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Inhibitory Effect of Gallic Acid and Its Esters on 2,2′-Azobis(2-amidinopropane)hydrochloride (AAPH)-Induced Hemolysis and Depletion of Intracellular Glutathione in Erythrocytes

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The protective effect of gallic acid and its esters, methyl, propyl, and lauryl gallate, against 2,2′-azobis(2-amidinopropane)hydrochloride (AAPH)-induced hemolysis and depletion of intracellular glutathione (GSH) in erythrocytes was studied. The inhibition of hemolysis was dose-dependent, and the esters were significantly more effective than gallic acid. Gallic acid and its esters were compared with regard to their reactivity to free radicals, using the DPPH and AAPH/pyranine free-cell assays, and no significant difference was obtained. Gallic acid and its esters not only failed to inhibit the depletion of intracellular GSH in erythrocytes induced by AAPH but exacerbated it. Similarly, the oxidation of GSH by AAPH or horseradish peroxidase/H_2O_2 in cell-free systems was exacerbated by gallic acid or gallates. This property could be involved in the recent findings on pro-apoptotic and pro-oxidant activities of gallates in tumor cells. We provide evidence that lipophilicity and not only radical scavenger potency is an important factor regarding the efficiency of anti-hemolytic substances.

KEYWORDS: Gallic acid; gallates; erythrocytes; hemolysis; glutathione; peroxyl radical; antioxidant; pro-oxidant activity

INTRODUCTION

Gallic acid and its derivatives are among the most abundant phenolic antioxidants in wines and green tea (*1*−*3*). This phytochemical is well-known for its antioxidant, antibacterial, anti-inflammatory, antimutagenic, and chemopreventive properties (*4*−*7*). The similar beneficial effects of the synthetic esters of gallic acid have been extensively studied, and in many cases, they are stronger than those of gallic acid itself. For instance, lauryl gallate (dodecyl gallate), a widely used food additive, is more efficient than gallic acid as an inhibitor of the enzyme xanthine oxidase, an endogenous source of the superoxide anion, and of mitochondrial lipid peroxidation induced by Fe^{III}−NADPH (*8*). Methyl gallate is more effective than gallic acid at inhibiting oral bacterial growth and the formation of *Streptococcus mutans* biofilms (*4*). Methyl gallate inhibits the production of leukotriene C4 and prostaglandin D2 by bone-marrow-derived mast cells (*9*). Nonyl gallate is more effective than gallic acid against the growth of *Salmonella choleraesuis* (*10*). Lauryl gallate protects against the formation of dimethylbenzanthracene-induced skin tumors in mice and kills, selectively, tumor cells in established tumors (*11*). Such biological activities have been correlated with the amphipathic feature of these ester derivatives (*12*), because the *in vitro* antioxidant potential is nearly the same as that of gallic acid. Hence, it may be supposed that the accessibility of the gallates to the intracellular medium could be a determining factor for their effects.

Another relevant property of gallates is their pro-oxidant characteristics. Indeed, the cytotoxic effect of octyl, dodecyl, and tetradecyl gallates on melanoma cells has recently been demonstrated (*13*, *14*). This biological effect has been associated with DNA fragmentation, activation of NF-κB, inhibition of cell adhesion, and alteration of the cell redox status by depletion of both glutathione (GSH) and ATP (*13*). Similarly, the inhibition of HeLa cell growth by propyl gallate was associated with depletion of intracellular GSH and increased production of the superoxide anion (*14*).

The hemolysis of erythrocytes has been extensively used as an *ex vivo* model for studying reactive oxygen species (ROS)-induced disruption of cell membranes and the protective effect of antioxidants. One of the most frequently studied models uses the water-soluble azo compound 2,2′-azobis(2-amidinopropane)hydrochloride (AAPH), which decomposes at physiological temperature (37 °C)

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to generate an alkyl radical (R•). In the presence of dissolved molecular oxygen, this radical is converted to the corresponding peroxyl radical (ROO•) (*15*). The mechanism of erythrocyte hemolysis induced by thermolysis of AAPH is not completely understood, but it has been correlated with lipid peroxidation and oxidation of membrane proteins (*16*). The incubation of erythrocytes with AAPH also provokes the depletion of intracellular GSH (*17*).

Because the above biological phenomenon is associated with the disruption of the cell membrane and in view of increasing evidence that the lipophilicity of gallic acid esters is decisive for their beneficial effects, here, we aimed to synthesize selected gallate esters and compare their efficacy as inhibitors of AAPH-induced hemolysis and GSH depletion. The objective was to retain the same oxidizable moiety while altering the lipophilicity. Additionally, we studied and compared the antioxidant and pro-oxidant effects of gallic acid and gallates in cell-free systems.

MATERIALS AND METHODS

Chemicals and Equipments. Gallic acid, vanillic acid, methyl vanillate, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (trolox), *N*-ethylmaleimide (NEM), (±)-α-tocopherol (vitamin E), 2,2′-azobis(2-amidinopropane) hydrochloride (AAPH), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), glutathione (GSH), 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (pyranine), 2,2-diphenyl-1-picrylhydrazyl (DPPH), *N*,*N*′-dicyclohexylcarbodiimide, cumene hydroperoxide (Cu−OOH), horseradish peroxidase (HRP) (EC 1.11.1.7), and *o*-phthalaldehyde (OPA) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Hydrogen peroxide (H_2O_2) was prepared by diluting a 30% stock solution and calculating its concentration from its absorption at 240 nm ($\varepsilon_{240} = 43.6\ M^{-1}\ cm^{-1}$) (*18*). Column chromatography was carried out over 0.06−0.20 mm silica gel (Acros Organics, Morris Plains, NJ). Gel permeation chromatography (GPC) was performed with Sephadex LH-20 (Pharmacia Biotech, Uppsala, Sweden). Preparative high-performance liquid chromatography (HPLC) was carried out in a Varian Prep-Star 400 system with a Phenomenex C-18 (250 × 21.2 mm) column. The monodimensional nuclear magnetic resonance (NMR) spectra were recorded on a Varian Inova 500 spectrometer (11.7 T) at 500 MHz (^{1}H) and 125 MHz (^{13}C), using $CDCl_3$ and DMSO-d_6 as solvents (Aldrich). All of the reagents used for buffers and mobile phases were analytical-grade. Stock solutions of antioxidants were prepared in ethyl alcohol. Ultrapure Milli-Q water from Millipore was used for the preparation of buffers and solutions.

Gallic Acid Source. Gallic acid was extracted from *Alchornea glandulosa*. The leaves were collected in the Biological Reserve and Experimental Station at Mogi Guaçu, São Paulo State, Brazil, in March 2005. A voucher specimen (SP319257) has been deposited in the herbarium of the Botanic Institute (São Paulo, SP, Brazil). The shade-dried plant material (1.5 kg) was ground and defatted with *n*-hexane (3.5 L × 3, at room temperature) and exhaustively extracted by maceration with MeOH (4.2 L × 3). The crude extract was concentrated under reduced pressure to yield 3.8 g of a syrupy residue. The concentrate was then diluted with MeOH/H_2O (4:1) and successively partitioned with EtOAc and *n*-BuOH. After solvent removal in a rotary evaporator, the partition phases yielded 2.5 and 0.8 g, respectively. The EtOAc residue (2.0 g) was chromatographed by gel permeation over Sephadex LH-20, eluted with methanol, to afford 11 fractions (A1−A11). Second-stage chromatographic purification of fraction A2 (730 mg) by reverse-phase (RP)-HPLC (7.5:92:0.5 MeOH/H_2O/HOAc, UV detection at 265 nm, and flow rate at 15 mL/min) yielded gallic acid (335 mg). The identification was based on analysis of ^{1}H and ^{13}C NMR data, as well as by a comparison to authentic material obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO).

Synthesis of Esters (Methyl, Propyl, and Lauryl). A 3 mL solution of *N*,*N*′-dicyclohexylcarbodiimide (DCC, 1.0 mmol) in *p*-dioxane was added to a cooled (5 °C) solution of 0.2 mmol of gallic acid and 20 mmol of methyl, propyl, or lauryl alcohol in 6 mL of *p*-dioxane. After the solution was stirred for 48 h, the solvent was removed under reduced pressure. The residue was partitioned 3 times with EtOAc and filtered. The filtrate was washed successively with saturated aqueous citric acid solution (3 times), saturated aqueous $NaHCO_3$ (3 times), and water (2 times), dried over anhydrous $MgSO_4$, and evaporated under reduced pressure. The crude products were purified over a silica gel column eluted isocratically with $CHCl_3$/MeOH (98:2). Structures of the semi-synthetic esters were established by ^{1}H and ^{13}C NMR spectral analysis.

Erythrocyte Suspension. Human erythrocytes from healthy donors were obtained from peripheral blood, centrifuged at 770*g* for 10 min, and washed 3 times with phosphate-buffered saline (PBS) at pH 7.4. The supernatant and buffy coat were removed by aspiration after each wash. The cells were resuspended to 20% (v/v) in PBS. The blood samples were taken from healthy volunteers. The study was approved by the faculty research ethics committee.

Hemolysis Assays. The hemolysis studies were performed as previously described (*19*), with modifications. Equal amounts of erythrocytes suspension and 100 mM AAPH in PBS were gently homogenized while being incubated for 6 h at 37 °C (blood tube rotator). Aliquots (75 μL) were removed at regular intervals, diluted 1:20 with PBS, and centrifuged at 4000 rpm for 10 min. The degree of hemolysis was measured in the supernatant by its absorbance at 540 nm. Reference values (100% hemolysis) were determined with the same aliquot of erythrocytes but diluted in 1500 μL of distilled water instead of PBS to provoke the total lysis of the erythrocytes. By diluting the 100% hemolysis sample in PBS, a calibration curve of percent hemolysis against absorbance was constructed for conversion of the absorbance measurement to degrees of hemolysis. The blank was PBS, and measurements were made in a UV-1240 spectrophotometer (Shimadzu, Japan). When used, 10 μL aliquots of inhibitors in ethanol were added at the beginning of the reaction. The same volume of ethyl alcohol (10 μL) was added to the negative (without AAPH) and positive (with AAPH) controls. In the studies where cumene hydroperoxide (Cu−OOH) was the hemolytic agent, this compound (500 μM) was used instead of AAPH.

Determination of Intracellular GSH. The intracellular concentration of GSH in erythrocytes was determined using the DTNB method (*19*), with modifications. The 20% suspension of erythrocytes (100 μL) was incubated with 100 μL of 50 mM AAPH at 37 °C for 2.0 h. After incubation, the suspension was diluted to 1000 μL with PBS and centrifuged at 6000 rpm for 10 min. The supernatant was removed, and the cell pellet was lysed by adding 700 μL of distilled H_2O. The protein in 700 μL of lysate was precipitated by adding 500 μL of an aqueous solution of 10% trichloroacetic acid (TCA). After 5 min, the protein precipitate was separated from the remaining solution by centrifugation at 14 000 rpm for 10 min. The supernatant (500 μL) was made alkaline by adding 187.5 μL of Na_2HPO_4 (300 mM) and 62.5 μL of NaOH (1 M). Next, 450 μL of Na_2HPO_4 (300 mM) and 100 μL of DTNB solution (0.2 mg/mL in 1.0% sodium citrate) were added to 450 μL of the alkaline mixture. In the blank, 500 μL of supernatant was mixed with 250 μL of PBS instead of 187.5 μL of Na_2HPO_4 and 62.5 μL of NaOH, after which 450 μL of PBS and 100 μL of DTNB were added to 450 μL of this mixture. Each sample test or control had its blank. The absorbance was read at 412 nm. A standard curve was constructed to measure the concentration of GSH. In this case, 700 μL of lysate was replaced by 700 μL of solutions of GSH at several concentrations. The hemoglobin content (Hb) in an aliquot of the lysate (prior precipitation with TCA) was determined by the cyanohemoglobin method. The GSH values were expressed as μmol/g Hb (*20*).

Oxidation of GSH by AAPH. A solution of GSH (100 μM) was incubated with 2.5 mM AAPH in PBS at 37 °C for 1.0 h (*21*). When present, gallic acid and gallates (100 μM) were added before the addition of AAPH. The remaining concentration of GSH was measured by the DTNB method as follows: 0.45 mL of the reaction mixture was added to 0.45 mL of 300 mM Na_2HPO_4 and 0.1 mL of DTNB solution (0.2 mg/mL in 1.0% sodium citrate). The absorbance at 412 nm was read against a blank consisting of 0.45 mL of PBS, 0.45 mL of 300 mM Na_2HPO_4, and 0.1 mL of DTNB. An analytical curve was produced using standard GSH solutions, which were submitted to the DTNB methods.

Oxidation of GSH by HRP/H_2O_2. A solution of GSH (100 μM) was incubated with 100 μM H_2O_2 and 0.1 μM HRP in PBS at 37 °C (*21*). When present, gallic acid and gallates (100 μM) were added before the addition of H_2O_2. The reactions were stopped by adding 10 μg/mL catalase. The remaining concentration of GSH was measured by the DTNB method as above.

Measurement of GSH and GSSG by HPLC. A solution of GSH (100 μM) was incubated with 100 μM H_2O_2 and 0.1 μM HRP in PBS at

37 °C. When present, apocynin, gallic acid, and gallates (100 μM) were added before the addition of H_2O_2. The reactions were stopped by adding 10 μg/mL catalase. The concentration of GSH and its oxidized form (GSSG) were determined by HPLC with fluorimetric detection, as previously described (*22*): For GSH determination, 50 μL of the above reaction mixture was added to 1.0 mL of 0.1% ethylenediaminetetraacetic acid (EDTA) in 0.1 M Na_2HPO_4 at pH 8.0. To a 20 μL aliquot of this mixture, 300 μL of 0.1% EDTA in 0.1 M Na_2HPO_4 at pH 8.0 and 20 μL of 0.1% OPA in methanol were added and this reaction mixture was incubated at 25 °C for 15 min in the dark in well-capped tubes, after which 20 μL of each sample was injected into the HPLC system. For GSSG determination, a 200 μL aliquot of the reaction mixture was incubated at 25 °C with 200 μL of 40 mM NEM, for 25 min, in the dark, to react with the GSH present in the sample. To this mixture, 750 μL of 0.1 M NaOH was added. A 20 μL portion of this mixture was taken for measurement of GSSG, using the procedure outlined above for the GSH assay, except that 0.1 M NaOH was employed as the diluent rather than 0.1% EDTA in 0.1 M Na_2HPO_4 at pH 8.0. The HPLC method for the determination of GSH and GSSG is based on derivatization with OPA to form a stable, highly fluorescent derivative (*23*). The derivatives of GSH and GSSG were separated by liquid chromatography (Varian ProStar in line with a fluorescence detector set at 350/420 nm). The analyses were carried out isocratically on a Luna C18 reversed-phase column (250 × 4.6 mm, 5 μm). The mobile phase consisted of 15% methanol in 25 mM Na_2HPO_4 at pH 6.0 (flow rate of 0.5 mL/min).

Measurement of Oxygen Consumption. The consumption of dissolved molecular oxygen during the oxidation of GSH was monitored in a YSI 5300A oxygen monitor (Yellow Spring, OH). The reaction mixture of 1 mM GSH, 100 μM H_2O_2, and 0.1 μM HRP in PBS was incubated at 37 °C in the presence or absence (control) of 10 μM apocynin or gallates. The reaction was initiated by adding H_2O_2. The oxygen consumption was monitored for 10 min.

Pyranine-Based Procedure for Evaluation of the Reactivity of Gallates with Peroxyl Radicals Generated by AAPH Thermolysis. This experiment was performed as described by Lissi et al. (*24*), with minor modifications. The experiments were based on the decay in the fluorescence of pyranine when it was submitted to oxidation by peroxyl radicals. The fluorescent compound pyranine (5 μM) was incubated with 20 mM AAPH in PBS at 37 °C in the absence (control) or presence of gallates in the wells of a microplate. The fluorescence bleaching of the pyranine was monitored at λ_{ex} = 460 nm and λ_{em} = 510 nm in a Spectramax M2 microplate reader (Molecular Devices, Sunnyvale, CA). The final reaction volume was 300 μL. The lag phase (induction time) obtained when the antioxidants were present was measured for various concentrations of each gallate. The slopes of the curves of induction time versus concentration were used to measure the relative reactivity of the gallates with AAPH-derived peroxyl radicals.

Free-Radical Scavenging Activity on DPPH/DPPH Bleaching Assay. The free-radical scavenger activity of gallic acid and its esters was compared by the DPPH bleaching assay (*25*). The antioxidants were incubated for 30 min with 100 μM DPPH in methyl alcohol in the dark. The scavenger activity was evaluated spectrophotometrically at 517 nm, using the absorbance of the unreacted DPPH radical as the control. The scavenger activity was calculated as ((absorbance of the control − absorbance of the sample)/absorbance of the control) × 100.

RESULTS

We studied the hemolysis of erythrocytes and the protective effect of gallic acid and its esters. The molecular structures of the compounds studied are shown in **Figure 1**. Hemolysis was provoked by thermolysis of the azo compound AAPH, which generates a low but constant flux of peroxyl radicals when incubated at 37 °C (*15*). **Figure 2** shows the kinetic profile of hemolysis and the protective effect of gallic acid and propyl gallate. The inhibition of hemolysis was dose-dependent (**Figure 3**), and the esters methyl gallate (G1), propyl gallate (G3), and lauryl gallate (G12) were more effective inhibitors than their acid precursor, gallic acid (G0) (**Table 1**).

Because the above results suggest a higher efficacy of the esters as free-radical scavengers, we also compared their relative

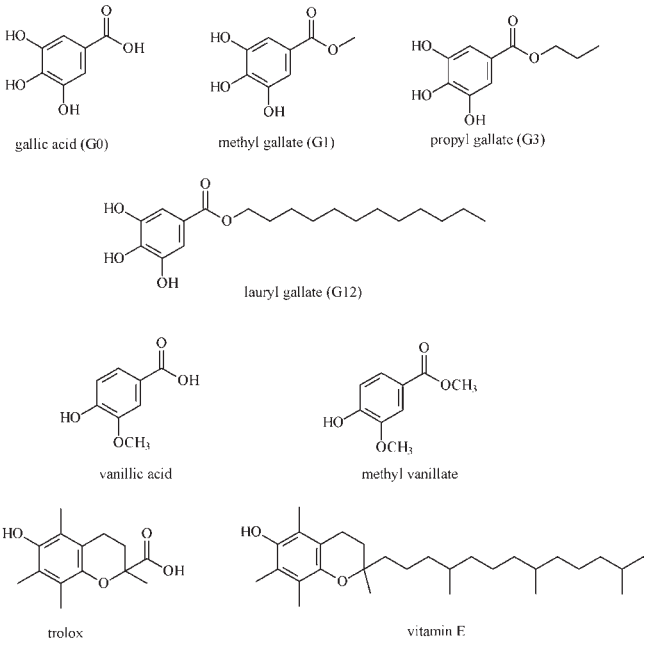


Figure 1. Chemical structures of the compounds used in this study.

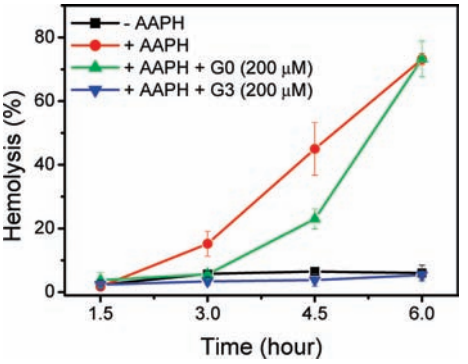


Figure 2. AAPH-induced hemolysis and the protective effect of gallic acid (G0) and propyl gallate (G3). The reaction mixture (positive control) contained 10% (v/v) erythrocytes and 50 mM AAPH in PBS at 37 °C. The results are mean ± standard error of the mean (SEM) of duplicates of three separate experiments.

potency in cell-free models. First, they were compared as scavengers of the stable free-radical DPPH. The results depicted in **Table 2** show that no significant difference was observed between gallic acid and its ester derivatives. In a second set of experiments, we studied the direct reactivity of gallic acid and its esters with AAPH-derived peroxyl radicals. In this assay, the relative reactivity of the antioxidants with peroxyl radicals is determined by the lag phase in the decay of pyranine fluorescence (*24*). **Figure 4** shows the typical time profile of the bleaching obtained when pyranine was incubated with AAPH in the presence of gallic acid and methyl gallate. The linear relation between the delay and the concentration of gallic acid or gallates was used to measure the reactivity of the tested compound with AAPH. In this design, the slope of the curve is directly proportional to the efficiency of the antioxidant as a scavenger of peroxyl radicals. **Figure 4** also shows the typical linear correlation between the concentration and lag phase for gallic acid and methyl gallate. The slopes obtained were 442 ± 30 for gallic acid, 499 ± 31 for methyl gallate, 453 ± 6 for propyl gallate, and 187 ± 7 for lauryl gallate (mean ± SEM, n = 3). As can be observed, only lauryl gallate presented a

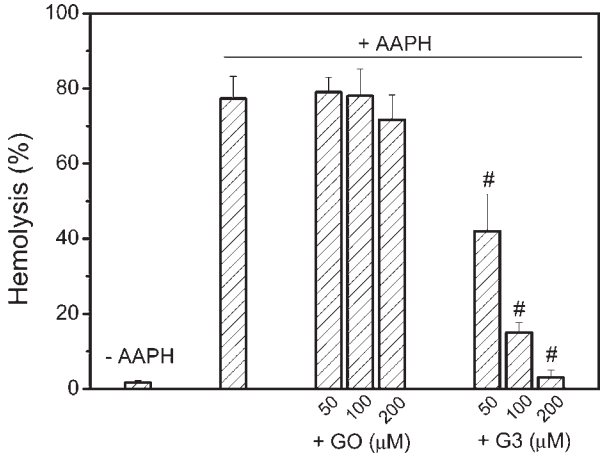


Figure 3. Concentration-dependent effect of gallic acid (G0) and propyl gallate (G3) on AAPH-induced hemolysis. The reaction mixture (positive control) contained 10% (v/v) erythrocytes and 50 mM AAPH in PBS at 37 °C. The results are mean ± SEM of duplicates of three separate experiments. Statistically significant difference relative to the positive control [$p < 0.05$, one-way analysis of variation (ANOVA) and Tukey multiple comparison test].

Table 1. AAPH-Induced Hemolysis and the Protective Effect of Antioxidants[a]

experimental condition		percent hemolysis (3 h)	percent hemolysis (6 h)
−AAPH		2 ± 1[b]	3 ± 1[b]
		17 ± 3[c]	78 ± 7[c]
	+G0	3 ± 2[d]	72 ± 6
	+G1	3 ± 2[d]	18 ± 5[d]
	+G3	2 ± 1[d]	9 ± 4[d]
+AAPH	+G12	16 ± 5	33 ± 5[d]
	+trolox	9 ± 2[d]	75 ± 6
	+vitamin E	7 ± 2[d]	42 ± 7[d]
	+vanillic acid	5 ± 2[d]	45 ± 2[d]
	+methyl vanillate	2 ± 1[d]	5 ± 2[d]

[a] Erythrocyte suspensions (10%) were incubated with 50 mM AAPH in the absence or presence of 200 μM antioxidants at 37 °C. The results are mean ± SEM of duplicates of three separate experiments. [b] Negative control without AAPH. [c] Positive control with AAPH. [d] Statistically significant difference relative to the positive control ($p < 0.05$, one-way ANOVA and Tukey multiple comparison test).

Table 2. Free-Radical Scavenging Capacity (DPPH Bleaching) of Gallic Acid and Gallates[a]

antioxidant (μM)		scavenging (%)
G0	5	23.0
	10	40.4
	20	99.2
G1	5	17.4
	10	57.2
	20	98.1
G3	5	23.3
	10	62.9
	20	99.6
G12	5	21.4
	10	59.0
	20	99.8

[a] The antioxidants were incubated for 30 min with 100 μM DPPH in ethyl alcohol in the dark. The absorbance was measured at 517 nm. The results are means of triplicates.

decreased reactivity with peroxyl radicals in this experimental model.

Considering the above cell-free studies, the stronger antihemolytic effect of the gallates, relative to that of gallic acid, suggests that lipophilicity is a determining factor in this experimental model. To verify this proposal, we also compared the antioxidant vanillic acid versus its methyl ester (methyl vanillate) and trolox, a more water-soluble form of vitamin E, versus vitamin E itself. The results with these two different classes of antioxidants followed the same pattern as that for gallates and gallic acid, reinforcing the suggestion that lipophilicity of antioxidants is crucial with respect to antihemolytic protection (**Table 1**).

The direct hemolytic capacity of gallic acid and gallates was also investigated, because these compounds could interact with and disrupt the erythrocyte membrane. In these experiments, gallic acid and gallates were incubated with erythrocytes without AAPH. The results in **Table 3** show that only the 12-carbon ester lauryl gallate caused significant hemolysis after incubation for 6 h.

The protective effect of gallates was not restricted to AAPH-induced hemolysis. The results given in **Table 4** show that gallates were also effective when the hemolysis was provoked by incubation of erythrocytes with cumene hydroperoxide. Again, there was a correlation with lipophilicity.

In a further experiment, the protective effect of gallic acid and gallates on the depletion of intracellular GSH induced by AAPH was also studied. Despite their antihemolytic properties, gallic acid and gallates were unable to inhibit the oxidation of GSH when erythrocytes were incubated with AAPH. On the contrary, the depletion of GSH was exacerbated by methyl and lauryl gallates (**Table 5**).

To clarify these findings, we studied the direct reaction between pure GSH and AAPH-derived peroxyl radicals and tested the effect of gallic acid and gallates on this reaction. Again, the presence of gallic acid and gallates tended to exacerbate the oxidation of GSH (**Figure 5**).

In another set of experiments, pure GSH was oxidized by H_2O_2 in a reaction catalyzed by HRP. Because GSH is a poor substrate for peroxidase, the efficiency of oxidation was low (*26*). However, the addition of gallic acid and gallates caused a strong increase in the oxidation of GSH (**Figure 6**).

It is well-known that some phenolic compounds are able to act as a co-catalyst in reactions of inefficient peroxidase substrates, such as GSH. In such situations, the oxidized intermediate phenolic radical is reduced back and GSH is converted to GSSG. Moreover, during this redox cycle, the dissolved molecular oxygen in the reaction mixture is consumed, being reduced to superoxide anion (*27*). For these reasons and in light of our results, we measured the production of GSSG and the oxygen consumption as GSH was oxidized by HRP/H_2O_2 in the presence of gallic acid or gallates. To ascertain whether our experimental conditions for assessing the co-catalytic effect of phenols upon the oxidation of GSH was correct, we used apocynin, which is able to initiate this process, as a reference compound (*28*). As can be seen in **Figure 7**, both gallic acid and apocynin exacerbated the oxidation of GSH; however, only in the presence of apocynin was GSSG obtained as a product. Similarly, only in the presence of apocynin was the consumption of dissolved oxygen observed (**Figure 8**). The other gallates showed the same results as gallic acid (data not shown).

DISCUSSION

The hemolysis of erythrocytes has been extensively used as an *ex vivo* model in the study of ROS-induced disruption of cell membranes. The inhibitory property of many antioxidants upon AAPH-induced hemolysis is well-documented (*29*−*32*). However, there is no clear relationship between the molecular structure of the antioxidants and their efficiency as inhibitors. Here, working with gallic acid and its ester derivatives, we found

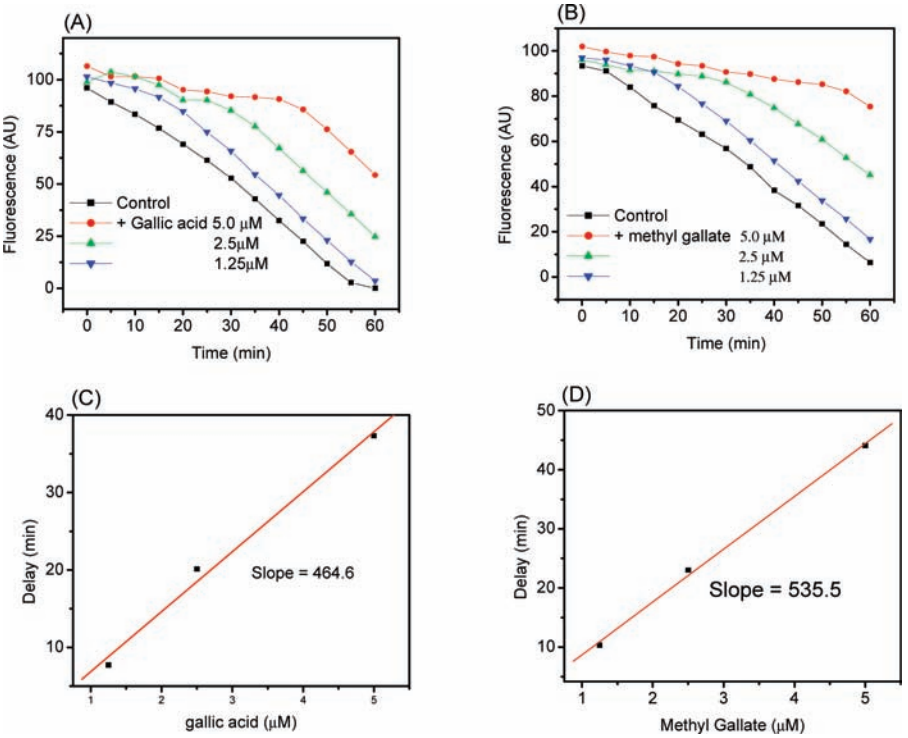


Figure 4. Gallic acid and methyl gallate as inhibitors of AAPH-mediated pyranine oxidation. The reaction system (control) consisted of 5 μM pyranine and 20 mM AAPH in PBS at 37 °C. Kinetics of pyranine bleaching and the lag phase provoked by the addition of (A) gallic acid and (B) methyl gallate. Correlation between the lag phase (delay) and concentration of (C) gallic acid and (D) methyl gallate.

Table 3. Induction of Hemolysis by Gallic Acid and Gallates[a]

experimental condition	percent hemolysis (6 h)
control	3.7 ± 1.2
+G0	4.7 ± 1.0
+G1	3.9 ± 1.8
+G3	4.9 ± 1.6
+G12	29.5 ± 2.1[b]

[a] Erythrocyte suspensions (10%) were incubated in the absence (control) or presence of gallic acid and gallates (200 μM) at 37 °C for 6 h. The results are mean ± SEM of duplicates of three separate experiments. [b] Statistically significant difference relative to the control ($p < 0.05$, one-way ANOVA and Tukey multiple comparison test).

Table 4. Cumene-Hydroperoxide-Induced Hemolysis and the Protective Effect of Gallates[a]

experimental condition			percent hemolysis (2 h)
−Cu−OOH			8.1 ± 4.9[b]
			72.9 ± 1.6[c]
+Cu−OOH	+G0 (μM)	10	67 ± 1.7
		20	17 ± 6.9[d]
	+G1 (μM)	10	11.3 ± 4.1[d]
		20	3.3 ± 1.0[d]
	+G3 (μM)	10	14.3 ± 6.1[d]
		20	4.2 ± 0.3[d]
	+G12 (μM)	10	4.2 ± 0.4[d]
		20	3.9 ± 0.3[d]

[a] Erythrocyte suspensions (10%) were incubated with 500 μM cumene hydroperoxide (Cu−OOH) in PBS at 37 °C for 2 h in the presence or absence of gallates. The results are mean ± SEM of duplicates of three separate experiments. [b] Negative control without Cu−OOH. [c] Positive control with Cu−OOH. [d] Statistically significant difference relative to the control ($p < 0.05$, one-way ANOVA and Tukey multiple comparison test).

that lipophilicity seems to be an essential characteristic of an efficient antihemolytic compound. Similar findings were reported by Wu et al. that ranked the antihemolytic efficiency of gallates as

Table 5. Depletion of Intracellular GSH in Erythrocytes by AAPH and Effect of Gallic Acid and Gallates[a]

experimental condition		GSH (μM/g Hb)
−AAPH		6.7 ± 0.2
		4.2 ± 0.4[b]
+AAPH	+G0	4.5 ± 0.3
	+G1	2.9 ± 0.6[c]
	+G3	4.0 ± 0.5
	+G12	1.6 ± 0.3[c]

[a] Erythrocyte suspensions (10%) were incubated with 25 mM AAPH in the absence or presence of antioxidants (100 μM) at 37 °C for 2.0 h. The results are mean ± SEM of duplicates of three separate experiments. [b] Positive control with AAPH. [c] Statistically significant difference relative to the positive control ($p < 0.05$, one-way ANOVA and Tukey multiple comparison test).

propyl > ethyl > methyl (*33*). Corroborating this proposal, the correlation between efficacy against AAPH-induced hemolysis and lipophilicity was not restricted to gallic acid and its ester derivatives but extended to other classes of molecules, such as vanillic acid versus its lipophilic derivative methyl vanillate and trolox versus vitamin E. Moreover, the benefit of lipophilicity was not restricted to AAPH-induced hemolysis, because it was also seen when the erythrocytes were challenged with cumene hydroperoxide.

Reinforcing the importance of lipophilicity, our result showed clearly that higher antihemolytic efficacy of the gallates was not related to their oxidizability. Indeed, except for lauryl gallate, no significant difference was observed between gallic acid and its esters in their potential as antioxidants in cell-free systems. Only the 12-carbon chain ester was relatively poor as a peroxyl radical scavenger in the cell-free systems. Despite that and again reinforcing the importance of lipophilicity, lauryl gallate was a more efficient inhibitor of hemolysis than gallic acid.

It is important to observe that any compound used as an antihemolytic agent when erythrocytes are challenged by free

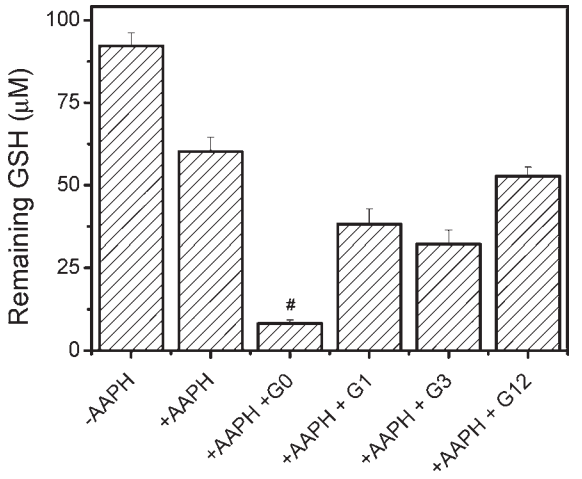


Figure 5. Oxidation of GSH by AAPH and effect of gallic acid and gallates. The reaction system (positive control) consisted of 2.5 mM AAPH and 100 μM GSH in PBS, incubated at 37 °C for 1 h. When present, the gallates were 100 μM. The results are mean ± SEM of duplicates of five separate experiments. (#) Statistically significant difference relative to the positive control ($p < 0.05$, one-way ANOVA and Tukey multiple comparison test).

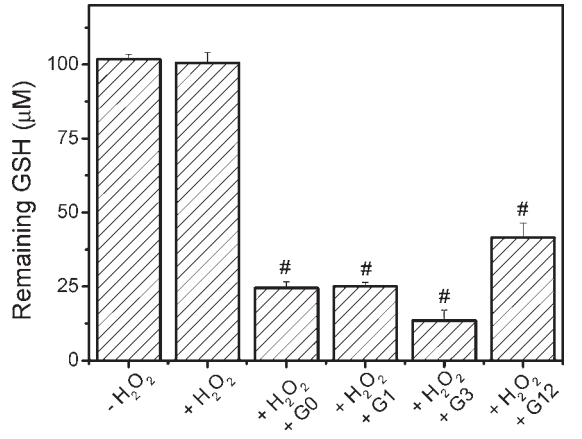


Figure 6. Oxidation of GSH by HRP/H_2O_2 and effect of gallic acid and gallates. The remaining GSH was analyzed by the DTNB method. The reaction mixture (control) consisted of 0.1 μM HRP, 100 μM H_2O_2, and 100 μM GSH in PBS and was incubated for 10 min at 37 °C. When present, the gallates were 100 μM. The results are mean ± SEM of duplicates of three separate experiments. (#) Statistically significant difference relative to the positive control ($p < 0.05$, one-way ANOVA and Tukey multiple comparison test).

radicals could also interact directly with the cell membrane and provoke its disruption. This effect has been described, and there is a relationship between lipophilicity and hemolytic capacity developed by many compounds (*34*, *35*). Accordingly, lauryl gallate, the most lipophilic compound used in this study, was the only ester capable of inducing hemolysis of erythrocytes under our experimental conditions.

Other important findings include the interaction of gallic acid and gallates with GSH. This chemical feature could explain or be involved in the recent findings about cellular pro-oxidant effects of gallates (*13*, *14*). This tripeptide is the main endogenous antioxidant in mammalian cells, and the ratio of reduced to oxidized glutathione (GSH/GSSG) reflects directly the redox status of the cell (*36*). It is well-known that redox homeostasis is crucial for cells to survive, and the pathophysiological mechanisms of several diseases are related to the exacerbated production

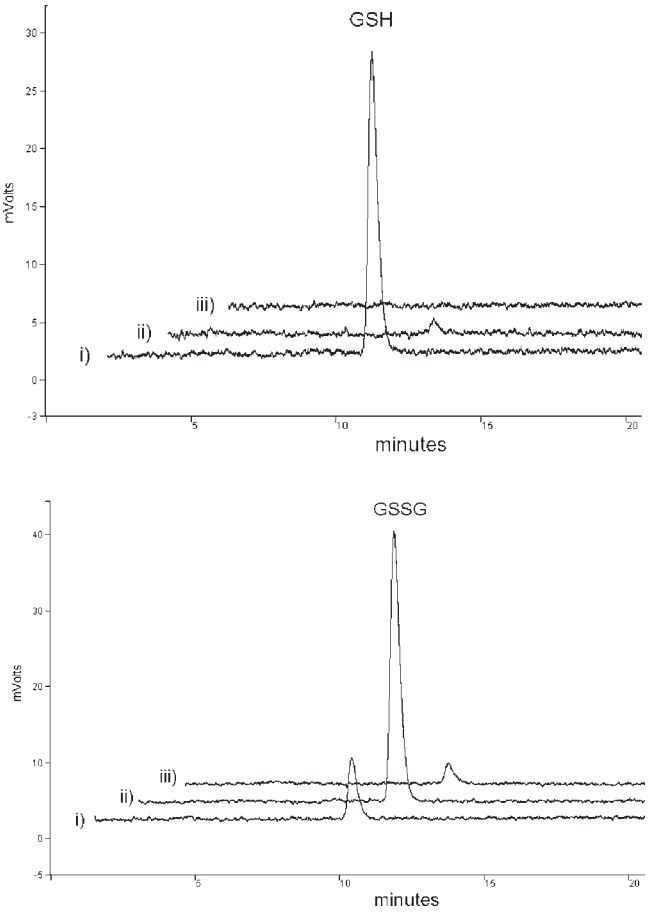


Figure 7. Oxidation of GSH and production of GSSG. The remaining GSH and the produced GSSG were measured by HPLC. The reaction mixture (control) consisted of 0.1 μM HRP, 100 μM H_2O_2, and 100 μM GSH in PBS and was incubated for 10 min at 37 °C. (Top) Analysis of remaining GSH: (i) chromatogram of the reaction mixture (control), (ii) chromatogram of the reaction mixture in the presence of apocynin, and (iii) chromatogram of the reaction mixture in the presence of gallic acid. (Bottom) Analysis of GSSG produced: (i) chromatogram of the reaction mixture (control), (ii) chromatogram of the reaction mixture in the presence of apocynin, and (iii) chromatogram of the reaction mixture in the presence of gallic acid.

of ROS and/or decreased antioxidant functions (*37*). In fact, apoptosis, which is altered in tumor cells, strongly depends upon the GSH/GSSG ratio (*38*, *39*). Two pathways could explanation the exacerbated oxidation of GSH when gallates are present in the reaction mixture or cellular medium. The first is the action of these compounds as co-catalysts, where the intermediate phenoxyl radical would be reduced back to gallate with concomitant oxidation of GSH. This phenomenon is well-established for phenolic compounds, such as tyrosine and apocynin (*27*, *28*), and the consequence of this redox cycle is the consumption of GSH, the formation of GSSG, and the reduction of oxygen to superoxide anion (*40*). This was exactly what we observed for apocynin, used as a model for a comparison to gallates in this study. However, when gallates were used, the consumption of GSH was not followed by the formation of GSSG and the depletion of dissolved oxygen. Hence, this mechanism does not seem to be responsible for the augmented depletion of GSH caused by gallic acid and gallates.

The second possible explanation for the involvement of gallic acid and gallates in the depletion of intracellular GSH is a chemical reaction between the oxidized trihydroxybenzene moiety, probably in its quinone form, and GSH via the Michael

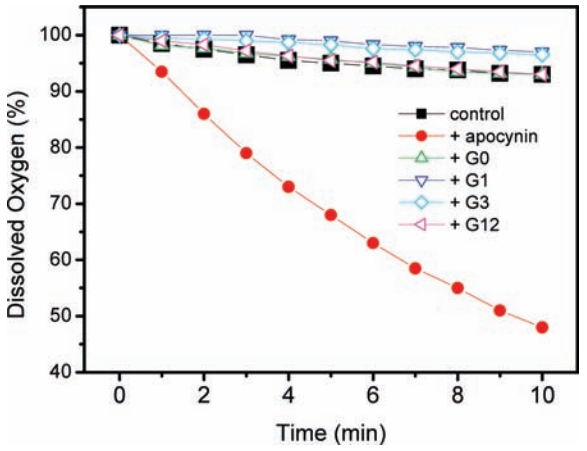
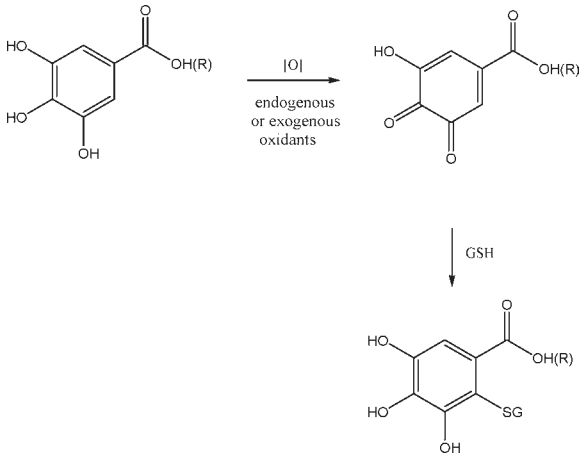


Figure 8. Consumption of oxygen during the oxidation of GSH and co-catalysis by phenolic compounds. The reaction mixture (positive control) consisted of 0.1 μM HRP, 100 μM H_2O_2, and 100 μM GSH in PBS at 37 °C. When present, the gallates and apocynin were 100 μM.

Scheme 1. Proposal for the Chemical Pathway by Which Intracellular GSH Is Depleted When Cells Are Treated with Gallic Acid and Gallates



reaction. This is a well-known reaction that involves electrophilic quinones and nucleophilic substances, such as GSH, leading to thiol adducts (*41*, *42*). In this case, the consumption of GSH is not followed by the formation of GSSG and oxygen consumption, as verified here. In summary, **Scheme 1** shows our proposal for the involvement of gallic acid and gallates when these compounds were oxidized in the presence of GSH, which could explain the depletion of intracellular GSH as verified here and by others (*13*, *14*).

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