

Apocynum venetum leaf extract protects against H₂O₂-induced oxidative stress by increasing autophagy in PC12 cells

YUELAI FENG¹, CHANG JIANG², FENG YANG³, ZIXIAN CHEN^{2*} and ZHENG LI^{2*}

¹Department of International Baccalaureate Diploma Program, Shanghai Pinghe School, Shanghai 200127;

²Department of Orthopaedics, ³Integrated Traditional and Western Medicine, Zhongshan Hospital of Fudan University, Shanghai 200032, P.R. China

Received December 4, 2019; Accepted April 29, 2020

DOI: 10.3892/br.2020.1313

Abstract. The effect of *Apocynum venetum* leaf extract (AVLE) on the nervous system has been widely studied, but its effect on injured neurons is not fully understood. In the present study, the protective effect of AVLE on injured neurons was determined. H₂O₂ was used to induce oxidative stress in PC12 cells and cell viability assays were used to determine the optimum concentration range of AVLE and its protective effects against oxidative stress. A live-dead assay was performed to confirm the effects of AVLE on oxidative stress. Subsequently, expression of apoptotic proteins including Bax and cleaved-caspase-3 were evaluated to determine whether AVLE affected apoptosis, and reactive oxygen species (ROS) levels were detected to determine the role of AVLE in H₂O₂ exposure. Furthermore, expression of autophagic proteins including LC3-II and p62 were detected to evaluate the effects of AVLE on autophagic activity, and cells were treated with 3-methyladenine (3-MA), an autophagic inhibitor, to identify the underlying protective mechanism of AVLE. The results showed that the optimum conditions to induce oxidative stress were treatment with 40 μM H₂O₂ for 2 h, and the suitable range of AVLE concentrations was shown to be 1-100 μg/ml. AVLE improved cell viability in PC12 cells following treatment with H₂O₂. AVLE reduced the expression of Bax and cleaved-caspase-3, and decreased ROS production. Furthermore, AVLE upregulated LC3-II expression and downregulated p62 expression, whereas treatment with 3-MA increased the levels of ROS and apoptotic proteins. These results suggest that AVLE may protect injured neurons against

oxidative stress-induced apoptosis, and this effect may be associated with the reduction of ROS by increasing autophagy.

Introduction

Nerve injury forms the pathological basis of the majority of neurological diseases, and is involved in spinal cord injury, brain injury and neurodegenerative diseases. There are several factors which result in nerve damage, among which oxidative stress serves an important role (1). Oxidative stress can result in the dysfunction of the mitochondria, the endoplasmic reticulum, lipid peroxidation, protein oxidation and subsequently lead to neuronal apoptosis or ferroptosis (2-4). Due to the 'non-renewable' nature of neurons, it is particularly important to improve the anti-oxidative capacity of neurons during potential crises. *Apocynum venetum* (sword-leaf dogbane) is a traditional Chinese herb used to treat hypertension, nephritis and neurasthenia, and has been shown to possess diuretic and sedative effects (5). *A. venetum* has become a popular herbal medicine in East Asia and North America (6,7). Numerous additional pharmacological properties of *A. venetum* leaf extract (AVLE) have been discovered, several of which are closely associated with the nervous system. In an *in vitro* model of ischemia-reperfusion induced by oxygen and glucose deprivation, administration of AVLE notably reduced apoptosis and morphological damage to neurons (8). Although several studies have shown that AVLE may protect neurons against injury and promote recovery, the underlying mechanisms of these pharmacological properties have not been shown, to the best of our knowledge.

Autophagy is an important mechanism of cell survival in eukaryotic cells under stressful conditions (9), which consists of several steps, from initiation of nucleation to formation of double membrane autophagosomes and finally to autophagosome/lysosome fusion and lysosomal enzyme-mediated degradation of the contents of the autophagosome (10). Autophagy involves degradation of long-lived proteins and damaged organelles, such as mitochondria, the endoplasmic reticulum, peroxisomes, and proteins damaged by oxidative stress, to prevent or slow down initiation of apoptosis. Blocking autophagy allows toxic proteins and damaged mitochondria to accumulate, which further aggravates oxidative stress (11,12). The role of autophagy in the nervous system

Correspondence to: Dr Zheng Li or Dr Zixian Chen, Department of Orthopaedics, Zhongshan Hospital of Fudan University, 180 Fenglin Road, Shanghai 200032, P.R. China
E-mail: 17111210053@fudan.edu.cn
E-mail: chen.zixian@zs-hospital.sh.cn

*Contributed equally

Key words: *Apocynum venetum* leaf extract, PC12, apoptosis, reactive oxygen species, oxidative stress, autophagy

has been widely investigated. Evidence from knockout mouse has shown that autophagy exerts a protective effect against neurodegeneration through clearance of intracytoplasmic aggregate-prone proteins (13). However, the effect of AVLE on autophagic activity has not been studied. In the present study, whether AVLE exerted protective effects on PC12 cells against H₂O₂-induced oxidative stress was determined. Additionally, autophagic activity was assessed to determine whether it was involved in the mechanism underlying the protective effects of AVLE.

Materials and methods

Reagents and antibodies. AVLE was donated by the Department of Integrative Medicine of Zhongshan Hospital, Shanghai, China. H₂O₂ was purchased from Sigma-Aldrich; Merck KGaA (cat. no. 323381). Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc. (cat. no. CK04). A live-dead detection kit was purchased from Thermo Fisher Scientific, Inc. (cat. no. R37601). A DHE cell reactive oxygen species (ROS) detection kit was purchased from Nanjing KeyGen Biotech Co., Ltd. (cat. no. KGAF019). 3-Methyladenine (3-MA; cat. no. S2767; Selleck Chemicals), was dissolved in double-distilled water. Primary antibodies for Bax (cat. no. 14796), caspase-3 (cat. no. 14220) and LC3-II (cat. no. 3868) were purchased from Cell Signaling Technology, Inc. (all used at 1:1,000). The primary antibody SQSTM1/p62 (cat. no. ab56416) was purchased from Abcam and the GAPDH antibody was purchased from ProteinTech Group, Inc. (cat. no. 60004-1-Ig; 1:5,000). The secondary antibodies peroxidase AffiniPure goat anti-mouse IgG (cat. no. 33201ES60) and peroxidase-conjugated goat anti-rabbit IgG (cat. no. 33101ES60) were purchased from Shanghai Yeasen Biotechnology, Co., Ltd.

Cell culture and treatment. PC12 cells were purchased from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences and were cultured in high-glucose DMEM (Nanjing KeyGen Biotech Co., Ltd.) supplemented with 10% FBS (cat. no. 16000-044; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin solution at 37°C and 5% CO₂. The cells were digested with 0.25% trypsin containing EDTA and passaged. When the cells reached 80% density, following 10 h of serum starvation, different concentrations of H₂O₂ or AVLE were added to the medium to screen for the optimum concentration. To evaluate the effect of AVLE on oxidative stress, the PC12 cells were pre-treated with AVLE for 2 h before subsequent experimentation and continued with treatment in the corresponding groups. To inhibit autophagy, cells were treated with 3-methyladenine (3-MA, 5 mM) prior to AVLE treatment.

Cell viability. A total of 1x10³ PC12 cells/well were added to a 96-well plate. The cells were treated with H₂O₂ (0, 10, 20, 40, 80 or 160 μmol/l) for 2 h to determine a suitable concentration for induction of oxidative stress. Subsequently, cells were treated with a series of concentrations of AVLE (0, 1, 10, 100, 250, 500 or 1,000 μg/ml) for 24 or 48 h to determine the safe pharmacological range, as AVLE is a herbal Chinese medicine with several complex constituents, thus it may be challenging for cells to metabolise or clear the compounds. The protective effects of AVLE against H₂O₂ were also measured (0 μg/ml AVLE,

10 μg/ml AVLE, 100 μg/ml AVLE, 40 μM H₂O₂, 40 μM H₂O₂ + 10 μg/ml AVLE and 40 μM H₂O₂ + 100 μg/ml AVLE). Each group had six repeat wells. After treatment, the cells were incubated with 10 μl CCK-8 at 37°C for 2 h to measure viability according to the manufacturer's protocol. The absorbance values were determined at 460 nm using a spectrophotometer. Cell viability was calculated as viability rate (%) = [optical density (OD) of the treatment group-OD blank group]/(OD control group-OD blank group) x100%.

Western blot analysis. Western blot analysis was performed as previously described (14). The cells were lysed with RIPA lysis buffer (cat. no. P0013B; Beyotime Institute of Biotechnology, Inc.) containing a protease inhibitor cocktail. A total of 30 μg protein was added to each lane of a 12.5% SDS gel, resolved using SDS-PAGE (cat. no. PG113; Epizyme) and transferred to a PVDF membrane (cat. no. ISEQ85R; EMD Millipore). The membrane was blocked in non-fat milk and incubated with cleaved caspase-3 and Bax primary antibodies at 4°C overnight. The membrane was incubated with secondary antibodies (1:5,000) at room temperature for 1 h. Enhanced chemiluminescence reagent was added for signal detection (Tanon Science and Technology Co., Ltd.), and densitometry analysis was performed using Quantity One software version 4.6.2 (Bio-Rad Laboratories, Inc.). GAPDH was used as the internal reference.

Immunofluorescence assay. The immunofluorescence assay was performed according to the manufacturer's protocol. For live-dead detection, cells were treated with H₂O₂ and AVLE (control, 10 μg/ml AVLE, 100 μg/ml AVLE, 40 μM H₂O₂, 40 μM H₂O₂ + 10 μg/ml AVLE, 40 μM H₂O₂ + 100 μg/ml AVLE), immersed in live-dead solution and incubated for 15 min at 20-25°C, and then imaged using a fluorescent microscope at x40 magnification (Nikon Corporation). For ROS detection, cells were treated with different combinations of H₂O₂, AVLE and 3-MA as follows: 0 μg/ml AVLE, 10 μg/ml AVLE, 100 μg/ml AVLE, 40 μM H₂O₂, H₂O₂ + 10 μg/ml AVLE, H₂O₂ + 100 μg/ml AVLE and vehicle + H₂O₂ + 100 μg/ml AVLE, 3-MA + H₂O₂ + 100 μg/ml AVLE. Cell medium was replaced with fresh medium containing 10 μM ROS detection solution, the cells were incubated at 37°C for 30 min and imaged using a fluorescent microscope. The images were analysed using ImageJ version 1.8.0 (National Institutes of Health) to compare the ROS expression.

Statistical analysis. Data are presented as the mean ± standard deviation. Analysis was performed using SPSS version 20.0 software (IBM, Corp.) and GraphPad Prism version 5.0 (GraphPad Software, Inc.). Comparisons between paired groups were determined using the Student's t-test. A one-way ANOVA followed by a post hoc Tukey's test was used for comparisons between multiple groups P<0.05 was considered to indicate a statistically significant difference.

Results

AVLE improves cell viability in PC12 cells treated with H₂O₂. Oxidative stress was induced in PC12 cells using different concentrations of H₂O₂ and a CCK-8 assay was used to detect

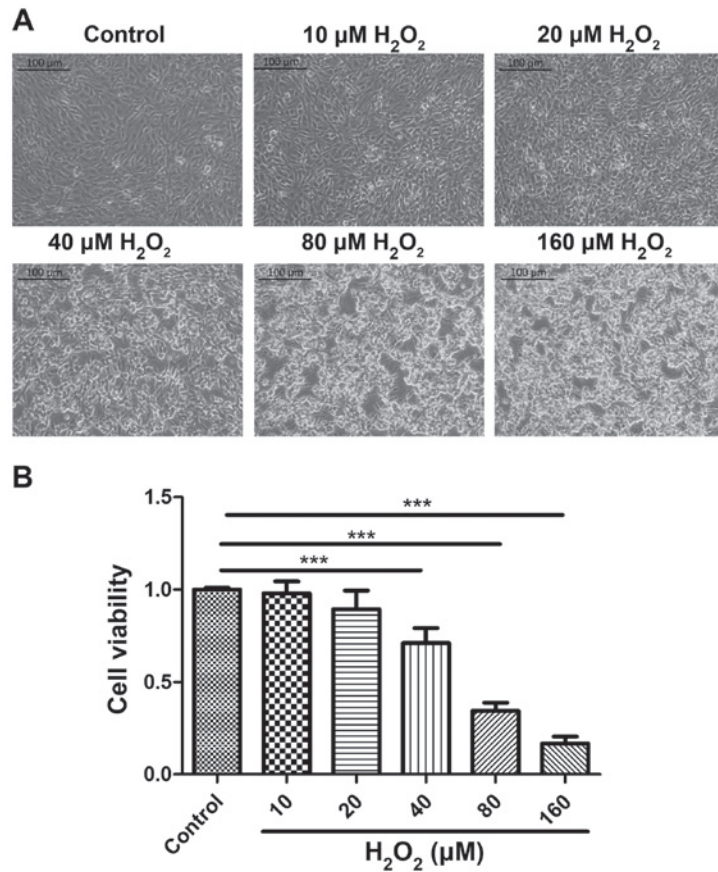


Figure 1. Generation of the oxidative stress model by treating PC12 cells with H₂O₂. (A) Microscopic images of cells treated with different concentrations of H₂O₂. Treatment with >40 μM H₂O₂ for 2 h resulted in cell shrinkage and loss of adhesion. Scale bar, 100 μm. (B) Cell Counting Kit-8 assay of the viability of cells treated with H₂O₂. Cell viability at H₂O₂ concentrations >40 μM was significantly lower compared with the control group. ***P<0.001.

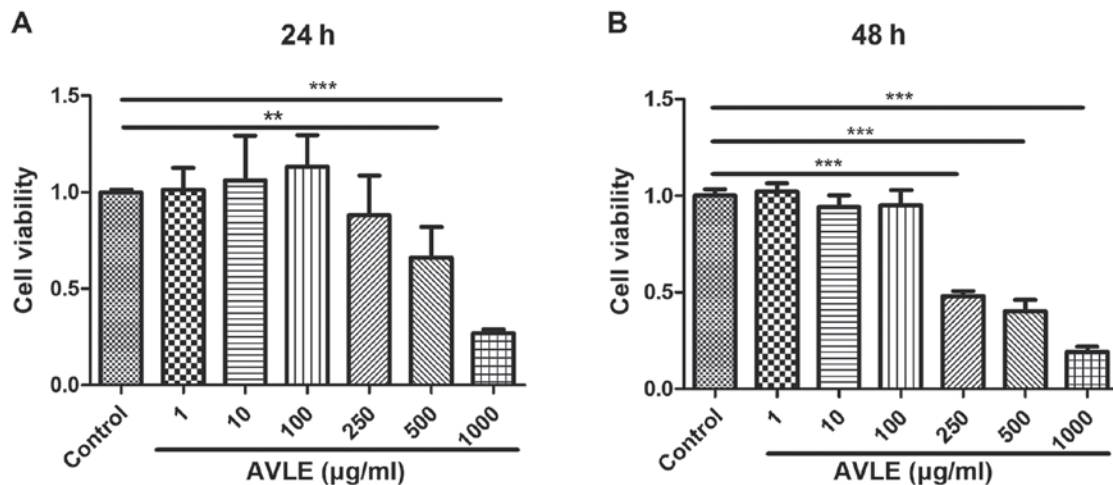


Figure 2. Determination of the safe AVLE concentration range using a CCK-8 assay. (A) CCK-8 assay of cells treated with different concentrations of AVLE for 24 h. Cell viability decreased significantly at AVLE concentrations ≥500 μg/ml. (B) CCK-8 assay of cell viability after 48 h of treatment. Cell viability decreased at AVLE concentrations ≥250 μg/ml but was not significantly affected at AVLE concentrations ≤100 μg/ml when compared with the control group. n=6. **P<0.01, ***P<0.001. CCK-8, Cell Counting Kit-8; AVLE, *Apocynum venetum* leaf extract.

cell viability. Cellular morphology was altered by H₂O₂ treatment, and treatment with 40 μM of H₂O₂ for 2 h caused cells to lose their adhesion and shrink (Fig. 1A). The CCK-8 assay confirmed that the viability of cells treated with H₂O₂ at concentrations >40 μM was significantly lower compared with the control group (P<0.001; Fig. 1B). Thus, the optimum

conditions for inducing oxidative stress were 40 μM H₂O₂ treatment for 2 h. The optimum AVLE concentration was also evaluated using a CCK-8 assay. Cell viability decreased significantly following exposure to AVLE at concentrations >500 μg/ml for 24 h (P<0.01), and cell viability continued to decrease at AVLE concentrations >250 μg/ml for 48 h

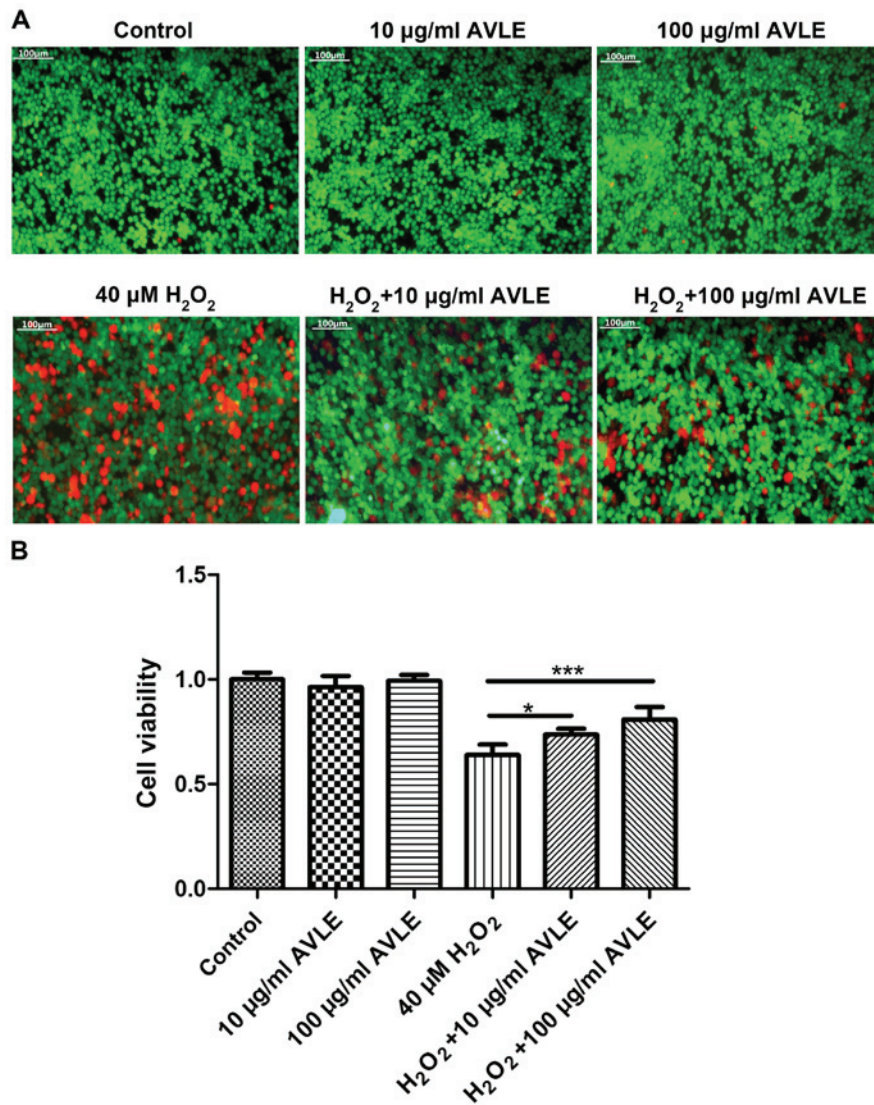


Figure 3. Live-dead assays of the effects of AVLE on cell viability in PC12 cells treated with 40 μM H₂O₂. (A) Fluorescence microscope images of the protective effects of AVLE against H₂O₂. AVLE treatment decreased red fluorescence (dead cells) and increased green fluorescence (live cells) notably. Magnification, x40. Scale bar, 100 μm . (B) A CCK-8 assay showed that cell viability improved significantly with increasing AVLE concentrations (0.64 \pm 0.05 for 40 μM H₂O₂ vs. 0.74 \pm 0.03 for 40 μM H₂O₂ + 10 $\mu\text{g/ml}$ AVLE, $P < 0.05$; 0.64 \pm 0.05 for 40 μM H₂O₂ vs. 0.81 \pm 0.06 for 40 μM H₂O₂ + 100 $\mu\text{g/ml}$ AVLE, $P < 0.001$). $n = 6$. * $P < 0.05$, *** $P < 0.001$. CCK-8, Cell Counting Kit-8; AVLE, *Apocynum venetum* leaf extract.

($P < 0.001$). Conversely, the cell viability was not significantly affected by AVLE at concentrations $< 100 \mu\text{g/ml}$ ($P > 0.05$). Thus, the safe AVLE concentration range was determined to be 1-100 $\mu\text{g/ml}$ (Fig. 2). The protective effects of AVLE against H₂O₂ were measured. Red fluorescence (dead cells) decreased and green fluorescence (live cells) increased significantly following AVLE treatment of cells exposed to H₂O₂, suggesting that AVLE reduced the number of dead cells, possibly through reduction of oxidative stress (Fig. 3A). In addition, cell viability increased significantly following AVLE treatment (0.64 \pm 0.05 for 40 μM H₂O₂ vs. 0.74 \pm 0.03 for 40 μM H₂O₂ + 10 $\mu\text{g/ml}$ AVLE; $P < 0.05$; 0.64 \pm 0.05 for 40 μM H₂O₂ vs. 0.81 \pm 0.06 for 40 μM H₂O₂ + 100 $\mu\text{g/ml}$ AVLE; $P < 0.001$; 0.35 \pm 0.04 for 80 μM H₂O₂ vs. 0.54 \pm 0.13 for 80 μM H₂O₂ + 100 $\mu\text{g/ml}$ AVLE; $P < 0.01$; Figs. 3B and S1).

AVLE protects PC12 cells against H₂O₂-induced apoptosis. Cells were treated with H₂O₂ and different concentrations of

AVLE (0 $\mu\text{g/ml}$ AVLE, 10 $\mu\text{g/ml}$ AVLE, 100 $\mu\text{g/ml}$ AVLE, 40 μM H₂O₂, 40 μM H₂O₂ + 10 $\mu\text{g/ml}$ AVLE, 40 μM H₂O₂ + 100 $\mu\text{g/ml}$ AVLE). Western blotting showed that the expression of the apoptotic proteins Bax and cleaved-caspase-3 increased following treatment with 40 μM H₂O₂ (Fig. 4). However, AVLE (10 or 100 $\mu\text{g/ml}$) significantly decreased apoptosis by reducing the expression of Bax compared with 40 μM H₂O₂ (both $P < 0.05$) and reduced the cleavage of the apoptosis-related protein caspase-3 (40 μM H₂O₂ + 10 $\mu\text{g/ml}$ AVLE vs. 40 μM H₂O₂, $P < 0.01$; 40 μM H₂O₂ + 100 $\mu\text{g/ml}$ AVLE vs. 40 μM H₂O₂, $P < 0.001$).

AVLE ameliorates ROS accumulation in H₂O₂-treated PC12 cells. The production of ROS was determined following H₂O₂ exposure and AVLE treatment (Fig. 5). Treatment with AVLE at 10 or 100 $\mu\text{g/ml}$ alone did not increase ROS production, indicating that it was safe for normal cells. ROS production was significantly increased by 40 μM H₂O₂ compared with

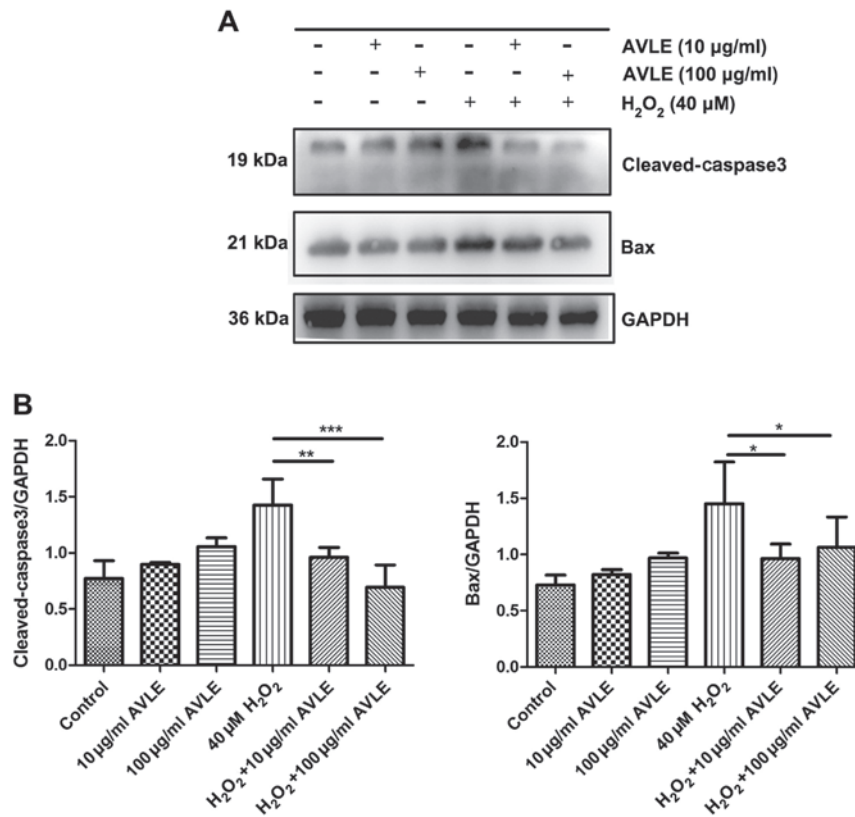


Figure 4. Western blot analysis of the protective effects of AVLE treatment against H₂O₂-induced apoptosis in PC12 cells. (A) Western blots and (B) densitometry analysis of Bax and cleaved caspase-3 expression following treatment with 40 µM H₂O₂ and AVLE. AVLE significantly reduced apoptosis by reducing Bax expression (P<0.05 vs. 40 µM H₂O₂ group) and reduced the cleavage of caspase-3 (n=4; P<0.01 for 40 µM H₂O₂ + 10 µg/ml AVLE vs. 40 µM H₂O₂; P<0.001 for 40 µM H₂O₂ + 100 µg/ml AVLE vs. 40 µM H₂O₂). *P<0.05, **P<0.01, ***P<0.001. AVLE, *Apocynum venetum* leaf extract.

the control cells, and treatment with 10 or 100 µg/ml AVLE significantly reduced ROS production compared with 40 µM H₂O₂ (P<0.001). The antagonistic effect of AVLE on ROS increased as the concentration of AVLE used AVLE increased.

AVLE protects against H₂O₂-induced oxidative stress by increasing autophagy. To confirm the mechanism underlying the protective effects of AVLE following H₂O₂ exposure, the protein expression levels of LC3-II and SQSTM1/p62 were measured to evaluate autophagic activity in the control, 40 µM H₂O₂, 100 µg/ml AVLE and 100 µg/ml AVLE + 40 µM H₂O₂ groups. LC3-II protein expression was significantly upregulated and SQSTM1/p62 expression was downregulated in the cells treated with 100 µg/ml AVLE + 40 µM H₂O₂ compared with those treated with 40 µM H₂O₂ (P<0.001; Fig. 6). Furthermore, administration of the autophagic inhibitor 3-MA significantly increased the expression of the apoptotic proteins cleaved caspase-3 and Bax in the 100 µg/ml AVLE + 40 µM H₂O₂ group compared with the control group (P<0.05; Fig. 7A and B) and increased the ROS levels compared with the control (P<0.001; Fig. 7C and D).

Discussion

AVLE has multiple pharmacological effects on the nervous system, including antidepressant effects, anxiolytic effects, and protective effects against stroke. Butterweck *et al* (5) reported that male rats treated with AVLE had significantly

shorter immobility times in a forced swimming test, suggesting possible antidepressant effects. AVLE also showed antidepressant-like effects in a chronic unpredictable mild stress model of depression in rats, and these effects were possibly caused by the suppression of neuronal apoptosis and increased expression of brain-derived neurotrophic factor (15). The anxiolytic effects of AVLE were measured in mice subjected to an elevated plus maze test, and mice treated with AVLE entered and spent more time in the open arms of the maze, whilst showing no other behavioural changes or motor dysfunction (7). AVLE has protective effects against neurological injuries such as stroke. AVLE (500 mg/kg/day) significantly reduced the cerebral infarct area by alleviating blood-brain barrier disruption in a rat model of cerebral ischemia-reperfusion injury (16). In the present study, an oxidative stress model was established by treating PC12 cells with 40 µM H₂O₂ for 2 h. The safe pharmacological range was established to be between 1-100 µg/ml. Since AVLE is a herbal Chinese medicine consisting of several complex components, when the drug concentration is too high, exceeding the metabolic or clearance capacity of cells, it will exhibit toxic effects on the cells, thus, 10 and 100 µg/ml AVLE were used as safe concentrations in the present study. Cell viability detection and a live-dead assay showed that AVLE reduced the number of injured PC12 cells. Oxidative stress is involved in several pathological processes, such as stroke, neurodegenerative disorders and spinal cord injury (1,17). AVLE also mitigated myocardial ischemia/reperfusion injury by inhibiting oxidative stress (18). The findings of the present

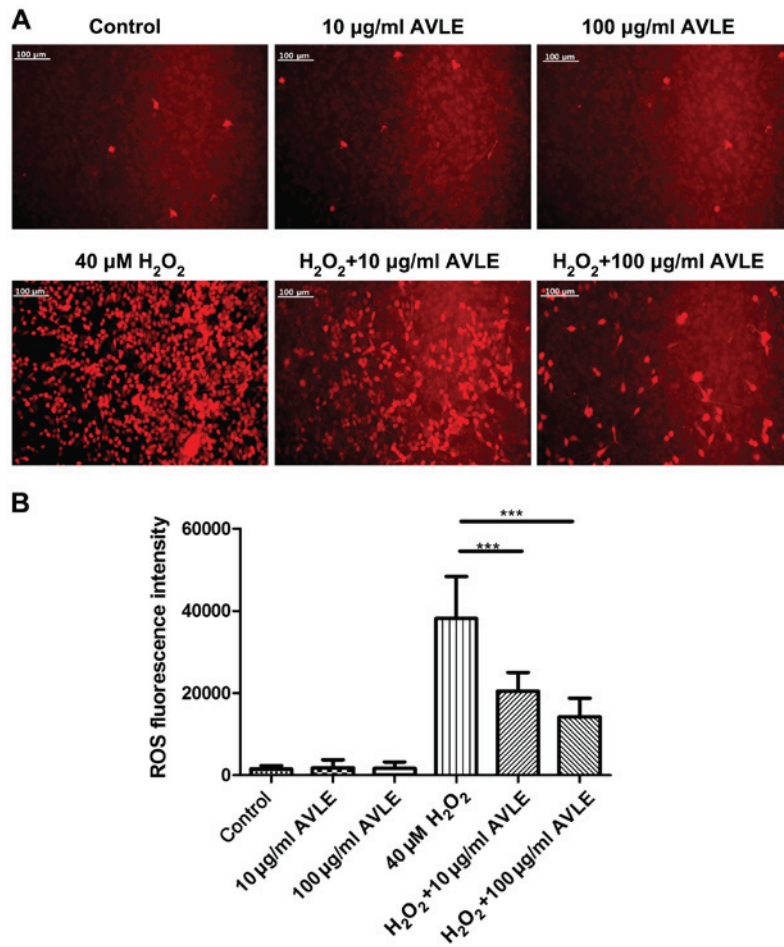


Figure 5. AVLE reduces ROS levels in PC12 cells treated with H₂O₂. (A) Fluorescence microscope images and (B) fluorescence intensity of cells treated with 40 µM H₂O₂ and 10 or 100 µg/ml AVLE. Scale bar, 100 µm. AVLE alone did not increase ROS production. Administration of 40 µM H₂O₂ significantly increased ROS accumulation compared with the control group. AVLE significantly reduced the levels of ROS compared with 40 µM H₂O₂ group, and the reduction in ROS expression increased with the AVLE concentration. ***P<0.001. AVLE, *Apocynum venetum* leaf extract; ROS, reactive oxygen species.

study suggest that AVLE may exert similar effects on nervous system diseases.

Oxidative stress induced by excessive accumulation of ROS serves an important role in the development and progression of nervous system diseases (3,19,20). Excess intracellular ROS can initiate apoptotic pathways, and following activation of the apoptotic pathway, the anti-apoptotic protein Bcl2 is inhibited and the pro-apoptotic protein Bax oligomerizes, which induces mitochondrial permeabilization and release of cytochrome c. Subsequently, caspase-3 is activated, leading to the fragmentation of PARP and ultimately apoptosis (19,21). In the present study, AVLE significantly inhibited H₂O₂-induced apoptosis in PC12 cells, and western blotting showed that AVLE reduced the expression of Bax and inhibited the cleavage of the apoptosis-related protein, caspase-3. ROS expression in the H₂O₂-treated cells was significantly reduced by AVLE. These results suggest that AVLE may inhibit apoptosis of neurons induced by oxidative damage, which may be achieved by reducing the levels of ROS, and subsequently blocking the activation of the pro-apoptotic proteins Bax and caspase-3.

ROS causes oxidative stress during apoptosis, and ~90% of ROS is produced by the mitochondrial membrane respiratory chain (22). Excessive amounts of ROS activates

autophagy by inhibiting the PI3K-Akt-mTOR pathway, and cells activate mitophagy to remove damaged mitochondria, and thus maintain low ROS levels (23,24). Natural antioxidants, including resveratrol, curcumin and apigenin, reduce oxidative stress by increasing autophagic activity (24-28). In the present study, in H₂O₂-treated cells, AVLE upregulated LC3-II expression and downregulated p62 expression. LC3-II may be attached to the membrane of an autophagosome, and is a labelled protein of the autophagosome, whereas p62 can recruit ubiquitinated proteins for autophagic degradation, and the aggregation of p62 often reflects disruption of autophagic flux (29,30). The expression of LC3-II and p62 indicated that autophagic activity was activated, whereas inhibition of autophagy with 3-MA increased the levels of ROS and apoptotic proteins. Autophagy is also maintained at low levels in normal cells and serves an important role in the physiological functioning of cells. Under stressful conditions, such as oxidative stress and inflammation, autophagy is readily activated, but activation is kept limited. certain autophagy activators, such as rapamycin and metformin, are often used to reduce stress in tissues (31,32). The results of the present study suggested that, under oxidative stress, AVLE increased autophagic activity, similar to the effects of rapamycin or metformin, and thus reduced intracellular ROS aggregation

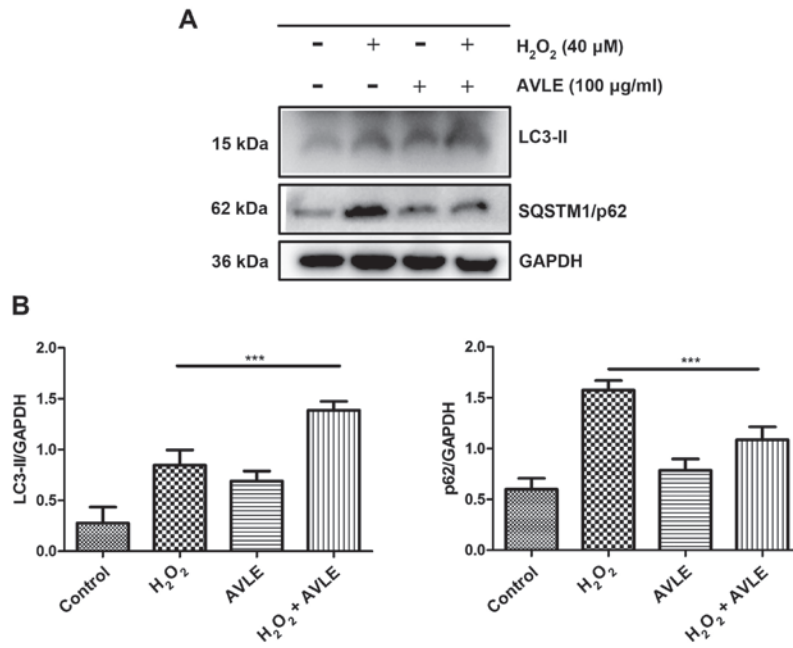


Figure 6. Western blot analysis of the effects of AVLE on autophagic activity of PC12 cells treated with 40 μM H₂O₂. (A) Western blots and (B) densitometry analysis of LC3-I, LC3-II and SQSTM1/p62 in cells treated with 40 μM H₂O₂ and 100 μg/ml AVLE. LC3-II protein expression was significantly upregulated and SQSTM1/p62 was downregulated in cells treated with AVLE + H₂O₂ compared with those treated with H₂O₂ alone (P<0.001 vs. 40 μM H₂O₂). ***P<0.001. AVLE, *Apocynum venetum* leaf extract.

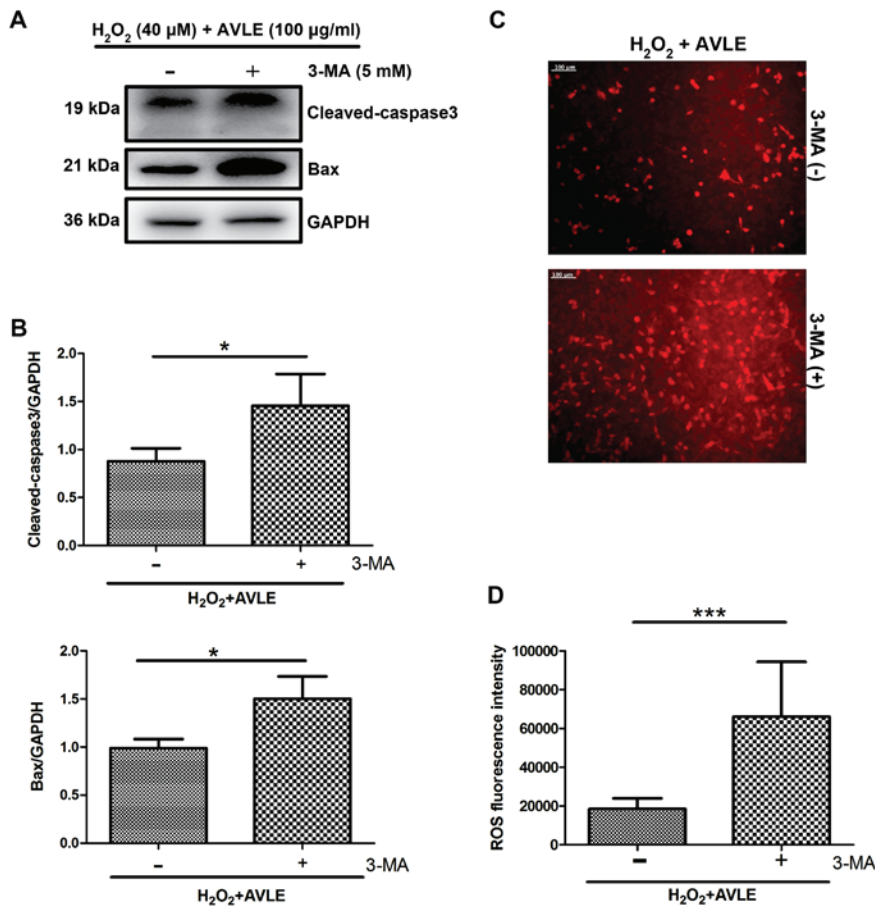


Figure 7. Effects of AVLE on apoptosis of PC12 cells treated with 40 μM H₂O₂. (A) Western blots and (B) densitometry analysis of cleaved-caspase-3 and Bax in cells treated with 40 μM H₂O₂ and 100 μg/ml AVLE with or without 3-MA. 3-MA significantly increased the expression levels of cleaved-caspase-3 and Bax. (C) Fluorescence microscopy and (D) fluorescence intensity analysis of cells treated with 40 μM H₂O₂ + AVLE with or without 3-MA. The number of red positive cells, indicative of the presence of ROS, was increased following 3-MA treatment, as was the fluorescence intensity demonstrating that 3-MA treatment significantly increased ROS levels. *P<0.05, ***P<0.001. Scale bar, 100 μm. AVLE, *Apocynum venetum* leaf extract; 3-MA, 3-Methyladenine; ROS, reactive oxygen species.

and cell apoptosis. These effects may be the result of AVLE itself being rich in antioxidants. *Apocynum venetum* contains an abundance of flavonoids, including hyperoside, quercetin, kaempferol and rutin (33,34), and hyperoside and quercetin have protective effects against oxidative damage on injured cells (35-38).

The present study provides further insights into the effects of AVLE on neurons, and the mechanism underlying the protective effects of AVLE against oxidative stress-induced apoptosis. The results of the present study may facilitate the development of novel treatments for nervous system diseases and injuries. However, there are some limitations. First, PC12 cells were used in the present study which are a highly differentiated cell line. PC12 cells are used as model of neurons, and thus the behaviour of PC12 cells may partially differ from that real neurons *in vivo*. It will be of value to repeat these experiments using primary cells and using *in vivo animal models* to further confirm the effects of AVLE. In addition, the environment of the cells *in vitro* differs from that of neurons *in vivo*, necessitating the need for *in vivo animal experiments*. Although the AMPK/mTOR pathway may participate in the protective effects of AVLE (39), the mechanism involved in the effects of AVLE in the nervous system still require further investigation. Additionally, the composition of AVLE is complex, thus it is difficult to distinguish or identify which components served the major roles in protecting PC12 cells against oxidative stress. Extraction of the functional components and identification of the effects of the individual components on the various signalling pathways is required to fully elucidate the effects of AVLE, and to provide more clinically relevant information. In future studies, the active ingredients will be further clarified with the aim of reducing the side effects of treatment with AVLE.

Acknowledgements

Not applicable.

Funding

The present study was supported by the National Natural Science Foundation of China (grant no. 81301047).

Availability of data and materials

The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ZL and ZC designed the study and drafted the manuscript. FY conducted the experiments and analyzed the data. YF and CJ contributed to the search of literature, analysis of data, and performed experiments. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

- Salim S: Oxidative stress and the central nervous system. *J Pharmacol Exp Ther* 360: 201-205, 2017.
- Ratan RR: The chemical biology of ferroptosis in the central nervous system. *Cell Chem Biol*: May 24, 2020 (Epub ahead of print). doi: 10.1016/j.chembiol.2020.03.007.
- Islam MT: Oxidative stress and mitochondrial dysfunction-linked neurodegenerative disorders. *Neurol Res* 39: 73-82, 2017.
- Höhn A, Tramutola A and Cascella R: Proteostasis failure in neurodegenerative diseases: Focus on oxidative stress. *Oxid Med Cell Longev* 2020: 5497046, 2020.
- Butterweck V, Nishibe S, Sasaki T and Uchida M: Antidepressant effects of *Apocynum venetum* leaves in a forced swimming test. *Biol Pharm Bull* 24: 848-851, 2001.
- Kobayashi M, Saitoh H, Seo S, Butterweck V and Nishibe S: *Apocynum venetum* extract does not induce CYP3A and P-glycoprotein in rats. *Biol Pharm Bull* 27: 1649-1652, 2004.
- Xie W, Zhang X, Wang T and Hu J: Botany, traditional uses, phytochemistry and pharmacology of *Apocynum venetum* L. (Luobuma): A review. *J Ethnopharmacol* 141: 1-8, 2012.
- Xiang J, Tang YP, Zhou ZY, Wu P, Wang Z, Mori M and Cai DF: *Apocynum venetum* leaf extract protects rat cortical neurons from injury induced by oxygen and glucose deprivation *in vitro*. *Can J Physiol Pharmacol* 88: 907-917, 2010.
- Codogno P and Meijer AJ: Autophagy and signaling: Their role in cell survival and cell death. *Cell Death Differ* 12 (Suppl 2): 1509-1518, 2005.
- Radad K, Moldzio R, Al-Shraim M, Kranner B, Krewenka C and Rausch WD: Recent advances in autophagy-based neuroprotection. *Expert Rev Neurother* 15: 195-205, 2015.
- Wu D and Cederbaum AI: Inhibition of autophagy promotes CYP2E1-dependent toxicity in HepG2 cells via elevated oxidative stress, mitochondria dysfunction and activation of p38 and JNK MAPK. *Redox Biol* 1: 552-565, 2013.
- Wang T, Wang Q, Song R, Zhang Y, Zhang K, Yuan Y, Bian J, Liu X, Gu J and Liu Z: Autophagy plays a cytoprotective role during cadmium-induced oxidative damage in primary neuronal cultures. *Biol Trace Elem Res* 168: 481-489, 2015.
- Menzies FM, Fleming A, Caricasole A, Bento CF, Andrews SP, Ashkenazi A, Füllgrabe J, Jackson A, Jimenez Sanchez M, Karabiyik C, *et al.*: Autophagy and neurodegeneration: Pathogenic mechanisms and therapeutic opportunities. *Neuron* 93: 1015-1034, 2017.
- Chen Z, Li Z, Jiang C, Jiang X and Zhang J: MiR-92b-3p promotes neurite growth and functional recovery via the PTEN/AKT pathway in acute spinal cord injury. *J Cell Physiol* 234: 23043-23052, 2019.
- Li X, Wu T, Yu Z, Li T, Zhang J, Zhang Z, Cai M, Zhang W, Xiang J and Cai D: *Apocynum venetum* leaf extract reverses depressive-like behaviors in chronically stressed rats by inhibiting oxidative stress and apoptosis. *Biomed Pharmacother* 100: 394-406, 2018.
- Xiang J, Lan R, Tang YP, Chen YP and Cai DF: *Apocynum venetum* leaf extract attenuates disruption of the blood-brain barrier and upregulation of matrix metalloproteinase-9/-2 in a rat model of cerebral ischemia-reperfusion injury. *Neurochem Res* 37: 1820-1828, 2012.
- Scholpa NE and Schnellmann RG: Mitochondrial-based therapeutics for the treatment of spinal cord injury: Mitochondrial biogenesis as a potential pharmacological target. *J Pharmacol Exp Ther* 363: 303-313, 2017.
- Wang W, Liang X, Fu D, Tie R, Xing W, Ji L, Liu F, Zhang H and Li R: *Apocynum venetum* leaf attenuates myocardial ischemia/reperfusion injury by inhibiting oxidative stress. *Am J Chin Med* 43: 71-85, 2015.
- Jabbour E, Ottmann OG, Deininger M and Hochhaus A: Targeting the phosphoinositide 3-kinase pathway in hematologic malignancies. *Haematologica* 99: 7-18, 2014.

20. Slimen IB, Najar T, Ghram A, Dabbebi H, Ben Mrad M and Abdrabbah M: Reactive oxygen species, heat stress and oxidative-induced mitochondrial damage. A review. *Int J Hyperthermia* 30: 513-523, 2014.
21. Sinha K, Das J, Pal PB and Sil PC: Oxidative stress: The mitochondria-dependent and mitochondria-independent pathways of apoptosis. *Arch Toxicol* 87: 1157-1180, 2013.
22. Mi Y, Xiao C, Du Q, Wu W, Qi G and Liu X: Momordin Ic couples apoptosis with autophagy in human hepatoblastoma cancer cells by reactive oxygen species (ROS)-mediated PI3K/Akt and MAPK signaling pathways. *Free Radic Biol Med* 90: 230-242, 2016.
23. Portal-Nunez S, Esbrit P, Alcaraz MJ and Largo R: Oxidative stress, autophagy, epigenetic changes and regulation by miRNAs as potential therapeutic targets in osteoarthritis. *Biochem Pharmacol* 108: 1-10, 2016.
24. Yuan H, Perry CN, Huang C, Iwai-Kanai E, Carreira RS, Glembocki CC and Gottlieb RA: LPS-induced autophagy is mediated by oxidative signaling in cardiomyocytes and is associated with cytoprotection. *Am J Physiol Heart Circ Physiol* 296: H470-H479, 2009.
25. Cho B, Kim T, Huh YJ, Lee J and Lee YI: Amelioration of mitochondrial quality control and proteostasis by natural compounds in Parkinson's disease models. *Int J Mol Sci* 20: E4208, 2019.
26. Sebori R, Kuno A, Hosoda R, Hayashi T and Horio Y: Resveratrol decreases oxidative stress by restoring mitophagy and improves the pathophysiology of dystrophin-deficient mdx mice. *Oxid Med Cell Longev* 2018: 9179270, 2018.
27. Guo S, Long M, Li X, Zhu S, Zhang M and Yang Z: Curcumin activates autophagy and attenuates oxidative damage in EA.hy926 cells via the Akt/mTOR pathway. *Mol Med Rep* 13: 2187-2193, 2016.
28. Sun H, Wang Z and Yakisich JS: Natural products targeting autophagy via the PI3K/Akt/mTOR pathway as anticancer agents. *Anticancer Agents Med Chem* 13: 1048-1056, 2013.
29. Ichimura Y, Kirisako T, Takao T, Satomi Y, Shimonishi Y, Ishihara N, Mizushima N, Tanida I, Kominami E, Ohsumi M, *et al*: A ubiquitin-like system mediates protein lipidation. *Nature* 408: 488-492, 2000.
30. Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, Kominami E, Ohsumi Y and Yoshimori T: LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *EMBO J* 19: 5720-5728, 2000.
31. Sekiguchi A, Kanno H, Ozawa H, Yamaya S and Itoi E: Rapamycin promotes autophagy and reduces neural tissue damage and locomotor impairment after spinal cord injury in mice. *J Neurotrauma* 29: 946-956, 2012.
32. Zhang D, Xuan J, Zheng BB, Zhou YL, Lin Y, Wu YS, Zhou YF, Huang YX, Wang Q, Shen LY, *et al*: Metformin improves functional recovery after spinal cord injury via autophagy flux stimulation. *Mol Neurobiol* 54: 3327-3341, 2017.
33. Kamata K, Seo S and Nakajima J: Constituents from leaves of *Apocynum venetum* L. *J Nat Med* 62: 160-163, 2008.
34. Song RJ and Zhou J: Microemulsion liquid chromatographic method for simultaneous separation and determination of six flavonoids of *Apocynum venetum* leaf extract. *J Chromatogr B Analyt Technol Biomed Life Sci* 995-996: 8-14, 2015.
35. Hao XL, Kang Y, Li JK, Li QS, Liu EL and Liu XX: Protective effects of hyperoside against H₂O₂-induced apoptosis in human umbilical vein endothelial cells. *Mol Med Rep* 14: 399-405, 2016.
36. Liu Z, Tao X, Zhang C, Lu Y and Wei D: Protective effects of hyperoside (quercetin-3-o-galactoside) to PC12 cells against cytotoxicity induced by hydrogen peroxide and tert-butyl hydroperoxide. *Biomed Pharmacother* 59: 481-490, 2005.
37. Rashidi Z, Aleyasin A, Eslami M, Nekoonam S, Zendedel A, Bahramrezaie M and Amidi F: Quercetin protects human granulosa cells against oxidative stress via thioredoxin system. *Reprod Biol* 19: 245-254, 2019.
38. Xiao HB, Lu XY, Liu ZK and Luo ZF: Kaempferol inhibits the production of ROS to modulate OPN- α v β 3 integrin pathway in HUVECs. *J Physiol Biochem* 72: 303-313, 2016.
39. Lu L, Zhang D, Sun B, Hu Y, Yan M, Liu K, Li X and Ren L: *Apocynum* leaf extract inhibits the progress of atherosclerosis in rats via the AMPK/mTOR pathway. *Pharmazie* 72: 41-48, 2017.



This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.