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Antioxidant properties of two gallotannins isolated from the leaves of *Pistacia weinmannifolia*

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Abstract

Pistacia weinmannifolia J. Poisson ex Franch (Anacardiaceae) is a shrub or arbor widely found in Yunnan province of China and its leaves are used as traditional Chinese medicine by herbalists. The leaves of *P. weinmannifolia* are rich in phenolic compounds, among which two novel gallotannins, Pistafolin A and Pistafolin B, are identified. In the present investigation, the antioxidant efficiency of Pistafolin A and Pistafolin B in preventing lipid, protein and DNA from reactive oxygen species-mediated damage was studied. Both Pistafolin A and Pistafolin B inhibited the peroxyl-radical induced lipid peroxidation of L-α-phosphatidylcholine liposomes dose-dependently and prevented the bovine serum albumin from peroxyl-induced oxidative damage. Pistafolin A and Pistafolin B also inhibited copper (II)-1,10-phenanthroline complex-induced DNA oxidative damage. Both Pistafolin A and Pistafolin B scavenged the hydrophilic 2,2′-azinobis(3-ethylbenzothiozoline-6-sulphonic acid) diammonium salt-free radicals and the hydrophobic 1,1-dipheny-2-picrylhydrazyl radicals effectively, suggesting they may act as hydrogen donating antioxidants. The protective effects of the two gallotannins against oxidative damage of biomacromolecules were due to their strong free radical scavenging ability. Pistafolin A with three galloyl moieties showed stronger antioxidant ability than Pistafolin B with two galloyl moieties.

Keywords: *Pistacia weinmannifolia*; Pistafolin A; Pistafolin B; Gallotannin; Antioxidant

1. Introduction

In our body, a number of biochemical reactions involve the generation of reactive oxygen species (ROS) [1]. Under normal conditions, the balance between the generation and diminution of ROS is controlled by the antioxidant defense system, which includes both enzymes with antioxidant activities and non-enzymatic factors. The former includes superoxide dismutase, catalase and glutathione peroxidase, while the latter includes some low molecular weight compounds, such as antioxidant vitamins (ascorbic acid, tocopherols, carotenoids, etc.) and some other antioxidant micronutrients (flavonoids, polyphenols, etc.) obtained from the diet [2,3]. Under certain pathological conditions, when ROS are not effectively eliminated by the antioxidant defense system, the dynamic balance between the generation and diminution of ROS is broken. Excessive ROS can attack lipids, carbohydrates, proteins, DNA, and result in oxidative stress. Several lines of evidence link ROS to the onset of a variety of pathologic events, such as coronary heart disease,

Abbreviations: ROS, reactive oxygen species; ABTS, 2,2′-azinobis(3-ethylbenzothiozoline-6-sulphonic acid) diammonium salt; DPPH, 1,1-dipheny-2-picrylhydrazyl; PC, L-α-phosphatidylcholine; BSA, bovine serum albumin; AAPH, 2,2′-azobis(2-amidinopropane)dihydrochloride; AMVN, 2,2′-azobis(2,4-dimethylvaleronitrile); Trolox, 6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid; OP, 1,10-phenanthroline; ESR, Electron Spin Resonance; HPLC, high-performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; IC_{50}, dose of 50% inhibition; TEAC, Trolox-equivalent antioxidant capacity

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cancer, central nervous system injury and the degenerative diseases associated with aging [4–6]. Theoretically, the damaging effects of ROS may be counteracted by antioxidants. Both epidemiological and in vitro studies have shown that a supplement of antioxidant agents that can enhance the antioxidant capacity of the body helps to attenuate the damage induced by oxidative stress. These effective antioxidants include antioxidant vitamins [7] (tocopherols, carotenoids) and other dietary micronutrients such as flavonoids and polyphenols [8].

Polyphenols occur widely in the plant kingdom and are especially common in leaves, flowering tissues and pollens. They are also abundant in woody parts such as stems and barks. Humans consume substantial amounts of polyphenols in fruits, vegetables, herbs and beverages. The average diet contains approximately 1 g/day of mixed polyphenols [9,10]. Traditional Chinese medicinal herbs are especially rich in flavonoids and polyphenols [11]. It has been found that flavonoids and other polyphenols possess antimutagenic/anticarcinogenic, antiallergic, antiplatelet, anti-ischemic and anti-inflammatory activities, but the mechanisms involved in these activities are not completely understood. Most of these biological effects are believed to be associated with their antioxidant properties [12,13]. Thus, it is of interest to examine the antioxidant capacity of flavonoids and polyphenols isolated from traditional Chinese medicinal herbs.

Pistacia weinmannifolia J. Poisson ex Franch (Anacardiaceae) is a shrub or arbor widely found in Yunnan province of China. The leaves of this plant are used as herb against dysentery, enteritis, influenza, traumatic bleeding, headache and lung cancer [14]. Phytochemical studies revealed that the leaves of *P. weinmannifolia* are rich in gallotannins and related phenolic compounds. In the previous research, Hou et al. isolated over eighteen different gallotannins and relevant compounds from the leaf extract of *P. weinmannifolia*, among which two novel gallotannins, Pistafolin A and Pistafolin B are identified [15]. Pistafolin A is a potent scavenger on both hydroxyl radicals and superoxide anion and protects neurons form oxidative damage [16]; however, its antioxidant mechanisms remain to be investigated. In the present investigation, we studied the antioxidant effects of Pistafolin A and Pistafolin B systematically. Their protection on biomacromolecules from oxidative damage was also assessed.

2. Materials and methods

2.1. Chemicals

2,2′-Azinobis(3-ethylbenzothiozoline-6-sulphonic acid) diammonium salt (ABTS), 1,1-dipheny-2-picrylhydrazyl (DPPH), L-α-phosphatidylcholine (PC, from soybean), bovine serum albumin (BSA; Fraction V) and calf thymus DNA were purchased from Sigma Chemical Co. (St. Louis, MO). 2,2′-Azobis(2-amidinopropane)dihydrochloride (AAPH) and 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN) were purchased from Wako Pure Chemicals (Osaka, Japan). 6-Hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid (Trolox) and 1,10-phenanthroline (OP) were purchased from Aldrich Chemical Co. Other chemicals and solvents used were of the highest grade commercially available.

2.2. Purification of Pistafolin A and Pistafolin B

The two novel gallotannins, Pistafolin A and Pistafolin B, were purified from the leaf extract of *P. weinmannifolia* by high-performance liquid chromatography (HPLC), as reported previously [15]. Their structures were shown in Fig. 1. Both of them contain the galloyl moieties: Pistafolin A contains three galloyl moieties whilst Pistafolin B contains two galloyl moieties.

2.3. $ABTS^{\bullet+}$ decolorization assay

The scavenging effects of Pistafolin A and Pistafolin B on the hydrophilic ABTS-free radicals ($ABTS^{\bullet+}$) was measured by the decolorization of $ABTS^{\bullet+}$ at 734 nm [17]. ABTS dissolved in water at a 7-mM concentration was reacted with 2.45 mM potassium persulfate in the dark at room temperature for 12–16 h. Then the $ABTS^{\bullet+}$ solution was diluted with 150 mM phosphate buffer (pH 7.3) to an absorbance of 0.700±0.020 at 734 nm and equilibrated at 30 °C. After addition of 1.0 ml of diluted $ABTS^{\bullet+}$ solution (A_{734nm}= 0.700±0.020) to 10 μl of gallotannin or Trolox solutions (final concentration 0–15 μM) the absorbance reading at 734 nm was taken exactly 5 min after initial mixing. The percentage scavenging of $ABTS^{\bullet+}$ was calculated by:

$$I = [(Ao - Ax)/Ao] \times 100\%$$

Here, Ax and Ao were the absorbance at 734 nm of samples with and without antioxidants, respectively.

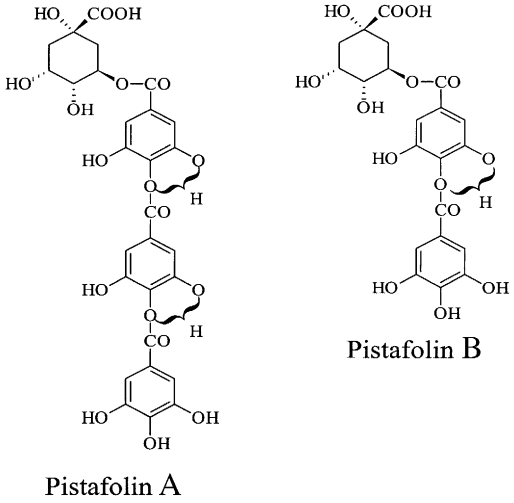

Fig. 1. Molecular structure of Pistafolin A and Pistafolin B.

2.4. DPPH radical assay

The scavenging of hydrophobic DPPH-free radicals by Pistafolin A and Pistafolin B was examined by Electron Spin Resonance (ESR) technique [18]. DPPH was dissolved in ethanol to give a 100-μM solution and mixed with equal volumes of antioxidants, then transferred to a quartz capillary which was inserted into the cavity of the Bruker ER200SRC ESR spectrometer. ESR spectra were recorded exactly 1 min after initial mixing. ESR measurement conditions were as follows: central field 3475 G, modulation frequency 100 kHz, modulation amplitude 2 G, microwave power 10 mW, scan width 200 G, gain 6.3×10^5, temperature 298 K. The scavenging effects of Pistafolin A and Pistafolin B on DPPH-free radical were calculated by:

$$I = [(Ho - Hx)/Ho] \times 100\%$$

Here, Hx and Ho were the ESR signal intensities of samples with and without antioxidants, respectively.

2.5. Lipid peroxidation

The inhibition of lipid peroxidation in PC liposomes by the two novel gallotannins was performed according to methods of Niki [19]. The multilamellar liposomes containing 5.15 mM PC and 1 mM AMVN were prepared by coevaporation from methanol, followed by vortex stirring and by freeze–thawing for 3 cycles in liquid nitrogen as previously reported [20]. Lipid peroxidation was initiated by heating the liposome solutions in open tubes placed in a 37 °C shaking water bath. After incubation at 37° for 30 min, aqueous solutions of gallotannins or Trolox were added into the liposome solutions. The kinetic formation of phosphatidylcholine hydroperoxide (PCOOH) was measured with HPLC by detection at 234 nm with an Supelco LC-Si column (particle size 5 μm, 4.6 mm × 25 cm) eluted with methanol: 40 mM phosphate (90:10, v/v) [21]. The increase of PCOOH concentration was plotted as a function against reaction time and the corresponding reaction rate of PCOOH formation was calculated. The percentage inhibition of lipid peroxidation was calculated by:

$$I = [(Ko - Kx)/Ko] \times 100\%$$

Here, Kx and Ko were the pseudo first-order rates of PCOOH formation with and without antioxidants, respectively.

2.6. Protein oxidation

The oxidation of BSA was carried out by using the azo compound AAPH as the peroxyl radical generator as reported previously [22]. BSA dissolved in air-saturated 150 mM phosphate buffer (pH 7.3) at a final concentration of 0.5 mg/ml was incubated with AAPH in the presence or absence of antioxidants. Reactions were carried out in open tubes placed in a shaking water bath at 37 °C. After incubation for 2 h, 4 μg of protein samples were subjected to electrophoresis in 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). Gels were stained with 0.15% Coomassie brilliant blue R-250 for 30 min, washed extensively, dried in a gel dryer for 45 min, and photographed. To determine the amount of protein damage, the density of each band was estimated and standardized with respect to the control group. Results showed the average of at least four different measurements.

2.7. DNA oxidation

Protective effects of Pistafolin A and Pistafolin B on DNA oxidative damage were studied by chemiluminescence [23]. Upon oxidation by H_2O_2 in the presence of copper (II)-1,10-phenanthroline complex [$Cu^{II}(OP)_2$], DNA gives a marked chemiluminescence with maximal emission wavelength around 420 nm. Addition of phenolic antioxidants causes significant decrease in the chemiluminescence intensity, which can be used as a criterion for the evaluation of antioxidant capacity against DNA oxidative damage. Experiments were carried out following procedures described previously [24]. Briefly, a 950 μl of reaction mixture containing 350 μM OP, 50 μM $CuSO_4$, 0.1 μg/ml calf thymus DNA, 0.3 mM L-ascorbic acid and certain concentrations of gallotannins were pre-mixed and inserted into the cavity of chemiluminescence detector (BPCL-4 Ultra Weak Chemiluminescence Analyzer equipped with a computerized high sensitive single-photon counter) for analysis. After the addition of 50 μl 2.4 M H_2O_2, the kinetic curve of chemiluminescence was recorded immediately. The percentage inhibition of DNA oxidation was calculated by:

$$I = [(Io - Ix)/Io] \times 100\%$$

Here, Ix and Io were the intensities of chemiluminescence with and without antioxidants, respectively.

2.8. Statistical analysis

All data shown are means of at least three different experiments using four samples per group. One-way ANOVA was performed to compare different groups, followed by a Student's t-test. Statistical significance was accepted when $P<0.05$.

3. Results

3.1. Scavenging of $ABTS^{\bullet+}$

The scavenging activities of Pistafolin A and Pistafolin B against hydrophilic $ABTS^{\bullet+}$ radicals were determined by measuring the decolorization of the $ABTS^{\bullet+}$ radicals at 734 nm. The extent of scavenging of the $ABTS^{\bullet+}$ was plotted as a function of antioxidant concentration, as shown in Fig. 2.

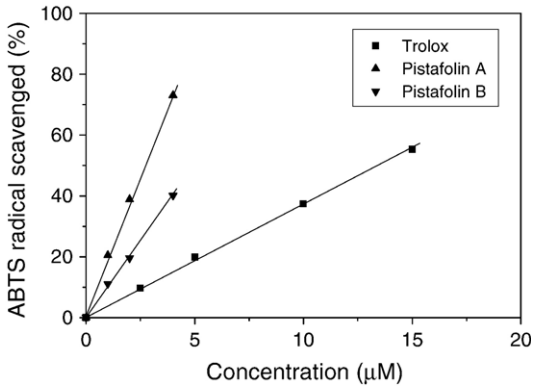

Fig. 2. Scavenging of $ABTS^{\bullet+}$ radicals by antioxidants. $ABTS^{\bullet+}$ solution was mixed with indicated concentrations of antioxidants for 5 min and the absorbance at 734 nm was determined. The scavenging effects were calculated from the absorbance at 734 nm and plotted against the concentration of antioxidants. Data are means of six samples.

Both Pistafolin A and Pistafolin B scavenged $ABTS^{\bullet+}$-free radicals dose-dependently. The IC_{50} values for Pistafolin A and Pistafolin B in scavenging $ABTS^{\bullet+}$ radical were 2.79 and 5.05 μM, respectively. Using Trolox as the control antioxidant, the TEAC (Trolox-equivalent antioxidant capacity) value which quantitatively represents the scavenging capacity of the $ABTS^{\bullet+}$ radical was calculated [17]. The TEAC values of Pistafolin A and Pistafolin B in scavenging $ABTS^{\bullet+}$ radicals were 4.94, and 2.71, respectively.

3.2. Scavenging of DPPH

A 5-lined ESR spectrum (pentad signal) was observed in the ethanolic solution containing 50 μM DPPH, as shown in Fig. 3A. Pistafolin B decreased the signal of DPPH radicals in a concentration-dependent manner and completely eliminated the DPPH radical signal at 15 μM. Pistafolin A, as well as the control antioxidant Trolox, also scavenged DPPH radicals dose-dependently as shown in Fig. 3B. The IC_{50} values for Pistafolin A and Pistafolin B in scavenging DPPH radicals were 3.37 and 5.48 μM, respectively. The TEAC values of Pistafolin A and Pistafolin B in scavenging DPPH-free radicals were 4.23 and 2.70, respectively.

3.3. Inhibition of lipid peroxidation

When incubating PC liposomes containing 5.15 mM of PC and 1 mM of the hydrophobic azo initiator AMVN at 37 °C, lipid peroxidation occurred. The lipid peroxidation product PCOOH increased time-dependently and reached a concentration of 224.6 μM after 240 min as measured by HPLC methods. The pseudo first-order rate for PCOOH formation was calculated as 1.56×10^{-8} M/s. Addition of Pistafolin A, Pistafolin B and Trolox into the reaction systems inhibited the peroxidation of PC liposomes significantly, as shown in Fig. 4A. After the addition of 5 μM Pistafolin A, the pseudo first-order rate for PCOOH formation decreased to 4.8×10^{-9} M/s. After the addition of 10 μM Pistafolin A, the formation of PCOOH was completely inhibited. The inhibition effects of the two gallotannins against the lipid peroxidation were shown in Fig. 4B. The IC_{50} values for Pistafolin A and Pistafolin B in inhibiting lipid peroxidation were 3.30 and 5.82 μM, respectively.

3.4. Inhibition of protein oxidation

Electrophoretic patterns of BSA after incubation with AAPH in the presence or absence of Pistafolin A and the corresponding densitometry were presented in Fig. 5A. The azo compound AAPH induced the oxidative degradation of BSA. Two hours after incubation with 40 mM of AAPH, the density of BSA band decreased to 46.5±5.8% of control. Pretreatment of BSA with 50 μM of Pistafolin A completely prevented the oxidative degradation of BSA. Pistafolin B also showed protective effects on BSA, but was less effective than Pistafolin A (Fig. 5B). Fifty micromolars of Pistafolin B

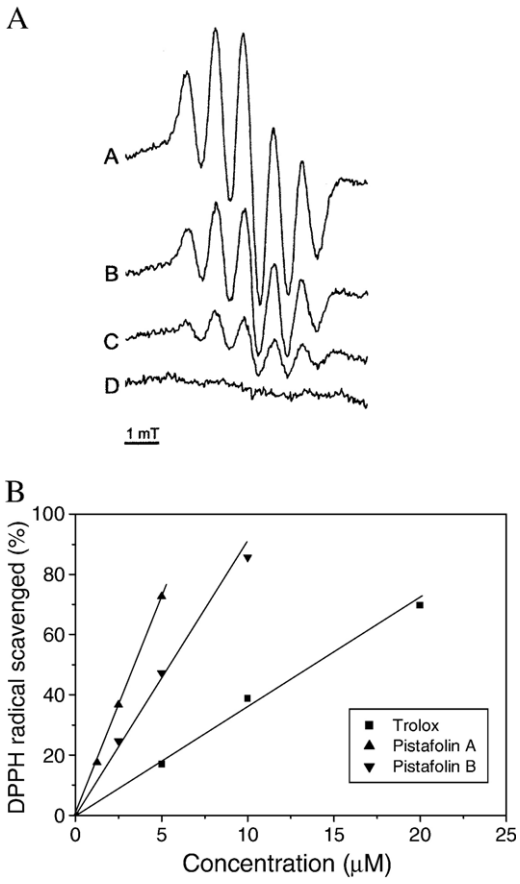

Fig. 3. Scavenging of DPPH radicals by antioxidants. (A) ESR spectra of DPPH-free radicals. DPPH dissolved in ethanol at a concentration of 50 μM was reacted with 0 (A), 2.5 (B), 5 (C) and 10 μM (D) of Pistafolin B for 1 min and the ESR spectra were recorded immediately. (B) Scavenging effects of antioxidants on DPPH radicals. DPPH dissolved in ethanol to a concentration of 50 μM was reacted with indicated concentrations of antioxidants for 5 min and the ESR spectra were recorded immediately. The scavenging effects were calculated from the ESR spectra and plotted against the concentration of antioxidants. Data are means of four samples.

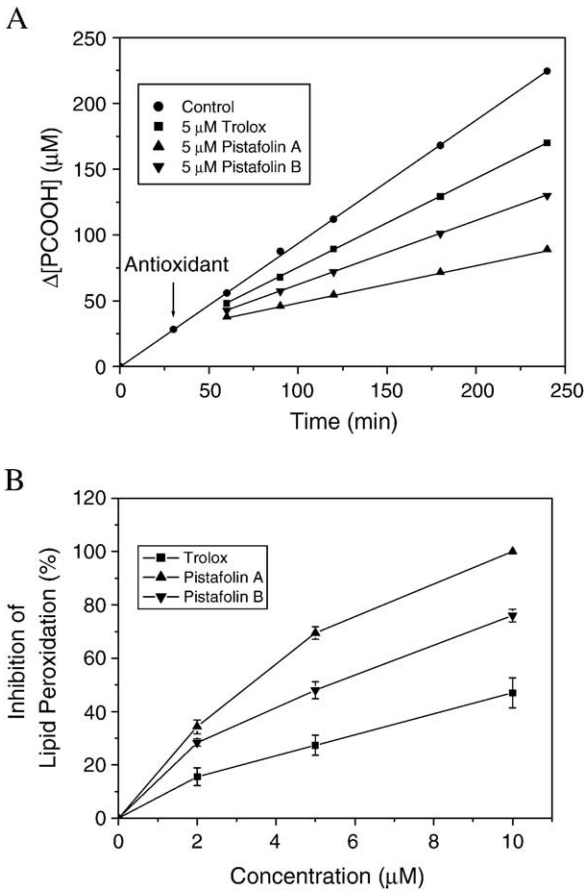

Fig. 4. Inhibition of lipid peroxidation by antioxidants. (A) Kinetics of AMVN-initiated lipid peroxidation. Liposomes containing 5.15 mM PC and 1 mM AMVN were incubated at 37 °C and lipid peroxidation was determined by monitoring the lipid peroxidation product PCOOH. (B) Quantitative determination of the inhibition effects against lipid peroxidation. Data are means ± S.E. of three samples.

restored the BSA band intensity to 76 ± 3.2% of control levels. Trolox exhibited a protective profile similar to that of Pistafolin B. Fifty micromolars of Trolox restored the BSA band intensity to 73 ± 3.7% of control levels (Fig. 5C).

3.5. Inhibition of DNA oxidation

In the presence of copper (II)-1,10-phenanthroline complex [$Cu^{II}(OP)_2$], oxidation of DNA by H_2O_2 gave a strong chemiluminescence (over 8000 counts/s) with maximal emission wave length around 400 to 420 nm, as shown in Fig. 6A. Addition of Pistafolin B caused significant decrease in the DNA-dependent chemiluminescence. In a reaction mixture containing 1 μM of Pistafolin B, the chemiluminescence intensity decreased to 5980 counts/s, suggesting that Pistafolin B effectively inhibited the oxidation of DNA. Pistafolin A also exhibited significant inhibition effect on DNA oxidative damage, as shown in Fig. 6B. The IC_{50} values for Pistafolin A and Pistafolin B in inhibiting DNA oxidative damage were 1.8 μM and 5.4 μM, respectively. Trolox showed no apparent effect on DNA oxidation.

4. Discussion

Biomacromolecules including lipids, proteins and DNA are prone to ROS-mediated oxidative damage. Oxidative damage to these molecules is associated with a variety of pathological processes, including atherosclerosis, arthritis, muscular dystrophy, cataractogenesis, pulmonary dysfunction, cancer and several neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis [4–6]. Therefore, it is essential to identify and characterize compounds that show pharmacological activity against oxidative damage of biomacromolecules.

P. weinmannifolia is a plant widely found in Yunnan province of China. The leaves of this plant, which can be used as a traditional Chinese medicinal herb, show anti-inflammatory and antimutagenic/anticarcinogenic activities [14]. Phytochemical studies revealed that the leaves of *Pistacia weinmannifolia* are rich in gallotannins and related phenolic compounds. Among them, two novel gallotannins, Pistafolin A and Pistafolin B, are identified [15]. We

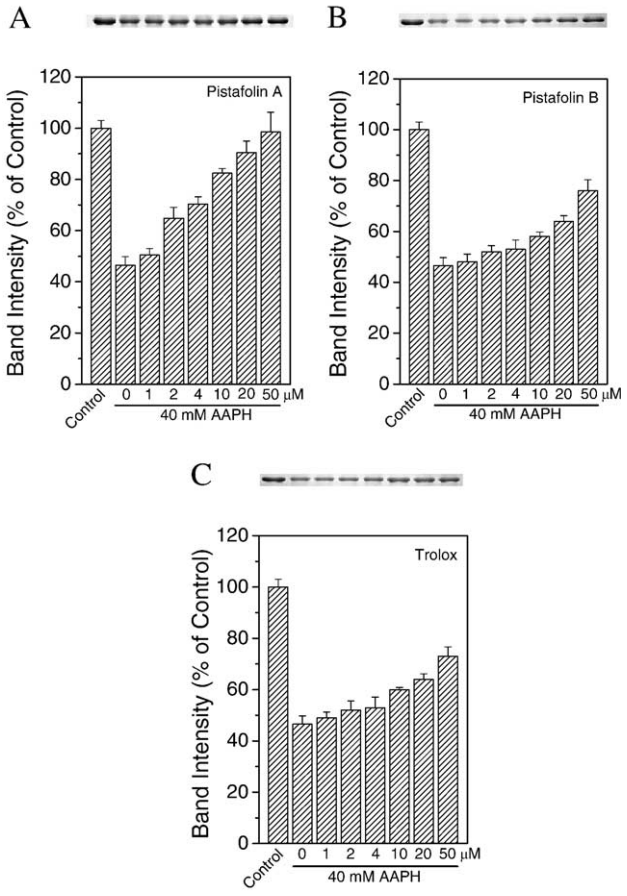

Fig. 5. Protection of BSA oxidative damage by antioxidants. BSA dissolved in phosphate buffer (pH 7.3) was preincubated with or without indicated concentrations of Pistafolin A (Fig. 6A), Pistafolin B (Fig. 6B) or Trolox (Fig. 6C) and then incubated with 40 mM AAPH at 37 °C for 2 h. The oxidative damage of BSA was quantified by SDS-PAGE. Data presented here show the typical electrophoretic pattern and the histogram of the means ± S.E. of four different experiments.

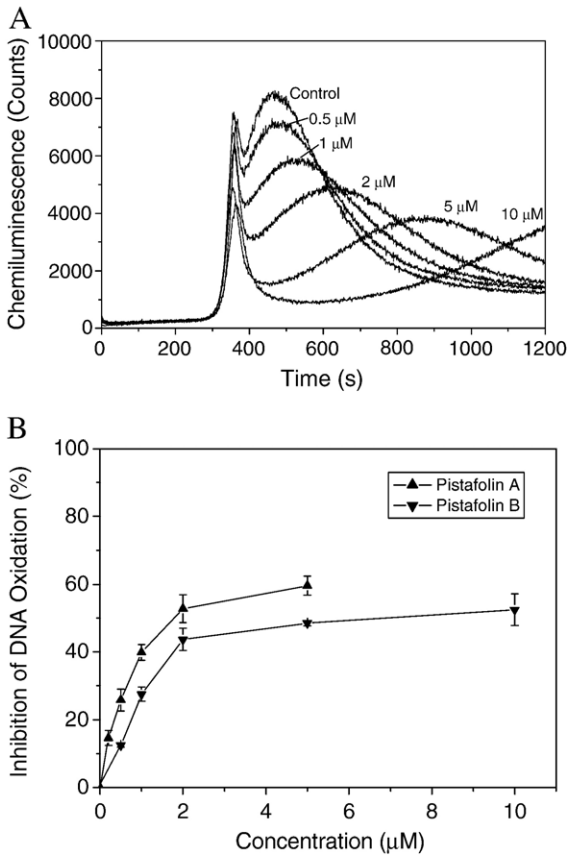

Fig. 6. Protection of DNA oxidative damage by antioxidants. (A) Kinetics of DNA-dependent chemiluminescence. Reaction mixture containing 350 μM OP, 50 μM $CuSO_4$, 0.1 μg/ml calf thymus DNA, 0.3 mM L-ascorbic acid was mixed with indicated concentrations of Pistafolin B and inserted into BPCL-4 Ultra Weak Chemiluminescence Analyzer. After the addition of 50 μl 2.4 M H_2O_2, the kinetics of Chemiluminescence was recorded immediately. (B) Quantitative determination of the inhibition effects against DNA oxidative damage. Data are means ± S.E. of three samples.

reported previously that Pistafolin A is a potent scavenger on both hydroxyl radicals and superoxide anion and protects neurons form oxidative damage [16]; however, its antioxidant mechanisms remain to be investigated.

We report here our studies on the antioxidant effects of the two novel gallotannins, Pistafolin A and Pistafolin B, isolated from the leaves of *Pistacia weinmannifolia*. The evaluation of their antioxidant effects was based on the protection of biomacromolecules against oxidative damage. First, the effect of Pistafolin A and Pistafolin B against peroxidation of lipids in PC liposomes was investigated. Compared to other cellular components, biomembranes rich in lipids are especially prone to oxidative damage. ROS attack the polyunsaturated fatty acid side chains of lipids and initiate a series of free radical-mediated chain reactions termed as lipid peroxidation. During lipid peroxidation, many kinds of cytotoxic compounds such as malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE) are generated. Furthermore, as the result of severe oxidative damage, the membrane integrity may be lost and the cellular function may be compromised. In the present investigation, the hydrophobic azo compound AMVN was used as the free radical initiator. AMVN can decompose to form carbon-centered radicals that react swiftly with O_2 to yield peroxyl radicals which initiate the lipid peroxidation chain reactions. Incubation of PC liposomes containing AMVN induced the peroxidation of lipids, as measured by monitoring the kinetic formation of lipid peroxidation product PCOOH. The peroxidation of PC was inhibited dose-dependently by the two novel gallotannins. The IC_{50} values for Pistafolin A and Pistafolin B in inhibiting lipid peroxidation were 3.30 and 5.82 μM, respectively.

We also investigated the effects of Pistafolin A and Pistafolin B on protein oxidation. ROS attack the polypeptide backbone of proteins and lead to the formation of alkyl, alkoxyl and alkylperoxyl radical intermediates, which set the stage for cleavage of the peptide bond via several means. Tryptophan, histidine and cysteine residues are especially prone to oxidative attack. The oxidatively modified proteins may undergo spontaneous protein fragmentation and cross-linking or exhibit a substantial increase in proteolysis. The inactivation of key enzymes or receptors by oxidative protein damage may cause the dysfunction of cells [25,26]. In the present investigation, we used the AAPH-induced BSA oxidative damage as the experimental model [22]. When incubating the protein BSA with the hydrophilic azo compound AAPH which generates peroxyl radicals, BSA underwent oxidative damage, which could be detected by SDS-PAGE. Both Pistafolin A and Pistafolin B effectively protected the protein from AAPH-induced oxidative damage. Fifty micromolars of Pistafolin A completely blocked the oxidative damage of BSA.

DNA is another sensitive biotarget for ROS-mediated oxidative damage [27]. ROS cause extensive base modification as well as single-strand breaks in both mitochondrial and genomic DNA. Oxygen-based reactants that escape detoxification by cellular antioxidant systems are responsible for roughly an estimated 10,000 DNA base modifications per cell per day [28], which have long been considered as an important underlying event in chronic inflammation leading to mutation and carcinogenesis [29]. In the present investigation, we investigated the protective effect of the two novel gallotannins on DNA oxidative damage by ultraweak chemiluminescence methods. Both Pistafolin A and Pistafolin B inhibited the chemiluminescence generated during the copper-catalyzed oxidative damage of DNA. The IC_{50} values for Pistafolin A and Pistafolin B in inhibiting DNA oxidative damage were 1.8 μM and 5.4 μM, respectively.

Polyphenols, including flavonoids, can exert their antioxidant activity by inhibiting the activities of enzymes, including lipooxygenase and cyclooxygenase, by chelating metal ions, and, most importantly, by scavenging free radicals [30]. Generally, polyphenols are potent free radical scavengers because phenolic groups are excellent nucleophiles [31]. To understand the mechanisms underlying the

antioxidant action of Pistafolin A and Pistafolin B, their direct scavenging effects on free radicals were measured.

We first investigated the elimination of stable hydrophilic $ABTS^{\bullet+}$-free radicals by Pistafolin A and Pistafolin B. Both of the two novel gallotannins scavenged the $ABTS^{\bullet+}$-free radicals dose-dependently and were more effective than the well-known antioxidant standard, Trolox. The Trolox-equivalent antioxidant capacities (TEAC value) of Pistafolin A and Pistafolin B in scavenging $ABTS^{\bullet+}$ radicals were 4.94, and 2.71, respectively. By the same method, the TEAC value of gallic acid was determined as 3.31. Rice-Evans et al. reported the TEAC value of Quercetin (a typical flavonoid) and Cyanidin (a typical anthocyanidin) were 2.77, and 2.30, respectively [17]. Compared with Quercetin, Cyanidin or gallic acid, Pistafolin A is much more effective in scavenging $ABTS^{\bullet+}$-free radicals.

The efficacy of Pistafolin A and Pistafolin B in scavenging hydrophobic-free radicals was also investigated. DPPH, a stable hydrophobic-free radical, was scavenged dose-dependently by both Pistafolin A and Pistafolin B. Compared with Trolox standard, the two novel gallotannins were more effective in the elimination of DPPH-free radicals. The Trolox-equivalent antioxidant capacity of Pistafolin A and Pistafolin B in scavenging DPPH radicals were 4.23, and 2.70, respectively.

By donating hydrogen in the active hydroxyl groups to form resonance-stabilized phenoxyl radicals, polyphenols are usually called hydrogen-donating antioxidants. In the present investigation, both Pistafolin A and Pistafolin B were potent hydrogen donors, which eliminated the stable $ABTS^{\bullet+}$- and DPPH-free radicals, and blocked the oxidative chain reactions of biomacromolecules by acting as chain-break antioxidants. Compared with Pistafolin B, Pistafolin A was a more effective antioxidant. The antioxidant efficiencies of Pistafolin A and Pistafolin B are closely related to their molecular structure. As shown in Fig. 1, Pistafolin A contains three galloyl moieties whilst Pistafolin B contains two galloyl moieties. The galloyl moieties containing active phenolic hydroxy groups were major contributors to the antioxidant capacity [32], so Pistafolin A with three galloyl moieties showed stronger antioxidant ability than Pistafolin B with two galloyl moieties. Our previous reports indicated that the stability of the resonance-stabilized phenoxyl radicals contributed significantly to the free radical scavenging ability of polyphenols compounds [33]. Because of the existence of the third galloyl moiety, the resonance-stabilized phenoxyl radicals formed by Pistafolin A should be more stable than the phenoxyl radicals formed by Pistafolin B. This may also influence the antioxidant ability of the two gallotannins.

5. Conclusion

Herein we reported the antioxidant properties of two novel gallotannins, Pistafolin A and Pistafolin B. Both of the gallotannins are potent free radical scavengers, and they prevented the biomacromolecules from oxidative damage effectively. Their antioxidant capacities are closely related to their molecule structures.

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