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Original Article

Moringa oleifera leaf extract suppresses TIMM23 and NDUFS3 expression and alleviates oxidative stress induced by Aβ1-42 in neuronal cells *via* activation of Akt

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Abstract

Background and purpose: Oxidative stress plays an important role in Alzheimer's disease (AD) pathogenesis. *Moringa oleifera* leaf (MOL) extract has been shown to have antioxidant activities. Here, we studied the antioxidative and anti-apoptotic effects of water-soluble MOL extract in an amyloid beta (Aβ)-induced oxidative stress model of AD.

Experimental approach: The effect of amyloid beta (Aβ)1-42 and MOL extract on differentiated SH-SY5Y cell viability was assessed by MTT assay. Cells were treated with Aβ1-42, MOL extract, or MOL extract followed by Aβ1-42. The mitochondrial membrane potential (ΔΨm) and the reactive oxygen species (ROS) were evaluated by flow cytometry and dihydroethidium (DHE) assay, respectively. Western blotting was used to assess the expression of mitochondrial proteins TIMM23 and NDUFS3, apoptosis-related proteins Bax, Bcl-2, and cleaved caspase-3 along with fluorescence analysis of caspase-3/7, and Akt phosphorylation.

Findings/Results: MOL extract pretreatment at 25, 50, and 100 µg/mL prevented ΔΨm reduction. At 100-µg/mL, MOL extract decreased TIMM23 and NDUFS3 proteins and DHE signals in Aβ1-42-treated cells. MOL extract pretreatment (25, 50, and 100 µg/mL) also alleviated the apoptosis indicators, including Bax, caspase-3/7 intensity, and cleaved caspase-3, and increased Bcl-2 levels in Aβ1-42-treated cells, consistent with a reduction in the number of apoptotic cells. The protective effects of MOL extract were possibly mediated through Akt activation, evidenced by increased Akt phosphorylation.

Conclusion and implications: The neuroprotective effect of MOL extract could be mediated *via* the activation of Akt, leading to the suppression of oxidative stress and apoptosis in an Aβ1-42 model of AD.

Keywords: Akt; Amyloid beta; Apoptosis; *Moringa oleifera* leaf extract; NDUFS3; Oxidative stress; TIMM23.

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disease that is the most common type of dementia. AD patients suffer from memory loss and cognitive impairment (1). However, there is currently no cure for AD and the most effective way is to slow down its progression. Thus, the development of therapeutics for AD is crucial for public health (2).

It is well known that the main neuropathological hallmarks of AD are an extracellular accumulation of amyloid plaques and intracellular neurofibrillary tangles in the brain, eventually causing neuronal dysfunction and cell death (3,4). Studies have shown that amyloid beta (Aβ) oligomers can interact with several receptors and membranes as well as with mitochondria. Once Aβ inserts the cell membrane, it transports into both outer and inner mitochondrial membranes through the translocase of the outer membrane and translocase of the inner membrane (TIM) complexes inducing mitochondrial dysfunction (5).

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Moreover, previous studies revealed that Aβ is directly or indirectly involved with the blocking of TIM complexes leading to a decrease in the mitochondrial membrane potential (ΔΨm) in oxidative phosphorylation (6). The disruption of oxidative phosphorylation machinery or one of its enzyme complexes by Aβ results in the opening of the mitochondrial permeability transition pore, which induces a decrease of the ΔΨm and an increase of reactive oxygen species (ROS) (7). During ROS generation, B-cell lymphoma-2 (Bcl-2)-associated X protein (Bax) is activated and further induces caspase-3, contributing to the apoptotic pathway (8). Nevertheless, apoptosis can be prevented by the protein kinase B (Akt), which has been shown to play an important role in the neuroprotective effect *via* the regulation of Bcl-2 family proteins (2). In addition, it has been reported that some natural herbs can protect neurons through the activation of the Akt protein (9-11).

Among the plant-derived chemical compounds, *Moringa oleifera* Lam. is well known for being a source of proteins and essential amino acids (12). Moreover, its parts, including the pods, seeds, blossoms, roots, and leaves contain many medicinal effects when extracted crudely (13). *Moringa oleifera* leaf (MOL) is rich in ascorbic acid, flavonoid, and carotenoid, which have been studied *in vitro* and *in vivo*, and it has beneficial properties such as antibacterial, antiviral, anti-inflammatory, anticancer, cardioprotective activities, and antioxidant effects (12). Depending on their chemical properties, plant-derived chemical compounds are often recovered using various solvent extraction methods. The most commonly used solvents are ethanol, methanol, and acetone. However, extracted chemicals with low water solubility tended to have therapeutic challenges, which included embolism and respiration failure due to the precipitation of the substances (14). In terms of safety, water extraction can be used as a clean and non-toxic technique, and due to the polarity of water, it can be used as an extraction solution for water-soluble products, which contain protein, sugar, alcohol, organic acid, and inorganic substances (15). The study by Jung in 2014 demonstrated that a cold water-soluble MOL extract produced antiproliferation, and antioxidant effects in several cancer cells with high specificity and fewer adverse effects (16).

In AD rat models, MOL extracts have been shown to exert the potential to prevent oxidative stress (17,18), enhance memory function (18), and attenuate Aβ pathology (19). Most studies focused on solvent extraction methods using methanol and ethanol. The effect of MOL extracted with cold water in a cellular model of AD has not been investigated. Here, we aimed to investigate the neuroprotective effect of cold-water-soluble MOL extract pretreatment on various indicators of oxidative stress, as well as its underlying mechanisms related to apoptosis in Aβ1-42-treated SH-SY5Y cells.

MATERIALS AND METHODS

Chemicals and reagents

Eagle's minimum essential medium (MEM), nutrient mixture ham's F12 culture media, and fetal bovine serum (FBS) were purchased from Gibco (ThermoFisher Scientific, USA). Penicillin-streptomycin, amphotericin B, and all-*trans* retinoic acid (RA) were purchased from HyClone, USA. Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), sodium pyruvate, paraformaldehyde, protease inhibitor, and Hoechst 33258 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Radioimmunoprecipitation assay (RIPA) buffer was purchased from Boster Bio (CA, USA). Dihydroethidium (DHE) and CellEvent caspase-3/7 green detection reagents were purchased from Invitrogen, USA. Antibodies against Akt, p-Akt, Bax, caspase-3, and microtubule-associated protein 2 (MAP2) were purchased from Cell Signaling Technology, USA. Aβ1-42, Aβ42-1, Alexa Fluoro 594 (IgG) goat anti-rabbit secondary antibody, and antibodies against Translocase of the inner mitochondrial membrane 23 (TIMM23), NADH dehydrogenase (ubiquinone) Fe-S Protein 3 (NDUFS3), and Bcl-2 were purchased from Abcam (Cambridge, UK). Anti-β-actin antibody was purchased from Sigma-Aldrich (St. Louis, MO, USA). Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG and HRP-conjugated anti-mouse IgG were purchased from Merck (Cambridge, UK) and Invitrogen (Eugene, USA), respectively.

Preparation of MOL extract

Dried MOL powder was purchased from MRM manufacture, USA. The MOL powder (150 mg) was immersed in 1 mL of cold water (4 °C), vortexed for 30 s, and refrigerated for 5 min, 30 min, 1 h, 3 h, and 24 h. The suspension was vortexed again for 1 min. The water-insoluble part was eliminated by centrifuging twice at 12,000 rpm for 10 min (16), and the supernatant was collected first through Whatman number 1 filter paper, and next through a 0.2-µm filter. The MOL extract was freeze-dehydrated using Supermodulyo-230 (ThermoFisher Scientific, USA) and stored at -20 °C. For experiments, the freeze-dehydrated MOL extract was resuspended in distilled water to make up various concentrations.

Preparation of Aβ

Aβ1-42 and Aβ42-1 reverse peptides were resuspended in DMSO to 5 mM to make up a stock solution. The stock was kept at -20 °C. When the experiments were required, the stock was diluted into 100 µM in phosphate-buffered saline (PBS; pH 7.4) and incubated at 37 °C for 24 h to allow the oligomerization before the cell treatment.

Cell culture

The human neuroblastoma SH-SY5Y cell line (ATCC® CRL-2266) was used in this study. Cells were grown in a 1:1 mixture of MEM, nutrient mixture ham's F12 medium, supplemented with 10% FBS; 5-mL/L amphotericin B, 10-mL/L penicillin-streptomycin, 1.5-g/L $NaHCO_3$, 50-µL/L non-essential amino acid, and 5-mL/L sodium pyruvate. Cells were propagated in a 25-cm^2 or a 75-cm^2 T flask in a 5% CO_2 incubator at 37 °C. For the experiments, cells were plated onto appropriate subculture plates and maintained to adhere to the plate for 24 h. After that, cells were differentiated with 10-µM RA in 1% FBS-containing media for 5 days before being used in experiments.

Cell viability assay

Cell viability was analyzed by using an MTT assay. SH-SY5Y cells were plated onto a 96-well plate to 80% cell confluence. The final volume of each well after any treatment was 100 µL. Cells were treated for 24 h with Aβ1-42 and Aβ42-1 at 0.3125, 0.625, 1.25, 2.5, 5, and 10 µM and with MOL extract at 25, 50, 100, 200, and 400 µg/mL. After the experiments, culture media were removed and replaced with fresh 1% FBS containing a 5 mg/mL MTT solution in the dark for 3 h. After that, the media were replaced with DMSO to dissolve the purple formazan. The absorbance was measured at 570 and 690 nm, using a Versamax microplate reader Softmax Pro 4.8 analysis software (Molecular Devices, Sunnyvale, CA, USA).

Immunofluorescence staining

SH-SY5Y cells were plated at a density of 6×10^4 cells/mL on a poly-L-lysine-coated coverslip in 24-well plates. After differentiation, cells were fixed with 4% paraformaldehyde for 30 min at 4 °C and permeabilized with 0.25% Triton X-100 in PBS. To block non-specific binding, cells were incubated with 1% bovine serum albumin (BSA), and 10% normal goat serum in PBS containing 0.1% Tween 20 for 1 h. Cells were then incubated overnight with 1:100 rabbit anti-MAP2 antibody. After washing with PBS, cells were incubated with Alexa Fluor-594 conjugated goat anti-rabbit IgG for 2 h at room temperature, followed by nuclear staining with Hoechst33258 for 30 min. Cells were visualized using fluorescence microscopy (Olympus BX53, Tokyo, Japan).

Hoechst 33258 staining

SH-SY5Y cells were placed at a density of 6×10^4 cells/coverslip. After treatment, cells were washed with PBS and fixed with 4% paraformaldehyde. Cells were washed with PBS and stained with 1-µg/mL Hoechst 33258 for 30 min at room temperature in a dark place. Next, cells were washed with PBS and mounted with an anti-fading mounting medium. The slides were stored at 4 °C and visualized under fluorescence microscopy.

Western blotting analysis

SH-SY5Y cells were seeded onto 6-well plates at 1×10^6 cells/well. After treatment, media were removed, and cells were washed

with PBS. Then, cells were trypsinized. After adding the media, cells were centrifuged to collect the pellet and washed with cold PBS. Next, the pellets were mixed with 50-µL cold RIPA buffer containing 1% protease inhibitor. Lysates were centrifuged at 14,000 *g* at 4 °C for 20 min. Supernatants were collected and kept at -80 °C until used. Protein concentration was determined using a BCA protein assay kit (Thermo Scientific, USA) and analyzed by SoftMax Pro 7 software (Molecular Devices, Sunnyvale, CA, USA). Equal amounts of protein samples were resuspended in a loading buffer and heated at 95 °C for 5 min before loading onto 6 or 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and subsequently transferred to polyvinylidene difluoride membrane. The membrane was blocked with either 5% skim milk or 5% BSA in Tris buffer saline containing 0.1% Tween® 20 for 2 h at room temperature and incubated at 4 °C overnight with 1:5,000 mouse anti-β-actin, 1:1,000 rabbit anti-MAP2, 1:1,000 rabbit anti-Bax, 1:1,000 rabbit anti-caspase 3, 1:1,000 rabbit anti-p-AKT, 1:1,000 rabbit anti-Akt, 1:1,000 rabbit anti-TIMM23, 1:1,000 rabbit anti-NDUFS3, or 1:500 mouse anti-Bcl-2 antibodies. Then, the membranes were incubated with HRP-conjugated anti-rabbit IgG or HRP-conjugated anti-mouse IgG for 90 min at room temperature. The blots were developed with Chemiluminescence imaging (Alliance Q9 Advanced, Uvitech, Cambridge, UK). The band intensity was measured using Image J software (National Institutes of Health, USA). The targeted proteins were normalized with β-actin or α-tubulin. The data are shown as the relative expression to the respective controls.

Mitochondrial membrane potential assay

SH-SH5Y cells were seeded onto 6-well plates at a density of 5×10^5 cells/mL. After treatment, cells were trypsinized and centrifuged to collect the pellet. Cells were resuspended in 500-µL JC-10 dye-loading solution and incubated at 37 °C in a 5% CO_2 atmosphere for 45 min in a dark place. The mitochondrial membrane potential was analyzed using the FACSCanto flow cytometry (BD Bioscience, Becton and Dickinson).

DHE assay

DHE was used to measure the generation of ROS. The DHE can penetrate the cell membrane and is oxidized by cellular $O_2^{\cdot-}$ producing red fluorescence (20). Cells were exposed to 10-µM DHE in FBS-free media in a 96-well plate for 30 min at 37°C in the dark. The ROS levels were measured using a fluorescent microplate reader (Tecan, Switzerland). The DHE staining on cells was evaluated under a live cell fluorescence microscopy (Olympus IX83) after a 30-minute incubation with DHE.

Activated caspase-3/7 staining

SH-SY5Y cells were plated onto 24-well plates at a cell density of 6×10^4 cells/coverslip. After treatment, media were removed, and cells were incubated with 5 µM of the CellEvent caspase-3/7 green detection reagent in PBS with 5% FBS. Cells were then stained with Hoechst33258 nuclear staining and incubated at 37 °C for 30 min. After mounting, cells were visualized under a fluorescence microscope (Olympus BX53, Tokyo, Japan).

Statistical analysis

Statistical analysis was performed with GraphPad Prism software version 8.0 (GraphPad Software, CA, USA). Data are presented as mean ± SEM or SD. The statistical differences between the groups were assessed using one-way or two-way ANOVA followed by Turkey's post hoc test. *P-values* < 0.05 were considered statistically significant.

RESULTS

Verification of neuronal differentiation of SH-SY5Y cells

To demonstrate that SH-SY5Y cells were differentiated into mature neuronal-like phenotypes, cells were treated with 10-µM RA in 1% FBS media for 5 days, and the morphological changes were observed under the inverted and fluorescent microscopies. On day 5, an increase in the length of neuronal processes and the pyramidal-shaped cell bodies were observed in RA-treated cells, whereas undifferentiated cells demonstrated the shorter process clusters of neuroblast-like cells (Fig. 1A).
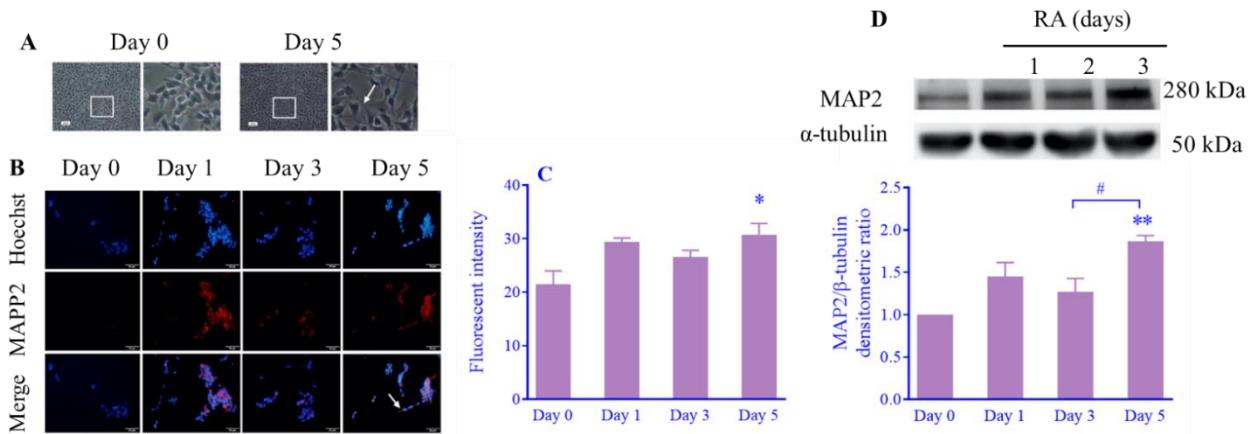
Fig. 1. RA-induced neuronal differentiation of SH-SY5Y cells. Cells were treated with 10-μm RA in 1% fetal bovine serum-culture medium for 5 days. (A) Representative phase contrast images. White boxed areas indicate magnified images shown on the right of each day. The arrow indicates neuritic extension. Bar = 40 μm. (B) Immunofluorescence staining with Hoechst33258 nuclear staining (blue) and the neuronal marker MAP2 (red). The arrow indicates a MAP2-positive cell. Bar = 50 μm. Imaging results are representative of 3 independent experiments. (C) Quantification of mean fluorescent intensity of MAP2-positive cells. Data are expressed as mean ± SD. (D) Immunoblots of MAP2 expression and band quantification were obtained from 3 independent experiments. The density of the bands was normalized with that of α-tubulin. Data are expressed as mean ± SEM of percentage to the control. $*P < 0.05$ indicates a significant difference in comparison with day 0 which was considered the control group; $^{##}P < 0.01$ implies the difference between designated groups. RA, Retinoic acid; MAP2, microtubule-associated protein 2.

In addition, MAP2 in neuronal-like cells was examined as a marker for mature neurons. The immunofluorescence staining showed that MAP2 was presented in the neuronal process from day 1 to day 5 (Fig. 1B), with a significant increase of mean fluorescent intensity at day 5 (Fig. 1C). The western blotting analysis also showed a significant increase of MAP2 protein expression at day 5 compared with day 0 and day 3 (Fig. 1D). Therefore, 5-day differentiation of SH-SY5Y cells was conducted in the subsequent experiment in this study.

Evaluation of the effect of Aβ and MOL extract concentrations on cell viability

To create a cellular model of AD, differentiated SH-SY5Y cells were treated with various concentrations of Aβ1-42 for 24 h. MTT assay was used to evaluate cell viability. Aβ42-1, a reverse form of Aβ, was used as a control peptide. The exposure to Aβ1-42 at any concentration (0.3125, 0.625, 1.25, 2.5, 5, and 10 μM) showed a significant decrease in cell viability compared to the control and vehicle (Fig. 2A). A significant difference was observed between the effect of Aβ1-42 and Aβ42-1 at 1.25 μM on cell survival, therefore, this concentration was used in further experiments. Next, we examined the effect of MOL extract

on SH-SY5Y cell viability. MOL extract was prepared in different extraction durations (5 min, 30 min, 1 h, 3 h, and 24 h). Cells were incubated with various concentrations of MOL extract obtained from each extraction duration. The results demonstrated that MOL extracts at 25, 50, 100, and 200 μg/mL in any extraction duration did not affect cell viability compared to the control (Fig. 2B). However, MOL extract at 400 μg/mL showed dramatically decreased cell viability compared to the control and to MOL extract at the concentrations of 25, 50, 100, and 200 μg/mL. We selected MOL extract at the concentrations of 25, 50, and 100 μg/mL with a 30-minute extraction duration for cell treatment in further experiments.

Effect of MOL extract pretreatment on the viability of Aβ1-42-treated SH-SY5Y cells

To investigate the protective effect of MOL extract, cells were pretreated with MOL extract for 24 h before 24-h Aβ1-42 exposure. Treatment with Aβ1-42 alone significantly decreased cell survival compared to the control, whereas MOL extract treatment alone at 100 μg/mL showed a significant increase in cell viability compared to Aβ1-42 treatment alone (Fig. 2C).
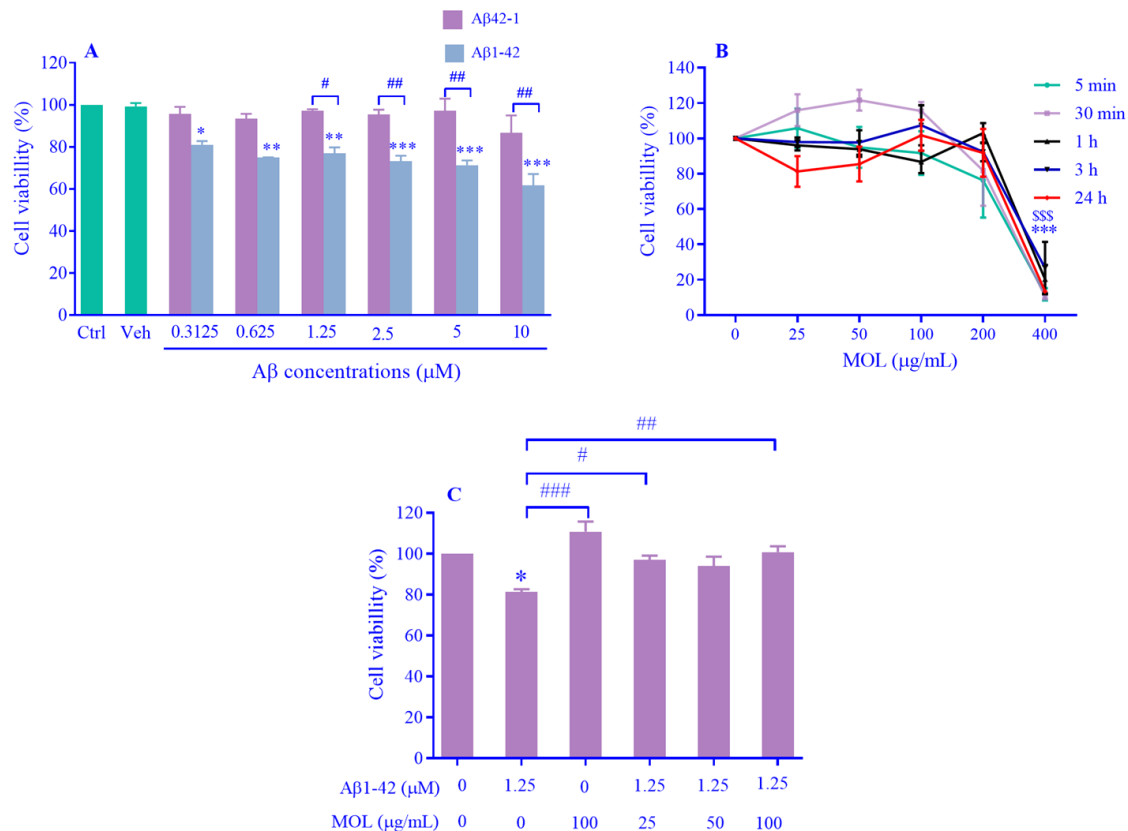
Fig. 2. Assessment of cell death in differentiated SH-SY5Y cells treated with Aβ1-42, MOL, and MOL with Aβ1-42. (A) Cell viability of SH-SY5Y cells treated with various concentrations of Aβ1-42 for 24 h. Aβ42-1 was used as a control peptide. (B) The viability of SH-SY5Y cells treated with MOL for 24 h. MOL was extracted at different extraction durations (5 min, 30 min, 1 h, 3 h, and 24 h). (C) The viability of SH-SY5Y cells pretreated with 30 min-extracted MOL for 24 h followed by Aβ1-42 for 24 h. All data were obtained from 3 independent experiments performed in quadruplicate and shown as the means ± SEM. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ indicate significant differences in comparison with the control group; #$P < 0.05$, ##$P < 0.01$, and ###$P < 0.001$ imply the differences between designated groups; and $$$P < 0.001$ *vs* MOL at concentrations of 25, 50, 100, and 200 μg/mL. Aβ, Amyloid beta; MOL, *Moringa oleifera* leaf.

In the pretreatment experiment, the pretreatment of MOL extract at 25 and 100 μg/mL before Aβ1-42 exposure significantly increased cell viability compared to Aβ1-42 treatment alone, and these levels were close to the level of cell viability in the control group. This result indicated that MOL extract pretreatment can protect against cell death in Aβ1-42-treated SH-SY5Y cells.

MOL extract pretreatment prevented the decrease of mitochondrial membrane potential in Aβ1-42-treated SH-SY5Y cells

As the inner mitochondrial membrane could be disrupted by Aβ1-42 causing the ΔΨm reduction (7), we further investigated the effect of MOL extract pretreatment in Aβ1-42-treated SH-SY5Y cells on ΔΨm changes, using the fluorescent JC-10 probe (Fig. 3A-F). Normally, JC-10 dye can enter the mitochondria, and

when it is excited upon membrane polarization, the dye color changes from green to red as the ΔΨm increases, suggesting the formation of JC-10 aggregates (Fig. 3A). In apoptotic and dead cells, JC-10 exists in the monomeric form and emits more green signals. In our JC-10 flow cytometry results, the Aβ1-42 treatment alone significantly decreased the ΔΨm compared to the control (Fig. 3B and G), and MOL extract treatment alone significantly increased the ΔΨm compared to the Aβ1-42 treatment alone (Fig. 3C and G). As expected, pretreatment of MOL extract before Aβ1-42 exposure significantly increased the ΔΨm compared to Aβ1-42 treatment alone, and these levels were close to the level observed in the control group (Fig. 3D-G). This finding suggested that MOL extract pretreatment could prevent the ΔΨm reduction in Aβ1-42-treated SH-SY5Y cells.
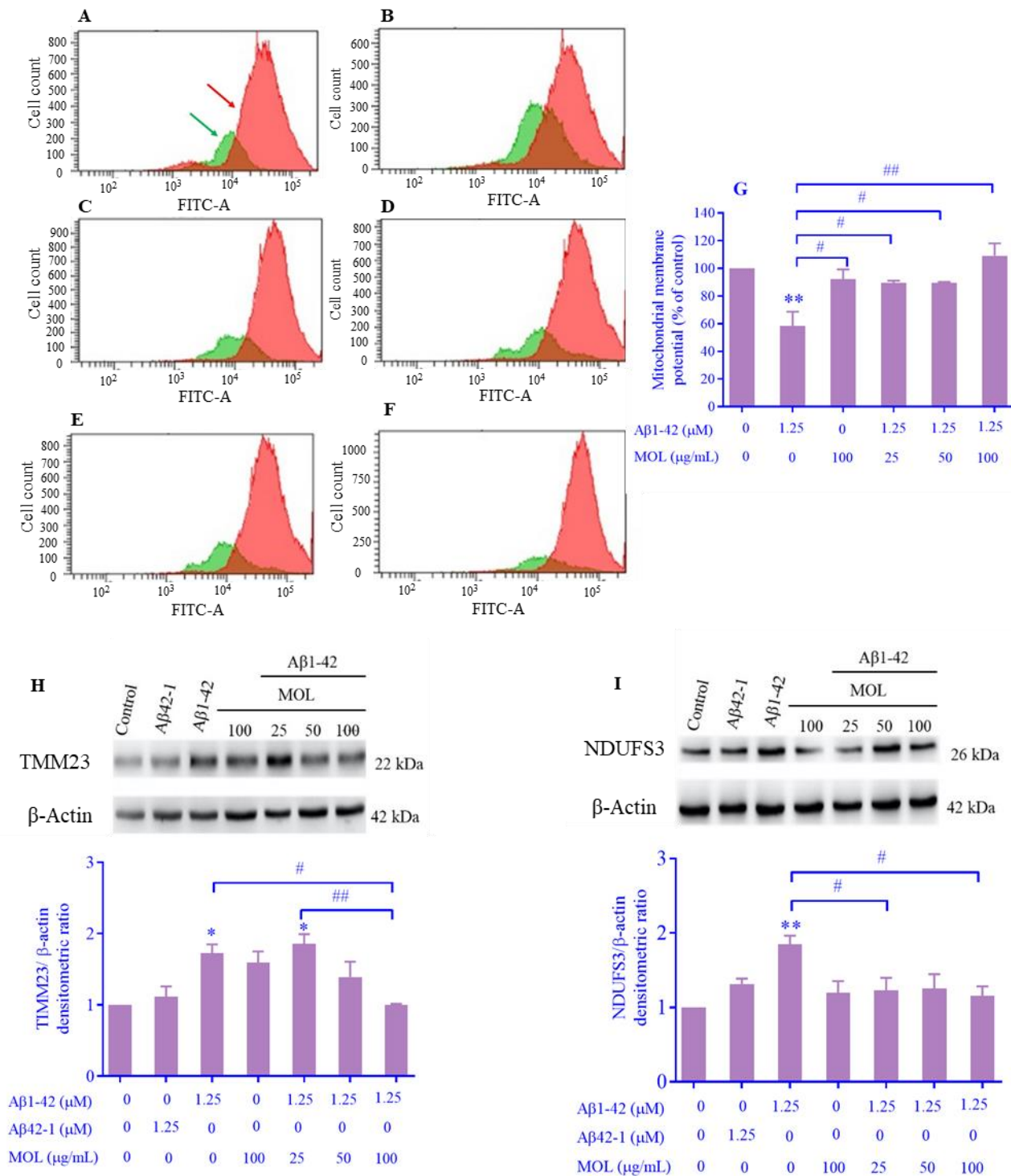
Fig. 3. Effects of MOL pretreatment on the mitochondrial membrane potential (ΔΨm) and mitochondrial proteins TIMM23 and NDUFS3 in Aβ1-42-treated SH-SY5Y cells. (A) Control; (B) Aβ1-42 treatment alone; (C) 100-µg/mL MOL alone; (D-F) 25, 50, and 100-µg/mL MOL pretreatment followed by Aβ1-42 exposure. The orange curve indicates the number of healthy cells (red arrow), and the green curve indicates the number of dead cells (green arrow). (G) Quantification of fluorescent JC-10 signals. Data are expressed as mean ± SEM from 3 independent experiments. (H and I) Immunoblots of TIMM23 and NDUFS3, respectively. Band quantification was obtained from 3 independent experiments. The density of the bands was normalized with that of β-actin. Data are expressed as mean ± SEM of percentage to the control. *P < 0.05 and **P < 0.01 indicate significant differences in comparison with the control group; #P < 0.05 and ##P < 0.01 imply the differences between designated groups. Aβ, Amyloid beta; MOL, *Moringa oleifera* leaf; TIMM23, translocase of the inner mitochondrial membrane 23; NDUFS3, NADH dehydrogenase (Ubiquinone) Fe-S protein 3.

MOL extract pretreatment suppressed an increase of TIMM23 and NDUFS3 in Aβ1-42-treated SH-SY5Y cells

The previous study has revealed that the clogging of TIM complexes decreased the import of other mitochondrial proteins coded in the nucleus, subsequently affecting the ΔΨm change in AD (6). In addition, the high level of NDUFS3, a component of mitochondrial complex I, can induce ROS generation leading to cell damage in AD (21). Therefore, the expression of TIMM23 and NDUFS3 proteins was investigated. The results showed that Aβ1-42 alone increased TIMM23 expression (Fig. 3H). However, the pretreatment of 100 μg/mL MOL extract before the Aβ1-42 exposure significantly decreased the TIMM23 expression compared to the Aβ1-42 alone group, and also significant when compared to the pretreatment of MOL extract at 25 μg/mL. Similarly, Aβ1-42 alone increased NDUFS3 expression, and the pretreatment of 100 μg/mL MOL extract before the Aβ1-42 exposure significantly decreased the NDUFS3 expression compared to the Aβ1-42 alone (Fig. 3I). These results suggested that 100 μg/mL MOL extract pretreatment could lower the mitochondrial protein TIMM23 and NDUFS3 in Aβ1-42-treated SH-SY5Y cells.

MOL extract pretreatment inhibited oxidative stress in Aβ1-42-treated SH-SY5Y cells

It is known that the decrease of ΔΨm, and insufficiency of ROS clearance cause excessive ROS accumulation, which extensively damages the cells (22). We next investigated the effect of MOL extract pretreatment in inhibiting oxidative stress in Aβ1-42 treated SH-SY5Y cells by evaluating the superoxide (O_2^-) production with DHE fluorescent staining (Fig. 4A-G). Treatment with Aβ1-42 alone significantly increased DHE intensity (Fig. 4C and H). Treatment with MOL extract at 100 μg/mL alone did not affect the DHE intensity compared to the control (Fig. 4D and H). In the pretreatment experiments, the MOL extract concentrations of 25, 50, and 100 μg/mL significantly decreased the DHE intensity compared to the Aβ1-42 alone group (Fig. 4E-H). These results demonstrated that MOL extract pretreatment could inhibit oxidative stress, resulting in the improvement of ΔΨm as found in the previous result (Fig. 3A-G).

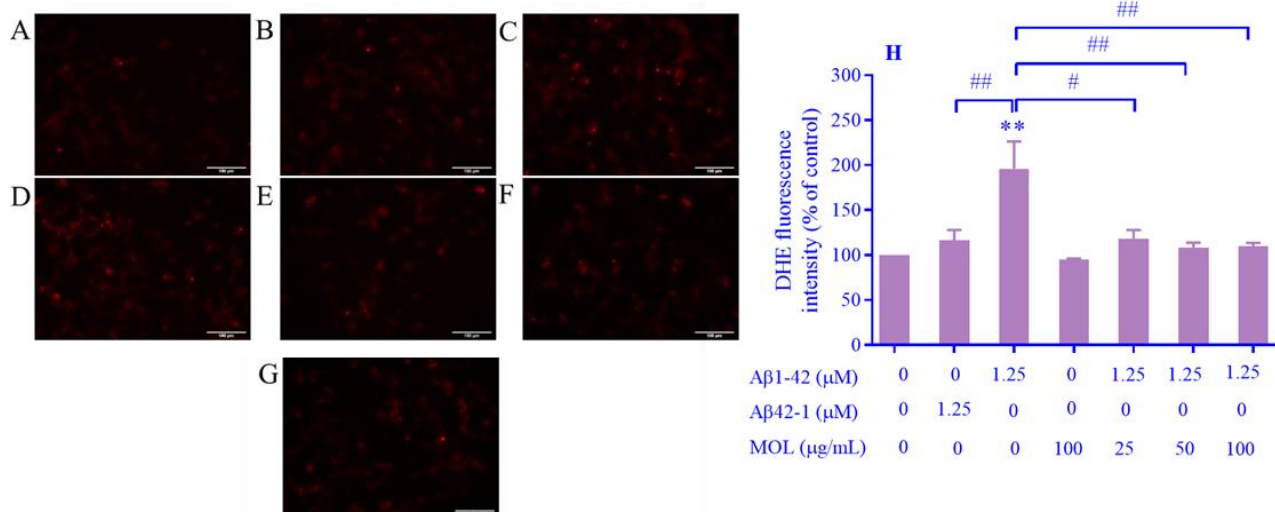**Fig. 4.** Assessment of ROS production in differentiated SH-SY5Y cells treated with Aβ1-42, MOL, and MOL with Aβ1-42. ROS production was evaluated with DHE staining. (A) Control; (B) Aβ42-1 treatment alone; (C) Aβ1-42 treatment alone; (D) 100-μg/mL MOL alone; (E-G) 25, 50, and 100-μg/mL MOL pretreatment followed by Aβ1-42 exposure. Bar = 100 μm. (G) Quantification of fluorescent DHE intensity. Data are expressed as mean ± SEM from 3 independent experiments. **$P < 0.01$ indicates a significant difference in comparison with the control group; #$P < 0.05$ and ##$P < 0.01$ imply the differences between designated groups. Aβ, Amyloid beta; MOL, *Moringa oleifera* leaf; DHE, dihydroethidium; ROS, reactive oxygen species.

Effect of MOL extract pretreatment on apoptosis in Aβ1-42-treated SH-SY5Y cells

Since the mitochondria are the center triggering the intrinsic apoptotic pathway, which plays an important role in AD (23), we further examined the effect of MOL extract pretreatment for preventing cell apoptosis in Aβ1-42-treated SH-SY5Y cells. Our results showed that Bax protein expression significantly increased in Aβ1-42-treated cells compared to the controls (Fig. 5A). Conversely, the MOL extract pretreatment significantly reduced Bax protein expression in any MOL extract concentration compared to the treatment of Aβ1-42 alone. Treatment of Aβ1-42 alone significantly decreased the Bcl-2 protein compared to the control (Fig. 5B). However, the pretreatment of MOL extract in each concentration significantly increased the Bcl-2 protein expression compared with the Aβ1-42 alone.

The activated caspase 3/7 green fluorescent staining was then examined. The treatment of Aβ1-42 alone showed a higher amount of bright green spots, indicating the activated caspase-3/7 than the control as supported by quantified intensity (Fig. 5C and D). Treatment with MOL extract at 100-μg/mL alone did not affect the caspase 3/7 green fluorescent intensity compared to the control. However, the pretreatment of MOL extract followed by Aβ1-42 exposure significantly decreased the caspase 3/7 green fluorescent intensity compared to the Aβ1-42 without MOL extract pretreatment. This result was supported by Western blot analysis of the cleaved-caspase 3 protein (Fig. 5E). Treatment with Aβ1-42 alone significantly increased the cleaved-caspase 3 protein expression compared to the control, whereas MOL extract alone treatment did not affect this expression. MOL extract pretreatment at 25, 50, and 100 μg/mL significantly decreased the cleaved-caspase 3 protein expression compared to the Aβ1-42 alone (Fig. 5F and G).
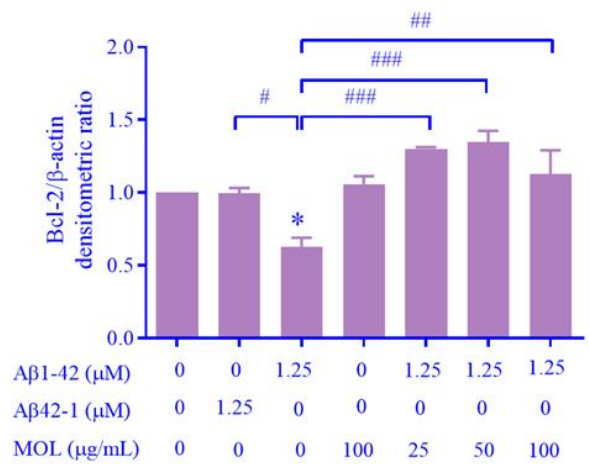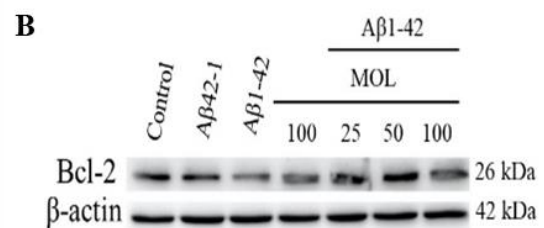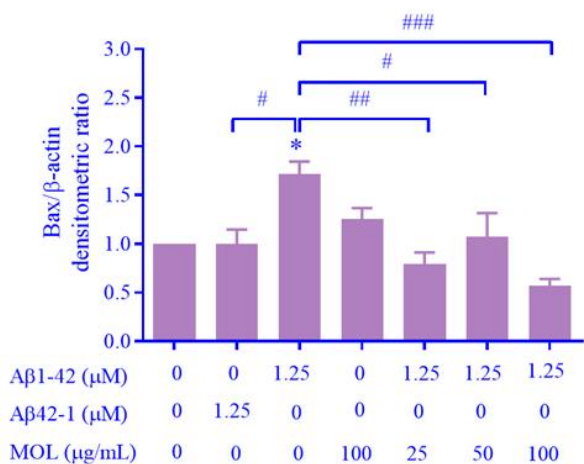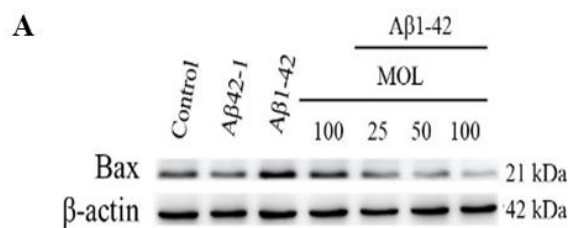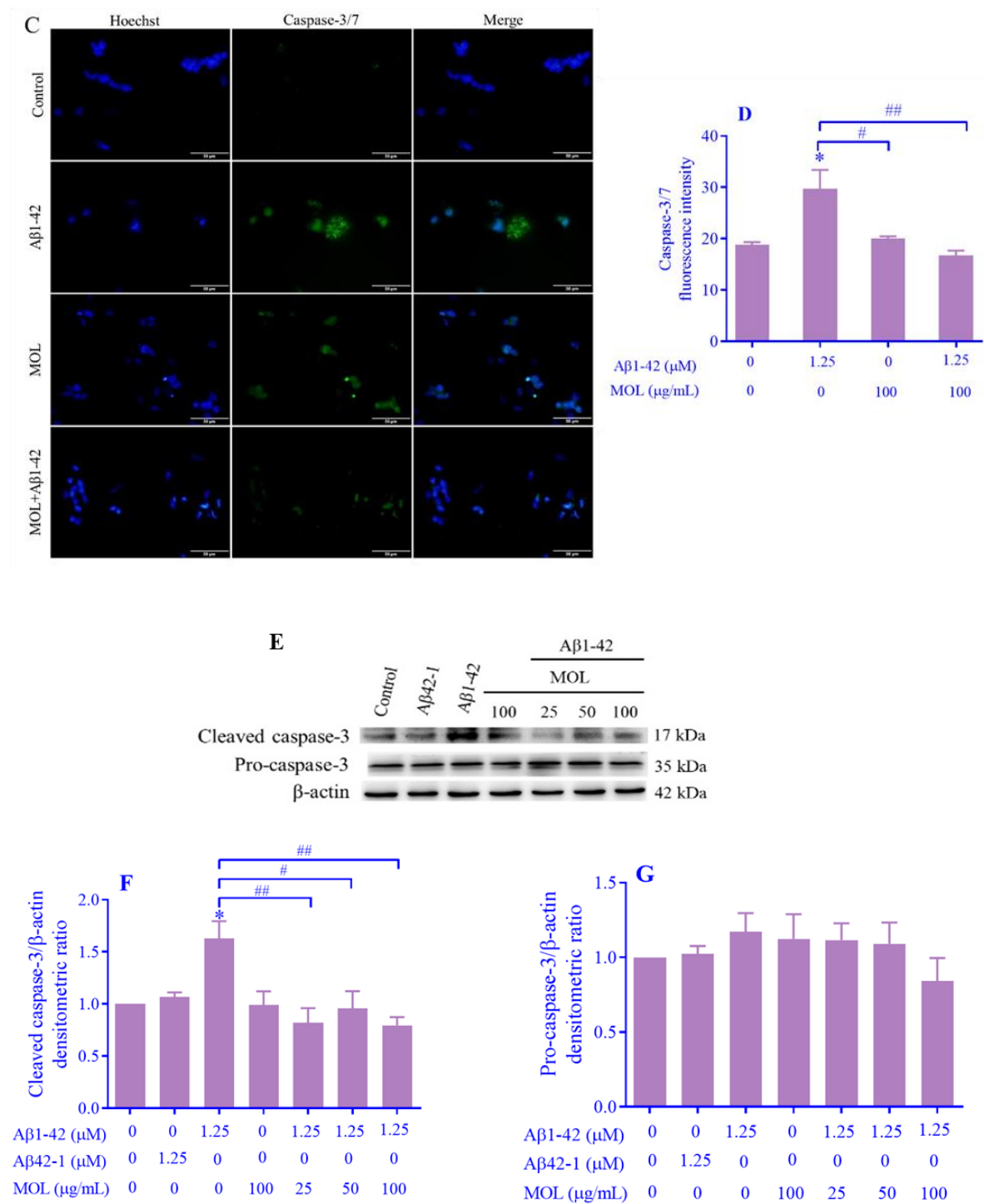
Fig. 5. Assessment of apoptotic indicators in differentiated SH-SY5Y cells treated with Aβ1-42, MOL, and MOL with Aβ1-42. (A and B) Immunoblots of Bax and Bcl-2, respectively. Band quantification was obtained from 3 independent experiments. The density of the bands was normalized with that of β-actin data are expressed as mean ± SEM of percentage to the control. (C) Immunofluorescence staining with Hoechst33258 nuclear staining (blue) and caspase-3/7 (green); Bar = 50 μm; imaging results are representative of 3 independent experiments. (D) Quantification of mean fluorescent intensity caspase-3/7 positive cells; data are expressed as mean ± SD. (E) Immunoblots of caspase-3. (F and G) Band quantification of cleaved caspase-3 and pro-caspase-3, respectively, was obtained from 3 independent experiments; the density of the bands was normalized with that of β-actin data are expressed as mean ± SEM of percentage to the control. *$P < 0.05$ indicates a significant difference in comparison with the control group; #$P < 0.05$, ##$P < 0.01$ and ###$P < 0.001$ imply the differences between designated groups. Aβ, Amyloid beta; MOL, *Moringa oleifera* leaf; DHE, dihydroethidium; Bax, B-associated X protein; Bcl-2, B-cell lymphoma-2.
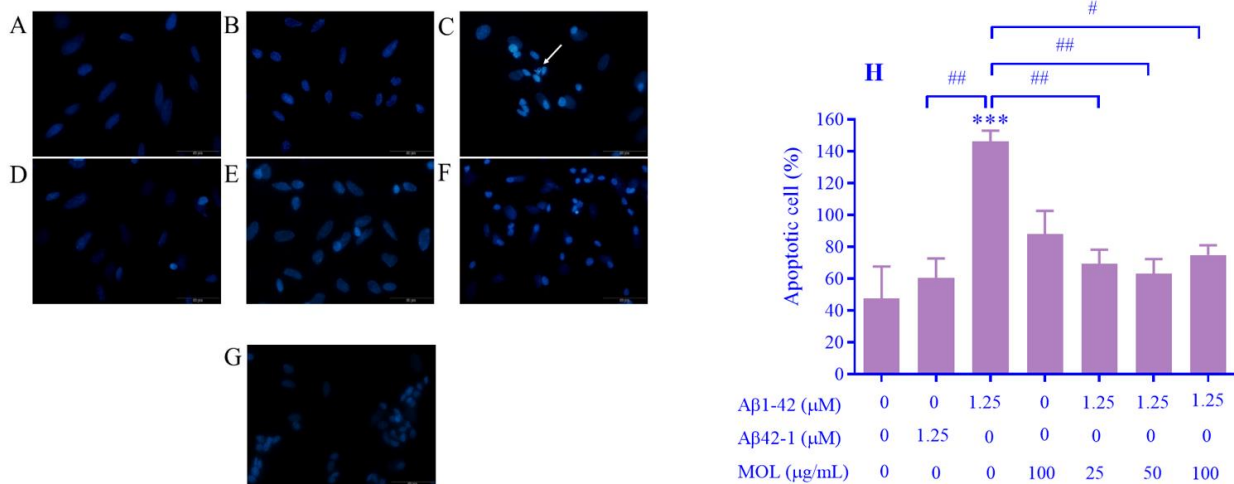
Fig. 6. Effects of MOL pretreatment on cell apoptosis in Aβ1-42-treated SH-SY5Y cells. Cells were stained with Hoechst33258. (A) Control; (B) Aβ42-1 treatment alone; (C) Aβ1-42 treatment alone; (D) 100-µg/mL MOL alone; (E-G) 25, 50, and 100-µg/mL MOL pretreatment followed by Aβ1-42 exposure. Condensed or fragmented nuclei (a white arrow) were considered apoptotic nuclei. (H) Quantification of apoptotic nuclei; data are expressed as mean ± SEM from 3 independent experiments. ***$P < 0.001$ indicates a significant difference in comparison with the control group; #$P < 0.05$ and ##$P < 0.01$ imply the differences between designated groups. Aβ, Amyloid beta; MOL, *Moringa oleifera* leaf.

It is known that caspase-3 activation could lead to DNA fragmentation. The treatment of Aβ1-42 alone showed an increased amount of fragmented bright blue spots of the chromatin condensation, as shown by Hoechst33258 staining (Fig. 6A-G), and supported by the quantified analysis of apoptotic cells (Fig. 6H). The pretreatment of MOL extract at the concentrations of 25, 50, and 100 µg/mL significantly decreased apoptotic cells compared to the Aβ1-42 alone (Fig. 6E-H). Overall, these results suggested that MOL extract pretreatment could decrease the pro-apoptotic Bax and cleaved-caspase3/7 proteins and enhance the anti-apoptotic Bcl-2, leading to decreased cell apoptosis.

Effect of MOL extract pretreatment on Akt protein expression in Aβ1-42-treated SH-SY5Y cells

Western blot analysis revealed that the p-Akt/t-Akt ratio was significantly decreased in the Aβ1-42 alone treatment compared to the control, whereas the ratio was not affected by the MOL extract alone treatment (Fig. 7). The pretreatment with MOL extract at 50 and 100 µg/mL significantly increased the p-Akt/t-Akt ratio compared to the Aβ1-42 alone. These suggested that MOL extract pretreatment could activate Akt in Aβ1-42-treated SH-SY5Y cells.
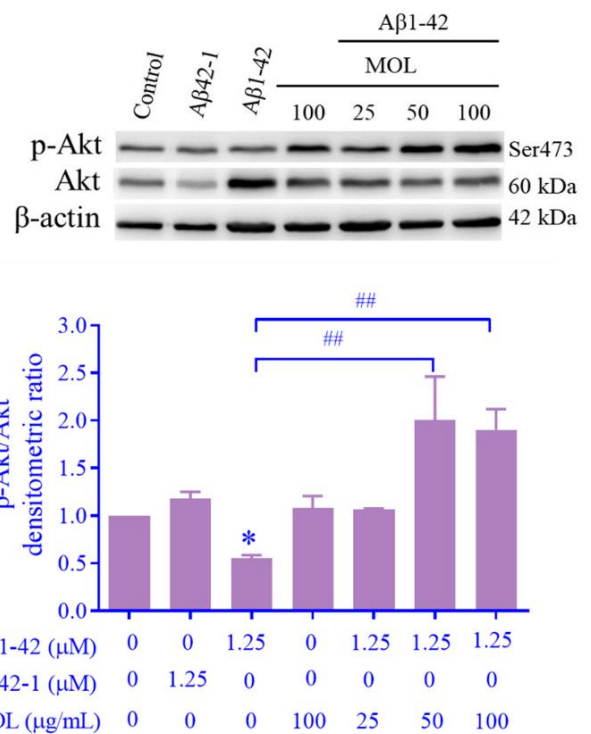

Fig. 7. Effects of MOL pretreatment on phosphorylation of Akt protein in Aβ1-42-treated SH-SY5Y cells. Quantification of immunoblots of p-Akt and total Akt obtained from 3 independent experiments. Data are expressed as mean ± SEM of percentage to the control. *$P < 0.05$ indicates a significant difference in comparison with the control group; ##$P < 0.01$ implies the differences between designated groups. Aβ, Amyloid beta; MOL, *Moringa oleifera* leaf; Akt, protein kinase B.

DISCUSSION

Although the treatment of AD has been developing, no new therapies have been approved for AD since 2002 (24). Nowadays, there are numerous studies of plant-derived chemical compounds, which demonstrate the beneficial effects in AD (25). Interestingly, it would be more profitable if those compounds could be applied as supplementary use before the development of AD in elderly people. Our present study demonstrated that a cold-water-soluble MOL extract has a neuroprotective effect against Aβ1-42-induced oxidative stress in SH-SY5Y cells. Components of MOL extract can pass through the blood-brain barrier (19,26). Analysis using liquid chromatography with tandem mass spectrometry revealed that metabolites of aqueous extracts of MOL have a good permeability of the blood-brain barrier with low toxicity (26). *In vivo,* studies involving experimental animals such as rodents and rabbits have been conducted to assess the safety and toxicity of MOL extracts. These studies did not reveal any prominent acute, sub-acute, or chronic toxicity or clinical symptoms associated with the use of aqueous MOL extracts (27).

Aβ, the component protein in amyloid plaque accumulating in the AD brain, is generated by protease cleavage of type I transmembrane amyloid precursor protein. In the amyloidogenic pathway, amyloid precursor protein is cleaved by β and γ secretase enzymes to produce Aβ1-40/42. However, previous studies found that Aβ1-42 in the core of amyloid plaque is neurotoxic and is more likely to be a key player in initiating AD pathogenesis (28). The oligomer form of Aβ1-42 is suggested to be a more toxic species for cells in AD patients' brains (29). In our study, Aβ1-42 was freshly prepared and allowed to oligomerize for 24 h at 37 °C before treating the cells. Similar to our preparation, Hettiarachchi and colleagues demonstrated by using electron microscopy that after incubation of Aβ1-42 for 24 h in SH-SY5Y cells, small globular assemblies were developed, and Aβ1-42 transformed into protofibrils (30). Moreover, their MTT assay supported our study that the exposure to Aβ1-42 for 24 h caused the concentration-dependent decrease in SH-SY5Y cell viability.

It is well established that mitochondria are targeted for Aβ. One of the defects in mitochondrial proteins, or its functions can induce the impairment of ΔΨm leading to ROS production (31), consistent with our results (Fig. 3B and G), which showed that the treatment of Aβ1-42 alone significantly decreased ΔΨm and increased oxidative stress. Aβ also interferes with the TIMM23 protein, which is the main component of the TIM23 complex, resulting in TIMM23 aggregation (6). In the present study, Aβ1-42 increased TIMM23 protein expression. However, the pretreatment of MOL extract at 100 μg/mL before Aβ1-42 exposure significantly decreased TIMM23 (Fig. 3H). Another essential mitochondrial membrane protein involved in AD is NDUFS3, which is one of the components in mitochondrial complex I. The study by Zhang *et al.* demonstrated that the NDUFS3 gene showed the highest degree of expression among several genes from the hippocampus of AD patients (21). A similar finding has been observed in rat models also showed that upregulated NDUFS3 could be mapped to several cellular pathways, including the oxidative phosphorylation and AD pathways (32). Our results showed that the treatment of Aβ1-42 alone significantly increased NDUFS3 protein expression, and we revealed the beneficial effect of the MOL extract pretreatment in reducing the TIMM23 and NDUFS3 proteins (Fig. 3H and I), which may lead to restoration of the ΔΨm in Aβ1-42-treated SH-SY5Y cells. The lack of a concentration-dependent effect of MOL extract on NDUFS3 expression in our results could be attributed to the varying expression levels of NDUFS3 in different subcomplexes of human mitochondrial complex I. Using an inducible NDUFS3-green fluorescent protein expression system, Vogel and colleagues demonstrated that NDUFS3 is present in four subcomplexes of complex I, and that the distribution of these complex I subcomplexes changes under conditions that promote complex I breakdown (33). A similar explanation could be applied to the absence of a concentration-dependent effect of MOL extract on the DHE intensity, as NDUFS3 regulates ROS-mediated biochemical alterations in cells (34).

Consistent with our results, several studies also showed that the treatment of Aβ1-42 disrupted mitochondrial membrane potential, promoted ROS production, and induced neuronal apoptosis through activation of Bax, cleaved-casepase3, and decrease the level of Bcl-2 protein expressions in SH-SY5Y cells, primary cultures of mouse cerebral cortical neurons, and mouse models (35-39). However, there have been few studies on MOL extract against ROS-induced cell apoptosis in AD. The studies by González-Burgos and colleagues, and Hashim and colleagues have revealed that the pretreatment of MOL extract prevented mitochondrial dysfunction by increasing ΔΨm and diminishing ROS production on H_2O_2-induced oxidative stress in SH-SY5Y cells (12,40). A previous study also showed that MOL extract promoted neurite outgrowth and increased the number and length of dendrites and axons resulting in the activation of synaptogenesis in the primary culture of hippocampal neurons (41).

It has been long proven that there is a relevance between oxidative stress and neuronal apoptosis in neurodegenerative disorders as well as AD (42). The excessive production of superoxide (O_2^-), hydroxyl radical (OH), and hydrogen peroxide (H_2O_2)

caused by dysregulation of the mitochondrial respiratory chain leads to ROS production (40). The overproduction of ROS and decrease of ROS elimination can trigger apoptotic cell death *via* lowering the anti-apoptotic Bcl-2 and promoting pro-apoptotic Bax proteins. Bax then induces the release of cytochrome c, and apoptosis-inducing factor to activate caspase 3 in the apoptotic downstream pathway (43). Previous studies have shown that Akt plays a crucial role in neuroprotection through a variety of mechanisms, including mediating Nrf2 activation to attenuate oxidative stress after resveratrol treatment (44), opposing the generation of ROS in PC12 cells (45), raising the ΔΨm of primary cortical neurons (46), and lowering ROS levels in H_2O_2-treated PC12 cells (47). Besides, activation of Akt can enhance the expression of antioxidant enzymes in neuronal cells (48). Previous studies also indicated that Akt could inhibit caspase-3 activation, enhance Bcl-2 expression, and suppress Bax to inhibit apoptosis in HMN1 and PC12 cells (49,50). In the present study, we showed that MOL extract pretreatment in Aβ1-42-treated SH-SY5Y cells decreased ROS generation, suppressed Bax, increased Bcl-2, and decreased cleaved caspase-3 levels, which could be mediated through activation of Akt.
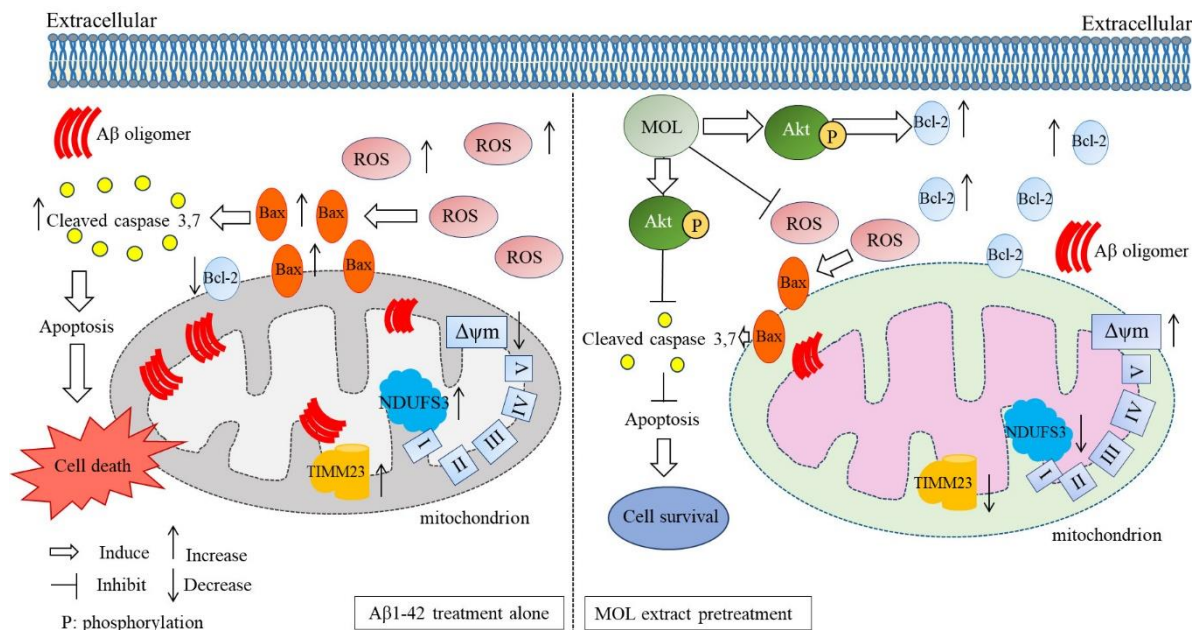

Fig. 8. The schematic of the possible underlying mechanisms of Aβ1-42 treatment alone versus the MOL pretreatment before Aβ1-42 exposure on the prevention of apoptotic cell death in neuronal cells. Akt, Protein kinase B; Aβ, amyloid beta; Bax, B-associated X protein; Bcl-2, B-cell lymphoma-2; MOLE, *Moringa oleifera* leaf extract; NDUFS3, NADH dehydrogenase (ubiquinone) Fe-S protein 3; P, phosphorylation; ROS, reactive oxygen species; TIM23, translocase of the inner mitochondrial membrane 23; ΔΨm, mitochondrial membrane potential.

Taken together, our results suggested that MOL extract pretreatment in Aβ1-42-treated SH-SY5Y cells could improve the ΔΨm, probably by restoration of the expression of TIMM23 and NDUFS3 proteins, which leads to attenuation of oxidative stress as shown by reducing DHE levels. The reduction of oxidative stress and inhibition of the apoptosis cascade by MOL extract could be mediated *via* the activation of Akt, leading to suppressed expression of Bax and cleaved caspase-3 and enhanced expression of Bcl-2 (Fig. 8). However, due to several bioactive substances in MOL extract, the lack of information regarding the beneficial properties of the pure bioactive substances present in water-extracted MOL associated with the neuroprotective effect is our limitation and remains to be investigated. MOL extracts contain several bioactive compounds and the antioxidant properties are often attributed to the presence of its phytochemicals, particularly the polyphenolic compounds (27,51,52). While our study demonstrated the neuroprotective effect of a cold-water-soluble MOL extract, our limitation is the lack of information regarding the specific beneficial properties of pure bioactive substances present in the water-extracted MOL associated with the observed effects. Further investigation is needed to fully elucidate the potential therapeutic benefits of these compounds.

CONCLUSION

AD is among the common neurodegenerative diseases that are expected to have a significant social impact and no definite treatment is currently available besides treatments to facilitate associated symptoms and conditions. Therefore, it is imperative to find potential alternatives to control the progression of the disease. As such, utilizing the medicinal properties of plants like *Moringa oleifera* as supplementation intake or nutraceuticals is one of the strategic approaches. The present study demonstrates possible mechanisms for the neuroprotection of MOL extract in a cellular model of AD. MOL extract pretreatment may protect neuronal cells against Aβ1-42-induced cell death through activation of the Akt/antioxidant and anti-apoptotic enzymes pathway. Additional studies on Akt-related defective mitophagy may provide further information regarding the application of MOL extract as a neuroprotective agent in AD.

Conflict of interest statement

The authors declared no conflicts of interest in this study.

Authors' contributions

T. Balit Performed the experiments and the statistical analyses, interpreted and analyzed the data, reviewed the literature, and wrote the original draft of the article. C. Thonabulsombat contributed to the interpretation and analysis of the data, reviewing and editing the manuscript. P. Dharmasaroja contributed to the research design, funding acquisition, review of the literature, interpretation and analysis of the data, supervision of the statistical analyses, writing, reviewing, and editing the manuscript.

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