

Oroxylum indicum (L.) extract protects human neuroblastoma SH-SY5Y cells against β -amyloid-induced cell injury

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Abstract. It has been reported that amyloid β peptide, the major component of senile plaques, serves a critical role in the development and progression of Alzheimer's disease (AD) by generating reactive oxygen species (ROS), leading to oxidative stress. The aim of the present study was to investigate the protective effect of *Oroxylum indicum* (L.) extract against A β 25-35-induced oxidative stress and cell injury using SH-SY5Y cells as a model, and at exploring the underlying mechanisms. The results revealed that the exposure of cells to 20 μ M A β 25-35 significantly increased cellular oxidative stress, as evidenced by the increased ROS levels. A β 25-35 treatment also increased caspase-3/7 activity and lactate dehydrogenase (LDH) release, and caused viability loss. *Oroxylum indicum* treatment not only attenuated the generation of ROS and suppressed caspase-3/7 activity but also reduced the neurotoxicity of A β 25-35 in a concentration-dependent manner, as evidenced by the increased cell viability and decreased LDH release. Treatment with *Oroxylum indicum* also increased superoxide dismutase (SOD) and catalase (CAT) activity, increased the phosphorylation of Akt and cAMP-responsive element binding protein (CREB), and contributed to the upregulation of Bcl-2 protein. In combination, these results indicated that *Oroxylum indicum* extract could protect SH-SY5Y cells against A β 25-35-induced cell injury, at least partly, by inhibiting oxidative stress, increasing SOD and CAT activity, attenuating caspase 3/7 activity and promoting the cell survival pathway, Akt/CREB/Bcl-2. The approach used in the present study may also be useful for preventing the neurotoxicity induced by A β in AD and related

neurodegenerative diseases. Further studies investigating the activity of *Oroxylum indicum* extract *in vivo* are now required.

Introduction

Alzheimer's disease (AD) is a multifactorial neurodegenerative disorder that mostly affects the elderly. Prevalence studies have revealed that >35 million people are suffering from AD worldwide, and this number is predicted to reach >100 million by the year 2050, if new preventive or neuroprotective therapies do not emerge (1). The neuropathology of AD is characterized by extracellular deposition of amyloid β (A β) plaques, and intracellular neurofibrillary tangles and loss of neurons in the brain (2). Although the mechanisms of neuronal cell death in AD still remain unknown, the deposition of A β has been reported to be neurotoxic both *in vitro* and *in vivo*, involving reactive oxygen species (ROS) generation, inflammation, and an increase in intracellular Ca²⁺ (3-8). A large number of studies have confirmed that the excessive production of A β itself leads to A β -induced free radical generation and elevated oxidative stress, leading to cell death (9). Thus, one promising preventive or therapeutic intervention in AD may be to attenuate or suppress oxidative stress-dependent, A β -mediated cytotoxicity.

Plants are a major component of diets that possess neuroprotective effects, including antioxidant and anti-inflammatory effects, and can improve memory and cognitive functions (10-13). The most important advantages of the medicinal use of plants are their minimal side effects and their relatively low cost, as compared to synthetic medicines. According to the World Health Organization, ~80% of the world's population currently uses medicinal plants for their primary healthcare (14), and the current trend is to conduct investigations into plant-based medicines. Therefore, searching for plants that can attenuate oxidative stress might be a useful strategy for preventing and/or treating A β -induced neurotoxicity. *Oroxylum indicum* (L.), also known as 'broken bones plant', 'Indian trumpet flower', 'Shyonaka' and 'Midnight horror', belongs to the Bignoniaceae family, which is widely distributed in tropical countries, such as India, Taiwan, Cambodia, Laos, Myanmar, Indonesia, Malaysia, Vietnam, Nepal, China, the Philippines and Thailand (15). *Oroxylum indicum* has been used in traditional herbal medicine

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in Asian countries for the treatment of various diseases over several centuries (16). Studies have indicated that almost all parts of the plant possesses medicinal properties (15), mainly antioxidant, anti-inflammatory, anticancer and immunomodulatory properties. Other effects, such as antibacterial and gastro-protective, have also been reported. The principal active components of this plant are the flavonoids chrysin, oroxylene A and baicalein (15,17). Other secondary metabolites, such as triterpene, carboxylic acid, ursolic acid, glycosides, tannins, alkaloids and terpenoids, have also been identified. Although many medicinal properties of *Oroxylum indicum* have been demonstrated, the effects of *Oroxylum indicum* extract on A β -induced oxidative stress have not, to our knowledge, been investigated. The aim of the present study was to investigate the protective effect of *Oroxylum indicum* (L.) fruit pod extract against A β 25-35-induced oxidative stress and injury in SH-SY5Y cells. The mechanisms underlying its neuroprotection were also investigated. Phenolic compounds and flavonoids have been shown to be very good antioxidants (18), thus their concentration in *Oroxylum indicum* extract was also determined. The fruit pod of *Oroxylum indicum* was selected for the present study, since this part is more readily edible than other parts (17). A β 25-35 was chosen because this fragment is an active toxic fragment of A β 1-42 peptides (19), and it has been reported that A β 25-35 and A β 1-42 have similar effects in inducing neuronal cell death and neuritic atrophy (20,21). SH-SY5Y cells were selected as a model, since they are commonly used in AD research and differentiated SH-SY5Y cells have displayed properties similar to those of mature neurons (22).

Materials and methods

Chemicals and antibodies. SH-SY5Y cell line was purchased from the American Type Culture Collection (Manassas, VA, USA; catalog number CRL-2266). Cell culture reagents, including penicillin/streptomycin were from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). DMEM/F12 medium was from Thermo Fisher Scientific, Inc. and fetal bovine serum (FBS) from Gemini Bio-Products (West Sacramento, CA, USA). Non-essential amino acids and all-trans retinoic acid (RA) were from Merck KGaA (Darmstadt, Germany). The ROS detection kit was also obtained from Merck KGaA. The catalase activity kit was from Biovision Inc. (Milpitas, CA, USA), and the superoxide dismutase (SOD) activity kit from Cayman Chemical Company (Ann Arbor, MI, USA). The lactate dehydrogenase (LDH) kit, together with A β 25-35 and 3-(3,4-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from Merck KGaA. The LDH assay kit was from Thermo Fisher Scientific, Inc., and the caspase 3/7 activity kit was from Promega Corporation (Madison, WI, USA). Antibodies against total- and phosphor(p)-Akt, and p-cAMP-responsive element binding protein (CREB) were from Cell Signaling Technology, Inc., (Danvers, MA, USA). Secondary anti-rabbit or anti-mouse antibodies and ECL detection kits were from GE Healthcare (Chicago, IL, USA). RIPA buffer, protease and phosphatase inhibitor cocktail, calcein-AM and quercetin were obtained from Merck KGaA. The BCA protein assay was from Thermo Fisher Scientific, Inc.

Plant material and extraction. Fruits of *Oroxylum indicum* were collected from Maha Sarakham Province, Thailand, in July 2016. Species identification was performed by members of the Applied Thai Traditional Medicine Department, Faculty of Medicine, Mahasarakham University. A specimen was deposited at the herbarium at the Faculty of Science, Mahasarakham University (specimen no. MSUT_7234). An ethanolic extract of *Oroxylum indicum* was prepared by drying the fruits, then weighing and chopping them, and macerating them in 95% (v/v) ethanol for 7 days at room temperature (RT). The extract was then filtered, concentrated using a rotary evaporator and lyophilized. The % yield of extract was 12.89% per dry weight of *Oroxylum indicum* fruits.

Determination of total flavonoid content. The total flavonoid content of *Oroxylum indicum* crude extract was determined by the aluminum chloride colorimetric method. In brief, 100 μ l of 1 mg/ml *Oroxylum indicum* crude extract was mixed with 0.9 ml flavonoid mixture (10% aluminum hydroxide, 1 M potassium acetate; dilution, 0.1, 0.1 and 4.3 ml). The mixture was incubated for 30 min at RT in the dark, and the absorbance intensity was measured at 450 nm. The total flavonoid content was calculated from a calibration curve and the result was expressed as mg rutin equivalent per g dry weight.

Determination of total phenol content. The total phenolic content of the *Oroxylum indicum* extract was determined using the Folin-Ciocalteu method. In brief, 100 μ l of 1 mg/ml *Oroxylum indicum* crude extract was mixed thoroughly with 0.45 ml Folin-Ciocalteu reagent for 5 min, followed by the addition of 0.45 ml of 60 g/l sodium bicarbonate. The mixture was kept in the dark for a further 1 h at RT, and the absorbance intensity was measured at 650 nm. The total phenolic content was calculated from the calibration curve, and the results were expressed as mg of gallic acid equivalent per g dry weight.

Cell culture. Human neuroblastoma SH-SY5Y cells were maintained in DMEM/F12 medium supplemented with 10% FBS, 1% penicillin-streptomycin and 1% non-essential amino acids at 37°C in a humidified atmosphere containing 5% CO₂. The culture medium was changed every 3 days. Cells were plated at an appropriate density according to each experiment. Cells were differentiated with 10 μ M all-trans retinoic acid over 6 days prior to treatments. At the beginning of each experiment, the culture medium in each well was completely removed and replaced with fresh medium containing A β with or without *Oroxylum* extract.

Preparation of A β 25-35 stock solution. A β 25-35 peptide was dissolved in deionized distilled water as a 1 mM stock solution and incubated at 37°C for 3 days. The solution was aliquoted into 1-ml tubes, kept at -20°C and thawed for subsequent use.

Cell viability assay. The *in vitro* cytotoxicity of A β 25-35 and *Oroxylum indicum* were determined using the MTT assay. SH-SY5Y cells were plated in 96-well plates at a density of 1x10⁴ cells/well and cultured as described above. Cells were treated with various concentrations of A β 25-35 (10-30 μ M) and *Oroxylum indicum* (0-100 μ g/ml) in 1% FBS for 24 h. To determine whether *Oroxylum indicum* protects against

A β -induced neurotoxicity, cells were treated with A β 25-35 with or without of *Oroxylum indicum* extract in 1% FBS for 24 h. Following 24 h of treatment, the medium was removed and replaced with MTT reagent at a final concentration of 0.5 mg/ml. Cells were then incubated for 4 h at 37°C in 5% CO₂ in an incubator. Following incubation, MTT reagent was aspirated and 100 μ l dimethyl sulfoxide was added to dissolve the insoluble purple formazan product. Absorbance was determined at 570 nm using a Synergy-4 plate reader (BioTek Instruments, Inc Winooski, VT, USA). Results were expressed as a percentage of the control.

Intracellular ROS assay. Cells were seeded at a density of 1x10⁴ cells/well in 96-well plates and cultured as described above. After 24 h, intracellular ROS levels were measured using the fluorescent probe 2',7'-dichlorofluorescein, as previously described (13). Data were expressed as the percentage of ROS relative to untreated controls.

Analysis of cell injury by the LDH assay. Cells were plated at a density 1x10⁴ cells/well in 96-well plates. Cells were then cultured and treated as described above. Following 24 h of treatment, A β -induced cell injury was measured by determining how much of the intracellular enzyme LDH had been released into the culture medium. Culture medium (100 μ l) was collected from each well and transferred to a new 96 well plate, and 100 μ l reaction mixture was added to each well and incubated for 30 min at 37°C. Absorbance was measured at 492 nm using a microplate reader. The quantity of LDH released was then expressed as a percentage of the untreated control.

Determination of catalase (CAT) activity assay. Cells were plated at a density 1x10⁵ cells/well in 6-well plates. Overnight cultured cells were treated as described above. Following 24 h of treatment, cells were harvested with a rubber policeman and collected by centrifugation (2,000 x g for 10 min at 4°C). The cell pellets were homogenized in cold assay buffer and centrifuged at 10,000 x g for 15 min at 4°C. The supernatant was also collected for the assay. Catalase activity was determined using a commercially available assay kit (Biovision Inc.), according to the manufacturer's instructions.

SOD activity assay. Cells (1x10⁵ cells/well) were plated in 6-well plates and were then cultured and treated as described above. Following 24 h of treatment, cells were harvested as described for the CAT activity assay. Cell pellets were then homogenized in 20 mM cold HEPES buffer, and centrifuged at 1,500 x g for 5 min at 4°C. The supernatant was also collected for the assay. Superoxide dismutase activity was measured using an assay kit from Cayman Chemical, according to the manufacturer's instructions. Results were expressed as a percentage of the untreated control.

Western blotting. Cells (1x10⁵ cells/well) were plated in 6-well plates, and then cultured and treated as described above. Following treatment, cells were collected and total protein concentration was determined using a BCA kit. Equal amounts of proteins were separated by 4-20% SDS-polyacrylamide gel and transferred onto nitrocellulose membranes. The

membranes were then incubated with primary antibodies against Bcl-2 (1:1,000), p-Akt, total Akt (1:1,000), p-CREB (1:1,000) and actin (1:5,000) overnight at 4°C. The membranes were then washed with TBST (Tris-buffered saline, 0.1% Tween 20), and probed with secondary antibody conjugated with HRP for 1 h at RT. Protein bands were detected using an enhanced chemiluminescence detection kit, and results were expressed as a fold change of the untreated control.

Detection of caspase-3/7 activity in cell culture. Caspase-3/7 activity was measured using Caspase-Glo[®] 3/7 kits from Promega Corporation, according to the manufacturer's instructions. Briefly, the caspase-GloR 3/7 buffer and lyophilized caspase-GloR 3/7 substrate were equilibrated at RT. The contents of the caspase-GloR 3/7 buffer were transferred into the bottle containing caspase-GloR3/7 substrate. Equal volumes of reaction mixture were added to samples and incubated for 30 min-2 h prior to the luminescence measurement. Results were expressed as a percentage of the untreated control.

Statistical analysis. All data are expressed as the mean \pm SEM from at least three independent experiments performed in triplicate. Multiple comparisons of data were evaluated by one-way ANOVA followed by Bonferroni post-hoc test. A P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of A β 25-35 on the viability of differentiated and undifferentiated SH-SY5Y cells. Since the SH-SY5Y cell line shares only a few properties with mature neurons (23), it is important to differentiate these cells with retinoic acid, so that they are comparable to *in vivo* models. Undifferentiated SH-SY5Y cells have been used as a model of cytotoxicity in several studies (24-26), but the comparative cytotoxic effects of A β on cell survival of RA-differentiated and undifferentiated SH-SY5Y cells has not yet been reported. Therefore, the purpose of this study was to compare the *in vitro* cytotoxicity of A β 25-35 between differentiated and undifferentiated SH-SY5Y cells. Both differentiated and undifferentiated cells were treated with various concentrations of A β 25-35 (0-30 μ M). As shown in Fig. 1A, A β 25-35 treatments were toxic to both cell groups, beginning at 10 μ M for undifferentiated cells and 20 μ M for differentiated cells. Although undifferentiated SH-SY5Y cells were more susceptible to A β 25-35 than differentiated cells, the differentiated cells possess more neuron-like properties, including neurite outgrowth and morphological changes of neurons in the brain. Therefore, RA-differentiated SH-SY5Y cells were selected for subsequent assays, and A β 25-35 was used at a concentration of 20 μ M.

Effects of *Oroxylum indicum* on the viability of SH-SY5Y cells. To determine whether *Oroxylum indicum* has any effect on the viability of SH-SY5Y cells, the cells were treated with various concentrations of the extract (0-100 μ g/ml). The results indicated that *Oroxylum indicum* extract at concentrations of 25 and 50 μ g/ml increased cell viability (139.45 \pm 7.89 and 130.61 \pm 5.83% of control value, respectively) and that concentrations of up to 100 μ g/ml were non-toxic to SH-SY5Y cells

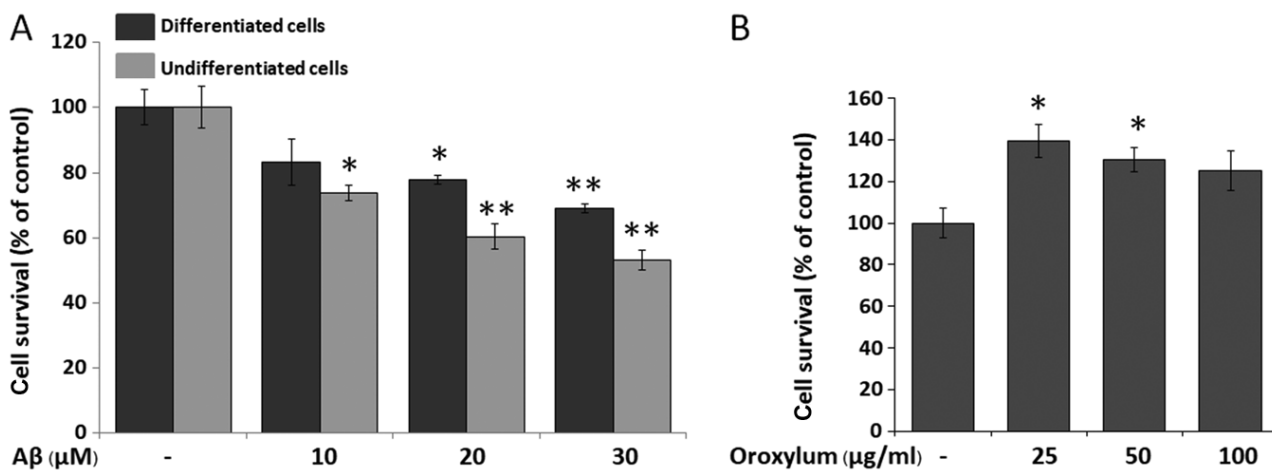


Figure 1. (A) Effect of Aβ25-35 on the viability of differentiated and undifferentiated SH-SY5Y cells and (B) effect of *Oroxylum indicum* on the viability of differentiated SH-SY5Y cells. Following 24 h of treatment, cell viability was determined by an MTT assay. Data are presented as the mean ± SEM from three independent experiments (n=3 per experiment); *P<0.05 and **P<0.01 vs. control group. Aβ, Amyloid-β; MTT, 3-(3,4-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

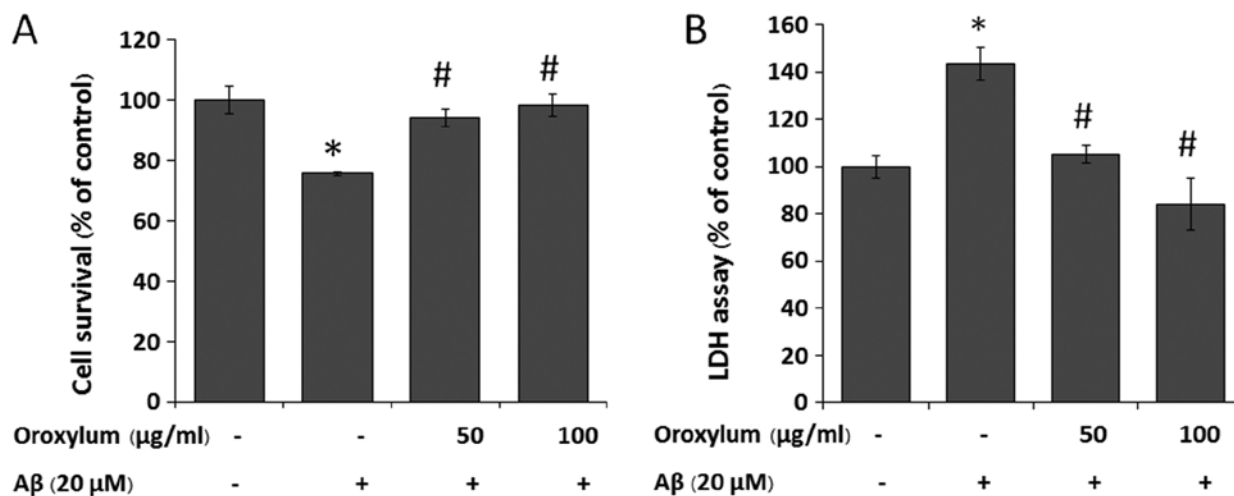


Figure 2. Effect of *Oroxylum indicum* on Aβ-induced cytotoxicity in SH-SY5Y cells. (A) Following 24 h of treatment, cell viability was determined by an MTT assay. (B) LDH release was determined using a cytotoxicity detection kit. Data are presented as the mean ± SEM from three independent experiments; *P<0.05 vs. control group; #P<0.05 vs. Aβ-treated group. Aβ, Amyloid-β; MTT, 3-(3,4-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; LDH, lactate dehydrogenase.

(Fig. 1B). Therefore, the highest non-toxic concentrations of *Oroxylum indicum* (50 and 100 μg/ml) were used in subsequent assays.

Oroxylum indicum protected SH-SY5Y cells against Aβ25-35-induced cytotoxicity. To determine the effect of *Oroxylum indicum* on Aβ25-35-induced cytotoxicity, SH-SY5Y cells were challenged with 20 μM Aβ25-35 in the presence or absence of 50 and 100 μg/ml *Oroxylum indicum* extract for 24 h. As shown in Fig. 2A, treatment of SH-SY5Y cells with 20 μM Aβ25-35 for 24 h induced cytotoxicity, as demonstrated by a cell viability reduction to 76.83±0.67%, when compared with the control group. When the cells were treated with *Oroxylum indicum* extract at concentrations of 50 and 100 μg/ml, cell viability was restored to 94.14±2.79 and 98.35±3.74%, respectively, indicating a concentration-dependent cytoprotective effect.

To further confirm the cytoprotective effect of *Oroxylum indicum*, LDH, another indicator of cell toxicity, was also examined. The results were similar to those determined by the MTT assay. The exposure of SH-SY5Y cells to 20 μM Aβ25-35 resulted in a 1.45-fold increase in LDH release in the medium, when compared to that in the control group. Treatment of cells with 50 and 100 μg/ml *Oroxylum indicum* extract reduced Aβ25-35-induced LDH release in a concentration-dependent manner (Fig. 2B).

Oroxylum indicum inhibited Aβ25-35-induced intracellular accumulation of ROS. ROS play a critical role in Aβ-dependent cell death. Therefore, in the present study, the effect of *Oroxylum indicum* on Aβ25-35-induced intracellular ROS production was examined. SH-SY5Y cells exposed to Aβ25-35 for 24 h exhibited elevated ROS levels (152.4±3.53%, as compared to the control group) (Fig. 3A).

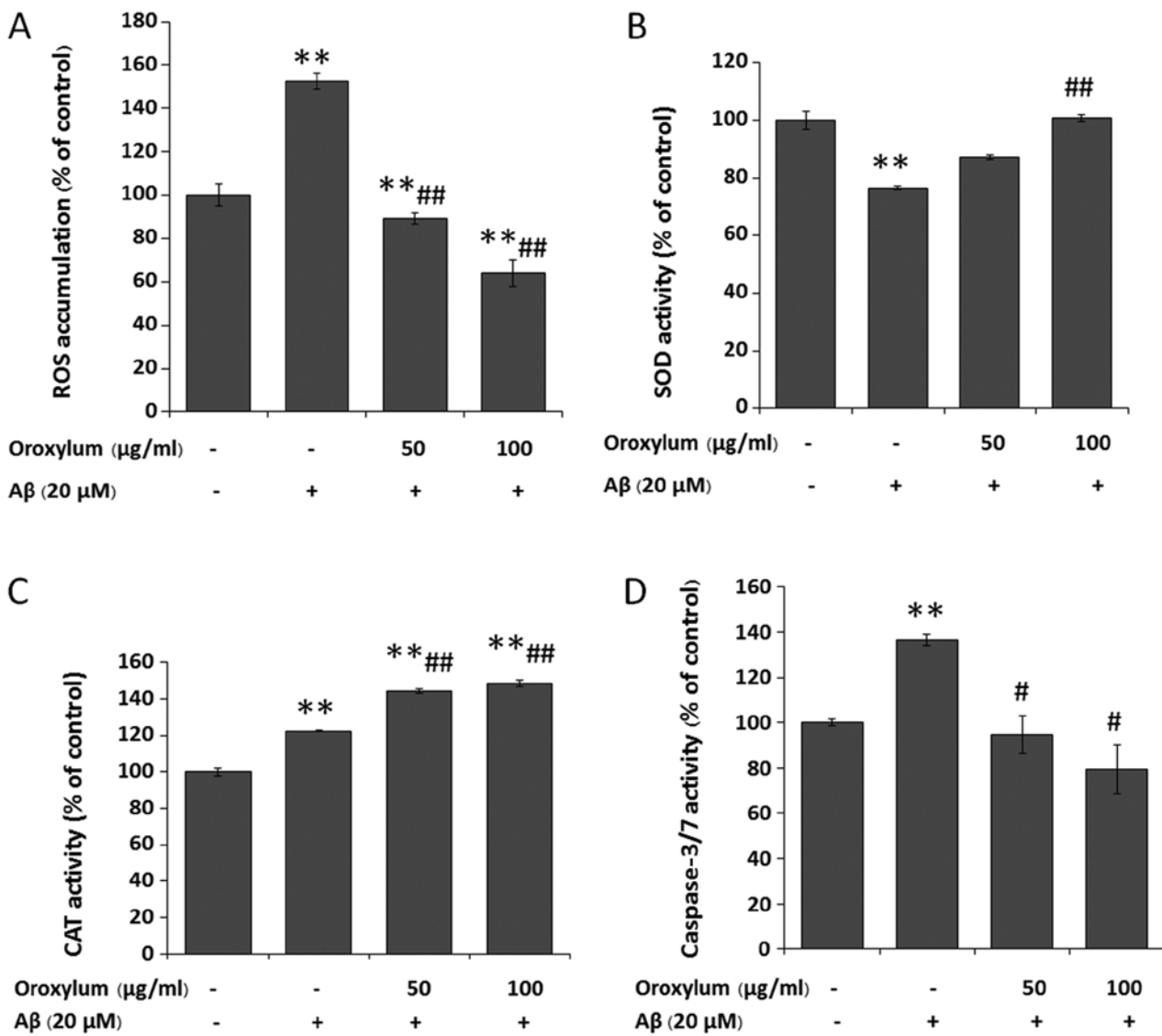


Figure 3. Effect of *Oroxyllum indicum* on (A) Aβ-induced free radical production in SH-SY5Y cells, as determined using the fluorescent probe DCF, and on Aβ-induced (B) SOD (C) CAT and (D) caspase-3/7 activity. Data represent mean ± SEM from three independent experiments; **P<0.01 vs. control group; #P<0.05 and ##P<0.01 vs. Aβ treatment group. Aβ, Amyloid-β; DCF, 2',7'-dichlorofluorescein; SOD, superoxide dismutase; CAT, catalase.

When the cells were treated with *Oroxyllum indicum* extract at concentrations of 50 and 100 μg/ml, there was a significant and concentration-dependent inhibition of Aβ25-35-induced intracellular ROS levels (89.17±2.46 and 64.25±6.21%, respectively).

Oroxyllum indicum increased the activity of anti-oxidative enzymes challenged by Aβ25-35. To determine whether the cytoprotective effect of *Oroxyllum indicum* is associated with the activity of anti-oxidative enzymes, SOD and CAT activity was determined. Following exposure to Aβ25-35 for 24 h, SOD activity was significantly decreased to 76.48±0.6% of the control value. Treatment of cells with 50 and 100 μg/ml *Oroxyllum indicum* extract for 24 h restored SOD activity to 87.30±0.81 and 100.76±1.19%, respectively (Fig. 3B). Following exposure to Aβ25-35, CAT activity was significantly increased to 122.45±0.029% of the control value. CAT activity following treatment with *Oroxyllum indicum* extract was significantly increased beyond that of the

Aβ treatment and control groups (144.15 and 148.4%, respectively) (Fig. 3C).

Oroxyllum indicum reduced Aβ25-35-induced caspase-3/7 activity. It has been reported that Aβ-induced neuronal cell death through the activation of the caspase pathway (27-31). To study the protective mechanism of *Oroxyllum indicum*, caspase-3/7 activity was measured. Following Aβ25-35 treatment, caspase-3/7 activity significantly increased to levels that were 1.4-fold higher than that of the control group. However, the induction of caspase-3/7 activity was blocked in the presence of *Oroxyllum indicum* (Fig. 3D)

Oroxyllum indicum enhanced the phosphorylation of Akt. The activation of Akt has been associated with the inhibition of the apoptotic cleavage of caspases, as well as the promotion of neuronal survival (32-35). We therefore hypothesized that *Oroxyllum indicum* can modulate the signaling of Akt. The results showed that treatment with Aβ25-35 for 15 min

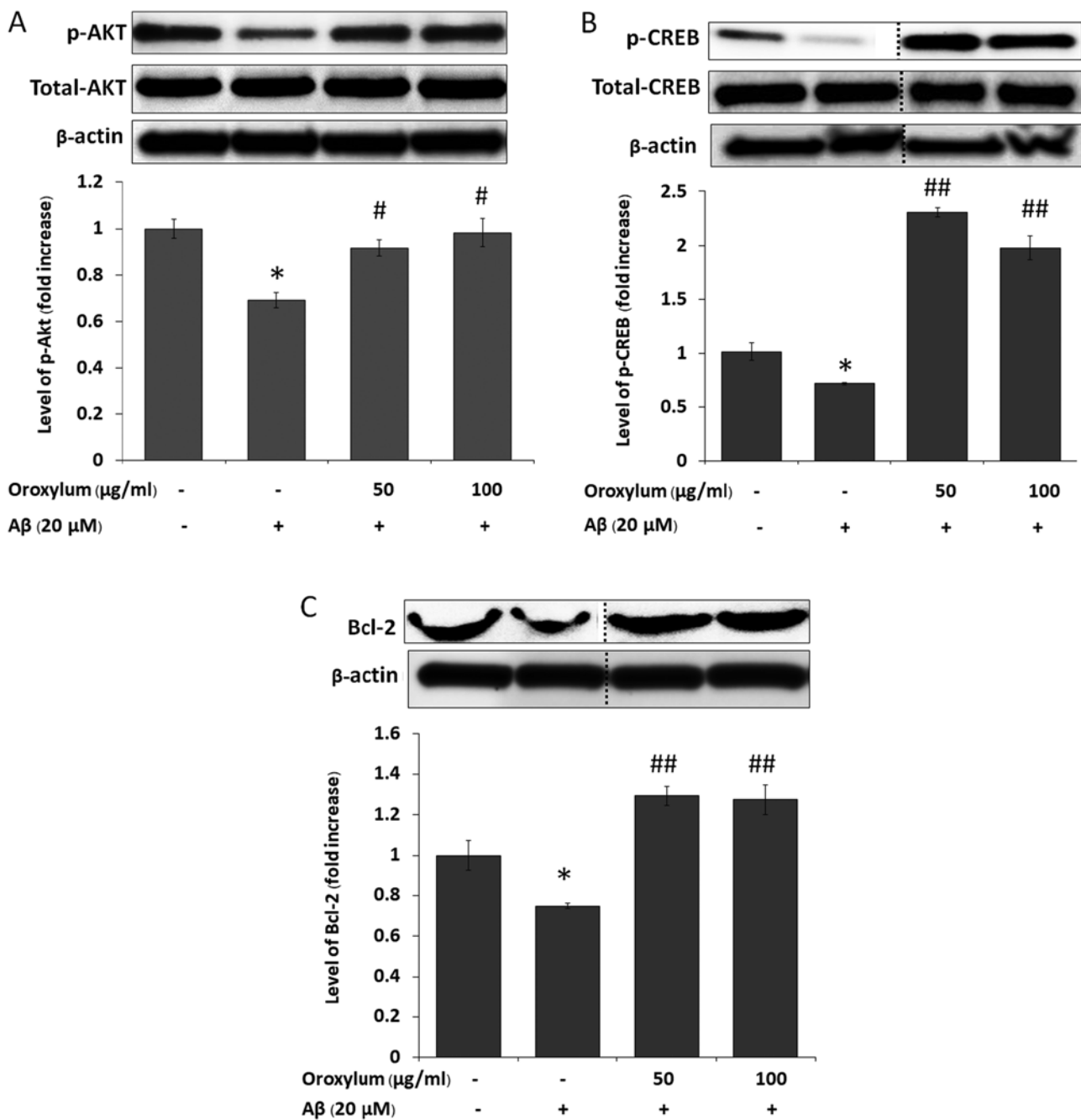


Figure 4. Effect of *Oroxylum indicum* on (A) p-Akt, (B) p-CREB and (C) Bcl-2 expression. Following treatment, total cell lysate was collected and the levels of anti-Akt, anti-p-Akt, anti-CREB, anti-p-CREB and anti-Bcl-2 were determined by western blotting. The levels of p-Akt, p-CREB and Bcl-2 were normalized to β -actin. Histograms represent the fold-increase relative to that of the untreated control. The dotted lines indicate the borders of cropped images between groups in the same blot. Data represent the mean \pm SEM from three independent experiments; * $P < 0.05$ when compared with control group; # $P < 0.05$ and ## $P < 0.01$ when compared with A β treatment group. p, phosphor; CREB, cAMP-responsive element binding protein; A β , Amyloid- β .

significantly decreased p-Akt, as compared to the untreated control (Fig. 4A). *Oroxylum indicum* treatments significantly increased the phosphorylation of Akt, as compared to A β 25-35 treatment.

Oroxylum indicum enhanced the phosphorylation of CREB. It has been reported that one element of the downstream signaling of Akt is CREB the transcription factor that regulates neuronal survival (35). We therefore examined whether CREB phosphorylation increased in SH-SY5Y cells following treatment with A β 25-35 in the presence of *Oroxylum indicum*. When

cells were treated with A β 25-35 for 1 h, CREB phosphorylation was significantly decreased, when compared with the untreated control. The phosphorylation of CREB was significantly increased following treatment with *Oroxylum indicum*, when compared to both the A β 25-35 treatment and control groups (Fig. 4B).

Oroxylum indicum enhanced Bcl-2 expression. The transcription factor CREB has been identified as a positive regulator of Bcl-2 (an apoptosis suppressor gene) expression (36,37). Therefore, these results demonstrated that A β 25-35 ameliorated

Bcl-2 expression, as compared with the control group. Treatment with *Oroxylum indicum* extract for 24 h caused a significant increase in Bcl-2 expression, as compared to the A β 25-35 group (Fig. 4C).

Total phenolic and flavonoid content of extracts from fruit pot of Oroxylum indicum. The *Oroxylum indicum* fruit extract was standardized using colorimetric methods to quantify the total phenolic content using gallic acid as a standard, and the total flavonoid content using rutin as a standard. The values obtained were 10.50 \pm 0.68 and 17.08 \pm 0.85 mg/g of dried extract, respectively (Table I).

Discussion

Accumulation of amyloid plaques in the brains of AD patients has been reported to induce cytotoxicity mediated through the generation of ROS and elevated oxidative stress (9). Therefore, the attenuation or suppression of this oxidative stress-dependent, A β -mediated cytotoxicity may be a promising strategy for preventive or therapeutic intervention in AD. Recently, natural antioxidants from medicinal and edible plants have attracted considerable attention as promising agents for reducing the risk of oxidative stress-induced neurological diseases. In the present study, RA-differentiated SH-SY5Y cells were selected as a model, since they displayed properties similar to those of mature neurons. It was demonstrated that A β 25-35-treated cells exhibited increased ROS production, which was consistent with previous reports (24,38,39). Treatment with *Oroxylum indicum* extract inhibited A β -induced ROS production in a concentration-dependent manner, indicating an antioxidant effect of *Oroxylum indicum*. Phenolic and flavonoid compounds in plants are reported to be very good antioxidants. Phenolics are effective hydrogen donors, while flavonoids act as scavengers of various oxidizing species, such as hydroxyl radicals, peroxy radicals and the superoxide anion (40). Therefore, it was reasonable to determine the quantities of these phytochemical classes in *Oroxylum indicum*. In the present study, it was demonstrated that the total phenolic and flavonoid content of *Oroxylum indicum* was 10.50 \pm 0.68 and 17.08 \pm 0.85 mg/g, respectively. Although *Oroxylum indicum* extract contained only low levels of phenolics and flavonoids, a marked decrease in ROS production was observed following treatment with the extract, suggesting that there is another antioxidant defense mechanism that can eliminate ROS. Under normal physiological conditions, ROS production is balanced by endogenous cellular antioxidant systems, including the cooperative action of SOD and CAT (41). SOD is the first line of defense against free radicals, its ROS-metabolizing activity occurring due to catalytic dismutation of the superoxide anion radical (O $_2^{\cdot-}$) into O $_2$ and H $_2$ O $_2$. H $_2$ O $_2$ is then converted into O $_2$ and H $_2$ O by CAT, another major primary antioxidant defense component (41). It was demonstrated herein that treatment with A β 25-35 decreases SOD activity, which was consistent with a previous report (42). A A β 25-35 treatment-induced SOD activity reduction in cultured cells is possibly due to its metabolites or a direct toxic effect of A β 25-35; however, unknown factors other than severe cell damage may also cause this effect. Of note, an increase in CAT activity was observed following exposure of cells to A β 25-35, which might have been either a direct

Table I. Total phenolic and flavonoid content of *Oroxylum indicum* fruit extract.

Total phenolic/flavonoid content	Concentrations (mg/g)
Total phenolic content ^a	30.50 \pm 0.68
Total flavonoid content ^b	17.08 \pm 0.85

^amg gallic acid equivalent/g dry weight. ^bmg rutin equivalent/g dry weight. Values are presented as the mean of three biological replicates.

induction or a compensatory mechanism against A β insult. When cells are treated with *Oroxylum indicum*, SOD and CAT activity is increased, suggesting that *Oroxylum indicum* may stimulate cells to increase SOD and CAT expression, leading to an increase in their enzymatic activity in order to cope with A β -induced ROS production or may result from its metabolites. Therefore, the mechanism(s) through which *Oroxylum indicum* attenuates ROS production may be due to its phenolic and flavonoid content and/or its ability to increase SOD and CAT enzyme activity.

It has been reported that A β -induced neurotoxicity is related to ROS generation and caspase activation (27-31), so we next investigated the effects of *Oroxylum indicum* on the activation of caspases-3/7, which are known to be effector caspases. The protective effect of *Oroxylum indicum* against A β -induced cytotoxicity was also determined. Treatment with *Oroxylum indicum* not only attenuated A β -induced caspase-3/7 activity, but also protected SH-SY5Y cells against A β -induced cytotoxicity, as determined by the MTT assay. The MTT assay was selected, since it has repeatedly been shown to be a very sensitive indicator of A β -induced cell death (43). The protective effect of *Oroxylum indicum* was further confirmed by LDH assay, another indicator of cell toxicity. The results reported above demonstrated that treatment with *Oroxylum indicum* reduced A β -induced LDH release. This finding indicated that *Oroxylum indicum* extract can protect SH-SY5Y cells against A β -induced cell injury.

The PI3K/Akt pathway is a key signal transduction pathway that mediates cell growth and promotes cell survival (44). The activation of Akt can phosphorylate CREB (45). Phosphorylated CREB is then translocated to the nucleus, where it upregulates the anti-apoptotic protein Bcl-2 (45). As Akt is an upstream signal that regulates p-CREB, and the phosphorylation process is rapid during protein translation, which occurs several hours following treatment, p-Akt and p-CREB were detected at 15 min and 1 h, respectively, and Bcl-2 at 24 h following treatment. Exposure of SH-SY5Y cells to A β decreased p-Akt. This finding was consistent with a previous report, which showed that intraneuronal A β accumulation leads to a decrease in the levels of phospho-Akt (46). Exposure of SH-SY5Y cells to A β also decreased CREB phosphorylation, as well as the protein expression of Bcl-2. However, treatment with *Oroxylum indicum* increased the phosphorylation of Akt and CREB, as well as the expression of Bcl-2 protein. These results suggested that the activation of Akt/CREB/Bcl-2 pathway also plays a critical role in *Oroxylum indicum*-induced neuronal protective effects against A β insult.

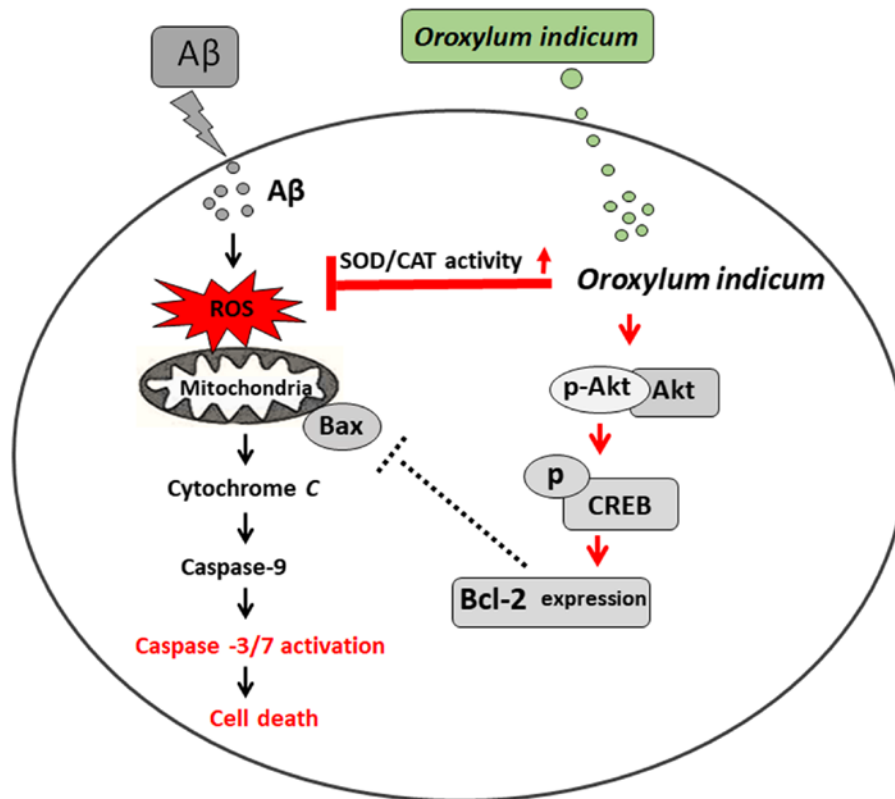


Figure 5. Hypothetical scheme illustrating signaling pathways through which *Oroxylum indicum* protects cells against A β -induced cell injury. It has been reported that A β causes ROS accumulation (52-53). ROS accumulation can induce the release of cytochrome c through several mechanisms. One mechanism is associated with the Bcl-2 family of proteins (Bcl-2, Bcl-X1, Bax and Bid) (54). Once cytochrome c is released, it can bind to caspase-9 to form a complex, which subsequently activates caspases-3/7 (and other caspases) and eventually causes cell death (54). *Oroxylum indicum* also increases SOD and CAT activity, decreases ROS production and decreases caspases-3/7 activity. Increases in SOD and CAT activity might contribute to inhibition of ROS production. If ROS production decreases, the activation of caspases-3/7 may decrease. It has been reported that the Akt/CREB/Bcl-2 pathway is important in promoting cell survival (45). *Oroxylum indicum* also increases the phosphorylation of Akt and CREB, and increases expression of the anti-apoptotic protein Bcl-2. Increasing phosphorylation of Akt might contribute to an increase in the phosphorylation of CREB, resulting in an increase in Bcl-2. Therefore, activation of the Akt/CREB/Bcl-2 pathway might also play a critical role in *Oroxylum indicum*-induced neuronal protective effects against A β insult. Information in red front represents finding from the current study. A β , Amyloid- β ; ROS, reactive oxygen species; SOD, superoxide dismutase; CAT, catalase; CREB, cAMP-responsive element binding protein.

To the best of our knowledge, there is no published study regarding the effects of *Oroxylum indicum* on A β exposure in neuronal cells. The present study was the first to show that *Oroxylum indicum* extract protects SH-SY5Y cells against A β ₂₅₋₃₅-induced cell injury. Although a cell line was used in this study, several studies have demonstrated the effects of *Oroxylum indicum* extract *in vivo*, including its protective effects against paracetamol-induced liver damage in experimental rats and cisplatin-induced renal injury in Wistar male albino rats, its hepatoprotective activity against CCl₄-induced liver damage in rats, as well as its anti-central nervous system-depressant activity in an animal model (47-51). We hope that this finding will encourage further investigations into the activity of *Oroxylum indicum* in AD and other neurodegenerative diseases.

In conclusion, the results of the present study suggested that *Oroxylum indicum* extract protects SH-SY5Y cells against A β ₂₅₋₃₅-induced injury, at least partly, by inhibiting oxidative stress, increasing SOD and CAT activity, attenuating caspase 3/7 activity and promoting cell survival pathways, such as Akt/CREB/Bcl-2 (Fig. 5). The present data suggested that *Oroxylum indicum* could be useful in the prevention of A β -induced neurotoxicity in AD and related neurodegenerative

diseases. Further studies into the activity of *Oroxylum indicum* extract *in vivo* are now required.

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Availability of data and materials

All the data generated and analyzed in the present study are available from the corresponding author on reasonable request.

Authors' contributions

NM was responsible for the conception and design of the study, as well as the acquisition of data and drafting of the manuscript. NM and BB performed the experiments. NM,

JRC and SYL were responsible for data analysis and interpretation, and revising the manuscript. The final version of the manuscript has been read and approved by all authors.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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