Title: Alpha-mangostin Ameliorates Apoptosis, Inflammation and Oxidative Stress in Cuprizone-Induced Demyelination in C57BL/6 Mice

Running Title: Protective Effects of Alpha-mangostin in Cuprizone-Induced Demyelination in C57BL/6 Mice

Authors: Sara Banaeeyeh[1], Bibi Marjan Razavi[1,2], Hossein Hosseinzadeh[1,3,*]

1. *Department of Pharmacodynamics and Toxicology, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran.*
2. *Targeted Drug Delivery Research Center, Pharmaceutical Technology Institute, Mashhad University of Medical Sciences, Mashhad, Iran.*
3. *Pharmaceutical Research Center, Pharmaceutical Technology Institute, Mashhad University of Medical Sciences, Mashhad, Iran.*

***Corresponding Author**: Hossein Hosseinzadeh, Department of Pharmacodynamics and Toxicology, School of Pharmacy, Pharmaceutical Research Center, Pharmaceutical Technology Institute, Mashhad University of Medical Sciences, Mashhad, Iran. Email: hosseinzadehh@mums.ac.ir

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Abstract:

Purpose: Alpha-mangostin (α-MG), the most prevalent xanthone found in *Garcinia mangostana* Linn, has been documented to possess antioxidant and anti-inflammatory properties. This study investigated the protective effects of α-MG against cuprizone (CZ)-induced demyelination in the corpus callosum (CC) of mice, as an animal model of multiple sclerosis.

Method: A chaw containing 0.4 % (w/w) CZ was used to feed adult female C57BL/6 mice for 5 weeks. Mice were divided into 6 groups: 1) The control group was fed a normal diet. 2) CPZ group. 3,4,5) Mice were fed with CZ diet + α-MG (20, 40 and 80 mg/kg by gavage). 6) Mice fed a normal diet + 80 mg/kg α-MG. After five weeks of administration, the levels of MDA and apoptotic markers, such as Bax, Bcl2, cleaved caspase-3, and inflammation factor TNF-α, were measured in CC.

Results: Compared with the control group, the CZ group lost weight ($p<0.0001$). In the CZ group, there was an increased amount of MDA ($p<0.01$) and noticeable increases in the Bax/Bcl2 ratio, cleaved caspase-3 ($p<0.0001$), and TNF-α ($p<0.001$) compared with the control group. Compared to the CZ group, using α-MG 80 mg/kg significantly increased body weight ($p<0.01$). In addition, α-MG at 20, 40, and 80 mg/kg decreased the MDA content ($p<0.01$, $p<0.001$, and $p<0.0001$, respectively). Administration of α-MG 80 mg/kg decreased the Bax/Bcl2 ratio, cleaved caspase-3 ($p<0.0001$), and TNF-α levels ($p<0.0001$).

Conclusion: Our data illustrate that α-MG can eliminate destructive CZ effects in CC by decreasing oxidative stress, inflammation and apoptosis**.**

Kew words: Multiple sclerosis, Cuprizune, Alpha-mangostin, Neuroprotective, Corpus callosum

Abbreviations:

MS: Multiple sclerosis

CNS: Central nervous system

CZ: Cuprizone

CC: Corpus callosum

α-MG: Alpha-mangostin

TNF-α: Tumor necrosis factor-α

BAX: BCL2 Associated X

BCL2: B-cell lymphoma 2

MDA: Malondialdehyde

EAE: Experimental Autoimmune Encephalomyelitis

AMPK: AMP-activated protein kinase

SIRT1: Silent mating type information regulation 2 homologue 1

NF-κB: Nuclear factor kappa B

Highlights

- Alpha-mangostin effects on carpus callosum were studied in a multiple sclerosis mice model.
- Cuprizone decreased weight and increased MDA, and alpha-mangostin inhibited this effect.
- Alpha-mangostin had an anti-inflammatory effect, by decreasing TNF-α level which increased by CZ.
- Alpha-mangostin inhibited CZ-induced apoptosis in the carpus callosum by reduction of Bax/Bcl2 ratio, and cleaved caspase-3.

Plain language summary

Multiple sclerosis (MS) is a debilitating condition affecting the central nervous system, comprising the brain and spinal cord. In MS, the immune system mistakenly attacks the protective myelin sheath surrounding nerve fibers, disrupting communication between the brain and the rest of the body.

However, several immune-mediated animal models of MS were developed throughout the 20th century. The cuprizone model is a well-known tool in the study of toxic demyelination, wherein it involves administering cuprizone to young mice to induce the desired effects. The cuprizone mouse model enables the study of the complicated molecular processes underlying spontaneous remyelination and non-autoimmune-mediated demyelination. There is no cure for multiple sclerosis. However, there are therapies to speed recovery from episodes, influence disease progression, and control symptoms. Recently, the focus on plant research worldwide has increased. α-Mangostin, a naturally occurring xanthone product, is extracted from the mangosteen tree as a secondary metabolite. It has attracted considerable attention owing to its wide-ranging effects. In this study, we found that feeding C57BL/6 mice with a 0.4% CZ-supplemented diet for 5 weeks consistently induced demyelination with increased oxidative stress, inflammation and apoptotic markers. Our data illustrate that α-Mangostin can eliminate the destructive effects of CZ. Based on these results, α-mangostin may be recommended as an adjuvant medication to complement mainline therapies for MS patients.

1) INTRODUCTION:

Multiple sclerosis (MS) is a chronic condition where the immune system mistakenly attacks and damages the protective covering of nerve fibers in the central nervous system (CNS), leading to the formation of numerous inflammatory lesions (Mauriz et al., 2013). The two main pathological features of multiple sclerosis are inflammation and demyelination. In addition, the CNS undergoes apoptosis of oligodendrocytes, axonal loss, and the activation of astrocytes and microglia as a result of repeated inflammatory attacks (Bjartmar, Wujek, & Trapp, 2003). Damaging myelin sheaths around the axons of the brain and spinal cord in MS leads to demyelination (Basoglu, Boylu, & Kose, 2013; Hurwitz, 2009). Clinical indications of axonal demyelination-induced neural conductance loss are mediated by an influx of inflammatory cells into the brain and spinal cord via the blood-brain barrier (BBB). Lymphocytes and macrophages are the two most common cell types in MS lesions. Although the exact cause of MS is still not clear, it is established that autoimmune processes are involved and T cells, macrophages, B cells, and dendritic cells (DCs) in the CNS contribute to immune-mediated damage (Frohman, Racke, & Raine, 2006). Additionally, oxidative stress and mitochondrial damage play important roles in the progression and deterioration of MS (Smirnova et al., 2011). Remyelination, which follows the pathological loss of myelin in MS, is a reparative procedure believed to improve neurodegeneration. Remyelination can be progressively imperfect, insufficient, or completely unsuccessful (Fiorio, Tinazzi, & Aglioti, 2006). Animal models are useful tools to report the creator mechanisms of demyelination and remyelination, and to study cellular responses during these processes. These animal models provide a good basis for clarifying therapeutic purposes. Several experimental demyelination models are considered to be suitable for studying MS pathogenesis. These models include genetic myelin mutations, immune-mediated, viral-induced demyelination and toxic demyelination models. It needs to be emphasized that the stages of MS are not entirely modeled in any sample; all these models mimic only a part of the MS pathology. The MS model, which is induced by cuprizone, has received considerable attention and admission in recent years (Kipp, Clarner, Dang, Copray, & Beyer, 2009; Skripuletz, Gudi, Hackstette, & Stangel, 2011). Cuprizone [oxalic acid bis (cyclohexylidene hydrazide) (CZ) is a well-known neurotoxic substance that works as a sensitive and selective copper-chelating substance. The substance has a documented toxic effect on oligodendrocytes, which ultimately leads to cell death and demyelination in various brain

regions, including the corpus callosum (CC) and superior cerebellar peduncles. Given that the CC is the largest myelinated tract in the brain, it is susceptible to demyelination and may also serve as a source of neural stem cells. This makes the CC an ideal location to monitor neuroprotective responses aimed at repairing demyelination.The CC, the brain's largest myelinated tract, is susceptible to demyelination, and can potentially provide neural stem cells. This is an ideal location for observing neuroprotective responses in demyelination recovery. This makes it a perfect place to observe neuroprotective reactions for the recovery of demyelination (Kipp, Nyamoya, Hochstrasser, & Amor, 2017; Matsushima & Morell, 2001). The CZ treatment induces "acute demyelination," which results in the CC being nearly demyelinated after 5–6 weeks. For several weeks after mice are given a regular diet, acute demyelination persists due to spontaneous remyelination (Kipp et al., 2009). On the other hand, persistent CZ administration (12 weeks or more) significantly reduces remyelination; this phenomenon is referred to as "chronic demyelination", but fails under continued CZ in the late stages of acute demyelination (Harsan et al., 2008; Tansey, Zhang, & Cammer, 1996; Torkildsen, Brunborg, Myhr, & Bø, 2008). The model is ideal for initiating and studying demyelination/remyelination processes due to its simplicity, high reliability, and reproducibility (Matsushima & Morell, 2001).

Alpha-mangostin (α-MG), a polyphenolic compound and the first xanthone isolated from the pericarp of the mangosteen fruit, Garcinia mangostana Linn, is a yellow agent (Pedraza-Chaverri, Cárdenas-Rodríguez, Orozco-Ibarra, & Pérez-Rojas, 2008). Several components of mangosteen, such as polyphenols, phenols, and xanthone derivatives, have been found to offer positive effects on various health conditions (Tousian Shandiz, Razavi, & Hosseinzadeh, 2017).

An extremely selective inhibitory effect against acid sphingomyelinase (ASMase) was demonstrated by α-MG, a typical natural product of the plant-derived xanthone class (Okudaira et al., 2000), in addition to various biological activities of this molecule (Chairungsrilerd, Furukawa, Ohta, Nozoe, & Ohizumi, 1996). It exhibits a broad range of biological activities, encompassing anti-inflammatory and antioxidant properties (Chen, Yang, & Wang, 2008; Devi Sampath & Vijayaraghavan, 2007). In some animal models of disease, α-MG can maintain the activity of several antioxidant enzymes, including glutathione-S-transferase (GST), glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT), and one of the major intracellular antioxidant systems, reduced glutathione (GSH) (Devi Sampath & Vijayaraghavan, 2007). The results of the study in Wistar rat brains indicate that α-MG acts as a neuroprotective agent in models of oxidative

damage (Márquez-Valadez et al., 2012). Furthermore, a study showed that treatment with α-MG and curcumin in primary cultures of CGNs provides neuroprotection against iodoacetate-induced oxidative stress. This protective effect was attributed to a reduction in reactive oxygen species (ROS) production caused by iodoacetate (Reyes-Fermín et al., 2012). In one study, using Rotenone as a cellular model of Parkinson's disease, SH-SY5Y cells treated with α-MG preserved dopaminergic neurons and decreased the accumulation of α-synuclein. These findings imply that α-MG protects neurons from neuronal damage linked to Parkinson's disease by preventing mitochondrial malfunction and synuclein aggregation (Hao, Li, Duan, & Li, 2017).

In this regard, we evaluated the effectiveness of α-MG in reducing clinical signs as well as inflammatory and oxidant processes in an animal model of CZ-induced MS in BALB/C57 mice over a 5-week experimental period. This is the first time that the potential neuroprotective properties of α-MG have been investigated within this context, and we also discussed the underlying mechanisms.

2) MATERIALS AND METHODS

2.1 – Animals

Adult female C57BL/6 mice (8-10 weeks old) weighing 18-22 g were procured from the School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran. All mice were housed (six animals per cage) under a 12-h light/dark cycle at 22 ± 2°C and a qualified humidity of 60 ± 5% in the animal room of the School of Pharmacy, Mashhad University of Medical Sciences, Iran. All experiments were authorized by the Animal Care and Use Committee of Mashhad University of Medical Sciences, Iran (ethical number: 960851) and carried out in accordance with the Internationally Accepted Principles for Animal Use and Care (Zimmermann, 1983).

2.2 Materials

The following reagents were obtained from various sources:

- CZ (Sigma-Aldrich, USA; CAS NO: 370810) and Alpha-mangostin were sourced from Trademax Pharmaceuticals & Chemicals Co., China, with a purity of greater than 90%.

- BSA (Solarbio, China), dry skim milk (Quetlab, UK), ethylene glycol tetraacetic acid (EGTA; Sigma, USA), ethylenediaminetetraacetic acid (EDTA; Pars Tous Biotechnology, Mashhad, Iran), sodium deoxycholate (Sigma, New Zealand), sodium orthovanadate (Na3VO4; Sigma, Madhya Pradesh, India), and other chemicals were purchased from Merck, Germany.
- Protease and phosphatase inhibitor cocktail and Pierce ECL Western blotting substrate were obtained from Thermo Fisher Scientific, USA.
- The protein assay kit (Bradford reagent) and polyvinylidene difluoride (PVDF) were sourced from Bio-Rad, USA.
- Fetal bovine serum was purchased from Gibco, USA.
- Malondialdehyde (MDA) and DZN were obtained from Fluca, Switzerland, and Shanghai Tosco Chemical Co., Shanghai, China, respectively.
- Rabbit polyclonal anti-Bax (Cell Signaling #2772, 1:1000), rabbit polyclonal anti-Bcl2 (Cell Signaling #2870, 1:1000), anti-cleaved caspase-3 (Cell Signaling #9664, 1:1000), anti-TNF-α (Cell Signaling #3707, 1:1000), anti-rabbit IgG labeled with horseradish peroxidase, and anti-mouse IgG labeled with horseradish peroxidase were purchased from Cell Signaling.
- Alliance Gel-doc (Alliance 4.7 Gel doc, UVtec UK) and UV Tec Software were also obtained from UVtec, UK.

2.3 Experimental Design

The CZ-mediated demyelination model was established in female C57BL/6 mice aged 8-10 weeks, as previously described by Kipp et al. (2009). The mice were fed a diet containing 0.4% (w/w) CZ mixed with milled chow. α-MG was dissolved in 0.9% normal saline (NS) with 2 drops of Tween 80% and administered by gavage at doses of 20, 40, and 80 mg/kg for five weeks. The administration of α-MG began on the first day of the test, concurrent with the CZ diet. The mice were randomly divided into six groups, each consisting of six animals:

(1) Normal group (Control): fed a normal diet + NS 0.9% + 2 droplets of Tween 80% by gavage for five weeks.
(2) CPZ group: fed with a 0.4% (w/w) CZ diet mixed into milled chow for five weeks.
(3) Mice fed with a 0.4% (w/w) CZ diet mixed into milled chow + 20 mg/kg α-MG by gavage for five weeks.
(4) Mice fed with a 0.4% (w/w) CZ diet mixed into milled chow + 40 mg/kg α-MG by gavage for five weeks.

(5) Mice fed with a 0.4% (w/w) CZ diet mixed into milled chow + 80 mg/kg α-MG by gavage for five weeks.
(6) Mice fed a normal diet + 80 mg/kg α-MG by gavage for five weeks.

2.4 Measurement of Body Weight

Body weight was assessed every day of the experiment according to the weight of the mice, but data were reported on 1st and last days of the experiment.

2.5 Tissue preparation

The mice were sacrificed at the end of five weeks. The corpus callosum (CC) of the brain was carefully removed and quickly stored at −80°C until processing.

2.6 Determination of MDA level in corpus callosum tissue

Malondialdehyde (MDA) was measured as a marker of lipid peroxidation. Elevated MDA levels indicated high levels of lipid peroxidation. The following procedure was used to measure MDA:

1. A 10% homogeneous solution was prepared from the tissue sample in 1.15% potassium chloride (KCl).
2. 0.5 ml of the homogenized tissue was mixed with:
   - 3 ml of 1% phosphoric acid
   - 1 ml of 6% thiobarbituric acid (TBA)
3. The mixture was boiled in water for 45 minutes.
4. After boiling, the tubes were cooled.
5. 4 ml of n-butanol was added to the cooled tubes, and the contents were vortexed for 1 minute.
6. The tubes were centrifuged at 3500 rpm for 10 minutes.
7. The supernatant was removed and its absorbance was measured at 532 nm.
8. A standard curve was prepared using MDA concentrations ranging from 0-100 nmol/ml.
9. MDA concentrations in the tissue samples were reported as nmol/g tissue (Uchiyama & Mihara, 1978).

2.7 Western blot assay

The Western blot analysis was performed on the protein extract to investigate the levels of apoptosis markers. After thawing, the CC samples were added to a lysis buffer solution containing 50 mM Tris-HCl (pH: 7.4), 2 mM ethylenediaminetetraacetic acid (EDTA), 2 mM egtazic acid (EGTA), 10 mM NaF, 1 mM sodium orthovanadate (Na3VO42H2O), 10 mM βglycerophosphate, 0.2% W/V sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, and a complete protease inhibitor cocktail (Roche, Mannheim, Germany). The samples were homogenized and sonicated on ice for three 10-second bursts at high intensity, with a 10-second cooling period between each burst, and then centrifuged at 10,000g for 10 minutes at 4°C. The Bradford assay kit (Bio-Rad; Bradford, 1976) was used to evaluate protein concentration and adjust sample contents. The adjusted samples were then combined in a 1:1 ratio with 2× sodium dodecyl sulfate (SDS) blue buffer, heated, and divided into smaller portions before being stored in a freezer at -80°C. Next, 100 μg of protein was loaded onto each lane of a 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) system for separation, and then transferred onto a polyvinylidene fluoride membrane (PVDF) from Bio-Rad.After blocking the PVDF membranes with skim milk for 2 hours, they underwent a process of incubation overnight at 4°C with the aid of various antibodies, including rabbit polyclonal anti-Bax, rabbit polyclonal anti-Bcl2 (Cell Signaling #2870, 1:1,000), anti-cleaved caspase-3 (Cell Signaling #9664, 1:1,000), anti-TNF-α (Cell Signaling#3707, 1:1,000), and either rabbit or mouse polyclonal anti-β-actin antibodies (Cell Signaling #4967, 1:1,000). Following three washes with Tris-Buffered Saline and Tween 20 (TBST), the membranes were incubated for another two hours with rabbit or mouse horseradish peroxidase-conjugate anti-IgG antibodies (Cell Signaling #7071, 1:2,000; Cell Signaling #7072, 1:2,000, respectively), at a dilution of 1:2,000. To visualize the peroxidase-coated bands, enhanced chemiluminescence (Pierce) was used, and the integrated optical densities of the bands were measured using the Alliance 4.7 Gel doc (UK). The analysis of protein bands was performed using UV Tec Software (UK), and the normalization of protein levels was done with respect to the corresponding bands of β-actin, which served as the control protein.

2.8 Statistical analysis

The Graph Pad Prism 8.0 Software (Inc., San Diego, CA, USA) was used for statistical analysis in this study. The data were presented as the mean ± SEM. For the statistical analysis of body weight,

western blot, and lipid peroxidation data, a one-way ANOVA was performed followed by the Tukey–Kramer test. A P-value of less than 0.05 was considered to be statistically significant.

3) RESULTS

3.1 . Effect of α-MG on Body Weight of CZ Treated Mice

On the first day of the experiment, there was no notable difference in body weight between the groups. However, by the end of the study period, a significant decrease in the average body weight was observed in the CZ group compared to the other groups. Five weeks of therapy with 80 mg/kg α-MG restored body weight loss relative to the group treated with CZ (Table 1).

3.2 Effect of α-MG on the Oxidative Stress Marker in CZ Treated Mice

At the end of the procedure, MDA, an oxidative stress marker, was evaluated in CC homogenates. The CZ group showed a significant increase in MDA levels compared to the control group ($p<0.01$). Five weeks of administration of α-MG (20, 40 and 80 mg/kg) resulted in a substantial reduction and restoration of MDA levels ($p<0.01$, $p<0.001$ and $p<0.001$ respectively) (Figure1).

3.3 Effect of α-MG on the Apoptotic Markers of CZ Treated Mice

Because of the superior effects of α-MG (80 mg/kg) on oxidative stress markers, this dose has been selected for further study. Cleaved caspase-3, Bax, and Bcl-2 protein levels were measured in the CC homogenates at the end of the protocol schedule. CZ-treated rats showed a significant increase in cleaved caspase-3($p<0.0001$) and Bax/Bcl-2 ratio ($p<0.0001$). Five weeks of administration of α-MG (80 mg/kg) resulted in a significant decrease in cleaved caspase-3 levels ($p<0.0001$) (Figures 2 A and B) and the Bax/Bcl-2 ratio ($p<0.0001$) (Figures 3 A and B).

3.4 Effect of α-MG on the inflammatory marker in CZ Treated Mice

CZ-induced demyelination was accompanied by an increased expression of the inflammatory cytokine TNFα ($p<0.001$). Five weeks of administration of α-MG (80 mg/kg) led to a significant reduction in TNF-α ($p<0.0001$) compared to that in CZ-treated mice (Figures 4A and B).

4) DISCUSSION

To our knowledge, this pioneering study is the first to investigate the effects of α-mangostin (α-MG) in a mouse model of cuprizone (CZ)-induced multiple sclerosis (MS), finding that feeding mice a diet containing 0.4% (w/w) CZ caused serious neurotoxicity, including a decline in body weight and increased oxidative stress, apoptosis, and inflammation in the corpus callosum (CC). Moreover, oral α-MG treatment (20, 40, and 80 mg/kg) for five weeks inhibited weight loss and reduced inflammation, apoptosis, and oxidative stress in the MS groups. Researchers have used some important experimental models in preclinical trials to diagnose MS and evaluate the effectiveness of therapy. None of these models fully represent the pathogenesis of MS. All of the brain's main white matter structures, including the CC, become demyelinated when CZ is added to the animal ration (Matsushima & Morell, 2001). As a result, the regulation of the demyelination and remyelination processes is kept apart in this model, which permits differentiation. The CZ model facilitates to create and refine techniques for visualizing locations that are compromised. Furthermore, this model assessed the potential effects of medication candidates on demyelination, enhancing the survival of demyelinated nerves, and promoting remyelination (Abakumova et al., 2015).

Previous studies have well-documented that when CZ is administered dietaryly, there is a dose-dependent and reversible drop in body weight (Hiremath et al., 1998; Skripuletz et al., 2011; Steelman, Thompson, & Li, 2012; Stidworthy, Genoud, Suter, Mantei, & Franklin, 2003). Drastic weight loss helps increase mortality rates at CZ doses higher than 0.3% (Hiremath et al., 1998; Stidworthy et al., 2003). CZ is a copper chelator in the body, so it can cause copper deficiency and weight loss in rats (Taylor, Bettger, & Bray, 1988) and mice (Prohaska, 1983). In the present study, there was a decline in body weight observed within the protocol plan compared to the control.

Administration of α-MG in a rat model of autism restored body weight, and there was a significant and dose-dependent increase in body weight compared to methylmercury administration (Sahu et al., 2022; Tiwari et al., 2021). In our study, chronic consumption of α-MG was efficient, and body weight was recovered. This finding showed that α-MG 80 mg/kg administration prevented weight loss in the treated groups of experimental MS.

According to several studies, that is true CZ increases oxidative stress in the CC by inducing mitochondrial dysfunction in oligodendrocytes (Kang et al., 2012), which is similar to pattern III lesions in MS patients (Lucchinetti et al., 2000). In addition, the generation of antioxidant enzymes

like SOD and GSH is decreased by CZ because it interferes with the electron transport chain and antioxidant system (Ghaiad, Nooh, El-Sawalhi, & Shaheen, 2017). CZ also increases MDA production, as a marker of free radical-mediated lipid peroxidation in CC, which eventually causes cell death (Kashani et al., 2014). Here, we showed that the CZ diet significantly increased MDA levels. This result is consistent with those of previous reports.

Studies have demonstrated that α-MG scavenges free radicals (superoxide and hydrogen peroxide) and indirectly mitigates these effects by stimulating antioxidants (Márquez-Valadez et al., 2012; Martínez, Galano, & Vargas, 2011), as well as reducing lipid peroxidation-induced toxicity (Tiwari et al., 2021). A mouse mammary organ culture assay showed that α-MG can scavenge peroxynitrite anion through its antioxidant effects. The preceding outcome provides backing for the utilization of α-MG as a botanical dietary supplement with antioxidant properties (Jung, Su, Keller, Mehta, & Kinghorn, 2006). In addition, α-MG has been shown to increase GSH levels and reduce MDA levels as an oxidative marker (Ghasemzadeh Rahbardar, Razavi, & Hosseinzadeh, 2020; G. Liu et al., 2018). Furthermore, α-MG also reduced oxidative stress in PC12 cells induced by cadmium and arsenic in an in vitro study (Ahmadian, Heidari, Razavi, & Hosseinzadeh, 2022). Following doxorubicin-induced cardiotoxicity in rat heart tissue, α-MG at all doses (50,100, and 200 mg/kg) moderately increased GSH levels while reducing MDA levels (Eisvand et al., 2022). The present study showed that α-MG exerts beneficial effects by acting as a potent antioxidant agent. α-MG treatment significantly reduced MDA levels at 20, 40, and 80 mg/kg doses during the demyelination stage.

In MS patients, the demyelinated regions of the CNS exhibit inflammatory infiltrates composed of myelin-specific T cells and B cells originating from the blood, which produce antibodies targeting myelin components. The pathophysiological explanations have resulted in the determination that MS is a persistent autoimmune inflammatory demyelinating disease affecting the CNS (Martino & Hartung, 1999). The neurotoxicant substance CZ induces demyelination and is an excellent model system for studying neuroinflammation in detail.

CZ induces a rapid and highly reproducible inflammatory response in mouse CC, including the activation and proliferation of microglia and astrocytes and their recruitment to their expected levelsat specific therapeutic stages. In the CZ model, inflammatory cells rarely appeared in the peripheral circulation. Therefore, microglial cells are an important source of inflammatory cytokines and pro-oxidant molecules (Arnett et al., 2002; Hillis, Davies, Mundim, Al-Dalahmah,

& Szele, 2016). Different experimental studies have provided evidence that taking CZ in the diet increases levels of inflammatory cytokines such as IL-1β, IL-6, and TNF-α in the CC and brain (Berghoff et al., 2017; Elbaz, Senousy, El-Tanbouly, & Sayed, 2018; Ghaiad et al., 2017; Rüther et al., 2017; Sanadgol et al., 2017; Vakilzadeh et al., 2015). Our findings suggest that the CZ increases TNF-α levels in the CC. Some investigations point to the anti-inflammatory ability of α-MG, illustrating that this agent acts via the inhibition of NO, PGE2, IL-1β, IL-6 and TNF-α (S.-H. Liu et al., 2012; Mohan, Syam, Abdelwahab, & Thangavel, 2018; Tiwari et al., 2021; Tousian, Razavi, & Hosseinzadeh, 2019, 2020). These results demonstrate that α-MG possesses anti-inflammatory properties and can be recommended as a complementary therapy alongside primary treatments (Chen et al., 2008). We also found that α-MG 80 mg/kg significantly alleviated high levels of TNF-α in mice during the demyelination stage. Therefore, α-MG has a reducing effect on neuroinflammatory processes.

Apoptosis is noticed as the principal mechanism of oligodedrocyte damage followed by immediated injury in MS (Barnett & Prineas, 2004). CZ, a pharmaceutical agent with copper-chelating properties, initiates apoptosis in oligodendrocytes and brings about the degradation of myelin by means of oxidative stress (Denic et al., 2011). Studies performed on C57BL/6 mice by adding CZ to their meals showed that the first pathological event was the apoptotic death of mature oligodendrocytes in the CC (Acs & Komoly, 2012; Blakemore, 1972). Three proteins with key roles in regulating apoptosis including Bax, Bcl2, and caspase-3, were studied to understand the effect of CZ on apoptosis. Several studies revealed that CZ can increase caspase-3 activation, diminish Bcl-2 anti-apoptotic protein levels, and increase Bax pro-apoptotic protein levels in CC (Sanadgol et al., 2020; Vakilzadeh et al., 2015; Vakilzadeh et al., 2016; Zahednasab et al., 2019). The results of our study showed increased protein levels of Bax, cleaved caspase-3, and the Bax/Bcl-2 ratio in CZ-fed mice compared to those in the control group.

These findings suggest that α-MG inhibits apoptosis. An investigation of the neuroprotective effect of α-MG against MPP+-induced apoptosis in SH-SY5Y cells showed that α-MG (10 μM) reduced ROS production, modulated the balance of pro- and anti-apoptotic genes, and suppressed caspase-3 activation (Janhom & Dharmasaroja, 2015). Searching for the potential anti-apoptotic value of α-MG (3, 6, and 12 μm) in rat chondrocytes, demonstrated that α-MG played a protective role in cartilage by blocking the apoptotic effects of Bax and caspase-3 (Pan et al., 2017). The protective effect of α-MG (100 mg/kg) on doxorubicin-induced cardiotoxicity in rats illustrated the anti-

apoptotic effect of α-MG by reducing caspase-3 and the ratio of Bax/Bcl2 in the heart tissue (Eisvand et al., 2022). Besides, by directly affecting α-MG on apoptotic and anti-apoptotic markers, it was reported to reduce apoptosis via modulating the function of the mitochondrial complex, thereby diminishing electron leak and free radical generation (Reiter, Tan, Manchester, & Qi, 2001). Moreover, α-MG reduces cadmium-induced apoptosis by reducing caspase-3 levels (Ahmadian et al., 2022). Doxorubicin causes cardiotoxicity in rats, and co-treatment with α-MG (100 mg/kg) reduces the ratio of Bax/Bcl-2 and cleaved caspase-3 in heart tissue (Eisvand et al., 2022). In our investigation, the administration of 80 mg/kg α-MG resulted in significant alterations in the Bax/Bcl-2 ratio and cleaved caspase-3 protein levels.

Our results suggest that α-MG has the potential to mitigate the detrimental effects of CZ in CC through the reduction of oxidative stress, inflammation, and apoptosis. This discovery could pave the way for the incorporation of α-MG as a supplementary treatment alongside conventional therapies for managing MS in patients.

Table 1: Effect of α-MG on body weight in CZ-treated mice.

Days	**Control group**	**Cuprizone group**	**Cup + α-MG 20**	**Cup + α-MG 40**	**Cup + α-MG 80**	**α-MG 80(mg/kg)**
Weight (g)						
1th day	20.33 ± 0.55 g	20.66 ± 0.42 g	20.5 ± 0.61 g	20.5 ± 0.42g	19.50 ± 0.56 g	19.66 ± 0.42 g
Last day	23.50 ± 0.56	14.33 ± 1.17 g ****	15.66 ± 0.42 g	15.66 ± 0.42	19.60 ± 1.47g ##	23.00 ± 1.00 g

The data shown in this research are reported as the mean ± standard error of the mean (SEM) from six mice in each experimental condition. Statistical significance was determined using one-way analysis of variance (ANOVA) with Tukey's multiple comparisons post hoc test. The results indicate: **** $p<0.0001$ Vs Control ## $p< 0.01$ Vs CZ; CZ: Cupeizone , α-MG: Alpha-mangostin

Figures:

Fig. 1

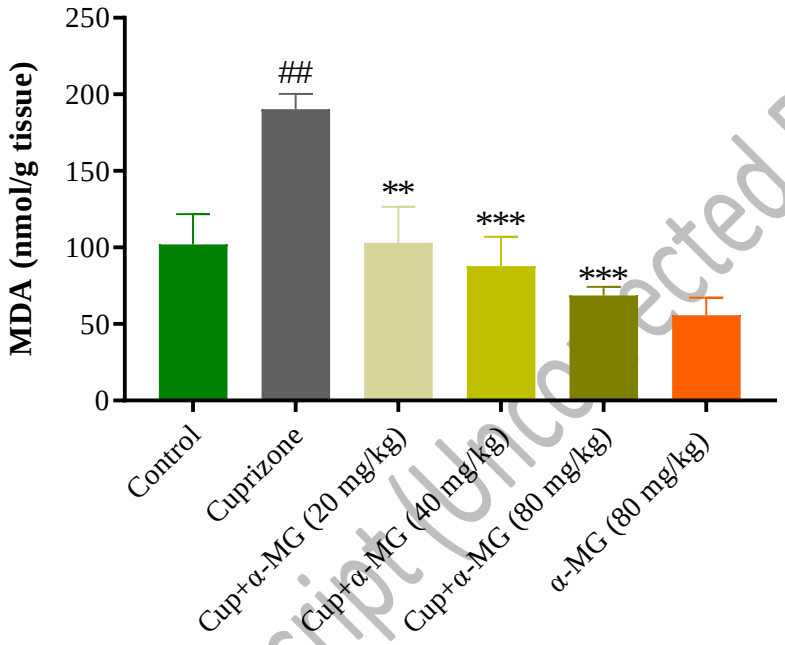


Fig. 2

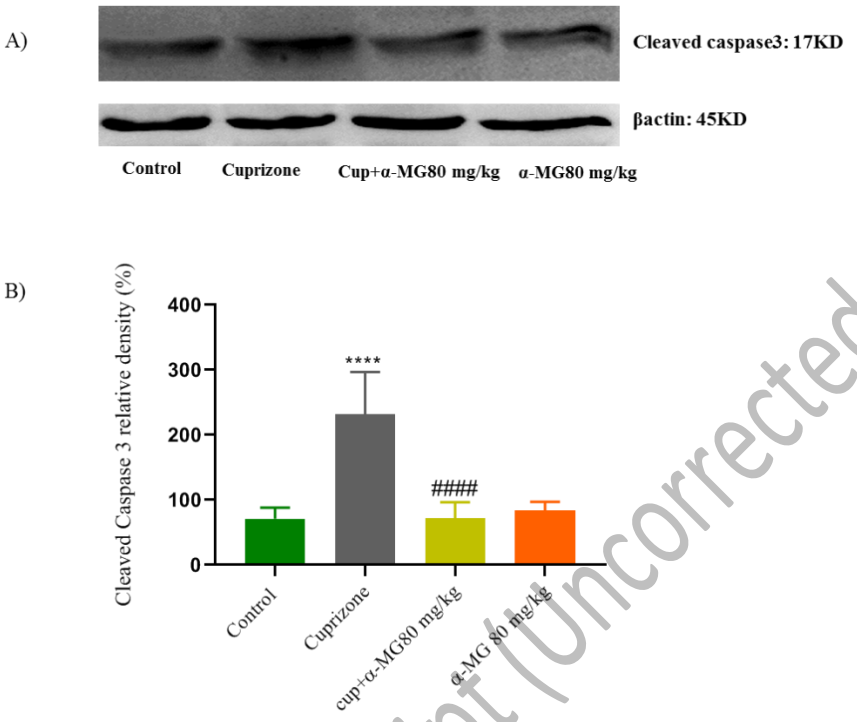
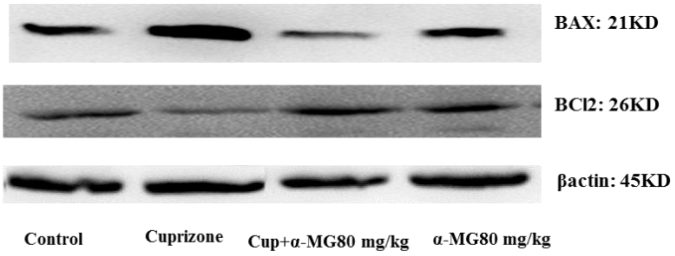


Fig. 3

A)



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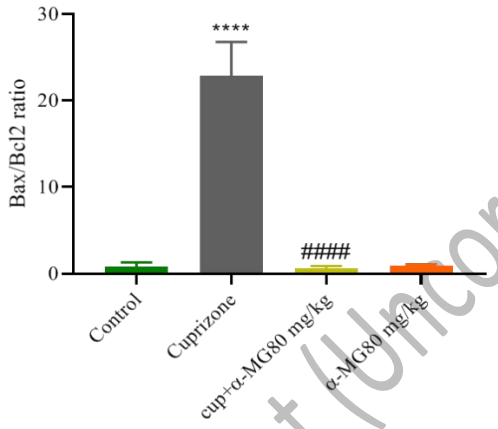
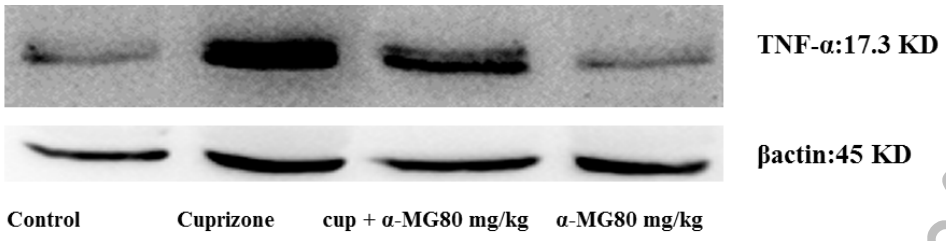
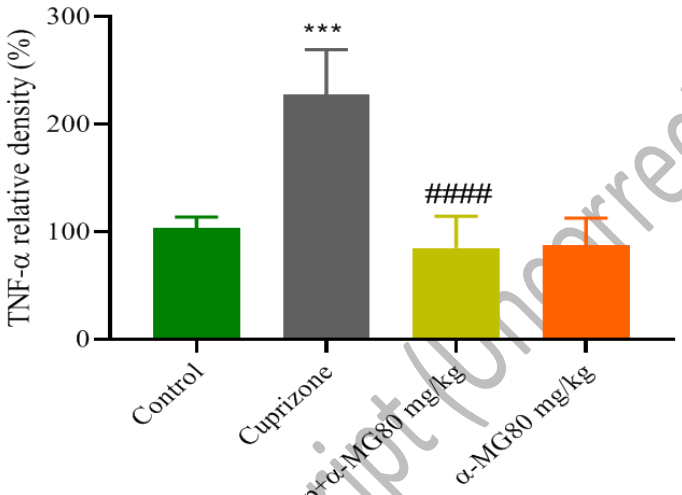


Fig. 4

A)



B)



Captions:

Fig. 1: Effect of α-MG co-administered with CZ for 5 consecutive weeks on lipid peroxidation in animals' corpus callosum. The control group was fed a normal diet + NS 0.9% + very low rate of Tween 80% by gavage The data are mean ± SD (n = 4). One-way ANOVA and post-test Tukey–Kramer were used for statistical analysis. ##p<0.01 vs. control ** p<0.01 and *** p<0.001 vs. CZ.

CZ: Cupeizone , α-MG: Alpha-mangostin

Fig. 2: Effect of α-MG on caspase-3 in the corpus callosum of animals co-administered with CZ for 5 consecutive weeks. The western blotting analysis yielded immunoblot bands that were represented in figure (A). Additionally, the quantitative presentation of these immunoblots was obtained from four independent experiments (B).The control group was fed a normal diet + NS 0.9% + very low rate of Tween 80% by gavage. The data are mean ± SD (n=4). One-way ANOVA and post-test Tukey–Kramer were used for statistical analysis. Beta-actin is the loading protein control. **** p< 0.0001 Vs Control , ##### p<0.0001 Vs CZ.

CZ: Cupeizone , α-MG: Alpha-mangostin

Fig. 3: Effect of α-MG (80 mg/kg by gavage) given concurrently with CZ for 5 weeks consecutive days on the protein levels of Bax, Bcl2, in the corpus callosum of animals. The western blotting analysis yielded immunoblot bands that were represented in figure (A). Additionally, the quantitative presentation of these immunoblots was obtained from four independent experiments (B).The control group was fed a normal diet + NS 0.9% + very low rate of Tween 80% by gavage. The data are mean ± SD (n=4). One-way ANOVA and post-test Tukey–Kramer were used for statistical analysis. Beta-actin is the loading protein control. **** p<0.0001 Vs Control. #### p<0.0001 Vs CZ.

CZ: Cupeizone , α-MG: Alpha-mangostin

Fig. 4: Effect of co-administration of α-MG (80 mg/kg by gavage) with CZ for 5 consecutive weeks on TNF-α protein levels in animals' corpus callosum. The western blotting analysis yielded immunoblot bands that were represented in figure (A). Additionally, the quantitative presentation

of these immunoblots was obtained from four independent experiments (B). The control group was fed a normal diet + NS 0.9% + very low rate of Tween 80% by gavage. The data are mean ± SD (n=4). One-way ANOVA and post-test Tukey–Kramer were used for statistical analysis. Beta-actin is the loading protein control. *** p< 0.001 Vs Control. ##### p<0.0001 Vs CZ.

CZ: Cupeizone , α-MG: Alpha-mangostin

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Contributions

Study conception and design, Hossein Hosseinzadeh, Bibi Marjan Razavi.

Material preparation, data collection and analysis, Sara Banaeeyeh.

The first draft of the manuscript writing, Sara Banaeeye.

Writing – Review & Editing, Hossein Hosseinzadeh, Bibi Marjan Razavi

Funding Acquisition, Hossein Hosseinzadeh

Resources

Supervision, Hossein Hosseinzadeh, Bibi Marjan Razavi

and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Ethics Approval

All experiments were authorized by the Animal Care and Use Committee of Mashhad University of Medical Sciences, Iran (ethical number: 960851), and carried out consistent with the Internationally Accepted Principles for Animal Use and Care (Zimmermann, 1983).

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Conflict of interest

The authors declare no competing interests.