Full Paper

Antioxidant Versus Cytotoxic Properties of Hydroxycinnamic Acid Derivatives – A New Paradigm in Phenolic Research

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Trihydroxycinnamic derivatives were synthesized and evaluated for their antioxidant and cytotoxic activities. The ester derivatives exhibited a higher radical-scavenging activity, when liposomes were used as target systems, a fact which may be related to their lipophilicity and conformational preferences. These compounds were found to display significant growth inhibition and cytotoxic effects towards a human cervix adenocarcinoma cell line (HeLa). The partition coefficients presently obtained for the trihydroxycinnamic derivatives correlate well both with their structural characteristics and with their antioxidant/cytotoxic activities. A positive structure-activity-property relationship between cytotoxic and antioxidant activities, which is intrinsically related with physico-chemical and conformational properties, is anticipated, as a noteworthy study that must be done for phenolic systems. As damage events are frequently correlated with oxidative stress, the prevalence of both properties in a single compound could be beneficial in terms of rationale preventive or therapeutic purposes.

Keywords: *Ab-initio* calculations / Antioxidant activity / Cytotoxic activity / Hydroxycinnamic acid derivatives / Lipophilicity

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Introduction

Polyphenols, bioactive substances commonly found in plants, are reducing agents supposed to be involved in the defense against oxidative damage due to their antioxidant and radical-scavenging characteristics [1 – 5]. Among polyphenolic compounds, hydroxycinnamic acids (*e.g.* ferulic and caffeic acids) are a well-known group of natural compounds, which are present in the human diet in representative amounts. Apart from being widely used as food additives [6, 7], some phenolic derivatives have been found to act as inhibitors of deleterious oxidative processes – *e.g.* in the prevention of cardiovascular and inflammatory diseases and cancer [8 – 15]. In fact, several phenolic compounds have been investigated as to their possible use as chemopreventive agents [16 – 18]. Cinnamic acid esters, in particular, were shown to display remarkable growth-inhibition properties towards some human cancer cell lines [17 – 20]. Nevertheless, the mechanisms underlying the protective action of phenolic compounds towards degenerative pathologies are not yet completely understood, although numerous

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Abbreviations: structure-property-activity relationships (SPAR); total antioxidant capacity (TAC); 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS); 2,2′-diphenyl-1-picrylhydrazyl (DPPH); trolox equivalent antioxidant capacity (TEAC); egg phosphatidylcholine (EPC); 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH); oxygen radical absorbance capacity (ORAC); diphenylhexatriene propionic acid (DPH-PA); large unilamellar vesicles (LUVs)

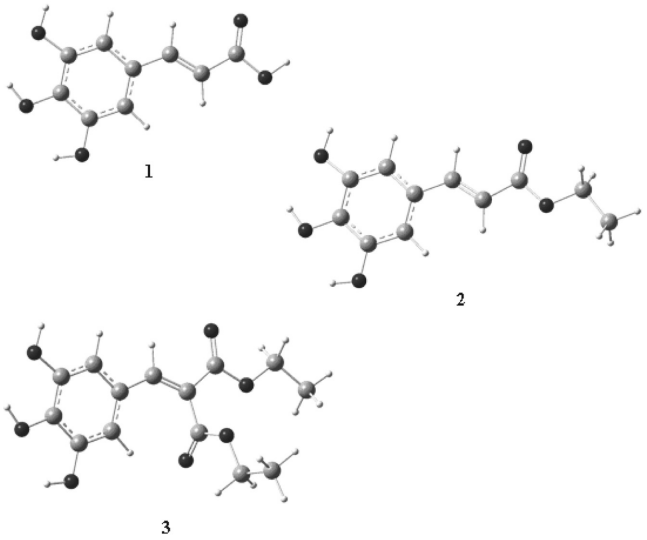


Figure 1. Schematic representation of the most stable geometries calculated for the phenolic compounds under study (at the B3LYP/6-31G** level): *trans*-3-(3,4,5-trihydroxyphenyl)-2-propenoic acid **1**, *trans*-ethyl-3-(3,4,5-trihydroxy-phenyl)-2-propenoate **2**, and diethyl 2-(3,4,5-trihydroxyphenylmethylene)malonate **3**.

evidence indicates that they are intrinsically linked to their antioxidant activity and strongly dependent on their structural characteristics [21 – 24]. Moreover, the biological activity of this type of compounds is thought to be determined by their rate of incorporation into the cell, which is directly related to their lipophilicity (also greatly influenced by their chemical and conformational preferences). In the last few years, this subject has become an emergent topic of research [25, 26], both in the field of medicinal and food sciences, in view of developing new and more effective phenolic agents suitable for both chemopreventive and therapeutic purposes (*e.g.* against neoplastic pathologies), or to be used as antioxidant additives (in food products).

Accordingly, an interactive project is being developed aiming at attaining a more reliable understanding of the structure-property-activity relationships (SPAR) underlying the biological function, particularly antioxidant and anticancer, of several phenolic systems, either of natural or synthetic origin, in order to acquire a better insight into their mechanisms of action. On the other hand, the co-administration of two compounds acting by different mechanisms may have a synergistic effect, resulting in a higher activity without raising the dose required to obtain the same effect with a single compound [27, 28]. Likewise, an agent that possesses more than one mechanism of action may have a net therapeutic effect greater than the sum of the effects due to the individual components.

In the present study, the antioxidant properties of the hydroxycinnamic acid derivative *trans*-3-(3,4,5-trihydroxyphenyl)-2-propenoic acid **1** and its esters *trans*-ethyl-3-(3,4,5-trihydroxyphenyl)-2-propenoate **2** and diethyl 2-(3,4,5-trihydroxyphenylmethylene)malonate **3** (Fig. 1) were evaluated, using total antioxidant capacity (TAC) and lipoperoxidation assays. Their cytotoxic activities were determined, in both cancer (cervix adenocarcinoma, HeLa) and non-neoplastic (skin fibroblasts, BJ) human cell lines. The partition coefficients (log P) of these compounds were also measured, in a liposome/buffer system. Furthermore, theoretical methods (*ab-initio* molecular orbital calculations) were used for the determination of the conformational preferences of the compounds under study [29], which are essential for a thorough interpretation of the biological results presently obtained, as well as for attaining reliable structure-activity or structure-property-activity relationships for this type of systems.

Results and discussion

Conformational analysis

The most stable geometries, relative energies, and populations at room temperature for the phenolic acid and ester derivatives under study were obtained by *ab-initio* molecular orbital calculations, at the DFT (density functional theory) level. Several structural parameters were varied, in order to determine their effect on the overall stability of these systems: i) orientation of the ethyl substituent relative to the aromatic ring; ii) orientation of the ring and the carbonyl group relative to the carbon-chain C=C bond, defining *trans* or *cis* isomers; iii) orientation of the ethyl moiety relative to the carbonyl, defining s-*cis* or s-*trans* conformations; iv) localization of the phenolic groups relative to the plane of the ring (either in-plane or out-of-plane).

For the three compounds investigated, a planar or quasi-planar geometry was found to be favored, due to the stabilizing effect of the π-electron delocalization between the coplanar aromatic ring and carbon chain C=C bond (Fig. 1). Regarding the orientation of the ring hydroxyl groups, it was verified that an identical position of the three OH's, coplanar with the ring, yield the most stable structures, since it allows a minimization of the steric repulsions between adjacent OH's and the formation of medium strength intramolecular O … H bonds ($d_{O\ldots H}$ ca. 218 pm). The data obtained are in accordance with that previously acquired for similar phenolic systems [30, 31].

This type of information is widely recognized to be important for a better understanding of the antioxidant activity, on a molecular basis, of phenolic compounds as

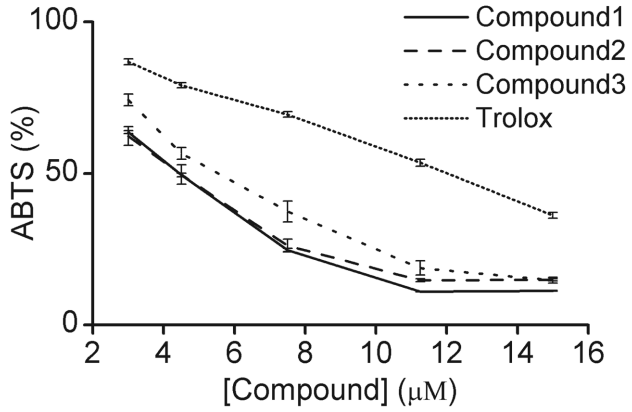


Figure 2. Dose/response curves for the scavenging of $ABTS^{+\bullet}$ by the phenolic compounds under study, after 20 min of reaction.

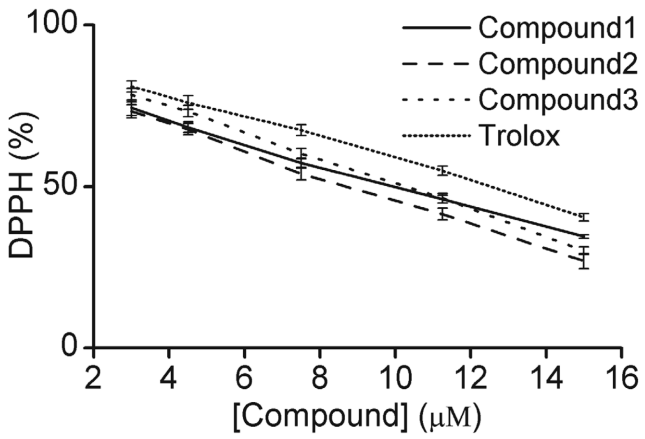


Figure 3. Dose/response curves for the scavenging of $DPPH^{\bullet}$ by the phenolic compounds under study, after 60 min of reaction.

electron or H-donating agents, since it can be related to the stabilization of the phenoxyl radical intermediates formed *in situ* [32]. Moreover, the knowledge of the true spatial arrangement of this kind of compounds is relevant for interpreting the biological data, mainly the interaction of these molecules with particular receptors and/or other types of targets (*e.g.* lipids, DNA, carbohydrates, or proteins).

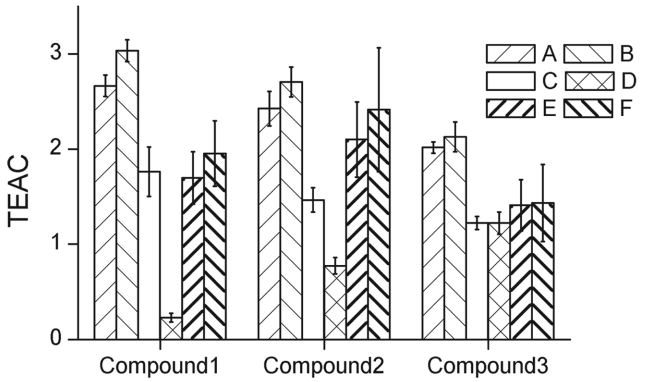
Antioxidant activity

Total antioxidant capacity assays

Total antioxidant capacity (TAC) assays were used in order to determine the hierarchy of radical scavenging abilities of the phenolic acid and its ester derivatives, by measuring their ability to scavenge 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic ($ABTS^{+\bullet}$) or 2,2′-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) radicals. These methods have the advantage of establishing an accurate ranking hierarchy of antioxidant activity of electron- or H-donating agents, since they are not affected by some factors which interfere in other model systems, such as metal chelation or partitioning abilities [21–23].

In addition, the effect of the phenolic compounds on the inhibition of lipid peroxidation in liposomes was also evaluated, using different and complementary methodologies. Trolox was introduced in all assays as a reference antioxidant, in order to get reliable data as trolox equivalent antioxidant capacity (TEAC) values.

Figures 2 and 3 display the results obtained in the TAC assays using $ABTS^{+\bullet}$ [33, 34] and $DPPH^{\bullet}$ [33, 35] radicals, after 20 and 60 min of reaction, respectively. The results depicted in Fig. 2 evidence noticeable antioxidant behaviors for the compounds under study, when compared to trolox. According to previous statements, this may be explained by the presence of hydroxyl groups in their



A: ABTS, after 5 min.; B: ABTS, after 20 min.; C: DPPH; D: lipoperoxidation, with DPH-PA as probe; E: peroxidation, with fluorescein as probe; F: lipoperoxidation, with fluorescein as probe.

Figure 4. TEAC values for the phenolic compounds under study, from TAC and peroxidation assays.

structures, which are intrinsically related with their antiradical properties [17, 18, 21]. It is noteworthy that an equilibrium was almost reached after 5 min (Fig. 4), rendering those compounds as fast as trolox as $ABTS^{+\bullet}$ scavengers. The DPPH assay yielded a similar antiradical tendency for the phenolic compounds studied (Fig. 3), although the difference in antioxidant activity between them was not so evident. The distinct chemical structure and reactivity of the radicals used in the two experimental evaluations might explain these discrepancies.

Lipoperoxidation assays

As lipid peroxidation is likely to be involved in the development of certain diseases such as cancer, atherosclerosis, and neuron impairment, evaluation of the antioxidant behavior of the compounds towards this specific tar-

get was carried out [36]. EPC (egg phosphatidylcholine) unilamellar liposomes were used as biomimetic membrane models [37], due to their similar structural characteristics. The advantage of using this type of systems, such as unilamellar *versus* multilamellar liposomes and a water-soluble radical azo-generator (2,2′-azobis(2-amidinopropane) dihydrochloride; AAPH), is largely documented in the literature [38]. 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.

One well-liked model for evaluation of the antioxidant efficiency towards the inhibition of lipid peroxidation is the oxygen radical absorbance capacity (ORAC) method. This assay was originally performed by measuring the fluorescence decay of β-phycoerythrin (β-PE) after AAPH oxidation [39]. However, Ou and co-workers [40] developed an improved ORAC assay in which β-PE is replaced by fluorescein, since the former has some drawbacks due to its photo(in)stability and the possibility of its interaction with the compounds under analysis. The method was also adapted to be employed with liposome systems and applied for 96-well microplate fluorescence readers [41 – 43], enabling the analysis of a broader range of compounds such as lipophilic antioxidants. In turn, as fluorescein, due to its hydrophilic properties, only allows evaluating the radical-scavenging activity of compounds in an aqueous phase, a different method was exploited using a lipophilic fluorescent probe, DPH-PA (diphenylhexatriene propionic acid), and the same peroxyl radical initiator [44].

In summary, protection against lipoperoxidation was presently evaluated using AAPH as a peroxyl radical azo-initiator [44] and two radical-sensitive fluorescent probes, one hydrophilic and one lipophilic (fluorescein and DPH-PA, respectively). The scavenging activity towards peroxyl radical was also assessed in a buffer solution, with the same azo-initiator and fluorescein. The results obtained in these three assays were expressed as TEAC values (Fig. 4).

The data gathered from these lipoperoxidation assays evidence that the ester derivatives **2** and **3** display an enhanced antioxidant activity when compared to their parent acid **1** (Fig. 4). Esterification of **1** seems to increase the activity of the acid when heterogeneous lipidic systems (liposomes) were used. Thus, it was believed that the capacity to scavenge peroxyl radicals can be intrinsically related to the lipophilicity difference between the compounds tested, a premise that was validated by the partition coefficient values of the compounds (see below; Section 2.3).

The protective effects of the phenolics under study on biomimetic membranes were mainly associated to the hydrophilicity/hydrophobicity of the compounds, since they possess the same number of hydroxyl groups and an identical aromatic pattern. The scavenging-activity results obtained in a buffer medium and in liposomes allow to conclude that this type of compounds act at the membrane surface. In addition, the antioxidant action of **3** may be related to the change in membrane fluidity, thus affecting the lipid ordering at a different depth in the bilayer (data not shown), as its activity is higher in the DPH-PA assay. Furthermore, the chemical structure of the phenolic compounds investigated has an intrinsic relationship with the membranes: their polar groups interact at the lipid-water interface with the polar head groups of the membrane phospholipids. This fact plays an important role on phenolic distribution in biological systems and consequently determines its local concentration, which then influences their capacity to regulate cellular events. Also, this kind of orientation on the membrane surface can affect the access of oxidants to the bilayer and/or control the rate of propagation of the free radical chain reactions occurring at the hydrophobic core of membranes, thus contributing to preserve the structure and function of biological membranes [45]. The results presently reported support the hypothesis that, besides the number of hydroxyl groups and the intramolecular hydrogen-bond formation, the exhibited activity is intrinsically related with the polar head group of lipids and the spatial arrangement of the compounds (see conformational analysis data).

Partition coefficients

The partition coefficient values (log P) were determined for the compounds studied in a liposome/buffer system (pH = 7.4), according to the method of Kitamura *et al*. [46], in order to correlate the results obtained in the heterogeneous systems (liposomes and cells) to the lipophilicity of the phenols. The log P values for **2** and **3** were 3.48 ± 0.02 and 3.12 ± 0,10, respectively (Figs 5A and 5B). As the phenolic acid **1** did not show any partition in these experimental conditions, its log P was determined by derivative spectrophotometry using micelles of 1-hexadecylphosphoryl-choline (HDPC) (2.18 ± 0,63 [47]). The phenolic acid is ionized at physiological pH and its antioxidant effect is believed to take place in the aqueous phase [47]. The ability of the phenolic ester **2** to interact with the membrane polar head groups, increasing their local concentration at the water-lipid interface of membranes, may be one factor leading to a higher antioxidant capacity.

Prevention of the initial reaction between aqueous radicals and membrane phospholipids is very important

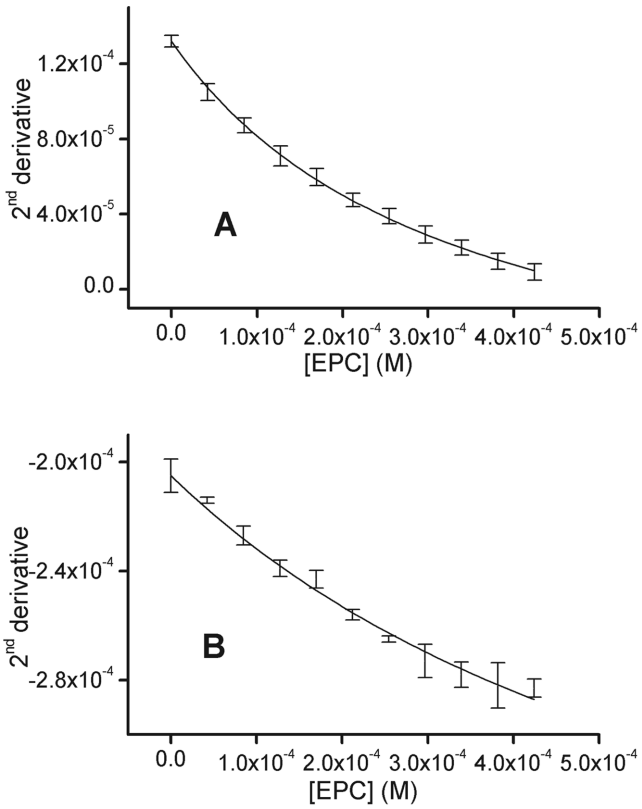


Figure 5A and 5B. Best fit of the second derivative at 350 nm, for the phenolic esters **2** (A) and **3** (B), for different concentrations of EPC.

since it plays an essential role in the antioxidant defense of biomembranes, which suffer a continuous attack by free radicals generated in the aqueous phase of cellular and subcellular fractions.

Antiproliferative and cytotoxic activity

The antineoplastic properties of the phenolic compounds investigated were evaluated in a human cervix adenocarcinoma cell line – HeLa (epithelial-like adherent line). In order to determine their degree of toxicity towards healthy cells, experiments were also performed in fibroblasts from human skin (BJ). All the results reported were gathered by two independent methods, which were found to be in very good agreement: cell density measurement (Trypan blue exclusion method) and cell viability evaluation (Alamar colorimetric assay). In all experiments, cisplatin (*cis*-diaminedichloroplatinum(II), CDDP), **4** was used for comparison purposes, since it is one of the most widely used chemotherapeutic drugs in clinical practice.

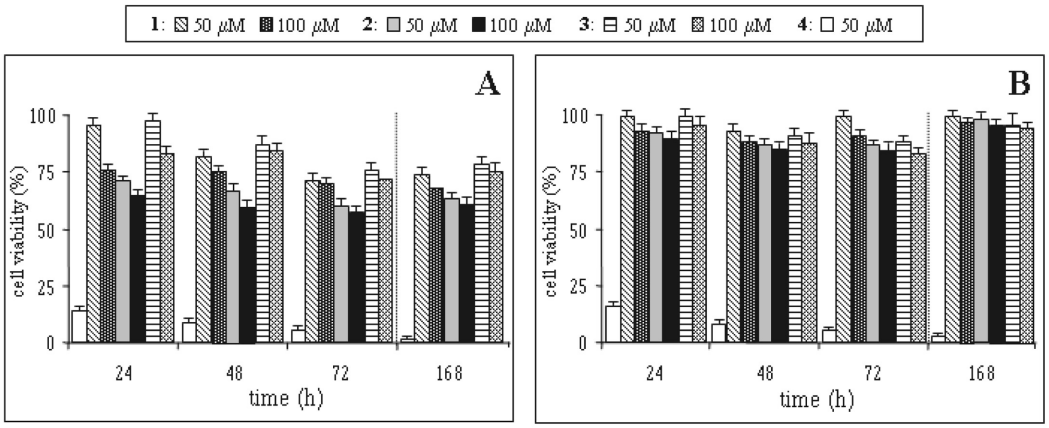
Figure 6 represents the cytotoxic effect of the phenolic compounds tested against the HeLa and BJ cell lines – cell viability variation as a function of the incubation time with the drug, for different drug concentrations (between 25 and 100 μM). From these time- and dose-response plots, it is possible to relate the structural characteristics of the compounds to their antiproliferative activity, thus learning on factors such as specificity of action and reversibility of the drug effect. The linear ester **2** showed to have the highest growth-inhibition and cytotoxic effects towards the HeLa cell line, followed by its acid **1** and diester **3** counterparts. This antineoplastic activity was determined as being of significance only after 72 hours of incubation with the drug, and it did not change much upon removal of the phenolic agents, thus evidencing a strong irreversibility of their effect (Fig. 6). In fibroblasts, in turn, none of the compounds tested displayed a significant toxicity (even after eight days of incubation). Moreover, upon removal of the compounds, the cell viability was found to reach ca. 100% (after 4 days in the absence of drug, Fig. 6) for all three phenols. As to the effect of drug concentration, the results clearly evidence that values above 50 μM should be used in order to get a significant growth-inhibition effect (up to ca. 50% viability decrease, for the monoester **2**, Fig. 6).

Overall, considering the effect on both HeLa cancer cells and fibroblasts, the ethyl ester **2** proved to be the most efficient cytotoxic agent as compared to the parent acid and diester analogue. This is suggested to be due to the more efficient transport mechanism of the linear ester into the cell, as compared to the charged molecule of the acid **1** and the bulkier branched diester molecule **3**. The results presently obtained for these hydroxycinnamic acid derivatives are in accordance with those previously reported for both caffeic and gallic esters [17, 18].

Conclusions

The results gathered from the studies developed in this area allow concluding that the antitumor activity of phenolic derivatives is highly dependent upon their conformational characteristics, which, in turn, determine their antioxidant properties. Moreover, researchers are placing increasing emphasis on identifying the biological mechanisms underlying the antiproliferative/cytotoxic activity of phenols [24]. In particular, it was shown that they can exert an effect on signal-transduction pathways, separately or sequentially, and that possible crosstalk between these pathways cannot be overlooked. Potential mechanisms of this type of compounds can be related with their protection against oxidative damage, either through their cytotoxic effect against malignant cells or *via* a pure scavenging-radical activity [24].

The present study on *trans*-3-(3,4,5-trihydroxyphenyl)-2-propenoic acid **1** and its esters *trans*-ethyl-3-(3,4,5-trihy-



Cells (5×10^5 cells/mL) were incubated with the drugs for periods of 24 to 72 h. Every 24 h, aliquots of the cell suspensions were removed and cell viability was evaluated by the Alamar blue colorimetric assay (as described in the Experimental, Section). In addition, the drug was removed 72 h after seeding and the cell viability was assessed following a further incubation of 96 h. The data are expressed as a percentage of the control Alamar reduction (100%) and represent the average ± mean standard deviation from two independent experiments carried out in triplicate. * Intergroup comparison: $p < 0.05$. (Values for cisplatin (CDDP, 4) are included for comparison).

Figure 6. Time-dependence of the cytotoxic effect of phenolic compounds **1**, **2**, **3** against HeLa (A) and BJ (B) cell lines.

droxyphenyl)-2-propenoate **2** and diethyl 2-(3,4,5-trihydroxyphenylmethylene)malonate **3** allowed to verify that the esterification of hydroxycinnamic precursors significantly improves their antioxidant capacity against lipoperoxidation, as well as their cytotoxic activity. The antioxidant activity towards peroxyl radicals exhibited by the esters may explain their capability to reduce carcinogenesis through cell protection against oxidative damage. In fact, the linear ester **2** was found to be the most efficient cytotoxic agent towards human adenocarcinoma, as compared to its parent acid and diester analogues. It is noteworthy that none of the compounds evaluated displayed a significant toxic effect against healthy cells. These results agree well with the lipophilic properties of the polyphenols, which are known to be closely related to their bioavailability at the biological target, thus ruling their cytotoxic activity.

The theoretical calculations carried out for these hydroxycinnamic compounds yielded their most stable structures, which are responsible for the biological activities presently evaluated. This information is essential for an accurate interpretation of these experimental results – lipophilicity, antioxidant, and growth-inhibition properties – as well as for achieving reliable structure-property-activity relationships (SPAR's), capable of explaining the biological role of this kind of systems.

Although the antioxidant and/or the anticancer activity of phenolic acids and derivatives has been evaluated in different model systems, few studies have been performed to this date in order to get an insight into the SPAR's underlying these functions. Moreover, physicochemical data obtained for these systems is important to enlighten some currently accepted models (and mechanisms) at the molecular level. In fact, the results gathered along this and other works [7, 17, 18, 21] have shown beyond doubt that the antioxidant and anticancer activities of the hydroxycinnamic derivatives are intrinsically dependent on their structural characteristics, namely the number of hydroxyl groups, and the presence of alkyl ester side chains and their chemical nature (*e.g.* linear *vs* branched, saturated *vs* unsaturated). Therefore if evident SPAR were found to rule the antioxidant/antiproliferative activities of these compounds, this being an effective approach for the design of new therapeutic agents as well as for the understanding of their mechanisms of action. Further research is presently in progress in order to find lead compounds displaying both antioxidant and growth-inhibition properties, thus being promising agents for cancer therapy.

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Experimental

Reagents

2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), egg L-α-phosphatidylcholine (EPC), 2,2′-diphenyl-1-picrylhydrazyl (DPPH), fluorescein sodium salt, 2-carboxy-2,5,7,8-tetramethyl-6-chromanol (trolox), 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) were obtained from Sigma-Aldrich-Fluka (Sintra, Portugal). Diphenylhexatriene propionic acid (DPH-PA) was obtained from Molecular Probes (Leiden, The Netherlands). All other reagents and solvents were pro-analysis grade, purchased from Merck (Lisbon, Portugal). Antibiotics (penicillin-streptomycin 100 × solution), cisplatin (*cis*-diaminedichloro-platinum(II)),

DMEM-HG culture medium, EDTA (ethylenediaminetetraacetic acid, disodium salt, dihydrate), glutamine, HEPES (*N*-[2-hydroxyethyl]piperazine-*N*′-[4-butane-sulphonic acid]), phenol red (phenol-sulfonphthalein), Trypan blue (0.4% solution, prepared in 0.81% sodium chloride and 0.06% dibasic potassium phosphate), trypsin, trisodium citrate, inorganic salts and acids (of analytical grade) were purchased from Sigma-Aldrich Chemical Co. (Madrid, Spain). Fetal calf serum was obtained from Biochrom KG, Berlin. Alamar blue was acquired from Accurate Chemical & Scientific Corporation, Westbury, NY, USA.

The HeLa and BJ cell lines were purchased from the European Collection of Cell Cultures (ECACC, United Kingdom).

Apparatus

Infrared spectra were recorded on an ATI Mattson Genesis Series FTIR spectrophotometer (Mattson Instruments, Inc., Madison, WI, USA) using potassium bromide disks. Only the most significant absorption bands are reported (ν_{max}, cm^{-1}). ^{1}H- and ^{13}C-NMR data were acquired, at room temperature, on a Bruker AMX 300 spectrometer (Bruker Bioscience, Billerica, MA, USA) operating at 300.13 and 75.47 MHz, respectively. Dimethylsulfoxide-d_6 was used as a solvent; chemical shifts are expressed in δ (ppm) values relative to tetramethylsilane (TMS) as an internal reference; coupling constants (*J*) are given in Hz. Assignments were also made from DEPT (distortionless enhancement by polarization transfer) (see underlined values). Electron impact mass spectra (EI-MS) were carried out on a VG AutoSpec instrument (Fisons Instruments, Mainz, Germany); the data are reported as *m*/*z* (% of relative intensity of the most important fragments). Melting points were obtained on a Köfler microscope (Reichert Thermovar, Vienna, Austria) and are uncorrected. UV/Vis and fluorescence measurements, for TAC, lipoperoxidation, and partition coefficient determination assays, were performed on a Bio-Tek Synergy HT multiplate reader (Biotek, Winooski,. VT, USA).

Synthesis

Trans-3-(3,4,5-trihydroxyphenyl)-2-propenoic acid ***1****, Trans-ethyl-3-(3,4,5-tri-hydroxyphenyl)-2-propenoate* ***2****, and Diethyl 2-(3,4,5-trihydroxyphenyl-methylene)malonate* ***3***

The phenolic compounds studied in the present work were synthesized according to an adaptation of the process described by Hübner *et al.* [48]. The reaction is of Knoevenagel type, occurring between the corresponding trihydroxybenzaldehyde (1 g) and malonic acid (1.2 g) or monoethylmalonate or diethylmalonate in pyridine (5 mL) using aniline (four drops) as catalyst. All reactions took place at 50°C for 20 h, and were followed by thin layer chromatography (TLC). The solvents were partially evaporated after the synthetic reaction; the product was diluted with diethyl ether and washed twice with 2 N HCl and water. The organic layer was then dried over anhydrous magnesium sulphate, filtered, and concentrated. The remaining residues were crystallized from dichloromethane/*n*-hexane or from methanol/water.

The newly synthesized compounds were identified by both NMR and EI-MS. The data obtained for **1** and **2** is in accordance with that described in Fiuza *et al.* [30].

Diethyl 2-(3,4,5-trihydroxyphenylmethylene)malonate ***3***

Yield 70%; IR: 3388, 3282, 1720, 1672, 1600, 1535, 1465, 1375,1326, 1272, 1234, 1149, 1078, 1032, 939, 837, 766, 731, 665, 631. ^{1}H-NMR δ: 1.23 (6H, t, $COOCH_2CH_3$), 4.19 (2H, q, $COOCH_2CH_3$), 4.30 (2H, q, $COOCH_2CH_3$), 6.48 (2H, s, H(2), H(6)), 7.37 (1H, s, H(b)), 9.20 (3H, s, OH); ^{13}C-NMR δ: 13.8 (OCH_2CH_3), 14.1 (OCH_2CH_3), 61.1 (OCH_2CH_3), 61.3 (OCH_2CH_3), 109.4 (*C*H(2, 6)), 121.7 (C(1)), 122.3(C(α)), 137.2 (*C*-OH), 142.0 (*C*H(β)), 146.0 (*C*-OH), 164.0 (C=O), 166.6 (C=O); EI-MS *m*/*z* (%): 296 [$M^{+\bullet}$] (100), 251 (36), 222 (38), 205 (49), 178 (38), 150 (58), mp. 182 – 184°C (156°C subl.).

Ab-initio molecular orbital calculations

The most stable conformations of the three phenolic compounds presently studied were obtained by *ab-initio* molecular orbital (MO) calculations. Full geometry optimization was performed using the GAUSSIAN 98W program [49], within the Density Functional Theory (DFT) approach in order to properly account for the electron correlation effects (particularly important in this kind of systems). The widely employed hybrid method denoted by B3LYP [50 – 55], which includes a mixture of HF and DFT exchange terms and the gradient-corrected correlation functional of Lee, Yang, and Parr [56, 57], as proposed and parameterized by Becke [58, 59], was used, along with the double-zeta split valence basis set 6-31G** [60, 61]. Molecular geometries were fully optimized by the Berny algorithm, using redundant internal coordinates [62]: the bond lengths to within ca. 0.1 pm and the bond angles to within ca. 0.10. The final root-mean-square (rms) gradients were always less than 3×10^{-4} hartree.bohr^{-1} or hartree.radian^{-1}. No geometrical constraints were imposed on the molecules under study.

Total antioxidant capacity assays

Total antioxidant capacity (TAC) assays were carried out using ABTS and DPPH as radicals. The experimental procedures were adapted from the literature, with slight modifications in order to be applied to a multiplate reader [63 – 67].

Before the measurements, the concentration of the $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ solutions was adjusted with ethanol to yield an absorbance of 0.45 ± 0.01 at 734 nm (at 30°C) and of 0.38 ± 0.01 at 515 nm (at 25°C), respectively, for 180 μL of solution in the plate reader.

Six solutions of each compound were prepared in ethanol, with concentrations between 1.5×10^{-5} and 1.5×10^{-4} M. The absorbances of 20 μL of each solution and 180 μL of radical (in quadruplicate) were recorded every 5 min, during 20 min, for $ABTS^{\bullet+}$ and every minute, during 10 min, then every 5 min, during the next 50 min, for $DPPH^{\bullet}$. 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.

The concentrations of the radicals ($ABTS^{\bullet+}$ and $DPPH^{\bullet}$) were plotted as a function of the phenolic concentration: after 5 and 20 minutes of reaction time for $ABTS^{\bullet+}$, and after 60 min of reaction time for $DPPH^{\bullet}$. Second degree polynomial regressions of the experimental points were generated, with a y-axis intercept at 100% of radical. TEAC value was the ratio between the trolox concentration needed to bleach 50% of the radical (IC_{50}) and the phenolic concentration needed to achieve the same effect, for each of the reaction times considered. The trolox equivalent antioxidant capacity (TEAC) value was the average of the TEAC values obtained from two independent determinations.

Lipoperoxidation assays

Liposomes preparation – EPC (15.75 mg, 2.25×10^{-5} mol) was dissolved in a 50 mL round flask containing 10 mL of a $CHCl_3$/CH_3OH (3 : 1) mixture. The solvent was evaporated on a rotava-

por 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, this film was vigorously shaken for 20 min in a vortex mixer with 15 mL of a Hepes solution (5 mM)/NaCl (0.1 M), 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).

Fluorescein assay

The antioxidant capacity of the compounds investigated was evaluated by an oxygen radical absorbance capacity (ORAC) assay, using fluorescein as the fluorescent probe and AAPH as the radical initiator. The determination was performed either in a buffer or in a liposome (LUVs)/buffer media. All solutions were prepared in a potassium phosphate buffer (75 mM, pH 7.4, ionic strength adjusted to 0.1 M with NaCl).

The final concentrations of the reagents placed in the 96-well plates were as follows: buffer or LUVs suspension – 0.3 mM; fluorescein – 40 nM; AAPH – 30 mM; phenolic compound – 1, 2 and 3 μM; acetonitrile – 0.15%. Prior to the addition of the radical initiator, the buffer (or the LUVs) and the phenol were incubated at 37°C, for 10 min, in the multiplate reader. The maximum of fluorescence emission (λ_{ex}: 485/20 nm, λ_{em}: 528/20 nm) was set to $\pm 8 \times 10^4$.

DPH-PA assay

EPC liposomes containing a radical sensitive fluorescent probe (DPH-PA) (38 μL of a 60% (w/v) methanolic solution) were used. The method of Arora *et al*. [44] was modified in order to be applied to a multiplate reader.

Reagents were introduced in 96-well plates as follows: 160 μL of the LUVs suspension, 70 μL of a solution of AAPH (64.27 mM in Hepes/NaCl solution), 70 μL of solutions of the polyphenol under study (6.42, 32.14, and 64.28 μM in Hepes/NaCl solution) containing 6.5% of ethanol or 70 μL of Hepes/NaCl solution. The final concentrations used were: 0.80 mM in LUVs, 15.00 mM in AAPH, 1.50, 7.50, and 15.00 μM in polyphenol, 1.5% in ethanol. Before the addition of the radical initiator, the LUVs and the polyphenol were shaken at 37°C for 10 min, in the multiplate reader. The maximum of fluorescence emission (λ_{ex}: 360/40 nm, λ_{em}: 460/40 nm) was set to $\pm 8 \times 10^4$ (by tuning the sensitivity of the fluorescence detector of the multiplate reader) and set to 100% of fluorescence emission (0% of oxidation).

For both fluorescein and DPH-PA assays AAPH is added and the fluorescence decay over time was recorded at regular intervals at 37°C for a 3-hour period. Trolox was used as a reference antioxidant.

The area under the curve of a control assay (without polyphenol) was subtracted from the area obtained for each concentration of polyphenol tested. For each compound, the reduced area was divided by the reduced area obtained for trolox, to yield TEAC values. Each assay was performed in triplicate.

Determination of partition coefficients (Kp)

Partition coefficients were determined for the two phenolic esters, at pH 7.4 and 25°C, using egg phosphatidylcholine (EPC) as the biomembrane model. The method of Kitamura *et al.* [46] was adapted in order to use a multiplate reader.

Solutions in 96-well plates contained: liposomes (LUVs) in buffer (Hepes: 0.005 M, I = 0.1 M (NaCl), pH 7.4) (from 0 to 500 μM), phenolic compound tested (50 μM) and ethanol (1.66%). The mixtures were stirred for 1 min and left standing for 30 min in the multiplate reader, at 25°C, in order to allow membrane partition. UV/Vis spectra of the solutions were recorded between 250 nm and 400 nm, every 2 nm. Blank spectra (solutions without phenolic compound) were measured and subtracted from the sample spectra for each corresponding liposome concentration. Data were treated according to Kitamura *et al.* [46]. The partition coefficient of the phenolic acid was previously determined by derivative spectrophotometry, using micelles of 1-hexadecylphosphoryl-choline (HDPC), at pH 2 and at 25°C [47].

Biological assays

Preparation of solutions: all compounds studied were water soluble in the concentration range used – from 2.5×10^{-5} M to 1.0×10^{-4} M. Samples were prepared in phosphate buffered saline solution (PBS): 132.0×10^{-3} M NaCl, 4.0×10^{-3} M KCl; 1.2×10^{-3} M NaH_2PO_4; 1.4×10^{-3} M $MgCl_2$; 6.0×10^{-3} M glucose; 1.0×10^{-2} M HEPES (*N*-[2-hydroxyethyl]piperazine-*N*′-[4-butanesulphonic acid]). Fresh solutions were prepared monthly and kept from light, in order to prevent oxidation. Trypan blue was used as a 0.04% (w/v) solution in PBS. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) was prepared, in a concentration of 0.5 mg/mL, in PBS solution containing 1.0×10^{-3} M $CaCl_2$.

Cell culture

Stock cultures of cells were maintained at 37°C, under 5% CO_2. HeLa and L-132 (grown in monolayers) were kept in Dulbecco's modified Eagle's high glucose (4500 mg/L) medium (DMEM-HG), supplemented with 10% heat-inactivated fetal calf serum, glutamine (1.168 g/L) and antibiotics (100 units of penicillin and 100 mg streptomycin). The cell lines were subcultured twice a week. HeLa cells were harvested using a dissociation medium composed of 136.9×10^{-3} M NaCl, 2.7×10^{-3} M KCl, 8.2×10^{-3} M Na_2HPO_4, 1.5×10^{-3} M KH_2PO_4, 4.0×10^{-4} M EDTA (ethylene-diaminetetraacetic acid, disodium salt, dihydrate) (pH 7.4) and containing 0.0004% (w/v) phenol red. L-132 cells were harvested with a trypsin / EDTA solution (0.05% trypsin, 0.35 mM EDTA 4 Na, reconstituted in balanced salt solution without Ca^{2+} or Mg^{2+}).

Cytotoxicity and cell growth inhibition evaluation

Cytotoxicity and cell density evaluation after drug exposure – for drug concentrations between 2.5×10^{-5} M and 1.0×10^{-4} M – was assessed with use of standard assays. Cells were plated at 5×10^5 cells/mL. In a time of 24 h hours after seeding, drug solutions were added to the medium and the cultures were incubated at 37°C. Cells were harvested and analyzed (both in controls and in drug-treated cultures) every 24 hours, for a total period of three days. Reversibility of the drug effect was tested by removing the drug and adding fresh culture medium in the last day of incubation with the drug, and assessing the cell viability following four more days of incubation. Cell density and viability were determined by Trypan blue exclusion on single-cell suspensions obtained from the monolayer cultures. Cell viability was further assessed by the Alamar blue colorimetric test [67, 17, 18]. All experiments were performed in triplicate.

All the results presented were obtained by two independent methods: cell density measurement – Trypan blue exclusion

method – and cell viability assessment – Alamar blue colorimetric assay [17, 18].

Data analysis

All experiments were performed in triplicate. The results are expressed as mean values ± SD, (the corresponding error bars being displayed in the graphical plots). Statistical analysis was performed using ANOVA, followed by post hoc test of Fisher's Protected Least Significant Difference. Statistical comparison between the data was based on the Pearson correlation coefficient, values less than 0.05 being considered as significant.

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