**Iranian Journal of Basic Medical Sciences**
*ijbms.mums.ac.ir*

# Rutin attenuates cuprizone-induced demyelination in C57BL/6 mice through activation of Nrf2 signaling and inhibition of NF-κB

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ARTICLE INFO

***Article type:***
Original

***Article history:***
Received: Dec 18, 2025
Accepted: Jul 18, 2026

***Keywords:***
Cuprizone
Demyelination
Mice
NF-kB
Nrf2
Rutin

ABSTRACT

***Objective(s):*** Multiple sclerosis (MS) is a debilitating demyelinating disease characterized by oxidative stress and neuroinflammation. This study investigated the therapeutic potential of rutin, a natural flavonoid, in a cuprizone (CPZ)-induced mouse model of demyelination.

***Materials and Methods:*** Male C57BL/6 mice were fed a 0.2% CPZ diet for six weeks to induce central nervous system demyelination and were concurrently treated with rutin (50 or 100 mg/kg, intraperitoneally, for the last 14 days). Behavioral assessments, histochemical examinations, and immunohistochemistry for Nrf2, HO-1, NQO-1, and NF-kB p65 were then performed.

***Results:*** Results: Behavioral tests revealed that rutin dose-dependently improved CPZ-induced motor coordination deficits in the pole, rotarod, and open-field tests. Histological analysis (LFB and H&E staining) demonstrated that rutin significantly attenuated demyelination in the corpus callosum. Mechanistically, immunohistochemistry showed that rutin treatment effectively up-regulated the key antioxidant pathway by increasing the expression of phosphorylated Nrf2 (p-Nrf2) and its downstream targets (HO-1 and NQO-1), while simultaneously suppressing the pro-inflammatory NF-κB p65 pathway.

***Conclusion:*** Our findings show that rutin protects against demyelination through a dual mechanism of enhancing endogenous antioxidant defenses and inhibiting neuroinflammation, highlighting it as a promising candidate for further studies on demyelinating disorders such as MS.

▶Please cite this article as:
Alavi SD, Shirooie S, Sureda A, Farzaei MH, Fakhri S, Asgary S. Rutin attenuates cuprizone-induced demyelination in C57BL/6 mice through activation of Nrf2 signaling and inhibition of NF-κB. Iran J Basic Med Sci 2026; 29:



## Introduction

Multiple sclerosis (MS) is a chronic neurodegenerative disease that affects the central nervous system (CNS) by damaging myelinated axons in the brain and spinal cord, leading to variable degrees of axonal degeneration (1).

The most common clinical form is relapsing-remitting MS (RRMS) (2). Current disease-modifying therapies, including interferonβ and glatiramer acetate, offer partial benefits but are limited by side effects and incomplete remyelination (3-5).

The cuprizone (CPZ) model is a well-established toxic demyelination paradigm used to study MS pathophysiology (6). Cuprizone is a copper chelator that induces mitochondrial dysfunction by inhibiting copper-dependent enzymes, particularly cytochrome c oxidase in the electron transport chain (7). This disruption leads to energy depletion and oxidative stress, ultimately triggering apoptosis of mature, myelinating oligodendrocytes. A hallmark of this model is region-specific demyelination in the corpus callosum and superior cerebellar peduncles, while sparing the spinal cord (8). Activated microglia and astrocytes contribute to the neuroinflammatory milieu by releasing reactive oxygen species (ROS) and proinflammatory cytokines that exacerbate oligodendrocyte injury (9, 10). Transcription factors such as nuclear factor erythroid-2-related factor 2 (Nrf2) and nuclear factor kappa B (NF-κB) regulate cellular defenses against oxidative and inflammatory damage (11). Nrf2 controls a battery of antioxidant and cytoprotective genes (including HO-1 and NQO-1) that limit ROS accumulation, whereas NF-κB drives the expression of inflammatory mediators (12).

The Nrf2 pathway controls a set of NADPH-generating enzymes that maintain redox balance, including glucose-6-phosphatedehydrogenaseandisocitratedehydrogenase-1(13, 14). Conversely, NF-κB activation in glial cells promotes

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transcription of pro-inflammatory cytokines such as IL-1, IL-6, and TNF-α, as well as inducible enzymes including iNOS and COX-2, which further exacerbate myelin loss (15, 16). Importantly, there is significant crosstalk between these pathways: NF-κB activation can antagonize Nrf2-driven transcriptional programs, and oxidative stress can activate NF-κB while Nrf2 activation reduces oxidative burden and can indirectly suppress NF-κB-driven inflammation(17-20). Thus, simultaneous activation of Nrf2 and inhibition of NF-κB is an attractive strategy to reduce both oxidative stress and neuroinflammation in demyelinating conditions(17, 18). One of the best strategies for targeting the Nrf2/NF-κB pathway is to use natural compounds, and flavonoids are among the best (21).

Flavonoids, a large class of polyphenolic compounds abundant in fruits and vegetables, demonstrate significant neuroprotective potential in neurodegenerative diseases such as MS, primarily through their multi-targeted actions on central pathogenic pathways. A cornerstone of their efficacy is their potent antioxidant and anti-inflammatory activity. Many flavonoids activate the Nrf2 pathway, up-regulating antioxidant enzymes such as HO-1 and NQO-1, which combat oxidative stress (22). Concurrently, they suppress the pro-inflammatory NF-κB pathway, reducing cytokines such as TNF-α and IL-6, thereby mitigating neuroinflammation (15).

Rutin, a widely distributed flavonoid glycoside found in fruits and vegetables, is one of the most important bioactive molecules and has been shown to alleviate inflammation and oxidative stress in various diseases (23). Rutin exerts antioxidant effects by modulating the endogenous antioxidant response via Nrf2. Upon oxidation, it forms a quinone, indicating that rutin exhibits protective effects primarily in regions experiencing oxidative stress (24). By down-regulating the p65 NF-κB subunit, rutin also reduces inflammatory and oxidative cell activation (25). Several studies have supported the neuroprotective effects of rutin in animal models of neurodegenerative disorders; for example, it has been demonstrated that rutin reduced the neurotoxic effects of aluminum by restoring mitochondrial function effectively, reducing the levels of nitrate and TNF-α, and elevating the antioxidant activity of enzymes (GPx, SOD, and catalase) in the brain of rats (26). In addition, *in vivo* and *in vitro* experiments indicated that rutin has beneficial effects in some neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease by multiple mechanisms, including reducing Aβ oligomer levels, protecting dopaminergic neurons from oxidative stress, and regulating microglial-mediated neuroinflammation (27). Given the numerous neuroprotective effects in various models of neurodegenerative diseases, we investigated the effect of rutin in an animal model of MS.

The doses of rutin used in this study (50 and 100 mg/kg, IP) were selected based on previous reports demonstrating neuroprotective effects of rutin in CNS injury models (24, 25). A recent study also showed that rutin at these doses exerts protective effects against cuprizone-induced demyelination by modulating the Nrf2 and NF-κB pathways (28).

The aim of the present study was to evaluate whether rutin attenuates cuprizone-induced demyelination and to investigate whether its effects involve modulation of the Nrf2 and NF-κB pathways.

## Materials and Methods

### *Chemicals*

Cuprizone (CPZ) was obtained from Merck KGaA (Germany). Antibodies include: Anti-Nrf2 (phospho S40) antibody (EP1809Y) ab76026, NFκB p65 (F-6): sc-8008, Anti-NQO1 antibody ab34173 Anti-NQO1 antibody ab34173, HemeOxygenase 1 (A-3): sc-136960 and m-IgGκ BP-HRP: sc-516102 as the secondary antibody. Dimethyl fumarate (DMF) and rutin were also supplied by Merck KGaA (Germany).

### *Animals*

A total of 35 male C57BL/6 mice (8 weeks old, weighing 20–25 g) were used in this study. The animals were obtained from the Pasteur Institute (Karaj, Iran) and housed in standard laboratory conditions (25±2 °C, 12-hr light/dark cycle, *ad libitum* access to food and water). All animal procedures complied with the guidelines of the Kermanshah University of Medical Sciences, Iran (Ethical Approval No. IR.KUMS.ACE.1400.004).

### *Grouping and cuprizone-induced demyelination and rutin treatment*

The mice were randomly divided into five groups (n = 7 per group): 1) Control group: healthy animals receiving only a standard diet for six weeks; 2) CPZ group: mice receiving 0.2% (w/w) cuprizone mixed with food pellets for six weeks; 3) CPZ + DMF group: mice receiving 0.2% cuprizone diet plus dimethyl fumarate (10 mg/kg) orally; 4) CPZ+rutin 50 group: mice treated with rutin (50 mg/kg, IP) daily for the last 14 days concurrently with 0.2% cuprizone diet, concurrently with 0.2% cuprizone diet; 5) CPZ+rutin 100 group: mice treated with rutin (100 mg/kg, IP) daily for the last 14 days, concurrently with 0.2% cuprizone diet (29). The cuprizone model of demyelination is well-established, with a standard protocol of feeding 0.2% (w/w) cuprizone for six weeks to induce demyelination in the corpus callosum of C57BL/6 mice (6-8). To induce demyelination, CPZ powder was thoroughly mixed with the food pellets (0.2% w/w) for 45 min and provided to the animals daily, with free access to food and water. This regimen specifically induces demyelination in the corpus callosum (30-32). Due to rutin poor aqueous solubility, it was prepared as a suspension in 0.5% (w/v) carboxymethylcellulose (CMC) in distilled water as the vehicle (33).

### *Body-weight measurement*

Body weight was recorded weekly throughout the experimental period. Because previous studies have shown that weight stability is critical for consistent demyelination in the CPZ model (34), changes in body weight before and after treatment were analyzed to evaluate general health and toxicity (35).

### *Behavioral tests*

At the end of the experimental protocol, pole, rotarod, and open-field tests were conducted to assess motor balance, coordination, and locomotor activity.

In the pole test, used to assess motor performance, each mouse was placed head-up at the top of a vertical pole (1 cm diameter, 50–60 cm height). The time required to descend to the base was recorded, with a cut-off time of 60 sec. The test was repeated three times per mouse with 5 min intervals between trials (36).

The Rotarod test was performed to evaluate motor learning and sensorimotor coordination in each mouse. The device's speed and the contact angle of the mice with the rotating cylinder were 25 rpm and 45 °, respectively. The test was repeated three times per mouse with 5 min intervals between trials (37).

In the open field test, locomotor activity, and general movement were measured in a 40×40 cm arena with 40 cm-high walls (38, 39). Number of movements during 5 min were recorded and analyzed.

### *Brain preparation*

Following behavioral assessments, animals were anesthetized with ketamine (50 mg/kg) and xylazine (10 mg/kg, IP) and then perfused transcardially with 10% formalin. Brains were fixed and processed for histological and immunohistochemical (IHC) studies, including hematoxylin–eosin (H&E) and Luxol Fast Blue (LFB) staining.

### *Hematoxylin and eosin (H&E) staining*

For general histopathological evaluation, paraffin-embedded sections (5 µm) from the prefrontal cortex were stained using hematoxylin (6 hr at 60 °C), differentiated with 10% acetic acid and 85% ethanol, rinsed, and counterstained with eosin. After bluing in saturated lithium carbonate solution, the slides were dehydrated and mounted for microscopic examination.

### *Myelin staining*

LFB staining was performed to evaluate myelin integrity in the corpus callosum (40, 41). Paraffin-embedded brain sections (5 µm thick, sagittal) were dried overnight at 37 °C, deparaffinized, cleared, and rehydrated through a graded ethanol series. Sections were stained with 0.1% LFB overnight, washed, and differentiated using 0.05% lithium carbonate and 70% ethanol. Slides were then dehydrated, cleared with xylene, and mounted. Myelin, neuropil, and nerve cells are dyed blue, pink, and purple, respectively, under light microscopy (42).

### *Immunohistochemistry (IHC) staining*

IHC was performed on 5 µm paraffin-embedded corpus callosum sections to determine the expression of p-Nrf2, HO-1, NQO-1, and NF-κB p65. Sections were deparaffinized with xylene, rehydrated, and subjected to antigen retrieval in citrate buffer (pH 6.0, 15 min, 90 °C). After blocking with 1% BSA at 37 °C for 1 hr, slides were incubated overnight at 4 °C with primary antibodies: Rabbit monoclonal anti-p-Nrf2 (phospho S40, 1:100, Abcam, USA) and mouse monoclonal antibody NFκB p65 (F-6): sc-8008 (1:100, SANTA CRUZ BIOTECHNOLOGY, USA), Rabbit monoclonal antibody HO-1 (1:100, SANTA CRUZ BIOTECHNOLOGY, 136960), Rabbit polyclonal antibody NQO-1 (1:100, Abckam, 34173). After washing with PBS, sections were incubated with 3% $H_2O_2$ for 12 min to quench endogenous peroxidase activity, followed by a biotinylated goat anti-rabbit secondary antibody (1:100, Abcam, USA). Visualization was performed using diaminobenzidine (DAB) substrate (Boster Biological Technology, USA, 10 min, room temperature), and slides were counterstained with hematoxylin (43, 44). Images were captured using a light microscope (Olympus BX51, Tokyo, Japan) equipped with a digital camera (Olympus DP72) at ×200 and ×400 magnifications. Immunohistochemical staining was quantified using ImageJ software (version 1.53, National Institutes of Health, USA). Briefly, images were converted using color deconvolution to separate the DAB (brown) channel. The mean optical density (or mean gray value) of the brown staining was measured in defined regions of interest (ROIs) within the corpus callosum. The average density value was calculated for each animal and used for statistical analysis. All analyses were performed in a blinded manner. This method provides an objective and continuous measure of staining intensity reflecting the amount of target protein.

### *Data analysis*

The statistical analysis was carried out with GraphPad Prism version 9.0 (GraphPad Software, San Diego, CA, USA). The normality of the data distribution was evaluated using the Shapiro–Wilk test, and homogeneity of variances was verified by Levene's test. One-way analysis of variance (ANOVA), followed by Tukey's post hoc test, was used to determine differences between groups. Results were expressed as mean±SEM, and $P<0.05$ was considered statistically significant. ImageJ software was used to quantify LFB and IHC staining intensities.

## Results

### *Body weight*

Rutin treatment (50 mg/kg) administered during the final two weeks partially reversed the weight loss induced by CPZ (Figure 1) ($P<0.05$ for Rutin 50 vs CPZ; $P<0.001$ for CPZ vs control).

### *Behavioral tests*

The pole test (Figure 2A) showed that CPZ-treated animals had significantly impaired motor coordination compared with controls ($P<0.05$). Rutin (50 and 100 mg/kg) improved performance compared with the CPZ group ($P<0.05$).

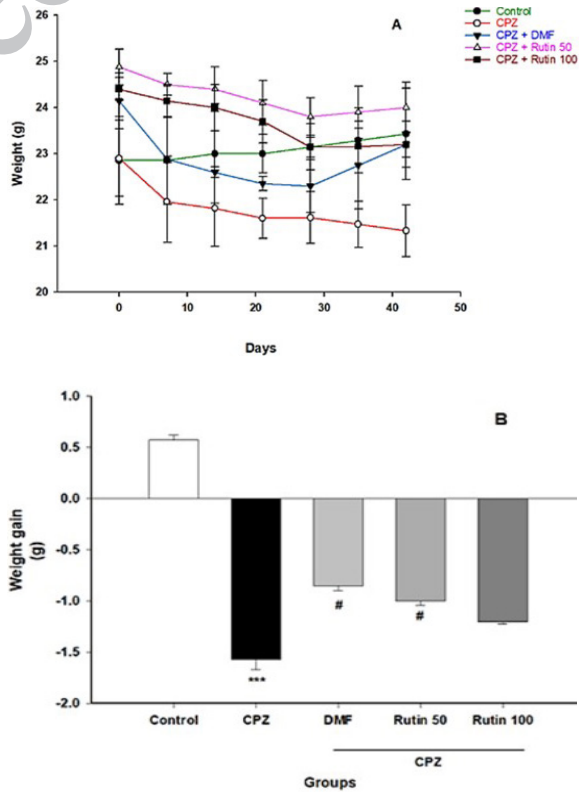


**Figure 1.** Weight gain of mice (A) and changes in body weight (B) throughout a six-week period

CPZ: cuprizone group; DMF group: dimethyl fumarate 10 mg/kg treated-CPZ group; CPZ+rutin 50: rutin 50 mg/kg treated-CPZ group; CPZ+rutin 100: rutin 100 mg/kg treated-CPZ group. (* $P<0.001$) compared with control group; (# $P<0.05$; ## $P<0.01$) compared with CPZ group.

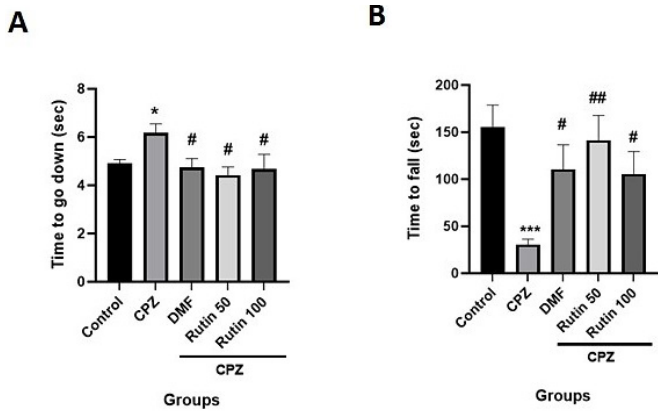


**Figure 2.** Motor function tests of mice: Pole test (A) shows the time to reach the floor, whereas Rotarod test (B) shows latency to fall
CPZ: cuprizone group; DMF group: dimethyl fumarate 10 mg/kg treated-CPZ group; CPZ+rutin 50: rutin 50 mg/kg treated-CPZ group; CPZ+rutin 100: rutin 100 mg/kg treated-CPZ group. (* $P$<0.05; *** $P$<0.001) vs control group; (# $P$<0.05; ## $P$<0.01) vs CPZ group.

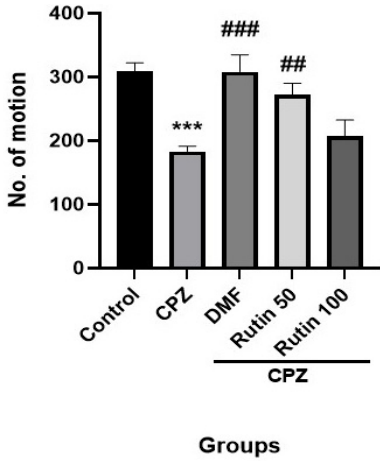


**Figure 3.** Open field test parameters in mice: (A) total distance traveled, (B) average speed, and (C) immobility time
CPZ: cuprizone; DMF: dimethyl fumarate. Data are presented as mean±SEM (n=7 per group). (*** $P$<0.001 vs control group; ## $P$<0.01, ### $P$<0.001 vs CPZ group).

Rotarod performance (Figure 2B) was reduced in CPZ mice ( $P$<0.001 vs control). Mice treated with rutin (50 and 100 mg/kg) remained on the rotating cylinder significantly longer than CPZ-only mice ($P$<0.01 and $P$<0.05, respectively). In the open field test (Figure 3), CPZ exposure markedly decreased locomotor activity and increased immobility time compared with controls ( $P$<0.001). Rutin treatment (50 mg/kg) partially restored locomotor activity and reduced immobility ( $P$<0.01, vs CPZ).

### *Myelin staining*

LFB staining of the corpus callosum (Figure 4A, B) showed a marked reduction in myelin staining intensity after CPZ treatment ( $P$<0.001 vs control), consistent with demyelination. Rutin-treated groups exhibited increased LFB staining intensity relative to the CPZ group, indicating preservation or partial recovery of myelin ($P$<0.01 and $P$<0.05 for rutin 50 and 100 vs CPZ, respectively).

### *Hematoxylin and eosin staining*

H&E staining of the prefrontal cortex revealed normal histology in controls, whereas CPZ-treated mice showed myelin degradation, chromatolysis, and vacuolated neurons (Figure 5). Rutin treatment reduced these CPZ-induced histopathological alterations, with improved cellular architecture.

### *Immunohistochemistry analysis*

IHC of the corpus callosum showed a significant decrease in HO-1 (Figure 6), NQO-1 (Figure 7), and p-Nrf2 (Figure 8) staining in CPZ mice compared with controls ($P$<0.01). Rutin 50 mg/kg administration increased the

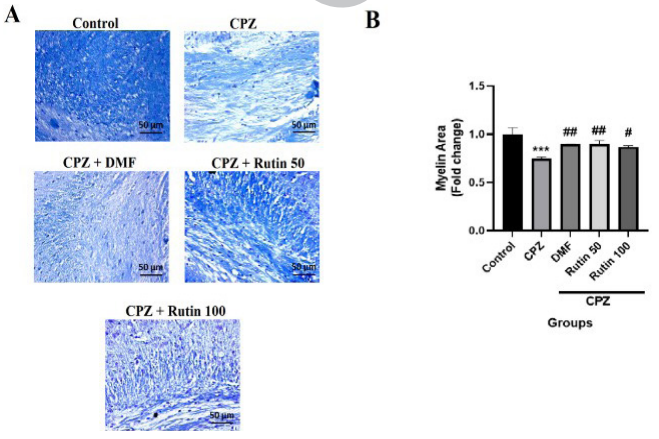


**Figure 4.** Representative photomicrographs of Luxol fast blue (LFB) staining of the corpus callosum (A). Magnification: ×100. Quantification of LFB staining optical intensity (B).
Data are mean±SEM (n=7). (***$P$<0.001 vs control group; #$P$<0.05, ##$P$<0.01, ### $P$<0.001 vs CPZ group).

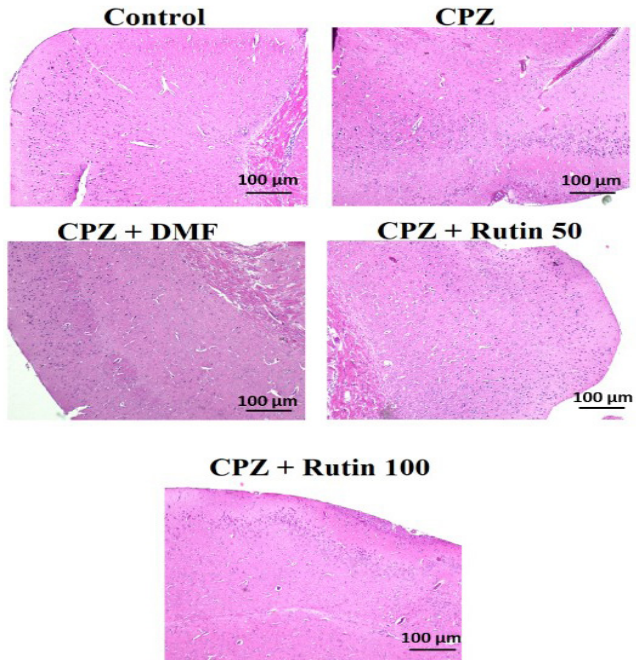


**Figure 5.** Photomicrograph of prefrontal cortex hematoxylin and eosin staining (Magnification: × 40) of mice
In the cuprizone group, compared to the control group, there is evident cell apoptosis and disruption of cellular organization. In the rutin-treated groups (especially at 50 mg/kgfirst), cell regeneration and re-establishment of normal cellular order can be observed. CPZ: cuprizone group; DMF group: dimethyl fumarate 10 mg/kg treated-CPZ group; CPZ+rutin 50: rutin 50 mg/kg treated-CPZ group; CPZ+rutin 100: rutin 100 mg/kg treated-CPZ group.

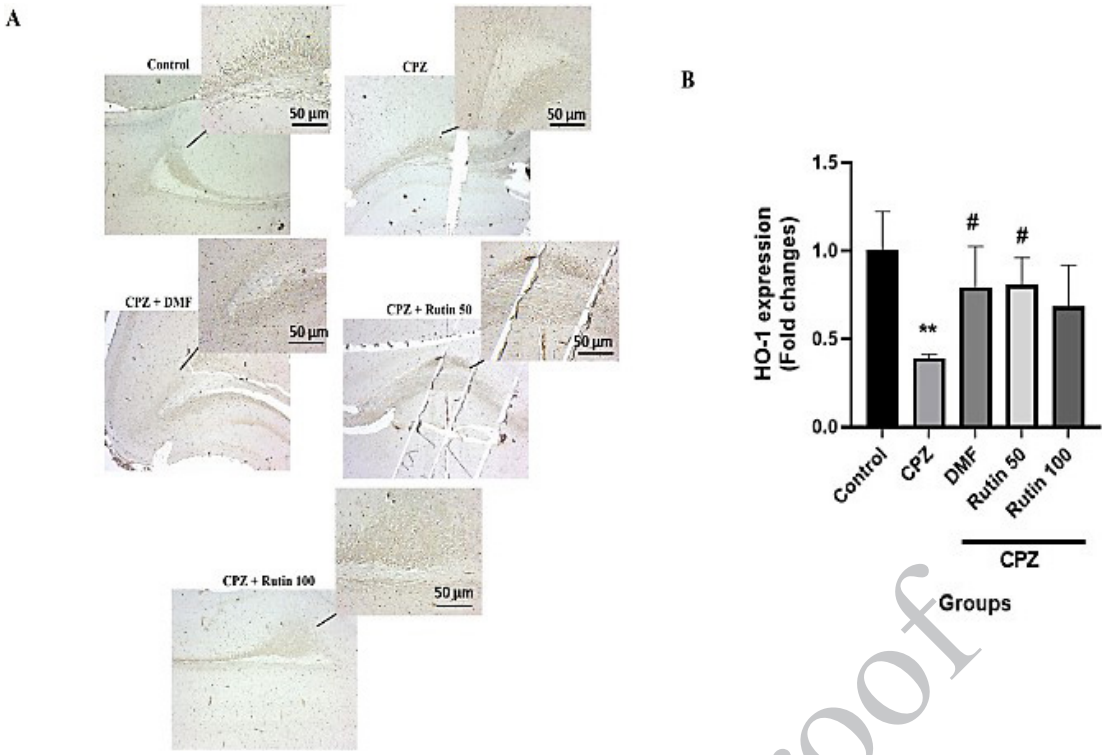


**Figure 6.** Representative photomicrographs of immunohistochemical staining for HO-1 in the corpus callosum of mice
Magnification: ×40 and ×100. CPZ: cuprizone; DMF: dimethyl fumarate. Quantification of HO-1 immunoreactivity (lower panel) is shown as mean optical density±SEM (n=7). (** $P$<0.01 vs control group; # $P$<0.05 vs CPZ group).

immunoreactivity of p-Nrf2 and its downstream targets compared with CPZ ($P$<0.05). In contrast, NF-κB p65 expression was elevated in CPZ mice and was reduced after rutin 50 mg/kg treatment (Figure 9).

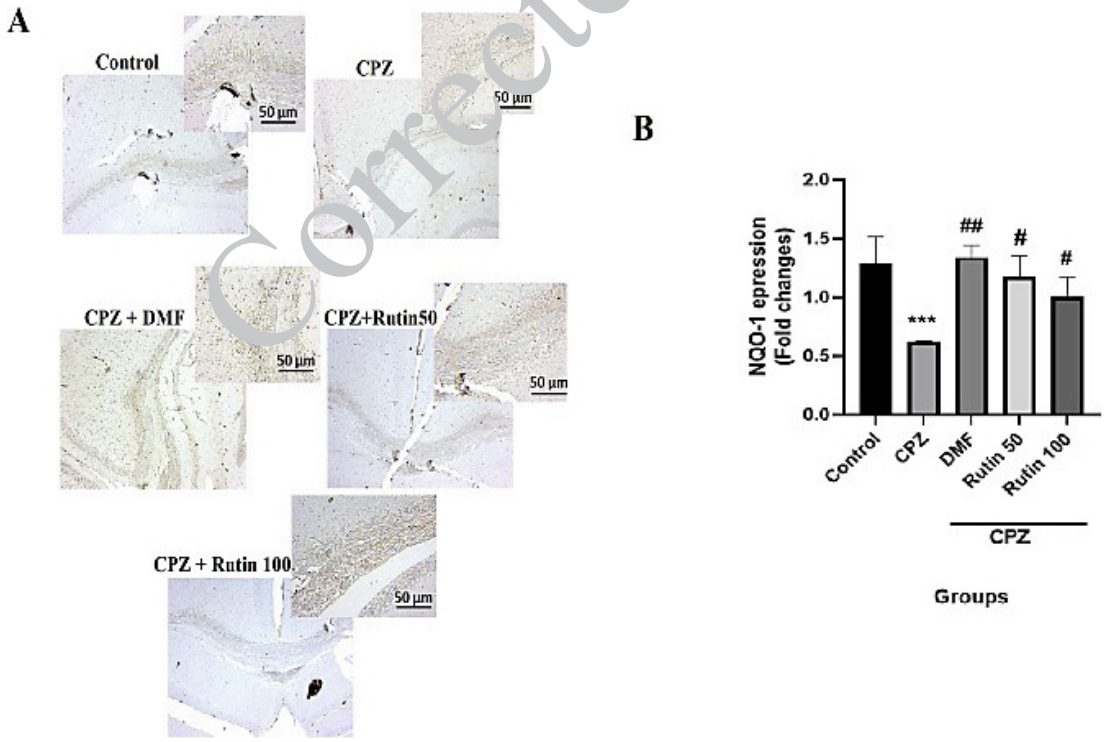


**Figure 7.** Immunohistochemistry of NQO-1 in the corpus callosum of mice
Magnification: ×40 and ×100. CPZ: cuprizone group; DMF group: dimethyl fumarate 10 mg/kg treated-CPZ group; CPZ+rutin 50: rutin 50 mg/kg treated-CPZ group; CPZ+rutin 100: rutin 100 mg/kg treated-CPZ group. (***: $P$<0.001) compared with control group; (#: $P$<0.05; ##: $P$<0.01) compared with the CPZ group.

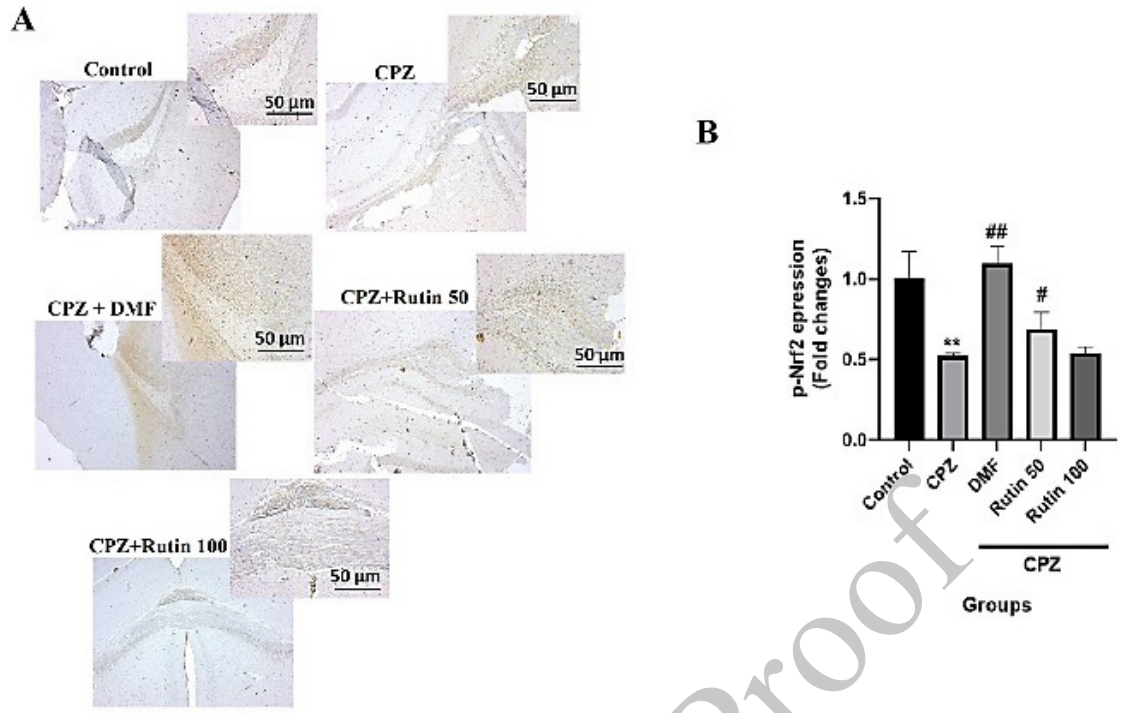


**Figure 8.** Immunohistochemistry of p-Nrf2 in the corpus callosum
Magnification: ×40 and ×100. CPZ: cuprizone group; DMF group: dimethyl fumarate 10 mg/kg treated-CPZ group; CPZ+rutin 50: rutin 50 mg/kg treated-CPZ group; CPZ+rutin 100: rutin 100 mg/kg treated-CPZ group. (** $P$<0.01) compared with control group; (# $P$<0.05, ## $P$<0.01) compared with CPZ group.

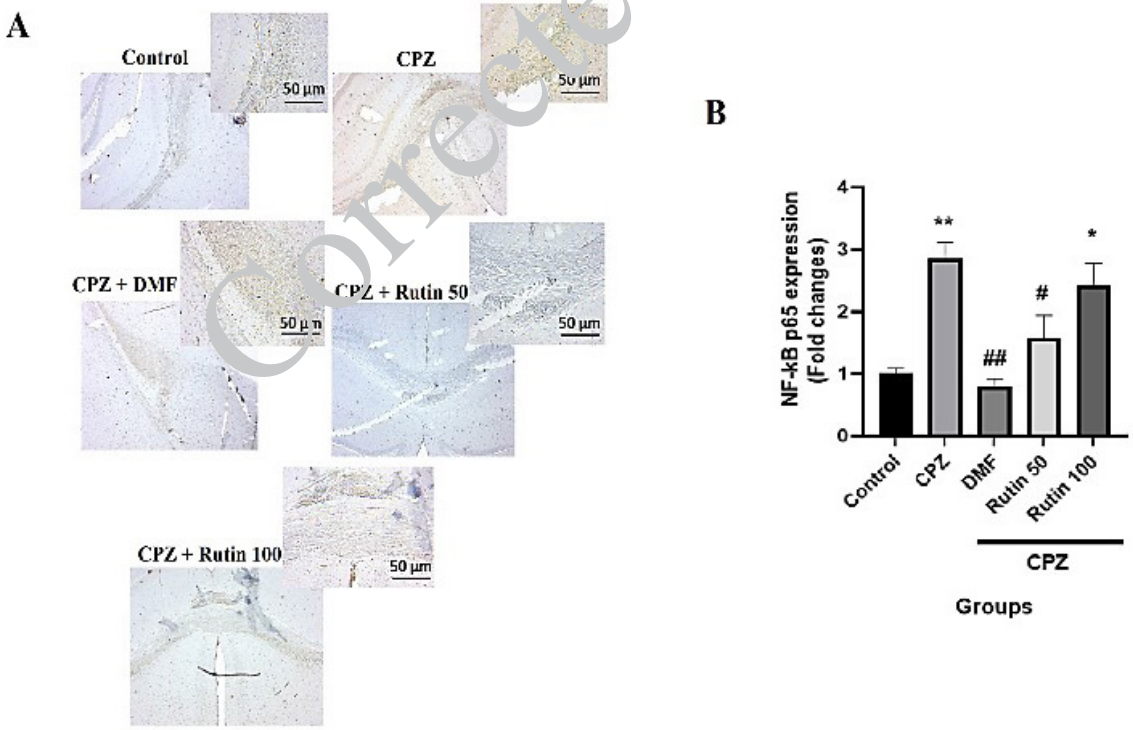


**Figure 9.** Immunohistochemistry of NF-κB p65 in the corpus callosum of mice
Magnification: ×40 and ×100. CPZ: cuprizone group; DMF group: dimethyl fumarate 10 mg/kg treated-CPZ group; CPZ+rutin 50: rutin 50 mg/kg treated-CPZ group; CPZ+rutin 100: rutin 100 mg/kg treated-CPZ group. (* $P$<0.05; ** $P$<0.01) compared with control group; (# $P$<0.05; ## $P$<0.01) compared with CPZ group.



## Discussion

This study demonstrates, for the first time, that rutin exerts dose-dependent neuroprotective and remyelinating effects in the cuprizone-induced mouse model of demyelination. Rutin treatment (especially at dose 50 mg/kg) significantly improved motor coordination deficits, preserved myelin integrity in the corpus callosum, and restored normal cellular architecture in the prefrontal cortex. Mechanistically, these

effects were mediated through concurrent activation of the Nrf2/HO-1/NQO-1 antioxidant pathway and suppression of the pro-inflammatory NF-κB p65 pathway. Although rutin exhibits poor aqueous solubility and relatively low bioavailability, several studies have demonstrated that it (and/or its metabolites) is able to cross the blood-brain barrier (BBB) and exert direct neuroprotective effects in the CNS. Following systemic administration (oral or intraperitoneal), rutin reaches measurable concentrations in brain tissue, sufficient to modulate neuroinflammation, oxidative stress, and demyelination processes (45-47).

In our study, CPZ-fed mice exhibited significant motor deficits in the pole test (increased descent time), rotarod test (reduced latency to fall), and open-field test (reduced locomotion and increased immobility). These findings are consistent with previous reports demonstrating that CPZ-induced demyelination in the corpus callosum leads to impaired motor coordination and bradykinesia (48, 49). Importantly, rutin treatment ameliorated all these behavioral deficits. Rutin 50 mg/kg produced greater improvement than the 100 mg/kg, Notably, the degree of behavioral recovery observed with rutin 50 mg/kg was comparable to that of the reference drug DMF (10 mg/kg, oral), indicating strong therapeutic potential.

The histological analysis using LFB staining confirmed extensive demyelination in the corpus callosum of CPZ-treated mice, as evidenced by a marked reduction in blue staining intensity. Rutin treatment significantly increased LFB intensity, indicating myelin preservation or reduced demyelination. These results align with the behavioral data, as improved motor performance correlated with greater myelin integrity. Furthermore, H&E staining of the prefrontal cortex revealed that rutin reduced CPZ-induced histopathological alterations, including vacuolation and chromatolysis, and promoted cellular regeneration.

The Nrf2 pathway is a master regulator of antioxidant defense. Under basal conditions, Nrf2 is bound to Keap1 in the cytoplasm and targeted for degradation. Upon oxidative stress, Nrf2 is released, translocates to the nucleus, and binds to the antioxidant response element (ARE), driving expression of cytoprotective enzymes including HO-1 and NQO-1 (14, 50). Rutin has shown neuroprotective effects by targeting Nrf2/Mac-1/caspase-1, improving postoperative cognitive dysfunction in animal models, and normalizing pyroptosis and microglial polarization (51). In another *in vivo* study in mice, rutin administration showed a beneficial role against diabetic retinopathy and corneal degeneration via targeting Nrf2, Sirt, and HO-1 (52). An *in vitro* study in primary neuronal cells (SH-SY5Y cells) indicated that rutin protected against lead (Pb)- induced cell death, oxidative stress, and inflammation by promoting the Nrf2/HO-1/NQO1 signaling pathway (53).

Our IHC data showed that CPZ feeding significantly reduced p-Nrf2, HO-1, and NQO-1 immunoreactivity in the corpus callosum, indicating impaired antioxidant capacity. Rutin treatment restored p-Nrf2 levels and up-regulated both HO-1 and NQO-1. This is consistent with previous studies showing that rutin activates Nrf2 in other models of oxidative stress (24, 54). Importantly, our study extends these findings to CPZ-induced demyelination, providing direct evidence that rutin enhances endogenous antioxidant defense in the demyelinated brain.

NF-κB is a central mediator of neuroinflammation. Upon activation, it translocates to the nucleus and promotes transcription of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) and inducible enzymes (iNOS, COX-2), which exacerbate oligodendrocyte injury and myelin loss (28,57,58). In the CPZ model, glial cells (microglia and astrocytes) become activated and release these inflammatory mediators (9, 10). A study demonstrated that rutin reversed neuroinflammation and neural death in a Tau-P301S mouse model of Alzheimer's disease by down-regulating the NF-κB pathway in mouse brains (33). In an experimental rat model of subarachnoid hemorrhage, rutin prevented neuroinflammation and showed neuroprotection by suppressing the NF-κB signaling pathway and reducing neuronal cell death in the cerebral cortex (55).

Our IHC data showed that CPZ increased NF-κB p65 immunoreactivity, confirming activation of this pro-inflammatory pathway. Rutin treatment significantly reduced p65 levels, indicating suppression of NF-κB signaling. This finding is consistent with previous reports showing that rutin downregulates NF-κB in other inflammatory conditions (25, 54). Rutin's concurrent suppression of NF-κB likely contributes to reduced glial activation and diminished neuroinflammation, thereby protecting oligodendrocytes from inflammatory damage.

An important finding of our study is that rutin simultaneously activates Nrf2 and suppresses NF-κB. These two pathways are known to antagonize each other: NF-κB activation can repress Nrf2-driven transcription by competing for shared transcriptional co-activators such as CBP/p300, while Nrf2 activation can suppress NF-κB by delaying IκBα degradation and reducing nuclear p65 levels (20, 50). Our data support a model in which CPZ-induced oxidative stress activates both pathways, but the net effect is detrimental because Nrf2 activation is inadequate and NF-κB activation is excessive. Rutin restores the balance by enhancing Nrf2 and inhibiting NF-κB. This dual mechanism is particularly attractive for MS therapy, as both oxidative stress and neuroinflammation contribute to disease pathogenesis.

Several previous studies have investigated the neuroprotective effects of rutin. It was reported that rutin prevented cognitive impairments in a rat model of Alzheimer's disease through antioxidant and anti-inflammatory mechanisms (56). The antioxidant properties of rutin and its potential for neurodegenerative disorders were reviewed (54). Hu *et al.* have indicated that rutin reduced neuroinflammation in zebrafish models induced by copper sulfate and lipopolysaccharide. The results showed that rutin reduced neutrophil and macrophage migration and improved locomotor ability by targeting the NF-κB signaling pathway (57). In an animal model of diabetes, rutin administration ameliorated mechanical hyperalgesia, thermal hyperalgesia, and cold allodynia and reduced oxidative stress in neurons through the Nrf2/HO-1 signaling pathway in dorsal root ganglia (13).

However, few studies have examined rutin in the context of demyelination. A recent study showed that rutin (50 and 100 mg/kg) protects against CPZ-induced demyelination through Nrf2 and NF-κB modulation, which is highly consistent with our findings. Our study independently confirms and extends these results by providing detailed behavioral, histological, and IHC evidence in the same model. Additionally, our study is the first to report the effects of rutin on both the corpus callosum and prefrontal cortex in the CPZ model.

DMF is an FDA-approved drug for MS that acts through Nrf2 activation (58, 59). In our study, DMF (10 mg/kg, oral) served as a positive control and produced significant protective effects, as expected. Rutin (50 mg/kg, IP) showed efficacy comparable to DMF in most parameters, particularly rotarod performance and LFB intensity. However, direct comparison between rutin and DMF is limited by the different routes of administration (IP vs oral) and the lack of pharmacokinetic data. Future studies should directly compare these agents using the same route and dose regimen.

***Limitations of the study***

Several limitations should be acknowledged. First, we did not include a vehicle control group for intraperitoneal injections, which may have introduced an additional stress variable. Second, the route of administration differed between rutin (IP) and DMF (oral), limiting direct comparison of efficacy. Third, we did not measure rutin bioavailability in brain tissue, so we cannot confirm that the observed effects are directly attributable to rutin reaching the CNS. Fourth, our study used only male mice; future studies should include both sexes to assess potential sex differences. Despite these limitations, our findings provide strong evidence for the neuroprotective effects of rutin in the CPZ model.

***Clinical implications and future directions***

The findings of the present study highlight the therapeutic potential of rutin as a promising neuroprotective agent in the cuprizone-multiple sclerosis model. By administering rutin intraperitoneally after demyelination induction, we demonstrated significant improvement in clinical scores, preservation of myelin, and reduction of inflammatory infiltration through activation of the Nrf2/HO-1/NQO-1 pathway and modulation of NF-κB. Unlike many previous studies that focused on prophylactic protocols, our therapeutic approach better mimics the clinical situation where treatment begins after disease onset. These results suggest that rutin could serve as a safe, natural, and cost-effective adjunct therapy to conventional MS treatments, potentially reducing neurodegeneration and improving quality of life in patients. Further clinical trials are warranted to validate these preclinical benefits in humans.

Unlike many current DMTs that primarily target the immune system, rutin acts through a dual mechanism that enhances antioxidant defenses and suppresses neuroinflammation, addressing two core pathological processes in MS. However, several steps are needed before clinical translation. First, pharmacokinetic studies should determine the optimal oral dose of rutin that achieves therapeutic brain concentrations. Second, the safety and efficacy of rutin should be tested in other MS models, such as experimental autoimmune encephalomyelitis (EAE). Third, clinical trials would be required to assess the efficacy of rutin in MS patients, either alone or in combination with existing therapies.

## Conclusion

The present study demonstrates that rutin exerts dose-dependent neuroprotective and remyelinating effects in a cuprizone-induced mouse model of demyelination. Our key findings are as follows:

Behavioral improvements: Rutin (50 and 100 mg/kg, IP) significantly improved motor coordination deficits, as evidenced by reduced descent time in the pole test, increased latency to fall in the rotarod test, and enhanced locomotor activity in the open-field test, compared with CPZ-treated mice.

Histological protection: Rutin treatment preserved myelin integrity, as evidenced by increased Luxol fast blue staining intensity in the corpus callosum, and restored normal cellular architecture, as visualized by H&E staining.

Molecular mechanism: Rutin concurrently activated the Nrf2/HO-1/NQO-1 antioxidant pathway (increasing immunoreactivity of p-Nrf2, HO-1, and NQO-1) and suppressed the pro-inflammatory NF-κB p65 pathway in the corpus callosum.

These findings position rutin as a promising therapeutic candidate for demyelinating disorders such as MS, acting through a dual mechanism that enhances endogenous antioxidant defenses and inhibits neuroinflammation. Future studies should validate these effects in additional experimental models (e.g., EAE) and investigate rutin's pharmacokinetic properties and optimal dosing regimens to support its clinical translation.

## Acknowledgment

This project was supported by the Kermanshah University of Medical Sciences, Iran, under grant number 4020106. A. Suredа was supported by the Instituto de Salud Carlos III, Spain, through the Fondo de Investigación para la Salud (CIBEROBN CB12/03/30038).

## Authors' Contributions

S S was responsible for conceptualization, methodology, writing the original draft, reviewing and editing, and supervision. SD A handled data curation and prepared the original draft. MH F contributed to validation, investigation, and writing, including review and editing. S S and A S worked on software development, validation, and writing, with a focus on review and editing. S A and S F participated in writing, specifically review and editing.

## Conflicts of Interest

The authors have no conflicts of interest.

## Declaration

We have not used any AI tools or technologies to prepare this manuscript.

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