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EVALUATION OF CURCUMIN AGAINST CUPRIZONE INDUCED MOUSE MODEL OF MULTIPLE SCLEROSIS

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Abstract:

Multiple sclerosis (MS) is a chronic demyelinating disorder that affects the neurons in the central nervous system. The current drugs used for the treatment of MS are expensive and involves toxicity related issues, so there is a need to discover inexpensive and less toxic approaches for the treatment of MS. Curcumin (CCM) is a phytochemical that was shown to exhibit beneficial effects due to its antioxidant and anti-inflammatory properties. CCM was reported to inhibit MS induced by Experimental autoimmune encephalomyelitis (EAE) in rodents. The present study focuses on ability of CCM to attenuate demyelination caused by the administration of CPZ, one of the most widely used model to study demyelination and remyelination process in MS. The parameters estimated were body weight, biochemical parameters, locomotor activity, luxol fast blue (LFB) staining. The animals administered with CPZ showed significant loss in body weight CCM attenuates this effect, it also normalized the behavioural changes and biochemical parameters that were altered in CPZ treated mice. It is concluded that CCM has the potential to attenuate CPZ induced demyelination.

Keywords: *Multiple sclerosis, Curcumin, Cuprizone, Demyelination, Luxol fast blue*

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1. INTRODUCTION:

Multiple sclerosis (MS) is a chronic demyelinating disorder that affects the neurons in the central nervous system. It is expected to have 2.5 million people affected by this disease worldwide[1]. There are 400,000 people suffering from this disease in the United states[1]. Most people with MS are between 20 to 50 years of age, with rare cases occurring after age 65 years[2]. The current drugs used for the treatment of MS are expensive and involves toxicity-related issues, so there is a need to find out inexpensive and less toxic approaches for the treatment of MS. Curcuma longa (Turmeric) of Zingiberaceae family is widely used for treatment of infections and inflammatory diseases[3]. Curcumin (CCM) is a yellow coloured pigment that is extracted from the rhizomes of Turmeric. CCM regulates immune system by cellular and humoral mediated immunity[3]. It is having many pharmacological activities like anticancer[4], immunosuppressant[5], antioxidant[6], antiarthritic and anti-inflammatory activities[7] and it is also capable of modulating the activities of several transcription factors such as NF-κB, Akt, BCL2, Caspases, PARP, Epidermal growth factor, Mitogen-activated protein kinase, Cyclooxygenase etc., responsible for the onset of many autoimmune disorders[7]. It is also reported that CCM has the potential to treat MS induced by Experimental autoimmune encephalomyelitis (EAE) method in rodents[8].

Biscyclohexanone oxaldihydrazone popularly known as Cuprizone (CPZ) is a copper chelating agent administered to induce demyelination in the brain which is the hallmark of the CNS disorders such as MS[9]. There are several animal models available for MS, but CPZ induced demyelination is the easiest and the most suitable method to investigate the demyelination and remyelination processes[10]. Thus, the present study aimed at investigating the potential benefits of Curcumin in Cuprizone induced MS in mice.

2.MATERIALS AND METHODS:

2.1. Chemicals

Cuprizone and luxol fast blue (LFB) stain were purchased from (Sigma Aldrich, St Louis, USA). Curcumin was purchased from (Lobachemie, Mumbai, India) lithium carbonate was purchased from (Otto chemie, Mumbai, India). All other chemicals used in this study were of analytical grade and of highest purity obtained from standard commercial sources in India.

2.2. Animals:

Healthy adult BALB/C mice of either sex weighing 20-30 grams were purchased from Mahaveera enterprises, Hyderabad, India. Animals were housed in appropriate cages in uniform hygienic conditions and fed with standard rodent chow diet and water ad libitum. The animals were well housed under standard well maintained 12:12 dark and light cycle in standard environment (temp 23 ± 10C) in Shri Vishnu College of Pharmacy with registration no: 439/PO/S/01/CPCSEA. The present study was approved by the Institutional Animal Ethical Committee (IAEC) bearing CPCSEA Ref Number-02/IAEC/SVCP/2018-19. Mice were randomly divided into four groups each containing six animals. Male and female mice were housed in separate cages. We used male and female in this study as the pattern of demyelination and remyelination is similar between genders and that there is little or no difference in the loss or repopulation of mature oligodendrocytes or accumulation of reactive glia[11,12]. An identification mark was made on the mice of each group using coloured marker by giving marks on the tail from one to six, following the standard procedure of animal identification marking. Each mouse was weighed and the doses were calculated accordingly.

2.3. MS Induction by Cuprizone (CPZ):

MS is induced by administering a powder diet or food pellets containing cuprizone[13]. It is difficult to administer the cuprizone to all animals uniformly by these methods and it is also prone to degradation by the environment[9]. In order to attain the dosage uniformity and keeping in view of stability, the induction protocol used by Zhen W et al.,[9] method is used in this current study. For oral gavage, CPZ powder was mixed in 1% methyl cellulose (MC) suspension and vortexed in order to obtain a homogenous CPZ - MC suspension. Mice were fed daily by oral gavage with 10 mL/kg volume. A stock solution of 1% MC was prepared weekly and stored at 4°C. CPZ did not react chemically with MC and just present in the suspension mixture. The vehicle group received 1% MC without cuprizone by gavage.

2.4. Preparation of CCM Suspension:

CCM is insoluble in water, for the ease of injection it was suspended in 0.5% MC suspension made by mixing 500 mg of MC in 100 mL distilled water. 0.25 µL of dimethyl sulfoxide (DMSO) was added and then it was homogenized for 5 minutes to obtain a uniform mixture. In case of i.p. The drug is more bioavailable than the gavage[14]. Due to the poor oral bioavailability of CCM we preferred to give the drug the through i.p. route.

2.5. Treatment Protocol:

The test compound and the standard drug were

administered one hour before the administration of CPZ for 5 weeks. **Group - I** fed with vehicle (Veh) MC suspension which served as normal control. **Group - II**, in which MS was induced by administering CPZ (400 mg/kg, p.o.) served as disease control. **Group - III**, in which MS was induced and treated with the standard drug (Fingolimod 0.125 µg/kg, p.o.). **Group-IV**, in which MS was induced and treated with the test compound (Curcumin 200 mg/kg, i.p.).

2.6. Body Weight Monitoring:
Most of the chemical inducing agents administration can lead to decrease in body weight of animals. To check whether administration of CPZ leads to decrease in body weight or not the animals were weighed daily.

2.7. Biochemical Analysis
To analyse the protective effects of CCM we determined MDA, GSH, levels in homogenates of the corpus callosum (CC), which are distinctive for oxidative stress. Mice were transcardially perfused with 0.1 M PBS. Following dissection of the brains, the CCs were isolated. After that, tissue samples were homogenized on ice. Tissues homogenates were produced in 0.15 M KCl (5% w/v homogenate). Microcentrifuge tubes each containing 0.6 mL were incubated 1 h at 37°C. Following that, 1.2 mL of 28% w/v trichloroacetic acid (TCA) solution (5%) was added, and by adding 1.2 mL of water, the final volume was made up to 3 mL. Subsequently, centrifuged at 3000 x g for 10 min and 2.5 mL of the supernatant was collected. After that, the colour was developed by addition of 0.5 mL of 1% w/v thiobarbituric acid dissolved in 0.05 N NaOH keeping the solution in boiling water bath for 15 minutes until the appearance of pink colour. Finally, the absorbance was read in a spectrophotometer at 532 nm. Malondialdehyde (MDA) contents were declared as nmol/g wet tissue. The GSH content of CC was also determined by spectrophotometer according to Sedlak et al.,[15] method. Briefly, proteins from homogenized tissues (10% w/v in PBS, pH 7.4) were removed, after adding an equal volume of 10% TCA, incubated at 4°C for 2 h. Then, the samples were centrifuged at 2000 x g for 15 min, and the supernatant was added to 2 mL of 0.4 M Tris buffer (pH 8.9) including 0.02 M EDTA (pH 8.9). After that, 0.01 M 5,5'-Dithio-bis 2-nitrobenzoic acid was added. Eventually, the mixture was diluted with 0.5 mL distilled water, and absorbance was read in a spectrophotometer at 412 nm. Results were shown as µg GSH/g wet tissue sample.

2.8. Locomotor Activity (Open Field Test):
Open field test apparatus is used to measure the locomotor activity and behavioural responses to a new environment. It comes in different sizes, in this study we have used a 50 x 50 Cm sized box with dark walls. The box was differentiated into two areas the open arm (central area) and closed arm (area in the periphery). The mice were placed in the open arm and they are allowed to move freely. The path of the animals was recorded with a video camera placed above the apparatus. Parameters such as total distance travelled, average speed was analysed as a measure of locomotor activity with the help of Maze master 1.2.0 software.

2.9. Luxol Fast Blue Staining:
Mice were euthanized by cervical dislocation and the brains were isolated and fixed with 10% neutral buffered formalin. The tissues were embedded with paraffin and 8 µm thick sections were cut with the help of microtome. The sections were deparaffinized and stained with LFB by incubating them in the staining solution overnight at 60°C. Following that sections were differentiated with lithium carbonate solution, rinsed with 95% and 70% ethanol in a series and then dehydrated with ethanol, mounted with xylene and viewed under the microscope to examine the extent of demyelination.

2.9. Statistical analysis:
Results were represented as Mean ± S.E.M., n=6/ group; data was analysed by one-way ANOVA followed by Tukey's multiple comparison test using GraphPad Prism version 8.2.0 (435) software; significance at ***/###p < 0.0001, **/##p < 0.001, */#p < 0.1. *P vs Veh, # vs Veh + CPZ.

3. RESULTS:

3.1. Effect of CCM on body weight:
Body weight of mice was measured daily throughout the experimental period (Fig 1). During the course vehicle-treated animals showed a slight increase in weight (1.45 ± 5.76). The animals treated with CPZ experienced severe weight loss (-22.33 ± 3.54). The animals treated with both CCM (-3.68 ± 5.22) and the standard drug Fingolimod (-1.12 ± 7.07) were shown a slight decrease in body weight.

3.2. Effect of CCM on biochemical parameters:
To analyse the effects of CPZ and CCM exposure on parameters for oxidative stress, we determined MDA and GSH levels in homogenates of brain (Fig 2). The results showed that administration of CPZ caused lipid peroxidation which was evidenced by increase in MDA levels (30.90 ± 4.36) in CPZ only treated group, administration of CCM significantly decreased

(18.61 ± 1.20) the lipid peroxidation. Administration of CPZ decreased the antioxidant enzyme GSH levels (56.91 ± 0.10) in the brain, treatment with CCM significantly attenuates this effect and increased GSH levels (70.13 ± 0.45) significantly.

3.3. Open field test:

Experimental animals were assessed in the open field test to evaluate locomotor activity (Fig 3). The total distance travelled, average speed was used as a measure of locomotor activity. The CPZ treated animal showed an increase in brain activity which was evidenced by increase in locomotor activity (distance travelled 1371.50 ± 680.75 and average speed 2.93 ± 1.33) treatment with CCM decreased (1202.98 ± 554.53, 2.52 ± 0.47) this phenomenon but it was not significant statistically.

3.4. Luxol fast blue staining:

Brain sections of each group were stained with LFB, in which the lipid rich myelin stains blue and the remaining area stands colourless. The myelin density is evaluated by intensity of colour in the stained sections, the more the intensity of the stain the lesser is the demyelination. We concentrated on the corpus callosum region because it is the widely studied region in demyelination model. LFB staining technique was carried out after 5 weeks of treatment protocol. The brain sections of the mice fed with CPZ exhibited patches of myelin loss and the colour intensity was also less when compared to the control group (Fig 4). The CCM treated group exhibited the colour intensity similar to that of control group and showed intact myelin.

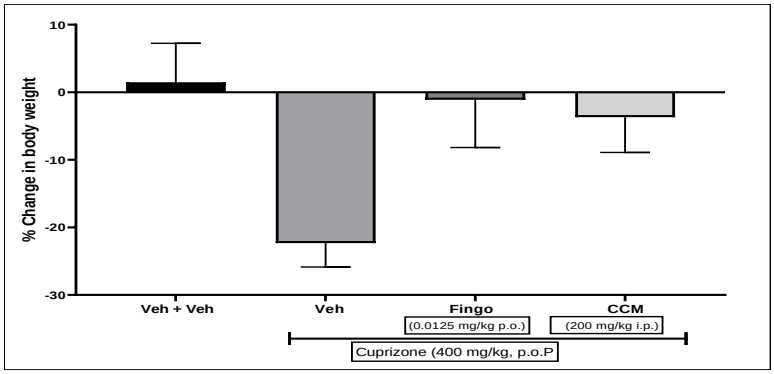

Fig 1: Effect of Curcumin on body weight. The body weight was measured daily during the course, the vehicle treated group showed slight increase in body weight. The animals administered with the only CPZ experienced severe weight loss, the animals treated with CCM and Fingolimod weshowed a slight decrease in body weight. It reveals that CCM attenuates the weight loss caused by CPZ administration

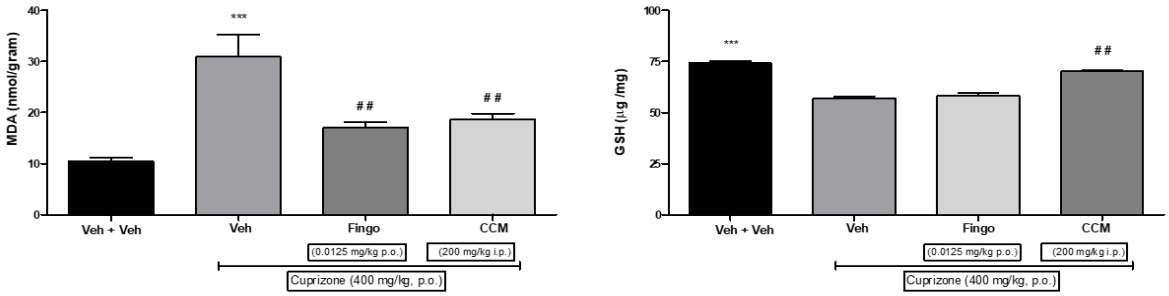

Fig 2: Effect of curcumin on biochemical parameters. The results showed that administration of CPZ caused lipid peroxidation which increased MDA levels (30.90 ± 4.36) in CPZ treated group. CCM administration significantly decreased (18.61 ± 1.20) lipid peroxidation. Administration of cuprizone decreased the reduced GSH levels (56.91 ± 0.10) in the brain region, curcumin administration significantly attenuates this effect and increased the levels of reduced GSH levels (70.13 ± 0.45) significantly.

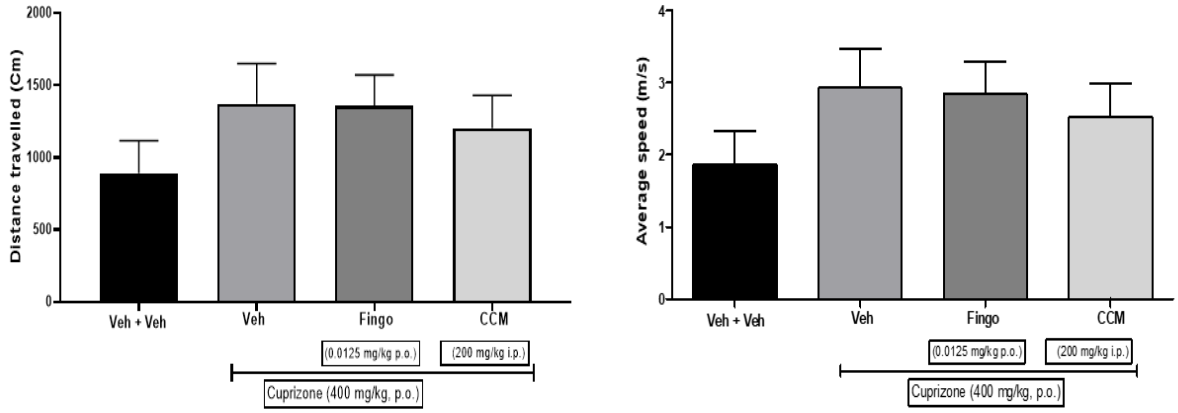

Fig 3: Effect of curcumin on locomotor activity. The cuprizone treated animals showed an increase in locomotor which is evidenced by the increase in total distance travelled and speed, treatment with curcumin decreased this phenomenon but it was not significant statistically.

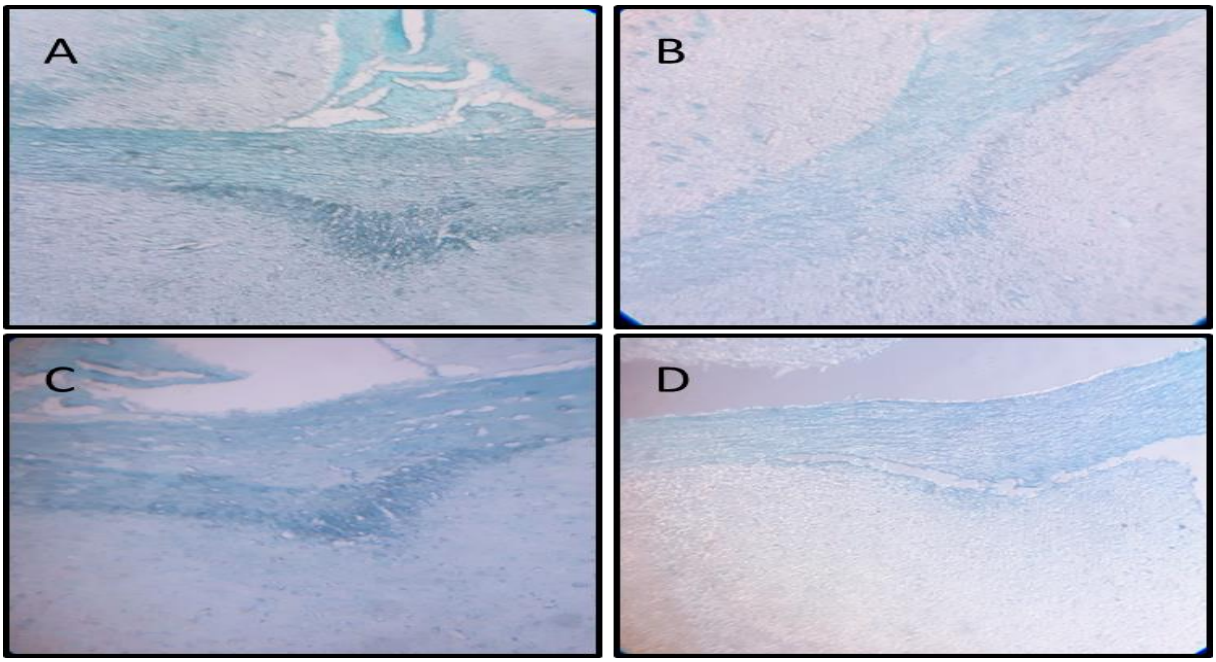

Fig 4: LFB staining of CC. Demyelination in the CC after 5 weeks of CPZ induction with or without CCM treatment. Myelin index was evaluated in LFB stained sections of the CC. A) Vehicle, B) CPZ + Vehicle, C) CPZ + Fingo, D) CPZ + CCM. In the diseased control group the colour was less intense when compared to the vehicle treated group and showed errosion of myelin. Both the standard drug and the test compound were near to the intensity of control group and showed intact myelin

4. DISCUSSION:

The present study addresses the anti-oxidant and anti-inflammatory effect of CCM and its protective role in CPZ induced MS in mice is evidenced by remarkable reduction of LPO products and increase in reduced GSH.

Multiple sclerosis (MS) is a disease of the central nervous system (CNS) involved with multiple factors, characterized by inflammation, demyelination, and axonal loss. It is considered to be biphasic disease with inflammatory relapsing-remitting (RR) and degen-erative secondary

progressive (SP) phases (Silvia BA et al., 2016).
The cuprizone model is a well-established instance to study demyelination and remyelination process in rodents. Usually, it is administered by mixing with powered or pelleted rodent chow. However, since it is sensitive to the environment and the intake of it varies between different animals, the major issue is the variance in demyelination of the animals. Administration of cuprizone at a dose of 400 mg/kg/day by oral gavage was found to be the best dosage to induce demyelination after 5 weeks of administration; while remyelination occurs after 9 days of cuprizone withdrawal (Zhen W et al., 2017). It has an advantage is that the consumption of cuprizone could be well controlled and the mice were exposed to the same dose of cuprizone. Thus, the variation in demyelination was minimized. This alternative dosing regimen minimizes the inter-animal variability on demyelination and consequently provides a consistent model for pharmacological evaluations (Zhen W et al., 2017).

Previous reports have shown that CPZ induces the formation of megamitochondria in oligodendrocytes was determined in CPZ after three weeks. Invitro experiments during which primary glial cultures of rats were treated with CPZ provided extra proof that neuroglia, astrocytes, and oligodendrocytes had shown signs of toxicity upon CPZ treatment (Praet J et al., 2014). CPZ intoxication clearly increased oxidative stress on oligodendrocytes. The formation of megamitochondria due to CPZ administration leads to the shortage of ATP and will increase ROS/RNS concentrations of that eventually disrupts the correct functioning of the Endoplasmic reticulum (ER)[17]. ER stress additionally reduces mRNA transcription/translation to avoid an accumulation of un or misfolded proteins. As such, the downregulation of myelin protein connected mRNAs are often determined already at intervals the primary week of CPZ treatment and continue till CPZ administration is halted (Praet J et al., 2014).

CCM suspended in MC administered at a dose of 200mg/kg through intraperitoneal route attenuates the weight loss (-3.68 ± 5.22) occurred due to the toxic effects of CPZ when compared to diseased group (-22.33 ± 3.54). Results have shown that CPZ feeding significantly increased MDA levels (30.90 ± 4.36) due to lipid peroxidation, curcumin treatment significantly reduced this effect and it also increased the reduced GSH (70.13 ± 0.45) levels when compared to the diseased group (56.91 ± 0.10). It is assumed that CCM can restore the normal balance between oxidative stress markers and endogenous antioxidant mechanisms that is often disrupted in neurodegenerative disorders. Mice given with CPZ for 5 weeks showed increased locomotor activity in the open field. Administration of CCM reduces the increased CNS activity but it was not statistically significant. The increase in locomotor activity is related to increased brain activity rather than myelin damage (Zhang H et al., 2013). The luxol fast blue staining of the corpus callosum in the diseased group showed the erosion in white matter, however, the fingolimod treated group and CCM treated group exhibited a similar pattern when compared to the vehicle treated group.

5. CONCLUSION:

In conclusion curcumin at this specified dose level of 200 mg/kg, i.p. in mice has normalized the biochemical, behavioural abnormalities in cuprizone induced multiple sclerosis. Further Luxol fast blue staining confirmed the neuroprotective activity of curcumin in cuprizone induced MS in mice. The actual mechanism of curcumin in cuprizone induced MS in mice is not clear with these studies. The action of curcumin in attenuating the demyelination and normalizing behavioral changes and other relevant mediators will be carried out in future to study its mechanism.

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Conflict of interest: The authors report no conflict of interest.

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