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Pectolinarin Against Amyloid-beta-induced Neuroinflammation and Apoptosis *In vitro*

Mei Tong He[1], Byeong Wook Noh[2], Hyun Young Kim[3], Ah Young Lee[3,*], and Eun Ju Cho[2,*]

[1]*College of Korean Medicine, Gachon University, Seongnam 13120, Republic of Korea*
[2]*Department of Food Science and Nutrition, Pusan National University, Busan 46241, Republic of Korea*
[3]*Department of Food Science and Nutrition, Gyeongsang National University, Jinju 52828, Republic of Korea*

Abstract – An excess of amyloid beta (Aβ) led to a rise in ROS production, which in turn caused inflammatory reactions and mitochondrial dysfunction, both of which accelerate the progression of Alzheimer's disease (AD). Natural flavonoids are proposed as possible agents for neurodegeneration. Pectolinarin is an important flavone mainly found in *Cirsium* species. In this study, we explored the potential neuroprotective effect of pectolinarin in $A\beta_{25-35}$-induced SH-SY5Y cells. The result demonstrated that pectolinarin enhanced cell viability. Pectolinarin treatment inhibited $A\beta_{25-35}$-induced ROS generation. Pectolinarin also suppressed NO generation by inhibiting the translocation of NF-κB and downregulating protein expression of iNOS and COX-2. Moreover, the expression of Bcl-2 increased while BAX protein decreased when the cells were exposed to pectolinarin, resulting in a decrease in the BAX/Bcl-2 ratio. Pectolinarin treatment also increased BDNF and its receptor TrkB protein expression. In conclusion, pectolinarin neuroprotected $A\beta_{25-35}$-induced inflammation and apoptosis. These findings suggest that pectolinarin may be a promising neuroprotective functional food in the protection of the neurodegenerative diseases, including AD.

Keywords – Pectolinarin, Amyloid beta, Neuroinflammation, Apoptosis, SH-SY5Y cells

Introduction

Alzheimer's disease (AD) is considered the most prevalent form of dementia.[1] One of the neuropathological hallmarks of AD is the deposition of senile plaques that are composed of beta-amyloid (Aβ), which is generated through an amyloidogenic processing.[1] High levels of Aβ induce the generation of reactive oxygen species (ROS), resulting in inflammatory reactions along with apoptotic cell death.[2,3] Previous studies revealed that Aβ promotes inflammatory response by releasing pro-inflammatory mediators and cytokines, such as inducible nitric oxide synthase (iNOS) and interleukins via the activation of nuclear factor-kappa B (NF-κB) signaling.[4,5] Moreover, studies have shown that Aβ can induce neuronal apoptosis *in vitro*.[6] Apoptosis involves the activation of B-cell lymphoma 2 (Bcl-2) protein family, including Bcl-2, an anti-apoptotic member, and Bcl-2-associated X (BAX), a pro-apoptotic member.[7,8] Therefore, neuroprotective effect could be related to the modulation of inflammatory reactions and apoptotic proteins.

In the nervous system, trophic support and synaptic plasticity are provided by brain-derived neurotrophic factor (BDNF) and its receptor tyrosine kinase B (TrkB).[9] Reduced BDNF has been indicated as a feature of AD and cognitive dysfunction.[10] Studies have shown that BDNF binds with TrkB to prevent Aβ-mediated cellular apoptosis by modulating BAX/Bcl-2 expression.[11,12] Therefore, modulation of BDNF/TrkB signaling may reverse the toxic effect induced by Aβ.

Pectolinarin is found in over 20 plant genera, including *Linaria*, *Kickxia*, *Cirsium*, and *Viburnum*, in which the content of pectolinarin in *Cirsium* is about 47.13%.[13] Cho et al. has indicated that the content of pectolinarin depends on the area and species of the plant, such as the aerial part of *C. chlorolepis* and *C. nipponcium* (110.65 mg/g extract and 61.44 mg/g extract, respectively), the root part of *C. chanroenicum* (0.479 mg/g extract), and the pappus of *C. japonicum* var. *maackii* (4.97 mg/g extract).[14] Several studies have indicated the neuroprotective potential of

*Author for correspondence
Ah Young Lee, Ph. D., Department of Food Science and Nutrition, Gyeongsang National University, 501, Jinju-daero, Jinju 52828, Republic of Korea
Tel: +82-55-772-1435; E-mail: aylee@gnu.ac.kr

Eun Ju Cho, Ph. D., Department of Food Science and Nutrition, Pusan National University, 2, Busandaehak-ro 63beon-gil, Geumjeong-gu, Busan 46241, Republic of Korea
Tel: +82-51-510-2837; E-mail: ejcho@pusan.ac.kr

pectolinarin in the management of neurodegenerative diseases.[15] A recent study confirmed that pectolinarin blocks the aggregation of $A\beta_{1-42}$ and thus reduces cytotoxicity in Neuro2a cells (a mouse neuroblastoma cell line).[16] Pectolinarin also showed protective effects on H_2O_2-induced cell death and oxidative stress.[17] However, the neuroprotective effects and mechanisms of pectolinarin against $A\beta_{25-35}$-induced inflammation and apoptosis in SH-SY5Y cells have not yet been determined. In this study, pectolinarin exhibited neuroprotective effects against $A\beta_{25-35}$ by reducing ROS and NO production, enhancing BDNF signaling, and suppressing the inflammation reaction and apoptosis through the regulation of NF-κB and Bcl-2 signaling.

Pectolinarin belongs to the flavones subclass, which is mainly derived from *Cirsium* and chemically similar to linarin.[18] Pectolinarin has attracted much attention due to its biological activities, such as antimicrobial, antioxidant, anti-inflammatory, and anti-diabetic effects.[13] Pectolinarin isolated from *C. setidens* has been shown to reduce H_2O_2-induced apoptotic cell death in SK-N-SH cells.[19] A previous study has revealed the protective effect of *C. japonicum* on Aβ-induced neuronal toxicity in SH-SY5Y cells, indicating that this effect may be related to pectolinarin.[20] However, the neuroprotective activity of pectolinarin has not been well studied. Here, the effects of pectolinarin against inflammation and apoptosis and its underlying mechanisms in Aβ-induced SH-SY5Y cells were explored.

Experimental

Chemicals and reagents – Pectolinarin (CFN99727) was purchased from Chem Faces (Wuhan, China). $A\beta_{25-35}$, 2',7'-dichlorofluorescein diacetate (DCF-DA), and Griess reagent were purchased from Sigma Chemical Co. (Saint Louis, MO, USA). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), and penicillin-streptomycin were obtained from Welgene Inc. (Daegu, Korea). 3-(4,5-Dimethylthiazol-2-yl)-2,3-diphenyl tetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Bio Basic Inc. (Toronto, Canada) and Daejung Chemicals & Metals Co., Ltd. (Siheung, Korea), respectively. Primary and secondary antibodies, including BAX (#2772; Cell Signaling Technology), Bcl-2 (ab196495; Abcam), NF-κB (ab28856; Abcam), pNF-κB (sc-136548; Santa Cruz Biotechnology), iNOS (#13120; Cell Signaling Technology), cyclooxygenase-2 (COX-2, #12282; Cell Signaling Technology), TrkB (ab33655; Abcam), BDNF (ab108319; Abcam), β-actin (#8457; Cell Signaling Technology), horseradish peroxidase (HRP)-conjugated anti-rabbit (#7074; Cell Signaling Technology), and anti-mouse (#7076; Cell Signaling Technology) IgG antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA), Abcam (Cambridge, UK), and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively. Pectolinarin stock solution was prepared in DMSO. $A\beta_{25-35}$ solution was prepared in double-distilled water and incubated for 72 h at 37°C to form aggregated oligomers.

Cell culture – Cell culture medium was composed of DMEM, 10% FBS, and 1% penicillin-streptomycin. The SH-SY5Y human neuroblastoma cell line was obtained from the American Type Culture Collection (Manassas, VA, USA), and the cells were cultured in cell culture medium at 37°C in an incubator with 5% CO_2. The cells were seeded in 96-well plates at a density of 2.5×10^5 cells/mL for MTT, DCF-DA, and nitric oxide (NO) assays. For Western blot analysis, cells were cultured in 6-well plates at a density of 1×10^6 cells/mL.

MTT assay – After the cells seeded at a density of 2.5×10^5 cells/mL on a 96-well plate, and pectolinarin at different concentrations (1, 2.5, and 5 μg/mL) was pretreated with the cells for 4 h, followed by $A\beta_{25-35}$ solution (50 μM) and incubated for another 24 h. After treatments, cell supernatant was removed and MTT solution (5 mg/mL) was added to the cells for 4 h at 37°C. The MTT formazan product was solubilized by DMSO. Absorbance was read at 540 nm using a microplate reader (Rayto Life and Analytical Sciences co., Ltd, Shenzhen, China).

DCF-DA assay – The cells were seeded at a density of 2.5×10^5 cells/mL in a 96-well black plate and incubated for 24 h. The cells were pretreated for 4 h with pectolinarin at 1, 2.5, and 5 μg/mL and then treated with $A\beta_{25-35}$ at a concentration of 50 μM. After 24 h of incubation, the media were removed and added with DCF-DA at 80 μM for 30 min. DCF-DA fluorescence was detected using a fluorescence spectrophotometer (BMG Labtech, Ortenberg, Germany) at 480 nm excitation and 535 nm emission.

NO assay – The cells were seeded on a 96-well plate and incubated for 24 h. Pectolinarin at 1, 2.5, and 5 μg/mL was pretreated for 4 h followed by treatment with $A\beta_{25-35}$ at 50 μM. Then 100 μL of the supernatant was mixed with 100 μL of Griess reagent in the dark and reacted for 10 min. The absorbance was measured at 540 nm using a microplate reader.

Western blot analysis – In a 6-well plate, the cells were seeded at a density of 1×10^6 cells/mL and pretreated with pectolinarin at 1, 2.5, and 5 μg/mL. Next, they were treated with $A\beta_{25-35}$ at 50 μM. Subsequently, the cells were collected and lysed in combination with a protease inhibitor cocktail, known as radioimmunoprecipitation

assay buffer, and centrifuged for 20 min at 4°C at 12,000 rpm. The protein concentration was determined by bovine serum albumin assay using Bradford Dye Reagent (#5000205; Bio-Rad Laboratories, Inc., Hercules, CA, USA).[21] An equal amount of protein (12.5 μg) was loaded onto 10% or 13% sodium dodecyl-sulfate polyacrylamide gel electrophoresis gels and then transferred to polyvinylidene difluoride membranes. The membranes were incubated with primary antibodies (BAX, 1:1000; Bcl-2, 1:200; NF-κB, 1:1000; pNF-κB, 1:1000; iNOS, 1:200; COX-2, 1:1000; TrkB, 1:1000; BDNF, 1:500; β-actin, 1:1000) overnight at 4°C. After the membranes were washed with PBST three times, they were incubated with the secondary antibodies (anti-rabbit, 1:1000 and anti-mouse, 1:1000) for 1 h at room temperature. Protein bands were detected by a chemiluminescent detection system (Davinch Chemi™, Seoul, Korea) and quantified by Image J software (v1.53, NIH, Bethesda, MA, USA).

Statistical analysis – The data was presented as mean ± SD. At least three independent experiments were performed. The data was analyzed using the Statistical Package for Social Sciences (version 26.0, SPSS Inc., Chicago, IL, USA). One-way ANOVA with Duncan's *post-hoc* analysis was used to compare the groups. It was determined that the *p*-value ($p < 0.05$) has statistical significance.

Results and Discussion

It has been reported that Aβ accumulation leads to increased toxic effect and ROS generation.[22] $A\beta_{25\text{-}35}$ is a short fragment of Aβ but retains the toxicity of the full-length $A\beta_{1\text{-}42}$. Therefore, $A\beta_{25\text{-}35}$ has been widely used for *in vitro* studies to analyze the potential mechanisms of new pharmacotherapies.[23,24] It has been revealed that the release of lactate dehydrogenase was significantly activated by $A\beta_{25\text{-}35}$ at 50 μM, but it did not affect it at 10 μM ($p > 0.05$) in neuronal cultures.[25] A previous study also showed that $A\beta_{25\text{-}35}$ at 50 μM significantly induced about 35% of cytotoxicity in SH-SY5Y cells.[26] Therefore, 50 μM of $A\beta_{25\text{-}35}$ was used to induce cytotoxicity in SH-SY5Y cells in the present study. The cytotoxicity of pectolinarin on SH-SY5Y cells at initial concentrations ranging from 0.5 to 50 μg/mL was measured by using an MTT assay. Results showed over 90% of cell viability at all concentrations which demonstrated the non-toxic effect of pectolinarin (Fig. 1A). Thus, pectolinarin at concentrations of 1, 2.5, and 5 μg/mL were used for the subsequent experiments. As shown in Fig. 1B, compared with the normal group (set as 100%), cell viability was decreased to 71.2 ± 3.6% in the $A\beta_{25\text{-}35}$-treated control group. In contrast, pretreatment

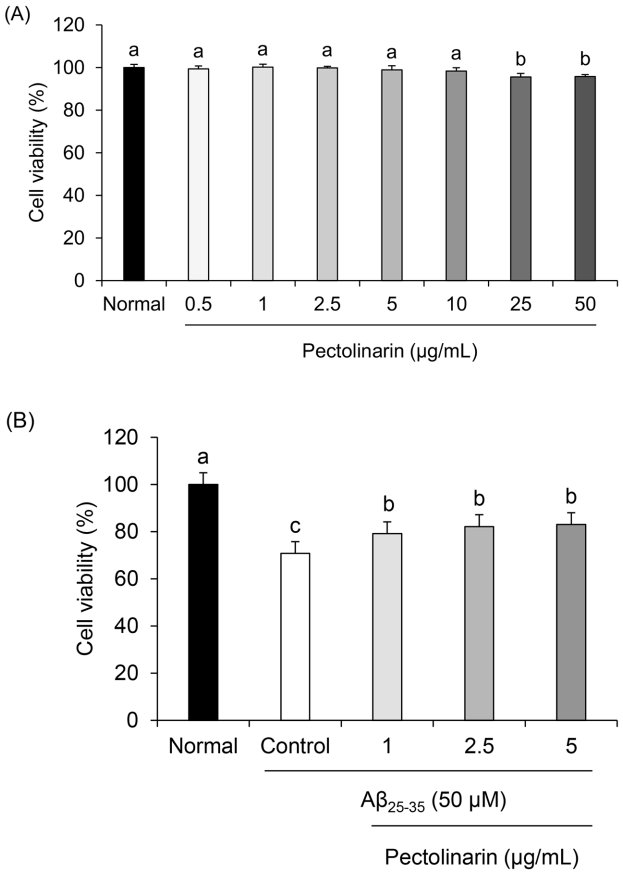

Fig. 1. Effect of pectolinarin on cell viability in $A\beta_{25\text{-}35}$-induced SH-SY5Y cells. (A) Cells were treated with various concentrations of pectolinarin (0.5–50 μg/mL) for 24 h and (B) 50 μM $A\beta_{25\text{-}35}$ for 24 h following 4 h pectolinarin pretreatment (1, 2.5, and 5 μg/mL). Values are mean ± SD ($n = 5$). Results of one-way ANOVA test with Duncan multiple range tests for multiple comparisons at $p < 0.05$. Means with the different letters (a, b, and c) are significantly different ($p < 0.05$) from each other. Means with the same letter are not significantly different.

with pectolinarin at 1, 2.5, and 5 μg/mL showed cell viability at 77.8 ± 4.4%, 80.8 ± 2.5%, and 81.2 ± 5.0%, respectively. These results suggest that pectolinarin may protect SH-SY5Y cells from $A\beta_{25\text{-}35}$-induced cytotoxicity.

It is well-established that excessive ROS results in protein oxidation, lipid peroxidation, and DNA mutation, leading to neurodegeneration.[27] Increased ROS production contributed to cellular death by $A\beta_{25\text{-}35}$ has been previously demonstrated in SH-SY5Y cells.[28] Previous studies have reported that pectolinarin at 5 and 10 μg/mL showed a decrease of ROS generation induced by H_2O_2 in SK-N-SH cells.[19] Consistently, $A\beta_{25\text{-}35}$ markedly elevated cellular ROS levels in the present study. As shown in Fig. 2A, the level of ROS significantly increased in the $A\beta_{25\text{-}35}$-treated control group, but it was decreased with the treatment of pectolinarin. At 60 min, ROS amounts were markedly

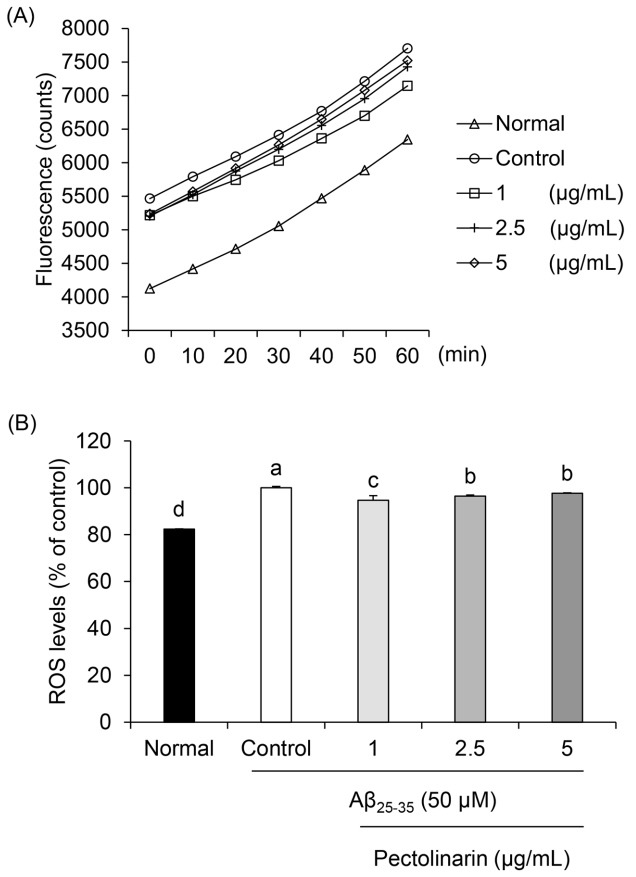

Fig. 2. Effect of pectolinarin on ROS scavenging in $A\beta_{25\text{-}35}$-induced SH-SY5Y cells. (A) Time course of change in intensity of DCF fluorescence during 60 min. (B) Intensity of ROS production at 60 min. Values are mean ± SD ($n = 4$). Results of one-way ANOVA test with Duncan multiple range tests for multiple comparisons at $p < 0.05$. Means with the different letters (a, b, c, and d) are significantly different ($p < 0.05$) from each other. Means with the same letter are not significantly different.

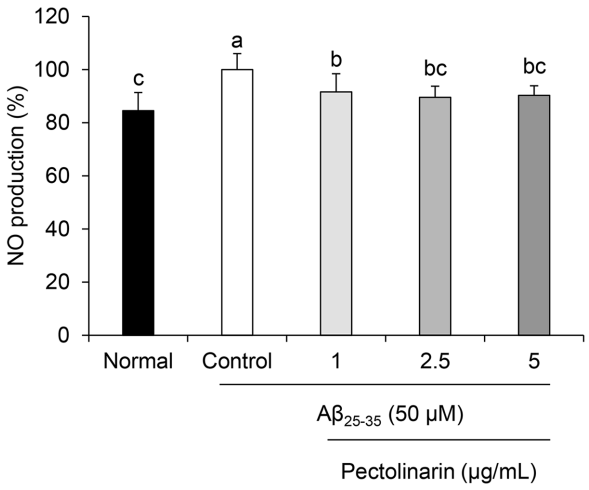

Fig. 3. Effect of pectolinarin on NO production in $A\beta_{25\text{-}35}$-induced SH-SY5Y cells. Values are mean ± SD ($n = 8$). Results of one-way ANOVA test with Duncan multiple range tests for multiple comparisons at $p < 0.05$. Means with the different letters (a, b, and c) are significantly different ($p < 0.05$) from each other. Means with the same letter are not significantly different.

decreased by pectolinarin at 1, 2.5, and 5 μg/mL with 94.7 ± 1.9%, 96.5 ± 0.5%, and 97.7 ± 0.2%, respectively, in comparison to that in the control group (100 ± 0.6%), and the normal group had a ROS level of 82.4 ± 0.1% (Fig. 2B). The result suggests that the protective effect of pectolinarin may be associated with its inhibition of ROS generation.

In addition to ROS, overproduction of NO by nitric oxide synthases (NOS) also promotes protein oxidation, involving neurotoxicity and triggering apoptotic neuronal death.[29,30] Significantly increased levels of NO resulted in memory impairment in $A\beta_{25\text{-}35}$-injected rats.[31] A previous study has demonstrated that pectolinarin effectively scavenged *in vitro* NO radicals and reduced NO generation in lipopolysaccharide-stimulated RAW 264.7 cells.[32] In this study, exposure of $A\beta_{25\text{-}35}$ to SH-SY5Y cells significantly increased NO generation (100 ± 6.0%) when compared with the normal cells (84.5 ± 6.8%) (Fig. 3). However, pretreatment of pectolinarin at 1, 2.5, and 5 μg/mL significantly reversed this feature that decreased to 91.6 ± 6.8%, 89.5 ± 4.2%, and 90.3 ± 3.6%, suggesting that pectolinarin could partially suppress the $A\beta_{25\text{-}35}$-induced NO production. In addition, NO overproduction is associated with the expression of iNOS in inflammatory condition.[33] iNOS is a neuroinflammatory marker known to involve AD progression.[34] A previous study has reported that deficiency of iNOS protected the AD-transgenic mice from Aβ deposition, demonstrating that iNOS may be a major factor in AD progression.[35] A high level of iNOS has been detected in Aβ-injected temporal lobe tissues.[36] In the present study, $A\beta_{25\text{-}35}$-induced iNOS protein expression (1.4-fold) was significantly inhibited by treatment with pectolinarin at 2.5 μg/mL (0.9-fold) and 5 μg/mL (0.3-fold) (Fig. 4). The iNOS inhibitory effect by pectolinarin did not show at 1 μg/mL. The result suggests that pectolinarin could suppress iNOS-mediated inflammatory response in AD. Another inflammatory marker, COX-2, has been considered an appropriate target for anti-inflammatory drugs due to its property for mediating neuroprotection.[37] The mRNA level of COX-2 increased with the exposure of Aβ in BV-2 microglia cells, mediating neuroinflammation, and in turn, neuron survival was enhanced after treatment with COX-2 inhibitors such as ibuprofen.[38] A previous study has revealed that Aβ-induced inflammation was

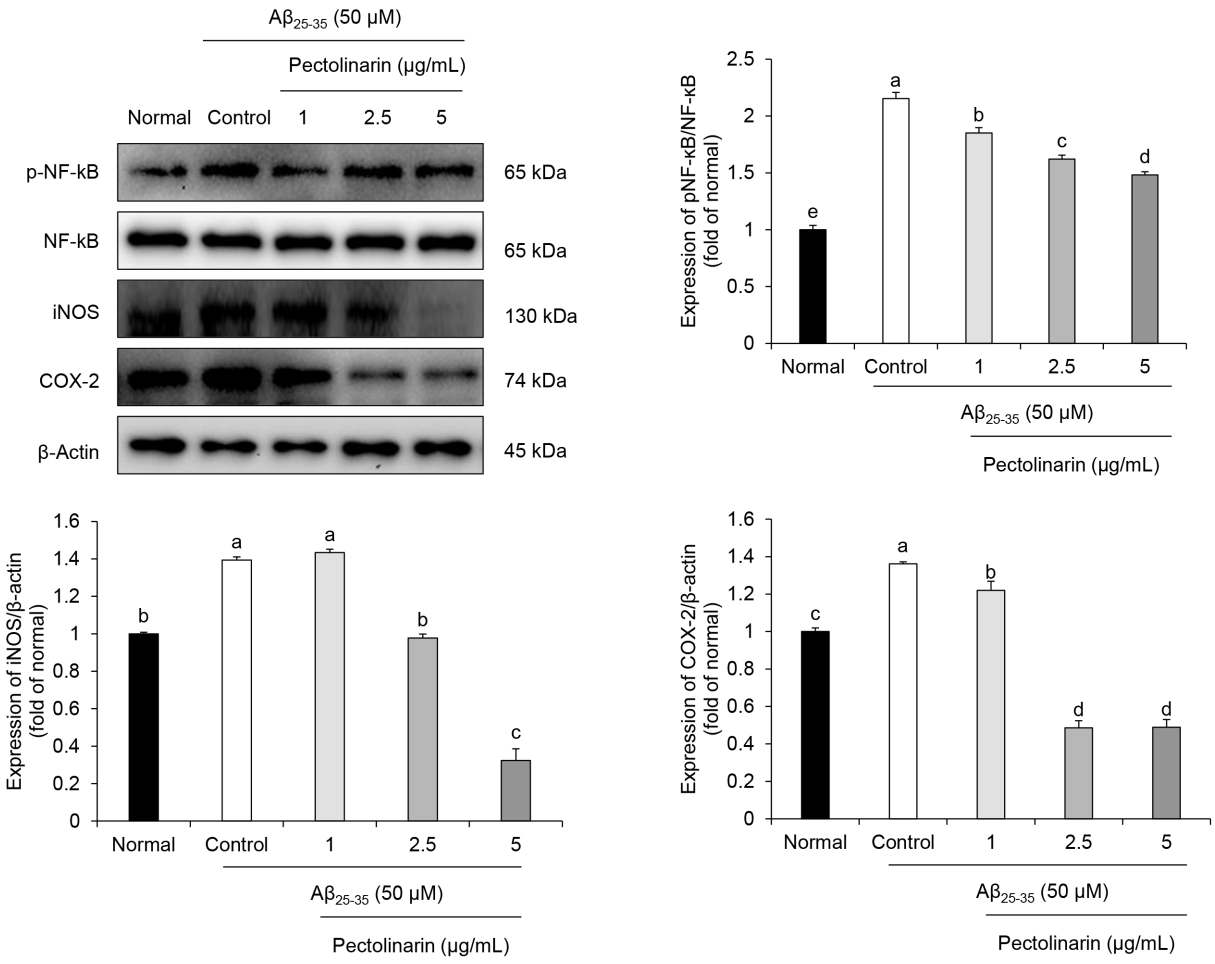

Fig. 4. Effect of pectolinarin on inflammation in $A\beta_{25-35}$-induced SH-SY5Y cells. Values are mean ± SD ($n = 3$). β-actin was used as a loading control. Results of one-way ANOVA test with Duncan multiple range tests for multiple comparisons at $p < 0.05$. Means with the different letters (a–e) are significantly different ($p < 0.05$) from each other. Means with the same letter are not significantly different. pNF-κB, phospho nuclear factor-κB; NF-κB, nuclear factor-κB; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2.

weakened in COX-2 deficient mice.[39] In Fig. 4, $A\beta_{25-35}$-increased COX-2 protein expression (1.4-fold) was significantly decreased by pectolinarin at 1 μg/mL (1.2-fold), 2.5 μg/mL (0.5-fold), and 5 μg/mL (0.5-fold), suggesting that pectolinarin could be used for attenuating COX-2-mediated inflammatory response. Moreover, NF-κB is a transcription factor that regulates DNA transcription. Once activated, NF-κB is released from IκB-α by a phosphorylation cascade into the nucleus, and consequently results in the activation of pro-inflammatory enzymes, including iNOS and COX-2.[40,41] It is thus known that NF-κB inhibition attenuates the inflammatory response. Flavonoids are strongly responsible for anti-inflammatory activities due to the presence of C2=C3 double bonds.[13] Linarin, which is structurally similar to pectolinarin, has been shown to inhibit NF-κB signaling in glioma cells.[42] In the present study, cytoplasm NF-κB protein expression was firstly measured, which was found to be that pectolinarin (1.9-fold at 1 μg/mL, 1.6-fold at 2.5 μg/mL, and 1.5-fold at 5 μg/mL) decreased the $A\beta_{25-35}$-induced phosphorylated NF-κB in SH-SY5Y cells (Fig. 4). These findings suggest that pectolinarin processes a protective effect on $A\beta_{25-35}$-mediated inflammatory response by modulating the NF-κB signaling pathway. However, there are still limitations in this study with respect to pectolinarin on more detailed molecular mechanisms in the changes in NF-κB signaling in the nucleus. More data need to be addressed for pectolinarin on its anti-inflammatory effect.

Modulation of pro- and anti-apoptotic proteins, including Bcl-2 and BAX, is associated with the neuronal apoptotic pathway.[43] Bcl-2 is a key member of the anti-apoptotic Bcl-2 family, playing a pivotal role in prolonging cell

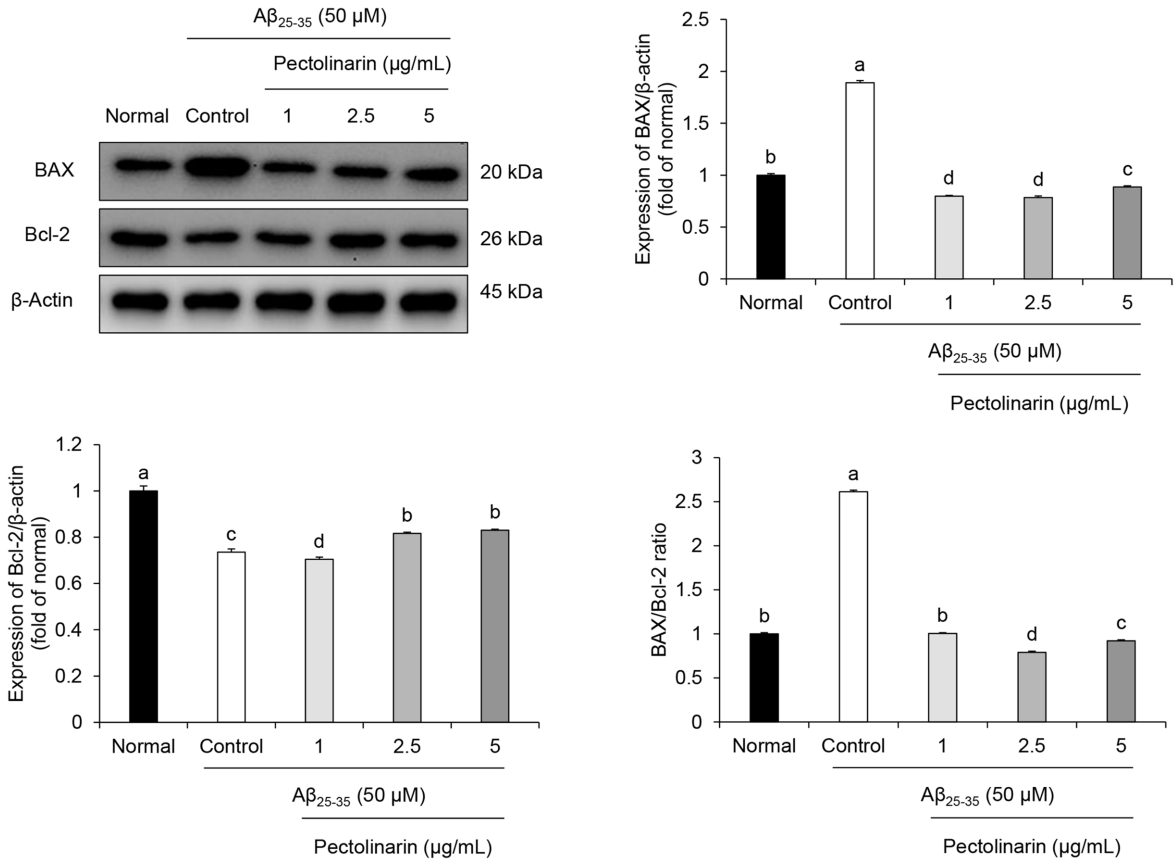

Fig. 5. Effect of pectolinarin on mitochondrial damage in $A\beta_{25-35}$-treated SH-SY5Y cells. Values are mean ± SD ($n = 3$). β-actin was used as a loading control. Results of one-way ANOVA test with Duncan multiple range tests for multiple comparisons at $p < 0.05$. Means with the different letters (a, b, c, and d) are significantly different ($p < 0.05$) from each other. Means with the same letter are not significantly different. BAX, B-cell lymphoma 2-associated X; Bcl-2, B-cell lymphoma 2.

survival by blocking apoptosis.[44] BAX promotes cell death and is considered to function as an antagonist of Bcl-2.[45] Several studies have shown that Aβ accumulation can induce neuronal damage and result in the imbalance between pro- and anti-apoptotic proteins.[6,8] As demonstrated in Fig. 5, in the $A\beta_{25-35}$-induced control group, a significant increase in the expression of BAX (1.8-fold) and a decrease in the expression of Bcl-2 (0.7-fold) were observed. The situation was reversed with the treatment of pectolinarin. However, pectolinarin at 1 μg/mL showed no significant difference in the expression of Bcl-2 when compared with the $A\beta_{25-35}$-induced control group. It has been indicated that the ratio between pro-apoptotic and anti-apoptotic proteins is a key marker to determine the apoptotic state of the cells.[6] The BAX/Bcl-2 ratio in the present study was significantly increased by $A\beta_{25-35}$ (2.6-fold); however, it was attenuated by pectolinarin at 1 μg/mL (1.0-fold), 2.5 μg/mL (0.8-fold), and 5 μg/mL (0.9-fold), showing a decreased expression ratio of BAX/Bcl-2. These results suggest that pectolinarin could provide a protective mechanism in $A\beta_{25-35}$-mediated neuronal apoptosis.

In many central nervous system cells, BDNF binding with its receptor TrkB plays a critical role in survival-promoting actions.[12] Evidence has shown that Aβ can directly downregulate BDNF expression by blocking TrkB.[46,47] Moreover, impairment of BDNF signaling causes synaptic dysfunction that contributes to memory deficits in AD.[48] To evaluate whether BDNF is regulated by pectolinarin, the protein expression of BDNF and its receptor TrkB were measured as shown in Fig. 6. $A\beta_{25-35}$ markedly reduced the protein expression of TrkB (0.5-fold) and BDNF (0.7-fold) compared to those in the normal group. By contrast, pectolinarin at 1, 2.5, and 5 μg/mL significantly increased TrkB (0.7-fold, 1.1-fold, and 1.3-fold, respectively) and BDNF (0.8-fold, 1.2-fold, and 1.4-fold, respectively) expression following the treatment of $A\beta_{25-35}$ in a concentration-dependent manner. The result suggests that pectolinarin could be used for protecting

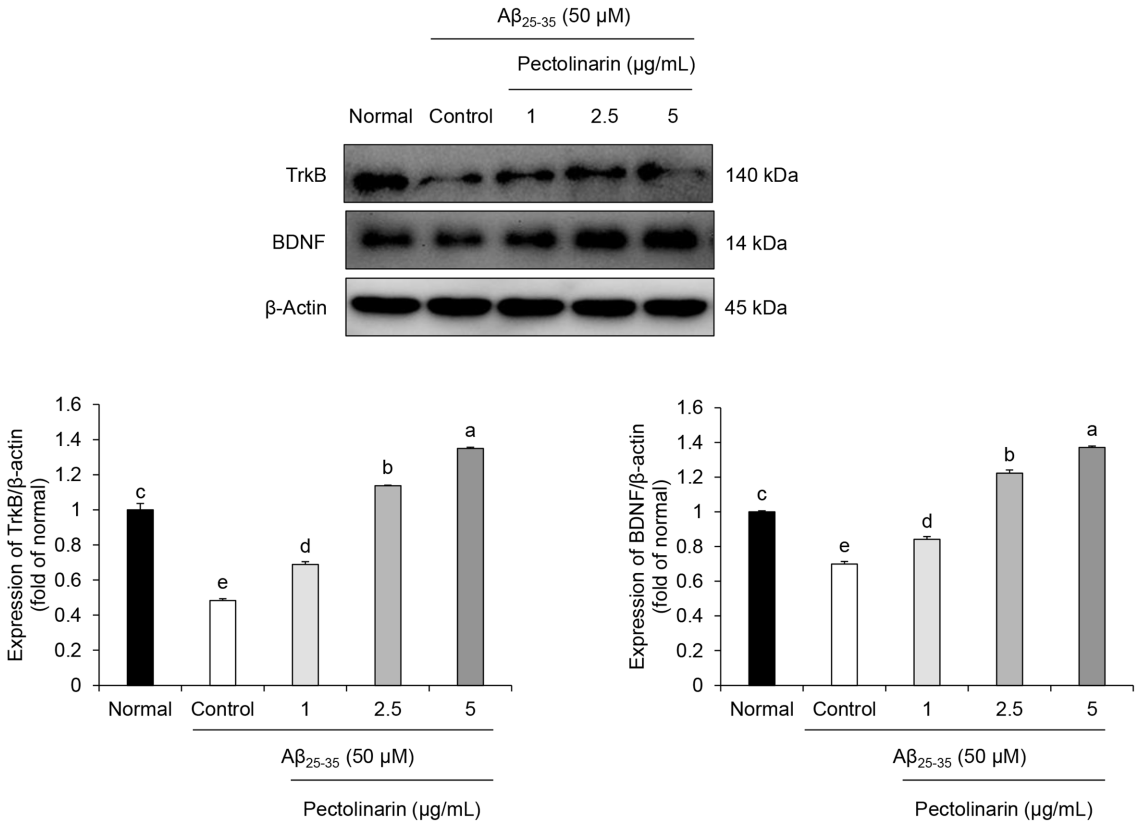

Fig. 6. Effect of pectolinarin on synaptic dysfunction in $A\beta_{25-35}$-treated SH-SY5Y cells. Values are mean ± SD (n = 3). β-actin was used as a loading control. Results of one-way ANOVA test with Duncan multiple range tests for multiple comparisons at $p < 0.05$. Means with the different letters (a–e) are significantly different ($p < 0.05$) from each other. Means with the same letter are not significantly different. TrkB, Tropomyosin receptor kinase B; BDNF, brain-derived neurotrophic factor.

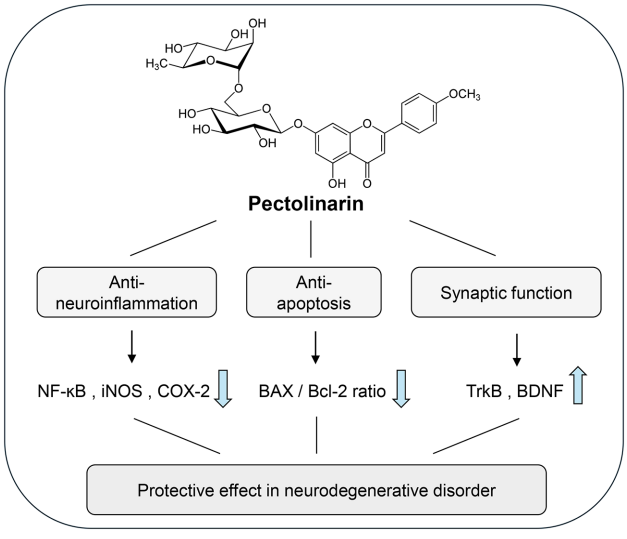

Fig. 7. Schematic diagram of the mechanism by which pectolinarin protects SH-SY5Y cells against $A\beta_{25-35}$.

$A\beta_{25-35}$-mediated synaptic dysfunction in SH-SY5Y cells.

In conclusion, the neuroprotective effects of pectolinarin in $A\beta_{25-35}$-induced SH-SY5Y cells were investigated, and their underlying mechanisms related to neuroinflammation and apoptosis (Fig. 7). Pectolinrain effectively inhibited $A\beta_{25-35}$-mediated inflammation by regulating the NF-κB signaling pathway. Moreover, pectolinarin exerts protective actions against $A\beta_{25-35}$-induced neuronal apoptosis and synaptic dysfunction through regulating the BAX/Bcl-2 ratio and BDNF/TrkB signaling, respectively. These findings suggest that pectolinarin could serve as a potential therapeutic agent in neurodegenerative disorders, including AD.

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Conflict of interest

The authors declare that there is no conflict of interest.

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