C-Methylflavonoids Isolated from *Callistemon lanceolatus* Protect PC12 Cells against Aβ-Induced Toxicity

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Key words
- *Callistemon lanceolatus*
- Myrtaceae
- β-amyloid
- 4′,5-dihydroxy-6,8-dimethyl-7-methoxyflavanone
- *C*-methylflavonoids
- apoptosis
- PC12 cells

Abstract

Increased beta-amyloid (Aβ) production and its aggregation to the oligomeric state is considered to be a major cause of Alzheimer's disease (AD). Therefore, reducing Aβ-induced neurotoxicity could provide a suitable means of prevention or intervention in the disease course of AD. The neuroprotective effects of isolates from *Callistemon lanceolatus* DC. (Myrtaceae) against Aβ were evaluated using PC12 cells. To evaluate the effects of Aβ on apoptotic cell death and the effects of Bcl-2 family proteins and caspase-3, TUNEL assays and Western blotting were performed, respectively. Substantial fractionation and purification of the EtOAc-soluble extract of the aerial parts of *C. lanceolatus* afforded six flavonoids, 4′,5-dihydroxy-6,8-dimethyl-7-methoxyflavanone (**1**), eucalyptin (**2**), 8-demethyleucalyptin (**3**), sideroxylin (**4**), syzalterin (**5**), and quercetin (**6**). Compounds **1, 5**, and **6** were found to protect PC12 cells effectively against Aβ-induced toxicity. In particular, compound **1** showed the most promising neuroprotective effect with an ED_{50} value of 6.7 µM in terms of decreasing Aβ-induced apoptotic cell death, and this was accompanied by a decrease in caspase-3 activation and an increase in Bcl-2/Bax ratio. These results suggest that compound **1** could be developed as a candidate anti-AD agent due to its attenuation of Aβ-induced apoptotic cell death.

Supporting information available online at http://www.thieme-connect.de/ejournals/toc/plantamedica

Introduction

Beta-amyloid peptide (Aβ) is a proteolytic product of amyloid precursor protein (APP) produced by β-secretase and γ-secretase [1]. The increased production of Aβ and its aggregation to oligomers induces its progressive accumulation in the form of senile plaques, which, by triggering neurotoxicity, oxidative damage, and inflammation, are a major cause of the pathology of Alzheimer's disease (AD) [2, 3]. The therapeutic approaches evaluated include reducing oxidative damage by administering antioxidants [2], and reducing inflammation with nonsteroidal anti-inflammatory drugs, such as, ibuprofen and COX-2 inhibitors [4], but these drugs have not been found effective in human trials. In addition, acetylcholinesterase inhibitors, such as tacrine or donepezil, have been shown to delay disease progression and ameliorate symptoms such as memory loss [5]. However, the lack or loss of therapeutic benefit, and safety and tolerability concerns have limited the duration of acetylcholinesterase inhibitor therapy [6]. Therefore, novel AD drugs with different mechanisms of action, such as reducing Aβ-induced neurotoxicity, might offer better approaches to prevention and therapeutic intervention in AD.

Callistemon lanceolatus DC. (Myrtaceae), which is native to Australia, is widely distributed in subtropical and tropical regions. Extracts of its leaves exhibit anti-inflammatory and analgesic effects, and essential oils from its leaves have antimicrobial and fungitoxic activity [7]. In addition, several triterpenoids, flavonoids, fatty acids, tannins, and phenolic compounds have been isolated from its leaves [8]. In our previous communication, we reported the isolation of nine triterpenoids from *C. lanceolatus* and the effects of these isolates on the inhibition of LPS-induced nitric oxide production in murine macrophage RAW264.7 cells [8]. In particular, of the isolates examined, betulinic acid 3-*O*-caffeate was found to exhibit a moderate inhibitory effect on nitric oxide production. In our search for neuroprotective agents of natural ori-

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gin against Aβ-induced toxicity in PC12 cells [9,10], six flavonoids were isolated from the EtOAc-soluble extract of the aerial parts of *C. lanceolatus*. Here, we report the identification of five *C*-methylflavonoids (**1**–**5**) and quercetin (**6**) (**Fig. 1**), and the results of biological evaluations of their neuroprotective potentials against Aβ-induced toxicity in PC12 cells.

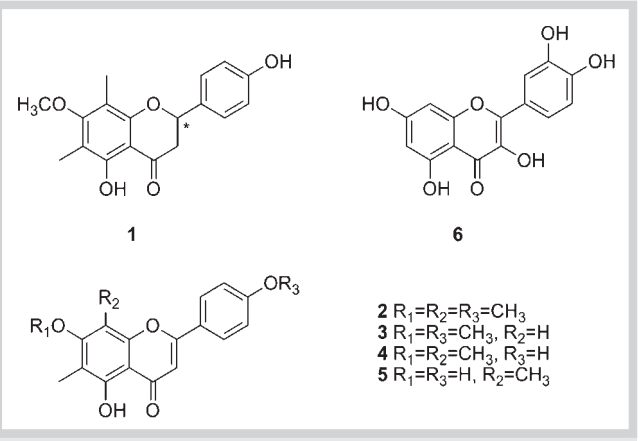

Fig. 1 Structures of compounds **1**–**6** isolated from *Callistemon lanceolatus*.

Materials and Methods

General experimental methods

UV spectra were obtained using a Mecasys Optizen 2120 UV. NMR spectra were obtained using a Varian 500 MHz NMR spectrometer with tetramethylsilane (TMS) as an internal standard (chemical shifts are expressed as δ values). ESI-mass spectra were recorded on a Waters Q-TOF micro mass spectrometer. Column chromatography was performed using silica gel (Kieselgel 60, 70–230 mesh and 230–400 mesh; Merck), and Sephadex LH-20 (18–111 µm; GE Healthcare). Preparative HPLC was carried out using a Varian system Prostar 210.

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Calbiochem), sodium dodecyl sulfate (SDS), trypan blue solution, and dimethyl sulfoxide (DMSO) were purchased from Sigma. $A\beta_{25-35}$ was purchased from Bachem California, Inc. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Invitrogen. Horse serum was obtained from Welgene, Inc.

Extraction and isolation

The aerial portion of *C. lanceolatus* was collected on Jeju Island, Korea, in September 2005, and identified by one of the authors (Y.T. Yang). A voucher specimen (2005–09-001-YTY) has been deposited with the Jeju Special Self-Governing Province Agricultural Research and Extension Services, Jeju, Korea. The air-dried aerial parts of *C. lanceolatus* (1.3 kg) were extracted with MeOH three times at room temperature (each time for 3 days). The combined MeOH extracts were then concentrated under vacuum at 40 °C to yield 180 g of residue, which was then suspended in water and partitioned using hexane (3 × 1.5 L) to afford a hexane-soluble extract (14 g). The remaining aqueous layer was partitioned with EtOAc to give an EtOAc-soluble extract (69 g).

The EtOAc extract (69 g) was chromatographed on a silica gel column (70–230 mesh; 10 × 60 cm) using a $CHCl_3$/MeOH ($CHCl_3$ → 1 : 4; 2 L for each eluent) gradient to afford 11 pooled fractions (F001 – F011). Fraction F004 was purified using a silica gel column (70–230 mesh; 5 × 60 cm) using a hexane/EtOAc (9 : 1 → 1 : 4; 400 mL for each eluent) gradient to afford fractions F012–F022. Compound **2** (18.4 mg) was isolated from F016 by silica gel column (230–400 mesh; 3 × 35 cm) chromatography using $CHCl_3$/acetone ($CHCl_3$ → 1 : 1; 100 mL for each eluent) gradient. F017 was purified using a Sephadex LH-20 gel column (18–111 µm; 4.5 × 150 cm) using $CHCl_3$/MeOH (1 : 1; 1.5 L) as eluant, to separate compounds **3** (5.3 mg) and **1** (5.7 mg). Fraction F005 was passed through a silica gel column (70–230 mesh; 5 × 60 cm) using a hexane/EtOAc (7 : 1 → 1 : 4; 400 mL for each eluent) gradient to afford fractions F044 – F058. Compound **4** (37.0 mg) was separated from F049 using a Sephadex LH-20 gel column (18–111 µm; 4.5 × 150 cm) and $CHCl_3$/MeOH (1 : 1; 1.5 L) as eluant. F050 was passed though a Sephadex LH-20 column (18–111 µm; 4.5 × 150 cm) using $CHCl_3$/MeOH (1 : 1; 1.5 L) as eluant to produce F082 – F092. Compound **5** (2.0 mg) was isolated from F091 by HPLC (YMC J'sphere ODS-H80, 4 µm, 250 × 20 mm i.d., 20–100% MeCN in H_2O, flow rate 8.0 mL/min). Fraction F009 was passed through a Sephadex LH-20 column (18–111 µm; 4.5 × 150 cm) using $CHCl_3$/MeOH (1 : 1; 1.5 L) as eluant, to produce eleven subfractions (F093 – F103). F102 was purified by HPLC (YMC J'sphere ODS-H80, 4 µm, 250 × 20 mm i.d., 20–80% MeCN in H_2O, flow rate 8.0 mL/min) to separate compound **6** (25.7 mg).

4′,5-Dihydroxy-6,8-dimethyl-7-methoxyflavanone (**1**): Brown resin; $[\alpha]_D^{28}$: +7.0° (*c* 0.1, MeOH); UV (MeOH): λ_{max} (log ε) = 282 (3.54), 357 nm (2.98); CD measurements gave no distinct Cotton effect indicating that compound **1** was obtained as an enantiomeric mixture in a different ratio; HR-ESI-MS: *m/z* = 315.1235 $[M + H]^+$, (calcd. for $C_{18}H_{19}O_5$: 315.1232); ^{1}H- and ^{13}C-NMR data, see Supporting Information.

Eucalyptin (**2**): Yellow needles; UV (MeOH): λ_{max} (log ε) = 282 (4.11), 323 nm (4.14); HR-ESI-MS: *m/z* = 327.1217 $[M + H]^+$ (calcd. for $C_{19}H_{19}O_5$: 327.1232); ^{1}H- and ^{13}C-NMR data, see Supporting Information.

8-Demethyleucalyptin (**3**): Pale yellow needles; UV (MeOH): λ_{max} (log ε) = 276 (4.11), 328 nm (4.18); HR-ESI-MS: *m/z* = 313.1069 $[M + H]^+$, (calcd. for $C_{18}H_{17}O_5$: 313.1076); ^{1}H- and ^{13}C-NMR data, see Supporting Information.

Sideroxylin (**4**): Yellow powder; UV (MeOH): λ_{max} (log ε) = 278 (4.47), 327 nm (4.55); HR-ESI-MS: *m/z* = 313.1066 $[M + H]^+$, (calcd. for $C_{18}H_{17}O_5$: 313.1076); ^{1}H- and ^{13}C-NMR data, see Supporting Information.

Syzalterin (**5**): Yellow powder; UV (MeOH): λ_{max} (log ε) = 279 (3.79), 330 nm (3.79); ESI-MS: *m/z* = 313.1066 $[M + H]^+$; ^{1}H- and ^{13}C-NMR data, see Supporting Information.

Quercetin (**6**): Yellow powder; UV (MeOH): λ_{max} (log ε) = 256 (4.32), 369 nm (4.30); ESI-MS: *m/z*: 303 $[M + H]^+$; ^{1}H- and ^{13}C-NMR data, see Supporting Information.

Cell cultures

Rat pheochromocytoma (PC12) cells were purchased from ATCC and cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% horse serum and 5% fetal bovine serum (FBS), at 37 °C under 5% CO_2. Cells in the exponential growth phase were utilized for experiments.

Preparation of $A\beta_{25-35}$ stock solution

$A\beta_{25-35}$ used in this study was preaggregated prior to use. Briefly, 1 mg of $A\beta_{25-35}$ was dissolved in 1 mL of DMEM and incubated at 37 °C in a water bath for three days to induce aggregation. The aggregated $A\beta_{25-35}$ was then diluted to 100 µg/mL (100 µM) and stored at − 20 °C before use.

MTT assay and trypan blue exclusion assay

The ability of compound **1** to protect PC12 cells from Aβ was determined by MTT or trypan blue assays. For MTT assay, PC12 cells (4×10^4 cells per well) were plated in 96-well tissue culture plates, after which the cells were pretreated with different concentrations of compound **1** (3, 6 and 12 µM), rosmarinic acid as a positive control [10,11] or DMSO as a negative (vehicle) control for 1 h. The cells were then incubated with 10 µM $A\beta_{25-35}$ for an additional 24 h, after which MTT solution (10 µL per well, 5 mg/mL stock solution in PBS) was added for 3 h at 37 °C. The cells were then lysed in the presence of 100 µL of lysis buffer (10% w/v of SDS in 0.01 N HCl) overnight at 37 °C. The optical density of the resulting solutions was determined colorimetrically at 570 nm using a microplate reader (Molecular Devices). For trypan blue assay, PC12 cells treated with Aβ in the presence or absence of compound **1** (12 µM) or rosmarinic acid (55 µM) were collected by centrifugation and incubated with trypan blue solution at 37 °C for 10 min, after which the number of viable cells and stained dead cells were counted under an inverted microscope (Optica). The data were obtained from three independent experiments.

Terminal dUTP nick-end labeling (TUNEL) assay

PC12 cells cultured on poly-L-lysine coated cover slips were treated with compound **1** (12 µM) in the presence or absence of 10 µM $A\beta_{25-35}$. After 24 h incubation, the cells were fixed in 4% formaldehyde for 15 min at room temperature, and DNA fragmentation resulting from apoptotic signaling were immunostained with commercially available TUNEL assay kit (Roche Applied Science). Then cells were mounted on slide glasses and examined under an inverted fluorescence microscope (× 400) (Nikon). Numbers of TUNEL positive cells were counted and results are expressed as percentages of total cells. The data were obtained from three independent experiments.

Western blot analysis

To evaluate the changes in apoptotic protein levels, PC12 cells were treated with 10 µM Aβ in the presence or absence of compound **1** (12 µM) for 24 h. Cultures were washed twice, scraped in Laemmli buffer and immediately boiled for 5 min. After the determination of protein concentration by Lowry method, proteins were separated on SDS-polyacrylamide gels and transferred to Immobilon membranes (Millipore). Immunodetection was then performed using the following antibodies: anti-α-tubulin (clone DM1A; 1 : 20 000; Sigma), anti-caspase-3 (1 : 1000; Cell Signaling Technology), anti-cleaved caspase-3 (1 : 1000; Cell Signaling Technology), anti-Bcl-2 (1 : 1000; Cell Signaling Technology) and anti-Bax (1 : 1000; Cell Signaling Technology). Proteins were detected using secondary antibodies conjugated to horseradish peroxidase (1 : 2500; Santa Cruz Biotechnology) and enhanced chemiluminescence reagents (Amersham Biosciences). Densitometry was then performed using a Bio-Rad 700 flatbed scanner and Molecular Analyst software (Bio-Rad). Densitometric values were normalized using α-tubulin as an internal control. Scanning of the Western blots showed the curve to be linear in the range used for each antibody.

Statistical analysis

All data in the text and figures are expressed as means ± SD. One-way analysis of variance (ANOVA) followed by Newman-Keuls post hoc test was performed to compare groups. Differences were considered statistically significant when p was < 0.05.

Supporting information

^{1}H- and ^{13}C-NMR data of compounds **1**–**6** are available as Supporting Information.

Results and Discussion

The EtOAc-soluble extract of the aerial parts of *C. lanceolatus* was subjected to repeated column chromatographic purifications and preparative HPLC to afford six known flavonoids (**1**–**6**) (**Fig. 1**). These six isolates were identified as 4′,5-dihydroxy-6,8-dimethyl-7-methoxyflavanone (**1**) [12], eucalyptin (**2**) [13], 8-demethyleucalyptin (**3**) [13], sideroxylin (**4**) [14], syzalterin (**5**) [15], and quercetin (**6**) [16], by interpreting spectral data and making comparisons with literature values (Supporting Information).

Although *C*-methylflavonoids have only rarely been reported from natural sources, they have been reported in certain species of Amaryllidaceae [15], Caesalpiniaceae [17], Myrtaceae [12–14], Pinaceae [18], Polypodiaceae [19,20], and Velloziaceae [21]. Furthermore, isolated compounds, such as piliostigmol from *Piliostigma reticulatum* (DC.) Hochst. and *C*-methylluteolins from goldenseal (*Hydrastis canadensis* L.), have been reported to exhibit antimicrobial activity [17,22], and matteuorienates and 2′-hydroxymatteucinol from *Matteuccia orientalis* (Hook.) Trevis. were found to have antidiabetic activity [19].

The neuroprotective effects of the five *C*-methylflavonoids and quercetin isolated from *C. lanceolatus*, and rosmarinic acid (positive control) were determined against Aβ-induced toxicity using PC12 cells. $A\beta_{1-42}$ insult to neuronal cells is one of the major causes of Alzheimer's disease [23], and $A\beta_{25-35}$ has been reported as an active toxic fragment of $A\beta_{1-42}$ [24]. Furthermore, $A\beta_{25-35}$ and $A\beta_{1-42}$ had similar effects on neuritic atrophy and cell death [25]. Therefore, we employed $A\beta_{25-35}$ as a neurotoxicant in this study. 55 µM of rosmarinic acid completely diminished the Aβ-induced toxicity in PC12 cells with ED_{50} value 23.6 µM. Syzalterin (**5**) and quercetin (**6**) were found to protect PC12 cells against Aβ-induced toxicity with ED_{50} values of 31.6 and 67.9 µM, respectively, whereas compounds **2**–**4** had no protective effect. In particular, compound **1** had the strongest protective effect against Aβ with an ED_{50} value of 6.7 µM. Accordingly, we further investigated the mechanism involved.

The neuroprotective effects of compound **1** were investigated by measuring the viability of PC12 cells incubated with Aβ (10 µM) in the presence or absence of compound **1** using MTT and trypan blue exclusion assays. For MTT assay, treatment with compound **1** (3, 6 and 12 µM) alone had minimal effects on cell viability as compared with DMSO treated controls (data not shown). On the other hand, treatment with 10 µM Aβ induced cell death of *ca.* 70% of the treated populations. However, pretreatment with 3, 6 or 12 µM of compound **1** significantly increased the viability of PC12 cells treated with Aβ. In particular, pretreatment of PC12 cells with 12 µM of compound **1** significantly blocked Aβ-induced

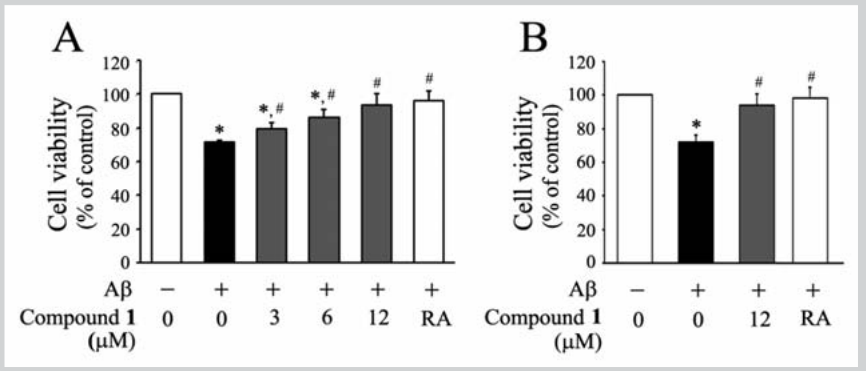

Fig. 2 Protective effects of compound **1** on Aβ-induced toxicity in PC12 cells. RA: rosmarinic acid. **A** PC12 cells were pretreated with 3, 6 or 12 μM of compound **1** for 1 h and then incubated with or without $A\beta_{25-35}$ (10 μM) for an additional 24 h, after which cell viability was determined using MTT assay. **B** The viability of cells treated with compound **1** (12 μM) with or without $A\beta_{25-35}$ (10 μM) for 24 h was evaluated using trypan blue exclusion assay. All data shown represent the mean ± SD. The values were obtained from five independent experiments. * $p < 0.05$, different from DMSO-treated control groups; # $p < 0.05$, different from Aβ-treated groups.

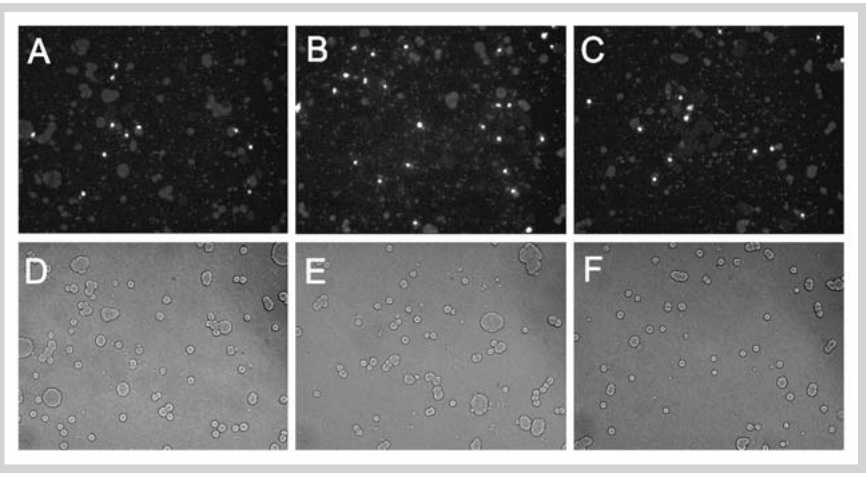

Fig. 3 Effect of compound **1** on Aβ-induced apoptotic cell death in PC12 cells. PC12 cells were treated with $A\beta_{25-35}$ (10 μM) in the presence or absence of compound **1** (12 μM) for 24 h and then labeled using a commercial TUNEL assay kit. Numbers of TUNEL positive cells were counted under an inverted fluorescence microscope (× 400). The pictures shown are representative images of control (**A, D**), Aβ-treated cells (**B, E**), and Aβ plus compound **1**-treated cells (**C, F**) as determined by three independent experiments. **D, E**, and **F** are phase-contrast images of **A, B** and **C**, respectively.

cytotoxicity as compared with control cells (**Fig. 2A**). Therefore, 12 μM of compound **1** were used for further investigation.

The determination of cell viability and proliferation by the MTT assay is a commonly employed method in biological screening. However, false positive results could occur due to the intrinsic reduction of MTT tetrazolium by the test compounds themselves [26]. Therefore, the viability of PC12 cells treated with Aβ in the presence or absence of compound **1** (12 μM) was further evaluated and confirmed using the trypan blue exclusion assay in our study. As shown in **Fig. 2B**, Aβ-treated cells showed a significant decrease in viable cell numbers as compared with DMSO-treated controls, while treatment with compound **1** prior to Aβ significantly increased viable cell numbers. These results are in agreement with results from the MTT assay.

In order to evaluate the effect of compound **1** on the apoptotic cell death induced by Aβ, a TUNEL assay was performed and positively labeled cells were counted under an inverted fluorescence microscope. As shown in **Fig. 3**, few DMSO-treated control cells (10.4 ± 2.5%) were TUNEL positive, whereas 33.2 ± 4.5% of Aβ-treated PC12 cells were positively stained by TUNEL staining. However, pretreatment with compound **1** (12 μM) prior to Aβ significantly decreased the number of TUNEL positive cells (14.8 ± 3.7%) as compared with Aβ-treated cells.

The effects of compound **1** on levels of Bcl-2 family proteins and caspase-3 were also examined by Western blot analysis (**Fig. 4**). PC12 cells treated with 10 μM Aβ showed significantly lower levels of the anti-apoptotic protein Bcl-2, and significantly higher levels of the pro-apoptotic protein Bax, which resulted in a marked decrease in the Bcl-2/Bax ratio compared to the controls.

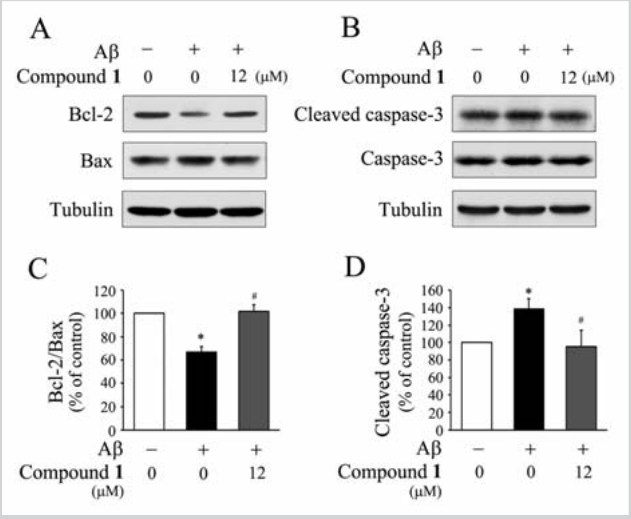

Fig. 4 Effect of compound **1** on the expressions of Bcl-2 family proteins and caspase-3 in Aβ-treated PC12 cells. Cells were pretreated with compound **1** (12 μM) for 1 h and then exposed to 10 μM $A\beta_{25-35}$ for 24 h. **A** Levels of the Bcl-2 family proteins, Bcl-2 and Bax, were determined by Western blot analysis. **B** Levels of cleaved caspase-3 and total caspase-3 were determined by Western blotting. **C, D** Graphs showing changes in the ratio of Bcl-2/Bax and the levels of cleaved caspase-3 in cells. All data represent the mean ± SD of three different experiments. * $p < 0.05$, different from the control groups; # $p < 0.05$, different from the Aβ-treated groups.

However, pretreatment with compound **1** (12 µM) significantly attenuated the increase of Bax and the decrease of Bcl-2 induced by Aβ, thus increasing the Bcl-2/Bax ratio compared to cells treated with Aβ. In addition, levels of activated (cleaved) caspase-3 in Aβ-treated cells were significantly higher than in DMSO-treated controls, whereas pretreatment with compound **1** significantly decreased the levels of activated (cleaved) caspase-3 induced by Aβ treatment.

Excess Aβ production in the brain and its aggregation into oligomers has been recognized as a major cause of AD. Although the mechanisms responsible for the neurotoxic effect of Aβ have not been fully elucidated, Aβ-induced apoptotic cell death is considered to be associated with neuritic degeneration and the onset of AD [27]. In the present study, compound **1** substantially decreased Aβ-induced apoptotic cell death by TUNEL assay. In addition, the anti-apoptotic effect of compound **1** was accompanied by a reduction in activated caspase-3, which plays a role in Aβ-induced apoptosis, and which has been reported to be upregulated in the AD brain [28]. Recently, purple sweet potato anthocyanins were reported to protect PC12 cells against Aβ by the reduction of apoptosis and caspase-3 activation [29]. In addition, piceatannol isolated from the seeds of *Euphorbia lagascae* were also neuroprotective against Aβ through the decreased rate of apoptosis and caspase-3 activation [30]. Therefore, the downregulation of activated caspase-3 by compound **1** could be responsible for its anti-apoptotic effect against Aβ.

The Bcl-2 protein family contains both the pro-apoptotic molecule Bax, and anti-apoptotic molecule Bcl-2 [31], and Aβ-induced apoptotic cell death in PC12 cells has been reported to be associated with Bcl-2 downregulation and Bax upregulation [32]. Some compounds derived from natural sources were reported to exhibit similar protective effects. For example, puerarin, an isoflavone purified from the root of the *Pueraria lobata* (Wild) Ohwi protected PC12 cells against Aβ accompanied with increased Bcl-2/Bax ratio and reduced caspase-3 activation [33]. In addition, isodojaponin D (CBNU06), a new diterpene isolated from *Isodon japonicus* (Burm.) Hara, also attenuated Aβ-induced toxicity by increasing the Bcl-2/Bax ratio [34]. In the present study, compound **1** prevented Bax upregulation and Bcl-2 downregulation induced by Aβ, which suggest that the neuroprotective effect of compound **1** is related to its inhibition of Aβ-induced apoptosis.

In summary, repeated column chromatographic purifications and preparative HPLC of the EtOAc-soluble extract of the aerial parts of *C. lanceolatus* afforded six known flavonoids, namely, 4′,5-dihydroxy-6,8-dimethyl-7-methoxyflavanone (**1**), eucalyptin (**2**), 8-demethyleucalyptin (**3**), sideroxylin (**4**), syzalterin (**5**), and quercetin (**6**), which were identified by interpreting spectral data and by making comparisons with literature values. Our evaluation of the neuroprotective effects revealed that compounds **1, 5,** and **6** effectively protected PC12 cells against Aβ-induced toxicity, and that compound **1** had the most potent neuroprotective effect (ED_{50} 6.7 µM). Furthermore, compound **1** decreased Aβ-induced apoptotic cell death accompanied with the decrease in caspase-3 activation and the increase of the Bcl-2/Bax ratio. These results suggest that compound **1** could be developed as an anti-AD agent.

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