

Original Research Article

Behavioural and oxidative effects of *Melissa officinalis* leaf ethanol extract in a valproic acid-induced rat model of autism spectrum disorder

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Sent for review: 29 August 2025

Revised accepted: 18 June 2026

Abstract

Purpose: To determine the effect of ethanolic leaf extract of *Melissa officinalis* on the behavioral and oxidative stress parameters in a prenatal valproic acid (VPA) induced rat model of autism spectrum disorder.

Methods: Pregnant Wistar rats were administered VPA (600 mg/kg, subcutaneously) or saline on gestational day 12.5. Control dams (saline group) received an equivalent volume of 0.9 % normal saline subcutaneously on the same gestational day. The male offspring were then administered 100 mg/kg of *M. officinalis* extract orally or vehicle (tap water) daily on postnatal days 35 - 82. Social behavior assessment was performed on postnatal day 1, and hippocampal biochemical analysis was conducted on the 10th day across four groups (n = 5 rats per group).

Results: Gas chromatography-mass spectrometry (GC-MS) phytochemical analysis revealed the presence of eight major constituents, with α -terpineol being dominant (29.898 %). Prenatal exposure to VPA resulted in severe social deficit with a negative sociability index (-0.286 ± 0.225 vs. 0.36 ± 0.116 in controls) and social novelty index (-0.33 ± 0.295 vs. 0.841 ± 0.089 in controls). This social deficit was completely restored by *M. officinalis* treatment ($p < 0.05$). Valproic acid administration reduced superoxide dismutase activity by 41.3 % and malondialdehyde by 70.3 % compared to control, thereby resulting in severe oxidative stress in the hippocampus due to reduction of glutathione levels by 17.4 %. This process was also reversed by the administration of *M. officinalis*.

Conclusion: *Melissa officinalis* extract reverses core autism-like social behavioral impairments and exhibits significant antioxidant and neuroprotective effects. This study justifies its ethnomedical therapeutic application.

Keywords: Autism spectrum disorder, Hippocampus, *Melissa officinalis*, Oxidative stress, Social behavior, Valproic acid

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INTRODUCTION

Autism spectrum disorder (ASD) is a multifaceted combination of neurodevelopmental disorders, characterized by severe impairments in social communication and interaction, as well as limited

and repetitive behavioral patterns and significant heterogeneity in clinical manifestation [1]. The prevalence rate of ASD is estimated to be about 1 in 100 children worldwide, and is continuously growing. The Centers for Disease Control and Prevention (CDC) in their 2023 surveillance data

reported a prevalence of 3.22% among children aged 8 years in the United States [2]. This increasing trend, especially in developed countries, has made ASD a major health issue of societal concern [3]. The etiology of ASD is still not clearly understood. Several factors are believed to be predisposing to it, which may be interrelated. There is a complex interplay of genetic susceptibilities, environmental factors and triggers, as well as dysregulation of synapses [4]. Antiepileptic drugs taken during neurodevelopmental stages in pregnancy, as well as environmental factors, are possible risk factors that can be modified [5]. The strongest of these is valproic acid (VPA), which is linked to the development of ASD in the event of administration during pregnancy, especially during the first trimester [6].

The disruption of neural pathways related to social cognition is the key pathophysiological factor in ASD, and the hippocampus is recognized as a key structure in social and emotional processing [7]. In addition to the already known functions of the hippocampus in learning and stereotyped behaviors, the hippocampus plays an important role in the modulation of social and emotional behaviors. A growing body of evidence points to the consistent hippocampal pathological changes in ASD, such as the aberrant neuronal layering, increased astrocytic reactivity, impaired synaptic plasticity, and disordered neurogenesis [8].

In the prenatal VPA rodent model, which accurately recapitulates key autism-like phenotypes such as impairments in sociability and stereotyped behaviors, there is a significant decrease in the expression of synaptophysin in CA3 hippocampal region, which correlates with the development of social and exploratory impairments in the juvenile stage [9]. Furthermore, changes in brain-derived neurotrophic factor (BDNF) and early GABAergic dysregulation trigger the faulty synaptic plasticity, which ultimately leads to a reduced integrity of hippocampal circuitry [10]. The existing pharmacological treatments (viz, risperidone and aripiprazole) are limited as they only treat irritability symptoms of ASD, but not the core symptoms [11]. The negative adverse effect profiles of these antipsychotics, such as metabolic disorders, weight gain, sedation, and extrapyramidal symptoms, are significant negative effects that limit their long-term usability and highlight the urgency of alternative therapeutic options that are relatively safe and tolerable [12]. This treatment gap has led to increased research in phytochemical interventions against key neuropathological

driver processes such as oxidative stress, neuro-inflammation, and impaired synaptic plasticity [13].

Melissa officinalis L. (Fam: Lamiaceae), also known as lemon balm, is a perennial herbaceous plant that is characterized by lemon-scented leaves and small white flowers. It is native to Europe, North Africa, and western Asia, and is traditionally used for its sedative and neuroprotective properties. The leaves are rich in Rosmarinus acid, flavonoids, and polyphenolic compounds, which have shown significant neuroprotective potential in several preclinical models [14,15]. This study aimed to determine the therapeutic potential of *Melissa officinalis* ethanolic leaf extract in autism-like social deficits in VPA-induced ASD rat model.

EXPERIMENTAL

Wistar male and female adult rats (178 - 268 g) were obtained from the animal house of the College of Pharmacy, University of Karbala, Iraq. The rats were kept in regularly cleaned cages under 12-hour light/dark cycles at 22 ± 2 °C, and 50 - 60 % relative humidity. Rats were allowed to acclimatize in the laboratory for 10 days before they were subjected to any procedures. They were given standard feed twice daily and were allowed free access to clean water *ad libitum*. The CPCSEA guidelines were followed in handling the animals [16].

All procedures were conducted in strict accordance with institutional and international ethical guidelines for the care and use of laboratory animals, specifically following the principles outlined in the Guide for the Care and Use of Laboratory Animals (8th Edition, National Research Council) and the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS No. 123) [17].

Ethical approval

All procedures involving animals were approved by the Scientific and Research Ethics Committee, College of Medicine, University of Karbala, Karbala, Iraq (approval no. 268_2024).

Mating and pregnancy verification

Mating was achieved by placing two sexually fit female rats in a cage together with one male rat for 24 hours. Vaginal cytology was used as a means of pregnancy confirmation. This was determined by collecting vaginal swabs and checking for the presence of spermatozoa. The

first time that the presence of sperm is noticed in the vaginal smears was regarded as gestational day (GD) 0.5. When pregnancy was confirmed, the pregnant females were isolated [18].

Valproic acid administration

Sodium valproate (VPA; Santa Cruz Biotechnology, USA, SC-202378) was utilized to induce the autism-like syndrome at a dose of 600 mg/kg body weight. The drug was dissolved in 2.5 mL of sterile normal saline (0.9 %w/v) immediately before subcutaneous administration. Pregnant dams received a single subcutaneous (S.C.) injection of VPA solution on gestational day (GD) 12.5, a critical period for neurodevelopment [19]. Control dams (saline group) received equivalent volumes of 0.9 % normal saline (1 mL/kg, S.C.) on GD 12.5, with injection volumes ranging from 0.44 to 0.67 mL for animals weighing 178 - 268 g.

Plant material and extract preparation

Melissa officinalis L. (lemon balm) leaves were thoroughly washed with distilled water and dried in the shade at room temperature for 7 - 10 days. The dried leaves were then ground into fine powder using an electric grinder and stored in sterilized opaque containers at 4 °C until extraction. Ethanol extraction was performed using a modified maceration protocol [20]. Dried *M. officinalis* leaf powder (100 g) was put in 400 mL of 70 % ethanol (v/v) and agitated every 24 hours in the dark at room temperature for 7 consecutive days. The extraction was done several times in order to maximize the production of bioactive compounds. The resultant extracts were then pooled and filtered using Whatman No. 1 filter paper and were concentrated using the rotary evaporator at reduced pressure. The concentrated extract was again dried in a hot-air oven at 40 °C for 72 hours in order to get a crude extract. The dried extract was reconstituted using distilled water to a final working solution of 100 mg/mL and stored at 4 °C before use.

Phytochemical characterization by Gas Chromatographic-Mass Spectrometry (GC-MS)

The analysis was done using Scion 436 GC-MS (Scion Instruments, USA). Samples were injected using helium as carrier gas at a flow rate of 1.0 mL/min. Additional operations were carried out as follows: Volume of injection, 5 µL; ratio of splits, 1:10; temperature of injector, 250 °C. The temperature of the column oven was initiated at 40 °C (held for 1 min). It was increased at a rate of 5 °C/min to 130 °C (held for 3 min), and further

to 150 °C (held for 3 min), and finally to 190 °C (held for 5 min) before ending the procedure. Compound identification was done through the comparison of mass spectral data and the NIST library databases.

Treatment

Following successful mating and pregnancy confirmation (GD 0.5), pregnant dams were randomly divided into two prenatal treatment groups (n = 5 dams/group). The VPA group received VPA (600 mg/kg, S.C.) on GD 12.5, and saline (SAL) group received 0.9 % normal saline (1 mL/kg, S.C.) on GD 12.5. Offspring were maintained with their respective dams until weaning, up to postnatal day (PND) 24. On PND 35, male offsprings from each prenatal treatment group were further subdivided into two postnatal treatment groups (n = 5 rats/group), yielding a total of four groups, namely: prenatally VPA-exposed offspring receiving tap water orally (VPA + vehicle group); prenatally VPA-exposed offspring receiving *M. officinalis* extract (100 mg/kg orally (VPA + MO); prenatally saline-exposed offspring receiving tap water orally (SAL + vehicle); and prenatally saline-exposed offspring receiving *M. officinalis* extract (100 mg/kg orally; SAL + MO). *Melissa officinalis* extract or vehicle (distilled water) was administered daily via oral intubation from PND 35 to 82.

Behavioral assessment (three-chamber social interaction test)

The three-chamber social interaction paradigm was used to assess social behavior on PND 83 [21], which is a well-validated assay of sociability and social novelty preference in rodents. The machine was a rectangular box made of Plexiglass (60 x 45 x 22 cm), partitioned into three interconnected chambers with removable doors that could move freely between the chambers. The male offspring of all four of the experimental groups (188 - 300 g in weight) were tested. Before the experiment, rats were subjected to 10 days of habituation to the apparatus environment in order to reduce anxiety due to novelty. The testing was done in a dimly lit environment to minimize anxiety-related behaviors that may confound the measurement of social interactions. The left and right chambers were equipped with two of the same inverted wire mesh cups, which only allowed auditory, visual and olfactory senses but not physical touch. After inter-trial, all surfaces of the apparatus and cups were wiped clean with 75 % ethanol to remove olfactory stimulation from any

prior subjects. The test had three sequential 10-minute blocks [22]:

Phase 1 (Habituation)

The test rat was placed in the central chamber and left to freely explore the three empty chambers for 10 minutes. Rats were carefully handled to minimize stress.

Phase 2 (Sociability)

After a short inter-trial interval, another age and sex matched unfamiliar conspecific rat (the Stranger 1) was introduced into one wire cup in a side chamber, and a second empty cup was enclosed in the other side chamber. The test rat was put in the middle chamber where the doors were opened, and the test rat was given a chance to explore freely within 10 minutes. The duration of stay in the respective chambers and duration of research on each cup were manually logged.

Phase 3 (Social Novelty Preference)

A second unfamiliar rat (Stranger 2) was introduced under the empty cup previously and was matched in terms of strain, age and sex. The test rat was once more provided with freedom of exploration for another 10 min. The time taken in each chamber and time taken to investigate each stimulus were noted (Stranger 1, now familiar, and Stranger 2, unfamiliar).

The parameters of behavior were calculated using Eqs 1 and 2 to obtain social indices [23].

$$\text{Sociability Index (SI)} = \frac{T_{\text{Stranger 1}} - T_{\text{Object}}}{T_{\text{Stranger 1}} + T_{\text{Object}}} \dots (1)$$

$$\text{Social Novelty Index (SNI)} = \frac{T_{\text{Stranger 2}} - T_{\text{Stranger 1}}}{T_{\text{Stranger 2}} + T_{\text{Stranger 1}}} \dots (2)$$

where T represents time spent investigating each stimulus. Positive index values indicate preference for social stimuli (SI) or novel social stimuli (SNI), while values near zero indicate no preference.

Tissue collection and processing

At PND 83, immediately after behavioral tests, rats were euthanized by deep intraperitoneal injection of a mixture of ketamine (100 mg/kg) and xylazine (20 mg/kg). The rats were decapitated after the surgical anesthesia (no evidence of pedal withdrawal reflex). The head was carefully removed and the brain was quickly

excised and carefully preserved to maintain tissue integrity. The brain was cut at the midsagittal plane under stereomicroscopic observation. The cerebrum and cerebellum were separated as well as the brainstem and extraneous portions (olfactory bulbs, thalamus) were excised. Each hemisphere of the hippocampus was carefully micro-dissected under ice-cold conditions. Dissected hippocampi were fixed in 10 % neutral buffered formalin overnight at room temperature and were used for histological and immunohistochemical studies. Hippocampi of the left hemisphere were snap-frozen in liquid nitrogen and stored at -80 °C and used for biochemical analysis [24].

Biochemical assessment of oxidative stress markers

One part of frozen hippocampal tissues was homogenized in 10 parts of ice-cold phosphate buffer (pH 7.4) at 10,000 rpm for 15 min and the supernatant was pooled and stored at -80 °C for further biochemical analyses [25].

Malondialdehyde (MDA) assay

Lipid peroxidation was determined by measuring the levels of MDA using the thiobarbituric acid reactive substances (TBARS) technique. Briefly, 125 µL of tissue homogenate was added to 1.5 mL of 1 % phosphoric acid in a test tube and vortexed. Then, 0.5 mL of 0.6 % thiobarbituric acid aqueous solution was added and incubated in boiling water for 45 min. Upon cooling, 1 mL of n-butanol was added, and the tubes were centrifuged at 7000 rpm for 10 min. Absorbance of the supernatant (organic layer) was determined spectrophotometrically at 532 nm. The MDA range was determined by using a standard calibration curve that was constructed from absorbances obtained from serial concentrations of 1,1,3,3-tetramethoxypropane [26].

Superoxide dismutase (SOD) activity

The assay of Superoxide Dismutase (SOD) was performed using an Enzyme-Linked Immunosorbent Assay (ELISA) kit. The SOD activity was determined using a commercial kit developed based on xanthine-xanthine oxidase method (Radox kit). The level of inhibition was determined spectrophotometrically at 560 nm [27].

Reduced glutathione (GSH) measurement

A modified Ellman method with 5,5'-dithiobis (2-nitrobenzoic acid; DTNB) was used to measure

GSH levels. The tissue homogenate was combined with trichloroacetic acid (5 %) in 1 % of Phosphate Buffered Saline (PBS), vortexed and centrifuged at 3000 rpm for 10 min. Phosphate buffer (pH 7.4) and DTNB reagent were added to the supernatant. Spectrophotometric analysis of the color formed was recorded at 412 nm. Standard curve was used to calculate the concentration of GSH. The concentration of proteins in the tissue homogenates was measured by Bradford procedure, where protein was compared with bovine serum albumin [28].

Statistical analysis

Data are presented as mean $\pm$ standard error of the mean (SEM). Statistical analysis was performed using SPSS software (version 27). Two-way ANOVA was used to compare the four groups, with prenatal treatment (VPA vs. saline) and postnatal treatment (*M. officinalis* vs. vehicle) independent variables. Post-hoc comparisons were conducted using the LSD test. Statistical significance was set at $p < 0.05$.

RESULTS

Phytochemical characterization of *Melissa officinalis* extract

Results of phytochemical analysis of the ethanolic extract of *Melissa officinalis* leaves using gas chromatographic-mass spectrometry showed the presence of eight major phytochemical constituents (Table 1).

Chromatographic result showed that α -Terpineol was predominant with 29.90 % of the total peak area, while cyclopentaneacetic acid, 3-oxo-2-pentyl, methyl ester, salicylic acid, and tert-butyl ester were the secondary and tertiary products, respectively. Other notable compounds include cyclohexene, 3-methyl-6-(1-methyldiene; 12.402 %), 7-Acetyl-6-ethyl-1,1,4,4-tetramethyltetralin (9.148 %), and 3-Buten-2-one, 4-(2,5,6,6-tetramethyl-1-cyclohexen-1-yl)- (7.703 %). The probability of identifying α -Terpineol was between 60 and 98 % and it had the highest

probability match of 98 % in the NIST library databases.

Effects of *M. officinalis* extract treatment on social behavior

Sociability Index (SI)

The three-chamber social interaction test demonstrated impacts of prenatal VPA exposure on sociability in which significant amelioration followed treatment using *M. officinalis* extract (Figure 1 A). Control group (SAL + vehicle) exhibited a positive sociability index (0.36 ± 0.116), thus showing that the animals preferred the room with social stimulus to empty room. Contrarily, prenatal VPA exposure resulted in social deficit shown by significantly negative SI in VPA + vehicle (-0.286 ± 0.225 , $p < 0.05$ vs. SAL + vehicle), indicating active avoidance of social interaction - a fundamental autism-like behavioral phenotype. Postnatal administration of *M. officinalis* extract to VPA-exposed offspring (VPA + MO group) significantly restored sociability, indicated by positive SI (0.656 ± 0.202), which was not significantly different from control ($p > 0.05$). The group of animals that portrayed the largest sociability index was the SAL + MO group (0.68 ± 0.090), which implies that the extract may have sociability-enhancing effects even in non-challenged animals. There were no significant differences among SAL + vehicle, VPA + MO and SAL + MO groups (LSD = 0.482599, $p > 0.05$), suggesting that *M. officinalis* administration was able to restore the biochemical enzyme levels and reverse ASD.

Social Novelty Index (SNI)

Results of analysis of social novelty preference, which is a more advanced sign of social memory and discrimination, demonstrated similar results (Figure 1). The SAL + vehicle (control) group showed significant likeness towards the new conspecific than the old one, as indicated by a positive SNI (0.841 ± 0.089).

Table 1: Phytochemical profiles of *Melissa officinalis* ethanolic extract identified using GC-MS retention time, relative peak area, identification probability and name of the compound

Peak no.	Retention time (min)	Area (%)	Probability (%)	Compound name
1	4.823	5.960	70	Cyclohexene, 1-methyl-4-(1-methylethenyl)-, (S)
2	8.754	29.898	98	α -Terpineol
3	8.867	12.402	75	Cyclohexene, 3-methyl-6-(1-methylethylidene)
4	19.715	22.270	73	Cyclopentaneacetic acid, 3-oxo-2-pentyl-, methyl ester
5	19.827	7.703	80	3-Buten-2-one, 4-(2,5,6,6-tetramethyl-1-cyclohexen-1-yl)
6	21.471	13.631	82	Salicylic acid, tert.-butyl ester
7	23.630	9.148	60	7-Acetyl-6-ethyl-1,1,4,4-tetramethyltetralin
8	23.823	5.149	-	Tonalid

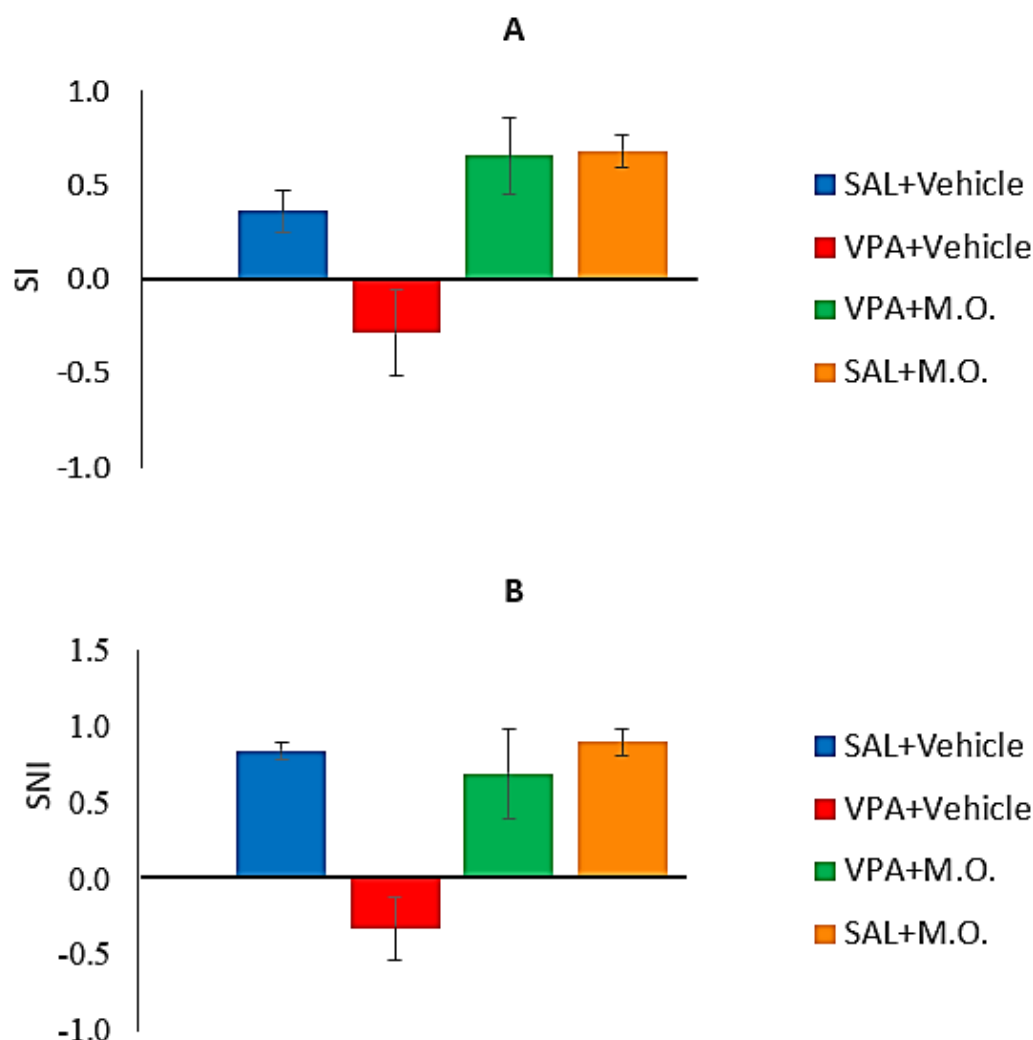


Figure 1: *Melissa officinalis* extract ameliorates VPA-induced social deficits in the three-chamber test. (A) Sociability Index (SI) showing time preference for social stimulus vs. empty chamber. (B) Social Novelty Index (SNI) showing preference for novel vs. familiar conspecific. Mean $\pm$ SE (n = 5 per group). VPA = valproic acid; MO = *Melissa officinalis*; SAL = saline

Prenatal exposure to VPA impaired this social recognition memory significantly, because VPA + vehicle group had a significantly negative SNI (-0.33 ± 0.295 , $p < 0.05$ vs. SAL + Vehicle), which suggests that social novelty was not detected or avoided in VPA + vehicle group. This deficit was significantly corrected with *M. officinalis* extract treatment. The VPA + MO group showed a significantly increased SNI (0.692 ± 0.206), which was statistically comparable with control group ($p > 0.05$), indicating that social novelty preference had been restored. The SAL + MO group had the best SNI value (0.9 ± 0.053), which also helped to substantiate the social cognition-enhancing potential of the extract. There was no significant variation between SAL + Vehicle, VPA + MO, and SAL + MO groups (LSD = 0.538695, $p > 0.05$).

Effect of *M. officinalis* extract treatment on hippocampal oxidative stress markers

Superoxide Dismutase (SOD) activity

The hippocampal tissue biochemical analysis showed that the antioxidant enzyme activity had changed significantly after VPA exposure and *M. officinalis* extract treatment (Figure 2 A). The SOD activity of SAL + Vehicle (control) group (44.826 ± 1.320 $\mu\text{g/mL}$) showed normal endogenous antioxidant defense capacity. There was a significant depletion of hippocampal SOD activity (26.302 ± 2.152 $\mu\text{g/mL}$) in VPA + vehicle group ($p < 0.05$ vs control), which is 41.3 %. This severe depletion of the hippocampal SOD activity was due to oxidative stress. Treatment with postnatal extract of *M. officinalis* significantly replenished the SOD activity in offspring of VPA-

exposed rats. The VPA + MO group had significantly high levels of SOD ($39.638 \pm 1.987 \mu\text{g/mL}$; $p < 0.05$ vs VPA + Vehicle). Likewise, SOD activity was high in SAL + MO group ($40.804 \pm 2.986 \mu\text{g/mL}$) and the high activity was statistically similar to that of the control group ($p > 0.05$).

Reduced Glutathione (GSH) levels

The hippocampal GSH, a non-enzymatic antioxidant and redox homeostasis marker, was also significantly altered by VPA exposure and *M. officinalis* treatment (Figure 2 B). Glutathione level in control group was within the physiological range. The Prenatal VPA exposure caused a significant decline of hippocampal GSH in VPA + vehicle group ($p < 0.05$ vs. control). The depletion of GSH caused by VPA was prevented by *M. officinalis* extract treatment. Glutathione levels in VPA + MO group ($14.698 \pm 0.264 \mu\text{g/L}$) were not significantly different from control ($p > 0.05$), which was evidence of a full restoration of the level of this antioxidant indicator. Interestingly, MO + SAL group exhibited very high levels of GSH ($17.976 \pm 0.880 \mu\text{g/L}$, $p <$

0.05 compared to all other groups showing that *M. officinalis* extract has inherent capacity to increase cellular antioxidant reserve even in non-challenged situations.

Malondialdehyde (MDA) levels

The extent of lipid peroxidation, which is a measure of cellular membrane oxidative damage, is a direct measure of oxidative damage (Figure 2 C). Control group showed low level of basal MDA ($26.602 \pm 2.313 \text{ mmol/L}$). Exposure to VPA in the prenatal period led to a significant increase in hippocampal MDA ($45.294 \pm 5.343 \text{ mmol/L}$ in VPA + vehicle group; $p < 0.05$ vs. control).

Lipid peroxidation was markedly reduced by the use of *M. officinalis* extract in the offspring of rats that received only VPA. The VPA + MO group also had lower MDA levels ($32.934 \pm 3.883 \text{ mmol/L}$; $p < 0.05$ vs. VPA + vehicle), representing a 27.3 % decrease in oxidative damage, while the levels were fairly high relative to controls.

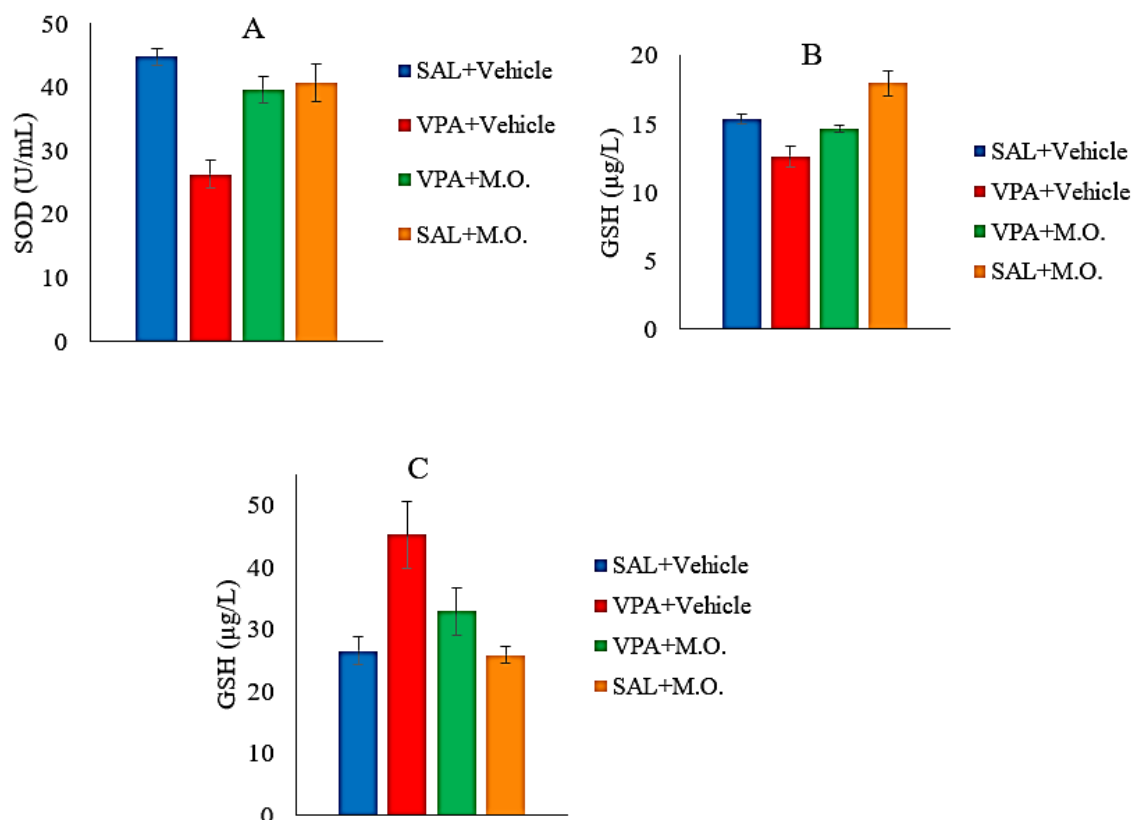


Figure 2: *Melissa officinalis* extract ameliorates VPA-induced hippocampal oxidative stress. (A) Superoxide dismutase (SOD) activity. (B) Reduced glutathione (GSH) levels. (C) Malondialdehyde (MDA) content. Mean $\pm$ SEM ($n = 5$ per group). Groups with different letters are significantly different at $p < 0.05$. VPA exposure induced oxidative stress (decreased SOD and GSH, increased MDA), which was significantly attenuated by *Melissa officinalis* extract treatment. VPA = valproic acid; MO = *Melissa officinalis*; SAL = saline

The SAL + MO group did not vary significantly when compared to SAL + vehicle (control) group ($p > 0.05$), ensuring that the extract in itself is not a pro-oxidant. These biochemical changes indicate that prenatal exposure to VPA resulted in severe oxidative stress in the hippocampus of offspring due to significantly declined SOD and GSH, and increased lipid peroxidation (MDA). The administration of postnatal *M. officinalis* extract resulted in antioxidant and neuroprotective effects in the hippocampus of VPA-exposed offspring.

DISCUSSION

The present study demonstrates that *Melissa officinalis* ethanolic extract attenuates behavioral deficits and neuropathological changes in the prenatal valproic acid (VPA)-induced rat model of autism spectrum disorder. These findings suggest that postnatal administration of *M. officinalis* effectively reversed VPA-induced impairments in social interaction, reduced hippocampal oxidative stress, and key pathophysiological features associated with ASD. These effects make *M. officinalis* a potential phytopharmaceutical candidate for developing regimens for ASD.

Impaired sociability is one of the diagnostic features of ASD that is not yet well addressed with current pharmacological interventions [11]. As shown in the results of the three-chamber test, VPA exposure resulted in significantly negative sociability and social novelty indexes in the offspring. This behavioral pattern is consistent with a previous report that prenatal exposure to VPA disrupts key neurodevelopmental pathways during the time of neural tube closure, leading to lifelong changes in neural circuitry underlying social cognition [6]. This study reports that *M. officinalis* extract activates the basic neurotrophic and neuroprotective mechanisms, which are in a position to restore the functional integrity of the compromised social processing networks. Therefore, both sociability index and social novelty preference were restored in VPA + MO animals when compared to control group.

Bioactive compounds in *M. officinalis*, especially Rosmarinus acid and flavonoids, are important in regulating neurotrophic factor (BDNF) expression and downstream TrkB-CREB signal pathways, which are essential in synaptic plasticity, neuronal life, and social behavior control [15]. The therapeutic effects of *M. officinalis* are therefore likely due to the neurotrophin-related synaptic restructuring and functional

reorganization of impaired neural networks. Since autism models induced by VPA are associated with BDNF downregulation, altered synaptic connectivity in prefrontal cortex areas and hippocampal areas subservient to social cognition [29].

The hippocampus has been known to be involved in learning and memory. It is also involved in social recognition memory and social novelty processing, which were measured in the three-chamber test [8]. The restoration of social novelty preference due to *M. officinalis* extract administration was due to recovered hippocampal networks that were important in differentiating familiar and novel conspecifics - a sophisticated kind of social memory that is usually impaired in ASD. Besides, the fact that *M. officinalis* increased social behavior in control group implied pro-social intrinsic attributes.

The processes that govern social behavioral recovery are probably diverse convergent pathways other than the direct function of neurotrophin modulation. To begin with, *M. officinalis* extracts regulate the equilibrium between inhibitory and excitatory neurotransmission by increasing the activity of the GABAergic system, which is critically dysregulated in autism. Secondly, the cholinergic effects of the extract, reported as acetylcholinesterase inhibition, could assist the attention process and social interaction.

Melissa officinalis possesses several phytochemical constituents identified in GC-MS analysis, with α -terpineol being the most abundant compound (29.898 %). The α -Terpineol possesses free radical-scavenging and antioxidant properties, which effectively neutralize reactive oxygen species and chelate transition metals [30]. The effects of the reduction in oxidative stress by *M. officinalis* are far-reaching in terms of preserving the functions of the synapse. Neuronal damage of synaptic proteins, lipid membranes, and mitochondria by oxidative damage is devastating to neurotransmitter release, receptor and energy metabolism processes critical to normal synaptic transmission and plasticity. The hippocampus is also susceptible to oxidative damage because of high metabolic activities and the presence of lipid-rich membranes. *Melissa officinalis* is able to preserve synaptic integrity by balancing redox functions; thus, this is the reason for the improvement in behavior in the social interaction tests.

Offspring of VPA-exposed animals exhibited decrease in SOD activity by 41.3 %. This is a crucial loss of the major enzymatic defense system against superoxide radicals. This reduction, in conjunction with 17.4 % drop in GSH and 70.3 % rise in MDA, results in cell damage, thereby causing structural damage to neuronal membranes.

The recovery of cellular antioxidant capacity, with *M. officinalis* restoring SOD activity to 88.4 % of control values, the total restoration of GSH and reduction of MDA by 27.3 % all point to clinically significant restoration of cellular antioxidant capacity. Interestingly, *M. officinalis* treatment in non-challenged animals increased the GSH levels above control, indicating that it may have a role in neuroprotective preconditioning studies, which needs to be further studied in prevention-based models. This observation indicates that the extract not only reverses oxidative stress but also increases cellular antioxidant reserves, which may provide future protection against oxidative stress [31]. Neurons of the hippocampal social recognition memory are highly reliant on optimal synaptic functioning, which depends on intact lipid membranes, appropriately folded proteins and sufficient ATP production, which is destroyed by oxidative stress. *Melissa officinalis* probably prevents downstream inflammatory and synaptic effects by inhibiting the initiation of oxidative damage.

Rosmarinus acid was absent in the GC-MS result of this study. Its presence in *M. officinalis* has been reported in an earlier study [20]. This difference may probably be due to the weaknesses of GC-MS in detecting non-volatile phenolic acids. The composition of botanical extracts greatly depends upon the state or condition of cultivation, time of harvest, method of extraction and storage.

CONCLUSION

This study has provided evidence that *Melissa officinalis* ethanolic leaf extract ameliorates autism-like behavioral and neuropathological features in the prenatal valproic acid rat model. Treatment with *M. officinalis* improved social interaction deficits, restored sociability and social novelty preference to levels comparable with controls.

The overall normalization of hippocampal redox homeostasis that includes restoration of enzymatic and non-enzymatic antioxidant defenses in addition to a significant decrease in lipid peroxidation proves that *M. officinalis* uses the basic cellular protection strategies that can

sustain the integrity of synaptic and neuronal viability in oxidative stress conditions.

DECLARATIONS

Acknowledgement/Funding

None.

Ethical approval

It is stated in the method.

Use of Artificial intelligence/Large language models

The authors confirm that AI-based tools were used solely for language editing and manuscript polishing purposes (e.g., grammar, phrasing, and clarity). No AI tools were used to generate content, interpret data, or influence study design, results, or conclusions. All intellectual contributions, study analyses, and interpretations reported in the manuscript are the original work of the authors.

Availability of data and materials

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

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the author(s) named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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