



NEUROMODULATORY EFFECT OF *CYNODON DACTYLON* ON CUPRIZONE (CPZ) INDUCED MULTIPLE SCLEROSIS IN WISTAR RAT CORPUS COLLUSUM- A PILOT STUDY ON COGNITIVE, BIOCHEMICAL AND HISTOPATHOLOGICAL APPROACH

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ABSTRACT

Demyelinating diseases like Multiple Sclerosis is often characterized by progressive cognitive impairment and motor deficient. In traditional medicine, numerous plants have been used to treat these neurological disorders including Alzheimers disease and Parkinson's disease. Henceforth, the aim of this study is to investigate the neuroprotective and antioxidant activities of the aqueous extract of *Cynodon dactylon*(AECD) against Cuprizone induced Multiple Sclerosis in rat brain. The animals were treated with AECD at two different doses (250 mg/kg and 500 mg/kg) for 6 weeks in different groups of rats against Cuprizone intoxication through oral gavage for the period of same 42 days. Further, biochemical and histopathological observations confirmed the beneficial roles of aqueous extract of *Cynodon dactylon* against Cuprizone induced MS in rat brain. The Cuprizone significantly disturbs the level of achetylcholinesterase, Lipid peroxidation and superoxide dismutase (SOD) along with the reduced level of glutathione-s-transferase (GST), glutathione (GSH) & glutathione peroxidase (GPx). Some of the neurobehavioral impairment also occur in MS model of rats which assessed by many cognitive and behavioural test. These values are retrieved significantly by the administration of AECD (500 mg/kg B/W). Thus, AECD might serve as a reliable neuroprotectant for Cuprizone induced Multiple sclerosis and may be promising drug for antioxidant deficit and neurodegenerative disease.

KEY WORDS

Multiple Sclerosis, Demyelination, Cuprizone, Antioxidant deficit, Aqueous extract of *Cynodondactylon*.

INTRODUCTION

Multiple sclerosis (MS), an autoimmune and inflammatory disorder of the CNS [1] involving the macrophages and microglia activation [2], thereby leads to oligodendrocyte loss, axonal damage, neuroinflammation and neuronal loss [3] which causes chronic demyelination in both white and grey matter of the brain. Cuprizone, CPZ [bis (cyclohexylidene hydrazide)] a copper chelator treatment of animals was

a murine model for investigating MS pathology which damage mature oligodendrocytes leads to microgliosis and astrogliosis [4] and attributed to neuropathological, neurobehavioral, neurophysical and neurochemical changes [5] resulting in cognitive decline and learning disabilities. Neuropathological and Neurochemical effect of cuprizone induced MS in *in vivo* model mainly causes the disturbances in antioxidant status and also elevate the level of Acetyl Choline Esterase (Ache), Nitrate (NO), Lipid Peroxidation (LPO) and Carbonylated

Proteins which leads to the neuroinflammation thereby plays a central role in demyelination for the progression of MS.

Despite of many clinical efficacy drugs against multiple sclerosis, *Cynodon dactylon* pers. (Family: Graminae) also known as *Vilfa stellata* which known for its constitutes of carbohydrates, crude proteins, minerals and oxides of some of the metals like magnesium, calcium, sodium, phosphorus and potassium. The CD extract was also comprising of vitamin C, hydroquinone, furfurals, linolenic acid and d-Mannose [6]. It has been proved therapeutically endowed with antidiabetic [7], antiepileptic [8], anti-inflammatory [9], immunomodulatory [10] and hepatoprotective activities [11]. Hence Aqueous extract of CD (AECD) examined for its neuroprotection against Parkinson disease by showing the reduction in the level of Thiobarbituric Acid Reactive substances and ameliorating the SOD, GPx levels [12] and attenuates the aluminium induced neurotoxicity by its scavenging activity of oxygen free radicals [13]. CD extract also possess the dual protection role against epilepsy and depression which probably associated with its GABA mimetic action [14]. Thus, for the first time in this explored pilot study CD was investigated against CPZ induced MS in corpus collusum of adult male Wister rats. In MS, behavioural impairment is a main factor which was significantly affected in rats are spatial memory loss [15], impairment of motor balance and coordination, abnormalities in social impairment, etc., [16]. Further, biochemical and histopathological observations confirmed the beneficial roles of AECD in an animal model of MS.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents

Cuprizone [bis (cyclohexylidene hydrazide)] were purchased from Sigma Aldrich. Glutathione reduced form (GSH), glutathione oxidized form (GSSG), 1-chloro-2, 4-dinitrobenzene (CDNB), 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) were purchased from SRL. β -Nicotinamide adenine dinucleotide phosphate reduced (NADPH) was purchased from CDH. All other chemicals used were of analytical grade.

2.2. Animals and Treatment Schedule

Male Wister rats weighing 300-350 g were obtained from Central Animal House, Dr. ALMPGIBMS, University of Madras, Tamil Nadu, India. Rats were accommodated

in polypropylene cages separately under hygienic conditions and fed standard pellet diet. Rats were placed on a 12 h light and dark cycles with *ad libitum* condition. All experiments and protocols described in the present study were approved by the Institutional Animal Ethics Committee (IAEC) (IAEC No: 02/18/2017) of Dr. ALMPGIBMS, University of Madras.

Group I - Control rats. Group II - Animals received Cuprizone (0.69g was dissolved in 1 liter of 1% sodium carboxy methyl cellulose (CMC-Na) and 69ml/kg of CPZ-CMC was fed daily) through oral gavage for 42 days [15]. Group III - Rats were treated with AECD (250 mg/kg b.w dissolved in d.d.H₂O) orally simultaneously to CPZ-CMC suspension (69ml/kg b.w) for 42 days. Group IV - Rats were treated with AECD (500mg/kg b.w dissolved in d.d.H₂O) orally simultaneously to CPZ-CMC suspension (69ml/kg b.w) for 42 days. Group V - Animals were treated with AECD (500 mg/kg b.w dissolved in d.d.H₂O).

2.3. Preparation of CPZ-CMC suspension and Aqueous extract of *cynodon dactylon*

The average consumption of daily rat chow was measured initially as 69±3g per kg of body weight. However, 0.69g of CPZ was mixed with one liter of 1% sodium carboxy methyl cellulose (CMC-Na) and the mixture was vortexed to obtain the CMC-CPZ suspension homogenously. The stock solution of 1%CMC was prepared weekly and stored in +4°C whereas CPZ-CMC suspension was made fresh daily. Hence 1% CMC-Na was used only for suspending CPZ it does not react chemically. From the liter of above 1%CMC-CPZ suspension, 69ml/kg b.w was given to animals through oral gavage for the period of 42 days [15]. The plant material was gathered at Chennai, Tamil Nadu, India and was authenticated by Dr. P. Brindha, Botanist, Captain Srinivasa Murthi Drug Research Institute for Ayurveda, Arumbakkam, Chennai, Tamil Nadu, India. About 500 g of the whole plant was extracted with boiling water for 48 h. The resulting extract was sieved and concentrated in rotavapour under reduced pressure. The concentrated extract was lyophilized to get a powder (yield 15.8%, w/w) [13]. Phytochemical screening of *C. dactylon* reveals the presence of Flavonoids and sterols [17,18].

2.5. Behavioral observations

All the behavioral studies were carried out at room temperature without any outside interference. All the experiments were conducted between 10.00 am and 6.00 pm.

2.5.1. Rotarod activity

The rotarod apparatus was utilized for the evaluation of motor co-ordination and grip performance of the animals. The prior training session was given to all animals to get acclimatize them to rotarod performance. Rats were placed on a rotating rod with a diameter of 7 cm (speed, 16 rpm). The cut-off time was 600 s, and each rat performed three separate trials after 5 weeks of CPZ administration. The average time of the fall was recorded [16].

2.5.2. Morris Water Maze test

It was performed to evaluate changes caused by CPZ on spatial memory and learning according to the method described [19]. The circular water tank which was 55 cm in height and 160 cm in diameter was used. To the tank escape platform was fixed in the center of the quadrant of pool and this was kept constant throughout the experiment. At the start of the experiment, the rat was placed at any of the quadrants expect the one with the escape platform, with its head facing the wall of the tank. Time taken by the rat to reach the platform (initial acquisition latency) was recorded.

2.5.3. Open field test

To access the abnormalities in motor function, spatial working memory, social interaction which was observed in nervous system dysfunction like demyelination diseases. Open field test is performed to examine the exploratory ability and anxiety of rats. Each animal was kept in the center of the open field apparatus which was a circle made of wood with 90cm in diameter. The time taken for the animal spent in central area was noted between the control and experimental rats [20].

2.6. Preparation of tissue homogenate for biochemical studies

On the day 42, after the neurobehavioral studies all the biochemical estimations were carried out. The animals were sacrificed by cervical decapitation and the brains were dissected out. The corpus collusum was separated and placed on ice. Using 0.1 M phosphate buffer (pH 7.4), 10% (w/v) tissue homogenate was prepared. The homogenate was centrifuged at $10,000 \times g$ for 15 min. Aliquots of the supernatant was separated and used for biochemical estimations.

2.6.1. Oxidative stress parameters

2.6.1.1. Estimation of lipid peroxidation level

In the eppendorf tube, 0.2 ml homogenate was pipetted and incubated at $37 \pm 1^\circ\text{C}$ in a metabolic water bath shaker at 120 strokes up and down for 60 min; In

another Eppendorf tube, 0.2 ml of homogenate was pipetted and placed at 0°C incubation. After 1 h of incubation 0.4 ml of 0.67% TBA and, 0.4 ml of 5% TCA was added in both samples (i.e., 0°C and 37°C) and centrifuged at $3500 \times g$ for 15 min by transferring the reaction mixture from the vial to the tube. The supernatant was transferred to another tube and placed in a boiling water bath for 10 min. Thereafter, the test tubes were cooled and the absorbance of the color was read at 535 nm. The rate of lipid peroxidation expressed as nmol of MDA released/min/mg protein. The method of Utley et al., 1967 was modified for the estimation of malondialdehyde (MDA) an end product of lipid peroxidation [21].

2.6.1.2. Determination of protein carbonyl content

The protein oxidative damage has been used as an indicator of protein carbonylation. In this, 100 μl of supernatant from brain homogenate was incubated with 0.5 ml of 2, 4-dinitrophenylhydrazine for 60 min. Subsequently, the protein was precipitated from the solution using 20% trichloroacetic acid. The pellet was washed after centrifugation ($3400 \times g$) with ethyl acetate: ethanol (1:1 v/v) mixture, to remove excess of 2, 4-dinitrophenylhydrazine. The final protein pellet was dissolved in 2.5 ml of 6 M guanidine [22]. Protein carbonyl content determination was based on the reaction of carbonyl groups with 2, 4-dinitrophenylhydrazine to form 2, 4-dinitrophenylhydrazone. The carbonyl content was evaluated in a spectrophotometer at 370 nm, the values were calculated using molar extinction coefficient ($22,000 \text{ M}^{-1}\text{cm}^{-1}$) and expressed as nmol per mg protein.

2.6.1.3. Estimation of Nitrite

By colorimetric assay using Greiss reagent (0.1% naphthyl ethylene diamine dihydrochloric acid and 1% sulfanilamide in 2.5% phosphoric acid) nitrite levels were determined. Equal volumes of Greiss reagent and supernatant were mixed, the mixture was incubated for 10 min at room temperature in dark and the absorbance was determined at 540 nm [23]. The concentration nitrite in supernatant was determined from a sodium nitrite standard and expressed as nmol per mg protein.

2.6.2. Antioxidant enzyme and non-enzyme parameters

2.6.2.1. Determination of superoxide dismutase activity

0.25 ml of ethanol and 0.15 ml of chloroform was added and kept in a mechanical shaker for 15 min and centrifuged. To 0.5 ml of supernatant, 2.0 ml of pyrogallol was added to 1 ml of homogenate. Changes

in optical density 0, 1, 2, 3 min at 420 nm were read in spectrophotometer. Against a buffer blank, control tubes containing 0.5 ml of water were also treated in a similar manner. The enzyme activity was expressed as units/mg protein. One unit is equivalent to the amount of SOD required to inhibit 50% of pyrogallol auto-oxidation was measured according to the method described [24].

2.6.2.2. Determination of catalase activity

The assay mixture consisted of 0.05 M phosphate buffer (pH 7.0), 0.019 M H₂O₂, and 0.05 ml PMS in a total volume of 3.0 ml. Changes in absorbance were recorded at 240 nm. Catalase activity was calculated in terms of $\mu\text{mol H}_2\text{O}_2$ consumed/min/mg protein Catalase by the method [25].

2.6.2.3. Assay for reduced glutathione content

To the 10% trichloroacetic acid equal quantity of homogenate was mixed and centrifuged to separate the proteins. To 0.1 ml of this supernatant, 0.4 ml of double distilled water and 2 ml of phosphate buffer (pH 8.4), 0.5 ml of 5, 5-dithiobis (2-nitrobenzoic acid) were added. The mixture was vortexed and the absorbance was read at 412 nm within 15 min. The concentration of reduced glutathione was expressed as μmol of GSH/min/mg protein, according to the method [26].

2.6.2.4. Determination of glutathione reductase activity

GR activity was measured by the method of Carlberg and Mannervik 1975. In a total volume of 2.0 ml, the assay system consisted of 0.1 M PB (pH 7.6), 0.5 mM EDTA, 1 mM GSSH, 0.1 mM NADPH and PMS (0.1 ml). At room temperature the enzyme activity was quantitated by measuring the disappearance of NADPH at 340 nm and was calculated as μmol NADPH oxidized/min/mg protein [27].

2.6.2.5. Determination of glutathione peroxidase activity

0.2 mM H₂O₂, 1 mM GSH, 1.4 unit of GR, 1.43 mM NADPH, 1 mM sodium azide, PMS (0.1 ml) and PB (0.1 M, pH 7.0) in a total volume of 2.0 ml was consider to be reaction mixture. The NADPH disappearance at 340 nm was recorded at room temperature. The enzyme activity was calculated as nmol NADPH oxidized/min/mg protein GPx activity was measured at 37°C by the coupled assay method [28].

2.6.2.6. Estimation of Glutathione-S-Transferase activity

Briefly, the assay mixture consisted of 2.7 ml of phosphate buffer, 0.1 ml of reduced glutathione, 0.1 ml 1-chloro-2, 4-dinitrobenzene (CDNB) as substrate and

0.1 ml of supernatant. The changes in absorbance were recorded at 340 nm, and the enzyme activity was calculated as nmol CDNB conjugate formed/min/mg protein was assayed by the method [29].

2.6.3.2. Acetylcholinesterase (AChE) activity

To the aliquot containing 2.85 ml phosphate buffer (0.1 M, pH 8.0), 50 μl of DTNB (10 mM), 50 μl sample and 20 μl acetylthiocholine iodide (78 mM) were added and the change in absorbance was monitored at 412 nm for 5 min in a spectrophotometer. The enzyme activity was expressed as nmol of substrate hydrolyzed/L/min/mg protein was determined according to the method [26].

2.6.3.3 Na⁺/K⁺ ATPase (Adenosine Triphosphatase)

The mixture containing 1.0ml of Tris-HCL buffer, 0.2ml each of magnesium, potassium chloride, sulphate, sodium chloride, ATP, EDTA and the tissue homogenate was made to incubated at 37°C for 15 minutes. By the addition of 1.0ml of 10% TCA the reaction was arrested with simultaneous mixing and centrifugation. The quantity of phosphorus was estimated and the enzyme activity was expressed as μmoles of phosphorus liberated/min/mg of protein [30].

2.6.3.4 Ca²⁺ ATPase (Adenosine Triphosphatase)

At 37 °C 0.1ml of Tris-HCL buffer, ATP, magnesium chloride and enzyme preparation was incubated for 15 minutes, after which the reaction was arrested by the addition of 1ml of 10%TCA. The amount of phosphorus liberated was estimated and the enzyme activity was expressed as μmoles of phosphorus liberated/min/mg of protein.

2.7. Histological evaluation of corpus collusum sections

On day 42, cervical decapitation was done by sacrificing the animal and the corpus collusum was removed and made fixed in 10% buffered formalin for 24 h then washing was done in the tap water and serial dilutions of alcohol, ethyl alcohol and absolute ethyl were used for dehydration. Specimens were cleared in xylene then embedded in paraffin at 56°C in hot water over 24 h. The paraffin bees wax tissue blocks were prepared for sectioning at 4 μm thickness by slide microtome. The obtained tissue sections were collected on glass slides, deparaffinized, stained by hematoxylin and eosin, and was examined using a light microscope [31].

3. RESULTS

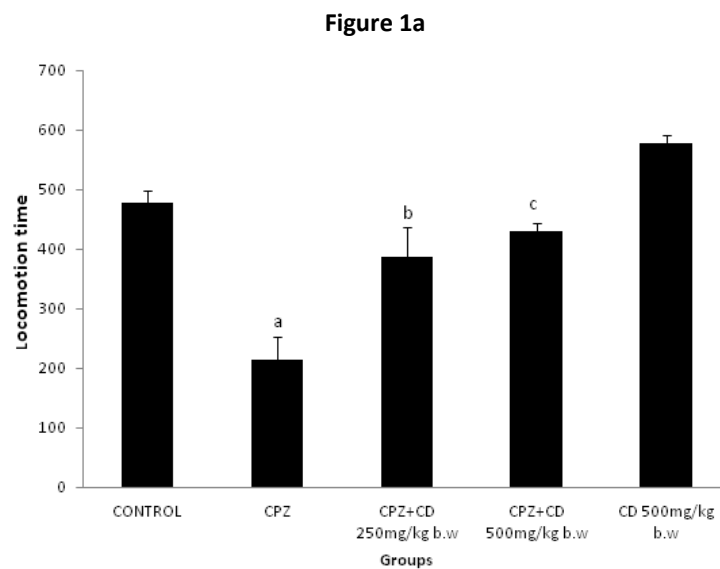
3.1. Behavioral assessments

3.1.1. Effect of AECD on CPZ induced Rotarod activity in control and experimental rats

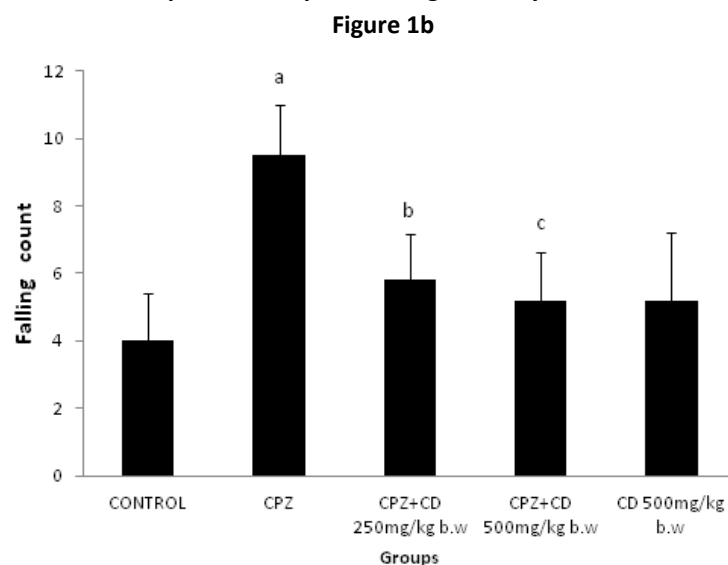
Oral induction of CPZ significantly affected the muscle grip performance which was assessed by rotarod test on 42nd day. Here, the CPZ administered rats showed a ($P<0.01$) significant impairment in the grip strength performances on 42nd days as compared to control group. Treatment with AECD at both the doses significantly ($P<0.05$ and $P<0.01$) enhanced the muscle strength (delayed fall of time) verses CPZ administered

group respectively (Figure 1a). Treatment with AECD also showed a significantly ($P<0.05$ and $P<0.01$) improved locomotion time on days 42nd day respectively when compared to CPZ administered group. However, the higher potentiation of grip strength were attained by the animals treated with AECD which have significant effect on grip strength performance (Figure 1b).

Figure 1: Effect of AECD on CPZ induced changes in motor dysfunction of control and experimental rats accessed by Rotarod test



a) Data represents locomotion time of the animals mean values \pm SD (n=6 animals each groups). ^a $p<0.01$, is significantly different from control. ^b $p<0.05$ and ^c $p<0.01$ is significantly different from CPZ exposed group.



b) Data represents falling count of the animals mean values \pm SD (n=6 animals each groups). ^a $p<0.01$, is significantly different from control. ^b $p<0.05$ and ^c $p<0.01$ is significantly different from CPZ exposed group; one-way ANOVA with Tukey's *post hoc* test was used for statistical analysis.

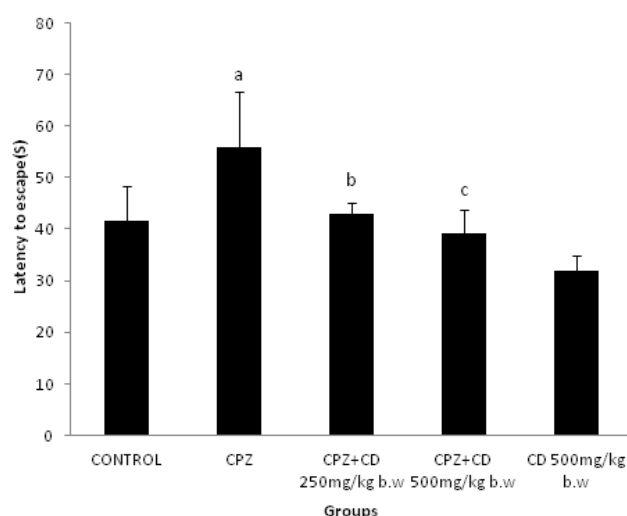
3.1.2. Effect of AECD on CPZ induced Spatial navigation task in control and experimental rats

The cognitive function was assessed by Morris water maze test CPZ intoxicated animals showed a significantly ($P < 0.01$) delayed mean latency on 42nd day as compared to the control group respectively. Subsequently, treatment with AECD (250 mg/kg) improved the memory performance on 42nd day significantly ($P < 0.05$) against CPZ administered group.

Also, treatment with AECD (500 mg/kg) significantly ($P < 0.01$) improves the cognitive function against CPZ induced group on 42nd day (Figure 2a). Besides, AECD (250mg/kg and 500 mg/kg) efficiently enhanced the seconds spent in target quadrant significance ($P < 0.05$ and $P < 0.01$) respectively on 42nd day compared to CPZ intoxication which significantly ($P < 0.01$) reduced. The memory defect and time spent in target quadrant were improvised well in AECD treated animals (Figure 2b).

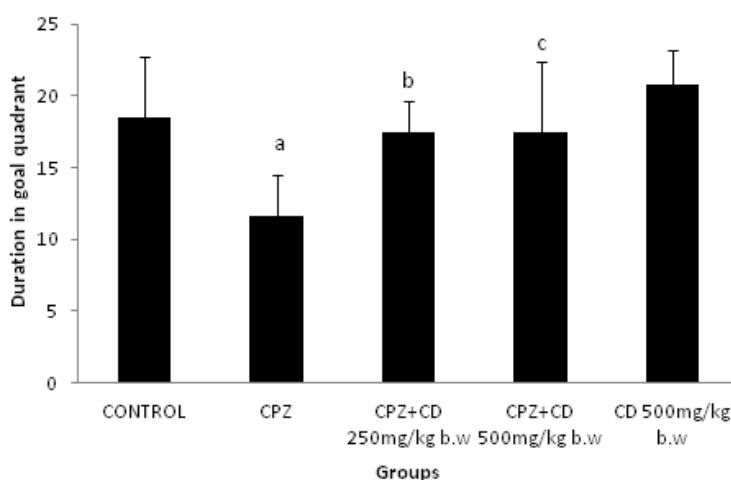
Figure 2: Effect of AECD on CPZ induced changes in spatial memory impairment of control and experimental rats accessed by Morris water Maze test

Figure 2a



a) Data represents escape latency of the animals mean values \pm SD (n=6 animals each groups). ^a $p < 0.01$, is significantly different from control. ^b $p < 0.05$ and ^c $p < 0.01$ is significantly different from CPZ exposed group;

Figure 2b



b) Data represents duration spent in target quadrant of the animals mean values \pm SD (n=6 animals each groups). ^a $p < 0.01$, is significantly different from control. ^b $p < 0.05$ and ^c $p < 0.01$ is significantly different from CPZ exposed group; one-way ANOVA with Tukey's post hoc test was used for statistical analysis.

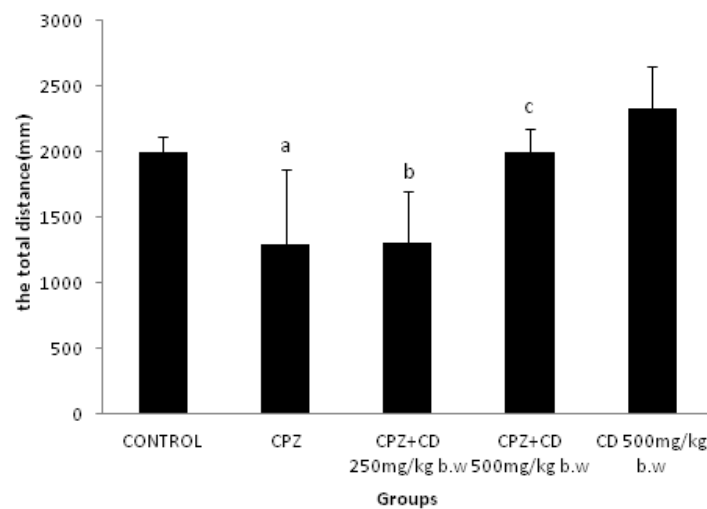
3.1.3. Effect of AECD on CPZ induced Open field test in control and experimental rats

The animals induced with CPZ showed a significant ($P < 0.01$) increase in the duration of immobility on 42nd day. Administration of 250mg/kg and 500mg/kg of AECD significantly ($P < 0.05$ and $P < 0.01$) respectively improved the mean time spent in central area of animals versus CPZ induced animals on 42nd day (Figure

3a). The total distance covered by CPZ induced animals in open field test was significantly ($P < 0.01$) reduced compared to control animals. While, in AECD treated groups (250 mg/kg and 500 mg/kg) shows the significant increase in total distance covered ($P < 0.05$ and $P < 0.01$) respectively on 42nd day compared to CPZ induced group (Figure 3b).

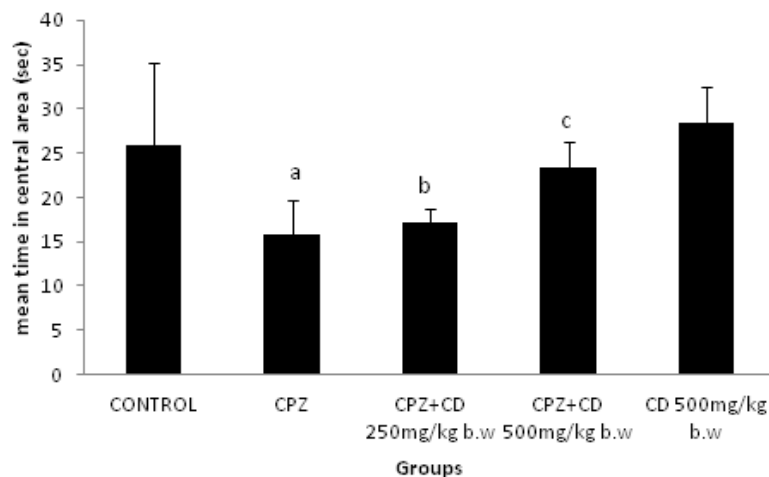
Figure 3: Effect of AECD on CPZ induced changes in behaviour and locomotory activity of control and experimental rats accessed by Open field test

Figure 3a



a) Data represents total distance travelled in open field of the animals mean values \pm SD ($n=6$ animals each groups). ^a $p < 0.01$, is significantly different from control. ^b $p < 0.05$ and ^c $p < 0.01$ is significantly different from CPZ exposed group;

Figure 3b



b) Data represents time spent in central area of the animals mean values \pm SD ($n=6$ animals each groups). ^a $p < 0.01$, is significantly different from control. ^b $p < 0.05$ and ^c $p < 0.01$ is significantly different from CPZ exposed group; one-way ANOVA with Tukey's post hoc test was used for statistical analysis.

3.2. Biochemical observations

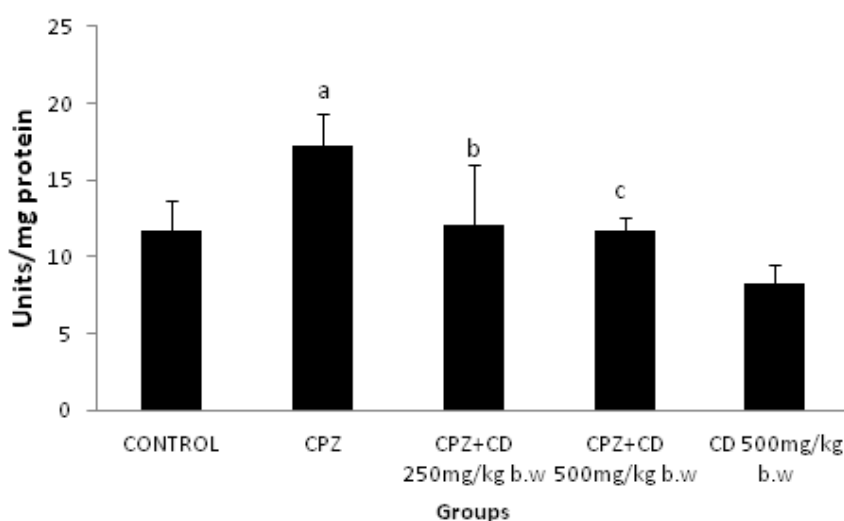
3.2.1. Effect of AECD on CPZ induced changes in the activities of LPO and GSH in the corpus collusum of control and experimental rats

The CPZ induced rats showed a significantly ($P<0.01$) increased level of LPO and decreased level of GSH when compared to control animals. The level of LPO was found to be decreased significantly ($P<0.05$ and $P<0.01$) respectively on treatment with AECD (250mg/kg and

500mg/kg) (Figure 4a). On the contrary, the level of GSH was found to be increased significantly ($P<0.05$ and $P<0.01$) upon treatment with AECD (250mg/kg and 500mg/kg) as compared to CPZ induced animals. Although, the oxidative stress indicator (LPO) and important non-enzymic antioxidant (GSH) levels were efficiently brought back to normal by the animals treated with AECD (250mg/kg and 500mg/kg) (Figure 4b).

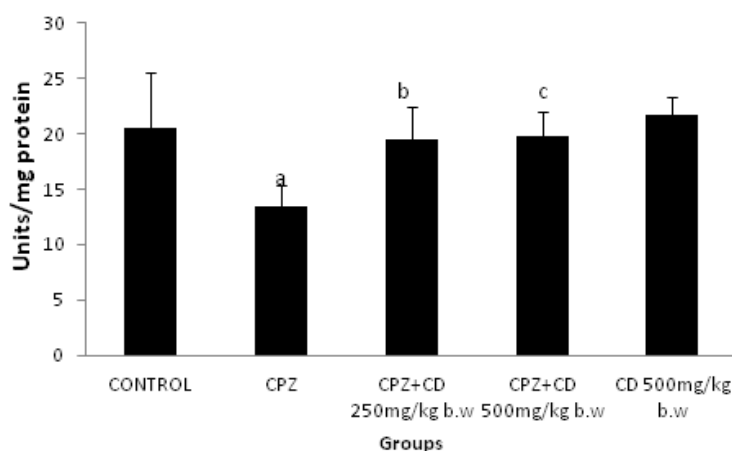
Figure 4: Effect of AECD on CPZ induced changes in the activities of GSH and LPO in corpus collusum of control and experimental rats accessed by Open field test

Figure 4a



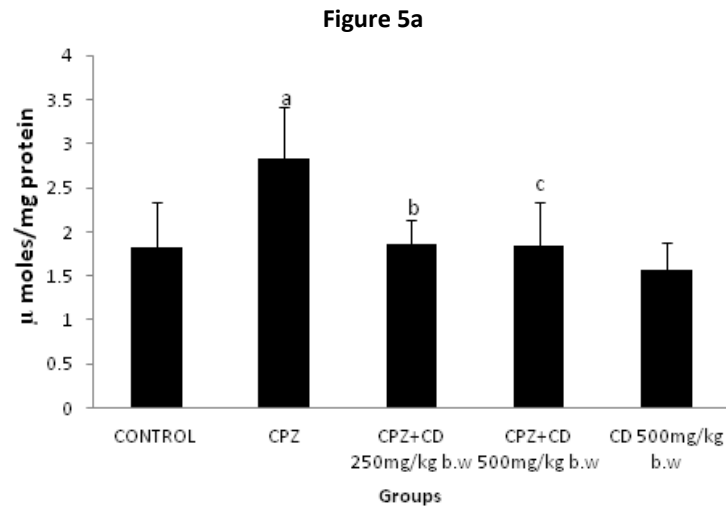
a) Data represents changes in LPO in corpus collusum mean values \pm SD (n=6 animals each groups). ^a $p<0.01$, is significantly different from control. ^b $p<0.05$ and ^c $p<0.01$ is significantly different from CPZ exposed group;

Figure 4b

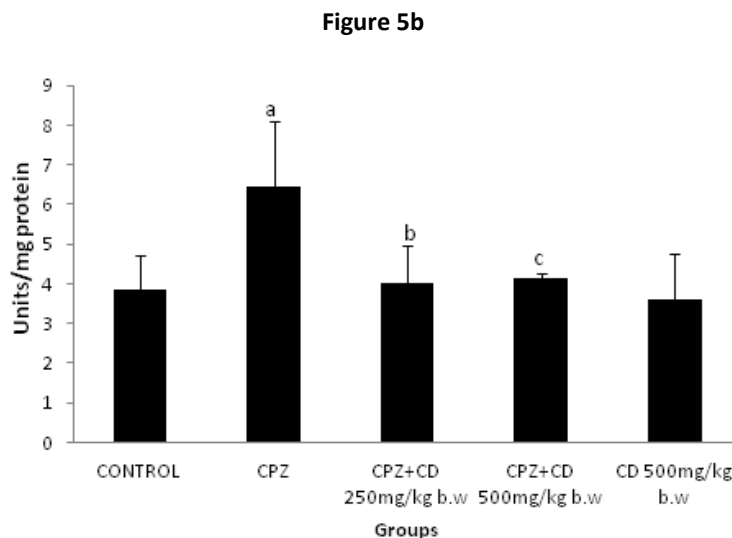


b) Data represents changes in GSH level in corpus collusum mean values \pm SD (n=6 animals each groups). ^a $p<0.01$, is significantly different from control. ^b $p<0.05$ and ^c $p<0.001$ is significantly different from CPZ exposed group one-way ANOVA with Tukey's *post hoc* test was used for statistical analysis.

Figure 5: Effect of AECD on CPZ induced change in the activities of NO and Protein Carbonyls in corpus collusum of control and experimental rats.



a) Data represents changes in NO level in corpus collusum mean values \pm SD (n=6 animals each groups). ^a $p < 0.01$, is significantly different from control. ^b $p < 0.05$ and ^c $p < 0.01$ is significantly different from CPZ exposed group;



b) Data represents alteration carbonylated protein level in corpus collusum mean values \pm SD (n=6 animals each groups). ^a $p < 0.01$, is significantly different from control. ^b $p < 0.05$ and ^c $p < 0.01$ is significantly different from CPZ exposed group; one-way ANOVA with Tukey's *post hoc* test was used for statistical analysis.

3.2.2. Effect of AECD on CPZ induced changes in activities of Protein carbonyls and Nitrite in the corpus collusum of control and experimental rats

Upon CPZ in-toxication, there was a significantly ($P < 0.01$) elevated levels of protein carbonyls and NO as compared to control group. However, treatment with AECD (250mg/kg and 500mg/kg) significantly ($P < 0.05$ and $P < 0.01$) respectively reduced those oxidative stress markers level when compared to CPZ administered animals (Figure 5a and 5b).

3.2.3. Effect of AECD on CPZ induced changes in activities of enzymic antioxidant CAT and SOD in the corpus collusum of control and experimental rats

Administration of CPZ caused significant ($P < 0.01$) reduction in the activities of CAT and SOD when compared to control animals. Upon simultaneous treatment with AECD (250mg/kg and 500mg/kg) significantly ($P < 0.05$ and $P < 0.01$) respectively improved their activities as compared to CPZ administered animals respectively (Figure 6a & 6b).

3.3 Glutathione family enzymes

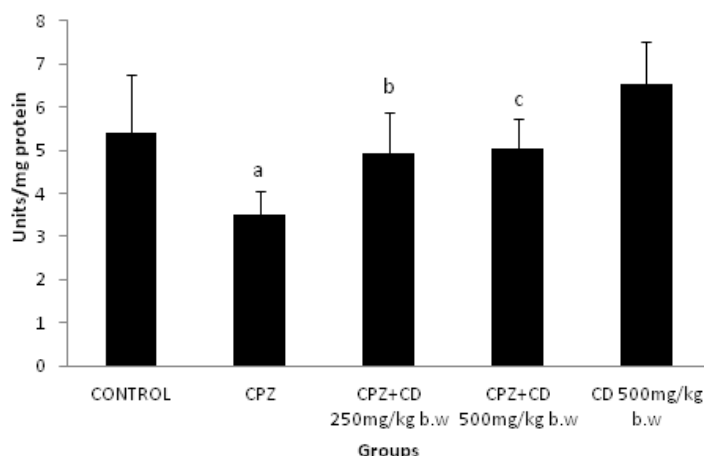
3.3.1. Effect of AECD on CPZ induced changes in activities of GPx and GR in the corpus collusum of control and experimental rats

Oral administration of CPZ resulted in significant ($P < 0.01$) loss of activities of Glutathione family enzymes

GPx and GR, as compared to control animals. Subsequent treatment with AECD (250mg/kg and 500mg/kg) raised those antioxidant enzyme activities significance of ($P < 0.05$ and $P < 0.01$) respectively when compared to CPZ induced animals respectively (Figure 7a and 7b).

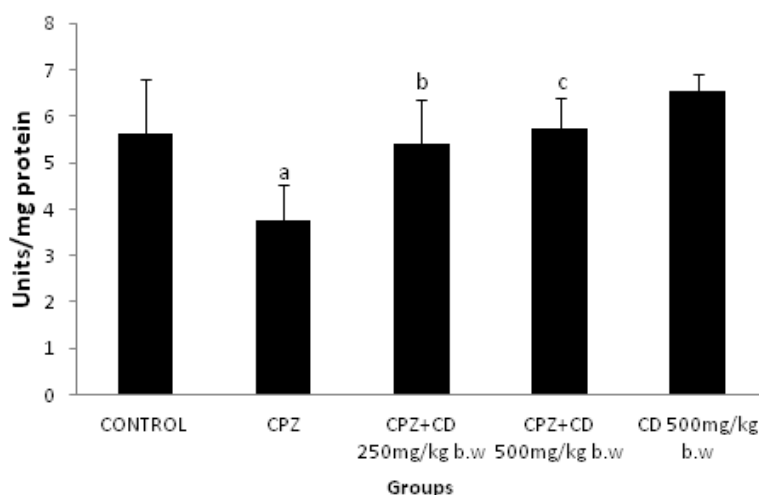
Figure 6: Effect of AECD on CPZ induced change in the activities of SOD and CAT in corpus collusum of control and experimental rats.

Figure 6a



a) Data represents changes in SOD antioxidant level in corpus collusum mean values \pm SD (n=6 animals each groups). ^a $p < 0.01$, is significantly different from control. ^b $p < 0.05$ and ^c $p < 0.01$ is significantly different from CPZ exposed group

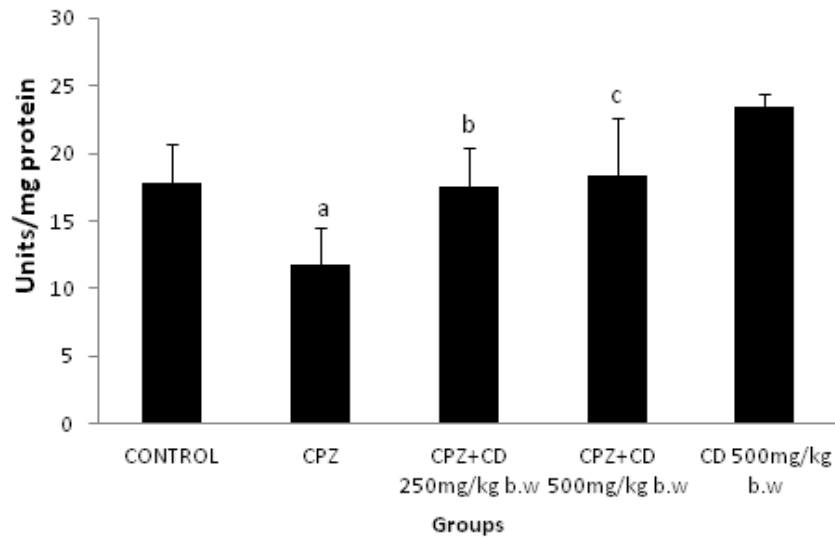
Figure 6b



b) Data represents alteration CAT level in corpus collusum mean values \pm SD (n=6 animals each groups). ^a $p < 0.01$, is significantly different from control. ^b $p < 0.05$ and ^c $p < 0.01$ is significantly different from CPZ exposed group; one-way ANOVA with Tukey's *post hoc* test was used for statistical analysis.

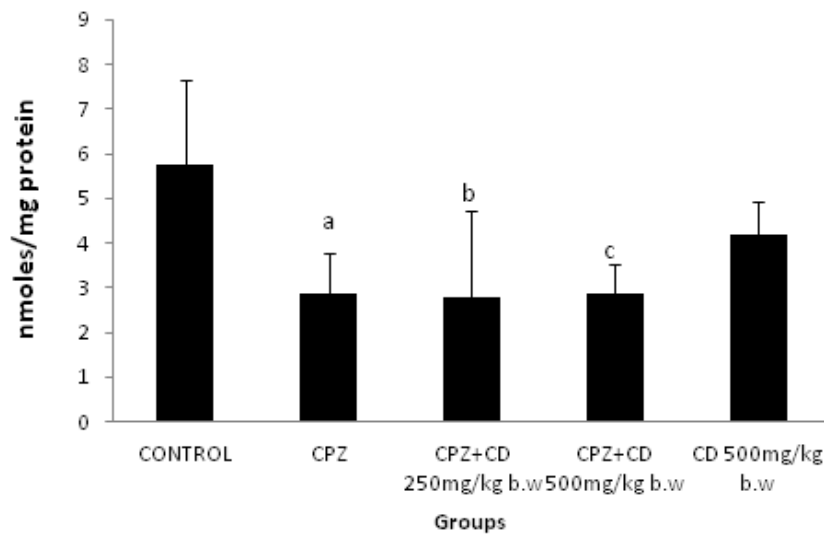
Figure 7: Effect of AECD on CPZ induced change in the activities of Gpx and GR in corpus collusum of control and experimental rats.

Figure 7a



a) Data represents changes in Gpx antioxidant level in corpus collusum mean values \pm SD (n=6 animals each groups). ^a $p < 0.01$, is significantly different from control. ^b $p < 0.05$ and ^c $p < 0.01$ is significantly different from CPZ exposed group;

Figure 7b



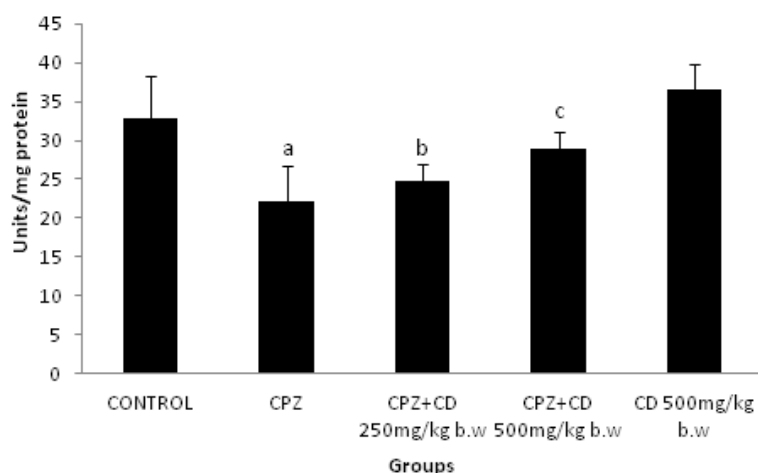
b) Data represents alteration GR level in corpus collusum mean values \pm SD (n=6 animals each groups). ^a $p < 0.01$, is significantly different from control. ^b $p < 0.05$ and ^c $p < 0.01$ is significantly different from CPZ exposed group; one-way ANOVA with Tukey's *post hoc* test was used for statistical analysis.

3.3.2. Effect of AECD on CPZ induced changes in activities of GST in the corpus collusum of control and experimental rats.

CPZ insulted group resulted in significant ($P < 0.01$) decrease in activities of GST, as compared to control

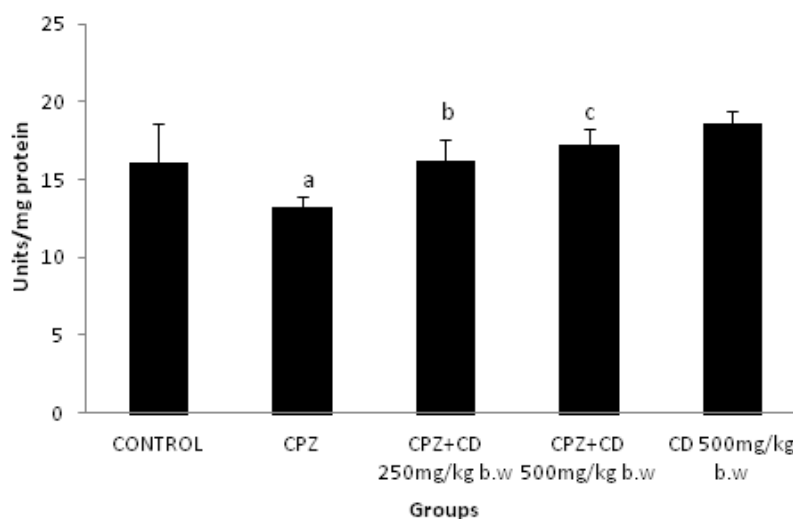
animals respectively. Subsequent treatment with AECD (250mg/kg and 500mg/kg) raised those antioxidant enzyme activities significantly ($P < 0.05$ and $P < 0.01$) when compared to CPZ induced animals (Figure 8).

Figure 8: Effect of AECD on CPZ induced change in the activities of GST in corpus collusum of control and experimental rats.



Data represents alteration GST level in corpus collusum mean values \pm SD (n=6 animals each groups). ^ap<0.01, is significantly different from control. ^bp<0.05 and ^cp<0.01 is significantly different from CPZ exposed group; one-way ANOVA with Tukey's post hoc test was used for statistical analysis.

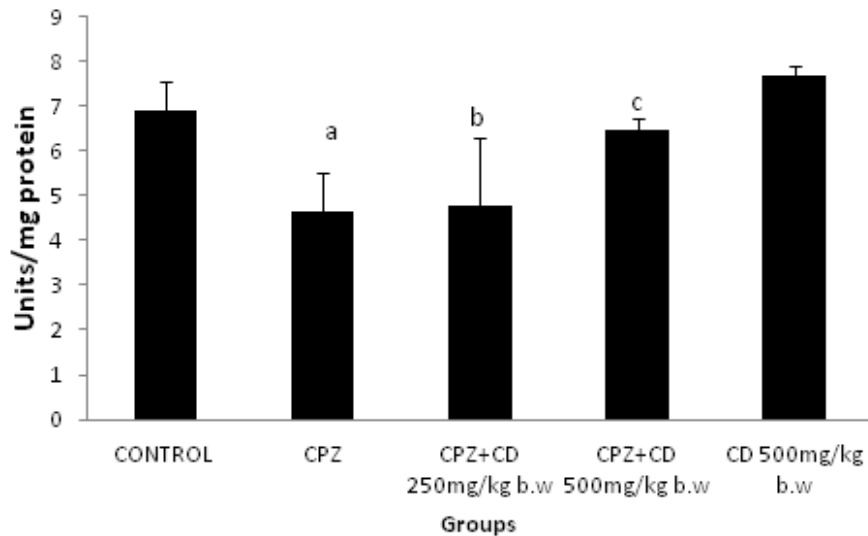
Figure 9: Effect of AECD on CPZ induced change in the activities of AChE in corpus collusum of control and experimental rats.



Data represents changes in AChE level in corpus collusum mean values \pm SD (n=6 animals each groups). ^ap<0.01, is significantly different from control. ^bp<0.05 and ^cp<0.01 is significantly different from CPZ exposed group.

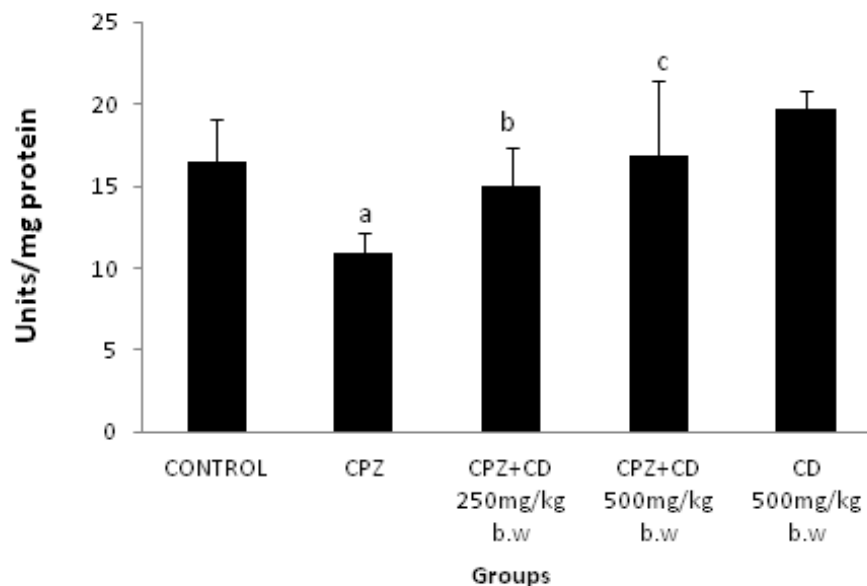
Figure 10: Effect of AECD on CPZ induced change in the Na⁺/K⁺ATPases and Ca²⁺ATPases activities in corpus collusum of control and experimental rats.

Figure 10a



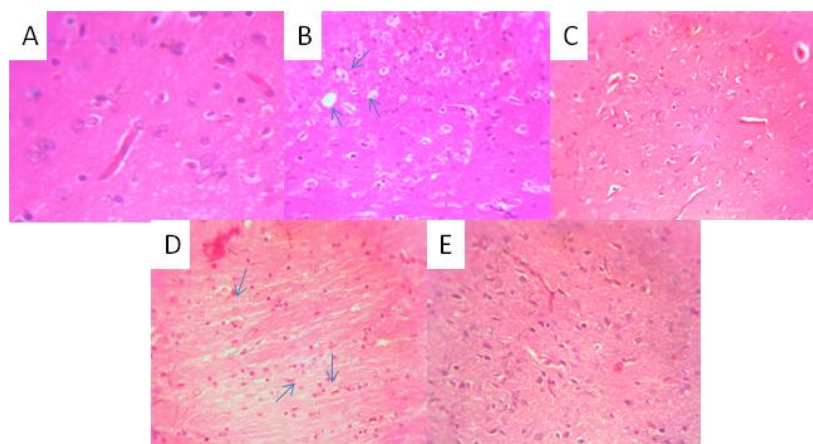
a) Data represents changes in Na⁺/K⁺ATPase level in corpus collusum mean values \pm SD (n=6 animals each groups). ^a $p < 0.01$, is significantly different from control. ^b $p < 0.05$ and ^c $p < 0.01$ is significantly different from CPZ exposed group;

Figure 10b



b) Data represents alteration Ca²⁺ATPase level in corpus collusum mean values \pm SD (n=6 animals each groups). ^a $p < 0.01$, is significantly different from control. ^b $p < 0.05$ and ^c $p < 0.01$ is significantly different from CPZ exposed group; one-way ANOVA with Tukey's *post hoc* test was used for statistical analysis.

Figure 11: Effect of AECD on CPZ induced pathological abnormalities in the corpus collusum of control and experimental rats



Haematoxylin and Eosin stained sections of striatum were visualized under light microscope at a magnification of 20x. Figure A: Control rats showing normal architecture of ECM. Figure B: CPZ induced rats showing severe Vacuole formation in ECM (denoted by arrow). Figure C & D: CPZ+AECD 250mg/kg and CPZ+AECD 500mg/kg showing a presence of healthy ECM and few damaged cells. Figure D: AECD 500mg/kg alone administered rats shows no pathological alterations they resemble that of control histology.

3.4 Effect of AECD on CPZ induced changes in the activities of AChE in the corpus collusum of control and experimental rats

Upon CPZ administration significantly ($P < 0.01$) decreased AChE activity as compared to control animals. Upon treatment with AECD (250mg/kg and 500mg/kg) raised those AChE activities significantly ($P < 0.05$ and $P < 0.01$) when compared to CPZ induced animals respectively (Figure 9).

3.5 Activity on ATPases

3.5.1 Effect of AECD on CPZ induced changes in the activities of $\text{Na}^+\text{K}^+\text{ATPases}$ and $\text{Ca}^{2+}\text{ATPases}$ in the corpus collusum of control and experimental rats

The animals present in CPZ administration group showed significantly ($P < 0.01$) decreased $\text{Na}^+\text{K}^+\text{ATPases}$ activity as compared to control animals. Upon treatment with AECD (250mg/kg and 500mg/kg) raised those activities in the significance of ($P < 0.05$ and $P < 0.01$) respectively when compared to CPZ induced animals (Figure 10a). The CPZ administration significantly ($P < 0.01$) decreased $\text{Ca}^{2+}\text{ATPases}$ activity as compared to control animals. Upon treatment with AECD (250mg/kg and 500mg/kg) raised those activities significantly ($P < 0.05$ and $P < 0.01$) when compared to CPZ induced animals respectively (Figure 10b).

3.6. Histological observation

3.6.1. Effect of AECD on CPZ induced pathological changes in the H&E stained corpus collusum sections of control and experimental rats

Hematoxylin and eosin stained corpus collusum sections of control and experimental groups were depicted in Fig. 11. Control group exhibited normal histology of corpus collusum (A). Section of CPZ induced group exhibited a prominent vacuole formation (B). Rats treated with AECD at 250 mg/kg and 500 mg/kg exhibited a presence of healthy granular cells and few damaged cells respectively (C, D). Animals received AECD (500 mg/kg) exhibited healthy granular cells which (E) resembles the control group.

4. DISCUSSION

Multiple sclerosis (MS) is a demyelinating disease which has a great challenge for treating its promotion in central nervous system [32,33,34] and characterized by perennial and cumulative demyelination/remyelination cascade, develops sclerosis in white and grey matter of the brain [35], damage to axons, neuronal loss and neuroinflammation [36,37]. During the progression of the disease the loss of myelin was accompanied by reduction of oligodendrocyte precursor cells, astrogliosis, mature oligodendrocyte loss and expropriation of macrophages/microglia and T lymphocyte [38]. The extent of mitochondrial abnormalities noticed in MS includes changes in cellular

distribution and structure with a wide range of molecular and biochemical abnormalities [39,40,41,42] such as lipid peroxidation and protein carbonyl, alteration in level of antioxidants such as superoxide dismutase(SOD), catalase, Glutathione peroxidase (GPx), Glutathione-S-transferase (GST), non-enzymatic antioxidants Glutathione reductase(GR) and nitrate finally may lead to changes in neurochemicals Na⁺-K⁺ ATPase, Ca²⁺ATPase which are increasingly considered to play a causative role in the pathogenesis and pathophysiology of developing multiple sclerosis [43]. The cuprizone (CPZ) model of MS, where CPZ is a copper chelator which damages predominantly the matured oligodendrocytes localized mainly in corpus collusum and causes the myelin sheath disruption [44]. Corpus collusum is a region of the brain which has a huge band of myelinated fibers that allows communication between the two hemispheres of the brain. In this study CPZ intoxication was given to adult wister rats through oral gavage for the period of six weeks which induces demyelination along with AECD in dosage of 250mg/kg/b.w and 500mg/kg/b.w was also given orally for a period of 42 days [15]. CPZ does not affect microglia, astrocytes and oligodendrocyte precursors cells (OPCs) but for mature oligodendrocyte show signs of toxicity because of its reduced mitochondrial transmembrane potential and decreased survival [45, 46]. CPZ intoxication to animals clearly exploit an increased oxidative stress on oligodendrocyte and disturbance of Cu homeostasis which causes neurotoxic effect due to enzyme inhibition thereby causing the changes in inflammatory markers i.e., LPO, Protein carbonyls and antioxidant enzymes like GST, GR, GSH, Catalase, SOD [5]. In this current study AECD was proved to be neuroprotective [12,13] against CPZ induced MS which was therapeutically possess anti-inflammatory due to the presence of phytoconstituents and immunomodulatory effect [9,10]. Hence the MS was said to be an inflammatory disorder due to the autoimmunisation of CNS.

The rats exposed with CPZ intoxication have undergone neurobehavioural changes which was elucidated by morris water maze, rotarod and open field test to examine the excellence of AECD because of its neuroprotective activity which on 42nd day reading was recorded by having the periodical trials on 7th, 14th, 21st, 28th, 35th day. In the present study, administration of CPZ for 42 days significantly raised the mean fall-off time in

rota rod task depicting the rigidity and movement disorders observed in MS. Similarly, CPZ delayed the transfer latency and reduced the time spent in target quadrant in morris water maze experiment, suggesting cognitive impairment. Our results in open field test of CPZ insulted rats showed greater period of immobility during induced condition compared to AECD treated rats which symptoms resembles limb paralysis and behavioral abnormalities in MS patients.

During CPZ treatment, the rate of O₂ •- scavenging and conversion to H₂O₂ is low in oligodendrocytes as the level of SOD was reduced [47, 48] whereas in AECD treated rats showed the significantly increased level of SOD.

Glutathione (GSH) was required by Glutathione peroxidase as an electron donor for the conversion of H₂O₂ to H₂O + O₂ which there is a reduction in GSH, GST and GPx levels during CPZ treatment [49] while in our results suggest that the level of antioxidant enzymes are markedly increased in AECD treated rats but distinguishly the level was higher in 500mg/kg/b.w AECD treated rats than 250mg/kg/b.w.

Previous *In vitro* study suggest that, lipid peroxidation can have noticed by an increase of malondialdehyde upon treatment of OLG cultures with CPZ [50]. For ATP production and lipid synthesis as an essential trophic factor, oligodendrocytes have a higher amount of Fe³⁺ inside ferritin which was sequestered as Fe intracellularly, however there is a reduction of sequestered Fe³⁺ to Fe²⁺ due to Excess O₂ •-, thereby releasing the latter into the cytosol or mitochondrial matrix. Subsequently lipid peroxidation through Fenton chemistry can be initiated by removed Fe from Fe³⁺ [47,51, 52]. In this study the level of LPO was apparently increased in CPZ treated rats which was perceivably decreased in AECD treated groups in dosage of 500mg/kg/b.w compared to 250mg/kg/b.w

The key players in β-oxidation and lipid synthesis are peroxisomes, produces higher amounts of H₂O₂ which is normally unloaded by peroxisome-bound catalase (CAT) and GPx. In vitro studies recommend that, decreased CAT activity in oligodendrocyte culture treated with CPZ [50]. Additionally, the number of peroxisomes increases significantly to allow the oligodendrocyte to synthesize the vast amounts of myelin sheath lipids upon oligodendrocyte precursor cell maturation, thereby increasing the production of H₂O₂ [51]. In this present study CPZ treated rats reported to

have decreased amount of CAT which was remarkably increased in AECD treated groups, the level was slightly higher in rats treated with 500mg/kg/b.w groups.

In our study the level of protein carbonyls are increased in CPZ treated rats, commonly there is an elevated of carbonylated protein in MS patients which is said to be an biomarker of oxidative injury within the CNS [53]. The AECD treated (500mg/kg/b.w) rats shows the decline in the level of carbonylated protein compared to induced group.

The level of nitric oxide was elevated in CPZ insulted rats mainly involved in oligodendrocyte injury, axonal degeneration and demyelination which higher concentration in cells such as macrophages and astrocytes are due to the appearance of the inducible form of nitric oxide synthase (iNOS) commonly seen in CSF, serum and urine of MS patients [55] Here the level of NO was subsequently recessed in AECD treated groups. In comparing, the dosage 250mg/kg/b.w and 500mg/kg/b.w, the higher dosage shows an apparent decline of NO.

The model of demyelination shows, lapse in Na⁺/K⁺ ATPase and Ca²⁺ ATPase level which would be expected to predispose demyelinated axons to import injurious levels of Ca²⁺. The ATPase predicted to have a protective effect, either by preventing axonal degeneration through direct action on axons or by blunting the activity of microglia and macrophages in MS [54]. In this study the level was decreased in CPZ treated groups whereas the level was abrogated in AECD treated group but significant increase was noticed in 500mg/kg/b.w treated groups.

The level of Ache was commonly reduced in CSF of MS patient, in CPZ model of MS also shows the decreased level of Ache while in AECD (250mg/kg/b.w and 500mg/kg/b.w) treated groups showed the variable difference compared to induced groups.

Using Haematoxylin & Eosin stain the changes in extracellular matrix (ECM) of corpus collusum [31] of rat induced with CPZ was depicted a heterogeneously composed ECM with vacuoles which significantly reduced in AECD treated rats. Hence pathological evidence at the dosage of 500mg/kg/b.w is more similar to control groups.

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6. Conclusion

From our result we are culminate that AECD in dosage of both 250mg/kg and 500mg/kg showed the significant neuroprotective effect which are established via biochemical estimation and histological evidence. Hence, we confirm that cynodonactylon may have a proficient property to decrease the progression of MS, Further research is required to prove the functional role of this plant at bioactive compound level against MS.

7. Conflict of interest

The authors declare that they have no conflict of interest.

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