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Comparative Influence of Chia Seed Oil and Astaxanthin in Mitigating Valproic Acid Induced Autism in Prenatal Rat Model

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ABSTRACT
To assess the potential of chia seed oil and astaxanthin in combination to reduce valproic acid-induced autism in a rat model used for prenatal research. Evaluation of the combinative effect of chia seed oil and astaxanthin by behavioral and biochemical estimations. Autism was induced by injecting 400 mg/kg valproic acid intraperitoneally to female pregnant Wistar rats on the gestation day 12th. Administration of VPA leads to autistic behavior in the pups which were confirmed by behavioral tests performed from PND 7 to 49. Subsequently treatment drugs were administered orally from PND 21 to 49. Biochemical estimations i.e., SOD, CAT, GSH and AchE were performed with serum obtained from brain homogenates. The results of this study showed that Chia seed oil and astaxanthin when taken in combination showed better results in reducing autistic characteristics rather than administered individually. In this study two drugs with different mechanisms were used which resulted in synergistic effects towards reducing Autism induced by valproic acid.
Keywords: chia seed oil, astaxanthin, VPA, SOD and Acetylcholinesterase (AChE).

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INTRODUCTION

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder causing disorder of brain development which is due to changes in the structure of brain (Figure 1) [1,2]. It is diagnosed by impairment in social interactions, lack of communication skills, having hypo or hyper reactivity for any sensory stimuli, restricted and repetitive behavioral pattern and interests [3,4]. These symptoms often coexist with intellectual impairment, seizures, attention-deficit hyperactivity disorder, sleep disorders, anxiety and other disorders

It is a disorder behavior parameter and is categorized under pervasive developmental disorders (PDDs). PDD is the term which is commonly used to describe developmental delays impacting social and communication skills. PDD category comprises of two conditions that are always linked to intellectual disability or mental retardation (Rett's syndrome and childhood Disintegrative Disorder) [5]. Similar symptoms but unrelated to language delay or general intellectual impairments include Asperger's syndrome.

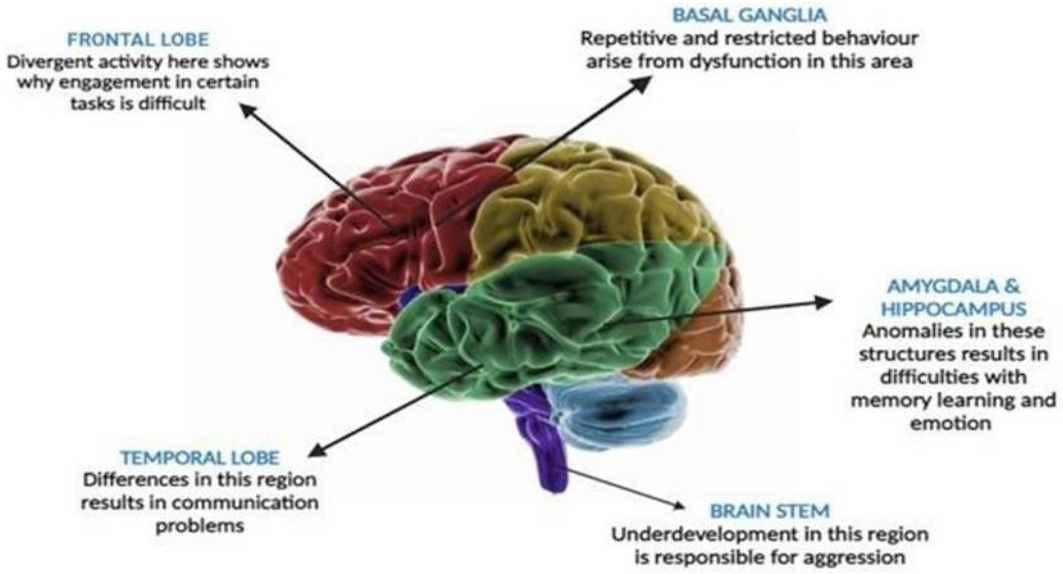

Figure 1: The Autistic Brain

The etiology of ASD is usually multifactorial consisting of genetic and non-genetic factors associated with it (Figure 2). There are non-syndromic forms of this illness. In essence, syndromic disorders are linked to chromosomal abnormalities or single gene changes. Rett syndrome, fragile X syndrome, and MECP2 are a few instances of syndromic ASD [6,7]. Unlike syndromic ASD, non- syndromic ASD is caused due to unknown genetic or environmental factors, oligogenic, polygenic and multifactorial mechanisms [8].

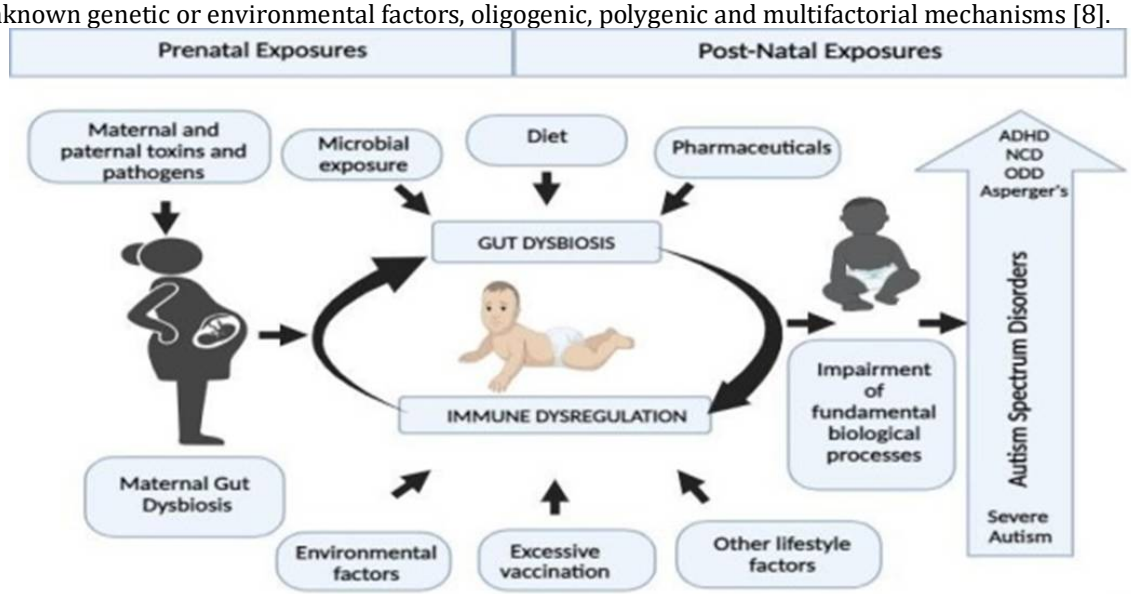

Figure 2: Etiology of Autism

Increased oxidative stress [9], hyperserotonemia [10] and loss of Purkinje cell integrity in the cerebellum [11] are the main pathological findings associated with autism.

MATERIAL AND METHODS

Drugs and chemicals

Sodium valproate injection- Encorate; Chia seed oil- Salvia: Astaxanthin- Vruksha Vitals.

Experimental Animals

Adult albino Wistar pregnant female rats weighing 180-200 g were used. Throughout the experiment, the animals were kept in appropriate environmental and nutritional conditions. The animals were kept in regular polypropylene cages with bedding from paddy husks. Standard lab conditions are maintained such as 23-25oc temperature, 35-60% relative humidity and a 12-hour light/dark cycle. The animals were provided with continuous supply of water and laboratory chow. Committee for the Purpose of control and supervision of experiments on animal (CPCSEA) guidelines were followed in all experimental

procedures. The institutional animal ethics committee (IAEC) authorized each and every experimental protocol. [Protocol No: I/IAEC/AU/10/2024/WR (♂♀)].

Induction Method – Valproic acid

Every day, female rats were observed for the development of a vaginal plug, which indicated the embryonic day (ED 0). A 450 mg/kg i.p dose of VPA was given to the pregnant rats on embryonic day 12.5 and they were closely watched until they gave birth.

Treatment

The off springs were divided into six groups of six animals in each group on the second day (PND 2). Each group was divided into control, negative control, standard group, treatment-1, and treatment- 2, combination group. The off springs were observed for postnatal development and behavioral changes from PND 7 to 49. Treatment was given to the pups from PND 21 to PND 49.

Experimental Design

Group-I: Control 0.9% normal saline p.o.
Group-II: Negative control group – Valproic acid (VPA 450mg/kg, i.p)
Group-III: Standard group – Aripiprazole (10mg/kg p.o.)
Group-IV: Chia seed oil (CSO 15ml/kg/day p.o.)
Group-V: Astaxanthin (2mg/kg p.o.)
Group-VI: Combination group – CSO (15ml/kg/day p.o.) + Astaxanthin (2 mg/kg)

EVALUATION OF BEHAVIORAL PARAMETERS

Motor coordination test

To evaluate motor coordination Rota rod apparatus is used. On PND 49 this test was performed for evaluating limb motor coordination and balance components of motor function in the pups. The device is made out of a horizontal, metal coated rod. Having a motor set to 40 rpm and a rubber disc of 3 cm in diameter connected to it. The pup was kept on roller, and 180 seconds was set aside as cutoff time, during which each animal was watched to see how long it lasted on the rotating rod [12]. Rota rod test is shown in figure 3.

Figure 3: Rota rod test

Postnatal growth and maturation

The offspring's weight, body length, and tail length were noted on PND 7, 21, 28. Eye opening score was recorded from PND 11-15 and was scored as 0 for both eyes closed, 1 for one eye opened, and 2 for both eyes opened.

The Morris water maze test

An aquarium filled with water (28-29^{o}C) was utilized for the learning and memory, latency period time on PND 32. Every animal was positioned in the middle of the tank and observed for 5-10mins. Computation of motor development and consolidation of Latency period time. **Figure 4** depict the swimming performance test.

Figure 4: The Morris water and maze test

Evaluation of biochemical parameters

Superoxide Dismutase

The pyrogallol oxidation method was used to measure the activity of SOD. Monitoring the rate of oxidation allowed for the determination of superoxide dismutase activity. Pyrogallol was added to start the reaction and the shift in optical density was measured at 420 nm [13].

Catalase

Hydrogen peroxide is broken down by the catalase enzyme present in the sample, indicating the enzyme's antioxidant capacity and serving as the fundamental foundation for catalase activity estimation [14].

Glutathione

The Ellman method is used in this spectrophotometric technique. It calculates that when 5,5′-dithiobis-(2-nitrobenzoic acid), or DTNB, is reduced by SH groups, one mole of 2- nitro-5-mercaptobenzoic acid is generated for every mole of SH groups.

Acetylcholinesterase

Levels of acetylcholine were measured to estimate the activity of acetylcholinesterase [15].

Histopathological examination

Histopathological examination is done using animal's brain by proper sacrificing methods, and fixed in formalin solution. H&E (Hematoxylin-eosin) staining procedure was used to examine under a microscope with 100x and 400x magnification.

Statistical analysis

The study's results were presented as mean ± SEM [Standard Error Mean], One-way and two-way analysis of variance were followed by Tukey's multiple comparison tests. ANOVA was employed in the statistical analysis. Graph Pad Prism software version 10 [16,17].

RESULTS

Estimation of superoxide dismutase

Superoxide dismutase levels are estimated between groups to evaluate the extent of antioxidant activity. showing a decreased level from Negative control group compared to control group ($P<0.001$). Combination group showed elevated levels of SOD with almost equally effective as standard group. There were no significant changes between Test groups I and II. **Table 1 shows** Effect of chia seed oil and astaxanthin on SOD, Catalase and GSH.

Table 1: Effect of chia seed oil and astaxanthin on SOD, Catalase and GSH

Group no.	Groups	SOD estimates	Estimates of Catalase	Estimates of GSH
I	Control group	0.53 ± 0.013	0.50 ± 0.26	0.65 ± 0.031
II	Negative Control group (VPA)	0.35 ± 0.16#	0.32 ± 0.06#	0.47 ± 0.12##
III	Standard group (Aripiprazole)	1.29 ± 0.75	0.91 ± 0.35	0.89 ± 0.15
IV	Chia seed oil (CSO)	0.95 ± 0.08.*	0.85 ± 0.07*	0.74 ± 0.62*
V	Astaxanthin	0.85 ± 0.12*	0.87 ± 0.009**	0.78 ± 0.14*
VI	Combination group (CSO + Astaxanthin)	1.24 ± 0.26**	0.88 ± 0.18**	0.89 ± 0.27**

The values represent the mean ± SEM of six distinct animals. Superscript letters denote the statistical significance of ANOVA, which is followed by Tukey multiple comparison test. In the comparison to the

control group, #P < 0.05, ##P < 0.01, ###P < 0.001 are used. In comparison to negative control group *P < 0.05, **P < 0.01, ***P < 0.001 are used.

Estimation of Catalase

Catalase levels were estimated in the brain homogenate serum. The significant increase from negative control group compared to control group (P<0.001). Catalase levels were improved in the groups III, IV, V and VI when compared to negative control group. There was no significant difference between Combination group, test group-1 and test group-2 when compared to group standard. Effect of Chia seed oil and Astaxanthin on Acetylcholinesterase are tabulated in **Table 2**.

Table 2: Effect of Chia seed oil and Astaxanthin on Acetylcholinesterase

Group no.	Groups	AChE Estimates
I	Control group	0.39 ± 0.02
II	Negative control group (VPA)	0.54 ± 0.01##
III	Standard group (Aripiprazole)	0.15 ± 0.01
IV	Chia seed oil (CSO)	0.25 ± 0.01*
V	Astaxanthin	0.33 ± 0.01**
VI	Combination group (CSO + Astaxanthin)	0.18 ± 0.01***

The values represent the mean ± SEM of six distinct animals. Superscript letters denote the statistical significance of ANOVA, which is followed by Tukey multiple comparison test. In the comparison to the control group, #P < 0.05, ##P < 0.01, ###P < 0.001 are used. In comparison to negative control group *P < 0.05, **P < 0.01, ***P < 0.001 are used.

Estimation of Body weight

Body weights of the pups were monitored on PND – 7,21,28 to evaluate maturation and growth of pups. Increase in the body weights from PND – 7 to 28 were observed in all the groups. Effect of chia seed oil and Astaxanthin on body weight is shown in Table 3.

Table 3: Effect of chia seed oil and Astaxanthin on body weight

Groups	PND-7	PND-21	PND-28
Control group	6.33 ± 0.66	14.30 ± 0.40	18.33 ± 0.35
Negative Control group (VPA)	7.25 ± 0.62#	13.58 ± 0.47#	17.63 ± 0.34#
Std group (Aripiprazole)	8.26 ± 0.50	15.12 ± 0.47	20.88 ± 0.22
Chia seed oil (CSO)	7.36 ± 0.17	14.37 ± 0.50*	19.30 ± 0.40*
Astaxanthin	7.86 ± 0.47*	12.12 ± 0.42	18.57 ± 0.18
Combination group	8.18 ± 0.15**	14.55 ± 0.25**	20.07 ± 0.41**

The values represent the mean ± SEM of six distinct animals. Superscript letters denote the statistical significance of ANOVA, which is followed by Tukey multiple comparison test. In the comparison to the control group, #P < 0.05, ##P < 0.01, ###P < 0.001 are used. In comparison to negative control group *P < 0.05, **P < 0.01, ***P < 0.001 are used.

Rotarod test

Rota rod test was performed to evaluate motor coordination and was compared between different groups, resulting an increase in the fall of time (sec) from negative control group to control group (P<0.001). Group II showed a shorter fall off time from the rotating rod compared to control group. Groups IV, V, VI showed comparatively less activity on rota rod than group III. **Table 4** shows the effect of Chia seed oil and Astaxanthin on motor coordination.

Table 4: Effect of Chia seed oil and Astaxanthin on motor coordination

Group no.	Groups	Fall off time (sec)
I	Control group	52.33 ± 2.17
II	Negative Control group (VPA)	20.83 ± 3.41
III	Standard group (Aripiprazole)	93.17 ± 1.42
IV	Chia seed oil (CSO)	63.50 ± 1.96**
V	Astaxanthin	52.50 ± 2.93*
VI	Combination group (CSO + Astaxanthin)	66.50 ± 2.87**

The values represent the mean ± SEM of six distinct animals. Superscript letters denote the statistical significance of ANOVA, which is followed by Tukey multiple comparison test. In the comparison to the control group, #P < 0.05, ##P < 0.01, ###P < 0.001 are used. In comparison to negative control group *P < 0.05, **P < 0.01, ***P < 0.001 are used.

The Morris water maze test

The Morris water maze test was performed to evaluate learning and memory in the pups and results were compared between groups. The time taken for negative control group to find the platform increased with that of control group (P<0.001). Whereas group VI showed equally effective as group II in finding the platform resulting in the enhanced memory of the pups with the combination of treatment drugs. **Table 5** show the effect of Chia seed oil and Astaxanthin on Learning and memory.

Table 5: Effect of Chia seed oil and Astaxanthin on Learning and memory

Group no.	Groups	Latency period (sec)
I	Control group	57.67 ± 3.15
II	Negative control group (VPA)	160.5 ± 8.73
III	Standard group (Aripiprazole)	35.17 ± 1.66
IV	Chia seed oil (CSO)	46.33 ± 1.22**
V	Astaxanthin	48.00 ± 2.49**
VI	Combination group (CSO + Astaxanthin)	34.50 ± 1.68***

The values represent the mean ± SEM of six distinct animals. Superscript letters denote the statistical significance of ANOVA, which is followed by Tukey multiple comparison test. In the comparison to the control group, #P < 0.05, ##P < 0.01, ###P < 0.001 are used. In comparison to negative control group *P < 0.05, **P < 0.01, ***P < 0.001 are used.

Histopathological evaluation

Figure 6(a) depicts the brain hippocampus of the negative control group, revealing decreased neuronal cell density. The image shows pyramidal cell degeneration, glial cell degeneration in the hilus of the dentate gyrus, and reduced neuronal cell density in the CA4 region of the cornu ammonis. Figure 6(b) illustrates the histopathology of the brain amygdala in the negative control group. This image demonstrates increased neuronal cell density and decreased neuronal cell size.

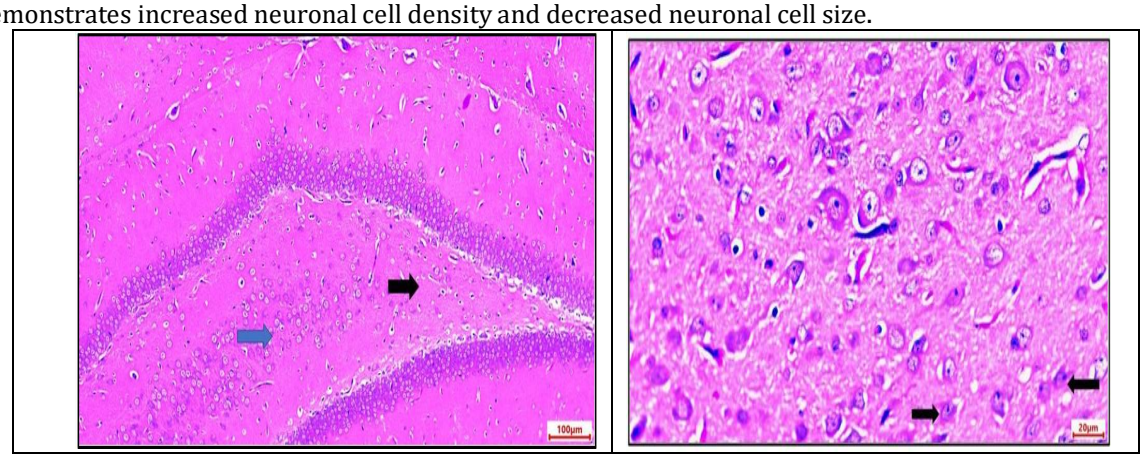

Figure 6 (a and b): Negative control group Brain hippocampus and Brain amygdala

Figure 7(a) depicts the histopathology of the hippocampus in the Standard Group Brain, revealing normal neuronal cell density. The CA4 region of the cornu ammonis exhibits typical neuronal cell density, while the hilus of the dentate gyrus shows regenerated pyramidal cells and glial cells. Figure 7(b) illustrates the histopathology of the amygdala in the Standard Group Brain, displaying normal morphology of neuronal cells and dendrites.

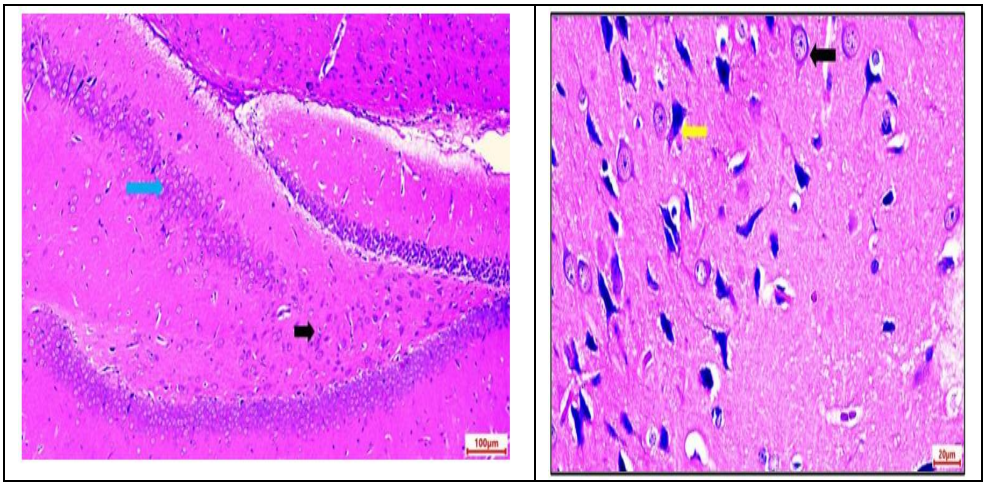

Figure 7 (a and b): Standard group Brain hippocampus and Brain amygdala

Figure 8(a) depicts the histopathology of the brain hippocampus in Test group-1 (CSO), revealing changes in neuronal cell density. A mild increase in neuronal cell density is observed in the CA4 region of the cornu ammonis, accompanied by a slight increase in pyramidal and glial cell density. Figure 8(b) illustrates the histopathology of the brain amygdala in Test group-1 (CSO), showing normal neuronal cell morphology and mildly regenerated dendrites.

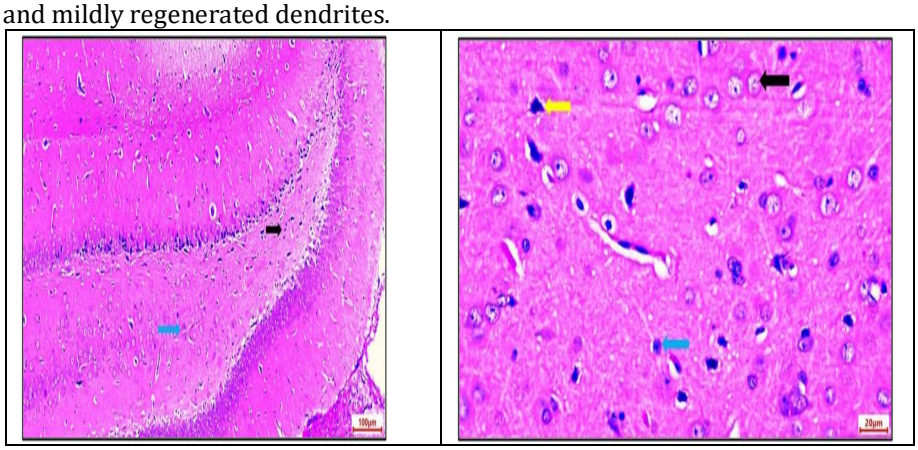

Figure 8(a and b): Test group-1 (CSO) Brain hippocampus and Brain amygdala

Figure 9(a) depicts the histopathology of the brain hippocampus neuronal cell density in Test group-2 (Astaxanthin). It reveals a mild increase in neuronal cell density within the CA4 region of the cornu ammonis, accompanied by a slight increase in pyramidal and glial cell density. Figure 9(b) illustrates the histopathology of the brain amygdala in Test group-2 (Astaxanthin), showing normal neuronal cell morphology with mildly regenerated dendrites and myelinated axons.

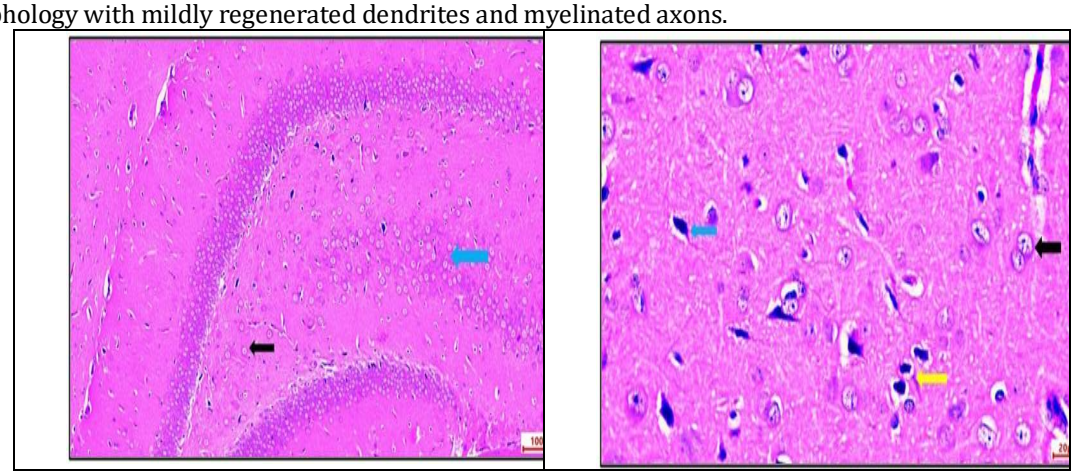

Figure 9(a and b): Test group-2(Astaxanthin) Brain hippocampus and Brain amygdala

Figure 10(a) depicts the histopathology of the combination group's brain hippocampus neuronal cell density. It shows a moderate increase in neuronal cell density in the CA4 region of the cornu ammonis and a mild increase in pyramidal and glial cell density. Figure 10(b) illustrates the histopathology of the combination group's brain amygdala, revealing normal morphology of neuronal cells and moderately regenerated dendrites and myelinated axons.

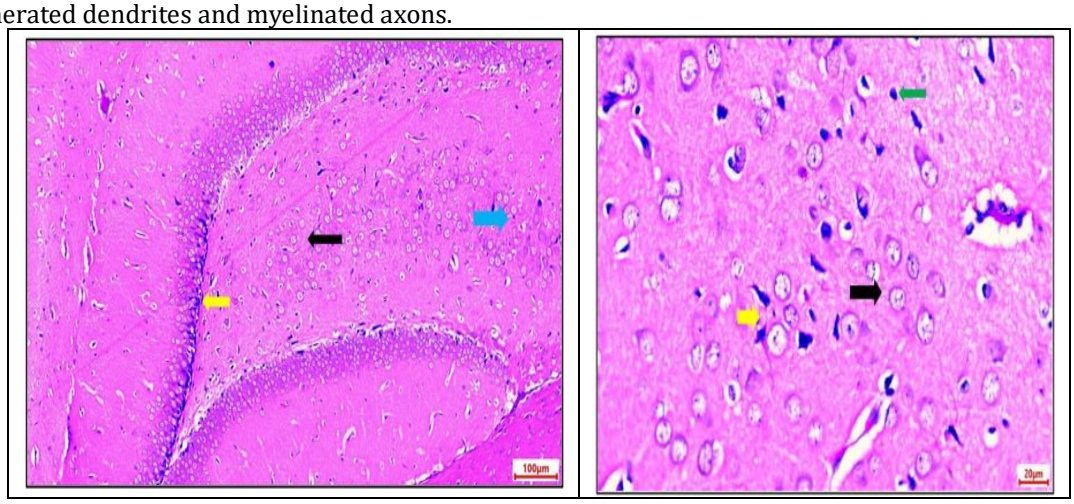

Figure 10(a and b): Combination group Brain hippocampus and Brain amygdala

DISCUSSION

Autism a complicated neurodevelopmental disorder which includes difficulties in communication not confined to repetitive and stereotyped patterns of behavior that are indicative of social impairment [18]. In this study prenatal rat model used in which pregnant female rats were induced with valproic acid (450 mg/kg i.p) on the gestation day 12, this leads to autistic symptoms in the pups.

Valproic acid is an antiepileptic drug and also a potent teratogen which causes neurotoxicity in CNS of children. VPA can alter the transcription levels of genes that are linked, which can create aberrant signaling pathways, synaptic dysfunction, and abnormalities in neurogenesis, all of which can result in ASD in the developing brain. ASD brought on by prenatal VPA exposure is also linked to altered brain-gut axis function, elevated oxidative stress, and neuroinflammation [19]. Exposure to valproic acid during critical times of neural development has been shown to be a well- documented environmental risk factor for the development of autism-like traits in offspring. The actual methods by which VPA generates these alterations are complicated, encompassing oxidative damage, neuroinflammation, and altered neurotransmitter systems.

The autistic behavior of pups was evaluated by behavioral tests and biochemical estimations [20]. Biochemical estimations include estimation of super oxide dismutase, which showed better results in the levels of SOD in the combination group when compared to negative control group. The combination of chia seed oil and astaxanthin showed better results in increasing the levels of Catalase which is an important enzyme in protecting cells from oxidative stress. Glutathione estimation is performed and the levels were increased in the combination group compared with standard group. Glutathione which is a crucial antioxidant, helps in detoxification, anti-inflammatory as well as a neurotransmitter regulator. Enhanced levels of glutathione help in reducing oxidative stress which plays key role in pathophysiology affecting autism [21]

Neurotransmitter acetylcholinesterase levels were also estimated as acetylcholinesterase is an enzyme which degrades acetylcholine levels in the synapse and reduces the availability of acetylcholine which may result in behavioral characteristics which are observed in autistic individuals. Chia seed oil and astaxanthin in combination showed better results in reducing the levels of acetylcholinesterase resulting in elevated levels of acetylcholine.

Rotarod test and Morris water maze test were performed. In rotarod test fall of time (sec) was noted as a parameter for motor coordination. Animals in the negative control group has fall off early showing less motor coordination, whereas animals in standard group and combination group stayed longer on the rotarod apparatus resulting in better coordination reflexes. Test group -1&2 animals had fall off from the apparatus within less time when compared to combination and standard groups.

The Morris water maze test was evaluated with trained animals where a platform is placed in the Morris apparatus and the animal is allowed to swim and find the platform. This test represents learning and memory, latency period time (sec) is the parameter [22,23]. Animals in negative control group took

longer time in identifying the platform, whereas in test group-1 (chia seed oil) showed less latency period time when compared to negative control and test group-2. Combination group showed less latency period time when compared to other group animals, and compared to standard group animals.
Body weight of the pups was recorded from PND-7,21 and 28, to observe the maturation and growth of the pups. The growth of pups has been increased from PND-7 to 28.

CONCLUSION

In conclusion the benefits of astaxanthin and chia seed oil in reducing the effects of valproic acid- induced autism in a rat prenatal model were identified. Both therapies showed signs of potential neuroprotective qualities, each with unique pathways that could lead to benefits in behavior and physiology. Chia seed oil, rich in omega-3 fatty acids, appears to increase neurodevelopment conditions and reduce inflammation, while astaxanthin's antioxidant activities may help alleviate oxidative stress and improved cognitive performance. Further studies are needed to examine these drugs underlying mechanisms, long term effects, and suitability for use in therapeutics. These findings underline the relevance of dietary interventions in addressing neurodevelopmental problems and pave the path for further study of natural phytoconstituents in autism mitigation techniques.

Funding

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Conflict of interest

None declared

Ethical approval

The institutional animal ethics committee (IAEC) authorized each and every experimental protocol. [Protocol No: I/IAEC/AU/10/2024/WR (♂♀)].

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