Lamiaceae often used in Portuguese folk medicine as a source of powerful antioxidants: vitamins and phenolics

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Abstract

Three Lamiaceae often used in Portuguese folk medicine: Ground ivy (*Glechoma hederaceae* L.), oregano (*Origanum vulgare* subsp. *virens* (Hoffmanns. & Link) Ietswaart) and mastic thyme (*Thymus mastichina* L.), revealed to be good sources of powerful antioxidants such as vitamins (ascorbic acid and tocopherols) and phenolics. The HPLC methodology developed for tocopherols analysis proved to be, after validation assays, sensitive and accurate. Ground ivy showed the highest levels of vitamins: ascorbic acid (168 ± 2 μg/g dry weight) and tocopherols (3692 ± 57 μg/g dry weight). The phenolics extraction was optimized using mastic tyme, and different conditions (water and ethanol:water 50% v/v, 30 min at 25ºC and boiling temperature; ethanol and methanol, 24h at 25ºC). The best methodology (25 ºC, 50 ml of methanol, 24h) was further applied to the other Lamiaceae. Oregano proved to have the highest radical scavenging and lipid peroxidation inhibition capacity. Particularly, the very low EC_{50} value (0.01 mg/ml) obtained for TBARS inhibition in brain homogenates is very promising, considering that brain is highly sensitive to oxidative damage. Significantly negative linear correlations were observed between phenolics, including flavonoids, and antioxidant activity EC_{50} values of the three Lamiaceae.

Keywords: Lamiaceae, Vitamins, HPLC validation, Radical scavenging capacity, Lipid peroxidation inhibition

1. Introduction

Many wild plants gathered from the scrubland were preserved and used for medicinal and food purposes in the north-eastern region of Portugal. Since they are important ingredients of the folk pharmacopoeia and traditional cuisine some of these wild botanicals have been semi-domesticated and are still cultivated in homegardens and present in every homesteads.

Several ethnobotanical surveys conducted in this Portuguese region have highlighted the use of three particular Lamiaceae (Carvalho 2005; Carvalho, Martins, & Frazão-Moreira, 2007; Pardo de Santayana et al., 2007). Ground ivy (*Glechoma hederaceae*), oregano (*Origanum vulgare* subsp. *virens*) and mastic thyme (*Thymus mastichina*) are widespread Mediterranean perennial herbs widely considered as medicinal plants, although it has also been reported some other common uses. Ground ivy leaves and oregano or thyme inflorescences are recommended to the respiratory and gastrointestinal systems. Infusions made of dried material are useful for relieving colds, coughs, throat irritations and abdominal pains. Herbal teas, prepared with fresh plant parts, are drunk for indigestion and stomachache. Decoctions of the leaves of each species have also been claimed to have anti-inflammatory and antiseptic properties and thus they are used for external inflammations and skin diseases. Some use-reports of ground ivy are concerned with beneficial effects in painful menstruations and emmenagogue properties. Ground ivy soup was claimed to have restorative effects: it was eaten by mothers after giving birth and given to newborns (Carvalho 2005; Carvalho et al. 2007; Frazão-Moreira, Carvalho, & Martins, 2007; Pardo de Santayana et al 2007).

In the present study we intend to evaluate the antioxidants content, radical scavenging effect and lipid peroxidation inhibition capacity of those three Lamiaceae often used in Portuguese folk medicine. As far as we know, there are no reports on vitamins C and E contents in the studies species. The antioxidant activity of *Glechoma hederacea* was reported, but in other countries, and relative to seeds (Kumarasamy et al., 2007) and grown plants in greenhouse (Kumarasamy, Cox, Jaspars, Nahar, & Sarker, 2002), and not in wild leaves and steams. For *Thymus mastichina* the reports on antioxidant activity are related to their essential oils (Miguel et al., 2004) and not to the phenolic extracts. The antioxidant activity of *Origanum vulgare* were also reported, but all from other countries such as USA (Zheng & Wang, 2001), Japan (Matsuura et al., 2003), Bulgaria (Ivanova, Gerova, Chervenkov, & Yankova, 2005) and Spain (López et al., 2007). Herein, we validated a method for the analysis of tocopherols and we developed an optimization procedure for the extraction of phenolics, being the best methodology applied to Lamiaceae gathered in North-eastern Portugal. Their lipid peroxidation inhibition capacity was accessed by biochemical assays used as models for the lipid peroxidation damage in biomembranes, namely inhibition of β-carotene bleaching in the presence of linoleic acid radicals and inhibition of thiobarbituric acid reactive substances (TBARS) formation in brain homogenates. The antioxidant properties were also evaluated through the reducing power determination and radical scavenging activity of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals.

2. Materials and methods

2.1. Samples

97 Samples of leaves and steams of *Glechoma hederaceae* (ground ivy; ~73 g of fresh
98 material) and inflorescences of *Origanum vulgare* subsp. *virens* (oregano; ~55 g of
99 fresh material) and *Thymus mastichina* (thyme; ~100 g of fresh material) were
100 gathered in Bragança, Trás-os-Montes, North-eastern Portugal. The selected sites and
101 gathering practices took into account local consumers gathering criteria for the
102 medicinal use of these species and the optimal growth stage of each species. The
103 plant material was collected early in the morning, in half shade sites at meadows'
104 edges: ground ivy and thyme in July 2008; oregano in September 2008.
105 Morphological key characters from Franco (1984) were used for plant identification.
106 Voucher specimens are deposited in the Herbarium of the Escola Superior Agrária de
107 Bragança. The material was lyophilized (Ly-8-FM-ULE, Snijders, HOLLAND) and
108 kept in the best conditions (-20ºC, ~30 days) for subsequent use.
109
110 *2.2. Standards and Reagents*
111 n-Hexane 95% and ethyl acetate 99.98% were of HPLC grade from Lab-Scan
112 (Lisbon, Portugal). Methanol was of analytical grade purity and supplied by Pronalab
113 (Lisbon, Portugal). Tocopherol standards (α, β, γ and δ), and the standard used in the
114 antioxidant activity assays, trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-
115 carboxylic acid), catechin and gallic acid were purchased from Sigma (St. Louis, MO,
116 USA). Racemic Tocol, 50 mg/ml, was purchased from Matreya (PA, USA). 2,2-
117 Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA,
118 USA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO,
119 USA). Water was treated in a Milli-Q water purification system (TGI Pure Water
120 Systems, USA).

121

122 *2.3. Determination of tocopherols*

123 *2.3.1. Preparation of standard solutions.*

124 Individual stock solutions (~5 mg/ml) of α, β, γ and δ tocopherols were prepared in
125 hexane and stored protected from light, at -20ºC. A stock standard mixture with the
126 four isomers was prepared in hexane with the final concentration of 1 mg/ml for each
127 isomer. Working standard mixture with concentration of 50 μg/ml was prepared from
128 the stock standard solution. Tocol was used as internal standard (IS), being prepared a
129 stock solution at 50 μg/ml in hexane, kept at -20ºC, protected from light, and diluted
130 to a working solution (2.0 μg/ml). BHT was prepared in hexane at a concentration of
131 10 mg/ml.

132

133 *2.3.2. Extraction procedure*

134 BHT solution (100 μl) and IS solution (250 μl) were added to the sample prior to the
135 extraction procedure. The samples (~500 mg) were homogenized with methanol (4
136 ml) by vortex mixing (1 min). Subsequently, hexane (4 ml) was added and again
137 vortex mixed for 1 min. After that, saturated NaCl aqueous solution (2 ml) was
138 added, the mixture was homogenized (1 min), centrifuged (5 min, 4000*g*) and the
139 clear upper layer was carefully transferred to a vial. The sample was re-extracted
140 twice with hexane. The combined extracts were taken to dryness under a nitrogen
141 stream, redissolved in 1 ml of n-hexane, dehydrated with anhydrous sodium sulphate,
142 filtered through a 0.22 μm disposable LC filter disk, transferred into a dark injection
143 vial and analysed by HPLC.

144

145 *2.3.3. HPLC analysis*

146 The HPLC equipment consisted of an integrated system with a Smartline pump 1000
147 (Knauer, Germany), a degasser system Smartline manager 5000, an AS-2057 auto-
148 sampler and a 2500 UV detector at 295 nm (Knauer, Germany) connected in series
149 with a FP-2020 fluorescence detector (Jasco, Japan) programmed for excitation at
150 290 nm and emission at 330 nm. Data were analysed using Clarity 2.4 Software
151 (DataApex). The chromatographic separation was achieved with a Polyamide II (250
152 x 4.6 mm) normal-phase column from YMC Waters (Japan) operating at 35ºC (7971
153 R Grace oven). The mobile phase used was a mixture of n-hexane and ethyl acetate
154 (70:30, v/v) at a flow rate of 1 ml/min, and the injection volume was 20 µl. The
155 compounds were identified by chromatographic comparisons with authentic
156 standards. Quantification was based on the fluorescence signal response, using the
157 internal standard method (0.075 to 16.0 µg/ml; α-tocopherol $y = 2.12899\ x$; β-
158 tocopherol $y = 0.51248\ x$; δ- tocopherol $y = 0.7359\ x$; γ- tocopherol $y = 0.65148\ x$).
159 Tocopherol contents in the samples are expressed in µg per g of dry matter.
160 The linearity and sensitivity of the HPLC analysis was determined and the method
161 was validated by the repeatability and accuracy, using *Thymus mastichina*.
162
163 *2.4. Determination of ascorbic acid*
164 Ascorbic acid was determined according to the method of Klein & Perry (1982). A
165 fine powder (20 mesh) of sample (150 mg) was extracted with metaphosphoric acid
166 (1%, 10 ml) for 45 min at room temperature and filtered through Whatman Nº 4 filter
167 paper. The filtrate (1 ml) was mixed with 2,6-dichloroindophenol (9 ml) and the
168 absorbance was measured within 30 min at 515 nm against a blank. Content of

ascorbic acid was calculated on the basis of the calibration curve of authentic L-ascorbic acid (0.006-0.1 mg/ml; $y = 3.0062x + 0.007$; $R^2 = 0.9999$), and the results were expressed as μg of ascorbic acid/g of dry weight.

2.5. Determination of phenolics and flavonoids

2.5.1. Optimization of the extraction conditions

A fine dried powder (20 mesh) of *Thymus mastichina* (~1g) was extracted using six different conditions:

i) Water (50 ml) at 25 ºC at 150 rpm for 30 min (E1); ii) Water (50 ml) at boiling temperature at 150 rpm for 30 min (E2); iii) Ethanol:water (50% v/v, 50 ml) at 25 ºC at 150 rpm for 30 min (E3); iv) Ethanol:water (50% v/v, 50 ml) at 80 ºC at 150 rpm for 30 min (E4); v) Ethanol (50 ml) at 25 ºC at 150 rpm for 12h, two times (E5); vi) Methanol (50 ml) at 25 ºC at 150 rpm for 12h, two times (E6). The samples were filtrated through Whatman nº 4 paper and the solvent was evaporated at 35ºC under reduced pressure (rotary evaporator Büchi R-210). The extracts (E1-E6) were re-dissolved in the corresponding solvent at a concentration of 50 mg/ml, and analysed for their content in phenolics, including total flavonoids, and for their antioxidant activity by DPPH radical scavenging and reducing power assays.

The best methodology was also applied to *Glechoma hederacea* and *Origanum vulgare* species. The samples were submitted to analysis of antioxidants (phenolics, flavonoids and ascorbic acid) and of antioxidant activity, measured by DPPH radical scavenging, reducing power, inhibition of β-carotene bleaching and inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS) assays.

192

193 *2.5.2. Phenolics and flavonoids determination*

194 Total phenolics were estimated by a colorimetric assay, based on procedures
195 described by (Wolfe, Wu, & Liu, 2003) with some modifications. The extract
196 solution (1 ml) was mixed with *Folin-Ciocalteu* reagent (5 ml, previously diluted
197 with water 1:10 v/v) and sodium carbonate (75 g/L, 4 ml). The tubes were vortexed
198 for 15 s and allowed to stand for 30 min at 40 °C for colour development.
199 Absorbance was then measured at 765 nm (Analytikijena 200-2004
200 spectrophotometer). Gallic acid was used to calculate the standard curve (0.05-0.8
201 mM; $y = 1.9799x + 0.0299$; $R^2 = 0.9997$), and the results were expressed as mg of
202 gallic acid equivalents (GAEs) per g of extract.
203 Total flavonoids contents were determined spectrophotometrically using the method
204 of Jia Tang, & Wu (1999) based on the formation of a complex flavonoid-aluminum,
205 with some modifications. An aliquot (0.5 ml) of the extract solution was mixed with
206 distilled water (2 ml) and subsequently with $NaNO_2$ solution (5%, 0.15 ml). After 6
207 min, $AlCl_3$ solution (10%, 0.15 ml) was added and allowed to stand further 6 min,
208 thereafter, NaOH solution (4%, 2 ml) was added to the mixture. Immediately,
209 distilled water was added to bring the final volume to 5 mL. Then the mixture was
210 properly mixed and allowed to stand for 15 min. The intensity of pink colour was
211 measured at 510 nm. (+)-Catechin was used to calculate the standard curve (0.0156-
212 1.0 mM; $y = 0.9186x - 0.0003$; $R^2 = 0.9999$) and the results were expressed as mg of
213 (+)-chatequin equivalents (CEs) per g of extract.

214

215 *2.6. Antioxidant activity.*

2.6.1. DPPH radical-scavenging activity

This methodology was adapted by us using an ELX800 Microplate Reader (Bio-Tek Instruments, Inc). The reaction mixture in each one of the 96-wells consisted of extract solution (30 µl) and aqueous methanolic solution (80:20 v/v, 270 µl) containing DPPH radicals ($6x10^{-5}$ mol/l). The mixture was left to stand for 60 min in the dark. The reduction of the DPPH radical was determined by measuring the absorption at 515 nm. The radical scavenging activity (RSA) was calculated as a percentage of DPPH discolouration using the equation: % RSA = $[(A_{DPPH}-A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution when the sample extract has been added at a particular level, and A_{DPPH} is the absorbance of the DPPH solution. The extract concentration providing 50% of radicals scavenging activity (EC_{50}) was calculated from the graph of RSA percentage against extract concentration. Trolox was used as standard.

2.6.2. Reducing power

This methodology was developed by us using the Microplate Reader described above. The extract solutions (0.5 mL) were mixed with sodium phosphate buffer (200 mmol/l, pH 6.6, 0.5 ml) and potassium ferricyanide (1% w/v, 0.5 ml). The mixture was incubated at 50 ºC for 20 min, and trichloroacetic acid (10% w/v, 0.5 ml) was added. The mixture (0.8 ml) was poured in the 48-wells, as also deionised water (0.8 ml) and ferric chloride (0.1% w/v, 0.16 ml), and the absorbance was measured at 690 nm. The extract concentration providing 0.5 of absorbance (EC_{50}) was calculated by

238 interpolation from the graph of absorbance at 690 nm against extract concentration.
239 Trolox was used as standard.
240
241 *2.6.3. Inhibition of β-carotene bleaching*
242 The antioxidant activity of the extracts was evaluated by the β-carotene linoleate
243 model system, as described previously by us (Barros et al., 2008). A solution of β-
244 carotene was prepared by dissolving β-carotene (2 mg) in chloroform (10 ml). Two
245 millilitres of this solution were pipetted into a round-bottom flask. After the
246 chloroform was removed at 40ºC under vacuum, linoleic acid (40 mg), Tween 80
247 emulsifier (400 mg), and distilled water (100 ml) were added to the flask with
248 vigorous shaking. Aliquots (4.8 ml) of this emulsion were transferred into different
249 test tubes containing different concentrations of the extracts (0.2 ml). The tubes were
250 shaken and incubated at 50ºC in a water bath. As soon as the emulsion was added to
251 each tube, the zero time absorbance was measured at 470 nm using a
252 spectrophotometer. A blank, devoid of β-carotene, was prepared for background
253 subtraction. β-Carotene bleaching inhibition was calculated using the following
254 equation: (β-carotene content after 2h of assay/initial β-carotene content) × 100. The
255 extract concentration providing 50% antioxidant activity (EC_{50}) was calculated by
256 interpolation from the graph of β-carotene bleaching inhibition percentage against
257 extract concentration. Trolox was used as standard.
258
259 *2.6.4. Inhibition of lipid peroxidation using thiobarbituric acid reactive substances*
260 *(TBARS)*

261 Brains were obtained from pig (*Sus scrofa*) of body weight ~150 Kg, dissected and
262 homogenized with a Polytron in ice-cold Tris–HCl buffer (20 mM, pH 7.4) to
263 produce a 1:2 (w/v) brain tissue homogenate which was centrifuged at 3000g for 10
264 min. An aliquot (0.1 ml) of the supernatant was incubated with the extracts solutions
265 (0.2 ml) in the presence of $FeSO_4$ (10 μM; 0.1 ml) and ascorbic acid (0.1 mM; 0.1
266 ml) at 37ºC for 1 h. The reaction was stopped by the addition of trichloroacetic acid
267 (28% w/v, 0.5 ml), followed by thiobarbituric acid (TBA, 2%, w/v, 0.38 ml), and the
268 mixture was then heated at 80 ºC for 20 min. After centrifugation at 3000g for 10 min
269 to remove the precipitated protein, the colour intensity of the malondialdehyde
270 (MDA)-TBA complex in the supernatant was measured by its absorbance at 532 nm.
271 The inhibition ratio (%) was calculated using the following formula: Inhibition ratio
272 (%) = [(A – B)/A] x 100%, where A and B were the absorbance of the control and the
273 compound solution, respectively. The extract concentration providing 50% lipid
274 peroxidation inhibition (EC_{50}) was calculated by interpolation from the graph of
275 TBARS inhibition percentage against extract concentration (Barros et al., 2008).
276 Trolox was used as standard.
277
278 *2.7. Statistical analysis*
279 For each one of the plant species three samples were analysed and also all the assays
280 were carried out in triplicate. The results are expressed as mean values and standard
281 deviation (SD). The results were analyzed using one-way analysis of variance
282 (ANOVA) followed by Tukey's HSD Test with $\alpha = 0.05$. This treatment was carried
283 out using SPSS v. 16.0 program. The regression analysis between phenolics and

284 flavonoids and EC_{50} values for antioxidant activity were performed using the same
285 statistical package.
286
287 **3. Results and discussion**
288 We studied the antioxidants content (vitamins and phenolics), free radical scavenging
289 activity and lipid peroxidation inhibition of three Lamiaceae often used in Portuguese
290 folk medicine: Ground ivy (*Glechoma hederacea*), oregano (*Origanum vulgare*
291 subsp. *virens*) and mastic thyme (*Thymus mastichina*).
292
293 *3.1. HPLC method validation for analysis of tocopherols*
294 The linearity and the limits of detection and quantification were determined and are
295 presented in **Table 1**. For each compound, 7-level calibration curve was constructed
296 using the peak-area ration between the tocopherol and tocol versus concentration of
297 the standard (ng/ml). The average of triplicate determinations for each level was used.
298 The correlation coefficients were always higher than 0.999 for all the compounds
299 (**Table 1**).
300 The limits of detection (LOD), calculated as the concentration corresponding to three
301 times the calibration error divided by the slope, ranged from 14.79 and 25.82 ng/ml.
302 The limits of quantification (LOQ) were calculated using the concentration
303 corresponding to ten times the calibration error divided by the slope, and ranged from
304 49.32 to 86.07 ng/ml.
305 In order to evaluate the instrumental precision, the sample (*Thymus mastichina*)
306 extract was injected six times. The chromatographic method proved to be precise
307 (CV% between 0.22 and 2.36%, **Table 2**). Repeatability was evaluated by applying

308 the whole extraction procedure 6 times to the same sample. All the obtained values
309 were low (CV% ranging from 2.18 to 4.45%, **Table 2**). The accuracy of the method
310 was evaluated by the standard addition procedure (% of recovery) with three addition
311 levels (0.5, 1.0 and 2.5 μg/ml, each one in duplicate). The standard mixture was
312 added to the sample, and all the extraction procedure was carried out. The results
313 demonstrate good recovery for the compounds under study (ranging from 89 to 95%).
314
315 *3.2. Vitamins content*
316 The vitamins (ascorbic acid and tocopherols) content in the three Lamiaceae samples
317 is presented in **Table 3**. *Glechoma hederacea* revealed higher content of tocopherols
318 (369 mg/100g) than ascorbic acid (16.8 mg/100g), while the other Lamiaceae
319 presented the opposite. Also, this species showed the highest levels of both vitamins.
320 The four tocopherols (α, β, γ, δ-tocopherols) were quantified in all the samples. α-
321 Tocopherol was the major tocopherol vitamer for *Glechoma hederacea* (272
322 mg/100g) and *Origanum vulgare* (10.1 mg/100g). For *Thymus mastichina*, γ-
323 tocopherol was the most abundant vitamer (3.8 mg/100g), revealing this species the
324 lowest total tocopherols content (4.1 mg/100g).
325
326 *3.3. Optimization of the phenolics extraction conditions*
327 There are several methodologies described in literature for the extraction of
328 phenolics, including different solvents (water, ethanol and methanol) and different
329 temperatures (room and boiling temperature). The most common solvent and
330 temperature extraction are boiling (Ivanova et al., 2005) and room temperature
331 (Wettasinghe & Shahidi, 1999) water, boiling and room temperature ethanol:water

1:1 (Wettasinghe & Shahidi, 1999), methanol (Kumarasamy et al., 2002; López et al., 2007) and ethanol (Conforti et al., 2008). Therefore, we decided to experiment those methodologies in *Thymus mastichina* species in order to achieve the best procedure that lead to highest contents in phenols and better antioxidant properties. The extraction yields, total phenolics including flavonoids, and antioxidant activity EC_{50} values of *Thymus mastichina* obtained using the different extraction conditions are presented in **Table 4**.

Two extractions with methanol at 25 ºC for 12h (E6) were considered the best conditions because the extract obtained revealed the highest contents in phenolics (165 ± 1 mg GAE/g) and the best antioxidant properties measured by chemical assays (EC_{50} values lower than 0.7 mg/ml). At all the tested concentrations, E6 showed the highest scavenging effects on DPPH radical's percentages (eg. 67% at 1 mg/ml) and reducing power values (eg. 1.7 at 1 mg/ml) (**Figure 1**). These chemical assays used to evaluate the extracts antioxidant activity were adapted in the present work using a Microplate Reader, which decreases the time consumed (preparation of material and reading time) and the volumes of the solutions used in the experiment, when compared with a standard spectrophotometer.

Curiously, one of the most used conditions (boiling water, E2) gave the worst results: 47.7 ± 0.1 mg GAE/g and EC_{50} values higher than 1.1 mg/ml. This extract lead to lower phenolics amounts than the extract obtained using 25 ºC water (E1). This could be explained by the destruction of the structures of polyphenols with boiling solvents which causes a decrease in the antioxidant activity (**Table 4**, as it was already described by us (Ferreira, Barros, & Abreu, 2009). The extract obtained with

355 ethanol:water 50% v/v at 80 ºC (E4; 121.7 ± 0.4 mg GAE/g) gave better results than
356 the same mixture at 25 ºC (E3; 109.1 ± 0.5 mg GAE/g) and only ethanol at 25 ºC
357 (E5; 109.7 ± 0.4 mg GAE/g). It was already described that a moderate heating might
358 increase the overall content of free polyphenolic and flavonoid compounds (Choi,
359 Lee, Chun, Lee, & Lee, 2006). The authors suggested that heat treatment might
360 produce changes in their extractability due to the disruption of the cell wall thus
361 bound polyphenolic and flavonoid compounds may be released more easily relative to
362 those of raw materials. Another reason for the improved antioxidant activity could be
363 the formation of novel compounds having antioxidant activities during heat treatment
364 or thermal processing.
365 It was not observed a relation between the extraction yields (measured as ration
366 between the extract weight and the dry weight of each sample) and the phenolics
367 contents or the antioxidant properties. The highest yield was obtained using water at
368 25 ºC, which proves that not all the extracted compounds are antioxidants.
369
370 *3.4. Phenolics content and antioxidant activity*
371 The extraction conditions used to obtain E6 of *Thymus mastichina* were also applied
372 to *Glechoma hederacea* and *Origanum vulgare*. **Table 5** presents the extraction
373 yields, bioactive compounds and antioxidant activity EC_{50} values of these Lamiaceae.
374 Among the analysed compounds, phenolics, and particularly flavonoids, were the
375 main antioxidant compounds found in the samples. *Origanum vulgare* gave the best
376 results in all the antioxidant activity assays (EC_{50} values ≤ 0.45 mg/ml), which is in
377 agreement with the highest content in phenolics (369 ± 18 mg GAE/g) and flavonoids

(224 ± 1 mg CE/g) found in this species. The same subspecies of *Origanum vulgare* was studied in Spain and the EC_{50} values obtained for methanolic extracts were 14.1 ± 0.3 µg/ml for inflorescences and 18.2 ± 1.4 µg/ml for steam and leaves (López et al., 2007). Our results were more similar to the ones obtained by the same authors with another subspecies, *Origanum vulgare* subs. *vulgare* (inflorescences 186 ± 12 µg/ml; steam and leaves 57 ± 7 µg/ml).

Portuguese methanolic extract of *Glechoma hederacea* leaves and steams revealed a higher DPPH EC_{50} value (0.39 mg/ml) than methanol extracts of cultivated *Glechoma hederacea* (plants grown in greenhouse) from United Kingdom (1.47×10^{-2} mg/ml; Kumarasamy et al., 2002) and than boiling methanol extract of its seeds (1.5×10^{-2} mg/ml; Kumarasamy et al., 2007).

Thymus mastichina inflorescences revealed the lowest phenolics (165 ± 1 mg GAE/g) and flavonoids (84 ± 1 mg CE/g) contents. Despite its lowest antioxidant activity (**Table 5**), the EC_{50} value obtained in the TBARS assay (0.43 ± 0.02 mg/ml) was much better than the results reported by Miguel et al. (2004) in the essential oils from the same species (38.9 ± 1.3% at 500 mg/ml).

Lipid peroxidation, a consequence of oxidative stress, is associated with progressive loss in membrane potential, increase in membrane permeability, and finally cell death. The formation of TBARS in brain homogenates is a consequence of lipid peroxidation. Therefore, the very low EC_{50} values (**Table 5**) obtained for TBARS inhibition in brain homogenates, in the presence of *Thymus mastichina*, *Glechoma hederacea* and mostly, *Origanum vulgare* are very promising. In fact, brain is considered highly sensitive to oxidative damage as it consumes a significant amount

401 of oxygen, is relatively deficient in antioxidant defences, is rich in oxidisable
402 substrates like polyunsaturated fatty acids and catecholamines (Chong, Li, & Maiese,
403 2005), and is abundant in redox active transition metal ions like iron, usually
404 involved in metal-catalyzed formation of reactive oxygen species (Ali, Barnham,
405 Barrow, & Separovic, 2004).
406 Despite the good results obtained in comparison with other natural sources, the EC_{50}
407 values obtained for the herbal extracts were higher than the values obtained for the
408 standard trolox (**Table 5**). Nevertheless, pure active compounds usually reveal more
409 activity than crude extracts, and mostly this specific standard which is a water soluble
410 derivative of vitamin E with powerful antioxidant activity. It must be considered that
411 these Lamiaceae EC_{50} values represent extract concentrations, where each one of the
412 antioxidant compounds is present in a lower final concentration.
413 Significantly negative linear correlations (**Figure 2**) were established between the
414 phenolics and flavonoids content, and EC_{50} values of DPPH scavenging activity
415 (determination coefficient 0.7931 for phenolics and 0.7454 for flavonoids; ***$P < 0.001$),
416 reducing power (determination coefficient 0.9647 for phenolics and 0.9515
417 for flavonoids; ***$P < 0.001$), β-carotene bleaching inhibition (determination
418 coefficient 0.873 for phenolics and 0.8468 for flavonoids, ***$P < 0.001$) and TBARS
419 inhibition (determination coefficient 0.6037 for phenolics and 0.5392 for flavonoids,
420 ***$P < 0.001$). This proves that the extraction methodology was adequate, being the
421 sample with the highest bioactive compounds content the most efficient in
422 antioxidant activity (with the lowest EC_{50} values). The correlations were slightly
423 more significant for phenolics than for flavonoids, and the highest determination

424 coefficients were obtained for reducing power and β-carotene bleaching inhibition
425 assays.
426
427 Overall, it is noteworthy to point out that the scientific literature contains no studies
428 that validate the possible beneficial effects of the Portuguese medicinal herbs,
429 particularly related to their antioxidant activity. Therefore, the report of the radical
430 scavenging activity and lipid peroxidation inhibition capacity of these Lamiaceae
431 from North-eastern Portugal could help in the explanation of their uses in folk
432 medicine against several chronic diseases known to be related to the production of
433 ROS and oxidative stress. This is also the first study reporting an optimization of the
434 determination of tocopherols in Lamiaceae; the method was sensitive and accurate.
435 Furthermore, the studied plants are a good source of powerful antioxidants such as
436 phenolics and vitamins (ascorbic acid and tocopherols).
437
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442
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510 **Table 1.** Analytical characteristics of the tocopherols analysis method.

Compound	R_t (retention time)		Correlation coefficient (r^2)	Linearity range (μg/ml)	Limit	
	min	CV, %(n=10)			LOD (ng/ml)	LOQ (ng/ml)
α-tocopherol	7.85	0.25	0.9996	0.075-8.0	18.06	60.20
β-tocopherol	13.22	0.23	0.9997	0.1-8.0	25.82	86.07
γ-tocopherol	15.24	0.23	0.9997	0.075-8.0	14.79	49.32
δ-tocopherol	19.75	0.29	0.9995	0.075-8.0	20.09	66.95
I.S. (tocol)	23.09	0.25	-	-	-	-

511 I.S. Internal standard.

512 **Table 2.** Method validation parameters obtained using *Thymus mastichina* sample.
513

Tocopherol	Precision CV, % (n=6)	Repeatability CV, % (n=6)	Recovery %
α-tocopherol	1.70	3.23	95 ± 4
β-tocopherol	1.86	4.47	89 ± 3
γ-tocopherol	2.36	2.18	92 ± 3
δ-tocopherol	0.22	2.71	91 ± 4

514

515 **Table 3.** Vitamins (ascorbic acid and tocopherols) composition (mg/100g dry weight)
516 of *Glechoma hederacea*, *Thymus mastichina* and *Origanum vulgare*. The results are
517 expressed as mean ± SD (n=3). In each column different letters mean significant
518 differences (*P<0.05).
519

Samples	Ascorbic acid	α-tocopherol	β-tocopherol	γ-tocopherol	δ-tocopherol	Total tocopherols
Glechoma hederacea	16.84 ± 0.22 a	272.42 ± 7.17 a	14.08 ± 1.70 a	78.55 ± 0.35 a	4.13 ± 0.00 a	369.18 ± 5.70 a
Origanum vulgare	17.07 ± 0.53 a	10.18 ± 0.43 b	0.45 ± 0.10 b	1.88 ± 0.06 b	0.15 ± 0.01 b	12.66 ± 0.55 b
Thymus mastichina	12.87 ± 0.22 b	0.35 ± 0.06 b	0.03 ± 0.00 b	3.75 ± 0.14 c	0.01 ± 0.00 c	4.14 ± 0.20 c

520 **Table 4.** Extraction yields, total phenolics and flavonoids, and antioxidant activity
521 EC_{50} values of *Thymus mastichina* obtained using different extraction conditions. The
522 results are expressed as mean ± SD (n=3). In each column different letters mean
523 significant differences (*P < 0.05).
524

Extracts	η (%)	Phenolics (mg GAE/g)	Flavonoids (mg CE/g)	DPPH EC_{50} (mg/ml)	Reducing Power EC_{50} (mg/ml)
E1	34.07 a	59.93 ± 0.06 d	8.63 ± 1.14 e	2.57 ± 0.54 b	0.70 ± 0.07 b
E2	16.82 d	47.71 ± 0.07 e	3.18 ± 0.94 f	4.22 ± 1.38 a	1.14 ± 0.01 a
E3	11.76 e	109.09 ± 0.46 c	13.04 ± 0.68 d	0.94 ± 0.04 d	0.53 ± 0.02 c
E4	31.60 b	121.73 ± 0.35 b	23.66 ± 1.46 c	0.86 ± 0.06 e	0.36 ± 0.00 e
E5	15.08 d	109.72 ± 0.35 c	50.89 ± 1.03 b	1.37 ± 0.12 c	0.43 ± 0.00 d
E6	21.61 c	165.29 ± 1.11 a	83.85 ± 1.42 a	0.69 ± 0.04 f	0.23 ± 0.00 f

525 E1 (H_2O, 25 ºC, 30 min), E2 (H_2O, b.t., 30 min), E3 (CH_3CH_2OH:H_2O 50% v/v, 25 ºC, 30 min), E4
526 (CH_3CH_2OH:H_2O 50% v/v, b.t., 30 min), E5 (CH_3CH_2OH, 25 ºC, 24h), E6 (CH_3OH, 25 ºC, 24h).
527

528 **Table 5.** Extraction yields, phenolics, flavonoids and antioxidant activity EC_{50} values of *Glechoma hederacea*, *Thymus*
529 *mastichina* and *Origanum vulgare* obtained using the best extraction conditions. The results are expressed as mean ± SD
530 (n=3). In each column different letters mean significant differences (*P < 0.05).
531

		Bioactive compounds		**Antioxidant properties (EC_{50} values; mg/ml)**			
Samples	η (%)	Phenolics (mg GAE/g)	Flavonoids (mg CE/g)	DPPH scavenging activity	Reducing power	β-carotene bleaching inhibition	TBARS inhibition
Glechoma hederacea	34.73 a	196.61± 6.09 b	95.02 ± 2.73 b	0.39 ± 0.02 b	0.22 ± 0.00 b	0.87 ± 0.10 a	0.11 ± 0.01 b
Origanum vulgare	30.69 b	368.58 ± 18.18 a	224.15 ± 0.96 a	0.16 ± 0.03 c	0.18 ± 0.00 c	0.45 ± 0.05 b	0.01 ± 0.00 c
Thymus mastichina	21.61 c	165.29 ± 1.11 c	83.85 ± 1.42 c	0.69 ± 0.04 a	0.23 ± 0.00 a	0.90 ± 0.09 a	0.43 ± 0.02 a
Trolox	-	-	-	0.04 ± 0.00	0.03 ± 0.00	0.003 ± 0.00	0.004 ± 0.00

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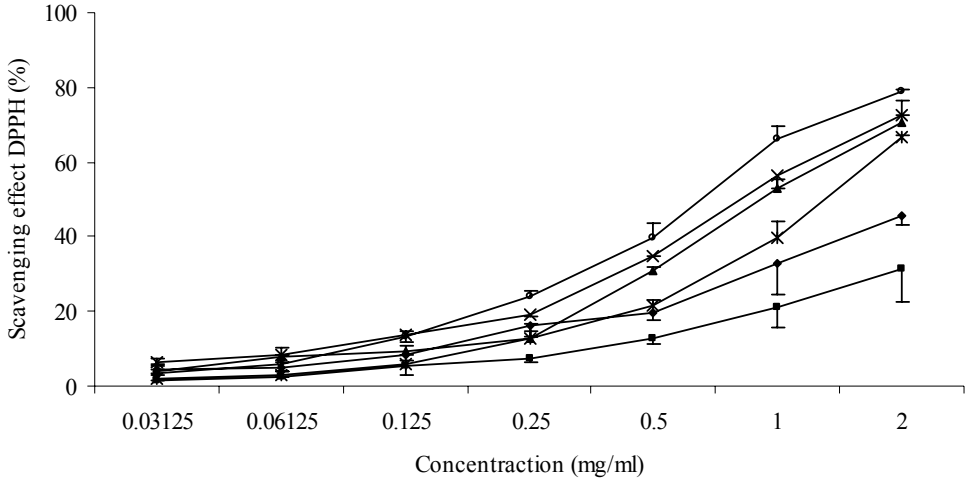

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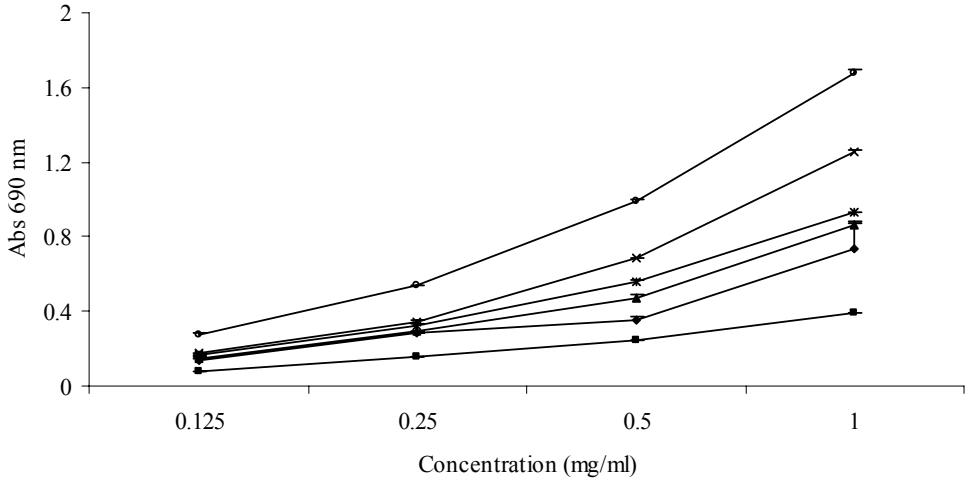

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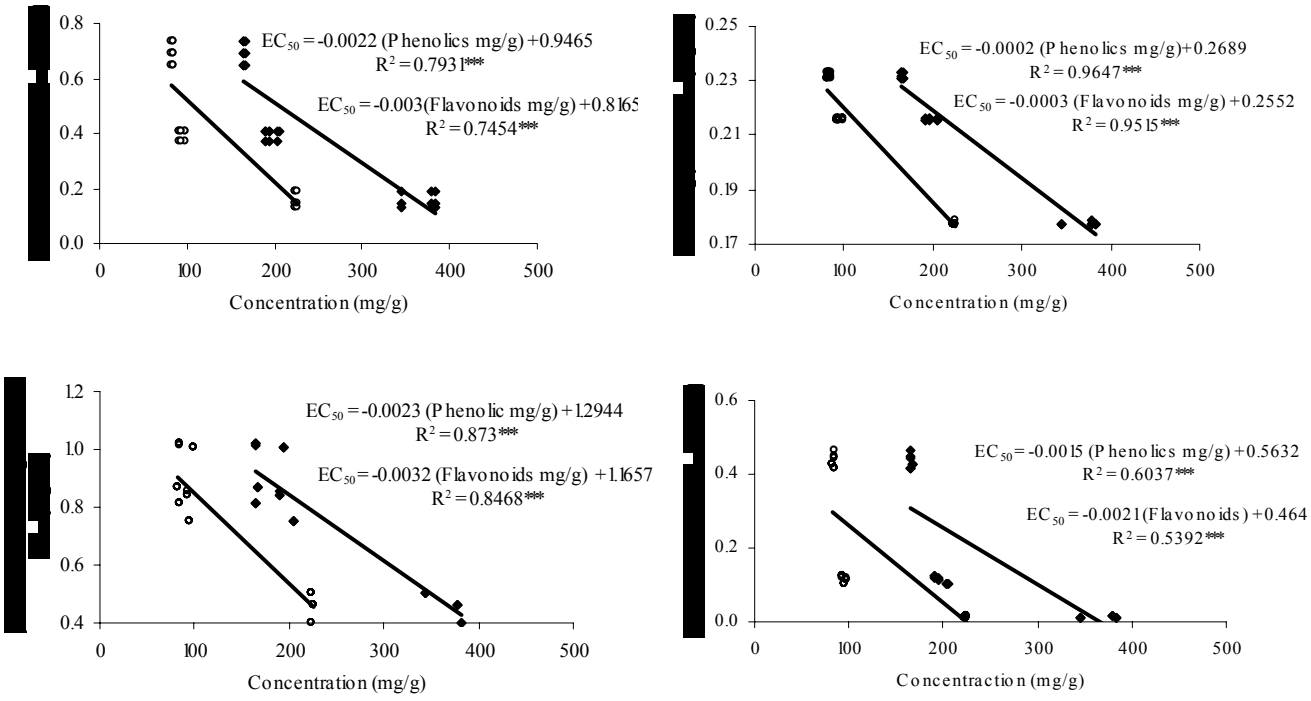
538 **Figure 1**. Scavenging activity on DPPH radicals (%) and reducing power of

539 *Thymus mastichina* obtained using different extraction conditions: (♦) E1; (■) E2;

540 (▲) E3; (×) E4; (*) E5 and (•) E6. Each value is expressed as mean ± SD (n=3).

541



552 **Figure 2**. Correlation established between total phenolics (♦) and flavonoids (○)
553 contents, and scavenging effect on DPPH radicals, reducing power, β-carotene
554 bleaching inhibition and lipid peroxidation inhibition.