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Original Article

Neuroprotective Effects of Melissa Officinalis in Chronic Constriction Injury Through Suppression of β-NGF and pro-inflammatory Cytokines

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ABSTRACT

Chronic constriction injury (CCI) is a well-established experimental model to induce neuropathic pain (NP). Evidence demonstrates that pro-inflammatory cytokines (IL - 1 β, TNF-α) and beta nerve growth factor (β NGF) are key contributors to the initiation and maintenance of NP. Melissa officinalis (MO) is a medicinal plant with recognized anti-inflammatory properties. In this investigation, we examine the neuroprotective effects of MO after CCI. The 32 rats were randomly assigned to four groups of eight animals per group, as follows: Sham (no surgery), CCI, CCI + vehicle (saline), and CCI + MO (150 mg/kg/day, p.o. gavage for 14 days after surgery). To evaluate NP, behavioral assessments of thermal hyperalgesia and Cold allodynia (N=8) were conducted on days 7, 14, and 21 after injury. Furthermore, H&E staining (N=5) was used to assess histopathological alterations within the dorsal horn of the spinal cord. Finally, the levels of IL - 1 β, TNF-α, β NGF, hepatotoxicity (ALT, AST), and nephrotoxicity (creatinine, Urea) markers were measured using standard biochemical and ELISA kits (N=3). MO Treatment significantly attenuated thermal hyperalgesia and cold allodynia at 14 and 21 days after CCI (Respectively, $p<0.05$, $p<0.01$). Furthermore, pro-inflammatory cytokine levels and β NGF expression were significantly decreased in the MO-treated group compared to the CCI groups ($p<0.05$). Furthermore, histological analyzes revealed that MO significantly decreased the number of dark neurons in the dorsal horn of the spinal cord ($p<0.001$). MO exerted possible protective effects in an animal model of CCI by improving behavioral tests, by reducing neuronal loss in the spinal dorsal horn, and by suppressing the expression of β-NGF protein and pro-inflammatory cytokines (TNF-α, IL-1β) in the spinal cord.

Keywords: Melissa officinalis, β NGF, Inflammation factor, Chronic constriction injury

INTRODUCTION

NP represents a severe and disabling clinical disorder, distinguished by increased pain sensitivity (hyperalgesia), pain in response to generally non-painful stimuli (allodynia), and spontaneous pain, typically resulting from damage to the peripheral nerves. The Chronic Constriction Injury model is widely applied to study NP in rodents, producing consistent behavioral manifestations such as thermal hyperalgesia and cold allodynia [1]. The neurotrophic protein known as NGF substantially contributes to the control of pain perception. β-NGF, as the active form of NGF, binds to TrkA and triggers signaling pathways that in the short term alter ion channel activity and in the long-term change gene expression and neuronal remodeling, playing an essential role in the onset and persistence of pain. These properties have made β-NGF an innovative and attractive remedial target to further improve chronic pain treatments [2]. Furthermore, pro-inflammatory cytokines such as TNF-α and IL-1β amplify nociceptive signaling and maintain neuroinflammation [3, 4]. After nerve injury, microglia in the spinal dorsal horn rapidly activate and release pro-inflammatory cytokines. IL-1β and IL-6 levels increase after CCI. TNF-α is released alongside IL-1β, driving central sensitization and neuropathic pain [5-8]. Pain signals originate in peripheral nociceptors, are transferred to dorsal horn interneurons, and are then conveyed by projection neurons in laminae I and V. These signals ascend primarily through the spinothalamic tract to the thalamus and the cerebral cortex, where pain is perceived. When the dorsal horn circuits are damaged or have maladaptive plasticity, the resulting central sensitization increases the transmission of nociceptive signals, thus promoting neuropathic pain syndromes [9, 10]. Given the restricted efficacy and adverse effects of standard analgesics, complementary and alternative approaches have attracted growing interest. MO (lemon balm) exhibits notable antioxidant, anti-inflammatory and neuroprotective properties [11, 12]. The CCI model of neuropathic pain induces cold allodynia, thermal hyperalgesia, and spontaneous pain-like behaviors, reflecting underlying neuroplastic changes. Repeated electroacupuncture exerts accumulative analgesia in CCI neuropathic pain through PKA-dependent neuroplasticity in hypothalamic-hippocampal regions, an effect that is diminished by cognitive impairment [13, 14]. Previous research has demonstrated that this model decreased the thermal and cold

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allodynia acetone threshold [15]. MO is a medicinal herb of the Lamiaceae family, traditionally used for digestive and sedative purposes. Modern studies show that it contains phenolic acids, flavonoids, terpenes, tannins, and essential oils, with rosmarinic acid as a key compound. These constituents confer multiple pharmacological properties, containing antioxidant, anti-inflammatory, antidiabetic, antiviral, antimicrobial, neuroprotective, and anticancer features. Evidence supports its use in anxiety, insomnia, Alzheimer's disease, cardiovascular disorders, and infections, making it a versatile medicinal plant with broad therapeutic potential [16-21]. Compared to previous studies, our work addresses several knowledge gaps: (1) examination of the dorsal horn of the spinal cord, a key region for neuropathic pain; (2) specific measurement of β-NGF in the spinal cord, given its role in spinal pain mechanisms; and (3) evaluation of hepatorenal safety of MO after CCI. Consequently, the current investigation was designed to examine the possible protective effects of MO on β-NGF expression and the spinal cord in a rat model of CCI.

MATERIALS AND METHODS

A total of 32 adult male Wistar rats (200–250 g), sourced from the Royan Institute of Iran, were housed under standard conditions (12-hour light/dark cycle) with free access to food and water. The rats were then randomly divided into four experimental groups, each containing eight animals (n=8): Control, CCI, CCI +vehicle (normal saline), and CCI + MO. In the CCI + MO group. Beginning on the first postoperative day after CCI induction, rats received Melissa officinalis L. extract (150 mg / kg / day) by oral gavage, with daily administration maintained for two consecutive weeks. The 150 mg/kg dose was selected as the optimal dose based on previous similar studies that reported significant pharmacological activity with this dose in relevant animal models [22]. The experimenter performing behavioral tests and data analysis was blinded to the groups.

Standardization and Phytochemical Analysis of Melissa Officinalis Extract

The extraction yield was calculated as the percentage of dry extract weight relative to the initial dry plant material weight. The extract was standardized by high performance liquid chromatography (HPLC) using an external standard method. The main active compounds identified and quantified were rosmarinic acid, with a retention time of approximately 20.78–20.90 minutes.

CCI Model Induction

The CCI model, originally developed by Bennett and Xie, was used to induce NP symptoms [1]. For anesthesia, rats were given intraperitoneal administration of a cocktail of ketamine (100 mg/kg) and xylazine (10 mg/kg). The hair overlying the sciatic nerve region was shaved and an incision was made in the right thigh. After the sciatic nerve was surgically exposed, four chromic ligatures (0.4 mm) were loosely placed around it at evenly spaced intervals. These ligatures did not obstruct blood flow within the epineurial vasculature. The muscle and skin layers were subsequently closed with nylon sutures. Following recovery from anesthesia, the animals were returned to their cages. The study received ethical approval from the University Ethics Committee (IR.IUMS.AEC.1402.130), and all experimental procedures followed the IASP committee's guidelines for research and ethical issues.

Enzyme-Linked Immunosorbent Assay (ELISA) for IL-1β, TNF-α, and β-NGF

R&D Systems (USA) provided the ELISA kits for detecting IL-1β, TNF-α, and β-NGF, which corresponded to the following catalog numbers: IL-1β (Cat. No. RLB00), TNF-α (Cat. No. RTA00), and β -NGF (Cat. No. DY556). IL-1β, TNF-α, and β-NGF levels were measured following the manufacturer's protocols. The absorbance was read at 450 nm, and the concentrations were calculated from standard linear regression curves. Samples from three animals in each experimental group (n = 3) were used for these analyzes. Twenty-one days after CCI, rats were sacrificed by cervical dislocation and the L4–L6 segments of the spinal cord were dissected and homogenized. The resulting supernatant was collected for biochemical quantification of IL-1β, TNF-α, and β-NGF.

Biochemical Assays for ALT, AST and Creatinine

Under anesthesia, blood was drawn through a cardiac puncture and centrifuged to separate the serum. Serum ALT and AST activities were measured to assess hepatic function, while serum creatinine levels were used to evaluate renal function.

Evaluation of Pain Sensitivity: Thermal Hyperalgesia and Cold Allodynia

Hot Plate Test

Thermal hyperalgesia was behaviorally assessed using Eddy's hot plate set at a temperature of 52–55 °C. The plantar surface of the right hind paw was placed in contact with the heated plate, and the latency to withdraw was determined based on jumping or paw-licking responses. To prevent tissue injury, a maximum cutoff time of 30 s was applied. Each animal was tested in three trials and assessments were conducted on days 7, 14, and 21 after CCI surgery [23].

Cold Allodynia (Acetone Test)

To determine the cold allodynia thresholds, the acetone test was used following established methods [24]. The animals were placed inside a clear Plexiglas enclosure equipped with a mesh floor, and a single drop of acetone was delivered to the plantar surface of the hind paw. The procedure was repeated five times, and the withdrawal of the paw was recorded as a positive response. The percentage of withdrawal responses was calculated according to the formula: (positive responses / total number of trials) × 100.

Histological Analysis

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Five rats were included in each group. Following perfusion, the L4–L6 segments of the spinal cord were dissected and post-fixed in 4% PFA for seven days. The tissues were then processed, embedded in paraffin and sectioned at a thickness of 6 µm using a microtome. After deparaffinization and rehydration, the sections were stained with H&E, dehydrated through ascending alcohols, cleared in xylene, and coverslipped. Using systematic random sampling, nine sections of each animal were selected and a dorsal horn field per section was imaged. Dark neurons, identified by their shrunken and dark cytoplasm, pyknotic nuclei, loss of nuclear–cytoplasmic distinction, and irregular cellular morphology, were counted by a blinded observer [25].

Statistical Analysis

All statistical analyzes were performed using GraphPad Prism. The normality of the data distribution was checked using the Shapiro–Wilk test. Values are presented as mean ± SEM. Differences among groups were analyzed by ANOVA with Tukey's post hoc comparison. The Levene test verified the homogeneity of the variances ($p > 0.05$), and significance was defined as $p \leq 0.05$. For behavioral assessments, a repeated-measures analysis was performed to account for within-subject variability over time.

RESULTS

Effect of MO Remedy on Thermal Hyperalgesia in CCI Rats

The impact of MO treatment on claw heat-hyperalgesia (CHH) was evaluated in the CCI sciatic rat model by using the hot plate test. A two-way repeated measures ANOVA revealed no significant therapeutic effect on day 7; however, significant improvements were observed on days 14 and 21 relative to the CCI group (#$p < 0.05$, Effect size (ηp^2): 0.13, ##$p<0.01$, Effect size (ηp^2): 0.15. CHH thresholds were significantly reduced in the CCI group compared to the control (days 7, 14, and 21; **** $p < 0.0001$). The reduction in hyperalgesia in MO-treated rats became evident after day 7 and persisted until day 21 (Fig. 1).

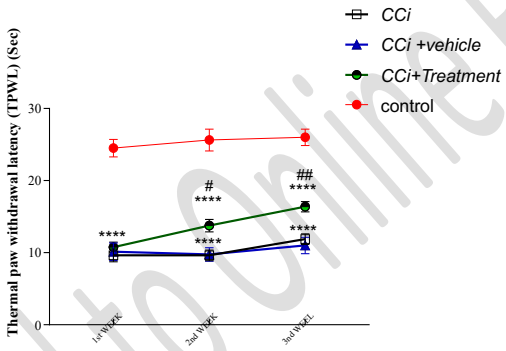

Fig. 1 Effects of MO remedy on claw heat hyperalgesia in CCI rats. The withdrawal thresholds of the right hind paw were measured in all groups on days 7, 14, and 21 following sciatic nerve CCI. Comparisons of withdrawal latencies between groups are indicated as ****$p < 0.0001$ versus control; #$p < 0.05$, ##$p < 0.01$ versus the CCI group. Data are presented as mean ± SEM (n = 8).

Effect of MO Remedy on Cold Allodynia in CCI Rats

The cold allodynia threshold was determined with a standard behavioral assay to evaluate hypersensitivity to cold stimuli in rodents. The frequency of claw withdrawal responses (CWR%) was significantly augmented in CCI rats relative to controls (****$p < 0.0001$ on days 7, 14, 21). A two-way repeated measures ANOVA revealed that MO treatment attenuated the CWR%, showing no differences compared to the CCI group on day 7, but significant reductions relative to the CCI group were observed on days 14 and 21 (#$p < 0.05$, Effect size: $\eta p^2 = 0.13$; ##$p < 0.01$, Effect size: $\eta p^2 = 0.20$; Fig. 2).

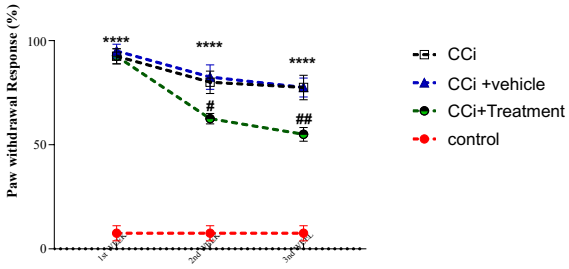

Fig. 2 Effects of MO remedy on cold allodynia (CWR%) in CCI rats. Claw withdrawal responses to acetone stimulation were measured on days 7, 14, and 21. ****$p < 0.0001$ versus control; #$p < 0.05$, ##$p < 0.01$versus CCI group. Data are mean ± SEM (n = 8).

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Effect of MO Remedy on β NGF Levels in CCI Rats

A significant elevation in β-NGF levels was observed in the CCI group compared with the control group (154.4 ± 10.74 vs. 61.65 ± 10.86) (***p<0.001). In contrast, β NGF levels in the CCI + Mo group were significantly reduced relative to the CCI group (108.4 ± 6.34 vs. 154.4 ± 10.74), (# p < 0.05), with an effect size ($\eta^2 = 0.892$) (Fig. 3).

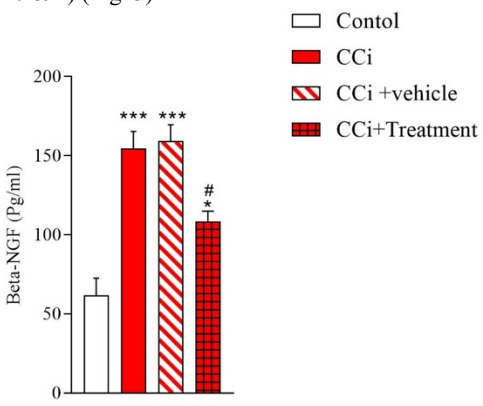

Fig. 3 β NGF levels in experimental groups measured by ELISA. Group comparisons are indicated as *p < 0.05, ***p < 0.001 versus control; #p < 0.05 versus the CCI group. Data are presented as mean ± SE (n = 3).

MO Remedy Reduces Elevated IL-1β and TNF-α Induced by CCI

Compared to control animals, the CCI group showed a significant increase in IL-1β and TNF-α levels. (20.71 ± 3.69 vs. 54.53 ± 4.45; 17.32 ± 3.06 vs. 48.65 ± 3.99, respectively) (**p<0.01). MO-treated rats exhibited reduced IL-1β and TNF-α levels relative to the CCI group (29.07 ± 4.15 vs. 52.15 ± 6.20; 24.40 ± 4.65 vs. 48.65 ± 3.99, respectively) (#p<0.05). Effect size ($\eta^2 = 0.83$ and 0.842, respectively) (Fig. 4a, b).

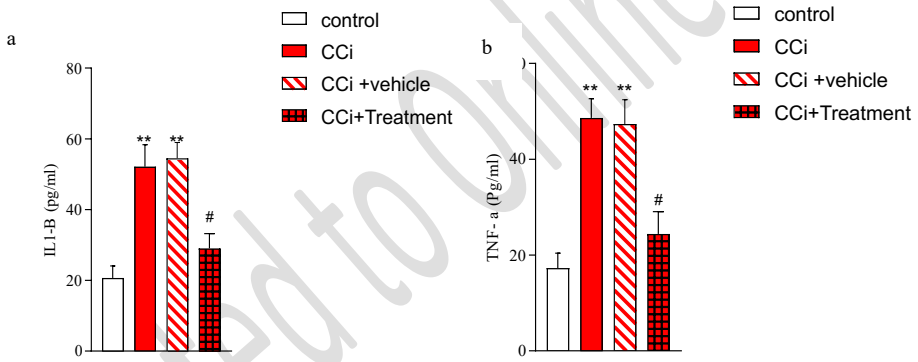

Fig. 4 Impacts of CCI and MO remedy on inflammatory markers. (A) IL-1β and (B) TNF-α levels were measured by ELISA. **p < 0.01versus control; #p < 0.05 versus the CCI group. Data are presented as mean ± SEM (n = 3).

MO Remedy Attenuates CCI-Induced Elevation of Liver Enzymes (ALT, AST)

In summary, the NP model (CCI) significantly elevated ALT and AST levels compared with the control group. (48.00 ± 2.30 vs. 97.00 ± 11.68; 105.00 ± 15.72 vs. 217.30 ± 9.73, respectively) (**p<0.01). Remedy with MO attenuated this increase, reducing ALT and AST levels relative to the CCI group (61.0 ± 3.21 vs. 97.00 ± 11.68; 154.0 ± 14.05 vs. 217.30 ± 9.73, respectively) (#p<0.05). Effect size ($\eta^2 = 0.824$ and 0.874, respectively) (Fig. 5a, b).

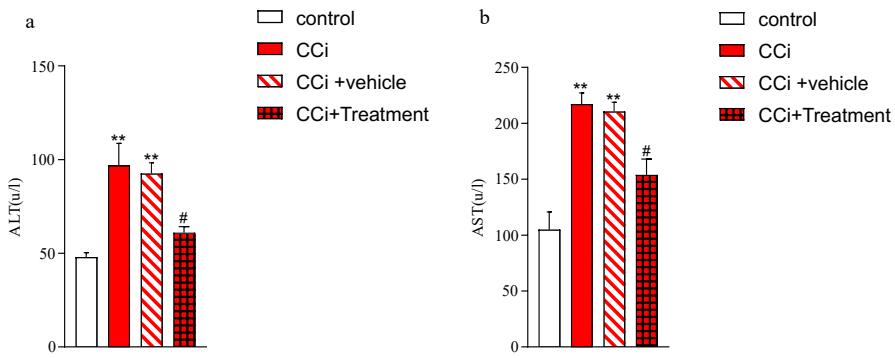

Fig. 5 Impacts of CCI and CCI + MO on ALT and AST markers. A) ALT, B) AST, levels in blood serum. **p < 0.01when relative to the control. #p < 0.05when relative to the CCI group. Data disclosed as mean ± SEM (n = 3).

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No Significant Changes in Creatinine and Urea Levels

No statistically significant alterations in creatinine and urea levels were observed in the CCI group relative to controls. (0.46 ± 0.03 vs. 0.55 ± 0.02; 43.00 ± 3.21 vs. 53.00 ± 3.21, respectively). Similarly, MO-treated rats exhibited no significant changes in creatinine and urea levels relative to the CCI group (0.48 ± 0.03 vs. 0.55 ± 0.02; 47.67 ± 3.52 vs. 53.00 ± 3.21, respectively) (Fig. 6a, b).

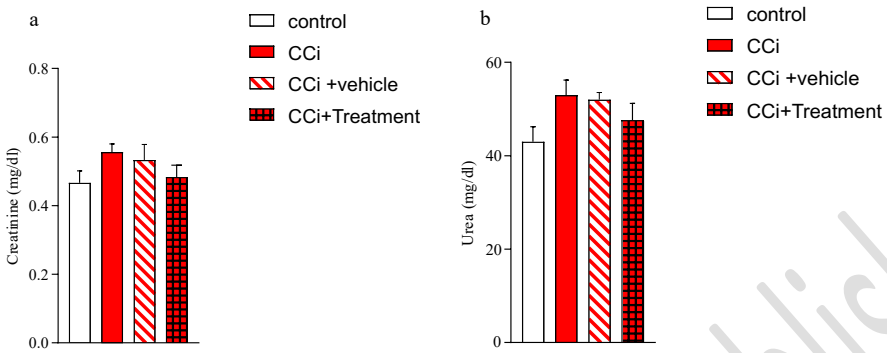

Fig. 6 Effects of CCI and MO remedy on renal function markers. (A) Creatinine and (B) urea levels were measured in serum.

MO Effect on the Number of Dark Neurons in the Dorsal Horn of the Spinal Cord

In this study, CCI significantly increased the number of dark neurons in the dorsal horn compared with the control group (***P < 0.001). On the contrary, MO therapy significantly reduced these numbers relative to the CCI group (38.11 ± 2.06 vs. 85.11 ± 2.39, ###p < 0.0001), with an effect size ($\eta^2 = 0.95$). (Fig. 7).

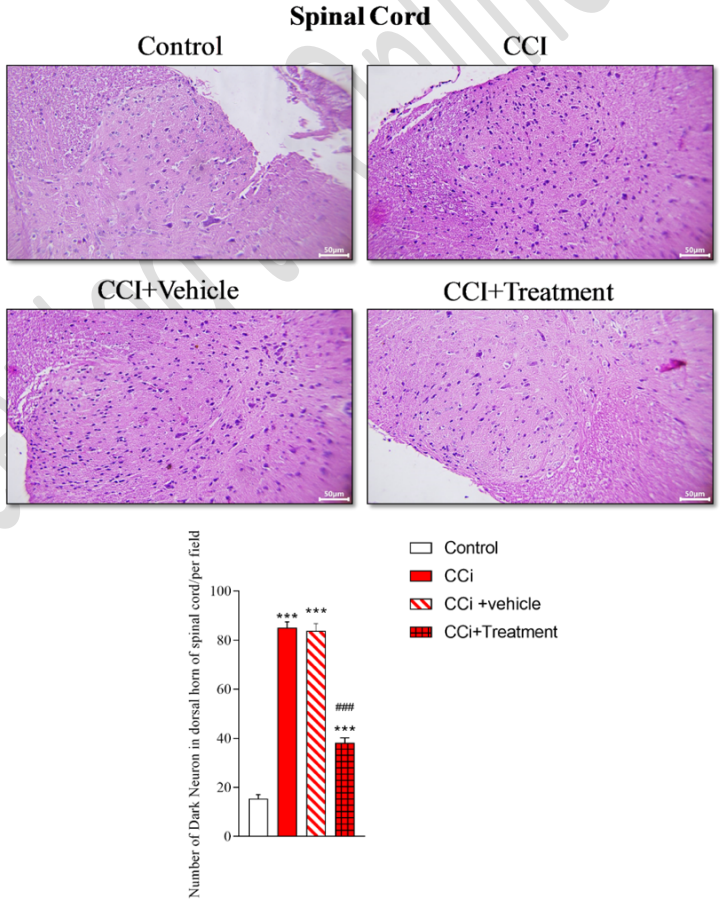

Fig. 7 Effects of CCI and MO remedies on the number of dark neurons in the dorsal horn. ***p < 0.001 when relative to the control; ###p < 0.001 versus the CCI group. Data are presented as mean ± SEM (n = 5).

DISCUSSION

One of the main clinical manifestations after sciatic neuropathy is chronic pain [26]. Transmission of pain, temperature, and touch sensations occurs through the spinothalamic tract. Primary afferent axons project into the spinal cord, where they synapse in the dorsal horn. Second-order neurons then decussate and ascend on the opposite side toward the thalamus, after which the information is transmitted to the somatosensory cortex for processing [27]. Within the spinal cord, pro-inflammatory cytokines (e.g., IL-6, IL-1β, TNF-α) together with neurotrophins (e.g., BDNF, NT-3/4, NGF) contribute significantly to the development and maintenance of enhanced NP [28, 29]. In another study, it was observed that IL-1β mRNA expression is markedly increased within the damaged sciatic nerve after partial nerve ligation [30]. Furthermore, ligation of the L5–L6 spinal nerves (SNL) led to increased expression of IL-6 mRNA in both the dorsal root ganglia and the spinal cord [31]. Furthermore, intrathecal injection of IL-6, IL-1β, and endoneural injection of TNF-α have been demonstrated to induce hyperalgesia and allodynia in rat models [29, 32, 33]. These cytokine cascades play a critical role in the pathogenesis of NP and may provide promising ideas for the improvement of novel and more effective analgesic remedies across multiple pain statuses [34]. In our experiment, the expression levels of TNF-α and IL-1β were significantly increased in the spinal cord following CCI. While the MO remedy was able to reduce the levels of these cytokines. This finding aligns with the known anti-inflammatory properties of Melissa officinalis constituents, particularly rosmarinic acid and flavonoids, which have been shown to suppress NF κB activation and subsequent cytokine production [35]. The observed analgesic and anti-inflammatory effects of Melissa officinalis are likely mediated by its major bioactive constituents. Rosmarinic acid, the predominant phenolic compound, has been shown to inhibit NF-κB activation and reduce pro-inflammatory cytokine production while enhancing Nrf2-driven antioxidant defense. Additionally, the flavonoids present in MO contribute to neuroprotection through their antioxidant properties and GABAergic modulation. Essential oil constituents, particularly monoterpenes such as geranial and neral, exert antinociceptive effects via the KATP, opioidergic, and serotonergic pathways [36-39].

Another essential marker of pain is the family of neurotrophic factors. While it is stated that these molecules play a critical role in modulating the maintenance and uniqueness of the nervous system, it is currently firmly established that they also exert a significant impact on the genesis and modulation of pain [40]. NGF is a critical neurotrophin for sensory neuron survival and maintenance, but its dysregulation leads to pain pathway sensitization and the upregulation of NP. NGF connects to the TrkA and p75NTR receptors to trigger signaling cascades underlying nociception, allodynia, and hyperalgesia [41]. In studies, NGF is upregulated in the dorsal root ganglion and spinal cord following CCI, while the anti-NGF remedy significantly alleviates NP. The anti-NGF remedy produced significant improvements in the threshold of thermal, cold, and mechanical sensitivity [42]. Our ELISA results showed that β-NGF protein levels were markedly elevated in the CCI group, while treatment with MO significantly attenuated β-NGF expression in the spinal cord.

According to previous results, our investigation showed that CCI leads to a marked augmentation in dark neurons within the dorsal horn [43]. The MO remedy effectively reduced the number of dark neurons. Furthermore, our outcomes demonstrated that administration of MO significantly alleviated the threshold of thermal and cold withdrawal in CCI rats. In pathological pain conditions, sensory neurons in the spinal dorsal horn undergo maladaptive changes. Normally, the dorsal horn integrates the input of different primary afferent fibers through a balance of excitatory and inhibitory interneurons. Following injury, this balance is disrupted: inhibitory interneurons lose function, excitatory circuits become overactive, and silent synapses are unmasked. These circuit-level changes lead to central sensitization, where innocuous stimuli can activate nociceptive projection neurons. Behaviorally, this manifests as pain from light touch and hyperalgesia (exaggerated pain responses to noxious stimuli) [44]. Considering the role of inflammatory cytokines (IL-1β, TNF-α), β-NGF, dorsal neurons of the spinal cord in neuropathic pain, and the effect of MO in reducing both inflammatory cytokines, β-NGF, and dark neurons in the dorsal horn, it can be suggested that MO could serve as a potential adjunct in the management of neuropathic pain. However, extensive clinical trials are still required to confirm its efficacy and safety.

In the present study, chronic sciatic nerve constriction injury was associated with a significant increase in serum ALT and AST levels. Chronic neuropathic pain induces systemic oxidative stress and inflammatory responses that can affect distant organs, including the liver. Elevated pro-inflammatory cytokines such as TNF-α and IL-1β following peripheral nerve injury may activate hepatic NF-κB signaling, leading to increased transaminase levels (ALT/AST) [45]. Consistently, a study on morphine-induced hepatotoxicity in a neuropathic pain model reported elevated liver enzymes, as this opioid increases reactive oxygen species (ROS), leading to systemic oxidative stress and subsequent hepatic damage[46]. Furthermore, the bidirectional liver-nervous system axis has been shown to mediate crosstalk between neuroinflammation and hepatic dysfunction under pathological conditions [47].

Our finding that serum urea and creatinine levels did not change significantly after chronic constriction injury (CCI) indicates that this model does not impair renal function. This is consistent with a previous study in which CCI failed similarly to alter these kidney markers, confirming that the observed neuropathic pain behaviors are not secondary to renal dysfunction [48]. Regarding safety, MO treatment did not elevate creatinine and urea levels. However, these findings should be interpreted conservatively, as creatinine and urea are insensitive markers of renal injury, and the absence of their elevation does not definitively exclude subclinical nephrotoxicity. Therefore, our safety conclusions are limited to preservation of glomerular filtration function under tested conditions and require confirmation using more sensitive biomarkers (e.g., NGAL, KIM-1) [49]. As a preclinical animal study, direct extrapolation of these findings to clinical settings is premature.

This study has several limitations that should be acknowledged. The First limitation is the small sample size used for ELISA-based quantification. Due to financial constraints, only three subjects were included in each group. Although such a restricted sample inherently reduces statistical power and may limit the detection of subtle biological variations, the effect sizes observed were consistent and biologically well-supported, thus reinforcing the credibility of our preliminary conclusions. Second, molecular confirmation techniques such as Western blot and qPCR were not performed, preventing verification of protein and gene-expression changes underlying the observed effects. Third, the

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study did not include immunohistochemistry, which would have allowed a more precise cell localization of the measured biomarkers. Finally, some conclusions should be interpreted with caution, given the preclinical nature of the work and the inherent limitations of animal models.

CONCLUSION

The present study demonstrates that Melissa officinalis (MO) exerts significant antinociceptive effects in the CCI model of neuropathic pain in rats, as evidenced by attenuation of thermal hyperalgesia and cold allodynia. These behavioral improvements were associated with suppression of spinal cord pro-inflammatory cytokines (IL-1β and TNF-α), reduction of β-NGF levels, and decreased dark neuron count in the dorsal horn. Our results provide preliminary evidence supporting the analgesic efficacy and acceptable short-term safety profile of MO in a rat model of neuropathic pain. Further confirmatory studies with larger sample sizes, dose-response designs, and comprehensive molecular validation are warranted before considering MO as a potential therapeutic candidate for neuropathic pain management.

Consent to Publication

All authors have read and agreed to the published version of the manuscript.

Conflict of interest

The authors declare that they have no conflicts of interest.

Financing Statement

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Author Contribution

Hamzeh Badeli Sarkala and Mohammad Reza Naghdi Badi contributed to the conceptualization and methodology of the study. Negin Khosravipour, Mohammad Reza Naghdi Badi, Mehrad Mahdavi, Mahdi Tavakolizadeh, and Ali Dadseresht carried out the investigation, while Negin Khosravipour and Mehrad Mahdavi curated the data. Formal analysis was performed by Mohammad Reza Naghdi Badi and Mahdi Tavakolizadeh. The original draft was prepared by Hamzeh Badeli Sarkala and Mohammad Reza Naghdi Badi, and the manuscript was reviewed and edited by Hamzeh Badeli Sarkala, who also handled visualization, supervision, and funding acquisition. All authors read and approved the final manuscript.

Ethical Statement

All experimental procedures involving animals were conducted in accordance with the ethical standards of the Iran University of Medical Sciences. The study protocol was reviewed and approved by the Institutional Animal Ethics Committee under approval code IR.IUMS.AEC.1402.089.

Data Availability

Data are available from the corresponding author upon reasonable request.

Acknowledgment

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