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Apios americana Medik flowers extract protects PC12 cells against H_2O_2 induced neurotoxicity via regulating autophagy

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ABSTRACT

Since flavonoids are antioxidant compounds, they could beneficially affect neurodegenerative diseases where reactive oxygen species are involved. In this study, we firstly isolated and identified fourteen compounds from the flowers of *Apios americana* Medik. Then, we tested whether *Apios americana* Medik flowers water extract (AFWE) exerts a protective effect on H_2O_2 induced PC12 cells injure. As expected, pretreatment with AFWE inhibited cytotoxicity and DNA condensation in H_2O_2induced PC12 cells. Exposure of PC12 cells to H_2O_2 resulted in reactive oxygen species accumulation and mitochondrial dysfunction, while AFWE alleviated these damages. AFWE obviously reversed the alternations as H2O2 increased Caspase-3 and decreased the ratio of Bcl-2/Bax expressions. Furthermore, autophagy in PC12 cells was further activated by AFWE, which was beneficial to resisting adversity. These results manifest that AFWE prevents H_2O_2 induced damage via regulating autophagy.

1. Introduction

Plenty of studies indicate that oxidative stress-induced cell apoptosis has been involved in most neurodegenerative diseases, such as Alzheimer's disease (AD) (Gibson and Huang, 2005), stroke (Allen and Bayraktutan, 2009), Parkinson's disease (PD) (Hwang, 2013)and Huntington's disease (Vaya et al., 2011). A large number of evidences show that a remarkable feature in neurodegenerative diseases is the accumulation of reactive oxygen species (ROS) (Barnham et al., 2004; Lin and Beal, 2006), which can lead to DNA damage, membrane lipid peroxidation and even injury or death of neuronal cells (Gackowski et al., 2008). In neuronal tissue, excessive ROS will easily attack neurons which have a low capacity of anti-oxidative systems, leading to cell damage (Boldyrev, 2000). The balance between ROS generation and antioxidant defense is critical in disease prevention. Thus, the strategies to release the oxidative damage of neurons by ROS could have enormous implications in promoting outcomes of neurodegenerative diseases (Uttara et al., 2009). Antioxidants are widely used to decrease neurons damage by restraining the development of free radicals. However, the chemical antioxidants are associated with toxicity and pathological changes in human bodies (Kahl, 1984). Studies suggested that plant-derived antioxidants can reduce the noxious effects of oxidative stress (Lobo et al., 2010). And the antioxidant activity could be associated with the multiple free radical scavengers such as vitamins, phenols, flavonoids, polysaccharides and terpenoids (Arnason et al., 2013). Therefore, the development of novel, non-toxic and effective antioxidants is crucial.

Apios americana Medik, a leguminous perennial plant, is native to North America and used as an staple food by native Americans. The tubers are believed to be very nutritious because of a high content of protein, oligosaccharides, polysaccharides, fatty acid, and amino acid can be found (Kim et al., 2018). It is also rich in isoflavones, such as genistein-7-O-gentiobioside, genistein and its derivative, which is similar to soybean (Takashima et al., 2013). Previous researches illustrated that Apios may inhibit the proliferation of cancer cells and cure chronic constipation, obesity, diabetes, hypertension, disorders before and after childbirth (Sohn et al., 2015). And the positive effect of Apios is partly related to its antioxidant activity. However, there is nearly no

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report about Apios flowers, whatever their chemical constitution or bioactivity on neurodegenerative diseases. PC12 cell line is derived from rat pheochromocytoma, which has been generally recognized as a neural cell model in research. Previous results showed that physiological and chemical oxidative stress inducers could result in numerous apoptosis (Klein and Ackerman, 2003). Among various ROS, H_2O_2 has been widely used to construct oxidative stress induced neurotoxicity by inducing lipid peroxidation, DNA damage and mitochondrial dysfunction in vitro (Neill et al., 2002). Mitochondria, the origin of major intracellular active oxygen species, play a key role on many neurological diseases(Blass et al., 2015). Moreover, previous evidence indicated that autophagy is involved in modulating the oxidative status and mitochondrial dysfunction (Scherz-Shouval and Elazar, 2007). Studies showed that flavonoids may activate the differentiation of PC12 cells (Sagara et al., 2004). Lately, it is illustrated that flavonoids stimulate the NRF2 pathways and relieve the ROS level in RAW 264.7 cells (Jeong et al., 2006). Also, Akt/mTOR, FoxO1 and other signaling pathways are involved in the antioxidant process of flavonoids (Zhang et al., 2012). And compounds from different plants may provide protection against oxidative stress to the PC12 cells via different pathways. However, autophagy is barely considered as an underlying protective mechanism of flavonoids against oxidative stress.

In this study, Flavonoids were firstly separated from dried *Apios americana* Medik flowers by water and identified main components by liquid chromatography – tandem mass spectrometry (LC – MS/MS) analysis. Subsequently, The aim of this study is to find out the underlying protective effects of *Apios americana* Medik flowers water extract (AFWE) against H_2O_2induced oxidative damage in PC12 cells and potential signal pathways involved.

2. Material and methods

2.1. Materials and reagents

Apios americana Medik flowers were obtained from Tianyu Ecological Agriculture Development Co., Ltd (Hangzhou, China). Thiazolyl Blue Tetrazolium Bromide (MTT), Hoechst 33342, 2′,7′-dichlorofluorescin diacetate (DCFH), dihydroethidium (DHE), Nonyl acridine orange (NAO), Rhodamine123 (Rh123) and Naphthalene-2,3-dicarboxal-dehyde (NDA) were purchased from the Sigma-Aldrich (St. Louis, MO, USA). Primary and secondary antibodies were obtained from Abcam (Shanghai, China), WB/IP cell lysis buffer, BCA Protein Assay Kit and β-actin were purchased from Beyotime Biotechnology (Jiangsu, China). The ECL Western blotting system was obtained from Yisheng Biological Technology (Shanghai, China). Other reagents were of reagent grade purchased from Aladdin (Shanghai, China).

2.2. Extraction and purification

The flowers of Apios americana Medik (100 g) were mashed, passed through a 200 mesh sieve and extracted for 4 h with 4 L of deionized water at 50 °C by ultra-sonication. After four times of extraction, the solution was evaporated at 50 °C (Shanghai Yarong, China) and centrifuged at 10000 g for 20 min to get the supernatant (Thermo Scientific, USA). Then, 100% ethanol was added to the final concentration of 80% and kept at for 24 h at 4 °C to remove polysaccharides. Subsequently, the supernatants were evaporated, applied to an AB-8macroporous resin column (Ø 3.2 × 60 cm) washed with 1 L of water and eluted with 80% ethanol at a flow rate of 1.0 mL/min. Finally, the eluent was collected, freeze-dried and stored at −80 °C for further analysis. The extract (AFWE) was dissolved in deionized water and filtered through a 0.22 μm membrane for the qualitative and quantitative analysis and cell experiments.

2.3. Identification of AFWE

An Ultra Performance Liquid Chromatography (UPLC) system (Waters, Milford, MA, U.S.A.) equipped with a triple- Time-of-Flight mass spectrometry (TOF/MS) system (AB SCIEX, Triple TOF 5600 +, Framingham, MA, U.S.A.) was used for qualitative analysis. For the UPLC conditions, a Promosil C18 column (4.6 mm × 250 mm, 5 μm) was used. The mobile phase consisted of 0.1% aqueous formic acid (A) and acetonitrile (B). The solvent gradient of phase A was as follows: 0–15 min, 0–95% B; 15–21 min, 95–85% B; 21–22 min, 85–72% B; 22–24 min, 72–60% B; 24–27 min, 60–40% B; 27–30 min, 40–95% B. The flow rate was set at 0.8 mL/min and the temperature was maintained at 40 °C. For the mass spectrometry conditions, the negative ion mode was applied for compound ionization and ions data were collected between m/z 100 and m/z 2000. The matched parameters were as follows: the source voltage (+4.5 kV) and drying gas temperature (550 °C).

2.4. Cell culture and treatments

PC12 cell line was purchased from Shanghai Institute of Cell Biology (Shanghai, China) and was cultured in DMEM supplemented with 12% fetal bovine serum, with penicillin (100 IU/mL), and streptomycin (100 IU/mL) at 37 °C, 5% CO_2 atmosphere. Then, the PC12 cells were treated with 0.25% trypsin in 0.02% EDTA solution after reaching 80% confluence. Subsequently, they were seeded and pre-incubated with different concentrations of AFWE for 24 h. Finally, 250 μM hydrogen peroxide (H_2O_2) was added for another 4 h. Cells untreated were used as the control, while cells incubated only with 250 μMH_2O_2 for 4 h served as the H_2O_2 group.

2.5. Cell viability

Cell viability was measured by the MTT methods (Zhao et al., 2018). PC12 cells were seeded in 96-well plates for 24 h and subsequently subjected to the AFWE with different concentrations (10, 20, 40, 80, 160, 320 and 640 μg/mL). After pre-incubation with AFWE for 24 h, 250 μM $H_2O_2$was added for 4 h. Then, the cultures were incubated with 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution for4 hours at 37 °C. Finally, the medium was removed and 200 μL DMSO was added to solubilize the formazan with shaking for 10 min. Absorbance at 570 nm was detected with a microplate reader (Molecular Devices, Shanghai, China). Cell viability of the control group not exposed to either H_2O_2 or AFWE was expressed as 100%.

2.6. Morphologic analysis

After treatments, PC12 cells were washed 3 times with PBS and changed to the medium without phenol red. Then, observed with a phase-contrast microscope (Nikon) to reveal possible changes of PC12 cells.

2.7. Fluorescent staining

Different fluorescent probes were used to determine intracellular DNA damage (Hochest 33342, 10 μM), ROS generation (DCFH 10 μM), GSH consumption (NDA, 40 μM), O_2^- formation (DHE, 10 μM), mitochondrial membrane potential alteration (RH123, 5 μM) and mitochondrial membrane lipid peroxidation (NAO, 20 μM). After treatments, cells were cultured in serum-free DMEM containing various probes for 30 min at 37 °C. Then, the 24-well plate was washed 3 times with PBS buffer and immediately analyzed using a fluorescence microscope (Nikon) with the same exposure time (Chu et al., 2017). The fluorescence intensity was quantified by Image-Pro Plus 6.0.

2.8. Transmission electron microscopy (TEM)

PC12 cells were collected and centrifuged at 1000 rpm for 5 min after treatments. Then performed with the TEM standard protocol and images were acquired with a transmission electron microscope (H-7650, Hitachi, Japan) operated at 15000 and 30000 magnifications.

2.9. Western blot

After treatments, the medium was discarded and cells were washed 3 times with cold PBS before lysed by WB/IPcell lysis buffer. The lysate was centrifuged at 4 °C, 12000 g for 10 min. The protein concentration of the supernatant was quantitated by the BCA Protein Assay Kit. Equal amounts of protein (30 mg) are separated on SDS-PAGE, and transferred to 0.22 μm PVDF membranes in transfer buffer. After blocking with 5% non-fat milk, the membrane was incubated with various primary antibodies at 4 °C overnight. Then, horseradish peroxidase-conjugated secondary antibodies were probed to the membrane for 2 h at room temperature. Finally, the membrane was incubated with the enhanced ECL immunoblotting detection kit and exposed with an X-ray film. The densitometry of bands was quantitated by the ImageJ software and the relative amount of target proteins in each lane was obtained after normalization with the β-actin values.

2.10. Statistical analyses

Results were expressed as means ± SD ($n \geq 3$) and significant differences between means of each group were assessed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test using the SPSS statistical software, version 19.0. And $P < 0.05$ was considered significant.

3. Result and discussion

3.1. Compounds identification of AFWE

The major compounds in AFWE were identified by HPLC–TOF-MS. As shown in Fig. 1A, fourteen major compounds were firstly identified in *Apiosamericana*Medik flowers based on the MS date and by comparison with previous studies: 4-(β-D-glucopyranosyloxy) hydroxybenzoic acid (peak 1) (Xu et al., 2011), 4-(β-D-glucopyranosyloxy)-3-methoxybenzoic acid (peak 2) (Borowiec et al., 2014), 1-O-(4-hydroxybenzoyl)-β-D-glucopyranose (peak 3) (Kaneko et al., 1998),3,4-Dihydroxybenzoic acid (peak 4) (Tang et al., 2015), kaempferol3- < β-D-glucopyranosyl(1- > 2)-β-D-glucopyranosyl(1- > 2)-β-D-glucopyranoside > (peak 5) (Xiao et al., 2006), 4-O-β-D-glucopyranosyl-p-coumaric acid (peak 6) (Johnsson et al., 2002), vicenin-2 (peak 7) (Ferreres et al., 2010), lucenin I (peak 8) (Ferreres et al., 2010), schaftoside (peak 9) (Colombo et al., 2010), apigenin (6-C-α-L-arabinopyranosyl)-8-C-β-D-glucopyranoside (peak 10) (Piccinelli et al., 2008), Luteolin-8-C-glucoside (peak 11) (F et al., 2003), apigenin 6,8-di-C-α-L-arabinopyranoside (peak 12) (Xie et al., 2003), kaempferol-3-O-sophoroside (peak 13) (K et al., 2010)and the iso(kaempferol-3-O-sophoroside) (peak 14) (K et al., 2010). Besides, the information of the peak identification, retention times, MS/MS date, and compound identification were summarized in Table 1.

3.2. AFWE alleviated H_2O_2 induced toxicity and DNA damage in PC12 cells

Previous studies have illustrated thatH_2O_2 could induce toxicity in various cells and animals (Rhee, 2006). However, the beneficial effect of *Apios americana* Medik flowers on H_2O_2 induced cytotoxicity is currently unclear. In this study, a PC12 cell model was involved in the identification of the protecting effect of AFWE against H_2O_2caused cell viability decrease. As shown in Fig. 1B, the cell viability of PC12 cells reduced to 72% in response to 250 μM hydrogen peroxide exposure (H2O2treated group) for 4 h. Also, compared with control group, PC12 cells shrank and turned smaller round in H2O2 treated group (Fig. 1C). All of those indicated that hydrogen peroxide treatment encouraged cell apoptosis. However, pretreatment of AFWE significantly reversed the decline of cell viability after exposed to H_2O_2.

We found that the cell viability was close to the control group in the presence of 80,160, 320, 640 μg/mL of AFWE. Besides, the cell morphological change of apoptosis was prevented at different AFWE concentrations. Based on these observations, Hoechst 33342, a specifically fluorescent probe sensitive to DNA, was applied to further explore whether AFWE could relieve the gene toxicity induced by H_2O_2 in PC12 cells. As displayed in Fig. 1D, pretreatment of AFWE could clearly decrease the number of small bright blue dots representing nuclear fragmentation compared with H_2O_2treated group, indicating that AFWE could effectively exert a protective effect against H_2O_2 induced toxicity in PC12 cells.

3.3. AFWE attenuated H_2O_2induced oxidative stress in PC12 cells

It is well known that oxidative stress plays a key role in neurodegenerative diseases and natural flavonoids may be a potential antioxidant. Therefore, we tested the effect of AFWE on H_2O_2 induced oxidative stress in PC12 cells. In the present PC12 cell model, the excessive ROS, O_2^- generation and Glutathione (GSH) depletion were measured in the presence or absence of AFWE by DCFH, DHE and NDA fluorescence assay, respectively. As shown in Fig. 2A and B, an increasing of fluorescence was observed in H_2O_2 treated cells, indicating that H_2O_2 indeed markedly promoted intracellular ROS and O_2^- levels, which was in accordance with previous studies. Interestingly, pretreatment with 50, 100, 200 μg/mL of AFWE dramatically decreased both ROS and O_2^- generation in a dose-dependent manner, with the fluorescence intensity close to control group, implying that AFWE can effectively decrease the overproduction of free radical.

Previous studies indicated that GSH is the most effective intracellular antioxidant that charge for keeping the intracellular redox homeostasis and the consumption of GSH contributed to the alleviation of oxidative stress-induced toxicity (Tong et al., 2004). It is illustrated that flavonoids increase the intracellular glutathione level by transactivation of the γ-glutamylcysteine synthetase catalytical subunit promoter (Myhrstad et al., 2002). Based on the aforementioned results, we assumed that the AFWE might maintain intracellular GSH level to defense the excess ROS. Hence, the intracellular GSH was measured by the NDA fluorescence probe in the presence or absence of AFWE. Interestingly, without pretreatments of AFWE, a visibly depletion of GSH was observed in H_2O_2treated group, with the fluorescence intensity declined to 0.31 (Fig. 2C). In contrast, pretreated with 100, 200 μg/mL of AFWE, the mean NDA fluorescence intensity were all significantly increased to 0.33 and 0.36. The mean DCF, DHE and NDA fluorescence intensities are shown in Fig. 2D, E and 2F, respectively. Taken the ROS and O_2^- results together, we can find that AFWE may protect PC12 cells against H_2O_2induced oxidative stress via resisting GSH depletion. Flavonoid compounds were reported to exert various bioactivities, both in vitro and in vivo models, because of their powerful ability to scavenge free radical. Luteolin-8-C-glucosideis the main compound of AFWE, which have been studied to possess anti-oxidative activity(Choi et al., 2014), may playing a key role in the cellular oxidative stress protective effect.

3.4. AFWE abated H_2O_2 induced mitochondrial dysfunction in PC12 cells

Mitochondrion, the main organelle that produces ROS, plays a key role in cellular ROS homeostasis (Zorov et al., 2006). Previous date has shown that excess ROS was always accompanied with mitochondrial dysfunction (Simon et al., 2000). However, the alteration of mitochondria membrane potential (MMP) and the peroxidation of mitochondrial membrane lipid are typical features of mitochondrial

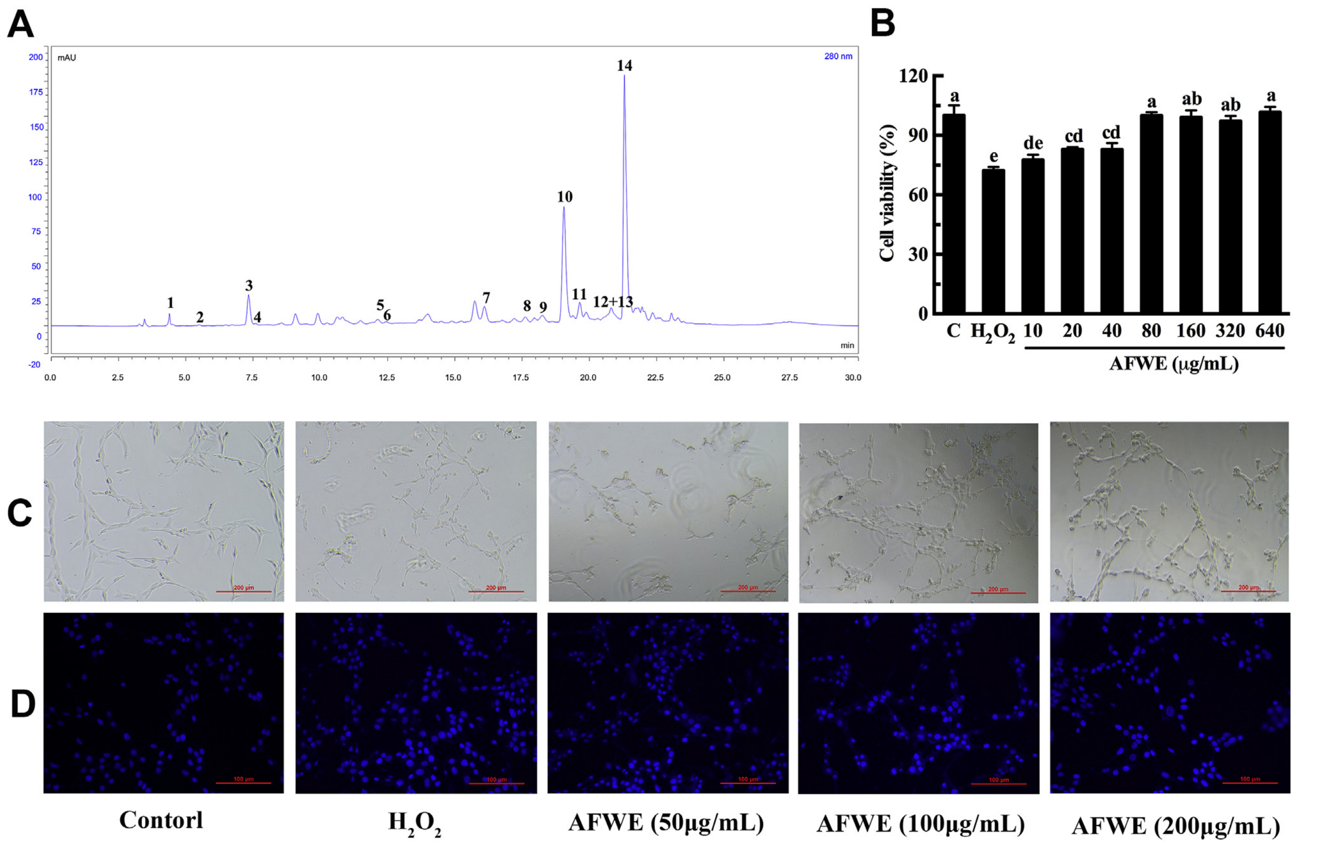

Fig. 1. HPLC elution profiles of compounds in*Apiosamericana*Medik flowers and effects of AFWE on H_2O_2 induced toxicity on PC12 cells. (A) HPLC elution profiles of AFWE. (B) MTT assay. (C) Morphological images of PC12 cells. (D) Effects of AFWE on H_2O_2 induced genotoxicity in PC12 cells measured by nuclear staining with Hoechst 33342.

dysfunction (Kadirvel et al., 2009). Previous studies performed on PC12 cells and HepG2 cells were convinced that H_2O_2 induced oxidative stress could lead to mitochondrial dysfunction (Kim et al., 2016; Mehmood et al., 2017). Consequently, we suspected that AFWE may alleviate H_2O_2 induced oxidative stress via recovering mitochondrial function. To verify our hypothesis, two sensitive fluorescence probes, Rh123 and NAO, were used to measure MMP and mitochondrial membrane peroxidation in PC12 cells, respectively. As we can see in Fig. 3A, compared with control group, a notable decrease (from 0.71 to 0.44) in mean Rh123 fluorescence intensity was appeared by H_2O_2treatment, implying that MMP was disintegrated. Conversely, 50, 100 and 200 μg/mL of AFWE could prevent the collapse of MMP in a dose-dependent manner, with the mean Rh123 fluorescence intensity increased to 0.59, 0.67 and 0.69 respectively. Subsequently, NAO, a fluorescence probe that could closely combine with the cardiolipin of mitochondrial membrane, was used to evaluate the peroxidation of mitochondrial membrane lipid. In our study, the mean fluorescence intensity was decrease (from 0.051 to 0.033) in H_2O_2treated group, while the decline was stopped and restored to 0.38, 0.46 and 0.50 through the pre-incubation of 50 μg/mL, 100 μg/mL and 200 μg/mL

Table 1
Tentatively identified compounds in AFWE.

No	Rt(min)	MS	MS/MS	Tentative identification
1	4.390	299. 08	93.04, 137.02	4-(β-D-glucopyranosyloxy) hydroxybenzoic acid
2	5.497	329.09	108.02, 123.05, 152.01, 167.03	4-(β-D-glucopyranosyloxy)-3-methoxybenzoic acid
3	7.333	299.08	59.02, 89.02, 119.03, 137.02, 151.04, 179.03, 209.04, 239.05	1-O-(4-hydroxybenzoyl)-β-D-glucopyranose
4	7.593	153.028	53.04, 91.02, 108.02, 109.03	3,4-Dihydroxybenzoic acid
5	12.130	771.208	285.04, 609.15, 610.15	kaempferol 3- < β-D-glucopyranosyl(1- > 2)-β-D-glucopyranosyl(1- > 2)-β-D-glucopyranoside >
6	12.450	325.10	117.04, 119.05, 163.04	4-O-β-D-glucopyranosyl-p-coumaric acid
7	16.100	593.15	297.08, 353.07, 383.08, 473.11, 503.12	vicenin-2
8	17.617	579.14	339.05, 369.06, 399.07, 429.08, 489.10, 519.12	Lucenin I
9	18.253	563.14	297.08, 353.07, 365.07, 383.08, 443.10, 473.11, 503.12	Schaftoside
10	19.053	563.14	297.08, 325.07, 353.07, 383.08, 413.09, 443.10, 473.11	Apigenin (6-C-α-L-arabinopyranosyl)-8-C-β-D-glucopyranoside
11	19.643	447.09	133.03, 284.03, 297.04, 327.05, 357.06, 429.08	Luteolin-8-C-glucoside
12	20.810	533.13	297.08, 325.07, 353.07, 383.08, 413.09, 443.10, 473.11, 515.12	Apigenin 6,8-di-C-α-L-arabinopyranoside
13	20.977	609.15	227.03, 255.03, 284.03, 285.04	Kaempferol-3-O-sophoroside
14	21.303	609.15	255.03, 284.03, 285.04	Kaempferol-3-O-sophoroside (isomerism)

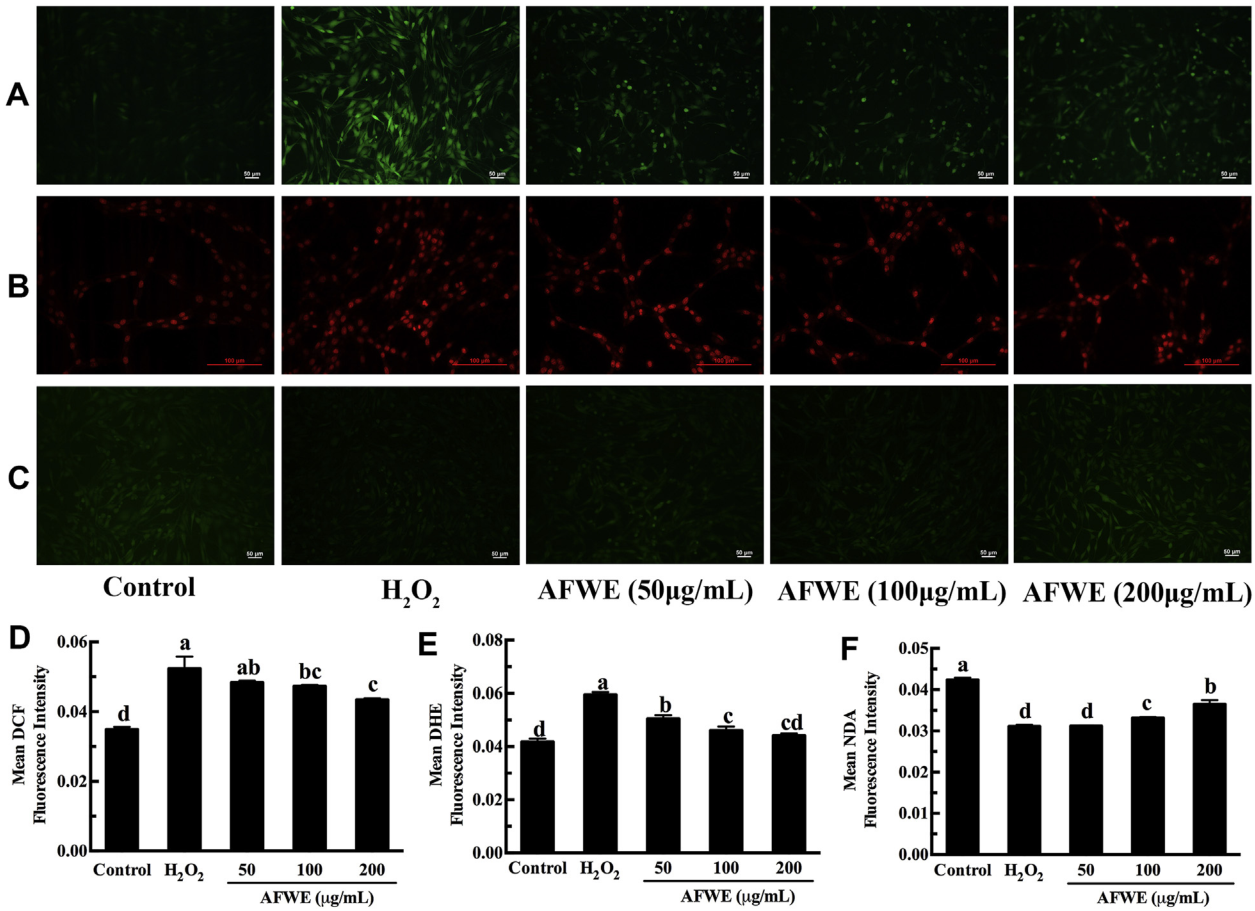

Fig. 2. Protective effects of AFWE on H_2O_2 induced oxidative stress in PC12 cells. (A) Effects of AFWE on ROS generation in PC12 cells measured by DCFH. (B) Effects of AFWE on O_2^- production in PC12 cells probed by DHE. (C) Effects of AFWE on GSH depletion in PC12 cells measured by NDA. (D), (E) and (F) are the quantitative results of panel (A), (B) and (C), respectively. Different letters show statistically significant differences among the groups, $p < 0.05$.

AFWE, respectively (Fig. 3B). The mean Rh123 and NAO fluorescence intensities are shown in Fig. 3C and D, respectively. Our preliminary results illustrated that AFWE could prevent H_2O_2induced ROS overproduction. Further results showed that AFWE not only suppressed MMP collapse but also restrained mitochondrial membrane peroxidation, suggesting that AFWE could offer protection against H_2O_2 induced oxidative stress by inhibiting ROS, O_2^- production and mitochondrial dysfunction.

3.5. Effects of AFWE on the ratio of Bcl-2/Bax and Caspase-3, Nrf2 expression in H_2O_2 induced PC12 cells

The Bcl-2 family proteins are important factors of apoptosis. According to previous research, Bcl-2 inhibits apoptosis and Bax promote apoptosis, over expression of Bcl-2 antagonizes the promotion effect of Bax to apoptosis, which could suppress apoptosis (Czabotar et al., 2014). And Caspase-3, a frequently activated cysteine protease, also connected with the mitochondrial apoptotic pathway (Mazumder et al., 2008). To investigate the protective of AFWE, the expressions of Bcl-2, Bax, and caspase-3 in PC12 cells after H_2O_2 exposure were measured by Western blot. As described in Fig. 4A and B, after treating with H_2O_2, the Caspase-3 expression was increased obviously in comparison with control group. However, pretreating with AFWE could dramatically converse the decline of Caspase-3 expression induced by H_2O_2. Previous data has illustrated that H_2O_2 exposure could result in cytotoxicity and the over expression of Caspase-3, while various flavonoids could alleviate the oxidative stress induced cell apoptosis via blocking the Caspase-3 (Williams et al., 2004; Spencer et al., 2001; Martín et al., 2010). Meanwhile, the ratio of Bcl-2/Bax was markedly down regulated by 250 μMH_2O_2treatment in PC12 cells, which was reversed in a dose-dependent manner by pre-incubation with AFWE for 24 h (Fig. 4A and C). The transcription factor Nrf2 is an essential mediator in the coordinated regulation of some cytoprotective genes. Western blot results showed that Nrf2 expression was improved notably increased while under H_2O_2 stress in comparison with that of control group. Interestingly, this increase was further enhanced to defense oxidative stress by pre-treating with 100 and 200 μg/mL of AFWE (Fig. 4A and D). These results suggested that AFWE could inhibit cell apoptosis by regulating the relative expression of caspase-3, Bcl-2, Bax and Nrf2.

3.6. AFWE activated autophagy in H_2O_2 induced PC12 cells

Autophagy, an evolutionarily conserved catabolic process, is capable of removing normal as well as damaged mitochondria, which are the production base of intracellular ROS (Filomeni et al., 2015). Excess ROS induce autophagy, and in turn autophagy renovates intracellular ROS levels (Li et al., 2015). Defective autophagy increases the amassment of damaged mitochondria and ROS, implicating in the pathogenesis of various diseases, such as cancer, aging, diabetes and neurodegenerative diseases (Jiang and Mizushima, 2014). According to our preliminary data, AFWE plays a positive role in H_2O_2 induced apoptosis, oxidative stress and mitochondrial dysfunction. Thus, we suspected that whether cell autophagy is involved in these physiological processes. To verify our conjecture, we examined the relative expression of some autophagy-related proteins, such as Atg4, Atg5 and Rab5.

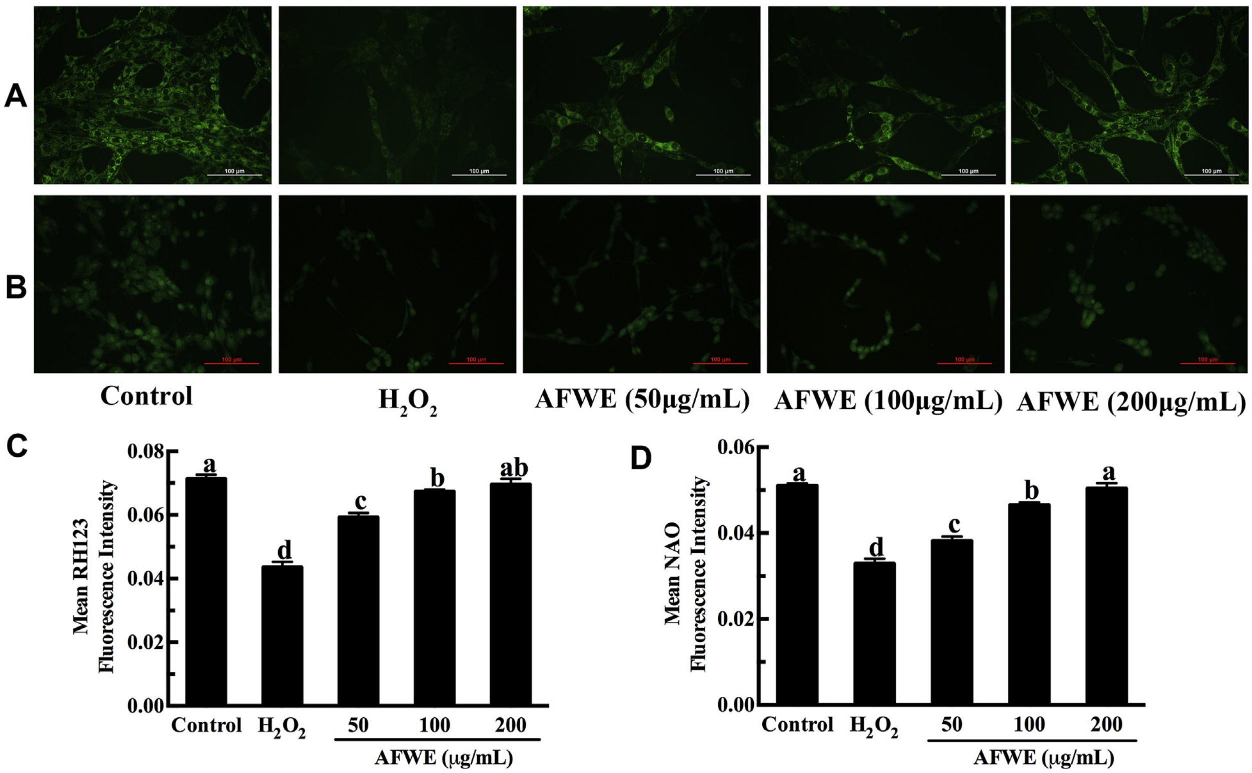

Fig. 3. Effects of AFWE on H_2O_2 induced mitochondrial dysfunction in PC12 cells. (A) Effect of AFWE on H_2O_2 induced mitochondrial membrane potential (MMP) decrease in PC12 cells. (B) Effect of AFWE on H_2O_2 induced mitochondrial membrane lipid peroxidation in PC12 cells. (C) and (D) are the quantitative results of panel (A) and (B), respectively. Different letters show statistically significant differences among the groups, $p < 0.05$.

As shown in Fig. 4E, F, 4G and 4H, a notable increase of Atg4, Atg5 and Rab5 expressions were appeared by pre-incubating with AFWE for 24 h in comparison with H_2O_2 solely treatment.

Furthermore, a TEM assay was performed to inspect autophagic vacuoles of PC12 cells (Fig. 5A). TEM of untreated PC12 cells showed an intact nucleus, with abundant mitochondria. While the H_2O_2group appeared uneven nuclear chromatin, numerous vacuoles in the cytoplasm, unclear organelle structure and increasing autophagosomes. In the H_2O_2 + AFWE group, alteration of the ultrastructure ameliorated and the number of autophagosomes was further increased. And this result is concordant with the changes measured by western blot (Fig. 4E), implying that autophagy was further activated by AFWE to resist excess ROS.

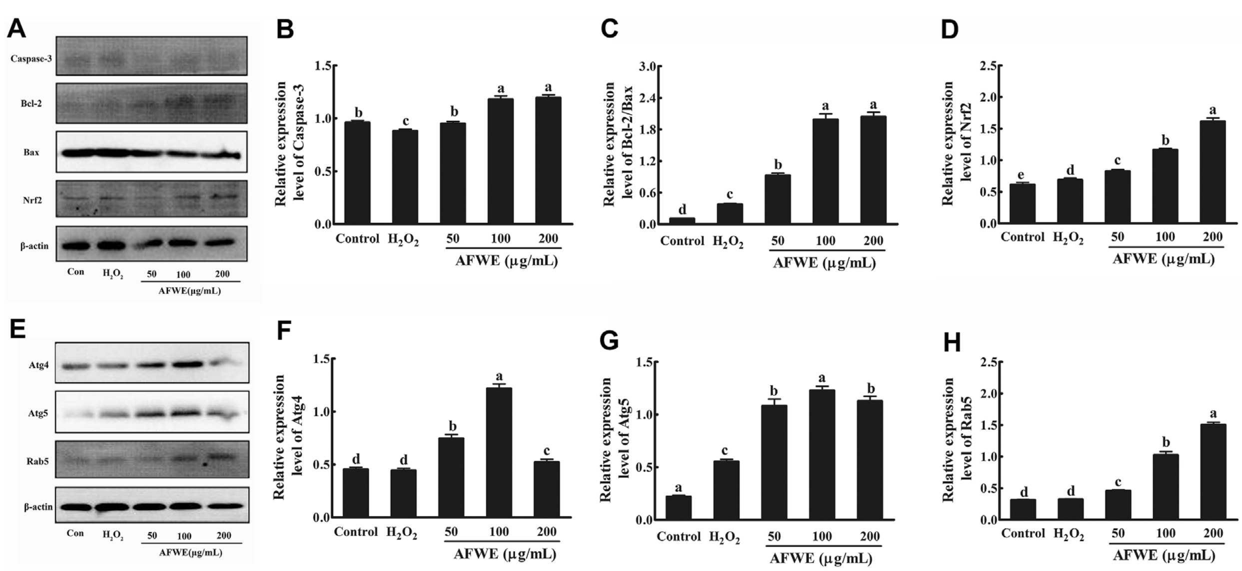

Fig. 4. AFWE pretreatment altered expressions of proteins associated with oxidative stress, apoptosis and autophagy. (A) Protein levels of Caspase-3, Bcl-2, Bax and Nrf2 in total cell extracts. (B), (C) and (D) Intensity of bands corresponding to Caspase-3, Bcl-2/Bax and Nrf2 were corrected by β-actin. (E) Effect of ALE on the intracellular distribution of Atg4, Atg5, and Rab5 in PC12 cells. (F), (G) and (H) Intensity of bands corresponding to Atg4, Atg5 and Rab5 were corrected by β-actin. Different letters show statistically significant differences among the groups, $p < 0.05$.

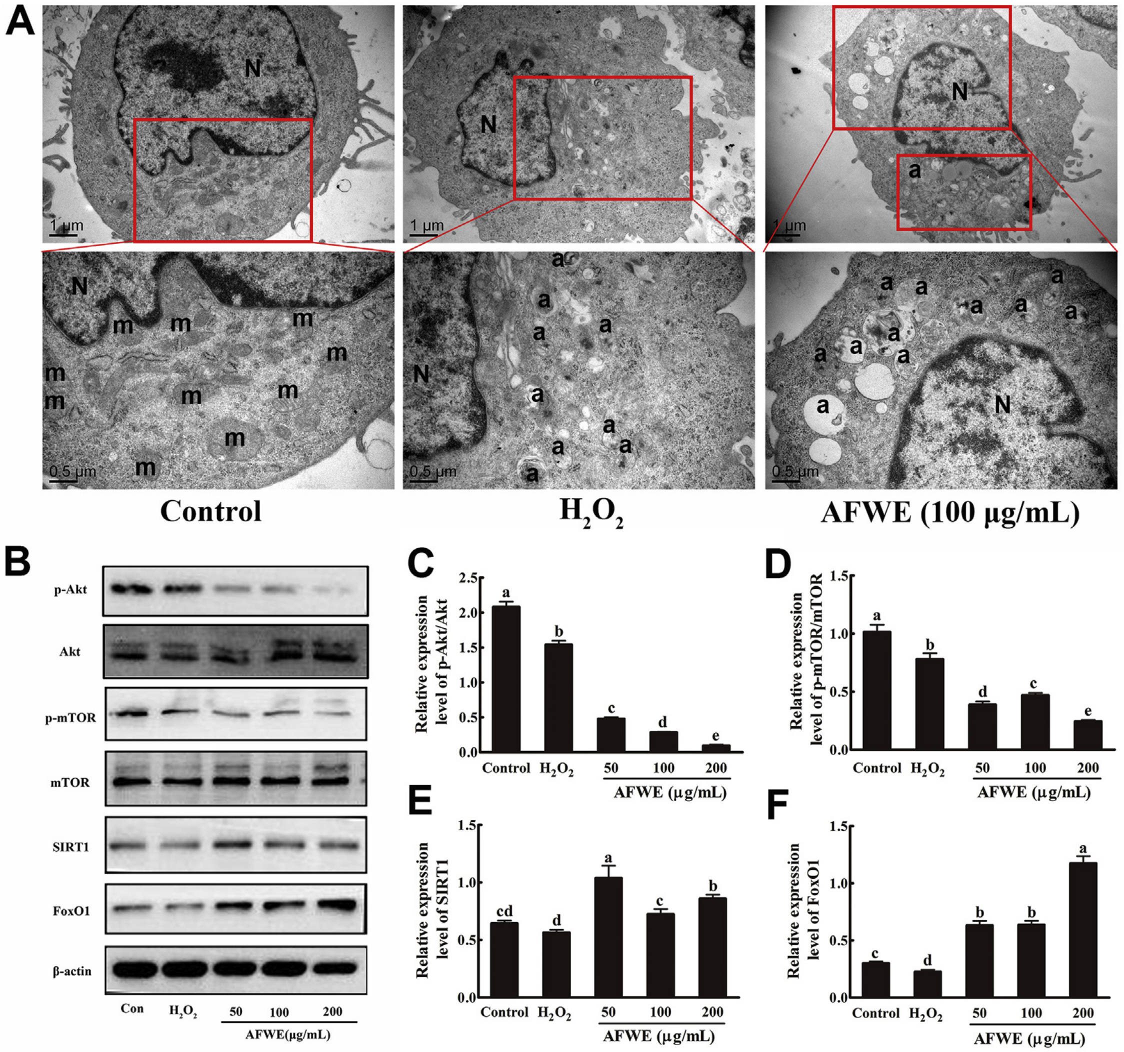

Fig. 5. AFWE pretreatment activated autophagy. (A) TEM images of PC12 cells, (N), (m) and (a) in the images represent cell nucleus, mitochondria and autophagosome, respectively. (B) Effect of ALE on the intracellular distribution of p-Akt, Akt, p-mTOR, mTOR, SIRT1, and FoxO1 in PC12 cells. (C), (D) Intensity of p-Akt, and p-mTOR were corrected by Akt, and mTOR to get the Akt, and mTOR phosphorylation amongsamples. (E), (F) Intensity of bands corresponding to SIRT1, and FoxO1 were corrected by β-actin. Different letters show statistically significant differences among the groups, $p < 0.05$.

3.7. Akt-mTOR and SIRT1-FoxO1 pathways are involved in AFWE-mediated autophagy activation in PC12 cells

Recently, the Akt-mTOR pathway has been considered to be an important regulator of a various intracellular physiological processes, such as maintaining the cell's viability, cell apoptosis, autophagy, anti-inflammation and antioxidant systems (Saxton and Sabatini, 2017). To further investigate whether AFWE induced autophagy by activating the Akt-mTOR pathway, we estimated intracellular Akt-mTOR pathway activation by evaluating phosphorylated AKT (p-AKT) and mTOR (p-mTOR) expression. Western blot results showed that the addition of AFWE resulted in the up-regulation of p-Akt/Akt, and p-mTOR/mTOR expressions. Meanwhile, the relative expression of Akt and mTOR appeared no significant changes (Fig. 5B, C and 5D). SIRT1,a histone deacetylase, plays a key role in regulating mitochondrial function, autophagy, cancer and aging (Wilking and Ahmad, 2015; Yuan et al., 2016). What's more, SIRT1 regulates autophagy-related genes Atg5, Atg7, Atg8, and FoxO1 expressions, which were related to the autophagy-lysosome pathway (Kitada et al., 2016). In this study, the relative expression of SIRT1 and FoxO1 were detected by western blot. Pretreatment of AFWE indeed enhanced the protein levels of SIRT1 and FoxO1, in comparison with the H_2O_2 group (Fig. 5B, E and 5F). These date suggested that AFWE might induce autophagy of PC12 cells by inhibiting Akt-mTOR pathway and activating SIRT1-FoxO1 signaling pathway.

4. Conclusion

Overall, in this study, fourteen compounds of *Apios americana* Medik flowers water extract (AFWE) were firstly identified by a LC-TOF-MS technique. Using in vitro studies, we firstly show that AFWE exerts the capacity of alleviating the cytotoxicity and oxidative stress induced by H_2O_2 in PC12 cells and this effect is related to the up-regulation of Nrf2. After in vitro pretreatment of AFWE, a protective performance on the generation of ROS and O_2^-, preventing GSH depletion, attenuating mitochondrial dysfunction. Furthermore, autophagy was significantly activated by AFWE via inhibiting Akt-mTOR and activating SIRT1-FoxO1 signaling pathways, which could disintegrate damaged mitochondria and maintain intracellular ROS homeostasis. This finding suggests that the flower of *Apios americana* Medik is a good source of antioxidant and providing some underlying mechanisms of AFWE on anti-oxidation, indicating that *Apios americana* Medik flowers can be served as a tactics of preventing neurodegenerative diseases.

Conflicts of interest

The authors declare no competing interests.

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