The Protective Effect of Kolaviron On Molecular, Cellular and Behavioral Characterization of Cerebellum in Rat Model of Demyelinating Diseases

Running Title: Kolaviron and cerebellar demyelination

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To appear in: Basic and Clinical Neuroscience

Received date: 2018/03/21

Revised date: 2018/10/7

Accepted date: 2018/11/27

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Please cite this article as:


DOI: 10.32598/bcn.9.10.300
Abstract

**Purpose of Study:** This study was aimed at assessing the protective mechanisms of Kolaviron on the cerebellum in a rat model of demyelination.

**Methods:** Twenty-eight male Wistar rats were used for the study. They were randomly divided into 4 groups and each group had 7 rats. Group A (control) received corn oil (0.5 ml/kg/day), Group B received 0.2% cuprizone (cpz), Group C was treated with 200 mg/kg/day of Kolaviron (kv), while Group D received 0.2% cuprizone (cpz) and 200 mg/kg/day Kolaviron (kv), for 6 weeks. Cuprizone powder was mixed with the regular diet while kv was dissolved in corn oil and administered orally. Behavioral test was conducted at the termination of the experiment. Thereafter, the animals were sacrificed and their brains were removed with excision of the cerebellum. Part of the cerebelli underwent tissue processing with a series of 5 µm thick sections cut from paraffin blocks for histological and immunohistochemical assessment, while the remaining cerebellar tissues were homogenized for spectrophotometric assays of oxidative stress parameters.

**Results:** Findings revealed minimal weight gain following CPZ treatment, but significant weight increase in kolaviron-treated rats. CPZ treatment was associated with reduction in number of line crossed, rearing frequency, rearing duration, center square entry and center square duration, but increase in freezing time, which were reversed significantly in kolaviron-treated animals. Oxidative markers such as SOD and GPx were reduced in CPZ-treated rats with elevated MDA level. However, these were reversed significantly by co-administration of CPZ with kolaviron. At the tissue level, the cerebellar cortex was characterized by poorly defined layers, cryptic granules, chromatolytic and pyknotic Purkinje cells with evidence of hypertrophic astrogliosis.
Conclusion: CPZ treatment significantly depresses locomotor and exploratory activities as well as increases oxidative stress and cerebellar toxicity; however, kolaviron intervention significantly enhances behavioral functions, ameliorates CPZ-induced cerebellar degeneration and considerably regulates oxidative stress markers in the cerebellum of rat model of demyelinating diseases.

**Keywords:** demyelinating disease, cerebellum, cuprizone, kolaviron, oxidative stress
1. Introduction

Demyelination is a condition that causes loss of myelin with relative preservation of axons. It results from diseases that damage myelin sheaths or the myelin-forming cells of the central nervous system (CNS), oligodendrocytes. Loss of myelin is considered as the cause of many diseases of the CNS (Love, 2006). Demyelination or focal white matter injury can occur in any location in the central nervous system (Ahmad, Satriotomo, Fazal, Nadeau & Doré, 2015). The most common form of demyelination is seen in multiple sclerosis (MS). Although the volume of white matter lesions in the cerebellum is small compared to other parts of the CNS, the lesions are detrimental to health (Ahmad et al., 2015). Recent insight into the function of the cerebellum indicates that the cerebellum not only control movement but also plays role in motor learning, cognitive behavior, sensation and adaptation following injury due to changes in the strength of the connections among its neurons (Houk and Miller, 2001; Diedrichsen and Bastian, 2014). It was reported that the defining clinical manifestation of MS is associated with cerebellar demyelination (Weinshenker, Issa & Baskerville, 1996; Rot, Ledinek & Jazbec, 2008). Despite these interesting findings, there is paucity of information regarding the molecular, cellular and behavioral characterization of the cerebellum in demyelinating diseases. Understanding such characterization could help identify the underlying mechanism involved in demyelination and potential therapeutic targets.

Due to the rise in the prevalence of some demyelinating diseases, various animal models have been used to study and find preventive measures or possible cure for these conditions. The cuprizone model, a toxin-induced demyelination, has been frequently used, as it represents a reversible demyelination and remyelination system (Kipp, Clarner, Dang, Copray & Beyer,
Cuprizone [oxalic acid bis(cyclohexylidenehydrazide)] (CPZ), is a copper chelator that induces demyelination in regions where white matter are located in the CNS of rodent (Sachs et al., 2014). Its underlying mechanism remains controversial; however studies have reported that rodents fed with cuprizone diet had megamitochondria, elevated level of free radicals due to (oxidative stress), an uncoupling of the oxidative phosphorylation process, oligodendrocyte apoptosis, disturbance of neurotransmitter homeostasis, synaptic dysfunction and axonal degeneration (Kipp et al., 2009; Norkute et al., 2009; Hesse et al., 2010; Skripuletz, Gudi, Hackstette & Stangel., 2011; Tandler and Hoppel, 1973; Wakabayashi, Asano, & Kurono, 1975).

Although cuprizone toxicity targets oligodendrocytes, other macroglia especially astrocytes could possibly be affected.

Astrocytes are specialised glial cells which provide structural and functional support for neurons (Şovrea and Boşca, 2013). They are involved in synaptogenesis, and in regulating the communication between already formed synaptic connections (Ota, Zanetti and Hallock., 2013). Astrocytes participate in the control of brain homeostasis, and the intrinsic brain defense system (Kettenmann and Verkhratsky), making them respond whenever an insult to the brain occurs.

On the contrary, treatment and management of complex demyelinating diseases could possibly be achieved through the use of the phytochemical constituents of certain plants with medicinal values (Omotoso, Gbadamosi, Afolabi, Abdulwahab & Akinlolu, 2018). Kolaviron (Kv) is one of such phytochemicals due to the properties attributed to it (Olaleye and Farombi, 2006; Farombi, Abarikwu, Adedara & Oyeyemi, 2007). Kv is a biflavonoid complex isolated from the seed of *Garcina kola* (bitter kola). The plant is used as herbal remedy for treating several disease.
conditions due to its antiviral, antibacterial, antifungal and analgesic properties, amongst other activities (Olaleye and Farombi, 2006; Farombi et al., 2007). Kolaviron inhibits excessive production of nitric oxide (NO), expression of cell death regulatory protein and neuronal cytoskeletal dysregulation (Olajide et al., 2016). The main aim of the study was to assess the cellular, molecular and behavioral changes associated with cuprizone toxicity in the cerebellum of Wistar rats, following kolaviron intervention.

2. Materials and Methods

Twenty eight male Wistar rats, 9 weeks old, were purchased from a private Animal Holding, Tanke, Ilorin. The rats were housed in the Animal House of the Faculty of Basic Medical Sciences, University of Ilorin and were allowed to acclimatize for 7 days before the onset of the experiment. They were fed on rat chow and water ad libitum. Ethical approval was sought for and obtained from the College of Health Sciences Ethical Committee, University of Ilorin, Nigeria. Animal handling and protocols were carried out according to the prescribed guidelines of the Ethical Committee. *Garcinia kola* seeds were procured from a market in Ilorin, Nigeria and verified at the herbarium of Botany Department, Faculty of Life Sciences, University of Ilorin with verification number ‘UILH/001/1217’. Cuprizone was procured from Sigma-Aldrich (Germany), while phosphate buffered solution (PBS; pH 7.0) was freshly prepared. Superoxide Dismutase (SOD), Glutathione Peroxidase (GPx) and Malondialdehyde (MDA) assay kits were purchased from Abcam®, USA. Rats anti- Glial Fibrillary Acidic Protein (GFAP) were acquired from Cell Signaling Technologies, Massachusetts, USA.

2.1 Isolation and identification of Kolaviron from *Garcinia kola*
The methods utilized in the isolation and identification of kolaviron were as earlier described by Farombi, Shrotiya, & Surh (2009) and Olajide et al. (2017). These involved drying of *Garcinia kola* seeds at room temperature, pulverization, extraction as well as the assessment and confirmation of the purity and identity of the extract (kolaviron) obtained from these processes.

### 2.2 Animal Grouping and Treatment

The rats were randomly grouped into four (4) classes labeled as A-D (n = 7). Group A received 0.5 ml of corn oil (CO) and served as the Control, while group B received 0.2% CPZ diet (Praet *et al*., 2015), group C received 200 mg/kg bw of Kv (Farombi *et al*., 2009; Omotoso, Olajide, Gbadamosi, Rasheed & Izuogu, 2018), and group D received both Kv (200 mg/kg bw) and CPZ (0.2%) diet. Corn oil served as the vehicle in which Kv was dissolved for ease of oral administration. Cuprizone diet was constituted to 0.2% by mixing 0.2 g CPZ with 100 g standard rat diet. Treatment lasted for 42 days.

### 2.3 Behavioral Study

The open field test was carried out to determine the locomotor activity and exploratory behaviours of rats. Each rat was placed at the center of an open-field box (Yan *et al*., 2015) and tested for 10 min; the rats in this location usually have freedom of movement. The activities of each rat were recorded by a video-camera situated above the area. Following the completion of the exercise, the following data were obtained: number of line crossed (NLC), center squared duration (CSD), center square entry (CSE), freezing duration (FD), rearing frequency (RF) and rearing duration (RD).

### 2.4 Tissue Preparation
Sequel to last administration and behavioral study, the rats meant for histological and immunohistochemical evaluation were anesthetized using 20 mg/kg bw ketamine intraperitoneally and subsequently perfused transcardially with 0.4 M phosphate buffer (PBS) followed by 4% paraformaldehyde (PFA). The brain tissues were thereafter excised, rinsed in 0.25 M sucrose solution thrice for 5 min each and then post-fixed in 4% PFA for 24 hours. The cerebella were excised and processed routinely to obtain paraffin wax-embedded blocks. The tissues were stained using Haematoxylin and Eosin and Cresyl fast violet, as described by Fischer, Jacobson and Rose (2005) and Bancroft and Stevens (1982) respectively. The rats used for enzyme studies were sacrificed by cervical dislocation. Their brains were immediately removed and rinsed in 0.25 M sucrose solution thrice for 5 min each and thereafter placed in 30% sucrose at 4°C.

2.5 Immunohistochemistry

Serial sections of the cerebellum were taken from paraffin blocks and processed as earlier described by Olajide et al. (2017). Immunohistochemistry was carried out according to the method described by Goldstein and Watkins (2008), while the tissues were stained with anti-glial fibrillary acidic protein (GFAP) antibody (as primary antibody) and thereafter treated with biotinylated secondary antibody (goat anti-rabbit) in order to detect the GFAP-positive cells (astrocytes) in the cerebellum. The mounted slides were viewed with the aid of an Olympus binocular research microscope (Olympus, New Jersey, USA) connected to an Amscope Camera (5.0 MP).

2.6 Preparation of Tissue Sample for Biochemical Analyses
Cerebellar tissue homogenate was prepared with cold 0.25 M sucrose solution using an automated homogenizer at 4°C. The tissue homogenate was centrifuged for 10 min in a microcentrifuge with a centrifugal force of 16099 ×g. The supernatants obtained were thereafter aspirated into plain bottles and analysed for the activities of superoxide dismutase (SOD), glutathione peroxidase (GPx) and malondialdehyde (MDA) according to the manufacturer’s instruction in the biochemical kits.

2.7 Data Analysis

All quantitative data were analyzed using the GraphPad Prism ® software (version 6). The data obtained were presented as mean and standard error of mean, using ANOVA with Tukey’s multiple comparisons test. Statistical significance was also determined, with p values less than 0.05 considered to be significant.

3. Results

3.1 Physical Observation and Weight Change

During the course of this study, the feeding pattern among rats treated with cuprizone reduced during the first two weeks of treatment, however their feeding pattern gradually returned to normal. Also those given Kolaviron experienced an increase in eaten habit but after the fourth week of administration their feeding habit dropped slightly. The animals treated with both cuprizone and Kolaviron showed increase feeding pattern during the early stage of administration however there was observable gradual decline in their feeding pattern. Animals treated with CPZ showed no appreciable weight gain (Fig. 1A and B). The group treated with Kv and those that received Kv and CPZ concomitantly showed a significant weight gain when...
compared with the group that was treated with CPZ only. However, when compared with the control group, there was a reduction in weight (Fig. 1A and B).
Figure 1: Weights of animals

Fig. 1A

Fig. 1B
3.2 Kolaviron Prevented Cuprizone-induced Locomotor and Exploratory Deficits

The outcomes of the open field test performed suggested that demyelinating and degenerative changes seen in CPZ caused a decrease in locomotor and exploratory activities (p< 0.001). Kolaviron treatment was found to be protective against cuprizone-induced behavioural deficits, which recorded a change in locomotor and exploratory activities when compared to the CPZ group (Fig 2).

Figure 2: Behavioral tests for locomotion and exploratory activities

(A) (B) (C)

(D) (E) (F)
3.3 Inhibition of cerebellar oxidative toxicity and enhancement of antioxidant defense system by Kolaviron

The activities of endogenous oxidative enzymes were assessed to show their involvement in cuprizone-induced demyelination and to understand the mechanistic inhibitory roles of Kv. Results from spectrophotometric assay of SOD profile from cerebellar homogenates showed a normal SOD level in the cerebellum of rats in the control and Kv groups with significant increase seen in group C. The SOD level significantly reduced in rats intoxicated with CPZ when compared with other groups. However, rats that received both CPZ and Kv concomitantly showed a significant increase in SOD level when compared with the group that received CPZ only (Fig. 3A). The GPx level of cerebellar lysates was examined (Fig. 3B) and findings revealed that the control and kolaviron-treated rats had elevated GPx levels compared to rats treated with CPZ and a combination of CPZ and Kv. Furthermore, animals co-treated with CPZ and Kv recorded a higher GPx level compared with the CPZ-treated rats. MDA level was also assessed to determine the degree of lipid peroxidation in cerebellar lysates across groups. The results (Fig. 3C) showed that Kv-treated rats had decreased expression of MDA in the cerebellar homogenate, while CPZ-treated had increased MDA level when compare to the other groups. The rats treated concomitantly with Kv and CPZ showed a significant downregulation of MDA expression when compared to the group that was treated with CPZ only.
Figure 3: Assessment of biochemical oxidative markers (superoxide dismutase, SOD; glutathione peroxidase, GPx and malondialdehyde, MDA)

![Bar charts showing SOD, GPx, and MDA levels in different groups.](image)

Figure 3 (A-C)

3.4 Kolaviron prevents demyelination, endoplasmic reticulum stress and cytoarchitectural degeneration induced by cuprizone intoxication

Histochemistry of the cerebellum was demonstrated using H & E (Haematoxylin and Eosin) and CFV (cresyl fast violet) staining methods. Figure 4 showed panoramic view and high power magnification of the microarchitecture of cerebellar cortices with distinct cell types and cell layers. Both the Control and Kv-treated rats had well arranged cerebellar layers with obvious soma and dendrites that project deep into the molecular layers, having a fan-like shape nucleus with well-stained white matter regions. Also the granular cell layers in the groups comprise of well-arranged small granule cells. Neuronal morphology of rats intoxicated with CPZ showed fragmented cerebellar layers with cryptic granules, degenerating Purkinje cells with pyknotic cell
bodies and short dendritic processes. However, rats treated with both CPZ and Kv showed cerebellar layers and neuronal morphology that are similar to that of the control groups.

**Figure 4: Histological demonstration of the cerebellar cortex of rats**

![Histological demonstration of the cerebellar cortex of rats](image)

**Figure 4**: Haematoxylin and Eosin (Magnification x40 and x400). Olympus binocular research microscope (Olympus, New Jersey, USA)
Figure 5: Histochemical demonstration of the cerebellar cortex and Immunohistochemical expression of astrocytes

Figure 5: A- Cresyl fast violet (Magnification x40 and x400); B: GFAP= glial fibrillary acidic protein immunohistochemical staining with anti-GFAP primary antibody (Magnification x400). Olympus binocular research microscope (Olympus, New Jersey, USA)
3.5 Kolaviron Prevents Cuprizone-Induced Cerebellar Astrogliosis

GFAP immunohistochemistry method was used in this study to demonstrate astrocytic morphology and distribution in the cerebellar cortex of Wistar rats. GFAP immunopositive cells within the cerebellar cortex of Control and Kv-treated rats appeared scanty around neurons and in between layers, with regular processes, distribution and sizes within the neuropil. However, increased astrocytic densities with reactive astroglia within granule cell layer and hypertrophic cells appeared within the cerebellar layers in CPZ treated rats. The expression of astrocytes within the cerebellar cortex of rats treated with Kv and CPZ had close similarities with the Control and those treated with Kv only. Astrocytic processes, cellular distribution and size were normal in Control and Kv-treated rats.

4. Discussion

The maintenance of body weight is dependent on normal energy flow and energy disruption is associated with the mechanism involved in cuprizone-induced toxicity (Gudi, Gingele, Skripuletz & Stangel, 2014). Eating and diets are important factors to consider in controlling body weight (Drapeau et al., 2004). Reduced feeding habit during the first 2 weeks of CPZ consumption and gradual improvement in feeding habit after CPZ diet withdrawal have been reported (Sachs et al., 2014; Praet et al., 2015; Steelman et al., 2015). Therefore, it is suggestive that the observed body weight reduction in this study was caused by reduced feeding pattern and disruption of energy normal flow. Kv treatment significantly prevented CPZ-associated weight
loss, probably by its ability to prevent normal energy disruption owing to its ability to mop up excess free radical that might be the reason for the disruption.

As a correlative test for cellular and neuropathological changes in the cerebellar cortex in this study, exploratory activities of rats were assessed using the open field test (Gould et al., 2010). Our results showed a significant reduction in exploratory activities in CPZ-treated rats, corroborating a previous study by Faizi et al. (2016). However, kolaviron significantly countered these effects. Therefore, it is deduced that locomotor and exploratory activities are dependent on intact integrity of myelin sheath. Report of increase in exploratory activities of rat following Kolaviron treatment after NaN₃-induced neurodegeneration has been documented (Olajide et al., 2016).

Decrease in SOD and GPx levels, as observed in the current study, has been implicated in the mechanism through which cuprizone induces demyelination (Biancotti, Kumar & de Vellis, 2008; Witherick et al., 2010; Praet et al., 2014). Faizi et al. (2016) reported a decrease in SOD level following administration of CPZ due to the reduced level of Cu²⁺ that was needed for proper functioning of SOD. Kv treatment improved these antioxidants’ levels within the cerebellar cortex of rats. SOD catalyzes the dismutation of superoxide (O₂⁻) radical (Sun and Trumpower, 2003; Hayyan et al., 2016); if upregulated by Kv, it could prevent the cytotoxic effects of O₂⁻ molecules, thereby preventing oxidative related damage. Glutathione peroxidase (GPx) is an enzyme capable of detoxifying reactive oxygen species (ROS) and nucleophilic compounds that have the ability to initiate lipid hydroperoxidases to their corresponding alcohols and water respectively (Muller et al., 2007). Lipid peroxidation and oxidative stress following CPZ treatment has been previously documented (Xuan et al., 2015). However, treatment of rats
with Kv showed a significant reduction in MDA level, suggesting that Kv was able to inhibit lipid peroxidation due to its antioxidant properties.

CPZ-induced cerebellar injury showed various degrees of structural damage to the cellular components and the normal architectural pattern of the cerebellum. These alterations in cellular morphology of the cerebellum following CPZ treatment adversely affects signal processing and the functionality of the synaptic complex and these are common features in patients with demyelinating diseases (Kutzelnigg et al., 2007; Rot et al., 2008).

Furthermore, CPZ intoxication causes the formation of megamitochondria and oxidative stress leading to energy flow disruption and shortage (Tandler and Hoppel, 1973). The energy flow disruption and depletion causes alteration in the proper functioning of the endoplasmic reticulum, which is important in neuronal protein synthesis. This often causes cellular degeneration and disintegration of oligodendrocytes perikaryon and myelin sheath (Praet et al., 2014). The neurotoxic nature of CPZ may explain the mechanisms by which it induces cellular degeneration and white matter demyelination. Interestingly, rats given kolaviron concomitantly showed cerebellar morphology that was similar to the Control. The ability of Kv to restore the chromatogenic nature of the Nissl substance following CPZ-induced damage might be due to its ability to prevent ER stress and inhibit the pathways that leads to failure of protein production owing to its antioxidative property and ROS scavenging ability.

Studies have shown an increase in astrocytic activity following one week CPZ intoxication (Zatta et al., 2005). Activation of astrocytes could result in extensive astrogliosis which may persist through the period of remyelination (Zaaraoui et al., 2008; Gudi et al., 2009). In the current work, changes observed on the cerebellum were characteristic of reactive astrogliosis,
which were however absent in the cerebellar cortex of rats treated with kolaviron, suggesting a cytoprotective role for kolaviron.

In conclusion, Kolaviron exhibited neuroprotection against behavioural deficit, oxidative stress, astrogliosis, demyelination and cortical neuronal damage induced by cuprizone in adult male Wistar rats. These findings provide a lead on potentials of kolaviron as a neuroprotective agent against demyelinating diseases.

**Acknowledgments**

The authors acknowledged the assistance and guidance of Dr O.J. Olajide of the Department of Anatomy, University of Ilorin. There was no financial supporter to this study.

**Conflict of Interest**

The authors hereby declare that there is no conflict of interest attached to this paper, nor any financial sponsorship received from any organization in support of this work.
Reference


http://dx.doi.org/10.1155/2013/185463.


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Legends

Figure 1: Body weight changes across treated groups: A = control, B = cuprizone, C = Kolaviron and D = cuprizone plus Kolaviron. Figure 1A showed the initial and final weights, with all the groups having increased weight difference, though the CPZ-group had very minimal weight difference. In Figure 1B, the control group (A) had the highest weight gain, while the CPZ-treated group (B) had the least weight gain with statistically significant difference compared to the control (p<0.001). Weight gain in Kv-treated group (C) was also high with statistically significant difference compared with the control (p<0.01); the difference between the Kv-treated group and CPZ-treated group was statistically significant (p<0.001); weight gain in CPZ+Kv group (D) was significantly higher than the CPZ-only group (B) (p<0.05), but lower than the control group (A) (p<0.001) and Kv-treated group (C) (p<0.01). Key: * = p <0.05, ** = p <0.01 and *** = p<0.001).

Figure 2 (A-F)

Figure 2: Locomotor and exploratory activities of rats across the groups. A = control, B = cuprizone, C = Kolaviron and D = cuprizone and Kolaviron. In comparison with CPZ and CPZ+Kv treatment groups, animals given kolaviron treatment had increase in number of line crossed (A) (p<0.001; p<0.01 respectively), rearing frequency (B) (p<0.001: Kv versus CPZ groups), rearing duration (C) (p<0.001; p<0.05 respectively), center square entry (D) (p<0.01: Kv versus CPZ groups)) and center square duration (E) (p<0.001: Kv versus CPZ groups), but there was a decrease in freezing time (F) when compared with the groups treated with cuprizone and Kv only (p<0.001; (p<0.05 respectively). Animals simultaneously treated with CPZ and
Kolaviron had generally significantly improved number of line crossed (A) \( (p<0.05) \), rearing frequency (B) \( (p<0.05) \), rearing duration (C) \( (p<0.01) \), center square entry (D) \( (p<0.05) \) and center square duration (E) \( (p<0.01) \) compared with the CPZ groups.

Figure 3A showed statistically significant decrease in superoxide dismutase (SOD) activity in CPZ-treated group B compared with the control \( (p<0.001) \), Kv-treated group C \( (p<0.001) \) and CPZ+Kv treated group D \( (p<0.05) \); Kv-treated group had the highest SOD level with statistically significant difference compared with the control \( (p<0.01) \) and CPZ-treated group B \( (p<0.001) \); while CPZ+Kv treated group D had a significant increase compared to CPZ-treated group B \( (p<0.05) \), and a significant decrease compared to the control \( (p<0.01) \) and Kv-treated groups \( (p<0.001) \).

Figure 3B showed the activity of glutathione peroxidase (GPx) across the groups. GPx activity was least in CPZ-treated group compared to the control \( (p<0.001) \), Kv-treated groups \( (p<0.001) \) and CPZ+Kv treated group D \( (p<0.05) \). Co-treatment of CPZ + Kv led to significant increase in GPx level when compared with CPZ group \( (p<0.05) \), though not reaching the enzyme level in Kv group \( (p<0.001) \).

In Figure 3C, the level of malondialdehyde (MDA) was significantly elevated in CPZ group compared with control \( (p<0.001) \) and Kv group \( (p<0.001) \), whereas simultaneous administration of cuprizone and kolaviron led to increased MDA level compared to the control \( (p<0.05) \) and Kv group \( (p<0.001) \) which had the least MDA level. However, CPZ+Kv group had a lower level of MDA compared with the CPZ-treated group, though not statistically significant.
**Figure 4:** Representative photomicrographs of the histology of the cerebellar cortices of rats. The different cortical layers were demonstrated: molecular cell layer (M), Purkinje cell layer (P), granule cell layer (G) and the medullary layer of white matter (W). The structure of the cerebellar cortices of the Control and Kv-treated rats appeared relatively normal. CPZ treatment resulted in fragmented granule cell layers and neuropils, cryptic granule cells, which were loosely arranged, degeneration of Purkinje cells and presence of short dendrites. Neuronal morphology and cerebellar layers in rats co-treated with Kv and CPZ appeared normal with the presence of neurons with normal somas, axons and dendrites. (H & E x40 and x400)

**Figure 5 (A and B):** Representative photomicrographs showing the Nissl profile (A) and immunohistochemical demonstration of astrocytes (B) using CFV and anti-glia fibrillary acidic protein (GFAP) respectively. **Fig. 5A** showed both panoramic view and high power magnification of the cerebellar cortex stained with CFV across groups. Rats treated with either CO (A) or Kv (C) showed well stained intensity and cellular density within granular layer and white matter layer with deeply stained and well expressed Purkinje cells (yellow dotted circles) and granule cells (yellow arrows). Rats fed with CPZ (B) presented poorly stained intensity and cellular density within the granular layer with chromatolytic Purkinje cells (red dotted circles) and granule cells (red arrows). However, rats treated with both CPZ and Kv showed improvement in the staining intensity and cellular density, also the deeply stained and well expressed Purkinje cells (yellow dotted circles) and granule cells (yellow arrows). GFAP immunopositive cells (**Fig. 5B**) (black arrows) appeared scanty around neurons in the Control and Kv-treated groups, with relatively normal architecture. CPZ-treated rats showed evidence of
increased astrocytic densities with reactive astroglia within the granule cell layer and hypertrophic cells. Meanwhile, the rats co-treated with CPZ and Kv had close similarities with those of the Control and Kv-treated groups. (Magnification CFV = x40 and x400; GFAP = x400)