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Determination of hydrogen peroxide scavenging activity of cinnamic and benzoic acids employing a highly sensitive peroxyoxalate chemiluminescence-based assay: Structure–activity relationships

Abdelhak Mansouri [a], Dimitris P. Makris [a,b], Panagiotis Kefalas [a,*]

[a] *Department of Food Quality Management & Chemistry of Natural Products, Mediterranean Agronomic Institute of Chania (M.A.I.Ch.), P.O. Box 85, 73100 Chania, Greece*

[b] *Department of Science of Nutrition Dietetics, Harokopio University, 70 El. Venizelou Str.,17671 Kallithea, Athens, Greece*

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Abstract

A series of cinnamic acids along with their corresponding benzoate analogues were tested for their ability to scavenge hydrogen peroxide (H_2O_2), by using a highly sensitive, peroxyoxalate chemiluminescence assay. Among benzoic acid derivatives, vanillic acid (3-hydroxy-4-methoxybenzoic acid) was found to be the most efficient H_2O_2 scavenger with its hydrogen peroxide scavenging activity (SA_{HP}) being 170.20 μM^{-1}, whereas protocatechuic acid (3,4-dihydroxybenzoic acid) exhibited the weakest activity (5.90 μM^{-1}). Caffeic acid (3,4-dihydroxycinnamic acid) was the strongest antioxidant amongst cinnamate derivatives with a $SA_{HP} = 8.2\ \mu M^{-1}$, as opposed to *m*-coumaric acid (2-hydroxycinnamic acid), which was found to be a poor hydrogen peroxide scavenger ($SA_{HP} = 0.18\ \mu M^{-1}$). Comparison between the two groups revealed that benzoate derivatives are much stronger hydrogen peroxide quenchers in relation with their cinnamate analogues, and this finding was discussed on a basis of structure–activity relationships and comparative assessment of other antioxidant characteristics.

Keywords: Antioxidants; Benzoates; Chemiluminescence; Cinnamates; Hydrogen peroxide; Polyphenols

1. Introduction

Currently, the concept of healthy nutrition has embraced the consumption of fresh fruit and vegetables, which have been proven to contribute in maintaining an improved antioxidant status, thus preventing the onset of degenerative processes [1,2]. One of the most prominent class of dietary antioxidants, polyphenols, have thoroughly been examined for their potency both in vitro and in vivo, but in many instances, the antioxidant profile of a given compound may lack consistency and completeness, because the credible evaluation of a compound to act as an antioxidant largely depends on the model system used, but also requires tests investigating different aspects of antioxidant activity [3,4].

Over the past few years, there has been a great amount of studies reporting on methods for assessing the antioxidant activity of various polyphenols, but it appears that most of the tests established for in vitro examination are rather oriented at the radical scavenging, whereas the ability of those substances in preventing oxidations through non-radical reactions has been given much less attention or disregarded [5,6]. This option, however, is of high significance as unilateral assessment may provide misleading or inadequate information, since many polyphenols exhibit multifunctionality, acting by more than one manner.

Hydrogen peroxide (H_2O_2) is a biologically relevant, non-radical, oxidising species, and may be formed in tissues

Abbreviations: CL, chemiluminescence; EtOAc, ethyl acetate; I_{CL}, chemiluminescence intensity; MeCN, acetonitrile; S.D., standard deviation; TCPO, bis(trichlorophenyl)oxalate; 9,10-DMA, 9,10-dimethylanthracene; 2,4,6-TCP, 2,4,6-trichlorophenol

* Corresponding author. Tel.: +30 28210 35056; fax: +30 28210 35001.
E-mail address: panos@maich.gr (P. Kefalas).

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through oxidative processes, but there has been limited information regarding its scavenging by polyphenolic antioxidants. The present study was undertaken to investigate the hydrogen peroxide scavenging activity of several simple phenolic compounds, including a series of benzoate derivatives and their hydroxycinnamate analogues. The choice was based on particular structural features, so as to distinguish plausible structure–activity relationships. The assessment was accomplished employing a previously established, peroxyoxalate chemiluminescence assay [7], which provides very high specificity, thus eliminating effects that might be caused by radical reactions. Furthermore, the assay employed also presents very high sensitivity, allowing the utilization of particularly low amounts of antioxidants, like those that may be encountered in biological matrices.

2. Materials and methods

2.1. Chemicals

Acetonitrile (MeCN) and ethyl acetate (EtOAc) were of HPLC grade. Water was nanopure. 9,10-Dimethylanthracene (9,10-DMA), imidazole, hydrogen peroxide (H_2O_2), bis(2,3,6-trichlorophenyl)oxalate (TCPO), gallic acid, protocatechuic acid, *p*-hydroxybenzoic acid, *m*-hydroxybenzoic acid, vanillic acid, caffeic acid, ferulic acid, *p*-coumaric acid, *m*-coumaric acid, hydrocaffeic acid and L-phenylalanine were from Sigma Chemical Co. (St. Louis, MO).

2.2. Preparation of reactants and solutions

All reactants for the chemiluminescence (CL) assay were prepared in EtOAc/MeCN (9:1). Solutions of test compounds were prepared in MeCN, with the exception of L-phenylalanine, which was dissolved in 0.05 mol L^{-1} NaOH.

2.3. Apparatus

CL measurements were carried out by using a JENWAY 6200 fluorimeter (Jenway Ltd., Gransmore, Essex, UK), keeping the lamp off and using only the photomultiplier of the apparatus.

2.4. Analytical protocol

A previously established methodology was used [7]. An aliquot of 1.8 mL DMA (0.5 mM) was mixed with 0.2 mL imidazole solution (4.5 mM) and 0.025 mL H_2O_2 (2.25 mM). This mixture was called as mixture A. In a 1 cm path length cuvette, 0.2 mL of TCPO solution (0.45 mM) and 0.05 mL of test compound were placed, and mixture A was immediately added and mixed well for 5 s. CL was continuously monitored until it reached a plateau. Reference samples were tested by adding 0.05 mL of solvent instead of antioxidant solution.

2.5. Determinations and statistics

By plotting I_0/I against concentration, an equation of the following type was obtained:

$$\frac{I_0}{I} = a \times C \pm b \quad (1)$$

where I_0 is the I_{CL} without antioxidant added and I is the I_{CL} after addition of a certain amount of antioxidant. Assignments "*a*" and "*b*" represent the gradient and the intercept of the equation, respectively. Setting $I_0/I = 2$, it was possible to calculate the amount of each antioxidant to provoke 50% reduction in I_{CL} (IC_{50}). Hydrogen peroxide scavenging activity (SA_{HP}) was defined as:

$$SA_{HP} = \frac{1}{IC_{50}} \quad (2)$$

SA_{HP} was expressed as μM^{-1}.

All measurements were performed at least in triplicate and values were averaged. Results are given as means ± standard deviation (S.D.). All correlations were established using linear regression analysis, employing Microsoft Excel™ 2000 software.

3. Results

In order to obtain credible information about the structural features that are involved in hydrogen peroxide scavenging by cinnamates, four representative derivatives were selected (Fig. 1, structures 1–4). In the same context, four corresponding benzoate analogues were chosen (structures 5–8), to assess the effect of the side chain. For comparison reasons, three other structurally similar compounds, L-phenylalanine, hydrocaffeic acid and gallic acid (structures 9–11) were also tested. Because the scavenging efficiency of the compounds varied largely, different concentration ranges were used to established correlations between I_0/I and concentration. The ranges, as well as correlation coefficients are given analytically in Table 1. For certain components including *m*-hydroxybenzoic, *p*-coumaric and *m*-coumaric acids no satisfactory linearity could be obtained, a phenomenon that has been observed for other CL reactions too (e.g. luminol), and attributed to the nature of the component under examination [18].

Within the group of benzoates, vanillic acid exhibited remarkable SA_{HP} of $170.20 \times 10^{-2}\ \mu M^{-1}$, a value which was 2.1-fold higher from that of gallic acid, the second most efficient benzoate derivatives (Table 2). The weakest activity was seen for protocatechuic acid ($5.90 \times 10^{-2}\ \mu M^{-1}$), which was 28.8-fold lower of vanillic acid. The monosubstituted *p*-hydroxybenzoic and *m*-hydroxybenzoic acids showed intermediate values, with the latter being slightly more effective. Overall, the ranking was vanillic acid > gallic acid > *m*-hydroxybenzoic acid > *p*-hydroxybenzoic acid > protocatechuic acid. The scavenging

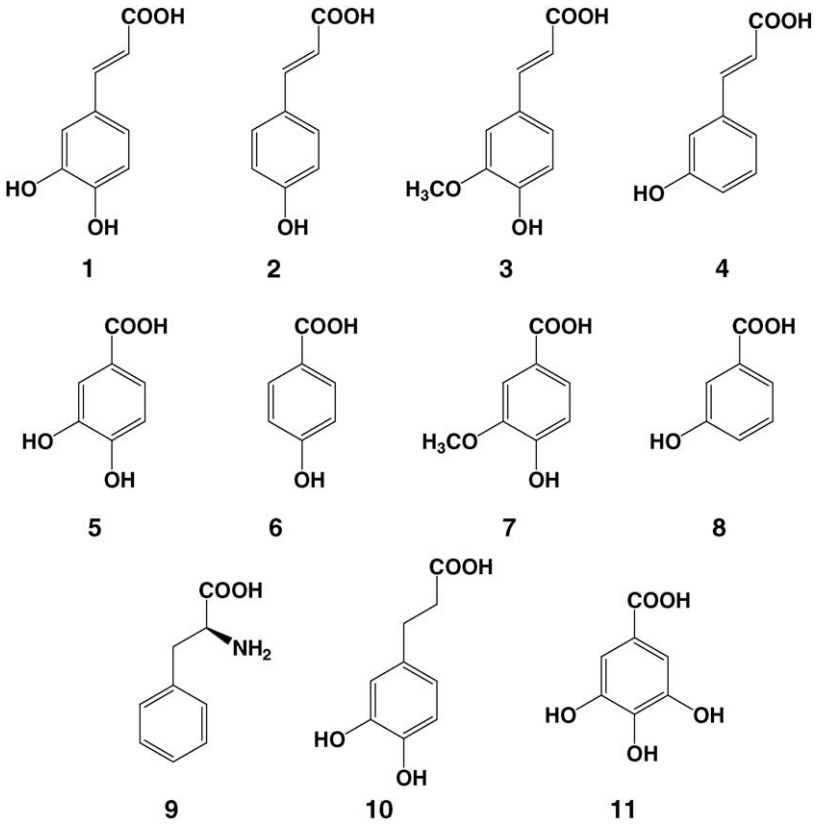

Fig. 1. Chemical structures of the phenolic acids used in this study. *Assignments:* (1) caffeic acid; (2) *p*-coumaric acid; (3) ferulic acid; (4) *m*-coumaric acid; (5) protocatechuic acid; (6) *p*-hydroxybenzoic acid; (7) vanillic acid; (8) *m*-hydroxybenzoic acid; (9) L-phenylalanine; (10) hydrocaffeic acid; (11) gallic acid.

pattern within the cinnamates group was completely different, evidencing a profound influence of the side chain. In particular, it was observed that the most active derivative was hydrocaffeic acid ($SA_{HP} = 12.6 \times 10^{-2}\ \mu M^{-1}$), which lacks the side chain double bond (Table 2). Caffeic acid had a SA_{HP} value 1.5-fold lower, whereas all the other derivatives exhibited activities that were from 11.5- to 70-fold lower than that of hydrocaffeic acid, except L-phenylalanine which had a 2.9-fold lower value. The order of efficacy was hydrocaffeic acid > caffeic acid > L-phenylalanine > ferulic acid > *p*-coumaric acid > *m*-coumaric acid. Comparison between the two groups indicated that benzoic acid derivatives appeared far more effective in scavenging hydrogen peroxide, but another point that should be emphasized is that SA_{HP} was differentially affected by the benzene ring substitution pattern within the two groups (Fig. 2).

Table 1
Analytical data used for the establishment of the calibration curves (I_0/I vs. concentration) for the determination of the hydrogen peroxide scavenging activity of the phenolic acids

Phenolic acid	Range (M)	Linearity (r^2)
Benzoates		
Gallic acid	0.04–0.22 ($\times 10^{-6}$)	0.9999
Protocatechuic acid	1–100 ($\times 10^{-6}$)	0.9627
p-Hydroxybenzoic acid	0.1–2 ($\times 10^{-6}$)	0.9549
m-Hydroxybenzoic acid	0.1–2 ($\times 10^{-6}$)	0.9043
Vanillic acid	0.075–0.75 ($\times 10^{-6}$)	0.9790
Hydroxycinnamates		
Caffeic acid	5–50 ($\times 10^{-3}$)	0.9979
p-Coumaric acid	50–500 ($\times 10^{-3}$)	0.9095
m-Coumaric acid	50–500 ($\times 10^{-3}$)	0.9026
Ferulic acid	10–100 ($\times 10^{-3}$)	0.9596
Hydrocaffeic acid	1–100 ($\times 10^{-6}$)	0.9997
L-Phenylalanine	2–50 ($\times 10^{-3}$)	0.9932

Table 2
Hydrogen peroxide scavenging activity (SA_{HP}) of the phenolic acids tested

Phenolic acid	IC_{50} (μM)	SA_{HP} ($\times 10^{-2}\ \mu M^{-1}$)
Benzoates		
Gallic acid	1.24 ± 0.02	80.50 ± 1.20
Protocatechuic acid	17.06 ± 1.08	5.90 ± 0.40
p-Hydroxybenzoic acid	2.24 ± 0.19	45.04 ± 3.92
m-Hydroxybenzoic acid	1.82 ± 0.04	55.0 ± 1.30
Vanillic acid	0.59 ± 0.05	170.2 ± 14.5
Hydroxycinnamates		
Caffeic acid	12.21 ± 0.48	8.20 ± 0.30
p-Coumaric acid	435.00 ± 35.35	0.23 ± 0.00
m-Coumaric acid	559.55 ± 49.30	0.18 ± 0.00
Ferulic acid	90.37 ± 8.11	1.10 ± 0.10
Hydrocaffeic acid	7.91 ± 0.66	12.6 ± 1.0
L-Phenylalanine	22.73 ± 1.74	4.40 ± 0.33

Results are means of triplicate determination ± S.D.

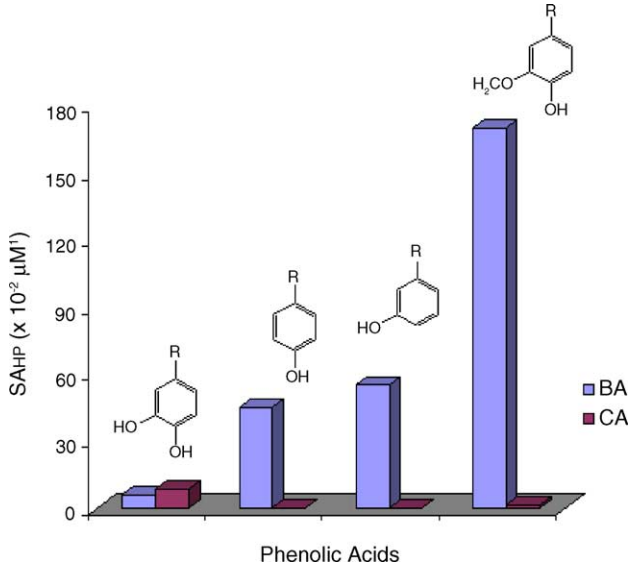

Fig. 2. Comparative diagram showing the hydrogen peroxide scavenging activities (SA_{HP}) of benzoate derivatives and their cinnamate analogues.

4. Discussion

Hydrogen peroxide is an oxidant that is being formed continuously in living tissues as a result of several metabolic processes, but its detoxification is very crucial in preventing it from reacting in deleterious Fenton-type reactions, which generate extremely reactive oxygen species, including hydroxyl free radical [8,9]:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^- + OH^\bullet \quad (3)$$

With respect to many dietary polyphenolic antioxidants, the radical–scavenger interactions have in many instances been thoroughly investigated [10,11], but the structural attributes that may play a prominent role in non-radical scavenging are yet to be defined. The examination of a few structurally diverse natural antioxidants, including β-carotene, α-tocopherol, quercetin and ascorbic acid in relation to their ability to scavenge hydrogen peroxide revealed that there is rather no correlation with lipophilicity, but the order of efficacy appeared to be dictated by other structural requirements [7]. In this regard, two series of simple phenolic acids that represent the most common benzoic and cinnamic acid derivatives were selected to carry out a comparative study, with the view to ascertain whether the criteria established for other aspects of the antioxidant activity hold true for hydrogen peroxide scavenging too.

Table 3
Comparative bibliographic data illustrating the antioxidant capacity of the most commonly encountered benzoic acids in different model systems

Compound	SA_{HP} ($\times 10^{-2}$ µM^{-1})[a]	EQ (mg L^{-1})[b]	AA[c]
Protocatechuic acid	5.90 ± 0.40	11.5	0.79 ± 0.03
p-Hydroxybenzoic acid	45.04 ± 3.92	>300	0.02 ± 0.00
m-Hydroxybenzoic acid	55.0 ± 1.30	–	–
Vanillic acid	170.2 ± 14.5	>300	0.15 ± 0.00

[a] Hydrogen peroxide scavenging activity (this study).
[b] Efficient quantity [12].
[c] Antioxidant activity (peroxyl radical scavenging) relative to Trolox™ [13].

In Table 3 are given some bibliographic data that may be critical for the evaluation of the findings on benzoates. Although the information available is rather limited, it can be seen that protocatechuic acid was proven far more potent in preventing methyl linoleate oxidation [12] and scavenging peroxyl radicals [13] than *p*-hydroxybenzoic and vanillic acids. Accordingly, in a number of different systems (Table 4), caffeic acid was a superior antioxidant compared with *p*-coumaric and ferulic acids, in inhibiting low-density lipoprotein (LDL) oxidation [14,15] but also quenching radicals [16] and singlet oxygen [17]. Taking into consideration the data in Tables 3 and 4, it can also be concluded that hydroxycinnamates always behave as more potent antioxidants compared with their benzoate analogues.

In both cases, the capacity lies on sequestering lipid radical species ($LOO^\bullet$), which largely depends on hydrogen-donating ability of the phenolics:

$$LOO^\bullet + PH \rightarrow LOO\text{–}H + P^\bullet \quad (4)$$

Phenoxyl radicals generated according to reaction (4) may be stabilized through resonance and/or intramolecular hydrogen bonding (Fig. 3) or combine to yield dimerization products,

Table 4
Comparative bibliographic data illustrating the antioxidant capacity of the most commonly encountered hydroxycinnamic acids in different model systems

Compound	SA_{HP} ($\times 10^{-2}$ µM^{-1})[a]	EQ (mg L^{-1})[b]	% Inhibition[c]	SAA (µM)[d]	AA[e]	SOQ (k_q)[f]	RSA (%)[g]
Caffeic acid	8.20 ± 0.30	8	97.9 ± 0.2	54.0	3.97 ± 0.05	40	49.6 ± 0.6
p-Coumaric acid	0.23 ± 0.00	126	40.7 ± 0.7	0.6	0.04 ± 0.00	6	7.0 ± 0.8
m-Coumaric acid	0.18 ± 0.00	–	–	–	–	7	–
Ferulic acid	1.10 ± 0.10	72	55.7 ± 5.9	8.5	0.90 ± 0.06	20	27.3 ± 0.8

[a] Hydrogen peroxide scavenging activity (this study).
[b] Efficient quantity [12].
[c] % Inhibition in LDL oxidation at 10 µM [14].
[d] Specific antioxidant activity in copper-mediated LDL oxidation [15].
[e] Antioxidant activity (peroxyl radical scavenging) relative to Trolox™ [13].
[f] Singlet oxygen quenching, given as rate constants k_q ($\times 10^5$ M^{-1} s^{-1}) [17].
[g] % Radical (DPPH) scavenging activity [16].

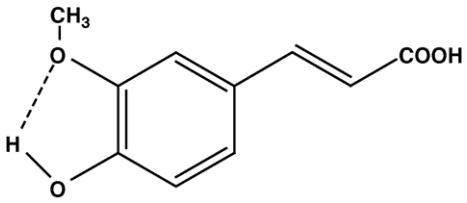

Fig. 3. Intramolecular hydrogen bonding of a phenolic acid (ferulic acid) that contributes to stabilization of the phenoxyl radical formed after hydrogen donation.

thus terminating the chain reaction:

$$P^{\bullet} + P^{\bullet} \rightarrow P\text{–}P \quad (5)$$

The scavenging mechanism of hydrogen peroxide by phenolic acids is not known, but from the relevant literature it is stressed that no radical reactions are involved [19–21]. From the results presented herein, it is evident that benzoates are remarkably more potent scavengers than their hydroxycinnamate analogues, which completely contrasts the findings for radical scavenging. It appears that the side chain of hydroxycinnamates is likely to hinder the relevant reaction(s) that lead to hydrogen peroxide quenching, or act as a destabilization factor for the reaction products. This is very well illustrated by comparison between caffeic and hydrocaffeic acid (Table 2). The latter is the saturated derivative of the former and exhibited an efficiency 1.5-fold higher, highlighting the negative impact of the side chain double bond. Furthermore, L-phenylalanine that lacks the vicinal phenolic hydroxyls of caffeic acid and bears a $–NH_2$ substituent instead of double bond also appeared considerably more effective than monosubstituted hydroxycinnamates (*p*- and *m*-hydroxyl derivatives), although almost two-fold less so than caffeic acid. This observation, however, is not alone enough to substantiate a profound influence of the phenolic hydroxyls, since the substitution pattern had not the same effect on benzoates and hydroxycinnamates. That is, the *m*-substituted benzoate was more powerful scavenger than the *p*-substituted one, but the order was inversed in hydroxycinnamates. Nevertheless, the fact that gallic acid, a tri-substituted benzoate, was demonstrated very active might indicate that the effect of phenolic hydroxyl should not be overlooked, as it can under certain condition be crucial.

5. Conclusions

The most important outcomes of this study may be summarized as follows:

1. Among the benzoic and cinnamic acid derivatives tested, the most active in scavenging hydrogen peroxide was vanillic acid and hydrocaffeic acid, respectively.
2. Benzoates were shown to be far more effective than their hydroxycinnamate analogues, which contrasts previous findings related with scavenging of radical species.
3. The side chain unsaturation in hydroxycinnamates appears to negatively affect their scavenging potency.
4. The structural requirements for efficient quenching of hydrogen peroxide are rather more complicated than those established for radical scavenging, and no assumptions can be made solely on the basis of the number and relevant position of phenolic hydroxyl on the aromatic nucleus.

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