ORIGINAL ARTICLE

Official Journal of the Neuroscience Society of Nigeria (NSN)

http://doi.org/10.47081/njn2020.11.2/001
ISSN 1116-4182

Ameliorative Effects of Kolaviron on Behavioural Deficits and Oxidative Damage in Prefrontal Cortex and Hippocampus of Cuprizone-Induced Demyelinated Mice

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Received: ……. July 2020
Accepted: ……. September 2020

ABSTRACT

Cuprizone neurotoxicity is commonly induced to mimic demyelinating disorders of the central nervous system, especially multiple sclerosis. This study assessed the role of kolaviron, a *Garcinia kola* biflavonoid, in restoring behavioural functions in cuprizone-induced neurotoxicity after termination of cuprizone treatment. Eighteen adult male Swiss albino mice aged between 6-8 weeks were randomly divided into 3 equal groups (A to C). Group A (control) mice were fed with normal rodent diet while groups B and C received 0.2% cuprizone diet for 5 weeks to induce demyelination; thereafter group B mice with cuprizone-induced demyelination were administered corn oil (0.5 mL), while group C mice with cuprizone-induced demyelination were administered kolaviron (200 mg/kg/d) for 14 days. The mice were assessed for learning, memory, anxiety and exploratory drive, and thereafter the concentration of malondialdehyde (MDA) and activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx) in the prefrontal cortex and hippocampus of the mice were evaluated. Findings revealed improved behavioural outcomes in mice treated with kolaviron compared to control and the untreated cuprizone-induced demyelinated mice. There was significant reduction in SOD and GPx activities with significant increase in MDA concentration in the untreated cuprizone-induced mice compared to controls. However, there was significant increase in SOD and GPx activities with significant reduction in MDA concentration in the kolaviron-treated cuprizone-induced mice compared to those of untreated cuprizone-induced mice. The results suggest that kolaviron may effectively ameliorate the oxidative damage and behavioural deficits associated with cuprizone-induced neurotoxicity.

Key words: *Cuprizone; Kolaviron; Oxidative stress; Lipid peroxidation; Behaviour*

INTRODUCTION

Cuprizone-induced neurotoxicity is of immense usefulness in studying the demyelination-remyelination phenomenon associated with multiple sclerosis (MS), due to its characteristic reversibility following withdrawal of the drug (Zendedel et al. 2013). Cuprizone is a copper chelating agent often formulated into diet (0.1% - 0.5% cuprizone diet) for oral consumption in experimental model and causes oligodendroglial cell death with subsequent demyelination (Silvestroff et al. 2012).

Remyelination is the process whereby new myelin sheaths are formed around axons after an initial demyelination (Hanafy and Sloane 2011). Using the cuprizone model, demyelination was completed after 5 weeks and remyelination process commenced immediately after cuprizone withdrawal (Gudi et al. 2014). In the absence of any intervention, new

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oligodendrocytes are produced from oligodendrocyte precursor cells to restore already depleted myelin sheath (Armstrong et al. 2016). It is quite possible for remyelination to occur during the course of MS lesion, although this is not adequate and does not also occur in all categories of patients with the disability (Chang et al. 2002). Hence, the potential for axonal remyelination during MS pathology differs from person to person. Some of the factors that could be responsible for impaired remyelination within MS lesions include problems associated with the processes of migration, proliferation and maturation of oligodendrocytes (Hanafy and Sloane 2011).

Natural medicinal products, including kolaviron and *Moringa oleifera* that possess antioxidant and anti-inflammatory properties, in experimental animal models of multiple sclerosis have been demonstrated to slow down the progression of the disorder (Tasset et al. 2013; Binyamin et al. 2015; Omotoso et al. 2018 a,b,c). These studies focused on using the natural antioxidants concurrently during the demyelinating process (Omotoso et al. 2018 a-c, 2019). Some of these natural products are able to scavenge free radicals while some in conjunction with this, are able to induce the antioxidant defense system *in vivo*. A typical example of these natural products which uses these dual mode of actions is kolaviron (Omotoso et al. 2018a). Kolaviron is a biflavonoid complex isolated from the seeds of *Garcinia kola* and experimental evidence showed it has anti-oxidative and anti-inflammatory properties (Farombi et al. 2004; 2009; 2013). Our previous studies demonstrated the anti-oxidative activity of 200 mg/kg body weight of kolaviron in rodents, corroborating other works that have used similar dose of kolaviron (Farombi et al. 2013; Olajide et al. 2016; Alabi et al. 2017; Omotoso et al. 2017, 2018a, 2018b). However, a study by Ayepola et al. (2013) demonstrated equal potential of kolaviron at a lower dose of 100 mg/kg body weight, while higher dose of 400 mg/kg body weight was reported to have adverse effect.

Meanwhile, there is paucity of information on the ability of kolaviron to enhance remyelination after the demyelinating agent is withdrawn. Thus, this study was set out to evaluate the activity of kolaviron on the rate of progression of remyelination after termination of cuprizone-induced demyelination.

MATERIALS AND METHODS

Ethical Approval

The study was approved by the University Ethical Review Committee, University of Ilorin, Ilorin, Nigeria.

Animal Handling

Following ethical approval, eighteen male albino Swiss mice aged between 6-8 weeks were obtained and used for the study. They were sheltered and acclimatized in the Animal Holding Unit of the Faculty of Basic Medical Sciences, University of Ilorin, Nigeria, under hygienic and favourable conditions. The mice had free access to pelletized rodent's feeds and water *ad libitum*.

Chemicals and Extraction of Kolaviron

Pelletized cuprizone diet (0.2%, 2018, Red TD 140804) was procured from Envigo®, Indianapolis, USA. *Garcinia kola* seeds were obtained from Oja-Oba market, Ilorin, Nigeria. The extraction process was carried out at the Central Research Laboratory of the University of Ilorin. The voucher number (UILH/001/1217) of the seeds of *Garcinia kola* was deposited in the Herbarium of the Department of Plant, University of Ilorin. The seeds were air-dried at room temperature and pulverized into fine powder (Farombi et al. 2009; 2013; Olajide et al. 2016; Omotoso et al. 2018a). Thereafter, the powder was treated with light petroleum ether (boiling point 40–60 ℃) in Soxhlet extractor. The defatted dried marc obtained was repacked and extracted with acetone (boiling point 56-60 ℃). The resulting product was concentrated and diluted to twice its volume with distilled water and extracted with ethyl acetate, yielding a yellow solid substance (kolaviron). The process of determination of purity and identity of kolaviron involved subjecting the extract to thin layer chromatography using silica gel GF 254-coated plates and solvent mixture of methanol and chloroform in a ratio 1:4 (v/v). Three bands were viewed under UV light at a wavelength of 254 nm with 'ratio to front' values of 0.48, 0.71 and 0.76 (Farombi et al. 2009; 2013). The structure of kolaviron is shown in Figure 1.

Animal Grouping and Drug Administration

The mice were randomly grouped into 3, with each group having 6 mice. Group A mice (control) received normal rodent chow for 5 weeks, while groups B and C mice received 0.2% cuprizone diet for 5 weeks to induce demyelination. After 5 weeks, all the groups were placed on normal rodent feed and water. In addition, group B mice were orally administered 0.5 mL of corn oil (as vehicle) (Carlini, ALDI Inc. Batavia), while those in group C were orally administered kolaviron (200 mg/kg, dissolved in 0.5 mL of corn oil) (Adaramoye et al. 2005) once daily for 14 days. Kolaviron was administered daily between 08:00 and 9.00 am.

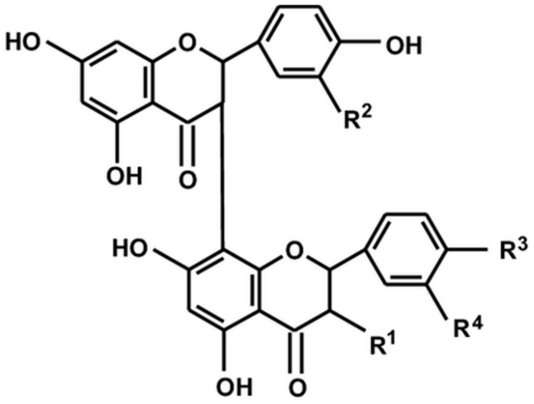
Behavioural Assessment

Learning and Memory

At the end of the treatment regimen, the learning capacity and memory indices of the experimental animals were obtained by subjecting the experimental animals to Morris water maze and Y-maze to evaluate their spatial and working memories respectively.

Morris Water Maze Test for Spatial Memory

This test was carried out to assess spatial learning and memory of mice in the different treatment groups. The procedure was done in accordance with the comprehensive description by Vorhees and Williams (2006). Briefly, the test was carried out with a water tank measuring 0.9 m in diameter and 0.6 m in depth. The water-filled tank was divided into four quadrants; North-East (NE), North-West (NW), South-East (SE) and South-West (SW) with an escape platform placed one inch deep at the centre of the NW quadrant. The animals were trained three days prior to the test day. During the training, the animals were placed in each of the other three quadrants (NE, SE and SW) for a maximum period of sixty seconds to find the escape platform. Animals that were unable to find the escape platform were guided to the escape platform. This process was repeated for another two days. On the test day, the animals were placed once in each of the three quadrants maximum time of one minute and the time taken to find the escape platform was recorded as the escape latency period. Animals that were unable to find the escape platform within sixty seconds were removed and the escape latency period was recorded as 60 s.



	R1	R2	R3	R4
GB1	OH	H	OH	H
GB2	OH	H	OH	OH
Kolaflavanone	OH	H	OCH_3	OH

Fig. 1: Chemical Structure of Kolaviron (Farombi et al. 2013)

Y maze Test for Working Memory

This test was used to examine working and cognitive memory in mice (Kim et al. 2013). The animals were placed in a Y-maze (without training, reward or punishment) which arms measured 75 cm in length and 15 cm in breath with an angle of 120º in between arms. The animals were allowed to explore the maze for duration of 10 min. The manner of alternation was recorded. The percentage correct alternation of each mouse was estimated as a ratio of the correct alternation to the total alternation multiplied by 100.

Anxiety and Exploratory Drive

Anxiety and exploratory drive test was carried out as previously described by Gould et al. (2009), using the open field apparatus. The apparatus was made from a plywood measuring 100 cm × 100 cm with walls 50 cm high. The floor was divided into square grids each measuring 25 cm in length with a blue marker and a centre 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 centre square and allowed to explore for 5 min, while the video was captured by a camera placed above the apparatus. Six behaviours were scored: the number of lines crossed, centre square entry, centre square duration, freezing duration, rearing frequency and stretch attend posture frequency.

Preparation of Homogenates of Brain Tissues and Biochemical Analysis

At the end of the experiments, the animals were sacrificed by cervical dislocation; each brain was removed and the prefrontal cortex and hippocampus were excised. Equal weighing brain tissues were homogenized in 0.25 M sucrose solution (1:5, w/v) with an automated homogenizer at 4 °C. The homogenates were frozen overnight to allow complete cell lysis and maximum release of enzymes (Akanji et al. 1993). The tissue homogenate was centrifuged for 10 min in a microfuge at 12,000 rpm to obtain the supernatant containing organelle fragments and synaptosomes. The supernatants were aspirated into plain labelled glass cuvette placed in ice. SOD (KT-60703), GPx (MBS-744364) activities and MDA (MBS-9389391) concentration in the supernatants of prefrontal cortex and hippocampus were assayed using spectrophotometric techniques (Thiha and Ibrahim 2015).

Statistical Analysis

All quantitative data were analysed using the GraphPad Prism® software (version 6). SOD, GPx, and MDA outcomes were plotted in analysis of variance (ANOVA) with Tukey's multiple comparisons test. Significance was set at $p < 0.05$ or 0.01. The outcomes were represented in bar charts with error bars to show the mean and standard error of mean (SEM).

RESULTS

Kolaviron Ameliorates Cuprizone-Induced Behavioural Deficits after Cuprizone Withdrawal

The findings from the behavioural analysis revealed that the untreated demyelinated mice exhibited a

significantly higher ($p<0.05$) escape latency (long-term memory index) period after 2 weeks post-demyelination compared to control (Fig. 2). The results also revealed that kolaviron significantly ($p<0.05$) reduced the escape latency period of mice that were treated with kolaviron for two weeks post-demyelination compared to the untreated demyelinated mice, restoring it to the range of control ($p>0.05$). However, the untreated demyelinated mice and kolaviron-treated demyelinated mice had percentage correct alternation which were not significantly different ($p>0.05$) from the control. The behavioural scores for the number of lines crossed, centre square entry, centre square duration and rearing frequency were significantly reduced ($p<0.05$) while the stretch-attend posture frequency and freezing duration of the untreated demyelinated mice were significantly increased ($p<0.05$) compared to controls (Fig. 3). The results revealed that kolaviron was able to revert the observed alteration in these parameters caused by cuprizone-induced demyelination to the range of controls ($p>0.05$).

Biochemical Parameters

Assessment of MDA level was used to determine the degree of lipid peroxidation. In this study, the results revealed that there was no significant alteration ($p>0.05$) in MDA concentrations in the prefrontal cortex and hippocampus of the untreated cuprizone-induced demyelinated mice and the kolaviron-treated cuprizone-induced demyelinated mice compared to controls (Fig. 4).

There was significant reduction ($p<0.05$) in SOD activities in both the prefrontal cortex and hippocampus of untreated cuprizone-induced demyelinated mice compared to those of kolaviron-treated cuprizone-induced mice and controls (Fig. 5). However, there was no significant change ($p>0.05$) in SOD activities in both the prefrontal cortex and hippocampus of kolaviron-treated cuprizone-induced mice compared to controls.

Glutathione peroxidase activities in the prefrontal cortex and hippocampus of the untreated demyelinated mice and the kolaviron-treated demyelinated mice were not significantly altered ($p>0.05$) compared to controls (Fig. 6).

DISCUSSION

The search for novel drugs for the treatment of MS has led to the development of a broad range of animal models of demyelination (Blakemore and Franklin 2008; Geurts and Barkhof 2008; Guo et al. 2018; Mecha et al. 2019). Nevertheless, the intricacy of MS pathogenesis makes none of these models reflect the whole spectrum of MS. The chemical model is the most used of the demyelination experimental models because of the predictable kinetics. Compared to other models, cuprizone model is remarkably appreciated in appraising and developing new re-myelination therapeutic approaches (Blakemore and Franklin 2008; Acs et al. 2013; Harlow et al. 2015; Ghaiad et al. 2017; Omotoso et al. 2018a, b). Several studies have reported various therapeutic approaches to the enhancement of re-myelination, such as enhancement of intrinsic antioxidant defence system (Hanwell and Banwell 2011; Tavakoli-Yaraki et al. 2018), induced neurotrophin recruitment (Zhang et al. 2011), immunomodulation (Hanafy and Sloane 2011) and modulation of intrinsic signalling pathways (Harlow et al. 2015). It is believed that any natural or synthetic product which can enhance re-myelination through any of these mechanisms will be of tremendous benefit in the treatment of MS.

Exploration of phytochemicals with medicinal properties and elucidating the molecular mechanisms underlying their pharmacological activities have been very important approaches in drug discovery which has continued to gain grounds over the past few decades (Szabados et al. 2004; Balunas and Kinghorn 2005; Harvey 2008; Libro et al. 2016). In the present study, kolaviron, which has been documented to have numerous medicinal properties (Farombi and Owoeye 2011; Adedara et al. 2015; Olajide et al. 2016), was evaluated for its potential to induce re-myelination through enhancement of intrinsic antioxidant defence system.

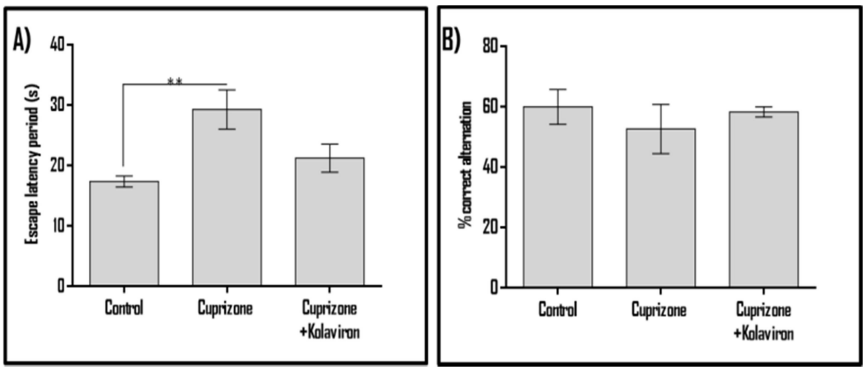


Fig. 2: Neurobehavioural Outcomes of Experimental Animals in the Morris Water (A) and Y- mazes (B). Cuprizone-treated mice had a significantly high escape latency period in the Morris water maze when compared to the control ($p<0.01$) and kolaviron-treated animals ($p>0.05$). For the Y-maze test, the cuprizone-treated mice presented with a lower percentage correct alternation relative to the control group and the group post-treated with kolaviron ($p>0.05$). ** is significant value of $p<0.01$. Control= regular diet for 5 weeks; Cuprizone group= cuprizone (5 weeks) + corn oil (2 weeks); Cuprizone + Kolaviron = cuprizone (5 weeks) + kolaviron (2 weeks).

Hippocampal demyelination is directly linked to cognitive impairment in MS patients (Geurts et al. 2007; Geurts and Barkhof 2008; Dutta et al. 2011). The results of this study also revealed that cuprizone treatment caused reduced escape latency of experimental animals suggesting impaired spatial memory, which has been reported to occur essentially through demyelination (Kouts-oudaki et al. 2009; Norkute et al. 2009), thus corroborating earlier reports (Geurts and Barkhof, 2008; Dutta et al. 2011). Functional re-myelination in the present study was inferred from behavioural analysis of the experimental animals treated with or without kolaviron following due demyelination. Memory enhancing properties of kolaviron have been extensively documented (Ishola et al. 2017; Omotoso et al. 2018b; 2019). The mice with cuprizone-induced demyelination treated with kolaviron had their escape latency and percentage correct alternation reverted to the range of controls, suggesting that kolaviron was able to enhance the spatial and working memories of the mice compared to the untreated demyelinated mice. It also suggests that kolaviron enhanced restoration of long- and short -term memory indices of the mice.

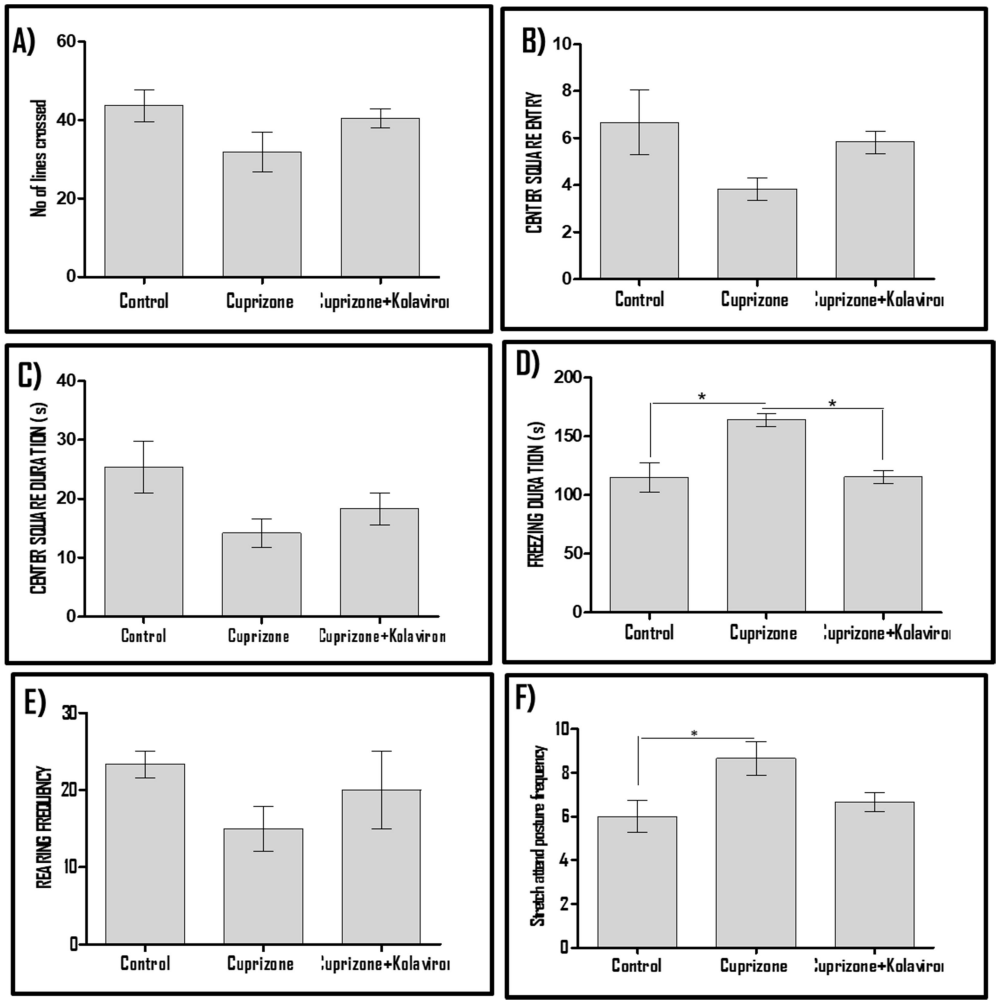


Fig. 3: Behavioural analysis of mice in the open field behavioral paradigm. Figures A to F represented number of lines crossed, center square entry, center square duration, freezing duration, rearing frequency and stretch attend posture frequency respectively. The behavioral scores for the number of lines crossed, center square entry, center square duration and rearing frequency were relatively higher in mice that received kolaviron post-cuprizone treatment relative to the untreated cuprizone-induced mice ($p>0.05$). The untreated cuprizone-induced group had a significantly higher freezing duration and stretch attend posture frequency relative to the control and kolaviron-treated group ($p<0.05$). * Significantly different at $p<0.05$.

Anxiety is one of the core clinical manifestations in patients suffering from demyelinating diseases (Simpson et al. 2016). High freezing duration and stretch-attend posture frequency are open field test parameters used to assess the presence of anxiety in rodents. In this study, untreated cuprizone-induced demyelinated mice had higher freezing duration and stretch-attend posture frequency compared to controls, suggesting anxiety in the animals. This finding corroborates previous reports on the anxiogenic properties of cuprizone (Franco-Pons et al. 2007; Zhang et al. 2013). The results also revealed that kolaviron effectively reversed the increased anxiety indices in demyelinated mice to the range of controls. This finding suggests that kolaviron did not only enhance quick and functional re-myelination, it also exhibited anxiolytic properties that counter-balanced cuprizone-induced anxiety. Spatial exploration indices were reduced in the untreated demyelinated mice compared to controls, suggesting cuprizone-induced perturbation

of spatial navigation. Treatment with kolaviron restored the spatial exploration indices to the range of controls.

Oxidative stress has been implicated in the pathogenesis of demyelinating diseases (Offen et al. 2004; di Penta et al. 2013; Wang et al. 2014; Lassmann and van Horssen 2016; Ghaiad et al. 2017). SOD and GPx are key antioxidant enzymes that scavenge superoxide radical and hydrogen peroxide respectively (MatÉs et al. 1999; Chiang.et al. 2006; Gbadamosi et al. 2016). Cuprizone is known to reduce the activities of these enzymes and mediate a cascade of biochemical reactions that lead to superfluous generation of reactive oxygen species, which culminates in lipid peroxidation (Omotoso et al. 2018a, b). MDA is a product derived from the peroxidation of polyunsaturated fatty acids and it is commonly used to assess lipid peroxidation (Gentile et al. 2017). In the present study, the MDA concentrations and GPx activities were not significantly altered in the untreated demyelinated mice and kolaviron-treated demyelinated mice compared to controls. However, SOD activities in the prefrontal cortex and hippocampus were significantly reduced in untreated demyelinated mice, but not changed in kolaviron-treated demyelinated mice compared to controls. These results suggest that in the absence of any intervention, withdrawal of cuprizone treatment does not immediately resolve the reduction in SOD activity which characterizes cuprizone neurotoxicity. However, kolaviron was able to reverse SOD activity to the range of control, thus

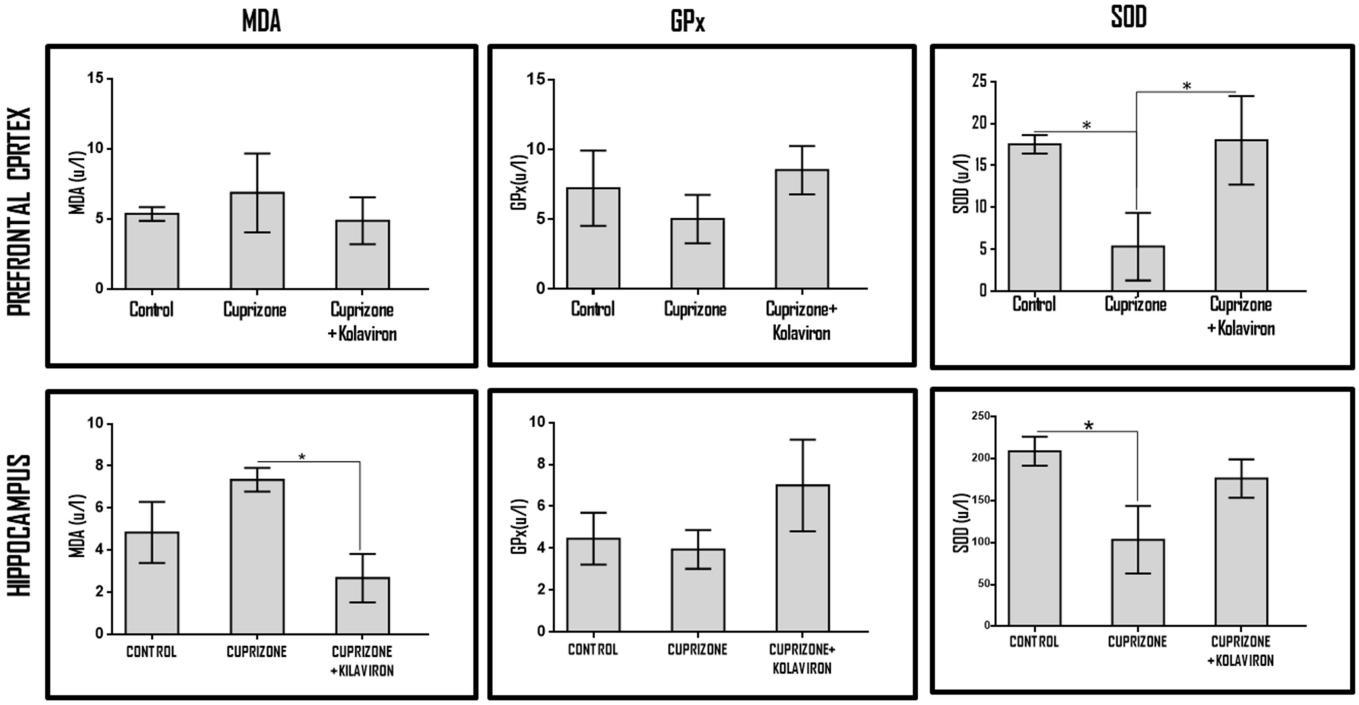


Fig. 4: Effects of kolaviron on malondialdehyde concentrations in the prefrontal cortex and hippocampus of cuprizone-induced demyelinated mice. MDA remained elevated in the prefrontal cortex and hippocampus of mice exposed to cuprizone for 5 weeks followed by regular diets for 2 weeks (cuprizone group), while mice that received kolaviron for 2 weeks immediately post-cuprizone treatment (cuprizone+kolaviron group) had the least levels of MDA in both prefrontal cortex and hippocampus ($p>0.05$). * Significantly different at $p<0.05$.

Fig. 5: Effects of kolaviron on superoxide dismutase activities in the prefrontal cortex and hippocampus of mice. SOD was reduced in untreated cuprizone-induced mice (cuprizone group) compared with kolaviron-treated cuprizone-induced mice (cuprizone + kolaviron) ($p<0.05$). *Significant difference at $p<0.05$.

Fig. 6: Effects of kolaviron on glutathione peroxidase activities in the prefrontal cortex and hippocampus of cuprizone-induced demyelinated mice. Both prefrontal cortex and hippocampus had reduced levels of GPx in untreated cuprizone-induced mice (cuprizone group), but increased values in kolaviron-treated cuprizone-induced mice (cuprizone + kolaviron) ($p>0.05$).

enhancing the first line of enzymic antioxidant defence to combat the oxidative stress induced by cuprizone.

The results of this study therefore suggests that kolaviron possesses the potential of enhancing re-myelination in MS subjects by inducing antioxidant defence system, thereby alleviating memory and behavioural deficits associated with MS.

Acknowledgment

The authors are grateful for the Institutional-based Intervention Research Grant received from the Tertiary Education Trust Fund through the University of Ilorin, Ilorin, Nigeria, to carry out the study.

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Cite as: Omotoso, G.O., Adebayo, J.O., Olajide, O.J., Gbadamosi, I.T., Enaibe, B.U., Akinola, O.B. and Owoyele, B.V. (2020). Ameliorative effects of kolaviron on behavioural deficits and oxidative damage in prefrontal cortex and hippocampus of cuprizone-induced demyelinated mice.. Nig. J. Neurosci. 11(2):53-61. http://doi.org/10.47081/njn2020.11.2/001