacturer's conditions in a 7900HT Fast Real-Time PCR System (Applied Biosystems). The TaqMan probes used were as follows: Hs99999029_m1 for IL-1β, Hs00174131_m1 for IL-6, Hs99999035_m1 for IL-10, Hs00174128_m1 for TNF-α, Hs00153133_m1 for COX-2 and Hs99999901_s1 for 18S rRNA. Expression of genes was normalised relative to 18S rRNA using SDS Software v2.4 (Applied Biosystems).

Statistical analysis

All data were expressed as the mean of three determinations ± SD. Data were analysed by one-way analysis of variance (ANOVA) followed by Dunnett's and Bonferroni tests, using Prism program for Windows (Version 5; GraphPad Software, USA). *P* values lower than 0.05 were considered significant.

Results

Analysis of supercritical rosemary extracts

First, the extraction yield of rosemary A and B was evaluated. Data shown that the extraction yield was higher when cosolvent was employed, 7.26% vs. 1.62% when only neat CO_2 was used. Analysis showed that higher carnosic acid and carnosol content was also obtained when ethanol was incorporated as a cosolvent during the extraction. Carnosic acid content was increased from 180 mg g^{-1} in rosemary A up to 256 mg g^{-1} in rosemary B, while carnosol content was 16 mg g^{-1} in rosemary A and 38 mg g^{-1} in rosemary B. These results indicated that both extracts presented a higher quantity of carnosic acid than the one presented by the supercritical extracts used to carry out previous anti-inflammatory studies (Peng *et al.*, 2007; Kuo *et al.*, 2011). Meanwhile, carnosol content was similar or slightly higher than that previously reported.

Besides, a characterisation by GC-MS of the extracts was carried out and the results are presented in Table S1, where a tentative identification has been performed based on the comparison of mass spectra. In both rosemary extracts, the main compounds present were 1,8-cineole and camphor. In terms of relative abundance, when a polar cosolvent (ethanol) is employed in the extraction procedure, few changes were reported in essential oil composition of supercritical rosemary extracts, less concentration of 1,8-cineole and verbenone detection.

Therefore, the incorporation of 5% of ethanol as a cosolvent caused an important increase in phenolic compounds in the extract, carnosic acid and carnosol, meanwhile the composition in essential oils was similar.

Effects of supercritical rosemary extracts and pure components on THP-1/M viability

The viability of the THP-1/M cells was assessed prior to anti-inflammatory studies to determinate the cytotoxicity of supercritical rosemary extracts, rosemary A and B and the main pure compounds presented in the extracts by MTT method. The results obtained indicated that 5 μg mL^{-1} of rosemary A and B was the highest concentration without significant decrease in cell viability. Similar results were obtained with pure standards, being 5 μg mL^{-1} the highest concentration that presented a 100% cell viability.

Effect of rosemary extracts and its main compounds on the cytokines release and gene expression in THP-1/M activated with lipopolysaccharide

The activation of THP-1/M was carried out with the incorporation of LPS into the medium. After 24 h of incubation, LPS treated cells shown an important increase in all pro- and anti-inflammatory cytokines measured (TNF-α, IL-1β, IL-6 and IL-10), compared with nonactivated controls (Fig. 1). These activated cells were considered as positive controls for all the cytokines tested. When the activation of THP-1/M was carried out in the presence of 5 μg mL^{-1} of rosemary extracts or pure standards (carnosic acid, carnosol, camphor and 1,8-cineole), an important decrease in TNF-α secreted level was observed (Fig. 1a) compared with levels obtained in the absence of extracts (positive control). Moreover, 5 μg mL^{-1} of rosemary B achieved an 80% of inhibition in TNF-α secretion, similar to that obtained with the anti-inflammatory drug and indomethacin. All pure standards were analysed also shown a significant reduction in TNF-α release, being carnosic acid and carnosol the most active, with a reduction of TNF-α secretion near to 50%.

Interleukin-1β secretion (Fig. 1b) was also reduced with the presence of 5 μg mL^{-1} rosemary extracts. Rosemary B decreased the secretion of this IL until 60%, similar to that produced by carnosic acid and carnosol. Camphor and 1,8-cineole did not change significantly its secretion levels.

The activation of macrophages in the presence of rosemary A or B also reduced the secretion of IL-6 in a dose-depended manner. Both rosemary extracts, at 5 μg mL^{-1}, showed a release of this cytokine close to basal levels of nonactivated cells. Carnosic acid and carnosol reduced IL-6 release up to similar values obtained with rosemary extracts, being both compounds more active than camphor and 1,8-cineole.

Regarding data obtained with IL-10, an anti-inflammatory cytokine, activated cells in the presence of rosemary A did not change its release, contrary rosemary B and all pure standards decreased significantly its secretion.

To determine whether the influence of supercritical extracts and pure standards in cytokine production was related to gene expression, total RNA was extracted from treated THP-1/M, and gene expression was calculated as relative quantification (RQ), being RQ value of 1 nonactivated cells or negative control. The effect of rosemary extracts and pure standards on TNF-α, IL-1β, IL-6 and IL-10 mRNA expression in

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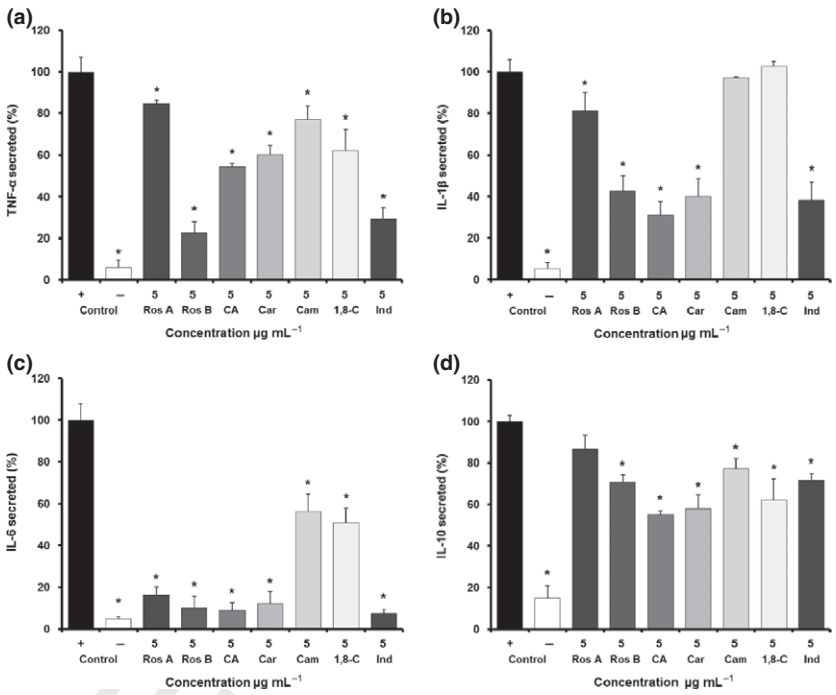

20, 24 **Figure 1** Levels of cytokines secreted by THP-1/M activated with LPS. Ros: rosemary; CA: carnosic acid; Car: carnosol; Cam: camphor; 1,8-C: 1,8-cineole; Ind: indomethacin. *Statistical differences between positive control and the other samples at $P < 0.05$.

THP-1 after 24 h of LPS activation are presented in Fig. 2. Gene expression of pro-inflammatory cytokines TNF-α, IL-1β and IL-6 was significantly reduced with rosemary A and B, as well as gene expression of IL-10 that was reduced with both extracts. Also, similar to cytokines release, carnosic acid and carnosol were more active, in terms of gene expression reduction, than camphor and 1,8-cineole.

In addition, gene expression of COX-2 (Fig. 2e) in the presence of rosemary extracts and its main compounds was also determined, as COX-2 is an inducible enzyme which expression is increased during the inflammatory process. Results indicated that the two extracts, carnosic acid and carnosol, reduced the gene expression of COX-2 up to basal level. Camphor and 1,8-cineole also decreased COX-2 expression, but in a lower level.

The decrease in cytokine and COX-2 gene expression by rosemary extracts was in agreement with the reduced cytokine release, which strengthen the anti-inflammatory activity of these extracts. Moreover, rosemary B, with a higher quantity of carnosic acid and carnosol than rosemary A, also presented a higher anti-inflammatory activity, with similar values to the anti-inflammatory drug and indomethacin. However, the anti-inflammatory activity of carnosic acid and carnosol pure standards was not as intense as that obtained with rosemary B. These results indicated that although carnosic acid and carnosol content in the extracts played a crucial role in their anti-inflammatory properties, it can be assumed a synergistic interaction of carnosic acid, carnosol and other compounds presented in rosemary B. In this sense, camphor and 1,8-cineole could be proposed as other components of the extracts that presented anti-inflammatory activity.

Effect of rosemary extracts and its main compounds on the cytokines release and gene expression in THP-1/M activated with oxidised low-density lipoproteins

Oxidised-LDLs were used to activate the inflammatory process in THP-1/M and carry out the second model of inflammation used in this research work. Figure 3 shows that ox-LDL-treated cells increased significantly the secretion of TNF-α, IL-1β, IL-6 and IL-10 compared with nonactivated cells. The incorporation of rosemary A and B and pure compounds promoted an important reduction in TNF-α release, up to basal levels with 5 μg mL^{-1} of rosemary B and carnosic acid and carnosol pure standards. IL-1β and IL-6 secretion were also significantly reduced up to basal levels with 5 μg mL^{-1} of rosemary B and carnosic acid and carnosol pure standards. Regarding to IL-10, only a small reduction was achieved with the highest concentration of all compound tested. Results obtained indicated that when THP-1/M cells were stimulated with ox-LDL, rosemary B presented a higher decrease in pro-inflammatory cytokines, compared with rosemary A. However, in this model, this decrease in the release of pro-inflammatory cytokines in the presence of extract B was similar to those obtained with carnosic acid and carnosol pure standards.

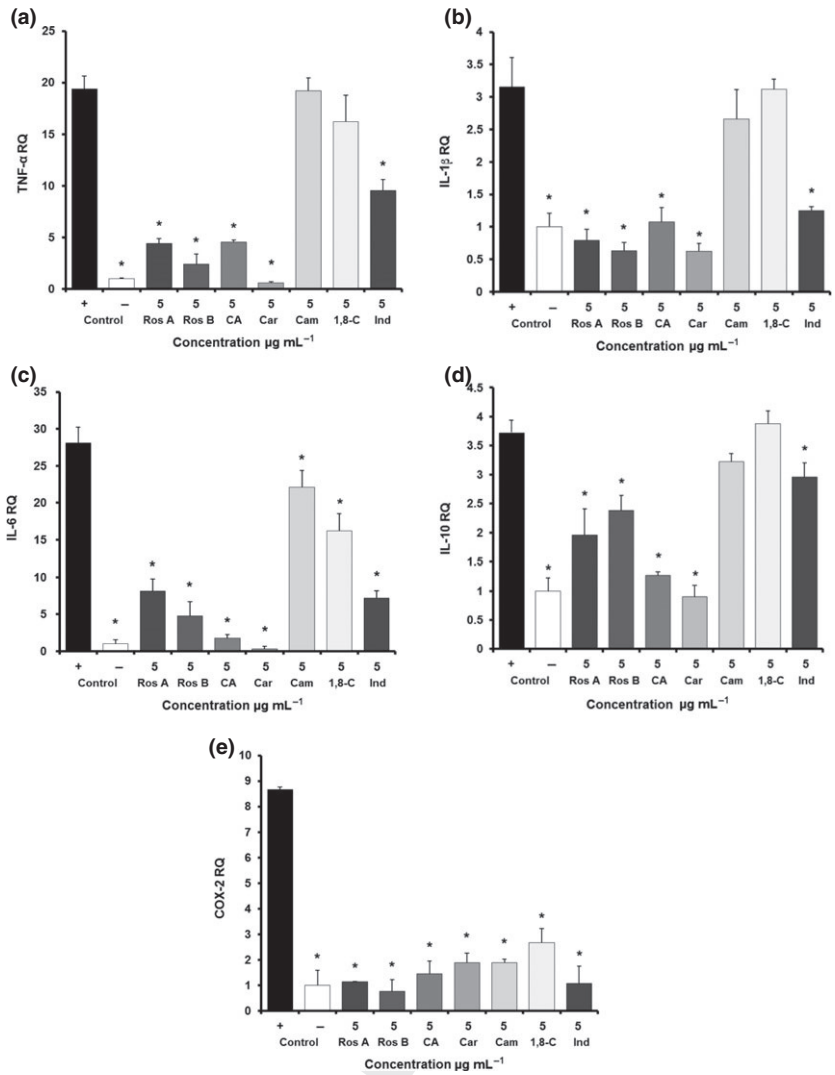

Figure 2 Gene expression of cytokines and COX-2 on THP-1/M stimulated with LPS. RQ: relative quantification; Ros: rosemary; CA: carnosic acid; Car: carnosol; Cam: camphor; 1,8-C: 1,8-cineole; Ind: indomethacin. *Statistical differences between positive control and the other samples at $P < 0.05$. 21

The effect of rosemary extracts and pure standards on TNF-α, IL-1β, IL-6 and IL-10 mRNA expression in THP-1 after 24 h of ox-LDL activation is presented in Fig. 4. Gene expression of pro-inflammatory cytokines TNF-α, IL-1β and IL-6 was significantly reduced in the presence of rosemary extracts and pure standards, although rosemary B and carnosic acid and carnosol presented the highest decreased. COX-2 gene expression was mainly reduced by rosemary B extract, until the levels obtained with indomethacin.

The anti-inflammatory activity of rosemary supercritical extracts when macrophages were activated with ox-LDL was also correlated with the amount of carnosic acid and carnosol presented in the extract, presenting a higher activity rosemary B, enriched in those compounds. However, also in this model, other compounds should be implicated in the final detected activity, like camphor and 1,8-cineole. It's also interesting to point out that rosemary B presented an important anti-inflammatory activity also in an atherosclerotic environment.

Discussion

Supercritical carbon dioxide extraction has been reported to be an efficient extraction technology to extract a high percentage of phenolic diterpenes, mainly carnosic acid and carnosol from rosemary leaves (Carvalho *et al.*, 2005; Chang *et al.*, 2008). The rosemary supercritical extracts proposed in this study presented a higher quantity of carnosic acid and carnosol than those used previously to determine the anti-inflammatory activity (Peng *et al.*, 2007; Kuo *et al.*, 2011). This work studied the anti-inflammatory

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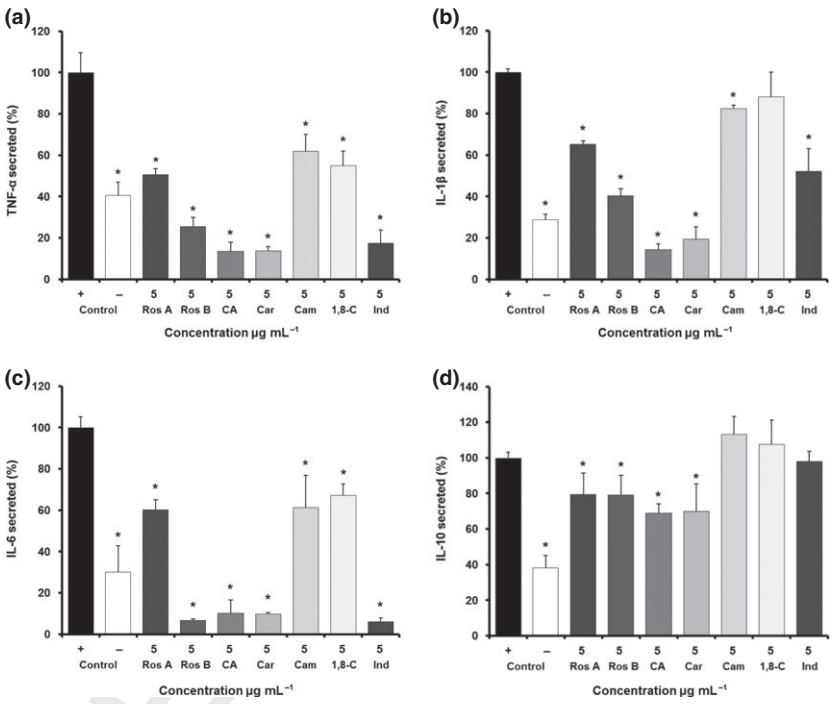

Figure 3 Levels of cytokines secreted by THP-1/M activated with ox-LDL. Ros: rosemary; CA: carnosic acid; Car: carnosol; Cam: camphor; 1,8-C: 1,8-cineole; Ind: indomethacin. *Statistical differences between positive control and the other samples at $P < 0.05$.

activity of two rosemary supercritical extracts with a high quantity of carnosic acid and carnosol and compared its anti-inflammatory activity with that of carnosic acid and carnosol, to increase the applicability of supercritical rosemary extracts in formulations for the prevention of inflammatory diseases. This anti-inflammatory activity was evaluated by two *in vitro* models of inflammation, using THP-1 human macrophages activated with LPS or human ox-LDL. The use of LPS to activate macrophages is a model commonly used to test anti-inflammatory properties of herbs extracts (Allen-Hall *et al.*, 2007). Ox-LDL activated macrophages simulate an atherosclerotic model of inflammation as low-density lipoprotein oxidation appears to be a fundamental event in the development of the atherosclerotic lesion and the initiation of the inflammatory cascade (Call *et al.*, 2004). The incorporation of ox-LDL and LPS in human macrophages activated both, secretion and gene expression of pro-inflammatory and anti-inflammatory cytokines, such as TNF-α, IL-1β, IL-6 and IL-10 (Kaperonis *et al.*, 2006; Wasaporn *et al.*, 2010). Gene expression of these cytokines and other pro-inflammatory factors are activated after NF-κB translocation to the nucleus. One of the most important pro-inflammatory enzymes that depends of NF-κB gene activation is COX-2 (cyclooxygenase-2), responsible of PGE-2 (prostaglandin E-2) synthesis, which secretion increases vasodilation and inflammatory progression (Huang *et al.*, 2000).

Considering the results presented, supercritical rosemary extracts showed an important anti-inflammatory activity, due to the inhibition of pro-inflammatory cytokines secretion and gene expression in ox-LDL and LPS activated macrophages. This anti-inflammatory activity was demonstrated due to an important reduction of TNF-α, IL-1β and IL-6 secretion obtained with 5 μg mL^{-1} of rosemary A and B. Moreover, this activity was higher after the incubation of activated macrophages with rosemary B, being the main different between rosemary A and B, its carnosic acid and carnosol content (A: 180 mg g^{-1} carnosic acid and 16 mg g^{-1} carnosol; B: 256 mg g^{-1} carnosic acid and 38 mg g^{-1} carnosol). These results indicated that carnosic acid and carnosol presented in the extract played a crucial role in the anti-inflammatory properties of the supercritical rosemary extracts. Thus, Kuo *et al.* (2011) demonstrated the anti-inflammatory activity of a supercritical rosemary extract with 107.7 mg g^{-1} of carnosic acid and 30 mg g^{-1} of carnosol in LPS activated macrophages, indicating that extract concentrations above 12.5 μg mL^{-1} reduced TNF-α secretion. Recently, Yu *et al.* (2013) also reported that 10 μg mL^{-1} of a rosemary extract obtained with methanol and later hexane fractionation, containing 184 mg g^{-1} of carnosic acid and 25 mg g^{-1} of carnosol also reduced the TNF-α secretion. These results supported our data, as 5 μg mL^{-1} of rosemary B, with the highest content in carnosic acid and carnosol between the compared extracts, reduced up to 75–80%, and the TNF-α

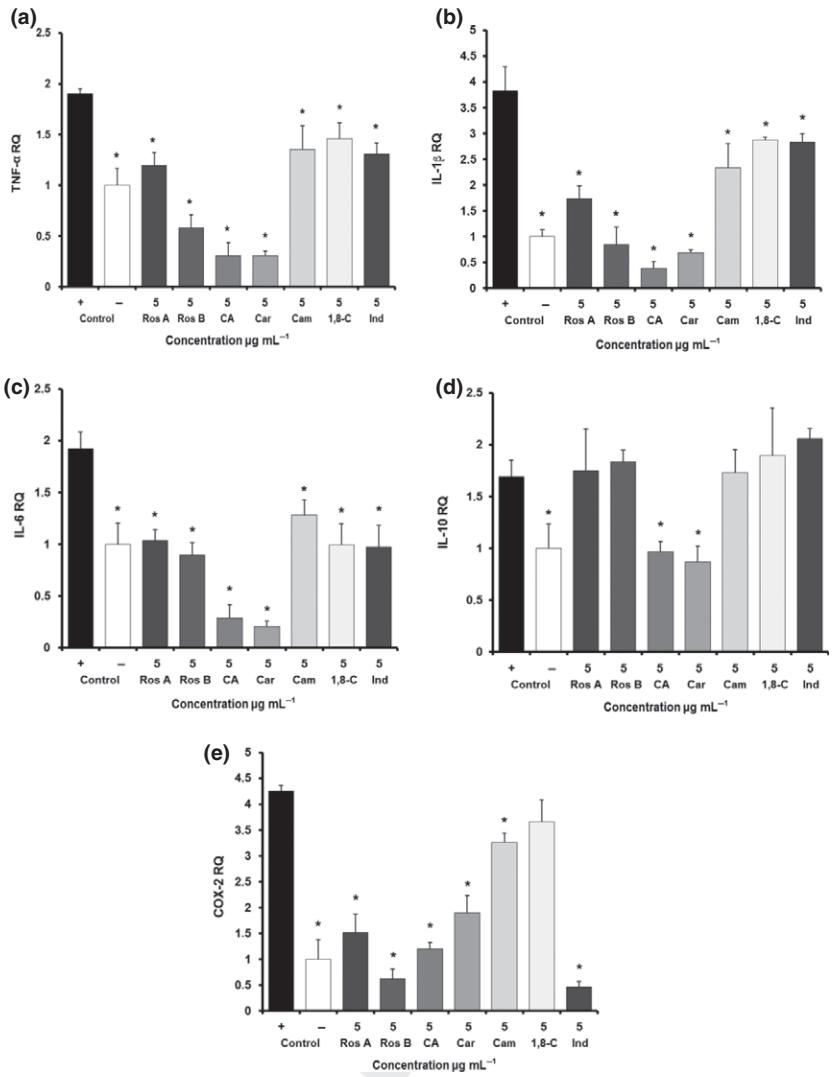

Figure 4 Gene expression of cytokines and COX-2 on THP-1/M stimulated with ox-LDL. RQ: relative quantification; Ros: rosemary; CA: carnosic acid; Car: carnosol; Cam: camphor; 1,8-C: 1,8-cineole; Ind: indomethacin. *Statistical differences between positive control and the other samples at $P < 0.05$. 23

secretion in macrophages activated with LPS or human ox-LDL. However, it is also interesting to emphasise that 5 μg mL^{-1} of rosemary B, containing 1.28 μg mL^{-1} of carnosic acid and 0.19 μg mL^{-1} of carnosol, produced a similar reduction in cytokines secretion and gene expression than 5 μg mL^{-1} of carnosic acid and carnosol pure standards, which indicates that other compounds presented in the extracts are also implied in the anti-inflammatory effect. This fact was also indicated by Yu *et al.* (2013) who reported that the anti-inflammatory effect of rosemary extracts results from the synergistic effects between carnosic acid and carnosol and other compounds presented in the extracts. Thus, our supercritical rosemary extracts presented an important quantity of camphor and 1,8-cineole, and compounds whose anti-inflammatory activity has been also shown in this work and reported by other authors (Santos & Rao, 2000; Ehrnhöfer-Ressler *et al.*, 2013). Consequently, these compounds could be proposed as the other components of the extracts that increased their anti-inflammatory activity.

Moreover, supercritical rosemary extracts and its main compounds caused an important reduction in COX-2 gene expression in both LPS and ox-LDL activated THP-1/M. Accordingly to our results, Mengoni *et al.* (2011) reported that carnosic acid and carnosol purified from fresh leaves of *Rosmarinus officinalis* inhibit COX-2 expression in an *in vivo* model. Yu *et al.* (2013) also studied COX-2 expression and showed a reduction in COX-2 protein expression with concentrations over 5 μg mL^{-1} using a rosemary extract with 184 mg g^{-1} of carnosic acid while no effect with 10 μg mL^{-1} of an extract with 93.8 mg g^{-1}. These authors related the reduction in COX-2 expression with the carnosic acid concentration in the extract, hypothesis that corroborates our results. How-

ever, as reported before, 5 μg mL^{-1} of rosemary B produced a similar reduction in COX-2 gene expression that 5 μg mL^{-1} of carnosic acid and carnosol pure standards, indicating that other compounds presented in the extracts were also implied in the anti-inflammatory effect. In this sense, camphor and 1,8-cineole pure standards also presented a reduction in COX-2 expression, in both anti-inflammatory models.

In conclusion, the results obtained in this work indicated that although carnosic acid and carnosol content in supercritical rosemary extracts played a crucial role in the anti-inflammatory properties of the extracts, it can be assumed a synergistic interaction of carnosic acid, carnosol and other compounds presented in rosemary supercritical extracts. Consequently, supercritical extracts reported in this work presented an important alternative to the use of carnosic acid and carnosol with a costly purification procedure. Furthermore, supercritical extracts used in this work also shown an important anti-inflammatory effect in an atherosclerotic environment and presented a highly potential application in the prevention of atherosclerosis. All the results obtained provided the basis for increasing the applicability of supercritical rosemary extracts in formulations for the prevention of inflammatory diseases.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. GC-MS identification, peak area contribution (normalized area percent) of compounds found in supercritical rosemary extracts.

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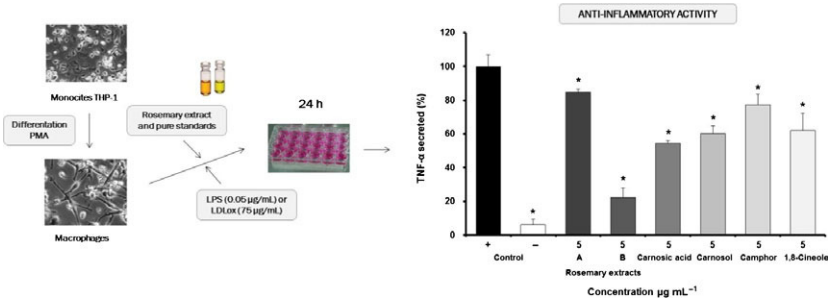

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