Title: Supercritical fluid extraction as an alternative process to obtain essential oils with anti-inflammatory properties from marjoram and sweet basil

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Abstract: The anti-inflammatory capacity of marjoram and sweet basil essential oils obtained by supercritical fluid extraction (SFE) was evaluated using two in vitro inflammation models. For that purpose, 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, besides to COX-2 and NFκB gene expression. Results indicated that 10 μg/mL of both essential oils markedly suppressed the production of pro-inflammatory cytokines in both models proposed. Moreover, both essential oils inhibited the ox-LDL induced production of pro-inflammatory cytokines, COX-2 and NFκB mRNA expression.
Main compounds presented in supercritical basil (linalool and eugenol) and marjoram (sabinene hydrate and terpineol) essential oils also presented an important anti-inflammatory activity, which allowed us to propose these compounds as responsible for the anti-inflammatory activity found in essential oils.
In conclusion, SFE allowed to obtain sage and marjoram essential oils with significant anti-inflammatory properties. Furthermore, these supercritical essential oils have also shown an important anti-inflammatory effect in an atherosclerotic environment and presented a highly potential application in the prevention of atherosclerosis

December, 2014

Prof. Marisol T. Berti

Editor in chief

Industrial Crops and Products

Dear Professor Berti,

Enclosed find the revised version and reviewer´s comments for the manuscript INDCRO-D-14-02072 entitled "Supercritical fluid extraction as an alternative process to obtain essential oils with anti-inflammatory properties from marjoram and sweet basil" by E. Arranz *et al*. All changes in the manuscript have been highlighted in red.

Yours sincerely,

Susana Santoyo

Manuscript INDCRO-D-14-02072 entitled "Supercritical fluid extraction as an alternative process to obtain essential oils with anti-inflammatory properties from marjoram and sweet basil"

Reply to reviewers' comments.

Have your paper read and revised by a native English speaker, the paper needs much improvement. The science is sound, but it is hard to understand your results and the value of them. In next version highlight all changes made.

> As referee suggested, the paper has been read and revised by a native English speaker and all the changes have been highlighted in red.

- *Highlight 4 is not clear. Please modify the sentence.*

> Highlight 4 has been modified.

- *Check for mistakes such as in Line 136.*

> The mistake found in Line 136 (Line 150 in the resubmitted version) has been corrected.

HIGHLIGHTS

- Anti-inflammatory capacity of extracts from marjoram and sweet basil was evaluated

- The extracts markedly suppressed the production of TNF-α, IL-1β, IL-6 and IL-10

- The extracts also inhibited COX-2 and NFκB mRNA expression

- Linalool, eugenol, sabinene hydrate and terpineol could be proposed as the compounds responsible for the anti-inflammatory activity

- The extracts could be used as products with anti-inflammatory and anti-atherogenic properties.

1 **Supercritical fluid extraction as an alternative process to obtain**

2 **essential oils with anti-inflammatory properties from marjoram and**

3 **sweet basil**

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ABSTRACT

The anti-inflammatory ~~capacity~~ potential of marjoram and sweet basil essential oils obtained by supercritical fluid extraction (SFE) was ~~evaluated~~ tested ~~using two~~ *in vitro* using THP-1 human macrophage cells.~~inflammation models. For that purpose,~~ THP-1 ~~macrophages~~ cells were activated ~~using~~ by lipopolysaccharide or human ox-LDL and the cytokine secretion and gene expression of respectively TNF-α, IL-1β, IL-6 and IL-10 along with COX-2 and NFκB gene expression were evaluated~~, besides to COX-2 and NFκB gene expression~~. Results indicated that ~~10 μg/mL of~~ both marjoram and sweet basil essential oils at a concentration of 10 μg/mL markedly suppressed the production of pro-inflammatory cytokines and gene expression in ~~in both models proposed~~LPS and ox-LDL THP-1 activation. ~~Moreover, both essential oils inhibited the ox-LDL induced production of pro-inflammatory cytokines, COX-2 and NFκB mRNA expression.~~The chemical composition of marjoram and basil extracts was evaluated and the activity of the main compounds was also tested for cytokine production and gene expression. We concluded that the anti-inflammatory activity of both oils is dedicated to their main compounds, respectively sabinene hydrate and terpineol for marjoram and linalool and eugenol for sweet basil extracts. Furthermore these results confirmed their application as anti-atherosclerotic agents.

~~Main compounds presented in supercritical basil (linalool and eugenol) and marjoram (sabinene hydrate and terpineol) essential oils also presented an important anti-inflammatory activity, which allowed us to propose these compounds as responsible for the anti-inflammatory activity found in essential oils.~~

~~In conclusion, SFE allowed to obtain sage and marjoram essential oils with significant anti-inflammatory properties. Furthermore, these supercritical essential oils have also~~

42   ~~shown an important anti-inflammatory effect in an atherosclerotic environment and~~

43   ~~presented a highly potential application in the prevention of atherosclerosis.~~

44   ***Key words*:** basil, marjoram, essential oils, anti-inflammatory activity, supercritical

45   extracts.

46

1. INTRODUCTION

Inflammation is a complex response of the immune system induced by a microbial infection or tissue injury: ischemic, toxic or autoimmune. ~~In T~~this process ~~exists~~ represent a complex ~~whole~~ of interactions between soluble factors and cells. Activated macrophages secrete several mediators such as cytokines with pro-inflammatory effect, as tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6 and anti-inflammatory, as IL-10~~, effect~~. During the inflammatory response, there is~~these cells~~ ~~also~~an increase in gene expression of pro-inflammatory enzymes such as cyclooxygenase-2 (COX-2)~~,~~ responsible ~~of~~ for prostaglandin E-2 synthesis ~~or~~ and the inducible form of nitric oxide synthase (iNOS)~~, which~~responsible for NO synthesis ~~increase NO levels~~ (Barton, 2008; Zhang, 2008). An acute inflammatory response recovers physiologica~~ly~~l homeostasis in a short period of time, while in an extended~~if the~~ inflammatory response ~~is extended~~, due to pathological diseases, it triggers a chronic inflammatory response that usually causes further damage. ~~Among principal diseases described, in which are involve a chronic inflammatory process, include~~Usually inflammatory processes induce cardiovascular diseases such~~,~~ as atherosclerosis, ~~obesity,~~ diabetes, cancer, rheumatoid arthritis or neurodegenerative diseases~~, as Alzheimer~~ (Medzhitov, 2008).

The functional role of herbs and spices and their constituents is a hot topic in food related plant research. Essential oils extracted from a wide variety of plants and species have been ~~related to~~shown to ~~present~~ exert many biological activities, such as antimicrobial, analgesic, sedative, anti-inflammatory and spasmolytic (Bakkali et al, 2008). Traditional approaches to recover essential oil from plants included steam ~~and,~~ hydro-distillation~~,~~ and liquid solvent-extraction. One of the disadvantages of steam-distillation and hydro-distillation methods is related ~~with~~ to the thermolability of the

72 essential oil constituents, which undergo chemical alteration due to the ~~effect of the~~ high

73 temperature~~s~~ applied. On the other side, the lipophilic character of essential oils

74 ~~required~~ requires the use of organic solvents to attain an adequate selectivity of

75 extraction. In this sense, the main drawback is the occurrence of organic toxic residues

76 in the extracted product applied (Diaz-Maroto et al, 2002). Among innovative process

77 technologies, supercritical fluid extraction (SFE) with carbon dioxide has been the most

78 widely studied application in order to isolate essential oils from plants. This technique

79 provides a high speed and efficiency of extraction; ~~extraction process can be carried~~

80 ~~out~~performance at relatively low temperature~~s~~, eliminates concentration steps and

81 avoids the use of organic solvents which are potentially harmful in terms of

82 environmental impact. Therefore, carbon dioxide is an ideal solvent for the extraction of

83 essential oils from plants because is non-toxic, non-explosive, readily available and easy

84 to remove from extracted products (Wenqiang et al, 2007).

85 Sweet basil (*Ocimun basilicum L.*) is a popular culinary herb grown in many parts of

86 the world that it is used for flavoring food, in cosmetics and in traditional medicine for

87 the treatment of respiratory and urinary tracts inflammation, cough or~~,~~ asthma~~, etc~~

88 (Makri and Kintzios, 2007). Several studies have also reported that different extracts

89 from sweet basil or its essential oil presented a high antioxidant, antimicrobial,

90 antihypertensive, antithrombotic and anti-inflammatory activities (Hussain et al, 2008;

91 Mueller et al, 2010; Umar et al, 2010). Associated with anti-inflammatory effects, Umar

92 et al. (2014) indicated that a butanol fraction ~~obtained from~~of *Ocimun basilicum*

93 ~~presented~~ displayed an inhibition of COX-2 gene expression and Mediratta et al. (2002)

94 also reported the immunomodulatory potential of *Ocimun sanctum* seed oil. ~~However,~~

95 ~~despite of these results, the anti-inflammatory activity of this plant remained~~

96 ~~understudied.~~ *Origanum majorana* (marjoram) is also a culinary herb widely used ~~to~~

97   ~~flavor foods products~~as a flavor in foods and alcoholic beverages. Its essential oil and

98   extracts have been indicated to possess antioxidant, antimicrobial, anticancer and anti-

99   inflammatory activities (Vági et al, 2005; Mueller et al, 2010; Roby et al, 2013). In this

100 sense, although several authors have employed supercritical fluid extraction with carbon

101 dioxide in order to isolate the essential oil from basil and marjoram (Leal et al, 2008;

102 Fornari et al, 2012; Filip et al, 2014), their anti-inflammatory activity ~~of these essentials~~

103 ~~oils obtained by SFE~~ needs to be explore further~~remained unstudied~~.

104 ~~The aim of this paper was to study~~In this work we present the *in vitro* anti-inflammatory

105 capacity of marjoram and sweet basil essential oils obtained by SFE. ~~The anti-~~

106 ~~inflammatory activity was evaluated using two *in vitro* models of inflammation with~~

107 ~~human macrophages. In the first model,~~ THP-1 human macrophages were activated

108 ~~using~~ by lipopolysaccharide (LPS), ~~which produced~~mimicking a general inflammatory

109 response. ~~In the other model, THP-1 macrophages were activated with~~Also, human ox-

110 LDL (oxidized low density lipoproteins) was used to induce an inflammation on human

111 macrophages as a model ~~that allow us~~ to determine the anti-inflammatory effect of the

112 extracts in an atherosclerotic environment ~~and could be useful to determine the potential~~

113 ~~activity of the extracts in the prevention of atherosclerosis~~. Further more ~~, this work~~

114 ~~analysed~~ the chemical composition of ~~the~~ both essential oils was evaluated ~~and intended~~

115 ~~to establish~~to better understand the role of their composition with respect to their anti-

116 inflammatory activity. ~~a relationship between the extracts' activity and their~~

117 ~~composition.~~

118 **2. MATERIAL AND METHODS**

6

2.1 Samples and chemicals

Marjoram (*Origanum majorana* L.) and sweet basil (*Ocimun basilicum* L.) samples

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.

Eugenol, linalool, sabinene hydrate and terpineol 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 40 ºC, with a CO_2 flow rate of 60

g/min. Temperature was set to 40 ºC 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 5 h (Fornari et al 2012).

2.3 GC-MS analysis

Characterization of the supercritical extracts was carried out by a GC-2010 (Shimadzu,

Kyoto, 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, Madrid, Spain) capillary column, 30 m x 0.32 mm I.D. and 0.25 µm phase thickness. Helium, 99.99% was used as a carrier gas at a flow of 1 mL/min. Oven temperature ~~programming was~~was first programmed at 60 ºC (kept for ~~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~~then increased to ~~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 spectrometer was used in TIC mode, and samples were scanned from 40 to 500 amu. Compounds linalool, eugenol, sabinene hydrate and terpineol 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 and by their linear retention index.

2.4 Isolation and oxidation of LDLs

LDLs were isolated from human plasma as described before (Havel et al 1995). Oxidation of LDLs was done by incubating LDLs with 5µM $CuSO_4$ for 3h at 37ºC. Oxidation degree was measured as the amount of thiobarbituric acid reactive substances (TBARS) produced (Yancey and Jerome, 1998).

2.5 Cell culture and treatment

8

166   Human THP-1 monocytes (American Type Culture Collection, ATCC, Barcelona,

167   Spain) were cultured in RPMI 1640 culture medium (Gibco, Madrid, Spain)

168   supplemented with 10% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin, 2 mM L-

169   glutamine and 0.05 mM β-mercaptoethanol at 37 ºC in 95% humidified air containing

170   5% CO_2. Cells were collected and plated at a density of $5x10^5$ cells/mL in 24 wells

171   plates. Differentiation to macrophages (THP-1/M cells) was induced by maintaining the

172   THP-1 cells for 48h in the presence of 100 ng/ml phorbol 12-myristate 13-acetate

173   (PMA) (Sigma, Madrid, Spain) for 48h. After differentiation, cells were washed with

174   PBS and incubated with 75 µg/mL ox-LDLs or 0.05 µg/mL LPS in presence of different

175   concentrations of supercritical extracts or pure standards for 6, 12 or 24h in a FBS free

176   medium. Then, the supernatant was frozen at -80ºC and cells RNA isolated.

177   *2.6 Cytotoxicity assays*

178   The cytotoxic effect of the extracts and pure standards on THP-1/M cells was tested

179   using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

180   (Sigma, Madrid, Spain), according to a published method (Mosmann, 1983). THP-1/M

181   cells in 24-multiwell plates were incubated with RPMI containing different

182   concentrations of the essential oils for 24h at 37ºC. Cells were then washed with PBS

183   and 0.5 mg/ml of MTT were added to each well and incubated 4h at 37ºC. Supernatants

184   were discarded and formazan crystals dissolved in an extraction solution (10% sodium

185   dodecyl sulphate in a mixture of dimethyl formamide and water (1:1 v/v), adjusted to

186   pH 4.7 with acetic acid) overnight at 37ºC. Formazan quantification was performed by

187   measuring the optical density at 570 nm using a multiscanner autoreader (Sunrise,

188   Tecan, Barcelona, Spain) with and the extraction solution was considered as a blank.

189   *2.7 Quantification of cytokines by ELISA*

190   The release of IL-1β, IL-10, IL-6 and TNF-α was measured in the supernatants of THP-

191   1/M cells treated with ox-LDL or LPS in presence of different concentrations of

192   essential oils and their main constituents as pure compounds standards using ELISA kits

193   (BD biosciences, Madrid, Spain), according to manufacturer's instructions. The color

194   generated was determined by measuring the OD at 450 nm using a multiscanner

195   autoreader (Sunrise, Tecan, Barcelona, Spain).

196   *2.8 RNA isolation and RT-PCR*

197   RNA was isolated from THP-1/M cells using Trizol® (Invitrogen, Madrid, Spain)

198   according to manufacturer's instructions. Reverse transcription (RT) of the RNA was

199   performed using High Capacity Archive Kit and GeneAmp PCR System 9700 (Applied

200   Biosystems, Carlsbad, CA, USA) according to the manufacturer´s instructions to obtain

201   20 ng/μL of cDNA. PCR amplification was conducted in a 10 μL reaction mixture with

202   cDNA, Taqman Gene Expression Master Mix (Applied Biosystems, Madrid, Spain) and

203   TaqMan probes (Applied Biosystems, Madrid, Spain) according to the manufacturer´s

204   conditions in a 7900HT Fast Real-Time PCR System (Applied Biosystems, Madrid,

205   Spain). The TaqMan probes used were as follows: Hs99999029_m1 for IL-1β,

206   Hs00174131_m1 for IL-6, Hs99999035_m1 for IL-10, Hs00174128_m1 for TNF-α,

207   Hs00153133_m1 for COX-2, Hs00921372_m1 for NFκB and Hs99999901_s1 for 18S

208   rRNA. Expression of genesGene expression was normalized relative to 18S rRNA using

209   SDS Software v2.4 (Applied Biosystems, Madrid, Spain).

210   *2.9 Statistical analysis*

211   All data were expressed as the mean of three determinations ± SD. Data were analyzed

212   by one-way analysis of variance (ANOVA) followed by Dunnett's and Bonferroni tests,

213 using Prism program for Windows (Version 5; GraphPad Software, San Diego, CA,

214 USA). P values lower than 0.05 were considered significant.

3. RESULTS

3.1 Supercritical fluid extraction of essential oils from marjoram and basil

217 The supercritical fluid extraction and fractionation conditions used in this work were

218 ~~supported by~~conducted based on ~~a~~ previous ~~study~~ studies ~~developed in~~of our research

219 group (Fornari et al, 2012). ~~Briefly, the extractor pressure was 30 MPa and temperature~~

220 ~~of extraction was maintained at 40ºC. Fractionation of the extracted material was~~

221 ~~accomplished by setting the pressure of the first separator (S1) to 10 MPa, while the~~

222 ~~second separator (S2) was maintained at the recirculation system pressure (5 MPa).~~

223 ~~These~~All the extraction conditions used allowed us to recover a ~~important~~significant

224 percentage of the essential oil compounds in S2 fractions, with respect to the total oil

225 recovered in S1 and S2 fractions, ~~(a~~respectively 97.7% of basil essential oil was

226 recovered in S2 and ~~a~~ 77.9% ~~of~~ in the marjoram essential oil~~)~~. ~~According to~~Since we

227 obtained a higher amount of the oil in the S2 fractions we estimated the ~~these results in~~

228 ~~this work, only the~~ anti-inflammatory activity ~~of the~~only for ~~S2 fractions was~~

229 ~~studied~~this fraction.

230 Besides~~,~~ a characterization by GC-MS of the S2 fractions was carried out and the results

231 are presented in Table 1, where ~~a~~ the ~~tentative~~ identification has been performed based

232 on the comparison of mass spectra. In S2 basil fraction the main compounds presented

233 were linalool, eugenol and α-bergamatone, representing a 64.84% of total composition

234 of the essential oil. These data were in agreement with those reported by Filip et al.

235 (2014), ~~since the major compounds identified in a basil essential oil obtained by~~

236 ~~supercritical fluid extraction were~~who also reported linalool, eugenol and α-

237 bergamatone as the main constituents, although the percentage of these compounds in

238 total oil composition was lower ~~than in our results~~than what we report here. Even

239 though these authors employed the same extraction conditions ~~This data could be~~

240 ~~explained because, although these authors employed extraction conditions~~ (pressure and

241 Tª), they did not use a fractionation step as we did.

242 The main components detected in marjoram S2 fraction were sabinene hydrate,

243 terpinen-4-ol and terpinene acetate (66.01% of total composition). Vági et al. (2005)

244 also indicated a ~~important~~ significant presence of terpinen-4-ol and terpinene acetate in

245 a~~n~~ marjoram SFE extract, ~~although they found a~~however the amount of sabinene

246 hydrate reported was low. ~~small amount of sabinene hydrate.~~ The conditions employed

247 by these authors were slightly different to ours and no fractionation step was used.

248 *3.2 Effects of supercritical extracts and pure components on THP-1/M viability*

249 The viability of the THP-1/M cells was assessed prior to anti-inflammatory studies to

250 determinate the cytotoxicity of supercritical extracts and the main pure compounds

251 presented in the extracts (linalool, eugenol, sabinene hydrate and terpineol) by MTT

252 method. The results obtained indicated that 20 µg/mL of basil and marjoram extracts

253 was the highest concentration without significant decrease in cell viability. Results

254 obtained with pure standards, indicated that they ~~presented~~ induced a higher

255 cytotoxicity, being 7.5 µg/mL the highest concentration ~~that presented a~~with 100% cell

256 viability.

257 *3.3 Effect of basil and marjoram extracts on the cytokine~~s~~ release in THP-1/M activated*

258 *with LPS.*

259   In a first assay, the activation of THP-1/M was carried out with the incorporation of

260   LPS into the cell medium during 6, 12 or 24h with or without ~~After all incubation times,~~

261   ~~LPS treated cells shown an important increase in all pro- and anti-inflammatory~~

262   ~~cytokines measured (TNF-α, IL-1β, IL-6 and IL-10), compared to non-activated~~

263   ~~controls (Fig. 1). These activated cells were considered as positive controls for all the~~

264   ~~cytokines tested. After 6 and 12h of activation in presence of~~ 10 µg/mL of basil and/or

265   marjoram essential oils. Cells incubated only with antigens were considered as positive

266   controls. An incubation of 6 and 12 h showed a small decrease in TNF-α secreti~~oned~~

267   ~~level was observed~~ (Fig. 1) compared with levels obtained in absence of

268   extracts~~(positive control)~~. ~~The decrease in this cytokine secreted levels in presence of~~

269   ~~essential oils was higher after 24h of incubation.~~ However~~,~~ this was not the case for IL-

270   1β, since ~~10 µg/mL of these~~the extracts ~~achieved~~ induced ~~a~~ 50-60% ~~of~~ inhibition ~~in IL-~~

271   ~~1β secretion~~ at 6h, although no significant differences were found between basil and

272   marjoram extracts. Similar results were also obtained after 12h of LPS incubation. ~~The~~

273   ~~activation of M~~macrophages activation at 6 and 12h in presence of supercritical extracts

274   was also ~~produced an important~~reduced significantly ~~reduction in the secretion of~~ for

275   IL-6 secretion~~.At 6 and 12h after LPS treatment, 10 µg/mL of marjoram extract,~~

276   ~~produced a release of this cytokine~~ , close to basal levels of non-activated cells.

277   Regarding the data obtained from~~with~~ IL-10~~, an anti-inflammatory cytokine,~~ after 12

278   and 24h of incubation, the release of this cytokine in presence of supercritical extracts

279   was lower than positive control. This ~~fact~~ could be explained since~~because~~ in presence

280   of supercritical extracts the inflammatory status in the cell was reduced and the

281   secretion of the~~an~~ anti-inflammatory cytokine was not as necessary as in the case of~~for~~

282   ~~the~~ positive control.

283 These data indicated that basil and marjoram essential oils obtained by supercritical

284 fluids presented an important anti-inflammatory activity in THP-1 macrophages

285 activated with LPS since, after 6h of incubation, a small quantity of these extracts (10

286 µg/mL) effectively inhibited the release of pro-inflammatory cytokines, mainly IL-1β

287 and IL-6. However no significant differences were observed between the two essential

288 oils.

289 *3.4 Effect of basil and marjoram extracts on the cytokines release and gene expression*

290 *in THP-1/M activated with ox-LDL*

291 Oxidized-LDLs were used to activate the inflammationory process in THP-1/M during

292 6, 12 or 24h. and carriedy out the second model of inflammation used in this research

293 work. As shown in Fig. 2 shows that ox-LDL treated cells increased significantly the

294 secretion of TNF-α, IL-1β, IL-6 and IL-10 compared to non-activated cells at all

295 incubation times (except for IL-10 at 6h). The incorporation of 10 µg/mL of basil and

296 marjoram extracts promoted an important reduction in TNF-α release, up to basal levels.

297 Moreover, marjoram essential oil inducedpresented a higher reduction in TNF-α

298 secretion than basil oil.

299 IL-1β and IL-6 secretion were also significantly reduced with 10 µg/mL of extracts.

300 Regarding to IL-10, a reduction was achieved only after 12 and 24h of ox-LDL

301 incubation.

302 Results obtained indicated that when THP-1/M were stimulated with ox-LDL both,

303 marjoram and basil extracts showedpresented a high decrease in pro-inflammatory

304 cytokines secretion. It is interesting to point out that, in this inflammation model, the

305 secretion of pro-inflammatory cytokines in presence of the extracts was reduced near to

306   basal levels, again indicating the anti-inflammatory effect of the extracts in an

307   atherosclerotic environment.

308   In order to determine if the influence of supercritical extracts in cytokine production

309   was related to gene expression, total cellular RNA was extracted from activated THP-

310   1/M and analyzed using RT-PCR. The effect of marjoram and basil extracts on TNF-α,

311   IL-1β, IL-6 and IL-10 mRNA expression in THP-1 after 6h of ox-LDL activation is

312   presented in Figure 3. Gene expression of pro-inflammatory cytokines TNF-α, IL-1β

313   and IL-6 was significantly reduced in presence of the extracts, withoutalthough

314   significantno differences were found between the two extracts. In addition, gene

315   expression of COX-2 and NFκB, in presence of supercritical extracts was determined.

316   As previously mentioned, since COX-2 is an inducible enzyme which expression is

317   increaseds during the inflammatory process whileand NFκB activation induces the

318   transcription of pro-inflammatory mediators such as COX-2, TNF-α, IL-1β and IL-6

319   (Wong and Tergaonkar, 2009). Results showed that COX-2 and NFκB gene expression

320   were also reduced by marjoram and basil supercritical extracts.

321   The decrease in cytokine, COX-2 and NFκB gene expression by marjoram and basil

322   extracts was in agreement with the reduced cytokines release, which furthermore

323   strengthen the anti-inflammatory activity of these extracts in an atherosclerotic

324   environment.

325   *3.5 Effect of basil and marjoram extracts' main components on the cytokines release in*

326   *THP-1/M activated with ox-LDL*

327   In an attempt to correlate the anti-inflammatory activity found in supercritical extracts

328   with their chemical composition, the cytotoxicity and anti-inflammatory activity of pure

329   standards of from the main components of found in the extracts (sabinene hydrate,

15

330   terpineol, linalool and eugenol) were examined in the same conditions. The pure

331   standards concentrations employed were 7.5 and 5 µg/mL, since 7.5 was the highester

332   concentration that presented a 100% cell viability. Besides, oxidized-LDLs were used to

333   activate the inflammatory process in THP-1/M due to extracts showed a higher anti-

334   inflammatory activity using this model. In this case the incubation time was 24h.

335   When the ox-LDL activation of THP-1/M was carried out in presence of sabinene

336   hydrate, terpineol, linalool and eugenol, an important decrease in TNF-α secreted level

337   was observed. At 7.5 µg/mL, all the standards decreased the TNF-α secretion to levels

338   lower thanbellow the basal levels. IL-β secretion was also reduced with 7.5 µg/mL of

339   the extracts, although in a lesser extent than TNF-α. In this case, eugenol seemed to

340   present a higher activity, but only when when employingusing 5 µg/mL. Regarding to

341   IL-6, all standards showed a remarklen important decrease in the secretion of this IL,

342   closenear to basal values, presenting no differences among themn. IL-10 secretion was

343   also decreased in the presence of 7.5 µg/mL of the standards.

344   These results indicated that the main compounds found in the SFE essential oils

345   presented an important anti-inflammatory activity, with a decrease in the release of pro-

346   inflammatory cytokines. For the Ddata obtained did not shown no significantimportant

347   differences were noticed onamong the activity of the four compounds. Accordingly,

348   sabinene hydrate, terpineol, linalool and eugenol could be proposed as the principal

349   compounds responsible for the anti-inflammatory activity found in marjoram and basil

350   supercritical essential oils. The similar activity found in four standards is agreement

351   Data also explainedwith the non significant differences onsimilar anti-inflammatory

352   activity found in both extracts, since the four standards presented a similar activity.

4. DISCUSSION

354 Supercritical carbon dioxide extraction has been reported to be an efficient extraction

355 technology in order to obtain essential oils from several species (Diaz-Maroto et al,

356 2002; Wenqiang et al, 2007). Some of these essential oils, obtained by SFE, have been

357 reported to possess anti-inflammatory properties (Ocaña-Fuentes et al, 2010; Arranz et

358 al 2014). Therefore, the aim of this paper was to study the anti-inflammatory capacity of

359 marjoram (*Origanum majorana* L.) and sweet basil (*Ocimun basilicum* L.) essential oils

360 obtained by SFE. This anti-inflammatory activity was evaluated by two *in vitro* models

361 of inflammation, using THP-1 human macrophages activated with lipopolysaccharide

362 (LPS) or human ox-LDL at different times (6, 12 and 24h). The use of LPS to activate

363 macrophages is a ~~model~~ commonly used model to test anti-inflammatory properties of

364 herb~~s~~ extracts (Allen-Hall et al, 2007). Ox-LDL activated macrophages simulate an

365 atherosclerotic model of inflammation since low-density lipoprotein oxidation appears

366 to be a fundamental event in the development of the atherosclerotic lesion and the

367 initiation of the inflammatory cascade (Call et al, 2004).

368 The incorporation of ox-LDL and LPS in human macrophages activated both~~,~~ secretion

369 and gene expression of pro-inflammatory and anti-inflammatory cytokines, such as

370 TNF-α, IL-1β, IL-6 and IL-10 (Kaperonis et al 2006; Wasaporn et al, 2010). During the

371 development of an inflammatory response, the pro-inflammatory cytokines TNF-α and

372 IL-1β secreted by macrophages play an~~d~~ important role in the initial amplification of the

373 response, while IL-6 secretion occurs later, after its stimulation by different signals, as

374 TNF-α or IL-1β (O´Shea and Nutman, 2001).Gene expression of these cytokines and

375 other pro-inflammatory factors are activated after NF-κB translocation to the nucleus.

376 One of the most important pro-inflammatory enzymes that depends o~~n~~f NF-κB gene

377 activation is COX-2 (cyclooxygenase-2), responsible of PGE-2 (prostaglandin E-2)

378 synthesis, which secretion increases vasodilation and inflammatory progression (Huang

379 et al, 2000). However during an inflammatory process exists a regulation though

380 secretion of anti-inflammatory mediators, as IL-10, preventing NF-κB translocation and

381 production of TNF-α and IL-6 due to miR-18 synthesis (Rossato et al, 2012).

382 Considering the results presented here, supercritical basil and marjoram essential oils

383 showned an important anti-inflammatory activity, due to the inhibition of pro-

384 inflammatory cytokines secretion and gene expression in activated macrophages. This

385 anti-inflammatory activity was demonstrated due to an important reduction of TNF-α,

386 IL-1β and IL-6 secretion obtained with 10 µg/mL of the extracts. According to these

387 results, Mueller et al. (2010) also reported that extracts obtained from several plants,

388 including basil and marjoram, were able to reduce the production of IL-6 in a LPS

389 stimulated macrophages model. However, these authors employed 0.2 and 0.5 mg/mL

390 of the extracts, concentrations too much higher than those reported in this work (10

391 µg/mL). Moreover, Choudhury et al. (2014) using several extracts of *Ocimum sanctum*

392 L. also reported a decrease in the secretion of TNF-α and IL-6 in an *in vitro* model. The

393 concentrations used by these authors were 0.5 and 1 mg/mL. Loizzo et al. (2009) also

394 indicated the anti-inflammatory activity of *Origanum ehrenbergii* and *Origanum*

395 *syriacum* L. essential oils. These oils inhibited the NO production in the murine

396 macrophage cell line Raw 264.7 activated with LPS with an IC_{50} value of 66.4 µg/mL.

397 However, it must be pointed out that the results presented in this work also shown that

398 supercritical essential oils were able to decrease the pro-inflammatory cytokine

399 secretion and gene expression in ox-LDL activated macrophages, a model employed to

400 determine the anti-inflammatory effect of the extracts in an atherosclerotic environment.

401 These data were in agreement with results presented by Arranz et al. (2014) using

402 supercritical sage extracts, although in that case 20 µg/mL of the supercritical extracts

403 were required to decrease pro-inflammatory cytokine release. Besides, Ocaña-Fuentes et

404   al. (2010) also reported an atherogenic effect of *Origanum vulgare* supercritical extracts

405   in an ox-LDL activated macrophages model.

406   Moreover, basil and marjoram supercritical extracts caused an important reduction in

407   COX-2 gene expression in ox-LDL activated THP-1/M. Likewise ~~Accordingly to our~~

408   ~~results,~~Umar et al. (2014) reported that a butanol fraction obtained from *Ocimun*

409   *basilicum* L. inhibit COX-2 expression in an *in vivo* model. However, Mueller et al.

410   (2010) did not found an inhibition in COX-2 expression when employing commercial

411   extracts. In that way, our results have been corroborated by the inhibition of NF-κB

412   gene expression with 10 µg/mL of the two essential oils, since gene expression of COX-

413   2 and other pro-inflammatory factors are activated after NF-κB expression.

414   The ~~M~~main compounds ~~of~~~~presented in~~ supercritical basil (linalool and eugenol) and

415   marjoram (sabinene hydrate and terpineol) essential oils also presented an important

416   anti-inflammatory activity in an ox-LDL activated macrophages model. 7.5 µg/mL of

417   these components showed an important decrease in TNF-α, IL-1β and IL-6 secretion.

418   Moreover, these components have been also reported to present a significant anti-

419   inflammatory activity on LPS activated macrophages (Mahapatra et al, 2011; Huo et al,

420   2013; Valente et al, 2013; Choudhury et al, 2014). These results allowed us to propose

421   linalool, eugenol, sabinene hydrate and terpineol, as the principal compounds

422   responsible ~~of~~ for the anti-inflammatory activity found in marjoram and basil

423   supercritical essential oils.

424   In conclusion, the results obtained in this work indicated that sage and marjoram

425   essential oils obtained by supercritical fluids presented important anti-inflammatory

426   properties. Furthermore, these supercritical essential oils also showe~~d~~~~n an~~

427   ~~important~~potential anti-inflammatory effect in an atherosclerotic environment and

428 presented a highly potential application in the prevention of atherosclerosis. All the

429 results obtained provided the basis for increasing the applicability of supercritical

430 essential oils from basil and marjoram in formulations for the prevention of

431 inflammatory diseases.

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539   **Table 1:** Essential oil composition (% area of GC-MS analysis) of the S2 fractions

540   obtained by SFE from marjoram and basil.

Retention time (min)	Compound	Basil	Marjoram
10.88	1,8-Cineole	5.75	-
12.89	Sabinene hydrate trans	0.68	7.41
14.67	Sabinene hydrate cis	0.71	37.00
14.91	Linalool	27.81	2.49
17.25	Camphor	0.66	-
18,5	Borneol	0.44	-
19,29	1-Terpinen-4-ol	1.62	12.81
20,1	α-Terpineol	3.03	8.10
21,12	Verbenone	0.06	0.89
23,84	Terpinene-4-acetate	-	16.20
25.6	Bornyl acetate	0.02	-
26.46	Carvacrol	-	1.74
30.3	Eugenol	24.76	0.88
32,05	Acid Cinamic methyl ester	11.36	0.59
34,5	Caryophyllene	0.80	4.99
36,1	α-Bergamatone	12.27	1.10
37,2	α-Caryophyllene	0.73	-
42,5	ɣ-Cadinene	7.34	-
48,12	Spathulenol	1.98	5.80

541

Figure legends

Figure 1: Levels of TNF-α, IL-1β, IL-6 and IL-10 secreted by THP-1/M activated with LPS in presence of basil and marjoram essential oils for 6, 12 and 24h. Each point is the mean of three determinations ± standard deviation.

**Figure 2:** Levels of TNF-α, IL-1β, IL-6 and IL-10 secreted by THP-1/M activated with ox-LDL in presence of basil and marjoram essential oils for 6, 12 and 24h. Each point is the mean of three determinations ± standard deviation.

Figure 3: Gene expression of TNF-α, IL-1β, IL-6, IL-10, COX-2 and NFκB on THP-1/M stimulated with ox-LDL in presence basil and marjoram essential oils. Each point is the mean of three determinations ± standard deviation. RQ: relative quantification.

Figure 4. : Levels of TNF-α, IL-1β, IL-6 and IL-10 secreted by THP-1/M activated with ox-LDL in presence of pure standards compounds for 24h. Each point is the mean of three determinations ± standard deviation.

555   **Figure 1**

TNF-α, IL-1β, IL-6, IL-10

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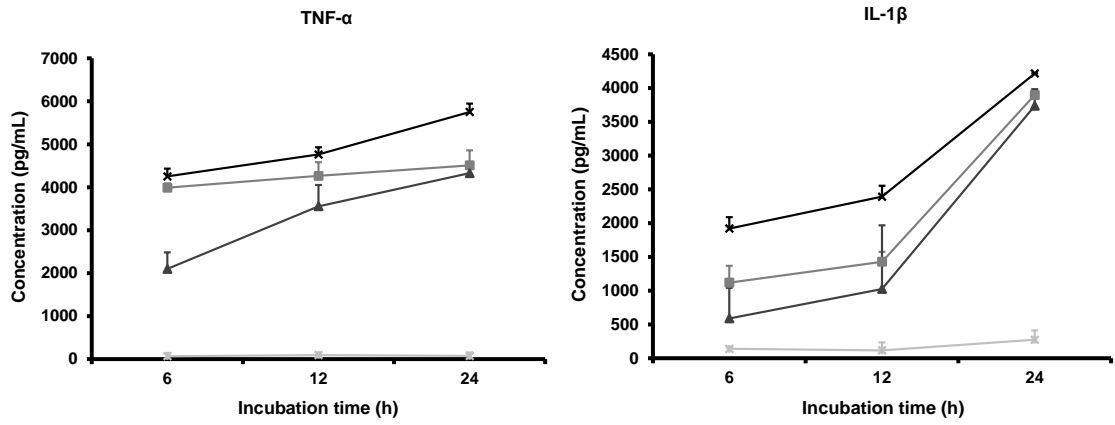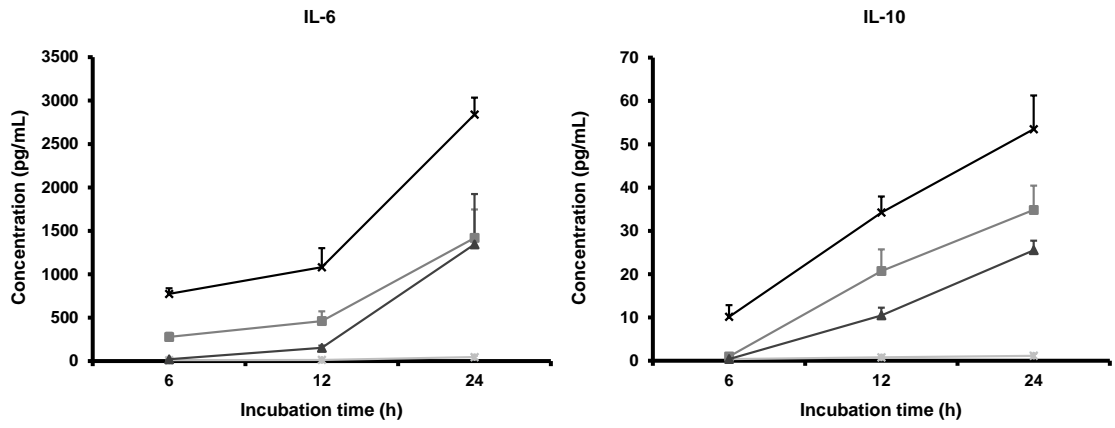
558   —✕— **+ Control LPS**   —✱— **- Control**   —■— **Basil 10 μg/mL**   —▲— **Marjoram 10 μg/mL**
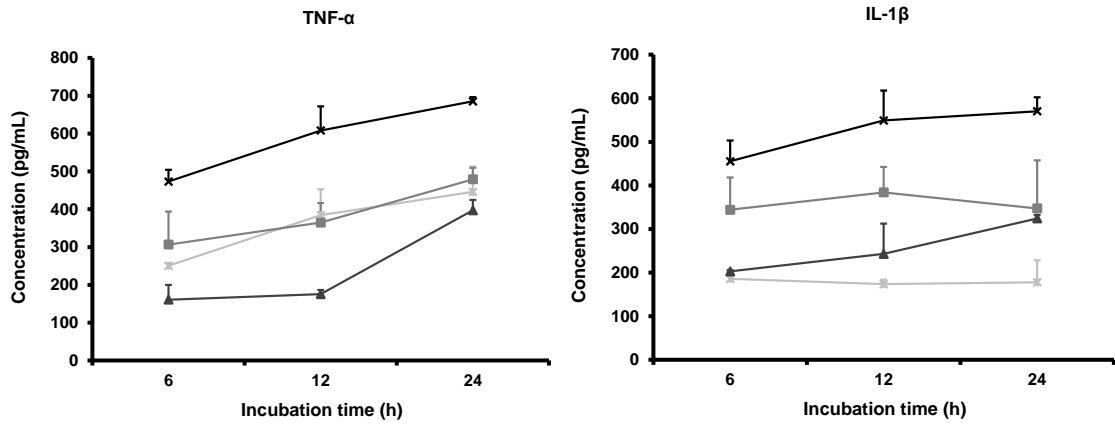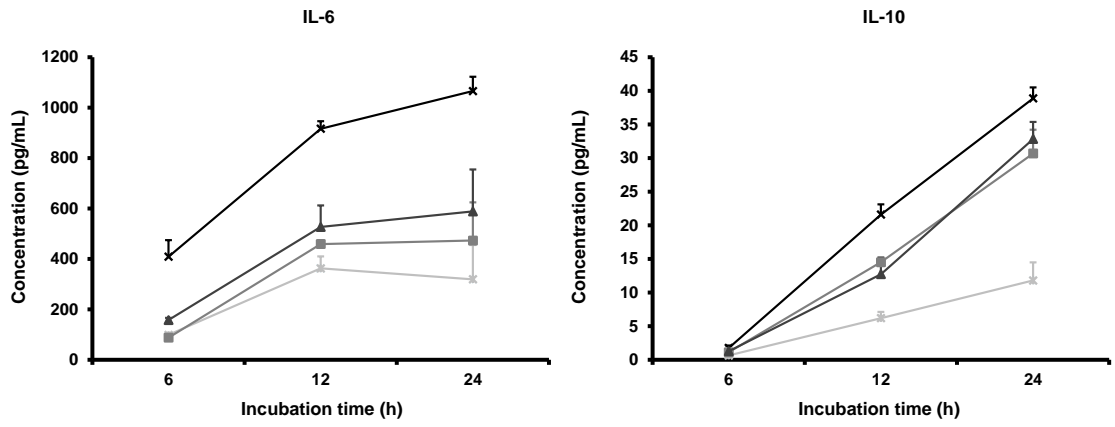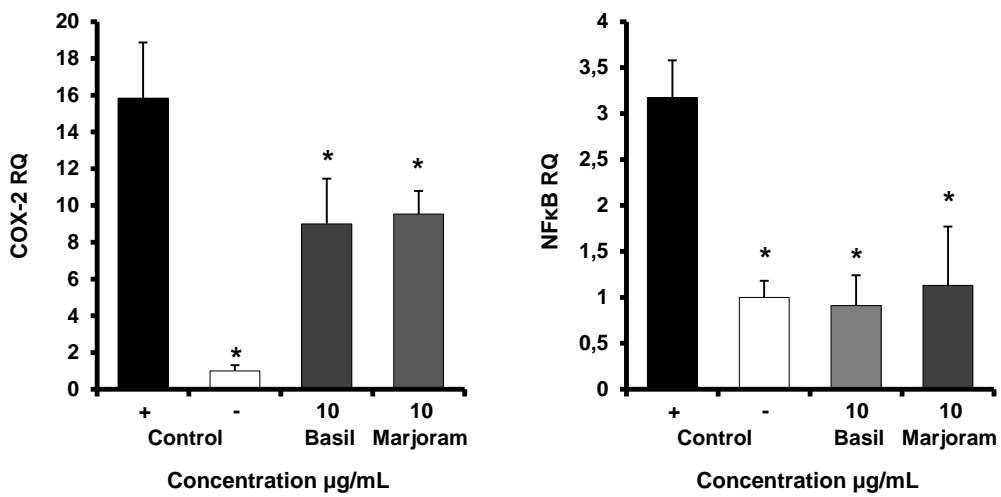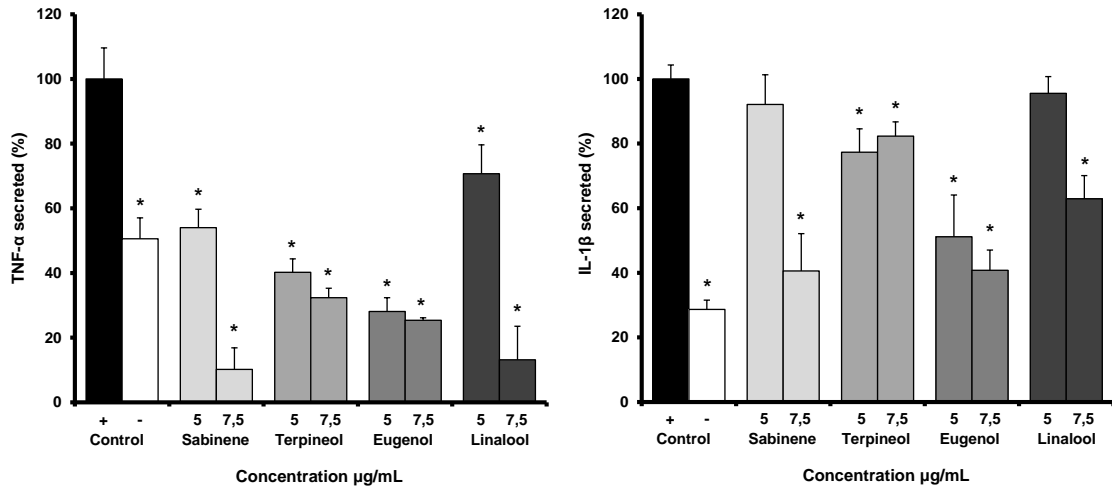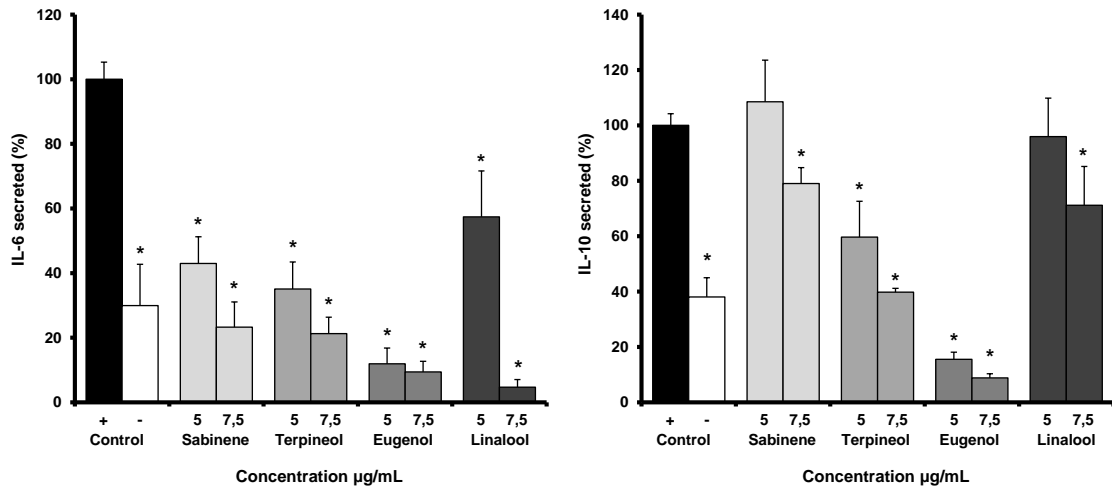
Figure 2

563   **Figure 3**
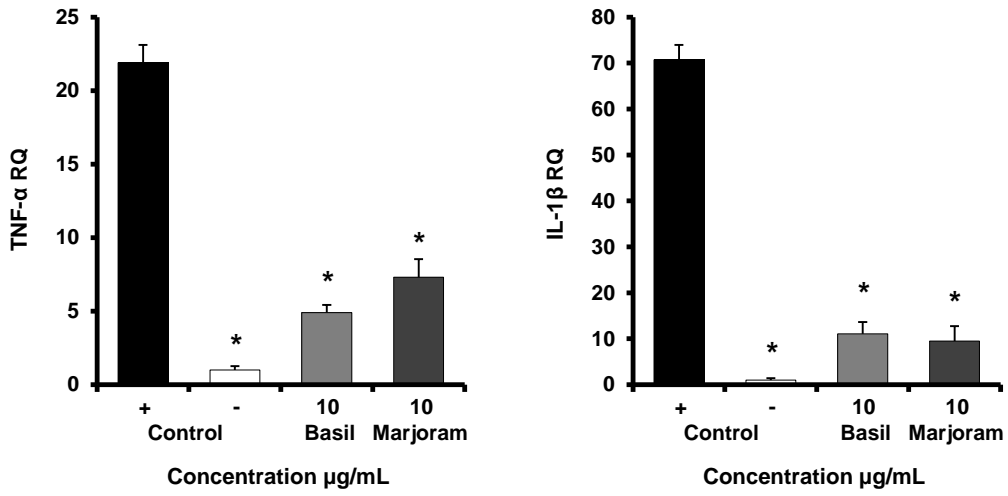
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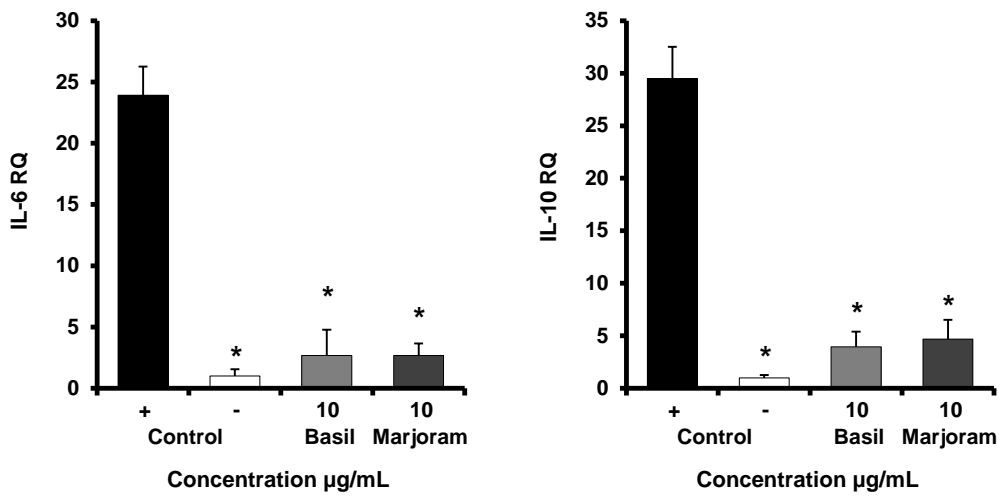
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567   **Figure 4**

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