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RESEARCH ARTICLE

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Synergistic effects of *Nepeta menthoides* and *Melissa officinalis* aqueous extracts on reserpine-induced depressive-like behaviors in mice

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Abstract

Nepeta menthoides Boiss. & Buhse and *Melissa officinalis* are extensively used in Persian medicine for the treatment of depression. Considering the active ingredients and main phenolic compounds of these plants and possible synergistic effects, this study examined the antidepressant and antioxidant activities of aqueous extract of *N. menthoides* (NM) and *M. officinalis* (MO) in reserpinized mice alone and combination. Mice were pretreated orally for 1-week with normal saline (10 ml/kg), fluoxetine (20 mg/kg), imipramine (10 mg/kg), NM (50–100–200–400 mg/kg), MO (150–350–550–750 mg/kg), and combination (NM 50 with MO 150 mg/kg). The behavioral changes were evaluated using forced swim, tail suspension, and open field tests, 24 hr after reserpine injection (4 mg/kg) on eighth day. The amounts of active components in the extracts and catalase (CAT) as a brain oxidative stress were measured with enzyme-linked immunosorbent assay. Data showed that this combination produced a synergistic action on behaviors and a significant increase in CAT activity. High-performance liquid chromatography results showed that rosmarinic acid contents in MO and NM were 6.42 ± 1.1 and 11.03 ± 2.16 mg/g of dried extract, respectively. Total flavonoid and phenolic contents of MO were higher than NM. The findings suggest that the present combination produces an antidepressant-like effect, which is possibly triggered by its antioxidant properties.

KEYWORDS
antidepressant effects, catalase activity, *Melissa officinalis*, *Nepeta menthoides*, Persian medicine, pharmacological synergy

1 | INTRODUCTION

Depression will be the most prevalent and pernicious disease in the world as per predictions by 2030 (Dzhavadian, 2020). Recent studies in the United States have shown that the pandemic of coronavirus of 2019 has reduced the age of depression and mental illness, and young people are more prone to depression (Daly, Sutin, & Robinson, 2021).

Abbreviations: CAT, catalase; DTNB, 5,5′-dithio-bis-2-nitro benzoic acid; ELISA, enzyme-linked immunosorbent assay; FLX, fluoxetine; FST, forced swim test; GABA, gamma-aminobutyric acid; HPLC, high-performance liquid chromatography; i.p., intraperitoneally; IMP, imipramine; MO, *Melissa officinalis* aqueous extract; NM, *Nepeta menthoides* aqueous extract; NS, normal saline; OFT, open field test; OS, oxidative stress; p.O., per orally; PM, Persian medicine; RA, rosmarinic acid; RES, reserpine; SSRI, selective serotonin reuptake inhibitors; TCA, tricyclic antidepressant; TST, tail suspension test; VMAT, the vesicular monoamine transporter.

Despite treatment with different antidepressants, one-third of the patients still suffer from treatment-resistant depression, while some of the others suffer from chronic side effects (Beardslee, Solantaus, Morgan, Gladstone, & Kowalenko, 2012). Three main monoamine neurotransmitters; serotonin, norepinephrine, and dopamine are very important in the mechanism of antidepressant medications currently used (Zheng, Fan, Shi, & Liu, 2013). Nevertheless, it seems depression is an illness with different etiopathogenesis, which remains to be fully elucidated. Therefore, the emergence of new drugs with mitigated side effects and different mechanisms may be promising therapeutic strategies (Yan et al., 2021).

Recent studies have shown that a high level of oxidative stress (OS) in the brain plays a vital role in triggering mood disorders such as depression (Feng et al., 2020). Medicinal plants with high amounts of antioxidants such as flavonoid components and rosmarinic acid (RA) develop new potent medicines with fewer side effects and more therapeutic efficacy (Rahbardar & Hosseinzadeh, 2020) (Figure S1). Synergistic combinations can increase the number of selective therapies using the current pharmacopeia and offer opportunities for more precise control of biological systems. Recent observations indicated that a combination of medicinal plant extracts changed the prescription pattern of antidepressant drugs (Keck et al., 2020).

Persian medicine (PM), as one of the complementary and integrative medical systems, provided helpful insights into the prevention and treatment of diseases; some of them have been demonstrated in recent research (Naseri et al., 2021). PM introduced various remedies in combination therapy for reducing the side effects of drugs and increasing their therapeutic effectiveness, which may be helpful in the management and control of depression (Araj-Khodaei et al., 2020; Jalali, Firouzabadi, & Zarshenas, 2021). *Nepeta menthoides* and *Melissa officinalis* are two of the best-known herbs for the treatment of depressive-like disorders in PM. *N. menthoides* and *M. officinalis* belong to the Lamiaceae family, which its genus has the highest amount of RA (Memariani, Rahimi, Farzaei, & Nejad, 2019; Shakeri, Sahebkar, & Javadi, 2016). Previous in vitro data suggest that RA inhibits neuronal cell death and possesses a neuroprotective impact, most probably by decreasing OS (Bhatt, Nagappa, & Patil, 2020). *N. menthoides* commonly grow in the northwest of Iran and *M. officinalis* is widely cultivated in Central Asia, Mediterranean regions, Europe, the United States, and the north of Iran (Memariani et al., 2019; Shakeri et al., 2016).

Several studies have shown the therapeutic effects of hydroalcoholic extracts of *N. menthoides* and *M. officinalis*, in mood disorders. In a few studies, the different doses of aqueous extracts of these medicinal plants have been investigated for the treatment of depression as a drug combination therapy (Memariani et al., 2019; Shakeri et al., 2016). Other investigators have shown that the combination of *N. menthoides* and *M. officinalis* improved the depression and anxiety in insomniacs; however, it is still not known whether the combination of aqueous extract of these two herbs is more effective in the treatment of depression compared to the effects of their extracts separately (Ranjbar et al., 2018). Dastmalchi et al. (2008) reported that *M. officinalis* contained phenolic and flavonoid compounds such as RA, but there are currently very limited studies investigating *N. menthoides* extract for the actual amount of these components. However, the effect of their combination on brain OS, such as catalase (CAT) activity in a mouse model of depression, still needs more research.

Based on the prioritization of herbal ingredients in PM and what we have found in our previous preclinical and clinical studies, these two medicinal plants may be more effective than commonly prescribed antidepressant drugs and show fewer side effects. We know that sufficient studies are needed to produce strong evidence regarding this hypothesis. It seems that these two plants in combination have good synergistic effects in controlling depression (Araj-Khodaei et al., 2020; Mozaffarpur et al., 2020).

To find differences in pathways between *N. menthoides* aqueous extract (NM) and *M. officinalis* aqueous extract (MO), different doses of these two plants, were compared with two different common antidepressant drugs. Fluoxetine (FLX), as a selective serotonin reuptake inhibitor (SSRI) and Imipramine (IMP), a tricyclic antidepressant (TCA) drug, are known to be effective for the treatment of depression. They are commonly used as monoaminergic antidepressants in preclinical screening as a positive control group for antidepressant activity. On the other hand, by predicting the high amounts of flavonoid and RA components in MO and NM, we decided to compare the antidepressant activity of the plant extracts with FLX and IMP. It has been suggested that antioxidants such as flavonoids restore the levels of monoamines in the brain either by inhibiting monoamine oxidase or by inhibiting the reuptake of these neurotransmitters (Can, Özkay, & Üçel, 2013). This study aimed to investigate the synergistic effect of combination therapy with lower doses of *N. menthoides* and *M. officinalis* on depressive-like behavior in mice. The content of chemical constitution including RA, flavonoid, and phenolic contents as well as antioxidant activity was also studied.

2 | MATERIALS AND METHODS

2.1 | Preparation of *N. menthoides* and *M. officinalis* aqueous extract

Dried aerial parts of *N. menthoides* and *M. officinalis* were purchased from Firoozeh botanical garden from Tehran, Iran. The samples of the plants were authenticated at Herbarium of Faculty of Pharmacy, Tehran University of Medical Sciences under the voucher specimen number PMP-1344 for *N. menthoides* and PMP-1343 for *M. officinalis*. Based on the method described in PM pharmacological, the aqueous extract of *N. menthoides* and *M. officinalis* was obtained by soaking 250 g of dried aerial part of the plants in 3 L hot water and heated at 60°C for 1 hr. The obtained extract was then filtered with Whatman paper No. 1, condensed, and dried (Tonekaboni, 2007).

2.2 | Measurement of active plant ingredients by high-performance liquid chromatography

High-performance liquid chromatography (HPLC) analysis for the phenolic acid compound of RA in the samples of NM and MO was

performed using a smart line HPLC instrument (Knauer, Germany) equipped with reverse phase C18 Eurospher-100 (5 μm particle, 125 mm × 4 mm) and a quaternary pump and a ultraviolet-visible detector (D-14163 model). The chromatographic data were processed using chrome gate software (version 3.1). Chemicals and standards all the analytical and HPLC-grade solvents were supplied from Merck Chemical Co. Ltd. (Darmstadt, Germany). RA standard was purchased from Aldrich and Fluka. Samples and standard solutions were filtered through 0.45 μm hydrophilic polytetrafluoroethylene membrane filters before injection. The sample injection volume was 20 μl and each sample was injected in triplicate. The solvent system was 0.1% (vol/vol) phosphoric acid in water (solvent A) and acetonitrile (solvent B). The flow rate used for column elution was 1 ml/min and peaks were monitored by UV detection at 280 nm. Identification of the RA in the chromatograms was performed by comparison of the RA retention time with a reference standard (Brera, Grossi, De Santis, & Miraglia, 2003). Determination of RA content was detected using the corresponding calibration curve with $y = 41{,}606x$ and $R^2 = 0.9889$ and expressed as mg/g dried extract.

2.3 | The Folin–Ciocalteu method for total phenol and flavonoids content

Total phenol contents of MO and NM were detected using spectrophotometry method, Folin–Ciocalteu's phenol reagent, and gallic acid (GA) as standard. The absorbance was measured at 765 nm and the concentration of phenolics was read from the GA calibration curve. The content of phenolics in extracts was calculated per mg equivalent of GA/g of the extract. The total flavonoid content of the extract was measured by the aluminum chloride colorimetric method. Catechin was used as standard and the absorbance was measured at 415 nm and the concentration of total flavonoid was calculated from the calibration curve and read from the GA calibration plot. The concentration of total flavonoid content in the test samples was plotted and expressed as mg Catechin equivalent (cat)/g extract. All of the tests were carried out in triplicate (Araj-Khodaei et al., 2020).

2.4 | Animals

Male NMRI mice (an outbred strain of albino mice, $n = 78$, weighting: 20–25 g) were purchased from the Razi Research Institute of Karaj. The mice were randomized into weight-balanced groups of six animals in each of them, housed in standard cages in a temperature-controlled room (21–25°C) under 12 hr of light and 12 hr of darkness cycles and 45–55% humidity with free access to water and food, for 1 week before the experiment. Animal care and handling were conducted in accordance with the guidelines of the NIH Animal Care and Use Committee (Council, 2010). The study protocol was reviewed and approved by the Animal care and ethical committee of Shahed University with code No. IR.SHAHED.REC.1398.065, 2019.

2.5 | Experimental protocol

The mice were randomly divided into 12 groups and received treatments for 7 days. Standard control groups were given FLX (20 mg/kg; Parsdarou Co, Iran) and IMP (10 mg/kg; Abidipharma Co, Iran) by intragastric gavage (Ozerov, Bagmetova, Chernysheva, & Tyurenkov, 2016). In PM, both NM and MO plants are used in decoction form. Therefore, we used the oral gavage method which allowed us a similar administration method, precise control over the dosage and timing of the plants' extract administration. Treatment groups were orally given NM 50, 100, 200, and 400 mg/kg and MO 150, 350, 550, and 750 mg/kg and a combination of NM 50 mg/kg and MO 150 mg/kg based on previous reports (Memariani et al., 2019; Shakeri et al., 2016). The measured LD_{50} was the prescribed dose criterion before the intervention. NM 50 mg/kg and MO 150 mg/kg were median effective doses (ED50), which had a specific effect on 50% of the treatment groups in the present study (Dimmitt, Stampfer, & Martin, 2017). Reserpine group (RES), as a positive control, was given normal saline (10 ml/kg) in oral administration (p.o.) during those 7 days (Tian et al., 2010). All treatment and control groups were injected intraperitoneally (i.p.) with RES (4 mg/kg, Sigma–Aldrich Co), 2 hr after receiving the last dose of drugs on the seventh day (Khurana & Bansal, 2019). On the eighth day, 24 hr after receiving RES, behavioral tests were performed. Each animal was submitted to a pre-training on the day before the test. Animals in the same groups were submitted to three behavioral tests 24 hr after receiving RES. The time interval between tests was 60 min for each animal. We have done behavioral tests on a saline control group (NS) treated only with normal saline 10 ml/kg (p.o.), at the beginning of the study. Control groups were also considered as a comparison group to isolate the independent variables in the experimental groups (Tian et al., 2010). The drugs were prepared fresh daily for oral treatment of the animal at room temperature. For each animal, 0.3 ml solution was prepared using a mixture of normal saline 0.9% and extract powder or FLX and IMP in accordance with the desired dose. RES was also dissolved in normal saline 0.9% for intraperitoneal administration (0.25 cc) (Ghazizadeh et al., 2020). The experimental framework is shown in Figure 1.

2.6 | Forced swim test

At 24 hr after RES treatment, the mice ($n = 6$ in each group) were gently placed individually into a cylindrical container made of glass (height 30 cm and diameter 16 cm) filled with 25 cm of 25 ± 2°C water and were forced to swim for 6 min. The total time of passive floating in the last 5 min of the test duration was recorded as immobility time, which is an indication of depressive behavior in the forced swim test (FST). The animals were dried with a towel, and the water was changed between each test. According to the previous day, animals were immersed in water for 15 min to get acquainted with the experiment (Porsolt, Le Pichon, & Jalfre, 1977). The animals were used only once in this test.

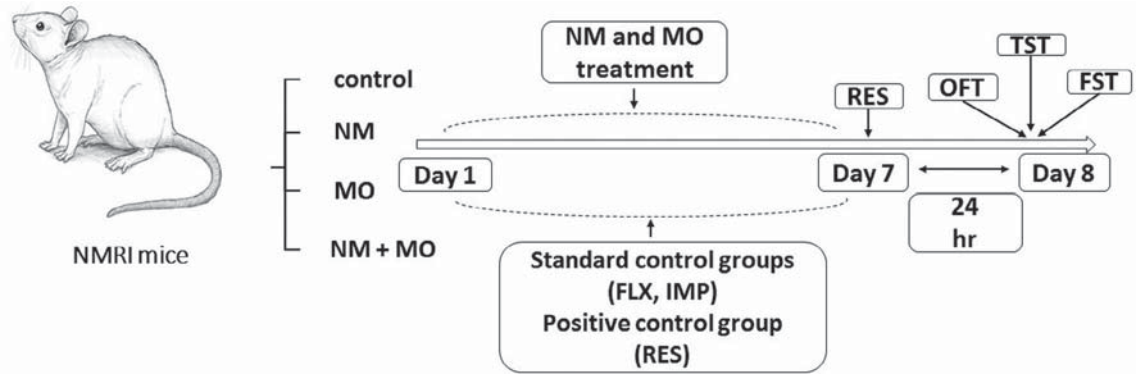


FIGURE 1 The experimental framework of animal treatments and behavioral tests. The mice (NMRI) were orally administered with aqueous extracts of *Nepeta menthoides* (NM 50–400 mg/kg), *Melissa officinalis* (MO 150–750 mg/kg), fluoxetine (FLX 20 mg/kg), imipramine (IMP 10 mg/kg), and normal saline (10 ml/kg) for 1 week prior to intraperitoneal injection with reserpine (RES, 4 mg/kg). FST, forced swim test; OFT, open field test; TST, tail suspension test

2.7 | Tail suspension test

The tail suspension test (TST) was conducted 24 hr after RES treatment. Mice ($n = 6$ in each group) were placed in a light and sound isolated room and suspended 40 cm above the floor using a tape placed approximately 2.5 cm from the end of the tail for 6 min. Immobility time was recorded during the last 5 min of the test duration whenever the mice hung passively. The longer immobility periods in TST are considered a sign of depression in the animals (Salehi-Sadaghiani et al., 2012). The animals were used only once in this test.

2.8 | Open field test

Open field test (OFT) was performed in a square-shaped open box (40 cm × 40 cm × 40 cm) with 16 small squares. The animals were gently placed into one of the corners and allowed to explore the open field freely for 5 min. The box was thoroughly cleaned with 70% alcohol between every animal. The total number of squares crossed (passing and entering another square with all four paws) was manually recorded by two trained observers as an indicator of locomotor activity. To dissociate the impact of locomotor capacity on the immobility time in the FST and TST, mice treated with drugs for 7 days, were tested in the OFT in our study (Porsolt et al., 1977).

2.9 | Determination of median lethal dose

To determine LD50 of NM and MO, 90 mice were randomly divided into nine groups ($N = 10$). The doses 250, 500, 1,000, 2,000, and 4,000 mg/kg of NM and 750, 1,500, 3,000, and 6,000 mg/kg MO were given to the animals via oral gavage in a single dose. They were observed for mortality up to 48 hr (Kpemissi et al., 2020). The measured LD_{50} was the prescribed dose criterion before the intervention.

2.10 | Enzyme-linked immunosorbent assay

After behavioral tests, the animals were anesthetized with ketamine (100 mg/kg, i.p.) and perfused through the ascending aorta with 50 ml of heparinized normal saline. Following the perfusion, the brains were removed from the skull and isolated, blotted dry, weighed, and made into a 10% tissue homogenate in cold normal saline containing protease inhibitor cocktail. After cooling, the samples were centrifuged at 5,000 rpm for 10 min at 40°C and supernatant was aliquoted and stored at −70°C until assayed (Deng et al., 2011).

For CAT activity, hydrogen peroxide (H_2O_2) was added to a mixture, which contained supernatant and potassium phosphate buffer (50 mM, pH 7.0). Then, the rate of H_2O_2 decomposition was assessed by recording the changes of absorbance at 240 nm for 2 min. The final results were expressed in unit/mg protein. In addition, the protein content of the supernatant was measured using the Bradford protein assay with bovine serum albumin as the standard (Bradford, 1976).

2.11 | Statistical analysis

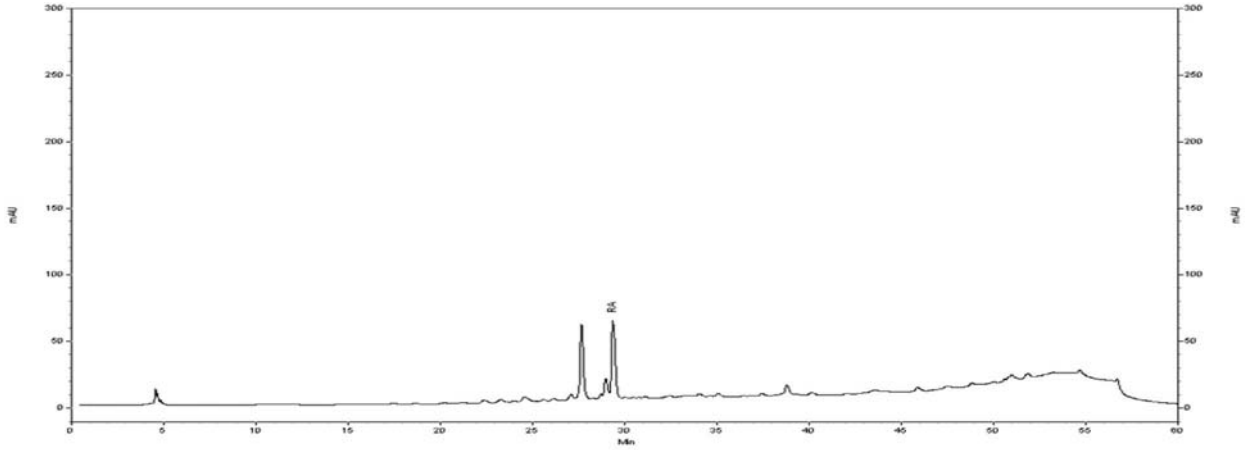
The data from these experiments were reported as mean ± standard error of mean (SEM) for each group. All statistical analyses were performed using GraphPad PRISM version 7.0. Inter-group differences were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test as a posttest to compare the group means if overall $p \leq 0.05$. Differences with a p-value of less than .5 were considered statistically significant.

3 | RESULTS

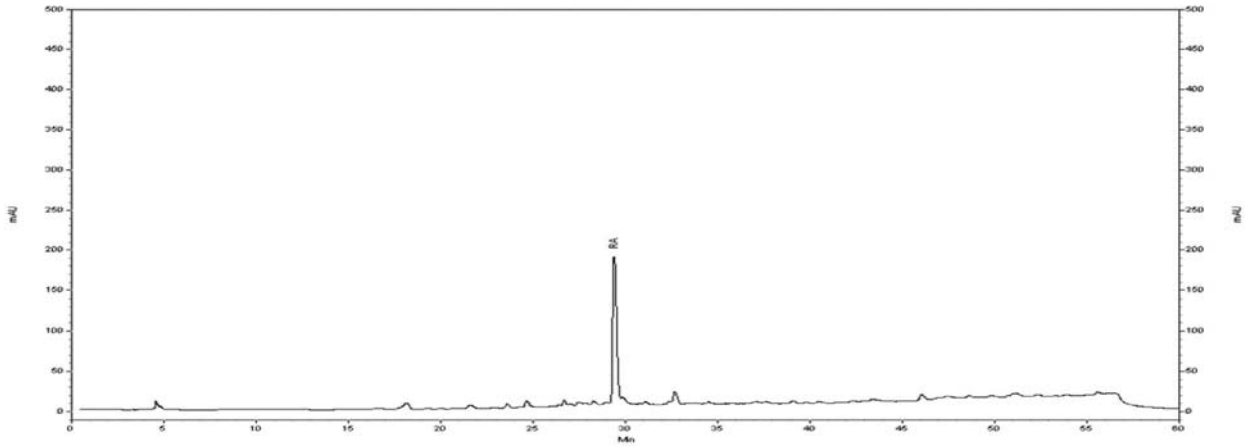
3.1 | Content of RA in *N. menthoides* and *M. officinalis* aqueous extract

HPLC analysis indicated that NM and MO contained RA. The content of RA in NM and MO, confirmed by comparison according to

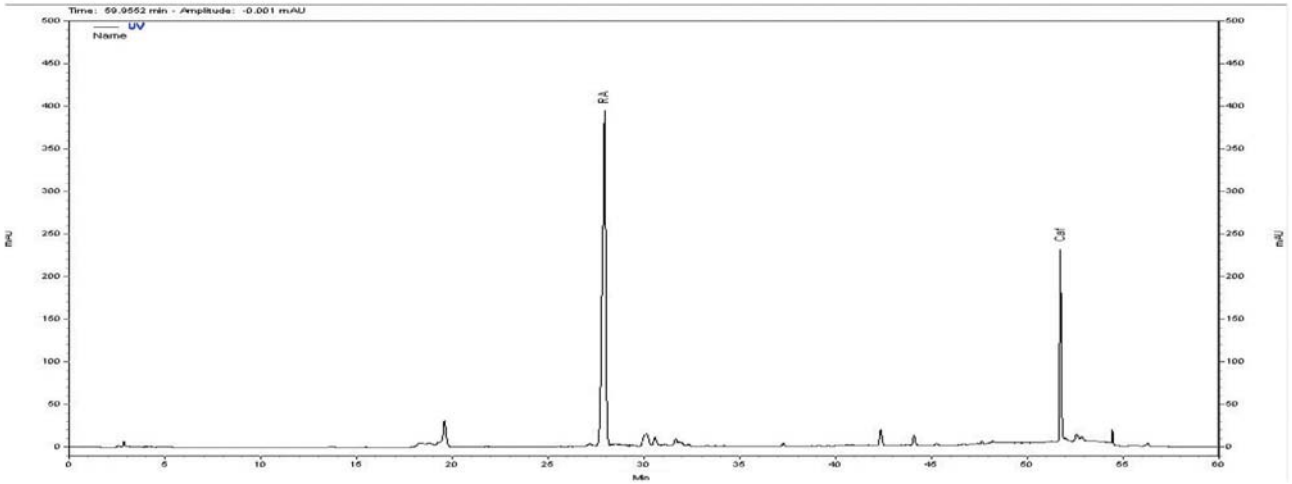
retention time and UV spectra, and HPLC chromatograms of standard RA, is shown in Figure 2. RA contents of NM was 11.02 ± 2.16 mg/g dried extract, which was higher than MO which was 6.42 ± 1.10 mg/g dried extract. Two peaks were found in the retention time of the NM chromatogram. The first peak had a retention time of ~27.183 min, whereas the second peak had a



(A) NM



(B) MO



(C)

FIGURE 2 HPLC chromatogram of rosmarinic acid in (A) *Nepeta menthoides* aqueous extract (NM), (B) *Melissa officinalis* aqueous extract (MO), and (C) HPLC chromatograms of standard rosmarinic acid. HPLC, high-performance liquid chromatography

Test name	NM	MO	Unit
Total flavonoid content	64.18 ± 1.02	346.71 ± 50.76	mg Catechin/g extract
Total phenol content	83.11 ± 7.17	311.99 ± 33.34	mg Gallic acid/g extract

Abbreviations: MO, *Melissa officinalis* aqueous extract; NM, *Nepeta menthoides* aqueous extract.

TABLE 1 Level of total phenol and flavonoid contents of aqueous extract of *Nepeta menthoides* and *Melissa officinalis*

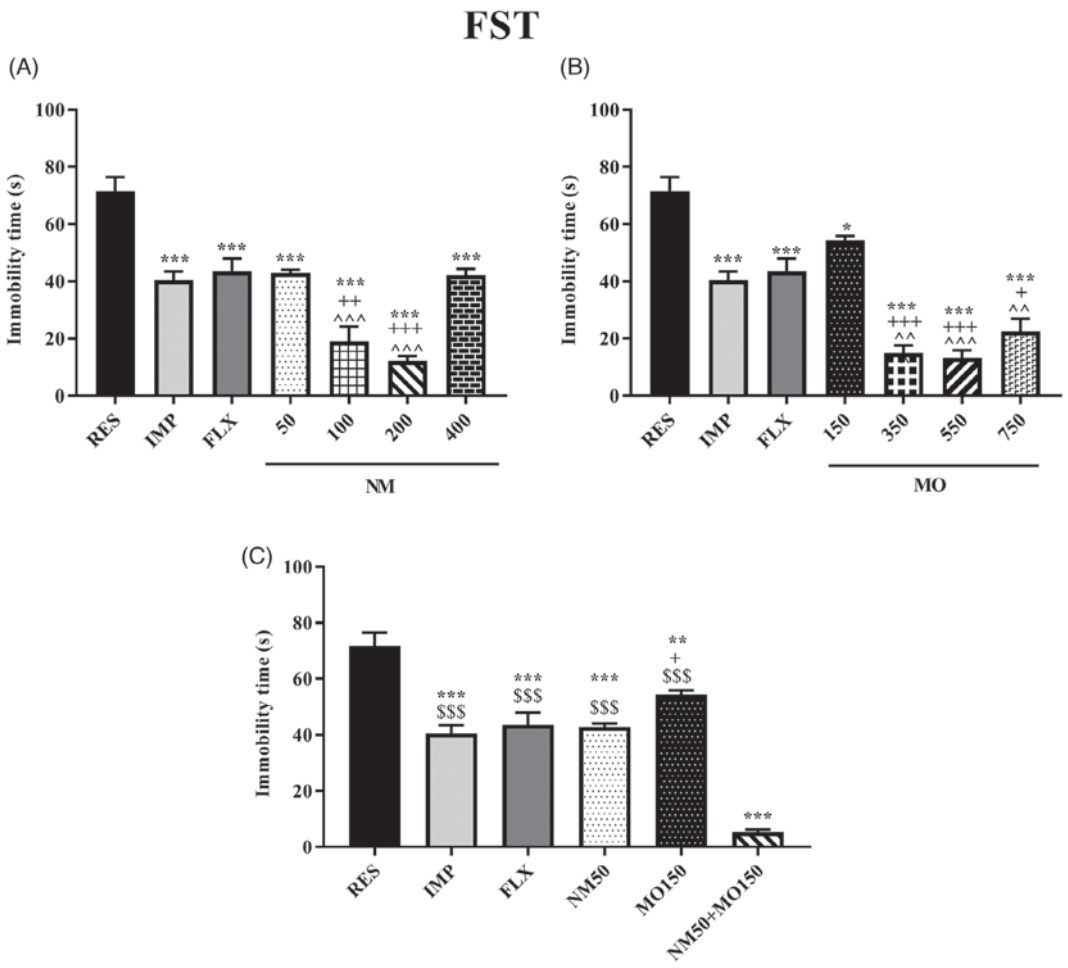


FIGURE 3 Effects of NM and MO alone and in a combination of NM with MO pretreatment on immobility time (s) in the FST. Each bar represents the mean response from 6 mice ± SEM. (A) Different doses of NM, (B) different doses of MO, (C) combination of NM with MO in comparison to doses of NM and MO. (*significant difference with reserpine, ^significant difference with fluoxetine, $^{+}$significant difference with Imipramine, $^{\$}$significant difference with a combination of NM with MO) (RES, reserpine 4 mg/kg; IMP, imipramine 10 mg/kg; FLX, fluoxetine 20 mg/kg; NM, *Nepeta menthoides* aqueous extract; MO, *Melissa officinalis* aqueous extract). $^{*,+}p < .05$. $^{++,\wedge\wedge}p < .01$. $^{***,+++,\wedge\wedge\wedge,\$\$\$}p < .001$

retention time of ~28.883 min. MO had one peak in retention time of ~28.933 min.

3.2 | Content of total phenolic and flavonoids of *N. menthoides* and *M. officinalis* aqueous extract

Total phenolic and flavonoids contents of NM and MO were determined as summarized in Table 1. Total phenol in MO was about four times more than NM (83.11 vs. 311.99) and flavonoid contents of MO were about five times more than NM (64.18 vs. 346.71).

3.3 | Behavioral assessments

3.3.1 | Effects of NM and MO and combination of NM and MO on immobility time in the FST

Treatment with all doses of NM (50–400 mg/kg) significantly reduced immobility time in FST compared to the RES group, which was somewhat dose-dependent ($p < .05$). The immobility time in RES ($n = 6$, 71.67 ± 4.821) was significantly higher than NS group ($n = 6$, 36 ± 2.62) at the beginning of the test ($p < .001$). Doses of 100 and 200 mg/kg NM, significantly induced a decrease in immobility time compared to

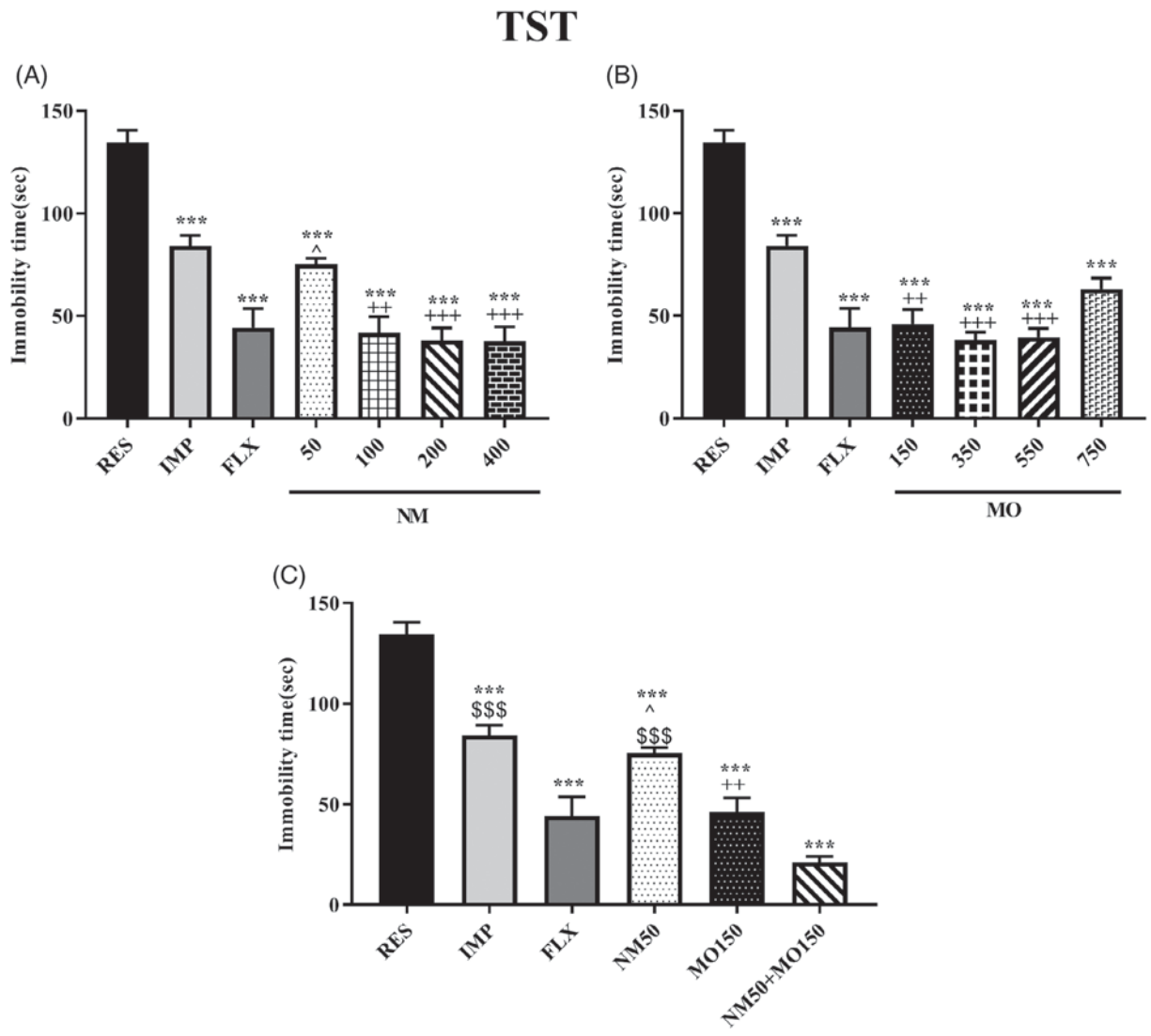


FIGURE 4 Effects of NM and MO alone and in a combination of NM with MO pretreatment on immobility time (seconds) in the TST. Each bar represents the mean response from 6 mice ± SEM. (A) Different doses of NM, (B) different doses of MO, (C) combination of NM with MO in comparison to doses of NM and MO (*significant difference with reserpine, ^significant difference with fluoxetine, $^{+}$significant difference with Imipramine, $^{\$}$significant difference with a combination of NM with MO) (RES, reserpine 4 mg/kg; IMP, imipramine 10 mg/kg; FLX, fluoxetine 20 mg/kg; NM, *Nepeta menthoides* aqueous extract; MO, *Melissa officinalis* aqueous extract). $^{\wedge}p < .05$. $^{++}p < .01$. $^{***,+++,\$\$\$}p < .001$

standard control groups (ANOVA, F [6, 35] = 29.45, $p < .001$, Figure 3A). A significant decrease in immobility time in FST was also observed in all doses of MO (150–750 mg/kg) in comparison to RES ($p < .05$). The reduction in immobility time in doses 350, 550, and 750 mg/kg MO was significant, compared to the FLX and IMP as standard drugs (ANOVA, F [6, 35] = 37.2, $p < .001$, Figure 3B). Combined treatment with NM 50 mg/kg with MO 150 mg/kg significantly decreased the immobility behavior in mice in comparison to each dose, alone and standard drugs (ANOVA, F [5, 30] = 50.73, $p < .001$, Figure 3C).

3.3.2 | Tail suspension test

Treatment with all doses of NM (50–400 mg/kg), significantly reduced immobility time in TST compared to the RES group ($p < .05$). The immobility time in RES (n = 6, 134.7 ± 5.89) was significantly higher than NS group (n = 6, 77 ± 9.94) at the beginning of the test ($p < .001$). Doses of 100 and 200 mg/kg, significantly induced a decrease in immobility time compared to the IMP (ANOVA, F [6, 35] = 29.58, $p < .001$, Figure 4A). A significant decrease in immobility time in TST was also observed in all doses of MO (150–750 mg/kg) in comparison to RES ($p < .05$). The reduction in immobility time in doses 350, 550, and 750 mg/kg MO, was significant compared to the IMP as a standard drug (ANOVA, F [6, 35] = 32.6, Figure 4B). Combined treatment with NM 50 mg/kg with MO 150 mg/kg significantly decreased the immobility behavior in mice in comparison to each dose, alone and IMP (ANOVA, F [5, 30] = 44.04, Figure 4C).

3.3.3 | Open field test

The results of OFT showed that all doses of NM (50–400 mg/kg) significantly increased the total number squares crossed compared to

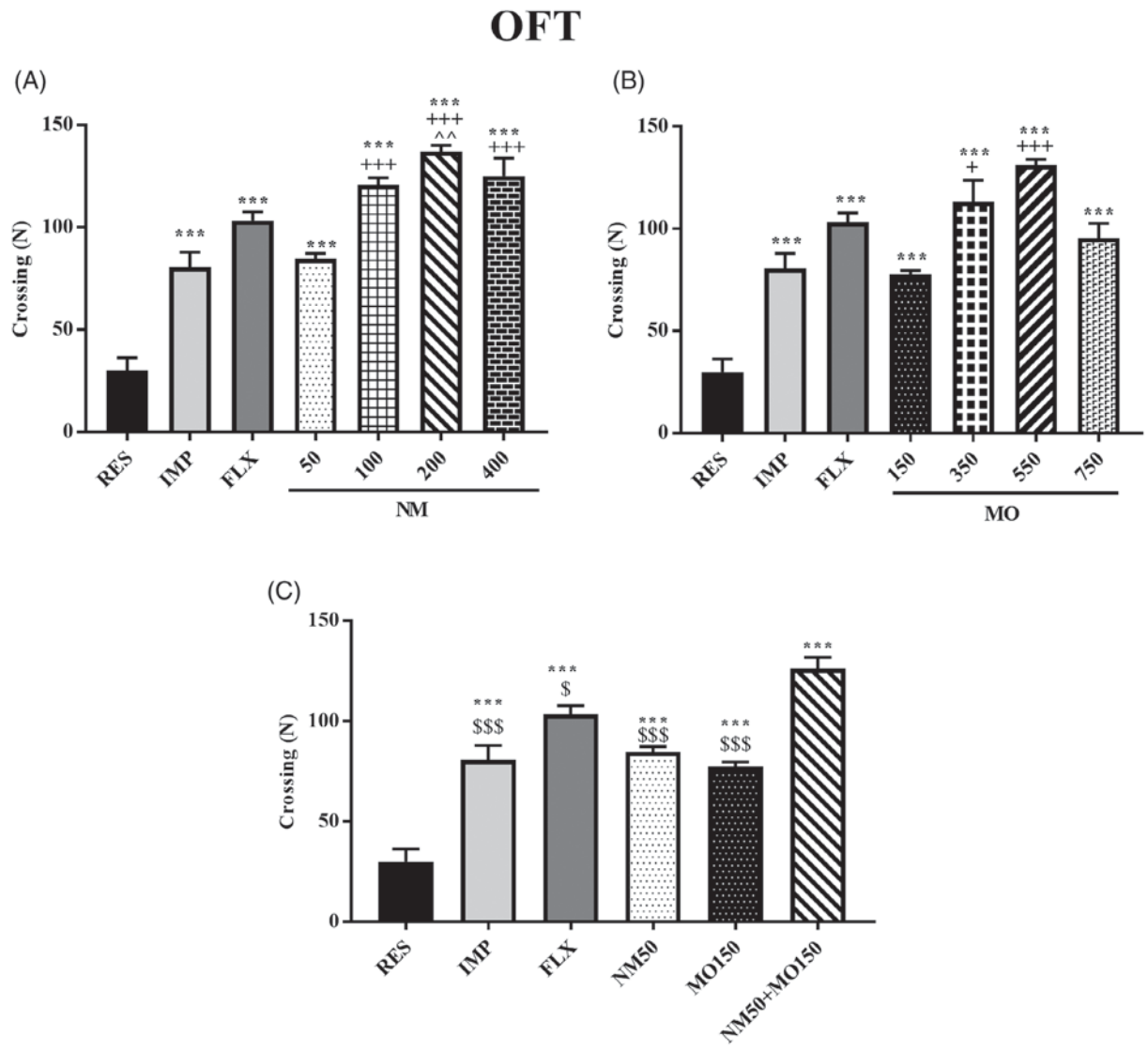


FIGURE 5 Effects of NM and MO alone and in a combination of NM with MO pretreatment on crossing numbers in OFT. Each bar represents the mean response from 6 mice ± SEM. (A) Different doses of NM, (B) different doses of MO, (C) combination of NM with MO in comparison to doses of NM and MO (* significant difference with reserpine, ^significant difference with fluoxetine, $^{+}$significant difference with imipramine, $^{\$}$significant difference with a combination of NM with MO) (RES, reserpine 4 mg/kg; IMP, imipramine 10 mg/kg; FLX, fluoxetine 20 mg/kg; NM, *Nepeta menthoides* aqueous extract; MO, *Melissa officinalis* aqueous extract), *N*: number of squares crossed. $^{\$,+}p < .05$. $^{\wedge\wedge}p < .01$. $^{***,+++,\$\$\$}p < .001$

the RES group, which was somewhat dose-dependent ($p < .05$). Total squares crossed in the NS group ($n = 6$, 96.17 ± 6.6) were significantly higher than RES ($n = 6$, 30 ± 6.29) at the beginning of the test ($p < .001$). All doses of NM significantly induced an increase in locomotor activity compared to IMP in OFT, but increasing squares, crossed in dose 200 mg/kg NM was significant compared to the IMP and FLX (ANOVA, F [6, 35] = 42.79, $p < .001$, Figure 5A). A significant increase in OFT was also observed in all doses of MO (150–750 mg/kg) in comparison to RES. Doses 350, 550,and 750 mg/kg MO were significant in increasing locomotor activity, compared to IMP as a standard drug (ANOVA, F [6, 35] = 25.17, $p < .001$, Figure 5B). Pretreatment with combination doses of NM 50 mg/kg with MO 150 mg/kg increased the number of squares crossed with a significant difference in comparison to those in standard and control groups (ANOVA, F [5, 30] = 39.64, $p < .001$).

3.4 | Median lethal dose (LD50)

No animal death occurred in the 48 hr of observation after gavage of different doses of NM (250–4,000 mg/kg) and MO (750–6,000 mg/kg) in none of the groups. The animals appeared healthy, attentive, and active.

3.5 | The effects of *N. menthoides* and *M. officinalis* aqueous extract on CAT activity in the brain

The findings of the present study showed a significant increase in the brain CAT activity in all NM groups compared to the RES group ($p < .05$). However, there was no significant difference between NM groups and standard drugs. NM 200 mg/kg showed more increased

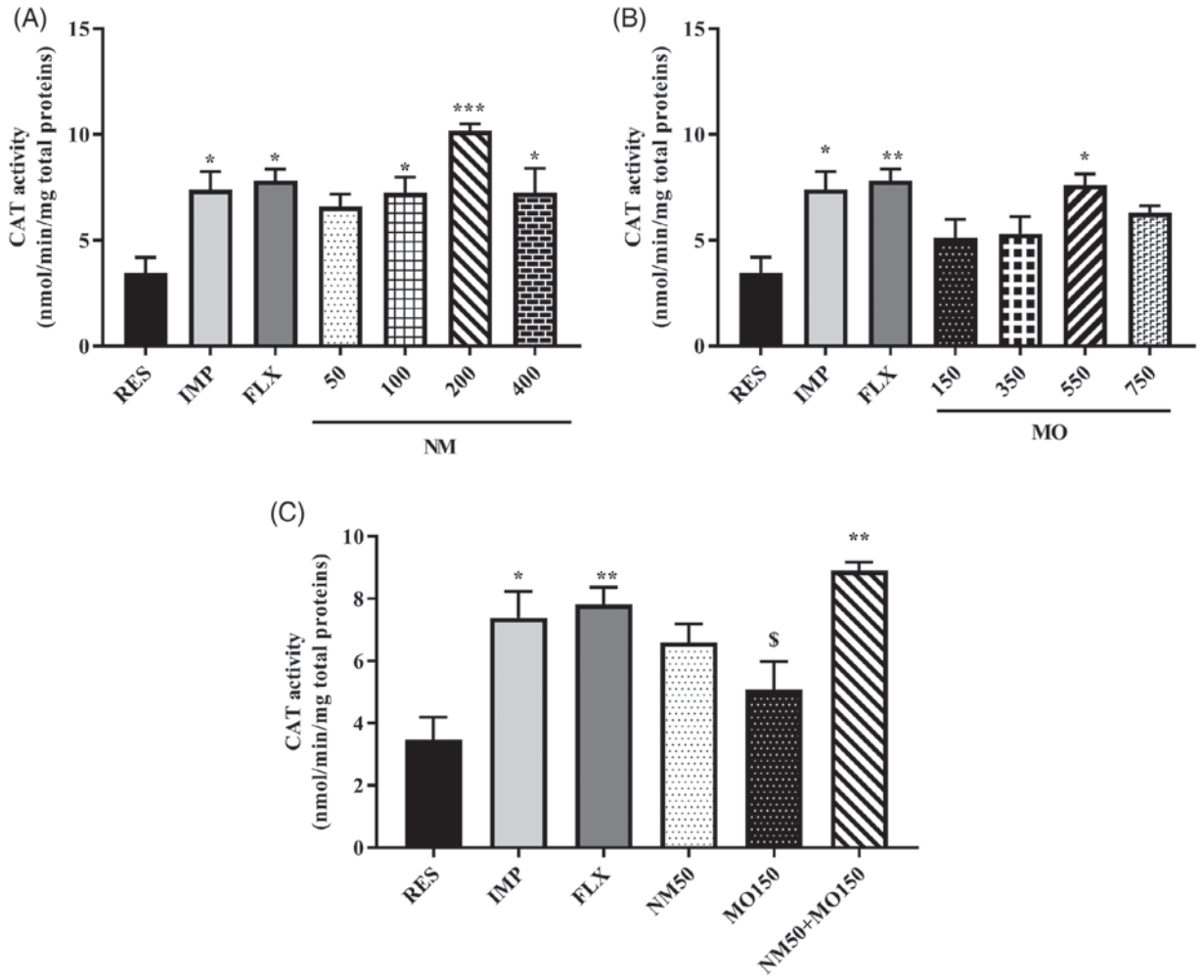


FIGURE 6 Effects of NM and MO alone and in a combination of NM with MO pretreatment on Catalase activity in the brain compared to control groups. Each bar represents the mean response from 4 mice ± SEM. (A) Different doses of NM, (B) different doses of MO, (C) combination of NM with MO in comparison to doses of NM and MO (*significant difference with reserpine, ^significant difference with fluoxetine, $^{+}$significant difference with imipramine, $^{\$}$significant difference with a combination of NM with MO) (RES, reserpine 4 mg/kg; IMP, imipramine 10 mg/kg; FLX, fluoxetine 20 mg/kg; NM, *Nepeta menthoides* aqueous extract; MO, *Melissa officinalis* aqueous extract). $^{*,\$}p < .05$. $^{**}p < .01$. $^{***}p < .001$

activity of CAT (ANOVA, F [6, 14] = 7.097, $p < .05$, Figure 6A). MO increased CAT activity in dose 550 mg/kg, compared to the RES (ANOVA, F [6, 14] = 5.304, $p < .05$, Figure 6B). A combination of NM 50 mg/kg with MO 150 mg/kg could increase CAT activity in comparison to RES, and its results were more significantly different than the other groups (ANOVA, F [5, 12] = 8.466, $p < .05$, Figure 6C).

4 | DISCUSSION

The combination strategy and pharmacological synergy between drugs, offers potential advantages, including fewer side effects and a more effective clinical response in depression management (Pagano et al., 2021). PM literature pursues combination therapy and suggests that plant extracts contain ingredients that potentiate each other's effects (Avicenna, 2005). Studying the active ingredients of medicinal plants and their mechanism of action in the treatment of depression can strengthen this view and develop traditional products as novel antidepressant drugs (Keck et al., 2020). The neuroprotective effects of *N. menthoides* and *M. officinalis* have been studied in some examinations, but there are a few scientific documents concerning their mechanisms of action in depression, when administered orally, alone, or in combination (Memariani et al., 2019; Shakeri et al., 2016). In this study, behavioral and biochemical approaches were applied to investigate the antidepressant and antioxidant effects of NM and MO, as two of the best-known herbs for the treatment of depression in PM, in combination therapy (Jalali et al., 2021; Keck et al., 2020). To find the different pathways in NM and MO, different doses of these two plants, compared with FLX and IMP, which are commonly used as positive controls in preclinical studies and have different mechanisms of action (Park et al., 2015).

After 1-week of pretreatment with the extracts, immobility time in TST and FST significantly decreased. The crossed squares in OFT also increased using a combined dose of plant extracts compared to

the single dose or standard control groups, remarkably. As in the standard groups, different treatment groups of NM and MO prevented the effects of RES on reducing CAT activity in the brain. It seems that the synergistic effects of combined therapy are more effective than NM and MO alone on depressive-like behaviors in mice. The level of total phenols and flavonoids and RA were measured in NM and MO, and the activity of CAT was estimated in the brain tissue in all treatment groups. Besides, we investigated the median lethal dose (LD50) of oral NM and MO in mice for the first time. Biochemical analysis has revealed the high amount of RA, total phenolic, and flavonoid contents in NM and MO.

Previously, it has been reported that NM, at the dose of 200 mg/kg, has antidepressant-like effects on the FST and TST compared to standard drugs (Rezaei, Rahmati, Malakian, Khalili, & Alizadeh, 2020). We used a wider range of different doses of NM (50–400 mg/kg) in our study and all doses had antidepressant effects. However, 200 mg/kg NM could prevent depressive-like behavior, better than the other doses. In this study, we showed MO at all doses tested had an antidepressant effect but MO at doses of 350 and 550 mg/kg had a better antidepressant effect on reserpinized mice compared to FLX, IMP (standard groups), and other treatment groups. These results differ from a previous study by Ghazizadeh et al. (2020) that proved dose of 150 mg/kg as more effective doses in their study. In Ghazizadeh et al.'s research, doses of 50–150 mg/kg MO were checked and they did not demonstrate the other doses. In the present study, 350 mg/kg MO has been one of the effective doses. It is in agreement with Lin et al. (2015) research who compared the dose of 300 mg/kg MO as more effective doses in their study with RA (36 mg/kg) and reported the serotonergic effect of 300 mg/kg MO on FST.

Our findings showed that the antidepressant effects of different doses of NM and MO were dose-dependent that were also consistent with the findings of a previous study by Bolandghamat, Moghimi, and Iranshahi (2011) who indicated that the antagonize effects of the extract on RES occurred in a dose-dependent manner. The antidepressant effect of MO (25, 75, 150, and 300 mg/kg) on FST in comparison to IMP was demonstrated by Emamghoreishi and Talebianpour (2015). Consistent with the present study, they suggested that MO might act on norepinephrine to control depression.

Our findings demonstrated synergistic interaction between the two plants and are in accordance with those of Fellah, Djenidi, and Chebout (2020) who suggested exerting synergistic antidepressant actions between two extracts by attenuating abnormalities in serotonergic and noradrenergic system functions. Ranjbar et al. (2018) proved that a combination of 400 mg NM and 1,000 mg MO per day, after 4 weeks had a better effect on insomnia severity, anxiety, and depression caused by insomnia in clinical application. They used a single dose of each plant and neither provide preclinical results nor mention the mechanism of action of these two plants in the treatment of depression. But, in the present study, we studied different doses of two plants and tried to provide preclinical data. It was also tried to determine the mechanism of action of the two plants. In addition, we measured biomarkers (CAT activity) in the brain. The method of isoboles was used to assess the synergism between two extracts. The dose-effect curve of two extracts was plotted and then the dose combination that gave the 50% effect was obtained. Many researchers use this method to combine doses of different drugs with each other, to represent a synergistic effect (Zhou et al., 2016). We showed that NM 50 mg/kg and MO 150 mg/kg (ED_{50}), individually produced a specific effect on 50% and the combined treatment of NM 50 mg/kg with MO 150 mg/kg showed more effects than the extracts alone. Although these doses were ineffective in preventing the effect of RES on CAT activity, but the combination of these two ineffective doses significantly increased CAT activity in the brain. Indeed, synergistic interaction allows the use of lower (not inactive) doses of the combination constituents, a situation that may reduce adverse reactions. We wanted to examine if the combination therapy with lower doses of two extracts shows more antidepressive effects. We used method of combination of ED50, which can either represent additive, synergistic, or antagonistic interactions, depending on the position of the dose of combination to the linear line. Based on this method, ED_{50} of each extract (not inactive doses), combined with each other, represents synergistic effect (Dimmitt et al., 2017). On the other hand, the biochemical analysis showed that the combination of ineffective doses significantly increased CAT activity in the brain. However, Mirghafourvand, Malakouti, Mohammad, Farshbaf, and Ghanbari (2016) reported that there was no statistically significant difference between extracts alone and their combinations in their clinical trial. It seems that further animal and clinical studies are needed to demonstrate that the combination of two plants has no difference with their single-use.

In the present study, the antidepressant effects of different doses of extracts were like FLX on FST and IMP on TST. Similar to our findings, Can et al. (2013) reported that serotonergic agents like FLX could decrease the immobility time in behavioral tests; so FLX and IMP were used as standard drugs in our study. In our animal study, antidepressant effects were evaluated in reserpine-induced mice. RES blocks the vesicular monoamine transporter, leading to a rapid decrease in monoamine concentrations, which is why it was used as a pharmacological model of depression in the current study (El-Marasy, El Awdan, Hassan, Ahmed-Farid, & Ogaly, 2021). We have done behavioral tests on a saline control group treated only with normal saline 10 ml/kg (p.o.), at the beginning of the study, to rule out the bias effect of locomotor activity on immobility time and the reserpine capability to induce depressive-like behavior. The results showed that the combination treatment with the extracts is more effective than tricyclic antidepressant and reuptake inhibitors of serotonin and norepinephrine to decrease depressive-like behaviors in mice. To evaluate the similar mechanism between the extracts and the standard drugs, we need more investigation such as measuring the neurotransmitter levels following plant extract administration.

Although different pathophysiological mechanisms have been described for depression such as hypothalamic–pituitary–adrenal axis hyperactivity, neuro-inflammation, and alteration of brain monoamine concentrations, it might be associated with a lowered concentration of antioxidants in plasma and increased OS (Bhatt et al., 2020; Khan, Perviz, Sureda, Nabavi, & Tejada, 2018). CAT is one of the

antioxidative enzymes observed to change their levels in the course of depression. The enzyme decomposes hydrogen peroxide (H_2O_2), whose overproduction is a result of many processes taking place in depression. So in this study, CAT activity in the brain was checked in the treatment groups at the end of experiments (Bhatt et al., 2020). NM and MO prevented reducing CAT activity in the brain of reserpinized-mice, which was similar to Ghazizadeh et al. (2020) research results. Reduction in CAT activity could trigger OS and depression in animals. Metabolized catecholamines are one of the triggers in the symptoms of depression, so a blockade of monoamine oxidase can prevent this effect. Many antidepressant drugs such as IMP and FLX cause selective inhibition and reabsorption of both norepinephrine and serotonin; thus, facilitating the treatment of depression. Previous studies reported that the intraperitoneal administration of IMP (10, 20, and 30 mg/kg) could increase CAT activity in the brain compared to control groups (Réus et al., 2010). It seems that NM and MO and their combinations can probably inhibit monoamine oxidase and reduce depression. These findings indicate that MO and NM increase the activity of antioxidant enzymes, thereby improving antioxidant defenses in the brain.

Some of the previous studies suggest that the phenol and flavonoid contents of NM and MO, especially RA, have an antidepressant effect because of their antioxidant activity. It has been suggested that RA has a variety of biological activities such as antiapoptotic, antioxidant, and antiinflammatory effects and possesses a neuroprotective impact, most probably by decreasing OS (Rahbardar & Hosseinzadeh, 2020).

Khan et al. (2018) demonstrated, due to antiinflammatory effects, antioxidants derived from flavonoids improved locomotor activity, increased neurogenesis, neuroplasticity, and restored monoamine levels. Moreover, they can act as antidepressant agents in plants, so high amounts of flavonoids in plants may lead to more antidepressant effects. In the present study, total phenol and flavonoid contents of NM was 83.11 ± 7.17, 64.18 ± 1.02 mg catechin/g extract and MO were 311.99 ± 33.34, 346.71 ± 50.76 mg catechin/g extract; so probably, these flavonoids could attenuate the decrease in monoamines and recover the inhibitory neurotransmission by GABA and reduce depressive-like symptoms in mice. Takeda, Tsuji, Inazu, Egashira, and Matsumiya (2002) proposed that 2 mg/kg RA declined the immobility time in the FST and showed antidepressant effects. Therefore, the RA amount in this study (11.02 ± 2.16 mg/g extract in NM and 6.42 ± 1.10 mg/g extract in MO) led to better antidepressant effects. Two peaks were found in the retention time of the NM chromatogram and MO had one peak in retention time of ~28.933 min. If the injected sample is diluted in a strong diluent compared to the mobile phase, the sample will elute faster than the mobile phase and may cause in doubling of the peaks. So, it seems two peaks of NM are due to this happening (Raval & Patel, 2020).

In our study, doses of 100 and 200 mg/kg NM and different doses of MO and a combination of these two extracts, showed better effects than FLX and IMP. It has been suggested that antioxidants such as flavonoids restore the brain levels of monoamines in the brain either by inhibiting monoamine oxidase or by inhibiting reuptake of these neurotransmitters (Can et al., 2013). Based on the previous studies, the mechanism of action of RA on depression is a proliferative influence on newly born cells in the hippocampus. So further research is necessary to show RA functions in the brain (Mirza, Amber, Hassan, Ahmed, & Zahid, 2021). It seems RA and total phenolic and flavonoids are the principal constituents of NM and MO for depression treatment and can probably inhibit monoamine oxidase activity. The results showed that NM and MO combination reversed reserpine-induced depressive-like behaviors significantly in mice. CAT level, as an antioxidant parameter, was reduced too. High amounts of antioxidants mainly flavonoid and RA components in MO have a crucial role in its antioxidant activity. The other possible mechanism of action for the antidepressant effect of flavonoid component is restoring the brain levels of monoamines, including serotonin and dopamine, an effect which is more than IMP and FLX (Hritcu et al., 2017). The size of the effect of FLX, IMP, and combination group as a quantitative measure of the magnitude of the experimental effect, is shown in Table 2. These results are based on Cohen's *d* (*standardized mean difference*), which formula is as follows (Sullivan & Feinn, 2012):

$$\text{Cohen's } d = \frac{[\text{Mean of experimental group}] - [\text{Mean of control group}]}{SD}.$$

The effect size of the combination of two extracts (NM50 + MO150) and IMP and FLX, were larger than 1.5 in three experiments ($p < .0001$). This effect size value shows the combination therapy of NM50 with MO150, had probably a large effect on depression. In a few studies, the different doses of NM and MO have been investigated for the treatment of depression, but they did not report the effect size in their results (Memariani et al., 2019; Shakeri et al., 2016).

Our study had some limitations. Indeed, this article is a part of PhD thesis. There was a time limit to thesis defense and the restriction of access to materials, animals, and lab facilities during the COVID-19 pandemic. Therefore, we did not succeed in doing all histological and pathological studies related to this research nor to measure other biomarkers to provide more evidence to understand the molecular mechanism. We are planning to do this in the near future studies. For the same restriction, we could not set up different animals for

TABLE 2 The effect size of control groups (FLX and IMP) and combination of *Nepeta menthoides* and *Melissa officinalis* (NM50 + MO150) in behavioral tests

	Effect size (Cohen's *d*)			
Groups	FST	TST	OFT	*p* value
RES-IMP	9.52	3.72	3.06	<.0001
RES-FLX	11.32	4.84	5.61	<.0001
RES-NM50 + MO150	9.5	10.3	6.51	<.0001

Abbreviations: FLX, fluoxetine; FST, forced swim test; IMP, imipramine; MO, *Melissa officinalis* aqueous extract; NM, *Nepeta menthoides* aqueous extract; OFT, open field test; RES, reserpine; TST, tail suspension test.

each test and use a large number of animals. Therefore, we considered a rational interval time to perform the second behavioral test in an animal study. The control group was used to compare the differences between groups. To evaluate both therapeutic and preventive effects of *N. menthoides* and *M. officinalis* on depression, we were using a preventive rather than a curative protocol.

According to the findings of this study, NM and MO, at dosage levels up to 4,000–6,000 mg/kg, are non-toxic and may be considered safe. In PM literature, both NM and MO have been introduced as effective medicinal plants in preventing and treatment of psychological diseases. There is not enough information about the safe doses of these plants. In *Makhzan-al-Advieh*, a book written by Aghili Khorasani, in 1771 AD, safe doses of the dried plant of *N. menthoides* are 6/4–16 g/day and *M. officinalis* is 32 g/day (Aghili Khorasani, 2011). LD50 of *M. officinalis* was reported 4.5 g/kg of hydroalcoholic extract (i.p.) and 2.57 g/kg of essential oil (p.o.) in previous studies. There is just one study that examined LD50 of NM (i.p.) and reported no death up to 60,000 mg/kg (Memariani et al., 2019; Shakeri et al., 2016). Our study examined the LD50 of MO and NM by gavage for the first time. Based on this information in PM and the results of this study, we did not use toxic doses. On the other hand, even edible plants could show adverse/toxic effects after overconsumption. Combination therapy allows to reduce the dose and get more pharmacological effects so we prescribed our doses based on these results and LD50. The doses of this study were 50–100–200–400 mg/kg for NM and 150–350–550–750 mg/kg for MO. Possible doses of these two extracts, which can be used in humans, are 4.05–32.4 mg/kg (NM) and 12.15–60.75 mg/kg (MO), which are translated using conversion tables between animals and humans (Nair & Jacob, 2016). However, further experiments are needed to confirm the active antidepressant effects of MO and NM and their corresponding mechanisms of action.

5 | CONCLUSION

This study demonstrates that pretreatment with NM and MO prevents depressive-like behavior induced by RES with more efficiency than FLX and IMP. Interestingly, the combination of ineffective doses of the two extracts show a synergistic effect and improves behavioral and biochemical parameters in the depressive-like state in mice. Based on our results, it seems that combination therapy with NM and MO would be a valuable candidate for the prevention of depression-like behavior. More investigations on biochemical, pharmacological, and toxicity aspects of this combination are needed to provide strong evidence for clinical application.

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CONFLICT OF INTEREST

The authors declare they have no competing interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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