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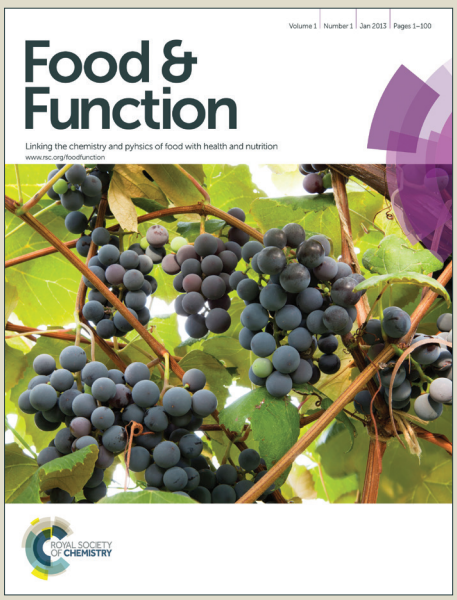

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Monomeric and oligomeric flavan-3-ols and antioxidant activity of leaves from different *Laurus* sp.

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Running title: Flavan-3-ols and antiradical activity of different *Laurus* sp. leaves

Abstract

The phenolic profile and antioxidant activity of three endemic *Laurus* sp. from Portugal were analysed. Dried leaves of *L. nobilis* L., *L. azorica* (Seub.) Franco, and *L. novocanariensis* Rivas Mart., Lousã, Fern. Prieto, E. Días, J. C. Costa & C. Aguiar, collected in the mainland and in the Azores and Madeira archipelagos, respectively, were used to prepare different extracts (aqueous, ethanolic and hydroalcoholic). They were studied regarding their DPPH• scavenging activity, total phenolics and flavonoids contents, and main phenolic compounds were identified by HPLC-DAD-ESI-MS/MS. Total flavonoid contents were 30.1, 46.3, and 36.7 mg of epicatechin equivalents/g of sample (dry weight), for *L. nobilis*, *L. azorica* and *L. novocanariensis*, respectively. Epicatechin was the major compound, representing ~12.1 % of total flavan-3-ols in *L. nobilis*, ~25.6 % in *L. azorica* , and ~19.9 % in *L. novocanariensis*. Although all samples presented a similar phenolic profile, significant differences were observed in total contents and antioxidant activity.

Keywords: *Laurus* sp.; Flavonoids; Proantocyanidins; HPLC-DAD-ESI-MS/MS; Antioxidant activity.

Introduction

Laurus is a genus of evergreen trees belonging to Lauraceae family, and three autochthonous species (*Laurus nobilis* L., *Laurus azorica* (Seub.) Franco and *Laurus novocanariensis* Rivas Mart., Lousã, Fern. Prieto, E. Días, J. C. Costa & C. Aguiar) are described in Portugal.

L. nobilis L., commonly known as bay leaves, is widely distributed in the Mediterranean area. It is usually used as a spicy fragrance and flavoring agent in culinary (particularly in traditional meat dishes, stews and rice)[1,2] and as an natural additive in cosmetics.[3] Several studies have already been performed in its leaves and extracts, appealing to their pharmacological properties and potential health benefits related to different phytochemical compounds. Infusions are generally used as carminative, diuretic, anti-rheumatic, among others.[4,5] Anti-inflammatory, anticonvulsive and antioxidant properties have also been reported in several studies for bay leaves and their extracts.[2,3,6,7]

L. azorica (Seub.) Franco, known as wild laurel, is a native shrub or small tree from the Azores archipelago.[8] It has been referenced in the red book of endangered species and, consequently, measures of conservation for the species have been suggested.[9] *L. azorica* leaves are commonly used in folk medicine owing to attributed anti-ulcer and blood depurative properties,[10] but contrary to *L. nobilis*, its use for culinary purposes is not recommended due to the toxicity of its leaves.[9]

L. novocanariensis is the endemic laurel from the Madeira archipelago. The leaves are used in traditional cuisine and its essential oil is used in folk medicine due to its cicatrizing and anti-rheumatic properties (topic preparations).[11]

The characterization of the polyphenolic profile of dry leaves of Portuguese endemic *Laurus* sp. (specially *L. azorica* and *L. novocanariensis*) are still, in some extent, unexplored. The aim of this work was, then, to compare the phenolic profile of these *Laurus* sp. with that of *L. nobilis* in order to evaluate the possibility to discriminate them chemically. Different extracts from *L. nobilis*, *L. azorica* and *L. novocanariensis* leaves were prepared and analysed regarding total phenolics, flavonoids contents and anti-radical activity. The phenolics profile of selected extracts was then analysed by HPLC-DAD-ESI-MS/MS and compared.

Results and discussion

Phytochemical contents

The type of the solvent is a key factor for the compounds extraction. It highly influences the kinectis of phytochemicals release from the solid matrix and, consequently, the chemical composition and antioxidant activity of the extracts.[12] Aiming to define the best solvent to maximize the extraction of the antioxidant compounds from the samples in study, an experimental design was assayed, in which three different extraction solvents were tested: 100% water, 100% ethanol, and a hydroalcoholic mixture (1:1). The extraction time and temperature used were 60 min and 40 ºC, respectively, based on previous studies performed in natural matrices that showed that the use of longer times and higher temperatures are not economically advantageous to extract this type of compounds.[13] The total phenolics and flavonoids contents were determined and the results are presented in Table 1. According to the type of solvent, a wide range of variability was found among the bioactive compounds

content ($p < 0.05$) of the different *Laurus* sp. analysed. The hydroalcoholic solvent showed the highest capacity to extract the phytochemical compounds in study, revealing a higher total content for *L. azorica*, followed by *L. novocanariensis* and *L. nobilis* (Table 1). Ethanol extracted less phytochemicals than the hydroalcoholic mixture. For comparison, distilled water was also used as extraction solvent, however the use of ethanolic and hydroalcoholic solvents resulted in better extraction rates. Our results are in agreement with those of other authors which claim that phenolic compounds are often more soluble in organic solvents that are less polar than water. [14-15] Indeed, previous studies conducted in dry leaves of *L. nobilis* using ethanol and water as solvents, reported higher phenolic contents in ethanolic extracts than in aqueous ones **(**132 mg GAE/g and 62 mg GAE/g, respectively).[7] More recently, Muñiz-Marquez et al. described a phenolics content of 17.32 mg/g of *L. nobilis* (from Mexico), by using an ultrasound-assisted extraction (35% ethanol) for 40 min.[5] The differences found among the results available in literature highlight, once more, that extraction conditions and polarity of solvents highly influence compounds quantification. Moreover, for the same plant species, the content and profile of phenolics (secondary metabolites) can be strongly influenced by the soil and climate conditions of each area.

In general, the samples studied in this work presented a similar profile of the analysed compounds: total phenolics > flavonoids (regardless of the solvent used). The results also show that *L. azorica* leaves contains significantly higher ($p<0.05$) amounts of these compounds, followed by *L. novocanariensis* and *L. nobilis*, which suggest differences between species.

Phenolic characterization by HPLC-DAD-ESI-MS/MS

The phenolic profiles of the extracts from *L. nobilis*, *L. azorica*, and *L. novocanariensis* leaves were investigated by chromatography coupled to a diode array detector and a mass spectrometer. A long chromatographic run (120 minutes), together with UV and mass spectra enabled us to identify the flavan-3-ols as the most abundant phenolics in the analysed laurel leaves. Figure 1 shows the chromatogram obtained for the *L. azorica* hydroalcoholic extract. Five main chromatographic peaks can be clearly observed, which were identified as peak 1, 3, 4, 5 and 6. The UV spectra of all these peaks exhibited a maximum at 277-280 nm, which suggested that they belong to the same family. Peak 4 (λ_{max} 238, 277 nm) showed an abundant protonated molecule at *m/z* 291. Collisional activation in MS^n experiments gave the fragment ions with *m/z* 273, 165, 139 and 123, which correspond to the fragmentation pathways of the monomeric flavan-3-ols catechin and epicatechin. Standards of (+)-catechin and (-)-epicatechin were injected and the peak was identified as epicatechin. Catechin (*m/z* 291 in the mass spectrum) was identified in Fig. 1 as peak 2. Only traces of this compound were found in the analysed laurel leaves (Table 2).

Peak 1 (λ_{max} 238, 280 nm) shows a pseudomolecular ion at *m/z* 1153 which fragments into 579 and 867 ions. This peak was ascribed to the tetrameric flavan-3-ol (E)C-(E)C-(E)C-(E)C and the fragments at *m/z* 579 and 867 correspond to the flavan-3-ol dimer and trimer, respectively. As with other mass spectrometric techniques no differentiation between stereoisomers is possible and no information about the position and stereochemistry of the interflavanoid linkage (4→6 or 4→8) is available.

The observed *m/z* 865 for the peak 3 (λ_{max} 238, 280 nm) in the Fig. 1 is consistent with the presence of a trimeric flavan-3-ol (E)C-(E)C-(E)C. MS^n experiments on this compound yielded five main fragments: *m/z* 713, 695, 533, 411, 287. Retro Diels-Alder (RDA) fission in the trimer resulted in the ion *m/z* 713 (neutral loss of 152 Da). Neutral

losses of 152 Da through RDA fissions are very common and were found to be the most important fragmentation for structure elucidation both for trimers and dimers. The fragment ion *m/z* 287 was formed by RDA fission of the dimer and subsequent neutral loss of 124 through H_2O/BFF (benzofuran-formin) fission of *m/z* 411, which is also found in the MS^n spectrum.

The pseudomolecular ion for peak 5 (λ_{max} 241, 277 nm) gave the *m/z* 865. The fragmentation pattern of this compound was found to be almost analogous to the previous compound (*m/z* 713, 695, 533, 411, 287) and thus a trimeric flavan-3-ol (E)C-(E)C-(E)C was ascribed to this peak.

The largest peak in the chromatogram (peak 6, Fig. 1) is characterized by maximum wavelengths of 241, 280 and 307 nm in the UV spectrum. This peak showed a protonated molecule at *m/z* 577 in the mass spectrum. The characteristic fragments at *m/z* 425 (RDA fission) was consistent with the presence of a dimeric flavan-3-ol (E)C-(E)C.

Proanthocyanidins can be divided into A-type and B-type.[16] The latter are flavan-3-ol oligomers and polymers linked mainly through C4→C8 and sometimes C4→C6 bonds, which cannot be elucidated here, as previously discussed. When an additional ether linkage is formed between C2→O→C7, the compounds are classified as A-type proanthocyanidins. The molecular mass of 2 units lower (*m/z* 577, 865, 1153) than that of the B-type analogous (*m/z* 579, 867, 1155 found for dimers, trimers and tetramers, respectively) and the similar fragmentation pathway are indicators that the proanthocyanidins here found and characterized are A-type proanthocyanidins.

Very recently, among several other bioactive compounds analysed, Dias et al. described a procyanidin trimer (B- and A-type linkages), (-)-epicatechin, a procyanidin dimer, and (+)-gallocatechin as the four major flavan-3-ols, by this order, in wild laurus, which

goes in accordance with our results.[17] It should be emphasized that our extracts have been further inspected for the presence of epigallocatechin (*m/z* 307), epicatechin-3-gallate (*m/z* 443) and epigallocatechin-3-gallate (*m/z* 459), other major flavonoids found in tea leaves.[18] However, these monomeric flavan-3-ols were not detected in the analysed extracts.

Quantification of low molecular proanthocyanidins

The content of monomeric and oligomeric flavan-3-ol found in the different species of *Laurus* is summarized in Table 2. A-type proanthocyanidins were the major phenolics present in the three *Laurus* leaves investigated whereas epicatechin was the dominant flavan-3-ol monomer. Catechin, on the other hand, was the minor flavanol constituent, ranging from 0.04 to 0.96 mg/g. The most abundant flavanol in the analysed *Laurus* extracts was the dimeric PA, followed by trimeric PA 2, trimeric PA 1 and tetrameric PA (Table 2). Epicatechin content ranged from 0.67 mg/ g (*L. nobilis* ethanolic extract) to 13.71 mg/ g (*L. azorica* hydroalcoholic extract).

Hydroalcoholic solvent allowed the highest recoveries of epicatechin from all samples according to the following order: *L. azorica* > *L. novocanariensis* > *L. nobilis* (13.71, 6.60 and 3.44 mg ECE/ g, respectively). Previous epidemiological research related with dietary interventions in humans, using high flavanol-containing foods, substantiates an inverse relationship between flavanol intake and the risk of cardiovascular diseases.[19] Several potential flavanol-mediated bioactivities, including vasodilatation,[20] insulin resistance, glucose tolerance,[21] and improvement of immune and antioxidant defense systems were also described.[22] The highest total flavan-3-ol content (*L. azorica*: 55.48

196 mg ECE/ g) was achieved with the hydroalcoholic solvent. The lowest content (*L.*
197 *nobilis*: 6.12 mg ECE/ g) was obtained with aqueous solvent.
198 Proanthocyanidins, better known as condensed tannins, are widely distributed
199 throughout the plant kingdom, and are present as the second most abundant class of
200 natural phenolic compounds after lignin. The estimated amount of total *Laurus*
201 proanthocyanidins in this work ranges from 2.4% (*L. nobilis*) to 3.9% (*L. azorica*) on a
202 dry weight basis, depending on the variety and geographic origin. These compounds
203 exhibit general toxicity toward fungi, yeast and bacteria and are linked with plant
204 defense mechanisms, organoleptic characteristics and stabilizing effects of pigments.[23]
205 The A-type proanthocyanidins here reported for the three endemic Portuguese *Laurus*
206 *sp*. leaves, with their unusual second ether linkage, may result from the oxidative
207 conversion of B- type into A-type, although this mechanism is still under
208 investigation.[15]
209
210 DPPH radical scavenging activity
211
212 The DPPH$^{\bullet}$ scavenging activity is commonly used as a basic screening method for
213 testing the antiradical activity of a large variety of compounds.[24]This method is
214 developed based on the ability of this stable free radical to change color in the presence
215 of antioxidants compounds. The DPPH$^{\bullet}$ contains an odd electron, which is responsible
216 for a visible deep purple color in alcoholic solution, and the respective absorbance can
217 be measured at 515 nm.
218 The antioxidant activity of *L. nobilis* leaves was previously reported by using different
219 solvents, including methanol/water extracts,[3] infusions,[4] ethanolic and aqueous
220 extracts,[7,25,26] but no similar study to this one was done so far, comprising two endemic

221 species coming from Madeira and Azores archipelagos (*L. novocanariensis* and *L.*
222 *azorica*, respectively).
223 In this work, the antioxidant activity of aqueous, ethanolic and hydroalcoholic extracts
224 of *L. nobilis*, *L. azorica*, and *L. novocanariensis* leaves were studied and results are
225 presented in Table 3. It can be pointed out that the extraction conditions also affected
226 significantly the antioxidant activity of the extracts, but contrary to what can be
227 observed in Table 1, the highest scavenging activity was achieved with ethanol, for all
228 the species in study**.** On the other hand, the hydroalcoholic mixture lead to intermediate
229 values (*L. azorica* ≅ *L. nobilis* > *L. novocanariensis*). According to the results presented
230 in Table 1, also the aqueous extracts exhibited the worst antioxidant activity. As
231 referred, ethanolic extracts presented higher antioxidant activity than the hydroalcoholic
232 ones, showing that other compounds than those quantified in this study are contributing
233 to the antiradical properties of the extract. This can be expected due to the complexity of
234 the matrix. Above all, the results of this study supports that the concentration of
235 bioactive compounds in plant extracts and their antioxidant activity vary according to
236 the type of solvent used, as well as the plant species. However, further detailed
237 examination of more methods would be advisable for the comprehensive assessment of
238 antioxidant activity.
239

240 **Experimental**

241

242 Reagents and standards

243

244 Gallic acid, catechin, epicatechin, sodium acetate, Folin-Ciocalteu's phenol reagent,
245 $DPPH^{\bullet}$ (2,2-diphenyl-1-picrylhydrazyl), sodium nitrite, aluminum chloride, and formic

246 acid (p.a.) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium
247 carbonate anhydrous, sodium hydroxide, acetonitrile (Lichrosolv HPLC grade), and
248 absolute ethanol were obtained from Merck (Darmstads, Germany). Ultrapure water
249 was treated in a Milli-Q water purification system (Millipore, Bedford, MA, USA) and
250 used to prepare all aqueous solutions. All other reagents are of analytical grade.
251
252 Samples and sample preparation
253
254 Three species of *Laurus* sp. identified as autochthonous plants in Portugal were
255 collected in August of 2012. L. nobilis L. was collected from the mainland, in the North
256 of Portugal, specifically in **the** district of Viana do Castelo (Latitude: 41º44' 31.57 N,
257 Longitude: 8º52' 32.68 W); L. azorica (Seub.) Franco from Pico island in Azores
258 archipelago (Latitude: 38°31' 35.7 N, Longitude: 28°19' 08.7 W); and L.
259 novocanariensis from Funchal, Madeira archipelago (Latitude: 32 39′ 39.0 N,
260 Longitude: 16°53′ 45.0 W). Leaves were dried in a D6450 Hanau oven (Heraeus,
261 Germany) at 25 ± 2 ºC for 3 weeks (in the dark). After, leaves were pulverized in a
262 grinder Grindomix GM 200 (Retsch, Germany), and stored into amber vials tightly
263 sealed, until further analysis.
264
265 Extracts preparation
266
267 In order to study the optimal extraction conditions, different procedures were tested by
268 varying polarity of the extraction solvent. Each sample (~5 g) was extracted by stirring
269 with 50 mL of solvent (100% water, 50% water/50% ethanol or 100% ethanol), for 1 h
270 on a heating plate (40 ºC at 600 rpm). Extracts were subsequently filtered through an

Whatman nº 4 paper and stored at -25 ºC until analysis. All extractions were performed in triplicate.

Determination of total phenolic compounds

Total phenolics were determined as described by Costa et al.[13] Briefly, 500 μL of extract were mixed with 2.5 mL of the Folin-Ciocalteu phenol reagent (1:10) and 2 mL of a sodium carbonate anhydrous solution (7.5%, m/v). The solution was incubated during 15 min at 45 ºC, followed by 30 min (room temperature) with absence of light. Absorbance was measured at 765 nm. Total phenolic compounds were expressed as mg gallic acid equivalents (GAE) per g of dry plant material, using a gallic acid calibration curve (r = 0.9990).

Determination of total flavonoids content

Total flavonoids were quantified by a colorimetric assay.[13] Briefly, aliquots (1 mL) of each extract were dilluted and mixed with 300 μL of sodium nitrite. After 5 min of incubation (room temperature), 300 μL of aluminum chloride were added, and after another minute, 2 mL of sodium hydroxide (1 M) and 2.4 mL of ultrapure water were also additioned. Absorbance was measured at 510 nm. A calibration curve was prepared with epicatechin (r = 0.9994) and the total flavonoids content was expressed as mg epicatechin equivalents (ECE) per g of dry weight.

HPLC-DAD-ESI-MS/MS analysis

296 The qualitative and quantitative analyses of the phenolic compounds in the extracts
297 were performed by HPLC coupled with an ion-trap mass spectrometer and diode array
298 detector (DAD). The HPLC system (Finnigan, Thermo Electron Corporation, San Jose,
299 CA,USA) consisted of a low-pressure quaternary pump (Thermo Finnigan Surveyor),
300 an auto-sampler (Thermo Finnigan Surveyor) with 200-vial capacity sample and a
301 photodiode array detector (Thermo Finnigan Surveyor). The compounds were separated
302 on a Phenomenex Synergi Hydro-RP C18 column (150 mm x 4.6 mm, 4 μm). A guard
303 column with the same characteristics was also used. The chromatographic conditions
304 were as follows: flow rate 0.3 mL/ min, sample injection volume of 25 μL, a mobile
305 phase A (acetonitrile) and a mobile phase B (0.1% aqueous formic acid). The following
306 gradient program was used: 10% A, 0 min; 10% to 25% A, over 80 min; 25% A to
307 100%, over 10 min; 100% A, for 5 min; back to 10% A in 10 min; and 15 min of
308 reconditioning before the next injection. An ion-trap mass spectrometer (Finnigan LCQ
309 Deca XP Plus) coupled with an electrospray ionization (ESI) source and Xcalibur
310 software Version 1.4 (Finnigan) were used for data acquisition and processing. The
311 interface conditions were applied as follows: capillary temperature, 325 ºC; source
312 voltage, 5.0 kV; capillary voltage, 4.0 V; sheath gas (N_2) flow at 90 arbitrary units and
313 auxiliary gas (N_2) flow rate at 25 arbitrary units. Data acquisition was performed
314 between *m/z* 200 and 1500. The positive ion polarity mode was selected due to a better
315 signal-to-noise ratio in comparison with negative ion mode. Tandem mass spectrometric
316 studies were performed (MS^2 and MS^3). For the MS^n analyses activation energy of 45%
317 was applied. The quantitative analysis was conducted at 280 nm for monomeric flavan-
318 3-ols (catechin and epicatechin) as well as for oligomeric flavan-3-ols. The
319 concentrations of individual phenolics in extracts were determined using external
320 standard calibration curves in the concentration range of 1 to 50 mg L^{-1}. The analytical

parameters of the calibration curves were calculated with the Excel program: catechin (y = 0.0901x - 0.02, r = 0.9998); epicatechin (y = 0.090x + 0.20, r = 0.9975). The molar-based detector responses of dimers were previously found to be twice that of monomers. Assuming a 3-fold molar response for trimers and 4-fold for tetramers, this allowed quantification of flavan-3-ols oligomers by the use of monomeric external standard. Epicatechin was used in this case and the results for oligomers are reported as mg epicatechin equivalentes (ECE) per g of dry sample, except for catechin (mg catechin/ g dry weight).

Antioxidant activity evaluation

Antioxidant activity of the extracts was evaluated by DPPH$^{\bullet}$ (2,2-diphenyl-1-picrylhydrazyl) radical scavenging ability, according to the method previously described by Brand-Williams et al.[27] with some modifications. An amount of 20 μL of each extract were mixed with 180 μL of a freshly prepared DPPH$^{\bullet}$ solution (6.0 x 10^{-5} mol/L in ethanol). The decrease in the absorbance at 515 nm (A_{515}) of DPPH was measured in equal time intervals of 3 min, in order to observe the kinetics reaction up to 20 min, using a 96-well microplate (GENS5). The DPPH$^{\bullet}$ radical scavenging activity (RSA) (%) was calculated by the following equation: [(Ac-As)/Ac)] x 100, where Ac is A_{515} of blank sample treated with no added extract and As is A_{515} of sample in the presence of extract.

Conclusions

In conclusion, the results of *in vitro* assays of examined *L. nobilis* L., *L. azorica* and *L. novocanariensis* expressed significant differences in phenolic contents. In addition, the hydroalcoholic mixture was selected as the best solvent for the extraction of the bioactive compounds analysed in this study. In turn, ethanolic extracts exhibited higher antioxidant activity, showing the possible contribution of additional compounds than those previously referred. *L. azorica* leaves**,** regardless of the solvent used**,** presented the highest content of total flavan-3-ols, followed by *L. novocanariensis* and *L. nobilis* L., respectively.

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References

1 D. J. M. Gómez-Coronado, C. Barbas, *J. Agric. Food Chem.I*, 2003, **51**, 5196-5201.

2 O. Ouchikh, T. Chahed, R. Ksouri, M. B. Taarit, H. Faleh, C. Abdelly, M. E. Kchouck, B. Marzouk, *J. Food Comp. Anal.*, 2011, **24**, 103-110.

3 F. Conforti, G. Statti, D. Uzunov, F. Menichini, *Biol. Pharm. Bull.*, 2006, **29**, 2056-2064.

4 S. Dall'Acqua, R. Cervellati, E. Speroni, S. Costa, M. Guerra, L. Stella, E. Greco, G. Innocenti, *J. Med. Food.*, 2009, **12**, 869-876.

5 D. B. Muñiz-Márquez, G. C. Martínez-Ávila, J. E. Wong-Paz, R. Belmares-Cerda, R. Rodríguez-Herrera, C.N. Aguilar, *Ultrason. Sonochem.*, 2013, **20**, 1149-1154.

6 M. Polovka, M. Suhaj, *Food Chem.*, 2010, **119**, 391-401.

7 C. Ramos, B. Teixeira, I. Batista, O. Matos, C. Serrano, N. R. Neng, J. M. F. Nogueira, M. L. Nunes, A. Marques, *Nat. Prod. Res.*, 2012, **6**, 518-529.

8 R. Ballabio, P. Goetz, *Phytothérapie*, 2010, **8**, 141-144.

9 R.F. Baptista [M.Sc. thesis] University of Coimbra: Portugal, 2012.

10 B. Ferrari, P. Castilho, F. Tomi, A. I. Rodrigues, M.C. Costa, J. Casanova, *Phytochem. Anal.*, 2005, **16**, 104-107.

11 D. Rivera, C. Obón, *J. Ethnopharmac*., 1995, **46**, 73-93.

12 I. Ignat, I. Volf, V. I. Popa, *Food Chem.*, 2011, *126*, 1821-1835.

13 A. S. G. Costa, R. C. Alves, A. F. Vinha, S. V. P. Barreira, M. A. Nunes, L. M. Cunha, M. B. P. P. Oliveira, *Ind. Crops Prod.*, 2014, 53, 350-357.

14 F. F. Liu, C. Y. Ang, D. Springer, *J. Agric. Food Chem.*, 2000, **48**, 3364-3371.

15 S. M. A. Soutinho, R. P. F. Guiné, A. M. Jordão, F. J. Gonçalves, *WASET*, 2013, **79**, 473-476.

16 D. Ferreira, D. Slade, *Nat. Prod. Rep.*, 2002, **19**, 517-541.

17 M. I. Dias, L. Barros, M. Dueñas, R. C. Alves, M. B. P. P. Oliveira, C. Santos-Buelga, I. C. Ferreira, *Food Chem.* 2014, **156**, 339-346.

18 M. S. Lee, Y. S. Hwang, J. Lee, M. G. Choung, *Food Chem.*, 2014, **158**, 351-357.

19 I. C. Arts, P. C. Hollman, *Am. J. Clin. Nutr.* 2005, **81**, 317S–325S.

20 M. Gómez-Guzmán, R. Jiménez, M. Sánchez, M. J. Zarzuelo, P. Galindo, A. M. Quintela, R. López-Sepúlveda, M. Romero, J. Tamargo, F. Vargas, F. Pérez-Vizcaíno, J. Duarte, *Free Rad. Biol. Med.*, 2012, *52*, 70-79.

21 A. Bettaieb, M. A. V Prieto, C. R. Lanzi, R. M. Miatello, F. G. Haj, C. G. Fraga, P. I. Oteiza, *Free Rad. Biol. Med.*, 2014, **72**, 247-256.

22 H. Sies, T. Schewe, C. Heiss, M. Kelm, *Am. J. Clin. Nutr.*, 2005, **81**, 304S-312S.

23 H. J. Li, M. L. Deinzer, *J. Agric. Food Chem.* 2006, **54**, 4048-4056.

24 O. P. Sharma, T. K. Bhat, DPPH antioxidant assay revisited. *Food Chem.* 2009, **113**, 1202-1205.

25 M. Elmastaş, I. Gülçin, Ö. Işildak, Ö. I. Küfrevioğlu, K. Ibaoğlu, H. Y. Aboul-Enein, *J. Ind. Chem. Soc.*, 2006, **3**, 258-266.

26 A. M. Emam, M. A. Mohamed, Y. M. Diab, N. Y. Megally, *Drug Discov. Ther.* 2010, **4**, 202-207.

27 W. Brand-Williams, M. E. Cuvelier, C. Berset, *LWT-Food Sci. Technol.* 1995, **28**, 25-30.

Table 1. Table 1. Total phenolics and flavonoids content of three endemic Portuguese *Laurus sp*. leaves (*L. nobilis* L.*, L. azorica*, and *L. novocanariensis*).

	Extracts	Phenolic (mg GAE/g)	Flavonoids (mg ECE/g)
L. nobilis	Aqueous	14.37 ± 0.79^{c}	14.12 ± 0.93^{c}
	Hydroalcoholic	43.03 ± 0.35^{a}	30.15 ± 0.25^{a}
	Ethanolic	31.09 ± 0.31^{b}	20.88 ± 0.88^{b}
L. azorica	Aqueous	26.29 ± 1.18^{b}	16.62 ± 0.49^{c}
	Hydroalcoholic	62.40 ± 0.68^{a}	46.32 ± 0.87^{a}
	Ethanolic	35.23 ± 0.92^{c}	25.59 ± 0.66^{b}
L. novocanariensis	Aqueous	25.42 ± 1.20^{c}	16.32 ± 0.49^{c}
	Hydroalcoholic	53.41 ± 0.62^{a}	36.71 ± 1.88^{a}
	Ethanolic	31.67 ± 1.38^{b}	25.44 ± 1.13^{b}

Data are reported as mean value ± standard deviation (three measurements). Values were significantly different when $p<0.05$ (Tukey's HSD test). Within each column, for each species, different letters indicate significant differences.

435 **Table 2.** Composition of monomeric (catechin and epicatechin) and oligomeric (proanthocyanidins A-type) flavan-3-ols in three endemic
436 Portuguese *Laurus sp*. leaves (*L. nobilis* L.*, L. azorica* (Seub.) Franco, and *L. novocanariensis*). Concentrations are reported in mg epicatechin
437 equivalents/ g dry weight, except for catechin (mg catechin/ g dry weight).

Flavan-3-ol	*L. nobilis*			*L. azorica*			*L. novocanariensis*		
	Aqueous	Hydroalcoholic	Ethanolic	Aqueous	Hydroalcoholic	Ethanolic	Aqueous	Hydroalcoholic	Ethanolic
(+)-Catechin	0.41	0.58	0.04	0.69	0.96	0.33	0.36	0.47	0.10
(-)-Epicatechin	0.99	3.44	0.67	5.81	13.71	6.12	1.37	6.60	2.43
Total monomers	1.40	4.02	0.71	6.50	14.67	6.45	1.73	7.07	2.53
Dimeric PA	1.49	16.97	5.25	2.51	23.5	7.47	2.88	15.70	6.13
Trimeric PA 1	0.48	1.24	0.32	1.95	2.91	2.11	0.40	1.86	0.60
Trimeric PA 2	1.73	5.05	2.46	4.79	10.47	7.10	1.71	7.62	3.83
Tetrameric PA	1.02	1.16	0.32	1.67	1.87	0.62	0.86	1.0	0.44
Total flavan-3-ols	6.12	28.44	9.06	17.42	53.48	23.74	7.58	33.24	13.53

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Table 3. Antioxidant activity measured by DPPH$^{\bullet}$ inhibition (%) of *Laurus* sp. species.

Laurus sp.	Type of extracts		
	Aqueous	**Hydroalcoholic (1:1)**	**Ethanolic**
L. nobilis	51.18 ± 1.51^{cB}	76.11 ± 0.09^{bA}	82.63 ± 0.00^{aC}
L. azorica	54.11 ± 0.00^{cA}	75.07 ± 0.09^{bA}	84.18 ± 0.05^{aA}
L. novocanariensis	23.65 ± 0.33^{cC}	64.35 ± 1.27^{bB}	83.10 ± 0.09^{aB}

Data are reported as mean value ± standard deviation (three measurements). Values were significantly different when $p<0.05$ (Tukey's HSD test). Within each column, different letters (A, B or C) indicate significant differences between plant species. Within each line, different letters (a, b or c) represent significant differences between different types of extract, for the same species.

464 **Figure Captions**

465 **Figure 1.** HPLC-UV profile of the *L. azorica* hydroalcoholic extract, obtained at 280
466 nm. Peak 1: tetrameric PA; peak 2: catechin; peak 3: trimeric PA 1; peak 4: epicatechin;
467 peak 5: trimeric PA 2; peak 6: dimeric PA.

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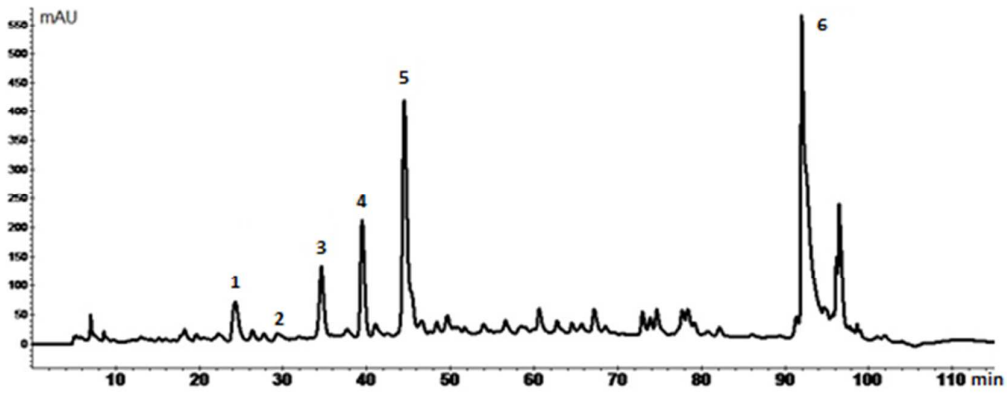

Figure 1.