Cannabinoid Extract Protects Against Valproic Acid Induced Autism Spectrum Disorder

Author

Faiza Ali

Reg. No. 00000329028

Supervisor

Dr. Adeeb Shehzad

Department of Biomedical Sciences and Engineering

School of Mechanical & Manufacturing Engineering (SMME)

National University of Sciences and Technology (NUST)

Islamabad, Pakistan

JULY, 2023

Cannabinoid Extract Protects Against Valproic Acid Induced Autism Spectrum Disorder

Author

Faiza Ali

Reg. No. 00000329028

A thesis submitted in partial fulfillment of the requirements for the degree of

MS Biomedical Sciences

Thesis Supervisor: Dr. Adeeb Shehzad

Thesis Supervisor's Signature: _____

Biomedical Sciences

School of Mechanical & Manufacturing Engineering (SMME)

National University of Sciences and Technology (NUST)

Islamabad, Pakistan

JULY, 2023

FORM TH-4

National University of Sciences & Technology

MASTER THESIS WORK

We hereby recommend that the dissertation be prepared under our supervision by: FAIZA ALI Reg No. 00000329028

Titled: **Cannabinoid Extract Protects Against Valproic Acid Induced Autism Spectrum Disorder** be accepted in partial fulfillment of the requirements for the award of **MS Biomedical Sciences** degree.

Examination Committee Members

1. Name: Dr. Syed Omer Gillani Signature: _____

2. Name: Dr. Muhammad Asim Waris Signature: _____

3. Name: _____ Signature: _____

Supervisor's name: Dr. Adeeb Shehzad Signature: _____

Date: 11/7/2023

_____ 24-7-23

Head of Department Date

COUNTERSINGED

Date: 24-7-23

Dean/Principal

THESIS ACCEPTANCE CERTIFICATE

Certified that final copy of MS thesis written by **Ms. FAIZA ALI (Registration No. 329028)**, of SMME (School of Mechanical and Manufacturing Engineering) has been vetted by undersigned, found complete in all respects as per NUST Statutes / Regulations, is within the similarity indices limit and is accepted as partial fulfillment for award of MS/MPhil degree. It is further certified that necessary amendments as pointed out by GEC members of the scholar have also been incorporated in the said thesis.

Signature: _____

Name of Supervisor:

Date: 11/7/2023

Signature (HOD): _____

Date: 24/7/23

Signature (Dean/Principal): _____

Date: 24-7-23

Declaration

I certify that this research work titled "***Cannabinoid Extract Protects Against Valproic Acid Induced Autism Spectrum Disorder***" is entirely my own work. The work has not been submitted or presented elsewhere for evaluation. The material that has been used from other sources it has been properly cited and acknowledged.

Signature of Student

FAIZA ALI

2023-NUST-MS-BMS-00000329028

Proposed Plagiarism Certificate

This certificate attests that the PhD/M.Phil/MS thesis titled "**Cannabinoid extract protects against valproic acid induced autism spectrum disorder**" by **Faiza Ali (Reg. No. 00000329028)** has been examined by us. We certify the following:

a. The thesis contains new and original work and knowledge that has not been previously published or submitted for publication elsewhere. No sentences, equations, diagrams, tables, paragraphs, or sections have been copied verbatim without proper citation and referencing.

b. There is no plagiarism in the work that is offered; it is the author's unique and original work. No methods, conclusions, or words that belong to someone else have been claimed as the author's own.

c. The information that has been collected and analysed is true; there has been no fabrication.

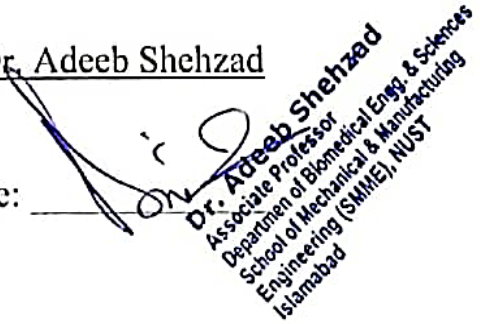
d. There are no modifications to the data or outcomes that might cause the research to be misrepresented in the research record, including falsification of research materials, tools, or procedures.

e. The thesis has been examined using TURNITIN (a copy of the originality report is included), and it has been determined that it complies with all applicable HEC plagiarism policies and guidelines.

Name & Signature of Supervisor

Name: Dr. Adeeb Shehzad

Signature: _____



Copyright Statement


بسم الله الرحمن الرحيم

Acknowledgement

All praise is to ALLAH Almighty, the most Beneficent, the most merciful who bestowed upon me with the sight to observe, the mind to think and the courage to work more. I could complete my research work only because of Allah's help, guidance and blessing.

My deepest and sincerest gratitude goes to my Supervisor, **Dr. Adeeb Shehzad**, whose support and guidance helped me through every problem I faced in research as well as in my life. Thank you for being the best mentor.

I am deeply indebted to my father **Amjad Ali** and my mother **Sajida Parveen**. I am grateful to those who gave me my name and my life, and everything in between. Though I am skilled with words, I struggle to express the extent of their efforts in giving me the life I have now. Their contributions are the reason I was able to accomplish this. I would like to acknowledge my siblings **Aliza, Rubab** and **Majid Ali** who always inspired me to achieve more and more in life.

I would like to express special thanks to my friends **Muhammad Ashraf**, **Dr. Touqeer Javed** and **Dr. Usama Ch.** who is the reason I thrive to be better and without whom I would be deprived of the love, support, and advice that helped me through all.

I am extremely grateful to my friends **Faiza Tariq, Mahum Khan, Maliha, Aroosa** and **Areej** without whom it was not an easy and beautiful journey full of memories I have now. I will forever be grateful for their constant support and care throughout my research work.

I would like to pay my gratitude to **Dr. Salman** (QAU), **Uncle Maqsood** (NIH) for being a listening to my problems, for helping me out wherever possible, and for inculcating positivity in me at every hurdle. I would like to acknowledge **Ms. Maria** and **Sir Irfan** for being so helpful throughout this journey. In conclusion, I would like to extend my sincere appreciation to all those who have provided valuable support throughout my research.

Faiza Ali

Dedication

I would like to Dedicate this thesis to my parents and adored siblings and friend Muhammad Ashraf, as they inspires me to be strong in every path of life and criticizes me to see me grow and face all the challenges in life to achieve every success that come along. All that I have done is because of their tremendous support, unfailing love and cooperation led me to this wonderful accomplishment.

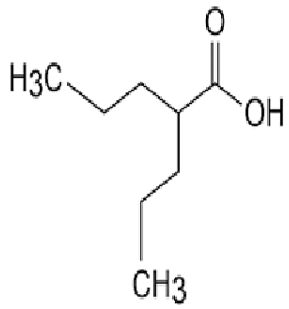
Abstract

Autism spectrum disorder is a complex behavioral disorder characterized by impairments in social interaction, communication, and repetitive patterns of behavior. The objective of this study was to create a mouse model of autism and examine how cannabis oil affected these animal models.

On gestational day 13 and pup postnatal day 14, valproic acid (600 mg/kg and 400 mg/kg) was subcutaneously injected into pregnant mice, resulting in poor behaviour in the offspring that is similar to the characteristics of autistic people. Two groups of valproic acid-exposed mice were used: one group received oral cannabis oil treatment (100 mg/kg), and the other group received oral risperidone (0.5 mg/kg). At the age of eight weeks, behavioural tests including the Y-maze, marble, open field, elevated plus maze, hot plate and social interaction were carried out. To assess the effects of cannabinoid oil, oxidative stress indicators including glutathione (GSH, GST), lipid peroxidation (LPO), nitric oxide (NO), and activity of superoxide dismutase (SOD) and catalase (CAT) were assessed on PND-58. The findings showed that mice exposed to valproic acid both prenatally and postnatally displayed autistic behaviour, including high levels of anxiety, prolonged latencies for responding to painful stimuli, and reduced social interaction. This study looked at the morphological and neuroanatomical abnormalities in the hippocampus, prefrontal cortex, and Purkinje cells in the cerebellum, as well as the neural tube dysfunction in offspring exposed to VPA. In the autistic mice model, it was determined that therapy with medical cannabis oil considerably ($p<0.05$) alleviated the behavioural abnormalities and it reduced oxidative stress by acting as an antioxidant.

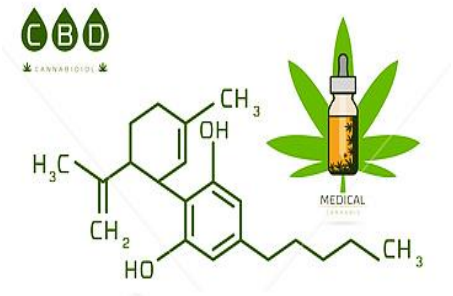
Keyword: Autism Spectrum Disorder, Valproic Acid, Cannabinoid, Risperidone, Oxidative Stress

Graphical Abstract

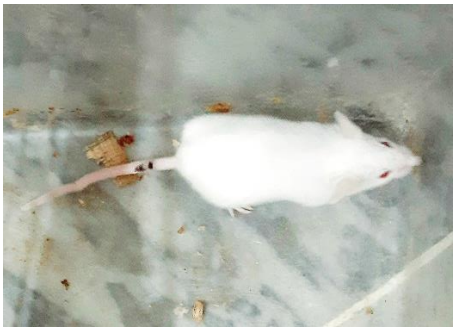


Valproic Acid

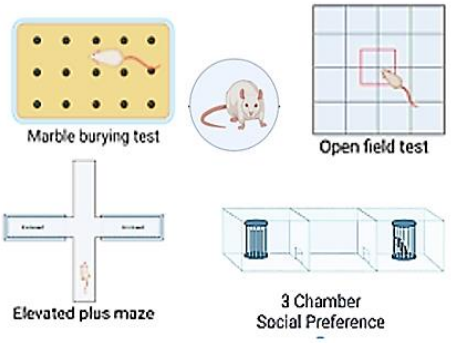
Valproic Acid Induced Autistic Balb/c Mice



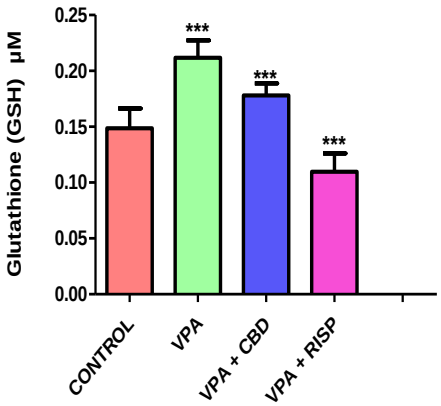
CBD
Neuroprotective effect of CBD oil from Cannabis Plant



Alleviation of Autistic Behavior in Mice



Behavioral Analyis



Biochemical Assessment

Table of Contents

List of Figures

List of Tables

List of Acronyms

ASD	Autism Spectrum Disorder
DSM-IV-TR	Diagnostic And Statistical Manual Of Mental Disorders-IV-Text Revision
PDDNOS	Pervasive Developmental Disorder Not Otherwise Specified
VPA	Valproic Acid
CDC	Centers For Disease Control And Prevention
GD	Gestational Day
EC	Endocannabinoid
CBD	Cannabidiol
THC	9-Tetrahydrocannabinol
RISP	Risperiodone
RELN	Reelin Gene
CNS	Central Nervous System
NLGN3 And 4	Neuroligin 3 And 4
Th	T-Helper Cells
PND	Postnatal Day
NRXN	Neurexins
CA	Cornu Ammonis 1
DG	Dentate Gyrus
GPCR	G-Protein-Coupled Receptor
NMDA	N-Methyl-D-Astarte Receptor I
AMPA	A-Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic Acid
CBDV	Cannabidivarin
CB1R	Type 1 Cannabinoid Receptor
NTD	Neural Tube Defect
NIH	National Institute Of Health
EPM	Elevated Plus Maze
DI	Discrimination Index
SPI	Social Preference Index

SI	Sociability Index
PFA	Paraformaldehyde
PBS	Phosphate Buffer Saline
GST	Reduced Glutathione Sulfo-Transferase
GSH	Glutathione
SOD	Superoxide Dismutase
LPO	Lipid Peroxidation
NO	Nitric Oxide
CAT	Catalase
CDNB	1-Chloro-2, 4-Dinitrobenzene
MDA	Malondialdehyde
ANOVA	Analysis of Variance

CHAPTER 1

1. INTRODUCTION

A group of neurodevelopmental abnormalities collectively known as autism spectrum disorder (ASD). ASD is typified by stereotypical or repetitive behaviors, disordered social interaction, and communication impairments. ASD is accompanied by a number of co-morbid features, such as anxiety, seizure activity, motor abnormalities, aggressive conduct, poor pain perception and sleep difficulties [1].

According to the Diagnostic and Statistical Manual of Mental Disorders-IV-Text Revision (DSM-IV-TR), ASD includes Asperger's Disorder, Pervasive Developmental Disorder Not Otherwise Specified (PDDNOS), and Autistic Disorder (commonly known as "classic" autism). The DSM-V draught states that the DSM-IV autism spectrum will be reclassified as a single category of ASD [2].Although numerous theories have surfaced in an effort to answer the autism puzzle and research has advanced, its etiology is still unknown, and there are no objective diagnostic standards or available treatments. Autism may be caused by genetic causes including mutations, deletions, and copy number variations [3]. Epidemiological research has shown that harmful environmental factors such maternal exposure to infections, ethanol, thalidomide, and Valproic Acid (VPA) increase the likelihood of having children with autism [4]. When combined with changes in diagnostic procedures, this may be one of the causes of the rising prevalence of autism. In reality, since the 1980s, there have been significantly more children diagnosed with autism. According to the Centers for Disease Control and Prevention (CDC), there are 1 in 88 children in the United States who have autism, costing the country $137 billion annually [5].

Currently, there is a lack of established pharmacological treatments for the main symptoms of autism spectrum disorder (ASD), which include persistent difficulties in social communication and repetitive, restricted patterns of behavior. Moreover, the effectiveness and tolerance of pharmacotherapies targeting associated disruptive behaviors that commonly coexist with ASD are comparatively limited. Various drugs, antipsychotics, anticonvulsants, and stimulants are commonly prescribed to treat individuals with autism. However, these medications do not completely eliminate the primary symptoms associated with autism spectrum disorder (ASD). Furthermore, there are potential negative effects of ASD medications, including an elevated risk of developing other conditions like obesity, dyslipidemia, diabetes and thyroid disorders.

An increased risk of autistic spectrum disorder (ASD) is allegedly linked to antiepileptic drug exposure during pregnancy, according to growing data [6]. A human study found a correlation between prenatal valproic acid (VPA) intake and poor adult cognitive function [7]. Considering this connection, animal models have proven to be a viable tool since they create morphological, behavioral, and pathophysiological changes that are comparable to those in humans. In animals, a single prenatal VPA injection leads to poor cognitive function [8], social interaction deficiencies [9], and stereotypical and repetitive behavior [10] throughout the postnatal period. In order to explore the impact of VPA during pregnancy, the possibility of developing an animal model of autism is presented by these findings. Previous studies have also revealed nociceptive alterations. On gestational day (GD) 12.5 a single dose of VPA (600 mg/kg) results in aberrant pain sensitivity in maturity, similar to human autistic people [11]. The cranial nerve neurons in rats are born at GDs 12 or 13, when the neural tube of the animal closes. This period of time (GD 12–13) is critical for the numerous sensory and motor nuclei nerves. When VPA is exposed during this period of pregnancy, cranial nerve neurons are reduced, behavior is impaired, and related gene expression is altered. Animal models of autism exhibit behavioral abnormalities like anxiety, hyperactivity, and impaired nociception in addition to the core symptoms [12].

The endocannabinoid (EC) system is a neuromodulating network that has previously been shown to be active in ASD patients with co-morbidities like anxiety and seizures [13]. The EC system controls behavioral reaction to context and social interaction as well as emotional responses [14]. The prospect of using medical cannabis to treat individuals with ASD is intriguing because EC imbalance is thought to be a probable mechanism causing the condition's primary symptoms and morbidities [15]. Medical cannabis oils are the most popular delivery method when using the drug to treat ASD patients. These oils are oral treatments made from cannabis plant extracts. Additionally, it is common practice to designate CBD-rich cannabis strains with low THC levels for the treatment of children and adolescents. Despite a sizable amount of evidence supporting the use of medical cannabis with added CBD to treat ASD [16], there are still significant knowledge gaps on the mechanisms underlying long-term medical cannabis use in ASD patients. Therefore, in order to efficiently manage autism, there is a need for safe and effective medications. This has led to the exploration of various natural plant-derived products that have therapeutic potential. Herbal treatments that prove to be effective can help alleviate clinical symptoms with minimal side effects, as demonstrated by recent studies.

This is the goal of our study to develop autistic mice model by pre and postnatal valproic acid exposure on gestational day 13 and postnatal day 14 of their development. After the development of diseased model, animals were treated with medical Cannabis oil obtained from cannabis sativa plant. This study will also examine the properties and potential application CBD oil. The current study aimed to investigate the efficacy of CBD oil and Risperidone as a standard drug to inhibit the oxidative stress and reduce the autistic symptoms in VPA exposed offspring. For this purpose, histopathological examination of brain hippocampus, prefrontal cortex and cerebellum and oxidative stress markers analysis were evaluated.

1.1. Objectives

The objectives of this study are as follows:

- To create a mouse model of autism through exposure to VPA during pregnancy and after postanatal period.
- To examine the impact of cannabinoid oil on offspring exposed to VPA
- To Compare of efficacy of CBD oil and Risperidone as standard drug for treating the symptoms associated with ASD
- To conduct different kinds of behavioral tests: a hot-plate test for nociception, an open field and elevated plus maze test for anxiety, a three-chamber social interaction device for studying social interaction , novel object recognition for exploration and memory, marble burying test for repetitive behaviors and y-maze test for spatial memory and reward related behaviors.
- To compare the histopathological changes in brain tissues (Cerebellum, Hippocampus and Pre-frontal Cortex) of VPA , CBD and Risperidone group
- Additionally, the adult offspring's oxidative stress indicators, including GST, GSH, SOD, LPO, CAT, and NO level, were assessd

CHAPTER 2

2. LITERATURE REVIEW

2.1 Autism Spectrum Disorder

The term autism spectrum disorder (ASD) refers to a diverse range of neurodevelopmental disorders with the typical triad of symptoms: impaired social interaction, abnormalities in language and communication, and stereotypical behavior. The prevalence of autism spectrum disorder (ASD) is increasing, making it one of the largest concerns in modern medicine. The CDC's Autism and Developmental Disabilities Monitoring Network has estimated that about 1 in 68 children have been diagnosed with ASD [17].

2.1.1 Pathophysiological Mechanisms of ASD

Animal studies and clinical data currently available suggest that ASD may relate to a collection of illnesses with complex etiologies. Numerous genes are probably implicated in the pathophysiology of the spectrum. Combining recent findings in the genetics of ASD with the application of animal models leads to the most common groups of underlying route mechanisms. One could claim that autistic patients' neurons don't communicate with each other in addition to having impaired social communication and linguistic difficulties. A number of processes, including the synapse's structural framework, activity-dependent modifications to protein production and degradation, the silencing of some genes, and the up-regulation of the expression of other genes, depend on normal brain activity to function properly. In this regard, if we combine the known genetic disorders with the high prevalence of ASD, we reveal numerous connected clusters, which show the pathways related to ASD: impaired growth of synapses, Impaired epigenetic control of protein expression and impaired protein turnover [17].

2.1.1.1 Neuronal Connectivity

Perhaps the two ideas that have received the most support for explaining the pathophysiology of autism are those that deal with defective synaptogenesis and impaired brain connection. The

significantly higher neuronal density in autistic patients compared to ordinary persons may have an impact on the structure and fine-tuning of brain circuits. The system must eliminate useless, non-functioning neurons to boost the strength of the active ones in order to build viable neural circuits. Thus, while the surviving neurons' connection is increasing daily in the brain of a child that is developing properly, there are less neurons overall. This mechanism appears to be disrupted in ASD youngsters, according to the findings [17].

2.1.1.2 Impaired Neural Migration

The previous hypothesis is very similar to the neuronal migration hypothesis. The impairment of neuronal migration during the prenatal period is also a contributing factor. The brain's ability to develop further may be hindered by initial neuronal implantation errors. Numerous Studies are supporting this fact. Reelin gene (RELN) mutation (rs362691) may considerably increase the incidence of ASD, according to a recently published meta-analysis. Reelin is among the most important proteins involved in the migration and proper positioning of neurons in the neocortex.
The thickened cortex and muddled borders with white matter tracts, both of which are common findings in ASD, may be caused by this and other genetic variables [17].

2.1.1.3 Damaged Synaptogenesis and Dendritic Morphognensis

During early development of the central nervous system (CNS), typically there is an excess of early synapse production followed by selective synapse removal. Synaptic maturation and control throughout the perinatal period are essential for optimal brain development. ASD is thought to be primarily characterized by defective synaptogenesis, which is supported by a number of lines of research.One of these indicators is morphological abnormality, which is characterised by altered dendritic morphology and dendritic spine abnormalities.
In a postmortem study, another research team discovered that layer V pyramidal neurons in the temporal lobes of persons with ASD had more dendritic spines and less developmental spine pruning. Correlative to the connection issues in ASD, Layer V pyramidal neurons are the main excitatory neurons that form cortical-cortical and cortical-subcortical projections. Additionally, they demonstrate a connection between these spine abnormalities and elevated mTOR activity and poor autophagy. On the presynaptic membrane, other sorts of abnormalities are also found.

Notably, the scaffolding proteins neuroligin 3 and 4 (NLGN3 and 4) and SHANK were discovered to be connected with autism, confirming this notion of ASD pathogenesis [17].

2.1.1.4 Immune Dysfunction and Neuroinflammation

The altered immune system, including both acquired and intrinsic immunity, is one of the most studied aspects of ASD. Despite significant research efforts, the etiology of immunological abnormalities identified in people with ASD is still unknown. One of the first important studies in this field, conducted on 31 patients with ASD, was published in the middle of the 1980s.

Numerous investigations using diverse methodologies and small ASD patient populations have found an increase in pro-inflammatory cytokines in peripheral blood. Leukocyte production of Th2-associated cytokines is higher in autistic people. Despite this, there is inconclusive evidence to suggest that children with ASD have a change in the total number of T cells in their peripheral blood. It turns out that the natural killer cells as well as the T cell subpopulations are imbalanced.

Neuroglial cells, including astrocytes and microglia, have a profound impact on the health and homeostasis of neurons. Endothelial cells and perivascular macrophages are also crucial. Microglia and astroglia primarily control the organisation of the cortex, neuroaxonal guidance, and synaptic plasticity. Neuroglial cells play a range of roles in regulating immune response in the central nervous system (CNS). For instance, astrocytes are crucial for the metabolism of glutamate. The detoxification of excessive excitatory amino acids, the preservation of the blood-brain barrier, the generation of neurotrophic factors, and other processes. The neuroglial response to damage or malfunction of the nervous system is strongly influenced by microglial and astrocyte activation. Microglia enable synaptic stripping, brain plasticity, and immune surveillance. According to the aforementioned lines of study, immunological dysfunction and abnormal synaptogenesis in the developing brain appear to be closely related mechanisms rather than two distinct processes as shown in (Fig. 1) [17].

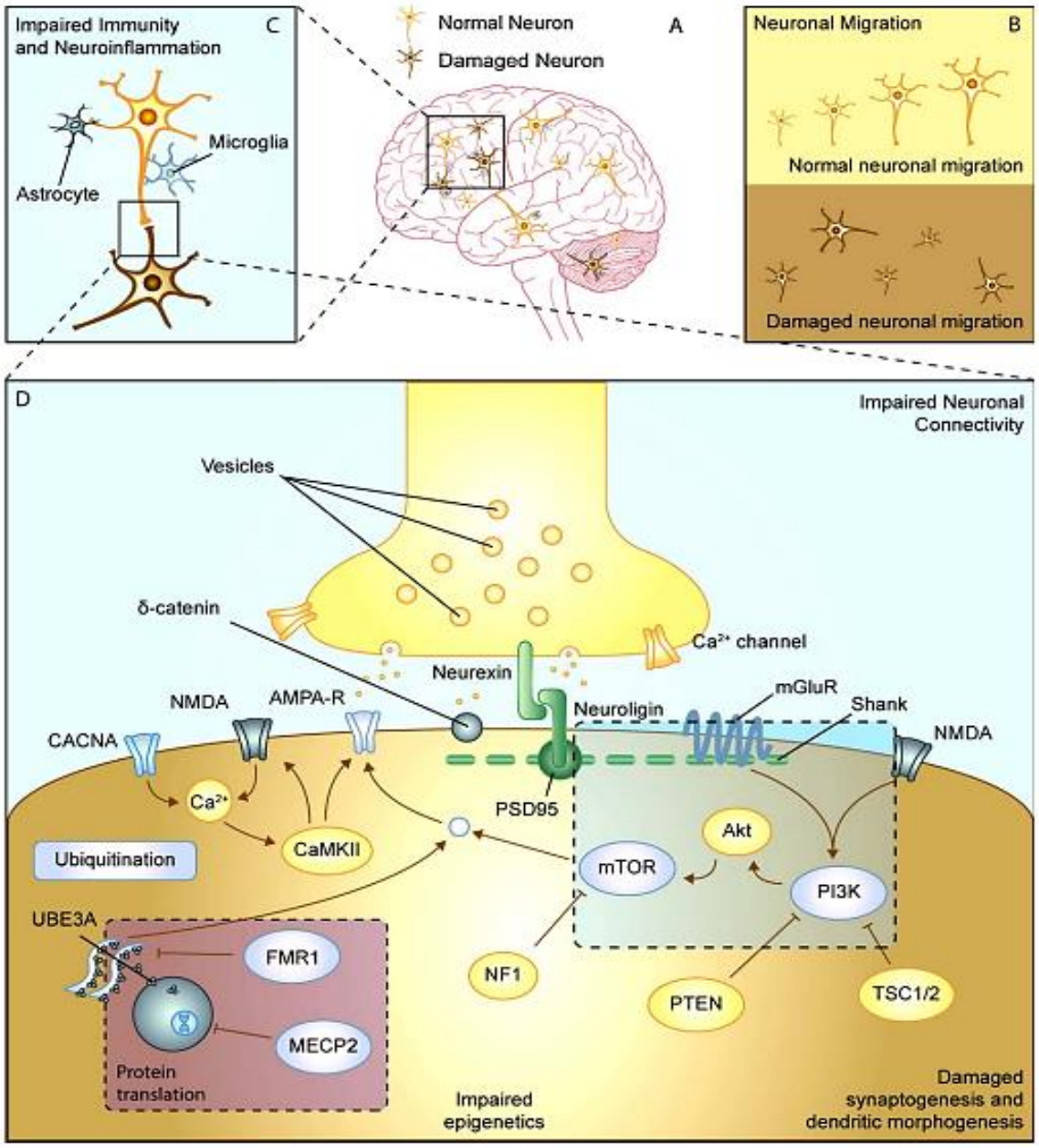


Fig. 1. Presents an Overview of the Converging Processes of Autism Spectrum Disorder. The pathophysiology of autism spectrum disorders (ASD) is depicted in this picture as multilayer abnormalities, which represent proposed processes. At the tissue level, it may result in altered minicolumns, impaired synaptogenesis with potential excitation–inhibition imbalances, changes in temporal dimension leading to a reduction in neuronal numbers (box A), and diminished effectiveness of interneuronal communication. Glial involvement (box C) results in an increase in inflammatory signalling along with these changes and neuronal damage. These could potentially interfere with neural migration problems (box B), preventing appropriate neuronal connection.

The abnormality of cellular growth and synaptic plasticity, abnormalities in protein synthesis, quality control, ubiquitination, and epigenetic regulation of protein expression, and impaired synapse construction are thought to be the causes of these changes at the cellular-molecular level (box D). The protein that is represented by a violet tiny circle and whose expression is controlled by the aforementioned pathways is one that is essential for effective synaptic plasticity, such as the AMPA receptor subunit. Even if they have been criticised, it's still possible that the aforementioned changes have a greater impact on some critical brain regions (box A) than others, for example, the cerebellar Purkinje cells or the prefrontal cortex with mirror neurons [17].

2.2 Oxidative Stress and ASD

Based on the already known research, it would seem logical to conclude that oxidative stress may result in cell damage and perhaps death, which in turn may be the root cause of autism diseases.
The cellular endogenous antioxidant glutathione may diminish as a result of oxidative stress, which can cause a fast alteration in the antioxidant system. Antioxidant therapy may enhance autistic behaviour, according to a double-blind, placebo-controlled trial.Therefore, as has been seen in autistic children, oxidative stress, which is typified by an increase in free radical and lipid peroxide levels, may play a significant role in autism.
An animal study showed that adult oxidative stress is brought on by prenatal VPA exposure. The signs of autism that appear in children following prenatal VPA exposure have been treated using a variety of methods. The Bacopa monniera (300 mg/kg, p.o. for 2 weeks) extract was tested on VPA-exposed autism and found to improve autistic behaviors, biochemical parameters and VPA-induced histopathological determinants in the brain. A green tea extract (75 and 300 mg/kg for 4 weeks) also shown improved motor coordination, nociception, locomotor and exploration, cognition, quantity of malondialdehyde (MDA), and undamaged purkinje cell layer.
Recently, Bambini and his coworkers suggested that taking prenatal VPA and resveratrol together may improved the poor social behaviour that is often observed in autism [12].

2.3 Valproic Acid

Numerous genetic changes linked to autism have been discovered through genetic investigations, however they only make up 25% of all instances of autism as of now. On the other hand,

environmental variables such exposure to xenobiotics like valproic acid (VPA) and thalidomide can either cause or contribute to the development of autism. According to a rodent prenatal VPA administration animal model, there is a link between human VPA exposure and ASD. Due to the model's extensive presentation of morphological and behavioral changes connected to the pathophysiology of autism, it has been shown to be a viable study tool over the years [18]. In animals, a single prenatal VPA injection leads to poor cognitive function, social interaction deficiencies [19], and stereotypical or repetitive behavior in the offspring [20].

Previous studies have also revealed nociceptive alterations. On gestational day (GD) 12.5 a single dose of VPA (600 mg/kg) results in aberrant pain sensitivity in maturity, similar to human autistic people [21]. Prenatal VPA administration on GD 12.5, resulted in neuroanatomical abnormalities in the progeny such as fewer neurons in the cranial nerve motor nuclei, cerebellar abnormalities [22], and less Purkinje cells in the cerebellar vermis [23]. After prenatal VPA administration to the rats, serotonin and dopamine levels have been shown to either reduce or rise in rats model [24, 25]. Genetic mutation, starvation, or exposure to teratogens during gestation can all cause neural tube abnormalities (NTD). In mice and rats, VPA induces crooked tail phenotypes, a moderate form of NTD, as well as aberrant behaviors resembling autism [26].

Thus, mice were treated to VPA either during pregnancy or after birth in the current research. According to George (2006), E13, the exposure time, corresponds to the mouse's Purkinje cell generation's final stages [27]. Previous research has shown that rodents treated with VPA on PND 14 caused intrusions, neurodevelopmental regressions, and cell degeneration in the cerebellum and hippocampus. These effects were manifested as numerous behavioral delays. Cerebellar dysfunction may be a significant factor in etiology of ASD, according to clinical data, neuroimaging, genetic diseases, and post-mortem studies. In both humans and animals, the cerebellum has a high rate of ASD diagnosis [28]. ASD animal models developed by VPA showed cerebellar atrophy, and abnormalities in the cerebellar cortex have been reported in 26 mice models of the ASD [29]. One of the families of serotonin receptors, G-protein-coupled receptor (GPCR) found on the surface of cell. In animals, these receptors are located in all areas of the brain, with the cortex, frontal cortex, limbic system, and cerebellum having the highest densities. As information about the brain's objectives is sent from higher levels, the cerebellum and cerebral cortex communicate continuously [30].

Induction of autism by VPA in mice during the developmental phases has indicated the onset of neurobehavioral problems similar to those reported in autistic individuals [31–36] in terms of cognitive impairments, anxiety, and chronic deficiencies in social behaviour.

On the 14th postnatal day for mice, which is thought to correspond to the third trimester of human development, neuron development , differentiation and migration are thought to take place in the cerebellum, striatum, and hippocampus.

The absence of gliosis after neuron loss in the structures described shows that the injury may have only happened extremely early in the development of the brain [36]. The exploratory behavior has been used to gauge rats' capacity for integrating spatial features and creating a spatial model of a novel environment [34].

Despite the fact that male animals have been the subject of numerous investigations on VPA-induced autism [38], few studies have used both male and female animals [31]. Men are 2 to 3 times more likely than women to be affected with ASD, according to earlier studies. The majority of male animals are used in Autism research because of its increased prevalence in men than in women, as the challenges that female animals face during the estrous cycle, particularly while conducting behavioral analyses. Mice with VPA-induced autism demonstrated aberrant postnatal behaviour in both the male and female pups [39].

2.4 Pharmacological Treatments for Autism Spectrum Disorder

2.4.1 Cannabinoid and ASD

2.4.1.1 Endocannabinoid System

Patients with ASD who have co-morbid conditions including stress and seizures have abnormalities in the endocannabinoid (EC) system [13, 40]. The EC system controls behavioral reaction to context and social interaction as well as emotional responses. Additionally, the EC system is crucial for brain development because it regulates the proliferation of neural progenitors, guides axonal migration, and maintains the inhibition/excitation ratio, among other things [41].

Cannabidiol (CBD) and 9-tetrahydrocannabinol (THC) are the two primary Phyto cannabinoids found in the cannabis plant. THC is a psychoactive cannabinoid that stimulates the type 1 cannabinoid receptor (CB1R) in the brain and can cause anxiety and psychosis. THC is a CB1R agonist, however CBD is an allosteric modulator of the CB1R and may lessen its effects. It has a

reasonably high threshold for toxicity and is not psychotropic. While consuming THC alone can increase the risk of addiction, cognitive deterioration, motivational loss, and psychosis, consuming CBD together with THC may lessen these dangers [42].

The euphoria that results from consuming THC is not experienced with CBD, which is recognised as a non-psychoactive phytocannabinoid. However, evidence suggests that CBD also has a number of other qualities, including anxiolytic [43, 44], anti-epileptic [45], and anti-inflammatory effects. An increased neuroinflammtion leads to ASD. In particular, the literature has previously discussed how astrocytes and microglia play a role in synaptic plasticity and neuroinflammation in ASD [46-47].

Medical cannabis oils are the most popular delivery method when using the drug to treat ASD patients. These oils are oral treatments made from cannabis plant extracts. Additionally, it is common practice to designate CBD-rich cannabis strains with low THC levels for the treatment of children and adolescents [48-51], there are still significant knowledge gaps on the mechanisms underlying long-term medical cannabis use in ASD patients.

2.4.1.2 Endocannabinoid System In Glutamatergic Synapses

Excitability toxicity, also known as "excitotoxicity," is caused by excessive stimulation of excitatory glutamatergic synapses in conjunction with a deficiency in glutamate absorption and has previously been linked to ASD. Comparatively to WT controls, InsG3680 Shank3 mutant mice exhibit abnormalities of glutamatergic signaling. Reducing glutamate signaling may be helpful in treating the symptoms of ASD because evidence suggests that excitotoxicity involves in the pathogenesis of this disorder.

In order to cause post-synaptic depolarization and Ca2+ influx, a neurotransmitter (such as glutamate) must be released from the pre-synaptic neuron and then bind to its Glu receptors in the postsynaptic neuron. The resulting rise in Ca2+ levels triggers the synthesis of endocannabinoids like anandamide (AEA) and 2-arachidonoylglycerol (2-AG). In the pre-synaptic neuron, these chemicals bind CB1 receptors (CB1R) after travelling retrogradely from the postsynaptic neuron. The CB1R is activated, which inhibits the release of further neurotransmitters. FAAH and MAGL, respectively, cause the rapid breakdown of AEA and 2-AG. Exogenous substances like the abundantly present phytocannabinoids THC and CBD in the cannabis plant can also activate CB1R. The majority of CBD's effects are therefore mediated by other mechanisms, such as the

inhibition of FAAH and a rise in AEA levels, since CBD has a very low affinity for the CB1R compared to THC. The proline-rich synapse-associated protein 2 (ProSAP2), also known as the protein SH3 and multiple ankyrin repeat domains 3 (SHANK3), is also depicted in this (Fig. 2). In the postsynaptic densities (PSDs), which link receptors to cytoskeletal signalling molecules, a scaffold protein by the name of SHANK3 links a number of ion channels, other scaffolding proteins, enzymes, and signalling molecules. Because it is prevalent in glutamatergic synapses, SHANK3 interacts with all well-known glutmate receptors, including NMDA, AMPA, and mGlu receptors. Another group that SHANK3 indirectly interacts with is the family of post-synaptic adhesion molecules known as Neuroligins (NLGN), which includes GKAP and Homer PSD95. Scientists are therefore interested in describing reliable genetic mouse models of ASD, particularly the several Shank3 mutant animal models. Spinogenesis, synapse development, and PSD structuring in glutamatergic synapses are all dependent on the protein SHANK3 [51].

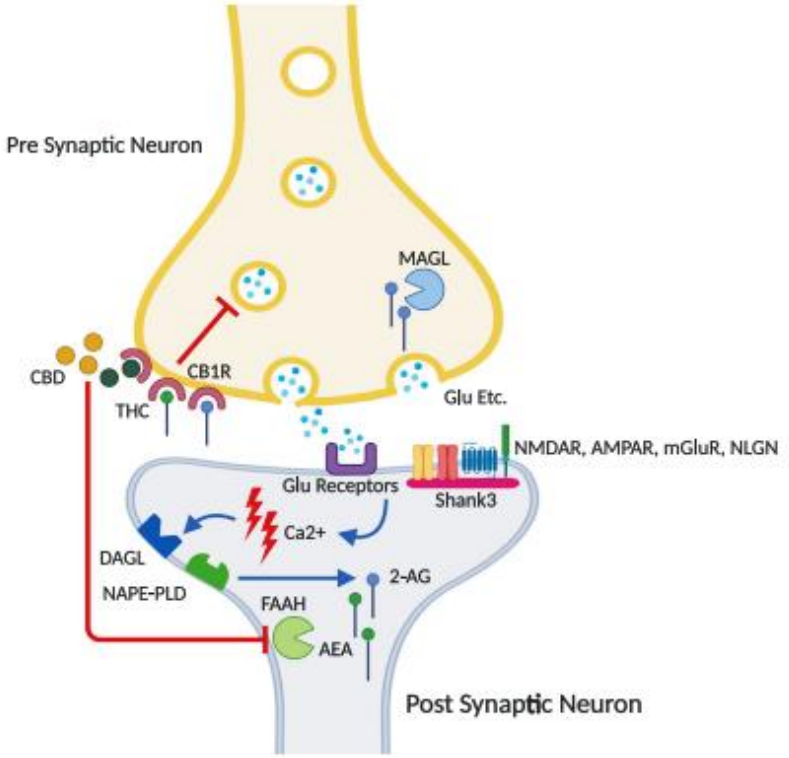


Fig. 2. Schematic Diagram of EC System in Glutamatergic Synapses [51]

2.4.2 Clinical Trials of CBD

Only a few research have examined the impact of repeated CBD exposure on healthy mice's developing brains. According to one study, giving C57BL/6J mice daily (3mg/kg and 20mg/kg))

CBD administration had no negative effects on behavioral tests. Given that mice have a shorter half-life of CBD than humans do and that children exposed to it for therapeutic purposes would experience consistently elevated levels throughout the day, it is critical that mouse studies of developmental CBD exposure more closely resemble the higher levels of CBD humans are achieving [52]. Putative CBD receptors are expressed in comparable quantities in the brains of Cln1/ mice and healthy animals, according to a proteomic investigation. For six months, Cln1/ mice were given an oral dose of CBD (100 mg/kg/day), and changes in seizure frequency and pathological illness indicators were monitored [53].

Recent research has demonstrated that the cannabinoid Cannabidivarin (CBDV), which is derived from plants, has positive effects on the neurological, motor abnormalities Rett syndrome animal model. The ability of CBDV to treat cognitive impairment, neurological and motor defects, and motor defects simultaneously in these animal models suggests that this Phyto cannabinoid may have intriguing but undiscovered therapeutic potential in ASD, prompting its investigation in animal models of this disorder. Here, we looked at how CBDV medication affected male rats that had been prenatally exposed to valproic acid and developing ASD-like behaviors. Both symptomatic and preventative CBDV protocols were used on the offspring [54].

2.5 Risperidone and ASD

Clinical trials have demonstrated that the atypical antipsychotic medications risperidone and aripiprazole, in addition to methylphenidate and atomoxetine, are useful in treating the signs and features of ASD. Previous studies on the effects of risperidone used ASD rodent models such Cntnap2 deletion mice, NMDA receptor NR1 subunit knockdown mice, and BTBR mice. However, in these mouse models of ASD, the medication does not address social problems. Therefore, there is little proof that antipsychotic medications reduce aberrant behaviors in mouse models of ASD. Risperidone (0.2 mg/kg), were given as part of chronic therapy, and behavioral assessment and a spine morphological study were conducted after 24 hours following the last dose [55]. Risperidone was diluted in saline (0.9% NaCl) containing 1% acetic acid (0.125, 0.250, and 0.500 mg/kg, Sigma Aldrich).30 minutes before to the commencement of the behavioral test sessions for the self-grooming, social approach, and open field behavioral tasks, male and female adult B6 and BTBR mice weighing 25–40 g underwent intraperitoneal (i.p.) injections of MPEP, risperidone, or saline vehicle [56].

CHAPTER 3

3. MATERIALS AND METHODOLOGY

3.1 Valproic Acid Preparation and Cannabinoid Oil

Valproic acid salt was purchased from pharmaceutical industries**.** Sodium valproate was dissolved in distilled water for injection at a concentration of 180mg/ml and 80mg/ml and administered subcutaneously at a dose of 600mg/kg and 400mg/kg per body weight. Pure 10ml CBD oil was purchased from CBD Pakistan industry which contains 100mg/ml CBD given at a dose of 100mg/kg for 3 consecutive weeks (5-8). Risperidone was used as a standard drug and Risperidone oral solution (1mg/ml) was used and given at a dose of 0.5mg/kg.

3.2 Experimental Design

Adult BALB/c male (n=1) and female (n=5) were placed together in each group and allowed to mate overnight. The vaginal plug was examined at 8am the following morning. Those females having plugs were denoted as embryonic day at 0.5 (E0.5). Pregnant Females were divided into two groups on embryonic day 13 (E13). Control group (n=5) was administered only water for injection (100µl) subcutaneously. The experimental group (n=5) was divided into further two groups.

Protocol I: (For Pre-natal Exposure)

In one group Pregnant females (n=5) were administered a single subcutaneous (s.c) injection of VPA at a dose of 600mg/kg on gestational day 13 (GD13) as in (Fig. 3).

Protocol II: (For Post-natal Exposure)

In other group, Pups of females (n=5) were administered a single subcutaneous (s.c) injection of VPA at a dose of 400mg/kg on post-natal day (PND-14) as shown in (Fig. 4).

The offspring were weaned on PND-21 and separated based on gender. Only males Pups were used in this study. Pups from Both groups were further divided into four groups. Twenty pups were selected for the postnatal experiments; control saline (n = 5, male = 5), VPA (n = 5, male = 5) , VPA + CBD (n = 5, male = 5) and VPA + RISP (n = 5, male = 5). Control group, diseased group, treated group was given with Cannabinoid oil 100mg/kg orally on PND-36 (5 weeks old)

for consecutive 3 weeks, and standard group was given with a Risperidone 0.5mg/kg on PND-36 for 3 consecutive weeks.

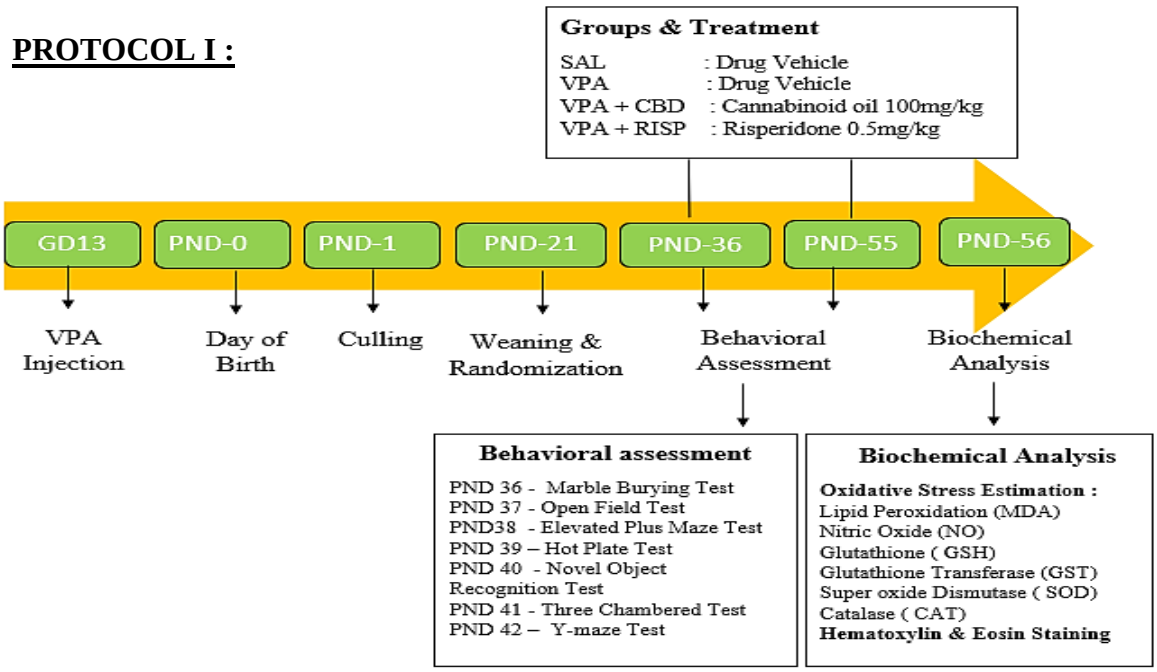


Fig. 3. A Comprehensive Study Plan illustrating VPA induced Autistic Mice Model (Protocol I)

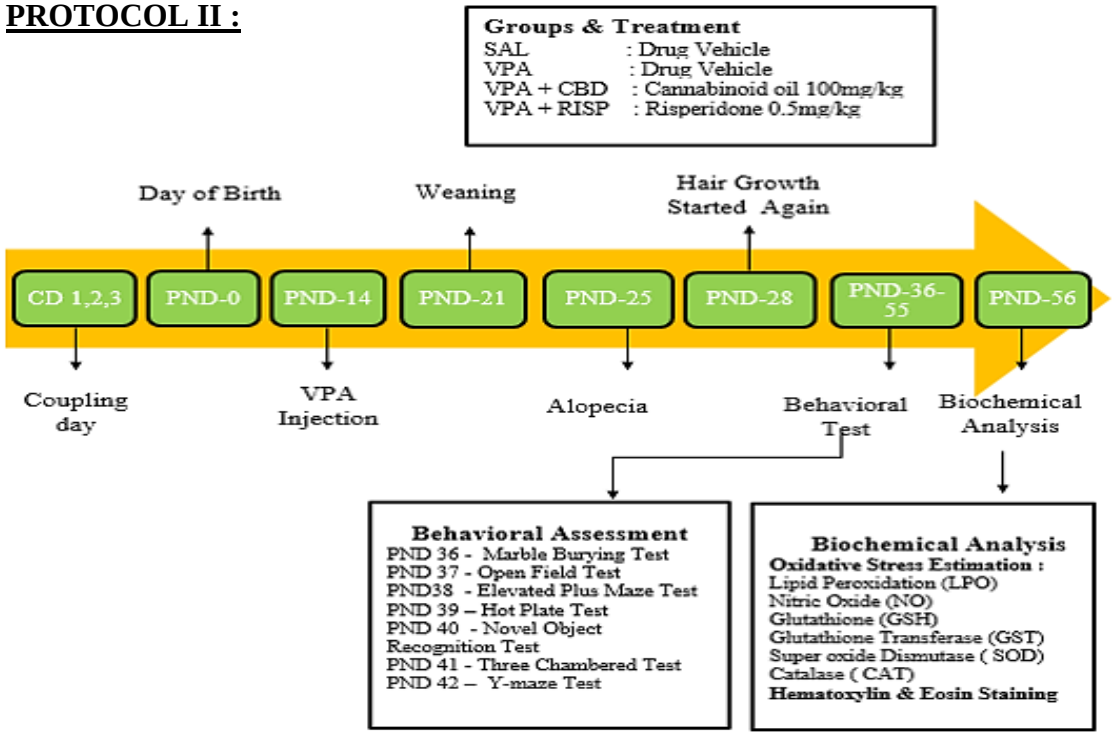


Fig. 4. A Comprehensive Study Plan illustrating VPA Induced Autistic Mice Model (Protocol II)

3.3 Animals

Adult male and female BALB/c mice (5-6 weeks old; weight: 25-30g) were purchased from National institute of Health (NIH) and 5 mice were group housed together in plastic cages with a 12 hr light/dark cycle, regulated humidity of 55%, and controlled temperature of 25°C. Fresh water and food pellets were freely available to mice. Prior to mating, mice underwent a one-week phase of acclimatization. The Pakistani Institutional Animal Ethics Committee (IRB No. 04-2021-02/36) examined and approved the experimental protocol. The treatment of the animals complied with the international standards governing their use and care (United States National Institute for Health Publication, 1985).

3.4 Behavioral Assessment

In behavioral analyses, animals were subjected to different behavioral tests at the age of 6-8 weeks. None of the mice were subjected to the same behavioral test again, so the behaviors at ages 6-8 weeks were evaluated using different animals.

3.4.1. Marble Burying Test

The defensive marble burying test was carried out as previously explained on PND-36 [57]. This test assesses anxious and repetitive activities, with a more number of marbles buried signifying higher degrees of anxiety. A 5-cm layer of bedding made of cedar wood chips was placed inside clean cages. On top of the bedding, fifteen glass marbles were distributed evenly in a 5×3 arrangement. Prior to testing, animals were given 30 minutes to get used to the testing environment. Each mouse was given 30 minutes to explore throughout the testing period as shown in (Fig. 5). After the animals had been in the area for 30 minutes, the number of marbles buried was counted and photographed.

Fig. 5. (A,B) Marble Burying Test

3.4.2. Open Field Test

The open field test apparatus was built using wood for open field testing. Animals are brought into the experimental space and given an hour to adjust before having their behavior studied. Periphery zone and center zone were established during the analysis, with the center zone having dimensions of 34.5 × 34.5 cm and animals are then each placed in the center of an open field box where a camera mounted above them was used to record their natural behavior for five minutes on PND-37 as in (Fig. 6). Following each experiment, animals are put back in their home cage. In between

experiments, the equipment is cleaned with 70% ethanol and allowed to dry. Using Pan Lab's Smart TM V 3.0 video tracking software, videos were manually analyzed [58,59].

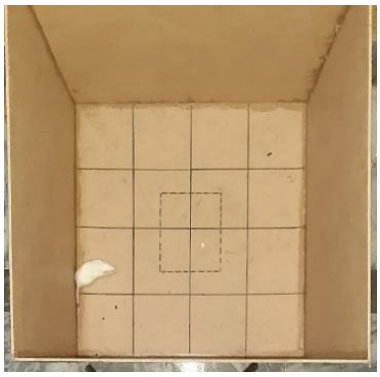


Fig. 6. Open Field Apparatus

3.4.3. Elevated Plus Maze Test

A behavioral test frequently used to look for symptoms of anxiety is the elevated plus maze (EPM). The device is made of plexiglass, and it has two open and two closed arms. The arms are each 50 cm long, 5 cm wide, and the closed arms are surrounded by a 15 cm wall according to (Fig. 7). To make the open arms more anxious, the equipment is hoisted one meter off the ground. In order to minimize visual cues, the device is divided from the rest of the room by matching white drapes. Additionally, the experiment is carried out in red light with predetermined light levels. An animal is introduced to the test by being positioned in the maze's "hub" and facing one of the open arms. It is then given five minutes to explore on PND-38. To eliminate olfactory cues from the preceding animal, the device is cleaned with 70% ethanol between subjects. A video camera immediately overhead is used to document the test. The test's scoring considered the total number of entries to open arm and the amount of time spent in each [59].

Fig. 7. Elevated Plus Maze Test

3.4.4. Hot Plate Test

An ASD phenotype is a reduction in sensitivity to unpleasant stimuli, and the animal model of experimental ASD does a good job of reproducing this phenotype. Paw withdrawal tests on hot plates with a clear glass enclosure and a temperature of 55 °C were used to assess the influence on nociception. Before starting test on PND-39, the animals were initially accustomed to the test area for 30 minutes. The maximum test period was limited to 22 seconds in order to minimize tissue injury as shown in (Fig. 8). We measured either the delay to lick the paw or the latency to remove the paw [60].

Fig. 8. Hot Plate Test

3.4.5. Novel Object Recognition Test

An open-field box made of Plexiglas measuring 43 x 32 cm was the experimental tool utilized for the object recognition test. It was set up in a room with low lighting. The experiment was run on PND-40, and the results were examined as previously mentioned [61]. Each test was carried out by an animal alone. Each animal spent 10 minutes in the arena (the familiarization phase) exploring two identical, previously unknown objects. Mice were brought back to the arena for the 10-min test phase (Training Phase) after a 3-min inter-trial break, where one of the two familiar objects was swapped out for a brand-new, never-before-seen object as shown in (Fig. 9). Between each animal, 70% ethanol was used to clean the arena. Two observers who were blind to the treatment

groups videotaped and separately recorded each participant's time spent examining the familiar (TF) and novel (TN) objects during the test phase. The following formula was used to determine the discrimination index (DI) and values are represented in the following table 1.

$$\text{Discrimination Index} = \frac{TN}{TF + TN} \times 100$$

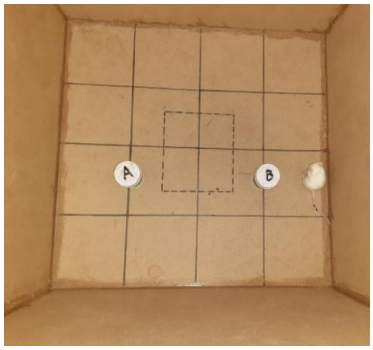


Fig. 9. Novel Object Recognition Test

Following table represents % of entries and % of time spent in the open arm during elevated plus maze test and during elevated plus maze (EPM) and time spent with familiar and novel object along with % discrimination index represents exploration memory during novel object recognition test.

Table 1: Shows the effect of Cannabinoid oil on Anxiety and Exploration Memory in EPM and Novel Object Recognition Test

Groups	Activity On Elevtaed Plus Maze Test		Novel Object Recognition Test				
			Familirization Phase		Training Phase		
	% Entries in open arm	% Time in open arm	Identical Object A	Identical Object B	Familiar Object A'	Novel Object N'	DI%
Control	66.23±4.76	53.13±2.73	56.4±7.29	70.6±4.32	47±5.78	70.2±7.80	63.08±0.11
VPA	48.09±4.69	36.05±2.89	45±7.39	42.16±4.76	111±4.56	37.5±6.54	25.69±0.11
VPA + CBD	52.56±4.55	47.16±2.71	66.8±9.80	65.8±9.76	48±5.87	62±7.54	62.08±0.12
VPA + RISP	50.52±4.60	44.33±2.97	39±8.0	36.2±6.97	36.5±6.54	43±8.65	51.87±0.16

3.4.6. Three-Chambered Test

The sociability test was performed on PND-41 as previously mentioned, [57]. This test is conducted in a rectangular box with three chambers—a smaller center chamber and ones on the left and right in (Fig. 10). Three ten-minute trials, completed one after the other, make up the test.
1. Habituation: The three chambers can be explored by animals for 10 minutes while the wire mesh cages in the left and right chambers are left vacant.
2. Sociability: Once more, animals are given ten minutes to explore an unknown mouse that is placed in one of the wire mesh cages and an object in the other.
3. Social novelty preference: for ten minutes of exploration, a new animal is introduced in its place while the previously known animal remains.
With an overhead camera, behavior was observed during each of the three stages, and the duration of interactions within each chamber was timed. The amount of time test animals spent in each side chamber was recorded. The following formula was used to determine the social preference index (SPI) and the sociability index (SI).

$$\text{Sociability index} = \frac{\text{Total Time in stranger Chamber}}{\text{Total Time in empty chamber}}$$

$$\text{Social Preference Index} = \frac{\text{Total Time in novel Chamber}}{\text{Total Time in familiar chamber}}$$

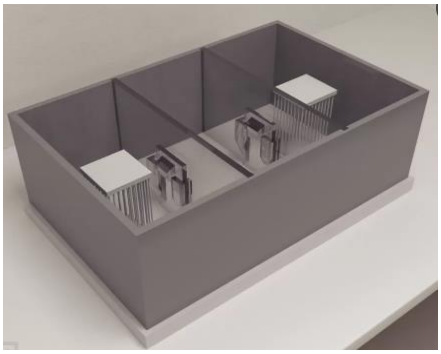


Fig. 10. Three Chambered Test

3.4.7. Y-Maze Test

The extent of % spontaneous alteration is regarded as a measure of stereotypical or repetitive behavior in animals. A y-maze apparatus was used to assess % spontaneous alterations. With three

equal-length arms that are each at a 120° angle from the other, the maze forms a Y shape as illustrated in (Fig. 11). The animals were positioned at the end of one of the arms, which was designated as the starting arm, with their heads looking in the direction of the maze's centre. The animals were put through a 5-minute test in the y-maze on PND-42. Three arms being explored in succession was regarded as one alternation [62]. For each animal's serial arm entries, % spontaneous alternations were calculated. The formula below can be used to compute the spontaneous alternation percentage.

$$\% \text{ Spontaneous Alternation} = \frac{\text{Total alternations}}{(\text{Total arm entries} - 2)} \times 100$$

Fig. 11. Y-Maze Test

The following table 2 shows the summary of behavioral analysis of mice along the brain regions and their related functions in the development of neurological disorders.

Table 2. Summary of Behavioral Analysis and Brain Functions and Associated Brain Regions

Behavioral Function	**Test**	**Brain Regions/ Systems Involved**	**Relevance to Human Psychopathology**
Repetitive Behavior	Marble Buryig	Cortical Basal Ganglia-Thalamic Pathway	Obsessive-compulsive disorder, ASD
Anxiety And Psychomotor Activity	Open Field	Nigrostriatal Dopamine System	Schizophrenia, bipolar disorder, depression
Anxiety / Fear	Elevated Plus Maze	Amygdala	Generalized anxiety disorder
Thermal Nociception	Hot Plate	Sensory Pathways of CNS	Diabetes, Autism spectrum disorder
Exploration And Memory	Novel Object Recognition	Hippoampus	Alzhiemer and other neurodegeberative disorders
Social Behavior	Three Chambered	PFC , Amygdala, VTA, Hypothalamus	Schizophrenia and Autism spectrum disorder
Spatial Working Memory	Y-Maze	PFC, Hippocampus, Septum, Basal Forebrain	Schizophrenia and various cognitive impairments such as Alzhiemer's disease

3.5 Tissue Preparation For Biochemistry

3.5.1 Harvesting of Brain Tissue

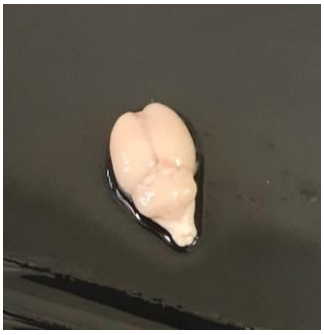


Fig. 12. Mice Whole Brain

3.5.2 Hematoxylin and Eosin Staining for Immunohistochemistry

Animals were killed by using Ketamine (0.1ml) after evaluation of the last behavioral analysis. For morphological analysis perfusion was done through the heart with ice cold 0.9% NaCl saline to remove blood from brain tissue followed by Cold 4% Paraformaldehyde (PFA) in 0.1M Phosphate Buffer Saline (PBS) (pH=7.4) The Whole brain was isolated and immediately kept in a 4% PFA solution for 24hr at 4℃ . The brain hemispheres were separated and cerebellum, hippocampus, and prefrontal cortex were isolated and incubated in 30% sucrose and then sequential 20µm Sagittal sections were obtained using a cryostat stored at 4℃. The slides were prepared by using Hematoxylin and Eosin staining for further analysis. Harris hematoxylin was used to stain the sections, and eosin diluted in 95% alcohol was used as a counterstain. The parts were mounted in paraffin wax after dehydration and cleaning. Using a photomicroscope, preparations for the section were photographed [63].

3.5.3 Preparation of Brain Tissue Homogenate

For other biochemical analysis, mice brain was isolated and quickly frozen in liquid nitrogen and stored at -80℃ for further assessment. The Frozen brain samples were thawed and hemispheres were isolated. Following brain tissues (200mg/ml) were homogenized in phosphate buffer (pH 7.4) with the use of a Polytron Homogenizer for 2 mints. The brain Homogenates were centrifuged at 12000rpm for 10 minutes and a clear supernatant was obtained as seen in (Fig. 13). For biochemical evaluation, supernatant of each homogenate was employed for biochemical analysis.

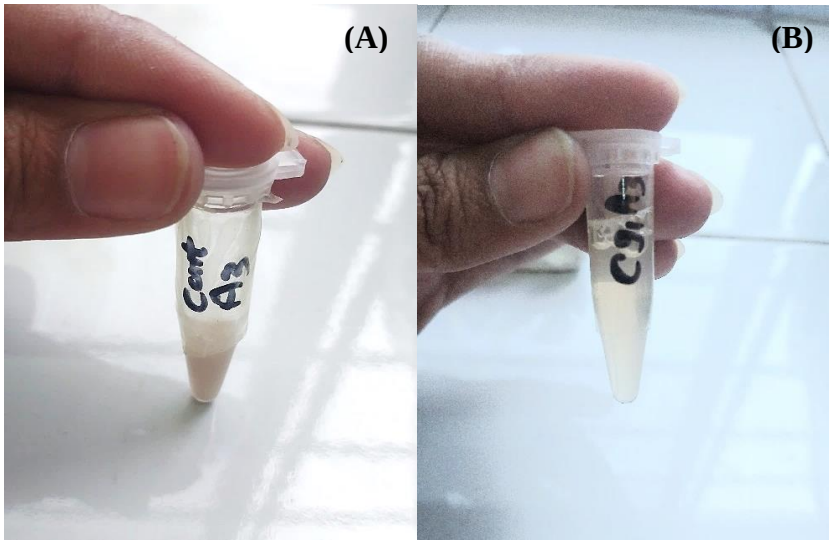


Fig. 13. Figure (A) shown Brain Tissue Hmogenate and (B) shows clear supernatant.

3.6 Biochemical Assessment

3.6.1 Determination of Antioxidant Levels

The indicators for oxidative stress are glutathione sulfo-transferase (GST), reduced glutathione (GSH), superoxide dismustase (SOD), lipid peroxidation (LPO), nitric oxide (NO) and catalase (CAT) activity [64]. To evaluate the oxidative stress in whole brain tissues, the levels of antioxidant enzymes were evaluated.

Ellman's technique was used to assess the reduced glutathione (GSH) activity, and absorbance at 450nm was determined. Results were represented as a µM [65]. When the absorbance at 340 nm was measured using the methods previously described and the absorbance was reported as a µM [66]. The SOD activity was also evaluated based on prior methodologies [67]. Results were given in percentages after the absorbance at 450 nm was determined. Additionally, catalase activity was estimated as a µM using the absorbance at 490 nm method, which estimates the rate of H_2O_2 decomposition following the addition of supernatant or sample [68].

3.6.2 Estimation of Lipid peroxidation

By quantifying the amount of malondialdehyde (MDA) in each sample, which gave an estimate of the level of lipid peroxidation (LPO), the amount of LPO was determined. The enzyme activity was reported as a µM and its absorbance was determined at 490 nm [69].

3.6.2 Determination of Nitric Oxide Production

The nitrite content of the prefrontal cortex, hippocampus, entorhinal cortex, and plasma was assessed using the Griess reagent. Griess reagent and supernatant were employed in equal amounts, and they were incubated for 15 minutes at 25 °C. Results of the 450 nm absorbance measurement were represented as a μM [70].

3.7 Statistical Analysis

To examine the impact of cannabis oil on the dependent variables in the control saline, VPA, VPA-CBD, and VPA-RISP groups, we used non-parametric t-test and one-way ANOVA. The Newman-Keuls post hoc multiple comparisons test was performed to compare between groups. To assess the effects of therapy on different dependent variables in the social interaction test, a two-way ANOVA was performed. All statistical parameters were calculated using GraphPad Prism programme. The findings were presented as Mean ± Standard deviation (S.D). Statistical significance was defined as a significance value with $p<0.05$.

CHAPTER 4

4. RESULTS

4.1 Effects of VPA Administration on GD 13 & PND 14

4.1.1 Delay in Eye-Opening

Our aim is to characterize the effects of pre-natal VPA exposure in the early development of mice model and observe that eye-opening was delayed from PND 14-16 in VPA exposed pups as compared to control pups in which eye-opening was seen in PND 11 (figure 14).

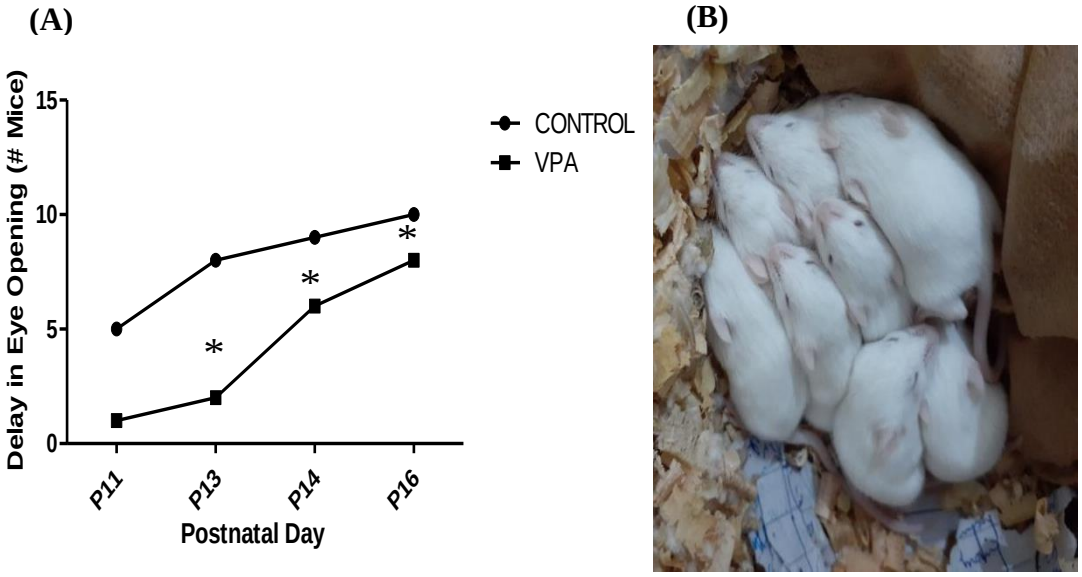


Fig. 14. Figure (A,B) shows delay in eye opening in VPA-exposed Mice.Values are represented as mean ± SD. For the comparison of the two groups, Two-way ANOVA test was performed. ($p < 0.05$); p=0.0219*.

4.1.2 Crooked Tail Phenotype

After weaning on PND-21, All controls animal pups showed normal tail but in comparison with the control , VPA-exposed Pups showed a crooked tail phenotypes from PND-25-56. This shows that VPA exposure during gestation results in neural tube defect (crooked tail) in pups after post-natal development (Fig 15).

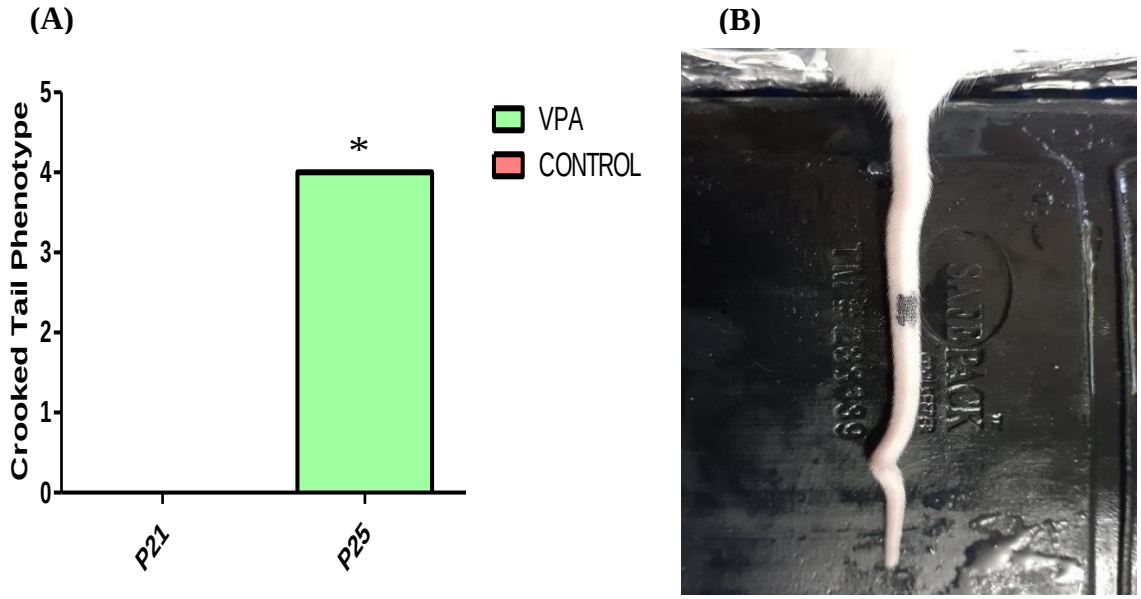


Fig. 15. (A,B) Crooked Tail phenotype in diseased (VPA) group as compared to control mice. Values are expressed as mean ± SD. For the comparison of the two groups, a non-parametric t-test was performed. ($p < 0.05$) ; $p = 0.016$*.

4.1.3 Alopecia

After PND-24, hair loss was started and complete alopecia symptoms were observed on PND-26 by post-natal VPA exposed male and female mice only. While again hair starts growing on PND-27 and complete hair growth was observed on PND-28 (Fig. 16).

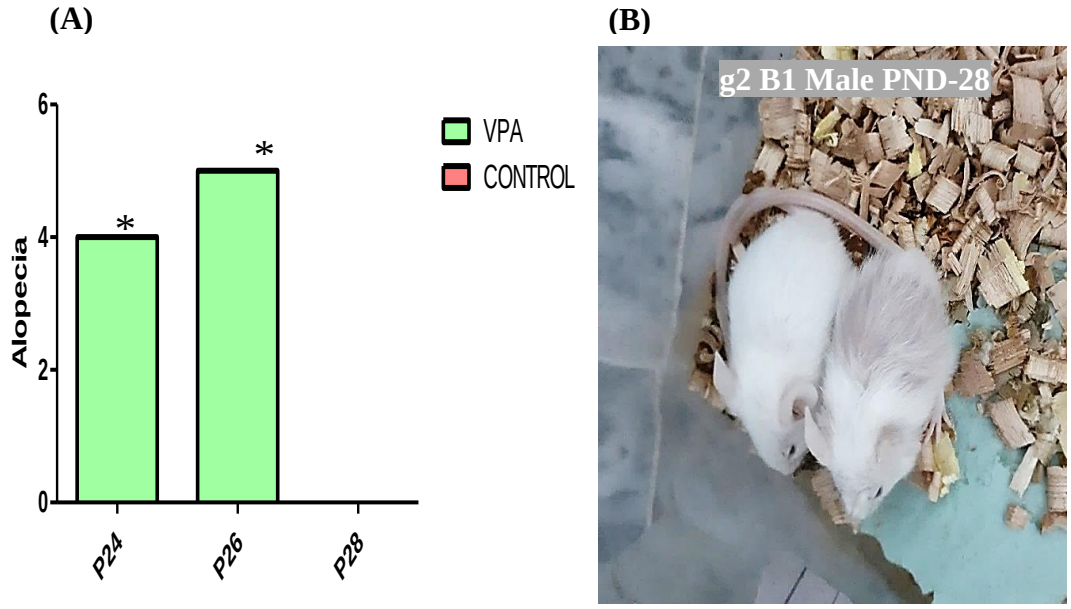


Fig. 16. Figure (A,B) represents Alopecia in VPA-exposed mice as compared to control mice. Values are expressed as mean ± SD. For the comparison of the two groups, Two-way ANOVA test was performed. ($p < 0.05$) ; $p = 0.0110$*.

4.2 Neurobehavioral Assessment

4.2.1 Cannabinid Oil Prevent Repetitive Behavior

During the defensive marble burying test, all control animals showed a decrease in repetitive behavior and less marbles were buried. In comparison with the control animals, VPA-exposed animals showed a significant increase in repetitive behavior and more marbles were buried. While CBD-treated animals revealed a considerable decline in marble burying compared to VPA-exposed mice on PND-36 (Fig. 17).

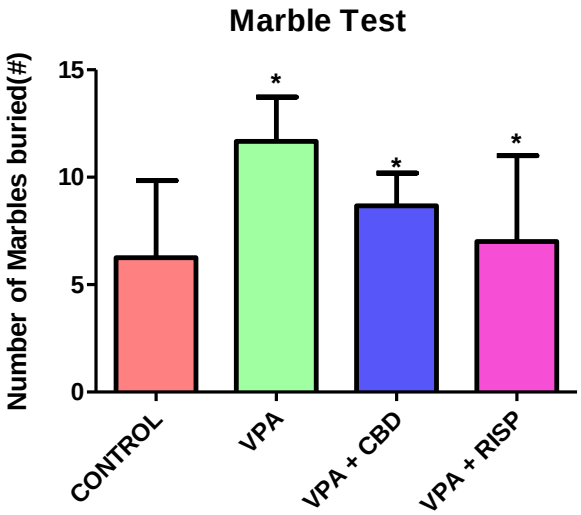


Fig. 17. Effect of cannabinoid oil treatment in marble burying test. Mice were grouped as control saline, VPA, VPA-CBD and VPA-RISP. Values are expressed as mean ± SD. To compare both groups, the Newman-Keuls (post hoc) test was performed. ($p < 0.05$) ; $p = 0.0491$*.

4.2.2 Cannabinoid Oil Decreased Anxiety and Locomotor Activity

Prenatal VPA-exposed mice have shown an increased level of mean speed, and total distance travelled as compared to their corresponding control group indicating that the anxiety has developed. Separate one-way ANOVA was conducted to compare the effect of treatment on time

spent in peripheral regions and center, mean speed, total distance travelled in the open field test in four groups. Treatment had a substantial impact on time spent in the centre (Fig. 18A), peripheral regions (Fig. 18B), total distance traveled (Fig. 18C), and mean speed (Fig. 18D), for four groups (control saline, VPA, VPA-CBD, VPA-RISP).

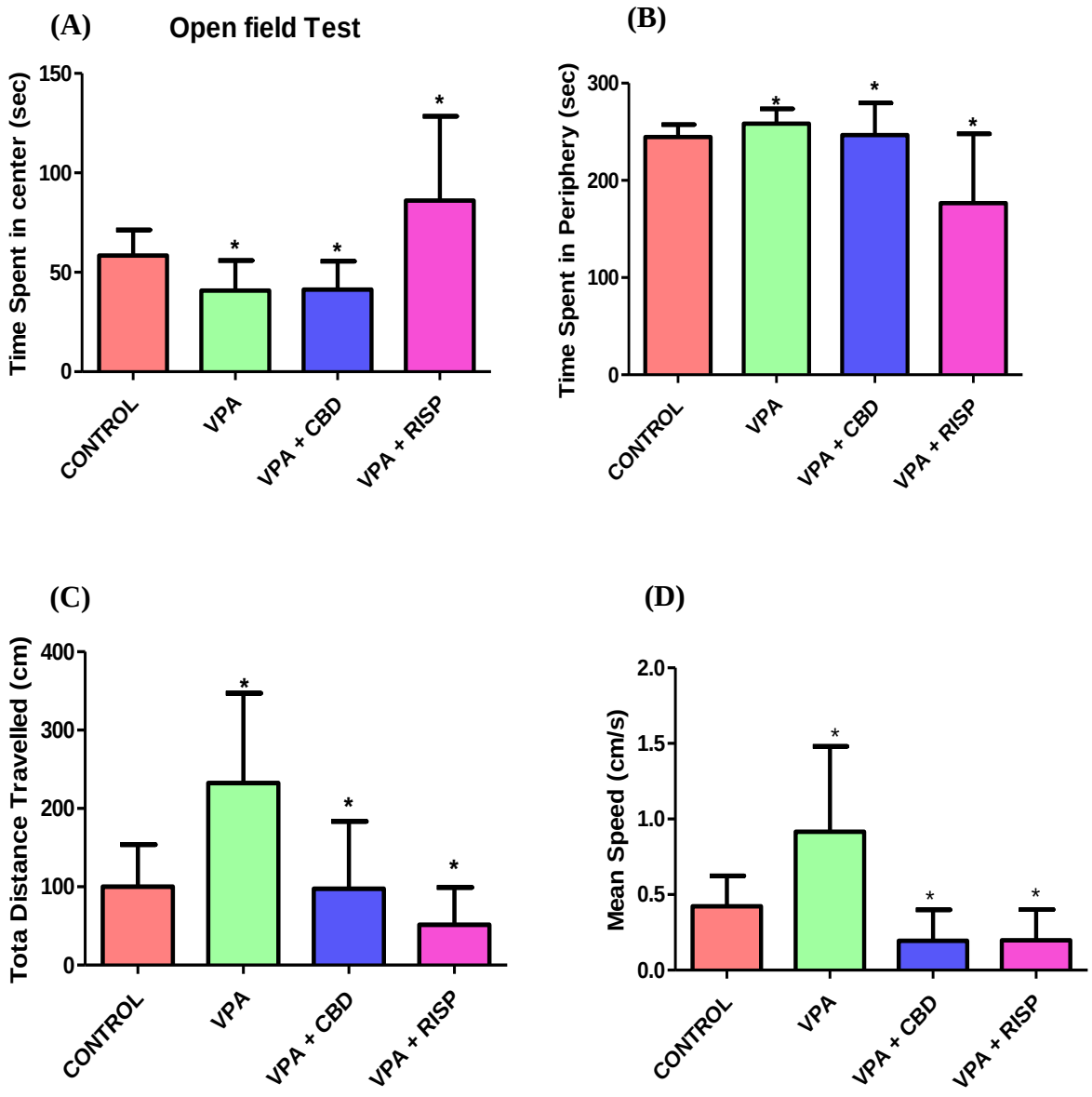


Fig. 18. Effect of cannabinoid oil treatment in open field test; (18A) time spent in periphery, (18B) time spent in center, (18C) total distance travelled and (18D) mean speed. Mice were grouped as control saline, VPA, VPA-CBD and VPA-RISP. Values are expressed as mean ± SD. The

Newman-Keuls (post hoc) test was performed to compare both groups. ($p < 0.05$) ; p = 0.0336*, p = 0.04*.

4.2.3 Cannabinoid Oil Reduces Stress Level

The percentage of time spent in open arm (Fig. 19A) and percentage of entries in open arm (Fig. 19B) were significantly decreased in VPA group, when compared with control group. Thus, reflecting anxiety like state in the animals in the VPA group when compared to the animals in the control group. In contrast, treatment with cannabinoid oil resulted in a significant increase in the % of time spent in open arm and % open arm entries as compared to the VPA-exposed group. As a result, the oral administration of cannabinoids to the VPA-exposed mice showed a reduction in anxiety.

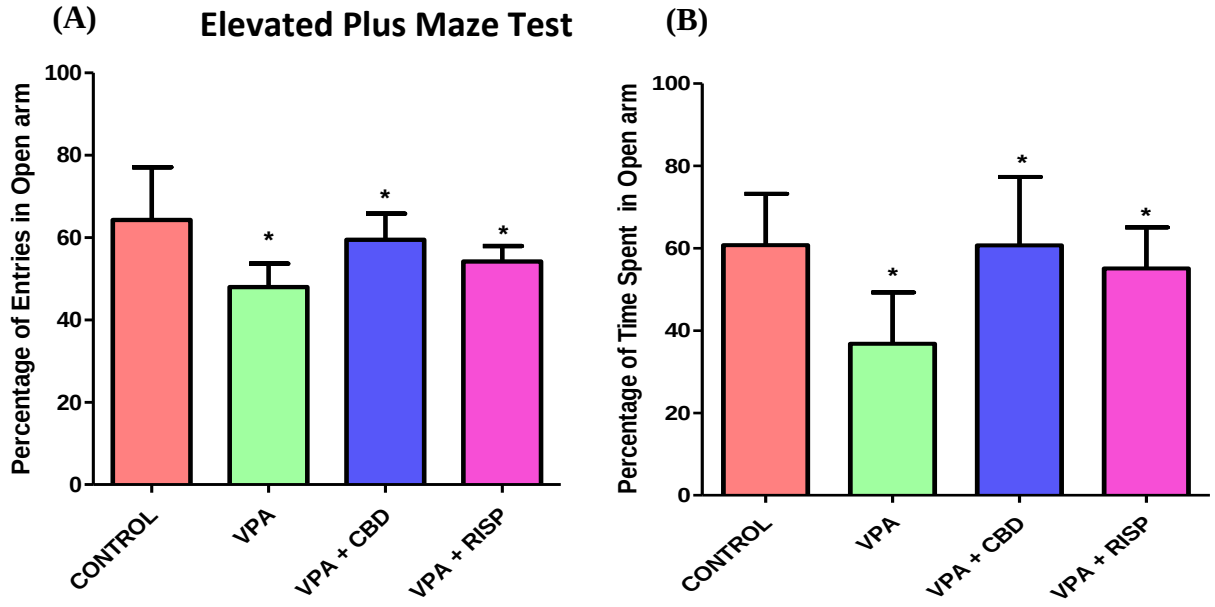


Fig. 19. Effect of cannabinoid oil treatment on the activity in elevated plus maze test ; (19A) % of entries in open arm, (19B) % of time spent in open arm. Mice were grouped as control saline, VPA, VPA-CBD and VPA-RISP. Values are expressed as mean ± SD. The Newman-Keuls (post hoc) test was performed to compare both groups. ($p<0.05$) ; p = 0.0444*, p = 0.0380*.

4.2.4 Cannabinoid Oil Improved Pain Sensitivity

Comparing the offspring of mice exposed to VPA during pregnancy to the control group, we discovered a statistically significant increase in the latency to withdraw the hind paw. On PND-39, treatment with CBD-treated animals resulted in a shorter delay to withdraw a foot than the group that had been subjected to VPA (Fig. 20).

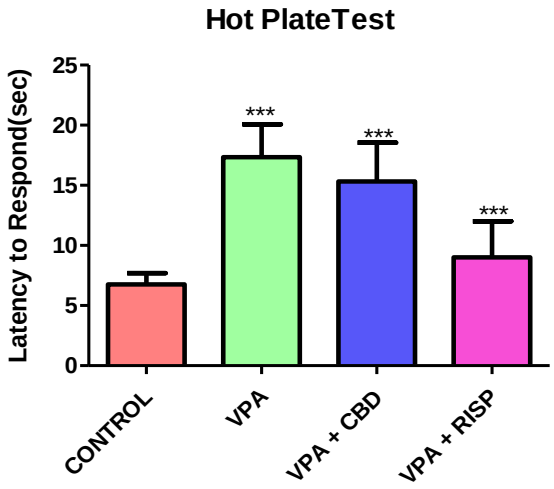


Fig. 20. Effect of cannabinoid oil treatment in hot-plate test. Mice were grouped as control saline, VPA, VPA-CBD and VPA-RISP. Values are expressed as mean ± SD. The Newman-Keuls (post hoc) test was performed to compare both groups. ($p < 0.05$) ; $p = 0.01$***.

4.2.5 Cannabinoid oil Enhances Exploration And Memory

Control animals gradually spend more time interacting with the novel object during the social preference phase than they do with the familiar chamber, which is an indication of typical social preference. Prenatal VPA exposure led to a large increase in time spent with the familiar object and a significant decrease in time spent with the novel item as well as a significant decline in the discrimination index (Fig. 21E). This suggests impairment in social preference in VPA-exposed animals when compared with control animals. Administration of cannabinoid oil and risperidone to VPA-group of mice significantly reduced time spent with familiar object and a significant increase in time spent with novel object (Fig. 21C and 21D). This suggests that cannabis oil is becoming more socially acceptable.

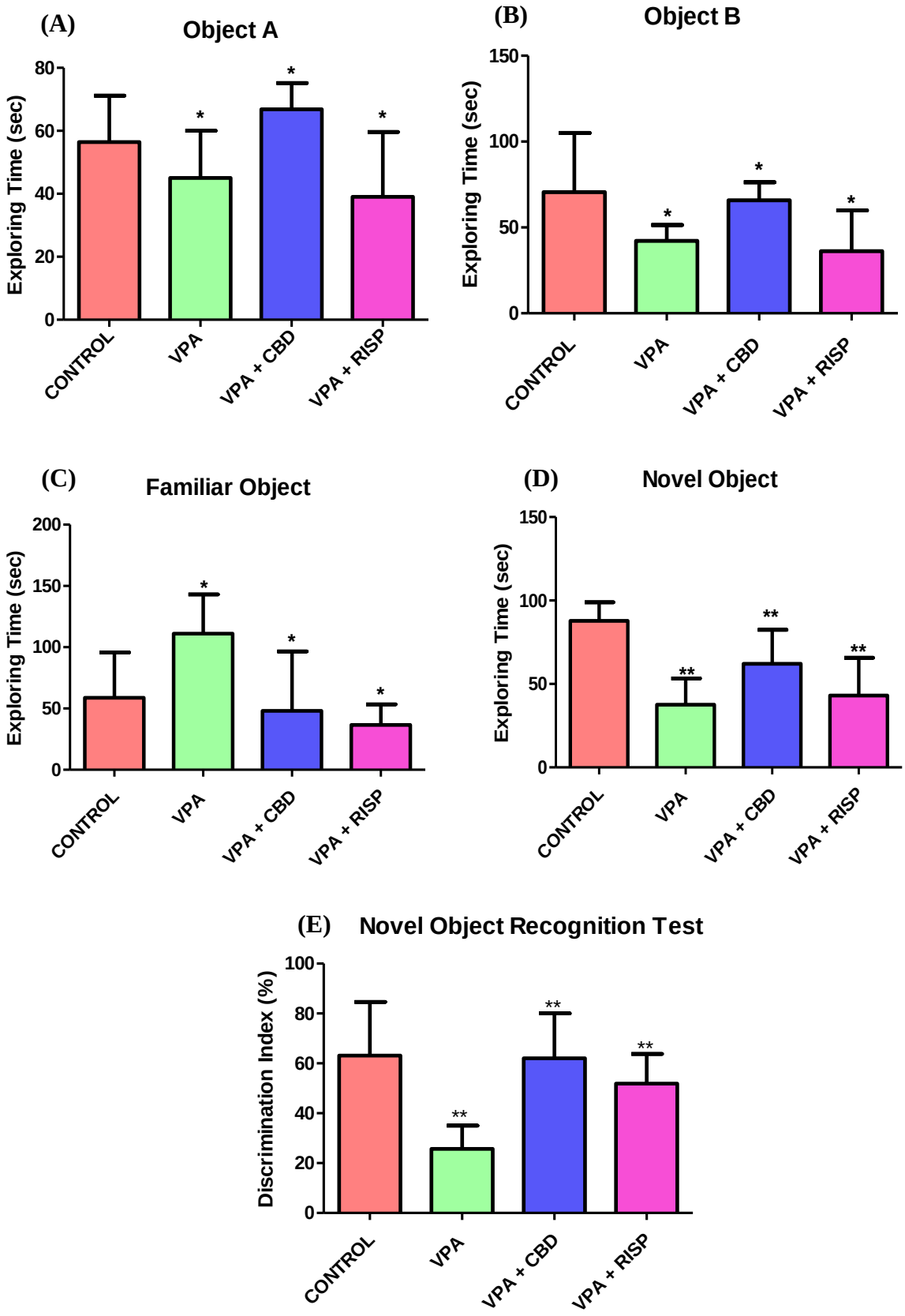
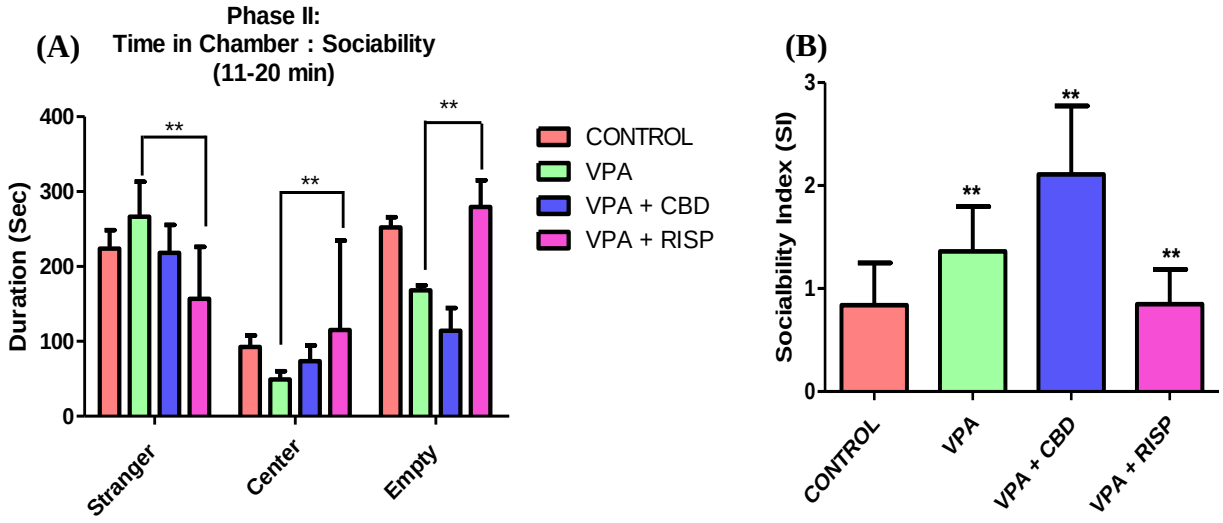

(A)
Object A
Exploring Time (sec)
80
60
40
20
0
CONTROL
VPA
VPA + CBD
VPA + RISP
(B)
Object B
Exploring Time (sec)
150
100
50
0
CONTROL
VPA
VPA + CBD
VPA + RISP
(C)
Familiar Object
Exploring Time (sec)
200
150
100
50
0
CONTROL
VPA
VPA + CBD
VPA + RISP
(D)
Novel Object
Exploring Time (sec)
150
100
50
0
CONTROL
VPA
VPA + CBD
VPA + RISP
(E)
Novel Object Recognition Test
Discrimination Index (%)
100
80
60
40
20
0
CONTROL
VPA
VPA + CBD
VPA + RISP

Fig. 21. Effect of cannabinoid oil treatment on exploration and memory in novel object recognition test, (21A) time spent with object A, (21B) time spent with Object B, (21C) Time with Familiar object, (21D) Time with Novel object and (21E) Discrimination Index. Mice were grouped as control saline, VPA, VPA-CBD and VPA-RISP. Values are expressed as mean ± SD. The Newman-Keuls (post hoc) test was performed to compare both groups. ($p < 0.05$) ; p= 0.0456*, 0.0492*, 0.0175*, 0.0039**, 0.0072**.

4.2.6. Cannabinoid Improved Social Behavior

A three-chamber social interaction test revealed that prenatal exposure to VPA mice spent significantly less time with unfamiliar mice than control mice. However, in mice exposed to VPA during pregnancy, therapy with cannabinoids greatly enhanced the quantity of social interactions and returned the mice to normal behaviour. The second portion of the social interaction test, which lasted between 11 and 20 minutes, was similarly affected by treatment for each group, according to the results of a two-way ANOVA (Fig. 22A). The four groups' sociability throughout the 11–20 minute period was significantly affected by treatment, according to one-way ANOVA (Fig. 22B). Further post hoc multiple comparisons revealed a significant difference in the social preference score and time spent in the stranger 2 chamber across groups (Fig. 22C), during the third stage of the social interaction test ((21-30 min), and social preference index in distinct groups (Fig. 22D).



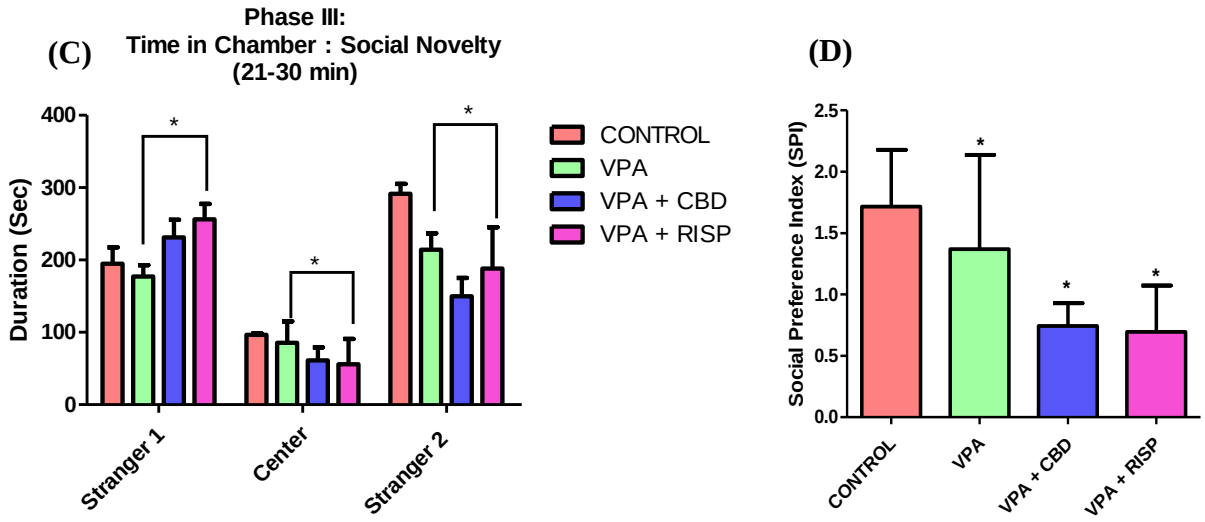


Fig. 22. Effect of cannabinoid oil treatment in social interaction test, sociability: time spent in three different chambers, sociability index , social novelty: social preference index. Mice were grouped as control saline, VPA, VPA-CBD and VPA-RISP. Values are expressed as mean ± SD. The Newman-Keuls (post hoc) test was performed to compare both groups. ($p < 0.05$) ; p = 0.03**,0.02** , p=0.035*, 0.045**.

4.2.7. Cannabinoid Oil Improved Spatial Memory and Reward Related Behavior

Comparing the animals in the VPA group to the control group, the spontaneous alteration percentage significantly decreased. In comparison to the VPA-treated mice in (Fig. 23), the oral administration of cannabinoids oil dramatically increased the percentage of spontaneous change in a dose-dependent manner. This indicated a reduction in repetitive behavior and spatial memory by cannabinoid treatment.

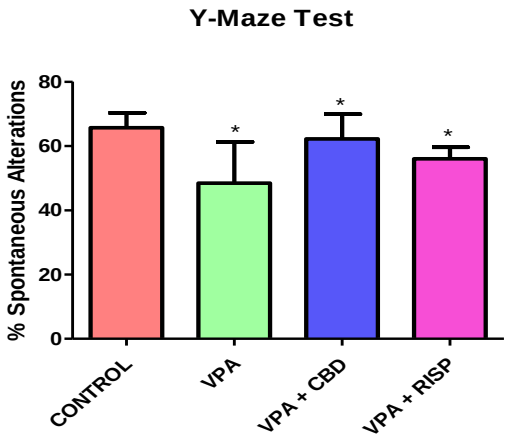
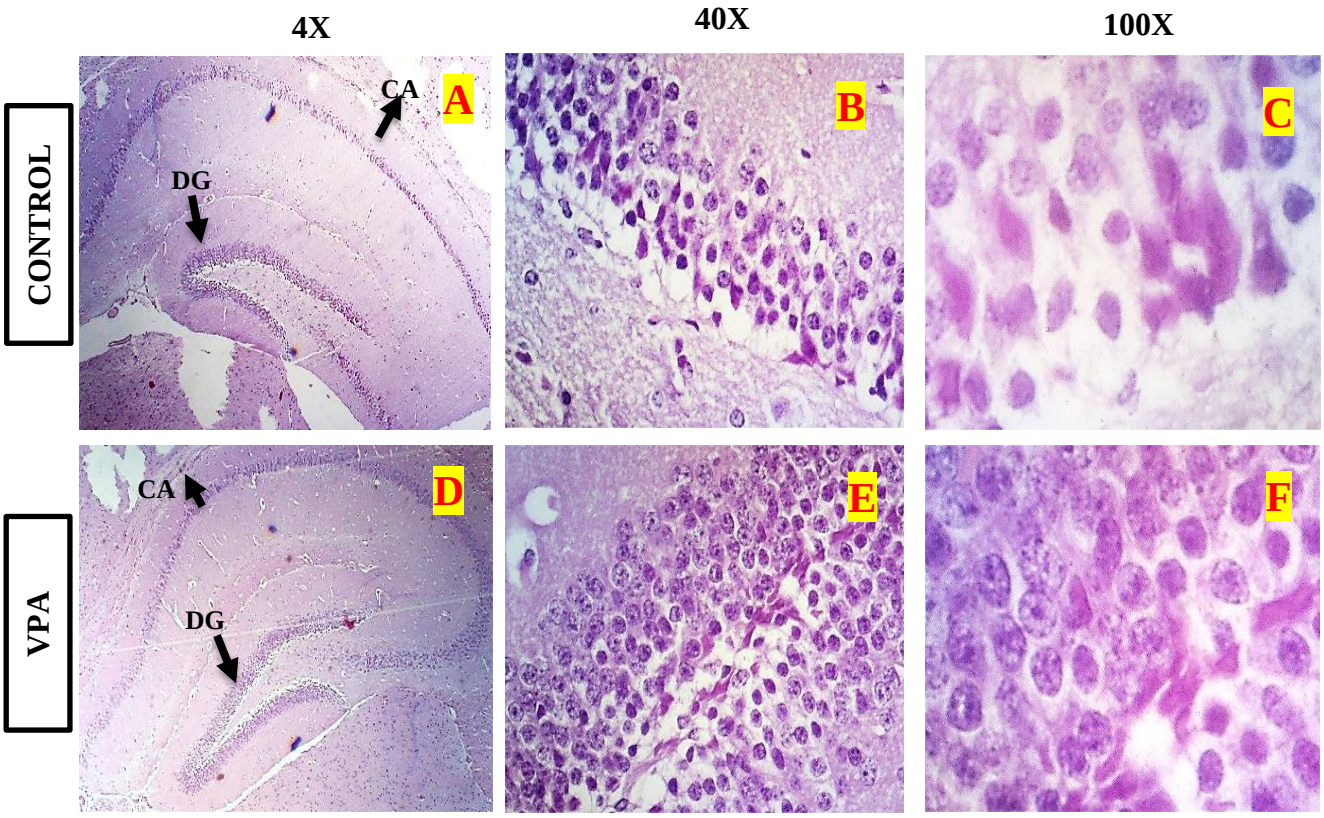


Fig. 23. Effect of cannabinoid oil treatment on % spontaneous alterations in y-maze test. Mice were grouped as control saline, VPA, VPA-CBD and VPA-RISP. Values are expressed as mean ± SD. The Newman-Keuls (post hoc) test was performed to compare both groups. (p = 0.05) ; p = 0.0402*.

4.3 Histopathological Studies

4.3.1. Cannabinoid Oil Improved Morphological Changes in Hippocampus

There was significant morphological changes were observed in the hippocampus of VPA-exposed male as compared to control animals. In male mice exposed to VPA, there was an increase in the number of atrophic hippocampus cells as compared to sex-matched controls. While the cannabinoid treated group showed a less significant neuroanatomical changes in the hippocampus. However, there were more atrophic cells seen in (Fig. 24) in VPA males as compared to control , cannabinoid treated group.



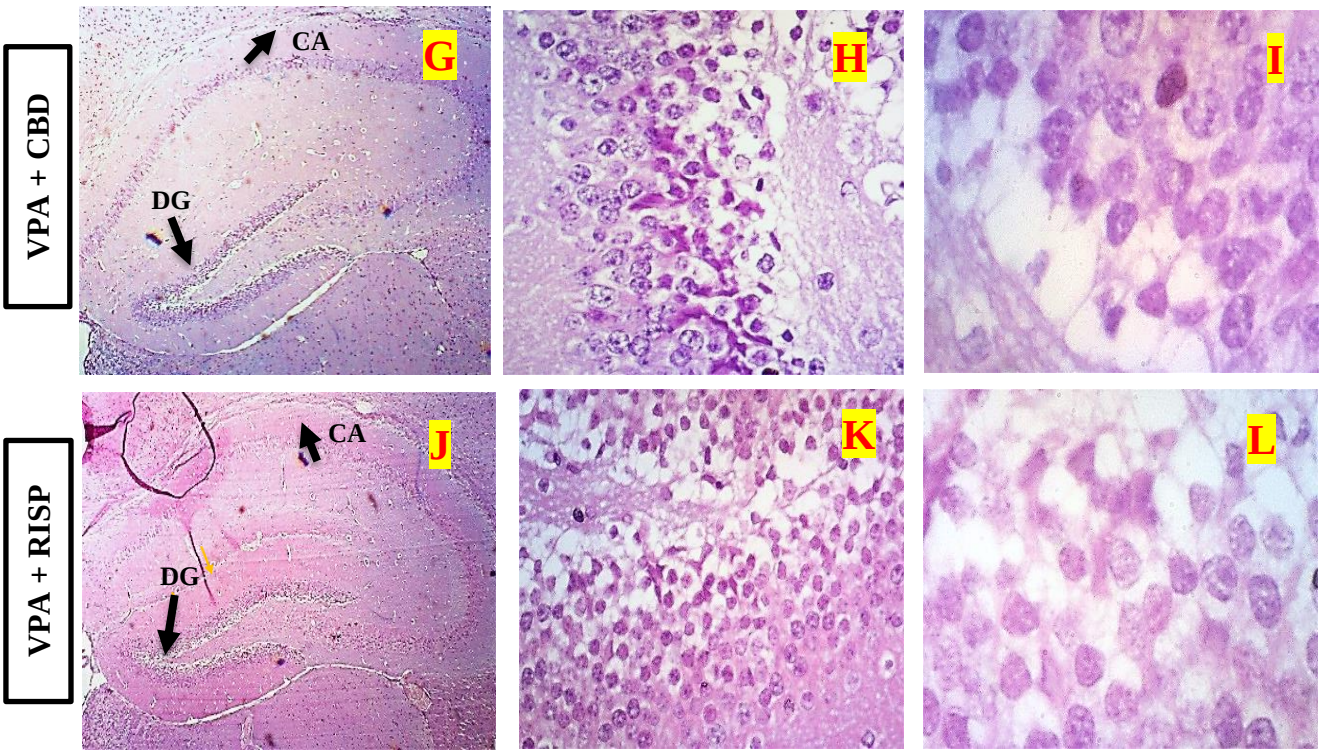


Fig. 24. Photomicrographs of hippocampus. (A-C) control groups showed normal hippocampal neurons, (D-F) VPA-exposed groups showed neuroanatomical changes in hippocampus and atrophic neurons, (G-I) VPA + CBD treated group showed less changes in the hippocampus and in neurons, (J-L) VPA + RISP treated group showed normal hippocampus similar to control, CA; Cornu Ammonis, DG; Dentate gyrus.

4.3.2. Cannabinoid Oil Involved Regeneration of Neurons in Prefrontal Cortex

Compared to sex-matched controls, male mice treated to VPA had a much higher rate of degenerating neurons. In the diseased group compared to the control, there were significantly more dark pyramidal cells. While the cannabinoid treated group showed a smaller number of degenerated neurons and less dark pyramidal cells in the prefrontal cortex as shown in (Fig. 25).

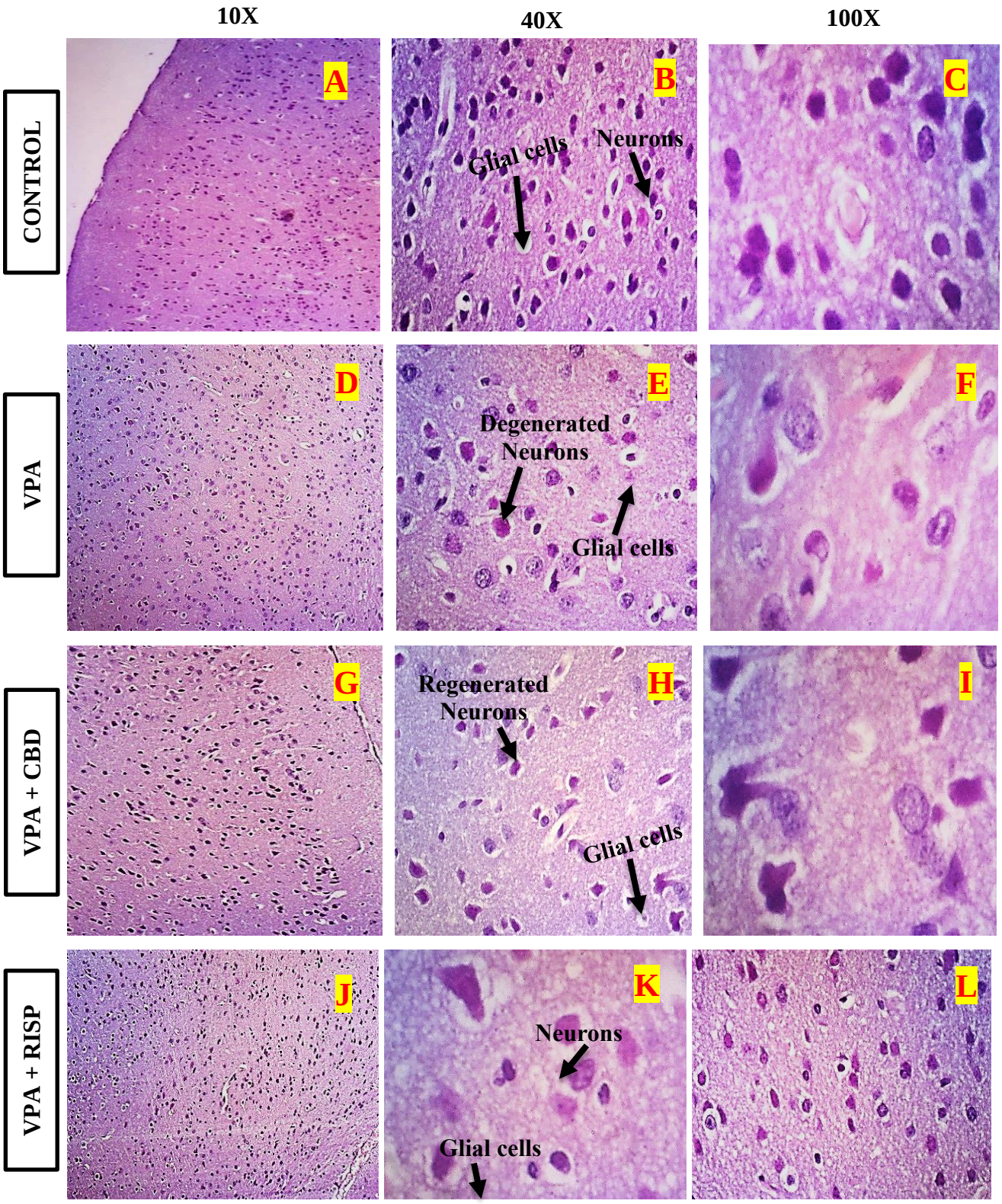
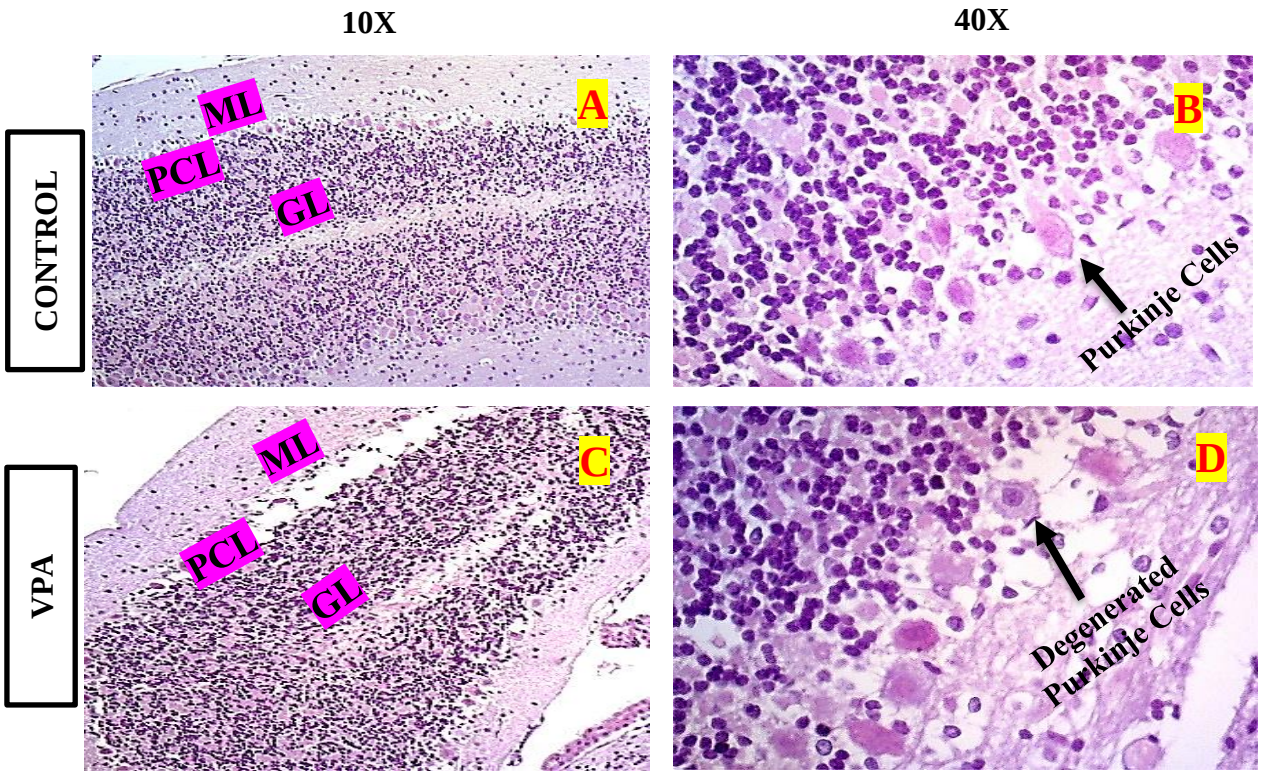

10X
40X
100X
CONTROL
VPA
VPA + CBD
VPA + RISP
A
B
C
D
E
F
G
H
I
J
K
L
Glial cells
Neurons
Degenerated Neurons
Glial cells
Regenerated Neurons
Glial cells
Neurons
Glial cells

Fig. 25. Photomicrographs of prefrontal cortex. (A-C) control groups showed normal hippocampal neurons, (D-F)) VPA-exposed groups showed decreased number of glial cells and degenerated neurons, (G-I) VPA + CBD treated group showed less degenerated neurons in the prefrontal cortex, (J-L) VPA + RISP treated group showed normal prefrontal cortex cells similar to control.

4.3.3. Cannabinoid Oil Inhibits Degeneration of Purkinje Cells in Cerebellum

When compared to sex-matched controls, male mice subjected to VPA showed a significant alteration in the Purkinje cell layers, such as ischemic degeneration of Purkinje cells. There was a smaller number of Purkinje cells in the diseased group as compared to control group. While the cannabinoid treated group showed an increased Purkinje cells count in the cerebellum (Fig. 26).



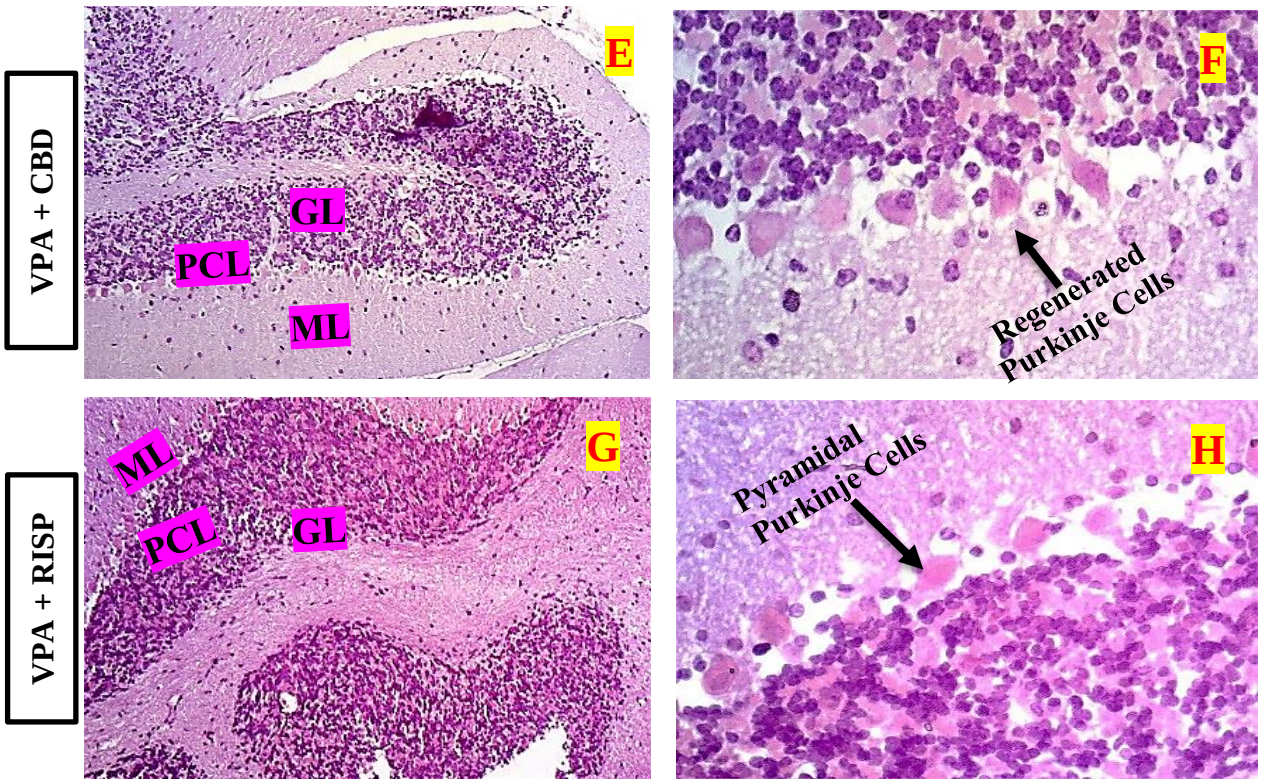


Fig. 26. Photomicrographs of Cerebellum. (A-B) control groups showed normal hippocampal neurons, (C-D) VPA-exposed groups showed atrophic or degenerated (dark) Purkinje cells in cerebellum, (E-F) VPA + CBD treated group showed regenerated pyramidal Purkinje cells in the cerebellum, (G-H) VPA + RISP treated group showed normal pyramidal Purkinje cells similar to control.

4.4. Biochemical Tests

4.4.1. Cannabinoid Oil Reduced Glutathione (GSH) Level

Mice exposed to prenatal VPA had levels of glutathione that were much higher than those in the control group. The level of glutathione is considerably reduced after receiving cannabis oil treatment in the mice brain in (Fig. 27) which shows imbalance of glutathione.

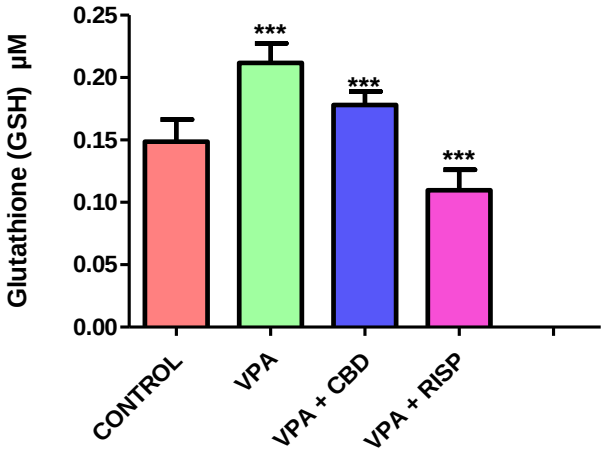


Fig. 27. Effect of cannabinoid oil treatment on glutathione (GSH). Mice were grouped as control saline, VPA, VPA-CBD and VPA-RISP. Values are expressed as mean ± SD. The Newman-Keuls (post hoc) test was performed to compare both groups. ($p < 0.05$) ; $p = 0.0002$***.

4.4.2. Cannabinoid Oil Increased Glutathione S-Transferase (GST) Level

Mice exposed with prenatal VPA didn't show a significant result in the glutathione (GST) levels in the brain. The diseased group showed a slight increase in glutathione s-transferase comparative to the control group. While the cannabinoid oil treatment showed an increase in GST level in (Fig. 28) which decreases oxidative stress in mice.

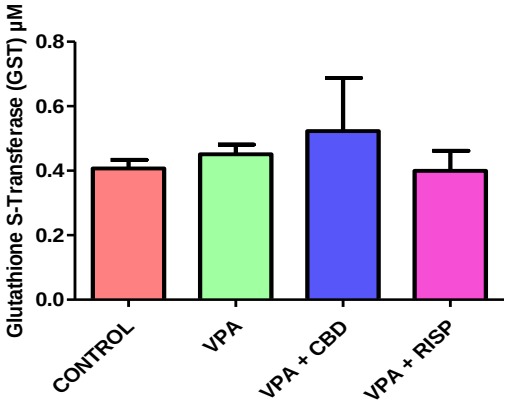


Fig. 28. Effect of cannabinoid oil treatment on glutathione s-transferase (GST). Mice were grouped as control saline, VPA, VPA-CBD and VPA-RISP. Values are expressed as mean ± SD. The Newman-Keuls (post hoc) test was performed to compare both groups. ($p < 0.05$) ; $p = 0.3793$.

4.4.3. Cannabinoid Oil Reduced Superoxide Dismutase (SOD) Level

Superoxide dismutase activity was noticeably higher in mice exposed to VPA than in the controlgroup. SOD levels in the brain were decreased after cannabis oil treatment (Fig. 29).

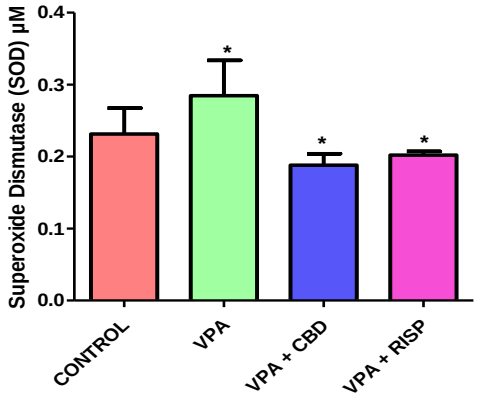


Fig. 29. Effect of cannabinoid oil treatment on superoxide dismutase (SOD). Mice were grouped as control saline, VPA, VPA-CBD and VPA-RISP. Values are expressed as mean ± SD. The Newman-Keuls (post hoc) test was performed to compare both groups. ($p < 0.05$) ; $p = 0.0236$*.

4.4.4 Cannabinoid Oil Slighty Increased Catalase (CAT) Level

The catalase activity in the brains of the mice exposed to VPA did not differ significantly from the control group. While the treatment with cannabinoid oil showed a slight increase in the catalase level in the mice brain (Fig. 30).

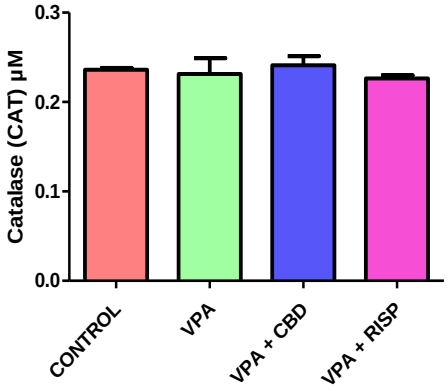


Fig. 30. Effect of cannabinoid oil treatment on catalase (CAT). Mice were grouped as control saline, VPA, VPA-CBD and VPA-RISP. Values are expressed as mean ± SD. The Newman-Keuls (post hoc) test was performed to compare both groups. ($p < 0.05$) ; $p = 0.4136$.

4.4.5 Cannabinoid Oil Decreased Lipid Peroxidation (LPO) Level

Mice exposed to prenatal VPA had higher levels of lipid peroxide than the control group. The amount of lipid peroxidation in the brain was significantly lowered after receiving cannabis oil treatment in (Fig. 31). This indicates reduction in oxidative stress in the mice brain.

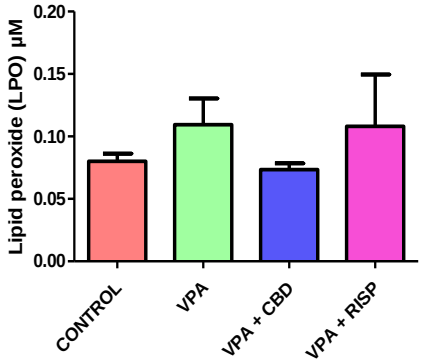


Fig. 31. Effect of cannabinoid oil treatment on lipid peroxide (LPO). Mice were grouped as control saline, VPA, VPA-CBD and VPA-RISP. Values are expressed as mean ± SD. The Newman-Keuls (post hoc) test was performed to compare both groups. ($p<0.05$) ; $p = 0.2123$.

4.4.6 Cannabinoid Oil Reduced Nitric Oxide (NO) Level

Nitric oxide levels were much lower in the animals exposed to prenatal VPA than in the control group. Compared to the mouse brains exposed to VPA, cannabis oil treatment considerably lowered the level of nitric oxide in (Fig. 32) and reduces the stress level.

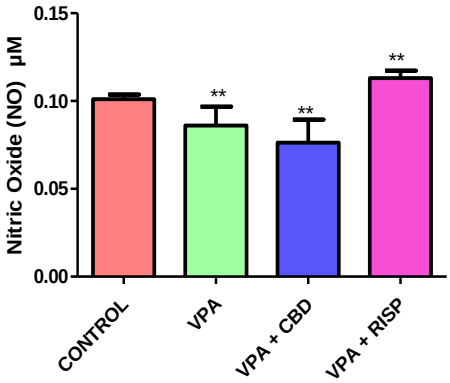


Fig. 32. Effect of cannabinoid oil treatment on nitric oxide (NO). Mic were grouped as control saline, VPA, VPA-CBD and VPA-RISP. Values are expressed as mean ± SD. The Newman-Keuls (post hoc) test was performed to compare both groups. ($p<0.05$) ; $p = 0.0044$**.

CHAPTER 5

5. DISCUSSION

Creating a mouse model of autism was the goal of the current investigation. Valproic acid was given to GD 13 and PND 14. Prenatal exposure led to (i) increased repetitive behaviour; (ii) increased locomotor activity, including greater levels of anxiety and decreased exploratory activity; (iii) deficits in pain perception (lower sensitivity to paw withdrawal); (iv) reduced sociability and social novelty; and (v) impairment in social preference; (vi) higher levels of oxidative stress. Cannabinoid oil treatment, on the other hand, improved the offspring's behavioral problem and decreased oxidative stress.

In current study, we observed a crooked tail phenotype in the male mice exposed to VPA on GD 13. This neural tube defect was also examined in mice and rats after post-natal development. In mice and rats, VPA induces crooked tail phenotypes, a moderate form of NTD, as well as aberrant behavior's resembling autism [9,10, 21]. However, Treatment with cannabinoid oil didn't reversed the neural tube defect.

One of the more noticeable signs of autism is increased repetition of behaviours [1]. The findings of the current investigation demonstrated that the offspring of VPA exposure exhibited higher repetitive behaviour (more protective marble burying). Anxiety and repeated behaviour may lead to more marble burying behaviour. But in the mice that had been prenatally exposed to VPA, cannabis oil treatment reduced the repeated behaviour. These results are once again compatible with the earlier research [13,14,55].

In both the elevated plus maze test and the open field test, we observed higher levels of anxiety in the VPA-exposed pups. In comparison to the matching control group, the VPA-exposed group of mice travelled farther at a faster mean speed and spent more time in the periphery zone. The elevated amygdala function in the VPA-exposed mice may be the cause of their elevated anxiety. Additionally, a human study reveals that patients with ASD exhibit worried behaviour. The earlier researches observed similar results [22,28,32-34].

In the prefrontal cortex of mice exposed to VPA, we found damaged cells. According to the authors, DSM-IV anxiety disorders affected 39.6% of young persons [2]. Cannabinoid oil therapy enhances anxious behaviour and locomotor activity in the elevated plus maze test and open field. Our current work has not examined the molecular basis of the anti-anxiety effect of cannabis oil.

But numerous research have previously shown that cannabinoids have calming effects [36,38, 43, 44]. Therefore , cannabinoid treatment may improve this condition by the endocannabinoid system through the reduction of oxidative stress.

In the present investigation, we discovered that male mice given VPA displayed a protracted delay in withdrawing their hind paw. When assessing autism in an animal model, lower pain sensitivity is an important issue to take into account. According to the findings of the current investigation, mice exposed to VPA during pregnancy exhibit impaired nociception in the hot plate test, which is consistent with earlier observations [22]. Studies on humans [1,15,16] and animals [9–14,22] have revealed a reduced susceptibility to painful stimuli. Sensory pathways are networks of neurons that connect the cerebral cortex to the sensory organ, and they are what allow us to perceive sensations [31–33]. The latency to remove the hind paw from painful stimuli is reduced as a result of cannabis therapy.

One of the primary signs of ASD is a deficit in social communication [1]. According to the study's findings, children who had been exposed to VPA exhibited poor social interaction (spending less time with strangers). Fear, anxiety, or a poor ability to interpret the communication cues from the unfamiliar mice could all contribute to poor social interaction. But in the rats who had been preferably exposed to VPA, cannabis oil administration enhanced the poor communication. With respect to the earlier investigations [10-12,52-54], these findings are once more inconsistent.

Within ASD exists a wide range of cognitive abilities from severe disability to high functioning individuals [8,9]. We used the novel object recognition paradigm as our test of cognitive ability. Previous studies demonstrated a reduced level of performance in animals in this test [8,9, 55,62]. In our study, the VPA-exposed group showed a preference for a known object over a novel object, and the control group and the VPA-exposed group discrimination indices were different. While cannabinoid treatment improved the performance of the VPA exposed group and this group showed more preference for a novel object over familiar object as compared to VPA group.

In our study , we conducted a y maze test to examine stereotypical or repetitive behavior and spatial memory in the mice model. We found a decrease in % spontaneous alteration in the VPA exposed offspring as compared to control group. The VPA exposed group showed an impairment in their spatial memory. Previous studies also reported stereotypical or repetitive behavior of mice and humans [13,14,1,52]. While the treatment with cannabinoid oil showed an increase in % spontaneous alteration as compared to the VPA exposed offspring.

In this study , we conducted a histopathological study of brains tissues including Hippocampus, prefrontal cortex, cerebellum. Due to their vital roles in the learning process, motor functions, anxiety, social functions, and emotions that are largely disrupted in autism [1,5,36], abnormalities in the cerebellum and hippocampus have been discovered to be significant in the pathophysiology of the illness.

The hippocampus is one region that has been implicated in ASDs [23, 25]. Based upon the recent data, ASD may be caused by multiple genetic factors that interrupt the development and function of brain circuits important for social recognition and language [3,4 , 9-14]. Prenatal VPA exposure increases embryonic neurogenesis which leads to a depletion of neural precursor cell pool and neuroanatomical changes in the hippocampus of the VPA group. We have observed hypocellularity in the hippocampal CA1 region. Our findings are in line with the hypocellularity in VPA mice that has been documented [27, 28, 55]. The cerebellum has two main neural circuits: purkinje cells and granules. Major connections between the cerebellum, cerebral cortex, and limbic system were visible in purkinje cells. Patients with autism have abnormalities in the cerebellum's Purkinje cell density and the cytoarchitecture of the cerebral cortex and subcortical systems [32]. In our study, decreased in number of Purkinje cells was observed in the brains of the VPA-exposed mice. The Purkinje cells also showed homogenized degeneration of Purkinje cells in Purkinje cell layer. These results are also inconsistent with the previous findings [24,25]. In our experiment, we observed damaged cells in the prefrontal cortex of the VPA exposed offspring. Prefrontal cortex areas of mice given VPA exposure showed more cortical alterations. This is the primary cause of the mice's social and behavioural deficiencies. However, Cannabinoid oil treatment significantly lessens the neuroanatomical changes in the hippocampus, increases the number of Purkinje cells in the cerebellum and a smaller number of degenerated neurons in prefrontal cortex were observed. Similarly, previous results were also consistent with the current study [25, 34, 53,54, 63].

According to earlier research, mice exposed to VPA exhibited an increase in oxidative stress. The group exposed to VPA had higher levels of reduced glutathione and glutathione GSH. These findings demonstrate unequivocally that a glutathione ratio increase causes an increase in oxidative stress, which interferes with the early phases of brain development in mice exposed to VPA during pregnancy. It has been shown that the susceptibility of neural cells to the effects of oxidative stress increases with brain development. Our results are in line with recent studies on neurodevelopmental problems in autism [12, 22, 64–66]. Enzymatic antioxidants as SOD and CAT

were additionally impacted. The VPA exposed group showed no differences in the CAT test. While VPA exposure at the prenatal period boosted SOD activity [67,68]. Lipid peroxidation were higher in the VPA-exposed offspring [69] . We also found a decreased level of NO in brain tissue [70].

Following the administration of cannabinoid oil, the behaviour of the offspring of mothers who had been exposed to VPA during pregnancy was better in the marble burying test, open field and elevated plus maze test, hot plate test, social interaction test, and test of new object identification. And in these animals, cannabinoid oil lessens oxidative stress. Our findings imply that Cannabinoid oil may possess neuroprotective and anxiolytic qualities, which are likely related to the antioxidant impact. After the administration of cannabinoid oil, the VPA exposed group showed improvement in our histological investigation on brain tissues from the hippocampus, cerebellum, and prefrontal cortex. Numerous studies show a connection between the antioxidant mechanism and the neuroprotective effects of cannabis oil [61–66].

6. Conclusion

In current study, GD 13 pregnant mice and pups of PND 14 were used to develop VPA-induced animal model of autism and autistic behavior was accessed. Moreover, histopathological analysis revealed the morphological and structural changes in the brain tissues of hippocampus, prefrontal cortex and cerebellum. The oxidative stress level was measured by several stress markers (GSH, GST, CAT, SOD, NO, LPO). Cannabinoid oil treatment decreased hypersensitivity, pain sensation, improved behaviors and social interaction of autism mice model. This therapeutic effect was shown to be strongly associated with decreased oxidative stress and neurodegeneration in the brain. Additional studies are required to gain more comprehensive understanding of potential of plant-based bioactive compounds as potential drugs for treating ASD.

REFERENCES

[1] Luhach, K., Kulkarni, G. T., Singh, V. P., & Sharma, B. (2021). Attenuation of neurobehavioural abnormalities by papaverine in prenatal valproic acid rat model of ASD. *European Journal of Pharmacology*, *890*, 173663. https://doi.org/10.1016/j.ejphar.2020.173663

[2] Gadia, C. A., Tuchman, R., & Rotta, N. T. (2004). Autism and pervasive developmental disorders. *Jornal de Pediatria*, *80*(7), 83-94. https://doi.org/10.2223/jped.1172

[3] Grigorenko, E. L. (2009). Pathogenesis of autism: A patchwork of genetic causes. *Future Neurology*, *4*(5), 591-599. https://doi.org/10.2217/fnl.09.29

[4] Arndt, T. L., Stodgell, C. J., & Rodier, P. M. (2005). The teratology of autism. *International Journal of Developmental Neuroscience*, *23*(2-3), 189-199. https://doi.org/10.1016/j.ijdevneu.2004.11.001

[5] Bristot Silvestrin, R., Bambini-Junior, V., Galland, F., Daniele Bobermim, L., Quincozes- Santos, A., Torres Abib, R., Zanotto, C., Batassini, C., Brolese, G., Gonçalves, C., Riesgo, R., & Gottfried, C. (2013). Animal model of autism induced by prenatal exposure to valproate: Altered glutamate metabolism in the hippocampus. *Brain Research*, *1495*, 52-60. https://doi.org/10.1016/j.brainres.2012.11.048

[6] Bromley, R. L., Mawer, G., Clayton-Smith, J., & Baker, G. A. (2008). Autism spectrum disorders following in utero exposure to antiepileptic drugs. *Neurology*, *71*(23), 1923-1924. https://doi.org/10.1212/01.wnl.0000339399.64213.1a

[7] Bromley, R. L., Mawer, G., Love, J., Kelly, J., Purdy, L., McEwan, L., Briggs, M., Smith, J. C., Sin, X., & Baker, G. A. (2010). Early cognitive development in children born to women with epilepsy: A prospective report. *Epilepsia*, *51*(10), 2058-2065. https://doi.org/10.1111/j.1528-1167.2010.02668.x

[8] Martin, H. G., & Manzoni, O. J. (2014). Late onset deficits in synaptic plasticity in the valproic acid rat model of autism. *Frontiers in Cellular Neuroscience*, *8*. https://doi.org/10.3389/fncel.2014.00023

[9] Cohen, O. S., Varlinskaya, E. I., Wilson, C. A., Glatt, S. J., & Mooney, S. M. (2013). Acute prenatal exposure to a moderate dose of valproic acid increases social behavior

and alters gene expression in rats. *International Journal of Developmental Neuroscience, 31*(8), 740-750. https://doi.org/10.1016/j.ijdevneu.2013.09.002

[10] Schneider, T., & Przewłocki, R. (2004). Behavioral alterations in rats Prenatally exposed to Valproic acid: Animal model of autism. *Neuropsychopharmacology*, *30*(1), 80-89. https://doi.org/10.1038/sj.npp.1300518

[11] Schneider, T., Labuz, D., & Przewłocki, R. (2001). Nociceptive changes in rats after prenatal exposure to valproic acid. *Polish journal of pharmacology*, *53*(5), 531–534.

[12] Al-Amin, M. M., Rahman, M. M., Khan, F. R., Zaman, F., & Mahmud Reza, H. (2015). Astaxanthin improves behavioral disorder and oxidative stress in prenatal valproic acid-induced mice model of autism. *Behavioural Brain Research*, *286*, 112-121. https://doi.org/10.1016/j.bbr.2015.02.041

[13] Zamberletti, E., Gabaglio, M., & Parolaro, D. (2017). The Endocannabinoid System and Autism Spectrum Disorders: Insights from Animal Models. *International journal of molecular sciences*, *18*(9), 1916. https://doi.org/10.3390/ijms18091916

[14] Poleg, S., Golubchik, P., Offen, D., & Weizman, A. (2019). Cannabidiol as a suggested candidate for treatment of autism spectrum disorder. *Progress in neuro-psychopharmacology & biological psychiatry*, *89*, 90–96. https://doi.org/10.1016/j.pnpbp.2018.08.030

[15] Folkes, O. M., Báldi, R., Kondev, V., Marcus, D. J., Hartley, N. D., Turner, B. D., Ayers, J. K., Baechle, J. J., Misra, M. P., Altemus, M., Grueter, C. A., Grueter, B. A., & Patel, S. (2020). An endocannabinoid-regulated basolateral amygdala-nucleus accumbens circuit modulates sociability. *The Journal of clinical investigation*, *130*(4), 1728–1742. https://doi.org/10.1172/JCI131752

[16] Aran, A., Cassuto, H., Lubotzky, A., Wattad, N., & Hazan, E. (2019). Brief Report: Cannabidiol-Rich Cannabis in Children with Autism Spectrum Disorder and Severe Behavioral Problems-A Retrospective Feasibility Study. *Journal of autism and developmental disorders*, *49*(3), 1284–1288. https://doi.org/10.1007/s10803-018-3808-2

[17] Yenkoyan, K., Grigoryan, A., Fereshetyan, K., & Yepremyan, D. (2017). Advances in understanding the pathophysiology of autism spectrum disorders. *Behavioural Brain Research*, *331*, 92-101. https://doi.org/10.1016/j.bbr.2017.04.038

[18] Bambini-Junior, V., Zanatta, G., Della Flora Nunes, G., Mueller de Melo, G., Michels, M., Fontes-Dutra, M., Nogueira Freire, V., Riesgo, R., & Gottfried, C. (2014). Resveratrol prevents social deficits in animal model of autism induced by valproic acid. *Neuroscience Letters*, *583*, 176-181. https://doi.org/10.1016/j.neulet.2014.09.039

[19] Bambini-Junior, V., Rodrigues, L., Behr, G. A., Moreira, J. C., Riesgo, R., & Gottfried, C. (2011). Animal model of autism induced by prenatal exposure to valproate: Behavioral changes and liver parameters. *Brain Research*, *1408*, 8-16. https://doi.org/10.1016/j.brainres.2011.06.015

[20] Schneider, T., Roman, A., Basta-Kaim, A., Kubera, M., Budziszewska, B., Schneider, K., & Przewłocki, R. (2008). Gender-specific behavioral and immunological alterations in an animal model of autism induced by prenatal exposure to valproic acid. *Psychoneuroendocrinology*, *33*(6), 728-740. https://doi.org/10.1016/j.psyneuen.2008.02.011

[21] Sandhya, T., Sowjanya, J., & Veeresh, B. (2012). Bacopa monniera (L.) Wettst ameliorates behavioral alterations and oxidative markers in sodium Valproate induced autism in rats. *Neurochemical Research*, *37*(5), 1121-1131. https://doi.org/10.1007/s11064-012-0717-1

[22] Rodier, P. M., Ingram, J. L., Tisdale, B., & Croog, V. J. (1997). Linking etiologies in humans and animal models: Studies of autism. *Reproductive Toxicology*, *11*(2-3), 417-422. https://doi.org/10.1016/s0890-6238(97)80001-u

[23] Ingram, J. L., Peckham, S. M., Tisdale, B., & Rodier, P. M. (2000). Prenatal exposure of rats to valproic acid reproduces the cerebellar anomalies associated with autism. *Neurotoxicology and Teratology*, *22*(3), 319-324. https://doi.org/10.1016/s0892-0362(99)00083-5

[24] Dufour-Rainfray, D., Vourc'h, P., Le Guisquet, A., Garreau, L., Ternant, D., Bodard, S., Jaumain, E., Gulhan, Z., Belzung, C., Andres, C. R., Chalon, S., & Guilloteau, D. (2010). Behavior and serotonergic disorders in rats exposed prenatally to valproate: A model for autism. *Neuroscience Letters*, *470*(1), 55-59. https://doi.org/10.1016/j.neulet.2009.12.054

[25] Narita, N., Kato, M., Tazoe, M., Miyazaki, K., Narita, M., & Okado, N. (2002). Increased monoamine concentration in the brain and blood of fetal thalidomide- and

Valproic acid–exposed rat: Putative animal models for autism. *Pediatric Research*, *52*(4), 576-579. https://doi.org/10.1203/00006450-200210000-00018

[26] Choi, C. S., Gonzales, E. L., Kim, K. C., Yang, S. M., Kim, J., Mabunga, D. F., Cheong, J. H., Han, S., Bahn, G. H., & Shin, C. Y. (2016). The transgenerational inheritance of autism-like phenotypes in mice exposed to valproic acid during pregnancy. *Scientific Reports*, *6*(1). https://doi.org/10.1038/srep36250

[27] Wagner, G. C., Reuhl, K. R., Cheh, M., McRae, P., & Halladay, A. K. (2006). A new Neurobehavioral model of autism in mice: Pre- and postnatal exposure to sodium Valproate. *Journal of Autism and Developmental Disorders*, *36*(6), 779-793. https://doi.org/10.1007/s10803-006-0117-y

[28] D'Mello, A. M., & Stoodley, C. J. (2015). Cerebro-cerebellar circuits in autism spectrum disorder. *Frontiers in Neuroscience*, *9*. https://doi.org/10.3389/fnins.2015.00408

[29] Ellegood, J., Anagnostou, E., Babineau, B. A., Crawley, J. N., Lin, L., Genestine, M., DiCicco-Bloom, E., Lai, J. K., Foster, J. A., Peñagarikano, O., Geschwind, D. H., Pacey, L. K., Hampson, D. R., Laliberté, C. L., Mills, A. A., Tam, E., Osborne, L. R., Kouser, M., Espinosa-Becerra, F., Xuan, Z., … Lerch, J. P. (2015). Clustering autism: using neuroanatomical differences in 26 mouse models to gain insight into the heterogeneity. *Molecular psychiatry*, *20*(1), 118–125. https://doi.org/10.1038/mp.2014.98

[30] Dougherty, J. P., & Aloyo, V. J. (2011). Pharmacological and behavioral characterization of the 5-HT2A receptor in C57BL/6N mice. *Psychopharmacology*, *215*(3), 581–593. https://doi.org/10.1007/s00213-011-2207-6

[31] Morakotsriwan, N., Wattanathorn, J., Kirisattayakul, W., & Chaisiwamongkol, K. (2016). Autistic-like behaviors, oxidative stress status, and histopathological changes in cerebellum of Valproic acid rat model of autism are improved by the combined extract of purple rice and silkworm pupae. *Oxidative Medicine and Cellular Longevity*, *2016*, 1-10. https://doi.org/10.1155/2016/3206561

[32] Bertolino, B., Crupi, R., Impellizzeri, D., Bruschetta, G., Cordaro, M., Siracusa, R., Esposito, E., & Cuzzocrea, S. (2017). Beneficial Effects of Co-Ultramicronized Palmitoylethanolamide/Luteolin in a Mouse Model of Autism and in a Case Report of

Autism. *CNS neuroscience & therapeutics*, *23*(1), 87–98. https://doi.org/10.1111/cns.12648

[33] Mirza, R., & Sharma, B. (2019). Benefits of Fenofibrate in prenatal valproic acid-induced autism spectrum disorder related phenotype in rats. *Brain research bulletin*, *147*, 36–46. https://doi.org/10.1016/j.brainresbull.2019.02.003

[34] Galani, R., Weiss, I., Cassel, J. C., & Kelche, C. (1998). Spatial memory, habituation, and reactions to spatial and nonspatial changes in rats with selective lesions of the hippocampus, the entorhinal cortex or the subiculum. *Behavioural brain research*, *96*(1-2), 1-1. https://doi.org/10.1016/S0166-4328(97)00197-6

[35] Yochum, C. L., Dowling, P., Reuhl, K. R., Wagner, G. C., & Ming, X. (2008). VPA-induced apoptosis and behavioral deficits in neonatal mice. *Brain research*, *1203*, 126–132. https://doi.org/10.1016/j.brainres.2008.01.055

[36] Gilles, F. H. (1983). Neural damage: inconstancy during gestation. In *The developing human brain*, pp. 227-243.

[37] Rice, D., & Barone, S., Jr (2000). Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models. *Environmental health perspectives*, *108 Suppl 3*(Suppl 3), 511–533. https://doi.org/10.1289/ehp.00108s3511

[38] Eissa, N., Azimullah, S., Jayaprakash, P., Jayaraj, R. L., Reiner, D., Ojha, S. K., Beiram, R., Stark, H., Łażewska, D., Kieć-Kononowicz, K., & Sadek, B. (2019). The dual-active histamine H3 receptor antagonist and acetylcholine esterase inhibitor E100 ameliorates stereotyped repetitive behavior and neuroinflammmation in sodium valproate induced autism in mice. *Chemico-biological interactions*, *312*, 108775. https://doi.org/10.1016/j.cbi.2019.108775

[39] Gouda, B., Sinha, S. N., Chalamaiah, M., Vakdevi, V., Shashikala, P., Veeresh, B., Surekha, V. M., Kasturi, V., & Boiroju, N. K. (2022). Sex Differences in Animal Models of Sodium-Valproate-Induced Autism in Postnatal BALB/c Mice: Whole-Brain Histoarchitecture and 5-HT2A Receptor Biomarker Evidence. *Biology*, *11*(1), 79. https://doi.org/10.3390/biology11010079

[40] Aran, A., & Cayam-Rand, D. (2020). Medical Cannabis in Children. *Rambam Maimonides medical journal*, *11*(1), e0003. https://doi.org/10.5041/RMMJ.10386

[41] Galve-Roperh, I., Palazuelos, J., Aguado, T., & Guzmán, M. (2009). The endocannabinoid system and the regulation of neural development: potential implications in psychiatric disorders. *European archives of psychiatry and clinical neuroscience*, *259*(7), 371–382. https://doi.org/10.1007/s00406-009-0028-y

[42] Aran, A., Harel, M., Cassuto, H., Polyansky, L., Schnapp, A., Wattad, N., Shmueli, D., Golan, D., & Castellanos, F. X. (2021). Cannabinoid treatment for autism: a proof-of-concept randomized trial. *Molecular autism*, *12*(1), 6. https://doi.org/10.1186/s13229-021-00420-2

[43] Bergamaschi, M. M., Queiroz, R. H., Chagas, M. H., De Oliveira, D. C., De Martinis, B. S., Kapczinski, F., Quevedo, J., Roesler, R., Schröder, N., Nardi, A. E., Martín-Santos, R., Hallak, J. E., Zuardi, A. W., & Crippa, J. A. (2011). Cannabidiol reduces the anxiety induced by simulated public speaking in treatment-naïve social phobia patients. *Neuropsychopharmacology*, *36*(6), 1219-1226. https://doi.org/10.1038/npp.2011.6

[44] Blessing, E. M., Steenkamp, M. M., Manzanares, J., & Marmar, C. R. (2015). Cannabidiol as a potential treatment for anxiety disorders. *Neurotherapeutics*, *12*(4), 825-836. https://doi.org/10.1007/s13311-015-0387-1

[45] Tzadok, M., Uliel-Siboni, S., Linder, I., Kramer, U., Epstein, O., Menascu, S., Nissenkorn, A., Yosef, O. B., Hyman, E., Granot, D., Dor, M., Lerman-Sagie, T., & Ben-Zeev, B. (2016). CBD-enriched medical cannabis for intractable pediatric epilepsy. *Seizure*, *35*, 41-44. https://doi.org/10.1016/j.seizure.2016.01.004

[46] Carbone, E., Manduca, A., Cacchione, C., Vicari, S., & Trezza, V. (2021). Healing autism spectrum disorder with cannabinoids: A neuroinflammatory story. *Neuroscience & Biobehavioral Reviews*, *121*, 128-143. https://doi.org/10.1016/j.neubiorev.2020.12.009

[47] Prata, J., Machado, A. S., Von Doellinger, O., Almeida, M. I., Barbosa, M. A., Coelho, R., & Santos, S. G. (2019). The contribution of inflammation to autism spectrum disorders: Recent clinical evidence. *Methods in Molecular Biology*, 493-510. https://doi.org/10.1007/978-1-4939-9554-7_29

[48] Fleury-Teixeira, P., Caixeta, F. V., Ramires da Silva, L. C., Brasil-Neto, J. P., & Malcher-Lopes, R. (2019). Effects of CBD-Enriched *Cannabis sativa* Extract on Autism

Spectrum Disorder Symptoms: An Observational Study of 18 Participants Undergoing Compassionate Use. *Frontiers in neurology*, *10*, 1145. https://doi.org/10.3389/fneur.2019.01145

[49] Adler, B. A., Wink, L. K., Early, M., Shaffer, R., Minshawi, N., McDougle, C. J., & Erickson, C. A. (2015). Drug-refractory aggression, self-injurious behavior, and severe tantrums in autism spectrum disorders: A chart review study. Autism, 19(1), 102–106. https://doi.org/10.1177/1362361314524641

[50] Bar-Lev Schleider, L., Mechoulam, R., Saban, N., Meiri, G., & Novack, V. (2019). Real life Experience of Medical Cannabis Treatment in Autism: Analysis of Safety and Efficacy. *Scientific reports*, *9*(1), 200. https://doi.org/10.1038/s41598-018-37570-yV

[51] Poleg, S., Kourieh, E., Ruban, A., Shapira, G., Shomron, N., Barak, B., & Offen, D. (2021). Behavioral aspects and neurobiological properties underlying medical cannabis treatment in Shank3 mouse model of autism spectrum disorder. *Translational psychiatry*, *11*(1), 524. https://doi.org/10.1038/s41398-021-01612-3

[52] Kaplan, J. S., Wagner, J. K., Reid, K., McGuinness, F., Arvila, S., Brooks, M., Stevenson, H., Jones, J., Risch, B., McGillis, T., Budinich, R., Gambell, E., & Predovich, B. (2021). Cannabidiol Exposure During the Mouse Adolescent Period Is Without Harmful Behavioral Effects on Locomotor Activity, Anxiety, and Spatial Memory. *Frontiers in behavioral neuroscience*, *15*, 711639. https://doi.org/10.3389/fnbeh.2021.711639

[53] Dearborn, J. T., Nelvagal, H. R., Rensing, N. R., Takahashi, K., Hughes, S. M., Wishart, T. M., Cooper, J. D., Wong, M., & Sands, M. S. (2022). Effects of chronic cannabidiol in a mouse model of naturally occurring neuroinflammation, neurodegeneration, and spontaneous seizures. *Scientific reports*, *12*(1), 11286. https://doi.org/10.1038/s41598-022-15134-5

[54] Zamberletti, E., Gabaglio, M., Woolley-Roberts, M., Bingham, S., Rubino, T., & Parolaro, D. (2019). Cannabidivarin Treatment Ameliorates Autism-Like Behaviors and Restores Hippocampal Endocannabinoid System and Glia Alterations Induced by Prenatal Valproic Acid Exposure in Rats. *Frontiers in cellular neuroscience*, *13*, 367. https://doi.org/10.3389/fncel.2019.00367

[55] Hara, Y., Ago, Y., Taruta, A., Hasebe, S., Kawase, H., Tanabe, W., Tsukada, S., Nakazawa, T., Hashimoto, H., Matsuda, T., & Takuma, K. (2017). Risperidone and

aripiprazole alleviate prenatal valproic acid-induced abnormalities in behaviors and dendritic spine density in mice. *Psychopharmacology*, *234*(21), 3217–3228. https://doi.org/10.1007/s00213-017-4703-9

[56] Silverman, J. L., Tolu, S. S., Barkan, C. L., & Crawley, J. N. (2010). Repetitive self-grooming behavior in the BTBR mouse model of autism is blocked by the mGluR5 antagonist MPEP. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*, *35*(4), 976–989. https://doi.org/10.1038/npp.2009.201

[57] Golubeva, A. V., Joyce, S. A., Moloney, G., Burokas, A., Sherwin, E., Arboleya, S., Flynn, I., Khochanskiy, D., Moya-Pérez, A., Peterson, V., Rea, K., Murphy, K., Makarova, O., Buravkov, S., Hyland, N. P., Stanton, C., Clarke, G., Gahan, C. G. M., Dinan, T. G., & Cryan, J. F. (2017). Microbiota-related Changes in Bile Acid & Tryptophan Metabolism are Associated with Gastrointestinal Dysfunction in a Mouse Model of Autism. *EBioMedicine*, *24*, 166–178. https://doi.org/10.1016/j.ebiom.2017.09.020

[58] Scott, K. A., Ida, M., Peterson, V. L., Prenderville, J. A., Moloney, G. M., Izumo, T., Murphy, K., Murphy, A., Ross, R. P., Stanton, C., Dinan, T. G., & Cryan, J. F. (2017). Revisiting Metchnikoff: Age-related alterations in microbiota-gut-brain axis in the mouse. *Brain, behavior, and immunity*, *65*, 20–32. https://doi.org/10.1016/j.bbi.2017.02.004

[59] Walsh, R. N., & Cummins, R. A. (1976). The Open-Field Test: a critical review. *Psychological bulletin*, *83*(3), 482–504. https://doi.org/10.1037/0033-2909.83.3.482

[60] Rodgers, R. J., & Dalvi, A. (1997). Anxiety, defence and the elevated plus-maze. *Neuroscience and biobehavioral reviews*, *21*(6), 801–810. https://doi.org/10.1016/s0149-7634(96)00058-9

[61] Zamberletti, E., Beggiato, S., Steardo, L., Jr, Prini, P., Antonelli, T., Ferraro, L., Rubino, T., & Parolaro, D. (2014). Alterations of prefrontal cortex GABAergic transmission in the complex psychotic-like phenotype induced by adolescent delta-9-tetrahydrocannabinol exposure in rats. *Neurobiology of disease*, *63*, 35–47. https://doi.org/10.1016/j.nbd.2013.10.028

[62] Markram, K., Rinaldi, T., La Mendola, D., Sandi, C., & Markram, H. (2008). Abnormal fear conditioning and amygdala processing in an animal model of autism. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*, *33*(4), 901–912. https://doi.org/10.1038/sj.npp.1301453

[63] Reddy, Y. A., Chalamaiah, M., Ramesh, B., Balaji, G., & Indira, P. (2014). Ameliorating activity of ginger (Zingiber officinale) extract against lead induced renal toxicity in male rats. *Journal of food science and technology*, *51*(5), 908–914. https://doi.org/10.1007/s13197-011-0568-9

[64] Naveed, M., Khan, S. Z., Zeeshan, S., Khan, A., Shal, B., Atiq, A., Ali, H., Ullah, R., Zia-Ur-Rehman, & Khan, S. (2019). A new cationic palladium(II) dithiocarbamate exhibits anti-inflammatory, analgesic, and antipyretic activities through inhibition of inflammatory mediators in in vivo models. *Naunyn-Schmiedeberg's archives of pharmacology*, *392*(8), 961–977. https://doi.org/10.1007/s00210-019-01645-y

[65] Khan, A., Khan, S., Ali, H., Shah, K. U., Ali, H., Shehzad, O., Onder, A., & Kim, Y. S. (2019). Anomalin attenuates LPS-induced acute lungs injury through inhibition of AP-1 signaling. *International immunopharmacology*, *73*, 451–460. https://doi.org/10.1016/j.intimp.2019.05.032

[66] Warholm, M., Guthenberg, C., von Bahr, C., & Mannervik, B. (1985). Glutathione transferases from human liver. *Methods in enzymology*, *113*, 499–504. https://doi.org/10.1016/s0076-6879(85)13065-x

[67] Marklund, S., & Marklund, G. (1974). Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *European journal of biochemistry*, *47*(3), 469–474. https://doi.org/10.1111/j.1432-1033.1974.tb03714.x

[68] Kazmi, Z., Zeeshan, S., Khan, A., Malik, S., Shehzad, A., Seo, E. K., & Khan, S. (2020). Anti-epileptic activity of daidzin in PTZ-induced mice model by targeting oxidative stress and BDNF/VEGF signaling. *Neurotoxicology*, *79*, 150–163. https://doi.org/10.1016/j.neuro.2020.05.005

[69] Khan, A. U., Muhammad Khan, A., Khan, A., Shal, B., Aziz, A., Ahmed, M. N., & Khan, S. (2020). The newly synthesized compounds (NCHDH and NTHDH) attenuates LPS-induced septicemia and multi-organ failure via Nrf2/HO1 and HSP/TRVP1 signaling in

mice. *Chemico-biological interactions, 329,* 109220. https://doi.org/10.1016/j.cbi.2020.109220

[70] Khan, S., Shehzad, O., Chun, J., & Kim, Y. S. (2013). Mechanism underlying anti-hyperalgesic and anti-allodynic properties of anomalin in both acute and chronic inflammatory pain models in mice through inhibition of NF-κB, MAPKs and CREB signaling cascades. *European journal of pharmacology, 718*(1-3), 448–458. https://doi.org/10.1016/j.ejphar.2013.07.039

Faiza Ali

ORIGINALITY REPORT

10%	9%	11%	1%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	docksci.com Internet Source	4%
2	Konstantin Yenkoyan, Artem Grigoryan, Katarine Fereshetyan, Diana Yepremyan. "Advances in understanding the pathophysiology of autism spectrum disorders", Behavioural Brain Research, 2017 Publication	1%
3	rcastoragev2.blob.core.windows.net Internet Source	1%
4	Kanishk Luhach, Giriraj T. Kulkarni, Vijay P. Singh, Bhupesh Sharma. " Vinpocetine amended prenatal valproic acid induced features of possibly by altering markers of neuronal function, inflammation, and oxidative stress ", Autism Research, 2021 Publication	1%
5	fjfsdata01prod.blob.core.windows.net Internet Source	1%

6	Submitted to Higher Education Commission Pakistan Student Paper	1 %
7	www.nature.com Internet Source	1 %
8	Md. Mamun Al-Amin, Md. Mahbubur Rahman, Fazlur Rahman Khan, Fahmida Zaman, Hasan Mahmud Reza. "Astaxanthin improves behavioral disorder and oxidative stress in prenatal valproic acid-induced mice model of autism", Behavioural Brain Research, 2015 Publication	1 %
9	daneshyari.com Internet Source	1 %

Exclude quotes	On	**Exclude matches**	< 1%
Exclude bibliography	On		