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Antioxidant properties of Thonningianin A, isolated from the African medicinal herb, *Thonningia sanguinea*

Maxwell Afari Gyamfi, Yoko Aniya*

Laboratory of Physiology and Pharmacology, Faculty of Medicine, School of Health Sciences, University of the Ryukyus, Nishihara, Okinawa 903-0215, Japan

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Abstract

The antioxidant properties of Thonningianin A (Th A), an ellagitannin, isolated from the methanolic extract of the African medicinal herb, *Thonningia sanguinea* were studied using the NADPH and Fe^{2+}/ascorbate-induced lipid peroxidation (LPO), electron spin resonance spectrometer and the deoxyribose assay. Th A at 10 μM inhibited both the NADPH and Fe^{2+}/ascorbate-induced LPO in rat liver microsomes by 60% without inhibitory effects on cytochrome P450 activity. Th A was similar to the synthetic antioxidant, tannic acid, as an inhibitor of both the NADPH and Fe^{2+}/ascorbate-induced LPO but potent than gallic acid, vitamin C and vitamin E. While Th A poorly scavenged the hydroxyl radical generated by the Fenton reaction it dose-dependently scavenged 1,1-diphenyl-2-picrylhydrazyl, superoxide anion and peroxyl radicals with IC_{50} of 7.5, 10 and 30 μM, respectively. Furthermore, Th A showed inhibitory effects on the activity of xanthine oxidase with an IC_{50} of 30 μM. In the deoxyribose assay both *T. sanguinea* and its methanolic component Th A showed only site-specific ($Fe^{3+} + H_2O_2$) but not non-site-specific ($Fe^{3+} + EDTA + H_2O_2$) hydroxyl radical scavenging suggesting chelating ability for iron ions. Spectroscopic studies showed that Th A enhanced absorbance in the visible region in the presence of Fe^{2+} ions. These results indicate that the antioxidant properties of Th A involve radical scavenging, anti-superoxide formation and metal chelation.

Keywords: Antioxidant; Lipid peroxidation; Reactive oxygen species; Xanthine oxidase; Electron spin resonance (ESR); Medicinal herb; Thonningianin A; *Thonningia sanguinea*

1. Introduction

T. sanguinea Vahl (Balanophoraceae) is a medicinal herb used prophylactically against bronchial asthma [1]. Extract of *T. sanguinea* has been shown to scavenge the stable nitrogen-centered radical, 1,1-diphenyl-2-picrylhydrazyl (DPPH), and to inhibit the formation of thiobarbituric acid reactive substances (TBARS) [2]. Studies using various hepatotoxic agents such as galactosamine, carbon tetrachloride and aflatoxin B_1 which cause liver injury *via* generation of free radicals, indicate that *T. sanguinea* extract has a potent hepatoprotective activity [2,3].

Besides free radical scavenging, the medicinal herb may mediate chemoprevention through modulation of activity of drug metabolizing enzymes [3,4]. While *T. sanguinea* extract increases erythromycin *N*-demethylase activity which is associated with cytochrome P450 (CYP) 3A1, it inhibits other CYP monoxygenase enzymes *in vitro* in rat liver microsomes [4]. Furthermore, *T. sanguinea* extract is a potent *in vivo* inhibitor of the constitutive CYP 1A2 enzyme activity [4]. The role of CYP 1A enzymes in the activation of procarcinogenic xenobiotics including benzopyrene and various heterocyclic amines have been reported [5,6]. Nyarko and Addy [1] demonstrated that the aqueous extract of *T. sanguinea* and some fractions from it possess anti-anaphylatic activity. However, the active component(s) in *T. sanguinea* responsible for these actions were not known.

It was of interest to examine the chemical components in *T. sanguinea* responsible for the pharmacologic actions observed in various animal models. In this endeavor two new tannic compounds, Th A and B from the methanolic extract of *T. sanguinea* have been isolated [7].

* Corresponding author. Tel.: +81-98-895-1251; fax: 81-98-895-1443.
E-mail address: yaniya@med.u-ryukyu.ac.jp (Y. Aniya).

Abbreviations: Th A, Thonningianin A; LPO, lipid peroxidation; TBARS, thiobarbituric acid reactive substances; DPPH, 1,1-diphenyl-2-picrylhydrazyl; ROS, reactive oxygen species; $O_2^{\bullet -}$, superoxide anion radical; $^{\bullet}OH$, hydroxyl radical; $RO_2^{\bullet}$, peroxyl radical; XO, xanthine oxidase; CYP, cytochrome P450; DTPA, diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid.

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Tannins are polyphenolic secondary metabolites of many plants. Multiple biologic effects of the polyphenols including antihepatotoxic, antimicrobial, antitumor and anti-lipidperoxidation have been reported [8–10]. It has been suggested that important properties underlying these biologic effects of the polyphenols include metal chelating, ability to bind macromolecules such as protein, antioxidant and free radical scavenging ability [9,11]. The biologic activity of tannins are also affected by the structure of the compound [8]. Since the structure of Th A, a mixture of gallic acid esters of glucose and dihydrochalcone, an ellagitannin has not been previously reported, it was important to examine its antioxidant properties.

To clarify this, we studied the effectiveness of one of the compounds isolated from *T. sanguinea*, Th A in preventing LPO, a chain reaction set into motion by reactive oxygen species (ROS) using several *in vitro* systems and the ability of Th A to interact with free radicals such as DPPH, superoxide anion ($O_2^{\bullet -}$), hydroxyl ($^{\bullet}OH$) and peroxyl ($RO_2^{\bullet}$) radicals in order to gain insight into the antioxidant specificity of Th A. Furthermore, the deoxyribose assay performed in the absence of di-sodium dihydrogen ethylenediamine tetraacetate dihydrate (EDTA) allowed us to evaluate the potential ability of Th A to chelate metal ions [12].

2. Materials and methods

2.1. Chemicals

Tannic acid, glucose 6-phosphate, ferrous sulphate, allopurinol, 1,1,3,3-tetramethoxypropane, SOD, D-mannitol, deferoxamine mesylate, 2-deoxy-D-ribose and β-nicotinamide adenine dinucleotide were purchased from Sigma Chemicals. DPPH and 2-thiobarbituric acid (TBA), hypoxanthine, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) and gallic acid monohydrate from Wako Pure Chemicals, glucose 6-phosphate dehydrogenase from Oriental Yeast, trichloroacetic acid from Kanto Chemicals, α-tocopherol (vitamin E), ascorbic acid (vitamin C) and EDTA from Nacalai Tesque, 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) and diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid (DTPA) from Dojindo Laboratories and xanthine oxidase (XO) (cow milk) from Boehringer Mannheim were used. All other reagents used were of analytical grade.

2.2. Preparation of medicinal plant extract

T. sanguinea roots were collected from the forest of the eastern region of Ghana by the staff of the Center for Scientific Research into Plant Medicine (CSRPM). A voucher specimen (CSRPM 406) of *T. sanguinea* is deposited at the herbarium of CSRPM, Akwapim-Mampong, Ghana and prepared as described previously [3]. Briefly, 1 g of pulverized oven-dried roots of *T. sanguinea* was boiled in 50 mL of distilled water for 1 hr and then centrifuged at 3000 rpm for 15 min. The supernatant was stored at 4° and used as the plant extract. The extract was pre-warmed to room temperature before use.

2.3. Isolation of antioxidants from T. sanguinea

Detailed description of isolation and structure elucidation of Th A (Fig. 1), the antioxidant in *T. sanguinea*, is published elsewhere [7]. Based on the isolation procedure used [7] 1 g of *T. sanguinea* powder should yield 2.6 mg Th A.

2.4. Measurement of antioxidant activity

Scavenging action for the DPPH, $O_2^{\bullet -}$, and $RO_2^{\bullet}$ radicals by Th A was evaluated by ESR spectrometer (JES-FR30, JEOL). ESR spectra were recorded at X-band with modulation frequency of 100 kHz and at 9.4 GHz resonant frequency. The detailed recording conditions for each spectrum are described as follows. Manganese oxide (MnO) was used as an internal standard.

The reaction mixture for the DPPH radical scavenging activity consisted of DPPH (100 μM) in ethanol and 1% methanol (control) or various concentrations of Th A (5–25 μM) dissolved in methanol and diluted with distilled water to give a final methanol concentration of 0.01–1%. The mixture (200 μL) was transferred to a flat cell for analysis of the DPPH. ESR spectra were recorded 40 s after taking the sample under the following conditions: power 4 mW, magnetic field center 335.350 mT, sweep time 5 min, modulation amplitude 1×0.1 mT, amplitude 2×100 and time constant 0.1 s. The signal intensity was evaluated by dividing the peak height of the third of the five line signals of the DPPH radical with the height of the MnO signal to give relative peak heights.

The $O_2^{\bullet -}$ radical was generated by the hypoxanthine–xanthine oxidase system. The reaction mixture consisted of 100 mM potassium phosphate buffer (pH 7.4) containing 100 μM DTPA, 675 mM DMPO, 0.5 mM hypoxanthine, 1% methanol (control) or various concentrations of Th A (10, 25 and 100 μM) and 0.4 U XO in a total volume of 200 μL. Spectra recording was started 60 s after taking sample from the reaction mixture under the following conditions: power 4 mW, magnetic field center 335.350 mT, sweep time 2 min, modulation amplitude 1×0.1 mT, amplitude 3.2×100 and time constant 0.1 s. SOD was used as a standard scavenger for the $O_2^{\bullet -}$ radical. The signal intensity was evaluated by dividing the peak height of the second of the 14 line signals of the $O_2^{\bullet -}$ radical with the height of the MnO signal to give relative peak heights. Both control and Th A samples contained small amount of methanol (0.01–1%), hence the 12 line signal of DMPO–OOH was increased to 14 due to the presence of $^{\bullet}OH$ radical.

The $RO_2^{\bullet}$ radical was generated at a constant rate by the thermal decomposition of water soluble azo-initiator, AAPH

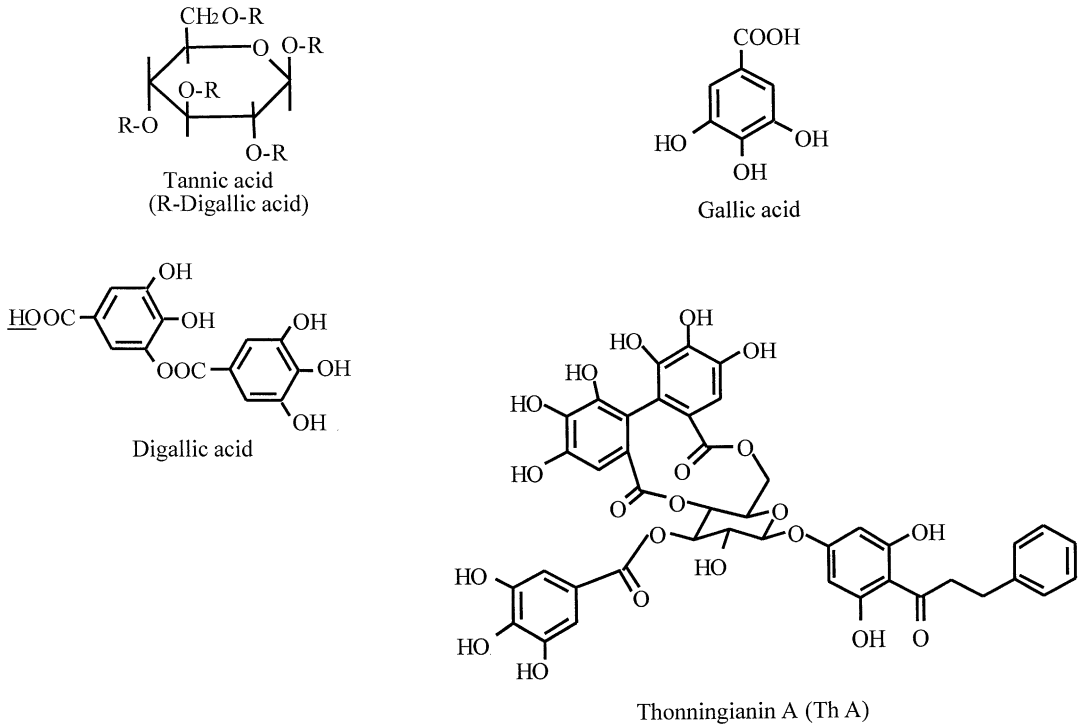

Fig. 1. Structure of the new ellagitannin, Th A isolated from the African medicinal herb, *T. sanguinea*, gallic acid and tannic acid.

[13,14]. The reaction mixture incubated at 37° for 20 min consisted of 100 mM potassium phosphate buffer (pH 7.4) containing 100 μM DTPA, 12.5 mM AAPH, 45 mM DMPO and 1% methanol (control) or various concentrations of Th A (10–100 μM) in a total volume of 200 μL. Spectra recording was started 60 s after taking the incubation mixture under the following conditions: power 4 mW, magnetic field center 335.350 mT, sweep time 1 min, modulation amplitude 0.1×0.79 mT, amplitude 1.25×100 and time constant 0.1 s. The signal intensity was evaluated by dividing the peak height of the first of the four line signals of the $RO_2^{\bullet}$ radical with the height of the MnO signal to give relative peak heights. The four line spectrum of the $RO_2^{\bullet}$ radical generated from AAPH was similar to that of the $^{\bullet}OH$ radical (DMPO–OH spin adduct) but the peak heights was not 1:2:2:1.

The $^{\bullet}OH$ radical scavenging action of Th A was investigated by the deoxyribose method. It has been suggested that some polyphenols are able to reduce Fe(III) to Fe(II) leading to increased degradation of deoxyribose. This assay has, therefore, been used to assess the pro-oxidant action of many compounds [15,16]. The principle of the assay is the quantification of the 2-deoxyribose degradation product, malonaldehyde by its condensation with TBA. The procedure is essentially as described by Halliwell *et al.* [17] with slight modifications. Briefly, the reaction mixture in a final volume of 1 mL contained 20 mM KH_2PO_4–KOH buffer (pH 7.4), 2.8 mM deoxyribose, 100 μM $FeCl_3$, 104 μM EDTA (when added), 300 μM H_2O_2, 0.001–0.025% methanol (control) or various concentrations of Th A (1–10 μM), *T. sanguinea* extract (×2000 to ×20 dilution), 10 mM mannitol, 200 μM DTPA and 100 μM ascorbic acid. Ferric chloride and EDTA (when added) were pre-mixed just before addition to the reaction mixture. Ascorbic acid was added to start the reaction. Reaction mixtures were incubated for 1 hr at 37°. At the end of the incubation period 1 mL of 1% TBA (w/v) in 0.05 M NaOH and 1 mL of 2.8% TCA (w/v) were added followed by heating for 20 min at 100°. After cooling, the extent of deoxyribose degradation by the formed $^{\bullet}OH$ radical was measured directly in aqueous phase at 532 nm. For all concentrations of Th A or *T. sanguinea*, controls in which deoxyribose was omitted from the reaction mixture were performed and revealed that Th A or *T. sanguinea* did not release TBA-reactive material when attacked by the $^{\bullet}OH$ radical.

2.5. Preparation of microsomes

Microsomes were prepared from livers of male Sprague–Dawley rats (200–250 g) by differential centrifugation as reported previously [2]. The microsomes obtained were kept at −80° until use.

2.6. Enzymatic LPO induced by NADPH

Rat liver microsomes (2.3 mg/mL) were incubated with the NADPH generating system (0.33 mM NADP, 8 mM G6P, 6 mM $MgCl_2$ and 0.5 U G6PDH) and 1% methanol (control) or tannic acid (1–10 μM), gallic acid (1–100 μM), vitamin C (1–5 mM), vitamin E (10–100 μM), Th A (1–25 μM) in 0.1 M potassium phosphate buffer (pH

7.4) at 37° for 30 min in a total volume of 1 mL. The extent of LPO was assayed by estimating TBARS formation after incubation, in a 200 μL of the incubation mixture in tubes containing 5% SDS. Following incubation, the peroxidation was terminated by 20% acetic acid. TBA (0.5% in water) was subsequently added and heated for 60 min at 100°. After cooling, 1-butanol was added followed by vigorous shaking and centrifugation. The absorbance of the butanol fraction was read at 532 nm to measure the TBARS. 1,1,3,3-Tetramethoxypropane was used as an authentic standard and results were expressed as nanomoles of malonaldehyde equivalents.

2.7. Non-enzymatic LPO induced by Fe^{2+}/ascorbate

Rat liver microsomes (2.2 mg/mL) were incubated with $FeSO_4$ (5 μM), ascorbate (500 μM) and indicated concentrations of tannic acid (1–10 μM), gallic acid (1–1000 μM), vitamin C (1–5 mM), vitamin E (10–1000 μM), Th A (1–50 μM) or 1% methanol (control) in 0.1 M Tris–HCl buffer (pH 7.4) at 37° for 20 min in a total volume of 1 mL. The extent of LPO was assayed by estimating TBARS formation after the incubation in a 200 μL of the incubating mixture in tubes containing 5% SDS as described above.

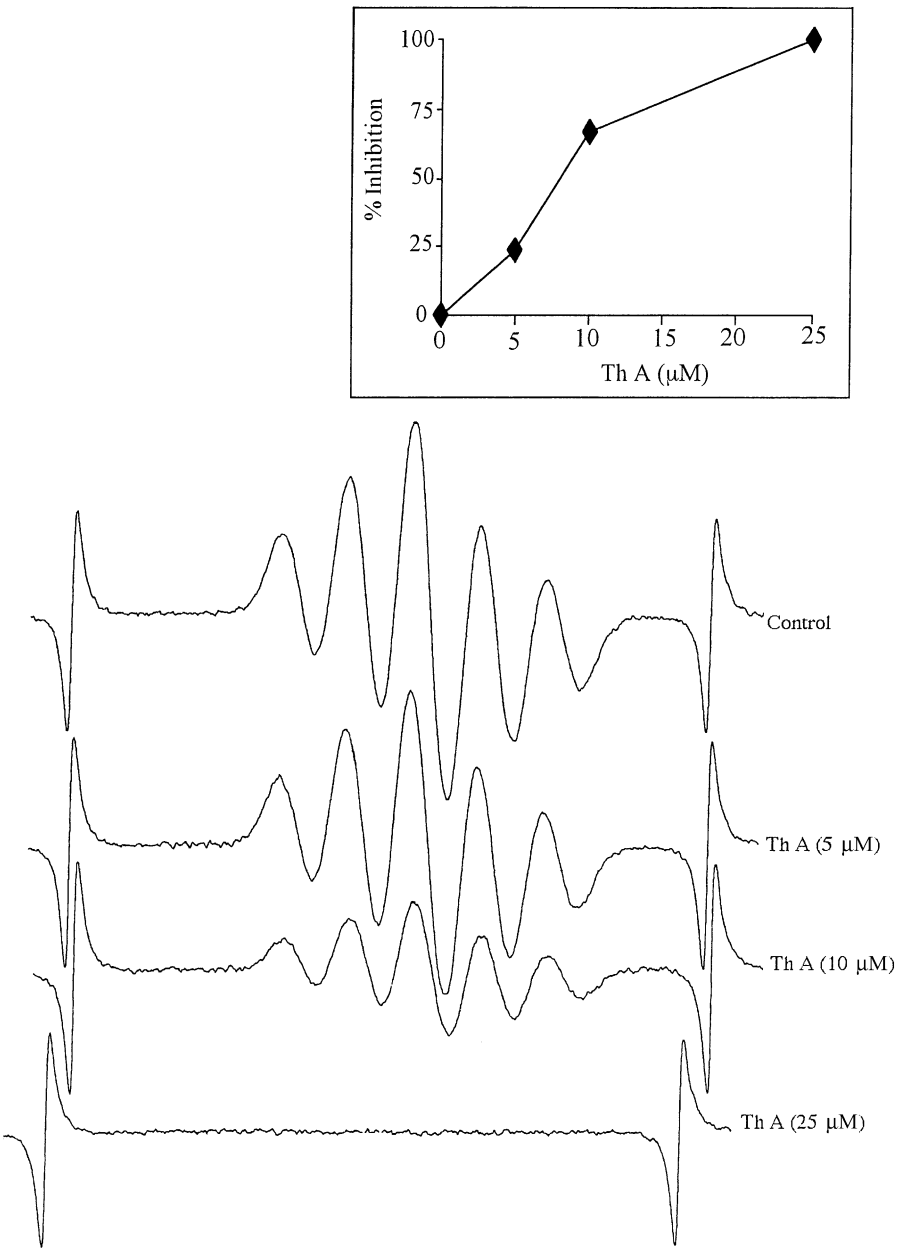

Fig. 2. Effect of Th A on ESR signals formed by the DPPH radical with hyperfine splitting constants of $g = 2.006$, $a^N = 0.876$ mT. Reaction and spectra recording conditions were as stated in the Section 2. Insert is the concentration dependence of percentage inhibition of the generation of the DPPH signal by Th A. Each point shows the mean of duplicate determinations.

2.8. Aniline hydroxylase activity and protein

Aniline hydroxylase activity in one washed microsomes was evaluated by estimating *p*-aminophenol (Imai *et al.* [18]). Protein concentration in each fraction was determined by the method of Lowry *et al.* [19].

2.9. Assay for XO activity

The XO activity with xanthine as the substrate was measured spectrophotometrically using the procedure of Marcocci *et al.* [20] with the following modifications described by Owen and Johns [21]. Briefly, the xanthine solution (0.15 mM) was prepared by boiling xanthine in distilled water. The XO solution was prepared by diluting XO to a final concentration of 0.2 U/mL in cold 50 mM potassium phosphate buffer (pH 7.5). The assay mixture consisted of 50 mM potassium phosphate buffer (pH 7.5, 535 μL), xanthine solution (330 μL) and 1% methanol or various concentrations of Th A. The reaction was initiated by adding 35 μL of the XO solution and the changes in absorbance recorded at 295 nm for 3 min at room temperature. Allopurinol was used as a standard inhibitor for XO.

2.10. Absorption spectra

Spectra of the complex of 50 μM Th A with 100 μM Fe^{2+} in 20 mM potassium phosphate buffer (pH 7.4) were determined at room temperature. The absorption spectra

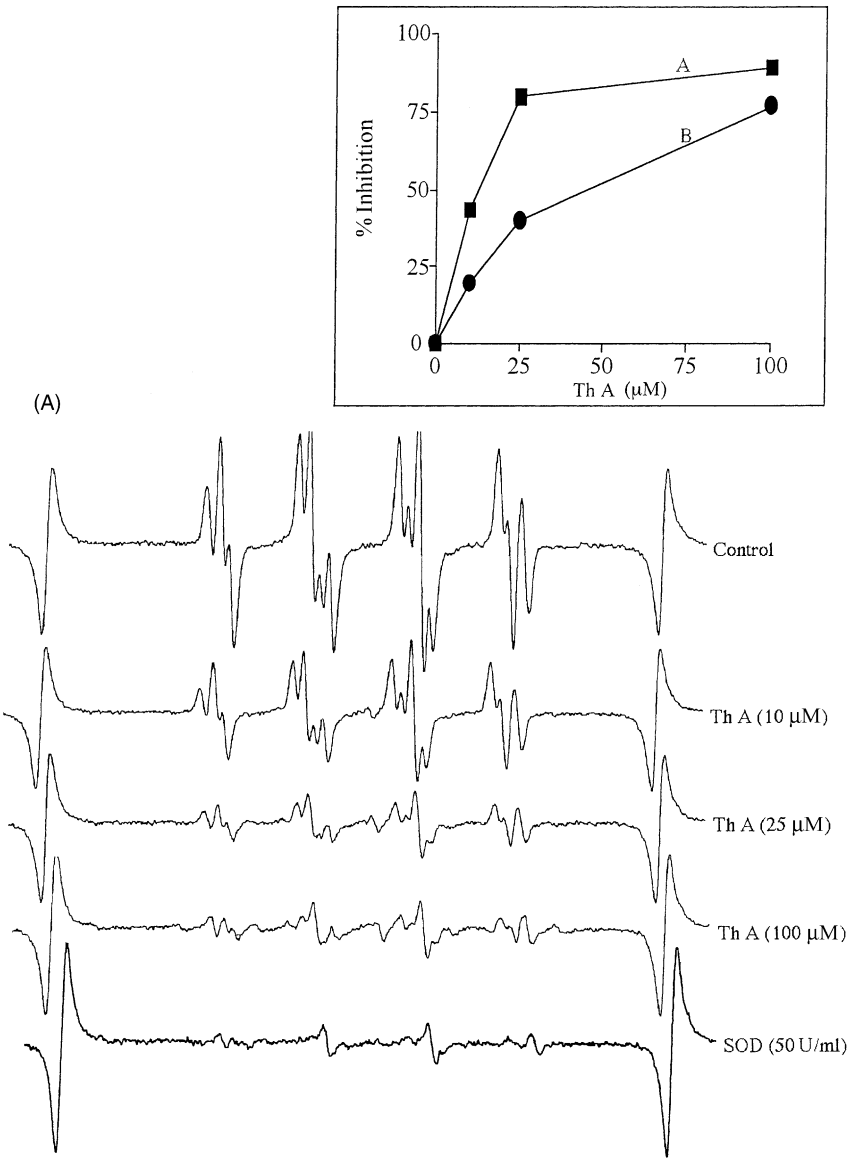

Fig. 3. Effect of Th A on ESR spectra of spin adducts of $O_2^{\bullet -}$ radical (A) with g = 2.006, $a^N = 1.32$ mT, $a^H_\beta = 1.16$ mT, $a^H_\gamma = 0.119$ mT and $RO_2^\bullet$ radical (B) having $a^N = 1.48$ mT, $a^H_\beta = 1.52$ mT observed with DMPO as spin-trap. Reaction and spectra recording conditions were as stated in the Section 2. Insert is the concentration-dependence of percentage inhibition of the generation of the $O_2^{\bullet -}$ radical signal by Th A (■, A) and the $RO_2^\bullet$ radical (●, B).

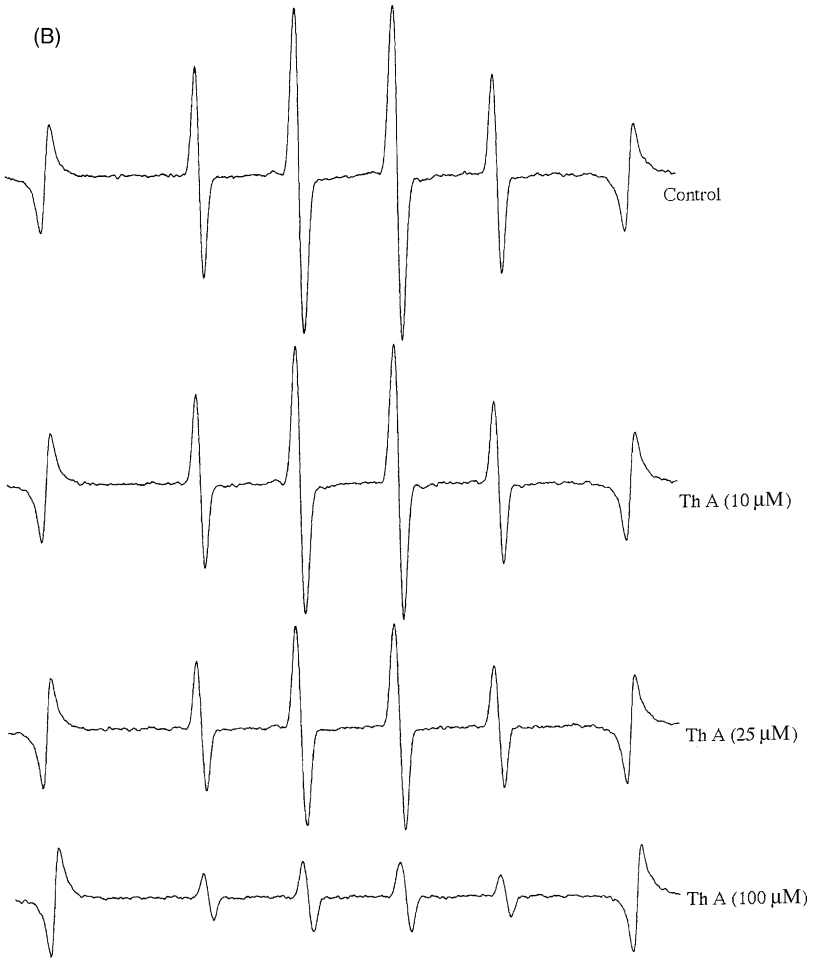

Fig. 3. (*Continued*).

(200–600 nm) was recorded using UV160A UV–VIS spectrometer.

2.11. Measurement of ESR spectra of semiquinone free radical of Th A

A solution containing 5 mM Th A in 0.5 M NaOH was transferred to a quartz cell for ESR measurement. Changes were recorded with time.

2.12. Stastistical analysis

Data were expressed as mean ± SD. Statistical analyses were performed using ANOVA. Dunnet's multiple range test was used to determine significant differences among means. The probability values of $P < 0.05$ were considered as significant.

3. Results

The scavenging effects of Th A on free radicals are shown in Figs. 2–4. DPPH is a stable free radical used extensively in ESR studies. We, therefore, investigated the effect of Th A on ESR signals formed by the DPPH radical (Fig. 2). Th A showed a dose-dependent decrease in the generation of the DPPH signal. This alteration was indicative of the potent scavenging action of the DPPH radical by Th A. The IC_{50} by Th A was 7.5 μM and completely scavenged with 25 μM as we previously reported [7].

Th A at 10 μM, scavenged 50% of the $O_2^{\bullet -}$ radical generated by the hypoxanthine–xanthine oxidase system (Fig. 3A). The $O_2^{\bullet -}$ radical was almost completely scavenged by 50 U/mL of SOD. Fig. 3B shows the effect of Th A on ESR signals formed by the $RO_2^{\bullet -}$. It was observed that Th A, at 30 μM concentration, scavenged 50% of the $RO_2^{\bullet -}$ radical, measured as DMPO spin adduct. The pattern of scavenging by Th A was dose-dependent for both the $O_2^{\bullet -}$ and $RO_2^{\bullet -}$ radicals (Figs. 3 and 4).

Figs. 4 and 5 show the ability of Th A or *T. sanguinea* extract to inhibit deoxyribose degradation in reaction mixtures with or without EDTA. The relative extents of inhibition of deoxyribose degradation will give an indication of $^{\bullet}OH$ scavenging and/or iron chelation action. Th A at 10 μM inhibited deoxyribose degradation by 15 and 77% in the presence and absence of EDTA, respectively, in a

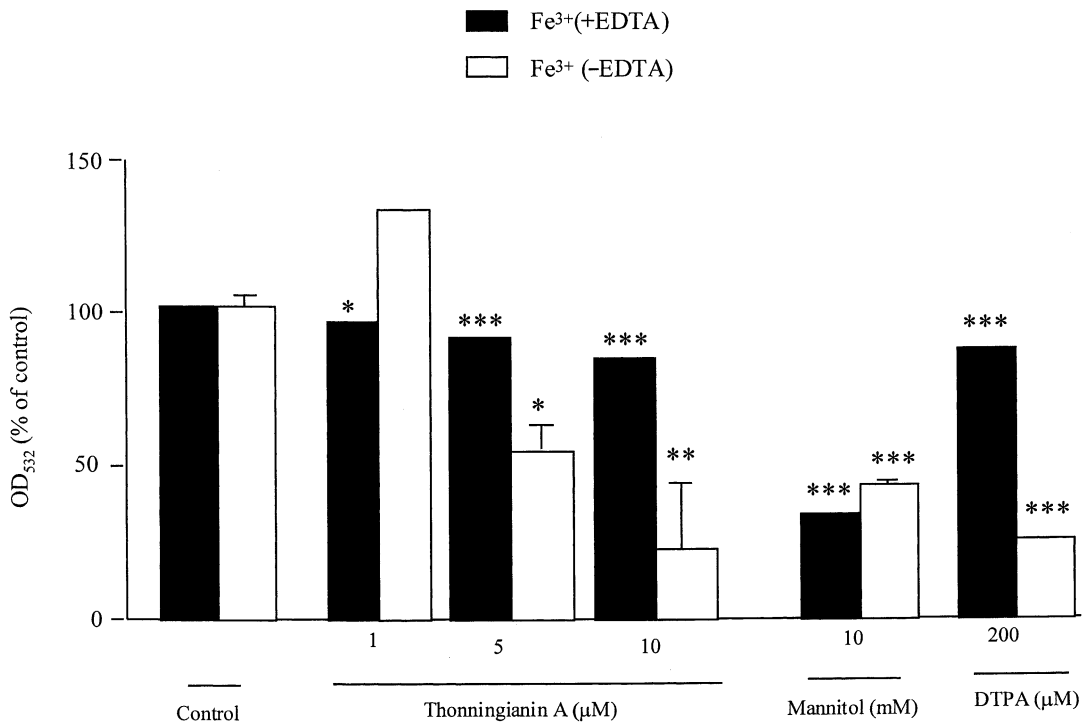

Fig. 4. Inhibition of deoxyribose degradation in the absence of EDTA (■) and presence of EDTA (□) by Th A, mannitol and DTPA. The reaction mixture in a final volume of 1 mL contained 20 mM KH_2PO_4–KOH (buffer pH 7.4), 2.8 mM deoxyribose, 100 μM $FeCl_3$, 104 μM EDTA (when added), 300 μM H_2O_2, or compounds tested and 100 μM ascorbic acid. Ascorbic acid was added to start the reaction. Reaction mixtures were incubated for 1 hr at 37°. At the end of the incubation period 1 mL of 1% TBA (w/v) in 0.05 M NaOH and 1 mL of 2.8% TCA (w/v) were added followed by heating for 20 min at 100°. The product of 2-deoxyribose degradation was measured at 532 nm. Values are means ± SD for triplicate determinations. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ vs. control.

reaction involving $FeCl_3$. This data suggest that Th A is a strong metal chelator and a moderate scavenger of •OH radicals (Fig. 4). Since Th A was first dissolved in methanol, a potent •OH radical scavenger and diluted with distilled water various control blanks were prepared using the content of methanol in the 1–10 μM Th A used for the assay. The absorbance produced by these blanks were each subtracted from the respective Th A absorbance to obtain

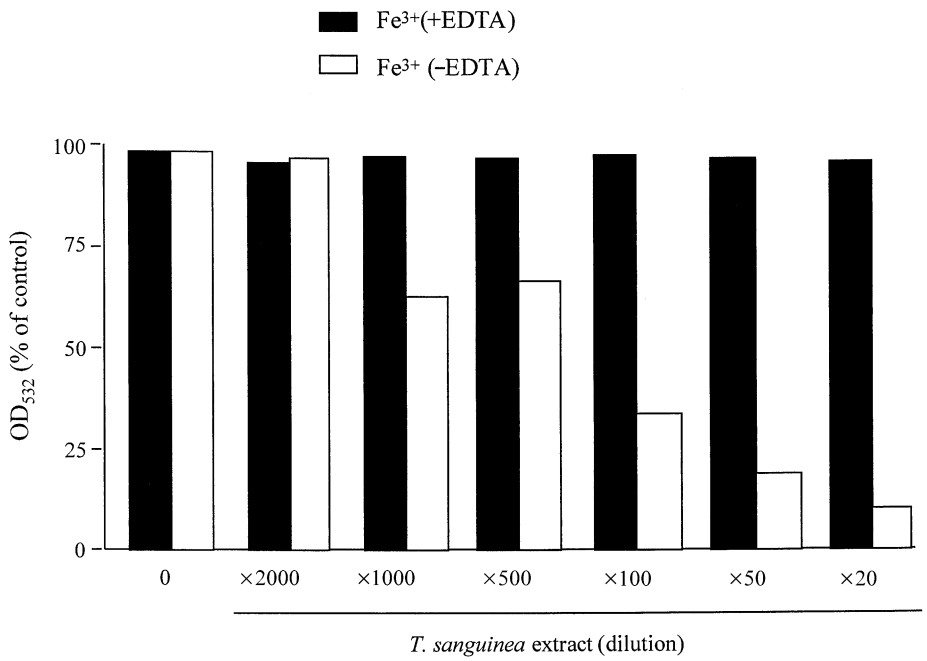

Fig. 5. Inhibition of deoxyribose degradation in the absence of EDTA (■) and presence of EDTA (□) by *T. sanguinea* extract. Experiments were conducted essentially as described in Section 2. Each point shows the mean of duplicate determinations.

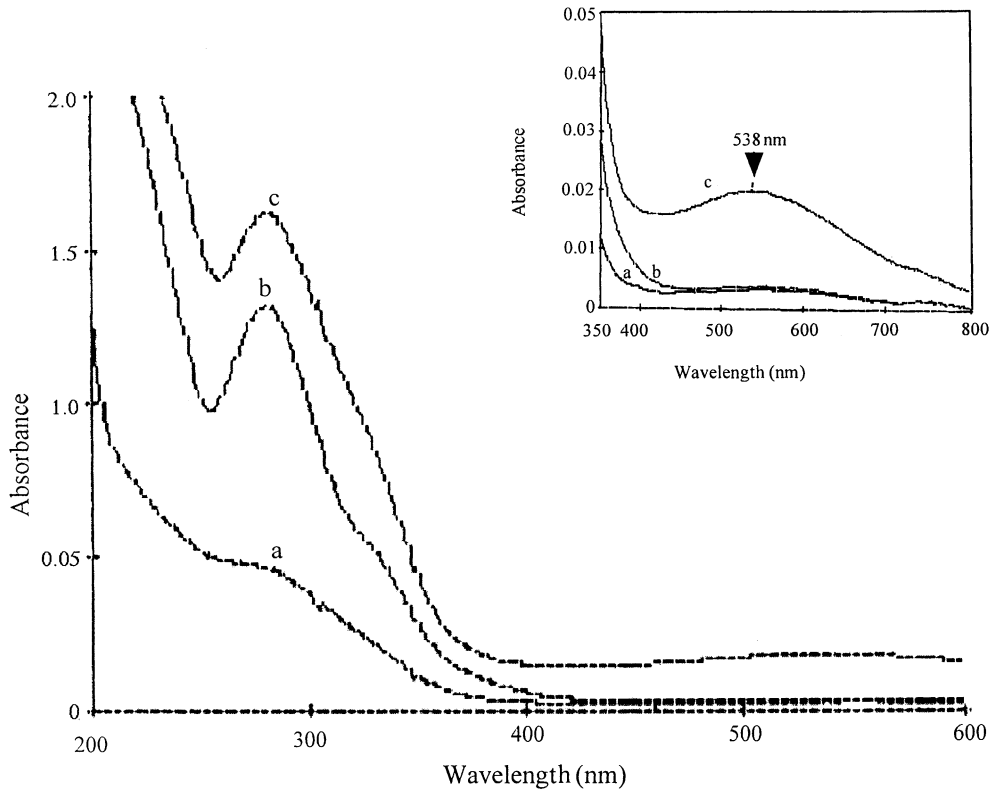

Fig. 6. Absorption spectra of Fe^{2+},Th A and Fe^{2+}–Th A complex in 20 mM potassium phosphate buffer (pH 7.4). Absorption spectra of (a) Fe^{2+}; (b) Th A and (c) Fe^{2+}–Th A complex. Insert is the spectra observed in the range from 350 to 800 nm.

net absorbance used in computation. Mannitol, a $^{\bullet}OH$ radical scavenger, at 10 mM inhibited deoxyribose degradation by 67 and 58% in the presence and absence of EDTA, respectively. While DTPA (200 μM), a chelating agent, inhibited deoxyribose degradation by 74% in the absence of EDTA, its inhibition was, however, 12% in the presence of EDTA. Similarly, the aqueous extract of *T. sanguinea* from which Th A was purified was not protective against deoxyribose degradation in the presence of EDTA, however, 1000 and 100 times diluted extract in the absence of EDTA showed 38 and 80% protection, respectively against deoxyribose degradation (Fig. 5).

Fig. 6 shows the absorption spectra of Th A and Fe^{2+}–Th A complex. Th A alone peaks at 281 nm while the Fe^{2+}–Th A complex peak at 282 and 538 nm.

The assay system we used to evaluate the scavenging action of Th A on the $O_2^{\bullet -}$ radical included XO. We, therefore, investigated the effect of Th A on generation of uric acid from xanthine to ascertain whether the potent scavenging observed for the $O_2^{\bullet -}$ radical might have resulted from inhibition of the XO enzyme. Our results revealed that Th A inhibited XO activity dose-dependently (Fig. 7). Th A at 10 μM inhibited XO activity by 18% with an IC_{50} of 30 μM. Allopurinol, a standard inhibitor of XO, showed 74% inhibition at 10 μM.

Th A was also tested for its inhibitory effects on both enzymatic and non-enzymatic LPO. In the NADPH-dependent LPO system, Th A at 10 μM inhibited TBARS formation by 61% compared to control (Fig. 8). Tannic acid (10 μM), gallic acid (10 μM), vitamin E (10 μM) and vitamin C (1 mM) inhibited the enzymatic LPO by 65, 7, 13 and 39%, respectively.

In the non-enzymatic LPO, stimulated by the $FeSO_4$–ascorbate, Th A inhibited TBARS formation dose-dependently. At a concentration of 10 μM, Th A inhibited TBARS formation by 65% (Fig. 9). Tannic acid (10 μM), gallic acid (10 μM), vitamin E (10 μM) and vitamin C (1 mM) inhibited TBARS formation by 84, 7, 12 and 5%, respectively.

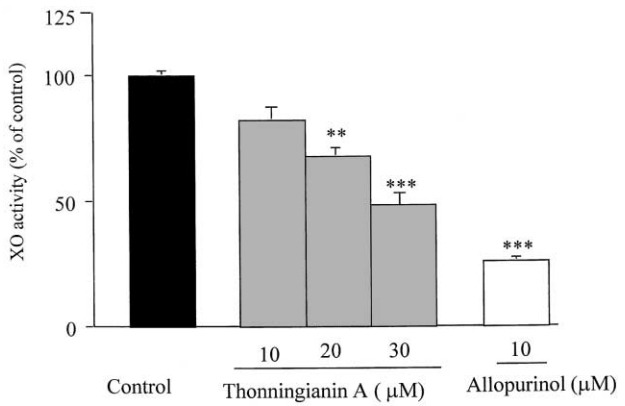

Fig. 7. Effect of Th A on XO activity. The XO activity was measured spectrophotometrically as described in Section 2. Values are means ± SD for triplicate determinations. $^{***}P < 0.001$, $^{**}P < 0.01$ vs. control (without Th A).

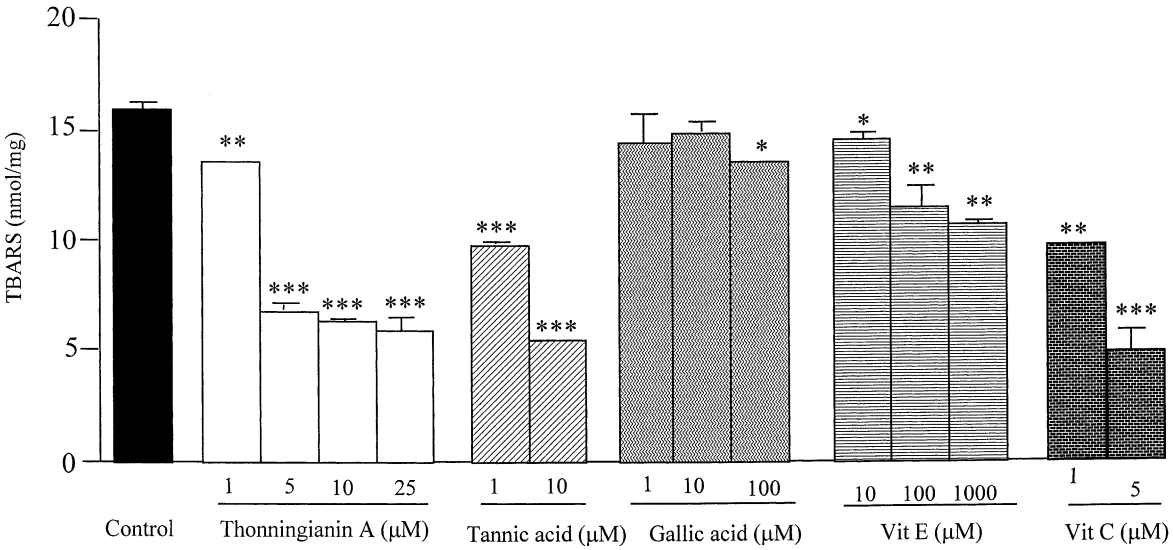

Fig. 8. Effect of Th A, tannic acid, gallic acid, vitamin C and vitamin E on enzymatic NADPH-dependent LPO. Microsomes (2.3 mg/mL) were incubated with the NADPH generating system and 1% methanol (control) or indicated concentrations of Th A, tannic acid, gallic acid, vitamin C and vitamin E in 100 mM potassium phosphate buffer (pH 7.4) at 37° for 30 min. The extent of LPO was assayed by estimating TBARS as described in Section 2. Values are means ± SD for triplicate determinations. $^{***}P < 0.001$, $^{*}P < 0.05$ vs. control.

We also studied the effect of Th A on CYP activity using aniline hydroxylase activity. Th A at 10 μM did not inhibit aniline hydroxylase activity, however, 25 and 100 μM inhibited the monooxygenase activity by 5 and 40%, respectively (Fig. 10). Tannic acid (10 μM) and gallic acid (100 μM) inhibited the monooxygenase activity by 74 and 23%, respectively.

The scavenging properties of polyphenolic compounds also depends on their ability to form stable radicals. These compounds in alkaline solution give rise to semiquinone free radicals stable enough to be detected by ESR spectroscopy. The ESR spectra of the Th A radical and its variation with time is shown in Fig. 11. The stability of the Th A radical decreased with time and was not detectable after 20 min.

4. Discussion

Involvement of ROS in LPO and many diseases are firmly established [22,23]. Substances termed antioxidants can influence the peroxidation process through either a simple or complex mechanisms including free radical scavenging, divalent metal chelation, etc. [10,24]. Tannins

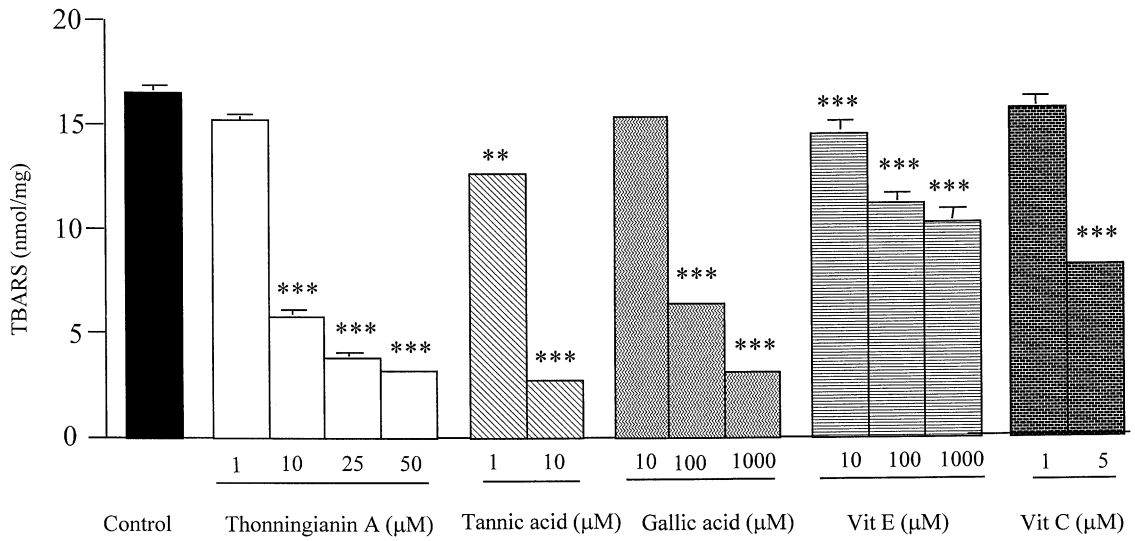

Fig. 9. Effect of Th A, tannic acid, gallic acid and vitamin E on non-enzymatic LPO induced by Fe^{2+}/ascorbate. Microsomes (2.2 mg/mL) were incubated with $FeSO_4$ (5 μM), ascorbate (500 μM) and 1% methanol (control) or indicated concentrations of Th A, tannic acid, gallic acid, vitamin C and vitamin E in 100 mM potassium phosphate buffer (pH 7.4) at 37° for 30 min. The extent of LPO was assayed by estimating TBARS as described in Section 2. Values are means ± SD for triplicate determinations. $^{***}P < 0.001$, $^{**}P < 0.01$ vs. control.

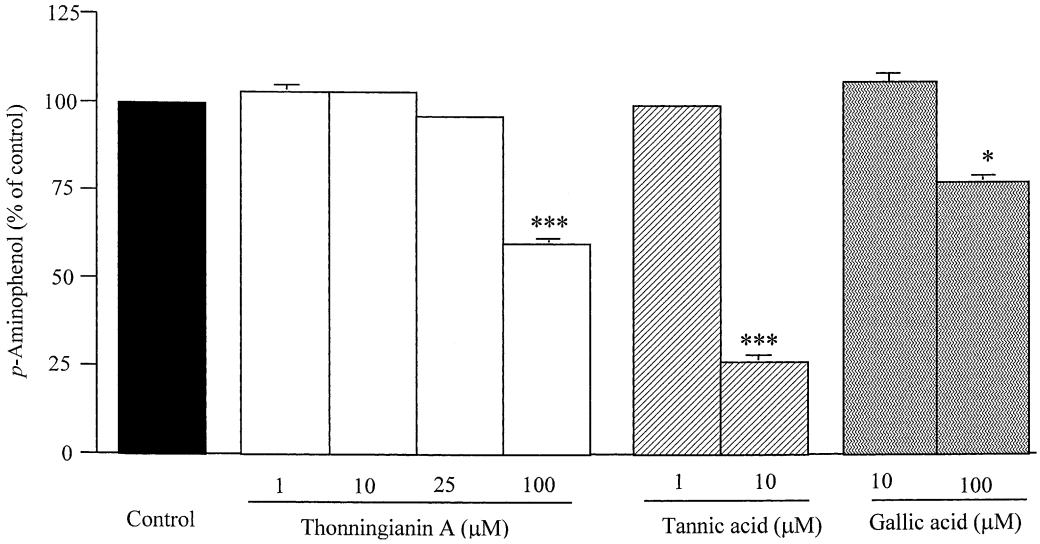

Fig. 10. Effect of Th A, tannic acid and gallic acid on aniline hydroxylase activity. Experiments were conducted essentially as described in Section 2. Values are means ± SD for triplicate determinations. $^{***}P < 0.001$, $^{*}P < 0.05$ vs. control.

are polyphenols widely present in plants with diverse biologic activity including inhibition of LPO.

In this study, using various *in vitro* assay systems, we examined the radical scavenging activity including inhibition of LPO by Th A, an ellagitannic compound (Fig. 1), which was recently isolated from the African medicinal herb, *T. sanguinea* [7]. We also compared the effect of Th A, a mixture of gallic acid esters of glucose and dihydrochalcone, on LPO with its structural component, gallic acid, a simple phenolic acid, tannic acid, a digallic acid

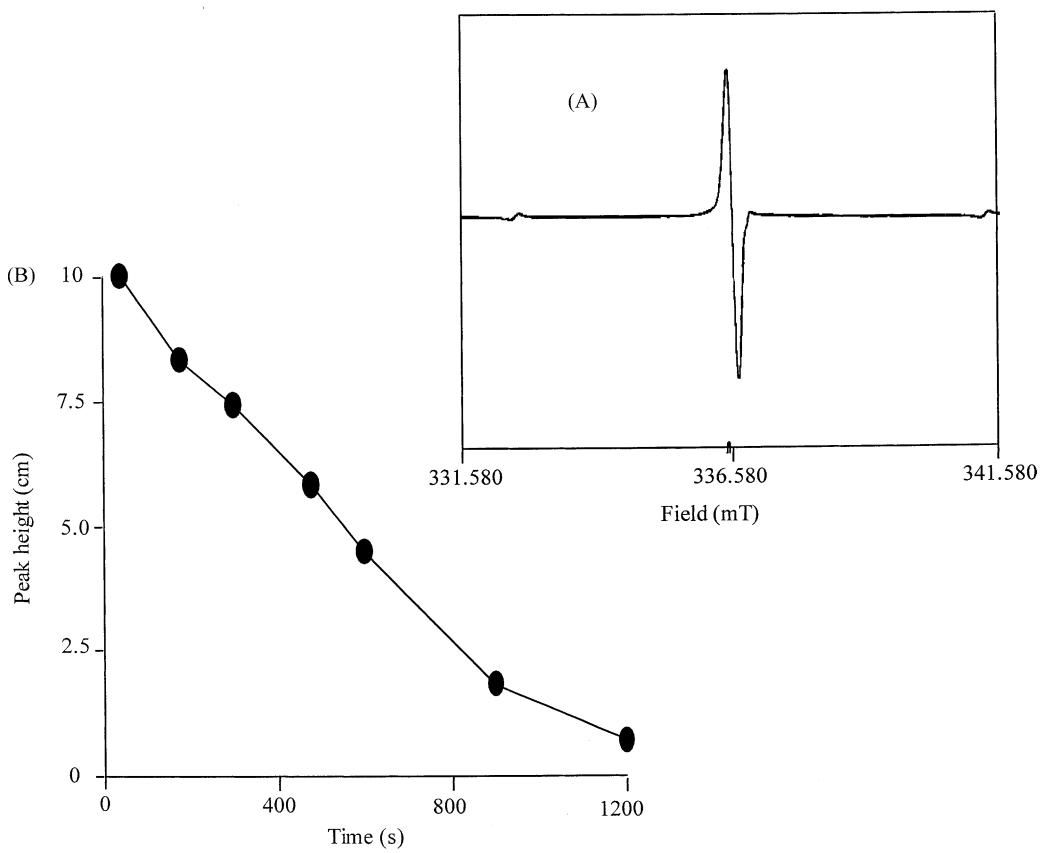

Fig. 11. ESR spectrum of the Th A radical (A) and variation of ESR spectrum of Th A radical with time in 0.5 M NaOH solution (B). The spectrum recording was started 40 s after taking sample from the reaction mixture under the following conditions: power 4 mW, magnetic field center 336.380 mT, sweep time 0.5 min, modulation amplitude 1 × 0.2 mT, amplitude 1 × 10 and time constant 0.1 s.

esters of glucose, and natural antioxidants such as vitamin E and vitamin C. The antioxidant activity of *T. sanguinea* extract has been previously reported [2,3].

Our results revealed that Th A dose-dependently scavenged DPPH, $O_2^{\bullet -}$, and $RO_2^{\bullet}$ radicals (Figs. 2 and 3). The $O_2^{\bullet -}$ and $RO_2^{\bullet}$ radicals are among the most important free radicals which are generated as harmful byproducts and have been implicated in LPO and human disease [25–28]. The $RO_2^{\bullet}$ radical which is generated in normal metabolic reactions by all aerobic organisms is also the source of the highly biologically reactive, $^{\bullet}OH$ radical which is capable of initiating LPO [29]. Besides, peroxylnitrite, a potent oxidizing and nitrating species, can be produced *in vivo* by the interaction of $O_2^{\bullet -}$ radical and nitric oxide [30]. Therefore, suppression of the $O_2^{\bullet -}$ radical as was seen in this study by Th A, either directly or indirectly, is probably one of the most effective defenses of a living organism against oxidative stress. Our data on the radical scavenging action of Th A suggested that the Th A, isolated from *T. sanguinea* might inhibit ROS-induced LPO. LPO, a prominent manifestation of ROS-induced cell injury was, therefore, measured to evaluate the protective value of Th A.

We used liver microsomes, a commonly used model procedure for evaluating LPO and NADPH (enzymatic) as well as Fe^{2+}/ascorbate (non-enzymatic), as inducers. Th A strongly inhibited both enzymatic NADPH-dependent LPO and non-enzymatic Fe^{2+}/ascorbate-dependent LPO stimulated in liver microsomes. It is known that certain substances by virtue of their phenolic compounds possess antioxidative properties [8,9]. These data confirmed that inhibition of LPO by Th A, a plant polyphenol was the result of its scavenging effect on NADPH and Fe^{2+}/ascorbate generated free radicals.

The NADPH-dependent LPO is catalyzed by the NADPH CYP reductase [31–34] and propagated by the CYP with the generation of intermediate free radicals, such as $O_2^{\bullet -}$ and $RO_2^{\bullet}$ radicals [32]. Since CYP is involved in the propagation step which accounts for about 90% [32] of peroxidation in the NADPH-dependent LPO, we studied the effect of Th A on CYP activity in uninduced rat liver microsomes. Our results showed that Th A at 10 μM did not inhibit CYP activity even though the same concentration inhibited NADPH-dependent LPO by 61% (Figs. 8 and 10). This suggested that the mechanism of inhibition of NADPH-dependent LPO by Th A could be ascribed solely to radical scavenging. It must be pointed out that we did not examine the inhibitory effect of Th A on the NADPH CYP reductase which is involved in the initiation of the NADPH–LPO in this study. Th A also inhibited Fe^{2+}/ascorbate LPO, a solely free radical-mediated process, to the same extent as the NADPH-dependent LPO. Furthermore, a scavenging action for DPPH, a stable radical seen in this study by Th A, demonstrates the direct radical scavenging activity by this ellagitannin.

It is also likely that the inhibition of the iron-dependent (Fe^{2+}/ascorbate) LPO may result from metal chelation, a characteristic feature of many tannic compounds [8,9]. To verify this, we examined the inhibitory action of the antioxidant on deoxyribose degradation which gives an indication of $^{\bullet}OH$ radical scavenging action and iron chelation ability [11]. Our results showed that while Th A at 10 μM slightly inhibited deoxyribose degradation in reaction mixtures containing EDTA, it showed potent inhibition in the absence of EDTA. In this assay without EDTA, the only substances that inhibit deoxyribose degradation are those that bind iron ions strongly enough to remove them from deoxyribose and forms complexes less reactive in generating $^{\bullet}OH$ radical. Our results suggest that Th A has the ability to bind iron ions with a moderate direct scavenging action for the $^{\bullet}OH$ radical in free solution. The mechanism of inhibition of both deoxyribose degradation and Fe^{2+}/ascorbate-dependent LPO we observed in this study may, therefore, involve formation of a complex between Th A and iron by the ''pull'' mechanism [35] leading to less generation of the $^{\bullet}OH$ radical because of unavailability of iron to react with H_2O_2 and hence inhibition of LPO. Spectroscopic studies revealed that Th A interacts with Fe (II) resulting in a blue–black complex with a new peak in the visible region (538 nm) (Fig. 6). It has been suggested that the gallic acid components of polyphenolic compounds are responsible for their ability to chelate iron ions [11,16]. It is suggested that the three galloyl components in Th A may be important for the iron chelating action of Th A.

Moreover, the slight inhibition of deoxyribose degradation we observed in the presence of EDTA by Th A (Fig. 4) is most likely due to iron chelation rather than $^{\bullet}OH$ radical scavenging since Th A (1–10 μM) might be competing with a high concentration of deoxyribose (2.8 mM) making $^{\bullet}OH$ radical scavenging unlikely. It is clear that Th A may offer protection against iron-catalyzed production of the $^{\bullet}OH$ radical which occurs in the presence of $O_2^{\bullet -}$ and H_2O_2 (metal-catalyzed Haber-Weiss reaction).

It has been suggested that oxidants such as $O_2^{\bullet -}$, H_2O_2, $^{\bullet}OH$ and hypochrorous acid are generated at the sites of inflammation [36–38] causing tissue injury and that most anti-inflammatory drugs has metal binding ability [38]. Since extracts of *T. sanguinea* has been reported to posses anti-inflammatory action [1,39], we examined its $^{\bullet}OH$ radical scavenging and iron-binding ability using the deoxyribose assay. Our results showed potent metal binding ability but poor $^{\bullet}OH$ radical scavenging ability by the crude extracts of *T. sanguinea* (Fig. 5). It is speculated that the anti-inflammatory activity of *T. sanguinea* reported earlier might involve metal binding properties in addition to the anti-histamic effect of the medicinal herb [1,39].

Polyphenols including tannins, are composed of one (or more) aromatic rings bearing one or more hydroxyl groups and capable of scavenging free radicals by forming resonance-stabilized phenoxyl radicals [40]. It is, therefore, reasonable to suggest that Th A, being polyphenolic with many hydroxyl groups (Fig. 1) is capable of donating

hydrogen atom in the initial stage of LPO to compete with polyunsaturated fatty acids and hence breaking the propagation chain. Indeed the chain-breaking activity of Th A was evident in the scavenging action for the $RO_2^{\bullet}$ radical and the inhibition of the NADPH-dependent LPO which involves generation of substantial amount of $RO_2^{\bullet}$ radicals. Besides, in the presence of NaOH and measured by ESR without spin-trapping agents, Th A generated a semiquinone radical which was stable for over 15 min (Fig. 11) suggesting that stability of the Th A radical contributes to its antioxidant activity.

XO is one of the major oxidative enzymes producing $O_2^{\bullet -}$ radical resulting in tissue injury and catalyzes the oxidation of hypoxanthine to xanthine and then uric acid in the presence of molecular oxygen to yield $O_2^{\bullet -}$ radical and H_2O_2 [41–43]. The accumulation of uric acid leads to hyperuricemia and gout [44,45]. XO-derived $O_2^{\bullet -}$ radical has also been linked to edema and post-ischemic tissue injury [46,47]. Inhibition of $O_2^{\bullet -}$ radical generation by the enzymatic pathway would be beneficial in these diseases including gout. The strong scavenging action of Th A for the $O_2^{\bullet -}$ radical (Fig. 3) we observed, might have resulted from direct inhibition of the XO enzyme which was used in our ESR study to generate the $O_2^{\bullet -}$ radical. We, therefore, examined the effect of Th A on the XO enzyme. Our results demonstrated inhibitory effects of Th A on the XO enzyme (Fig. 7) suggesting that besides direct scavenging, inhibition of the XO enzyme may contribute to the scavenging action of the $O_2^{\bullet -}$ radical by Th A.

It was also evidenced from the IC_{50} values obtained that the scavenging action of Th A was greatest for the DPPH radical (7.5 μM), followed by the $O_2^{\bullet -}$ radical (10 μM) and then the $RO_2^{\bullet}$ radical (30 μM). The differences in radical scavenging of Th A might reflect the fact that strength of radical scavenging effects of tannins and related polyphenols is influenced by the nature of the radical species, type of phenolic groups and their numbers in the molecule [48,49]. Among the three polyphenolic compounds studied here, it was evident that tannic acid with numerous phenolic groups or gallic acid molecules showed strongest inhibition of LPO followed by Th A and then gallic acid.

In summary, results presented here, reveal that Th A is an efficient antioxidant in biologic systems susceptible to free radical-mediated reactions. The activity of Th A was similar to the synthetic antioxidant, tannic acid but more potent than gallic acid, vitamin E and vitamin C in both the NADPH and Fe^{2+}/ascorbate-induced LPO. Although, additional studies are needed to characterize this property of Th A *in vivo*, its antioxidant property may be important for the inhibition of free radical-mediated diseases. Our results also show the metal chelating ability of *T. sanguinea* extract and this property has potential for treatment in iron-overload diseases. We conclude that Th A, isolated from the methanolic extract of *T. sanguinea*, exhibit anti-lipid peroxidation, anti-superoxide formation, metal chelating and radical scavenger activities.

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