ORIGINAL ARTICLE

Fisetin potentiates anxiolysis in rat models of aluminium chloride-induced Alzheimer-like disease

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Abstract

Background: Alzheimer's disease is a chronic, irreversible neurological disease that affects brain cells. *Aim and Objectives:* To investigate the remediatory benefits of fisetin against Aluminum chloride induced anxiogenic and behavioural deficits in adult male Wistar rats. *Material and Methods:* Thirty-two adult rats with average weight of 170 g were grouped into 8 (1-8), consisting of 4 rats each. Group 1 received -1 ml of PBS, Group 2 received 100 mg/kg body weight of $AlCl_3$, Group 3-25 mg/kg body weight fisetin, Group 4- 50 mg/kg body weight of fisetin, Group 5-75 mg/kg body weight of fisetin, Group 6- 25 mg/kg body weight fisetin + 100 mg/kg body weight of $AlCl_3$, Group 7- 50 mg/kg body weight fisetin + 100 mg/kg body weight of $AlCl_3$ and Group 8-75 mg/kg body weight fisein + 100 mg/kg body weight of $AlCl_3$ orally for 21 days. Histochemical analysis using Congo red stain and neurobehavioural studies were carried out. *Results:* It showed the inhibitory roles of fisetin against $AlCl_3$–induced anxiogenic cascades. Significant differences ($p< 0.05$) in closed and open arm entries were observed across the group compared with control. This showed that rats tarried longer in the maze's closed arm which is indicative of increased levels of anxiety. Fisetin normoregulated the escape period of rats induced with $AlCl_3$. Furthermore, evidences of amyloidsis were seen by increased amyloid substances in neuronal axons and dendrites which corresponded to apoptotic changes observed in the Congo red stain sections. Interestingly, fisetin (50 and 75 mg/kg daily for 14 days) significantly attenuated behavioural deficits in rats by inhibiting cellular stressor proteins activated by $AlCl_3$. *Conclusion:* These findings suggest that endogenous fisetin may protect against Alzheimer-like disease-related anxiogenesis and behavioural alterations.

Keywords: Aluminum chloride, Alzheimer-like disease, Fisetin

Introduction

Human interactions with the ubiquitous presence of Aluminum (Al) metal in the environment are on the increase. Reports by some schools of thoughts have implicated Al as a causative substance of neurodegeneration and can be linked to the causes of some neurodegenerative diseases such as Alzheimer's Disease (AD) [1]. Various human contact and interaction with this substance such as in food, water, medicine and antiperspirants remain the major routes Al toxicity occurs [2].

A neurotoxicant like Al permeates and alters the blood-brain barrier by producing changes in the cholinergic, ionic, noradrenergic and dopaminergic neurotransmission in the central nervous system. These alterations are associated with attention, visual, perception, memory, learning, cognitive and learning abilities [3]. Reports have shown that Al exposure led to mitochondrial dysfunction *in-vitro* and *in-vivo*, as well as it destroys the antioxidant defense system by

decreasing the status of antioxidant enzymes [4]. Neurodegenerative diseases are characterized by loss of function and structure in the circuit of neurons leading to behavioral impairment. Therefore, the science of behavior helps to detect several symptoms of neurodegenerative diseases as well authenticates the veracity and efficacy of neuropharmacologic agents [5]. Behavioral tests like Morris water maze for spatial memory and learning and elevated plus maze for anxiogenic cascades have been utilized to assess comprehensively neurological functions in neurodegenerative diseases [5].

Previous studies have shown the efficacious and beneficial effects of flavonoid in the treatment of neurodegenerative diseases. Fisetin (3, 3, 4', 7-tetrahydroxyflavone), a flavonoid found in vegetables and fruits, like strawberry, mango, kiwi, cucumber, grape, apple, and tomatoes [6, 7]. The number of evidences using renovate strategies have showed pharmacological potentials of fisetin, which includes antioxidant [8] anti-inflammatory [9], anti-metastatic [10] hepatoprotective [11], free radical scavenger [12], cardio-protective [13], anti-carcinogenic [14] and anti-diabetic [15]. This study aimed to investigate the remediatory benefits of fisetin against Aluminum chloride induced anxiogenic and behavioural deficits in adult male Wistar rats.

Materials and Methods

Ethical approval was sought from the College of Medicine Ethical Committee, University of Nigeria Enugu Campus, Enugu State, Nigeria. All protocols and treatment procedures complied strictly with the Institutional Animal Care and Use Committee guidelines. Adult male Wistar rats were bred at the animal holdings of the College of Medicine, Faculty of Basic Medical Sciences, University of Nigeria Enugu Campus (UNEC), Enugu State. The rats were kept in clean separate cages where they were served rat feed and water *ad-libitum*. Fisetin and aluminum chloride ($AlCl_3$) crystalline salts were procured from Sigma-Aldrich (USA). Phosphate-buffered saline (PBS; pH 7.8) was freshly prepared.

Crystalline salt of $AlCl_3$ (20 mg/ml) was dissolved in distilled water and adjusted to pH 7.8 with 0.1 M PBS. The solution was freshly prepared each morning of administration and kept at 4°C before use. Fisetin (100 g/ml) was dissolved in distilled water. Oral administration of treatment solutions (Fisetin and $AlCl_3$) to rats across all groups was done using a modified oral cannula. In this study, Adults male Wistar rats (32, with average weight of 170 g) were used. The rats were assigned randomly to 8 different groups (A–H), consisting of 4 rats each.

The groups were treated as follows: Group 1 received 1 ml of PBS daily for 21 days; Group 2 received 100 mg/kg/bw of $AlCl_3$ daily for 21 days; Group 3 received 25 mg/kg/bw of fisetin (**low dose**) daily for 21 days; Group 4 received 50 mg/kg/bw of fisetin (**medium dose**) daily for 21 days; Group 5 received 75 mg/kg/ bw of fisetin (**high dose**) daily for 21 days; Group 6 received 25 mg/kg/bw of fisetin daily for 14 days followed by treatment with 100 mg/kg/bw of $AlCl_3$ for the subsequent 7 days; Group 7 received 50 mg/kg/bw of fisetin daily for 14 days followed by treatment with 100 mg/kg/bw of $AlCl_3$ for the subsequent 7 days; Group 8 rats received 75 mg/kg/bw of fisetin daily for 14 days followed by treatment with 100 mg/kg/bw of $AlCl_3$ for the subsequent 7 days. Rats were weighed weekly, beginning from the first

week of administration using Gallenkramp weighing balance. Treatment doses adopted in this study were previously reported by He *et al.* [16] and Gbadamosi *et al.* [17].

Differences in the anxiety levels of rats was evaluated across treatment groups using the elevated plus maze method. The rats were introduced into an elevated plus apparatus that stood 45 cm tall with two open arms and two closed arms for an exploration time of 5 min. The open arm duration was estimated as total time the rats spent in the open arm of the elevated plus maze for the duration of the study. Morris water Maze test was carried out to assess memory and spatial learning of rats across the groups. The procedure was done in accordance with the comprehensive description [18]. Briefly; the water maze was a pool of water measuring 30 cm deep and 100 cm in diameter which was kept at room temperature. An escape platform of about an inch deep from the surface of the water was placed in one of the quadrants, outside of which were visual cues. Rats were trained for 3 days prior to the actual test. During the training, each rat undertook 4 trials and was placed in each of the other three quadrants for a maximum of 60 seconds to find the escape platform at an interval of 15 min between quadrants until the escape latency period reduced to less than 20 seconds. During the test, the pool was colored and the rats were placed in each of the three quadrants different from the escape platform quadrant at an interval of 15 minutes. The time taken to find the escape platform was recorded as the escape latency period.

Coronal sections of PFC were obtained stereotaxically (- 3 mm and +4 mm from the bregma respectively) from each brain. Subsequently, sections were processed routinely to obtain paraffin wax embedded blocks for histology and Congo red staining to amyloid deposits in neurons. Histochemical demonstration of amyloid substances was done with slight modification to the method [19]. All quantitative data were analyzed using the GraphPad Prism® software (version 9.3.1). Neurobehavioral outcomes using one way ANOVA with Tukey's multiple comparisons test. Significance was set at $p<0.05$*, $p<0.01$**, $p<0.001$***, $p<0.0001$****. The outcomes were represented in bar charts with error bars to show the mean and standard error of mean respectively. Light microscopic studies of the PFC sections on glass slides were captured using Olympus binocular research microscope (Olympus, New Jersey, USA) which was connected to a 5.0 MP Amscope Camera (Amscope Inc, USA.).

Results

Inhibitory roles of fisetin against $AlCl_3$–induced Alzheimer-like anxiogenic cascades

Fisetin antipanic and anxiolytic potentials in inhibiting anxiogenic cascades caused by $AlCl_3$ induction was assessed using the elevated plus maze as described in the methods section. Reports showed that rats treated with fisetin had similar patterns of maze activities with the control group as shown in (Fig. 1A). In the open arm group, rats administered fisetin tarried longer in the maze's open arm which was indicative of reduced levels of anxiety. Comparatively, rats that were administered $AlCl_3$ alone had an insignificant increase in anxiety levels ($p > 0.05$) when compared to both the fisetin and control groups. In the closed arm group, an insignificant difference ($p > 0.05$) was also observed in Groups 2, 3 and 4

compared to control group. At day 7 of administration in the closed arm group, a significant increase ($p< 0.05$) was observed in Group 2 compared to the control while a significant reduction ($p< 0.05$) in Group 8 was observed when compared to control (Fig. 1b). This showed that rats tarried longer in the maze's closed arm which was indicative of increased levels of anxiety. In the open arm group, an insignificant reduction was observed in Group 2 when compared to other groups at day 7 of administration.

The open arm group of day 21 of administration showed a significant decrease ($p<0.001$) in Group 2 compared to control while significant reductions ($p<0.01$, $p<0.05$) in Groups 4-8 were observed when compared to control. The difference observed in Group 2 may be attributed to the animals' aversion to the open arm of the maze. Though a significant difference was recorded in other groups but the values were much higher when compared to Group 2, this could be as a result of the neuro-protective effect of fisetin and the rats' affinity for open spaces. In the closed arm group, a significant increase ($p<0.05$) in Groups 2 and 3 (L.D fisetin) was observed when compared to control while a significant reduction ($p<0.05$) in Group 6 was observed when compared to Group 2.

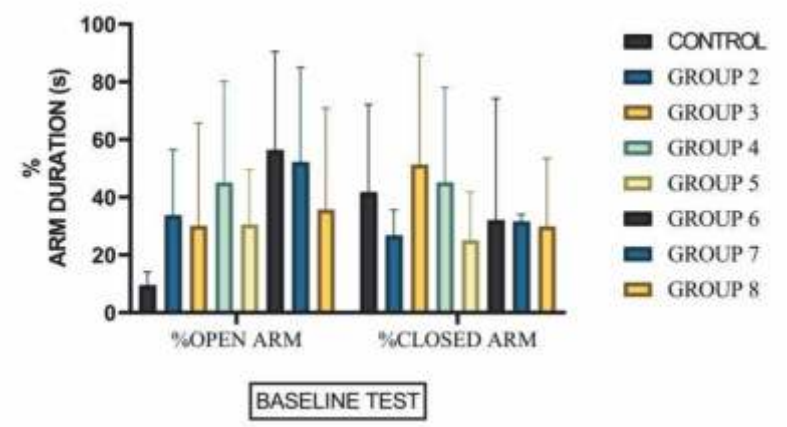

Figure 1a: Baseline test for the percentage arm time (Elevated plus maze)

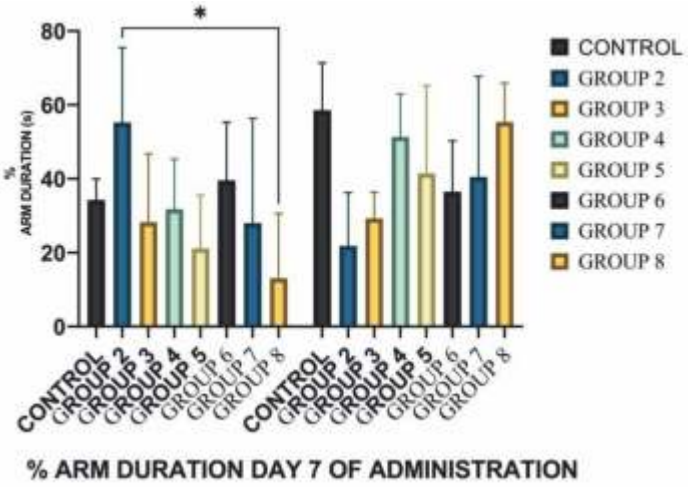

Figure 1b: Showing Day 7 of percentage arm time (Elevated plus maze)

In this study, the increase in values observed in Groups 3-5 administered varying dosages of fisetin could possibly point to the fact fisetin had no effect in these groups and that was why rats tarried longer in the maze's closed arm (Fig. 1c). However, it contrasted with values observed in groups 6-8 that was administered different doses of fisetin plus $AlCl_3$ which was in tandem with the rats' affinity for possibly the open arm thereby pointing out the neuroprotective effects of fisetin on $AlCl_3$ induced damage. These results further highlighted the anxiolytic properties of fisetin administration on aluminum chloride-induced Alzheimer like disease.

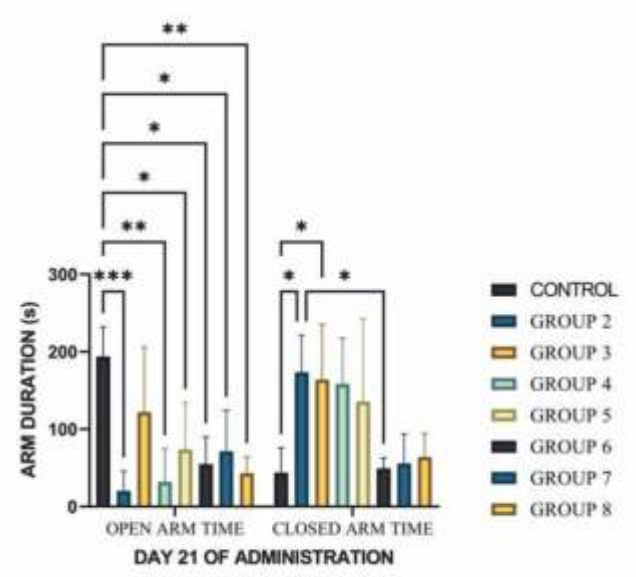

Figure 1c: Showing the day 21 of percentage arm time (Elevated plus maze)

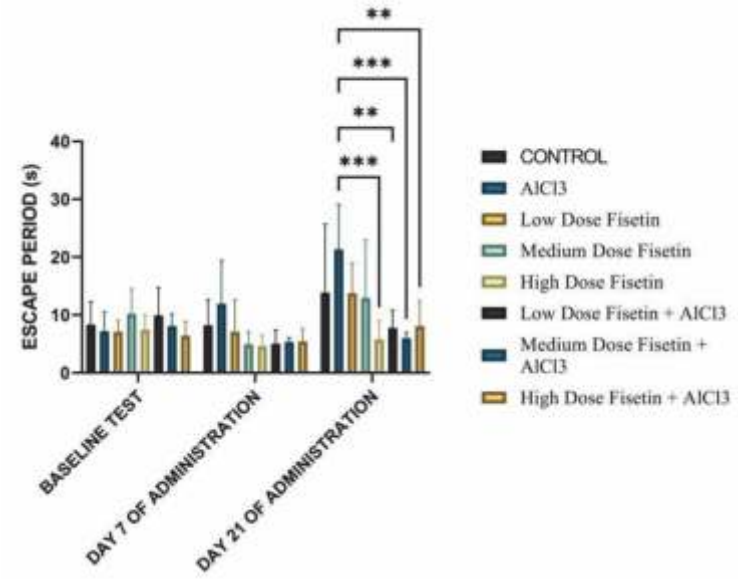

Figure 2: Analysis of mean escape period activity levels of rats across all groups

Fisetin administration normoregulated the escape period of rats induced with $AlCl_3$

This study showed the potential ability of fisetin in the therapeutic management of memory and learning disorder. Escape period of rats in all groups were comparatively assessed using the MWM test. In fig. 2, no significant difference in escape period was observed across all groups during the baseline test. On day 7 of administration, no significant difference was observed across all groups but Groups 4-8 were at par with one another. These showed that rats administered fisetin discovered the platform on time leading to less time spent exploring the maze. On day 21 of administration, significant decrease ($p < 0.01$, $p < 0.001$) in Groups 5-8 were observed when compared to control group. This may be attributed to the rat spending lesser time in the maze and also down to the neuroprotective effects of fisetin. The increase in values observed in Group 2 may be attributed to the detrimental effects of $AlCl_3$ on spatial learning in rats. The results were in concordance with fact that rats found it difficult to locate the escape platform resulting in longer time spent in the maze.

Further evaluation to measure comparative differences between the groups that received $AlCl_3$ alone and FST + $AlCl_3$ demonstrated a palliative role of fisetin which revealed some degree of positive variance in the FST + $AlCl_3$ groups (Fig. 2). However, it was made apparent by the significant reductions ($p <0.01$, $p< 0.001$) in escape period of rats in the FST+$AlCl_3$ groups when compared to the $AlCl_3$ treated group. The escape periods were at par between Groups 1 and 3 and also between Groups 5 and 7 with analysis showing that these two groups were identical comparatively (Fig. 2).

Fisetin cytoprotective roles against $AlCl_3$-induced cortical degeneration

This study demonstrated structural and cytological disposition of cells revealing pertinent mechanisms-arising from physical and chemical alterations that precipitate behavioral deficits in conditions such as neurodegeneration. This provides a good base to seek for therapeutic interventions that are effective. In this experiment, the Prefrontal Cortex (PFC) morphology was demonstrated using histochemical (Congo red staining) techniques.

Analysis of Congo red sections of the prefrontal cortex morphological demonstration (fig. 3) showed regular neuronal population, normal cytoarchitecture and elaborated cortical layers with healthy neurons while Congo red stain did not pick any signs of amyloid aggregates across these profiles. In contrast, the PFC sections from $AlCl_3$ treated group had several distortions in the histo-architecture across the neuropil with numerous observable signs of amyloid aggregates across the profiles. Groups 3-5 had same histoarchitectural characteristics observed when compared to the control. Group 6 had a few degenerative changes and few observable signs of amyloid aggregates within the PFC sections of rats that received fisetin after treatment with $AlCl_3$ (fig. 3f). There were notable improvements in cell sub-population, morphology and architecture in groups 7 and 8 across cortical layers when compared to Group 2. Another thing observable in these groups was the mild diffused presence of amyloid protein aggregates (yellow arrow in figs. 3g and h). These results further highlighted the neuroprotective effects of fisetin against $AlCl_3$ induced Alzheimer-like disease.

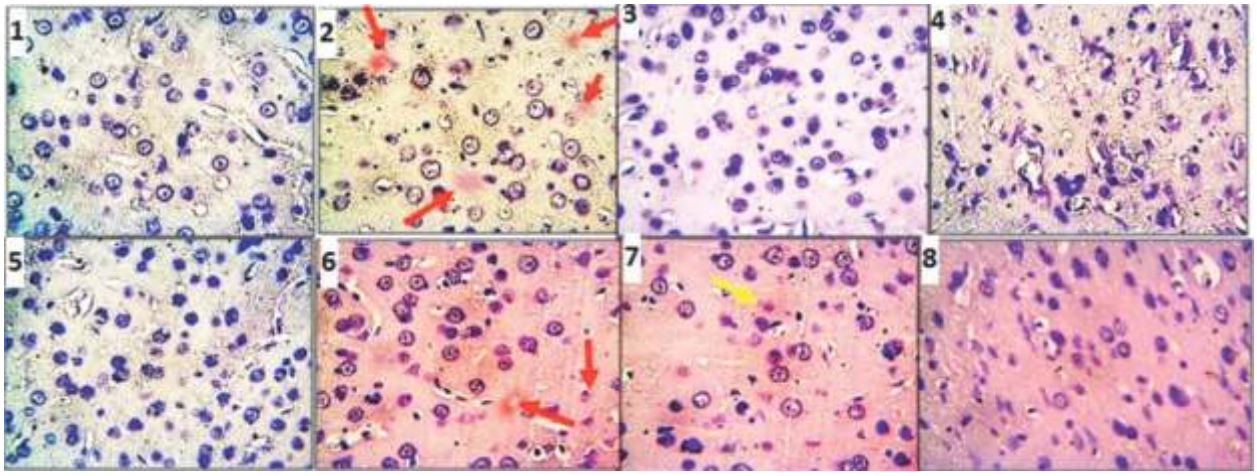

Figure 3: Showing the magnified views of the prefrontal cortex general micromorphological presentations in Wistar rats across the study groups 1-8. The external pyramidal layer (III), Internal granular layer (IV) is demonstrated across study groups. Red arrows indicate profiles with a significant appearance of amyloid aggregations, while yellow arrows indicate profiles with a mild presence of amyloid protein aggregation. Congo Red staining (scale bar- 40µm).

Discussion

The harmful side effects of clinical AD treatment drugs contribute immensely to its morbidity and mortality, thereby underscoring an urgent need for safer remedies. Reports have shown that aluminum chloride, a known chemical substance, exhibited Alzheimer-like phenotypes following chronic and acute toxicity [20-21]. It has been reported to promote chromatolysis [22], Reactive Oxygen Species (ROS) production [23], disrupt cholinergic neurotransmission [24], increase lipid peroxidation [25], trigger neuroinflammation [26], by stimulating glial activation [27] and finally promote neuronal cell death, which leads to increased anxiety, cognitive decline and memory loss. Most AD researches over the past decades have concentrated on disease-modifying treatments that will change the course of the disease rather than the symptoms alone. The lack of effective disease-modifying medications from these trials illustrates the challenges of developing a therapeutic agent that possess the ability to alter the course of the disorder as nuanced as AD. Therefore, new therapies against AD pathologies are necessary, and treatments using plant-derived natural substances have recently become a trend. Natural products extracted from medicinal plants or fruits showed promising activities in treating it by targeting multiple signaling pathways.

Flavonoids possess numerous antioxidant activities that are innocuous in the treatment of neurodegenerative disorders [28]. Recently, the immense potential of flavonoids have gained so much interest for their ability to modulate neuronal function and influence learning and memory processes [29].

Fisetin (3, 7, 3, 4-tetrahydroxyflavone), a flavonoid present in numerous fruits and vegetables possess that potential against Al induced Alzheimer-like disease in Wistar rats [30].

In this study, we showed that the processes of neurodegeneration caused by Al toxicity within the PFC and hippocampus can be reversed by the oral administration of fisetin through restoration and protection of behavioral and cellular parameters to the normal levels. Interestingly, these two parameters may provide important clues regarding the relationship between both brain structures and executive functioning while the administration of fisetin potentially provides a protective and therapeutic approach against behavioral decline and neuronal cell death aligned with different neurodegenerative disorders like Alzheimer's disease [31-32].

Evaluating the anxiolytic properties using the elevated plus maze test, this study further showed fisetin's ability to protect Al-induced anxiogenicity as shown by the data obtained from the study. Several studies that reported alterations in behavior associated with AD symptoms have similarly documented fear and anxiety as a major impediment common to all disease sufferers [31-32]. Flavonoids with potentials to protect against panic and anxious behaviours such as fisetin are described to have strong inhibitory properties against pathways involved in molecular toxicity and neurodegeneration. Evaluating spatial memory and learning in the Morris Water Maze (MWM) test showed similar results as the escape period of rats was significantly highest in the group administered $AlCl_3$ while treatment with fisetin protected against such impairment. Al toxicity causes dysfunction in cognition and learning in rodents by interfering with oxidative redox and causing severe infractions [32]. Fisetin improved memory and learning in this study by inhibiting toxicity and metabolic impairment. Neuronal excitotoxicity as a result of Al toxicity initiates several neurochemical imbalances [17], suggesting an ameliorative role of fisetin.

Neural cell structural disposition confirmed that $AlCl_3$ initiated the deposition of amyloid substances within the prefrontal sections of rat brains. Neurons in these key brain areas showed signs of cell death, hallmarked by cytoplasmic condensation, chromatolysis, nuclear degradation and amyloidosis. This was in consonance with Mishra *et al.* and Gbadamosi *et al.* [33-34] who reported that $AlCl_3$ treated rat brain showed the presence of dead and dense neuronal cells, pyramidal cells, pyknosis, vacuolation with the presence of neurofibrillary tangles and, focal hemorrhage indicating neuron degeneration. Another evidence of $AlCl_3$-induced neurodegeneration observed in this study was the damage to the dendrites and axon. These axons determine several complex operations within the nervous system that control the processing of signal of different circuits in the brain its synapses and timing. The structural alteration in the morphology of the axons caused by $AlCl_3$ administration led to the loss of signal processing and amyloid deposition in the cortex, and thus accounted for the behavioral deficits seen in the EPM and MWM tests.

Protection against cell death by fisetin observed in the groups administered fisetin + $AlCl_3$ highlighted its roles in preserving antioxidant defense system

and also the prevention of neuron bioenergetics dysregulation which is a neurodegenerative factor involved in the multiplication of amyloid beta in the extracellular space [34]. Reports observed that Al enhanced the polymerization of AβP (1–40) [35]. These results suggested that Al-aggregated AβPs have a strong affinity for membrane surfaces as a result of protease degradation. Fisetin protected neurons against the biochemical activities that activated protease enzymes which destroyed molecules responsible for cell survival. Therefore, the potentials showed by fisetin administration in the inhibition of behavioral deficits in our study may be through the protection and/or prevention against amyloidosis and synaptic dysfunction.

Conclusion

This study identified the connections between the neuroprotective potentials of fisetin in inhibiting alterations of behaviour and targets within the machineries involved in cell death. Fisetin improved the cellular degeneration and behavioral activities caused by Al toxicity within the hippocampus and PFC of adult Wistar rats. Therefore, fisetin supplements may provide a protective intervention against AD if further developed.

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