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Lipophilic phenolic antioxidants: Correlation between antioxidant profile, partition coefficients and redox properties

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ABSTRACT

Lipophilic compounds structurally based on caffeic, hydrocaffeic, ferulic and hydroferulic acids were synthesized. Subsequently, their antioxidant activity was evaluated as well as their partition coefficients and redox potentials. The structure–property–activity relationship (SPAR) results revealed the existence of a clear correlation between the redox potentials and the antioxidant activity. In addition, some compounds showed a proper lipophilicity to cross the blood–brain barrier. Their predicted ADME properties are also in accordance with the general requirements for potential CNS drugs. Accordingly, one can propose these phenolic compounds as potential antioxidants for tackling the oxidative status linked to the neurodegenerative processes.

1. Introduction

Neurodegenerative diseases (ND) are a group of illnesses with diverse clinical importance and etiologies. ND include motor neuron disease such as amyotrophic lateral sclerosis (ALS), cerebellar disorders, Parkinson's disease (PD), Huntington's disease (HD), Alzheimer's disease (AD) and schizophrenia. Neurodegeneration appears to be a multifactorial process where a complex set of toxic reactions, including oxidative stress, leads to the demise of neurons.[1]

The molecular mechanisms of neuronal degeneration remain largely unknown and effective therapies are not currently available. Yet, it is widely accepted that oxidative stress increases with age, and that age is a major risk factor for several neurodegenerative diseases.

Reactive oxygen species (ROS) are particularly active in the brain and neuronal tissue and aggressive to glial cells and neurons. In-built endogenous antioxidant system plays its decisive role in prevention of any loss due to free radicals. However, imbalanced defense mechanism of antioxidants, overproduction or incorporation of free radicals from environment to living system leads to serious penalty and therefore to neuronal death.[1] Neuronal system is particularly susceptible to ROS owing to the high content of unsaturated fatty acids that are more susceptible to oxidative damage, namely lipid peroxidation. To note that brain consumes an inordinate fraction (20%) of total oxygen for its relatively small weight (2%) and it is not particularly enriched in endogenous antioxidant defenses.[1]

Exogenous antioxidants are nowadays considered a promising therapeutic approach in neurodegenerative diseases since they could play an important role in preventing and/or minimizing neuronal oxidative damage.[1,2] Recent interest has been focused on dietary phenolic antioxidants, such as carotenoids, cinnamic acids and flavonoids, as potentially useful agents in ND.[2,3] However, few of them have shown efficiency in animal models or in clinical studies, a drawback that is usually related with their inability to cross the blood–brain barrier (BBB) after systemic administration.[4]

In fact, BBB acts as a regulatory interface that selectively limits drug delivery to the brain. From the positive and negative crosswalks lipid solubility is believed to be a key feature in determining the rate at which a drug passively crosses the BBB and thus correspond to a major challenge in the rational design of novel neuroprotective antioxidants. However, it is important to mention

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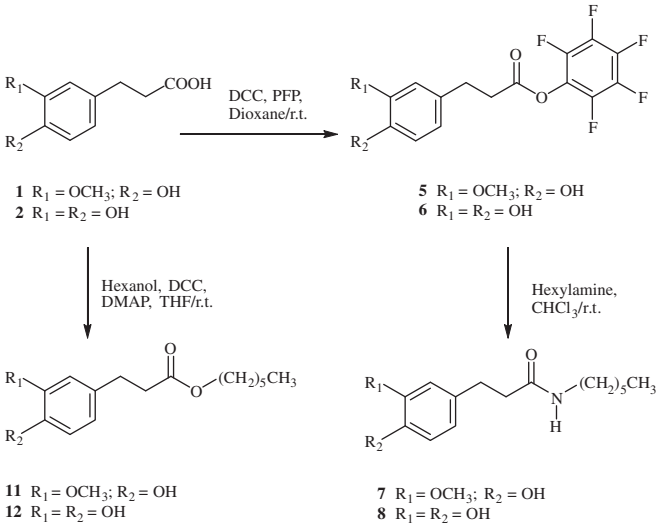
that other physicochemical drug properties such as solubility, molecular weight, and pK_a have also a noticeably influence on drug permeability and hence in drug bioavailability.

In this context, this work reports the rational design and synthesis of a new set of lipophilic phenolic antioxidants structurally based on the natural antioxidants caffeic and ferulic acids, and their saturated counterparts (Scheme 1). With this purpose, hexylamides and hexylesters of cinnamic and hydrocinnamic acids were synthesized in which the lipophilicity was essentially improved by an introduction of an additional alkyl side chain (Schemes 2 and 3). The antioxidant profile of the synthesized amides and esters, as well as the corresponding acid precursors, was determined by different methods, specifically throughout a lipoperoxidation assay.[5,6] The overall information obtained together with the assessment of the lipophilicity and redox potentials of the compounds allow acquiring significant information to establish a correlation between the physicochemical properties and the activity, herein called SPAR (structure–property–activity relationship). In addition, important data to predict the BBB permeation of the new chemical entities was attained using in silico models relying on the physicochemical parameters (e.g., solubility, lipophilicity, molecular size and hydrogen-bonding capacity).



1 $R_1 = OCH_3$; $R_2 = OH$
2 $R_1 = R_2 = OH$
5 $R_1 = OCH_3$; $R_2 = OH$
6 $R_1 = R_2 = OH$
11 $R_1 = OCH_3$; $R_2 = OH$
12 $R_1 = R_2 = OH$
7 $R_1 = OCH_3$; $R_2 = OH$
8 $R_1 = R_2 = OH$

Scheme 2. Synthetic strategies used for the obtention of hydrocinnamic amides and esters.

2. Results and discussion

2.1. Chemistry

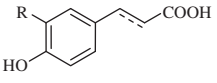
The synthesis of hexylamides (**7** and **8**) (Schemes 1 and 2) starts with the corresponding hydrocinnamic acids (**1** and **2**) which were previously converted into the intermediates pentafluorophenyl esters (**5** and **6**) by reaction with pentafluorophenol (PFP) and dicyclohexylcarbodiimide (DCC), in dioxane, at room temperature.[7] Subsequent reaction of the resulting activated esters with hexylamine, in chloroform and at room temperature, provided the required hexylamides (**7** and **8**).[7] This method was useful for the amidation of saturated acids containing phenolic groups.[8,9]

In the synthesis of unsaturated hexylamides (**9** and **10**) (Scheme 3), the cinnamic acids (**3** and **4**) reacted straightforward with hexylamine, in dimethylformamide (DMF), in the presence of the coupling agent (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP), at room temperature. This is a particularly suitable method for the direct amidation of α,β-unsaturated acids.[10]

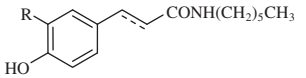
All the adopted strategies for the preparation of phenolic esters are outlined in Schemes 2 and 3. As the classic acid-catalyzed Fisher esterification is not appropriate when using long chain alkyl alcohols, compounds **11** and **12** (Scheme 2) were obtained by using DCC/DMAP (dimethylaminopyridine) method from the corresponding parent hydrocinnamic acids (**1** and **2**).[11] In contrast, compounds **13** and **14** (Scheme 3) were prepared by base-catalyzed alkylation from the corresponding dihydroxycinnamic acids (**3** and **4**) with in situ generation of the carboxylate anions and subsequent reaction, at room temperature, with hexylbromide.[12] Efforts were made to drive both reactions to a satisfactory yield by changing either the coupling reagent or the nature of the base. However, fairly moderate yields were obtained in all cases.

2.2. Effect of the phenolic derivatives on lipid peroxidation

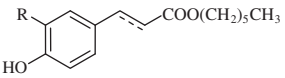
Lipid peroxidation is an uncontrolled deleterious process that occurs in cellular and subcellular membranes, causing or enhancing the formation of lipid hydroperoxides. These reactive species are cytotoxic and capable of reacting with numerous cellular components providing one of the mechanisms underlying the toxicity of several drugs. The use of model membranes such as unilamellar liposomes has lately been encouraged as a helpful tool for understanding the effect of drugs, such as antioxidants, in membrane phospholipid bilayers. The advantage of using this type of systems, including unilamellar versus multilamellar liposomes, and a water-soluble radical azo-generator (AAPH), is largely documented in the literature.[13] In AAPH induced peroxidation of unilamellar



1 Hydroferulic acid **R=OCH_3**
2 Hydrocaffeic acid **R=OH**
3 Ferulic acid **R=OCH_3**
4 Caffeic acid **R=OH**

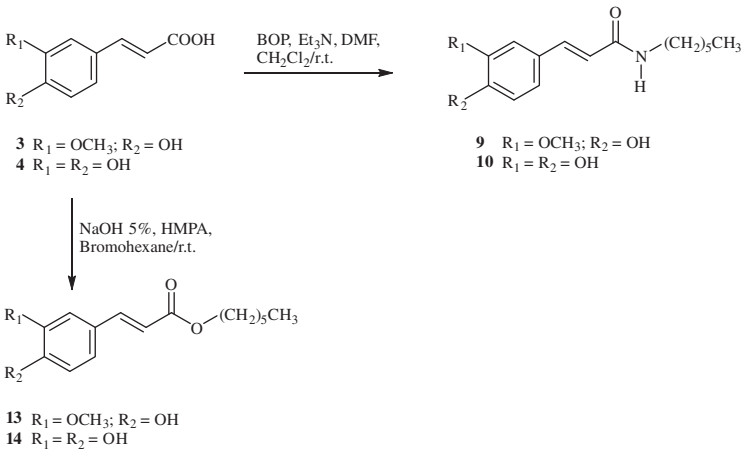


7 Hydroferuloylhexylamide **R=OCH_3**
8 Hydrocaffeoylhexylamide **R=OH**
9 Feruloylhexylamide **R=OCH_3**
10 Caffeoylhexylamide **R=OH**



11 Hexylhydroferulate **R=OCH_3**
12 Hexylhydrocaffeate **R=OH**
13 Hexylferulate **R=OCH_3**
14 Hexylcaffeate **R=OH**

Scheme 1. Phenolic acids and their derivatives.



3 $R_1 = OCH_3$; $R_2 = OH$
4 $R_1 = R_2 = OH$

9 $R_1 = OCH_3$; $R_2 = OH$
10 $R_1 = R_2 = OH$

13 $R_1 = OCH_3$; $R_2 = OH$
14 $R_1 = R_2 = OH$

Scheme 3. Synthetic strategies used for the obtention of cinnamic amides and esters.

liposomes the chain initiating radical is generated in the aqueous phase and the chain-propagating lipid peroxyl radicals are located within the membranes. In the assay, DPH-PA was used as fluorescence probe and Trolox as chain-breaking antioxidant standard.[14]

The data obtained for the phenolic acids and derivatives are shown in Table 1. From the results one can conclude that hydroxycinnamic acids are more effective than their saturated counterparts (**3** vs **1** and **4** vs **2**) and that the catecholic moiety plays an important role in the antioxidant activity (**4** and **2** vs **3** and **1**). The observed tendency is in accordance with data reported in the literature.[15] In addition a ranking antioxidant order could be inferred from the data of catecholic cinnamic derivatives: hexylcaffeate (**14**) presented higher antioxidant activity than caffeoylhexylamide (**10**) and this last better than caffeic acid (**4**). An identical succession was observed for the antioxidant profile of hydrogenated analogues (hexylhydrocaffeate (**12**) > hydrocaffeoylhexylamide (**8**) > hydrocaffeic acid (**2**)). From these results it was also possible to infer that the cinnamic series present better antioxidant activity than the saturated ones.

In general, for catecholic systems it can be concluded that esters (**14** and **12**) are better antioxidants than amides (**10** and **8**) and that amides have a superior antioxidant efficacy than acids (**4** and **2**), a fact that could be related with the intrinsic lipophilicity of each system (Table 2).

Regarding the monohydroxylated derivatives the following antioxidant outlines were obtained for the cinnamic acid and derivatives and for their saturated analogues: feruloylhexylamide (**9**) > hexylferulate (**13**) > ferulic acid (**3**) and hydroferuloylhexylamide (**7**) > hexylhydroferulate (**11**) > hydroferulic acid (**1**), respectively (Table 1). As stated above it was also possible to infer that the monohydroxylated cinnamic derivatives are more effective as antioxidants than the saturated ones. In this series the monohydroxylated amide derivatives (**9** and **7**) were slightly better antioxidants than the correspondent esters (**13** and **11**).

In summary, taking into account the lipid peroxidation data one can conclude that in general the catecholic derivatives are effective lipophilic antioxidant candidates. The performed chemical modifications (introduction of an ester or amide spacer and an alkyl side chain) do not diminish the antioxidant properties relatively to the natural precursor acids but on contrary, in some cases, it led to a huge activity enhancement. Dihydroxycinnamic hexylester and its saturated analogue look like to be the greatest antioxidant agents

2.3. Evaluation of the total antioxidant capacity (TAC) of the catecholic derivatives

Total antioxidant capacity (TAC) assays have been often used to determine the hierarchy of radical-scavenging abilities of potential phenolic antioxidant compounds that work either through electron- or H-donating mechanisms. Classically, Trolox, a water-soluble vitamin E analogue, is used as reference. The results of this type of assays are usually expressed as trolox equivalent antioxidant capacity (TEAC).[16,17]

Accordingly, the TAC assays (ABTS and DPPH) were applied to assess the radical-scavenging ability of the most outstanding antioxidant compounds (compounds **2**, **4**, **8**, **10**, **12** and **14** Scheme 1).

Table 1
TEAC results for the lipoperoxidation, ABTS and DPPH assays of phenolic acids and derivatives

Phenolic compounds	Lipoperoxidation DPH-DA	ABTS		DPPH
		5 min	20 min	
Caffeic acid (**4**)	2.23 ± 0.05	1.14 ± 0.02	1.27 ± 0.01	1.29 ± 0.05
Hexylcaffeate (**14**)	3.55 ± 0.05	0.91 ± 0.02	0.93 ± 0.01	0.97 ± 0.05
Caffeoylhexylamide (**10**)	2.41 ± 0.07	1.04 ± 0.04	1.05 ± 0.01	1.11 ± 0.07
Hydrocaffeic acid (**2**)	1.75 ± 0.04	1.16 ± 0.03	1.41 ± 0.01	2.05 ± 0.06
Hexylhydrocaffeate (**12**)	3.49 ± 0.04	0.96 ± 0.02	0.96 ± 0.01	0.99 ± 0.05
Hydrocaffeoylhexylamide (**8**)	2.08 ± 0.05	1.01 ± 0.02	1.02 ± 0.01	1.00 ± 0.03
Ferulic acid (**3**)	0.79 ± 0.01	–	–	–
Hexylferulate (**13**)	1.13 ± 0.03	–	–	–
Feruloylhexylamide (**9**)	1.22 ± 0.03	–	–	–
Hydroferulic acid (**1**)	0.22 ± 0.02	–	–	–
Hexylhydroferulate (**11**)	0.57 ± 0.01	–	–	–
Hydroferuloylhexylamide (**7**)	0.68 ± 0.02	–	–	–

Table 2
Partition coefficients and redox potentials of the phenolic acids and derivatives

Phenolic compounds	miLog *P*/Log *D* (Theor., pH 5)[a]	Log *P* (Theor.)[b]	Log *P* (Exp., pH 4.7)	E_p
Caffeic acid (**4**)	1.53/0.16	1.15	–	0.183
Hexylcaffeate (**14**)	4.12	3.49	3.91 ± 0.08[c]	0.175
Caffeoylhexylamide (**10**)	3.16	2.81	3.61 ± 0.02[d]	0.162
Hydrocaffeic acid (**2**)	1.45/0.26	1.17	_	0.139
Hexylhydrocaffeate (**12**)	3.81	3.51	3.79 ± 0.06[c]	0.125
Hydrocaffeoylhexylamide (**8**)	3.08	2.83	2.62 ± 0.14[d]	0.125
Ferulic acid (**3**)	1.67/0.42	1.42	–	0.350
Hexylferulate (**13**)	4.27	3.76	4.05 ± 0.04[c]	0.328
Feruloylhexylamide (**9**)	3.30	3.08	3.58 ± 0.04[d]	0.322
Hydroferulic acid (**1**)	1.59/0.51	1.44	–	0.410
Hexylhydroferulate (**11**)	3.95	3.78	3.60 ± 0.08[c]	0.434
Hydroferuloylhexylamide (**7**)	3.22	3.10	2.83 ± 0.17[d]	0.388

[a] Determined with Molinspiration Calculation software.[31]
[b] Determined with ChemDraw software.[30]
[c] Determined in liposomes.
[d] Determined in micelles.

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).

The data obtained is depicted in Table 1. From the results obtained in the ABTS assay one can infer that, in general, the parent acids (**2** and **4**) have a higher antioxidant activity towards $ABTS^{\cdot+}$ than the corresponding amides (**8** and **10**) and esters (**12** and **14**), particularly after 20 min of reaction. From this assay, and in opposition to what was observed in the lipid peroxidation assay, the saturated cinnamic acids (**2**) showed a higher antioxidant activity (after 20 min) than the unsaturated ones (**4**). To note that the phenolic acids are still reacting after the first 5 min of reaction. All the lipophilic compounds (**8**, **10**, **12** and **14**) exhibit approximately the same antioxidant performance, reaching their maximum antioxidant capacity after 5 min. The presence of a double bond (compounds **4**, **10** and **14**) does not seem to markedly influence the antioxidant capacity of the esters and amides when compared to their saturated homologues (**2**, **8** and **12**). The results are in accordance with the data obtained with similar systems.[15]

Concerning the DPPH assay (Table 1) it was observed that, in general, phenolic acids (**2** and **4**) have higher antioxidant activity than the corresponding amides (**8** and **10**) and esters (**12** and **14**). The saturated acid (**2**) showed a higher antioxidant activity than the unsaturated one (**4**). In these experimental conditions the phenolic derivatives reveal to be less active than their parent compounds.

The DPPH results follow the same tendency than those obtained with the ABTS assay. However, a larger dissimilarity in the antioxidant potency was observed between the acids and their derivatives (esters and amides), a fact that could be related with to steric hindrance caused by the bulkiness of alkyl groups.[17]

The radical-scavenging ability data obtained for hydroxycinnamic acids and derivatives against DPPH and ABTS radicals were in good agreement with the expected activities of this type of phenolic systems: higher when a catechol group is present (caffeic or hydrocaffeic series) and lower when the *meta*-hydroxyl function is substituted by a methoxyl group (ferulic and hydroferulic series) (see Table 1).

From the results obtained one must conclude that the data obtained from TAC assays could be used only for a qualitative and rapid antioxidant screen. In fact, as demonstrated herein quantitative data must be carefully analysed mainly if the compounds under analysis present either intrinsic steric hindrance or lipophilic properties. The results are in accordance with previous studies that concluded that the TAC assays may give an indication for the presence of antioxidants in a certain system but SAR cannot be readily inferred.[6]

2.4. Evaluation of the redox properties of the phenolic derivatives

Many antioxidants are proven to undergo an electron-transfer mechanism upon exerting their antioxidant functions. As a result, electrochemistry has been employed to evaluate antioxidant activity since it can provide direct and detailed information on the oxidation of these compounds. Quantitative correlations have been already established between the antioxidant activities and redox potentials suggesting that this physicochemical parameter could be considered a good measure of the antioxidant activity.[18–20] Hence, it was found important to study the electrochemical properties of the synthesized caffeic and ferulic acid and their derivatives (Scheme 1). Thus, the redox properties of the synthesized phenolic compounds were studied, at physiological pH 7.3, by differential pulse and cyclic voltammetry, using a glassy carbon working electrode.

The differential pulse voltammograms of dihydroxylated cinnamic series, caffeic and hydrocaffeic acid derivatives (Scheme 1), present only one well-defined anodic peak at physiological pH (Fig. 1A and B). The oxidation peaks observed for these cinnamic compounds are intrinsically related with the oxidation of catechol group. Cyclic voltammograms were also recorded at different sweep rates. The cyclic voltammograms obtained are characteristic of an electrochemical reversible reaction showing only one anodic peak and one cathodic peak on the reverse scan (Fig. 2A and B). Linear plots of peak current (I_p) as a function of the square root of scan rate (v) were obtained indicating that the oxidation processes are diffusion controlled.[21] The ratio of the anodic to cathodic peak heights raise gradually with increasing potential scan rate from 10 to 100 mVs^{-1} and the calculated current functions ($I_p \cdot v^{-1/2}$) diminish gradually with the potential scan rate, showing the classical behaviour of an oxidation process coupled with a slow subsequent chemical reaction. The results are in agreement with the literature in which concerns the oxidative behaviour of the parent compounds.[22–25] Electrochemical studies on the caffeic acid (**4**) oxidation mechanism have shown that the first oxidation step involves two electrons *per* molecule which likely correspond to the formation of the caffeic acid *ortho*-quinone, an intermediate that is quickly decomposed at pH higher than 7.4.[22–24]

The differential pulse voltammetric study of ferulic acid (**3**) (Scheme 1), revealed the presence of two convolved anodic peaks

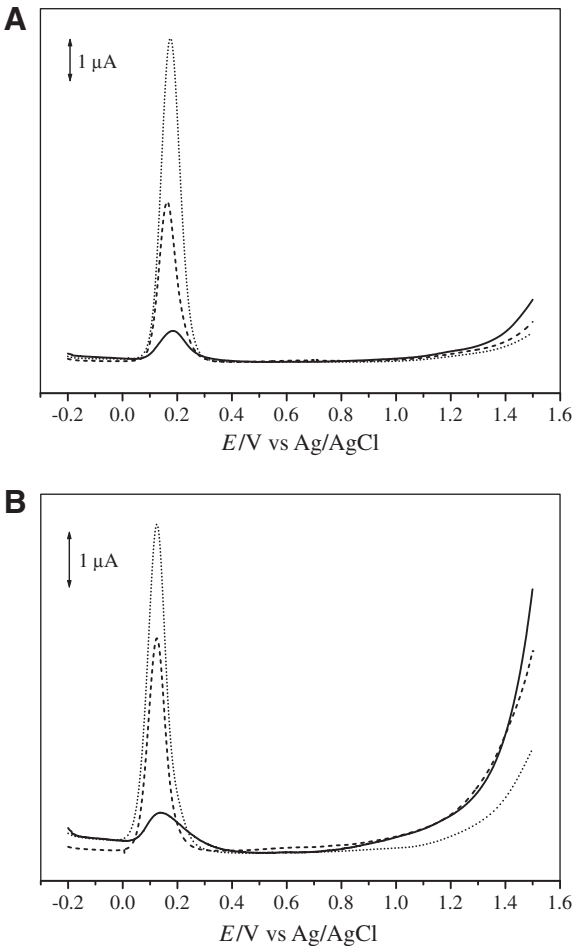


Figure 1. Differential pulse voltammograms for 0.1 mM solutions of (A) (—) caffeic acid, (——) caffeoylhexylamide, (▪▪▪▪) hexylcaffeate and (B) (—) hydrocaffeic acid, (——) hydrocaffeoylhexylamide, (▪▪▪▪) hexylhydrocaffeate, in physiological pH 7.3 supporting electrolyte. Scan rate: 5 mV s^{-1}.

at physiological pH (Fig. 3A). The oxidation peaks at E_p = +0.335 V and E'_p = +0.443 V are related with the oxidation of the phenolic group present in the structure. The results may be interpreted by assuming that cinnamic acids oxidation takes place by electron transfer for both free and adsorbed forms. The free form corresponds to the first peak, E_p, while the strongly adsorbed form, which is consequently stabilized, is oxidized at a more anodic potential, E'_p. The appearance of an adsorption peak, E'_p, has been described in literature in electrochemical studies involving ferulic acid analogues.[25,26]

For ferulic (**3**) and hydroferulic acid (**1**) derivatives (Scheme 1), only one anodic wave was observed at physiological pH using differential pulse voltammetry (Fig. 3A and B). The oxidation peaks observed are also related with the oxidation of the phenolic group present in the structure. The large wave shape observed could be a sign of a higher superimposition of the peaks from both free and adsorbed forms or might indicate a lesser adsorption propensity of this molecule. The cyclic voltammograms obtained for ferulic acid have also shown two convolved anodic peaks. The anodic peaks obtained for ferulic and hydroferulic series appear to correspond to irreversible processes (Fig. 2C and D). Plots of peak current (I_p) as a function of the square root of scan rate (v) gave a straight line indicating that the oxidation processes are diffusion controlled.[21] The assembled data corroborate the results found in literature regarding ferulic acid oxidative behaviour. The proposed mechanism involves a one-electron transfer from the phenolate ion followed by one irreversible dimerisation process due to a radical–radical coupling reaction between two phenoxyl radicals.[22,26]

The voltammetric results showed that the higher the number of hydroxyl substituents on the aromatic ring, the lower the electrochemical potential (Table 2). Redox potentials of caffeic and hydrocaffeic acid derivatives (**8**, **10**, **12** and **14**) are lower than those of ferulic and hydroferulic acid derivatives (**7**, **9**, **11** and **13**), a fact that could be related with the presence of the cathecol moiety. The methoxylation of the *meta*-hydroxy group of the cinnamic derivatives (ferulic and hydroferulic acid derivatives) shifted the peak potentials toward more positive values (Table 2).

Extension of the conjugation via the side chain also affects considerably the redox potentials of the cinnamic acids and derivatives studied. This effect is more noticeable in caffeic acid and its derivatives, due to an increase of the resonance effects on the stability of the intermediate radical.[27–29]

The introduction of an alkylamide or an alkylester groups in the conjugated side chain did not significantly change the oxidation potentials, when compared to the parent compounds. The data obtained strongly suggest that the structural modifications performed result in modest or even no effect on the electron density of the phenol or catechol ring. Hence, the introduction of these groups did not significantly influence the energetic of the electron transfer as can be ascribed from the similarity of the E_p values for these derivatives.

From the electrochemical results one can conclude that the structural principles governing the redox potentials of the cinnamic acids and its derivatives under study were found to be the presence of a phenolic group, preferentially a catechol.

2.5. Partition coefficient measurements

In view of better correlate the overall properties of the antioxidant compounds the lipophilicity, expressed as the octanol–water partition coefficient and herein called log *P* was calculated either theoretically (according to Broto's fragmentation method and using Molinspiration property calculation program) or by derivative spectrophotometry in two mimetic systems (liposomes and in micelles) (see Table 2).[30–33] As expected the log *P* (exp), at pH 4.7, could not be determined for the acids (**1**–**4**) because at this pH they are ionized, a fact that is in accordance with the theoretical values (see Table 2). From the data obtained, one can notice that the studied acids (**1**–**4**) are not able to cross membranes effectively, once they have a log $P \leqslant 1$. On the contrary, the synthesised amides (**7**–**10**) and esters (**11**–**14**), possessing an additional lipophilic alkyl chain showed a superior lipophilicity, with log *P* values compatible with those required to cross membranes, particularly the blood–brain barrier (log *P* between 1 and 3).[34]

In general, the experimental values of log *P* for the esters and amides studied are in accordance with the theoretical ones, and esters are more lipophilic than amides. The hexylesters (**13**, **14**) are more lipophilic than the correspondent hexylhydroesters (**11**, **12**). The same ranking order is observed for the hexylamides (**9**, **10**) versus hexylhydroamides (**7**, **8**). From all of compounds studied, hexylferulate (**13**) (log *P* = 4.05) is the most lipophilic which is in agreement with the predicted theoretical values. The hexylhydrocaffeic amide (**8**) and the hexylhydroferulic amide (**7**), with a log *P* of 2.62 and 2.83, respectively, seem to have the ideal lipophilicity to cross the blood–brain barrier.[34] In addition, the theoretical prediction of ADME properties of all compounds was carried out (see Tables 2 and 3). From the data obtained it can be observed that no violations of Lipinski's rule (molecular weight, log *P*, number of hydrogen donors and acceptors, and number of rotatable bonds) were found making the cinnamic derivatives promising antioxidant agents. Topological polar surface area (TPSA), described to

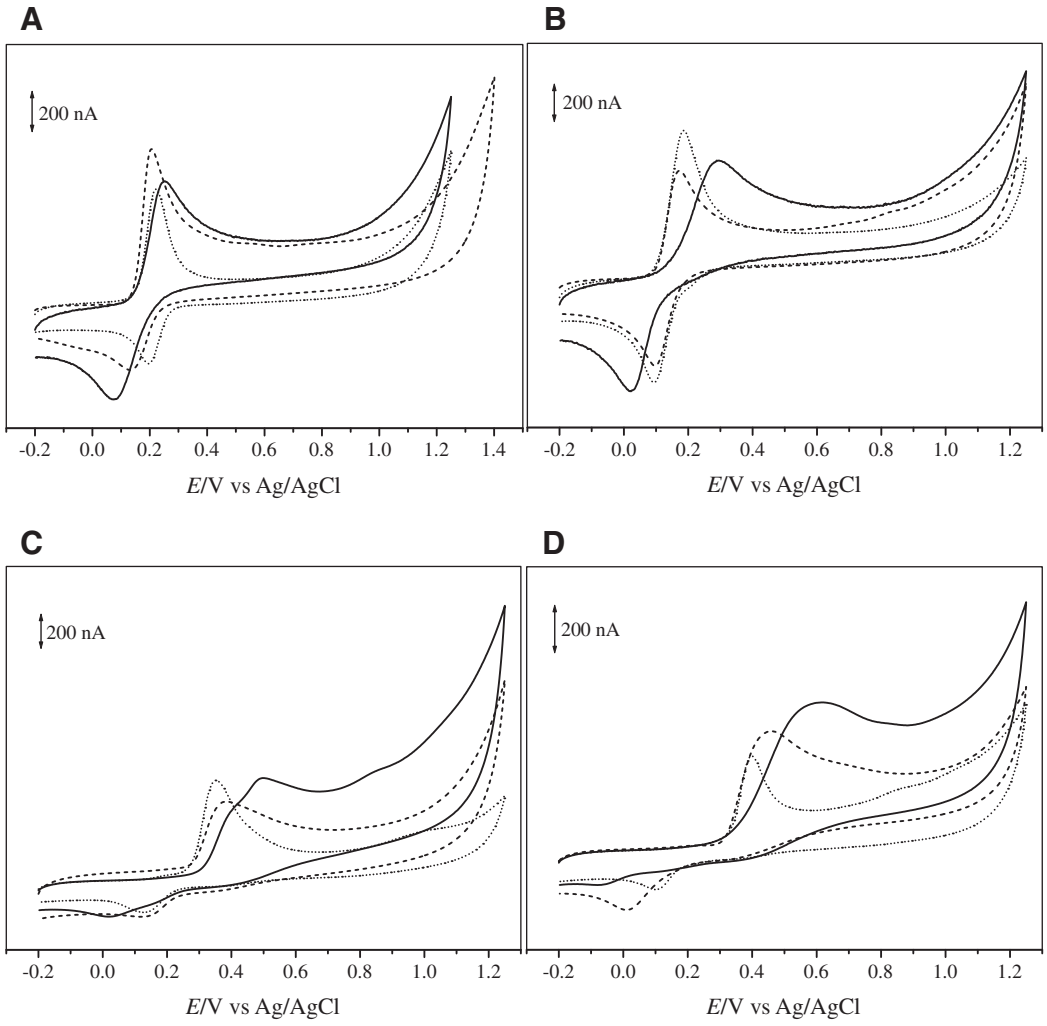


Figure 2. Cyclic voltammograms for 0.1 mM solutions of (—) acids, (——) hexylamides and (....) hexylesters for (A) caffeic, (B) hydrocaffeic, (C) ferulic and (D) hydroferulic series, in physiological pH 7.3 supporting electrolyte. Scan rate: 50 mV s^{-1}.

be a predictive indicator of blood–brain barrier penetration, is also found to be positive for these potential CNS drugs.[35–37]

2.6. Structure–property–activity relationships (SPAR)

The development of novel lipophilic antioxidants structurally based on the natural models—ferulic (**3**) and caffeic (**4**) acids and their corresponding saturated counterparts (**1** and **2**, respectively) was performed by introducing alkyl groups in the side chain and a diverse spacer either of amide or ester type. In accordance with the aim of the work the physicochemical properties as well as antioxidant activity of the compounds were examined in order to carry out structure–property–activity relationship studies as an upgrading tool for the rational design of lipophilic antioxidants.[5,6,25]

The antioxidant efficacy of the phenolic derivatives is higher when the redox potential is lower (as seen in the caffeic and hydrocaffeic series). The presence of a catechol group leads to an increase of the activity due mainly to resonance stabilization of the phenoxyl radical intermediate with subsequent *ortho*-quinone formation.[6,15] A redox potential increase and a decrease of the antioxidant activity was obtained in monohydroxylated compounds (ferulic and hydroferulic series). The higher lipophilicity of the alkylamides and alkylesters in comparison with their parent acids lead to an increase of activity since they have the ability to interact with the polar head groups of the membrane and attain a local concentration at the water–lipid interface.

The overall structure–property–activity outcome allow to conclude that the best antioxidant candidates are the dihydroxycinnamic hexylester (**14**) and its saturated analogue (**12**) since they possess a suitable redox potential (E_p 0.175–0.125) supplemented by a proper lipophilicity (log *P* 3.91–3.79) (Table 2).

3. Conclusions

From the results obtained it is possible to infer that the synthesized antioxidants of amide or ester type possess an amplified lipophilicity, increasing the antioxidant activity relatively to the precursor acids. The esters revealed to be more active than the homologous amides. The hexylamides (**10** and **8**) and hexylesters (**14** and **12**) of caffeic and hydrocaffeic acids showed to be the most active of all the phenolic compounds investigated. The hexylesters (**14** and **12**) were found to be the most promising compounds, a fact that can be related with their lipophilicity and redox behaviour.

From the electrochemical results one can conclude that the structural features governing the redox potentials of the cinnamic acids and derivatives were found to be a phenolic group, preferentially a catechol, as well as the additional resonance-effective substituents in the aromatic ring. Furthermore, the determined partition coefficients have shown the desired increased lipophilicity of the synthesized amides and esters relatively to the precursor acids, giving special emphasis on their brain permeable properties. In addition, the ADME properties predicted for these antioxidants

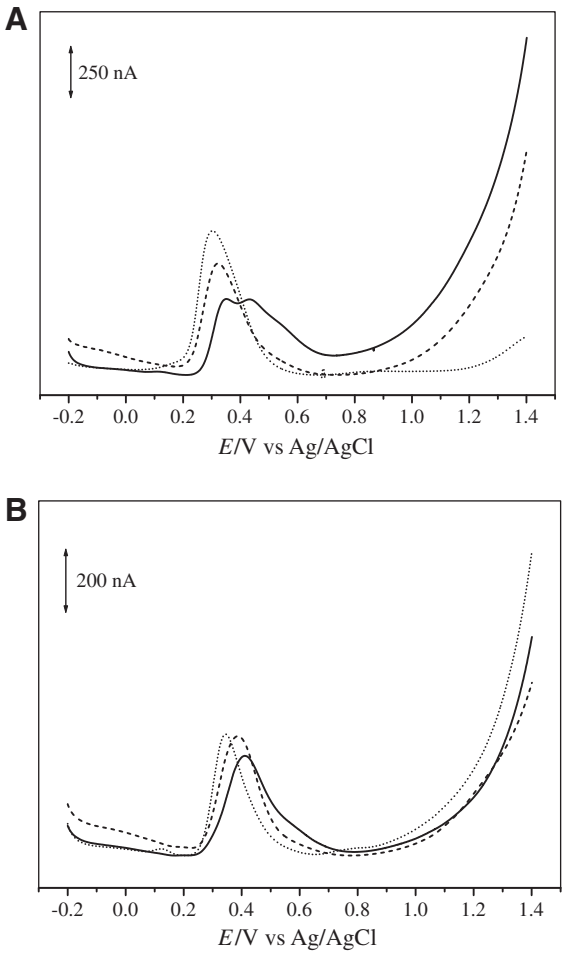


Figure 3. Differential pulse voltammograms for 0.1 mM solutions of (A) (—) ferulic acid, (——) feruloylhexylamide, (....) hexylferulate and (B) (—) hydroferulic acid, (——) hydroferuloylhexylamide, (....) hexylhydroferulate, in physiological pH 7.3 supporting electrolyte. Scan rate: 5 mV s^{-1}.

are in accordance with the general requirements for potential CNS drugs.

The results gathered along this work provide a rational approach to the design of membrane-target antioxidants that could be effective candidates for preventing or reducing the oxidative status associated with the neurodegenerative processes.

4. Experimental

4.1. Chemicals

2,2′-Azobis(2-amidinopropane)dihydrochloride (AAPH), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid hemisodium salt (Hepes), egg L-α-phosphatidylcholine (EPC), 2-carboxy-2,5,7,8-tetramethyl-6-chromanol (Trolox) and 1-hexadecylphosphorylcholine (HDPC) were obtained from Sigma. Diphenylhexatriene propionic acid (DPH-PA) was obtained from Molecular Probes. All the other chemicals were purchased from Aldrich, Fluka and Merck and used as supplied by the manufacturers. Flash column chromatography was performed with Silica Gel 60—particle size: 0.040–0.063 mm.

4.2. Apparatus

Melting-points were determined on a Reichert Thermopan or Thermovar hot block apparatus and were not corrected. IR spectra were recorded on a Jasco 420FT/IR spectrometer. The ^{1}H and ^{13}C NMR spectra were recorded at 300.13 and 75.6 MHz, respectively, on a Bruker-AMX 300 spectrometer using deuterated dimethyl sulfoxide (DMSO-d_6) as a solvent. The ^{1}H and ^{13}C NMR spectra of compound **9** were recorded at 599.7 and 150.8 MHz, respectively, on a Varian 600 MHz VNMRX spectrometer. Chemical shifts were recorded in δ (ppm) values downfield from TMS as internal standard for ^{1}H and ^{13}C NMR. Coupling constants (J) are given in Hertz. Assignments were also made from DEPT (distortionless enhancement by polarization transfer) (underlined values). Electron impact mass spectra (EI-MS) were carried out on a VG AutoSpec instrument; the data are reported as m/z (% of relative intensity of the most important fragments).

UV/vis and fluorescence measurements were carried out on a Bio-Tek Synergy HT multiplate reader) in flat-bottomed 96-well microplates (Orange Scientific).

Liposomes extrusion was performed at room temperature with an extruder Lipex (Lipex Biomembranes, Vancouver, Canada) under high pressure of nitrogen (N_2).

Voltammetric studies were performed using an Autolab PGSTAT 12 potentiostat/galvanostat (Eco-Chemie, Netherlands) and a one-compartment glass electrochemical cell. Voltammetric curves were recorded at room temperature using a three-electrode system. A glassy carbon working electrode (GCE) (d = 2 mm), a platinum wire counter electrode and an Ag/AgCl saturated KCl reference electrode were used. A Crison pH-meter with glass electrode was used for the pH measurements (Crison, Spain).

Table 3
Structural properties of the phenolic acids and derivatives[a]

Phenolic compounds	Molecular weight	*n*-ROTB	*n*-ON acceptors	*n*-ONNH donors	TPSA
Caffeic acid (**4**)	180.16	2	4	3	77.76
Hexylcaffeate (**14**)	264.32	8	4	2	66.76
Caffeoylhexylamide (**10**)	263.34	7	4	3	69.55
Hydrocaffeic acid (**2**)	182.18	3	4	3	77.76
Hexylhydrocaffeate (**12**)	266.34	9	4	2	66.76
Hydrocaffeoylhexylamide (**8**)	265.35	8	4	3	69.55
Ferulic acid (**3**)	194.19	3	4	2	66.76
Hexylferulate (**13**)	278.35	9	4	1	55.76
Feruloylhexylamide (**9**)	277.36	8	4	2	58.56
Hydroferulic acid (**1**)	196.20	4	4	2	66.76
Hexylhydroferulate (**11**)	280.36	10	4	1	55.77
Hydroferuloylhexylamide (**7**)	279.38	9	4	2	58.56

[a] *n*-ROTB, number of rotatable bonds; *n*-OHNH, number of hydrogen bond donors; *n*-ON, number of hydrogen acceptors; TPSA, topological polar surface area. Determined with Molinspiration Calculation software.[31]

4.3. Synthesis of phenolic compounds

4.3.1. General procedure to obtain the pentafluorophenyl esters (5–6)

According previous descriptions by the authors.[7]

4.3.2. General procedure to obtain the hydrocinnamic hexylamides (7–8) from the pentafluorophenyl esters (5–6)

According previous descriptions by the authors.[7]

4.3.3. General procedure to obtain the cinnamic hexylamides (9–10)

In order to synthesize the unsaturated amides, cinnamic acids (**3** and **4**) (5.0 mmol) were dissolved in 10 mL of DMF containing 0.7 mL of triethylamine. The solution was then cooled in an ice-water bath and hexylamine (0.67 mL, 5.0 mmol) was added, followed by a solution of BOP (2.21 g, 5.0 mmol) in 10 mL of dichloromethane (CH_2Cl_2). The mixture was stirred at 0 °C for 30 min and at room temperature for additional 4 h. Dichloromethane was removed under reduced pressure and the remaining solution was diluted with 100 mL of water. The mixture was then extracted with 2 × 100 mL of ethyl acetate. The extracts were washed with 1 N HCl (2 × 100 mL), water (2 × 100 mL), $NaHCO_3$ 5% (3 × 100 mL), and finally with water (2 × 100 mL), dried over $MgSO_4$, filtered and concentrated. The obtained residues were purified by flash column chromatography to provide the corresponding hexylamides (**9** and **10**).

4.3.3.1. *N*-Hexyl-3-(4-hydroxy-3-methoxyphenyl)-2-propenamide (9). Chromatographic solvent: petroleum ether/ethyl acetate (from 9:1 to 5:5); yield: 70% (as oil); IR ν_{max} (NaCl plates, cm^{-1}): 3426 (O–H), 3284 (N–H stretch), 1620 (C=O); ^{1}H NMR δ: 0.86 (3H, *t*, *J* = 6.8, C*H*$_3$), 1.26 (6H, m, 3 × C*H*$_2$(3′–5′)), 1.42 (2H, m, C*H*$_2$(2′)), 3.14 (2H, m, C*H*$_2$(1′)), 3.80 (3H, s, OC*H*$_3$), 6.43 (1H, d, *J* = 15.7, C*H*(α)), 6.78 (1H, d, *J* = 8.2, C*H*(5)), 6.97 (1H, dd, *J* = 8.2; 1.9, C*H*(6)), 7.10 (1H, d, *J* = 1.9, C*H*(2)), 7.30 (1H, d, *J* = 15.7, C*H*(β)), 7.92 (1H, t, *J* = 5.6, N*H*), 9.40 (1H, s, O*H*); ^{13}C NMR δ: 14.0 *C*H$_3$, 22.1, 26.2, 29.2, 31.0 *C*(2′)–*C*(5′), 55.5 OCH$_3$, 38.6 *C*(1′), 110.6 *C*(2), 115.6 *C*(5), 119.1, 121.5 *C*(6) and *C*(α), 126.4 *C*(1), 138.7 *C*(β), 147.8 *C*(4), 148.2 *C*(3), 165.2 *C*=O. EI-MS *m/z*: 277 ($M^{+\cdot}$). For structural data comparison, see Ref. 38.

4.3.3.2. *N*-Hexyl-3-(3,4-dihydroxyphenyl)-2-propenamide (10). Chromatographic solvent: petroleum ether/ethyl acetate (from 8:2 to 5:5); yield: 65%; IR ν_{max} (ATR, cm^{-1}): 3324 (O–H and N–H stretch), 1608 (C=O); ^{1}H NMR δ: 0.87 (3H, t, *J* = 6.7, C*H*$_3$), 1.36 (8H, m, 4 × C*H*$_2$(2′–5′)), 3.14 (2H, m, C*H*$_2$(1′)), 6.32 (1H, d, *J* = 15.7, C*H*(α)), 6.74 (1H, d, *J* = 8.1, C*H*(5)), 6.83 (1H, dd, *J* = 8.1; 1.8, C*H*(6)), 6.93 (1H, d, *J* = 1.8, C*H*(2)), 7.22 (1H, d, *J* = 15.7, C*H*(β)), 7.96 (1H, t, *J* = 5.5, N*H*), 9.14 (1H, s, O*H*(3)), 9.37 (1H, s, O*H*(4)); ^{13}C NMR δ: 14.1 *C*H$_3$, 22.2, 26.3, 29.3, 31.1 *C*(2′)–*C*(5′), 38.6 *C*(1′), 113.9 *C*(2), 115.9 *C*(5), 118.8, 120.5 *C*(6) and *C*(α), 126.6 *C*(1), 139.0 *C*(β), 145.7, 147.4 *C*(3) and *C*(4), 165.4 *C*=O; EI-MS *m/z* (%): 263 ($M^{+\cdot}$, 5), 178 (36), 163 (100), 145 (14), 134 (14), 117 (12), 89 (18), 77 (10); mp 130–133 °C. For structural data comparison, see Ref. 39.

4.3.4. General procedure to obtain the hydrocinnamic hexylesters (11–12)

The hydrocinnamic acids (5.0 mmol), 1-hexanol (0.627 mL, 5.0 mmol), *N*,*N*′-dicyclohexylcarbodiimide (DCC) (1.03 g, 5.0 mmol), 4-(dimethylamino)pyridine (DMAP) (1.22 g, 10.0 mmol) and 30 mL of anhydrous tetrahydrofuran (THF) were stirred in a one-neck round bottom flask at room temperature for 24 h. The mixture was then cooled to 0 °C, and the solid side product, dicyclohexylurea, was separated and removed by vacuum filtration. The filtrate was taken to dryness and the remaining residue was purified by flash column chromatography.

4.3.4.1. Hexyl (*E*)-3-(4-hydroxy-3-methoxyphenyl)propanoate (11). Chromatographic solvent: chloroform/methanol (9:1); yield: 23% (oil); ^{1}H NMR δ: 0.86 (3H, t, *J* = 6.4, C*H*$_3$), 1.26 (6H, m, 3 × C*H*$_2$(3′–5′)), 1.53 (2H, m, C*H*$_2$(2′)), 2.58 (2H, t, *J* = 7.4, C*H*$_2$(α)), 2.74 (2H, t, *J* = 7.4, C*H*$_2$(β)), 3.74 (3H, s, OC*H*$_3$), 3.99 (2H, t, *J* = 6.6, C*H*$_2$(1′)), 6.58 (1H, dd, *J* = 8.0; 1.8, C*H*(6)), 6.67 (1H, d, *J* = 8.0, C*H*(5)), 6.78 (1H, d, *J* = 1.8, C*H*(2)), 8.72 (1H, s, O*H*); ^{13}C NMR δ: 13.9 *C*H$_3$, 22.0, 25.1, 28.2, 30.9 *C*(2′)–*C*(5′), 28.2 *C*(β), 35.6 *C*(α), 55.5 OCH$_3$, 63.7 *C*(1′), 112.4 *C*(2), 115.3 *C*(5), 120.2 *C*(6), 131.3 *C*(1), 144.8 *C*(4), 147.4 *C*(3), 172.4 *C*=O; EI-MS *m/z* (%): 280 ($M^{+\cdot}$, 5), 178 (21), 151 (14), 137 (100), 122 (9), 107 (14), 91 (18), 77 (12), 65 (8). For structural data comparison, see Ref. 40.

4.3.4.2. Hexyl (*E*)-3-(3,4-dihydroxyphenyl)propanoate (12). Chromatographic solvent: diethyl ether; yield: 22%; ^{1}H NMR δ: 0.85 (3H, t, *J* = 6.5, C*H*$_3$), 1.25 (6H, m, 3 × C*H*$_2$(3′–5′)), 1.51 (2H, m, C*H*$_2$(2′)), 2.49 (2H, t, *J* = 7.3, C*H*$_2$(α)), 2.65 (2H, t, *J* = 7.3; C*H*$_2$(β)), 3.97 (2H, t, *J* = 6.6, C*H*$_2$(1′)), 6.42 (1H, dd, *J* = 8.0; 2.0, C*H*(6)), 6.56 (1H, d, *J* = 2.0, C*H*(2)), 6.60 (1H, d, *J* = 8.0, C*H*(5)), 8.64 and 8.71 (2H, s, O*H*(3) and (4)); ^{13}C NMR δ: 13.9 *C*H$_3$, 22.0, 25.0, 28.1, 30.9 *C*(2′)–*C*(5′), 29.8 *C*(β), 35.6 *C*(α), 63.7 *C*(1′), 115.4 *C*(5), 115.6 *C*(2), 118.7 *C*(6), 131.2 *C*(1), 143.5 *C*(4), 145.0 *C*(3), 172.4 *C*=O; EI-MS *m/z* (%): 266 ($M^{+\cdot}$, 55), 182 (44), 138 (41), 136 (79), 123 (100). (lit.[29] mp 67–68 °C). For structural data comparison, see Ref. 29.

4.3.5. General procedure to obtain the cinnamic hexylesters esters (13–14)

Cinnamic acids (11.0 mmol), 25 mL of hexamethylphosphoramide (HMPA) and 2.28 mL of aqueous NaOH 5% were placed in a 100 mL one-neck round bottom flask. After vigorous stirring for about 1 h, a solution of bromohexane (6.18 mL, 44 mmol) in HMPA (10 mL) was added dropwise. The solution was stirred for additional 2 h. After quenching the reaction with addition of water/ice mixture, it was extracted with diethylether (2 × 50 mL). The organic layer was washed twice with HCl 1 M and water, dried over anhydrous magnesium sulphate, filtered and finally evaporated to dryness. The remaining residue was purified by flash column chromatography.

4.3.5.1. Hexyl (*E*)-3-(4-hydroxy-3-methoxyphenyl)-2-propenoate (13). Chromatographic solvent: chloroform/methanol (8:2); yield: 27% (oil); ^{1}H NMR δ: 0.85 (3H, t, *J* = 6.4, C*H*$_3$), 1.28 (6H, m, 3 × C*H*$_2$(3′–5′)), 1.58 (2H, m, C*H*$_2$(2′)), 3.82 (3H, s, OC*H*$_3$), 4.10 (2H, t, *J* = 6.6, C*H*$_2$(1′)), 6.46 (1H, d, *J* = 15.9, C*H*(α)), 6.79 (1H, d, *J* = 8.1, C*H*(5)), 7.10 (1H, dd, *J* = 8.2; 1.9, C*H*(6)), 7.32 (1H, d, *J* = 1.9, C*H*(2)), 7.54 (1H, d, *J* = 15.9, C*H*(β)), 9.59 (1H, br s, O*H*). ^{13}C NMR δ: 13.9 *C*H$_3$, 22.1, 25.2, 28.3, 31.0 *C*(2′)–*C*(5′), 55.6 OCH$_3$, 63.7 *C*(1′), 111.1 *C*(2), 114.5 *C*(5), 115.5 *C*(α), 123.2 *C*(6), 125.6 *C*(1), 145.0 *C*(β), 147.9 *C*(4), 149.3 *C*(3), 166.7 *C*=O; EI-MS *m/z* (%): 278 ($M^{+\cdot}$, 70), 194 (100), 177 (47), 150 (30), 145 (16), 137 (13), 117 (10), 89 (11), 77 (7), 55 (8). The compound was also previously reported by Murakami et al.[41]

4.3.5.2. Hexyl (*E*)-3-(3,4-dihydroxyphenyl)-2-propenoate (14). Chromatographic solvent: chloroform/methanol (9:1); yield: 8%; ^{1}H NMR δ: 0.88 (3H, t, *J* = 6.5, C*H*$_3$), 1.33 (6H, m, 3 × C*H*$_2$(3′–5′)), 1.62 (2H, m, C*H*$_2$(2′)), 4.11 (2H, t, *J* = 6.6, C*H*$_2$(1′), 6.25 (1H, d, *J* = 15.9, C*H*(α)), 6.75 (1H, d, *J* = 8.1, C*H*(5)), 7.00 (1H, dd, *J* = 8.2; 2.0, C*H*(6)), 7.05 (1H, d, *J* = 2.0, C*H*(2)), 7.47 (1H, d, *J* = 15.9, C*H*(β)); ^{13}C NMR δ: 13.9 *C*H$_3$, 22.0, 25.1, 28.2, 30.9 *C*(2′)–*C*(5′), 63.7 *C*(1′), 113.8 *C*(2), 114.7 *C*(5), 115.7 *C*(α), 121.4 *C*(6), 125.3 *C*(1), 145.1 *C*(β), 145.7 *C*(3), 148.7 *C*(4), 166.7 *C*=O; EI-MS *m/z* (%): 264 ($M^{+\cdot}$, 45), 181 (11), 180 (100), 163 (55), 136 (15), 134

(12),117 (7), 89 (10). mp 129–132 °C. For structural data comparison, see Ref. 29.

4.4. Determination of antioxidant activity

4.4.1. 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.[13] Peroxyl radicals were generated as a consequence of thermal decomposition of AAPH.[14] The experimental method was adapted in order to use a multiplate reader.

4.4.1.1. Liposomes preparation. EPC (15.75 mg, 2.25×10^{-5} mol) and DPH-PA (38 μL of a 60% (*m*/*v*) methanolic solution) were 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 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 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) containing the DPH-PA fluorescent probe.

4.4.1.2. Liposomes oxidation. Polyphenol solutions (6.42, 32.14 and 64.28 μM) were prepared 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 LUV/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 was used in the experiment as reference antioxidant.

4.4.1.3. 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 yielding TEAC values:

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

4.4.2. Total antioxidant capacity (TAC) assays

Total antioxidant capacity assays were performed using ABTS and DPPH as radicals. The experimental procedures were adapted from the literature,[16,17,42] in order to use a multiplate reader.

4.4.2.1. ABTS solution. An aqueous solution (25 mL) of ABTS (96.02 mg, 1.75×10^{-4} mol) and potassium persulfate (16.55 mg, 6.12×10^{-5} mol) was left standing overnight allowing to develop the deep blue–green colour of $ABTS^{\cdot+}$.

4.4.2.2. DPPH solution. An ethanolic solution (25 mL) of DPPH (19.13 mg, 4.85×10^{-5} mol) was prepared yielding 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 get absorbance values of 0.45 ± 0.01 at 734 nm and 30 °C, and 0.38 ± 0.01 at 515 nm and 25 °C, when 180 μL samples were placed in the plate reader.

Six different ethanolic solutions of each polyphenol (with concentrations ranging from 1.5×10^{-5} to 15×10^{-5} M) were prepared in duplicate. Each solution (20 μL) was 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 plus 180 μL of radical) was set as 100% of radical (0% bleaching). Trolox was used as a reference antioxidant.

4.4.2.3. 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).

$$\mathrm{TEAC} = \frac{\mathrm{IC}_{50}(\mathrm{trolox})}{\mathrm{IC}_{50}(\mathrm{compound})}$$

4.5. Determination of redox potentials

Solutions used in the electrochemical determinations were obtained as follows: 10 mM stock solutions of the studied compounds were prepared by dissolving them in an appropriate amount in ethanol. The voltammetric working solutions were prepared, in the electrochemical cell, by diluting 100 μL of the stock solution in 10 mL of the supporting electrolyte in order to get a final concentration of 0.1 mM.

The pH 7.3 supporting electrolyte was prepared by diluting 6.2 mL of 0.2 M dipotassium hydrogen phosphate and 43.8 mL of 0.2 M potassium dihydrogen phosphate to 100 mL.

Deionised water with conductivity less than 0.1 μS cm^{-1} was used throughout. Buffer solutions employed were 0.2 M in the pH range 1.2–12.2.[15]

4.6. Determination of partition coefficients

Partition coefficients were determined at pH 7.4 and 25 °C, using liposomes of EPC or micelles of HDPC at pH 2, as previously described.[32,33]

4.7. Statistical analysis

Each experiment was performed at least three times. Results were expressed as the mean ± SEM. Data were analyzed by a one-way analysis of variance (ANOVA). Differences were considered significant where $p < 0.05$.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.06.090. These data

include MOL files and InChiKeys of the most important compounds described in this article.

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