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Therapeutic effects of Melissa officinalis extract on hippocampal oxidative stress and body weight in a valproic acid-induced autism model in albino rats

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Abstract

Autism spectrum disorder (ASD) is an autistic neurodevelopmental disorder where humans have social communication problems, limited interests, and repetitive behaviors and the prevalence is growing in the world. This paper examined the therapeutic impact of Melissa officinalis alcoholic extract on body weight variation and signs of oxidative stress in an autism model induced by valproic acid (VPA) on male rats. Pregnant Wistar rats were injected with VPA (600mg/kg, subcutaneously) or saline on gestation day 12.5, and the male offspring were injected with Melissa officinalis extract (100mg/kg, orally) or vehicle on postnatal day 35 to 82. The body weights were analyzed after every week and the biomarkers of oxidative stress in the hippocampal were measured biochemically in the four experimental groups (n=5/group). The experimental VPA dosage has a significant effect on the body weight, causing a 71 percent weight gain on average of 246.88±2.50 g at week 7 over baseline of 144.15±3.83 g in controls. Exposure to VPA caused severe oxidative stress in the hippocampal which included reduction of superoxide dismutase activity (41% vs. 44.826±1.320 U/mL) reduced levels of reduced glutathione (17% vs. 15.346±0.366 ug/l) and an increase in malondialdehyde concentration (70% vs. 26.602±2.313 mmol/L). Melissa officinalis extract treatment was significant in improving the conditions of oxidative stress by replacing the activity of superoxide dismutase up to 88% of the levels of control (39.638±1.987 U/mL), returning glutathione concentrations (14.698±0.264 µg/L) to normal level, and lowering the amount of lipid peroxidation by 27% (32.934±3.883 mmol/L), yet exhibited slight effic The above findings indicate that Melissa officinalis extract has strong neuroprotective actions against oxidative stress induced by VPA due to its increased antioxidant enzyme activity and abated lipid peroxidation, which can be used as a complementary medicine in the management of oxidative stress-related pathology of autism spectrum disorders.

Keywords: Autism spectrum disorder, Valproic acid, Melissa officinalis, Oxidative stress, Body weight, Antioxidants.

Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental disorder that is complicated by difficulties related to social communication, loss of interests, and repetitive behaviors. Recent epidemiological surveys have shown that ASD has become more prevalent in the world, with about 1 in 36 children affected **[1]**. This increase in prevalence has increased the research endeavors to elucidate the underlying pathophysiological processes and come up with therapeutic interventions that are effective.

Various etiological factors that can lead to ASD development can be identified as genetic predisposition, environmental factors, prenatal

exposure to different teratogenic agents [2]. Prenatal exposure of rodents to valproic acid (VPA) is currently one of the most well-proven experimental models to cause autism-like behaviors [3]. VPA, which is a widely used anticonvulsant and mood stabilizer, is a risk factor of ASD when taken during pregnancy, especially in critical neurodevelopmental periods [4]. VPA-induced autism model has good face, construct, and predictive validity that reproduce key behavioral, neuroanatomical and biochemical phenomena in human ASD [5].

It has recently been indicated that oxidative stress is a significant pathogenesis factor in ASD [6]. ASD is often characterized by high levels of oxidative damage such as an enhanced production of lipid peroxidation products and a decrease in antioxidant capacity [7]. Oxidative damage is especially susceptible to the developing brain since it has a high oxygen demand, is rich in polyunsaturated fatty acids, and has a relatively weak antioxidant defense system [8]. VPA exposure has been revealed to cause severe oxidative stress on the neural tissues that cause neuronal damage and abnormalities in behavior [9].

Another major issue in ASD populations is the metabolic changes that include abnormalities in weight gain [10]. ASD kids experience more obesity than neurotypical kids do, and several of its factors are interconnected such as medication, issues with selectivity of diets, and lack of physical activity [11]. VPA therapy in its own right is linked to a high level of weight gain and dysfunction in metabolis.

The multifactorial nature of ASD and lack of pharmacological options to address core symptoms have led to the increased interest in the use of complementary therapies, especially medicinal plants [12]. The traditional uses of Melissa officinalis L. (lemon balm), which belongs to the Lamiaceae family, include neurological and psychiatric disorders. Its neuroprotective, antioxidant, and anti-inflammatory effects are due to its bioactive compounds, namely, rosmarinic acid, caffeic acid, flavonoids, and triterpenes. The studies prove the antioxidant properties of Melissa officinalis by free radical scavenging, stimulation of endogenous enzymes, and inhibition of lipid peroxidation [13]. It further demonstrates anxiogenesis and cognitive enhancement in animal and human research, posing a possibility of neurodevelopmental illnesses, as well as, behavioral loss.

Although it has a good profile, studies on Melissa officinalis in the models of autism, especially on the metabolic and oxidative alterations induced by VPA, are scarce. This paper investigated the effects of Melissa officinalis alcoholic extract on the body weight and the oxidative stress markers in VPA-induced rat models of autism and assumed that it would reduce the VPA-induced weight gain and improve the oxidative stress levels of the hippocampus of the rat models through increased antioxidant activity and reduced lipid peroxidation.

Materials and Methods

Plant Material and Extract Preparation

The leaf of Melissa officinalis was harvested in the month of January through April 2025 among the Iranian herbalists. The leaves were dried in controlled laboratory conditions, washed clean with a lot of water and the leaves were ground in an electric grinder to a fine form, it was stored in sterile dark bags at a temperature of 4°C until extraction. Preparation of Alcoholic Extract: 100 g of powdered Melissa officinalis dried leaf was dissolved in 400 mL of 70% ethanol in a dark tube in a 24 h soaking process. The process of extraction was done repeatedly in order to get the highest yield of bioactive compounds. A rotary evaporator was used to concentrate the combined extracts and then dried them in an oven at a temperature of 40° C over a period of 72 h. The dry extract was re-dissolved in distilled water to make a final 100 mg/mL volume and kept at 4°C until use [13].

Animals

The rats used in this study were adult male and female Wistar rats (178-268 g) which were acquired at the Animal House, College of Pharmacy, University of Karbala. Animals were kept in wooden cages whose bedding was made of sawdust and was regularly washed and disinfected. Rats were kept in normal laboratory environment (12 h light/dark cycle) and they had ad libitum access to food and water. The food composition was in compliance with CPCSEA Guidelines (2003) specifications Feed intake was 25-35 g/day which was offered in two meals. The animals were subjected to 10-day acclimatization before experiment procedures. At Karbala Veterinary Hospital, veterinary examination was done to confirm health conditions and disease-free status.

Ethical Approval and Guideline (ARRIVE)

All the laboratory work was performed following the internationally acceptable ethics in dealing with laboratory animals. The experiment was conducted in accordance with the National Institutes of Health (NIH) Guide to the Care and Use of Laboratory Animals and in accordance with the ARRIVE guidelines on reporting animal research. All efforts have been taken to ensure that the suffering of animals was kept to the minimal, the number of animals used was kept to the minimum

and all the protocols in the experiment were structured in order to reach the scientific goals of the research and at the same time to uphold the highest standards of animal care.

Mating and Pregnancy Confirmation

Mating was done through co-housing of the female sexually fit two with one male rat in each cage over a period of 24 h. Diagnosis of pregnancy was made by use of vaginal smear to identify spermatozoa. When sperm was detected (marked as gestational day 0.5), pregnant females were placed in isolation and standard diet and water was fed to them during gestation.

VPA-Induced Autism Model

The valproic acid (VPA, Santa Cruz Biotechnology) was made at a concentration of 600 mg/kg body weight by dissolving the powder in 0.9% normal saline. Pregnant women were injected on gestation day 12.5 with one S/C injection with sterilized medical syringes. The volume given was calculated by the formula:(weight of animal in grams × 0.025)/10. Pregnant rats (174-229 g), were used as controls and trial rats (178-268 g). Control group and trial rats received the same volumes (0.44-0.67 mL) of 0.9% normal saline and at the same route and time.

Experimental Design

The experiment was carried out in a period of 82 days in the Laboratories at the college of education of Pure Sciences and at the animal house of the college of Pharmacy at the University of Karbala. Based on mating and confirmation of pregnancy at gestational day 0.5, animals were placed in experimental groups as follows.

Prenatal Phase: VPA Group: On gestational day 12.5, pregnant rats were treated with VPA (600mg/kg, S/C). Saline (SAL) Group: On gestational day 12.5, pregnant rats were given normal saline (1 mL/kg, S/C).

Postnatal Phase: Weaning was done on postnatal day (PND) 24 where children were left with the mothers. After the weaning procedure, males were kept in a standard laboratory environment and provided with an ad libitum food and water. The treatment commenced at PND 35 and proceeded on a daily basis until PND 82 (treatment period: 48 days).

Treatment Groups (n=5 per group):

1. Normal Saline Control (NORMALSALINE): The offspring of saline-injected mothers that were fed on oral tap water.
2. VPA Control (VPA): The children of VPA-injected mothers fed on oral tap water. VPA + Extract (VPA+Extract): Pups born of VPA-inoculated mothers that were fed Melissa officinalis extract (100 mg/kg body weight) orally.
3. Normal Saline + Extract (NORMALSALINE + Extract): The offspring of saline-injected mothers were fed the oral Melissa officinalis extract (100 mg/kg body weight).

Extract Administration Melissa officinalis extract was given orally between PND 35 and PND 82. The formula to calculate the dose (100 mg/kg body weight) was (Grams of animal weight/0.025)/10. The calculated volume was dissolved using 2.5 mL of distilled water and orally gavaged. As an illustration, 0.5 ml extract with 2.5 ml distilled water was administered to a 200g rat.

Body Weight Monitoring

The calibrated electronic scale was used to measure body weights every week between weeks 1 and 7 of the treatment period (Al-nargelchi, Russia). Measurements were also taken at the same time of day to eliminate the effects of circadian variation.

Animal Sacrifice and Tissue Collection

Rats were put to rest on PND 83 (24 hours after the last treatment) with ketamine (10%, 100 mg/mL) and xylazine (2%, 20 mg/mL) by intraperitoneal injection as per body weight. Animals were decapitated after deep anesthesia (7-15 min) and the brain had to be removed quickly. Hippocampus Dissection: Brains were put on ice-cooled surfaces as soon as possible in order to ensure that tissue is rigid. A longitudinal incision was made to separate right and left hemispheres which were inverted to reveal surfaces on the ventral sides. In order to isolate the hippocampus, the cerebellum, brainstem, diencephalon, olfactory bulb and thalamus were removed. The hippocampus was sub-divided into two parts with one part instantaneously immersed in 10% neutral buffered formalin to prepare the hippocampus histologically and the other portion frozen instantly at -80°C in dry ice in order to conduct biochemical analyses (oxidative stress biomarker assays). The dissection process was performed on the brain tissues on ice to preserve the integrity of the tissues

Biochemical Analyses

Hippocampal Tissue Homogenization

Processing of frozen hippocampal tissues was done on biochemical assays according to protocol of Bodzon-Kulakowska et al. (2007) **[14]** with some modifications. One tablet of Complete Mini Protease Inhibitor Cocktail (Roche) was dissolved in 10 ml of T-

PER (Tissue Protein Extract Reagent, Thermo Scientific) to prepare protease inhibitor solution.

Homogenization Procedure

The pieces of the tissues were left to thaw on ice, weighed and put back to ice. Scissors were used to chop tissues in small bits (about 1 mm) at cold temperature. The pieces of the tissues were put into glass homogenization tubes. T-PER was used at 5mL /250 mg tissue. A pestle was used to homogenize tissues in ice until no longer lumps were present. Homogenates were placed in 15 mL Falcon tubes (2.5 mL each) and placed on ice. Ice was used to homogenize the sample ultrasonically 5-10 times (30% cycle, 3 output control) to a foamy state. The samples were placed on ice and were allowed to lysate cells in 10 minutes. The samples were pipetted into 1.5 mL Eppendorf tubes and centrifuged at 14,000 g and 4°C within 10 min. Supernatants were cautiously transferred to PCR tubes (about 200 µL in each tube) The samples were kept at -80°C until biochemical analysis.

Superoxide Dismutase (SOD) Activity Assay

SOD activity was determined using the pyrogallol autoxidation inhibition method (Marklund and Marklund, 1974) **[15]**. This method is based on the enzyme's ability to inhibit pyrogallol autoxidation in the presence of EDTA at pH 8.2.

Reagent Preparation:

- Tris-EDTA buffer (pH 8.2): 2.85 g Tris and 1.11 g EDTA-Na_2 dissolved in 1L distilled water
- Pyrogallol solution (0.2 mM): 0.252 g pyrogallol dissolved in 0.6 mL concentrated HCl, diluted to 1 liter with distilled water

Assay Procedure:

1. Spectrophotometer was zeroed using Tris-EDTA buffer
2. Test and control tubes were prepared as follows:

Reagent	Test (µL)	Control (µL)
Sample supernatant	50	-
Tris-EDTA buffer	1000	1000
Distilled water	-	50
Pyrogallol	1000	1000

3. Absorbance was measured at 420 nm at time zero and after 1 minute of pyrogallol addition

Calculations:

- % Inhibition of pyrogallol autoxidation = (ΔA control - ΔA test) / ΔA control × 100%
- SOD Activity (U/mL) = % Inhibition / 50%. One unit of SOD activity is defined as the amount of enzyme required to inhibit pyrogallol autoxidation by 50%.

Reduced Glutathione (GSH) Assay

GSH levels were measured according to the method of Moron et al. (1979) **[16]**. This assay is based on the reaction of acid-soluble sulfhydryl groups (primarily reduced glutathione) with dithionitrobenzoate (DTNB) to form a yellow complex.

100 µL supernatant was mixed with 100 µl of 25% trichloroacetic acid (TCA), mixture was kept on ice for several minutes, samples were centrifuged at 3000 rpm for 5 min, then 300 µL supernatant was mixed with 700 µL of 0.2 M sodium phosphate buffer (pH 8.0) and 2 mL of 0.6 mM DTNB (prepared in 0.2 M buffer, pH 8.0), yellow color development was measured at 412 nm after 10 min, blank consisted of 0.1 mL 5% TCA instead of sample supernatant GSH concentration was calculated using a standard curve and expressed as µg/L.

Malondialdehyde (MDA) Assay

Lipid peroxidation was assessed by measuring malondialdehyde (MDA) using the thiobarbituric acid (TBA) method (Buege and Aust, 1978) **[17]**. This method measures MDA as an aldehyde decomposition product of lipid peroxides.

Principle: One molecule of MDA reacts with two molecules of thiobarbituric acid to form a red MDA-TBA complex measurable at 535 nm.

TCA-TBA-HCl Reagent Preparation

Chemical solutions: 15% w/v trichloroacetic acid, 0.375% w/v thiobarbituric acid, 0.25 N HCl (2.1 mL concentrated HCl in 100 mL), Solution was heated slightly to dissolve TBA. The preparation as follows: 0.6 ml TCA-TBA-HCl reagent was added to 0.4 mL sample, mixture was vortexed and incubated in boiling water bath for 10 min, after cooling, 1.0 mL freshly prepared 1N NaOH was added, pink color absorbance was measured at 535 nm, blank contained distilled water instead of sample, MDA concentration was calculated and expressed as mmol/L.

Statistical Analysis

Data are given as mean ± SE. Statistical analysis performed using SPSS software (version 27). Two-way ANOVA used to compare the four experimental groups, with prenatal treatment (VPA vs. saline) and postnatal treatment (*M. officinalis* vs. vehicle) independent variables. Posthoc comparisons were conducted using the LSD test. Statistical significance at $p < 0.05$.

Results

Effects of VPA and Melissa officinalis Extract on Body Weight

Two-way ANOVA revealed significant main effects of both Group ($F = 30.12$, $p < 0.001$) and Week ($F = 25.16$, $p < 0.001$) on body weight. However, the Group × Week interaction was not significant ($F = -0.48$, $p > 0.05$), indicating that the pattern of weight gain over time was similar across all groups, with primary differences attributable to treatment effects rather than differential growth trajectories.

Bidirectional ANOVA, Table 1, showed significant main effects of Group ($F = 30.12$, $p < 0.001$) and Week ($F = 25.16$, $p < 0.001$) on the body weight. The Group × Week interaction was however not significant ($F = -0.48$, $p > 0.05$) which implies the same pattern of weight gain over time among all the groups with primary differences being due to treatment effects and not different growth patterns.

Table 1. ANOVA Summary for Body Weight Analysis.

Source	Sum of Squares (SS)	Degrees of Freedom (df)	Mean Square (MS)	Fvalue	p-value
Group	48751.32	2	24375.66	30.12	p < 0.001
Week	101810.82	5	20362.16	25.16	p < 0.001
Group × Week	-3868.27	10	-386.83	-0.48	ns
Error	77688.86	96	809.26	-	-
Total	224382.72	113	-	-	-

ns = not significant. Source: Own authorship.

LSD post-hoc comparison showed that the groups had different patterns of weight gain during the seven weeks of the experiment (Table 2). Normal Saline Control group showed a consistent, physiologically adequate weight gain, which rose to 101.63 ± 2.84 g in week 1 to 144.15 ± 3.83 g in week 7. In sharp contrast, VPA group showed very high body weights commencing as early as week 2 (144.43 ± 1.13 g) than controls with the differences increasing and reaching high levels of 246.88 ± 2.50 g (week 7). This is 71 % higher than the baseline which is a significant increase that goes way above the normal developmental weight gain pattern observed in the control animals. The VPA + Extract group showed a small yet significant reduction in VPA induced weight gain. Although the body weights were considerably higher than normal controls during the weeks 3, onwards, this group experienced a smaller weight compared to the VPA only group, which experienced a weight of 243.47 ±5.09 g at week 7. This is a little but significant loss of weight gain associated with VPA.

Table 2. Effect of Treatment with Valproic Acid (VPA) and Melissa officinalis Extract on Body Weights (g) of Male Rats Across Seven Weeks.

Group	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7
NORMALSALINE (CONTROL)	101.63 ± 2.84 Aa	107.92 ± 2.3 Ab	114.21 ± 2.2 Ab	123.5 ± 2.73 Ab	133.71 ± 3.77 Ac	138.56 ± 4.73 Ac	144.15 ± 3.83 Ac
VPA	119.62 ± 1.95 Aa	144.43 ± 1.13 Bb	176.99 ± 1.27 Bc	211.35 ± 2.09 Bd	233.97 ± 1.21 Be	245.37 ± 2.09 Be	246.88 ± 2.5 Be
VPA + Extract	121.36 ± 4.43 Aa	137.13 ± 2.81 Ba	172.88 ± 2.99 Bb	207.59 ± 3.83 Bc	231.97 ± 5.94 Bd	238.93 ± 4.44 Bd	243.47 ± 5.09 Bd
NORMALSALINE + Extract	243.47 ± 5.09 Bd	158.47 ± 5.95 Ba	193.06 ± 2.26 Bb	210.23 ± 2.21 Bc	215.54 ± 3.0 Bc	219.25 ± 2.91 Bc	221.32 ± 2.66 Bc

Source: Own authorship.

LSD values: Between groups (within same week) = 35.7; Between weeks (within same group)

= 18.0; α = 0.05. Different uppercase letters (A, B) indicate significant differences among groups within the same week. Different lowercase letters (a, b, c, d, e) indicate significant differences among weeks within the same group. Values are presented as Mean ± SE.

Figure 1 shows line graph showing body weight progression (g) over seven weeks for four experimental groups. The VPA group (red line) shows marked weight gain exceeding controls. VPA + Extract group (green line) demonstrates slightly reduced weight gain compared to VPA alone. NORMALSALINE control (blue line) shows normal developmental weight gain. NORMALSALINE + Extract (orange line) shows elevated weights throughout the study period. Data points represent Mean ± SE.

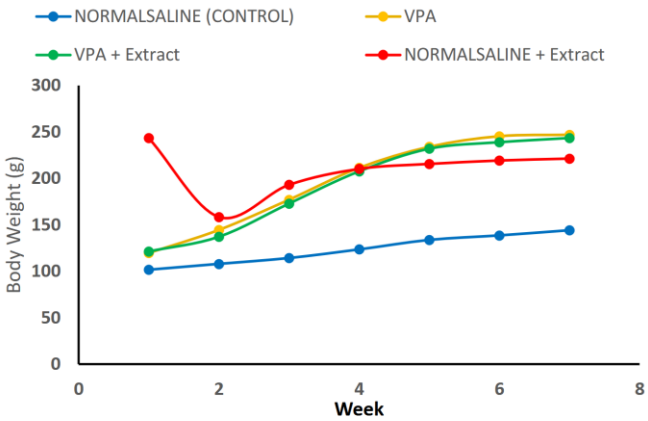

Figure 1. Effect of Treatment on Animal Body Weight Over 7 Weeks. Source: Own authorship.

Effects of VPA and Melissa officinalis Extract on Oxidative Stress Markers

The status of oxidative stress in the tissue of the hippocampal was fully examined by assessing three major biomarkers: superoxide dismutase (SOD) activity, decreased glutathione (GSH) levels, and concentration of malondialdehyde (MDA). ANOVA conducted on a single sample showed that there was a

significant difference between the experimental groups on all three markers ($p<0.05$), Table 3.

Table 3. Effect of VPA and Melissa officinalis Extract on Oxidative Stress Markers in Rat Hippocampal Tissue.

Groups	SOD (U/mL)	GSH (µg/L)	MDA (mmol/L)
NORMALSALINE (CONTROL)	44.826 ± 1.320 A	15.346 ± 0.366 B	26.602 ± 2.313 B
VPA	26.302 ± 2.152 B	12.67 ± 0.764 C	45.294 ± 5.343 A
VPA + Extract	39.638 ± 1.987 A	14.698 ± 0.264 B	32.934 ± 3.883 B
NORMALSALINE + Extract	40.804 ± 2.986 A	17.976 ± 0.880 A	25.91 ± 1.270 B
LSD	**6.576**	**1.874**	**10.662**
p-value	**0.05**	**0.05**	**0.05**

Source: Own authorship.

Superoxide Dismutase (SOD) Activity

One of the most important markers of antioxidant defense capacity was the SOD activity, which demonstrated significant changes within experimental groups. The highest activity of SOD was seen in the Normal Saline Control group (44.826 ± 1.320 U/mL), which is considered as the best activity of antioxidant enzyme when physiological conditions are normal. VPA group showed a drastic and statistically significant cut in SOD activity (26.302 +- 2.152 U/mL, $p < 0.05$) which constituted about 41% reduction than controls. Such a significant decrease is a sign of intense malfunction of antioxidant defense mechanisms caused by prenatal exposure to VPA. Melissa officinalis extract treatment was found to have a significant effect of reversing this SOD depletion

caused by VPA. VPA + Extract group exhibited significantly higher SOD activity (39.638 ± 1.987 U/mL) than VPA-only group with approximately 88 percent control level as compared to normal control level. The statistical analysis showed that there was no significant difference in VPA + Extract group and the Normal Saline Control group, which showed that the antioxidant enzyme capacity has been restored to a great extent. The group of Normal Saline + Extract was also found to have much greater SOD activity (40.804 ± 2.986 U/mL), similar to normal controls and indicating that Melissa officinalis extract increases endogenous antioxidant defenses even without an oxidative stress.

Reduced Glutathione (GSH) Levels

GSH, which is a large non-enzymatic antioxidant, and an important aspect of cellular redox homeostasis was significantly depleted after VPA exposure. Normal Saline Control group had physiologically adequate levels of GSH (15.346 ± 0.366 µg/L). Such a decrease in the concentration of GSH (12.67 ± 0.764 ug/L, $p < 0.05$) or about 17 percent as compared to control was observed in the VPA group. This decrease is an indication of impaired antioxidant ability and predisposition to oxidative injury. The VPA + Extract group showed significant GSH recoveries (14.698 ± 0.264 µg/L) with no significant difference which was shown with normal controls. Such recovery to almost normal values suggests that Melissa officinalis extract is successfully able to preserve the non-enzymatic antioxidant capacity. Notably, the levels of GSH were remarkably high in the group of Normal Saline + Extract (17.976 ± 0.880 µg/L) as compared to all other groups including normal controls. This 17 % increase over baseline indicates that Melissa officinalis extract is actively involved in increasing the synthesis of GSH or decreasing its use, thus increasing the total antioxidant reserve capacity.

Malondialdehyde (MDA) Levels

A trustworthy biomarker of lipid peroxidation and oxidative damage of cell membranes, MDA, indicated the existence of strong oxidative stress in the case of VPA-exposed animals. Normal Saline Control group had low MDA (26.602 ± 2.313 mmol/L), which showed that there was little lipid peroxidation in the normal conditions. VPA group recorded a statistically significant high and dramatic increase in the MDA concentration (45.294 ± 5.343 mmol/L, $p<0.05$) of about 70% over controls. This significant increase confirms that there have been serious oxidative damages to the lipid membranes by prenatal exposure to VPA. Melissa officinalis extract treatment had a significant effect of reducing lipid peroxidation. VPA + Extract animals showed significantly lower levels of MDA (32.934 ± 3.883 mmol/L) than VPA-only animals, which was about 27 % decrease in lipid peroxidation. Although this is still somewhat elevated relative to normal controls, this is a very large decrease, suggesting that there is plenty of protection against oxidative damage of membranes. The Normal Saline + Extract group showed no significant difference to normal controls (MDA level is 25.91 ± 1.270 mmol/L) meaning that Melissa officinalis extract does not cause oxidative stress condition in healthy animals and prevent lipid peroxidation in distressed conditions.

Values are presented as Mean ± SE. Different letters (A, B, C) within the same column indicate statistically significant differences among groups ($p<0.05$). SOD = Superoxide dismutase; GSH = Reduced glutathione; MDA = Malondialdehyde; LSD = Least Significant Difference.

All these results indicate that prenatal VPA

treatment causes severe oxidative stress in hippocampal tissue, which is characterized by a loss of antioxidant enzyme activity (SOD), nonenzymatic antioxidant reserves (GSH) and increased lipid peroxidation (MDA). Melissa officinalis extract can be used to effectively counter these oxidative changes, improve antioxidant status and substantially decrease the markers of oxidative damage, and as such offers neuroprotection to oxidative injury induced by VPA.

Discussion

The significant weight gain of VPA-exposed offspring is consistent with clinical and preclinical studies of VPA-weight gain as one of the major adverse effects **[18]**. Our findings showed that VPA animals gained weight much higher than controls, which started to grow in week 2 and continued to increase the weight during the experimental period, and at the end of the experiment achieved weights that were 71 percent more than on the baseline.

A variety of processes lead to the VPA-induced weight gain, such as the change of energy metabolism, insulin resistance, dysregulation of leptin, and the change in hypothalamic circuitry controlling appetite **[19]**. VPA stimulates the activation of AMP-activated protein kinase (AMPK) in the hypothalamus, which increases food intake and reduces energy expenditure as well as influences lipid metabolism by inhibiting fatty acid b-oxidation and increasing lipogenesis **[20]**.

These results go well beyond the VPA model to autism spectrum disorders in general. The prevalence of obesity among children with ASD is always higher than in neurotypical children according to the epidemiological studies, where prevalence was estimated between 19-30% **[11]**. The risk of obesity is an indication of intricate interplay between genetic vulnerability, metabolic issues, dietary choosiness, decreased exercise, and medication influence **[21]**. The administration of Melissa officinalis extract had a small, yet statistically insignificant effect on reducing the weight gain rate in VPA-exposed animals. Although the final weights in the VPA + Extract group (243.47 g) were slightly less than the final weights in VPA (246.88 g), the difference was not statistically significant, which indicated that the extract at the dosage used (100 mg/kg) was not very effective in reversing the effects of VPA on weight. Surprisingly, the use of the Melissa officinalis extract on the normal saline-treated animals also led to the increase of body weights indicating that the extract as such can modify the metabolic parameters regardless of VPA exposure, and that future metabolic and hormonal profiling is required.

We found a significant biochemical change of the tissue of the hippocampal on exposure to prenatal VPA. Significant improvement of the SOD activity, GSH levels and MDA concentration were noticed in the VPA group (reducing by 41, 17 and 70 %, respectively, versus controls) which clearly indicated the presence of intense oxidative imbalance due to weakened antioxidant defenses and excessive lipid peroxidation. The hippocampus is especially sensitive to oxidative injury because of its high metabolic rates, a large percentage of polyunsaturated fatty acids, and a comparatively low level of antioxidants **[22]**. The pathophysiology of autism spectrum disorders has been associated with oxidative stress in the hippocampus **[23]**. There are a number of interrelated processes leading to VPA-related oxidative stress. VPA is metabolized to reactive oxygen species (ROS) by the b-oxidation pathways in the mitochondria and biotransformation by cytochrome P450 **[9]**. Also, VPA suppresses the activity of histone deacetylases (HDACs), which causes changes in the expression pattern of the genes potentially affecting the production of antioxidant enzymes and the performance of mitochondria **[24]**. VPA also interrupts the mitochondrial energy metabolism by blocking the transport of fatty acids using carnitine and disrupting the electron transport chain, leading to the increased production of ROS and the reduced production of ATP **[25]**.

The high decrease in the SOD activity of animals that were exposed to VPA is an indicator of the disruption of enzymatic antioxidant protection, which exposes neurons to damage through superoxide **[26]**. Equally, the depletion of GSH undermines non-enzymatic antioxidant activity, which suppresses protective processes and increases vulnerability to oxidative damage **[22]**. The dramatic increase in levels of MDA is direct evidence of oxidative injury to membrane lipids, which is evidence of severe membrane injury that probably leads to dysfunction of the neurons and impaired behavior in the VPA autism model **[27]**.

The most interesting results were associated with the high neuroprotective properties of Melissa officinalis extract against oxidative stress in VPA. The extract restored SOD activity to 88% of normal levels of control, normal GSH concentrations, and decreased MDA levels by 27 compared to VPA-alone animals, indicating multi-factorial effects on antioxidant and cytoprotective effects.

The GC-MS analysis revealed several bioactive compounds that are known to be antioxidants. A-terpineol (29.9 per cent) is the main constituent that has a strong free radical scavenging effect and neuroprotective properties **[28]**. In addition to the

identifications made by GC-MS, Melissa officinalis contains phenolic acids (especially rosmarinic acid and caffeic acid) and flavonoids, all of which explain the strong antioxidant property of the plant [29]. The effects of the Melissa officinalis extract on restoring SOD activity probably represents both anti-oxidative inactivation protection and improved expression of genes. In Melissa officinalis, phytochemicals, especially rosmarinic acid and flavonoids, stimulate transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) which promotes the expression of antioxidant response element (ARE)-controlled genes such as SOD, catalase, and glutathione-related enzymes [30].

The high rise in the GSH level after extract treatment is indicative of the boosted GSH production and /or decreased oxidative degradation. Nrf2 activation may enhance the GSH formation by regulating g-glutamylcysteine ligase (GCL) by Melissa officinalis constituents [31]. Also, direct ROS scavenging of phenolic compounds inhibits the oxidation of GSH and maintains the reduced GSH pool. The Melissa officinalis extract inhibited the lipid peroxidation, and this is an indicator of both the inhibition of the ROS-mediated peroxidation and the potential chainbreaking antioxidant. Phenolic compounds have the ability to donate hydrogen atoms to lipid peroxyl radicals and disrupt the lipid peroxidation chain sequence [32]. In addition to the direct antioxidant action, Melissa officinalis extract could have neuroprotective effects by other complementary mechanisms. In a number of studies, anti-inflammatory efficacy, such as proinflammatory cytokine inhibition (TNF-a, IL-1b, IL-6) and inhibition of inflammatory signaling pathways (NF-kB) have been reported [33,34].

Anti-inflammatory effects can also be a part of neuroprotection since in the VPA model of autism neuroinflammation and oxidative stress are tightly coupled with each other. Important implications of the shown Melissa officinalis extract capacity to alleviate oxidative stress in the VPA autism model to future therapeutic use of ASD. It is becoming more and more clear that people with ASD show enhanced levels of oxidative stress, such as high levels of lipid peroxidation and low levels of antioxidant capacity [35]. Such oxidative changes are associated with the severity of symptoms and can be part of core ASD pathophysiology.

Conclusion

This research paper showed that prenatal exposure to valproic acid causes major metabolic changes and excessive oxidative stress in hippocampal tissue of rat pups that has been exposed to prenatal valproic acid and reflects the main characteristics of autism spectrum disorders. The use of VPA led to excess weight gain and oxidative imbalance in the form of reduced antioxidant enzyme (SOD), empty glutathione (GSH) and increased lipid peroxidation (MDA). Neuroprotection against oxidative stress caused by VPA in the alcoholic extract treatment of Melissa officinalis was significant, recovering the activity of antioxidant enzymes, the levels of glutathione and lipid peroxidation had largely decreased. These preventing effects were probably due to the multi-constituent nature of Melissa officinalis specifically the phenolic compounds and terpenes which had strong antioxidant and neuroprotective properties. Although the extract was not very effective in preventing VPA-related weight gain, its antioxidant and neuroprotective properties are very strong implying that it can be used to alleviate oxidative stress-related pathology in autism spectrum disorders. The findings justify further research on Melissa officinalis as a complementary treatment option in ASD where future studies are necessary to understand more about the specific mechanisms at the molecular level, the dosage regimen, and determine its clinical efficacy in humans. The current research can be added to the existing body of evidence on the role of oxidative stress in the pathophysiology of autism and the potential of medicinal antioxidants found in plant sources as safe and convenient therapeutic agents. The lack of pharmacological interventions to counteract core ASD symptoms, the already established safety profile of Melissa officinalis, and the potential of the given botanical intervention in enhancing the results of patients with autism spectrum disorders makes the ongoing research of the specified botanical intervention an exciting opportunity.

CRediT

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Not applicable.

Ethical Approval

All the laboratory work was performed following the internationally acceptable ethics in dealing with laboratory animals. The experiment was conducted in accordance with the National Institutes of Health (NIH) Guide to the Care and Use of Laboratory Animals and in accordance with the ARRIVE guidelines on reporting

animal research. All efforts have been taken to ensure that the suffering of animals was kept to the minimal, the number of animals used was kept to the minimum and all the protocols in the experiment were structured in order to reach the scientific goals of the research and at the same time to uphold the highest standards of animal care.

Informed Consent

Not applicable.

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Data Sharing Statement

The datasets created and analyzed during this study are available upon reasonable request from the responsible author.

Conflict of Interest

The authors declare no competing interests.

Similarity Check

It was applied by Ithenticate[@].

Application of Artificial Intelligence (AI)

Not applicable.

Peer Review Process

It was performed.

References

1. Maenner MJ. Prevalence and characteristics of autism spectrum disorder among children aged 8 years—Autism and Developmental Disabilities Monitoring Network, 11 sites, United States, 2020. MMWR. Surveillance Summaries, 2023. 72.
2. Modabbernia AE. Velthorst, and A. Reichenberg, Environmental risk factors for autism: an evidence-based review of systematic reviews and meta-analyses. Molecular Autism, 8 (1), 13. 2017, BioMed Central Ltd. https://doi.org/10.1186/s13229-017-0121-4.
3. Nicolini C, M Fahnestock. The valproic acid-induced rodent model of autism. Experimental neurology, 2018. 299: p. 217-227.
4. Hernández-Díaz SL, Straub BT, Bateman Y, Zhu H, Mogun KL, Wisner KJ, Gray B, Lester CJ, McDougle, E DiCesare. Risk of autism after prenatal topiramate, valproate, or lamotrigine exposure. New England Journal of Medicine, 2024. 390(12): p. 1069-1079.
5. Roullet FI, JK Lai, JA. Foster, In utero exposure to valproic acid and autism—a current review of clinical and animal studies. Neurotoxicology and teratology, 2013. 36: p. 47-56.
6. Bjørklund G, NA Meguid, MA El-Bana, AA Tinkov, K Saad, M Dadar, M Hemimi, AV Skalny, B Hosnedlová, R Kizek. Oxidative stress in autism spectrum disorder. Molecular neurobiology, 2020. 57(5): p. 2314-2332.
7. Frustaci A, M Neri, A.Cesario, JB Adams, E Domenici, B Dalla Bernardina, S Bonassi. Oxidative stress-related biomarkers in autism: systematic review and meta-analyses. Free Radical Biology and Medicine, 2012. 52(10): p. 2128-2141.
8. Teng M, TJ Wu, X Jing, BW Day, KA Pritchard Jr, S Naylor, RJ Teng. Temporal dynamics of oxidative stress and inflammation in bronchopulmonary dysplasia. International Journal of Molecular Sciences, 2024. 25(18): p. 10145.
9. Mehra SA. Ul Ahsan, E Seth, M Chopra. Critical evaluation of valproic acid-induced rodent models of autism: current and future perspectives. Journal of Molecular Neuroscience, 2022. 72(6): p. 1259-1273.
10. Li YJ, XN Xie, X Lei, YM Li, X. Lei. Global prevalence of obesity, overweight and underweight in children, adolescents and adults with autism spectrum disorder, attentiondeficit hyperactivity disorder: A systematic review and meta-analysis. Obesity Reviews, 2020. 21(12): p. e13123.
11. Healy S, CJ Aigner, JA Haegele. Prevalence of overweight and obesity among US youth with autism spectrum disorder. Autism, 2019. 23(4): p. 1046-1050.
12. Rossignol DA, RE Frye. Evidence linking oxidative stress, mitochondrial dysfunction, and inflammation in the brain of individuals with autism. Frontiers in physiology, 2014. 5: p. 150.
13. Hassan RS, Abotaleb H, Hamed MS, Eldeen. Antioxidant and antimicrobial activities of Melissa officinalis L.(lemon balm) extracts. Journal of Agricultural Chemistry and Biotechnology, 2019. 10(9): p. 183-187.
14. Bodzon-Kulakowska AA, Bierczynska-Krzysik T, Dylag A, Drabik P, Suder M, Noga J, Jarzebinska

J, Silberring. Methods for samples preparation in proteomic research. Journal of Chromatography B, 2007. 849(1-2): p. 1-31.

15. Marklund S, G Marklund. Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. European journal of biochemistry, 1974. 47(3): p. 469-474.
16. Moron MS, JW Depierre, B Mannervik. Levels of glutathione, glutathione reductase and glutathione S-transferase activities in rat lung and liver. Biochimica et biophysica acta (BBA)-general subjects, 1979. 582(1): p. 67-78.
17. Buege JA, SD. Aust, **[30]** Microsomal lipid peroxidation, in Methods in enzymology. 1978, Elsevier. p. 302-310.
18. Torlasco M, M Estrin. Valproic acid and weight gain in patients receiving antiepileptic treatment: a systematic review. Rev Inform Cientif, 2023. 102: p. 1-16.
19. Tamura Y, T Omura K. Toyoshima, and A. Araki, Nutrition management in older adults with diabetes: a review on the importance of shifting prevention strategies from metabolic syndrome to frailty. Nutrients, 2020. 12(11): p. 3367.
20. Chu MC, HF Wu, CW Lee, CC Wu, H Chi, CY Ko, YC Lee, CW. Tang, P.S. Chen, and H.-C. Lin, Soluble epoxide hydrolase deletion rescues behavioral and synaptic deficits by AMPK-mTOR pathway in autism animals. Progress in Neuro-Psychopharmacology and Biological Psychiatry, 2025. 136: p. 111190.
21. Shedlock K, A Susi GH Gorman, E Hisle-Gorman, CR Erdie-Lalena, and CM Nylund. Autism spectrum disorders and metabolic complications of obesity. The Journal of pediatrics, 2016. 178: p. 183-187. e1.
22. Liu XJ, Lin H, Zhang NU, Khan J, Zhang X, Tang XCao, L Shen. Oxidative stress in autism spectrum disorder—current progress of mechanisms and biomarkers. Frontiers in psychiatry, 2022. 13: p. 813304.
23. Frye RE. Mitochondrial dysfunction in autism spectrum disorder: unique abnormalities and targeted treatments. in Seminars in pediatric neurology. 2020. Elsevier.
24. Choi CS, EL Gonzales, KC Kim, SM Yang, JW Kim, DF Mabunga, JH Cheong, SH Han, GH Bahn, CY Shin. The transgenerational inheritance of autism-like phenotypes in mice exposed to valproic acid during pregnancy. Scientific reports, 2016. 6(1): p. 36250.
25. Tursunov AD, Vasilyev, N Nalivaeva. Molecular mechanisms of valproic acid action on signalling systems and brain functions. Journal of Evolutionary Biochemistry and Physiology, 2023. 59(5): p. 1740-1755.
26. Carvalho AN, C Marques, RC Guedes, M Castro-Caldas, E Rodrigues, J Van Horssen, MJ Gama, S-Glutathionylation of Keap1: a new role for glutathione S-transferase pi in neuronal protection. FEBS letters, 2016. 590(10): p. 1455-1466.
27. Chen S, L Li, C Peng, C Bian, PE Ocak, JH Zhang, Y Yang, D Zhou, G Chen, Y Luo. Targeting oxidative stress and inflammatory response for blood–brain barrier protection in intracerebral hemorrhage. Antioxidants & Redox Signaling, 2022. 37(1-3): p. 115-134.
28. Rădulescu M, C Jianu, AT Lukinich-Gruia, M Mioc, A Mioc, C Șoica, LG Stana. Chemical composition, in vitro and in silico antioxidant potential of Melissa officinalis subsp. officinalis essential oil. Antioxidants, 2021. 10(7): p. 1081.
29. Petrisor GL, Motelica LN, Craciun OC, Oprea D, Ficai A. Melissa officinalis: Composition, pharmacological effects and derived release systems—A review. International journal of molecular sciences, 2022. 23(7): p. 3591.
30. Nadeem MM, Imran T, Aslam Gondal A, Imran M, Shahbaz R. Muhammad Amir, M. Wasim Sajid, T. Batool Qaisrani, M. Atif, and G. Hussain, Therapeutic potential of rosmarinic acid: A comprehensive review. Applied Sciences, 2019. 9(15): p. 3139.
31. Shahcheraghi SH, F Salemi W Alam, H Ashworth L, Saso H, Khan, M.Lotfi. The role of NRF2/KEAP1 pathway in glioblastoma: pharmacological implications. Medical Oncology, 2022. 39(7): p. 91.
32. Bhagwat PH, Soni G, Singh S, Tandon V, Kumar SK, Lale CV, Narasimhaji S, Mathapati N, Srikanth, R Acharya. Prebiotic potential of selected plants, fruits, vegetables and herbs– An in-vitro study. Food and Humanity, 2025. 5: p. 100678.
33. Abo-Zaid OA, Moawed FS, Taha EF, Ahmed ES, Kawara RS. Melissa officinalis extract suppresses endoplasmic reticulum stress-induced apoptosis in the brain of hypothyroidism-induced rats exposed to γ-radiation. Cell Stress and Chaperones, 2023. 28(6): p. 709-720.
34. Majeed Z, Sharhan A. Efficiency of Melissa officinalis leaf extract in amelioration of oxidative status and histological changes in male albino rats with induced hypothyroidism. Regulatory Mechanisms in Biosystems, 2024. 15(4): p. 985-991.

35. Akhtar A, Rahaman SB. The interplay of oxidative Stress, mitochondrial Dysfunction, and neuroinflammation in autism spectrum disorder: behavioral implications and therapeutic strategies. Brain Sciences, 2025. 15(8): p. 853.