Contribution of Essential Oils and Phenolics to the Antioxidant Properties of Aromatic Plants

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ABSTRACT

Different *in vitro* assays characterise most of the essential oils and phenolic compounds as antioxidants. These molecules can be found in a variety of aromatic plants and have been related to their bioactive properties. For the first time, a comparative study between the antioxidant properties of essential oils and phenolic extracts from *Cistus ladanifer* leaves, *Citrus latifolia* fruit peels, *Cupressus lusitanica* foliage and *Eucalyptus gunnii* leaves was performed. Overall, the antioxidant properties of phenolic extracts (unless scavenging activity of *Citrus latifolia*) were excellent and better than those obtained from the essential oils extracts, and even for the standards BHA (2-*tert*-butyl-4-methoxyphenol) and α-tocopherol. The better EC_{50} values for all the assays (scavenging activity, reducing power and lipid peroxidation inhibition) were obtained in the *Eucalyptus gunnii* phenolic extract (less than 0.1 mg mL^{-1}). Among the essential oils extracts, the best contribution was given by *Cistus ladanifer*.

***Keywords*:** Aromatic plants; Essential oils; Phenolics; Antioxidant activity.

1. Introduction

Essential oils are volatile, natural compounds with a strong odour and formed by aromatic plants as secondary metabolites. Since the middle ages, essential oils have been widely used for bactericidal, virucidal, fungicidal, antiparasitical, insecticidal, medicinal and cosmetic applications, especially nowadays in pharmaceutical, sanitary, cosmetic, agricultural and food industries. Particularly, they are used in embalmment, preservation of foods and as antimicrobial, analgesic, sedative, anti-inflammatory, spasmolytic and locally anesthesic remedies. Because of the mode of extraction, mostly by distillation (steam or hydro-distillation) from aromatic plants, they contain a variety of volatile molecules such as terpenes and terpenoids, phenol-derived aromatic components and aliphatic components. *In vitro* physicochemical assays characterise most of them as antioxidants (Bakkali et al., 2008).

Phenolic compounds are aromatic hydroxylated compounds commonly found in vegetables, fruits and many food sources that form a significant portion of our diet, and some of which are among the most potent and therapeutically useful bioactive substances. Natural phenolic compounds accumulate as end-products from the shikimate and acetate pathways and can range from relatively simple molecules (phenolic acids, phenylpropanoids, flavonoids) to highly polymerised compounds (lignins, melanins, tannins), with flavonoids representing the most common and widely distributed sub-group (Bravo, 1998). In our diet, they might provide health benefits associated with reduced risk of chronic diseases that may be due to their ability to reduce agents by donating hydrogen and quenching singlet oxygen (Nijveldt et al., 2001). Antioxidant properties of phenolic compounds also play a vital role in the

52 stability of food products, as well as in the antioxidative defence mechanisms of
53 biological systems (Macheix and Fleuriert, 1998).
54 In Portugal, *Cistus ladanifer* (systematic family Cistaceae) is widely distributed, being
55 one of the most abundant species in the southern part of the country, occurring in large
56 areas as pure dense stands (Amaral, 1971; Teixeira et al., 2007). The "Cistus" products
57 are particularly appreciated for their balsamic odour, as well as for their fixative
58 properties (Moyler and Clery, 1997). *Cistus* species are used as an antidiarrheics, as
59 general remedies in folk medicine for treatment of various skin diseases, and as anti-
60 inflammatory agents (Attaguile et al., 2000). Several research works have been reported
61 in the literature on *C. ladanifer* L. volatile compounds, due to the great importance of
62 this raw material for the fragrance industry (Teixeira et al., 2007). Phytochemical
63 studies on different *Cistus* species have also revealed the presence of several flavonoid
64 compounds that are considered to be chain-breaking antioxidants (Danne et al., 1994).
65 *Cupressus lusitanica*, commonly known as cedar of Goa, Mexican cypress and
66 Portuguese cypress, belongs to the systematic family Cupressaceae. The leaves of this
67 plant are used in indigenous practice to treat catarrh and headache. The essential oil of
68 the leaves is used against rheumatism, whooping cough, and styptic problems (Kuiate et
69 al., 2006). The chemical analysis of three samples of this oil from Portugal showed that
70 it contains monoterpenes, sesquiterpenes and diterpenes, with abietadiene as major
71 component (Adams et al., 2001). Nevertheless, its phenolic composition and antioxidant
72 activity was not reported yet.
73 *Eucalyptus* species are fast growing trees exploited mainly for paper pulp but also as a
74 source for various essential oils. For the production of phytopharmaceuticals, essential
75 oils rich in 1,8-cineole (called also ''eucalyptol''), are of special importance. These

products are applied for relief of head colds, rheumatism, muscular pain, and as expectorant in cases of bronchitis (added to cough syrups) (Lassak and McCarthy, 1983). Only the antioxidant activity and phenolic contents of *Eucalyptus globulus* bark aqueous extracts were reported (Vázquez et al., 2009).

Lime is the second most important citrus fruit, in both fresh consumption and industrial uses; it is the fifth largest crop in harvested area worldwide. The Persian lime (*Citrus latifolia*) is one of the main varieties in the production of this fruit. Total phenolics content and antioxidant activity of this lime species peels were reported by Urbando-Rivera et al. (2005), while nothing was described in its essential oils composition.

In this work, the antioxidant properties of four different aromatic plants (*Cistus ladanifer* leaves, *Citrus latifolia* fruit peels, *Cupressus lusitanica* foliage and *Eucalyptus gunnii* leaves) were evaluated, and compared, for the first time, considering two fractions: volatile fraction (essential oils) and phenolic fraction. To access the different contributions of both extracts, their DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity, reducing power, and inhibition of lipid peroxidation by decreasing the β-carotene bleaching were evaluated.

2. Materials and Methods

2.1. Standards and reagents

All the solvents were of analytical grade purity; methanol was supplied by Lab-Scan (Lisbon, Portugal). The standards used in the antioxidant activity assays: BHA (2-*tert*-butyl-4-methoxyphenol), TBHQ (tert-butylhydroquinone), L-ascorbic acid, α-tocopherol, gallic acid and (+)-catechin were purchased from Sigma (St. Louis, MO,

USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). The standard butylated hydroxytoluene (BHT) was purchased from Merck (Darmstadt, Germany). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

2.2. Plant material

Cistus ladanifer fresh leaves from flowering steams were collected randomly, from wild plants growing in the Natural Park of Montesinho (Northeastern Portugal) in July 2008. *Cupressus lusitanica* foliage and *Eucalyptus gunnii* matured alternate leaves were randomly gathered from the crown of trees cultivated in the experimental farm of the school of agriculture (Escola Superior Agrária, ESA), in Bragança (Northeastern Portugal) in July 2008. *Citrus latifolia* fruits were obtained from a local supermarket in December 2008, and the bioactive compounds were extracted from the peels.

2.3. Essential oils extraction

The essential oil samples were isolated from the fresh material (~100 g leaves/peels plus 350 mL of distilled ultra pure water) by hydro-distillation for 3 h, using a Clevenger-type apparatus. The extracts were dried with anhydrous sulphate and concentrated under reduced pressure by rotatory evaporator, until water evaporation (Fakhari et al., 2005). The extraction yield was calculated in mL of oil per 100 g of fresh material. The collected oil was weighed, dissolved in methanol at a concentration of 500 mg mL^{-1}, and stored in sealed vials at -20ºC for further use.

124 *2.4. Phenolics extraction*

125 Lyophilized (Ly-8-FM-ULE, Snijders, HOLLAND) powdered samples (typically 3 g)
126 were extracted by stirring with 50 mL of methanol at 25ºC at 150 rpm for 12h and
127 filtered through Whatman nº 4 paper. The residue was then extracted with one
128 additional 50 mL portion of the methanol. The extracts were evaporated (rotary
129 evaporator Büchi R-210) to dryness and redissolved in methanol at a concentration of 5
130 mg mL^{-1}, and stored at 4ºC for further use.

131 For phenolics estimation, the extract solution (1 mL) was mixed with Folin and
132 Ciocalteu's phenol reagent (1 mL). After 3 min, saturated sodium carbonate solution (1
133 mL) was added to the mixture and adjusted to 10 mL with distilled water (Singleton and
134 Rossi, 1965). The reaction was kept in the dark for 90 min, after which the absorbance
135 was read at λ=725 nm (Analytikijena 200-2004 spectrophotometer). Gallic acid was
136 used to calculate the standard curve (0.01-0.4 mM; Y=2.8557X-0.0021; R^2=0.9999) and
137 the results were expressed as mg of gallic acid equivalents (GAE) per g of extract.

138

139 *2.5. Antioxidant activity*

140 *2.5.1. DPPH radical-scavenging activity*

141 Various concentrations of the extracts (0.3 mL) were mixed with 2.7 mL of methanolic
142 solution containing DPPH radicals ($6x10^{-5}$ mol L^{-1}). The mixture was shaken vigorously
143 and left to stand for 60 min in the dark (until stable absorption values were obtained).
144 The reduction of the DPPH radical was determined by measuring the absorption at 517
145 nm (Hatano et al., 1988). The radical scavenging activity (RSA) was calculated as a
146 percentage of DPPH discoloration using the equation: % RSA = $[(A_{DPPH}-A_S)/A_{DPPH}] \times$
147 100, where A_S is the absorbance of the solution when the sample extract has been added

148 at a particular level, and A_{DPPH} is the absorbance of the DPPH solution. The extract
149 concentration providing 50% of radicals scavenging activity (EC_{50}) was calculated by
150 interpolation from the graph of RSA percentage against extract concentration. BHA and
151 α-tocopherol were used as standards.
152
153 *2.5.2. Reducing power*
154 Various concentrations of the extracts (1.0 mL) were mixed with 1.0 mL of 200 mmol
155 L^{-1} sodium phosphate buffer (pH 6.6) and 1.0 mL of 1% potassium ferricyanide. The
156 mixture was incubated at 50ºC for 20 min. After 1.0 mL of 10% tricloroacetic acid
157 (w/v) were added, the mixture was centrifuged at 1000 rpm for 8 min (Centorion
158 K24OR- 2003 refrigerated centrifuge). The upper layer (1 mL) was mixed with 1 mL of
159 deionised water and 0.2 mL of 0.1% of ferric chloride, and the absorbance was
160 measured spectrophotometrically at 700 nm: higher absorbance indicates higher
161 reducing power (Oyaizu, 1986). The extract concentration providing 0.5 of absorbance
162 (EC_{50}) was calculated by interpolation from the graph of absorbance at 700 nm against
163 extract concentration. BHA and α-tocopherol were used as standards.
164
165 *2.5.3. Inhibition of β-carotene bleaching*
166 The antioxidant activity of the extracts was evaluated by the β-carotene linoleate model
167 system. A solution of β-carotene was prepared by dissolving β-carotene (2 mg) in
168 chloroform (10 mL). Two millilitres of this solution were pipetted into a round-bottom
169 flask. After the chloroform was removed at 40ºC under vacuum, linoleic acid (40 mg),
170 Tween 80 emulsifier (400 mg), and distilled water (100 mL) were added to the flask
171 with vigorous shaking. Aliquots (4.8 mL) of this emulsion were transferred into
172 different test tubes containing different concentrations of the extracts (0.2 mL). The

tubes were shaken and incubated at 50ºC in a water bath. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm using a spectrophotometer. Absorbance readings were then recorded at 20-min intervals until the control sample had changed colour. A blank, devoid of β-carotene, was prepared for background subtraction (Shon et al., 2003). Lipid peroxidation (LPO) inhibition was calculated using the following equation: LPO inhibition = (β-carotene content after 2h of assay/initial β-carotene content) × 100. The extract concentration providing 50% antioxidant activity (EC_{50}) was calculated by interpolation from the graph of antioxidant activity percentage against extract concentration. TBHQ was used as standard.

2.6. Statistical analysis

For each one of the plants three samples were analysed and also all the assays were carried out in triplicate. The results are expressed as mean values and standard deviation (SD). The statistical differences represented by letters (**Table 1**) were obtained through one-way analysis of variance (ANOVA) followed by Tukey's honestly test, coupled with Welch's statistical analysis using SPSS software version 16.0.

3. Results and Discussion

Radical scavenging effects of phenolic and essential oils extracts of the aromatic plants *Cistus ladanifer*, *Citrus latifolia*, *Cupressus lusitanica* and *Eucalyptus gunnii* were examined and compared (**Figure 1**). Results are expressed as a percentage of the ratio of the decrease in the absorbance at 517 nm to the absorbance of DPPH solution in the absence of sample at 517 nm. The extracts scavenging effects on DPPH radicals increase with the concentration and the results are very good for phenolic extracts,

197 specially for *Eucalyptus gunnii* sample (95.96 ± 0.14% at 0.31 mg mL^{-1}), being much
198 higher than the scavenging effects of the standards BHA (96% at 3.6 mg mL^{-1}) and α-
199 tocopherol (95% at 8.6 mg mL^{-1}) (**Figure 1**). Nevertheless, this effect drastically
200 decreases in the corresponding essential oils extracts and, curiously, the lowest values
201 were obtained for the *Eucalyptus gunnii* sample (68.18 ± 1.86% at 500.00 mg mL^{-1}). In
202 the essential oils extracts, the best contribution to the antioxidant activity was achieved
203 by *Cistus ladanifer* (86.88 ± 0.76% at 153.60 mg mL^{-1}).
204 Reducing power of the aromatic plants was examined as a function of their
205 concentration in phenolics or essential oils (**Figure 2**). Reducing power of the samples
206 increased with the increase of concentration. A high value of absorbance at 700 nm is
207 related to a high reducing power. For phenolic extracts the absorbance values at 700 nm
208 were higher than the ones observed for essential oils extracts. The reducing power of
209 *Eucalyptus gunnii* phenolic extract was the highest (1.77 ± 0.07 at 0.31 mg mL^{-1}) while
210 for the lime extract was the lowest (0.02 ± 0.00 at 0.31 mg mL^{-1}). For essential oils
211 extracts the best reducing power was obtained for *Cistus ladanifer* sample (0.60 ± 0.00
212 at 4.80 mg mL^{-1}) and the worst was obtained for *Eucalyptus gunnii* (0.13 ± 0.00 at 4.80
213 mg mL^{-1}). Most of the samples were better than the standards (only lime extract gave a
214 higher EC_{50} value than one of the standards, BHA); reducing power of BHA at 3.6 mg
215 mL^{-1} and α-tocopherol at 8.6 mg mL^{-1} was only 0.12 and 0.13, respectively.
216 Lipid peroxidation inhibition, measured by the bleaching of β-carotene, is also
217 presented in **Figure 3**. β-Carotene undergoes a rapid decolourization in the absence of
218 an antioxidant since the free linoleic acid radical attacks the β-carotene molecule, which
219 loses the double bonds and, consequently loses its orange colour. The results obtained
220 for phenolic extracts were excellent and even better than the standard TBHQ (82.2% at

221 2.00 mg mL^{-1}). For *Eucalyptus gunnii*, phenolic extracts gave the highest β-carotene
222 bleaching inhibition percentages (e.g. 84.71 ± 6.96% at 0.31 mg mL^{-1}) while *Cupressus*
223 *lusitanica* gave the lowest (e.g. 43.51 ± 0.37% at 0.31 mg mL^{-1}). Once more, in the
224 essential oils extracts, the best contribution was given by *Cistus ladanifer* (66.32 ±
225 1.39% at 0.60 mg mL^{-1}) and the worst by *Citrus latifolia*.
226
227 For an overview of the results, EC_{50} values (mg mL^{-1}) obtained in the different
228 antioxidant activity assays performed for phenolic and essential oils extracts from the
229 aromatic plants are shown in **Table 1**. Both kind of extracts of *Cistus ladanifer*, *Citrus*,
230 *Cupressus lusitanica* and *Eucalyptus gunnii* revealed interesting antioxidant properties.
231 Nevertheless, the phenolic extracts revealed better antioxidant properties (significantly
232 lower EC_{50} values; $p \leq 0.05$) than essential oils extracts. After distillation extraction from
233 aromatic plants, several volatile molecules can be present in the samples such as
234 terpenes and terpenoids, aliphatic components, but also phenol-derived aromatic
235 components. Nevertheless, it is already known that thermal degradation of compounds
236 is one of the most significant disadvantages of the distillation extraction methodology
237 (Teixeira et al., 2007). In fact, our research group also reported the destruction of the
238 structures of polyphenols by heat which causes a decrease in the antioxidant activity
239 (Barros et al., 2007). This could explain the decrease in the antioxidant activity of
240 essential oils fraction (loss of phenolics and remain of the other volatile compounds)
241 relatively to the phenolic fraction (extracted at room temperature). Also, phenols might
242 be better antioxidants than terpenes, terpenoids or aliphatic components, since they
243 easily donate hydrogen atoms to quench the radicals formed in the DPPH and lipid

peroxidation inhibition assays, or electrons to reduce Fe^{3+}/ferricyanide complex to the ferrous form (Fe^{2+}) in the reducing power assay.

The extraction yields obtained in essential oils extractions were much lower than those obtained for the phenolic extraction (**Table 1**). Nevertheless, it was not observed any relation between the extraction yields and the antioxidant activity EC_{50} values obtained in the different assays. Phenolic extracts (unless for DPPH scavenging properties of lime extracts) revealed excellent antioxidant properties, even better than the well-known standards BHA and TBHQ. These synthetic antioxidant compounds are added to an extensive variety of foods in order to prevent or retard oxidation, so they are widely used in the food industry (Adegoke et al., 1998) and are included in the human diet (Leclercq et al., 2000). Nevertheless, the use of naturally occurring antioxidants has been promoted because of concerns regarding the safety of synthetic antioxidants. The possible activity of synthetic antioxidants as promoters of carcinogenesis has become a concern. BHA and related antioxidants have been suggested to have toxic effects like liver damage and mutagenesis (Grice, 1986; Wichi, 1988). Therefore, replacing synthetic antioxidants with natural alternatives has attracted great interest over the last years, and aromatic plants seem to be good candidates.

Eucalyptus gunnii phenolic extract revealed EC_{50} values lower than 0.1 mg mL^{-1}, which can be explained by the higher phenolic contents (176.07 ± 0.18 mg GAE g^{-1}; **Figure 4**). This value is similar to the content found in a previous study (Vázquez et al., 2009) on aqueous *Eucalyptus globulus* bark extracts from Galicia (180.9 mg GAE g^{-1}). *Citrus latifolia* revealed the lowest phenolic content (14.19 ± 0.48mg GAE g^{-1}; **Figure 4**) which is in agreement with the highest EC_{50} values obtained in all the antioxidant activity assays. This value was lower than the phenolics found in another study

(Urbando-Rivera et al., 2005) in *Citrus latifolia* obtained from Mexico (20 mg GAE g^{-1}). The EC_{50} values obtained with the studied aromatic plants are very promissory being better than the values obtained by our research group in different natural products such as mushrooms (Barros et al., 2008), honey (Ferreira et al., 2009) or chestnut fruits (Barreira et al., 2008). Furthermore, there are several reports on antioxidant properties of phenolics and essential oils but, as far as we know, this is the first study comparing the antioxidant properties of both extracts of these four aromatic plants.

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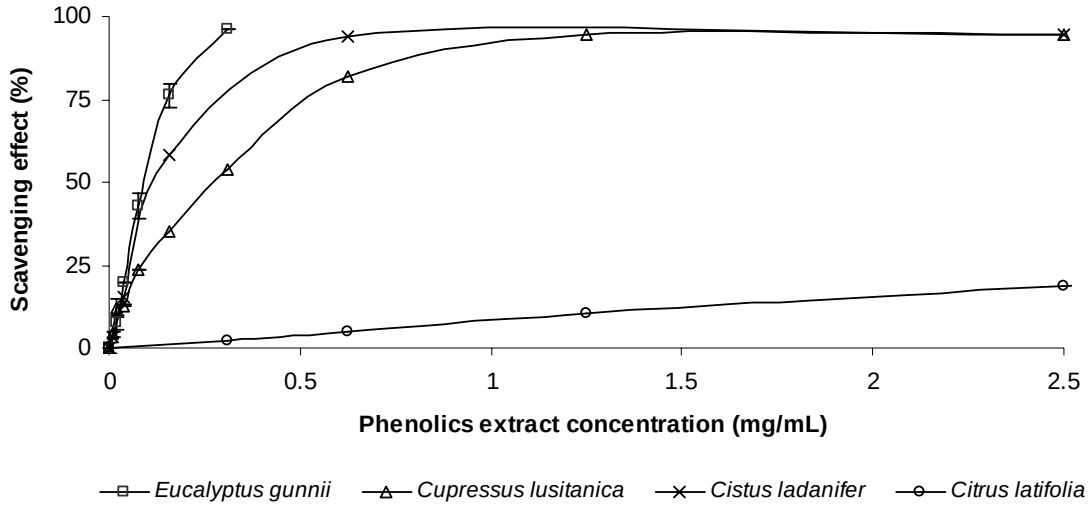
356

357 **Table 1.** Extraction yields (%) and EC_{50} values (mg mL^{-1}) obtained for the antioxidant
358 activity of the aromatic plants (mean ± SD; n=3). In each column different letters mean
359 significant differences.

Samples		Extraction yield	DPPH scavenging activity	Reducing power	Lipid peroxidation inhibition
Essential oils extract	*Cistus ladanifer*	0.63 ± 0.09 d	36.28 ± 0.36 d	4.00 ± 0.01 c	0.12 ± 0.01 d
	Citrus latifolia	1.26 ± 0.16 b	156.92 ± 0.40 b	23.64 ± 0.02 b	4.51 ± 0.03 b
	Cupressus lusitanica	0.80 ± 0.11 c	53.46 ± 0.70 c	4.38 ± 0.02 c	0.75 ± 0.08 c
	Eucalyptus gunnii	5.00 ± 0.89 a	272.93 ± 1.20 a	24.95 ± 0.07 a	5.49 ± 0.04 a
ANOVA		$p \leq 0.05$	$p \leq 0.05$	$p \leq 0.05$	$p \leq 0.05$
Phenolic extract	*Cistus ladanifer*	41.24 ± 8.15 a	0.13 ± 0.02 c	0.19 ± 0.01 c	0.12 ± 0.01 c
	Citrus latifolia	15.18 ± 0.20 d	7.53 ± 0.76 a	19.25 ± 0.09 a	0.65 ± 0.02 a
	Cupressus lusitanica	29.84 ± 2.21 c	0.28 ± 0.01 b	0.47 ± 0.00 b	0.39 ± 0.01 b
	Eucalyptus gunnii	38.33 ± 3.12 b	0.10 ± 0.31 c	0.08 ± 0.00 d	0.05 ± 0.00 d
ANOVA		$p \leq 0.05$	$p \leq 0.05$	$p \leq 0.05$	$p \leq 0.05$



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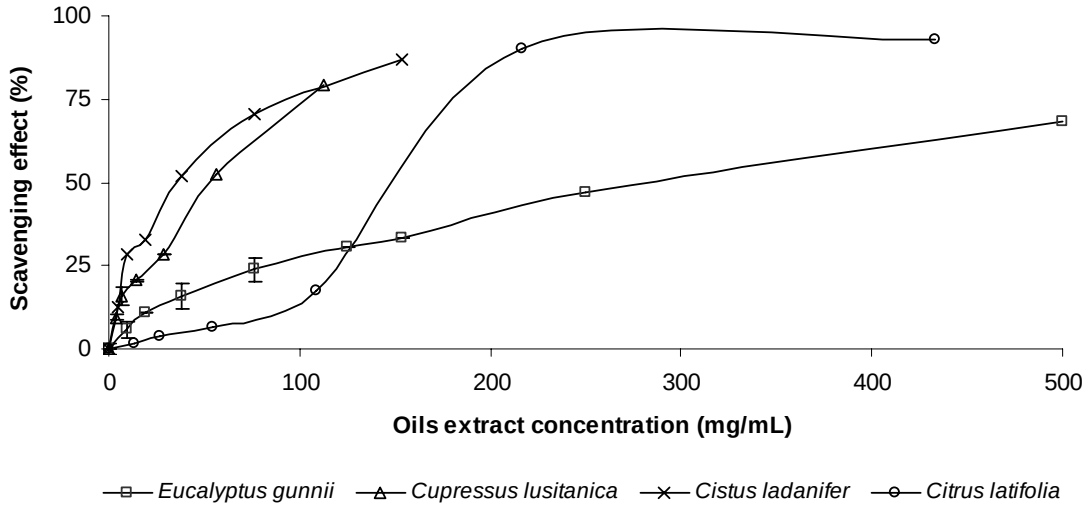

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362 **Figure 1.** Scavenging activity on DPPH radicals (%) of essential oils and phenolic
363 extracts of aromatic plants. Each value is expressed as mean ± SD (n=3). Standards:
364 BHA (96% at 3.6 mg mL^{-1}) and α-tocopherol (95% at 8.6 mg mL^{-1}).

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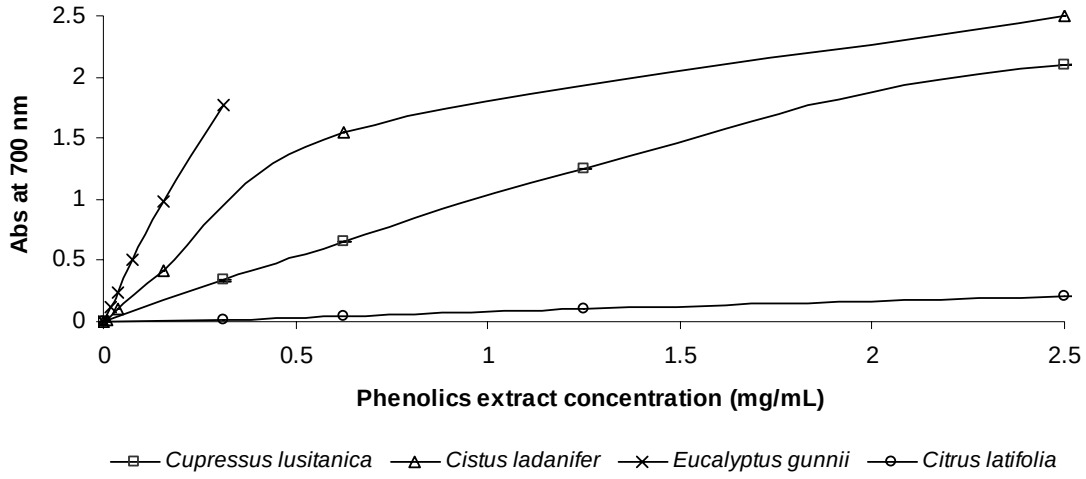

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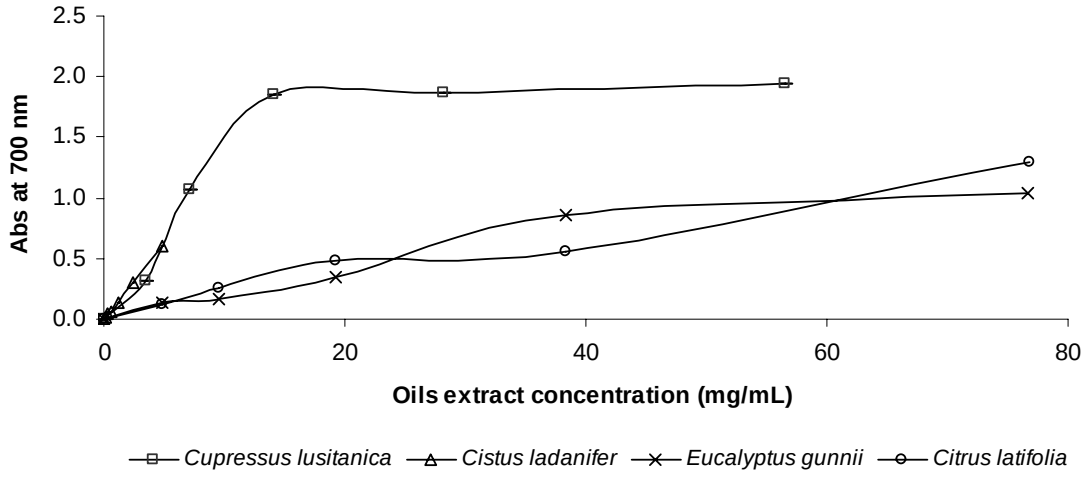
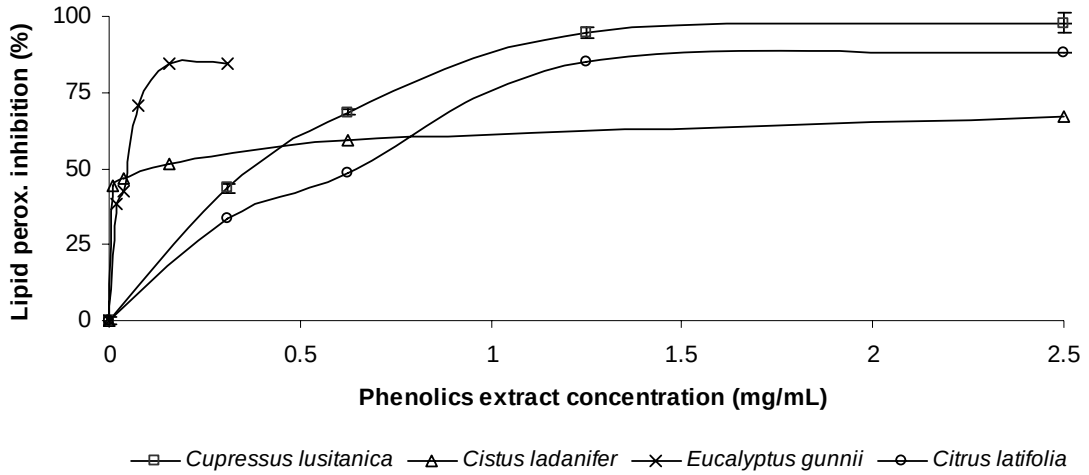


367

368 **Figure 2.** Reducing power of essential oils and phenolic extracts of aromatic plants.
369 Each value is expressed as mean ± SD (n=3). Standards: BHA (0.12 at 3.6 mg mL^{-1})
370 and α-tocopherol (0.13 at 8.6 mg mL^{-1}).



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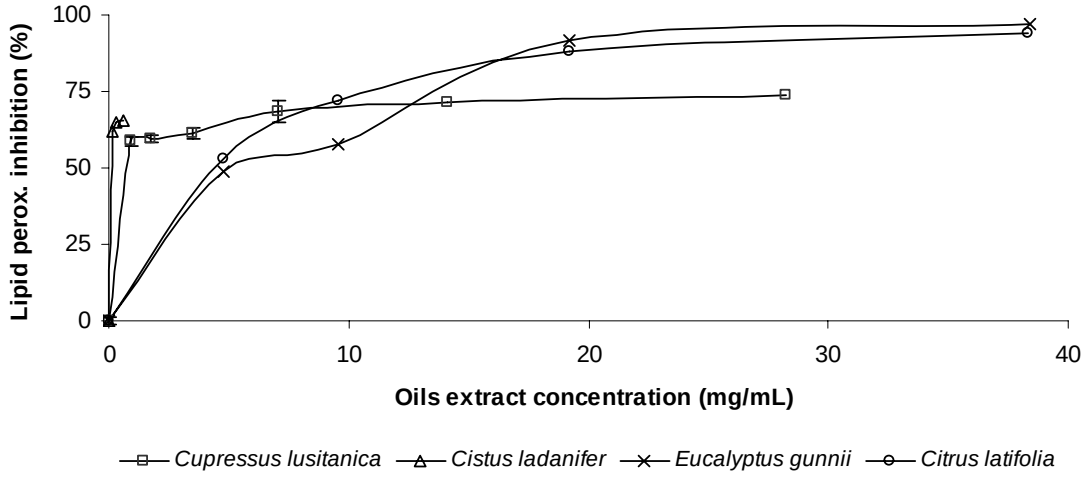

372

373 **Figure 3.** Lipid peroxidation inhibition of essential oils and phenolic extracts of
374 aromatic plants. Each value is expressed as mean ± SD (n=3). Standard: TBHQ (82.2%
375 at 2 mg mL^{-1}).

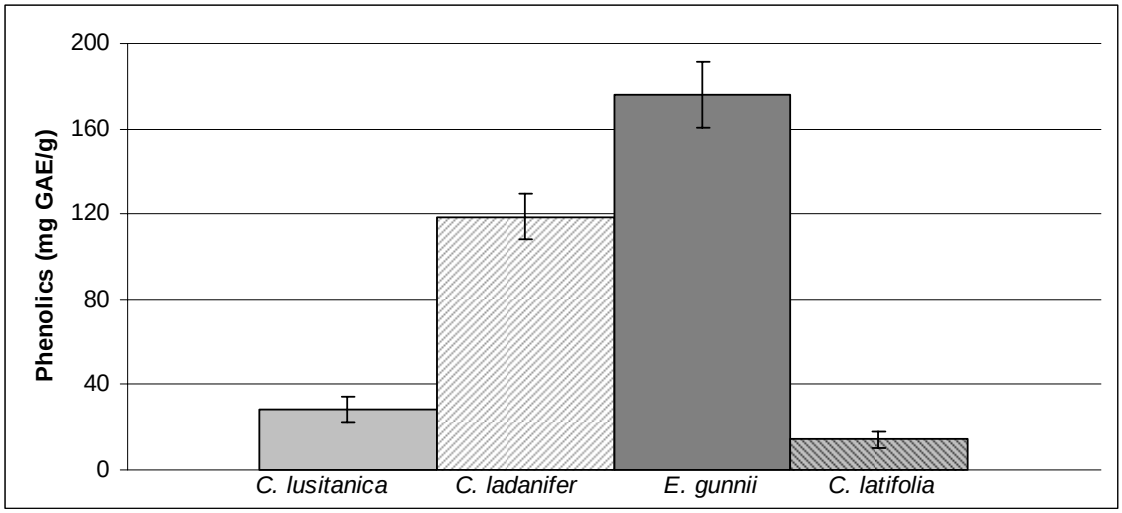

Figure 4. Total phenolic content in the aromatic plants.