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# The antioxidant effect of tannic acid on the in vitro copper-mediated formation of free radicals

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**Abstract**

Tannic acid (TA) has well-described antimutagenic and antioxidant activities. The antioxidant activity of TA has been previously attributed to its capacity to form a complex with iron ions, interfering with the Fenton reaction [Biochim. Biophys. Acta 1472, 1999, 142]. In this work, we observed that TA inhibits, in the micromolar range, in vitro Cu(II) plus ascorbate-mediated hydroxyl radical (·OH) formation (determined as 2-deoxyribose degradation) and oxygen uptake, as well as copper-mediated ascorbate oxidation and ascorbate radical formation (quantified in EPR studies). The effect of TA against 2-deoxyribose degradation was three orders of magnitude higher than classic ·OH scavengers, but was similar to several other metal chelators. Moreover, the inhibitory effectiveness of TA, by the four techniques used herein, was inversely proportional to the Cu(II) concentration in the media. These results and the observation of copper-induced changes in the UV spectra of TA are indications that the antioxidant activity of TA relates to its copper chelating ability. Thus, copper ions complexed to TA are less capable of inducing ascorbate oxidation, inhibiting the sequence of reactions that lead to 2-deoxyribose degradation. On the other hand, the efficiency of TA against 2-deoxyribose degradation declined considerably with increasing concentrations of the ·OH detector molecule, 2-deoxyribose, suggesting that the copper–TA complex also possesses an ·OH trapping activity.


*Keywords:* Tannic acid; Polyphenol; Copper; Chelator; Antioxidant; Hydroxyl radical; Ascorbate radical

Tannic acid (TA, penta-*m*-digalloyl-glucose) is a hydrolyzable polyphenol formed from the secondary metabolism of plants. It is most commonly found in the bark and fruits of many plants and has a structure consisting of a central carbohydrate (glucose) and 10 galloyl groups [1,2]. Several authors have demonstrated that TA and other polyphenols have antimutagenic and anticarcinogenic activities [2–8]. Moreover, the consumption of polyphenol-rich fruits, vegetables, and beverages, such as tea and red wine, has been linked with inhibitory and preventive effects in various human cancers and cardiovascular diseases [2,5,7–13], which may be related—at least in part—with the antioxidant activity of polyphenols [8–11].

Polyphenols (including flavonoids) have the capacity to quench lipid peroxidation, prevent DNA oxidative damage, and scavenge hydroxyl radical (·OH) [14–20]. However, polyphenols can be toxic as well, with prooxidant effects [21–25], which seem to be related with the nature of either the polyphenol molecule or the

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oxidizing agent used, including the presence or absence of transition metals. The ability of several polyphenols to chelate iron or copper ions has been ascribed to their antioxidant [7,15,17,19,26–33] or prooxidant [7,21–25,27,31,32,34–37] activity.

A few years ago, we demonstrated that TA is able to prevent ·OH generation and 2-deoxyribose oxidative damage induced by Fenton reagents by forming a Fe(II) complex [30]. In the present work, we present evidence that TA inhibits ·OH damage to 2-deoxyribose induced by the Cu(II)/ascorbate/dissolved $O_2$ system, as well as Cu(II)-mediated oxygen uptake, ascorbate oxidation, and ascorbate radical formation (in EPR studies). The antioxidant effect of TA was attributed to its capacity to form a complex with copper that inhibits ascorbate oxidation and subsequent ·OH formation, as well as ·OH scavenging of the TA complex with copper.

## Materials and methods

### *Reagents*

Ascorbic acid, catalase, deferoxamine mesylate (DFO), 2-deoxyribose, EDTA, ellagic acid, gallic acid, rutin, tannic acid (TA), and thiobarbituric acid (TBA) were purchased from Sigma (St. Louis, MO). Other reagents were of analytical grade. Stock solutions of gallic acid, rutin, TA (1 mM), and ellagic acid (see Table 1) were freshly prepared in 20 mM phosphate buffer (pH 7.2). Pyridoxal isonicotinoyl hydrazone (PIH, donated by Dr. Prem Ponka, Montreal), copper sulfate, and TBA stock solutions were prepared as described in [38]. All the solutions were made with Milli-Q deionized water.

### *Determination of 2-deoxyribose oxidative degradation*

The formation of ·OH radicals was measured based on a 2-deoxyribose oxidative degradation assay, producing malonaldehyde [30,38,39]. Typical reactions were started by adding 0.02 mL ascorbate (0.5 mM final concentration) to 0.48 mL solutions containing (as final concentrations) 20 mM phosphate buffer (pH 7.2), 5 mM of 2-deoxyribose, Cu(II) (5 or 15 μM), and 0–50 μM TA. The solutions were preincubated for 5 min before adding ascorbate. Reactions were carried out for 8 min at room temperature (25 ± 1 °C) and were terminated by the addition of 0.5 mL of 4% phosphoric acid (v/v), followed by 0.5 mL of 1% TBA solution. After boiling for 15 min, the solutions' absorbance was recorded at 532 nm. "Zero time" absorbance values (where ascorbate is added to solutions after phosphoric acid and TBA [38,39]) were subtracted from the values obtained for each experimental condition.

### *Ascorbate oxidation, $O_2$ uptake, and absorption spectra of copper–TA complexes*

The oxidation of ascorbate was performed at room temperature in 1 mL solution, and was started by the addition of ascorbate; it was monitored at 265 nm for 3 min [38]. The solutions were preincubated for 5 min before the addition of ascorbate. Absorbance was read against blanks: phosphate buffer only (for controls) or buffer plus TA. Oxygen uptake was measured using a Clark-type electrode in a 3 mL glass chamber equipped with magnetic stirring, as previously described [38]. Spectra of the complexes of 10 μM TA with Cu(II) were obtained with a Hitachi U-2001 spectrophotometer. The mixtures were incubated in phosphate buffer (20 mM, pH 7.2) for 2 min and were measured against a blank containing only buffer.

### *EPR spectroscopy*

A Bruker ESP 300 spectrometer equipped with an ER 4102 ST resonator and operating in the X-band (9.416 GHz) was utilized in our investigations. The spectral parameters were as follows: microwave power, 20 mW; modulation frequency, 100 KHz; modulation amplitude, 0.3 G; magnetic field scan, 14 G; sweep time, 84 s; detector time constant, 41 ms; and receiver gain, $1.6 \times 10^5$. The temperature was kept at 25 °C. The solutions were preincubated for 5 min before adding ascorbate. The samples were transferred to the capillary tube and the spectra were recorded exactly 3 min after the reaction started. Since the shape of the EPR spectral line of the ascorbate radical (ascorbyl·) [39] was kept essentially the same in all the experiments, ascorbyl· was quantified by measuring the intensity (as arbitrary units) of the first resonance line.

## Results and discussion

### *Copper-mediated 2-deoxyribose degradation*

Preliminary studies determined the time course of oxidative damage to 5 mM 2-deoxyribose induced by 5 μM Cu(II) and 0.5 mM ascorbate. The addition of 10 μM TA at time zero of incubation, i.e., before the addition of ascorbate, caused a marked inhibition of 2-deoxyribose degradation. The rate of 2-deoxyribose degradation was linear for approximately 10 min in the absence or in the presence of TA (data not shown; $n = 3$). Therefore, we selected an 8 min incubation period for the following experiments.

A titration curve of the TA inhibition of oxidative damage to 2-deoxyribose induced by Cu(II) (5 or 15 μM) plus 0.5 mM ascorbate is shown in Fig. 1. The inhibition by TA against 2-deoxyribose degradation

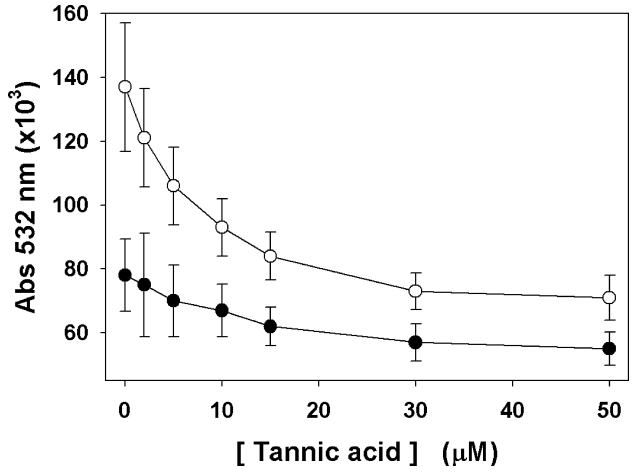


Fig. 1. Dependence of TA concentration on 2-deoxyribose (5 mM) oxidative degradation in 20 mM phosphate buffer (pH 7.2) containing 5 μM Cu(II) (●) or 15 μM Cu(II) (○). The solutions were preincubated for 5 min before adding 0.5 mM ascorbate to initiate the free radical reaction. Reactions were then carried out for 8 min and terminated as described in Materials and methods (this preincubation/incubation protocol was also employed in experiments depicted in Figs. 2–4). The increase of 0.05 in $A_{532}$ represents the formation of ~1 μM malonaldehyde in the 0.5 mL reaction media. Values are means ± SD ($n = 5$).

was concentration dependent, reaching saturation at about 30 μM TA. An $I_{50}$ value for TA of 5.3 ± 0.8 μM was obtained in the presence of 15 μM Cu(II) (the $I_{50}$ value could not be calculated with 5 μM copper). Such a low $I_{50}$ value for TA is incompatible with an antioxidant mechanism involving only ·OH trapping, since molecules with ·OH scavenger activity are effective only in the millimolar range in totally aqueous systems [30,40]. Indeed, TA was about three orders of magnitude more efficient than the classical ·OH scavengers DMSO and ethanol ( $I_{50} = 20$–40 mM) in preventing 2-deoxyribose degradation induced by 10 μM Cu(II) and 3 mM ascorbate [38], suggesting that the antioxidant mechanism of TA is not related mainly to ·OH trapping activity.

The efficiency of TA in inhibiting copper-mediated damage to 2-deoxyribose was also compared with those of other known metal chelators and phenolic compounds. As observed in Table 1, the antioxidant action of TA was similar to those of DFO, EDTA, PIH, and ellagic acid; in contrast, gallic acid (a TA structural component) and rutin presented a very poor antioxidant effect. Other metal chelators (D-penicillamine, and the PIH analogues salicylaldehyde isonicotinoyl hydrazone (SIH) and pyridoxal benzoyl hydrazone (PBH)) also displayed a similar efficiency ($I_{50} = 5$–11 μM; see [38]), when compared with TA, in protecting 2-deoxyribose from oxidative degradation induced by 10 μM Cu(II) and 3 mM ascorbate.

The increase in Cu(II) concentrations (0–30 μM) in the presence of 0.5 mM ascorbate resulted in an increase in 2-deoxyribose degradation (Fig. 2). The very minor level of 2-deoxyribose damage observed in the absence of Cu(II) was probably due to contaminating metals, possibly ferric iron (at submicromolar levels [41]). The oxidative damage to 2-deoxyribose was inhibited by TA (1 and 10 μM) in all the Cu(II) concentrations. The antioxidant efficiency of TA at 1 μM was diminished by about 20% with the augmentation in Cu(II) concentration from 15 μM to 30 μM (Fig. 2 (inset)).

Table 1
Comparison of the antioxidant efficiency of TA and several metal chelators or phenolic compounds

| | |
|---|---|
| Control | 0.110 ± 0.015[a] |
| Compounds at 10 μM | |
| TA | 0.062 ± 0.005 (43.6)[a] |
| EDTA | 0.081 ± 0.007 (26.4) |
| DFO | 0.080 ± 0.008 (27.3) |
| PIH | 0.083 ± 0.008 (24.5) |
| Ellagic acid[b] | 0.066 ± 0.004 (40.0) |
| Gallic acid | 0.104 ± 0.012[c] (5.5) |
| Rutin | 0.109 ± 0.010[c] (0.9) |
| Compounds at 100 μM | |
| Gallic acid | 0.096 ± 0.006[c] (12.7) |
| Rutin | 0.100 ± 0.003[c] (9.1) |

Damage to 2-deoxyribose induced by 0.5 mM ascorbate and 15 μM Cu(II) was tested under standard incubation conditions.

[a] Values represent the $A_{532}$ (± SD; $n = 4$) and the percent protection against 2-deoxyribose damage (in parentheses).

[b] The stock solution of ellagic acid was originally prepared at 100 nmol/mL; due to partial insolubility at pH 7.2, the soluble concentration of the stock solution was 67 ± 2 μM (calculated from an extinction coefficient of 16.2 ± 0.4 mM$^{-1}$ at 360 nm, pH 7.4 [49]).

[c] Values not significantly different (unpaired one-tailed $t$-test) from the control value (as $A_{532}$); the % protection in these cases is irrelevant.

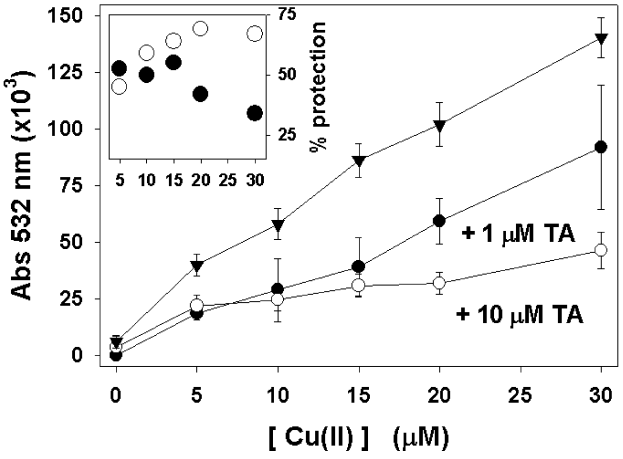


Fig. 2. Dependence of Cu(II) concentration on 2-deoxyribose (5 mM) degradation in media containing 20 mM phosphate buffer (pH 7.2), 0.5 mM ascorbate and in the absence (▼) or presence of TA at 1 μM (●) or 10 μM (○). Inset: replot of data from the main panel, showing the percent inhibition of 2-deoxyribose degradation by TA (1 or 10 μM). Values are means ± SD ($n = 5$).

However, at 10 μM TA, the efficiency of the polyphenol was unaffected by 15–30 μM copper. This indicates that, at 1 μM TA, "free" Cu(II) (as aqueous Cu(II) ($Cu(II)_{aq}$)) is available for oxyradical formation when the copper concentration is high, which was not the case in the presence of 10 μM TA. These results suggest that the mechanism of TA antioxidant activity is due to Cu(II) chelation, forming a copper–TA complex that is less active in the participation of oxyradical formation.

The concentration dependence of 2-deoxyribose on its degradation induced by 15 μM Cu(II) and 0.5 mM ascorbate, in the absence or presence of 10 μM TA, is illustrated in Fig. 3A. Hydroxyl radical scavengers are expected to show a substantial reduction in antioxidant effectiveness with increasing 2-deoxyribose concentrations (from 1 to 50 mM) because the ·OH detector molecule (2-deoxyribose) competes with the scavenger for ·OH trapping. We observed a significant drop in the antioxidant efficiency of TA (from 80 to 42%) with the increase in 2-deoxyribose concentrations (Fig. 3B). A significant decrease in the efficiency of TA at 1 μM also occurred when 2-deoxyribose was raised from 2 mM to 70 mM (the protection declined from 54 to 38%) in media containing 5 μM Cu(II) and 0.5 mM ascorbate (data not shown; $n = 4$). These results indicate that ·OH trapping activity is relevant for the antioxidant mechanism of TA. However, considering the previous results (Figs. 1 and 2), we can postulate that antioxidant activity of TA against 2-deoxyribose oxidation behaves in a hybrid manner: (i) ·OH scavenging activity and (ii) copper-chelating activity, forming a copper–TA complex that inhibits oxyradical attack to 2-deoxyribose. Lopes et al. [30] also reported an ·OH trapping activity of the iron complex with TA.

The effect of ascorbate concentration on copper-mediated 2-deoxyribose degradation was analyzed in the absence or presence of 10 μM TA (Fig. 4). The protection

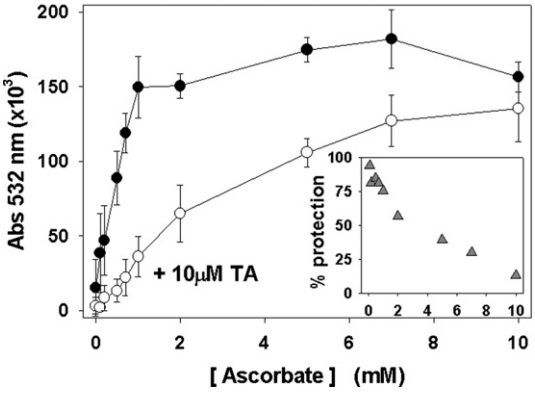


Fig. 4. Dependence of ascorbate concentration (0–10 mM) on 2-deoxyribose (5 mM) degradation in media containing 15 μM Cu(II), and in the absence (●) or presence of 10 μM TA (○). Inset: replot of data from the Main Panel, showing the percent inhibition of the 2-deoxyribose degradation by TA. Values are means ± SD ($n = 5$).

afforded by TA against 2-deoxyribose degradation dropped from 85% at 0.5 mM ascorbate to 30% at 7 mM ascorbate (Fig. 4 (inset); the effect of TA was not significant at 10 mM ascorbate). Such a decrease in antioxidant efficiency with the augmentation in ascorbate concentration was also observed for the effect of PIH on copper-mediated 2-deoxyribose damage [38].

Hadi's group [31,34] observed that TA (0.25–50 μM) plus 100 μM Cu(II)–EDTA, in the absence of ascorbate, induces salicylate hydroxylation (a technique for ·OH quantification) in phosphate-buffered solutions (pH 8.0) after 2 h of incubation. However, we observed that 5 μM Cu(II) and TA (zero or 10 μM), in the absence of ascorbate, caused no relevant degradation of 5 mM 2-deoxyribose in 5 min to 2 h incubations ($A_{532}$ values ranged only 0.02–0.03). The addition of EDTA (50 or 100 μM) to these reactions (with 5 or 50 μM copper; without TA or ascorbate) failed to affect 2-deoxyribose degradation (data not shown; $n = 3$).

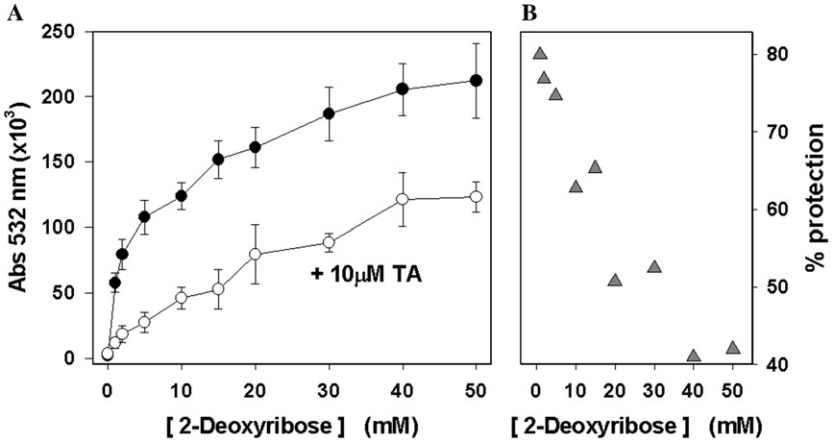


Fig. 3. (A) Dependence of 2-deoxyribose concentration (0–50 mM) on its oxidative degradation in phosphate buffer (pH 7.2) induced by 15 μM Cu(II) and 0.5 mM ascorbate, in the absence (●) or presence of 10 μM TA (○). (B) Replot of data from (A), showing the percent inhibition of 2-deoxyribose degradation by TA. Values are means ± SD ($n = 3$).

*Cu(II)-mediated ascorbate oxidation and ascorbyl• formation*

Oxidation of ascorbate to ascorbyl• mediated by Cu(II) is the first step in a series of reactions leading to •OH formation and 2-deoxyribose degradation (Eqs. (1)–(6)) [32,38,42,43]. Previous reports state that tannins and other polyphenols, including TA, are able to inhibit Cu(II)-mediated ascorbate oxidation [15,44].

$$\mathrm{Cu(II)} + \text{ascorbate} \rightarrow \mathrm{Cu(II)}\text{-ascorbate} \rightarrow \mathrm{Cu(I)} + \text{ascorbyl}^{\bullet} \quad (1)$$

$$\mathrm{Cu(I)} + \mathrm{O_2} \rightarrow \mathrm{Cu(II)} + \mathrm{O_2^{\bullet -}} \quad (2)$$

$$\mathrm{Cu(I)} + \mathrm{O_2^{\bullet -}} + 2\mathrm{H^+} \rightarrow \mathrm{Cu(II)} + \mathrm{H_2O_2} \quad (3)$$

$$2\mathrm{O_2^{\bullet -}} + 2\mathrm{H^+} \rightarrow \mathrm{O_2} + \mathrm{H_2O_2} \quad (4)$$

$$\mathrm{H_2O_2} + \mathrm{Cu(I)} \rightarrow \mathrm{Cu(II)} + \mathrm{OH^-} + {}^{\bullet}\mathrm{OH} \quad (5)$$

$${}^{\bullet}\mathrm{OH} + \text{2-deoxyribose} \rightarrow \text{measurable products} \quad (6)$$

Fig. 5 depicts a titration curve of TA on the oxidation of 100 μM ascorbate induced by 15 μM Cu(II) in phosphate-buffered solutions. The $I_{50}$ value for TA was about 5 μM (considering 25 μM TA as saturating in the inhibition curve), which is essentially the same $I_{50}$ value observed in the 2-deoxyribose degradation reaction. A titration curve of ascorbate oxidation employing 5 μM Cu(II) yielded an $I_{50}$ value of ~1 μM AT (data not shown; $n = 4$). The oxidation of ascorbate (100 μM) was also determined with different concentrations of Cu(II) (from 5 to 50 μM), in the absence or presence of 10 μM TA. TA inhibited ascorbate oxidation under all the experimental conditions; however, its efficiency declined significantly from 90% (at 5 μM copper) to 76% at 50 μM copper (data not shown; $n = 4$).

The formation of ascorbyl• from copper (5 or 15 μM) and 0.5 mM ascorbate was also determined in EPR experiments (see Eq. (1)). The EPR signal for ascorbyl• was significantly weaker in the presence of 1 μM TA when compared with the controls without TA (Fig. 6; note that TA has no effect on the EPR signal parameters, only on its intensity). Fig. 7 depicts titration curves

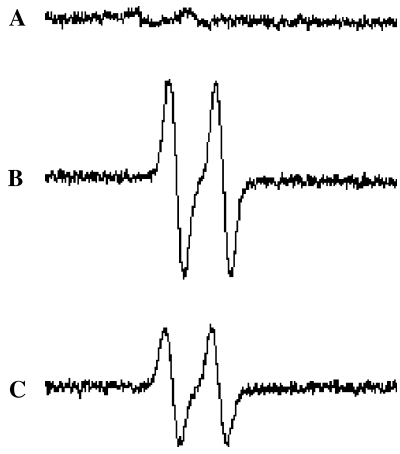


Fig. 6. Effects of copper ions and TA on EPR ascorbyl• signal. (A) Shows the EPR spectrum of 20 mM phosphate buffer (pH 7.2) containing 0.5 mM ascorbate, in the absence of copper and TA. EPR signals were recorded 3 min after the addition of ascorbate; this spectrum shows only background signals. (B) Spectrum of a solution containing buffer and 5 μM Cu(II); 0.5 mM ascorbate was added after 5 min of preincubation and the EPR signals were recorded after 3 min. (C) Same as in (B), but with the addition of 1 μM TA before the preincubation period (there is a 43% inhibition in EPR signal intensity). (A–C) Representative spectra of experiments performed three times.

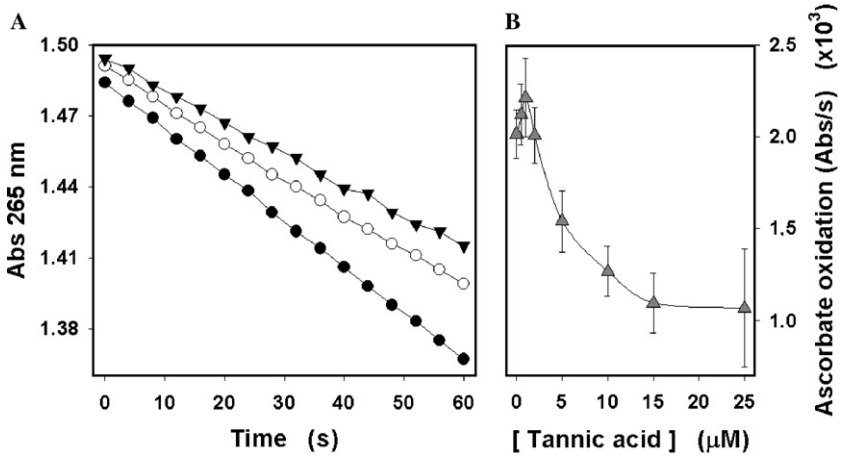


Fig. 5. (A) Kinetics of 100 μM ascorbate oxidation induced by 15 μM Cu(II), in 20 mM phosphate buffer (pH 7.2) in the absence (●) or the presence of 5 μM (○) and 10 μM TA (▼); the solutions were preincubated for 5 min before adding ascorbate. (A) A representative result of experiments performed four times. Based on the extinction coefficient of ascorbate at 265 nm (at neutral pH), the loss of 0.1 absorbance corresponds to the oxidation of 6.9 μM ascorbate [39]. (B) Effect of TA concentration on the rate of ascorbate oxidation (calculated as loss of $A_{265}$/s). Values are mean ± SD ($n = 4$).

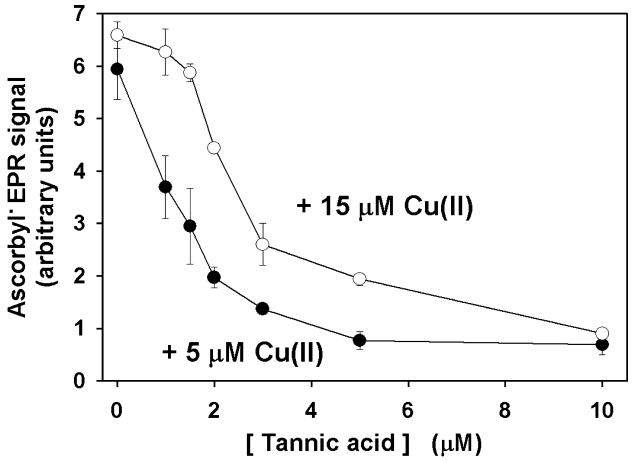


Fig. 7. Dependence of TA concentration on the intensity of the EPR ascorbyl• signal in 20 mM phosphate buffer (pH 7.2) and TA (0–10 μM), in the presence of 5 μM (●) or 15 μM Cu(II) (○). Reaction media were preincubated at room temperature for 5 min before adding 0.5 mM of ascorbate. Values are means ± SD ($n = 3$). The baseline EPR signal in all the determinations was 0.5 arbitrary units.

of TA on the intensity of ascorbyl• EPR signal generated from Cu(II) (5 or 15 μM) and 0.5 mM ascorbate. The $I_{50}$ values for TA were 1.4 ± 0.1 and 2.6 ± 0.2 μM in reactions containing 5 or 15 μM Cu(II), respectively. The difference in the $I_{50}$ values demonstrated that the inhibitory efficiency of TA was inversely proportional to the copper concentration.

In addition, the effect of 1 μM TA on the ascorbyl• EPR signal was examined with different concentrations of ascorbate (0.1–1 mM), in the presence of a fixed amount of copper. Similar to the findings depicted in Fig. 4, the inhibitory efficiency of TA decreased as the ascorbate concentration increased (Table 2).

### *Copper-mediated $O_2$ uptake*

Figs. 8A and B show that TA inhibits $O_2$ uptake induced by Cu(II) (5 or 15 μM) and 0.5 mM ascorbate in a concentration dependent way. The calculated $I_{50}$ values for TA on the rates of $O_2$ uptake (see Materials and methods; rates of $O_2$ uptake in the absence of TA were approximately 0.7 and 1.2 μM per second for reactions containing 5 or 15 μM copper, respectively) were 2.8 and 4.6 μM in the reactions containing 5 or 15 μM copper, respectively. The values of $I_{50}$ were essentially the same as those in the experiments of 2-deoxyribose degradation (with 15 μM copper), ascorbate oxidation,

Table 2
Effect of ascorbate concentration on the intensity of EPR ascorbyl• signal

| Ascorbate (mM) | Intensity of EPR signal | | Percent inhibition by TA (significance in parentheses) |
|---|---|---|---|
| | Without TA | TA (1 μM) | |
| 0.10 | 3.82 ± 0.65 | 2.17 ± 0.52 | 43% ($P < 0.025$) |
| 0.30 | 4.90 ± 0.31 | 3.10 ± 0.23 | 37% ($P < 0.001$) |
| 0.50 | 6.04 ± 1.15 | 3.99 ± 0.41 | 34% ($P < 0.025$) |
| 0.75 | 6.29 ± 0.28 | 5.40 ± 1.05 | 14% (not significant) |
| 1.0 | 6.87 ± 0.17 | 5.50 ± 0.35 | 20% ($P < 0.005$) |

The solutions contained 20 mM phosphate buffer (pH 7.2), 5 μM Cu(II), with or without TA, and varying concentrations of ascorbate. Reaction media were preincubated for 5 min before the addition of ascorbate. EPR signals (as arbitrary units) were determined 3 min after the addition of ascorbate. The significance of TA inhibitory effect was checked using an unpaired one-tailed $t$ test. Values are means ± SD ($n = 3$).

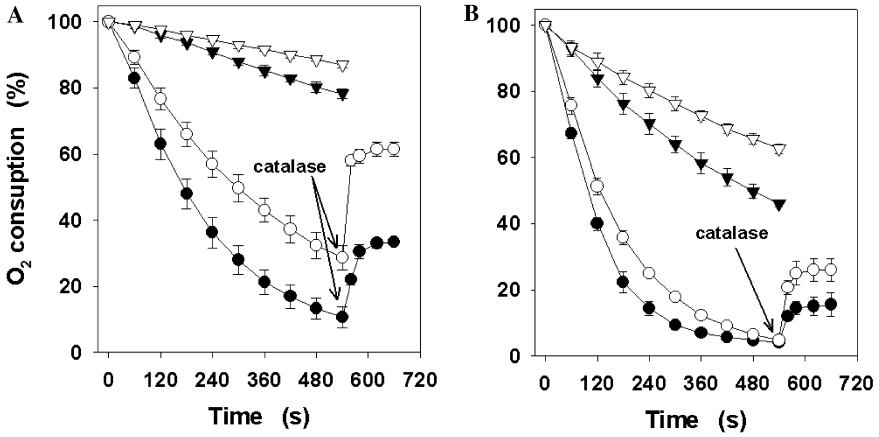


Fig. 8. (A) Time course of $O_2$ uptake in 20 mM phosphate buffer (pH 7.2) containing 5 μM Cu(II), TA (0–10 μM) and 0.5 mM ascorbate. The TA concentration was represented as: zero (●, control), 1 (○), 5 (▼), and 10 μM (▽). Catalase (1000 U/mL) was added after 500 s to controls and to solutions containing 1 μM TA. (B) The same conditions as in (A), except that Cu(II) was 15 μM. Data in (A) and (B) are means ± SD ($n = 4$).

and ascorbyl• formation. This strongly suggests that inhibition of copper-mediated $O_2$ uptake by TA may be a consequence of the inhibition of ascorbate oxidation (see reaction (1)).

Release of $O_2$ is observed after the addition of catalase (after 9 min of reaction) to media containing 0.5 mM ascorbate, Cu(II) (5 or 15 μM), and dissolved $O_2$ (Figs. 8A and B). As expected, $H_2O_2$ is formed as an intermediary of these reactions (see reactions (3) and (4), above). Based on the yield of $O_2$ recovery, we estimated that 100 μM $H_2O_2$ was present in reaction media containing 5 μM copper after 9 min incubation; in the presence of 1 μM TA, the concentration of $H_2O_2$ was 45% higher than in its absence. Moreover, in media containing 15 μM Cu(II), the concentration of $H_2O_2$ in the presence of TA was about 85% higher than in the absence of TA (51 μM versus 94 μM $H_2O_2$). These results show that more $H_2O_2$ is present in solutions containing TA ($H_2O_2$ is formed as in Eq. (3)) than in the absence of polyphenol.

Furthermore, the presence or absence of TA (at 10 μM) in 20 mM phosphate-buffered solutions containing 5 or 50 μM Cu(II) (no ascorbate present) produced no detectable $O_2$ uptake within 1 h of continuous observation. The addition of 50 μM EDTA to the reacting media (with 50 μM copper) also induced no $O_2$ uptake, in the absence or presence of 10 μM TA (data not shown; $n = 3$). These data imply that the copper–TA complex cannot undergo autoxidation and generation of $O_2^-$ and $H_2O_2$, as proposed by Bhat and Hadi [22,34].

*Copper–TA complex characterization*

The wavelength spectra of the complex formed between TA (10 μM) and copper (5–50 μM) revealed absorbance peaks at 235 and 325 nm (Fig. 9). The increase in Cu(II) concentration caused a linear increase in the absorbances at 235 and 325 nm up to 60 μM Cu(II), and saturation was attained at 80–90 μM Cu(II) (data not shown; $n = 4$). These results suggest that 1 mol of TA is able to chelate 6–8 mol of copper ions, which is consistent with a 6:1 stoichiometry for the copper–TA complex reported elsewhere [22]. We also determined that the formation of the complex between 10 μM TA and 5 μM Cu(II) (followed at 235 and 325 nm) was completed within 10 s in 20 mM phosphate buffer (data not shown; $n = 3$).

Fig. 9 also shows the formation of three isosbestic points at 223, 262, and 304 nm. The isosbestic point at 304 nm shifted to 292 nm in the presence of 40 and 50 μM Cu(II). This could also be explained by the reduction of Cu(II)–Cu(I) by TA, with the concomitant oxidation of the TA molecule Eq. (7). A proposed oxidation such as this (also suggested in [22]) may cause structural changes in the TA molecule.

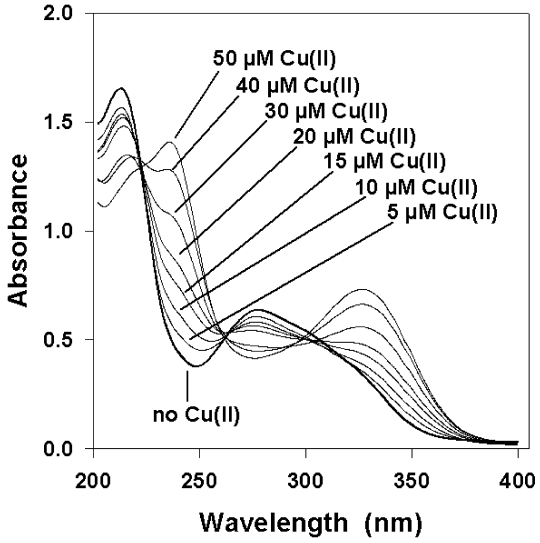


Fig. 9. Spectra of the complexes formed between 10 μM TA and several concentrations of Cu(II) (zero to 50 μM) in 20 mM phosphate buffer (pH 7.2). The spectrum shown is representative of three independent determinations.

$$n\mathrm{Cu(II)} + \mathrm{TA} \rightarrow \mathrm{Cu(II)_n{-}TA} \rightarrow \mathrm{Cu(I)_n{-}TA_{(oxidized)}} \quad (7)$$

## Conclusions

We have demonstrated that TA inhibits •OH formation and, thus, 2-deoxyribose degradation induced by the ascorbate/Cu(II)/$O_2$ system. Since TA inhibits Cu(II)-mediated ascorbate oxidation, it is possible that the "pathway" of •OH formation (Eqs. (1)–(6)) is halted by TA in its first step (Eq. (1)). The complexation of Cu(II) by TA (see Fig. 9 and [45]; the complex may be converted to $Cu(I)_n$–$TA_{(oxidized)}$ [22]) may inhibit the reaction of Cu(II) with ascorbate. Thus, we can postulate that TA causes a decrease in the reduction potential of the $Cu(II)_{aq}/Cu(I)_{aq}$ pair. A similar process was postulated for the effect of PIH on the reaction of ascorbate with Cu(II) [38] or Fe(III)–EDTA [39].

Moreover, the effectiveness of TA in inhibiting ascorbyl• formation (see below) proved to be inversely dependent on the Cu(II) concentration (Fig. 7); the same was observed for copper-mediated ascorbate oxidation (see Results and discussion). This suggests that TA and ascorbate may compete in copper chelation and reduction of Cu(II)–Cu(I) (ascorbate forms a weak complex with Cu(II) before the production of Cu(I) and ascorbyl• [46]). This hypothesis of a competition between TA and ascorbate appears to be true due to the loss of antioxidant efficiency of TA with increasing ascorbate concentration on 2-deoxyribose damage and in EPR experiments (see Fig. 4 and Table 2). Moreover,

millimolar amounts of ascorbate exert a relevant free radical quenching activity, which also competes with the antioxidant effect of a fixed concentration of TA. Analogous to our observations, it has been shown that resveratrol protects copper-mediated oxidation of glutathione and inhibits ·OH formation and DNA damage induced by glutathione/Cu(II)/$H_2O_2$ [32].

We also observed that TA can inhibit $O_2$ uptake and that the effectiveness of TA is inversely dependent on the Cu(II) concentration. Since TA also inhibits the copper-dependent oxidation of ascorbate, TA should diminish the concentration of $Cu(I)_{aq}$ (formed from reaction (1) that is able to reduce oxygen to $H_2O_2$ (Eqs. (2) and (3)). This would also leave less $Cu(I)_{aq}$ to destroy $H_2O_2$ via the copper-Fenton reaction (Eq. (4)). The observation that more $H_2O_2$ is present in solutions with TA (than without TA; see the effect of catalase in Fig. 8) is congruent with these ideas. Moreover, it is also possible that the copper–TA complex has a superoxide dismutase-like activity (as observed in several copper-complexes [47,48]), which might increase the steady state concentration of $H_2O_2$.

The possibility that TA may act only as a "classical antioxidant" (·OH scavenger) against 2-deoxyribose degradation was discarded based on several factors: (i) the TA titration experiments using two concentrations of Cu(II) in EPR studies (Fig. 7) and $O_2$ uptake assays (Fig. 8); (ii) the effect of Cu(II) concentration versus the effectiveness of TA (at 1 μM) on 2-deoxyribose degradation (Fig. 2) and ascorbate oxidation (see Results and discussion); (iii) the quantitative difference (three orders of magnitude) in the antioxidant activity of TA against 2-deoxyribose degradation and classical ·OH scavengers; and (iv) the similarity in effectiveness of TA and metal chelators (including ellagic acid, a phenolic metal chelator [49], and DFO) against 2-deoxyribose degradation. However, the significant component of ·OH scavenging activity of TA observed in the experiment depicted in Fig. 3 suggests a hybrid behavior, in which ·OH generated in the vicinity of the copper complex with TA (·OH would be formed from the reaction of $H_2O_2$ with $Cu(I)_n$–TA) possibly reacts with the polyphenol structure. Under these conditions, hypothetically, TA may form a radical species (reaction 8). Indeed, Bors et al. [50] observed EPR signals of TA radical formed from horseradish peroxidase, $H_2O_2$, and TA (1 mM).

$$\text{copper}_n\text{–TA} + {}^{\bullet}\text{OH} \rightarrow \text{copper}_n\text{–TA}_{(\text{radical})} + H_2O \qquad (8)$$

As proposed by Satoh and Sakagami [51], TA may also react directly with ascorbyl·, which could be an alternative explanation for the inhibitory action of TA in the ascorbyl· EPR signal. However, a relevant scavenging effect of TA was only observed with far higher concentrations than ours, i.e., 0.1–10 mg/mL [51,52], approximately 60 μM to 6 mM. Moreover, this scavenging effect of TA (at 10 mg/mL) was ineffective or only slightly efficient at ascorbate levels above 0.1 mM [52]; this contrasts with the fact that 0.5 mM ascorbate was employed in most of our experiments. Thus, if any TA-induced ascorbyl· scavenging activity is present in our experimental conditions, this may be of minor relevance.

One could also suppose that the TA-dependent decrease in the EPR ascorbyl· signal would occur, at least in part, because $Cu(II)_n$–TA oxidizes ascorbyl· to dehydroascorbate easier (and/or faster) than $Cu(II)_{aq}$. However, our suggestion that TA decreases the reduction potential of copper seems to rule out the above possibility. Indeed, when following the kinetics of the ascorbyl· EPR signal disappearance (linear from 3 to 8 min, with 5 μM copper), the rate of the signal disappearance was 2-fold slower in the presence of TA (1 μM) than in its absence [53]; the reason for this is still unclear. Further studies employing stopped-flow EPR are needed to gain a better understanding of the effect of TA on copper-mediated ascorbyl· formation and disappearance.

Our results on the antioxidant activity of TA do, however, conflict with those from Hadi's group and coworkers [22,31,34,54], who observed that TA in the presence of Cu(II)–EDTA and in the absence of ascorbate is able to generate $O_2^{\bullet-}$, $H_2O_2$, and ·OH [31,34], with resulting DNA strand breaks and bacteriophage inactivation [22,34]. The possibility that the copper complex with TA, in the absence of ascorbate, could induce formation of $O_2^{\bullet-}$ and ·OH radicals under our experimental conditions was discarded due to the lack of copper–TA-dependent $O_2$ uptake and 2-deoxyribose degradation (see Results and discussion). At this time, we are still unable to explain the reason for the discrepancies between our observations and those of Hadi's group. However, TA plus copper may intercalate with the DNA structure, causing strand breaks [22,54], as in the case of other polyphenols in the presence of copper ions [21,24,37]. Indeed, preliminary results from our laboratory showed that TA (10–50 μM) significantly augments strand breaks in plasmid pUC-18 induced by 15 μM Cu(II) plus 1 mM ascorbate [53]. The chemical mechanism behind this dual activity of the copper–TA complex (antioxidant and DNA damaging effects) is still to be unearthed. On the other hand, TA has been found to protect DNA plasmids from strand breaks caused by Fe(II)–EDTA, ascorbate, and $H_2O_2$ [31] and Fenton reagents (Fe(II) plus $H_2O_2$) [53].

Finally, we conclude that copper ions complexed to TA are less redox-active for reactions with ascorbate and, possibly, other reducing agents. The TA-mediated inhibition of reaction (1) would be the key factor for the overall diminution in copper-mediated ·OH formation. We also hypothesize that ·OH formed from any reaction between $H_2O_2$ and $Cu(I)_n$–TA is also trapped by the polyphenol structure. The current in vitro observations contribute toward understanding the intricate

antimutagenic mechanisms of TA and related polyphenols, and their in vivo antioxidant effects. Moreover, the observation that TA has an in vitro antioxidant effect comparable to those of other metal chelating agents (Table 1), and modest toxicity (toxic at >1–2 g/kg in mice [55]), could make it relevant for the in vivo control of copper-mediated oxidative stress.

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## References

[1] E. Haslam, Chemistry and Pharmacology of Natural Products. Plant Polyphenols. Vegetable Tannins Revisited, Cambridge University Press, Cambridge, UK, 1989.
[2] A. King, G. Young, J. Am. Diet Assoc. 99 (1999) 213–218.
[3] M. Athar, W.A. Khan, H. Mukhtar, Cancer Res. 49 (1989) 5784–5788.
[4] A. Kaul, K.L. Khanduja, Nutr. Cancer 32 (1998) 81–85.
[5] Y. Kuroda, Y. Hara, Mutat. Res. 436 (1999) 69–97.
[6] H.U. Gali-Muhtasib, S.Z. Yamout, M.M. Sidani, Nutr. Cancer 37 (2000) 73–77.
[7] L.R. Ferguson, Mutat. Res. 475 (2001) 89–111.
[8] B. Frei, J.V. Higdon, J. Nutr. 133 (2003) 3275S–3284S.
[9] L.O. Dragsted, M. Strube, J.C. Larsen, Pharmacol. Toxicol. 72 (1993) 116–135.
[10] K.T. Chung, T.Y. Wong, C.I. Wei, Y.W. Huang, Y. Lin, Crit. Rev. Food Sci. Nutr. 38 (1998) 421–464.
[11] J.H. Weisburger, Proc. Soc. Exp. Biol. Med. 220 (1999) 271–275.
[12] C.S. Yang, J.M. Landau, M.T. Huang, H.L. Newmark, Annu. Rev. Nutr. 21 (2001) 381–406.
[13] J.D. Lambert, C.S. Yang, J. Nutr. 133 (2003) 3262S–3267S.
[14] H. Chimi, J. Cillard, P. Cillard, M. Rahmani, J. Am. Oil Chem. Soc. 68 (1991) 307–312.
[15] L. Ramanathan, N.P. Das, Int. J. Food Sci. Technol. 28 (1993) 279–288.
[16] J.A. Laranjinha, L.M. Almeida, V.M. Madeira, Biochem. Pharmacol. 48 (1994) 487–494.
[17] Q. Guo, B. Zhao, M. Li, S. Shen, W. Xin, Biochim. Biophys. Acta 1304 (1996) 210–222.
[18] P. Leanderson, A.O. Faresjo, C. Tagesson, Free Radic. Biol. Med. 23 (1997) 235–242.
[19] R. Pulido, L. Bravo, F. Saura-Calixto, J. Agric. Food Chem. 48 (2000) 3396–3402.
[20] M. Lodovici, F. Guglielmi, C. Casalini, M. Meoni, V. Cheynier, P. Dolara, Eur. J. Nutr. 40 (2001) 74–77.
[21] R. Zaidi, S.M. Hadi, J. Biochem. Toxicol. 7 (1992) 213–217.
[22] R. Bhat, S.M. Hadi, Mutat. Res. 313 (1994) 39–48.
[23] S.C. Sahu, G.C. Gray, Cancer Lett. 85 (1994) 159–164.
[24] S.C. Sahu, G.C. Gray, Food Chem. Toxicol. 35 (1997) 443–447.
[25] A. Malik, S. Azam, N. Hadi, S.M. Hadi, Phytother. Res. 17 (2003) 358–363.
[26] I. Morel, G. Lescoat, P. Cogrel, O. Sergent, N. Pasdeloup, P. Brissot, P. Cillard, J. Cillard, Biochem. Pharmacol. 45 (1993) 13–19.
[27] J.F. Moran, R.V. Klucas, R.J. Grayer, J. Abian, M. Becana, Free Radic. Biol. Med. 22 (1997) 861–870.
[28] J.E. Brown, H. Khodr, R.C. Hider, C.A. Rice-Evans, Biochem. J. 330 (1998) 1173–1178.
[29] M. Yoshino, K. Murakami, Anal. Biochem. 257 (1998) 40–44.
[30] G.K.B. Lopes, H.S. Schulman, M. Hermes-Lima, Biochim. Biophys. Acta 1472 (1999) 142–152.
[31] N.S. Khan, A. Ahmad, S.M. Hadi, Chem. Biol. Interact. 125 (2000) 177–189.
[32] M.J. Burkitt, J. Duncan, Arch. Biochem. Biophys. 381 (2000) 253–263.
[33] I.B. Afanas'eva, E.A. Ostrakhovitch, E.V. Mikhal'chik, G.A. Ibragimova, L.G. Korkina, Biochem. Pharmacol. (2001) 677–684.
[34] R. Bhat, S.M. Hadi, Mutat. Res. 313 (1994) 49–55.
[35] K. Fukuhara, N. Miyata, Bioorg. Med. Chem. Lett. 8 (1998) 3187–3192.
[36] N. Hoshino, T. Kimura, T. Ando, Free Radic. Biol. Med. 27 (1999) 1245–1250.
[37] M. Yoshino, M. Haneda, M. Naruse, H.H. Htay, S. Iwata, R. Tsubouchi, K. Murakami, Toxicol. In Vitro 16 (2002) 705–709.
[38] M. Hermes-Lima, M.S. Gonçalves, R.G. Andrade Jr., Mol. Cell. Biochem. 228 (2001) 73–82.
[39] A.Q. Mauricio, G.K.B. Lopes, C.S. Gomes, R.G. Oliveira, A. Alonso, M. Hermes-Lima, Biochim. Biophys. Acta 1620 (2003) 15–24.
[40] S.M. Klein, G. Cohen, A.I. Cederbaum, Biochemistry 20 (1981) 6006–6012.
[41] M. Hermes-Lima, E.M. Wang, H.M. Schulman, K.B. Storey, P. Ponka, Mol. Cell. Biochem. 137 (1994) 65–73.
[42] M.J. Zhao, L. Jung, Free Radic. Res. 23 (1995) 229–243.
[43] J.E. Biaglow, Y. Manevich, F. Uckun, K.D. Held, Free Radic. Biol. Med. 22 (1997) 1129–1138.
[44] T. Yoshida, S. Koyama, T. Okuda, Yakugaku Zasshi 101 (1981) 695–699.
[45] B.H. Cruz, J.M. Díaz-Cruz, C. Arino, R. Tauler, M. Esteban, Anal. Chim. Acta 424 (2000) 203–209.
[46] K. Hayakawa, Y. Hayashi, J. Nutr. Sci. Vitaminol. (Tokyo) 23 (1997) 395–401.
[47] L.L. Costanzo, G. De Guidi, S. Giuffrida, E. Rizzarelli, G. Vecchio, J. Inorg. Biochem. 50 (1993) 273–281.
[48] S. Guner, S. Karabocek, J. Biochem. Mol. Toxicol. 12 (1998) 53–59.
[49] P.E. Bock, K.R. Srinivasan, J.D. Shore, Biochemistry 20 (1981) 7258–7266.
[50] W. Bors, C. Michel, K. Stettmaier, Arch. Biochem. Biophys. 374 (2000) 347–355.
[51] K. Satoh, H. Sakagami, Anticancer Res 16 (1996) 2885–2890.
[52] K. Satoh, Y. Ida, M. Ishihara, H. Sakagami, Anticancer Res 19 (1999) 4177–4186.
[53] R.G. Andrade Jr., Mecanismos de ação antioxidante e pró-oxidante do polifenol ácido tânico na presença de íons cobre, MSc Thesis, Universidade de Brasília, Brazil, 2004.
[54] N.S. Khan, S.M. Hadi, Mutagenesis 13 (1998) 271–274.
[55] J. Zhu, L.J. Filippich, M.T. Alsalam, Res. Vet. Sci. 53 (1992) 280–292.