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# Evaluation of Antiradical Capacity by $H_2O_2$−Hemin-Induced Luminol Chemiluminescence

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This paper describes a screening method for antioxidant potential determination based on luminol/hemin/hydrogen peroxide chemiluminescence. The emission depletion, caused by an antiradical compound added during the chemiluminescence decay, is proportional to the number of reactive species trapped. Therefore, the difference between the areas of the emission decay curves, obtained in the absence and in the presence of the potential antioxidant, is a measure for the antiradical capacity of the sample. The technique has been applied to measure the antiradical capacity of pure compounds and complex mixtures from natural origin, providing reliable results that indicate the method's feasibility.

**KEYWORDS: Luminol; hemin; TRAP; antioxidant; antiradical; free radicals**

## INTRODUCTION

Several in vitro methods have been developed in the last 15 years to assess the antioxidant capacity of pure compounds, food extracts, and diverse biological matrixes (*1*, *2*). However, considering the complex nature of the balance between oxidant and antioxidant species in living organisms, the biological significance of this method is doubtful (*3*−*7*). Even so, these tests may still be used as screening methods for antiradical compounds in complex matrixes, although positive results do not necessarily indicate in vivo antioxidant activity (*8*).

The TRAP assay, proposed by Wayner et al., is based on the generation of alkyl peroxyl radicals by the thermolysis of the azoinitiator ABAP in a lipid dispersion (*9*, *10*). The rate of radical-induced lipid peroxidation is measured by oxygen consumption, providing an indirect measure for the ability of plasma to inhibit the reaction. The length of the induction period (lag phase), induced by plasma, on the rate of oxygen consumption is compared with that induced by a known amount of the water soluble vitamin E analogue trolox. The original TRAP assay has been modified using alternative radical generation and detection methods to provide more suitable routine analysis protocols (*2*).

The chemiluminescence of luminol can be induced by free radical and/or redox processes, and the simplicity and sensitivity of this technique are taken as an advantage in the development of analytical methods for the monitoring of free radical production or consumption (*11*−*15*). Most of the methods used for measuring total antioxidant capacity of plasma or serum employ azoinitiators, due to their capacity to decompose, producing a peroxyl radical flow at a constant rate and luminol chemiluminescence (*3*).

The first chemiluminescence TRAP assay, reported by Metsä-Ketelä in 1991 (*16*), is based on the oxidation of luminol using ABAP as a free radical source. A few months later, Lissi et al. proposed a very similar method, having discussed the mechanism of luminol chemiluminescence induced by ABAP (*17*). In both cases, the inhibition potential is also compared with that obtained by trolox to access TRAP values. Whitehead et al. reported an enhanced chemiluminescent assay for determination of the antioxidant capacity in biological fluids (*18*). The method consists of the enhanced luminol reaction with hydrogen peroxide or perborate in the presence of HRP, which produces long-lasting, slow chemiluminescence decay. The continuous light output depends on constant production of free radical intermediates derived from *p*-iodophenol, luminol, and oxygen. For this reason, the light emission is sensitive to interference by radical-scavenging compounds but will be restored after antioxidant consumption. The sample is added during the chemiluminescent reaction, and the period of light suppression is related to the amount of antioxidant present. The main advantage of this method is that antioxidants are exposed to reactive oxygen species that are known to occur in vivo, differently from the alkyl peroxyl radicals formed by thermolysis of azo compounds (*5*).

In this work, we propose a reliable method, based on the chemiluminescent oxidation of luminol by hydrogen peroxide in the presence of hemin catalyst, as a screening assay to assess the antiradical capacity of pure compounds and complex mixtures of natural origin.

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## MATERIALS AND METHODS

**Chemicals.** Luminol (5-amino-2,3-dihydro-phthalazine-1,4-dione) was obtained from Merck (Darmstadt, Germany), and a stock solution (10 mM) was prepared in 1 M NaOH and stored at 4 °C for up to 10 days. The working solution was a 1:100 dilution in phosphate buffer (0.1 M, $Na_3PO_4/Na_2HPO_4$), pH 11.6. The final luminol concentration was determined spectrophotometrically at 347 nm ($\epsilon = 7600\ M^{-1}\ cm^{-1}$). Hydrogen peroxide (Peróxidos do Brasil, São Paulo, Brazil) was obtained as a 60% w/w unstabilized aqueous solution. The final concentration after dilution with demineralized water (18 MΩ, Milli-Q, Millipore) was determined spectrophotometrically as described by Cotton and Dunford (*19*). Hemin (ferriprotoporphyrin IX chloride) was purchased from Sigma (St. Louis, MO). A stock solution (8 $\mu$M) was prepared by dissolving 2.5 mg of hemin in 5 mL of 1 M NaOH. The working solution was a 1:100 dilution with 1 M NaOH. The concentration was determined spectrophotometrically using $\epsilon = 58\ 400\ M^{-1}\ cm^{-1}$ at 385 nm (*20*).

Trolox was obtained from Aldrich (Milwaukee, WI) and was used as an antiradical standard. Uric acid, quercetin, and ascorbic acid were from Sigma and were used without further purification. 4-Nerolidylcatechol was extracted from *Pothomorphe umbellata* (L.) roots, collected in the Campus of the Universidade de São Paulo (Brazil), and purified as described in the literature (*21*). Hesperidin was a gift from Prof. Massayoshi Yoshida (Universidade de São Paulo). *Ginkgo biloba* extract was obtained from Santos Flora (São Paulo, Brazil). Stock solutions were prepared before use, with a 10 mM NaOH solution saturated with nitrogen, and stored at 4 °C protected from light. All antioxidants were monitored for alterations in absorption spectra in the pH range between 8 and 11.6.

**Instrumentation.** The antioxidant assay was performed on a SPEX Fluorlog 1681 spectrofluorimeter with a PMT voltage of 750 V, an emission wavelength of 420 nm, and a slit width of 4 mm. Absorption spectra were recorded using a Shimadzu Multispec 1500 UV−visible spectrophotometer.

**Antioxidant Assay Procedure.** In a 10 mm quartz fluorescence cell with a magnetic stirrer, thermostated at 25.0 ± 0.2 °C, 30 $\mu$L of a 10 mM luminol solution and 30 $\mu$L of a 8 $\mu$M hemin solution were added to 2.88 mL of a 0.1 M phosphate buffer, pH 11.6, $\mu = 0.1$ M. The chemiluminescent reaction was initiated by adding 30 $\mu$L of a 1 mM hydrogen peroxide solution, and 30 $\mu$L of the antiradical sample solution were added after 100 s. The final reaction mixture contained 80 nM hemin, 0.1 mM luminol, and 10 $\mu$M hydrogen peroxide in a 3 mL final volume.

**Quantification.** For a light emission whose intensity varies with time, the number of photons emitted per second at any time $t$ will be $N(t)$, and the total light emitted ($S_{blank}$) will be proportional to the emission intensity ($I$) (eq 1).

$$S_{blank} = \int_0^\infty N(t)\,dt = \int_0^\infty I\,dt \qquad (1)$$

The area under the chemiluminescence decay curve of the standard reaction, which represents the total light emitted ($S_{blank}$), also represents the total amount of the reactive species present in the reaction media, which leads to light emission. Radical scavenging compounds will consume these reactive species and therefore suppress the light emission. Thus, the difference between the areas obtained in the presence and in the absence of an antiradical compound ($S_{supp}$) represents the number of reactive species consumed by the antiradical (**Figure 1**) and the value obtained is proportional to the concentration of antiradical added to the system and a measure for its antiradical capacity. The plot of the antiradical concentration vs the suppression area ($S_{supp}$) is predicted to yield a straight line, and the antiradical capacity of the sample compound is determined by comparison of the slope obtained with a sample compound ($\alpha_S$) and the slope obtained with trolox ($\alpha_T$; **Figure 2**). As trolox is capable of scavenging two radicals per molecule (*22*−*24*), the number of radicals trapped by one sample molecule ($n_s$) is obtained using eq 2.

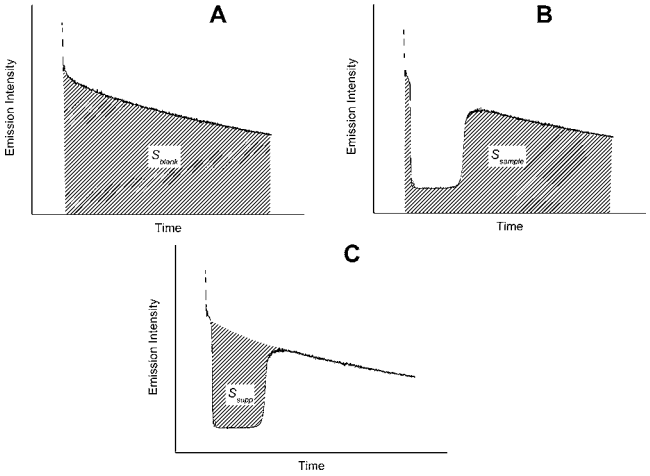



**Figure 1.** Suppression of light emission by addition of the standard antiradical trolox. (**A**) Light emission kinetics of the luminol/$H_2O_2$/hemin system in standard conditions (dashed area corresponds to $S_{blank}$). (**B**) Light emission kinetics of the system in **A** with addition of trolox (1 $\mu$M) after 100 s (dashed area corresponds to $S_{sample}$). (**C**) Determination of the suppression area $S_{supp} = S_{blank} - S_{sample}$ (dashed area) by numerical subtraction of the emission area obtained in the presence of antiradical from that obtained in the absence of antiradical.

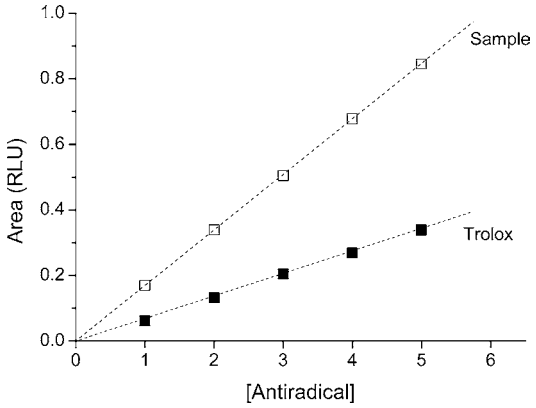



**Figure 2.** Linear correlation between the antiradical concentration and the suppression area ($S_{supp}$) for the standard trolox and quercetin, as an example for a sample compound.

$$n_S = \frac{\alpha_S}{\alpha_T} \times 2 \qquad (2)$$

The antiradical capacity of complex mixtures, like natural products or food extracts, can also be evaluated by this methodology from the linear correlation between the suppression area and the sample concentration. The antiradical capacity of the complex sample is most conveniently expressed, in this case, as the concentration (mg/L) with the same capacity as 1 $\mu$M trolox (**Figure 3**). The numerical value is, therefore, inversely proportional to the antiradical capacity.

**Statistical Data.** All values are expressed as mean ± standard deviation (SD) of at least three independent experiments. Statistical data analysis was achieved by one way analysis of variance. The level of statistical significance was taken at $P < 0.05$. All calculations were performed using Microcal Origin 5.0 (1998).

## RESULTS AND DISCUSSION

Luminol oxidation by hydrogen peroxide in the presence of hemin (protoferrihaem) as catalyst results in chemiluminescence (*25*−*27*). Although the mechanism of this reaction is not completely understood (*11*), it is known that the emission results

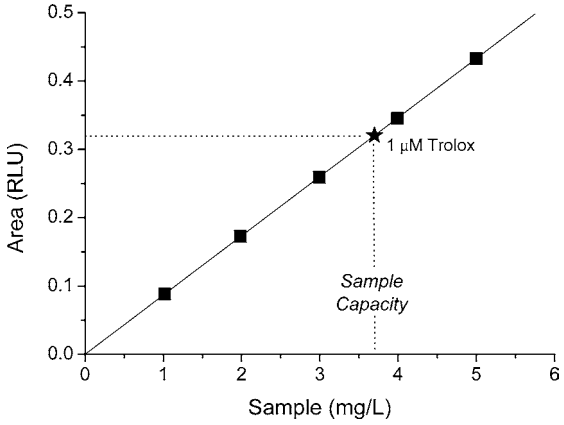


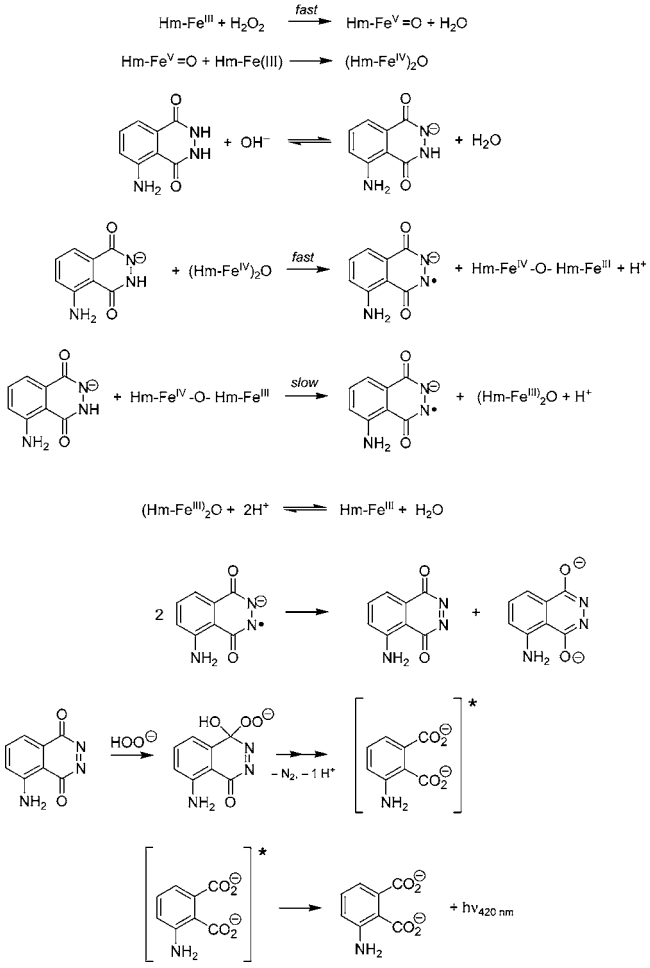

**Figure 3.** Linear correlation between the concentration of a complex mixture and the suppression area for the determination of the antiradical capacity of complex mixtures. The area value obtained with 1 $\mu$M trolox corresponds to the capacity in mg/mL of the sample.

from excited 3-aminophthalate generated by the reaction of luminol with an oxidizing intermediate formed in the reaction of hemin with hydrogen peroxide (*26*). The mechanism of catalysis by haem-containing peroxidase enzymes such as catalases, HRP, and heme-mono-oxygenase enzymes (cytochrome P-450) in the presence of hydrogen peroxide involves the formation of oxidizing intermediates that are formally "Fe(V)" species (*28*). In most cases, the structure is an Fe(IV)═O (ferryl) center, combined with a porphyrin $\pi$-cation radical. In the oxidation of protoferriheme, the Fe(V) intermediate is not observed because it is quickly reduced by unoxidized protoferriheme to a formal Fe(IV) species, which is possibly a dimeric Fe(III)−porphyrin $\pi$-cation radical complex. This species is supposed to be responsible for luminol oxidation and the initiation of the reaction sequence leading finally to the production of exited 3-aminophthalate, with subsequent light emission (**Scheme 1**) (*26*).

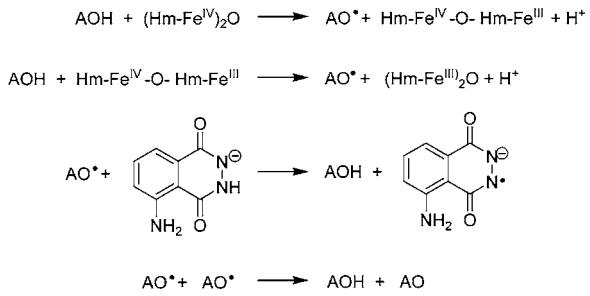
To use the luminol chemiluminescence as an antiradical assay, the kinetics should consist in slow light emission decay. This system behavior indicates a constant, or nearly constant, production of free radical intermediates derived from luminol, hemin, and hydrogen peroxide. As the kinetics of the reaction can be modified by varying the hemin or hydrogen peroxide concentration, low hydrogen peroxide concentrations are employed in order to obtain slow emission decay and a relatively slow consumption of the antiradical, turning the assay more accurate and highly sensitive (*25*, *26*, *29*). These assay conditions allow the quantitative determination of the antiradical capacity of compounds at micromolar and submicromolar concentrations (*30*). It must be pointed out here, however, that the catalytic activity of ferrihaem in the formation of peroxidatically active intermediates is strongly influenced by its dimerization. As the monomer/dimer equilibrium constant is pH- and concentration-dependent, the preparation of hemin stock solutions is an important step in this assay and it should be performed exactly as described in order to obtain slow decaying emission kinetics. The procedure described leads to a solution with a constant monomer/dimer ratio, as judged by its absorption spectrum, providing reproducible results.

An antiradical (AOH) added to the system will compete with luminol for the hemin-derived oxidizing intermediates and any other oxidant species present in the reaction mixture, as well as intercept luminol-derived free radical species and oxidants. Therefore, chemiluminescence emission will be suppressed until all antiradical compounds in the system are completely consumed. The main pathway for disappearance of the phenoxyl radicals is its dismutation, leading to the formation of the quinoid analogues (AO), as well-known for the case of trolox (*31*−*37*) (**Scheme 2**). Thus, a delay period in chemiluminescence emission, proportional to the antiradical concentration and its capacity, results from the competitive oxidation of the nonchemiluminescent substrate (*5*). Trolox has been used as a reference compound because it is stable for many hours in solution at room temperature (*18*) and its addition to the luminol system results in almost complete suppression of the light emission, which is restored immediately after total consumption (**Figure 4**).

**Scheme 1**




**Scheme 2**




Several chemiluminescence methods are based on the measurement of a lag time, induced by a sample added to the system before the initiation of the reaction or the delay period caused by sample addition during the course of the chemiluminescent reaction. The value used by the delay period method for antioxidant capacity is the difference between the time of the

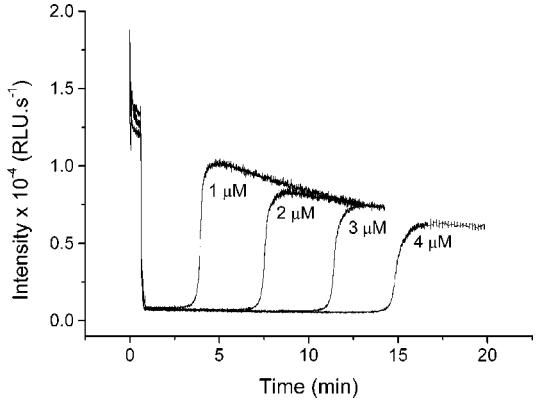



**Figure 4.** Emission kinetics obtained from luminol/$H_2O_2$/hemin system upon addition of different concentrations of trolox. Concentrations: [luminol], 0.1 mM; [$H_2O_2$], 10 $\mu$M; [hemin], 80 nM; phosphate buffer ($\mu = 0.1$), pH 11.6.

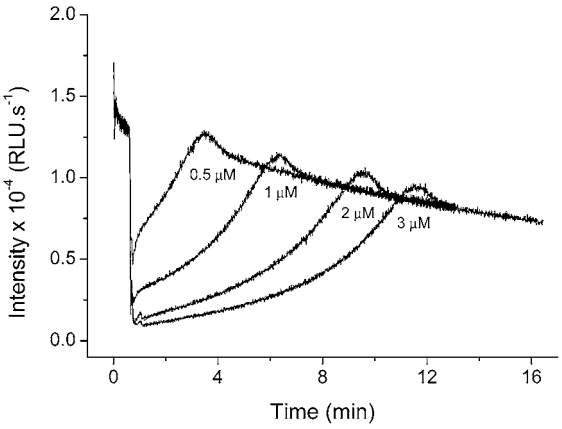



**Figure 5.** Emission kinetics obtained from luminol/$H_2O_2$/hemin system upon addition of different concentrations of uric acid. Concentrations: [luminol], 0.1 mM; [$H_2O_2$], 10 $\mu$M; [hemin], 80 nM; phosphate buffer ($\mu = 0.1$), pH 11.6.

antiradical addition and that when the light intensity increases to an arbitrary percentage, typically 30%, of the maximum emission intensity (*38*). This method tends to be inexact when comparing compounds with different quenching behaviors in a given oxidizing system and is suitable only for compounds whose behavior results in a "square-wave" appearance after its consumption (*18*). This behavior is obtained for trolox (**Figure 4**) but not for many other antioxidants, e.g., uric acid (**Figure 5**). In this case, the determination of the antioxidant capacity by the delay time method does not appear to be adequate, and the results can be significantly different depending on the manner in which the method is applied.

The different kinetical behavior of the antioxidants may be related to their reaction mechanism and reactivity. Although attempts have been made to quantify the antioxidant reactivity by the TAR (total antioxidant reactivity) index (*17*, *39*, *40*), there is no consensus, however, about what is being measured by this parameter (*40*, *41*). In view of these problems, an alternative and more precise method for the determination of the antiradical capacity is proposed here, which uses the suppression area ($S_{supp}$) of the luminol chemiluminescence as a parameter for the sample antiradical capacity. The main advantage of the present method is that it is applicable to antiradicals that show different kinetic behaviors (**Figures 4** and **5**); therefore, it can be employed to any antiradical compound (or complex mixture), independent of the kinetic emission profile.

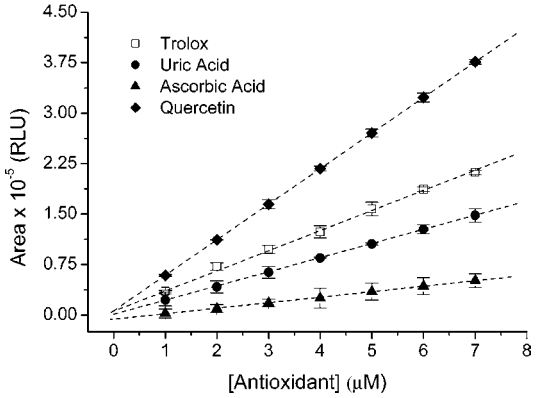



**Figure 6.** Correlation of the suppression area with the antioxidant concentration. Concentrations: [luminol], 0.1 mM; [$H_2O_2$], 10 $\mu$M; [hemin], 80 nM; phosphate buffer ($\mu = 0.1$), pH 11.6. The dashed lines correspond to the linear fitting. Trolox: $A = 5910 \pm 1520$, $B = (2.98 \pm 0.05) \times 10^{10}$, $R = 0.99964$, SD = 0.72331, $N = 7$. Uric acid: $A = 930 \pm 5410$, $B = (2.09 \pm 0.12) \times 10^{10}$, $R = 0.99993$, SD = 0.08887, $N = 7$. Ascorbic acid: $A = -6430 \pm 8150$, $B = (8.18 \pm 1.67) \times 10^9$, $R = 0.99962$, SD = 0.06059, $N = 7$. Quercetin: $A = 5910 \pm 1210$, $B = (5.29 \pm 0.05) \times 10^{10}$, $R = 1.00000$, SD = $1.12 \times 10^{-14}$, $N = 7$.

**Table 1.** Number of Radicals Trapped (*n*) by an Inhibitor Molecule, Obtained Using Different Methods

| | | antioxidant | | | |
|---|---|---|---|---|---|
| method | ref | quercetin | trolox | uric acid | ascorbic acid |
| TRAP oxygen electrode | *9* | | 2.0 | 1.3 | 1.7 |
| TRAP luminol/ABAP | *30* | | 2.0 | 1.2 | 0.4 |
| TRAP phycoerythrin (PE) | *44* | | 2.0 | 1.7 | 1.5 |
| TRAP lysozyne/ABAP | *45* | | 2.0 | 1.7 | 1.3 |
| TRAP luminol/SIN-1 | *46* | | 1.0 | 1.1 | 0.96 |
| ORAC phycoerythrin (PE) | *42* | 2.1 | 1.0 | | 0.4 |
| ORAC phycoerythrin (PE) | *47* | | 2.0 | 1.8 | 1.0 |
| TOSC KMBA | *48* | | 2.0 | 1.4 | 0.9 |
| DPPH luminol/Co(II)EDTA | *15* | 3.8 | 2.0 | | 1.9 |
| TRAP luminol/hemin/$H_2O_2$ | this work | $3.6 \pm 0.12$ | 2.0 | $1.4 \pm 0.15$ | $0.5 \pm 0.22$ |

To show the applicability and reliability of the new methodology, we have determined the number of radicals quenched by some antiradical compounds, which have previously been reported as antioxidants and have had this antioxidant capacity determined by a variety of experimental methods. Quercetin, a flavonol reported as a very efficient antioxidant, was included to represent an antioxidant compound originating from natural products (*15*, *42*, *43*). The suppression areas show high linear correlation with the antiradical concentrations for all compounds studied (**Figure 6**). The results obtained by our method are in good agreement with literature values, although quite different results are obtained for ascorbic acid using different assays (**Table 1**).

The results for ascorbic acid can be rationalized in light of a fast oxidation of this compound even at low oxygen concentrations and its tendency to decompose in the presence of light. Otherwise, the prooxidant activity of ascorbic acid in vivo is

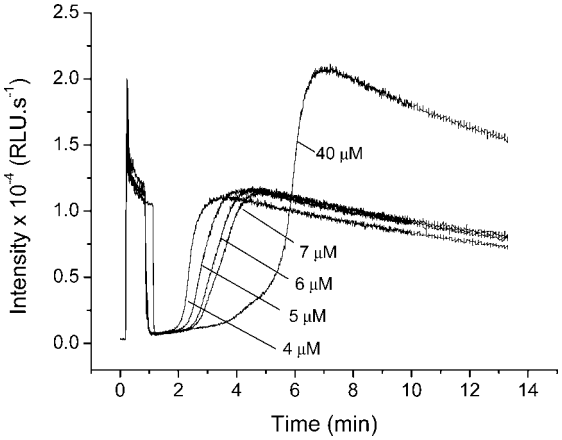



**Figure 7.** Emission kinetics obtained from luminol/$H_2O_2$/hemin system upon addition of different concentrations of ascorbic acid. Concentrations: [luminol], 0.1 mM; [$H_2O_2$], 10 $\mu$M; [hemin], 80 nM; phosphate buffer ($\mu$ = 0.1), pH 11.6.

widely discussed in the literature being attributed to radical chain reactions mediated by ascorbyl radical (*49*−*52*). The results using high concentrations of ascorbic acid (>10 mM) to access a calibration curve with this antioxidant are intriguing (**Figure 7**). After the delay time, the light emission is increased, and the suppression area correlates with ascorbic acid concentration only for low concentrations (**Figure 6**). The enhancement of the emission intensity after the lag phase in the presence of high ascorbate concentrations may be attributed to an enhancer effect of ascorbate, which may react faster than luminol with the FeIII−O−FeIV species, increasing the hemin recycling rate and resulting in enhancement of the emission intensity (*53*−*59*). Additionally, the ascorbyl radicals may act as redox mediators accelerating luminol oxidations by formation of luminol radicals (*35*, *53*, *54*), although this reaction may not be very important due to its low oxidation potential (*60*, *61*). Ascorbate is well-known to act as antioxidant as well as prooxidant in several systems (*49*, *51*, *52*, *62*−*68*).

As further proof for the applicability of the assay, we describe here the determination of the antiradical capacity of natural products. Two isolated compounds, 4-NC and hesperidin, and a plant extract used as phytomedicine were utilized as model substances. The antioxidant effect of plant extracts has been related to the prevention of coronary diseases and cancer, as well as age-related degenerative brain disorders. This activity is mainly due to phenolic substances, which are widely distributed in the plant kingdom and have been reported to possess a broad range of other biological effects (*69*, *70*). Furthermore, natural antioxidants may constitute alternatives to synthetic compounds used in food and pharmaceutical industries.

Extracts of *P. umbellata* (L.) Miq. have been shown to reduce oxidative damage when tested in in vitro lipid peroxidation models (*21*), and it appears that 4-nerolidylcatechol is the main compound responsible for this activity (*71*, *72*). Hesperidin is a glycosilated flavanone, present in citric fruits, especially orange, and has shown a wide range of therapeutic properties for medical and clinical applications such as antiinflammatory, antihypertensive, diuretic, analgesic, and hypolipidemic activities (*73*, *74*). *G. biloba* extracts are used as phytomedicine in several pathological disorders and possess two groups of active substances, flavonol glycosides and terpene lactones, including ginkgolides A, B, and C, bilobalide, rutin (quercetin-3-O-rutinoside), and kaempferol-3-O-rutinoside. The flavonoids in *Ginkgo* extracts are supposed to function as antioxidants (*75*−*79*).

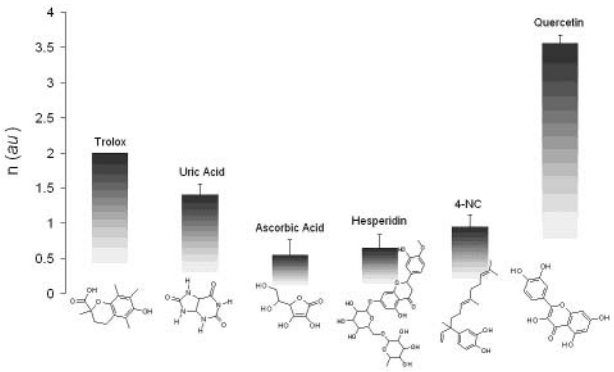



**Figure 8.** Antioxidant capacity of several antiradical compounds expressed as the number of radicals trapped (*n*) by inhibitor molecule, obtained using the luminol/$H_2O_2$/hemin system.

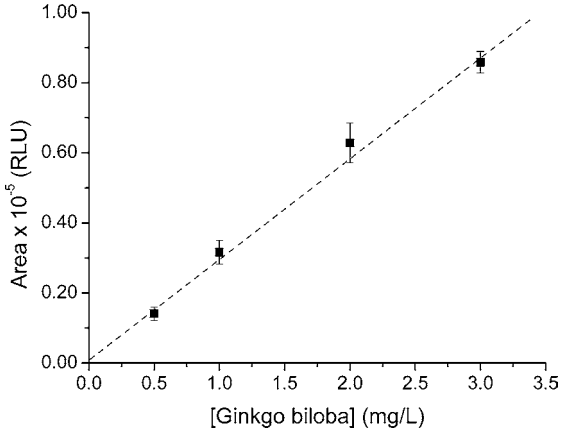



**Figure 9.** Linear correlation between *G. biloba* concentration and the suppression area. The area value obtained with 1 $\mu$M trolox in identical experimental conditions is 35 342 ± 1254 and corresponds to the *G. biloba* concentration of 1.2 mg/mL. Concentrations: [luminol], 0.1 mM; [$H_2O_2$], 10 $\mu$M; [hemin], 80 nM; phosphate buffer ($\mu$ = 0.1), pH 11.6. The dashed line corresponds to the linear fitting: $A$ = 445 ± 2206, $B$ = (2.89 ± 0.14) × $10^7$, $R$ = 0.99827, SD = 0.85565, $N$ = 4.

The number of radicals trapped by 4-nerolidylcatechol, calculated using the luminol/$H_2O_2$/hemin chemiluminescence assay, is 0.95. Desmarchelier et al. have reported that 4-NC can trap 0.71 radicals per molecule using the luminol/ABAP TRAP assay (*71*). In view of considerable variations in TRAP values obtained with different methodologies, these values are in reasonable agreement. The number of radicals quenched by hesperidin determined in this work is $n$ = 0.65, which is low when compared with quercetin, a nonglycosilated flavonoid. Despite these results, however, it may have utility as a commercial antioxidant due to its availability in considerable amounts being generated as waste in the citric fruit juice industry (*74*). **Figure 8** depicts the summary of the results obtained in the study of the number of radicals trapped by the antiradicals with different structures, including the SDs, and demonstrating the viability and accuracy of the methodology utilized.

The assay is also applied to the determination of the antiradical capacity of a sample of *G. biloba* extract. The capacity determined from the calibration curve of *G. biloba* (**Figure 9**) is 1.2 mg/mL, corresponding to the sample concentration necessary to obtain the same suppression area ($S_{supp}$) as 1 $\mu$M trolox, and this value can be compared to that obtained for other complex mixtures. Therefore, this method can be used to evaluate the antiradical capacity of any complex mixture, even though its composition is not known.

In summary, this method offers a reproducible and low cost alternative for the screening of pure compounds and complex mixtures from natural origins, such as natural products and food, for utilization as possible antioxidants in vivo. However, total antioxidant capacity assays need to be interpreted based on the source of oxidative stress, essentially the oxidizing species generated, and the target of the damage. Thus, it is important to realize that there is no assurance that a given reductant acting like an antiradical compound in in vitro tests corresponds to an antioxidant compound in vivo.

## ABBREVIATIONS USED

4-NC, 4-nerolydilcatechol; ABAP, 2,2′-azobis-(2-amidinopropane) dihydrochloride; HRP, horseradish peroxidase; RLU, relative light unit; TRAP, total radical-trapping antioxidant parameter; trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxilic acid.

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