IJBPAS, June, 2024, 13(6): 2630-2650

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

DIOSMIN AMELIORATES ALUMINIUM INDUCED NEUROTOXICITY IN THE HIPPOCAMPUS AND CORTEX OF MALE WISTAR RATS: A BEHAVIOURAL, BIOCHEMICAL AND MOLECULAR APPROACH

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Received 15th April 2023; Revised 8th June 2023; Accepted 6th Sept. 2023; Available online 1st June 2024

https://doi.org/10.31032/IJBPAS/2024/13.6.8055

ABSTARCT

Background: Aluminium is an omnipresent metal on the Earth's crust that has wide application in human lives. This increases the burden of Aluminium metal exposure and consequent deposition of it within the body. Several animal studies have shown Aluminium deposition in the brain over a chronic period leads to neurotoxicity, and eventually trigger other neurodegenerative diseases particularly Alzheimer's disease. In the emerging need of new therapeutic agents, Diosmin, a plant flavone was chosen to study the anti-neurotoxicity effects against Aluminium neurotoxicity. The present study aimed in evaluating the effect of Diosmin on Aluminium Chloride induced behavioural impairments, and its consequent changes in the biochemical parameters. neurotransmitter enzyme, and neurotrophic factors were also assessed in the study.

Methods: Briefly, Diosmin (50mg/Kg) was administered orally 1 hour prior to the induction of Aluminium Chloride (4.8mg/Kg) through the intraperitoneal route for 28 days. At every 7th day the behavioural paradigms were conducted. On the completion of the treatment schedule, the animals were sacrificed and, the Hippocampus and the Cortex regions were dissected and taken for biochemical and molecular analysis.

Results: Our results indicate a statistically significant impairment in the Spatial and Learning Memory in the Aluminium Chloride induced rats, followed by diminished Motor Coordination. The biochemical assessments indicated an elevation in Oxidative Stress markers with

subsequent decrease in Antioxidant Status. The neurotrophic factors presented with decreased expression. These changes were substantially attenuated by the administration of Diosmin.

Conclusion: Through our study, we conclude Diosmin as a potential flavone against Aluminium Chloride induced neurotoxicity.

Keywords: Neurotoxicity, Aluminium Chloride, Diosmin, Oxidative Stress, Neurotrophic Factors, Neuroprotection

INTRODUCTION

Aluminium (Al) is one of the most abundant metal ubiquitously present on the Earth's crust; however is a nonessential metal in the biological system with no acknowledged function **[1]**. Therefore, any amount of Aluminium present in the brain is relatively considered neurotoxic in nature. Aluminium is theorized to enter in to the human system through dermal absorption **[2]**, inhalation **[3]**, food **[4]** and water consumption **[5]** over a chronic period. The systemic uptake of Aluminium is elusive however it could possibly be influenced by the type of compound it is present as, e.g. Aluminium in their respective lactate, citrate, and fluoride forms show increased uptake as compared to their silicates and phosphates compounds **[4]**. The distribution of Aluminium in the brain has been hypothesized to be non-homogenous with increased burden in the Blood-Brain-Barrier (BBB) **[6]**. In a study conducted by Julka **[7]** the deposition of Aluminium in rat brain happened with maximum localization in the Striatum > Hippocampus (HC) > Brain Stem > Cerebral Cortex (CBC) > Cerebellum (CB).

Aluminium is a known potent pro-oxidant **[8 & 9]**. Chronic exposure and subsequent deposition of Aluminium epidemiologically leads to severe neurodegenerative conditions including Alzheimer's Disease (AD) and Dementia. Aluminium has been known for misfolding of proteins leading to an increased expression in Senile Plaques (SP), Neurofibrillary Tangles (NFT), Amyloid Beta Precursor Protein (APP) and Amyloid Beta (Aβ). This serves as a key pathological marker during the progression of Aluminium neurotoxicity to AD **[10]**. Aluminium in animal studies have also shown decreased Copper (Cu) and Zinc (Zn) levels in the brain, suggesting a decrease in Cu/Zn dependent Super Oxide Dismutase (SOD) enzymes. It has been proposed that these disparities in trace element pool triggers the oxidative stress imbalance **[5, 8 & 11]**. Therefore, Aluminium directly or indirectly compromises the brain chemistry, closely accompanied with free radical generation, and neuronal damage.

Due to the complexity of Aluminium neurotoxicity progression and its

multifaceted approach across various parameters, natural molecules are selectively studied due to its promising multifunctional properties to act parallel on various pathways targeting diverse pathological markers. Diosmin (DOS), chemically known as 3′,5,7-trihydroxy-4′-methoxyflavone-7-rutinoside. It is a glycosyl-flavone, naturally found in Citrus plants and chemically derived from Hesperidin **[12]**. DOS once absorbed through the gastrointestinal tract is rapidly hydrolysed into its aglycone form called Diosmetin by intestinal microbial flora **[13]**. This form of DOS is easily absorbed and distributed throughout the body. DOS has a high pKa (10) value making it hard to pass through the cell membrane. In contrast, Diosmetin has a low pKa (6-8) value that helps in its absorption by passive diffusion. The glucuronide derivative of Diosmetin was the major metabolite found in human plasma. The possible glucuronide derivatives of Diosmetin present in plasma are Diosmetin-3-O-glucuronide, diosmetin-7-Oglucuronide, and diosmetin-3,7-O-glucuronide **[14]**.

Diosmin has been studied for its antioxidant, anti-inflammatory, anti-apoptotic, anticancer, antibacterial properties. DOS has been reported in several neuronal studies namely AD **[15]**, Parkinson's Disease (PD) **[16]**, Huntington Disease (HD) **[17]** Traumatic Brain Injury (TBI) **[18]**, and Arsenic Neurotoxicity **[19]**.

Therefore, the present study evaluated the alterations in the brain Hippocampus and Cerebral Cortex induced by $AlCl_3$ on the oxidative stress parameters, endogenous and exogenous antioxidant homeostasis and the changes in the brain neurotrophic factors. This study also recorded the various behavioural paradigms that were diminished during Aluminium neurotoxicity followed by the alleviating effect of Diosmin respectively.

MATERIALS AND METHODS

Animals

The study was carried out using male Wistar rats weighing 200g-250g each. They were obtained from the Central Animal House facility of Dr. ALM Post Graduate Institute of Basic Medical Science, University of Madras, Taramani campus, Chennai-113, Tamilnadu, India. The animals were housed under standard laboratory conditions and maintained on natural light and dark [12:12] cycle. They had free access to food and water *ad libitum*. The animals were acclimatized to various laboratory conditions and handling prior to the treatment schedule. All experimental protocols were approved by the Institutional Animal Ethic Committee **[IAEC NO: 01/12/22]**, Dr. ALM Post Graduate Institute of Basic Medical Science, University of

Madras, Taramani campus, Chennai-113, Tamilnadu, India.

Treatment Schedule

All rats were randomly divided into four groups, with six animals in each group. The treatment schedule was done as described in **(Table 1)**.

Behavioural Paradigms

Open Field Test [OFT]

The OFT was carried out to observe the general locomotory and explorative behaviour of the rats. The open field was equally divided into squares and the rats were habitualised to the testing arena 24 hours prior to the experiment. The rats were placed in the center of the open field and its general explorative behaviour was observed. The total number of squares crossed per minute for each trial was measured **[20]**.

Rotarod Task

The motor coordination of the rats were assessed using rotarod apparatus. Briefly, the rats were mount on the rotarod apparatus and allowed to run at a steady rpm. The number of falls of each rat from the rotating bar was recorded per minute. Each rat was subjected to three trials with adequate intervals between each trial **[21]**.

Morris Water Maze [MWM]

The MWM was performed to evaluate the spatial memory and cognitive performance of the rats. Water at room temperature [23±1° C] was filled to a depth of 20cm and a steady platform was submerged at any randomly selected quadrant. The platform was significantly marked by a colored paper for spatial reference. The test was divided into two phases (i) the habitualisation phase and the (ii) testing phase. The habitualisation phase lasted 24hrs to 48hrs prior to the test phase. During the habitualisation phase the rats were trained to swim across the water maze and find the 'hidden platform' based on the colored cue under the shortest time possible. The experiment was conducted in clear water. The trial lasted 60 secs, if the rats failed to reach the submerged platform in the target quadrant, they were guided towards the platform. This was repeated by changing the submerged platform at different quadrants. On the day of the test, the water was made opaque by the addition of non-fat milk powder. The ability of the rats to swim to the submerged platform in the target quadrant at the shortest time based on the colored cue (escape latency) within a period of 60 secs was recorded. The rats that had the shortest escape latency was considered to have successful retention in spatial memory and conventional cognitive performance **[22]**.

Forced Swim Test [FST]

The forced swim test was performed to reflect the behavioural despair of the rats, based on their active and immobile phases. A vertical glass cylinder of 40cm in height and 20cm in diameter was filled with water

to a depth of 25cm. Once the rats were place inside the tank the stop clock started immediately. The test was recorded for three minutes each trial. The tendency of rat to persist the 'escape behaviour' and continued to be mobile was assessed. The time period of 'immobility' during the trial was considered to have shown behavioural despair **[23]**.

Tissue Preparation

At the end of the treatment schedule and behavioural assessments, all animals were sacrificed and the brain was removed by decapitation. The brain was washed with ice cold 0.9% NaCl to remove any residual blood. The brain was then immediately frozen at -20℃ for 1 hour. The Hippocampus and Cerebral Cortex was then carefully dissected and subsequently homogenised [10% w/v] in ice-cold 0.1 M solution of phosphate buffer at pH 7.4. The homogenate was centrifuged at 10,000 rpm at 4℃ for 15 minutes and the aliquots of supernatant were used for biochemical estimation.

Protein Estimation

The aliquots of the supernatant was taken for estimation of protein according to the method of **[24]** with slight modifications.

Estimation of Lipid Peroxidation (LPO)

Lipid peroxidation products were estimated by the method of **[25]** with slight modifications

Determination of Nitric Oxide (NO)

Nitric oxide was determined by the method of **[26]** with slight modification.

Assay of Superoxide Dismutase (SOD) (EC 1.15.1.1)

The enzyme was assayed according to the method of **[27]** with slight modifications.

Assay of Catalase (CAT) (EC 1.11.1.6)

The catalase activity was assayed by the method of **[28]** with slight modifications.

Determination of Reduced Glutathione (GSH)

Reduced glutathione was determined by the method of **[29]** with slight modifications.

Estimation of Vitamin C

Vitamin C was estimated by the method of **[30]** with slight modifications.

Assay of Total ATPase

The activity of Total ATPase was assessed in accordance with the previously described method **[31]** with slight modifications.

Acetylcholine Esterase (AChE) Activity (E.C.3.1.1.7)

The activity of AChE was estimated using previously described protocols **[32]** with slight modifications.

Quantification of Neurotrophic Factors using Enzyme Linked Immunosorbent Assay (ELISA)

Brain Derived Neurotrophic Factor (BDNF) and Glial Derived Neurotrophic Factor (GDNF) levels were measured using

ELISA as previously described **[33]** with slight modifications. About 10mg of Hippocampus and Cerebral Cortex tissues were weighed and subsequently homogenised in Radioimmunoprecipitation assay buffer (RIPA). The samples were then centrifuged at 3500 rpm at 4ºC for 3 minutes. The supernatant was then quantified for protein present. High affinity 96 micro well plates were coated with the sample in bicarbonate buffer and left overnight for incubation at 4ºC. The plates were then washed with washing buffer (0.15 M of phosphate buffered saline (PBS) at pH 7.4), thrice and then blocked using 200 µl/well blocking buffer (PBS containing 1% BSA) for a period of about 1 hour at room temperature. After incubation at room temperature, plates were washed with wash buffer twice. Primary antibodies BDNF & GDNF diluted to 1:300, 100µl/well was added and incubated overnight at 4ºC. After which the plates were further washed. Following, 100µL of Horseradish Peroxidase (HRP) conjugated secondary antibody diluted 1:5000 in blocking buffer were added and then kept for incubation at room temperature for an hour. After further washing and a final rinse with PBS, 1µL/well of 30% H_2O_2 was added immediately followed by the addition of 50µL/well 2,2'-azino-bis (3-ethylbenzothiazoline-6- sulphonic acid) (ABTS) substrate for about 15 minutes at 37ºC (in dark). The reaction was arrested by the addition of 50µl/well 2M H_2SO_4. The optical densities were subsequently determined at 405 nm by utilizing a plate ELISA reader.

Statistical Analysis

Data were represented as mean ± Standard Deviation (SD) of the results obtained from the average of at least six concordance. Results were calculated by one-way analysis of variance (ANOVA) using SPSS software for Windows Operating System (Version 20.0), if values were found significant, the 'P' values were calculated using the Tukey's *Post-hoc* test. A 'P' value lesser than 0.05 ($P < 0.05$) was found to be statistically significant. A 'P' value ($P < 0.001$) is considered more significant than ($P < 0.01$) is considered more significant than ($P < 0.05$).

RESULTS

Effect of Diosmin on $AlCl_3$ induced changes in Open Field Test (OFT) of control and experimental rats

The number of square crossings in the $AlCl_3$ induced group was significantly decreased as the toxicity progressed on days 7, 14, 21 and 28 $^a(P < 0.001)$ as compared to the control group. The number of square crossing was shown to be increased by the administration of Diosmin (50mg/kg b.w.t) days 7, 14 $^b(P < 0.05)$, 21 and 28 $^b(P < 0.01)$

respectively, as compared to the induced group. There was no significant variations found in the Diosmin alone administered group in comparison with control group **(Figure 1)**.

Effect of Diosmin on $AlCl_3$ induced changes in Rotarod activity in control and experimental rats

Aluminium Chloride induced neurotoxicity in rats showed motor impairment when assessed using the Rotarod test. The $AlCl_3$ induced group showed a gradual increase in number of falls/min on the Rotarod on days 7 $^a(P < 0.05)$ and 28 $^a(P < 0.01)$ as compared to the control group. The rats treated with Diosmin (50 mg/kg b.wt) showed significantly decreased number of falls/min on day 7 and 28 $^b(P < 0.05)$ as compared to the induced group. There was no significant deviations found in the Diosmin alone administered group in comparison with control group (**Figure 2)**.

Effect of Diosmin on $AlCl_3$ induced memory defects in Morris Water Maze (MWM) test in control and experimental rats

In the present study, the administration of Aluminium Chloride for 28 days produces memory defects and cognitive dysfunction which significantly increases the escape latency of $AlCl_3$ induced rats on days 7 $^a(P < 0.01)$, 14, 21 and 28 $^a(P < 0.001)$ respectively, as compared to the control group. Diosmin (50mg/Kg) was able to significantly decrease the escape latency on days 7, 14, 21 $^b(P < 0.05)$ and 28 $^b(P < 0.01)$ respectively as compared to induced group. There was no significant changes found in the Diosmin alone administered group in comparison with control group **(Figure 3)**.

Effect of $AlCl_3$ induced Behavioural Despair in Force Swim Test in control and experimental rats

The assessment of forced swim test showed that the data represents increased immobility time in $AlCl_3$ induced group on days 7, 14, 21 and 28 $^a(P < 0.001)$ respectively as compared to control group. Diosmin (50 mg/kg b.w.t) administration significantly decreased immobility time as compare to the $AlCl_3$ induced group on day 7, 14, 21 and 28 $^b(P < 0.01)$. There was no statistically substantial variations found in the DOS administered group as compared to the control group **(Figure 4)**.

Effect of Diosmin on $AlCl_3$ induced changes in the level of Lipid Peroxidation in the Hippocampus and Cortex of control and experimental rats

The induction of Aluminium Chloride for 28 days resulted in elevated lipid peroxidation levels in the brain Hippocampus and Cortex $^a(P < 0.001)$ as compared to the control group. The lipid peroxidation levels were severely mitigated $^b(P < 0.01)$ in the Hippocampus and Cortex

when treated with Diosmin (50mg/Kg) as compared to the induced group. Treatment with DOS alone did not show any statistically significant alterations in the levels of LPO as compared to the control group **(Figure 5)**.

Effect of Diosmin on $AlCl_3$ induced changes in level of Nitric Oxide in the Hippocampus and Cortex of control and experimental rats

The induction of Aluminium Chloride lead to in a significant escalation in NO levels in brain Hippocampus and Cortex $^{a}(P < 0.01)$ as compared to the control group. The DOS (50mg/Kg) treated group presented a substantial decrease in the NO levels $^{b}(P < 0.05)$ in the Hippocampus and Cortex as compared to the induced group. Standalone treatment of DOS did not show any statistically significant change as compared to the control group **(Figure 6)**.

Effect of Diosmin on $AlCl_3$ induced changes in Super Oxide Dismutase activity in the Hippocampus and Cortex of control and experimental rats

Aluminium Chloride induction showed a pronounced reduction in the SOD activity in brain Hippocampus $^{a}(P < 0.01)$ and Cortex $^{a}(P < 0.001)$ as compared to the control group. Diosmin (50mg/Kg) treatment was able to significantly improve the SOD activity in Hippocampus $^{b}(P < 0.05)$ and Cortex $^{b}(P < 0.01)$ as compared to the induced group. Disomin alone treated group did not show any notable change in the levels of SOD as compared to the control group **(Figure 7)**.

Effect of Diosmin on $AlCl_3$ induced changes in the activity of Catalase in the Hippocampus and Cortex of control and experimental rats

The induction of Aluminium Chloride for 28 days resulted in a remarkable decrease in the level of CAT in the Hippocampus $^{a}(P < 0.01)$ and Cortex $^{a}(P < 0.001)$ as compared to the control group. However, treatment with Diosmin (50mg/kg) restored the activity of CAT in Hippocampus $^{b}(P < 0.05)$ and Cortex $^{b}(P < 0.01)$ as compared to the Aluminium Chloride treated group. There was also no evident variation in the Diosmin alone treated group as compared to the control group **(Figure 8)**.

Effect of Diosmin on $AlCl_3$ induced changes in level of Reduced Glutathione in the Hippocampus and Cortex of control and experimental rats

The induction of Aluminium Chloride for 28 days showed important alterations in GSH levels. The Hippocampal and Cortex GSH content were found to be decreased $^{a}(P < 0.01)$ as compared to the control group. Treatment with Diosmin (50mg/Kg) showed $^{b}(P < 0.01)$ significant elevation in Hippocampus and $^{b}(P< 0.05)$ significant elevation in Cortex as compared to the induced group. Besides, there was no

evident difference in the GSH levels of Diosmin alone treated group as compared to the control group **(Figure 9)**.

Effect of Diosmin on $AlCl_3$ induced changes in levels of Vitamin C in the Hippocampus and Cortex of control and experimental rats

Vitamin C levels was observed to be severely decreased [a](P<0.001) post 28 days of $AlCl_3$ induction. Treatment with Diosmin showed a partial restoration in the Vitamin C levels in Hippocampus and Cortex [b](P < 0.01) as compared to the induced group. There was no evident change in the Vitamin C levels in the Diosmin alone treated group as compared to the control group **(Figure 10)**.

Effect of Diosmin on $AlCl_3$ induced changes in the activity of Total ATPase in Hippocampus and Cortex of control and experimental rats

The induction of Aluminium Chloride resulted in a considerable decrease in Total ATPase activity in Aluminium Chloride induced rats in the Hippocampus [a](P < 0.001) and Cortex [a](P < 0.01) as compared to control animals. However, it was observed that treatment with Diosmin (50mg/Kg) showed a significant increase in the activity of Total ATPase levels in the Hippocampus and Cortex [b](P < 0.05) as compared to the induced group. Besides, it was observed as Diosmin (50mg/Kg) treatment alone did not show any statistically important change in the levels of Total ATPase as compared to the control group **(Figure 11 a & b)**.

Effect of Diosmin on $AlCl_3$ induced changes in AChE activity in the Hippocampus and Cortex of control and experimental rats

The induction of Aluminium Chloride for 28 days showed an elevation of Acetylcholine Esterase activity in the Hippocampus and Cortex [a](P < 0.05) as compared to the control group. When treated with Diosmin (50mg/Kg) it was observed that, DOS was able to suggestively partially restore the activity of AChE in the Hippocampus and Cortex [b](P < 0.05) as compared to the induced group. Changes in the activity of AChE was not observed to be significant in the DOS alone treated group as compared to the control group **(Figure 12)**.

Effect of Diosmin on Aluminium Chloride induced changes in the levels of BDNF and GDNF in the Hippocampus of control and experimental rats.

The Hippocampus Neurotrophic Factors i.e., Brain Derived Neurotrophic Factor (BDNF) and Glial Derived Neurotrophic Factor (GDNF) were quantified using ELISA. The induction of Aluminium Chloride for 28 days showed a remarkable decrease in the BDNF and GDNF levels in the Hippocampus [a](P < 0.05) as compared to the control group. The

Aluminium Chloride induced decrease in the neurotrophic levels was restored partially by the treatment with Diosmin (50mg/Kg) in the Hippocampus [b](P < 0.05) as compared to the induced group. The treatment with Diosmin alone did not show any leading changes in the BDNF and GDNF levels as compared to the control group **(Figure 13)**.

Table 1: Treatment Schedule

GROUPS	TREATMENT SCHEDULE
Group I [Control]	**The rats were untreated of vehicle, inducer, and treatment drug.**
Group II [Induced]	**The rats were induced with Aluminium Chloride ($AlCl_3$) (4.8 mg/kg/i.p) [48] from day 1 to day 28.**
Group III [Treatment]	**The rats were administered with Diosmin (DOS) (50mg/kg/p.o) [15] 1 hour prior to $AlCl_3$ induction (4.8 mg/kg/i.p) [48] from day 1 to day 28.**
Group IV [Drug alone]	**The rats were treated with DOS (50 mg/kg/p.o) [15] alone from day 1 to day 28**

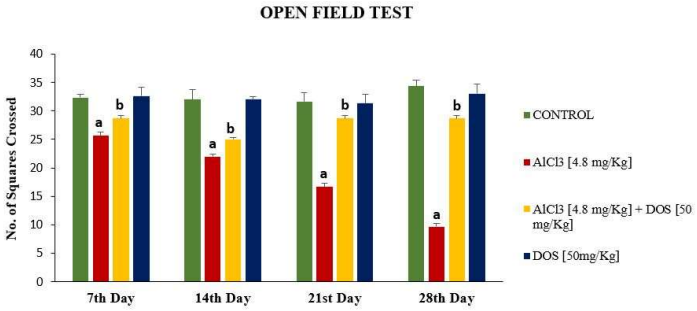

Figure 1: Effect of Diosmin on $AlCl_3$ induced changes in Open Field Test in control and experimental rats

Data represents mean ± SD of 6 rats in each group. The values are statistically significant on days 7, 14, 21and 28 [a](P<0.001) as compare to control group, and on days 7, 14 [b](P<0.05), 21 and 28 [b](P<0.01) as compare to induced group. The data was analysed through One Way ANOVA. When ANOVA values were found to be statistically significant (P < 0.05), the results were followed by Tukey's *Post hoc* test

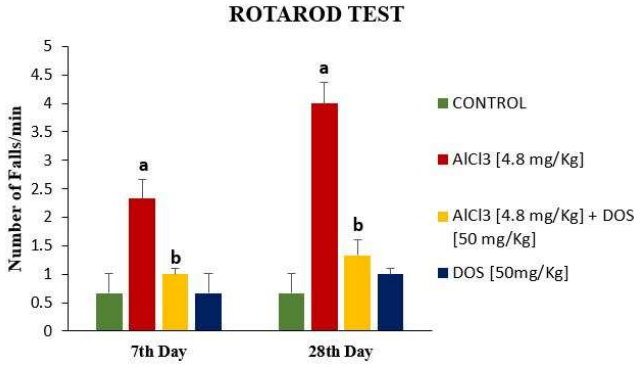

Figure 2: Effect of Diosmin on $AlCl_3$ induced changes in Rotarod activity in control and experimental rats

Data represents mean ± SD of 6 animals in each group. The values are statistically significant on days 7 [a](P<0.05) and 28 [a](P<0.01) as compare to control group, and on days 7 and 28 [b](P<0.05) as compare to induced group. The data was analysed through One Way ANOVA. When ANOVA values were found to be statistically significant (P < 0.05), the results were followed by Tukey's *Post hoc* test

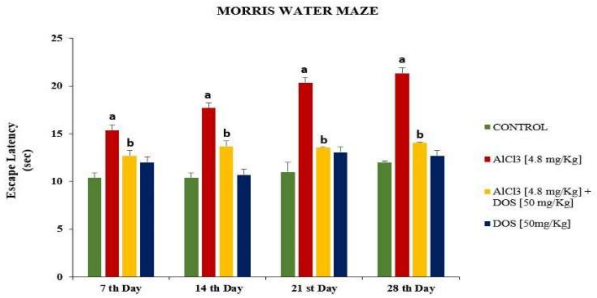

Figure 3: Effect of $AlCl_3$ induced memory defects in Morris Water Maze test in control and experimental rats

Data represents mean ± SD of 6 animals in each group. The values are statistically significant on days 7 [a](P<0.01), 14, 21and 28 [a](P<0.001) respectively as compare to control group, and on days 7, 14, 21 [b](P<0.05) and 28 [b](P<0.01) respectively as compare to induced group. The data was analysed through One Way ANOVA. When ANOVA values were found to be statistically significant (P < 0.05), the results were followed by Tukey's *Post hoc* test

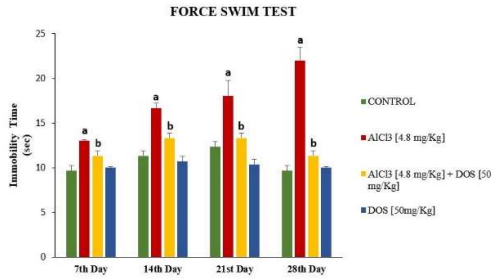

Figure 4: Effect of $AlCl_3$ induced Behavioural Despair in Force Swim Test in control and experimental rats

Data represents mean ± SD of 6 rats in each group. The values are statistically significant on days 7, 14, 21 and 28 [a](P<0.001) as compare to control group, and on days 7, 14, 21 and 28 [b](P<0.01) as compared to induced group. The data was analysed through One Way ANOVA. When ANOVA values were found to be statistically significant (P < 0.05), the results were followed by Tukey's *Post hoc* test

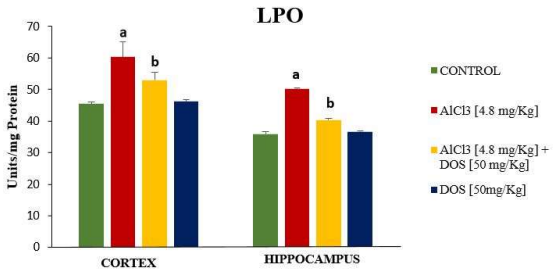

Figure 5: Effect of Diosmin on $AlCl_3$ induced Lipid Peroxidation in the Hippocampus and Cortex of control and experimental rats

Data represents mean ± SD of 6 rats in each group. The statistics were found to be significant in the brain Hippocampus and Cortex [a](P < 0.001) as compared to the control group and [b](P<0.01) as compared to induced group. The data was analysed through One Way ANOVA. When ANOVA values were found to be statistically significant (P < 0.05), the results were followed by Tukey's *Post hoc* test

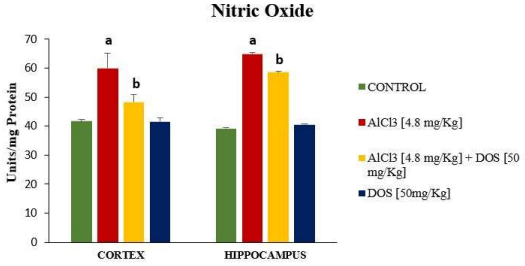

Figure 6: Effect of Diosmin on $AlCl_3$ induced changes in level of Nitric Oxide in the Hippocampus and Cortex of control and experimental rats

Data represents mean ± SD of 6 rats in each group. The statistics were found to be significant in the brain Hippocampus and Cortex a (P < 0.001) as compared to the control group and b (P<0.01) as compared to induced group. The data was analysed through One Way ANOVA. When ANOVA values were found to be statistically significant (P < 0.05), the results were followed by Tukey's *Post hoc* test

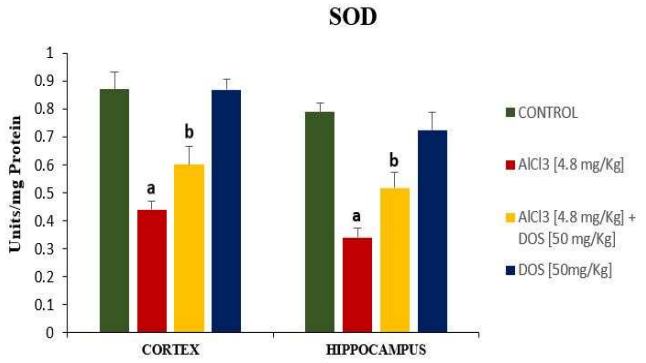

Figure 7: Effect of Diosmin on $AlCl_3$ induced changes in Super Oxide Dismutase activity in the Hippocampus and Cortex of control and experimental rats

Data represents mean ± SD of 6 rats in each group. The statistics were found to be significant in the brain Hippocampus [a](P < 0.01) and Cortex [a](P < 0.001) as compared to the control group. The values of the treatment group was found to be significant in the brain Hippocampus [b](P < 0.05) and Cortex [b](P < 0.01) as compared to the induced group. The data was analysed through One Way ANOVA. When ANOVA values were found to be statistically significant (P < 0.05), the results were followed by Tukey's *Post hoc* test

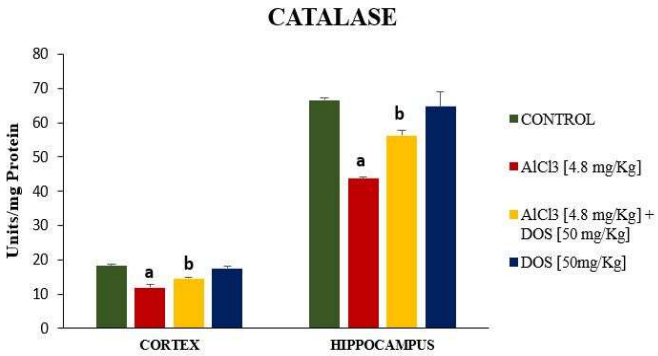

Figure 8: Effect of Diosmin on $AlCl_3$ induced changes in the activity of Catalase in the Hippocampus and Cortex of control and experimental rats

Data represents mean ± SD of 6 rats in each group. The statistics were found to be significant in the brain Hippocampus [a](P < 0.01) and Cortex [a](P < 0.001) as compared to the control group. The values of the treatment group was found to be significant in the brain Hippocampus [b](P < 0.05) and Cortex [b](P < 0.01) as compared to the induced group. The data was analysed through One Way ANOVA. When ANOVA values were found to be statistically significant (P < 0.05), the results were followed by Tukey's *Post hoc* test

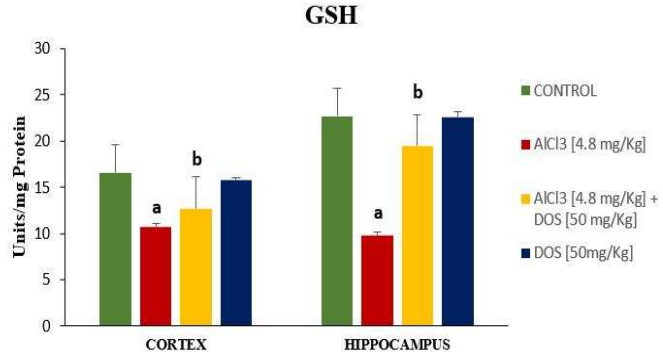

Figure 9: Effect of Diosmin on $AlCl_3$ induced changes in level of GSH in the Hippocampus and Cortex of control and experimental rats

Data represents mean ± SD of 6 rats in each group. The statistics were found to be significant in the brain Hippocampus and Cortex [a](P < 0.01) as compared to the control group. The values of the treatment group was found to be significant in the brain Hippocampus [b](P < 0.01) and Cortex [b](P < 0.05) as compared to the induced group. The data was analysed through One Way ANOVA. When ANOVA values were found to be statistically significant (P < 0.05), the results were followed by Tukey's *Post hoc* test

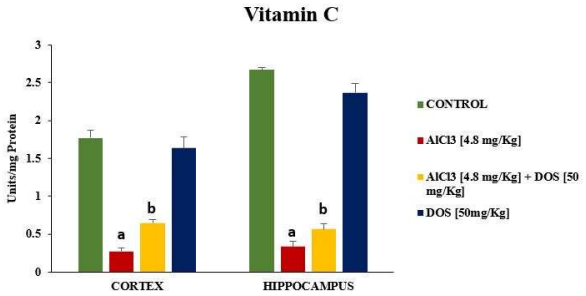

Figure 10: Effect of Diosmin on $AlCl_3$ induced changes in levels of Vitamin C in the Hippocampus and Cortex of control and experimental rats

Data represents mean ± SD of 6 rats in each group. The statistics were found to be significant in the brain Hippocampus and Cortex [a]($P < 0.001$) as compared to the control group and [b]($P<0.01$) as compared to induced group. The data was analysed through One Way ANOVA. When ANOVA values were found to be statistically significant ($P < 0.05$), the results were followed by Tukey's *Post hoc* test

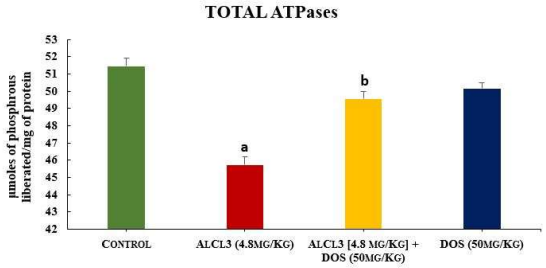

Figure 11a: Effect of Diosmin on $AlCl_3$ induced changes in activity of Total ATPase in Hippocampus control and experimental rats

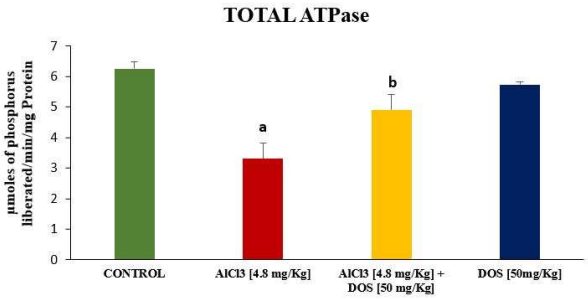

Figure 11b: Effect of Diosmin on $AlCl_3$ induced changes in activity of Total ATPase in Cortex control and experimental rats

Data represents mean ± SD of 6 rats in each group. The statistics were found to be significant in the brain Hippocampus a and Cortex a ($P < 0.001$) as compared to the control group and b ($P<0.01$) as compared to induced group. The data was analysed through One Way ANOVA. When ANOVA values were found to be statistically significant ($P < 0.05$), the results were followed by Tukey's *Post hoc* test

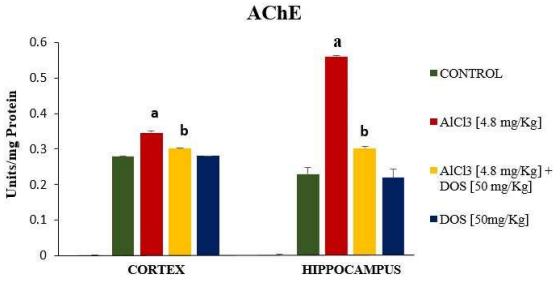

Figure 12: Effect of Diosmin on $AlCl_3$ induced changes in Acetyl Choline Esterase activity in the Hippocampus and Cortex of control and experimental rats

Data represents mean ± SD of 6 rats in each group. The statistics were found to be significant in the brain Hippocampus and Cortex [a]($P < 0.05$) as compared to the control group and [b]($P<0.05$) as compared to induced group. The data was analysed through One Way ANOVA. When ANOVA values were found to be statistically significant ($P < 0.05$), the results were followed by Tukey's *Post hoc* test

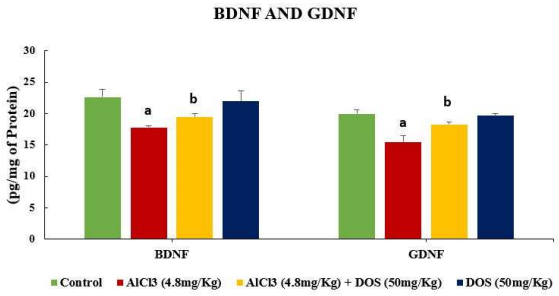

Figure 13: Effect of Diosmin on Aluminium Chloride induced changes in the levels of BDNF and GDNF in the Hippocampus of control and experimental rats

Data represents mean ± SD of 6 rats in each group. The statistics were found to be significant in the brain Hippocampus [a](P < 0.05) as compared to the control group and [b](P<0.05) as compared to induced group. The data was analysed through One Way ANOVA. When ANOVA values were found to be statistically significant (P < 0.05), the results were followed by Tukey's *Post hoc* test

DISCUSSION

The Chronic administration of Aluminium Chloride resulted in progressive decline in the square crossing after the performance in the Open Field Test (OFT). This was significantly increased in the DOS treated group suggesting a possible increase in ambulatory ability due to decrease in anxiousness and emotionality behaviour. This study was in accordance with the results of **[33]** who reported a notable decrease in locomotory behavior in the Aluminium induced group with alterations in the secondary parameters such as rearing and grooming. Okubo **[34]** have shown similar results in accordance with the present study (DOS 50mg/Kg by increased line crossing with DOS 25mg to 100mg administration in a Ketamine induced group. There results signified a decreased immobility time in the DOS administered groups in a dose dependent manner suggesting a decrease in the emotionality and increase in ambulatory ability. This was further reflected in the Forced Swim Test (FST), which assessed the anxiety and behavioural despair. Aluminium induced group showed high immobility time in the FST due to decreased escape behaviour suggesting a possibility of 'learned helplessness'. DOS at 50mg/kg significantly improved the escape behaviour suggesting a possible anti-stress paradigm. These results were also in accordance with Okubo **[34]**.

The results of Morris Water Maze (MWM) and Rotarod showed that Al deposition in the Hippocampus interferes with the cognition and memory function. Aluminium induced group showed decreased motor coordination and balance marked by the increase in no of falls at short intervals of time on the Rotarod and showed a gradual decline in recollection and recognition memory leading to diminished spatial orientation in the MWM. The result was similarly observed by Khan **[35]** in an Aluminium induced dementia like model. They reported a substantial decrease in the

recall and acquisition memory in the MWM test. Diosmin administration at 50mg/Kg showed improvement in the overall motor balance and grip strength followed by significant elevation in the escape latency implying a possible restorative effect on the cognition and motor functions. Sahreh Shaban **[14]** documented a similar result with Diosmin administration at 50mg/Kg and 100mg/Kg in a Scopolamine induced Dementia model. The study concluded that Diosmin administration reduced the mean latency time in a dose dependent manner, paralleling improving the spatial and learning memory functions.

Aluminium shows significant toxicity interfering the neurotransmitter enzymes such as Acetylcholine Esterase **[36-40]**. One of the consistent neurotransmitter alterations found in Aluminium neurotoxicity is AChE. The chronic exposure of Aluminium, leading to Alzheimer's disease, which causes an overexpression of AChE and disruption in the calcium homeostasis **[41]**. However, Aluminium tend to have biphasic expression during Al intoxication. Based on the days of exposure the AChE tends to increase or decrease **[33]**. As it stands, our results tend to disagree with a few studies. We observed that the treatment of DOS significantly decreased the over expression of AChE in the Hippocampus & Cortex brain region. This result was in accordance with the reports of Taïr **[33]** and Okubo **[34]**.

Oxidative stress is a key burden in during neurotoxicity. It has been well documented that Aluminium exposure disrupts the oxidative stress and antioxidant levels. Therefore oxidative stress markers LPO and NO, endogenous antioxidants such as CAT, SOD, GSH and non-endogenous antioxidants such as Vitamin C and the levels of total ATPases were assessed in the present Aluminium induced neurotoxicity study. Through our studies, it was evident that Aluminium increased the oxidative stress markers in the Hippocampus and Cortex region of the brain, considering its sensitivity towards ROS. A significant increase in the levels of LPO and NO was noticed. This was mitigated by the administration of DOS at 50mg/Kg possibly due to the anti-peroxidative effects of DOS along with reactive nitric oxide scavenging properties. This study was in accordance with the results obtained by Okubo **[34]**. The SOD and CAT activity were considerably decreased on the administration of Al possibly due to the disturbance in the enzymatic antioxidant homeostasis in the Hippocampal and Cortex region and decrease in their respective substrates. Under the oxidative stress conditions, SOD presents a crucial role against superoxide as it converts the superoxide anion to H_2O_2 and O_2 **[42]** which is then converted to H_2O by

CAT at the expenditure of GSH. Therefore, the increased lipid peroxidation may be interpreted as an inhibition in the activity of SOD and CAT leading to membrane damage and neuron death **[42 & 43]**. In the current study, we were able to establish that DOS at 50mg/Kg b.wt was able to notably increase the levels of SOD and CAT in the Hippocampus and Cortex. This was backed by the reduction in the levels of LPO as previously discussed possibly due to the radical scavenging effect of Diosmin, which was able to attenuate oxidative stress and directly or indirectly increase the levels of antioxidants in the Hippocampus and Cortex. The results obtained were in accordance with Sati **[44]** and Okubo **[34]**. GSH, a ubiquitous tripeptide glutathione and a non-enzymatic antioxidant which is a reductant in redox reaction results in formation of GSSG. The results obtained in the present study showed decreased levels of GSH in the Hippocampus and Cortex of Aluminium treated group. It was observed that Diosmin treatment enhance the activity of GSH in the Hippocampus and Cortex of male rats. This was in accordance with the study of Jyoti **[45]** and Anil Kumar **[46]**. In Vitamin C test, the induced group shows decreased activity when compared with control group. Diosmin treated group shows increased in the activity of Vitamin C.

The decreased levels of BDNF and GDNF expression are known to play a role in the progression of Neurodegenerative diseases like AD, PD, and HD. These neurotrophic factors are crucial in the development and maturing of neuronal cells. The levels of BDNF and GDNF was found to be severely diminished in the brain Hippocampus and Cortex. These results were in accordance with previously described reports **[47]**. Diosmin administration at 50mg/Kg was significantly able to elevate the neurotrophic factors.

CONCLUSION

The results of the present study signify that the exposure of Aluminium Chloride decreases the status of antioxidants leading to increased oxidative stress, cognitive and motor impairments in the Hippocampus and Cortex of the rat brain. However, pre-treatment with Diosmin significantly attenuated these changes by reducing the levels of oxidative stress markers, increase in status of enzymatic, non-enzymatic antioxidants and neurotrophic factors, decrease in activity of AChE, and improvement in cognitive and motor performance. Hence, Diosmin is suggested as a promising flavone against $AlCl_3$ neurotoxicity.

Conflicts of Interest

The authors declare that no conflicts of interests exist.

Acknowledgement

The Scholar thanks **Dr. T. Sumathi, Associate Professor**, Department of

Medical Biochemistry, Dr. ALM Post Graduate Institute of Basic Medical Sciences, University of Madras, Taramani, Chennai- 600113, for giving an opportunity to conduct the research work in her laboratory, and aiding with the necessary requirements and chemicals.

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