EFFECT OF ETHANOLIC EXTRACT OF *ALTERNANTHERA SESSILIS* IN CUPRIZONE-INDUCED DEMYELINATION IN WISTAR RATs

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Abstract

One of the most prevalent neurological disorders is multiple sclerosis (MS). In this condition, the immune system assaults the central nervous system's oligodendrocyte cells and myelinated neurons, destroying them. These ailments cause poor nerve impulse conduction and show symptoms like weakness, exhaustion, and visual and movement problems. The purpose of this study was to determine whether the ethanol extract of *Alternanthera sessilis* Linn. (EEAS) could mitigate the behavioural and histological alterations in male rats brought on by Cuprizone. By administering 0.4% Cuprizone (CPZ) by oral gavagefor six weeks, demyelination was induced. For the latter two weeks of treatment, EEAS 200mg/kg was administered once daily. In comparison to the control group, therapy with CPZ caused weight loss over the course of 6 weeks, while EEAS administration stopped. A decline in motor coordination and balance was seen in the group receiving CPZ treatment in behavioural tests (pole test, narrow beam test and y-maze) (P 0.01). These motor impairments have improved with EEAS treatment over the past two weeks. The CPZ group showed increased demyelination, which was reduced by the use of EEAS, according to histopathological investigation.

Keywords: Multiple sclerosis, cuprizone, myelin sheath, fatigue, visual and motor disorders

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1.0 Introduction

Axons covered by a unique sheath of ectodermal origin make up nerve fibres. The brain, spinal cord, and peripheral nerves are made up of tracts of nerve fibres. Depending on whether they are a member of the central or peripheral nervous system, nerve fibres have different surrounding sheaths. Oligodendrocytes and Schwann cells serve as the sheath cells in peripheral and central nerve fibres, respectively (Junqueira Lc et al., 1995). Myelin sheaths surrounding the axons of the brain and spinal cord are destroyed in the inflammatory autoimmune illness known as multiple sclerosis (MS), which results in demyelination (Compston A and Coles A, 2002, Hurwitz Bj, 2009, Polman Ch et al., 2010). Myelin's role in the central nervous system (CNS) is to accelerate saltatory axonal conduction, which speeds up electrical impulse conduction. Numerous clinical and biochemical characteristics of multiple sclerosis are explained by the consequence of demyelination for saltatory conduction. Axons that have lost some myelination conduct impulses more slowly. Experimental autoimmune encephalomyelitis (EAE), the Theiler's virus, murine hepatitis virus, and toxic models of demyelination using lysolecithin, ethidium bromide, and cuprizone are at least three animal models for demyelination (Rodriguez M 2007). A powerful copper chelator is cuprizone (CPZ), bis-cyclohexanone oxaldihydrazone. In the CPZ model, oligoden-drocytes in the animals are fed CPZ, which results in consistent demyelination (Torkildsen O et al., 2008, Morell P et al., 1998, Matsushima Gk and Morell P 2001, Franco-Pons N et al., 2007, Adamo Am et al., 2006). Additionally, cuprizone elimination from an animal's diet triggers remyelination (Morell P et al., 1998). As a result of several CPZ experiments, a mouse MS model was produced (Skripuletz T et al., 2008, Lindner M et al., 2008), but conflicting results for rat MS models have been reported by researchers. For instance, Adamo et al discovered demyelination in the corpus callosum (CC) of Wistar rats (Adamo Am et al., 2006) despite Love S. notifying that the CPZ model did not operate at the Wistar rat strain. Additionally, a few recent research (Love S 1998, Franco P et al., 2008, Silvestr off L et al., 2012, Kanno T et al., 2012) describe the CPZ model in Wistar rats. However, Love S. asserted that Wistar rats could not develop central demyelination whereas other rat strains could. In every prior Wistar rat CPZ model, milled chow containing CPZ was fed to the animals. Because CPZ is a powder and can be gathered on the bottom of the manger, rats can mix the milled chow by sniffing it, which allows them to consume less of the CPZ portion. Because of this, the amount of CPZ that each animal consumes may vary, which may affect how demyelinating each animal is. As a result, we believed that we could successfully administer a daily standard dose of CPZ to animals by oral gavage. In this study, we also developed a new approximation. Both the brain and the spinal cord contain oligodendrocytes, which are destroyed by CPZ.

2.0 Materials and methods

2.1 Animals:

24 male wistar rats were obtained from Gentox Bio Services Pvt Ltd, Thumukunta, Yadadri Dist., and kept in a controlled environment with a room temperature of 252 C, a humidity level of 55%, a 12-hour light/dark cycle, and access to food and water. Additionally, all animal experimentation techniques were carried out in accordance with CPCSEA standards. The G.Pulla reddy college of pharmacy in Hyderabad, India, Institutional Animal Ethics Committee reviewed and approved this work (GPRCP//IAEC-1/24/08/2022/PCL/AE-2).

2.2 Multiple sclerosis

2.2.1 Cuprizone-induced demyelination in wistar rats

Experimental design

Group 1 receive vehicle designated as Normal control (NC)

Group 2 receive vehicle and cuprizone (0.4 mg/ kg , s.c.) designated as Disease control (DC)

Group 3 receive orally A*lternanthera sessilis* 100 mg/kg, respectively and cuprizone, designated as Treatment 1 (T1)

Group 4 receive orally A*lternanthera sessilis* 200 mg/kg, respectively and cuprizone, designated as Treatment 2 (T2)

2.3 Cuprizone (CPZ) Administration

In order to create animal models of demyelination, several CPZ doses have been reported in the literature. Every week, 1% CMC stock solution was prepared and kept at +4°C. Every day, CMC and CPZ suspension was made and consumed. To create a uniform CMC-CPZ suspension, this combination was vortexed. CPZ simply suspends in the CMC mixture; it does not chemically react with it. Animals started losing weight when it reached 20% of their total body weight, at which point CPZ application was stopped for a day and resumed the next day. Only a few of the animals

lost weight at the end of the CPZ application period, and none of them lost more than one day's worth of body weight. Animals were given gavage and had their weights monitored.

2.4 Behavioural tests

2.4.1 Pole test

The locomotor coordination of all animals was evaluated using a pole test on the final day of the study (training of the pole test on rats was carried out every day) [Khaledi E et al., 2021]. Each rat was placed on the top of a vertical pole (8 m 55 cm), and the duration till it touched the ground was noted. The test had a 1-minute time limit. For each rat, three consecutive trials were performed every five minutes.

2.4.2 Narrow beam test

The first step is to position the animals in one corner of the thin beam and let them to traverse it at least three times. The 1-3 cm wide narrow beam is elevated between their home cage and a pole (to draw the rat to the finish point). A steady baseline measurement can be attained by using this training phase. Each trial's number of foot slips and the amount of time it took to cross the beam are noted. Animals can be encouraged to walk by tapping their tails if they are hesitant to do so. To keep testing sessions comparable, this must be done on all animals. Additionally, a ledge that can be used as a crutch adjacent to the thin beam can be built, allowing the handicapped limb to be supported there. With this system in place, healthy animals are then able to cross the beam without the need for further assistance, as opposed to animals with impaired fore- or hindlimb function, who frequently need the ledge as a support.

2.4.3. Y-maze task

The Y-maze is a Y-shaped wooden holding cage with 40 cm length, 30 cm height and 15 cm width consisting of three arms at an angle of 120^0 between each arm. Rats were allowed to freely walk the maze for 8 min duration. Alteration was defined as successive entries into the three arms on overlapping triplet sets. The alteration percentage was calculated as the ratio of actual to possible alternation (defined as the total number of arm entries minus two)

2.5 Histopathological Tissue Evaluation

The slides were labelled according to the type of hematoxylin and type of bluing solution and were kept in hot air oven at 70^0C for 20 minutes. Deparaffinization was done by changing the xylene 3 times each 5 minutes. Rehydration by using graded alcohols (100%, 70%, 50%) each 3 minutes followed by washing with distilled water 2 times each 2 minutes. Now, Hematoxylin was added to the slides and incubated for desired period of time. The slides were rinsed under running tap water for 1-2 minutes. Dip the th slides in 1% Acid alcohol 2 times quickly and place the slides under running water for 1-2 minutes. Now, dip the lides in Lithium carbonate bluing solution for 30-60 sec and then rinse the slides with running tap water for 1-2 minutes. Dip the slides in Eosin working solution for 10-20 seconds. Dehydrate the tissues by placing the slides in graded alcohols (95%, absolute/100%) and xylenes was changed 3 times for each 2 minutes. Coverslip the slides using DPx moutant and checked for results under the microscope.

2.6 Assessment of Demyelination by Luxol Fast Blue

Animals were sacrificed by deep anaesthesia using ketamine/xylazine (50/10 mg/kg, i.p.). First, the cerebrum and cerebellum were separated after the entire brain (including both) had been removed. Cerebrum divided into the right, left, and corpus callosum hemispheres. 48 hours were spent fixing the tissues in 10% neutral buffered formaldehyde solution. Following regular tissue processing, samples were paraffin embedded. In order to gauge the level of myelination, 5 m cross sections cut from paraffin blocks were dyed with luxol fast blue (LFB). Olympus, Tokyo, Japan, used the BX51 microscope to analyse the sections, and the DP 20 digital camera, which was mounted to the microscope, was used to capture images.

2.7 Statistical Analysis

All the data were expressed as mean ± SEM. Statistically significant differences were determined using a one way analysis of variance (ANOVA) and Dunnet Multiple Comparison test to compare relations between control and other groups by using GraphPad Prism 5.0 software. Value of $p < 0.01$ was considered as significant.

3.0 Results and Discussion

3.1 Pole test results

Table 1: Effect of EEAS on simple motor function activity in Cuprizone induced multiple sclerosis in Wistar rats

Time (in sec) taken to reach floor by Pole test				
	NC	**DC**	**T1 (100mg/kg)**	**T2 (200mg/kg)**
1st Week	41.33±1.282	22.50±1.118 α	27.17±0.9458 a	32.50±1.565 a
2nd Week	36.00±1.317	13.00±0.9661 α	19.00±0.5164 a	19.17±0.9458 a
3rd Week	23.17±0.7923	7.333±0.6667 α	13.00±0.5774 a	17.50±0.7638 a
4th Week	13.67±0.8028	2.000±0.3651 α	6.500±0.5627 a	11.83±0.6540 a

Data are expressed as mean ± SEM (n=6). The data were analysed by One Way ANOVA followed by Tukey/s Test for Multiple Comparisons of Means. αP<0.01when compared to Normal control group, aP<0.01when compared to Disease control group.

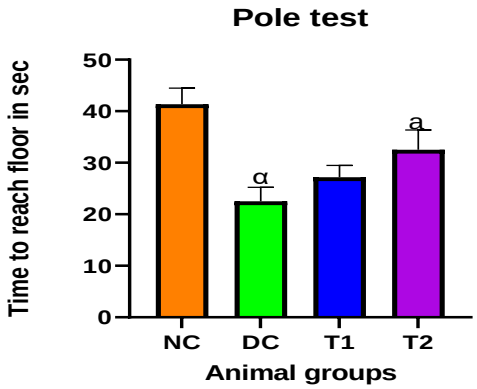

Figure 1: Simple motor function activity in Cuprizone induced multiple sclerosis in Wistar rats in 1st week
(ANOVA) followed by Tukey/s Test for multiple comparison of means.
αP<0.01when compared to Normal control
aP<0.01when compared to Disease control

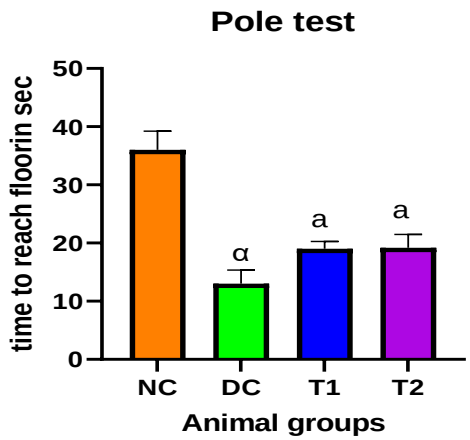

Figure 2: Simple motor function activity in Cuprizone induced multiple sclerosis in Wistar rats in 2nd week

(ANOVA) followed by Tukey/s Test for multiple comparison of means.
αP<0.01when compared to Normal control
aP<0.01when compared to Disease control

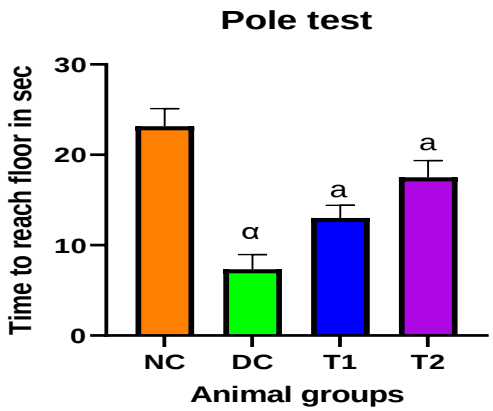

Figure 3: Simple motor function activity in Cuprizone induced multiple sclerosis in Wistar rats in 3rd week
(ANOVA) followed by Tukey/s Test for multiple comparison of means.
αP<0.01when compared to Normal control
aP<0.01when compared to Disease control

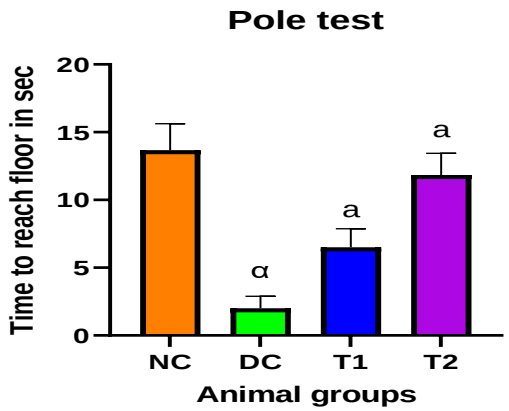

Figure 4: Simple motor function activity in Cuprizone induced multiple sclerosis in Wistar rats in 4th week
(ANOVA) followed by Tukey/s Test for multiple comparison of means.
αP<0.01when compared to Normal control

[a]P<0.01when compared to Disease control

The results of the pole test performed on the 1st, 2nd. 3rd, and 4th week of the study are presented in **Figure1-4**. Feeding CPZ significantly increased the latency time to fall compared with the control animals.

3.2 Narrow beam test

Table 2: Effect of EEAS on motor coordination disability activity in Cuprizone induced multiple sclerosis in wistar rats

Score achieved to cross beam by Narrow beam test				
	NC	**DC**	**T1 (100mg/kg)**	**T2 (200mg/kg)**
1st Week	0.000	0.000 [α]	0.000 [a]	0.000 [a]
2nd Week	0.000	0.000 [α]	0.000 [a]	0.000 [a]
3rd Week	0.000	1.333± 0.3333 [α]	0.000 [a]	0.000 [a]
4th Week	0.000	2.500± 0.2236 [α]	0.000 [a]	0.000 [a]

Data are expressed as mean ± SEM (n=6). The data were analysed by One Way ANOVA followed by Tukey/s Test for Multiple Comparisons of Means. [α]P<0.01when compared to Normal control group, [a]P<0.01when compared to Disease control group.

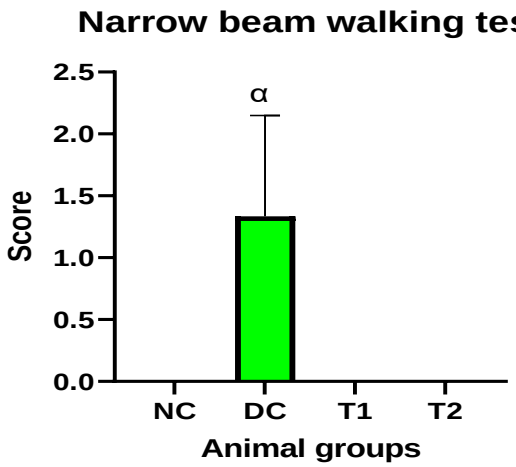

Figure 5: Motor coordination disability activity in Cuprizone induced multiple sclerosis in Wistar rats in 3rd week

(ANOVA) followed by Tukey/s Test for multiple comparison of means.

[α]P<0.01when compared to Normal control
[a]P<0.01when compared to Disease control

3.3 Y-maze task

Table 3: Effect of EEAS on working memory activity in Cuprizone induced Multiple sclerosis in wistar rats for last week.

Groups	**Percentage of correct alternation**	**Number of entries**
Normal control	61.37±4.086	1.583±0.2289
Disease control	5.550±5.550 [α]	0.6667±0.1880 [α]
Treatment 1 (100mg/kg)	36.08±9.037 [a]	1.333±0.1880 [a]
Treatment 2 (200mg/kg)	49.98±4.299 [a]	1.500±0.2303 [a]

Data are expressed as mean ± SEM (n=6). The data were analysed by One Way ANOVA followed by Tukey/s Test for Multiple Comparisons of Means. [α]P<0.01when compared to Normal control group, [a]P<0.01when compared to Disease control group.

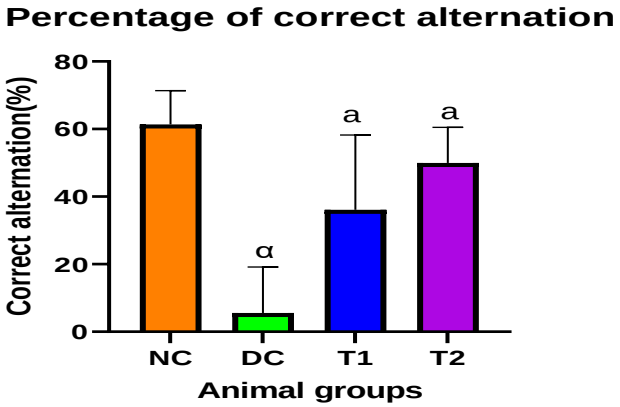

Figure 6: Effect of EEAS on working memory activity in Cuprizone induced Multiple sclerosis in wistar rats for last week

(ANOVA) followed by Tukey/s Test for multiple comparison of means.
$^{\alpha}$P<0.01when compared to Normal control
^{a}P<0.01when compared to Disease control

3.4 EESA-Mediated Neuroprotective effect against Cuprizone induced histopathological changes:

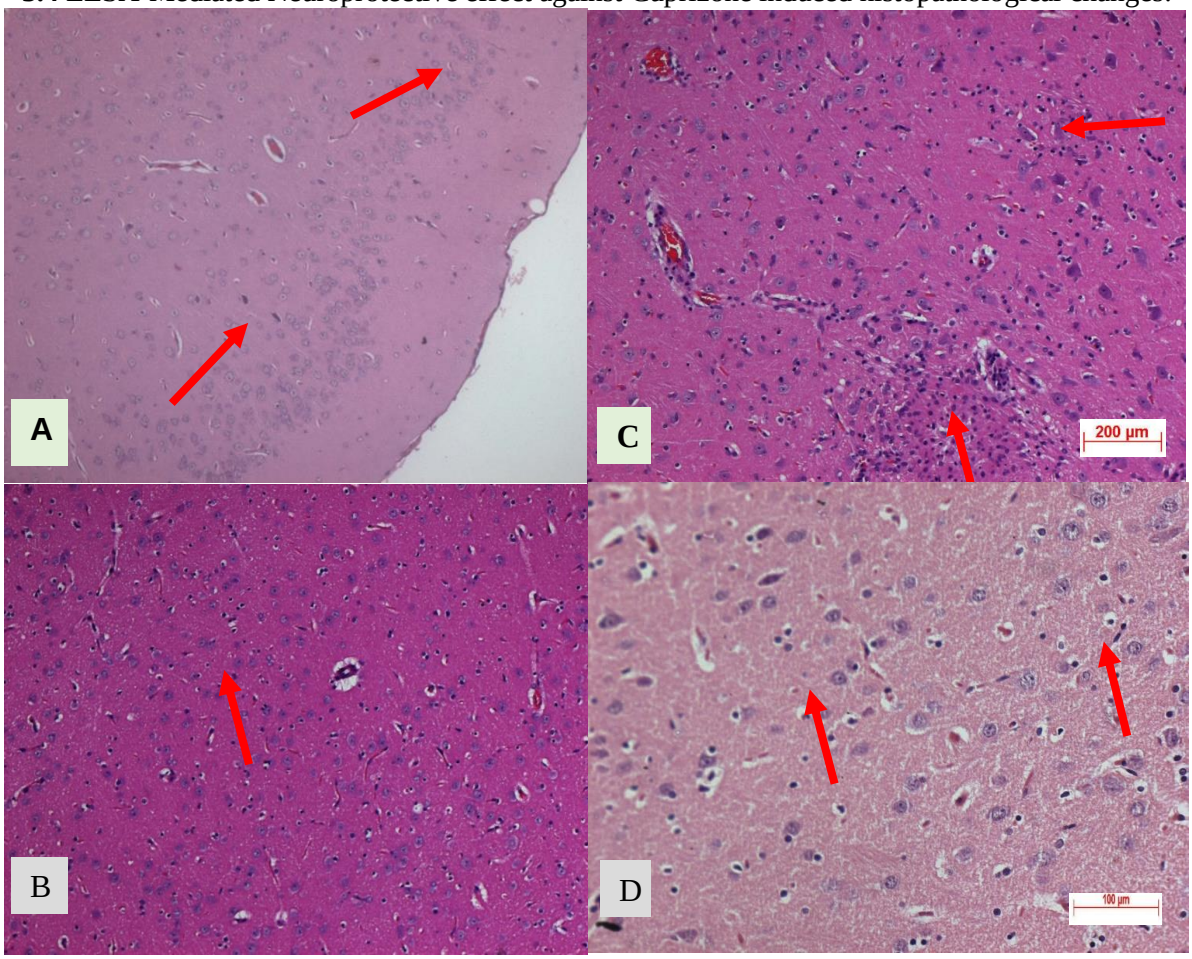

Figure 7: Saggital sections of cerebral cortex stained with H&E of different groups

A. Normal morphology of Frontal/Cerebral cortex of brain indicated by red arrows in normal control group
B. Severe multifocal necrosis with infiltration of inflammatory cells observed in cerebral cortex of brain indicated by red arrows in disease control group.
C. Multifocal necrosis with infiltration of inflammatory cells observed in cerebral cortex of brain indicated by red arrows in treatment 1

group of low dose of *Alternanthera sessilis* (100mg/kg)

D. Morphology of cerebral cortex with normal morphology of pyramidal neurons indicated by red arrows in treatment 2 group of high dose of *Alternanthera sessilis*(200mg/kg)

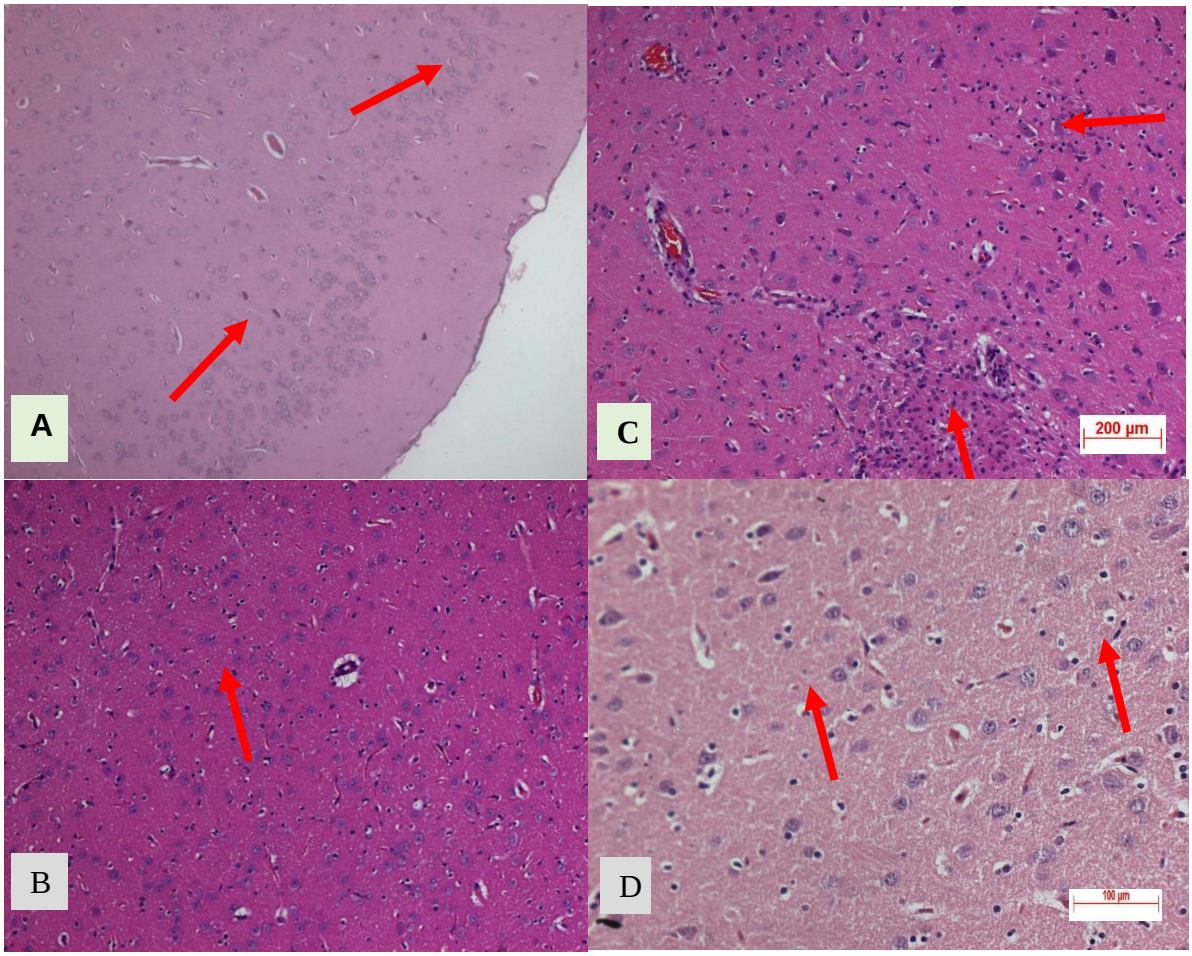

Figure 8: Saggital sections of hippocampus stained with H&E staining of different groups

A. Normal morphology of hippocampus of brain indicated by green arrows in normal control group
B. Multifocal necrotic/apoptotic neurons with demyelination observed in hippocampus indicated by green arrows in disease control group
C. Mild demyelination observed in hippocampus indicated by red arrows in treatment 1 group of low dose of *Alternanthera sessilis* (100mg/kg)
D. Normal morphology of hippocampus of brain indicated by red arrows in treatment 2 group of high dose of *Alternanthera sessilis* (200mg/kg)

3.5 EEAS-Mediated Neuroprotective effect against Cuprizone induced Demyelination:

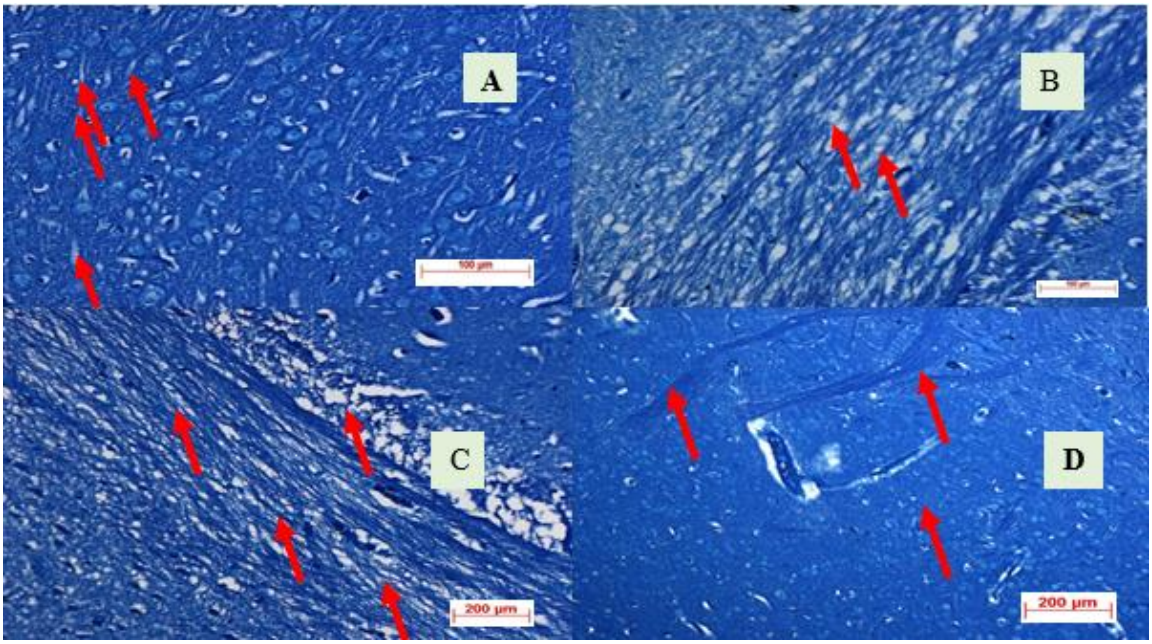

Figure 9: Luxol fast blue staining of mid brain

A. Normal morphology of myelinated neurons associated in mid brain as indicated by red arrows in normal control group
B. Severe demyelination was observed in mid brain as indicated by red arrows in disease control group
C. Mild demyelination in mid brain as indicated by red arrows in treatment 1 group of low dose of *Alternanthera sessilis* (100mg/kg)
D. Normal morphology of myelinated fibres and neurons in mid brain as indicated by red arrows in treatment 2 group of high dose of *Alternanthera sessilis* (200mg/kg)

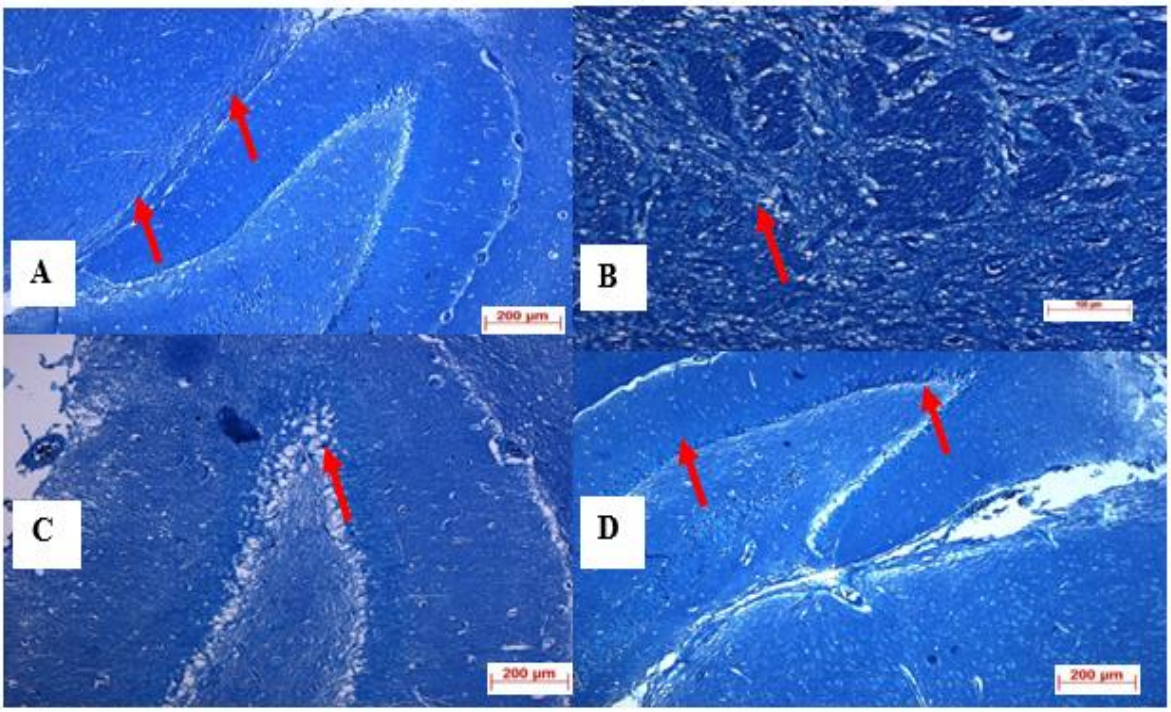

Figure 10: Luxol fast blue staining of hippocampus region of brain

A. Normal morphology of myelinated neurons as noticed in hippocampus of brain as indicated by red arrows in normal control group
B. Demyelination observed in hippocampus region of brain as indicated by red arrows in diseases control group
C. Mild demyelination in hippocampus region of brain as indicated by red arrows in treatment 1 group of low dose of *Alternanthera sessilis* (100mg/kg)
D. Normal morphology of myelinated neurons in hippocampus as indicated by red arrows in treatment 2 group of high dose of *Alternanthera sessilis* (200mg/kg)

3.6 Discussion

It is evident that administering CPZ to young rats causes demyelination, particularly in the corpus callosum (Jurevics H et al., 2001, Liebetanz D and Merkler D 2006). This study's goal was to assess the demyelination characteristics brought on by CPZ treatment in the Wistar rat strain. This is the first study to administer CPZ via gavage to adult animals that are 5-6 week old in order to administer a standard dose. In every prior study, 2-3 week old weanling animals were administered CPZ-containing feed that was milled or powdered. Although CPZ has been the subject of numerous experiments, the mechanism by which CPZ induces demyelination is still unknown. The behaviour of animals given copper-chelated cuprizone remained unchanged. The prevalent belief is that CPZ, a copper chelator, promotes demyelination based on copper shortage in the central nervous system (Matsushima Gk and Morell P 2001). It has been established that CPZ caused spongy chances in the test animals' brains. Only the rat brains that had been treated with CPZ for 5 and 7 weeks were vacuolated in our study. No vacuolization (Kanno T et al., 2012) was seen in the animal corpus callosum 4 weeks after CPZ application. In the cerebellar white matter, dentate nucleus hilum, and superior cerebellar peduncle of Wistar rats, CPZ results in intramyelinic edoema. Despite the ongoing administration of CPZ, only sporadic oligodendrocyte degradation and substantial axonal regrowth were seen despite the edema's association with pyknosis. Here, we describe the first electrophysiological and histological findings demonstrating demyelination is a side effect of CPZ treatment for six weeks. But if it were thought of as axonal degradation, our 4-week CPZ treatment results led us to believe that remyelination begins after the sixth week, which is consistent with Love S. findings. Similar to these investigations, the histopathological findings from our study demonstrate that giving Wistar rats 1% of their daily caloric intake as CPZ by gavage results in partial central nervous system injury. Oligondendroglial response to CPZ is not age dependent because the rate and extent of de- and remyelination in affected adults is similar to that found in intoxicated neonates (Politis Mj et al., 1980).The age of the animal and the length of time it was exposed to CPZ, on the other hand, are crucial factors, according to Matsushima and Morell 8's findings. According to Irvine and Blackmore17, demyelination was distributed similarly in young and old mice, although its severity was slightly higher in the young adult mice. Additionally, the number of axons was decreased in both young adult and old mice, but the loss of axons at the demyelination stage was significantly greater in the old animals. These findings lead us to believe that the adult rats we utilised in this study, which were one year old, may also have the same effect. Our histological findings show that the corpus callosum exhibits demyelination. Additionally, on the slices taken from the rat cerebellum, localised demyelinated regions and vacuolization could be identified. On 4-week-old rats receiving CPZ, localised demyelinated regions and vacuolization were clearly visible. The cerebellum of CPZ-administered rats that were 6 weeks old showed no vacuolization, but the demyelinated regions were larger than those of the rats that were 5 and 7 weeks old. This suggests that at least a portion of the oligodendrocytes are still capable of producing myelin sheath. These results imply that remyelination occurred in the 7-week-old CPZ-treated rats. Similar to this, Mason et al (Mason Jl et al., 2001) report that long-term CPZ administration to rats causes both demyelination and remyelination to be visible. This relapsing-remitting event is a common characteristic of multiple sclerosis.

4.0 Conclusion

The present study offers evidence for the neuroprotective effect of A*lternanthera sessilis* against demyelination and locomotor dysfunction induced by CPZ. The protective effect of A*lternanthera sessilis* may be correlated to its antioxidant and anti-inflammatory effects protecting oligodendrocytes from the oxidative stress induced by CPZ. The present results revealed the demyelinating effects of CPZ intake as a study model for MS, and the protective effects of EEAS. The results of behavioural tests evidenced that EEAS can improve movement

disorders by ameliorating demyelination in rats exposed to CPZ. Mechanisms that may be involved in the protective function of EEAS include activation of the Nrf2 signaling pathway and inhibition of the NF-kB pathway. .

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