Neuroprotective Effects of a Novel Peptide Purified from Venison Protein

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A novel antioxidative peptide (APVPH I, antioxidative peptides from venison protein hydrolysates I) was purified from venison by enzymatic hydrolysis, column chromatography of DEAE-Sephacel, and high-performance liquid chromatography. The molecular mass of the purified peptide was found to be 9,853 Da and the amino acid sequences of the purified peptide was Met-Gln-Ile-Phe-Val-Lys-Thr-Leu-Thr-Gly. The purpose of this study was to evaluate the effects of APVPH I against H₂O₂-induced neuronal cells damage in PC-12 cells. Antioxidative enzyme levels in cultured neuronal cells were increased in the presence of the peptide. In addition, APVPH I inhibited productions of nitric oxide (NO), reactive oxygen species (ROS), malondialdehyde (MDA), and cell death against H₂O₂-induced neuronal cell damage in PC-12 cells. It was presumed to be APVPH I involved in regulating the apoptosis-related gene expression in the cell environment. The present results indicate that APVPH I substantially contributes to antioxidative properties in neuronal cells.

Keywords: Venison, enzymatic hydrolysis, antioxidative peptide, neuronal cell, neuroprotective effects

ROS have been implicated in more than one hundred diseases, including malaria, acquired immunodeficiency syndrome, heart disease, stroke, arteriosclerosis, diabetes, cancer, and gastric ulcer [16]. In addition, excess production of ROS in the brain has been implicated as a common underlying factor for the etiology of a number of neurodegenerative diseases, including Alzheimer’s disease (AD), Parkinson’s disease (PD), and stroke. Therefore, the development and utilization of effective antioxidants are desired.

APtosis occurs mainly through two pathways. In the death receptor pathway, receptor ligation leads to activation of caspase-8. In the mitochondrial pathway, on the other hand, various apoptosis-inducing triggers such as DNA damage and oxidative stress cause activation of caspase-9. In these two different pathways, activated initiators caspase-8 and caspase-9 finally activate caspase-3, the downstream caspase. The mitochondrial pathway is regulated by Bcl-2 family proteins, the pro-apoptotic Bax and the antiapoptotic Bcl-2 [24].

Recently, other potent antioxidative and radical-scavenging peptide sequences have been identified from animal resources such as porcine mussel and milk casein hydrolysates, and some amino acids are important for antioxidation [29]. The potential of milk to release biologically active peptides has been widely studied [8], but less is known about other protein-rich foods such as meat. Since little is known about peptides in meat, we investigated the antioxidative peptide of venison hydrolysates. In a previous paper [17], we described the process to obtain the purified peptide of venison protein by enzymatic hydrolysis, column chromatography of DEAE-Sephacel, and high performance liquid chromatography. The free-radical scavenging activity of APVPH I, which was estimated using an electron spin resonance spectrometer, was even more high than Vitamin C. It was presumed that the free-radical scavenging activity of APVPH I could be attributed to the presence of a hydrophobic amino acid, such as Met, Val and Leu, in the identified peptide.

In the present study, we aimed to evaluate the protective effects of APVPH I against the neuronal cell death induced by oxidative stress on PC-12 cells.

MATERIALS AND METHODS

Materials
PC-12 (ATCC CRL-1721), the standard model for neuronal function studies, was obtained from the American Type Culture Collection (Manassas, VA, U.S.A.). Propidium iodide (PI), Glutathione Peroxidase

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Cellular Activity Assay Kit, Catalase Assay Kit, Glutathione S-transferase Assay Kit, Bicinchoninic Acid (BCA) Protein Assay Reagent Kit, and Hoechst 33342 were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Dulbecco’s Modified Eagle’s Medium (DMEM), fetal bovine serum (FBS), penicillin–streptomycin, and TRizol were obtained from Invitrogen Corporation (Carlsbad, CA, U.S.A.). Annexin V-fluorescein Isothiocyanate (FITC) Apoptosis Detection Kit I was purchased from BD Science (San Diego, CA, U.S.A.). RNase A and Tween-20 were supplied by Novagen (Madison, WI, U.S.A.) and USB (Cleveland, OH, U.S.A.), respectively. Antibodies for iNOS, Bcl-2, Bax, and cytochrome c were purchased from Santa Cruz Biotechnology (Delaware, CA, U.S.A.). Anti-cleaved caspase-3 was supplied by Cell Signaling (Beverly, MA, U.S.A.), and anti-actin was purchase from Sigma (St. Louis, MO, U.S.A.). Anti-rabbit and mouse Ig conjugated with horseradish peroxidase and the Enhanced Chemiluminescence (ECL) Advanced Detection Kit were obtained from Amersham Bioscience (Uppsala, Sweden). Fluorescein anti-rabbit IgG was obtain from Vector Laboratories, Inc. (Burlingame, CA, U.S.A.). All other reagents were of the highest grade available commercially.

Cell Culture
PC-12 cells were cultured and maintained in DMEM supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% FBS and maintained at 37°C under a humidified atmosphere with 5% CO₂. All the treatments were performed at 30% confluence.

Determination of Antioxidative Enzyme Activity
Catalase (CAT). CAT activity was evaluated by the rate of hydrogen peroxide (H₂O₂) decomposition [9]. The method was based on H₂O₂ degradation by the action of CAT contained in the examined samples. In this procedure, 50 nM phosphate buffer (pH 7.0) was used and 30 mM H₂O₂ as substrate. CAT activity was expressed as µM H₂O₂/min/mg protein.

Glutathione peroxidase (GPx) activity assay. The activity of GPx was determined following the oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) with t-butyl hydroperoxide as a substrate [32]. This reaction was proceeded by the action of GPx contained in the samples examined on t-butyl hydroperoxide (3 mM) as substrate in 0.5 M phosphate buffer, pH 7.0, at 37°C. The activity of GPx was expressed as nM NADPH/min/mg protein.

Glutathione S-transferase (GST). The activity of GST towards 1-chloro-2,4-dinitrobenzene (CDNB) was determined by the method of Habig et al. [13]. The method was based on the reaction of CDNB with the -SH group of GSH catalyzed by GST contained in the samples. The reaction proceeded in the presence of 1 mM GSH in phosphate buffer (pH 6.5) at 37°C. GST activity was expressed as nM GSH/min/mg protein. The GST Assay Kit utilized CDNB, which is suitable for the broadest range of GST isozymes. Upon conjugation of the thiol group of glutathione to the CDNB substrate, there was an increase in the absorbance at 340 nm.

Measurement of Oxidative Stress-Related Factors in Neuronal Cell
Nitrile oxide assay. Cells were grown in 96-well plates and then incubated with or without H₂O₂ (1 mM) in the absence or presence of various concentrations of APVPH I for 24 h. The nitrile accumulation in the supernatant was assessed by Griess reaction [25]. Each 50 µl of culture supernatant was mixed with an equal volume of Griess reagent [0.1% N-(1-naphthyl)-ethylenediamine, 1% sulphanilamide in 5% phosphoric acid] and incubated at room temperature for 10 min. The absorbance at 550 nm was measured in a microplate absorbance reader, and a series of known concentrations of sodium nitrite was used as a standard.

Measurement of ROS generation. DCF-DA can be deacetylated in cells, where it can react quantitatively with intracellular radicals (mainly hydrogen peroxide) to convert into its fluorescent product DCF, which is retained within the cells. Therefore, DCF-DA was used to evaluate the generation of ROS in oxidative stress [18]. Cells were incubated with 5 µM DCF-DA for 30 min at room temperature. The uptake of DCF-DA fluorescence was measured by a flow cytometer.

Lipid peroxidation assay. MDA is an endogenous genotoxic product of enzymatic and oxygen radical-induced lipid peroxidation [12]. PC-12 cells (2.1×10⁵ cells/well) in 6-well plates were first incubated with various concentrations of APVPH I for 1 h, and then incubated with 1 mM H₂O₂ for 24 h. Two hundred µl of each medium supernatant was mixed with 400 µl of TBARS solution and then boiled at 95°C for 30 min. The absorbance at 532 nm was measured. MDA levels were measured by a colorometric method using malondialdehyde tetrahydroxy-tetramethylaminium as the standard and results are given as µM.

Determination of Apoptosis-Related Factor in Neuronal Cells
Cell cycle and apoptosis analysis. For cell cycle analysis, the harvested cells were fixed with ethanol (with 0.5% Tween-20) for 24 h, incubated with 50 µg/ml PI and 1 µg/ml RNase A at 37°C for 30 min, and analyzed by flow cytometry, using a FACScan (BD, Franklin Lakes, NJ, U.S.A.). The cells belonging to sub-G1 population were considered as apoptotic cells, and the percentage of each phase of the cell cycle was determined. The percentage of cells undergoing apoptosis was also determined by Annexin V-FITC. Cells were harvested and double-stained with Annexin V-FITC (10 µg/ml) and PI (20 µg/ml). The proportions of viable, necrotic, early apoptotic, and late stage of apoptotic cells were determined by a flow cytometer.

Western blot analysis. Whole cell lysates were prepared by incubating cell pellets in lysis buffer consisting of 30 mM NaCl, 0.5% Triton X-100, 50 mM Tris-HCl (pH 7.4), 1 mM Na₂VO₄, 25 mM NaF, 10 mM Na₂P₂O₇, and protease inhibitor cocktail for 60 min on ice. After insoluble fractions were removed by centrifugation at 14,000 rpm (4°C) for 30 min, the supernatants were collected and the protein concentration was determined with a BCA protein assay kit. The membranes were incubated with corresponding primary antibodies in Tris-buffered saline containing 0.05% Tween-20 (pH 7.4) in the presence of 5% non-fat dry milk for 2 h at room temperature. After the membranes were washed in TBS-T three times (5 min each), secondary antibody reactions were performed with an appropriate source of antibody labeled with horseradish peroxidase for 90 min at room temperature. The signals were detected with an ECL advanced detection kit by using the LAS-3000 detector (Fujifilm, Tokyo, Japan). Immunoblotting for actin was performed as an internal control.

Immunocytochemistry. Cells were attached onto slide glass by cytospin centrifugation for 2 min at 900 rpm using Cellspin (Hanil, Seoul, Korea) and then fixed with 4% paraformaldehyde at room temperature for 20 min. Fixed cells were washed three times with phosphate-buffered saline (PBS) for 10 min and incubated with 0.2% Triton X-100 for 15 min. After three washes with PBS, cells were incubated with an appropriate primary antibody in 1% bovine
serum albumin (BSA) at room temperature for 2 h. For secondary reaction, cells were incubated with an appropriate FITC-conjugated secondary antibody at room temperature for 2 h. Cells were observed under a confocal microscope (Carl Zeiss, Jena, Germany).

**Morphological analysis of nucleus.** Morphological change of apoptotic cells was assessed by staining nuclei of cells with Hoechst 33342 as described by Hasegawa et al. [14]. Cells were collected and washed in PBS. Washed cells were fixed in 4% paraformaldehyde for 2 h at 4°C, washed in PBS, added to 20 µl of PBS containing 0.8 µg/ml Hoechst, and incubated for 30 min at 37°C. Hoechst-stained cells were analyzed with a fluorescence microscope (Olympus Co., Tokyo, Japan).

**Statistical Analysis**
The data are presented as mean±SD, which were made in triplicate experiments. The paired t-test was used for comparisons between oxidative stress group and APVPH I-treated group. All analyses were performed using an SAS system (SAS Institute, Cary, NC, U.S.A.).

**RESULTS AND DISCUSSION**

**Antioxidant Enzyme Activity of Oxidative Peptide**

**Catalase activity.** Catalase catalyzes the decomposition of hydrogen peroxide (H$_2$O$_2$) to water and oxygen. Hydrogen peroxide is highly deleterious to the cell and its accumulation causes oxidation of cellular targets such as DNA, proteins, and lipids, leading to mutagenesis and cell death [1]. Removal of the H$_2$O$_2$ from the cell by catalase provides protection against oxidative damage to the cell. Its role in oxidative-stress-related diseases has been widely studied [1]. CAT activity was significantly higher on the APVPH I treated-group compared with the oxidative stress group (Fig. 1A). These results indicate that APVPH I increases the CAT level on PC-12 cells.

**Glutathione peroxidase activity.** The GPx reaction plays a crucial role in protecting cells from damage by free radicals, which are formed by peroxide decomposition. GPx enzymes reduce peroxides to alcohols using glutathione, thus preventing the formation of free radicals. GPx provides a mechanism for detoxification of peroxides in living cells [21]. GPx activity (Fig. 1B) was significantly higher in the APVPH I treated-group compared with the oxidative stress group. In addition, the enzyme activity increased in a dose-dependent manner. These results show that APVPH I stimulates secretion of GPx on PC-12 cells.

**Glutathione S-transferase activity.** GST is a group of enzymes that are important in the detoxication of many different xenobiotics in mammals. The enzymes protect cells against toxicants by conjugating the thiol group of the glutathione to electrophilic xenobiotics, and thereby defend cells against the mutagenic, carcinogenic, and toxic effects of the compounds [21]. A similar trend was obtained for GST activity (Fig. 1C), and CAT and GPx activities. GST activity was significantly higher in the APVP I treated-group compared with the oxidative stress group.

**Changes of Oxidative Stress in Neuronal Cells**

**Effect of oxidative peptide on NO production.** NO is a free radical generated by NO synthases (NOS). NO has a
short half-life and is rapidly oxidized to the stable, inactive end-products, nitrite and nitrate. NO may contribute to many pathologic disorders such as autoimmune disease, tumor growth, and metastasis, and septic shock [22]. Fig. 2A, shows the effect of APVPH I on NO production in PC-12 cells. It indicates that the NO production was increased by \( \text{H}_2\text{O}_2 \) compared with the control group. However, APVPH I protected neuronal cells by inhibition of NO generation compared with the oxidative stress group (*\( p<0.05 \)).

Effect of oxidative peptide on ROS generation. The intracellular ROS was measured using an oxidation-sensitive fluorescent probe, DCFHDA. As per the result in Fig. 2B, \( \text{H}_2\text{O}_2 \)-treated cells led to increased DCF fluorescence against the control group. However, the generation of ROS was decreased by APVPH I. These results imply that accumulated ROS is an important mediator of \( \text{H}_2\text{O}_2 \)-induced apoptosis, and the generation of DCFHDA was reduced by APVPH I.

Inhibition effect on lipid peroxidation of oxidative peptide. To inhibit lipid peroxidation, it may be an important hydrophobic property of the APVPH I sequence to scavenge on lipid-derived radicals. Based on the sequence, APVPH I has high hydrophobic moiety [17]. Because of this property, APVPH I could interact with lipid molecules and could scavenge by donating protons to lipid-derived radicals. APVPH I significantly reduced MDA levels on PC-12 compared with the oxidative stress group (Fig. 2C).

Changes of Apoptosis in Neuronal Cells

Cell cycle and apoptosis analysis by flow cytometry. The neuroprotective effect of APVPH I was determined with sub-G1 analysis by a flow cytometer. The cells were treated with various concentrations of APVPH I prior to 1.0 mM \( \text{H}_2\text{O}_2 \) for 24 h. Damage of neuronal cells against \( \text{H}_2\text{O}_2 \) was inhibited significantly by APVPH I (Fig. 3A). The percentage of apoptotic cells observed was 53.52% at 1.0 mM \( \text{H}_2\text{O}_2 \), but the percentages of APVPH I-treated cells were 44.43%, 28.25%, and 27.42% at 2.5, 5.0, and 10 \( \mu \text{g/ml} \), respectively (Fig. 3A). Finally, Annexin-V staining was applied to the cells to identify the type of cell death evoked by \( \text{H}_2\text{O}_2 \). The treatment of \( \text{H}_2\text{O}_2 \) caused the migration of viable cell population (region IV) to early apoptotic cell population (region III, 41.2%) on the oxidative stress group. However the treatment of APVPH I caused the migration of early apoptotic cell population (region III, 18.5%) to viable cell population (region IV) (Fig. 3B). These results indicate that APVPH I protects neuronal cells against \( \text{H}_2\text{O}_2 \)-induced oxidative stress.

Changes of apoptosis-related gene expression. The expression of iNOS on PC-12 cells was evaluated by Western blot analysis. As shown in Fig. 4, the expression of iNOS tended to increase in the \( \text{H}_2\text{O}_2 \)-treated group.
compared with the control group. Contrary to the oxidative stress group, the H₂O₂-treated with APVPH I group showed lower expression of iNOS compared with the H₂O₂-treated group. As shown in Fig. 4, the expression of Bax tended to increase in the H₂O₂-treated group compared with the control group. Contrary to the oxidative stress group, the H₂O₂-treated with APVPH I group showed lower expression of Bax compared with the H₂O₂-treated group. In addition, expression of Bcl-2 tended to decrease on the H₂O₂-treated group compared with the control group. On the other hand, the H₂O₂-treated with APVPH I group showed higher expression of Bcl-2 than the H₂O₂-treated group. In addition, the expression of cleaved caspase-3 tended to increase in the H₂O₂-treated group compared with the control group. Contrary to the oxidative stress group, the H₂O₂-treated with APVPH I group showed lower expression of cleaved caspase-3 compared with the H₂O₂-treated group as well as Bax.

**Immunocytochemical localization of cytochrome c.**
Most of the cytochrome c was in the mitochondria in the control group. On the other hand, most of the cytochrome c was released into the cytosol (Fig. 5) in the H₂O₂-treated group. Meanwhile, APVPH I suppressed the release of cytochrome c in a dose-dependant manner on the H₂O₂–APVPH I co-treated group. We assumed the reason was due to inhibition of the expression of iNOS, Bax/Bcl-2, and cleaved caspase-3 by APVPH I.

**Morphological analysis of nucleus.** Nuclear staining was performed as another marker of apoptosis on PC-12 cells, as described above. When the nucleus was exposed to oxidative stress, it shrank and fragmentized. On the contrary, in the APVPH I-treated group, the nucleus was preserved from oxidative damage in a dose-dependent manner (Fig. 6). These results show that APVPH I protects neuronal cells against H₂O₂-induced oxidative stress.

A similar phenomenon was shown for the activities of three antioxidative enzymes. The activities of antioxidative enzymes were significantly higher in the oxidative stress group compared with the control group. These results are opposite to those of Liu et al. [20] but are the same results...
of Sibel and Canan [30]. This was likely due to the concentration of hydrogen peroxide. That is, when the concentration of hydrogen peroxide is high (≥1.0 mM), the activity of antioxidative enzyme tends to increase on the hydrogen peroxide-induced oxidative stress group compared with the control group; on the other hand, when the concentration of hydrogen peroxide is low (<1.0 mM), the activity of antioxidative enzyme tends to decrease on the hydrogen peroxide-induced oxidative stress group compared with the control group.

iNOS expression also increases in activated glia during neuroinflammatory processes [26]. Such increased iNOS expression is associated with local release of substantial amounts of NO, which synergistically contributes to the detrimental effects of other mediators of neuronal damage released from inflammatory foci [5]. In addition, Cantarella et al. [6] showed that the redundancy between TRAIL and NO in activated astrocytes synergistically contributes with other factors, bringing about neuronal damage. It was shown the APVPH I inhibited NO generation (Fig. 2A). Thus, APVPH I could be considered for pharmaceutical therapy of neurodegenerative disease.

2,7-Dichlorodihydrofluorescein (DCFH), like other members of the fluorescein family, can be chemically reduced to the corresponding colorless, nonfluorescent leuco dye [4]. It can thus serve as a fluorogenic probe for detecting oxidative activity in cells and tissues [2]. However, this oxidation may not easily discriminate between the various reactive oxygen species [15]. H$_2$DCFHDA is commonly used to detect the generation of reactive oxygen intermediates in neutrophils and macrophages. Recent investigations suggest that the cell permeant DCFHDA may also be extremely useful for assessing the overall oxidative stress in toxicological phenomenon. Since H$_2$O$_2$ was known to act via induction of intracellular ROS, the relationship between intracellular ROS content and apoptosis was assessed. It reduced generation of DCF fluorescence on the APVPH I-treated group compared with the oxidative stress group (Fig. 2B). These results showed that APVPH I suppressed accumulation of ROS.

MDA is an important lipid peroxidative product that can reveal the degree of lipid peroxidation in organisms and indirectly the degree of cell degeneration. These free radicals may cause cellular damage by initiating lipid peroxidation through covalent binding combined with macrocellular molecules (protein, nucleic acid, and lipid), and thus ultimately lead to cell death [35]. Antioxidative properties that prevent peroxidation of essential fatty acids has been attributed to certain amino acid sequences [31]. High amounts of histidine and some hydrophobic amino acids are related to the antioxidantive potency [28]. The addition of hydrophobic amino acids (Met, Val, or Leu) to the N-terminus of the peptide will enhance its antioxidative activity. In this study, therefore, we speculate that the inhibition activity of MDA generation for APVPH I could be attributed to the presence of Met, Val, and Leu in the identified peptide.
Accumulating evidence indicates that NO, a gaseous molecule possessing neurotransmitter/neuromodulator properties in the brain, plays an important role in the pathophysiology of epilepsy, producing both anti- and pro-convulsant effects in various experimental seizure models in rodents [11]. NO is produced by the oxidation of l-arginine by NO synthase existing in three distinct isoforms: neuronal (nNOS), inducible (iNOS), and endothelial (eNOS) [23]. Considerable progress has been made in the examination of the role of NO in the brain during seizures after discovery that synthetic l-arginine analogs and nitroindazole derivatives inhibit the NOS activity in the brain [33]. In addition, NO, which is synthesized by iNOS, has been implicated as an inducer of apoptosis in a number of different cell types [33].

Regulation of apoptotic signaling is achieved in general by expression of distinct protein families, such as the Bcl-2 family. The Bcl-2 family consists of two subfamilies: pro-apoptotic members such as Bax, Bad, Bim, Bik, or Bcl-xs, which initiate or promote the apoptotic signal, and anti-apoptotic members such as Bcl-2, Bcl-xl, Mcl-1, or A1, which block the activation of effector caspasess, such as caspase-3 and caspase-2, which transduce the apoptotic signals [34]. Bcl-2, present in the outer mitochondria membrane, has been suggested to block apoptosis by inhibiting the release from mitochondria of apoptosis-inducing factors, such as cytochrome c [7]. Contrary to the Bcl-2, Bax plays a major role in initiating the release of cytochrome c. Because Bax has been shown to form channels in synthetic membranes [3], Bax may form a pore in the outer membrane of the mitochondria that allows cytochrome c to leak out. Cell survival in the early phases of the apoptotic cascade depends mostly on the balance between the Bax and Bcl-2. The Bax/Bcl-2 ratio may better predict the apoptotic fate of the cell than the absolute concentrations of either [10]. Therefore, the Bax/Bcl-2 ratio might be important in the mitochondria-dependent apoptosis cascade for the release of cytochrome c. In addition, it appears to be the relative relation of Bcl-2 and Bax that determines the fate of a cell, rather than the absolute concentrations of either [27]. In this study, the ratio of Bax/Bcl-2 was reduced significantly by APVPH I against H$_2$O$_2$-induced damage (Fig. 4B). These results suggest that APVPH I protects PC-12 through regulation of the Bcl-2 family.

Oxidation of cardiolipin could disrupt electrostatic and hydrophobic interactions to create a soluble pool of cytochrome c that can pass into the cytosol in a Bax-dependent process [19]. Oxidized cytochrome c may enhance its own release through its acquired peroxidase activity, which leads to increased cardiolipin oxidation. Characterization of the chemical modification will provide insight into the loss of function reported following exposure of cytochrome c to O$_2$. Further identifying modifications of amino acid residues in cytochrome c that would be uniquely attributable to reaction with O$_2$ could provide a specific marker for this ROS and its mitochondrial production. In this study, inhibition of ROS generation by APVPH I treatment suppressed the release of cytochrome c into the cytosol (Fig. 5). These results imply that accumulated ROS is an important mediator of O$_2$-induced apoptosis and APVPH I-reduced generation of ROS against oxidative stress on PC-12 cells.

The induction of death needs its enzymatic activity and is characterized by externalization of phosphatidylserine, cytochrome c release, nuclear condensation, DNA fragmentation, and so on. Therefore, we conducted a morphological analysis of the nucleus in PC-12 cells. As shown in Fig. 6, APVPH I preserved neuronal cells against oxidative stress.

In this study, the antioxidative activity of APVPH I was evaluated using different in vitro systems as direct free-radical scavenging activity generation assay of oxidative stress-related and apoptosis-related factors. In addition, the treatment of APVPH I increased PC-12 proliferation in a dose-dependent manner (data not shown). These results showed that APVPH I had a potent antioxidative activity owing to reduced generation of ROS. However, further detailed studies on APVPH I in regard to its antioxidative activity in vivo are needed.

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References


