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Antioxidant profile of dihydroxy- and trihydroxyphenolic acids-A structure–activity relationship study

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Abstract

Eight structurally similar dihydroxy and trihydroxyphenolic acids (protocatechuic acid, 3,4-dihydroxyphenylacetic acid, hydrocaffeic acid, caffeic acid, gallic acid, 3,4,5-trihydroxyphenylacetic acid, 3-(3,4,5-trihydroxyphenyl)propanoic acid and 3-(3,4,5-trihydroxyphenyl)propenoic acid) were examined for their total antioxidant capacity (TAC). Furthermore, their ability to scavenge peroxyl radicals, generated by AAPH in liposomes, was determined. The antioxidant/pro-oxidant activity of the compounds was screened using the 2′-deoxyguanosine assay. All compounds behave as radical scavengers, with 3,4,5-trihydroxyphenylacetic acid being the most potent. Nevertheless, in the lipid peroxidation assay an inverse ranking order was observed, 3,4-dihydroxyphenylacetic acid being the most effective compound. All the dihydroxylated compounds showed a pro-oxidant behaviour leading to an increase of 50% in 8-OH-dG induction. From the structure–antioxidant activity relationship studies performed it may be concluded that the number of phenolic groups and the type of the alkyl spacer between the carboxylic acid and the aromatic ring strongly influence the antioxidant activity.

Keywords: *Dihydroxyphenolic acid, trihydroxyphenolic acid, ABTS, DPPH, lipoperoxidation, 2′-deoxyguanosine*

Introduction

Phenolic acids have been widely investigated as potential models for the development of new primary antioxidants, which can prevent or delay *in vitro* and/or *in vivo* oxidation processes [1–5]. The antioxidant activity of this type of phenolic compounds has attracted much attention in relation to their physiological functions. In particular, dietary hydroxybenzoic and hydroxycinnamic acids are expected to be useful in the prevention of coronary heart disease, cancer and inflammation, since epidemiological studies have shown an inverse relationship between the intake of dietary phenolic acids and the occurrence of this type of pathologies [6–12]. These compounds have been described as chain-breaking antioxidants, probably acting through radical scavenging, which is related to their hydrogen or electron donating capacity, and their ability to delocalize/stabilize the resulting phenoxyl radical within the structure [2,13].

The overproduction of reactive oxygen species (ROS) or reactive nitrogen species (RNS) can result in oxidative damage to cell lipids, proteins and DNA [14]. Lipid peroxidation causes damage of unsaturated fatty acids, usually causing a decrease in the membrane fluidity, leading to pathological events such as arteriosclerosis and cancer. On the other hand,

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oxidative DNA damage may involve several lesions such as strand breaks, base modifications and DNA-protein cross-links, which may disrupt DNA replication, transcription and translation, thus causing mutations and neoplastic disorders.

Although the antioxidant and/or the antiradical activity of phenolic acids has been evaluated in different model systems, few studies have been performed to date in order to get an insight into the structure–activity relationships (SARs) underlying these functions. In fact, this may be a rather efficient approach for the design and development of new antioxidant agents and/or for the understanding of their mechanisms of action.

In this work, the antioxidant activity of eight structurally similar polyphenolic acids, either of natural or synthetic origin, (3,4-dihydroxybenzoic acid (1), 3-(3,4-dihydroxyphenyl)ethanoic acid (2), 3-(3,4-dihydroxy)propanoic acid (3), 3-(3,4-dihydroxyphenyl)propenoic acid (4), 3,4,5-trihydroxybenzoic acid (5), 3-(3,4,5-trihydroxyphenyl)ethanoic acid (6), 3-(3,4,5-trihydroxy)propanoic acid (7) and 3-(3,4,5-trihydroxyphenyl)propenoic acid (8)) (Figure 1), was evaluated by different methods, in order to correlate their antioxidant/pro-oxidant behaviour to their chemical structures.

Total antioxidant capacity (TAC) assays were used in order to determine the hierarchy of radical scavenging abilities of the polyphenolic acids under study as electron- or H-donating agents, by measuring their ability to scavenge $ABTS^{\cdot+}$ or $DPPH^{\cdot}$. The antioxidant effect of the compounds against lipid peroxidation was evaluated, using liposomes as a biomembrane mimetic model [15,16]. In order to better understand the results obtained by the lipoperoxidation experiments, the partition of the compounds between a heterogeneous phase (liposomes) and an aqueous phase (buffer) was also assessed [17,18]. Finally, a DNA damage model reaction was used in order to evaluate the antioxidant/pro-oxidant capacities of the polyphenols investigated, through the oxidation of 2′-deoxyguanosine (2′-dG) to 8-hydroxy-2′-deoxyguanosine (8-OH-2′-dG) by hydroxyl radicals ($HO^{\cdot}$) generated by a Fenton reaction.

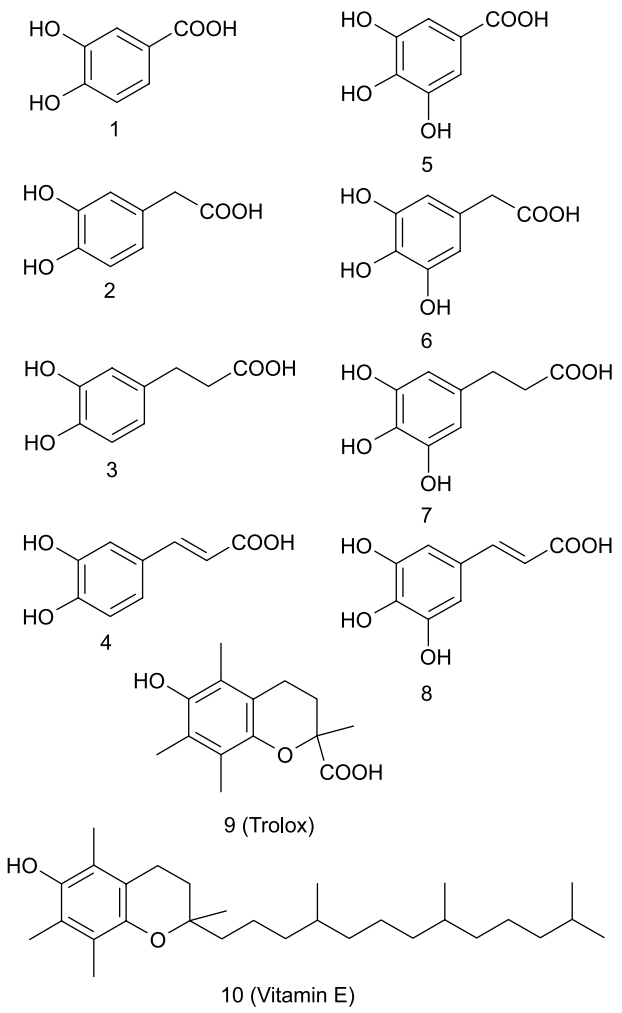

Figure 1. Structural representation of the polyphenolic acids under study.

Materials and methods

Chemicals

2′-Deoxyguanosine (2′-dG), 8-hydroxy-2′-deoxyguanosine (8-OH-2′-dG), 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid hemisodium salt (Hepes) and egg L-α-phosphatidylcholine (EPC) were obtained from Sigma (Sintra, Portugal). 1-hexadecylphosphorylcholine (HDPC) was obtained from Cayman Chemical (Ann Arbos, USA). 2-Carboxy-2,5,7,8-tetramethyl-6-chromanol (trolox), 2,2′-Azobis (2-amidinopropane) dihydrochloride (AAPH), 3,4-dihydroxybenzoic acid and 3-(3,4-dihydroxyphenyl)ethanoic acid were obtained from Fluka (Sintra, Portugal). 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 3-(3,4-dihydroxyphenyl)propanoic acid, 3-(3,4-dihydroxyphenyl)propenoic acid and 3,4,5-trihydroxybenzoic acid were purchased from Aldrich (Sintra, Portugal). Diphenylhexatriene propionic acid (DPH-PA) was obtained from Molecular Probes (Leiden, The Netherlands). Methanol LiChrosolv was purchased from Merck (Lisbon, Portugal). Water was double deionized (conductivity less than $0.1\,\mu S\,cm^{-1}$). All other reagents were of analytical grade and used without any further purification.

Apparatus

UV/Vis and fluorescence measurements were carried out on a Bio-Tek Synergy HT multiplate reader.

HPLC analyses were conducted at room temperature, on a Merck/Hitachi—LaChrom 7000 series equipped with a DAD detector (L-7455), a pump (L-7100) and a Waters Spherisorb ODS2 (4.6 × 250 mm, 5 μm) column with pre-column. Liposome extrusions were performed with an extruder Lipex (Lipex Biomembranes, Vancouver, Canada).

Synthesis of polyphenolic acids 6, 7 and 8

3-(3,4,5-trihydroxyphenyl)ethanoic acid (6) and 3-(3,4,5-trihydroxyphenyl)propanoic acid (7) were synthesized according to the procedure described by Gomes et al. [9]. The demethylation agent used in all the synthetic procedures was hydrogen bromide.

3-(3,4,5-Trihydroxyphenyl)propenoic acid (8) was synthesized as described by Fiuza et al. [19]. The synthesis was a Knoevenagel-type reaction between the corresponding trihydroxybenzaldehyde and malonic acid, using aniline as catalyst.

Total antioxidant capacity (TAC) assays

TAC assays were performed using ABTS and DPPH as radicals. Experimental procedures [20–23] were adapted from the literature in order to use a multiplate reader.

ABTS solution. An aqueous solution (25 ml) of ABTS (96.02 mg, 0.175 mmol) and potassium persulfate (16.55 mg, 0.0612 mmol) was left standing overnight to develop the deep blue–green colour of $ABTS^{\cdot+}$.

DPPH solution. An ethanolic solution (25 ml) of DPPH (19.13 mg, 0.0485 mmol) was prepared to obtain a deep purple solution of $DPPH^{\cdot}$.

Prior to the measurements, the concentration of the $ABTS^{\cdot+}$ and $DPPH^{\cdot}$ solutions were adjusted with different volumes of ethanol in order to have absorbance values of 0.45 ± 0.01 at 734 nm at 30°C, and 0.38 ± 0.01 at 515 nm at 25°C, respectively, when 180 μL of sample were placed in the plate reader.

Six different ethanolic solutions of each polyphenol (with concentrations ranging from 15 to 150 μM) were prepared in duplicate. A total of 20 μL of each were added to 180 μL of radical solution (in quadruplicate) and absorbances were recorded: for $ABTS^{\cdot+}$, every 5 min for a 20 min period; for $DPPH^{\cdot}$ every minute for a 10 min period, followed by every 5 min for the next 50 min. The absorbance of a blank control (20 μL ethanol + 180 μL of radical) was set as 100% of radical (0% bleaching). Trolox was used as a reference antioxidant.

Data analysis. The radical concentrations (both $ABTS^{\cdot+}$ and $DPPH^{\cdot}$) were plotted as a function of the concentration of the phenolic compounds, for 5 and 20 min of reaction time for $ABTS^{\cdot+}$, and for 60 min of reaction time for $DPPH^{\cdot}$. Second-degree polynomial regressions of the experimental points were generated, with a *y*-axis intercept at 100% of radical. The TEAC value was considered as the ratio between the trolox concentration corresponding to a 50% bleach of the radical (IC_{50}) and the concentration of phenol needed to achieve the same effect (for the different reaction times considered):

$$\mathrm{TEAC} = \frac{IC_{50}(\mathrm{trolox})}{IC_{50}(\mathrm{compound})}.$$

Lipoperoxidation assay

Antioxidant activity against lipoperoxidation was estimated in liposomes of EPC, containing a radical sensitive fluorescent probe, according to the procedure described by Arora et al. [15]. Peroxyl radicals were generated as a consequence of the thermal decomposition of AAPH. The experimental method was adapted in order to use a multiplate reader.

Liposomes preparation. EPC (15.75 mg, 22.5 μmol) and DPH-PA (38 μL of a 60% (m/v) methanolic solution) were dissolved in a 50 ml round flask with 10 ml of a $CHCl_3/CH_3OH$ (3:1) mixture. The solvent was evaporated on a rotavapor at 30°C, under a nitrogen flow in a light-protected environment, leaving a homogeneous lipidic film on the flask wall. The film was kept in a dessicator, under vacuum and protected from light, until further use. Before the measurements, the film was vigorously shaken for 20 min in a vortex mixer with 15 ml of a Hepes (5 mM)/NaCl (0.1 M) solution, in order to obtain a suspension of MultiLamellar Vesicles (MLVs). This suspension was extruded ten times through a 100 nm pore polycarbonate filter (Nucleopore, Whatman), yielding a suspension of Large Unilamellar Vesicles (LUVs) containing the DPH-PA fluorescent probe.

Liposomes oxidation. Polyphenol solutions (0, 6.42, 32.14 and 64.28 μM) were made in HEPES/NaCl solution, containing 6.5% of ethanol. Reagents were introduced in the 96 wells plate as follows: 160 μL of the LUVs suspension, 70 μL of polyphenol solution under study and 70 μL of a AAPH solution. The final concentrations were: 0.80 mM of LUVs, 1.50, 7.50 and 15.00 μM of polyphenol, 1.5% of ethanol and 15.00 mM of AAPH. Each assay was conducted in duplicate. Before the addition of the radical initiator, the LUVs/polyphenol mixtures were shaken for 10 min at 37°C in the multiplate reader. The maximum of fluorescence emission (λ_{ex}: 360/40 nm, λ_{em}: 460/40 nm) was set to 100% (0% of oxidation). AAPH was added and the fluorescence decay over time was recorded at 37°C, at regular intervals, for 3 h.

Trolox (9) and Vitamin E (10) were introduced in the experiment as reference antioxidants.

Data analysis. The area under the curve of a control assay (without polyphenol) was subtracted from the area obtained for the polyphenol and trolox assays. For a given concentration, the area obtained for the polyphenol was divided by the one obtained for trolox giving TEAC values:

$$\mathrm{TEAC} = \frac{\mathrm{AREA}_{(\mathrm{compound})} - \mathrm{AREA}_{(\mathrm{control})}}{\mathrm{AREA}_{(\mathrm{trolox})} - \mathrm{AREA}_{(\mathrm{control})}}.$$

Determination of partition coefficients in mimetic systems by derivative spectrophotometry

Partition coefficients were determined at pH 7.4 and at 25°C, using liposomes of egg phosphatidylcholine (EPC), as previously described [17]. Partition coefficients were determined by derivative spectrophometry using micelles of HDPC at pH 2 and at 25°C, as previously described [24].

2′-deoxyguanosine oxidation assay

In order to evaluate the antioxidant/pro-oxidant capacity of the compounds studied, the oxidation of 2′-dG *in vitro* by hydroxyl radicals ($HO^{\cdot}$) generated by a Fenton reaction was performed, in the presence of a radical scavenger, according to a method adapted from Yen et al. [25,26]. The level of 8-OH-2′-dG detected was compared to a control assay (without radical scavenger).

A reaction mixture (1.5 ml) containing 2′-dG (1 mM), potassium phosphate buffer (20 mM, pH 7.4), phenolic compound (from 5 μM to 1 mM), EDTA (6.5 mM), $FeCl_3$ (1.3 mM), ascorbic acid (0 or 15 mM), CH_3CN (2%) and H_2O_2 (50 mM) were incubated at 37°C for 45 min. Ethanol (20 μl) was then added, in order to stop the oxidation process, and the samples were kept in an ice bath until further analysis. The product of the oxidation, 8-OH-2′-dG, was detected by HPLC at 254 nm. The volume of sample injected was 20 μL. A total of 85% KH_2PO_4 (50 mM) and 15% methanol was used as the mobile phase, at a flow rate of 1 mL/min. Compounds were identified by comparison of their retention times with those of known standards. In the HPLC assays, 2′-dG and 8-OH-2′-dG had retention factors (k) of 1.77 and 2.50, respectively.

Data analysis. The area ratio (8-OH-2′-dG_{sample}/8-OH-2′-$dG_{control}$) was plotted as a function of the sample concentrations used. Values under 1 represent an antioxidant effect, while values over 1 correspond to a pro-oxidant effect (for that particular concentration). Trolox was used as a reference antioxidant.

Results and discussion

In order to study the influence of the chemical structure on the antioxidant activity of the phenolic systems investigated, a structure activity relationship (SAR) study was developed, for which a group of phenolic acids structurally related were either synthesized or commercially acquired. Two series of compounds were defined and are shown in Figure 1. One series is constituted by dihydroxylated (catechol moiety) molecules and the other by trihydroxylated (pyrogallol moiety) phenolic acids. The nature of the link between the carboxylic acid and the aromatic nucleus was also taken into account, including the type of spacer and its saturation degree.

Evaluation of the total antioxidant capacity (TAC) of the polyphenolic acids

In order to evaluate the radical-scavenging ability of the phenolic compounds under study, either of synthetic or natural origin (Figure 1), TAC assays (ABTS and DPPH) were used. These methods have the advantage of establishing an accurate ranking hierarchy of antioxidant activity since some factors which interfere in other model systems, such as metal chelation, partitioning abilities, etc. are absent. Classically, trolox, a water-soluble vitamin E analogue, is used as reference standard. The results are usually expressed as trolox equivalent antioxidant capacity (TEAC).

Figure 2 shows the TEAC values obtained for the polyphenols under study. From the results obtained in the ABTS assay, two sets of compounds can be recognized: one group containing the catechol moiety and another with the pyrogallol one. It is clear that trihydroxylated compounds have a higher antioxidant activity towards $ABTS^{\cdot+}$ than their dihydroxylated homologues. For each group, a correlation is evident between the activity measured and the type of spacer linking the aromatic ring to the carboxylic acid group. The presence of a methylene group ($-CH_2-$) (2 and 6) seems to enhance the radical scavenging properties against $ABTS^{\cdot+}$ for this type of compounds. These results are in agreement with those reported by Rice-Evans et al. when comparing 3,4-dihydroxyphenylacetic acid (2) to 3,4-dihydroxybenzoic acid (1) and 3-(3,4-dihydroxyphenyl)propenoic acid (4) [27]. This behaviour may be explained by the fact that the benzylic position plays an important role in the chemistry of radicals due to a high stabilization by resonance (Figure 3) [28]. This type of stabilization has been already proposed for similar antioxidant compounds in the literature [23,29]. For both series, the presence of a double bond (4 and 8) does not seem to influence the antioxidant capacity, in this type of assay when compared to their saturated homologues (3 and 7, respectively).

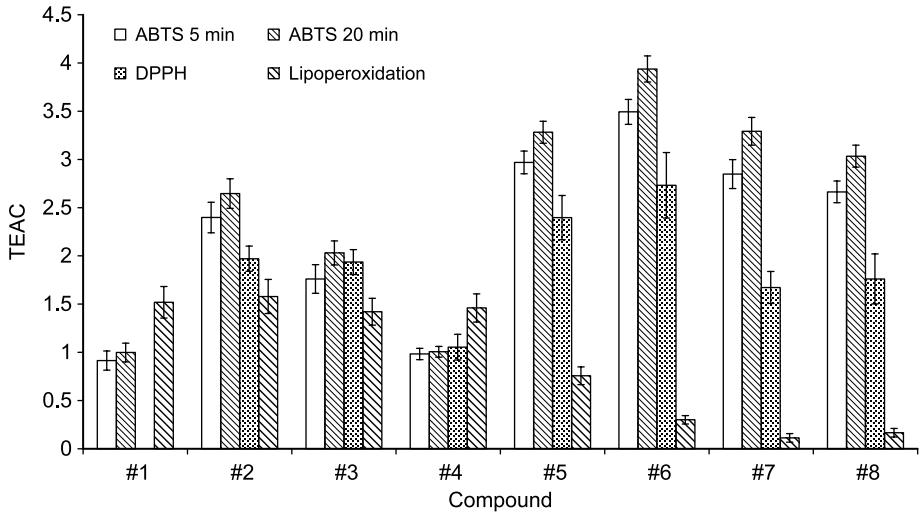

Figure 2. Graphical representation of the TEAC values obtained in the TAC and lipoperoxidation assays. The results represent the mean value of four assays ± standard error (SE). Compound 1 does not have a TEAC value associated to the DPPH assay because the IC_{50} was not reached for this compound after the considered reaction time.

The levels of $ABTS^{\cdot+}$ were recorded after 5 and 20 min of reaction, in order to determine the kinetics of each compound in the neutralization step of the radical (data not shown). It was found that the majority of the compounds tested is still reacting after the first five minutes of reaction. However, caffeic acid (4), 3,4-dihydroxybenzoic (1) acid and trolox (9) reached their maximum antioxidant capacity after 5 min.

The difference in antioxidant activity between dihydroxylated and trihydroxylated phenolic acids is also evidenced by the results obtained in the DPPH assay (Figure 2). It should be pointed out that 3-(3,4,5-trihydroxyphenyl)ethanoic acid (6) is still the best of the series. All the compounds were found to be radical scavengers in a dose-dependent manner.

For the sake of clarity, the entire set of results obtained was not represented graphically. Graphs can be, however, reproduced by following the text hereafter.

As the relation between the concentration of radical species and the concentration of compounds under study (response/dose) is not always a linear response, a polynomial regression model of the second degree was used to obtain a better fit of the experimental data, instead of a linear regression.

The coefficients of the polynomial regressions ($y = \boldsymbol{a}x^2 + \boldsymbol{b}x + 100$) obtained for ABTS and DPPH are presented in Tables I and II, respectively. From these values several observations can be made. (i) The bigger the **a** coefficient, the more the polynomial regression is breaking away from a linear function. In other words, when that breaking point is reached, the concentration of the radical (either $ABTS^{\cdot+}$ or $DPPH^{\cdot}$) is less influenced by the concentration of the antioxidant when compared to the effect at lower concentration of the compound under study. (ii) If a linear regression is considered, the **b** coefficient may be associated to its slope. Therefore, when **b** increases (in absolute value), the concentration of antioxidant needed to reach one particular level of radical concentration decreases. (iii) The difference between the value obtained for **b** after 5 min of reaction and the one measured after 20 min (see the case of ABTS, for example) reflects the kinetic of the reaction occurring between the antioxidant and the radical; the bigger the difference, the slower the reaction.

A hierarchical antioxidant potency order can be established for the phenolic systems investigated, according to the results obtained for ABTS (Figure 2): $6 > 5 \geq 7 > 8 > 2 > 3 > 4 \geq 1$. The results obtained for DPPH were not found to contradict this sequence.

It must be pointed out that in the two TAC assays trihydroxylated compounds showed a better antioxidant capacity than the dihydroxylated ones. The main structural difference between di- and trihydroxylated compounds is related to an additional –OH group on the position 5 of the aromatic ring.

Although, the correlation of the antioxidant properties of polyphenols to the number of hydroxyl groups

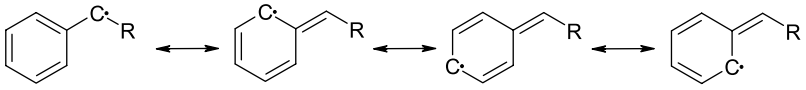

Figure 3. Schematic representation of the resonance stabilization of a radical in the benzylic position [28].

Table I. Coefficients of the polynomial regression obtained in the ABTS assay, after 5 and 20 min of reaction time. The results represent the mean value of eight assays ± standard error (SE).

ABTS compound	5 min		20 min	
	a	**b**	**a**	**b**
1	0.000 ± 0.045	−3.517 ± 0.311	0.070 ± 0.048	−4.833 ± 0.363
2	0.332 ± 0.058	−10.956 ± 0.867	0.426 ± 0.048	−12.316 ± 0.676
3	0.151 ± 0.003	−7.823 ± 0.511	0.235 ± 0.041	−9.465 ± 0.752
4	0.018 ± 0.042	−3.450 ± 0.396	0.032 ± 0.052	−4.310 ± 0.533
5	0.558 ± 0.072	−14.291 ± 0.978	0.626 ± 0.039	−15.241 ± 0.501
6	0.678 ± 0.052	−15.937 ± 0.676	0.768 ± 0.013	−17.194 ± 0.108
7	0.515 ± 0.083	−13.670 ± 1.146	0.622 ± 0.051	−15.177 ± 0.679
8	0.413 ± 0.021	−12.170 ± 0.313	0.535 ± 0.012	−13.947 ± 0.166
9	0.029 ± 0.007	−3.643 ± 0.301	0.025 ± 0.019	−4.260 ± 0.183

is often described in the literature another explanation of this property must be considered in the light of the latest works in the area. At least two mechanisms have been recognized for the radical scavenging activity of phenolic antioxidants: a direct hydrogen-transfer process (HAT) (Equation 1) and a proton-coupled electron-transfer process (SET) (Equation 2) [30–34]

$$R\cdot + ArOH \rightarrow RH + ArO\cdot \quad (1)$$

$$R\cdot + ArOH \rightarrow R^{-} + ArOH\cdot^{-} \rightarrow RH + ArO\cdot. \quad (2)$$

It is believed that the first pathway occurs in non-polar solvent and is characterized by the O–H bond dissociation enthalpy (BDE) while the second should happen in polar solvents (like ethanol) and is governed by the ionization potential (IP). Therefore, several studies have been performed to determine the BDE and IP of polyphenols and to correlate the values to antioxidant properties [31,32,35,36]. It has been proposed that a good antioxidant must have a low O–H BDE to facilitate the H-abstraction from a radical and that the radical generated by a HAT process must be stable [35]. Wright and co-workers analyzed the effects of the substituents of the aromatic ring on the parameters mentioned above [32]. They showed that a –OH group in *ortho* position to a phenol tends to lower the BDE value by stabilizing the radical formed thus enhancing the antioxidant capacity. Other parameters, referred in the literature as playing a role in the antioxidant capacity of polyphenols, are the intra-molecular hydrogen bonds that can exist either in the catechol or pyrogallol moieties (Figure 4) [32,35,37] and the inter-molecular hydrogen bonds between these functional groups with polar protic solvents (H_2O, CH_3OH, CH_3CH_2OH, ···) [38,39].

From the above statements, it can be concluded that a pyrogallol unity present in a compound could lead to be a better antioxidant activity than a catechol moiety mainly to the possibility of an extra stabilization, due to the presence of an additional hydroxyl group, as it was observed in the TAC results.

Effect of the polyphenolic acids on lipid peroxidation

Lipid peroxidation is an uncontrolled deleterious reaction that occurs in cellular and subcellular membranes, causing or enhancing the formation of lipid hydroperoxides. These species are cytotoxic and capable of reacting with numerous cellular components, providing one of the mechanisms underlying the toxicity of reactive species (ROS and RNS).

The use of model membranes such as unilamellar liposomes (e.g. of EPC) has lately been encouraged since it is helpful for understanding the effect of

Table II. Coefficients of the polynomial regression obtained in the DPPH assay, after 5, 15 and 60 min of reaction time. The results represent the mean value of eight assays ± standard error (SE).

DPPH Compound	5 min		15 min		60 min	
	a	**b**	**a**	**b**	**a**	**b**
1	0.053 ± 0.002	−1.368 ± 0.090	0.078 ± 0.014	−2.031 ± 0.268	0.180 ± 0.046	−4.726 ± 0.705
2	0.125 ± 0.007	−3.737 ± 0.488	0.173 ± 0.016	−6.087 ± 1.107	0.228 ± 0.012	−9.021 ± 0.176
3	0.106 ± 0.008	−3.120 ± 0.060	0.154 ± 0.001	−4.842 ± 0.418	0.228 ± 0.012	−8.918 ± 0.181
4	0.124 ± 0.016	−3.877 ± 0.272	0.132 ± 0.003	−4.761 ± 0.212	0.132 ± 0.026	−6.021 ± 0.239
5	0.186 ± 0.022	−5.033 ± 0.441	0.262 ± 0.040	−7.515 ± 0.723	0.403 ± 0.039	−11.361 ± 0.709
6	0.216 ± 0.015	−6.774 ± 0.251	0.257 ± 0.055	−9.040 ± 0.998	0.484 ± 0.095	−12.791 ± 1.326
7	0.181 ± 0.003	−6.349 ± 0.185	0.175 ± 0.001	−7.000 ± 0.251	0.237 ± 0.001	−8.265 ± 0.327
8	0.181 ± 0.021	−6.502 ± 0.644	0.211 ± 0.018	−7.423 ± 0.688	0.291 ± 0.024	−8.971 ± 0.794
9	0.072 ± 0.033	−4.193 ± 0.519	0.053 ± 0.025	−4.297 ± 0.511	0.088 ± 0.030	−5.004 ± 0.491

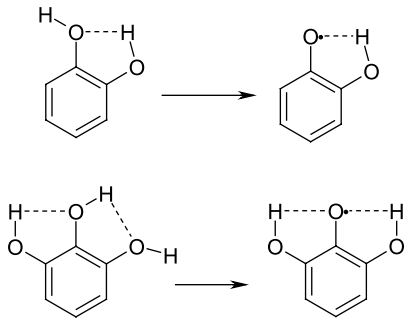

Figure 4. Intramolecular hydrogen bond in the catechol and pyrogallol moieties and radical stabilization.

antioxidants in phospholipid bilayers of membrane. The advantage of using this type of systems, including unilamellar vs multilamellar liposomes, and a water-soluble radical azo-generator (AAPH), is largely documented in the literature. In AAPH induced peroxidation of unilamellar liposomes, the chain-initiating radical is generated in the aqueous phase and the chain-propagating lipid peroxyl radicals are located within the membranes. Trolox and α-tocopherol are typical chain-breaking antioxidants used as standards.

The results obtained in the lipoperoxidation assays are shown in Figure 2. In this type of assay, an inverse ranking order was observed when compared to the results of the ABTS and DPPH assays. The dihydroxylated compounds presented a better anti-oxidant activity than the trihydroxylated ones. One explanation for this behaviour can lie on the reactivity difference towards the bulky AAPH radical species, as previously proposed by Arora et al. for flavonoids [15]. An identical sequence was obtained for flavonoids possessing a catechol vs pyrogallol moiety in their chemical structure [6,7].

The antioxidant effect of the phenolic acids is believed to take place in the aqueous phase, since the carboxylic acid, ionized at physiological pH, is hydrophilic [16]. This hypothesis is supported by the absence of data in the liposomes/buffer partition coefficient assay, as no changes in the spectra of the compounds upon an increase in the lipid concentrations was observed (data not shown). No clear interaction of the polyphenols with the liposomes was observed, which leads to the hypothesis that they may be present solely in the aqueous phase. This assumption is also supported by the results from the lipoperoxidation assay, using vitamin E (10) as antioxidant.

Vitamin E is the lipophilic equivalent of trolox (9) and presented a three-fold higher TEAC value (2.90 ± 0.24). As the ability of vitamin E to penetrate the lipidic bilayer is higher than for trolox, it was assumed that this behaviour is directly linked to Vitamin E better anti-peroxidation capacity. AAPH being a hydrophilic radical initiator, it was concluded that the antioxidant activity of the eight compounds studied should take place in the aqueous phase, as they do not display the lipophilic characteristics that would allow them to penetrate the lipidic bilayer.

Table III. Partition coefficients values of phenolic compounds determined by derivative spectrophometry in HDPC micelles.

Compound	log *P*	Compound	log *P*
1	3.10 ± 0.05	5	nd†
2	3.54 ± 0.02	6	2.67 ± 0.03
3	3.67 ± 0.02	7	nd†
4	2.64 ± 0.05	8	2.18 ± 0.63

† nd—not determined.

In addition, it may be assumed that, for the compounds tested, the phenolic groups are essential for their activity, the remaining part of the molecule having no active role in the antioxidant effect (1–4 vs 5–8 series). In fact, catecholic compounds were found to be better antioxidants against lipoperoxidation than pyrogallol ones, regardless of the other substituent groups present in these molecules. This observation may be related with the intrinsic physicochemical properties of the compounds, especially their lipophilicity (log *P*). Partition coefficients were determined by derivative spectrophotometry using micelles of HDPC. The results obtained are presented in Table III.

The results obtained show that there is a tendency of the dihydroxylated molecules to have higher partition coefficients than their trihydroxylated counterparts. This tendency and the values of logP (octanol/water) for the compounds 1 and 2 (1.15 and 0.70, respectively) found in the literature [40] support the hypothesis that dihydroxylated compounds are more lipophilic than the trihydroxylated ones and that they can then give a better protection against lipoperoxidation. Phenolic acids are more hydrophilic than α-tocopherol and, therefore, less effective than this official antioxidant. Nevertheless, prevention of the initial reaction between aqueous radicals and membrane phospholipids is very important since it plays an essential role in the antioxidant defence of biomembranes, which suffer a continuous attack by free radicals generated in the aqueous phase of cellular and subcellular fractions. Thus, radical scavenging by phenolic derivatives in the aqueous interface must be considered as an effective way of inhibiting peroxidation of membrane phospholipids.

Effect of the polyphenolic acids on the oxidation of 2′-deoxyguanosine

2′-dG oxidation assays in the absence of ascorbic acid were conducted in the present work, in order to evaluate the pro-oxidant capacity of the polyphenols under study. From the results obtained, shown in

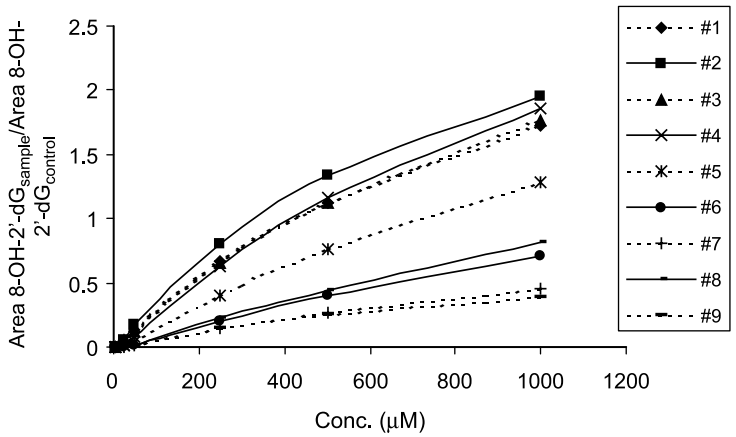

Figure 5. Pro-oxidant behaviour of the compounds under study in the oxidation of 2′-dG by a Fenton reaction, in the absence of ascorbic acid.

Figure 5, it was concluded that all the compounds were able to interact with the system, generating ferrous ions, needed for the Fenton reaction. In this experiment, compounds displaying an *ortho*-dihydroxyl moiety were found to possess a higher pro-oxidant activity than the pyrogallol ones, as evidenced by the 8-OH-2′-dG$_{sample}$/8-OH-2′-dG$_{control}$ levels detected. A pro-oxidant effect was to be expected whenever transition metals were involved in the production of the deleterious radicals, due to the probable chelating and reducing capacities of the catechol group present in this type of polyphenols. It thus seems that the catechol structure plays an important role in the reductive capacity of the polyphenols towards the ferrous state of iron, as previously proposed by Mira et al. [41] for flavonoids. The fact that catechol can complex transition metal ions seems also to be of paramount importance. The lower reactivity of the trihydroxylated phenolic acids can be explained either by their redox potential values and/or by the fact that the pyrogallol system possesses three hydroxyl groups, two of them chelating one iron atom, and the third being left free to contribute for the antioxidant effect.

When ascorbic acid is present in the reaction medium, the polyphenolic compounds do not show the expected antioxidant effect, except for trolox. Higher levels of 8-OH-2′-dG than the control reaction are still reached, evidencing that the pro-oxidant stage is not yet overcome (Figure 6). Under these conditions, no antioxidant capacity was detected for the range of concentration tested.

Conclusions

The trihydroxylated phenols showed a better antioxidant activity against $ABTS^{\cdot+}$-type radicals. A similar structure–activity trend, although not as noticeable, was verified from the results obtained with the DPPH method. For the series of phenolic acids studied, it was concluded that the type of spacer between the carboxylic acid and the aromatic ring markedly influences their antioxidant profile: the highest activity was achieved by introducing

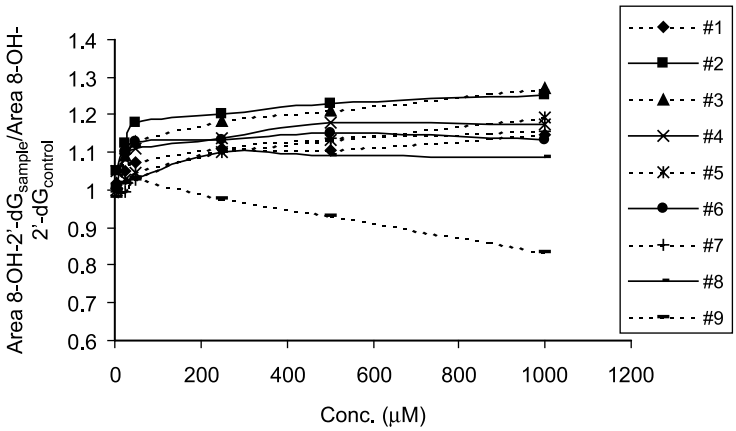

Figure 6. Antioxidant behaviour of the compounds under study in the oxidation of 2′-dG by a Fenton reaction, in the presence of ascorbic acid.

a methylenic ($-CH_2-$) spacer group, followed by an ethylenic ($-CH_2CH_2-$) one and an unsaturated chain ($-CH=CH-$).

In the lipoperoxidation assay an inverse ranking order was observed: the *ortho*-dihydroxylated compounds presented a higher antioxidant activity than the trihydroxylated ones apparently due to their higher lipophilicity. The results obtained for the peroxidation studies are in agreement with the statement of Zhang and co-authors, which propose solubility as key factor of an enhanced antioxidant potency for phenolic compounds, a parameter that improves the mobility of the antioxidant between membranes [35]. In these experimental conditions, dihydroxyphenolic acids were found to act as unique antioxidants in phospholipids bilayers. Their radical-scavenging activity is much lower than that of α-tocopherol, a major lipophilic chain-breaking antioxidant in biomembranes. The hydrophilic properties of these phenolic acids might facilitate their localization at the interface of the bilayers and thereby an effective inhibition of the initial attack by aqueous radicals is expected. The presence of a catechol vs a pyrogallol moiety seems to be determinant of the antioxidant activity, regardless of the presence of other chemical substitutions in the compounds considered in this study. The extra hydroxyl group present showed to be the key feature in the difference of antioxidant properties observed between di- and trihydroxylated compounds. Phenolic acids can then act as chain-breaking antioxidants by scavenging chain-propagating peroxyl radicals, since they possess phenolic hydrogens responsible for the peroxyl radical-scavenging activity.

A pro-oxidant effect can also be expected when transition metals are involved in the production of the deleterious radicals, due to the probable chelating and reducing capacities of the phenolic groups of this type of polyphenols. Catechol and pyrogallol moieties, responsible for their pro-oxidative effect, seems to mediate Fe(III)/Fe(II) redox cycle playing a principal role in the generation of reactive species, which lead to site-specific oxidative injuries in DNA. It should be noted that this effect was also found for ascorbic acid, an hydrophilic antioxidant and effective reducing agent of the Fe^{3+}-EDTA complex, as well as for other antioxidants, and does not reflect a potential DNA-damaging activity *in vivo*.

The present results are in agreement with the reported antioxidant and pro-oxidant profile of phenolic compounds, allowing to understand the structural basis of their chain-breaking properties, specifically aiming at elucidating the effect of chemical substitution on these properties. The gathered data corroborates the results reported for similar systems [2,3,9,10,19], allowing concluding that the number of phenolic groups and the type of spacer strongly influence the antioxidant activity. It is important to notice that phenolic acids and their analogues, varying considerably in their backbone structures and type of functional groups, have showed to be rather promising anticancer agents, due to their antioxidant/pro-oxidant activity.

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References

[1] Madhavi DL, Desphande SS, Salunkhe DK, editors. Food antioxidants—technological, toxicological and health perspectives. New York: Marcel Dekker Inc. 1996.

[2] Silva FAM, Borges F, Ferreira MA. Effects of phenolic propyl esters on the oxidative stability of refined sunflower oil. J Agric Food Chem 2001;49:3936–3941.

[3] Silva FAM, Borges F, Guimarães C, Lima JLFC, Matos C, Reis S. Phenolic acids and derivatives: Studies on the relationship among structure, radical scavenging activity, and physicochemical parameters. J Agric Food Chem 2000;48:2122–2126.

[4] Son S, Lewis B. Free radical scavenging and antioxidative activity of caffeic amide and ester analogues: Structure–activity relationship. J Agric Food Chem 2002;50:468–472.

[5] Rajan P, Vedernikova I, Cos P, Berghe DV, Augustyns K, Haemers A. Synthesis and evaluation of caffeic acid amides as antioxidants. Bioorganic & Medicinal Chemistry Letters 2001;11:215–217.

[6] Bors W, Heller W, Michel C, Stettmaier K. Flavonoids and polyphenols: Chemistry and biology. New York: Marcel Dekker Inc. 1996.

[7] Rice-Evans C. Screening of phenolics and flavonoids for antioxidant activity. In: Packer L, Hiramatsu M, Yoshikawa T, editors. Antioxidants food supplements in human health. London: Acadmic Press; 1999. p 239–253.

[8] Chung WY, Jung YJ, Surh YJ, Lee SS, Park KK. Antioxidative and antitumor promoting effect of [6]-paradol and its homolog. Mutat Res 2001;496:199–206.

[9] Gomes CA, Cruz TGd, Andrade JL, Milhazes N, Borges F, Marques MPM. Anticancer activity of phenolic acids of natural or synthetic origin: A structure–activity study. J Med Chem 2003;46:5395–5401.

[10] Fiuza SM, Gomes C, Teixeira LJ, Cruz MTGd, Cordeiro MNDS, Milhazes N, Borges F, Marques MPM. Phenolic acid derivatives with potential anticancer properties—A structure–activity relationship study. Part 1: Methyl, propyl and octyl esters of caffeic and gallic acids. Bioorg Med Chem 2004;12:3581–3589.

[11] Surh YJ. Molecular mechanisms of chemopreventive effects of selected dietary and medicinal phenolic substances. Mutat Res 1999;428:305–327.

[12] Galati G, O'Brien PJ. Potential toxicity of flavonoids and other dietary phenolics: Significance for their chemopreventive and anticancer properties. Free Radic Biol Med 2004;37:287–303.

[13] Silva AMS, Santos CMM, Cavaleiro JAS, Tavares HR, Borges F, Silva FAM. NMR studies on the antiradicalar mechanism of phenolic compouds towards 2,2-diphenyl-1-picrylhydrazyl radical. In: Webb GA, Belton PS, Gil AM, Delgadillo I, editors. Magnetic resonance in food science—a view to the future. 8th ed. London: Royal Society Chemistry; 2001. p 110–116.

[14] Halliwell B, Gutteridge JMC. Free radicals in biology and medicine. Oxford: Oxford University Press; 1999.
[15] Arora A, Nair MG, Strasburg GM. Stucture–activity relationships for antioxidant activities of a series of flavonoids in a liposomal system. Free Radic Biol Med 1998; 24:1355–1363.
[16] Niki E. Free radical initiators as source of water- or lipid-soluble peroxyl radicals. Method Enzymol 1990;186:100–108, Academic Press Inc..
[17] Kitamura K, Imayoshi N, Goto T, Shiro H, Mano T, Nakai Y. Second derivative spectrophotometric determination of partition coefficients of chlorpromazine and promazine between lecithin bilayer vesicles and water. Anal Chim Acta 1995;304:101–106.
[18] Ferreira H, Lúcio M, Castro Bd, Gameiro P, Lima JLFC, Reis S. Partition and location of nimesulide in EPC liposomes: A spectrophotometric and fluorescence study. Anal Bioanal Chem 2003;377:293–298.
[19] Fiuza SM, Besien EV, Milhazes N, Borges F, Marques MPM. Conformational analysis of a trihydroxylated derivative of cinnamic acid—A combined Raman spectroscopy and *Ab initio* study. J Mol Struct 2004;693:103–118.
[20] Miller NJ, Rice-Evans CA. Factors influencing the antioxidant activity determined by the $ABTS^{+}$ radical cation assay. Free Radic Res 1997;26:195–199.
[21] Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic Biol Med 1999;26:1231–1237.
[22] Cos P, Rajan P, Vedernikova I, Calomme M, Pieters L, Vlietninck AJ, Augustyns K, Haemers A, Berghe DV. *In vitro* antioxidant profile of phenolic acid derivatives. Free Radic Res 2002;36:711–716.
[23] Brand-Williams W, Cuvelier ME, Berset C. Use of free radical method to evaluate antioxidant activity. Food Sci Technol—LEB 1995;28:25–30.
[24] Teixeira S, Siquet C, Alves C, Boal I, Marques MP, Borges F, Lima JLFC, Reis S. Structure-property studies on the antioxidant activity of flavonoids present in diet. Free Radic Biol Med 2005;39:1099–1108.
[25] Yen G-C, Chen H-Y, Peng H-H. Antioxidant and pro-oxidant effects of various tea extracts. J Agric Food Chem 1997;45:30–34.
[26] Hsieh C-L, Yen G-C. Antioxidant actions of Du-Zhong (*Eucommia ulmoids* oliv.) towards oxidative damage in biomolecules. Life Sci 2000;66:1387–1400.
[27] Rice-Evans CA, Miller NJ, Paganga G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radic Biol Med 1996;20:933–956.
[28] McMurry J. Organic chemistry. Belmont: Brooks/Cole; 1992.
[29] Bondet V, Brand-Williams W, Berset C. Kinetics and mechanisms of antioxidant activity using the DPPH free radical method. Food Sci Technol—LEB 1997;30:609–615.
[30] Foti MC, Daquino C, Geraci C. Electron-transfer reaction of cinnamic acids and their methyl esters with the DPPH radical in alcoholic solutions. J Org Chem 2004;69:2309–2314.
[31] Giacomelli C, Miranda FdS, Gonçalves NS, Spinelli A. Antioxidant acitivity of phenolic and related compounds: A density functional theory study on the O–H bond dissociation enthalpy. Redox Rep 2004;9:263–269.
[32] Wright JS, Johnson ER, DiLabio GA. Predicting the activity of phenolic antioxidants: Theoretical method, analysis of substituent effects, and application to major families of antioxidants. J Am Chem Soc 2001;123:1173–1183.
[33] Zhang H-Y, Sun Y-M, Wang X-L. Electronic effects on O-H proton dissociation enerigies of phenolic cation radicals: A DFT study. J Org Chem 2002;67:2709–2712.
[34] Kong L, Sun Z-L, Zhang H-Y, Yao S-D. Theoretical elucidation of the radical-scavenging-activity difference of hydroxycinnamic acid derivatives. Helv Chim Acta 2004;87:511–515.
[35] Zhang H-Y, Sun Y-M, Wang X-L. Substituent effects on O–H bond dissociation enthalpies and ionization potentials of catechols: A DFT study and its implications in the rational design of phenolic antioxidants and elucidation of structure–activity relationships for flavonoid antioxidants. Chem-Eur J 2003;9:502–508.
[36] Chandra AK, Uchimaru T. The O–H bond dissociation energies of substituted phenols and proton affinities of substituted phenoxide ions: A DFT study. Int J Mol Sci 2002;3:407–422.
[37] Nenadis N, Zhang H-Y, Tsimidou MZ. Structure-antioxidant activity relationship of ferulic acid derivatives: Effect of carbon side chain characteristic groups. J Agric Food Chem 2003;51:1874–1879.
[38] Litwinienko G, Ingold KU. Abnormal solvent effects on hydrogen atom abstractions. 1. The reactions of phenols with 2,2-diphenyl-1-picrylhydrazyl (dpph.) in alcohols. J Org Chem 2003;68:3433–3438.
[39] Singh N, O'Malley PJ, Popelier LA. Mechanistic aspects of hydrogen abstraction for phenolic antioxidants. Electronic structure and topological electron density analysis. Phys Chem Chem Phys 2005;7:614–619.
[40] Hansch C, Leo A, Hoekman D. Exploring QSAR hydrophobic, electronic, and steric constants. Washington, DC: American Chemical Society; 1995.
[41] Mira L, Fernandez MT, Santos M, Rocha R, Florêncio MH, Jennings KR. Interactions of flavonoids with iron and copper ions: A mechanism for their antioxidant activity. Free Radic Res 2002;36:1199–1208.