Neuroprotective role of Asiatic acid in aluminium chloride induced rat model of Alzheimer's disease

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TABLE OF CONTENTS

1. ABSTRACT

Alzheimer's disease (AD) is the most common form of dementia, characterized by memory loss, cognitive impairment and personality disorders accompanied by diffuse structural abnormalities in the brain of elderly people. The current investigation explored the neuroprotective potential of asiatic acid (AA), a natural triterpene of *Centella asiatica* on aluminium chloride (AlCl$_3$) induced rat model of AD. Oral administration of AlCl$_3$ (100 mg/kg b.w.) for 42 days significantly elevated the levels of Al, activity of acetyl cholinesterase and expressions of amyloid precursor protein, amyloid beta$_{1-42}$, beta and gamma secretases, glial fibrillary acidic protein, ionized calcium binding adaptor molecule 1, interleukins -1β, 6, 4, 2, tumor necrosis factor alpha, inducible nitric oxide synthase, nuclear factor- k beta and cyclooxygenase-2 in the hippocampus and cortex compared to the control group. Our observations suggested that AA treatment mitigated AlCl$_3$ induced AD associated pathologies, which might be due to its multiple pharmacological actions. Further studies are necessary in order to explore the link between AlCl$_3$-mediated oxidative stress and associated apoptosis to establish its neuroprotective role in AD.

2. INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disease, associated with the loss of memory, language and other cognitive impairments in aged population. Accumulation of extracellular amyloid β peptide (Aβ) containing plaques through amyloidogenic pathway and intraneuronal fibrillary tangles in the brain are two critical features in the neuropathogenesis of AD, consequently destroys the neurons slowly. The cause of AD is multifactorial and apparently entails numerous factors including head trauma, genetics, oxidative stress, inflammation, proteosomal dysfunction and environmental features such as aluminium (Al) toxicity. Al is an abundantly existing neurotoxin, extremely exposed to humans and shown to accumulate in AD vulnerable brain regions such as cortex and hippocampus (1-3). Furthermore, Al diverts amyloid precursor protein (APP) metabolism from its non-amyloidogenic pathway to amyloidogenic pathway, thereby favours the formation of β-amyloid oligomers, fibrils and plaques (4).

Al inhibits long-term potentiation, induces inflammatory responses, affects the slow and fast axonal transports and causes synaptic structural abnormalities, resulting in profound memory loss (5). Becaria *et al.* (6) and Ghribi *et al.* (7) found that Al exposure activated nuclear factor kappa B (NF-κB), which is the regulator of inflammatory markers such as cyclooxygenase- 2 (COX-2), IκB, TNF-α, cyclin D1, intercellular adhesion molecule-1 (ICAM-1), inducible nitric oxide synthase (iNOS) and interleukins-6 and, -8 (IL-6, IL-8) by inducing the degradation of inhibitor kappa B (IκB) (8).

In Ayurvedha, Mandukaparni (*Centella asiatica*) is described as "Medhya Rasayana" or brain tonic, which possesses various effects on CNS such as nerve stimulatory tonic, rejuvenator of memory, learning, sedative, tranquilizer and intelligence promoting properties. *Centella asiatica* (CA) has been shown to enhance the capability of rats in performing several memory tasks, including the Morris water maze and passive avoidance test (9). It is demonstrated that CA protected Aβ toxicity in *in vitro* and *in vivo* conditions (10, 11). The neuroprotective effect of CA was explored against aluminium-induced mitochondrial dysfunction, oxidative stress, apoptosis and cognitive impairment in experimental AD rats (12). Based on the numerous studies, the medicinal values of this plant are mainly attributed due to the presence of several triterpenes, namely asiatic acid, madecassic acid, asiaticoside and madecassoside (13). Krishnamurthy *et al.* (14) reported that asiatic acid (AA), a tri-terepene of CA could transverse the blood-brain barrier and mitigated mitochondrial injury in a mouse model of focal cerebral ischemia. Many previous studies revealed that this compound was a potent protective agent for brain against various neurotoxins (15-19).

Though the Al exposure is involved in the development of clinical and pathological features of AD in rats (2, 3, 20, 21), the underlying mechanism by which AA exerts its neuroprotective action in animal model is still unravelling. Based on this background, the present study was carried out to explore the neuroprotective effect of AA against $AlCl_3$ induced alterations on the level of aluminium, activity of AChE and expressions of APP, $A\beta_{1-42}$, β- and γ-secretases and inflammatory indices in hippocampus and cortex of rats.

3. MATERIALS AND METHODS

3.1. Animals

Male Albino Wistar rats (200–225 g) were procured from Central Animal House, Rajah Muthiah Medical College & Hospital, Annamalai University and were maintained at standard conditions with food and water *ad libitum*. The experimental protocols were approved by the Institutional Animal Ethics Committee (Reg. No. 160/1999/CPCSEA, Proposal No. 1006) and met with the guidelines of Indian National Science Academy, New Delhi, 2000.

3.2. Chemicals

Aluminium chloride, anti-amyloid precursor protein, anti-β-amyloid$_{1-42}$, anti-γ-, β- secretases and horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG were purchased from Sigma-Aldrich, Bangalore, India and used in this study. Anti-NF-kB, anti- IL-2, anti- IL-4, anti-glial fibrillary acidic protein (GFAP), anti-cyclooxygenase-2 (COX-2) and anti-β-actin were procured from Cell Signaling technology, USA. Anti-inducible nitric oxide synthase (iNOS), interleukins (IL)-1β, IL-6, IL-4, IL-2 anti-tumor necrosis factor alpha (TNF-α) and anti-ionized calcium binding adaptor molecule 1 (Iba-1) were procured from Santa Cruz Biotechnology, USA. All other chemicals used were of analytical grade.

3.3. Experimental design

3.3.1. Preparation of Asiatic acid

AA (Sigma-Aldrich, St. Louis, MO) was suspended in 0.5.% carboxymethylcellulose (CMC) and administered orally for 6 weeks.

3.3.2. Phase I experiment

After a week acclimatization period, thirty six rats were divided into six groups of each containing six animals. Group I rats received CMC (0.5.% in d/w).

Group II animals were treated with AlCl$_3$ (100mg/kg *b.w.*, oral) for 6 weeks (12). Group III rats were treated AlCl$_3$ as group II and subsequent oral treatment with AA (30 mg/kg *b.w.*) (14). Group IV received AlCl$_3$ as group II and subsequent oral treatment with AA (75 mg/kg *b.w.*) (14). Group V rats received AlCl$_3$ as group II and subsequent oral treatment with AA (150 mg/kg *b.w.*) (14). Group VI animals were treated with AA (150 mg/kg *b.w.*) alone.

After performing Morris water maze test, animals were sacrificed; brain tissues (cortex and hippocampus) were excised and utilized for the determination of Al and AChE. No significant changes were found in the activities of AChE between 75 mg/kg *b.w.* and 150 mg/kg *b.w.* AA co-treated rats. Hence, the dose 75 mg/kg *b.w.* AA has been used for further studies.

3.3.3. Phase II experiment

Forty eight rats were randomized and divided into four (n = 18) groups: control, AlCl$_3$, AlCl$_3$+AA (75 mg/kg *b.w.*) and AA (75 mg/kg *b.w.*) for 6 weeks. The neuroprotective efficacy of AA against AlCl$_3$ induced neurotoxicity was determined by performing passive avoidance test and the protein expression studies of Aβ biosynthesis related and inflammatory markers.

3.3.4. Morris water maze test

The apparatus consists of a large circular swimming pool (150 x 45 cm; water filled up to the depth of 30 cm), which is divided into four equal quadrants. During the acquisition phase, each and every rat was gently placed in the different quadrants of swimming pool for each trial, facing the wall of pool and permitted 120 seconds (s) to locate the small visible platform that was placed about 1 cm above the water level. Rats were permitted to stay in the platform for 20 s. The failed animals were guided to reach the destination and allowed to remain there for next 20 s. On 19th and 20th days, the animals were permitted to go two consecutive training sessions. The mean time taken to reach the visual platform was calculated as acquisition latency. On 21st and 42nd days, after AlCl$_3$ administration, mean time taken to locate the hidden platform was recorded as first and second retention latencies respectively (22).

3.3.5. Passive avoidance task

The apparatus consisted of a light and dark chamber that was separated by a wall that contains a door. In the acquisition trial, rat was independently placed in the light chamber and allowed to enter into the dark chamber. Immediately electric shock (40 V, 0.5. mA for 1 s) was delivered to the feet of the animal through the metal grid. Then the rat was taken from the apparatus and returned to the cage. After 24 h, each and every rat was placed individually again in the light chamber and the time taken by them to enter into the dark chamber was considered as step-through latency. If the rat did not enter the dark chamber within a 5-minute test period, the test was ceased and the step-through latency was noted as 300 seconds (23).

3.3.6. Determination of Al concentration

Brain tissues (cortex and hippocampus) were weighed and put into polytetrafluoroethene (PTFE), then added 0.0.5 ml nitric acid and 0.2. ml H$_2$O$_2$ per 30 mg tissue and incubated at 120°C for 2 h. Al levels in cortex and hippocampus of control and experimental rats were measured by atomic absorption spectrophotometer (24).

3.3.7. Activity of AChE

The AChE activity was measured in the studied brain regions by spectrophotometer at 412 nm (25).

3.3.8. Western blot analysis

Brain tissues were homogenized in cold suspension lysis Hepes–KOH buffer (Hepes 25 mM; MgCl 5 mM; EDTA 5 mM in double distilled H$_2$O) and the homogenates were centrifuged (10,000 rpm/15 min/4°C). The protein content of the supernatant was assayed (26) and diluted to give equal protein concentration of 50 μg. Samples containing equal cellular protein were loaded on 10% SDS-polyacrylamide gel electrophoresis and resolved. The gel was transferred on to a polyvinylidene difluoride membrane (Millipore). To assess the expression of specific protein, the membranes were incubated with Aβ$_{1-42}$, β-,γ- secretases, GFAP, Iba-1, IL-1β, IL-6, TNF-α, iNOS, NF-kB, COX-2 and β-actin IgG in 5% BSA in Tris-buffered saline and 0.0.5% Tween-20 (TBST) with mild shaking overnight (4°C) (27). Then the membranes were incubated for 2 h at room temperature with their corresponding HRP conjugated secondary antibodies and washed. Immunoreactive proteins were visualized by the chemiluminescence protocol (GenScript ECL kit, USA) and the densitometric analysis was performed with gel image analysis program. Data were normalised by background subtraction and β-actin was used as internal control. Bands were scanned using a scanner and quantitated by Image J, a public domain Java image processing software, which of control was set to 100.

3.3.9. Data analysis

Data were expressed as mean ± Standard Error (SEM) of six rats for behavioural and biochemical studies and of three for protein expression studies.
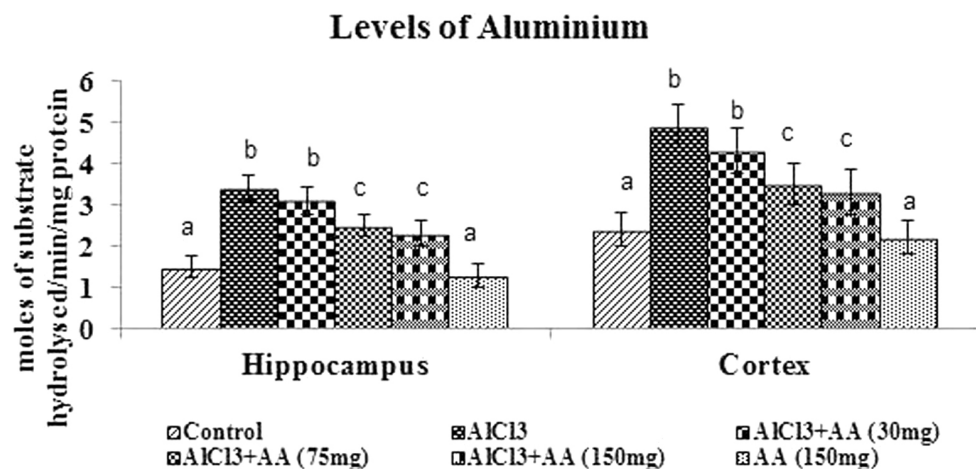
Levels of Aluminium

Figure 1. AlCl$_3$ treatment enhanced the levels of Al as compared to control, whereas AA (30, 75 and 150 mg/kg b.w.) pretreatment dose dependently attenuated AlCl3 induced Al accumulation. Data are expressed as mean ± SD (one-way ANOVA followed by DMRT).Values not sharing same alphabet differ significantly.
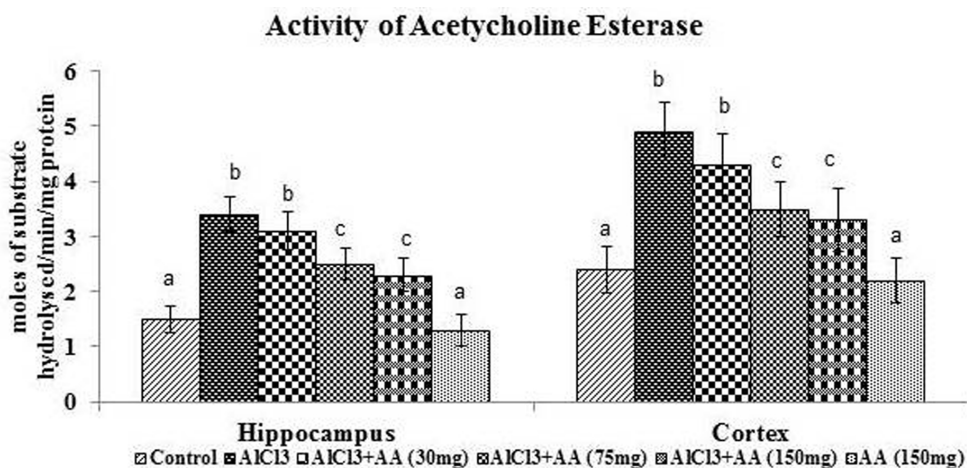
Activity of Acetycholine Esterase

Figure 2. AlCl$_3$ treatment enhanced the levels of activities of AChE in hippocampus and cortex of control and experimental rats as compared to control, whereas AA (30, 75 and 150 mg/kg b.w.) pretreatment dose dependently reversed these changes.

The statistical significance was calculated by one-way analysis of variance (ANOVA) using SPSS version 15.0. and the individual comparisons were obtained by Duncan's Multiple Range Test (DMRT). A value of P< 0.0.5 was considered to indicate a significant difference between groups and the values sharing a common alphabet do not differ significantly with each other.

4. RESULTS

4.1. AA dose dependently attenuated the AlCl$_3$ induced Al load and AChE activities

A preliminary study was conducted with three different doses of AA (30, 75 and 150 mg/kg b.w.) to determine the dose-dependent effect of AA in AlCl$_3$ induced AD rats. Rats treated with AlCl$_3$ (100 mg/kg b.w. oral) for 42 days showed a significant increase in the levels of Al (Figure 1) and activities of the AChE (Figure 2) in hippocampus and cortex. AA co-treatment dose dependently and significantly attenuated the Al levels and AChE activities in AlCl$_3$ induced rats. However middle (75 mg/kg b.w.) and high dose (150 mg/kg b.w.) of AA showed non-significant attenuation in Al levels and AChE activities, but more significantly than low dose (30 mg/kg). So 75 mg/kg b.w. of AA is chosen as optimum dose for further studies.

4.2. AA attenuated AlCl$_3$ induced learning and memory deficits

AlCl$_3$ treated rats took more time to reach the visible platform on 20th day in Morris water maze test
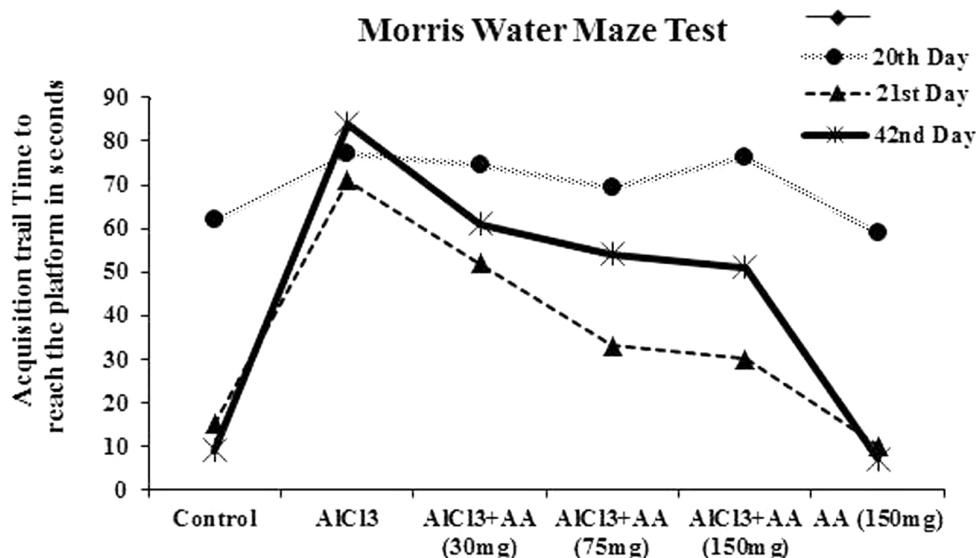
Morris Water Maze Test

Figure 3. Effect of AA on memory and learning deficit in Morris water maze test. $AlCl_3$ treatment significantly enhanced time taken to reach the visible and hidden platform as compared to control, whereas AA treatment diminished memory impairments. Data are expressed as mean ± SD (one-way ANOVA followed by DMRT).
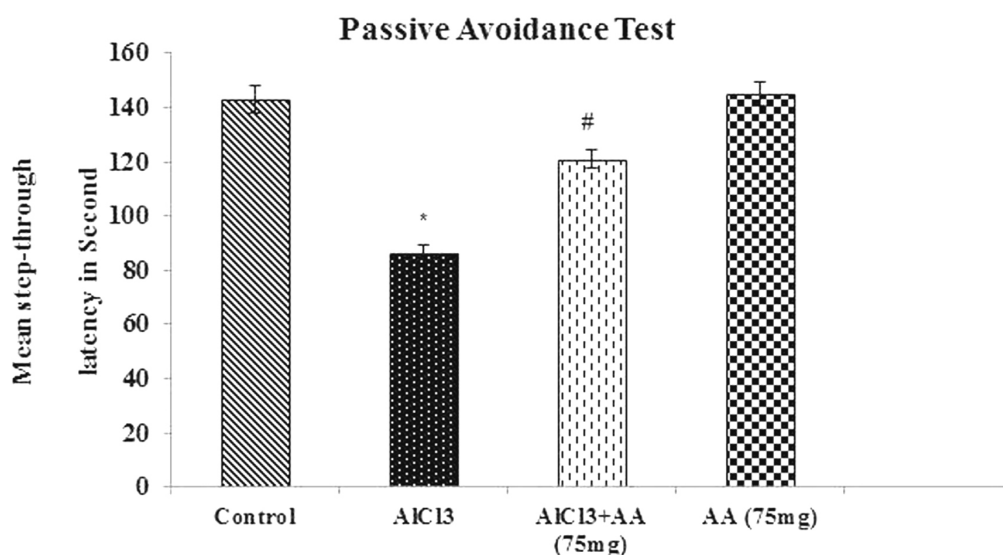
Passive Avoidance Test

Figure 4. Effect of AA memory deficit in Passive avoidance test. $AlCl_3$ treatment reduced the time taken to enter the dark room in the passive avoidance test as compared to control, whereas AA pretreatment reduced the memory impairments. Data are expressed as mean ± SEM (one-way ANOVA followed by DMRT) for six rats in each group. Values not sharing the same symbol differ significantly−*p < 0.05 compared to the control, #p < 0.05 compared to the AlCl3 treated rats.

(Figure 3) and exhibited a diminished step-through latency in passive avoidance task (Fig 4) than the control group. Co-treatment of AA dose dependently and significantly reversed the $AlCl_3$ induced memory and learning deficits as compared to $AlCl_3$ alone treated rats. Moreover $AlCl_3$ treatment significantly diminished the 1st and 2nd retention latencies (on days 21 and 42 respectively), whereas AA treatment significantly enhanced both the retention latencies as compared to $AlCl_3$ alone treated rats. AA alone treated rats showed

no significant differences in behavioural patterns when compared to control animals (Figure 3, 4).

4.3. AA protected $AlCl_3$ induced Aβ burden

We have performed western blot analysis to examine changes in β- amyloid metabolic markers in $AlCl_3$ animal models with or without AA treatment. $AlCl_3$ injection enhanced the protein expressions of APP, $Aβ_{1-42}$, β and γ secretases as compared to the
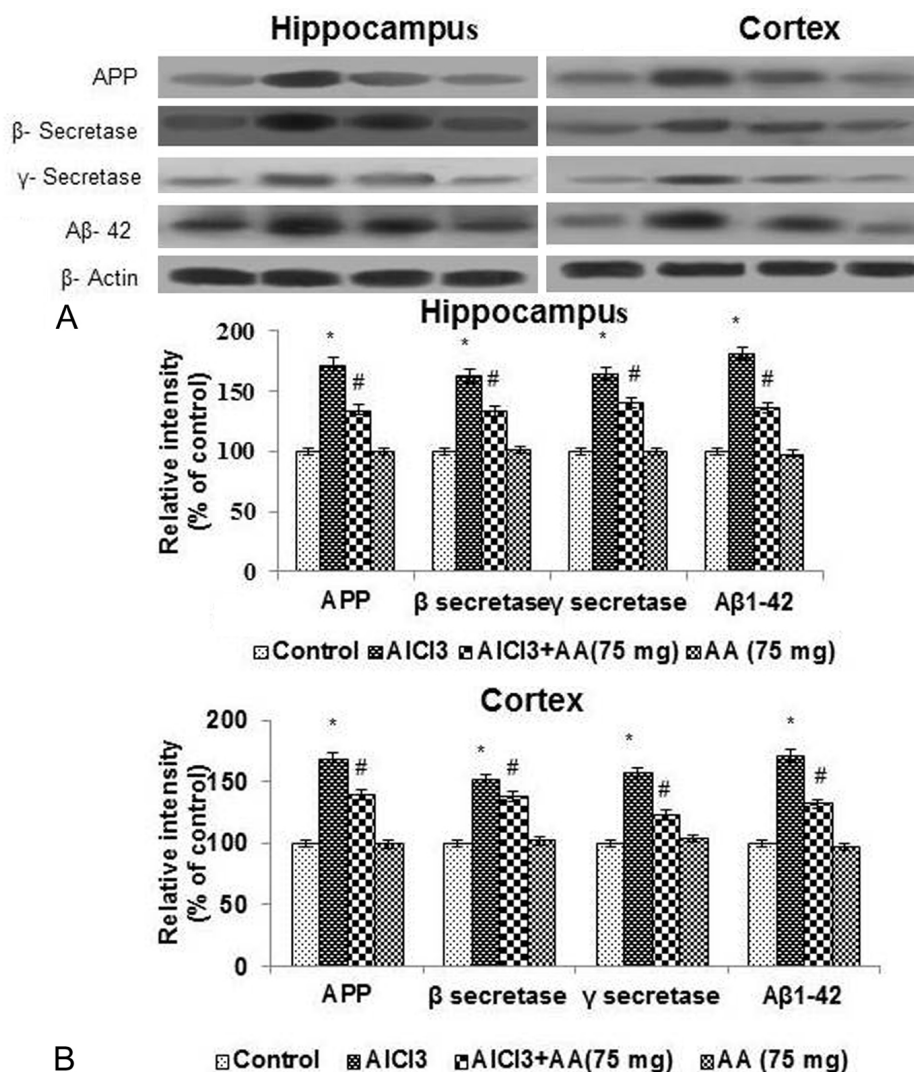
266

Figure 5. AlCl3 diminished protein expressions of APP, Aβ1–42, β and γ secretases as compared to control rats, whereas AA treatment reversed these changes. Evaluation of protein expression by using western blot. Data are expressed as mean ± SEM (one-way ANOVA followed by DMRT) for three rats in each group. Values not sharing the same symbol differ significantly−*$p < 0.05$ compared to the control, #$p < 0.05$ compared to the AlCl$_3$ treated rats.

control group, thereby favouring Aβ plaque formation. Oral administration of AA to AlCl$_3$ treated rats showed diminished expressions of APP, Aβ$_{1-42}$, β and γ secretases as compared to AlCl$_3$ alone treated rats (Figure 5).

4.4. AA ameliorated AlCl$_3$ influenced Inflammation

To explore the anti-inflammatory effect of AA, the protein expressions of GFAP and Iba-1 (markers of activated microglia and astrocyte) (Figure 6) were studied in the hippocampus and cortex of control and experimental rats. The expressions of these markers were found to be enhanced in the AlCl$_3$ treated rats, whereas their expressions were reduced by AA treatment. Activation of astrocytes and microglia by

AlCl$_3$, leads to the enhanced production of iNOS, NF-kB, TNF-α, IL-1β, IL-6, IL-4, IL-2 and COX-2, whereas AA treatment attenuated these indices through its anti-inflammatory properties (Figure 7,8).

5. DISCUSSION

Results of our current study indicated that the AlCl$_3$ administration significantly increased Al levels in hippocampus and cortex of rats. As Al^{3+} and Fe^{3+} ions are similar in size, the former occupies iron-binding sites in transferrin and circulates throughout the body (28). Al first enters into the endothelial cells of blood brain barrier and then into brain cells via transferrin dependent and independent mechanisms (29). Prolonged half-life of Al (i.e. 150 days in rats) raises
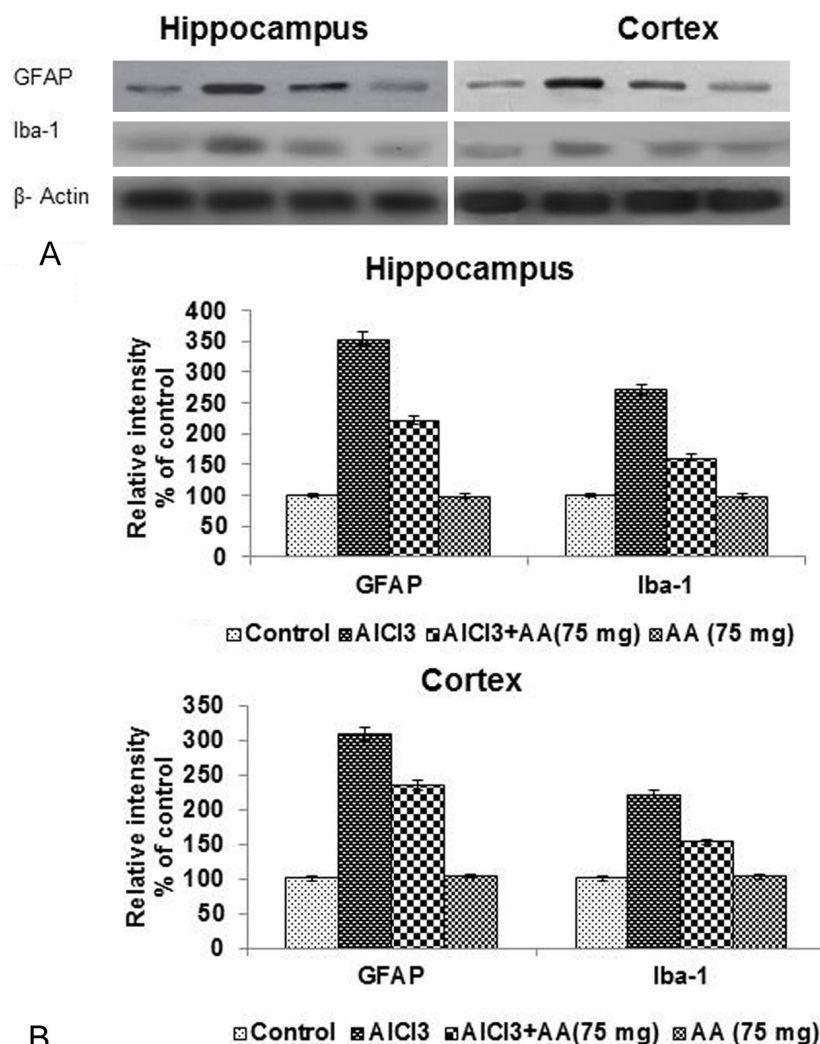
267

Figure 6. AlCl$_3$ injection enhanced the protein expressions of GFAP and Iba-1, markers of activated microglial and astrocytes as compared to control, whereas AA pretreatment diminished the expressions of these markers. Evaluation of protein expression by using western blot. Data are expressed as mean ± SEM (one-way ANOVA followed by DMRT) for three rats in each group. Values not sharing the same symbol differ significantly−*p < 0.05 compared to the control, #p < 0.05 compared to the AlCl$_3$ treated rats.

the risk of accumulation in all the regions of rat brain, particularly in hippocampus and cortex, which are the sites responsible for memory and learning (30). In our study, enhanced Al levels were found in hippocampus and cortex of AlCl$_3$ treated rats, whereas AA offer neuroprotection in AD animals by reducing the levels of Al. Mohd Salim *et al.* (31) demonstrated that the metal chelating ability of CA, which may be attributed due the presence of active components including AA. Previous studies from our lab reported that the plant extracts such as *Emblica officinalis* (3)*, Fenugreek* (23) and phytochemicals including hesperidin (2) offer neuroprotection against Al induced toxicity by diminishing their levels.

Acetylcholine (ACh) is the key neurotransmitter involved in the learning and memory processes and alterations on the cholinergic activity is the main event in the neurochemical changes of AD (32). AChE is the marker enzyme for cholinergic activity that degrades and terminates the physiological action of ACh. Zatta *et al.,* (33) indicated that Al can enhance the activity of AChE by interacting with its peripheral sites and modifying the secondary structure. In the present study, the observed elevated activity of AChE in the AlCl$_3$ treated group is ascribed due to the direct effect of Al. We found that the AA co-administration caused a significant reduction in hippocampal and cortical AChE activity. AChE has two special reactive sites: anionic and esteratic sites. Nasir *et al.,* (34) demonstrated that the inhibition of AChE activity by AA in *in vitro* condition was due to an active competition with ACh that occurs at the esteratic site on the enzyme, which is in consistent with our study.
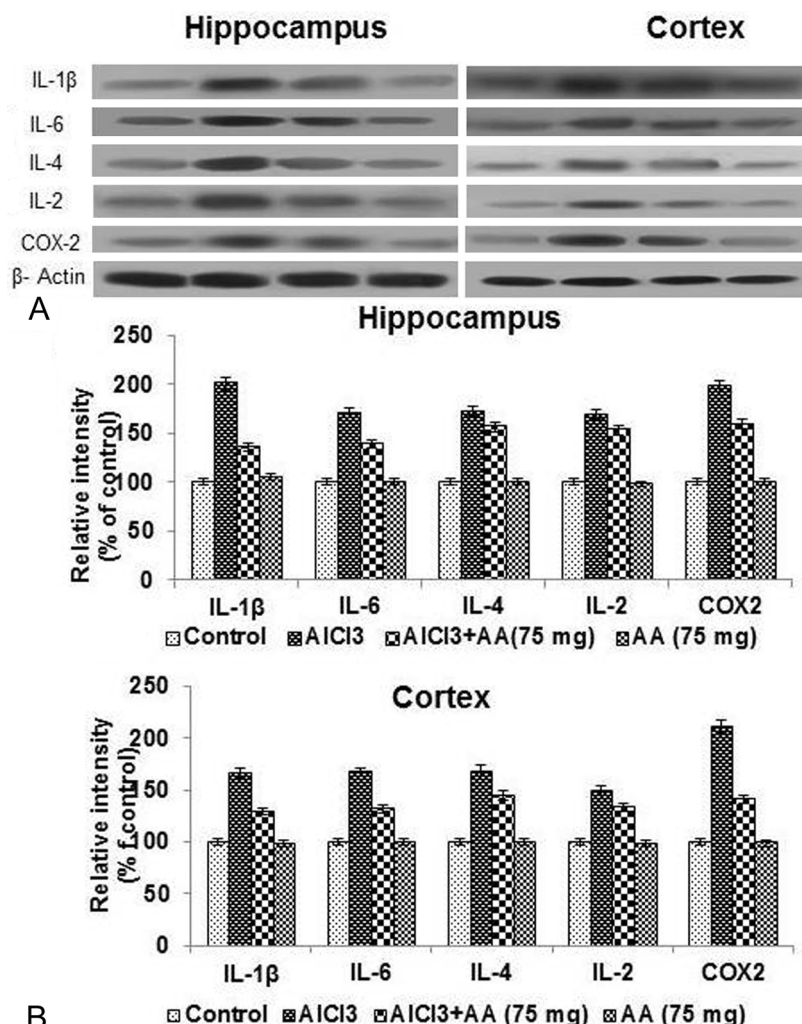
Figure 7. AlCl$_3$ treatment enhanced the protein expressions of IL-1β, IL-6, IL-4, IL-2 and COX-2 as compared to control, whereas AA treatment attenuated these inflammatory markers. Evaluation of protein expression by using western blot. Data are expressed as mean ± SEM (one-way ANOVA followed by DMRT) for three rats in each group. Values not sharing the same symbols differ significantly−*p < 0.05 compared to the control, #p < 0.05 compared to the AlCl$_3$ treated rats.

Measurement of behavioural changes is a more sensitive indicator of neurotoxicity during Al exposure (35). West (36) demonstrated that the Morris water maze test is used to measure the memory deficits in AD like conditions by inspecting the hippocampal function. In this test, AlCl$_3$ treated rats took more time to reach the visible platform than the control group, indicating memory deficits, whereas administration of AA significantly enhanced memory performance as compared to AlCl$_3$ alone treated group. Initially animals are learnt to locate the hidden platform by using spatial cues and the time taken to locate the hidden platform after several days can be measured as spatial memory. We found that the administration of AlCl$_3$ resulted in the loss of spatial as well as contextual memory, whereas AA treatment improved these cognitive impairments. In the passive avoidance task, the animal must learn to avoid or escape from an electric shock exposure

in darkness. All the nocturnal animals including rats naturally prefers dark environment, but the animal has to suppress this tendency by remembering the negative stimulus. AA markedly improved learning and memory of rodents injected with scopolamine (37) and monosodium glutamate (18), which is corroborated with our present results.

Amyloid cascade hypothesis demonstrated that the abnormal aggregation of Aβ in the brain is the main cause of AD that occurs due to an imbalance between Aβ production and Aβ clearance. Aβ$_{1-42}$ is formed after the sequential cleavage of amyloid precursor protein (APP) by β and γ secretases in the amyloidosis pathway, whereas in the nonamyloid pathway, APP is acted by α and γ secretases, which prevents Aβ generation and seems to be a protective pathway (38). Previous studies from our lab indicated
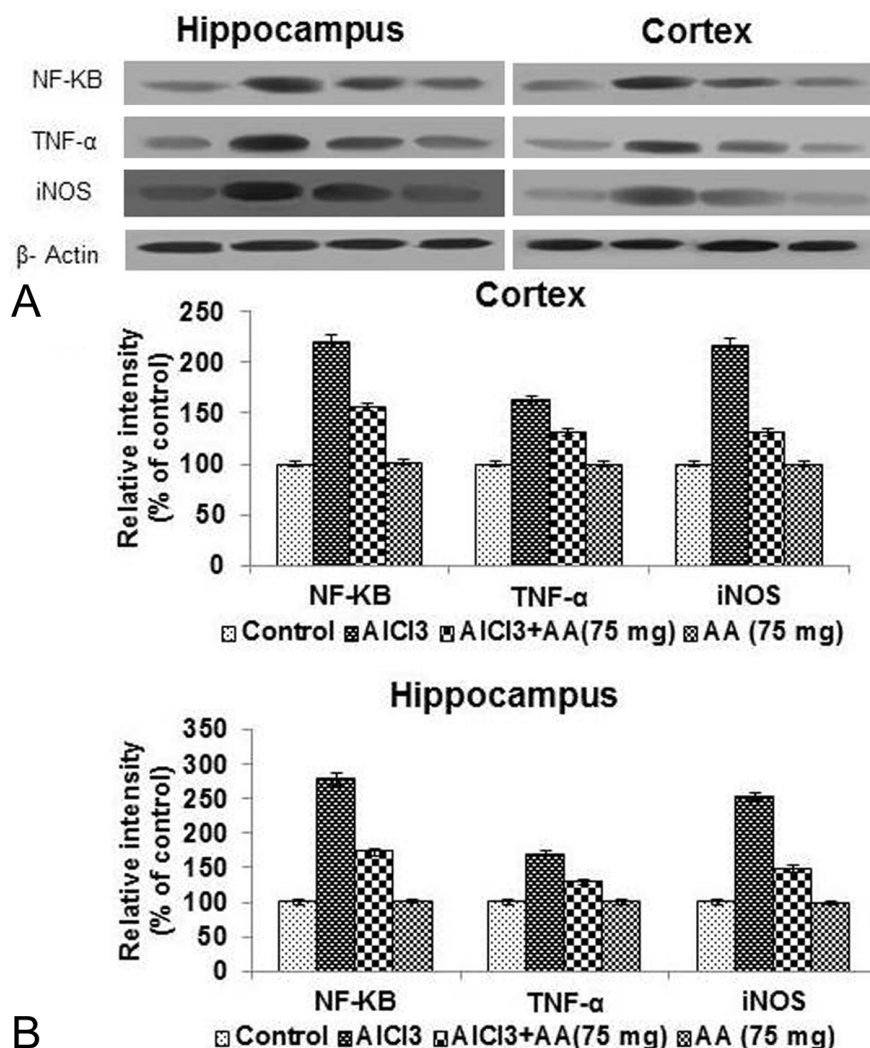
Figure 8. AlCl$_3$ elevated the protein expressions of NF-kB, TNF-α and iNOS as compared to control, whereas AA treatment attenuated these inflammatory markers. Evaluation of protein expression by using western blot. Data are expressed as mean ± SEM (one-way ANOVA followed by DMRT) for three rats in each group. Values not sharing the same symbols differ significantly−*$p < 0.05$ compared to the control, #$p < 0.05$ compared to the AlCl$_3$ treated rats.

that the chronic exposure to AlCl$_3$ accelerated Aβ generation and diminished its degradation through enhancing the expression of APP, Aβ$_{1-42}$, β and γ secretases (2, 3, 39, 40). Exley, (41) reported that Al increases the Aβ burden in experimental animals through a direct impact on Aβ anabolism or direct or indirect influence on Aβ catabolism. In the present study, AA attenuated the Al induced Aβ toxicity by lowering the expressions of APP, Aβ$_{1-42}$, β and γ-secretases. AA inhibited the Aβ-induced cell death under *in vitro* conditions, through its anti-oxidative and anti-apoptotic actions (42). Jew *et al.*, (19) indicated that various derivatives of AA exhibited protective activity at different levels against Aβ-induced neurotoxicity *in vitro*. AA significantly down-regulated the expression of β-secretase and up-regulated the expression of α-secretase in primary

rat cortical neurons (43), which is corroborated with our present results.

Neuroinflammation is considered as a key feature in the pathology of AD and current target for therapeutic interventions. In AD, microglia, astrocytes and few neurons having significant functions in the homeostasis and function of the brain are the major players involved in the inflammatory process (44, 45). Reactive gliosis is a phenomenon during which astrocyte and microglia get activated in response to an array of toxins that contribute to the pathogenesis of neuroinflammation and neurodegeneration. GFAP is an astrocyte-specific intermediate filament protein essential for homeostasis of central nervous system and Ionized calcium-binding adapter molecule 1 (Iba-1) is a microglial specific protein, also known

as allograft inflammatory factor-1 (Alf1), are up-regulated in reactive gliosis following Al exposure (12). In our study, the enhanced expression of inflammatory markers in the hippocampus and cortex in AlCl$_3$ alone treated rats, demonstrated the reactive gliosis, whereas the attenuated expression of GFAP and Iba-1 during AA treatment suggested its anti-inflammatory role.

Previous studies indicated that the administration of various Al salt complexes can aggravate glial and astrocyte and further endorse an inflammatory cascade in the brain (12, 40, 46). In the present study, AlCl$_3$ administration enhanced the expressions of pro-inflammatory cytokines such as IL-1β (key regulator of inflammation during host defence response), IL-4 (enhanced during the sustained IL-1β neuroinflammation), IL-2 (numerous effects on hippocampal neurons, where its receptors are enriched, thus improving cognitive performances), IL-6 (mediator of the inflammatory and immune responses), TNF-α (trigger for other cytokines) (46, 47), whereas AA treatment attenuated the expressions of above said indices. Li *et al.*, (48) indicated that the treatment of AA attenuated lipopolysaccharide -induced TNF-α, IL-6 and IL-1β production in acute lung injury in mice. Enhanced TNF-α expression activates the transcription factor NF-κB. COX-2 expression in neurons can be induced by excitotoxic insults, transient global cerebral ischemia and oxidative stress as well as inflammatory mediators such as TNF-α (49). Proinflammatory cytokines exaggerated the oxidative/nitrosative stress by inducing iNOS gene expression through NF-κB activation. In the CNS, NF-κB transcription factors are key players in a number of physiological processes such as neurogenesis (50), neuritogenesis (51) and synaptic plasticity which related to learning and memory (52). Haung *et al.*, (53) revealed that AA decreased the expressions of iNOS, COX-2 and NF-κB against λ-Carrageenin-induced edema. Diminished expressions of TNF-α, IL-1β, COX-2 and iNOS were found in the cortex and hippocampus of rats treated with AA, which demonstrates that AA exerts anti-inflammatory effect through regulating the expression of NF-κB.

To conclude, our observations suggested that AA treatment mitigated AlCl$_3$ induced AD associated pathologies including Al overloading, AChE hyperactivity, behavioural impairment, Aβ burden and inflammation, which may due to its multiple pharmacological action. It is suggested that the herbs having anti-inflammatory, antioxidant and anti-apoptotic activities could be useful in the treatment of AD (54, 55). Further studies are warranted to explore the link between AlCl$_3$-mediated oxidative stress and associated apoptosis to establish the neuroprotective role of AA in AD.

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