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Effect of Curcumin alone and in Combination with Eugenol or Rosemary Acid in Valproic Acid-induced Autism in Mice: Postnatal Model

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ABSTRACT

Autism is a neurological disorder that impairs the cognitive, emotional, social, and physical well-being of an affected individual. Several phytomolecules have therapeutic efficacy in ameliorating autistic conditions. Curcumin is a potent neuroprotective molecule, which can able to hinder the toxicity of valproic acid (VPA). Although the bioavailability of curcumin is low because of poor absorption. This study aims to design the combination effect of curcumin with eugenol and rosemary acid in the VPA-induced postnatal method of autism in swiss albino mice. On a postnatal day (PND) 14 separate mice were into 5 groups, each group with 6 animals. VPA at a dose of 400 mg/kg was given by i.p route to all groups except group-I. upon treatment with the combination of curcumin + eugenol and curcumin + rosemary acid the combination treatment has given significant improvement in ameliorating autism symptoms and biochemical abnormality in autistic mice. Thus, we conclude that curcumin combination of eugenol or rosemary acid has a potent neuroprotective, improves cognitive ability, and reduces anxiety.

Keywords: Autism, Curcumin, Eugenol, Rosemary acid.

1. INTRODUCTION

Autism is a neurological disorder with different skills and abilities than normal kids. Progress in age may lead to disability in verbal, non-verbal, and social communication portrayed by a constrained creative mind with limited and redundant patterns of behavior and activities.[1,2] About 1.5% of children are diagnosed to have autism as of 2017 in developed countries with higher chances of males being affected.[3]

Autism possesses a solid genetic foundation, while its underpinnings are multifarious and uncertain. The role of many neurotransmitters in early mental development might validate to be important for understanding autism. Conventional therapies and other treatment options can help patients alleviate the symptoms as there is no cure for autism. The brain of autistic individuals presents several structural and functional abnormalities, which could be caused by risk factors such as environmental factors and maternal exposures during pregnancy.[4-6]

Valproic acid (VPA) is a carboxylic acid that increases the levels of γ-aminobutyric acid in the brain, probably by inhibiting its catabolism. Autism diagnosis is more likely when VPA exposure occurs during pregnancy. It is thought that synaptic connection is disrupted as a result of VPA-mediated developmental impairments of nerve cells in the cerebellar, limbic, and medulla oblongata regions and decreased Purkinje fiber density.[7-9]

Since ancient times, due to the enormous inexhaustible potential of bioactive molecules, plants have always been used in African, Ayurvedic, and Chinese medicine for health care. In this way, our study is focused on plants to explore their neuropsychiatric profile. In the literature review, many bioactive compounds from plants have shown psychotropic properties among which curcumin, eugenol, and rosemary acid have gained importance.[10]

Curcumin is a diarylheptanoid, a curcuminoid phenolic compound produced by the *Curcuma longa* plant. Curcumin inhibits apoptosis and has scientifically-proven anti-inflammatory, antioxidant, and psychotropic activities.[11,12] Though curcumin has poor bioavailability due to its low absorption, it shows a substantial pharmacological effect in reducing neurological damage in autism brought about by valproic acid.[11]

Several methods have already been tried to render curcumin more bioavailable which include, nanotech-

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Table 1: Animal Grouping

Group	*Treatment (14th – 40th Day)*
I	Vehicle treated group (0.9 % NaCl)
II	Autistic group
III	Curcumin-treated group (50 mg/kg, p.o.)
IV	Curcumin + Eugenol treated group (50 mg/kg + 5 mg / kg, p.o.)
V	Curcumin + Rosemary acid treated group (50 mg/kg + 5 mg/kg, p.o.)

nology-based formulations like curcumin-encapsulated PLGA (Poly lactide-co-glycolide), curcumin-based nanoparticles, heat-extracted curcumin, and microencapsulation.[12-14]

Eugenol is a phenolic molecule extracted from the flower buds of the *Syzygium aromaticum* and has been found to have anti-inflammatory, free-radical scavenging, neuroprotective, antimycotic, antinociceptive, and antipyretic activities.[15] Previous studies have shown that eugenol can increase cutaneous curcumin uptake by 2.2 to 2.5 times, suggesting that eugenol may be beneficial as an adjuvant in improving the bioavailability of curcumin.[16]

Rosemary acid is obtained from the leaves of the *Rosmarinus officinalis* and is known to have analgesic, anti-inflammatory, as well as psycho-protective properties.[17] Latest investigations have demonstrated that co-administration of curcumin with certain antioxidants like rosmarinic acid, caffeic acid, gallic acid, and ascorbic acid, decreases the degradation of curcumin and thus increasing its bioavailability.[18]

In the present study, it was hypothesized that the bioavailability of curcumin can be increased when it is administered along with eugenol and rosemary acid.

The present work was carried out to evaluate the behavioral improvement of VPA-induced mice in autism by using curcumin alone and as a combination of curcumin with eugenol or curcumin with rosemary acid.

2. MATERIALS AND METHODS

2.1 Chemicals

Sodium valproate injection was acquired from Sun Pharmaceuticals, Hyderabad, India. Curcumin capsules were obtained from Himalaya Pharmaceuticals, Bengaluru, India. Clove oil 100% pure and natural was obtained from I & Z Essentials Hyderabad, India. Rosemary acid was procured from Seyal Naturals, Hyderabad, India.

2.2 Experimental Animals

Swiss albino mice were obtained from Vyas Labs, Hyderabad, mice were kept under a constant temperature of 22ºC with a 12-hour light-dark cycle, and allowed to drink water and food ad libitum. All the experiments were carried out according to the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines. The Institutional Animal Ethical Committee (IAEC) authorized all the methodological approaches (Protocol No: I/IAEC/AGI/002/2019 SM).

2.3 Experimental Design

On postnatal day 14 autism was induced by a single intraperitoneal injection of VPA at a dose of 400mg/kg. Then mice were divided randomly into 5 groups each group containing 6 animals. Groups namely, Group I (Normal control) was a vehicle-treated (0.9 % NaCl) group, Group II (Autistic group) received VPA (400 mg/kg, i.p.), Group III was treated with curcumin (50 mg/kg, p.o.), Group IV was treated with curcumin and eugenol (50 mg/kg + 5 mg /kg, p.o.), Group V was treated with curcumin and rosemary acid (50 mg/kg + 5 mg/kg, p.o.), all the treatments were provided from the 14th to the 40th day (Table 1).

2.4 Evaluation of Behavioral Parameters

To evaluate the therapeutic potency of a combination of drugs on autism treatment assessed for five important behavioral patterns: negative geotaxis (NG), learning and memory test, motor coordination test, habituation test, and elevated plus-maze test.

2.4.1 Negative Geotaxis[19]

Negative geotaxis was done by placing mice on a 45° slanted platform and the average duration to spin 180° was observed on the inclined surface was noted. This test was performed on PND 14-19.

2.4.2 Learning and Memory Test[20]

Learning and Memory of animals were performed by Morris's water maze test, apparatus consisting of a round white pool of about 94 cm in diameter and 31 cm deep. It was filled with water (25 ± 0.5°C) up to 30 cm and made opaque with white non-toxic paint. The escape platform was positioned in the middle, about 15 cm away from the pool's corner, and immersed 1 cm under the water level. Throughout the learning visual cue tests, the platform stayed in place, and during the probe test, it was taken out of the pool. Around the pool, different distal additional-maze markers like a traffic cone, a colorful poster, and two grayscale construction sheet patterns were kept. Animals were placed on any one corner of the maze and allowed to identify the hidden platform. This test was performed on PND 38–40.

2.4.3 Motor Coordination Test[21]

This test was performed on PND 38–40 by using rotarod apparatus. The apparatus was configured to run at 4 rpm and then increased to 20 rpm per minute speed.

Each animal was placed on a rotating rod after 10 sec, acceleration increased to 20 rpm until mice slipped off and the fall of time from the rotating rod was noted. The experiment was repeated at least three times.

2.4.4 Open Field Habituation[22, 23]

The test was performed on PND 38-40 on an open field where it was divided into equal quadrants on a 42 × 42 × 42 cm polyvinyl chloride box, this test is used to study the line crossings and dippings. The computer was used to quantify the duration of time it took for covering the overall distance and the average time in the central zone, the number of times the animal entered the central and peripheral zones, along with the frequency of ambulation.

2.4.5 Elevated Plus Maze Test[24,25]

The test was performed on PND 38-40, the experiment relies upon mice's innate inclination to extensively investigate an unfamiliar area. The maze has a height of 50 cm above the ground and comprises two enclosed arms of 21 cm in length, 40 cm high sidewalls, and two open arms. Mice were given five-minute trials on plus-maze. They were provided full freedom to investigate the maze by being placed in the center section. Time taken and the number of movements into open and closed arms was recorded.

2.5 Histopathology

Animals were sacrificed on the last day of the experiment (PND 40); brains were collected and immersed in 10% formalin solution (neutral buffered), processed, followed by encasing in paraffin. Sagittal 5 µm thick cerebellar segments were stained with hematoxylin and eosin and examined the cerebellum for changes using a light microscope.

2.6 Statistical Analysis

The data is represented as Mean ± S.E.M. One-way analysis of variance (ANOVA) as well as the Tukey's test was used to determine the significance of differences between the various groups with the help of Graph pad PRISM software and $p < 0.05$ was deemed significant.

3. RESULTS

3.1 Behavioral Parameters

3.1.1 Effect of Curcumin alone and Combination on Negative Geotaxis

Autistic group animals took more time to reorient on an inclined plain than other groups on all postnatal days ($^{\alpha}p < 0.001$) as compared to the control group as shown by the data in Table 2. On the other hand, the curcumin alone treated group had a significantly decreased time to reorient. However, in the early phase, treatment with a combination of curcumin with eugenol or rosemary acid had shown an increased time ($^{**}p < 0.01$) however, as PNDs progressed, the pattern did not persist and a significantly decreased time was reported ($^{***}p < 0.001$) in comparison with autistic mice.

3.1.2 Effects of curcumin alone and combination on Learning and Memory Test

Data shown in Table 3 revealed that the time taken by animals to reach the hidden platform was decreased in the autistic group in comparison with the vehicle-treated group ($^{\alpha}p < 0.001$). Curcumin alone and the combination had shown significant improvement in time to reach the hidden platform ($^{***}p < 0.01$) when compared with the autistic group.

Table 2: Effect of curcumin alone and combination on Negative Geotaxis.

Groups	*PND 14*	*PND 15*	*PND 1w6*	*PND 17*	*PND 18*	*PND 19*
Vehicle group	6.20 ± 0.36	7.65 ± 0.66	5.34 ± 0.80	6.58 ± 0.84	5.48 ± 0.36	5.3 ± 1.05
Autistic group	12.3 ± 0.61$^{\alpha}$	13.0 ± 1.24$^{\alpha}$	13.5 ± 0.70$^{\alpha}$	14.0 ± 0.55$^{\alpha}$	14.3 ± 0.80$^{\alpha}$	14.5 ± 0.71$^{\alpha}$
Curcumin group	10.35 ± 0.42**	12.33 ± 0.99***	11.35 ± 0.60***	10.35 ± 0.56**	8.69 ± 0.80**	8.50 ± 0.47***
Curcumin +Eugenol	11.35 ± 0.47	12.3 ± 0.71*	11.4 ± 0.84**	10.65 ± 0.56**	9.5 ± 0.85***	8.4±0.7***
Curcumin + Rosemary acid	12.33 ± 0.42	10.50 ± 0.99**	8.53 ± 0.60**	8.50 ± 0.56***	7.60 ± 0.80***	7.0 ± 0.47***

Data expressed as mean ± SEM, using a one-way analysis of variance followed by Tukey's test. Significance was reported as $^{\alpha}p < 0.001$ compared to the vehicle-treated group. ***p < 0.001, **p < 0.01, and *p < 0.05 compared with the autistic group.

Table 3: Effect of Curcumin Alone and Combination on Learning and Memory Test.

Groups	*PND 38*	*PND 39*	*PND 40*
Vehicle group	55.12 ± 1.32	15.78 ± 0.85	10.23 ± 0.8
Autistic group	150.24 ± 2.36$^{\alpha}$	135.06 ± 3.24$^{\alpha}$	55.96 ± 1.3$^{\alpha}$
Curcumin group	90.08 ± 1.3**	46.65 ± 1.35***	30.35 ± 1.33***
Curcumin +Eugenol	75.56 ± 1.2**	30.81 ± 1.0***	18.34 ± 1.22***
Curcumin + Rosemary acid	70.24 ± 1.1**	35.36 ± 1.1***	20.86 ± 0.9***

Data expressed as mean ± SEM, using a one-way analysis of variance followed by Tukey's test. Significance was reported as $^{\alpha}p < 0.001$ compared to the vehicle-treated group. ***p < 0.001 and **p < 0.01, compared with the autistic group.

3.1.3 Effects of curcumin alone and combination on Motor Coordination Test

Data shown in Table 4 revealed that the least motor coordination and balance activity was seen in the autistic group than vehicle-treated group ($^{\alpha}p < 0.001$). Treatment with curcumin alone and in combination with eugenol or rosemary acid had shown high motor coordination (***p < 0.001) in comparison with the autistic group.

3.1.4 Effects of curcumin alone and combination on Open Field Habituation Test

Data shown in Table 5 revealed that autistic animals have shown an increase in line crossings and head dippings than with vehicle-treated group ($^{\alpha}p < 0.001$). curcumin alone or in combination significantly improved the results (***p < 0.001) when compared to the autistic group.

3.1.5 Effect of curcumin alone and combination on Elevated Plus Maze Test

Data shown in Table 6 revealed that the number of entries in the open arm by autistic group animals was higher than the vehicle-treated group ($^{\alpha}p < 0.001$). However, curcumin administration alone as well as in combination reduced the open arm exploration (***p < 0.001) in comparison to the autistic group.

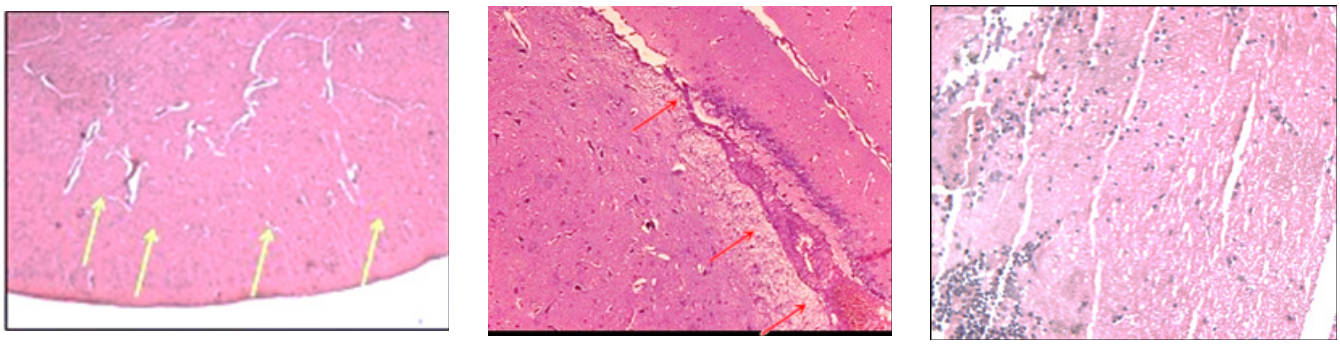
3.2 Histopathology Results

The vehicle group was found to have a regular cerebral cortex of cerebral hemispheres. Degeneration and demyelination were noticed surrounding the ventricle in the VPA-alone treated group (Figure 1). Animals of curcumin alone and in combination with eugenol or rosemary acid have shown normal hippocampus and no degenerative changes were observed.

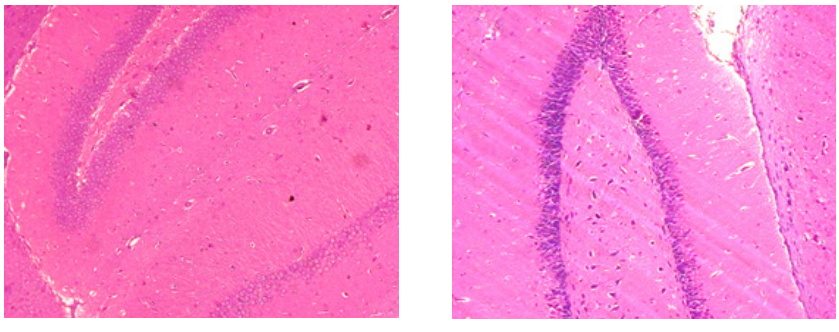
Table 4: Effect of Curcumin Alone and Combination on Motor Coordination

Groups	*PND 24*	*PND 25*	*PND 26*
Vehicle group	16.25 ± 0.2	18.66 ± 0.23	17.50 ± 0.08
Autistic group	7.4 ± 0.11$^{\alpha}$	8.30 ± 0.11$^{\alpha}$	6.50 ± 0.03$^{\alpha}$
Curcumin group	9.65 ± 0.22*	13.25 ± 0.08***	13.70 ± 0.06***
Curcumin + Eugenol	8.55 ± 0.33*	12.50 ± 0.15**	14.56 ± 0.04***
Curcumin + Rosemary acid	10.35 ± 0.4*	13.22 ± 0.09***	13.41 ± 0.07***

Data expressed as mean ± SEM, using a one-way analysis of variance followed by Tukey's test. Significance was reported as $^{\alpha}p < 0.001$ compared to the vehicle-treated group. ***p < 0.001, **p < 0.01, *p < 0.05 compared with the autistic group.



(a) Vehicle-treated Group (b) Autistic Group (c) Curcumin



(d) Curcumin + Eugenol Group (e) Curcumin + Rosemary acid

Figure 1: Histopathology of Cerebellum.

Table 5: Effect of Curcumin Alone and Combination on Open Field Habituation

Groups	Open Field Habituation	
	Line Crossings	Head Dipping
Vehicle group	84.17 ± 2.05	25.67 ± 1.62
Autistic group	127.17 ± 2.18 α	46.17 ± 1.35 α
Curcumin group	98.5 ± 5.89***	25.50 ± 0.46***
Curcumin +Eugenol	83.3 ± 4.64***	22.50 ± 0.67***
Curcumin + Rosemary acid	82.7 ± 4.64**	20.0 ± 0.96**

Data expressed as mean ± SEM, using a one-way analysis of variance followed by Tukey's test. Significance was reported as $^{\alpha}p < 0.001$ compared to the vehicle-treated group. ***p < 0.001, **p < 0.01, *p < 0.05 compared with the autistic group.

Table 6: Effect of Curcumin Alone and Combination on Elevated Plus Maze Test

Groups	No. of Entries in Open Arms	No. of Entries in Closed Arms
Vehicle group	4.25 ± 0.63	6.5 ± 0.61
Autistic group	14.17 ± 0.70 α	2.66 ± 0.33 α
Curcumin group	6.0 ± 0.73***	6.83 ± 0.60***
Curcumin +Eugenol	4.66 ± 0.49***	6.45 ± 0.44***
Curcumin + Rosemary acid	4.12 ± 1.05***	6.14 ± 0.42***

Data expressed as mean ± SEM, using a one-way analysis of variance followed by Tukey's test. Significance was reported as $^{\alpha}p < 0.001$ compared to the vehicle-treated group. ***p < 0.001, compared with the autistic group.

4. DISCUSSION

Autism is a non-progressive neurological spectrum disorder that influences the cognitive, emotional, social, and physical well-being of an affected individual. Conventional therapies and other treatment options can alleviate the symptoms as there is no cure for autism. Medicines from natural sources have become a center of research due to most of their beneficial secondary metabolites.[1,6]

According to the current research, VPA may successfully cause autism in mouse neonates at a dosage of 400 mg/kg, intraperitoneally, and the behavioral abnormalities appeared similar to those seen in autistic children. To evaluate the effectiveness of numerous pharmacological drugs in autism, the postnatal technique is applicable. Group III, IV, and V animals substantially acquired autistic behaviors after receiving VPA on PND 14. Various behavioral parameters were assessed.[7-9] Curcumin is a promising, natural agent for ameliorating valproic acid-induced autism. But the problem associated with curcumin is its poor bioavailability. Eugenol and rosemary acid possess incredible benefits in treating autism spectrum disorder by alleviating the symptoms. The addition of eugenol and rosemary acid to curcumin reduces the hydrophilicity of curcumin thus increasing its bioavailability.[15,19,20]

A negative geotaxis test was performed for evaluating brain development and functioning. The autistic animals showed a rise in the length of duration needed to realign on an inclined surface. Administration of curcumin alone or a blend of eugenol or rosemary acid effectively reduced the readjustment period from PND 17 ahead, proving its neuroprotection. Neuromuscular coordination was evaluated by a rotarod test in which decreased time of retention in VPA-administered animals was revealed to be due to cerebellar damage. Curcumin alone and a combination with eugenol or rosemary acid increased the latencies to drop off the rotarod. Anxiety and apprehension pose a significant impact on animals' motor activities in addition to being associated with improved amygdala functioning.[26] Administration of curcumin alone and a combination with eugenol or rosemary acid enhanced the frequency of entries and length of time the animal remained in open arms.

The mitigating action of curcumin, eugenol, or rosemary acid on behavioral abnormalities was most probably because of their neuroprotective actions on the cerebellum and hippocampus. Histological analyses in the research demonstrated that VPA disrupted the Purkinje epithelium and resulted in the loss of cerebellar granule cells. Meningitis with severe meningeal hemorrhages was also observed. Purkinje epithelium integrity as well as recovery of meningeal hemorrhages were restored with curcumin with eugenol or rosemary acid treatment indicating neuroprotective action. Thus, the findings of this study are in agreement with a previous study carried out on post-natal mice.[27]

5. CONCLUSION

In the current research work, it was shown that VPA administration leads to remarkable behavioral alterations similar to autistic features via severe oxidative stress and neuronal vulnerability during the early stages of brain development. Curcumin with eugenol or rosemary acid administration was found to increase the bioavailability of curcumin, which was effective in improving behavioral imperfections and various biochemical parameters. Effective action against autism can only be seen in treatment with a combination of curcumin with eugenol or rosemary acid rather than taking curcumin separately. This protective effect could be responsible for increased bioavailability, antioxidant, and neuroprotection properties. Hence the administration of curcumin along with eugenol or rosemary acid ameliorates the symptoms of autism and promises a beneficiary action.

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