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Original Article

Pharmacology Section

Modulatory Role of *Mucuna pruriens* against Aluminium Fluoride Induced Neuronal and Behavioural Alterations in Rats

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ABSTRACT

Introduction: Aluminium Fluoride (AlF_3) intake produces oxidative stress resulting in brain damage. Although *Mucuna pruriens* (Velvet bean) is commonly used in ayurvedic system of medicine for different neurological disorders, its usefulness in oxidative stress induced behavioural and neuronal damage has received little attention.

Aim: This study was conducted to evaluate the neuroprotective role of *Mucuna pruriens* against AlF_3 induced behavioural and neuronal changes in rats.

Materials and Methods: Thirty six male Wistar albino rats were divided into six groups as: I-Normal saline; II-AlF_3; III-25 mg/kg Quercetin and AlF_3; IV, V and VI received 50, 100 and 200 mg/kg *Mucuna pruriens* methanolic extract and AlF_3 (600 ppm). Extract was given for 10 days followed by AlF_3 treatment for next seven days. Behavioural parameters were assessed such as T-maze test, actophotometer, and rotarod followed by histopathological studies.

Results: A reduction in the locomotor activity, spatial learning and motor coordination was observed in the animals of Group II which reversed when treated with *Mucuna pruriens*. The histopathological studies of hippocampal CA1 region also showed the neuroprotective role of this plant.

Conclusion: This study suggests the potential neuroprotective ability of *Mucuna pruriens* against the change in behaviour and neuronal damage which occurred due to induction of AlF_3.

Keywords: Neuroprotection, Oxidative stress, Stroke

INTRODUCTION

Aluminium and Fluoride are found in abundance on earth. It gets access to our body mainly through drinking water, food stuff, drugs, and utensils. Continuous exposure of aluminium in drinking water can lead to age-associated neurological problems like Alzheimer's disease and Parkinson's disease [1]. Similarly, fluoride is also a biologically highly active compound. High level of exposure to it produces oxidative stress, DNA impairment and neuronal damage, which also results in decreased learning and memory ability [2]. When drinking water contains both Fluoride and Aluminium, then there will be the formation of fluoroaluminium complex (AlF_3) in the stomach. Its absorption and passage in the bloodstream is more compared to that of its ionic forms and later crosses the Blood Brain Barrier (BBB) [3]. The AlF_3 complex produces cell death in the hippocampal CA1 region which is the site of learning and memory when given as 0.1 ppm through drinking water. This also induces abnormal behaviour of the animal, altered neuronal and cerebrovascular integrity [4]. These changes are due to the generation of free radicals, lipid peroxidation and altered antioxidant defense system. Natural products such as flavonoids exert remarkable antioxidant activity which protects from damage occurred due to Aluminium fluoride-induced oxidative stress [5]. The flavonoids normalise the brain damage occurred due to oxidative stress, may be because of its ability to decrease the glutamate-induced increase in intracellular Ca^{2+} level [6].

Mucuna pruriens (M. pruriens) Linn. which is commonly called as velvet bean or Kapikacchu is one of the widely studied plants for its medicinal effects. It is well-known for its aphrodisiac activities as it is known to increase the sperm count and testosterone levels in the body [7]. It has also been used to treat arthritis, nervous disorder, atherosclerosis, as an antipyretic, analgesic and in Parkinson's disease [8]. The plant seed contains large amounts of phenolic compounds which exhibit high antioxidant and free radical scavenging activities. As this plant is a natural source of antioxidants, it might be helpful in preventing the oxidative stress. The alcoholic extract shows significant antioxidant activity which was comparable with standard ascorbate and total phenol content [9,10]. However, to date, there are no reports on the effect of *Mucuna pruriens* against the change in behaviour and neuronal damage which occurred due to AlF_3. Taking antioxidant property into the consideration, it can be hypothesised that the extract of *Mucuna pruriens* might show a protective effect against AlF_3 exposure on behavioural and neurological integrity. So, the present study was conducted to assess the possible neuroprotective potential of *Mucuna pruriens* from the stress occurred due to AlF_3 exposure.

MATERIALS AND METHODS

Preparation of alcoholic plant extract: The Mucuna pruriens plant seeds were powdered and passed through a 40-mesh sieve. First extracted with petroleum ether (-80°C), and then with methanol (95%) for 72 hours. Then this extract was transferred, filtered and lyophilised (-40°C) to attain dry extract which is used for this study.

Animals

The present preclinical experimental study was conducted on 36 male Wistar albino rats each weighing 200 gm-250 gm which were obtained from Central Animal Research Facility, Manipal Academy of Higher Education, Manipal, Karnataka, India. The study was conducted for about four months from January 2016 to April 2016. The rats were accommodated at standard room temperature (23±2°C) in Central Animal House with food and water ad libitum. The protocol of this experiment was accepted by the Institutional

Animal Ethics Committee (No. IAEC/KMC/59/2014) and was carried out according to the committee guidelines.

Experimental Design

In present study, we chose six groups with six rats each and allowed to familiarise for seven days prior to the experiment.

Group I-Normal saline, 10 mL/kg, orally.

Group II-Aluminium fluoride (negative control).

Group III-25 mg/kg Quercetin (standard), AlF_3 induced stress.

Group IV-50 mg/kg extract, AlF_3 induced stress.

Group V-100 mg/kg extract, AlF_3 induced stress.

Group VI-200 mg/kg extract, AlF_3 induced stress.

Group IV, V and VI rats were fed with extract and Group III rats fed with Quercetin for 10 days. Eleventh day onwards all the rats except Group 1 were treated with AlF_3 through the drinking water for seven days in dosage of 600 ppm [6]. After 24 hours, the following tests were conducted:

Spatial learning (T-maze) test: This is a spatial memory task, as described earlier [11] where the ability of the rats to differentiate the left or right arm of the T-maze apparatus to procure food as a reward was observed. In spontaneous alternation test, the mouse was kept in the start box, and allowed to enter into the stem and choose any one of the arms. The arm chosen by the rat in each trial was noted and the percentage bias for each animal was calculated. This was followed by rewarded alternation test which had two parts: forced run and choice run. In the forced run, we closed one arm so that the animal is forced to go to the other arm. In the next step which is a choice run, both arms were made available for the animal. We considered a "correct response" only when a rat enters opposite to the forced arm. Percentage of correct response was calculated.

Evaluation of locomotor activity using actophotometer: The locomotor activity in animals was measured using a digital actophotometer (INCO, Ambala, India). Locomotor activity can be an index of wakefulness (alertness) of mental activity. The movement of the animal cuts off a beam of light falling on the photo cell and a count was recorded and displayed digitally. First the animals were weighed and then numbered. Each mouse was placed individually in the actophotometer for about 3 minutes to habituate the animal. Later for next 10 minutes the basal activity score was recorded. The area was cleaned with dilute alcohol and dried between trials to maintain hygienic condition. Decreased activity score was taken as an index of CNS depression [12].

Rotarod test: We conducted this test (Panlab, Harvard apparatus) to assess motor coordination and balance, similar to a previous study [13]. The instrument was adjusted for the speed of 15 rpm and the animal was separately placed on the rotating rod. The latency at which each mouse falls off the rod was documented for a maximum cut-off time for 300 seconds. The procedure was repeated for a total of three trials separated by 15 minutes inter-trial intervals.

Histopathology: After 10 days of behavioural tests the animals were euthanised and the brain was fixed in formalin, embedded and 5 µm thick sections were made, stained with Haematoxylin and Eosin (H&E) for histopathological observation. The hippocampal CA1 region was observed for viable cells which were rounded cells with clear cytoplasm and nuclei. Non viable cells which were shrunken and had fragmented nuclei, were excluded from the count.

STATISTICAL ANALYSIS

The results are represented as mean±SEM with one-way Analysis of variance (ANOVA) and Tukey's multiple comparisons test as a post-hoc test using a GraphPad Prism version 5.0. The statistical significant value was considered wherever $p<0.05$.

RESULTS

Spatial Learning (T-maze) Test

Spontaneous alternation tests: During the spontaneous alternation test, animals treated with AlF_3 (Group II) showed significant ($p<0.05$) impairment in spatial learning, in the form of less number of alternations and more percentage bias when compared to the Normal group (Group I). There was an increased number of alternations and decreased percentage bias observed in 50, 100 and 200 mg/kg of extract treated groups (Group IV, V and VI) when compared to that of AlF_3 treated group (Group II). A significant difference was observed only in 200 mg/kg of extract treated group (Group VI) and Quercetin treated group (Group III) when compared to AlF_3 treated group (Group II) [Table/Fig-1].

Rewarded alternation test: During rewarded alternation test, animals treated with AlF_3 (Group II) showed significant ($p<0.05$) impairment in spatial learning, by decreased percentage correct response in comparison to the Normal group (Group I). A dose-dependent increase in percentage correct response was observed in extract treated groups (Group IV, V and VI) as compared to that of AlF_3 (Group II) treated group. The increased percentage correct response was also observed in the group treated with Quercetin (Group III) [Table/Fig-1].

Evaluation of locomotor activity using actophotometer: A reduction in the locomotor activity was observed in the animals of Aluminium fluoride-treated group (Group II) in comparison with the animals of the control group (Group I). A dose-dependent significant ($p<0.05$) increase in locomotor activity was observed in the animals with *Mucuna pruriens* treated group (Group IV, V and VI) as compared to AlF_3 (Group II) treated (negative control) group. Increase in locomotor activity was also observed in Quercetin treated group (Group III) in comparison with that of AlF_3 (Group II) treated group [Table/Fig-1].

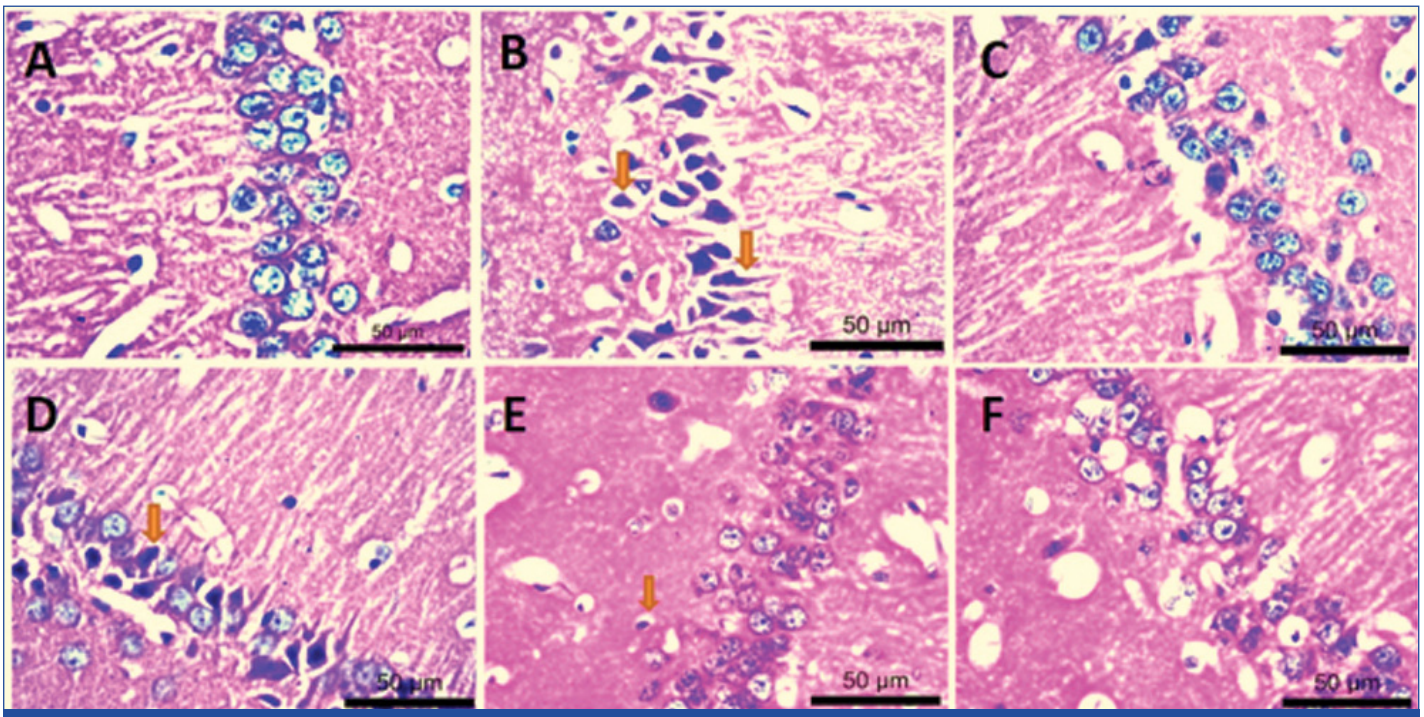
Instrument Used	Parameters	Normal	AlF_3	Quercetin+AlF_3	MP (50 mg/kg)+AlF_3	MP (100 mg/kg)+AlF_3	MP (200 mg/kg)+AlF_3
T-maze	Number of Alternations	14.00±1.00	6.67±0.88^a	12.00±1.16^b	9.33±0.67^a	9.67±0.88^a	12.67±0.67^b
	Percentage bias	51.39±1.39	70.83±4.17^a	55.55±1.39^b	63.72±3.52	65.11±3.55	55.55±3.68^b
	Percentage correct response	76.22±2.94	51.39±1.39^a	67.78±4.94	55.55±1.39^a	58.16±6.20^a	74.83±2.55^{bc}
Actophotometer	Locomotor	377.00±4.51	188.70±2.96^a	363.30±2.33^b	248.00±3.22^{ab}	330.30±3.38^{abc}	345.00±3.61^{abc}
Rotarod	Motor coordination	193.30±7.37	130.30±6.06^a	176.30±4.33^b	138.30±9.70^a	140.30±5.36^a	179.30±5.49^{bcd}

[Table/Fig-1]: Effect of *Mucuna pruriens* methanolic extract on AlF_3 induced behavioral alterations.
Data presented as mean±SEM (n=6), where ^{a}p<0.05 compared to normal group, ^{b}p<0.05 compared to AlF_3 treated group and ^{c}p<0.05 compared to MP (50 mg/kg)+AlF_3 group and ^{d}p<0.05 compared to MP (100 mg/kg)+AlF_3 group. p-value calculated by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. For the number of alternations in T-maze the p-value=0.0010 and F (DFn, DFd): F (5,12)=8.953. For the percentage bias in T maze the p-value=0.0077 and F (DFn, DFd): F (5,12)=5.433. For the percentage correct response in T maze the p-value=0.0016 and F (DFn, DFd): F (5,12)=7.994. For the locomotor activity the p<0.0001 and F (DFn, DFd): F (5,12)=476.4. For the motor coordination the p<0.0001 and F (DFn, DFd): F (5,12)=15.93.

Rotarod test: A reduction in motor coordination and the balance was seen in the rats of Aluminium fluoride-treated group (Group II) in comparison with that of the control group (Group I). A significant ($p<0.05$) increase in motor coordination and the balance was

observed in the animals with *Mucuna pruriens* treated group (200 mg/kg) (Group VI) as compared to AlF_3 treated (negative control) group (Group II). Increase in motor coordination was also seen in Quercetin treated group (Group III) in comparison with that of AlF_3 treated group (Group II) [Table/Fig-1].

Histopathology: Haematoxylin and eosin stained sections of the hippocampal CA1 region showed significantly decreased ($p<0.05$) number of viable neurons in Aluminium fluoride-treated group (Group II) in comparison with that of the control group (Group I) [Table/Fig-2A,B]. Non viable neurons were dark stained, small irregular cells which were distinguished from viable neurons having prominently rounded cells, with clear cell membrane and nuclei. A dose-dependent significant ($p<0.05$) increase in viability of cells was seen in the animals with *Mucuna pruriens* treated group (Group IV, V and VI) as compared to AlF_3 treated (negative control) group (Group II). Increase in viable cell number was also seen in Quercetin treated group (Group III) in comparison with that of AlF_3 treated group (Group II) [Table/Fig-2C-F,3]. Overall, the animals treated with AlF_3 showed the neuronal damage. It also reflected the safety of *Mucuna pruriens* as well as quercetin treated groups.



[Table/Fig-2]: High power (40X) photomicrographs showing haematoxylin and eosin (Ehrich's H&E yellowish) stained sections of hippocampal CA1 region. A) Normal saline; B) AlF_3; C) Quercetin+AlF_3; D-F) 50 mg/kg, 100 mg/kg, and 200 mg/kg of extract respectively+AlF_3.

Test	Histopathology
Parameters	Viable neurons of CA1 region
Normal	140.0±4.61
AlF_3	42.00±4.04[a]
Quercetin+AlF_3	125.3±4.48[b]
MP (50 mg/kg)+AlF_3	51.67±4.49[a]
MP (100 mg/kg)+AlF_3	101.7±6.01[abc]
MP (200 mg/kg)+AlF_3	123.3±3.75[bc]

[Table/Fig-3]: Effect of *Mucuna pruriens* extract on AlF_3 induced histopathological changes.
Data presented as mean±SEM (n=6), one-way Analysis of Variance (ANOVA) and Tukey's multiple comparison as post-hoc test where [a]$p<0.05$ compared to normal group, [b]$p<0.05$ compared to AlF_3 treated group and [c]$p<0.05$ compared to MP (50 mg/kg)+AlF_3 group. The $p<0.0001$ and F (DFn, DFd): F (5,12)=79.12.

DISCUSSION

It has been reported earlier that chronic aluminium exposure can result in cognitive [14] and locomotor [15,16] impairment. Fluoride anions influence the activity of a variety of enzymes. Fluoroaluminium complex mimics the action of a majority of the neurotransmitters, hormones and growth factors [17]. Fluoride gets collected in the brain and muscle which causes stress and inhibits auto-oxidation mechanism. Thus, it results in oxidative damage of neural and muscular tissue which alters the function of brain and muscle [18]. The present study was conducted to assess the therapeutic potential of previously reported antioxidant principals of methanolic extract of *Mucuna pruriens* [9] in ameliorating these structural and functional neuromuscular changes induced by AlF_3 in Wistar rats.

AlF_3 can affect learning and memory [2]. Spatial learning and memory assessment help in evaluating the functional status of neurons. In the present study, the spatial learning ability of the different group of animals was assessed by the T-maze test. Significant learning and memory deficits were found in aluminium fluoride-treated group in the form of less number of alternations, more percentage bias and decreased percentage correct response when compared to the normal group. The treatment with *Mucuna pruriens* extract showed an increase in spatial learning ability compared to AlF_3 control. The reversal of memory deficits by the extract treatment might be due to its neuroprotective effect.

AlF_3 affect the cell membrane and integrity of purkinje fiber which results in a decline in motor function [19]. In the present study, we found a similar decline in locomotor activity in AlF_3 treated control animals compared to normal control. The reversal in the declined motor activity was observed in *Mucuna pruriens* extract treated groups when compared with AlF_3 treated group. This could be due to intact neuronal integrity which might have prevented a decline in the motor function.

Mucuna pruriens has shown improvement in behavioural profile in various conditions including sexual behavioural profile. These effects indicate alterations in neurotransmitters levels like noradrenaline, dopamine etc., and reports are available for the same [20].

Although, oxygen is necessary for human life, it is also a precursor to the formation of harmful Reactive Oxygen Species (ROS) [21]. The free radicals generated in the body can lead to the formation of ROS which damages the protein, lipid and nucleic acid thus leading to enzyme inactivation, altering the genetic material and cell death [22]. Fluoride is biologically highly active compound and its excessive intake can lead to fluorosis which mainly affects muscles and brain [6]. Studies have also shown that fluoride accumulates in the hippocampal region of rat brain resulting in oxidative stress and neuronal degeneration [2]. Neuronal death may be due to apoptosis

or autophagic pathways. This is due to increased glutamate release, the release of ROS, mitochondrial dysfunction or inflammation [23]. In the present study, the hippocampal CA1 region showed a significant decrease ($p<0.05$) in a number of viable neurons in AlF_3 treated group as compared to the animals of the control group.

Natural products with antioxidant properties can be chosen for its maximum therapeutic effect with minimal risk of iatrogenic adverse effects. The neuronal protection is observed in *Mucuna pruriens* extract treated group which may be due to the antioxidant property of this plant against free radicals. Further studies are required to elucidate the exact mechanism of action.

LIMITATION

The study could find out behavioural changes only. However, a detailed study for the change in the behavioural parameters at the nuclear level will give a better insight. The functional status of neurons needs to be evaluated using neurotransmitters in the various parts of the brain.

CONCLUSION

The present study results showed a possible protective role of *Mucuna pruriens* against AlF_3 induced behavioural (such as cognitive deficit and locomotor impairment) and neuronal damage in rat brain. However, the molecular mechanism of the neuroprotective potential of this plant can be assessed by pursuing advanced studies on this plant extract.

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