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RESEARCH PAPER

Melissa officinalis L. hydro-alcoholic extract inhibits anxiety and depression through prevention of central oxidative stress and apoptosis

Javid Ghazizadeh[1,2] | Sanaz Hamedeyazdan[3] | Mohammadali Torbati[4] | Fereshteh Farajdokht[1] | Ali Fakhari[5] | Javad Mahmoudi[1] | Mostafa Araj-khodaei[2,6] | Saeed Sadigh-Eteghad[1]

[1]Neurosciences Research Center (NSRC), Tabriz University of Medical Sciences, Tabriz, Iran

[2]Department of Persian Medicine, Faculty of Persian medicine, Tabriz University of Medical Sciences, Tabriz, Iran

[3]Department of Pharmacognosy, Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran

[4]Department of Food Science and Technology, Faculty of Nutrition, Tabriz University of Medical Sciences, Tabriz, Iran

[5]Research Center of Psychiatry and Behavioral Sciences, Tabriz University of Medical Sciences, Tabriz, Iran

[6]Aging Research Institute, Tabriz University of Medical Science, Tabriz, Iran

Correspondence
Mostafa Araj-khodaei, Aging Research Institute, Tabriz University of Medical Science, Golgasht Street, Azadi Avenue, Tabriz, Iran 5166614756.
Email: mostafaa33@gmail.com

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Abstract

This study evaluated the effects of a hydro-alcoholic extract of *Melissa officinalis* (HAEMO) on anxiety- and depressive-like behaviours, oxidative stress and apoptosis markers in restraint stress-exposed mice. In order to induce a depression-like model, mice were subjected to restraint stress (3 h day^{-1} for 14 days) and received normal saline or HAEMO (50, 75 and 150 mg kg^{-1} day^{-1}) for 14 days. The administered doses of HAEMO were designated based on the concentration of one of the main phenolic compounds present in the extract, rosmarinic acid (2.55 mg kg^{-1} at lowest dose); other phytochemical analyses including assays for anti-oxidant activity, total phenols and flavonoids were also carried out. The behavioural changes in an open field task, elevated plus maze, tail suspension and forced swimming tests were evaluated. Also, malondialdehyde (MDA) levels and enzyme activities of superoxide dismutase and glutathione peroxidase, and total antioxidant capacity were assessed in the prefrontal cortex and hippocampus. Moreover, levels of Bcl-2, Bax and caspase 3 in the brain as well as serum concentration of corticosterone were evaluated. HAEMO (75 and 150 mg kg^{-1}) significantly reversed anxiety- and depressive-like behaviours. Also, HAEMO reduced MDA levels, enhanced enzymatic antioxidant activities and restored serum levels of corticosterone. An immunoblotting analysis also demonstrated that HAEMO decreased levels of pro-apoptotic markers and increased anti-apoptotic protein levels in the prefrontal cortex and hippocampus of restraint stress-exposed mice. Our findings suggested that HAEMO reduced inflammation and had anxiolytic and anti-depressant effects in mice.

KEYWORDS

anxiety, apoptosis, depression, *Melissa officinalis*, oxidative stress, restraint stress

1 | INTRODUCTION

Stress is a normal response to negative challenges in daily life and affects profoundly every system in the body, leading to a state of constant physiological arousal. Given the deleterious effects of stress on the various physiological systems, the World Health Organization (WHO) has categorized it as a worldwide epidemic (World Health Organization, 2007).

Anxiety and depression are the most common stress-related psychiatric diseases which disturb the normal physiological equilibrium of the body, and they impose a high cost on public health. According to the WHO statistics, 4.4% of the world's population suffer

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from depression, and by 2030, it will be the leading cause of disability (Lépine & Briley, 2011; World Health Organization, 2017).

Prolonged stress, physical or psychological, causes structural and neurochemical changes in the structures controlling mood and cognitive functions, including the hippocampus (HIP) and prefrontal cortex (PFC) (Kim, Pellman, & Kim, 2015). The hypothalamus–pituitary–adrenal (HPA) axis and sympathetic nervous system are major neuroendocrine systems affected by stressors (Smith & Vale, 2006; Yang et al., 2015). Prolonged stress dysregulates the HPA axis, increasing circulating levels of stress hormones – cortisol in human and corticosterone (CORT) in rodents – thereby causing mood disorders (Chiba et al., 2012; McEwen, 2008).

The exact molecular and cellular mechanisms by which stress induces the neuronal damage and thereby psychiatric disorders are still a matter of debate. Accumulating evidence indicates that oxidative stress in the brain plays a causative role in the pathophysiology of mood disorders evoked by chronic stress (Hovatta, Juhila, & Donner, 2010; McEwen, Nasca, & Gray, 2016; Smaga et al., 2015). Several preclinical and clinical studies also demonstrated that depressive symptoms are accompanied by increased levels of reactive oxygen species (ROS), protein oxidation and lipid peroxidation (T. Liu et al., 2015; Lopresti, 2019; Maes, Galecki, Chang, & Berk, 2011), and agents with antioxidant properties can alleviate the symptoms (S.-Y. Lee et al., 2013). Moreover, depression is associated with diminished enzymatic and non-enzymatic antioxidant defences (Maes et al., 2019).

Despite many advances in neuroscience and pharmacology, optimal treatments have not yet been elucidated for some of the disorders. On the other hand, resistance to available synthetic drugs as well as side effects has led to the use of medicinal plants as an alternative source of therapeutic agents.

Melissa officinalis (MO) of the Lamiaceae family is a popular medicinal herb that is widely cultivated in Europe, the USA, the Mediterranean region as well as the north of Iran. MO has been assigned to the FDA 'Generally Recognized as Safe' (GRAS) list in the USA. No serious side effects have been reported (Ulbricht et al., 2005). According to the literature, MO contains different classes of phytochemicals such as essential oils, terpenoids and polyphenolic compounds such as flavonoids, rosmarinic acid (RA) and tannins (Dastmalchi et al., 2008; De Sousa et al., 2004). In traditional medicine, MO has been extensively used for treatment of many psychological disorders such as nervous sleeping disorders, stress, anxiety and depression (Awad, Muhammad, Durst, Trudeau, & Arnason, 2009; Heydari, Dehghani, Emamghoreishi, & Akbarzadeh, 2018; Ibarra, Feuillere, Roller, Lesburgere, & Beracochea, 2010; Khodaei et al., 2017; Lin et al., 2015). It has anti-inflammatory, anti-microbial, anti-oxidative, sedative and neuroprotective actions (López et al., 2009; Miraj, Rafieian, & Kiani, 2017). Many of these pharmacological activities have been mainly attributed to its phenolic and flavonoid constituents, mainly RA. RA is considered one of the major polyphenolic ingredients in this plant; it could cross the blood–brain barrier (Falé, Madeira, Florêncio, Ascensão, & Serralheiro, 2011) and has been reported to shorten immobility time in a forced swimming test in mice (Takeda, Tsuji, Inazu, Egashira, & Matsumiya, 2002). Also it has been shown that RA administration produced an anti-depressive activity in rats exposed to chronic unpredictable stress via up-regulation of hippocampal brain-derived neurotrophic factor (Jin, Liu, Yang, Zhang, & Miao, 2013).

In this study, we investigated the effect of a hydro-alcoholic extract of *Melissa officinalis* (HAEMO) on behavioural and molecular changes in the HIP and PFC of mice that were induced by chronic restraint stress, with a particular focus on oxidative stress status and apoptosis markers.

New Findings

- **What is the central question of this study?**
  How does an extract of *Melissa officinalis* L. ameliorate anxiety- and depressive-like behaviour of mice?
- **What is the main finding and its importance?**
  An extract of *Melissa officinalis* L. possessed anxiolytic and anti-depressant effects, which could mainly be mediated through its antioxidant and anti-apoptotic properties.

2 | MATERIALS AND METHODS

2.1 | Ethical approval

All experimental procedures complied with the principles for the care and use of laboratory animal and were approved by the Animal Ethics Committee of Tabriz University of Medical Sciences (Ethics Approval ID: IR.TBZMED.VCR.REC.1397.219, date: 8 October 2018). We have taken all steps to minimize the animals' pain and suffering.

2.2 | Extract preparation

Fresh MO aerial parts were collected from an organic farm in Tekmeh Dash (longitude: 46° 55′ 57″ and latitude: 37° 44′ 38″), East Azerbaijan (Iran); after certification of the plant's identity and quality, a voucher specimen of the plant was deposited at the herbarium of the Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran (NO: Fph-Tbz 4031). Then aerial parts of the plant were dried in the shade at room temperature and mechanically powdered using a blender. For the preparation of the extract, 100 g of the powdered material was carefully macerated and extracted with ethanol–distilled water (70:30) v/v for different maceration periods with occasional shakings at room temperature. After filtration of the extract, the solvent was evaporated to dryness using a rotary evaporator under vacuum. The yielded dried extract was stored in a dark bottle inside a refrigerator (4°C) until further analysis.

2.3 | Extract phytochemical analysis

2.3.1 | Total rosmarinic acid contents

Reversed-phase HPLC (Shimadzu, Kyoto, Japan) analysis with a C_{18} Knauer column (250 mm length × 4.6 mm I.D.) was performed to determine the RA content of the HAEMO. The mobile phase (flow

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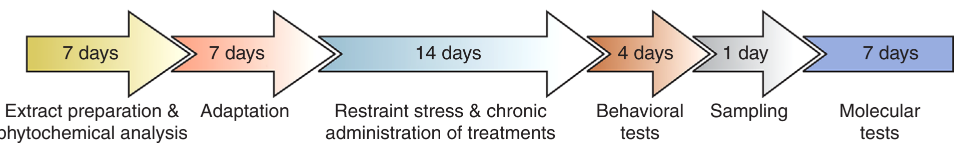

FIGURE 1 Schematic representation of the timeline of the study

rate: 15 ml min^{-1}) consisting of acetonitrile (A) and 5% trifluoroacetic acid in water (B) was used for the chromatographic separations. The separation was performed in a linear gradient elution with the following programme: 0–15 min linear gradient of 0–10% (A) and 5% (B), 15–25 min isocratic 10% A and 25–50 min linear gradient 10–55% A. The detector was set at a wavelength of 280 nm.

RA (PubChem CID: 5281792) was purchased from Sigma-Aldrich (St Louis, MO, USA). The standard solutions of RA were prepared at different concentrations through dilution of the stock standard RA solution. After injection of the standards to the HPLC, the peak areas of RA were identified and compared with the samples using the corresponding standard curve of RA (Asghari et al., 2019).

2.4 | *In vitro* antioxidant activity assay

The antioxidant activity of HAEMO was evaluated *in vitro* using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) protocol, in which the ability of the extract to scavenge DPPH free radicals was determined. Briefly, 3 ml of 8% DPPH solution (v/v) was mixed separately with 3 ml of HAEMO in different concentrations and the obtained relative absorbance was recorded at 517 nm using a spectrophotometer (Shimadzu UV-2100) after 30 min of incubation at 25°C in the dark. Quercetin was used as the standard and the same procedure was applied for quercetin. The percentage of DPPH-free radical scavenging activity of the extract (%RSA) was calculated by the following formula: %RSA = absorbance of the control (quercetin) − absorbance of sample/absorbance of the control × 100. RC_{50} values, representing the concentration of the sample that inhibits 50% of the DPPH radicals, were calculated for the HAEMO and quercetin. (Alizadeh Behbahani & Shahidi, 2019).

2.5 | Total phenol content

The total phenol content (TPC) of the HAEMO was assessed according to the Folin–Ciocalteu (FC) method, as previously described (Chemsa et al., 2018). Briefly, 1 ml of FC reagent was mixed with HAEMO (1 mg ml^{-1}) and Na_2CO_3 7.5% (2 ml) and incubated in the dark for 2 h at room temperature. Gallic acid was used as the standard. Subsequently, the absorbance was read at 765 nm. The results were expressed as mg gallic acid equivalents (GAE)/100 mg HAEMO.

2.6 | Total flavonoid content

The total flavonoid content (TFC) of the HAEMO was assessed with a colorimetric method using $AlCl_3$ reagent. For this, 2 ml of $AlCl_3$ was mixed with 1 mg ml^{-1} of HAEMO solution and incubated for 10 min at room temperature. Then the absorbance was read at 415 nm. A quercetin calibration curve (10–100 μg ml^{-1}) was used to determine the TFC of the HAEMO as the standard. The results were expressed as mg quercetin equivalents (QE)/100 mg HAEMO.

2.7 | Animals

We used 60 male albino BALB/c mice, weighing 25–28 g, from the animal house of Tabriz University of Medical Sciences (Tabriz, Iran). After transferring to the animal house of the Neuroscience Research Center, the mice were housed five per cage in poly-carbon cages and kept at a constant temperature (21–25°C) on a 12/12 h light–dark cycle with free access to water and food.

2.8 | Experimental design and grouping

Following a week of adaptation to the new conditions, animals were randomly assigned to five groups (n = 12/group): control, restraint stress, restraint stress+M50, restraint stress+M75, and restraint stress+M150. Animals in the control group were kept in their home cages and received daily 0.9% normal saline by gavage. Restraint-stress exposed groups were horizontally immobilized (3 h day^{-1} for 2 weeks, from 08.00 to 11.00 h) in a well-ventilated 50 ml falcon tube and only received normal saline by gavage after cessation of stress procedure. The restraint stress+M50, restraint stress+M75 and restraint stress+M150 groups were chronic administered orally 50, 75 and 150 mg kg^{-1} day^{-1} of HAEMO, respectively, for 14 days. The dosages of administration chosen was based on previous reports (Emamghoreishi & Talebianpour, 2009; Taiwo et al., 2012; Jin et al., 2013; Lin et al., 2015). Also, since the major neuroactive component of MO is RA, the RA content (2.55, 3.825 and 7.65 mg kg^{-1} day^{-1}) was considered in dosage optimization (Komes et al., 2011; Lin et al., 2015; Mahboubi, 2019; Pereira et al., 2005). Figure 1 shows the timeline of the study.

2.9 | Behavioural analysis

All behavioural tests were carried out in a quiet room by an experimenter blinded to the group treatments. Animals were transferred to the room and allowed to adapt to the room at least 45 min before the test. Anxiety-like behaviours were evaluated in an open field test (OFT) and elevated plus maze (EPM), and depressive-like behaviours were assessed in a forced swimming test (FST) and tail suspension test (TST). All test sessions were recorded by a digital video camera and subsequently analysed using a video tracking program EthoVision^{TM} (Noldus, Wageningen, The Netherlands).

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2.10 | Open field test

The apparatus was a square arena made of black Plexiglas (33 × 33 × 20 cm) which was divided into peripheral and central zones. Each mouse was gently placed in the central zone, and the total distance travelled, as an index of locomotor activity, and the time spent in the central area were recorded for 10 min (Salehpour et al., 2019). After each test, the arena was cleaned with 70% ethanol to remove the residual odour.

2.11 | Elevated plus maze

The EPM apparatus was a wooden device consisting of two opposite open arms (30 × 5 cm and 0.5 cm edge) and two opposite closed arms (30 × 5 cm and 15 cm high wall) which was elevated 50 cm from the ground. Each animal was placed in the centre of the apparatus facing an open arm. The behaviours of the animal were recorded for 5 min and the percentage of time spent in the open arms (%OAT) and the percentage of entries into open arms (%OAE) were calculated (Majdi et al., 2018).

2.12 | Forced swimming test

The FST was performed as previously described by Juszczak, Lisowski, Śliwa, & Swiergiel (2008). The mice were separately placed into a vertical glass cylinder (diameter 14 cm, height 20 cm), containing 10 cm of water at 25 ± 2°C, and left to swim for 6 min. Total immobility time was the time in which animal ceased struggling and remained floating motionless in the water making basic movements to hold its head above water, and it was calculated in the last 4 min. Then the mice were removed, dried, and returned to their home cage. Following each test, the water of the tank was renewed.

2.13 | Tail suspension test

In this test, the tip of the tail of each mouse was fixed to a metal hook attached to the centre of a wooden panel (50 cm above the floor) using an adhesive tape and suspended for 6 min. The immobility time during the last 4 min of the test session was calculated (Mahmoudi, Farhoudi, Talebi, Sabermarouf, & Sadigh-Eteghad, 2015).

2.14 | Sampling

After the last behavioural test, animals were anesthetized with ketamine (80 mg kg^{-1}) and xylazine (8 mg kg^{-1}) via intraperitoneal injection. We took all steps to minimize the animals' pain and suffering. Blood samples were collected from the heart. To separate serum samples, the blood was centrifuged at 1500 *g* for 10 min at 4°C. Animals were killed by decapitation; the brains were excised immediately, and PFC and HIP were cautiously isolated on an ice-cold plate and then maintained at −70°C for further use.

2.15 | Serum levels of CORT

An enzyme-linked immunosorbent assay (ELISA) kit (Abcam, ab108821, Cambridge, UK) was used for the measurement of serum CORT level according to the manufacturer's protocol.

2.16 | Brain biochemical assessments

2.16.1 | Homogenization

First, frozen PFC and HIP tissue samples were thawed to 4 °C and then homogenized in 1.15% potassium chloride (KCl) solution using a tissue homogenizer. Next, the solution was centrifuged at 9375 *g* for 10 min at 4°C, and the supernatant was collected. The protein content was determined by the Bradford method.

2.17 | Malondialdehyde concentration

The malondialdehyde (MDA) level is a biomarker of oxidative stress and an index of lipid peroxidation. The MDA level was evaluated using the thiobarbituric acid reaction (TBAR) colorimetric assay, and optical density of the supernatant was read at 540 nm in a plate reader and presented as nmol (mg protein)$^{-1}$ (Pourmemar et al., 2017).

2.18 | Superoxide dismutase and glutathione peroxidase (GPx) activities

The enzyme activity of superoxide dismutase (SOD) was assessed using a RANSOD kit (Randox Laboratories Ltd, Crumlin, UK) based on the manufacturer's guidelines. The absorbance of the solution was measured at 505 nm by a spectrophotometer at 37°C, and the results were expressed as U (mg protein)$^{-1}$.

The enzyme activity of glutathione peroxidase (GPx) was also measured using the RANSEL laboratory kit (Randox Laboratories Ltd). The reduction in absorbance was read at a wavelength of 340 nm using a spectrophotometer at 37°C, and GPx concentration was expressed as U (mg protein)$^{-1}$.

2.19 | Total antioxidant capacity level

The total antioxidant capacity (TAC) was determined according to the 2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) method using a Randox total antioxidant status kit (Randox Laboratories Ltd). The absorbance was measured at 600 nm using a spectrophotometer and expressed as nmol l^{-1} (Pourmemar et al., 2017).

2.20 | Apoptosis markers

Immunoblotting was performed for detection of the protein expressions of apoptosis markers, including Bax, Bcl-2 and caspase 3 in the PFC and HIP tissues. Briefly, frozen PFC and HIP tissues were homogenized in 100 μl RIPA lysis buffer containing protease inhibitor cocktail (Roche, Germany) using a tissue homogenizer. To obtain supernatant, the homogenate was centrifuged at 12,000 *g* for 15 min at 4 °C, and total protein concentration in the

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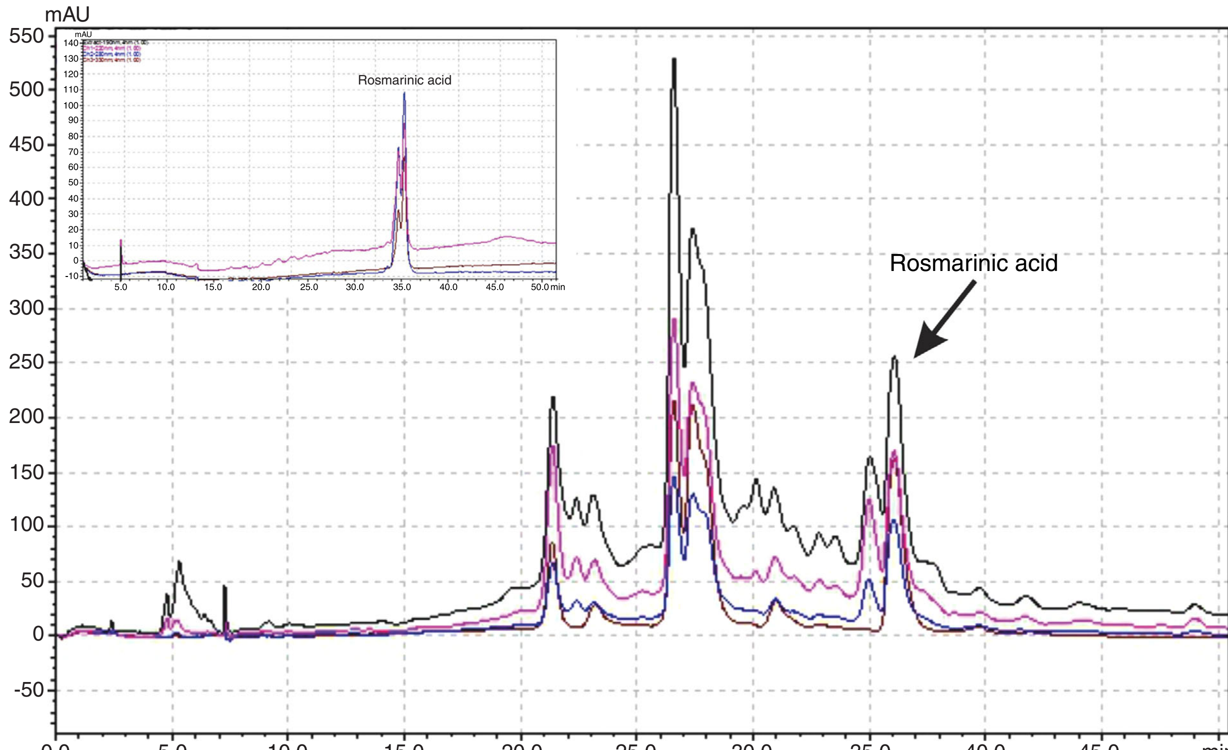

FIGURE 2 HPLC chromatograms of standard rosmarinic acid (inset) and *M. officinalis* hydro-alcoholic extract demonstrating rosmarinic acid at retention time of 36 min

supernatant was estimated using the Bradford method. Next, 20 μg of protein samples were loaded to 12.5% SDS-polyacrylamide gel and separated by electrophoresis, then transferred onto a polyvinylidene difluoride (Roche, UK) membrane. We also blocked non-specific binding reactions by incubation of the membranes with 3% BSA in Tris-buffered saline (pH 7.5) for 2 h at room temperature. Subsequently, the membranes were probed overnight with mouse primary antibodies (Santa Cruz Biotechnology, Dallas, TX, USA) including anti-Bax (sc-70405), anti-Bcl-2 (sc-7382), anti-caspase-3 (sc-56053) and anti-β-actin (sc-47778) and then with horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (1:5000, sc-2005) for 2 h at room temperature. Finally, the membrane was washed with phosphate-buffered saline then soaked in enhance chemiluminescence (ECL) detection reagents (GE Healthcare, Buckinghamshire, UK) and exposed to X-ray film (Kodak, Rochester, NY, USA). The density of protein bands was quantified by Image J (version 1.62, NIH, Bethesda, MD, USA) software and then normalized to the corresponding internal control, β-actin.

2.21 | Statistical analysis

The results are expressed as means ± standard deviation (SD). The statistical analysis was performed using GraphPad Prism 6.01 (GraphPad Software Inc., La Jolla, CA, USA). Experimental data were subjected to one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test to evaluate the differences among the groups. A *P*-value < 0.05 was regarded as significant. Pearson's correlation analysis was conducted to detect the correlation of variables.

3 | RESULTS

3.1 | Preliminary phytochemical analysis

3.1.1 | RA content of the HAEMO

The results of HPLC analysis of HAEMO demonstrated that the RA content was 5.1% (w/w) of the dried extract at the retention time of 36 min (Figure 2).

3.1.2 | Total phenol and flavonoid, and DPPH radical scavenging activity of the HAEMO

Maceration of the aerial parts of MO with 70% ethanol led to the extraction of a greenish residue with a yield of 20.85% (w/w).

The total phenolic content of MO was 62.085 ± 1.136 mg GAE/100 mg dry weight (DW) HAEMO or 129.45 ± 2.37 mg GAE/g DW aerial parts of MO (n = 3) (Figure 3a). The total flavonoid content of MO was 7.612 ± 1.368 mg QE/100 mg DW HAEMO or 15.87 ± 2.85 mg quercetin/g DW aerial parts of MO (n = 3) (Figure 3b).

The antioxidant potential, the DPPH free radical scavenging activity (RC_{50}), for quercetin and HAEMO was 4.01 and 15.64 μg ml^{-1}, respectively (Figure 3c). The RSA value for HAEMO was 3.9.

3.2 | Anti-depressant and anxiolytic effects of the HAEMO

Although there was no significant difference in the locomotor activity among groups in the OFT (Figure 4a), the time spent in the central zone

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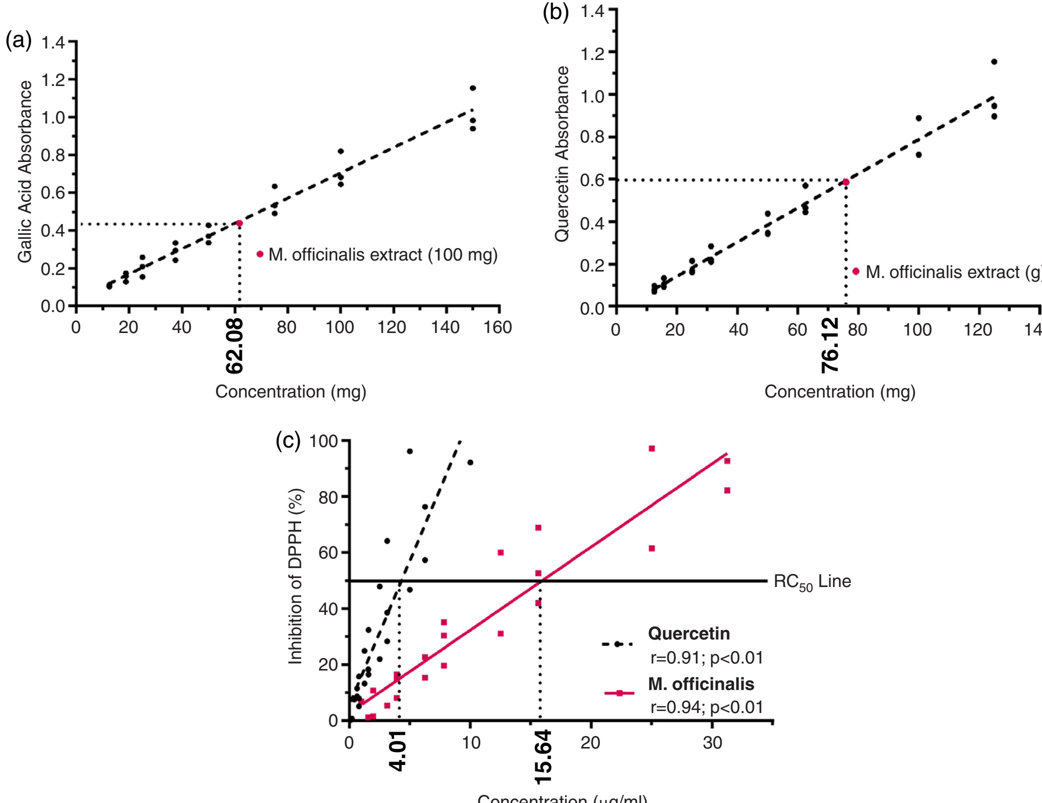

FIGURE 3 Preliminary phytochemical analysis of hydro-alcoholic extract of *Melissa officinalis*. (a) Total phenol content, (b) flavonoid content, and (c) DPPH radical scavenging activity of the hydro-alcoholic extract of *Melissa officinalis* along with relative standards

was markedly decreased in the restraint group, which was significantly increased by HAEMO administration at a dose of 150 mg kg^{-1} ($P < 0.001$; Figure 4b).

Furthermore, the results of the EPM test showed that restraint stress exposure significantly decreased %OAT ($P < 0.01$; Figure 4c, left panel) and %OAE ($P < 0.001$; Figure 4c, right panel) compared to the control animals. On the other hand, animals treated with HAEMO at doses of 75 and 150 mg kg^{-1} had longer OAT ($P < 0.001$ for both doses) and increased OAE ($P < 0.001$ for both doses) compared to the restraint-exposed mice.

The results of the behavioural tests revealed that restraint stress markedly increased immobility time in the FST ($P < 0.001$; Figure 4d, right panel) and TST ($P < 0.001$; Figure 4d, left panel) compared to the control mice. However, MO-treated animals showed shorter immobility time in the FST ($P < 0.01$ at dose of 75 mg kg^{-1} and $P < 0.001$ at dose of 150 mg kg^{-1}) and TST ($P < 0.01$ at dose of 75 mg kg^{-1} and $P < 0.001$ at dose of 150 mg kg^{-1}), indicating anti-depressant effect of MO.

3.3 | HAEMO regulates serum concentration of CORT

ELISA results revealed an increase in the serum levels of CORT in the normal saline-treated group ($P < 0.001$), which was significantly reduced in the MO-treated groups in doses of 75 ($P < 0.05$) and 150 mg kg^{-1} ($P < 0.001$, Figure 5).

3.4 | HAEMO modulated lipid peroxidation and the antioxidant defence system

The result of the biochemical assay also demonstrated that restraint stress exposure significantly increased MDA levels (Figure 6a), an index of lipid peroxidation, in the PFC and HIP ($P < 0.001$ for both regions) regions of the normal saline group compared to the control group. Moreover, restraint stress significantly decreased enzymatic activities of SOD ($P < 0.001$ for both regions; Figure 6c) and GPx ($P < 0.001$ for PFC, $P < 0.01$ for HIP; Figure 6d), and reduced TAC levels ($P < 0.001$ for both regions, Figure 6b) in the PFC and HIP regions of the normal saline-treated group compared to the control group. However, administration of HAEMO caused a significant decrease in the levels of MDA and increase in the SOD (PFC: $P < 0.05$ at dose 150 mg kg^{-1}; HIP: $P < 0.01$ at dose 150 mg kg^{-1}) and GPx (PFC: $P < 0.001$ at dose 150 mg kg^{-1}; HIP: $P < 0.05$ at dose 150 mg kg^{-1}) activities and TAC levels in the PFC and HIP regions (PFC: $P < 0.05$ at dose 75 mg kg^{-1} and $P < 0.001$ at dose 150 mg kg^{-1}; HIP: $P < 0.05$ at dose 75 mg kg^{-1} and $P < 0.01$ at dose 150 mg kg^{-1}) compared with that of the normal saline group.

3.5 | Behavioural indexes and antioxidant defence system correlation analysis

According to the results of Pearson's correlation analysis, immobility time in the TST and FST exhibited a significant positive correlation

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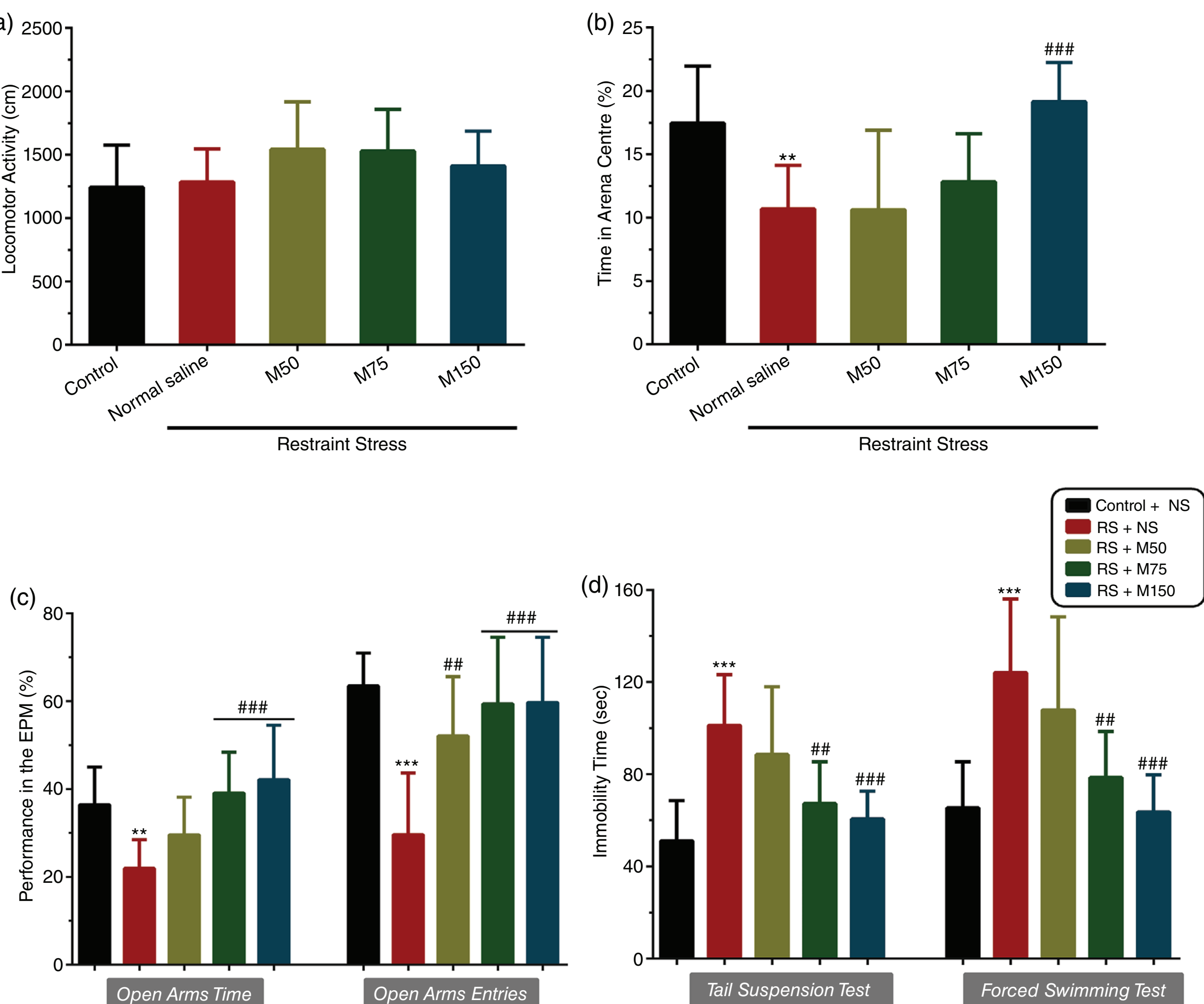

FIGURE 4 Effects of hydro-alcoholic extract of *Melissa officinalis* on the anxiety- and depressive-like behaviours assessed in the experimental groups. (a,b) Locomotor activity (a) and time spent in the arena centre (b) of the open field test. (c) The percentage of open arm time (left panel) and open arm entries (right panel) in the elevated plus maze. (d) Immobility time in the forced swimming test (FST, right panel) and tail suspension test (left panel). Data are expressed as means ± SD ($n = 12$). $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* control group. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs.* normal saline-treated group. M50, M75 and M150: 50, 100 and 150 mg kg^{-1} hydro-alcoholic extract of *Melissa officinalis*

with MDA levels and a negative correlation with TAC levels in the HIP and PFC. In addition, a significant positive correlation between time spent in the arena centre with TAC levels and a negative correlation with MDA levels in the HIP and PFC were observed. Furthermore, a significant positive correlation between open-arm entries and TAC levels and a negative correlation with MDA levels were identified in the HIP and PFC regions (Figure 7).

3.6 | HAEMO regulated mitochondria-mediated pro- and anti-apoptotic markers

The protein levels of Bcl-2 were significantly decreased in the normal saline group in the PFC ($P < 0.05$, Figure 8b, left panel) and HIP ($P < 0.05$, Figure 8b, right panel) of the normal saline-receiving group. Restraint stress exposure significantly increased protein levels of Bax (PFC: $P < 0.05$, Figure 8a, left panel; HIP: $P < 0.01$, right panel) and the cleaved caspase 3/pro-caspase 3 ratio ($P < 0.001$ for both regions. Figure 8c) in the PFC and HIP compared to the control animals. HAEMO at all three doses caused a significant decrease in the protein levels of Bax ($P < 0.05$ for PFC and $P < 0.01$ for HIP) and cleaved caspase 3/pro-caspase 3 ratio ($P < 0.001$ for both regions). It also increased the protein expression of Bcl-2 (PFC: $P < 0.05$ for all three doses; HIP: $P < 0.01$ at a dose of 75 mg kg^{-1} and $P < 0.001$ at a dose of 150 mg kg^{-1}) in the PFC and HIP of restraint stress-subjected mice.

4 | DISCUSSION

The results of the present study demonstrated that chronic treatment with HAEMO, at doses of 75 and 150 mg kg^{-1}, attenuated restraint stress-induced anxiety- and depressive-like behaviours through the amelioration of oxidative stress and apoptosis. Immobilization/restraint stress is considered to be the most severe type of stress in rodent models, and induces both an emotional and a

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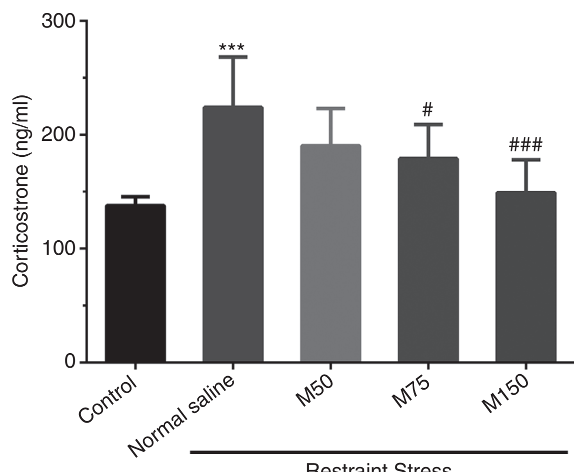

FIGURE 5 Effects of hydro-alcoholic extract of *Melissa officinalis* on serum concentration of corticosterone in the experimental groups. Data are expressed as means ± SD ($n = 10$). $^{***}P < 0.001$ *vs.* control group. $^{\#\#\#}P < 0.001$ *vs.* normal saline-treated group. M50, M75 and M150: 50, 100 and 150 mg kg^{-1} hydro-alcoholic extract of *Melissa officinalis*

physical response (Christoffel, Golden, & Russo, 2011; Wong & Licinio, 2004).

Nowadays, stress is an inevitable consequence of modern life that increases the risk of both physical and mental illnesses in some people. Stress-related mood disorders, such as anxiety and depression, result from abnormal responses to acute or prolonged stressors (Khan & Khan, 2017).

Our results showed that chronic restraint stress caused anxiety-like behaviours, indicated by decreased %OAT and %OAE in the EPM test and less time spent in the arena centre of the OFT, and depressive-like behaviours, presented by increased immobility time in the TST and FST. Nevertheless, administration of HAEMO, at doses of 75 and 150 mg kg^{-1} (containing 3.825 and 7.650 mg RA), could efficiently attenuate these behavioural changes. Moreover, these results were accompanied by elevated serum levels of CORT, which was decreased by HAEMO treatments.

A previous study has reported anti-depressant-like activity of an aqueous extract of MO similar to imipramine in the FST in non-stressed mice (Emamghoreishi & Talebianpour, 2009). Taiwo et al. also showed gender- and administration length-dependent anxiolytic

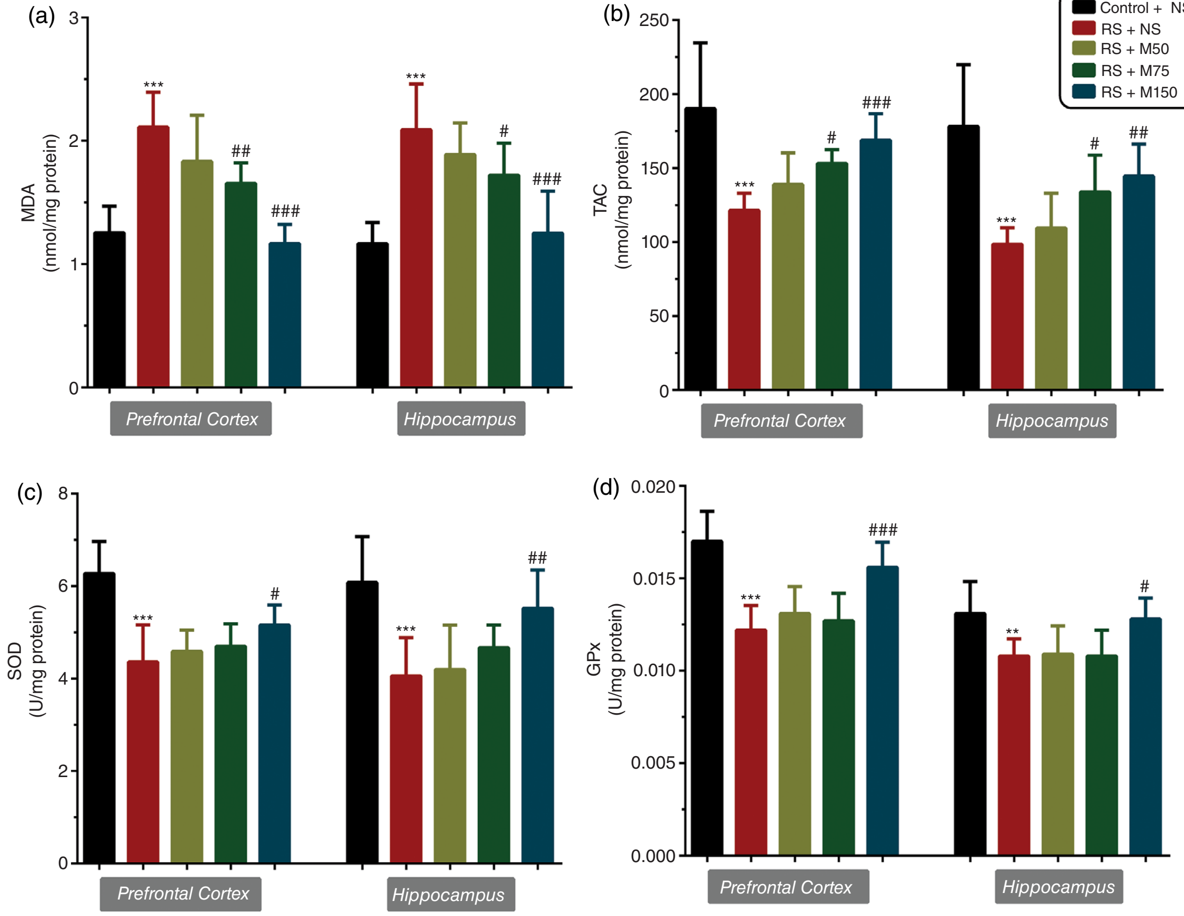

FIGURE 6 Effects of hydro-alcoholic extract of *Melissa officinalis* on (a) malondialdehyde (MDA) levels, (b) total antioxidant capacity (TAC), (c) superoxide dismutase (SOD), and (d) glutathione peroxidase (GPx) activities in the experimental groups. Data are expressed as means ± SD ($n = 10$). $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* control group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs.* normal saline-treated group. M50, M75 and M150: 50, 100 and 150 mg kg^{-1} hydro-alcoholic extract of *Melissa officinalis*

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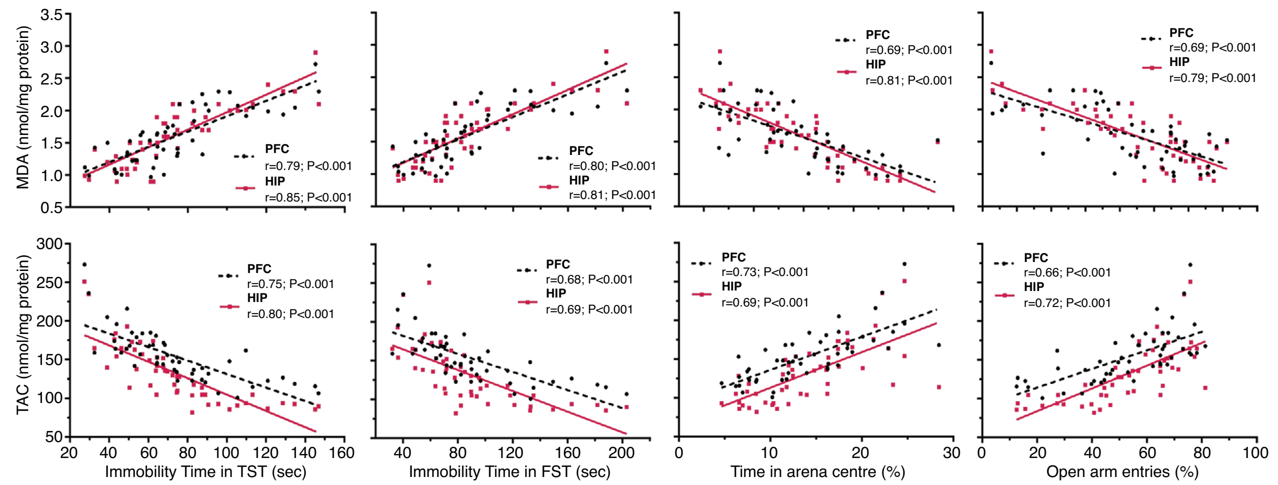

FIGURE 7 Correlation of anxiety and depressive-like behavioural indexes with malondialdehyde (MDA) level (upper row) and total antioxidant capacity (TAC) (lower row) in the hippocampus (HIP) and prefrontal cortex (PFC) regions

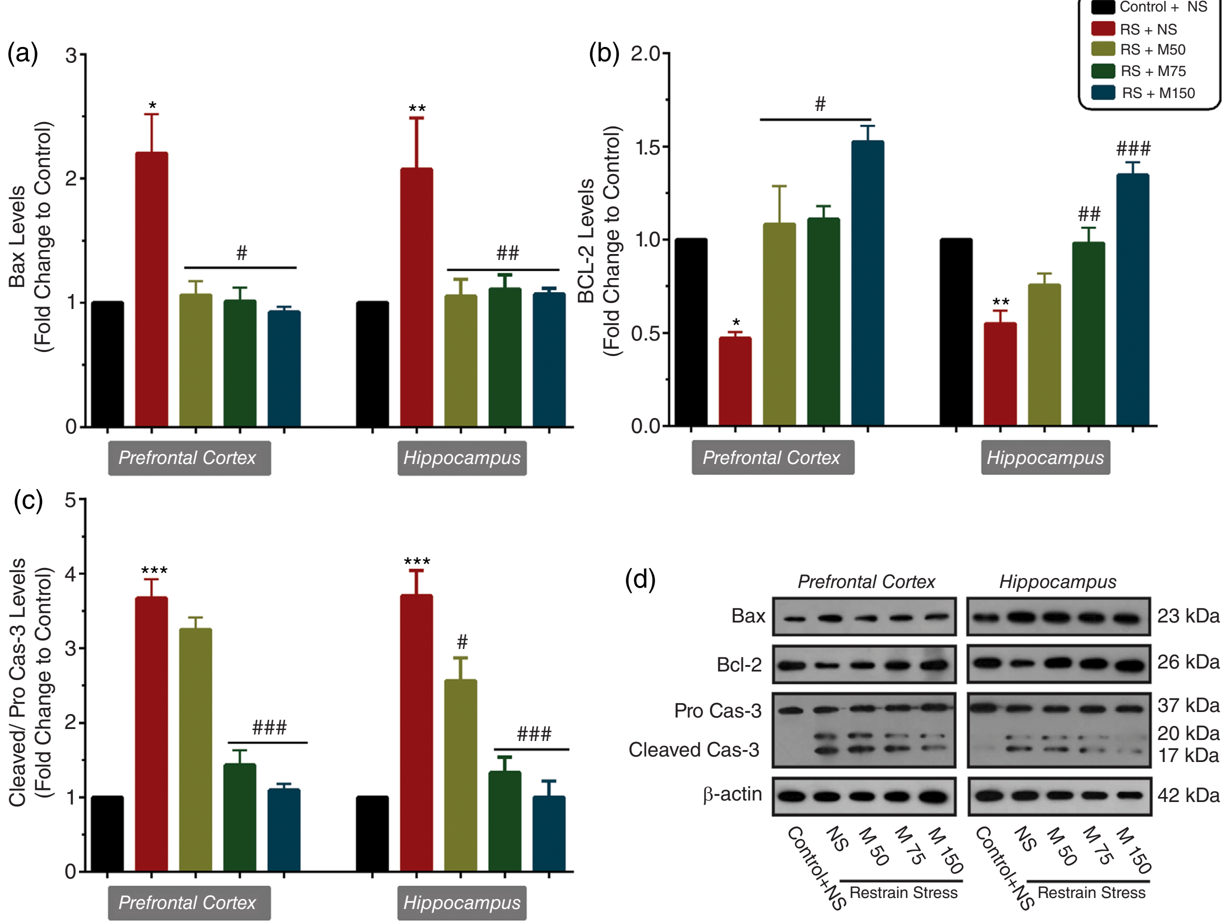

FIGURE 8 Effects of hydro-alcoholic extract of *Melissa officinalis* treatment on protein expression of apoptosis markers in the prefrontal cortex (PFC) and hippocampus (HIP) of the experimental groups. (a–c) Protein levels of Bax (a) and Bcl-2 (b), and cleaved caspase 3/pro-caspase 3 ratio (c) in the PFC and HIP regions. (d) Representative images of the protein bands assessed by immunoblotting. From top to bottom Bax, Bcl-2, pro-caspase 3, cleaved caspase 3 and β-actin. Data are expressed as means ± SD ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* control group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs.* normal saline-treated group. M50, M75 and M150: 50, 100 and 150 mg kg^{-1} hydro-alcoholic extract of *Melissa officinalis*

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(similar to diazepam) and anti-depressant (less than fluoxetine) effects of subacute administration of MO in non-stressed rats (Taiwo et al., 2012). Moreover, Lin et al. reported that MO decreased immobility time in the FST acute model through modulation of the serotonergic system (Lin et al., 2015). A clinical trial also reported that 8 weeks' administration of MO markedly attenuated stress, anxiety, depression and insomnia (Haybar et al., 2018). To the best our knowledge, the effect of monotherapy with MO in the treatment of stress-induced anxiety and depression has not been investigated.

Following chronic stress, stress hormones are persistently released from the adrenal glands, and mainly affect brain structures implicated in the regulation of emotions and mood. Clinical studies show that depressed patients demonstrate overactivity of the HPA axis and thereby hypercortisolaemia (Keller et al., 2017; Murphy, 1991). In line with previous reports (Chu et al., 2016; Mohammadi et al., 2019; Pandian Selvan & Rajan, 2016; Torres et al., 2001), it was found that chronic restraint stress elevated serum CORT levels, a biomarker of stress. Moreover, we found that HAEMO reduced serum CORT levels in restraint stress-exposed animals. Likewise, Yoo et al. have indicated that MO (50 or 200 mg kg^{-1} for 3-week) decreased serum CORT levels (Yoo et al., 2011). The combination of MO and *Passiflora caerulea* has also been shown to decrease plasma glucose and CORT levels in a chronic restricted movement stress model in mice (Feliú-Hemmelmann, Monsalve, & Rivera, 2013). RA has the most abundant content in MO, and has been shown to reduce anxiety- and depressive-like behaviours along with attenuation of serum CORT levels (Kondo, El Omri, Han, & Isoda, 2015; Makhathini, Mabandla, & Daniels, 2018). We suggest that the observed anxiolytic and anti-depressant effects, as well as diminishing serum levels of CORT, of MO are in part due to its high content of RA.

Dysregulation in oxidative stress systems is linked to the maladaptive consequences of chronic stress. A robust increase in oxidative stress markers is reported following physical or psychological stress (Maes et al., 2011; Schiavone, Jaquet, Trabace, & Krause, 2013). In fact, excessive ROS production and free radical levels disrupt the balance between the oxidant and antioxidant systems, which impairs mitochondrial function resulting in neuronal damage (Guo, Sun, Chen, & Zhang, 2013). Evidence also shows that HPA axis hyperactivity along with elevated catecholamine levels increases glucose availability and metabolic rate, enhancing free radical production and hence causing oxidative damage in the brain (Spiers, Chen, Sernia, & Lavidis, 2015; Teague et al., 2007). On the other hand, neurons are more vulnerable to oxidative stress due to their poor expression of endogenous anti-oxidants (Salim, 2017). Results of the present study also revealed that restraint stress increased oxidative factors and diminished enzymatic antioxidant activities and TAC in the PFC and HIP. These results were accompanied by increased serum concentration of CORT. It seems that during stress exposure, limited detoxification capacity of the antioxidant defence system in the neurons along with excessive production of free radicals leads to oxidative damage and thereby causes anxiety- and depressive-like behaviours. Previous studies, by our lab and others, have demonstrated that restraint stress, acute or chronic, increased lipid peroxidation and decreased SOD and GPx activity and TAC levels in the brain (Atif, Yousuf, & Agrawal, 2008; Fontella et al., 2005; Mohammadi et al., 2019; Salehpour et al., 2019). Moreover, clinical studies also established that anxiety and depression are linked to diminished levels of endogenous antioxidant capacity (T. Liu et al., 2015). In our study, there was a significant positive correlation between anxiety- and depressive-like behaviours and MDA levels and a negative correlation between these behaviours and TAC levels in the PFC and HIP regions.

The benefits of antioxidant supplementation in inhibiting stress-induced oxidative damage in the brain and depressive-like behaviours has been established (Chakraborti, Gulati, Banerjee, & Ray, 2007; Ghadrdoost et al., 2011; Moretti et al., 2013; Zaidi, Al-Qirim, & Banu, 2005). We also found that MO protected the PFC and HIP against oxidative stress induced by restraint stress, presented by diminished MDA levels and an enhanced enzymatic antioxidant defence system. Saberi et al. also reported that MO inhibited lipid peroxidation and increased activity of SOD and GPx in the indomethacin-induced gastric ulcer rat model (Saberi et al., 2016). Moreover, Martins et al. showed that MO aqueous extract (100 mg kg^{-1} day^{-1}) reduced lipid peroxidation, increased total thiol content, and restored SOD and catalase activities in the hippocampus and striatum of manganese-exposed mice (Martins et al., 2012). Several lines of work have confirmed the direct antioxidant and radical scavenging activities of MO, which is related to its chemical compounds including RA, flavonoids, gallic acid and phenolic compounds (Miraj et al., 2017; Moacă et al., 2018). In this study, the phytochemical analysis of HAEMO also revealed high amounts of flavonoids, total phenolic content and RA, as well as high ability to scavenge the free radical DPPH. Soodi et al., in an *in vitro* study, have found that MO extract attenuated amyloid β-induced oxidative stress and apoptosis in cerebellar granule neurons (Soodi, Dashti, Hajimehdipoor, Akbari, & Ataei, 2017). Therefore, the protective effects of HAEMO may stem from enhancing the antioxidant enzyme activities and inhibition of lipid peroxidation, or from its potent natural antioxidant components, such as RA.

Apoptosis has been proposed as an essential mechanism contributing to neurodegeneration in stress-induced depression (Kubera, Obuchowicz, Goehler, Brzeszcz, & Maes, 2011). Our results showed increased levels of Bax and cleaved caspase 3 in the PFC and HIP of stressed mice, while Bcl-2 was reduced, which suggest the activation of apoptosis pathways induced by stress. These findings were consistent with previous studies (Huang et al., 2015; Mohammadi et al., 2019; Woo, Hong, Jung, Choe, & Yu, 2018). Furthermore, MO reversed these changes, confirming that MO could protect against the restraint stress-mediated neural apoptosis in the PFC and HIP. These results were obtained at the end of the behavioural tests, just 19 days after the onset of stress and treatment. Hamza et al. have reported that MO pretreatment decreases Bax and caspase-3 protein levels in the cardiac tissue of rats (Hamza, Ahmed, Elwey, & Amin, 2016).

Several lines of studies have demonstrated the neuroprotective effect of MO, which is attributed to its anti-apoptotic property. Bayat et al. found that MO decreases caspase 3 activity, DNA fragmentation and apoptotic cell death in a cortical neuron culture as well as in the

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hippocampal CA1 subfield, which was attributed to its antioxidant effect (Bayat et al., 2012). Likewise, Hassanzadeh et al. have reported that MO decreases caspase 3 activity and apoptotic cell death in the hippocampal primary culture exposed to ecstasy (Hassanzadeh et al., 2011).

Oxidative stress is one of the major factors that induce mitochondria-mediated neuronal apoptosis (Méndez-Armenta, Nava-Ruíz, Juárez-Rebollar, Rodríguez-Martínez, & Yescas Gómez, 2014; Poh Loh et al., 2006). Besides, evidence shows that prolonged exposure to high concentrations of CORT can induce mitochondrial dysfunction and neuronal apoptosis (Gong, Zhang, Guo, & Fu, 2018; B. Liu et al., 2011; Woo et al., 2018). Additionally, the neuroprotective effect of RA, the main phytochemical content of MO, has been demonstrated in a large body of studies, and is mainly through suppression of oxidative stress and the apoptosis pathway (Cui et al., 2018; Khamse et al., 2018; H. J. Lee et al., 2008; Taram, Ignowski, Duval, & Linseman, 2018). It seems that both the increase in CORT and ROS overproduction could activate apoptotic signalling pathways, which might be ameliorated by MO through relieving the oxidative stress and CORT levels.

5 | CONCLUSION

In summary, the results of this study implicated the protective effect of higher doses of HAEMO on restraint stress-induced anxiety and depression by inhibiting the oxidative stress and apoptosis pathways in the PFC and HIP of mice.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

All Authors performed the experiments, interpreted the results, and wrote the first draft manuscript. S.S.-E., M.A.-K. and J.G.H. designed the experiments. J.G.H., S.S.-E. and M.A.-K. critically interpreted data and critically revised and the manuscript. All authors have read and approved the final version of this manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

ORCID

Javid Ghazizadeh https://orcid.org/0000-0002-8327-4925
Mostafa Araj-khodaei https://orcid.org/0000-0002-2590-8467

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