

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

**Running title:**

***Melissa officinalis* extract inhibits anxiety and depression in mice**

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**New Findings**

**What is the central question of this study?**

Prolonged stress exposure induces detrimental changes in the brain structure, and increases the vulnerability to develop psychiatric disorders. The central question of this study is how *Melissa officinalis* L. ameliorates anxiety- and depressive-like behavior of mice.

**What is the main finding and its importance?**

*Melissa officinalis* L. possessed anxiolytic and anti-depressant effects, which could mainly mediate through its antioxidant and anti-apoptotic properties.

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**Abstract**

**Objective:** This study evaluated the effects of hydro-alcoholic extract of *Melissa officinalis* (HAEMO) on anxiety- and depressive-like behaviors, oxidative stress, and apoptosis markers in the restraint-stress exposed mice.

**Methods:** In order to induce depression-like model, mice were subjected to restraint-stress (3 h/day for 14 days) and received normal saline or HAEMO (50, 75, and 150 mg/kg/day) for 14 days. The administered doses of HAEMO were designated based on one the main phenolic compounds present in the extract, rosmarinic acid (RA), concentration (2.55 mg/kg at lowest dose), other phytochemical analysis including assays for antioxidant activity, total phenols, and flavonoids contents were also carried out. The behavioral changes in the open field task, elevated plus maze, tail suspension, and forced swimming tests were evaluated. Also, malondialdehyde (MDA) levels and enzyme activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx), and total antioxidant capacity were assessed in the prefrontal cortex (PFC) and hippocampus (HIP). Moreover, levels of Bcl-2, Bax, and caspase 3, in the brain as well as serum concentration of corticosterone (CORT), were evaluated.

**Results:** HAEMO (75 and 150 mg/kg) significantly reversed anxiety- and depressive-like behaviors. Also, the HAEMO reduced MDA levels, enhanced enzymatic antioxidant activities, and restored serum levels of CORT. The immunoblotting analysis also demonstrated that HAEMO decreased levels of pro-apoptotic markers and increased anti-apoptotic protein levels in the PFC and HIP of restraint-stress exposed mice.

**Conclusion:** Our findings suggested that HAEMO reduced inflammation and had anxiolytic and antidepressant effects in mice.

**Keywords:** *Melissa officinalis*, Restraint-stress, Anxiety, Depression, Oxidative stress, Apoptosis

## Introduction

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

Anxiety and depression are the most common stress-related psychiatric diseases which disturb the normal physiological equilibrium of the body and impose a high cost to the public health. According to the statistics of WHO, 4.4% of the world's population worldwide suffer from depression, and by 2030, it will be the leading cause of disability ([Lépine & Briley, 2011](#); [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 HPA axis increasing circulating levels of stress hormones, cortisol in human and corticosterone (CORT) in rodents, thereby cause mood disorders ([Chiba et al., 2012](#); [Bruce S 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](#); [Bruce S. 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 defenses ([Maes et al., 2019](#)).

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

*Melissa officinalis* (MO) Lamiaceae family is a popular medicinal herb that is widely cultivated in Europe, United States, 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 United States. No serious side effects have been reported ([Ulbricht et al., 2005](#)). According to the literatures, MO contained different classes of phytochemicals like; 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, ant oxidative, sedative, and neuroprotective actions ([López et al., 2009](#); [Miraj, Rafeian, & Kiani, 2017](#)). Many of these pharmacological activities were mainly attributed to its phenolic and flavonoids constituents, mainly RA. RA, is considered as the one of major polyphenolic ingredients in this plant which could cross the blood brain barrier ([Falé, Madeira, Florêncio, Ascensão, & Serralheiro, 2011](#)) and has been reported to shorten immobility time in the 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 hydro-alcoholic extract of *Melissa officinalis* (HAEMO) on behavioral and molecular changes in the HIP and PFC of mice that induced by chronic restraint-stress with a particular focus on oxidative stress status and apoptosis markers.

## Materials and Methods

### Ethical Approval

All experimental procedures were 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: 2018-10-08. We have taken all steps to minimize the animals' pain and suffering.

### **Extract preparation**

Fresh MO aerial parts were collected from an organic farm placed 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 Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran (NO: Fph-Tbz 4031). Then aerial parts of the plant were dried in 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) for different maceration periods with occasional shakings at room temperature. Thereafter filtration of the extract, the solvent was evaporated to dryness using a rotary evaporator under *vacuum*. The yielded dried extract was stored in dark bottle inside a refrigerator (4 °C) until further analysis.

### **Extract phytochemical analysis**

#### **Total Rosmarinic Acid contents**

The reversed-phase high-pressure liquid chromatography (HPLC: Shimadzu-Japan) analysis consisting of an analytical column, C<sub>18</sub> Knauer column (250 mm L. × 4.6 mm I.D.) was performed to determine the RA content of the HAEMO. The mobile phase (flow rate: 15 mL/min) consisted 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 program: 0-15 min linear gradient of 0-10% acetonitrile (A) and 5% trifluoroacetic acid in water (B), 15-25 min isocratic 10% (A) and 25-50 min linear gradient 10-55% A. The detector was set at the wavelength of 280 nm. RA (PubChem CID: 5281792) was purchased from Sigma-Aldrich (USA). The standard solutions of RA were prepared in different concentrations through dilution of the stock standard RA solution. Later than 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](#)).

#### ***In-vitro* Antioxidant Activity Assay**

The antioxidant activity of HAEMO was evaluated *in-vitro* using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) protocol, which determined the ability of the extract to scavenge the DPPH free radicals. Briefly, 3 mL of DPPH solution 8% (V/V) were mixed separately with 3 mL of HAEMO in different concentrations and the obtained relative absorbance were

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. Eventually, the  $RC_{50}$  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](#)).

#### **Total phenols content (TPC)**

The TPC content of the HAEMO was assessed according to the Folin-Ciocalteu (FC) method, as previously described ([Chemsal et al., 2018](#)). Briefly, 1 mL of FC reagent was mixed with HAEMO (1 mg/mL) and  $Na_2CO_3$  7.5% (2 mL) and incubated in a dark place for 2 h at room temperature. Gallic acid was used as a standard. Subsequently, the absorbance was read at 765 nm. The results were expressed as mg Gallic acid equivalents (GAE)/100mg of the HAEMO.

#### **Total flavonoids content (TFC)**

The TFC of the HAEMO was assessed in a colorimetric method using  $AlCl_3$  reagent. For this purpose, 2 mL of aluminum trichloride was mixed with 1 mg/mL of HAEMO solution and incubated for 10 min at room temperature. Then, the absorbance was read at 415 nm. The quercetin calibration curve (10-100  $\mu$ g/mL) was used to determine the TFC of the HAEMO as the standard. The results were expressed as mg Quercetin equivalents (QE)/100mg of the HAEMO.

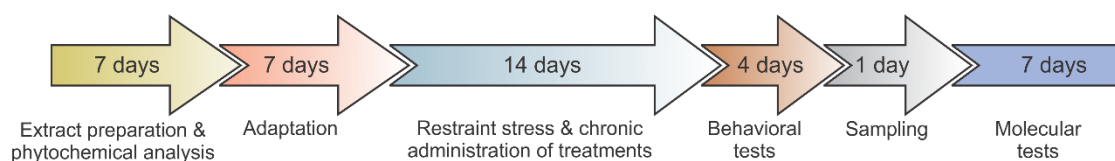
#### **Animals**

We used sixty albino BALB/c male mice, weighing 25-28 g, from Animal House of Tabriz University of Medical Sciences (Tabriz, Iran). After transferring to the animal house of Neuroscience Research Center, mice housed 5 mice 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.

#### **Experimental design and grouping**

Following a week of adaptation to the new condition, animals were randomly assigned into 5 groups (n=12/group) as follows: control, restraint-stress, restraint-stress+M50, restraint-stress+M75, and restraint-stress+M150 groups. 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 for two weeks, from 8:00 a.m. to 11:00 a.m.) 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/day of HAEMO for 14 days. The dosages of administration chosen based on previous reports ([Emamghoreishi & Talebianpour, 2009](#)), Taiwo et al. (2012), Xiang Jin et al. (2013), Shin-Hang 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/day) considered for dosage optimization ([Komes et al., 2011](#); [Lin et al., 2015](#); [Mahboubi, 2019](#); [Pereira et al., 2005](#)). Fig. 1 shows the timeline of the study.



**Fig. 1.** Schematics show the timeline of the study.

### Behavioral analysis

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

### Open field test (OFT)

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 traveled, 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 odor.

### **Elevated plus maze (EPM)**

The EPM apparatus was a wooden device consisted 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 center of the apparatus facing an open arm. The behaviors of the animal were recorded for 5 min and the percentage of time spent in the open arms (%OAT), as well as the percentage of entries into open arms (%OAE), were calculated ([Majdi et al., 2018](#)).

### **Forced swimming test (FST)**

The FST test was performed as previously described by Juszcak et al. ([Juszcak, 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  $25 \pm 2$  °C, and left to swim for 6 min. Total immobility time, time which animal ceased struggling and remained floating motionless in the water and making basic movements to hold the head above water was calculated in the last 4 min. Then mice were removed, dried, and returned to their home cage. Following each test, the water of the tank was renewed.

### **Tail suspension test (TST)**

In this test, the tip of the tail of each mouse was fixed to a metal hook attached to the center 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](#)).

### **Sampling**

After the last behavioral test, animals were anesthetized with ketamine (80 mg/kg) and xylazine (8 mg/kg) via intraperitoneal injection. We have taken 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 4000 rpm for 10 min at 4°C. Animals were sacrificed 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.



### **Serum levels of CORT**

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

### **Brain biochemical assessments**

#### **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 10000 rpm for 10 min at 4 °C, and the supernatant was collected. The protein content was determined by the Bradford method.

#### **Malondialdehyde (MDA) concentration**

The 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 ([Pourmemar et al., 2017](#)).

#### **Superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities**

The enzyme activity of SOD was assessed using a RANSOD kit (Randox Laboratories Ltd, Crumlin, United Kingdom) 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.

The enzyme activity of GPx was also measured using the RANSEL laboratory kit (Randox Laboratories Ltd, Crumlin, United Kingdom). 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.

#### **Total antioxidant capacity level**

The TAC was determined according to the 2'-azinobis [3-ethylbenzothiazoline-6-sulfonic acid] (ABTS<sup>+</sup>) method using a Randox total antioxidant status kit (Randox Laboratories Ltd, Crumlin, United Kingdom). The absorbance was measured at 600 nm using a spectrophotometer and expressed as nmol/l ([Pourmemar et al., 2017](#)).

### 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  $\mu$ l RIPA lysis buffer containing protease inhibitors cocktail (Roche, Germany) using a tissue homogenizer. To obtain supernatant, the homogenate was centrifuged at  $12,000 \times g$  for 15 min at 4 °C, and total protein concentration in the supernatant was estimated using the Bradford method. Next, 20  $\mu$ g of protein samples were loaded to 12.5% SDS-polyacrylamide gel and separated by electrophoresis then transferred onto a polyvinylidene difluoride (PVDF) (Roche, United Kingdom) membranes. We also blocked non-specific binding reactions by incubation of the membranes with bovine serum albumin [BSA] 3% in Tris-buffered saline [pH 7.5] for 2 h at the room temperature. Subsequently, the membranes were probed overnight with mouse primary antibodies (Santa Cruz Biotechnology, U.S.A) including anti-Bax (sc-70405), anti-Bcl-2 (sc-7382), anti-caspase-3 (sc-56053), and anti- $\beta$ -actin (sc-47778) and then with horseradish peroxidase-conjugated (HRP) goat anti-mouse IgG secondary antibody (1:5000, sc-2005) for 2 h at room temperature. Finally, the membrane was washed with PBS then soaked in enhance chemiluminescence (ECL) detection reagents (Amersham, UK) and exposed to X-ray film (Kodak, USA). The density of protein bands was quantified by Image J (version 1.62, National Institutes of Health, Bethesda, MD, USA) software and then normalized to the corresponding internal control,  $\beta$ -Actin protein.

### Statistical analysis

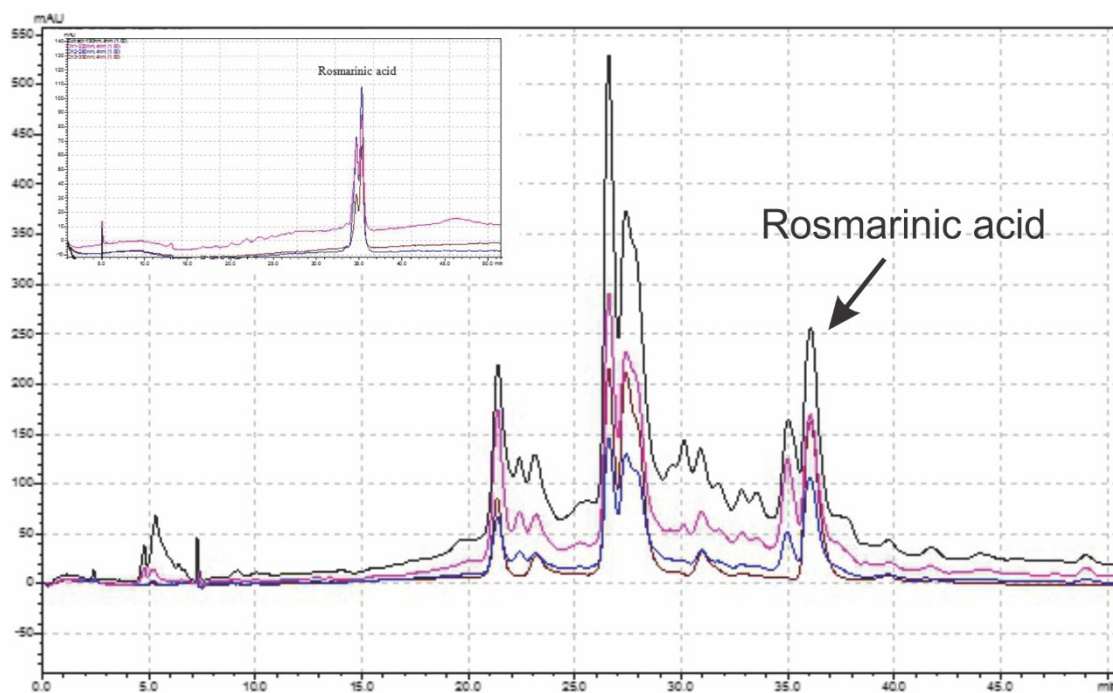
The results were expressed by mean  $\pm$  Standard Deviation (SD). The statistical analysis was performed using Graph Pad Prism 6.01 (Graph Pad Software Inc., La Jolla, CA, USA). Experimental data were subjected to one-way analysis of variance (ANOVA) followed by Tukey *post-hoc* test to evaluate the differences among the groups. A p-value  $<0.05$  was regarded to be significant. A Pearson's correlation analysis was conducted to detect the correlation of variables.

### Results

#### Preliminary phytochemical analysis

##### *RA contents of the HAEMO*

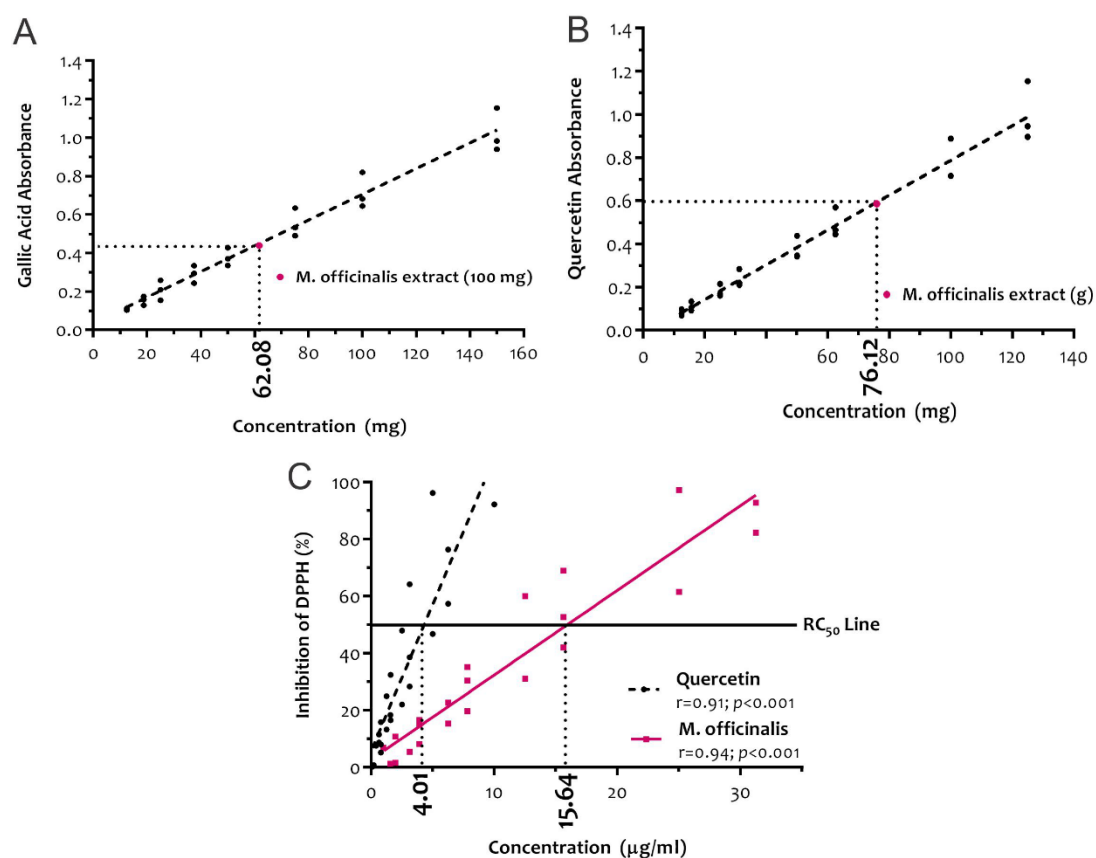
The results of HPLC analysis of HAEMO demonstrated that RA content of the HAEMO was 5.1% (w/w) of the dried extract in the retention time of 36 min (Fig. 2).



**Fig. 2** HPLC chromatograms of standard rosmarinic acid (Left up) and *M. officinalis* hydroalcoholic extract demonstrating rosmarinic acid at retention time of 36 min.

#### ***Total phenol and flavonoid, and DPPH radical scavenging activity of the HAEMO***

Maceration of the aerial parts of MO with ethanol 70% led to the extraction of greenish residue with a yield of 20.85% (W/W). Moreover, Total phenolic and total flavonoid contents of the MO were  $62.085 \pm 1.136$  mg GAE/100 mg (dry wt.) HAEMO. In fact  $129.45 \pm 2.37$  as mg GAE /g (dry wt.) aerial parts of MO (n=3) (Fig. 3A) and  $7.612 \pm 1.368$  mg QE/100 mg (dry wt.) HAEMO. In fact,  $15.87 \text{ mg/gr} \pm 2.85$  as mg Quercetin/g (dry wt.) aerial parts of MO (n=3) (Fig. 3B), respectively. In addition, antioxidant potential, DPPH free radical scavenging activity ( $RC_{50}$ ), for quercetin and HAEMO were 4.01 and 15.64  $\mu\text{g/mL}$ , respectively (Fig.3 C). RSA value for HAEMO was 3.9.



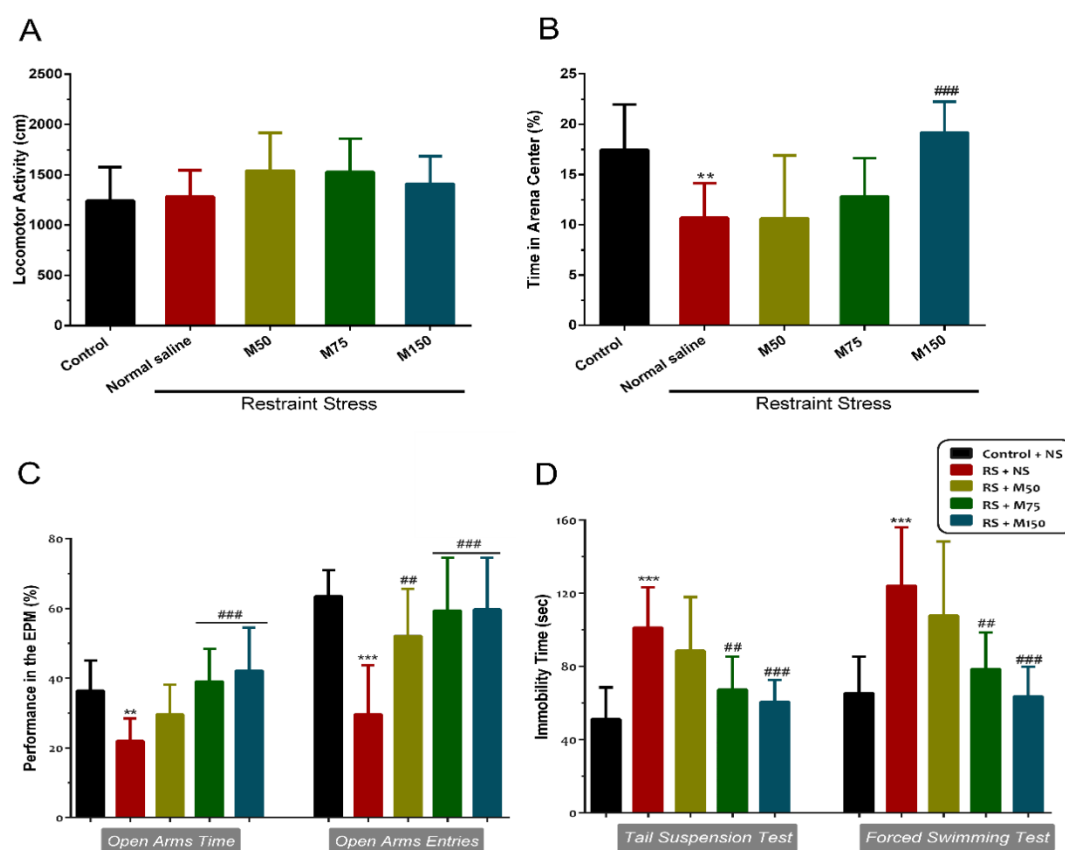
**Fig. 3** Preliminary phytochemical analysis of hydro-alcoholic extract of *Melissa officinalis*. **A)** Total phenols content, **B)** flavonoids content, and **C)** DPPH radical scavenging activity of the hydro-alcoholic extract of *Melissa officinalis* along with relative standards.

#### Anti-depressant and anxiolytic effects of the HAEMO

Although there was no significant difference in the locomotor activity among groups in the OFT (Fig. 4A), the time spent in the central zone was markedly decreased in the restraint group, which was significantly increased by MO administration at dose of 150 mg/kg ( $p<0.001$ ; Fig. 4B).

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

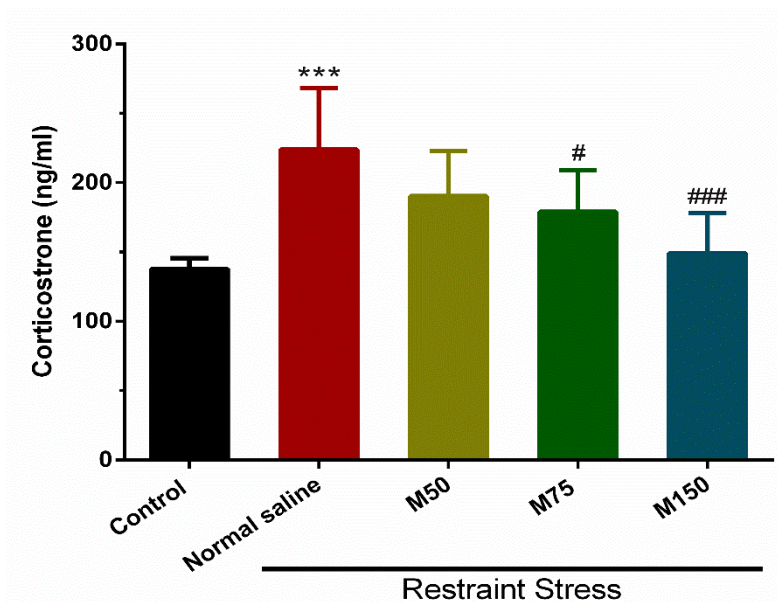
The results of the behavioral tests revealed that restraint-stress markedly increased immobility time in the FST ( $p < 0.001$ ; Fig. 4D, right panel) and TST ( $p < 0.001$ ; Fig. 4D, left panel) compared to the control mice. However, MO-treated animals showed shorter immobility time in the FST ( $p < 0.01$  at dose of 75 mg/kg and  $p < 0.001$  at dose of 150 mg/kg) and TST ( $p < 0.01$  at dose of 75 mg/kg and  $p < 0.001$  at dose of 150 mg/kg), indicating anti-depressant effect of MO.



**Fig. 4** Effects of hydro-alcoholic extract of *Melissa officinalis* on the anxiety- and depressive-like behaviors assessed in the experimental groups. **A)** Locomotor activity and **B)** time spent in the arena center of the open field test (OFT). **C)** The percentage of open arm time (OAT, left panel) and open arm entries (OAE, right panel) in the elevated plus maze (EPM). **D)** Immobility time in the forced swimming test (FST, right panel) and tail suspension test (TST, left panel). Data are expressed as mean  $\pm$  SD ( $n=12$ ). \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  vs. control group. ##  $p < 0.01$ , ###  $p < 0.001$  vs. normal saline-treated group. [M50: hydro-alcoholic extract of *Melissa officinalis* 50 mg/kg; M75: hydro-alcoholic extract of *Melissa officinalis* 100 mg/kg; M150: hydro-alcoholic extract of *Melissa officinalis* 150 mg/kg].

### HAEMO regulates serum concentration of CORT

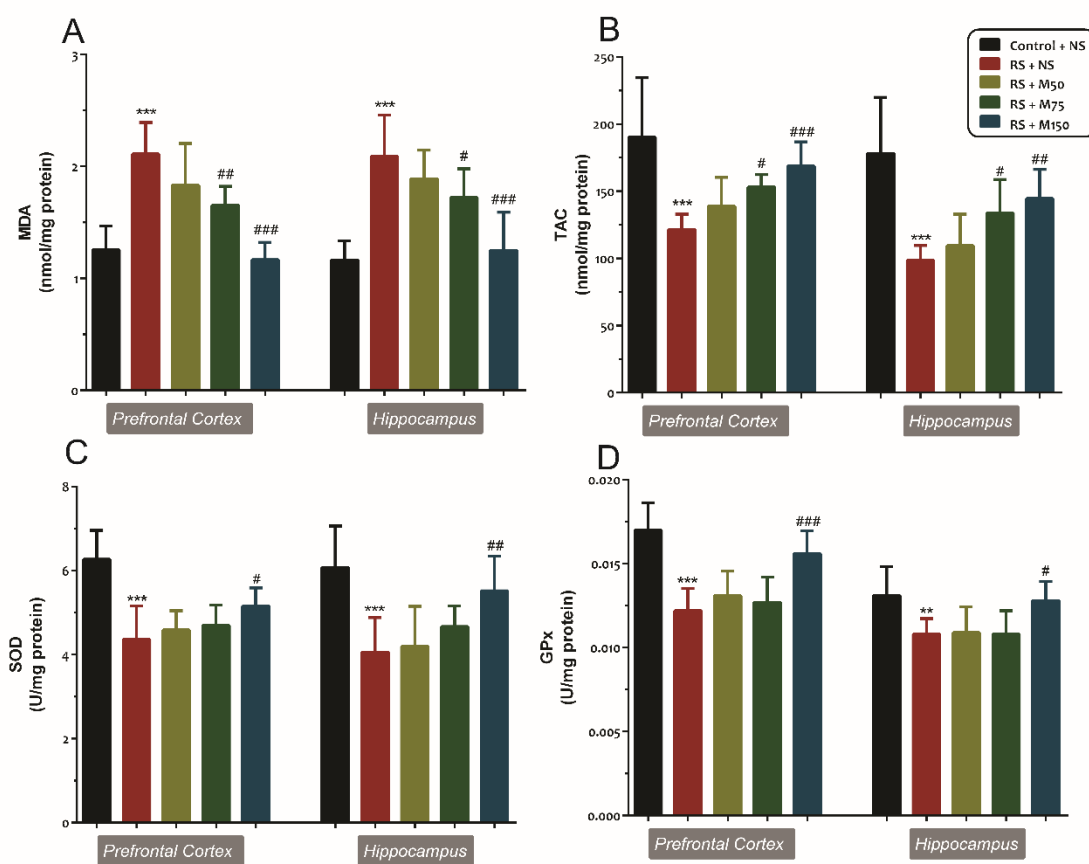
The result of ELISA revealed an increase in the serum levels of CORT in the normal saline-treated group ( $p < 0.001$ ), which was significantly declined in the MO-treated groups in doses of 75 ( $p < 0.05$ ) and 150 mg/kg ( $p < 0.001$ , Fig. 5).



**Fig. 5** Effects of hydro-alcoholic extract of *Melissa officinalis* on serum concentration of corticosterone in the experimental groups. Data are expressed as mean  $\pm$  SD ( $n=10$ ). \*\*\* $p < 0.001$  vs. control group. ### $p < 0.001$  vs. normal saline-treated group. [M50 hydro-alcoholic extract of *Melissa officinalis* 50 mg/kg; M75: hydro-alcoholic extract of *Melissa officinalis* 75 mg/kg; M150: hydro-alcoholic extract of *Melissa officinalis* 150 mg/kg].

### HAEMO modulated lipid peroxidation and antioxidant defense system

The result of the biochemical assay also demonstrated that restraint stress exposure significantly increased MDA levels (Fig. 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, restraints stress significantly decreased enzymatic activities SOD ( $p < 0.001$  for both regions; Fig. 6C) and GPx ( $p < 0.001$  for PFC,  $p < 0.01$  for HIP; Fig. 6D), and reduced TAC ( $p < 0.001$  for both regions, Fig. 6B) levels 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; HIP:  $p < 0.01$  at dose 150 mg/kg) and GPx (PFC:  $p < 0.001$  at dose 150 mg/kg; HIP:  $p < 0.05$  at dose 150 mg/kg) activities and TAC levels in the PFC and HIP regions (PFC:  $p < 0.05$  at dose 75 mg/kg and  $p < 0.001$  at dose 150 mg/kg; HIP:  $p < 0.05$  at dose 75 mg/kg and  $p < 0.01$  at dose 150 mg/kg) compared with that of the normal saline group.



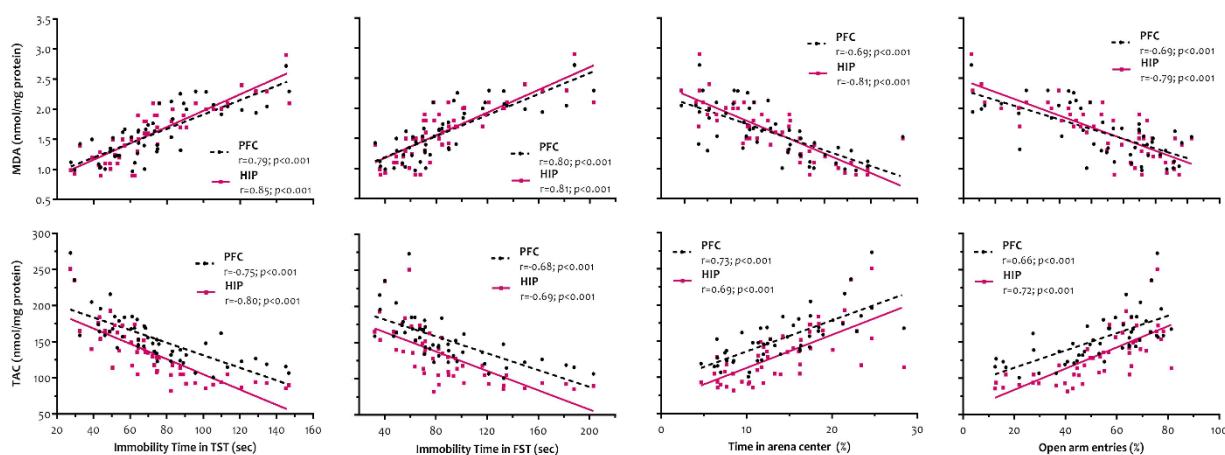
**Fig. 6** Effects of hydro-alcoholic extract of *Melissa officinalis* on **A)** malondialdehyde (MDA) levels, **B)** total antioxidant capacity (TAC), **C)** superoxide dismutase (SOD), and **D)** glutathione peroxidase (GPx) activities in the experimental groups. Data are expressed as mean  $\pm$  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: hydro-alcoholic extract of *Melissa officinalis* 50 mg/kg; M75: hydro-alcoholic extract of *Melissa officinalis* 75 mg/kg; M150: hydro-alcoholic extract of *Melissa officinalis* 150 mg/kg].

### Behavioral indexes and antioxidant defense system correlation analysis

According to the results of Pearson's correlation analysis, immobility time in the TST and FST exhibited a significant positive correlation 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 center with TAC levels and a negative correlation with MDA levels in the HIP and PFC were observed. Furthermore, a significant positive correlation between open



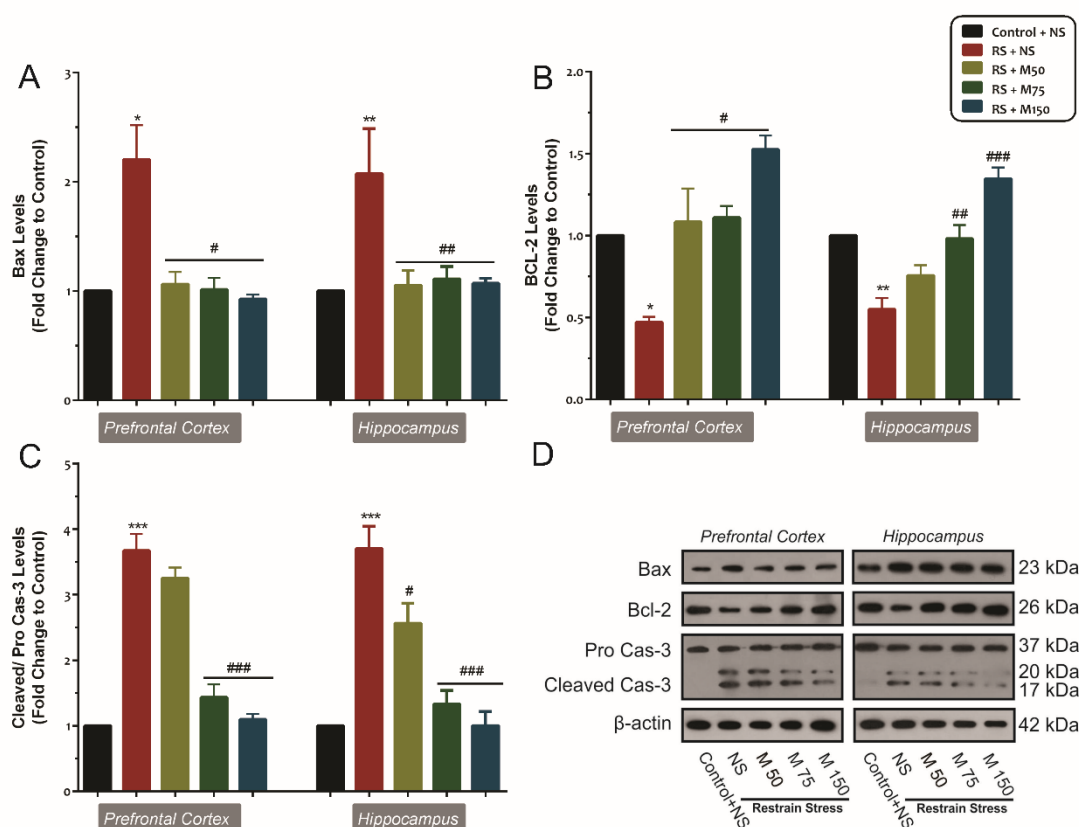
arms entries and TAC levels and a negative correlation with MDA levels were identified in the HIP and PFC regions (Fig. 7).



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

### HAEMO regulated mitochondria-mediated pro- and anti-apoptotic markers

As shown in Fig. 5, the protein levels of Bcl-2 were significantly decreased in the normal saline group in the PFC ( $p < 0.05$ , Fig. 8B, left panel) and HIP ( $p < 0.05$ , Fig. 8B, right panel) of the normal saline received group. Whereas, restraint-stress exposure significantly increased protein levels of Bax (PFC:  $p < 0.05$ , Fig. 8A, left panel; HIP:  $p < 0.01$ , right panel) and cleaved caspase 3/pro-caspase 3 ratio ( $p < 0.001$  for both regions, Fig. 8C) in the PFC and HIP compared to the control animals. MO 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 and  $p < 0.001$  at a dose of 150 mg/kg) in the PFC and HIP of restraint-stress-subjected mice.



**Fig. 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. Protein levels of **A)** Bax and **B)** Bcl-2, **C)** Cleaved caspase3/pro-caspase 3 ratio in the PFC and HIP regions. **D)** Representative images of the protein bands from top to the bottom Bax, Bcl-2, pro-caspase 3, cleaved caspase 3, and  $\beta$ -actin assessed by immunoblotting. Data are expressed as mean  $\pm$  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: hydro-alcoholic extract of *Melissa officinalis* 50 mg/kg; M75: hydro-alcoholic extract of *Melissa officinalis* 75 mg/kg; M150: hydro-alcoholic extract of *Melissa officinalis* 150 mg/kg].

## Discussion

The results of the present study demonstrated that chronic treatment with HAEMO, at doses of 75 and 150 mg/kg, attenuated restraint-stress-induced anxiety and depressive-like behaviors through the amelioration of oxidative stress and apoptosis. Immobilization/restraint-stress is considered as the most severe type of stress in rodent models, which induces both emotional and physical endurance ([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, resulting from abnormal responses to acute or prolonged stressors ([Khan & Khan, 2017](#)).

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

A previous study has reported anti-depressant-like activity of aqueous extract of MO similar to imipramine in the FST in non-stressed mice ([Emamghoreishi & Talebianpour, 2015](#)). Taiwo et al. also showed gender and administration length-dependent anxiolytic (similar to diazepam) and anti-depressant (less than fluoxetine) effects of sub-acute 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-week administration of MO markedly attenuated stress, anxiety, depression, and insomnia ([Haybar et al., 2018](#)). To the best of 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, which 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, hypercortisolemia ([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 for 3-week) decreased serum CORT levels ([Yoo et al., 2011](#)). Combination of MO and *Passiflora caerulea* has also been shown to decrease plasma glucose and CORT levels in chronic restricted movement stress model in mice ([Feliú-Hemmelmann, Monsalve, & Rivera, 2013](#)). The RA is the most abundant content of MO, which has been shown to reduce anxiety and depressive-like behaviors 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 by MO, is 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 system, which impair mitochondrial function resulting in neuronal damage ([Guo, Sun, Chen, & Zhang, 2013](#)). Evidence also shows that HPA axis hyperactivity along with elevated catecholamine levels increase glucose availability and metabolic rate enhancing free radical productions and hence cause 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 antioxidants ([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 defense system in the neurons along with excessive production of free radicals leads to oxidative damage and therefore causes anxiety and depressive-like behaviors. Previous studies, by our lab and others, have demonstrated that restraint-stress, acute or chronic, increased lipid peroxidation and decreased SOD, GPx, 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 antioxidants capacity ([T. Liu et al., 2015](#)). In our study, there was a significant positive correlation between anxiety- and depressive-like behaviors and MDA levels and a negative correlation between these behaviors and TAC levels in the PFC and HIP regions.

The beneficial of antioxidants supplementation in inhibiting the stress-induced oxidative damage in the brain and depressive-like behaviors has been established ([Chakraborti, Gulati, Banerjee, & Ray, 2007](#); [Ghadroost 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 enhanced enzymatic antioxidant defense 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/day) 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 studies 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 contents, and RA, as well as high ability to scavenge free radical DPPH. Soodi et al., in an *in vitro* study, have found that MO extract attenuated amyloid beta-induced oxidative stress and apoptosis in cerebellar granule neurons ([Soodi, Dashti, Hajimehdipour, Akbari, & Ataei, 2017](#)). Therefore, the protective effects of HAEMO may stem from enhancing the antioxidant enzyme activities and inhibition of lipid peroxidation, or 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 behavioral 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. have found that MO decreases caspase 3 activity, DNA fragmentation, and apoptotic cell death in the cortical neuron culture as well as in the 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 mitochondrial-mediated neuronal apoptosis ([Méndez-Armenta, Nava-Ruiz, 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, which is mainly through suppression of oxidative stress and apoptosis pathway ([Cui et al., 2018](#); [Khamse et al.](#); [H. J. Lee et al., 2008](#); [Taram, Ignowski, Duval, & Linseman, 2018](#)). It seems that the both increase in CORT and ROS overproduction could activate apoptotic signaling pathways, which might be ameliorated by MO through relieving the oxidative stress and CORT levels.

### **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.

### **Additional Information**

#### **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. SS-E, MA-K and JGH designed the experiments. JGH, SS-E, and MA-K critically interpreted data and critically revised and approved the manuscript. All authors approved the final version of the manuscript for publication.

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