Study on Neuroprotective Capacity of Key Flavonoids of *Ampelopsis grossedentata*

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Abstract *Ampelopsis grossedentata* could be of potential significance for health food applications in view of the abundant flavonoids, most of which could be effective in preventing and curing senile diseases. To evaluate the neuroprotective effects of flavonoids in *A. grossedentata*, model of $A\beta_{25-35}$ -induced PC12 cells apoptosis was established, and methods as MTT assay for determining cell viability, DCFH-DA fluorescence analysis for the detection of intracellular reactive oxygen species (ROS) induced by $A\beta_{25-35}$, and the DPPH radical scavenging assay for evaluating the antioxidant activity were applied. Cells co-incubated with flavonoids (100 μ M) and $A\beta_{25-35}$ showed a significantly higher survival rate ($P < 0.01$ or $P < 0.05$) than with $A\beta_{25-35}$ only, flavonoids in particular kaempferol, rutin, kaempferol-3-O- β -D-glucopyranoside and isoquercitrin exerted better neuroprotective effects than the positive control donepezil HCl. ROS induced by $A\beta_{25-35}$ in PC12 cells decreased after exposure to 100 μ M flavonoids and extracts, which inoculated with the data of DPPH assay. In the way of inhibiting ROS production, flavonoids in *A. grossedentata* could attenuate cellular apoptosis induced by $A\beta_{25-35}$, and it is concluded that when administered in soluble preparation, *A. grossedentata* being rich in flavonoids that structurally related to dihydromyricetin could become a mine for the development of a new generation of food to be clinically effective in dementia.

Keywords: flavonoids, *ampelopsis grossedentata*, $A\beta_{25-35}$, PC12 cells, neuroprotective capacity, ROS

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1. Introduction

Alzheimer's disease (AD), with the clinical symptoms cognitive declining, irreversible memory losing, disorientation, language impairment, etc, threatened millions of elderly people [1,2]. It has been well established that the cytotoxic beta-amyloid ($A\beta$) was a trigger of the pathological cascade of events leading to AD [3,4,5,6]. $A\beta_{1-40}$ is the most common peptide found in amyloid plaques, and the partial fragment, $A\beta_{25-35}$ can be produced in brains of AD patients by enzymatic cleavage of the naturally occurring $A\beta_{1-40}$ [7,8]. $A\beta_{25-35}$ mimics many of the toxicological and oxidative properties of the native full-length peptide $A\beta$, the monomeric form of $A\beta_{25-35}$ may be cytotoxic [9], and has often been chosen as a model for full-length $A\beta$ in structural and functional studies [10], and as a convenient alternative to induce cell death for preparing in vitro and in vivo models in AD investigations [11-15].

Neurotoxicity exerted by $A\beta$ might involve reactive oxygen species (ROS) generation [16-21], which could damage sugars, proteins, polyunsaturated lipid, and DNA, thus leading to degenerative processes and diseases [22,23], furthermore, oxidative stress produced by ROS [24,25] was critically involved in AD [26]. Therefore, the

inhibition of $A\beta$ aggregation and neurotoxicity, and reducing the generation of ROS, were attractive therapeutic and preventive strategies in the development of disease modifying drugs for AD [27]. Plant secondary substances especially flavonoids have been suggested as potential alternatives for AD therapy [28,29], and most activities of flavonoids analogues were generally known to be associated with anti-oxidative or free radical scavenging properties [30-34].

Ampelopsis grossedentata (Hand-Mazz) W.T. Wang, known as rattan tea, approved as a new food resource [35], is also a health beverage in China. The leaves are rich of flavonoids such as dihydromyricetin, myricetin, rutin, quercetin, kaempferol, taxifolin, etc [36,37,38]. Although many subsequent studies have highlighted the potential role of polyphenol components in rattan tea, such as dihydromyricetin, myricetin, which have been reported to possess antioxidant activity [39,40,41], the effects on alleviating $A\beta$ toxicity [42,43,44,45], and have the potential to prevent AD [46-50], the neuroprotective effects of *A. Grossedentata* extracts and its key flavonoids on $A\beta_{25-35}$ induced neurocytotoxicity in neuronal cells had not yet been investigated. Therefore, the aim of the present study was to investigate the potential of *A. Grossedentata* and its key flavonoids to protect against $A\beta_{25-35}$ -induced neurotoxicity in PC12 cells. We performed a study of a group of flavonoids from *A.*

Grossedentata in a PC12 cell culture model. As the cellular insult we have chosen $A\beta_{25-35}$, a widely used agent for studies of neural cell damage [9-15], which is a conspicuous event in apoptosis [3-8]. Furthermore, this work attempted to better understand the mechanisms behind the neuroprotective effects of the flavonoids.

2. Materials and Methods

2.1. Preparation of Materials

PC12 cells were obtained from CCTCC, Donepezil HCl (Sigma), $A\beta_{25-35}$ (Sigma), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (Sigma), RPMI-1640 (Hyclone), fetal bovine serum (FBS) (Hyclone) and Trypsinase (Gibco) were purchased from suppliers listed respectively, all other related chemical reagents and solvents were purchased from commercial suppliers and used as received unless otherwise specified.

Compounds Dihydromyricetin, Myricetin, Rutin, Isoquercitrin, Kaempferol, Taxifolin, Apigenin, Hesperetin, Kaempferol-3-O- β -D-Glucopyranoside and Naringenin were purchased from Shanghai Tauto Biotech Co., Ltd, 98% purity by HPLC, and were dissolved in dimethylsulfoxide (DMSO) with the final concentration 1.0×10^{-1} mol/L (100 mM), diluted with fresh medium if needed.

$A\beta_{25-35}$ was dissolved in deionized distilled water at a concentration of 1 mM, stored at -20°C until use, dilute with PBS (0.1 M, pH 6.0) if needed. Donepezil HCl was dissolved in DMSO (final concentration 100 mM, diluted if needed), stored at -20°C . Trypsinase (Gibco) 0.25g was dissolved in 100 mL PBS, pH 7.4, treated by filtration and without heating sterilization. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was prepared in methanol buffered with acetic acid buffer (0.1 M, pH 5.5), the buffered methanol was prepared by mixing 40 mL of 0.1 M acetate buffer (pH 5.5) with 60 mL methanol. 2', 7'-Dichlorofluorescein diacetate (DCFH-DA) was dissolved in DMSO (final concentration 1 mM).

The leaves of *A. Grossedentata* were collected in Guizhou Province of China, and authenticated by Professor Zhengwen Yu of Guizhou Normal University. Voucher specimens were deposited in the author's laboratory. The powdered leaf thallus of *A. Grossedentata* (100 g) were refluxed with MeOH (3×1 L) for 3 hours each, and the filtrates were concentrated to dryness in vacuo at 40°C to render the MeOH extracts (38.5 g). These MeOH extracts were separately suspended in distilled H_2O and sequentially partitioned with CH_2Cl_2 , EtOAc, n-BuOH to yield CH_2Cl_2 (1.5 g), EtOAc (24.6 g), n-BuOH (10.9 g), and H_2O (1.5 g) fractions respectively. In the experiment, the crude MeOH extracts and fractions were diluted with DMSO at 50 mg/mL, dilute to 0.5, 0.2, 0.1, 0.05, 0.01 and 0.005 mg/mL with culture medium when needed.

2.2. Cell Culture

PC12 cells were cultured in RPMI-1640 supplemented with 10% heat-inactivated (heat-treated for 30 min at 56°C) horse serum, 5% fetal bovine serum, and 0.1% penicillin/streptomycin at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cultures were fed

twice a week with complete medium. When they reached near-confluent state one week later, subculturing was carried out (the split ratio was 1:5) [51]. Cells ($100 \mu\text{L}$, 2×10^5 cells/mL, digested by trypsinase) were pre-seeded into 96-well polystyrene plates and incubated at 37°C in a water-saturated atmosphere of 5% CO_2 for 48 hours.

2.3. Cell Proliferation Assay

The cell viability was measured by the quantitative colorimetric MTT method [52,53]. Cell viability was determined with addition of $20 \mu\text{L}$ MTT solution (dissolved in fresh complete medium, final concentration 0.5 g/L) for 4 hours at 37°C . Supernatants were then aspirated off, the dark-blue formazan crystals formed were dissolved in DMSO, and the optical density (OD) was measured with microplate reader (BioMedical Technology Solutions, Inc., POLARstar OPTIMA) at 570 nm. The values of different absorbances were expressed as a percentage of control.

2.4. Model of PC12 Cell Damage Induced by $A\beta_{25-35}$

The inhibition on PC12 cells of $A\beta_{25-35}$ was measured using MTT method, with the premise that the treating-time and concentration being investigated. Model group were treated with 1-20 μM $A\beta_{25-35}$ for 1 hour, tri-washed with D-Hanks solution, and the survival rate of PC12 cells was measured. At the fixed concentration of $A\beta_{25-35}$, the treating-time (1-48 hours)-cell viability was studied with the MTT method.

For the cytoprotective assessment of Donepezil HCl, cells were co-incubated with 1, 5, 10, 50, 100 and 500 μM Donepezil and $A\beta_{25-35}$ (10 μM) at 37°C for 1 hour, treatment was removed and incubated for an additional 12 hours, cell viability was determined by the MTT colorimetric assay following the mentioned protocols.

2.5. Cytoprotective Activity Assay

2.5.1. Cytotoxicity of Target Flavonoids

Flavonoids (150 and 30 μL of each concentration) was diluted with fresh medium to 300 μL and added to individual wells, the final concentrations were 500, 100, 50, 10, 5 and 1 μM . Controls were exposed to 0.50% (v/v) DMSO in culture medium, and then incubated with PC12 Cells at 37°C . Then the cell viability measured to evaluate the cytotoxicity. The cytotoxicity (%) was calculated as $[(\text{OD of control}) - (\text{OD of flavonoids treatment})] / (\text{OD of control}) \times 100$.

2.5.2. Effects of the Materials on $A\beta_{25-35}$ -induced Cytotoxicity

To assess the effects of the compounds on $A\beta_{25-35}$ -induced cytotoxicity, PC12 cells were cultured as described above and then pre-incubated with compounds at concentration of 0, 1, 5, 10, 50, 100 and 500 μM prior to being exposed to 10 μM $A\beta_{25-35}$ for 12 hours. The diluted extracts 150 μL were added and co-incubated with cells as described above. Donepezil HCl was used as treatment positive control. The cytoprotective activity (%) was calculated as $[(\text{OD of control}) - (\text{OD of flavonoids treatment})] / (\text{OD of control}) \times 100$.

2.6. DPPH Radical Scavenging Assay in vitro

The antioxidant activity of selected compounds and extracts was measured using DPPH radical scavenging assay [54]. The DPPH concentration was 50 μ M, compounds concentration was 1, 5, 10, 50, 100 and 500 μ M, extracts of MeOH, CH_2Cl_2 , EtOAc, n-BuOH and H_2O fractions were diluted to 0.5, 0.2, 0.1, 0.05, 0.01 and 0.005 mg/mL, ascorbic acid was used as standard. The reaction tubes, in triplicates, were wrapped in aluminum foil and kept at 30°C for 30 min in dark, and the absorbance was measured at 517 nm. The DPPH radical scavenging activity (%) was calculated as $[(\text{OD of DPPH radical}) - (\text{OD of DPPH radical with flavonoids treatment})] / [(\text{OD of DPPH radical}) - (\text{OD of control})] \times 100$.

2.7. Effects on $\text{A}\beta$ -induced ROS Generation in PC12 Cells

ROS generation was assessed using the ROS-sensitive fluorescence indicator DCFH-DA [55]. To determine intracellular ROS inhibitory activity, PC12 cells were seeded in a black 96-well plate. After 24 hours, the cells were treated with selected compounds and fractions for 1 hour and then incubated for 12 hours with $\text{A}\beta_{25-35}$ (10 μ M) to induce ROS generation. And after the 30-min-incubation with DCFH-DA (15 μ L DCFH-DA diluted with 275 μ L PBS), the cells were washed twice with ice-cold PBS, followed by suspension in the same buffer, and the fluorescence intensity was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm using a fluorescence microplate reader (BioMedical Technology Solutions, Inc., POLARstar OPTIMA). Treatments with $\text{A}\beta_{25-35}$ (10 μ M) only were measured as

the control and the culture without any treatment served as the blank control. The intracellular ROS scavenging activity (%) was calculated as $[(\text{OD of } \text{A}\beta_{25-35}) - (\text{OD of } \text{A}\beta_{25-35} \text{ with flavonoids treatment})] / [(\text{OD of } \text{A}\beta_{25-35}) - (\text{OD of control})] \times 100$.

2.8. Statistical Analysis

All experiments were performed at least in triplicate and repeated two times to ensure reproducibility. The results were expressed as the mean $\pm$ SD, and the statistical analyses were performed using the SPSS statistical package (IBM Inc.). In all cases the confidence coefficient was set at 0.05.

3. Results

3.1. Effects of $\text{A}\beta_{25-35}$ induced Cytotoxicity in PC12 Cells

The cytotoxicity of $\text{A}\beta_{25-35}$ in PC12 cells was measured using the MTT assay. As shown in Figure 1, when PC12 cells were exposed to $\text{A}\beta_{25-35}$ (0, 1, 5, 10, 15 and 20 μ M) for 1 hour, the rate of MTT reduction decreased in a dose dependent manner. Therefore, a fixed concentration of 10 μ M $\text{A}\beta_{25-35}$ was used to determine $\text{A}\beta_{25-35}$ induced toxicity. Results in time-course (Figure 2) experiments demonstrated that the survival rate was markedly decreased after treated with 10 μ M $\text{A}\beta_{25-35}$ for 12 hours compared with control group. Quantitative analysis of cells revealed that treatment with 10 μ M $\text{A}\beta_{25-35}$ for 12 hours evoked marked cell apoptosis. In the present of Donepezil HCl, cytotoxicity of $\text{A}\beta_{25-35}$ was minimized (Figure 3).

Figure 1. The survival rate of PC12 cells after treatment with various concentrations (n=12) of $\text{A}\beta_{25-35}$ one hour. The survival rate decreased by 41.82% at the concentration of 10 μ M, below which it changed little on survival rate. **p values < 0.01 when compared with the control

Figure 2. Effect of 10 μ M $\text{A}\beta_{25-35}$ on survival rate of PC12 cells for various times (1, 6, 12, 24, 48 hours; n=12). Data demonstrated that PC12 cells exposure to $\text{A}\beta_{25-35}$ for 12 hours resulted in significant levels of apoptosis. **p values < 0.01 when compared with the control

Figure 3. Effect of Donepezil HCl on PC12 cells lesion induced by 10 μM A β_{25-35} . Pretreatment of PC12 cells with 100 μM Donepezil HCl, prior to A β_{25-35} exposure, significantly decreased cell apoptosis, whereas the 1 μM and 5 μM Donepezil HCl slightly decreased A β_{25-35} -induced cell apoptosis compared with A β_{25-35} alone. **p values < 0.01 when compared with the treatment of A β_{25-35} alone (n=6)

Figure 4. Cytotoxicity on PC12 cells of different compounds. After pretreatment of PC12 cells with 500, 100, 50, 10, 5 and 1 μM (n=6) Donepezil HCl and flavonoids of *A. Grossedentata*, the 500 μM concentration of Dihydromyricetin, Myricetin, Taxifolin, Apigenin, Naringenin, Hesperetin, Kaempferol and Kaempferol-3-O- β -D-Glucopyranoside exerted marked cell apoptosis, whereas the lower slightly induced cell apoptosis. On the other side, Rutin and Isoquercitrin expressed none cytotoxicity at all the concentrations; Apigenin, had strong ability at 500, 100 and 50 μM . As a treatment positive control, Donepezil HCl was non-cytotoxicity

Figure 5. The effects of extraction fractions on PC12 cells. (a) The inhibitory of PC12 cells lesion induced by A β_{25-35} after treatment with various concentrations (0.5, 0.2, 0.1, 0.05, 0.01, 0.005 mg/mL) of the fractions demonstrated that the EtOAc fraction could be of the best capacity, followed by n-BuOH, MeOH, H₂O and CH₂Cl₂ fractions. (b) Effect of *A. Grossedentata* extracts on 10 μM A β_{25-35} induced intracellular ROS generation in PC12 cells. Data showed that PC12 cells exposure to A β_{25-35} resulted in significant levels of ROS generation. The different fractions of *A. Grossedentata* MeOH extracts inhibited intracellular ROS generation. (c) DPPH radical scavenging activity of the fractions was basically identical, and in a dose dependent manner

Table 1. The Effects of Compounds Tested in this Study

Compounds	Concentration (mol/L)	Inhibition on PC12 Cells/%	Inhibition on Cell death/%	Inhibition on ROS generation/%	Scavenging of DPPH radical/%
dihydromyricetin	5×10^{-4}	53.64±0.99 (A*)	n. d.**	n. d.	98.79±0.44 (A)
	1×10^{-4}	2.24±1.65 (a)	33.08±4.54 (A)	51.16±7.92 (A)	58.70±0.69 (A)
	5×10^{-5}	2.03±0.33 (A)	27.38±2.10 (A)	28.75±3.19 (A)	46.42±0.69 (A)
	1×10^{-5}	0	11.55±1.76 (A)	20.65±3.65 (A)	29.48±1.31 (A)
	5×10^{-6}	0 (A)	0	14.36±2.32 (A)	17.20±1.31 (A)
	1×10^{-6}	0	0	10.25±2.09 (A)	8.27±0.85 (A)
myricetin	5×10^{-4}	54.22±0.73 (A)	n. d.	n. d.	100.10±0.16 (A)
	1×10^{-4}	0 (A)	30.81±3.71 (A)	77.07±4.39 (A)	46.09±1.28 (A)
	5×10^{-5}	0 (A)	25.09±3.27 (A)	37.91±1.58 (A)	39.52±1.18 (A)
	1×10^{-5}	0	10.34±4.18 (a)	25.33±2.98 (A)	31.74±0.82 (A)
	5×10^{-6}	0	10.09±2.59 (A)	18.78±1.70 (A)	27.40±0.79 (A)
	1×10^{-6}	0	0	12.99±2.82 (A)	22.56±0.63 (A)
Taxifolin	5×10^{-4}	58.31±0.61 (A)	n. d.	n. d.	60.50±0.48 (A)
	1×10^{-4}	0 (a)	36.11±3.99 (A)	57.80±5.48 (A)	36.14±0.46 (A)
	5×10^{-5}	0 (A)	22.48±0.94 (A)	43.24±2.02 (A)	28.89±0.81 (A)
	1×10^{-5}	0 (A)	10.23±5.66 (A)	25.23±4.31 (A)	21.40±0.28 (A)
	5×10^{-6}	0 (a)	4.39±2.57 (A)	21.46±1.93 (A)	20.25±0.25 (A)
	1×10^{-6}	0 (a)	0.33±4.15	18.12±3.34 (A)	18.90±0.34 (A)
Apigenin	5×10^{-4}	49.69±3.74 (A)	n. d.	n. d.	41.61±0.34 (A)
	1×10^{-4}	47.08±2.52 (A)	n. d.	n. d.	31.20±0.61 (A)
	5×10^{-5}	26.24±1.27 (A)	11.99±1.48 (A)	24.55±3.78 (A)	17.12±0.27 (A)
	1×10^{-5}	0 (a)	6.69±2.14 (A)	7.33±2.89 (A)	4.75±0.34 (A)
	5×10^{-6}	0 (a)	0.51±3.65 (A)	4.14±1.49 (a)	0.29±0.75
	1×10^{-6}	0	0	1.20±1.81	1.27±0.48 (A)
Naringenin	5×10^{-4}	66.86±0.44 (A)	n. d.	n. d.	53.42±0.58 (A)
	1×10^{-4}	2.59±3.83	8.66±3.76 (A)	35.97±4.25 (A)	37.45±0.31 (A)
	5×10^{-5}	0 (a)	5.19±1.40 (A)	15.71±1.20 (A)	24.51±0.66 (A)
	1×10^{-5}	0 (A)	1.37±0.92 (a)	8.87±3.14 (A)	12.92±0.31 (A)
	5×10^{-6}	0	0	3.16±0.68 (A)	12.22±0.45 (A)
	1×10^{-6}	0	0	2.41±1.67 (a)	11.90±0.54 (A)
Kaempferol	5×10^{-4}	12.27±2.74 (A)	130.96±4.79 (A)	78.67±4.45 (A)	88.49±0.56 (A)
	1×10^{-4}	3.60±2.20 (a)	102.56±3.15 (A)	34.44±5.14 (A)	19.04±0.49 (A)
	5×10^{-5}	0 (A)	58.41±2.84 (A)	22.98±0.94 (A)	14.23±0.46 (A)
	1×10^{-5}	0	13.27±3.49 (A)	17.32±4.24 (a)	11.67±0.35 (A)
	5×10^{-6}	0 (A)	7.46±1.39 (A)	6.34±0.80 (a)	10.46±0.38 (A)
	1×10^{-6}	0	0	7.99±3.36 (A)	8.82±0.45 (A)
Hesperetin	5×10^{-4}	35.49±1.09 (A)	n. d.	n. d.	51.09±0.41 (A)
	1×10^{-4}	3.39±1.67 (a)	26.83±3.31 (A)	32.68±4.39 (A)	26.31±0.34 (A)
	5×10^{-5}	0.69±0.35 (a)	18.75±1.25 (A)	26.17±1.28 (A)	17.22±0.95 (A)
	1×10^{-5}	0 (A)	0.73±1.77	8.82±2.95 (a)	12.10±0.38 (A)
	5×10^{-6}	0	0 (a)	3.30±0.80 (A)	10.91±0.83 (A)
	1×10^{-6}	0	0	1.76±1.61 (a)	8.05±0.34 (A)
Isoquercitrin	5×10^{-4}	0	48.54±4.39 (A)	99.54±3.03 (A)	100.12±0.16 (A)
	1×10^{-4}	0 (a)	48.39±5.32 (A)	93.28±2.70 (a)	31.08±0.79 (A)
	5×10^{-5}	0	48.21±1.48 (A)	70.20±5.09 (a)	27.09±0.59 (A)
	1×10^{-5}	0 (a)	45.94±4.40 (A)	46.61±4.97 (A)	22.17±0.68 (A)
	5×10^{-6}	0	45.50±1.50 (A)	26.17±1.10	15.48±0.61 (A)
	1×10^{-6}	0	42.51±6.99 (A)	10.13±3.05 (A)	11.38±0.73 (A)
Rutin	5×10^{-4}	0 (A)	89.29±2.34 (A)	97.62±2.22 (A)	100.08±0.24 (A)
	1×10^{-4}	0 (A)	43.49±5.59 (A)	67.98±4.84 (A)	31.88±0.70 (A)
	5×10^{-5}	0 (a)	42.29±1.45 (A)	50.59±1.59 (A)	25.23±0.86 (A)
	1×10^{-5}	0 (a)	38.19±4.20 (A)	43.08±2.46 (A)	18.88±0.48 (A)
	5×10^{-6}	0	23.90±2.01 (A)	28.69±0.94 (A)	14.37±0.65 (A)
	1×10^{-6}	0	14.66±4.25 (A)	12.67±3.83 (A)	12.80±0.50 (A)
KG	5×10^{-4}	15.62±2.71 (A)	85.71±5.60 (A)	76.22±4.20 (A)	44.80±0.73 (A)
	1×10^{-4}	5.35±2.76 (a)	42.62±4.87 (A)	58.65±3.10 (A)	23.73±0.50 (A)
	5×10^{-5}	0 (a)	39.25±1.58 (A)	49.22±1.30 (A)	16.58±0.54 (A)
	1×10^{-5}	0 (A)	31.69±2.68 (A)	35.85±2.86 (A)	12.74±0.58 (A)
	5×10^{-6}	0	21.09±1.16 (A)	24.43±1.81 (A)	9.36±1.03 (A)
	1×10^{-6}	0 (A)	10.93±5.58 (a)	7.00±2.70 (A)	7.82±0.46 (A)
Donepezil-HCl	5×10^{-4}	1.39±4.65	77.85±4.21 (A)	67.28±4.45 (A)	n. d.
	1×10^{-4}	0.63±4.02	40.64±4.77 (A)	40.76±2.41 (A)	n. d.
	5×10^{-5}	0	34.43±3.46 (A)	19.76±1.77 (A)	n. d.
	1×10^{-5}	0 (a)	13.49±2.44 (A)	12.92±3.32 (A)	n. d.
	5×10^{-6}	0 (a)	3.80±3.38 (a)	4.49±1.31 (A)	n. d.
	1×10^{-6}	0 (A)	1.39±3.51	0	n. d.

*. a. $p < 0.05$; A. $p < 0.01$; **. n. d. for Not detected. Ascorbic acid was set as positive control in DPPH radical scavenging assay, data in each concentration was 100.12%±0.16% (A, 500 μM), 21.97%±1.19% (A, 100 μM), 11.94%±0.45% (50 μM), 2.72%±0.54% (10 μM), 1.45%±0.42% (a, 5 μM), 0.04%±0.44% (1 μM).

Table 2. The Effects of Fractions Tested in this Study

Fractions	Concentration (mg/mL)	Inhibition on Cell death/%	Inhibition on ROS generation/%	Scavenging of DPPH radical/%
MeOH	0.5	86.48±3.26 (A*)	72.94±1.47 (A)	76.60±0.94 (a)
	0.2	61.15±1.42 (A)	56.58±8.09 (a)	52.03±0.94 (A)
	0.1	29.46±1.78 (A)	46.55±1.34	35.89±0.65 (A)
	0.05	7.09±1.70 (A)	38.71±0.90 (A)	17.24±0.74
	0.01	3.55±1.64 (A)	18.77±1.42 (A)	8.19±0.53
	0.005	0	8.44±1.79	0
n-BuOH	0.5	76.10±0.84 (A)	76.42±2.93 (A)	72.28±0.82 (A)
	0.2	70.21±2.16 (A)	60.47±3.49 (A)	44.47±0.71
	0.1	36.66±1.77 (A)	44.11±4.13 (a)	27.64±0.78 (A)
	0.05	18.64±0.80 (A)	34.33±2.81 (A)	17.57±0.69
	0.01	8.99±1.51 (A)	23.35±2.61	7.15±0.48
	0.005	1.50±0.96 (a)	16.60±1.92	4.30±0.59
EtOAc	0.5	96.20±1.12 (A)	85.04±2.00 (A)	86.57±0.94 (A)
	0.2	79.28±2.67 (A)	76.69±1.88 (a)	49.28±0.99 (A)
	0.1	50.29±1.33 (A)	65.96±2.27 (a)	28.87±0.66
	0.05	21.64±1.16 (A)	41.19±2.49 (A)	20.09±0.61
	0.01	13.45±1.63 (A)	31.92±1.81	12.39±1.06
	0.005	0	19.09±1.74 (A)	6.41±0.84 (A)
H ₂ O	0.5	64.80±1.31 (A)	40.86±4.17 (A)	53.62±0.63 (A)
	0.2	59.39±1.64 (A)	26.16±3.51 (A)	48.36±0.46 (a)
	0.1	28.58±1.43 (A)	23.00±1.99	34.64±0.52 (a)
	0.05	13.93±2.16 (A)	16.80±3.37	28.71±0.87
	0.01	6.69±1.55 (A)	8.74±1.39	19.55±0.93
	0.005	1.75±0.99 (A)	3.33±0.74 (a)	7.25±0.98
CH ₂ Cl ₂	0.5	31.14±1.43 (A)	17.48±1.21 (a)	37.04±0.86 (A)
	0.2	3.62±0.90 (A)	10.88±1.15 (A)	35.50±0.89
	0.1	0	6.21±1.03 (a)	27.54±0.97 (A)
	0.05	0 (A)	4.53±1.41	17.71±1.10
	0.01	0 (A)	0	12.10±0.99 (a)
	0.005	0 (A)	0 (a)	0

*. a. p<0.05; A. p<0.01

3.2. Neuroprotective Effects of the Selected Compounds and Extracts Fractions on A β ₂₅₋₃₅ induced Cell Apoptosis

3.2.1. Cytotoxicity of the flavonoidson Cells

To investigate if there be cytotoxicity, six concentrations of each monomer were used: 1, 5, 10, 50, 100 and 500 μ M. The absorbance value of each well was determined after PC12 cells were cultured with each monomer for 12 hours using a MTT assay. Data in Table 1 showed that at the concentration of 500 μ M, all of the compounds expressed strong cytotoxicity except for isoquercitrin, rutin. And the 100 μ M would be the appropriate concentration for most flavanoids where only apigenin expressed cytotoxicity on PC12 cells (Figure 4), although it differed from data *in vitro* [13]. At 50, 10, 5 and 1 μ M levels, none showed an inhibition on cell viability, that is to say, the selected compounds worked non-cytotoxicity on PC12 cells at low concentrations.

3.2.2. Neuroprotective Effects Assay

With regard to the neuroprotective effect, myricetin and dihydromyricetin and the analogues all had the potentiality, and among the flavonoids tested above, kaempferol (102.56±3.15%; 100 μ M) was the most potent inhibitor of the model on A β ₂₅₋₃₅ inducing PC12 cells lesion, and followed by isoquercitrin (48.39±5.32%; 100 μ M), KG (42.62±4.87%; 100 μ M) and rutin (43.49±5.59%; 100 μ M), that they exerted better activities on protecting cells than the positive control, 100 μ M Donepezil HCl (40.64±4.77%). Isoquercitrin showed good neuroprotective effect while non-cytotoxicity at high

concentration-level. In the meantime, apigenin and naringenin expressed poor neuroprotective activity.

The four fractions of MeOH extracts had the obvious activity (Table 2) on PC12 cells lesion. Data revealed that the EtOAc fraction might be of the best activity (Figure 5a), probably for the more flavonoids compounds and content, and the CH₂Cl₂ fraction was on the contrary.

3.3. Inhibitory Effects on A β ₂₅₋₃₅ induced Intracellular ROS Generation in PC12 Cells

3.3.1. Effects of the compounds in A. Grossedentata on A β ₂₅₋₃₅-induced ROS Generation

Recent studies have shown that ROS are involved in A β -induced apoptosis [17-21]. To determine whether the compounds from A. *Grossedentata* attenuated cell death by blocking ROS generation or not, we examined the effects of compounds on the accumulation of ROS in A β ₂₅₋₃₅ treated PC12 cells via the CM-H₂DCFDA fluorescence dye method. In previous MTT reduction experiment, we have demonstrated that most flavonoids had adverse effect on cell viability at the concentration over 500 μ M. Therefore, for further experiments, the experimental concentration ranges of flavonoids were adjusted from 1 to 100 μ M that did not affect cell viability. Data showed that treatment with 10 μ M A β ₂₅₋₃₅ increased ROS accumulation by 49.82% in DCF fluorescence, whereas flavonoids significantly decreased ROS formation in a dose-dependent manner (Table 1). Donepezil HCl (100 μ M), which was used as a positive control, inhibited A β ₂₅₋₃₅ induced ROS generation by 40.76%. Flavonoids greatly exerted protective effect in scavenging ROS in A β ₂₅₋₃₅ induced PC12 cells at 100 μ M beside apigenin,

which was proved to inhibit intracellular ROS generation *in vitro* [13].

3.3.2. Effects of the *A. Grossedentata* extracts on $A\beta_{25-35}$ induced ROS Generation

The inhibitory activity of *A. Grossedentata* extracts on $A\beta_{25-35}$ induced intracellular ROS generation in PC12 cells was evaluated. The MeOH extract as well as its solvent soluble fractions of *A. Grossedentata* inhibited intracellular ROS generation induced by $A\beta_{25-35}$ and these effects were shown to be concentration-dependent (Figure 5b). The ROS generation inhibitory activity potential of the individual fraction at the tested concentrations was in the following order: EtOAc > n-BuOH > MeOH > H₂O > CH₂Cl₂.

3.4. DPPH Scavenging Activity Assay

The concentrations of compounds and extracts were set the same as ROS test described. The DPPH was 50 μ M, and ascorbic acid was used as standard. Data in Table 1 showed that in the concentration of 100 μ M, all of the compounds expressed strong DPPH radical scavenging activity. The scavenging property was quite inosculated with the data of preventing ROS generation, between which the correlation was 0.659 with statistical significance. Note that flavonoids with greater effects on scavenging DPPH expressed better inhibitory effects on ROS-generation.

For the extracts, it changed little between the individual fractions (Figure 5c). Besides the concentration dependent manner, the DPPH radical scavenge activity potential of the individual fractions were in the same following order with ROS-generation inhibitory: EtOAc > n-BuOH > MeOH > H₂O > CH₂Cl₂. Ascorbic acid (100 μ M), which was used as a positive control, scavenged DPPH radical by 21.97%, when compared with the control.

4. Discussion

In the current study, we were able to detect high levels of polyphenolic compounds as dihydromyricetin and myricetin in *A. Grossedentata* growing natively in China [56,57]. We also observed that the level of dihydromyricetin was higher in the leaves of all species compared to the respective fruits and stems [58,59]. The leaves also had much higher total antioxidant capacity than the fruits and stems, as indicated by radical scavenging activity, which was consistent with our previous findings in *A. Grossedentata* extracts [58]. These biochemical data were generally in line with the biological activities of the extracts on $A\beta_{25-35}$ exposed PC12 cells. For example, although the individual flavonoids of *A. Grossedentata* were highly protective against $A\beta_{25-35}$ toxicity, the higher concentration levels were not protective since the cytotoxicity was great. However, the extracts fractions from leaves were highly neuroprotective in our cell culture model (Table 2).

4.1. Effects of the Bioactive Constituents of *A. Grossedentata* on $A\beta_{25-35}$ -induced Cell Death

In the present study, exposure of PC12 cells to $A\beta_{25-35}$ was shown to significantly decrease cell viability and the

effect was concentration-dependent as reported earlier [60]. Our result confirmed that in PC12 cells, $A\beta_{25-35}$ induced cell death and that selected flavonoids (100 μ M) of *A. Grossedentata* and the extracts pretreatment markedly mitigated these changes. As established in the experiments, $A\beta_{25-35}$ at a dose of 10 μ M was able to reduce the viability. In contrast, cultures exposed to the same amount of $A\beta_{25-35}$ in the presence of selected flavonoids (100 μ M) and fractions appeared remarkably preserved, indicating that these compounds significantly prevented neuronal cells death, and extracts of *A. Grossedentata* was surely protecting PC12 cells from $A\beta_{25-35}$ induced apoptosis. This protective effect was also demonstrated when cells were pretreated with Donepezil HCl (100 μ M), although with a lower efficacy when it was compared to kaempferol, isoquercitrin, rutin and KG.

4.2. Molecular Weight and Hydroxyl Group Dictated the Effects

4.2.1. Effects of Molecular Weight and Hydroxyl Group Number on Inducing PC12 Cell Death

Flavonoids at high level concentrations could induce cell death whereas the lower tested less or non-cytotoxicity, and the molecular weight and -OH number seemed to greatly affect the activity. Cytotoxicity data of the compounds (Table 1 and Figure 4) revealed that more than half of the compounds had cytotoxicity on PC12 cells, almost near the 50 percent inhibitory, and it came to the opposite when the concentration turned down. And the data revealed a negative correlation ($r = -0.758^*$, $p < 0.05$; see Table 3) between the individual molecular weight, i. e., lower molecular weight flavonoids as apigenin (270) and naringenin (272) exhibited the stronger cytotoxicity, while the heavier molecular isoquercitrin (464), rutin (610), and kaempferol-3-O- β -D-glucopyranoside (448) expressed non-cytotoxicity, as shown in Table 1. In the meanwhile, the cytotoxicity performed a correlated character ($r = -0.655^*$, $p < 0.05$; see Table 3) with the -OH number being similar with molecular weight. Hydroxyl groups in apigenin (3), hesperetin (3) and naringenin (3) ended a stronger cytotoxicity, which changed when came to isoquercitrin (8), rutin (10), and kaempferol-3-O- β -D-glucopyranoside (8).

Table 3. Correlations of flavonoids at 500 μ M

		Cytotoxicity
-OH number	Pearson Correlation	-.655*
	Sig. (2-tailed)	.040
	N	10
Molecular Weight	Pearson Correlation	-.758*
	Sig. (2-tailed)	.011
	N	10

*. Correlation is significant at the 0.05 level (2-tailed). **. Correlation is significant at the 0.01 level (2-tailed).

4.2.2. Effects of Molecular Weight and Hydroxyl Group Number on PC12 Cell Death Induced by $A\beta_{25-35}$

Neuroprotective results of individual flavonoids suggested that the molecular weight and hydroxyl groups presented in the molecules played a pivotal role in dictating neuroprotective effects against $A\beta_{25-35}$ induced cytotoxicity. The neuroprotective data of flavonoids revealed a positive correlation ($r = 0.434^{**}$, $p < 0.01$)

between the individual molecular weight, i. e., lower molecular weight compounds exhibited weaker neuroprotective capacity than the heavier ones, as shown in Table 1, lower molecular weight flavonoids apigenin (270) and naringenin (272) exhibited marginal effect on A β_{25-35} -induced cytotoxicity, while higher molecular weight-flavonoids isoquercitrin (464), rutin (610), and kaempferol-3-O- β -D-glucopyranoside (448) exerted neuroprotective effects.

Flavonoids with fewer hydroxyl groups and without 3-OH as apigenin, hesperetin and naringenin exerted poor neuroprotective effects, whereas flavonoids with the 3-OH group as dihydromyricetin, myricetin and kaempferol showed remarkable anti- A β_{25-35} -induced apoptosis activity, and the pearson correlation was 0.452 with statistical significance (Table 4), as proved in previous study [61]. Flavonoids with large number of -OH exerted better neuroprotective capacity, as isoquercitrin (8), rutin (10), KG (8), dihydromyricetin (6), myricetin (6), taxifolin (5) and kaempferol (4) performed.

Table 4. Correlations^a

		Neuro-protective
DPPH Scavenging	Pearson Correlation	.301*
	Sig. (2-tailed)	.034
	N	50
ROS Scavenging	Pearson Correlation	.653**
	Sig. (2-tailed)	.001
	N	50
-OH number	Pearson Correlation	.452**
	Sig. (2-tailed)	.001
	N	50
Molecular Weight	Pearson Correlation	.434**
	Sig. (2-tailed)	.002
	N	50

a. Concentration of the data various from 100 μ M to 1 μ M.

b. *.Correlation is significant at the 0.05 level (2-tailed). **.Correlation is significant at the 0.01 level (2-tailed).

4.3. DPPH Scavenging Activity and ROS-generation Inhibitory Effects Dictated the Neuroprotective Effects

Since A β_{25-35} -induced neurotoxicity is mediated by the oxidative stress [16-21], and ROS play a critical role in enhancing neuroinflammation [62], we further investigated the effects of compounds and fractions, which showed potent neuroprotective activities, on the antioxidative defense system in A β_{25-35} -injured PC12 cells. ROS production inhibitory activities of these flavonoids were evaluated, the increased cellular peroxides induced by the A β_{25-35} insult were effectively decreased by the treatment of flavonoids, with a positive correlation 0.653** (Table 4) between neuroprotective activities. Isoquercitrin, rutin, KG, dihydromyricetin, myricetin and taxifolin, which revealed potent neuroprotective activities, showed the significant ROS production inhibitory activities at the concentration ranging from 1 to 100 μ M. In line with many previous studies reporting antioxidative activities of flavonoids, the selected flavonoids exhibiting perfect DPPH scavenging activity were found to possess outstanding neuroprotective activities in PC12 cells ($r=0.301^*$, $*p<0.05$, Table 4), likewise, the greater ROS production inhibitory activities ($r=0.659^{**}$, $**p<0.01$).

Generally, the required structural criteria for high antioxidant activity of the flavonoids was known to include the 3',4'-ortho-dihydroxy group in the B-ring [62], and the large number of hydroxyl groups. In this respect, apigenin, naringenin, kaempferol and hesperitin which are neither orthohydroxy in the B-ring nor many hydroxyl groups seem to have weaker radical scavenging activities than the other flavonoids in terms of chemical structure itself. Actually, it was tested that they showed weak radical scavenging activities in the DPPH assay system (Table 2). However, in our experiments, these compounds showed potent antioxidant activities by intracellular ROS scavenging activity in PC12 cells, whereas weaker than the other 6 flavonoids.

These results could be stated that the protective effects of these flavonoids in PC12 cells depended on indirect induction of inhibitory effects on A β_{25-35} -induced ROS generation as well as simply the direct radical scavenging activities due to chemical structure itself. On the basis of above results, we anticipate that *A. Grossedentata* and the flavonoids might be therapeutic candidates as neuroprotective agents to attenuate the progression of AD and the other neurodegenerative diseases.

5. Conclusions

The present study has verified that extractions of *A. Grossedentata* inhibit apoptosis of PC12 cells induced by A β_{25-35} by blocking the accumulation of ROS, so did the individual flavonoids. The findings suggested that consumption of *A. Grossedentata* or rattan tea could have a positive effect on human health. For example, the high flavonoids content and antioxidant capacity of the plant would be potentially beneficial for the prevention of neurologic disease. It is possible that the consumption of tea made from the leaves of the plants, as well as supplements produced from the extracts of the leaves could slow brain aging or inhibit the development of neurodegenerative disorders.

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Statement of Competing Interests

The authors declare no competing financial interest.

List of Abbreviations

Alzheimer's disease (AD)
Beta-amyloid (A β)
Reactive Oxygen Species (ROS)
3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl-Tetrazolium Bromide (MTT)
Dimethylsulfoxide (DMSO)

Optical Density (OD)
Kaempferol-3-O- β -D-Glucopyranoside (KG)

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