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Antiinflammatory and antioxidant activities from the basolateral fraction of Caco-2 cells exposed to a rosmarinic acid enriched extract

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ABSTRACT

The potential use of *Origanum majorana* L. as a source of bioavailable phenolic compounds, specifically rosmarinic acid (RA), has been evaluated. Phenolic bioavailability was tested using an *in vitro* digestion process followed by a Caco-2 cellular model of intestinal absorption. The HPLC-PAD-MS/MS analysis showed the main components in the extract were 6-hydroxyluteolin-7-O-glucoside and rosmarinic acid, followed by luteolin-O-glucoside. After digestion process, the amount of total phenolic compounds (TPC) only decreased slightly, although a remarkable reduction in RA (near 50%) was detected. Bioavailable fraction contained 7.37±1.39 mg/L digested extract of RA with small quantities of lithospermic acid and diosmin and presented an important antioxidant activity (0.89±0.09 mmol Trolox/L digested extract). Besides, this bioavailable fraction produced a significant inhibition in TNF-α, IL-1β and IL-6 secretion, using a human THP-1 macrophages model. Therefore, RA content in the basolateral compartment could play an important role in the antioxidant and anti-inflammatory activities found.

Key words: anti-inflammatory activity, antioxidant activity, rosmarinic acid, *in vitro* digestion, Caco-2 cells.

INTRODUCTION

Origanum majorana L. (marjoram) is a culinary herb often used in foods. Its essential oil and extracts have been indicated to possess antioxidant, antimicrobial, anticancer and anti-inflammatory activities.[1,2] These activities have been attributed to the presence of a high percentage of phenolic acids and flavonoids in marjoram leaves[3] particularly, to rosmarinic acid (RA), an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid naturally occurring in marjoram.[4,5] Antioxidant activity of RA has been generally admitted, since this compound may act as free radical scavenger.[6,7] Related to RA anti-inflammatory effects, Jiang et al.[8] showed that this compound down regulated the levels of TNF-α, IL-6 and high mobility box 1 protein in bacterial lipopolysaccharide (LPS) induced RAW264.7 cells, indicating that RA might inhibit activation of the nuclear factor-κB pathway by inhibiting IκB kinase activity. Accordingly, Zdarilová et al.[9] reported that RA inhibited LPS-induced up-regulation of Il-1β, IL-6, TNF-α and suppressed expression of iNOS in human gingival fibroblasts. Further, Lembo et al.[10] indicated that RA produced a significant reduction in IL-1β, IL-6, IL-8 and TNF-α gene expression in HaCat cells after UVB irradiation.

In order to extrapolate results founded *in vitro* to *in vivo* situation, it is important to know the bioavailability of bioactive compounds. Therefore, the use of an *in vitro* digestion/Caco-2 cell culture model has been proposed by several authors as an economical and useful alternative to study the bioavailability of these compounds.[11,12] Thus, although the transepithelial transport of RA in intestinal Caco-2 cells monolayers has been studied,[13] it is crucial to investigate the effect that plant matrix plays on its bioavailability, since plant material matrix may alter absorption and bioavailability of phytochemicals.[14] For that matter, it would be very interesting to determine the anti-inflammatory and antioxidant effect of the bioavailable fractions of marjoram extracts

enriched in RA, in order to corroborate the biological activities described for these extracts.

Solid –liquid extraction (SLE) is the most traditional technology used to extract active compounds from plant matrix. It is widely known that higher temperatures enhance the solubility of the solute in the solvent and thus improve its recovery. Nevertheless, the SLE temperature is limited by solvent boiling and, in some cases, by the loss of volatile compounds. In this regard, pressurized liquid extraction (PLE) allows the use of solvents in a liquid state at higher temperatures. Furthermore, a compression effect is made on vegetal particles, which also contributes to improve extraction yield; moreover, lower amount of solvent is required, extraction is faster and volatiles loss is minimized.[15] Thus, several studies proposed PLE extraction as an alternative to conventional solid/liquid extraction in order to obtain phenolic compounds from herbs and spices.[16,17]

However, it is hard to obtain a highly concentrated extracts in phenolic compounds using only PLE, due to the complexity and the presence of impurities in crude extracts of herbal raw materials. Nowadays, the use of adsorption resins has been proposed as one of the most useful tools for selective enrichment of phenolic compounds from plant material, such as naringenin recovery from orange juice[18] or anthocyanins from grapes.[19]

For this purpose, some of the most commonly employed resins are XAD-2, XAD-7, XAD-16 and Oasis HLB, that have been successfully used for phenolic compounds enrichment from natural extracts.[20,21] Among them, XAD-7 has been proposed for rosmarinic acid enrichment from *Lavandula vera*[22] or *Rabdosia serra.*[23]

The aim of this work was to study the anti-inflammatory and antioxidant properties of the bioavailable fraction of PLE extracts enriched in rosmarinic acid. In order to increase the quantity of RA in PLE extracts of marjoram an amberlite XAD-7HP resin was employed. The bioavailability of both, original and enriched extracts, was determined by using an *in vitro* digestion/Caco-2 cell culture model. Thus, the anti-inflammatory and antioxidant activity of the basolateral fraction was measured.

MATERIALS AND METHODS

Chemicals

Ethanol of analytical grade and Folin-Ciocalteu's reagent were obtained from Panreac (Madrid, Spain). (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox, <97%), 2,2-Diphenyl-1-picrylhydrazyl free radical (DPPH), gallic acid for titration (<97,5%), and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma Aldrich (Madrid, Spain). Formic acid (99%) was obtained from Acros Organics (Madrid, Spain) and acetonitrile HPLC grade from Macron Fine Chemicals (Madrid, Spain). Reference substances (chromatographic purity ≥ 95%) for phenolic compounds identification such as cryptochlorogenic acid, neochlorogenic acid and rosmarinic acid were purchased from Sigma Aldrich (Madrid, Spain). Apigenin, apigenin 7-O-glururonide, caftaric acid, diosmin, lithospermic acid, luteolin, salvianolic acid and vicenin 2 were from Phytolab (Madrid, Spain). Ethyl gallate, apigenin 7-O-glucoside, caffeic acid, luteolin 7-O-β-D-glucoside, p-coumaric acid and protocatechuic acid were obtained from Extrasynthese S.A. (Genay, France). Finally, luteolin-7-O-B-D-glucuronide were from HWI Analytic GmbH (Rülzheim, Germany). The water used in this study was ultrapure type 1 (Millipore, Madrid, Spain).

114

115 **Marjoram samples and PLE extraction**

116 Marjoram sample consisted of dried leaves obtained from an herbalist shop (Murciana
117 herboristería, Murcia, Spain). The sample was grinded in a knife mill (Grindomix GM
118 200, Retsch, Llanera, Spain) and the particle size was determined by sieving the ground
119 plant material to the appropriate size (<500 μm). The whole sample was stored at -20ºC
120 until use.

121 Extractions were carried out in an ASE 350 system from Dionex Corporation
122 (Sunnyvale, CA, USA) equipped with a solvent controller unit. Each extraction cell (11
123 mL of capacity) was filled with a mixture of 1g of solid sample and 4 g of sea sand.
124 Then, the cell was filled with the solvent (a mixture of ethanol: water, 70:30) up to a
125 pressure of 1500 psi and heated to 100ºC. Static extractions were performed for 10 min.
126 The extracts were recovered in glass vials, ethanol was eliminated by evaporation and
127 extracts were lyophilized. The dried samples were stored at 4ºC in the dark until
128 analysis.

129

130 **Enrichment in phenolic compounds by resin column**

131 Enrichment experiments were carried out in a glass column (3 cm x 50 cm) packed with
132 XAD-7HP resin (Sigma-Aldrich, St. Louis, MO, USA). In order to remove the
133 monomers and pyrogenic agents trapped inside the pores during the synthesis process, a
134 pre-treatment of this resin was realized following the method described by Lin et al.[23]

135 After pre-treatment process, the resin was rehydrated overnight with ethanol at 4ºC and
136 packed with a bed volume (BV) of 174 mL. In order to remove the ethanol, a distillated
137 water washing was placed (4BV) at constant flow of 4BV/h. PLE extract was dissolved
138 in acid water (pH 3) (15 mg/mL) and 45 mL was applied onto the column. After

139 absorption equilibrium was reached (1h), the column was washed with 2BV of distilled
140 water and eluted with 3BV of 80% ethanol at a constant flow of 2 BV/h. The eluted
141 fraction was collected, evaporated to remove methanol, freeze-dried and stored at -20ºC
142 until evaluation.
143
144 **Determination of total phenolic content (TPC) and antioxidant activity**
145 In order to determine the TPC, Folin-Ciocalteau reagent method was applied as
146 described by Singleton et al.[24] The results were expressed as mg of gallic acid
147 equivalents (GAE)/g extract. Antioxidant activity was determined by DPPH method.
148 This method was applied according to Brand-Williams et al.[25] protocol. The results were
149 expressed as TEAC value (mmol trolox/g extract or L of digested extract). All analyses
150 were done in triplicate.
151
152 **Chemical characterization of samples**
153 HPLC-PAD analysis of phenolic compounds was performed by using an Agilent HPLC
154 1260 Infinity series system with photodiode-array detector (Agilent Technologies Inc.,
155 Santa Clara, CA, USA). A reverse phase ACE Excell 3 Super C18 column (150 mm x
156 4.6 mm, 3 μm particle size) protected by a guard column ACE 3 C18-AR (10 mm x 3
157 mm) was used at 35ºC. 20 μL of sample (extract, digested extract, apical or basolateral
158 fractions) was injected previously filtered by 0.45 μm PVDF filter.
159 Chromatographic separation was achieved using solvent A (99.9/0.1 water/formic acid
160 v/v) and solvent B (ACN) at a flow rate of 0.5 mL/min as follows: 0 min, 0% B; 1 min,
161 0% B; 6 min, 15% B; 21 min, 25% B; 26 min, 35% B; 36 min, 50%; 41 min, 50% B; 44
162 min, 100% B; 49 min, 0% B. Chromatograms were recorded at 280 nm, 320 nm and
163 360 nm. Peaks were tentatively identified according to its retention time and UV-Vis

spectrum by comparison with analytical standards. Ethyl gallate was added as internal standard in each analyze sample before it was filtered.

Confirmation of the identified compounds was carried out by HPLC-MS analyses. HPLC 1100 (Agilent Technologies Inc., Santa Clara, CA, USA) coupled to a hybrid quadrupole-time of flight mass spectrometer (QTOF, QSTAR pulsar i, ABSciex) equipped with a turbo ion electrospray source was used. MS experiments in negative mode were carried out in TOF/MS and MS/MS mode. The instrumental parameters were set as follows: mass range 50-2000 Da; ion spray voltage (IS) -4500 V; ion source gas pressure (GS1): 65 psi; (GS2): 65psi; curtain gas pressure (Cur): 20 psi; declustering potencial (DP): 30 V; focusing potencial (FP): 210 V; declustering potencial 2 (DP2): 15 V; Collision gas: 3 psi in MS experiments and 5 psi in MSMS experiments. In TOF/MS experiments, just before separation, an external calibration in the mass spectrometer was performe with a mixture of phosphazenes and verified after the assays. The maximum error accepted to calibrate in the whole range of mass was 5 ppm. In MSMS product ion experiments, the ion precursor was selected and the collision energy was fixed to 35 eV. Just before each MS/MS experiment, the instrument was calibrated with taurocholic acid and verified after the experiment. The accurate masses obtained were processed using the elemental compositon calculator incorporated in the Analyst Software (Applied Biosystems). A margin of error up to 5 ppm for unknown compounds was allowed. Chromatographic conditions were similar as for HPLC-PAD analysis.

Quantification of identified compounds was carried out by using calibration curves of its authentic standard (Extrasynthese S.A., Genay, France) at five levels in triplicate by HPLC-PAD. Moreover, 6-hydroxyluteolin-7-O-glucoside, luteolin-O-glucoside,

188 lithospermic acid isomer and salvianolic acid isomer were quantified by the calibration
189 curve of luteolin-7-o-glucoside, lithospermic acid and salvianolic acid, respectively.
190 Validation of the chromatographic method (LOD, LOQ, precision, repeatability,
191 stability and recovery) was previously done (data not shown).
192
193 ***In vitro* digestion**
194 The digestion process was carried out following a previously published protocol[12]
195 slightly modified. Briefly, each extract (100 mg) was dissolved in 5 mL of ethanol:
196 water (50:50), mixed with 0.1 mL α- amylase from human saliva type XIII-A (Sigma-
197 Aldrich, St. Louis, MO, USA) (9.3 mg in Cl_2Ca 1 mM) and shaking for 2 min at 37ºC
198 (oral phase). Stomach and intestinal phases were carried out employing a titrator Titrino
199 Plus 877 (Methrom AG, Herisau, Switzerland). Thus, oral phase was mixed with 25 mL
200 of a gastric solution (127 mg of porcine pepsin from porcine mucosa, 536 U/mg,
201 (Sigma-Aldrich, St. Louis, MO, USA) at pH 2 (adjusted with 0.1M HCL) and shaking
202 for 1h at 37ºC. After gastric digestion, samples were adjusted to pH 7 with 1M NaOH
203 prior to the pancreatic step. Next, a pancreatic-bile extract containing 9.3 mg pancreatin
204 (Sigma-Aldrich, St. Louis, MO, USA) and 115.7 mg of bile salts in 2.8 mL of 10mM
205 trizme-maleate buffer was incorporated and incubated for 2h at 37ºC. At the end of
206 digestion, the enzyme reaction was stopped immediately by cooling the samples in ice
207 and samples were kept at -20ºC until analysis.
208
209 **Caco-2 experiments**
210 Caco-2 cells (American Type Culture Collection (ATCC), Manassas, VA, USA) were
211 maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10%
212 fetal bovine serum (FBS), 100 U/mL penicillin, 100 mg/mL streptomycin, 1%

nonessential amino acids and 2 mM L-glutamine (Gibco, Paisley, UK) at 37 ºC in at humidified atmosphere containing 5% CO_2. The cytotoxic effect on Caco-2 cells of the extracts after digestion process was tested using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay following Mosmann[26] method. Briefly, Caco-2 cells were plated in 24-well plates until cell monolayers were obtained. Then the medium was aspirated and cells were treated with different concentrations of digested extracts for 6h. After the medium was removed and cells washed and incubated with MTT for 2-3h. The medium was removed and 500 µL of DMSO were added. The absorbance was measured at 570nm.

For transport experiments, Caco-2 cells were seeded onto six-wells Transwell® plates (0.4 µm pore size, inserts of 24 mm diameter, Costar, Corning, Madrid, Spain) at a density of $3x10^5$ cells per insert. The cells were maintained along 21 days, once the monolayer was formed, during which time culture medium was replaced every three days. The Caco-2 monolayer was used when transepithelial electrical resistance (TEER) (EVOM2, World Precision Instruments, Hitchin, UK) values were larger than 350 Ω cm^2. ~~The integrity of the monolayer was checked by measuring the transepithelial electrical resistance (TEER) (EVOM2, World Precision Instruments, Hitchin, UK).~~ Apical and basolateral compartments were washed once with PBS and then incubated with 1.5 mL and 2.6 mL of supplement DMEM without FBS. 150 µL of digested extracts were incorporate in the apical compartment and incubated for 6 h at 37 ºC. TEER value was measured twice before and after experiment to monitor the integrity of the Caco-2 monolayer. Then apical and basolateral samples were freeze-dried and stored at – 20ºC prior analysis.

Anti-inflammatory activity of basolateral samples from Caco-2 experiments

Human THP-1 monocytes (ATCC, Manassas, VA, USA) were cultured in RPMI 1640 culture medium supplemented with 10% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin, 2 mM L-glutamine (Gibco, Paisley, UK) and 0.05 mM β-mercaptoethanol (Sigma-Aldrich, Madrid, Spain) at 37ºC in 95% humidified air containing 5% CO_2. Cells were plated at a density of $5x10^5$ cells/mL in 24 wells plates. Differentiation of monocytes to macrophages (THP-1/M cells) was induced by maintaining the cells with 100 ng/ml of phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, Madrid, Spain) for 48 h. The viability of THP-1/M cells in presence of basolateral medium from Caco-2 experiments was tested used the MTT assay following Mosmann[26] method. The assays were performed in triplicate.

After differentiation, cells were washed with PBS and incubated with 0.05 µg/mL of LPS from E. Coli O55:B5 (Sigma-Aldrich, Spain) in presence of the basolateral medium from Caco-2 experiments for 24 h. Then, the supernatant was frozen at -20ºC.). Positive controls represented THP-1/M cells incubated with LPS but without basolateral medium and negative controls indicated cells non stimulated with LPS and without basolateral medium.

The release of TNF-α, IL-1β and IL-6 was measured in the supernatants of THP-1/M cells using ELISA kits (BD Biosciences, Aalst, Belgium), according to manufacturer's instructions. The color generated (yellow) was quantified by measuring the optical density at 450 nm with substrate correction at 570 nm using a multiscanner autoreader (InfiniteM200 Tecan, Barcelona, Spain). The results were expressed as the mean of three determinations ± standard deviation.

Statistical analysis

Experimental results are expressed as means ± standard deviation (SD). One-way analysis of variance (ANOVA) followed by least significant difference test was used at $p \leq 0.05$ to look for differences between means. Statistical analyses were performed using Statgraphics v. Centurion XVI software for Windows (Statpoint Inc., Warrenton, VA, USA).

RESULTS AND DISCUSSION

Phenolic and functional characterization of PLE and enriched extracts

PLE extracts from marjoram were carried out using 70% ethanol, 100ºC and 10 min. These extraction conditions were supported by previous studies (data not shown) developed to obtain extracts with a high content of TPC and an important antioxidant activity. The PLE obtained extract presented a TPC of 265.9 mg GAE/g extract and a TEAC value of 1.81 mmol Trolox/g extract.

For the purpose to achieve extract enrichment in phenolic compounds, XAD-7HP resin was used. The effectiveness of this kind of resins for rosmarinic acid enrichment has been previously reported.[23] As Table 1 shows, the use of this resin achieved a TPC 1.5-fold superior to the original PLE extract. ~~representing a 39% of the extract~~. Thus, the amount of phenolic compounds presented in the new extract represented a 39% of total extract. Moreover, this enriched extract also presented significantly higher antioxidant activity (Table 1).

The HPLC-PAD-MS-QTOF analysis of the phenolic compounds (Table 2) resulted in the identification of 17 compounds in the extracts (original and enriched), whereas p-coumaric and neochlorogenic acids were also identified in the digested extracts. All the identified compounds in the extracts had been previously described in marjoram extracts (Kawabata et al., 2003; Taamalli et al, 2015; Vallverdú-Queralt et al., 2015).

287 Moreover, according to its specific λ_{max}, accurate mass and MS/MS fragments 6-
288 hydroxyluteolin-7-O-glucoside, luteolin-O-glucoside, lithospermic acid isomer and
289 salvianolic acid isomer were tentatively identified in the extracts (de Beer et al., 2011;
290 Greenham et al., 2003; Taamalli et al., 2015).
291 Both extracts showed a similar qualitative composition, although important quantitative
292 differences were found (Table 3). Therefore, enriched extract presented, in general, an
293 increase between 1.8-1.5 times in the quantity of all compounds in relation to the
294 original extract. Accordingly, the main components in both extracts were 6-
295 hydroxyluteolin-7-O-glucoside and rosmarinic acid, followed by a luteolin-O-glucoside
296 and an isomer of salvianolic acid.
297 This phenolic composition was consistent with other research works where marjoram
298 extracts had been characterized by an extended composition in phenolic acids and
299 flavonoids. These works reported that, among phenolic acids, RA was the main
300 compound detected. In addition, luteolin and its glucosil derivatives have been also
301 detected as the main flavonoids.[3,16]
302
303 **Phenolic composition and antioxidant activity of the extracts during *in vitro***
304 **digestion**
305 Digestion effect in TPC and antioxidant activity for both extracts is shown in Table 1.
306 After digestion process, the amount of TPC only decreased slightly for both extracts, as
307 well as antioxidant activity. Notwithstanding, digestion step produced a remarkable
308 reduction of RA (approx. 50%) in both original and enriched extracts (Table 3). This
309 decrease in RA content during gastrointestinal digestion was in agreement with other
310 studies that reported RA degradation or transformation into other compounds during *in*
311 *vitro* gastrointestinal digestion.[27,28] Moreover, Zoric et al.[29] showed that gastrointestinal

stability of RA was highly influenced by plant matrix, indicating that the presence of some flavonoids such as luteolin or apigenin enhances the stability rate of RA during digestion process.

~~On the other hand~~ Moreover, the quantitative analysis of phenolic composition after the digestion process (Table 3) showed that most compounds, presented in both extracts, were affected by digestion step to a lesser or greater extent. For that matter, although RA was reduced after digestion, it represented the main compound in both digested extracts, followed by luteolin 7-O-glucoside, 6-hydroxyluteolin-7-O-glucoside and luteolin-O-glucoside. Among these compounds, 6-hydroxyluteolin-7-O-glucoside and luteolin-O-glucoside were most affected by the digestion step, meanwhile, luteolin 7-O-glucoside and glucuronide, were much less affected, even more apigenin 7-O-glucoside and glucuronide increased after digestion. It should be also noted the appearance of neochlorogenic and p-coumaric acids in both digested extracts, compounds not detected in the non-digested extracts, and whose presence could be attributed to isomerization and degradation processes of initial phenolic acids. Accordingly, Xie et al.[30] noted isomeric transformations of chlorogenic acids, where cryptochlorogenic acid was mainly turned into chlorogenic acid at pH 7 and 37 ºC, in agreement with intestinal conditions. Moreover, some studies have shown the presence of coumaric acid as a related metabolite of rosmarinic acid in human and animal plasma.[31,32]

Caco-2 cell transport experiments.

Transport experiments of digested extracts were carried out using an *in vitro* model of the intestinal barrier: Caco-2 cells differentiated to enterocytes[33]. Prior to transport experiments, the cytotoxicity of the digested extracts was evaluated at 6h. The results

showed that 150 µL of the digested extracts was the maximum concentration that did not significantly affected cell viability (data not shown). In addition, the integrity of the Caco-2 monolayer during exposure experiments was evaluated as TEER-value.

Total phenolic compounds content recovered in apical and basolateral fractions for both digested extracts was measured to determine the quantity of these compounds unabsorbed and bioavailable, respectively (Table 4). For both extracts, only a small amount of the phenolic compounds presented in the digested extracts was detected in the bioavailable fraction. Thereby, the bioavailable fraction from enriched extract presented a quantity of 84.8 mg GAE/L digested extract, meanwhile for original extract only 68.5 mg GAE/L digested extract was detected in basolateral fraction.

The phenolic compounds recovered in apical and basolateral fractions for both digested extracts were also analysed by HPLC in order to determine the bioavailability of individual components (Table 5). The results indicated that, for both extracts, the main component detected in basolateral fraction was RA, although enriched extract presented a quantity 1.6 times superior. Besides RA, lithospermic acid isomer and diosmin were the major components of basolateral fraction in both extracts, meanwhile 6-hydroxyluteolin-7-O-glucoside and luteolin 7-O-glucoside, compounds with an important presence after digestion process, only were presented in less than 5% in that fraction.

The permeability of RA across Caco-2 cells monolayer has been studied by several authors. Accordingly, Konishi and Kobayashi[13] reported that RA transport throughout Caco-2 cells was mainly *via* paracellular diffusion and its intestinal absorption efficiency was low. These authors supported this idea with the fact that RA transport

362 increased linearly with the concentration and did not reach a plateau even at 30 nM.
363 However, more recently, Qiang et al.[34] suggested that RA was absorbed across Caco-2
364 cells *via* both paracellular and transcellular diffusion. Moreover, Falé et al.[35] reported
365 that the transport of RA across these cells was increased in presence of a mixture of
366 luteolin and apigenin, since these flavonoids may interfere in several mechanisms
367 involved in the permeation of RA, such as uptake and efflux mechanisms, indicating
368 that the flux of RA may be mediated by transport systems. Data obtained in this work
369 for RA were consistent with the idea that RA transport throughout Caco-2 cells was
370 mainly *via* paracellular diffusion, since in this case the transport increased linearly with
371 the concentration. Thus, enriched extract (after digestion) with a quantity of rosmarinic
372 acid 1.6 times higher than original extract, also presented in basolateral fraction 1.6 fold
373 of RA than original extract. However, it could not be discarded the influence of the
374 flavonoid content in the absorption efficiency of RA obtained in this work (approx.
375 14%).
376
377 In this work, the bioavailability of luteolin and apigenin derivatives was, in general,
378 lower than for RA, although, in this case the transport also increased linearly with the
379 concentration. However, in this study is noteworthy that, after 6h of experiment, the
380 amount of luteolin and apigenin aglycone in the apical compartment substantially
381 increased in relation to the amount of these components at t=0. According to those
382 results, Yasuda et al.[36] suggested that luteolin glucoside is partially hydrolysed by LPH
383 (lactase-phlorizin hydrolase), and moreover, only a fraction of the released aglycone is
384 absorbed inside the cells, where it is converted to its glucuronide conjugate, and
385 subsequently secreted to basolateral compartment. In this study, glucoside and
386 glucuronide forms of luteolin and apigenin were detected in basolateral solution after 6

h, inferring that a portion of glycoside derivatives are also transported in an unchanged way across Caco-2 cells.[37,38] Nevertheless, for a better comprehensive absorption path for flavones, further analysis should be submitted.

In addition, antioxidant activity for both digested extracts was also measured in apical and basolateral compartments and data are shown in Table 4. As could be observed, the antioxidant activity detected in basolateral fraction from enriched extract was ~~a 13%~~ significantly higher than that measured in this fraction when original extract was used. This result was in agreement with the greater amount of phenolic compounds detected in the basolateral chamber for this extract. Thus, mainly RA, lithospermic acid and diosmin presented in the bioavailable fraction could be responsible of its antioxidant activity.

Anti-inflammatory activity of basolateral samples from Caco-2 experiments

The activation of THP-1/M was carried out by LPS incorporation into the medium. After 24h of incubation, LPS treated cells shown an important increase in all pro-inflammatory cytokines measured (TNF-α, IL-1β and IL-6), compared to non-activated controls (Fig. 1). These activated cells were considered as positive controls for all the cytokines tested. The cytotoxicity assays (data not shown) indicated that 20 μL of the basolateral fraction was the maximum concentration that did not affected THP-1 viability. Thus, when the activation of THP-1/M was carried out in presence of 20 μL of basolateral medium from Caco-2 experiments, an important decrease in TNF-α secreted level was observed compared with levels obtained in absence of extracts (positive control). Moreover, basolateral samples from enriched extract after digestion achieved an 80% of inhibition in TNF-α secretion, higher to that obtained with the original

412 extract (40%). IL-1β and IL-6 secretion were also reduced (60%) in presence of 20 μL
413 basolateral fractions of original digested extracts (with respect to positive control). For
414 both interleukins, the enriched extract decreases its release in a greater extent than
415 original extract, a 70% for IL-1β and an 85% for IL-6. Besides, the basolateral fraction
416 from control digestion did not reduced significantly the secretion of any of the pro-
417 inflammatory cytokines studied, compared to positive control.
418 Considering these results, basolateral fractions of both digested extracts presented an
419 important anti-inflammatory activity, although enriched extract showed a higher
420 inhibition in the release of all pro-inflammatory cytokines studied. In this regard,
421 several studies have reported that RA, either as pure standard or incorporated into a
422 vegetable matrix, inhibited LPS-induced up-regulation of IL-1 β, TNF-α and IL-6 in
423 different cells lines.[11,12] Besides, luteolin and its derivatives have also been reported to
424 be able to inhibit the production of pro-inflammatory cytokines, such as TNF-α and IL-
425 1β.[39] However, none of these studies measured the inhibition in the production of these
426 cytokines by extracts or pure compounds after a digestion and absorption processes.
427 In conclusion, this study showed the potential use of marjoram extracts as a source of
428 bioavailable compounds with an important antioxidant and anti-inflammatory activities.
429 Thus, the results indicated that PLE extracts from *Origanum majorana* L. represented a
430 rich source of bioavailable RA, especially when using the enriched extract. Besides RA,
431 luteolin derivatives, lithospermic acid isomer and diosmin were also detected in the
432 basolateral fraction in both extracts. Moreover, the bioavailable fractions of both
433 extracts showed a remarkable antioxidant and anti-inflammatory activities, being more
434 prominent when using enriched extract. Thus, RA could have an important role in these
435 activities ~~could be mainly related with RA~~, although other phenolic compounds detected in

436 the basolateral fractions could also interact synergistically. ~~and with the presence of other~~

437 ~~phenolic compounds in a lesser extent.~~

438

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443

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445

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Figure captions

Figure 1: Levels of TNF-α, IL-1β and IL-6 secreted by THP-1/M, activated with LPS, in presence of bioavailable fractions from extracts. Positive control (cells stimulated with LPS but in absence of extract), negative control (cells in contact just with RPMI media), control digestion (bioavailable fraction from a digestion without extract, only digestion juices). Each bar is the mean of three determinations ±S.D. * Denotes statistical differences when compares with positive control. [a,b,c] Different letters indicate statistical differences among original extract, enriched extract and digestion control. Significance level at $p \leq 0.05$ with LSD procedure.

Tables

Table 1. TPC content and antioxidant activity in original and enriched extracts.

		TPC (mg GAE/g)	TEAC value (mmol Trolox/g)
Original extract	initial	266 ± 4.80 $^{a}{}_{2}$	1.81 ± 0.04 $^{a}{}_{2}$
	after digestion	2220 ± 12.6 $^{b}{}_{2}$	1.71 ± 0.05 $^{b}{}_{2}$
Enriched extract	initial	389 ± 38.6 $^{a}{}_{1}$	2.81 ± 0.01 $^{a}{}_{1}$
	after digestion	312. ± 4.86 $^{b}{}_{1}$	2.67 ± 0.03 $^{b}{}_{1}$

Data shown represent means ± S.D. (n=3).

[a,b] Different superscript letters denote significant differences within the same extract before and after digestion ($p \leq 0.05$).

[1,2] Different subscript numbers denote significant differences between both extracts in the same condition (before or after digestion) ($p \leq 0.05$).

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Table 2. Phenolic compounds identified in the samples using HPLC-PAD-MS/MS.

Peak	Compound	R_t (min)	λ_{max} (nm)	Acc. mass (m/z)	Error (ppm)	MS^2 (m/z)
1	Neochlorogenic acid*	12.9	300, 326	353.0878	+1.3	191(100), 179(75), 135(30)
2	Protocatechuic acid*	13.4	260, 294	153.0193	+1.2	153(30), 109(100)
3	Caftaric acid*	13.7	300, 328	311.0408	+2.5	149(100)
4	Cryptochlorogenic acid*	15.1	300, 326	353.0878	+4.0	191(100), 179(75), 135(30)
5	Vicenin II*	15.9	272, 336	593.1511	-1.8	503(20), 473(100), 383(20), 353(30)
6	Caffeic acid*	17. 8	299, 324	179.0349	+2.6	135(100)
7	6-hydroxyluteolin-7-O-glucoside	19.8	282, 344	463.0882	-4.1	463 (20), 301(100)
8	p-coumaric*	22,9	310	163.0400	+2.1	119(100)
9	Luteolin-O-glucoside	23.4	282, 334	447.0932	-3.4	285(100), 151(20)
10	Luteolin 7-O glucoside*	23.8	254, 266, 348	447.0932	-2.6	285(100), 151(20)
11	Luteolin 7-O-glucuronide*	24.0	254, 266, 348	461.0725	-4.7	285(100), 151(20)
12	Diosmin*	27.1	352, 266, 346	607.1668	-2.7	607(10), 299(100), 284(10)
13	Apigenin 7-O-glucoside*	27.6	266, 336	431.0983	-4.6	431(20), 269(100)
14	Apigenin 7-O-glucuronide*	28.1	266, 336	445.0776	-4.3	445(20), 269(100)
15	Rosmarinic acid*	28.9	288, 330	359.0772	+0.7	197(20), 161(100), 179(30), 135(20)
16	Lithospermic acid	29.7	288, 310, 334	537.1038	-4.9	493(10), 359(30), 295(30), 197(20), 161(100), 135(80)

	isomer					
17	Salvianolic acid isomer	30.4	288, 310, 334	717.1461	-2.8	717(10), 519(30), 475(10), 359(100), 295(10), 179(10)
18	Luteolin*	33.7	254, 266, 348	285.0404	-3.0	285(20), 217(35), 175(100), 151(50)
19	Apigenin*	37.0	266, 336	269.0444	-3.8	269(20), 225(30), 151(100), 119(50)

*Comparison with standards

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Table 3. Effect of *in vitro* gastrointestinal digestion process in extracts composition.

Compound	Original extract (mg/g extract)		Enriched extract (mg/g extract)	
	Initial	After digestion	Initial	After digestion
Neochlorogenic acid	N.D. [b]	0.57 ± 0.01 *	N.D.	0.88 ± 0.02 *
Protocatechuic acid	0.17 ± 0.01	0.19 ± 0.00 *	0.30 ± 0.01	0.18 ± 0.00 *
Caftaric acid	0.06 ± 0.00	0.04 ± 0.00 *	0.10 ± 0.01	0.09 ± 0.00
Cryptochlorogenic acid	0.86 ± 0.00	0.53 ± 0.00 *	1.49 ± 0.01	0.76 ± 0.00 *
Vicenin II	2.32 ± 0.02	2.24 ± 0.03 *	4.00 ± 0.01	3.73 ± 0.01 *
Caffeic acid	0.93 ± 0.07	0.57 ± 0.01 *	1.32 ± 0.01	0.88 ± 0.01 *
6-hydroxyluteolin-7-O-glucoside	33.9 ± 0.09	14.5 ± 0.18 *	60.9 ± 0.04	21.0 ± 0.23 *
p-coumaric	N.D.	0.36 ± 0.00 *	N.D.	0.52 ± 0.00 *
Luteolin-O-glucoside	25.2 ± 0.03	10.5 ± 0.12 *	44.7 ± 0.02	15.3 ± 0.17 *
Luteolin 7-O glucoside	15.5 ± 0.08	14.6 ± 0.02 *	25.5 ± 0.01	21.8 ± 0.04 *
Luteolin 7-O-glucuronide	5.69 ± 0.02	5.38 ± 0.02 *	9.29 ± 0.01	8.17 ± 0.04 *
Diosmin	6.48 ± 0.04	6.46 ± 0.03	10.3 ± 0.01	10.8 ± 0.06 *
Apigenin 7-O-glucoside	2.45 ± 0.02	3.22 ± 0.03 *	4.69 ± 0.01	4.90 ± 0.04 *
Apigenin 7-O-glucuronide	3.64 ± 0.49	5.74 ± 0.06 *	7.20 ± 0.01	8.70 ± 0.02 *
Rosmarinic acid	33.9 ± 0.05	19.0 ± 0.11 *	57.2 ± 0.05	29.0 ± 0.34 *
Lithospermic acid isomer	9.10 ± 0.02	6.91 ± 0.11 *	15.9 ± 0.05	11.0 ± 0.11 *
Salvianolic acid isomer	17.2 ± 0.35	2.60 ± 0.14 *	24.9 ± 0.16	4.27 ± 0.09 *
Luteolin	1.39 ± 0.08	0.55 ± 0.03 *	2.07 ± 0.01	0.73 ± 0.02 *
Apigenin	0.45 ± 0.01	0.22 ± 0.00 *	0.75 ± 0.01	0.31 ±0.04 *

[a] N.D. = non detected. * Denotes significant difference when compares initial and after digestion concentration within same extract ($p \leq 0.05$). Data represent means ± S.D. (n= 4).

Table 4. TPC content and antioxidant activity in original and enriched extracts after caco-2 absorption experiments.

		TPC (mg GAE/L digested extract)	TEAC value (mmol Trolox/ L digested extract)
Original extract	After digestion	$502 \pm 18.1^{a}{}_{2}$	$3.41 \pm 0.16\ ^{a}{}_{2}$
	Apical fraction	$358 \pm 26.7\ ^{b}{}_{2}$	$1.77 \pm 0.21^{b}{}_{2}$
	Basolateral fraction	$68.5 \pm 6.40\ ^{c}{}_{2}$	$0.73 \pm 0.01\ ^{c}{}_{2}$
Enriched extract	After digestion	$684 \pm 31.1^{a}{}_{1}$	$4.78 \pm 0.58\ ^{a}{}_{1}$
	Apical fraction	$556 \pm 40.5\ ^{b}{}_{1}$	$3.74 \pm 0.36\ ^{b}{}_{1}$
	Basolateral fraction	$84.8 \pm 7.86\ ^{c}{}_{1}$	$0.89 \pm 0.09\ ^{c}{}_{1}$

Data shown represent means $\pm$ S.D. (n=3).

[a,b,c] Different superscript letters denote significant differences within the different fractions of the same extract ($p \leq 0.05$).

[1,2] Different subscript numbers denote significant differences among the same fractions of both extracts ($p \leq 0.05$).

Table 5. Extracts phenolic composition after caco-2 absorption experiments.

Compound	Original extract (mg/L digested extract)			Enriched extract (mg/L digested extract)		
	Initial	Apical	Basolateral	Initial	Apical	Basolateral
Neochlorogenic acid	1.45 ± 0.41	0.94 ± 0.04	0.31 ± 0.03	2.09 ± 0.03	1.77 ± 0.09	0.41 ± 0.07
Protocatechuic acid	0.45 ± 0.03	0.37 ± 0.00	N.D. [b]	0.43 ± 0.03	0.39 ± 0.04	N.D.
Caftaric acid	0.06 ± 0.00	N.D.	N.D.	0.15 ± 0.03	0.05 ± 0.00	N.D.
Cryptochlorogenic acid	1.27 ± 0.00	0.81 ± 0.08	0.13 ± 0.03	1.75 ± 0.02	1.39 ± 0.09	0.21 ± 0.07
Vicenin II	4.81 ± 0.54	4.07 ± 0.52	0.63 ± 0.09	8.35 ± 0.14	7.74 ± 0.42	1.00 ± 0.40
Caffeic acid	1.51 ± 0.17	1.08 ± 0.10	0.37 ± 0.03	2.39 ± 0.01	1.86 ± 0.07	0.54 ± 0.09
6-hydroxyluteolin-7-O-glucoside	17.1 ± 1.04	2.66 ± 0.35	0.47 ± 0.29	20.5 ± 0.43	6.69 ± 1.09	1.01 ± 0.62
p-coumaric	0.80 ± 0.15	0.50 ± 0.03	0.30 ± 0.01	1.38 ± 0.05	0.81 ± 0.01	0.45 ± 0.06
Luteolin-O-glucoside	12.8 ± 0.84	2.81 ± 0.10	0.42 ± 0.21	15.9 ± 0.41	6.61 ± 0.89	1.02 ± 0.58
Luteolin 7-O glucoside	17.2 ± 1.35	2.69 ± 0.41	0.54 ± 0.32	23.84 ± 0.58	8.34 ± 1.22	1.03 ± 0.05
Luteolin 7-O-glucuronide	7.03 ± 0.99	2.26 ± 0.04	0.74 ± 0.06	8.36 ± 0.23	4.71 ± 0.28	1.26 ± 0.03
Diosmin	10.5 ± 0.19	8.56 ± 0.02	1.06 ± 0.21	15.7 ± 0.17	18.6 ± 1.36	1.96 ± 0.62
Apigenin 7-O-glucoside	4.25 ± 0.10	1.66 ± 0.02	0.17 ± 0.06	6.20 ± 0.08	3.72 ± 0.14	0.46 ± 0.21
Apigenin 7-O-glucuronide	9.03 ± 0.36	3.23 ± 0.38	0.75 ± 0.16	13.4 ± 0.24	7.33 ± 0.19	1.40 ± 0.47
Rosmarinic acid	32.2 ± 2.30	23.1 ± 4.05	4.45 ± 0.54	51.6 ± 2.61	42.4 ± 5.63	7.37 ± 1.39
Lithospermic acid isomer	11.4 ± 1.02	8.53 ± 1.37	1.45 ± 0.29	18.3 ± 0.76	14.4 ± 0.08	2.13 ± 0.42
Salvianolic acid isomer	4.27 ± 0.41	3.20 ± 0.33	0.61 ± 0.08	6.44 ± 0.10	4.82 ± 0.10	0.87 ± 0.10
Luteolin	0.23 ± 0.02	1.92 ± 0.75	0.15 ± 0.01	0.45 ± 0.00	3.82 ± 0.69	0.61 ± 0.47
Apigenin	0.04 ± 0.00	0.70 ± 0.39	N.D.	0.07 ± 0.00	1.48 ± 0.37	0.17 ± 0.00

[a] *Data represent means ± S.D. (n= 4).* [b] *N.D. = non detected.*

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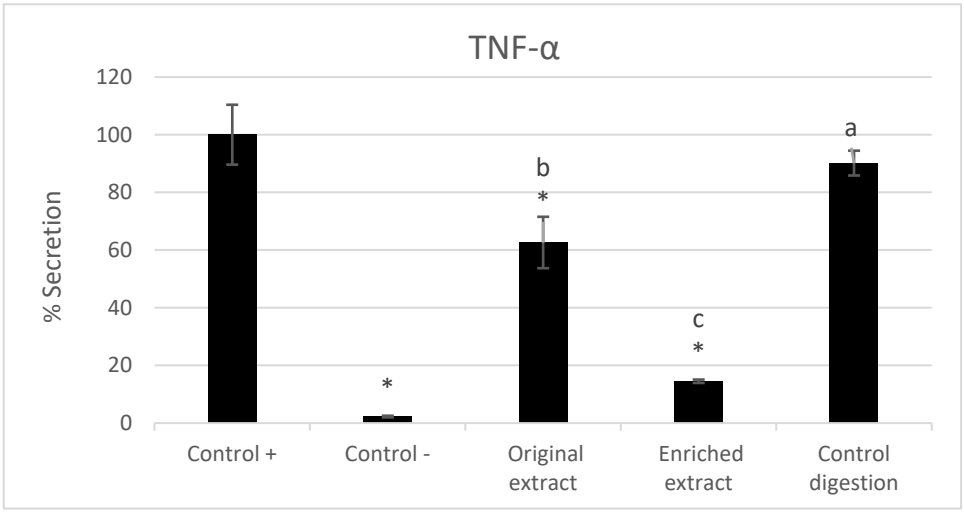
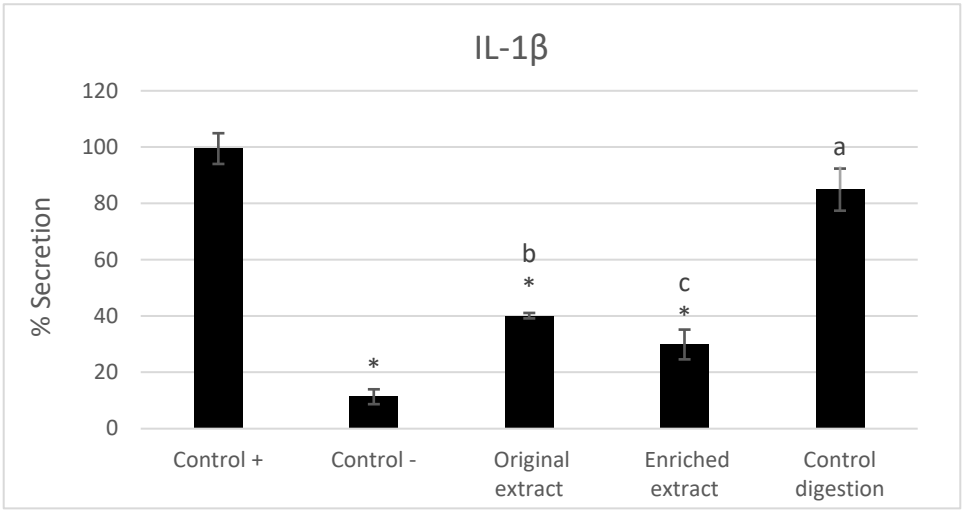
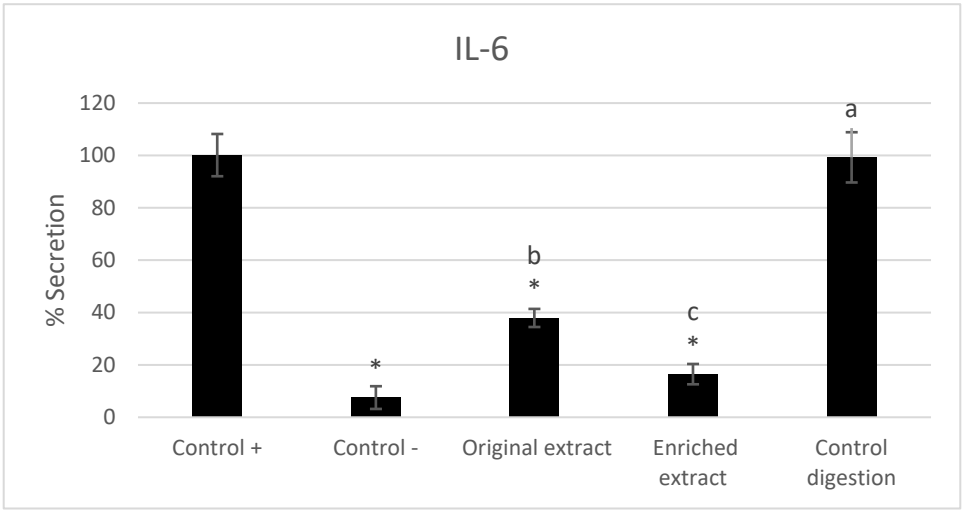
Figures

Figure 1







Graphics

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