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Polyphenol tannic acid inhibits hydroxyl radical formation from Fenton reaction by complexing ferrous ions[1]

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Abstract

Tannic acid (TA), a plant polyphenol, has been described as having antimutagenic, anticarcinogenic and antioxidant activities. Since it is a potent chelator of iron ions, we decided to examine if the antioxidant activity of TA is related to its ability to chelate iron ions. The degradation of 2-deoxyribose induced by 6 μM Fe(II) plus 100 μM H_2O_2 was inhibited by TA, with an I_{50} value of 13 μM. Tannic acid was over three orders of magnitude more efficient in protecting against 2-deoxyribose degradation than classical $^{\bullet}OH$ scavengers. The antioxidant potency of TA was inversely proportional to Fe(II) concentration, demonstrating a competition between H_2O_2 and AT for reaction with Fe(II). On the other hand, the efficiency of TA was nearly unchanged with increasing concentrations of the $^{\bullet}OH$ detector molecule, 2-deoxyribose. These results indicate that the antioxidant activity of TA is mainly due to iron chelation rather than $^{\bullet}OH$ scavenging. TA also inhibited 2-deoxyribose degradation mediated by Fe(III)-EDTA (iron = 50 μM) plus ascorbate. The protective action of TA was significantly higher with 50 μM EDTA than with 500 μM EDTA, suggesting that TA removes Fe(III) from EDTA and forms a complex with iron that cannot induce $^{\bullet}OH$ formation. We also provided evidence that TA forms a stable complex with Fe(II), since excess ferrozine (14 mM) recovered 95–96% of the Fe(II) from 10 μM TA even after a 30-min exposure to 100–500 μM H_2O_2. Addition of Fe(III) to samples containing TA caused the formation of $Fe(II)_n$–TA, complexes, as determined by ferrozine assays, indicating that TA is also capable of reducing Fe(III) ions. We propose that when Fe(II) is complexed to TA, it is unable to participate in Fenton reactions and mediate $^{\bullet}OH$ formation. The antimutagenic and anticarcinogenic activity of TA, described elsewhere, may be explained (at least in part) by its capacity to prevent Fenton reactions.

Keywords: Iron; Tannin; Tannic acid; Polyphenol; Antioxidant; Hydroxyl radical; Oxidative stress

1. Introduction

Polyphenols are plant secondary metabolites consisting of hydrolyzable and condensed forms. Tannic acid (TA), which is part of the first group, has a structure consisting of a central carbohydrate (glucose) and 10 galloyl groups. It occurs in the bark and fruits of many plants [1]. Tannic acid and other polyphenols have antimutagenic, anticarcinogenic and antioxidant activities, but the mechanisms involved in these activities are not completely understood [2–6]. Polyphenols are $^{\bullet}OH$ radical scavengers because phenolic groups are excellent nucleophiles [6–8] and

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[1] This study is dedicated to the memory of Botany Professor Luiz F.G. Labouriau (1921–1996).

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are also able to quench lipid peroxidation, acting as chain break antioxidants [6–12].

Tannic acid chelates iron due to its ten galloyl groups and it diminishes intestinal non-heme iron absorption [13,14]. It might also be able to inhibit iron-mediated oxyradical formation like other iron chelators, such as desferrioxamine (DFO), 1,10-phenanthroline and pyridoxal isonicotinoyl hydrazone (PIH) [15–17]. Iron chelators, such as DFO, prevent various processes of oxidative stress in vivo, including damage from heart reperfusion [18–20] and liver injury in chronic iron overload [20,21].

The antioxidant activity of several polyphenols involving prevention of $^{\bullet}OH$ formation and lipid peroxidation has been correlated with their iron chelating properties [12,22–26]; however, as far as we know, this has not been established with TA. Here we present the results of studies on the antioxidant activity of TA in preventing the degradation of 2-deoxyribose induced by $^{\bullet}OH$ radicals which were produced by the Fenton reagents Fe(II) and H_2O_2 [27,28] or via Fe(III)-EDTA/ascorbate/O_2 [16,29]. We also report on the possible stability of complexes of TA and Fe(II) in the presence of H_2O_2, and on the ability of TA to reduce Fe(III) to Fe(II).

2. Materials and methods

2.1. Reagents and solutions

2-Deoxyribose, DMSO, DFO, EDTA, ferrozine, HEPES, H_2O_2, 1,10-phenanthroline, penta-*m*-digalloyl-glucose (TA), thiourea and thiobarbituric acid (TBA) were purchased from Sigma (St. Louis, MO). PIH was a gift from Dr. Prem Ponka, Jewish General Hospital, Montreal, Canada (see [30]). Other reagents were of analytical grade.

Stock solutions of ferrous ammonium sulfate (0.2 mM), DFO (1 mM), EDTA (1 mM) and ferrozine (28 mM) were freshly prepared in water. Stock solutions of EDTA were neutralized with HCl/NaOH. Tannic acid stock solutions (1 mM) were freshly prepared in 20 mM phosphate buffer (pH 7.2). Ferric chloride solutions were prepared daily in 10 mM HCl. Stock solutions of PIH and 1,10-phenanthroline (1 mM each) were freshly prepared in 0.1 M NaOH and then neutralized with HCl. Stock solutions of 1% TBA were prepared in 50 mM NaOH and used within 1 week. All solutions were made with milli-Q deionized water.

2.2. The assay of 2-deoxyribose degradation

The formation of $^{\bullet}OH$ radicals from Fenton reagents was quantified using 2-deoxyribose oxidative degradation. The principle of the assay is the quantification of the 2-deoxyribose degradation product, malonaldehyde, by its condensation with TBA [29,31]. Typical reactions were started by the addition of Fe(II) (6 μM final concentration) to solutions (0.5 ml of final volume) containing 5 mM 2-deoxyribose, 100 μM H_2O_2, iron chelator (0 or 10 μM) and 20 mM phosphate buffer (pH 7.2). Reactions were carried out for 10 min at room temperature (25 ± 1°C) and were stopped by the addition of 0.5 ml 4% phosphoric acid (v/v) followed by 0.5 ml 1% TBA (w/v, in 50 mM NaOH). After boiling for 15 min, the absorbance of solutions was measured at 532 nm.

The formation $^{\bullet}OH$ radicals from Fe(III)-EDTA, ascorbate and O_2 was performed as described above. The samples containing 20 mM phosphate buffer (pH 7.2), 5 mM 2-deoxyribose, Fe(III)-EDTA (50 μM iron) and TA (0 or 200 μM) were pre-incubated for 30 min at room temperature before addition of ascorbate (100 μM). The reactions were carried out for 10 min and were stopped as described above. Products of 2-deoxyribose degradation were measured at 532 nm.

2.3. Absorption spectra

Spectra of the complexes of TA with Fe(II) or Fe(III) were obtained with a Hitachi U-2001 spectrophotometer. The complexes were pre-incubated in phosphate buffer (pH 7.2) for 1 h before measurement against a blank containing TA, in the absence of iron, or against a blank containing buffer only.

2.4. Ferrozine assay

The ferrozine assay for Fe(II) [32] was performed by addition of excess ferrozine (14 mM) to phosphate buffered media (pH 7.2) containing iron (Fe(II) or Fe(III)) or iron plus TA. The stable com-

plexes between ferrozine and Fe(II) were quantitated by measuring absorbance at 562 nm.

3. Results and discussion

3.1. TA inhibits the Fenton reaction

Fig. 1 depicts the time course of the oxidative degradation of 5 mM 2-deoxyribose induced by Fenton reagents, 6 μM Fe(II) plus 100 μM H_2O_2, in phosphate buffer (pH 7.2). Because the Fenton reaction is very fast [28] there was no increase in $^{\bullet}OH$ formation and 2-deoxyribose degradation after 5 s. The presence of 10 μM TA in the incubation media inhibited 2-deoxyribose degradation to an extent which remained unchanged for up to 30 min (Fig. 1). Addition of 10 μM TA after 10 min of exposure of 2-deoxyribose to the Fenton reagents had induced no inhibitory effect (data not shown).

Increasing Fe(II) concentrations, from 6 to 100 μM, in the presence of 100 μM H_2O_2 caused an increase in 2-deoxyribose degradation (Fig. 2A). Tannic acid (10 μM TA) decreased 2-deoxyribose degradation at all Fe(II) concentrations, but the antioxidant efficiency of TA fell from ~60% to ~40% with increasing Fe(II) concentrations (Fig. 2B). This demonstrates that the antioxidant efficiency of TA depends on the concentration of Fe(II) and suggests that TA inhibits $^{\bullet}OH$ formation by chelating iron.

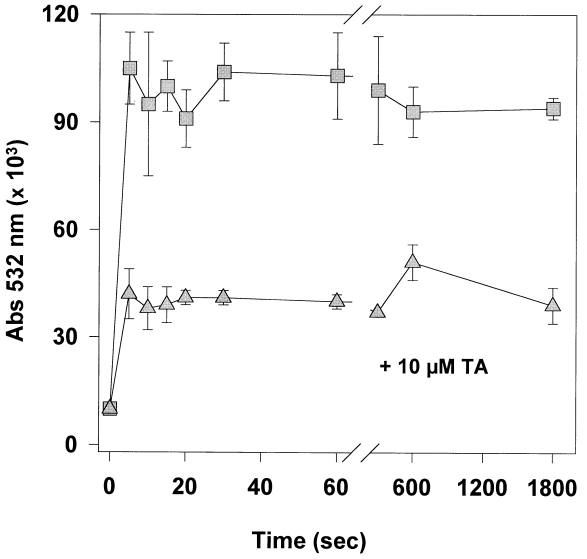

Fig. 1. Time course of 2-deoxyribose oxidative degradation induced by Fenton reagents as measured by absorbance at 532 nm. The solutions with a final volume of 0.5 ml contained 5 mM 2-deoxyribose, 100 μM H_2O_2, 20 mM phosphate buffer (pH 7.2) and in the absence or presence of 10 μM TA. Reactions were started by addition of ferrous ammonium sulfate (6 μM final concentration) and quenched by 0.5 ml of 4% phosphoric acid (see Section 2). In the case of the first time point (0 s), Fe(II) and H_2O_2 were included after the addition of phosphoric acid. Values are means ± S.D. ($n = 4$).

Table 1
Comparison of the efficiencies of TA and antioxidants (iron chelators and $^{\bullet}OH$ scavengers) in preventing 2-deoxyribose degradation[a]

Effectors	Absorbance at 532 nm[b]
None	0.355 ± 0.008
Iron chelators (10 μM)	
TA	0.161 ± 0.010 (55%)[c]
PIH[d]	0.326 ± 0.004 (8%)
DFO	0.284 ± 0.004 (20%)
1,10-Phenanthroline	0.216 ± 0.003 (39%)
EDTA	0.283 ± 0.016 (20%)
Scavengers of $^{\bullet}OH$ (10 mM)	
DMSO	0.337 ± 0.005 (5%)
Ethanol	0.335 ± 0.007 (6%)
Thiourea	0.109 ± 0.005 (69%)

[a]Incubations contained 5 mM 2-deoxyribose, 6 μM Fe(II), 100 μM H_2O_2, in the absence or presence of effectors (iron chelators or $^{\bullet}OH$ scavengers), in 20 mM phosphate buffer (pH 7.2).
[b]Values of absorbance are mean ± S.D. ($n = 5$; except for controls where $n = 4$).
[c]Values in parentheses are the % inhibition of 2-deoxyribose degradation.
[d]Inhibition of 2-deoxyribose degradation with 60 μM PIH was 65% of controls [37].

Titration curve of the TA inhibition of oxidative degradation of 2-deoxyribose induced by Fenton reagents is shown in Fig. 3. An I_{50} value of 13 μM was obtained. Such a low I_{50} value is not compatible with an antioxidant mechanism involving only $^{\bullet}OH$ trapping, since molecules which scavenger $^{\bullet}OH$ radicals are effective only in the mM range in aqueous systems [16,33]. Since phosphate, which was used as a buffer, is an iron chelator it could have interfered with the antioxidant efficiency of TA. Therefore, we repeated the TA titration curve using HEPES, a buffer with a lower affinity for iron [34]. The effective-

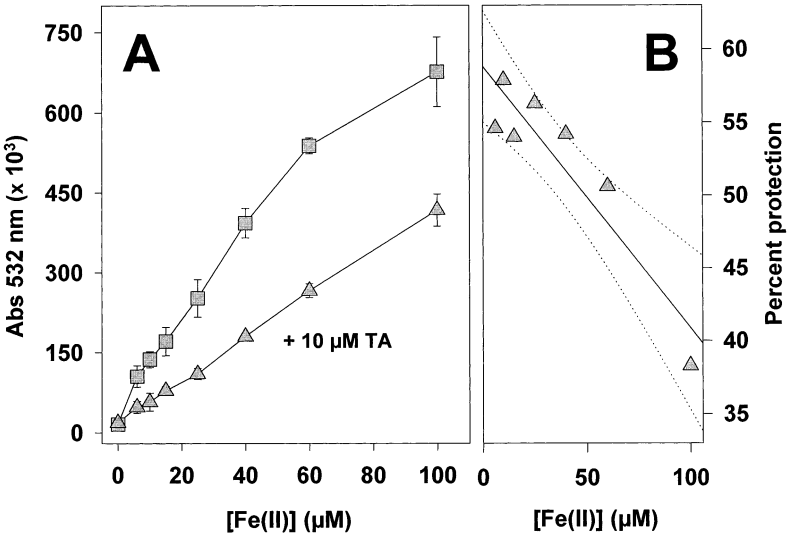

Fig. 2. Dependence of Fe(II) concentration on 2-deoxyribose degradation induced by Fenton reagents (100 µM H_2O_2), in 20 mM phosphate buffer (pH 7.2), in the absence or presence of 10 µM TA (A). Values are means ± S.D. ($n = 4$). (B) Replot of data from A showing the % protection of 2-deoxyribose by TA. A first-order regression line is shown ($r^2 = 0.85$; $P < 0.005$); dotted lines represent the 95% confidence interval.

ness of TA against 2-deoxyribose degradation increased in HEPES buffer, especially at 10–20 µM TA (inset to Fig. 3). However, the mechanism of the antioxidant action of TA should not be affected by HEPES or phosphate.

Tannic acid was a more efficient antioxidant in preventing 2-deoxyribose degradation than other iron chelators (EDTA, 1,10-phenanthroline, PIH and DFO) and than the classical •OH scavengers, DMSO, ethanol and thiourea (Table 1). Interestingly, TA was at least three orders of magnitude more effective than the •OH scavengers.

The concentration dependence of 2-deoxyribose on its oxidative degradation induced by Fenton reagents in the absence or presence of 10 µM TA is shown in Fig. 4A. If TA acted mainly as an •OH scavenger we would have expected that its effectiveness would have diminished with increasing 2-deoxyribose concentrations (from 3 to 70 mM) because the •OH detector molecule (2-deoxyribose) would have competed with TA for •OH trapping. Fig. 4B shows that there was just a slight reduction in the antioxidant efficiency of TA (from 64 to 58.5%) with increasing 2-deoxyribose concentrations. The effect of the classical •OH scavenger thiourea on the concentration dependence of 2-deoxyribose was also tested. We verified that the antioxidant effectiveness of 10 mM thiourea at in-

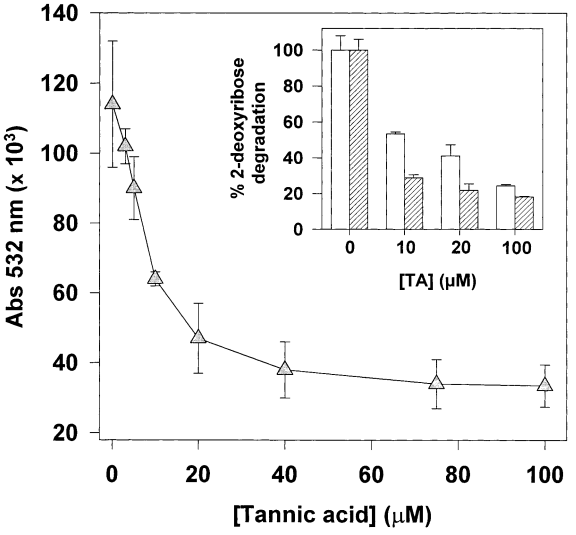

Fig. 3. Effect of TA concentration on the oxidative degradation of 2-deoxyribose induced by 6 µM Fe(II) plus 100 µM H_2O_2, in 20 mM phosphate buffer (pH 7.2). Values are means ± S.D. $n = 4$. Inset: effect of TA concentration on 2-deoxyribose degradation in 20 mM phosphate buffer (open bars) or 20 mM HEPES buffer (hatched bars). Results are means ± S.D. ($n = 4$) of % levels of 2-deoxyribose degradation. Absorbance at 532 nm for controls in phosphate and HEPES buffer were 0.123 ± 0.010 and 0.140 ± 0.009, respectively.

creasing 2-deoxyribose concentrations (from 3 to 70 mM) was affected more than we found with TA. Under these conditions the antioxidant efficiency of thiourea fell from 76.3 to 42.7% (first-order regression line with: $n=7$, $r^2=0.979$, $P<0.001$; data not shown). Taken together, these data, strongly suggest that the main mechanism by which TA inhibits 2-deoxyribose degradation is not by trapping •OH radicals, but by chelating Fe(II).

The time course of 2-deoxyribose degradation induced by Fenton reagents plus 1 mM ascorbate is shown in Fig. 5. Since ascorbate recycles Fe(III) to Fe(II), the formation of •OH leveled off only after 15–20 min. Tannic acid at 10 μM inhibited 2-deoxyribose degradation, and the yield of •OH formation in the presence of TA was unchanged during the incubation period. These results are consistent with preliminary observations that TA inhibits the rate of oxidation of 0.1 mM ascorbate (followed at 265 nm) mediated by ferric iron [35]. Thus, it is possible that the redox cycling of iron induced by ascorbate is blocked by TA, causing the observed inhibition in •OH formation and 2-deoxyribose degradation.

Table 2
Removal of Fe(II) from iron–TA complexes by ferrozine

Experimental conditions[a]	Absorbance at 562 nm[b]
With Fe(II)	
No TA[c]	1.487 ± 0.028 (3)
No ferrozine[d]	0.139 ± 0.009 (3)
45 min[e]	1.433 ± 0.002 (3)
With Fe(III)	
No TA[f]	0.050 ± 0.008 (3)
No ferrozine[g]	0.118 ± 0.005 (3)
45 min[e]	1.473 ± 0.112 (3)
Without iron[h]	0.009 ± 0.002 (4)

[a]Samples in 10 mM phosphate buffer (pH 7.2) containing 10 μM TA and 50 μM iron (Fe(II) or Fe(III)), added in that order, were pre-incubated for 60 min before the addition of ferrozine (14 mM final concentration).
[b]Values of absorbance are means ± S.D. (*n* values in brackets).
[c]Control sample, defined as the absorbance of the complex between ferrozine and Fe(II).
[d]The absorbance at 562 nm of the $Fe(II)_n$–TA complex (which remained constant for 60 min).
[e]Incubation time after ferrozine addition.
[f]The absorbance at 562 nm of sample containing ferrozine and Fe(III).
[g]Sample containing TA and Fe(III).
[h]Sample containing ferrozine and TA.

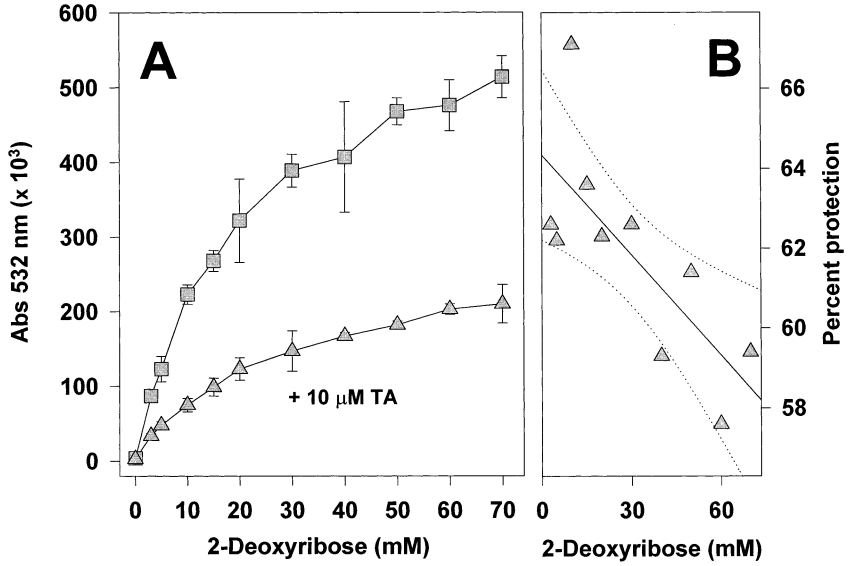

Fig. 4. Effect of 2-deoxyribose concentration on the oxidative degradation of 2-deoxyribose by 6 μM Fe(II) and 100 μM H_2O_2, in the absence or presence of 10 μM TA, in 20 mM phosphate buffer (pH 7.2) (A). Values are means ± S.D. ($n=3$–4). (B) Replot of data from A depicting the % protection induced by TA. A first-order regression line is shown in B ($r^2=0.565$; $P<0.025$); dotted lines represent the 95% confidence interval.

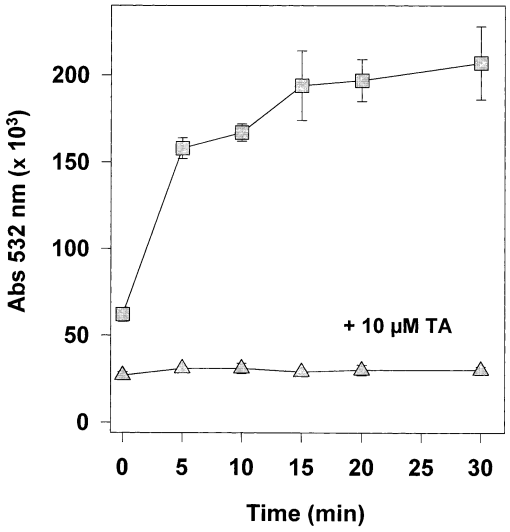

Fig. 5. Time course of 2-deoxyribose degradation induced by 6 μM Fe(II), 100 μM H_2O_2 and 1 mM ascorbate, in the absence or presence of 10 μM TA, in 20 mM phosphate buffer (pH 7.2). Reactions were started by Fe(II) addition. The first time point was 5 s. Values are means ± S.D. ($n = 4$–5).

3.2. Characteristics of the iron–TA complexes

We investigated the mechanism of TA antioxidant activity acting as an iron chelator. It is possible that TA increases the rate of Fe(II) oxidation to Fe(III), thus decreasing the concentration of Fe(II) available for the Fenton reaction (hypothesis A). The iron chelator PIH seems to inhibit $^{\bullet}OH$ formation from Fe(II) plus H_2O_2 by this mechanism [36,37]. On the other hand, TA might form a complex with Fe(II) that is unreactive with H_2O_2, thus preventing its participation in the Fenton reaction (hypothesis B). A similar mechanism has been established for the antioxidant activity of 1,10-phenanthroline [15]. The following experiments were designed to distinguish between these two possible mechanisms.

Fig. 6 shows the spectra of the complexes of TA (10 μM) with different concentrations (6–100 μM) of Fe(II) in phosphate buffer (pH 7.2). Three absorbance peaks were observed at 235–340, 316–323 and 520–550 nm. The peaks in the UV range moved from 240 to 235 nm and from 323 to 316 nm with increasing Fe(II) concentrations while the peak in the visible

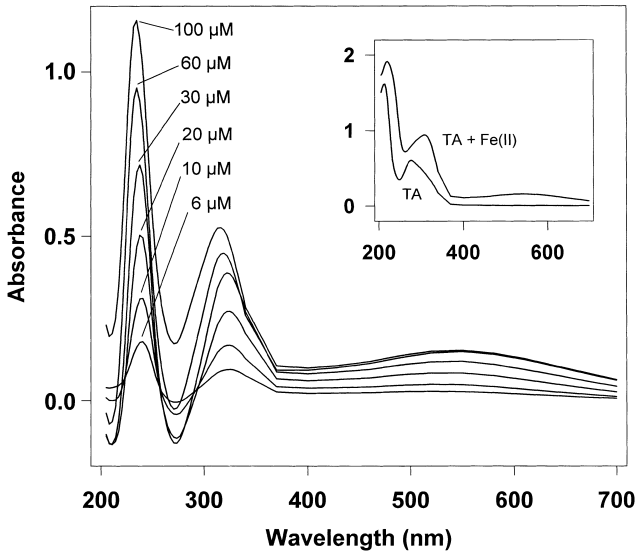

Fig. 6. Spectra of the complexes formed between 10 μM TA and several concentrations of Fe(II) in 20 mM phosphate buffer (pH 7.2). The spectra of the complexes were recorded with 10 μM TA as the blank. Samples were pre-incubated for 1 h at room temperature prior to spectral analysis. Spectra shown are representative of four independent determinations. Inset: spectra of TA (10 μM) and TA plus Fe(II) (100 μM) recorded with buffer as the blank.

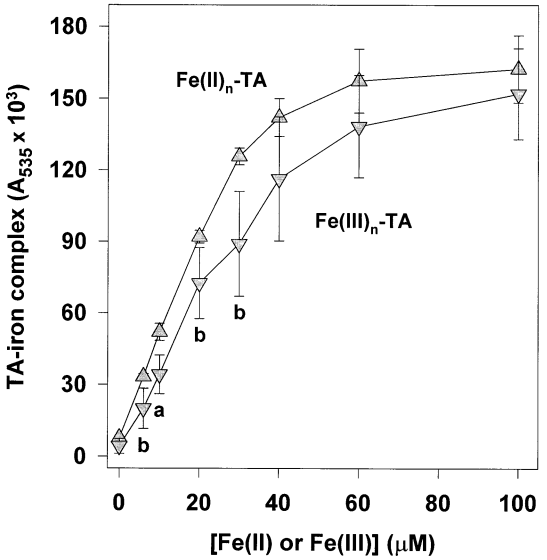

Fig. 7. Effect of different Fe(II) or Fe(III) concentrations on complex formation with TA in 20 mM phosphate buffer (pH 7.2). The complexes between TA (10 μM) and iron were quantified by measuring the absorbance at 535 nm with buffer as the blank. Samples were pre-incubated for 1 h before measuring the absorbances. Values are means ± S.D. ($n = 3$–4). [a]Significantly different from corresponding value at $P < 0.01$ (one-tailed t-test); [b]$P < 0.05$.

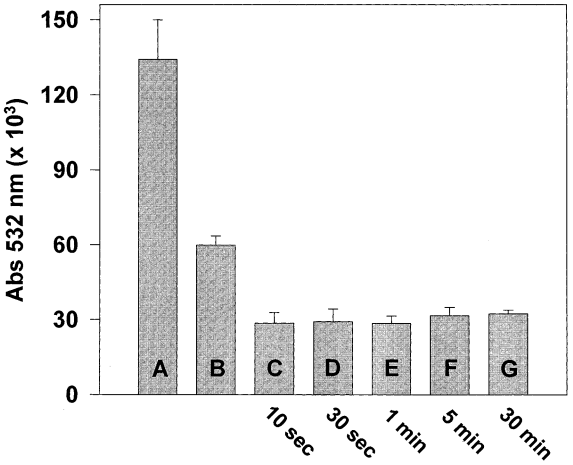

Fig. 8. Determination of the degradation of 5 mM 2-deoxyribose caused by the reaction of $Fe(II)_n$–TA with H_2O_2. Columns A and B ('standard reactions' as described in Section 2): 2-deoxyribose degradation induced by 6 μM Fe(II) plus 100 μM H_2O_2 in 20 mM phosphate buffer (pH 7.2), in the absence (A) or presence of 10 μM TA (B). Columns C–G: six μM Fe(II) was added to solutions containing 10 μM TA and phosphate buffer which were then pre-incubated for 10 s to 5 min. Then 2-deoxyribose and 100 μM H_2O_2 where added and the solutions were incubated for 10 min. Values are means ± S.D. ($n = 6$). The absorbance value of B is significantly different from all the other absorbance values ($P < 0.005$, Student–Newman–Keuls (SNK) comparison test).

range moved from 520 to 550 nm. Even though the absorbance data in Fig. 6 were obtained after 30 min of incubation, complexation was completed within a few seconds following the addition of Fe(II) to solutions containing TA. Interestingly, the spectra shown in Fig. 6 are nearly the same as the spectra of TA with different concentrations of Fe(III) (data not shown). Small differences were detected only in the 520–550 nm peak. This is demonstrated when the absorbances at 535 nm are measured as a function of Fe(II) or Fe(III) concentration (Fig. 7).

The data in Fig. 7 also indicate a maximum ratio of iron ions to TA of ~10:1 for the complex at pH 7.2. The higher antioxidant efficiency of TA compared with other iron chelators (see Table 1) could be partially explained by the greater number of iron ions that can be chelated by TA. It has been demonstrated that iron ions form complexes with DFO, PIH and 1,10-phenantroline with ratios of 1:1, 1:2 and 1:3, respectively [15,20,36–38].

The lack of major spectral differences between the two complexes (TA complexed to Fe(II) or to Fe(III)) may have been due to oxidation of Fe(II) to Fe(III) by TA or due to the reduction of Fe(III) to Fe(II) by TA. To examine these possibilities we used ferrozine to measure the levels of Fe(II) in solutions containing TA. Ferrozine forms a stable complex with Fe(II) that can be measured by its absorbance at 562 nm [32]. Ferrozine was added to a phosphate buffered (pH 7.2) solution of 10 μM TA and 50 μM Fe(II) and a yield of 96% of Fe(II) was observed within 45 min (Table 2). Since ferrozine does not induce reduction of Fe(III), the data indicate that complexes of Fe(II) and TA ($Fe(II)_n$–TA) are not oxidized to $Fe(III)_n$–TA in aqueous solution for at least 45 min.

Since TA does not oxidize Fe(II), the lack of major spectral differences between the complexes of TA with Fe(II) or Fe(III) might be explained by the reduction of Fe(III) to Fe(II) by TA. It has been reported that several polyphenols can reduce Fe(III) to Fe(II) [26,39]. Addition of ferrozine (14 mM) to samples containing 10 μM TA and 50 μM Fe(III), in phosphate buffer (pH 7.2), resulted in significant levels of Fe(II)–ferrozine complexes (Table 2). About 99% of the iron in the samples formed a chelate with ferrozine, indicating that TA reduced Fe(III), forming $Fe(II)_n$–$TA_{oxidized}$ complexes (Eqs. 1 and 2).

$$TA + nFe(III) \rightarrow Fe(III)_n\text{–}TA \quad (1)$$

$$Fe(III)_n\text{–}TA \rightarrow Fe(II)_n\text{–}TA_{oxidized} \quad (2)$$

Table 3
Effect of H_2O_2 on the stability of $Fe(II)_n$–TA complexes

$[H_2O_2]$ (μM)	Absorbance at 562 nm	
	Plus TA[a]	No TA[b]
0	1.320 ± 0.094[c]	0.051 ± 0.012
100	1.315 ± 0.093	0.044 ± 0.001
200	1.273 ± 0.143	0.051 ± 0.016
500	1.259 ± 0.164	0.039 ± 0.003

[a]Samples in phosphate buffer (pH 7.2) containing 10 μM TA and 50 μM Fe(II) (added in this order) were pre-incubated for 60 min before addition of H_2O_2. Then the reaction mixture was incubated for 30 min before ferrozine addition. Samples without H_2O_2 were also incubated for 30 min.

[b]H_2O_2 was added to samples without TA immediately after addition of Fe(II). Then the reaction mixture was incubated for 30 min followed by ferrozine addition.

[c]Values are means ± S.D. ($n = 4$). Absorbances were recorded 45 min after ferrozine addition (see Table 2).

We also tested the effect of H_2O_2 on the stability of $Fe(II)_n$–TA complexes. Ferrozine was added to solutions containing 10 μM TA and 50 μM Fe(II) which had been incubated for 30 min with several concentrations of H_2O_2 (0, 100, 200 and 500 μM). Nearly full recovery of Fe(II) (from samples containing 10 μM TA) was obtained at all H_2O_2 concentrations tested, as compared with the samples in the absence of H_2O_2 (Table 3). In the absence of TA, 96–97% of the Fe(II) was oxidized, suggesting that $Fe(II)_n$–TA complexes are unreactive with H_2O_2 (up to 500 μM).

3.3. 2-Deoxyribose degradation induced by the reaction of $Fe(II)_n$–TA complexes with H_2O_2

We also examined whether or not the reaction of $Fe(II)_n$–TA with H_2O_2 could induce damage to 2-deoxyribose. Tannic acid at 10 μM was pre-incubated with 6 μM Fe(II) for periods ranging from 10 s to 30 min, allowing $Fe(II)_n$–TA complexes be formed, before the addition of 2-deoxyribose and 100 μM H_2O_2 (in that order). The mixture was incubated at room temperature for a further 10 min. The 2-deoxyribose degradation products formed under these conditions were compared with those formed under standard experimental conditions (H_2O_2 plus Fe(II), in the absence or presence of 10 μM TA). Under standard experimental conditions, TA protected 2-deoxyribose from degradation by 55,4%. On the other hand, when TA was previously complexed with Fe(II), a 76–79% reduction in 2-deoxyribose degradation was observed (Fig. 8). These experiments give further support to the hypothesis that the formation of complexes between Fe(II) and TA is crucial for the antioxidant activity of TA.

3.4. The effect of TA on 2-deoxyribose degradation induced by Fe(III)-EDTA/ascorbate/O_2

The inhibitory action of TA on 2-deoxyribose degradation mediated by Fe(III)-EDTA, 100 μM ascorbate and O_2 was also investigated. Fe(III)-EDTA/ascorbate/O_2 slowly generates $^{\bullet}OH$ radicals, causing 2-deoxyribose degradation [16,29]. When a 1:1 ratio of Fe(III) (50 μM) and EDTA (50 μM) was employed, TA protected 2-deoxyribose against degradation by 49% (Table 4). However, when the ratio was 1:10 (500 μM EDTA), this decreased to only 21% (Table 4). These data indicate that TA inhibits 2-deoxyribose degradation by removing Fe(III) from EDTA, forming a complex with iron that cannot participate in Fenton reactions. With high levels of EDTA (500 μM), less iron is available for complexation with TA resulting in less protection against 2-deoxyribose degradation.

Table 4
Effect of TA on 2-deoxyribose degradation induced by ascorbate plus Fe(III)-EDTA

[EDTA] (μM)	Deoxyribose degradation (absorbance at 532 nm)	
	No TA	Plus 200 μM TA
50	0.219 ± 0.012[a]	0.112 ± 0.011 (48.9%)[b]
500	0.213 ± 0.011	0.169 ± 0.020 (20.9%)

Samples in 20 mM phosphate buffer (pH 7.2) containing 5 mM 2-deoxyribose, 50 μM Fe(III)-EDTA (50 or 500 μM EDTA) and TA (0 or 200 μM), added in this order, were pre-incubated at room temperature for 30 min before addition of 100 μM ascorbate. Reactions were carried out for 10 min at room temperature. The detection of 2-deoxyribose degradation products is described in Section 2.

[a]Values are means ± S.D. ($n = 4$).

[b]Values between brackets are the % protection induced by TA.

4. Conclusions

The activity of TA in preventing 2-deoxyribose oxidative degradation induced by $^{\bullet}OH$ radicals generated from Fenton reagents was analyzed. The mechanism of antioxidant action of polyphenols has usually been attributed to $^{\bullet}OH$ scavenging activity [5] and this could be true for TA. However, our results strongly indicate that TA does not block 2-deoxyribose degradation by simply trapping $^{\bullet}OH$ radicals. Rather, TA seems to act as an antioxidant by complexing iron, forming complexes with Fe(II) ($Fe(II)_n$–TA) that cannot participate in Fenton reactions (Table 3 and Fig. 8). Grinberg et al. [24] also concluded that the protective activity of tea polyphenols against $^{\bullet}OH$-dependent salicylate hydroxylation was due to iron chelation.

The fact that TA is able to reduce Fe(III) to Fe(II) suggests that redox cycling of Fe(III) formed by the Fenton reaction is possible. Ferric iron formed by

the Fenton reaction would be complexed to TA, forming an intermediate complex $Fe(III)_n$–TA, which would then be converted to $Fe(II)_n$–$TA_{oxidized}$ (see Eqs. 1 and 2). However, the complex of TA with Fe(II) may not react with H_2O_2 (Table 3 and Fig. 8). Alternatively, it is possible that H_2O_2 induces redox cycling of Fe(II) complexed to TA ($Fe(II)_n$–$TA_{oxidized}$ and $Fe(II)_n$–TA), producing complexes of TA with Fe(III), which can be reduced back to $Fe(II)_n$–$TA_{oxidized}$. In this case, Fe(II) would be bound by excess ferrozine (as observed in Table 3) and TA would trap $^{\bullet}OH$ radicals that are being formed, since degradation of 2-deoxyribose is highly inhibited by TA (Eq. 3).

$$Fe(II)_n\text{–}TA + H_2O_2 \rightarrow Fe(III)_n\text{–}TA_{hydroxylated} + OH^- \quad (3)$$

We also observed that 200 μM TA inhibits 2-deoxyribose degradation mediated by Fe(III)-EDTA/ascorbate/O_2 (see Table 4). Fe(III)-EDTA is a more biologically relevant pro-oxidant, which simulates cellular low molecular weight iron complexes, such as iron–ATP and iron–citrate [40,41]. Tannic acid may inhibit 2-deoxyribose damage by removing Fe(III) from EDTA, forming an intermediate complex $Fe(III)_n$–TA, which can be converted to $Fe(II)_n$–$TA_{oxidized}$ (see Eq. 2). We propose that this complex is unable to react with H_2O_2, which is formed from Fe(III)-EDTA/ascorbate/O_2, thus preventing $^{\bullet}OH$-dependent 2-deoxyribose degradation. TA also inhibits ascorbate oxidation and the conversion of Fe(III)-EDTA into Fe(II)-EDTA [35], a key step in the formation of H_2O_2 and $^{\bullet}OH$ [29]. Accordingly, we have proposed that PIH prevents 2-deoxyribose damage (induced by Fe(III)-EDTA/ascorbate/O_2) and Fe(III)-EDTA-dependent ascorbate oxidation by removing Fe(III) from Fe(III)-EDTA and forming Fe(III)-PIH_2 that cannot participate in Haber–Weiss reactions [16].

The present observations on the antioxidant activity of TA might explain, at least in part, its antimutagenic and anticarcinogenic activity [2–4,6,42], since DNA can be damaged by $^{\bullet}OH$ radicals formed from Fe(II) and H_2O_2 [43,44]. By chelating Fe(II), TA would protect DNA from oxidative damage. It is tempting to propose the use of TA for prevention and/or experimental therapy of disorders related to iron-mediated oxidative stress, such as colorectal cancer [45,46]. Since TA has been shown to be absorbed by the gastrointestinal tract in mice and sheep and reach the plasma [47,48], it is possible that TA could be taken up by several cell types and protect DNA against iron-mediated oxidative damage. Indeed, Athar and coworkers [42] observed that dietary TA reduced the incidence of lung and forestomach tumors induced by benzopyrene in mice. Further research must be done in vivo on the iron chelating and antioxidant activities of μM amounts of TA since excess TA, i.e. > 1–2 g/kg b.wt., is highly toxic to mammals [48].

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