Journal of Integrative Neuroscience

J. Integr. Neurosci. **2023**; 22(4): 98
https://doi.org/10.31083/j.jin2204098

Original Research

Malic Acid Improves Behavioral, Biochemical, and Molecular Disturbances in the Hypothalamus of Stressed Rats

Khaled M. M. Koriem[1,*], Hatem A. K. Tharwat[2]

[1]Department of Medical Physiology, Medical Research and Clinical Studies Institute, National Research Centre, Dokki, 12622 Giza, Egypt
[2]Faculty of Veterinary Medicine, Cairo University, 12211 Giza, Egypt
*Correspondence: kkoriem@yahoo.com (Khaled M. M. Koriem)
Academic Editor: Gernot Riedel
Submitted: 27 March 2023 Revised: 7 May 2023 Accepted: 9 May 2023 Published: 17 July 2023

Abstract

Background: Stress can lead to emotional and mental symptoms such as anxiety, sadness, panic attacks, and depression. Malic acid was chosen due to malic acid has the ability to improve antioxidant activity and improves liver damage. This study evaluates malic acid anti-depressant activity in the hypothalamus of stressed rats. **Methods**: Thirty-six male albino rats were divided into 2 equal groups; Normal and chronic mild stress (CMS) rats. Normal rats were divided into 3 equal groups; control, malic acid, and venlafaxine drug groups: normal rats were administered orally with 1 mL of saline solution, 250 mg/kg of malic acid, and 20 mg/kg of venlafaxine drug, respectively. CMS rats were divided into 3 equal groups; CMS, CMS + malic acid, and CMS + venlafaxine drug: CMS rats were administered orally with 1 mL of saline solution, 250 mg/kg of malic acid, and 20 mg/kg of venlafaxine drug, respectively. All the above-mentioned treatments were administered once a day by oral gavage for 6 weeks. **Results**: The obtained results revealed that the animal behavioral tests such as forced swimming test, tail suspension test, sucrose preference test, and open-field test (center square entries test, center square duration test, and distance travelled test), norepinephrine, dopamine, serotonin, γ-aminobutyric acid, nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase activity, oxidative index, conjugated dienes, catalase, glutathione peroxidase, superoxide dismutase, malondialdehyde, interleukin-6, tumor necrosis factor-α, interleukin-10, interleukin-1β, sodium/potassium-ATPase activity, and histamine-N-methyl transferase (*Hnmt*) and tyrosine hydroxylase (*TH*) enzymes in the hypothalamus of stressed rats, were returned to approaching the normal state in the stressed group after treating with malic acid for 6 weeks. **Conclusions**: Malic acid ameliorated stressed-related symptoms and it inhibited superoxide anion and neuro-inflammation in the hypothalamus of stressed rats.

Keywords: stress; antioxidants; neurotransmitters; inflammation; histology

1. Introduction

Changes in physiology, the nervous system, hormones, and behavior are all a result of stress. Continuous excessive stress led to obsessive brain damage and serious neurological malfunction [1]. Human memory and the brain are impacted by stress.

By interfering with biological hormones and proteins within the human body, it changes the brain and memory and thus leads to human neurological dysfunction [2].

Prolonged stress throughout adolescence increases the risk of developing neuropsychiatric disorders because it alters neuronal structure and behavior in all major brain regions [3]. The hypothalamus was selected for this study because it is a more precise target for stress or depression [4] due to its involvement in two main axes: (1) the hypothalamic-pituitary-adrenal (HPA) axis, which is a major factor in the neural/hormonal cycle that reacts to both internal and external stressors. Human mental and physical states are completely under the control of the HPA axis, and dysregulation of this axis has been linked to a number of mental and physical diseases [5]. (2) Stress or depression also inhibits the hypothalamic-pituitary thyroid (HPT) axis. Many interactions exist between the aminergic systems that play a role in stress or depression and the HPA- and HPT-axis [6]. Emotional and mental symptoms like anxiety or irritation, melancholy, panic attacks, and depression can be brought on by stress. Stress on an ongoing internal and external level leads to depression. Over 350 million people worldwide suffer from depression [7]. Depression is characterized by a decrease in brain neurotransmitter levels, an increase in the hypothalamic-pituitary-adrenal axis, elevated inflammatory markers, and changes in the gut flora [8].

The potential processes underlying the link between stress and depression have been the subject of numerous recent investigations. These research [9] demonstrated links between stress and depression, including those involving the immune system, the microbiota, hormones, and brain abnormalities. In the USA, depression affects adolescents and accounts for one-third of adolescent fatalities [10]. There are many animal models that mimic human depression, but the chronic mild stress (CMS) model is the most widely used model that replicates the main symptoms of human depression [11]. In this model, rats are exposed to multiple stressors, which alters their behavior and biochemistry by altering the chemistry, plasticity, expression, and function of their neurons, neuroreceptors, and neurotrophin [12].

Depression is a chronic illness that lasts for years; as a result, it requires affordable and secure treatment. Given its high antioxidant activity, malic acid was selected for this investigation from natural herbs, which are a viable source in this regard [13]. In this regard, malic acid enhanced brain neural connection function, reduced inflammation, improved amino acid metabolism, energy metabolism and neurotransmitter metabolism, and decreased dopaminergic degeneration [14], Malic acid is used to treat fibromyalgia [15], where it has a neuroprotective role against the loss of glutamic acid decarboxylase activity, depletion of γ-aminobutyric acid level, increased propidium iodide uptake, and increased protein level [16]. Malic acid is a naturally occurring acid with the chemical formula $C_4H_6O_5$ that is produced by numerous species [17,18]. Malates are the names for the salts and esters of malic acid [19]. Malic acid is a prominent component of several fruits, including blueberries, cherries, blues, apricots, blackberries, plums, grapes, pears, mirabelles, peaches, and quince. Malic acid is also a minor component of citrus [20]. A new kind of phytopharmaceutical with cardioactive properties is malic acid [21]. Malic acid increases catalase and ascorbate peroxidase by acting as an antioxidant. In the plant, it binds to cadmium and lowers the concentration and toxicity of the metal [22]. Malic acid can increase antioxidant activity and slow down ageing [23]. Malic acid has a clear therapeutic effect on treating liver damage, and as a result, it protects the liver [24]. Malic acid is used to treat the consequences of diabetes mellitus (type 2), such as xerostomia [25]. Malic acid is a crucial component of clinical nanomedicine since it is utilized to diagnose and treat diseases [26].

This study aims to study the anti-depressant activity of malic acid through modifying animal behavior, neurotransmitters, oxidative stress, inflammation, sodium/potassium-ATPase activity, and histamine-N-methyl transferase (*Hmnt*) and tyrosine hydroxylase (*TH*) enzymes in the hypothalamus of CMS rats.

2. Materials and Methods

2.1 Materials

DL-malic acid (99% pure malic acid, 6915-15-7, Sisco Research Laboratories (SRL) Pvt. Ltd., New Delhi, India). Ethylene glycol-bis(2-amino-ethylether)-N,N,N',N'-tetraacetic acid (EGTA), nicotinamide-adenine dinucleotide phosphate (NADPH), potassium phosphate monobasic (KH_2PO_4), lubrol, and lucigenin (9, 9'-bis[N-methyl acridinium nitrate) were purchased from Sigma-Aldrich Co (Merck Group, St. Louis, MO, USA). Venlafaxine (93413-69-5, a standard anti-depressant drug used for depression treatment) was obtained from the International Drug Agency for Pharmaceutical Industry, Cairo, Egypt. All kits reagents used for biochemical analysis were purchased from Bio-diagnostics Company (Birmingham, UK), by a local Egyptian branch.

2.2 Animals

The animal house of the National Research Centre, Dokki, Giza, Egypt provided this study by thirty-six male albino rats of *Sprague Dawley* strain (130 $\pm$ 10 g, 12 weeks old). The experimental plastic cages held the animals. They were fed commercial rat food and drank tap water. The study was carried out following receipt of the approval sheet (approval number 12041126) from the National Research Centre of Egypt's ethics committee. The research used laboratory animals with the appropriate handling and care (NIH publication no. 85:23, revised 1985).

2.3 Study Protocol

Thirty-six male albino rats were divided into 6 equal groups (6 rats/group) as follows: Control group: normal rats were administered orally with 1 mL of saline solution. Malic acid-treated group: normal rats were administered orally with 250 mg/kg of malic acid [27] dissolved in 1 mL distilled water. This dose increases the motor activity and excitatory processes in the sensory motor brain areas. This dose increases carbohydrate reserves and decreases oxygen consumption of the brain tissues [27]. Venlafaxine drug-treated group: normal rats were administered orally with 20 mg/kg of the venlafaxine drug [28] dissolved in 1 mL distilled water. CMS group: CMS animals were administered orally with 1 mL of saline solution. CMS + malic acid (250 mg/kg)-treated group: CMS rats were administered orally with malic acid (250 mg/kg of malic acid dissolved in 1 mL water). CMS + Venlafaxine drug (20 mg/kg)-treated group: CMS rats were administered orally with 20 mg/kg of Venlafaxine drug dissolved in 1 mL distilled water.

During 6 weeks, oral gavage was used to provide all of the aforementioned therapies once daily. All rats were observed throughout the entire study for any aberrant symptoms, such as rat hair loss, skin patches, convulsions, and a reduction of normal locomotors activity, as well as, any deaths.

2.4 Chronic Mild Stress Induction

The authors have already conducted this stress animal model [29–31]. According to a predetermined plan, the normal rats were exposed to 1 or 2 stressors everyday as follows: The first day involved forced swimming in hot water (45 °C for 5 minutes); the second day cage tilting and wet bedding; the third day animal shaking for 10 minutes; the fourth day nipping the tail for 1 minute; the fifth day involved forced swimming in cool (4 °C for 5 minutes); the sixth day food deprivation for 24 hours and overnight illumination; the seventh day water deprivation for 24 hours and all the above aforementioned stressors were applied once a week for 6 weeks, after which time all rats underwent the following tests: (1) sucrose preference test, (2) open-field test (which included the distance travelled test, the center square entries test, and the center square duration test), (3) tail suspension test, and (4) forced swimming test.

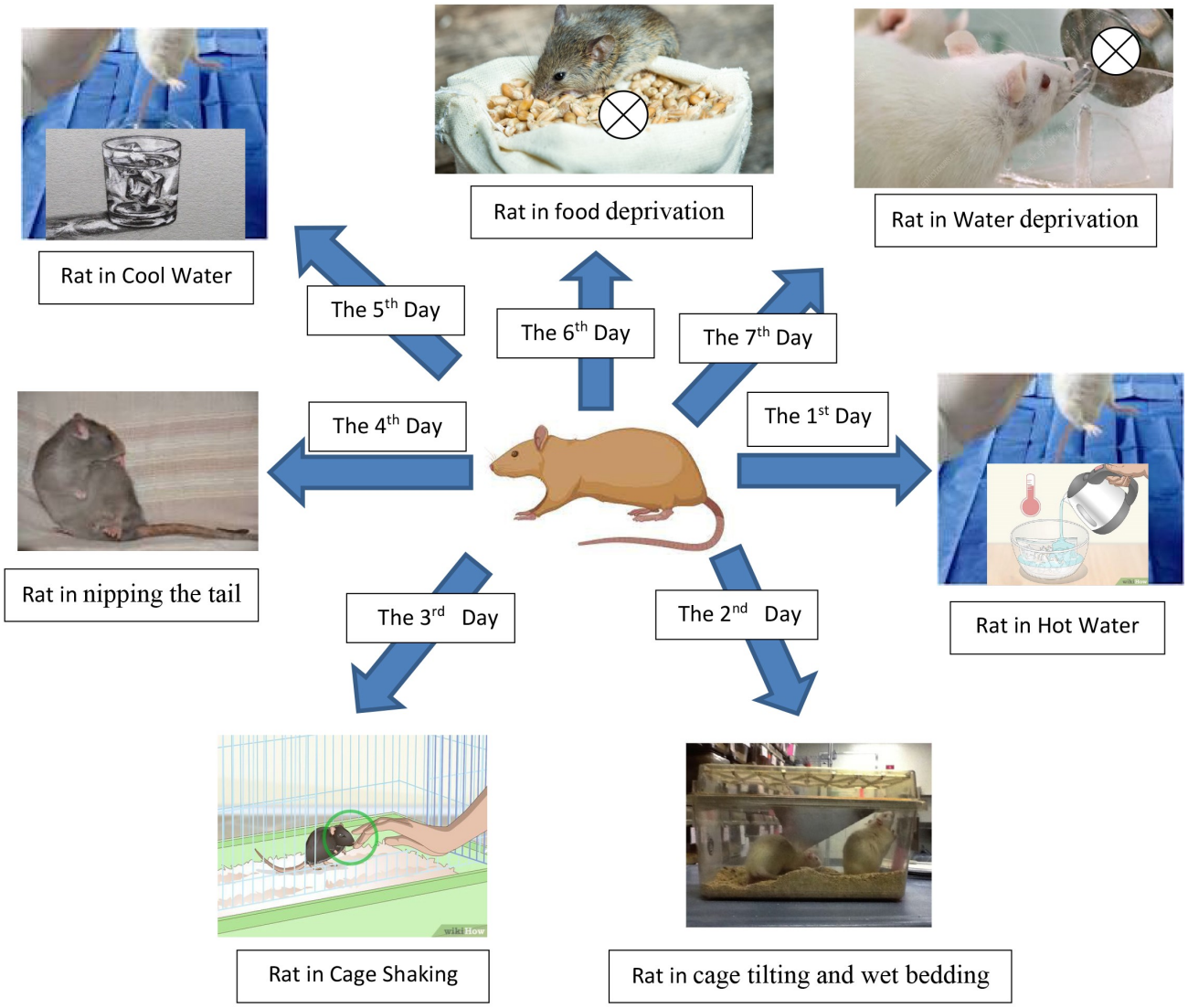

Fig. 1. Chronic mild stress (CMS) induction. Summarize the program for chronic mild stress induction as follows: The first day involved forced swimming in hot water (45 °C for 5 minutes); the second day cage tilting and wet bedding; the third day animal shaking for 10 minutes; the fourth day nipping the tail for 1 minute; the fifth day involved forced swimming in cool (4 °C for 5 minutes); the sixth day food deprivation for 24 hours and overnight illumination; the seventh day water deprivation for 24 hours and all the above aforementioned stressors were applied once a week for 6 weeks.

Fig. 1 could serve as a summary of the induction of mild chronic stress in rats.

2.5 Hypothalamus Tissue Preparation

The rats were inhaled a solution of diethyl ether as an anesthesia after the final dose of each treatment. The rats' heads were detached, and then they were dissected. The tissue from the brain's hypothalamus was taken and soaked in saline solution. This procedure followed Palkovits' [32] instructions for isolating the hypothalamus. The entire hypothalamic tissue, which has 3 regions (the supraoptic, tuberal, and mammillary regions) was examined in the study.

The filter papers were used to dry this tissue. Two pieces of hypothalamic tissue were separated, and the first piece was dissolved in 2.5 mL of Tris buffer solution. Thereafter, for 10 minutes at room temperature, hypothalamus tissue was homogenized in an automated homogenizer. The supernatant, which was used to calculate the biochemical parameters, was removed from the tissue by centrifuging it for 15 minutes at –4 °C and 7000 rpm. The second portion of the tissue from the hypothalamus was used for histological analysis. The hypothalamus tissue was sufficient to conduct biochemical, molecular, and histological examinations despite the fact that the hypothalamus is a small tissue.

Table 1. Effect of malic acid on physiological measures of CMS rats.

Parameters	Control	Malic acid	Venlafaxine drug	CMS rats	CMS rats + Malic acid	CMS rats + Venlafaxine
Total body weight (g)	165 ± 6.24	167 ± 6.13 (1.21%)	164 ± 6.52 (–0.61%)	128 ± 5.09^a (–22.42%)	163 ± 6.24^b (27.34%)	162 ± 6.24^b (26.56%)
Food consumption (g/day)	11.6 ± 1.2	11.4 ± 1.4 (–1.72%)	11.5 ± 1.6 (–0.86%)	7.2 ± 1.3^a (–37.93%)	11.3 ± 1.6^b (56.94%)	11.2 ± 1.5^b (55.56%)
Water intake (mL/day)	12.4 ± 1.7	12.6 ± 1.5 (1.62%)	12.3 ± 1.2 (–0.81%)	7.8 ± 1.3^a (–37.09%)	12.2 ± 1.7^b (56.41%)	12.1 ± 1.7^b (53.85%)
Liver weight (g/100 g bw)	2.7 ± 0.08	2.6 ± 0.06 (–3.70%)	2.5 ± 0.09 (–7.41%)	1.8 ± 0.05^a (–33.33%)	2.4 ± 0.08^b (33.33%)	2.3 ± 0.08^b (27.78%)
Kidney weight (g/100 g bw)	0.36 ± 0.03	0.37 ± 0.05 (1.67%)	0.35 ± 0.04 (–2.78%)	0.21 ± 0.02^a (–41.67%)	0.33 ± 0.06^b (57.14%)	0.34 ± 0.05^b (61.90%)
Brain weight (g)	0.56 ± 0.07	0.57 ± 0.08 (1.79%)	0.58 ± 0.06 (3.57%)	0.39 ± 0.04^a (–30.36%)	0.53 ± 0.09^b (35.90%)	0.54 ± 0.06^b (38.46%)
Pancreas weight (g/100 g bw)	0.25 ± 0.03	0.27 ± 0.02 (8.00%)	0.24 ± 0.04 (–4.00%)	0.16 ± 0.02^a (–36.00%)	0.23 ± 0.03^b (43.75%)	0.22 ± 0.03^b (37.50%)
Spleen weight (g/100 g bw)	0.34 ± 0.06	0.33 ± 0.04 (–2.94%)	0.36 ± 0.05 (5.88%)	0.21 ± 0.03^a (–38.24%)	0.32 ± 0.05^b (52.38%)	0.31 ± 0.07^b (47.62%)
Heart weight (g/100 g bw)	0.36 ± 0.04	0.35 ± 0.06 (–2.78%)	0.38 ± 0.05 (5.56%)	0.23 ± 0.03^a (–36.11%)	0.34 ± 0.03^b (47.83%)	0.33 ± 0.03^b (43.49%)
Adrenal gland weight (mg/100 g bw)	5.6 ± 0.19	5.4 ± 0.16 (–3.57%)	5.5 ± 0.17 (–1.79%)	3.6 ± 0.13^a (–35.71%)	5.3 ± 0.19^b (47.22%)	5.2 ± 0.19^b (44.44%)
Urinary volume (mL/100 g/8 h)	0.96 ± 0.19	0.94 ± 0.16 (–2.08%)	0.97 ± 0.18 (1.04%)	0.64 ± 0.13^a (–33.33%)	0.93 ± 0.17^b (45.31%)	0.95 ± 0.18^b (48.43%)
Fecal pellet count	38 ± 4.07	37 ± 3.86 (–2.63%)	36 ± 4.20 (–5.26%)	25 ± 3.14^a (–34.21%)	35 ± 4.35^b (40.00%)	34 ± 4.26^b (36.00%)

Number of animals = 6 rats/group. Data are represented as mean ± SEM. CMS, Chronic mild stress. ^{a} Highly significant change compared to control. ^{b} Highly significant change compared to CMS rats. (%): Percentage of change compared to control or CMS rats.

Table 2. Effect of malic acid on behavioral tests of CMS rats.

Parameters	Control	Malic acid	Venlafaxine drug	CMS rats	CMS rats + Malic acid	CMS rats + Venlafaxine
Sucrose preference test (%)	89.2 ± 1.7	88.3 ± 1.5 (–1.01%)	87.9 ± 1.6 (–1.46%)	48.8 ± 1.5^a (–45.29%)	86.3 ± 1.4^b (76.84%)	89.7 ± 1.2^b (83.81%)
Distance traveled test (cm)	226.7 ± 90.1	220.0 ± 74.1 (–2.96%)	223.7 ± 80.5 (–1.32%)	70.0 ± 16.3^a (–69.12%)	220.6 ± 78.0^b (215.14%)	224.7 ± 44.3^b (221.0%)
Center square entries test (/5 minutes)	1.40 ± 1.8	1.50 ± 0.97 (7.14%)	1.46 ± 1.6 (4.29%)	0.20 ± 0.10^a (–85.71%)	1.36 ± 0.15^b (580.0%)	1.39 ± 0.72^b (595.0%)
Center square duration test (seconds)	3.60 ± 1.3	3.43 ± 1.4 (–4.72%)	3.54 ± 1.7 (–1.67%)	7.18 ± 11.9^a (99.44%)	3.62 ± 1.1^b (–49.85%)	3.64 ± 0.9^b (–49.30%)
Tail suspension test (seconds)	19.33 ± 2.4	18.80 ± 2.2 (–2.74%)	18.71 ± 2.4 (–3.21%)	54.70 ± 2.8^a (182.98%)	19.54 ± 3.0^b (–64.28%)	20.35 ± 2.6^b (–62.79%)
Forced swimming test (seconds)	83.7 ± 3.6	83.4 ± 3.9 (–0.36%)	82.9 ± 3.2 (–0.96%)	134.4 ± 5.5^a (60.57%)	81.37 ± 3.7^b (–39.46%)	84.16 ± 3.4^b (–37.38%)

Number of animals = 6 rats/group. Data are represented as mean ± SEM. CMS, Chronic mild stress. ^{a} Highly significant change compared to control. ^{b} Highly significant change compared to CMS rats. (%): Percentage of change compared to control or CMS rats.

Table 3. Effect of malic acid on antioxidants levels in hypothalamus of CMS rats.

Parameters	Control	Malic acid	Venlafaxine drug	CMS rats	CMS rats + Malic acid	CMS rats + Venlafaxine
Superoxide dismutase (U/g tissue)	3250 ± 60	3240 ± 50 (–0.31%)	3245 ± 55 (–0.15%)	1170 ± 40^a (–64.0%)	3230 ± 50^b (176.0%)	3220 ± 70^b (175.2%)
Glutathione peroxidase (U/g tissue)	785 ± 19	780 ± 24 (–0.64%)	785 ± 21 (0.0%)	365 ± 18^a (–53.50%)	775 ± 16^b (112.32%)	770 ± 21^b (110.96%)
Catalase (µmol H_2O_2/min/mg tissue)	0.18 ± 0.05	0.19 ± 0.04 (5.56%)	0.17 ± 0.06 (0.0%)	0.09 ± 0.03^a (–50.0%)	0.17 ± 0.06^b (88.89%)	0.16 ± 0.05^b (77.78%)
Malondialdehyde (µmol/g tissue)	8.52 ± 0.60	8.49 ± 0.72 (–0.35%)	8.50 ± 0.69 (–0.23%)	19.38 ± 0.54^a (127.46%)	9.54 ± 0.83^b (–50.77%)	9.58 ± 0.90^b (–50.56%)
NADPH oxidase activity (mg/mg protein × 10^5)	12.3 ± 1.18	12.5 ± 1.54 (1.62%)	12.4 ± 1.37 (0.81%)	7.6 ± 1.31^a (–38.21%)	11.6 ± 1.25^b (52.63%)	11.9 ± 1.26^b (56.57%)
Conjugated dienes (µmol/g tissue)	1.45 ± 0.19	1.42 ± 0.17 (–2.06%)	1.43 ± 0.15 (–1.37%)	2.13 ± 0.14^a (46.89%)	1.46 ± 0.18^b (–31.46%)	1.47 ± 0.17^b (–30.98%)
Oxidative index (A_{233}/A_{215} ratio)	0.49 ± 0.03	0.47 ± 0.05 (–4.08%)	0.50 ± 0.03 (2.04%)	0.82 ± 0.04^a (67.34%)	0.53 ± 0.04^b (–35.36%)	0.51 ± 0.03^b (–37.80%)

Number of animals = 6 rats/group. Data are represented as mean ± SEM. CMS, Chronic mild stress; NADPH, nicotinamide-adenine dinucleotide phosphate. ^{a} Highly significant change compared to control. ^{b} Highly significant change compared to CMS rats. (%): Percentage of change compared to control or CMS rats.

2.6 Behavioral and Biochemical Tests

2.6.1 Behavioral Tests in a Rat Model by Using Automated Software

2.6.1.1 Sucrose Preference Test. The Strekalova and Steinbusch [33] technique was used for this test. In this technique, the rats were trained to absorb sucrose by being housed in cages with two sucrose bottles (1% w/v) for a period of 72 hours. One bottle was then swapped out for one that contained tap water. To determine the sucrose preference, the amount of water and sucrose that were consumed were both measured.

2.6.1.2 Forced Swimming Test. This test was located using the Zhang *et al*. [34] technique. We used a plastic cylinder that was 25 cm in diameter and 50 cm high, and we filled it with water that was 23–25 °C to a specific height (= 45 cm). For 5 minutes, every single rat was submerged in this cylinder. The rat was taken out of the water and allowed to dry. Each and every rat was returned to its cage. Each rat's immobility period was determined by measuring how long it spent floating in the water without making any effort to maintain its head above the surface.

2.6.1.3 Tail Suspension Test. The results of this test were calculated using the techniques proposed by Belovicova *et al*. [35] and Castagné *et al*. [36]. The animals were suspended independently from their tails for 5 minutes, 58 cm from the ground. After the struggle phase, the rat became immobile and immobile time of each rat was detected.

2.6.1.4 Open Field Test. According to Zhang *et al*. [34] technique, the open field test was conducted using the distance travelled test, center square entries test, and center square duration test. Twenty-five squares made up an extra-large cage with dimensions of 75 cm × 75 cm × 40 cm. Each animal was evaluated separately by being left in the center for 5 minutes so that it could learn its surroundings. Each session included calculations for the number of crossings, rearing, and central square entrances times.

2.6.2 Biochemical Tests

2.6.2.1 Hypothalamus Antioxidants Determination. The activity of superoxide dismutase (SOD) was measured using the Suttle [37] method. The Pagalia and Valentine [38] method was used to measure the activity of glutathione peroxidase (GPx). Catalase (CAT) activity was calculated using the Aebi [39] technique. Malondialdehyde was measured as a sign of lipid peroxidation using the Ohkawa *et al*. [40] method. Following the instructions in the kit booklets, all of the aforementioned antioxidants were detected using spectrophotometry.

To detect conjugated dienes (CD), the method of Kogure *et al*. [41] was used. Hypothalamus homogenate was added to a solution containing 1 mL of 10 mmol/L phosphate buffer (pH 7.4) and 1% Lubrol (0.01 mg of protein). Using a spectrophotometer, conjugated dienes were measured using the absorbance ratio A_{233}/A_{215} (oxidative index) [42,43].

Superoxide radical (O_2^-) production in NADPH oxidase activity was examined using a chemiluminescence assay to measure NADPH oxidase activity [44]. Hypothalamus homogenate (250 µL) and phosphate buffer (50 mmol/L KH_2PO_4, 1 mmol/L EGTA, 150 mmol/L sucrose, pH 7.4) were the solutions to which NADPH (0.1 mmol/L) was added. The aforementioned solution was supplemented with lucigenin (5 mmol/L). NADPH oxidase activity was determined using a multimode microplate fluorometer at 30 °C/5 seconds for a period of 10 minutes [45].

2.6.2.2 Hypothalamus Neurotransmitters Determination. The Kitagawa [46] method was used to measure the serotonin level. The Guo *et al*. [47] method was used to determine the dopamine level. The norepinephrine level was determined using the Kapoor and Chalmers [48] technique. To measure the level of γ-aminobutyric acid (GABA), the Sciotti *et al*. [49] method was used. For all of the aforementioned neurotransmitters, enzyme-linked immunosorbent assay (ELISA) kits (20386, SinoGeneClon, Hangzhou, China) were utilized, and the kit guidelines were followed.

2.6.2.3 Hypothalamus Inflammatory Markers Determination. Tumor necrosis factor-α (TNF-α) was detected using the Matalka *et al*. [50] method. The Interleukin-1β (IL-1β) level was measured using the DeCicco *et al*. [51] method. Both Interleukin-6 (IL-6) and Interleukin-10 were calculated using the Stelmasiak *et al*. [52] method. All of the aforementioned inflammatory indicators were detected using ELISA kits in accordance with the instructions provided in the kit booklets.

2.6.2.4 Hypothalamus Sodium/Potassium-ATPase Determination. The primary solution was made up of the following ingredients: NaCl (80 mM), Tris HCl (50 mM, pH 7.4), $MgCl_2$ (5 mM), KCl (20 mM), and ATP disodium salt (3 mM). Sodium/potassium-ATPase activity was then determined. To start the reaction, 50 mL of hypothalamus homogenate was added to the aforementioned solution, which was then incubated at 37 °C for 10 minutes. Trichloroacetic acid (50 mL) was then added to terminate the reaction. After that, the solution was centrifuged for 15 minutes (at 3000 rpm). Then, the supernatant (1 mL) was collected and added to the mixture of ascorbic acid, trichloroacetic acid, and ammonium molybdate (250 mL of each ingredient). A spectrophotometer was used to measure the acquired color at 680 nm [53].

2.6.2.5 Detection of Hypothalamus Histamine-N-methyl transferase (*Hnmt*) and Tyrosine Hydroxylase (*TH*) Enzymes Expression by Using Real Time PCR (4385610, Hercules, CA, USA). RNA was extracted from the hypothalamus using an Invitrogen® Pure.Link® RNA Mini Kit (b12183018A, Invitrogen™, ThermoFisher Scientific

Company, Waltham, MA, USA). This study's primary methodology is the use of the RNA-to-cDNATM Kit to convert cDNA to RNA (Applied Biosystems kit reagents, Biorad Company, Hercules, CA, USA). Tyrosine hydroxylase is activated by the primer (Rn Th 1 SG), Histamine N-methyl transferase is activated by the primer (Rn Hnmt 1 SG), and Glyceraldehyde 3-phosphate dehydrogenase is activated by the primer (Rn GAPDH 1 SG) (GAPDH). The Qiagen Corporation (Qiagen, Hilden Town, Germany) was used to purchase all of the primers indicated above. The mRNA for the *GAPDH* gene was identified using 2-CT, and the fold change from the control is expressed [54].

2.7 Histopathological Investigation

Hypothalamus specimens were preserved in 10% formalin. It was a routine task to embed hypothalamic tissue in paraffin blocks. Five meters of partitioning separated these blocks. Hematoxylin and eosin was used to stain all of the blocks before they were examined under a light microscope.

2.8 Statistical Analysis

The results were presented as mean standard error mean in tables (SEM). In this study, the normal distribution or the Gaussian distribution was used. The SPSS 13 application (IBM Corp., Chicago, IL, USA) was used to run a one-way Analysis of Variance (ANOVA) test. p-values ≤ 0.05 were considered significant when using the fisher least significant difference (FLSD) test in post-hoc analysis among all treatment groups.

3. Results

3.1 Physiological Measures Results

Table 1 exhibits that there is a significant decrease in total body weight, food consumption, water intake, organ (liver, kidney, brain, pancreas, spleen, heart, adrenal gland) weight, and urine and fecal output in CMS rats with percentage of change equal to –22.42%, –37.93%, –37.09%, –33.33%, –41.67%, –30.36%, –36.00%, –38.24%, –36.11%, –35.71%, –33.33%, and –34.21% compared to the control group. On the contrary, malic acid and venlafaxine drug oral administration to CMS rats returned the total body weight, food consumption, water intake, organ (liver, kidney, brain, pancreas, spleen, heart, adrenal gland) weight, and urine and fecal output to approach the control values with percentage of change equal to 27.43% and 26.56%, 5.94% and 55.56%, 56.41% and 53.85%, 33.33% and 27.78%, 57.14% and 61.90%, 35.90% and 38.46%, 43.75% and 37.50%, 52.38% and 47.62%, 47.83% and 43.49%, 47.22% and 44.44%, 45.31% and 48.43%, and 40.00% and 36.00%, respectively compared to CMS rats. No loss of rat hair, no skin patches, no rat convulsions, and no animal deaths were observed in any of the groups studied during the experimental phase of the study.

3.2 Behavioral Results

Table 2 reveals malic acid effect on behavioral tests in CMS rats. It is clear that CMS caused a highly significant decrease ($p \leq 0.01$) in the sucrose preference test, distance travelled test, and center square entries test with percentages of change equal to –45.29%, –69.12%, and –85.71% compared to the control group. A highly significant increase ($p \leq 0.01$) was observed in the center square duration test, tail suspension test, and forced swimming test with percentages of change equal to 99.44%, 182.98%, and 60.57% compared to the control group. On the other hand, malic acid and venlafaxine drug oral administration to CMS rats pushed the above-mentioned behavioral tests (sucrose preference test, distance travelled test, center square entries test, center square duration test, tail suspension test, and forced swimming test) to be near the control values with percentages of change equal to 76.84% and 83.81%, 215.14% and 221.0%, 580.0% and 595.0%, –49.85% and –49.30%, 64.28% and –62.79%, and –39.46% and –37.38% of these tests compared to CMS rats. Moreover, the oral administration of malic acid or venlafaxine drug to normal rats without behavioral change in all tests applied through the study period.

3.3 Antioxidants Results

Table 3 shows malic acid effect on antioxidant levels in the hypothalamus of CMS rats. It can be concluded from the table that CMS induced a highly significant decrease ($p \leq 0.01$) in superoxide dismutase, glutathione peroxidase, catalase activities, and NADPH oxidase activity with percentages of change equal to –64.0%, –53.50%, –50.0%, and –38.21% compared to the control group. A highly significant increase ($p \leq 0.01$) was recorded in malondialdehyde, conjugated dienes, and oxidative index with percentages of change equal to 127.46, 46.89, and 67.34%, respectively, compared to the control group. Furthermore, oral administration of malic acid or venlafaxine to CMS rats pushed antioxidant tests to approach the control levels with a percentage of change equal to 176.0% and 175.2%, 112.32% and 110.96%, 88.89% and 77.78%, 52.63% and 56.57%, –50.77% and –50.56%, –31.46% and –30.98%, –35.36% and –37.80% for superoxide dismutase, glutathione peroxidase, catalase activities, NADPH oxidase activity, malondialdehyde, conjugated dienes, and oxidative index, compared to CMS rats. In the contrary, malic acid or venlafaxine drug oral administration in normal rats did not show any change in any of the antioxidants used in this study.

3.4 Neurotransmitters Results

Fig. 2 exhibits malic acid effect on neurotransmitter levels in the hypothalamus of CMS rats. It is obvious that CMS caused a highly significant decrease ($p \leq 0.01$) in serotonin, dopamine, norepinephrine, and γ-amino butyric acid levels with percentages of change equal to –54.13, –47.14%, –47.22%, and –47.06% compared to the control group. On the other hand, malic acid or venlafax-

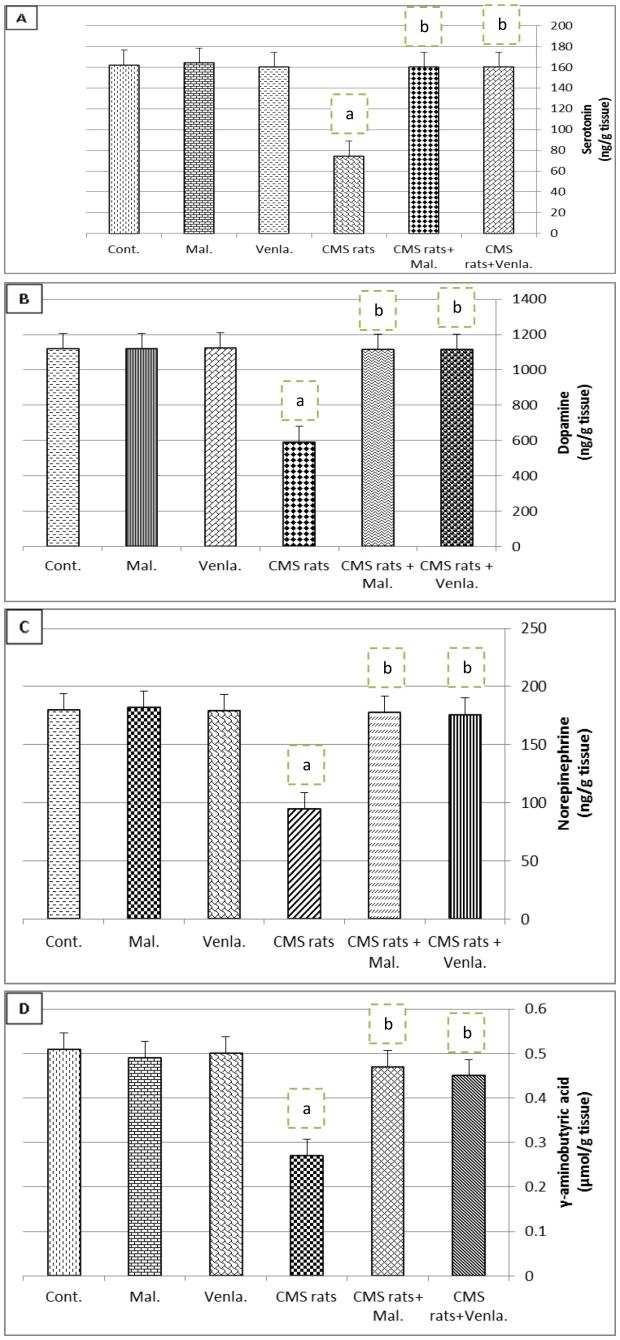

Fig. 2. Effect of malic acid on neurotransmitters levels in hypothalamus of chronic mild stress (CMS) rats. Fig. 2 explains hypothalamus neurotransmitters. (A) Serotonin (ng/g tissue). (B) Dopamine (ng/g tissue). (C) Norepinephrine (ng/g tissue). (D) Gamma amino butyric acid (µmol/g tissue). Number of animals = 6 rats/group. Data are represented as mean ± SEM. [a] Highly significant change compared to control. [b] Highly significant change compared to CMS rats. Cont., Control; Mal., Malic acid; Venla., Venlafaxine drug; CMS, Chronic mild stress; CMS rats + Mal., CMS rats + Malic acid; CMS rats + Venla., CMS rats + Venlafaxine drug.

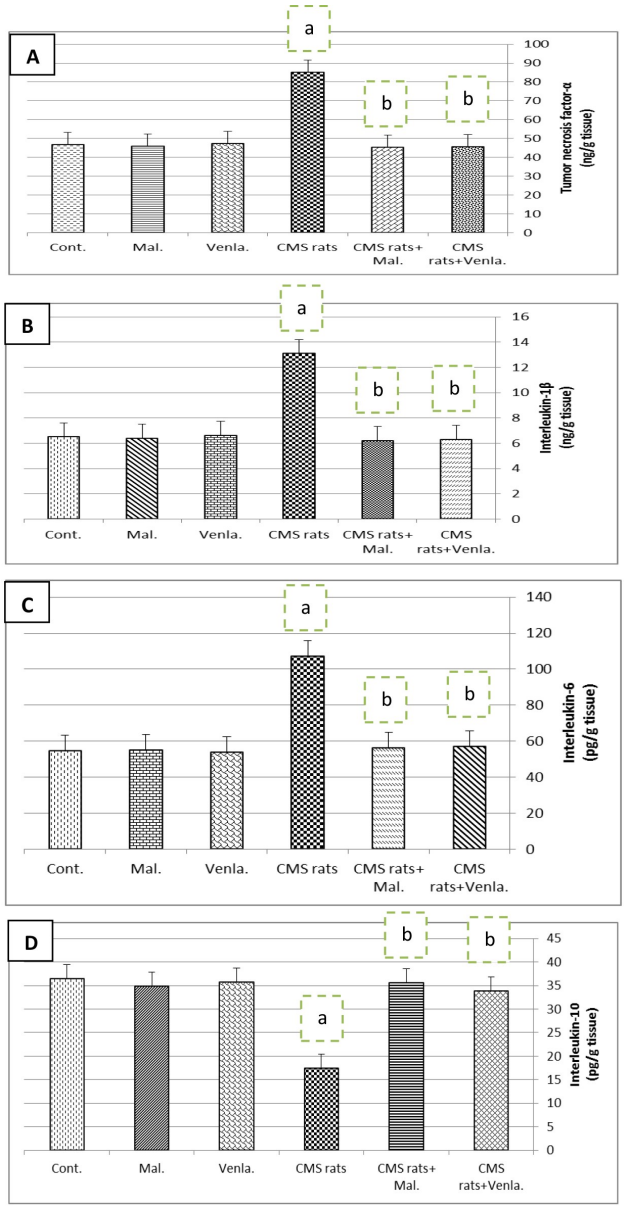

Fig. 3. Effect of malic acid on inflammatory markers in hypothalamus of CMS rats. Fig. 3 shows hypothalamus inflammatory markers. (A) Tumor necrosis factor-α (ng/g tissue). (B) Interleukin-1β (ng/g tissue). (C) Interleukin-6 (pg/g tissue). (D) Interleukin-10 (pg/g tissue). Number of animals = 6 rats/group. Data are represented as mean ± SEM. [a] Highly significant change compared to control. [b] Highly significant change compared to CMS rats. Cont., Control; Mal., Malic acid; Venla., Venlafaxine drug; CMS, Chronic mild stress; CMS rats + Mal., CMS rats + Malic acid; CMS rats + Venla., CMS rats + Venlafaxine drug.

ine drug oral administration to CMS rats pushed the above-mentioned neurotransmitters to be near the control values with percentages of change equal to 116.7% and 115.3%, 88.51% and 88.17%, 87.36% and 85.26%, and 74.07% and 66.67% compared to CMS rats. Moreover, the oral administration of malic acid or venlafaxine drug to normal rats without change in all neurotransmitters in this research.

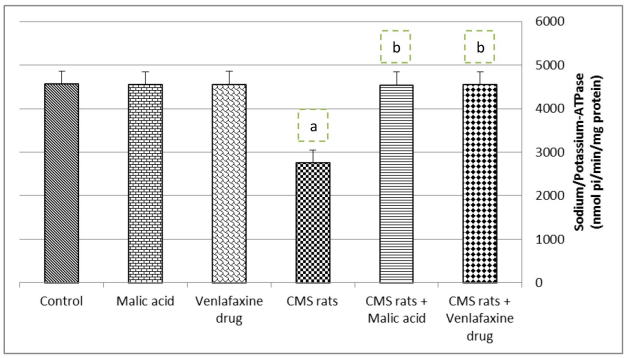

Fig. 4. Effect of malic acid on sodium/potassium-ATPase activity in hypothalamus of CMS rats. Fig. 4 reveals hypothalamus sodium/potassium-ATPase activity (nmol pi/min/mg protein). Number of animals = 6 rats/group. Data are represented as mean ± SEM. CMS, Chronic mild stress. [a] Highly significant change compared to control. [b] Highly significant change compared to CMS rats.

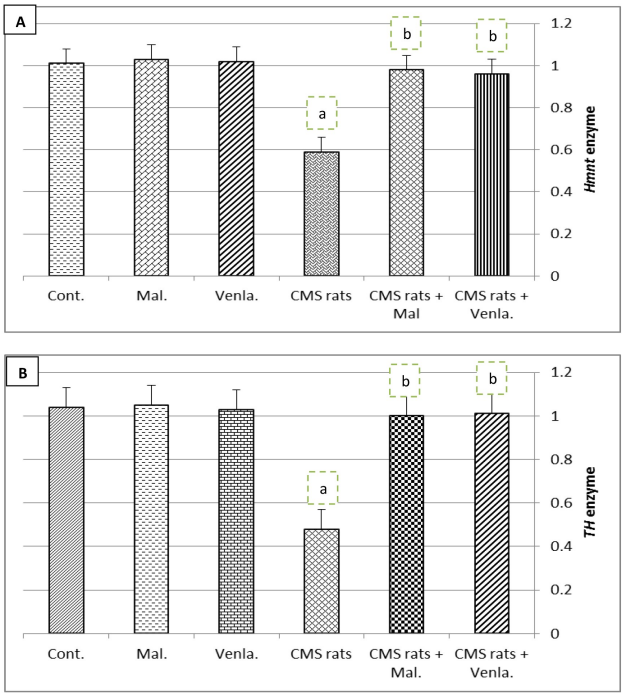

Fig. 5. Effect of malic acid on fold change of histamine-N-methyl transferase (*Hmnt*) and tyrosine hydroxylase (*TH*) enzymes expressions in hypothalamus of CMS rats. Fig. 5 exhibits hypothalamus histamine-N-methyl transferase (*Hmnt*) and tyrosine hydroxylase (*TH*) enzyme expressions. (A) Fold change of *Hmnt* enzyme. (B) Fold change of *TH* enzyme. Number of animals = 6 rats/group. Data are represented as mean ± SEM. [a] Highly significant change compared to control. [b] Highly significant change compared to CMS rats. Cont., Control; Mal., Malic acid; Venla., Venlafaxine drug; CMS, Chronic mild stress; CMS rats + Mal., CMS rats + Malic acid; CMS rats + Venla., CMS rats + Venlafaxine drug.

3.5 Inflammatory Markers Results

Fig. 3 shows malic acid effect on inflammatory markers in the hypothalamus of CMS rats. It can be estimated that depression induced a highly significant increase ($p \leq 0.01$) in tumor necrosis factor-α, interleukin-1, and interleukin-6 levels with percentages of change equal to 82.05%, 101.53%, and 95.97% compared to control levels. A highly significant decrease ($p \leq 0.01$) was found in interleukin-10 level with percentages of change equal to –51.92% compared to the control group. Furthermore, oral administration of malic acid or venlafaxine drug to CMS rats pushed these inflammatory markers to approach the control levels with percentage changes equal to –46.83% and –46.47%, 52.67% and –51.90%, –47.57% and –46.72%, and 103.42% and 93.71%, respectively, compared to CMS rats. Furthermore, the oral administration of malic acid or venlafaxine drug to normal rats without change in all inflammatory markers in the experimental study.

3.6 Sodium/Potassium-ATPase Results

Fig. 4 exhibits the effect of malic acid on sodium/potassium-ATPase activity in the hypothalamus of CMS group. It is obvious from this figure that CMS induced a highly significant decrease ($p \leq 0.01$) in sodium/potassium-ATPase activity with a percentage of change equal to –39.69% compared to the control group. On the other hand, oral administration of malic acid or venlafaxine to CMS rats pushed the above-mentioned sodium/potassium-ATPase activity to near the control values with a percentage of change equal to 65.09% and 61.82% compared to CMS rats. Moreover, oral administration of malic acid or venlafaxine drug to normal rats without change in sodium/potassium-ATPase activity during the study period.

3.7 Histamine-N-methyl transferase (Hnmt) and Tyrosine Hydroxylase (TH) Enzymes Results

Fig. 5 reveals malic acid effect on the fold change of *Hnmt* and *TH* enzyme expressions in the hypothalamus of CMS rats. The data indicate that CMS caused a highly significant decrease ($p \leq 0.01$) in *Hnmt* and *TH* enzymes, with percentages of change equal to –41.58% and –23.08%, respectively, compared to the control group. Moreover, malic acid or venlafaxine drug oral administration to CMS rats pushed the above-mentioned *Hnmt* and *TH* enzymes to approach the control values with a percentage of change equal to 66.10% and 62.71% and 108.33% and 110.42% compared to CMS rats. Furthermore, the oral administration of malic acid or venlafaxine drug to normal rats without change in *Hnmt* and *TH* enzymes.

3.8 Histology Results

Fig. 6 reveals hypothalamus tissue in the control group, showing the control neurons with large nuclei (pale-stained neurons) (black arrows) (Fig. 6A). Hypothalamus

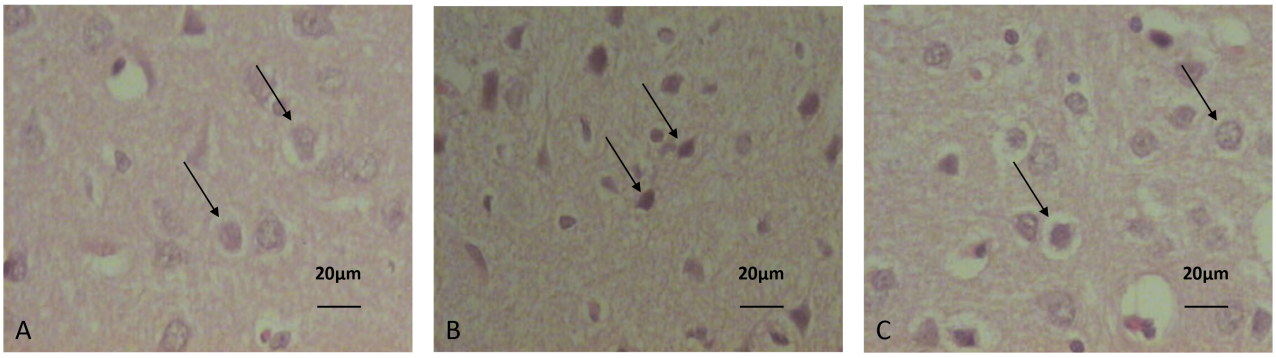

Fig. 6. Hypothalamus histology in control, CMS, CMS+ malic acid (250 mg/kg)-, and CMS+ venlafaxine (20 mg/kg)-treated groups. (A) Hypothalamus sections of control rats showing the highly active nerve cells that have huge nuclei that are relatively pale-stained (black arrows). (B) Hypothalamus sections from CMS rats showed small cells that are missing the normal large normal cells and their normal nuclei; these small cells appear like rings, and the recently dead neurons appear dark (black arrows). (C) The CMS rats treated with malic acid (250 mg/kg bw) or venlafaxine drug (20 mg/kg bw) revealed large normal cells with normal nuclei that looked like control cells. Scale bar = 20 µm.

sections of the CMS rats showed small cells that were missing the normal large cells and their normal nuclei. These small cells appear as rings and these neurons contain newly dark dead neurons (black arrows) (Fig. 6B). CMS rats treated with malic acid (250 mg/kg bw) or venlafaxine drug (20 mg/kg bw) showed large normal neurons with normal nuclei that looked like the control group (Fig. 6C).

4. Discussion

Stress causes depression by increasing brain superoxide anion and neuro-inflammation due to stimulation of the hypothalamus-pituitary-adrenal axis [55]. The pathogenesis and symptoms of human depression are comparable to those shown in the CMS animal model [56]. In comparison to the control group, the study found a reduction in food intake, water consumption, urine production, and organ weights. CMS significantly affects the animal's desire for food and water, which in turn causes the animal's total and organ weights to decrease. This decrease in animal appetite is brought on by the CMS rats' lower levels of insulin and leptin, and these findings concur with those of Aluko and Umukoro [57], Flak *et al.* [58], and Wang *et al.* [59]. CMS caused a decline in the sucrose preference test, travel distance test, and center square entries test, but a rise in the center square duration test, tail suspension test, and forced swimming test. In tests using inescapable cases like the tail suspension test and the forced swimming test to force the rat to confront depressing and challenging circumstances, CMS is associated with stress exposure in rats. These studies assessed the animal's management strategy in these circumstances. At the conclusion of each rat's struggling phase throughout these tests, the immobility time is recorded [57]. In the forced swimming test and the tail suspension test, CMS rats showed an increase in the immobility period. These findings explain the animal's management strategy in an unavoidable circumstance that closely resembles evident depressive symptoms, and they are corroborated by earlier investigations [34,60].

CMS makes people respond more anxiously, which has an impact on their automatic behavior. By introducing the rat to a novel habitat and observing the animal's adaptability, risk assessment-correlated behavior, and exploratory activities like the open field test, anxiety in rats was studied [35]. In the open field test, CMS rats display a decrease in the number of rearing, crossed lines, and central square entries. These findings, which related to anxiety and a poorer risk assessment behavior, are corroborated by other studies [34,59]. Anhedonia simulates the loss of pleasure that is a hallmark of depressive patients' symptoms [61]. In depressive individuals, anhedonia is associated with indoleamin 2,3-dioxygenase I expression. The sucrose preference test can be used to measure hedonic behavior in rats. CMS rats show a decrease in this test, and the degree of the decline depends on the length of CMS exposure. These results are consistent with those of Zhang *et al.* [34] and Filho *et al.* [62].

The decrease of distance travelled test in CMS rats is due to 2 reasons; (1) the decrease of hypothalamus neurotransmitters serotonin, dopamine, norepinephrine, and γ-aminobutyric acid in CMS rats compared to that in control rats [63]; where rats after 5 weeks of chronic unpredictable stress had depressive-like behaviors such as decreased total travel distance and decreased open field test [64,65] and (2) depressive-like behaviors in CMS rats change the concentration of mood-related hormones, and cause immune/endocrine dysfunction [66].

CMS caused also a decrease in hypothalamus neurotransmitters, where CMS is characterized by disturbances of both psychological and physiological conditions [67], chronic mild stress decreased 5-hydroxytryptamine levels [68]. Malic acid oral administration to CMS rats amended the behavioral disturbances and hypothalamus neurotrans-

mitters in CMS rats. This observation is related to the antidepressant activity of malic acid, which, in accordance with many previous studies such as Tanasiewicz *et al*. [69], stated that malic acid treated xerostomia, which led to depression. Also, Ferreira *et al*. [70] showed that malic acid treats patients with fibromyalgia, and the acid reduces the patient's pain and its depressive symptoms. Moreover, von Eggelkraut-Gottanka *et al*. [71] revealed that malic acid plays a key role in the treatment of mild/moderate depressive disorders. Furthermore, Lian *et al*. [72] reported that malic acid has an antidepressant effect.

CMS induced oxidative stress in the hypothalamus of the CMS group, where CMS increased hydrogen peroxide production and neuroinflammation [73]. Malic acid oral administration to CMS rats jumped the antioxidant enzyme activities in the hypothalamus of those rats. This effect is related to the antioxidant activity of malic acid. Many previous studies agree with these results. As Quiroga *et al*. [74] stated, malic acid has antioxidant activity. Also, Yan *et al*. [75] found that malic acid improves the antioxidants in pigs. Moreover, Mousavi *et al*. [22] revealed that malic acid increases catalase, ascorbate peroxidase, and many antioxidants but it decreases cadmium toxicity inside the plant. Furthermore, Calvo *et al*. [76] and Taher *et al*. [77] proved that malic acid has antioxidant, antihypertensive, and hypoglycemic activities.

CMS increased the inflammatory markers in the hypothalamus of CMS rats, where CMS induce neuroinflammation [73]. The oral administration of malic acid to CMS rats decreased these markers in the hypothalamus. This observation is related to the anti-inflammatory activity of malic acid, where Tang *et al*. [78] stated that malic acid reduced serum levels of tumor necrosis factor-α and platelet aggregation, and consequently, malic acid has anti-inflammatory and antiplatelet aggregation properties. Also, Barragán-Zarate *et al*. [79] revealed that malic acid exhibits antioxidant and/or anti-inflammatory activity. Moreover, Sahpaz *et al*. [80] reported that malic acid exerts anti-inflammatory activity. Furthermore, Añibarro-Ortega *et al*. [81] showed that malic acid inhibits neuro-inflammation, free radicals in the brain, microbes, and tyrosinase in the neurons.

CMS induced a decrease in sodium/potassium-ATPase activity in the hypothalamus of CMS rats, where CMS induced oxidative stress that caused the depletion of antioxidant enzymes. The imbalance in the oxidative state caused a deficiency in sodium/potassium-ATPase activity [82]. The oral administration of malic acid to CMS rats amended sodium/potassium-ATPase activity in the hypothalamus of CMS rats. This observation is related to the finding that malic acid increases oxygen consumption and decreases glucose secretion [83]. Also, malic acid increases the K^+ accumulation inside the cell, which activates the plasma membrane H^+-ATPase and phosphoenolpyruvate carboxylase cycle [84] and consequently restores sodium and potassium-ATPase activity in the hypothalamus of CMS rats. Finally, sodium/potassium-ATPase activity was elevated secondary to the alleviation of oxidative stress by malic acid [22].

CMS caused a decrease in *Hnmt* and *TH* enzyme overexpression in the hypothalamus of CMS rats. Brain histamine is a neurotransmitter and regulates many physiological functions, and histamine depletion causes many neurological disorders such as sleeping disorders, depression, and Parkinson's disease. *Hnmt* is a histamine-metabolising enzyme expressed in the brain. The deficiency causes the depletion in the concentration of catecholamines because it is an important rate-limiting enzyme in their biosynthesis. *Hnmt* is an essential enzyme for the elimination of histamine [85,86]. The *TH* and *Hnmt* genes were inhibited in CMS rats. The decrease of *Hnmt* and *TH* enzymes will perturb the synthesis of neurotransmitters and enhance neuroinflammation. The oral administration of malic acid to CMS rats restored *Hnmt* and *TH* enzymes in the hypothalamus of CMS rats to levels approaching the control levels due to malic acid's inhibition of neuro-inflammation and hydrogen peroxide production in the neurons [22,78,80].

Histological investigation showed acid cytoprotective on the hypothalamus of CMS rats, and these results are related to the fact that malic acid inhibits neuro-inflammation and hydrogen peroxide production in the neurons. Such an observation is supported by Martin-Nizard *et al*. [87], who revealed that malic acid inhibits low-density lipoprotein-induced cellular toxicity. Also, Bhattacharya and Tulsawani [88] showed that malic acid has maximum cytoprotection against the toxicity of potassium cyanide.

Exposing animals to many stressors causes CMS rats. CMS was associated with disturbances of animal behaviors such as decreasing sucrose preference testing, distance travelled testing, and center square entry testing but increasing center square duration testing, tail suspension testing, and forced swimming tests. Depression decreased hypothalamus antioxidants such as catalase and NADPH oxidase activities but increased malondialdehyde, conjugated dienes, and the oxidative index. It also decreased hypothalamic neurotransmitter levels. Moreover, it increased hypothalamic inflammatory marker levels. Furthermore, it decreased hypothalamic sodium/potassium-ATPase activity. Finally, it decreased hypothalamic *Hnmt* and *TH* enzymes in CMS rats compared to the control group. Malic acid or venlafaxine drug oral administration to CMS rats pushed the above-mentioned behavioral tests, antioxidants, neurotransmitters, inflammatory markers, sodium/potassium-ATPase activity, and *Hnmt* and *TH* enzymes in the hypothalamus of depressed rats to approach the control values.

Malic acid's neuroprotective mechanism depends on a rise in adenosine triphosphate (ATP) levels and a fall in propidium iodide (PI) nucleic acid levels. Malic acid's neuroprotective function stops the degenerative degeneration of the neurons as well as the loss of mitochondrial O_2 consumption. Malic acid thus participates in anaerobic phosphorylation [89]. However, the neuroprotective effect of

venlafaxine drug is dependent on changes in calcium homeostasis and neurotransmitter levels in the neurons of CMS rats [90].

Malic acid is a potent antioxidant, yet it has no effect on all the parameters tested in the normal rats. This effect is due to the acid's preservation properties, which allow food goods to be preserved for months without experiencing any change in the composition of the contents. Malic acid is listed as a food additive with the E number (E296). The European Union [91], the United States [92], Australia, and New Zealand [93] all utilize it as a food additive for years.

The limitation of the study includes small sample size was used. Also, all other study factors, including internal and external variables, are under control. A small sample size and thorough control of the experimental circumstances also make it simple to determine the neuroprotective impact of malic acid on CMS rats. Consequently, there is a better and accurate result obtained from this study but this result is difficult to apply in depressive human subjects due to external, psychological, and internal variables, as well as, small sample size used in the study.

5. Conclusions

CMS modified animal behaviors, as well as, neurotransmitters, antioxidants, inflammatory markers, sodium/potassium-ATPase activity, and *Hnmt* and *TH* enzymes in the hypothalamus of CMS rats. Malic acid was given orally to CMS rats to treat these CMS-related symptoms. This result is associated with malic acid's ability to reduce neuro-inflammation and the generation of hydrogen peroxide in the neurons. The clinical study that will be conducted as the second stage of this research will examine the impact of malic acid on depressive patients, and the results will be positive if malic acid may reduce depressive-like symptoms in those individuals.

Availability of Data and Materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

KMMK selected the topic and created the research plan. The experiments for the study were conducted by KMMK and HAKT. KMMK carried out the study's statistical analysis and collected the outcome data. The initial and final forms of the work were written by KMMK. The article's final form was authorized by KMMK and HAKT. Both authors contributed to editorial changes in the manuscript. Both authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

This study and all experimental methods were authorized by the National Research Centre Institutional animal care and use committee; the approval code was 12041126. This study was conducted at the National Research Centre, which adhered to strict guidelines for the housing of animals and all other experimental study procedures, particularly the protocol for the care and use of animals.

Acknowledgment

Not applicable.

Funding

This research received no external funding.

Conflict of Interest

The authors declare no conflict of interest.

References

[1] Guo H, Zheng L, Xu H, Pang Q, Ren Z, Gao Y, *et al*. Neurobiological Links between Stress, Brain Injury, and Disease. Oxidative Medicine and Cellular Longevity. 2022; 2022: 8111022.

[2] Schwabe L, Hermans EJ, Joëls M, Roozendaal B. Mechanisms of memory under stress. Neuron. 2022; 110: 1450–1467.

[3] Wang ZJ, Shwani T, Liu J, Zhong P, Yang F, Schatz K, *et al*. Molecular and cellular mechanisms for differential effects of chronic social isolation stress in males and females. Molecular Psychiatry. 2022; 27: 3056–3068.

[4] Zheng Z, Guo C, Li M, Yang L, Liu P, Zhang X, *et al*. Hypothalamus-habenula potentiation encodes chronic stress experience and drives depression onset. Neuron. 2022; 110: 1400–1415.e6.

[5] Leistner C, Menke A. Hypothalamic-pituitary-adrenal axis and stress. Handbook of Clinical Neurology. 2020; 175: 55–64.

[6] Swaab DF, Bao AM, Lucassen PJ. The stress system in the human brain in depression and neurodegeneration. Ageing Research Reviews. 2005; 4: 141–194.

[7] Herrman H, Patel V, Kieling C, Berk M, Buchweitz C, Cuijpers P, *et al*. Time for united action on depression: a Lancet-World Psychiatric Association Commission. Lancet. 2022; 399: 957–1022.

[8] Chen J, Li J, Qiao H, Hu R, Li C. Disruption of IDO signaling pathway alleviates chronic unpredictable mild stress-induced depression-like behaviors and tumor progression in mice with breast cancer. Cytokine. 2023; 162: 156115.

[9] LeMoult J, Battaglini AM, Grocott B, Jopling E, Rnic K, Yang L. Advances in stress and depression research. Current Opinion in Psychiatry. 2023; 36: 8–13.

[10] Grossberg A, Rice T. Depression and Suicidal Behavior in Adolescents. The Medical Clinics of North America. 2023; 107: 169–182.

[11] Willner P. Validity, reliability and utility of the chronic mild stress model of depression: a 10-year review and evaluation. Psychopharmacology. 1997; 134: 319–329.

[12] Hill MN, Hellemans KGC, Verma P, Gorzalka BB, Weinberg J. Neurobiology of chronic mild stress: parallels to major depression. Neuroscience and Biobehavioral Reviews. 2012; 36: 2085–2117.

[13] Qiu K, He W, Zhang H, Wang J, Qi G, Guo N, *et al*. Bio-Fermented Malic Acid Facilitates the Production of High-Quality Chicken via Enhancing Muscle Antioxidant Capacity of Broilers. Antioxidants. 2022; 11: 2309.

[14] Li G, Huang P, Cui SS, Tan YY, He YC, Shen X, *et al*. Mechanisms of motor symptom improvement by long-term Tai Chi

training in Parkinson's disease patients. Translational Neurodegeneration. 2022; 11: 6.
[15] Thorpe J, Shum B, Moore RA, Wiffen PJ, Gilron I. Combination pharmacotherapy for the treatment of fibromyalgia in adults. The Cochrane Database of Systematic Reviews. 2018; 2: CD010585.
[16] Gramsbergen JB, Sandberg M, Kornblit B, Zimmer J. Pyruvate protects against 3-nitropropionic acid neurotoxicity in corticostriatal slice cultures. Neuroreport. 2000; 11: 2743–2747.
[17] "ChemBlink Database of Chemicals from Around the World". Available at: https://www.chemblink.com/ (Accessed: 6 May 2022).
[18] Dawson RMC, Elliott DC, Elliott WH, Jones KM. Data for biochemical research. 3rd edn. Clarendon Press: Jones Oxford University Press, Oxford. 1986.
[19] Merck Company. Safety Data Sheet for DL-Malic acid 100383. 2021. Available at: https://www.merckmillipore.com/INTL/en/product/DL-Malic-acid,MDA_CHEM-100383 (Accessed: 27 April 2022).
[20] Duarte AM, Caixeirinho D, Miguel MG, Sustelo V, Nunes C, Fernandes MM, *et al*. Organic acids concentration in citrus juice from conventional versus organic farming. Acta Horticulturae. 2012; 933: 601–606.
[21] Palacios J, Paredes A, Cifuentes F, Catalán MA, García-Villalón AL, Borquez J, *et al*. A hydroalcoholic extract of Senecio nutans SCh. Bip (Asteraceae); its effects on cardiac function and chemical characterization. Journal of Ethnopharmacology. 2023; 300: 115747.
[22] Mousavi A, Pourakbar L, Siavash Moghaddam S. Effects of malic acid and EDTA on oxidative stress and antioxidant enzymes of okra (Abelmoschus esculentus L.) exposed to cadmium stress. Ecotoxicology and Environmental Safety. 2022; 248: 114320.
[23] Li N, Li Q, He X, Gao X, Wu L, Xiao M, *et al*. Antioxidant and anti-aging activities of Laminaria japonica polysaccharide in Caenorhabditis elegans based on metabonomic analysis. International Journal of Biological Macromolecules. 2022; 221: 346–354.
[24] Wang Y, Zhang N, Zhou J, Sun P, Zhao L, Zhou F. Protective Effects of Several Common Amino Acids, Vitamins, Organic Acids, Flavonoids and Phenolic Acids against Hepatocyte Damage Caused by Alcohol. Foods. 2022; 11: 3014.
[25] Muhamed SA, Moussa EM, Aboasy NK, Gaweesh YY. Effect of 1% malic acid spray on diabetes mellitus-induced xerostomia: A randomized clinical trial. Oral Diseases. 2022. (online ahead of print)
[26] Huang X, Xu L, Qian H, Wang X, Tao Z. Polymalic acid for translational nanomedicine. Journal of Nanobiotechnology. 2022; 20: 295.
[27] Dunaev VV, Tishkin VS, Milonova NP, Belaĭ IM, Makarenko AN. Effect of malic acid salts on physical work capacity and its recovery after exhausting muscular activity. Farmakologiia i Toksikologiia. 1988; 51: 21–25. (In Russian)
[28] Ratajczak P, Kus K, Skurzyńska M, Nowakowska E. The influence of aripiprazole and venlafaxine on the antidepressant-like effect observed in prenatally stressed rats (animal model of depression). Human & Experimental Toxicology. 2018; 37: 972–982.
[29] El-Shamy KA, Koriem KMM, Fadl NN, El-Azma MHA, Arbid MSS, Morsy FA, *et al*. Oral supplementation with geranium oil or anise oil ameliorates depressed rat-related symptoms through oils antioxidant effects. Journal of Complementary & Integrative Medicine. 2019; 17: 20190028.
[30] Koriem KMM, Fadl NN, El-Zayat SR, Hosny EN, El-Shamy KA, Arbid MSS, *et al*. Geranium oil and anise oil inhibit brain cerebral cortex and hippocampus inflammation in depressed animal model. Nutration & Food Science. 2021; 51: 439–456.
[31] El-Azma MHA, El-Beih NM, El-Shamy KA, Koriem KMM, Elkassaby MI, El-Sayed WM. Pumpkin seed oil and zinc attenuate chronic mild stress perturbations in the cerebral cortex of rats. Nutration & Food Science. 2022; 52: 1070–1082.
[32] Palkovits M. Isolated removal of hypothalamic or other brain nuclei of the rat. Brain Research. 1973; 59: 449–450.
[33] Strekalova T, Steinbusch HWM. Measuring behavior in mice with chronic stress depression paradigm. Progress in Neuropsychopharmacology & Biological Psychiatry. 2010; 34: 348–361.
[34] Zhang L, Liu C, Yuan M, Huang C, Chen L, Su T, *et al*. Piperlongumine produces antidepressant-like effects in rats exposed to chronic unpredictable stress. Behavioural Pharmacology. 2019; 30: 722–729.
[35] Belovicova K, Bogi E, Csatlosova K, Dubovicky M. Animal tests for anxiety-like and depression-like behavior in rats. Interdisciplinary Toxicology. 2017; 10: 40–43.
[36] Castagné V, Moser P, Roux S, Porsolt RD. Rodent models of depression: forced swim and tail suspension behavioral despair tests in rats and mice. Current Protocols in Neuroscience. 2011; Chapter 8: Unit 8.10A.
[37] Suttle NF. Copper deficiency in ruminants; recent developments. The Veterinary Record. 1986; 119: 519–522.
[38] Paglia DE, Valentine WN. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. The Journal of Laboratory and Clinical Medicine. 1967; 70: 158–169.
[39] Aebi H. Catalase *in vitro*. Methods in Enzymology. 1984; 105: 121–126.
[40] Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Analytical Biochemistry. 1979; 95: 351–358.
[41] Kogure K, Watson BD, Busto R, Abe K. Potentiation of lipid peroxides by ischemia in rat brain. Neurochemical Research. 1982; 7: 437–454.
[42] Klein RA. The detection of oxidation in liposome preparations. Biochimica et Biophysica Acta. 1970; 210: 486–489.
[43] Kaplán P, Doval M, Majerová Z, Lehotský J, Racay P. Iron-induced lipid peroxidation and protein modification in endoplasmic reticulum membranes. Protection by stobadine. The International Journal of Biochemistry & Cell Biology. 2000; 32: 539–547.
[44] Matsui H, Shimosawa T, Uetake Y, Wang H, Ogura S, Kaneko T, *et al*. Protective effect of potassium against the hypertensive cardiac dysfunction: association with reactive oxygen species reduction. Hypertension. 2006; 48: 225–231.
[45] Vokurková M, Rauchová H, Řezáčová L, Vaněčková I, Zicha J. NADPH oxidase activity and reactive oxygen species production in brain and kidney of adult male hypertensive Ren-2 transgenic rats. Physiological Research. 2015; 64: 849–856.
[46] Kitagawa Y. Simple method for UV spectrophotometric assay of serotonin oxidation by mitochondrial monoamine oxidase from hog kidney cortex. The Showa University Journal of Medical Sciences. 1994; 6: 75–83.
[47] Guo L, Zhang Y, Li Q. Spectrophotometric determination of dopamine hydrochloride in pharmaceutical, banana, urine and serum samples by potassium ferricyanide-Fe(III). Analytical Sciences. 2009; 25: 1451–1455.
[48] Kapoor V, Chalmers JP. A simple, sensitive method for the determination of extracellular catecholamines in the rat hypothalamus using in vivo dialysis. Journal of Neuroscience Methods. 1987; 19: 173–182.
[49] Sciotti MA, Hasan L, Scholer A, Jermann TM, Weber JM, Gygax D. Development and characterization of an enzymatic method for the rapid determination of gamma hydroxybutyric acid. Chimia. 2010; 64: 793–798.

[50] Matalka KZ, Tutunji MF, Abu-Baker M, Abu Baker Y. Measurement of protein cytokines in tissue extracts by enzyme-linked immunosorbent assays: application to lipopolysaccharide-induced differential milieu of cytokines. Neuro Endocrinology Letters. 2005; 26: 231–236.
[51] DeCicco LA, Rikans LE, Tutor CG, Hornbrook KR. Serum and liver concentrations of tumor necrosis factor alpha and interleukin-1beta following administration of carbon tetrachloride to male rats. Toxicology Letters. 1998; 98: 115–121.
[52] Stelmasiak Z, Kozioł-Montewka M, Dobosz B, Rejdak K, Bartosik-Psujek H, Mitosek-Szewczyk K, *et al*. Interleukin-6 concentration in serum and cerebrospinal fluid in multiple sclerosis patients. Medical Science Monitor. 2000; 6: 1104–1108.
[53] Gamaro GD, Streck EL, Matté C, Prediger ME, Wyse ATS, Dalmaz C. Reduction of hippocampal Na+, K+-ATPase activity in rats subjected to an experimental model of depression. Neurochemical Research. 2003; 28: 1339–1344.
[54] Doleshal M, Magotra AA, Choudhury B, Cannon BD, Labourier E, Szafranska AE. Evaluation and validation of total RNA extraction methods for microRNA expression analyses in formalin-fixed, paraffin-embedded tissues. The Journal of Molecular Diagnostics. 2008; 10: 203–211.
[55] Bakunina N, Pariante CM, Zunszain PA. Immune mechanisms linked to depression via oxidative stress and neuroprogression. Immunology. 2015; 144: 365–373.
[56] Strekalova T, Liu Y, Kiselev D, Khairuddin S, Chiu JLY, Lam J, *et al*. Chronic mild stress paradigm as a rat model of depression: facts, artifacts, and future perspectives. Psychopharmacology. 2022; 239: 663–693.
[57] Aluko OM, Umukoro S. Role of purinergic signaling pathways in the adaptogenic-like activity of methyl jasmonate in rats exposed to unpredictable chronic mild stress. Drug Metabolism and Personalized Therapy. 2020; 35.
[58] Flak JN, Jankord R, Solomon MB, Krause EG, Herman JP. Opposing effects of chronic stress and weight restriction on cardiovascular, neuroendocrine and metabolic function. Physiology & Behavior. 2011; 104: 228–234.
[59] Wang W, Yang J, Xu J, Yu H, Liu Y, Wang R, *et al*. Effects of High-fat Diet and Chronic Mild Stress on Depression-like Behaviors and Levels of Inflammatory Cytokines in the Hippocampus and Prefrontal Cortex of Rats. Neuroscience. 2022; 480: 178–193.
[60] Shi W, Zhang S, Lu Y, Wang Y, Zhao J, Li L. T cell responses in depressed mice induced by chronic unpredictable mild stress. Journal of Affective Disorders. 2022; 296: 150–156.
[61] Cai L, Mu YR, Liu MM, Tang WJ, Li R. Antidepressant-like effects of penta-acetyl geniposide in chronic unpredictable mild stress-induced depression rat model: Involvement of inhibiting neuroinflammation in prefrontal cortex and regulating hypothalamic-pituitaryadrenal axis. International Immunopharmacology. 2020; 80: 106182.
[62] Filho CB, Jesse CR, Donato F, Giacomeli R, Del Fabbro L, da Silva Antunes M, *et al*. Chronic unpredictable mild stress decreases BDNF and NGF levels and Na(+),K(+)-ATPase activity in the hippocampus and prefrontal cortex of mice: antidepressant effect of chrysin. Neuroscience. 2015; 289: 367–380.
[63] Park BK, Kim NS, Kim YR, Yang C, Jung IC, Jang IS, *et al*. Antidepressant and Anti-Neuroinflammatory Effects of Bangpungtongsung-San. Frontiers in Pharmacology. 2020; 11: 958.
[64] Boyko M, Kutz R, Grinshpun J, Zvenigorodsky V, Gruenbaum SE, Gruenbaum BF, *et al*. Establishment of an animal model of depression contagion. Behavioural Brain Research. 2015; 281: 358–363.
[65] Matallah A, Guezi R, Bairi A. Repeated restraint stress induced neurobehavioral and sexual hormone disorders in male rats. AIMS Neuroscience. 2022; 9: 264–276.
[66] Park BK, Kim YR, Kim YH, Yang C, Seo CS, Jung IC, *et al*. Antidepressant-Like Effects of *Gyejibokryeong-hwan* in a Mouse Model of Reserpine-Induced Depression. BioMed Research International. 2018; 2018: 5845491.
[67] Guo Y, Chen X, Gong P, Li Z, Wu Y, Zhang J, *et al*. Advances in the mechanisms of polysaccharides in alleviating depression and its complications. Phytomedicine. 2023; 109: 154566.
[68] Lorenzo EC, Kuchel GA, Kuo CL, Moffitt TE, Diniz BS. Major depression and the biological hallmarks of aging. Ageing Research Reviews. 2023; 83: 101805.
[69] Tanasiewicz M, Hildebrandt T, Obersztyn I. Xerostomia of Various Etiologies: A Review of the Literature. Advances in Clinical and Experimental Medicine. 2016; 25: 199–206.
[70] Ferreira I, Ortigoza Á, Moore P. Magnesium and malic acid supplement for fibromyalgia. Medwave. 2019; 19: e7633.
[71] von Eggelkraut-Gottanka SG, Abu Abed S, Müller W, Schmidt PC. Quantitative analysis of the active components and the by-products of eight dry extracts of Hypericum perforatum L. (St John's Wort). Phytochemical Analysis. 2002; 13: 170–176.
[72] Lian B, Xia J, Yang X, Zhou C, Gong X, Gui S, *et al*. Mechanisms of ketamine on mice hippocampi shown by gas chromatography-mass spectrometry-based metabolomic analysis. Neuroreport. 2018; 29: 704–711.
[73] Bhatt S, Devadoss T, Jha NK, Baidya M, Gupta G, Chellappan DK, *et al*. Targeting inflammation: a potential approach for the treatment of depression. Metabolic Brain Disease. 2023; 38: 45–59.
[74] Quiroga PR, Nepote V, Baumgartner MT. Contribution of organic acids to α-terpinene antioxidant activity. Food Chemistry. 2019; 277: 267–272.
[75] Yan E, Wang Y, He L, Guo J, Zhang X, Yin J. Effects of Dietary L-malic Acid Supplementation on Meat Quality, Antioxidant Capacity and Muscle Fiber Characteristics of Finishing Pigs. Foods. 2022; 11: 3335.
[76] Calvo MM, Martín-Diana AB, Rico D, López-Caballero ME, Martínez-Álvarez O. Antioxidant, Antihypertensive, Hypoglycaemic and Nootropic Activity of a Polyphenolic Extract from the Halophyte Ice Plant (*Mesembryanthemum crystallinum*). Foods. 2022; 11: 1581.
[77] Taher MA, Tadros LK, Dawood DH. Phytochemical constituents, antioxidant activity and safety evaluation of Kei-apple fruit (Dovyalis caffra). Food Chemistry. 2018; 265: 144–151.
[78] Tang X, Liu J, Dong W, Li P, Li L, Lin C, *et al*. The cardioprotective effects of citric Acid and L-malic Acid on myocardial ischemia/reperfusion injury. Evidence-based Complementary and Alternative Medicine. 2013; 2013: 820695.
[79] Barragán-Zarate GS, Lagunez-Rivera L, Solano R, Pineda-Peña EA, Landa-Juárez AY, Chávez-Piña AE, *et al*. Prosthechea karwinskii, an orchid used as traditional medicine, exerts anti-inflammatory activity and inhibits ROS. Journal of Ethnopharmacology. 2020; 253: 112632.
[80] Sahpaz S, Garbacki N, Tits M, Bailleul F. Isolation and pharmacological activity of phenylpropanoid esters from Marrubium vulgare. Journal of Ethnopharmacology. 2002; 79: 389–392.
[81] Añibarro-Ortega M, Pinela J, Barros L, Ćirić A, Silva SP, Coelho E, *et al*. Compositional Features and Bioactive Properties of *Aloe vera* Leaf (Fillet, Mucilage, and Rind) and Flower. Antioxidants. 2019; 8: 444.
[82] Novaes LS, Dos Santos NB, Dragunas G, Perfetto JG, Leza JC, Scavone C, *et al*. Repeated Restraint Stress Decreases Na,K-ATPase Activity via Oxidative and Nitrosative Damage in the Frontal Cortex of Rats. Neuroscience. 2018; 393: 273–283.
[83] Nikiforov AA, Ostretsova IB. Stimulation of weak organic acid uptake in rat renal tubules by cadmium and nystatin. Biochemical Pharmacology. 1994; 47: 815–820.

[84] Outlaw WH, Jr, Du Z, Xia Meng F, Aghoram K, Riddle KA, Chollet R. Requirements for activation of the signal-transduction network that leads to regulatory phosphorylation of leaf guard-cell phosphoenolpyruvate carboxylase during fusicoccin-stimulated stomatal opening. Archives of Biochemistry and Biophysics. 2002; 407: 63–71.
[85] Yoshikawa T, Yanai K. Histamine Clearance Through Polyspecific Transporters in the Brain. Histamine and Histamine Receptors in Health and Disease. 2017; 173–187.
[86] Lu Q, Mouri A, Yang Y, Kunisawa K, Teshigawara T, Hirakawa M, *et al*. Chronic unpredictable mild stress-induced behavioral changes are coupled with dopaminergic hyperfunction and serotonergic hypofunction in mouse models of depression. Behavioural Brain Research. 2019; 372: 112053.
[87] Martin-Nizard F, Sahpaz S, Furman C, Fruchart JC, Duriez P, Bailleul F. Natural phenylpropanoids protect endothelial cells against oxidized LDL-induced cytotoxicity. Planta Medica. 2003; 69: 207–211.
[88] Bhattacharya R, Tulsawani R. In vitro and in vivo evaluation of various carbonyl compounds against cyanide toxicity with particular reference to alpha-ketoglutaric acid. Drug and Chemical Toxicology. 2008; 31: 149–161.
[89] Mazzio E, Soliman KFA. The role of glycolysis and gluconeogenesis in the cytoprotection of neuroblastoma cells against 1-methyl 4-phenylpyridinium ion toxicity. Neurotoxicology. 2003; 24: 137–147.
[90] Cao Y, Ng C. Absorption, distribution, and toxicity of per- and polyfluoroalkyl substances (PFAS) in the brain: a review. Environmental Science. Processes & Impacts. 2021; 23: 1623–1640.
[91] UK Food Standards Agency. Current EU approved additives and their E Number. 2020. Available at: https://www.food.gov.uk/business-guidance/approved-additives-and-e-numbers (Accessed: 27 March 2022).
[92] FDA. “Food Additive Status List”. 2021. Available at: https://en.wikipedia.org/wiki/Malic_acid (Accessed: 5 May 2022).
[93] FSANZ. “Australia New Zealand Food Standards Code”. Standard 1.2.4 - Labelling of ingredients. 2003. Available at: https://www.foodstandards.gov.au/code/Pages/default.aspx (Accessed: 27 October 2022).