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Identification of flavonoids from *Flammulina velutipes* and its neuroprotective effect on pheochromocytoma-12 cells

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ABSTRACT

The flavonoids from *Flammulina velutipes* (FVF) were isolated and the chemical composition of FVF was identified by high-performance liquid chromatography/mass spectrometry (HPLC/MS). The antioxidant activity and the neuroprotective effect against H_2O_2-induced pheochromocytoma-12 cells (PC12) cytotoxicity of FVF was investigated. Six flavonoids from FVF were identified as arbutin, epicatechin, phillyrin, apigenin, kaempferol and formononetin. Obtained results suggested that FVF possesses strong antioxidant properties in 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging activities as well as reducing power. Pretreatment of PC12 cells with FVF significantly increased the survival rate of cells, glutathione level and superoxide dismutase activity. Conversely, the pretreatment reduced the release of lactate dehydrogenase and the accumulation of both intracellular reactive oxygen species and malondialdehyde. These findings collectively suggested that the protective effects of FVF against H_2O_2-induced neurotoxicity in PC12 cells as well as the potential for neuroprotection of FVF could be due to its antioxidant action.

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1. Introduction

Flammulina velutipes is one of the most important edible mushrooms available commercially, and has widely been cultivated and consumed in oriental countries owing to its high nutritional value and attractive taste. A number of bioactive compounds have been identified in *F. velutipes* such as flavonoid glycosides, proteins, polysaccharides and phenols (Kang, Zeng, Ye, Lin, & Guo, 2014). Recently, flavonoids have been considered as a promising approach to slow down ageing and exert powerful protection against neuronal injury involved in Parkinson's disease (PD) and Alzheimer's disease (AD).

Flavonoids exist widely in fruits, vegetables and flowers of plants. According to their various structural characteristics, more than 4000 different flavonoids have been identified and broadly categorized into flavones, flavanonols, flavonols, isoflavonoids, anthocyanins and flavanones (Fu, Chen, Li, Zheng, & Li, 2013). The role of flavonoids such as apigenin, quercetin, eriodictyol, 3-hydroxyflavone, luteolin, rutin, taxifolin, naringenin, and kaempherol in neuroprotection has been recognized. (Sasaki, Toda, Kaneko, Baba, & Matsuo, 2003). However, limited information is available on the protective effects of flavonoids from *F. velutipes* (FVF) towards neurodegenerative diseases.

It is well established that oxidative stress-induced apoptosis is associated with lactate dehydrogenase (LDH) release, generation of reactive oxygen species (ROS) and initiation of caspase cascades which may subsequently lead to neurodegenerative diseases such as Parkinson's and Alzheimer's disease (Shokoohinia, Rashidi, Hosseinzadeh, & Jelodarian, 2015). Moreover, 7,8-dihydroxyflavone (7,8-DHF) has been demonstrated to protect PC12 cells against 6-hydroxydopamine-induced cytotoxicity through its free radical scavenging and enhanced superoxide dismutase (SOD) activity (Han et al., 2014). There is growing evidence from animal and human studies showing that flavonoids can reduce memory dysfunction and neuronal injury caused by oxidative stress (Pavlica & Gebhardt, 2010; Wang, Peng, Zhu, & Ren, 2007). Collectively, this evidence shows that FVF have a strong antioxidant ability in scavenging various types of free radicals Bao, Ochiai, and Ohshima (2010). Therefore, it is interesting to explore the neurodegenerative bioactivities of FVF by attenuating oxidative stress.

In the current study, FVF prepared from ultrasonic-assisted extraction was further purified using AB-8 macroporous resin column chromatography and Sephadex LH-20 column chromatogra-

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phy. The flavonoid content, profiles and antioxidant activities were investigated. To better understand the protection of FVF on PC12 cells, the viability of the cells induced by hydrogen peroxide was evaluated using 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and LDH assay in vitro. The morphological changes, levels of malondialdehyde (MDA) and glutathione (GSH), SOD activities, and intracellular ROS production were also analyzed.

2. Materials and methods

2.1. Materials and chemicals

F. velutipes (CCMSSC 00110), was purchased from a local agricultural trade market (Nanjing, China). The mushrooms were selected according to their whiteness, developmental stage, closed veil, and shape (stipe length of 10–16 cm). PC12 cells were obtained from Cell Bank of Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). AB-8 macroporous adsorption resin and Sephadex-LH20 were purchased from Chemical Plant of Nankai University (Tianjin, China) and Pharnacia Co. (Sweden), respectively. Rutin, ascorbic acid, H_2O_2, dimethylsulfoxide (DMSO), and MTT were from Sigma Chemical Co. (St. Louis, MO, USA). The kits used for MDA, LDH, ROS and GSH assays were obtained from Jiancheng Bioengineering Institute (Nanjing, China). Dulbecco's minimal essential medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were purchased from Invitrogen (Carlsbad, CA, USA). All solvents and the other chemicals used were of analytical grade.

2.2. Extraction and isolation of FVF

Fresh *F. velutipes* was freeze-dried at 25 °C, powdered and sieved through NO. 300 mesh. The *F. velutipes* powder was extracted with 95% ethanol under reflux (60 °C) twice for 3 h. The extraction condition was optimized as following: ultrasonic power of 600 W, ultrasonic time of 25 min, and ultrasonic temperature of 60 °C. The extracts were combined and evaporated to dryness under vacuum at 45 °C. Crude flavonoids extract was then extracted with acetic ether 3 times and the extract liquid was purified by AB-8 macroporous resin and eluted with 60% ethanol (Wan et al., 2014). The elutes were further purified on a Sephadex LH-20 column (2.6 cm×60 cm) and eluted with methanol at a flow rate of 0.2 ml/min (Guimarães et al., 2015). The collected fractions were freeze dried to obtain the pure flavonoids and stored at 4 °C for the further experiments.

2.3. Determination of total flavonoids content

The total flavonoid content in *F. velutipes* was measured according to Zhishen, Mengcheng, and Jianming (1999) with some modifications. Rutin was used as a standard. A 1.0 ml sample solution was added to a 10 ml volumetric flask followed by addition of 0.5 ml $NaNO_2$ solution (1:20, w/v). After 6 min, another 0.5 ml Al $(NO_3)_3$ (1:10, w/v) was added, mixed thoroughly and kept at room temperature for 6 min. Finally, 4 ml NaOH (1 M) was added to the solution, which was then topped up to the mark with 60% ethanol. The mixed solution was left to stand at room temperature for a further 12 min before reading the absorbance at 510 nm (UV 2550, Shimadzu, Japan). A calibration curve was obtained by plotting the absorbance of rutin against its concentration. A solution containing all the reagents without the sample was used as a blank. Data obtained were expressed as mg of rutin equivalents per gram dry weight of extract. The calibration equation was $A = 4.807C - 0.004$, $R^2 = 0.9996$ where A was the absorbance and C was the concentration of rutin equivalent (RE) mg/ml.

2.4. HPLC/MS analysis

Determination of FVF was performed on an Agilent 1100 HPLC (Agilent Technologies, Palp Alto, CA, USA) series equipped with a dual pump, an auto sampler, a vacuum degasser, a diode array detector (DAD), and an Agilent HPLC workstation. The sample was separated on an Agilent Zorbax SB-C18 column (250 × 4.6 mm, 5 μm) and the temperature maintained at 30 °C. The mobile phase constituted of (A) 0.1% formic acid and (B) 95% acetonitrile in 0.1% formic acid. A gradient elution program was applied at a flow rate of 0.6 ml/min and the injection was 20 μl. The percentage of B was kept at 2% from 0 to 5 min, then raised to 10.0% from 5 to 10 min, 20% from 10 to 15 min, 40% from 20 to 25 min, 60% from 25 to 30 min, lowered to 15% from 30 to 35 min and finally 2% from 35 to 40 min. Before the next run, the column was equilibrated with 98% mobile phase A and 2% mobile phase B for 10 min.

MS analyses were performed by a Mass spectrometer 1100 Series LC/MSD Trap-SL (ion trap) equipped with electro spray ionization/mass spectrometry (ESI/MS) and running on Agilent Chem-Station software. ESI/MS conditions were optimized as follows: negative ion mode; drying gas (N_2), 9 ml/min; nebulizer pressure 60 pis; drying gas temperature, 320 °C; capillary voltage 3500 V; mass range (m/z) 100–1500.

2.5. Evaluation of antioxidant activity in vitro

2.5.1. DPPH free radical scavenging assay

The DPPH free radical scavenging activity of FVF was measured according to the method described by Hsu, Zhang, Peng, Travas-Sejdic, and Kilmartin (2008) with some modifications. Briefly, a 150 μM solution of DPPH in 100% methanol was prepared. To 1.5 ml of this solution 1.5 ml of FVF (dissolved in methanol) was added at different concentrations (2–20 μg/ml). The mixture was incubated in the dark at room temperature for 30 min before measuring its absorbance at 517 nm. Ascorbic acid and rutin with the same concentrations as the samples were used as reference standards. The radical scavenging activity (RSA) was calculated by the following equation:

$$\mathrm{RSA}(\%) = \left[\frac{A_0 - A_1}{A_0}\right] \times 100 \quad (1)$$

where A_0 is absorbance of pure DPPH, and A_1 is the absorbance of samples. Each experiment was replicated three times and the reproducibility was expressed as % CV (RSD < 5.0%). The result was presented as the effective concentration of FVF needed for 50% RSA (EC_{50}).

2.5.2. ABTS radical scavenging activity assay

The ABTS radical scavenging activity assay as described by Siddhuraju (2006) was used with slight modifications. The method is based on the decolonization reaction between an antioxidant with ABTS radical cation ($ABTS^{\cdot+}$) produced by mixing an ABTS stock solution (7 mM in water) with 2.45 mM potassium persulfate and leaving in the dark at room temperature for 12 h before using. For each analysis, the ABTS solution was diluted 60 times with phosphate buffered saline (PBS) (pH 7.4) at 37 °C. A 100 μl of FVF was added to 900 μl of ABTS solution and incubated in the dark at room temperature. Absorption was measured at 734 nm after 60 min of reaction. The $ABTS^{\cdot+}$ scavenging activity was calculated by the following equation:

$$\mathrm{RSA}(\%) = \left[\frac{A_0 - A_1}{A_0}\right] \times 100 \quad (2)$$

where A_0 is absorbance of pure ABTS, and A_1 is the absorbance of samples. Ascorbic acid and rutin with the same concentrations as the samples were used as reference standards. Each experiment was replicated three times.

2.5.3. Ferric-reducing antioxidant power (FRAP) assay

The ferric-reducing antioxidant power was determined using the method of Zhang, Zhang, and Cheung (2009). Briefly, 2.5 ml of different concentrations of FVF (10–80 μg/ml) suspended in 2.5 ml of 0.2 M phosphate buffer (pH 6.6) were mixed with 2.5 ml of 1% potassium ferricyanide and incubated at 50 °C. At 20 min, 2.5 ml of 10% trichioroacetic acid was added to the mixture to terminate the reaction. Then the solution was centrifuged at 4500 rpm for 15 min. Distilled water (2.5 ml) and 0.5 ml of 0.1% ferric chloride were mixed with 2.5 ml of the supernatant and the absorbance measured at 700 nm. Ascorbic acid and rutin with the same concentrations as the samples were used as reference standards. Increase in absorbance of the reaction mixture indicated increased reducing power.

2.6. Effects of FVF on viability and morphology of H_2O_2-induced PC12 cells

To investigate the protective effects of FVF against H_2O_2-induced cytotoxicity, the cell viability of PC12 cells was evaluated using the MTT assay (Crispo et al., 2010). The cells were grown in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin and maintained at 37 °C in a humidified incubator at 5% CO_2. Briefly, PC12 cells were seeded at a density of 2×10^5 cells/ml in a 96-well plate for 24 h. The cells were pretreated with various concentrations of FVF (0, 1.25, 2.5, 5, 10 and 20 μg/ml) for 12 h and then exposed to 200 μM H_2O_2 for 2 h. At the end of the exposure, 10 μl of MTT (5 mg/ml) was added to each well and incubated for a further 4 h. Following aspiration of the culture media, 100 μl DMSO was added to each well to dissolve the formazan crystals. The absorbance was read at 570 nm with an ELISA reader (Thermo multiskan Mk3, Thermo Fisher Scientific Inc, USA).

To observe the morphological changes, PC12 cells were seeded at a density of 2×10^5 cells/ml in 24-well plate and incubated for 24 h. The cells were pretreated with various concentrations of FVF (0, 1.25, 2.5, 5, 10 and 20 μg/ml) for 12 h and then exposed to 200 μM H_2O_2 for 2 h. After the treatment, the cells were observed under inverted phase contrast microscopy and cell images were recorded (Zhu, Guo, Guo, Peng, & Zhou, 2013).

2.7. LDH assay

Cytotoxicity was evaluated by LDH assay based on the measurement of the leakage of cytoplasmic LDH enzyme activity to the cell supernatants. The LDH leakage was detected by using a commercial LDH assay kit (Jiancheng BioEngineering, Nanjing, China) according to the manufacturer's instructions. Absorbance was read at 450 nm with an ELISA reader (Thermo multiskan Mk3, Thermo Fisher Scientific Inc, USA). LDH leakage was expressed using the following equation:

$$\text{LDH leakage}(\%) = \left(\frac{\text{LDH activity in the culture medium}}{\text{total LDH activity}}\right) \times 100 \quad (3)$$

2.8. Measurement of intracellular ROS level, MDA, GSH and SOD

The levels of intracellular ROS were examined by a fluorometry assay using DCFH-DA according to Cheng, Lu, Bai, and Chen (2013). PC12 cells were seeded at a density of 2×10^5 cells/ml in 6-well plate for 24 h and exposed to various concentrations of FVF (0, 1.25, 2.5, 5, 10 and 20 μg/ml) for 12 h before exposure to 200 μM H_2O_2 for 2 h. Cells were washed with PBS buffer (pH 7.4) and incubated with 50 μM DCFH-DA for 30 min at 37 °C in the dark. The relative levels of intracellular ROS were measured using a fluorescence microplate reader with excitation at 485 nm and emission at 530 nm. Control group cells treated with serum-free medium and the results were expressed as a percentage of control.

The content of MDA, the level of GSH and the activity of SOD were measured by using commercial assay kits (Jiancheng BioEngineering, Nanjing, China). The tests were done according to the manufacturer's instructions.

2.9. Statistical analysis

All experiments were done in triplicate and all data were expressed as means ± standard deviation. One-way analysis of variance (ANOVA) was conducted to determine the variations between groups and within groups. The SPSS program (version 18.0 SPSS Inc., Chicago, IL, USA) and Origin 8.5 (OriginLab, USA) were used for the statistical and graphical analysis. Statistical significance was set at $P < 0.05$ and $P < 0.01$.

3. Results and discussion

3.1. Total flavonoids content

Total flavonoids content in *F. velutipes* was quantified using the calibration curve. The acetic ether extract contained 3.68 ± 0.20 mg RE/g of total flavonoids, and the purity of total flavonoids was increased from 23.81 ± 1.78% to 65.18 ± 2.16% following purification by AB-8 macroporous. The purity of total flavonoids purified by repeating Sephadex-LH20 was 96.16 ± 1.83%. Kang et al. (2014) reported that the levels of the total flavonoids in different *F. velutipes* strains varied. The extract of TF71 strain contained 15.97 mg/kg while in the other two strains TF7H and F7, the total contents were 18.33, and 28.06 mg/kg, respectively. In addition, flavonoids were reported in numerous mushrooms and fruits, such as *white rot fungi* (3.27 mg RE/g), guava (1.13 mg RE/g), and R. tomentosa berries (5.21 ± 0.20 mg RE/g). (Gąsecka, Mleczek, Siwulski, Niedzielski, & Kozak, 2015; Wu et al., 2015) Compared with other fungi and fruits, *F. velutipes* was rich in flavonoids.

3.2. Identification of flavonoids in F. velutipes

HPLC-DAD-ESI/MS were used to identify the purity of flavonoids prepared by repeating Sephadex-LH20 column. The mass spectrum for tentative flavonoids were shown in Fig. 1. The formula, retention times (t_R), MW, MS and MS^2 fragmentation ions of the flavonoids are listed in Table 1. The purification procedure employed in this study has aided isolation of six major flavonoids from *F. velutipes*: arbutin (0.09 ± 0.01 mg RE/g), epicatechin (0.13 ± 0.01 mg RE/g), phillyrin (0.11 ± 0.02 mg RE/g), apigenin (0.17 ± 0.01 mg RE/g), kaempferol (0.10 ± 0.01 mg RE/g) and formononetin (0.18 ± 0.01 mg RE/g). The specific proportion of arbutin, epicatechin, phillyrin, apigenin, kaempferol and formononetin in FVF were 10.71%, 14.12%, 10.91%, 18.07%, 24.84% and 19.43%, respectively, as determined by HPLC analysis.

Compound **1** (t_R = 10.4 min) showed pseudo molecular ions of m/z 271 $[M-H]^-$ and m/z 573 $[2M-H]^-$. Its negative ion mode of MS^2 spectra revealed 161 $[(M-H)-110]^-$, 109 $(M-H)-162]^-$. The ions at m/z 161 and m/z 109 indicated that it was tentatively identified as arbutin (Panusa, Petrucci, Marrosu, Multari, & Gallo, 2015),

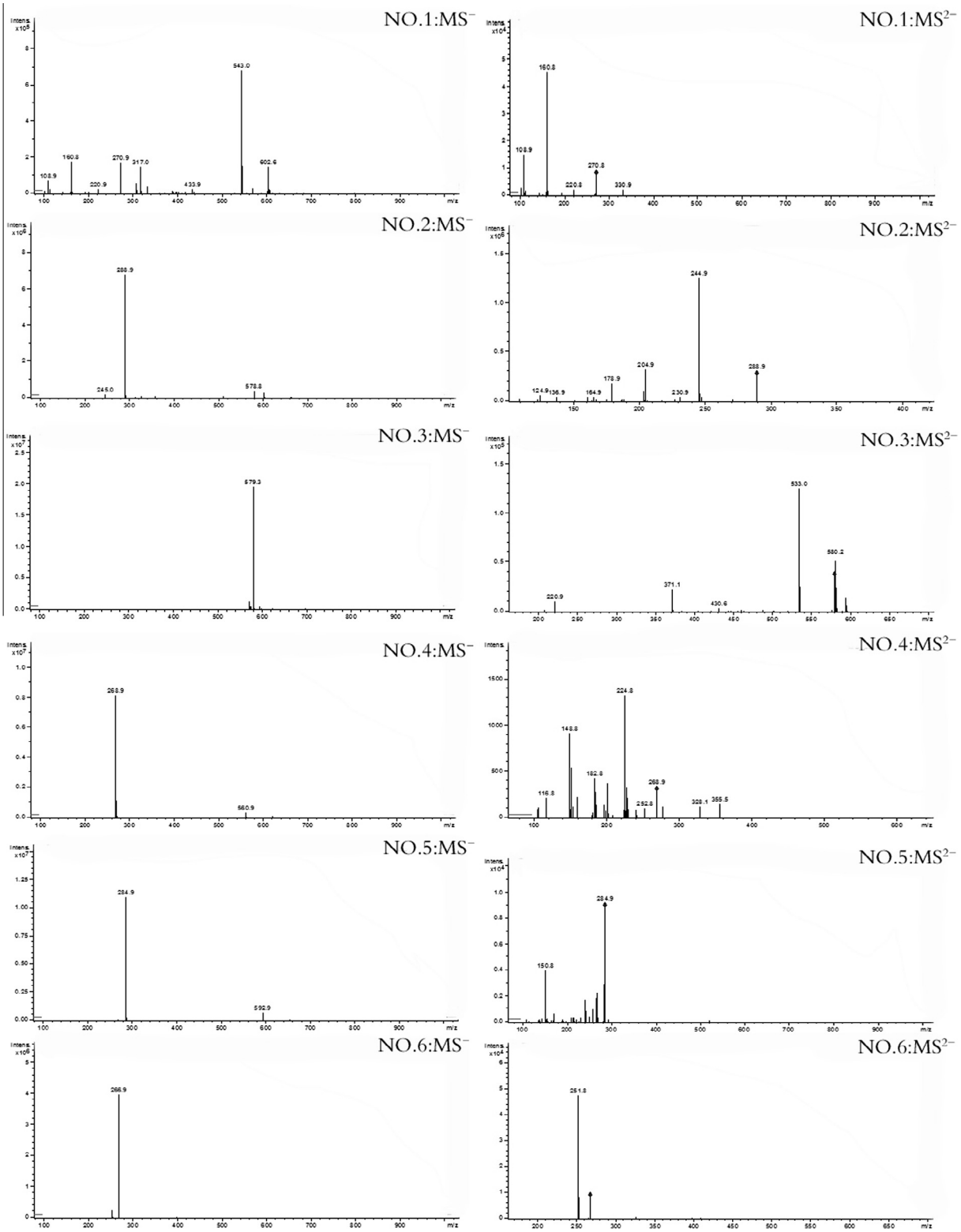


Fig. 1. The extraction ion chromatograms for six tentative flavonoids in FVF analysis by HPLC-DAD-ESI/MS. NO. 1: arbutin; NO. 2: epicatechin; NO. 3: phillyrin; NO. 4: apigenin; NO. 5: kaempferol; NO. 6: formononetin.

which has been reported in *Arctostaphylos pungens*, however, it has not been reported in *F. velutipes* previously.

Compound **2** (t_R = 23.1 min), with $[M-H]^-$ ion at *m*/*z* 289. Its MS^2 spectrum produced ions at *m*/*z* 245 $[(M-H)-44]^-$, 205 $[(M-H)-84]^-$, 179 $[(M-H)-110]^-$, 165 $[(M-H)-124]$, 137 $[(M-H)-152]^-$, 125 $[(M-H)-164]^-$. It was tentatively assigned to epicatechin with two fragment ions at *m*/*z* 245 and 179 (Falleh et al., 2011; Jaitz et al., 2010; Zhu et al., 2012).

Compound **3** (t_R = 26.5 min) showed $[M-HCOO]^-$ at *m*/*z* 579. The fragmentation ions by MS^2 was observed with *m*/*z* 533

Table 1
Retention times, MS, and MS^2 fragmentation ions of the FVF.

No.	t_R(min)	MW	MS-(m/z)	MS^2 fragment ions (m/z)	Identification
1	10.4	272	270.8(M−H+)	221,161,109	Arbutin
2	23.1	290	288.9(M−H+)	245,231,205,179,165,137,125	Epicatechin
3	26.5	534	579.3(M−HCOO−)	533,431,371,221	Phillyrin
4	29.8	270	268.9(M−H+)	253,225,193,149,117	Apigenin
5	30.2	286	284.9(M−H+)	151	Kaempferol
6	32.8	268	266.9(M−H+)	252	Formononetin

$[(M-H)-46]^-$, 371 $[(M-H)-208]^-$, 221$[(M-H)-356]^-$. The predominant product ion of m/z 371 ($[M\text{-(glucose-}H_2O)\text{-}H]^-$) tentatively indicated that compound **3** was phillyrin (Jiao et al., 2013; Ye et al., 2012; Zhang, Wei, & Wang, 2012).

Compound **4** (t_R = 29.8 min) produced a $[M-H]^-$ ion at m/z 269 which was fragmented into three product ions at m/z 183 $[(M-H)-86]^-$, 149 $[(M-H)-120]^-$ and 117 $[(M-H)-152]^-$. The characteristic fragment ion at m/z 117 corresponded to $[M-H-C_7H_4O_4]^-$ tentatively indicating that compound **4** was apigenin (Fattahi et al., 2013; Sun et al., 2013).

Compound **5** (t_R = 30.2 min) yielded a molecular ion $[M-H]^-$ at m/z 285 was tentatively assigned to kaempferol with its MS^2 spectrum showing a characteristic fragment ion at m/z 151 $[(M-H)-134]^-$ (Chang et al., 2012; Coppin et al., 2013).

Compound **6** (t_R = 32.8 min) had a pseudomolecular ion at m/z 267 and fragment ion at 252 $[(M-H)-15]^-$ tentatively indicating that compound **6** was formononetin (He, Cheng, & Chen, 2013; Singh et al., 2010; Wen et al., 2009).

3.3. Evaluation of the antioxidant activity in vitro

3.3.1. DPPH radical scavenging ability

It is well known that antioxidants have a strong protective function against oxidative damage by their ability to quench the free radicals. The DPPH method has been extensively used to determine the free radical scavenging activity of antioxidants (Jiao et al., 2013). The DPPH scavenging activities of FVF are shown in Fig 2A. The radical scavenging ability of FVF at the concentrations of 2–20 μg/ml ranged from 14.40% to 56.11%, while that of ascorbic acid and rutin were 20.90–86.61% and 10.81–23.70%, respectively. EC_{50} values of rutin, FVF, and ascorbic acid were 43.62 ± 0.26 μg/ml, 17.25 ± 0.14 μg/ml and 7.95 ± 0.21 μg/ml, respectively, which indicated that DPPH radical scavenging ability of FVF was significantly higher than rutin. Additionally, FVF had a better DPPH radical scavenging ability compared to extracts from other mushroom such as *Pachyornis australis*, *Lentinula edodes*, and *Pleurotus ostreatus* (Ren et al., 2014; Woldegiorgis, Abate, Haki, & Ziegler, 2014).

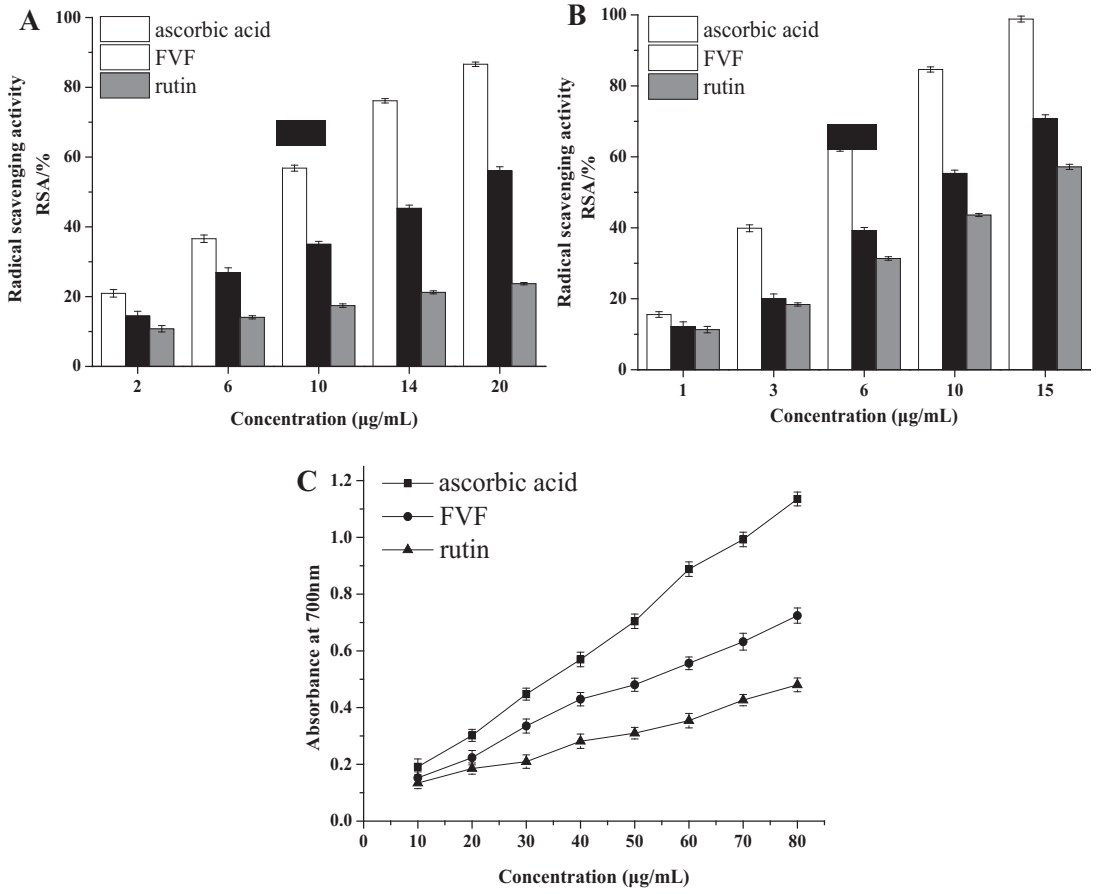


Fig. 2. The antioxidant activities of FVF. Data was presented as mean ± SD. (n = 3). (A) DPPH free radical scavenging ability; (B) ABTS radical scavenging ability; (C) ferric-reducing antioxidant power assay.

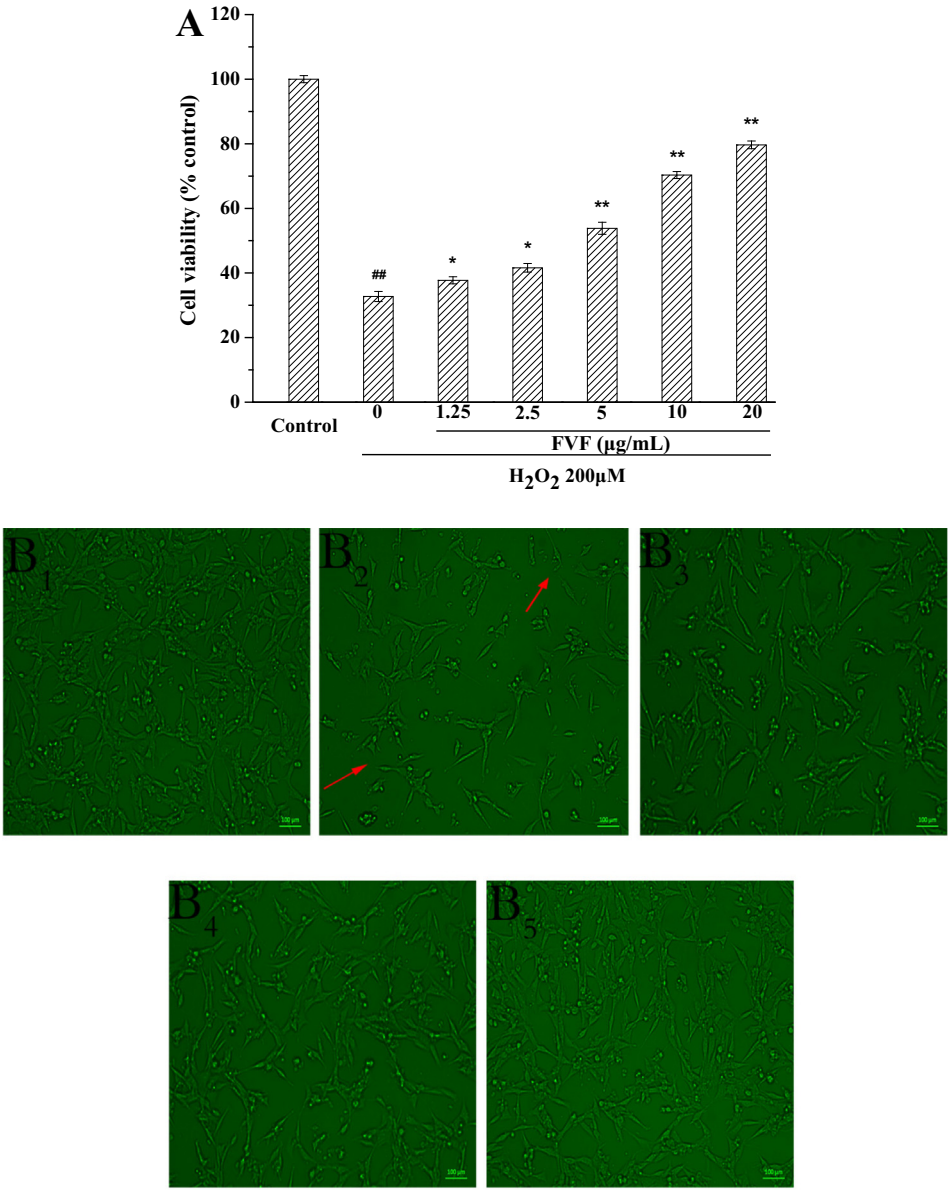


Fig. 3. Effects of FVF on cell viability (A) and cell morphology (B) in PC12 cells under hydrogen peroxide condition. Cells were incubated with H_2O_2 (200 μM) for 2 h. FVF (1.25–20 μg/ml) was added to the culture 12 h prior to H_2O_2. (B_1) untreated PC12 cells; (B_2) PC12 cells exposed to H_2O_2 (200 μM) for 2 h; (B_3) PC12 cells pro-incubated with 1.25 μg/ml FVF for 12 h and exposed to H_2O_2 for 2 h; (B_4) PC12 cells pro-incubated with 5 μg/ml FVF for 12 h and exposed to H_2O_2 for 2 h; (B_5) PC12 cells pro-incubated with 20 μg/ml FVF for 12 h and exposed to H_2O_2 for 2 h. Scale bar = 100 μm. Data are presented as mean ± SD ($n = 3$). ($^{\#\#}$) $P < 0.01$ indicate statistically significant differences versus blank control group. (*) $P < 0.05$ and (**) $P < 0.01$ indicate statistically significant differences versus H_2O_2 group without FVF pretreatment.

3.3.2. ABTS radical scavenging ability

The $ABTS^{\cdot+}$ scavenging assay is based on evaluating the capacity of an antioxidant to scavenge the stable ABTS radical in vitro. As shown in Fig. 2B, the ABTS radical scavenging ability of FVF was concentration-dependent, and the scavenging rate of FVF at 15 μg/ml was up to 70.70%. The EC_{50} value of rutin, FVF, and ascorbic acid were 13 μg/ml, 9.02 μg/ml, and 4.5 μg/ml, respectively. ABTS radical scavenging activity of *L. edodes* was shown to be correlated with their flavonoid compounds (Jiang, Luo, & Ying, 2015), which indicated FVF was an important antioxidant component in *F. velutipes*.

3.3.3. FRAP assay

FRAP method is based on the ability of transforming Fe^{3+}/ferricyanide into Fe^{2+} ferrous complex. The reducing power was monitored by measuring the formation of Perl's Prussian blue at 700 nm. In Fig. 2C, dose–response curves were found in reducing activity of ascorbic acid, FVF and rutin. At 80 μg/ml, the reducing power of FVF, rutin and ascorbic acid were 0.72, 0.48 and 1.14, respectively. The reducing ability of FVF (EC_{50} = 52 μg/ml) was significantly lower than ascorbic acid (EC_{50} = 36 μg/ml), but significantly higher than rutin (EC_{50} = 83 μg/ml). A study by Liu et al. (2012) concluded that the reducing abilities (EC_{50}) of ethanol extracts of five wild mushrooms were in the order *Laccaria amethystea* (2.84 ± 0.92 mg/ml) > *Craterellus cornucopioides* (2.91 ± 0.75 mg/ml) > *Stropharia rugoso-annulata* (4.71 ± 0.62 mg/ml) > *Cycnoches ventricosum* (5.57 ± 0.72 mg/ml) > *Cucurbita maxima* (6.39 ± 0.17 mg/ml). Compared to other edible mushrooms, FVF was shown to provide higher reducing power.

3.4. Effect of FVF on viability and morphology of PC12 cells

ROS have been associated with apoptosis and necrosis of PC12 cells by elevating oxidative stress (Liu, Han, Zhang, & Yang,

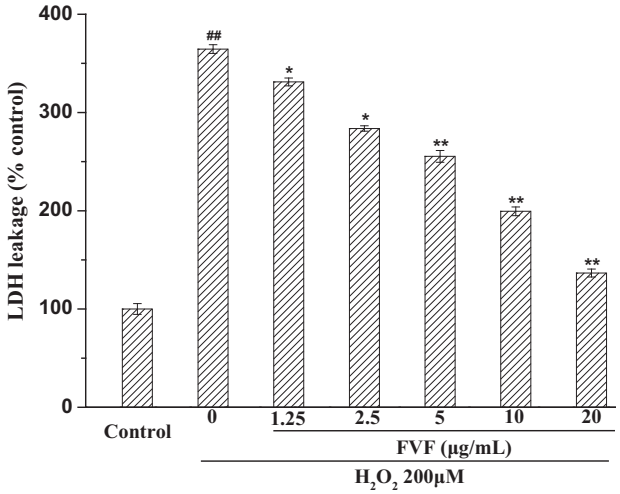


Fig. 4. Effects of FVF on LDH release in PC12 cells under hydrogen peroxide condition. Cells were incubated with H_2O_2 (200 μM) for 2 h. FVF (1.25–20 μg/ml) was added to the culture 12 h prior to H_2O_2. Data are presented as mean ± SD (n = 3). ($^{\#\#}$) $P < 0.01$ indicate statistically significant differences versus blank control group. (*) $P < 0.05$ and (**) $P < 0.01$ indicate statistically significant differences versus H_2O_2 group without FVF pretreatment.

2011). In this study, H_2O_2 was used to induce PC12 cells injury in order to assess the neuroprotective effects of FVF. Cell viability and morphological alteration of PC12 cells are shown in Fig. 3A. There was a 67.3% decrease in cell viability treated with 200 μM H_2O_2 compared to control. However, pretreatment with FVF (1.25–20 μg/ml) resulted in increased PC12 cells viability, and the increase was concentration-dependent. PC12 cell viability treated with 20 μg/ml FVF increased to 79.66%, suggesting a significant neuroprotective effect of FVF against H_2O_2-induced injury.

The morphological alteration of PC12 cells was observed using inverted phase-contrast microscopy. The control PC12 cells grew well and showed an intact morphology (Fig. 3B_1). However, after exposure to H_2O_2 for 2 h, the cell viability significantly declined and the dendritic networks were mostly disrupted (Fig. 3B_2), which suggested 200 μM H_2O_2 induced the oxidant damage to PC12 cells. These injuries were significantly ameliorated by FVF pretreatment at different concentrations, and most of PC12 cells kept a dense plexiform dendritic network without adherent reducing ability (Fig. 3B_3–B_5). These observations from MTT assay indicated the protective effects of FVF on oxidative damage to PC12 cells induced by H_2O_2.

3.5. Effect of FVF on LDH release of PC12 cells

LDH, a steady cytosolic enzyme present in cells, is released into the cell culture medium when the cell membrane is damaged. Therefore, the leakage level of LDH is an important index to evaluate the integrity of cell membrane as well as the degree of cell damage (Lobner, 2000). As shown in Fig. 4, the percentage of LDH leakage of H_2O_2-induced group significantly increased to 364.57 ± 4.42% compared to control group. LDH leakage was inhibited by FVF in a

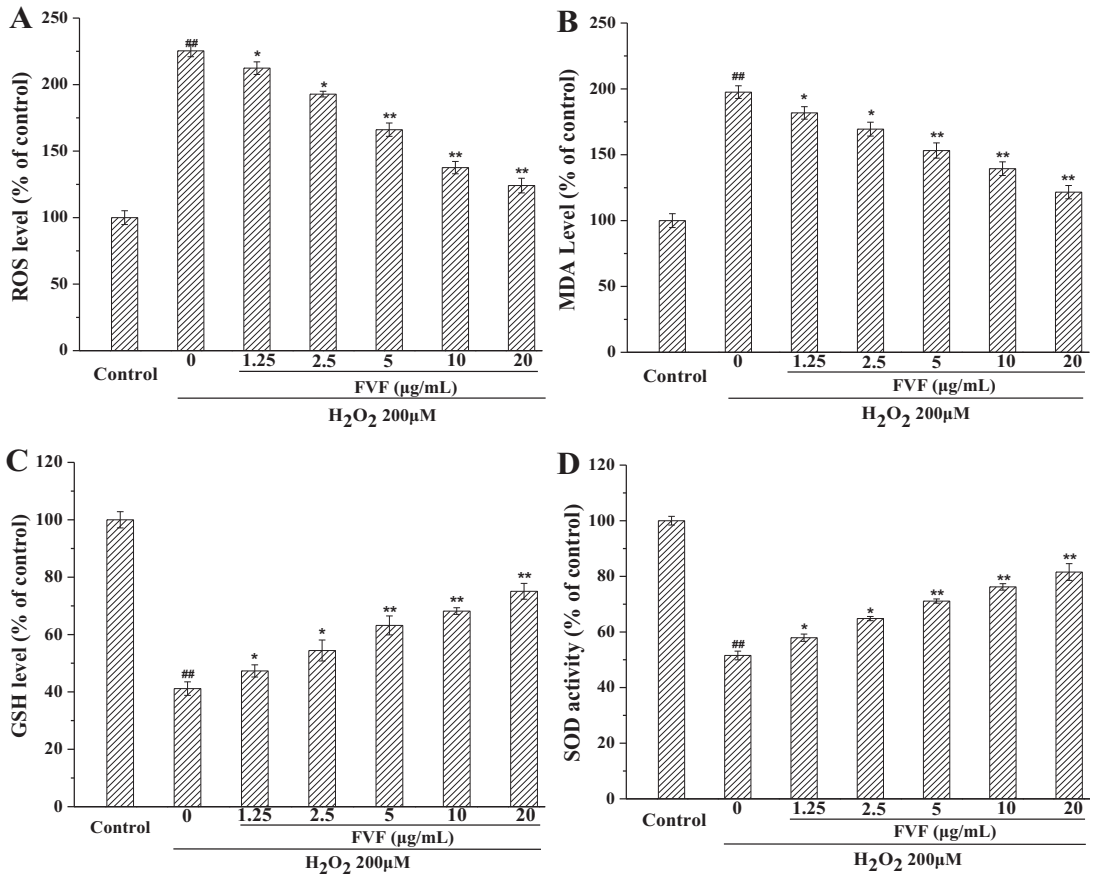


Fig. 5. Effect of FVF on intracellular ROS level, MDA, GSH and SOD in H_2O_2-induced PC12 cells. Cells were incubated with H_2O_2 (200 μM) for 2 h. FVF (1.25–20 μg/ml) was added to the culture 12 h prior to H_2O_2. Then, ROS level (A), MDA content (B), GSH level (C), and SOD activity (D) in the cells were determined. Data are presented as mean ± SD (n = 3). ($^{\#\#}$) $P < 0.01$ indicate statistically significant differences versus blank control group. (*) $P < 0.05$ and (**) $P < 0.01$ indicate statistically significant differences versus H_2O_2 group without FVF pretreatment.

concentration-dependent manner, and the percentage of leakage was 136.64 ± 4.06% after treatment with 20 μg/ml of FVF. These data strongly indicate that pretreatment of PC12 cells with FVF could prevent the lesion in cell membrane caused by H_2O_2.

3.6. Effect of FVF on intracellular ROS level, MDA, GSH and SOD

Generation of intracellular ROS is another mechanism of oxidative damage to PC12 cells. Therefore, the levels of intracellular ROS were examined using the fluorescence probe DCFH-DA to explore the protective effects of FVF. The effects of FVF on intracellular ROS production in PC12 cells exposed to H_2O_2 is shown in Fig. 5A. Exposure of PC12 cells to 200 μM H_2O_2 for 2 h led to intracellular ROS overproduction (225.40 ± 4.43% of control group). Lipid peroxidation is a significant marker of oxidative damage occurring in the cell membranes. MDA, as one of its degradation products, has been widely studied as an index of cell membrane damage (Si et al., 2013). Increased MDA content compared to control group (197.54 ± 4.82%) was observed after 200 μM H_2O_2 treatment (Fig. 5B), which is due to the overproduction of ROS. Pretreatment with FVF markedly counteracted ROS production consequently leading to low MDA content. The reduction of ROS generation and MDA content suggested that antioxidant activities of FVF might be an important factor in protecting PC12 cells against H_2O_2-induced apoptosis.

Glutathione peroxidase and SOD are antioxidant defense systems in cells to prevent ROS damage. GSH, a substrate of glutathione peroxidase, could scavenge ROS *in vivo*. Therefore, the GSH level and SOD activity were measured to evaluate the neuroprotective effects of FVF (Fig. 5C and D). Results suggested that H_2O_2 treatment of PC12 cells significantly decreased the GSH level (41.16 ± 2.36%) and SOD activity (51.55 ± 1.57%) compared to the control group, which were significantly attenuated by FVF pretreatment. The GSH level and SOD activity in cells treated with 20 μg/ml FVF were restored to 75.07% and 81.54%, respectively.

4. Conclusions

In this study, FVF was extracted under ultrasonic assistance and purified by AB-8 macroporous resin column chromatography and Sephadex LH-20 column chromatography. Six flavonoids (arbutin, epicatechin, phillyrin, apigenin, kaempferol and formononetin) were identified. The antioxidant potential of FVF were tested using different antioxidant methods such as DPPH, ABTS radicals scavenging capabilities and reducing power. FVF showed the potential in protecting PC12 cells from the H_2O_2-induced injury by diminishing the LDH release, ROS over production and MDA generation. The activities of antioxidant enzymes such as GSH level and SOD were also enhanced by FVF exposure in PC12 cells. Our results suggested that protective effect of FVF on PC12 cells injury induced by H_2O_2 are mainly ascribed to its antioxidant activity.

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