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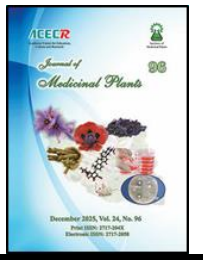

**Research Article**

# Evaluation of the antidepressant-like activity of essential oil from *Citrus medica* fruits (L.) in mice: Involvement of CREB and BDNF

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ABSTRACT

**Background:** Major depressive disorder affects more than 300 million people worldwide. *Citrus medica* L. essential oil (CMEO), rich in terpenes such as limonene, has shown antidepressant-like effects in preclinical models, potentially through hippocampal BDNF/CREB signaling, and may offer a safer natural alternative to conventional antidepressants. **Objective:** This study aimed to investigate the antidepressant-like effects of CMEO on depression-like behaviors induced by lipopolysaccharide (LPS) in mice and to elucidate potential underlying mechanisms involving hippocampal BDNF/p-CREB signaling and brain nitric oxide (NO) levels. **Methods:** CMEO composition was characterized by gas chromatography–mass spectrometry (GC-MS). Depression-like behavior was induced by intraperitoneal LPS injection (1 mg/kg). Mice were randomly assigned to four groups: Control, LPS, CMEO 50 mg/kg + LPS, and CMEO 100 mg/kg + LPS. Behavioral assessments included the Forced Swimming Test (FST), Tail Suspension Test (TST), and Open Field Test (OFT). Biochemical analyses measured hippocampal phosphorylated CREB (p-CREB) and brain-derived neurotrophic factor (BDNF), as well as brain NO levels. **Results:** GC-MS identified ten CMEO constituents, with limonene as the dominant compound (93.96%). CMEO at 50 and 100 mg/kg significantly reduced immobility time in the FST and TST versus the LPS group, indicating attenuated depressive-like behavior. CMEO also restored hippocampal BDNF and p-CREB levels and significantly reduced LPS-elevated NO. **Conclusion:** CMEO alleviates depression-like behaviors in an LPS-induced mouse model, potentially via upregulation of hippocampal BDNF and p-CREB and reduction of NO. The high limonene content may contribute to these effects, supporting CMEO as a promising natural candidate for managing depressive disorders.

*Abbreviations:* CMEO, Citrus medica essential oil; GC-MS, Gas chromatography-mass spectrometry analysis; LPS, lipopolysaccharide; FST, Forced swimming test; TST, Tail suspension test; OFT, open field test; P-CREB, Phosphorylated cAMP response element-binding protein; BDNF, brain-derived neurotrophic factor; NO, nitric oxide; MDD, Major depressive disorder; NGF, Nerve growth factor

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## 1. Introduction

Major depressive disorder (MDD) is a prevalent psychiatric condition and ranks as the second leading cause of disability globally. According to a report published by the World Health Organization on March 31, 2023, MDD impacts over 300 million individuals worldwide and has a lifetime prevalence rate of 3.8 % [1]. Some biomarkers, such as inflammation, oxidative stress, hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis, and alterations in functional neurotrophic factors, are associated with the pathophysiological mechanisms underlying MDD [2, 3].

Brain-derived neurotrophic factor (BDNF), a constituent of the nerve growth factor (NGF) family, is abundantly expressed in the adult brain and is integral to neuronal development and neuroplasticity. [4]. Many studies have shown that BDNF plays a role in neuropsychiatric disorders. The activity-dependent secretion of BDNF protein was reduced with the substitution of methionine for valine in the BDNF gene. As a result, neuropsychiatric diseases such as depression occurred in the mice, and these conditions were not improved by antidepressants [5]. Evidence suggests that BDNF may mediate the effects of antidepressants [6, 7]. Conventional antidepressants increase concentration of neurotransmitters such as norepinephrine and serotonin in the synapses of neurons within hours, but therapeutic responses are achieved after 2 to 3 weeks. This could be due to alterations expression of fctors such as BDNF in hippocampal and cortical neurons [8].

CREB is a nuclear transcription factor. CREB is inactive in its non-phosphorylated form, which is located in the cytoplasm. Phosphorylation of CREB is carried out by various protein kinases, including PKA and $Ca^{2+}$/calmodulin-dependent protein kinases [9]. CREB is activated by binding to the cAMP response element (CRE) in multiple cells, including neurons, and subsequently translocates to the nucleus. CREB activity is associated with neurogenesis and neuronal plasticity [10]. Neuronal apoptosis is regulated via the phosphoinositide 3-kinase/Akt and the Ras/MAPK pathways. Ribosomal S6 kinases (Rsks) phosphorylate CREB through the Ras/MAPK cascade. The increase in p-CREB leads to enhanced neuroprotection [11].

The neurotrophic hypothesis of depression posits that prolonged exposure to chronic stress results in a reduction of brain-derived neurotrophic factor (BDNF) levels and promotes neuronal atrophy, particularly within the hippocampal region. [2, 12-14]. These effects likely occur, in part, through decreased CREB activity [15].

According to previous studies, the level of BDNF is reduced in the hippocampus of depressed patients [16-18] and animal models of depression [19-22]. In contrast, antidepressant treatment improved the level of BDNF[23]. Experimentally, mice with LPS-induced depression exhibited depressive behaviour in the forced swim test (FST), tail suspension test (TST), and sucrose preference test (SPT), which is a predictive model for depression [24-26].

*Citrus medica* L. (Rutaceae) frequently recognized as Citron. The Citron tree usually grows to be 8 to 15 feet tall. It is commonly known as 'Balang' in Iran. A literature survey revealed the presence of terpenes in the essential oil of C. medica. Iso-limonene, citral, and limonene represents the principal components of citrus essential oils [27]. Preclinical studies have demonstrated that both essential oil and extract of citrus fruits exert several pharmacological activities, such as cardioprotective [28],

antioxidant [29, 30], anti-inflammatory and hypoglycemic potential, and anticholinesterase activity [31, 32], antibacterial [33], anticancer [34], anxiolytic [35], antidepressant-like Effects [36], and analgesic activity [37]. Considering the high prevalence of depression and the numerous side effects associated with current antidepressants, including gastrointestinal, sexual, and cardiovascular issues, it is crucial to explore alternative treatments that offer high efficacy and minimal side effects.

Therefore, this study aimed to investigate the potential antidepressant-like effects of essential oil extracted from Citrus medica. Following GC-MS characterization of the oil, its effects were evaluated in an LPS-induced depression model in mice using behavioral assays, along with immunohistochemical analysis of BDNF and CREB expression in the brain.

## 2. Materials and methods

### *2.1. Research Ethical Committee*

All experimental processes involving animals received approval from the Research Ethics Committee for Laboratory Animals at Kermanshah University of Medical Sciences (IR.KUMS.AEC.1401.003). All behavioural assessments were performed by a researcher blinded to the treatment groups to reduce bias in data collection

### *2.2. Chemicals*

*Escherichia coli* lipopolysaccharide was purchased from Sigma-Aldrich (Buchs, Switzerland).

### *2.3. Plant material*

The fruits of *C. medica* were collected in May 2022 from Gilan province, located in the north of Iran.

### *2.4. Extraction of essential oil and its analysis using gas chromatography–mass spectrometry (GC–MS)*

The essential oil was extracted from fresh *C. medica* fruits via water distillation for 3 hours employing a Clevenger-type apparatus.To prepare the essential oil, 50 grams of *C. medica* fruit were placed in a round-bottom flask along with 500 ml of distilled water. The obtained oil was dried over anhydrous sodium sulphate and stored in a dark glass container at 4 °C to prevent oxidation until GC-MS analysis was performed.[38].

GC/MS analysis was performed at the Institute of Medicinal Plants in Karaj, Alborz Province, Iran. The chemical composition of *C. medica* essential oil was characterized by gas chromatography–mass spectrometry (GC-MS). The gas chromatography device used was an Agilent 6890 model with a capillary column (30 m × 0.25 mm; 0.25 µm thickness) of BPX5 type. The sample, diluted with n-hexane to a volume of 1 µl, was injected into the MS/GC machine to identify the essential oil components. The temperature of the injector was 250 °C with a split ratio of 1:35. Helium was used as the carrier gas at a flow rate of 0.5 mL/min. The mass spectra were taken at a voltage of 70 V. The constituents of the essential oil were determined by comparing their mass spectra and retention indices (RIs) with data reported in the literature and with authentic reference samples [39, 40].

### *2.5. Animals*

The experiments were performed employing adult male mice (22-25 g) provided by the University of Kermanshah Animal Care Unit. Animals were housed at the Animal House, Faculty of Pharmacy, Kermanshah University, Kermanshah, Iran, under a 12-hour light/dark

cycle, a temperature of 20 ± 2 °C, and a humidity-controlled environment. Ad libitum access to food and water was maintained for all animals. Animals were allowed a one-week acclimation period to the laboratory environment prior to the start of the experiments. All experimental procedures were conducted between 9:00 a.m. and 2:00 p.m

Animals were randomly assigned to treatment groups using a simple randomization method. A random number table was used to assign animals to one of four groups, ensuring that each animal had an equal chance of being assigned to any group. Randomization was performed by an investigator who was not involved in the behavioural experiments or data analysis to reduce selection bias.

*2.6. Experimental protocols*

A total of 28 animals were included in the study and were randomly assigned to four experimental groups. Group I served as the control and received normal saline. Group II received LPS (1 mg/kg, i.p.) [41]. Group III received LPS (1 mg/kg, i.p.). +CMEO (50 mg/kg; orally, 30 minutes before LPS). Group IV received LPS (1 mg/kg, i.p.). +CMEO (100 mg/kg; orally, 30 minutes before LPS). The doses of CMEO used in the present experiment were chosen based on a pilot study. These doses (50 and 10 mg, administered orally) were selected based on our initial findings, which showed antidepressant-like effects at the dose without observable toxicity. In addition, several studies have reported antidepressant-like effects of *C. medica* essential oil at similar doses. One study showed that Citrus medica essential oil ameliorates anti-cancer-like behaviours and their consequences, for example, through neuroprotection at α7-nicotinic acetylcholine receptors. Furthermore, a separate investigation demonstrated that the hydroalcoholic extract of Citrus medica exhibited antidepressant-like effects in rats at dosages between 250 and 750 mg/kg. These findings support the use of 50 and 100 mg doses in our study [42].

The behavioural tests, including the Open Field Test (OFT), Forced Swim Test (FST), and Tail Suspension Test (TST), were conducted 24 hours following the administration of lipopolysaccharide (LPS). Subsequently, the mice were anesthetized using a combination of ketamine and xylazine, with dosages of 65.0 mg/kg and 9.9 mg/kg, respectively, as described by Mees et al. (2018). Following anesthesia, the brains of the animals were extracted. Half of the brains from each experimental group were fixed in 10 % formalin for immunohistochemical (IHC) assays, while the other half were stored at -80 °C for nitrite assays.

*2.7. Nitrite assay*

Riefly, brain tissues were homogenized and subjected to centrifugation. $ZnSO_4$ was added to the resulting supernatant to precipitate proteins, and the samples were incubated at room temperature for 15 minutes. 100 µl of the supernatant was placed in a well of a microplate. Subsequently, 100 µl of vanadium (III) chloride solution (8 mg/ml) was introduced into each well to facilitate the reduction of nitrate to nitrite. After that, Griess reagents were added, including 50 µl of sulfanilamide (2 %) and 50 µl of N-(1-naphthyl) ethylenediamine dihydrochloride (0.1 %). The absorbance was determined at 540 nm using an ELISA plate reader. After incubating the brain samples at 37 °C for 30 minutes, the concentration of NOx was determined using a standard curve [43].

*2.8. Forced swimming test (FST)*

The forced swim test (FST) is among the most widely employed animal models for investigating depressive-like behavior. The escape-related mobility behaviour is measured by placing mice in an inescapable glass jar (25 cm × 12 cm × 15 cm) filled with water (25 °C) for 6 minutes. The time the mouse spent floating with minimal movements is considered immobilization [44].

*2.9. Open-field test (OFT)*

To analyze the locomotor activity of mice, an open-field test (OFT) was conducted. Animals were randomly placed in the center of the open field apparatus (40 × 60 × 50 cm) for 5 minutes. Mice's time spent in the central square indicates lower levels of depression [45].

*2.10. Tail suspension test (TST)*

Another well-established model for evaluating antidepressant efficacy is the tail suspension test (TST). In this method, each mouse is suspended using a fixed adhesive tape, approximately 1cm from the end of its tail, at a height of half a meter above the ground. The total duration of immobility for each animal was considered a measure of depression over 6 minutes [46].

*2.11. Immunohistochemistry assay*

Immunohistochemical analysis was employed to assess the expression levels of BDNF and phosphorylated CREB (p-CREB) within the hippocampal region of mice. Hippocampal sections were fixed in 10 % buffered formaldehyde for 24 hours and then in 70 % ethanol. Sections were then embedded in paraffin wax for processing onto appropriate slides. The formalin-fixed paraffin tissue section was deparaffinized and hydrated. Then, a sufficient number of drops of hydrogen peroxide were added to block the endogenous peroxidase. Subsequently, the samples were incubated with the specific primary antibodies targeting BDNF (ab108319, rabbit monoclonal antibody) and CREB (ab32515, rabbit monoclonal antibody. After washing the slides with PBS four times, they were incubated with a biotinylated secondary antibody for 10 minutes at room temperature. The samples were subsequently washed again with PBS and incubated with streptavidin-peroxidase for 10 minutes at room temperature. They were incubated in a 0.1 % DAB solution after being rewashed in PBS. Finally, a counterstain was applied, and the slides were dehydrated [47].

*2.12. Statistical analyses*

For data analysis, PRISM software version 9 was used. All data were represented as mean ± SEM. One-way analysis of variance was conducted, followed by a Tukey post-hoc test for group comparison. 95 % confidence intervals (CIs) were calculated and reported alongside p-values where applicable, to improve statistical transparency. A p-value of less than 0.05 indicates that the effect was statistically significant.

## 3. Results

*3.1. Standardization of C. medica essential oil*

The results of the GC-MS analysis are presented in Table 1. The main constituents of *C. medica* essential oil are primarily Monoterpene Hydrocarbons. Ten constituents were identified in the essential oil of *C. medica*, representing 99.18 % of the total oil. The volatile oil contained 96.91 % monoterpene hydrocarbons, 1.66 % sesquiterpene hydrocarbons, and 0.61 % oxygenated monoterpenes. The most abundant components were Limonene (93.96 %), Myrcene (1.79 %),

and beta-bisabolene (1.04 %), respectively (Table 1).

*3.2. Effects of CMEO on the Immobility Time in FST and TST*

The results indicated that animals in the LPS-treated group exhibited an increased immobility time in both the FST and TST compared to the control group (95 % CI: -209.4 to -59.62; $P < 0.001$ and 95 % CI: -117.8 to -0.9122; $P < 0.05$, respectively).CMEO at doses of 50 and 100 mg/kg reduced immobility time in both the FST and TST compared to the LPS group (95 % CI: 62.76 to 236.6; $P < 0.001$ (FST for dose 50 mg/kg)), (95 % CI: 11.47 to 161.3; $P < 0.05$ (FST for dose 100 mg/kg)) and (95 % CI: 18.15 to 140.0; $P < 0.01$ (TST for dose 50 mg/kg), 95 % CI: 62.05 to 165.9; $P < 0.01$ (TST for dose 100 mg/kg)). This demonstrates that CMEO attenuated depressive-like behaviour in mice with depression (Fig. 1).

*3.3. Effects of CMEO on locomotor activity in OFT*

OFT was performed to exclude any possible effects of depression induction and treatment with CMEO on locomotor activity. Treatment with CMEO (50 or 100 mg/kg) showed no significant impact on the number of crossed squares in either the LPS or control mice (95 % CI: -52.23 to 159.4; 95 % CI: -78.23 to 102.2; $P > 0.05$, respectively). This indicates that CMEO has antidepressant effects without stimulating motor function (Fig. 1).

*3.4. Effects of CMEO on brain nitrite level*

Nitrite concentrations in the brains of mice were markedly increased following LPS administration relative to the control group (95 % CI: - 47.11 to - 26.22; $P < 0.001$). CMEO treatment in both doses decreased the level of nitrite in the brains of mice (95 % CI: 14.57 to 35.47 (for dose 50 mg/kg); 95 % CI: 17.03 to 37.92 (for dose 100 mg/kg); $P < 0.001$), indicating that CMEO reduced stress (Fig. 1).

*3.4. Effects of CMEO on hippocampal BDNF and p-CREB level*

Immunohistochemical techniques were used.to assess the protein expression of BDNF and phosphorylated CREB (p-CREB) in the mouse hippocampus. The results showed that the intraperitoneal injection of lipopolysaccharide significantly decreased the protein expression levels of BDNF and p-CREB in the hippocampus of mice compared to the control group (95 % CI: 0.2201 to 0.6104; $P < 0.001$, 95 % CI: 0.3641 to 0.7287; $P < 0.001$, respectively). Treatment with both doses of CMEO (50 or 100 mg/kg) significantly reversed these changes (95 % CI: 0.06466 to 0.2729; $P < 0.01$, 95 % CI: -0.3687 to -0.004127; $P < 0.05$ both for 50 mg/kg, 95 % CI: 0.02966 to 0.2354; $P < 0.05$, 95 % CI: -0.3852 to -0.02066; $P < 0.05$, respectively both for 100 mg/kg) (Fig. 2-3).

**Table 1 .**Gas chromatography-mass spectrometry (GC-MS) Analysis

| NO. | RT (MIN) | % | COMPONENTS | KI | TYPE |
|---|---|---|---|---|---|
| **1** | 11.32 | 0.63 | $\alpha$-Pinene | 930 | MH |
| **2** | 14.22 | 1.79 | Myrcene | 991 | MH |
| **3** | 16.16 | 0.12 | $\alpha$-Phellandrene | 1002 | MH |
| **4** | 16.45 | 93.96 | Limonene | 1029 | MH |
| **5** | 20.11 | 0.41 | Terpinolene | 1089 | MH |
| **6** | 25.08 | 0.20 | $\alpha$-Terpineol | 1189 | MO |
| **7** | 28.52 | 0.13 | Nerol | 1230 | MO |
| **8** | 33.23 | 0.28 | Geranyl acetate | 1381 | MO |
| **9** | 35.45 | 0.62 | $\alpha$-cis Bergamotene | 1413 | SH |
| **10** | 38.63 | 1.04 | $\beta$-Bisabolene | 1506 | SH |

MH: Monoterpene Hydrocarbons; MO: Oxygenated Monoterpenes; SH: Sesquiterpene Hydrocarbons; SO: Oxygenated Sesquiterpenes; RT: Retention Time (minutes); KI: Kovats Index

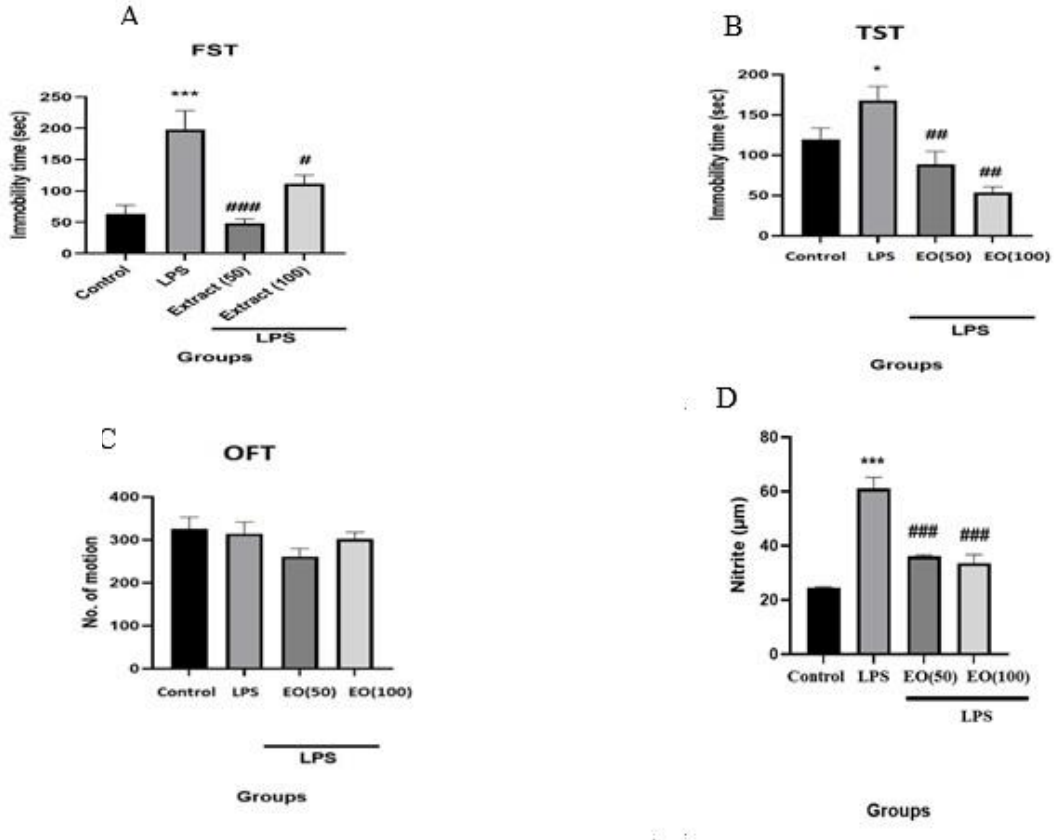


**Fig. 1.** Effects of *C. medica* essential oil (CMEO) on the Immobility Time in FST (A) and TST (B) (N = 7), Effects of CMEO on locomotor activity in OFT (C), Effects of CMEO on brain nitrite level (D) (N = 3).

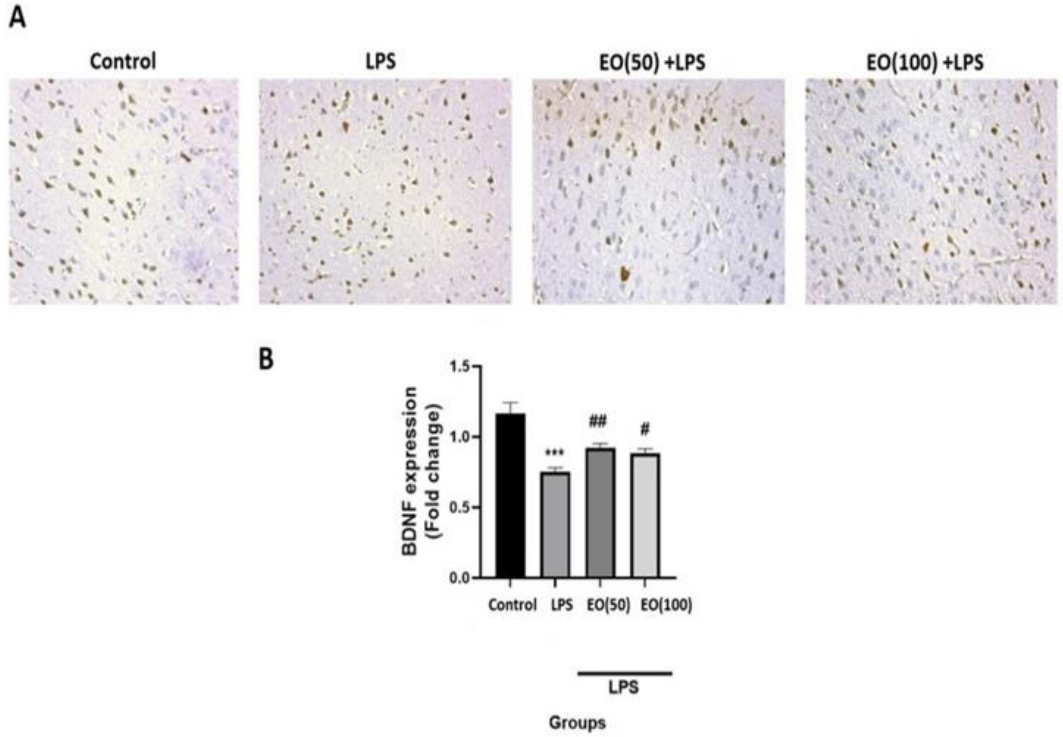


**Fig. 2.** Effects of *C. medica* essential oil (CMEO) on hippocampal BDNF levels (N = 4).

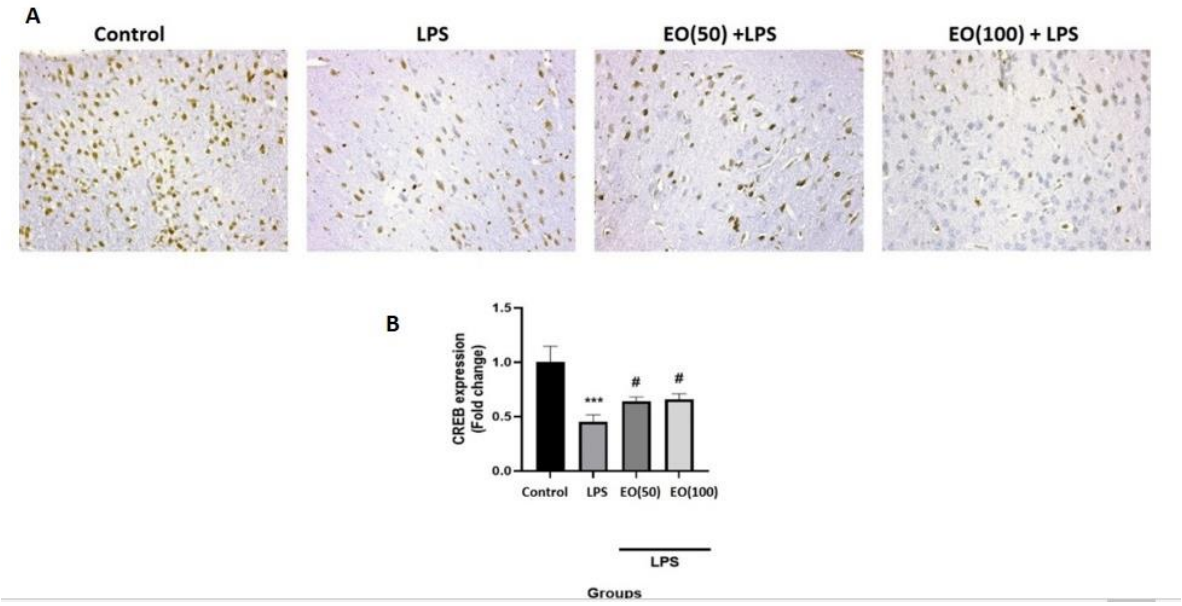


**Fig. 3.** Effects of *C. medica* essential oil (CMEO) on hippocampal CREB levels (N = 4)

## 4. Discussion

In this study, *C. medica* (CM) essential oil at doses of 50 and 100 mg/kg significantly reduced immobility time in both the FST and TST, indicating an improvement in depressive-like behavior in mice compared to the LPS-treated group.

Numerous studies have underscored the pivotal role of the BDNF and CREB signaling pathways in mediating the behavioral effects of antidepressant treatments. BDNF is essential for synaptic plasticity, neurogenesis, and neuronal survival, processes that are often disrupted in depression. Increased BDNF expression in the hippocampus has been consistently linked to reduced depressive-like behaviours in various animal models [12, 48].

CREB, a key transcription factor, regulates the expression of BDNF and other genes involved in neuronal growth and plasticity. Activation (phosphorylation) of CREB leads to increased transcription of BDNF, which in turn supports neuronal resilience and synaptic connectivity [49]. Therefore, the increase in p-CREB and BDNF levels observed in this study likely contributes to the improvement in behavioural outcomes (reduced immobility in the FST and TST) by increasing neuroplasticity in the hippocampus.

Furthermore, inflammatory processes induced by LPS administration reduce BDNF levels and disrupt CREB activity, leading to depressive-like behaviours. By restoring BDNF and p-CREB expression, CM essential oil may counteract the neuroplasticity deficits caused by inflammation. This mechanism is consistent with the observed decrease in brain nitric oxide levels, which is known to interfere with neurotrophic signalling [50].

Taken together, these molecular changes provide a neurobiological basis for the antidepressant-like effects of CM essential oil, linking biochemical modulation with behavioural improvement.

“Depression is a serious psychiatric disorder that negatively impacts patients’ health and quality of life, leading to physical, social, and psychological impairments [51]. While conventional antidepressants can effectively treat depression, they also have drawbacks. These medications often cause unwanted side effects, including nausea, drowsiness, weight gain, and sexual dysfunction, which can seriously affect a person's quality of life. In addition, abruptly stopping antidepressants can result in withdrawal symptoms. [52]. The utilization of medicinal plants presents a promising natural approach that may offer a safer alternative for the treatment of depression.

Administration of LPS as a depression model in rodents causes an overproduction of inflammatory mediators, such as NO, cytokines, free radicals, and oxidative stress [53] as well as a decrease in neurotrophins (such as BDNF and VEGF) [54] in the hippocampus and prefrontal cortex [55].

In 1997, Duman et al. proposed the "neurotrophin hypothesis of depression," which states that a lack of neurotrophic support causes depression. According to this hypothesis, antidepressant treatments may alleviate depressive symptoms by increasing the levels of neurotrophins such as NGF and BDNF. One of the most abundant neurotrophic factors in the brain, which has been the focus of numerous studies, is BDNF [56]. Based on previous studies, chronic antidepressant treatment may also upregulate the transcription factor "CREB" in the hippocampus [57]. BDNF and CREB are both critical factors in the development of depression. BDNF is a protein that promotes the survival and growth of neurons, as well as the development of neuronal connections in the brain.

On the other hand, CREB functions as a transcription factor regulating gene expression critical for neuronal growth and plasticity. The relationship between CREB and BDNF is bidirectional. CREB can influence the level of BDNF expression, and CREB activity can be regulated by BDNF [58]. Reduced expression of

brain-derived neurotrophic factor (BDNF) in the hippocampus and prefrontal cortex (PFC) in response to diverse stressors constitutes a critical factor underlying the pathophysiology of depression. Several studies have reported that the increase in various growth factors, such as BDNF, VEGF, and VGF, in the hippocampus of rodents after treatment with antidepressants is likely mediated through the actions of CREB or other transcriptional regulators. As a result, the antidepressant treatment affects neurogenesis [13, 59]. Thus, according to this mechanism, a potential treatment for depression is a medication that restores BDNF levels and promotes CREB activity.

Citrus fruits are rich in bioactive compounds, including vitamin C and flavonoids, which have been reported to exert positive effects on mental health. The antioxidant and anti-inflammatory properties of these compounds may help reduce symptoms of depression [29]. Studies show that citrus fruits might possess antidepressant effects through mechanisms such as the reduction of inflammatory mediators and the upregulation of neurotrophic factors. Limonene ($C_{10}H_{16}$) is one of the most abundant natural terpenes and constitutes the main component of peel oils in citrus fruits, including lemons, oranges, tangerines, and grapefruits [60]. D-limonene reduces oxidative stress and inflammation[61]. GC-MS analysis of CM essential oil showed that the most abundant compound was limonene (93.96 %). Zhang et al. reported that the inhalation of *C. sinensis* (L.) Osbeck essential oil, as well as its primary constituent limonene, markedly ameliorated depressive behaviors induced by chronic unpredictable mild stress (CUMS). Limonene modulates HPA-axis hyperactivity, decreases monoamine neurotransmitter levels, and downregulates BDNF expression in the hippocampus [62].

The potential antidepressant-like activity of *C. reticulata* essential oil was examined in FST and TST in mice with reserpine-induced depression. Inhalation of *C. reticulata* essential oil led to a reduction in immobility duration across the behavioral tests. It has been noted that the essential oil of *C. reticulata* increases the expression levels of BDNF, glucocorticoid receptors, and 5-hydroxytryptamine1A receptors in the brain tissue of mice treated with reserpine. Based on GC-MS analysis of *C. reticulata* essential oil, the main component was d-limonene [63]. Furthermore, the results of Lopes Campêlo's study have shown that the essential oil of citrus limon (Burn) leaves, when administered in doses of 50, 100, and 150 mg/kg, has significant antidepressant effects in behavioural tests. The GC-MS analysis of *C. limon* essential oil revealed that the predominant constituents were limonene (52.77 %), geranyl acetate (9.92 %), and trans-limonene oxide (7.13 %). [64].

Kim et al. studied the effect of *C. medica* essential oil on LPS-stimulated inflammation in a murine macrophage cell line, finding that it could relieve inflammation by blocking the JNK, ERK, and NF-κB signalling pathways. It has the capacity to markedly diminish the levels of inflammatory mediators, including tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), interleukin-1 beta (IL-1β), nitric oxide (NO), and prostaglandin E2 (PGE2) [65]. Another study by Keyvanara et al. has demonstrated the anti-inflammatory effects of Hydroalcoholic Extracts of *C. medica* on a rat model of Acute Colitis by down-regulating myeloperoxidase (MPO) activity and malondialdehyde (MDA) levels in the colonic tissue [66]. To more clearly establish limonene as the principal active constituent underlying the antidepressant effects of *C. medica* essential oil, future research should evaluate the isolated effects

of pure limonene in appropriate depression models. Such research will help clarify whether the observed behavioural and molecular changes are primarily related to limonene or the synergistic action of multiple components present in the essential oil.

Immunohistochemical analysis revealed that treatment with *C. medica* essential oil at dosages of 50 and 100 mg/kg significantly elevated the expression levels of BDNF and p-CREB factors in comparison to the group administered LPS exclusively. Furthermore, several studies have shown that elevated plasma nitrate levels—reflecting increased nitric oxide production—may contribute to the pathophysiology of depression The CM essential oil also reduced levels of nitric oxide in the brains of mice compared to the group treated with LPS. In another study, limonene reduced inflammatory mediators and nitrite levels in the brain of a mouse model of depression [67].

Indeed, we have once again demonstrated that mice treated with LPS display depressive behaviour and exhibit pathological changes commonly associated with depression. Depression, a chronic disease, requires long-term medication. However, associations have been found between first-line antidepressants and adverse events, which can complicate patient compliance.

The limitations of this study include the need for further research to establish the generalizability of the findings beyond mice to other animal models or humans. In addition, this study may have limitations regarding the translation of results from animal models to clinical applications, underscoring the need for human trials to confirm the efficacy and safety of citrus essential oil for treating depression. Additionally, this study may not have information about potential side effects, optimal dosages, and long-term effects of using *C. medica* (Rutaceae) essential oil as an antidepressant.

Furthermore, it is essential to acknowledge the limitations of translating results from animal models to clinical settings. Although the LPS-induced depression model in mice is widely accepted and provides valuable insights into the pathophysiology associated with inflammation, it only mimics certain aspects of human depression. It may not fully reflect its multifactorial nature. In addition, there are significant physiological, metabolic, and neurochemical differences between rodents and humans that could affect both the pharmacodynamics and pharmacokinetics of plant compounds such as *C. medica* essential oil. Effective doses in mice may not be directly translatable to humans due to variations in metabolism, bioavailability, and compound interactions. Furthermore, the controlled laboratory environment is highly variable, with complex psychosocial and environmental factors that influence depression in humans. Consequently, although our results indicate a neurobiological mechanism that may underlie the antidepressant-like effects of CMEO, additional preclinical investigations using diverse animal models, along with rigorously designed clinical trials, are required to confirm its efficacy, safety profile, optimal dosage, and therapeutic potential in human subjects.

## 5. Conclusion

Overall, the results of this study indicate that administration of *C. medica* essential oil alleviated depression-like behaviors in mice. These effects appear to be associated with enhanced hippocampal expression of BDNF and phosphorylated CREB, along with attenuation of inflammatory processes, as evidenced by reduced nitric oxide levels in the brain tissue of LPS-treated animals. The main component of

*C. medica* essential oil, limonene, may be involved in this process. However, understanding the mechanisms of CMEO antidepressant activity requires further research on changes in cellular and molecular parameters.

**Author contributions**

N.K. designed the study, conducted the experiments, and analyzed the data. S.S. contributed to the methodology and behavioural assessments. M.T. conducted the statistical analyses and assisted in interpreting the results. M.H.F. supervised the project, provided critical revisions, and contributed to the final manuscript preparation. All authors reviewed and approved the final version of the manuscript.

**Conflicts of interest**

The authors declare no conflict of interest.

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