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Antioxidant properties of a North American ginseng extract

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Abstract

A North American ginseng extract (NAGE) containing known principle ginsenosides for *Panax quinquefolius* was assayed for metal chelation, affinity to scavenge DPPH-stable free radical, and peroxyl ($LOO^{\bullet}$) and hydroxyl ($^{\bullet}OH$) free radicals for the purpose of characterizing mechanisms of antioxidant activity. Dissociation constants (Kd) for NAGE to bind transition metals were in the order of $Fe^{2+} > Cu^{2+} > Fe^{3+}$ and corresponded to the affinity to inhibit metal induced lipid peroxidation. In a metal-free linoleic acid emulsion, NAGE exhibited a significant ($p \leq 0.05$) concentration (0.01–10 mg/mL) dependent mitigation of lipid oxidation as assessed by the ammonium thiocyanate method. Similar results were obtained when NAGE was incubated in a methyl linoleate emulsion containing haemoglobin catalyst and assessed by an oxygen electrode. NAGE also showed strong DPPH radical scavenging activity up to a concentration of 1.6 mg/mL ($r^2 = 0.996$). Similar results were obtained for scavenging of both site-specific and non site-specific $^{\bullet}OH$, using the deoxyribose assay method. Moreover, NAGE effectively inhibited the non site-specific DNA strand breakage caused by Fenton agents, and suppressed the Fenton induced oxidation of a 66 Kd soluble protein obtained from mouse brain over a concentration range of 2–40 mg/mL. These results indicate that NAGE exhibits effective antioxidant activity in both lipid and aqueous mediums by both chelation of metal ions and scavenging of free radicals. (Mol Cell Biochem **203**: 1–10, 2000)

Key words: lipid oxidation, hydroxyl radical, peroxyl radical, chelation, DNA, Fenton reaction

Introduction

Panax ginseng (Asian ginseng), *Panax pseudo-ginseng* (Japanese ginseng) and *Panax quinquefolius* (North American ginseng) represent primary sources of the herb commonly referred to as ginseng. North American ginseng is currently grown in both Canada and Eastern U. S and has been recognized as a valuable tonic similar to *Panax ginseng* C.A. Mayer.

Ginseng is composed of a mixture of glycosides, essential oils, and a variety of complex carbohydrates and phytosterols as well as amino acids and trace minerals [1]. The principle active ingredient of ginseng are believed to be a complex mixture of over 30 triterpenoid saponins commonly referred to as ginsenosides (Fig. 1), which are present in leaf, stem and berries in addition to the traditionally harvested root [2]. Former studies have shown ginseng to enhance the immune system [3], carbohydrate and lipid intermediary metabolism [4], and anti-stress properties [5]. There are also reports on antioxidant activity of *Panax ginseng* [6] and its activity towards the transcription of a primary antioxidant enzyme Cu-Zn superoxide dismutase [7]. These later studies are potentially relevant to the reported anti-tumour activity of ginseng [8, 9].

In recent years, considerable focus has been given to identifying antioxidant properties of plant materials that may be used for human consumption [10–13]. The comprehensive evaluation of antioxidant activity of natural products using a battery of test methods has been shown to be important in identifying both the antioxidant and potential prooxidant activity of these compounds in both lipid and aqueous mediums [14–15]. On the other hand, such studies are also

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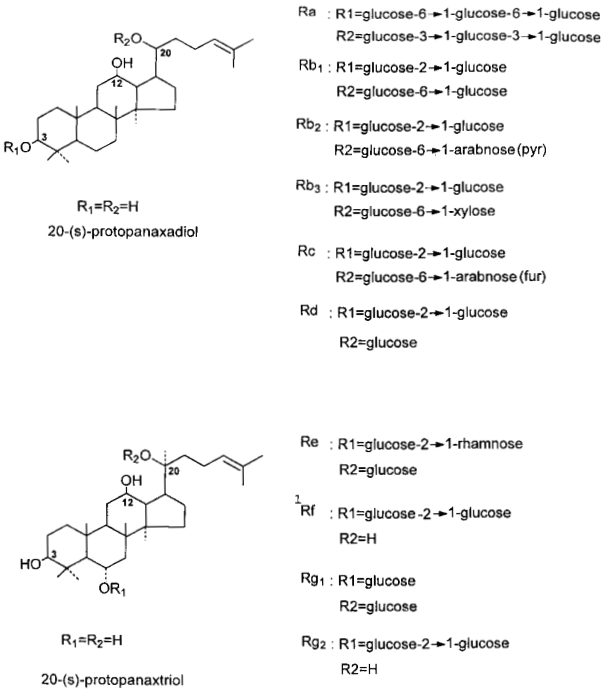

Fig. 1. Structures of different ginsenosides present in North American ginseng extract. [1]Rf is absent; $Rb_1/Rb_2 > 5$.

useful in identifying the mechanism of activity of the antioxidant agent, particularly in determining whether an antioxidant compound decreases the •OH generation by chelating metal ions or by scavenging of hydroxyl and peroxyl radicals [15]. The purpose of this study was to evaluate the antioxidant activity of a standardized North American ginseng extract (NAGE), using a number of antioxidant assay systems.

Materials and methods

Materials

All chemicals and reagents used were of highest purity. Cupric sulphate pentahydrate ($CuSO_4.5H_2O$), ferrous sulphate ($FeSO_4$), ferric sulphate [$Fe_2(SO_4)_3$], potassium chloride (KCl), ferrous chloride ($FeCl_2$), ferric chloride ($FeCl_3$), potassium di-hydrogenorthophosphate (KH_2PO_4), di-potassium hydrogen orthophosphate (K_2HPO_4), tetramethyl murexide (TMM), hexamine, linoleic acid, ammonium thiocyanate, Tween-20, haemoglobin, L-ascorbic acid, trichloroacetic acid (TCA), potassium ferricyanide, ethylenediaminetetraacetic acid (EDTA), ethidium bromide, 1-diphenyl-2-picrylhydrazyl (DPPH), electrophoresis grade agarose, chelex-100, pBR322 plasmid DNA, bromophenol blue, xylene cyanol FF, 15% ficoll, ovalbumin, bovine serum albumin (BSA), phosphorelase-B, β-galactosidase, myosin, hydrogen peroxide and molecular biology grade Trizma base were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Electrophoresis grade sodium dodecyl sulfate (SDS), metal-free micro-centrifuge tubes, pipette tips and polaroid type 665 positive films were obtained from BioRad Laboratories (Richmond, CA, USA). Hydrochloric acid and ethanol were obtained from BDH Chemical Co. (Toronto, ON, Canada).

North American ginseng extract

A North American Ginseng extract, CNT2000, was obtained from Chai-Na-Ta, Corp. Ltd. (Langley, BC, Canada). CNT2000 is a trademarked extract produced by a proprietary process. The NAGE was standardized to 8% (w/v) ginsenosides and confirmed as North American ginseng by the absence of Rf and the presence of a typical Rb_1 to Rb_2 ratio [16, 17]. The reducing activity of the ginseng extract was measured according to the method of Wijewickreme and Kitts [18].

Metal chelating activity of NAGE

The metal chelating activity of NAGE was assessed by a complexometric method using tetramethyl murexide [19]. All buffers and distilled water was treated with chelex-100 to remove any adventitious metal ions present. Solutions consisting of $CuSO_4$, $FeSO_4$, or $Fe_2(SO_4)_3$ (50–400 µmol), NAGE (300 µg/mL), and TMM (1 mM) were prepared in 10 mM hexamine HCl buffer (pH 5) containing 10 mM KCl. The aliquots (1 mL) of NAGE were individually incubated with 1 mL of 50–400 µmol $CuSO_4$, $FeSO_4$, or $Fe_2(SO_4)_3$ and 0. 1 mL of TMM for 10 min at room temperature and the absorbance was read at 460 and 530 mn. The amount of free cupric, ferric or ferrous in the samples were read from a standard curve, where the absorbancy ratio (A_{460}/A_{530}) of a solution of 1 mL $CuSO_4$, $FeSO_4$, or $Fe_2(SO_4)_3$ (50–400 µmol), 1 mL of hexamine HCl buffer, and 0. 1 mL TMM was plotted as a function of the amount of total ferrous, ferric or cupric in the sample. The difference between the absorbance ratio of the standard and the NAGE added sample indicated the concentration of metal bound to the ginseng extract. The dissociation constants (K_d) of NAGE for different metal ions were calculated from Scatchard plots, where the ratio of bound metal per mg NAGE to free metal was plotted vs. the bound metal per mg NAGE [19].

Ammoniumthiocyanate assay

With metal ions

A linoleic acid pre-emulsion was made by vortexing 3 mL of linoleic acid with 3 mL of Tween-20 in 200 mL of 30% (v/v) ethanol [20]. The reaction mixture consisted of 5 mL of pre-emulsion, 0.5 mL of NAGE in water (0.001–10 mg/mL) and 0.5 mL of $CuSO_4$, $FeSO_4$, or $Fe_2(SO_4)_3$ in water (10, 100 μM). The total volume of the reaction was adjusted to 10 mL with deionized distilled water. All reactions were incubated at 37°C in a 25 mL test tube with plastic caps. Aliquots from the incubated mixture were withdrawn at 30 min of incubation and tested for lipid peroxidation. The assay was conducted by adding 2.5 mL of 75% ethanol, 0.05 mL of ammonium thiocyanate solution (30% w/v), and 0.05 mL, of ferrous chloride (0. 1% w/v) to 0.05 mL of sample. The colour development in the reaction mixture was measured at 500 nm against ethanol contained in a reference cell. The results were plotted as the absorbance at 500 nm vs. the ratio of [NAGE]/[metal].

Without metal ions

A similar procedure devoid of metal ions was also used to assess the inhibition of lipid peroxidation by NAGE. Samples contained linoleic acid pre-emulsion (5 mL) and NAGE (0.5 mL) at various concentrations (0.01–10 mg/mL). The total volume of the reaction mixture was adjusted to 10 mL with deionized distilled water and the reaction was monitored for lipid peroxidation using the thiocyanate method [20].

Oxygen consumption measurements

The rate of oxygen consumption in a linoleic acid emulsion with haemoglobin as the prooxidant was measured in the presence of NAGE using an oxygen electrode [21]. A pre-emulsion for this experiment was prepared by vortexing 1.5 g of linoleic acid with 0.4 g of Tween-20 in 40 mL of potassium phosphate buffer (0.1 M, pH 7.0). The working solution consisted of 1.5 mL of pre-emulsion, 15 mL of phosphate buffer, 600 μL of haemoglobin (0.3%, w/v), and 600 μL of NAGE (0.1–10 mg/mL). The percentage of oxygen remaining in the chamber (vol = 600 μL) was recorded every 30 sec. Rate of oxygen depletion in an emulsion devoid of NAGE was used as the control. The antioxidative effect of NAGE was reported as a protective index (PI), where,

$$PI = \frac{\text{Time taken for 50\% of the oxygen remaining in the chamber to deplete with added NAGE}}{\text{Time taken for 50\% of the oxygen remaining in the chamber to deplete without NAGE}}$$

The control sample had a PI value of 0, and the compounds without antioxidant or prooxidant activity had a PI value of 1. Antioxidant and prooxidant compounds had PI > 1 or PI < 1, respectively.

DPPH radical scavenging activity of NAGE

The scavenging of NAGE for DPPH, a stable free radical, was assayed spectrophotometrically [22]. DPPH in ethanol (0. 1 mM) (control) was mixed thoroughly with a concentration range of (0–1.6 mg/ML) NAGE and the absorbance was read at 519 nm. The degree of DPPH radical scavenging activity of NAGE was calculated as a percentage of inhibition (% inhibition), where:

$$\% \text{ Inhibition} = [(A_{519\ \text{control}} - A_{519\ \text{sample}}) / A_{519\ \text{control}}] \times 100$$

Deoxyribose assay for site-specific and non site-specific $^{\bullet}OH$ scavenging activity

Non-site specific $^{\bullet}OH$ radical scavenging activity of NAGE was measured using the deoxyribose assay method [23]. Solutions of $FeCl_3$ and ascorbate were prepared in deaerated water immediately before use. One mL of the final reaction solution consisted of aliquots (500 μL) of NAGE (0.8–4.9 mg/mL), $FeCl_3$ (100 μM), EDTA (100 μM), H_2O_2 (1 mM), deoxyribose (3.6 mM), and L-ascorbic acid (100 μM) in potassium phosphate buffer (pH 7.4). The reaction mixture was incubated for 1 h at 37°C and further heated in a boiling water bath for 15 min after addition of 1 mL of TCA (10%, w/v) and 1 mL of 2-TBA (0.5%, w/v, 2-TBA in 0.025 M NaOH containing 0.02%, w/v, BHA). The colour development was measured at 532 mn against a blank containing phosphate buffer.

The procedure for measuring site-specific hydroxyl radical scavenging activity was similar to the above method with the exception that EDTA was replaced with a similar volume of buffer [24].

DNA nicking assay for non site-specific $^{\bullet}OH$ scavenging activity

All experiments were conducted in 50 mM phosphate buffer (pH 7.4) under ambient oxygen pressure. To assess the non-site specific $^{\bullet}OH$ radical scavenging activity of NAGE, 2 μL of NAGE (0. 1 and 1 mg/mL) was mixed with 2 μL each of EDTA-Na_2 (30 mM), KH_2PO_4 buffer (50 mM, pH 7.4), H_2O_2 (30 mM), $FeSO_4$ (16 mM), and pBR 322 plasmid DNA (0.1 μg/mL) in a 500 μL microcentrifuge tube. The molar ratio of $FeSO_4$/EDTA was kept at 0.53 [25]. The final volume of the

reaction mixture was brought to 12 µL with deionized distilled water and incubated for 1 h at 37°C. Following incubation, 2 µL of loading dye (25%, w/v, bromophenol blue; 0.25%, w/v, xylene cyanol FF; and 15%, w/v, ficoll in water) was added to the incubated mixture, and 12 µL was loaded onto a 0.7% (w/v) agarose gel. Electrophoresis was conducted at 60 volts in Tris-Acetate-EDTA•Na_2 (TAE) buffer (0.04 M tris-acetate and 1 mM EDTA, pH 7.4) using a DNA subcell (Bio-Rad). The agarose gel was stained with ethidium bromide (0.5 µg/mL deionized distilled water) for 20 min. DNA bands were visualized under illumination of UV light using an UV transilluminator and photographed with a Bio-Rad polaroid camera using type 665 positive films. Photographs were scanned by a Bio-Rad imaging densitometer using the Bio-Rad Molecular Analyst™/PC image analysis program (Version 1.0) to quantitate DNA breakage as percentages of total absorbance bands of Form I (supercoiled) and Form II (nicked circular) DNA.

Inhibition of autoxidation of mouse brain homogenate by NAGE

Whole brains were obtained from CD-1 male mice (body wt = 20–21 g) by cervical dislocation, washed with ice cold phosphate buffer, and homogenized in 10 mL of ice cold phosphate buffer (50 mM, pH 7.4) using a glass homogenizer. Aliquots (1 mL) from homogenate were diluted with 4 mL of phosphate buffer (50 mM) containing NAGE (2–40 mg/mL) and a peroxidizing reagent [Fe^{3+} (0.1 mM) and H_2O_2 (0–1 mM)]. Brain homogenate with peroxidizing agent but without NAGE was used as the control. Following 30 min of incubation at 37°C, aliquots were assayed for total protein content using microtitre plates with bovine serum albumin as the standard [26]. The samples were also electrophoresed using a 10% (w/v) sodium dodecyl sulphate-polyacrylamide gel (SDS-P AGE) at 100 V at room temperature using a mini-Protean II electrophoresis apparatus (Bio-Rad) and a model 200/2.0 power supply (Bio-Rad) [27]. The gels were stained with Coomassie blue R-250. Ovalbumin (MW = 45,000), bovine serum albumin (MW = 66,000), phosphorylase B (MW = 92,500), β-galactosidase (MW = 116,250), and myosin (MW = 200,000) were used as the molecular weight markers.

Statistics

All results are expressed as mean ± S.D. One way analysis of variance followed by Tukey test (Systat Inc., Evanston, IL, USA) was used to test the differences among treatments. The level of confidence required for significance was selected at $p \leq 0.05$.

Results

Metal binding and reducing activity of NAG

Table 1 reports the respective dissociation constants (K_d) of NAGE for Fe^{2+}, Fe^{3+}, and Cu^{2+} as calculated from individual metal binding Scatchard curves. NAGE possessed a higher K_d value for Cu^{2+} and Fe^{3+} relative to Fe^{2+}. Since K_d value is an indicator of the strength of metal ion binding to components of NAGE, both Cu^{2+} and Fe^{3+} were shown to exhibit relatively weaker binding to NAGE than Fe^{2+}. Determining the concentration of ascorbic acid required to contribute to 100% reducing activity, the reducing activity of the NAGE was determined to be equivalent to 6 µM of ascorbic acid.

Metal catalyzed linoleic acid peroxidation as assayed by ammonium thiocyanate

In this test method, free metal ions Fe^{2+}, Fe^{3+} or Cu^{2+} were added to a linoleic acid emulsion in order to understand the relative effectiveness of NAGE to inhibit metal ion catalyzed lipid peroxidation. The results of these tests are given in Figs 2A, 2B and 2C, for Fe^{2+}, Fe^{3+}, and Cu^{2+} respectively. The NAGE effectively mitigated the peroxidation caused by all three metal ions, although the strongest effect was seen above a lignan to metal ion ratio of 6. A slight prooxidant activity of NAGE was only observed at 0.001 mg/mL NAGE with 100 µM Cu^{2+}.

Haemoglobin catalyzed linoleic acid peroxidation as measured by an oxygen electrode

The time course depletion of oxygen in a haemoglobin-linoleic acid emulsion system containing NAGE is shown in Fig. 3. The rate of initial (0–6 min) oxygen depletion was lowest at the highest NAGE concentration (10 mg/mL) (Fig. 3, inset). Pronounced reductions in oxygen consumption over an extended duration of incubation (100 min) was also seen with NAGE as the concentration was increased from 0.1–10 mg/mL, compared to the control. As a result, an increase in PI value was also observed and recorded for each increasing

Table 1. Dissociation constants of Fe^{2+}, Fe^{3+} and Cu^{2+} for NAGE[1]

Metal ion	Dissociation constant (K_d) (µmol)
Fe^{2+}	12.7 ± 1.1
Fe^{3+}	42.6 ± 5.4
Cu^{2+}	55.1 ± 3.8

[1]Values represent mean ± S.D of 3 independent experiments.

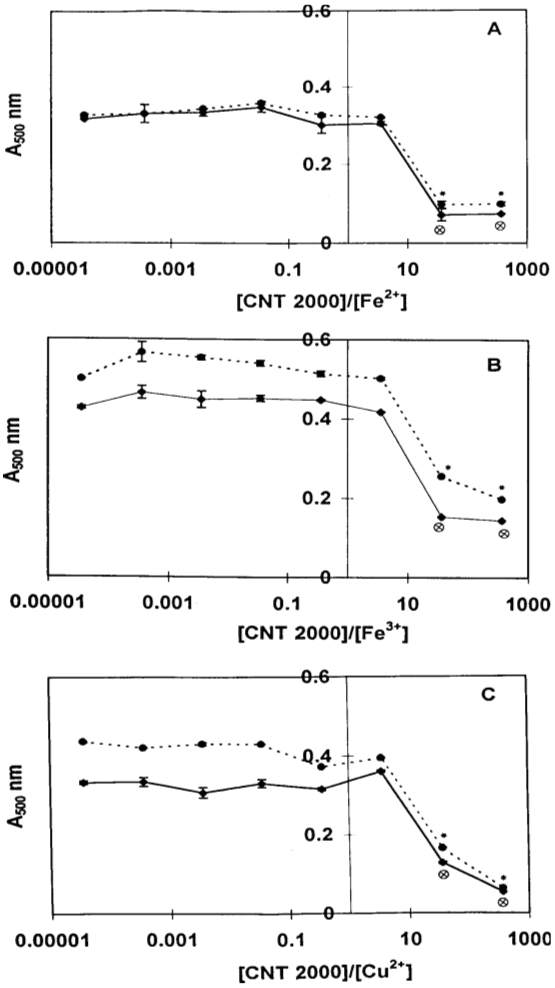

Fig. 2. The effect of ligand (NAGE) to metal ion concentration ratio on degree of lipid oxidation. A – Fe^{2+}; B – Fe^{3+}; C – Cu^{2+}. ● – 100 μM of metal ion; ♦ – 10 μM of metal ion. Lipid oxidation was measured by ammonium thiocyanate assay, as described under Materials and methods using a linoleic acid emulsion. Results represent mean ± S.D. of 3 separate experiments. * and ⊗ – values are significantly different.

concentration of ginseng extract present in the haemoglobin emulsion.

Metal-free linoleic acid peroxidation as assayed by ammonium thiocyanate

Unlike the previous two assay methods, the affinity of NAGE to restrict the peroxidation of a linoleic acid emulsion over an extended period of incubation at 37°C was tested using a metal free emulsion (Fig. 4). Significant ($p < 0.01$) peroxyl radical scavenging activities were observed for extract concentrations exceeding 0.01 mg/mL for all time periods.

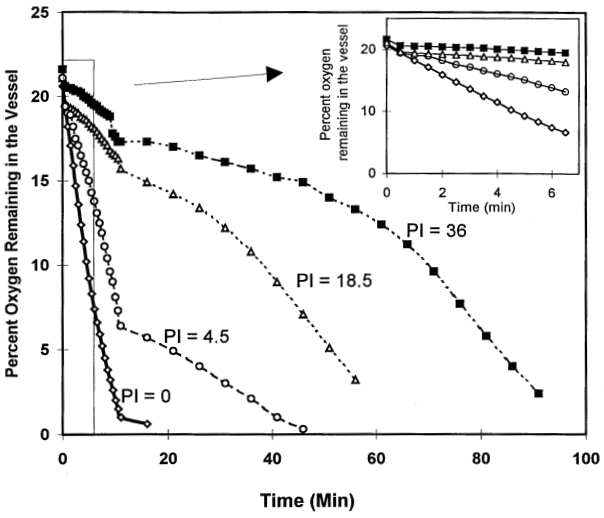

Fig. 3. Depletion of oxygen in a linoleic acid emulsion containing haemoglobin catalyst in the presence of three NAGE concentrations as measured by an oxygen electrode. ◊ – control emulsion without NAGE; ■ – emulsion containing 10 mg/mL NAGE; Δ – emulsion containing 1 mg/mL NAGE; ○ – emulsion containing 0.1 mg/mL NAGE.

The strongest inhibitory activity against lipid peroxidation was observed at the highest concentration (10 mg/mL) of NAGE.

Free radical scavenging activity of NAGE.

DPPH radical scavenging activity

Figure 5 shows the concentration dependent DPPH radical scavenging activity of NAGE. A linear relationship ($r^2 = 0.996$) between the concentration of NAGE and the scavenging activity of DPPH radical was obtained over a concentration range of 0–1.6 mg/mL.

Site specific and non site-specific scavenging activity of NAGE

Site specific and non site-specific ·OH scavenging activity of NAGE was measured using both deoxyribose and DNA scission assays. NAGE was effective in suppressing deoxyribose oxidation by both site-specific and non site-specific ·OH scavenging, in a concentration dependent manner (Fig. 6). Suppression of deoxyribose ·OH damage by NAGE was greatest in the site specific than in the non site-specific protocol. A similar result was obtained for concentration dependent protection against Fenton induced ·OH damage of pBR 322 plasmid DNA using the non site-specific ·OH assay (Fig. 7). As given in Fig. 7, Fenton agents caused the breakage of supercoiled DNA (lane 1) to nicked circular (Form II) or

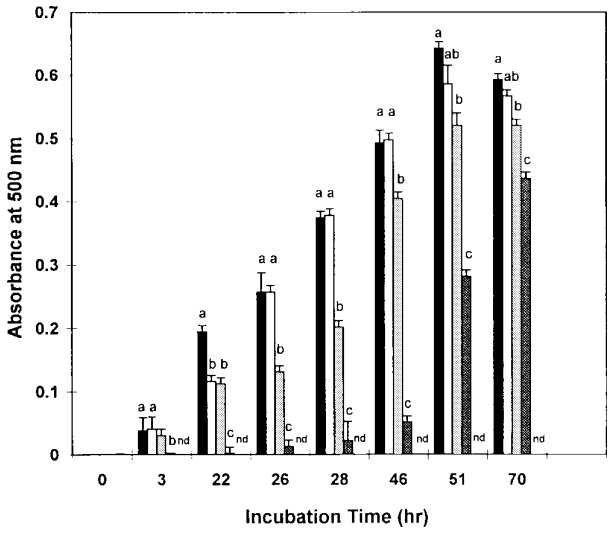

Fig. 4. The effect of NAGE on lipid oxidation occurring in a metal-free linoleic acid emulsion. Values represent mean ± S.D. of 3 separate experiments. Lipid oxidation was measured by ammonium thiocyanate assay, as described under Materials and methods. Letters a–d denote the Tukey test results. Experiments with different letters are significantly different at a given time. ■ – control emulsion without NAGE; □ – emulsion with 0.01 mg/mL NAGE; ⊠– emulsion with 0.1 mg/mL NAGE; ⊡– emulsion with 1 mg/mL NAGE; nd – absorbance at 500 mn was not detected for emulsion with 10 mg/mL NAGE.

degraded forms (smear) (lane 2). However, the application of NAGE at both 0.1 and 1 mg/mL concentrations retained DNA in its original supercoiled form (Form I, lanes 3 and 4 respectively) in the presence of peroxidizing Fenton agents. These results clearly demonstrated that NAGE is an effective agent in mitigating oxidative stress to susceptible biomolecules such as DNA.

Inhibition of protein oxidation in rat brain homogenate

The effect of NAGE to inhibit peroxidation of soluble proteins present in mouse brain homogenate exposed to Fenton reactants is shown in Fig. 8. The soluble protein profile of whole mouse brain is shown in lane 2. Incubating brain homogenate with Fenton reactants resulted in the peroxidation of numerous soluble proteins (lane 3). The presence of NAGE together with the same peroxidizing Fenton agents produced a concentration dependent retention of a 66 kDa protein (lanes 4–9).

Discussion

Our results show that the North American ginseng extract (NAGE) exhibits antioxidant activity by both chelating transition metal ions as well as by scavenging free radicals. The principle active ingredients of ginseng are expected to be a complex mixture of over 20 triterpenoid saponins, commonly referred to as ginsenosides [28–29]. Although, quantitative HPLC analysis of ginsenosides has been reported by other workers for *Panax ginseng* [18, 30], only recently have similar evaluations been reported for North American ginseng [2, 16, 18]. The differences in the content of both total

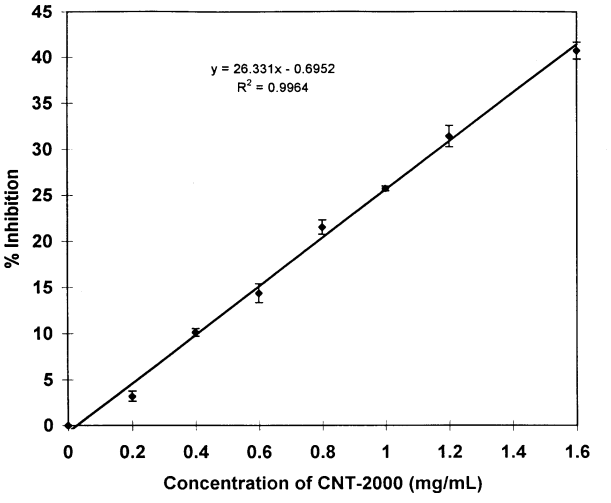

Fig. 5. DPPH (1-diphenyl-2-picrylhydrazyl) radical scavenging activity of NAGE. Concentration of DPPH = 0.1 mM. Values represent mean ± S.D. of 3 separate experiments.

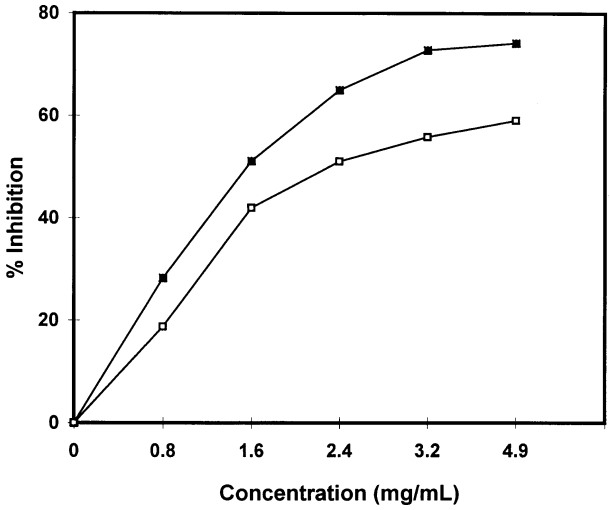

Fig. 6. Site-specific and non site-specific hydroxyl radical (·OH) scavenging activity of NAGE as measured by deoxyribose assay. ■ – site-specific ·OH scavenging activity;□ – non site specific ·OH scavenging activity.

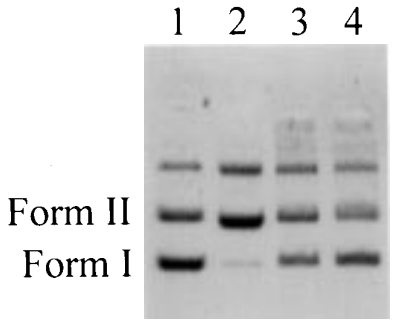

Fig. 7. Non site-specific ·OH scavenging activity of NAGE as measured by DNA nicking assay. Supercoiled double stranded pB322 DNA from *Escherichia coli,* ATCC 37017 was used. Lane 1 – control pBR322 DNA; lane 2 – control DNA + EDTA (30 mM) + H_2O_2 (30 mM) + Fe^{2+} (16 mM); lanes 3 and 4 – reaction mixture used for lane 2 + 0.1 and 1 mg/mL NAGE, respectively. Form I – supercoiled double stranded DNA; Form II – nicked circular DNA.

and individual ginsenosides have been shown to vary with the plant age and size, plant growing location, and growing and harvesting conditions [2]. A distinctive difference in North American ginseng from *Panax ginseng* is the presence of a markedly higher Rb_1/Rb_2 ratio and the absence of Rf [16, 18]. The determination of the characteristic ginsenoside profile of NAGE used in this study confirmed that it was produced from North American ginseng.

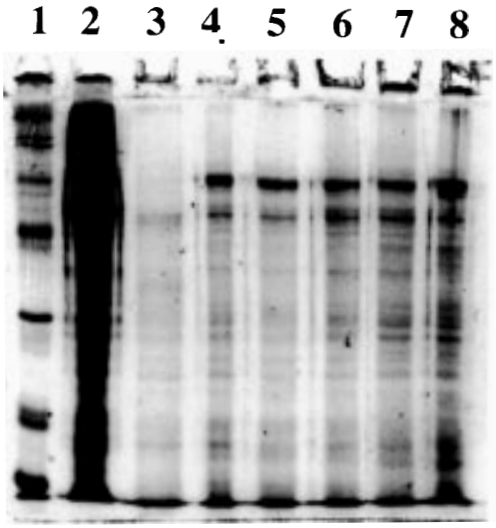

Fig. 8. The retention of mouse brain proteins in the presence of a peroxidizing agent (Fe^{2+} + H_2O_2). Electrophoresis was conducted in a 10% (w/v) sodium dodecyl sulphate polyacrylamide gel. Lane 1 – molecular weight standards; lane 2 – control brain proteins; lane 3 – brain proteins with a peroxidizing agent; lanes 4–8 – reaction mix in lane 3 with 2, 2.5, 4, 10, and 20 mg/mL NAGE, respectively.

Our lipid oxidation studies, performed in the presence of metal ions, demonstrated that both the source and valence of the free transition metal is an important factor in the catalysis of oxidation in model lipid systems. Both Fe^{2+} and Cu^{2+} ions catalyze ·OH radical formation and thereby accelerate lipid oxidation [31–33]. In our study, NAGE displayed strong binding activity and low reducing activity for free Fe^{2+}, Cu^{2+}, and Fe^{3+} ions. This, in part, explained the reason for the observed antioxidant rather than prooxidant activity of NAGE in the metal containing linoleic acid emulsions. Chelating agents, such as phytic acid, and desferal, diethylenetriamine pentaacetic acid (DTPA) are also known as effective antioxidants as they occupy all the aquo-coordination sites on transition metal ions required for ·OH generation [35]. The slight increase in lipid peroxidation observed with Cu^{2+} and Fe^{3+} (Fig. 2) in combination with very low concentrations of NAGE was an indication of the biphasic (antioxidant – prooxidant) mechanism of ginseng components in the presence of free metals. Similar observations have been reported for Cu^{2+} and ascorbic acid [35]. The results of our [ligand]/[metal] ratio experiment (Fig. 2), which showed that the highest antioxidant activity of NAGE occurred against Fe^{2+}, can be further explained by our findings in Table 1 that show Fe^{2+} to also have the strongest binding affinity for NAGE. The ability of NAGE to inhibit the reduction of Fe^{3+} to Fe^{2+} and Cu^{2+} to Cu^{+}, and the inability of Fe^{3+} and Cu^{2+} to initiate peroxidation reactions accounts for the absence of prooxidation from these metal ions.

The depletion of oxygen in a commercial haemoglobin (largely met-haemoglobin) added lipid emulsion can be explained by two possible mechanisms. First, in the absence of H_2O_2, the reaction of Fe^{2+}-Hb with possible traces of peroxides already present in the lipid emulsion source cannot be overlooked to produce ·OH (Eq. 1). It would result in the depletion of oxygen at a very early (initiation phase) phase of the action (Fig. 3, inset).

$$Hb\text{-}Fe^{2+} + LOOH \rightarrow Fe^{3+} + LOO^{\cdot} + {}^{\cdot}OH \quad (1)$$

Second, peroxyl radicals produced at a latter stage during the propagation phase (Eq. 2) would account for the depletion of oxygen during more advanced phases of incubation.

$$LOO^{\cdot} + LH \rightarrow LOOH + L^{\cdot} \quad (2)$$

Since H_2O_2 was not added as a reactant to the emulsion system in this particular experiment, the initial fast phase of oxygen depletion (Fig. 3, inset) could not be solely attributed to the formation of ·OH by Fenton reactants (Eq. 3).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\cdot}OH + OH^{-} \quad (3)$$

The suppression of oxygen depletion by NAGE was shown to correspond to the mitigation of ·OH generation which

typically occurs at very early (first 30 sec) stage of the reaction [36]. The slower phase of oxygen depletion examined in the experiment can be explained by either the direct generation of hydroperoxides (LOOH) from the haemoglobin bound Fe^{2+} (e.g. Eq. 1) [37], or by the indirect generation of lipid peroxides through Fe^{2+} released from intact haemoglobin (e.g. Eq. 3) [38]. The theory that the chelating activity of NAGE decreases the heme-induced lipid oxidation observed in this study is supported by other studies that have shown lipid-soluble iron chelators to be effective agents at preventing oxidative injury [39]. The observation that the concentration-dependent inhibition of oxygen depletion by NAGE was more pronounced during the slower phase of oxidation than during the fast phase of oxidation strongly suggests that the metal chelation property of NAGE was not the sole reason for observed antioxidant activity.

To further understand the mechanism of NAGE in suppressing the generation of lipid hydroperoxides by means other than metal chelation, a linoleic acid emulsion system free of metal ions was used. The concentration-dependent inhibition of lipid oxidation by NAGE over an extended incubation period was a strong indicator of its affinity to scavenge peroxyl radicals. Similar findings for ginseng have been reported with a water soluble extract of *Panax ginseng* protected arachidonic acid (C20:0), a polyunsaturated fatty acid particularly susceptible to autooxidation [40]. The observed slight to moderate suppression of lipid oxidation in our study by NAGE at both 0.0 l to 0. 1 mg/mL concentrations suggested possible chain breaking antioxidant activity of ginseng components. The markedly greater inhibition of lipid peroxidation at 10 mg/mL concentration indicated that the antioxidant constituents present in NAGE were effective at inhibiting initiation as well as propagation steps in lipid oxidation. Moreover, the active constituents of NAGE did not appear to be affected by diffusion or dissociation rates that otherwise potentially limit effective concentrations at different phase locations in a heterogeneous system [41]. Although the complex composition of NAGE precludes a structure-function activity assessment of ginseng for its antioxidant activity at this time, our results do show that sufficient hydrophobic and solubility characteristics of active components present in NAGE enabled effective antioxidant activity in the emulsion system used.

In addition to using a lipid media to assess antioxidant activity, the deoxyribose assay was also used to measure the site-specific (e.g. Fe^{2+} + H_2O_2) and non site-specific (Fe^{2+} + EDTA + H_2O_2) $^{\bullet}OH$ scavenging activity of NAGE [23, 24] in an aqueous medium. The concentration dependent affinity of NAGE to inhibit site-specific damage to deoxyribose further substantiated the chelation power of NAGE towards Fe^{2+} and a mechanism for catalyzed inhibition of $^{\bullet}OH$ formation. Due to the affinity of deoxyribose for Fe^{2+}, the presence of free form of Fe^{2+} in the site-specific assay enables Fe^{2+} to directly attack deoxyribose before the generation of $^{\bullet}OH$ from Fenton reactants [23]. However, the presence of EDTA in the non site-specific assay decreases the Fe^{2+} binding to deoxyribose and thus the formation of $^{\bullet}OH$ predominates in solution. As a result, the antioxidant activity of NAGE in this experiment is directly related to its affinity to scavenge $^{\bullet}OH$ radicals in the non site-specific assay. Thus, the comparatively less inhibitory activity of ginseng towards non-site specific $^{\bullet}OH$ damage denotes that the chelation of transition metal by NAGE may surpass $^{\bullet}OH$ scavenging activity. Similar findings for ginger and thymol using this assay have been reported [41]. Due to the complex composition of NAGE, it is not possible to calculate the chemical rate constants for NAGE induced $^{\bullet}OH$ inhibition in this assay. Our observation that NAGE was similarly effective at scavenging the stable DPPH radical, supports the conclusion that $^{\bullet}OH$ was scavenged in the site-specific and non site-specific deoxyribose assays. These finding are also similar to an earlier observation made using electron spin resonance that demonstrated an affinity of a *Panax ginseng* extract to quench Fe^{2+} induced $^{\bullet}OH$ generation [6].

In addition to deoxyribose, oxidizing agents also damage DNA [42], as evidenced by strand breakage observed in supercoiled double stranded DNA when exposed to Fenton reactants. As a result, the attack of DNA by $^{\bullet}OH$ degrades supercoiled DNA into nicked circular or linear forms [17]. The effect of NAGE towards protecting plasmid DNA strand scissions at both 0.1 and 1 mg/mL provides further evidence of antioxidant activity exhibited by hydrophilic constituents in ginseng. It is noteworthy that the genoprotection offered by NAGE in this study also corresponds to significant bioactivity obtained from the same extract used in preventing breast cancer growth *in vitro* [8].

The oxidation of brain membrane proteins by Fenton reactants occurred by the following scheme (Eq. 4) [43].

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + O_2 + 2H^+ \quad (4)$$

The generation of $^{\bullet}OH$ from reduced Fe^{2+} resulted in the subsequent disappearance of many low (less than 40 Kda) to intermediate (40–70 Kda) molecular weight soluble brain proteins. A similar observation has been reported with membrane proteins exposed to the oxidative effects of ultraviolet irradiation [44]. Incubation of tissues with Fenton reagents in the presence of NAGE produced a concentration dependent retention of low molecular weight proteins and, in particular, a specific protein with a molecular weight of approximately 66 kDa. Although the identification of this protein is unknown, it is of great interest that similar molecular weight proteins have been protected from irradiation induced oxidation reactions by the antioxidant properties of the plant extract *Ginko biloba* [EGb 761] [44]. It is plausible that $^{\bullet}OH$ scavenging and metal chelating

antioxidant activities of NAGE were associated with the protection of the 66 kDa protein since oxidative damage of protein will result in protein fragmentation [45], as a consequence of oxidation of thiols in proteins [46].

In conclusion, the *in vitro* findings of the present study demonstrate that the constituents of the North American ginseng extract, NAGE, exhibit both lipid-soluble and water-soluble antioxidant activity by chelating transition metal ions and scavenging of free radicals. Although a chemopreventative effect of ginseng against tumerogenesis has been shown [9], further work is required to characterize the structure-activity relationship of NAGE for its observed antioxidant activity as well as the bioactive properties of NAGE in relation to absorption efficiencies of metabolically active compounds.

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