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Original Article

Combating Neuroinflammation and Neurotoxicity with Curcumin-Piperine Nanoformulations

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ABSTRACT

Background: Neuroinflammation plays a critical role in the progression of neurodegenerative diseases such as multiple sclerosis, Alzheimer's, and Parkinson's disease. The Cuprizone (CPZ)-induced model is widely used to study neuroinflammatory mechanisms, as it selectively targets oligodendrocytes, causing demyelination and oxidative stress. **Objectives:** This study investigates the neuroprotective effects of curcumin nanoformulations prepared in *Zanthoxylum rhetsa* seed oil against CPZ-induced neurotoxicity in Swiss albino mice. **Materials and Methods:** The study assessed antioxidative enzyme activities and inflammatory markers, including catalase, superoxide dismutase, glutathione peroxidase, GFAP, MCP-1, MIP-1, and CCL-5. **Results:** The results indicated that CPZ exposure significantly reduced catalase and SOD levels while increasing oxidative stress markers such as Malondialdehyde (MDA) and inflammatory cytokines. The administration of curcumin nanoformulations demonstrated substantial neuroprotection by restoring antioxidative enzyme levels, reducing inflammation, and mitigating myelin degradation. Notably, the combination of curcumin with Piperine (PFZ) exhibited the highest neuroprotective potential, as it significantly enhanced catalase and SOD activity while suppressing inflammatory markers like TNFα and GFAP. Furthermore, PFZ-treated mice displayed improved Myelin Basic Protein (MBP) levels, indicating enhanced remyelination. **Conclusion:** The findings suggest that curcumin-based nanoformulations, particularly those combined with piperine, hold promise as therapeutic strategies for mitigating neuroinflammation and oxidative damage associated with demyelination. These results provide valuable insights into the potential of plant-derived antioxidants in treating neurodegenerative conditions.

Keywords: Cuprizone, Cytokines, GFAP, Inflammation, MCP-1, MIP-1, Neurotoxicity,

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INTRODUCTION

Neuroinflammation is a multifaceted immune response within the Central Nervous System (CNS) that plays a crucial role in the pathogenesis of various neurodegenerative diseases, including Multiple Sclerosis (MS), Alzheimer's disease, and Parkinson's disease.[1] This inflammatory response is primarily mediated by the activation of resident immune cells, such as microglia and astrocytes, which, when stimulated, release a cascade of pro-inflammatory cytokines, chemokines, and Reactive Oxygen Species (ROS).[2] These inflammatory mediators contribute to neuronal damage, synaptic dysfunction, and the progression of neurodegenerative conditions by exacerbating oxidative stress and impairing cellular homeostasis. To investigate the mechanisms underlying neuroinflammation and demyelination, researchers frequently employ the Cuprizone (CPZ)-induced model, which selectively targets oligodendrocytes, leading to myelin degradation and mimicking several pathological features of MS.[3] CPZ exposure results in substantial oxidative stress, disrupting redox equilibrium and triggering glial cell activation. This process is marked by the upregulation of key inflammatory

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and glial markers, such as Glial Fibrillary Acidic Protein (GFAP) and Chemokine Ligand 5 (CCL-5), which serve as indicators of neuroinflammatory responses.[4]

Oxidative stress plays a pivotal role in the development and progression of neuroinflammatory conditions. The excessive accumulation of ROS leads to cellular damage and impairs the body's intrinsic antioxidant defense mechanisms.[5] Antioxidant enzymes, including catalase, Glutathione Peroxidase (GPX), and Superoxide Dismutase (SOD), are essential in counteracting oxidative stress by neutralizing ROS and preserving redox balance in neural tissues.[6] Given the impact of oxidative damage on neuroinflammation, augmenting the antioxidative defense system has emerged as a potential therapeutic strategy for mitigating CPZ-induced neurotoxicity and inflammation.

In recent years, natural compounds have garnered considerable attention for their neuroprotective and antioxidative properties. *Zanthoxylum rhetsa*, a medicinal plant rich in bioactive compounds, has been shown to possess significant anti-inflammatory and antioxidant activities, making it a promising candidate for neuroprotection.[7] Likewise, curcumin, the principal active ingredient in turmeric (*Curcuma longa*), has demonstrated potent anti-inflammatory, antioxidative, and neuroprotective effects, which can attenuate neuroinflammatory responses and reduce CPZ-induced neuronal damage.[8] However, despite its therapeutic potential, curcumin exhibits poor bioavailability, which limits its clinical efficacy. To overcome this limitation, co-administration with piperine, a major alkaloid derived from black pepper (*Piper nigrum*), has been shown to significantly enhance curcumin absorption and bioavailability.[9] While curcumin and piperine are well-established for their neuroprotective and antioxidant effects, the present study introduces a novel approach by formulating these compounds using *Zanthoxylum rhetsa* seed oil (ZRO) within a Self-Nanoemulsifying Drug Delivery System (SNEDDS). To the best of our knowledge, this is the first report to utilize ZRO a natural oil with its own documented anti-inflammatory and antioxidant properties as a carrier for curcumin-piperine nanoformulations in a neurodegenerative disease model. This innovative formulation strategy offers potential synergistic benefits and enhances bioavailability, providing a new avenue for plant-based neurotherapeutics.

Given the intricate relationship between neuroinflammation, oxidative stress, and glial activation, exploring the neuroprotective effects of natural antioxidants in CPZ-induced neurotoxicity is of paramount importance. This study aims to evaluate the therapeutic potential of various curcumin nanoformulations prepared in *Zanthoxylum rhetsa* seed oil by examining their effects on antioxidative enzyme activities and inflammatory markers, including GFAP, MIP-1, MCP-1, and CCL-5, in a CPZ-induced neuroinflammatory model. By elucidating these mechanisms, this research seeks to contribute to the development of novel, plant-derived therapeutic strategies for combating neuroinflammation and neurodegenerative disorders.

MATERIALS AND METHODS

Animals

Seventy-five Swiss Albino Male Mice (SWR/J) weighing between 21 and 25 grams were acquired from the King Fahd Medical Research Center (KFMRC) animal housing unit at King Abdulaziz University in Jeddah, Saudi Arabia. A 12-hr light/dark cycle, with the light cycle occurring between 7:00 am and 7:00 pm, was implemented to maintain mice at a suitable room temperature of 23±2°C and humidity level of 65%. Food and water were freely available to every mouse. Animal studies were carried out in compliance with KFMRC's animal unit committee rules. The biomedical ethics research committee at King Abdulaziz University authorized the study protocol (approval No. ACUC-22-1-2), which complied with the guidelines established by the KFMRC's Animal Care and Use Committee. The research adhered to the "System of Ethics." The study was authorized by Royal Decree No. M/59 dated August 24, 2010, and it conformed with the "System of Ethics of Research on Living Creatures" rules created by King Abdulaziz City for Science and Technology. From June 1, 2022, until July 16, 2022, the study was conducted at the King Fahad Medical Research Center in Jeddah, Saudi Arabia.

Cuprizone and nanoformulations

Mice were administered 0.2% w/w Cuprizone (CPZ) (purchased from Sigma-Aldrich, Bangalore, India) mixed with ground rodent chow for five weeks to induce acute demyelination, as previously described.[10] A curcumin nanoformulation, with and without piperine, was developed by dissolving it in *Z. rhetsa* Seed Oil (ZRO), following previously published methods.[11] The nanoformulations were prepared by combining various ratios of surfactant, cosurfactant, and/or co-solvent with ZRO. A fixed amount of cosurfactant (I988) and Tween 85 was used to examine the effect on formulation performance. The mixture was homogenized and stored for later use. This formulation is essentially a Self-Nanoemulsifying Drug Delivery System (SNEDDS). The SNEDDS solubility values for curcumin and piperine were 19.0 mg/g and 48.22 mg/g, respectively. The drug-free SNEDDS had a droplet size of 607.1 nm, while the curcumin-piperine loaded SNEDDS measured 700.86 nm. Zeta potentials of the curcumin-piperine loaded SNEDDS (-36.35 mV) and drug-free SNEDDS (-36.9 mV) indicated good physical stability of the resulting emulsion. The zeta potential remained consistent after curcumin-piperine loading. Curcumin was administered at 10 mg/kg, a dosage based on previous research.[12] The blank and curcumin-piperine nanoformulations were diluted in normal saline to achieve concentrations of 3 mg/kg piperine and 10 mg/kg curcumin per 0.1 ml for each mouse. Daily intraperitoneal injections of 0.1 ml of the formulation were administered to each mouse between 11:00 a.m. and 1:00

p.m. Dosages were adjusted weekly based on the mice's weight measurements.

Experimental Design

The study lasted for seven weeks in total in which cuprizone administered mixed with rodent chow for first 5 weeks to all groups except control. After Week 5, the cuprizone was removed from diet and lasted for next two Weeks (Week 6 and 7). A total of fifteen mice each group were randomly assigned to five primary groups. (1) For seven weeks, the control group was given standard chow and 0.1 ml of saline intraperitoneally. The second group (CPZ) given cuprizone-mixed chow for five weeks and 0.1 ml of normal saline intraperitoneally. The third group received 0.1 ml of ZRO-based blank formulation administered intraperitoneally along with CPZ-mixed chow daily for 5 weeks. The fourth group was given CPZ-mixed chow and a ZRO-based formulation of curcumin (0.1 ml) intraperitoneally for 5 weeks. The fifth group was given CPZ-mixed chow and a ZRO-based nanoformulation of curcumin with piperine for 5 weeks. Cuprizone feeding was discontinued in all groups at the conclusion of the week 5 (cuprizone toxicity stage), but treatment with various nanoformulations persisted until the end of week 7. Group names and designations: 1) Control group, CNT; 2) Cuprizone (CPZ) group; 3) blank formulation of ZRO, BFZ group; 4) nanoformulation of curcumin, CFZ group; and 5) curcumin-Piperine Formulation (PFZ) group.

Sample Collection

By the conclusion of the fifth week, a random selection of 7 mice from each group was used to induce isoflurane anaesthesia and decapitate them in order to end their lives. The whole brain of each mouse was then obtained by dissecting it. To get certain brain areas, including the hippocampus, frontal cortex, cerebral cortex, brainstem, and cerebellum, the obtained brains were further dissected. These brain slices were kept for future research at -80ºC after being immersed in RNA-later solution. At the conclusion of the study (Week 7), the remaining mice underwent the same process.

Biochemical analysis

Tissues were homogenized in RIPA lysis buffer (R-0278; Sigma) containing PMSF at the prescribed doses, Halt™ phosphatase inhibitor (Thermo-Fisher Scientific), and protease inhibitor cocktail (cOmplete™, Roche) for use in various biochemical assays. After centrifuging the homogenate at 15000 × g for 20 min, the supernatant was separated and stored for further biochemical examination. With the use of a colorimetric assay kit obtained from SolarBio (Beijing, China), the SOD and CAT levels were determined. ELISA kits (colorimetric) from Sunlong Biotech Co. Ltd., (Hangzhou, China) were used to measure the levels of GSH, GPX, GFAP, MCP-1, MIP-1 and CCL-5 in accordance with the manufacturer's instructions.

Statistical analysis

The changes and comparisons between different parameters at the end of toxicity stage (week 5) and healing stage (week 7) were statistically analyzed using One-way Analysis of Variance (ANOVA) followed by Tukey's analysis. Alterations in the results were considered significant when the p value was ≤0.05. Most of the statistical analyses were performed using Microsoft Excel and Social Science Statistics freely available at https://www.socscistatistics.com/

RESULTS

Impact of CPZ and ZRO-Based Nanoformulations on Catalase Levels

In the fifth week of the study, catalase activity was measured in different treatment groups to examine the effect of Cuprizone (CPZ) on enzyme levels. The control group exhibited a baseline catalase level of 17.31±1.55 U/mg protein (Figure 1). In stark contrast, the CPZ group demonstrated a significant reduction in catalase level, measuring only 5.59±1.21 U/mg protein, indicating a detrimental effect of cuprizone on enzyme activity. Among the treatment groups that also received cuprizone, both the Blank Formulation group (BFZ) and the curcumin group (CFZ) showed significantly higher catalase levels compared to the CPZ group. Specifically, BFZ recorded a catalase activity of 8.47±1.17 U/mg protein, while CFZ exhibited 9.95±1.26 U/mg protein. Notably, the group treated with curcumin-Piperine Nanoformulation (PFZ) displayed the highest catalase level at 14.22±0.34 U/mg protein. At the conclusion of the study (Week 7), following the cessation of cuprizone treatment after Week 5, catalase levels were reassessed (Figure 1). The control group demonstrated a catalase activity of 12.35±1.12 U/mg protein, indicating a moderate level of enzyme activity that remained stable over the two-week period. In contrast, the CPZ group continued to show suppressed catalase activity at 8.94±0.84 U/mg protein, indicating a continued suppression of enzyme activity even after the cuprizone was discontinued Among the treatment groups, BFZ recorded an increase in catalase activity to 11.37±1.45 U/mg protein, reflecting some recovery yet remaining below the control levels. The CFZ group showed further improvement, with catalase activity measuring 12.81±1.05 U/mg protein. The PFZ group continued to exhibit the highest catalase level

Impact of CPZ and ZRO-Based Nanoformulations on SOD Levels

The levels of Superoxide Dismutase (SOD) were assessed across different treatment groups at the conclusion of Week 5. The CNT group exhibited SOD activity of 19.93±1.23 U/mg protein, serving as a reference for the other groups (Figure 1). In contrast, the group receiving only cuprizone (CPZ) displayed a significant reduction ($p \leq 0.001$) in SOD activity compared to the control, measuring just 10.25±0.56 U/mg protein. Among the treatment groups that

also received cuprizone, the BFZ recorded the lowest SOD level at 8.41±1.45 U/mg protein. The CFZ group showed a slightly higher SOD level of 9.51±0.92 U/mg protein units however, it remains significantly lower than the control ($p \leq 0.001$). Notably, the PFZ group exhibited an improved SOD level of 12.94±1.88 U/mg protein units compared to the other treatment groups. At the end of Week 7, the group that had received Cuprizone (CPZ) until the end of Week 5 and now recovering spontaneously showed a significant increase in SOD level to 16.96±1.26 U/mg protein compared to the control, suggesting a recovery in antioxidant enzyme levels after the discontinuation of cuprizone treatment. The group treated with the combined curcumin-Piperine Nanoformulation (PFZ) displayed an SOD level of 12.64±0.59 U/mg protein, which was lower than the SOD activity observed in the CFZ group but still comparable to the control and BFZ groups (Figure 1). The comparison of SOD levels between Weeks 5 and 7 highlights the impacts of cuprizone on oxidative stress and the potential for recovery after treatment discontinuation.

Impact of CPZ and ZRO-Based Nanoformulations on GSH Levels

At Week 5, the GSH levels varied significantly among the different groups. The PFZ group exhibited the highest GSH level (159.18±8.74 ng/mg protein) even in the presence of cuprizone (Figure 2). On the other hand, the CPZ group had GSH level 74.42±5.37 ng/mg protein, which is significantly lower ($p \leq 0.05$) than the control group (102.71±7.59 ng/mg protein). After Week 5, cuprizone was withdrawn from rodent diet but treatment with different nanoformulations continued for two more Weeks. The cessation of cuprizone resulted in an increase in GSH level (91.22±4.68 ng/mg protein) in CPZ group compared to its level at Week 5. The CFZ and PFZ group continued to maintain higher GSH levels than the control group at the end of week 7. The results indicate significant changes in GSH levels within the brainstem region across different treatment groups and time points (Figure 2). At Week 5, the PFZ group had markedly higher GSH levels compared to all other groups.

Impact of CPZ and ZRO-Based Nanoformulations on GPX Levels

During Week 5, the control group exhibited the highest GPX activity, measuring 4.34±0.06 ng/mg protein (Figure 2). The CPZ group, which was exposed to cuprizone until the end of week 5, had a significantly lower GPX activity (2.24±0.09 ng/mg protein) compared to the CNT group ($p \leq 0.001$), indicating cuprizone-induced toxicity. The BFZ (3.04±0.21 ng/mg protein) and PFZ (2.84±0.09 ng/mg protein) groups showed moderate GPX activity. The CFZ group exhibited the lowest GPX activity among the treated groups. At week 7, after CPZ was discontinued at the conclusion of Week 5, a general reduction in GPX activity was observed in all groups. The CNT group showed a significant decrease from 4.34±0.06 ng/mg protein at Week 5 to 1.16±0.05 ng/mg protein at Week 7. The CPZ group exhibited a minor reduction (from 2.24±0.09 to 1.24±0.06 ng/mg protein), indicating persistent oxidative stress effects. The BFZ group showed a moderate decrease, while the CFZ group decreased from 1.91±0.09 to 1.39±0.04 ng/mg protein. Notably, the PFZ group had the smallest decrease in GPX activity (from 2.84±0.32 to 2.35±0.13 ng/mg protein). Across all groups, a decline in GPX level was observed from Week 5 to Week 7 (Figure 2). The control group exhibited the most significant decrease. The CPZ group showed a minimal decrease despite CPZ discontinuation. The BFZ, CFZ, and PFZ groups showed a protective trend, with PFZ retaining the highest GPX activity at week 7.

Impact of CPZ and ZRO-Based Nanoformulations on AChE Levels

At the conclusion of Week 5, the Acetylcholinesterase (AChE) levels in the brainstem was measured across different treatment groups. The results are presented in Table 1. The CPZ group exhibited a significant reduction ($p \leq 0.0001$) in AChE level compared to the control group. The BFZ group demonstrated partial restoration of AChE level relative to CPZ. In contrast, the CFZ and PFZ groups showed markedly elevated AChE levels. After Week 5, cuprizone was stopped in all groups but treatment with nanoformulations continued until the end of Week 7. At the end of Week 7, the CPZ group (now going on spontaneous recovery) showed an increase in AChE level compared to Week 5, but levels remained lower than in the control group. The BFZ group exhibited a further increase in AChE activity. Notably, the CFZ and PFZ groups found with significantly increased level of AChE compared to the control and CPZ groups at Week 7. Comparing the AChE levels of Week 5 with Week 7, the CPZ group exhibited the most dramatic increase (103.31%) in AChE level. The BFZ group showed a modest 25% increase. In contrast, the CFZ and PFZ groups showed significant reductions (61.58% and 42.36%, respectively from Week 5 to Week 7), though their levels were still significantly high compared to the control and CPZ groups within Week 7 (Table 1).

Impact of CPZ and ZRO-Based Nanoformulations on MDA Levels

Malondialdehyde (MDA) is a well-established marker of lipid peroxidation and oxidative stress. The CPZ-treated group exhibited the highest MDA levels (3.20±0.81 nmol/mg protein) compared to the control group as determined at the end of Week 5 (Table 1). The BFZ group also showed elevated MDA levels (2.78±0.93). The CFZ and PFZ groups had slightly lower MDA levels (2.22±0.75 and 2.56±0.83 nmol/mg protein respectively) but were still elevated compared to the control. Compared to Week 5, the control group in Week 7 showed a marked increase in MDA levels (from 1.47 nmol/mg protein to 3.73 nmol/mg protein). The CPZ-treated group (now recovering spontaneously) exhibited equivalent MDA levels compared to the control. The

BFZ group displayed the significantly high MDA levels compared to the control and CPZ group ($p \leq 0.01$). The CFZ group (3.56±0.65 nmol/mg protein) had values close to the control whereas PFZ group had the lowest MDA levels among the treated groups (2.99±0.97 nmol/mg protein) The percent change in MDA levels from Week 5 to Week 7 was also calculated. The control group exhibited the highest percentage increase (153.74%) (Table 1). The CPZ-treated group showed a 27.50% increase. Although the CFZ group showed a substantial rise (60.36%) in MDA levels from Week 5 to Week 7 but it was observed to be close to the MDA level of control group within Week 7. The PFZ group exhibited the lowest increase (16.80%).

Impact of CPZ and ZRO-Based Nanoformulations on MBP Levels

The levels of Myelin Basic Protein (MBP) in the brainstem for different treatment groups in Week 5 are summarized in Table 1. The CPZ-treated group exhibited the lowest MBP levels (27.7±3.018 pg/mg protein) among all the groups. The BFZ group showed moderate MBP levels (50.03±6.12 pg/mg protein), slightly lower than the control. The CFZ and PFZ groups demonstrated relatively higher MBP levels. The withdrawal of cuprizone from diet after Week 5 resulted in a significant (184.94%) increase in MBP levels (from 27.7 to 78.92 pg/mg protein). The BFZ group also showed an increase of 30.8% from Week 5 to Week 7. The CFZ group maintained relatively stable MBP levels (from 72.7 to 76.51 pg/mg protein). The PFZ group, which had the highest MBP levels at Week 5, showed a slight decrease (from 97.22 to 92.37 pg/mg protein) but still maintained the highest levels among all groups.

Impact of CPZ and ZRO-Based Nanoformulations on TNFα Levels

At the end of Week 5, TNFα level was found to be significantly increased ($p \leq 0.01$) in the CPZ group compared to the control group (Table 1). On the other hand, the blank formulation group

Table 1: Levels of AChE, MDA, MBP and TNFα in brainstem at the end of Week 5 and Week 7.

Group	AChE (U/mg protein)			MDA (nmol/mg protein)		
	Week 5	Week 7	% change	Week 5	Week 7	% change
Control	32.88±2.81	30.46±6.12	7.36↓	1.47±0.26	3.73±0.87	153.74↑
CPZ	9.36±1.19	19.03±2.15	103.31↑	3.20±0.81	4.08±0.94	27.50↑
BFZ	20.48±2.43	25.60±3.14	25.00↑	2.78±0.93	5.23±1.26	88.13↑
CFZ	146.29±12.84	56.21±4.92	61.58↓	2.22±0.75	3.56±0.65	60.36↑
PFZ	136.45±15.73	78.65±6.93	42.36↓	2.56±0.83	2.99±0.97	16.80↑
p value	****CNT × CPZ, CFZ, PFZ ****CPZ × PFZ, CFZ ****BFZ × CFZ, PFZ	**CNT × CPZ, CFZ ***CNT × PFZ ***CPZ × PFZ **CFZ × BFZ NS=CNT × BFZ; BFZ × CPZ		**CNT × CPZ *CNT × BFZ, CFZ, PFZ NS=PFZ × CFZ, BFZ	**CNT × BFZ **BFZ × CFZ, PFZ *CPZ × BFZ, PFZ NS=CNT × CPZ, PFZ, CFZ	
Group	**MBP (pg/mg protein)**			**TNFα (pg/mg protein)**		
	Week 5	**Week 7**	**% change**	**Week 5**	**Week 7**	**% change**
CNT	56.08±4.37	69.89±5.26	24.63↑	418.97±28.67	505.85±39.43	20.74↑
CPZ	27.70±3.18	78.92±6.74	184.91↑	649.69±56.31	601.83±52.76	7.37↓
BFZ	50.03±6.12	65.44±7.52	30.80↑	623.03±45.36	575.20±54.38	7.71↓
CFZ	72.70±6.59	76.51±8.14	5.24↑	500.46±61.92	403.20±24.55	19.43↓
PFZ	97.22±8.43	92.37±9.61	4.99↓	326.02±23.84	356.94±19.84	9.48↑
p value	****CPZ × PFZ ***CNT × CPZ, PFZ; CPZ × CFZ; BFZ × PFZ **CPZ × BFZ *BFZ × CFZ NS=CNT × BFZ	**PFZ × CNT, BFZ *PFZ × CPZ, CFZ NS=CNT × CPZ, BFZ, CFZ		***PFZ × BFZ, CPZ **CNT × CPZ, BFZ *CNT × CFZ, PFZ NS=CPZ × BFZ	***PFZ × CPZ **CNT × PFZ **CFZ × CPZ *CNT × CPZ NS=BFZ × CPZ; CNT × BFZ	

One-way ANOVA with Tukey's multiple comparison test was done to compare the variance between groups. *$p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$; ns=nonsignificant.

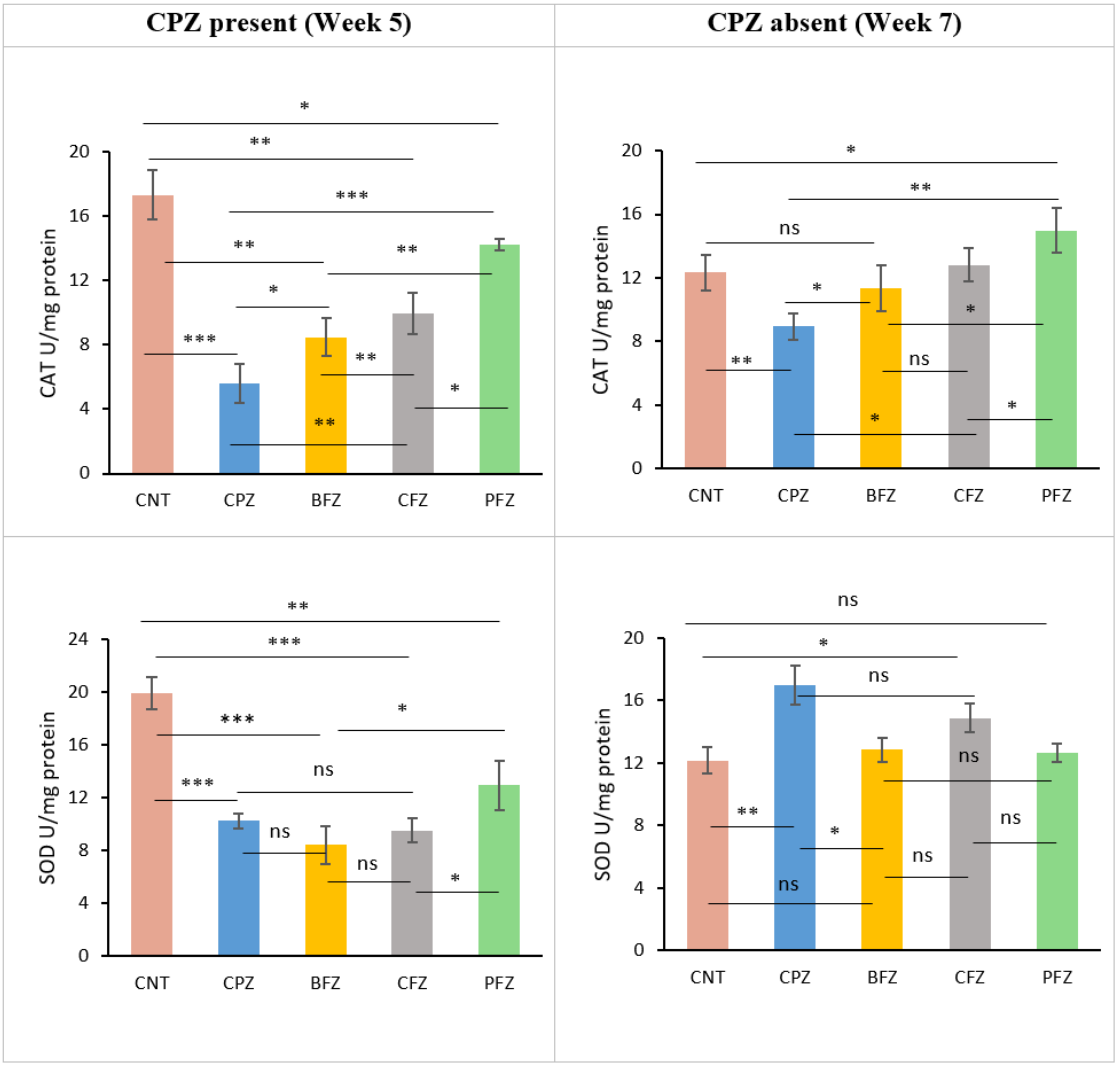

Figure 1: Level of Catalase and SOD in brainstem at the end of demyelination stage (Week 5) and at the end of remyelination stage (Week 7). One-way ANOVA with Tukey's multiple comparison test was done to compare the variance between groups. *$p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$; ns=nonsignificant.

(BFZ) found to contain equivalent level of TNFα compared to the CPZ group. The addition of curcumin to blank formulation resulted in slight improvement in TNFα level in CFZ group (500.46±61.92 pg/mg protein) compared to the CPZ group (649.69±56.31 pg/mg protein). Among all the treatment groups, curcumin-piperine formulation proved best is suppressing the TNFα level not even below the CPZ group but also even below the level of TNFα in control group at the conclusion of Week 5. At Week 7, the CPZ-treated group (now going on spontaneous recovery) exhibited the highest TNFα levels (601.83±52.76 pg/mg protein). The BFZ group also demonstrated elevated TNFα levels (575.20±54.38 pg/mg protein), albeit slightly lower than CPZ. However, CFZ (403.20±24.55 pg/mg protein) and PFZ (356.94±19.84 pg/mg protein) groups displayed lower TNFα levels compared to the control group (505.85±39.43 pg/mg protein). Among all groups, PFZ exhibited the lowest TNFα levels (Table 1).

Impact of CPZ and ZRO-Based Nanoformulations on GFAP Levels

At the conclusion of week 5, GFAP levels were assessed across all experimental groups (Figure 3). The CNT group, which was not exposed to Cuprizone (CPZ), exhibited a GFAP level of 35.17±4.68 pg/mg protein, representing baseline astrocyte activation. In contrast, the CPZ-treated group showed a significant increase in GFAP expression (100.48±9.84 pg/mg protein). The BFZ group, which received the blank formulation of *Zanthoxylum rhetsa* seed oil alongside CPZ, displayed a moderate decrease in GFAP levels (89.33±6.94 pg/mg protein) compared to the CPZ group. In contrast, groups receiving curcumin formulations, CFZ and PFZ, exhibited GFAP levels comparable to the control group. By the end of week 7, GFAP levels continued to reflect the recovery phase following the cessation of CPZ exposure at week 5 (Figure 3). The CNT group maintained a relatively stable GFAP level (48.25±7.25 pg/mg protein), while the CPZ group, which underwent spontaneous recovery without additional

treatment, still exhibited elevated GFAP expression (82.29±4.38 pg/mg protein). The BFZ group, which continued receiving *Z. rhetsa* seed oil without curcumin, showed a moderate reduction in GFAP expression (70.32±6.84 pg/mg protein) compared to the CPZ group, yet levels remained substantially higher than in the curcumin-treated groups. In contrast, the CFZ (24.38±2.15 pg/mg protein) and PFZ (38.64±5.93 pg/mg protein) groups exhibited a marked decrease in GFAP levels.

Impact of CPZ and ZRO-Based Nanoformulations on MCP-1 Levels

MCP-1 levels in the brainstem were measured across different groups at Week 5. The control group, which did not receive cuprizone, exhibited an MCP-1 level of 401.44±19.84 pg/mg protein (Figure 4). The CPZ group showed a similar MCP-1 level as observed in control. The BFZ group (361.69±32.14 pg/mg protein) displayed a slight decrease in MCP-1 levels compared to both the CNT and CPZ groups. The PFZ group (383.14±28.32 pg/mg protein), which was administered the curcumin-piperine combination formulation, exhibited MCP-1 levels slightly lower than the control but not significantly different from the CPZ group. The trend of MCP-1 expression indicates that the brainstem is largely unaffected by cuprizone exposure. At the conclusion of the study (Week 7), the CPZ group demonstrated slightly elevated but non-significant MCP-1 levels (571.15±49.3 pg/mg protein) compared to the control (Figure 4). The BFZ

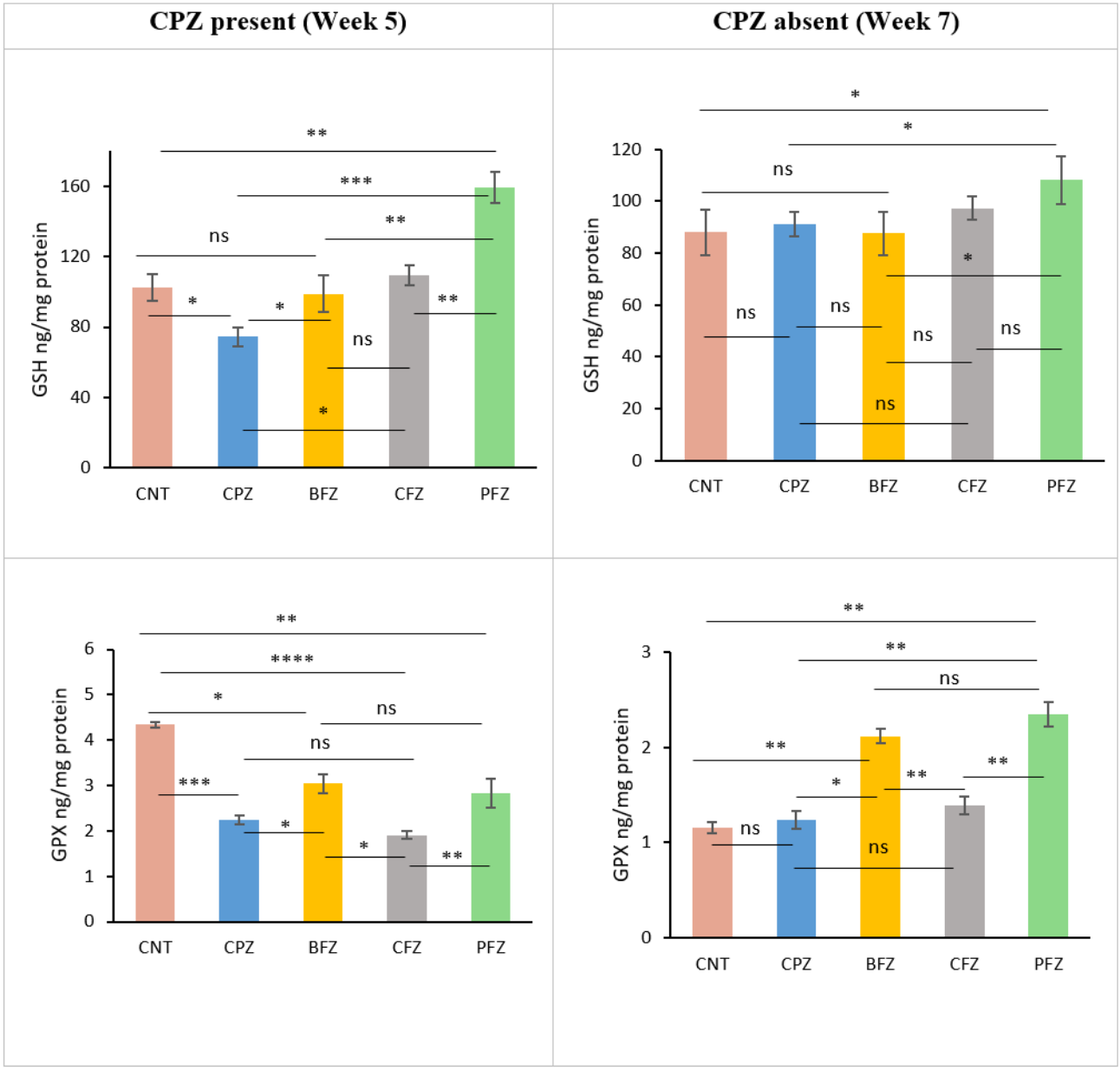

Figure 2: Level of GSH and GPX in brainstem at the end of demyelination stage (Week 5) and at the end of remyelination stage (Week 7). One-way ANOVA with Tukey's multiple comparison test was done to compare the variance between groups. *$p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$; ns=nonsignificant.

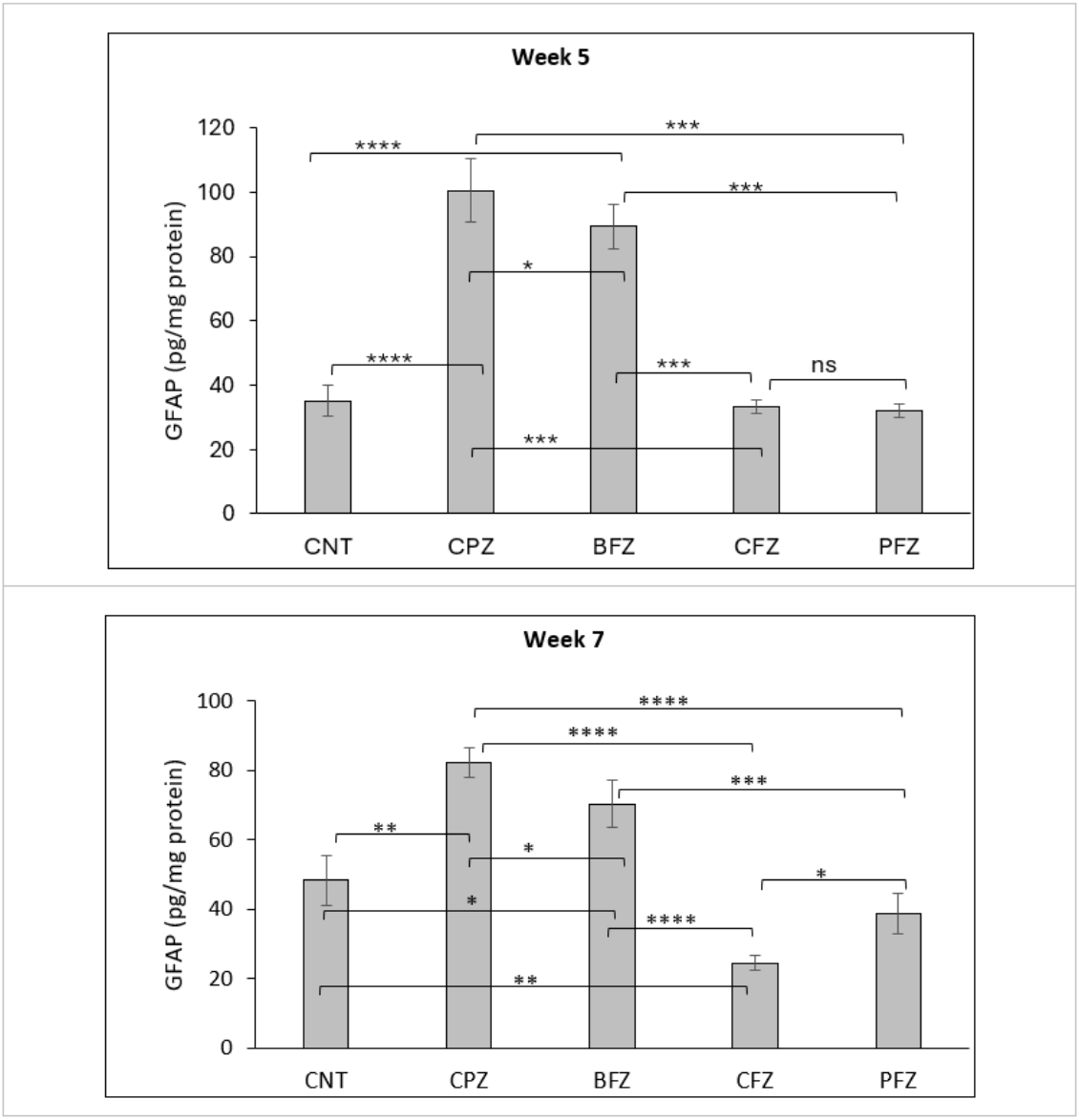

Figure 3: Levels of GFAP in brainstem regions at the end of demyelination stage (Week 5) and at the end of remyelination stage (Week 7). One-way ANOVA with Tukey's multiple comparison test was done to compare the variance between groups. *$p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$; ns=nonsignificant.

group showed a slight decrease in MCP-1 levels compared to the control and CPZ groups. However, the CFZ and PFZ groups exhibited MCP-1 levels slightly non-significantly higher than both the control and CPZ groups.

Impact of CPZ and ZRO-Based Nanoformulations on MIP-1 Levels

At the conclusion of week 5, the MIP-1 level in the control group was recorded at 238.89±16.87 pg/mg protein. The CPZ group exhibited a significant increase ($p \leq 0.001$) in MIP-1 levels (516.50±46.12 pg/mg protein) compared to the control group (Figure 5). The BFZ group showed an MIP-1 level of 229.44±21.36 pg/mg protein, which was slightly lower than the control but significantly lower than the CPZ group. The CFZ group demonstrated a further reduction in MIP-1 levels (184.65±14.38 pg/mg protein), while the PFZ group, which received the curcumin-piperine combination formulation, exhibited the lowest MIP-1 level (150.44±16.78 pg/mg protein). At the end of week 7, the CPZ group continued to display significantly elevated MIP-1 levels (481.18±46.52 pg/mg protein) compared to the control group ($p \leq 0.001$) The BFZ, CFZ, and PFZ groups exhibited significant but varying degrees of MIP-1 reduction (Figure 5).

Impact of CPZ and ZRO-Based Nanoformulations on CCL-5 Levels

The CPZ group exhibited a significant increase in CCL-5 levels (1244.84±119.45 pg/mg protein) compared to the control group (726.33±53.21 pg/mg protein) at the end of Week 5 (Figure 6). The BFZ (807.38±66.54 pg/mg protein) and CFZ (1021.49±94.24 pg/mg protein) groups displayed significantly reduced CCL-5 levels compared to the CPZ group but still remained elevated

relative to the control. Notably, the PFZ group demonstrated the lowest CCL-5 level (603.87±79.48 pg/mg protein) At the end of week 7, CCL-5 levels were reassessed across groups following the cessation of cuprizone (CPZ) treatment at the end of week 5 (Figure 6). The CPZ group, which was allowed to recover spontaneously, exhibited a persistently elevated CCL-5 level (1519.53±165.72 pg/mg protein). The BFZ group displayed a moderate CCL-5 level (921.22±126.85 pg/mg protein), signifying a mild reduction compared to the CPZ group but still elevated. In contrast, the CFZ group showed a further decline in CCL-5 levels. Interestingly, the PFZ group, which received the curcumin-piperine combination, exhibited a CCL-5 level of 949.17±110.49 pg/mg protein, reflecting an increase from its week 5 level but still lower than the CPZ group

DISCUSSION

The Cuprizone (CPZ) model is a well-established and reproducible model for studying demyelination and neuroinflammation. It selectively induces oligodendrocyte apoptosis, leading to myelin loss, astrocytosis, microglial activation, and oxidative stress, a key pathological feature observed in MS. Unlike immune-mediated models, the CPZ model allows for the investigation of central nervous system-specific mechanisms without peripheral immune system involvement, making it especially useful for evaluating neuroprotective and remyelination therapies.[13]The decrease in catalase activity suggests that cuprizone has neurotoxic effects, inducing oxidative stress and disrupting antioxidant defenses.[13] Among the treatment groups that also received cuprizone, both the Blank Formulation group (BFZ) and the Curcumin group (CFZ) exhibited significantly higher catalase levels compared to

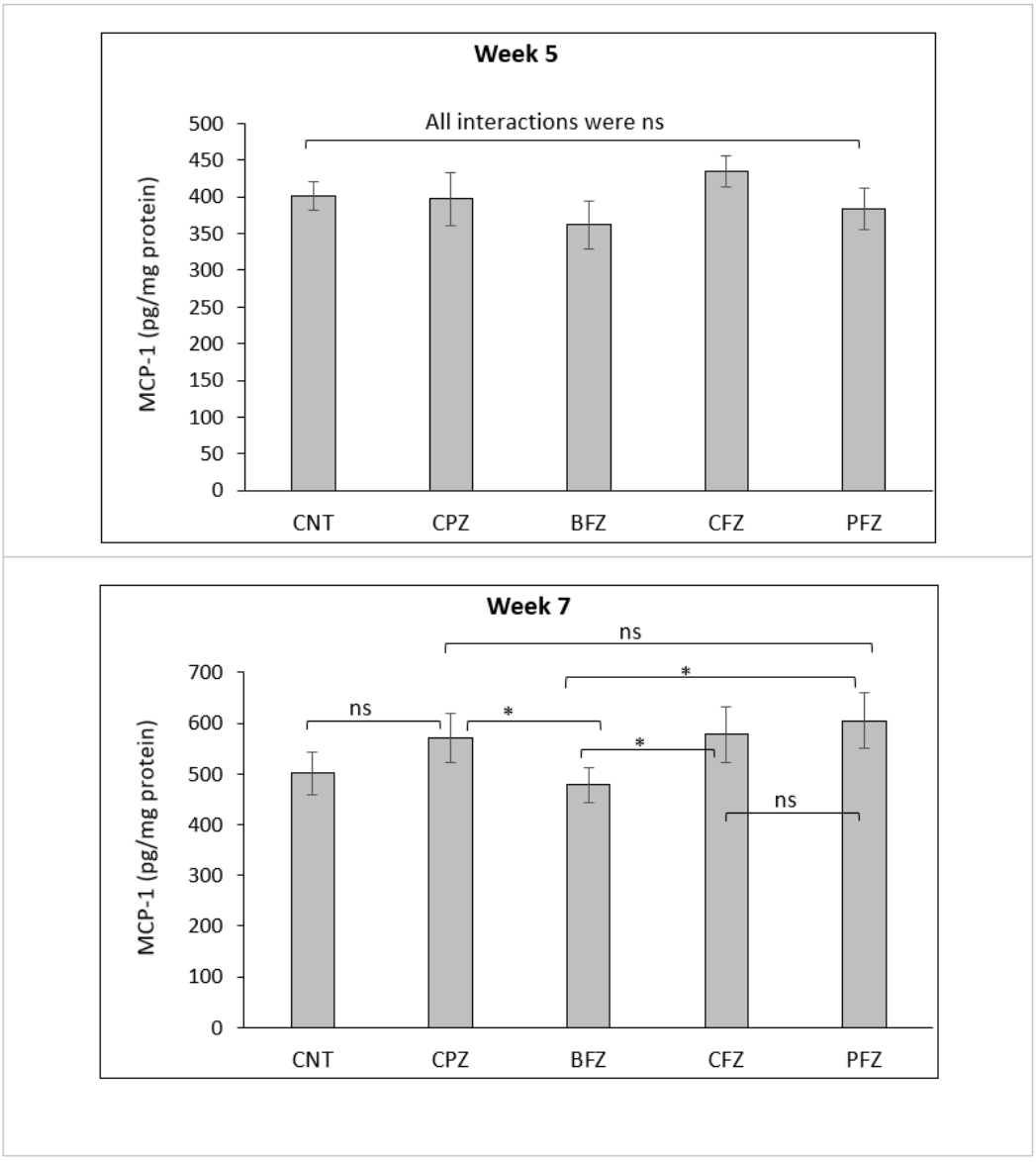

Figure 4: Levels of MCP-1 in brainstem regions at the end of demyelination stage (Week 5) and at the end of remyelination stage (Week 7). One-way ANOVA with Tukey's multiple comparison test was done to compare the variance between groups. *$p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$; ns=nonsignificant.

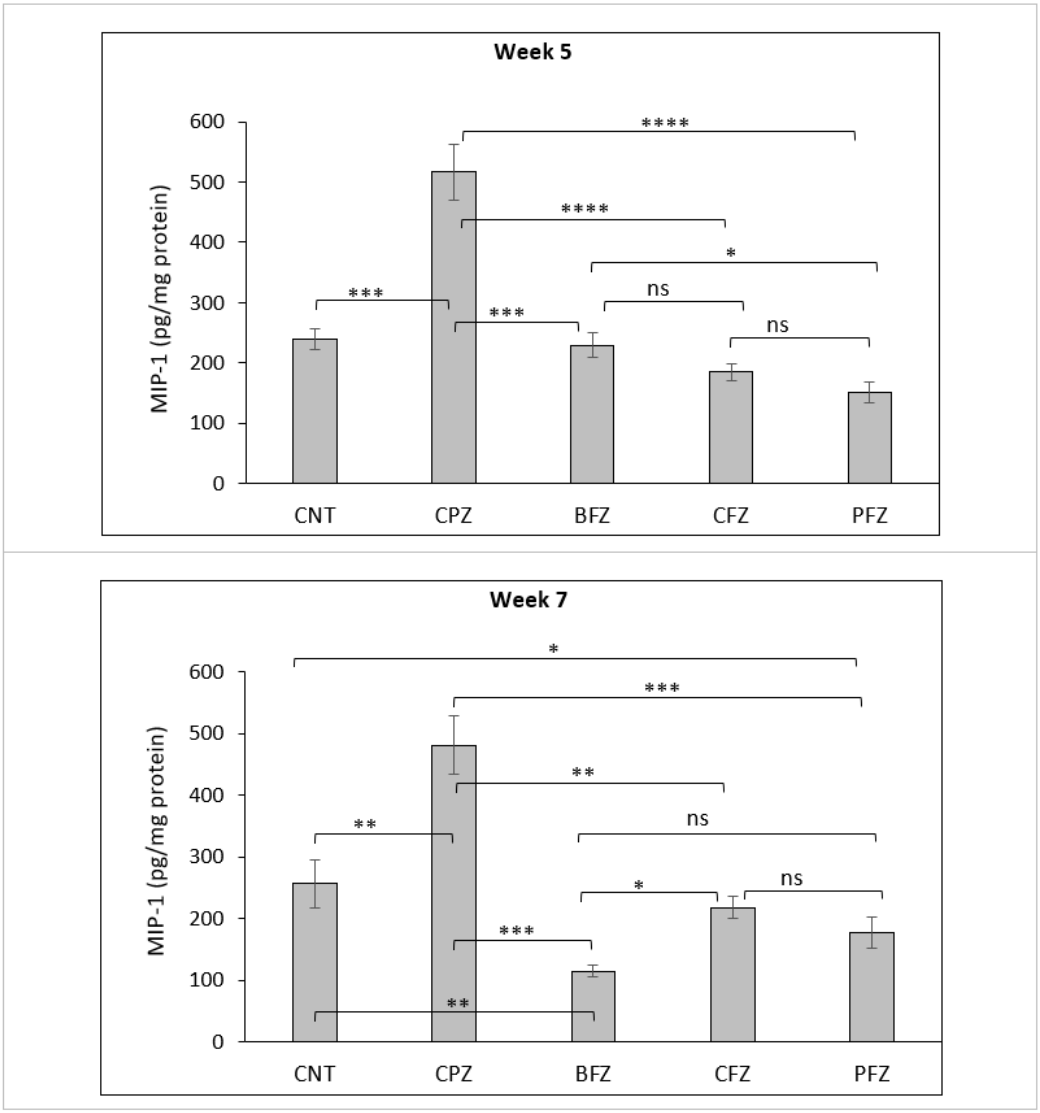

Figure 5: Levels of MIP-1 in brainstem regions at the end of demyelination stage (Week 5) and at the end of remyelination stage (Week 7). One-way ANOVA with Tukey's multiple comparison test was done to compare the variance between groups. *$p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$; ns=nonsignificant.

the CPZ group. These results imply that these formulations may provide some protection against cuprizone-induced toxicity, as evidenced by the relatively higher catalase levels.[14] Notably, the curcumin-Piperine Nanoformulation group (PFZ) showed the highest catalase levels among all treatment groups, indicating a synergistic protective effect, likely due to piperine's ability to enhance curcumin's bioavailability.[15]

Superoxide Dismutase (SOD) levels were measured in different treatment groups at the end of Week 5, revealing a significant decline that confirms cuprizone's effect on antioxidant enzyme levels. The PFZ group exhibited improved SOD levels compared to the other groups, suggesting a potential synergistic effect in enhancing antioxidant defense. At Week 7, the CPZ group, which had received cuprizone until Week 5 and was now recovering spontaneously, showed a notable increase in SOD compared to the control group, indicating recovery in antioxidant enzyme levels after cuprizone withdrawal. Comparing SOD levels between Weeks 5 and 7 underscores the effects of cuprizone on oxidative stress and the potential for recovery once cuprizone is withdrawn. These findings suggest that antioxidant strategies, especially those involving curcumin, could be beneficial in reducing oxidative damage in neurodegenerative conditions.[9]The significant reduction in GSH and GPX in the CPZ group indicates the harmful effects of cuprizone on antioxidant levels. However, from Week 5 to Week 7, the CPZ group showed a significant increase in GSH, suggesting spontaneous recovery even without any treatment. At Week 5, the PFZ group had substantially higher GSH levels compared to all other groups, supporting previous research that curcumin and piperine enhance antioxidant defenses.[16] The low GSH in the CPZ group likely reflects increased oxidative stress, consistent with known cuprizone effects.[17] Both the BFZ and PFZ groups showed moderate GPX levels, suggesting partial protection. The CFZ group had the lowest GPX activity, indicating a limited effect of curcumin alone in mitigating oxidative stress caused by cuprizone. By Week 7, the control group had the

most significant drop in GPX, likely due to natural aging. These findings align with previous research on cuprizone's oxidative effects and the neuroprotective potential of curcumin-based formulations.[18,19] Curcumin's antioxidant properties, enhanced by piperine, may help mitigate oxidative stress.[20]The reduction in Acetylcholinesterase (AChE) levels in the CPZ group suggests a build-up of acetylcholine in the brainstem.[21] The BFZ group showed a partial restoration of AChE levels compared to the CPZ group, indicating some mitigation of the effects. The CFZ and PFZ groups displayed markedly elevated AChE levels, suggesting that the curcumin formulations prevented AChE degradation, thus reducing acetylcholine accumulation.[22] By Week 7, the CPZ group showed increased AChE levels compared to Week 5, but levels remained lower than the control group, suggesting incomplete recovery. AChE is crucial for breaking down acetylcholine, a neurotransmitter involved in cognitive and neuromuscular functions.[23] The significant reduction in AChE in the CPZ group at Week 5 confirms cuprizone's impairment of cholinergic neurotransmission.[21] The BFZ group showed partial restoration of AChE activity, indicating some protective effect, while the CFZ and PFZ groups displayed a significant increase in AChE, highlighting the neuroprotective benefits of curcumin and piperine.

Malondialdehyde (MDA), a marker of oxidative stress and lipid peroxidation, revealed that cuprizone induces oxidative damage in all groups, to varying degrees. The nanoformulations appeared partially successful in limiting the detrimental effects of cuprizone in terms of MDA. At Week 7, the control group showed a marked increase in MDA levels, reflecting the natural rise in oxidative stress associated with aging. The CPZ group, now recovering, exhibited similar MDA levels to the control, suggesting some

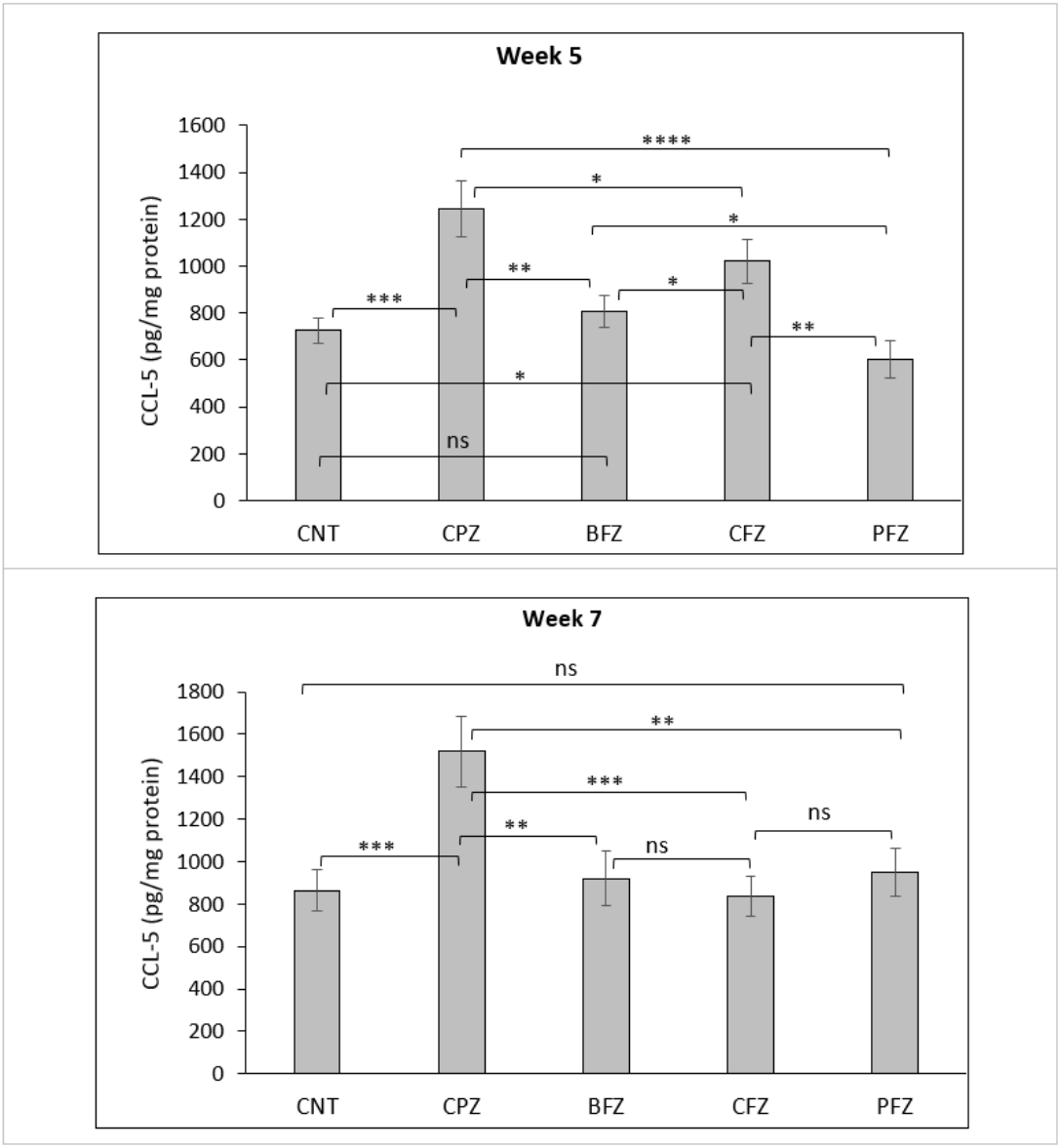

Figure 6: Levels of CCL-5 in brainstem regions at the end of demyelination stage (Week 5) and at the end of remyelination stage (Week 7). One-way ANOVA with Tukey's multiple comparison test was done to compare the variance between groups. *$p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$; ns=nonsignificant.

spontaneous recovery after cuprizone withdrawal. The CFZ group had MDA levels close to the control, while the PFZ group had the lowest MDA levels, suggesting a potential protective effect. These results align with previous studies indicating that oxidative stress increases with aging and environmental factors,[24] with PFZ showing the least oxidative damage.

The CPZ group showed the lowest Myelin Basic Protein (MBP) levels, indicating significant damage to myelin, possibly due to demyelination. The CFZ and PFZ groups exhibited higher MBP levels, suggesting that these formulations helped protect myelin from cuprizone toxicity. These findings indicate that cuprizone has the most damaging effect on MBP, while PFZ appears most effective in preserving myelin integrity. The significant increase in TNFα in the CPZ group compared to the control group suggests that cuprizone induces neuroinflammation. Among the treatment groups, the Curcumin-Piperine Formulation (PFZ) showed the greatest reduction in TNFα, even below the control levels by Week 5, suggesting strong anti-inflammatory effects.

In contrast to the control group, the CPZ-treated group exhibited significant astrocytosis and glial activation, reflected by increased GFAP expression. The BFZ group showed a moderate reduction in GFAP compared to the CPZ group, though not enough to suggest significant neuroprotection. However, the CFZ and PFZ groups exhibited GFAP levels similar to the control, suggesting that curcumin, especially in combination with piperine, helped reduce astrocyte activation and neuroinflammation. By Week 7, the CPZ group still had elevated GFAP expression, indicating incomplete recovery from cuprizone-induced astrocytosis. The CFZ and PFZ groups showed pronounced reductions in GFAP, reinforcing the neuroprotective and anti-inflammatory effects of curcumin-based formulations. These results support the potential of curcumin as a therapeutic strategy to reduce astrocyte activation and promote CNS repair after demyelination.

MCP-1 levels in the CPZ and control groups were similar, suggesting that cuprizone exposure did not significantly alter MCP-1 expression in the brainstem. This trend suggests that certain brain regions may be more resilient to cuprizone-induced inflammation.[25,26] The CPZ group showed a significant increase in MIP-1 levels after 5 weeks of cuprizone feeding, indicating inflammation. The BFZ, CFZ, and PFZ groups showed varying levels of MIP-1, suggesting their potential anti-inflammatory effects.[7] Various parts of *Zanthoxylum rhetsa* are known for their anti-inflammatory properties, including its seed oil.[27-29] Finally, the CPZ group exhibited a strong inflammatory response in terms of CCL-5 compared to the control group, highlighting cuprizone-induced neurotoxicity. The PFZ group had the lowest CCL-5 levels, suggesting that the curcumin-piperine combination may have a stronger anti-inflammatory effect than the other treatments.[22]This study demonstrates that the curcumin-Piperine-ZRO Nanoformulation (PFZ) provides enhanced neuroprotection compared to Curcumin Alone (CFZ), particularly by more effectively reducing oxidative stress and neuroinflammatory markers. Our results offer quantitative support for the synergistic action of curcumin and piperine within a novel ZRO-based SNEDDS platform. The comprehensive analysis of markers such as GFAP, TNFα, MBP, and AChE in the brainstem adds translational value and distinguishes this work from prior studies.

A key novelty of this study lies in the use of ZRO as the delivery base in our SNEDDS formulation. While curcumin-piperine combinations have been previously investigated, the incorporation of ZRO a bioactive-rich oil with established anti-inflammatory potential distinguishes our approach from earlier works. This natural oil not only serves as a solubilizing medium but may also contribute additional neuroprotective effects, potentially enhancing the efficacy of curcumin and piperine through synergistic mechanisms. These findings highlight the promise of ZRO-based nanoformulations as an innovative platform for neuroinflammatory disease intervention.

Although mechanistic pathway analysis was not within the scope of this study, the differential expression of MIP-1, MCP-1, and CCL-5 across treatment groups provides translational insight into glial signaling dynamics often overlooked in CPZ models. The enhanced efficacy of the PFZ formulation suggests modulation of key inflammatory and neuroprotective pathways, including NF-κB, MAPK, and CREB. Future studies will focus on delineating these pathways to better understand the molecular mechanisms underlying the observed therapeutic synergy.

CONCLUSION

This study highlights the detrimental effects of cuprizone-induced neurotoxicity and the therapeutic potential of curcumin nanoformulations in counteracting oxidative stress and neuroinflammation. The results demonstrate that curcumin, especially in combination with piperine, significantly improves antioxidative enzyme activities, reduces inflammatory markers, and preserves myelin integrity. The findings suggest that curcumin-based nanoformulations could serve as effective neuroprotective agents against demyelination and neurodegeneration. Further research is warranted to explore the clinical applicability of these formulations in neurodegenerative disease treatment.

TRANSLATIONAL RELEVANCE AND STUDY LIMITATIONS

This study highlights the potential of curcumin-piperine nanoformulations, delivered via a *Zanthoxylum rhetsa*-based SNEDDS, as a promising therapeutic strategy for neuroinflammatory conditions. The formulation's ability to modulate oxidative and inflammatory markers supports its relevance for early-stage intervention in neurodegenerative disorders. However, limitations include the use of intraperitoneal

administration rather than the clinically preferred oral route, absence of long-term follow-up, and lack of mechanistic validation through pathway analysis. Future studies should address bioavailability *in vivo*, explore underlying molecular pathways, and evaluate therapeutic efficacy in chronic models and behavioral domains relevant to human pathology.

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ABBREVIATIONS

CPZ: Cuprizone; **CAT:** Catalase; **SOD:** Superoxide dismutase; **GSH:** Glutathione; **GPX:** Glutathione peroxidase; **GFAP:** Glial fibrillary acidic protein; **MCP-1:** Monocyte chemoattractant protein-1; **MIP-1:** Macrophage Inflammatory Proteins; **CCL-5:** Chemokine (C-C motif) ligand 5; **MDA:** Malondialdehyde; **TNFα:** Tumor Necrosis Factor-alpha; **CNS:** Central nervous system; **MS:** Multiple Sclerosis; **ROS:** Reactive oxygen species; **ZRO:** *Zanthoxylum rhetsa* Seed Oil; **SNEDDS:** Self-nanoemulsifying drug delivery system; **CNT:** Control group; **BFZ:** Blank formulation of ZRO; **CFZ:** Curcumin nanoformulation in ZRO; **PFZ:** Curcumin-piperine formulation; **RNA:** Ribonucleic Acid; RIPA: Radioimmunoprecipitation assay; **PMSF:** PhenylMethylSulfonyl Fluoride; **ELISA:** Enzyme-linked immunosorbent assay; **ANOVA:** Analysis of variance; **AChE:** Acetylcholinesterase; **MBP:** Myelin Basic Protein.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

FUNDING

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ETHICAL APPROVAL

Animal studies were carried out in compliance with KFMRC's animal unit committee rules. The biomedical ethics research committee at King Abdulaziz University authorized the study protocol (approval No. ACUC-22-1-2), which complied with the guidelines established by the KFMRC's Animal Care and Use Committee. The research adhered to the "System of Ethics." The study was authorized by Royal Decree No. M/59 dated August 24, 2010, and it conformed with the "System of Ethics of Research on Living Creatures" rules created by King Abdulaziz City for Science and Technology. From June 1, 2022, until July 16, 2022, the study was conducted at the King Fahad Medical Research Center in Jeddah, Saudi Arabia.

SUMMARY

The study examined the effects of Cuprizone (CPZ) and various ZRO-based nanoformulations on oxidative stress markers and neuroinflammation. CPZ exposure significantly reduced antioxidant enzyme activities (catalase, SOD, GSH, and GPX) while increasing Markers of oxidative Damage (MDA) and Neuroinflammation (TNFα, GFAP, MCP-1, MIP-1, and CCL-5). Among treatment groups, the curcumin-Piperine Nanoformulation (PFZ) consistently showed the most protective effects, improving antioxidant enzyme levels, reducing oxidative stress markers, and suppressing inflammatory responses. After CPZ withdrawal (Week 7), some spontaneous recovery was observed, but PFZ-treated groups exhibited the most significant restoration of enzymatic activity and reduction in inflammation compared to control and CPZ groups.

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