Amjad S and Umesalma S, J Pharmacol Pharmaceut Pharmacovigil 2018, 2: 007
DOI: 10.24966/PPP-5649/100007

HSOA Journal of
Pharmacology, Pharmaceutics & Pharmacovigilance

Research Article

Centella Asiatica Extracts Regulates Aluminium Chloride-Induced Neurotoxicity in Rats: Impact on Inflammation, Apoptosis and Biogenic Amine Levels

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Abstract

Objectives and design: Aluminium (Al) is an environmental neurotoxin that affects cerebral functions and causes health complications. The antioxidant and memory enhancing effects of *Centella Asiatica* (CA) are well known in the past few decades. Therefore, the present study has been designed to investigate the therapeutic potential of CA against chronic Aluminium Chloride ($AlCl_3$) exposure induced rats. Wistar albino rats were segregated into four groups: group 1-control rats, group 2-rats received $AlCl_3$ (300 mg/kg body weight, every day orally) for 60 days, rats in group 3-received CA (500 mg/kg body weight, orally) and group 4 rats were initiated with both $AlCl_3$ and CA treatment.

Material and methods: Neurotoxicity was assessed by measuring brain biogenic amines, Al concentration in blood and brain, the levels of pro-inflammatory cytokines and later the apoptotic related components. Significant depletion of neither brain Dopamine (DA), Epinephrine (E), Nor Epinephrine (NE) nor 5-Hydroxytryptamine (5-HT) levels was observed following $AlCl_3$ exposure. Administration of $AlCl_3$ raised the pro-inflammatory cytokines levels (tumor necrosis factor alpha, TNF-α and inducible nitric oxide synthase, iNOS) and modulated the levels of apoptotic proteins cytochrome c, caspases 3, with altered Bax/Bcl-2 ratio in favour of apoptosis in cortex that are determined by western blotting and immunohistochemistry.

Results: Administration of CA significantly increased biogenic amine levels, decreased Al concentration compared to Al-induced animals. CA ameliorates $AlCl_3$-induced inflammatory niche. Further, treatment with CA ameliorated neuronal apoptosis by reducing cytochrome c, caspase- 3 expressions significantly and by modulating Bax/Bcl-2 ratio.

Conclusion: The findings of this study suggest that CA protects the cerebral cortex against $AlCl_3$-induced neurotoxicity. This neuroprotective effect is partially mediated by its anti-oxidant, anti-inflammatory and anti-apoptotic activities as well as elevating biogenic amine levels.

Keywords: Aluminium chloride; Apoptosis; *Centella asiatica*; iNOS; TNF-α

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Citation: Amjad S, Umesalma S (2018) *Centella Asiatica* Extracts Regulates Aluminium Chloride-Induced Neurotoxicity in Rats: Impact on Inflammation, Apoptosis and Biogenic Amine Levels. J Pharmacol Pharmaceut Pharmacovigil 2: 007.

Received: October 24, 2017; **Accepted:** January 09, 2018; **Published:** January 24, 2018

Introduction

Aluminum (Al) is a potent environmental neurotoxin and has been associated with several disorders. Al and its salts are widely exposed to human body due to their presence in several products including cosmetics, drugs, food additives (salt, spices, yellow cheese and herbs) and a powerful flocculants in water purification. Al has the potential to be toxic for humans and its compounds have neurotoxic manifestations which further shown to contribute several age-related neurodegenerative diseases [1]. Chronic exposure to Al causes pathogenesis of age-related illnesses such as the Alzheimer's Disease (AD), the Parkinson's disease, the Huntington's disease, inflammation, Amyloid Beta (Aβ) deposition and plaque formation in the brain and suggested that this excess might be a possible contributing factor for the aging process by inflicting oxidative damage [2]. Evidence suggests that inhaled Al not only causes neurologic signs, which mimic progressive neurodegeneration, but also results in neurofilamentous changes in the cerebral cortex focal region where more Al accumulation takes place [3,4]. Previous studies have shown that, the excessive concentration of Al in neurotic deposits, plaques and neurofibrillary tangles in the brain of Alzheimer's patients [5].

Al is a well-documented and experimental neurotoxicant that enhances neuroinflammatory events in the brain by multiple mechanisms. Accumulating evidence suggests that Aluminium Chloride ($AlCl_3$) promote free radical generation by potentiating the pro-oxidant properties of transition metals (iron and copper) [6]. In addition, $AlCl_3$ exerts its own pro-oxidant effect by altering calcium flux and homeostasis and to facilitate peroxidation of membrane lipids and antioxidant enzymes [7]. Beside this, various Al salt complexes can alter neuronal signal transduction pathways associated with glial cell activation, which further promote an uncontrolled inflammatory cascade in the brain. These activated glial cells secrete cytotoxic agents including Reactive Oxygen Species (ROS), Nitric Oxide (NO), inflammatory cytokines Like Tumor Necrosis Factor alpha (TNF-α), inducible Nitric Oxide Synthase (iNOS) which are toxic and inturn

cause synaptic abnormalities ultimately related to memory dysfunction. Earlier study has evidenced that $AlCl_3$ activates astrocytes and glial cells which releases different inflammatory mediators due to induction of apoptosis [8,9]. Therefore, overall effects are to reduce pro-inflammatory mediator's production by activated microglia and astrocytes should be useful for prevention of neuroinflammation and eventually neuroprotection.

Apoptosis under mitochondrial control has been implicated in a progressive and selective loss of neurons, which involve the release of cytochrome c into the cytoplasm and initiation of the apoptosis cascade. $AlCl_3$ is a potent cholinotoxin and causes aberrant apoptotic neuronal loss by stimulating ROS production, which is a characteristic symptom of neurodegeneration [10-12]. It induces apoptosis of neurons and astrocytes *via* involvement of caspases activation in rodent and *in vitro* models [13,14]. Reactivation of caspases has been implicated in mediating neural death during pathological processes causing neuronal loss in acute and chronic neurodegenerative diseases. Caspase-induced apoptosis has been documented in Amyloid-β-induced Alzheimer's and 6-hydroxydopamine-induced Parkinson's neuronal apoptosis [15,16]. Restriction of apoptosis and its associated factors during neurodegeneration is presumed to be beneficial for the survival of neurons. Thus clinical trials are in progress that anti caspase approach in neurodegeneration will be an exciting avenue for therapeutic neuroprotective interventions [17].

The antioxidant capabilities of *Centella Asiatica* (CA) have been documented previously in different experimental models. *Centella Asiatica* (CA), also known as gotu kola and Indian pennywort, is a perennial herbaceous creeper of the Apiaceae family. Recently, CA has been reported as a neuroprotectant against different degenerative disorders. CA modulate the immune system [18], act as antioxidant [19], prevent alleviation of oxidative stress [20], act as anti-inflammatory agent [21], and inhibit proliferation of cancer cells [22]. Yet, the most widely reported health benefit of this herb is in improving the brain function, particularly related to learning and memory. In fact, CA has been reported to stimulate nerve regeneration *in vitro* [23], which could explain its brain protective effect. In humans, it has been reported to improve mental ability of the mentally retarded children and decrease the amyloid-β levels in the brain [24,25]. Previously, we have demonstrated that CA inhibited cognitive impairment and improves learning and memory in Al-induced rats by reducing oxidative stress and Acetyl Cholinesterase (AChE) activity [26]. Based on this background, the present study was carried out to investigate the protective effect of CA against $AlCl_3$ induced inflammation and oxidative stress associated mitochondria apoptosis. Therefore in this study, we have investigated the efficacy of CA in terms of biogenic amines, expression of pro-inflammatory cytokines TNF-α, iNOS and apoptotic inducing factors in cortex of rat brain.

Materials and Methods

Animals

Wistar albino rats of either sex (120-150g) were procured from King Institute and Central Animal House Facility (CAHF) of University of Madras, Chennai, India. Animals were fed with commercial pellet diet and water ad libitum and maintained at an ambient temperature of 25 ± 2°C with a 12-hr light: dark cycle. The experiments were conducted in accordance with guidelines of Institutional Animal Ethical Committee (IAEC) governed by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines, Government of India.

Drugs and experimental design

Aluminum Chloride Hexahydrate ($AlCl_3$. $6H_2O$) was purchased from Sd fine-chem, Mumbai, India. All other chemicals and reagents used were of analytical grade.

Preparation of the plant extract: Fresh leaves of CA were collected from regular vendor and species identification was done from Center for Advanced Studies in Botany, University of Madras, Chennai, India. The leaves were cleaned, shade dried and coarsely ground with grinder. The coarse powder of plant was extracted with 8 parts of distilled water under boiling for 5 hrs and was filtered through a 400 mesh cloth to collect the extract. The extract was concentrated and freeze dried to get a powder of greenish brown in color. The percentage w/w yield of the extract was 41%. Standardized aqueous extract of CA and $AlCl_3$ solutions (made freshly at the beginning of each experiment daily). For oral administration, $AlCl_3$ and CA were administrated in a dose of 0.5 ml/100 g body weight. The animals were divided into four groups (n=6). Group 1 control rats. Group 2 rats administered with $AlCl_3$ (300 mg/kg) for 60 days. Group 3 rats received CA alone (dissolved in water, 500 mg/kg). Group 4 rats co-treated with CA and induced with $AlCl_3$ simultaneously for 60 days. The doses of $AlCl_3$ [26,27] and CA [26] were selected based on published report of our own laboratory.

Biochemical and cellular assessments

The animals were anaesthetized by diethyl ether and killed by cervical decapitation after an overnight fast. Brains were dissected out by making midline incision to view the skull. Then, a small incision from the caudal part of parietal bone and a firm cut in the anterior part of the frontal bone were made to remove the brain more easily. Isolated brains were rinsed with ice-cold isotonic saline then kept on ice (using large and small curved serrated forceps) to obtain cerebral cortex [28]. The isolated brain and its parts were homogenized using 0.1% Triton X-100 buffer (pH - 7.4), in a Potter-Elvehjem homogenizer and then centrifuged at 10,000xg (4°C) for 30 min. Aliquots of the supernatants were separated, stored at -80°C and used for further biochemical assays.

Aluminium estimation

The aluminium was analyzed by wet acid digestion method of Zumkley *et al.,* in serum and cortex of brain. A mixture of 2.5 ml of perchloric acid/nitric acid (1:4 in volume) was added to brain parts [29]. Then, mixture with brain sample was placed in sand bath for 44h until the point of a white ash or residue was obtained. Then residues were dissolved in 2.5 ml of 10mm nitric acid. Then this sample (in liquid form) was placed in the sample holder of atomic absorption spectrophotometer (Perkin Elmer, India). The total concentration of aluminium was calculated in µg/gm of tissue or PPM.

Assessment of brain monoamine neurotransmitters levels

The level of biogenic Amines Dopamine (DA), Epinephrine (E), Norepinephrine (NE) and 5-Hydroxytryptamine (5-HT, serotonin) were assessed in brain homogenate of the different treated groups by the method described by Byers *et al.,* [30]. Briefly, for the estimation of monoamine neurotransmitter level, brain tissue was homogenized in homogenizing solution containing the internal standard Dihydroxy

Benzylamine (DHBA). Following tissue disruption, the samples were centrifuged, then filtered using 0.22 µm filter (Millipore, USA). The concentrations of the monoamine contents DA, E, NE and 5-HT were measured by reverse-phase High-Performance Liquid Chromatography with Electrochemical Detector (HPLC-ECD). The mobile phase consisted of 50mm phosphate buffer (pH - 3.5) containing 80mg/L Octane Sulfonic Acid (OSA), 25 mg/L Ethylenediamine Tetraacetic Acid (EDTA) and 10% v/v methanol. The mobile phase flow rate was 1ml/min. The applied oxidation potential was set at 750mv. The concentration of the neurotransmitters was determined by external standard method using peak areas. A linear standard curve was constructed by plotting peak areas versus the corresponding concentrations.

Protein extraction and Western Blotting

The proteins were isolated from cerebral cortex region from brain tissue and were homogenized in ice-cold HEPES (N-2- Hydroxyethylpiperazine-N-2-Ethane Sulfonic acid) lysis buffer (HEPES 25 mm; $MgCl_2$ 5 mm; EDTA 5 mm in dd. H_2O). The homogenates were centrifuged (10,000 rpm/15 min/4˚C), and the protein content of the supernatant was assayed and diluted to give equal protein concentrations of 30 µg. The protein content was estimated by *Lowry et al.,* using BSA as standard [31]. Western blot analysis was performed according to the methods of *Towbin et al.,* [32]. Equal concentration of protein from each sample was (prepared with 6X Laemmli Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS PAGE). Sample loading buffer 0.5 mm Tris-HCl, pH 6.8; 10 % glycerol v/v; 10 % SDS w/v, 0.2 % β-mercaptoethanol v/v, 0.05 % bromophenol blue (w/v) boiled for 5 min prior to loading onto 12 % gels, resolved at a constant supply of voltage and transferred onto PVDF (Poly Vinylidene Fluoride) membranes (Millipore Corp., Bedford, MA, USA). To assess the expression of specific proteins, membranes were incubated overnight at 4˚C in 10 ml PBS/Tween (0.1 % v/v) containing 1 % BSA with one of the following antibodies: goat polyclonal TNF-α, caspase-3, rabbit polyclonal iNOS, Bax, Bcl-2 and cyt c, (1:500) (Santacruz Biotech, USA). Following incubation, immunoreactive bands were detected by incubating with respective Horse Radish Peroxidase (HRP)-conjugates. Protein– antibody complexes were visualized by the addition of Diaminobenzidine (DAB) as a substrate. Relative band intensities were quantified using Image-J analysis software.

Immunohistochemical analyses

Brain sections were fixed in 4% cold para formaldehyde in 100-mm Phosphate Buffered Saline (PBS) and were made into coronal (4µm) coated on clean slides. The sections were de-paraffinized in fresh xylene and rehydrated using graded ethanol solutions. The slides were dipped in freshly prepared solution of 1 % H_2O_2 in methanol for 20 min to quench endogenous peroxidase. Slides were rinsed in PBS and incubated for 1 hr in blocking solution (3 % BSA, 0.1 % Tween-20 in PBS) at Room Temperature (RT). Sections were incubated with goat polyclonal TNF-α, (1:3,000), rabbit polyclonal iNOS and cyt c (1:500) in blocking solution for 12 h at 4˚C re-equilibrated to room temperature and washed with PBS, incubated with Horse Radish Peroxidase (HRP) antibody conjugates (1:2,500) in blocking solution without Tween-20 for 2 hrs at RT. Sections were washed with PBS and incubated with 0.2 % solution of 3,3'-Diaminobenzidine (DAB) until desired stain intensity develops at RT followed by washing in distilled water. Sections were counter stained with hematoxylin and mounted with Di-N-Butylphthalate-Polystyrene-Xylene (DPX). Sections were viewed by two independent observers blinded to experimental conditions and the total number of positively stained cells was counted and presented graphically.

Statistical analysis

Statistical analysis of the data was performed using SPSS/10.0 software. Data are expressed as mean ± Standard Deviation (SD). Significance of differences between groups' mean was analyzed using two-way ANOVA followed by Least Significant Difference (LSD) test. Statistical significance was set below 5 % level ($p<0.05$ was considered significant).

Results

Effect of CA on Al concentration in serum and brain tissue

The concentration levels of Al detected in serum and brain tissue samples from control and experimental rats are illustrated in figure 1. $AlCl_3$ treatment significantly increased concentration level of Al in brain cortex areas and serum as compared to control rats. However, chronic CA treatment significantly attenuated the rise in Al concentration in both as compared to control rats ($p<0.05$) and CA exposed groups which showed the lowest concentrations of Al.

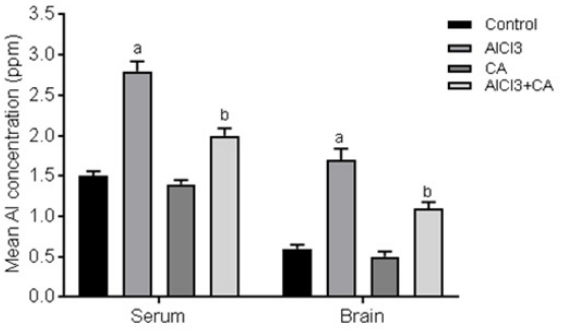

Figure 1: Effect of CA supplementation on Al concentration in serum and brain tissue of control and experimental rats subjected to $AlCl_3$. Results were expressed as mean ± SD (n=6). Values are statistically significant at $p<0.05$; compared with $^{a}AlCl_3$ vs. control; $^{b}AlCl_3$+ CA vs. $AlCl_3$.

Effect of CA on $AlCl_3$-induced rat brain neurotransmitters

The effects of $AlCl_3$ on brain neurotransmitters like DA, E, NE, 5-HT and their response to treatment with CA are shown in figure 2. Significant depression ($p<0.05$) of DA, E, NE and 5-HT neurotransmitters levels in brain homogenates of Al-exposed rats compared to control group. A remarkable induction of neurotransmitters was achieved in the $AlCl_3$ +CA co-treated group whilst not restored to the control level. A significant enhancement of brain neurotransmitters was observed in $AlCl_3$+CA co-treated group compared with Al exposed group.

CA treatment decreases the $AlCl_3$-induced expressions of TNF-α and iNOS

Activated microglia and astrocytes release proinflammatory cytokines that may eventually cause neuroinflammatory events and neuronal death [33]. We have studied the expressions of pro-inflammatory

cytokines TNF-α and iNOS, as these are implicated in excitotoxicity [34]. Figure 3 shows the immunohistochemical analysis of TNF-α protein expression in the cortex of control group of rats. $AlCl_3$-induced rats (Figure 3B) showed significantly ($p<0.05$), increased TNF-α expression in rat brain. CA-treated groups of rats showed significantly ($p<0.05$), reduced expressions for TNF-α in cortex as compared to $AlCl_3$-induced groups (Figure 3D). No adverse changes were noticed in cortex of control and CA-alone-treated groups of rats (Figure 3C).

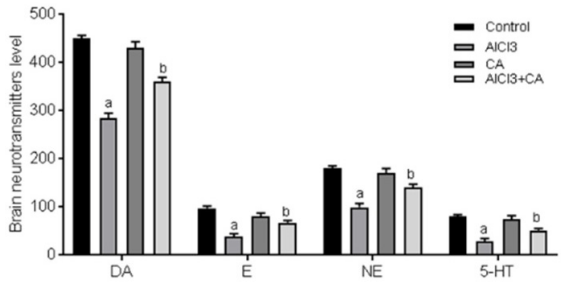

Figure 2: Graph represents the effect of CA supplementation on the levels of brain monoamine neurotransmitters in the rats of control and experimental groups. Dopamine (DA, ng/g), epinephrine (E, ng/100g), norepinephrine (NE, ng/100g) and 5-hydroxytryptamine (5-HT, ng/g). Results were expressed as mean ± SD (n=6). Values are statistically significant at $p<0.05$; compared with $^{a}AlCl_3$ vs. control; $^{b}AlCl3$+ CA vs. $AlCl_3$.

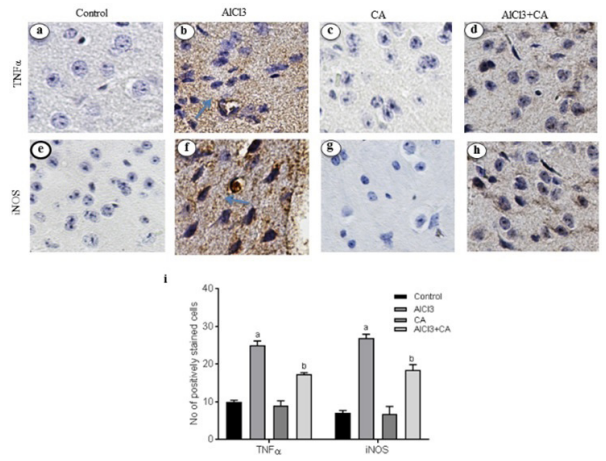

Figure 3: Representative micrographs of immune stained specimens of cortex from control and experimental groups of rats. Brain specimens were probed with antibody specific for TNFα and iNOS. a) TNF-α expression in cortex of control group; b) TNF-α expression in cortex of $AlCl_3$-induced group; c) CA alone (drug control) group; d) TNF-α expression in cortex of $AlCl_3$-induced and CA treated group; e) iNOS expression in cortex of control group; f) iNOS expression in cortex of $AlCl_3$-induced group; g) CA alone (drug control) group; h) iNOS expression in cortex of $AlCl_3$-induced and CA treated group; Scale bar 100 µm, magnification ×20. (i) Immunoreactivity was assessed and measured objectively by two independent observers. For quantitation, data expressing the respective protein expression were quantitated in ten fields/section and an average was used to denote the no. of positively stained cells and is represented in graph. Arrows indicate the positively stained cells. Hypothesis testing method included two-way Analysis of Variance (ANOVA) followed by Least Significant Difference (LSD). Results are given as statistically significant at $p<0.05$; compared with $^{a}AlCl_3$ vs. control; $^{b}AlCl_3$+ CA vs. $AlCl_3$.

An abundant activation of iNOS (Figure 3F) positive cells was evident in cerebral cortex tissue from rats exposed to $AlCl_3$ ($p<0.05$), in contrast to an almost negligible expressions was found in control and CA-alone treated animals (Figure 3E). CA-treated group of rats exhibited an evident reduction of expression of iNOS ($p<0.05$) (Figure 3H) when compared with $AlCl_3$-induced and control group. No adverse changes were noticed in cortex of control and CA-alone-treated groups of rats (Figure 3G). The total number of immunoreactive cells was quantified and is represented in graph (Figure 3I).

Western blotting analyses of TNF-α and iNOS, is shown in figure 4A, which was found to be significantly ($p<0.05$) increased in rat brain cortical region of $AlCl_3$-induced group when compared to TNF-α and iNOS protein levels in control and CA alone groups. CA treatment significantly ($p<0.05$) reduced protein expressions of TNF-α and iNOS. Control and drug control groups showed similar levels of TNF-α and iNOS expressions. Further, quantitative data expressing the corresponding protein levels were assessed using Image-J (NIH software), relative to β-actin, and are graphically represented (Figure 4B). Thus, CA exerts beneficial effects against neuroinflammation by limiting inflammatory cytokine production [21].

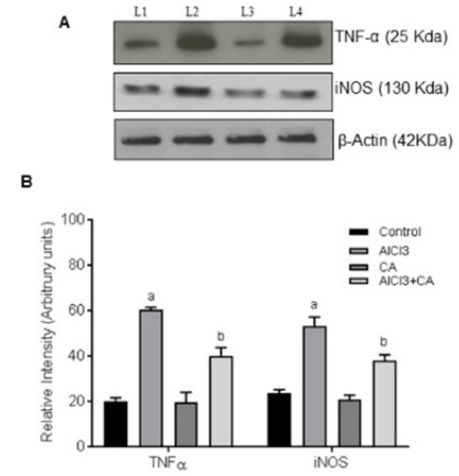

Figure 4: Representative immunoblots of TNF-α and iNOS protein expressions in the cortex of control and experimental groups of rats. Western blot analyses were performed using probes specific for TNF-α, iNOS, and β-actin as indicated. Lane 1 Control; Lane 2 $AlCl_3$-induced; Lane 3 CA alone; Lane 4 $AlCl_3$-induced and CA treated. Data expressing the corresponding protein levels were assessed using densitometry and is expressed in relative intensity (arbitrary units). Hypothesis testing method included two-way Analysis of Variance (ANOVA) followed by Least Significant Difference (LSD). Results are given as statistically significant at $p<0.05$; compared with $^{a}AlCl_3$ vs. control; $^{b}AlCl_3$+ CA vs. $AlCl_3$.

CA treatment suppressed cytochrome c expression in $AlCl_3$-induced rat brain

The mitochondria-mediated apoptotic pathway is characterized by mitochondrial dysfunction with the release of caspase activators including cytochrome c. Figure 5 shows the immunohistochemical analysis of cytochrome c in the control and experimental groups of rat brain. Level of cytochrome c was significantly ($p<0.05$) increased in cortex of $AlCl_3$-induced rat as compared to controls. CA treatment significantly ($p<0.05$) reduced the cytochrome c levels in cortex as compared to $AlCl_3$-administered groups of rats. Control and

CA-alone administered groups of rats showed no appreciable changes in cytochrome c expression levels. Quantitative data expressing the corresponding protein levels were assessed using densitometer and the results were expressed as relative intensity arbitrary unit (Figure E).

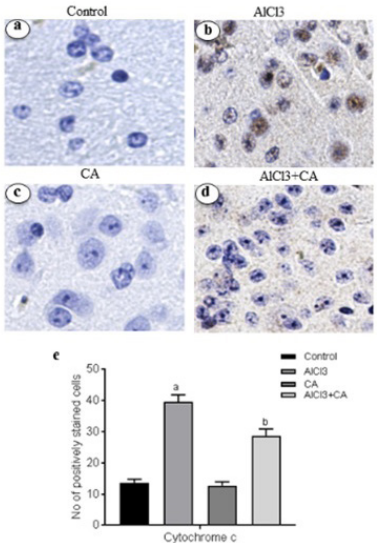

Figure 5: (A) Representative micrographs of immune stained sections of cytochrome c expression in cortex from control and experimental groups of rats. Brain specimens were probed with antibodies specific for cytochrome c (Magnification ×40). Immunoreactivity was assessed and measured objectively by two independent observers. B) For quantitation, data expressing total number of positively stained cells quantification were performed by counting the positively stained cells in 10 fields/section by two independent observers in blinded fashion, and the average was used to denote the total number of positively stained cells. Hypothesis testing method included two-way Analysis of Variance (ANOVA) followed by Least Significant Difference (LSD). Results are given as statistically significant at $p<0.05$; compared with $^{a}AlCl_3$ vs. control; $^{b}AlCl_3$+ CA vs. $AlCl_3$.

CA treatment suppressed apoptotic-inducing proteins in $AlCl_3$-induced rat brain

The mitochondria-mediated pathway is controlled by anti-apoptotic Bcl2 and pro-apoptotic Bax. The balance between Bcl2/Bax determines cell survival or death. Figure 6a shows the levels of Bax, Bcl2 and caspase-3 in the cortex of control and experimental groups of rats. Levels of Bax/Bcl2 were significantly ($p<0.05$) tuned in favor of apoptosis in $AlCl_3$-induced rats as compared to control rats. Treatment with CA caused significant ($P<0.05$) decrease in the protein level of Bax, caspase-3 and increased level of Bcl-2 protein as compared to $AlCl_3$-induced rats. Quantification of Bax/Bcl2 and caspase-3 expressions were represented graphically (Figure 6B) and the levels show that CA significantly protected cortex from $AlCl_3$-induced neuronal apoptosis.

Discussion

Al is a ubiquitous metal and implicated in the pathophysiology of neurodegenerative diseases. Reports suggest that $AlCl_3$ alters cytoskeletal dynamics of neurons and astrocytes leading to pathogenesis of neurodegeneration. In mammals, Al accumulation in the brain has been linked to neurodegenerative diseases such as PD and AD [4, 35]. Understanding the etiology of neurodegeneration and identifying ways to achieve early prevention are being considered as important approaches in the management of neurological diseases. Counteracting attempts to prevent/inhibit neurodegeneration and enhance neuronal survival has been greatly summoned. Recently, the traditional medicinal plant CA and its extracts have gained much interest for their ability to modulate neuronal function and influence learning and memory processes [36,37]. Hence, the present study was an attempt to understand the reduction of neuroinflammatory signals and apoptotic mechanisms underlying neuroprotective efficacy of CA in a rat model of neurodegeneration.

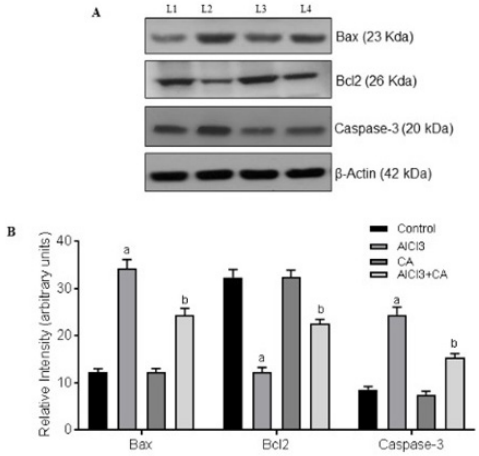

Figure 6: Representative immunoblots of Bax, Bcl2 and caspase-3 protein expressions in the cortex of control and experimental groups of rats. Western blot analyses were performed using probes specific for Bax, Bcl2, caspase-3 and β-actin as indicated. Lane 1 Control; Lane 2 $AlCl_3$-induced; Lane 3 CA alone; Lane 4 $AlCl_3$-induced and CA treated. Data expressing the corresponding protein levels were assessed using densitometry and is expressed in relative intensity (arbitrary units). Hypothesis testing method included two-way Analysis Of Variance (ANOVA) followed by Least Significant Difference Difference (LSD). Results are given as statistically significant at $p<0.05$; compared with $^{a}AlCl_3$ vs. control; ^{b}AlCl3 + CA vs. $AlCl_3$.

Brain is considered a preferential site of Al accumulation mainly at some cortical regions and hippocampus via crossing the Blood-Brain Barrier (BBB) [38]. The obtained data demonstrated that the serum Al level was elevated in Al exposed rats, indicated an occurrence of serum hyper-aluminemia which resulted in an elevation of brain tissue Al content. This elevation appears to be a consequence of an alteration in the permeability of the BBB, it has been shown that Al somehow interacts with the BBB via interfering with its function at the endothelial cell level, whereas positively charged Al ions neutralize the anionic sites of the endothelial cell surface and also may consequently altering the function of the BBB [39]. Concomitant treatment with CA was associated with a significant reduction in serum and brain Al content this depletion may be due to CA's free radical scavenging potential [26]. This may preserve cell membrane function including ion transport and membrane fluidity.

In the current study, the effect of chronic Al exposure on brain monoaminergic systems as stress response indicators was assayed. Primarily, the data presented in this work elicited a marked depletion

in the brain monoaminergic system (5-HT, DA, E, NE and 5-HT) levels, which negatively correlated with the brain Al level. 5-HT is an excitatory neurotransmitter synthesized from tryptophan via the intermediate 5-hydroxytryptophane. The decrease in the brain 5-HT levels are associated with a decrease in the synthesis of 5-HT and loss of serotonergic neurons [40]. The decline in the levels of DA, E and NE neurotransmitters may be linked to their closely related synthetic route. These neurotransmitters are derived from tyrosine, which is converted to DA by the enzymes tyrosine hydroxylase and DOPA decarboxylase. DA is converted through the action of dopamine-β-hydroxylase to NE [41]. Similar decreases were previously reported by Fernandez-Davila *et al.,* [42]. In the current study CA supplementation might be useful in maintaining brain neurotransmitters levels to some extent.

Al intoxication leads to marked astrocytes and microglial activation that contribute to the pathogenesis of neuroinflammation and neurodegeneration [43]. Chronic administration of $AlCl_3$ causes a greater inflammatory response over immunoreactivity of astrocytes and microglia associated with cognitive dysfunction and disease [44]. Reactive gliosis and the associated proinflammatory cytokines/chemokines could be result of inflammation and neuronal injury in the rat brain [45]. Inhibition of pro-inflammatory mediators would be a better approach in regulating the progression of neurodegenerative disorders. The pro-inflammatory cytokines, TNF-α and iNOS, contribute to neuronal damage and death *in vivo* and *in vitro* [46]. Activation of iNOS in glial cells is a key step in neuroinflammation and is often related with TNF-α release. A potent proinflammatory cytokine TNF-α is synthesized by microglia, astrocytes and neurons [47]. TNF-α together with other transcription factors (NF-κB, nuclear factor kappa B) involved in cell responses including inflammation, proliferation, cell migration and apoptosis [48]. NO, the enzymatic product of iNOS, is associated with neuronal death by inhibiting mitochondrial respiration and excitotoxicity [49]. iNOS is normally not expressed in the brain, but is induced by pro-inflammatory cytokines and pathogenic components like endotoxins. To explore the mechanism of CA on the attenuation of $AlCl_3$-induced neuroinflammation, the expression of TNF-α and iNOS was detected in the rat brain. Reduced expressions of TNF-α and iNOS were observed in the cortex of rats treated with CA, which demonstrates that CA exerts anti-inflammatory effect. Hence, we assume that CA regulates focal microglia activation and proinflammatory cytokine production in cortex and this antioxidant is an effective experimental tool to reduce the brain lesions associated with inflammatory responses [21]. It also influences memory performance and, hence, CA can be utilized as a protective agent against neurodegenerative disorders [26].

Apoptosis is one of the mechanisms contributing to neuronal loss in $AlCl_3$ induced neurodegeneration. Aβ oligomers are majorly contributed to neuronal cell death by inducing apoptotic proteins during $AlCl_3$ administration. Bcl-2 is a member of apoptotic regulator protein that plays a major role in the regulation of cell death under both the physiological as well as pathological conditions. Bcl-2 over expression is perceived as a survival factor to protect against neurons during brain injury [12,13]. In the present study, induction of $AlCl_3$ resulted in a decrease in the activity of Bcl2 where as increased the activity of Baxin cerebral cortex region of rat brain. The ratio of Bax/Bcl2 levels in cortex was turned in favour of apoptosis in $AlCl_3$-administered rats that denotes that $AlCl_3$ induces neuronal apoptosis incortex region. Results of the present study are supported by documented report denoting the involvement of Bax, Bcl2 in $AlCl_3$-induced apoptosis in rabbit [13,50]. Further, we demonstrate that treatment with CA reduces Bax/Bcl2 ratio thereby antagonizing apoptotic niche in cortex of $AlCl_3$-administered rat brain. Mobilization of proapoptotic Bcl2 family signaling protein, Bax from cytosol to mitochondria causes mitochondrial outer membrane permeabilisation leading to cytochrome c expulsion from mitochondrial inter-membrane space leading to Caspase activation [51]. In this study, protein levels of cytochrome c and expressions of caspase-3 were significantly increased upon $AlCl_3$-exposure in the cortex denotes that $AlCl_3$-induces neuronal apoptosis. Results of present study are in accordance with documented reports denoting the involvement of caspase activation in many neurodegenerative disorders [52]. Treatment with CA attenuates $AlCl_3$-induced cytochrome c expression and also its downstream effectors caspases-3. This indicates that CA exerts neuroprotective effect by inhibiting neuronal apoptosis and is further supported that dietary flavonoids are capable of inhibiting intrinsic apoptosis of neurons [53].

In summary, the present investigation sheds light on the potent neuroprotective potential of CA in $AlCl_3$-induced neurotoxicity. Our observations suggest that CA helps to maintain brain neurotransmitters levels and further regulates apoptotic neuronal cell death, focal microglia activation and inflammation in the cortex. Therefore, it is sensible to suggest from the previous and the present studies that CA may be used as therapeutic agents in the treatment of cognitive dysfunction-associated disorders such as Alzheimer's disease. Future research may be directed towards the use of CA in clinical trials to evaluate its neuroprotective effect on humans.

Conflict of interest:

The authors declare no conflict of interest.

Acknowledgment

This work was supported by "National facility for Neurotoxicity Research and Drug Development" sanctioned by Department of Science and Technology (DST-DPRP), New Delhi, Govt of India.

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