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Cuprizone toxicity and *Garcinia kola* biflavonoid complex activity on hippocampal morphology and neurobehaviour

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ABSTRACT

Cuprizone-induced neurotoxicity has been employed to study the biology of remyelination in experimental models of multiple sclerosis. This study was aimed at determining the role of kolaviron, a biflavonoid from *Garcinia kola*, in mitigating the damaging effects of cuprizone on behaviour and the hippocampus. Twenty-four male albino mice aged 6–8 weeks were categorised into 4 equal groups: Group A (Control) received regular diet; Group B received 200 mg/kg/d of kolaviron in addition to their regular diet; Group C received 0.2% cuprizone diet only, while Group D received both kolaviron and cuprizone diet. The treatment lasted for 35 days after which behavioural tests (Morris water maze, Y maze and open field tests) were conducted and brain tissues were processed for histology, histochemistry (Nissl staining), immunohistochemistry (glial fibrillary acidic protein) and biochemistry (malondialdehyde, superoxide dismutase and glutathione peroxidase). Results showed that cuprizone toxicity led to weight loss, impairment in memory and exploratory drive, oxidative stress, chromatolysis and reactive astrocytosis; meanwhile administration of kolaviron prevented cuprizone-induced weight loss, memory decline, oxidative stress and neuromorphological alterations. In conclusion, administration of kolaviron might be useful in limiting the effects of cuprizone toxicity on the morphology and functions of the hippocampus.

1. Introduction

Cuprizone is a copper chelator and one of the neurotoxicants commonly used to induce demyelination, thereby providing the opportunity to study the biology of remyelination (Blakemore and Franklin, 2008). Cuprizone-induced demyelination is characterised by oligodendrocyte loss and microglial activation, with subsequent initiation of a cascade of signals that stimulates astrocytes to express signaling molecules which contribute to demyelination (Hibbits et al., 2012; Kang et al., 2012).

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) characterised by demyelination and neurodegeneration (Calabrese et al., 2009). The associated depletion in myelin is usually due to the dysfunction of oligodendrocytes, the cells responsible for myelin production in the CNS, or as a result of direct damage to myelin through immune-mediated processes (Mahad et al., 2008). MS is a devastating disorder with the global prevalence varying between 20 and 150 per 100,000, and affects commonly the young adults (Rosati, 2001).

Although the aetiopathogenesis is not well understood, MS is regarded as an inflammatory disorder, controlled by T cell-mediated autoimmune reaction against the myelin sheath (Maña et al., 2009). However, aside the effect on the white matter constituted by myelin, the gray matter is also affected, causing major cortical changes and tissue loss, leading to varying degrees of impairment in cognitive functions (Calabrese et al., 2009; Geurts and Barkhof, 2008).

Garcinia kola Heckel of the family *Guttiferaceae*, called by different names such as kola bitter, bitter kola (due to its characteristic bitter and resinous taste), false or male kola, is commonly consumed locally (Adegbehingbe et al., 2008). The seed is eaten raw for its acclaimed health benefits and for the belief that it promotes longevity (Adaramoye, 2012). Phytochemically, the extract of the seed of *G. kola is rich in* phenolic acids, flavonoids, vitamin C, cardiac glycosides, tannins and saponins (Ogunmoyole et al., 2012; Stanley et al., 2014). The

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antimicrobial nature of *G. kola* seed has been attributed to the benzophenone and flavonones components of the plant while the anti-inflammatory effect is believed to result from the inhibition of the cyclooxygenase enzyme (Stanley et al., 2014). The pharmacodynamic mechanism of *G. kola* action is anchored on kolaviron.

Kolaviron is a biflavonoid complex found in *G. kola* (Ijomone and Obi, 2013) and is responsible for the strong antioxidant properties of *G. kola* which limits the oxidative conversion of amino acid by reactive oxygen species to other damaging fatty acid products (Adaramoye et al., 2005). Various studies have highlighted the numerous health benefits of kolaviron. The study by Adaramoye (2012) showed that oral administration of kolaviron reduced hyperglycaemic indices associated with streptozotocin-induced diabetes in rats. It has a hypocholesterolaemic effect, thereby conferring on it an anti-atherogenic property in rats fed on cholesterol diet (Adaramoye, 2012). A few works have explored the antioxidative potential of kolaviron in preventing neurotoxicity. In their study, Ijomone and Obi (2013) reported that kolaviron inhibited acetylcholinesterase activities in the brain, and therefore suggested that it could have a place in the management of neurodegenerative disorders especially those associated with cholinergic dysfunction. Ijomone et al. (2012) and Ojo et al. (2019) also described the protective roles of kolaviron in mitigating neurotoxicity especially via its antioxidant activities. We also observed from our recent study that prefrontal cortical neurotoxicity due to cuprizone intoxication was ameliorated by the use of kolaviron by enhancing the intrinsic antioxidant mechanisms (Omotoso et al., 2018a).

Various works have used different doses of kolaviron, such as 100 mg/kg, 200 mg/kg and 400 mg/kg (Ayepola et al., 2013; Farombi et al., 2013, 2018; Olajide et al., 2016; Alabi et al., 2017; Omotoso et al, 2017, 2018a, 2018b). Many of these studies reported that both 100 mg/kg and 200 mg/kg doses have beneficial effects, with effective antioxidant and anti-inflammatory properties, amongst other potentials (Ayepola et al., 2013; Farombi et al., 2013, 2018; Olajide et al., 2016; Omotoso et al., 2018a, 2018b), but no reference was made to adverse effects of kolaviron on body organs at these doses. However, with the use of doses as high as 400 mg/kg, Alabi et al. (2017) reported detrimental effects on body weights, liver morphology and biochemistry as well as oxidative status. The current work explored the possibility of a 5-week cuprizone-induced neurotoxicity and intervention using kolaviron, on the pattern of weight change, cognitive function, oxidative status and morphology of hippocampus. This study is critical to show experimental evidence that supports the ability of kolaviron to mitigate neurodegenerative changes associated with cuprizone toxicity in mice hippocampus.

2. Materials and methods

2.1. Animal care and use

The study was approved by the University Ethical Review Committee. Twenty-four male albino mice aged 6–8 weeks were used. They were sheltered in the Animal House of the Faculty of Basic Medical Sciences, University of Ilorin, under hygienic and favourable conditions. Feeds and water were provided *ad libitum*.

2.2. Isolation of kolaviron from Garcinia kola

Garcinia kola seeds were obtained from the central market in Ilorin, Nigeria. Seeds of *Garcinia kola* deposited in the Herbarium of Plant Biology Department of the University of Ilorin have the voucher number UILH/001/1217. The seeds were air-dried at room temperature and pulverized into fine powdery form (Farombi et al., 2009; Olajide et al., 2016; Omotoso et al., 2018a). This was then extracted with light petroleum ether in Soxhlet extractor which was placed in an electric water bath. The defatted, dried marc was then repacked and extracted with acetone in the water bath. The extract was concentrated and diluted to twice its volume with distilled water and extracted with ethyl acetate. The concentrated ethyl acetate fraction gave a yellow solid substance, that is, 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 can be viewed under UV light at a wavelength of 254 nm with Rf (ratio to front) values of 0.48, 0.71 and 0.76.

2.3. Cuprizone diet

Cuprizone diet (0.2%, 2018, Red TD 140804) was procured from Envigo®, Indianapolis, USA. Two groups of mice, as shown in the experimental design below received solely cuprizone diet throughout the experimental period.

2.4. Experimental design

The mice were grouped into 4, with each group having 6 mice. Group A: Control, received regular diet; Group B: received kolaviron (200 mg/kg/d) (Omotoso et al., 2017, 2018a; 2018b) in addition to their regular diet; Group C: received 0.2% cuprizone diet only; while Group D: received a combination of kolaviron (200 mg/kg/d) and 0.2% cuprizone diet. The mice were monitored closely throughout the 35 days treatment.

2.5. Behavioural assessment

Prior to termination of the experiment, the mice underwent neurobehavioural tests to study learning and memory using Morris water maze and Y maze tests (Kim et al., 2013; Vorhees and Williams, 2006) and, fear and anxiety using the open field apparatus (Kenney and Gould, 2008; Gould et al., 2009).

2.6. Morris water maze test

Morris water maze (MWM) was used to assess spatial learning and memory of mice. 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 3 ft in diameter and 2 ft 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 in the centre of the NW quadrant. During the training sessions prior to the main test, the mice were placed in each of the other three quadrants (NE, SE and SW) for a maximum period of 60 sec to find the escape platform. Those that were unable to find the escape platform were guided to the escape platform. On the test day, they were placed once in each of the three quadrants for a maximum time of 1 min and the time taken to find the escape platform was recorded as the escape latency period. Those that were unable to find the escape platform within 60 s were removed and the escape latency period was recorded as 60 s.

2.7. Y maze test

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

2.8. Open field test (OFT)

The open field apparatus (Kenney and Gould, 2008; Gould et al., 2009) was constructed using plywood measuring 100 cm by 100 cm and

height of 50 cm. 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 using a red marker. Each mouse was picked by the tail and dropped in the centre square and allowed to explore for 5 minutes while the video was captured by a camera from above the apparatus. Five behavioural patterns were scored: number of lines crossed, centre square entry, centre square duration, rearing frequency and stretch attend posture. The number of lines crossed was the frequency with which the mouse crossed one of the grid lines with all four paws; the centre square entry was the frequency with which the mouse crossed one of the red lines with all four paws into the central square; the centre square duration was the total time spent in the center square; the rearing frequency was the number of times the mouse stood on its hind limbs; while the stretch attend posture was the frequency with which the mouse demonstrated forward elongation of the head and shoulders followed by retraction to the original position.

2.9. Preparation of Brain Tissue for Histology, Histochemistry and Immunohistochemistry

At the end of the experiments, the animals were anaesthesised with intramuscular ketamine, and transcardially perfused with 4% paraformaldehyde (PFA). The brain was removed and the hippocampus was excised and processed for tissue histology using haematoxylin & eosin and cresyl fast violet stains (Fallone et al., 1995; Kádár et al., 2009). Serial sections (10 μ) of embedded hippoccampal tissue were obtained for immunohistochemistry and the protein cross-linkages were removed using 0.1% trypsin to retrieve the antigens. Hydrogen peroxide was used to block endogenous peroxidase, while 5% bovine serum albumin (BSA) was used to reduce nonspecific protein reactions. Primary antibody (anti-glial fibrillary acidic protein: GFAP) dilution was done in blocking buffer (10% calf serum with 1% BSA and 0.1% Triton X-100 in 0.1 M PBS) at 1:100. Thereafter, secondary biotinylated antibody was desalted and diluted in PBS (pH 8.0) and applied on tissue sections. The sections were incubated; immunogenic reaction was developed using 3,3′-diaminobenzidine and intensified using methenamine silver kit (used according to manufacturer's instruction). The sections were counterstained in haematoxylin, and subsequently treated in 1% acid alcohol to reduce the counter-stain intensity (Acs and Kalman, 2012).

2.10. Biochemical studies

Fresh brain tissues were also collected into cold sucrose solution to assess lipid peroxidation using malondialdehyde (MDA) and the activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx) enzymes, as markers for oxidative stress. Biochemical assay kits for MDA (MBS-9389391), GPx (MBS-744364) and SOD (KT-60703) were used. Carefully dissected hippocampus (in 0.25M sucrose at 4 °C) from mouse brain were weighed and pulverised in ice with the aid of an automated homogeniser. Lysates from each brain regions were centrifuged for 10 min in a cold microfuge (IEC: CENTRA-GP8R, DJB Labcare, UK) at 12,000 rpm to obtain supernatants containing organelle fragments and synaptosomes. The supernatants were aspirated into plain labelled glass cuvette placed in ice to determine MDA level and GPx and SOD activities according to the manufacturer's instruction in the assay kit pack. For MDA: 1 ml of supernatant was added to 2 ml of MDA reagent and boiled at 100 °C for 15 min. The reaction mixture cooled down to room temperature and then centrifuged to obtain the supernatant. The supernatant absorbance was read at 532 nm against blank. MDA was calculated using the molar extinction coefficient for MDA-thiobarbituric acid-complex of 1.56×10^5 M cm. For GPx: 0.1 ml of the supernatant was added to 3.0 ml of glutathione peroxidase substrate solution in a test cuvette. 0.5 ml of H_2O_2 was then added and mixed. The change in absorbance was recorded every 30 seconds up to 3 minutes in a spectrophotometer adjusted to read at 430 nm. One unit of peroxidase was defined as the change in absorbance/minute at 430 nm using the coefficient of 0.00622 $Nm^{-1}cm^{-1}$. For SOD, 20 μL of standard and supernatants (test samples) were added to SOD-specific antibodies pre-coated well plates. 20μL of SOD horseradish peroxidase (HRP)-conjugated detection antibody were then added to the microplate wells and incubated at room temperature for 45 min. After the removal of unbound proteins by washing, 3,3′,5,5′-Tetramethylbenzidine (TMB) was added and catalyzed by HRP to produce a blue colour product that changes to yellow after addition of an acidic stop solution. The plate absorbance was then read at 430 nm. A standard curve was plotted and the values of SOD in the test samples were obtained from the standard curve.

2.11. Statistical analysis

Statistical analysis was performed by one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test using the GraphPad Prism® software. The data were reported as means ±SEM, while differences between means at $p < 0.05$, 0.01 or 0.005 were considered significant.

3. Results

3.1. Kolaviron prevents excessive loss of body weight induced by cuprizone

The first one week of the control mice was characterised by a rapid increase in body weight as against the three treatment groups that had weight reduction of different degrees. However, after the initial rapid increase, weight increase became gradual and progressive till the termination of the experiment (Fig. 1A). Cuprizone consumption led to a marked decrease in body weight within the first two weeks of treatment compared with the control and other treatment groups, followed by a week of minimal change in weight; the last two weeks (week 4 and 5) was characterised by a gradual increase in the body weight, which however did not attain to the initial weight at the onset of experiment; hence, loss of weight. Kolaviron-treated mice showed consistent increase in body weight compared with other groups, while the mice co-treated with cuprizone and kolaviron had slight decrease in weight in the first week, followed by a sudden increase in the second week which was maintained till the end of the third week. Thereafter, there was reduction in weight for the next one week, while the last one week (week 5) was characterized by another episode of rise in body weight beyond the initial body weight at the onset of the experiment. The weights gain was estimated as a difference between the final weight and initial weight (Fig. 1B). Weight gain over the 35 day treatment was most prominent in kolaviron -treated mice, with significant difference from the cuprizone-treated mice; those co-treated with cuprizone and kolaviron also gained weights compared with the cuprizone group, though not as high as in mice given kolaviron only.

3.2. Kolaviron mitigates cuprizone-induced perturbation of hippocampal-dependent spontaneous alternation, learning and memory activities.

Two neurobehavioural paradigms - Y-maze and Morris water maze (MWM), were used to quantify short- and long-term memory in the present study (Fig. 2). In the MWM, the escape latency period was used to describe the learning and memory status (Fig. 2A). Analysis revealed that cuprizone treatment led to a significantly higher escape latency period when compared to the control ($p < 0.01$) and kolaviron-treated ($p < 0.01$) mice. This showed that cuprizone caused a decline in the long-term memory index. Meanwhile, kolaviron treatment prevented this alteration as we observed that co-treatment with cuprizone and kolaviron resulted in a better long-term memory index which was significantly lower than that of the cuprizone-treated mice ($p < 0.05$). Observation from spontaneous

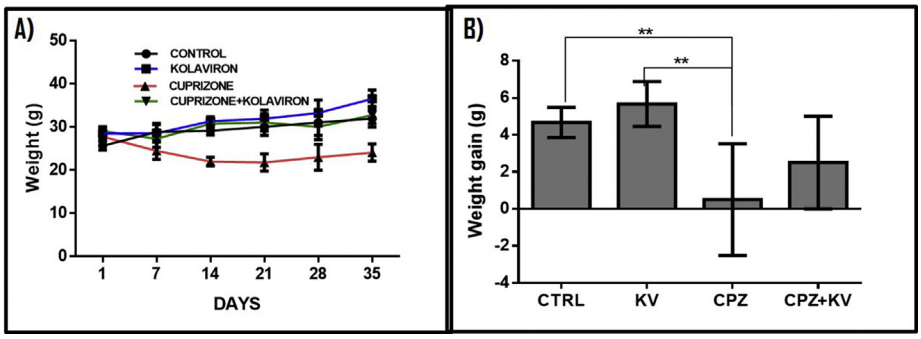

Fig. 1. Changes in the body weight of mice over a period of 35 days. In Figure A, the control mice had an initial rapid increase in body weight within the first 7 days (compared to all other treated groups that had reduction in body weights during the same period), followed by a less rapid but progressive weight increase till the end of the experiment. Kolaviron treatment caused only very slight body weight reduction in the first 7 days (compared to mice co-treated with cuprizone and kolaviron, and cuprizone group which had the lowest body weight), and the weight increased after day 7 till day 35. Body weight was highest in kolaviron-treated mice compared to other groups. Mice treated with cuprizone suffered a sharp reduction in body weight between the 1st day and day 14 while the weight remained relatively constant thereafter till day 21 and then increased slightly till day 35. However, the weight remained consistently lower than body weights of other groups. In mice co-treated with cuprizone and kolaviron, body weight dynamics showed an initial decrease in the first 7 days, then an increase in the next 7 days, followed by a slightly sustained weight between day 14 and 21 (during which period the body weight of cuprizone mice was also slightly sustained); a slight reduction between day 21 and 28 (compared to other groups whose body weights increased within same period), and a raised body weight similar to other groups, but more than the final weight of the cuprizone-treated mice. In Figure B, the weight gain of animals in the cuprizone group was significantly lower than those of control ($p < 0.01$), kolaviron ($p < 0.01$) and cuprizone + kolaviron groups. Cuprizone + kolaviron group had a higher weight gain than the cuprizone-treated animals ($p > 0.01$), though not as high as the weight gain observed in the control and the kolaviron-treated mice which had the highest weight gain. ** is significant value of $p < 0.01$.

alternation test conducted using the Y-maze test for short-term memory showed that cuprizone-treated mice had a lower percentage correct alternation compared to control and the kolaviron-treated mice, with particularly significant difference when compared to the kolaviron group ($p < 0.05$) (Fig. 2B). Mice that received a combined treatment of cuprizone and kolaviron had a higher percentage correct alternation compared to the cuprizone –treated mice ($p > 0.05$), showing that kolaviron activity helped to restore the short-term memory. Cumulatively, kolaviron use in the present study prevented cuprizone-induced memory loss.

3.3. *Kolaviron prevents anxiogenic properties of cuprizone and sustained exploratory drive*

The open field test was used to score exploratory drive and anxiety (Fig. 3). Cuprizone significantly reduced the number of lines crossed - a measure of exploratory drive-relative to the control, kolaviron group ($p < 0.05$) and the group co-treated with cuprizone and kolaviron. The group that received a combined treatment of cuprizone and kolaviron presented with number of lines crossed similar to the control. Furthermore,

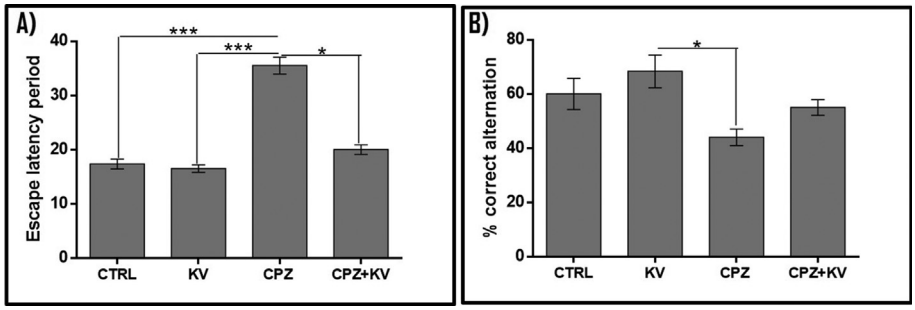

Fig. 2. Neurobehavioural outcomes of mice in the Morris water maze (Fig. A) and Y-maze (Fig. B). KV, CPZ and CPZ + KV are the kolaviron, cuprizone and the cuprizone + kolaviron groups respectively. Cuprizone-treated mice had a significantly high escape latency period in the Morris water maze when compared to the control ($p < 0.01$), kolaviron ($p < 0.01$) and cuprizone + kolaviron ($p < 0.05$); the reduction in escape latency period in mice co-treated with cuprizone and kolaviron was however slightly higher when compared with mice of the control and kolaviron groups. Kolaviron-treated mice had the highest percentage correct alternation, while cuprizone-treated mice had the least percentage correct alternation compared to the control group ($p > 0.05$), kolaviron group ($p < 0.05$) and cuprizone + kolaviron group ($p > 0.05$); however, the percentage correct alternation in cuprizone + kolaviron group was lower compared to the control and kolaviron groups. * and *** are significant values at $p < 0.05$ and $p < 0.005$ respectively.

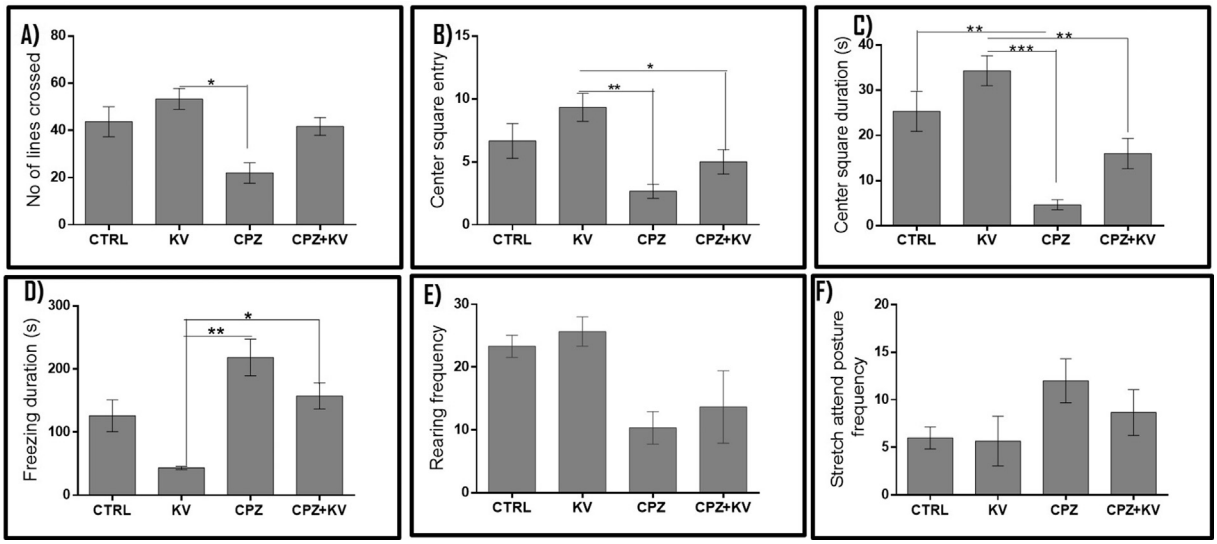

Fig. 3. Performances of mice in the open field test behavioural paradigm. A, B, C, D, E and F represented number of lines crossed, centre square entry, centre square duration, freezing duration, rearing frequency and stretch attend posture frequency respectively, while KV, CPZ and CPZ + KV are the kolaviron, cuprizone and cuprizone + kolaviron groups respectively. In Fig. 3A, number of lines crossed was highest in KV-treated mice, while CPZ-treated mice had significant reduction compared to KV ($p < 0.05$) and control groups; number of lines crossed was higher in CPZ + KV group compared to CPZ group ($p > 0.05$). Centre square entry (Fig. 3B) was low in CPZ group compared to control and KV groups with a more marked difference when compared with KV group ($p < 0.01$) which had the highest entry; the difference in entry between the KV and CPZ + KV groups was significant ($p < 0.05$), while the difference between the latter and the CPZ group was not significant statistically. Centre square duration (Fig. 3C) was least in CPZ group compared to the control and other groups and highest in KV group ($p < 0.005$); the difference between the CPZ and control groups was significant ($p < 0.01$); though the duration was higher in CPZ + KV group compared to the CPZ group, it was still lower than the centre square duration of KV-treated group ($p < 0.01$). As shown in Fig. 3D, the freezing duration was highest in CPZ group and least in KV group with a statistically significant difference ($p < 0.01$); CPZ + KV group also recorded a high freezing duration but lower than the CPZ group and higher than the control and KV groups ($p < 0.05$); the difference between the control and CPZ group was not statistically significant. Rearing frequency (Fig. 3E) was least in CPZ group and very high in the control and KV group, with the latter being highest; increased rearing frequency was only slightly higher in CPZ + KV group than the CPZ group. No significant changes occurred in the stretch attend posture frequency (Fig. 3F) even though the CPZ group had the highest frequency followed by the CPZ + KV group; KV group was only slightly lower than the control. *, ** and *** are significant values of $p < 0.05$, 0.01 and 0.005 respectively.

cuprizone -treated animals showed a reduction in centre square entry, centre square duration and rearing frequency while increasing the freezing duration and stretch attend posture frequency compared to the control, suggesting that cuprizone caused anxiety-related behaviour. Co-treatment with cuprizone and kolaviron showed behavioural scores that were similar to the baseline control.

3.4. *Kolaviron counterbalances cuprizone-induced hippocampal disturbance of neurochemical redox*

The hippocampus was assessed for the levels of SOD and GPx enzymes, which are in the frontline of antioxidant defense system of the CNS (Fig. 4). The level of SOD was depleted in the cuprizone group when compared with kolaviron group ($p < 0.05$) and the control ($p > 0.05$); the group co-treated with cuprizone and kolaviron had a marked reduction compared with control ($p > 0.05$) and kolaviron-treated group ($p < 0.05$), but slightly higher than the SOD activity in the cuprizone-treated mice ($p > 0.05$). Likewise, GPx activity was also depleted in the cuprizone group when compared with control ($p > 0.05$), kolaviron group ($p < 0.05$) and the group co-treated with cuprizone and kolaviron ($p > 0.05$). Correspondingly, cuprizone diminished SOD and GPx activities in the cuprizone group which had a significantly lower levels relative to the control ($p > 0.05$) and kolaviron group ($p < 0.05$). These findings suggested that cuprizone induced an excessive generation of reactive oxygen

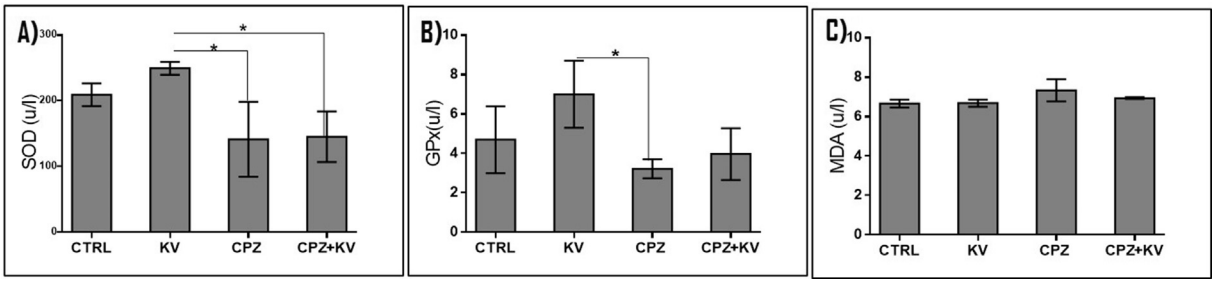

Fig. 4. Activity of superoxide dismutase (SOD), glutathione peroxidase (GPx) and malondialdehyde (MDA). SOD activity reduced in cuprizone-treated mice (Fig. 4A) compared with the control and kolaviron group which had the highest level of enzyme activity ($p < 0.05$); significant reduction occurred in cuprizone + kolaviron group compared to the kolaviron group ($p < 0.05$) though SOD activity in cuprizone + kolaviron group was only slightly above the cuprizone group. The activity of GPx (Fig. 4B) was raised in the kolaviron group compared with the control and other treated groups, with a significant reduction in cuprizone group compared with the kolaviron group ($p < 0.05$); although the enzyme activity was higher in the cuprizone + kolaviron group compared with the cuprizone group, the difference was not statistically significant ($p > 0.05$). The levels of MDA in the control and kolaviron groups were about the same (Fig. 4C), while the cuprizone group had a higher MDA level compared with all the other groups ($p > 0.05$); cuprizone + kolaviron group had a reduced MDA level compared with the cuprizone group but higher than the control and kolaviron groups ($p > 0.05$).

species which could cause oxidative stress in the brain. We also quantified MDA, a marker of lipid peroxidation. Expectedly, the cuprizone treatment led to increased lipid peroxidation when compared to both control and kolaviron group. These findings suggest that the exacerbated level of reactive species generation triggered by cuprizone culminated in heightened level of lipid peroxidation in the hippocampus. This perturbation of biochemical redox could result into structural modification of the brain and the perceived decline in memory index of the cuprizone-treated mice.

3.5. Kolaviron maintains hippocampal histomorphology against cuprizone-induced microarchitectural alterations

Micro slices of hippocampus were obtained and stained with Haematoxylin & Eosin for characterization of histomorphological changes (Fig. 5A). Hippocampal sections revealed granule neurons in properly delineated dentate gyrus of control and kolaviron groups. Hippocampal neuropil of cuprizone-treated mice was characterised by numerous fragmentations and poor staining characteristics. The hippocampus of mice co-treated with cuprizone and kolaviron showed mildly dispersed neurons, especially in the dentate network and the general cellular disposition shared similar features with those of the control. The observed changes in the general microarchitecture of the hippocampus of cuprizone-treated animals can be attributed to the exacerbated lipid peroxidation which occurred as a result of superfluous reactive species and impaired antioxidant defense system.

3.6. Cuprizone-induced chromatolysis and astrogliosis ameliorated by kolaviron

Demonstrating the cytoarchitectural integrity, structural modifications and intactness of Nissl substance in the hippocampus (Fig. 5B) would help substantiate the perceived cuprizone-mediated behavioural decline, neurochemical redox imbalance and histomorphological alterations within the brain structure. Findings from the Nissl profiling showed high Nissl intensity within well-delineated neuronal soma in the hippocampus of control and kolaviron groups whereas the cuprizone-treated animals presented with varying degree of central chromatolysis in the sparsely expressed degenerating neurons (Fig. 5B, C). Expectedly, the animals co-treated with cuprizone and kolaviron presented with a Nissl profile similar to that seen in the control group, except for the expression of a few mild chromatolytic neurons.

Immunohistochemical characterisation of the hippocampus of cuprizone-treated mice showed marked increase in the expression of activated astrocytes (Fig 6A, B). In contrast, mice co-treated with cuprizone and kolaviron manifested low expression of astrocytes, similar to those of the control and kolaviron-treated mice.

4. Discussion

Continuous exploration of therapeutic advantages of phytomedicinal substances has contributed immensely to the process of drug discovery. In the present study, we explored the therapeutic role of kolaviron in

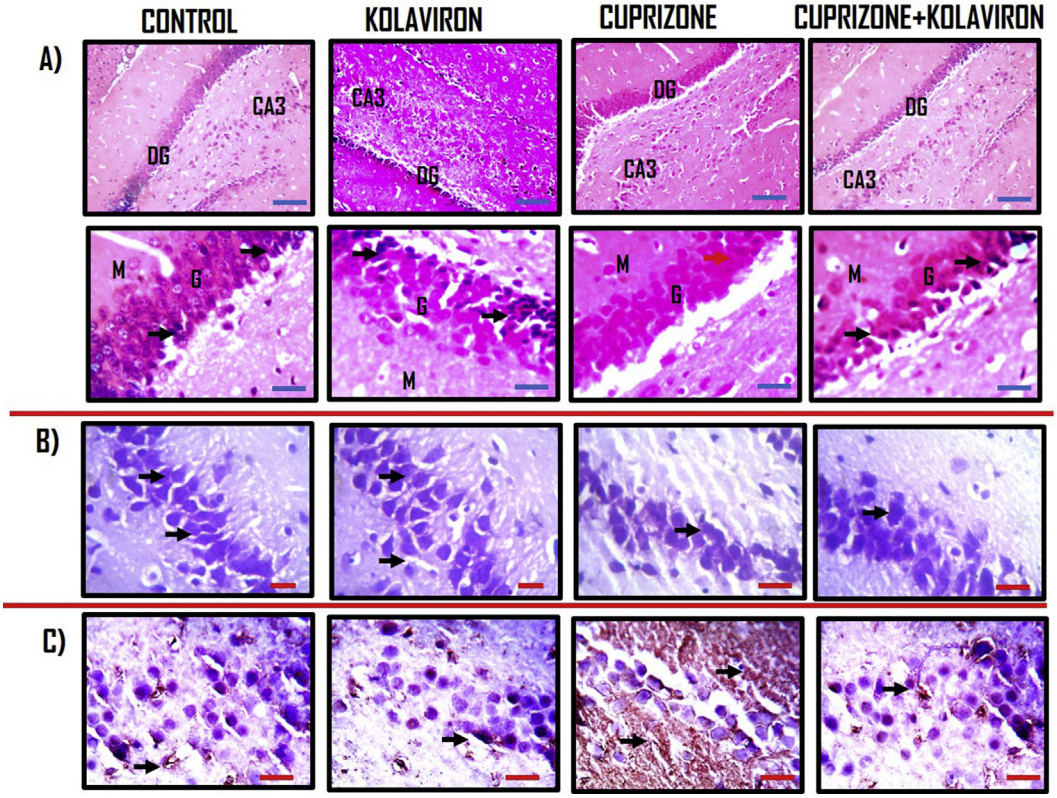

Fig. 5. Representative photomicrographs of the histomorphological manifestation of the dentate gyrus of mice. A) showed a panoramic view of the dentate gyrus (100 μm scale bar). The dentate gyrus and *cornu ammonis* 3 (CA3) of the hippocampus were well appreciated. The control, kolaviron and cuprizone + kolaviron groups manifested normal cytoarchitectural assortment with succinct expression of cells within the CA3. The cuprizone-treated groups present with poor staining intensity and cellular fragmentation. The granular layer of the dentate gyrus showed the control, kolaviron and cuprizone + kolaviron groups presented with characteristic cellular delineation deeply stained nuclei of inherent granule cells with apical and basal dendrites jotting out of the soma (black arrows) whereas the granular cells of those of cuprizone-treated animals were poorly characterized (red arrow). B): Representative photomicrographs of the hippocampus showing the CA3, characteristic staining profile of neuronal cells (black arrows) of the control and kolaviron groups, and chromatolytic cells (red arrow heads) in the cuprizone and cuprizone + kolaviron groups. Scale bar is 25 μ. C): Representative photomicrographs of the hippocampus (CA3) stained with anti-GFAP for expression of astrocytes. Expressed astrocytes (black arrow) were more in the CPZ group relative to other experimental group. Scale bar is 25 μ.

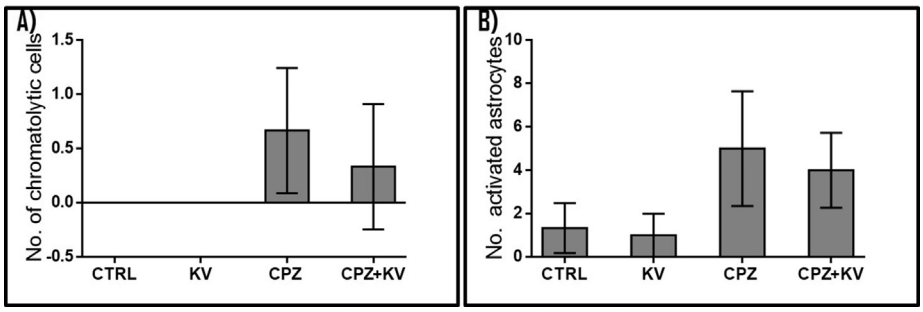

Fig. 6. A and B: Amount of chromatolytic cells (A) and activated astrocytes (B) per unit area of the hippocampus; CTRL, KV, CPZ and CPZ + KV are the control, kolaviron, cuprizone and cuprizone + kolaviron groups respectively. Number of chromatolytic cells was higher in CPZ group compared with CPZ + KV group, while the amount was negligible in the control and KV groups (A). Activated astrocytes were minimal in KV compared to control and other groups, while the number was markedly raised in CPZ group compared to CPZ + KV and other groups (B).

cuprizone-induced neurotoxicity. The pattern of weight change in mouse model of cuprizone demyelination was critically observed. This was made possible by daily monitoring of body weights of experimental animals. Weight decrease associated with cuprizone toxicity occurred within the first two weeks of induction. The role of poor dietary intake in body weight emaciation has previously been documented (Ogden et al., 2014; Tobias et al., 2016). Expectedly, kolaviron counteracted the weight loss actions of cuprizone in the mice that received a combined treatment of cuprizone and kolaviron as they presented with a linear increase in body weight from the second week of intervention, and a final weight gain that was similar to the control. This finding is consistent with our previous report of reduction in body weight induced by cuprizone which was attributable to reduction in food intake as well as increase in basal metabolic rate, both of which were prevented by kolaviron intervention (Omotoso et al., 2018a). This study has been able to demonstrate the dynamics that occur in body weight patterns during cuprizone toxicity and following kolaviron intervention.

General declination in long- and short-term memory and other forms of neurobehaviour have been implicated in experimentally induced demyelinating diseases (Chun and Hartung, 2010; Vorhees and Williams; 2006; Omotoso et al., 2018a). In the present study, we characterized learning and memory parameters by using Morris water maze and Y-maze to measure spatial memory and working memory respectively. Our findings showed that cuprizone perturbed the memory index of experimental animals. In the Morris water maze behavioural paradigm, cuprizone treatment resulted in a significantly higher escape latency period (long-term memory index), while the percentage correct alternation (a short-term memory index) measured in the Y-maze behavioural paradigm was markedly reduced. These findings implicated cuprizone in disturbance of memory and learning activities of mice. Concomitant administration of cuprizone and kolaviron to mice prevented memory decline observed in cuprizone-treated mice. Thus, kolaviron intervention prevented cuprizone-induced memory loss and sustained the learning capacity of the animals.

Cuprizone is a well-known copper chelator that attacks matured oligodendrocytes which are responsible for the myelination of neurons (Kipp et al., 2009; Acs and Kalman, 2012; Acs et al., 2013). The mechanism by which cuprizone exerts this effect to some extent has been delineated. It has been reported to cause demyelination in the brain through the generation of reactive oxygen species which cumulates in oxidative stress (Omotoso et al., 2018c), thus causing apoptosis in mature oligodendrocytes. Copper is an essential cofactor for cuproenzymes such as superoxide dismutase which is the first line of enzymic antioxidant defense against reactive oxygen species and plays a vital role in dismutating superoxide anion. Three isoforms of SOD exist which are cytosolic (Cu, Zn-SOD), mitochondrial (Mn-SOD) and extracellular (EC-SOD) SOD, with Cu,Zn-SOD being, by far, the most abundant (Marklund, 1984). Thus, a deficit in copper ion concentration in the brain will adversely affect the function of superoxide dismutase which invariably will lead to oxidative stress and lipid peroxidation in cell membranes. The oxidative stress within the cell in conjunction with the activation of the microglial cells/macrophages (which also produce a lot of ROS) and the decrease in cytochrome oxidase and monoamine oxidase activities in the mitochondria leads to apoptosis of the oligodendrocytes and neuronal cell death (Kipp et al., 2009; Liu et al., 2015). In short, cuprizone has been reported to cause increase in hydrogen peroxide and malondialdehyde levels and apoptosis in oligodendrocytes with corresponding decrease in SOD, GPx activities in the brain of mice (Acs et al., 2013; Xuan et al., 2015). The results of this study corroborate these earlier reports as evidenced by the observed increase in MDA concentrations (which is a marker for lipid peroxidation) and reduction in SOD and GPx activities in the hippocampus. The administration of kolaviron was able to reverse the activities of SOD and GPx and malondialdehyde concentration to the range of controls. This finding corroborates our earlier report that kolaviron protects against ROS and their subsequent cellular damage in the brain (Omotoso et al., 2018b). This protective effect of kolaviron could be as a result of its intrinsic antioxidant activity (Farombi et al., 2004).

The hippocampus is an important brain structure in memory formation and consolidation, hence any pathological alteration in the histomorphological integrity will affect the normal physiological functions of the brain. Histological and histochemical findings support reports that cuprizone induces memory decline and oxidative stress in the brain. This was inferred from the observed cellular clusters with pyknotic characteristics, which were observed in the CA3 region of the hippocampus, and the poorly stained granule cells in the granular layer of the dentate gyrus of cuprizone-treated mice. Expectedly, cuprizone exacerbated levels of cellular expression of chromatolysis. Nissl granules are rough endoplasmic reticulum present in the soma of neurons. Morphological alterations affecting their availability and distribution could impair neuronal protein and cell membrane synthesis (Raivich, 2009) with consequent impairment in overall neurologic functions. This situation has the potential of further complicating impaired function arising as a result of axonal demyelination seen in multiple sclerosis. However, the introduction of kolaviron through its antioxidant property and ability to protect the cell membrane from ROS-related insults, significantly preserved Nissl granules, thereby sustaining their function in neuronal protein synthesis.

Astrocytes are critical for the control of brain homeostasis and the intrinsic brain defense system (Kettenmann and Verkhratsky, 2011). In brain pathology, they are activated and thereafter undergo transformation in their molecular expression and morphology (Sofroniew, 2009; Hamby and Sofroniew, 2010). The high expression of astrocytes in the hippocampus of cuprizone-exposed mice was an indication of ongoing reactive astrogliosis. Although reactive astrogliosis is a nonspecific response, it is highly characteristic, and involves various morphological and molecular changes (Sun and Jakobs, 2012). Meanwhile, reactive astrocytes were sensitive to kolaviron intervention resulting in the reduction of astrocytic count in mice co-treated with cuprizone and kolaviron, and this could aid axonal repair and quick recovery of neuronal function.

These pathological alterations induced by cuprizone can be attributed to the copper chelating properties of cuprizone which in turn resulted in exacerbated ROS accumulation which the intrinsic antioxidant defense

system could not manage. The reactive species gained access to the intracellular environment by acquiring electron from the poly-unsaturated lipid bilayer of the neuronal cell wall which resulted to lipid peroxidation and caused cell membrane degradation and neuro-inflammation. Kolaviron intervention regulates neuronal physiology despite the presence of neurotoxicant. It has been suggested that the molecular mechanisms underlying the protective action of kolaviron are activated by suppressing certain pro-inflammatory genes whose expression have been shown to be regulated by transcription (Farombi et al., 2009; Olajide et al., 2016).

5. Conclusion

Administration of kolaviron isolated from *Garcinia kola* limits the damaging effect of cuprizone toxicity on the morphology and functions of the hippocampus of mouse model of multiple sclerosis. To the best of our knowledge, our study is the first to show line of evidences to support the ability of kolaviron to mitigate degenerative changes associated with cuprizone-induced neurotoxicity in mice. Being protective against ROS-related insults, kolaviron preserves the integrity of cell membrane and Nissl bodies, thereby promoting neuronal protein and cell membrane synthesis and consequently enhancing normal hippocampal functions in demyelinating conditions; this could help improve cognitive functions and also delay disease progression.

Declarations

Author contribution statement

Omotoso G. O: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Olajide O. J: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Gbadamosi I. T: Analyzed and interpreted the data; Wrote the paper.

Adebayo J. O, Owoyele B.V: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Enaibe B.U, Akinola O.B: Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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