Protective Effect of Coconut Oil and *Nigella sativa* on Alcohol Induced Neurochemical and Behavioral Changes in Animal Model

PhD Thesis

By

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FINAL APPROVAL

It is certified that we have read the thesis submitted by Ms. Beenish Asrar and it is our judgement that this thesis is of sufficient standard to warrant its acceptance by the International Islamic University, Islamabad for the award of PhD degree in Biosciences.

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DECLARATION

I hereby declare that this dissertation, neither as a whole nor as a part there of has been copied out from any source. It is further declared that this dissertation is entirely based on my personal efforts and experiments made under the sincere guidance of my supervisor.

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FORWARDING SHEET BY RESEARCH SUPERVISOR

The dissertation entitled **"Protective Effect of Coconut Oil and *Nigella sativa* on Alcohol Induced Neurochemical and Behavioral Changes in Animal Model"** submitted by **Beenish Asrar** in partial fulfillment of Doctor of Philosophy (PhD) degree in Biosciences, has been completed under my guidance and supervision. I am satisfied with the quality of student's research work and allow her to submit this dissertation for further process to graduate with PhD degree in Biosciences from Sulaiman Bin Abdullah Aba Al-Khail, Center for Interdisciplinary Research in Basic Sciences (SA-CIRBS), as per IIUI rules and regulations.

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Commitments transfer dreams into realities!

I am dedicating my PhD thesis to

"My Fabulous Family"

***"Mom and Dad,** who always supported me and believed in me"*

***"Brothers,** who inspired me to fly towards my dream"*

***"Sisters,** who always treated me like their daughter"*

***"Brothers and Sisters in Law**, who supported me unconditionally and treated me like a sister"*

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TABLE OF CONTENTS

List of Tables

List of Figures

List of abbreviations

4-DOPAL	4-Dihydroxyphenylacetaldehyde
5-HIAA	5-Hydroxyindole acetic acid
5-HT	5-hydroxytryptamine or serotonin
5-HTP	5-Hydroxy tryptophan
$NaH_2PO_4.2H_2O$	Sodium dihydrogen phosphate dihydrate
AD	Alzheimer's disease
ANOVA	Analysis of variance
ARF	Animal Resource Facility
BDNF	Brain-derived neurotrophic factor
CNS	Central nervous system
CPP	Conditioned place-preference
DA	Dopamine
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DOPAC	Dihydroxyphenyl acetic acid
ECD	Electrochemical detection
ECL	ECL
EDTA	Ethylenediaminetetraacetic acid disodium salt 2-hydrate
ELISA	Enzyme-linked immunosorbent assay
ENSO	Ethanol + *Nigella sativa* oil

EPM	Elevated plus maze
EVCO	Ethanol + Virgin coconut oil
EW	Ethanol + Water
GABA	Gamma aminobutyric acid
GIT	Gastrointestinal Tract
GPCR	G-protein coupled receptor (GPCR)
HP	Hippocampus
HPLC	High Pressure liquid chromatography
HRP	Enzyme horseradish peroxidase
HT	Hypothalamus
HVA	Homovanillic acid
ICCBS,	International center for chemical and biological sciences
IgG	Immunoglobulin G
L-DOPA	L-dihydroxy phenylalanine
LTM	Long term memory
MAO	Monoamine oxidase
MCT	Medium Chain Triglycerides
MDA	Malondialdehyde
MS	Multiple sclerosis
MWM	Morris water maze
NA	Noradrenaline

NMDA	N-Methyl-D-aspartate
NS	*Nigella sativa*
NSO	*Nigella sativa* Oil
OCD	Obsessive Compulsive Disorders
OD	Optical density
ODS	Octadecyl silane - 2
OF	Open Field
OSS	Ocetyl sodium sulphate
PBS	Phosphate buffer saline
PCMD	Panjwani center for molecular medicine and drug research
PD	Panic Disorder
PFC	Prefrontal Cortex
PTSD	Posttraumatic Stress Disorders
PVDF	Polyvinylidene fluoride
RMD	Repeated measures design
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RP	Reverse phase
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SOS	Sodium octyl sulfate
ST	Striatum

STM	Short term memory loss
T-CHO	Total cholesterol (T-CHO)
TG	Triglyceride (TG)
UK	United Kingdom
VCO	Virgin Coconut Oil
WHO	World Health Organization (WHO)
WNSO	Water + *Nigella sativa* Oil
WVCO	Water + Virgin Coconut Oil
WW	Water + Water

Abstract

The neurological disorders represent the global health priority in terms of social and medical problems. Globally, the use of addictive substances or drugs is increasing day by day and roughly more than two billion people are using different drugs of abuse. Alcohol is one of the commonly available and used drugs of abuse regardless of knowing the facts of negative impacts on health. The chronic alcohol use in drinking water in animal model particularly in rats leads to the symptoms of anxiety, depression and cognitive impairments such as learning and memory. So, different approaches or neuroprotective strategies are adopted to overcome or reduce the negative consequences of alcohol on central nervous system. The aim and objectives of the current project were to evaluate the neuroprotective effects of *Nigella sativa* oil (NSO) and Virgin *Cocos nucifera* oil (VCO) in Wistar male rats of chronic alcohol consumption model. The total number of male rats (n=48) were divided in to two groups, a water drinking group and an ethanol drinking group. Each group was having 24 rats and then further divided into 4 groups having 6 rats each, i.e. two vehicle control groups (w) for *Nigella sativa* oil and (NSO) treated group and virgin coconut oil (VCO) treated group. Initially, the physical parameters such as body weight, food intake and fluid intake were measured in all groups. Rats were examined for anxiety by elevated plus maze test, spatial learning and memory by Morris water maze test, exploratory activity by open field test. Neurotransmitters like serotonin, dopamine, noradrenaline with their metabolite's levels in prefrontal cortex, hippocampus and midbrain were measured by high pressure liquid chromatography- electrochemical detector. Furthermore, BDNF level in hypothalamus was evaluated by western blotting. Catalase enzyme and peroxide concentrations in striatum were evaluated by colorimetric assay. Concentrations of some of the important blood biomarkers such as serum glucose, total cholesterol and triglycerides were assessed by colorimeter/spectrophotometer and serum insulin was measured by Elisa. Our results showed that chronic alcohol reduced the body weight, food and fluid intake. NSO and VCO treatment of ethanol drinking animals exacerbated the effects of ethanol on body weight, food and fluid intake. VCO treatment also reduced food intake in water drinking animals. NSO significantly improved spatial memory and reduced anxiety in ethanolic rats where as VCO treatment led to anxiety in water and ethanol drinking rats but

significantly improved memory in water drinking group only. Brain serotonin and its metabolite concentrations were elevated in NSO treated water and ethanolic rats whereas prefrontal serotonin (5-HT) and its metabolite "5-HIAA" levels were increased by VCO treatment in water drinking animals only. Brain 5-HT level was reduced in VCO treated ethanolic rats. Brain dopamine (DA) metabolism was enhanced caused increase in DA's metabolite "DOPAC" level in water and ethanol drinking rats treated with NSO. VCO treatment led to reduced prefrontal DA levels in water and ethanolic rats and increased DOPAC level only in water drinking animals. NSO treated water drinking rats showed reduced hippocampal noradrenaline and serum glucose whereas results were vice versa for VCO treated water drinking rats but VCO acted as hypoglycemic agent in the ethanolic rats. NSO and VCO treated groups showed reduced total cholesterol in ethanol drinking group. Triglycerides level was increased in VCO treated water and ethanolic rats. The present studies provide enough evidence that chronic alcohol leads to memory impairment, anxiety with impaired neurotransmission. In conclusion, this study has provided enough evidences that chronic alcohol leads to anxiety and impaired memory by altered neurotransmission. The findings of this study also explored that NSO counteracts the impaired memory, anxiogenic effects of chronic ethanol by improving the 5-HT and DA neurotransmission. VCO can be useful to improve memory in water drinking animals only by increased prefrontal serotonin and noradrenalin levels. Ethanol drinking rats treated with VCO showed anxiety and impaired memory with reduced serotonin, dopamine and DOPAC. In future, the active compounds from VCO and NSO will be tested on same parameters tested and would aid knowledge to scientific world.

CHAPTER # 1

INTRODUCTION

1. Introduction

1.1. Background

Neuroscience reveals the untold facts about structural, chemical, molecular and physiological basis of the most important system of body that is central nervous system (CNS). With this brain efficacy in terms of functionality, behaviors and development of diseases are demonstrated (Anup *et al.*, 2015; John *et al.*, 2000; Hunt, 1961). The CNS consist of most vital organs "brain and spinal cord". CNS is responsible to maintain various physiological processes like movement, emotions, cognition and senses. The brain is a delicate organ made up of neurons which are nerve cells. CNS receives and sends signals between neurons or between neurons and sensory organs and respond to environment. A typical neuron consists of a cell body, dendritic field and an axon. There are two main modalities of synaptic transmission: electrical and chemical. Although, these 2 modalities are different in functions but are closely linked. Neurotransmission or neuronal connections in central nervous system depend on interactions between electrical and chemical synapses. This interaction has pathological implications. Recapitulation of interactions between these two synaptic transmissions has been found in brain injury and imbalance of electrical synapses by chemical synapses or neurotransmitters led to cognitive impairment (Alberto, 2014). Diseases associated to central nervous system are comprised of neurodevelopmental diseases, neurodegenerative diseases and traumatic injuries (Anup *et al.*, 2015). Common name used to indicate such diseases is "neurological disease".

1.2. Neurological Disorders

Neurological disorder, a broad term used to give information about various conditions that can affect the central nervous system. Neurological disorders pose a large burden on worldwide health. According to "Global Burden of Disease Study", 3% of all the disease burden include neurological diseases including Schizophrenia, Multiple sclerosis, Alzheimer's disease, Attention-deficit/hyperactivity, Epilepsy, headache disorders and Addiction (Murray *et al.*, 2012). Below is the brief description of each of these disorders.

Schizophrenia, a mental disorder characterized by several signs and symptoms including hallucinations, impaired cognition, delusions, abnormal behavior and disorganized speech. Early onset of Schizophrenia makes it a disabling condition for patients and their families (Lavretsky, 2008). Disability often results from impaired memory in which affected subject is not able to concentrate or memorize due to impaired cognition and negative symptoms (Crismon, Argo and Buckley, 2014). Positive symptoms may also cause relapse due to hallucinations, suspiciousness and delusions (Crismon, Argo and Buckley, 2014; Lavretsky, 2008). Multiple sclerosis (MS) is a neurological disease characterized by autoimmune and inflammatory disorder of brain (Hauser and Goodwin, 2008; Calabresi, 2004). In MS, myelinated axons are destroyed (Olek, 2011; Weinshenker, 1996). About 350,000 people in United States of America are suffering from MS (Singh, Mehrotra and Agarwal, 1999) and 50 % of them are not able to even walk alone in next 15 years after the development of MS (Navikas and Link, 1996). It is most common in adults of age between 20 to 45 years and rarely found in childhood and middle age (Cree, 2007). Genetics, environment, metabolism and viral infections are the key factors that lead to the autoimmunity that attack immune system in central nervous system and develop MS (Cree, 2007).

About 48% of the world population is having some type of dementia. Dementia can be defined as the "degeneration of neurons" (World Alzheimer Report, 2016). Most common Alzheimer's disease (AD) also causes dementia. AD accounts for almost 80% of all the dementia cases (Barker *et al.*, 2002). AD, a neurodegenerative condition which is marked by impaired cognition and memory deficits including abnormal behavior, speech, impaired motor and balance activities, and visuospatial orientation (Schultz and Del Tredici, 2004). This neurological disease AD is linked with aging (Masters *et al.*, 2015). Neuropathological of AD include occurrence of neurofibrillary tangles and plaques consisting of amyloid protein (Scheltens *et al.*, 2016). Genetic modifications of amyloid precursor protein, presenilin 1 or *PSEN2* genes can lead to the development of AD (Ryman *et al.*, 2014).

Attention deficit hyperactivity disorder is characterized by lack of attention, impulsiveness plus hyperactivity which interfere with function, starts at early ages to usually till adulthood (Bellinger, 2012; Grandjean *et al.*, 2012; Ricceri *et al.*, 2008). Another neurological disease is Epilepsy. It is one of the most common neurological condition of unprovoked seizures. Development of Epilepsy has different causes, each reflecting brain dysfunction (Shorvon *et al.*, 2011). Each year about 50 new cases of Epilepsy are found in population of 1000,00 (Hauser and Hersdorffer, 1990).

Addiction is also a condition associated with brain characterized by insobriety of drug, unmanageable usage, desire, symptoms associated with withdrawal and sensitization. Addictive drugs are known as "psychoactive substances" as these drugs are capable to change behaviors and mind thoughts. World Health Organization (WHO) reports that more than 2 billion population is using addictive substances like alcohol, tobacco and many other substances including cocaine, heroin and cannabis (Monteiro, 2001). It costs the society 179 billion Euros annually. In 2005, "Department of Health and Human Services of United State of America", conducted survey on drug use at national level. According to which 90.7 million Americans which is about 37.4% of population are binge alcohol users or use illicit drugs (Rockville, 2005).

This present study has been conducted on addiction. Most common substances and drugs of abuse are alcohol, nicotine, cocaine, cannabis, opiates, tobacco, barbiturates and heroin (Cami and Farre, 2003; Monteiro, 2001). Among all the addictive drugs, alcohol/ethanol is most used and available drug in world.

1.3. Alcohol Addiction

Alcohol is one of the most easily available and widely used beverages in the World, despite of the risks of harmful effects on health. Each year, 3 million people die worldwide due to harmful effects of alcohol use, and this death ratio is equal to 5.3% of all deaths (WHO, 2018). Studies have demonstrated that genetics may also lead to alcohol associated disorders (Lu *et al.*, 2004; Nurnberger *et al.*, 2004). Its interaction with external environment plays critical role in alcohol consumption intoxication and preference due to

addiction behavior (Barrett *et al.*, 2004). Alcohol addiction causes the craving and desires for alcohol and addicted person's only goal is to drink alcohol despite of care and realizing its adverse consequences on health (Hyman, 2005; Hser *et al.*, 2001; Mc Lellan *et al.*, 2000).

1.3.1. Alcohol and Behavior

Alcohol exerts its effects on almost each organ of the body. It exerts it effects on metabolism by prompting the impaired lipid metabolism. At behavioral level it results in anxiety (Homes *et al.*, 2012), imbalanced motor activity, impaired cognition (Wixted, 2005; White, 2003; Hartzler and Fromme, 2003; Samson and Harris, 1992; Deitrich *et al.*, 1989; Ollat *et al.*, 1988).

1.3.2. Alcohol and Neurotransmitters

1.3.2.1. Serotonin in addiction and behavior

Serotonin (5HT) is entangled in addiction pathophysiology (Kirby *et al.*, 2011; Commons, 2008; Rothman *et al.*, 2006; Vazquez *et al.*, 2002). Receptors for serotonin 5HT1A plays part in psychostimulant prompted behaviors (Muller *et al.*, 2005). Behavioral sensitization and hyperactivity are associated with diminished activation of 5HT1A receptors and serotonin release (Broderick *et al.*, 2004; Przegalinski *et al.*, 2000). It has major role in regulating many of the functions including cognition, stress, appetite, mood, sleep (Hornung, 2003) anxiety, depression, and addiction (Hauser *et al.*, 2014; Savitz, Lucki and Drevets, 2009; Lanfumey *et al.*, 2008; Gordon and Hen, 2004; Young and Leyton, 2002; Bell, Abrams and Nutt, 2001; Barnes and Sharp, 1999). Increased serotonin level improves cognition (Laercio *et al.*, 2004; Levkovitz *et al.*, 2003;), and reduced serotonin and tryptophan is associated with impaired cognition (Khaliq *et al.*, 2006; Lieben *et al.*, 2004; Porter, Lunn and O'Brien, 2003).

1.3.2.2. Dopamine in addiction and behavior

Increased brain dopamine was released after alcohol consumption in rats (Boileau *et al.*, 2003). Increased dopamine level is involved in self-preference of addictive substance

(Oswald *et al.*, 2005). Alcohol exposure increases the dopamine level by increased release from nucleus accumbens through increased activity of mesolimbic dopaminergic neurons. It possesses affinity towards N-Methyl-D-aspartate (NMDA) and Gamma aminobutyric acid ($GABA_A$). Dopamine and serotonin release in striatum relies on synergistic interaction between NMDA receptors and alcohol, which is relevant to alcohol induced potentiation of hyperlocomotion (Jin *et al.*, 2008) and alcohol mediated behavior deficits. In prefrontal cortex and hippocampus, dopamine alters cognition by regulating plasticity and synaptic transmission (De Bundel *et al.*, 2013). Dopamine act as a moderator in forming emotional memory (Shohamy and Adcock, 2010).

1.3.2.3. Noradrenaline in addiction and behavior

At brain level it disturbs central nervous systems by blocking the chemical signals between neuronal cells. It interacts with psychological stress to affect noradrenaline. Noradrenaline (NA) is a fight and flight response hormone. Alcohol exposure reduces activity of dopamine beta-hydroxylase, which is important for NA formation, ultimately reduces NA level (Yamanaka, 1982) and mediates the alcohol prompted anxiety (Karoum *et al.*, 1976).

1.3.3. Alcohol and Metabolic Syndrome

Alcohol or ethanol is the only psychoactive drug that provides energy. One gram of ethanol possesses 7.1 kcal or 29 kJ (Triraversi and Chaput, 2015; WHO, 2004; Block, 2004). Because of this much energy, it is supposed that when consumed in excess, alcohol enhances the risk of weight-gain (Wakabayashi, 2009). Enhanced calorie intake by consuming ethanol makes it a risk factor for the development of obesity (Yeomans *et al.*, 2003). A clinical study has shown that there is a connection between alcohol use and obesity or weight gain (Sayon *et al.*, 2011). In contrast, Fromenty and colleagues (2009) reported that chronic long-term alcohol usage significantly decreased the body weight and diminished type 2 diabetes in obese mice. In another study decreased body weight in alcohol drinking rats was recorded because of malnutrition that resulted from decreased nutrient absorption in intestine (Macdonald *et al.*, 2010). Finally, alcohol consumption has been

reported to alter glucose levels and impair its metabolism causing hypercholesterolemia and insulin resistance (Jennifer *et al.*, 2015; Toffolo *et al.*, 2012; Qiwei, 2010; Yu *et al.*, 2000; Wilkes, 1996).

Up to date no effective treatment or therapy has been developed to cure alcohol related disorders. The common methods that might be use for this are (Carol , 2019):

1) Alcohol detox
2) Alcohol counseling
3) Medication
4) Group therapy

1.4. Plants in Health

World health organization (WHO) has given the opinion that medicinal plants are one of the best sources to get diverse classes of medicines. Use of plants and herbs for disease prevention and treatment of various disease is gaining attention because of the safety and inexpensiveness (Kumar *et al.*, 2012; Yadav *et al.*, 2009). Medicinal herbs are best known sources of medicines, food supplements, pharmaceutical intermediates, nutraceuticals, modern or traditional medicines, and chemical entities for synthetic medicines. Drugs derived from medicinal plants do not have many side effects that is why approximately 3.5 to 4 billion world population rely on plants for drugs. A total of 75% population worldwide depends on plants and other tools of traditional medicine as they are the best source of development of new drugs. These medicines have efficiently contributed to the health and have remedies for almost all diseases (Haque *et al.*, 2015).

1.4.1. Therapeutics Effects of Drugs or Compounds Extracted from Medicinal Plants

Ferulic acid is one of the phenolic compounds, mainly found in coconut oil, has the potential reduce oxidative stress and inflammatory responses also prevent amyloid beta aggregation (Ono *et al.*, 2008; Zhao and Moghadasian, 2008).

In a study (Ullah *et al.*, 2012), thymoquinone and metformin played neuroprotective role by reducing the ethanol induced mitochondria dependent apoptosis in rat primary cortical neuron. Another research (Gülşen *et al.*, 2015) demonstrated that thymoquinone has a healing impact on neural cells followed head injury and this role reconciled by reducing malondialdehyde (MDA) levels in the mitochondrial membrane and nuclei of neurons.

Linalool, a monoterpene is found in essential oils of many aromatic plant species. Many of these species are used traditionally for medicine and aromatherapy to soothe symptoms and to cure various chronic or acute illnesses (Batista *et al.*, 2010). It has numerous pharmacological benefits. Antioxidant, anti-inflammatory, antimicrobial, anti-cardiovascular effects have been observed in hypertensive and normotensive rats (Beier *et al.*, 2014; Wu *et al.*, 2014; Anjos *et al.*, 2013).

Troxerutin is a derivative of naturally occurring bioflavonoid named rutin. Troxerutin is evidenced to have very important biological roles, like anti-oxidative and anti-inflammatory role (Zhang *et al.*, 2009). Latest reports also provide evidences about the neuroprotective roles of troxerutin against oxidative damage. Furthermore, they also mention that troxerutin prevent the end products *in vivo* plus increase insulin signaling (Lu *et al.*, 2010 b, d). Advanced generation of advanced glycation end products is the protein modification and activator of increased reactive oxygen species (ROS) and elevated abnormal oxidative stress .It brings about to the initial phases of aging associated disease i.e. diabetes, Alzheimer's disease and arteriosclerosis (Tian *et al.*, 2005).

Lu *et al.*, (2011), demonstrated protective function of troxerutin on high cholesterol induces cognitive deficits in mice model. Troxerutin blocked the endoplasmic reticulum (ER) stress pathway by suppressing the level of protein carbonyl, ROS and advanced glycation end-product as troxerutin possess anti-inflammatory and antioxidant characteristics. Troxerutin also prevent the stimulation or activation of IKKb/NF-kB that was prompted from ER stress and it also reduces insulin resistance in hippocampus of obese mice. This obesity of mice was due to high cholesterol/fat supplementation and consumption. Troxerutin inhibited obesity, recovered normal glucose, fatty acid and

cholesterol metabolism and decreased ER stress stimulated apoptosis of neurons in hippocampus and improved the cognition that was caused by high cholesterol diet.

Luteolin, is a naturally occurring flavonoid, present in vegetables and fruit like carrots, green pepper, chamomile tea and celery. Diverse evidences have demonstrated the anti-inflammatory role of luteolin, *in vivo and in vitro* (Kim and Jobin, 2005; Kotanidou *et al.*, 2002). Luteolin also possess protective impact on neurodegeneration. Previous researches described that luteolin can alleviate the cognitive impairment in diabetic rats by modulating the neuroinflammation and oxidative stress in brain (Tian *et al.*, 2013).

This proposed research work includes two organic oils from plants *Nigella sativa* and *Cocos nucifera* respectively to evaluate their effect and potential mechanism against the above discussed behavioral, neurochemical and biological changes induced by alcohol consumption in male Wistar rats.

1.4.2. *Nigella sativa* (Fennel Flower)

Nigella sativa is placed among best herbal medicines (Juma and Hayfaa, 2011). This annual flowering herb is often known as "*Black cumin, nutmeg or fennel flower*" (Qidwai *et al.*, 2009), belongs to Family Ranunculaceae with 5 to 10 petals and 20 to 90 cm of height (Ahmad *et al.*, 2014). This plant is natively belonging to North Africa, Southern Europe and Asia Minor whereas is also cultivated in Pakistan and India. This plant is capable to do miracle because of its amazing healing power and used as treatment to several diseases. There are reports that *Nigella sativa* possess many therapeutics properties. For example, *in vitro, in vivo* and preclinical studies have proven that *Nigella sativa* has anti-inflammatory (Ahmad and Beg, 2013; Alemi *et al.*, 2013; Pichette *et al.*, 2012), cytoprotective, neuroprotective (Javanbakht *et al.*, 2013; Alhebshi *et al.*, 2013; Dariani *et al.*, 2013; Kanter, 2008), antitumor (Bai *et al.*, 2013; Woo *et al.*, 2012; Majdalawieh *et al.*, 2010; Salim, 2010), antimicrobial and immunopotentiation (Saleem, 2005), and antioxidative effects (Ashraf *et al.*, 2011). In addition, *Nigella sativa* is useful in the treatment of phenotypes that may have gone awry in mental illnesses. For instance, *Nigella sativa* reduces anxiety-like behavior (Ajao *et al.*, 2016) and enhances spatial

memory in rats (Anaeigoudari *et al.*, 2018; Farimah *et al.*, 2016; Imam *et al.*, 2016). Increased motor activity has been reported with use of *Nigella sativa* (Ajao *et al.*, 2016; Parveen *et al.*, 2009). Researches also revealed that *Nigella sativa* oil has the capability to lower body weight, lipid and glucose levels and to ameliorate abnormal insulin levels in rats (Alli-oluwafuyi *et al.*, 2017; Al-Jasass and Al-Jasser, 2012; El-Dakhakhny *et al.*, 2002b).

1.4.3. *Cocos nucifera*

Cocos nucifera, commonly known as coconut, is a family member of Arecaceae (Gardens, 2014; Janick and Paull, 2008). It is distinctive among dietary fats as its main composition is saturated fatty acids (about 92%) with 62% being Medium Chain Triglycerides (MCT), (Krishna *et al.*, 2010). Whereas, high proportion is also composed of phenolic acids, sometimes called polyphenols. Phenolic acids are famous for their excellent antioxidant properties. Few major phenolic acids present in coconut oil are ferulic acid, catechin acid, caffeic acid and p-Coumaric acid, (Marina *et al.*, 2009).

In current time period coconut oil is being use as dietary supplement due to a huge number of benefits because of its variety of biological activities like anti-obesity and antibacterial (Assunçao *et al.*, 2009; Nevin and Rajamohan, 2004), antioxidant and anti-inflammatory (Vysakh *et al.*, 2014; Intahphuak *et al.*, 2010). Coconut oils are made up of MCTs, which is a powerful brain food because brain uses nearly 20% of the caloric energy you eat (Marcus and Debra, 2001). Almost 2% of human body weight is comprised of brain and it needs 20 % of oxygen and calories (Clark *et al.*, 1999). Virgin coconut oil taken out from fresh coconut milk, has been shown to enhance the cognitive functioning of normal adult Wistar rats by enhancing synaptic transmission (Rahim *et al.*, 2017). Virgin Coconut oil has also been reported to be capable of significantly decreasing the brain-derived neurotrophic factor (BDNF) protein expression (Patricia *et.al.*, 2018; JS Rao, 2007). In contrast, medium chain fatty acid containing coconut oil is a ketogenic diet and ketogenic diet is able to increase the BDNF expression (Vizuete *et al.*, 2013).

Up to date, no investigation has been conducted which estimated the effects of *Nigella sativa* oil (NSO) and Virgin Coconut oil (VCO) supplementation for alcohol induced cognitive deficits, neurochemical changes and biochemical markers for metabolic syndrome or obesity and diabetes. In the current project we have find out the impact of NSO and VCO supplementations on alcohol induced changes on behavioral (spatial learning and memory, anxiety and locomotion), neurochemical (Serotonin, dopamine and noradrenaline) and biochemical (Insulin, glucose, triglycerides, total cholesterol) changes.

1.5. Animals Models of Addiction

Various animal models have been used so for, that explained the mechanism of addiction. Uses of animal models have led to easy and attainable detection of the neuropharmacological mechanism of addiction. Use of animal models can help to explore new therapeutics for various conditions like neurochemical and cognitive dysfunction as a result of addiction. Drug administration and addiction can be done by 2 main ways, 1) Self-administration and 2) Conditioned place-preference (CPP)

1.5.1. Self-Administration of Drug

Animal models for self-administration of drug are widely used in preclinical addiction research. Self-administration drug models are classified according to pharmacokinetics and behavioral aspect of drug. Pharmacokinetic aspect is based on route of the drug administration. Drug can be administered orally, intravenously or intraperitoneally. This yield different pharmacokinetics and ultimately different pharmacodynamics. Example is opioid drug, which gives different effects when taken orally or intravenously by humans. From behavioral aspect, self-administration of drug can be operant. Nonoperant method is restricted to self-administration of drugs orally. This method is very commonly used in alcohol research. It is also used for other illicit drugs like as amphetamine, cannabinoids, nicotine, opioids and cocaine (Meisch, 2001).

1.5.2. Conditioned Place Preference Test

This is a model/test to check rewarding impacts of any medicine in animals. The CPP is made up of 3-compartments separated by a sliding door. In CPP test, drug is injected to rats and coupled with a specific side or compartment. A second compartment is paired with vehicle injections and groups. Post repeated drug administration, a compartment preference is achieved. Then, this acquired preference is extinguished with repeated vehicle injections in both the previously drug-paired compartment and the vehicle -paired compartment. Then addiction effects are monitored.

1.6. Aim and Objectives

1.6.1. Aim

Aim of the current research is to find out potential of natural compounds from natural food like *Nigella sativa* and coconut that can reduce the risk and can be used to cure the most common health problems that developed by consuming alcohol in safe and inexpensive way.

1.6.2. Objectives

1) To explore the effects of chronic alcohol on behavior, neurotransmission (neurotransmitters' level), metabolic markers, catalase and hydrogen peroxide level in striatum.
2) To figure out the effects of *Nigella sativa oil* (NSO) and Virgin coconut oil (VCO) on memory, anxiety, locomotion in alcoholic male rats.
3) To find out the effects of NSO and VCO on neurotransmitters' level in alcohol drinking rats.
4) To find out the effects of NSO and VCO on biochemical markers of metabolic syndrome/obesity biochemical in alcoholic rats.
5) To find out the effects of NSO and VCO on catalase and hydrogen peroxide level in striatum of alcohol drinking rats.

CHAPTER # 2 LITERATURE REVIEW

2. Literature Review

2.1. Addiction

Addiction is a psychological and neurological disease (Koob *et al.*, 2004; Hser *et al.*, 2001). Addiction is characterized by insobriety of drug, unmanageable usage, desire, symptoms associated with withdrawal and sensitization. Addictive drugs are known as "psychoactive substances" as these drugs are capable to change behaviors and mind thoughts. Cocaine, nicotine, alcohol, amphetamine, opiates, barbiturates and cannabis are most abused drugs (World Drug Reports, 2018; Cami and Farre, 2003). Greater than 2000 million world population are addicted to cocaine, heroin, alcohol, cannabis and tobacco (Monteiro, 2001).

Addictive substances i.e. ethanol, heroin and morphine affect neurotransmitters levels in the parts of the brain. Addictive and abused drugs increase dopaminergic neurotransmission in pedunculopontine nucleus, frontal cortex, hippocampus, ventral tegmental and pedunculopontine nucleus (Vetulani, 2001). Increased dopamine transmission in nucleus accumbens is linked to drug-reward (Corominas-Roso *et al.*, 2007). Other than dopamine, serotonin (5-HT) also has a part in rewarding effects of illicit drugs. Preclinical studies conducted on dopamine transmitter knock out mice have proven the involvement of serotonin in reinforcing role of the self- administered cocaine (Sora *et al.*, 2001).

Among all the addictive drugs, alcohol is most commonly consumed drug and no other substance has been as comprehensively studied like alcohol, not only because it is one of the most abused drugs, but also because of its unique and interesting pharmacology.

2.2. Alcohol

Alcohol is a colorless, clear and volatile liquid at room temperature. The concentrated form possesses burning and strong taste, whereas the diluted form tastes sweet. It is insoluble in fats. Alcohol slows down communication of brain to rest of body

which results in unstable movement, garble speech, slow response and perceptions (Brands *et al.*, 1998). Alcohol beverages are formed of ethanol which is another form of alcohol.

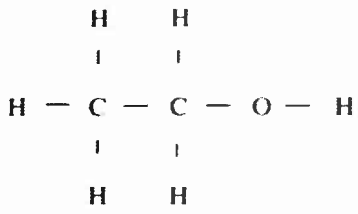


Figure 2.1: Biochemical structure of Alcohol

2.2.1. Alcohol pharmacokinetics

Absorption and distribution: Once ingested, alcohol is absorbed by gastrointestinal tract to distribute in body. Absorbance in stomach is affected by amount of food. After absorption, alcohol is uniformly and evenly distributed throughout the fluids and tissues. Distribution of alcohol in body water relies on gender (Jones, 2019). Female possess lower water content than males. If male and female consume similar amount of alcohol, the alcohol level would be higher in blood of females.

Metabolism: Alcohol is metabolized and oxidized mainly by the liver. Brain, stomach and pancreas also metabolize alcohol and less than 10% of alcohol is removed through kidneys, lungs and sweat. Alcohol metabolism in body takes place by three mechanisms:

1) The principle pathway involves cytosolic enzymes which help to metabolize and break alcohol molecule, making it possible to be eliminated it from the body. Metabolism of alcohol is catalyzed by cytosolic alcohol dehydrogenase enzyme and transformed to highly toxic and carcinogenic substance "acetaldehyde". This acetaldehyde is oxidized to acetate by mitochondrial aldehyde dehydrogenase enzyme (Purohit, Gao and Song, 2009; Sozio and Crabb, 2008). Acetate is broken down into water and carbon dioxide for easy elimination.
2) Ethanol-inducible cytochrome P-450 2E1 is responsible for microsomal oxidation of alcohol (Koop *et al.*, 1982).

3) Peroxisomes' catalase plays a minor function in ethanol metabolism by catalyzing < 2% of ethanol oxidation. This metabolism may increase during chronic ethanol consumption (De Craemer, Pauwels and Van den Branden, 1996).

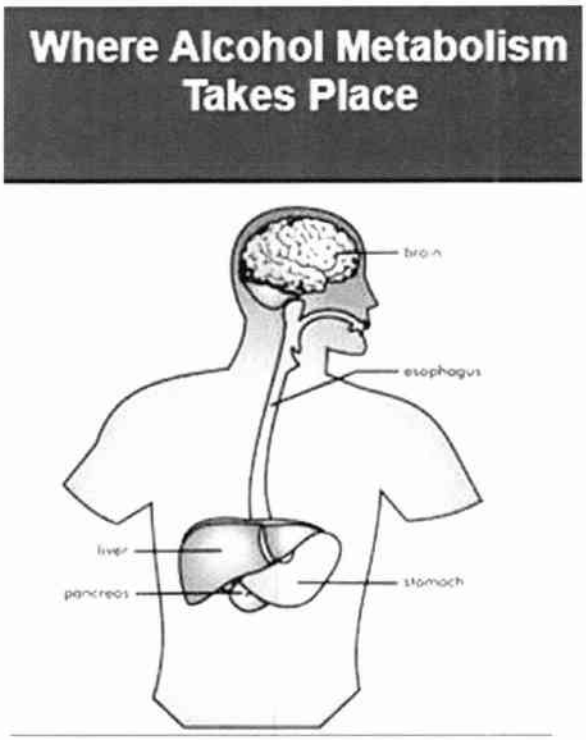


Figure 2.2: Alcohol Absorption and distribution. (Pictures adopted from https://www.emaze.com e)

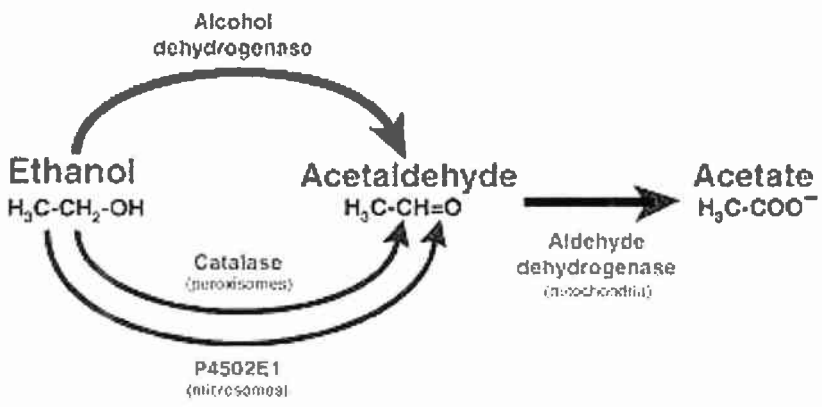


Figure 2.3: Alcohol Metabolism. Picture adopted from (https://pubs.niaaa.nih.gov/publications/arh27-4/285-290.htm)

2.2.2. Pharmacodynamics

Alcohol exerts its effects on almost each organ of the body. It exerts it effects on metabolism by prompting the impaired lipid metabolism. The alcohol at behavioral level results in anxiety, imbalanced motor activity and impaired memory. At brain level it disturbs CNS systems by blocking the chemical signals between neuronal cells.

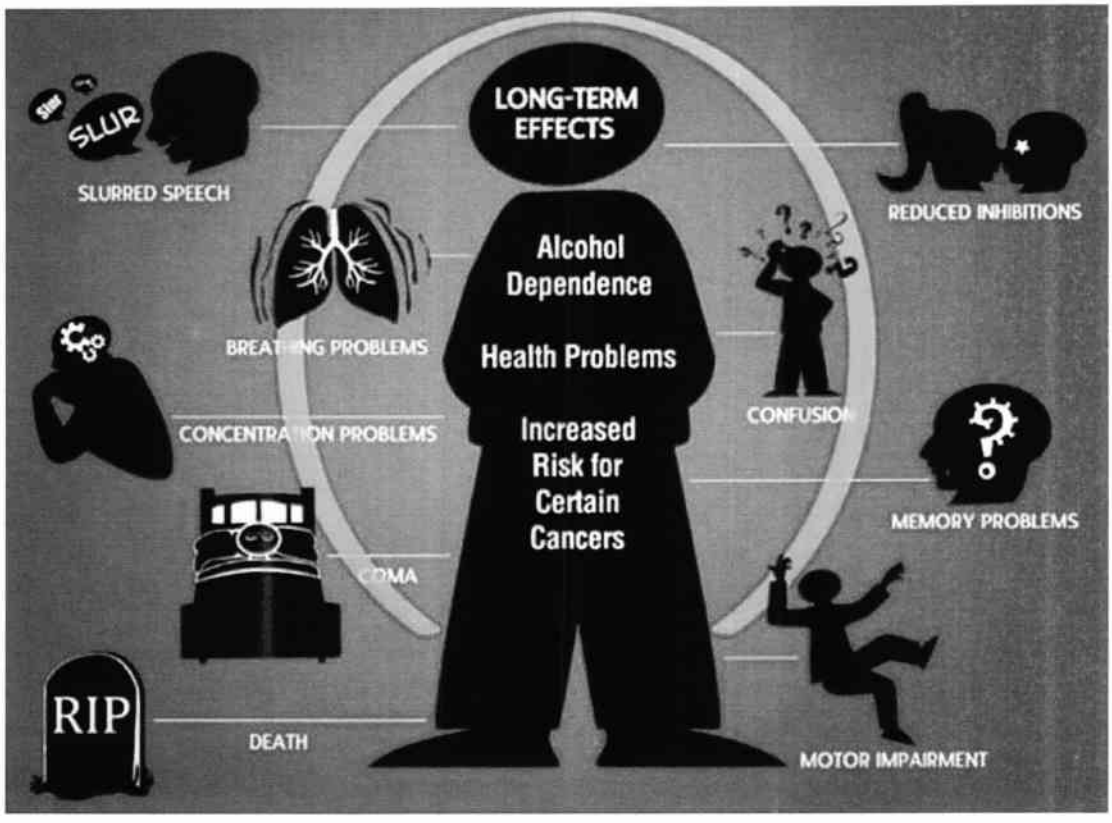


Figure 2.4: Alcohol Pharmacodynamics and Consequences of Alcohol. Picture adopted from https://www.horizon-health.org/blog/2015/04/the-short-and-long-term-effects-of-alcohol-on-the-body/

Glutamate: Excitatory neurotransmitter glutamate enhances brain functionality. Ethanol ceases glutamate secretion and lead to reduced activity and efficiency.

Gamma aminobutyric acid (GABA): GABA makes everything calm and smooth by lowering the brain energy level. Sedative substances increase GABA production in the brain. So ethanol mimics GABA's. It binds to its receptors and inhibits neuronal signaling.

Serotonin: Acute alcohol consumption makes you happy by increasing serotonin concentrations, whereas chronic alcohol consumption causes decrease in serotonin concentration. This reduced serotonin results in depression. **Dopamine:** Alcohol elevates dopamine concentrations in brain and affect reward system. Addictive drugs increases dopamine synthesis and release.

2.3. Alcohol and Behavior

Alcohol use leads to various unusual diseases and conditions, including mental to behavioral disorders. Studies have demonstrated that many factors including genetics play part in developing alcohol linked diseases (Nurnberger *et al.*, 2004; Liu *et al.*, 2004). Alcohol use is linked to increased anxiety and memory loss (Kristeen and Tim, 2016; Holmes *et al.*, 2012; Wixted, 2005; Hartzler and Fromme, 2003; White, 2003). Clinical and preclinical studies have described the alcohol and cognition relationship (Brown *et al.*, 2010; Hoffmann and Matthews, 2001). Adults are prone to alcohol adverse effects mainly on cognition (Swartzwelder *et al.*, 1995) and spatial memory (Rajendran and Spear, 2004), but young are more susceptible to ethanol hazards. Impaired cognition in aging is believed to be caused by altered synapses and plasticity (Seidler *et al.*, 2010). These changes might occur due to various ways and mechanism and examples like altered neurotransmitters level, damaged hippocampus, a brain structure involved in learning and memory by promoting apoptosis, changes in cholesterol and lipoprotein metabolism or oxidative stress.

Many harmful effects on metabolism have been reported with alcohol consumption and these risks are widely known in persons having familial predisposition to hyperlipidemia (Sane *et al.*, 1984). Impairment in cholesterol metabolism results in high fat accumulation in the body, and thereby obesity. In a recent study, it has been demonstrated that longterm ethanol use increases homocysteine concentration which causes Hyperhomocysteinemia. Hyperhomocysteinemia increases endoplasmic reticulum stress by promoting oxidative stress. Epigenetic changes lead to impaired cognition (George *et al.*, 2018).

2.4. Learning and Memory

Learning can be defined as a method of gaining any new information while storing and saving for later is called memory. This is a psychological mechanism performed by synaptic neurons in brain. Cognition is all the mental abilities like critical thinking, languages, reasoning and is mainly relies on memory (Kandel, Schwartz and Jessell, 2000).

There are three stages or phases that tell about the structure and mechanism of memory formation (Atkinson and Shiffrin, 1968). The three stages are:

I. **Encoding**: acquiring any new information
II. **Storage:** The saving of memory in system based on a kind of memory.
III. **Memory Retrieval:** recovery of memory from system.

Based on these phases, following models and types of memory were proposed.

i. **Sensory memory:** Memory formation starts with observations and information like, a touch, a sound or vision received during sensory stage.

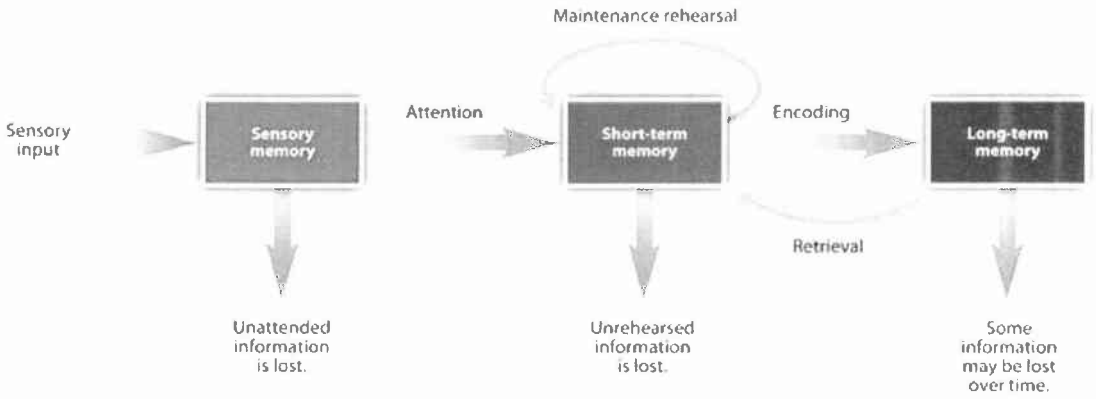


Figure 2.5: Stages of Memory.

Picture adopted from https://opentextbc.ca/introductiontopsychology/chapter/8-1-memories-as-types-and-stages/

ii. **Short term memory:** The information stored for short duration of time is known as short-term memory (STM). Sensory data is transformed into STM. It takes time

to convert from STM to long term memory (LTM). Stabilization and retention of this memory traces are known as consolidation. This phenomenon is called memory acquisition.

iii. **Long-term memory (LTM):** It is recovered STM after a long time, recovery of storage memory to work. Usually sensory memory and STM data is diminished and faded easily after short time period but working memory LTM can gather data for long time. It is recognized as memory retention,

2.5. Anxiety

Anxiety is characterized by feelings for future danger or misfortune with dysphoria signs of tension and focusing on threat (Martin and Richard, 1996; American Psychiatric Association, 1994). There are 6 major types of anxiety.

1) Panic Disorders (PD)
2) Obsessive Compulsive Disorders (OCD)
3) Generalized Anxiety
4) Posttraumatic Stress Disorders (PTSD)
5) Specific Phobia
6) Social Phobia

Acute anxiety can increase heart rate and blood flow. This make you able to cop up in intense situation, but chronic one might cause mental and physical health problems.

2.5.1. Anxiety and Central nervous system

Chronic anxiety can lead to increased release of stress hormones from brain which causes depression, dizziness and headaches. These stress hormones: cortisol, adrenaline and noradrenaline might be beneficial in acute and sever stress/anxiety but in chronic anxiety, exposure to these hormones is dangerous for the physical health in long term. For example, increased cortisol for long term can lead to weight gain.

Neurotransmitters are involved in the development and maintenance of anxiety or fear. For example, in OCD, the serotonergic system is important (Liebowitz and Hollander, 1991), in PD the noradrenergic system (Charney *et al.*, 1990) and in generalized anxiety GABA benzodiazepine system (Cowley and Roy-Byrne, 1991) have been most thoroughly studied. Dopamine is involved in reduced social exposure and social phobia (Levin, Schneier, and Liebowitz, 1989).

2.5.2. Anxiety and Respiration

Anxiety increases breathing rate to increase the oxygen level and supply. It exacerbates Asthma. There is increased danger and death risk in chronic obstructive pulmonary disease in combination with anxiety.

2.5.3. Anxiety and Cardiovascular system

Anxiety increases heart function, palpitation with pain in chest. It is a risk factor for high blood pressure and cardiac problems. In combination with any cardiac problem, anxiety disorders might cause coronary events (Celano *et al.*, 2016).

2.5.4. Anxiety effects on Digestive system

Anxiety exerts adverse effects on digestion and excretion. It decreases appetite, and causes nausea, stomach pain, diarrhea. Anxiety is also linked irritable bowel syndrome after a bowel infection. Irritable bowel syndrome can cause vomiting, constipation or diarrhea (Pasquali, 2012; Block *et al.*, 2009; Torres and Nowson, 2007).

2.5.5. Anxiety effects on Immune system

Anxiety triggers flight-or-fight stress response and secretes increased hormones and neurochemicals in system (Stein, Keller and Schleifer, 1988). This increases breathing and pulse rate to supple an adequate oxygen supply to brain. This enable one to face an intense situation. In this situation, the immune system may also get boosted. When the body is returned to normal functioning after stress is gone, immune system also returned

to normal. In chronic anxiety, the body never returned to normal functioning, the immune system gets weaken and making body vulnerable to infections and illnesses. Anxiety also makes you resistant to vaccines.

2.6. Alcohol and Neurochemistry

2.6.1. Dopamine in Alcohol addiction and behavior

Alcohol mediates effects on the reward system via dopaminergic systems (Aminoff, 2007) by releasing dopamine in ventral tegmental and nucleus accumbens regions of limbic system (Gilpin and Koob, 2008; Zaleski *et al.*, 2004; Castro LA, Baltieri, 2004). Dopamine is released in synaptic cleft and its binding with nerve cells' receptors D-1 and D-2 activates reward system and lead to addiction (Martins *et al.*, 2005; Castro and Baltieri, 2004; Zaleski *et al.*, 2004).

Alcohol exposure increase the dopamine level by interacting with NMDA receptors and increased release from nucleus accumbens through increased activity of mesolimbic dopaminergic neurons. It possesses affinity towards the N-Methyl-D-aspartate and $GABA_A$, like alcohol prompted potentiation (Jin *et al.*, 2008) and alcohol mediated behavior deficits.

2.6.2. Serotonin in Alcohol addiction and behavior

Studies have demonstrated role of serotonin (5-HT) in mood elevation during alcohol intoxication (Bittencourt, 2000; Lovinger, 1997; Boullosa and Lopez-Mato, 1995). Studies have demonstrated the alcohol effects on serotonin transmission, in various brain regions. In acute alcohol consumption, increased serotonin has been observed while the withdrawal caused reduced ion in its neurotransmission (Vengelien *et al.*, 2008; Laranjeira *et al.*, 2000). Decreased serotonin neurotransmission in dependent animals might be relapse. Like, reduced serotonin and alcohol consumption has been reported by administration of $GABA_A$ receptor agonist (Lee, Petty and Coccaro, 2008). There are several serotonin receptors subtypes (Hoyer, Hannon and Martin, 2002) and alcohol intake depends on serotonin receptor activation (Vengelien *et al.*, 2008; Lovinger, 1997).

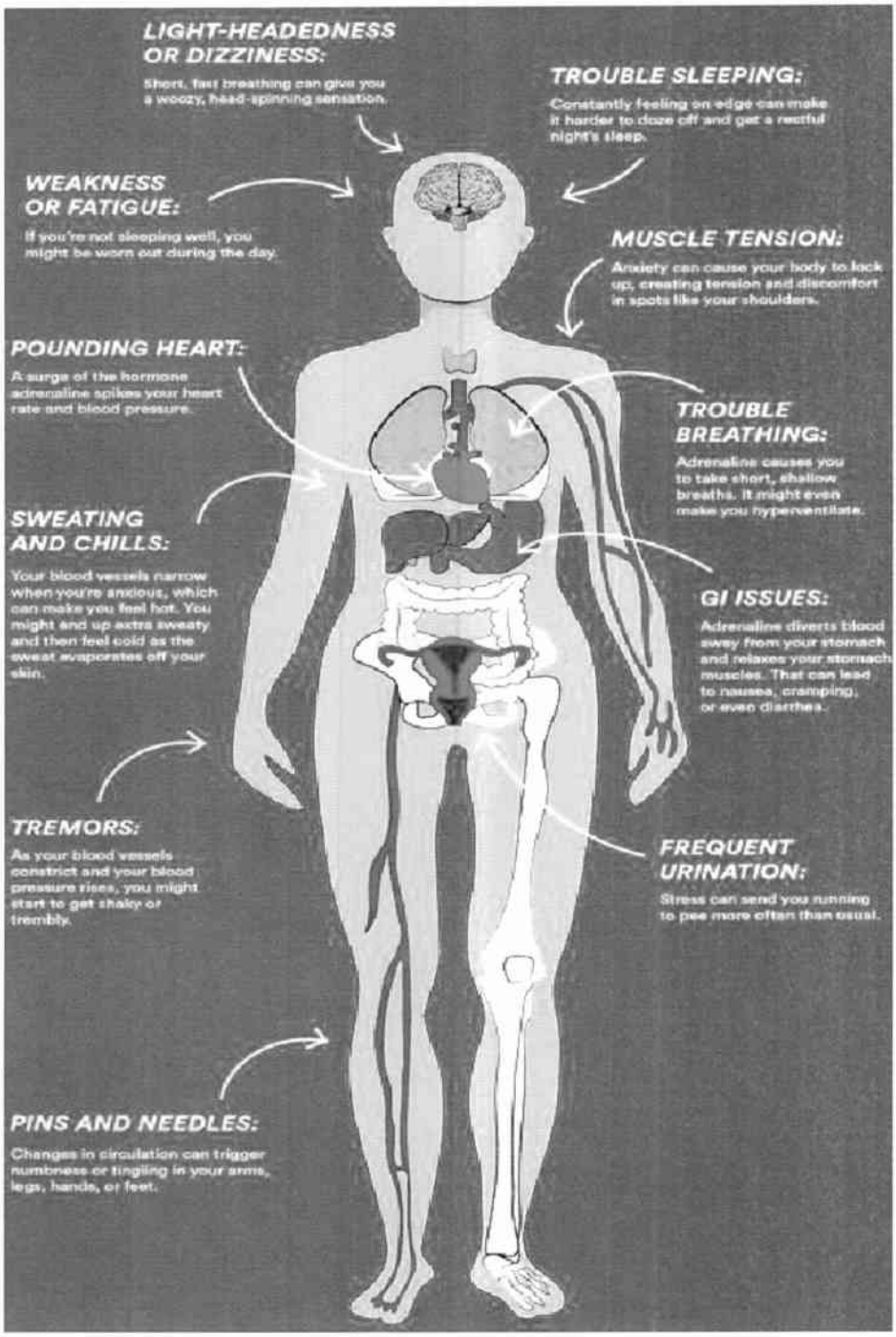


Figure 2.6: Physical symptoms of anxiety and its consequences on health. Adopted from https://greatist.com/health/physical-symptoms-of-anxiety

For example, activated 5-HT1A or 5-HT2C receptors decreases the alcohol intake and consumption (Vengelien *et al.*, 2008; Sartania and Strange, 2002; Tomkins *et al.*, 2002;

Lovinger, 1997). The role of 5-HT1B receptor is quite controversial, its increased or decreased synthesis can lead to increased alcohol consumption (Vengelien *et al.*, 2008; Blednov *et al.*, 2007; Risinger, Doan and Vickery, 1999; Lovinger, 1997), but elevated 5-HT1B receptors are believed to exert much significant effects. Inhibition of 5-HT3 receptors also been known to decrease ethanol intake (Hodge *et al.*, 2004).

Alcohol can activate serotoninergic pathways indirectly by affecting dopaminergic system (Carboni, Frau and Di Chiara, 1989). The alcohol and 5-HT3 receptor relationship have been shown in researches which explored the mechanism of how serotonin is associated with alcoholism (Lovinger, 1991). Few drugs' component, i.e. 5-HT3 antagonist ondansetron has successfully decreased alcohol intake (Kranzler *et al.*, 2003; Ait-Daoud e*t al.*, 2001). A 5-HT1A receptor agonist buspirone can also inhibit increased alcohol intake (Kranzler *et al.*, 1994). Collectively, researches are clarifying that serotonin receptors' location and functions determine their roles in managing the alcohol usage (Bittencourt, 2000).

2.6.3. Noradrenaline in behavior

Alcohol administration may interact with psychological stress to affect noradrenaline signaling. Noradrenaline is a fight and flight response hormone. Some researchers have found that acute alcohol use elevates norepinephrine concentration and results in arousing nervous system. But Continuous alcohol consumption decreases brain noradrenaline and reduces the activity of the enzyme dopamine beta-hydroxylase responsible for noradrenaline synthesis in rat hippocampus (Yamanaka, 1982). Such changes in noradrenaline levels may mediate the alcohol-induced increases in anxiety (Karoum *et al.*, 1976).

2.6.4. GABA

Ethanol can alter the $GABA_A$ receptor expression. Two subunits of $GABA_A$ receptors are there, the large one and smaller one. Phosphorylation state of long subunit has major part in inhibitory effects of alcohol (Lovinger, 1993). The preclinical studies on

alcohol have demonstrated the significant reduction in messenger RNA of the a1 and a2 subunits with increased messenger RNA concentrations of a4, g1 and g2S subunits (Sieghart, 1995).

In chronic alcohol consumption, downregulated GABA receptors and their expression has been observed, this clarify the alcohol tolerance (Zheng *et al.*, 2009; Beresford *et al.*, 2006). The lack of GABA receptors causes withdrawal symptoms (Lüddens, Seeburg and Korpi, 1994). Beresford *et al.*, 2006 has reported that chronic alcohol intake impaired memory by acting like GABA and decreased hippocampal mass.

2.6.5. Glutamate

Receptors for glutamate affects alcohol sensitivity towards glutamatergic neurotransmission. The calcium signals in activation of glutamate subtypes receptors ionotropic (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, N-methyl-D-aspartate and kainate) and metabotropic (rM1 and rM8) are different in sensitivity to alcohol (Castro and Baltieri, 2004). Chronic alcohol usage elevates glutamatergic receptors in hippocampus which is most important for memory and seizures (Charlton *et al.*, 2002).

2.7. Alcohol Consumption and Metabolic Syndrome

Alcohol metabolism reduces uric acid excretion which produces secondary hyper-uricemia and also impairs glucose metabolism and results in hypoglycemia. Development of steatosis is linked with impaired fat metabolism: H^+ ions replace two-carbon fragments derived from fatty acids as main energy source in hepatocyte mitochondria and also depress citric acid cycle (Tinakos, Anstee and Burt, 2018). Long-term ethanol use depresses protein synthesis together with impaired liver cell secretory function, causes retention of lipoproteins and results in accumulation of fat in hepatocytes.

Alcohol enhances the risk of weight-gain (Wakabayashi, 2009). Increased energy intake from alcohol is a risk factor for obesity (Yeomans *et al.*, 2003). In contrast, Fromenty and colleagues (2009) reported that chronic alcohol use significantly decreased body weight and

diminished type 2 diabetes in obese (ob/ob) mice. In another study decreased body weight in alcohol drinking rats was recorded because of malnutrition that resulted from decreased nutrient absorption in intestine (Macdonald *et al.*, 2010). At blood level, alcohol consumption has been reported to alter glucose levels and impair its metabolism causes hypercholesterolemia and insulin resistance (Jennifer *et al.*, 2015; Toffolo *et al.*, 2012; Qiwei, 2010; Yu *et al.*, 2000; Wilkes, 1996).

2.8. Alcohol and Oxidative Stress

Abnormal ethanol metabolism effects might occur because of oxidative stress. This causes cellular modifications linked with inflammation which eventually cause cell cycle arrest or cell death (Barcia *et al.*, 2017). In tissues, alcohol mediated effects are present due to oxidative stress (Bondy and Guo, 1995), lipid peroxidation and free radical formation (Flores-Bellver *et al.*, 2014; Almansa *et al.*, 2013; Ramachandran *et al.*, 2003; Bosch-Morell *et al.*, 1998; Sun *et al.*, 1997) catalase or hydrogen peroxide enzymes. The most toxic property of ethanol is to favor the formation of reactive oxygen species (ROS). The reaction of ROS with macromolecules, for example membrane lipids, forms aldehydes like Malondialdehyde (MDA) and 4-Hydroxynonenal. ROS and aldehydes affect DNA and proteins via transcription-repression of genes. Studies have confirmed this with the antioxidant supplement experiments, the supplements prevented cellular alterations with alcohol use (Herrera *et al.*, 2003; Bati *et al.*, 2015; Han *et al.*, 2015).

2.9. Dopamine Neurotransmission

Dopamine (DA), a neurotransmitter made from non-essential amino acid called tyrosine. It also known as hydroxy tyramine and comes under the category of catecholamines. Dopamine serves many vital function (Schultz, 2007: Vallone, Picetti and Borrelli, 2000).

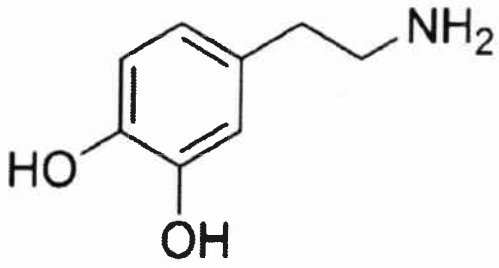


Fig 2.7: Chemical structure of dopamine.

Dopaminergic neurons: Hypothalamus and midbrain regions of brain have enormous number of dopaminergic neurons (Bjorklund and Dunnett, 2007). Dopamine is synthesized in ventral tegmental and substantia nigra regions (Cossette, Lecomte and Parent, 2005) and it reaches to other brain areas (Grace *et al.*, 2007; Chinta and Andersen, 2005).

Synthesis and storage: Dopamine synthesis takes place in dopaminergic neurons initially from L-DOPA, which itself is formed from tyrosine (Daubner, Le and Wang, 2011; Dunkley *et al.*, 2004) and L-phenylalanine (Lou, 1994). An enzyme called phenylalanine hydroxylase transformed phenylalanine amino acid into tyrosine amino acid when enough of tetrahydrobiopterin and oxygen is present. Once tyrosine enters neurons, an enzyme tyrosine hydroxylase transforms tyrosine into L-DOPA in presence of tetrahydrobiopterin, molecular oxygen and iron (Lindstrom *et al.*, 1999). In presence of pyridoxal phosphate L-DOPA is transformed into dopamine by DOPA decarboxylase (Cumming *et al.*, 1995). Further, dopamine is antecedent of epinephrine (Napolitano *et al.*, 1999). The conversion of dopamine into epinephrine is done by action of enzyme dopamine β-hydroxylase in adequate bioavailability of co-factor L-ascorbic acid (Zabetian *et al.*, 2001).

Secretion to metabolism: Vesicles releases DA into synaptic cleft by exocytosis in calcium dependent manner (Fon *et al.*, 1997; Eshleman *et al.*, 1994). After the dopamine release, it is removed from extracellular environment by presynaptic transporters or degenerate in synapse. In neurons, it binds with the receptors in synaptic cleft. Dopaminergic neuron takes back some unbound DA with help of reuptake receptors, (Haenisch and Bonisch, 2011; Gainetdinov *et al.*, 1998). The monoamine oxidase (MAO)

degrade and remove the dopamine (Carradori and Silvestri, 2015) from extracellular spaces.

Metabolism of dopamine is performed by aldehyde dehydrogenase Catechol-O-methyltransferase and MAO (Meiser, Weindl and Hiller, 2013) and the metabolites formed are DOPAC, 3-methoxytyramine, ,4-Dihydroxyphenylacetaldehyde (DOPAL) and homovanillic acid (HVA) (Elsworth and Roth, 1997). HVA is the final metabolite and product which is removed from body through excretion (Sher *et al.*, 2006; Lambert *et al.*, 1991).

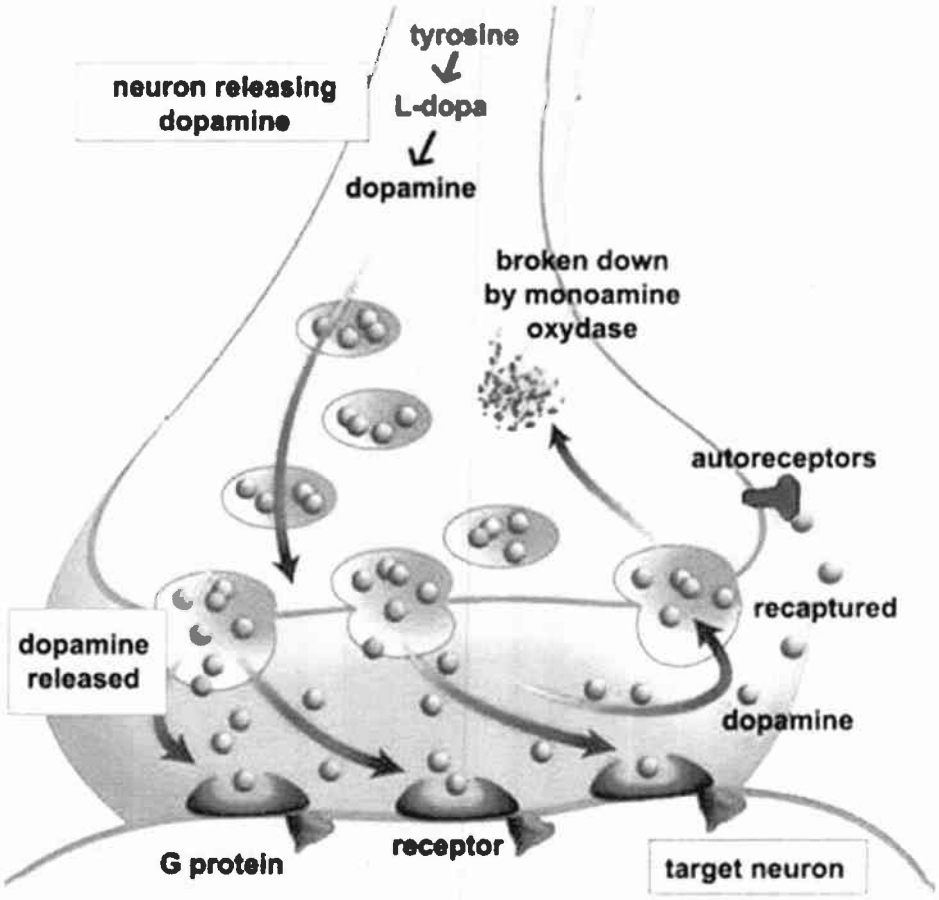


Figure 2.8: Biosynthesis and secretion of DA.

Picture adopted from: www.thebrain.mcgill.ca.

Receptors: Dopamine physiological effects and functions are achieved by binding of dopamine to 5 different receptors (Abi-Dargham, 2004: Vallone, Picetti and Borrelli, 2000). There are 2 subtypes of dopamine receptors, DA1 like and DA2 like receptors. D1 and D5 comes under the DA1 like receptors and activates adenylyl cyclase. The D2, D3 and D4 are under DA 2 like receptors and inhibits adenylyl cyclase (Feuerstein, 2008: Usiello *et al.*, 2000).

2.9.1. Dopamine and Addiction

For addiction of drugs, dopamine plays a crucial role. Recent research work depicts dopamine's involvement in addicted side-effects of exploited drugs. During the smoking of cigarette, an increase in Dopaminergic activity was observed (Barrett *et al.*, 2004). In amphetamine induced reinforcement, an increase in DA levels were reported (Oswald *et al.*, 2005). In the nucleus accumbens, the alcohol and amphetamine amplified the DA activity (Boileau *et al.* 2003, Leyton *et al.*, 2002), which signified the nucleus accumbens role in rewarding effects.

Dopamine works in three different pathways: mesocortical, nigrostriatal and mesolimbic. Inside the substantia nigra cell bodies of nigrostriatal pathway are present and leads to caudate, whereby motor activities are controlled by these pathways (Bergquist, Shahabi and Nissbrandt, 2003; Timmerman and Abercrombie, 1996; Robertson *et al.*, 1989; Ungerstedt, 1976). Natural or drug prompted reward are due to the mesolimbic pathway, this pathway's cell bodies are placed in ventral tegmental area and leads to nucleus accumbens (Haleem, 2013 and 2015). Mesocortical pathway starts from ventral tegmental area and innervating at frontal cortex has an impact on cognition (Masana *et al.*, 2012).

2.9.2. Role of Dopamine in Memory Enhancement

Dopamine regulates cognition by adjusting the plasticity and synaptic transmission in prefrontal cortex and hippocampus (De Bundel *et al.*, 2013). Dopamine act as mediator for generation of memory from original information and emotional experiences (Shohamy,

and Adcock, 2010). Dopamine neurons enhance ventral tegmental area because of novel reward (Cohen *et al.*, 2012; Nakahara *et al.*, 2004) and boost dopamine release in prefrontal cortex and hippocampus (Bassareo, De Luca and Di Chiara, 2002). Activation of D1/5 receptor in various regions enhances synaptic neurotransmission (Xu and Yao, 2010; Ortiz *et al.*, 2010; Gurden, Takita and Jay, 2000) which generates memory (Neves, Cooke and Bliss, 2008). Dopamine has been found to responsible for the cognitive deficits i.e., attention deficits and impaired memory (Millan *et al.*, 2012; Disner *et al.*, 2011). Prefrontal cortex and hippocampal functional abnormalities are also linked to these deficits (Clark, Chamberlain and Sahakian, 2009). Furthermore, dopamine level rises in prefrontal cortex and hippocampus by administration of reboxetine (Linnér *et al.*, 2001) and of other drugs like methylphenidate (Carmack *et al.*, 2014).

2.9.3. Role of Dopamine in Anxiety

For anxiety and depression, dopamine contributes essentially (Hamner and Diamond, 1996). Cognitive and behavioral disorders can be due to deficiency of tyrosine (Grattan- Smith *et al.*, 2002). Exogenous supply of tyrosine significantly ameliorates behavioral disorders including impaired memory and anxiety. McTavish, Cowen and Sharp, 1999, have reported the improvements in stress and depression when tyrosine was given to clinical subjects. Dopamine also efficiently controls locomotion and balance (Brooks, 2001). Serotonin neurotransmission is renounced to effect DA neurotransmission (Alex and Pehek, 2007).

Dopamine possess several physiological roles that are summarized in Table 2.1 (taken from Missale *et al.*, 1998).

Table 2.1 Physiological functions of DA.

Receptors Name	Region specific roles
D1	**Location**: Central nervous system and kidney. **Regulatory Roles**: Voluntary control, growth, development, hunger, reproduction, sleep, learning behavior.
D2	**Location**: Central nervous system, kidney, heart, adrenal gland, Gastrointestinal tract and sympathetic ganglia. **Regulatory roles**: Gastrointestinal tract (GIT), blood pressure, locomotion, cognition, rewarding effect.
D3	**Location**: Central nervous system **Regulatory roles**: hormones, cognition, emotions, locomotory activities.
D4	**Location**: Central nervous system, kidney, heart, adrenal gland, gastrointestinal tract and sympathetic ganglia. **Regulatory roles**: Vasodilation, GIT motility, renal and cognition.
D5	**Location**: Central nervous system, kidney, heart, adrenal gland, gastrointestinal tract, dental gyrus and sympathetic ganglia. **Regulatory roles**: Hormones and pain.

2.10. Serotonin Neurotransmission

Serotonin is made from L-tryptophan. In literature, other terms used for this are called 5-hydroxytryptamine (5-HT). This monoamine and hormone found in skin, central nervous system, gastrointestinal tract, lungs, liver and blood spleen (Markus, 2008). Serotonin play part in physiological functions (Lucki, 1998: Markus, 2008).

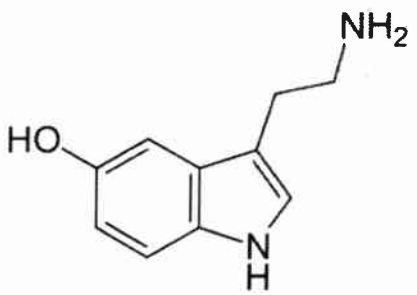


Figure 2.9: Biochemical structure of serotonin

Serotonergic neurons: Serotonin is formed in neurons of raphe nuclei (Lucki, 1998). Axons distribute it to other brain areas (Dorocic *et al.*, 2014; Lucki, 1998). Descending fibers project into mid bran, spinal cord, pontine and cerebellum (Dorocic *et al.*, 2014; Zhuang *et al.*, 2005). Ascending fibers project into remaining brain (Zhuang *et al.*, 2005). Researches have also showed the uneven distribution of serotonin in brain (Kocsis *et al.*, 2006; Hornung, 2003).

Synthesis: Carrier proteins carrie tryptophan from blood to brain (Birdsall, 1998). 5-HT level and synthesis relies on Tryptophan concentration in blood (Meyer and Brinck, 1999).

In brain, tryptophan is transformed into 5-hydroxy tryptophan (5-HTP) with help of hydroxylase rate limiting enzymes (Martínez, Knappskog and Haavik, 2001) which is also known as Trp-5-monooxygenase. This enzyme has 2 isoforms (Walther *et al.*, 2003: Zhang *et al.*, 2004). Pteridine and oxygen are mandatory for the reaction (Walther *et al.*, 2003). In addition to this, decarboxylase enzymes transform 5-HTP into 5-HT (Hyland *et al.*, 1992). The enzyme needs pyridoxal phosphate to react. After release, vesicles keep 5-HT stored.

Release and reuptake: Presynaptic vesicles discharge serotonin into synaptic cleft by exocytosis in calcium-dependent manner (De-Miguel and Trueta, 2005; Chaouloff, 2000) and this calcium influx is because of an action potential. After release, 5-HT binds with receptors.

Once the 5-HT effects are diminished, reuptake transporters broken down or takes 5-HT back again by presynaptic neuron (Decker and Lehmann, 2007; Reivich *et al.*, 2004). The MAO enzyme degrade serotonin to form 5-hydroxyindole acetaldehyde (5-HT), which further converted to 5-hydroxyindole acetic acid (5-HIAA) by the action of an enzyme aldehyde dehydrogenase (Benedetti, 2001; Sarre *et al.*, 1997). In minute quantity, 5-hydroxyindole acetaldehyde may also transformed into 5-hydroxytryptophol with alcohol reductase (Omahony *et al.*, 2015; Sarre *et al.*, 1997).

Receptors and Roles: Sometimes few physiological processes are regulated by 5-HT by receptors binding in central and peripheral nervous systems. (Berger, Gray and Roth, 2009; Naughton, Mulrooney and Leonard, 2000). The seven 5-HT receptors are there (Buhot, 1997) which are classified as G-protein coupled receptor (GPCR) and ligand gated ion channels (Wacker *et al.*, 2013; Stahl, 1998). Except 5-HT3, all remaining receptors are GPCRs. As 5-HT3 are ligand gated channel receptor, act as natural ligands and agonist and antagonist can also form bond with 5-HT3 receptors (Anguelova, Benkelfat and Turecki, 2003).

5-HT also possess significant function in psychological and physiological systems. It controls and can exert effects on hormones release from anterior pituitary gland, bodyweight, food intake hunger, mood, sleep, anxiety, memory, locomotion and depression (Rogers, 2011; Steiner, 2011). Impaired learning and memory have been associated with reduced 5-HT levels in brain. (Buhot, Martin and Segu, 2000; Harvey, 2003). Table 2.2 summarize the important features of 5-HT receptors (Table adopted from Mohammad Zadeh, Moses and Gwaltney- Brant, 2008).

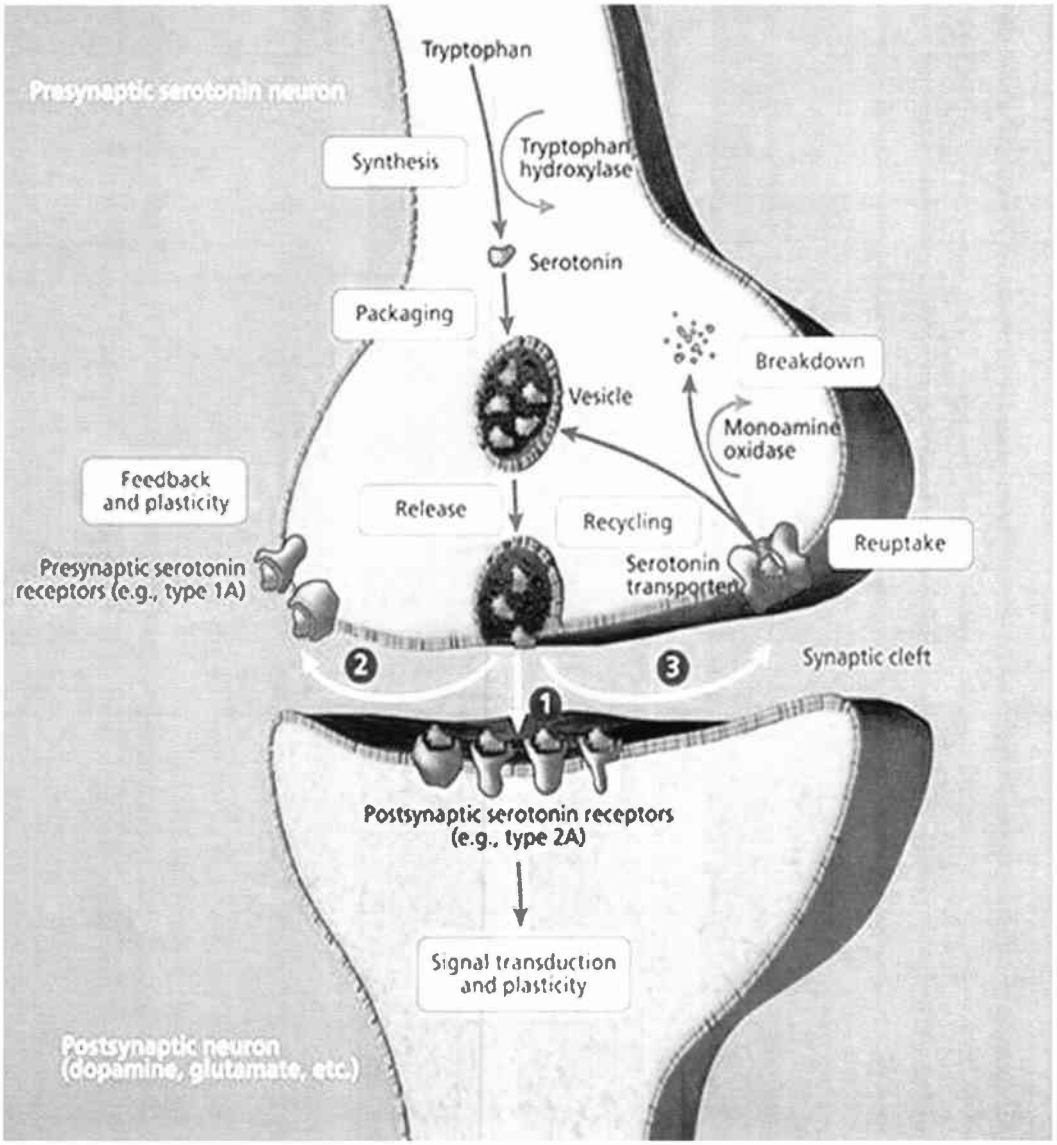


Figure 2.10: Biosynthesis, release and reuptake of serotonin.

Picture adopted from: Aanhetrot, Mathew and Charney, 2009.

2.10.1. Serotonin-1A (5-HTIA) Receptors

5-HT1 receptor family includes three receptor sub-types namely 5-HT1A, 5-HT1B and 5-HT1C. (Barnes and Sharp, 1999). These receptors play a significant part in maintaining the weight and food consumption, stress, mood and pain (Albert and Lemonde, 2004; Barnes and Sharp, 1999). Serotonergic neuronal cell bodies present raphe nuclei possess 5-HT1A auto-receptors. It inhibits somato-dendritic auto-receptor (Lanfumey and Hamon, 2000). Once activated, it inhibits the 5-HT and lead to decreased release and level of serotonin (Bose *et al.*, 2011; Hoyer, Hannon and Martin, 2002).

2.10.2. 5-HTIA Receptors in Rewarding Effects of Abused Drugs

Previously, it has been proven that alterations in action mechanism of somatodendritic serotonin-1A receptors affects the rewarding and addictive impacts of apomorphine and ethanol (Haleem and Farhan, 2015; Haleem, Ikram and Haleem, 2014, Ikram and Haleem, 2011; Haleem *et al.*, 2002).

2.10.3. 5-HT1A Receptors in The Modulation of Addiction and behavior

The 5-HT1A receptors are crucial for the development of drug addiction and behavioral sensitization (Katsidoni, Anagnostou and Panagis, 2013; Ikram and Haleem, 2011; Lanteri *et al.*, 2009; Muller *et al.*, 2007) and also in alteration of dopamine transmission via 5-HT1A agonists (Haleem, 2013; Haleem, Inam and Haleem, 2014). In a preclinical study, reduced sensitization has been observed by stimulating the activation of 5-HT1A receptors by amino tetralin which is agonist of 5-HT1A (Przegalinski *et al.*, 2000) and activated 5-HT1A receptors also enhanced the cognition particularly memory (King, Marsden and Fone, 2008). 5-HT1A receptor agonist also possess anxiolytic and antidepressant properties (Blier and Ward, 2003; Hensler, 2003).

5-HT1A receptors with 5-HT2A receptors have part in feeding habits and anxiety (Celada *et al.*, 2004; Graeff, Viana and Mora, 1997). Anxiety is a profound behavioral deficits sign of chronic alcoholism. Anxiety and hyperactivity are found in almost all chronic alcoholics. Depression found in patients can be resulted from decreased serotonin

neurotransmission (Gauthier *et al.*, 2014; Brewerton and Jimerson, 1996). Preclinical researches have reported tremendous increase in 5-HT1A auto receptor in anorexia nervosa disease (Akimova, Lanzenberger and Kasper, 2009) because of reduction in 5-HT transmission (Lam *et al.*, 2010). Increased sensitivity of 5-HT1A receptors reduces serotonin bioavailability in hypothalamus has been observed (Li *et al.*, 2000) and insensitivity towards 5-HT1A receptors has also been observed in stressed and anxious animals (Flugge *et al.*, 1998).

Table 2.2 Location and functions of 5-HT receptors.

Receptors' Name	Regional effect
5-HT1A	**Area**: Central nervous system **Roles**: maintenance of anxiety, feeding and sleep behavior.
5-HT1B	**Area**: Central nervous system **Roles**: Behaviors and neuronal inhibition.
5-HT1C	**Area**: Central nervous system and blood vessels **Roles**: Vasoconstriction and locomotion.
5-HT2A	**Area**: Central nervous system, smooth muscles, platelets. **Roles**: Muscular contractions, behaviors
5-HT2B	**Area**: Enteric nervous system. **Effects**: Motility of gut.
5-HT2C	**Area**: Central nervous system **Effects**: Anxiety like behavior and hunger.
5-HT3	**Area**: Sensory nerves **Effects**: Vomiting

5-HT4	**Area**: Central nervous system and Enteric nervous system **Roles**: Motility of gut.
5-HT5A	**Area**: Central Nervous system **Roles**: Not known
5-HT6	**Area**: Central nervous system **Roles**: Not known
5-HT7	**Area**: Central nervous system, blood vessels and Enteric nervous system. **Roles**: Not known

2.11. *Nigella sativa*

Among herbal medicinal plants *Nigella sativa* (NS) is placed in list of most evidences-based plant medicines (Juma and Hayfaa, 2011). *Nigella sativa* is often known as "*Black cumin, nutmeg or fennel flower*" (Qidwai *et al.*, 2009), belongs to Family Ranunculaceae with 5 to 10 petals and 20 to 90 cm of height (Ahmad *et al.*, 2014).

This plant is natively belonging to North Africa, Southern Europe and Asia Minor whereas is also found in Pakistan and India. It is considered as a miracle plant because of its amazing healing power and used as treatment to several diseases. There are reports that *Nigella sativa* possess many therapeutics properties. For example, in vitro, in vivo and preclinical studies has proven that *Nigella sativa* has anti-inflammatory (Pichette *et al.*, 2012; Ahmad and Beg, 2013; Alemi *et al.*, 2013), cytoprotective, neuroprotective (Javanbakht *et al.*, 2013, Alhebshi *et al.*, 2013; Dariani *et al.*, 2013, Kanter, 2008), antitumor (Majdalawieh *et al.*, 2010; Salim, 2010; Woo *et al.*, 2012; Bai *et al.*, 2013), antimicrobial and immunopotentiation (Saleem, 2005), and antioxidative effects (Ashraf *et al.*, 2011). In addition, *Nigella sativa* is useful in the treatment of phenotypes that may have gone awry in mental illnesses. For instance, NS reduces anxiety-like behavior (Ajao *et al.*, 2016) and enhances spatial memory in rats (Farimah *et al.*, 2016; Imam *et al.*, 2016; Anaeigoudari *et al.*, 2018). NS also increase motor activity (Parveen *et al.*, 2009; Ajao *et al.*, 2016). Many researches also revealed that NS oil has the capability to lower body weight, lipid and glucose levels, and to ameliorate abnormal insulin levels in rats (Alli-oluwafuyi *et al.*, 2017; El-Dakhakhny *et al.*, 2002b; Al-Jasass and Al-Jasser, 2012).

2.12. *Cocos nucifera*

Cocos nucifera, commonly known as coconut, a family member of Arecaceae (Janick and Paull,2008; Gardens,2014). It is distinctive among dietary fats as its main composition is saturated fatty acids (about 92%) with 62% being medium chain triglycerides (MCT), (Krishna *et al.*, 2010), Whereas high proportion is also composed of phenolic acids, sometimes called polyphenols. Phenolic acids are famous for their excellent antioxidant properties. Few major phenolic acids present in coconut oil are ferulic acid, p-

Coumaric acid, catechin acid and caffeic acid (Marina *et al.*, 2009). Main constituents of fatty acids in coconut oil is lauric acid (45–53%).

Coconut oils are made up of MCTs, which is a powerful brain food. The powerful brain food is because brain uses nearly 20% of the caloric energy you eat (Raichle and Giscard, 2002). Almost 2% of human body weight is comprised of brain and it needs 20% of oxygen and calories (Clark *et al.*, 1999).

Currently, period coconut oil is using as dietary supplement due to a huge number of benefits because of its variety of biological activities like antioxidant, anti-inflammatory (Intahphuak *et al.*, 2010; Vysakh *et al.*, 2014), anti-obesity and antibacterial (Nevin and Rajamohan, 2004; Assunçao *et al.*, 2009). Virgin coconut oil taken out from fresh coconut milk, has been shown to enhance the cognitive functioning of normal adult Wistar rats by enhancing synaptic transmission (Rahim *et al.*, 2017). VCO is has also been reported to capable to significantly decrease the BDNF expression (Patricia *et.al.*, 2018; Rao, 2007), but it is also reported that the medium chain fatty acid containing coconut oil is a ketogenic diet and ketogenic diet is able to increase the BDNF expression (Vizuete *et al.*, 2013).

The VCO is the best source of lauric acid. After absorption, coconut oil is quickly metabolized to transport lauric acid is. Researches have reported that most of the consumed lauric acid is transported to liver where it converts into energy with metabolite instead of storing as fat. Ketone bodies are the main metabolites and tissues like liver, heart and brain utilize it as energy. Studies have shown the least contribution of lauric acid in fat accumulation. It possesses antimicrobial properties as well. It can act as antimicrobial agent for various kinds of fungi, virus and for gram positive bacteria. (Dayrit, 2015).

2.13. Animal Models to Monitor Addiction and Behaviors

Use of animal models in research help in development of pharmacotherapeutics and improving treatment regimes. We aim to use Wistar rats because they are considered as the standardized rat strain for laboratories. Since long time, animal models have been

important for understanding the physiological and cognitive mechanisms of different diseases including neurobiology of addiction (Carroll and Overmier, 2001). Studies conducted to check animal behavior have described link of various memory system in competition, in combination or in parallel-depending condition on the kind of assignment and psychological needs (Gold 2002; White and McDonald, 2002; Kim and Baxter, 2001). Till now, different models have been developed and used for exploring the mechanism of different behaviors. The most widely used animal models are Elevated plus-maze, Morris water-maze test, two compartment test, Barnes circular-maze test, Shuttle box avoidance, Radial arm-maze, Passive avoidance, Runway avoidance and T-maze delayed alternation task.

CHAPTER # 3

MATERIAL AND METHODS

3. Materials and Methods

3.1. Animals

Wistar male rats of average body weight ranging from 120-180 g (n=48) were purchased from the Animal Research Facility (ARF), Dr. Panjwani Center for Molecular Medicine and Drug Research, University of Karachi. All rats were placed individually in opaque cages in controlled humidity of about 55±10 %, temperature of (22-24 °C) and in 12h:12h light and dark period. Animals were acclimatized for 7 days before the experiment started and was given unlimited access to food and water during entire experimental period. The protocol design was based on the international rules and guidelines for care and use of animals in research. This study was approved by Institutional Animal Care and Use committee, International Center for Chemical and Biological Sciences (ICCBS), University of Karachi.

Figure 3.1: Individual rat Housing

3.2. Oils, Chemicals and Drugs

Absolute alcohol was purchased from Merck (ET00072500). It was diluted with tap water to make 5 % and 10 % (*v/v*) alcohol. Mature seeds of *Nigella sativa* (NS) plant were purchased from a local herbal store of Sukkur. NS crude oil was obtained by seed pressing procedure described by the Al-okbi *et al.*, 2013. Virgin coconut oil (VCO) was extracted from fresh coconut using the wet process method as previously described (Neela and Prasad, 2012; Hamid *et al.*, 2011) with some modifications. Briefly, fresh coconuts were purchased from a local shop in Karachi. After removing the shell, the water was removed. The coconut was cut into small pieces, and grinded with lukewarm water. A new cheese cloth was spread on a bowl and a small amount of grounded coconut meat was carefully transferred to the cheese cloth. It was mechanically squeezed to obtain coconut milk. This step was repeated for all the remaining grounded meat. The whole process was repeated from grinding to squeezing. The coconut milk was chilled in the fridge for 24 hours to separate the coconut butter and water. The emulsified coconut butter was carefully taken and mildly heated in a non-stick pan. Once the non-oil fractions are separated, VCO was collected and filtered to remove any suspended solid.

Kits for Biochemicals Assays: For the measurements of insulin, total cholesterol (Catalog No: E-BC-K10) and triglycerides (Catalog No: EBC-K238) enzyme linked immunosorbent assays (ELISA) kits were purchased from (Elabscience Biotechnology Inc, USA). Whereas, Glucose (Catalog number GL-2623 /GL2614/ GL2610) colorimetric kit was purchased from (Randox laboratory limited, country Antrim, UK). For the estimation and measurements of neurotransmitters the chemicals and reagents used were purchased from (Sigma-Aldrich St. Louis, Mo, USA). All the reagents used were of analytical grade.

Chemicals for Western Blotting: Protein extraction reagent, enzyme inhibitor, protein assay kit, protein ladder, Mouse anti-beta actin primary antibody and secondary antibodies were purchased from (Invitrogen, Thermo Fisher scientific). Recombinant BDNF protein and mouse anti-BDNF primary antibody were purchased from (Bio-Connect). ECL western blotting detection reagents were from (GE Healthcare).

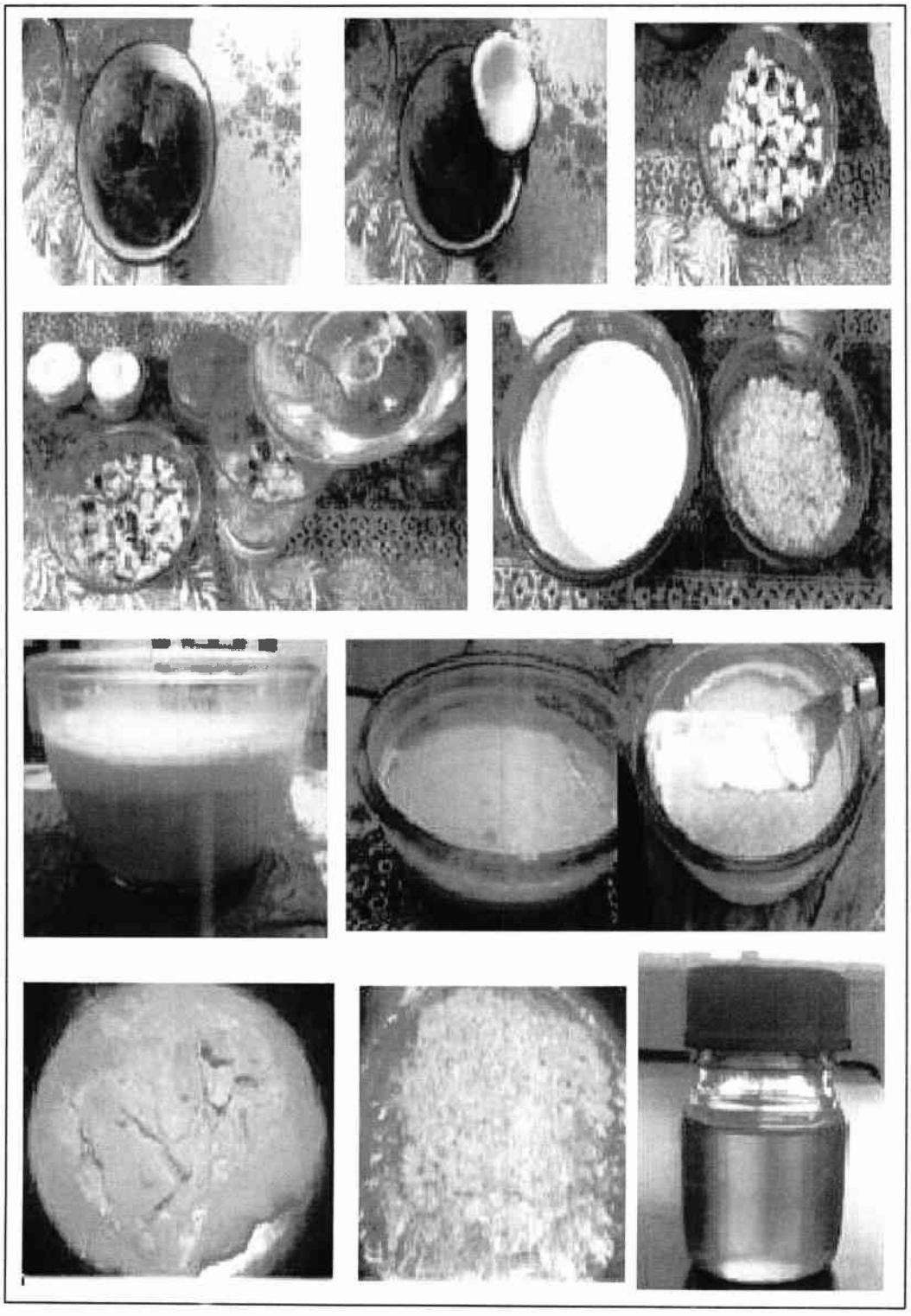


Figure 3.2: Virgin Coconut Oil Extraction Method

3.3. Experimental Design and Protocol

3.3.1. Alcohol and *Nigella sativa* Oil Experiment

Twenty-four Wistar male rats were divided into two experimental groups each having (n=12): (i) Water drinking, (ii) Alcohol drinking. The animals in water drinking group were provided tap water and the animals in alcohol drinking group were given alcohol. Initially the rats were administered 5 % ethanol (for 7 days) followed by 10 % ethanol in drinking water from day 8 to 26. After 16 days, the water and alcohol treated animals were subdivided into 4 groups (n=6): (i) Water + Water (WW), (ii) Water + *Nigella sativa* oil (WNSO), (iii) Alcohol + Water (EW) and (iv) Alcohol + *Nigella sativa* oil (ENSO). Food intake, water intake and body weights were monitored daily from 9:30 to 10:00 h. Water drinking + *Nigella sativa* oil and alcohol drinking + *Nigella sativa* oil groups were administered *Nigella sativa* oil (0.5 mL/kg) by oral gavage for 10 days (day 17 to 26) and water drinking + water and alcohol drinking + water groups received same dose of tap water by same route from 10:00 to 10:30 h. Behavioral experiments were conducted at 11:00 h onwards. On day 1,9,17 and 27 anxiety level were tested by elevated plus maze (EPM).

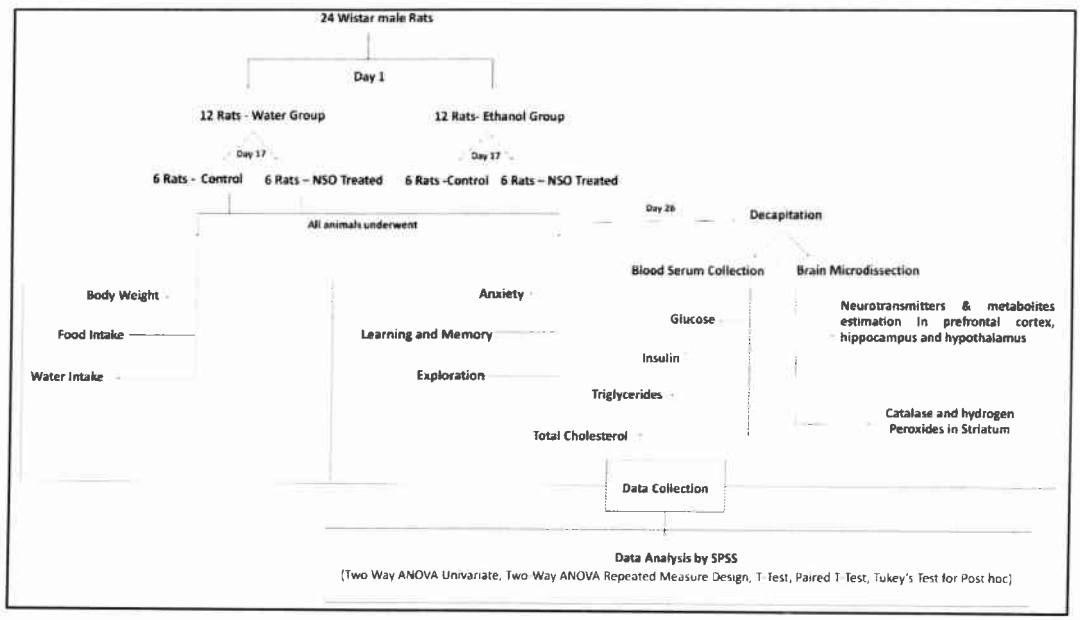


Figure 3.3: Study Design of Alcohol and *Nigella sativa* Oil Experiment

On day 2, 3 and 16 and 26 learning and memory were examined by Morris water maze (MWM) test. Activity was assessed in the open field on day 5, 12 and 25. On day 26, animals were decapitated. Blood was collected, and brains were immediately dissected. The samples were stored at -80 °C till further use.

3.3.2. Alcohol and Virgin Coconut Oil Experiment

Twenty-four Wistar male rats were divided into two experimental groups each having (n=12): (i) Water drinking, (ii) Alcohol drinking. The animals in water drinking group were supplied with tap water and the animals in alcohol drinking group were initially given alcohol. Initially the rats were administered 5 % ethanol (for 7 days) followed by 10 % ethanol in drinking water from day 8 to 28. After 16 days, the water drinking (n=12) and alcohol drinking (n=12) groups were subdivided (n=6): (1) Water drinking + Water treated, (2) Water drinking + VCO treated, (3) Alcohol drinking + Water treated and (4) alcohol drinking + VCO treated. Body weight, food and fluid consumption for each group were noted from 9:00 to10:00 h. Water drinking + VCO treated and alcohol drinking + VCO groups were supplied VCO (10 mL/kg) via gavage for 12 days and Group 1 and 3 received same dose of tap water by same route at 10:15 to 10:30 h.

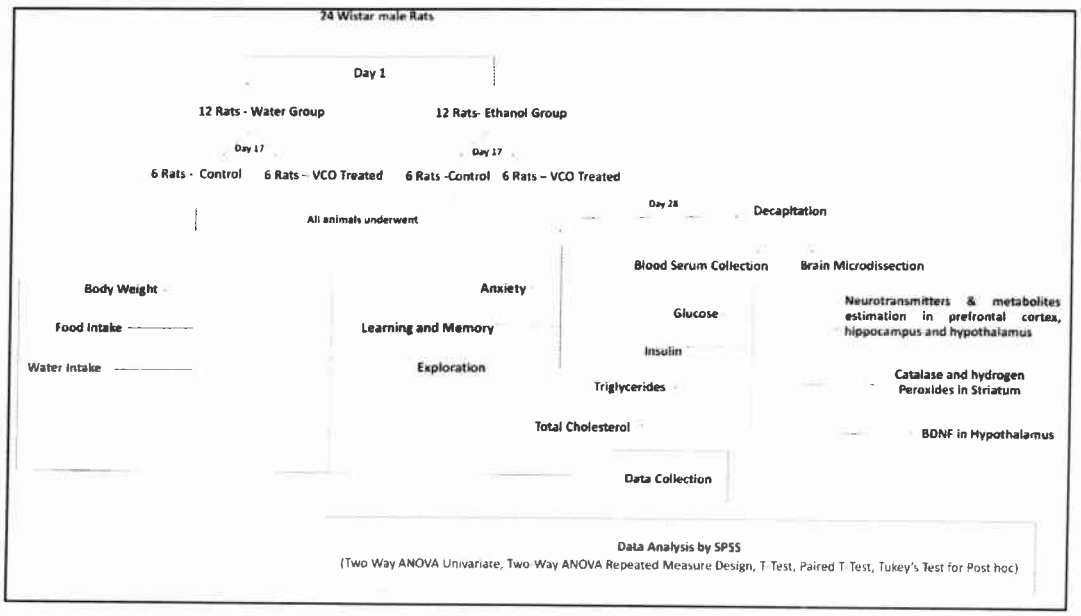


Figure 3.4: Study Design of Alcohol and Virgin Coconut Oil Experiment

On day 28 the animals were sacrificed by decapitation, blood collected, and brains were immediately dissected. The brain tissues samples were isolated from dissected brains and preserved in -80 °C till further use.

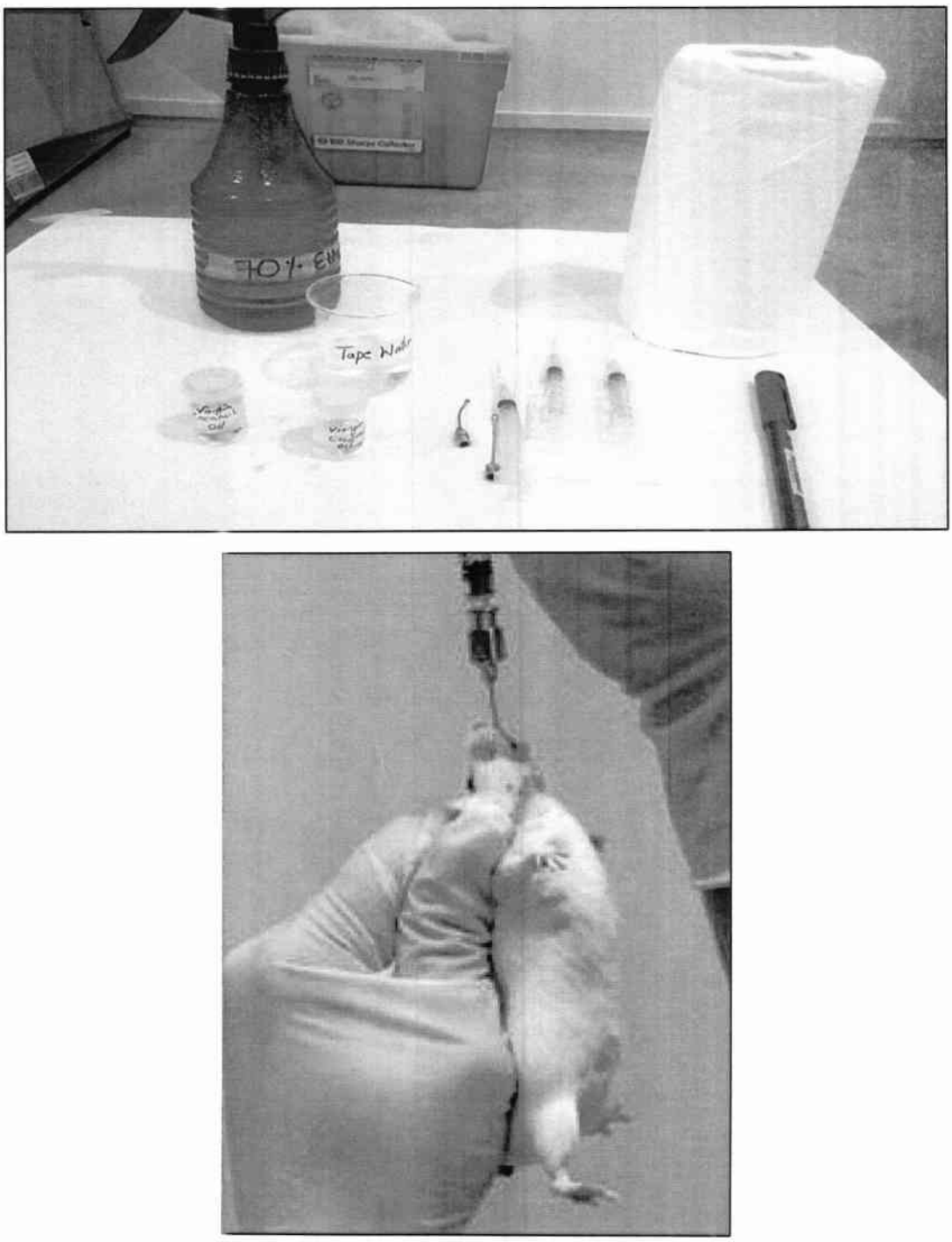


Figure 3.5: Administration of oils by oral gavage

3.4. Measurements of Body Weight, Food and Fluid Intake

3.4.1. Body Weights

Body weights of animals were monitored for 26 days by using digital balance machine. Animals were placed in an empty pan of balance. Initial and after treatment body weights of animals were compared.

3.4.2. Food Intake

Animals were given a known amount of food. Daily food intake in grams (g) was calculated by measuring remained food in cage hopper. Cumulative food consumption was estimated by calculating the food consumption collectively for each rat and group.

3.4.3. Fluid Intake

Animals were given a known amount of water and alcohol in glass feeding bottles. Daily water intake in milliliter (mL) was calculated by measuring the amount of remaining liquid in bottles. Cumulative water intake was estimated by calculating the water consumption collectively for each rat and group.

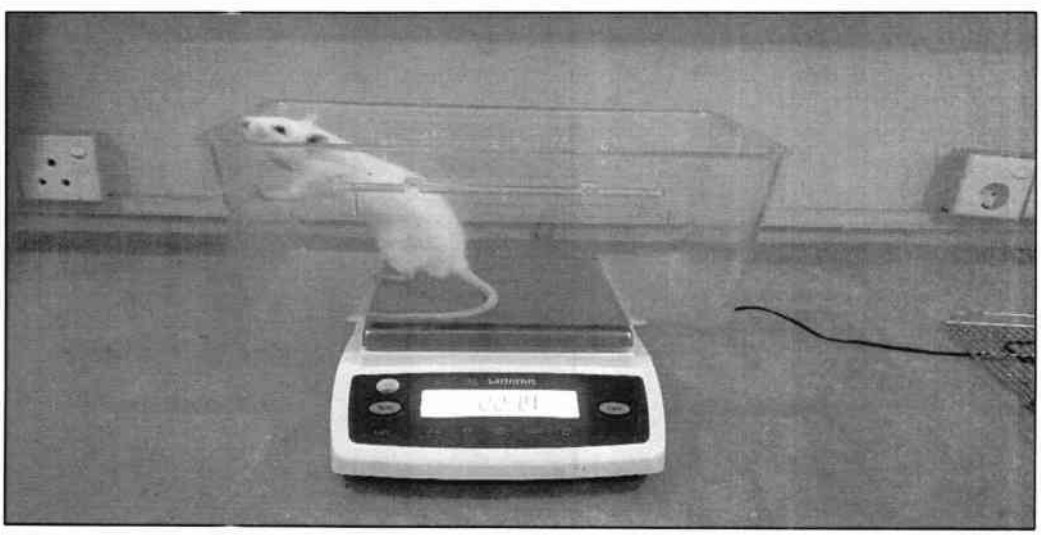
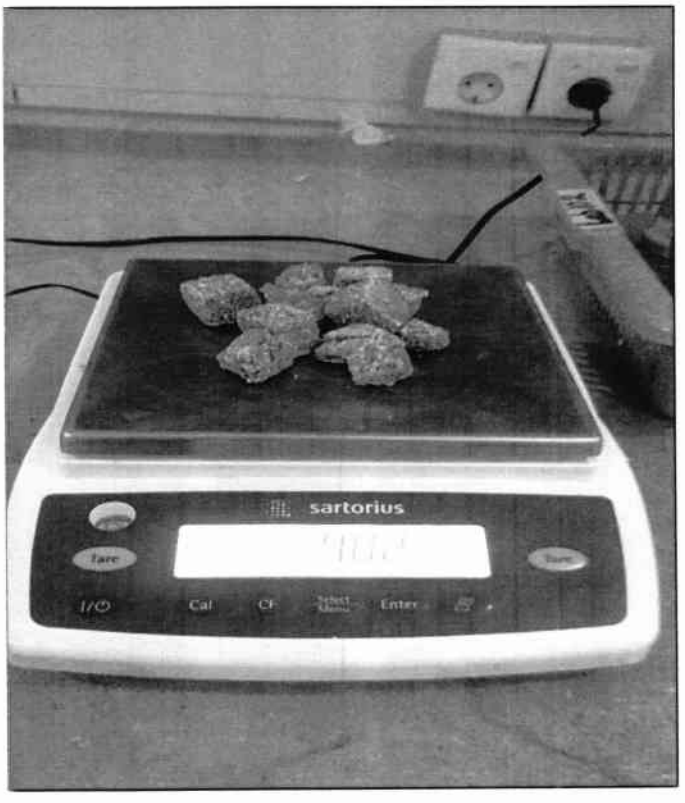


Figure 3.6: Measurement of body weight of animal



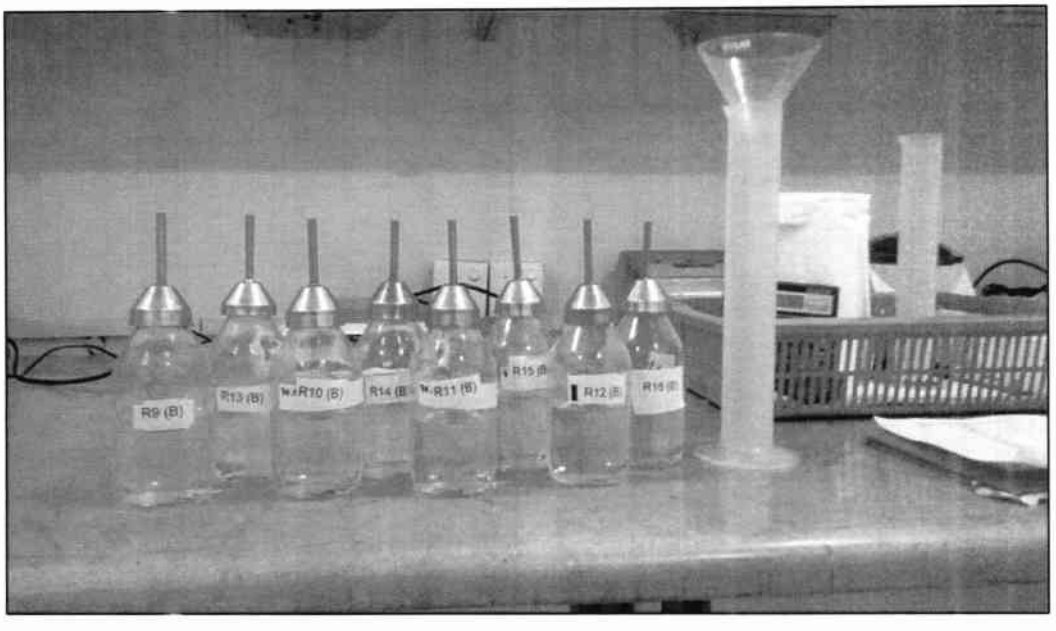


Figure 3.7: Estimating Food in hopper and fluid in bottles

3.5. Behavioral Studies

3.5.1. Elevated Plus Maze

EPM test was considered to explore anxiolytic effects of NSO in water and alcohol drinking rats in way described by Haleem *et al.*, 2015. The EPM consisted of 4 arms (16 x 5 cm), 2 open and 2 closed arms placed 60 cm high from ground. Rodent was placed in middle of apparatus and stay there for five minutes. Entries and time spent in open arm were noted.

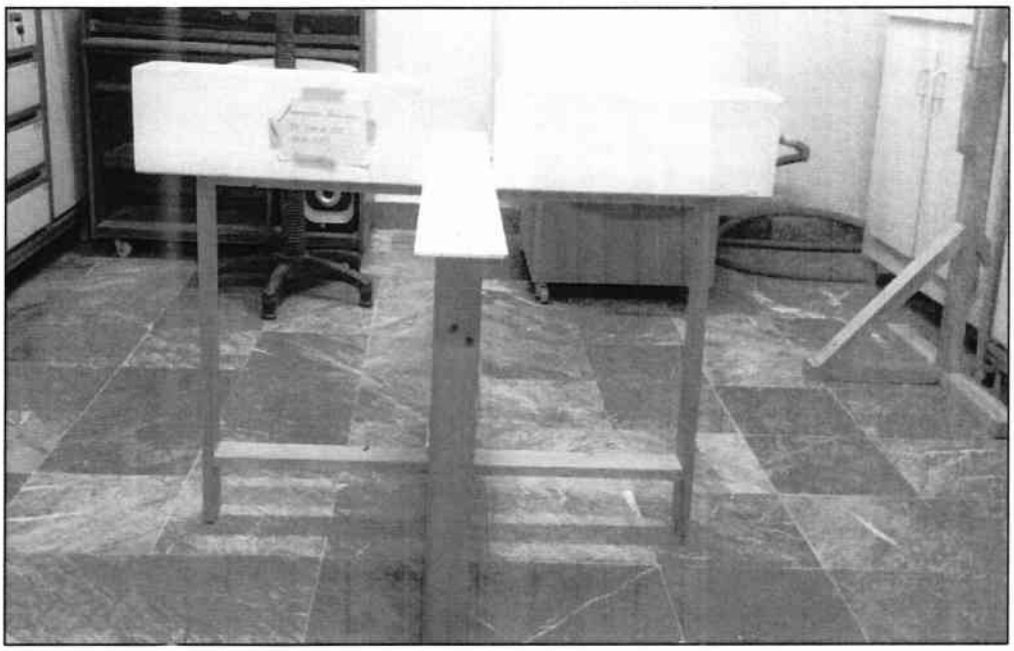


Figure 3.8: Elevated plus maze to check anxiety like behavior

3.5.2. Morris Water Maze Test (MWM)

Spatial memory was evaluated by an apparatus named Water Maze as described by Morris, (1984, 1981). Apparatus was made up of a circular pool of (90 cm/dm, 37 cm height) and platform of 10 x 10cm. Test was conducted in same way explained by Haleem *et. al.*, 2015. Pool was filled with opaque water (30 cm depth) and platform was submerged (2cm) in water dedicated to fixed direction. The maze was given 4 directions or quadrants named North, South, East and West. Animals underwent trial in which they were trained

to escape hidden platform from three directions (except the platform quadrant) followed by acquisition after 2 hours of last trial to evaluate short term memory (STM). Retention and long-term memory (LTM) were checked weekly. Escape Latency time was recorded to assess the acquisition and retention results.

For Probe test, platform was removed from maze and animal was placed in the center of pool facing wall from same direction as it was placed before to test various memory stages. Animals swim in pool for 60 seconds. Latency to enter, entries and time spent in platform quadrant were recorded.

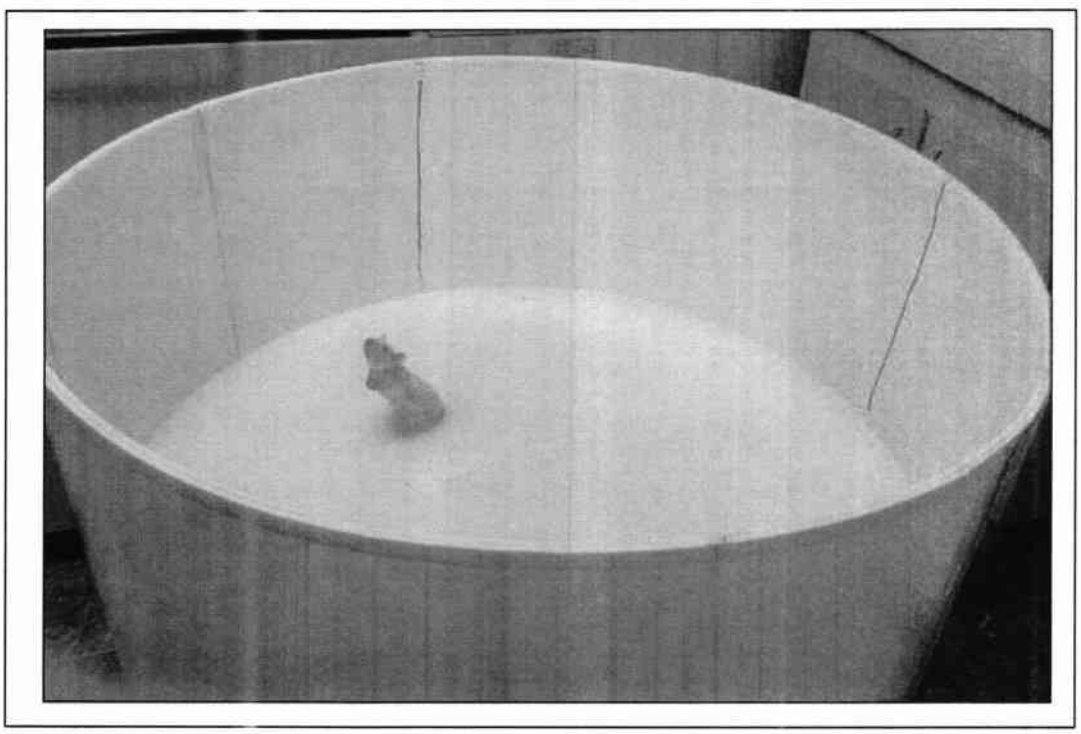


Figure 3.9: Morris water maze used to examine learning and memory

3.5.3. Activity in An Open Field (OF)

To explore effects of NSO on exploratory activity in water and alcohol drinking animals the OF was conducted as described by Kennett *et al*., 1985 and Ikram and Haleem, 2011. OF apparatus is made up of opaque plastic being 42 cm in height and covering a 76X76 cm square area. The base of the open field consists of 25 equal squares. Rats were

placed one by one in middle of the OF and left there for five minutes then squares crossed was noted.

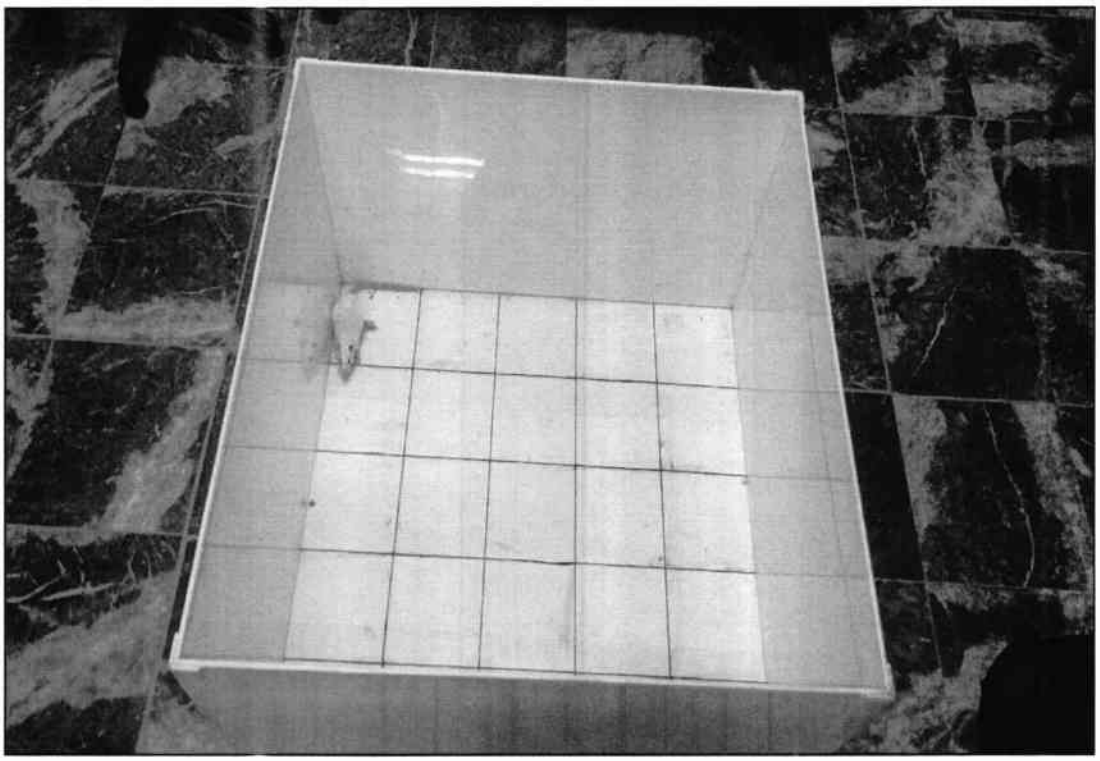


Figure 3.10: Open Field test used to examine exploratory activity

3.6. Decapitation to collect Brain Tissues and Serum Collection

3.6.1. Decapitation

Animals were sacrificed by decapitation in a surgery specified room at animal's research facility of ICCBS, UOK. Rats head were cut from the neck area in conscious mental condition with the help of a decapitator with sharp blades that smoothly cut the tissues and bones and separated the head from the rest of body.

3.6.2. Blood Sample

Blood sample was taken from the decapitated neck area. Chopped animals were placed over funnels, dropping the whole blood in 20 mL sterile falcon tube for each rat.

3.6.3. Serum Collection

Serum was taken from previously collected whole blood. Blood samples were centrifuged for ten minutes with speed of 12,000 rpm. Supernatants were taken out in new tubes then preserved at -80 ^{0}C till the biochemical tests were performed.

3.6.4. Brain Removal

Amputated head were immediately rinsed by ice cold 0.9 % saline. First, head skin tissues were peeled off and skull bones were centrally broken and separated from lambda to nasal bone regions with pointed surgical sharp scissors. Skull was divided into two symmetrical regions. Skull bone was carefully tuned out and twisted disclose brain. Whole brain was gently taken out from skull with help of spatula and set on slicer cavity. This whole procedure of placing brain in cavity was completed within 30 seconds.

3.6.5. Brain Micro-Dissection

After sacrificing the animals, fresh brains were immediately removed from skull. Brain was placed in brain slicer and washed with ice chilled 0.9% saline to make the brain rigid and to remove excessive blood clots from surface. Stainless sharp blades were used to make slices. Blades were fixed by insertion in graduation present in brain slicer. Slices were taken with the help of forceps and different brain regions including hippocampus, prefrontal cortex, midbrain, striatum and hypothalamus were extracted. Regions were immediately transferred in 2.5 mL Eppendorf or cryo-tubes which then stored in -80 °C freezer. Whole process of region collection was complete within 2 to 2.5 minutes after decapitation.

3.7. Neurochemistry in Prefrontal Cortex, Hippocampus and Midbrain

Biogenic amines called neurotransmitters i.e., Serotonin (5HT), Dopamine (DA), Noradrenaline (NA). Serotonin with metabolite 5-hydroxy indole acetic acid (5HIAA), dopamine with metabolites homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid

(DOPAC), and noradrenaline levels were estimated in hippocampus, prefrontal cortex and midbrain by using high performance liquid chromatography-electrochemical detector (HPLC-EC) as described previously (Haleem *et al.*, 2014). Instrument and software were supplied by Water Alliances e2695 HPLC system.

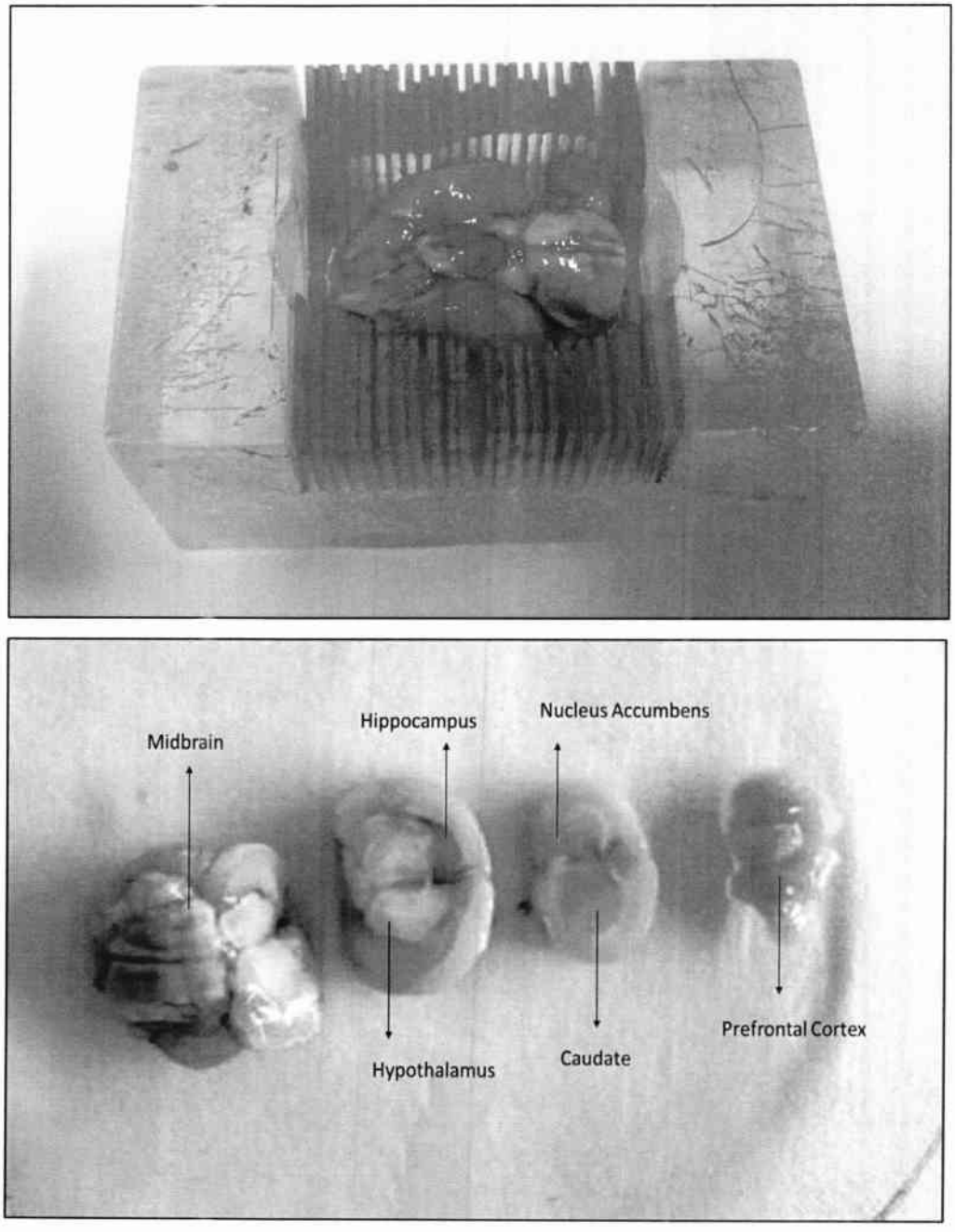


Figure 3.11: Brain Isolation and regions separation

3.7.1. High Pressure Liquid Chromatography (HPLC)

This technique is based on separation of a substance between mobile and stationary phases (Venn, 2008) resulting in separation from sample mixture. Separation of biogenic amines plus their metabolite's separation by HPLC is based on the on their affinity and hydrophobicity with stationary phase made up of silica gel. This is one of the best and sensitive separation techniques that uses a column consisting of stationary phase in which mobile phase passes at high pressure (Kazakevich and Lobrutto, 2007). The separation relies on composition of mobile phase (organic solvents) and content of salts. The HPLC machine used very high pressure i.e. 2000–3000 psi to run samples.

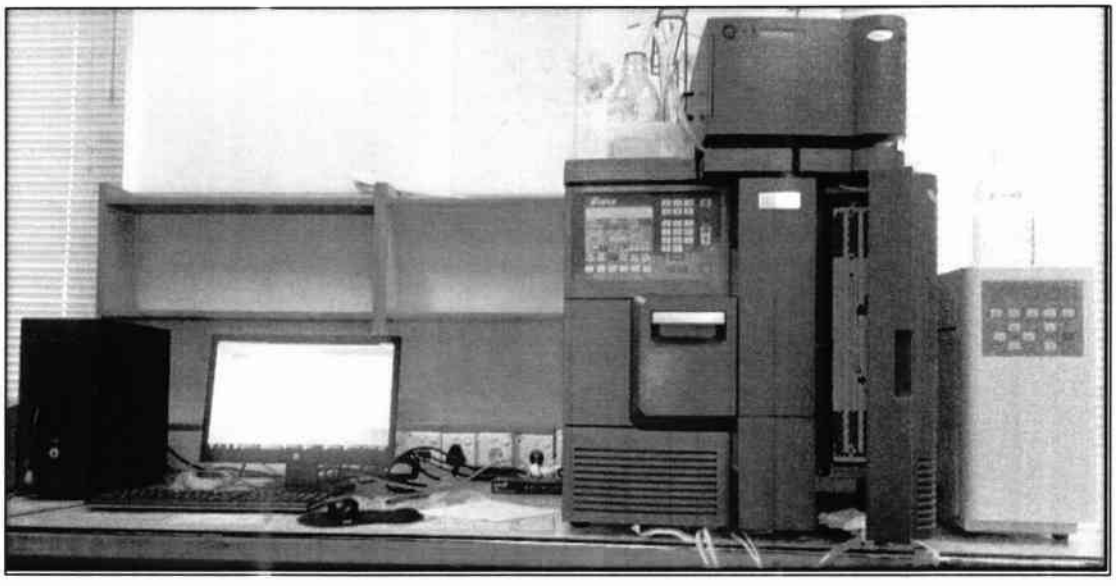


Figure 3.12: HPLC Machine with system

Brain tissues contains minute quantities of biogenic amines and HPLC is capable to detect such this minute quantities from nanogram (ng) to picogram (pg.). We used C18 Octadecyl silane-2 (ODS) stationery column. 20 µL of the supernatant and standard was filtered and auto-injected on top of the column to detect the levels by reverse phase HPLC (RP-HPLC). The high pressure supplied by high pressure pumps help to move the solvent via column and it remove the separated substance. Strong binding affinity between a substance being eluted and silica stationary phase, increases the elution/extraction time

(Snyder, Kirkland and Glajch, 2012). After passing through stationary phase, analyte reaches electrochemical detector (ECD).

Voltaic cell of detector comprises of reference and working electrodes made of KCl-calomel and glassy carbon respectively. Each electrode is placed in cell and difference between electrodes are maintained. This possible difference causes ionization of substance (analyte) which enable it to give up an electron (Swartz, 2010). Concentration of biogenic amines and their metabolites in sample is estimated by intensities of these charged electrons and electrons production.

3.7.2. Mobile Phase and Stationary Phase

It was made up of 0.1M phosphate buffer containing 10% methanol with pH 2.9. The chemicals were added and dissolved in 500 mL HPLC grade water. 2.9 Ph was adjusted by adding ortho-phosphoric acid. Afterwards, water was added to make 1000 mL volume. Commercially available waters Spherisorb column of 5mu (4.6 X 150mm) packed with C18 - ODS was used as stationary phase.

3.7.3. Standard preparation

Individual standards for serotonin, dopamine, noradrenaline, 5-HIAA, HVA and DOPAC were made by mixing each in extraction buffer (100 mg/mL). First, standards were run on machine to find out the elution time for each standard. Then samples were run and compared with standards.

3.7.4. Extraction of neurotransmitters and sample preparation

To extract monoamines from brain tissues, extraction medium was used. The hippocampus, prefrontal cortex and midbrain tissues were weighed and 5X volume (mL) of extraction medium to the sample was added. Tissues were homogenized in this extraction medium. The homogenates were centrifuged 15 minutes at 4 ^{0}C with 12000 rpm. Supernatant samples were collected and were spinned down again for five minutes. The

clear supernatants were taken out and used to find out the unknown concentrations of NA, DA, DOPAC HVA, 5HIAA and 5HT.

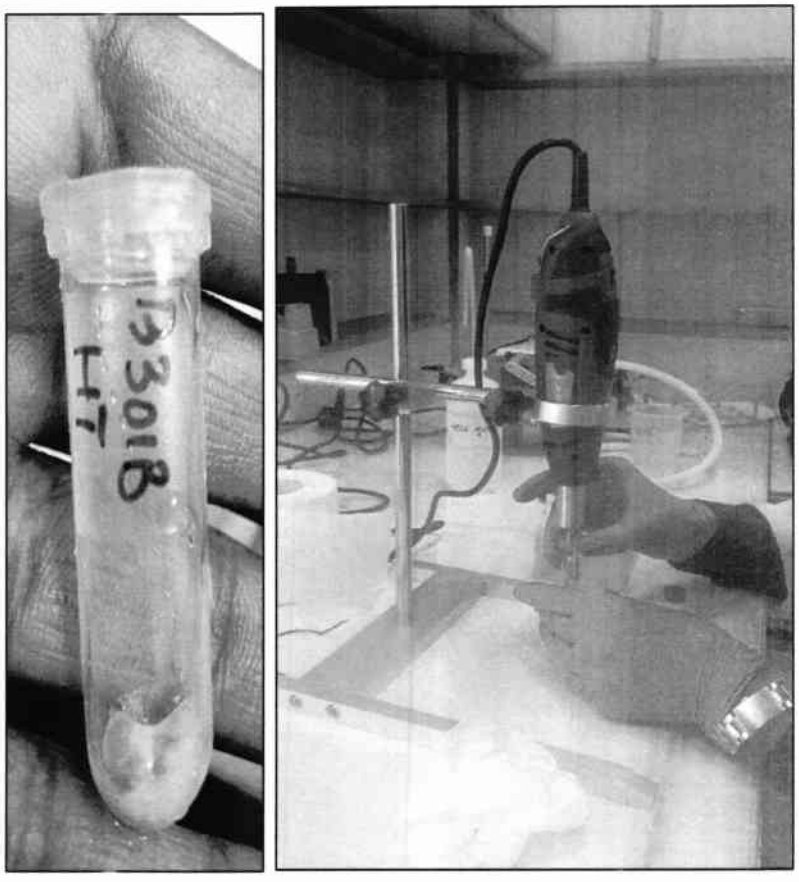


Figure 3.13: Sample preparation by homogenization for HPLC-EC

3.7.5. Calculations

The chromatograms of standards and samples showing monoamines were interpreted by the Empower™ 3 software by Water Alliance HPLC system. The peaks area and retention time were noted. Unknown concentration of monoamines in samples was measured with help of formula given below:

$$\frac{\text{Known concentration of standards x curve area of samples x Dilution factor}}{\text{Area of Standard}}$$

3.8. Protein Extraction and Western Blotting for Brain Derived Neurotropic Factor (BDNF) in Hypothalamus

Hypothalamic tissues were weighed and homogenized in lysis buffer (1000 µL per 100 µg of tissue) made up of Neuronal lysis agent 8N-PER, Neuronal Protein Extraction Reagent (Thermo Scientific, cat: 87792), and enzyme inhibitor HALT (Thermo Scientific, cat: 87785). The homogenates were centrifuged for ten minutes with speed of 10,000 g at 4 °C and supernatants were separated. This supernatant was used to estimate the total protein concentration by Pierce TM BCA Proteins Assay kits (Thermo Scientific, cat: 23225). Absorbance was measured at 562 nm and standard curve was made to determine the level of the total proteins in samples. Samples were heated 3 minutes at 100 °C loading buffer. Samples, ladder (Thermo fisher Scientific) and recombinant human/murine/rat BDNF protein (Bio-connect) were loaded in wells of 10 % gel. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was run for 30 minutes on 50 V first and then increased to 100 V for 60 minutes. Afterwards, from gel, proteins were shifted to Polyvinylidene fluoride (PVDF) membrane on 300 mA for 90 minutes. Blots were washed (3X * 10 minutes) with washing buffer and unbound areas were blocked in blocking buffer (1-2 hour at room temperature). After blocking, PVDF membranes were incubated overnight at 4 °C with two primary antibodies, mouse monoclonal antibody against human BDNF Immunoglobulin G (IGg1) (1:2000, Bio-Connect, Icosegen) and mouse monoclonal antibody IGg2b for beta actin (1:5000, Invitrogen Thermo Fisher Scientific). Blots were washed (3X * 10 minutes) with washing buffer to remove unbound proteins/antibodies. Then membranes were incubated with goat anti-mouse IGg1 (1:1000, Invitrogen, Thermo Fisher Scientific) and Goat anti-mouse IGg2b (1:2000, Invitrogen, Thermo Fisher Scientific) horseradish peroxidase (HRP) conjugated secondary antibodies for 1 hour at room temperature. After that, visualization of membranes was done using a Luminescence Image Analyzer (LAS4000) and bands were analyzed by software Image J wine 54. BDNF expression levels were measured and normalized against beta actin levels.

3.9. Catalase and Peroxide levels estimation in Striatum

3.9.1. Determination of rat striatal Catalase by Colorimetric Assay

Catalase in rat striatum was calculated by colorimetric assay kit according to the protocol provided with kit. Briefly, brain was homogenized in cold phosphate buffer saline (PBS) (10 mg/200 µL). Homogenate was centrifuged and supernatant was collected. 10 µL of samples and positive controls were added in microwells and 10 µL of assay buffer as blank. 50 µM H_2O_2 substrate was added to wells to start catalase activity and incubated for 30 minutes.

Now make the four concentrations of standards according to the manufacture protocol. Briefly, 4.8 mM H_2O_2 (40 µL) with distilled water (440 µL) was mixed to make 400 µm H_2O_2. Then 100 µL, 60 µL, 30 µL and 0 µL of this 400 µm H_2O_2 was pipetted in wells and water was added to make 100 µL volume in each well. Finally, 90 µL of assay buffer in standard well was poured and 100 µL the detection reagent was pipetted in each well. Content in wells were mixed and incubated for 10 minutes. After incubation the optical density (OD) or absorbance at 570 nm (550 to 585 nm) was measured. To calculate the level of catalase, blank values was subtracted from each standard and sample value.

3.9.2. Determination of rat striatal Peroxide by Colorimetric Assay

Peroxide level in striatum was measured by colorimetric assay kit as described in kit protocol. Briefly, 10 % tissue homogenate in PBS was prepared. Diluted standard, detection reagent and 30 µm premix from diluted standard were freshly made. The premix was serially diluted. Then 40 µL of diluted standards and samples were added in each well of microwell plate and 200 µL of detection reagent was also poured in all wells. Plate was incubated for 30 minutes. Afterwards, optical density at 540 to 610 nm was measured.

To calculate the peroxide concentration, blank absorbance values from standards and sample absorbance values were subtracted. Standard curve from H_2O_2 OD was generated and helped in evaluating the hydrogen peroxide level.

3.10. Biochemical Analysis

Concentrations of insulin, glucose, cholesterol and triglycerides were measured in blood serum. Insulin was estimated by ELISA as described by manufactures' protocol. glucose, total cholesterol and triglyceride levels were evaluated by colorimetric assay performed using a microplate reader.

3.10.1. Determination of Rat Serum Insulin by Enzyme linked immunosorbent assay

Rat insulin (INS) enzyme linked immunosorbent assay (ELISA) kit was utilized to determine serum insulin level. This sandwich ELISA kit was purchased from Elabscience Biotechnology Inc, USA. The 96 well microplate supplied in kit was precoated with an antibody for Rat INS. The 100 µL of standard, blank and samples were added to each microwell, plate was covered and incubated for 90 minutes. Liquid was removed from wells and detection antibody was added. Plate was covered, shake and incubated again followed by addition of HRP conjugated enzyme. Liquid was removed from well and washing was performed. Subtract was added and plate was incubated for 10 minutes. Reaction was stopped by adding 50 µL of stop solution and absorbance at 450 nm was recorded. From OD standard curve generated and the concentration of insulin was estimated.

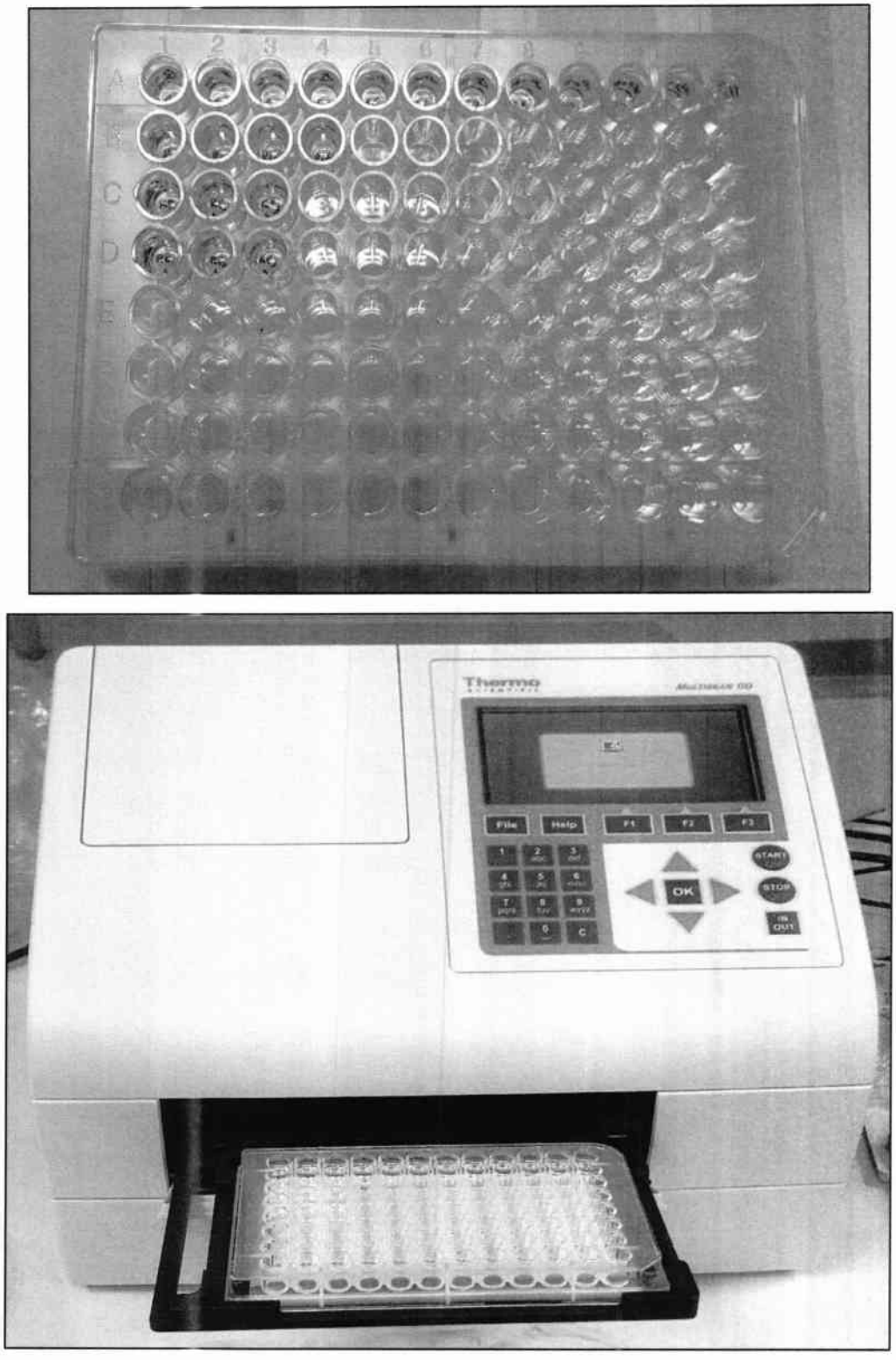


Figure 3.14: 96 Microwell plate and ELISA plate reader

3.10.2. Determination of Rat Serum Glucose by Colorimetric Technique

Glucose assay kits was used to estimate glucose level in rat serum. Kit was purchased from Randox laboratory limited, country Antrim, UK. Kit applies the GOD-PAP Assay without deproteinization as method. The 10 µL of given standard mixture and samples were pipetted in wells. Then 1000 µL of working solution was added in all wells. Content was mixed by gently tapping the plate or placing it on shaker for few seconds and plate was incubated for 25 minutes at 15-25 °C. Absorbance of standards and samples at 500 nm was measured and glucose level was estimated by using following formula.

Serum Glucose (mmol/L) = OD Sample / OD Standards x concentration of standard

3.10.3. Determination of Rat Serum Total cholesterol by Colorimetric Technique

Total cholesterol (T-CHO) assay kit used was purchased from Elabscience Biotechnology Inc, USA. This detection was based on the COD-PAP method and can be used to determine the *in vitro* total T-CHO level. Briefly, 10 µL of given standard, serum sample and blank were pipetted in wells. Distilled water was used as blank control. Then 1000 µL of working solution was added in all wells followed by mixing the content. Plate was incubated for 10 minutes at 37 °C. Afterwards, absorbance at 510 nm was measured and level of total cholesterol (mmol/L) in serum was calculated by using following formula.

^{OD} Sample – ^{OD} Blank / ^{OD} Standard – ^{OD} Blank × Concentration of standard (5.17 *mmol/L*)

3.10.4. Determination of Rat Serum Triglycerides by Colorimetric Technique

Triglyceride (TG) Assay Kit "Catalog No: EBC-K238" was purchased from Elabscience Biotechnology Inc, USA. The kit applies GPO-PAP method and can be useful for *in vitro* determination of TG level. Briefly, 2.5 µL of given standard, serum sample and blank were pipetted in wells. Distilled water was used as blank control. Then 250 µL of working solution was added in all wells followed by mixing the content. Plate was

incubated for 10 minutes at 37 °C. Afterwards, absorbance at 510 nm was measured and level of serum TG (mmol / L) was calculated by using formula given below:

OD Sample – OD Blank / OD Standard – OD Blank × Concentration of standard ($mmol/L$).

3.11. Statistical Analysis

Statistical analysis was done using the IBM SPSS version 16. All data is presented as mean ± SD. Two-way ANOVA (univariate) or two-way ANOVA repeated measures design (RPM) and T-test have been used to analyses the data based on the nature of data and number of groups. A value of $p < 0.05$ was considered significant. Individual comparisons were made by Tukey's post-hoc or by paired t-test.

CHAPTER # 4

RESULTS AND DISCUSSION

(Alcohol and *Nigella sativa* Oil Experiment)

4. Results and Discussion (Alcohol and *Nigella sativa* Oil Experiment)

4.1. Experimental Design

Twenty-four Wistar male rats were divided into 2 groups (n=12): (i) Water drinking, (ii) Ethanol drinking. The animals in water drinking group were supplied with tap water and the animals in ethanol drinking group were given ethanol. Initially the rats were administered 5 % ethanol (for 7 days) followed by 10 % ethanol in drinking water from day 8 to 26. After 16 days, the water and ethanol treated animals were subdivided into 4 groups (n=6): (i) Water + Water (WW), (ii) Water + *Nigella sativa* oil (WNSO), (iii) Ethanol + Water (EW) and (iv) Ethanol + *Nigella sativa* oil (ENSO). Body weights, food and water intake were monitored daily from 9:30 to 10:00 h. To the water drinking + *Nigella sativa* oil and ethanol drinking + *Nigella sativa* oil groups, *Nigella sativa* oil (0.5 mL/kg) were administered orally by gavage from day 17 to 26 (10 days) and water drinking + water and ethanol drinking + water groups received same dose of tap water by same route from 10:30 to 11:00 h.

Three behavioral tests were performed, anxiety, learning and memory and exploratory. Behavioral tests were performed at 11: 30 h onwards.

1) Anxiety was tested by elevated plus maze (EPM). EPM experiments on day 1, 9 and 17 were done to see alcohol's effects while day 26 EPM experiment explored the NSO effects.
2) Memory was tested by Morris water maze (MWM) test. MWM test were performed on day 2, 3 and 16 to check STM, retention and extinction respectively in alcoholic rats. MWM on day 26th, explored the effects of NSO on long term memory.
3) Exploratory activity was tested by open field (OF). Activity was assessed in the OF on day 5, 12, and 25.
On Day 26th, animals were decapitated. Blood was collected, and brains were immediately dissected to isolate prefrontal cortex (PFC), hippocampus (HP), midbrain (MB) and striatum (ST). All samples were then preserved at -80 °C.

Neurotransmitters' level in "PFC, HP and MB' were measured by using high performance liquid chromatography - electrochemical detector (EC-HPLC) as performed previously (Haleem *et al.*, 2014).

Catalase and hydrogen peroxide in "ST" were estimated by ELISA and colorimetric assay technique.

Blood was centrifuged. Clear supernatant was collected in new tubes and used to calculate the serum levels of insulin (by ELISA), glucose, total cholesterol and triglycerides by colorimetric assay performed using a microplate reader.

Statistical data analyses were done with the help of the IBM SPSS software version 16. All data was presented as means ± SD. Physiological, neurochemistry, biochemical and behavioral data analyses was done by two-way ANOVA univariate and repeated measures design. T-test was used to analyze memory results of ethanolic rats. Comparisons among groups were done by Tukey's and paired t-tests. The $p< 0.05$ was taken as significant value.

4.2. Results

4.2.1. Effect of *Nigella sativa* oil on Body Weights, Food Intake and Water Intake in Alcohol Drinking Rats

The figure 4.1 is showing the effects of ethanol drinking and *Nigella sativa* oil (NSO) treatment on body weight. Two-way ANOVA, repeated measure design (RMD) revealed a significant impact of ethanol consumption ($F=4.79$, $df=1,22$, $p<0.05$), repeated measure ($F=104.373$, $df=2,44$, $p<0.01$) and significant ethanol consumption and repeated measure interaction effect ($F=54.32$, $df=2,44$, $p<0.01$). Post hoc tests showed that ethanol drinking groups significantly ($p<0.01$) lost body weight than water drinking animals. Water drinking animals significantly ($p<0.01$) gained weight and had higher values from initial weight (4.1A).

Figure. 4.1B is showing the effects of *Nigella sativa* oil (NSO) treatment on body weights of water and ethanol drinking animals. To analyze the data on body weight after NSO treatment (10 days) on water and ethanol drinking animals, two-way ANOVA was

used. We found significant impacts of NSO ($F=56.096$, $df=1,20$, $p<0.01$). Post-hoc testing for groups comparisons and differences revealed that the NSO treatment of ethanol drinking animals significantly ($p<0.01$) reduced weight relative to the respective water drinking groups. These data show that NSO reduced the weigh only in ethanol drinking animals but did not alter the weight in water dinking animals. Surprisingly, the difference in weight of ethanol drinking rats was almost like ethanol drinking control rats.

The figure. 4.2A represents the role and impacts of NSO treatment on food intake in ethanol and water drinking animals. Data on food intake were statistically analyzed by using two-way ANVOVA. We found significant impact of NSO treatment ($F=16.345$, $df=1,20$, $p<0.01$) on food consumption in the ethanolic rats. No significant interaction ($F=855$, $df=1,20$) was found. Post-hoc testing of *Nigella sativa* oil treatment of water and ethanol drinking animals revealed that ethanol drinking + *Nigella sativa* oil treated rats showed significant difference ($p<0.01$) and ate less amount of food from water drinking + NSO rats. It also revealed significant ($p<0.05$) decreased intake than ethanol drinking + water treated animals. These data indicate that NSO treatment of ethanol drinking animals reduced the hunger which ate reduced amount of food compared to ethanol drinking control and water drinking + *Nigella sativa* oil treated animals.

The figure. 4.2B represents the role and impacts of NSO treatment on fluid intake in ethanol and water drinking animals. Data analysis revealed a significant impact of NSO treatment ($F=28.121$, $df=1,20$, $p<0.01$). Post-hoc testing shows that the NSO treated ethanol drinking animals consumed a significantly lower amount of fluid as compared to NSO treated water drinking rats ($p<0.01$) plus ethanol drinking + water treated group ($p<0.05$). The difference in fluid intake in water drinking + water and water drinking + *Nigella sativa* oil treated groups was insignificant. Ethanol drinking + *Nigella sativa* oil showed significant decrease in fluid intake compared to Ethanol drinking + *Water and* water drinking + *Nigella sativa* oil. The data revealed that NSO reduced the thirst in ethanol dinking animals which resulted in reduced water intake.

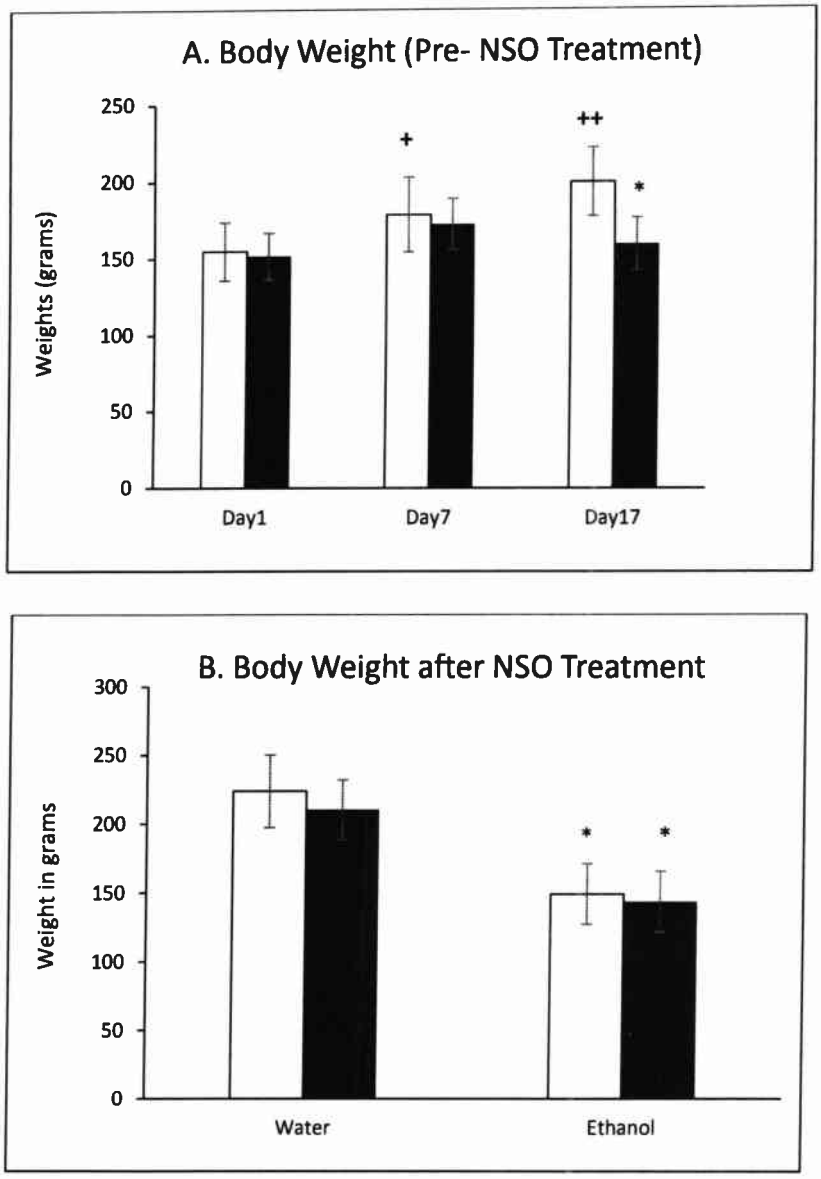
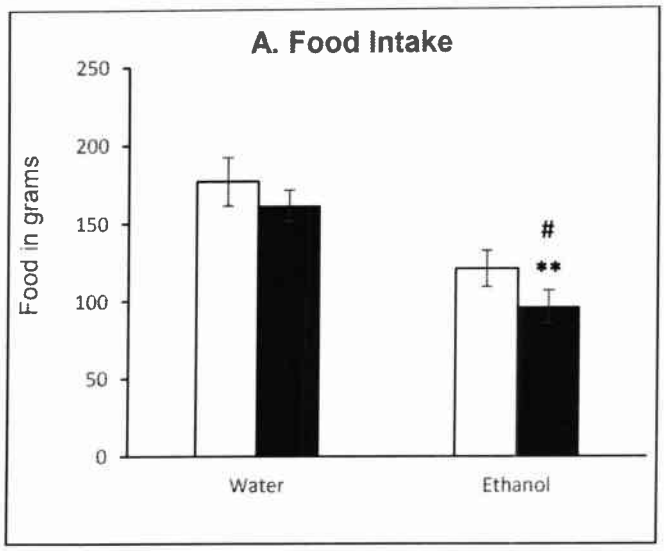


Figure 4.1: Effects of Ethanol consumption and NSO treatment on body weight. Values represent mean ± SD. (A) Ethanol's Effect. Body weight at day 1, 7 and 17. *p<0.01 different from similar day water drinking control animals; Body weights of water drinking control animals on day 7 and 17 was +p<0.05 and ++p<0.01 different from first day respectively. (B) Effects of NSO treatment of ethanol drinking animals. *p<0.01 different from water drinking + *Nigella sativa* oil treated animals and water drinking + water treated control animals.



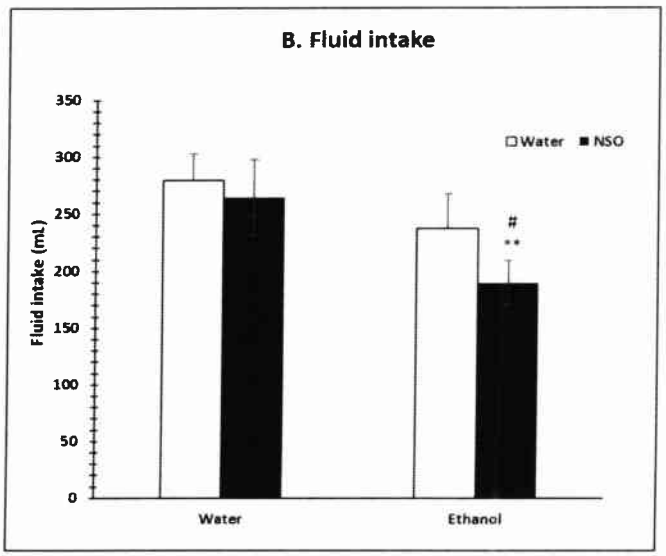


Figure 4.2: Effects of *Nigella sativa* oil (NSO) treatment (10 days) of water and ethanol drinking animals on accumulative 10 days food and fluid intake measured on last day of experiment. Values are means ± SD. (A) Food intake. $^{**}p<0.01$ different from water drinking + *Nigella sativa* oil; $\#p<0.05$ different from ethanol drinking + water treated control animals. (B) Fluid intake. **p<0.01 different from water drinking + *Nigella sativa* oil group; $^{\#}p<0.05$ different from ethanol drinking + water treated group.

4.2.2. Behavioral Study

4.2.2.1. Effects of Nigella sativa oil on anxiety in alcohol drinking rats

Figure. 4.3 is showing the ethanol effects on anxiety observed in elevated plus maze (EPM). Two-way ANOVA-RMD demonstrated significant impacts of alcohol consumption ($F=6.609$, $df=1,22$, $p<0.05$) and repeated measure (RM) ($F=240.669$, $df=2,44$, $p<0.01$) on open arms (OA) entries. There was also alcohol consumption and repeated measure interaction effect ($F= 100.151$, $df=2,44$, $p<0.01$). Post-hoc test revealed significant higher number of entries into open arms of the EPM on day 1 and reduced number of entries on day 17 in alcohol drinking rats relative to water drinking animals. The difference in number of entries in water and alcohol drinking animals on day 9 was not significant. In every elevated plus maze test, alcohol drinking animals showed significantly reduced ($p<0.01$) entries in the OA relative to previous days (entries in day 17 < day 9 < day 1). In contrast, water drinking group showed reduced ($p<0.01$) entries in the OA only on day 9 compared to day 1 (Figure 4.3A).

Data analysis revealed significant impact of alcohol consumption ($F=6.144$, $df=1,22$, $p<0.05$), and RM ($F=277.872$, $df=2,44$, $p<0.01$) on time spent in an OA. A significant alcohol consumption and repeated measure interaction effect ($F= 50.500$, $df=2,44$, $p<0.01$) was also found. Post-hoc analysis revealed a significant increase ($p<0.01$) in time spent in the OA of the EPM test on day 1 and a significant reduction ($p<0.01$) in time spent in the OA on day 17 in alcohol drinking rats relative to water drinking animals. There was no significant difference of time spent in open arms between alcohol and water drinking animals was observed on day 9. The alcohol drinking group revealed continuous significant decrease in the time spent in OA. In every EPM test, alcohol drinking animals spent lesser time ($p<0.01$) in the OA relative to the previous test (day 17 < day 9 < day 1). The water drinking animals showed significant difference between day 9 and day 1 only ($p<0.01$) (Figure. 4.3B).

Figure 4.4 is showing the impacts of 10 days NSO administration on anxiety behavior in water and ethanol drinking groups tested on day 26th. Data analyzed by two-way ANVOVA. A significant interaction effect between alcohol consumption and NSO

administration ($F=32.527$, $df=1,20$, $p<0.01$) on entries in OA was found. Post-hoc testing shows that alcohol drinking animals showed significant ($p<0.01$) less entries into the OA than water drinking animals. The OA entries were significantly increased ($p<0.01$) in alcohol drinking + *Nigella sativa* oil treated group compared to alcohol drinking + water treated control animals. NSO treatment of water drinking animals decreased ($p<0.01$) OA entries than water drinking + water treated animals (Figure 4.4A). These data indicate that *Nigella sativa* oil ameliorates the anxiety like effects of alcohol drinking.

Time spent in an open arm data was also analyzed by the help of two-way ANOVA. We found a significant interaction between alcohol consumption and *Nigella sativa* oil treatment ($F=61.184$, $df=1,20$, $p<0.01$) on time spent in OA. Post-hoc testing showed that alcohol drinking animals spent a significant lower ($p<0.01$) time in OA compared to water drinking animals. *Nigella sativa oil* treatment significantly ($p<0.01$) enhanced the time spent the open arms in alcohol drinking rats and decreased ($p<0.01$) time spent OA in water drinking animals as compared to water treated control animals. These data indicate that *Nigella sativa oil* treatment decreased anxiety-like behavior in alcohol drinking animals (Figure. 4.4B).

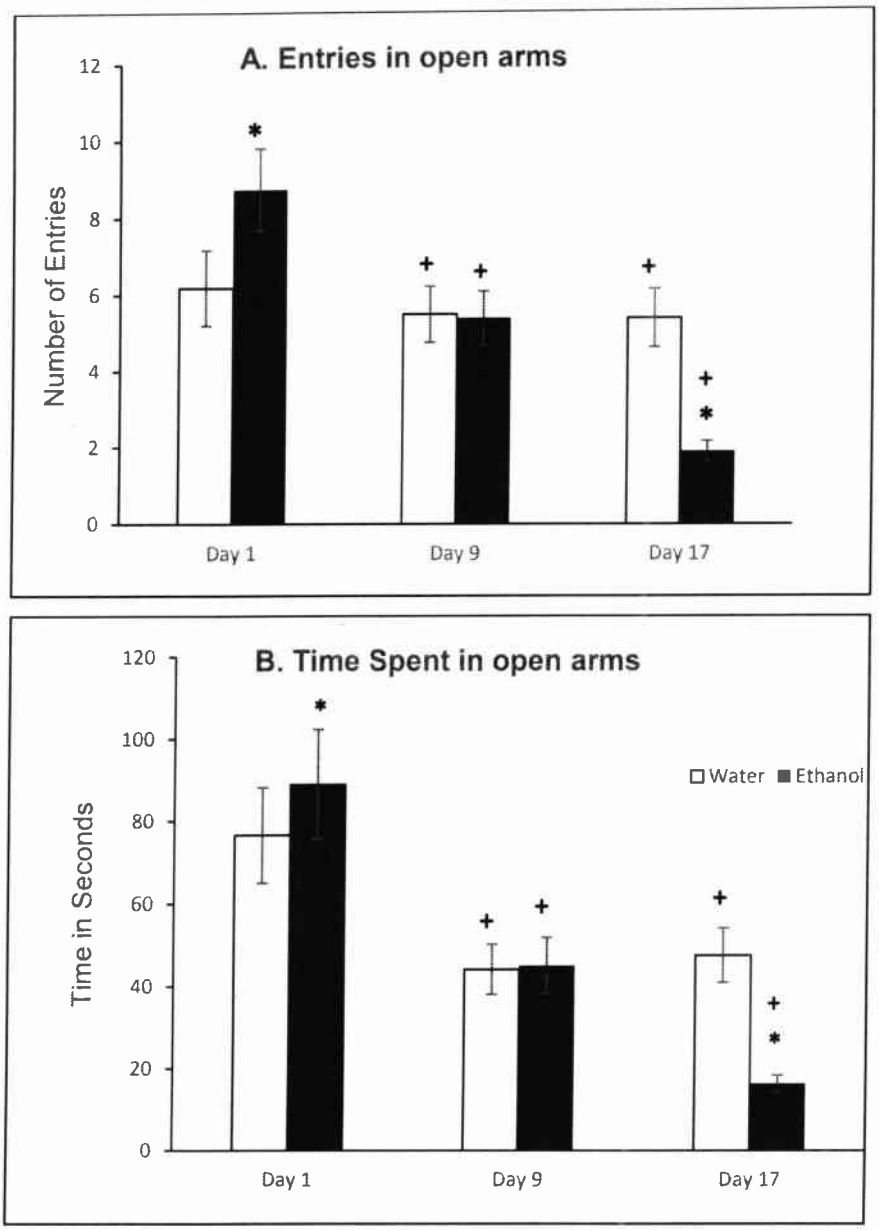


Figure 4.3: Effects of alcohol consumption on anxiety like behavior observed in the elevated plus maze from 1st day of experiment to 17th day. Values are means ± SD. (A) The number of open arm entries. *p<0.01 on day 1 and on day 17 different from relevant water drinking animals, +p<0.01 different than previous days. (B) Time spent in the open arms. *p<0.01 on day 1 and on day 17 different from respective water drinking animals, significant difference +p<0.01 different from previous days results.

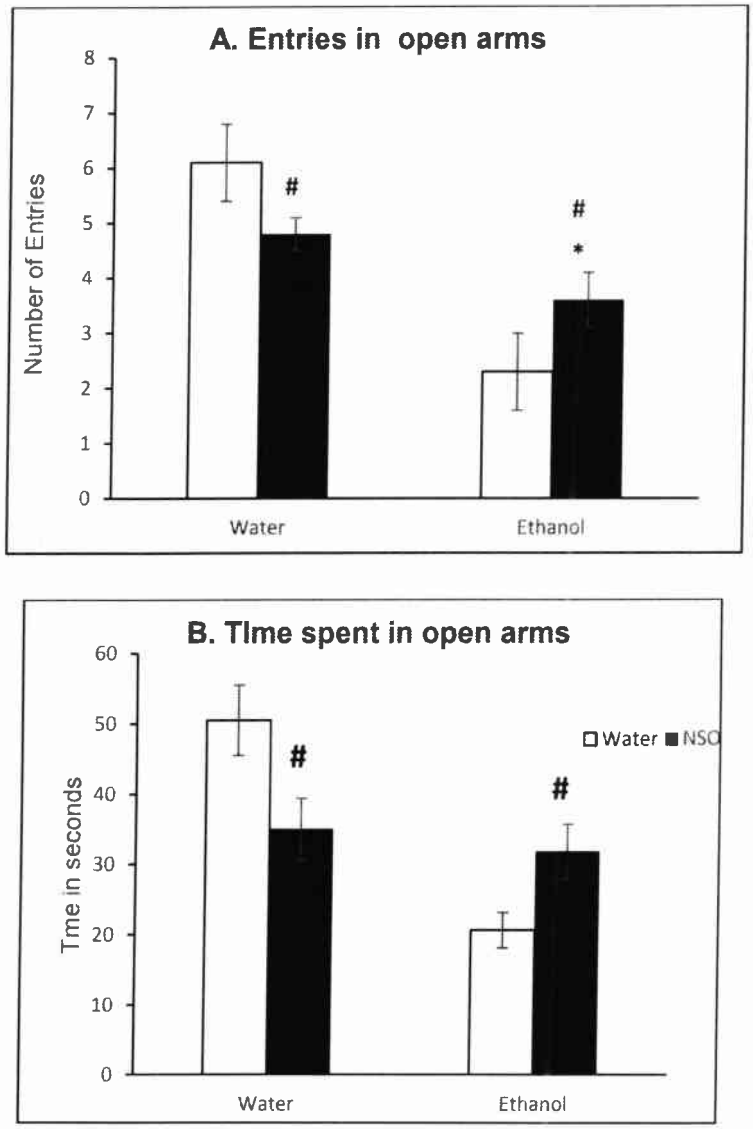


Figure 4.4: Effects of *Nigella sativa* oil (NSO) treatment (10 days) of water and ethanol drinking animals on anxiety as measured in the elevated plus maze. Values represent means ± SD. (A) The number of open arm entries. *$p<0.05$ different from water drinking + *Nigella sativa* oil treated animals, #$p<0.01$ different from respective water treated controls. (B) Time spent in open arms. # $p<0.01$ different from water treated controls of alcohol and water drinking animals

4.2.2.2. Effects of Nigella sativa oil on learning and memory in alcohol drinking rats

Figure 4.5 Independent sample t-tests were conducted to compare the spatial learning and memory of water and alcohol drinking groups as acquisition, retention and extinction.

Acquisition: T-test analysis of acquisition data showed that there was a significant difference in the escape latency time for water drinking (M=29.5833, SD= 4.86375) and alcohol drinking (M=36.0833, SD=5.28202) groups; t (22) = -3.136,22, $p<0.01$.

Retention: T-test analysis of retention data showed that there was a significant difference in the escape latency time for water drinking (M=27.4167, SD=3.9856) and alcohol drinking (M=52.3333, SD=8.2188) groups; t (22) = -9.449, $p<0.01$.

Extinction: T-test analysis of extinction data showed that there was a significant difference in the escape latency time for water drinking (M=12.0833, SD=1.60841) and alcohol drinking (M=50.6667, SD=7.28340) groups; t (22) = -17.919, $p<0.01$.

Paired t-test analysis of results showed that alcohol drinking animals showed delayed escape latency in acquisition, retention and extinction compared to water drinking animals ($p<0.01$). When the individual data was compared to own previous results, it showed that in retention period alcohol drinking rats could not recall the memory and took significantly ($p<0.01$) more time to find platform. Whereas no such difference was observed in water drinking controls. In Extinction period, the water drinking animals took significantly less time to reach platform from previous days showing the habituation effect and reformation of previous memory whereas no such habituation effect was observed in alcohol drinking animals and they took showed significant delayed escape latency.

Figure 4.6 is showing results of NSO treatment of water and ethanol drinking animals on memory. Two-way ANVOVA used to analyze the data showed significant impact of ethanol consumption (F= 209.716, df =1,20, $p<0.01$), NSO treatment (F=125.980, df=1,20, $p<0.01$) and fluid consumption and NSO treatment interaction (F= 116.623, df=1,20, $p<0.01$) on latency to locate platform in Morris water maze (MWM). Post-hoc testing shows that alcohol drinking animals took more time and showed increased

latency time ($p<0.01$) compared to controls. *Nigella sativa* oil treatment of alcohol drinking animals improved the memory and latency time was reduced ($p<0.01$) compared to alcohol drinking + water administered animals.

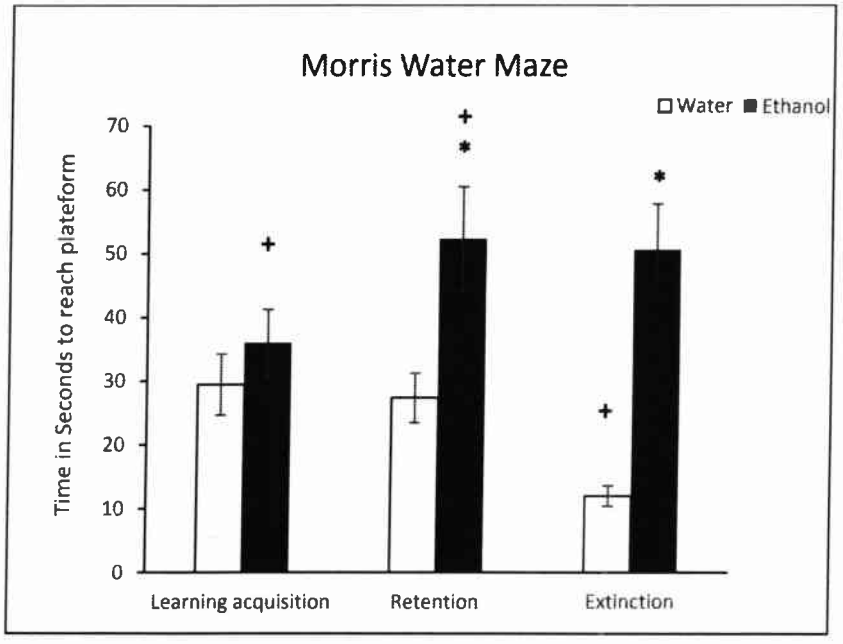


Figure 4.5: Effects of alcohol consumption on learning and memory observed in the MWM on Day 2nd (Acquisition), Day 3rd (Retention) and Day 16th of the experiment. Values are means ± SD. *$p<0.01$ on retention and extinction different from respective water drinking controls, +$p<0.01$ on retention and extinction different from previous days.

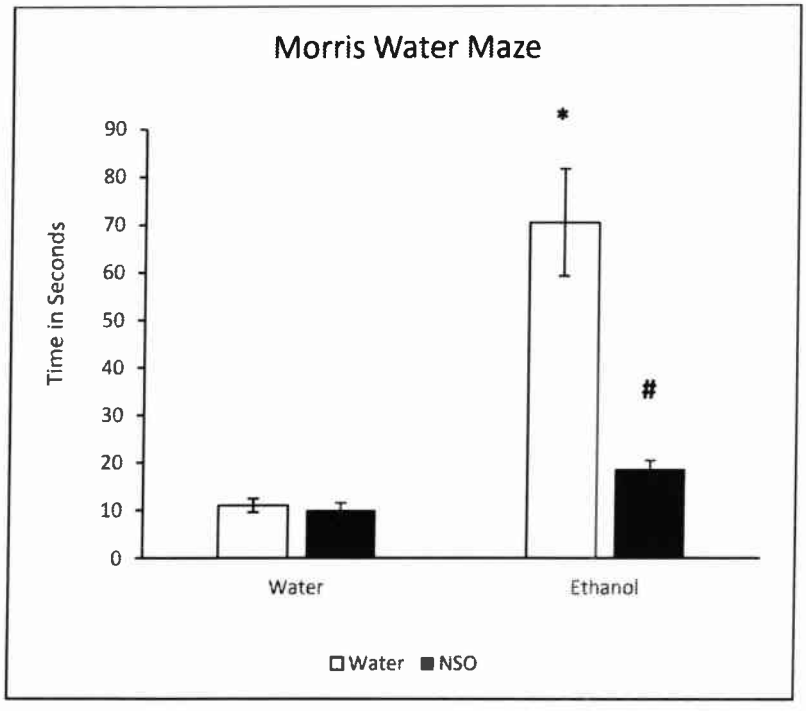


Figure 4.6: Effects of *Nigella sativa* oil treatment (NSO) of alcohol and water drinking rats on long term memory reformation on day 26th. The values are representing means ± SD. #p<0.01 different from respective alcohol drinking + water treated control animals and *p<0.01 than respective water drinking animals

4.2.2.3. Effects of alcohol and Nigella sativa oil on exploratory activity in rats

The figure 4.7 is showing impacts of ethanol drinking on exploration and motor skills in an open field (OF). We found significant effects of alcohol consumption ($F=20.983$, $df=1,22$, $p<0.01$), RM ($F=197.311$, $df=1,22$, $p<0.01$) and fluid consumption and RM interaction ($F=14.744$, $df=1,22$, $p<0.01$) effects on exploratory activity. Post-hoc analysis revealed that alcohol drinking animals crossed a significant ($p<0.01$) less boxes of the open field (OF) compared to boxes crossed by water drinking animals on day 12. There was no difference in the number of boxes crossed between groups on day 5. Number of boxes crossed by alcohol and water drinking animals on Day 12 was significantly ($p<0.01$) reduced from Day 5.

The figure 4.8 is showing the impact of NSO treatment on exploration and motor skills of ethanol and water drinking animals. There was significant impact of ethanol drinking ($F=380.046$, $df=1,20$, $p<0.01$), NSO treatment ($F=35.166$, $df=1,20$, $p<0.01$) and interaction ($F=64.975$, $df=1,20$, $p<0.01$) between water and NSO treatment on locomotion. Post-hoc test shows that exploratory activity on Day 25th of experiment was significantly ($p<0.01$) reduced in alcohol drinking + water and alcohol drinking + *Nigella sativa* oil treated groups from respective water drinking + water and water drinking + *Nigella sativa* oil treated groups. The activity was also decreased ($p<0.01$) in water drinking + *Nigella sativa* oil treated group as compared to water drinking + water groups. These results showed that NSO treatment resulted in insignificant increase in activity in ethanolic rats but reduced exploration activity in water drinking animals.

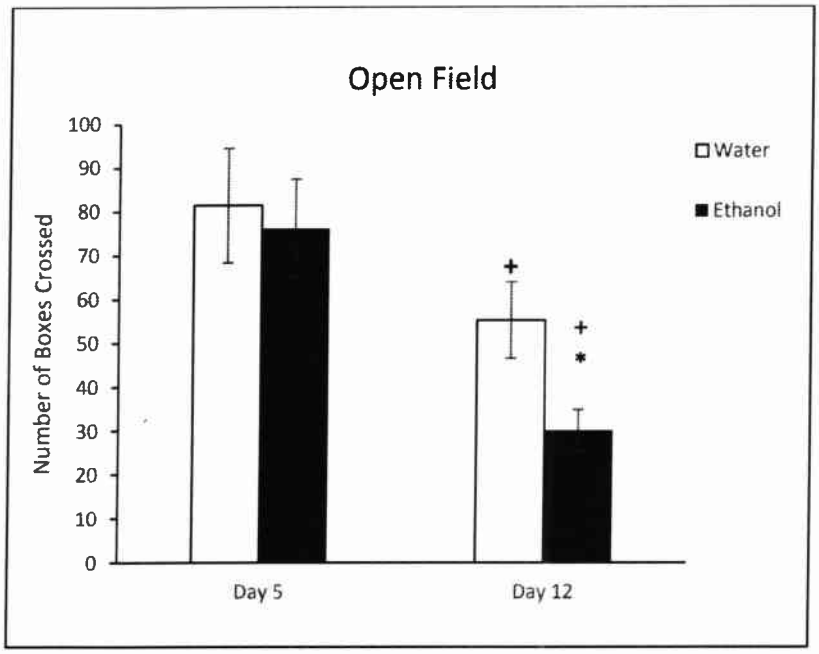


Figure 4.7: Effect of alcohol consumption on exploratory activity. Values represent means ± SD. *p<0.01 different from previous days control animals; +p<0.01 different compared to previous day 5 results in same groups.

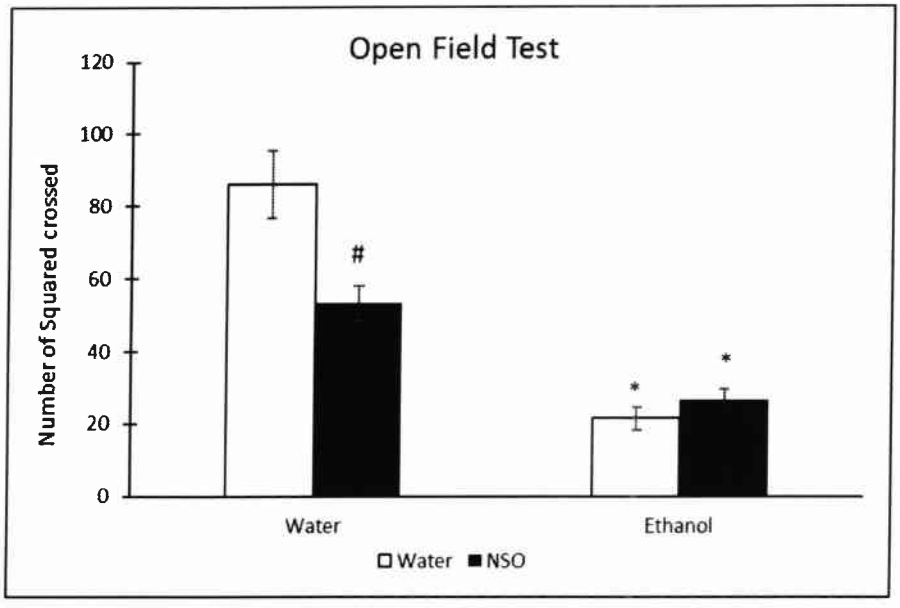


Figure 4.8: Effects of *Nigella sativa* oil (NSO) treatment (day 25) on exploratory activity of water and alcohol drinking animals observed in open field test. Values represent means ± SD; *p<0.01 different to respective water drinking + *Nigella sativa* oil treated animals and water drinking + water treated control group; #*p*<0.01 different from water drinking + water treated animals.

4.2.3. Neurochemistry

4.2.3.1. Effects of Nigella sativa oil on neurotransmitters and their metabolites level in prefrontal cortex of alcohol drinking rats

Figure 4.9 is showing the of NSO treatment effect on 5-HT and its metabolite 5-HIAA concentration in prefrontal cortex of water and ethanol drinking rats. 5-HT level data analysis by two-way ANOVA revealed significant impact of alcohol consumption ($F=49.936$, $df=1,20$, $p<0.01$), NSO treatment ($F=40.745$, $df=1,20$, $p<0.01$) and alcohol consumption and NSO treatment interaction ($F=60.015$, $df=1,20$, $p<0.01$). Post-hoc test for 5-HT level data showed that serotonin in EW group was reduced significantly ($p<0.01$) than WW and ENSO group had significantly ($p<0.01$) elevated serotonin levels than WNSO.

Data on 5-HIAA level in prefrontal cortex was also analyzed by using two way ANOVA and revealed significant impacts of alcohol drinking ($F=7.8581$, $df=1,20$, $p<0.05$), NSO treatment ($F=77.338$, $df=1,20$, $p<0.01$) and alcohol drinking + NSO treatment ($F=19.367$, $df=1,20$, $p<0.01$) interaction . Post hos tests for 5-HIAA data showed that WNSO and ENSO groups had significantly lower ($p<0.01$) 5-HIAA concentrations than WW and EW respectively in prefrontal cortex.

The figure 4.10 is showing the impacts of NSO treatment on DA, DOPAC and HVA levels in prefrontal cortex of water and ethanol drinking rats. Prefrontal DA data analysis revealed significant impacts of alcohol consumption ($F=135.41$, $df=1,20$, $p<0.01$), NSO treatment ($F=5.803$, $df=1,20$, $p<0.05$) and alcohol consumption and NSO treatment interaction ($F=15.517$, $df=1,20$, $p<0.01$). By post hoc test significant increase ($p<0.01$) in DA levels in prefrontal cortex of EW and ENSO than WW and WNSO respectively were found. Significant ($p<0.01$) reduction was observed in Da level of ENSO group compared to EW.

Prefrontal cortical DOPAC level analysis revealed significant roles of alcohol drinking ($F=15.245$, $df=1,20$, $p<0.01$), NSO treatment ($F=144.971$, $df=1,20$, $p<0.01$) and alcohol drinking and NSO treatment ($F=17.523$, $df=1,20$, $p<0.01$) interaction . By using

post hoc test significant ($p<0.01$) increase in DOPAC level in prefrontal cortex of WNSO and ENSO than WW and EW respectively were noticed. EW group had significantly ($p<0.01$) increased DOPAA levels than WW.

Prefrontal cortical HVA level analysis revealed significant impact of alcohol drinking ($F=9.528$, $df=1,20$, $p<0.01$) and ethanol drinking and NSO treatment ($F=528.188$, $df=1,20$, $p<0.01$) interaction. Insignificant impact of NSO treatment ($F=3.828$, $df=1,20$, $p>0.05$) on prefrontal HVA concentration was found. Post-hoc analysis showed significant increase ($p<0.01$) in prefrontal HVA concentrations of WNSO and EW than WW. The HVA level in ENSO group was significantly ($p<0.01$) reduced compared to WNSO and EW.

The figure 4.11 is showing the impacts of NSO treatment on Noradrenaline level in prefrontal cortex. Analysis revealed significant impacts of alcohol consumption ($F=131.271$, $df=1,20$, $p<0.01$), NSO treatment ($F=424.32$, $df=1,20$, $p<0.01$) and alcohol consumption + NSO treatment interaction ($F=26.282$, $df=1,20$, $p<0.01$). Post hoc test found significant ($p<0.01$) increase in noradrenaline concentration in prefrontal cortex of *Nigella sativa* oil treated water and alcohol drinking rats compared to respective controls. When alcohol drinking + *Nigella sativa* oil and water drinking + *Nigella sativa* oil groups were compared, we found that this increase was more significant ($p<0.01$) in alcohol drinking than water dinking rats, which is showing the interaction of *Nigella sativa* oil and alcohol drinking. These data indicated that *Nigella sativa* oil is capable to increase noradrenaline level in the prefrontal cortex.

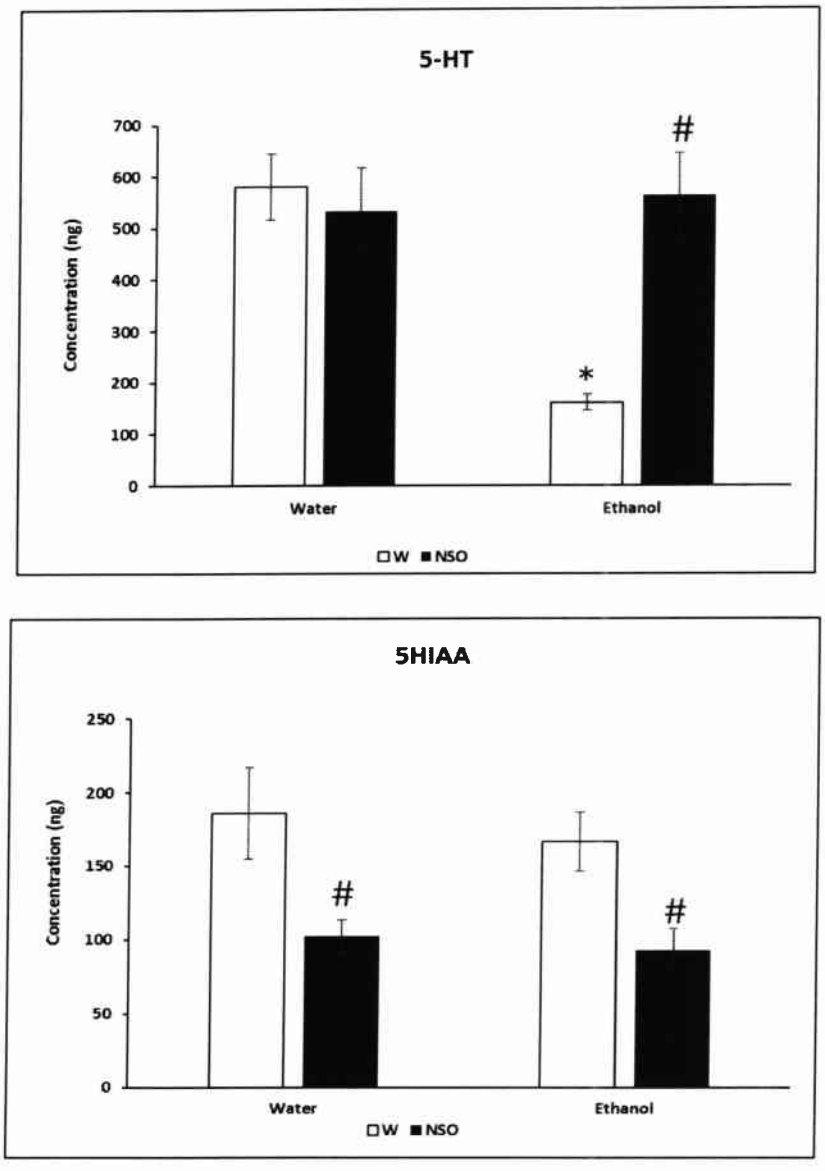


Figure 4.9: Effects of NSO treatment (0.5 mL/kg) on 5-HT and 5-HIAA levels in pre-frontal cortex of water and ethanol drinking groups. Values represent means ± SD. **(5HT)** *$p<0.01$ and #$p<0.01$ different from water drinking + water treated group and ethanol drinking + water treated group respectively. **(5HIAA)** #$p<0.01$ different from respective water treated groups.

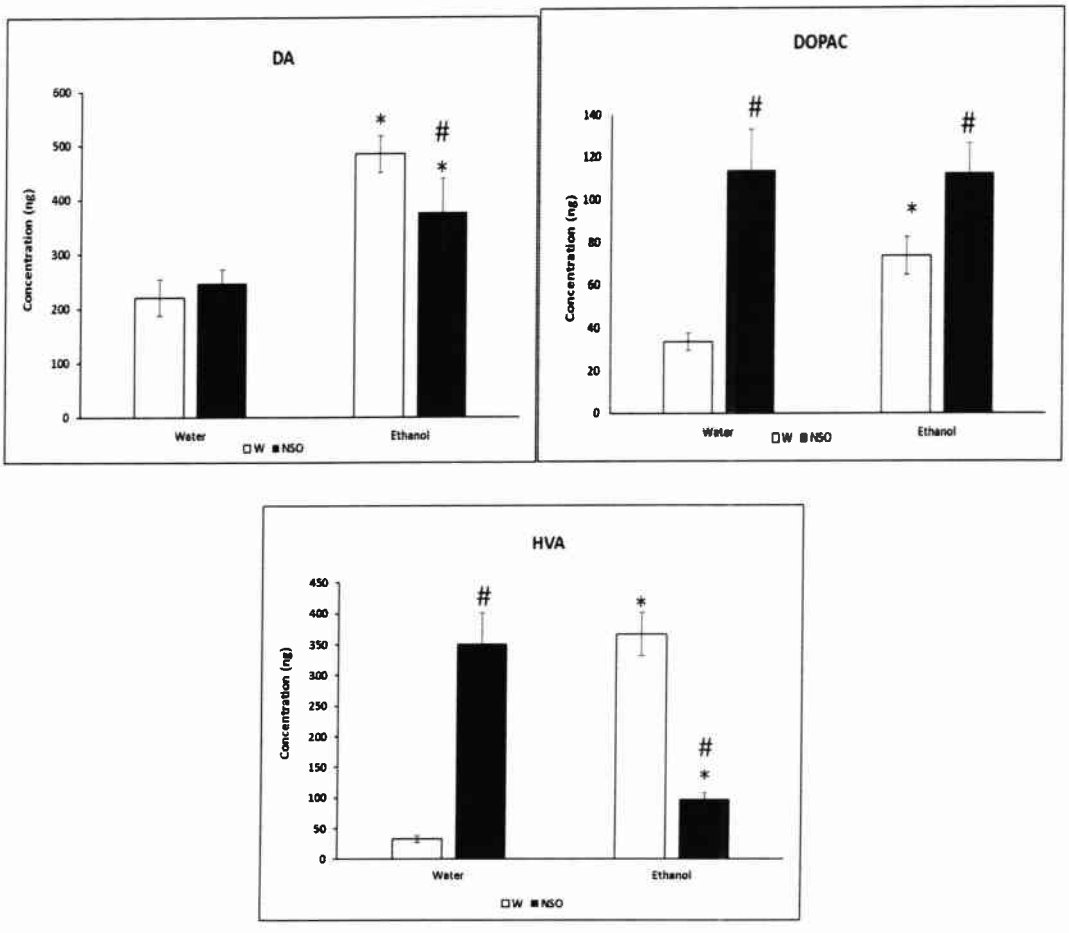


Figure 4.10: Effects of NSO treatment on levels of DA and its metabolites DOPAC, and HVA in PFC of water and ethanol drinking groups. Values represent means ± SD. **(DA)** *p<0.01 different from water drinking + water treated controls, water drinking +NSO treated animals; #p<0.01 different from ethanol drinking control group. **(DOPAC)** *p<0.01 different from water drinking + water treated controls and water drinking + NSO treated animals; #p<0.01 different from respective control groups. **(HVA)** *p<0.01 different from water drinking + water treated controls and water drinking + NSO treated animals; #p<0.01 different from respective control groups.

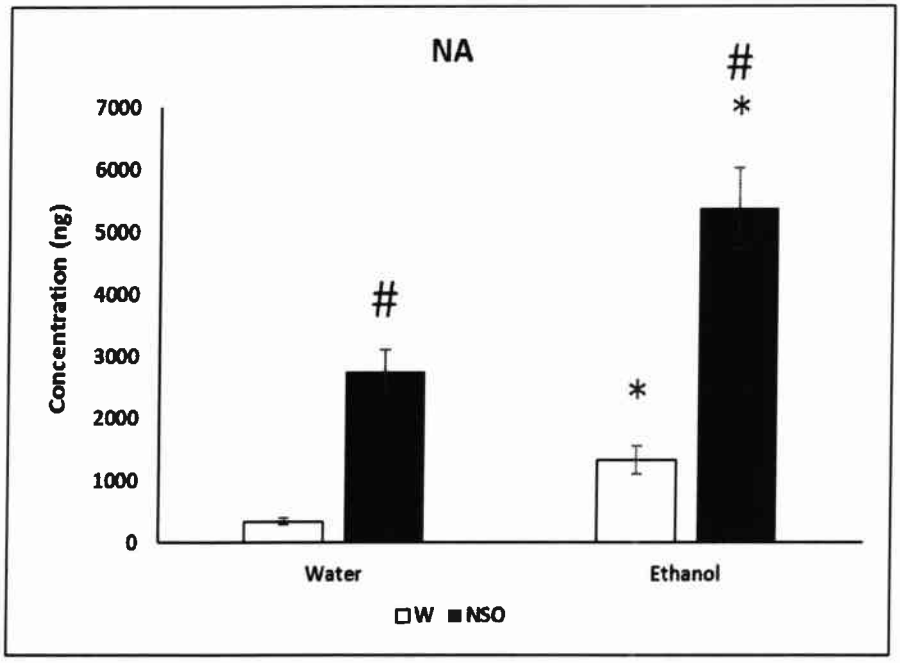


Figure 4.11: Effects of NSO treatment on noradrenaline level in the PFC of water and ethanol drinking groups. Values represent means ± SD. #p<0.01 and *p<0.01 different from water drinking + water control group; #p<0.01 and *p<0.01 different from water drinking + *Nigella sativa* oil treated animals.

4.2.3.2. Effects of Nigella sativa oil on neurotransmitters and their metabolites level in hippocampus of alcohol drinking rats

Figure 4.12 shows the effects of NSO treatment on 5-HT and 5-HIAA level in hippocampus of water and ethanol drinking groups. Data on hippocampal 5-HT and 5-HIAA levels was analyzed using two-way ANOVA. Results from 5-HT data showed significant impacts of alcohol consumption ($F= 7.858$, $df=1,20$, $p<0.05$), NSO treatment ($F=77.338$, $df=1,20$, $p<0.01$) and alcohol consumption and NSO treatment ($F=19.367$, $df=1,20$, $p<0.01$) interaction. By using post hoc test significant ($p<0.01$) increase in serotonin levels in WNSO and ENSO than WW and EW respectively were observed. This increased 5-HT level in ENSO also possess significant ($p<0.05$) difference from EW.

Results from 5-HIAA data revealed significant effect of NSO treatment ($F=77.338$, $df=1,20$, $p<0.01$) on 5-HIAA levels. But no significant impact of alcohol consumption ($F=3.053$, $df=1,20$, $p>0.05$) or alcohol drinking and NSO treatment ($F=.305$, $df=1,20$, $p>0.05$) interaction found. Post-hoc test for 5-HIAA levels showed significant increased 5-HIAA levels in WNSO and ENSO than WW and EW groups respectively.

The figure 4.13 is showing the impacts of NSO treatment on DA, DOPAC and HVA levels in hippocampus of water and ethanol drinking rats. Hippocampal DA data analysis by using two-way ANOVA revealed significant impact of alcohol consumption ($F=18.466$, $df=1,20$, $p<0.01$) on DA concentrations in hippocampus. Alcohol consumption and NSO treatment ($F=9.52$, $df=1,20$, $p<0.01$) interaction effect was found. NSO treatment effect on hippocampal DA was not significant ($F=.154$, $df=1,20$, $p>0.05$). Post hoc analysis showed significant ($p<0.01$) reduction in DA level in hippocampus of ENSO group compared to WNSO.

Data analysis on hippocampal DOPAC level revealed significant impact of alcohol drinking ($F=129.397$, $df=1,20$, $p<0.01$), NSO treatment ($F=233.834$, $df=1,20$, $p<0.01$) and alcohol drinking and NSO treatment ($F=176.141$, $df=1,20$, $p<0.01$) interaction. Post-hoc test revealed that WNSO had significantly ($p<0.01$) elevated DOPAC level compared to WW. ENSO group possess significantly reduced DOPAC level than WNSO.

Hippocampal HVA data analysis showed revealed significant impact of alcohol drinking ($F=25.891$, $df=1,20$, $p<0.01$) and NSO treatment ($F=133.994$, $df=1,20$, $p<0.01$) on HVA level. No interaction between alcohol drinking and NSO treatment ($F=3.644$, $df=1,20$, $p>0.05$) on HVA level was observed.

Post hoc test of HVA concentrations in hippocampus data showed that WNSO and ENSO had significantly ($p<0.01$) lower HVA concentration than WW and EW respectively. ENSO group also showed significant ($p<0.10$) difference from EW and had decreased HVA level.

The figure 4.14 is showing the impacts of NSO treatment on Noradrenaline level in hippocampus of water and ethanol drinking groups. Data analysis by two-way ANOVA showed significant impact of alcohol consumption ($F= 16.645$, $df=1,20$, $p<0.01$), NSO treatment ($F=9.107$, $df=1,20$, $p<0.01$) and a significant ethanol consumption and NSO treatment interaction effect ($F=33.28$, $df=1,20$, $p<0.01$).

Post-hoc data analysis revealed a significant ($p<0.01$) decrease in noradrenaline levels in the hippocampus of water drinking + *Nigella sativa* oil treated rats compared to water drinking + water treated rats. No significant difference in noradrenaline concentration was observed in alcohol drinking + *Nigella sativa* oil treated animals and alcohol drinking + water treated animals. alcohol drinking + water treated animals displayed significant ($p<0.01$) lower noradrenaline levels compared to water drinking + water treated animals. These data indicate that *Nigella sativa* oil can reduce noradrenaline level in the hippocampus.

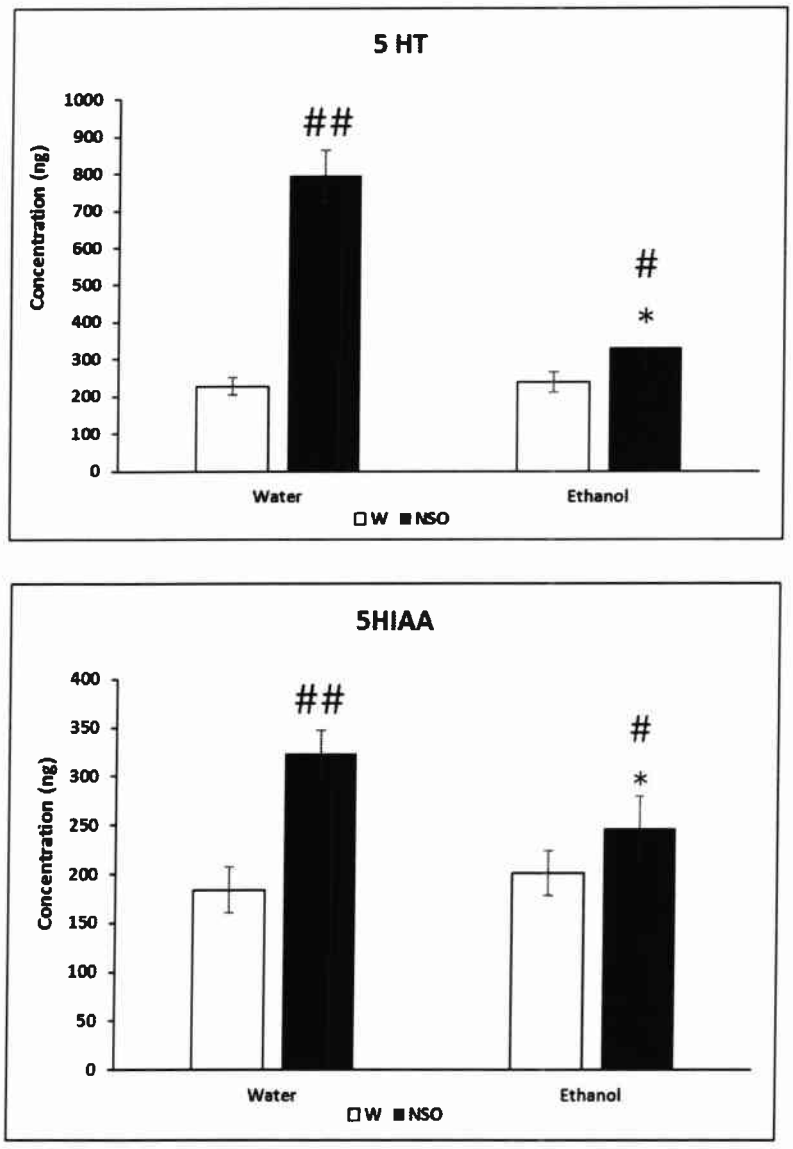


Figure 4.12: Effects of NSO treatment on serotonin and 5-HIAA levels in hippocampus (HP) of water and ethanol drinking groups. Values represent means ± SD. **(5HT)** ##p<0.01 and *p<0.01 different from water drinking + water treated control animals and water drinking + NSO treated group respectively; #p<0.05 different from water drinking + NSO treated animals. **(5HIAA)** ##p<0.01 and *p<0.01 different from water drinking + water treated controls and water drinking + NSO treated rats respectively; #p<0.05 different from water drinking + NSO treated group.

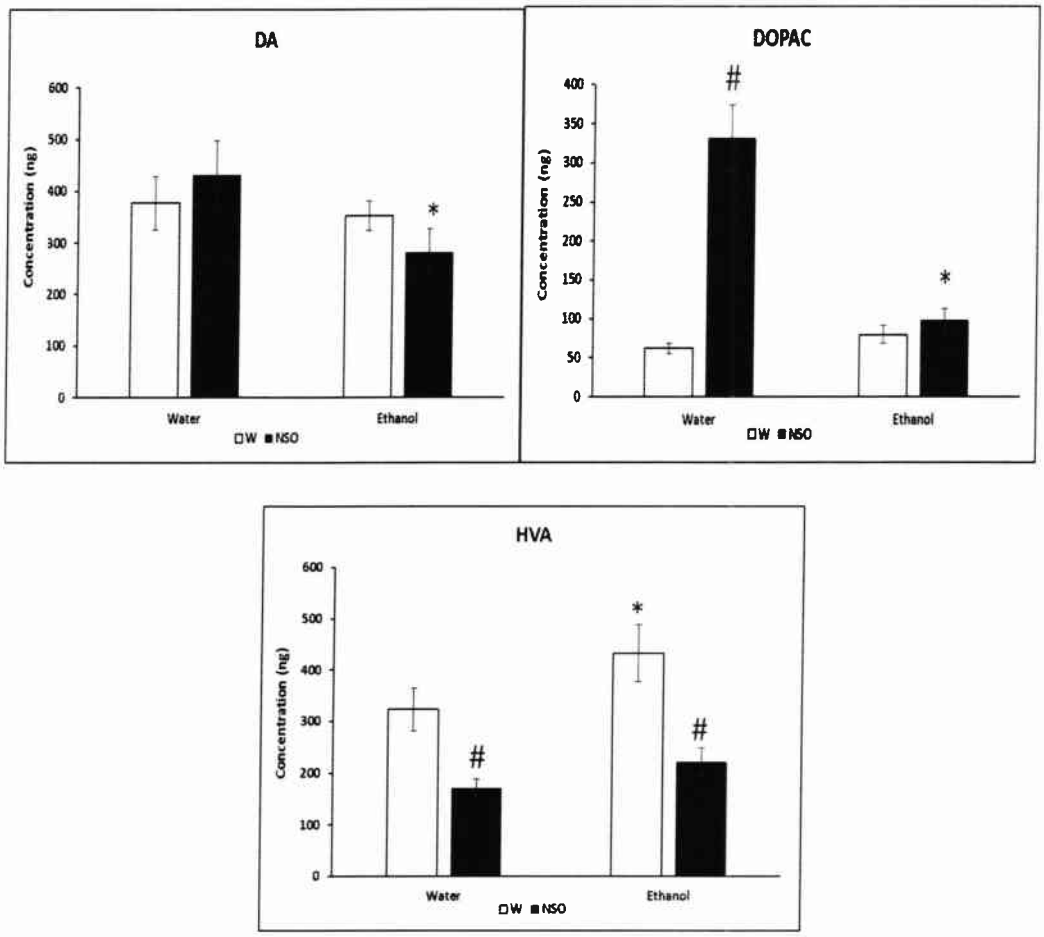


Figure 4.13: Effects of NSO treatment on dopamine, DOPAC, and HVA levels in hippocampus of water and ethanol drinking groups. Values represent means ± SD. **(DA)** *p<0.01 different from water drinking + NSO treated group. **(DOPAC)** *p<0.01 different from water drinking + *Nigella sativa* oil treated animals and #p<0.01 different from water drinking +water treated controls. **(HVA)** *p<0.01 different from water drinking + water treated group; #p<0.01 different from respective water treated control groups.

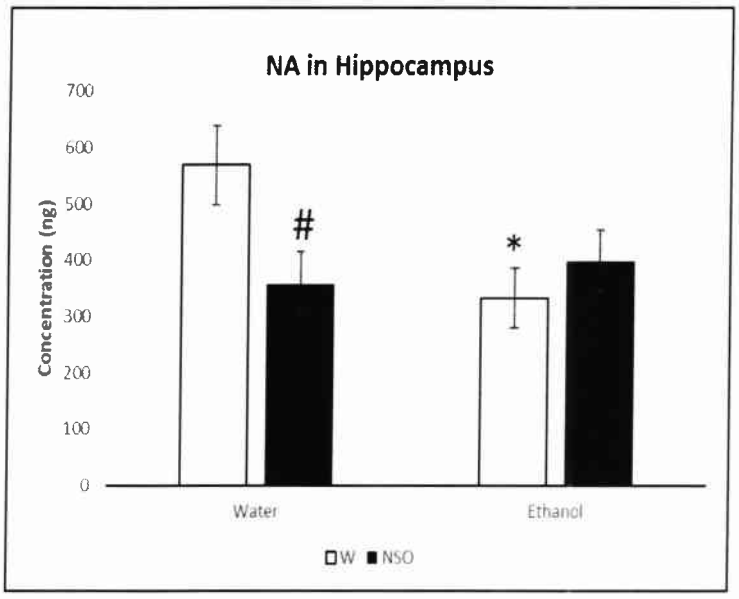


Figure 4.14: Effects of NSO treatment on noradrenaline level in the hippocampus of water and ethanol drinking groups. Values represent means ± SD. #p<0.01 and *p<0.01 different from water drinking + water treated controls.

4.2.3.3. Effects of Nigella sativa oil on neurotransmitters and their metabolites level in midbrain of alcohol drinking rats

The figure 4.15 is showing the impacts of NSO treatment on Noradrenaline level in midbrain of water and ethanol drinking groups. Data analyzed showed significant impact of ethanol consumption ($F=508.251$, $df=1,20$, $p<0.01$), NSO treatment ($F=31.516$, $df=1,20$, $p<0.01$) and a significant ethanol consumption and NSO treatment interaction effect ($F=30.162$, $df=1,20$, $p<0.01$). Post-hoc analysis indicated that noradrenaline levels in the midbrain were significantly ($p<0.01$) decreased in the water drinking + *Nigella sativa* oil treated group compared to the water drinking + water treated group. No significant difference in noradrenaline concentration was observed in the alcohol drinking + *Nigella sativa* oil treated and alcohol drinking + water treated group. However, noradrenaline was deceased ($p<0.01$) in alcohol treated animals than water drinking animals.

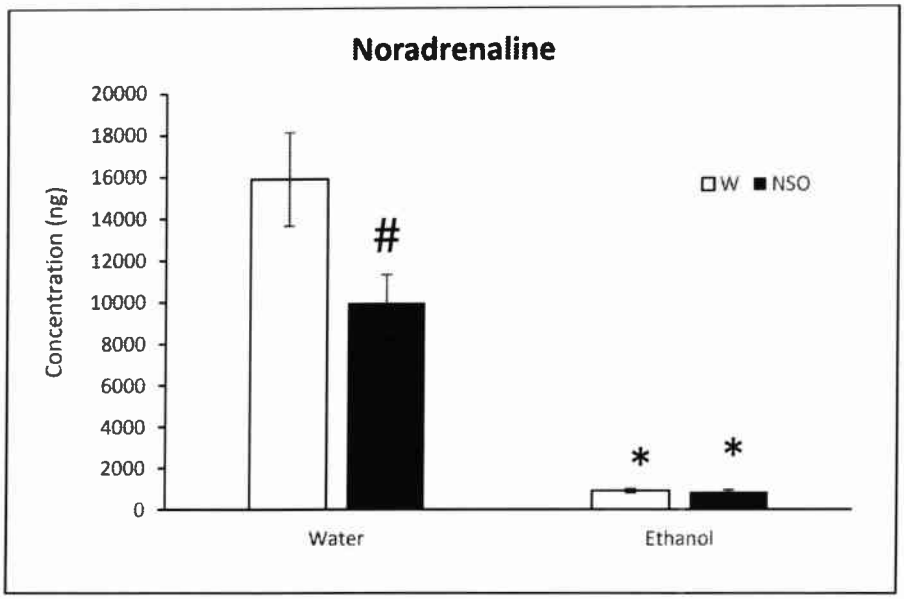


Figure 4.15: Effects of NSO treatment on noradrenaline concentration in midbrain of water and ethanol drinking groups. Values represent means ± SD. #p<0.01 different from water drinking + water treated controls; *p<0.01 different from water drinking + water treated controls; *p<0.01 different from water drinking + *Nigella sativa* oil treated control group.

4.2.4. Effects of *Nigella Sativa* on Catalase and Peroxide in Alcoholic Rats

Figure 4.16 shows the results of alcohol consumption and NSO treatment on striatal catalase level. Data analysis was done using two-way ANOVA showed insignificant impact of alcohol consumption ($F=.228$, $df=1,20$, $p>0.05$), NSO treatment ($F=.467$, $df=1,20$, $p>0.05$) or fluid type and NSO treatment ($F=.592$, $df=1,20$, $p>0.05$) interaction was on striatal catalase level.

Figure 4.17 shows the results of alcohol consumption and NSO treatment on Peroxide levels in Striatum. Data analysis done by Two-way ANOVA and showed insignificant impact of alcohol consumption ($F=.382$, $df=1,20$, $p>0.05$), NSO treatment ($F=.783$, $df=1,20$, $p>0.05$), interaction effect ($F=.309$, $df=1,20$, $p>0.05$) on peroxidase level.

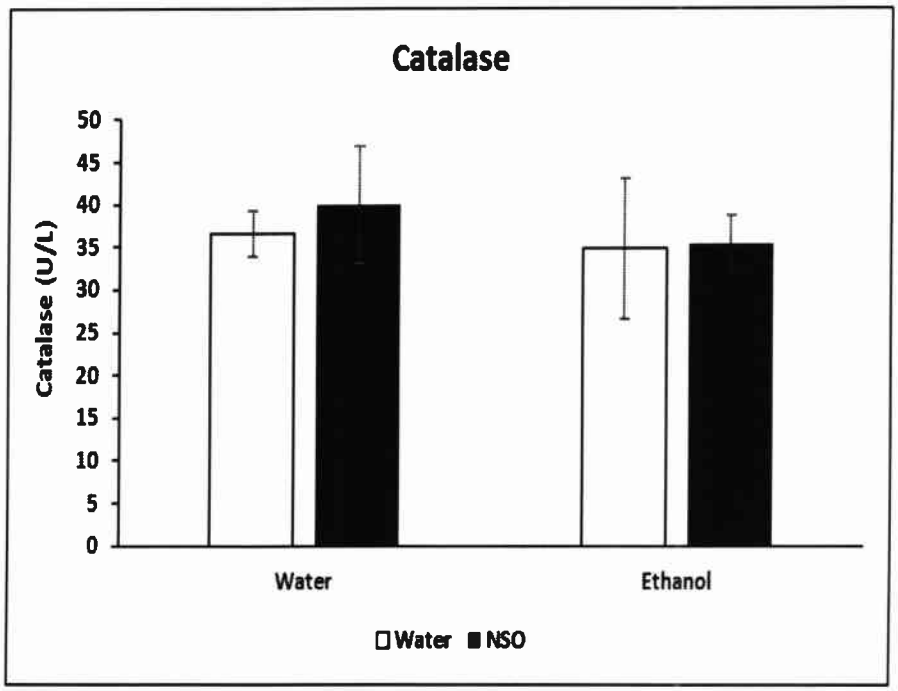


Figure 4.16: Effects of NSO treatment (0.5 mL/kg) on Striatal catalase levels of water and ethanol drinking groups. Values represents means ± SD.

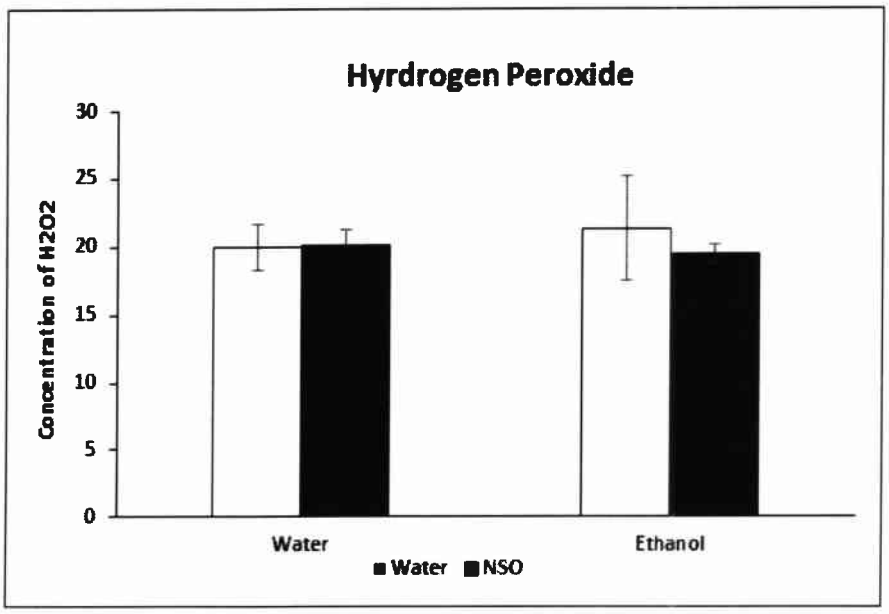


Figure 4.17: Effects of NSO treatment (0.5 mL/kg) on Striatal peroxide levels of water and ethanol drinking groups. Values represents means ± SD.

4.2.5. Biochemical Studies

4.2.5.1. Effects of Nigella sativa oil on serum insulin, glucose, total cholesterol and triglycerides levels in alcoholic rats.

Figure 4.18 is showing the impact of alcohol consumption and NSO treatment on serum insulin levels. Two-way ANOVA analysis revealed that there were no significant effects of alcohol consumption ($F=.619$, $df=1,20$, $p>0.05$) and NSO treatment ($F=.000$, $df=1,20$, $p>0.05$). No significant ($F=.022$, $df=1,20$, $p>0.05$) interaction effect was found in any of the group.

Figure 4.19 shows the results of alcohol consumption and NSO treatment on serum glucose levels. Two-way ANOVA analysis revealed significant effects of alcohol consumption (19.287, $df=1,20$, $p<0.01$) and NSO administration ($F= 12.682$, $df=1,20$, $p<0.01$) on glucose concentration in water drinking animals. Post-hoc testing revealed that the water drinking + NSO treated group had significant lower ($p<0.05$) serum glucose level compared to water drinking + water treated controls. Furthermore, glucose levels were decreased ($p<0.01$) in alcohol drinking + water treated compared to water drinking + water treated controls. The difference between alcohol drinking + water and alcohol drinking + NSO was not significant. These results demonstrate that NSO exerts hypoglycemic effects in controls.

Figure 4.20 is showing the results of alcohol consumption and NSO treatment on total cholesterol levels in serum. Data analysis revealed significant impact of alcohol consumption ($F=12.105$, $df=1,20$, $p<0.01$) and a significant alcohol consumption and NSO treatment interaction effect on total cholesterol levels in serum ($F=4.861$, $df=1,20$, $p<0.05$). NSO treatment effect on cholesterol was insignificant ($F=3.789$, $df=1,20$, $p>0.05$). Post hoc test revealed that the alcohol drinking + water treated control animals had significantly ($p<0.01$) higher concentration than water drinking + water. When alcohol drinking + water was compared to alcohol dinking + NSO groups, the alcohol dinking + NSO treated animals showed significantly ($p<0.05$) lower levels of total cholesterol in their serum. These results show that NSO is capable to reduce total cholesterol level during alcohol drinking.

Figure 4.21 is showing the impact of alcohol consumption and NSO treatment on serum triglycerides levels. Two-way ANOVA revealed that there were no significant effects of alcohol consumption ($F=1.099$, $df=1,20$, $p>0.05$) and NSO treatment ($F=3.132$, $df=1,20$, $p>0.05$). There was also no significant ($F=.735$, $df=1,20$, $p>0.05$) interaction effect was found in any of the group.

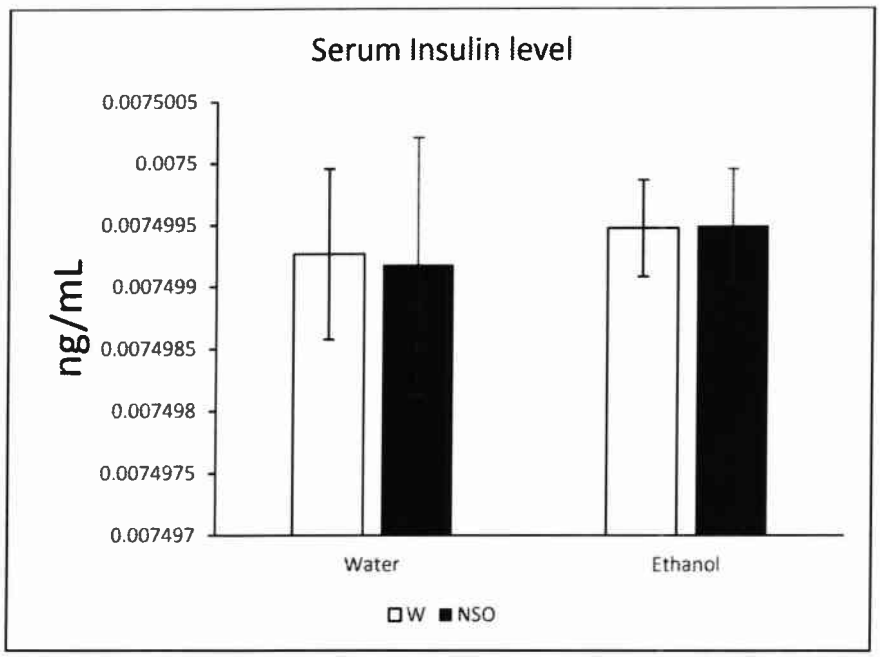


Figure 4.18: Effects of NSO treatment (0.5 mL/kg) on serum insulin levels of water and ethanol drinking groups. Values represent means ± SD.

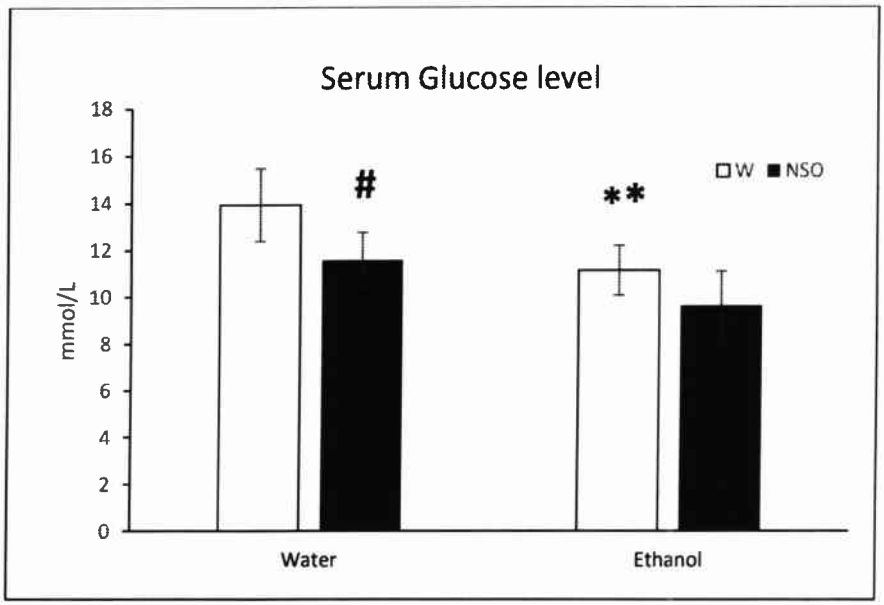


Figure 4.19: Effects of NSO treatment (0.5 mL/kg) on serum random glucose levels of water and ethanol drinking groups. Values represent means ± SD. **p<0.01 and #*p*<0.05 were different from water drinking + water treated control groups.

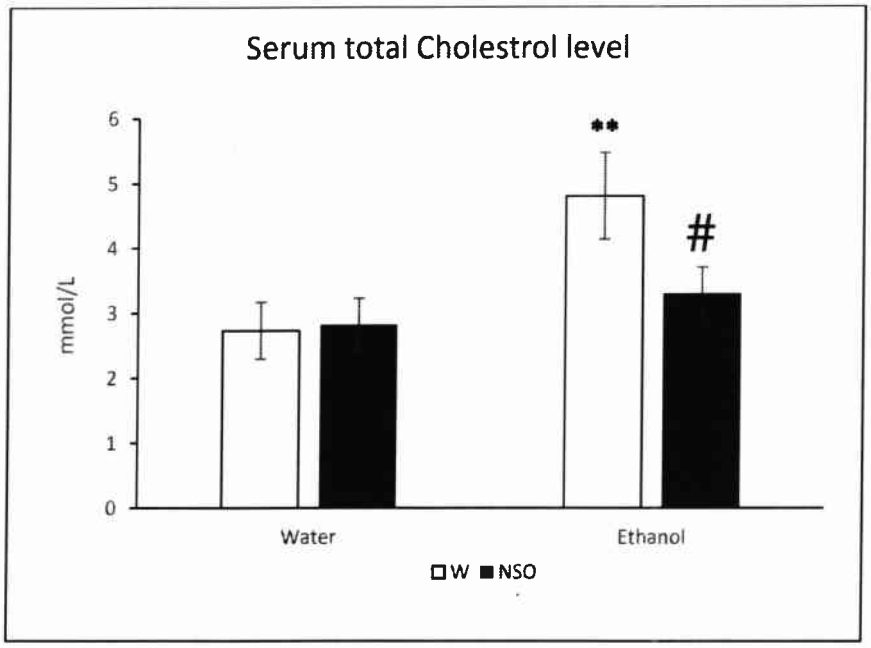


Figure 4.20: Effects of NSO treatment (0.5 mL/kg) on serum total cholesterol levels of water and ethanol drinking groups. Values represents means ± SD. **p<0.01 different from water drinking + water treated group; #p< 0.05 different from alcohol drinking + NSO group.

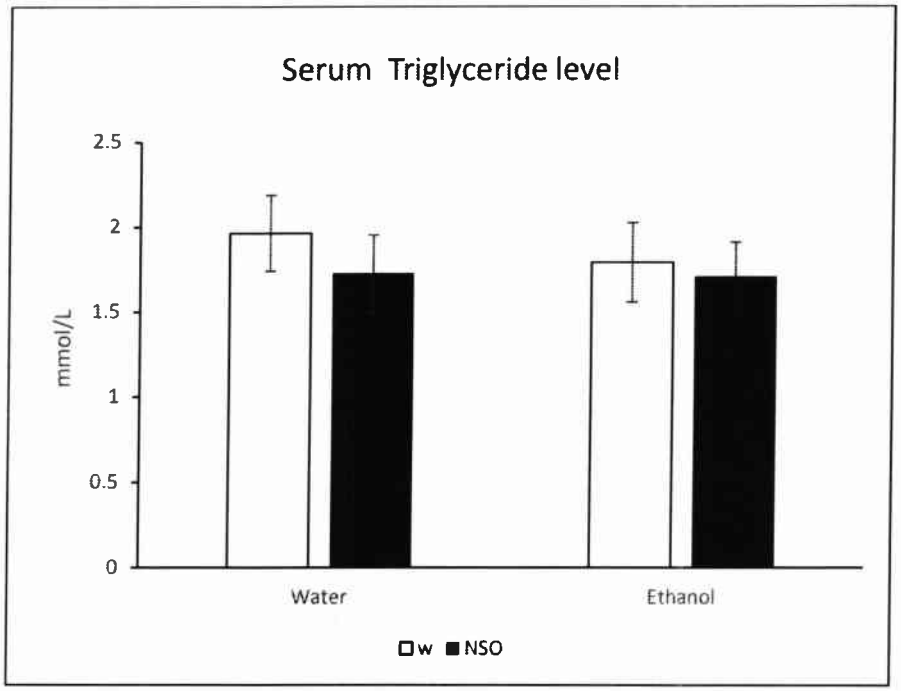


Figure 4.21: Effects of NSO treatment (0.5 mL/kg) on serum triglycerides levels of water and ethanol drinking groups. Values are means ± SD.

4.3. DISCUSSION

Featured findings of current study are:

1. *Nigella sativa* oil (NSO) treatment of ethanol drinking animals exacerbated the effects of ethanol on body weight, food and fluid intake.
2. NSO improves spatial memory and reduces anxiety in ethanolic rats.
3. Brain serotonin and its metabolite concentrations were elevated by NSO treatment in water and ethanolic rats.
4. Brain dopamine (DA) level was decreased in NSO treated ethanolic rats.
5. DOPAC was increased in NSO treated water and ethanol drinking groups.
6. NSO increased noradrenaline levels in the prefrontal cortex and slightly increased in hippocampus of water and ethanol consuming rats.
7. NSO reduced the total cholesterol levels in ethanol drinking rats.
8. NSO acted as hypoglycemic agent in the water drinking group.

In current study, ethanol consumption lead to reduced body weight, food and fluid intake in rats. Fromenty and colleagues, (2009) and Macdonald and coworkers, (2010) also reported that chronic ethanol intake reduced body weight. The effect of ethanol on body weight is biphasic, with smaller amount of ethanol that have no effect at all, moderate quantities lead to increased body weight whereas excessive ethanol consumption has been reported to reduced body weight (Suter, 1997). Acute ethanol intake has only a small effect on meal size (Poppitt *et al.*, 1996) but chronic ethanol consumption inhibits growth by decreasing the food intake and consumed meal size (Strbak *et al.*, 1998, Seeley *et al.*, 1997; Reidelberger *et al.*, 1996).

As mentioned above ethanol drinking resulted in reduced body weight, food and fluid intake. *Nigella sativa* oil (NSO) treatment of animals exacerbated the effects of ethanol and caused further decrease in body weights, food intake and water intake. There were no significant changes in body weight, food and fluid intake observed in NSO treated water drinking control animals. Also not significant difference was shown before and after NSO treatment. A previously published research (Cheema *et al.*, 2016) is favoring our

results with no significant effects of NSO on body weight, food or fluid intake in control Wistar male rats.

Ethanol drinking rats showed anxiety-like behavior in the elevated plus maze compared to water drinking controls. Holmes and coworkers, (2012) also reported that in most individuals, chronic ethanol use increases anxiety. NSO treatment of ethanol drinking animals reduced anxiety and spent more time in open arms of elevated plus maze compared to ethanol drinking controls. Ajao and coworkers, (2016) reported that NSO reduces anxiety and associated behavior. Many studies have demonstrated the effect of *Nigella sativa* oil on anxiety, but this is the first study to demonstrate the protective effects of NSO on anxiety induced by chronic ethanol consumption.

A previous study reported that when rats were treated with *Nigella sativa* oil (0.4 mL / kg) for a month, anxiety was reduced. This was associated with an increase in activity (Parveen *et al.*, 2009). In contrast, smaller doses of *Nigella sativa* oil, in the range of 0.1 and 0.25 mL/kg for 5 weeks, did not affect anxiety in rats (Cheema *et al.*, 2016). In our study, the dose 0.5 mL/kg was used, and it was found that oral administration of NSO for 10 days produced significant anxiolytic effects and reduced ethanol-induced anxiety. Our results demonstrated that *Nigella sativa* oil can reduce anxiety when given at bit higher dose.

Ethanol influences brain functioning, leading to behavioral and cognitive impairments (Ollat *et al.*, 1988; Deitrich *et al.*, 1989; Samson and Harris, 1992). In our study, ethanol resulted in impaired spatial learning and memory compared to water drinking animals. There are reports that chronic ethanol consumption is associated with impaired memory (White, 2003, Hartzler and Fromme, 2003; Wixted, 2005). These studies are in line with our results. When animals were treated with *Nigella sativa* oil, we found that the ethanol drinking + *Nigella sativa* oil treated group showed improved memory and gave quick response in MWM test which is showing the memory reformation. Many studies are in line with our results and revealed that NSO enhances memory in rats (Farimah *et al.*, 2016; Imam *et al.*, 2016; Anaeigoudari *et al.*, 2018). Our results are showing the protective role of NSO on ethanol prompted impaired spatial memory.

Alcohol consumption exerts negative effects on behavior. Most of the associated behaviors are associated to alterations in serotonin, DA and NA transmission in various brain regions. Neurotransmitters influence the functioning of neuroanatomic circuits that offer support to cognition, anxiety and fear. Number of studies has proven a relationship and linked between abnormal behavioral and reduced serotonin level in different brain areas (Berger, Gray and Roth, 2009; Muller and Jacobs, 2009; Lucki, 1998). Researches also suggest a significant role of dopamine in behavior (Dunlop and Nemeroff, 2007). Continuous ethanol consumption decreases brain noradrenaline (Yamanaka and Egashira, 1982).

Altered levels and transmission of 5-HT and DA between neurons have been found in patients with anxiety and hyperactivity (Alex and Pehek, 2007). Reduced DA transmission is linked with unusual neuropsychological conditions (Dailly *et al.*, 2004). Impaired DA concentrations are observed in depression and anxiety (Kafka, 2003). These studies give a possible indication of the pathogenesis of behavioral and phycological disorders. Many researches have demonstrated the role of noradrenaline in a range of psychological and physiological processes including memory, learning, anxiety, sleep, adaptation and arousal (Montgomery, 1997).

Previous researches have shown that 5-HT neurotransmission play a significant part in behavioral deficits (Gingrich and Hen 2001; Lucki, 1998). Reduced serotonin transmission is linked to impaired memory (Buhot, Martin and Segu, 2000), anxiety (Stein and Stahl, 2000), stress (Chaouloff, Berton and Mormede, 1999), and mood disorders (Ruhe, Mason and Schene, 2007). This current study reports reduced prefrontal and hippocampal 5-HT levels in ethanol drinking rats and these rats have shown impaired memory with anxiety.

The only precursor for 5-HT is tryptophan, an essential amino acid. Deficiency of tryptophan is linked with malnutrition (Young and Leyton, 2002). Decreased tryptophan might be a reason to decrease 5-HT neurotransmission. Exogenous supplements of tryptophan alleviate 5-HT level and neurotransmission (Neumeister, 2003; Delgado *et al.*, 1995). Researches have demonstrated memory enhancing (Haider, Khaliq and Haleem,

2007), antidepressant (Miller *et al.*, 1992) and anxietolytic (Gauthier *et al.*, 2014) properties of tryptophan supplements (Haider, Khaliq and Haleem, 2007). 5-HT also help to manage psychiatric diseases (Bell, Abrams and Nutt, 2001).

5-HT1A, 5-HT1B and 5-HT1C receptors belong to 5-HT1 receptor family (Barnes and Sharp, 1999). All three types of these receptors have an important function in regulation of food intake, maintaining body weight, anxiety, mood control and pain (Albert and Lemonde, 2004; Barnes and Sharp, 1999). In raphe nuclei (midbrain), the serotonergic neurons' cell bodies are found to have 5-HT1A auto-receptors. It prevents the excessive 5-HT transmission in somato-dendritic (Lanfumey and Hamon, 2000). Once activated, this exert inhibitory role leading to reduction in serotonin secretion (Bose *et al.*, 2011). The 5-HT1A and 5-HT2A receptors serves remarkable part in mood, anxiety, and feeding behavior (Celada *et al.*, 2004; Graeff, Viana and Mora, 1997). Anxiolytic and antidepressant drugs are agonist of 5-HT1A in nature (Blier and Ward, 2003; Hensler, 2003).

Depression in patients can be resulted from reduced 5-HT neurotransmission (Gauthier *et al.*, 2014; Brewerton and Jimerson, 1996). Preclinical research has demonstrated relative increase of somatodendritic 5-HT1A auto receptor in anxiety disorders (Akimova, Lanzenberger and Kasper, 2009), linked to the decreased 5-HT level and transmission (Lam *et al.*, 2010). Activated 5-HT1A receptors decreases brain 5-HT (Li *et al.*, 2000). Reduced response to 5-HT1A has been observed in anxiety model of animal (Flugge *et al.*, 1998). In present study, alcohol drinking caused reduced food intake and have shown impaired memory and anxiety.

Increased dopamine level is involved in self-preference of addictive substance (Oswald *et al.*, 2005). Alcohol consumption increases dopamine level through increased activity of mesolimbic dopaminergic neurons (Boileau *et al.*, 2003). Dopamine possesses affinity towards N-Methyl-D-aspartate (NMDA) and $GABA_A$. 5-HT and dopamine release relies on synergistic interaction between NMDA receptors and alcohol, which is relevant to alcohol induced potentiation of hyperlocomotion (Jin *et al.*, 2008) and alcohol mediated behavior deficits like anxiety (Homes *et al.*, 2012), cognitive impairments (Wixted, 2005;

White, 2003; Hartzler and Fromme, 2003; Samson and Harris, 1992; Deitrich *et al.*, 1989; Ollat *et al.*, 1988) and hyperactivity. Researches report important part of dopamine in behavioral deficits (Wahlstrom *et al.*, 2010; Moghaddam, 2002). Enormously reduced dopamine transmission is linked to depression and hypoactivity in rodents (Gershon, Vishne and Grunhaus, 2007). Abnormal D1 plus D2 receptors' expression has been reported in psychological issues, anxiety and depression (Vallone, Picetti and Borrelli, 2000). In present research, we found similar results with previous researches that alcohol increased the brain dopamine level and caused impaired memory, anxiety and hypoactivity.

Several studies have found relationship between DA and 5-HT transmission (Zangen *et al.*, 2001; Ichikawa and Meltzer, 1999). Zhou, Lesch and Murphy, (2002) reported that DA neurons also takes up some amount of serotonin. Although, DA reuptake sites have low affinity for 5-HT (Alex and Pehek, 2007) but persistent increase in extra-neuronal level of 5-HT in mice lacking 5-HT reuptake sites, causes DA reuptake sites to reuptake 5-HT (Zhou, Lesch and Murphy, 2002). Sometime long exposure to DA cause 5-HT level to decrease (Stansley and Yamamoto, 2014).

NSO treatment of alcohol drinking animals showed increased 5-HT and NA levels with reduced DA. Results also showed reduced 5-HIAA and increased DOPAC levels. These rats have also shown improved memory and reduced anxiety in MWM and EPM tests. This means NSO treatment improved memory and behavior by preventing serotonin metabolism and increasing dopamine metabolism in alcoholic rats. NSO administration reduced anxiety and depression related symptoms by increasing the 5-HT and decreasing the 5-HIAA levels (Parveen *et al.*, 2014 and 2009). The memory enhancing effects are also linked with increased 5-HT, DOPAC and HVA with reduced 5-HIAA and DA levels (Cheema *et al.*, 2016).

Noradrenaline is one of the best-known neurotransmitters that modulate the synaptic functions. In the central nervous system, the primary source of noradrenaline is the locus coeruleus (Sara *et al.*, 1994; Kitchigina *et al.*, 1997), a structure located in the brain stem that sends noradrenergic projections to many brain regions (Jones *et al.*, 1977).

Yamanaka and Egashira, (1982) demonstrated that acute to moderate ethanol intake had no effect on noradrenaline level in hippocampus, midbrain, hypothalamus, striatum or medulla oblongata. Continuous ethanol exposure resulted in reduced noradrenaline in hypothalamus, midbrain and hippocampus. These results are in favor of our results where ethanol drinking rats showed decreased noradrenaline levels in the hippocampus and midbrain. This reduction in noradrenaline levels may be interpreted as a manifestation of an accelerated noradrenaline turnover (Pohorecky, 1974; Karoum *et al.*, 1976) or an enhanced release of noradrenaline (Thadani and Truitt, 1997). Several clinical and scientific research have demonstrated the role of noradrenaline in a range of psychological and physiological processes including memory, learning, anxiety, sleep, adaptation and arousal (Montgomery, 1997). Continuous ethanol consumption decreases brain noradrenaline and reduces the activity of the enzyme dopamine beta-hydroxylase responsible for noradrenaline synthesis in rat hippocampus (Yamanaka and Egashira, 1982). Such changes in noradrenaline levels may mediate the ethanol-induced increases in anxiety (Karoum *et al.*, 1976). In contrast to the ethanol-induced decreases in noradrenaline levels in the hippocampus and midbrain, increases in noradrenaline were found in the prefrontal cortex. Prefrontal cortex possesses higher dopamine-β hydroxylase varicosities relative to sensory cortical regions (Agster *et al.*, 2013). This makes clear by pointing the fact that prefrontal cortex areas might be subjected greater levels of noradrenaline in synaptic transmission and explain why the prefrontal cortex is sensitive to anxiety and stress (Park and Moghaddam, 2017). We furthermore found that *Nigella sativa* oil treatment decreased noradrenaline levels in the hippocampus and midbrain of water drinking animals and in the midbrain of ethanol drinking animals, and significantly increased noradrenaline levels in the prefrontal cortex.

By looking at the hippocampus data, it shows that ethanol reduced noradrenaline levels in the hippocampus and that *Nigella sativa* oil tends to antagonize that in the ethanolic rats because there is no further decrease. The hippocampus has major role in integrating and processing spatial information important in formation and retrieval of memory (Chen *et al.*, 2017; Hansen, 2017; Jin and Maren, 2015; Maren *et al.*, 2013; Bouton *et al.*, 2006), further locus coeruleus projections to hippocampus has effect on learning

(Wagatsuma *et al.*, 2018). Noradrenaline can influence the function of hippocampus and locus coeruleus stimulation (Yavich *et al.*, 2005; Hajós-Korcsok *et al.*, 2003; Abercrombie *et al.*, 1988). Many studies have suggested that noradrenaline increase long-term potentiation (LTP) in hippocampus, specifically in CA1 and dentate gyrus, which is dependent upon βAR and α1 procedures (Harley, 2007; Yang *et al.*, 2002; Izumi and Zorumski, 1999; Chaulk and Harley, 1998; Katsuki *et al.*, 1997; Dunwiddie *et al.*, 1992; ; Segaletal., 1991; Dahl and Sarvey,1989; Hopkins and Johnston, 1988; Lacaille and Harley, 1985; Stanton and Sarvey, 1987 and 1985a,b; Blissetal,1983; Neuman and Harley,1983). High level of noradrenaline enhances excitatory post synaptic potentials, reduce spike on set latency, and enhances the population spike amplitude. These effects promote long-term potentiation induction and are important for memory formation (Harley, 2007; Lacaille and Harley,1985; Neuman and Harley, 1983).

Park and Moghadda, (2017) reported that impaired cognition is a hallmark deficit associated with anxiety. In line with this results anxiety with impaired memory was observed in ethanolic rats. *Nigella sativa* oil treatment ameliorated the ethanol induced anxiety and improved memory by increasing hippocampal and prefrontal noradrenaline. These results showed that higher level of hippocampal noradrenaline decreased anxiety and improved spatial memory by increasing the hippocampal long-term potentiation.

This study demonstrated that chronic ethanol exposure resulted in disturbed total cholesterol level in rat serum. This is in line with the finding of wang and coworkers, (2012) where 4 weeks ethanol exposure resulted in disturbed total cholesterol homeostatic in rats. They provided the evidence that ethanol prompted hypercholesterolemia by hyperactivation proprotein convertase subtilisin/kexin type 9 (PCSK9) levels in the liver and deactivation of Extracellular Signal-Regulated Kinase (ERK) which lead to increased cholesterol biosynthesis. They also reported that chronic ethanol intake causes hyperlipidemia by promoting the cholesterol due to reduced low density lipids receptors in liver. Cholesterol, a lipid as major component of cell membrane plays many functional and structural roles and is required for viability and growth of mammalian cells as well. Dysregulated cholesterol balance and hyperlipidemia homeostasis is a risk factor for the

development of atherosclerotic cardiovascular disease (Castelli, 1996; Davignon and Cohn, 1996). In the present study, ethanol drinking resulted in higher cholesterol levels and *Nigella sativa* oil treatment of ethanolic animals showed decreased total cholesterol level. There are number of studies showing protective effects of *Nigella sativa* oil on cholesterol, but this is the very first study showing the effects of *Nigella sativa* oil treatment on ethanolic rats. El-Bolkiny and coworkers, (2006) showed that pre- and post-treatment of rats with *Nigella sativa* oil tended to ameliorate and reduce total cholesterol plasma levels (49.31 % and 59.26 % respectively) that were upregulated by injecting Cyclophosphamide, an alkaline drug that increased cholesterol levels. The water drinking control animals from the present study showed, like the control animals in the study of El-Bolkiny and colleagues, no effect on total cholesterol. Likewise, El-Dakhakhny and colleagues (2000) and Badary and colleagues (2000) investigated the effect of *Nigella sativa* oil and showed that oral administration of *Nigella sativa* oil resulted in reduced hyperlipidemia in serum including total cholesterol, triglycerides, and low-density lipids. These results are favoring the protective effects of *Nigella sativa* oil with reduced cholesterol levels in ethanolic rats.

We also observed that ethanol consumption resulted in decreased glucose levels compared to water controls. After *Nigella sativa* oil treatment of animals, serum glucose levels were reduced further, but this was significant only in water drinking animals. Our result suggested that *Nigella sativa* oil treatment is not affective to treat ethanol induced decreased glucose levels, whereas in water drinking controls, it altered the normal sugar level and causes hypoglycemia. The later can be considered as a side effect of NSO.

4.4. Conclusion:

The findings of this experiment have explored that ethanol drinking resulted in reduced body weight, food and fluid intake. NSO treatment of ethanol drinking animals exacerbated these effects of ethanol on body weight, food and fluid intake. Ethanol drinking impaired memory and developed anxiety. NSO treatment counteracts the impaired memory, anxiogenic effects of chronic ethanol by increasing the 5-HT and DA level and neurotransmission.

CHAPTER # 5

RESULTS AND DISCUSSION

(Alcohol and Virgin Coconut Oil Experiment)

5. Results and Discussion (Alcohol and Virgin Coconut Oil Experiment)

5.1. Experimental Design

Twenty-four male Wistar rats were divided into 2 groups (n=12): (i) Water drinking, (ii) Ethanol drinking. The animals in water drinking group were supplied with tap water and the animals in ethanol drinking group were initially given ethanol. Initially the rats were administered 5 % ethanol (for 7 days) followed by 10 % ethanol in drinking water from day 8 to 28th. After 16 days, the water and ethanol treated animals were subdivided into 4 groups (n=6): (i) Water + Water (WW), (ii) Water + Virgin Coconut Oil (WVCO), (iii) Ethanol + Water (EW) and (iv) Ethanol + Virgin Coconut Oil (EVCO). Body weights, food and water intake were monitored daily from 9:30 to 10:00 h. Water drinking + virgin coconut oil and ethanol drinking + virgin coconut oil groups were administered with virgin coconut oil (VCO) at dose of 10 mL/kg by oral gavage for 12 days (day 17 to 28) and water drinking + water and ethanol drinking + water groups received same dose of tap water by same route from 10:00 to 10:30 h.

Three behavioral tests were performed, Anxiety tests, memory tests and exploratory tests. Behavioral tests were performed at 11:00 h onwards.

1) Anxiety was tested by elevated plus maze (EPM).
2) Memory was tested by Morris water maze (MWM) test.
3) Exploratory activity was tested by open field (OF).

On Day 28th, animals were decapitated. Blood was collected, and brains were immediately dissected to isolate prefrontal cortex (PFC), hippocampus (HP), midbrain (MB), hypothalamus (HT) and striatum (ST). The samples were preserved at -80 °C.

Neurotransmitters' level in "PFC, HP and MB' were measured by high performance liquid chromatography with electrochemical detector (EC-HPLC) as described previously (Haleem *et al.*, 2014). From "HT" tissues, BDNF protein levels were estimated by western

blotting. Enzymes such as catalase and hydrogen peroxide in "ST" were estimated by colorimetric assay technique.

Blood was centrifuged. Clear supernatant was collected in new tubes and used to calculate the serum levels of insulin (by ELISA), glucose, total cholesterol and triglycerides by colorimetric assay performed using a microplate reader of all animal groups.

Statistical analyses were done using the IBM SPSS software version 16. Data are presented as mean ± SD. Physiological, neurotransmitter and behavioral data were analyzed by two-way (repeated measures) ANOVA. T-test analysis was done for Memory results of ethanolic rats. Individual comparisons were made by Tukey's Post-hoc Test and paired t-test. A value of $p<0.05$ was considered significant.

5.2. Results

5.2.1. Effects of Virgin Coconut Oil on Body Weights, Food Intake and Water Intake in Alcohol drinking Rats

Figure 5.1 is showing the effects of ethanol drinking on body weight. Data analyzed by independent t-test. T-test analysis showed significant ($F=2.288$, $df=1,22$, $p<0.01$) effect of ethanol on body weight compared to water drinking control animals. Individual comparisons were made by paired t-test, which showed that body weight in alcohol drinking group was significantly decreases ($p<0.01$) than water drinking animals. When the weight of groups was compared with their pretreatment weight/initial weight, significant ($p<0.01$) increase was observed in both the group. These results indicated that alcohol causes decrease in body weight.

Figure 5.2 is showing the body weight results of animals treated with Virgin Coconut Oil (VCO) for 12 days. Data analysis revealed that there was significant impact of alcohol drinking ($F=73.581$, $df=1,20$, $p<0.01$) on body weight. Insignificant impact of VCO treatment ($F=3.628$, $df=1,20$, $p>0.05$) with interaction effects ($F=2.918$, $df=1,20$, $p > 0.05$) on body weight was found. Post hoc analyses revealed that EW and EVCO group

animals significantly reduced (*$p<0.01$) weight as compared to WW and WVCO groups respectively. There was no difference in weight was observed in WW and WVCO or EW and AVCO. These results indicated that VCO treatment had no effect on body weight. Reduced body weight in alcoholic rats was due to alcohol consumption.

Figure 5.3 represent the impacts of alcohol consumption on fluid intake and food intake. Fluid intake data analysis by using two-way ANOVA revealed significant ($F=13.020$, $df=1,20$, $p<0.01$) impact of ethanol on fluid intake. There were insignificant impacts of VCO treatment ($F=.262$, $df=1,20$, $p>0.05$) or interaction ($F=1.647$, $df=1,20$, $p>0.05$) between fluid intake and VCO treatment observed.

Food intake data analysis showed significant impact of ethanol drinking ($F=131.573$, $df=1,20$, $p<0.01$) and VCO administration ($F=73.746$, $df=1,20$, $p<0.01$) on food intake. There was insignificant interaction effect ($F=2.064$, $df=1,20$, $p>0.05$) between any group was observed. Post hoc analysis revealed significant differences among groups. When EW was compared with WW, significantly ($p<0.01$) reduced food intake was noticed which revealed that alcohol drinking significantly reduced food intake. WVCO and EVCO consumed significantly ($p<0.01$) lower amount of food compare to WW and EW groups respectively. This means VCO can reduce hunger.

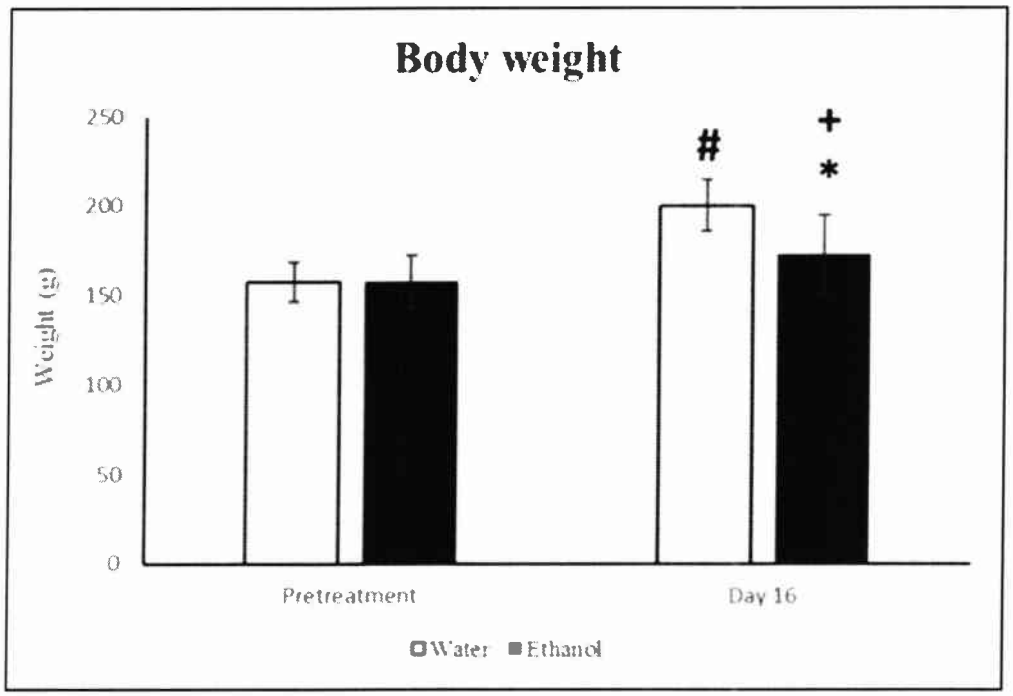


Figure 5.1: Effects of Ethanol consumption on body weight. Values are means ± SD. *p< 0.01 than water drinking controls; #p<0.01 and +p<0.01 than pre-treatment weight.

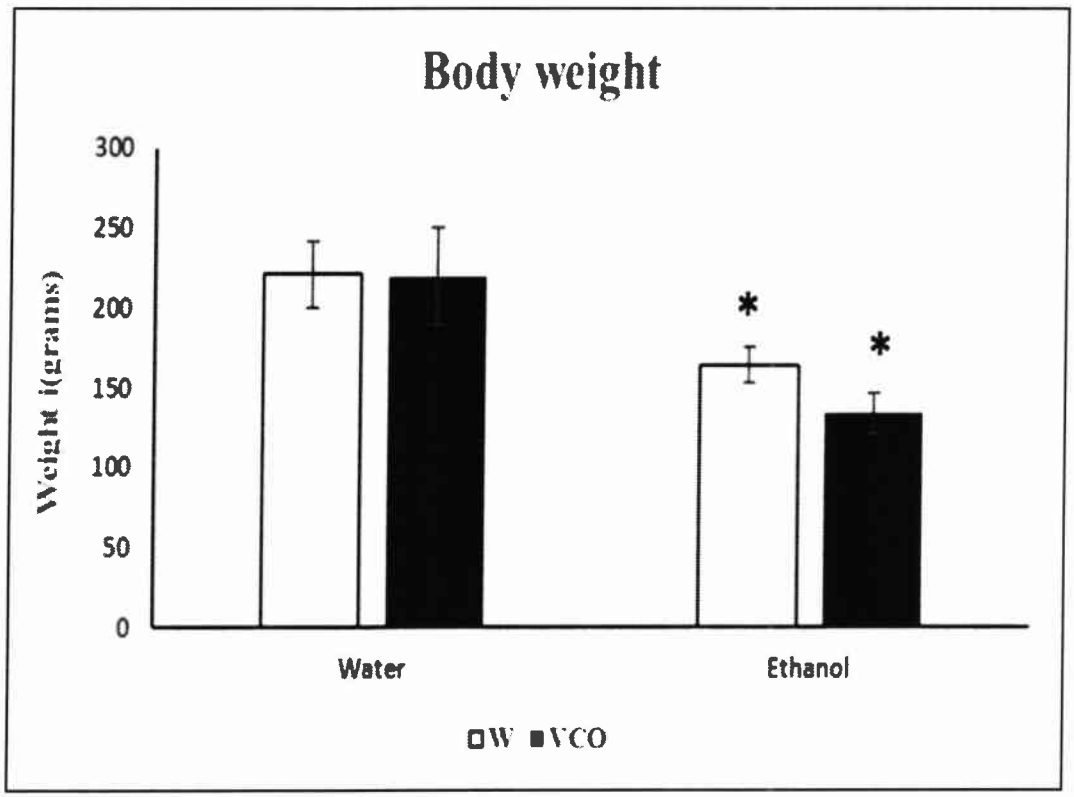
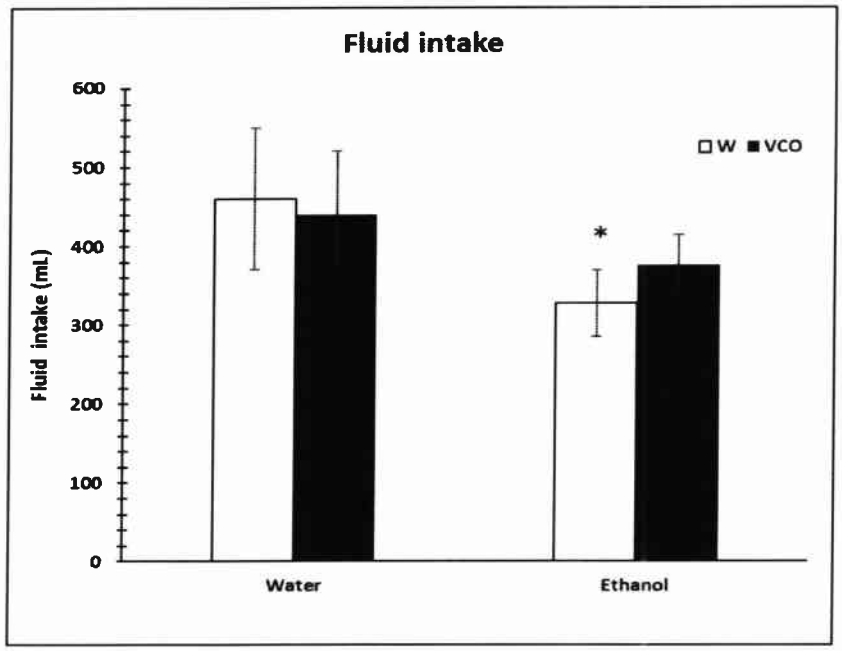


Figure 5.2: Effects of Virgin Coconut Oil (VCO) treatment on body weight in water and ethanol drinking animals. Values are means ± SD. *p<0.01different from water drinking respective controls.



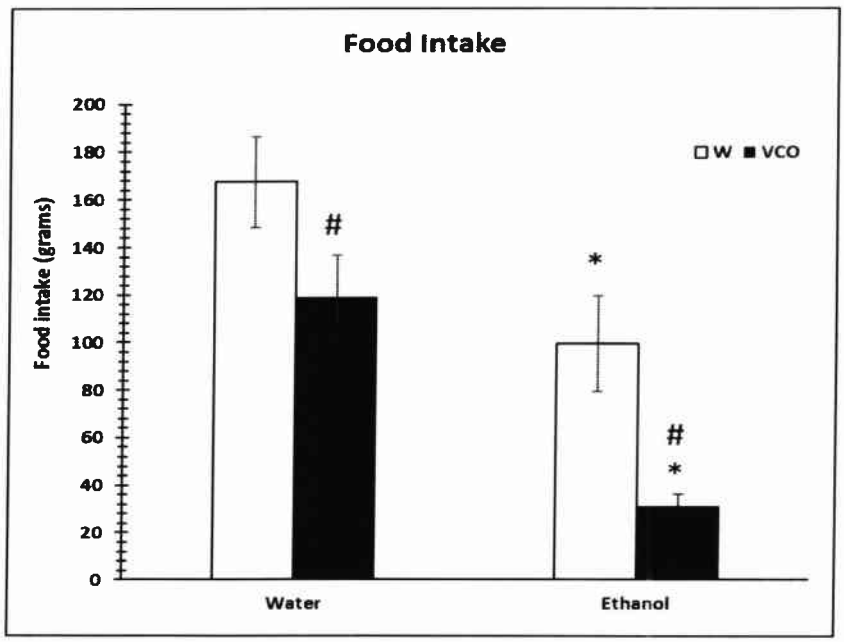


Figure 5.3: Effects of VCO treatment of water and ethanol drinking animals on accumulative (12 days) fluid and food intake measured on last day of experiment. Values are means ± SD. (Fluid intake): *p<0.01 from Water drinking + water treated group. (Food Intake): *p<0.01 from WW and WVCO; #p<0.01 different from WW and EW.

5.2.2. Behavioral Study

5.2.2.1. Effects of Virgin coconut oil on anxiety in alcohol drinking rats

Figure 5.4 is showing the effects of VCO treatment on anxiety like behavior in water and ethanol drinking groups, observed in EPM as entries and time spent (B) in open arms. Data for entries in an open arm was analyzed by two-way ANVOVA. Results showed significant effects of ethanol consumption ($F=133.819$, $df=1,20$, $p<0.01$) and VCO treatment ($F=18.564$, $df=1,20$, $p<0.01$) on entries in open arms whereas no significant interaction effect was found ($F=.836$, $df=1,20$, $p>0.05$). Post hoc comparison analyses showed that EW group animals showed significantly (*$p<0.01$) smaller number of entries in open arms of EPM compared to WW. AVCO group showed much (*$p<0.01$) OA entries than WVCO. When AVCO group was compared with EW, AVCO showed significant (#$p<0.01$) increase in number of entries in open arms of EPM. This means VCO treatment reduced fear like behavior in alcohol drinking animals. When WVCO group was compared with WW, decreased number of entries in open arms was observed in WVCO than WW. This indicated that VCO treatment developed some anxiety like behavior in water drinking animals (5.4A).

Data for time spent in open arms was also analyzed using two-way ANVOVA. Results revealed significant impact of alcohol drinking ($F=5.117$, $df=1,20$, $p<0.01$), VCO treatment ($F=62.682$, $df=1,20$, $p<0.05$) and alcohol drinking and VCO treatment interaction ($F=184.211$, $df=1,20$, $p<0.01$) on time spent in open arms of EPM. Post hoc comparison analyses showed that EW and AVCO group animals spent significantly (*$p<0.01$) less time in open arms compared to WW and WVCO respectively. When AVCO group was compared with EW, significant (#$p<0.01$) decreased time spent in open arms was observed. Whereas no such difference was found between WVCO and WW groups. we found that VCO led WVCO group animals to spend decrease time in open arms, but the difference was not significant (#$p<0.01$). WW spent more time in open arms (5.4B).

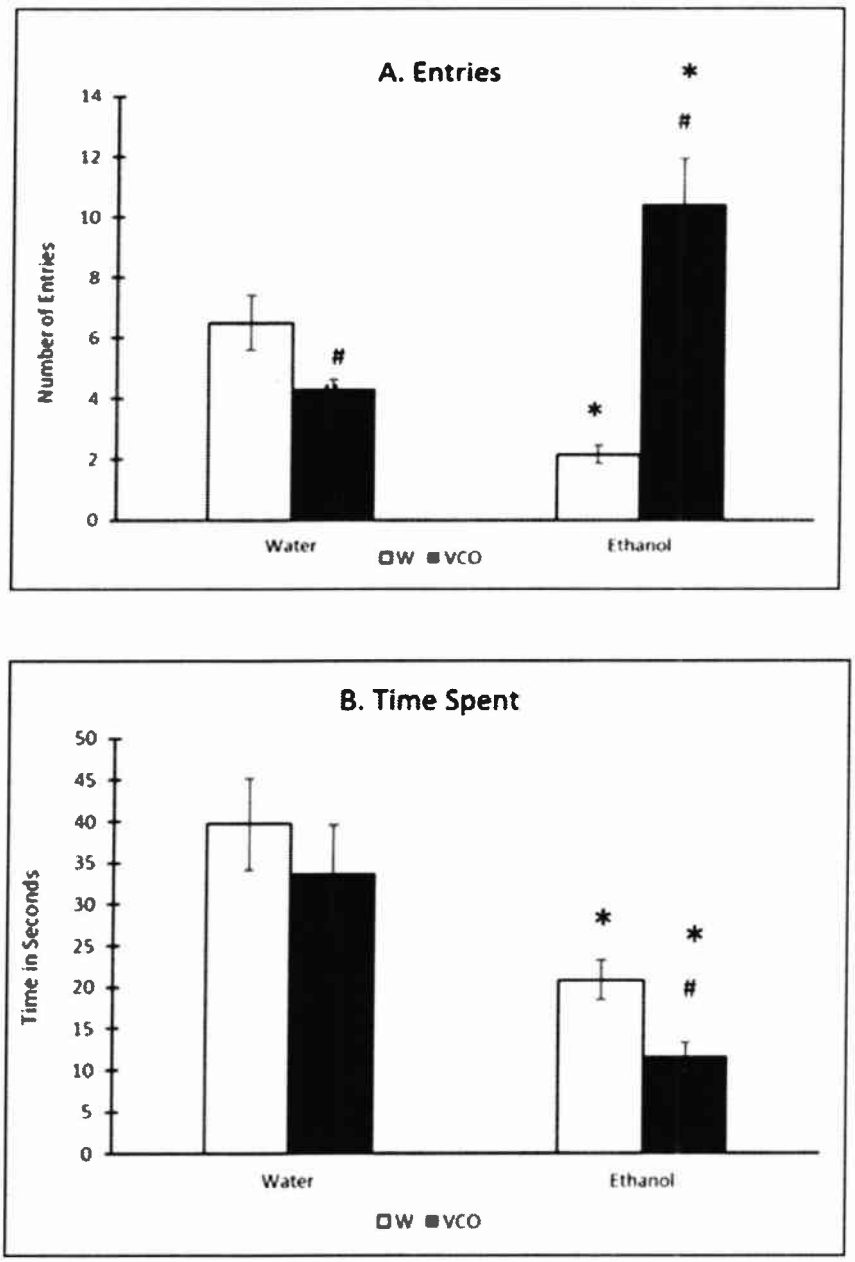


Figure 5.4: Effects of VCO treatment on anxiety like behavior in water and ethanol drinking group observed in the elevated plus maze. Values represent means ± SD. (A) Entries: #p<0.01 different from respective WW and EW groups; *p<0.01 different from respective WW and WVCO groups. (B) Time spent: #p<0.01 different from EW group; *p<0.01 different from respective WW and WVCO groups.

5.2.2.2. *Effects of alcohol and Virgin coconut oil on learning and memory in alcoholic rats*

Figure 5.5 is representing the effects of ethanol on learning and memory in rats. Data for spatial learning and memory of water and ethanol drinking animals was analyzed by independent sample t-tests. Acquisition, retention and extinction stages of memory were considered to determine the STM, LTM, and extinction.

Acquisition: Observed data of acquisition showed that there was a significant effect of alcohol drinking ($t(22) = .931$, $p<0.01$) on STM and significant ($p<0.01$) difference in the escape latency time for water drinking ($M=21$, $SD= 3.794$) and alcohol drinking ($M=28.75$, $SD=4.476$) groups.

Retention: Data analysis showed the significant effect of alcohol drinking ($t(22) = 8.295$, $p<0.01$) on LTM and significant ($p<0.01$) difference in the escape latency time for water drinking ($M=15.083$, $SD=2.896$) and alcohol drinking ($M=24.916$, $SD=4.515$) groups.

Extinction: Extinction data analyses showed that there was no significant ($t(22) = .054$, $p>0.05$.) effect of alcohol drinking and no difference in the escape latency time for water drinking ($M=20.75$, $SD=3.518$) and alcohol drinking ($M=17.333$, $SD=3.247$) groups.

Paired t-test analysis showed that alcohol drinking groups showed delayed escape latency in acquisition and retention compared to respective control groups ($p<0.01$). Individual data in comparison to own previous results, showed retrieval of memory at retention phase in both the groups (water and alcohol drinking) and latency time was significantly ($p<0.01$) reduced from acquisition time. Extinction phase result, the water drinking animals could not retrieve memory and latency to locate platform was significantly increased compared to retention. Whereas reconsolidation of memory was seen in alcohol drinking group which showed significant decreased escape latency time compared to retention time. This is indicating habituation effect in alcohol drinking rats.

Figure 5.6 is showing the effects of VCO administration on memory in water and ethanol drinking animals. Memory data analysis was done by using two-way ANVOVA. Results represented significant impact of alcohol drinking ($F=5.018$, $df=1,20$, $p<0.01$), VCO treatment ($F=18.365$, $df=1,20$, $p<0.01$) and significant interaction effect ($F=204.980$,

df=1,20, p<0.01) between alcohol drinking and VCO treatment on escape latency time in MWM. Post-hoc testing of MWM data showed that alcohol drinking group showed significantly (p<0.01) reduced escape latency compared to water drinking group and located the platform quickly. VCO treatment of water and alcohol drinking animals gave different results. WVCO group showed reduced latency where as AVCO group showed significant impaired memory. Results indicated that VCO treatment helped in reconsolidation of memory only in water drinking controls and it impaired the memory in alcohol drinking group.

Figure 5.7 is showing the impact of VCO on prob test in water and ethanol drinking animals. Data collected from probe trial data a and analysis was done by using two- way ANOVA.

Latency: Results demonstrated significant impact of alcohol drinking (F=8.458, df=1,20, p<0.01), VCO treatment (F=47.921, df=1,20, p<0.01) and alcohol drinking and VCO treatment interaction (F=2.820, df=1,20, p<0.05) on time to locate and reach to the platform quadrant. Post hos analyses showed that the VCO treated water and alcohol drinking animals took significantly (p<0.01) lesser time to reach the hidden platform quadrant in probe trial than WW and EW respectively. When latency time for WVCO and AVCO groups were compared we found that AVCO group took significantly (p<0.05) more time to reach platform quadrant (A).

Entries in Platform quadrant: Data analyses showed significant role of VCO administration (F=10.071, df=1,20, p<0.01) on entries in platform quadrant. No significant effect off alcohol drinking or interaction between alcohol drinking and VCO treatment was found. Post hos analyses showed that the WVCO group's entries in platform quadrant were higher (p<0.05) than WW. Insignificant difference in entries was observed between WW and EW or EW and AVCO groups (B).

Time spent in Platform quadrant: Data analyses revealed significant impact of VCO administration (F=8.528, df=1,20, p<0.01) on time spent in platform quadrant. No significant effect off alcohol drinking or interaction between alcohol drinking and VCO treatment was found. Post hos analyses showed that the WVCO group spent much time

($p<0.01$) in platform quadrant than WW. Insignificant difference in time spent was noted between WW and EW or EW and AVCO groups (C).

These results indicated that VCO possess memory enhancing and improving abilities in water drinking animals, where retrieval and reconsolidation of memory was improved.

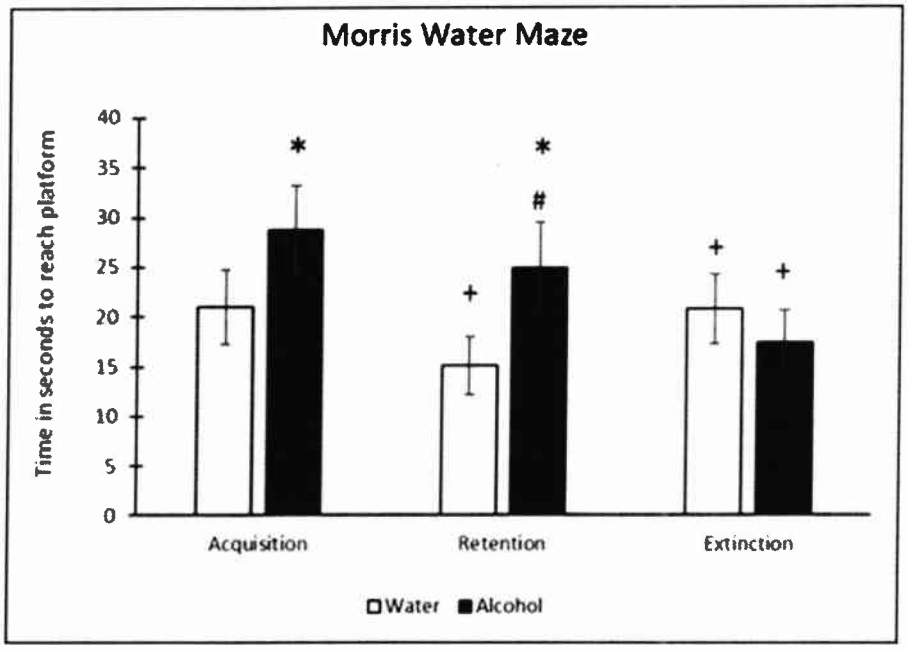


Figure 5.5: Effects of alcohol consumption on spatial learning and memory observed in the Morris water maze on Day 2nd (Acquisition), Day 3rd (Retention) and Day 16th of the experiment. Values are means ± SD. *p<0.01 on acquisition and retention different from respective water drinking controls, +p<0.01 on acquisition, retention and extinction different from previous days.

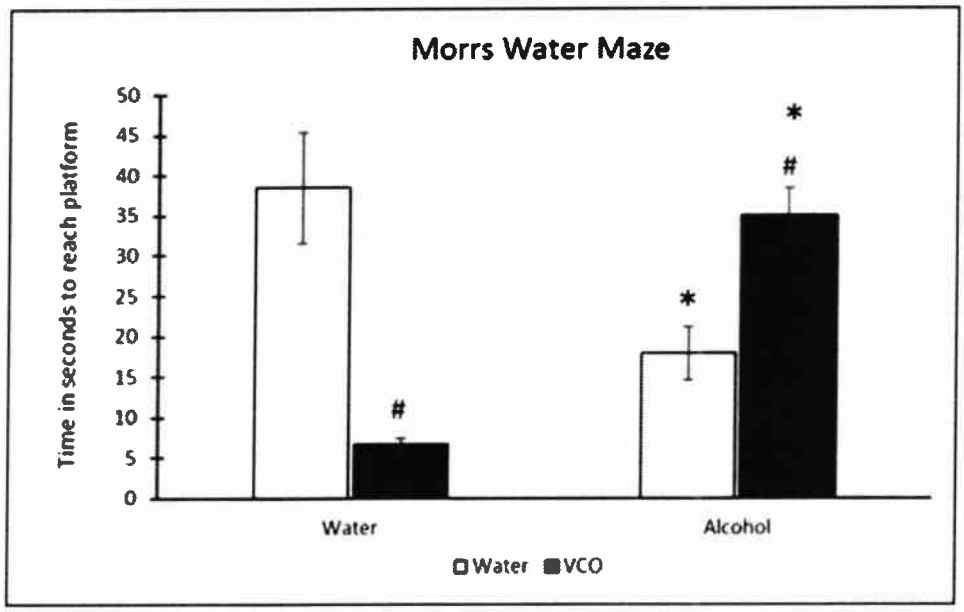


Figure 5.6: Effects of VCO treatment on memory retrieval in alcohol and water drinking groups. Values represent means ± SD. #p<0.01 different from respective WW and EW groups and *p<0.01 from respective water drinking control animals.

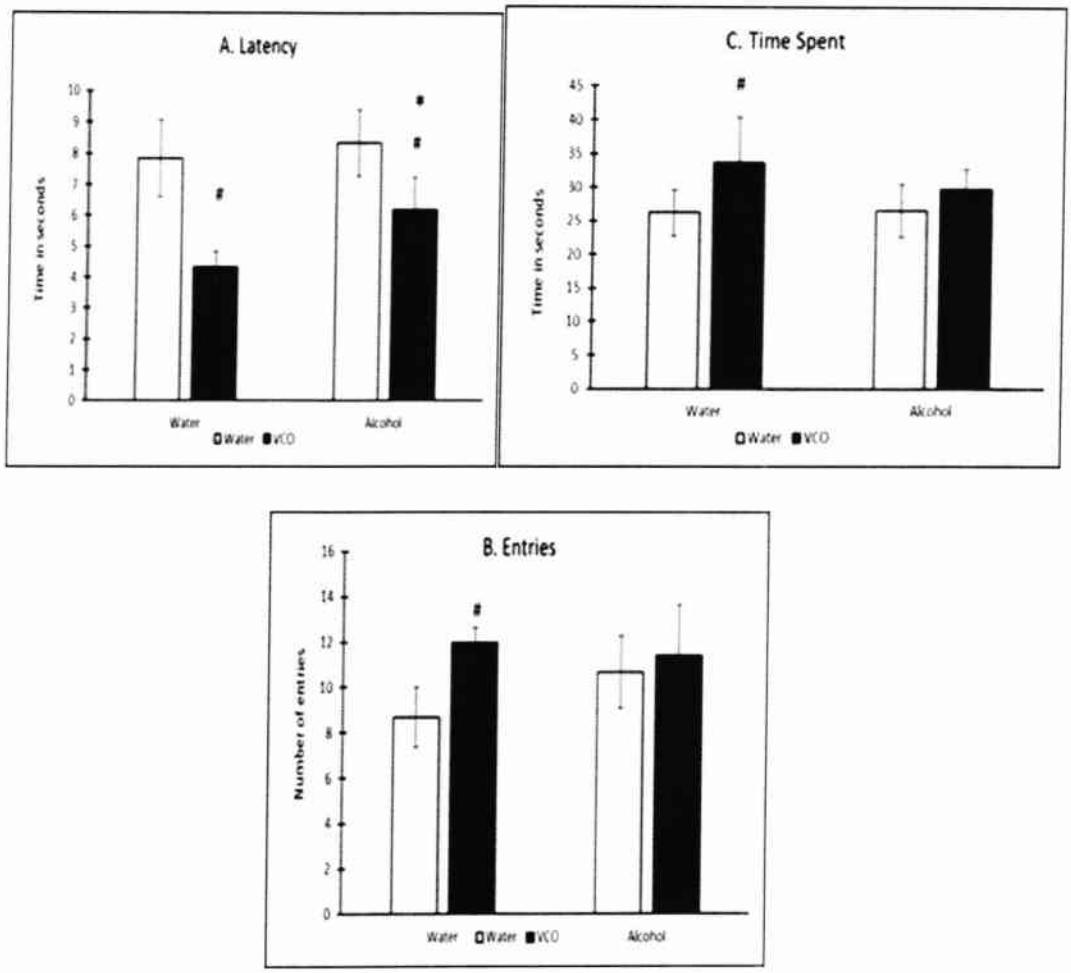


Figure 5.7: Effects of VCO treatment on probe trial in alcohol and water drinking groups. Values represent means ± SD. (A) #p<0.01 different from respective WW and EW groups; *p<0.05 different from WVCO. (B) #p<0.05 different from WW. (C) #p<0.01 different from WW.

5.2.2.3. *Effects of Virgin coconut oil on exploratory activity in alcohol drinking rats*

Figure 5.8 is showing the effects of VCO treatment on exploratory activity in novel environment in water and ethanol drinking groups, observed in open field. Data analyzed by two-way ANOVA repeated measure design showed that there was significant effects of alcohol drinking ($F=57.803$, $df=1,20$, $p<0.01$), VCO treatment ($F=78.325$, $df=1,20$, $p<0.01$) and interaction between alcohol drinking and VCO treatment ($F=56.010$, $df=1,20$, $p<0.01$) on exploratory activity in OF. Further, analysis showed significant interaction between time and VCO administration ($F=26.992$, $p<0.01$) and time, alcohol drinking and VCO treatment ($F=17.849$, $p<0.01$) on locomotor activity in the open field test. Post hoc analyses showed that EW and EVCO group animals crossed less (*$p<0.01$) squares in an OF than WW and WVCO respectively. This decrease was observed in both weeks. This is representing the effect of alcohol drinking. When AVCO group was compared with EW, no significant (#$p>0.05$) difference was found in any of the weeks and they crossed almost similar number of squares. When WVCO group was compared with WW, we found that VCO led to significant (#$p<0.01$) decrease in squares crossed and WW group animals crossed much squares in OF in both weeks. When each group data was compared with its own previous result, significant difference was observed only in WW and WVCO groups. Group WW crossed less (+$p<0.01$) squares in OF than week 1 whereas, group WVCO crossed more (+$p<0.01$) squares in OF than week 1. This might be the habituation effect and retrieval of memory in WVCO group.

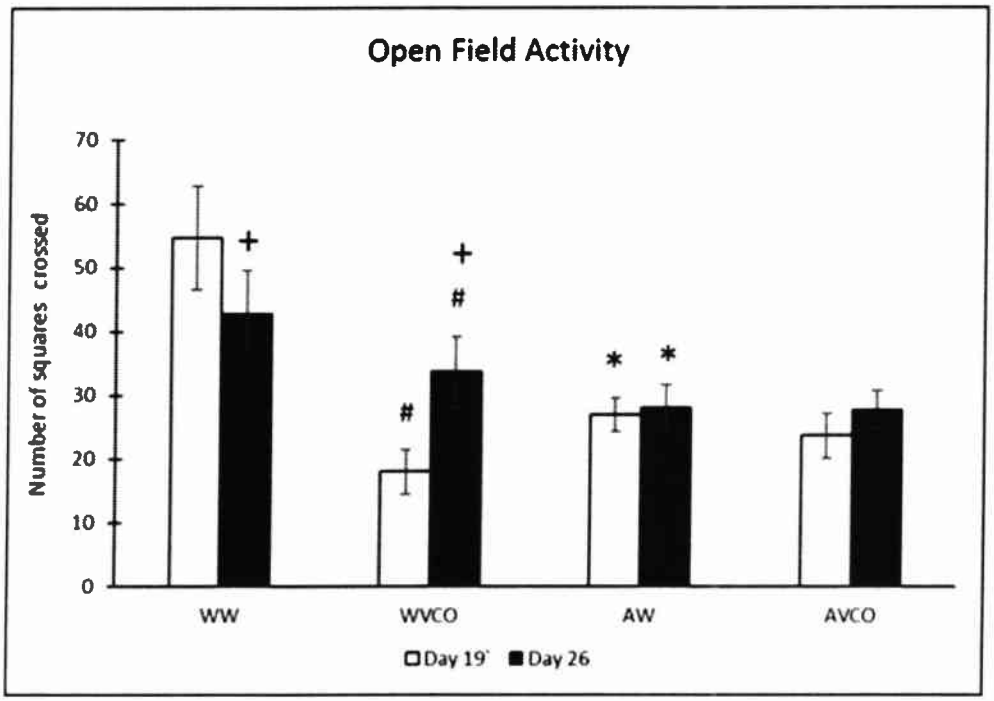


Figure 5.8: Effects of VCO treatment on exploratory activity of water and ethanol drinking group in an open field. values represent means ± SD. #p<0.01 different from respective WW groups; *p<0.01 different from respective WW and WVCO groups and +p<0.01 different from own previous days results

5.2.3. Neurochemistry

4.4.1.1. Effects of Virgin coconut oil on neurotransmitters and their metabolites level in prefrontal cortex of alcohol drinking rats

Figure 5.9 is showing the results of VCO treatment on serotonin (5-HT) and 5-HIAA concentrations in prefrontal cortex of water and alcohol drinking rats. Data analysis of 5HT level in prefrontal cortex demonstrated significant impacts of VCO treatment ($F=50.925$, $df=1,20$, $p<0.01$) and alcohol consumption and VCO treatment interaction ($F=200.375$, $df=1,20$, $p<0.01$). Insignificant impact of alcohol consumption ($F=0.058$, $df=1,20$, $p>0.05$) on 5-HT levels was observed in prefrontal cortex. Post hoc tests for 5-HT data showed significant ($p<0.01$) difference between vehicle and VCO treated water and alcohol drinking groups. WVCO group showed significant increase ($p<0.01$) in 5-HT level than WW, whereas EVCO group showed significantly ($p<0.01$) reduced 5-HT level compared to EW and WVCO. 5-HT level in EW group was elevated ($p<0.01$).

Data on prefrontal 5HIAA level analyzed, by two way ANOVA revealed significant impact of alcohol drinking ($F=26.712$, $df=1,20$, $p<0.01$) and VCO treatment ($F=4.777$, $df=1,20$, $p<0.05$) on 5HIAA levels but insignificant interaction effect ($F=3.437$, $df=1,20$, $p>0.05$) between alcohol drinking and VCO treatment observed. Post hoc test for 5-HIAA level in prefrontal cortex showed significant increased 5-HIAA level in WVCO ($p<0.05$) and EW ($p<0.01$) compared to WW group.

Figure 5.10 is showing the effects of VCO treatment on DA, DOPAC and HVA levels in prefrontal cortex of water and alcohol drinking rats. Data on DA level in prefrontal cortex was statistically checked by using two-way ANOVA showed significant impacts of VCO treatment ($F=251.563$, $df=1,20$, $p<0.01$) and alcohol consumption and VCO treatment interaction ($F=8.656$, $df=1,20$, $p<0.01$). Insignificant impact of alcohol consumption ($F=.266$, $df=1,20$, $p>0.05$) on DA level was observed in prefrontal cortex. Post hoc test for DA level in prefrontal cortex data showed significantly reduced ($p<0.01$) DA level in WVCO than WW and EVCO than EW.

Data on DOPAC level in prefrontal cortex was also analyzed by two way ANOVA and showed significant effect of alcohol drinking ($F=79.951$, $df=1,20$, $p<0.05$), VCO treatment ($F=73.968$, $df=1,20$, $p<0.01$) and alcohol drinking and VCO treatment interaction ($F=260.836$, $df=1,20$, $p<0.01$) on DOPAC level in prefrontal cortex. Post hoc test for DOPAC in prefrontal cortex showed interesting results. The DOPAC level was significantly reduced ($p<0.01$) in WVCO and EW from WW. Whereas the level was higher in EVCO ($p<0.01$) than EW.

Similarly data on HVA level in prefrontal cortex was also checked by using two-way ANOVA and demonstrated significant impact of alcohol drinking ($F=59.179$, $df=1,20$, $p<0.01$) and VCO treatment ($F=61.844$, $df=1,20$, $p<0.01$). No significant interaction effect ($F=.201$, $df=1,20$, $p>0.05$) was found between alcohol drinking and VCO treated groups. Post hoc test for HVA level in prefrontal cortex showed that HVA level was significantly reduced ($p<0.01$) in WVCO and EVCO from WW and EW respectively. But when WW and EW groups were compared, EW showed significantly ($p<0.01$) increased level than WW. When WVCO and EVCO groups were compared, EVCO also showed significant ($p<0.01$) increase in HVA level than WVCO.

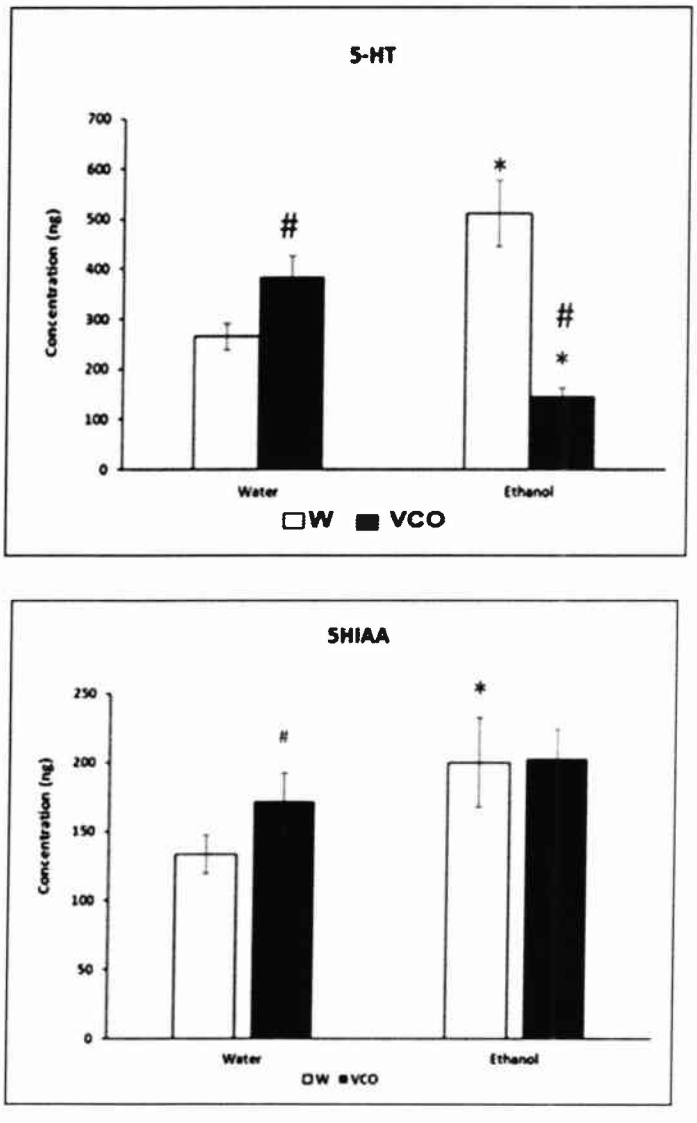


Figure 5.9: Effects of VCO treatment (10 mL/kg) on serotonin (5-HT) and 5-HIAA levels in pre-frontal cortex (PFC) of water and ethanol drinking groups. Values represent means ± SD. **(5HT)** #p<0.01 different from respective water treated group and *p<0.01 different from water drinking +water and water drinking + VCO treated animals. **(5HIAA)** #p<0.05 and *p<0.01 different from water drinking + water treated animals.

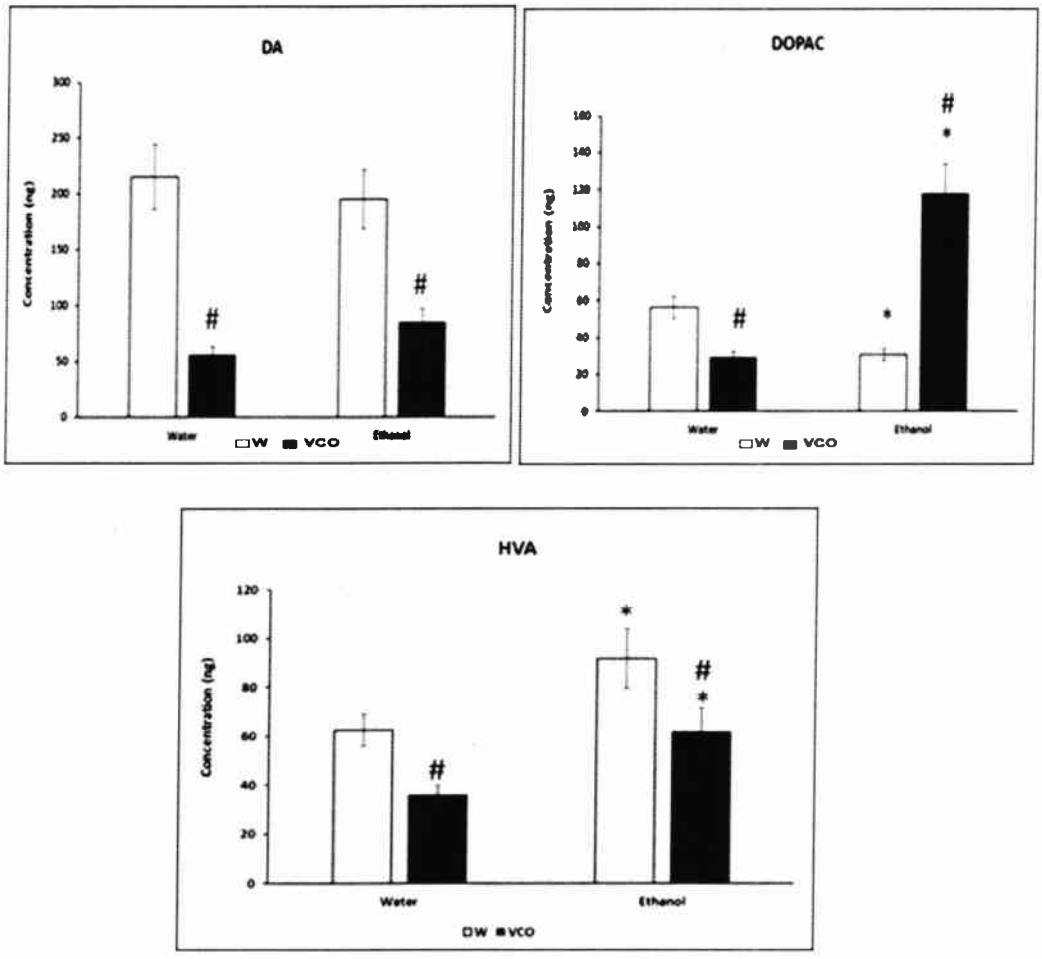


Figure 5.10: Effects of VCO treatment (10 mL/kg) on levels of dopamine, DOPAC, and HVA in PFC of water and ethanol drinking groups. Values represent means ± SD. **(DA)** #p<0.01 different from respective water treated animals. **(DOPAC)** #p<0.01 different from respective water treated animals; *p<0.01 different from water drinking + water treated and water drinking + VCO treated animals. **(HVA)** #p<0.01 different from respective water treated animals; *p<0.01 different from water drinking + water treated and water drinking + VCO treated groups.

5.2.3.1. *Effects of Virgin Coconut oil on neurotransmitters and their metabolites level in hippocampus of alcohol drinking rats*

The figure 5.11 is showing the impacts of VCO treatment on serotonin (5-HT) and its metabolite "5-HIAA" level in hippocampus region of water and ethanol drinking groups. Data on hippocampal 5-HT and 5-HIAA levels was analyzed using two-way ANOVA. Results from 5-HT data revealed significant VCO treatment ($F=28.474$, $df=1,20$, $p<0.01$) and alcohol consumption and VCO treatment interaction ($F=35.641$, $df=1,20$, $p<0.01$). But insignificant impact of alcohol consumption ($F= 2.766$, $df=1,20$, $p>0.05$) on hippocampal 5HT level was observed. Post hoc test for 5-HT level in hippocampus showed that the water drinking vehicle and VCO treated group did not show significant ($p>0.05$) difference between them. But EW and EVCO groups showed significant ($p<0.01$) difference between them and had decreased 5-HT level in EVCO than EW. When EW group was compared with WW, we found that EW group had higher ($p< 0.01$) serotonin level in hippocampus than WW. The comparison between EVCO and WVCO groups revealed significant ($p<0.01$) reduction in 5-HT level in hippocampus of WVCO.

Results from 5-HIAA data demonstrated the significant impact of alcohol drinking ($F=49.387$, $df=1,20$, $p<0.01$) VCO treatment ($F=38.142$, $df=1,20$, $p<0.01$) and alcohol drinking and VCO treatment interaction ($F=87.372$, $df=1,20$, $p<0.01$) on 5-HIAA levels hippocampus. Post hoc test for 5-HIAA level in hippocampus showed significant ($p<0.01$) difference between EW and WW. 5-HIAA level in EW was increased compared to WW. When EW was compared with EVCO, significantly ($p<0.01$) reduced 5-HIAA level was observed in hippocampus of EVCO.

Figure 5.12 is showing the impacts of VCO treatment on dopamine (DA) and its metabolites DOPAC and HVA levels hippocampus of water and ethanol drinking rats. Data on DA level in hippocampus, analyzed by using two-way ANOVA revealed significant impacts of alcohol consumption ($F=6.7$, $df=1,20$, $p<0.05$), VCO treatment ($F=27.479$, $df=1,20$, $p<0.01$) and alcohol consumption and VCO treatment interaction ($F=57.635$, $df=1,20$, $p<0.01$) on DA levels in hippocampus. Post hoc test for DA level in

hippocampus showed that EW had significantly ($p<0.05$) increased DA level compared to WW and EVCO group had significantly ($p<0.01$) lower Da level than WVCO and EW.

Hippocampal DOPAC level analysis demonstrated impact of alcohol drinking ($F=7.504$, $df=1,20$, $p<0.05$) on hippocampal DOPAC level. There was no significant effect of VCO treatment ($F=.04$, $df=1,20$, $p>0.05$) or alcohol drinking and VCO treatment interaction ($F=2.616$, $df=1,20$, $p>0.05$). Post-hoc test for DOPAC level in hippocampus showed that there was significant ($p<0.05$) increased DOPAC hippocampus of EW than WW.

Similarly data analysis of HVA level in hippocampus was done by using two-way ANOVA and demonstrated significant impacts of VCO treatment ($F=28.474$, $df=1,20$, $p<0.01$) and alcohol drinking and VCO treatment interaction ($F=35.642$, $df=1,20$, $p<0.01$). Insignificant impact of alcohol drinking ($F=2.766$, $df=1,20$, $p>0.05$) on HVA level was found in hippocampus. Post hoc tests for HVA level in hippocampus showed that no significant differences were found between WW and WVCO, and EW and EVCO groups. Results showed very significant ($p<0.01$) decreased HVA level in EW and EVCO compared to WW and WVCO respectively.

Figure 5.13 is showing the impacts of VCO treatment on hippocampal NA concentration in water and ethanol drinking rats. Data analysis of NA level in hippocampus was performed by using two-way ANOVA and results demonstrated significant roles of alcohol consumption ($F=68.005$, $df=1,20$, $p<0.01$), VCO treatment ($F=44.196$, $df=1,20$, $p<0.01$) and alcohol consumption and VCO treatment interaction ($F=21.67$, $df=1,20$, $p<0.01$). Post hoc test for NA data in hippocampus showed that WVCO group had higher ($p<0.01$) NA level in midbrain than WW. Similar results were observed in ethanol drinking rats which showed significantly increased NA level in hippocampus of EVCO group than EW group.

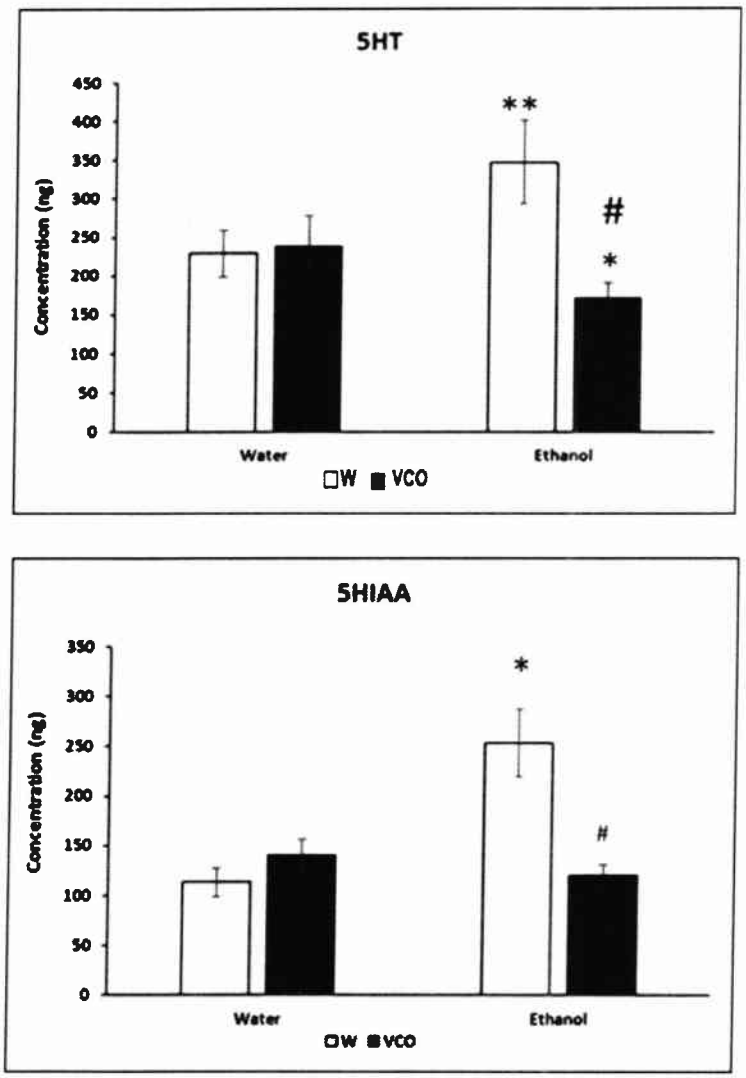


Figure 5.11: Effects of VCO treatment on 5-HT and 5-HIAA levels in hippocampus of water and ethanol drinking groups Values represent means ± SD. **(5HT)** **p<0.01 and *p<0.05 different from water drinking + water treated controls and water drinking + VCO treated animals respectively; #p<0.01 different from ethanol drinking + water treated animals. **(5HIIAA)** #p<0.01 and *p<0.01 different from water drinking + water treated controls and ethanol drinking +VCO treated animals respectively.

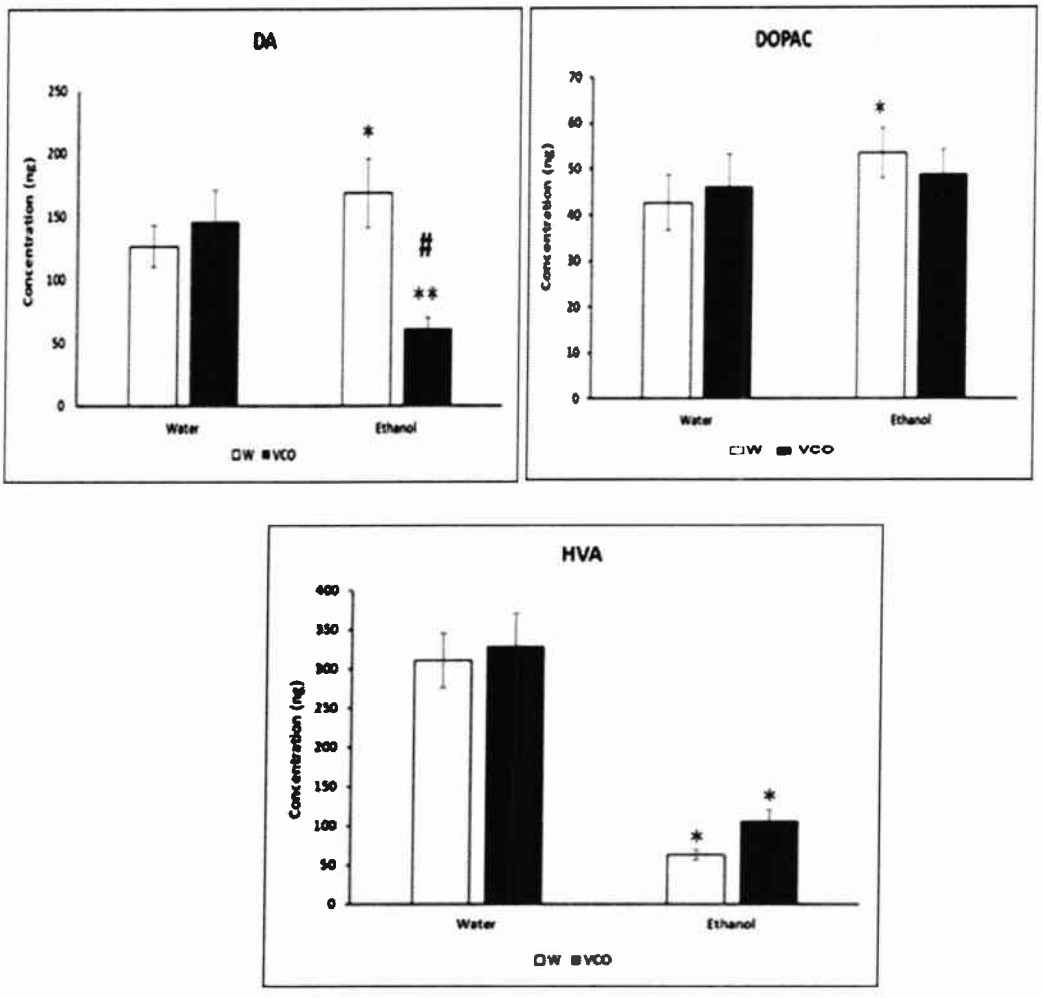


Figure 5.12: Effects of VCO treatment on levels of dopamine, DOPAC, and HVA in hippocampus of water and ethanol drinking groups. Values represent means ± SD. **(DA)** *p<0.05 and **p<0.01 different from respective water drinking groups; #p<0.01 different from ethanol drinking + water treated controls. **(DOPAC)** *p<0.05 different from water drinking + water treated controls. **(HVA)** *p<0.01 different from water drinking + water treated and water drinking + VCO treated animals.

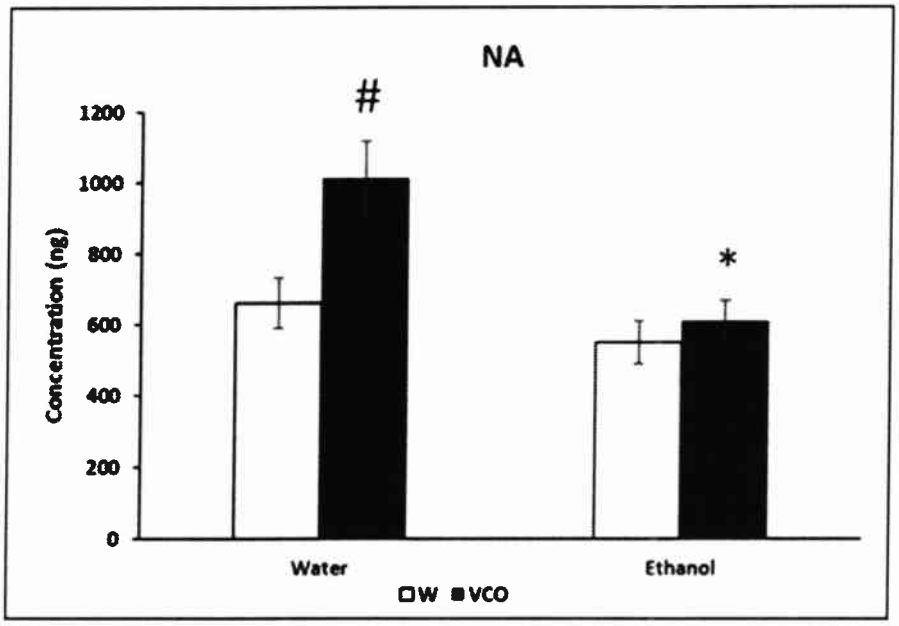


Figure 5.13: Effects of VCO treatment on levels of noradrenaline level in the hippocampus of water and ethanol drinking groups. Values represent means ± SD. #p<0.01 and *p<0.01 different from water drinking + water treated controls and water drinking + VCO treated groups respectively.

5.2.3.2. *Effects of Virgin coconut oil on neurotransmitters and their metabolites level in midbrain of alcohol drinking rats*

Figure 5.14 is showing the impact of VCO treatment on serotonin and its metabolite "5-HIAA" concentrations in midbrain of water and ethanol drinking groups. Data on serotonin and 5-HIAA levels in midbrain was statistically tested using two-way ANOVA. Results from 5-HT data revealed insignificant effects of alcohol drinking ($F=.085, df=1,20, p>0.05$), VCO treatment ($F=2.389$, $df=1,20$, $p>0.05$) and alcohol consumption and VCO treatment ($F=.345$, $df=1,20$, $p>0.05$) interaction. Post-hoc test for 5-HT level in midbrain showed no significant ($p>0.05$) difference between any groups.

Results from 5-HIAA data revealed significant impact of alcohol drinking ($F=9.12$, $df=1,20$, $p<0.01$) VCO treatment ($F=75.041$, $df=1,20$, $p<0.01$) and alcohol drinking and VCO treatment ($F=4.804$, $df=1,20$, $p<0.05$) interaction on 5-HIAA levels midbrain. Post hoc test for 5-HIAA level in midbrain showed that WVCO and EVCO groups had significantly increased 5-HIAA levels than WW and EW groups respectively. Significant ($p<0.01$) reduction in 5-HIAA level was noted in EW than WW.

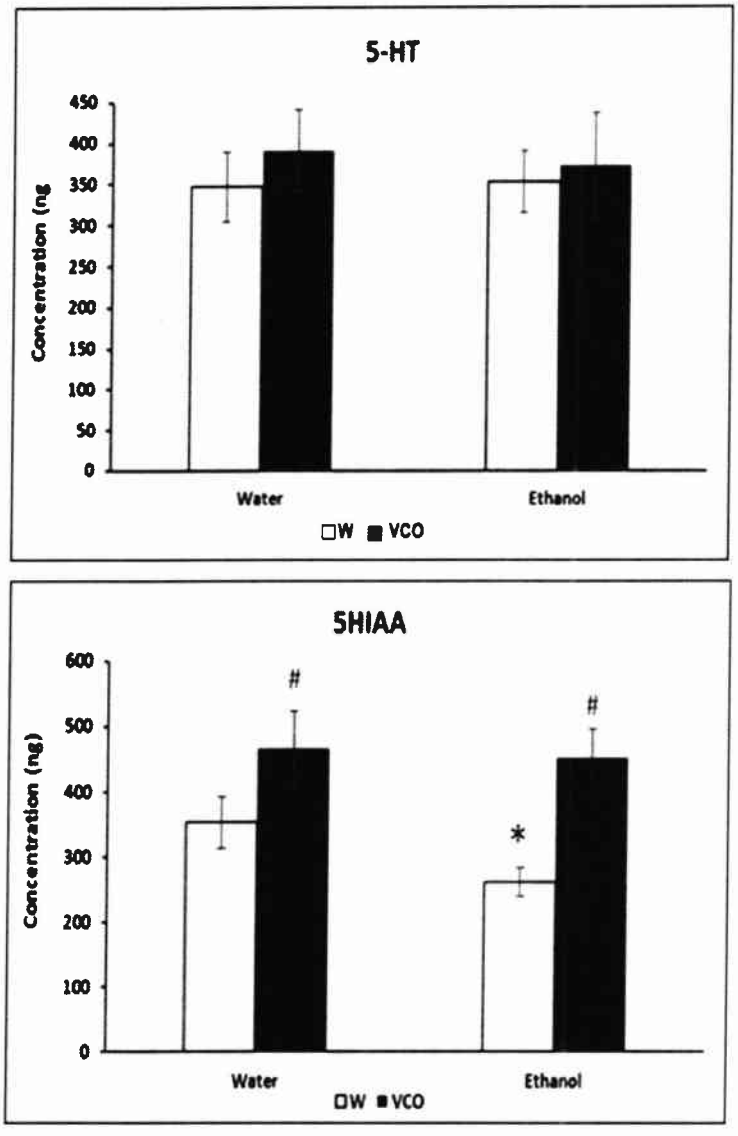
Figure 5.15 is showing the impacts of VCO treatment on dopamine, DOPAC and HVA concentration in midbrain of water and ethanol drinking rats. Data analysis of DA level in midbrain by using two-way ANOVA demonstrated significant impacts of alcohol consumption ($F=139.278$, $df=1,20$, $p<0.01$), VCO treatment ($F=156.479$, $df=1,20$, $p<0.01$) and alcohol consumption and VCO treatment interaction ($F=165.241$, $df=1,20$, $p<0.01$) . Post hoc test for DA levels in midbrain revealed significant ($p<0.01$) tremendous increase in EW than WW. Results also showed significant decreased Da level in EVCO than EW. This means VCO treatment lead to reduction in DA levels of ethanolic rats.

Data analysis of DOPAC levels in midbrain by using of two-way ANOVA demonstrated significant impacts of alcohol drinking ($F=53.236$, $df=1,20$, $p<0.01$), VCO treatment ($F=.8.002$, $df=1,20$, $p<0.05$) and alcohol drinking and VCO treatment interaction ($F=29.098$, $df=1,20$, $p<0.05$). Post hoc test for DOPAC level in midbrain showed increased

($p<0.01$) DOPAC level in WVCO than WW. Significant reduction ($p<0.01$) in DOPAC levels in midbrain were found in EW than WW group.

Similarly data on HVA level in midbrain was also analyzed by using two-way ANOVA and showed significant roles of alcohol drinking ($F=266.784$, $df=1,20$, $p<0.01$), VCO treatment ($F=17.888$, $df=1,20$, $p<0.01$) and alcohol drinking and VCO treatment interaction ($F=70.133$, $df=1,20$, $p<0.01$). Post hoc test for HVA levels in midbrain showed that WVCO groups had significantly ($p<0.01$) higher HVA level in WVCO than WW whereas EVCO group had significantly lower HVA level than EW. EW and EVCO groups showed significant reduction in HVA level when compared to WW and WVCO groups respectively.

Figure 5.16 is showing the effects of VCO treatment on NA concentrations in midbrain of water and ethanol drinking rats. Data analysis was done by the help of two-way ANOVA and results showed significant impacts of alcohol consumption ($F=8.003$, $df=1,20$, $p<0.05$), VCO treatment ($F=45.118$, $df=1,20$, $p<0.05$) and alcohol consumption and VCO treatment interaction ($F=9.004$, $df=1,20$, $p<0.01$) on NA levels in midbrain. Post hoc test for NA levels in midbrain showed that the WVCO and EW groups had reduced ($p<0.01$) NA levels in midbrain than WW.



5.14: Effects of VCO treatment on 5-HT and 5-HIAA levels in mid brain of water and ethanol drinking groups. Values represent means ± SD. **(5HIAA)** #p<0.01 different from respective water treated controls and *p<0.01 different from water drinking + water treated animals.

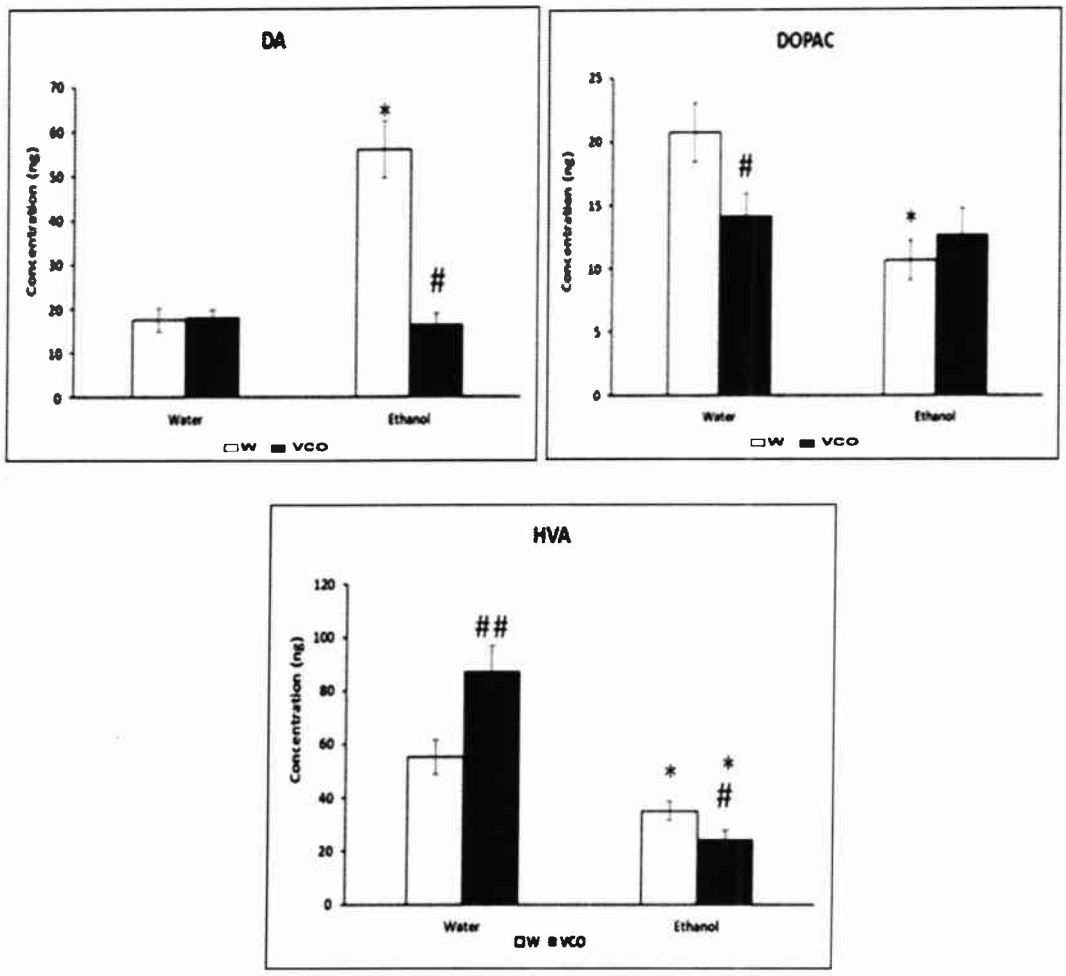


Figure 5.15: Effects of VCO treatment on levels of dopamine, DOPAC, and HVA in mid brain of water and ethanol drinking groups Values represent means ± SD. **(DA)** #p<0.01 and *p<0.01 different from water drinking + water treated controls and ethanol drinking + water treated animals. **(DOPAC)** #p<0.01 and *p<0.01 different from water drinking + water treated control. **(HVA)** ##p<0.01 and #p<0.05 different from respective water treated groups; *p<0.01 different from water drinking + water treated animals and water drinking + VCO treated animals.

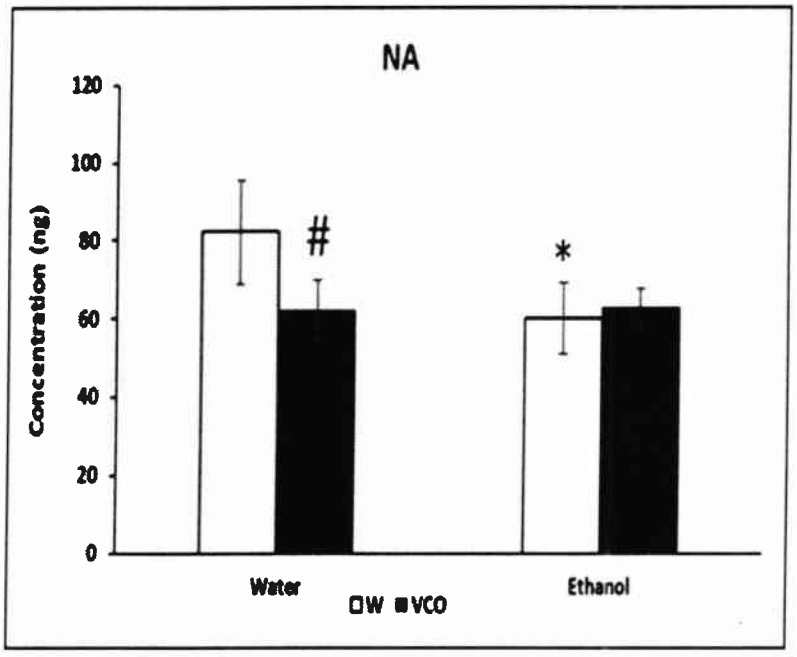


Figure 5.16: Effects of VCO treatment on levels of noradrenaline level in the midbrain of water and ethanol drinking groups. Values are present as means ± SD. #p<0.01 and *p<0.01 different from water drinking + water treated controls.

5.2.4. Effects of Virgin Coconut Oil on levels of Striatal Catalase, Striatal Peroxide and hypothalamic Brain Derived Neurotropic Factor (BDNF)

Figure 5.17 shows the results of alcohol consumption and VCO treatment on striatal catalase level. Data was analyzed by using two-way ANOVA and showed insignificant impacts of alcohol consumption ($F=000$, $df=1,20$, $p>0.05$) or VCO treatment ($F=0.006$, $df=1,20$, $p>0.05$) on striatal catalase level but significant interaction ($F=9.397$, $df=1,20$, $p<0.01$) effect between water consumption and VCO treatment was observed.

Figure 5.18 shows the results of alcohol consumption and VCO treatment on peroxide levels in Striatum. The two-way ANOVA analysis showed significant impacts of alcohol consumption ($F=3.584$, $df=1,20$, $p<0.01$) on peroxidase level in striatum. VCO treatment effect on peroxidase was not significant ($F=1.1015$, $df=1,20$, $p>0.05$). Further, no significant interaction effect ($F=.309$, $df=1,20$, $p>0.05$) was observed in any of the groups.

Figure 5.19 is showing the impacts of ethanol consumption and VCO treatment on BDNF concentration in the hypothalamus of water and ethanol drinking animals. Data analysis was done with help of two-way ANOVA revealed significant impacts of ethanol consumption ($F=51.527$, $df= 1,20$, $p<0.01$), VCO treatment ($F=300.559$, $df=1,20$, $p<0.01$) and ethanol consumption and VCO treatment interaction ($F=34.525$, $df=1,20$, $p<0.01$). Post hoc test showed that BDNF level was significantly reduced in EW group than the WW group, and in the WVCO and EVCO groups than WW and EW.

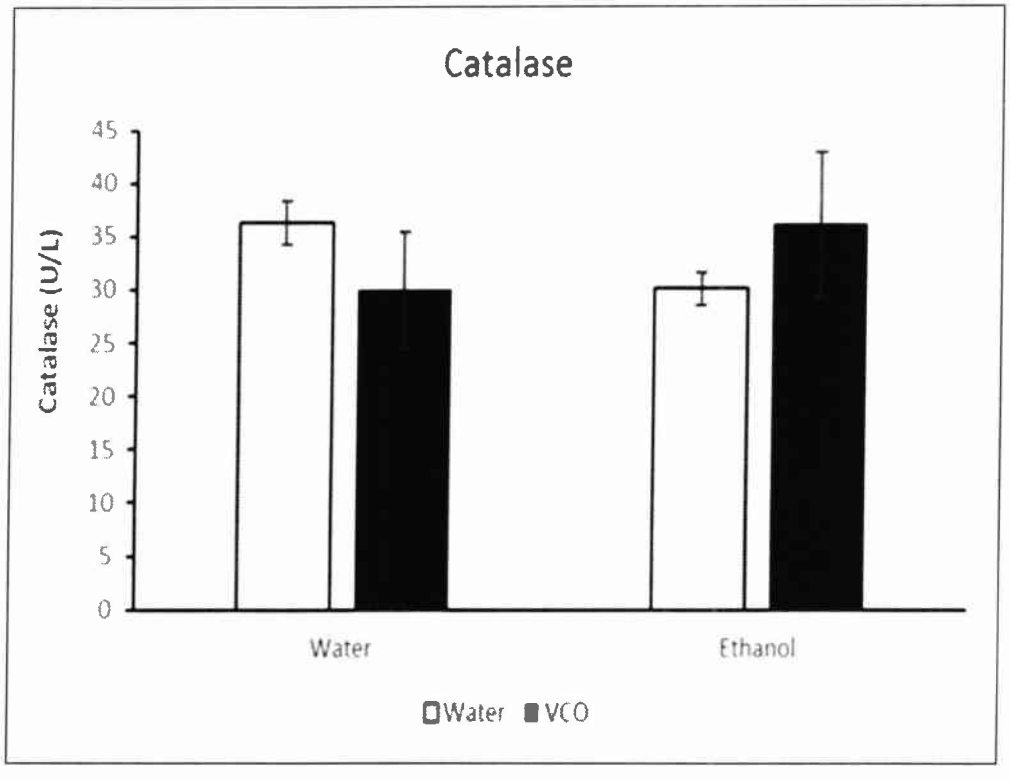


Figure 5.17: Effects of VCO treatment on Striatal catalase levels of water and ethanol drinking groups. Values are means ± SD. No significant difference was observed among groups.

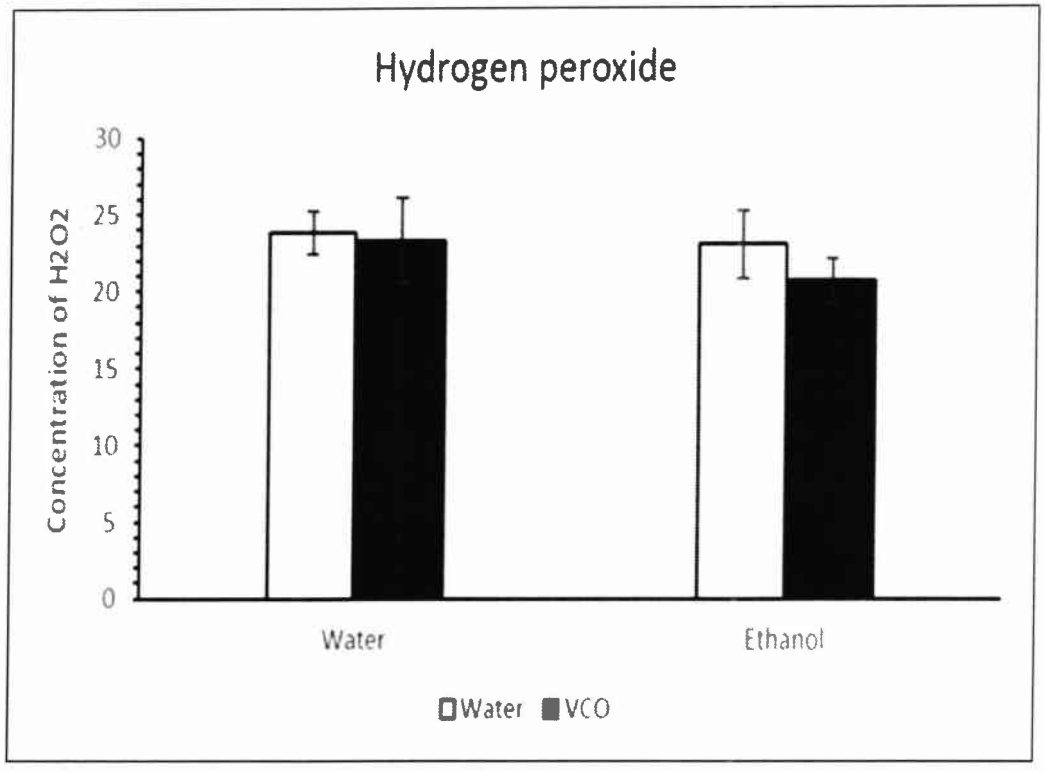
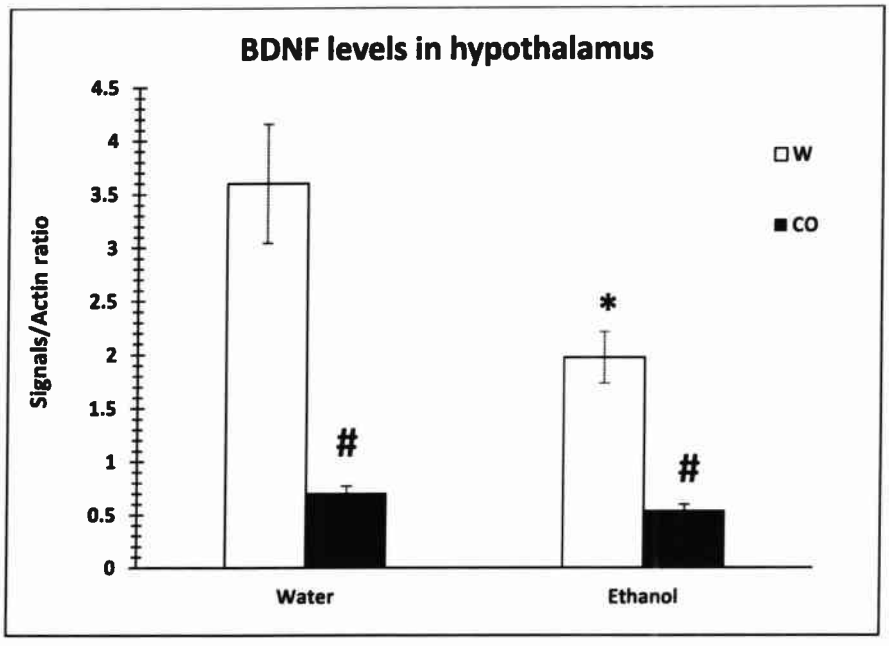
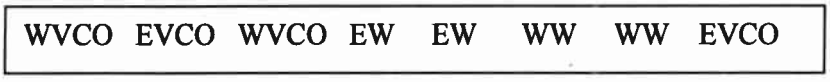


Figure 5.18: Effects of VCO treatment on Striatal peroxide levels of water and ethanol drinking groups. Values are means ± SD. No significant difference was observed among groups.





BDNF 28 KDA

Beta- actin 42 KDA

Figure 5.19: Effects of VCO treatment on BDNF protein level in hypothalamus of water and ethanol drinking groups. Values are represented as mean and standard deviations. #p<0.01 and #p<0.01 from respective control groups.

5.2.5. Biochemical Studies

5.2.5.1. Effects of Virgin Coconut oil on serum glucose, insulin, triglycerides and total cholesterol levels in alcoholic rats

Figure 5.20 is showing the results for serum glucose level measured by colorimetric method. Data analysis using two-way ANOVA revealed significant roles of ethanol consumption ($F=25.579$, $df=1,20$, $p<0.01$), VCO treatment ($F=6.323$, $df=1,20$, $p<0.05$) and water drinking and VCO treatment interaction ($F=21.505$, $df=1,20$, $p<0.01$) on serum glucose levels. Post hoc analyses showed insignificant difference in glucose level between EW and EVCO but EVCO had significant ($p<0.01$) lower glucose level than WVCO. WVCO had significantly higher ($p<0.05$) glucose level than WW. These results revealed that VCO might be harmful for the normal glucose level.

Figure 5.21 is showing the effects of ethanol consumption and VCO treatment on serum insulin concentrations. Data analyzed by using two-way ANOVA revealed insignificant impacts of ethanol consumption and VCO treatment. No significant differences between controls and ethanol drinking groups were observed.

Figure 5.22 is showing the results for serum triglycerides levels. Data analyzed by using two-way ANOVA and revealed significant impacts of VCO treatment ($F=116.444$, $df=1,20$, $p<0.01$) and ethanol consumption and VCO treatment interaction ($F=16.803$, $df=1,20$, $p<0.01$) on serum triglycerides level. Post hoc analyses for groups comparison showed that VCO treatment resulted in increased serum triglycerides level in both the groups WVCO and EVCO showed significantly ($p<0.01$) increased triglycerides level than WW and EW respectively. EVCO groups showed significant ($p<0.01$) difference with WVCO by having increased level of serum triglycerides.

Figure 5.23 is representing results for total cholesterol concentrations in serum. Analysis showed significant effects of ethanol consumption ($F=4.293$, $df=1,20$, $p<0.05$), VCO treatment ($F=7.951$, $df=1,20$ and $p<0.05$) and water consumption and VCO treatment interaction ($F=17.250$, $df=1,20$, $p<0.01$) on total cholesterol levels. Post hoc analyses showed significant differences. WVCO showed increased total cholesterol levels as

compared to WW. EVCO showed decreased total cholesterol concentration compared to EW and WVCO but this decrease was significant only from WVCO. These results revealed that VCO can decrease the total cholesterol in ethanolic rats and it might be harmful for water drinking animals by increasing the cholesterol.

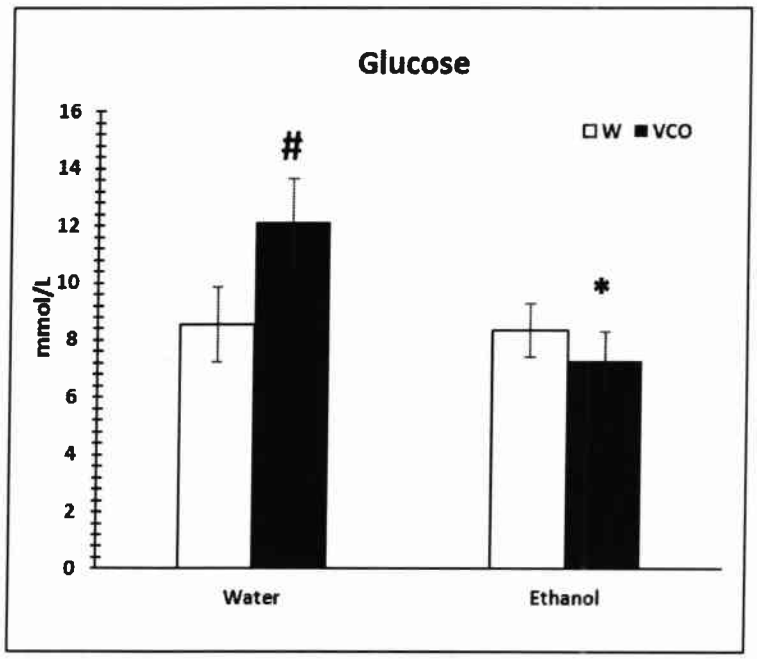


Figure 5.20: Effects of VCO treatment on serum random glucose levels of water and ethanol drinking groups. Values are means ± SD. Significant difference by post hoc test: •p<0.01 and #p<0.05 from respective controls

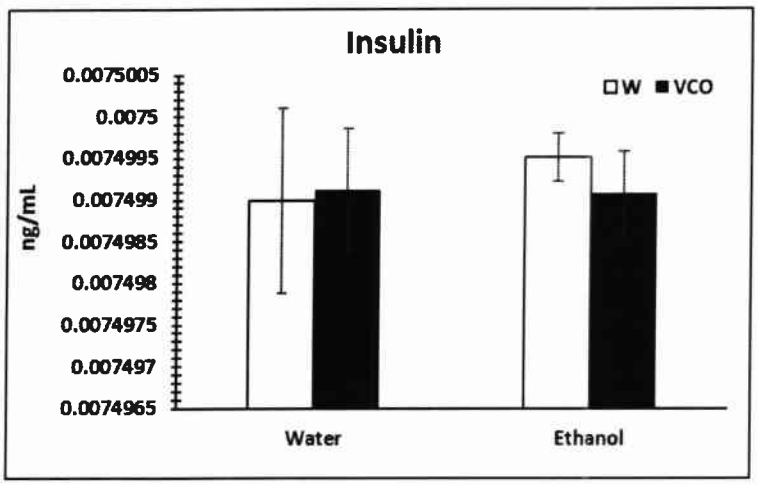


Figure 5.21: Effects of VCO administration on serum insulin levels of water and ethanol drinking groups. Values are means ± SD. Insignificant difference was observed among groups.

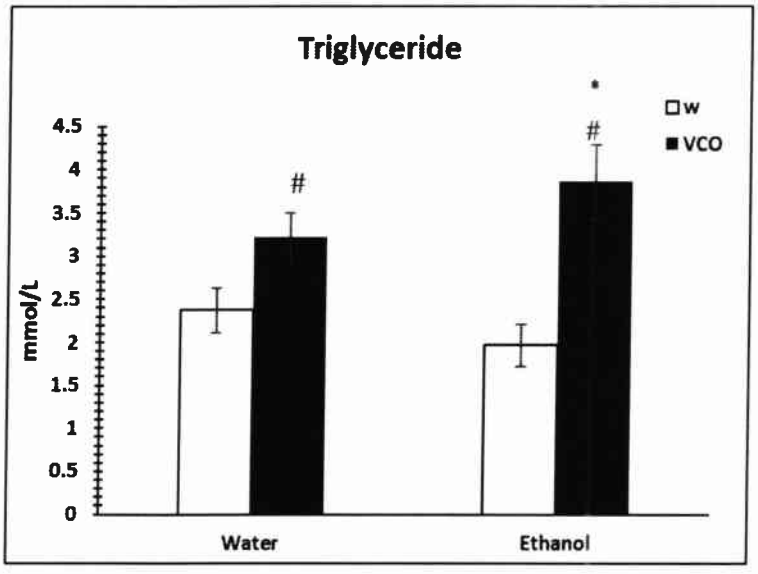


Figure 5.22: Effects of VCO treatment on serum triglycerides levels of water and ethanol drinking groups. Values are means ± SD. Significant difference of ***p<0.01** and #p<0.01 observed by posthoc testing.

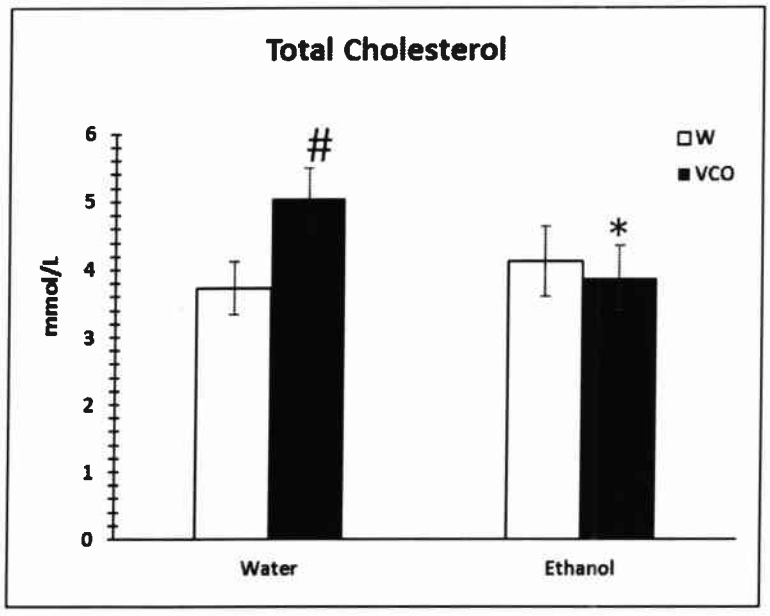


Figure 5.23: Effects of VCO treatment on serum total cholesterol levels of water and ethanol drinking groups. Values are means ± SD. Significant difference by post hoc test: *p<0.05 and #p<0.05 from respective water treated control animals.

5.3. Discussion

Important results and finding of current study are:

9. Virgin coconut oil (VCO) treatment of ethanol drinking animals exacerbated the effects of ethanol on body weight.

10. VCO treatment lead to decreased food intake in water and ethanol drinking animals. It means VCO treatment exacerbated the effects of ethanol on food intake.

11. Ethanol drinking resulted in decreased fluid intake but VCO treatment of ethanolic rats caused slight increase in fluid intake.

12. VCO acted as anxiogenic in water and ethanol drinking animals.

13. VCO treatment improved the memory in water drinking animals but impaired memory in ethanolic rats.

14. VCO treatment reduced locomotory activity in water drinking animals.

15. Brain serotonin (5-HT) level in VCO treated ethanol drinking (EVCO) group was reduced.

16. Prefrontal 5-HT and 5-HIAA levels were increased by VCO treatment in water drinking animals.

17. VCO treatment caused decreased dopamine level in prefrontal cortex (PFC) of water and ethanol drinking rats.

18. VCO treatment caused decreased DOPAC level in PFC of water drinking rats but increased in ethanolic rats.

19. VCO treatment caused decreased dopamine with HVA concentrations in ethanolic animals.

20. Hippocampal serotonin and metabolite concentrations were reduced in EVCO.

21. VCO treatment of water drinking animals increased hippocampal noradrenaline (NA) level.

22. VCO treatment increased 5-HT metabolism in mid brain and caused increases 5-HIAA.

23. VCO acted as hypoglycemic agent in the ethanolic rats but hyperglycemic agent in water drinking group.

24. Triglycerides level was increased in VCO treated water and ethanolic rats.

25. VCO reduced the total cholesterol levels in ethanol drinking rats and increased in water drinking animals.
26. Finally, VCO treatment lead to decreased BDNF level in hypothalamus of ethanol and water drinking animals.

In current study, ethanol consumption lead to reduced body weight, food and fluid intake in rats. Fromenty and colleagues, (2009) and Macdonald and coworkers, (2010) also reported that chronic ethanol intake reduced body weight. The effect of ethanol on body weight is biphasic, with smaller amount of ethanol that have no effect at all, moderate quantities lead to increased body weight whereas excessive ethanol consumption has been reported to reduced body weight (Suter, 1997). Acute ethanol intake has only a small effect on meal size (Poppitt *et al.*, 1996) but chronic ethanol consumption inhibits growth by decreasing the food intake and consumed meal size (Strbak *et al.*, 1998, Seeley *et al.*, 1997; Reidelberger *et al.*, 1996). VCO treatment of animals led to further reduction in body weight of ethanolic rats. Interestingly reduced food intake was observed in both groups treated with VCO.

Anxiety like behavior in animals is assessed by EPM where exploratory activity and aversion in open arms describes the mental state of animals. Animals with anxiety prefer to spend more time in closed arms whereas, animals without anxiety like behavior prefer to explore environment in EPM (Pellow *et al.*, 1985). However, in the OF, exploratory activity and anxiety-like behavior is assessed through number of lines or squares crossed (Asano, 1986). Centre of any maze can be taken as anxiogenic because an anxious animal travel and remain close to the edges and wall of maze and this makes them feel safe (Crusio *et al.*, 1989). Anxious animals stay in corner of maze as well. A huge literature is available which have demonstrated the anxiogenic role of alcohol by using these behavioral tests (Whimbey and Denenberg, 1976; Lister, 1990).

Alcohol drinking group showed anxiety like behavior in EPM test. In line with this, Holmes and coworkers, (2012) have also demonstrated the anxiety in binge alcohol drinking. Cognition deficit is co-morbid with anxiety (Park and Moghadda, 2017). In our study, impaired cognition and anxiety were observed together. VCO treatment of alcohol

drinking animals resulted in increased number of entries but could not help to increase time spent in open arms. This shows that VCO treatment of alcohol drinking animals lead to anxiety and entries were increased due to panic situation. In line with our results, increased anxiety by coconut oil has also been observed previously (Curezone, 2011). However, Joette Calabrese, reported that coconut oil reduced anxiety in patients (Calabrese, 2014). VCO treatment of water drinking animals did not alter or act as anxiogenic

Reduced locomotory activity was observed in alcohol drinking rats. In line with our results, Kumar and co-workers, (2013) have reported the significant reduced locomotory activity by a reduced crossing of squares in OF with less entries in OA of EPM by alcohol drinking rats compared to water drinking controls. This reduced activity or hypo locomotion induced by chronic alcohol consumption is similar with few previous researches (Overstreet *et al.*, 2003; Rasmussen, 2001). VCO treatment of alcohol drinking rats did not alter the locomotory activity and did not show any significant difference compared to EW controls.

VCO treatment of water drinking animals caused the habituation effect by crossing a greater number of squares compared to water controls. This might be due to retrieval of memory to similar environment or habituation effects.

Alcohol drinking affects brain functioning and lead to behavioral deficits i.e. cognitive impairments (Deitrich *et al.*, 1989; Ollat *et al.*, 1988; Samson and Harris, 1992) and anxiety (Holmes *et al.*, 2012). Long term alcohol exposure exerts harmful effects on cognition, characterized by impaired learning and memory (Hartzler and Fromme, 2003; White, 2003; Wixted, 2005). In our study, we also observed impaired learning and memory in alcohol drinking rats with significant increased escape latency time in MWM test. STM, LTM was impaired by consuming alcohol for long term.

VCO treatment of alcohol dinking animals exacerbated the impaired memory and the took significant more time escape latency time in last memory test compared to EW and water controls. They could not perform well and did not show any improvement in prob test as well. Whereas VCO treatment of water drinking animal enhanced the memory

reconsolidation and showed improved memory retrieval in prob tests with decreased escape latency and increased entries with time spent in platform quadrant compared to WW. Olanrewaju and coworkers, (2018) have also found the memory enhancing role of VCO in their Alzheimer's like dementia in rats. Da Silva et *al.*, (2018) also reported the similar effects of VCO treatment.

In previous 2nd chapter we have discussed neurotransmitters role in alcohol addiction and behavior in detail. To the best of our knowledge very few researches have done which describe role of VCO on neurotransmitters. This is the first study to provide data on this regard.

Alcohol consumption exerts negative effects on behavior. Most of the associated behaviors are associated to changes in neurotransmitters level and neurotransmission in various brain regions. Neurotransmitters influence the functioning of neuroanatomic circuits that offer support to cognition, anxiety and fear. A few studies have proven a relationship between reduced 5-HT neurotransmission and behavioral disorders (Berger, Gray and Roth, 2009; Muller and Jacobs, 2009; Lucki, 1998). Researches have suggested an important part of dopamine in behavioral disorders (Dunlop and Nemeroff, 2007). Continuous ethanol consumption decreases brain noradrenaline (Yamanaka and Egashira, 1982).

Altered 5-HT and DA levels and neurotransmission have been found in patients with anxiety and hyperactivity (Alex and Pehek, 2007). Reduced DA transmission is linked with neuropsychological disorders (Dailly *et al.*, 2004), for example, impaired DA have been found in anxiety and depression (Kafka, 2003). These studies give a possible indication of the pathogenesis of behavioral and phycological disorders. Many researches have demonstrated the role of noradrenaline in a range of psychological and physiological processes including memory, learning, anxiety, sleep, adaptation and arousal (Montgomery, 1997).

Previous studies have shown that 5-HT neurotransmission play an important role in behavioral deficits (Gingrich & Hen 2001: Lucki, 1998). Reduced 5-HT is linked to

mood (Ruhe, Mason and Schene, 2007), impaired memory (Buhot, Martin and Segu, 2000), anxiety (Stein and Stahl, 2000), stress (Chaouloff, Berton and Mormede, 1999). This present study found reduced 5-HT levels in prefrontal cortex and hippocampus of ethanol drinking rats and these rats have shown impaired memory and anxiety.

Increased dopamine level is involved in self-preference of addictive substance (Oswald *et al.*, 2005). Alcohol consumption increases dopamine level through increased activity of mesolimbic dopaminergic neurons (Boileau *et al.*, 2003). Studies have showed function of dopamine transmission in abnormal behaviors (Wahlstrom *et al.*, 2010; Moghaddam, 2002). Enormous reduction in DA neurotransmission is linked with depression and hypoactivity in rodents (Gershon, Vishne and Grunhaus, 2007). In present study, we found similar results with previous researches that alcohol increased the brain dopamine level and caused impaired memory, anxiety and hypoactivity.

VCO treatment of alcohol drinking animals showed reduced 5-HT, DA and NA levels. Results also showed reduced 5-HIAA and increased DOPAC levels. These rats have also shown impaired memory and anxiety in EPM tests. VCO treatment of water drinking animals improved memory and showed elevated serotonin levels in PFC and midbrain with reduced or normal brain DA levels. This means VCO treatment improved memory by increasing serotonin level and promoting dopamine metabolism in water drinking rats. The memory enhancing effects are also linked with increased DOPAC, HVA with reduced 5-HIAA and DA levels (Cheema *et al.*, 2016).

Noradrenaline is one of the best-known neurotransmitters that modulate the synaptic functions. In the central nervous system, the primary source of noradrenaline is the locus coeruleus (Kitchigina *et al.*, 1997; Sara *et al.*, 1994), a structure located in the brain stem that sends noradrenergic projections to many brain regions (Jones *et al.*, 1977).

VCO treated water drinking group showed increased hippocampal NA. The hippocampus has major role in integrating and processing spatial information important in formation and retrieval of memory (Chen *et al.*, 2017; Hansen, 2017; Jin and Maren, 2015; Maren *et al.*, 2013; Bouton *et al.*, 2006) and locus coeruleus projections to hippocampus

has impact on learning (Wagatsuma *et al.*, 2018). Noradrenaline can influence the function of hippocampus and locus coeruleus stimulation (Yavich *et al.*, 2005; Hajós-Korcsok *et al.*, 2003; Abercrombie *et al.*, 1988). Many studies have suggested that noradrenaline increase long-term potentiation (LTP) in hippocampus, specifically in CA1 and dentate gyrus, which is dependent upon βAR and α1 mechanisms (Harley, 2007; Yang *et al.*, 2002; Izumi and Zorumski, 1999; Chaulk and Harley, 1998; Katsuki *et al.*, 1997; Dunwiddie *et al.*, 1992; Segal *et al.*, 1991; Dahl and Sarvey,1989; Hopkins and Johnston, 1988; Stanton and Sarvey, 1987 and 1985a,b; Lacaille and Harley, 1985; Blissetal,1983; Neuman and Harley,1983). High level of noradrenaline enhances excitatory post synaptic potentials, reduce spike on set latency, and enhances the population spike amplitude. These effects promote long-term potentiation induction and are important for memory formation (Neuman and Harley, 1983; Lacaille and Harley,1985; Harley, 2007).

Park and Moghadda, (2017) reported that impaired cognition is a hallmark deficit associated with anxiety. In line with this results anxiety with impaired memory was observed in ethanolic rats. VCO treatment could not ameliorated the ethanol induced anxiety and improved memory, and EW and EVCO group showed significantly reduced NA compared to WW and WVCO groups. These results showed that higher level of hippocampal noradrenaline improved spatial memory by increasing the hippocampal long-term potentiation in water drinking rats.

Clinical and preclinical studies have demonstrated the effects of alcohol consumption on glucose level. This might cause increase, decrease or no change in circulating glucose level. A recent report on meta-analysis of intervention researches has demonstrated the no effect of alcohol consumption on the glucose concentration in nondiabetic individuals (Schrieks *et al.*, 2015), which was supported by previous studies (Davies *et al.*, 2002; Kiechl *et al.*, 1996). In line with these studies, no change in glucose level was observe in ethanol drinking rats of present study.

VCO is capable to reduce glucose level (Sadikot, 2005; Garfinkel *et al.*, 1992). Likely, in this current study reduced glucose level was observed in VCO treated ethanolic rats. Previously hypoglycemic role of VCO has also been published by Bolanle and

coworkers, (2013) and Mahadevappa and coworkers, (2011). Our result suggested that VCO treatment is effective to reduce glucose levels in ethanol drinking.

Cholesterol, a lipid as major component of cell membrane plays many functional and structural roles and is required for viability and growth of mammalian cells as well. Dysregulated cholesterol balance and hyperlipidemia homeostasis is a risk factor for the development of atherosclerotic cardiovascular disease (Castelli, 1996; Davignon and Cohn, 1996). Chronic ethanol exposure results in disturbed total cholesterol level in rat serum (wang *et al.*, 2012). In contrast, in present experiment ethanol drinking did not alter the cholesterol level. It also showed that VCO treatment of ethanolic animals showed decreased total cholesterol level and increased in water drinking rats.

Chronic ethanol exposure s linked with higher triglycerides level (Klop, do Rego and Cabeza, 2013). In contrast our results showed no significant effect of alcohol on serum triglycerides and showed similar results with controls. But VCO treatment resulted in increased level. VCO has been known to suppress hypolipidemic effects (Weiss, Maker and Lehrer, 1980) and reduces circulating lipids level (Cohen, Dembiec and Marens, 1970). VCO metabolizes faster and prevent fat accumulation (Evans, 2007; Nevin and Rajamohan, 2004). Mahadevappa and coworkers, (2011) found that VCO ameliorated the total cholesterol, triglycerides, low density lipids and very low-density lipoprotein in diabetic and nondiabetic rats. These results are supporting with our results where VCO treatments resulted in reduced serum total cholesterol and increased serum triglycerides level in nondiabetic rats. In contras Akinjide *et al.*, 2014) showed reduction in triglycerides level in VCO fed diabetic rats.

Ethanol consumption had significant effect on level of BDNF which was reduced as compared to water treated animals. These results are also in consistent with Tapia *et al.*, (2001), they have reported that chronic ethanol intoxication decreases brain-derived neurotrophic factor mRNA expression in discrete regions of the rat in the hypothalamus. In the literature, there are studies that report lower peripheral BDNF levels in ethanol-addicted patients and it has been suggested that chronic ethanol drinking leads to a reduction in BDNF levels compared to those of healthy controls, implying that BDNF may

have a role in the development of ethanol dependence (Sercin *et al.*, 2012; Roberta *et al.*, 2011; Joe *et al.* 2007).

Coconut oil is enriched in saturated fat (Badolato *et al.*, 1992). Many investigators have demonstrated that high fat rich in saturated fatty acids give rise to lower levels of BDNF in the hippocampal formation (Molteni *et al.*, 2004) and in cerebral cortex (Wu *et al.*, 2003). Results of current study also tells BDNF expression was significantly reduced in the VCO treated water drinking and ethanol drinking groups compared to respective water treated controls (WW) and (EW) respectively. Our results are consistent with those of Patricia *et al.*, 2018 and Rao, 2007, which reported that VCO can decrease BDNF expression. In their study, coconut oil has been used as a model for docosahexaenoic acid (DHA) restricted diet. Coconut oil diet reduced BDNF. Decreased BDNF may reduce neuronal and glial cell number as observed in the brain of bipolar patients (Rajkowska, 2001), and BDNF knockdown/knockout mice display an increase in depression- behavior (Berton *et al.*, 2006; Monteggia *et al.*, 2006). These data show that coconut oil can modulate synaptic plasticity and neuronal development by decreasing the BDNF expression (Patricia *et al.*, 2018; Rao, 2007). Studies have found effects of VCO on BDNF level in different brain regions but data in hypothalamic BDNF was lacking. This research has demonstrated the reduced BDNF level in hypothalamus of water drinking and ethanolic rats.

5.4. Conclusion

The findings of this experiment have explored that ethanol drinking resulted in reduced body weight, food and fluid intake. VCO treatment of ethanol drinking animals exacerbated these effects of ethanol on body weight and food. Ethanol drinking impaired memory and developed anxiety. VCO treatment improved memory in water drinking animals only by increased prefrontal serotonin and noradrenalin levels whereas the effects were in contrast in ethanol drinking rats. They showed anxiety and impaired memory with reduced serotonin, dopamine and DOPAC.

CHAPTER # 6

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List of publications

PUBLISHED

1) Asrar B., Khan I.U., Homberg J.R., and Haleem D.J., (2020) *Nigella sativa* oil ameliorates chronic ethanol induced anxiety and impaired spatial memory by modulating noradrenaline levels, Pak Vet J, 40(3): 350-354. http://dx.doi.org/10.29261/pakvetj/2020.029

2) Schipper P., Brivio P., Leest D.D., Madder L., Asrar B., Rebuglio F., Verheij M.M., Kozicz T., Riva M.A., Calabrese F., Henckens M.J., and Homberg J.R., (2019) Impaired Fear Extinction Recall in Serotonin Transporter Knockout Rats Is Transiently Alleviated during Adolescence, Brain Sciences, 9, 118.

SUBMITTED (IN REVIEW):

3) *In silico* and *in vitro* screening of suitable substrate for phytase activity.
4) Trends and strategies for lipid peroxidation: Salinity triggers defensive switches in aero-terrestrial green microalgae
5) *Immunological view of osteoporosis*

Three (03) papers from PhD thesis are in pipeline which will be submitted in relevant journals soon.