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Adlay hull extracts attenuate β-amyloid-induced neurotoxicity and oxidative stress in PC12 cells through antioxidative, anti-inflammatory, and antiapoptotic activities

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ABSTRACT

Alzheimer's disease (AD) is characterized by accumulation of β-amyloid (Aβ) in senile plaques, contributing to oxidative stress, mitochondrial diseases, and synaptic atrophy, consequently leading to the deterioration of brain function. Adlay (*Coix lacryma-jobi* L.) is an annual botanical. Here, a 95% ethanol extract of adlay hull (AHEE) was partitioned by ethyl acetate (AHEAE), *n*-butanol (AHBUE), and water (AHWE), and the effects of these extracts on lipopolysaccharide (LPS)-induced RAW264.7 cells and Aβ-induced PC12 cells, as experimental models of neurotoxicity, were evaluated. The expression of anti-inflammatory and antiapoptosis-related proteins was investigated and AHEE, AHEAE, and AHWE were found to exert anti-inflammatory effects. AHWE exhibited antiapoptotic effects and inhibited inducible nitric oxide synthase expression and nitric oxide production. We investigated the protective effects of AHWE against Aβ-induced neurotoxicity in dPC12 cells and explored the underlying mechanism. Pretreatment with AHWE significantly attenuated cell death and Aβ-mediated increase in B cell lymphoma (Bcl)-2/Bax ratio. AHWE significantly inhibited Aβ and enhanced protein kinase B (Akt) level in dPC12 cells, suggesting that its protective effect against Aβ-induced apoptosis in dPC12 cells was mediated through upregulation of the phosphoinositide 3-kinases (PI3K)/Akt signaling pathway. These extracts and its bioactive compound K36–21 may be potentially useful to treat neurodegenerative disorders.

1. Introduction

Alzheimer's disease (AD) is an age-related neurodegenerative disorder and the most common cause of dementia in the elderly population. It causes memory loss and cognitive decline, which strongly interferes with normal daily functions. AD is a neurodegenerative disorder associated with protein misfolding and aggregation [1]. The generation of oxygen free radicals, reactive oxygen species (ROS), and oxidative stress is thought to be associated with the pathogenesis of neurodegenerative disorders [2,3]. Neurodegenerative diseases are characterized with apoptosis/necrosis and neuronal cell dysfunction, leading to malignant effects on the neural system [4]. AD manifests as the deposition of protein aggregates, extracellular amyloid plaques (Aβ), intracellular tau (τ), neurofibrillary tangles (NFTs), and loss of synaptic connections in specific regions of the brain [5–7]. Growing evidence suggests a link between the polymorphic nature of Aβ oligomers and fibrils and the specific mechanisms underlying neurodegeneration [8]. β-amyloid peptides and their aggregates are the main components of senile

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plaques, and evidence from *in vitro* and *in vivo* experiments have revealed the contribution of Aβ-induced neurotoxicity to the pathogenesis and progression of AD [9,10]. Aβ is neurotoxic to neural cells both *in vivo* and *in vitro* [11,12]. Exposure of neural cells to Aβ in the culture medium caused neurotoxicity by increasing cellular oxidative stress and apoptosis [13,14]. Aβ protein-targeted therapeutic strategies are, thus, promising disease-modifying approaches for the treatment and prevention of AD [15]. Acetylcholine esterase (AChE) is known to promote the formation of Aβ fibrils and plaques [16], and could be a potential therapeutic target for the treatment of AD and other diseases. It participates in the breakdown of acetylcholine (ACh) neurotransmitters and in ACh-mediated neurotransmission. Acetylcholine esterase inhibitors (AChEIs) are the most effective candidates for treating AD [17]. Therefore, inhibition of AChE and Aβ-induced neuronal apoptosis may serve as a possible approach for AD prevention and treatment.

Adlay (*Coix lacryma-jobi L. var. ma-yuen* Stapf) is an annual herb of the Gramineae Coix genus and is often used as an edible or medicinal grain in Asian countries. Adlay has long been used in traditional Chinese medicine (TCM) and as a nourishing food for the treatment of warts, chapped skin, rheumatism, and neuralgia. The knowledge of the phytochemical profiles and antioxidant activity of adlay may provide an insight into its potential beneficial health effects [18]. Recent studies have indicated that adlay is rich in phenolic compounds, including ferulic acid, rutin, *p*-coumaric acid, and quercetin, which contribute to antioxidant functions [19]. In addition, studies have shown that the fraction with high phenolic and flavonoid contents from ethanol extracts of adlay bran could suppress lipopolysaccharide (LPS)-stimulated interleukin (IL)-6 and tumor necrosis factor (TNF)-α secretion in a concentration-dependent manner in RAW 264.7 cells and murine peritoneal macrophages [20]. Other studies have shown that adlay extracts and their physiological ingredients have antioxidative [21,22], anti-inflammatory [23,24], antiproliferative [25], and antitumor activities [26]. We have previously shown that eriodictyol and ceramide from adlay hull extracts exerted anti-inflammatory properties in RAW264.7 macrophages [27]. However, the effects of adlay hull extracts on Aβ peptide-mediated oxidative damage and neuroinflammation in neuronal cells are unknown.

In this study, we investigated the protective effects of adlay hull extracts against Aβ peptide-induced oxidative stress by assessing free radical-scavenging activity, cell viability, and inflammatory mediator levels. This study used a cellular model to investigate the physiological changes and the possible mechanism underlying the effects of adlay hull agricultural waste on neurodegenerative diseases caused by Aβ peptide-induced oxidative stress and neuroinflammation [28]. We hope that this study highlights the multifunctional nutritional and health benefits of adlay hull, and innovates the value of agricultural products to facilitate the reuse of agricultural waste for the development of sustainable agriculture. In the future, there is an opportunity to develop agricultural waste into neuroprotective health food or drugs for AD treatment [29].

2. Materials and methods

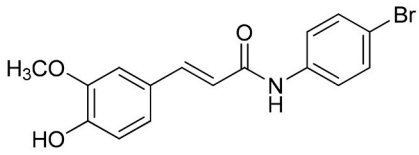
2.1. Experimental sample preparation

The experimental samples were taken from the Taichung No. 3 adlay seed of the Caotun Adlay Production Cooperative in Nantou County, Taiwan. The seed coat was removed using a screening machine to obtain the adlay hull, as shown in Fig. 1a. The obtained adlay hull was powdered with a grinder for extraction. In the extraction method, the adlay hull powder was soaked in 95% ethanol for 3 days to obtain AHEE. After suction and filtration, ethanol was removed using a rotary evaporator. The sample was re-dissolved in deionized water and placed in a separatory funnel. Ethyl acetate and *n*-butanol were partitioned, extracted, and then concentrated under rotary evaporator to obtain AHEAE, AHBUE, and AHWE. The obtained organic extracts were re-dissolved in absolute alcohol to prepare stocks, while the aqueous extract was re-dissolved in phosphate-buffered saline (PBS) to prepare stocks. The extracts were stored at −20 °C until experimental analysis.

2.2. Chemicals

The compounds 2,2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid, LPS (*E. coli* serotype 0127-8B), dexamethasone (DXMS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), huperzine A, and Aβ peptide were purchased from Sigma (Sigma-Aldrich Corporation, St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO), ethanol or double-distilled water at appropriate concentrations. After preparation, the solutions were stored at −20 °C until use. Stock solutions were diluted to desired concentrations immediately before use. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), nerve growth factor (NGF), L-glutamine, antibiotics (penicillin and streptomycin), and trypsin-ethylenediaminetetraacetic acid (EDTA) were procured from Gibco BRL (Thermo Fisher Scientific Corporation, Grand Island, NY, USA). Acetylthiocholine iodide (ATCh) and 5, 5-dithio-bis-2-nitrobenzoic acid (DTNB) were obtained from AAT Bioquest® Inc. (Sunnyvale, CA, USA). In addition, a synthetic caffeamide derivative (K36-21) was derived from Professor Kuo's laboratory using the amide-binding coupling method, as previously described [30,31], and its chemical structure is shown in Fig. 1b. It is a structural analog of ceramide, the bioactive ingredient of the adlay hull, primarily responsible for the anti-inflammatory activity [27].

(a)



(b)

Fig. 1. (**a**) Experimental sample of adlay hull. (**b**) Chemical structure of a synthetic caffeamide derivative (K36-21), (*E*)-*N*-(4-bromophenyl)-3-(4-hydroxy-3-methoxyphenyl) prop-2-enamide.

2.3. Determination of antioxidative capacity

2.3.1. Total phenol content measurement

The Folin-Ciocalteu assay was used to evaluate the total phenol content in samples. Each sample was diluted with ethanol to 2 mg/mL concentration, and 50 μL of the diluted sample was added to 50 μL Folin-Ciocalteu phenol reagent, mixed well, and treated with 700 μL deionized water. The reactants were mixed and allowed to stand for 3 min, and then treated with 200 μL of a 20% sodium carbonate (Na_2CO_3) solution in a water bath at 100 °C for 1 min in the dark. The sample was allowed to cool and its absorbance was measured at 700 nm wavelength. Using the same method, different concentrations of gallic acid were used as samples, and the absorbance value changes were calculated to obtain the regression equation of the standard curve. The absorbance value of the sample was substituted to calculate gallic acid equivalent.

2.4. DPPH radical-scavenging activity

The experimental samples were diluted with ethanol to concentrations of 0.25, 0.5, 1, and 2 mg/mL, and the same concentration of gallic acid was used as the control group. The DPPH solution was mixed with all samples and gallic acid at a ratio of 1:4, and the reaction was protected from light. After 30 min, an enzyme-linked immunosorbent assay (ELISA) reader (Molecular Devices, USA) was used to detect changes in absorbance at a wavelength of 517 nm. The lower the absorbance value, the stronger is the ability of the sample to remove DPPH.

2.5. Cell culture and treatment

The murine macrophage cell line RAW264.7 was obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA) and cultured in DMEM supplemented with 2 mM L-glutamine, antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin), and 10% FBS at 37 °C and 5% CO_2. The cells were treated with adlay hull extracts and the bioactive compound 36-21 in the presence of LPS (100 ng/mL) for 24 h to further evaluate their anti-inflammatory effects.

Another cell line PC12, was obtained from ATCC (Rockville, MD, USA) and maintained in 85% Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 10% heat-inactivated horse serum and 5% FBS, 100 units/mL penicillin and 100 μg/mL streptomycin. These cells were differentiated with 50 ng/mL NGF in RPMI medium with 1% FBS and penicillin/streptomycin (1% FBS-NGF medium) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

To prepare the aggregated $A\beta_{25\text{-}35}$ and $A\beta_{1\text{-}42}$ fibrils, Aβ were dissolved in double-distilled water and incubated at 37 °C for 7 days to induce its aggregation, respectively [12]. After aggregation, the stock solutions (20 mM) were stored at −20 °C until use. The solutions were diluted to desired concentrations immediately with medium prior to use. The medium was replaced every 3 days, and PC12 cells were differentiated for 7–10 days before treatment. In all experiments, d-PC12 cells were seeded in 24-well plates (1×10^5 cells/well). After 24 h, the cells were pretreated with or without adlay hull extracts and bioactive compound K36-21 for 24 h, followed by incubation with $A\beta_{1\text{-}42}$ (20 μM) for an additional 24 h to evaluate the neuroprotective effect.

2.6. Cell viability test

Cell viability was estimated by the MTT reduction assay, which is based on the conversion of MTT to formazan crystals by mitochondrial dehydrogenases. Following incubation for 24 h at 37 °C and 5% CO_2, the supernatant was removed and each well was treated with 100 μL DMSO to dissolve the formazan crystals. Absorbance was measured using ELISA reader at a wavelength of 570 nm. Cell viability was normalized to relative percentages as compared with untreated controls.

2.7. Determination of anti-inflammatory capacity

Confluent RAW264.7 cells were trypsinized, diluted with culture medium to 4×10^5 cells/mL, and seeded in a 96-well plate in 100 μL volume. The cells were cultured in a 37 °C, 5% CO_2 incubator for 18–24 h. The original medium was replaced and the test sample was treated with LPS (100 ng/mL) in a total volume of 100 μL/well. After incubating for 18 h in an incubator, the supernatant from each well was transferred to another 96-well plate and treated with mixed Griess reagent (100 μL/well) for 10 min in the dark. An ELISA reader was used to detect changes in absorbance at 540 nm. The absorbance was measured against a standard curve to calculate the NO content. Assays were performed independently in triplicates.

2.8. Antioxidative enzyme activity analysis

2.8.1. SOD activity determination

After collecting a certain number of cells, 1 mL of cell lysate (20 mM HEPES buffer, pH 7.2, containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose) was centrifuged at 1500×*g* for 5 min, and the supernatant was collected at 4 °C for use. Later, 200 μL diluted radical detector and 10 μL sample supernatant were added to each well of a 96-well plate, and mixed with 20 μL of diluted xanthine oxidase to stop the reaction at 25 °C for 30 min. The absorbance of each well was measured at 450 nm using ELISA reader. Different concentrations of SOD stocks were used to calculate the absorbance value change of the standard to obtain the regression equation of the standard curve, and the absorbance value of the sample was substituted to obtain SOD activity.

2.8.2. CAT activity determination

After collecting a certain number of cells, 1 mL of cell lysate (50 mM potassium phosphate, pH 7.0, containing 1 mM EDTA) was centrifuged at 10,000×*g* for 15 min and the supernatant was collected at 4 °C for further use. In a 96-well plate, 100 μL assay buffer, 30 μL methanol, and 20 μL samples were added to each well. The reaction was started by quickly adding 20 μL of hydrogen peroxide (H_2O_2) to each well. The plate was shaken at room temperature for 20 min to initiate the reaction. Then, 30 μL potassium hydroxide (KOH) and 30 μL CAT were added, and the mixture was shaken at room temperature for 10 min. Each sample was then treated with 10 μL of catalase potassium periodate to stop the reaction, shaken well at room temperature for 5 min, and analyzed at 540 nm wavelength using ELISA reader. The regression equation of the standard curve was calculated using the absorbance change of different concentrations of CAT stocks as the standard. The absorbance of the sample was then substituted to obtain CAT activity.

2.9. AChE activity analysis

The levels of AChE in the culture medium were determined using Amplite™ Colorimetric assay kits (AAT Bioquest®) according to the manufacturer's protocol. The supernatant was used as an enzyme source to measure AChE activity. In brief, 50 μL enzyme and 50 μL ATCh (5 mM) were incubated at 30 °C for 15 min, and then the reaction was terminated with 0.9 mL of 0.125 mM DTNB phosphate-ethanol reagent as a thiol indicator. Color change was immediately detected at 412 nm using ELISA reader. Huperzine A is a sesquiterpene alkaloid derived from the Chinese firmoss *Huperzia serrata*. It is an effective AChE inhibitor that can cross the blood-brain barrier and has recently been used to treat AD and other forms of dementia [32]. It was used as a positive control in this analysis.

2.10. Western blot analysis

Treated or untreated cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris-HCl, 150 mM sodium chloride [NaCl], 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate

[SDS], pH 7.6, Sigma-Aldrich) to prepare protein extracts, and protein concentrations were determined using the Bradford protein assay method. Equal amounts of total cell proteins were loaded and separated by 10% SDS polyacrylamide gel electrophoresis (PAGE) and transferred onto polyvinylidene fluoride membranes by electroblotting. After blocking in 5% non-fat milk for 1 h at room temperature, the membranes were probed with primary antibodies (*anti*-iNOS, *anti*-Bcl-2, *anti*-Bax, *anti-p*-Akt, anti-PI3K; 1:1000, Santa Cruz) for overnight at 4 °C. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin (1:2000, Abcam) were used as controls for protein loading in each lane. The blots were washed, incubated with a horseradish peroxidase-conjugated secondary antibody (1:10000) for 2 h at room temperature, and then subjected to autoradiography using an enhanced chemiluminescence (ECL) western blotting detection kit (Millipore, USA). Signals were visualized using a chemiluminescence image analysis system to develop color (Bio-Rad, USA).

2.11. Statistical analysis

Data are presented as mean ± standard deviation (SD) of three replicates. Values of $P < 0.05$ were considered as statistically significant by one-way analysis of variance with Duncan's multiple range test using SPSS (Statistics Package Social Science) software®. A Student's *t*-test was performed to analyze differences between groups, and $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ indicated statistically significant difference.

3. Results

3.1. Yields of different extraction fractions of adlay hull

A sample of 6105 g of adlay hull was dried, ground into powder, and extracted with ethanol to obtain 12.21 g of ethanol extract (AHEE), corresponding to a yield of 0.2%. The ethanol extract was subjected to partition extraction to obtain 5.08 g ethyl acetate fraction extract (AHEAE), 1.46 g n-butanol fraction extract (AHBUE), and 4.21 g water fraction extract (AHWE) at a yield of 41.6%, 12.0%, and 34.5%, respectively (Table 1).

3.2. Antioxidative effects of adlay hull extracts

The results of total phenol content of different extracts of adlay hull are shown in Fig. 2a. The content of total phenol was significantly higher in AHBUE than in other extracts. Each gram of AHBUE contained 70.86 mg gallic acid equivalent of phenol. The results of DPPH free radical-scavenging activity of different adlay hull extracts are shown in Fig. 2b. The scavenging ability of AHEAE, AHBUE, or AHWE was equal to that of the positive control gallic acid at a concentration of 2 mg/mL, while AHEAE and AHBUE could scavenge free radicals at low concentrations of 0.5 and 1 mg/mL, respectively.

Table 1
Yields of different adlay hull extracts and partition fractions.

Item	Original sample weight (g)	Net weight of extract (g)	Yield[1] (%)
AHEE	6105	12.21	0.2
AHEAE[2]	12.21	5.08	41.6
AHBUE[2]	12.21	1.46	12.0
AHWE[2]	12.21	4.21	34.5

AHEE: Adlay hull ethanol extract; AHEAE: AHEE partitioned with ethyl acetate; AHBUE: AHEAE partitioned with *n*-butanol; AHWE: AHBUE partitioned with water.

[1] Yield (%) = (net weight of extract/original sample weight) × 100.

[2] Means the extracts were partitioned from AHEE.

3.3. Anti-inflammatory effect of adlay hull extracts in RAW264.7 cells

LPS (100 ng/mL) was used to induce inflammation in RAW264.7 cells, which were then treated with different extracts of adlay hull to observe the production of nitric oxide (NO), an inflammatory mediator, and their effects on cell survival. All adlay hull extracts inhibited the formation of NO. The relative NO levels were 38.2%, 52.3%, 70.4%, and 59.1%, after treatment with 100 μg/mL of AHEE, AHEAE, AHBUE, and AHWE, respectively (Fig. 3a). AHEE had the best anti-inflammatory effect, which was equal to that of the anti-inflammatory drug dexamethasone (DXMS). AHEE significantly reduced the expression of inducible nitric oxide synthase (iNOS) protein, and its inhibitory effect was better than that of the positive control DXMS. Among the different extracts of adlay hull, AHEE was the most potent in reducing iNOS protein expression, followed by AHEAE, AHWE, and AHBUE. The results are shown in Fig. 3b.

3.4. Neuroprotective effect of adlay hull extracts in d-PC12 cells

To compare the cytotoxic effects of $A\beta_{1\text{-}42}$ and $\beta_{25\text{-}35}$ fragments on PC12 cells, we performed the MTT assay and found that treatment with 20 μM $A\beta_{1\text{-}42}$ for 24 h induced significantly higher damage to d-PC12 cells. The result of cell survival rates are shown in Fig. 4a. Therefore, $A\beta_{1\text{-}42}$ (20 μM) was used to induce neurotoxicity in d-PC12 cells, which were then treated with different extracts of adlay hull for 24 h to evaluate its neuroprotective effect. The effect is shown in Fig. 4b, which reveals that both AHBUE and AHWE protected neuronal cells at a concentration of 200 μg/mL, and that their protective effects were better than those of AHEE and AHEAE.

3.5. Effect of adlay hull extracts on $A\beta_{1\text{-}42}$-induced antioxidative enzymes in d-PC12 cells

To determine whether the protective effects of adlay hull extracts on neuronal cells were associated with the activity of antioxidative enzymes, the levels of superoxide dismutase (SOD) and catalase (CAT) were assessed. Exposure of cells to $A\beta_{1\text{-}42}$ (20 μM) for 24 h led to a decrease in the levels of SOD and CAT to 40.5% and 52.3%, respectively, as compared to that observed in vehicle treatment group. The pretreatment of d-PC12 cells with different adlay hull extracts for 24 h led to a significant increase in the levels of antioxidative enzymes. The SOD activities of cells treated with different adlay hull extracts are shown in Fig. 5a. AHBUE treatment group had the highest SOD activity at a concentration of 200 μg/mL, and the SOD level was 84.5% of the vehicle group. The CAT activities of cells treated with different adlay hull extracts are shown in Fig. 5b. AHEE and AHBUE groups had higher CAT activity at 200 μg/mL, and the levels in these groups were 90.1% and 92.2%, respectively, of the vehicle group.

3.6. Effect of adlay hull extracts on antiapoptotic protein expression in d-PC12 cells

The effects of different adlay hull extracts on phosphatidylinositol 3-kinase (PI3K), p-protein kinase B (Akt), B cell lymphoma (Bcl)-2, and Bax protein expression levels in $A\beta_{1\text{-}42}$-treated PC12 cells were analyzed by western blotting and are shown in Fig. 6a. The expression of PI3K protein was significantly upregulated in d-PC12 cells after treatment with different adlay hull extracts. In addition, AHWE activated PI3K protein and showed the best effect. The expression of *p*-Akt protein in AHWE-treated d-PC12 cells was significantly higher than that in the control group. AHWE was the most potent in activating *p*-Akt protein. The expression of Bcl-2 in AHWE-treated d-PC12 cells was significantly higher than that in the control group. AHWE treatment induced maximum activation of Bcl-2. Bax protein expression was significantly downregulated in AHWE-treated d-PC12 cells as compared to that in the control group. The best inhibitory effect on Bax protein expression was

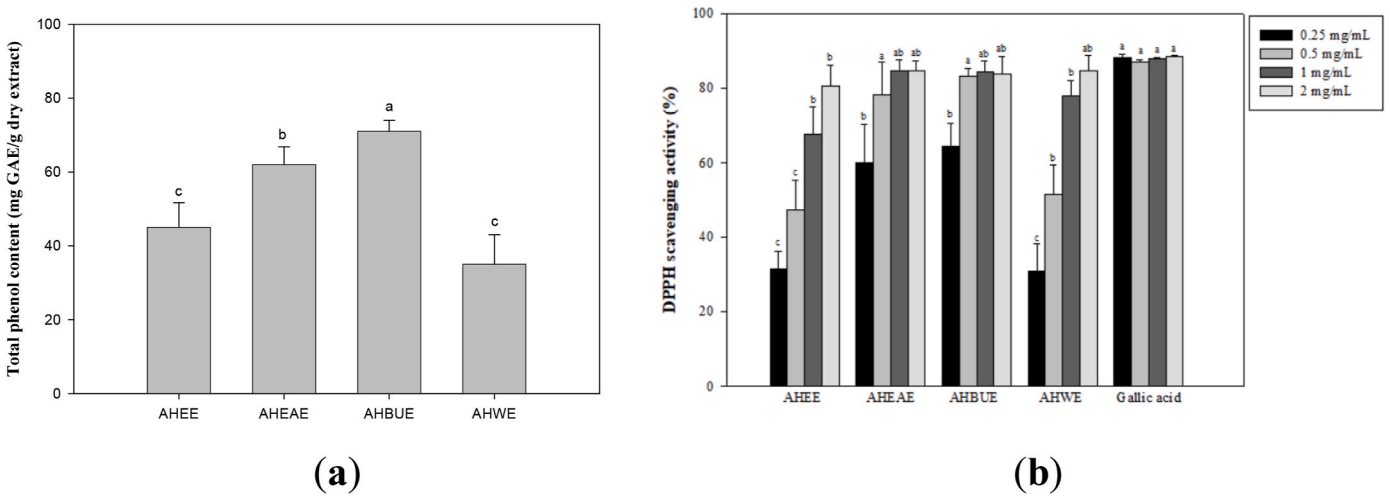


Fig. 2. (**a**) Total phenol contents of adlay hull extracts. (**b**) DPPH radical-scavenging activities of adlay hull extracts. Values are means ± SD (n = 3), as analyzed by one-way ANOVA followed by Duncan's new multiple range test. Superscript letters indicate significant differences ($P < 0.05$). *GAE*: gallic acid equivalent.

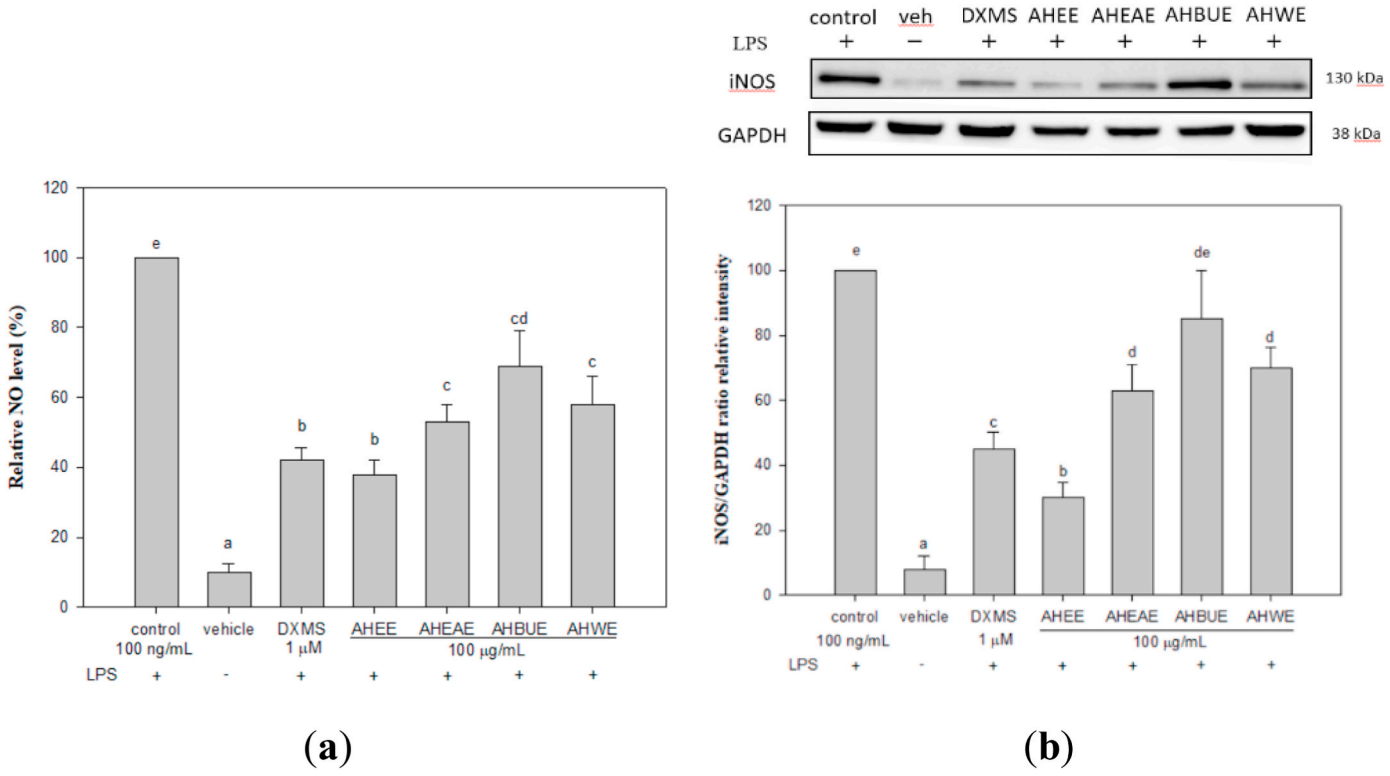


Fig. 3. Effect of adlay hull extracts on nitric oxide production (**a**) and iNOS protein expression (**b**) in RAW264.7 cells. Values are means ± SD (n = 3), as analyzed using one-way ANOVA followed by Duncan's new multiple range test. Bars that do not share a common letter are significantly different ($P < 0.05$) from each other. Dexamethasone (DXMS) at 1 μM concentration was used as a positive control; RAW264.7 cells were treated with 100 ng/mL LPS to induce inflammation.

observed with AHWE, as shown in Fig. 6b. After exposure to 20 μM $A\beta_{1\text{-}42}$ for 24 h, the ratio of Bcl-2 to Bax reduced to 71% of that observed after vehicle treatment. The pretreatment of d-PC12 cells with different adlay hull extracts for 24 h led to a significant increase in the ratio of Bcl-2 to Bax as compared with that in the control treatment, as shown in Fig. 6c. AHWE had the best antiapoptotic effect.

3.7. Inhibitory effect of adlay hull ethanol extract and its bioactive compound on AChE activity in d-PC12 cells

The inhibitory effect of AHEE and its bioactive compound K36-21 on AChE activity is shown in Table 2. Huperzine A is an effective AChEI that can cross the blood-brain barrier, and was used as a positive control. AChE activity was inhibited by 81.3% (±0.86%) following treatment with 100 mg/mL AHEE. Within a certain concentration range, the inhibition rate showed a dose-dependent effect that was significantly different from the effect observed with the positive control huperzine A. AChE activity was inhibited by 65.8% (±3.68%) at 25 mg/mL concentration of the bioactive compound K36-21, and this effect increased to 89.7% (±0.71%) at 100 mg/mL concentration. The AChE inhibitory activity of K36-21 was better than that of AHEE.

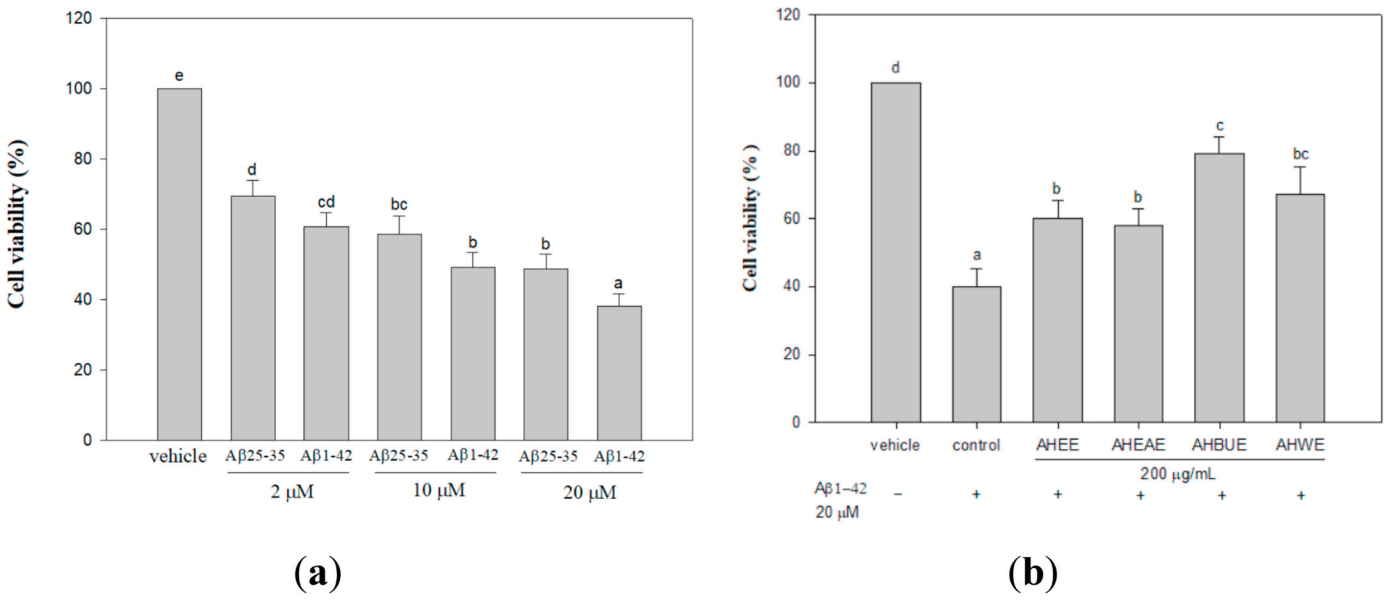


Fig. 4. Effects of different β-amyloid fragments on the viability of cells treated with different concentrations (**a**). Neuroprotective effect of adlay hull extracts against $A\beta_{1\text{-}42}$-induced cytotoxicity in d-PC12 cells (**b**). The cells were treated with 20 μM $A\beta_{1\text{-}42}$ for 24 h to induce neurotoxicity. Subsequently, cell viability was determined by the MTT assay. Values are means ± SD (n = 3), as analyzed using one-way ANOVA followed by Duncan's new multiple range test. Bars that do not share a common letter are significantly different ($P < 0.05$) from each other.

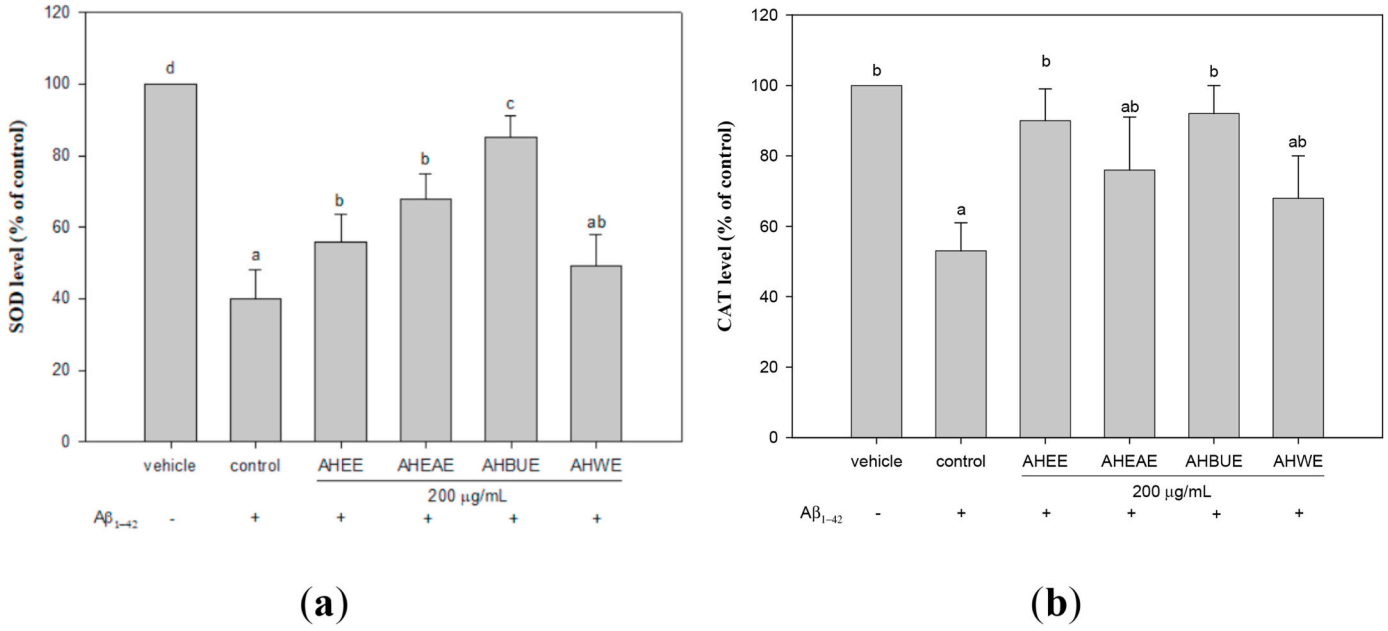


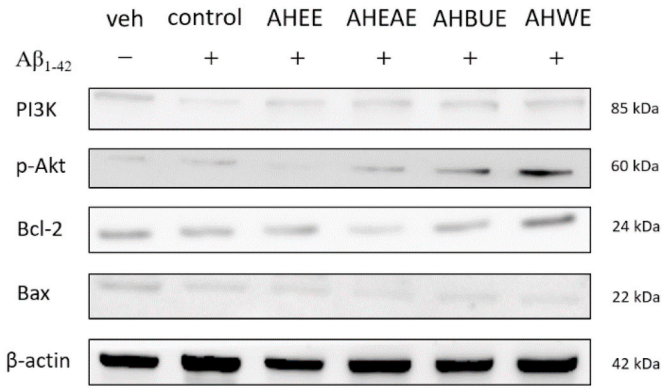
Fig. 5. Effects of adlay hull extracts on the activities of antioxidative enzymes SOD (**a**) and CAT (**b**) in d-PC12 cells. Values are means ± SD (n = 3), as analyzed using one-way ANOVA followed by Duncan's new multiple range test. Bars that do not share a common letter are significantly different ($P < 0.05$) from each other. Cells were exposed to 20 μM $A\beta_{1\text{-}42}$ for 24 h to induce oxidative damage.

4. Discussion

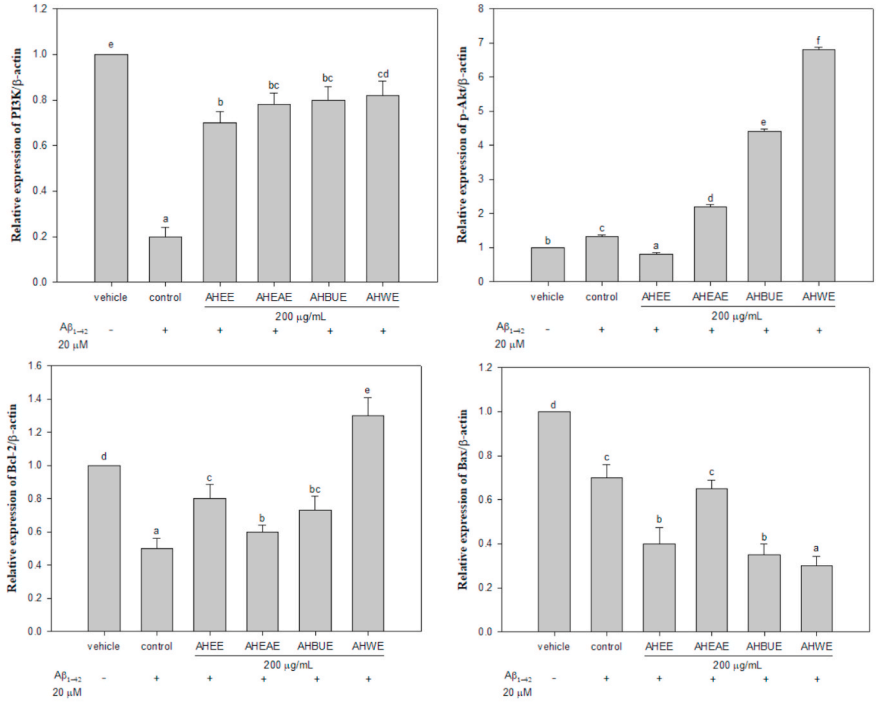
4.1. Antioxidative abilities of different extracts of adlay hull

The analysis of the *in vitro* antioxidative capacity of different adlay hull extracts showed that AHBUE fraction had significantly better activity than that did the other fractions in terms of scavenging of DPPH free radicals and total phenolic content. The adlay hull, which covers the seed, plays a major role in the physical and chemical defense system of seeds. Adlay is rich in phenolic acids such as gallic acid, chlorogenic acid, *p*-hydroxybenzoic acid, vanillic acid, *p*-coumaric acid, and ferulic acid, which are widely accepted as important antioxidant compounds [33,34]. Adlay also contains other active ingredients such as coniferyl alcohol, triolein, rutin, syringaresinol, 4-ketopinoresinol, campesterol, β-sitosterol, coixol, *p*-hydroxybenzaldehyde, and syringaldehyde [35]. Phenolic compounds and flavonoids can provide hydrogen ions in chemical reactions and inhibit the oxidation of free radicals, thereby contributing to the process of antioxidation. Coniferyl alcohol, syringic acid, ferulic acid, syringaresinol, 4-ketopinoresinol, and mayuenolide have been identified as DPPH-scavenging active components in adlay hull [21]. The total phenolic and flavonoid contents of AHBUE were significantly higher than those of the other extracts, and together these

(**a**)



(**b**)



(**c**)

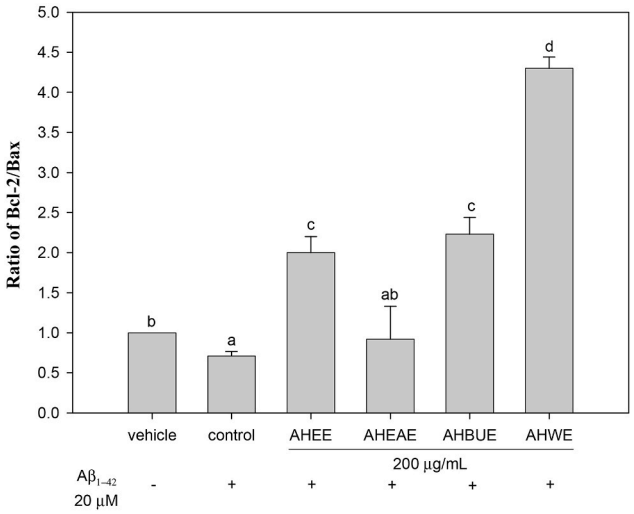


Fig. 6. Effects of adlay hull extracts on PI3K, *p*-Akt, Bcl-2, and Bax protein expression in $A\beta_{1\text{-}42}$-treated PC12 cells was analyzed by western blotting (**a**) and are quantitatively shown (**b**). Quantification of Bcl-2/Bax ratio in d-PC12 cells (**c**). Total cellular protein (20 μg/lane) was separated on a 10% SDS-PAGE, transferred to a PVDF membrane, and stained with antibodies. Values are means ± SD (n = 3), as analyzed using one-way ANOVA followed by Duncan's new multiple range test. Bars that do not share a common letter are significantly different ($P < 0.05$) from each other. The cells were pretreated with the indicated concentrations of adlay hull extracts for 24 h, followed by exposure to $A\beta_{1\text{-}42}$ (20 μM) for an additional 24 h to induce neurotoxicity.

Table 2
Inhibitory effect of adlay hull ethanol extract and the bioactive compound K36-21 on AChE activity.

Sample	Concentration (mg/mL)	AChE inhibition (%)
AHEE	100	81.3 ± 0.86**
	50	70.2 ± 2.25**
	25	48.9 ± 2.90***
K36-21	100	89.7 ± 0.71*
	50	77.4 ± 2.33*
	25	65.8 ± 3.68**
Huperzine A[1]	100	97.8 ± 0.52
	50	91.3 ± 1.33
	25	86.6 ± 2.69

[1] Huperzine A was used as positive control. Values are means ± SD (n = 3), and statistical analysis was performed using the Student's *t*-test. Values significantly different as compared to the positive control, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

compounds exhibited effective antioxidative functions. We also found that AHBUE increased the expression of antioxidative enzymes SOD and CAT in d-PC12 cells, probably owing to its high phenolic and flavonoid contents.

4.2. Anti-inflammatory functions of different extracts of adlay hull

Neuroinflammation has been identified as an important process in the pathogenesis of AD [36]. A previous study reported the action of flavonoids on the enzymes and pathways involved in anti-inflammatory processes [[37]]. Flavonoids in adlay bran contribute to anti-inflammatory effects in RAW264.7 cells and murine peritoneal macrophages [20]. This study showed that adlay hull extracts inhibited NO production and that AHEE was the most potent fraction in inhibiting NO production at low concentrations such that its activity was equal to that of the anti-inflammatory drug DXMS. It is important to note that iNOS plays an important role in inflammation, and is mostly expressed in macrophages, lymphocytes, and vascular smooth muscle cells. It synthesizes a large amount of NO upon stimulation with LPS [[38]], causes cell damage and excessive vasodilation, and finally induces severe inflammation and complications. A previous study confirmed the presence of six phenolic compounds in the adlay hull that exhibited strong free radical-scavenging activities [21]. Among these antioxidative components, coniferyl alcohol and 4-ketopinoresinol could significantly inhibit iNOS protein expression in RAW264.7 cells. In this study, we found that AHEE and extract fractions could inhibit iNOS protein expression. Therefore, we speculate that the anti-inflammatory bioactive ingredients in adlay hull are coniferyl alcohol and 4-ketopinoresinol.

4.3. Antiapoptotic and neuroprotective abilities of different extracts of adlay hull

AD is a neurodegenerative disease characterized with two neuropathological hallmarks, extracellular deposition of amyloid plaques and intracellular neurofibrillary tangles [[39]]. The deposition of β-amyloid leads to the formation of free radicals, causing initial inflammation and damage and consequently nerve cell death. The Aβ-induced neuronal cell death has been shown to occur by several modes such as apoptosis, necrosis, and necroptosis [[40]]. Aβ has been implicated as a key molecule in the neurodegenerative cascades of AD. Aβ directly induces neuronal apoptosis, suggesting the important role of Aβ-induced neurotoxicity in AD neurodegeneration. The neuroprotective phenolic compounds exhibit a strong antioxidant potential and may neutralize the toxic effects of Aβ and protect nerve cells from neurotoxin-induced apoptosis. Resveratrol was shown to reduce apoptosis, decrease oxidative status, and alleviate mitochondrial damage in Aβ-treated PC12 cells [[41]]. We used $A\beta_{1\text{-}42}$ to induce neurotoxicity in d-PC12 cells, simulating the pattern of nerve cell damage in AD. We found that AHBUE and AHWE had the best neuroprotective effects and excellent antioxidant enzyme activities. We speculate that the active ingredients in this sample that protected nerve cells are phenolic compounds and flavonoids [[42]].

The main molecular components involved in nerve cell apoptosis [[43]] are the family of apoptotic proteins (caspase), Bcl-2 [[44]] protein family, and apoptotic protease activating factor 1 (Apaf-1) [[45]]. The PI3K signaling pathway promotes cell survival and has been reported to participate in apoptosis in the central nervous system. Akt, a serine/threonine protein kinase, is the primary protein effector downstream of the PI3K signaling pathway [[46]]. Neurotrophic factors regulate cell apoptosis key protein kinase signals, such as the PI3K/Akt and mitogen-activated protein kinase (MAPK) pathways [[47]]. In neurodegenerative diseases, similar cell death signaling pathways are activated by abnormal structural proteins such as β-amyloid peptides [[48]]. The exact mechanism of action of adlay hull extract against Aβ-induced neurotoxicity is yet unclear but thought to be related to the inhibition of the PI3K/Akt signaling pathway [[49]]. Studies have suggested that the activation of the PI3K/Akt signaling pathway may prevent Aβ-induced neurotoxicity [[50]]. The ratio of Bcl-2 to Bax is known to be correlated with apoptosis [[51]]. To investigate the neuroprotective mechanisms of adlay hull extracts, the ratio of Bcl-2/Bax in d-PC12 cells was measured by western blotting, and AHWE was found to significantly activate PI3K/Akt signaling, upregulate the expression of the antiapoptotic protein Bcl-2, and suppress the expression of the pro-apoptotic protein Bax. These effects may result from the combined actions of different compounds, indicating the possibility of a matrix effect from the phenolic compounds and flavonoids in adlay hull extracts.

In addition, we found the novel compound K36-21, a structural analog of ceramide (the bioactive ingredient of adlay hull), with antioxidative, anti-inflammatory, and AChE inhibitory effects. According to the above results, adlay hull extracts activated the PI3K/Akt pathway and upregulated the Bcl-2/Bax ratio that prevented $A\beta_{1\text{-}42}$-induced apoptosis of d-PC12 cells, thereby serving as potential therapeutic agents for neurodegenerative diseases. Future studies with animal experiments (including transgenic AD mouse model) will be informative and helpful for understanding the underlying pathogenic mechanism.

5. Conclusions

In conclusion, the present study demonstrates that phenolic compound and flavonoid-rich AHBUE have higher antioxidative enzyme activities that can significantly scavenge DPPH free radicals and mediate neuroprotective effects. AHEE can effectively inhibit iNOS protein expression and NO production in RAW264.7 cells, thus mediating anti-inflammatory effects. AHEE and the compound K36-21 can significantly inhibit AChE activity and potentially be useful for AD treatment. AHWE can activate the PI3K/Akt pathway, upregulate the Bcl-2/Bax ratio, inhibit Aβ-induced neuronal apoptosis, and exert neuroprotective effects. These results indicate that adlay hull extracts and the bioactive compound K36-21 attenuated β-amyloid-induced neurotoxicity and oxidative stress in PC12 cells through antioxidative, anti-inflammatory, and antiapoptotic activities, highlighting their potential applications for the treatment of neurodegenerative diseases such as AD.

Author contributions

GJT and CYC conceived the experiments. GJT, YTL, and CHH performed the experiments, analyzed the results, and made figures and tables. FYT and CYC contributed to designing the experiments. CYC wrote the paper and supervised the study. YHK and YTL contributed to writing-review and editing. All authors have read and approved the final manuscript.

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Not applicable.

Informed consent statement

Not applicable.

Declaration of competing interest

All of the authors of this manuscript declare that they do not have any conflicts of interest, and there is nothing to disclose.

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