Vol. 24 (1) 15-22 June 2012

Biokemistri

An International Journal of the Nigerian Society for Experimental Biology

Original Article

Polymerization of gallic acid enhances its antioxidant capacity: Implications for plant defence mechanisms

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Received: 25 April 2012

ABSTRACT: **Gallic acid (3, 4, 5-trihydroxybenzoic acid, GA) and its polymer, tannic acid (TA) are ubiquitous phytochemicals and are found to co-exist in plants. However, the rationale for the polymerisation of GA in plants is rather obscure. Hence, the present study compared the free radical scavenging ability, iron chelating potency, ferric reducing antioxidant power and inhibitory potential of both GA and TA against deoxyribose and lipid oxidative assaults *in vitro*. The results show that TA exhibited marked free radical scavenging ability as well as chelate Fe^{2+} and reduce Fe^{3+} more effectively than GA. Furthermore, this differential antioxidant capacity is also observed in the ability of both polyphenols to protect against hydroxyl radical induced deoxyribose degradation in the Fenton reaction. Similarly, in comparison with GA, TA also exerted marked inhibitory effect against mammalian cerebral and hepatic lipids assaulted with different prooxidants possessing diverse mechanisms of action. The present finding suggests that the polymerisation of GA to form a more efficient radical scavenger may have be associated with the need for increased antioxidant capacity required to cope with disturbed redox balance associated with pathological conditions in plants in which free radical induced oxidative stress may be implicated in their etiologies.**

KEYWORDS: *Gallic acid, tannic acid, antioxidant, oxidative stress, polymerisation*

INTRODUCTION

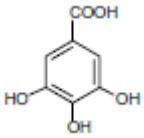
The plant kingdom abounds with vast arrays of daunting structurally related chemical compounds. It appears that for generic phytochemicals, a simpler chemical compound initially emerge in the family as a pharmacological necessity for the survival of these plants. Subsequently, other modified forms of the initial chemical compound are produced by these plants, as further defensive needs. A recent study involving the comparison of the antioxidant properties of two related plant chemicals, namely, quercetin and its rhamnosyl glucoside derivative, rutin revealed that while quercetin generally exhibited profound antioxidant effects than rutin, the latter exhibited marked iron chelating ability than the former [Omololu *et al*., 2011], suggesting that quercetin may have been synthesized first and that rutin possibly emerged as a consequence of a possible increase in the level of oxidative-linked transition metals such as iron in such highly-oxidizing environments.

Although plant chemicals exhibit a wide variety of biological activities, comparison of the antioxidant properties of these chemical compounds may be a laudable approach since reports have indicated that these chemicals are largely polyphenols which have hitherto been demonstrated to exhibit antioxidant properties.

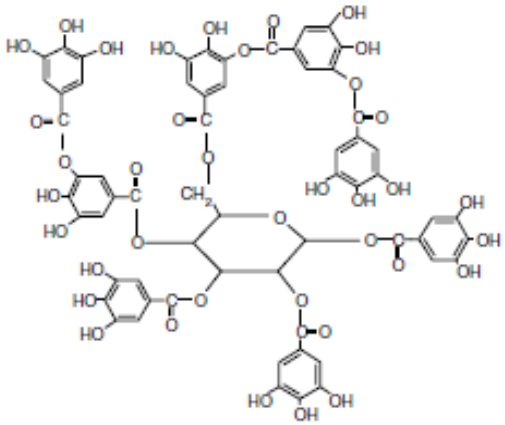
In fact, biochemical and pharmacological studies have shown that these polyphenols play important roles in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides [Anderson *et al*., 2001]. In addition, quite a number of human ailments have been linked to free radicals that induce oxidative assaults to biological macromolecules consequently leading to dysfunction in the physiology and biochemistry of these macromolecules, thus resulting in a disease state [Halliwell *et al*., 1999; Haddad, 2002]. Furthermore, epidemiological evidences have also shown that there is an inverse relationship between the consumption of polyphenols and free radical related diseases. Consequently, understanding the structure-activity relationship of plant phytochemicals could improve drug design to combat such oxidative-linked degenerative diseases in man.

Therefore, in the quest to further understand the order of emergence of these phytochemicals, the antioxidant properties of two ubiquitous and structurally related polyphenols: gallic acid

(GA, 3,4,5-trihydroxybenzoic acid) and its polymeric form tannic acid (TA) (Scheme 1) were compared in the present study.



Gallic acid



Tannic acid

SCHEME 1 **Chemical structure of gallic and tannic acids**

MATERIALS AND METHODS

Chemicals

Gallic acid (GA) and tannic acid (TA) (Scheme 1), thiobarbituric acid (TBA), 1,10 phenanthroline, 2-deoxyribose, and 2, 2–diphenyl -2 picrylhydrazyl (DPPH) were obtained from Sigma (St. Louis, MO). All other chemicals were of analytical grade and were obtained from standard commercial suppliers.

Animals

Male adult Wistar rats (200–250 g) from our own breeding colony were used. Animals were kept in separate animal cages, on a 12-h light: 12-h dark cycle, at a room temperature of 25-28 ^{0}C, and with free access to food and water. The animals were used according to standard guidelines of the Committee on Care and Use of Experimental Animal Resources.

Free radical scavenging ability

The free radical scavenging ability of the polyphenols against DPPH radicals was evaluated as described by [Gyamfi *et al*., 1999]. Briefly, 600 µl of the polyphenols (0-10 µM) were mixed with 600 µl, 0.4 mM methanolic solution containing DPPH radicals. The mixture was left in the dark for 30 min before measuring the absorbance at 516 nm.

Reducing property

The reducing property of both GA and TA were determined by assessing their ability to reduce $FeCl_3$ solution as described by [Pulido *et al*., 2000]. Briefly 250 µl of either GA or TA (0-10 µM) was mixed with 250 µl, 200 mM sodium phosphate buffer (pH 6.6) and 250 µl of 1 g/100 ml potassium ferrocyanide. The mixture was incubated at 50 ^{0}C for 20 min, thereafter 250 µl, 10 g/100 ml trichloroacetic acid was added and subsequently centrifuged at 650 rpm for 10 min. 250 µl of the supernatant was mixed with equal volume of water and 100 µl of 0.1 g/100 ml ferric chloride. The absorbance was later measured at 700 nm. A high absorbance indicates a higher reducing power.

Fe^{2+} Chelating assay

The Fe^{2+} chelating ability of both GA and TA were determined using a modified method of [Puntel *et al*., 2005]. Freshly prepared 500 µmol/l FeSO4 (150 µl) was added to a reaction mixture containing 168 µl of 0.1 mol/L Tris-HCl (pH 7.4), 218 µl saline and either GA and TA (0-10 µM). The reaction mixture was incubated for 5 min, before the addition of 13 µl of 0.25% 1, 10-phenanthroline (w/v). The absorbance was subsequently measured at 510 nm in a spectrophotometer. The Fe (II) chelating ability was subsequently calculated with respect to the reference (which contains all the reagents without the phenolic acids).

Deoxyribose degradation

Deoxyribose degradation was determined as described in literature [Halliwell *et al*., 1987]. Deoxyribose is degraded by hydroxyl radicals with the release of thiobarbituric acid (TBA) reactive substances. Deoxyribose (2 mM) was incubated at 37 ^{0}C for 30 min with 50 mM potassium phosphate (pH 7.4) plus ferrous sulphate (0.1 mM) and/or H_2O_2 (1 mM) to induce deoxyribose degradation, and either GA or TA at a concentration of 0-10 µM. After incubation, 0.4 ml of TBA 0.8% and 0.8 ml of TCA 2.8% were added, and the tubes were heated for 20 min at 100 ^{0}C and spectrophotometrically measured at 532 nm.

Tissue preparation for Thiobarbituric acid reactive species (TBARS) assay

Rats were decapitated under mild ether anesthesia and the whole brain and liver were rapidly removed, placed on ice and weighed. Tissues were immediately homogenized in cold 50 mM Tris–HCl, pH 7.4 (1/10, w/v). The homogenate was centrifuged for 10 min at 4,000 g to yield a pellet that was discarded and a low-speed supernatant (S1).

TBARS assay

An aliquot of 100 µl of S1 was incubated for 1 hour at 37 ^{0}C in the presence of either GA or TA (concentrations range of 0-10 µM; pH 7.4), with and without the prooxidants; iron (final concentration (10 µM) and sodium nitroprusside (SNP) (final concentration 3 µM) H_2O_2 (2 mM) and sodium oxalate (2 mM). One rat brain and liver were used per experiment. Productions of TBARS were determined as described by method of Ohkawa *et al*. (1979) excepting that the buffer of color reaction has a pH of 3.4. The color reaction was developed by adding 300 µl 8.1% SDS to S1,

followed by sequential addition of 500 µl acetic acid/HCl (pH 3.4) and 500 µl 0.8% of thiobarbituric acid (TBA). This mixture was incubated at 95 ^{0}C for 1 h. TBARS produced were measured at 532 nm.

Statistical analysis

All values obtained were expressed as mean ± SEM. The data were analyzed by appropriate ANOVA followed by Duncan's multiple range tests where appropriate and this is indicated in the text of results. The differences were considered significant when $p<0.05$.

RESULTS AND DISCUSSION

General Observation

It is noteworthy that the antioxidant properties of both GA and TA were also compared at different pH conditions and the pattern of results obtained is similar to what is presented in this report (data not shown). In addition, both polyphenols were heated at a constant temperature of 100 ^{0}C for 10 minutes and the various antioxidant assays were also evaluated on the heated polyphenols. Likewise, the pattern of results obtained for all antioxidant assays carried out on the heated polyphenols is equally similar to the results presented in this report (data not shown). The possible differential antioxidant properties of both TA and GA were evaluated under these two conditions (pH and temperature) in order to determine if any of these conditions possibly elicited the polymerization of GA consequently producing TA in physiologically challenging conditions.

Free radical scavenging properties of polyphenols

Figure 1 depicts the free radical scavenging properties of both polyphenols. Individual one-way ANOVA analysis revealed that both GA and TA scavenged free radicals in a concentration dependent manner. Equally, two-way comparison of the free radical scavenging ability of both polyphenols revealed that TA exerted a marked free radical scavenging properties than GA ($p < 0.05$).

Fe^{3+} reducing properties of GA and TA

A comparison of Fe^{3+} reducing properties of TA and GA is presented in Figure 2. One-way ANOVA revealed that both polyphenols exhibited marked Fe^{3+} reduction in a concentration dependent manner with TA exerting marked reductive effect than GA in all the concentration tested ($p < 0.05$). In fact, two-way ANOVA (2 polyphenols types X 5 (concentrations)) show that the reducing ability of TA is more than that of its monomeric form, GA and this difference is significant at all concentration range tested ($p < 0.05$).

Comparison of Fe^{2+} chelating ability of polyphenols

Figure 3 shows the Fe^{2+}-chelating properties of both TA and GA. It is noteworthy that one-way ANOVA revealed that GA exhibited Fe^{2+} chelating ability. However, two-way ANOVA (TA/GA X 5 (concentrations)) showed that the polymerization of GA conferred marked ability on its chelating ability of the transition metal ($p < 0.05$).

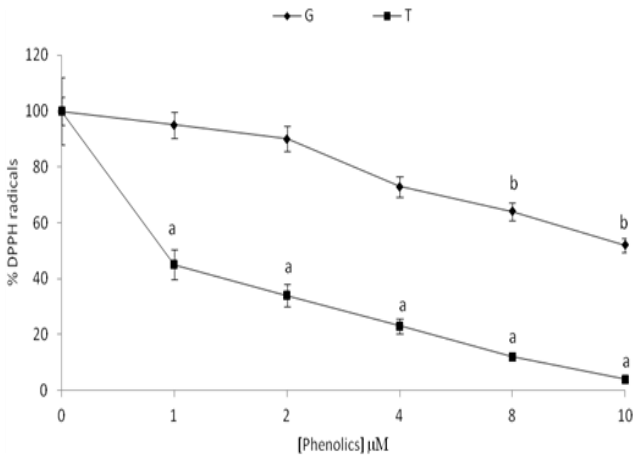


FIGURE 1 Free radical scavenging activities of GA and TA. Data represent mean±SEM values averages from 3 to 4 independent experiments performed in triplicate and were tested by two way ANOVA followed by the Duncan's test. [a,b]Indicates a significant difference from control at $p < 0.05$.

Effect of both polyphenols on deoxyribose degradation

Figure 4 depicts the oxidative degradation of 2 mM 2-deoxyribose induced by Fenton reagents in phosphate buffer (pH 7.4). Separate statistical analysis were carried out on the results obtained when $^{\bullet}$OH assault deoxyribose sugar in the presence or absence of both TA and GA. In this regard, One-way ANOVA [and two-way ANOVA (2 TA/GA X 5 concentrations)], show that both polyphenols do not degrade deoxyribose sugar (data not shown). However, in the presence of prooxidant, three-way ANOVA [(2 polyphenols types X with or without prooxidants X 5 concentrations) show that TA exerted profound inhibitory effect on the degradation of deoxyribose sugar at all concentrations tested.

Effect of polyphenols on inhibiton of induced lipid peroxidation

Figure 5 show the inhibitory effect of TA and GA on lipid peroxidation induced with various prooxidants. Separate one-way ANOVA indicate that both GA and TA exerted considerable inhibitory effect on the oxidation of cerebral and hepatic lipids. However, Three-way ANOVA (2 (TA/GA) X 5 concentrations X 2 (basal/prooxidant) clearly show that TA protected all lipid types against all prooxidants assaults employed in the present study in a fashion markedly different from the pattern observed with GA ($p < 0.05$).

DISCUSSION

The rationale for the observed diverse arrays of chemical compounds in the plant kingdom is far from being completely understood. Strikingly, plant chemicals are organized in a family-like arrangement with a common ancestral parental chemical compound [Omololu *et al.*, 2011]. Hence, it is rational to speculate that the emergence of a phytochemical is as a result of pharmacological necessity. Consequently, our understanding of the

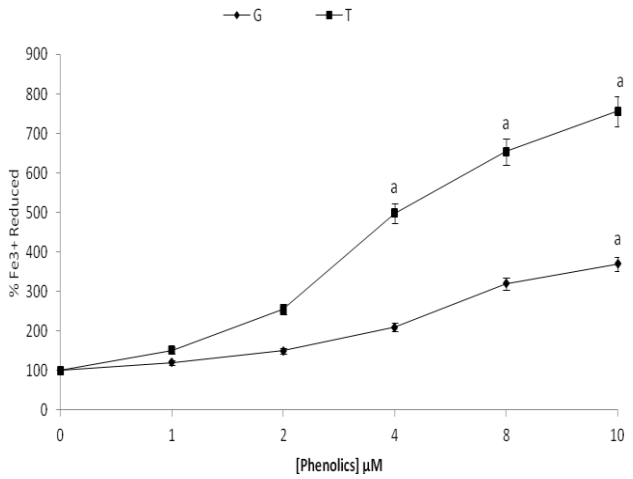


FIGURE 2 Reducing properties of both polyphenols; [a,b]Indicates a significant difference from control at p ‹ 0.05.

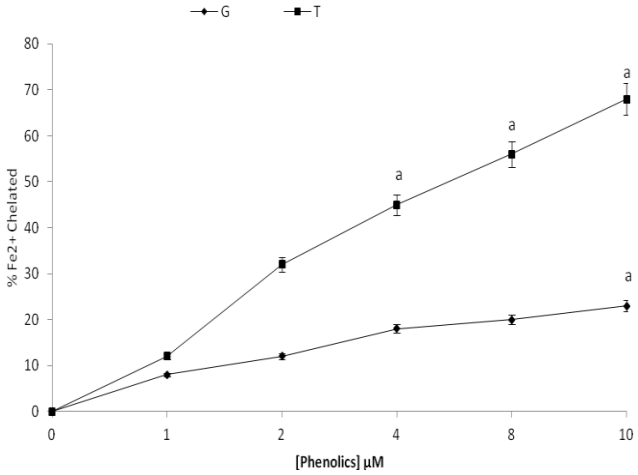


FIGURE 3 Iron (II) chelating ability of GA and TA; [a,b]indicates a significant difference from control p ‹ 0.05

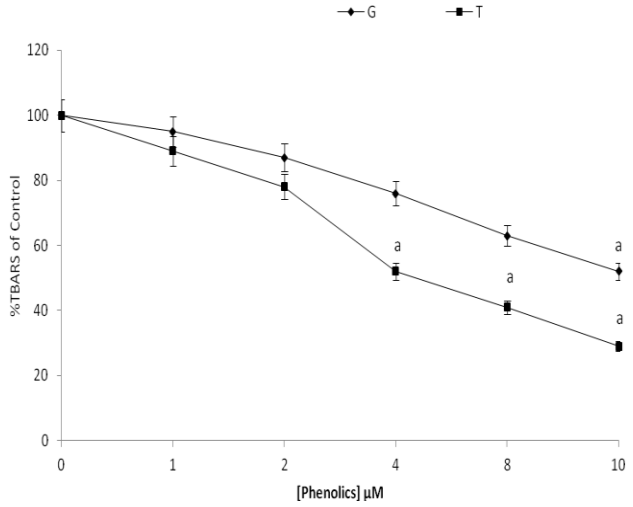


FIGURE 4a Effect of different concentrations of both polyphenols on Fe^{2+}-induced deoxyribose degradation. The deoxyribose was incubated for 20 min with Fe2+ in the presence or absence of both polyphenols. Letter indicates significant difference from control p ‹ 0.05.

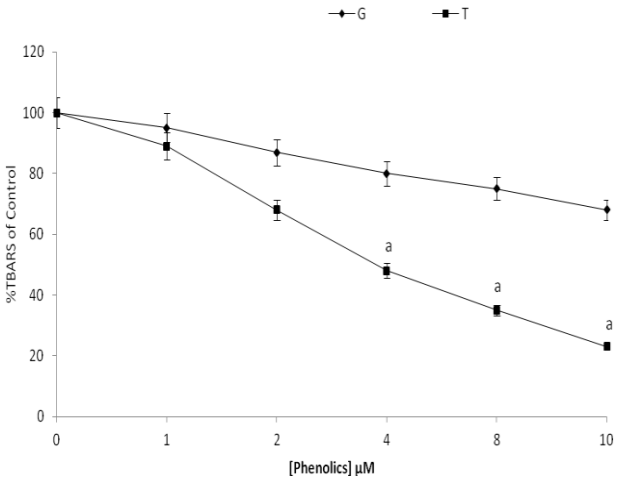


FIGURE 4b Effect of different concentrations of GA and TA on H_2O_2-induced deoxyribose degradation. Deoxyribose was incubated for 20 min with H_2O_2 in the presence or absence of both GA and TA. Letters indicate significant difference from control (p ‹ 0.05).

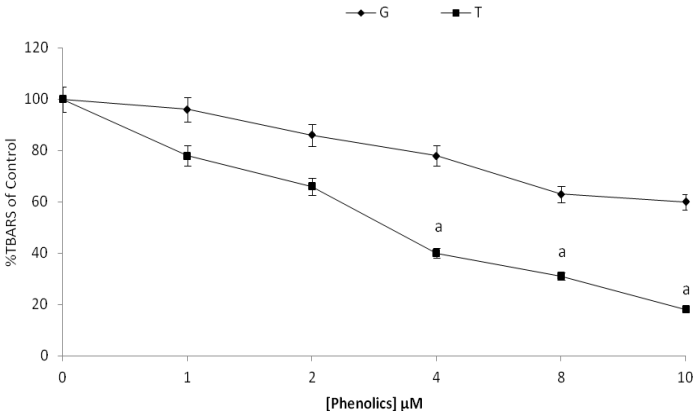


FIGURE 4c Effect of different Ga and TA on Fe2+/H2O2-induced deoxyribose degradation. The deoxyribose was incubated for 20 min with Fe2+/H2O2 in the presence or absence of both polyphenols. Letter indicates significant difference from control at p ‹ 0.05.

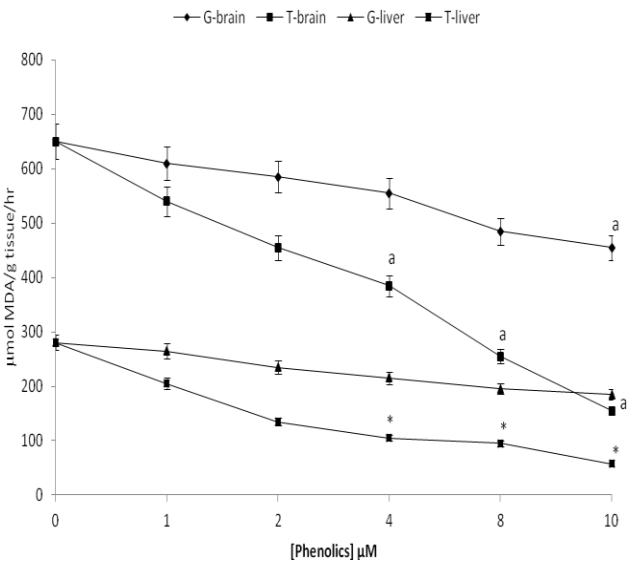


FIGURE 5a Effect of different concentrations of polyphenols on iron (II)-induced TBARS production in brain and liver homogenates. Data show mean±SEM values average from 4 independent experiments performed in quadruplicate on different days. Letters and symbols indicate significant difference from the control.

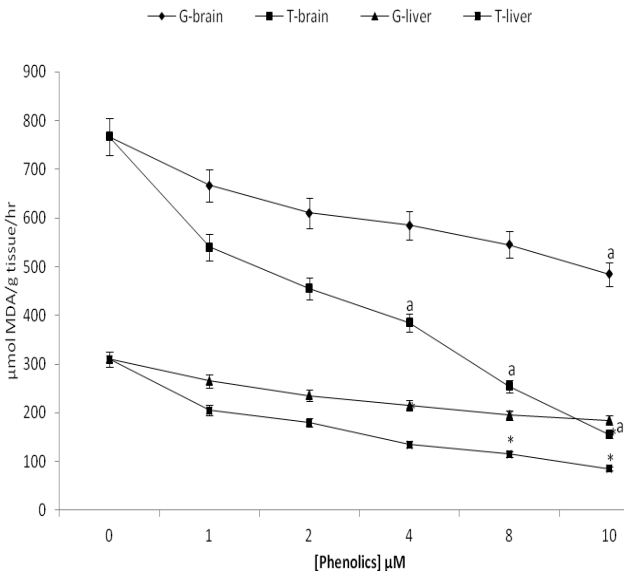


FIGURE 5b Effect of different concentrations of polyphenols on sodium nitroprusside (3 µM) – induced TBARS production in liver and brain homogenates. Data shows mean±SEM values average from 4 independent experiments performed in quadruplicate in different days. Letter and symbol indicates significant difference from the control.

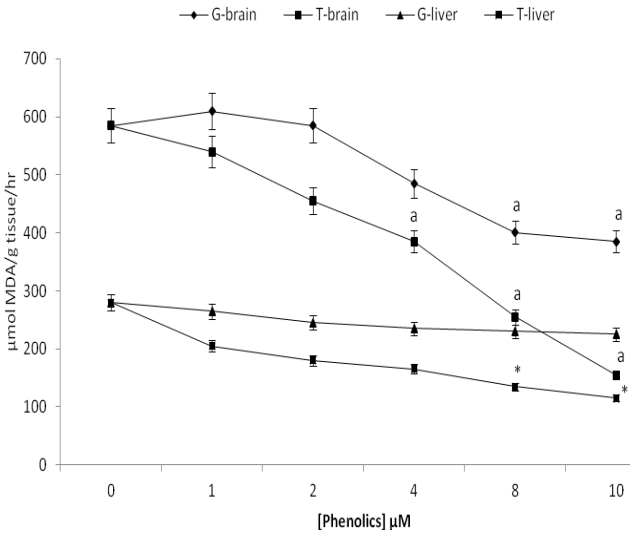


FIGURE 5c Effect of different concentrations of polyphenols on hydrogen peroxide–induced TBARS production in liver and brain homogenates. Data shows mean±SEM values average from 4 independent experiments performed in quadruplicate in different days. Letter and symbol indicate significant difference from the control.

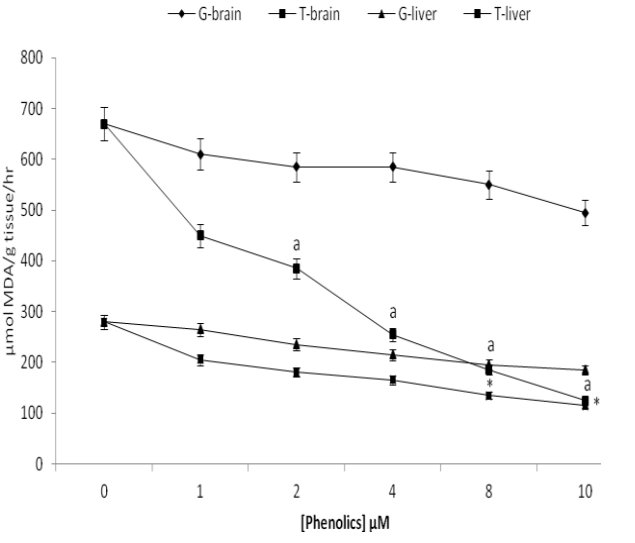


FIGURE 5d Effect of different concentrations of polyphenols sodium oxalate–induced TBARS production in liver and brain homogenates. Data shows mean±SEM values average from 4 independent experiments performed in quadruplicate in different days. Letter and symbol indicate significant difference from the control.

sequential emergence of these chemical structures may provide a novel approach in the design of drugs to combat human ailments.

It is apparent in Figure 1 that TA exerted marked free radical scavenging ability than GA. Although the mechanisms involved in their antioxidant activities are not completely understood, several postulations can help in the explanation of this observed effect. Firstly, reports have shown that the nature of polyhydroxyls groups of these polyphenols can be intrinsically related to their antioxidant action [Letan, 1966; Morel *et al*., 1993]. Consequently, it appears that the substitution of polyhydroxyl groups on benzene rings and subsequent polymerisation of such structural compound elicited an increased free radical scavenging capacity in TA (Figure 1). Secondly, from chemical point of view, the free radical scavenging activity of antioxidants is also related to the dissociation energy of the phenolic O–H bond and ionization potential of the antioxidant [Van *et al*., 1993; Zhang, 1998; Cheng *et al*., 2003; Lien *et al*., 1999; Bakalbasis *et al*., 2001; Soffers *et al*., 2001; Wright *et al*., 2001; Leopoldini *et al*., 2004]. Hence we could speculate that polymerization of GA induces such conformational change and spatial rearrangements of the hydroxyl group thus rendering TA with high free radical scavenging capacity.

The prominent role of free chelatable iron in initiating reactive oxygen species (ROS) dependent oxidative stress and its accumulation in progressive degenerative diseases has been well discussed in the literature [Warshawsky *et al*., 2000]. Generally as a rule, transition metals such as iron tend to amplify oxidant damage especially in organs with very active mitochondria. In fact, there is little evidence of iron per se exerting toxic effects in the absence of agents that affect the active oxidation and reduction of the metal. Thus, "antioxidants" or chelators which keep iron in one valence state will effectively block iron-mediated oxidant damage [Graf *et al*., 1987; Graf *et al*., 1984]. In this regard, antioxidant agents are also tested for their ability to act as iron chelators. These chelators would have two advantages; firstly they would prevent iron-hydrogen peroxide initiated generation of reactive OH. (Fenton chemistry), secondly they might mobilize free chelatable iron from the tissues and thus become protective. Figure 3 shows that TA exhibits marked iron chelating ability than GA. The increased iron chelating ability of TA over GA may stem from the polymerization of the benzyl ring and also the incorporation of a sugar moiety to the polymer. This assumption is consistent with our recent report where we observed that the addition of a rhamnosyl glucoside moiety confers potent iron chelating ability on quercetin [Omololu *et al*., 2011].

In a related mechanism, potent antioxidant agents that help to reduce or keep a transition metal that can potentially amplify oxidative damage in one valence state can be considered as an antioxidant mechanism. Generally as a rule, reducing power is related to the ability of antioxidant agents to transfer electron or hydrogen atom to oxidants or free radicals. In the present study, the reducing power was evaluated based on the ability of both polyphenols to reduce Fe^{3+} to Fe^{2+} (Figure 2), with the results showing that TA exerts more profound reducing property than GA.

In order to establish whether GA and TA will exhibit similar differential antioxidant capacity in their protection of oxidative-linked macromolecular damage in mammalian tissues, both polyphenols were used to protect against oxidant-induced deoxyribose degradation and lipid peroxidation. It has been established that oxidant induced damage to deoxyribose sugar is greatly enhanced by iron [Jackson *et al*., 1987; Schraufstatter *et al*., 1988] and the damage to deoxyribose caused by iron is decreased or absolutely prevented by effective iron chelators which fill all six coordination positions and make the iron chemically unreactive [Graf *et al*., 1984]. Result presented in Figure 4 shows that while both polyphenols markedly inhibited deoxyribose degradation, TA showed a marked effect in comparison with GA. The mechanism for the observed inhibitory effect of TA on deoxyribose degradation is not clearly understood, however Lopes, [Lopes *et al*., 1999] reported that TA did not block 2-deoxyribose degradation by simply trapping the OH^{*} radical; rather, TA appears to act as an antioxidant by forming complexes with Fe (II) [Fe (II)n-tannic acid] that are unable to participate in Fenton reactions. [Grinberg *et al*., 1997] also suggested that the protective activity of TA against OH^{-} dependent degradation of deoxyribose may be as a result of iron chelation.

Similarly, evidences have shown that cell damage and death is associated with a combination of iron load and or exogenous oxidant (such as H_2O_2), peroxidation of polyunsaturated fatty acids (PUFA) within membrane phospholipids is possibly a crucial event. In fact, short-term experiments have demonstrated that antioxidants that prevent PUFA oxidation will also block cell death [Balla *et al*., 1990a; Balla *et al*., 1990b; Balla *et al*., 1991]. Consequently, survival of primordial plants may necessarily require the protection of the cell membrane lipids and other critical lipids. The results obtained in our present study show that under prooxidants-induced oxidative assaults, both GA and TA exert potent inhibitory effect on peroxidation of lipids in both the liver and brain tissues. However, in all concentrations tested TA exerted profound inhibitory effect on lipid peroxidation in comparison to GA. It is noteworthy that the possible mechanisms employed by SNP to oxidize lipids have been well discussed in previous reports [Kade *et al*., 2008; Kade *et al*., 2009]. Hence, we can conclude that in addition to chelating and reducing iron, both phenolic acids could also scavenge other free radicals as a component of their antioxidant mechanisms and in the case of SNP, TA effectively scavenged the $NO^{\bullet}$ generated.

However, since experimental data have shown that both GA and TA and their derivatives have antimicrobial activities [Fiuza *et al*., 2004; Klein and Weber, 2001], it is not clear whether TA is synthesized to combat increased microbial assaults in plants. From the foregoing, however, it appears that the rationale for the polymerization of GA is largely for pharmacological reasons and from the present data; the need for increased antioxidant defense may be a plausible speculations. In any case, we could deduce from the present results that even if microbial attack on plants is the foremost basis for the production of TA, however, such microbial attack would possibly involve disturbed redox balance which

would necessitate the need to increase the antioxidant capacity of GA. This possibility is of further research interest.

Acknowledgements *IJK and JBTR acknowledge the financial support by CNPq-PROAFRICA. JBTR sincerely thank UFSM, CAPES, CNPq and FAPERGS FINEP, "Rede Instituto Brasileiro de Neurociência (IBN-Net)" # 01.06.0842-00, INCT for Excitotoxicity and Neuroprotection-CNPq and FAPERGS/CNPq (PRONEX) # 10/0005-1 for their financial support..*

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