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Study of phenolic compounds as natural antioxidants by a fluorescence method

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Abstract

Much work has been carried out in recent years on the beneficial effect of phenolic compounds as natural antioxidants which help to neutralize free radicals. In fact, researchers have focused their attention on the pathological role of free radicals in a variety of diseases, among which the most important are atherosclerosis and cancer. Thus, among the components of the so-called 'Mediterranean Diet', phenolic compounds have received increased attention as epidemiological studies have shown that consumption of foods and beverages rich in phenolics is correlated with reduced incidence of heart disease. In this study, four phenolic compounds: (1) 3,4,5-trihydroxybenzoic acid (gallic acid); (2) *trans* 3,4′,5-trihydroxystilbene (*trans*-resveratrol); (3) 3,3′,4′,5,7-pentahydroxyflavone (quercetin) and its glycoside (4) 3,3′,4′,5,7-pentahydroxyflavone–3-rutinoside (Rutin) have been subjected to antioxidant study by a fluorimetric assay. In this method, the rate of peroxidation induced by 2,2′-azobis (2-methylpropionamidine) dihydrochloride was monitored through the loss of fluorescence of the protein B-phycoerythrin (B-PE). Under appropriate conditions, the loss of B-PE fluorescence in the presence of reactive species is an index of oxidative damage of the protein. The inhibition of the action of reactive species by phenolic compounds, reflected in the protection against the loss of B-PE fluorescence in the fluorimetric assay, is a measure of its antioxidant capacity against the reactive species. The antioxidant effects of phenolic compounds have been investigated at different concentrations to relate activity to structural effects. It has been observed that the fluorescence decay due to peroxyl radical attack on B-PE decreases exponentially with time. As a reference compound for antioxidant capacity we used 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylicacid (trolox), a water soluble tocopherol analogue. This compound reacts rapidly with peroxyl radicals, and, until the trolox is consumed, no loss in phycoerythrin fluorescence is observed. A linear correlation of the net protection value with the concentration of trolox was demonstrated. The phenolic compounds studied react with peroxyl radicals in a similar way to trolox. Quercetin and rutin were shown to have strong antioxidant activities. The results obtained here are in agreement with previous studies confirming that quercetin is the most antioxidant of the four polyphenolics.

Keywords: Polyphenolics; Antioxidant activity; Fluorimetric method

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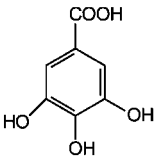
1. Introduction

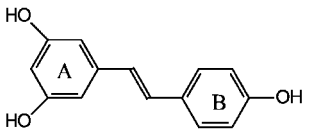
There is increasing interest in the use and measurement of antioxidant capacity in the food, pharmaceutical, and cosmetic industries. This interest is derived from the overwhelming evidence for the importance of reactive oxygen/nitrogen species (ROS/RON) in aging and pathogenesis. Accumulated evidence indicates that reactive oxygen species (ROS), such as peroxyl radicals ($ROO^{\bullet}$), hydroxyl radicals ($HO^{\bullet}$), superoxide ion ($O_2^{-\bullet}$), and singlet oxygen ($^1O_2$), are involved in the pathophysiology of aging and a multitude of diseases, such as cancer, Alzheimer's disease, and Parkinson's disease [1,2]. The antioxidant compounds can neutralize free radicals and may be of great importance in the prevention of these diseases.

Resveratrol and gallic acid are polyphenolic non-flavonoids, widely distributed in the plant kingdom [3], and are present in tea, red wine, fruits, beverages and various medicinal plants [4]. There is now considerable evidence that wine phenolics, particularly resveratrol, inhibit low-density lipoprotein oxidation [5,6] and reduce platelet aggregation [7], two major parameters implicated in atherothrombogenesis. This provides a possible explanation for the cardioprotective action of wine [8]. Furthermore, resveratrol has been shown to reduce the hepatic synthesis of cholesterol and triglyceride in rats [9], to inhibit the synthesis of eicosanoids and rat leukocytes [10], to interfere with arachidonate metabolism [11], to inhibit the activity of some protein kinases [12], and is a more powerful antioxidant than vitamin E in preventing LDL oxidation [13]. The demonstrated biological and pharmacological activities of resveratrol are attributed to its antioxidant property [14].

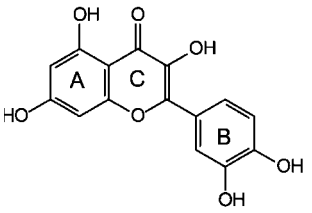
Gallic acid (3,4,5-trihydroxybenzoic acid) is often obtained by alkaline or acid hydrolysis of tannins or from hydrolysis of spent broths from *Penicillium glaucum* or *Aspergillus niger*. Gallic acid and its derivates are often present in the diet



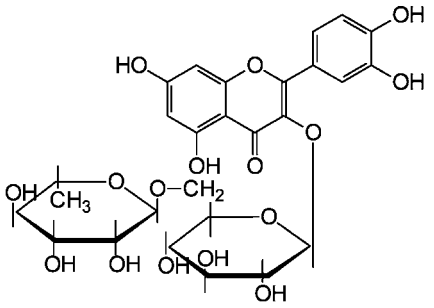
Gallic acid
[3,4,5 – Trihydroxybenzoic acid]



Trans-resveratrol
[Trans 3,4′,5 – Trihydroxystilbene]



Quercetin
[3,3′,4′,5,7-pentahydroxyflavone]



Rutin
[3,3′,4′,5,7-pentahydroxyflavone–3-rutinoside]

Fig. 1. Chemical structures of phenolics studied.

and in wine [15]. This acid phenolic compound is included in the list of existing food additives as natural antioxidants in some countries [4].

Flavonoids are low molecular weight polyphenolic compounds that are widely distributed in vegetables and fruits [16]. Quercetin is the main aglycone found in foods [17]. Many flavonoids have been shown to have antioxidant [18], anti-inflammatory, antiallergic, anticancer, and antihemorragic properties [19]. Recently, flavonoids have attracted increasing attention for their antioxidant properties, which may help to explain the protective effect of vegetable-rich diets on coronary heart disease (CHD) [20]. Among beverages, red wine has been reported to be more protective against CHD than other alcoholic beverages, thus confirming a possible role of red wine polyphenols in reducing thrombotic and atherogenic processes. Moreover, recent clinical studies have shown that moderate consumption of red wine increases the total antioxidant capacity of human serum [21]. Consequently, the great interest in these phenolic constituents of red wine, including gallic acid, *trans*-resveratrol, quercetin and rutin (Fig. 1), has been stimulated by the potential beneficial effects on health.

Furthermore, the antioxidants are of great interest because of their involvement in important biological and industrial processes. Phenols and flavonoids have recently gained significant interest among various antioxidants.

In previous papers we studied the separation of these phenolic compounds in red wines using reversed-phase liquid chromatography and photodiode array detection [22]. We later studied 'in vitro' antioxidant effects of these polyphenolics by a spectrophotometric method [23]. The aim of the current work was to study the antioxidant effects of the phenolic components gallic acid, *trans*-resveratrol, quercetin and its glucoside rutin using the fluorimetric method. This assay measures the effect of antioxidant compounds on the decline in B-phycoerythrin (B-PE) fluorescence induced by a peroxyl radical generator, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH). The objective was to determine whether differences existed between the relationship structure–antioxidant activity for the compounds mentioned.

2. Experimental

2.1. Reagents

All phenolic compounds, B-phycoerythrin (B-PE), 2,2′-azobis (2-methylpropionamidine) dihydrochloride 97% (AAPH) and 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid (trolox) was obtained from Sigma–Aldrich Quimica S.A. (Madrid, Spain).

2.2. Material

All fluorimeter analyses were performed on a Perkin–Elmer luminescence spectrometer (Beaconsfield, UK). The operating conditions were at 37 °C.

2.3. Methods

2.3.1. Antioxidant activity determination

Methods that have been developed for the measurement of the antioxidant activity are essentially inhibition methods: a free radical species is generated, there is an end point by which the presence of the radical is detected, and the antioxidant activity of the added sample inhibits the end point by scavenging the free radical.

2.3.1.1. Fluorimetric assay. The procedures for the fluorimetric assay on phenolic compounds were modified from a method described previously by Cao et al. [24]. Free radicals are produced by AAPH and the fluorescent indicator protein B-PE is subsequently oxidized. All reagents were prepared in 75 mM phosphate buffer, pH 7.0, and trolox (0–5 μM) was used as standard. Phenolic compounds were first dissolved in methanol and then diluted with buffer. Reaction mixtures consisted of 5 μl of B-PE (1.7×10^{-6} M) in 445 μl phosphate buffer (pH 7.0) preincubated for 15 min at 37 °C, 2.5 μl of test compound and 50 μl of AAPH (40 mM). The assay is carried out at 37 °C in fluorimeter cuvettes. The reaction was started by the addition of AAPH and fluorescence was measured every 5 min for 120 min with emission and excitation wavelengths of 575 and 545 nm, respectively, using a Perkin Elmer LS 55 luminis-

cence spectrometer. The antioxidant activity value refers to the net protection area under the quenching curve of B-PE in the presence of an antioxidant.

Final results of antioxidant activity were calculated by using a regression equation between the trolox concentration and the net area under the quenching curves of B-PE and were expressed as trolox equivalents. The area under curve (AUC) was calculated as:

$$\mathrm{AUC} = (0.5 + f_5/f_0 + f_{10}/f_0 + f_{15}/f_0 + \ldots f_i/f_0)\times 5 \quad (1)$$

where f_0 is the initial fluorescence reading at 0 min and f_i is the fluorescence reading at time i.

The net AUC was obtained by subtracting the AUC of the blank from that of the sample.

3. Results and discussion

Four different polyphenolics were measured for antioxidant properties in this study. The selection of these compounds was based on chemical structure characteristics, availability, and prevalence in plant foods.

3.1. Fluorimetric assay

The linear relationship between the net area and the antioxidant concentration was evaluated using trolox as standard at different concentrations. By integrating the areas under the fluorescence decay curve, it is possible to quantify the hydroxyl radical inhibition capacity of antioxidants. The inhibition capacity is expressed as trolox equivalents, which is quantified by the integration of the area under the curve (AUC). Fig. 2 illustrates the B-PE fluorescence decay curves in the presence of trolox and AAPH. Table 1 summarizes the correlation coefficient, slope, and intercept of the trolox standard curve and shows the nearly perfect linear relationship ($R^2 > 0.99$) between the trolox concentration and the AUC. The linearity range of the assay is between 0.1 and 5 μM.

Quenching curves of B-PE fluorescence illustrate the peroxyl radical absorbance ability of phenolic compounds studied compared to that of the standard trolox. The antioxidant effects of phenolic compounds have been investigated at different concentrations to relate activity to structural effects (Fig. 3). In addition, it has been observed that the loss of B-PE fluorescence in the presence of reactive species, which include peroxyl radicals generated from AAPH, does not follow zero-order kinetics (i.e. linear with time). Figs. 2 and 3 show that the fluorescence decay due to peroxyl radical attack on B-PE decreases exponentially with time.

The reaction kinetics differed between the phenolic compounds, exhibiting a concentration-dependent inhibition of the fluorescence decay. This distinct behaviour of the antioxidants suggests that different reaction mechanisms may be operative, depending on the antioxidant concentration. Table 2 shows the net areas corresponding to the different concentrations of representative phenolic antioxidants. Fig. 4 presents the type of concentration-dependent data obtained from each of the polyphenolics tested. The regression and correlation coefficients for the four phenolic compounds that were tested are presented in Table 3. Least-squares regression lines were computed between polyphenolic concentration and antioxidant activity expressed as trolox equivalents.

Phenolic compounds are widely distributed in nature. The antioxidant potential of these compounds depends on the number and arrangement of the hydroxyl groups and the extent of structure conjugation [25,26]. For flavonoid compounds (quercetin and rutin), *O*-dihydroxy groups in the B-ring, the presence of a C 2–3 double bond in conjunction with 4-oxo in the C-ring, and 3- and 5-hydroxy groups and the 4-oxo function in the A- and C-rings are associated with antioxidant activity [27]. For phenolic acids, the activity improves as the number of hydroxyl and methoxyl groups increases, the number of hydroxyl groups being more important [28]. The structural feature responsible for the antioxidative and free radical scavenging activity of gallic acid is the three hydroxyl groups [25]. The chemical criteria essential for the antioxidant activity of *trans*-resveratrol

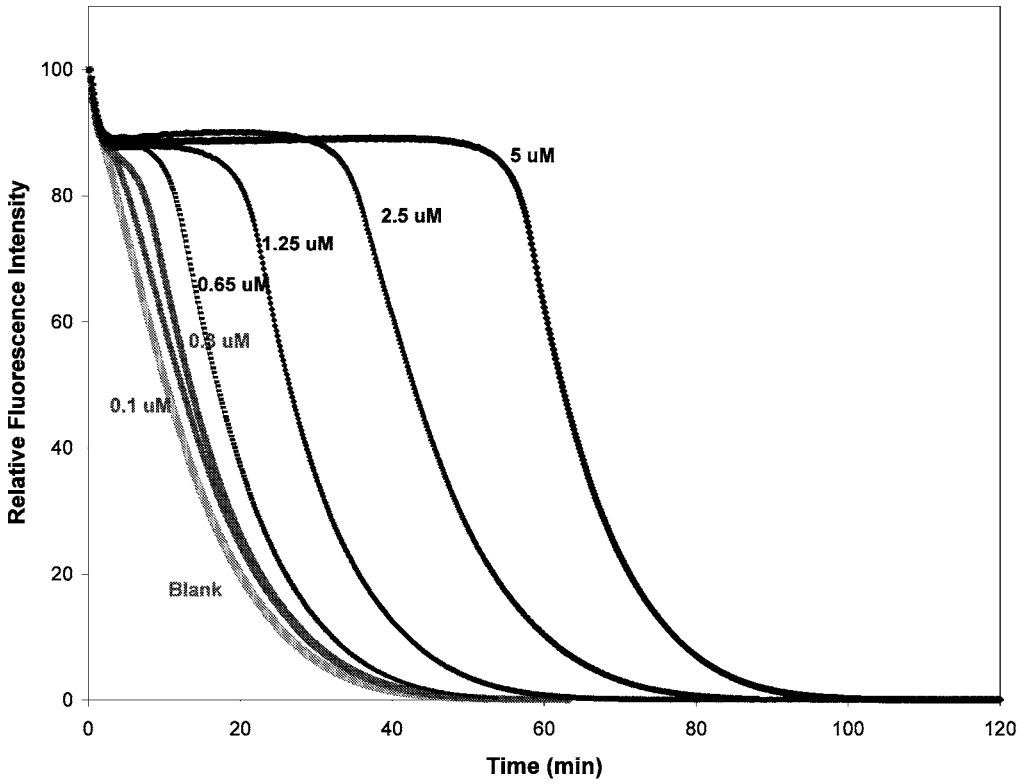


Fig. 2. Effect of trolox concentration on B-PE fluorescence decay curve.

(stilbene) are (1), the presence of the catechol structure or 4′-hydroxy in ring B, and (2), the presence of the *meta*-hydroxy structure in ring A. Polyphenols possess ideal structural chemistry for free radical-scavenging activities, and have been shown to be more effective antioxidants in vitro than vitamins E and C on a molar basis [29].

Flavonoids are known to inhibit lipid oxidation through both metal chelating and free radical scavenging mechanisms, whereas phenolic acids act as antioxidants via free radical trapping mechanisms [30].

The fluorescence method uses AAPH as a free radical-generating system. AAPH undergoes spontaneous decomposition and produces peroxyl radicals with a rate determined primarily by temperature. The analyzed antioxidant samples are not likely to affect this rate, particularly when the chemical structure of AAPH and the very high molar ratio of AAPH to an antioxidant sample are considered; thus, the fluorescence assay measures the capacity of an antioxidant to directly quench peroxyl radicals. The fluorescence method is more sensitive than the spectrophotometric assay as the final standard concentration required in the fluo-

Table 1
Summary of trolox calibration curve[a]

	Slope (b)	Intercept (a)	R^2
1	0.1094	−0.0811	0.9956
2	0.1593	−0.099	0.9889
3	0.0914	−0.2444	0.991
4	0.0736	0.2424	0.9929
5	0.1012	−0.1137	0.9995
Average	0.10698 ± 0.0321[b]	−0.05916 ± 0.1805[c]	0.9936 ± 0.0041

[a] [Y (μM) = $a+bx$ (net area)].
[b] Standard error of estimate.
[c] Standard error of the y-intercept.

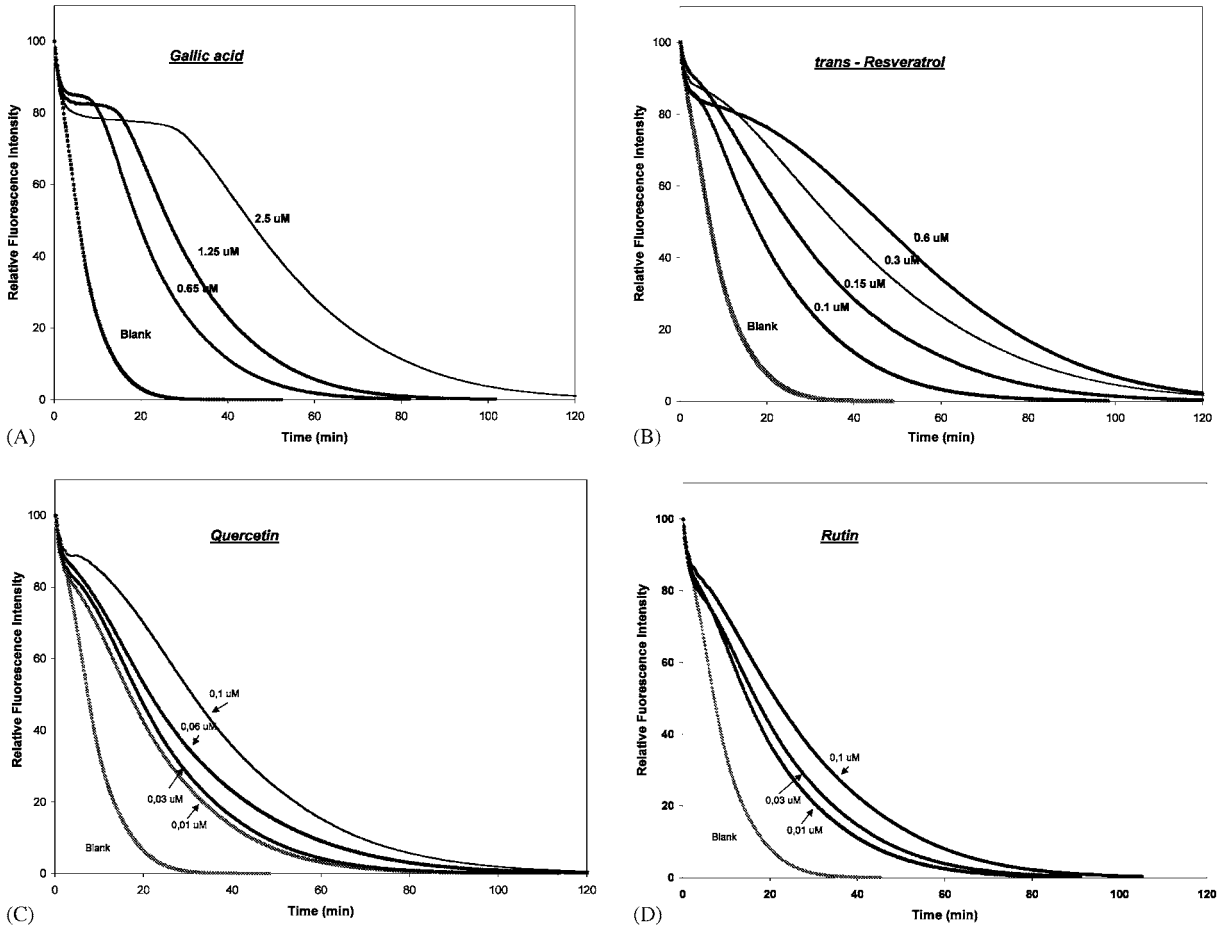


Fig. 3. B-PE fluorescence decay curve induced by AAPH in the presence of phenolic compounds at different concentrations.

rescence assay is much lower than those required in the spectrophotometric assay [23]. In addition, spectrofluorometric assays, such as the fluorimetric assay have been reported to be 100–1000 times more sensitive than spectrophotometric techniques [31].

The fluorimetric assay is a relatively simple but sensitive and reliable method. The uniqueness of this method is that it takes a free radical reaction to completion and uses an area-under the curve (AUC) technique for quantifying antioxidant capacity, thus combining both inhibition time and inhibition degree of free radical action by antioxidants into a single quantity [32]. The relative antioxidant capacity of these compounds evaluated by fluorimetric assay was as follows: quercetin > rutin > *trans*-resveratrol > gallic acid. Phenolic acids, in general, had lower antioxidant activities against peroxyl radicals than *trans*-resveratrol and flavonoids that contained multiple hydroxyl groups. Fig. 3 and Table 2 show that at the same final concentration, quercetin has the largest AUC and thus the greatest antioxidant activity. It has been observed that rutin (quercetin–3-*O*-rutinose) showed lower antioxidant capacity than its aglycon, quercetin, in both sectrophotometric and fluorimetric assays [23]. For flavonoids, glycosilation of the hydroxyl group at C-3 does not seem to change antioxidant activity notably, as reported by Teissedre et al.

Table 2
Net area under the curve corresponding to different concentrations of phenolic compounds

Compound	Concentration (μM)	Net AUC	AA[a]
Gallic acid	0.65	14.64	1.51
	1.25	26.14	2.74
	2.5	37.44	3.95
trans-Resveratrol	0.1	12.53	1.28
	0.15	21.83	2.28
	0.3	31.58	3.32
	0.6	38.70	4.08
Quercetin	0.01	11.90	1.21
	0.03	13.76	1.41
	0.06	18.20	1.89
	0.1	26.04	2.72
Rutin	0.01	10.04	1.02
	0.03	12.02	1.23
	0.1	17.02	1.76

[a] AA: antioxidant activity values are expressed as trolox equivalent calculated based on Eq. (1).

[33]. However in our case, the glycosylation of the 3-hydroxyl group in flavonols clearly decreased the antioxidant activity. This decrease may be due to the steric hindrance created by the saccharides. Our results are similar to those of previous studies [25,34] confirming that quercetin has the greater antioxidant activity of the compounds studied in this paper.

Table 3
Regression coefficients of phenolic compounds concentration (μM) (X) and antioxidant activity (Y) (trolox equivalents, μM)

Compound	Coefficients[a]		
	Slope (b)[b]	Intercept (a)	R[c]
Gallic acid	1.4924 ± 0.2274[d]	0.4796 ± 0.3950[e]	0.98
trans-Resveratrol	5.7675 ± 0.7967	0.9937 ± 0.1441	0.95
Quercetin	16.0735 ± 0.9685	0.6787 ± 0.2795	0.99
Rutin	9.7949 ± 1.6605	0.915 ± 0.0402	0.99

[a] Regression coefficients: Y (antioxidant activity) $= a + bX$ (concentration, μM).
[b] All b coefficients are significantly greater than zero.
[c] Multiple correlation coefficient.
[d] Standard error of estimate.
[e] Standard error of the y-intercept.

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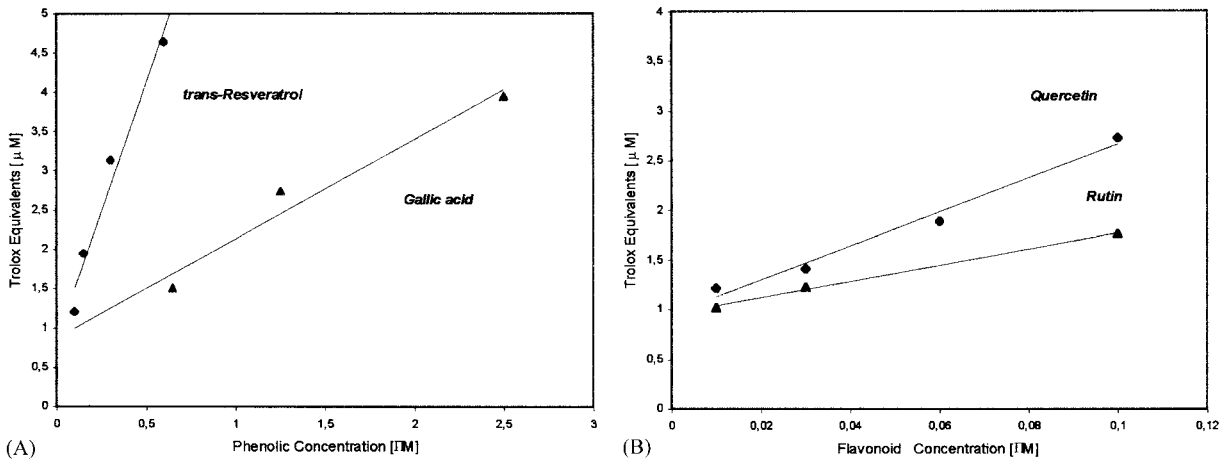

Fig. 4. Relationship between antioxidant activity (trolox equivalents, μM) and concentration (μM) of (A) non-flvanoids compounds [gallic acid ($R^2 = 0.96$) and *trans*-resveratrol ($R^2 = 0.97$)] and (B) flavonoids compounds [quercetin ($R^2 = 0.98$) and Rutin ($R^2 = 0.99$)].

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