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Roles of kolaviron on the prefrontal cortex of adult female Wistar rats exposed to aluminum chloride

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ABSTRACT

Aluminium is a widely recognized neurotoxic agent that can affect the central nervous system and other parts of the body. In this study, effects of kolaviron were investigated on the prefrontal cortex of adult female Wistar rats treated with Aluminium chloride. Twenty – five adult female Wistar rats were purchased and acclimatized for 2 weeks. They were assigned into five groups, each containing five (5) rats, and administered the following respectively; Group A: distilled water, Group B: corn oil, Group C: 200 mg/kg body weight of kolaviron, Group D: 100 mg/kg body weight of Aluminium chloride and Group E: 100 mg/kg body weight of Aluminium chloride + 200 mg/kg body weight of kolaviron. Administration was done consecutively for 14 days with the use of oral cannula. Behavioural assessments (Open field test and Morris water maze) were carried out after which the animals were sacrificed by cervical dislocation and the brain excised. Immunohistochemical techniques using anti-glial fibrillary acidic protein (GFAP) antibody and tumor necrosis factor alpha (TNF-α) were also carried out. Findings revealed that the kolaviron-treated group showed improved learning and memory activities and this was supported by observations in the immunohistochemical assessments following responses to oxidative stress and inflammatory stress markers respectively.

Keywords: kolaviron, aluminium chloride, Morris water maze test, Open field test, GFAP, TNF-α

INTRODUCTION

Aluminum is the most abundant metallic element in the earth's crust[1]. It is the third after oxygen and contains 8.8% of the earth's crust which can be found in numerous amounts in rocks[2]. Aluminum can react with other metals in the environment to create different complexes[3]. Exposure to aluminum over time and its consequences has evoked a profound sense of concern in human health and disease[4] especially as it has been established that it is not essential to human metabolism at any concentration[5] but has been found to be a potential neurotoxic agent, hence harmful to brains of both humans and animals[6].

Food source, food storage, type and cooking method are some ways through which humans are exposed to various concentrations of aluminum [2]. The most common form through which this occurs is by absorption through the gastrointestinal tract[7]. It can also be absorbed through the nasal cavity when exposed for instance during mining activities, creating a direct pathway to the brain[5]. Humans can therefore ingest a significant amount of aluminum not only because of the aluminum existence in food but also as a result of cooking with aluminum utensils, food packaging with aluminum foil, and food stored in aluminum cans[4].

Aluminum has been found to be implicated in human diseases, including Alzheimer's disease (AD), autism spectrum disorder, Parkinson's disease, dialysis encephalopathy and alcohol use disorder [8]. This is made possible through its ability to cross the blood brain barrier (BBB) via transferrin receptor (TfR)–mediated endocytosis [8]. This could result in oxidative stress owing to an imbalance between antioxidants and cellular reactive oxygen species (ROS) production [9, 10]. Oxidative stress and poor long-term

memory performances were observed in oral exposure of aluminum chloride ($AlCl_3$) to rats as a result of increased concentrations of aluminum in the hippocampus and prefrontal cortex [11].

Kolaviron (KV) is an ethanolic extract of *Garcinia kola* which contains a mixture of flavonoids high in anti-oxidative and anti-inflammatory properties. It has numerous therapeutic potentials against cancer, genotoxicity and hepatotoxicity[12,13,14,15]. Kolaviron exhibits anti-oxidative properties through protection against oxidative stress induced by toxins while demonstrating favorable pharmacological effects in suppressing oxidative stress in various experimental animal models [16]. Kolaviron is protective against neurotoxicity associated with cuprizone in the cerebellum, prefrontal cortex, and hippocampus of male rats [15, 17].

The ubiquitous nature of aluminum both in foodstuffs and in the environment makes exposure to it practically impossible [18]. Therefore, there is need to pay more attention to health hazards associated with human exposure to aluminum toxicity. This research therefore investigated the roles of kolaviron on neurotoxicity associated with aluminum chloride particularly in non-pregnant female population of Wistar rats.

MATERIALS AND METHODS

Animal Care

Twenty-five (25) female Wistar rats weighing 200 - 220 g were procured from an authorized vendor (MCTEMMY Laboratory Animals Concept, Oshogbo, Nigeria) for the purpose of this study. Animals were housed in the animal holding of the Faculty of Basic Medical Science, College of Health Sciences, University of Ilorin, under a 12- hour light and 12- hour dark cycle at room temperature, in a well -ventilated environment. They were fed with standard animal pellet (Ogo-Oluwa Feeds, Ilorin, Nigeria) and water *ad-libitum*.

Seeds of fresh *Garcinia kola* were obtained locally in Ilorin, Nigeria and certified by the curator in the Department of Plant Biology, Faculty of Life Sciences, University of Ilorin, where a voucher specimen is available in the herbarium of the same institution (Specimen voucher number: UILH/001/1217). About 4 kg of the peeled seeds of *Garcinia kola* were cut into pieces and air-dried for 2 weeks at room temperature (28-30˚C). The dried seeds were pounded to fine powder with a mortar and pestle. The powdered seeds were defatted using light petroleum ether (boiling point: 40-60°C) for 48 hrs in the Laboratory of Anatomy Department. The defatted dried marc was further extracted with acetone (boiling point: 56-60°C) in a soxhlet extractor for 24 hrs. The yield was then concentrated and diluted twice its volume with distilled water and then extracted with ethyl acetate which yielded a golden yellow solid known as kolaviron. This procedure was carried out according to the method of Iwu [19] as modified by Farombi *et al.*[20] and Olajide *et al.* [21]. Purification and validation of kolaviron was determined by subjecting it to thin layer chromatography (TLC) in the laboratory of Prof. E. O. Farombi at the Drug Metabolism Unit, Faculty of Basic Medical Sciences, University of Ibadan, Nigeria. It was achieved through the use of silica gel GF 254-coated plates and solvent mixture of methanol and chloroform in a ratio 1:4 v/v. The separation revealed the presence of three bands which were viewed under UV light at a wavelength of 254 nm with RF values of 0.48, 0.71 and 0.76 respectively.

Experimental design

Twenty-five (25) female Wistar rats were assigned into five groups with five rats each: Group A: control, Group B: corn oil, Group C: Kolaviron, Group D: aluminum chloride ($AlCl_3$), Group E: Kolaviron + aluminum chloride ($AlCl_3$). Administration was done orally with an oral cannula. Each rat was carried gently from its cage with the bulk of its back lying on one palm using the fingers to carefully keep the animal in position to prevent injury and choking during administration, while the other hand was used to administer the solution with the cannula by carefully inserting it into its mouth. A 30-minute interval was given between administrations of aluminum chloride and kolaviron to the rats in group E.

Termination of study

Behavioural studies were carried out on the day of last administration to assess learning and memory and exploratory drive of the rats (using Morris water maze and open field tests). Twenty-four hours after, the rats were sacrificed by cervical dislocation. The brain was carefully excised and fixed in 4% paraformaldehyde for tissue preservation in preparation for histological and immunohistochemical analysis of the prefrontal cortex.

Behavioural studies

Open field maze test: The open field apparatus was made from plywood measuring 100×100 cm with walls 50 cm high. The floor was divided into square grids, each measuring 25 cm in length lined with a black pen, and a center square of the same length was drawn with a red marker. During the test, the rats were picked by their tails and dropped in the center square and allowed to explore for 5 minutes while a video was being captured by a camera on a fixed device. The

parameters assessed were number of lines crossed which was assessed by the frequency with which the rats crossed one of the grid lines with all four paws, fecal boli which was assessed by the total number of fecal boli count deposited in the box during the allotted time and the center square duration which was the time spent in the center square of the box.

Morris water maze: This test is done to assess spatial learning and memory in rats. A black circular pool filled with water at varied temperature was used for this investigation. It was divided into four equal quadrants as North (N), South (S), East (E), and West (W). This test was conducted in two phases, the acquisition phase with a hidden platform and the probe trial without platform. Protocol was carried out according to Zheng *et al*. [22] and Cao *et al*. [23]

Histochemical technique for histomorphology

Cresyl fast violet stain is used to demonstrate neuronal morphology and Nissl or chromatophilic bodies aggregation, to represent ribosomes of the rough endoplasmic reticulum in the cytoplasm of neurons. The reagents used for this procedure included 70% and 90% Ethanol, 2 drops glacial acetic acid in 95% ethanol, cresyl violet acetate 0.2% in acetate buffer. The slides containing the paraffin sections were made to undergo deparaffinization in 3 changes of xylene of 3 min each and then it was rehydrated in 100% alcohol for 3 min each. The sections were stained in 0.1% cresyl violet for 15 min. A quick rinse in tap water was done to remove excess stain and then washed in 70% ethanol to remove the stain. It was dehydrated in 50%, 70%, 90% and absolute ethanol for 3 mins each then cleared in xylene and mounted on DePeX and allowed to dry in the fume hood.

Immunohistochemical preparation for astrocyte expression

The non-biotin, enzymatic, one-step detection kit, ImmPRESS™ (Polymerized Reporter Enzyme Staining System) provides very high sensitivity staining with very minimal background interference in immunohistochemical applications. The reagents included ImmPRESS™ (Peroxidase) Polymer Anti-Rabbit IgG Reagent (made in horse, ready-to-use) and 2.5% Normal Animal (Horse) Serum for blocking (ready-to-use) supplied by MP-7401; Vector®Labs, USA. The ImmPRESS™ Reagent is ready to-use and it requires no mixing or tittering of the ImmPRESS™ reagent to obtain optimal immunohistochemical staining. The staining procedure was performed at room temperature. For optimal performance, the ImmPRESS™ Reagent was equilibrated to room temperature before use. Phosphate buffered saline was used as wash buffer.

Immunohistochemical preparation for tumor necrosis factor alpha

The staining was performed using the Thermo Scientific Pierce Peroxidase IHC Detection Kit (36000, Thermo Sciencific, USA) with slight modification of the procedure. Endogenous peroxidase activity was quenched by incubating tissue for 30 minutes in Peroxidase Suppressor, washed three times in Wash Buffer. Blocking buffer was added to the slides and incubated for 30 minutes. Excess buffer was blotted from the sections of the prefrontal cortex, before addition of the primary antibody, TNFα at a dilution of 1:100, and left overnight in a humidified chamber at 4°C.

Afterward, slides were washed two times for 3 minutes with Wash Buffer. The tissue sections were treated with Biotinylated Secondary Antibody and incubated for 30 minutes. The slides washed three times for 3 minutes each with Wash Buffer, treated and incubated with the Avidin/Streptavidin-HRP for another 30 minutes, and washed three times for 3 minutes each with Wash Buffer.

The tissues were incubated with Metal Enhanced DAB (3.3 diaminobenzidine) Substrate Working Solution for 5 minutes for desired staining to be achieved. The slides were rinsed with distilled water and drained. Adequate amount of Mayer's hematoxylin stain was dropped on the slide to cover the entire tissue surface and incubated for 1-2 minutes at room temperature. Hematoxylin was drained off and the slide was washed several times with distilled water. The slides were mounted with cover slips and DPX mountant.

Statistical analysis

Data were expressed as mean ± standard error of mean (SEM) using one-way analysis of variance (ANOVA) and Tukey's post hoc test using GraphPad Prism (version 8) (GraphPad Inc. USA) where $p < 0.05$ was considered statistically significant.

RESULTS

Neurobehavioural studies: open field test and Morris Water Maze

This study explored the open-field test (OFT) paradigm via assessments of parameters such as line crossing, fecal boli and center square entry. This model is known to investigate behaviours related to anxiety and locomotive impairments. Fecal boli was investigated to probe for anxiety while line crossing and center square entry were analyzed to examine locomotion and exploratory behaviours respectively.

There was no significant difference (p>0.05) seen in the frequency of lines crossed across the five groups. However, it was observed that there were more lines crossed by the treatment group when compared to the lines crossed by the $AlCl_3$ and control groups respectively. This showed that kolaviron was able to promote locomotory activities although, it was not statistically significant (p>0.05). For the fecal boli count, it was observed that the $AlCl_3$ group recorded the least number of fecal boli while the treatment group recorded the highest number. Although this was also found to be statistically insignificant (p>0.05), the kolaviron group recorded fewer boli count when compared to the control group.

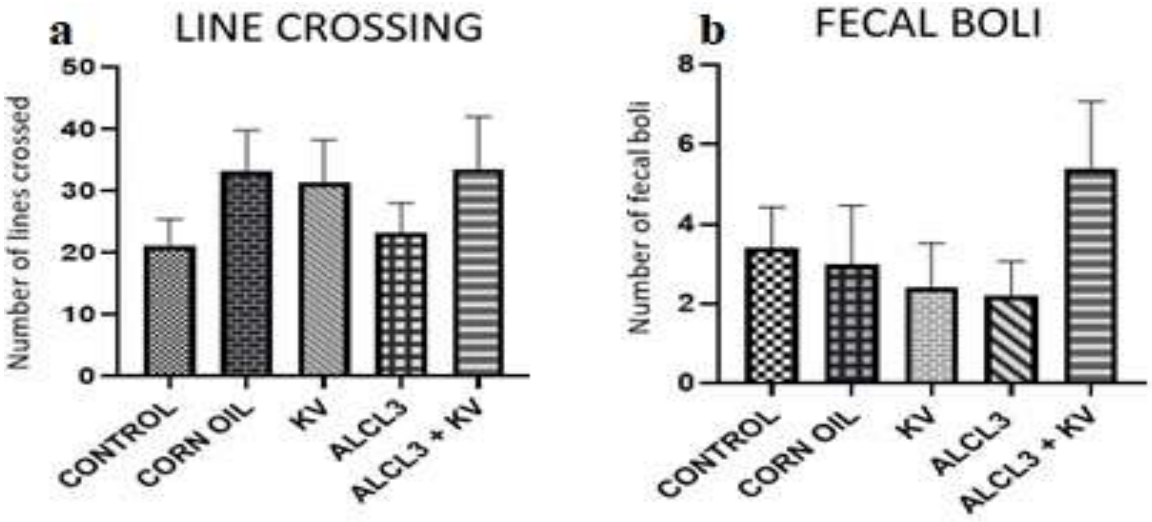

Figure 1a: Line crossing as an index of locomotor activity in female rats. There was no statistically significant difference (p>0.05) between the $AlCl_3$ group and the control group. However, there was a decrease in the number of lines crossed by rats in the $AlCl_3$ group when compared to lines crossed in the treatment group **1b:** Number of fecal boli: no significant difference (p>0.05) across the groups. Aluminium chloride had the least boli count compared to the count observed in all other groups.

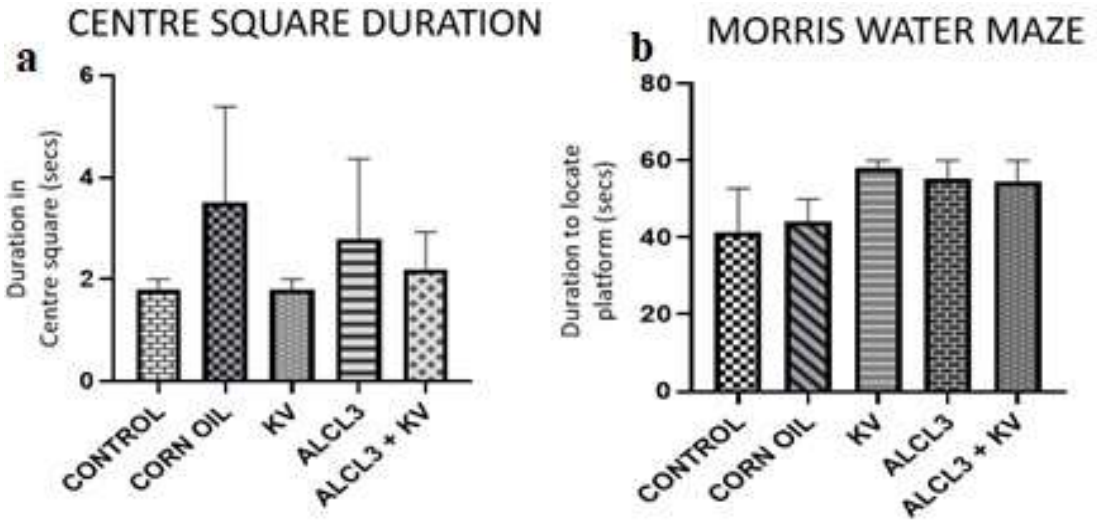

Figure 2a: Duration in centre square in the Open Field Test box. Although, the $AlCl_3$ group recorded an increase in time spent in the centre square when compared to the control, kolaviron and treatment groups respectively, there was no significant statistical difference (p>0.05) between the $AlCl_3$ group and the other groups. **2b:** Assessment of spatial memory using Morris Water Maze. An increase in time to locate the platform was observed in the $AlCl_3$ group when compared to the control group; there was no significant statistical difference (p>0.05) between the $AlCl_3$ group, the control and treatment groups.

Results from these assessments revealed that the $AlCl_3$ group recorded the longest time spent in the centre square when compared to the control, kolaviron and treatment groups (Fig. 2a). It showed that kolaviron in the treatment group was not able to minimize the time spent in the centre square when compared to the time spent by the $AlCl_3$ group, although the result was not statistically significant across all groups. Similarly, assessment of Morris Water Maze showed a similar trend as it is known to play an important role in the assessment of spatial learning and memory in rats. There was no significant statistical difference ($p>0.05$) between the $AlCl_3$ group when compared to all groups. Although an increase in time to locate the platform was observed in the $AlCl_3$ group when compared to the control group, it appeared the treatment group narrowly experienced a shorter period of time to locate the platform when compared to the $AlCl_3$ group. This very narrow margin signifies the role of kolaviron in its attempt to improve their spatial learning ability.

Histochemical evaluation of the PFC assessed the presence of Nissl substances which are expressed subject to protein synthesis in the rough endoplasmic reticulum. In this study, groups administered $AlCl_3$ showed less expression of Nissl substances hence did not stain as deeply as the control or kolaviron groups. There was very mild improvement in staining intensity in the treatment group when compared to the $AlCl_3$ group (Figure 3). Antibody against glial fibrillary acidic protein (GFAP) was used for astroglial expression. It was observed that the $AlCl_3$ group showed an increased expression of GFAP across all layers of the prefrontal cortex when compared to all other groups. Kolaviron was able to minimize GFAP expression levels slightly as the treated group showed reduced expression, hence reduced reactive astrocytes not as much as seen in the $AlCl_3$ group (Figure 3). Tumor necrosis factor alpha (TNF-α) was found to be highly expressed in the group that received $AlCl_3$ when compared to other groups. Although its activity seemed to be expressed among all groups, it appeared to be more activated around the external pyramidal and internal pyramidal layer of the tissue. Minimal expression of TNF- α was seen around the molecular and external granular layers in rats treated with kolaviron (Figure 3).

Morphological evaluation of the prefrontal cortex

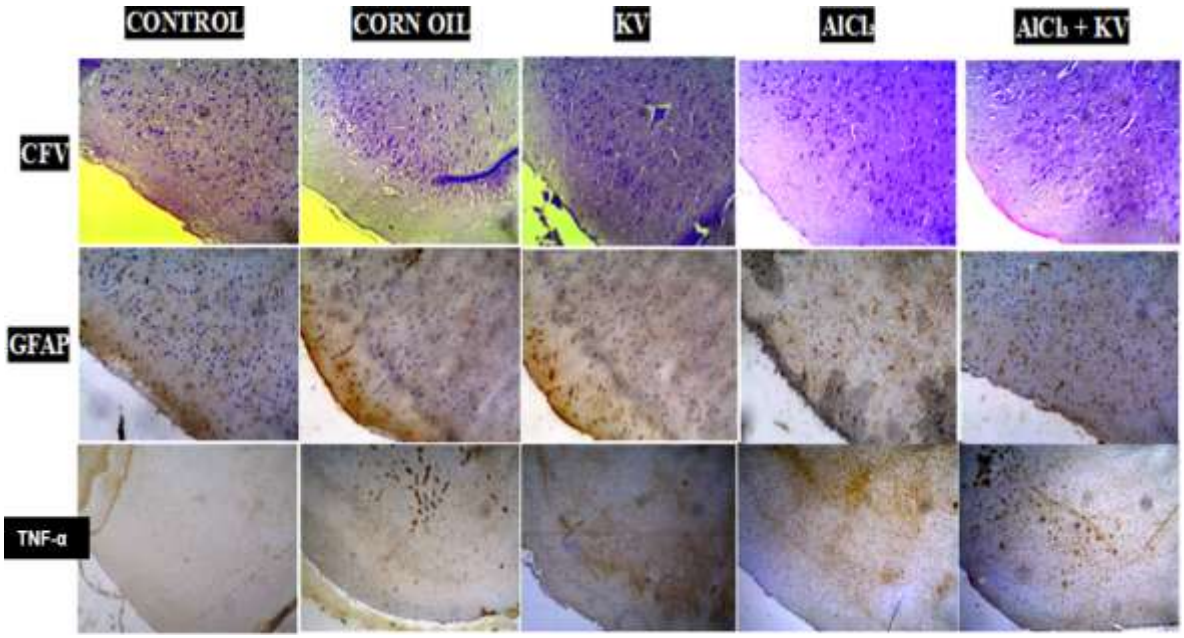

Figure 3: Photomicrographs showing histochemical/immunohistochemical changes in the prefrontal cortex of female Wistar rats. CFV: Cresyl fast violet; GFAP: Glial fibrillary acidic protein; TNF-α: Tumor necrosis factor. PFC cells showed deeply stained cells in the treated group when compared to the $AlCl_3$ group. There was increased expression of GFAP in the $AlCl_3$ group when compared to the control while there was also an increased expression of TNF-α in the $AlCl_3$ group as well when compared to the control. The KV-treated group showed a decrease in the expression of GFAP and TNF-α (mag. x 100).

DISCUSSION

Line crossing, fecal boli, time spent in the centre square and Morris Water Maze were investigated to assess locomotor activity, anxiety, exploratory behaviours and both spatial and learning memory respectively. No significant statistical difference was seen across all the parameters assessed. However, there were improvements in the kolaviron-treated group which indicated that kolaviorn was able to improve each component of the behavioural model investigated. However, it was surprising to have recorded a decrease in the time to locate the platform in the $AlCl_3$ group when compared to the kolaviron-treated group. Results from the behavioural assessments of this research suggested that kolaviron was not able to restore spatial memory as seen in the treatment group. Besides the use of female animals in this study, there might be need to carry out a dose-dependent study to determine their effects on the behavioural parameters as the present study explored the role of kolaviron at 200 mg/kg body weight. Temitayo et al. [24] reported anxiety, reduced motor functions and poor performances in line crossing, entry into the central square, central square duration, rearing frequency, stretch and attend posture in male rats administered $AlCl_3$.

Tissue sections of the control group animals showed neurons with deeply stained Nissl substances throughout the layers. There was less expression of CFV stains and loss of Nissl substance stain in most of the neurons with chromatolytic appearance in the aluminum chloride-treated rats when compared with the control, indicative of a reduction in metabolic activities. This is in agreement with Olajide *et al.* [25] who reported that the modification in the arrangement of Nissl bodies could be attributed to the use of chemicals including drugs, toxins and lack of oxygen, which influenced metabolic activities and affected protein synthesis and neural function. Reports show poorly stained neurons indicative of signs of early degeneration resulting from dissociation of ribosomes from rough endoplasmic reticulum [26]. In this present work, kolaviron was able to preserve the integrity of Nissl substance in the prefrontal cortex of female Wistar rats thereby expressing its protective properties.

Astrocytes play very vital roles within the central nervous system (CNS) such as metabolic maintenance of extracellular ionic homeostasis, modulation of neurotransmitter actions, protection of the CNS from peripheral system through the blood-brain barrier and energy support [27, 28]. Studies have extensively investigated the roles of astrocytes in supporting learning and memory formation, synaptic formation and neurogenesis. Morphological changes and synaptic invasion of astrocytes are known to be required for controlling synaptic strength [29]. Increase in GFAP expression, an inflammatory response has been reported to occur during oxidative stress [30]. An increase in reactive astrocytes was demonstrated by GFAP expression in Aluminum-induced rats in the present study. This observation was seen to be minimal in the KV-treated rats which revealed that KV improved antioxidant profiles and scavenged cytotoxic free radicals within the cells of the prefrontal cortex which may explain its ameliorative roles in astrocyte hypertrophy.

Microglial cells are the occupant pro-inflammatory cells of the central nervous system. They are usually inactive, but can be activated in response to inflammatory conditions. TNF-α is regarded as the extensively inspected pro-inflammatory mediator in the brain and influence in the pathological progress of memory and learning deficits in AD [31]. The findings of this investigation revealed an increase in the expression of TNF-α in the group treated with aluminum chloride; however, kolaviron supplementation appreciably diminished the pro-inflammatory modulator TNF-α status in the prefrontal cortex of female rats exposed to aluminum chloride.

In conclusion, the morphological, histological and immunohistochemical findings of the present study suggest the ameliorative roles of kolaviron in aluminum-induced neurotoxicity in female Wistar rats, via its anti-oxidative and anti-inflammatory properties.

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