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Asiatic acid nullified aluminium toxicity in *in vitro* model of Alzheimer's disease

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1. ABSTRACT

Aluminium (Al) is a ubiquitously distributed environmental toxicant that lacks biological functions; however, its accumulation in the brain has been demonstrated to be linked to several neuropathological conditions particularly Alzheimer's disease (AD). Asiatic acid (AA), a triterpene extracted from *Centella asiatica*, has been reported to cross the blood brain barrier and also displayed antioxidant and neuroprotective activities. The present study was aimed to explore the neuroprotective effect of AA against aluminium maltolate (Al(mal)$_3$) induced neurotoxicity by assessing cell viability, mitochondrial membrane potential, levels of reactive oxygen species (ROS), DNA damage and apoptosis (Hoechst and dual staining, comet assay; expressions of pro-apoptotic, anti-apoptotic and signaling indices) via AKT/GSK-3β signaling pathway in SH-SY 5Y neuroblastoma cells. Pre-treatment with AA significantly enhanced cell viability, attenuated rotenone-induced ROS, mitochondrial membrane dysfunction and apoptosis regulating AKT/GSK-3β signaling pathway. Downregulation of Al induced neurodegeneration may be one of the approaches to control the impairment of metal ion homeostasis leading to neuronal injury in early development of

AD. However, more extensive work in animal model is desirable to confirm its neuroprotective action.

2. INTRODUCTION

Aluminium (Al) is an abundantly distributed metallic element and its exposure gets increased due to lifestyle modifications. It enters into humans through soil, water, food and pharmaceutical agents and accounted to cause various adverse effects on the axonal transport, neurotransmitter synthesis, synaptic transmission, phosphorylation or dephosphorylation of proteins, protein degradation, gene expression and inflammatory responses of mammalian Central Nervous System. Al has been linked to neurotoxicity (1,2) and implicated as a possible causative or contributing factor in neurodegenerative disorders particularly Alzheimer's disease (AD) (3). Maltolate is found in human diets including coffee, chocolate milk, bread, cakes, baked cereals and browned foods. Maltolate readily reacts with Al due to its high affinity and forms aluminium maltolate $Al(mal)_3$ in the intestine (4). $Al(mal)_3$ enhances the levels of Al in the brain, thereby initiating apoptosis by inducing oxidative stress and mitochondrial dysfunction (5).

Centella asiatica (CA), belonging to the family of Apiaceae, also known as Mandookaparni or Brahmi, is considered as a rejuvenating herb in Ayurvedic medicine. CA has been reported to promote cognitive performance due to its wound healing, memory enhancing and anti oxidative, anti inflammatory and anti apoptotic properties (6,7). Asiatic acid (AA), the triterpenoid of CA exhibited its neuroprotective properties in *in vitro* and *in vivo* studies such as glutamate-induced excitotoxicity, beta-amyloid neurotoxicity and rotenone- or H_2O_2-induced injury and mouse model of focal cerebral ischemia (8-11). Patil *et al.*, (12) reported that AA modulated multiple targets associated with amyloid-β precursor protein processing and amyloid-β protein clearance. However, the underlying mechanistic pathways by which AA protects neuronal cell death in $Al(mal)_3$ are largely unknown.

The PI3K/AKT/GSK-3β signaling pathway plays a vital role in promoting neuronal survival by augmenting the cell proliferation and inhibiting apoptosis. SH-SY 5Y, the most widely used human neuroblastoma cell line displays some of the molecular and cellular processes like AD and widely used as a *in vitro* model for various neurodegenerative diseases. Understanding those regulations may provide further insight towards the better understanding of therapeutic applications of AA against $Al(mal)_3$-induced cell death in in SH-SY 5Y cells. Hence, the present study was aimed to shed light on the protective effect of AA by analyzing its antioxidant, mitochondrial protective and antiapoptotic role via AKT/GSK3β signaling pathways against $Al(mal)_3$-induced cytotoxicity in SH-SY 5Y cells which were unexplored till now.

3. MATERIALS AND METHODS

Aluminium chloride hexahydrate, Maltol, Asiatic acid, 2,5-diphenyl tetrazolium bromide (MTT) dye, 2,7-dichlorofluorescein diacetate (DCFHDA), rhodamine 123 (Rh-123), propidium iodide (PI), acridine orange/ethidium bromide (AO/EB), DMEM/F12 cell culture medium, trypsin–EDTA, fetal bovine serum (FBS) and 100X antibiotic and antimycotic solution were purchased from Sigma Chemicals Co. (St. Louis, USA). Bax, Bcl-2, cyto-c, caspase-3, caspase-9, p-AKT, AKT, p-GSK3β, GSK3β, and β-actin antibodies, anti-mouse and anti- rabbit secondary antibodies were procured from Cell Signaling (USA).

3.1. Cell culture

Human neuroblastoma SH-SY 5Y cell line was obtained from National Centre for Cell sciences (NCCS) Pune, India, and cells were grown in DMEM F12 Hams (1:1) medium with 10% fetal bovine serum, 1% antibiotic and antimycotic solution and maintained at 37°C, 5% CO_2. Cell culture medium was changed thrice in a week.

32. Preparation of $Al(mal)_3$

According to the method followed by Berthold *et al.*, (13), $Al(mal)_3$ was synthesized from maltol (3-hydroxy-2-methyl-4-H-pyran-4-one) and aluminium chloride hexahydrate. For 10- 15 g of complex, 40.9. mM (9.9. g) of $AlCl_3$.$6H_2O$ and 122.8. mM (15.5. g) of maltol were dissolved in 160 ml of deionized water by mild heating and the pH was adjusted to 8.3. While heating the mixture to 65°C, precipitate was formed by stirring the solution and after cooling off-white crystals were filtered, washed with acetone and dried in a vacuum-dessicator for overnight.

3.3. MTT Assay

The mitochondrial integrity and proliferation of cells were determined by MTT assay (14). Cells were seeded in 96 well plate at a density of $3x10^3$ cells per well, incubated for 24 h and introduced to different concentrations of $Al(mal)_3$ (100, 200, 400, 500 and 600 µM) and AA (0.0.1, 0.1., 5, 10 and 100 nM) for 24 hours. To evaluate therapeutic efficacy of AA against $Al(mal)_3$ toxicity, cells were pretreated with different concentrations of AA (0.0.1, 0.1., 5, 10 and 100 nM) for 2 h and then incubated with $Al(mal)_3$ (effective dose) for 24 h followed by addition of MTT (5 mg/ml) for 4 h. Media was removed after the incubation, and 100 µL of DMSO was added to dissolve the formazan crystals. The absorbance of formazan product was examined by spectrophotometer at 570 nm using a microplate reader. Based on the results obtained from cell viability

assay, the effective dose of AA against Al(mal)$_3$ toxicity was employed to study the effect of AA by evaluating various parameters.

3.4. Measurement of intracellular ROS levels assay

The levels of ROS found in control and experimental cells were determined by using fluorescence dye DCFH-DA (15). SH-SY 5Y cells (1x 10^5) were pretreated with AA (10 nM) for 2 h and then incubated with Al(mal)$_3$ (400 µM) for 24 h, followed by 25 µM DCFH-DA for 30 mins at 37ºC, washed twice with PBS and visualized using fluorescent microscope. Percentage changes in ROS production of the treated groups were determined by comparing to the untreated control.

3.5. Measurement of mitochondrial membrane potential

MMP changes were determined by the mitochondrial specific fluorescent dye - Rh-123. Cells were cultured in 6-well plate (1x 10^5) and were treated with AA for 2 h and Al(mal)$_3$ for 24 h, followed by incubation of Rh-123 (5 mmol/ml) for 15 minutes (16). Then washed with PBS and fluorescence was quantified by using blue filter (450-490 nm) and fluorescence intensity was measured by using spectrofluorometer at 535 nm.

3.6. Determination of apoptosis using the acridine orange/ethidium bromide dual staining assay

Apoptosis was analyzed by treating the control and experimental cells with fluorescent dyes (AO/EB) and quantified by using fluorescence microscope. Cells were incubated with Al(mal)$_3$ alone, AA and Al(mal)$_3$, AA (10 nM) alone. After incubation period, cells were washed and followed by the addition of AO/EB reagent for 10 mins. Cells were observed by using fluorescence microscopy, live cells show normal green nuclei, whereas early apoptotic cells shows bright red nuclei and late apoptotic cells illustrates orange colored chromatin (17).

3.7. Analysis of cellular and nuclear morphology using Hoechst 33258 staining assay

SH-SY5Y cells were seeded in 6-well plate and then treated with Al(mal)$_3$ and AA. After the treatment, cells were washed and stained with Hoechst 33258 dye for 10 mins in the dark (18). Then dye was removed and the cells were washed three times with PBS. Finally, the cells were visualized under fluorescence microscope.

3.8. Comet assay analysis

Alkaline comet assay was performed as described by Nataraj *et al.*, (18). Cells were washed with PBS, trypsinized and centrifuged at 1200 rpm for 5 mins. Subsequently, 100 µl cell suspension containing 1x 10^5 cells were mixed with 900 µl 0.7.5 % low melting point agarose and instantly spread on frosted microscopic slides precoated with high melting point agarose, leave it to solidification for 10 mins at 4°C. After that cells were immersed in ice-cold lysis solution for 1 h at 4°C. Slides were then placed in freshly prepared electrophoresis buffer for 20 mins to allow DNA unwinding before electrophoresis. Electrophoresis was run for 20 mins at 25 V (300 mA). After electrophoresis, slides were neutralized, washed and stained with propidium iodide (2.5. µg/ml). Then dried slides were observed under fluorescence microscope. The amount of DNA damage was measured by % head DNA, tail length, tail movement and olive tail movement in normal, Al(mal)$_3$, AA+Al(mal)$_3$ and AA alone treated cells.

3.9. Western blot analysis

Cells in 6-well plate were harvested, washed with PBS and lysed in 100 µl lysis buffer (20 mM Tris–HCl, pH 7.4., 150 mM NaCl, 1 mM EDTA, 30 µg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride) and centrifuge at 1000 g for 5 mins at 4°C. The supernatant was preserved and the pellets solubilized in the same volume of lysis buffer kept on ice and vortex for 20 mins followed by pelleting at 10000 g for 10 mins at 4°C and subjected to 12.5.% polyacrylamide gel electrophoresis (18). A total volume of 40 µg of protein was loaded per lane. The separated proteins were blotted onto a nitrocellulose membrane. After blocked for 1 h with 5% BSA, blots were incubated with primary antibody against Bcl-2, Bax at a dilution of 1: 500, cyto c (1: 1000), p-AKT, AKT, p-GSK3β, GSK3β, Caspases-3, and 9 (1:1000) and β-actin (1:1000) overnight at 4°C. After washing, membrane was incubated with anti-rabbit and anti-mouse HRP conjugated secondary antibody (1:2000) and bands were detected by treating the membranes with 3,3′-diaminobenzidine tetrahydrochloride and densitometry was done by using Image J analysis software.

3.10. Statistical analysis

Statistical analysis was performed by one-way analysis of variance followed by Duncan's multiple range test (DMRT) using Statistical Package for the Social Science (SPSS) software package version 12.0. Results were expressed as mean ± SD for four experiments in each group. $p < 0.0.5$ were considered significant.

4. RESULTS

4.1. Effect of AA on Al(mal)$_3$ induced cytotoxicity

Cells exposed to different concentration of Al(mal)$_3$ showed a significant ($p<0.0.5$) and dose

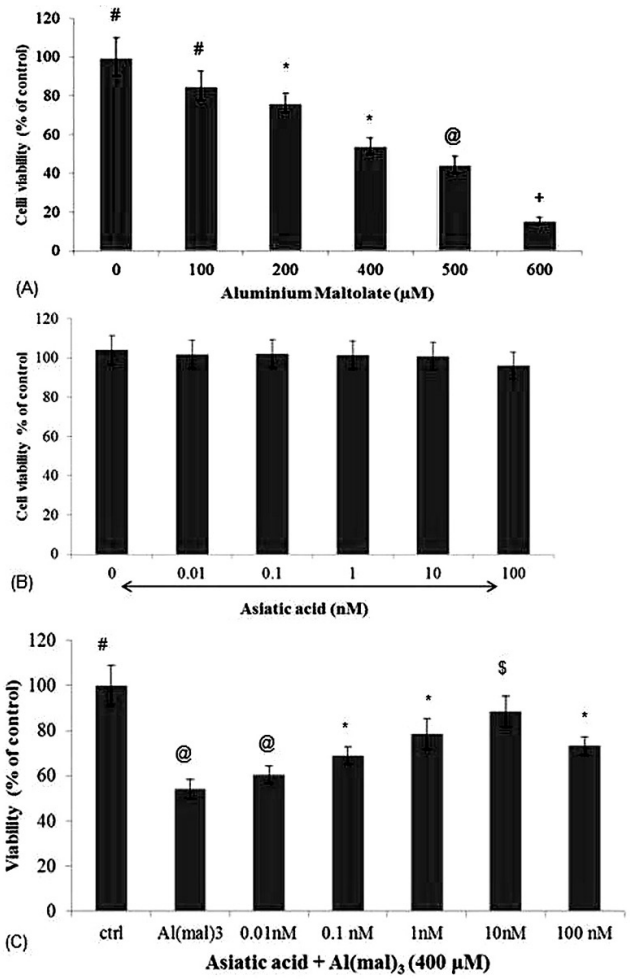

Figure 1. Al(mal)$_3$ (0, 100, 200, 400, 500 and 600 μM) treatment dose dependently diminished the cell viability as compared to control (A). AA (0.01, 0.1, 5 and 10 nM) alone treatment did not altered the cell viability, whereas high dose (100 nM) diminished the cell viability slightly (B). AA pretreatment (0.01, 0.1, 5 and 10 nM) dose dependently enhanced the cell viability against Al(mal)$_3$ toxicity, whereas 100 nM of AA reduced cell viability significantly (C). Values are presented as mean ± SD in four experiments each groups. Values not sharing a common symbol differ significantly ($p<0.05$).

dependent cyto-toxicity. At a dose of 400 μM, it caused ~ 50% of cell death as compared with control and considered as inhibitory dose. Different concentrations of AA (0.0.1, 0.1., 5, 10 and 100 nM) reduced toxicity caused by Al(mal)$_3$ and maximum protection was offered at 10 nM concentration and was taken as protective dose (Figure 1). So 400 μM of Al(mal)$_3$ and 10 nM of AA were used as effective doses and used for further studies.

4.2. Effect of AA on Al(mal)$_3$ induced intracellular ROS production

Exposure of SH-SY 5Y cells to Al(mal)$_3$ (400 μM) increased the green fluorescence significantly ($p<0.0.5$), an indicator of high levels of ROS, whereas 10 nM AA pretreatment to Al(mal)$_3$ (400 μM) exposed cells reduced green fluorescence significantly ($p<0.0.5$), an indicator of decreased intracellular ROS levels (Figure 2).

4.3. Effect of AA on Al(mal)$_3$ induced reduction in MMP

Rh-123 steadily penetrates the normal cells, stains mitochondria and exhibits high fluorescent intensity. Al(mal)$_3$ exposure diminished the intracellular green fluorescence significantly ($p<0.0.5$), indicating the mitochondrial membrane depolarization. However, incubation with 10 nM AA attenuated Al(mal)$_3$ induced mitochondrial membrane depolarization significantly ($p<0.0.5$), which is revealed by increase in fluorescence intensity (Figure 3).

4.4. mpact of AA on Al(mal)$_3$-mediated apoptosis

SH-SY 5Y cells treated with Al(mal)$_3$ and AA were used to determine apoptosis by using AO/EB in dual staining method. In, cells exposed to Al(mal)$_3$ induced the formation of orange/red luminescent apoptotic cells significantly ($p<0.0.5$) whereas

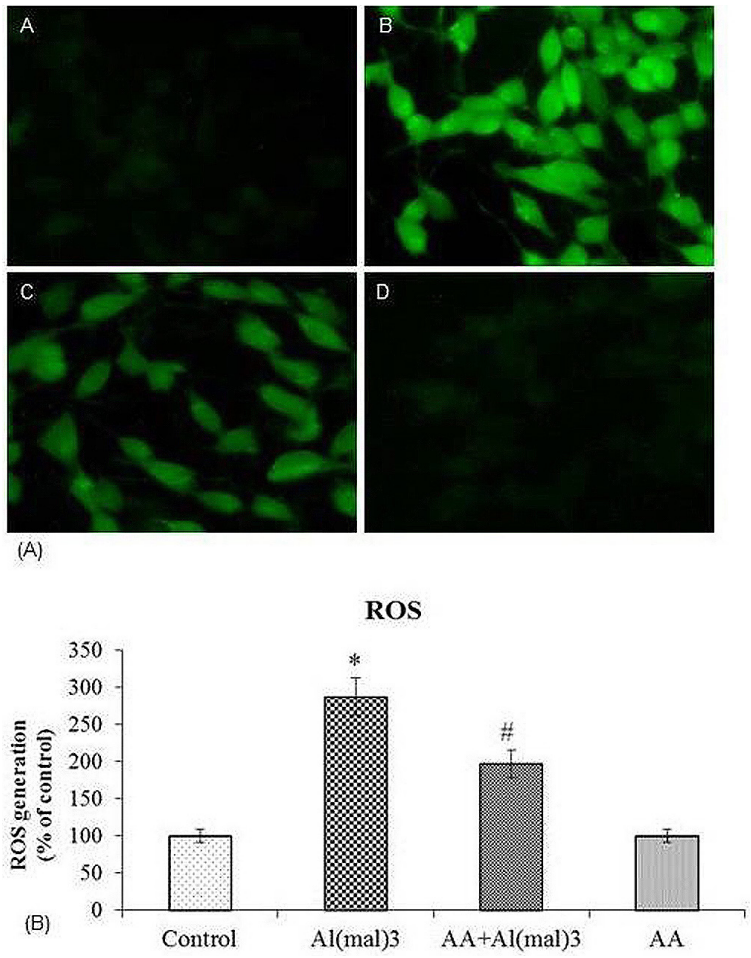

Figure 2. Al(mal)$_3$ treatment enhanced the ROS levels as compared to control, whereas AA pretreatment attenuated the levels of ROS in SH-SY 5Y neuroblastoma cells. Values are given as mean ± SD of four experiments in each group. *p<0.05 compared to control; p<0.05 compared to Al(mal)$_3$ group (Duncan's multiple range test-DMRT).

pretreatment of AA to Al(mal)$_3$ exposed cells, increased the cell viability and decreased apoptotic cell death significantly (p<0.0.5) as compared to Al(mal)$_3$ alone exposed cells (Figure 4).

4.5. Effect of AA on Al(mal)$_3$ induced morphological changes

Hoechst 33258 staining showed significant (p<0.0.5) DNA condensation and nuclear fragmentation in cells treated with Al(mal)$_3$ as compared to control cells. However, pretreatment with AA significantly inhibited significantly (p<0.0.5) the characteristics of apoptosis (Figure 5).

4.6. Impact of AA on Al(mal)$_3$ induced DNA damage

DNA damage induced by Al(mal)$_3$ was studied by comet assay (Figure 6). Al(mal)$_3$ significantly (p<0.0.5) increased % head DNA, tail length, tail moment and OTM, whereas AA treatment prior to Al(mal)$_3$ exposure significantly (p<0.0.5) reduced the DNA damage. Results were analyzed by Comet Assay Software Project (CASP).

4.7. Effect of AA on Al(mal)$_3$ induced imbalance in the expression of apoptotic and signaling markers

Western blot analysis showed that Al(mal)$_3$ treatment significantly (p<0.0.5) downregulated the expression of Bcl-2 and increased the expression of Bax (Figure 7). AA pretreatment significantly (p<0.0.5) attenuated the Al(mal)$_3$ induced reduction in the expression of Bcl-2 and enhancement in the expression of Bax. We also observed that Al(mal)$_3$ treatment significantly (p<0.0.5) increased release of cyto c in cytosol and the expression of active caspases-3 and 9 compared with control. Pretreatment with AA restored Al(mal)$_3$ toxicity specifically by withdrawing the release of cyto c in cytosol and the expression of apoptotic proteins significantly (p<0.0.5). In order to reveal the mechanism of Al(mal)$_3$ induced cell death and protective effective of AA, the protein expression studies of signaling molecules were performed.

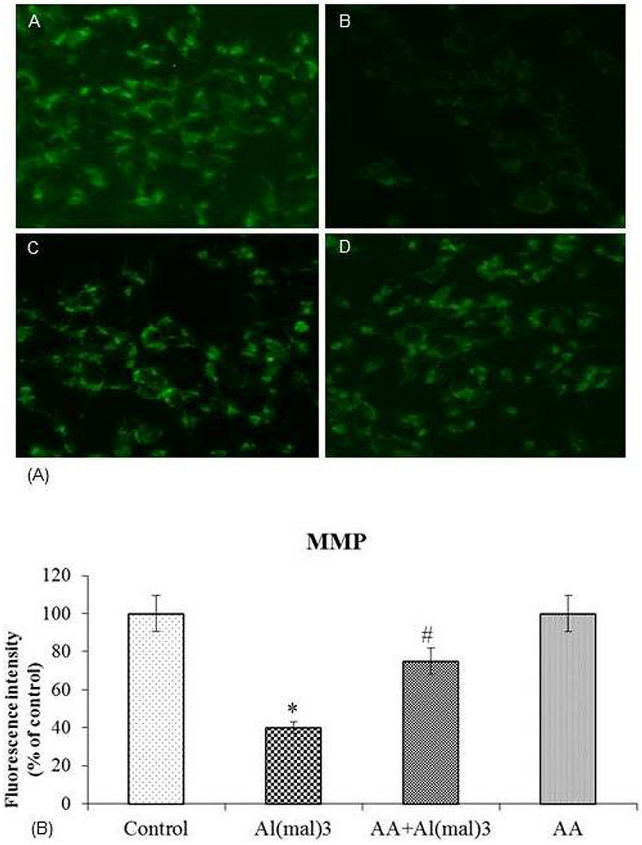

Figure 3. Al(mal)$_3$ treatment reduced mitochondrial membrane potential as compared to control, whereas AA pretreatment enhanced mitochondrial membrane potential in SH-SY 5Y neuroblastoma cells. Values are given as mean ± SD of four experiments in each group. *p<0.05 compared to control; #p<0.05 compared to Al(mal)$_3$ group (Duncan's multiple range test-DMRT).

Expressions of p-AKT and p-GSK3β were significantly (p<0.0.5) decreased after Al(mal)$_3$ treatment as compared with control. There is no significant reduction was found in the expressions of total AKT and total GSK3β after Al(mal)$_3$ treatment as compared with control. Pretreatment with AA significantly (p<0.0.5) increased the expressions of p-AKT and p-GSK3β as compared with. However, treatment with AA alone unaltered the expression of p-AKT and p- GSK3β as compared to control (Figure 8).

5. DISCUSSION

The mitochondrial dehydrogenases catalyse the formation of blue formazan product by the reduction of the MTT tetrazolium salt and this assay is widely used for evaluating cellular survival (19). Neuronal apoptosis is a significant characteristics of neurodegeneration and well-known form of cell death in many NDDs including AD (20). The observation of Hoechst 33342 and dual staining techniques confirms these apoptotic changes in Al(mal)3 treated cells. Previous studies indicated that the treatment with Al(mal)$_3$ induced cell death in brain (21,22) and as well in *in vitro* studies (23-25), which is corroborated with our study. The neuroprotective effect of AA in MTT assay paralleled the morphological analyses obtained with Hoechst- 33258 and dual staining. AA exposure increased the survival of PC12 cells against oxygen-glucose deprivation/reoxygenation injury (26), SH-SY5Y cells against glutamate toxicity (27) and cultured rat hepatocytes against D-galactosamine or carbon tetrachloride injury (28), which strengthen our finding.

Al disrupts the homeostasis of magnesium, calcium and iron (29, 30) and effectively mimics these metals in their respective biological functions, thereby triggering biochemical abnormalities. The accumulation of Al obstructs the activities of electron transport chain (ETC) complexes I, III and IV, which are heavily loaded with Fe–S clusters and hemes (31), thus limiting ATP synthesis via the inhibition of oxidative phosphorylation. The mitochondrial ROS production occurs mainly at two distinct points in the ETC: complexes I (NADH dehydrogenase) and III (ubiquinone–cytochrome c reductase) (32, 33).

Dichlorodihydrofluorescein diacetate (DCFH-DA) staining revealed that Al(mal)$_3$ treatment induced ROS generation which could be suppressed by 10 nM AA pretreatment (Figure 2) These effects are reversed by antioxidants like N-acetyl cysteine (34,

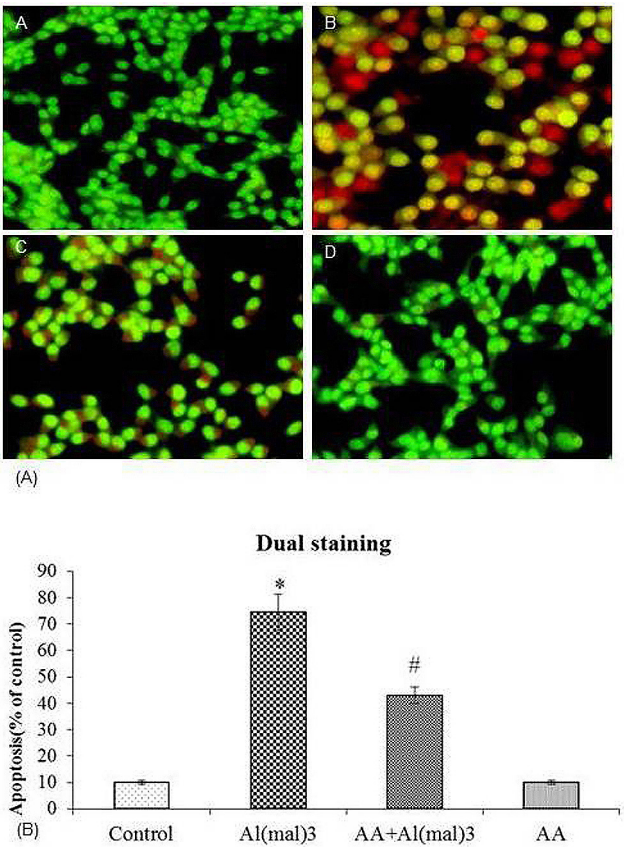

Figure 4. Al(mal)$_3$ treatment enhanced apoptosis as compared to control, whereas AA pretreatment showed antiapoptotic effect in SH-SY 5Y neuroblastoma cells. Values are given as mean ± SD of four experiments in each group. *p<0.05 compared to control; #p<0.05 compared to Al(mal)$_3$ group (Duncan's multiple range test-DMRT).

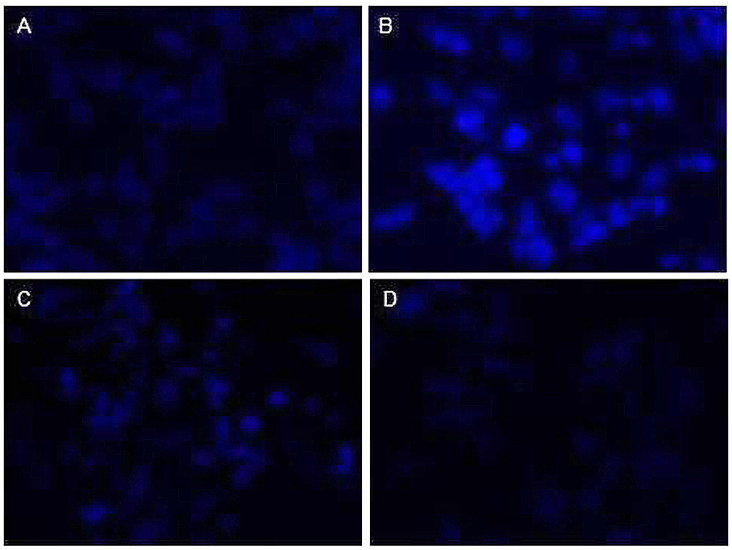

Figure 5. Al(mal)$_3$ treatment showed morphological changes in SH-SY 5Y neuroblastoma cells as compared to control, whereas AA pretreatment reversed these changes. Values are given as mean ± SD of four experiments in each group. *p<0.05 compared to control; #p<0.05 compared to Al(mal)$_3$ group (Duncan's multiple range test-DMRT). Figures indicate treatment with A) Control, (B) Al(mal)$_3$ (C) AA + Al(mal)$_3$ and (D) AA.

35). AA exhibited significant neuroprotective effect against age related (36), glutamate (9) and rotenone (18) induced oxidative stress due to its antioxidant potential. Mitochondrial energization induces quenching of Rho 123 fluorescence and the rate of fluorescence decay is proportion to the ΔΨm (37). Our results demonstrated that the cells treated with Al(mal)$_3$ decreased the integrity of mitochondrial membrane,

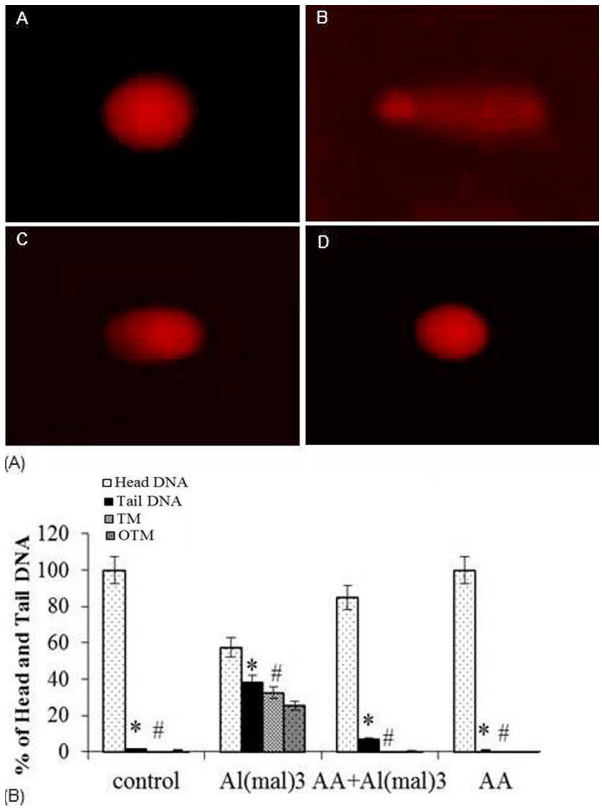

Figure 6. Al(mal)$_3$ treatment showed oxidative changes in SH-SY 5Y neuroblastoma cells as compared to control, whereas AA pretreatment nullified these changes. Values are given as mean ± SD of four experiments in each group. *p<0.05 compared to control; #p<0.05 compared to Al(mal)$_3$ group (Duncan's multiple range test-DMRT).

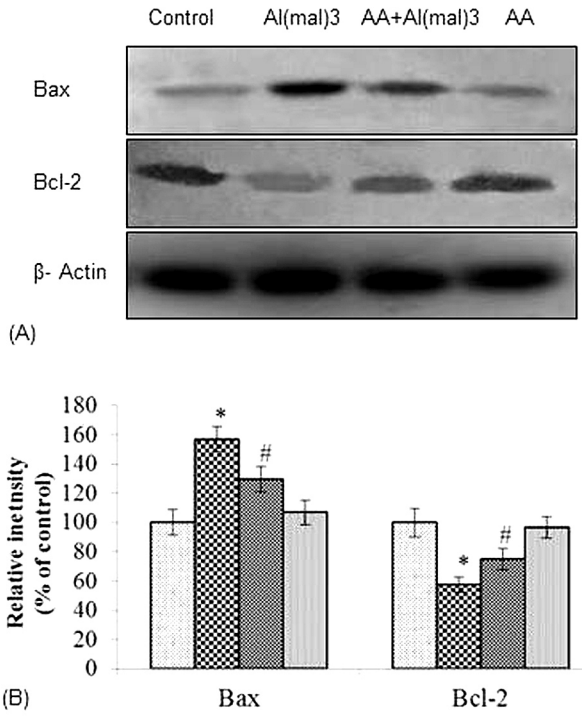

Figure 7. Al(mal)$_3$ treatment altered the expressions of Bcl-2, Bax, cyto c, caspases-3 and 9 in SH-SY 5Y neuroblastoma cells as compared to control, whereas AA pretreatment showed antiapoptotic effects. Values are given as mean ± SD of four experiments in each group. *p<0.05 compared to control; #p<0.05 compared to Al(mal)$_3$ group (Duncan's multiple range test-DMRT).

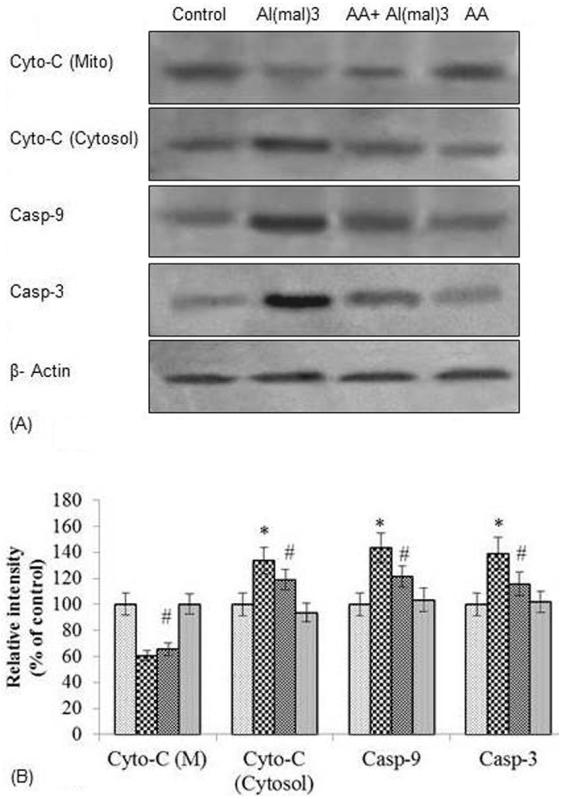

Figure 8. Al(mal)$_3$ treatment altered the expressions of AKT, p-AKT, GSK3β and p-GSK3β in SH-SY5Y cells as compared to control, whereas AA pretreatment reversed these changes. Values are given as mean ± SD of four experiments in each group. *p<0.05 compared to control; #p<0.05 compared to Al(mal)$_3$ group (Duncan's multiple range test-DMRT).

whereas cells pretreated with AA prevented the loss of mitochondrial membrane integrity (Figure 3). The neuroprotective effect of AA against ΔΨm caused by rotenone, a mitochondrial inhibitor has been shown by our lab recently, is consistent with the present results (18). The loss in ΔΨm leads to the opening of the permeability transition (PT) pore (38), which facilitates the release of cyto c into the cytosol. In cytoplasm, cyto-c binds to Apaf-1, activates caspase 9 with subsequent activation of the death-inducing caspase 3 (39). The Bcl-2 family of proteins is essential in controlling ΔΨm (40). The anti-apoptotic proteins Bcl-2 located in the outer mitochondrial wall and inhibit the release of cyto c. The pro-apoptotic Bcl-2 proteins such as Bad, Bid, Bax and Bim exist in the cytosol but translocate to mitochondria following ΔΨm loss, where they promote the release of cyto c. In the present study, we observed that, Al (mal)$_3$ induces cyto c translocation from mitochondria into the cytosol, Bcl-2 down-regulation, Bax up-regulation and caspases -9 and 3 activation, which indicates the progression of apoptosis. Moreover Al was shown to induce mitochondrial permeability transition by reducing ΔΨm (41) and bounding to the PT pore. It held the pore in an intermediate state, partially open position, which is sufficient to trigger cyto c release. Caspases are important mediators of apoptosis, and caspase activation has been demonstrated in Al(mal)$_3$-induced neurodegeneration (21,42). We found that AA pre treatment enhanced the Bcl-2 expression with diminished Bax expression and reduced cyto c release from mitochondria, thereby inhibiting the expressions of caspase-9 and -3 as compared to Al(mal)$_3$ alone exposed cells. Huang *et al*., (43) indicated that the treatment of AA inhibited myocardial ischemic reperfusion injury induced apoptotic cell death by downregulating the activities of caspase-3 and -9 and reverting Bax/Bcl-2 ratio in hypoxic H9c2 cells.

The activation of Akt pathway promotes neuronal survival (44, 45), while its inhibition induces neuronal death (46). Akt exerts a wide range of biological effects mainly by promoting the phosphorylation of Bax (one of the apoptosis promoters of the Bcl-2 family), mTOR (mammalian target of rapamycin), glycogen synthase kinase-3 (GSK-3), and other downstream substrates (47). PI3K activates phosphorylation of Akt at Ser 473, which activate the phosphorylation of GSK-3β at serine 9 (GSK-3β Ser9), thereby preventing the activity GSK-3β (48, 49). *AKT* down regulation with elevated GSK-3β activity is associated to brain dysfunctional pathogenesis. Al(mal)$_3$ is reported to reduce the phosphorylation of Akt and thereby increasing the activity of GSK-3β (49). In the active form, GSK-3*β* translocates from the cytoplasm to the nucleus and then participates in the development of

apoptosis activities. GSK-3*β* also induces apoptosis through phosphorylating Bax, which then enters the mitochondria and induces the release of cyto C to the cytoplasm. $Al(mal)_3$ exposure decreased the expressions of Ser 9 pGSK3β in this study, which is consistent with our previous findings (50). AA co-treatment upregulated the expression of Akt, thereby induces the phosphorylation of serine 9 pGSK3β that results in the inactivation of GSK-3β, which prevents cell apoptosis and confers neuroprotection. These findings demonstrated that AA treatment could alleviate Al induced apoptosis in *in vitro* model of AD likely by modulating oxidative stress, mitochondrial dysfunction and signaling pathways.

In conclusion, AKT/GSK-3β pathway is considered as a functional molecular event that prevent the learning and memory impairments induced by Al to a certain degree and to exploring some valid targets to treat diseases correlating to Al. Down regulation of Al induced neurodegeneration may be one of the approaches to control the impairment of metal ion homeostasis leading to neuronal injury in the early development of AD. However, pre clinical and clinical studies are warrented to elucidate the neuroprotective efficacy of AA in the management of AD.

6. REFERENCES

1. Levesque L, Mizzen CA, McLachlan DR, Fraser PE: Ligand specific effects on aluminum incorporation and toxicity in neurons and astrocytes. Brain Res 877:191-202 (2000)
DOI: 10.1016/S0006-8993(00)02637-8

2. Tsubouchi R, Htay H, Murakami K, Haneda M, Yoshino M: Aluminum-induced apoptosis in PC12D cells. Biometals 14:181-185 (2001)
DOI: 10.1023/A:1016677319875

3. Strong MJ, Garruto RM: Chronic aluminum-induced motor neuron degeneration: clinical, neuropathological and molecular biological aspects. Can J Neurol Sci 18:428-431 (1991)
DOI: 10.1017/S0317167100032601

4. Kaneko N, Takada J, Yasui H, Sakurai H: Memory deficit in mice administered aluminum-maltolate complex. Biometals 19:83-89 (2006)
DOI: 10.1007/s10534-005-6965-7

5. Mustafa Rizvi SH, Parveen A, Verma AK, Ahmad I, Arshad M, Mahdi AA: Aluminium induced Endoplasmic reticulum stress mediated cell death in SH-SY5Y neuroblastoma cell line is independent of p53. PLos one 9 e98409 (2014)
DOI: 10.1371/journal.pone.0098409

6. Kumar A, Dogra S, Prakash A: Neuroprotective effects of Centella asiatica against intracerebroventricular colchicine-induced cognitive impairment and oxidative stress. Int J Alzheimer's Dis 2009: 972178 (2009)
DOI: 10.4061/2009/972178

7. Gray NE, Harris CJ, Quinn JF, Soumyanath A: Centella asiatica modulates antioxidant and mitochondrial pathways and improves cognitive function in mice. J Ethnopharmacol 180:78-86 (2016)
DOI: 10.1016/j.jep.2016.01.013

8. Krishnamurthy RG, Senut MC, Zemke D, Min J, Frenkel MB, Greenberg EJ, Yu SW, Ahn N, Goudreau J, Kassab M, Panickar KS, Majid A: Asiatic acid, a pentacyclic triterpene from *Centella asiatica* is neuroprotective in a mouse model of focal cerebral ischemia. J Neurosci Res 87:2541-2550 (2009)
DOI: 10.1002/jnr.22071

9. Lee MK, Kim SR, Sung SH, Lim D, Kim H, Choi H, Park HK, Je S, Ki YC: Asiatic acid derivatives protect cultured cortical neurons from glutamate-induced excitotoxicity. Res Commun Mol Pathol Pharmacol 108:75-86 (2000)

10. Jung IM, Shin JE, Yun SH, Huh K, Koh JY, Park HK, Jew SS, Jung MW: Protective effects of asiaticoside derivatives against beta-amyloid neurotoxicity. J Neurosci Res 58:417-425 (1999)
DOI: 10.1002/(SICI)1097-4547(19991101)58:3<417::AID-JNR7>3.3.CO;2-7

11. Xiong Y, Ding H, Xu M, Gao J: Protective effects of asiatic acid on rotenone- or H_2O_2-induced injury in SH-SY5Y cells. Neurochem Res 34:746-754 (2009)
DOI: 10.1007/s11064-008-9844-0

12. Patil PS, Sarah M, Khedkar AS, Rigby CA, Christina C: Withanolide A and asiatic acid modulate multiple targets associated with amyloid-β precursor protein processing and amyloid-β protein clearance. J Nat Prod 73:1196-1202 (2010)
DOI: 10.1021/np900633j

13. Berthold RL, Herman MM, Savory J , Carpenter RM, Sturgill BC, Katsetos CD,

Vandenberg SR, Wills MR: A long term intravenous model of aluminum maltol toxicity in rabbits: tissue distribution, hepatic, renal, and neuronal cytoskeletal changes associated with systemic exposure. Toxicol Appl Pharmacol 15:58-74 (1989)
DOI: 10.1016/0041-008X(89)90134-8

14. Dhanalakshmi C, Manivasagam T, Nataraj J, Justin Thenmozhi A, Essa MM: Neurosupportive role of vanillin, a natural phenolic compound on rotenone induced neurotoxicity in SH-SY5Y neuroblastoma cells. Evid Based Complement Alternat Med 1-11 (2015)
DOI: 10.1155/2015/626028

15. Tamilselvam K, Braidy N, Manivasagam T, Essa MM, Prasad NR, Karthikeyan S, Justin Thenmozhi A, Selvaraju S, Guillemin GJ: Neuroprotective effects of hesperidin, a plant flavanone, on rotenone-induced oxidative stress and apoptosis in a cellular model for Parkinson's disease. Oxid Med Cell Longev 1-11 (2013)
DOI: 10.1155/2013/102741

16. Kavitha M, Manivasagam T, Essa MM, Tamilselvam K, Selvakumar GP, Karthikeyan S, Justin Thenmozhi A, Subash S: Mangiferin antagonizes rotenone: induced apoptosis through attenuating mitochondrial dysfunction and oxidative stress in SK-N-SH neuroblastoma cells. Neurochem Res 39:668-676 (2014)
DOI: 10.1007/s11064-014-1249-7

17. Jayaraj RL, Tamilselvam K, Manivasagam T , Elangovan N: Neuroprotective effect of CNB-001, a novel pyrazole derivative of curcumin on biochemical and apoptotic markers against rotenone-induced SK-N-SH cellular model of Parkinson's disease. J Mol Neurosci 51:63-70 (2013)
DOI: 10.1007/s12031-013-0075-8

18. Nataraj J, Manivasagam T, Justin Thenmozhi A, Essa MM: Neuroprotective effect of asiatic acid on rotenone-induced mitochondrial dysfunction and oxidative stress-mediated apoptosis in differentiated SH-SYS5Y cells. Nutr Neurosci 1-9 (2016)
DOI: 10.1080/1028415X.2015.1135559

19. York JL, Maddox LC, Zimniak P, McHugh TE, Grant DF: Reduction of MTT by glutathione S-transferase. Biotechniques 25:622-624 (1998)

20. Honig LS, Rosenberg R.N: Apoptosis and neurologic disease. Am J Med 108: 317-330 (2000)
DOI: 10.1016/S0002-9343(00)00291-6

21. Ghribi O, Herman MM, Spaulding NK, Savory J: Lithium inhibits aluminum-induced apoptosis in rabbit hippocampus, by preventing cytochrome c translocation, Bcl-2 decrease, Bax elevation and caspase-3 activation. J Neurochem 82:137-145 (2002)
DOI: 10.1046/j.1471-4159.2002.00957.x

22. Savory J, Rao JK, Huang Y, Letada PR, Herman MM: Age related hippocampal changes in Bcl-2:Bax ratio, oxidative stress, redox-active iron and apoptosis associated with aluminum-induced neurodegeneration: Increased susceptibility with aging. Neurotoxicology 20:805-817 (1999)

23. Johnson VJ, Kim SH, Sharma RP: Aluminum maltolate induces apoptosis and necrosis in neuro-2a cells: potential role for p53 signaling. Toxicol Sci 83:329-339 (2005)
DOI: 10.1093/toxsci/kfi028

24. Griffioen KJ, Ghribi O, Fox N, Savory J, DeWitt DA: Aluminum maltolate induced toxicity in bNT2 cells occurs through apoptosis and includes cytochrome c release. Neurotoxicology 25:859-867 (2004)
DOI: 10.1016/j.neuro.2003.12.004

25. Chen TJ, Cheng HM, Wang DC, Hung HS: Nonlethal aluminum maltolate can reduce brain-derived neurotrophic factor-induced Arc expression through interrupting the ERK signaling in SH-SY5Y neuroblastoma Cells. Toxicol Lett 200:67-76 (2011)
DOI: 10.1016/j.toxlet.2010.10.016

26. Yuan JP, Lu JM, Lu Y: The protective effect of asiatic acid against oxygen-glucose deprivation/reoxygenation injury of PC12 cells. Yao Xue Xue Bao 48:38-42 (2013)

27. Xu MF, Xiong YY, Liu JK, Qian JJ, Zhu L, Gao J: Asiatic acid, a pentacyclic triterpene in *Centella asiatica*, attenuates glutamate-induced cognitive deficits in mice and apoptosis in SHSY5Y cells. Acta Pharmacol Sin 33:578-587 (2012)
DOI: 10.1038/aps.2012.3

28. Ma KF, Zhang XY, Qi LY: Protective effects of triterpenoids on primarily cultured rat hepatocytes injured by D-galactosamine

and carbon tetrachloride. Zhejiang Da Xue Xue Bao Yi Xue Ban 36:247-254 (2007)

29. Kawahara M, Negishi MK: Link between aluminum and the pathogenesis of Alzheimer's disease: the integration of the aluminum and Amyloid cascade hypothesis. Int J Alzheimer's Dis 2011: 276393 (2011). DOI: 10.4061/2011/276393

30. Harris WR, Berthon G, Day JP, Exley C, Pender T, William F: Speciation of aluminium in biological systems. J Toxicol Environ Health 48:543-568 (1996) DOI: 10.1080/009841096161069

31. Rouault TA, Tong WH: Iron-sulphur cluster biogenesis and mitochondrial iron homeostasis. Nat Rev Mol Cell Biol 6:345-351 (2005) DOI: 10.1038/nrm1620

32. Turrens JF: Mitochondrial formation of reactive oxygen species. J Physiol 552:335-344 (2003) DOI: 10.1113/jphysiol.2003.049478

33. Finkel T: Signal transduction by reactive oxygen species. Cell Biol 194:7-15 (2011) DOI: 10.1083/jcb.201102095

34. Richards SA, Muter J, Ritchie P, Lattanzi G, Hutchison CJ: The accumulation of un-repairable DNA damage in laminopathyprogeria fibroblasts is caused by ROS generation and is prevented by treatment with N-acetyl cysteine. Hum Mol Genet 20:3997-4004 (2011) DOI: 10.1093/hmg/ddr327

35. Zhang X, Banerjee A, Banks WA, Ercal N: N-Acetylcysteine amide protects against methamphetamine-induced oxidative stress and neurotoxicity in immortalized human brain endothelial cells. Brain Res 1275:87-95 (2009) DOI: 10.1016/j.brainres.2009.04.008

36. Subathra M, Shila S, Devi MA, Panneerselvam C: Emerging role of *Centella asiatica* in improving age-related neurological antioxidant status. Exp Gerontol 40:707-715 (2005) DOI: 10.1016/j.exger.2005.06.001

37. Lee MK, Kim SR, Sung SH, Lim D, Kim H, Choi H, Park HK, Je S, Ki YC: Asiatic acid derivatives protect cultured cortical neurons from glutamate-induced excitotoxicity. Res Commun Mol Pathol Pharmacol 108:75-86 (2000)

38. Baracca A, Sgarbi G, Solaini G, Lenaz G: Rhodamine 123 as a probe of mitochondrial membrane potential: evaluation of proton flux through F(0) during ATP synthesis. Biochim Biophys Acta 1606:137-146 (2003) DOI: 10.1016/S0005-2728(03)00110-5

39. Brown GC, Nicholls DG, Cooper CE: Mitochondria and cell death. New Jersey: Princeton University Press (1999)

40. Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X: Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell 91:479-489 (1997) DOI: 10.1016/S0092-8674(00)80434-1

41. Kawahara M, Negishi MK, Hosoda R, Imamura L, Tsuda M, Kuroda Y: Brain-derived neurotrophic factor protects cultured rat hippocampal neurons from aluminum maltolate neurotoxicity. J Inorg Biochem 97:124-131 (2003) DOI: 10.1016/S0162-0134(03)00255-1

42. Toninello A, Clari G, Marcon M, Tognon G, Zatta P: Aluminum as an inducer of the mitochondrial permeability transition. J Biol Inorg Chem 5:612-623 (2000) DOI: 10.1007/s007750000144

43. Ghribi O, DeWitt DA, Forbes MS, Herman MM, Savory J: Co-involvement of mitochondria and endoplasmic reticulum in regulation of apoptosis: Changes in cytochrome c, Bcl-2 and Bax in the hippocampus of aluminum-treated rabbits. Brain Res 903:66-73 (2001a) DOI: 10.1016/S0006-8993(01)02406-4

44. Haung X, Zuo L, Chen C, Yang Y, Xin H, Li Y, Qian Y: Asiatic acid attenuates Myocardial Ischemia/Reperfusion Injury via Akt/GSk-3β/HIF-1α Signalling in Rat H9c2 Cardiomyocytes. Molecules 21(9): E1248 (2016) DOI: 10.3390/molecules21091248

45. Namikawa K, Honma M, Abe K, Takeda M, Mansur K, Obata T: Akt/protein kinase B prevents injury-induced motor neuron death and accelerates axonal regeneration. J Neurosci 20:2875-2886 (2000)

46. Zhao H, Sapolsky RM, Steinberg GK: Phosphoinositide-3-kinase/akt survival signal pathways are implicated in neuronal survival after stroke. Mol Neurobiol 34:249-270 (2006)
DOI: 10.1385/MN:34:3:249

47. Ji L, Chauhan A, Wegiel J, Essa MM, Chauhan V: Gelsolin is proteolytically cleaved in the brains of individuals with Alzheimer's disease. J Alzheimer's Dis 18:105-111 (2009)
DOI: 10.3233/JAD-2009-1127

48. Yang L, Xie S, Jamaluddin MS, Altuwaijri S, Ni J, Kim E, Chen YT, Hu YC, Wang L, Chuang KH, Wu CT, Chang C: Induction of androgen receptor expression by phosphatidylinositol 3-kinase/Akt downstream substrate, FOXO3a, and their roles in apoptosis of LNCaP prostate cancer cells. J Biol Chem 280: 33558-33565 (2005)
DOI: 10.1074/jbc.M504461200

49. Lee KY, Koh SH, Noh MY, Kim SH, Lee YJ: Phosphatidylinositol-3-kinase activation blocks amyloid beta-induced neurotoxicity. Toxicology 243:43-50 (2008)
DOI: 10.1016/j.tox.2007.09.020

50. Ma T, Du X, Pick JE, Sui G, Brownlee M, Klann E: Glucagon-like peptide-1 cleavage product GLP-1 (9-36) amide rescues synaptic plasticity and memory deficits in Alzheimer's disease model mice. J Neurosci 32:13701-13708 (2012)
DOI: 10.1523/JNEUROSCI.2107-12.2012

51. Justin Thenmozhi A, Dhivya Bharathi M, Manivasagam T, Essa MM: Tannoid principles of *Emblica officinalis* attenuated aluminum chloride induced apoptosis by suppressing oxidative stress and tau pathology via Akt/GSK-3β signaling pathway. J Ethnopharmacol 194:20-29 (2016)
DOI: 10.1016/j.jep.2016.08.047

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