

Full Length Article

Neuroprotective role of kolaviron in striatal redo-inflammation associated with rotenone model of Parkinson's disease

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ABSTRACT

Parkinson's disease is the most prevalent movement disorder. Currently, therapies are palliative with associated irreversible behavioural incompetence. Here, we investigated the ability of kolaviron (KV), an anti-inflammatory biflavonoid isolated from *Garcinia kola* seeds, to rescue striatal neuronal damage and redo-inflammation in rats exposed to rotenone (ROT). Aged rats exposed to 11 days of rotenone intoxication were treated with KV either concurrently or for 18 days. The 18-day regimen included 7 days of pre-treatment prior 11-day concurrent ROT-KV treatment.

Rotenone-exposed rats lost weight appreciably and travelled less distance with reduced speed, decline efficiency to maintain a straight path, enhanced freezing, increased immobile episodes and poor hole recognition. The motor incompetence was attributed to enhanced striatal neurodegeneration, increased alpha synuclein formation and reduced tyrosine hydroxylase expression. ROT intoxication significantly increased reactive species production, which co-existed with induction of striatal antioxidant system and damage to biomolecules. ROT additionally upregulated COX-2 expression, enhanced myeloperoxidase activity and increased concentration of striatal interleukin-6 (IL-6), IL-1 β and tumour necrosis factor (TNF- α). Treatment with kolaviron reversed the rotenone-associated locomotor impairment and exploratory deficits, motor/neuromuscular incompetence, striatal neurodegeneration, neurobiochemical imbalance, altered antioxidant defence system and neuroinflammation. KV-treated rats showed improved capacity to maintain efficient gait with minimal rigidity and enhanced coordination.

Taken together, kolaviron exhibited neuroprotective properties, which may be beneficial for the prevention and management of Parkinson's disease, via antioxidant, anti-inflammatory and anti-apoptotic mechanisms.

1. Introduction

Parkinson's disease (PD) is a chronic, progressive, syndromal and multifactorial neurological disorder which clinically manifest around 60 years of age (Dorsey et al., 2007). PD is rapidly becoming a global communal illness with 7–10 million Parkinsonians globally. It is estimated that 8.7–9.3 million individuals with the disease will be domicile in the top 10 most populous nation in the world by 2030. In Nigeria, PD cases constitute 79% of the overall 2% hospital frequency of neurological cases (Dorsey et al., 2007; Okubadejo et al., 2010, 2006; Parkinson Disease Foundation, 2016). PD is cardinally characterised by

resting tremor, prolonged reaction times, bradykinesia, rigidity, postural instability and freezing of gait, which may degenerate to tightened facial expression and unconscious facial movement (Blandini, 2013; Chaudhuri and Schapira, 2009). PD symptoms are evident when 50–70% of the dopaminergic neurons in the substantia nigra per compacta (SNc) and striatum have degenerated (Gao et al., 2011). Exposure to occupational and environmental toxins such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and agrochemicals, including paraquat (a herbicide) and rotenone (a pesticide), have been attributed to increase the risk of PD (Cabras et al., 2002; Costello et al., 2009).

Prior to clinical presentation, several years of pre-symptomatic

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neuronal morbidity which progressively results in neurodegeneration may have occurred. This period is characterised by clandestine events including biochemical abnormalities, mitochondrial complex I insufficiency, unabated free radical generation, persisting neuroinflammation and compromised degradation of protein, which gradually fuel pathological formation of intraneuronal inclusions such as alpha synuclein, oxidative stress and energy crisis. When unabated, this progresses to impairment of intracellular functions, gliosis, neuronal death via apoptosis, behavioural dysfunction and characteristic motor symptoms (Blandini, 2013; Chao et al., 2014; Style, 2013) due to reduced concentration of available dopamine in the nigrostriatal pathway (Barzilai and Melamed, 2003).

Currently, gold standard interventions for PD are based on enhancing nigrostriatal functions through constant supply of dopamine, and associated measures to prevent its biotransformation and consequently sustain its bioavailability in brain tissues. Unfortunately, these measures have been relegated to merely motor-symptomatic relief, thus precluding their use for associated non-motor issues especially during the pre-symptomatic phase. They also lack preventive and curative purposes because of their inability to halt the progression of the disease (Kalinderi et al., 2011; Style, 2013). Despite the monumental improvement of motor issues, the therapy of choice Levodopa (L-dopa), a precursor of dopamine, is associated with severe abnormalities in performing voluntary muscle movements (dyskinesia) and motor functions when used consistently for more than five years (Jankovic and Stacy, 2007). The use of dopamine agonists substitutes is also characterized with hallucinations and heart complications such as valvulopathy (Schapira, 1999). Furthermore, safety of several inhibitors of peripheral L-Dopa metabolism such as Dopa decarboxylase, monoamine oxidase B and catechol-O-methyltransferase inhibitors and other strategies has been a subject of concern because of reported hepatotoxicity and minimal efficacy (Antonini and Poewe, 2007; Kalinderi et al., 2011). Meanwhile, alternative strategies such as stem cell therapy, neuro-transplantation, and deep brain stimulation are invasive, expensive, not easily available, and not readily accessible (Brundin et al., 2010).

Thus, the exigent need for novel, non-invasive, and non-toxic alternatives to dopamine dependent and/or related drugs is obvious. Long term strategies with broad spectrum efficacy against implicated progressive neuronal degeneration have been suggested as neuroprotective agents capable of interfering, preventing, mitigating and/or reversing the cardinal pathological cascades involved in nigrostriatal and extra-nigra neuronal death associated with PD (Chaudhuri and Schapira, 2009; Schapira, 1999; Schapira et al., 2006). As modes of action, these proposed agents should be able to prevent apoptosis, attenuate inflammation, dissipate free radicals, mitigate oxidative stress/damage and/or shield mitochondria from internal and external assaults (Barnham et al., 2004; Chao et al., 2014; Style, 2013).

Kolaviron (KV), a biflavonoid, is a naturally occurring antioxidant and anti-inflammatory phytochemical derived from the seed of *Garcinia kola* with a range of pharmacological activities (Awogbindin et al., 2017; Farombi and Owoeye, 2011). Studies have shown that KV is neuroprotective against vanadium-induced neurotoxicity (Igado et al., 2012). KV also protected against atrazine-induced apoptotic neuronal damage in human dopaminergic SH-SY5Y and PC 12 cells (Abarikwu et al., 2011a; Abarikwu et al., 2011b). Additionally, Onasanwo et al. (2016) reported a remarkable inhibition of BV2 microglia neuroinflammation by KV. Mechanistically, KV elicits these chemopreventive properties majorly by its intrinsic free radical scavenging, metal chelating, antioxidant and anti-inflammatory characteristics (Farombi and Owoeye, 2011). Recently, we reported that KV increased longevity and attenuated rotenone-induced toxicity in *Drosophila melanogaster* (Farombi et al., 2018). In this study, we therefore investigated the ability of KV to interfere with rat Parkinsonism induced by rotenone, a potent inhibitor of mitochondrial complex I of the electron transport chain.

2. Materials and methods

2.1. Chemicals, reagents and antibodies

High purity rotenone (< 98%), rat-specific IL-1 β , TNF- α , IL-6 ELISA kits, were obtained from Abcam, Cambridge UK. NL493-conjugated anti-rabbit was purchased from R&D systems MN, USA. Anti- α -synuclein monoclonal antibodies was purchased from Sino Biological Inc. (Beijing China). Cyclooxygenase (COX)-2, and Caspase-3 antibodies were obtained from Elabscience (Texas, USA). 4',6-diamidino-2-phenylindole (DAPI) solution and N-propyl gallate were purchased from Novus Biologicals, CO USA and AK Scientific CA USA, respectively while Ellman reagent, 5-5'-dithiobis-(2-dinitrobenzoic acid) DTNB and N-(1-naphthyl) ethylenediamine dihydrochloride were products of Alfa Aesar, Heysham Lancs and British Drug House (Poole, Dorset UK), respectively. Blot-qualified bovine serum albumin (BSA) was purchased from Promega, Madison USA. Glutathione, xylenol orange, sorbitol, 1-chloro- 2, 4-dinitrobenzene (CDNB), epinephrine, O-dianisidine dihydrochloride were sourced from Sigma Aldrich, St. Louis, USA. Coomassie brilliant blue G-250 was purchased from Bio-Basic Canada Inc. Triton-X was obtained from Consolidated Chemicals, Allentown, Pennsylvania USA. All other reagents were of the highest analytical grade and purity from Sigma-Aldrich, St. Louis, USA and British Drug Houses, Dorset, Poole, UK.

2.2. Experimental animals

Male Wistar rats weighing 255 g to 270 g were purchased from the Central Animal House of College of Medicine, University of Ibadan, Nigeria. The rats, fifteen per group, were habituated for two weeks prior the study. All animals were housed in plastic suspended cages and subjected to 12-h light/dark cycle in a well-ventilated animal house where they were provided with satisfactory quantity of rat pellets (obtained from Ladokun Feeds Nig. Limited) and adequate supply of clean water throughout acclimatization and administration periods. All animals were treated with humaneness and the experiment was conducted according to the protocols and approval of the University of Ibadan institutional Animal Care and Use Research Ethics Committee (ACUREC). The ACUREC protocol is in accordance with Guide for the Care and Use of Laboratory Animals of the National Academy of Science published by the National Institute of Health.

2.3. Kolaviron

Garcinia kola seeds were purchased from Ile-Ife, peeled, sliced and spread on a laboratory bench until dryness after which they were milled. According to the modified method of Iwu et al. (1987) as reported by Farombi et al. (2004). The powdered seeds were defatted with n-hexane using soxhlet apparatus. The defatted marc was dried, repacked into the apparatus and extracted with methanol. Kolaviron was obtained from the methanol extract by a twin purification process of dilution, requiring twice its volume of distilled water, and chloroform extraction to give a golden yellow solid. The identity of KV was confirmed as earlier reported (Farombi et al., 2004) with a thin layer chromatography process using Silica gel GF 254 coated plates by a chloroform/methanol solvent mixture (80:20) which revealed the presence of three compounds GB1, GB2 and kolafavanone identified by their RF values when compared with reference compounds and from the analysis of their spectra data- ¹H Nuclear Magnetic Resonance (NMR), ¹³C NMR and electron ionization (EI)-mass spectral results (Iwu, 1982). The purity of KV was 96.5%.

2.4. Experimental treatments

The experimental animals were randomly distributed into five groups. Rotenone (2.5 mg/kg) was freshly prepared in dimethyl

sulfoxide (DMSO)-polyethylene glycol (PEG) solution (1/1 v/v) and administered to a group of rats (ROT) intraperitoneally for 11 consecutive days to establish Parkinsonism in rats. A group of rats (KV (11) + ROT) received KV (200 mg/kg) concurrently with rotenone for 11 days while another group of rats (KV (18) + ROT) was pre-treated for seven days with KV prior ROT-KV concurrent treatment. The remaining two groups received 2 ml/kg of DMSO/PEG vehicle (Control) or KV for 18 days.

2.5. Behavioural testing

Beginning from the first day of rotenone administration, each experimental animal was weighed prior to daily treatment. Behavioural assessments to monitor locomotory, exploratory, motor and neuromuscular changes following treatments were conducted before the experimental animals were euthanized on days 9 and 10 post rotenone intoxication between 9:00 am and 2:00 pm. These included open field assessment, fore limb grip test, hole and board test, staircase test and negative geotaxis.

2.6. Open field assessment

The procedure of Owoeye et al. (2018) was followed in an open chamber using a 56 × 56 × 20 cm open chamber. The floor of the apparatus was partitioned evenly into sixteen squares with a superimposed central square of 18 × 18 cm equally spaced around the fusion point of the four innermost squares. Seven rats were randomly selected from each group and placed individually into the arena's central square to explore the arena. The animals were cam-recorded (DNE webcam, Porto Alegre, Brazil) from an elevated position above the apparatus for five minutes. The recordings were analysed with ANY-maze video tracking software (Stoelting CO, US) to assess indices of locomotion (Distance covered, path efficiency, latency to mobility and speed), exploration (distance and absolute turn angle by head, meandering, angular velocity and rotations), and motor/neuromuscular dysfunction (freezing, grooming and rearing).

2.7. Assessments of fore-limb grip strength, negative geotaxis and hole and board test

The limbs strength of the experimental rats was evaluated with wire grip test. Animals were suspended with their fore paws on a horizontal 4 mm diameter metal rod at about 12 cm above a padded ground level. Each rat was timed for five minutes and observed for its ability to hang on the metal rope. However, the latency to fall (sec) was recorded. The motor competence and compensatory responses of the experimental animals to re-orient and ply uphill an incline plank was measured by the negative geotaxis method described by Motz and Alberts, (2005). Individually, the animals were placed downhill on a rough wooden plank inclined at 45° angle and were observed for directional movement against gravitational cues. The time taken to re-orient (sec) was recorded. For the hole and board test, seven rats per group were placed at the centre of a 40 cm X 40 cm board with 16 evenly spaced holes of 3 cm diameter for 3 min to assess nose poking behaviour. Nose poking ability indicates curiosity and inclination to explore. The number of nose pokes and head dipping is measured.

2.8. Tissue processing

Twenty-four hours after the last treatment, nine (9) rats from each group were euthanized and the brain tissues were immediately excised, weighed and sectioned. Striatum sections from each group were homogenized in four volumes of ice-cold 0.1 M phosphate buffer and further processed for the determination of oxidative stress, antioxidant and inflammatory markers, DNA fragmentation assay and fluorometric quantitation of total reactive oxygen species. For immunofluorescence

and immunohistochemistry analyses of COX-2, Caspase-3, α -synuclein and tyrosine hydroxylase expressions, animals (n = 6) were transcardially perfused in situ with PBS first, to wash off blood from the brain parenchyma, followed by 2% buffered formalin.

2.9. Biochemical estimation of oxidative stress status and inflammatory markers

Striatal homogenate was centrifuged at 13,000 g for 10 min at 4 °C, to obtain post mitochondrial fraction. The supernatant was collected and used to determine striatal antioxidant capacity, oxidative stress status, inflammatory indices and acetylcholine esterase activity. Protein concentration was estimated using Coomassie brilliant blue-dependent Bradford assay (Bradford, 1976). Superoxide dismutase (SOD) activity was determined by monitoring inhibition of adrenaline auto-oxidation in a basic milieu according to a revised method of Misra and Fridovich, 1972. Catalase (CAT) activity was assayed using hydrogen peroxide (H₂O₂) as a substrate according to the method of Clairborne (1995). Hydrogen peroxide generation was quantified using a ferrous ions (Fe²⁺) oxidation and sorbitol colour amplification system according to the method of Wolff, (1994). The Ellman's reagent-dependent method of Jollow et al. (1974) was employed to assess the concentration of reduced glutathione. Oxidative damage was determined according to the procedure described by Varshney and kale (Varshney and Kale, 1990) by estimating the level of lipid peroxidative product, malondialdehyde after forming a pink coloured chromogen upon reacting with 2-thiobarbituric acid. Striatal nitric oxide (NO) radical level was determined by measuring the end product of nitric oxide metabolism, nitrite, using Griess reaction method of Crespo et al. Crespo et al. (1999). Myeloperoxidase activity was assayed by monitoring the oxidation of O-dianisidine in the presence of hydrogen peroxide according to the method of Bradley et al. (1992). Activity of acetylcholinesterase was determined according to the method described by Owoeye et al. (2018).

2.10. DNA fragmentation assay

Striatal level of fragmented DNA was determined according to the method of Al-Okbi et al. (2011) using a diphenylamine (DPA)-based colorimetric assay. Striatal tissue from left side of the brain (n = 5) was homogenized in hypotonic lysis buffer and centrifuged. The lysate supernatant, containing small DNA fragments, and the pellet, containing large pieces of DNA, were subsequently processed independently for DPA assay after their DNA was extracted with 10% Trichloroacetic acid (TCA). Two volumes of freshly prepared DPA reagent was later added to the extracted soluble DNAs and the solutions were stored at 4 °C for 48 h for the development of colour, which was measured at 578/600 nm. Fragmented DNA was expressed as percentage of optical density ratio of the supernatant to that of pellet and supernatant.

2.11. Determination of total reactive species generation by DCFH-DA method

Total reactive species was quantitated in the striatal samples as an index of general oxidative stress according to the method described by Abolaji et al. (2014). The assay is based on the ability of reactive species in the sample such as NO, H₂O₂ and hypochlorous acid to oxidize 2', 7'-dichlorodihydrofluorescein (H₂DCF) to a fluorophore, 2', 7'-dichlorofluorescein (DCF), following the cleavage of acetate from H₂DCF diacetate (H₂DCF-DA) by the ubiquitous non-specific cellular esterases in the sample. The reaction milieu consists of 450 μ l of H₂DCF-DA (20 μ M) in 0.1 M phosphate buffer and 150 μ l of the sample. Using a CytoFluor 95 fluorescence spectrophotometer (Molecular Devices, USA), DCF was excited at 498 nm and its emission intensity was monitored at 530 nm for 10 min at 30 s interval. The rate of formation of DCF was expressed as percentage of vehicle-treated control samples.

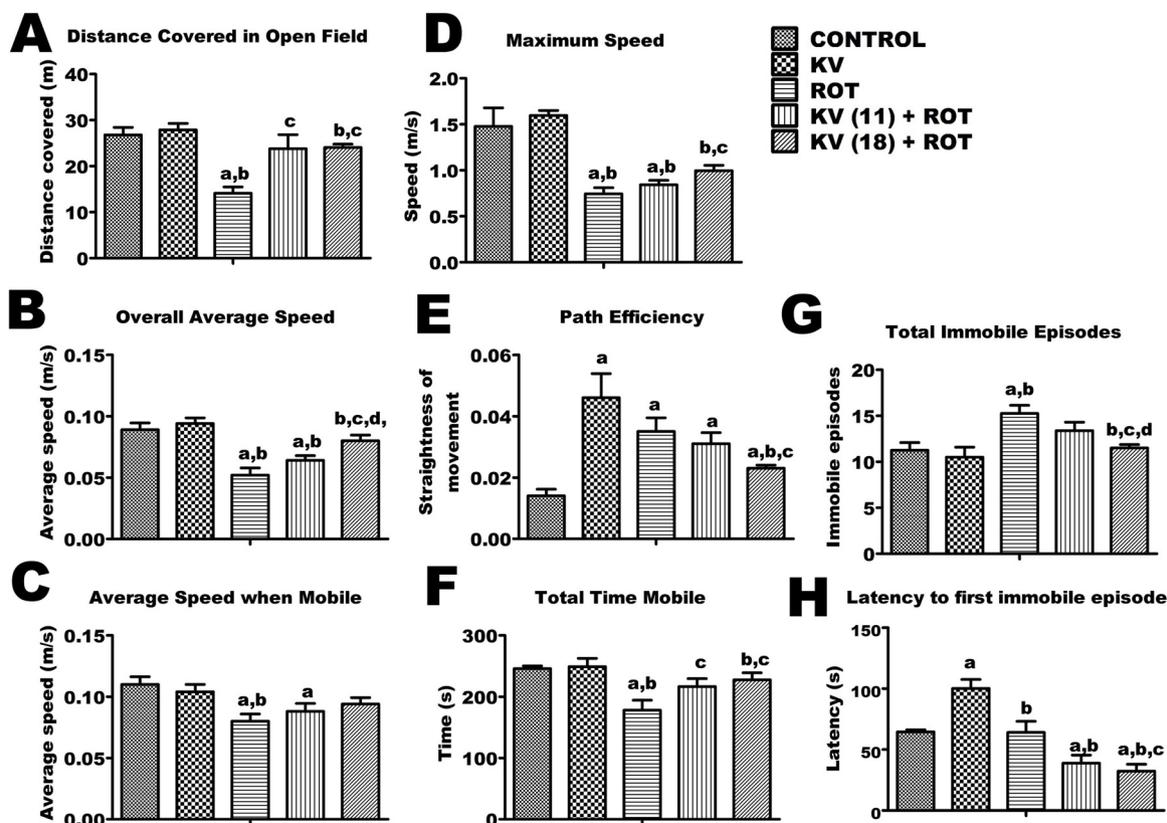


Fig. 1. Locomotory profile of rats challenged and treated with rotenone and kolaviron, respectively. Values of locomotor activity in the open field measured as distance covered (A), average speed (B), speed when mobile (C), maximum speed (D), path efficiency (E), total mobile time (F), total mobile episodes (G) and latency to first immobile episodes are presented as Mean \pm SEM ($n = 7$). a, b, c and d represent statistical differences ($p < 0.05$) when compared with the control, KV, ROT and KV (11) + ROT groups, respectively.

2.12. Serological determination of IL-1 β , IL-6 and TNF- α titres by ELISA

Striatal levels of the secreted interleukin 1beta (IL-1 β), IL-6 and tumour necrosis factor alpha (TNF- α) were quantitatively determined using enzyme-linked immunosorbent assay (ELISA) kits (Abcam, Cambridge UK) according to the manufacturer's instructions.

2.13. Immunohistochemistry

For immunohistochemical evaluation of tyrosine hydroxylase and alpha synuclein in both SNc and striatum, animals ($n = 5$) were perfused through the aorta with PBS followed by 2% buffered formalin. After the brains were removed, they were post-fixed in 2% buffered formalin for 30 min, transferred into 30% sucrose solution and stored at 4 °C. Coronal sections were cut through the SNc and the striatum using a cryostat at 30 μ m and kept at 4 °C until processing. Sections were incubated with 70% methanol containing 2% hydrogen peroxide for 10 min to quench the endogenous peroxidase. Following this, the sections were incubated with primary antibodies (rabbit anti-tyrosine hydroxylase, 1:250; rabbit anti-alpha Synuclein, 1:250) for 90 min at room temperature. Antibody detection was performed with a universal polymer system containing specific primary antibody-detecting HRP-conjugated probe (BioCare Medical, USA) for 30 min and the immunoreaction was visualized by 3, 3'-diaminobenzidine (DAB) for 7 min. Sections were counterstained with haematoxylin, dehydrated in ascending grades of ethanol and finally cleared in xylene before they were mounted. Images were acquired with Leica DM 500 digital light microscope (Germany) with a mounted ICC50 E digital camera (Germany) at X100 and X400 magnification. Both striatal and SNc images were processed with Fiji-ImageJ (National Institute of Health, USA) to determine the staining optical density. The number of TH

positive neurons in the SNc was also determined with image J at X100. All analyses in image J were carried out using 5 sections per sample.

2.14. Immunofluorescence

Brain tissues from perfused animals were excised and placed in 2% buffered formalin for another 30 min before it was sectioned coronally to reveal striatum. Unspecific sites were blocked with 1% BSA solution in PBS-Triton-X-100 (PBST) solution for 1 h at room temperature (RT). Tissue sections were incubated with anti-COX-2 and anti-Caspase-3 primary antibodies (5 μ g/ml) dissolved in blocking buffer at RT for 90 min. Following this, the tissues were stained with NL-493-CONjugated secondary antibody (1:200) in the dark for 1 h at RT. Slides were washed and counterstained with 1 μ g/ml DAPI solution in PBS before being mounted with NPG mounting medium containing N-propyl gallate in glycerol-PBS solution. Sections were visualized with SP-98-FL inverted fluorescent microscope (Brunel Microscope Limited). Exposure settings and light intensity were uniformly applied to all the images. After acquisition, the images were taken to ImageJ software (NIH), segmented into individual channels, processed for background noise removal and quantification of fluorescence signal.

2.15. Statistical analysis

Data were analysed using one-way analysis of variance with Bonferroni post hoc test and presented as mean \pm standard error. Values were considered as statistically significant at $p < 0.05$.

3. Results

3.1. Kolaviron prevented locomotory impairment associated with rotenone-induced Parkinsonism

Exposure to rotenone significantly caused reduced locomotor ability as evident by reduction in total distance covered in the open field when compared with rats in both vehicle-treated and kolaviron control groups (Fig. 1A). Rotenone-treated rats additionally demonstrated decline in the recorded maximum and average speed, even when mobile (Fig. 1B–D), as well as reduced efficiency to maintain a straight path (Fig. 1E) and total mobile time (Fig. 1F). While rotenone significantly increased the total number of immobile episodes (Fig. 1G), latency to first immobile episode remained unchanged when compared with the vehicle-treated animals (Fig. 1H). However, kolaviron treatment reversed the rotenone-associated locomotory impairment. The modulatory role of kolaviron was more pronounced when kolaviron pre-treatment continued concurrently with rotenone challenge. Notably, kolaviron alone markedly enhanced path efficiency and increased the length of time before the animal experienced first immobility.

3.2. Kolaviron improved exploratory deficits in rotenone challenged rats

Similar to the locomotory profile, exploratory deficit was also observed in rats treated with rotenone. These rats were unable to explore the four walls of the open field apparatus (Fig. 2A). The rats also demonstrated poor hole recognition profile comparable to the control rats. The poor exploratory pattern was associated with reduced angle between each movement per metre (meandering; Fig. 2C) or per seconds (angular velocity; Fig. 2D), significant reduction in absolute head turn angle (Fig. 2E) and reduced efficiency to rotate clockwise (Fig. 2F) thereby culminating in reduced total distance travelled by head (Fig. 2G). On the other hand, kolaviron treatment for 18 days significantly protected the animals from rotenone-induced exploratory deficits with a remarkable increase in the walking traces in the open field and the number of poked holes.

(Fig. 2F) thereby culminating in reduced total distance travelled by head (Fig. 2G). On the other hand, kolaviron treatment for 18 days significantly protected the animals from rotenone-induced exploratory deficits with a remarkable increase in the walking traces in the open field and the number of poked holes.

3.3. Pre- and/or concurrent treatment with kolaviron ameliorated rotenone-mediated motor incompetence and neuromuscular dysfunctions

To determine whether the associated locomotory and exploratory deficit were associated with motor dysfunction we assessed neuromuscular functions using a battery of apparatuses. Fig. 3 shows the influence of kolaviron on the indices of motor and neuromuscular competence, such as negative geotaxis, grip test, freezing, grooming and rearing, in rats exposed to rotenone. Overall, rotenone induced severe motor incompetence and neuromuscular dysfunction in the animals compared with the control rats. Rotenone-challenged rats were inept to reorient against gravity on an inclined board within a limited time (Fig. 3A). The animals also demonstrated reduced tenacity to hang on a wire (Fig. 3B). Additionally, these rats showed enhanced freezing period characterized with minimal activity as evident by the considerably reduced grooming and rearing duration (Fig. 3C–E). However, kolaviron treatment preserved the behavioural deficit with evidence of recovery of functions. Kolaviron enhanced the motor coordination of the parkinsonian rats with increase in muscle/grasp strength and minimal rigidity. Above all, the magnitude of kolaviron effect was comparable with the control.

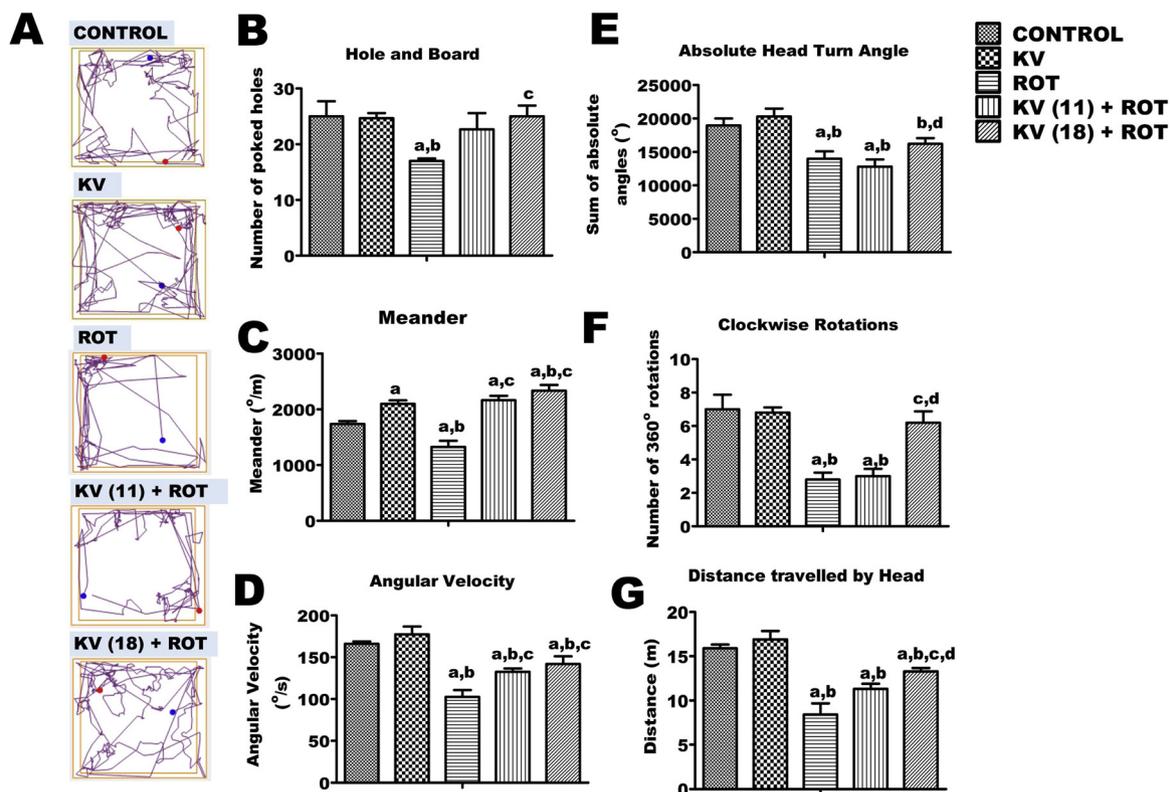


Fig. 2. Exploratory patterns of rats challenged and treated with rotenone and kolaviron, respectively. Exploratory profile of the experimental rats was determined with open field (A, C–G) and hole and board (B) apparatuses. Measured parameters include path travelled indicated on a track plot (A), number of poked holes (B), meandering (C), angular velocity (D), absolute head turn angle (E), clockwise rotations (F) and distance travelled by head. Except the track plots, values are presented as Mean \pm SEM ($n = 7$). a, b, c and d represent statistical differences ($p < 0.05$) when compared with the control, KV, ROT and KV (11) + ROT groups, respectively.

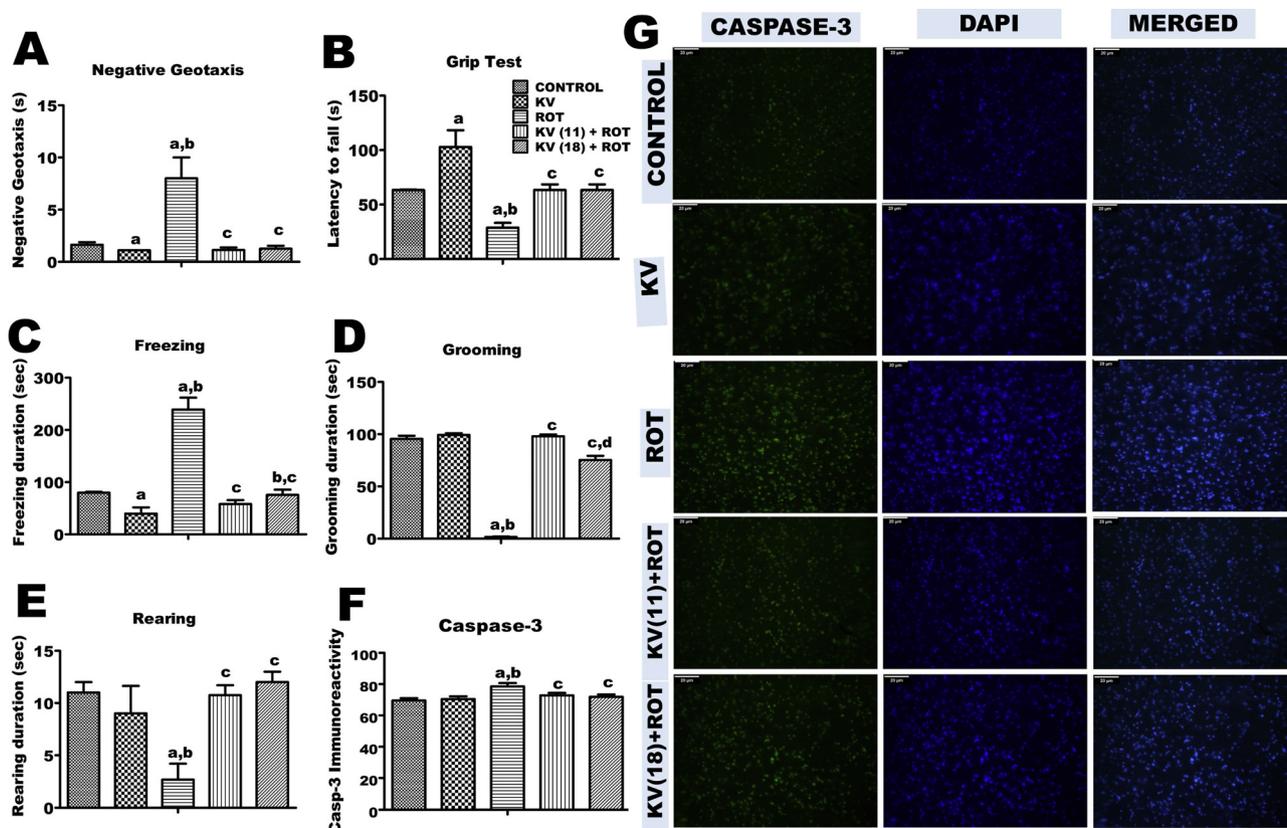


Fig. 3. Motor activities of rats challenged and treated with rotenone and kolaviron, respectively. Negative geotaxis (A), grasp strength (B) and durations of freezing (C) grooming (D) and rearing (E) in an open field were used as indices of motor and neuromuscular competence. Values are presented as Mean \pm SEM (n = 7). a, b, c and d represent statistical differences ($p < 0.05$) when compared with the control, KV, ROT and KV (11) + ROT groups, respectively. Representative immunofluorescent images of caspase-3 (n = 6) and its fluorescence intensities were presented in G and F, respectively. Scale bars = 20 μ m.

3.4. Kolaviron mitigated nigro-striatal neurodegeneration in rats exposed to rotenone

To establish a link between behavioural impairment and rotenone-associated degeneration of striatal neurons, we performed a range of post-mortem staining procedures for α -synuclein, tyrosine hydroxylase, and Caspase-3. Typical of rotenone intoxication, exposure to rotenone induced systemic toxicity resulting in rapid body weight loss (Fig. 4A) and a significant effect on the brain as indicated by reduced brain weight (Fig. 4B) when compared with KV and vehicle-treated control animals. We observed that administration of rotenone resulted in a significant upregulation of caspase 3 expression (Fig. 3F-G) and enhanced level of fragmented DNA in the striatum (Fig. 4C) while these were barely observed in the control and kolaviron alone groups. However, our results show that concurrent kolaviron treatment, particularly when additionally pre-treated prior rotenone intoxication, significantly prevented loss of body and brain weight. Caspase-3 immunoreactivity was significantly reduced in these KV-treated rotenone-challenged rats indicating protection from neurodegeneration associated with rotenone toxicity. Substantiating the evidence that rotenone induced degeneration of dopaminergic neurons in the nigrostriatal pathway, rotenone administration caused a marked loss of tyrosine hydroxylase immunoreactivity in both SNc and striatum (Fig. 4D). Rotenone resulted in about 50% and 75% reduction in SNc (Fig. 4E) and striatal (Fig. 4G) TH staining intensity, respectively and about 50% depletion of TH-positive cells in the SNc (Fig. 4F). However, the intensity analysis showed that KV pre-treatment preserved more than 70% and 75% of dopaminergic terminal in the striatum and TH-positive neurons in the SNc, respectively. While α -synuclein immunoreactivity was barely detected in striatal neuropil of KV- and vehicle-treated rats,

increased α -synuclein immunostaining was evident in the striatum of rotenone-exposed rats (Fig. 4G). However, α -synuclein expression was reduced significantly in the striatum of kolaviron-treated rats exposed to rotenone.

3.5. Kolaviron reversed the oxidative imbalance associated with rotenone

Assessment of oxidative and antioxidant status revealed obvious oxidative imbalance in the striatum of animals exposed to rotenone as presented in Fig. 5. This is characterized by elevated concentration of total reactive oxygen species as evident by sustained increase in reactive species-mediated DCF fluorescence, when compared with the control. While Fig. 5A shows the fluorescence plot for every value obtained at 30 s interval, the change in emission intensity of DCF fluorescence following H_2DCF oxidation over 10 min (emission at 10th minute minus that of 0th minute) is presented in Fig. 5B as percentage of control. Increase in striatal H_2O_2 production in the parkinsonian rats (Fig. 5C) substantiates the total reactive species results. In response to rotenone administration, activities of SOD (Fig. 5D) and CAT (Fig. 5E) were markedly enhanced by 75% and 49%, respectively. Damage to striatal lipids was increased by 24%, when compared to control (Fig. 5F). Rotenone exposure for 11 days also induced an increase of 85% in the level of reduced glutathione in the experimental animals (Fig. 5G). However, pre-treatment and/or co-administration of kolaviron significantly attenuated the oxidative imbalance and ameliorated the oxidative damage in the striatum of rotenone-exposed rats. Notably, a more remarkable effects of kolaviron on ROS generation, lipid peroxidation and catalase activity were observed when experimental rats were treated with kolaviron for a week before the 11-day rotenone-kolaviron co-administration. Additionally, rotenone activity altered the

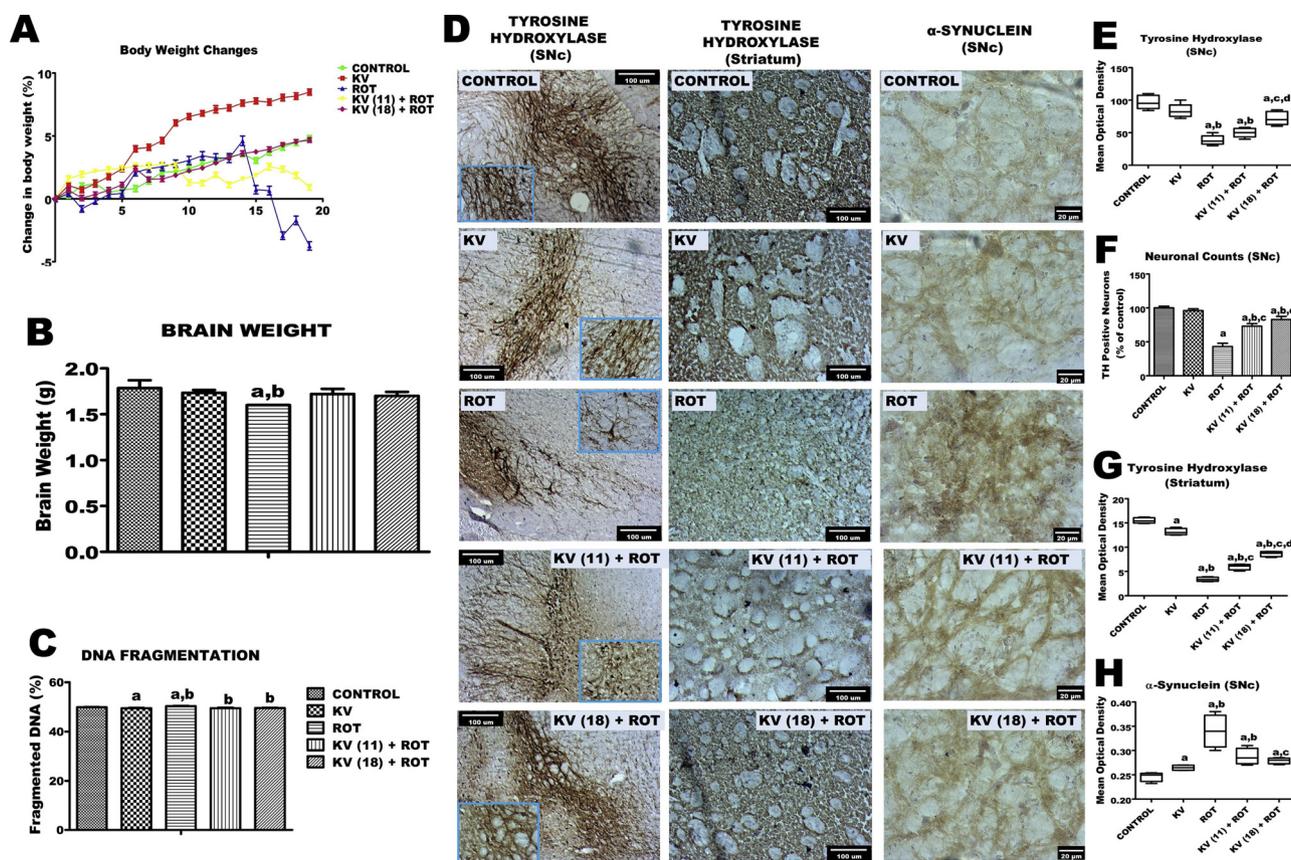


Fig. 4. Rotenone elicited characteristic features of Parkinson's disease and these were modulated by kolaviron treatments. Daily body weight changes (A) were monitored after kolaviron and rotenone treatments. On day 12 post rotenone challenge, whole brain was immediately weighed (B; $n = 9$), sectioned and processed for DNA fragmentation assay (C; $n = 5$). Some rats ($n = 6$) were perfused through the aorta and processed for tyrosine hydroxylase (TH) and α -synuclein immunohistochemistry. Quantification of TH immunoreactivity and TH-positive cells in SNc and striatum (E–G), and α -synuclein immunoreactivity in SNc (H) showing neuroprotective effect of kolaviron on rotenone-induced alterations. a, b, c and d represent statistical differences ($p < 0.05$) when compared with the control, KV, ROT and KV (11) + ROT groups, respectively. Scale bars: TH = 100 μ m; α -synuclein = 20 μ m.

activity of acetyl cholinesterase in the striatum of rotenone-exposed rats whereas kolaviron treatment modulated the induction (Fig. 5H).

3.6. Kolaviron mitigated rotenone-mediated striatal neuroinflammation

In the light of the established oxidative imbalance associated with inhibition of striatal and SNc tyrosine hydroxylase, we probed further the involvement of neuroinflammation in rotenone-induced striatal loss of function and also assessed the influence of kolaviron treatment. We detected an enhanced expression of COX-2 in the striatum of rotenone challenged rats, when compared to KV alone and sham treated groups (Fig. 6A–B). Corroborating the immunostaining result, we observed elevated level of nitric oxide and enhanced secretion of IL-1 β , IL-6 and TNF- α in the striatum of rats intoxicated with rotenone (Fig. 6C–E), when compared with the control, whereas pre- and/or co-administration of kolaviron significantly attenuated the generation of these pro-inflammatory mediators. Moreover, administration of rotenone significantly elevated nitric oxide level and enhanced striatal myeloperoxidase activity. However, kolaviron treatment significantly inhibited the rotenone-induced generation of nitric oxide and activation of myeloperoxidase. Meanwhile kolaviron treatment alone elicited marked inhibitory effect on the secretion of cytokines except on IL-6 level, which was comparable to the rotenone-intoxicated group.

4. Discussion

Although the precise mechanism underlying the pathogenesis of PD remains incomplete, oxidative stress and neuroinflammation have been

strongly implicated in the degeneration of dopaminergic neurons in the SNc leading to depletion of striatal dopamine (Heneka et al., 2014). Moreover, deficiency of striatal dopamine and direct assault to striatal neurons have been reported to result in Parkinsonism. More importantly, susceptibility of striatal terminals to degeneration have been shown to precede the death of SNc cell body and predispose substantia nigra to depletion of dopaminergic neurons (Herkenham et al., 1991; Wu et al., 2003). Meanwhile, the use of rotenone to induce PD has been faulted because of its non-selective toxicity and widespread inhibition of complex 1 in the brain parenchyma, even at low dose. However, rotenone still remains a preferred model that consistently simulate the neuropathological features of PD because of its ability to reproduce the progressive nature of PD with characteristic slowness of cell death and motor impairment and evidence of PD pathologic hallmark, α -synuclein – an intra-cytoplasmic inclusion (Zeng et al., 2018). This has given substantial advantage to the use of the model to assess a disease modifying therapeutic intervention relevant for PD.

The above-mentioned considerations have been supported by our findings as rotenone, despite its administration for 11 days, was found to elicit alterations in striatal cholinergic system, as evident by acetyl cholinesterase result, albeit with significant expression of α -synuclein and alterations in the antioxidant system in the rotenone-intoxicated rats. This suggests that rotenone administration for 11 days induced a damaging toxicity to nigrostriatal neurons, including the tyrosine hydroxylase-positive neurons, leading to striatal dopamine depletion, which may cause alterations in the striatal dopaminergic terminals and basal ganglia neurotransmission, the underlying mechanism responsible for the onset of akinesia, hypokinesia and bradykinesia. In

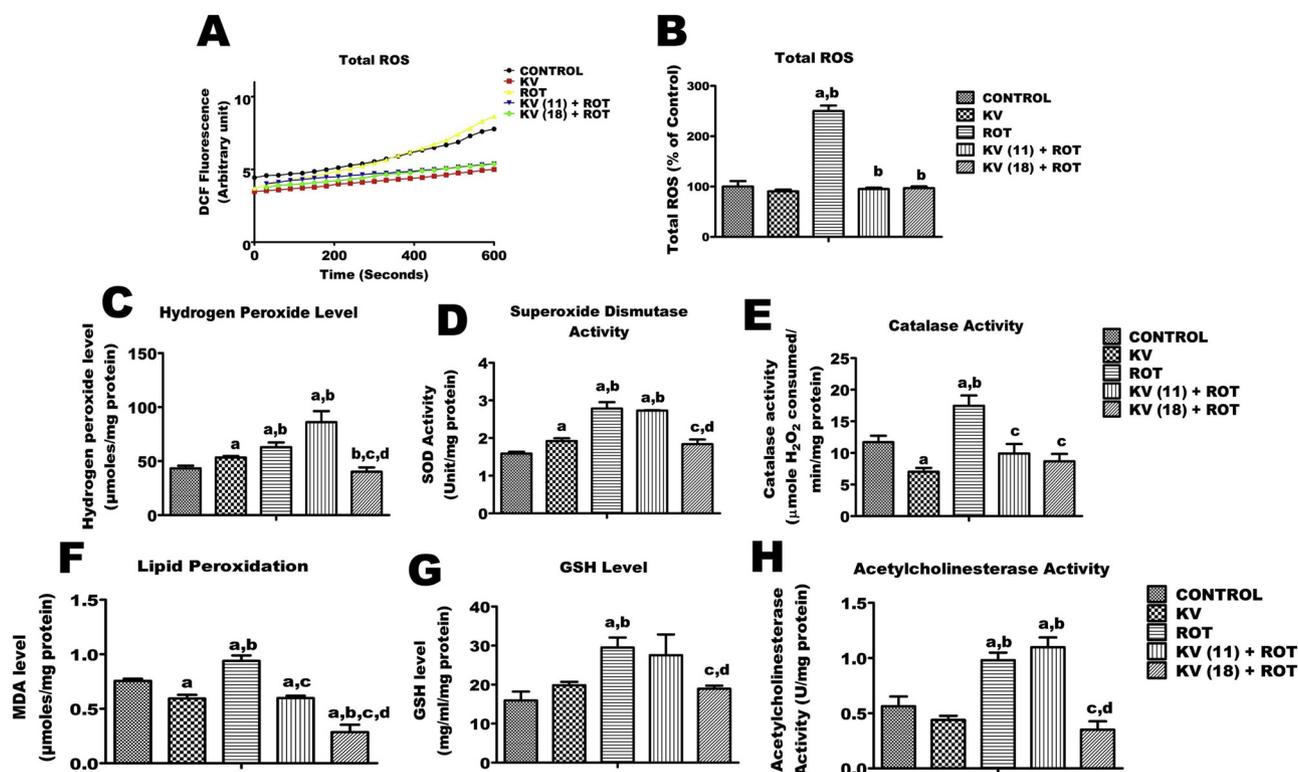


Fig. 5. Kolaviron restored the progressive striatal oxidative stress associated with rotenone administration in rats. Influence of kolaviron on rotenone induced oxidative stress was determined using striatal total ROS level (A–B), hydrogen peroxide concentration (C), superoxide dismutase activity (D), catalase activity (E), lipid peroxidative product (malondialdehyde) level (F), reduced glutathione level (G) and acetyl cholinesterase (H) as endpoints. Fig. 5A shows the fluorescence plot for every value obtained at 30 s interval. Fig. 5B shows the change in emission intensity of DCF fluorescence following H₂DCF oxidation over 10 min presented in Fig. 5B as percentage of control. a, b, c and d represent statistical differences ($p < 0.05$) when compared with the control, KV, ROT and KV (11) + ROT groups, respectively.

affirmation, rotenone intoxicated rats demonstrated locomotory impairment, exploratory deficits, and motor/neuromuscular incompetence. Kolaviron treatment remarkably increased the expression of tyrosine hydroxylase in the terminals of nigrostriatal neurons and mitigated rotenone-associated behavioural deficit and formation of α -synuclein.

Other studies have reported that marked inhibition of antioxidant enzymes and significant depletion of reduced glutathione are associated with rotenone intoxication, especially when administered for several weeks (Khatri and Juvekar, 2016; Ojha et al., 2015). However, the observed induction of both enzymic and non-enzymic antioxidant following 11-day rotenone challenge in this study signifies an adaptive response by striatal tissue as an attempt to counter the persisting rotenone assaults. Notably, co-existence of adaptive response with significant damage to neuronal biomolecules and resultant neuronal degeneration is an indication of the progressive nature of rotenone induced Parkinsonism, particularly at the early stage of the disease. Kolaviron mediated neuroprotection in the striatum by reversing the neurochemical alterations and biochemical imbalance while preserving the striatal cells from oxidative damage and subsequent neurodegeneration.

Evidence from neurotoxic animal model and post mortem tissue indicates that oxidative stress is partly responsible for neurodegeneration through mechanism associated with neuroinflammation and apoptosis but driven by microglia activation and striatal dopamine mismanagement (Kaur et al., 2017; Zhang et al., 2011). Although a previous study has demonstrated the inhibitory effect of kolaviron on LPS-associated microglial activation using BV2 cell (Onasanwo et al., 2016), the enhanced nitric oxide level and pronounced COX-2 expression observed in the striatum of rotenone intoxicated rats as opposed to minimal or no expression in the kolaviron treated groups is an

indication of striatal neuroinflammation (Hirsch and Hunot, 2009; Teismann et al., 2003). Increased concentration of secreted cytokines and reactive nitrogen species in the striatum of rotenone-exposed rats, which was remarkably reversed by kolaviron treatment, corroborate this observation.

In conclusion, few studies have reported kolaviron as a neuroprotective agent and the beneficial effects was attributed to its antioxidant and anti-inflammatory properties although majority of them focused on cortex, cerebellum and hippocampus (Igado et al., 2012; Olajide et al., 2017; Omotoso et al., 2018). Parallel to these studies, our data further confirmed the relevance of kolaviron in neurodegenerative disorders. In the present study, we have demonstrated with compelling evidence that behavioural deficit associated with rotenone intoxication in rats were markedly attenuated by kolaviron. In addition, we showed that kolaviron treatment mitigated the molecular processes and pathological features associated with Parkinson's disease in the striatum via mechanisms related to its antioxidant, anti-inflammatory and anti-apoptotic properties. Thus, our data suggest the relevance and usefulness of kolaviron in the prevention and management of neurodegenerative disorders, which present with behavioural impairment, including Parkinson's disease.

Conflict of interest statement

The authors declare no competing financial statements.

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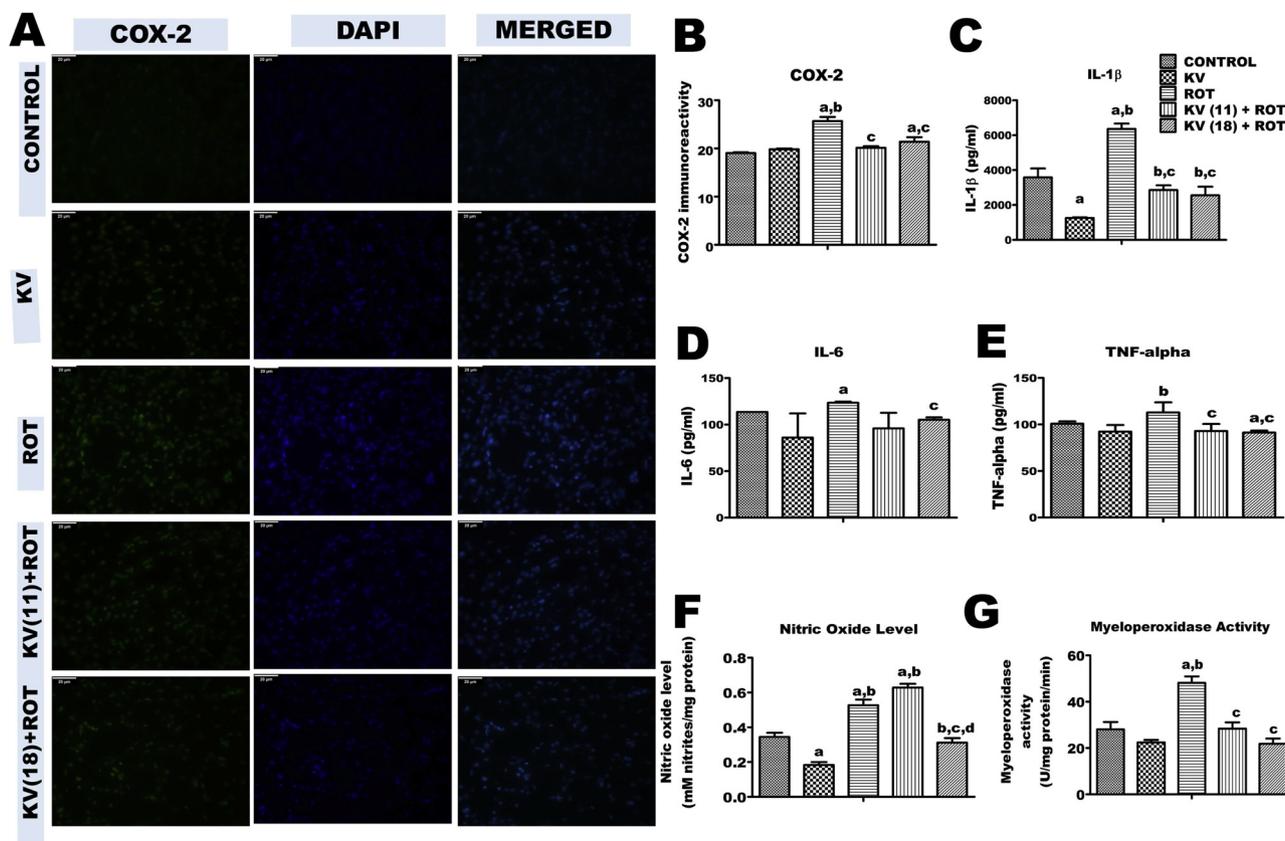


Fig. 6. Kolaviron treatment influences rotenone-induced striatal neuroinflammation. COX-2 immunostaining (A) and fluorescence intensity (B) in the striatum of kolaviron-treated and rotenone exposed rats showed the neuroprotective effect of kolaviron. levels of IL-1 β (C) IL-6 (D), TNF- α (E) and nitric oxide (F) as well as activity of myeloperoxidase (G) and were also determined in striatal homogenate. a, b, c and d represent statistical differences ($p < 0.05$) when compared with the control, KV, ROT and KV (11) + ROT groups, respectively.

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