Phenolic Acids and Derivatives: Studies on the Relationship among Structure, Radical Scavenging Activity, and Physicochemical Parameters[†]

Francisco A. M. Silva,[‡] Fernanda Borges,*,[§] Carla Guimarães,[#] José L. F. C. Lima,[#] Carla Matos,[#] and Salette Reis[#]

CEQOFFUP/Departamento de Química Orgânica, Faculdade de Farmácia, Universidade do Porto, 4050-047 Porto, Portugal, and CEQUP/ Departamento de Química-Física, Faculdade de Farmácia, Universidade do Porto, 4050-047 Porto, Portugal

The antiradical activity of caffeic acid (**1**), dihydrocaffeic acid (**5**), and their corresponding *n*-alkyl esters was evaluated by using the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•) method. Dihydrocaffeic acid (**5**) was the most potent compound, having an antiradical effect higher than that of (±)-α-tocopherol, whereas caffeic acid (**1**) was less efficient. Esterification of the carboxyl group of dihydrocaffeic acid (**5**) had a dramatic effect on its antiradical potency, but similar effects were not observed for caffeic acid (**1**) derivatives. The *n*-alkyl esters of both phenolic series had similar potencies, and their antiradical activities were independent of the alkyl chain length. Dose-dependent scavenger effects were found in both series. Acid–base properties of the compounds, evaluated by using potentiometry and spectrophotometry, showed that the catechol moiety had pK_{a2} and pK_{a3} values of 9.24–9.02 and 11.38–10.99 in the dihydrocaffeic series and 8.48–8.24 and 11.38–11.07 in the caffeic series, respectively. Antiradical activity and pK_a values of the compounds were not related.

Keywords: *Caffeic acid; dihydrocaffeic acid; n-alkyl esters; antiradical activity; 2,2-diphenyl-1-picrylhydrazyl radical; dissociation constants; structure–property–activity*

INTRODUCTION

Among naturally occurring phenolic compounds, phenolic acids and flavonoids are of particular interest because of their potential biological properties, such as anti-inflammatory, antiallergic, antimicrobial, anticarcinogenic, and antiviral activities (Castellucio et al., 1996; Rice-Evans et al., 1996; Laranjinha et al., 1994).

Many phenolic acids (e.g., cinnamic acids) are also known to be potent antioxidants, probably through their radical scavenging activity, although other mechanisms may be involved. The antiradical activity of phenolic compounds depends on their molecular structure, that is, on the availability of phenolic hydrogens and on the possibility for stabilization of the resulting phenoxyl radicals formed by hydrogen donation (Mathiesen et al., 1997; Rice-Evans et al., 1996). In fact, preliminary structure–activity relationship studies on cinnamic acids and derivatives have pointed out the importance of the catechol group to the antiradical efficacy (Moon and Terao, 1998; Chen and Ho, 1997; Brand- Williams et al., 1995; Graf, 1992). The role of the ethylenic side chain of this type of phenolic compounds in their radical scavenging properties remains controversial. Some studies suggest that this structural feature is important for the activity because it could participate in the stabilization by resonance of the phenoxyl radical formed in the process, whereas others claim that the conjugated olefinic double bound is not a requirement for their efficacy (Chen et al., 1999; Moon and Terao, 1998; Chen and Ho, 1997; von Gadow et al., 1997; Cuvelier et al., 1992).

As the information on this area of research is sparse and not fully understood, a fundamental study on the structure–activity of cinnamic compounds was deemed to be necessary to clarify some aspects related with their reactivity. Therefore, the aim of our work was to synthesize phenolic acid derivatives, (re)evaluate their antiradical properties, and try to elucidate the relationship among their activity, chemical structure, and physicochemical parameters.

The present study was performed with caffeic acid [*trans*-3-(3,4-dihydroxyphenyl)-2-propenoic acid] (**1**) and its metabolite, a hydrogenated analogue known as dihydrocaffeic acid [3-(3,4-dihydroxyphenyl)propanoic acid] (**5**) (Petrou, 1993). Structure modification of the lead compounds was done by homologation. The homologous series of *n*-alkyl esters synthesized is found to be suitable for the establishment of a ranking order of efficacy and to define the chemical features required for antiradical activity (Figure 1). (±)-α-Tocopherol, a known native chain-breaking antioxidant, was used as reference in this comparative study.

The efficiency of the phenolic acids and their alkyl esters (Figure 1) as radical scavengers was evaluated

* Address correspondence to this author at Faculdade de Farmácia do Porto Rua Aníbal Cunha 164, 4050-047 Porto, Portugal (telephone 351-22-2078900; fax 351-22-2003977; e-mail fborges@ff.up.pt).

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[‡] Permanent address: Instituto Superior de Ciências da Saúde–Norte, Rua Central de Gandra 1317, 4580 Paredes, Portugal.

[§] CEQOFFUP.

[#] CEQUP.

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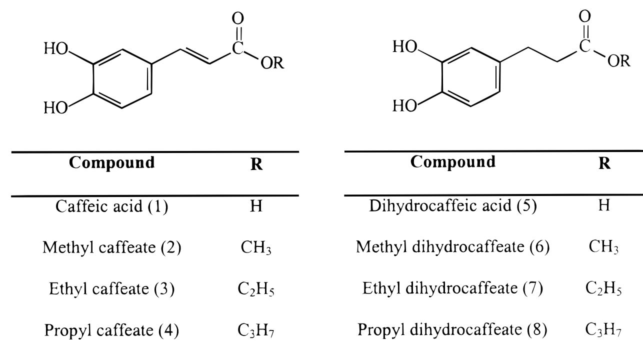

Compound	R
Caffeic acid (1)	H
Methyl caffeate (2)	CH_3
Ethyl caffeate (3)	C_2H_5
Propyl caffeate (4)	C_3H_7

Compound	R
Dihydrocaffeic acid (5)	H
Methyl dihydrocaffeate (6)	CH_3
Ethyl dihydrocaffeate (7)	C_2H_5
Propyl dihydrocaffeate (8)	C_3H_7

Figure 1. Chemical structures of phenolic acids and alkyl esters.

by their reactivity toward a stable free radical, 2,2-diphenyl-1-picrylhydrazyl (DPPH•). The DPPH• test is a nonenzymatic method currently used to provide basic information on the reactivity of compounds to scavenge free radicals (Nanjo et al., 1996; Brand-Williams et al., 1995; Bors et al., 1984). The electronic effects of the substituents of these series of compounds on antiradical activity were evaluated by assessing their dissociation constants by potentiometry and spectrophotometry.

MATERIALS AND METHODS

Chemicals. Caffeic acid (**1**), dihydrocaffeic acid (**5**), (±)-α-tocopherol, and DPPH• were obtained from Sigma-Aldrich Quimica S.A. (Sintra, Portugal). All other reagents and solvents were of pro analysis grade, purchased from Merck (Lisbon, Portugal).

Apparatus. Synthesized compounds were identified by FTIR, UV, NMR, and EI-MS. Infrared spectra were recorded on a ATI Mattson Genesis series FTIR spectrophotometer using potassium bromide disks; only the most significant absorption bands are reported (υ_{max}, cm^{-1}). Ultraviolet spectra were acquired on a UV−vis Varian Cary 1E spectrophotometer; absorption bands (λ_{max}) are reported in nanometers (ethanolic solutions). ^{1}H and ^{13}C NMR data were acquired, at room temperature, on a Brüker AMX 300 spectrometer operating at 300.13 and 75.47 MHz, respectively. Dimethyl sulfoxide-d_6 was used as solvent; chemical shifts are expressed in δ (parts per million) values relative to tetramethylsilane (TMS) as internal reference; coupling constants (J) are given in hertz. Electron impact mass spectra (EI-MS) were obtained on a VG AutoSpec instrument; the data are reported as m/z (percent of relative intensity of the most important fragments). Melting points were obtained on a Köfler microscope (Reichert Thermovar) and are uncorrected.

General Synthetic Procedure. The alkyl esters of caffeic and dihydrocaffeic acids were synthesized by Fischer esterification following the procedure described in Borges and Pinto (1994). The structural data of ethyl caffeate (**3**) were in accordance with those reported in the literature (Borges and Pinto, 1994).

*Methyl Caffeate (**2**).* Yield, 65%; FTIR υ_{max} (cm^{-1}) 3476, 2944, 2364, 2338, 1675,1607, 1535, 1441, 1277, 1247, 1183, 1105, 1038, 981; UV λ_{max} (nm) (log ϵ) 332 (4.2), 245 (4.0), 217 (4.1), 202 (4.0); ^{1}H NMR δ 3.68 (3H, s, OCH$_3$), 6.27 [1H, d, J = 15.9, H(α)], 6.76 [1H, d, J = 8.2, H(5)], 7.00 [1H, dd, J = 8.2, 2.1, H(6)], 7.05 [1H, d, J = 2.1, H(2)], 7.48 [1H, d, J = 15.9, H(β)], 9.33−9.45 (2H, br s, OH); ^{13}C NMR δ 51.2 OCH$_3$, 113.7 C(2), 114.8 C(α), 115.7 C(5), 121.4 C(6), 125.5 C(1), 145.2 C(4), 145.6 C(3), 148.4 C(β), 167.0 (C=O); EI-MS, m/z (%) 194 (M$^{+•}$, 100), 163 (100), 136 (22), 135 (28), 134 (36), 123 (10), 117 (23), 89 (29), 77 (23); mp, 156−159 °C.

*Propyl Caffeate (**4**).* Yield, 62%; FTIR υ_{max} (cm^{-1}) 3462, 2969, 2895, 1665, 1605, 1534, 1476, 1442, 1319, 1278, 1239, 1184, 1155, 1111, 1037, 982; UV λ_{max} (nm) (log ϵ) 331 (4.2), 218 (4.2), 202 (4.3); ^{1}H NMR δ 0.91 (3H, t, J = 7.4, CH$_3$), 1.63 (2H, m, CH$_2$), 4.06 (2H, t, J = 6.7, OCH$_2$), 6.26 [1H, d, J = 15.9, H(α)], 6.75 [1H, d, J = 8.0, H(5)], 7.00 [1H, dd, J = 8.1, 2.0, H(6)], 7.04 [1H, d, J = 2.0, H(2)], 7.47 [1H, d, J = 15.9, H(β)], 9.19−9.56 (2H, br s, OH); ^{13}C NMR δ 10.3 CH$_3$, 21.7 CH$_2$, 65.2 OCH$_2$, 113.9 C(2), 114.8 C(α), 115.7 C(5), 121.4 C(6), 125.5 C(1), 145.0 C(4), 145.6 C(3), 148.4 C(β), 166.6 (C=O); EI-MS, m/z (%) 222 (M$^{+•}$, 90), 180 (81), 163 (100), 136 (54), 135 (37), 134 (43), 123 (15), 117 (24), 89 (40), 77 (22); mp, 125−127 °C.

*Methyl Dihydrocaffeate (**6**).* Yield, 68%; FTIR υ_{max} (cm^{-1}) 3481, 3309, 2944, 1711, 1607, 1516, 1443, 1378, 1342, 1311, 1276, 1205, 1181, 1106, 984; UV λ_{max} (nm) (log ϵ) 283 (3.5), 204 (4.4), ^{1}H NMR δ 2.51 [2H, t, J = 7.4, H(α)], 2.66 [2H, t, J = 7.3, H(β)], 3.56 (3H, s, OCH$_3$), 6.42 [1H, dd, J = 8.0, 2.1, H(6)], 6.56 [1H, d, J = 2.0, H(2)], 6.61 [1H, d, J = 8.0, H(5)], 8.71 (2H, br s, OH); ^{13}C NMR δ 29.7 C(α), 35.4 C(β), 51.2 OCH$_3$, 115.5 C(2)*, 115.6 C(5)*, 118.7 C(6), 131.3 C(1), 143.5 C(4), 145.0 C(3), 172.8 (C=O) (*, assignment interchangeable); EM-IE, m/z (%) 196 (M$^{+•}$, 82), 165 (22), 137 (23), 136 (85), 123 (100), 91 (22), 77 (24); mp, 69−71 °C.

*Ethyl Dihydrocaffeate (**7**).* Yield, 65%; FTIR υ_{max} (cm^{-1}) 3490, 3290, 2981, 1701, 1608, 1516, 1446, 1373, 1342, 1269, 1215, 1176, 1107, 953; UV λ_{max} (nm) (log ϵ) 283 (3.5), 204 (4.4); ^{1}H NMR δ 1.15 (3H, t, J = 7.1, CH$_3$), 2.46 [2H, br t, H(α)], 2.65 [2H, t, J = 7.4, H(β)], 4.02 (2H, m, J = 7.1, OCH$_2$), 6.43 [1H, dd, J = 8.0, 1.7, H(6)], 6.56 [1H, d, J = 1.7, H(2)], 6.60 [1H, d, J = 8.0, H(5)], 8.69−8.75 (2H, br s, OH); ^{13}C NMR δ 14.2 CH$_3$, 29.8 C(α), 35.6 C(β), 59.8 OCH$_2$, 115.5 C(2)*, 115.7 C(5)*, 118.8 C(6), 131.3 C(1), 143.5 C(4), 145.0 C(3), 172.4 (C=O) (*, assignment interchangeable); EI-MS, m/z (%) 194 (M$^{+•}$, 100), 163 (100), 136 (22), 135 (28), 134 (36), 123 (10), 117 (23), 89 (29), 77 (23); mp, 49−51 °C.

*Propyl Dihydrocaffeate (**8**).* Yield, 70%; FTIR υ_{max} (cm^{-1}) 3427, 3350, 2969, 2883, 1710, 1609, 1523, 1448, 1356, 1279, 1197, 1112, 984; UV λ_{max} (nm) (log ϵ) 282 (3.5), 204 (4.4); ^{1}H NMR δ 0.84 (3H, t, J = 7.4, CH$_3$), 1.54 (2H, m, CH$_2$), 3.93 (2H, t, J = 6.6, OCH$_2$), 2.50 [2H, t, J = 7.5, H(α)], 2.66 [2H, t, J = 7.3, H(β)], 6.43 [1H, dd, J = 8.0, 2.1, H(6)], 6.57 [1H, d, J = 2.1, H(2)], 6.61 [1H, d, J = 8.0, H(5)], 8.69 (2H, br s, OH); ^{13}C NMR δ 10.3 CH$_3$, 21.5 CH$_2$, 65.3 OCH$_2$, 29.8 C(α), 35.6 C(β), 115.4 C(2)*, 115.6 C(5)*, 118.8 C(6), 131.3 C(1), 143.5 C(4), 145.0 C(3), 172.4 (C=O) (*, assignment interchangeable); EI-MS, m/z (%) 224 (M$^{+•}$, 95), 182 (49), 165 (84), 137 (54), 136 (93), 123 (100), 91 (48), 77 (31); slightly yellow viscous oil.

Free Radical Scavenging Activity on DPPH•. The free radical scavenging activities of tested compounds were measured using the DPPH radical method. The experimental procedure was adapted from Ohnishi et al. (1994). Special care was taken to minimize the loss of free radical activity of the DPPH• solution, as recommended by Blois (1958). Spectrophotometric data were acquired at room temperature using a UV-160 Shimadzu dual-beam spectrophotometer and disposable cells from ATI Unicam (Porto, Portugal). For each compound and concentration tested (50, 100, 200, 400, and 800 μM), the reduction of DPPH• was followed by monitoring the decrease of absorbance at 517 nm until the reaction reached a plateau (steady state). The percentage of remaining DPPH• was then calculated, and the radical scavenging effects of the tested compounds were compared on the basis of 1/IC$_{50}$ (IC$_{50}$ represents the concentration needed to reduce 50% of the initial amount of DPPH•, and it was expressed as the molar ratio of each compound to radical). All tests were performed in triplicate. Statistical comparisons were by one-way analysis of variance (ANOVA), followed by Fisher's PLSD test. The level of significance was set at $P < 0.05$.

Potentiometric Determination of Dissociation Constants. Potentiometric measurements were carried out with a Crison 2002 pH meter and 2031 buret controlled by a personal computer, which was also used for data manipulation. The electrode assembly was made up of an Orion 900029/4 AgCl/Ag reference electrode and a Russell SWL glass electrode. System calibration was performed according to the Gran method (Gran, 1952) in terms of hydrogen ion concentration, using strong acid/strong base titrations [HCl (0.001 M)/NaOH (≈0.02 M)] with solutions having adjusted (with NaCl) ionic strengths of 0.1 M. Titrations were always carried out under

Table 1. Scavenging Effects of Phenolic Acids and Alkyl Esters on the 2,2-Diphenyl-1-picrylhydrazyl Radical

compound	time to reach steady state[a] (min)	$1/IC_{50}$[b] (mean value ± SD)
1	10−35	2.50 ± 0.04[a]
2	2−32	3.94 ± 0.05[b]
3	3−22	4.22 ± 0.02[c]
4	16−27	4.12 ± 0.02[d]
5	8−35	7.58 ± 0.04[e]
6	3−35	3.77 ± 0.02[f]
7	4−30	4.03 ± 0.01[g]
8	4−33	3.85 ± 0.01[h]
(±)-α-tocopherol	7−20	3.94 ± 0.03[b]

[a] In the range of concentrations (50−800 μM) (see Materials and Methods). [b] Values with the same superscript are not significantly different at $P < 0.05$.

a nitrogen atmosphere at 25 °C in a double-walled glass cell. Acidity constants of the compounds were obtained by titrating 20 mL of acidified solutions (1 mM HCl) of the phenols (0.8−1 mM) with NaOH (≈0.02 M). All titrations were performed at 25 °C under nitrogen, and for all solutions the ionic strength was adjusted to 0.1 M with NaCl. System calibration was always performed by titration of HCl with NaOH, before and after each determination. Calculations were performed with data obtained from at least six independent titrations, each with >30 points, and the experimental titration data were analyzed using the computer program Superquad (Gans et al., 1985). The reported errors were calculated according to the method of Albert and Serjeant (1971) as the maximum difference between the logarithm of the average of the antilogarithms of the calculated pK_a values and their individual values.

Spectrophotometric Determination of Dissociation Constants. All absorption spectra were recorded with a Hitachi U-2000 dual-beam spectrophotometer using quartz cells with 1 cm path length that were thermostated at 25 °C. Dissociation constants of the compounds were obtained from UV data of solutions of phenols (5×10^{-5} M), for which the ionic strength was adjusted to 0.1 M with NaCl. Aliquots of strong base or strong acid were added to 20 mL of the stock solution to adjust $-\log[H^+]$ to the desire value; $-\log[H^+]$ measurements and system calibration were performed by potentiometry as described above. The calculations were performed with the program SQUAD 85 (Legget and MacBryde, 1975) by using data from at least two independent experiments, each with more than six solutions, and in the range from 200 to 500 nm at 2 nm intervals.

RESULTS AND DISCUSSION

Antiradical Activity. The phenolic compounds (**1**−**8**) and (±)-α-tocopherol were examined for their radical scavenging activity toward the stable free radical DPPH•.

All of the compounds (Table 1) had significant antiradical scavenger activity compared with (±)-α-tocopherol. Dihydrocaffeic acid (**5**) was the most potent compound, having an antiradical effect higher than that of (±)-α-tocopherol, whereas caffeic acid (**1**) was less efficient. These results are in agreement with those of Chen et al. (1999), which reinforces the idea that the ethylenic side chain of the aromatic ring may not be an important factor influencing the antiradical behavior of this family of compounds.

The structural modification of the carboxyl group by esterification affected the antiradical activity of phenolic acids **1** and **5** in a different way. Caffeates (**2**−**4**) had a higher antiradical potency when compared to the corresponding phenolic acid, whereas esterification of dihydrocaffeic acid markedly led to a dramatic decrease in its scavenging activity. The alkyl esters of both phenolic series had similar efficacies, in a range of values placed between the activities of their precursors. The activity was independent of the alkyl chain length.

The steady state of the reaction between DPPH• and the phenolic compounds or (±)-α-tocopherol was reached in <35 min. (Table 1). Dose-dependent scavenging effects were found in both series. However, the kinetics of the reaction was dependent on the concentration and structural type of the compound. Figure 2 shows the kinetic behavior of caffeic (**1**) and dihydrocaffeic (**5**) acids as well as their ethyl esters **3** and **7**, respectively.

Although further studies on structure−activity are required to confirm the previous findings, it is our belief that molecular conformation of the phenolic compounds could be one of the factors affecting their antiradical activity, which is intrinsically related to DPPH•.

Dihydrocaffeic acid (**5**) has a side chain connected to the aromatic ring by single bonds, which allows the phenyl group to have a certain flexibility to rotate. Therefore, the phenomena observed could be interrelated with the folding of the side chain of **5** onto the phenyl ring, whereas caffeic acid (**1**) has a coplanar conformation. When the carboxyl group of **5** was esterified, the rotation of the phenyl moiety may have been restrained to a degree that depends on the nature of the substituents and their size and position, leading to conformational modification. Studies of molecular modeling on the compounds of the dihydrocaffeic series showed that the potential energy levels (kilocalories per mole) associated with the lowest energy conformation were 11.15 (**5**), 13.12 (**6**), 14.39 (**7**), and 14.24 (**8**), which was in agreement with previous statements.

Dissociation Constants. To have insight into the mechanism that controls the antiradical activity of the phenolic compounds **1**−**8** (Figure 1), their dissociation constants were evaluated by potentiometry and spectrophotometry. Spectrophotometric determinations were done to validate the pK_{a3} value obtained by potentiometry.

Table 2 shows that the acidity of the phenols under study was affected by electronic influences such as substituent dipolar field/inductive properties, π-electron delocalization, and polarizability effects. The catechol moiety of the phenolic compounds had pK_{a2} and pK_{a3} values of 9.24−9.02 and 11.38−10.99 in the dihydrocaffeic series and 8.48−8.24 and 11.38−11.07 in the caffeic series, respectively. The pK_{a3} values of the catechol group were similar in both series.

The dissociation constants of dihydrocaffeic acid (**5**) and caffeic acid (**1**) and those found in the literature were similar (Petrou, 1993; Bell et al., 1991; John et al.,1990; Linder and Voyé, 1987; Bizri et al., 1985). The pK_{a2} of the hydroxyl group of caffeic acid (**1**) was more acidic than the corresponding group in dihydrocaffeic acid (**5**), suggesting that some electron-withdrawing effect of the carboxyl moiety was operative across the double bond of the side chain. Therefore, the dissociation constants of the catechol group in caffeic acid were assigned as $pK_{a2} = 8.48$ (*p*-OH) and $pK_{a3} = 11.38$ (*m*-OH), which contrast to those proposed by John et al. (1990).

These studies on structure−property−activity indicate no relationship between the antiradical activity and pK_a values of the compounds. It can be concluded that this parameter is apparently not a major determining factor for the activity of the phenolic compounds and that other physicochemical properties, for instance,

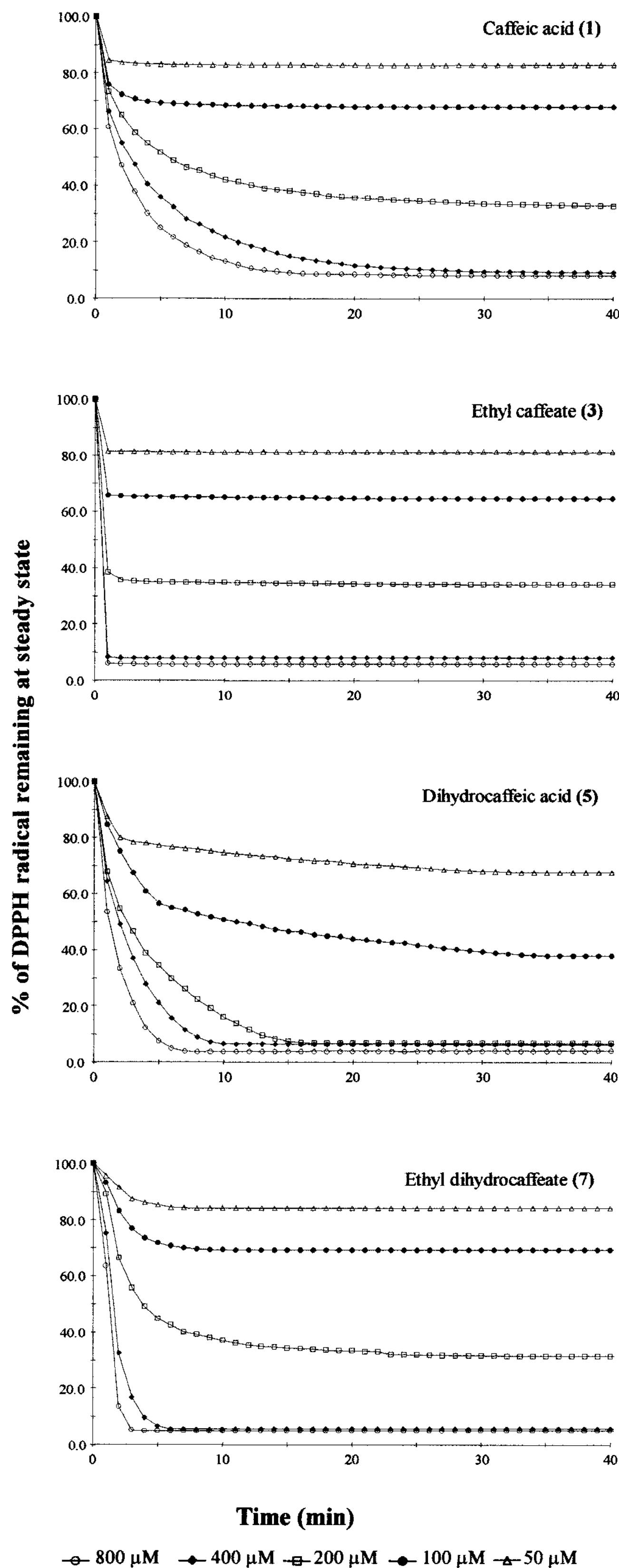

Figure 2. Time course for DPPH• scavenging by phenolic acids and derivatives.

redox potential, could control their antiradical activity. Nevertheless, these data could be a useful tool for bioavailability and pharmacokinetics studies because some of these compounds are intrinsic components of diet (Laranjinha et al., 1994).

Knowledge of the driving forces related with antiradical and/or antioxidant behavior of these compounds is worthy of research because it could be a very important basis to explain some of their biological properties, especially those related with deleterious oxidative processes. As the literature affords only very limited studies on the structure−property−antiradical and/or antioxidant activity relationships, it is our belief that more information is needed to understand the mechanism of their antiradical action. The evaluation of other physicochemical parameters such as partition properties and redox potentials is being carried out to obtain a suitable database to achieve the goal.

Table 2. Dissociation Constants of Phenolic Acids and Alkyl Esters

compound	pK_{a1}[a]	pK_{a2}[a]	pK_{a3}[a]	pK_{a3}[b]
1	4.36 ± 0.03	8.48 ± 0.05	11.17 ± 0.30	11.38 ± 0.02
2		8.35 ± 0.05	11.40 ± 0.30	11.22 ± 0.03
3		8.29 ± 0.02	11.98 ± 0.90	11.17 ± 0.01
4		8.24 ± 0.03	11.24 ± 0.10	11.07 ± 0.04
5	4.43 ± 0.02	9.24 ± 0.02	11.38 ± 0.20	11.38 ± 0.02
6		9.18 ± 0.01	11.13 ± 0.18	11.21 ± 0.02
7		9.16 ± 0.03	11.12 ± 0.05	11.14 ± 0.01
8		9.02 ± 0.01	10.84 ± 0.21	10.99 ± 0.02

[a] Dissociation constants obtained by potentiometry at 25 °C and I = 0.1 M in NaCl. [b] Dissociation constants obtained by spectrophotometry at 25 °C and I = 0.1 M in NaCl.

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