Original Article

Lavender and Rosemary Essential Oils as a Promising Intervention for Valproic acid induced Autism: Behavioral and Oxidative Stress Mitigation

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ABSTRACT

Background: Autism spectrum disorder (ASD) represented by challenges in cognition, communication skills and showed repetitive activities. Valproic acid (VPA) is a prescribed medication used for managing epilepsy, migraine and mood disorders. However, prenatal VPA exposure increased the risk of ASD in children.

Methods: This experiment involved Wister albino rats which are equally divided into five groups. Normal control group contain rat pups from untreated female rats and the remaining four groups were from female rats that received 600 mg/kg of VPA at gestation day of 12.5, and classified as an untreated VPA-induced autism group, risperidone-treated group, LEO-treated group and REO-treated group. Treatments were administered from PND 15 to PND 45. Neurobehavioral tests and anti-oxidant activity were examined at the end of experiment along with histopathological and immunohistochemical examination.

Results: At PND-45, treatment with risperidone, lavender essential oil, and rosemary essential oil significantly improved alternation performance and transfer latency relative to untreated VPA group ($p < 0.001$), indicating partial restoration of working memory function. Treatment with risperidone, lavender essential oil, and rosemary essential oil significantly increased mobility time relative to the untreated VPA group ($p < 0.001$), indicating improved motor and exploratory behavior. At PND-45, all treated VPA-induced autism groups showed a significant increase in time spent in the open arm compared to both the control and untreated VPA groups ($p < 0.001$), indicating reduced anxiety-like behavior. In open field the treatment with RISP, LEO, and REO significantly reduced crossings compared to the untreated VPA group ($p < 0.001$).

Conclusion: Lavender and Rosemary Essential Oils showed beneficial effects by reducing oxidative stress and improved different behavioral outcomes. Future studies should focus on clarifying the precise molecular mechanisms through which lavender and rosemary essential oils exert their neuroprotective effects in valproic acid-induced autism models.

Keywords: *Autism, Lavender, Risperidone*

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INTRODUCTION

Autism spectrum disorder (ASD) is mainly categorized by complications in communication and social interactions, alongside repetitive behaviors and interests. As of the most recent prevalence data, ASD affects over 2% of the population, marking a significant public health concern[1]. A global survey conducted in 2022 reported an ASD prevalence rate of 0.6% worldwide, with rates of 0.4% in Asia, 0.5% in Europe, 1% in both America and Africa, and 1.7% in Australia[2].

It has been found in the researches that oxidative stress is involved mainly in the pathogenies of ASD. Although, oxidative stress and inflammation are normal physiological processes of body but their over-stimulation may result in cell and tissue damage. The mechanism behind the oxidative stress is mitochondrial dysfunction, rise in the level of markers of oxidative stress, increase peroxidation of lipids and deposition of glycation products in peripheral blood. Moreover, inflammation and stress are interconnected to each other, as inflammation also induces oxidative stress and mitochondrial dysfunction[3].

Valproic acid (VPA) is a prescribed medication used for managing epilepsy, migraine and mood

disorders. Among available antiepileptic drugs, the use of valproic acid (VPA) during reproduction years and especially during pregnancy, has raised up significant issues. Clinical research has recognized numerous risks linked to VPA including congenital malformations, birth defects, delayed development, reduced cognitive abilities, and, more recently, a heightened risk of autism[4].

Lavendar essential oil has a profound impact on reducing oxidative stress and neuroinflammation, by preventing neurodegeneration and stimulating activation of microglial cells. Lavender essential oil have possessed ability to regulate neurotransmitter systems i.e. gamma-aminobutyric acid (GABA), this neurotransmitter displays a critical role in diminishing neuronal excitability and promoting relaxation. Moreover, LEO also exhibited significant anti-inflammatory potential by preventing the release of interleukin-6, tumor necrosis factor-alpha, as their levels has raised during stress and neuroinflammation. This property has enabled LEO to be used in managing various psychiatric disorders. The anxiolytic and anti-inflammatory activity of LEO was reported due to the presence of chemical compounds like linalool and linalyl acetate[5].a

Rosemary essential oil has contained the high percentages of phenolic compounds like carnosic acid and rosamarinic acid, which accounts for the anti-oxidant potential of REO and involved in removing reactive oxygen species, reducing oxidative stress and managing the levels of superoxide dismutase, catalase and glutathione peroxidase. Furthermore, regulate the signaling mechanism of pro-inflammatory and inflammatory pathways. REO also possess the ability to diminish the levels of serum corticosterone and to elevate the dopamine levels in brain, these activities help in mitigating the stress[6]. 1,8-cineole is also an important bioactive compound with has effect on the central nervous system and promote relaxation and reduces anxiety, the mechanism lie behind this activity is their interaction with the GABAA receptor- benzodiazepine site. Linalool present in REO has the ability to interact with the postsynaptic 5-HT1A receptors and α2-receptors, both of them play a crucial role in stress- induced behavioral changes[7].

As per the author's knowledge, studies exploring the potential of lavender and rosemary essential oil in improving memory, anxiety and depression in autistic individual are scarce. This study hypothesizes that lavender and rosemary essential oils ameliorate behavioral and oxidative stress abnormalities in VPA-induced autistic rats.

METHODOLOGY

Pregnancy Determination

Female rats were paired with male for the whole night in the ratio of 1:3. After the confirmation of pregnancy by vaginal plug the day was considered equivalent to 0.5 days of gestation, and pregnant females were kept individually in cages with unlimited access to food and water.[8]

Pregnancy Population

Twenty-four female rats were paired with 12 male rats and after pregnancy divided into two groups (control group that received normal saline and have 5 female pregnant rats and VPA group that received valproic acid and have 19 female rats. The 15 pups from 5 female pregnant rats were grouped under the name of control groups, while 60 pups' group under the name of VPA induced group, Risperidone treated group, LEO treated group and REO treated group were come from the 19 pregnant female rats.

Valproic acid model

The VPA group was administered with 600 mg/kg of valproic acid intraperitoneally on 12.5 gestation day, while the control group received an equivalent volume of saline. All pregnant rats were kept individually until delivery. Following delivery, the pups of pregnant female rats receiving VPA were randomly assigned to four equal groups (n=15) as describe below.

Grouping of pups

Control group (n=15): Normal rat's pups received normal saline orally till PND-45.

VPA induced group (n=15): Autistic rats' pups received normal saline orally till PND-45.

Risperidone treated group (n=15): Autistic rats' pups received risperidone (0.1 mg/kg) orally till PND-45[10].

LEO treated group (n=15): Autistic rats' pups received 0.5ml/100g lavender essential oil orally till 45[11].

REO treated group (n=15): Autistic rats' pups received 0.5 ml/100g rosemary essential oil orally till PND-45.[11]

The dose of risperidone, REO and honey were started from the PND 15 until the end of the experiment PND-45, because before weaning period (the period during which pups start taking food other the milk) pups were unable to received drugs.

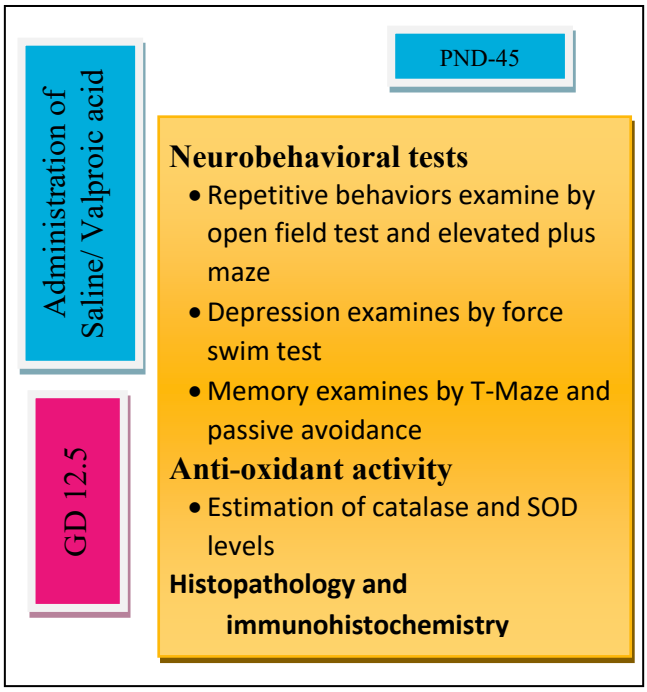

Figure 1: Experimental Design and Study Workflow

Neurobehavioral tests

The effectiveness of lavender and rosemary essential oil in alleviating autism symptoms was assessed using a range of neurobehavioral tests, such as the T-Maze, passive avoidance test, open field test, elevated plus maze and force swim test at PND 45. All procedures were conducted during daylight, and the animals were given at least one hour to acclimate to the testing environment prior to beginning the behavioral assessments.

T-Maze

The T-maze comprises of a start arm and two goal arms arranged at a 90-degree angle, separated by a guillotine door. Initially, the animal was positioned in the start arm and allowed to explore for 5 min. Once it entered one of the goal arms, the animal was immediately removed and returned to the starting point. Behavior was quantified by counting the number of entries into each goal arm. A higher number of alternations between the left and right arms indicates normal behavior, while fewer alternations suggest memory impairment. Animal performance was rated on a scale from 0 to 4 (0 indicating no alternation and 4 indicating four alternations between the two goal arms)[12].

Passive Avoidance Test

The apparatus comprised two compartments (25×25×35 cm), one illuminated by a 45-watt bulb and the other dark, with a metal-grill floor connected to an electrical supply, separated by a guillotine door. Over four days, animals were tested. During the first two days, they were trained by allowing free movement between compartments for 5 minutes. On 3rd day, animals were placed in the light compartment, and after 20 seconds, door unlocked. Entry into the dark compartment triggered a 0.5 A electric shock for 2 minutes, with the latency to enter recorded as t1. On the fourth day, the same procedure was repeated without the shock, and t2 was recorded which is the latency to enter the dark compartment[13].

Force swim test (FST)

The FST is a widely used behavioral test in animal models, primarily to assess the levels of depression-like behavior. It includes placing a rodent in a cylindrical container filled with water, from which the animal cannot escape. A higher amount of immobility suggests depression-like behavior, while increased swimming or climbing behavior is often interpreted as an indicator of a more active or resilient response.[14]

Open field test

Animals were located in the center of Plexiglass which is equally divided into 16 squares. After exploring the apparatus for five minutes following parameters were observed i.e. the total number of crossed slots, time spent in the central area and rearing (standing on two paws)[15].

Elevated plus maze

This apparatus comprises of two open and close arms, each measured (49 l × 10 w × 50 h) cm, and positioned 50 cm above the ground. Animals were placed at the interaction of four arms and allowed to explore the apparatus for five minutes. The factors to be observed were transitions between

the open and close arm and time duration of animal spent in each arm[16].

Preparation of hippocampus homogenate and estimation of anti-oxidant activity

At the PND 45, hippocampal tissues of the rodents' brains were homogenized in a 10% chilled phosphate-buffered saline solution with a 7.4 Ph and a 10 mM of concentration. After homogenization the sample centrifugation for 15 min at 4,000 rpm. After discarding the pellet, the supernatant was collected and analyzed for superoxide dismutase and catalase concentration in the hippocampal tissues, according to the manufacturer's guidelines mentioned in the ELISA kits[17].

Histopathological and immunohistochemical findings

At the neurobehavioral testing, animals were euthanized and sacrificed. The tissues were initially preserved in formaldehyde overnight, followed by dehydration with 70% isopropanol and 100% xylene. After dehydration, the tissues were embedded in paraffin wax to prepared 5 µm sections, stained with hematoxylin and eosin and analyzed under a microscope at 100x magnification for any hippocampal abnormalities[18].

For immunohistochemistry, tissue sections were initially rinsed with PBS and washed with 0.1% hydrogen peroxide (H_2O_2). After that sections were incubated in a blocking solution of 5-10% normal serum. A primary antibody targeting GFAP, diluted in the blocking buffer, was applied to the sections, which were then incubated either overnight at 4°C. After washing the sections with PBS, a secondary antibody was added, and the sections were kept in the dark for 1-2 hours. The sections were washed again and treated with a substrate solution containing streptavidin-horseradish peroxidase for 10 minutes. The antibody-antigen interaction was pictured using 3,3-diaminobenzidine and PBS, with 0.03% H2O2 as the substrate. After a final PBS wash, the tissues were optionally counterstained with hematoxylin to enhance contrast. The slides were then dehydrated using a graded ethanol series in the concentration of 70%, 95%, 100%, clean in xylene and mounted with histomount before applying coverslips. Five sections were randomly selected and examined under a microscope at 100x magnification[17].

Statistical Analysis

Group differences were examined with one-way ANOVA, followed by a post-hoc Bonferroni test. SPSS version 26 was used for statistical analysis. Statistical significance was significant ($p \leq 0.05$) and highly significant ($p \leq 0.001$).[19]

RESULTS

Neurobehavioral parameters

Effect of LEO AND REO on T-Maze

At PND-45, the number of alternations was significantly decreased in the untreated VPA-induced autism group (5.06 ± 1.56; n = 15) versus control group (12.02 ± 1.81; n = 15; $p < 0.001$), indicating impaired working memory (Figure 2). In contrast, postnatal treatment with RISP, LEO, and REO give rise to significant increase in alternation behavior relative to untreated VPA group (RISP: 8.37 ± 1.40, $p < 0.001$; LEO: 7.62 ± 1.06, $p < 0.01$; REO: 9.33 ± 1.41, $p < 0.001$, n = 15 per group), demonstrating partial **restoration of working memory performance**.).

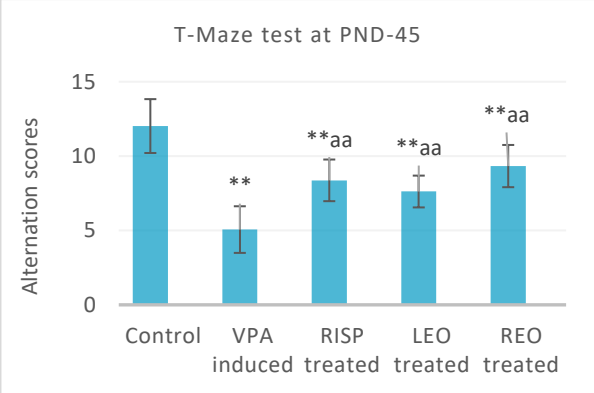

Figure 2: Effect of rosemary and lavender essential oil on T-Maze alternation score

Significant difference measured by Bonferroni test (n=15)

- ***** = significant versus Control group ($p < 0.05$)
- ****** = Highly significant versus Control group ($p < 0.001$)
- **a** = Significant versus VPA group ($p < 0.05$)
- **aa** = Highly significant versus VPA group ($p < 0.01$)

Effect of LEO AND REO on Passive avoidance

At PND-45, the transfer latency was significantly decreased in the untreated VPA-induced autism group (153.25 ± 6.71 sec; n = 15) compared to

the control group (280.16 ± 6.61 sec; n = 15; p < 0.001), indicating impaired learning and memory (Figure 3). Postnatal treatment with RISP, LEO, and REO resulted in a significant increase in transfer latency relative to the untreated VPA group (RISP: 230.16 ± 5.45 sec, p < 0.001; LEO: 215.75 ± 4.15 sec, p < 0.001; REO: 214.25 ± 5.47 sec, p < 0.001; n = 15 per group), demonstrating improvement in cognitive performance and restoration of memory function.

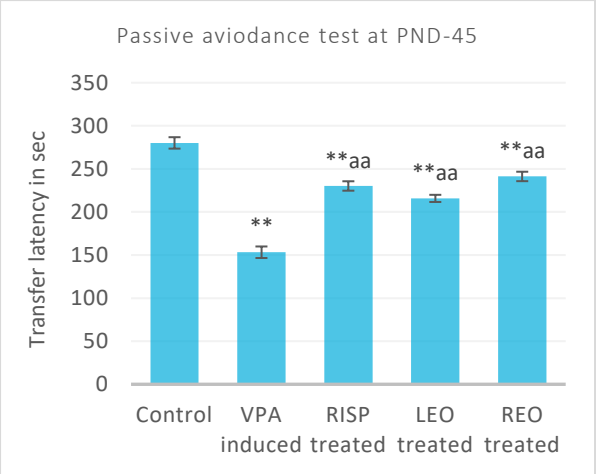

Figure 3: Effect of rosemary and lavender essential oil on Passive avoidance transfer latency

Significant difference measured by Bonferroni test (n=15)

- * = significant versus Control group (p < 0.05)
- ** = Highly significant versus Control group (p < 0.001)
- **a** = Significant versus VPA group (p < 0.05)
- **aa** = Highly significant versus VPA group (p < 0.01)

Effect of LEO and REO on Force Swim Test

Figure 4 demonstrates that at PND-45, the mobility time in the untreated VPA-induced autism group was significantly decreased (150.08 ± 3.09 sec, n = 15) versus control group (299.08 ± 1.32 sec, n = 15; p < 0.001). Similarly, the RISP-treated VPA group also showed a significant reduction in mobility time (200.33 ± 2.13 sec, n = 15; p < 0.001 vs. control).

However, treatment with RISP, LEO, and REO resulted in significantly increased mobility time relative to the untreated VPA group, with values of 200.33 ± 2.13 sec (RISP; p < 0.001 vs. VPA), 297.50 ± 1.97 sec (LEO; p < 0.001 vs. VPA), and 298.25 ± 1.08 sec (REO; p < 0.001 vs. VPA), respectively. These results indicate that LEO and REO were more effective than RISP in restoring motor and exploratory behavior in VPA-induced autism.

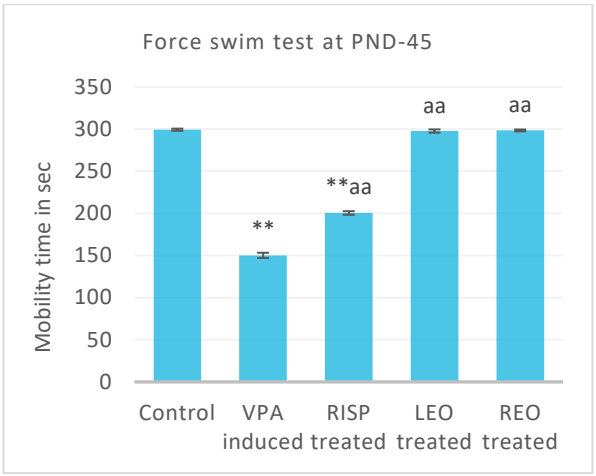

Figure 4: Effect of rosemary and lavender essential oil on Force swim mobility time

Significant difference measured by Bonferroni test (n=15)

- * Significant vs. Control group (p < 0.05)
- ** Highly significant vs. Control group (p < 0.001)
- **a** Significant vs. VPA group (p < 0.05)
- **aa** Highly significant vs. VPA group (p < 0.01)

Effect of LEO and REO on open field test

The results of the Open Field Test at PND-45 are presented in Table 1. The untreated VPA group exhibited a significant decrease in the time spent in the central area (0.12±0.14 sec; n = 15) compared to the control group (1.84 ± 2.75 sec; n = 15; p < 0.05), indicating increased anxiety-like behavior. Similarly, the REO-treated VPA group also demonstrated a significant reduction in central area time versus control (0.25± 0.55 sec; p < 0.05).

In contrast, the total number of crossed squares was significantly increased in both untreated and treated VPA-induced groups (VPA: 80.08 ± 4.58; RISP: 67.16 ± 3.27; LEO: 63±3.30; REO: 68.33±5.26) relative to the control group (50.08±3.89; p < 0.001), reflecting hyperactivity. However, treatment with RISP, LEO, and REO resulted in a significant decrease in the total number of crossings compared to the untreated

VPA group ($p < 0.001$), suggesting partial restoration of locomotor regulation.

Regarding rearing behavior, the untreated VPA group showed a highly significant decrease (2.08±0.79; $p < 0.001$ vs. control). Conversely, when compared with the untreated VPA group, REO treatment produced a highly significant increase in rearing (5.25±1.42; $p < 0.001$ vs. VPA), while RISP and LEO also resulted in significant improvements ($p < 0.05$ vs. VPA). These findings suggest that postnatal treatment with essential oils, particularly REO, mitigates anxiety-like and motor impairments associated with VPA-induced autism-like behavior at PND-45.

Table 1: Effect of rosemary and lavender essential oil on repetitive/stereotypic-like behaviors in OFT at PND-45

Groups	Time spent in central area	Total number of crossed slots	Rearing
Control	1.84±2.75	50.08±3.89	7.25±2.05
VPA induced	0.12±0.14*	80.08±4.58**aa	2.08±0.79**
RISP treated	0.41±0.52	67.16±3.27**aa	4.33±1.30*a
LEO treated	0.34±0.53	63±3.30**aa	4.16±1.4*a
REO treated	0.25±0.55*	68.33±5.26**aa	5.25±1.42*aa

Significant difference measured by Bonferroni test (n=15)

***** = significant versus Control group ($p < 0.05$)

****** = Highly significant versus Control group ($p < 0.001$)

a = Significant versus VPA group ($p < 0.05$)

aa = Highly significant versus VPA group ($p < 0.01$)

Effect of LEO and REO on elevated plus maze

At PND-45, the untreated VPA-induced autism group showed a significant reduction in the duration of time spent in the open arm (40.66 ± 3.31 sec; n = 15) compared to the control group (100.25 ± 6.22sec; n = 15, $p < 0.001$). In contrast, all treated VPA-induced groups (RISP, LEO, and REO; n = 15 each) demonstrated a significant increase in open arm time when compared to both the control and untreated VPA groups (RISP: 220.83 ± 4.5 sec, $p < 0.001$ vs VPA; LEO: 230 ± 2.4 sec, $p < 0.001$ vs VPA; REO: 250.66 ± 4.5 sec, $p < 0.001$ vs VPA), indicating an amelioration of anxiety-like behavior (Table 2).

Table 2: Effect of rosemary and lavender essential oil on repetitive/stereotypic-like behaviors in EPM at PND-45

Groups	No. of entries in open arm	No. of entries in close arm	Time duration in open arm	Time duration in close arm
Control	4±5.6	4±4.5	100.25±3.62.5	200.91±
VPA induced	10.33±3.5**	9±1.9**	40.6±2.4**aa	270.16±3.4**aa
RISP treated	6.37±2.5*aa	6.25±2.5*aa	220.83±4.5**aa	81.75±3.5**aa
LEO treated	5.79±2.4aa	5.95±1.8*aa	230±2.4**aa	70.08±1.6**aa
REO treated	4.2±2.7aa	5.08±1.8aa	250.66±4.5**aa	50.91±4.6**aa

Significant difference measured by Bonferroni test (n=15)

***** Significant vs. Control group ($p < 0.05$)

****** Highly significant vs. Control group ($p < 0.001$)

a Significant vs. VPA group ($p < 0.05$)

aa Highly significant vs. VPA group ($p < 0.01$)

Effect of LEO and REO on anti-oxidant activity

At PND-45, catalase concentration was significantly increased in the RISP-treated VPA-induced autism group compared to the untreated VPA group (Control: [0.65 ± 0.16]; VPA: [0.19 ± 0.13]; RISP: [0.5 ± 0.2]; $p < 0.05$]). Additionally, SOD levels were markedly elevated in the RISP,

LEO, and REO-treated VPA groups compared to both control and untreated VPA groups (Control: [72.37 ±7.44]; VPA: [27.3 ± 6.73]; RISP: [51.57 ± 7.53]; LEO: [48.5 ± 6.23]; REO: [37.03 ± 7.09]; $p < 0.001$, indicating enhanced antioxidant activity.

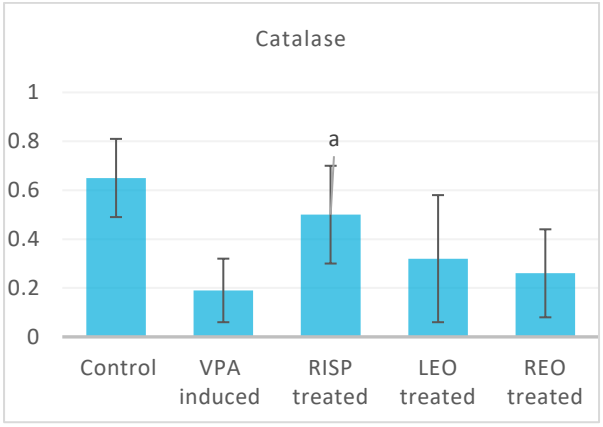

Figure 5: Effect of rosemary and lavender essential oil on catalase

Significant difference measured by Bonferroni test (n=15)

* = significant versus Control group ($p < 0.05$)

** = Highly significant versus Control group ($p < 0.001$)

a = Significant versus VPA group ($p < 0.05$)

aa = Highly significant versus VPA group ($p < 0.01$)

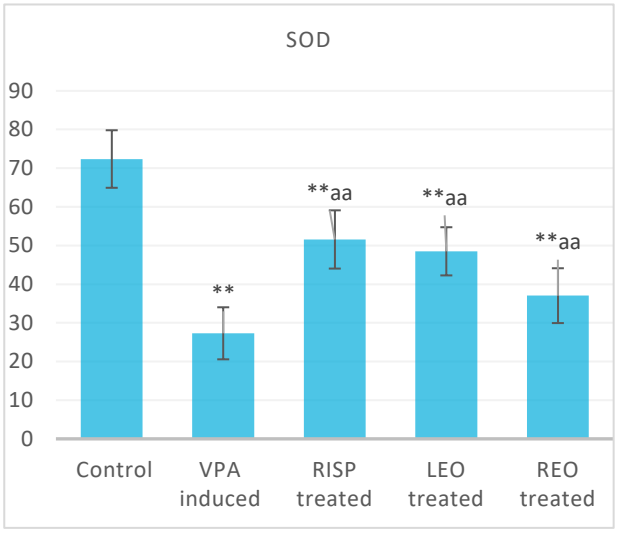

Figure 6: Effect of rosemary and lavender essential oil on SOD

Significant difference measured by Bonferroni test (n=15)

* = significant versus Control group ($p < 0.05$)

** = Highly significant versus Control group ($p < 0.001$)

a = Significant versus VPA group ($p < 0.05$)

aa = Highly significant versus VPA group ($p < 0.01$)

Histopathological examination

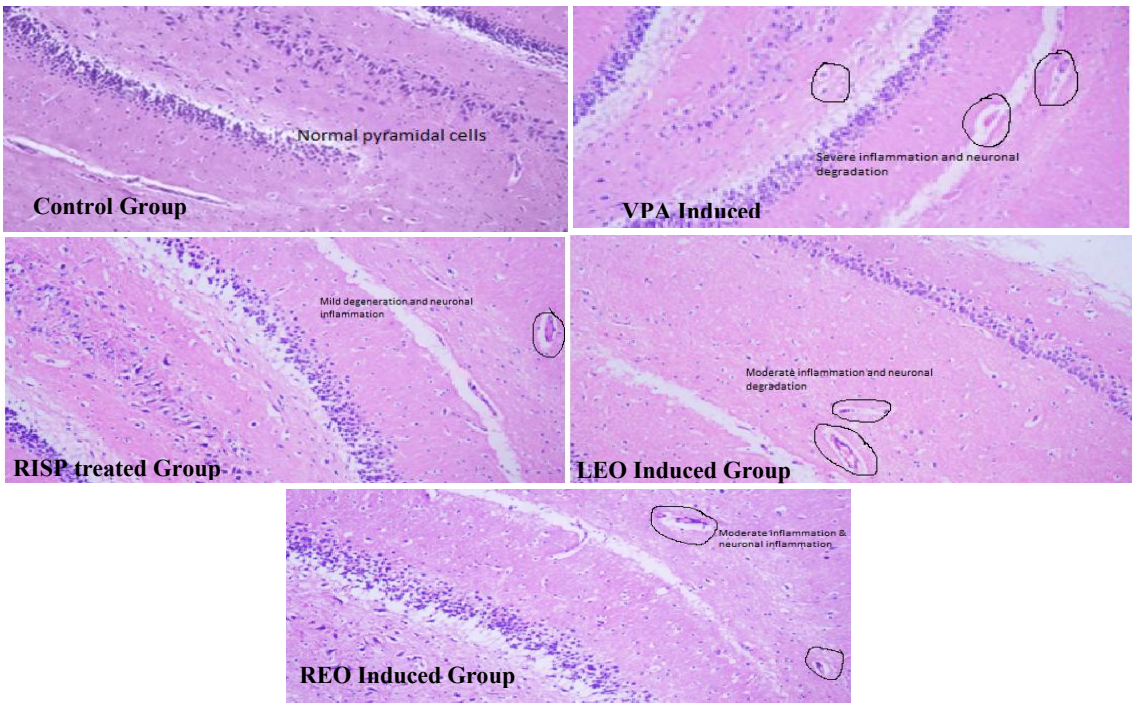

Figure 7: H& E stained of hippocampus 5µm (H&E X100). (A): Group 1 revealing normal pyramidal cell layer formed of densely packed rounded neurons (B): Group 2 showing many degenerated neurons and severe inflammation (C): Group 3 showing some degenerated neurons and mild inflammation (D): Group 4 & 5 showing some degenerated neurons with moderate inflammation

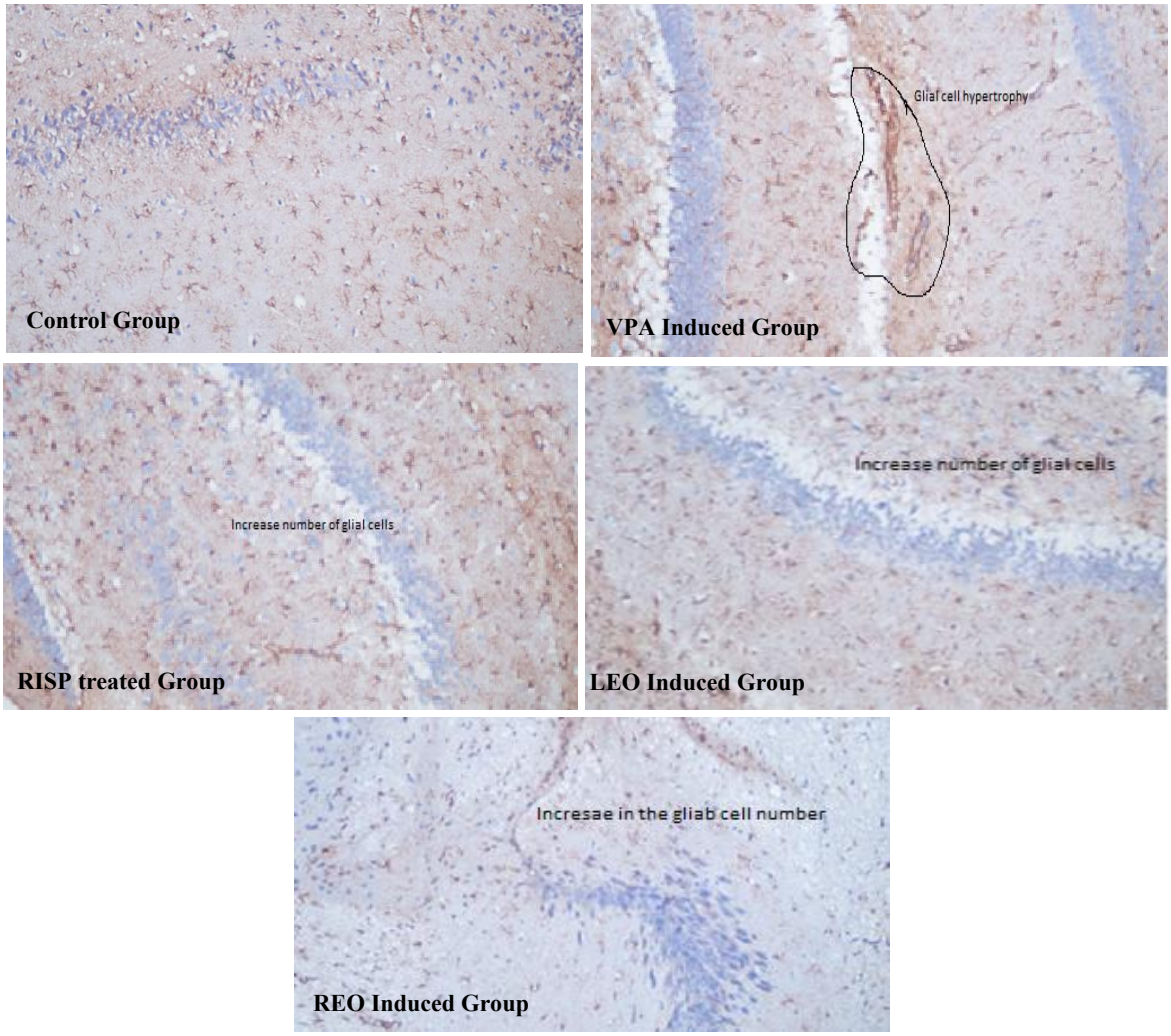

Figure 8: GFAP staining of hippocampus (H&E X100). (A): Group 1 revealing no sign of hypertrophied glial cells (B): Group 2 showing increase glial cells count (C): Group 3 showing mild increase in glial cells count (D): Group 4 & 5 showing moderate increase in glial cells count.

DISCUSSION

In the current study, different parameters (behavioral, biochemical, and histopathological) have been evaluated in a well-established VPA-induced autism model, providing a direct comparison between the lavender and rosemary essential oils alongside a standard therapeutic agent risperidone[20].

In this study, lavender and rosemary essential oil established prominent progresses in neurobehavioral parameters, including working of memory performance, anxiety and depression-like symptoms. The mechanism lie behind these improvements might be due to alteration in neurotransmitter systems i.e. serotonergic, cholinergic and GABAergic pathways. There are also various bioactive constituents highlighted in these essential oils like linalool in LEO and 1,8-cineole in REO which accounts for the anxiolytic and neuromodulatory effects[5,6].

Increased rate of lipid peroxidation and reduction in the levels of glutathione are trademark of oxidative stress in ASD[7]. In the current study LEO and REO improves the level of anti-oxidant enzymes like superoxide dismutase and catalase in hippocampus, thus restoring redox balance by reducing neuroinflammation and improves neural functioning and display a critical role in managing oxidative stress which contributes mainly to pathophysiology of ASD.

Moreover, LEO and REO displayed comparable efficacy to risperidone, so these essential oils offer natural option with less side effects, though

further clinical studies are required to confirm these findings.

Histopathological and immunohistochemical findings in treated groups further support a neuroprotective effect, evidenced by improved hippocampal architecture and reduced neuronal degeneration. These structural improvements align with the biochemical data, indicating that antioxidant and anti-inflammatory pathways may support the functional recovery observed.

Although the current study provides promising evidence for the neuroprotective and behavioral benefits of LEO and REO, further studies will be required to measure neurotransmitter levels (e.g., GABA, serotonin, acetylcholine, glutamate) as well as molecular signaling analyses (e.g., inflammatory cytokines, BDNF, MAPK, or NF-κB pathways) in order to elucidate mechanisms of neuroprotection or to explore synergistic or combinational therapies. The sample size in the present sturdy was limited, which leads to reduce statistical power and generalizability. Moreover, the therapeutic effects of essential oils were evaluated only at single dose in the present study, if varying concentrations may use it may give different or even opposing results. Furthermore, different neurochemical assays were not part of the current study like evaluation of neurotransmitter levels, inflammatory markers.

CONCLUSION

In conclusion, the satisfactory results exhibited by Lavendar and rosemary essential oils in managing the symptoms of depression, memory loss, repetitive behavior and oxidative stress, marked these candidates as a supportive therapeutic choice in ASD-related behavioral problems. These results offer a foundation for translational research, highlighting the need for well-designed clinical trials to confirm their efficacy, safety, and underlying mechanisms in human populations.

Ethical Approval

The research protocol was approved by the Institutional Animal Ethics Committee at the Faculty of Pharmacy, Hamdard University, under Ethics Code: 2023-17

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Author Contributions

SJK: Conception & Design, Manuscript Writing, Critical Revision
RI: Data Collection, Data Analysis & Interpretation, Critical Revision
All authors approved the final version of the manuscript to be published.

Conflict of Interests

No conflict of interest.

Data Availability

Data will be available upon request.

Funding Source

No sources

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