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Antioxidant and pro-oxidant properties of ascorbic acid and gallic acid

Gow-Chin Yen[a,*], Pin-Der Duh[b], Hui-Ling Tsai[a]

[a]Department of Food Science, National Chung Hsing University, 250 Kuokuang Road, Taichung 40227, Taiwan
[b]Department of Food Health, Chia Nan University of Pharmacy and Science, 60 Erh-jen Road, Section 1, Pao-An, Jen-te Hsiang, Tainan Hsien, Taiwan

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Abstract

The antioxidant and pro-oxidant properties of ascorbic acid (AA) and gallic acid (GA) were investigated. AA and GA, at a concentration of 1.65 mM, accelerate the oxidation of deoxyribose induced by Fe^{3+}–EDTAJH_2O_2. The reducing power of these two compounds increased upon increasing the concentration. AA and GA showed no chelating ability toward iron (II). At a concentration of 4.17 mM, AA and GA exhibited 42.1 and 43.9% scavenging effects on DPPH radicals, respectively. They exhibited 60% scavenging effects on hydrogen peroxide at a concentration of 4.17 mM. No toxicity was found in AA and GA toward human lymphocytes. AA, at 0.82 mM, and GA, at 0.6 mM, exhibited the maximal DNA damage, the means of tail DNA% were 14.8 and 28.8%, respectively. When AA and GA were mixed with H_2O_2, they exhibited a slight inhibitory effect on DNA damage induced by H_2O_2 on pre-incubating both the compounds with human lymphocytes for 30 min before exposure to H_2O_2. The antioxidant activities of AA and GA at a higher concentration were mainly due to the scavenging of hydrogen peroxide in this system. The pro-oxidant mechanism for AA and GA acid is most likely due to the strong reducing power and weak metalchelating ability.

Keywords: Ascorbic acid; Gallic acid; Deoxyribose; Comet assay; DNA damage; H_2O_2

1. Introduction

Reactive oxygen species are thought to be involved in cancer and other diseases. This species may not only have an impact on DNA damage but also influence DNA messengers to modulate DNA replication and cell cycle (Hu, Kurland, Ma, & Roush, 1995).

The oxygen consumption inherent in cell growth leads to the generation of a series of free radicals of oxygen, which are the most abundant and characteristic species in the phenomenon known as "oxidative stress". The "oxidative stress" increases formation of superoxide radical and hydrogen peroxide, which can directly promote cellular damage. These oxygen-related products can also interact in the presence of suitable transition metal catalysts to form highly toxic hydroxyl radicals and other oxidizing species. As a result, NADH, GSH and ATP are depleted, whereas calcium ion is increased, inducing cell damage and causing diseases such as atherosclerosis, cancer, and ischaemia (Smith, Halliwell, & Aruoma, 1992). Oxidative DNA damage is a good marker of cancer (Aces & Gold, 1991; Floyd, 1990). There is a considerable amount of epidemiological evidence revealing natural antioxidants, including ascorbic acid (AA), polyphenol, and β-carotene, which may function as free radical interrupters. AA, a well-known antioxidant, has been suggested to act synergistically with tocopherol to regenerate the tocopheryl radicals. AA may scavenge peroxyl radical and inhibit cytotoxicity induced by oxidants. In addition, AA can reduce or prevent H_2O_2-induced lipid peroxidation and the formation of OH-deoxyguanosine (Retsky & Frei, 1995; Tsou, Chen, Liu, & Yang, 1996). However, some authors have raised questions on the potential side effects of the intake of large doses of AA. AA can cause strand breakage in DNA in the presence of oxygen and can initiate cell death in tissue culture, possibly through the generation of H_2O_2. Reactions of AA with metals

* Corresponding author. Tel.: +886-4-2287-9755; fax: +886-4-2285-4378.
E-mail address: gcyen@mail.nchu.edu.tw (G.-C. Yen).

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such as Cu^{2+} are thought to lead to the production of H_2O_2 (Zhao & Jung, 1995).

Plant polyphenols are well known to show biological activity, such as antimutagenicity, anticarcinogenicity and antioxidative activity. Gallic acid (GA), a naturally occurring plant phenol, was also found to be a strong antioxidant in emulsion or lipid systems (Madsen & Bertelsen, 1995; Nakatani, 1992) and exhibits antimutagenicity. GA is used in processed food, cosmetics and food packing materials to prevent rancidity induced by lipid peroxidation and spoilage. It is almost as effective as the tocopherol analogue Trolox and even more effective than several water-soluble antioxidants, such as AA (Cholbi, Pays, & Alcaraz, 1991). Recently, GA was described as having a cytotoxic effect on isolated hepatocytes (Nakagawa & Tayama, 1995). Sakagami and Satoh (1997) showed AA and GA acting as pro-oxidants for the induction of apoptotic cell death in human glioblastoma cells. Inoue et al. (1995) demonstrated that gallic acid could cause cell death in HL-60. Thus, this study evaluated pro-oxidant and antioxidant properties of gallic and ascorbic acid by using deoxyribose assay, scavenging of free radicals and chelation of ferrous ions; and effects on oxidative DNA damage to human lymphocytes using single cell gel electrophoresis.

2. Materials and methods

2.1. Materials

Gallic acid, ascorbic acid, ferrozine, ferric chloride hexahydrate, trypan blue, triton X-lOO, ethidium bromide (EtBr) and N-lauroyl sarsosinatei were purchased from Sigma Co. Ltd. (St Louis, MO, USA). Iron (II) chloride tetrahydrate, thiobarbituric acid (TBA), trichloroacetic acid (TCA), and potassium ferricyanide were obtained from Merck Co. Ltd. (Darmstadt, Germany). Hydrogen peroxide (H_2O_2) was obtained from Wako Co. Ltd. (Japan). Histopaque 1077 was obtained from Pharmacia Biotech (Uppsala, Sweden). Dutch modified RPMI 1640 medium, Ultrapure low melting point agarose and normal melting point agarose (both electrophoresis) were obtained from Gibco Ltd. (Grand Island, NY, USA). Frosted microscope slides was obtained from Richardson Co. Ltd. (London, UK). Blood samples were obtained from healthy volunteers.

2.2. Deoxyribose assay

The assay was carried out as described by Smith et al. (1992). The reaction mixtures contained, in a final volume of 3.5 ml, the following reagents at the final concentrations stated: deoxyribose (3 mM), hydrogen peroxide (3 mM), KH_2PO_4–KOH buffer pH 7.4 (20 mM), $FeCl_3$ (50 μM), EDTA (100 μM) and different compounds. Test tubes were incubated at 37 °C for 1 h, 0.5 ml 2.8% (w/v) trichloroacetic acid and 0.5 ml 1% thiobarbituric acid were then added, and the tubes were heated in a water bath maintained at 100 °C for 20 min. The measurement of the absorbance, at 532 nm, of resulting chromogen gives a measure of ·OH-dependent damage to deoxyribose. The concentration of TBARS was calculated by using molar absorption coefficient of 1.56×10^5 M^{-1} cm^{-1}.

2.3. Determination of the reducing power

The reducing power of AA and GA was determined according to the method reported by Oyaizu (1988). Compounds (2.5 ml, the final concentration was 0.004–4.17 mM) were added to sodium phosphate buffer (0.2 M, pH 6.6, 2.5 ml) containing potassium ferricyanide $[K_3Fe\ (CN)_6]$(1%, 2.5 ml). The reaction mixture was incubated at 50 °C for 20 min, at the end of which 2.5 ml of trichioroacetic acid (10%) were added, and the mixture was centrifuged at 3000 rpm for 10 min. The supernatant (5 ml) was mixed with distilled water (5 ml) and $FeCl_3$ (0.1%, 1 ml), and the absorbance was measured at 700 nm. An increase in absorbance of the reaction mixture was taken as a measure of the reducing power of compounds.

2.4. Chelating effect of AA and GA on Fe^{2+}

The chelating activity of AA and GA on Fe^{2+} was measured according to the method of Dinis, Madeira, and Almeida (1994). AA and GA (0.004–4.17 mM) were reacted with $FeCI_2$ (2 mM, 0.2 ml) and ferrozione (5 mM, 0.2 ml) for 10 min, and the spectrophotometric absorbance was determined at 562 nm. A lower level of absorbance indicated a stronger chelating activity.

2.5. Determination of DPPH radical

The assay was carried out as described by Shimada, Fujikawa, Yahara and Nakamura (1992). Compound (the final concentration was 0.004–4.17 mM) was added to 1 ml of 2×10^4 M αα-diphenyl-β-picryihydrazyl (DPPH) in methanol. The mixture was shaken vigorously and allowed to stand for 30 min; the absorbance of the resulting solution was measured at 517 nm with a spectrophotometer (Hitachi U-2000). All tests and analyses were run in three replicates and averaged.

2.6. Hydrogen peroxide assay

The procedure is a modification of that reported by Rinkus and Taylor (1990). Phenol red (sodium salt) was prepared in 0.2 M potassium phosphate, pH 6.2, at a final concentration of 7.5 mM. HRPase was prepared in

0.2 M potassium phosphate pH 6.2, at a final concentration of 0.5 mg/ml. Just before use, one volume of HRPase solution and two volumes of phenol red solution were combined. Sample was incubated with 0.4 ml H_2O_2 for 20 min and treated with 0.6 ml HRPase/phenol red solution. The tubes were vortexed and then allowed to sit for 10 min, then immediately placed on an ice bath. This reaction mixture was monitored at 610 nm.

2.7. Cytotoxicity of AA and GA toward human blood lymphocytes

2.7.1. Cell preparation

Human lymphocytes were isolated from fresh whole blood by adding blood to RPMI 1640, then underlaying it with Histopaque 1077 before centrifuging at 1600 rpm for 10–15 min. Lymphocytes were separated as a pink layer at the top of the Histopaque 1077. Lymphocytes were washed in RPMI 1640. Cell number and viability (Trypan blue exclusion) were determined using a Neubauer Improved Haemocytometer before treatment. Human lymphocytes were incubated at a density of 5×10^5/ml and viability was over 90%.

2.7.2. Cytotoxicity

Cell suspensions were incubated with different concentrations of compounds (the final concentration was 0.41–4.17 mM) for 30 min at 37 °C in a dark incubator together with untreated control samples. Samples were then centrifuged at 800–900 rpm, the lymphocytes were resuspended in RPMI 1640 and 0.4% trypan blue, and viable and dead cells were scored.

2.7.3. Genotoxicity of AA and GA toward human lymphocytes

Cells were incubated with different concentrations of compounds (the final concentration was 0.41–4.17 mM) for 30 min at 37 °C in a dark incubator together with untreated control samples. Samples were then centrifuged at 800–900 rpm; the lymphocytes were resuspended in low melting point agarose for comet analysis.

2.7.4. Effects of AA and GA on oxidative DNA damage to human lymphocytes

Cells were incubated with different concentrations of compounds and 50 μmol/l hydrogen peroxide for 30 min at 37 °C in the dark. Samples were then centrifuged at 800–900 rpm. Control samples were treated with PBS alone without hydrogen peroxide. (1) samples + cell + H_2O_2: cells were treated with samples and H_2O_2 for 30 min; (2) samples + cells→ + H_2O_2: cells were pretreated with samples for 30 min, then incubated with H_2O_2 (3) samples + H_2O_2 + cells: samples were pretreated with H_2O_2 for 30 min then incubated with cells.

2.7.5. Comet assay

Three layers of agarose were prepared. For the first layer 75 μl of 1% NMP agarose were prepared at 40 °C in PBS, then dispensed onto fully frosted slides and covered with a 22×22 mm (no.1) cover slip. To solidify the agarose, the slides were kept at 4 °C for 10 min. Lymphocytes were suspended in 1% LMA agarose in PBS (prepared at 37 °C) and 75 μl were plated onto the first layer of agarose, covered with a cover slip and kept for 10 min at 4 °C to solidify. After the cover slips were removed, 75 μl of 1% LMA were plated onto the second layer of agarose, covered with a cover slip and kept for 10 min at 4 °C. Then the slides were immersed in freshly-prepared cold lysing solution.

Slides were treated at 4 °C for 60 min (vertically without a cover slip) with lysis solution (2.5 mol NaCl/l, 100 mol Na_2EDTA/l, 10 mM Tris/l, 1% N-lauroyl sarsosinatei), adjusted to pH 10 with sodium hydroxide and 1% (v/v) Triton X- 100 plus 10% DMSO (added immediately before use). After the slides were removed from the lysis solution they were placed in an electrophoresis tank horizontally, side by side. Slides were covered with fresh electrophoresis buffer (300 mol NaOH/l and 1 mol Na_2EDTA/l) at 4 °C for 20 min in dark conditions. The electrophoresis was run at 25 V for 20 min at 4 °C, covered with black paper against light.

After electrophoresis, slides were placed vertically, without a cover slip, in a neutralizing tank and gently washed three times for 5 min with neutralizing buffer (0.4 mol Tris/l adjusted to pH 7.5 with hydrochloric acid) at 4 °C in the dark. Twenty microlitres of 20 μg ethidium bromide/ml was dispensed directly onto slides and covered with a cover slip.

Slides were examined at on a Nikon EFD-3 fluorescence microscope (Japan) with excitation filter BP at 543/10 nm and a 590 nm emission barrier filter. Objective measurements of the distribution of DNA were performed for a sample of cells by using a Komet 3.1 (Kinetic Imaging Ltd., Liverpool, UK). One hundred cells on each slide (scored at random) were classified according to the relative intensity of fluorescence in the tail. The degree of DNA damage was scored by determining the percentage of DNA in the tail, Tail DNA% = [Tail DNA/(Head DNA + Tail DNA)] ×100.

2.8. Statistical analysis

All analyses were run in triplicate and averaged. Statistical analyses were performed according to the SAS Institute User's Guide. Analyses of variance were performed using the ANOVA procedure. Significant differences ($P<0.05$) between the means were determined using Duncan's multiple range test.

3. Results and discussion

3.1. The effects of AA and GA on oxidative damage of deoxyribose

Deoxyribose degradation is often directed to ·OH-dependent oxidation to yield products that can be quantitatively measured as TBA–malondialdehyde (MDA) adducts. Hence, the deoxyribose assay has become a useful experimental tool for investigating the ability to react with •OH. The effects of AA and GA on deoxyribose damage induced by Fe^{3+}–H_2O_2, measured by the thiobarbituric acid method, are plotted in Fig. 1. The thiobarbituric acid reactive substance (TBARS) formation was increased with increasing concentration of GA, in the range 0.004–0.82 mM, and reached a maximum TBARS when the concentration of GA was greater than 1.65 mM; then the formation of TBARS was decreased with increasing concentration. AA, in the range 0.004–0.24 mM, shows that TBARS formation was increased with increasing concentration of AA. The TBARS formation reached a maximum when the concentration of AA was 1.65 mM, and above 1.65 mM the TBARS formation decreased with increasing concentration. This implies that pro-oxidative activity of AA and GA, at lower concentration, may be attributed to Fe^{3+} that was reduced to Fe^{2+} by AA and GA, and stimulated the ·OH formation. However, at higher concentration, AA and GA may markedly scavenge ·OH and reduce the oxidative damage of deoxyribose.

3.2. Reducing ability of AA and GA

The reducing ability of AA and GA was measured and was found to increase with increasing concentration of AA and GA (Fig. 2). The reducing ability of GA, in the range 0–0.24 mM, was greater than that of AA. However, no significant difference ($P > 0.05$) in the reducing ability was found between GA and AA at a concentration of 4.17 mM. Sakagami and Satoh (1997) noted that the addition of AA and GA to distilled water resulted in a rapid increase of the reducing potential, which a few seconds later was replaced with much higher oxidation potential. Consequently, the cell apoptosis induction might be related to ascorbyl radical- and gallic acid radical-producing activity. These results show that the intermediates, generated by the redox reaction of AA or GA, bind with deoxyribose and cause the pro-oxidation action.

3.3. Chelating activity of AA and GA on ferrous ions

Satoh and Sakagami (1997) reported that the reaction of AA and GA with metal ions, such as $FeCl_3$, $CuCl_2$ or $FeCl_2$, might enhance the degradation of AA and GA, and increase the ascosbyl radical and gallic acid radical concentrations. Duh, Yen, Yen, and Chang (2001) reported that water extracts of unroasted barley were as liposome-, deoxyribose- or protein-protectors, possibly as a result of minimizing the concentration of metal in the Fenton reaction. However, in the present study, the chelating effect of AA and GA on ferrous ions was not significant (data not shown), suggesting that the pro-oxidative action of AA and GA may be related to their weak chelating activity toward ferrous ions. Based on the data obtained, the pro-oxidative action of AA and GA at lower concentration may be due to a weak metal-chelating effect and strong reducing ability.

3.4. Scavenging effect of extracts on DPPH radical

DPPH is a stable free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The reduction in DPPH radical was determined by the decrease in its absorbance at 517 nm induced by antioxidants. Hence, DPPH radical is usually used as a substrate to evaluate the antioxidative action of antioxidants (Shimada et al., 1992). Fig. 3 illustrates the scavenging effect of AA and GA on DPPH radical. The scavenging effect increased with increasing concentration of BHA in the range 0–0.24 mM. With BHA at a concentration above 0.24 mM, no significant difference in scavenging effect ($P > 0.05$) was observed. As for the AA and GA in the range 0–0.02 mM, the scavenging effect on DPPH radical increased with increasing concentration. When GA, at a concentration up to 0.24 mM, was added, the scavenging effect on the DPPH radical was found to decrease. With AA, at a concentration above 0.02 mM, no significant difference ($P > 0.05$) in scavenging effect on the DPPH radical was observed. These results show that AA and GA at higher concentration exhibited antioxidant activity which might be mainly due to their scavenging

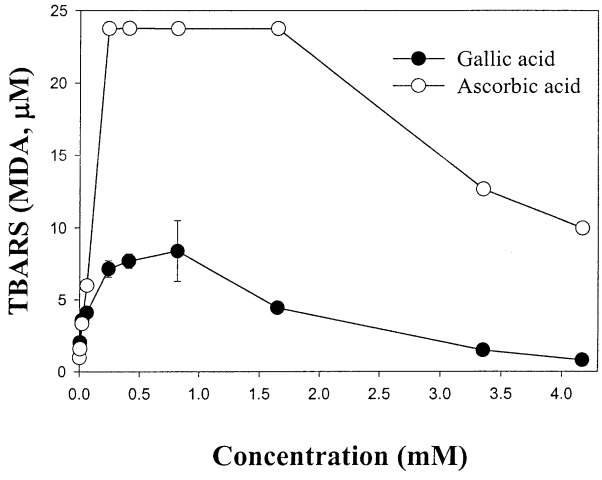

Fig. 1. Effects of ascorbic acid (AA) and gallic acid (GA) on deoxyribose damage induced by Fe^{3+}–H_2O_2. The reaction mixtures were treated by 50 μM $FeC1_3$–3 mM H_2O_2 for 1 h at 37 °C. Each value is the mean ± standard deviation ($n = 3$).

effect on the hydroxyl radical, but not due to their hydrogen-donating ability.

3.5. Determination of the effects on hydrogen peroxides

The scavenging effect of AA and GA toward hydrogen peroxide is shown in Fig. 4. AA and GA, at a concentration of 4.0 mM, exhibited scavenging effects of 60 and 62% toward hydrogen peroxide, respectively. On the whole, the scavenging effect of GA toward hydrogen peroxide was greater than that of AA. In addition, AA and GA in a range of 0.004–0.24 mM show the induction of hydrogen peroxide, suggesting that AA and GA at low concentration enhanced the levels of hydrogen peroxide.

3.6. Effects of AA and GA on DNA damage of lymphocytes

The cytotoxicities of AA and GA against lymphocytes, incubated at 37 °C for 30 min were evaluated. No significant difference ($P > 0.05$) in cell viability (%) was found when AA and GA, in the range 0–4.17 mM, were added. The cell viabilities (%) were 97.6 and 98.7% for AA and GA, respectively (data not shown). Several reports (Inoue et al., 1995; Sakagami et al., 1996) show that AA and GA had cytotoxicity against cancer cells and induced apoptotic cell death. However, in the present research, AA and GA did not show any cytotoxicity toward normal lymphocytes, suggesting that AA and GA showed cytotoxicity against cancer cells but no cytotoxicity against normal lymphocytes.

Fig. 5 shows the DNA damage in lymphocytes treated with different concentrations (0–4.17 mM) of AA and GA at 37 °C for 30 min and measured by means of comet assay. The extent of DNA damage in the model

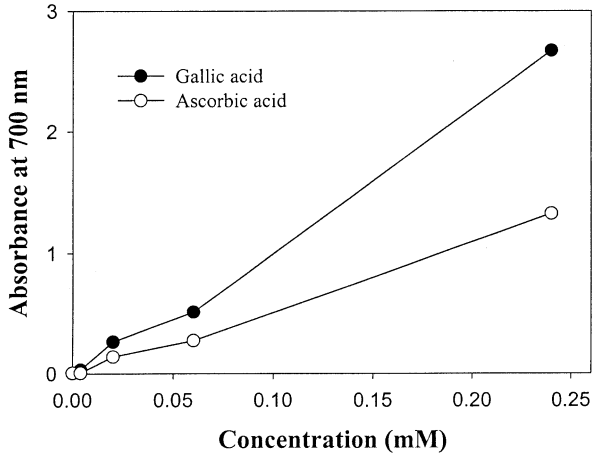

Fig. 2. Reducing power of ascorbic acid (AA) and gallic acid (GA). Each value is the mean±standard deviation ($n = 3$).

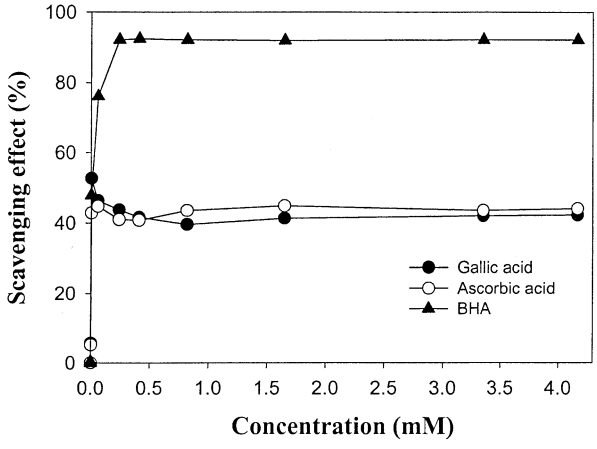

Fig. 3. Scavenging effects of ascorbic acid (AA) and gallic acid (GA) on DPPH radical. Scavenging effect % (capacity of scavenging the DPPH) = [(absorbance of control at 517 nm)−(absorbance of sample at 517 nm)/(absorbance of control at 517 nm)]×100. Each value is the mean±standard deviation ($n = 3$).

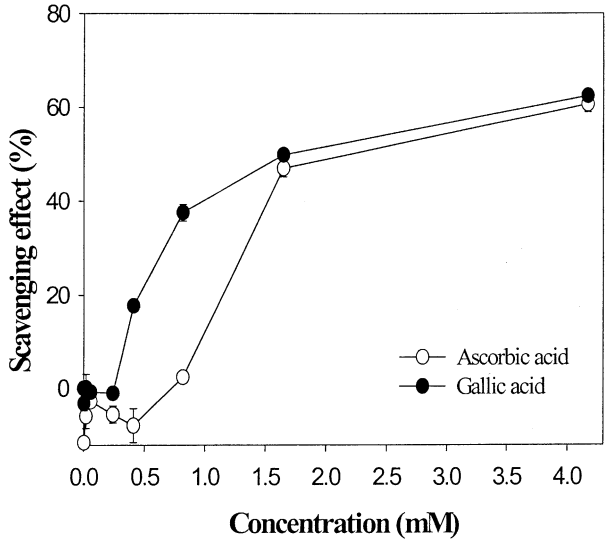

Fig. 4. Scavenging effects of ascorbic acid (AA) and gallic acid (GA) on hydrogen peroxide. Each value is the mean±standard deviation ($n = 3$).

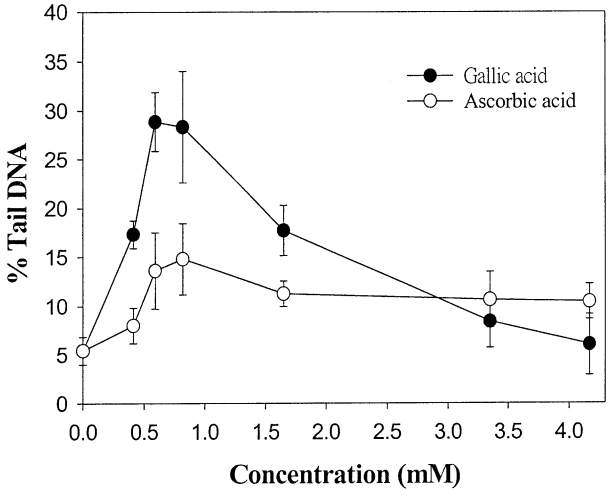

Fig. 5. DNA damage of human lymphocytes treated with ascorbic acid (AA) and gallic acid (GA). Human lymphocytes were incubated for 30 min at 37 °C with different concentrations of AA and GA. Results are mean±standard deviation for $n \geqslant 4$.

system tested was expressed as % Tail DNA. The results indicated that the control (0 mM) showed very slight DNA damage, with 5.4% Tail DNA. AA, at 0.82 mM, and GA at 0.6 mM, showed the highest DNA damage, with 14.8 and 28.8% Tail DNA, respectively.

After that, the DNA damage decreased with increasing concentration of AA and GA. The DNA extents of damage of AA and GA, at a concentration of 4.17 mM, as compared with the control, were 10.4 and 6.0% Tail DNA respectively. Deutsch (1998) reported that the pro-oxidant properties of AA may be due to the simultaneous formation of the semi-DHA free radical during AA oxidation to DHA, which generates hydrogen peroxide. Hence, AA causes strand breakage in DNA in the presence of oxygen and can induce cell death in tissue culture, possibly through the generation of hydrogen peroxide. This result is in accordance with the results in Fig. 1, suggesting that a marked reducing ability and a weak metal-chelating effect of AA and GA might cause the pro-oxidant action of AA and GA.

3.7. Effect of AA and GA on lymphocyte DNA damage induced by hydrogen peroxide

Hydrogen peroxide is one of the reactive products of oxygen metabolism. Accumulation of hydrogen peroxide can show adverse effects on cells (Deutsch, 1998). AA is a water-soluble vitamin which reacts with hydrogen peroxide. AA reduces or prevents hydrogen peroxide-induced lipid peroxidation, and the formation of OH deoxyguanosine, acting as a free radical scavenger (Deutsch, 1998). AA and GA, at lower concentration, show pro-oxidant property; however, whether these compounds, in the presence of high concentrations of free radical or active oxygen, show the pro-oxidant action is still unclear. Fig. 6 shows the effects of GA on lymphocyte DNA, damage induced by hydrogen peroxide. The results indicate that the inhibitory effect of GA on lymphocyte DNA damage was 40% at a concentration of 4.17 mM as compared with the control, when lymphocyte cells were incubated with GA and hydrogen peroxide for 30 min. The inhibitory effect of GA on lymphocyte DNA damage was 67% at a concentration of 4.17 mM when lymphocyte cells were preincubated with GA before exposure to hydrogen peroxide for 30 min. Under the third condition, the inhibitory effect of GA on lymphocyte DNA damage was 78% at a concentration of 4.17 mM when GA was preincubated with hydrogen peroxide for 30 min before the incubation with lymphocyte cells. As shown in Fig. 7, the inhibitory effect of AA on lymphocyte DNA damage was 40% at a concentration of 4.17 mM for the three model systems used as in Fig. 6.

Results obtained in this study clearly demonstrate that the pro-oxidant action of AA and GA at lower concentrations may be due to their weak metal-chelating effects and their strong electron-donating effects (reducing ability), as well as their stimulation of oxidative effects. Further studies on the in vivo evidence of pro-oxidant activity of AA and GA are required.

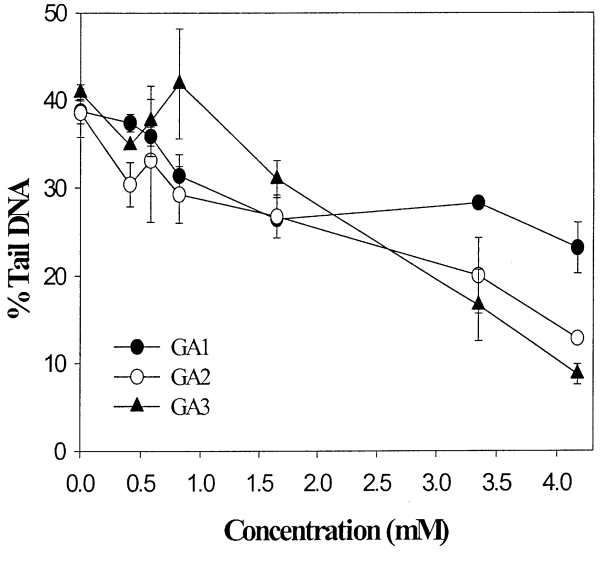

Fig. 6. Effect of gallic acid (GA) on lymphocyte DNA damaged by H_2O_2. Results are means±standard deviation for $n \geqslant 3$. GA1: Human lymphocytes were incubated with GA and hydrogen peroxide (50 μM) for 30 min. GA2: Human lymphocytes were preincubated with GA before exposure to hydrogen peroxide for 30 min. GA3: GA was preincubated with hydrogen peroxide for 30 min before incubation with human lymphocytes.

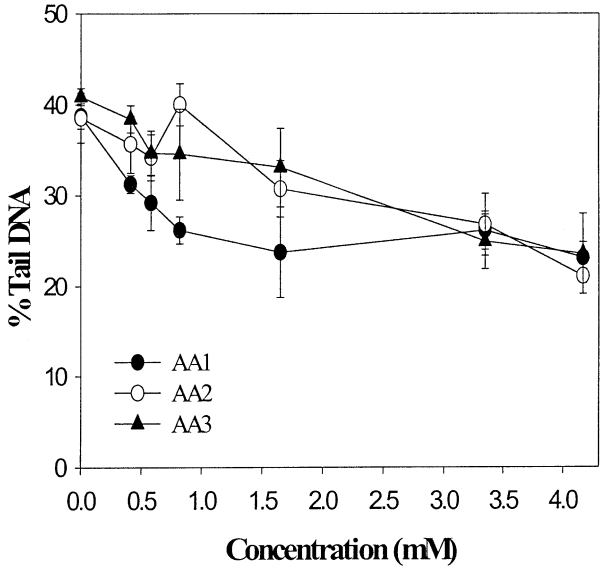

Fig. 7. Effect of ascorbic acid (AA) on lymphocyte DNA damaged by H_2O_2. Results are means±standard deviation for $n \geqslant 3$. AA1: Human lymphocytes were incubated with AA and hydrogen peroxide (50 μM) for 30 min. AA2: Human lymphocytes were preincubated with AA before exposure to hydrogen peroxide for 30 min. AA3: AA was preincubated with hydrogen peroxide for 30 min before incubation with human lymphocytes.

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