Supercritical sage extracts as anti-inflammatory food ingredients

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Abstract

The anti-inflammatory capacity of supercritical CO_2 extracts (S1 and S2) obtained from sage (*Salvia officinalis*) was evaluated using THP-1 human macrophages activated with human ox-LDL, a specific *in vitro* model to determine the anti-inflammatory effect of the extracts in an atherosclerotic environment. The expression of pro-inflammatory cytokines, with an important role in the atherogenic process, such as TNF-α, IL-1β and IL-6, in presence of different extracts concentrations was evaluated. Results showed that 30 μg/mL of both supercritical extracts (S1 and S2) markedly suppressed the ox-LDL induced production of TNF-α, IL-1β and IL-6, as well as their mRNA expression. Data showed that S1 presented a higher anti-inflammatory activity than S2.

A characterization by GC-MS of sage extracts identified 16 compounds, mainly camphor, borneol and 1,8-cineole. These three compounds represented a 62.4% of S1 and a 48.1% of S2. Camphor, borneol and 1,8-cineole presented an important anti-inflammatory activity in the proposed model, with a decrease in the release and gene expression of TNF-α, IL-1β and IL-6 and an increase in IL-10 expression. These results explained the higher activity found in S1.

This study suggested that supercritical sage extracts could be used as food ingredients in the development of anti-inflammatory/anti-atherogenic products.

1. Introduction

Atherosclerosis consists of cholesterol deposition in the intima of large and medium size arteries, accompanied by a chronic inflammatory process (Barter, 2005). A large body of evidence supports the key role of oxidized low-density lipoproteins (ox-LDL) in the early inflammatory and more advances stages of the atherosclerosis lesions. Ox-LDL are not recognized by the LDL receptor apo (B/E), but taken up in a non-regulated manner by the scavenger receptors in monocytes-macrophages and endothelial cells. This process leads to the accumulation of cholesterol in the macrophages, forming foam cells, the hallmark of the atherosclerosis lesion (Chouinard *et al*., 2008). In addition, these ox-LDLs can induce the expression of adhesion molecules, pro-inflammatory cytokines (such as TNF-α, Il-1β, and IL-6) and other mediators of inflammation in macrophages and endothelial cells (Kaperonis *et al*., 2006).

The functional role of herbs and spices and their constituents is a hot topic in food related plant research. Herbs and spices, that have been added to foods since ancient times, mainly to modify or improve their flavor, are nowadays also appreciated for their biological activities. Extracts and essential oils obtained from several herbs and spices present different biological activities, including antioxidant, anti-inflammatory, antitumoral, antiviral and antimicrobial activities (Hamdy *et al.*, 2013; Chan *et al.*, 2011; Tajkarimi *et al.*, 2010; Kaefer and Milmer, 2008; Santoyo et al., 2006a; Santoyo et al., 2006b; Shin and Kim, 2002). The genus *Salvia* is, with about 900 species, one of the most widespread members of the *Lamiaceae* family. Although sage is a popular kitchen herb, it has one of the longest histories of use as medicinal herb. The positive benefits of *Salvia officinalis* (common sage) to health are reputed throughout Ancient Romans times to the Middle Ages; thus this herb has been commonly used for treating

colds, coughs and bronchial infections. Some studies have reported that species of the genus *Salvia* possess specific characteristics which could be used as anti-inflammatory agents. The *in vitro* anti-inflammatory activity of several essential oils and solvent extracts of *Salvia* species was evaluated using the 5-lipoxygenase assay, indicating that, in general, essential oils exhibited better anti-inflammatory activity than solvents extracts (Kamatou *et al*., 2005). Kamatou *et al*. (2008) reported the anti-inflammatory activity of the essential oils of *Salvia rapens, Salvia runcinata* and *Salvia stenophylla* measuring the inhibition of cyclo-oxigenase-1 (COX-1) and COX-2, two key enzymes in the inflammation process. The results indicated that the three essential oils inhibited COX-2 enzyme, being *Salvia stenophylla* the most active. Also Mueller *et al*., (2010) indicated that an extract from *Salvia officinalis* presented anti-inflammatory activity, since this extracts moderately reduced the secretion of pro-inflammatory cytokines like IL-6 and TNF-α. In the same way, Wang *et al*. (2006), reported that an extract from *Salvia miltiorrhiza* was able to suppress LPS-induced TNF-α in human peripheral blood leukocytes and RAW 264.7 macrophage cell line. Using the same inflammation model, more recently Abu-Darwish *et al.* (2013) described a reduction in NO production after the incubation of activated cells with common sage extracts obtained by hydrodistillation. Besides, Ehrnhöfer-Ressler *et al.* (2013) indicated the anti-inflammatory activity of a *S. officinalis* infusion was due to a reduction of IL-6 and IL-8 release in activated human gingival fibroblasts. Using an *in vivo* model of inflammation, carrageenan-induced edema in rats, different extracts obtained from *Salvia* species showed anti-inflammatory and anti-edematous activity (Çadirci *et al.*, 2012), specifically hydroalcoholic extracts showed anti-inflammatory activity, correlated with inhibition of inflammatory mediators release (Alves *et al.*, 2012).

In recent years, supercritical fluid extraction (SFE) has received increased attention as an important alternative to the traditional solvent extraction methods, since this technique provides a high speed and efficiency of extraction, eliminates concentration steps and avoids the use of organic solvents which are potentially harmful in terms of environmental impact. SFE is an extraction/fractionation method that exploits the unique properties of gases above their critical points to extract soluble components from a raw material. Carbon dioxide is an ideal solvent for the extraction of some classes of natural substances from food because is non-toxic, non-explosive, readily available and easy to remove from extracted products. In that way, the quality of supercritical fluid extracts is higher than those obtained by extraction solvents, water or steam distillation, since these methods can induce thermal degradation or present the problem of toxic residual solvent in the products (Diaz-Maroto *et al*., 2002). In particular, SFE has been applied to obtain extracts and fractions from *S. officinalis* essential oils with a higher terpenoids content than when employing traditional techniques (Aleksovski and Sovová 2007; Gañán and Brignole, 2013; Maksimovic *et al*., 2013). Thus, the combination of SFE and other extraction methods, such as ultrasound-assisted extraction, have been also used to concentrated sage extracts in diterpenes, the main valuable components (Glisic *et al.*, 2011).

The aim of this paper was to study the anti-inflammatory capacity of *Salvia officinalis* supercritical extracts using THP-1 human macrophages activated with human ox-LDL. This model of inflammation allowed 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. Also, this model has been used only in few studies, since most of anti-inflammatory studies reported used

lipopolysaccharide to activate macrophages. Further, this work analysed the chemical composition of the extracts and intended to establish a relationship between the extracts' activity and their composition.

2. Material and methods

2.1 Samples and chemicals

Sage (*Salvia officinalis*) sample consisted of dried leaves obtained from a herbalist´s shop (Murcia, Spain). Cryogenic grinding of the samples was performed under liquid nitrogen. The size of the particle was determined by passing the ground plant material through sieves between 1000-500 µm (CISA, Barcelona, Spain). The whole sample was stored at – 20ºC until use.

1,8-cineole, camphor and borneol standards were purchased from Sigma (Madrid, Spain). CO_2 (N38 quality) was supplied from Air Liquid (Madrid, Spain).

2.2 Extraction methods

Supercritical extractions were carried out using a pilot-plant supercritical fluid extractor (Thar Technology, Pittsburgh, PA, USA, model SF2000), 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 0.6 kg of the cryogenically milled and sieved plant particles. Extraction assays were performed at 30 MPa and 313 K, with a CO_2 flow rate of 50 g/min. Temperature was set to 313 K in both S1 and S2 separators. In the first separator (S1) the pressure was maintained at 10 MPa, while in the second separator (S2) the

pressure was ambient pressure. The cascade decompression system produced two different extracts with different composition which were collected in separator 1 (S1) and separator 2 (S2) respectively. According to previous kinetic studies the overall extraction time was set to be 8 h (García-Risco *et al*., 2011).

Solid-liquid (S/L) extractions using ethanol and methanol with chloroform were carried out according to the methods describe by Kamatou *et al*. (2010, 2005), with several modifications. For each experiment, 2 g of cryogenically milled and sieved plant particles were dissolved in 100 mL of 100% of ethanol or 100 mL of methanol:chloroform (1:1) to obtain two different extracts. In both cases the extraction time was 4 h at 310 K with shaking (200 rpm) and the process was repeated 3 times. The extracts were obtained by removing the solvents with a rotary evaporator at 40 °C.

2.3 GC-MS analysis

Characterization of the supercritical sage extracts 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 GCMS Solution software. The column used was a ZB-5 (Zebron) capillary column, 30 m x 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 ºC isothermal for 4 min, increased to 64 ºC at 1 ºC/min, then increased to 106 ºC at 2.5 ºC/min. Oven temperature was then increased from 106 ºC to 130 ºC at 1 ºC/min, and then to 200 ºC at 5 ºC/min, and then to a final temperature of 250 ºC/min at 8 ºC/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 ºC and MS ion source and interface temperatures were 230 and 280 ºC, respectively. The mass

161 spectrometer was used in TIC mode, and samples were scanned from 40 to 500 amu.
162 Compounds 1,8-cineole, camphor, borneol and linalool were identified by comparison
163 with standard mass spectra obtained in the same conditions and compared with the mass
164 spectra from library Wiley 229. The rest of the compounds were identified by
165 comparison with the mass spectra from Wiley 229 library and by their linear retention
166 index.

167

168 *2.4 Isolation and oxidation of LDLs*

169 LDLs were isolated from human plasma as described before (Havel *et al*., 1995).
170 Oxidation of LDLs was done by incubating LDLs with 5µM $CuSO_4$ for 3h at 37ºC.
171 Oxidation degree was measured as the amount of thiobarbituric acid reactive substances
172 (TBARS) produced.

173

174 *2.5 Cell culture and treatment*

175 Human THP-1 monocytes (American Type Culture Collection, ATCC) were cultured in
176 RPMI 1640 culture medium (Gibco, Spain) supplemented with 10% FBS, 100 U/mL
177 penicillin, 100 mg/mL streptomycin, 2 mM L-glutamine and 0.05 mM β-
178 mercaptoethanol at 37ºC in 95% humidified air containing 5% CO_2. Cells were
179 collected and plated at a density of $5x10^5$ cells/mL in 24 wells plates. Differentiation to
180 macrophages (THP-1/M cells) was induced by maintaining the THP-1 cells in the
181 presence of 100 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma, Spain) for 48h.
182 After differentiation, cells were washed with PBS and incubated with 75 µg/mL ox-
183 LDLs in presence of different concentrations of supercritical extracts, S/L extracts or
184 pure standards for 24h in a FBS free medium. Then, the supernatant was frozen at -80ºC

and cells RNA isolated. Indomethacin (4 μg/mL), an anti-inflammatory drug, was used as a reference.

2.6 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, Spain), according to a publish method (Mosmann, 1983). THP-1/M cells in 24-multiwell plates were incubated with RPMI containing different concentrations of the extracts for 24h at 37ºC. Cells were then washed with PBS and 0.5 mg/ml of MTT were added to each well and incubated 4h at 37ºC. Supernatants were discarded and formazan crystals dissolved in an extraction solution (10% sodium dodecyl sulphate in a mixture of dimethyl formamide and water 1:1 v/v, adjusted to pH 4.7 with acetic acid) overnight at 37ºC. Formazan quantification was performed by measuring the optical density at 570 nm using a multiscanner autoreader (Sunrise, Tecan) with the extraction solution as a blank. The data were plotted as dose-response curves, from which the concentration required to reduce 50% the number of viable THP-1/M (CC_{50}) after 24 h of incubation with the different extracts were obtained.

2.7 Quantification of cytokines by ELISA

The release of IL-1β, IL-10, IL-6 and TNF-α was measured in the supernatants of THP-1/M cells treated with ox-LDL in presence of different concentrations of sage extracts and standards using ELISA kits (BD biosciences, Spain), according to manufacturer's instructions. The color generated was determined by measuring the OD at 450 nm using a multiscanner autoreader (Sunrise, Tecan).

209

210 *2.8 RNA isolation and RT-PCR*

211 RNA was isolated from THP-1/M cells using Trizol® (Invitrogen, Spain) according to
212 manufacturer's instructions. Reverse transcription (RT) of the RNA was performed
213 using High Capacity Archive Kit and GeneAmp PCR System 9700 (Applied
214 Biosystems, Carlsbad, CA, USA) according to the manufacturer´s instructions to obtain
215 20 ng/µL of cDNA. PCR amplification was conducted in a 10 µL reaction mixture with
216 cDNA, Taqman Gene Expression Master Mix (Applied Biosystems) and TaqMan
217 probes (Applied Biosystems) according to the manufacturer´s conditions in a 7900HT
218 Fast Real-Time PCR System (Applied Biosystems). The TaqMan probes used were as
219 follows: Hs99999029_m1 for IL-1β, Hs00174131_m1 for IL-6, Hs99999035_m1 for
220 IL-10, Hs00174128_m1 for TNF-α and Hs99999901_s1 for 18S rRNA. Expression of
221 genes was normalized relative to 18S rRNA using SDS Software v2.4 (Applied
222 Biosystems).

223

224 *2.9 Statistical analysis*

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

229

230 **3. Results**

231 Extraction of sage leaves by using supercritical carbon dioxide was carried out on a
232 pilot scale plant. Usually, the first step was to determine both, the working pressure and

temperature, since the optimisation of the experimental conditions represent a critical step in the development of a SFE process. In this work, the extraction pressure and temperature were set to 30 MPa and 313 K respectively, using pure CO_2 since previous experiments using extraction pressures of 12, 18 and 35 MPa resulted in extracts with a lower quantity of essential oils than using 30 MPa (data not shown). These results were also in agreement with previous studies done in our laboratory with rosemary, oregano and laurel leaves (García-Risco *et al.*, 2011; Ocaña-Fuentes *et al.*, 2010; Santoyo *et al.*, 2006a; Santoyo *et al.*, 2006b). As already mentioned, the extraction system employed in the present study allows a cascade of depressurization providing two different extracts: S1 and S2. The main difference between S1 and S2 extracts was the fractionation pressure, which brought about a gradual precipitation of the extracted compounds on the basis of their solubility in the extracting agent at the conditions set in each separator. These two extracts are expected to present different characteristics in terms of both, composition and functional activity. Therefore, in this work, the anti-inflammatory activity of S1 and S2 extracts of sage using an *in vitro* model of atherosclerosis was investigated.

3.1 Effects of supercritical sage extracts on THP-1/M viability

Sage supercritical extracts (S1 and S2) were initially evaluated for cytotoxicity on THP-1/M cells by MTT method. The CC_{50} data obtained (Table 1) indicated that extract recovered in separator 2 presented a lower cytotoxicity than extract from separator 1. However, at the higher concentration used in the (anti-inflammatory) assays, 30 µg/mL, both extracts (S1 and S2) presented no cytotoxicity. Also S/L extracts at 30 µg/mL, presented no cytotoxicity.

258 *3.2 Effect of sage extracts on the cytokines release*

259 The activation of THP-1/M was carried out with the addition of ox-LDLs to the
260 medium. These ox-LDLs treated cells showed, after an incubation period of 24h, an
261 important increase in the release of all pro- and anti-inflammatory cytokines tested
262 (TNF-α, IL-1β, IL-6 and IL-10) compared to non-activated controls (Figure 1). These
263 activated cells were considered as positive controls for all the cytokines tested. When
264 the activation of THP-1/M was carried out in presence of sage extracts, a decrease in
265 TNF-α secreted level was observed (Figure 1A), compared with levels obtained in
266 absence of extracts (positive control). Moreover, a very significant decrease in the
267 amount of TNF-α secreted was obtained with 30µg/mL of extracts, where TNF-α
268 secretion was even lower than basal level in non-activated cells. At this concentration,
269 S1 extract produced a lower TNF-α secretion than S2 extract. Regarding to IL-1β
270 secretion by activated cells in presence of sage extracts (Figure 1B), it can be also
271 observed an important decrease in the secretion of this cytokine, with values close to
272 basal levels presented by non-activated cells. However, in this case, the different
273 concentrations of sage extracts employed presented a similar decrease in IL-1β release,
274 indicating that the decrease was not related to the extract concentration. This data could
275 be explained because the lowest concentration employed in this work (10µg/mL) was
276 already able to decrease the IL-1β release to basal levels. In this case, S1 extract
277 induced a lower secretion of IL-1β than S2 extract too. The activation of macrophages
278 in presence of supercritical extracts also produced a decrease in the IL-6 release (Figure
279 1C), although an extract concentration higher than 20µg/mL was required. With this
280 cytokine, S1 and S2 extracts presented a similar activity. Regarding IL-10, an anti-
281 inflammatory cytokine, the presence of extracts did not increase its release.

These data indicated that supercritical sage extracts presented anti-inflammatory activity in THP-1 human macrophages activated with human ox-LDL, since these extracts effectively inhibited the release of pro-inflammatory cytokines as TNF-α, Il-1β and IL-6.

In order to compare the results obtained with supercritical extracts with S/L extracts, 30 µg/mL of these S/L extracts were added to THP-1/M activated with ox-LDLs (data not included). Results shown that methanol:chloroform extract decreased the secretion of TNF-α and IL-6 compared to positive control, meanwhile IL-1 β was not affected. However, ethanol extract only reduced the secretion of IL-6. When compared these results with those obtained with supercritical extracts, it can be concluded that S/L extracts presented a lower decrease of pro-inflammatory cytokines secretion.

3.3 Effect of supercritical sage extracts on the cytokines mRNA expression

In order to determine if the influence of the supercritical sage extracts in cytokine production was related to gene expression, total cellular RNA was extracted from activated THP-1/M and available for RT-PCR. The effect of sage extracts (30µg/mL) on TNF-α, IL-1β, IL-6 and IL-10 mRNA expression in THP-1/M after 24h of ox-LDLs activation is presented in Figure 2. TNF-α gene expression was significantly reduced by both S1 and S2 extracts, compared to the expression of activated cells in absence of the extracts. Similar results were obtained for IL-1β and IL-6, where extracts significantly decreased the expression of these interleukins. The decrease in TNF-α, IL-1β and IL-6 gene expression was in agreement with cytokine release indicated before, which strengthen the anti-inflammatory activity of sage extracts.

305 Regarding data obtained with IL-10, sage extracts significantly increase its mRNA
306 expression, although, as shown previously, these extracts did not increase the release of
307 IL-10. This result could be explained due to IL-10 presented a slower response to ox-
308 LDL activation than other interleukins tested (Wasaporn *et al.*, 2012).

309

310 *3.4 GC-MS characterization of supercritical sage extracts*

311 In an attempt to identify the compounds responsible for the anti-inflammatory activity
312 found in supercritical sage extracts, a characterization by GC-MS of these samples was
313 carried out. Results obtained are shown in Table 2, where a tentative identification has
314 been performed based on the comparison of mass spectra and retention index (RI). As
315 can be observed, 16 compounds were identified. Some of them were detected in large
316 amounts like camphor, borneol and 1,8-cineole. The sum of these three compounds
317 represented a 62.4% of the S1 extract; meanwhile extract obtained on separator 2
318 contained a smaller quantity (48.1%) of these compounds. In order to correlate the anti-
319 inflammatory activity found in the supercritical extracts with their chemical
320 composition, the cytotoxicity and anti-inflammatory activity of pure standards of these
321 three main components of the extracts (camphor, borneol and 1,8-cineole) were also
322 examined at the same conditions. The cytotoxicity assays revealed that these
323 compounds were more cytotoxic than supercritical extracts, specially 1,8-cineole and
324 borneol (Table 1). The concentrations used to carry out the experiments (7.5 and 5
325 µg/mL) presented 100% cell viability in all cases.

326 When the activation of THP-1/M was carried out in presence of camphor, borneol and
327 1,8-cineole, an important decrease in TNF-α secreted level was observed, especially
328 when employing 7.5 µg/mL (Figure 1A). At this concentration, borneol and 1,8-cineole

were more active than camphor, since they produced a lower TNF-α secretion. Figure 1B also showed an important decrease in the secretion of IL-1β, with values close to basal levels, with 7.5 µg/mL of camphor, 1,8-cineole and borneol. In this case, no differences were found among the three compounds. The IL-6 release (Figure 1C), also presented a great decrease in presence of camphor, 1,8-cineole and borneol. These data indicated that the three compounds presented an important anti-inflammatory activity. However, camphor, 1,8-cineole and borneol did not increase the release of the anti-inflammatory cytokine IL-10 (Figure 1D) at the concentrations used.

Also, formulation of model mixtures with pure standards, mimicking the composition of the S1 and S2 extracts based on the compositions of Table 2, were carried out. When the activation of THP-1/M was studied in presence of these mixtures, at 30 µg/mL, an important decrease in all cytokines studied was found (Figure 1). When these results were compared with those obtained with supercritical extracts, no significant differences were found.

The effect of camphor, 1,8-cineole and borneol (7.5µg/mL) on TNF-α, IL-1β, IL-6 and IL-10 mRNA expression in THP-1/M after 24h of ox-LDLs activation was also studied (Figure 2). TNF-α, IL-1β and IL-6 gene expression was significantly reduced by the three compounds tested. Regarding IL-10 gene transcription, an important increase was observed when activation was carried out in presence of camphor, borneol and 1,8-cineole. Surprisingly, this important increase in IL-10 gene expression was not related with data obtained in the secretion of these IL-10 measured by ELISA. This result, found also for sage extracts, could be related with the time of the treatment, since a 24h treatment could be able to increase the gene expression but not the release of this

cytokine to medium, due to IL-10 presented a slower response to ox-LDL stimulation than other cytokines.

Thus, these compounds presented an important anti-inflammatory activity in the proposed model, with a decrease in the release and gene expression of pro-inflammatory cytokines and an increase in gene expression of IL-10, an anti-inflammatory cytokine. However, data did not show important differences among the activity of the three compounds. Accordingly borneol, camphor and 1,8-cineole could be proposed as the principal compounds responsible for the anti-inflammatory activity found in supercritical sage S1 and S2 extracts. Data also explained the higher activity found in extract obtained in S1, since this fraction contained a higher quantity of borneol, camphor and 1,8-cineole than extract S2). TNF-α, IL-1β and IL-6 gene expression using model mixtures of pure standards did not presented significant differences compare with results obtained when using supercritical extracts (data not shown).

4. Discussion

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). Ox-LDL can induce the expression of pro-inflammatory cytokines, such as IL-1β, TNF-α and IL-6 from monocytes and macrophages. IL-1β and TNF-α are primary pro-inflammatory cytokines, which are mainly produced by monocytes and macrophages, and play a crucial role in the initial amplification of the inflammatory response. *In vivo* and *in vitro* research has highlighted their potential pro-atherogenic action. In particular, it has been shown that both IL-1β and TNF-α could induce the

expression of adhesion molecules by endothelial cells, promote secretion of different cytokines and chemokines by monocytes, and may enhance the smooth muscle cells proliferation and migration (Rattazzi *et al.*, 2004). They can also actively participate in the process of foam cell formation mainly through the induction of grown factors. These cytokines also play a crucial role in the later stage of atheroma development by hampering plaque stability (Young *et al.*, 2002). IL-6 is a secondary inflammatory cytokine produced by different kinds of cellular elements such as activated macrophages, lymphocytes, fibroblast, and vascular smooth muscle cells under stimulation by IL-1 and TNF-α. IL-6 may exert an important direct pathogenic role in atherosclerosis development and progression, especially through inflammatory elements recruitment and smooth muscle cells activation (Rattazzi *et al.*, 2004).

The aim of this study was to investigate the anti-inflammatory capacity of supercritical *Salvia officinalis* extracts using THP-1 human macrophages activated with human ox-LDL, since this model of inflammation allowed 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. The results suggested that supercritical sage S1 and S2 fractions may act as effective inhibitors of ox-LDL induced pro-inflammatory cytokines (TNF-α, IL-1β and IL-6). Moreover, a very significant decrease in the secretion of these cytokines was obtained with 30 µg/mL of sage extracts, since pro-inflammatory cytokines secretion levels with this concentration were even lower than its basal level in non activated cells. These results were also in agreement with the ones obtained regarding the transcription of cytokines genes at 24 h of incubation. In that way, Mueller *et al.* (2010), also reported that an extract from sage leaves was able to reduce the secretion of pro-inflammatory cytokines

399 TNF-α and IL-6 in a lipopolysaccharide-stimulated macrophage model, although the
400 extract concentrations employed in this study were 0.2-0.5 mg/mL. Other authors also
401 reported the anti-inflammatory activity of sage extracts, although the anti-inflammatory
402 activity was evaluated using the 5-lipoxigenase enzyme (Kamatou *et al.*, 2010). The
403 IC_{50} values reported for sage extracts against 5-lipoxygenase enzyme were greater than
404 100µg/mL, with the exception of *Salvia radula* (IC_{50} value: 78.8µg/mL). Besides, a
405 *Salvia miltiorrhiza* extract also suppressed LPS-induced TNF-α in human peripheral
406 leukocytes and the Raw 264.7 macrophage cell line (Wang *et al.*, 2006). Therefore, it is
407 likely that small doses of supercritical sage extracts (30 µg/mL) inhibit ox-LDL induced
408 TNF-α, IL-1β and IL-6 production by THP-1 human macrophages preventing cytokine
409 stimulation, which may contribute to the anti-inflammatory effect in atherosclerotic
410 condition.

411 IL-10 is an anti-inflammatory cytokine that inhibits the production of IL-2 and INF-γ by
412 Th1 cells, reduces pro-inflammatory cytokines production and down-regulated
413 eosinophil function and activity (Konno *et al.*, 2006). Results obtained in this work
414 indicated that sage supercritical extracts significantly increase IL-10 mRNA expression
415 by the activated macrophages, although these extracts did not produce a significant
416 increased in IL-10 release at 24h of stimulation. According to these data, Chen *et al.*
417 (2011), reported that Salvianolic acid B, a compound isolated from dry root of *Salvia*
418 *miltiorrhiza* enhanced the expresion of IL-10.

419 Main compounds presented in supercritical sage extracts were camphor, borneol and
420 1,8-cineole. These compounds represented a 62.4% in S1 extract and a 48.1% in S2
421 extract. These results indicated that the fractionation step increased the oxygenated
422 monoterpenes composition in the extract obtained in the first separator. Similar results

were reported by Fornari *et al.* (2012) using a similar procedure. Gañán and Brignole (2013) also showed that a SFE fractionation of *S. officinalis* essential oil enriched sage extract in oxygenated monoterpenes, being 1,8-cineol, α-thujone, β-thujone and camphor the main compounds.

A concentration of 7.5 μg/mL of 1,8-cineol, camphor and borneol showed an important decrease in both soluble levels and gene expression of TNF-α, IL-1β and IL-6 by stimulated THP1/M. IL-10 gene expression at 24h was also up-regulated by the presence of 7.5 μg/mL camphor, borneol and 1,8-cineole. The anti-inflammatory activity showed by these components was in agreement with the results showed by Juergens *et al*. (2004), who reported 1,8-cineole as a strong inhibitor of TNF-α, IL-1β and IL-6 production by human unselected lymphocytes and LPS-stimulated monocytes. Santos and Rao (2000), also reported the inhibitory effect of 1,8-cineole, a terpenoid oxide present in many plant essential oils, on the pro-inflammatory cytokines production by stimulated monocytes. Regarding borneol, Tung *et al.* (2008), indicated that this compound exhibited excellent anti-inflammatory activity in suppressing nitric oxide production by LPS-stimulated macrophages. In addition, camphor has been also reported to have significant anti-inflammatory activity (Yoon *et al.*, 2010; Chainy *et al.*, 2000). The mixtures prepared with camphor, 1,8-cineol and borneol in order to simulate the composition of extract S1 and S2 have showed a similar anti-inflammatory effect than the supercritical extracts. These results were expected since these pure standards represented a 62.4% of the S1 extract and a 48.1% of S2 and these compounds, when using individually, have demonstrated a potent anti-inflammatory activity.

Concluding, supercritical fluid extraction allowed the production of sage extracts with an important anti-inflammatory activity using an atherosclerotic environment model.

These extracts act as effective inhibitors of ox-LDL induced pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) and therefore have a great potential to be used as anti-inflammatory agents in the prevention of atherosclerosis.

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578

Table 1. THP-1/M cell viability in presence of supercritical sage extracts and pure standards. CC_{50} (cytotoxic concentration 50%): concentration required to reduce 50% the number of viable THP-1/M cells after 24 h of incubation with the compounds. [a,b,c,d] Different superscript letters indicated significant differences ($p<0.05$) among data.

Sample	**CC_{50} (µg/mL)**
Sage S1	66.70 ± 5.41 [a]
Sage S2	80.24 ± 7.58 [b]
Camphor	55.75 ± 6.12 [c]
1,8-Cineole	35.32 ± 4.21 [d]
Borneol	26.93 ± 2.78 [d]

598 **Table 2.** GC-MS identification, peak area contribution (normalized area percent), and
599 retention index (RI) of compounds found in supercritical extracts (S1 and S2) from sage
600 (*Salvia officinalis*). NI: non-identified compound, n.d.: non detected.

601

Retention time (min)	RI	Compound	S1 normalized area (%)	S2 normalized area (%)
13.33	1029	1,8-Cineole	13.34	4.64
17.38	1099	Linalool	1.16	1.10
19.64	1138	Cis-sabinol	2.05	n.d.
19.78	1140	Camphor	41.78	33.9
21.07	1163	Borneol	7.28	9.56
22.55	1188	Alpha-terpineol	1.16	1.81
26.44	1253	Linalyl acetate	4.86	5.81
28.19	1282	Endobornyl acetate	3.19	3.46
28.70	1291	Sabinyl acetate	5.00	5.94
32.60	1345	α-terpinenyl	3.68	4.71
37.84	1412	E-cariophyllene	2.33	2.97
40.65	1484	α-humulene	1.75	2.41
43.05	1535	Geranyl propionate	1.25	1.91
51.19	1575	Spathulenol	1.01	2.05
51.48	1579	Cariophyllene oxide	1.03	1.61
52.04	1586	viridiflorol	2.00	4.02
56.61	1685	NI	2.11	5.06

602

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605

606 **Figure legends**

607 **Figure 1:** Levels of TNF-α (1A), IL-1β (1B), IL-6 (1C) and IL-10 (1D) secreted by
608 THP-1/M activated with ox-LDL in presence of supercritical sage extracts, pure
609 standards or mixtures of pure standards simulate sage S1 and S2 extracts. Each bar is
610 the mean of three determinations ± standard deviation. * Denotes statistical differences
611 between control+ox-LDL and the other samples at $p<0.05$. a,b Columns with different
612 superscript letters are significantly different at $p<0.05$ (comparison between S1 and S2
613 sage extracts and mixtures of pure standards at 30 μg/mL).

614

615 **Figure 2:** mRNA expression of TNF-α (2A), IL-1β (2B), IL-6 (2C) and IL-10 (2D) on
616 THP-1/M stimulated with ox-LDL in presence of supercritical sage extracts or pure
617 standards. Each bar is the mean of three determinations ± standard deviation. RQ:
618 relative quantification. * Denotes statistical differences between control+ox-LDL and
619 the other samples at $p<0.05$.

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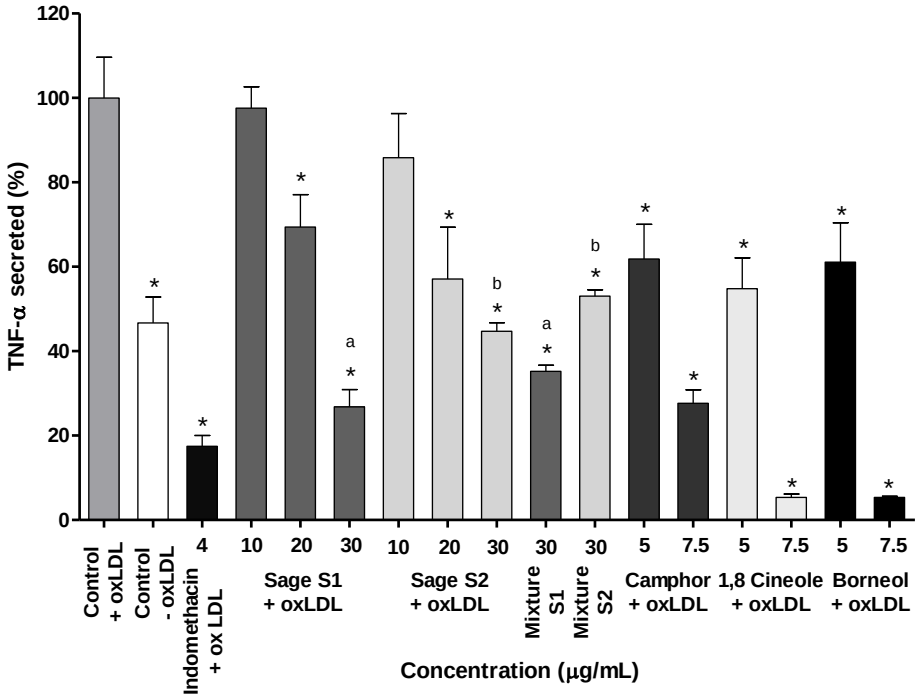
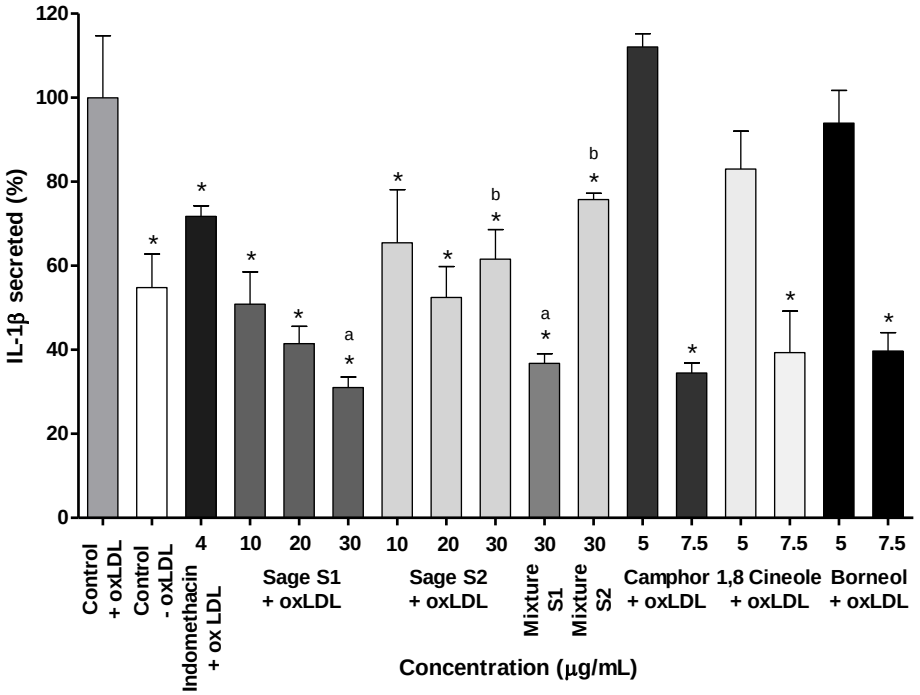
625

626

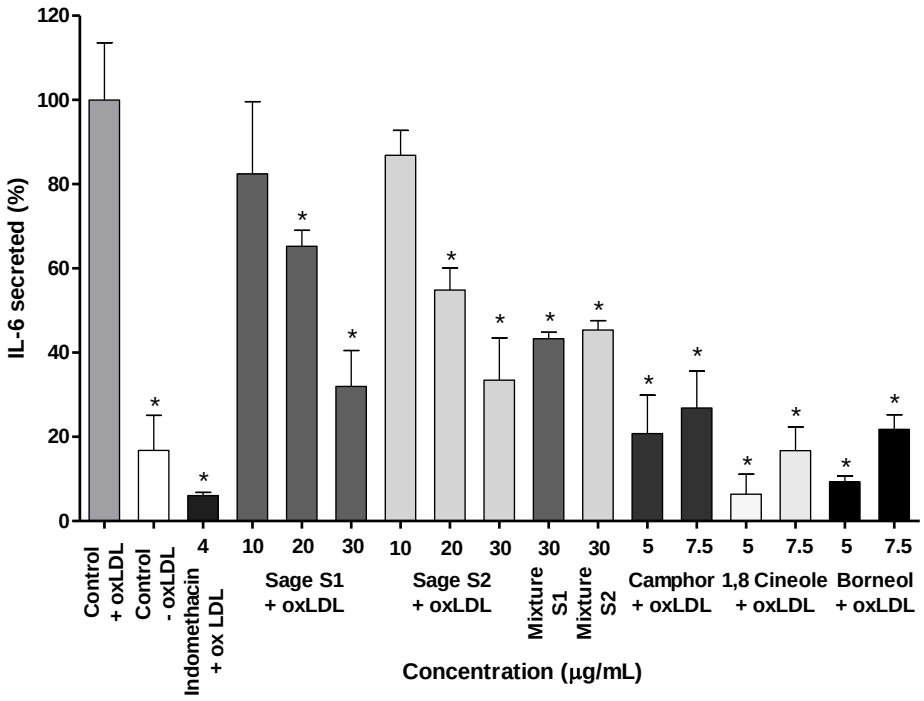
Figure 1


TNF-α secreted (%)
120
100
80
60
40
20
0
Control + oxLDL
Control - oxLDL
Indomethacin + ox LDL
4
10
20
30
Sage S1 + oxLDL
10
20
30
Sage S2 + oxLDL
30
Mixture S1
30
Mixture S2
5
7.5
Camphor + oxLDL
5
7.5
1,8 Cineole + oxLDL
5
7.5
Borneol + oxLDL
Concentration (µg/mL)

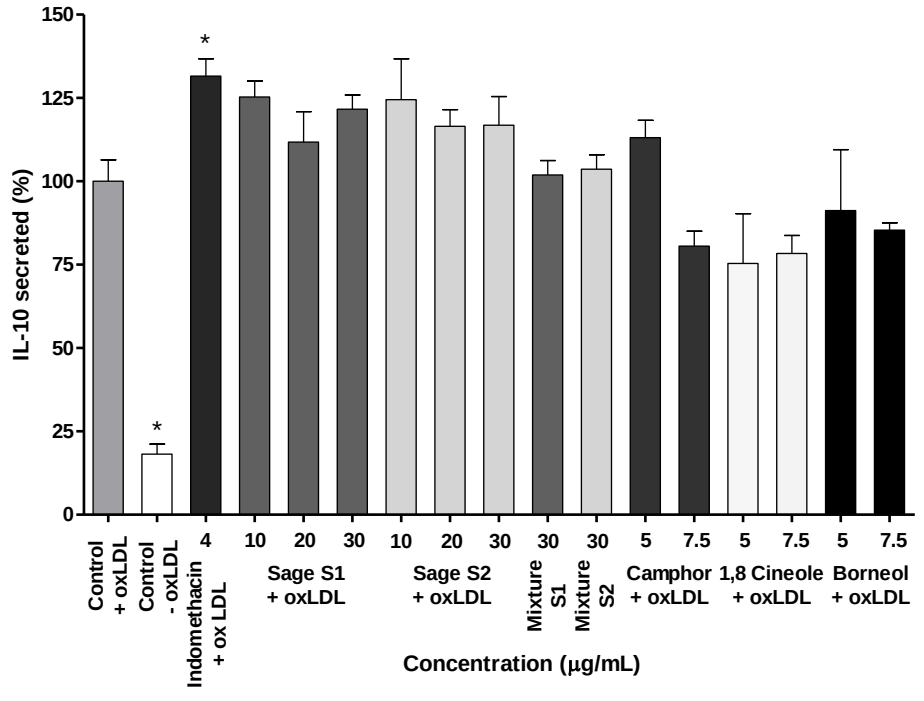
(A)


IL-1β secreted (%)
120
100
80
60
40
20
0
Control + oxLDL
Control - oxLDL
Indomethacin + ox LDL
4
10
20
30
Sage S1 + oxLDL
10
20
30
Sage S2 + oxLDL
30
Mixture S1
30
Mixture S2
5
7.5
Camphor + oxLDL
5
7.5
1,8 Cineole + oxLDL
5
7.5
Borneol + oxLDL
Concentration (µg/mL)

(B)

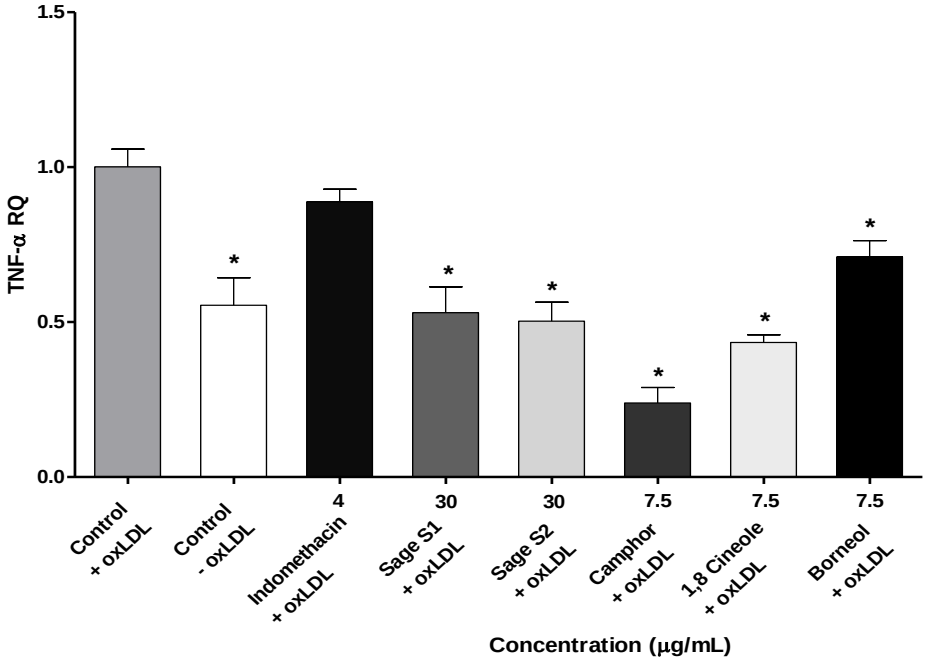

IL-6 secreted (%)
120
100
80
60
40
20
0
Control + oxLDL
Control - oxLDL
Indomethacin + ox LDL
4
10
20
30
Sage S1 + oxLDL
10
20
30
Sage S2 + oxLDL
30
30
Mixture S1
Mixture S2
5
7.5
Camphor + oxLDL
5
7.5
1,8 Cineole + oxLDL
5
7.5
Borneol + oxLDL
Concentration (µg/mL)

(C)

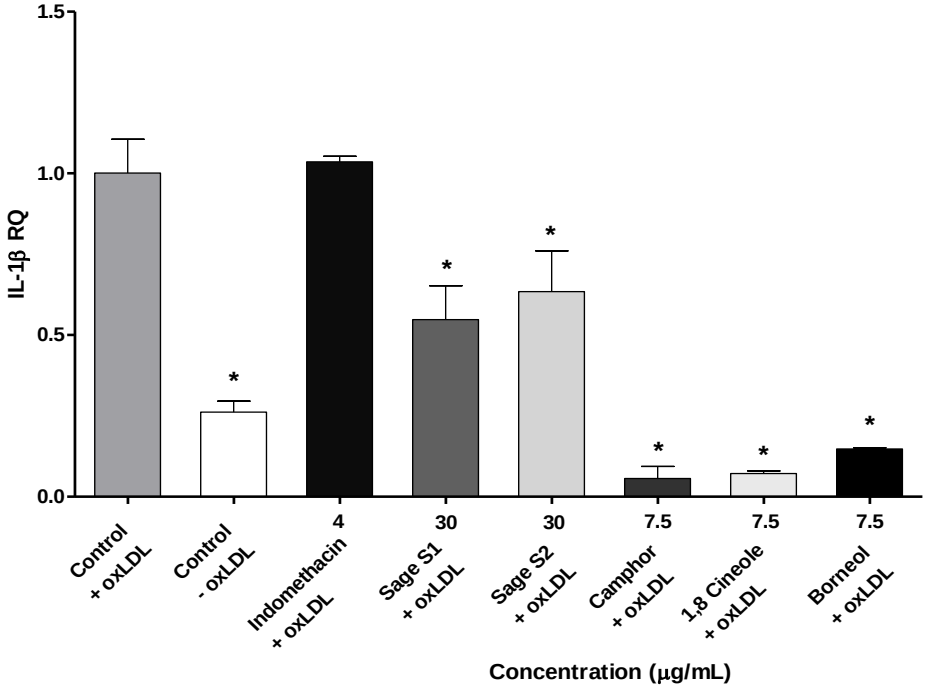

IL-10 secreted (%)
150
125
100
75
50
25
0
Control + oxLDL
Control - oxLDL
Indomethacin + ox LDL
4
10
20
30
Sage S1 + oxLDL
10
20
30
Sage S2 + oxLDL
30
30
Mixture S1
Mixture S2
5
7.5
Camphor + oxLDL
5
7.5
1,8 Cineole + oxLDL
5
7.5
Borneol + oxLDL
Concentration (µg/mL)

(D)

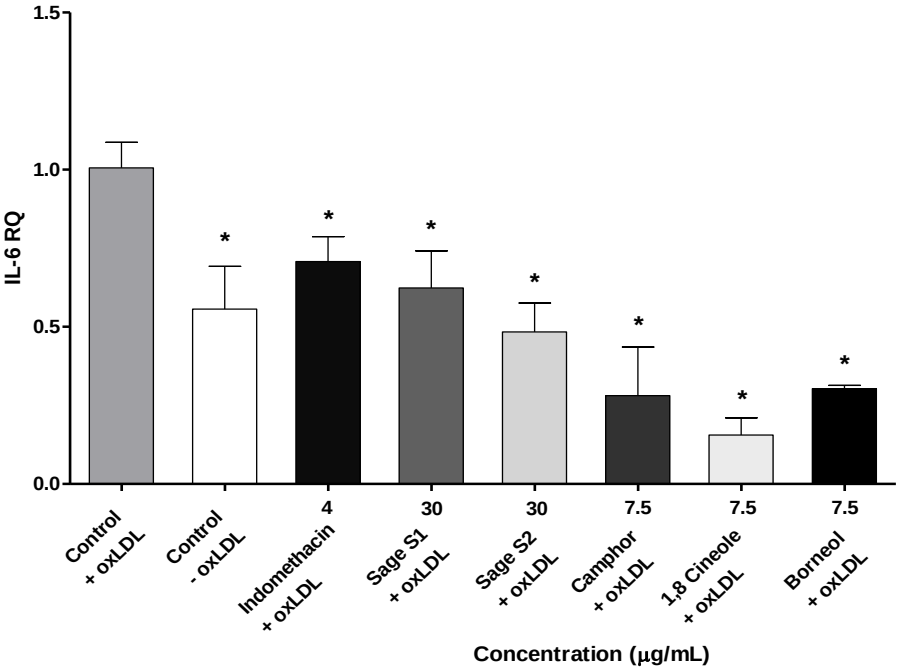
Figure 2


TNF-α RQ
1.5
1.0
0.5
0.0
*
Control + oxLDL
Control - oxLDL
Indomethacin + oxLDL
Sage S1 + oxLDL
Sage S2 + oxLDL
Camphor + oxLDL
1,8 Cineole + oxLDL
Borneol + oxLDL
4
30
30
7.5
7.5
7.5
Concentration (μg/mL)

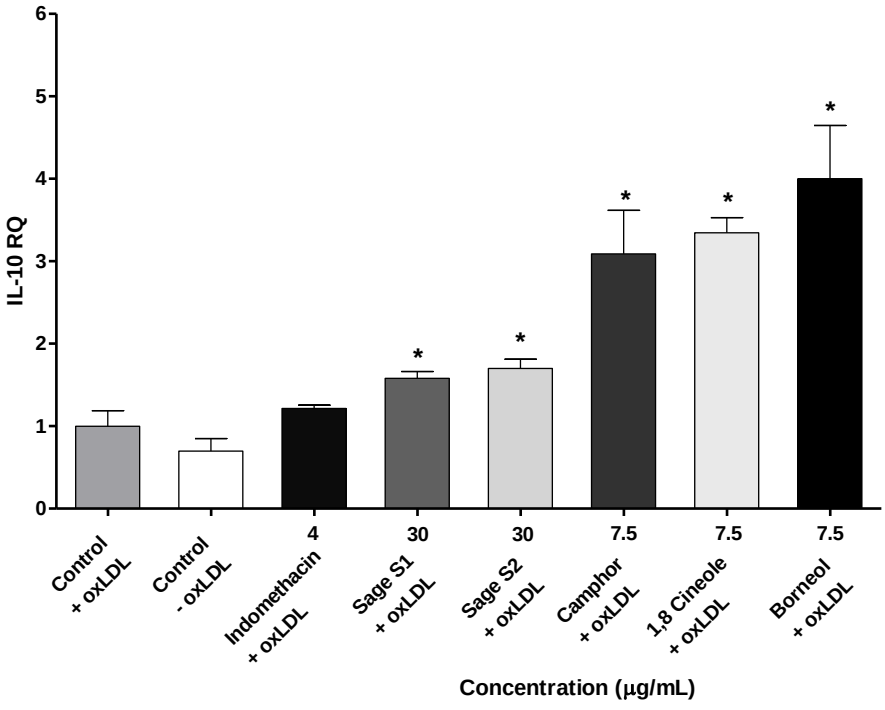
(A)


IL-1β RQ
1.5
1.0
0.5
0.0
*
Control + oxLDL
Control - oxLDL
Indomethacin + oxLDL
Sage S1 + oxLDL
Sage S2 + oxLDL
Camphor + oxLDL
1,8 Cineole + oxLDL
Borneol + oxLDL
4
30
30
7.5
7.5
7.5
Concentration (μg/mL)

(B)


IL-6 RQ
1.5
1.0
0.5
0.0
*
Control + oxLDL
Control - oxLDL
4
Indomethacin + oxLDL
30
Sage S1 + oxLDL
30
Sage S2 + oxLDL
7.5
Camphor + oxLDL
7.5
1,8 Cineole + oxLDL
7.5
Borneol + oxLDL
Concentration (µg/mL)

(C)


IL-10 RQ
6
5
4
3
2
1
0
*
Control + oxLDL
Control - oxLDL
4
Indomethacin + oxLDL
30
Sage S1 + oxLDL
30
Sage S2 + oxLDL
7.5
Camphor + oxLDL
7.5
1,8 Cineole + oxLDL
7.5
Borneol + oxLDL
Concentration (µg/mL)

(D)