

Ability of scavenging free radicals and preventing lipid peroxidation of some phenols and ascorbic acid

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ARTICLE INFO

Article history:

Received on: 06/05/2015

Revised on: 05/06/2015

Accepted on: 17/06/2015

Available online: 28/08/2015

Key words:

Phenolic acids; eugenol;
thymol; ascorbic acid;
antioxidant.

ABSTRACT

The capacity for scavenging free radicals and preventing lipid peroxidation of gallic, caffeic, and *p*-coumaric acids, thymol, eugenol and ascorbic acid were evaluated. The capacity for scavenging DPPH[•] free radicals were performed in ethanol 96% and ethanol 70%. In the same assay, the activity estimation was followed at 10, 20 and 30 minutes. In this assay, eugenol presented the best activity (IC₅₀ ranging from 2.10 mg/mL to 9.74 mg/mL. In the opposite site, *p*-coumaric had the lowest activity, in which the IC₅₀ values were not possible to determine. Generally, 10 minutes of reaction provided lower scavenging activities than 30 minutes. The sole exception was ascorbic acid in which the activities were independent on the time of reaction. Ascorbic acid, eugenol and thymol possessed higher ability for scavenging DPPH free radicals in ethanol 70% than in ethanol 96%. Gallic and *p*-coumaric acids as well as thymol revealed to be the best scavengers of ABTS^{•+} free radicals in contrast to ascorbic acid. The capacity for preventing lipid peroxidation was dependent on the concentration of samples. The assay showed that higher concentrations of gallic acid, thymol and *p*-coumaric acid added to sunflower oil (from 0.3 to 0.6%) induced higher lipid peroxidation with higher peroxide values. In contrast, increasing the percentages of caffeic acid and eugenol induced lower peroxidation of the sunflower oil. The percentage of samples added to this fat did not influence the index of *p*-anisidine. In this test, gallic acid had the best capacity for preventing the formation of 2,4-dienals and 2-alkenals decadienals able to react with *p*-anisidine.

INTRODUCTION

Phenols have been reported as being antioxidants and therefore with an important role of protecting organisms against the harmful effects of reactive oxygen species (ROS). These free radicals are very important in the organisms. Oxygen radicals are involved in diverse biochemical activities of great importance in cells such as signal transduction and gene transcription (Uttara *et al.*, 2009).

In addition, human beings produce oxygen free radicals (superoxide and hydroxyl radicals) and other oxygen reactive species (hydrogen peroxide, nitric oxide, peroxy nitrile and hypochlorous acid) due to diverse physiological and biological

processes as a result of aerobic metabolism (Uttara *et al.*, 2009). These free radicals may be responsible for oxidative damage of lipids, proteins and nucleic acids, which can lead to pathologies such as rheumatoid arthritis, diabetics, cancer, atherosclerosis cardiovascular diseases among other chronic and degenerative diseases (Yoshikawa; 1993; Freidovich, 1999; Uttara *et al.*, 2009). However, only an overproduction of free radicals may contribute to the appearance of those diseases. In this way, organisms possess antioxidant defence systems to counteract oxidative damage not only produced by reactive species due normal biochemical and physiological processes but also to radicals of environmental origin (Uttara *et al.*, 2009). When the antioxidant defence systems fail and/or an overproduction of radicals occur such lead to the damage of biomolecules and consequently disease. For this reason, an intake of antioxidants may be necessary which can be supplied through diet. Vitamins E and C, β -carotene and coenzyme Q are some examples of antioxidants obtained through diet.

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Nevertheless other components also present in foodstuffs of plant origin possess antioxidant activity, as for instance, phenols (Stratil *et al.*, 2006; Pereira *et al.*, 2009; Uttara *et al.*, 2009). The antioxidant ability of phenols is also important in the prevention of food degradation, particularly due to the oxidation of fats. Those components when added to foods may retard rancidity, maintaining their nutritional quality and, consequently increasing their shelf-life (Fukumoto and Mazza, 2000).

Due to the importance of phenols as antioxidants, there is a great interest in these compounds as well as in the methods for the estimation of that property (Dawidowicz and Olszowy, 2012). Such activity may be followed by direct and indirect methods. In the indirect approach, the evaluation of antioxidant activity is based on the capacity for scavenging free radicals. Among the most used indirect methods one can cite the scavenging of DPPH (1,1-diphenyl-2-picrylhydrazyl) and ABTS^{•+} [2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)] free radicals (Dawidowicz and Olszowy, 2012). Such methods are not associated with the real oxidative degradation (Roginsky and Lissi, 2005).

Direct methods are based on the effect of the sample with potential antioxidant activity on the oxidative degradation of a tested substrate (individual lipids, lipid mixtures, proteins, nucleic acids, lipid containing biologically relevant species such as blood plasma, lipoproteins of low density, biological membranes, among other substrates) (Roginsky and Lissi, 2005). Lipid oxidation is generally evaluated determining the peroxide value thiobarbituric acid reactive substances (TBARS), conjugated dienes, anisidine value or checking volatile compounds (Kristinová *et al.*, 2009).

Generally indirect methods are mostly used, such as DPPH and ABTS methods, although their poor repeatability and the lack of certitude for the capability of samples to inhibit oxidative processes. Reagent concentrations, time of incubation, solvents used, matrix of sample, among other factors may be determinant on the results obtained which may vary strongly, do not permitting compare results (Roginsky and Lissi, 2005; Pérez-Jiménez and Saura-Calixto, 2006).

Although direct methods are more adequate, particularly those based on the model of the chain controlled reaction, they are generally time-consuming methodologies and therefore they are not suitable for routine testing natural products (Roginsky and Lissi, 2005). These authors even consider that direct methods may be recommended to use for calibrating indirect methods.

In the present work the capacity of cinnamic acid derivatives (*p*-coumaric acid and caffeic acid), benzoic acid derivative (gallic acid), phenylpropene (eugenol), aromatic monoterpene (thymol) and ascorbic acid (vitamin C) for scavenging DPPH and ABTS^{•+} free radicals were evaluated. The type of solvent and course time reaction in DPPH method was also assayed. At the same time, the capacity of the same compounds for

preventing lipid peroxidation was also estimated, using sunflower oil as lipidic substrate.

MATERIAL AND METHODS

Scavenging free radical ability

1,1-Diphenyl-2-picrylhydrazyl (DPPH) free radical-scavenging activity

A 25 μ L aliquot of each sample dissolved in 96% ethanol or ethanol 70% was mixed with 975 μ L DPPH 60 μ M dissolved in 96% ethanol or ethanol 70%, respectively. After 10, 20 or 30 min reaction period, the absorbance (Abs) was recorded at 517 nm (Brand-Williams *et al.*, 1995). Several sample concentrations were prepared for the determination of the percentage of inhibition that was calculated using a graph of the scavenging effect percentage against sample concentrations. The sample concentration providing 50% inhibition (IC₅₀) was calculated from that graph. The scavenging effect percentage was calculated from the formula: Percentage of inhibition = $[(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}) / \text{Abs}_{\text{control}}] \times 100$.

In the control, the volume of sample is replaced by the same volume of solvent. The percentage was plotted against the samples, and IC₅₀ values were estimated (therefore, concentration of samples able to scavenger 50 % of the DPPH free radical).

2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) free radical-scavenging activity

The ABTS radical was generated by reaction of a 7 mM ABTS aqueous solution with K₂S₂O₈ (2.45 mM) in the dark for 16 h and adjusting the absorbance at 734 nm to 0.7 at room temperature (Re *et al.*, 1999). Samples (10 μ L) were added to 990 μ L of ABTS and the absorbance at 734 nm was read after 6 min (Antunes *et al.*, 2010). Several sample concentrations were prepared and the percentage inhibition was calculated from the formula:

$$\text{Percentage of inhibition} = [(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}) / \text{Abs}_{\text{control}}] \times 100.$$

IC₅₀ values (concentration of sample able to scavenger 50% of the ABTS free radical) were determined as reported in DPPH method. In the control, the volume of sample is replaced by the same volume of solvent.

Sample preparation for the estimation of lipid oxidation

Four hundred and fifty or nine hundred milligrams of each sample were added separately to 150 g of the sunflower oil. Each mixture was stirred for 5 min at room temperature, placed in closed transparent glass bottles (250 mL), at 60 °C, and kept in the dark. After 26 days, the peroxide value and *p*-anisidine were evaluated as reported below.

Peroxide value

A mixture of sunflower oil and chloroform/acetic acid (3:2) (v/v) was left to react in darkness with saturated potassium iodine solution. The free iodine was titrated with a sodium thiosulphate solution (0.01 M). Peroxide value was determined in milliequivalents of active oxygen per kilogram of oil (mEq O₂/kg),

according to the following formula: $I_p = 10 \times (n_1 - n_2) / m$ where n_1 was the mL of sodium thiosulphate consumed in sample titration, n_2 was the mL of sodium thiosulphate consumed in blank titration and m was the amount of sample in g (Neves *et al.*, 2011).

***p*-Anisidine value**

p-Anisidine value was determined as reported previously (Neves *et al.*, 2011). Briefly, it was carried out by the determination of the absorbance increase, measured at 350 nm, of a sample solution of 0.5 g of sunflower oil (m) in *iso*-octane (25 mL), before (A_1) and after reaction with *p*-anisidine (A_2) in glacial acetic acid in the dark. The *p*-anisidine value was determined with the following formula: $25 \times (1.2 A_2 - A_1) / m$.

Statistical Analysis

Each treatment consisted of 3 replications. Statistical analysis was carried out with the SPSS 20.0 software (IBM, Inc.). Two-way ANOVA and Duncan's Multiple-Range Test ($P < 0.05$) for comparisons among treatments over time was performed.

RESULTS AND DISCUSSION

Scavenging free radical ability

TEAC (Trolox Equivalent Antioxidant Capacity) assay is also known as ABTS [2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonate)] radical cation ($ABTS^{*+}$) method. ABTS is a compound that after oxidation by peroxy radicals or other oxidants originates the radical cation $ABTS^{*+}$, which is blue and absorbs light at 734 nm. An antioxidant reacts directly with the radical cation $ABTS^{*+}$, reducing the blue colour (Prior *et al.*, 2005). DPPH $^{\bullet}$ is a very stable organic nitrogen radical of deep purple colour, which absorbs light at 515 nm. There is a loss of colour when this radical reacts directly with an antioxidant (Prior *et al.*, 2005).

Those two methods were used for evaluating the antioxidant ability of *p*-coumaric and caffeic acids (both cinnamic acid derivatives), gallic acid (benzoic acid), eugenol (phenylpropanoid), thymol (phenolic monoterpene) and ascorbic acid (vitamin). These compounds were chosen based on their known antioxidant capacity, albeit their different chemical structures.

p-Coumaric acid had the best capacity for scavenging the radical cation $ABTS^{*+}$, nevertheless the worst one for scavenging DPPH $^{\bullet}$ radicals (Table 1). Such results are in accordance to those already reported by several authors (Nenadis and Tsimidou, 2002; Nenadis *et al.*, 2004; Kristinová *et al.*, 2009; Kiliç and Yeşilouglou, 2013; Xie and Schaich, 2014).

p-Coumaric acid, thymol and gallic acid presented the best capacity for scavenging $ABTS^{*+}$. *p*-Coumaric acid also presented better capacity for scavenging $ABTS^{*+}$ than caffeic and ascorbic acids according to the results previously reported by Re *et al.* (1999). The results obtained in our work did not coincide with those found when DPPH $^{\bullet}$ method was used for evaluating the

capacity for scavenging free radicals (Table 1). In DPPH assay, *p*-coumaric acid was the worst compound for scavenging DPPH $^{\bullet}$, not even being possible to determine IC_{50} values, whereas in TEAC method, the same phenolic compound was one of the best scavengers of $ABTS^{*+}$. The IC_{50} values found for *p*-coumaric acid, thymol and gallic acid were lower when compared to those obtained in DPPH method, such meaning better antioxidant activity when measured through TEAC method. In contrast, the IC_{50} values found for ascorbic acid were practically similar to those in which the DPPH method used ethanol 70% as solvent (around 40 mg/mL) (Table 1). Eugenol had worse ability for scavenging $ABTS^{*+}$ than DPPH $^{\bullet}$, according to the IC_{50} values found for this compound in both methods (Table 1).

The different trend of antioxidant activity observed in the present work was also reported by Khanan *et al.* (2012), when they evaluated antioxidant abilities of diverse leafy vegetables. Those authors reported that the presence of different phenols in the samples and not identified in their work, could contribute to such results. According to the experiments of Arts *et al.* (2004) with pure flavonoids, they reported that some products resulting from the oxidation of flavonoids in the presence of $ABTS^{*+}$ had higher antioxidant capacity, reacting faster with $ABTS^{*+}$ than with the parent compound. Other example given by the same authors was the trolox quinone formed during scavenging $ABTS^{*+}$ from trolox, which did not react with $ABTS^{*+}$. According to these results, the authors suggested that TEAC assay did not necessarily reflect the antioxidant ability of only one structure, that is, the reaction products may have a considerable contribution to the TEAC method.

However, the same has also been reported in DPPH method: the formation of complex mixtures of dimeric compounds originated by different coupling processes as observed for eugenol, caffeic acid, among other compounds, which also possesses capacity for acting with DPPH $^{\bullet}$ free radicals (Bortolomeazzi *et al.*, 2010; Gülçin *et al.*, 2012). According to these findings, the ability of the polyphenols to act as antioxidants not only depends on the redox properties of their phenolic hydroxyl groups and the potential for electron delocalization across the chemical structure, as also of the possible reactivity of the reaction products formed during the reaction with DPPH and ABTS. For this reason, some authors consider that this contribution of the reaction products to the radical scavenging activity limits those methods to evaluate structure-activity relationship as well as correlate the results with the antioxidant activity obtained by other methods (Bortolomeazzi *et al.*, 2010). In fact and only considering the ABTS and DPPH methods used in the present work, it is very difficult to explain the results obtained in both methods, since there is no correlation of activities between the two methods.

However, some authors had reported that compounds with lower redox potential possess higher antioxidant activity and therefore they considered that redox potentials could be considered as a good measure of antioxidant activity (Teixeira *et al.*, 2013). In our case, such is not as evident.

According to Kristinová *et al.* (2009) the different capacity of phenolic compounds to scavenge free radicals depending on the method used may be due to the specific reactions between the different assay reagents and the antioxidant samples and unrelated reactions of phenols (dimerization, polymerization) that occur during the reactions.

Kiliç and Yeşilouğlu (2013) found that the concentration 30 µg/mL had 55.6% capacity for scavenging DPPH radicals. In our case, only from 1.84 mg/mL the capacity for scavenging DPPH radicals was > 50% (50.7%), when the solvent of DPPH radicals was ethanol 70% (Fig. 1). When the solvent was ethanol 96%, higher concentration of *p*-coumaric was needed to reach 50% activity (2.46 mg/mL) (Fig. 2).

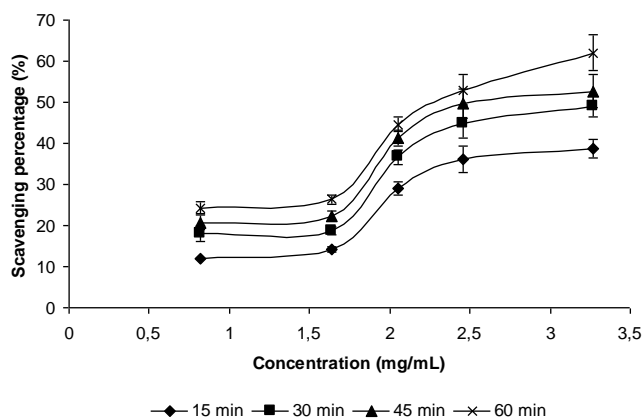


Fig. 1: DPPH scavenging ability of *p*-coumaric acid when the assay was carried out in ethanol 96%.

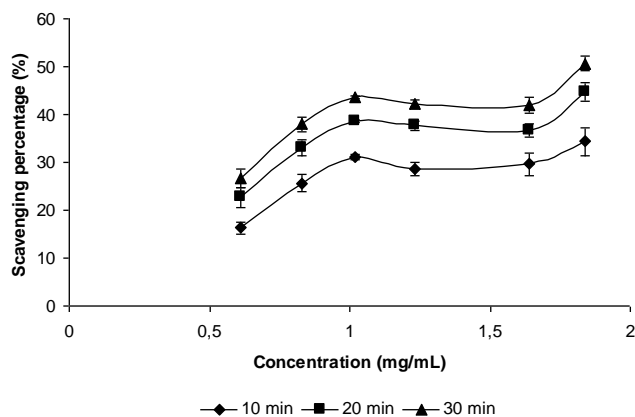


Fig. 2: DPPH scavenging ability of *p*-coumaric acid when the assay was carried out in ethanol 70%.

In addition, these activities were highly dependent on the reaction time (Fig. 1 and 2). *p*-Coumaric acid is a mono-hydroxyl derivative of cinnamic acid and according to some authors, aromatic acids containing only one OH-group does not react with DPPH (Roginsky and Lissi, 2005; von Gadow *et al.*, 1997) or never react with more than 75% of the initial DPPH radicals, even after several hours of reaction time and at very high concentrations (Brand-Williams *et al.*, 1995). These authors considered, therefore, that this phenolic compound presents a slow kinetic behaviour. Our results demonstrated that the activity of *p*-coumaric for

scavenging DPPH[•] radicals depend on the concentration, type of solvent used and reaction time (Fig. 1 and 2).

Thymol was the second worst phenolic compound for scavenging DPPH[•] radicals (Table 1) and, such as *p*-coumaric acid, its activity was dependent on the type of solvent and reaction time (Fig 3 and 4). Antioxidant activity of thymol after 30 minutes of reaction was better than that determined soon after 10 minutes (Table 1). Thymol also had better capacity for scavenging DPPH[•] free radicals when the solvent ethanol 70% for solubilise DPPH[•] was used than ethanol 96% (Table 1). Without significant differences, thymol had similar capacity for scavenging radical cation ABTS^{•+} to that found for *p*-coumaric acid (Table 1).

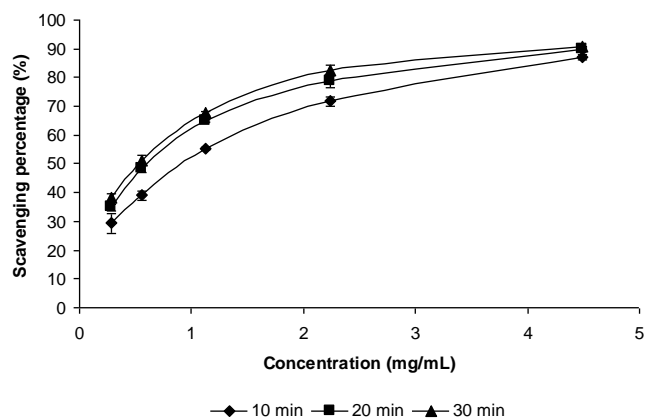


Fig. 3: DPPH scavenging ability of thymol when the assay was carried out in ethanol 96%.

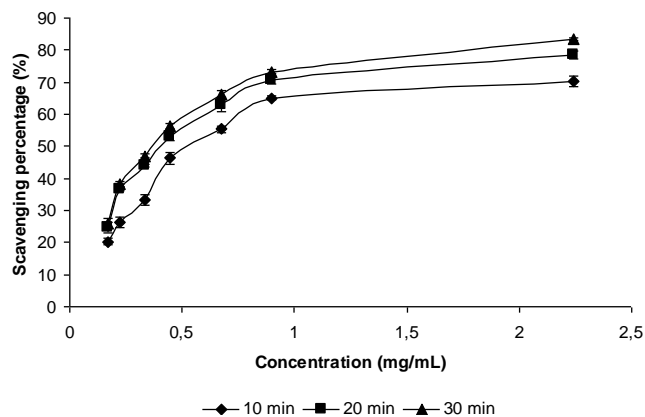


Fig. 4: DPPH scavenging ability of thymol when the assay was carried out in ethanol 70%.

The main methods for evaluating *in vitro* antioxidant activities are grouped in two categories: hydrogen atom transfer (HAT) reaction and electron transfer (ET) reaction-based methods. For example, ORAC (oxygen radical absorbance capacity) assay measures the capacity of an antioxidant for scavenging peroxy radicals (ROO[•]) by a hydrogen atom transfer reaction. TEAC (Trolox equivalent antioxidant capacity) and 2,2-diphenyl-1-picrylhydrazyl (DPPH[•]) methods have been classified as SET reactions (Prior *et al.*, 2005). Nevertheless, these free radicals may react with antioxidants either by direct reduction via electron transfer or by hydrogen atom transfer reaction (Jiménez *et al.*,

2004; Prior *et al.*, 2005). The contribution of a direct abstraction of phenol H-atom by the free radical (DPPH[•]) (HAT reaction) or of an electron-transfer process from the phenol (ArOH) or its phenoxide (ArO⁻) to the free radical (DPPH[•]) (ET reaction) depends on the nature of the solvent and/or the redox potentials of the species involved (Foti *et al.*, 2004). Generally, HAT mechanisms predominate in apolar solvents, but with very strong oxidizing radicals (Cl₃COO[•]), ET may be the preferential mechanism even in apolar solvents (Foti *et al.*, 2004). According to some authors (Foti *et al.*, 2004; Foti *et al.*, 2008), the best capacity of esters of cinnamic acids than cinnamic acid derivatives for scavenging DPPH[•] radicals is due to the fact that dissociation of the carboxylic group reduces the quantity of phenoxide (ArO⁻) in equilibrium with the phenol (ArOH). This fact may partly explain the weak capacity of *p*-coumaric acid to react with DPPH[•] in contrast to the remaining samples assayed (Table 1).

SET and HAT mechanisms may occur together being the balance determined by antioxidant structure and pH (Prior *et al.*, 2005; Karadag *et al.*, 2009). In DPPH[•] method, several works have demonstrated that the mechanism involving hydrogen atom transfer may occur in at least four different sequences: hydrogen-atom transfer (HAT), proton-coupled electron transfer (PCET), sequential proton-loss electron transfer (SPLET), and electron-transfer proton-loss (ET-PT) (Foti *et al.*, 2008). These authors consider that the reaction between esters of cinnamic acid or cinnamic acid derivatives and DPPH[•] occur by SPLET in solvents with high dielectric constant. The reaction between phenols and DPPH[•] may occur by a combination of PC-ET and SPLET mechanisms. PC-ET dominates in non-polar solvents of low dielectric constant and of low basicity, while SPLET mechanism occurs preferentially in alcohols such as methanol or ethanol, which have higher dielectric constant and high ability to solvate phenolic anions.

PC-ET is a slower mechanism than SPLET one (Dawidowicz and Olszowy, 2012). Higher dielectric constant of water than ethanol may be responsible for the best capacity of *p*-coumaric or thymol for scavenging DPPH[•] radicals in the solvent ethanol 70%. In addition, this mixture probably has better capacity for solvating phenolic anions. The combination of these two factors may partly explain the best antioxidant activities found when DPPH[•] radicals were solubilised in the solvent ethanol 70%. Eugenol and gallic acid presented the best capacity for scavenging DPPH[•] radicals (Table 1). When the solvent assayed was ethanol 96%, the reaction time did not influence the activity of eugenol (Fig. 5).

Nevertheless when ethanol 70% was used as solvent, activity differences were obtained, after 10 minutes of reaction the activity was worse than 20 and 30 minutes (Fig. 6, Table 1). Such results demonstrated that the behaviour of eugenol in the presence of DPPH[•] radicals is dependent on the solvent. Such as reported for *p*-coumaric acid and thymol, in eugenol, the presence of water ameliorated its activity, maybe due to the higher dielectric constant of water. The capacity for scavenging DPPH[•] radicals of phenolic

compounds increases with increasing degree of hydroxylation (Rice-Evans *et al.*, 1996; Scherer and Godoy, 2009). Such may partly explain the worst activity of thymol and *p*-coumaric acid with only one –OH attached to the aromatic ring. Nevertheless eugenol with also only one –OH presented the best activity. Such means that other groups attached to the aromatic ring and their positions have a role in the antioxidant activity. The electron donating groups, such as –CH₂–CH=CH and –OCH₃ attached to the aromatic ring of eugenol in positions *p*- and *o*-, respectively seem to contribute to the best activity found for this phenolic compound when measured through the DPPH method (Table 1). Methoxyl groups in an *ortho*-position to a hydroxyl group resulted in an increase of the antioxidant activity in studies performed by Teixeira *et al.* (2013).

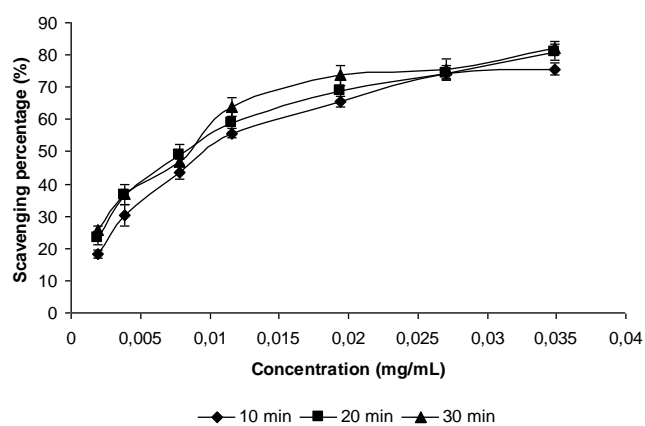


Fig. 5. DPPH scavenging ability of eugenol when the assay was carried out in ethanol 96%.

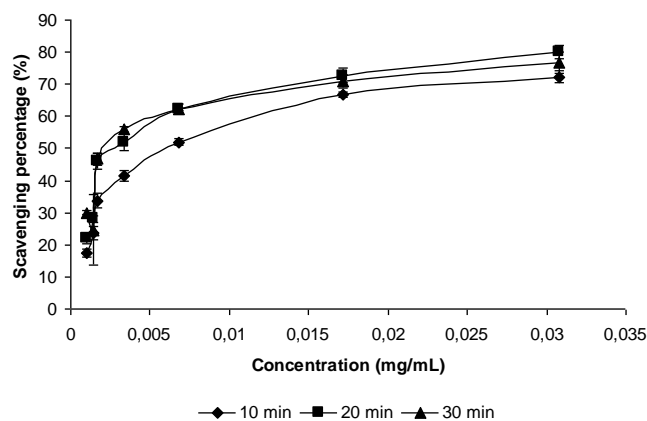


Fig. 6. DPPH scavenging ability of eugenol when the assay was carried out in ethanol 70%.

In the case of gallic acid, the effect of 3-OH attached to the aromatic ring superposes to the electron withdrawing of –COOH, which makes it stronger as scavenger than *p*-coumaric and caffeic acids.

Ascorbic acid is not an aromatic compound but presents a good capacity for scavenging DPPH[•] radicals (Table 1). Such activity and according to Nenadis *et al.* (2007) may be attributed to the possible formation of a quinone from its ene-diol group by donating two hydrogen atoms to the free radical.

Table 1: Antioxidant activity of some phenols and ascorbic acid expressed as IC₅₀ (mg/mL) for DPPH and ABTS method, and as mEq O₂/kg for peroxide value, and index of *p*-anisidine.

Sample	Peroxide value (0.6 %)	Peroxide value (0.3 %)	<i>p</i> -Anisidine (0.6 %)	<i>p</i> -Anisidine (0.3 %)	ABTS	DPPH (ethanol 96%) (30 min)
Gallic acid	22.48±2.30ab	12.59±5.48b	28.43±3.40b	27.45±3.63b	1.13±2.09c	13.35±0.02c
Thymol	27.74±2.30 ^a	14.56±5.48b	33.09±3.40ab	33.50±3.63ab	1.39±2.09c	580.86±0.02 ^a ,B
Caffeic acid	15.85±2.30b	29.52±5.48a	34.94±3.40ab	39.16±3.63a	17.89±2.09b	40.38±0.02bc,A
<i>p</i> -Coumaric acid	20.21±2.30b	12.36±5.48b	36.40±3.40ab	34.51±3.63ab	0.83±2.09c	-
Ascorbic acid	18.77±2.30b	12.97±5.48b	37.00±3.40 ^a	31.28±3.63ab	41.11±2.09 ^a	88.07±0.02b,A
Eugenol	17.64±2.30b	31.55±5.48a	35.76±3.40ab	31.68±3.63ab	22.58±2.09b	8.03±0.02c,A

	DPPH (ethanol 96%) (20 min)	DPPH (ethanol 96%) (10 min)	DPPH (ethanol 70%) (30 min)	DPPH (ethanol 70%) (20 min)	DPPH (ethanol 70%) (10 min)
Gallic acid	nd	nd	14.89±2.28d	nd	nd
Thymol	669.25±20.05 ^a ,B	934.66±20.87 ^a ,A	371.22±2.28 ^a ,C	412.31±9.91 ^a ,B	567.87±6.22 ^a ,A
Caffeic acid	49.70±20.05bc,A	59.38±20.87bc,A	49.95±2.28b,B	53.54±9.91b,B	71.66±6.22b,A
<i>p</i> -Coumaric acid	-	-	-	-	-
Ascorbic acid	89.04±20.05b,A	88.37±20.87b,A	38.67±2.28c,A	40.00±9.91b,A	40.69±6.22c,A
Eugenol	8.23±20.05c,A	9.74±20.87c,A	2.10±2.28e,B	2.61±9.91c,B	7.19±6.22d,A

Values in the same column followed by the same lower case letter are not significant different by the Duncan's Multiple-Range Test ($p < 0.05$).

For DPPH analysis, values in the same row followed by the same upper case letter are not significant different by the Duncan's Multiple-Range Test ($p < 0.05$).

In contrast to the remaining compounds, the capacity for scavenging DPPH[•] radicals was independent on the reaction time (Fig. 7 and 8).

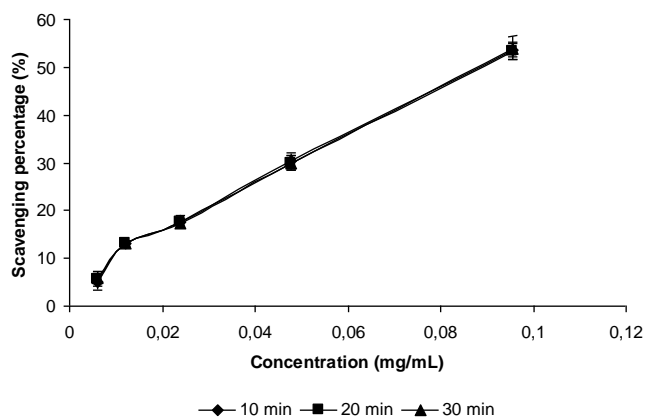


Fig. 7. DPPH scavenging ability of ascorbic acid when the assay was carried out in ethanol 96%.

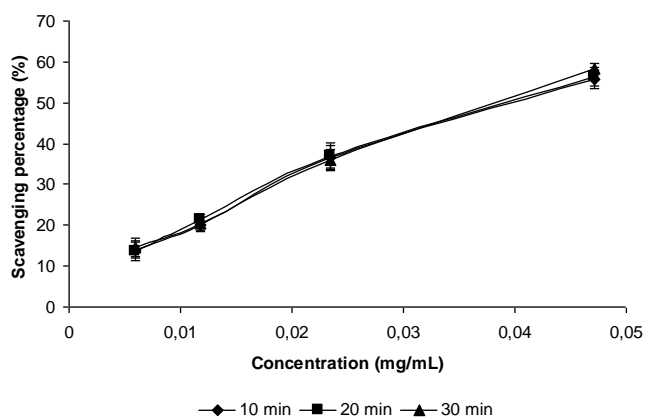


Fig. 8. DPPH scavenging ability of ascorbic acid when the assay was carried out in ethanol 70%.

This behavior was observed in both solvents; nevertheless the activity of vitamin C was significantly better

when the solvent used was ethanol 70% such as for thymol and eugenol. In ethanol 70%, ascorbic acid was even better antioxidant than caffeic acid (Table 1). The best activity of ascorbic acid in ethanol 70%, such as observed for thymol and *p*-coumaric acid, may also be explained by the SPLET mechanism induced by the proton transfer of the ascorbic acid to the solvent, as reported above for thymol and *p*-coumaric acid.

Lipid oxidation

The values presented in peroxide and *p*-anisidine values are those obtained after 26 days of fat storage at 60°C, whereas the remaining results are presented as IC₅₀ values (Table 1).

For a concentration of 0.6% of samples added to the sunflower oil, caffeic acid, eugenol, ascorbic and *p*-coumaric acids presented the best capacity for preventing the formation of hydroperoxides. Nevertheless, after the formation of these oxidation intermediates, these compounds are not sufficiently strong to prevent the formation of the secondary oxidation products such as 2,4-dienals and 2-alkenals decadienals, measured through the *p*-anisidine method (Table 1). In this case, gallic acid reveals to be the most effective (Table 1). However, some care must be taken with gallic acid, because some authors detected that this phenol compound exhibit significant prooxidant effect on total oxidation in the pH 3.0 emulsions (Osborn and Akoh, 2003). In fact, in our case, when the concentration of this benzoic acid derivative was doubled (from 0.3% to 0.6%), the formation of hydroperoxides also increased (from 12.59 to 22.81 mEq O₂/kg, respectively) (Table 1). Other phenolic compounds also presented this trend: *p*-coumaric acid (from 12.36 to 20.21 mEq/O₂ kg, respectively), and thymol (from 14.56 to 27.84 mEq/O₂ kg, respectively). There are some examples that show that some phenols considered antioxidants may have pro-oxidant activity in high concentrations or in the presence of metal ions (Bouayed and Bohn, 2010; Yordi *et al.*, 2012). On the contrary, increasing the amounts of caffeic acid or eugenol in sunflower oil, the peroxidation of the vegetable oil decreased. The peroxide values

found were 29.52 and 15.85 mEq O₂/kg, for caffeic acid at 0.3 and 0.6%, respectively; and for eugenol, the values found were 31.55 and 17.64 mEq O₂/kg (Table 1). These results suggest an antioxidant ability of these two phenols, in contrast to *p*-coumaric and gallic acids, and thymol.

The capacity of *p*-coumaric acid for preventing lipid peroxidation was already reported by Kiliç and Yeşilouğlu (2013), although using a different method. The inhibition of lipid peroxidation was determined according to the thiocyanate method and using linoleic acid emulsion in potassium phosphate buffer as lipidic substrate. They found that *p*-coumaric acid was better than the controls butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT).

In bulk methyl linoleate or in fish muscle, Kikuzaki *et al.* (2002) and Medina *et al.* (2007), respectively, reported that caffeic acid had the capacity for preventing the formation of hydroperoxides, in contrast to *p*-coumaric acid which had a very weak capacity, as observed in the DPPH method or in the ferric reducing/antioxidant power (FRAP). Such results should be expected since both methods utilize the same single-electron transfer mechanism, according to Medina *et al.* (2007). Using other lipidic substrate such as egg yolk phosphatidylcholine liposomes which hydroperoxides were induced by AAPH [2,2'-azobis(2-amidinopropane) dihydrochloride], Kikuzaki *et al.* (2002) found that gallic acid had the lowest capacity for preventing hydroperoxides immediately followed by caffeic acid and *p*-coumaric acid. In this lipid substrate, ascorbic acid had lower capacity for preventing lipid peroxidation than gallic acid. This is not coincident with our results, in which *p*-coumaric acid was a very weak scavenger of DPPH[•] free radicals, but with similar capacity to those of caffeic and ascorbic acids for preventing hydroperoxide formation when their concentration in sunflower oil was 0.6% (Table 1). Such results are somehow different to those found in our experimental work, but such may partly be explained by the type of lipid substrate.

CONCLUSIONS

Several methods may be assayed for evaluating the antioxidant activity of samples; nevertheless diverse factors may contribute to originate distinct results. In the present work, the same assay (DPPH method) but using different solvents led to different results. Time of reaction also produced diverse results. When comparing two different methods but both based on the capacity for scavenging free radicals (DPPH[•] or ABTS^{•+}) the results were not coincident. Some authors, as reported in the Introduction, suggest that methods based on the lipid oxidation may be recommended to use for calibrating those methods which are based on scavenging free radicals. In the present work such was not as evident because some of the compounds with good capacity for preventing hydroperoxide formation were not the same which possessed the best capacity for scavenging some free radicals. For example, eugenol, was the best antioxidant in terms of DPPH[•] scavenging ability, whereas *p*-coumaric acid, thymol

and gallic acid were the best compounds for scavenging ABTS free radicals. These compounds were also the best ones for preventing hydroperoxidation of a lipidic substrate but along with caffeic acid.

Higher concentrations of thymol, gallic acid and *p*-coumaric acid induced higher peroxidation of triglycerides of sunflower oil than at lower concentrations. On the contrary, caffeic acid and eugenol presented better capacity for preventing peroxidation at higher concentration than at lower ones. Several factors (methods, concentrations, matrices, among others) determine different results which need be carefully understood and enlightened.

Conflicts of interest

All authors declare no conflicts of interest.

ACKNOWLEDGEMENTS

Authors are grateful to FCT-Fundação para a Ciência e Tecnologia (PEst-OE/EQB/LA0023/2013).

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How to cite this article:

Jaqueline Badanai, Celi Silva, Denise Martins, Dulce Antunes, Maria Graça Miguel. Ability of scavenging free radicals and preventing lipid peroxidation of some phenols and ascorbic acid. *J App Pharm Sci*, 2015; 5 (08): 034-041.