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Scavenging of reactive oxygen species and prevention of oxidative neuronal cell damage by a novel gallotannin, Pistafolia A

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Abstract

Pistafolia A is a novel gallotannin isolated from the leaf extract of *Pistacia weinmannifolia*. In the present investigation, the ability of Pistafolia A to scavenge reactive oxygen species including hydroxyl radicals and superoxide anion was measured by ESR spin trapping technique. The inhibition effect on iron-induced lipid peroxidaiton in liposomes was studied. The protective effects of Pistafolia A against oxidative neuronal cell damage and apoptosis induced by peroxynitrite were also assessed. The results showed that Pistafolia A could scavenge both hydroxyl radicals and superoxide anion dose-dependently and inhibit lipid peroxidation effectively. In cerebellar granule cells pretreated with Pistafolia A, peroxynitrite-induced oxidative neuronal damage and apoptosis were prevented markedly. The antioxidant capacity of Pistafolia A was much more potent then that of the water-soluble analog of vitamin E, Trolox. The results suggested that Pistafolia A might be used as an effective natural antioxidant for the prevention and cure of neuronal diseases associated with the production of peroxynitrite and related reactive oxygen species.

Keywords: Pistafolia A; gallotannin; free radical; cerebellar granule cell; oxidative stress; apoptosis

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Introduction

In our body, a number of biochemical reactions involve the generation of reactive oxygen species (ROS). Under normal conditions, the balance between the generation and diminishing of ROS is controlled by the antioxidant defense system, which includes both enzymes with antioxidant activities and nonenzymatic factors. The former includes superoxide dismutase, catalase, and glutathione peroxidase. The latter includes some low molecular weight compounds such as antioxidant vitamins (ascorbic acid, tocopherols, and carotenoids) and some other antioxidant micronutrients obtained from diet [1]. Under certain pathological conditions, when ROS are not effectively diminished by the antioxidant defense system, oxidative stress results. It has been well accepted that oxidative stress is linked to the onset of various diseases such as coronary heart disease, rheumatoid arthritis, cancer and the degenerative diseases associated with aging [2,3]. Accordingly, supplement of dietary antioxidant vitamins and micronutrients that can enhance the antioxidant capacity of the body will help to attenuate the damage of body induced by oxidative stress, and can be used as potential therapeutic or preventive drugs for diseases associated with oxidative stress.

Recently, the antioxidant properties of natural flavonoids and related phenolic compounds extracted from dietary or herb plants have aroused much attention. Plants rich in these compounds are ideal source of natural antioxidants. *Pistacia weinmannifolia* J. Poisson ex Franch. (Anacardiaceae) is a shrub or an arbor widely found in Yunnan province of China. The leaves of this plant are used as daily drink and herb against dysentery, enteritis, influenza, traumatic bleeding, headache and lung cancer [4]. Phytochemical studies revealed that the leaves of *Pistacia weinmannifolia* are rich in gallotannins and related phenolic compounds. In the previous research, Hou et al. isolated over 18 different gallotannins and relevant compounds from the leaf extract of *Pistacia weinmannifolia*, among which two new gallotannins, pistafolins A and B are identified [5]. In general, phenolic compounds isolated from plants could scavenge free radicals effectively in vitro [6]. However, the free radical scavenging ability of gallotannin components isolated from the leaf extract of *Pistacia weinmannifolia* has not been examined systematically so far. In the present investigation, we studied the free radical scavenging effects of pistafolin A, and its protective effect on oxidative neuronal cell damage were assessed. We found that pistafolin A could scavenge both hydrophilic and hydrophobic free radicals effectively, and could protect neuronal cells from oxidative damage as well. The cytotoxic effect of pistafolin A is quite low. The present investigation suggested that pistafolin A might be used as a potential drug for neuronal diseases associated with the excessive generation of free radicals.

Materials and methods

Materials

Albino Wistar rats were purchased from the Experimental Animal Center of Peking University Medical School, China. Acridine orange (AO), propidium iodide (PI), 3-morpho-

linosydnonimine-*N*-ethylcarbamide (SIN-1), dilinoleoyl-L-α-phosphatidylcholine (DLPC), poly-L-lysine, hypoxanthine, xanthine oxidase and spin traps 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) and α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (POBN) were purchased from Sigma. 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was purchased from Aldrich. Dulbecco's modified Eagle medium (DMEM), cell culture supplements, fetal bovine serum, and trypsin (1:250) were products of Gibco BRL. Pistafolin A isolated from the leave extract of *Pistacia weinmannifolia* is purified by HPLC and identified by spectral methods in the Laboratory of Phytochemistry, Kunming Institute of Botany, Chinese Academy of Science, as reported previously [5]. Its structure was shown in Fig. 1. Other chemicals were made in China.

Scavenging of Hydroxyl radicals

The scavenging effect of the gallotannin Pistafolin A on hydroxyl radicals was studied by ESR spin trapping using DMPO as the spin trap. Hydroxyl radicals were generated by Fenton reaction. Briefly, reaction mixtures containing 0.3 mM $FeSO_4$, 0.05 M DMPO and indicated concentration of Pistafolin A were premixed in a test tube, and the reaction was initiated by the addition of H_2O_2 (final concentration 0.25 M). Then the reaction mixture was transferred into quartz capillary and fitted into the cavity of Varian E109 ESR spectrometer for ESR measurement. The spectrum were recorded exactly 90 s after the addition of H_2O_2.

Scavenging of Superoxide Anion

Superoxide radicals were generated with hypoxanthine and xanthine oxidase and determined by ESR spin trapping [6]. Reaction mixtures containing 0.5 mM hypo-

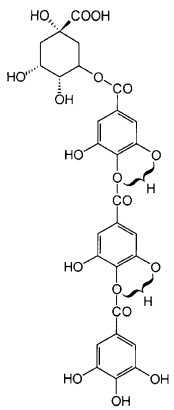

Fig. 1. Structure of Pistafolin A.

xanthine, 0.96 mM DETAPAC, 0.1 M DMPO and indicated concentration of Pistafolin A were premixed in a test tube, and the reaction was initiated by the addition of 0.05 U/ml xanthine oxidase. Then the reaction mixture was transferred into quartz capillary for ESR measurement. The ESR spectra were recorded 40 s after the addition of xanthine oxidase.

Inhibition of lipid peroxidation

Inhibition effect of the gallotannin Pistafolin A on lipid peroxidation in aqueous multilamellar phospholipid liposomes prepared from dilinoleoyl-L-α-phosphatidylcholine (DLPC) was monitored by ESR spin trapping technique using α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (POBN) as the spin trap. The signal intensity of POBN-lipid radical spin adducts was used as an index of lipid peroxidation. Briefly, liposomes (DLPC concentration 6 mM) were mixed with 0.3 mM $FeSO_4$, 0.9 mM $FeCl_3$ and 0.03 M POBN. After incubation at 37 °C for 1 h, the reaction mixture was transferred into quartz capillary and the ESR spectrum was recorded immediately.

ESR measurtement conditions

All ESR spectra were recorded at room temperature (25 °C) by a Varian E-109 spectrometer with measurement conditions as: X-band, central magnetic field 325 mT, sweep width 20 mT, microwave power 20 mW, 100 kHz modulation with amplitude 0.1 mT (DMPO as spin trap) or 0.2 mT (POBN as spin trap), time constant 0.128 s.

Cell culture and drug treatment

Primary cultures of rat cerebellar granule cells were obtained from 7-day-old Wistar rats following procedures described previously with slight modification [7]. Cells were plated on 35 mm Petri-dishes (2.5×10^6 cells in 2 ml/dish) or 24-well multidishes (5×10^5 cells in 0.4 ml/well) precoated with poly-L-lysine. Culture medium consisted of DMEM supplemented with KCl (19.6 mM), glutamine (2 mM), HEPES (10 mM) and fetal bovine serum (10%, v/v). Cells were maintained at 37 °C in a humidified 5% CO_2–95% air atmosphere. Twenty-four h after plating, the growth of non-neuronal cells was suppressed by the addition of cytosine arabinoside (10 μM). Mature cerebellar granule cells (10 days in vitro) were used in the present investigation.

After 10 days in vitro, cerebellar granule cells were exposed to peroxynitrite by treatment with SIN-1, which generates nitric oxide and superoxide anion simultaneously upon decomposition. Briefly, cells were washed twice with Hanks' buffered salt solution (HBSS) and incubated in HBSS at 37 °C for 15 min. Aliquots of freshly prepared stock solution of SIN-1 were added to cells. After incubation at 37 °C for 1 h, cells were washed twice with HBSS, the original culture medium was restored, and cells were cultured for indicated time. In some experiments, cells were pre-incubated with Pistafolia A for 15 min, washed twice with HBSS, and then exposed to SIN-1.

Measurement of oxidative neuronal cell damage

The loss of cell membrane integrity, which characterizes the irreversible damage of cell, was measured by determining the lactate dehydrogenase (LDH) released from the injured cells into the culture medium [8]. After treatment with 1 mM SIN-1 for 1 h and being cultured in fresh medium for indicated time, the LDH activity in the culture supernatant was determined using an LDH clinical diagnosis kit (Zhongsheng Hightech Bioengineering, Beijing, China). The percentage of LDH released into the medium was defined as the ratio of LDH activity in the culture medium to the total LDH activity, where the total LDH activity represents the LDH activity in the cells and the medium. The total LDH activity was measured in sister cultures by freezing/thawing cells rapidly.

Morphological observation

The nuclear morphology of cerebellar granule cells was observed by fluorescence microscopy. In brief, after treatment with 1 mM SIN-1 for 1 h and being cultured in fresh medium for 8 h, cells were stained with 1 μM Acridine orange, and the image of Acridine orange fluorescence was observed with a Bio-Rad MRC 1024MP laser confocal scanning microscope at 488 nm excitation and 520 nm emission.

Flow cytometry analysis of apoptosis

The number of apoptotic cells was determined by flow cytometry. In brief, after treatment with SIN-1 and being cultured in fresh medium for 8 h, cells were collected into cold PBS, washed twice and fixed with 70% ethanol at 20 °C overnight. Then cells were treated with 0.1 mg/ml RNase A at 37 °C for 30 min, and stained with PI. The DNA content of 10,000 cells was determined with a Beckman Dickinson FACScan flow cytometry. The rate of apoptotic nuclei was calculated with the standard software provided by the manufacturer.

Statistical analysis

Each experiment was performed at least three times and results are presented as mean ± SD. Statistical analyses were performed using one-way ANOVA or Student's *t*-test. A probability of <0.05 was considered significant.

Results

Scavenging of hydroxyl radicals

Pistafolia A scavenged hydroxyl radicals dose-dependently as shown in Fig. 2. Trolox, a water-soluble analog of vitamin E, also shows scavenging effect on hydroxyl radicals. The

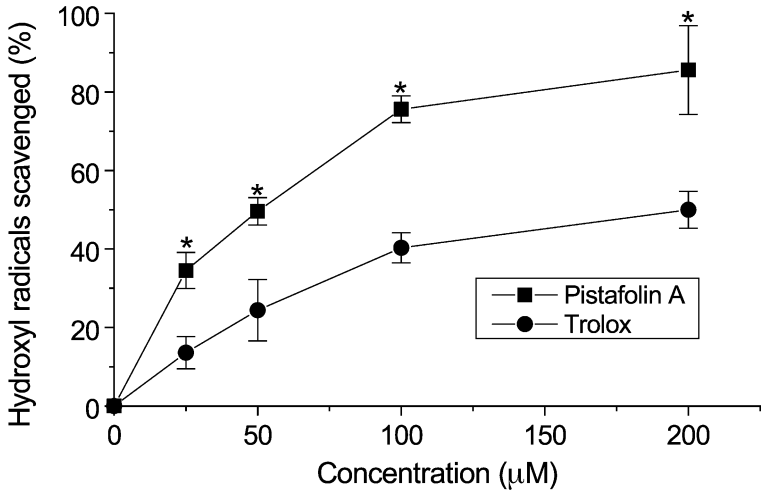

Fig. 2. Scavenging effect of Pistafolin A on hydroxyl radicals. Data are mean ± SD of four samples. *: $p<0.05$ in comparison with Trolox.

IC50 of Pistafolia A in scavenging of hydroxyl radicals is 50.4 μM, which is much more lower than that of Trolox (202.6 μM).

Scavenging of superoxide anion

Fig. 3 shows the scavenging effect of Pistafolia A on superoxide anion generated by hypoxanthine/xanthine oxidase. Pistafolia A scavenged superoxide anion with an IC50 of

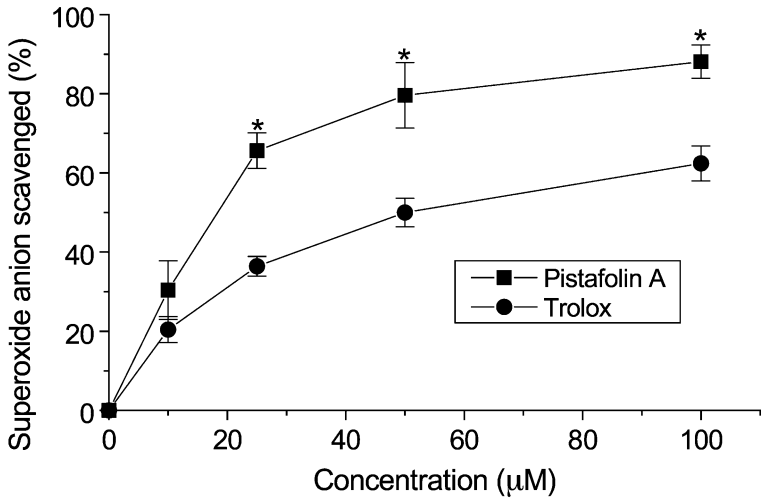

Fig. 3. Scavenging effect of Pistafolin A on superoxide anion. Data are mean ± SD of four samples. *: $p<0.05$ in comparison with Trolox.

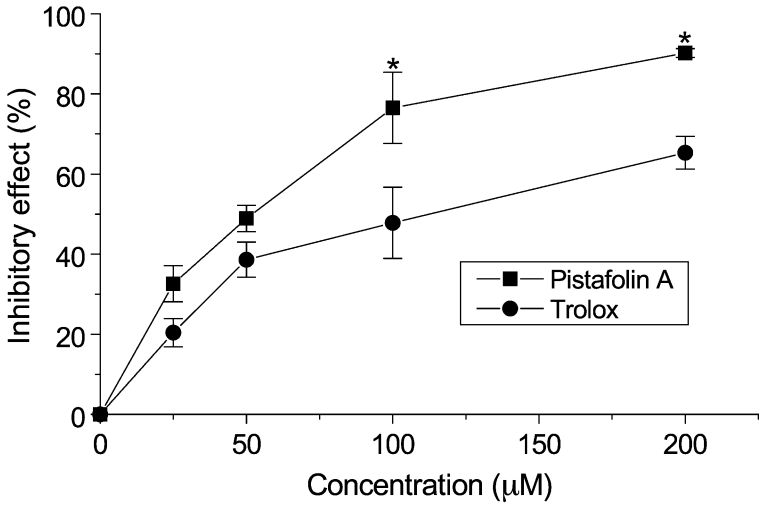

Fig. 4. Inhibitory effect of Pistafolin A on lipid peroxidation in DLPC liposomes. Data are mean ± SD of four samples. *: $p<0.05$ in comparison with Trolox.

14.4 μM. Trolox shows less efficacy in scavenging of superoxide anion, the IC 50 of which is 50.2 μM.

Inhibition of lipid peroxidation

Pistafolia A effectively inhibited iron-induced lipid peroxidation in DLPC liposomes as shown in Fig. 4. The IC50 of Pistafolia A in scavenging of hydroxyl radicals is

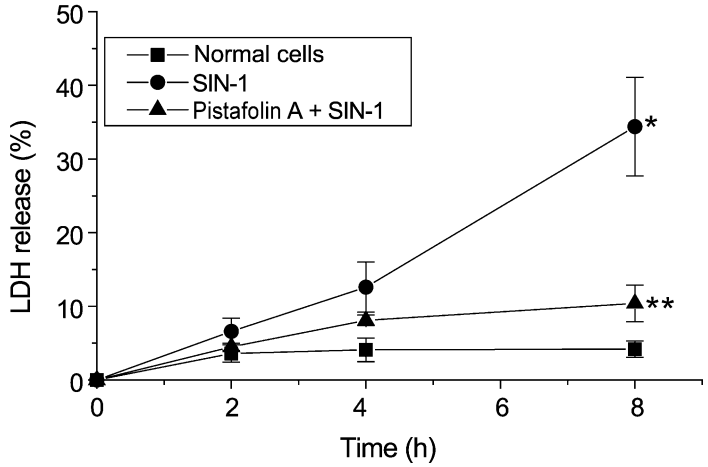

Fig. 5. Time-course of cell death induced by SIN-1. Cells were treated with 1 mM SIN-1 for 1 h, cultured in fresh medium for indicated time, and the cell membrane integrity that characterized the viability of cells was assessed by LDH efflux assay. Data are means ± SD of six samples. *: $p<0.05$ in comparison with normal cells; **: $p<0.05$ in comparison with cells treated with 1 mM of SIN-1.

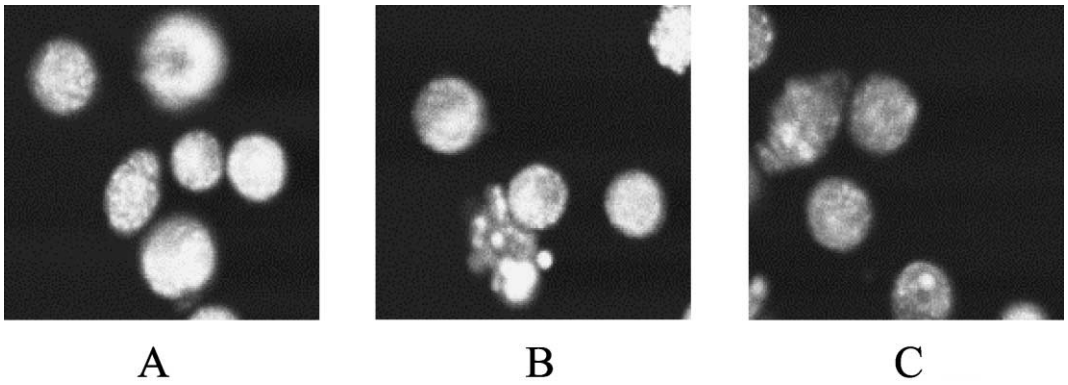

Fig. 6. Nuclear morphology. Cells were treated with 1 mM SIN-1 for 1 h, cultured in fresh medium for 8 h, stained with 1 μM Acridine Orange, and observed under a Bio-Rad Model MRC1024EC Laser Confocal Scanning Microscope. A, Nuclear morphology of normal neurons; B, cells treated with SIN-1, which showed typical morphology of apoptosis (nuclear fragmentation); C, cells pretreated with 20 μM of Pistafolin A, which protected neurons from apoptosis effectively.

48.3 μM. Trolox also effectively inhibited lipid peroxidation, the IC50 of which is 100.5 μM.

Prevention of oxidative neuronal damage

When the cell membrane integrity is damaged, cytoplasmic enzyme LDH is released into the culture medium. Exposure of cells to SIN-1 (peroxynitrite donor) causes time-dependent LDH leakage as shown in Fig. 5, suggesting that peroxynitrite induced cell

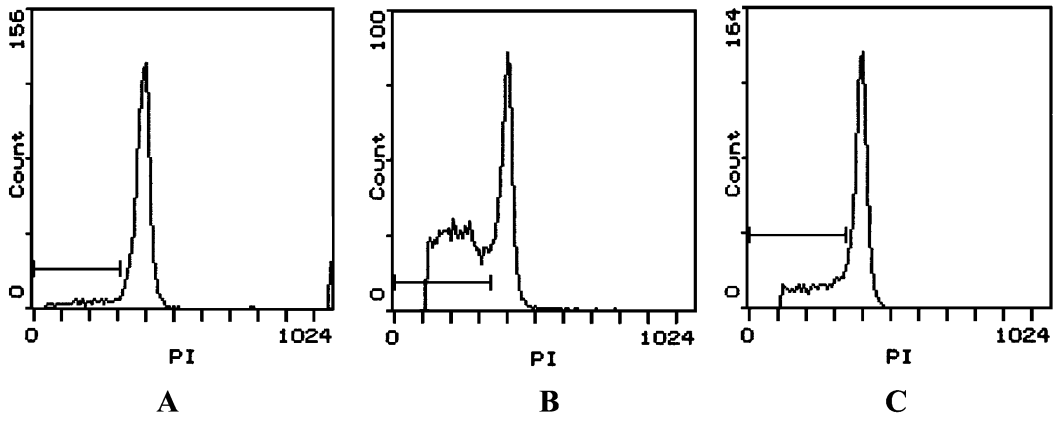

Fig. 7. Typical DNA content histograms. Cells were treated with 1 mM SIN-1 for 1 h, cultured in fresh medium for 8 h, and the cellular DNA content was determined by flow cytometry. A, normal cells; B, cells treated with SIN-1; C, cells pretreated with 20 μM of Pistafolin A.

death. Pretreating cells with 20 μM of Pistafolia A prevents cells from peroxynitrite-induced lysis effectively. Trolox did not show protective effect on neuronal cells even at high concentration (1 mM).

Morphological characteristics of apoptosis

To assess whether neuronal cells underwent apoptosis after exposure to the peroxynitrite donor SIN-1, we examined the nuclear morphology by Laser Confocal Scanning Microscopy. Morphological observation showed typical apoptotic features such as nuclear fragmentation and formation of apoptotic bodies in SIN-1-treated neuronal cells (Fig. 6B), suggesting peroxynitrite induced apoptosis in cerebellar granule cells. In cells pretreated with 20 μM of Pistafolia A, no apparent morphological alteration occurred (Fig. 6C).

Quantitative determination of apoptotic percentage

The number of apoptotic cells was quantified by flow cytometry. Fig. 7 showed the typical DNA content histograms of cerebellar granule cells. The percentages of normal (diploid DNA content) and apoptotic cells (subdiploid DNA content) were calculated from the histogram according to the DNA content. In normal cultures, only 8.3 ± 3.3% of total cells underwent apoptosis (A); in cultures treated with SIN-1, up to 38.7 ± 11.3% of cells are apoptotic cells (B); in cultures pretreated with 20 μM of Pistafolia A, the percentage of apoptotic cells is 16.8 ± 4.2%.

Disscussion

Recently, the antioxidant properties of various flavonoids and related phenolic compounds have been studied extensively by Rice-Evans and coworkers [9,10] as well as by many other research groups including our laboratory [11,12]. Generally, flavonoids are potent antioxidants in vitro. However, many other natural polyphenols such as gallotannins also show potent free radical-scavenging effect [13,14]. *Pistacia weinmannifolia* is a plant widely found in Yunnan province of China. The leaves of this plant can be used as daily drink and a substitute of tea. *Pistacia weinmannifolia* is also used as a herb against dysentery, enteritis, influenza, traumatic bleeding, headache and lung cancer [4]. Phytochemical studies revealed that the leaves of *Pistacia weinmannifolia* are rich in gallotannins and related phenolic compounds, suggesting that the pharmacological effects of *Pistacia weinmannifolia* might be, at least in part, due to the fact that gallotannins in the leaf are potent free radicals. However, the antioxidant properties of gallotannins extracted from the leaves of *Pistacia weinmannifolia* have not been studied yet. In this regard, studying the antioxidant effect of leave extract of *Pistacia weinmannifolia* is very significant. In the present investigation, we systematically examined the antioxidant scavenging properties of Pistafolia A, a novel gallotannin isolated from the leaf extracted of *Pistacia weinmannifolia* for the first time. The results showed that Pistafolia A is a potent scavenger on free radicals. In comparison with the

water-soluble analog of α-tocophorol, Trolox, Pistafolia A is several folds more effective in scavenging hydroxyl radicals and superoxide anion.

We also investigated the protective effects of Pistafolia A against peroxynitrite-induced oxidative neuronal damage. Under certain pathologic conditions (for example, ischemia/reperfusion injury), simultaneous production of superoxide anion and nitric oxide may occur in the central nervous system (CNS), potentially leading to the formation of peroxynitrite [15]. Both peroxynitrite and its breakdown product hydroxyl radical-like species are potent oxidants, which can oxidize biomacromolecules including membrane, protein and DNA, and cause oxidative injury and induce apoptosis in different cell types. It has been proposed that peroxynitrite-induced neuronal injury might be involved in different neuronal disorders such as brain ischemia, Alzheimer's disease, Parkinson's disease and multiple sclerosis. Accordingly, antioxidants may be effective drugs for these diseases associated with ROS [16]. In the present investigation, we used peroxynitrite-induced oxidative neuronal cell damage as the experimental model. Exposure of cerebellar granule cells to peroxynitrite donor SIN-1 induced oxidative damage, and resulted in apoptosis finally. Pre-treatment with the novel antioxidant EPC-K1 effectively attenuated peroxynitrite-induced oxidative damage and apoptosis. We also tested the cytotoxic effect of Pistafolia A on cerebellar granule cells. Pistafolia A showed only slight influence on the viability of neuronal cells up to 100 μM. The results of present study suggested that the extract of *Pistacia weinmannifolia* might be used as effective antioxidants for neuronal diseases associated with the over production of free radicals.

Conclusion

A novel gallotannin isolated from the leaf extract of *Pistacia weinmannifolia*, Pistafolia A is a potent scavenger on free radicals and an effective inhibitor on lipid peroxidation. Pistafolia A also attenuates the oxidative neuronal cell damage in primary cultures of cerebellar granule cells induced by peroxynitrite. The results of present investigation suggested that the gallotannin-rich *Pistacia weinmannifolia* extract might be used as potential drugs for the therapy and prevention of neuronal diseases associated with the overproduction of ROS.

Acknowledgments

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