

Naringin Attenuates Aluminum Induced Cognitive Deficits in Rats

Orsu Prabhakar

GITAM Institute of Pharmacy, GITAM (Deemed to be University), Visakhapatnam, Andhra Pradesh, INDIA.

ABSTRACT

Aim: The exposure of aluminum for longer period in brain can lead to impaired cognition and behavioral changes. The present study was aimed to investigate the aluminum induced cognitive deficits and possible protective outcomes of naringin. **Materials and Methods:** Aluminum chloride in a dose of 100 mg/kg *i.p* was employed for 60 days to induce cognitive deficits in Westar rats. The animals were divided into groups each group contain six animals, as normal, aluminum treated, naringin treated (25, 50, 100 mg/kg). These animals were used to estimate behavioral characters such as Open field, eight arm radial maze, Morris water maze and Rota-rod tests and also used to assess the biochemical parameters such as acetylcholine esterase activity, malondialdehyde, superoxide dismutase and catalase. **Results:** Aluminum intoxicated rats showed significantly reduced exploratory and spontaneous locomotors activities in open field test and significant impairment in working and learning memory assessed by Morris water maze and radial arm maze tests. It also significantly increased acetylcholine esterase activity and malondialdehyde. The superoxide dismutase and catalase were also decreased significantly. Naringin was given (25, 50 and 100 mg/kg *PO*) to the aluminum intoxicated rats for a period of 30 days. The naringin treatment has shown significant improvements in spontaneous locomotors and exploratory activities and memory performance tasks of rats. **Conclusion:** The oxidative stress (reduced lipid peroxidation, restored reduced superoxide dismutase and catalase) and acetylcholine esterase activity were significantly decreased in the naringin group. Thus, these findings supported that naringin has showed potential neuroprotection against aluminum induced oxidative stress and cognitive deficit.

Key words: Acetylcholine esterase, Aluminum, Alzheimer, Cognitive impairment, Oxidative stress, Naringin.

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INTRODUCTION

Aluminum (Al) is well known and most abundant metal in nature. It is a constituent in drinking water, medicines and cooking utensils. It can be accessed into the human body through the water, food and air. Al can produce pathological and clinical features which were in resemblance to those features observed in dementia. In many cases the patients with dementia showed Al traces in their brains which were deposited in the neurofibrillary tangles and senile plaques of neurons. Several researchers also demonstrated the Al intoxication in experimental animals and humans cause neurotoxicity

and cognitive deficits.¹⁻⁴ Therefore, in this study Al intoxication in animals was considered as experimental model. Generally, memory process and learning process in the brain are maintained by the cholinergic system. In dementia brain, the cholinergic system is one of the key area which is affected mostly in brain especially the projections from basal forebrain to cortex and hippocampus.

The superoxide semi-reduced radical ions formed due to Al intoxication can cause cognitive deficiency and dementia in the brain,⁵ suggesting the influence of Al and superoxide radical interactions in

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Correspondence:

Dr. Orsu Prabhakar

Assistant Professor,
Department of
Pharmacology, GITAM
Institute of Pharmacy, GITAM
(Deemed to be University),
Visakhapatnam-530045,
Andhra Pradesh, INDIA.
Phone: +91 9912988579
E-mail: prabhakar.orsu@
gitam.edu



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the promotion of superoxide associated biological oxidation.^{6,7} Furthermore, the exposure of Al can result in its CNS accumulation and induces the reactive oxygen species (ROS) formation. This results in increased lipid peroxidation and altering the brain oxidative mechanisms that might promote β -amyloid peptide deposition and formation.

Al chronic exposure can induce cholinergic terminals degeneration in the cortical areas and depletion of cells in its cortex and hippocampus. In the cingulate bundle, more Al accumulation was found and thereby induces learning deficits.⁸ The expression of antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) are induced by the exposure of Al and associated oxidative stress.^{9,10} Thus, Al promotes aggregation of insoluble amyloid- β -protein, hyperphosphorylated tau-protein and causes more pathological changes in cholinergic system.^{11,12} This might be the possible way by which aluminum involving in pathological process of dementia.

In the management of dementia, acetylcholinesterase inhibition and oxidative stress inhibition are the important therapeutic strategies. There are very good number of pragmatic evidences supporting that the plant-derived antioxidants, especially flavonoids are useful for the control of neurodegenerative disease. Naringin is one of the flavonoids which has been reported as a potent antioxidant, anti-inflammatory and neuroprotective agent.¹³⁻¹⁶ Therefore, this study was involved to explore the behavioral and biochemical changes of Al intoxicated brain and the protective effects of naringin. Open field, Eight arm radial maze, Morris water maze and Rota-rod tests were selected to evaluate the behavioral characters.

MATERIALS AND METHODS

Animals

Either sex Westar rats (180–210 g) were obtained from the NIN, Hyderabad, Telangana, India. Animals were maintained in an ambient temperature ($24 \pm 1^\circ\text{C}$) colony room under a 12/12-hr light/dark cycle. Animals were taken care in compliance with the CPCSEA guidelines, New Delhi and experiments were conducted by the approval of the Institutional Animal Ethics Committee, GITAM Deemed to University, Visakhapatnam.

Experimental Design

Drugs and Chemicals

Aluminum chloride (CDH, India), Thiopentone sodium (Neon-labs, Mumbai), Thiobarbituric acid (Merck India), 1,1,3,3-tetraethoxy-propane (arrow India),

nitroblue tetrazolium (Merck India), Nicotinamide adenine dinucleotide phosphate reduced form (Sigma-Aldrich, India), Naringin (Sigma-Aldrich India), All other reagents and chemicals used in the experiment were analytical grade.

Treatment schedule

AlCl_3 was administered intraperitoneally at a dose of 100 mg/kg/day for 60 days. This was followed by oral administration of naringin at different doses (25, 50 and 100 mg/kg/day) for 30 days. At the beginning of experiment, before aluminum or naringin administration the animals were weighed followed by weighing on daily basis until the end of behavioral tests.

Experimental design

Group I: Normal, Group II: Al+S, Group III: Al+NG25 (Naringin 25 mg/kg treated), Group IV: Al+NG50 (Naringin 50 mg/kg treated), Group V: Al+NG100 (Naringin 100 mg/kg treated), Group VI: Al+Donepezil (Donepezil treated 0.75 mg/kg) with each group consisting of 6 animals. This study was conducted for a period of 90 days.

Biochemical Assessments

After completion of behavioral tests the animals were sacrificed by cervical dislocation. The rat's brains were removed and rinsed in ice-cold isotonic saline solution. Further, the brain tissues were homogenized with ice-cold 150 mM KCl. The obtained homogenate (10% w/v) was then centrifuged at 11,000 rpm for 15 min. The supernatants of centrifuged tissues were used for biochemical estimations.

Estimation of Acetylcholinesterase (AChE) activity

According to the standard method of Ellman *et al.* the activity of acetylcholinesterase was measured in the centrifuged brain tissue supernatant.¹⁷ The absorbance changes were recorded at 412nm by UV spectrophotometer and the findings were expressed in μmoles of acetylcholine iodide hydrolyzed/min/mg protein.

Estimation of oxidative stress markers

The isolated brain tissues were used for measuring oxidative stress parameters in it, such as malondialdehyde (MDA), superoxide dismutase (SOD) and catalase.

The lipid peroxidation concentration (MDA) in the serum was determined by the Ohkawa *et al.* MDA levels were articulated in nMol/lit.¹⁸ SOD levels were measured by the superoxide radicals which are generated by xanthine and xanthine oxidase, using Kakkar *et al.* In this method, with 2-(4-iodophenyl)-3-(4-nitro phenol-s-

phenyl tetrazolium) chloride to form a red formazon dye. SOD activity is then measured by the degree of inhibition of this reaction.¹⁹

Catalase concentration was measured by the degradation rate of H₂O₂ using Aebi's method. The rate of H₂O₂ desertion was observed spectrophotometrically at 230 nm. The assay consisted of 50 µl 1 M Tris HCl buffer (pH 8), 930 µl 10 mM H₂O₂, 930 µl deionized water and 20 µl serum sample. One unit of Catalase activity is defined as the amount of enzyme causing about 90% destruction of the substrate in 1 min in a volume of 1 ml. Catalase activity in the serum was expressed as Unit/ml.²⁰

Behavioral tests

Open field method

The method followed was described by Abdel-Aal *et al.* 2011. In brief, the equipment consisted of a square wooden box in which the base was divided into 49 squares. Individual animal was tested for 5 min in the instrument. The number of squares crossed by the animal gives the locomotor activity. The rearing movements of the animals were assessed by vertical activity.

Morris water maze test

This test was carried as described in Abdel-Aal *et al.* 2011. The apparatus consisted of a circular stainless steel tank. This tank was divided by 4 quadrants and fixed 4 points on its perimeter. This test assesses the nootropic activity in terms of latency period. The latency time recorded in terms of time taken for the animal to reach the hidden platform.

Radial arm Maze test

In an eight-arm maze, this test was conducted. This method was executed according to Abdel-Aal *et al.* 2011. The rats entering at least half of the length of the arm were recorded as arm choices. The rats choosing all baited arms or spent 10 min was noted as completion of task. Rats were trained twice daily for 6 days/week upto 2.5 weeks i.e. each rat received 30 trials. The number of entries to unbaited arms or re-entering a baited arm was noted as errors. In every trail, the time required to complete the task was also noted.

Rota-rod test

On the rota-rod apparatus rats were placed for 5 min. The time latency was noted in terms of motor coordination and integrity which was assessed by the time required to fell from the rota-rod. This method was described by Abdel-Aal *et al.* 2011.

Statistical analysis

All values of experiment results were represented as mean±S.E.M and analyzed by one-way ANOVA or two way ANOVA by the Bonferroni test, $P \leq 0.005$ was considered to be significant.

RESULTS

Effect of naringin on open field test

Horizontal (crossing) activity

The effect of naringin (25, 50 and 100 mg/kg) on horizontal crossing activity based on number of crossings was demonstrated in Figure 1A. One way ANOVA has revealed the significant difference among the all experiment groups in terms of number of crossings [F (6, 52) =13.41, $P \leq 0.05$]. The number of crossings were significantly reduced in Al+ S group relative to control [t=8.14, $P \leq 0.05$]. In contrast, increased number of crossings was observed in naringin treated groups significantly than Al+ S group.

Rearing activity

Among all experiment groups One-way ANOVA showed significant differences in the number of rearing [F (6, 52) = 10.82, $P \leq 0.05$]. The number of rearings were decreased significantly in Al+ S group with relative to control [t=5.53, $P \leq 0.05$]. However, naringin (25, 50 and 100mg/kg) treated groups were shown increased number of rearings significantly rather than the Al+ S group. Results were showed in the Figure 1B.

Role of naringin on morris water maze test

The effect of naringin (25, 50 and 100 mg/kg) on latency period to find the platform was demonstrated in Table 1. In comparison with control group, there

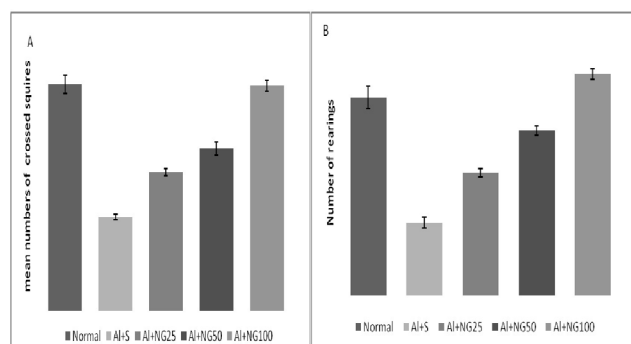


Figure 1: Effect of Naringin (25,50, 100mg/kg) on crossings (A), the number of rearings (B) against Aluminum intoxication rats.

All values expressed mean±S.E.M. $P \leq 0.05$, a= Al+S Vs Normal, b= Al+S Vs A+NG25, c= A+NG25 Vs Al+S, d= Al+NG100 Vs Al+ S. NG = Naringin, S=Saline, Al= Aluminum

Table 1: Effect of Naringin (25, 50,100 mg/kg) on the time (seconds) in Morris water maze test against Aluminum intoxication rats.

	Day 1	Day 2	Day 3	Day 4	Day 5
Normal	46.0±0.856	32.7±0.802	24.8±0.654	23.7±0.843	19.5±0.612
Al+S	64.5±1.522	56.0±0.683 ^a	53.5±0.847 ^a	50.7±1.085 ^a	50.7±1.085 ^a
Al+NG25	57.5±0.619	45.17±0.477 ^b	41.5±0.428 ^b	38.17±0.307 ^b	34.17±0.703 ^b
Al+NG50	52.2±0.654	42.2±0.703 ^b	33.5±0.671 ^b	31.2±0.601 ^b	29.8±0.703 ^b
Al+NG100	45.7±0.843 ^b	35.7±0.558 ^b	29.3±0.715 ^b	24.5±0.428 ^b	18.5±0.619 ^b

All values expressed mean±S.E.M. $P \leq 0.005$, a= Al+S Vs Normal, b= Al+NG25 Vs Al+S, Al+NG50 Vs Al+S and Al+ NG100 Vs Al+S. NG =Naringin, S=Saline, Al= Aluminum

Table 2: Effects of Naringin (25, 50 and100 mg/kg) on the mean number of errors in radial arm maze against aluminum intoxication.

Normal	Al+S	Al+NG25	Al+NG50	Al+NG100
3.67±0.21	6±0.26 ^a	3.83±0.17 ^b	2.83±0.17 ^c	1.83±0.17 ^d
2.98±0.19	5.24±0.24 ^a	2.89±0.19 ^b	2.65±0.17 ^c	1.86±0.15 ^d
3.12±0.21	5.59±0.20 ^a	2.16±0.15 ^b	2.05±0.17 ^c	1.79±0.25 ^d
2.96±0.19	4.94±0.96 ^a	2.25±0.16 ^b	1.96±0.25 ^c	1.89±0.12 ^d
2.54±0.15	4.52±0.25 ^a	2.21±0.19 ^b	1.89±0.32 ^c	1.29±0.13 ^d

All values expressed mean±S.E.M. $P \leq 0.05$, a= Al+S Vs Normal, b=Al+S Vs A+NG25, c=Al+ NG50 Vs Al+S, d=Al+NG100 Vs Al+S. NG Indicate Naringin, S=Saline, Al= Aluminum

was significant increased latency period in finding the platform in the Al+ S treated group [F (1, 60)= 64.5, $P \geq 0.05$], significant differences were obtained in day 2 [t=6.21, $P \leq 0.05$], 3 [t=6.71, $P \leq 0.05$], 4 [t=9.21 $P \leq 0.05$] and 5 [t=8.22, $P \leq 0.05$]. Whereas, naringin treated group showed a significant decrease in time latency when compared to Al+ S treated group.

Role of naringin on Radial arm maze test

Reference memory

The effects of naringin on reference memory based on the number of errors using radial arm maze test was demonstrated in Table 2. The results showed a significant increased number of errors among the Al+S treated groups when compared to control group [factor aluminum treatment; F(1, 56)=65.06, $P \leq 0.05$] in blocks 2 [t=.473, $P \leq 0.05$], 3 [t=5.07, $P \leq 0.05$], 4 [t=7.18, $P \leq 0.05$] and 5 [t=7.78, $P \leq 0.05$]. Repeated measures ANOVA showed significant differences between Naringin treated groups and Al+ S treated group [factor drug treatment: F (4, 140) =8.12, $P \leq 0.05$].

Time required ending task in maze

The effect of naringin on working memory based on ending the task in the maze was depicted in Figure 2. The results showed a significant differences in between groups which were analysed by the one-way ANOVA [F (5, 42) = 6.65, $P \leq 0.05$]. Al+ S treated group has showed significantly increased mean time in ending the task compared to control group [t=6.12, $P \leq 0.05$]. In contrast naringin treated groups has shown a

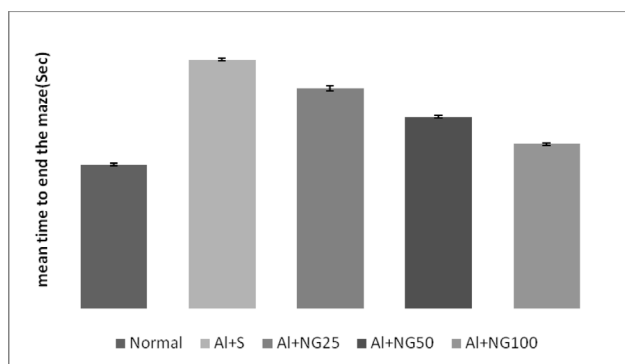


Figure 2: Effects of Naringin (25, 50 and100 mg/kg) on mean time to end the task in radial arm maze against aluminum intoxication.

All values expressed mean±S.E.M. $P \leq 0.05$, a= Al+S Vs Normal, b= Al+NG25 Vs Al+S, c= Al+ NG50 Vs Al+S, d= Al+NG100 Vs Al+S. NG = Naringin, S=Saline, Al= Aluminum

significant reduction in time required to end the task in maze when compared to Al+ S treated group.

Role of naringin in Rota-rod test

As demonstrated in Figure 3, the groups didn't varied significantly in terms of time required to fall from rota rod. This results were analysed by one-way ANOVA [F (5, 42)=2.11, $P \geq 0.05$].

Role of naringin on AChE activity

Effect of naringin on brain AChE activity was demonstrated in Table 3. The results shown that there was a significant increase in AchE levels among Al+ S rats [181.3 ± 2.525, $P \leq 0.05$] when compared to control rats

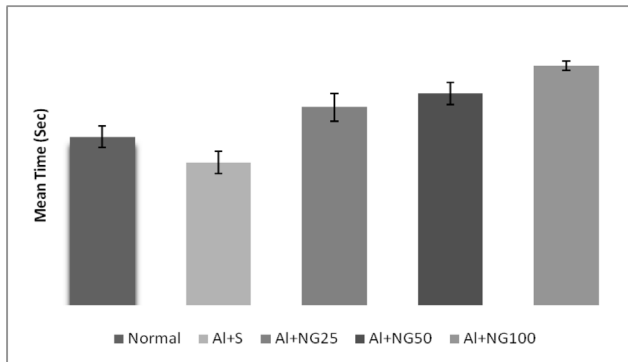


Figure 3: Effect of naringin on Rota rod test against aluminum intoxication rats.

All values expressed mean±S.E.M. $P>0.05$, No significance difference, NG= Naringin, S=Saline, Al= Aluminum.

Table 3: Effect of Naringin on Acetylcholine esterase activity against aluminum intoxication rats.

Groups	AchE moles/min/gm tissue
Normal	105.3 ± 1.667
Al+S	181.3 ± 2.525*
Al+NG 25mg/kg	141.5± 2.964*
Al+NG 50 mg/kg	112 ±3.088*
Al+NG 100mg/kg	91.33 ± 3.18*
Al+ Donepezil 0.75mg/kg	91.33 ± 3.18

Data represent the mean±SEM, the asterisk indicates $P\leq 0.05$, No of animals used in each group =6. NG=Naringin, Al=Aluminum, S= Saline

Table 4: Effect of Naringin on oxidative markers in Aluminum Intoxication rats.

ASSESSMENTS	NORMAL	Al+S	Al+NG25	Al+ NG50	Al+ NG100	Al+ Donazapil
MDA (nMol/gm of wet tissue)	181.5±2.287	636.3±3.902	361.8± 71.67*	341.00± 11.85	233.7±5.919	217.9±3.64
SOD (Units/mg of protein)	9.372±0.356	3.607±0.1439	5.223±0.1628*	7.923± 0.1653	9.477±0.203*	8.985±0.2599
CAT (Units/mg of protein)	110.9±3.225	27.00±0.7948	39.00±1.005*	84.17± 0.9627	98.83±2.501*	93.82±3.873

Data represent the mean±SEM, the asterisk indicates $P\leq 0.05$, statistically significant difference from control group, MDA indicates Malondialdehyde, SOD indicates Superoxide dismutase, CAT indicates catalase. No of animals used in each group =6, NG=Naringin Al=Aluminum S= Saline.

[105.3 ± 1.667]. However, the AchE activity was significantly reduced in naringin (25, 50 and 100 mg/kg) treated groups 141.5± 2.964, 112± 3.088, 91.33 ± 3.18, $P\leq 0.05$ respectively compared to Al+ S rats. Moreover naringin (100mg/kg) treated rats has shown decreased AchE levels (91.33 ± 3.18) in comparison with donezapil treated rats (98.33 ± 2.28).

Role of naringin on oxidative stress

SOD, MDA and CAT levels in the brain were quantified to evaluate the oxidative stress. The levels of MDA were significantly increased with significantly decreased SOD, CAT levels in Al+S rats [636.3±3.902, 3.607±0.1439, 27.00±0.7948, $P\leq 0.05$] when compared to control group. Further, significantly reduced MDA levels and increased SOD and CAT levels were observed in naringin (25, 50 and 100 mg/kg) treated rats when compared to Al+S rats. These results were shown in Table 4.

DISCUSSION

Naringin activity against impaired cognitive and locomotor functions in aluminum intoxication rats was investigated in this study. Rats are exposing to Al for long term which leads to accumulation in brain, especially in the hippocampus. It is the learning and memory center. Al intoxication alters endogenous antioxidant enzyme activity and neuronal apoptosis.^{21,22} When it encountered with brain cells, cholinergic deficits will occur that are reduced mRNA muscarinic receptors expressions, change in cholinesterase activity, degeneration of cholinergic terminals in hippocampus and decreased neuronal choline uptake.^{23,24} Contemporarily, several researchers focus on natural antioxidants, in particularly plant derived poly-phenolic compounds with neuroprotective potential²⁵⁻²⁷ and also reported the bio applicable properties of naringin, like anti-cancer, antioxidant, anti-inflammatory, cardiovascular protection and neuro protective effects.²⁸⁻³⁰

In our study, we observed that the rats with Al-exposure for 60 days did not gain weight when we compared to control rats. Also, we clearly noticed the performance in food searching behavior of animals which are treated

with Al for 60 days did not have a significant difference when we compare with control group. Thus, this findings supports that, Al does not decrease in motivation for food when the animals were subjected to trails for the learning the experiment.^{31,32} In Al treated rats, the time required to finish the task was significantly increased in comparison with the control group, this may be due to exploring more wrong arms before finish the task. Therefore, the animals' performance might be declined typically due to impairment in memory function rather than being related to decrease in motivation for food consumption in radial arm maze tests.³³

In the open field test, Al treated group rats performance was not improved significantly in comparison to control group with repetitive administration of trials that undoubtedly reflected the Al-induced impairment in spontaneous locomotive capacity, exploratory behavior, memory and learning.³⁴ Rota-rod test is usually used to assess the motor function, fortitude and balance of muscle. In the experiment, we noticed that there is no significant alteration of motor performance in Al treated rats. These results implicate the effect of Al precisely on cognitive decline rather than alterations in motor function in rats.³¹

The significant results obtained from the eight arm radial maze and water morris maze tests that were demonstrated the potential effects of naringin in inverting the cognitive decline with the improved performance in intoxicated rats. The exploratory behavior and locomotors activity of naringin treated rats in open field test were improved significantly than Al treated. These results were in accordance with earlier reports.⁸ Recently, some of the studies also demonstrated the significant cognitive impairment was caused by chronic exposure of Al in the rats.^{2,3} Chronic administration of Al decreased the spontaneous locomotors activity and exploratory activity without any significant collision on motor coordination.^{31,33} In this experiment, we noticed the naringin treated rats had a significant reduction in the escape latencies during both hidden and visible platform sessions when compared to Al treated rats.

In comparison with Al treated rats, there was a significant improvement among naringin treated rats in the aspect of working and reference memories by ending their missions faster as demonstrated by radial arm maze test. It was found that chronic Al intoxication can decline reference and working memory.³⁵ Therefore, in passive avoidance test, naringin was thought to improve the performance of Al-intoxicated rats as it increased the step-through latency significantly. This indicates that naringin treated animals were able to

repossess and keep hold of the information. Even though, the fecal balls were significantly increased in the rats treated with large dose of naringin (100 mg/kg/day), the latency to kick off movement was not changed significantly because of naringin didn't affect anxiety in Al treated rats. This can be explained by naringin mediated peripheral acetylcholine esterase activity.

Acetylcholine (ACh) is a key factor in learning and memory functions of brain. AChE enzyme degrades the ACh and terminates its action. In addition, the onset of few neurodegenerative diseases was involved with AChE enzyme.⁷ Chronic exposure of naringin (25 mg/kg) produces a minimal effect on the performance of rats in the redial and water maze tasks; while in large dose (100 mg/kg) formed its maximum responses. The cholinergic system plays an active role in several physiological processes like learning, memory and synaptic plasticity.^{36,37} To understand exact mechanisms of Alzheimer, the activity of brain AChE was determined. The results showed that levels of AChE were increased significantly in chronic Al-exposed rats. The cholinergic neurotransmission disruptions observed in this study can be correlated with behavioral impairments in the Al exposed rats. AChE levels were significantly reduced in chronic naringin exposed rats suggesting the naringin activity against AchE. These findings were in accordance with the earlier reports.^{8,38} Dose dependent effect of naringin was expressed in terms of AChE activity resulting as the levels of AChE is inversely proportional to the dose. Therefore, chronic supplementation of naringin clearly alleviated the AChE enzyme in the. This demonstrates the protective effects of naringin against the aluminum intoxication.

Oxidative stress is one of the major risk factor in Alzheimer disorder. Aluminum induced oxidative stress mechanism destructs the physiological antioxidant/ pro oxidant turmoil in favor of ROS generation.^{39,41} Al may bind with superoxide anions and form Al-superoxide anions and these oxidants can lead to reduced activity of mitochondrial superoxide dismutase.^{4,6} In the Al treated rats, significantly increased MDA level and decreased CAT, SOD activities were noticed. Further, chronic supplementation of naringin significantly enhanced the antioxidant enzyme activity (SOD, CAT) and reduced the level of MDA. The protective activity of naringin on neurodegenerative disorders was advanced by the reduced activity of brain AChE enzyme and lipid peroxidation.

CONCLUSION

This study demonstrates that Al treated rats were associated with behavioral changes and cognitive deficits. Naringin reverse these deficits at dose dependent manner. Therefore, we conclude that naringin had a protective role in the aluminum-induced deficits in the brain which is mostly related to oxidative stress and cholinergic dysfunction.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ABBREVIATIONS

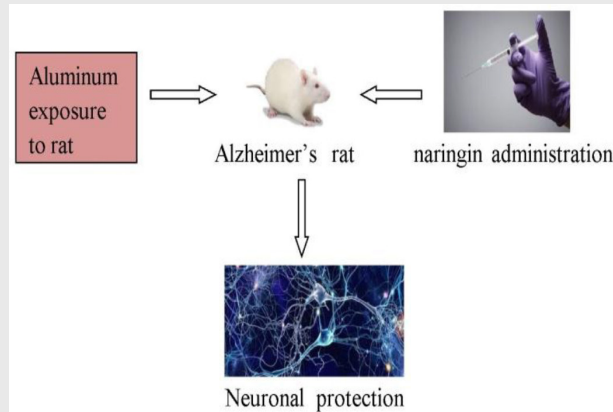
Al: Aluminium; **ROS:** Reactive oxygen species; **SOD:** Superoxide Dismutase; **CAT:** Catalase; **AChE:** Acetylcholinesterase; **MDA:** Malondialdehyde.

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PICTORIAL ABSTRACT



SUMMARY

The effects of aluminum on the cognition and development of Alzheimer's has been demonstrated. The positive effect of naringin in Alzheimer's through reducing the oxidative stress and acetylcholine esterase activity has been reported. The improvement of cognition among aluminum induced alzheimer's by naringin has been reported.

About Authors



Dr. Orsu Prabhakar working as an Assistant Professor In GITAM Institute of Pharmacy, GITAM Deemed to be University. He had extensive research and teaching experience in the field of Pharmacology and Toxicology.

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