Title: Bioaccessibility and biological activity of *Melissa officinalis*, *Lavandula latifolia* and *Origanum vulgare* extracts: influence of an *in vitro* gastrointestinal digestion.

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ABSTRACT

Melissa officinalis, *Lavandula latifolia* and *Origanum vulgare* are widely used medicinal plants and spices. Their extracts were evaluated as potential antioxidants for functional food formulations. After being submitted to an *in vitro* gastrointestinal digestion, all the extracts showed antioxidant activity (measured by DPPH, ABTS, FRAP, TPC). Furthermore, their main polyphenols maintained high stability. Biological activity was measured using *Caenorhabditis elegans,* which is a widely used model in this context. Treatments of 50 and 100 µg/mL of *M. officinalis* significantly attenuated juglone-induced stress in the survival assay performed; moreover, all tested concentrations decreased intracellular ROS. *Lavandula latifolia* and *O. vulgare* had no significant effect against acute stress in the survival assay, but significantly decreased ROS basal levels. GST-4 expression under juglone-induced oxidative stress was significantly down-regulated by treatment with the three plant extracts (up to 63%). Besides, similar biological activity of all digested extracts was demonstrated in all *in vivo* assays.

Keywords:

Polyphenols, *Caenorhabditis elegans*, antioxidants, lemon balm, oregano, lavender, plant extract.

1. INTRODUCTION

Medicinal plants have long been consumed for their therapeutic effect. Plants of the *Lamiaceae* family, such as *Melissa officinalis* L., *Origanum vulgare* L. and *Lavandula latifolia* Medicus are traditionally used in Spain and other European countries for different purposes (spices, medicinal plants). *Melissa officinalis*, also known as lemon balm, has been used for its sedative, carminative, antispasmodic, neuroprotective and anticancer properties (Barros et al., 2013; López et al., 2009; Saraydin et al., 2012; van Wyk & Wink, 2015). Oregano is a widely consumed spice, with antiviral, anti-inflammatory and cancer prevention activities (González, Lanzelotti, & Luis, 2017; van Wyk & Wink, 2015; van Wyk & Wink, 2017; Zhang et al., 2014). In the case of *L. latifolia*, commonly known as spike lavender, it is mainly used due to its aromatic properties and its medical properties, such as antispasmodic, sedative, antihypertensive, antiseptic, healing and anti-inflammatory (Herraiz-Peñalver et al., 2013; van Wyk & Wink, 2015). Most of these health benefits have been attributed to different bioactive compounds and, in particular, to phenolic compounds, which are molecules associated with antioxidant actions among others (Embuscado, 2015; López et al., 2007; Milevskaya, Temerdashev, Butyl'skaya, & Kiseleva, 2017; Shan, Cai, Sun, & Corke, 2005; van Wyk & Wink, 2015).

The over-accumulation of reactive oxygen species (ROS) in the cells, such as superoxide anion radicals ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-), is known as oxidative stress and is considered to be a promoter of chronic and degenerative illnesses, including cancer, autoimmune disorders, aging, cataracts, rheumatoid arthritis, and cardiovascular and neurodegenerative diseases (Pham-Huy, He, & Pham-Huy, 2008; van Wyk & Wink, 2015). To reduce oxidative damage, the cellular defence mechanisms include an enzymatic redox system, with catalase, superoxide dismutase, glutathione peroxidase and glutathione-reductase as its major enzymes (Bhattacharyya, Chattopadhyay, Mitra, & Crowe,

2014). Furthermore, flavonoids and phenolic acids may also contribute, along with antioxidant vitamins, to the total antioxidant defence system of the human body and to minimize the onset of ROS-related diseases (Saxena, Saxena, & Pradhan, 2012; H. Zhang & Tsao, 2016).

Several *in vitro* analytical assays have traditionally been employed to measure the antioxidant activity of plant-derived antioxidants, such as DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) and FRAP (ferric reducing antioxidant power) (Gülçin, 2012). In recent years, an increasing number of studies have focussed on the assessment of the *in vivo* antioxidant effects using the nematode *Caenorhabditis elegans* (Abbas & Wink, 2010; Abbas & Wink, 2009; Chen et al., 2016; Link, Roth, Sporer, & Wink, 2016; Ma et al., 2016; Sobeh et al., 2016). Some of the advantages of this animal model, as compared to rodent models, are its short lifespan, morphological simplicity, and ease of maintenance and genetic manipulation (Gruber, Ng, Poovathingal, & Halliwell, 2009). Indeed, in contrast to cellular models, it allows to study whole organisms, with many different organs and tissues and increases the chance of identifying synergistic and/or off-target effects (Kaletta & Hengartner, 2006).

It has been described that bioaccessibility/bioavailability of dietary compounds can be influenced by many factors, such as chemical structure (glycosylation, esterification, and polymerization), food matrix, and excretion back into the intestinal lumen (Manach, Scalbert, Morand, Rémésy, & Jiménez, 2004). Therefore, to further understand the potential beneficial effects of the dietary antioxidants, it is important to examine their stability under gastro-intestinal conditions during digestion. In this sense, *in vitro* digestion techniques have been described as good models for predicting compound bioaccessibility (amount of compound available for absorption), and consequently able to be biologically active within the body (Gayoso et al., 2016; Minekus et al., 2014).

Currently, there is growing interest in the identification and characterization of plant-based compounds with high bioaccessibility and bioactivity for their application in the food industry (Barba et al., 2017; Costa et al., 2015). Thus, the objective of this work was to evaluate three plant extracts of common medicinal and aromatic herbs (*M. officinalis*, *O. vulgare* and *L. latifolia*) as potential sources of bioactive compounds. We determined their antioxidant activity using *in vitro* tests and an *in vivo* model (*C. elegans*), before and after a simulated gastrointestinal digestion. Moreover, characterization of their main phenolic compounds was performed by LC-MS.

2. MATERIAL AND METHODS

2.1. Material and chemicals

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid 97%), DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), Folin-Ciocalteu reagent, gallic acid, juglone (5-hydroxy-1,4-naphthalenedione), H_2DCF-DA (2′,7′-dichlorodihydrofluorescein diacetate), EGCG [(-)-epigallocatechin gallate] and rosmarinic acid (RA) were purchased from Sigma-Aldrich (Steinheim, Germany), and sodium azide from AppliChem GmbH (Darmstadt, Germany).

In vitro digestion: alpha-amylase from human saliva (A1031, 852 units/mg protein), pepsin from porcine gastric mucosa (P7000, 674 units/mg protein), pancreatin from porcine pancreas (P1750, 4 × United States Pharmacopeia specifications) and bile extract (B8631) were purchased from Sigma-Aldrich (Steinheim, Germany).

2.2. Plant extracts

Dried leaves of *Melissa officinalis* were purchased from Plantaron S.L. (Barcelona, Spain). *Lavandula latifolia* and *Origanum vulgare* were collected in Navarra (Spain), dried at room temperature and deposited in the PAMP Herbarium at the School of Science of University of

Navarra (voucher number 21581 and 21637, respectively). These three plants were selected based on their use in the traditional Spanish medicine.

Aqueous extract of *M. officinalis* was prepared as described by Berasategi et al. (2014). A purification of a hydro-alcoholic extract of *M. officinalis* was made after washing with ethanol three times, and the solid residue was redissolved in water and lyophilized.

A methanol extract of *L. latifolia* was obtained by sequential cold maceration with dichloromethane, ethyl acetate and methanol (Pinacho, Cavero, Astiasarán, Ansorena, & Calvo, 2015). The extract was concentrated using a rotary evaporator and lyophilized.

A methanol extract of *O. vulgare* was obtained by immersing 10 g of dried plant into 250 mL of methanol and heating until boiling by refluxing for 30 min. The extraction process was repeated 3 times with 250 mL of methanol. The extracts obtained after filtration were combined, concentrated using a rotary evaporator, and lyophilized.

2.3. *In vitro* gastrointestinal digestion

The three plant extracts were digested *in vitro* following the procedure described by Gayoso et al. (2016) with some modifications. Stock solutions of the enzymes were freshly prepared (1.3 mg/mL of alpha-amylase solution in 1 mM $CaCl_2$; 160 mg/mL of pepsin solution in 0.1 M HCl;4 mg of pancreatin + 25 mg of bile extract mL/solution in 0.1 M $NaHCO_3$ for the pancreatin-bile extract). The digestion was performed in Falcon tubes inside a water bath at 37 °C with magnetic stirring. 500 mg of plant extract were mixed with 12.5 mL of water (40 mg/mL) and after a preheating at 37 °C, 125 µL of amylase were added and its pH was adjusted to 6.5 in order to start the oral phase (2 min). Afterwards, the gastric step followed by the addition of 165 µL of pepsin at pH 2.5, for 2 h. Finally, in the intestinal step, 1250 µL of pancreatin-bile extract were added and its pH was adjusted to 7.5. After 2 h, the intestinal digestion mixture was centrifuged (4000 rpm, 40 min) and the supernatant (soluble fraction) was collected and lyophilized (Cryodos-50, Telstar, Barcelona, Spain). The pH adjustments

were performed with 1 M $NaHCO_3$ (oral and intestinal step) or 3 M HCl (gastric step). Each plant extract was digested in triplicate.

2.4. *C. elegans* strains and maintenance

Wild type (N2) and CL2166 (dvIs19 [(pAF15)gst-4p::GFP::NLS]) strains were used in this work. Nematodes were grown on nematode growth medium (NGM) inoculated with living *Escherichia coli* OP50 (*E. coli* OP50) and incubated at 20 °C (Stiernagle, 2006). All used *C. elegans* strains and *E. coli* OP50 were provided by the *Caenorhabditis* Genetic Center (CGC, University of Minnesota, MN, USA).

2.4.1. Worm synchronization

Age-synchronized worms were obtained by hypochlorite treatment (Peixoto, Roxo, Krstin, Röhrig, et al., 2016). Adults were treated with 1.5 mL of 5M NaOH and 0.5 mL of 5% NaOCl and the suspension was vigorously shaken for 5 min. The lysate was then pelleted by centrifugation (1200 rpm, 1 min). Consecutively, the supernatant was removed and 5 mL of sterile water and 5 mL of sucrose solution 60% (w/v) were added. The eggs were separated from the lysed worms and other debris by a sucrose density gradient centrifugation (1200 rpm, 4 min). The upper layer, in which the eggs float, was transferred to a new tube, mixed with 10 mL of sterile water and centrifuged (1200 rpm, 2 min) in order to remove the sucrose. The supernatant was then removed and the eggs resuspended in M9 buffer. 24 h after synchronization, L1 larvae were transferred to S-medium inoculated with living *E. coli* OP50 (OD_{600}=1.0) and treated with different concentrations of plant extracts (before and after the *in vitro* digestion).

2.5. *In vivo* antioxidant activity

Samples [plant extracts (before and after the *in vitro* digestion process), RA and EGCG] were dissolved in water and added to the plate with S-medium. Control plates had the same final volume but lacked sample solutions. The final concentrations tested in the plate were 50, 100

and 200 µg/mL for *L. latifolia* and *O. vulgare* extracts. In the case of *M. officinalis*, a dose of 200 µg/mL was toxic for the worms, so the concentrations tested were 50, 100 and 150 µg/mL. During the *in vitro* digestion, there is a decrease in the concentration of the plant extract due to a dilution effect that occurs during the digestive process (Gayoso et al., 2016). For this reason, samples of the plant extracts after digestion were tested in a concentration equivalent to the amount of extract tested in the plant extract before digestion (100, 200, 400 µg/mL equivalent to 50, 100 and 200 µg/mL of extract from *L. latifolia* after digestion, respectively; 109, 216 and 435 µg/mL equivalent to 50, 100 and 200 µg/mL of extract from *O. vulgare* after digestion, respectively; 250 and 500 µg/mL equivalent to 95 and 190 µg/mL of extract from *M. officinalis* after digestion, respectively). The concentrations for the positive controls were 50 µg/mL for EGCG and 50 and 100 µg/mL for RA.

2.5.1. Intracellular ROS levels

Intracellular ROS accumulation was measured in wild type worms using H_2DCF-DA (Peixoto, Roxo, Krstin, Wang, & Wink, 2016). Age-synchronized worms (L1 stage) were treated with various concentrations of samples (plant extracts before and after *in vitro* digestion), except for the control group. After 48 h, 50 µM of H_2DCF-DA were added to the plate, which was then incubated for 1 h at 20 °C, protected from light. Afterwards, worms were placed onto a glass slide with a 10 mM sodium azide drop for their paralysis. Images were taken in a fluorescence microscope at a constant exposure time using a 10X objective lens (BIOREVO BZ-9000, Keyence, Deutschland GMBH, Neu-Isenburg, Germany) and analysed with ImageJ 1.50i software (Wayne Rasband, National Institute of Health, USA). Fluorescence intensity correlated with the ROS level. Results (mean fluorescence intensity) were presented as mean ± SEM of four independent experiments with 30 worms/ experiment.

2.5.2. Survival assay

The survival assay was conducted as described by Peixoto, Roxo, Krstin, Röhrig, et al. (2016). Synchronized wild type L1 larvae were treated with various concentrations of samples (plant extracts before and after *in vitro* digestion), except for the control group. After 48 h, approximately 80 worms were picked up and transferred to new plates with S-medium, treated again with the respective sample, except for the control group, and exposed to a lethal dose of juglone (80 µM). Survivors were counted 24 h after the lethal oxidative stress induced by juglone. Worms not responding to touch with a platinum wire were counted as dead. The assay was repeated at least three times and the results were expressed as percentage of survival (mean ± SEM).

2.5.3. Glutathione-S-transferase under juglone-induced oxidative stress

CL2166, a transgenic strain expressing green fluorescent protein (GFP), was used to quantify the expression of glutathione-S-transferase (GST-4). Age-synchronized worms (L1 stage) were treated with various concentrations of samples (plant extracts before and after *in vitro* digestion), except for the control group. Worms were exposed to 20 µM juglone, 24 h before being analyzed by fluorescence microscopy. An additional control group was included without juglone. After 72 h, the worms were placed onto a glass slide with a 10 mM sodium azide drop and the analysis by fluorescence microscopy was carried out (BIOREVO BZ-9000, Keyence, Deutschland GMBH, Neu-Isenburg, Germany). Images were taken at constant exposure time using a 10X objective lens and analysed with ImageJ 1.50i software (Wayne Rasband, National Institute of Health, USA). Results (mean fluorescence intensity) were presented as mean ± SEM of four independent experiments.

2.6. I*n vitro* antioxidant activity

Lyophilized samples (plant extracts before and after digestion) were dissolved in methanol/water/formic acid (79.9/20/0.1; v/v/v) and total phenolic compounds (TPC), DPPH and ABTS were determined following the procedures described by García-Herreros, García-

Iñiguez, Astiasarán and Ansorena (2010). Ferric Ion Reducing Antioxidant Power Assay (FRAP) was determined by the method proposed by Benzie and Strain (1996) but adapted to a 96 well plate, and dissolving samples in water.

2.7. HPLC and UPLC analyses

High performance liquid chromatography with diode array (HPLC-DAD) and Ultra-performance liquid chromatography/mass spectrometry (UPLC-MS) have been used to identify the main phenolic compounds in the samples. HPLC-DAD analysis was performed with a Waters HPLC (Milford, Massachusetts, USA) 600E multi-solvent delivery system, a Waters U6K sampler and a Waters 991 photodiode-array detector. Chromatography was performed on a C18 reversed phase column (Nova-Pak, 150 mm × 3.9 mm, 4 µm, Waters) at 25 °C. Detection range was between 210 and 550 nm. The mobile phase was composed by acetonitrile (A) and acidified water adjusted to pH 2 with formic acid (B). The elution system was performed by different % of B: 0-10 min, 95%; 10-20 min, 95-90%; 20-35 min, 90-80%; 35-45 min, 80-60%; 45-50 min, 80-20% and then 95% in 5 min. Afterwards, the gradient was maintained for 5 more min until the end of the analysis to re-equilibrate the column. The flow rate employed was 1 mL/min. Quantification of individual phenolic compounds was performed with calibration curves. In particular, quantitative determination of phenolic acids, luteolin derivatives and 4-(3,4-dihydroxybenzoyloxymethyl)-phenyl-β-D-glycopyranoside was performed with caffeic acid, luteolin-7-*O*-glucoside and 2,5-dihydroxybenzoic acid as external standards, respectively. The amount of the identified compounds (µg/mg extract) before and after digestion was used to calculate the bioaccessibility (percentage of compound that remains after intestinal digestion). The chromatographic profile of the peaks was similar before and after intestinal digestion in all plant samples. The analysis was run in triplicate for each sample.

Ultra-performance liquid chromatography/mass spectrometry (UPLC-MS) analysis was performed using a Waters Acquity UPLC, a Waters Acquity BEH C18 column (100 mm × 2.1 mm, 1.7 µm) and a Waters LCT Premier XE ESI-TOF-MS (electrospray ionization- time-of-flight- mass spectrometry). UPLC conditions were: flow rate 300 µL/min, column temperature 30 ºC, mobile phase: methanol (A) and 0.1% aqueous formic water (B); gradient elution (different % of B): 0-0.5 min 95%, 0.5-16 min 95-1%; 16-20 min 1%. Mass spectrometry operation conditions were: negative and positive ionisation mode; capillary voltage, 1000 V; cone voltage, 100 V; source temperature, 120 ºC; desolation temperature, 350 ºC; cone gas flow, 50 L/h; desolation gas flow, 600 L/h; MS range, 100-1000 *m/z*.

2.8. Statistical analysis

ROS induction, survival rate and glutathione-S-transferase expression graphs were generated with GraphPad Prism, Version 6.01 (GraphPad Software, La Jolla, CA, USA). Statistical analysis was performed using Stata 12 software (StataCorp LP, Texas, USA). Means were compared by Student's t-test or one-way analysis of variance (ANOVA) followed by Bonferroni *post hoc* test. Pearson correlation coefficients between TPC and DPPH, ABTS and FRAP were calculated.

3. RESULTS AND DISCUSSION

3.1. Characterization of extracts

Chemical characterization of *M. officinalis*, *L. latifolia* and *O. vulgare* was performed using the retention time, the UV-visible absorption spectra and the molecular weight of the mass spectra. Table 1 shows the tentative identification of the main compounds found in the plant extracts. UPLC-DAD chromatograms are included as supplementary material (Figure S1, S2, S3). RA (**1**), with a *m/z* 359[M-H]$^-$ and the identical UV data and retention time as the pure standard, was present in the three plant extracts. This compound has been widely described in *Lamiaceae* species (Petersen & Simmonds, 2003). In *M. officinalis* extract, compound **1** was

the most abundant one, whereas the other detected compound (**2**) was luteolin-3´-*O*-glucuronide. The negative ion mode in this compound showed a peak with *m/z* 461[M-H]$^-$ and a fragment at *m/z* 285 [M-H-176]$^-$ (loss of a glucuronyl moiety), confirming the lutein aglycone. Our results are in agreement with previous publications showing the presence of both compounds, **1** and **2**, in this plant (Barros et al., 2013; Heitz, Carnat, Fraisse, Carnat, & Lamaison, 2000).

Regarding, *L. latifolia* analysis, its extract led to the separation of five major compounds. Compounds **3** and **5** were tentatively identified as glycoside derivatives of coumaric acid, and compound **4** as a glycoside derivative of ferulic acid. These three compounds were previously described in another lavender species (*Lavandula x intermedia* Emeric *ex* Loiseleur) and their molecular ion peaks (*m/z*) were the same as reported in Torras-Claveria, Jauregui, Bastida, Codina and Viladomat (2007). Compound **6** was identified as luteolin-7-*O*-glucoside, with a typical flavonoid UV-visible spectrum, including two absorbance bands at 254 and 348 nm. MS analysis in positive mode showed a molecular ion [M+H]$^+$ at *m/z* 449 and one fragment ion at *m/z* 287 [M+H-162]$^+$, corresponding to aglycone.

At last, the chromatographic profile of *O. vulgare* showed three main compounds. Compounds **7** and **8** were tentatively identified as luteolin glycoside and 4-(3,4-dihydroxybenzoyloxymethyl)-phenyl-β-D-glycopyranoside. The presence of these compounds in this plant, and our results of their UV absorbance and their molecular ions, are in agreement with previous publications for different *Origanum* subspecies (Fernandes et al., 2017; González, Luis, & Lanzelotti, 2014).

3.2. Compound stability after *in vitro* gastrointestinal digestion

In vitro models have been extensively used to simulate the bioaccessibility of secondary plant metabolites like polyphenols (Alminger et al., 2014). In our work, the extracts were submitted to an *in vitro* intestinal digestion process in order to evaluate their stability under

gastrointestinal conditions. Table 1 shows the bioaccessibility data (% of each compound), namely, the percentage of a compound that remained stable after the digestion process. Notably, RA in *M. officinalis*, coumaric acid-*O*-glycoside in *L. latifolia* and 4-(3,4-dihydroxybenzoyloxymethyl)-phenyl-β-D-glycopyranoside and RA in *O. vulgare* were quite stable during the gastrointestinal digestion (% of remaining compound was 98, 101, 93 and 83, respectively). This fact is in line with other studies where high stability of phenolic compounds has been reported after *in vitro* digestive processes (Costa, Grevenstuk, Rosa da Costa, Gonçalves, & Romano, 2014; Gayoso et al., 2016; Tagliazucchi, Verzelloni, Bertolini, & Conte, 2010). Nevertheless, the loss of other compounds, for instance luteolin-7-*O*-glucoside and RA in *L. latifolia* or luteolin glycoside in *O. vulgare*, was apparent, in agreement also with other studies that have described substantial losses of individual compounds under mild alkaline conditions (Celep, Charehsaz, Akyüz, Acar, & Yesilada, 2015; Pinacho et al., 2015; Siracusa et al., 2011; Vallejo, Gil-Izquierdo, Pérez-Vicente, & García-Viguera, 2004). Food matrix composition, in which polyphenols are located, seems to be an important factor that affects stability and digestibility. Previous data (Ortega, Reguant, Romero, Macià, & Motilva, 2009; Sengul, Surek, & Nilufer-Erdil, 2014) and our results point out the protective effect of the plant extract matrix on its components, as reported by Siracusa et al. (2011).

3.3. *In vitro* antioxidant activity

The potential antioxidant properties of plant extracts are mainly related to the presence of polyphenols and carotenoids, compounds that possess antioxidant activity, mainly due to their role as reducing or chelating agents, hydrogen donors and singlet oxygen quenchers (Embuscado, 2015). The *in vitro* antioxidant activity of the *M. officinalis*, *L. latifolia* and *O. vulgare* extracts was assessed before and after the digestion by different methods (Table 2). A significant positive correlation was observed between the TPC and DPPH ($r = 0.9712$, p

< 0.0001), ABTS ($r = 0.8838$, $p < 0.0001$), and FRAP ($r = 0.9176$, $p < 0.0001$), indicating that polyphenols contribute significantly to the antioxidant activity of the extracts. In fact, the chemical structure of identified compounds (Figure S4) was linked with antioxidant activity. In particular, the radical scavenging capacity of RA, luteolin-7-*O*-glucoside and 4-(3,4-dihydroxybenzoyloxymethyl)-phenyl-β-D-glycopyranoside) has been previously demonstrated (Goulas et al., 2010, Liang et al., 2012; Shahidi & Chandrasekara, 2010).

The *M. officinalis* extract was the one with the highest activity for all assays (531.54 µg TE/mg extract in ABTS; 278.66 µg TE/mg extract in DPPH,382.05 µg GA/mg extract in TPC 9.21 mM Fe II/mg extract in FRAP. Previous studies have already described the antioxidant *in vitro* capacity of these plants and have been successfully used as natural antioxidants in the formulation of functional foods and ingredients (Berasategi et al., 2014; Gayoso et al., 2017; López et al., 2007). In all techniques, we observed a similar trend, where no decrease (in *O. vulgare* samples) or a slight loss (up to 18% in *M. officinalis* and up to 19% in *L. latifolia* samples) in the antioxidant activity after gastrointestinal digestion was detected. Thus, indicating that the bioaccessible fraction is potentially bioactive. This result matches our bioaccessibility data, where the main compounds showed high stability. These results are consistent with previous studies, where a slightly degradation or even an increase in antioxidant activity was observed after digestion of grapes (Tagliazucchi et al., 2010), fruit extracts (Pavan, Sancho, & Pastore, 2014), lavender extracts (Costa et al., 2014) or fruit juices (Attri, Singh, Singh, & Goel, 2017). However, other authors reported a large decrease in antioxidant activity after digestion of different food (Carbonell-Capella, Buniowska, Esteve, & Frígola, 2015; Pinto et al., 2016; Rodríguez-Roque, Rojas-Graü, Elez-Martínez, & Martín-Belloso, 2013). This controversy points out the complexity of interpreting this type of results due to the multiple factors that affect the antioxidant activity of polyphenols, such as assay conditions, solubility of the digested sample, matrix effect, digestive enzymes and

alkaline conditions (Bouayed, Hoffmann, & Bohn, 2011; Pineda-Vadillo et al., 2016). In any case, despite some losses resulting from gastrointestinal digestion, the antioxidant potential of dietary polyphenols at intestinal level, independently from their bioavailability, could have local effects within the gastrointestinal tract and offer protection against other food components and/or oxidative stress at the intestinal cells (Holst & Williamson, 2008; Pineda-Vadillo et al., 2016).

3.4. *In vivo* antioxidant activity (*C. elegans)*

In addition to *in vitro* assays for antioxidant activity, it is interesting to study this activity at the *in vivo* level. In this work, intracellular ROS levels, survival assay and GST-4 expression under juglone-induced oxidative stress were assessed in *C. elegans* to explore the biological activity of the extracts obtained from *M. officinalis*, *L. latifolia* and *O. vulgare*.

Juglone is a superoxide-generating naphthoquinone from walnut (*Juglans regia*) that has been used in assays for oxidative stress resistance (Possik & Pause, 2015). Figure 1 shows the survival rate under a lethal dose of juglone, where wild type worms (N2) were incubated in the presence of different concentrations of the three plant extracts, before and after being subjected to the *in vitro* digestion process. Only *M. officinalis* significantly attenuated the deleterious effects of juglone, except at the highest dose tested. In fact, doses higher than 200 µg/mL were toxic for the worms, as previously reported by other studies where toxic effects of high concentrations of polyphenols and plant extracts in *C. elegans* were described (Jara-Palacios et al., 2013; Saul, Pietsch, Stürzenbaum, Menzel, & Steinberg, 2011). Some polyphenols were also reported to have hormetic activity (Saul et al., 2011). For instance, Zamberlan et al. (2016) observed that treatment with 10 and 100 µg/mL of *Rosmarinus officinalis* extract did not have any effect on juglone-induced mortality, in contrast with 25 and 50 µg/mL, being the most effective 25 µg/mL. In our study, the most effective dose of *M. officinalis* was 50 µg/mL, which showed a survival rate similar to one of the samples tested

as positive control EGCG (60% *vs* 63-65%, respectively). RA, the major compound of *M. officinalis*, did not show a protective effect in comparison to the control group. This suggests that the minor components in *M. officinalis* might play a role in the observed oxidative stress resistance. Information regarding RA behavior in *C. elegans* is still scarce. Data by Pietsch et al. (2011) showed RA-mediated lifespan extension in a hormetic dose-response manner and imposed a negative effect in the survival rate in presence of the oxidative stressor paraquat.

To explore the protective mechanisms of the plant extracts against oxidative damage, the intracellular ROS accumulation was also measured in *C. elegans* using H_2DCF-DA. This compound is able to cross cell membranes where it becomes deacetylated (H_2DCF) by intracellular esterases. Then, H_2DCF is subjected to oxidation in the presence of intracellular ROS. The oxidized compound emits fluorescence and its intensity has been correlated with intracellular ROS levels (Peixoto, Roxo, Krstin, Röhrig, et al., 2016). Wild type worms were pre-treated with different concentrations of the plant extracts before and after digestion, and we found a significant decrease (p <0.0001) in intracellular ROS accumulation among treated worms in comparison with the untreated control (Figure 2). *L. latifolia* and *O. vulgare* had no significant effect against acute stress in the survival assay, but showed a significant influence on the basal level of ROS accumulation. The decrease in ROS ranged between 36-16% in crude extracts and 27-17% in digested samples, thus samples before and after digestion effectively reduced endogenous ROS levels. Similar ROS-scavenging activity has been observed for other antioxidants, such as guarana extract (reduction about 30%) (Peixoto et al., 2017), lignans from *Arctium lappa* (reduction up to 21%) (Su & Wink, 2015), and grape pomace (reduction of 29-18%) (Jara-Palacios et al., 2013).

Glutathione S-transferases (GSTs) are major cellular detoxification enzymes and *C. elegans* GST-4 is upregulated in response to oxidative stress (Leiers et al., 2003). We used GST-4-GFP-expressing transgenic CL2166 worms to measure the GFP intensity in response to 20

µM juglone after treatment with different concentrations of plant extracts before and after digestion. The expression of GST-4 under basal conditions was also checked, confirming that under stress conditions, GST-4 is strongly induced in *C. elegans* to compensate the pro-oxidative conditions (Figure 3). Treatment with the plant extracts led to a significant reduction (p <0.0001) in GST-4 expression, indicating a reduction in the oxidative stress (Figure 3). The highest reduction in GST-4 expression in comparison to the control was found at 200 µg/mL of *O. vulgare* (62% in crude extract and 63% in digested extract) and *L. latifolia* (55% in crude extract and 61% in digested extract). *Melissa officinalis* showed a reduction of 55% of the control fluorescence in the extract before digestion at 150 µg/mL and 54% in the extract after digestion at 190 µg/mL. These results point out that the antioxidant capacity of the extract before and after digestion is actually comparable.

Other authors have described a down-regulation of GST-4 after treatment with antioxidant compounds, such as quercetin (Büchter et al., 2015; Kampkötter, Nkwonkam, et al., 2007) or *Ginkgo biloba* extract (Kampkötter, Pielarski, et al., 2007). In this sense, as suggested by Büchter et al. (2015), the modulation of GST-4 by these compounds could be by a direct reduction of oxidative stress that would reduce the amount of antioxidant enzymes or by an indirect antioxidant effect of the compounds by specific activation of the antioxidant response system of the nematode.

4. CONCLUSIONS

Melissa officinalis, *L. latifolia* and *O. vulgare* are rich sources of bioactive compounds, including phenolic acids and flavonoids as the most abundant ones. All the analysed extracts, before and after digestion, exhibited antioxidant activity *in vitro* and *in vivo*. Our findings suggest that the antioxidant activity is not only achieved by scavenging radicals but also by modulation of the expression of the GST-4 antioxidant enzyme. Therefore, lemon balm,

lavender and oregano could be a bioaccessible and bioactive source of natural antioxidants for the development of new nutraceutical and functional foods.

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6. CONFLICT OF INTEREST

There are no conflicts of interest to declare.

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Table 1.Tentative identification of the main compounds detected in the plant extracts by LC-MS, their quantification in µg/mg plant extract (mean ± SD) before digestion and their bioaccessibility (%) after *in vitro* digestion process.

Plant extract	Compound	Identification	Reference	µg/mg plant extract	Rt (min)	Mw	$[M-H]^-$ (m/z)	UV λ max (nm)	Bioaccessibility (%)
Melissa officinalis	1	Rosmarinic acid	Barros et al., 2013	38.59± 2.31	8.131	360	359.0865	329	98
	2	Luteolin 3´-*O*-glucuronide	Barros et al., 2013	29.81± 4.56	8.745	462	461.0952	245, 329	58
Lavandula latifolia	3	Coumaricacid-*O*-glycoside	Torras-Claveria et al., 2007	79.86 ±1.20	4.931	326	325.1089	263	101
	4	Ferulicacid-*O*-glycoside	Torras-Claveria et al., 2007	8.39 ± 0.08	5.588	356	355.1297	302	107
	5	Coumaric acid-*O*-glycoside	Torras-Claveria et al., 2007	8.76 ± 0.10	6.509	326	325.1173	276	98
	6	Luteolin-7-*O*-glucoside		29.23 ± 0.36	7.473	448	447.1137	254, 348	36
	1	Rosmarinic acid	Petersen & Simmonds, 2003	15.21 ± 0.10	8.174	360	359.1036	329	48
Origanum vulgare	7	Luteolinglycoside	Fernandes et al., 2017; González, Luis, &Lanzelotti, 2014	29.64 ± 0.39	6.377	nd	nd	255, 345	41
	8	4-(3,4-dihydroxybenzoyloxymethyl)-phenyl-β-D-glycopyranoside	Fernandes et al., 2017; González, Luis, &Lanzelotti, 2014	29.07± 2.10	7.296	422	421.1244	262, 294	93
	1	Rosmarinic acid	Petersen & Simmonds, 2003	39.68 ± 2.51	8.175	360	359.0988	329	83

Rt: retention time; Mw: molecular weight; $[M-H]^-$: molecular ion; nd: not determined.

Bioaccessibility(%) =(amount of compound after digestion / amount of compound before digestion) x100

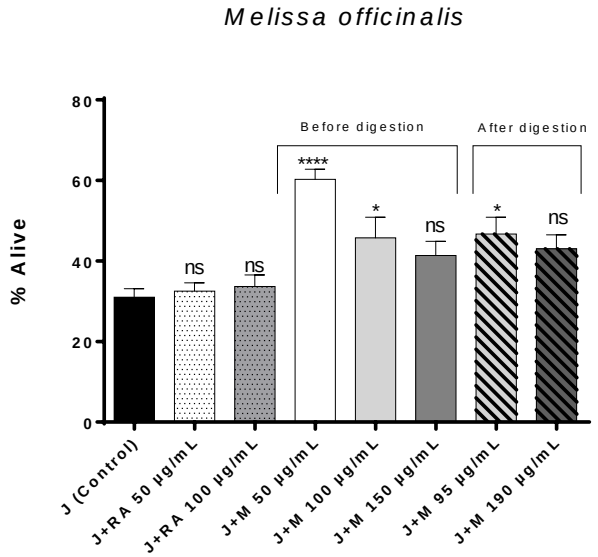
Table 2. Antioxidant activity: ABTS, DPPH, TPC and FRAP in the plant extracts before and after an *in vitro* digestion process (mean ± SD).

		ABTS		**DPPH**		**TPC**		**FRAP**	
		µg TE/mg extract	% Remaining antioxidant activity	µg TE/mg extract	% Remaining antioxidant activity	µg GA/mg extract	% Remaining antioxidant activity	mM Fe II/mg extract	% Remaining antioxidant activity
Melissa officinalis	Before digestion	531.54 ± 23.62 [a]		278.66 ± 15.13 [b]		382.05 ± 12.72 [b]		9.21 ± 0.42 [b]	
	After digestion	491.67 ± 16.67 [a]	92.50	233.49 ± 19.35 [a]	83.79	350.13 ± 7.44 [a]	91.65	7.53 ± 0.55 [a]	81.76
Lavandula latifolia	Before digestion	169.68 ± 5.22 [b]		95.45 ± 7.38 [a]		126.04 ± 4.56 [b]		3.28 ± 0.26 [b]	
	After digestion	137.80 ± 2.72 [a]	81.21	90.43 ± 3.28 [a]	94.74	117.27 ± 2.86 [a]	93.04	2.73 ± 0.11 [a]	83.23
Origanum vulgare	Before digestion	422.06 ± 9.61 [a]		173.67 ± 6.91 [a]		315.82 ± 29.23 [a]		9.64 ± 0.96 [a]	
	After digestion	415.6 ± 12.18 [a]	98.47	171.58 ± 8.30 [a]	98.80	310.56 ± 8.41 [a]	98.33	9.63 ± 0.21 [a]	99.90

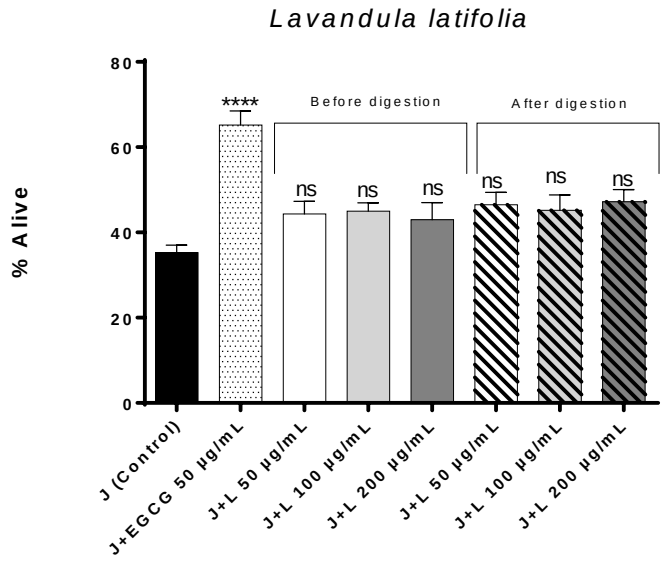
Different letters indicate significant differences (p <0.05) among samples before and after digestion.

Figure 1. Survival rate of *C. elegans* N2 under a lethal dose of juglone (80 µM) (J) at different doses of extracts from *M. officinalis* (a), *L. latifolia* (b) and *O. vulgare* (c). The results are expressed as mean ±SEM. Statistical significance of differences between control ant treated groups was determined by one-way ANOVA followed by Bonferroni (*post-hoc*) (* p <0.05, ***p <0.001, ****p <0.0001). RA: Rosmarinic acid, EGCG: epigallocatechin gallate; M: *M. officinalis*; L: *L. latifolia*; O: *O. vulgare;* ns: not significant (p >0.05*)*.

a)



b)



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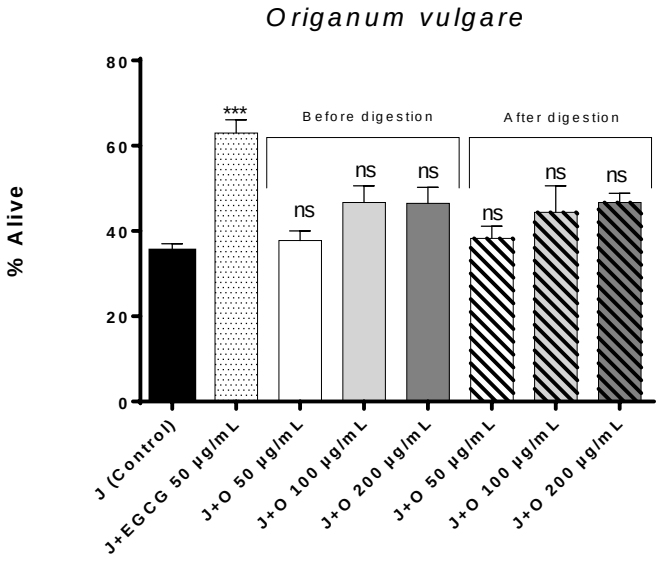
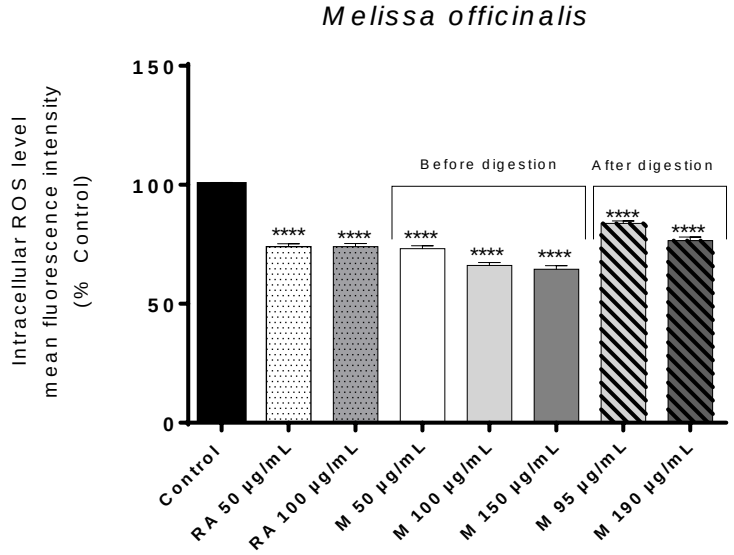
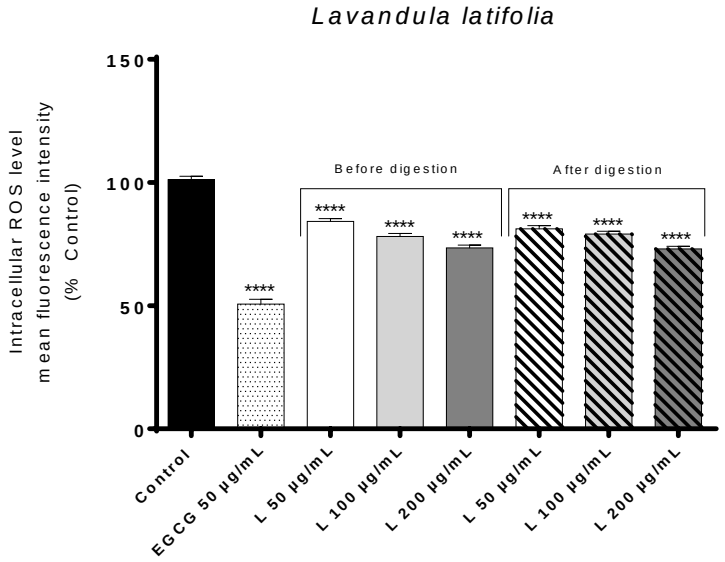

Origanum vulgare
% Alive
80
60
40
20
0
***
Before digestion
After digestion
ns
ns
ns
ns
ns
ns
J (Control)
J+EGCG 50 µg/mL
J+O 50 µg/mL
J+O 100 µg/mL
J+O 200 µg/mL
J+O 50 µg/mL
J+O 100 µg/mL
J+O 200 µg/mL

Figure 2. Effect of *M. officinalis* (a), *L. latifolia* (b) and *O. vulgare* (c) on ROS production in *C. elegans* N2 using H_2DCF-DA. The results are expressed as mean ±SEM. Statistical significance of differences between control ant treated groups was determined by one-way ANOVA followed by Bonferroni (*post-hoc*) (****p <0.0001). RA: Rosmarinic acid, EGCG: epigallocatechin gallate; M: *M. officinalis*; L: *L. latifolia*; O: *O. vulgare*

a)



b)



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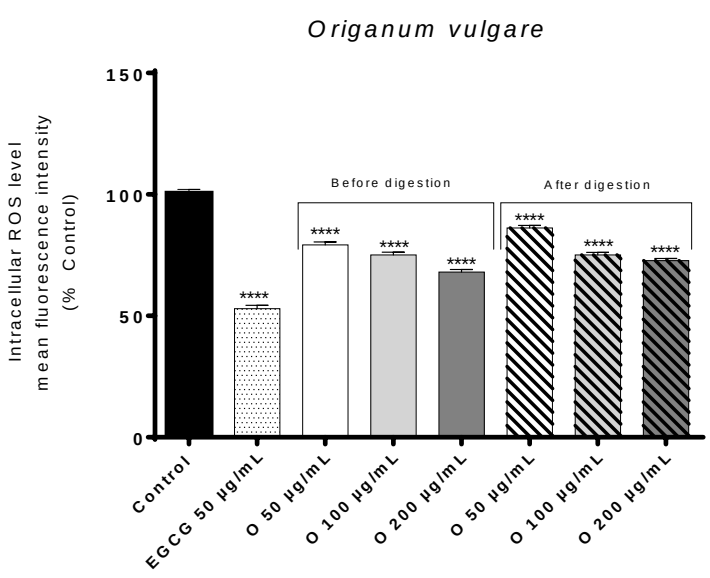
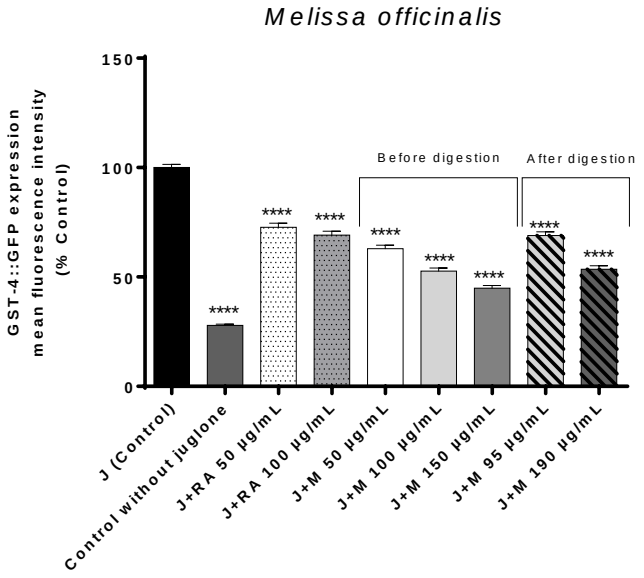
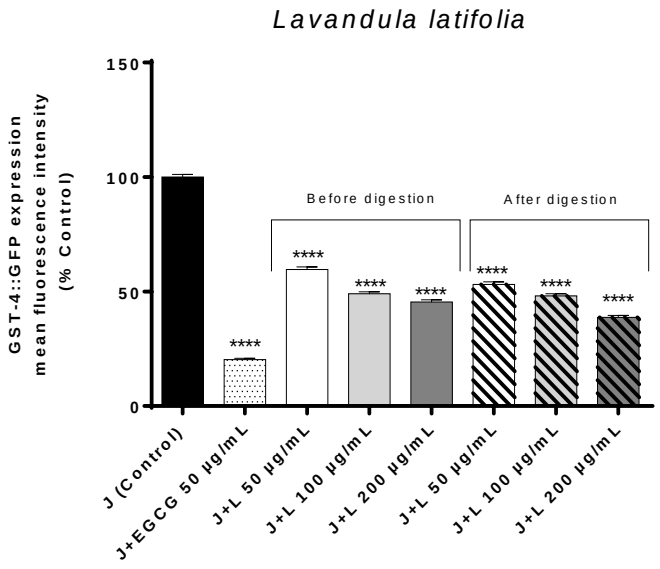

Origanum vulgare
Intracellular ROS level
mean fluorescence intensity
(% Control)
150
100
50
0
Before digestion
After digestion
****
Control
EGCG 50 µg/mL
O 50 µg/mL
O 100 µg/mL
O 200 µg/mL
O 50 µg/mL
O 100 µg/mL
O 200 µg/mL

Figure 3. Effect of extracts from *M. officinalis* (a), *L. latifolia* (b) and *O. vulgare* (c) on GST-4 expression in CL2166 worms, under juglone-induced oxidative stress (20 µM for 24 h) (J). The results are expressed as mean ± SEM. Statistical significance of differences between control ant treated groups was determined by one-way ANOVA followed by Bonferroni (*post-hoc*) (****p <0.0001). RA: Rosmarinic acid, EGCG: epigallocatechin gallate; M: *M. officinalis*; L: *L. latifolia*; O: *O. vulgare*.

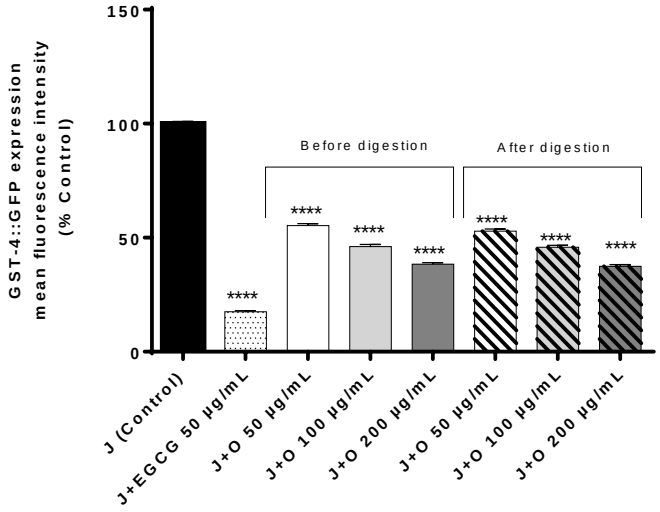
a)



b)

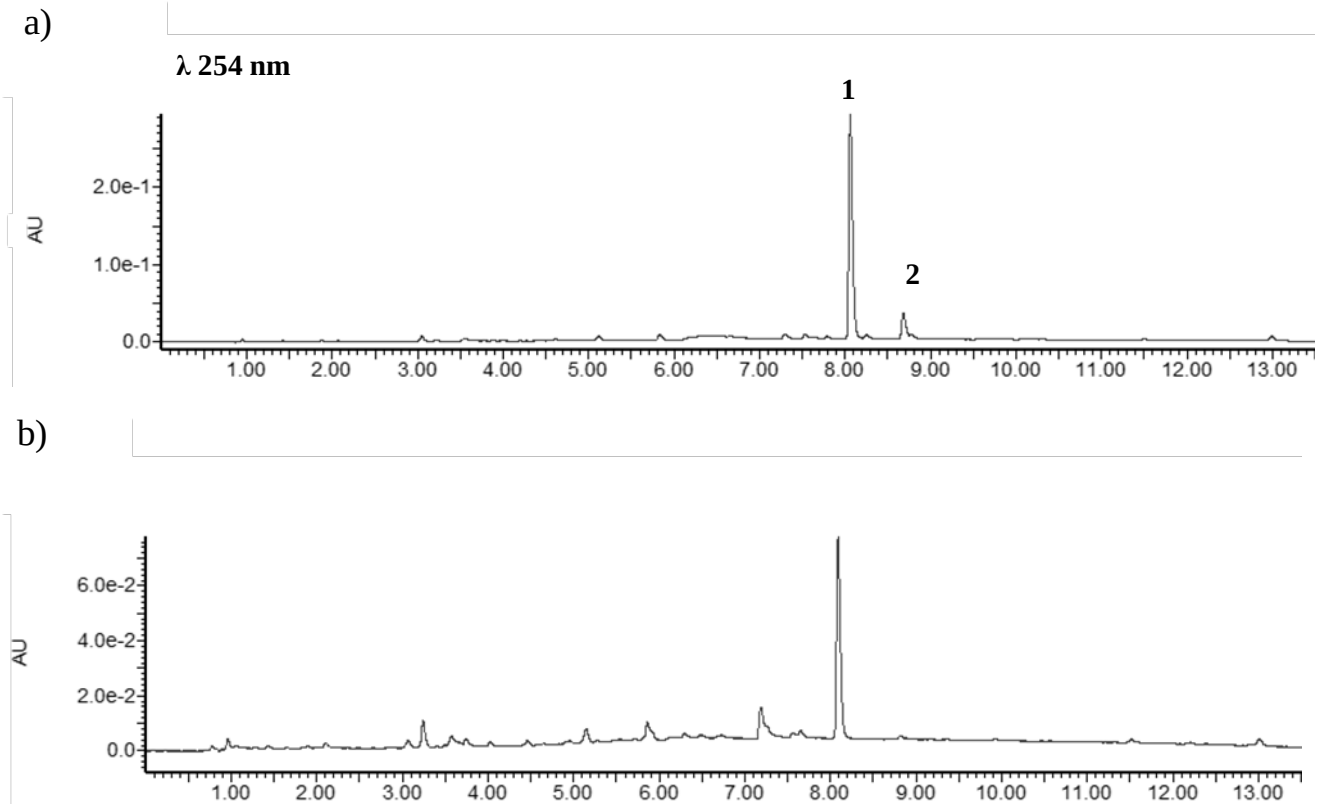


c) *Origanum vulgare*



Supplementary information.

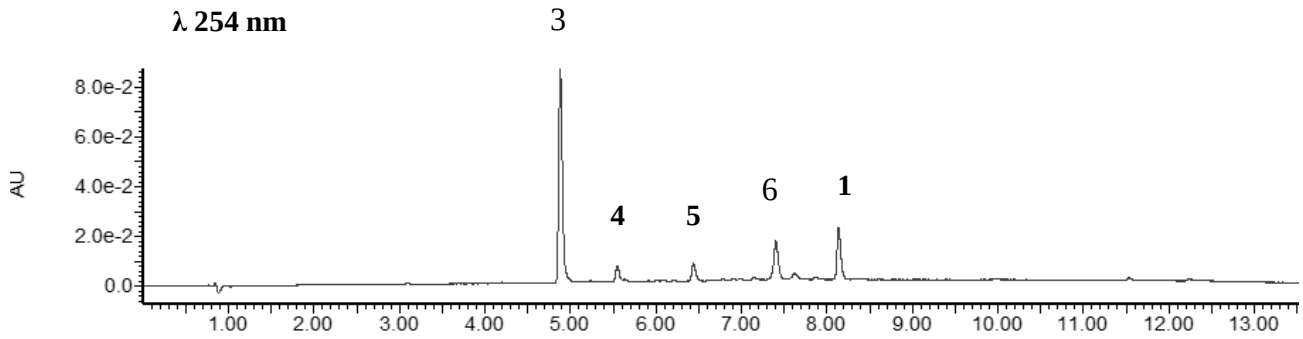
Figure S1.UPLC-DAD chromatogram of *Melissa officinalis* extract before (a) and after (b) an *in vit*ro digestion



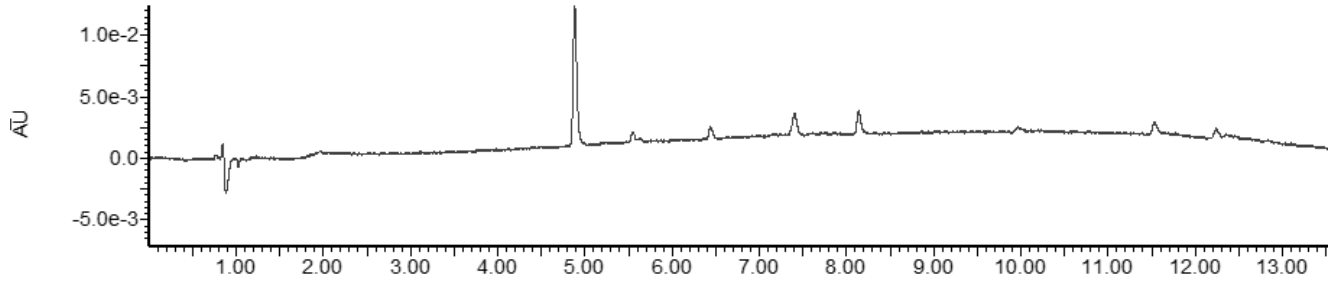
Supplementary information.

Figure S2. UPLC-DAD chromatogram of *Lavandula latifolia* extract before (a) and after (b) an *in vit*ro digestion

a)

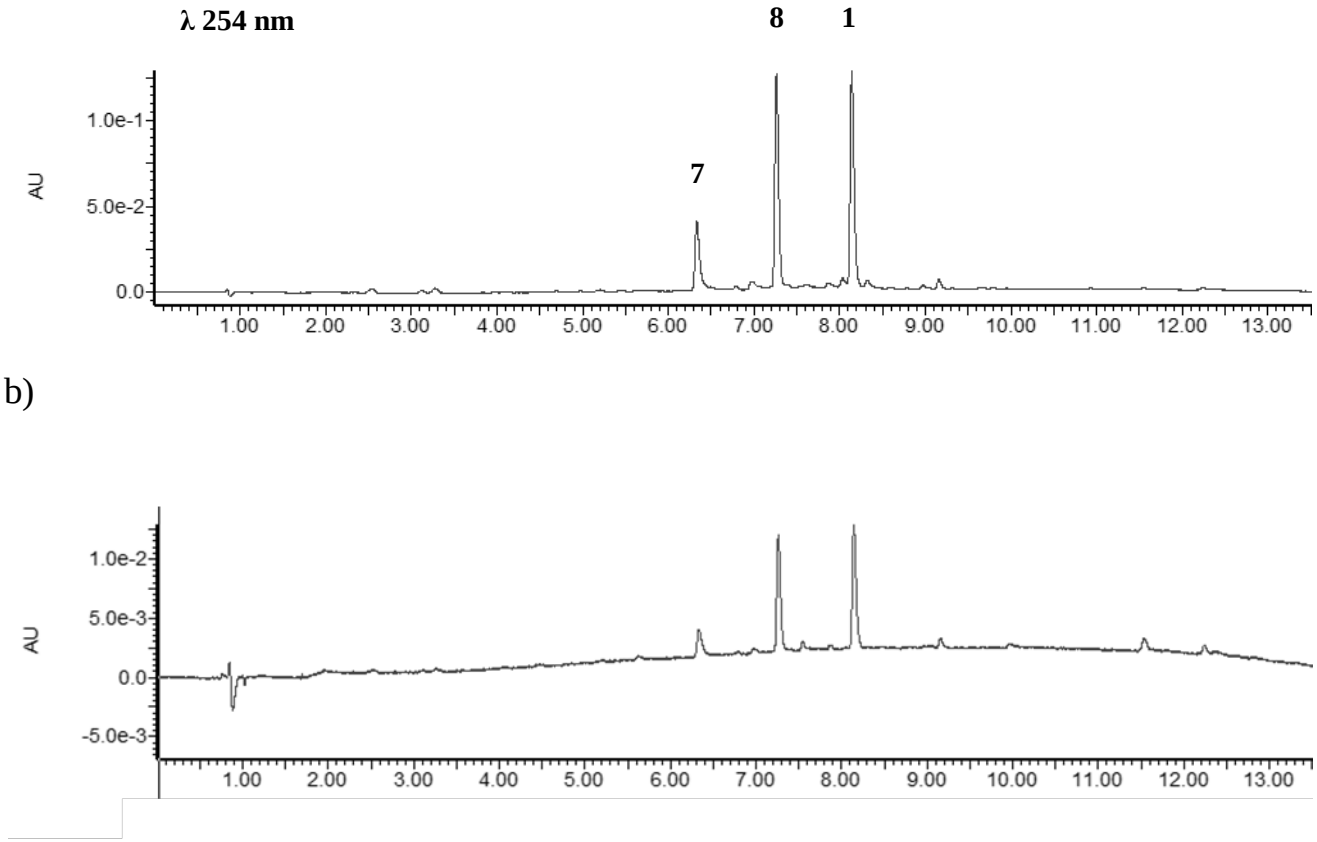


b)



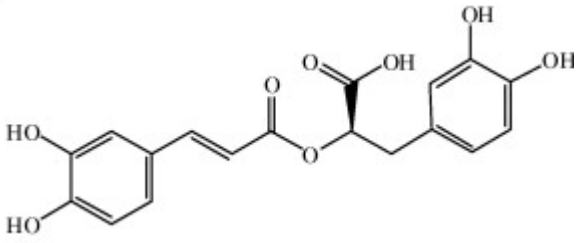
Supplementary information.

Figure S3. UPLC-DAD chromatogram of *Origanum vulgare* extract before (a) and after (b) an *in vit*ro digestion

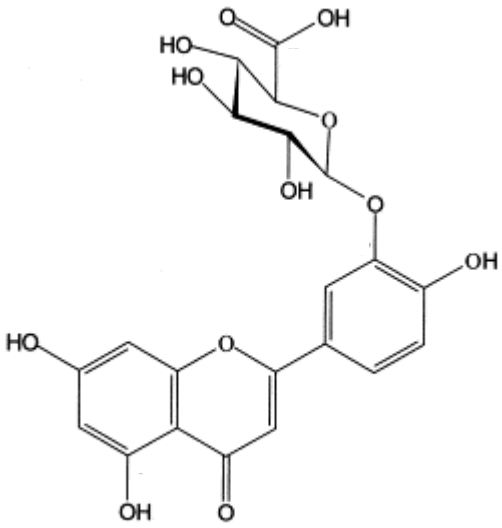


Supplementary information.

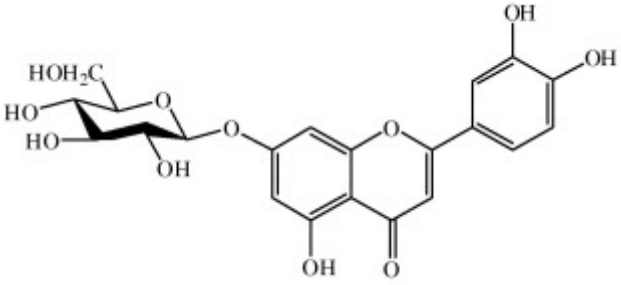
Figure S4. Chemical structures of the identified compounds in *M. officinalis*, *L. latifolia* and *O. vulgare* extracts.



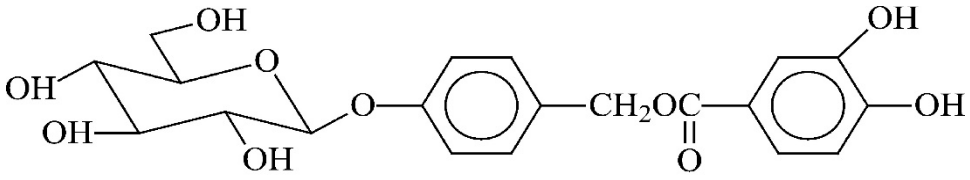
Rosmarinic acid (**1**)



Luteolin 3′-*O*-glucuronide (**2**)



Luteolin-7-*O*-glucoside (**6**)



4-(3,4-dihydroxybenzoyloxymethyl)-phenyl-β-D-glycopyranoside (**8**)

Source: (**1**) and (**6**) Costa et al. 2011; (**2**) Heitz et al (2000); (**8**) Liang et al. (2012).