EVALUATION OF CO-ADMINISTRATION OF ETHYL ACETATE FRACTION OF *Cannabis sativa* LEAVES AND ETHANOL ON LEARNING, MEMORY AND THE HISTOMORPHOLOGY OF THE HIPPOCAMPUS IN MICE (*Mus musculus*)

BY

Bilkisu Talatu, ILIYA
B.Sc (ABU, 2011)
(P13MDHP8006)

A THESIS SUBMITTED TO THE COLLEGE OF HEALTH SCIENCES, FACULTY OF BASIC MEDICAL SCIENCES,
DEPARTMENT OF HUMAN PHYSIOLOGY,
AHMADU BELLO UNIVERSITY,
ZARIA

DECEMBER, 2017

EVALUATION OF CO-ADMINISTRATION OF ETHYL ACETATE FRACTION OF *Cannabis sativa* LEAVES AND ETHANOL ON LEARNING, MEMORY AND THE HISTOMORPHOLOGY OF THE HIPPOCAMPUS IN MICE (*Mus musculus*)

BY

Bilkisu Talatu, ILIYA
B.Sc (ABU, 2011)
(P13MDHP8006)

A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES,
AHMADU BELLO UNIVERSITY,
ZARIA

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTER DEGREE IN HUMAN PHYSIOLOGY

DEPARTMENT OF HUMAN PHYSIOLOGY,
FACULTY OF BASIC MEDICAL SCIENCES,
COLLEGE OF HEALTH SCIENCES
AHMADU BELLO UNIVERISTY
ZARIA NIGERIA

DECEMBER, 2017

DECLARATION

I declare that the work in this dissertation entitled "**EVALUATION OF CO-ADMINISTRATION OF ETHYL ACETATE FRACTION OF *Cannabis sativa* LEAVES AND ETHANOL ON LEARNING, MEMORY AND THE HISTOMORPHOLOGY OF THE HIPPOCAMPUS IN MICE (*Mus musculus*)"** was carried out by me in the Department of Human Physiology, Faculty of Medicine, Ahmadu Bello University, Zaria, under the supervision of Dr M.I.A Saleh and Dr. A. ALHASSAN. The information derived from the literature has been duly acknowledged in the text and list of references provided. No part of this dissertation was previously presented for another degree or diploma at any university.

Bilkisu Talatu, ILIYA _____ _____

Signature **Date**

CERTIFICATION

This dissertation entitled **"EVALUATION OF CO-ADMINISTRATION OF ETHYL ACETATE FRACTION OF *Cannabis sativa* LEAVES AND ETHANOL ON LEARNING, MEMORY AND THE HISTOMORPHOLOGY OF THE HIPPOCAMPUS IN MICE (*Mus musculus)"*** by **ILIYA, TALATU BILKISU** meets the regulations governing the award of the degree of Master of Science in Human Physiology at Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literary presentation.

_____ _____

Dr. M.I.A SALEH, MBBS, MSc, PhD ***Date***
Chairman, Supervisory Committee

_____ _____

Dr. A. ALHASSAN, BSc, MBBS, MSc, PhD ***Date***
Member, Supervisory Committee

_____ _____

Dr. Maryam B. Akor-Dewu, BSc, MSc, PhD ***Date***
Head of Department

_____ _____

Prof. S.Z. ABUBAKAR, B. Eng., MSc, PhD ***Date***
Dean, Postgraduate School

DEDICATION

This work is dedicated to God Almighty for guidance and protection in my life and all my family and friends.

ACKNOWLEDGEMENT

I profoundly acknowledge my creator for His faithfulness, mercy and for giving me the privilege to attain this level of my academic pursuit. I acknowledge my supervisors, Dr M.I.A Saleh and Dr. A. Alhassan for their valuable input to my work. I immensely appreciate their contribution. Also I immensely appreciate the Post Graduate Co-ordinator, Dr. Y. Tanko for his encouragement and support. I am also grateful to my course mates, Emmanuel Solomon, Munira Aliyu, Jibril Zubairu, and Nabila Sada, for their encouragement and moral support. I am very grateful to Ogunbodede Gbenga who patiently assisted me in the laboratory and Omoniyi Akinyemi who assisted with running the statistical analysis for my data. I am very grateful to my Husband, Dr. Richard Anche for his encouragement and perseverance during my studies. I am also grateful to my brother, Dr. Ibrahim Iliya and my entire family for their support. Finally, am grateful to all those who have contributed in encouraging and praying for me to ensure the successful completion of this work. I appreciate you all. Remain blessed.

ABSTRACT

Substance abuse especially amongst young adults constitutes a global problem. *Cannabis sativa* is a commonly abused illicit drug. Some of its effects include impaired short memory, attention and reduced motor skills. Ethanol is a multiple action depressor of the central nervous system and under its influence, confused and disorganized thinking results. Both drugs have a detrimental effect on prospective memory in adults but users may not be aware of this. This study was therefore aimed at evaluating the co-administration of ethyl acetate fraction of *Cannabis sativa* leaves and ethanol on Learning, Memory and the histomorphology of the Hippocampus in Mice (*Mus Musculus)* using the Y-maze and Novel Object Recognition (NOR) paradigms. Fourty eight apparently healthy male albino mice of 20-28 g body weight were assigned into two major groups consisting of four sub-groups each; A, B, C, and D. Each sub group had six mice each (n=6). Sub-groups A, B, C, and D were administered distilled water 1 ml/kg body weight (bwt) (control), 145 mg/kg bwt ethyl acetate fraction of *Cannabis sativa* leaves, ethanol 20% v/kg bwt and co-administration of 145 mg/kg bwt ethyl acetate fraction of *Cannabis sativa* leaves and 20% v/kg bwt of alcohol respectively, orally for 21 days. Neurobehavioural assessment was carried out using the Y-maze and Novel Object Recognition (NOR) Paradigms. The Hippocampal histology and volumes were obtained using H & E staining technique and Cavalieri estimator respectively. Data were expressed as mean±SEM. One way ANOVA and Kruskal Wallis tests were used to compare the means at $p<0.05$. The result of Y-maze test showed a significant increase ($P<0.05$) in the mean rank of the co-administered group followed by the cannabis group indicating memory impairment when compared to control. NORT result showed a significant decrease ($P<0.05$) in discrimination and retention indexes in the co-administered group followed by the *Cannabis sativa* treated group when compared to control. A non-statistical significant ($P>0.05$) decrease was observed in Hippocampal volume when compared to the control. Histopathological evaluation revealed disorganization and focal necrosis of pyramidal cells of the hippocampus in the co-administered group when compared to the control. It can therefore be inferred from the findings of this study that, co-administration of ethyl acetate fraction of *Cannabis sativa* leaves with ethanol is more detrimental to hippocampal histoarchitecture with a corresponding memory impairment relative to administration of either.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF PLATES

LIST OF TABLES

LIST OF ABBREVIATIONS

ADH	Alcohol Dehydrogenase
AIHW	Australian Institute of Health and Welfare
AMPA	α-Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic Acid
CB1-R	Cannabinoid Receptor 1
CB2-R	Cannabinoid Receptor 2
CDC	Center for Disease Control
EC	Entorhinal Cortex
etOH	Ethanol
FAEE	Fatty Acid Ethyl Ester
GABA-R	Gamma Amino Butyric Acid Receptor
NDLEA	National Drug Law Enforcement Agency
NIDA	National Institute on Drug Abuse
NMDA	N-Methyl-D-Aspartate
NORT	Novel Object Recognition Test
THC	Tetrahydrocannabinol
W.H.O	World Health Organization

CHAPTER ONE

1.0 INTRODUCTION

Substance abuse, also known as drug abuse, is a patterned use of a drug in which the user consumes the substance in amounts or with methods which are harmful to themselves or others, and is a form of substance-related disorder (Kris and Oakley Ray, 2002). Widely differing definitions of drug abuse are used in public health, medical and criminal justice contexts. In some cases criminal or anti-social behavior occurs when the person is under the influence of a drug, and long term personality changes in individuals may occur as well (Kris and Oakley Ray, 2002). *Cannabis sativa* and alcohol are among the most commonly abused illicit drugs (Fontes *et al*., 2011).

Cannabis sativa, commonly known as marijuana (prepared leaves), is one of the most commonly used illicit drugs for recreational purposes. *Cannabis sativa* contains a unique group of chemicals called cannabinoids. Its main psychoactive constituent is tetrahydro cannabinol (THC). The plant is known to contain about sixty cannabinoids; however, most of these 'minor' cannabinoids are only produced in trace amounts (Ashton, 2001). The psychoactive substance in cannabis, delta-9-tetrahydrocannabinol (THC), stimulates cannabinoid receptors (CBRs), located on the surface of neurons, to produce the psychoactive effects.

Alcohol is also a psychoactive substance based on its abilities to alter human consciousness. It is commonly consumed as a recreational drug. It also acts as 'gateway' drug to the use of other substances like cocaine, heroin, amphetamine, inhalants and hallucinogens (Makanjuola *et al*, 2014). Alcohol has effects on every organ in the drinker's body. Intoxication can impair brain function and motor skills; chronic use can increase risk of cancers, stroke, and liver disease. Alcoholism or alcohol dependence is a diagnosable disease characterized by a strong craving for

alcohol, with continued use despite harm or injury. Alcohol abuse is a pattern of drinking that result in harm to one's health, interpersonal relationships, or ability to work (NIDA, 2015).

1.2 Statement of Research Problem

Globally, substance use has become a major public health issue (Makanjuola *et al.,* 2014). According to the World Drug Report 2014, drug-related death is the most extreme form of harm that can result from substance use. It was reported that there were 183,000 (range: 95,000-226,000) drug-related deaths in 2012, corresponding to a mortality rate of 40.0 (range: 20.8-49.3) deaths per million persons aged 15-64 (UNODC, 2014). Worldwide, there has been an increase in drugs and substance abuse especially amongst young adults, with no exclusion to Nigeria. The youths have been identified as a high risk group for the use of psychoactive substances as reported by Makanjuola *et al.,* (2014), with alcohol and *Cannabis sativa* as the most popular of these substances (Amresh *et al*., 2013). Despite the preventive governmental policies; substance use has continued to increase among the youth especially among university students (Owoaje and Bello, 2010). *Cannabis sativa* use has a detrimental effect on prospective memory ability in young adults but users may not be aware of these deficits (Bartholomew *et al.,* 2011). The abuse of alcohol is the leading risk factor for death in males 15-59 years of age (Krista *et al*., 2007).

1.2.1 Justification

Some substance use continues to be amajor risk behaviour among youth, with consequent physical and or mental health complications (Oshodi *et al*., 2010). Despite the frequency with which young adults engage in alcohol and marijuana use, research on the combined effects of these substances is limited, and it is unclear whether concomitant use of alcohol and marijuana could necessarily result in compounding the deficits associated with each substance (Amresh *et al*., 2015). Evidence suggests that the hippocampus may be particularly vulnerable to structural

damage caused by heavy alcohol or marijuana use, especially during adolescence (Krista *et al.*, 2007). The use of these substances does not only pose a threat to their health but also to the health and social wellbeing of their families and the society at large (Makanjuola *et al.*, 2014). It is therefore imperative to investigate the effects of co-administration with *Cannabis sativa* and alcohol on learning, memory and histomorphology of the hippocampus.

1.3 Aim of the study

This study is aimed at investigating the effects of co-administration with *Cannabis sativa* and alcohol on learning, memory and histomorphology of the hippocampus in Mice.

1.3.1 Objectives of the study

i. To evaluate the effects of *Cannabis sativa* on learning and memory in mice, using the Y-maze and Novel Object Recognition Test.
ii. To determine the effects of alcohol on learning and memory in mice, using the Y-maze and Novel Object Recognition Test.
iii. To determine the effects of co-administration of *Cannabis sativa* and alcohol on cognition in mice, using Y-Maze and Novel Object Recognition Test.
iv. To estimate volumes of the hippocampus in the control and experimental mice using the cavalieri estimator point counting grid method
v. To determine the histological effects of co-administration of *Cannabis sativa* and alcohol on hippocampal structure of mice

1.4 Research Hypothesis

H_0; *Cannabis sativa* or co-administration of *Cannabis sativa* and alcohol does not have any effect on learning, memory and histomorphology of the hippocampus in mice.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Physiology of Learning and Memory

2.2 Learning

Learning is the act of acquiring new, or modifying and reinforcing existing knowledge, behaviors, skills, values, or preferences and may involve synthesizing different types of information (Karban, 2015).

Learning ranges from simple forms such as habituation and classical conditioning seen in many species, to more complex activities seen only in relatively intelligent animals and humans. Generally, learning can be either a conscious or non-conscious process (Mather and Anderson, 1998).

For instance, in small children, non-conscious learning processes are as natural as breathing. In fact, there is evidence for behavioral learning prenatally, in which habituation was observed as early as 32 weeks into gestation, indicating that the central nervous system is sufficiently developed for learning and memory to occur early on in development (Sandman *et al*., 1997).

The brain is a complex structure with about one hundred billion nerve cells (Tessier-Lavigne and Goodman, 1996). These nerve cells, called neurons, are microscopic in size and interconnected in innumerable ways. Some neurons receive information from the rest of the body, while others synthesize and interpret the information; still others send messages that tell the body how to respond to its present circumstances. However, neurons don't actually touch one another. Neurons use a variety of substances known as neurotransmitters, which send chemical messages to their neighbours across the tiny spaces called 'synapses'. A single neuron may have up to hundreds or even thousands of synaptic connections with other neurons (Lichtman and Fraser, 2001). Complex thinking, learning, and knowledge are located primarily in the upper and outer

parts of the brain known as the cortex, which rests on the top and sides of the brain (Smith, 2009). The prefrontal cortex, which is the portion of the cortex located near the forehead, is largely responsible for a wide variety of human activities, including reasoning, sustained attention, decision making, planning, inhibiting nonproductive thoughts and behaviors and coordinating complex activities. Other parts of the cortex are important such as areas being actively involved in interpreting visual and auditory information, are important in identifying the spatial characteristics of objects and events, and keeping track of general information about the world (Yeo *et al*., 2011).

Physiologically, the basis for learning lies in changes in the interconnections among neurons. In particular, learning may involve strengthening existing synapses or forming new ones (Merzenich, 2001). However, in some instances, learning may actually involve eliminating synapses. Effective learning requires not only that people think and do certain things but also that they not think or do other things, in other words, they inhibit tendencies to think or act in certain ways (Julie and Greenough, 2006).

Until recently, it was thought that all the neurons are produced in the first few weeks after conception, long before the person is even born. Researchers are finding, however, that some formation of new neurons continues throughout life in the hippocampus and possibly also in some areas of the cortex (Thompson and Nelson, 2001).

2.2.1 Neural Networks and Connections

Neuroplasticity is the single most important concept in terms of learning and the brain. Our brain is constantly changing and growing and cortical plasticity extends throughout the human

lifespan. Learning is not just changing of external behavior, but changing the very wiring of the brain as it relates to those behaviors (Ronen *et al*., 2011).

2.2.2 Hebbian Synapses

In 1949, Canadian psychologist Donald Hebb proposed that learning may exist at the level of synapses. His proposition led to the well-known phrase "neurons that fire together wire together". Two cells that are strengthened in this way are called a 'Hebbian synapse' (Hebb, 1950). His hypothesis began to be confirmed in the early 1970's, through neuroscientific research, and gained further support as new methods and tools emerged. What he hypothesized and what has since been proven is that neurons that are repeatedly used grow stronger synapses and more effective neuronal networks. And the more they fire, the more they send out new branches, formimg new and useful connections. From this emerged the concept of "attention density" which states that Focused attention and repetition helps neurons fire solidly together, creating new learning as proposed by Jeffrey Schwartz (Lavigne *et al*., 2016).

Learning affects the brain in two different ways; by altering existing connections or by creating brand new connections. New connections lead to an increase in overall synaptic density, while altering connections makes existing pathways more efficient or suitable. In either case, the brain is remodeled to take in new information and, if useful, it retains it (Louis and Susan, 2006).

Learning and memory are based on two plausible principles: one is concerned with the activity (neuronal firing) and the other with the plasticity of the neurons (i.e. sprouting of new axons and dendrites, and formation of new synapses) (Gulpinar and Yegen, 2004). Neuronal plasticity is the basic process by which the brain acquires sensory, cognitive, emotional, social, and endocrine inputs, or combinations of these data, and makes the appropriate adaptive responses (Duman, 2002).

Long-term potentiation (LTP) is a phenomenon that has been recently shown to be associated with the formation of new synapses, is regarded as one of the best and universally accepted model of learning and memory formation (Arendt, 2001). The discovery of long lasting potentiated synapses provided a possible cellular mechanism for learning and memory. In addition to its duration, which lasts for several hours in vitro, several weeks in vivo, LTP is rapidly induced, strengthened by repetition and demonstrates specificity and associativity (Kim and Yoon, 1998). However, LTP alone cannot provide enough explanation for the synaptic model of learning and memory. During this process, along with these increases in synaptic efficacy in the form of LTP, decreases in synaptic efficacy, termed as long-term depression (LTD), are also needed. Both processes occur prominently in the hippocampus and appear to be mediated by mechanisms that are triggered by Ca^{2+} influx via activation of N-methyl-d-aspartate (NMDA) receptors. In LTD, low levels of intracellular Ca^{2+} lead to the activation of phosphatases that depress the synapse, while in LTP, high levels of Ca^{2+} lead to the activation of kinases, which potentiate the chemical synapse (Kim and Yoon, 1998).

Among some of the neurotransmitters involved in learning and memory processes are, acetylcholine (ACh), a well-known neurotransmitter of the basal forebrain cholinergic neurons that is associated with cognitive processes. These cholinergic neurons innervate the limbic and the cortical structures, such as the hippocampus, anterior cingulate cortex, olfactory bulb, amygdala, frontal, parietal and temporal cortices. Also, the basal forebrain cholinergic system contains many non-cholinergic neurons, such as g-aminobutyric acid-releasing (GABA) interneurons, which make synaptic connections with the cholinergic neurons. The involvement of glutamate, its receptor, NMDA, and its modulation by experience in learning and memory is also well established (Zhou and Baudry, 2006).

2.3 Memory

Memory is the process by which information is encoded, stored, and retrieved. Memory is also the ability of the brain to store, retain, and subsequently recall information (Reingold, 2002). It is defined as a behavioral change caused by an experience (Okano *et al.*, 2000). Human memory may be regarded as a system that stores and retrieves information acquired through the visual and auditory senses. Functionally, memories are stored in the brain by changing the basic sensitivity of synaptic transmission between neurons due to previous neural activity (Guyton and Hall, 2006).

.

2.3.1 Classification of Memory

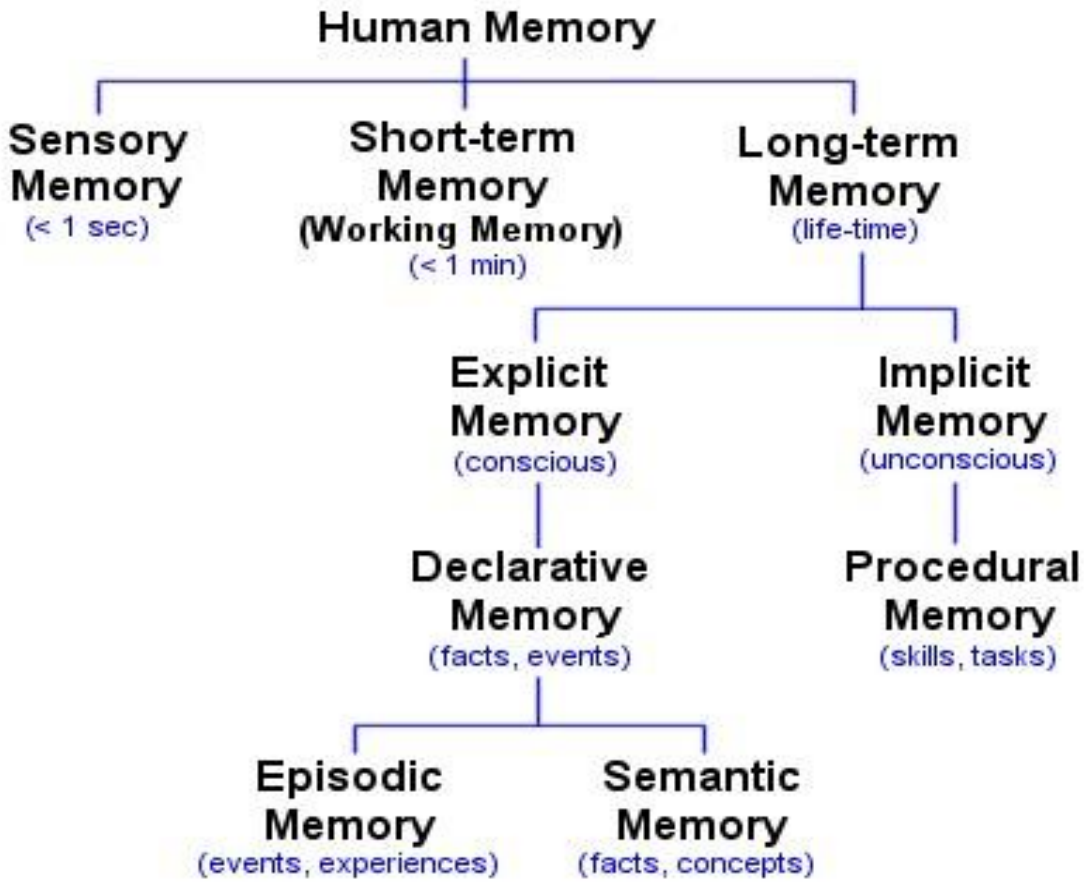


Figure 2.1: Types of Human Memory adopted from Luke Mastin, 2010.

2.3.1 Classification of Memory

2.3.1.1 Sensory Memory

Sensory memory is the shortest-term element of memory. It is the ability to retain sensory impressions of information after the original stimuli received via the five senses, have ended. It is very brief but accurate. It is regarded as ultra-short term memory and represents an essential step in storing information in short term memory (Luke Mastin, 2010).

2.2.1.2 Short Term (Working) Memory

Short-term memory implies a memory that lasts a fraction of a second to several seconds (Baddeley and Warrington, 1970). It is the component that does most of the mental work of the memory system, hence the name, working memory. It is the component of memory where attended-to information stays for a short while so that we can make better sense of it. Basically, it is also where much of our thinking, or cognitive processing, occurs. It is where we think about the content of a lecture, understand a textbook passage, or solve a problem. Information stored in working memory does not last long, perhaps five to twenty seconds at the most (Baddeley, 2001)

2.3.1.2 Long-term Memory

Long-term memory describes the ability to identify a stimulus for days, months, or years after exposure (Sherwin, 2003). It is classified into Implicit/Non-declarative memory and Explicit/Declarative memory.

Implicit/Non-declarative memory

This refers to information storage to perform various reflexive or perceptual tasks. It is referred to as non-declarative or implicit memory because it is recalled unconsciously. When we use implicit memory, we act automatically and we are not aware of recalling memory traces. Implicit memory is a heterogeneous collection of memory functions and types of learned behaviors such as reflexive learning (sensitization, habituation), classical conditioning, fear conditioning,

procedural memory (for skills and habits) and priming (the recall of words or objects from a previous unconscious exposure to them) (Okano *et al.,* 2000; Ganong, 2005).

Explicit/Declarative memory

This second form of memory is called declarative or explicit memory because it is recalled by a deliberate and conscious effort. It concerns factual knowledge of persons, things, notions and places. Declarative memory can be further classified as episodic or autobiographic memory and semantic memory (Ganong, 2005; Benfenati, 2007).

Episodic memory refers to the ability to recall in a deliberate manner recent episodes or events. It is often assessed by delayed recall tasks, such as the ability to recall items from a word list or narrative details from a paragraph story, tested after some delay of minutes or longer. Normal episodic memory performance depends particularly on integrity of the hippocampus and adjacent areas of the medial temporal lobes (Squire, 2009). It allows us to remember personal events and experience and, being a link between what we are and what we have been, gives us the sense of our individuality. On the other hand, semantic memory is a sort of public memory for facts and notions whether general or autobiographical. Often, over time, autobiographical memory shades into semantic memory so that the experience of an event is remembered as the simple occurrence of such event (Squire, 2009).

While explicit memory fades relatively rapidly in the absence of recall and refreshing, implicit memory is much more robust and may last for all our life even in the absence of further practice (Benfenati, 2007).

2.4.0 Hippocampus

The hippocampus is a major component of the brains of humans and other mammals, located in the medial temporal lobe, beneath the cortical surface. It belongs to the limbic system and plays important roles in long-term memory and spatial navigation. Similar to the cerebral cortex, with which it is closely associated, it is also a paired structure, with mirror-image halves in the left and right sides of the brain. The primary cells in the hippocampus are called pyramidal cells due to their shape (ZolaMorgan *et al*. 1986).

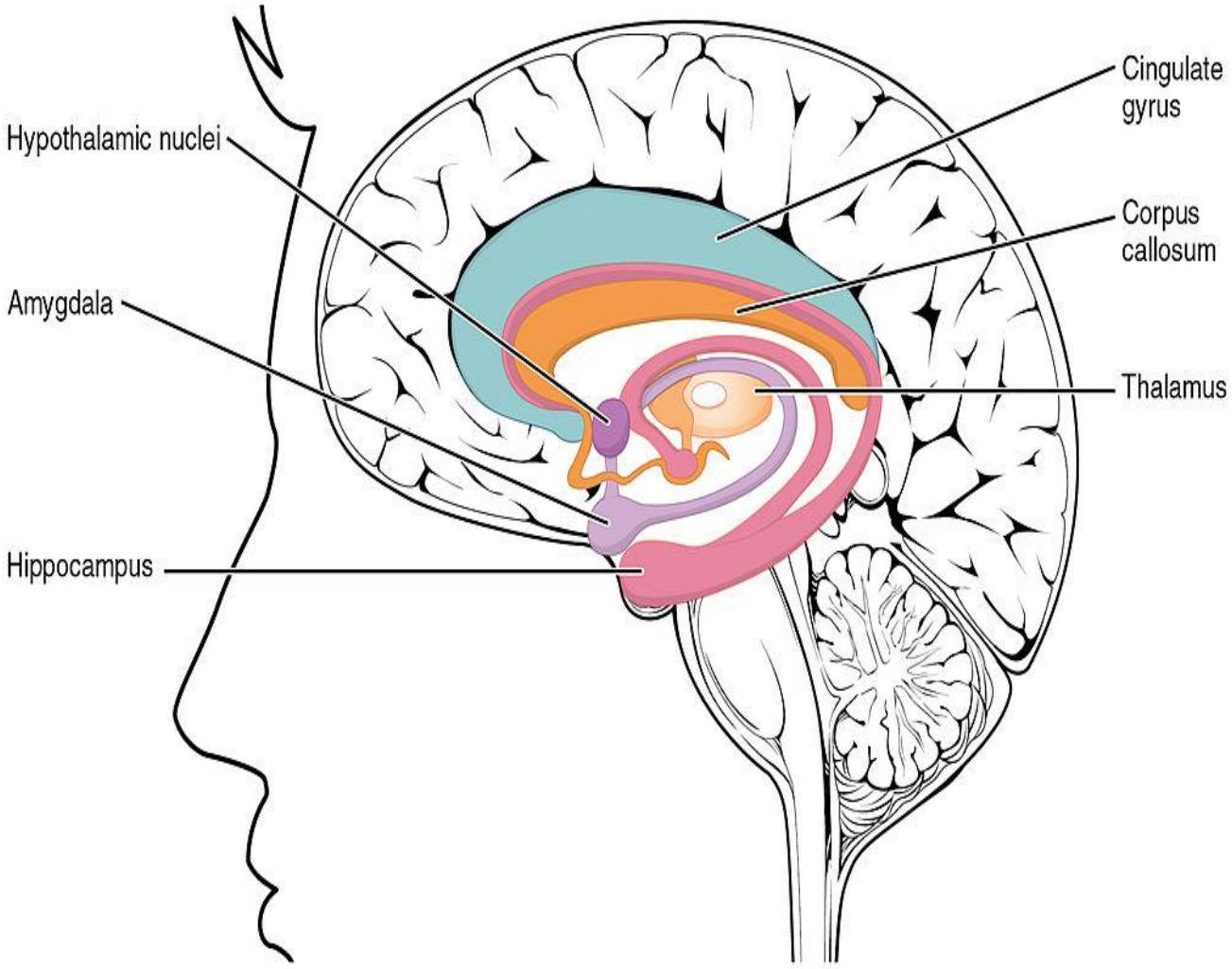


Figure 2.2: Anatomy of the brain adopted from Stockley *et al*., 1999

2.4.1 Intra-Hippocampal Connections and Flow of Information in the Hippocampus

The entorhinal cortex (EC) is the greatest source of hippocampal input as well as the target of hippocampal output. It is divided into medial (MEC) and lateral (LEC) areas and reciprocally connected with several parts of the cerebral cortex, and thereby serves as the basic 'interface'

between the hippocampus and other parts of the brain. Within the hippocampus, the flow of information is largely unidirectional, with signals propagating through a series of tightly packed cell layers, first to the dentate gyrus, then to the CA3 layer, then to the CA1 layer, then to the subiculum, then out of the hippocampus to the EC. Each of these layers also contains composite intrinsic circuitry and extensive longitudinal connections (Amaral and Lavenex, 2006). The EC has six well-defined layers; the neurons in layers II and III send major inputs to the hippocampus, DG, and the subiculum, and the neurons in layers V and VI receive feedback from CA1 and subiculum (Naber, 2001). The hippocampus also receives modulatory input from the serotonin, norepinephrine, and dopamine systems, and from nucleus reuniens of the thalamus. Important projection comes from the medial septal area, which sends cholinergic and GABAergic fibers to all parts of the hippocampus (Amaral and Lavenex, 2006).

2.4.2 Hippocampus and Memory Formation

Research has found that the hippocampus has an important role in the formation of new memories about experienced events (i.e. episodic or autobiographical memory) (Squire and Schacter, 2002). It is involved in the detection of novel events, places and stimuli (VanElzakker *et al*., 2008). Severe damage to the hippocampus results in great difficulties in forming new memories (anterograde amnesia), and this often also affects memories formed before the damage (retrograde amnesia). Although the retrograde effect normally extends some years before the brain damage, in some cases older memories are intact. The sparing of some of these older memories has led to the idea that consolidation over time involves the transfer of memories out of the hippocampus to other parts of the brain (Squire and Schacter, 2002).

However, damage to the hippocampus does not affect other types of memory, such as the ability to learn new motor or cognitive skills, for instance playing a musical instrument, or solving

certain types of puzzles (i.e. procedural memory). According to research, conscious recollection of information depends on the hippocampus while familiarity, depends on portions of the medial temporal cortex (Diana *et al*., 2007).

2.5 *Cannabis sativa*

2.5.1 Scientific classification

Kingdom: Plantae

Order: *Rosales*

Family: Cannabaceae

Genus: Cannabis

Species: C. sativa

Cannabis sativa is an annual herbaceous plant in the Cannabis genus, a species of the Cannabaceae family. People have been cultivating *Cannabis sativa* throughout recorded history as a source of industrial fibre, seed oil, food, recreation, religious and spiritual moods, as well as medicine. Each part of the plant is harvested differently, depending on the purpose of its use. The species was first classified by Carl Linnaeus in 1753 (Greg, 2005).

Cannabis sativa exerts its effects on the body by interacting with specific endogenous receptors, cannabinoid receptors 1 and 2 (CB1 and CB2) (AIHW, 2002). These receptors normally modulate neuronal activity by affecting second messenger and ion transport systems. CB1 receptors are found in the hippocampus, cerebral cortex, limbic areas, basal ganglia, cerebellum and thalamic areas, explaining the mental health effects of cannabis on endogenous cannabinoid receptor system in the human body (AIHW, 2002). There are three main forms of *Cannabis sativa* namely, marijuana, hashish and hash oil. Marijuana is the most common (Iversen, 2000).

Figure 2.3: *Cannabis sativa* leaves adopted from Andre *et al*., 2016.

When smoked approximately 50 percent of the THC is absorbed through the lungs and enters the bloodstream, from where THC reaches the brain within seconds; its effects are apparent within

minutes. Peak levels of THC occur within 10 minutes of smoking and decline to 5 to 10 percent of initial levels within an hour. When ingested, the amount of cannabis absorbed is 25 to 30 percent less than that of smoking the same amount due to the first-pass metabolism by the liver (Ashton, 2001). Therefore, the onset of the effects is delayed by about 30 minutes to two hours, but the duration of effects is prolonged (Iversen, 2000; Kumar *et al.*, 2001).

Few statistics on production of cannabis for narcotic purposes exist as the cultivation, possession, preservation; transportation and trade are illegal in most countries. Cannabis drugs (herbal and resin) are by far the most commonly consumed illegal drugs in the world and an estimated 161 million people used cannabis in 2003, of which 37 million in Africa. Average annual consumption is estimated at 200g of the herbal drug or 150g resin per user. In 2003 worldwide cannabis herb production was estimated to be more than 40,000 tonnes, of which 30,000 tonnes reached end users, valued at retail level at about US$ 113,000 million. Africa was a large producer (28%) with as major exporters Nigeria, South Africa, Malawi, Lesotho, Swaziland and Tanzania. However, Morocco remains the the world's largest producer of cannabis with 38,000tonnes being produced annually (UNODC 2017). The major importer is Europe, but the largest market is North America. Worldwide cannabis resin production is estimated at 7400tonnes, of which 6000tonnes reached end users, valued at retail level at about US$ 28,000 million, with northern Africa (Morocco 40%), Turkey and the Middle East (30%) as major suppliers (NIDA, 2015).

Major importers are Europe (78%), followed by Africa (9%) and Asia (8%). In India cannabis is cultivated for 'ganja' by a few licensed growers, and the drug is a monopoly of the Indian Government. In 2004 world production of hemp fibre and tow was 66,000tonnes from 50,000 hectares, of which 40,000 tonnes was produced in Asia, mainly in China and Korea. World

production of cannabis fruits in 2004 was about 30,000tonnes, of which 24,000tonnes was in China (Gaby *et al*., 2008).

2.5.2 Pharmacology of *Cannabis sativa*

Cannabis is very complex in its chemistry due to the vast number of its constituents and their possible interaction with one another. These constituents represent almost all of the chemical classes, for example; sesquiterpenes, sugars, hydrocarbons, steroids, flavonoids, nitrogenous compounds and amino acids, among others (Elsholy and Slade, 2005).

About 489 natural compounds have been identified, of which 70 are known as cannabinoids which are C_{21} terpenophenolic compounds. They are the best-known and the most specific class of Cannabis constituents with delta-9 –trans-tetrahydrocannabinol (Δ9 -THC) being the most psychoactive constituent (Elsholy and Slade, 2005; Aizpurua-Olaizola *et al*., 2016).

The cannabinoids are further classified into 11 categories (Kudus *et al*., 2013);

- Cannabigerol type (7),
- Cannabichromene type (5),
- Cannabidiol type (7),
- Δ9-trans-Tetrahydrocannabinol type (9),
- Δ8-trans- Tetrahydrocannabinol type (2),
- Cannabicyclol type (3),
- Cannabielsoin type (5),
- Cannabinol type (7),
- Cannabinodiol type (2)
- Cannabitriol type (9)
- Cannabitriol type (9)

Besides THC, another cannabinoid produced in high concentrations by some plants is cannabidiol (CBD), which is not psychoactive but has recently been shown to block the effect of THC in the nervous system. Differences in the chemical composition of Cannabis varieties may produce different effects in humans (Russo, 2011).

2.5.3 Metabolism of *Cannabis sativa*

Metabolism is via the hepatic cytochrome P_{450} (CYP) system. Delta-9 THC is metabolized into an active compound, 11-hydroxy-THC (11-OH-THC), which is further metabolized into another inactive form, (8-11-DiOH-THC). The half-life ranges from 2-57 hours following intravenous use and inhalation. The half-life of 11-OH-THC, the active metabolite, is 12-36 hours (*Russo et al*., 2016). The long life of this active metabolite is explained by the incorporation of the compound in lipid storage depots and similar storage sites in muscle tissue (Genen *et al.*, 2017). About 60% of THC, in all forms, is excreted in feces; the remaining amount is excreted in urine (Russo *et al.*, 2016).

2.5.4 Common uses of *Cannabis sativa*

Its seeds are chiefly used to make hempseed oil which can be used for cooking, lamps, lacquers, or paints. They can also be used as caged-bird feed, as they provide a moderate source of nutrients for most birds. The flowers (and to a lesser extent the leaves, stems, and seeds) contain psychoactive chemical compounds known as cannabinoids that are consumed for recreational, medicinal, and spiritual purposes (Greg, 2005). Preparations of flowers (marijuana), leaves and preparations derived from resinous extract (e.g., hashish) are consumed by smoking, vaporizing and oral ingestion. Historically, tinctures, teas, and ointments have also been common preparations. In traditional medicine of India in particular C. sativa has been used as hallucinogenic, hypnotic, sedative, analgesic, and anti-inflammatory agent (Wang *et al*., 2014).

2.5.5 Effects of *Cannabis sativa* on the body

2.5.5.1 Neurological Effects

The psychoactive substance in cannabis, delta-9-tetrahydrocannabinol (THC), stimulates cannabinoid receptors (CBRs), located on the surface of neurons, to produce the psychoactive effects. THC mimics anandamide, binding with the CBRs, but the effects of THC are more potent and longer acting than the endogenous ligands. Animal studies have indicated that THC exposure increases the release of noradrenaline, causing anxiety like behavior in rodents. The rewarding effects of cannabis may be due to an increase of serotonin, while GABA is responsible for memory deficits including the inability to form new memories, promoted by. The after effects of THC can last up to 28 days period of abstinence from the drug (Hall and Degenhard, 2009). Chronic abusers have also been found to have reduced volumes of the hippocampus and amygdala (Krista *et al.*, 2007).

2.5.5.2 Cardiovascular Effects

Delta-9 tetrahydrocannabinol (THC) increase heart rate, peripheral vasodilation, postural hypotension; which may lead to dizziness or syncope and elevation in both systolic and diastolic blood pressures in supine position. Cardiac output increases, and peripheral vascular resistance and maximum exercise performance decrease (Jones 2002). Tachycardia is believed to be as a result of increased sympathetic nervous system activity after marijuana use. However, tolerance to most of the initial cardiovascular effects appears rapidly. With repeated exposure, supine blood pressure decreases slightly, orthostatic hypotension disappears, blood volume increases, heart rate slows, and circulatory responses to exercise are diminished, consistent with centrally mediated, reduced sympathetic, and enhanced parasympathetic activity. Receptor-mediated and probably non-neuronal sites of action account for cannabinoid effects. The endocannabinoid

system appears important in the modulation of many vascular functions (Jones 2002). Cardiac risk factors are lowest among young, healthy users as compared to adults and people well advanced in age, although occasional myocardial infarction, stroke, and other adverse cardiovascular events are reported in young users (Afshar *et al*, 2017). Marijuana smoking by people with cardiovascular disease poses health risks because of the consequences of the resulting increased cardiac work, increased catecholamine levels, carboxyhemoglobin, and postural hypotension (Jones 2002).

2.5.5.3 Respiratory Effects

Regular marijuana smoking leads to bronchial epithelial ciliary loss, impairs the microbicidal function of alveolar macrophages and causes visible and microscopic injury to the large airways that is consistently associated with an increased likelihood of symptoms of chronic bronchitis .Several case reports have implicated marijuana smoking as an etiologic factor in pneumothorax/pneumomediastinum and bullous lung disease. However, habitual use of marijuana alone does not appear to lead to significant abnormalities in lung function, except for possible increases in lung volumes and modest increases in airway resistance of unclear clinical significance. Therefore, no relationship to chronic obstructive pulmonary disease has been established. Although marijuana smoke contains a number of carcinogens, there is paucity of information on increased risk for the development of cancer from moderate use, although evidence is mixed concerning possible carcinogenic risks of heavy, long-term use (Tashkin, 2013).

2.5.5.4 Effects on the Immune System

The cannabinoid system has been linked to immunosuppressive effects attributed to delta-9-tetrahydrocannabinol (THC), the major psychoactive component of cannabis sativa. Both in-vivo and in-vitro studies have revealed that THC affects cell-mediated immunity, humoral immunity and cellular defences against infectious agents. Cannabinoids affects the immune systems by altering functional capabilities of immunocytes (Cabral, 2001).

2.5.6 Pathophysiology of *Cannabis sativa*

The cannabinoids can activate their receptors; cannabinoid receptor 1 (CB1) and CB2 receptor. However, about 60 additional substances have been isolated from cannabis that does not activate the cannabinoid receptors. Delta-9-tetrahydrocannabinol (THC) serves as a neuromodulator by potently activating the G-protein–coupled cannabinoid receptors CB1 and CB2 (Burstein and Zurier, 2009; Gertsch *et al*., 2010).

The CB1 receptors are predominant and widely distributed in the brain. They are densely concentrated at the frontal cerebral cortex (higher functioning), hippocampus (memory, cognition), basal ganglion and cerebellum (movement), and striatum (brain reward). They are also found in regions responsible for anxiety, pain, sensory perception, motor coordination, and endocrine function. The CB2 receptor, on the other hand, is found at the periphery, specifically, the immune system (splenic macrophages, T and B lymphocytes), peripheral nerves, and the vas deferens.

Both the CB1 and CB2 receptors inhibit adenylate cyclase and stimulate potassium channels. As a result, the CB1 receptors inhibit the release of several neurotransmitters, including acetylcholine, glutamate, norepinephrine, dopamine, serotonin, and gamma–aminobutyric acid (GABA). CB2 receptor signaling is involved in immune and inflammatory reactions.

2.6 Alcohol

Ethanol (Ethyl alcohol), is an intoxicating agent found in beer, wine, and liquor. It is produced by the fermentation of yeast, sugars, and starches. It is a central nervous system depressant that is rapidly absorbed from the stomach and small intestine into the bloodstream. A standard drink equals 0.6 ounces of pure ethanol, or 12 ounces of beer; 8 ounces of malt liquor; 5 ounces of wine; or 1.5 ounces (a 'shot') of 80-proof distilled spirits or liquor (e.g., gin, vodka, or whiskey) (NIDA, 2015).

Alcoholism or alcohol dependence is a diagnosable disease characterized by a strong craving for alcohol, with continued use despite harm or injury. Alcohol abuse is a pattern of drinking that result in harm to one's health, interpersonal relationships, or ability to work (NIDA, 2015).

Alcohol is a multiple-action depressor of the Central Nervous System, and the depression caused by it is dose-dependent. Although alcohol is mainly used because of its stimulating action, this action is only apparent and happens only with moderate doses. It results from the depression of inhibitory controlling mechanisms. Under the effect of alcohol, the cortex is freed from its integrative role, thus resulting in confused and disorganized thinking, as well as disruption of adequate motor control (Martin *et* al., 2003).

Ethanol diffuses through lipids, modifying proteins fluidity and functions. High concentrations of ethanol can decrease the eletron-transporting functions of the $Na^{+}K^{+}$/ATPase pump, thus impairing electrical conduction in the nerve cells (Chandler *et al*., 1998).

2.6.1 Absorbtion/Metabolism of Alcohol

Ethanol is a small two carbon alcohol that, due to its small size and alcoholic hydroxyl group is soluble in both aqueous and lipid environments. This allows ethanol to freely pass from bodily fluids into cells. Since the portal circulation from the gut passes first through the liver, the bulk

of ingested alcohol is metabolized in the liver. The process of ethanol oxidation involves at least three distinct enzymatic pathways. The most significant pathway, responsible for the bulk of ethanol metabolism, is that initiated by alcohol dehydrogenase, ADH. ADH is an NAD^+-requiring enzyme expressed at high concentrations in hepatocytes (Arthur, 2012). Animal cells (primarily hepatocytes) contain cytosolic ADH which oxidizes ethanol to acetaldehyde. Acetaldehyde then enters the mitochondria where it is oxidized to acetate by mitochondrial aldehyde dehydrogenase (ALDH). A cytosolic ALDH exists but is responsible for only a minor amount of acetaldehyde oxidation. The second major pathway for ethanol metabolism is the microsomal ethanol oxidizing system (MEOS) which involves the cytochrome P450 enzyme CYP2E1 and requires NADPH instead of NAD^+ as for ADH. The MEOS pathway is induced in individuals who chronically consume alcohol. The third pathway involves a non-oxidative pathway catalyzed by fatty acid ethyl ester (FAEE) synthase (Chales, 2004). This latter pathway results in the formation of fatty acid ethyl esters and takes place primarily in the liver and pancreas, both of which are highly susceptible to the toxic effects of alcohol. Oxidation of ethanol can also occur in peroxisomes via the activity of catalase. However, this oxidation pathway requires the presence of a hydrogen peroxide (H_2O_2) generating system and as such plays no major role in alcohol metabolism under normal physiological conditions (Arthur, 2012).

2.6.2 Alcohol's effects on the body

2.6.2.1 Short term effects

The cell membrane is highly permeable to alcohol, therefore, once alcohol gets into the blood stream it diffuses nearly into every cell in the body leading to short term effects such as

anterograde amnesia, decrease in anxiety and motor skills and even unconsciousness. The blood alcohol content (BAC), i.e the concentration of alcohol in the blood, determines the extent of intoxication. Extreme consumption of alcohol exacerbates sleep disorders, can lead ashyxation from vomit, alcohol poisoning and death (Feige *et al.,* 2007).

2.6.2.2. Long term effects

Chronic alcohol consumption can damage nearly every organ and system in the body such as a higher detrimental rate of cardiovascular disease (NIAAA, 2000), damage to the central nervous system and peripheral nervous system (Testino, 2008). Large levels of alcohol intake can increase the risk of alcoholism, malnutrition, chronic pancreatitis, liver cirhosis and cancer. A developing fetus may Suffer Fetal Alcohol Spectrum Disorders (FASDs), if the mother consumed alcohol while pregnant (Guerri and Pascal, 2010).

2.7 Drug Abuse

Drug misuse is a term used commonly when prescription medication with sedative, anxiolytic, analgesic, or stimulant properties are used for mood alteration or intoxication ignoring the fact that overdose of such medicines have serious adverse effects. It often involves drug diversion from the individual for whom it was prescribed. Prescription misuse has been defined differently and rather inconsistently based on status of drug prescription, the uses without a prescription, intentional use to achieve intoxicating effects, route of administration, co-ingestion with alcohol, and the presence or absence of dependence symptoms (McCabe *et al.*, 2012). Chronic use leads to a change in the central nervous system which means the patient has developed tolerance to the medicine that more of the substance is needed in order to produce desired effects. When this happens, any effort to stop or reduce the use of this substance would cause withdrawal symptoms to occur. The rate of prescription drug abuse is fast overtaking illegal drug abuse in the United

States. According to the National Institute of Drug Abuse, 7 million people were taking prescription drugs for nonmedical use in 2010. Among 12th graders, prescription drug misuse is now second only to cannabis (Nathaniel *et al.*, 2013).

2.7.1 Epidemiology

The initiation of drug and alcohol use is most likely to occur during adolescence, and some experimentation with substances by older adolescents is common. For example, results from 2010 Monitoring the Future survey, a nationwide study on rates of substance use in the United States, show that 48.2% of 12th graders report having used an illicit drug at some point in their lives. In the 30 days prior to the survey, 41.2% of 12th graders had consumed alcohol and 19.2% of 12th graders had smoked tobacco cigarettes. In 2009 in the United States about 21% of high school students have taken prescription drugs without a prescription (Johnston, 2011). Earlier in 2002, the World Health Organization estimated that around 140 million people were alcohol dependent and another 400 million with alcohol-related problems (Rudd *et al*., 2016).

2.7.2 Endogenous Cannabinoid system and Memory

The endogenous cannabinoid (eCB) system is a communication network that plays an important role in memory. The eCB system, is composed of its endogenous ligands, anandamide and 2-arachidonoyl-glycerol (2-AG), usually synthesized on demand through cleavage of membrane precursors (Piomelli, 2003), and its receptors, cannabinoid receptor-1 and cannabinoid receptor-2(CB1-R and CB2-R). The CB1 receptors are the most abundant metabotropic receptors in the brain, located on presynaptic terminals in regions involved in cognition, especially learning and memory, specifically in hippocampus, prefrontal cortex, basal ganglia and cerebellum (NIDA, 2010). The eCBs mediate the flow of information in the brain through retrograde signaling, modulating inhibitory and excitatory neurotransmitter release crucial for synaptic plasticity, long

term potentiation, and hence learning, memory (Piomelli, 2003; Howlett *et al*., 2004 and Alger, 2005). Research has demonstrated alterations in the functioning of the brain in CB1 rich regions and in cognitively-relevant neuromodulator systems (e.g., dopaminergic, cholinergic, serotonergic, GABAergic, glutamatergic) as a result of exposure to cannabinoids (Iversen, 2003). Alterations in the functionality of the eCB system, such as receptor downregulation, desensitization and downstream effector changes accompanying the development of tolerance, dependence and resultant regional neuro-adaptations, occur following chronic administration of cannabinoids (Sim-Selley, 2003).

2.7.3 Alcohol and the Brain

Alcohol is a threat to global health, accounting for 4% of the global health burden, a proportion that is comparable to tobacco and hypertension (Rehm *et al*., 2009). Dysfunctions of multiple organs brought on by chronic alcohol use have long been the focus of medical concern, and are well documented in the medical literature. Nevertheless, alcohol continues to be a part of human culture. For a long time, the effect of alcohol was thought to be a generalized depression of neural activity causing global impairment of cognitive, psychological, and behavioral domains (White *et al*., 2000; White *et al*., 2004). However, the blackout, characterized by amnesia during episodes of intoxication where the subject is conscious and able to carry on conversations or even drive a vehicle (Goodwin, 1995, Jennison and Johnson, 1994), is a manifestation of the selective effects of alcohol on specific brain systems. Ethanol, a short chain lipid soluble compound, was previously thought to affect cells by a nonspecific lipid membrane disordering effect (Matthew and Silver, 2004). Alcohol interacts with specific neurotransmitter receptors, and the current consensus is that specific regions of the brain are selectively vulnerable to acute effects of alcohol (Oscar-Berman and Marinkovic, 2003). Progress has been made in elucidating

the mechanism of various memory systems and how they are affected by alcohol. The molecular mechanisms of the effects of alcohol on the hippocampus are not clear. However, one leading candidate for a cellular substrate of memory formation is long-term potentiation (LTP), which is the establishment of long lasting heightened responsiveness to signals from other cells (Schummers and Browning, 2001). Alcohol inhibits establishment of LTP by potently antagonizing N-Methyl-D-Aspartate (NMDA) receptor activity (White and Best, 2000). The NMDA receptor is necessary for LTP induction in area CA1 of the hippocampus. Alcohol's effect on LTP in area CA1 of the hippocampus is thought to involve both inhibition of the NMDA receptor and potentiation of the γ-Aminobutyric Acid A (GABAA) receptor transmission, which leads indirectly to further NMDA receptor inhibition (White, 2004).

2.7.4 Alcohol and the Hippocampus

The primary cells in the hippocampus are called pyramidal cells. The hippocampus can be divided into several areas, and studies in humans have found that in some patients with an inability to form new explicit memories, brain damage was limited to a single region of hippocampal neurons called the CA1 region (ZolaMorgan *et al*. 1986). In rodents, the activity of CA1 cells correlates strikingly with behavior: Each CA1 neuron tends to emit signals primarily when the animal is in a specific area of its environment. For example, cell A may be active predominantly when the animal is in the northeast corner of its cage, whereas cell B may become active when the animal enters the southwest corner of the cage. As a result, these cells can play a very strong and specific role in spatial learning (e.g., the ability to learn the path through a maze or the location of a certain item, such as a food reward) (ZolaMorgan *et al*. 1986).

Researchers have used this characteristic of the CA1 cells to assess the effects of alcohol exposure and other interventions on hippocampal cell activity in intact, living rodents. In one

study, electrodes were implanted in the hippocampus of rats and allowed to move freely around their cages. After the animals were administered alcohol, the activities of their CA1 cells were measured. This study found that the activity of the CA1 cells was reduced when alcohol levels reached at least 0.5 grams per kilogram (g/kg) of body weight and ceased almost completely at higher alcohol doses (White and Best 2000). This finding is consistent with the hypothesis that alcohol can interfere with the formation of new explicit memories by disrupting hippocampal function.

2.7.5 Alcohol's Effects on Long Term Potentiation (LTP)

Alcohol impairs other hippocampal functions. It affects a process called Long Term Potentiation (LTP). This is an experimentally induced adaptation of the nerve cell connections in response to repeated activation. To illustrate this, imagine two neurons in the hippocampus, a CA1 neuron and a neuron from a region called CA3: that connect in the hippocampus, with the CA3 neuron sending signals to the CA1 neuron. To transmit the signals, the CA3 neuron releases a neurotransmitter, which then interacts with receptors on the surface of the CA1 neuron, resulting in the formation of a new nerve signal in the CA1 neuron (White *et al*., 2004).

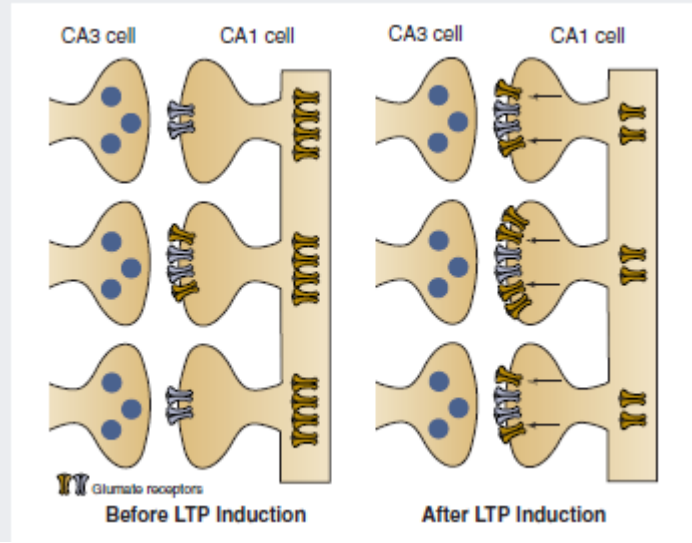


Figure 2.4: Schematic representation of the long term potentiation (LTP) process. Adopted from Sussanne and Scott, 2005

When a hippocampal CA3 cell is initially stimulated, it releases the neurotransmitter glutamate, which binds to NMDA receptors on a CA1 cell and induces a response of a certain size (baseline response). The intensity of this signal depends on various factors, including the number of receptors on the CA1 neuron. When the CA3 neuron first is exposed to a stimulus, it will emit a signal that leads to a certain level of response in the CA1 neuron. This is called the baseline response. The CA3 neuron then can be stimulated experimentally in a specific pattern, a process that resembles what happens during actual learning events. If the original stimulus subsequently

is reapplied to the CA3 neuron, it will evoke a response in the CA1 neuron that is substantially greater than the response that occurred after the initial stimulation (i.e., the response is potentiated). In other words, as the result of the patterned stimulation, the CA1 cell becomes more responsive to signals emitted by the CA3 cell. This potentiated response often persists for a long period of time, hence the name "long-term" potentiation. There is accumulating evidence that something like LTP occurs naturally during learning and memory formation. Alcohol has been shown to interfere with LTP during experiments using hippocampal brain slices from rats. In these experiments, alcohol concentrations corresponding to those achieved in humans after consuming only one or two drinks interfered with the establishment of LTP (Blitzer *et al*. 1990). The brain slices were kept in a special fluid, and two electrodes were introduced into the tissue, one that allowed stimulation of the CA3 cells and one that recorded the responses of the CA1 cells. If sufficient alcohol was present in the surrounding fluid during the repeated patterned stimulation of the CA3 cells, LTP was not detected in the CA1 cells, that is, their response remained at the baseline level. However, adding alcohol to the fluid after the patterned stimulation had no effect on LTP, which is consistent with the observation that alcohol consumption does not impair recall of previously established memories (White *et al.*, 2004). In addition to impairing balance, motor coordination, and decision making; alcohol interferes with the ability to form memories. However, it does not impair all types of memory equally. Alcohol interferes more with a person's ability to form new, lasting memories much more than it interferes with the ability to recall previously established memories or to hold new data in memory for just a few seconds (White *et al*., 2004). Alcohol particularly affects the ability to form explicit memories (Sussanne and Scott, 2005).

One neurotransmitter system involved in the establishment of LTP is the excitatory neurotransmitter glutamate and its NMDA receptor. When this receptor is activated by glutamate, it allows calcium to enter the cells. Repeated calcium influx, in turn, sets off a chain reaction leading to long lasting changes in the structure and/or function of the cells that cause LTP. Alcohol has been shown to interfere with activation of the NMDA receptor, thereby reducing calcium influx and, thus, the subsequent changes in cell function that result in LTP. Researchers think that this is the main mechanism through which alcohol prevents establishment of LTP, although other neurotransmitter systems also may play a role (White *et al.*, 2004).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Animals

Forty-eight (48) apparently healthy adult mice, weigh between 20 to 28g, were purchased from the Animal House of the Faculty of Pharmaceutical Sciences, A.B.U. Zaria. The animals were

housed in the Department of Human Physiology, Faculty of Medicine, Ahmadu Bello University Zaria, where they were preconditioned for two weeks prior to commencement of the experiment. They were housed in cages containing dust-free sawdust bedding and were allowed free access to pellets made from grower's mash and water *ad libitum*.

3.1.2 *Cannabis sativa*

Cannabis sativa was obtained from the National Drug Law Enforcement Agency (NDLEA) office at Kaduna and was identified by Mallam Namadi Sunusi (Botanist), Department of Biological Sciences Ahmadu Bello University, Zaria and voucher number of 2438 was assigned to it. It was then taken to the Department of Pharmacognosy, Ahmadu Bello University, Zaria where it was extracted and fractionated into four fractions. The ethyl acetate fraction with the highest concentration of delta-9 tetrahydrocannabinol (a terpenoid) was used for the experiment.

3.1.3 Ethanol

Absolute Ethanol was purchased from Steve Moore Chemical Stores, Samaru, Zaria. It was diluted to 20% for the experiment.

The 20% of the ethanol solution was prepared by mixing 20ml of absolute ethanol with 80ml of distilled water. Volume administered was based on body weight of the animals (Nagy, 2008)

3.2 METHODS

3.2.1 Experimental Groupings

The animals were grouped into four groups. Each group comprised of six mice each. According to the reported oral LD_{50} of *Cannabis sativa* in mice (482mg/kg) (Philips *et al*., 1971), doses were administered based on body weight of the animals via the oral route for 21days.

Group I: Animals received distilled water (control group) (1ml/oral route/21days).

Group II: Animals received 30% LD_{50} (145 mg/kg/oral route/21days *Cannabis sativa* extract), (Philips *et al*., 1971).

Group III: Animals received 0.18 mg/kg bwt of 20% ethanol (etOH) (oral route/21days), (Nagy, 2008).

Group IV: Animals received 0.18 mg/kg bwt of 20% etOH +145 mg/kg *Cannabis sativa* extract (oral route/21days)

3.2.2 Neurobehavioural Assessments

3.2.2.1 Y- maze

The Y-maze discriminates learning, spatial reference memory and spatial working memory which, are related to the hippocampus and prefrontal brain regions in rodents (Xu *et al*., 2013). The evaluation of spontaneous alternation was used to investigate short term spatial working memory in mice.

The Y−maze apparatus was made of black PVC, consisting of three equal arms (length 50cm, height 20cm, and width 10cm), interconnected at 120 degrees.

The test included two sessions; the first session measures working memory in mice by scoring the number of alternations the mouse does in Y-maze when the animal visits all three arms without going into the same arm twice in a row. The animals were habituated for three days. Each mouse was allowed to explore the apparatus for 5minutes and then the apparatus was cleaned with 5% ethanol before the next mouse was introduced.

The sequence of arm entries is manually recorded, the arms being labelled A, B, or C. An alternation is defined as entry into all three arms consecutively (i.e. ACB, ACB, BAC, etc). a mouse was considered inside a specific arm when it had all the four paws inside that arm. The number of maximum spontaneous alternations is then the total number of arms entered minus

two, and the percentage alternation is calculated as {(actual alternations /maximum alternations) x 100}. For instance, if a mouse performed CABBCACBACCBACB, the number of arm entries would be 15-2=13, and spontaneous alternations: CAB, BCA, ACB, CBA, BAC, CBA, BAC, ACB. Therefore the percentage alternation was calculated as [8/(13)]x100= 61.5%] (Olakunle *et al*., 2012).

3.2.2.2 The Novel Object Recognition Test (NORT)

The NOR task evaluates the rodent's ability to recognize a novel object in the environment. This test accesses the natural preference for novel objects displayed by rodents (Antunes and Biala, 2012). The test was conducted in the open field box (72 x 72 cm). All animal test was conducted under dim lighting conditions via a 60-Watt red light bulb. The task procedure consisted of three phases; habituation, familiarization and test phase. In the habituation phase, the animals were habituated by allowing each animal explore freely the open field arena in the absence of objects. The animal was then removed from the arena and placed in holding cage. During the familiarization phase, a single animal was placed in the open-field arena containing two identical sample objects (A+A), for 5 minutes. To prevent coercion to explore the objects, the animal was released against the center of the opposite wall with its back to the objects. After a retention interval, during the test phase, after 60seconds, the animal is returned to the open-field arena with two objects, one was identical to the sample and the other is novel (A+B) for 5minuites. The new object was similar in size but different to the familiar object in order to reduce preference for either object. All objects and the apparatus were cleaned using 70% alcohol to eliminate olfactory stimuli (Mathiasen, 2010).

Difference $\mathbf{(D_1) = T_n - T_f}$

Where $\mathbf{T_n}$ = Time (s) spent with new object.

$\mathbf{T_f}$ = Time (s) spent with familiar object

The difference D_1 is the first measure for habituation. It is the difference in exploration time for novel versus familiar object as shown in the formula above (Mathiasen, 2010).

Discriminatory Index (DI)

This is the second index measured. It quantifies discrimination between the novel object and familiar objects. It is computed as follows:

$$\mathbf{DI = T_n - T_f \div T_n + T_f}$$

T_n and T_f are same as above.

Recognition Index (RI)

This s the percent of time spent exploring the novel object relative to the total time spent exploring both objects. This is the measure of novel object recognition. It is the main index of retention. It is calculated thus:

$$\mathbf{RI = T_n \div T_n + T_f}$$

Where T_n and T_f are same as above.

3.3.3 Histological Procedure

Brain tissue was removed from the skull of the mice under light anesthesia in a Chloroform enclosed Chamber) and was fixed in Bouin's fluid.

Fixed tissues were processed and stained with routine histology stain (H and E) for the general architecture of the hippocampus and a special stain, Cresyl fast violet for nuclear changes (Shafri *et al*., 2012).

3.3.4 Stereological Estimation of Volume of Hippocampus in Mice.

3.3.4.1 Sampling

Isotropic uniform random (IUR) samples were obtained by the orientator method. Hippocampus region from each cerebral hemisphere were obtained by dissection. First each hippocampal tissue was placed at the center of a circle with equal divisions. A random number 2 was selected from the random number table and samples were cut along the number 2. Each cut sample tissue was again placed on a second circle with unequal divisions. Another random number was chosen from the random number table (6 was chosen) and samples were cut again into equal divisions (Gundersen *et al*., 1998).

Tissues were then processed and embedded in paraffin wax. Serial Sections were cut with a rotatory microtome at 10µ (Leica). A random number 5 was selected from the random number table and sections were randomly picked (1, 6, 11, 16,21, 26, 31, 36, 41, 46) and stained with H and E. Photomicrographs were taken with a light microscope (Leitz Wetzlar) and a digital microscope camera (DCM 510) at ×250.

A test point counting grid (Cavalieri estimator) was superimposed on hippocampal tissue sections and single test points hitting the different parts of the hippocampus (CA1, CA2, CA3, Subiculum) were counted and summed. Hippocampal volumes were calculated from the formula $V\ (mm^3) = \square \times a/p \times \sum Pi$ after imputation as shown in the following table 1 below. ($\square$ = distance from the 1st section to the 46th section (50mm); a/p = area per point on the counting grid= $1mm^2$; ∑Pi = sum of test point (Gundersen *et al*., 1998).

3.3.5 Statistical analysis

Statistical analysis was carried out using Statistical Package for Social Scientists version 20). Data obtained from Y-maze test were analysed using Kruskal Wallis and Mann-Whitney U test and the results expressed as Mean ranks. Data from the other tests were analysed using ANOVA

and Tukey's post-hoc test. Values obtained were expressed as Mean ± SEM. Values with $P \leq 0.05$ were considered significant.

CHAPTER FOUR

4.0 RESULTS

4.1 Effect of alcohol, cannabis and co-administration of alcohol and cannabis on Percentage Alternation in mice.

The percentage alternation (fig 4.1) showed a statistically significant difference ($P < 0.05$) in the cannabis treated and alcohol + cannabis treated groups compared to the control; (48.52 vs 62.57) % and (79.44 vs 62.57) % respectively. Percentage alternation decreased significantly in cannabis treated group compared to control (65.24 vs 48.52) %. There was also a significant increase ($P < 0.05$) observed in alcohol + cannabis treated group compared to the control (79.44 vs 48.52) %.

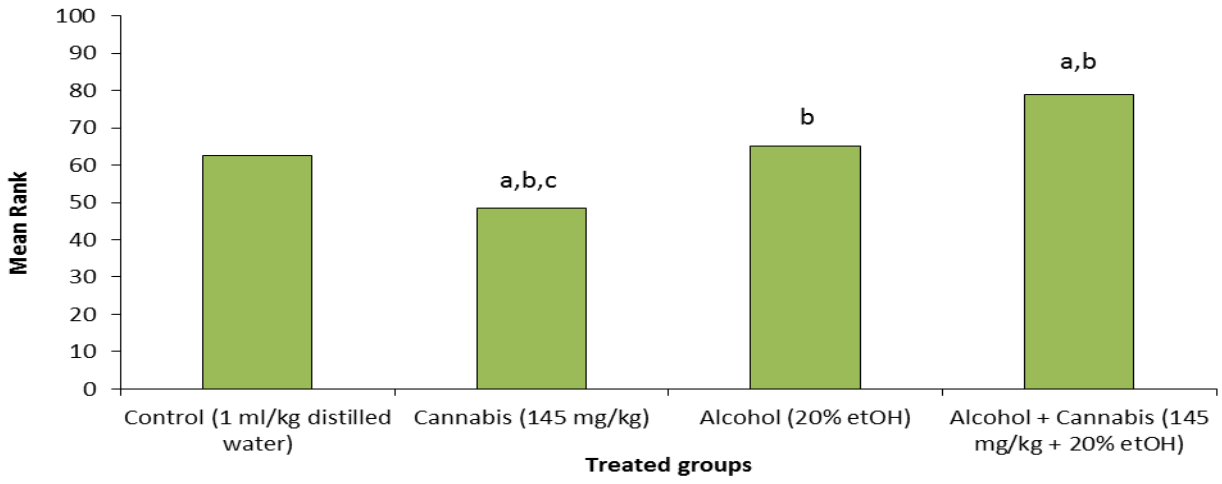

100
90
80
70
60
50
40
30
20
10
0
Mean Rank
a,b,c
b
a,b
Control (1 ml/kg distilled water)
Cannabis (145 mg/kg)
Alcohol (20% etOH)
Alcohol + Cannabis (145 mg/kg + 20% etOH)
Treated groups

Figure 4.1: Showing mean rank of percentage alternation in mice treated as follows: Group I; Control, Group II; Cannabis 145 mg/kg, Group III; Alcohol 20% b/w and Group IV; (Alcohol + Cannabis) for 21 days. Superscripts (a), (b), (c) and (d) indicate statistical significance compared to Groups I, II, III and IV respectively ($p \leq 0.05$)

4.2 Effect of alcohol, cannabis and co-administration of alcohol and cannabis on Discrimination Index (DI)

The result of alcohol, cannabis and the co-administration of alcohol and cannabis on Discrimination index (DI) (fig 4.2) showed a statistically significant ($P<0.05$) decrease in DI in the groups treated with co-administration of alcohol and cannabis compared to the control; (-0.09±0.21 vs 0.35±0.64) respectively. This group also showed a significant decrease in DI when compared to the groups treated with cannabis and alcohol alone; (-0.09±0.21 vs 0.35±0.64) and (-0.09±0.21 vs 0.43±0.09) respectively.

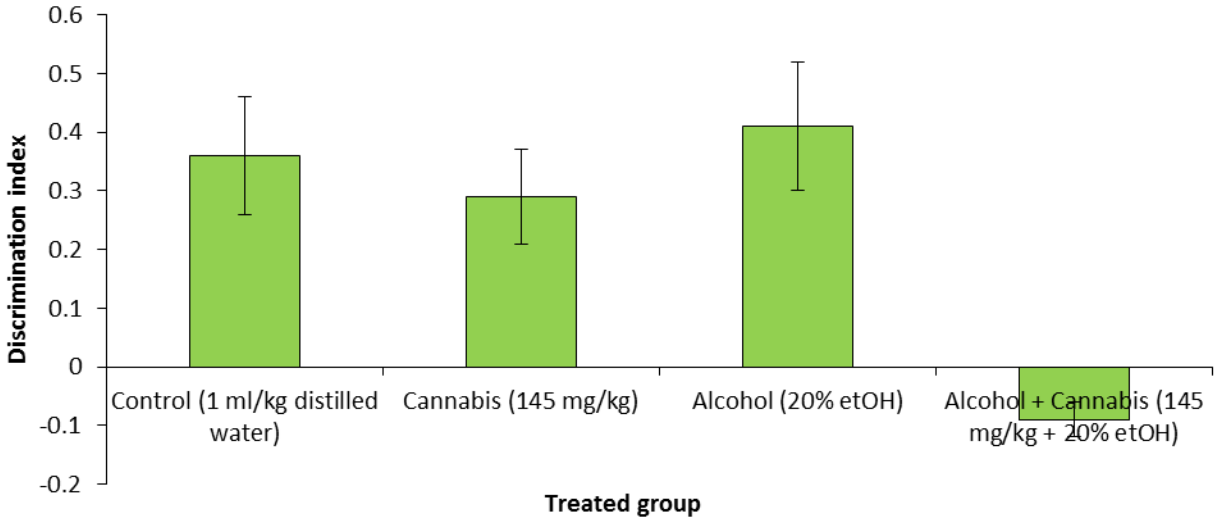


Figure 4.2: Showing discrimination index in mice treated as follows: Group I; Control, Group II; Cannabis 145 mg/kg, Group III; Alcohol 20% b/w and Group IV; (Alcohol + Cannabis) for 21 days. Superscripts (a), (b), (c) and (d) indicate statistical significance compared to Groups I, II, III and IV respectively ($p \leq 0.05$)

4.3 Effect of alcohol, cannabis and co-administration of alcohol and cannabis on Recognition index (RI)

Figure 4.3 showed a statistically significant decrease in recognition index (RI) ($P< 0.05$) in the group treated with alcohol and cannabis compared to the control; (0.46±0.10 vs 0.68±0.05). Although there was a decrease in the other treated groups compared to the control, it was however not statistically significant ($P> 0.05$). Groups treated with cannabis and alcohol separately showed a non-statistically significant increase ($P> 0.05$) compared to alcohol + cannabis treated group; 0.71±0.05 vs 0.46±0.10 and 0.67±0.10 vs 0.46±0.10 respectively.

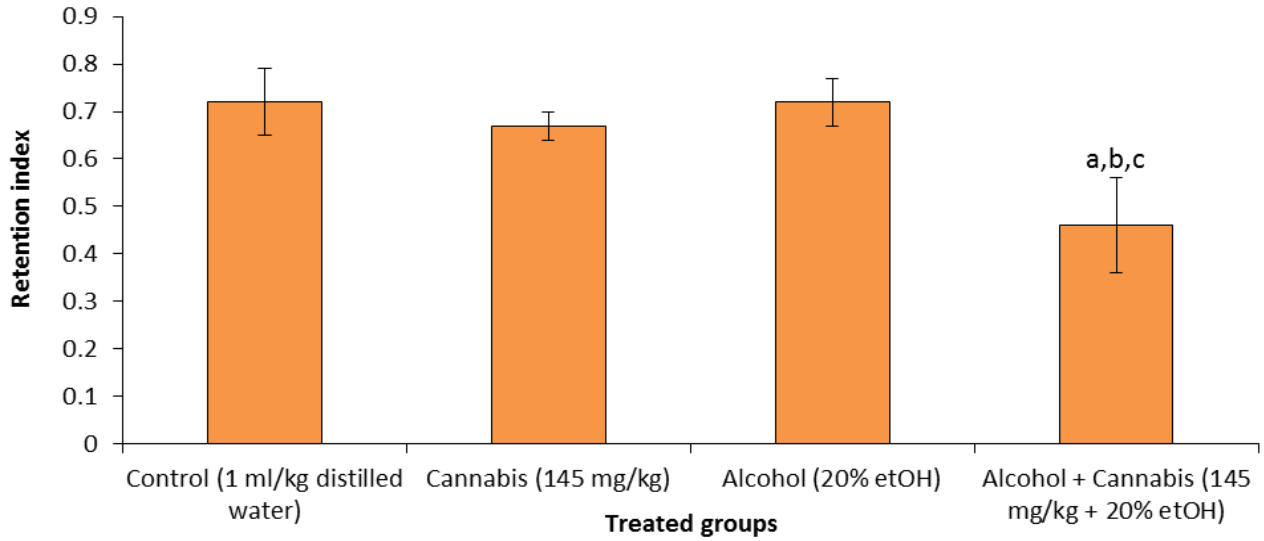


Figure 4.3: Showing recognition index in mice treated as follows: Group I; Control, Group II; Cannabis 145 mg/kg, Group III; Alcohol 20% b/w and Group IV; (Alcohol + Cannabis) for 21 days. Superscripts (a), (b), (c) and (d) indicate statistical significance compared to Groups I, II, III and IV respectively ($p \leq 0.05$)

4.4 Shows the result of volume estimation of hippocampus in mice treated with alcohol and cannabis

Table 4.1 There was a decrease in volume of hippocampus in the cannabis and alcohol treated groups as compared to the control (10250 vs 11100) mm^3 and (10400 vs 11100) mm^3,

respectively. There was also a decrease in volume of hippocampus in the alcohol + cannabis group as compared to the control (9750 vs 11100) mm^3 though not statistically significant.

Table 4.1: Volume estimation of hippocampus in mice treated with alcohol and cannabis

	Group1 (Control)	**Group2 (Cannabis)**	**Group3 (Alcohol)**	**Group4 (Both)**
Volume (mm^3)	11,100	10,250	10,400	9,750
Noise	215.56	188.34	196.10	173.28
VAR_{SURS}	-14,873.68	-13,211.02	-13,906.30	-10,989.82
TVAR	-14,658.12	-13,022.68	-13,710.19	-10,816.55
CE	0.54	0.55	0.56	0.53

VAR_{SURS}= Variance of the systematic uniform random sampling, TVAR = Total variance

CE = Coefficient of error

4.5 Photomicrograph sections of the hippocampus following administration of *cannabis sativa,* alcohol, and alcohol + *cannabis sativa* in mice treated for 21 days

Plate I: Group 1 (Control): Photomicrograph section from the mice hippocampus showing the normal general histoarchitecture: CA 1, CA 2, CA 3; DG-dendate gyrus; Subiculum. Plate II: Group 2 (*Cannabis sativa*) Photomicrograph of section of mice hippocampus showing no significant histological change compared to Control. Plate III: Group 3 (Alcohol) Photomicrograph section of mice hippocampus showing no significant histological change Compared to Control. Plate IV: Group 4(Both) Photomicrograph section of mice hippocampus showed delamination of hippocampal cells in the CA2 portion of the hippocampus and loss of cells in the subiculum portion of the hippocampus.

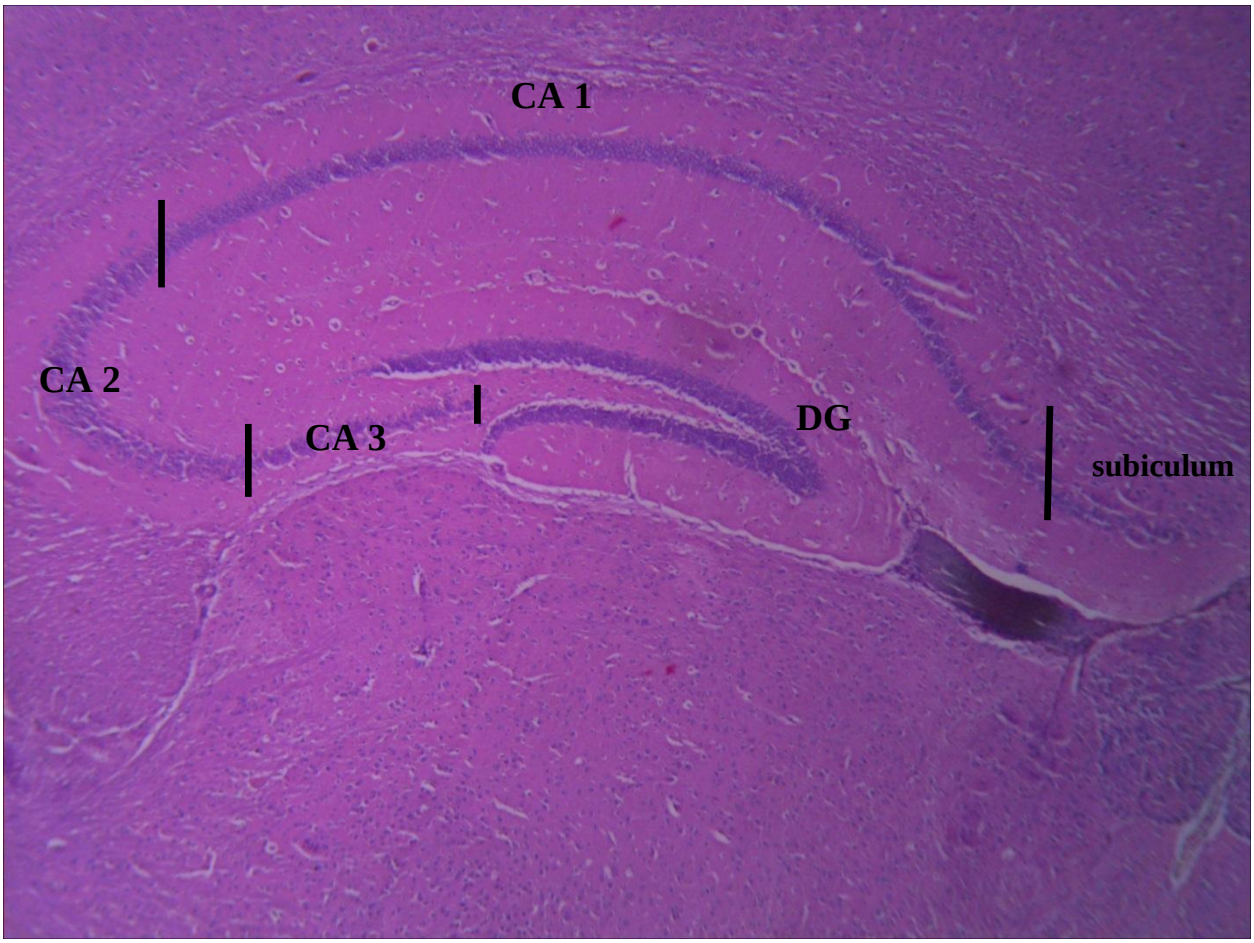


Plate I: Group 1 (Control) (1ml/kg distilled water): Photomicrograph section from the mice hippocampus showing the normal general histoarchitecture: CA 1, CA 2, CA 3; DG-dendate gyrus; Subiculum (H and E; x40).

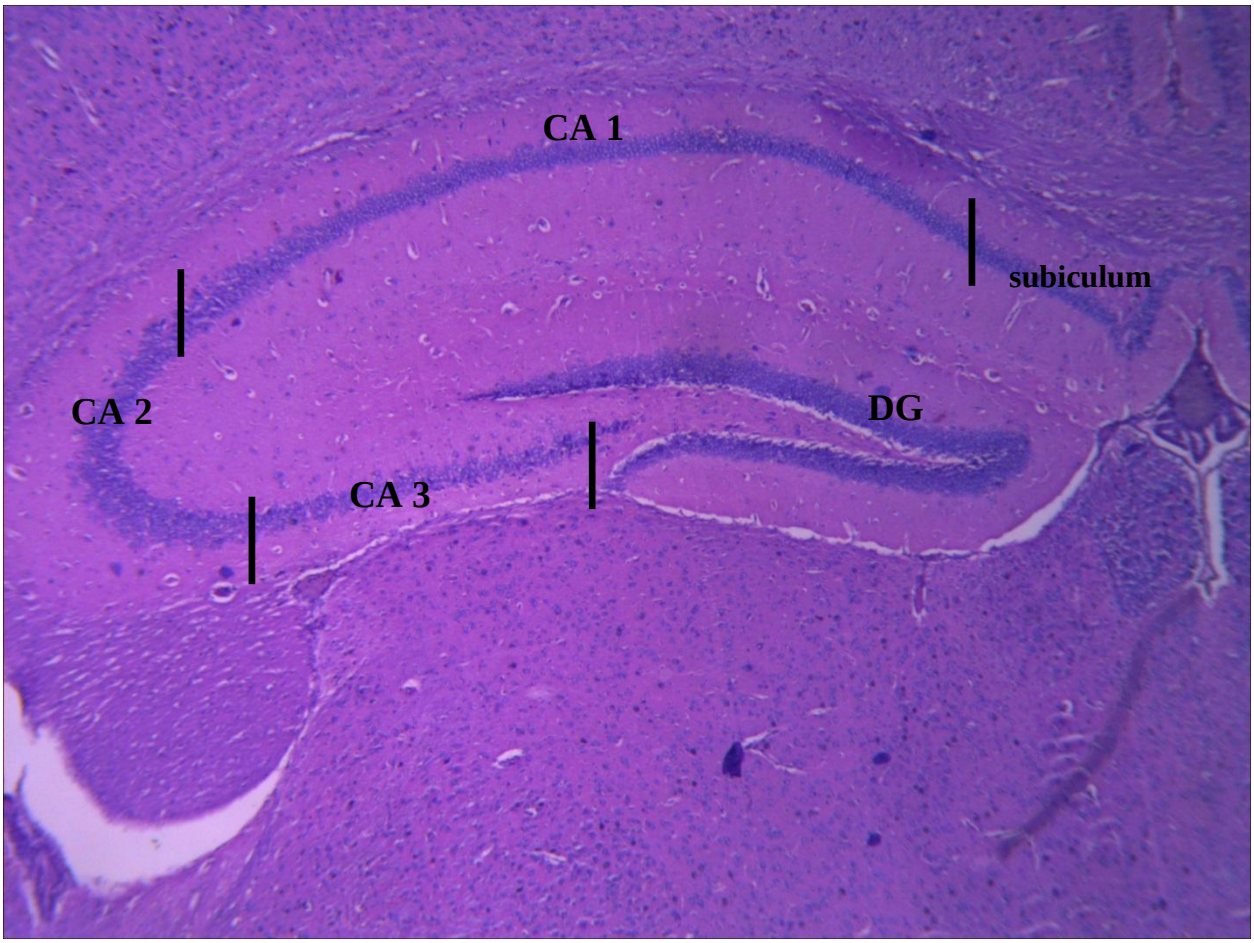


Plate II: Group 2 (Cannabis) (145mg/kg) Photomicrograph of section of mice hippocampus showing no significant histological change compared to Control (H and E; x40).

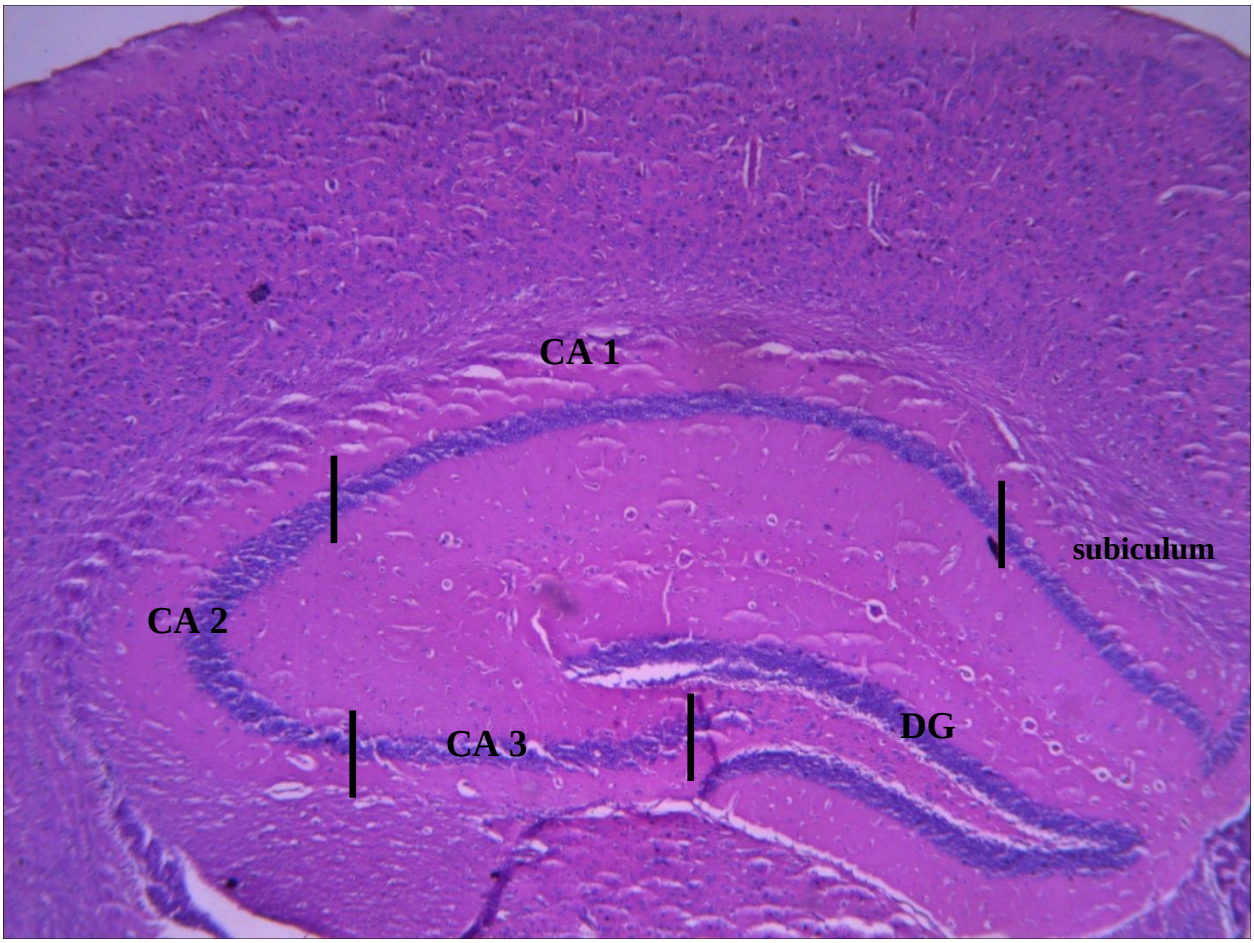


Plate III: Group 3 (Alcohol)(20% etOH) Photomicrograph section of mice hippocampus showing no significant histological change Compared to Control (H and E; x40).

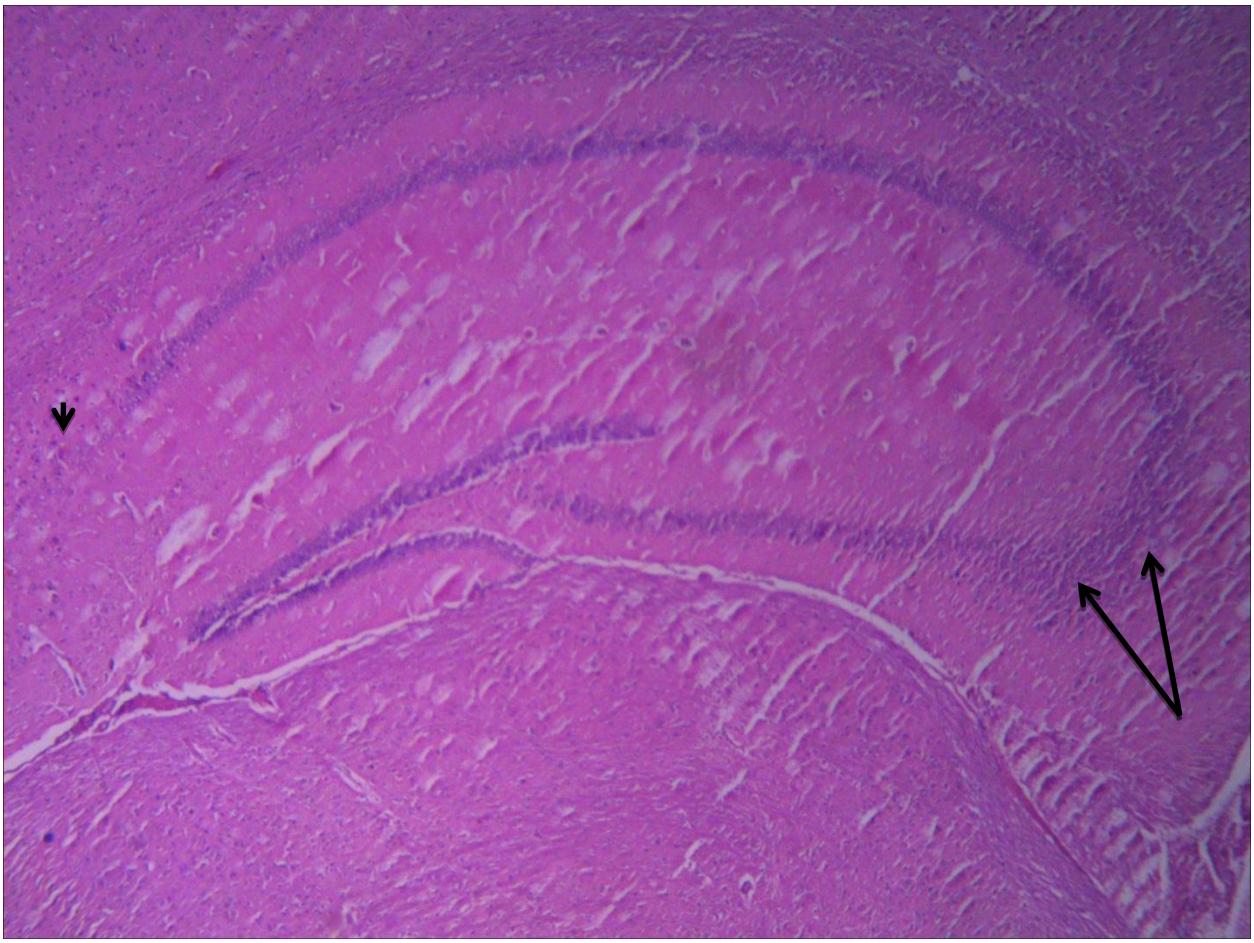


Plate IV: Group 4 (*Cannabis sativa* 145mg/kg + 20% etOH) Photomicrograph section of mice hippocampus showing scattering/disorganization of hippocampal cells in the CA2 portion (double head arrow) and loss of some neural cells in the subiculum portion (black arrow head) of the hippocampus (H and E; x40).

CHAPTER FIVE

5.0 DISCUSSION

Learning and memory are closely related concepts which play vital roles in our day to day life as data are consistently being acquired, processed, stored and retrieved for purposes like decision

making or recognizing a familiar face etc. There are several substances however that could have detrimental effects to this intricate function of the human brain when abused. Some of which include *cannabis sativa* and alcohol, which is why this study was designed to evaluate the effects of the administration of ethyl acetate fraction of *cannabis sativa (δ-9 thc)* and alcohol on learning, memory and histology of the hippocampus in *mus musculus* (mice) using the Y-Maze, and Object Recognition neurobehavioral models for memory.

The results from this study of percentage alternation showed a significant increase in Alcohol + Cannabis treated when compared to Cannabis treated group. An increase in mean rank indicates memory impairment while a decrease indicates memory enhancement. This cognitive deficit could have been activation of the prostanoid synthesis pathway and subsequent generation of free radicals by cyclooxygenase from cannabis administration. Excessive stimulation of CB1 receptors within the hippocampus stimulates the production of arachidonic acid which is catalyzed by cyclooxygenase enzymes to form prostaglandins, thromboxanes and reactive oxygen species which stimulates peroxidation of lipids, proteins and DNA resulting in destruction of hippocampal cells and consequent cognitive impairment (Ameri, 1999 and Banjara, 2015).

More so, alcoholism causes thiamine deficiency (vitamin B1) which can result in memory impairment. Thiamine plays vital role in the synthesis of neurotransmitters like glutamic acid and gamma amino butyric acid (GABA) and can also serve directly as a neuromodulator. Alcohol administered could have reduced thiamine synthesis resulting in memory impairment (Wilhelm *et al.,* 2008). The activity of cannabis in this study could also have been due to Delta-9 THC, which acts via activation of CB1 receptors found in the hippocampus resulting in decreased glutamate release at the synapse, thereby interrupting the process of Long Term Potentiation,

which ultimately impairs memory. This result is consistent with the findings of Young *et al*., (2006) who reported impairment in spatial and non-spatial memory, following administration of delta-9 THC for 21days in wistar rats. The result of alcohol only treated group showed an insignificant change in percentage alternation compared to control. This could have been due to tolerance, which is the diminished response to alcohol or other drugs over a course of repeated or prolonged exposure (Andrzej and Steven, 2008). The result could also have been from the direct action of alcohol on ion channels leading to modification of gated channels, which regulates the ratio between channel open states (during which ions flow through) and closed states (during which ions cannot flow through) (Dopico and Lovinger, 2009). Consequently, this increases the contribution of long openings and decreases the contribution of long closures, thereby making a channel more active (Harris *et al*., 2008), thus increasing enhancing memory. Another possible mechanism through which alcohol elicited activity as observed from this result could have been via the phosphorylation of protein, resulting in alterations in receptors and subsequent development of tolerance. Alcohol also enhances memory by selectively altering mRNA and protein expression of selected subunits of membrane proteins such as NMDA and GABA receptors, thereby increasing their surface expression and targeting to synapses (Qiang *et al*., 2005). This could also have been a possible pathway for the development of tolerance in the alcohol treated group.

Discriminatory index (DI), showed a statistically significant ($P<0.05$) decrease in the groups treated with co-administration of alcohol and cannabis compared to the control. This lack of preference to novel object displayed by this group indicates disrupted short-term object recognition memory. This result could have been from a possible decrease in brain derived neurotrophic factor (BDNF) release or synthesis as a result of the interraction between cannabis

and alcohol. In the hippocampus BDNF is well characterized as a key modulator of hippocampal synaptic plasticity. Therefore the interaction between cannabis and alcohol could have led to the loss of hippocampal BDNF surge and cognitive deficits in this study (Di forti *et al*., 2009). This memory deficit may also be explained by the extensive damage observed in the hippocampus of these animals from the histology. The hippocampus is the main brain region responsible for recognition memory (Dere *et al*., 2007; Broadbent *et al*., 2010). The Novel object recognition test explores deficits in non-spatial working memory and is based on rodents' innate preference to examine novel rather than familiar objects (Dere *et al*., 2007; Broadbent *et al*., 2010).

There was a statistically significant decrease of recognition index in the group treated with co-administration of alcohol and cannabis compared to control and other treated groups. RI is the measure of novel object recognition. It is the main index of retention. It is recorded as a percentage (Wang *et al*., 20014; Antunes and Biala, 2012). This result could have been due to alcohols propensity to potentiate the effects of other drugs when they are co-administered with it (Althobaiti and Sari, 2016). Alcohol metabolism and its metabolites might have increased the blood concentration of delta-9 tetrahydrocannabinol, thereby potentiating the effects of cannabis on memory impairement. This is consistent with the work of Johannes *et al*. (2011) who reported greater impairment on memory when alcohol was co-administered with *Cannabis sativa*. However, this is in contrast to the work carried out by Ortiz *et al*., 2004 who reported enhancement in memory when alcohol was co-administered with cannabis sativa. Impairement of memory seen in this group is supported by our histological results which revealed the delamination of hippocampal cells in the CA2 portion and loss of cells in the subiculum portion of the hippocampus and reduction in volume of hippocampus.

Significant change was also observed in the photomicrograph section of the hippocampus in group four that received alcohol and cannabis; this is evident by the delamination and loss of cells in the CA2 and Subiculum regions, respectively. This result explains the decrease in Stereological volume estimation of the hippocampus in this group as compared to the control, though it was not statistically significant.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

In this present study, it can be concluded that *Cannabis sativa* impairs learning and memory more than Alcohol does and Co-administration of *Cannabis sativa* with Alcohol has greater impairement on learning and memory than their single administration.

6.2 Recommendation

Based on the findings of this study, the following recommendations are therefore suggested.

1. Molecular Studies should be conducted on Co – administration of *Cannabis sativa* and Alcohol.
2. Other neurobehavioural models should use to assess learning and memory.
3. Other types of animals should be used to study Co – administration of *Cannabis sativa* and Alcohol.

6.3 Contributions to Knowledge

1. The percentage alternation observed in Mice co-administered with *Cannabis* and alcohol was significantly higher compared to Mice administered with either of them.
2. The Discrimination index (DI) in NORT observed in Mice administered with *Cannabis sativa* and Mice administered with alcohol were significantly higher compared to Mice administered with either of them
3. Co-administration of *cannabis sativa* and Alcohol decreased the hippocampal volume compared to the control, meaning delamination and loss of cells in the hippocampus leads to memory impairement.

REFERENCES

Aizpurua-Olaizola, O., Soydaner, U., Ozturk, E., Schibano, D., Simsir, Y., Narraro, P., Efxebarria, N. and Usobiaga, A. (2016). Evolution of the Cannabinoid and Terpene content during the growth of *Cannabis sativa* plants from different chemotypes. *Journal of Natural Product,*79(2): 342-331.

Alger, B.E. (2005). Endocannabinoid identification in the brain: *Science Signal Transduction Knowledge Environment*, 309:51.

Althobaiti, Y. S., & Sari, Y. (2016). Alcohol Interactions with Psychostimulants: An Overview of Animal and Human Studies. *Journal of Addiction Research & Therapy*, *7*(3), 281. http://doi.org/10.4172/2155-6105.1000281

Amaral, D. and Lavenex, P. (2006). *Hippocampal Neuroanatomy In*: Auferson, P., Morris, R., Amaral, D., Bless, T. and O'Keefe, J., Eds. The *Hippocampal Book*, Oxford University Press, Oxford, pp 37-115.

Ameri A. 1999. The effects of cannabinoids on the brain. *Progress in neurobiology*, 58(4):315-348

Amresh, S., Boylan, J., Bureau, Y., De Sousa, A. and Shah, N. (2013). Biological Trajectory for psychosocial risk factors in psychiatric disorders-A concept based review. *Open Journal of Psychiatry*, 4(7): 456-467.

Andre, C.M., Hausman, J.F. and Guerriero, G. (2016). Cannabis Sativa: The plant of the thousand and one molecules. *Frontiers in Plant Science,* 10(19): 33-35.

Andrzej, P. and Steven, N.T. (2008). The molecular basis of tolerance. *Alcohol Research and Health*, 31(4): 298-308.

Andrzej, Z.P. and Steven, N.T. (2008).The Molecular Basis of Tolerance. *National Institute on Alcohol Abuse and Alcoholism,* 31(4): 23-27.

Antunes, M. and Biala, G. (2012). The novel objects recognition memory: neurobiology, test procedure, and its modifications. *Springer Open Choice.*13(2): 93–110.

Arendt, T. (2001). Alzheimer's disease as a disorder of mechanisms underlying structural brain self-organization. *Neuroscience,* 102(4): 723-765.

Arthur, I.C. (2012). Alcohol Metabolism. *Journal of Biomedical Life Sciences,* 16(4): 667-685.

Ashton, C.H. (2001). Phamacology and effect of Cannabis: A brief review. *The British Journal of Psychiatry*, 128: 101-106.

Australian Institute of Health and Welfare (2003). Statistics on drug use in Australia, AIHW cat. No: 43. Cambera.

Baddeley, A.D. (2001). Is working memory still working? *American Journal of Psychology*, 56(11): 851-864.

Banjara, M. (2015). Marijuana: Neurotoxic or Neuroprotective? *Global Journal of Medical Research*, 15(2): 2249-4618

Bartholomew, J. (2011). The effect of Cannabis use on prospective memory processes in young adults. *Journal of Psychophamacology,* 13(6): 67-69.

Benfenati, E. (2007). Synaptic plasticity as the Neurobiology of Learning and Memory. *Acta Biomedica,* 78(1): 58-66.

Blitzer, R.D., Gil, O., Landau, E.M.(1990). Cholinergic Stimulation enhances Long-term Potentiation in CA1 region of rat hippocampus. *Neuroscience Letter* 119:207-210.

Broadbent, N.J., Gaskin, S., Squire, L.R., Clark, R.E., 2010. Object recognition memory and the rodent hippocampus. *Learning and Memory*. 17, 5e11

Caan, Woody; Belleroche, Jackie de, eds. (2002). Drink, Drugs and Dependence: From Science to Clinical Practice (1st ed.). Routledge. pp. 19–20. ISBN 978-0-415-27891-1

Chales, S. Lieber. (2004) The Discovery of the Microsomal Ethanol Oxidizing System and Its Physiologic and Pathologic Role. *Drug Metabolism Reviews* 36:511-529.

Chandler, L.J., Haris, R.A. and Grews, F.T. (1998). Ethanol tolerance and synaptic Plasticity. *Trends in Phamacological Sciences,* 19: 491-495.

Cowan, N. (1995). *Attention and Memory; an integrated frame network*. New York: Oxford University Press, pp167.

Dere, E., Huston, J.P., De Souza Silva, M.A., 2007. The pharmacology, neuroanatomy and neurogenetics of one-trial object recognition in rodents. *Neuroscienc and Biobehavioral Review*. 31, 673e704

Di Forti, M., Morgan, C., Dazzan, P., Pariante, C.,Mondelli, V., Marques, T.R., Handley, R., Luzi, S., Russo, M., Paparelli, A., et al.(2009) High-potency cannabis and the risk of psychosis. *British Journal of Psychiatry*, 195, 488–491

Diana, R.A., Yonelinas, A.P. and Ranganath, C. (2007). Imaging recollection and familiarity in the medial temporal lobe: a three-component model. *Trends in Cognitive Science* 11(9): 379–386.

Duman, R. S. (2002). Pathophysiology of depression: the concept of synaptic plasticity. *European psychiatry*, *17*, 306-310

Feige, B., Scaal, S., Hornyak, M., Gann, H. and Riemann, D. (2007). Sleep electroencephalographic spectral power after withdrawal from alcohol in alcohol-dependent patients. *Alcohol and Clinical Experimental Research*, 31(1): 19-20

Fontes, M.A., Bolla, K.I., Cunha, P.J., Almeida, P.P., Jungerman, F., Laranjeita, R.R., Bressan, R.A. and Lacerda, A.L. (2011). Cannabis use before age 15 and subsequent executive functioning. *British Journal of Psychiatry*, 198(6): 442-447.

Global Burden of Disease. (2013). A systematic analysis for the global burden of disease study. *Lancet*, 385(9965): 117-171.

Goodwin, D.W., Crane, J.B. and Guze, S.B. (1993). Alcoholic blackouts: a review and clinical study of 100 alcoholics. *American Journal of Psychiatry*, 126: 191-198.

Greg, G. (2005). *The Cannabis Breeder's Bible*. Green Candy Press, pp. 15-16

Gudersen, K. (1998). Determination of muscle contractile properties: the importance of the nerve. *Acta Physiologica,* 162(3): 333-341.

Guerri, C.; Pascual, M.A. (2010)."Mechanisms involved in the neurotoxic, cognitive, and neurobehavioral effects of alcohol consumption during adolescence". *Alcohol* 44 (1) 15–26.

Gulpinar, M.A. and Yegen, B.C.(2004). The physiology of learning and memory: role of peptides and stress. *Current Protein Peptides Science,* 5(6): 1-17

Hall, W. and Degenhardt, L. (2009). Adverse health effects of non medical cannabis use. *Lancet,* 374(9698): 1383-1391.

Harris, R.A., Trudell, J.R. and Mihic, S.J. (2008). Ethanol's molecular targets. *Science Signalling,* 1(28): 7.

Hebb, D. O. (1950) Organization of behavior. *Journal of Clinical Psychology* 6(307)pp.335

Howlett, A.C., Breivogel, C.S., Childers, S.R., Deadwyler, S.A., Hampson, R.E. and Porrino, L.J. (2004). Cannabinoid physiology and pharmacology: 30 years of progress. *Neuropharmacology,* 47: 345-58.

Iversen, L. (2000). *The Science of Marijuana.* 1st Ed. Oxford Unviversity Press, Cambridge.

Jenningson, K.M. and Johnson, K.A. (1994). Drinking induced blackouts among young adults; results from a national longitudinal study. *International Journal of Addiction,* 29(1): 23-51.

Johannes, G.R., Eef, T., Stefan, W.T., Manfred, R.M., and Gerhold, K. (2011). Tolerance and cross-tolerance to neurocognitive effects of THC and alcohol in heavy cannabis users. *Psychopharmacology,* 214(2): 391–401.

Johnston, L. D., O'Malley, P. M., Bachman, J. G., and Schulenberg, J. E. (2011). Monitoring the Future national results on adolescent drug use: Overview of key findings, Ann Arbor: Institute for Social Research, The University of Michigan.

Julie, A.M. and Greenough, W.T. (2006). Experience-driven brain plasticity; beyond the synapse. *Neuron Glia Biology,*1(4): 351-363.

Karban, R. (2015). *Plant Learning and Memory. In: Plant Sensing and Communication.* Chicago and London: The University of Chicago Press, pp.31-44.

Kim, J.J. and Yoon, K.S. (1998). Stress: metaplastic effects in the hippocampus. *Trends in Neuroscience,* 21(12): 505-509.

Krista, M.L., Alecia, D., schwansburg, M.A., and Susan, F.T. (2007). Effects of Alcohol and combined Marijuana and Alcohol use during adolescence on Hippocampal volume and asymmetry. *Neurotoxicology and Teratology,* 29(1): 141-152.

Ksir, Oakley Ray (2002). *Drugs, society, and human behavior* (9th ed.). Boston [u.a.]: McGraw-Hill.

Kumar, R.N., Chambers, W.A. and Pertwee, R.G. (2001). Pharmacological actions and therapeutical uses of Cannabis and Cannabinoids. *Anaesthesia,* 56(11): 1059-1068.

Lavigne, F., Longree, D., Mayaffre, D. and Mellet, S. (2016). Semantics integration by pattern priming: Experimental and cortical network model. *Journal of Cognitive Neuroscience,* 10(6): 513-531.

Litchman, J.W. and Fraser, S.E. (2001). The Neuronal naturalist; Watching Neurons in their Native Habitat. *National Neuroscience,* 4: 1215-1220.

Loius, C. and Susan, S. (2006). Neuroscience and adult learning. *Wiley Online Library,* 110: 11-19.

Makanjuola, A.B., Abiodun, O.A. and Sajo. S. (2014). Alcohol and psychoactive substances use among medical students of university of Illorin, Nigeria. *European Scientific Journal,* 10(8): 1857-1881.

Martin, P.R., Singleton, C.K. and Hiller-Stumhofel, S. (2003). The role of thyroxine deficiency in Alcoholic Brain disease. *Alcoholic Research and Health,* 27(2): 134-142.

Mather, J. A. and Anderson R. C. (1998). What behavior can we expect of octopuses? In: The Cephalopod Page. Wood, J. B. Ed.

Mathiasen, J.R. and Dicamillo, A. (2010). Novel object recognition in rat: A faile assay for cognitive function. *Current Protocol in Pharmacology,* 5(5): 49.

Matthews, D.B.; Silvers, J.R. (2004) The use of acute ethanol administration as a tool to investigate multiple memory systems. *Neurobioliology of Learning and Memory* 82, 299-308.

Mc Carbe, S.E., Wert, B.T., Teter, C.J. and Boyd, C.J. (2012). Co-ingestion of prescription Opioids and other drugs among High School seniors; Results from a national study. *Journal of Biomedical and Life sciences,* 126(1-2): 65-70.

Mehta, R.L., Kellum, J.A., Shah, S.O., Molitoni, B.A., Ronco, C., Warmock, D.G. and Lewin, A. (2007). Acute Kidney injury Network: Repot of an initiative to improve outcomes in Acute Kidney injury. *Critical Care,* 11(2): R31.

Merzenich, M.M. (2001). Cortical plasticity contributing to child development in mechanisms in cognitive development, eds. Mcclelland, J. and Seigler, R. pp 67-96. San Fracisco: Parnauss publishing.

Naber, P.A., Lopes, D.A., Silva, F.H. and Witter, M.P. (2001). Reciprocal connections between the entorhinal cortex and hippocampal fields CA1 and the subiculum are in register with the projections from CA1 to the subiculum. *Hippocampus,* 11:99–104.

Nagy, J. (2008). Alcohol related change in NMDA receptor functions. *Current* Neuropharmacology, 6(1): 39-54.

Nathaniel, P., Birbaum, H., Mernan, M.J., Freedman, J.D., Gilmore, G.P., Jay, D., Kenna, G.A., Maras, B.K., Lisa, M., Weiss, R.D. and White, A.G. (2013).Prescription Opioids abuse: Challneges and opportunities for payers. *Americam Journal of Medical Care,* 19(4): 295-302.

National Institute of Drug Abuse (2010). Drug abuse and addiction research strategic priorities for the next five years on prevention and treatment. *National Institute of Health,* 10-6119.

National Institute on Alcohol Abuse and Alcoholism (NIAAA). (2000). Health risks and benefits of alcohol consumption.*Alcohol Research Health,* 24(1): 5-11

Olakunle, J.O., Adejoke Y.O., Tolulope J.M., Onigbinde O.A., and Oyedele, R.A. (2012). Elevated Plus Maze and Y-Maze Behavioral Effects of Subchronic, Oral Low Dose Monosodium Glutamate in Swiss Albino Mice. *Journal of Pharmacy and Biological Sciences,* 3(4): 2278-3008

Ortiz, S., Oliva, J.M., Perez-Rial, S., Palomo, T. and Marizanares, J. (2004). Difference in basal cannabinoid CB1 receptor function in selective brain areas and vulnerability to voluntary alcohol consumption in Fawn hooded and wistar rats. *Alcohol,* 39: 297-302.

Oscar-Berman, M. and Marinkovic, K. (2003). Alcoholism and the brain: an overview. *Alcohol Research and Health*, 27: 125-133.

Oshodi, O.Y., Aina, O.F. and Onajole, A.T. (2010). Substance use among secondary school students in an urban setting in Nigeria; Prevalence and associated factor. *African Journal of Psychiatry,* 13: 53-57.

Owoaje, E.T. and Bello, J. (2010). Psychoactive substance use among undergraduate students of the university of Ibadan, Niggeria. *Tropical Journal of Health Sciences,* 17(2).

Philips, R.N., Turk, R.F. and Forray, R.B. (1971). Acute toxicity of delta-9-tetrahydrocanabinol in rats and mice. *Proceedings of Society of Experimental Biological Medicine,* 136: 260-263.

Piomelli, D. (2003). The molecular logic of endocannabinoid signalling. *National Review Neuroscience*, 4: 873-84.

Qiang,M., Rani, C.S., Ticku, M.K. (2005) Neuron-restrictive silencer factor regulates the N-methyl-D-aspartate receptor 2B subunit gene in basal and ethanol-induced gene expression in fetal cortical neurons. *Molecular Pharmacology*.67:2115.

Rehm, J., Mathers, C., Popova, S., Thavorncharoensap, M., Teerawattananon, Y., Patra, J. (2009). Global burden of disease and injury and economic cost attributable to alcohol use and alcohol-use disorders. *Lancet*, 373, 2223-2233.

Reingold, E.M. (2002). On the perceptual specificity of memory representations. *Memory*, 10: 365-379

Reingold, E.M., Charnes, N., Pomplun, M. and Stampe, D.M. (2001). Visual span in expert chess players: Evidence from eye movements. *Psychological science*, 12: 49-56.

Ronen, G.M., Fayed, N. and Rosenbaum, P.L. (2011). Outcome in pediatrics Neurology: A review of conceptual issue and recommendations. The 2010 Ronnie mac Keith Lecture Review. *Journal of Developmental Medicine and Child Neurology*, 53(4): 305-312.

Rudd, R.A., Seth, P., David, F. and Scholl, L. (2016). Increase in drug and opioid-involved overdose deaths in United States. *Morbidity and Mortality Weekly Report*, 65(50-51): 1445-1452.

Russo, E.B. (2011). Taming THC: Potential Cannabis synergy and Phyto-cannabinoid-terpenoid entourage effects. *British Journal of Phamacology*, 163(7): 1344-1364.

Russo, E.B. (2011). Taming THC: potential cannabis synergy and phytocannabinoid-terpenoid entourage effects. *British Journal of Pharmacology*, 163 (7): 1344–1364.

Schrijver, N.C., Bahr, N.I., Weiss, I.C. and Würbel, H. (2002). *Pharmacology and Biochemistry of Behaviour*, 73: 209–224.

Schummers, J.; Browning, M.D. (2001). Evidence for a role for GABA(A) and NMDA receptors in ethanol inhibition of long-term potentiation. *Molecular. Brain Research.*, 94, 9-14.

Shafri, M.A., Abdulmanan M. J., Jaffri M. J., Kim M.K. and Farahidah, M. (2012). Cresyl Violet Staining to Assess Neuroprotective and Neuroregenerative Effects of Haruan Traditional Extract Against Neurodegenerative Damage of Ketamine. *International Journal of Pharmacy and Pharmaceutical Sciences*, (4): 0975-1014.

Sim-Selley, LJ. (2003). Regulation of cannabinoid CB1 receptors in the central nervous system by chronic cannabinoids. *Critical Reviews in Neurobiology*; 15: 91-118.

Smith, S.M., Peter, T.F., Karlal, M., Glahn, D.C., Fox, P.M., Mackay, C.E., Fillipini, N., Watkins, K.E., Toro, R., Laird, A.R. and Beckmann, C.F. (2009). Correspondence of the Brain's Functional architecture during activation and rest. *Proceedings National Academia of Science U.S.A*, 106(31): 13040-13045.

Squire, L.R. and Schacter, D.L. (2002). *The Neuropsychology of Memory* (3rd edition) New York: Guilford Press, pp65.

Stockley, C., Chris, O. and Jane, W. (1999). *The Usborne illustrated dictionary of Science* (Rev. ed.). London: Usborne, pp304.

Sussanne, H., Scott H.S. (2005). Alcohol and Adolescent Brain; Animal models. *Alcohol research and Health:*28(4), P. 216.

Tessier-Lavingne, M. and Goodman, S. (1996). The molecular biology of Axon guidance. *Sciences,* 274(5290): 1123-1133.

Testino, G. (2008). Alcoholic diseases in hepato-gastroenterology: a point of view. *Hepatogastroenterology.* 55: 371-377.

Thompson, R.A. and Nelson, C.A. (2001). Developmental Science and the media. Early brain development. *Journal of American Psycchology,* 56(1): 5-15.

United Nations Office on Drugs and Crime, World Drug Report 2014 (United Nations publication, Sales No. E.14.XI.7).

VanElzakkter, M., Rebecca, D.F. and Robert, L.S. (2008). Environmental novelty is associated with a selective increase in Fos expression in the output elements of the hippocampal formation and perrihiral cortex. *Learning and Memory,* 15(12): 899-908.

Wang, G.S., Roosevelt, G., Lelait, M.C., Martinez, E.M., Baucher-Bartelson, B., Bronstein, A.C. and Heard, K. (2014). Association of unintentional pediatric exposures with decriminalization of Marijuana in the United States. *Annals of Emergency Medicine,* 63(6): 684-689.

White, A.M. and Best, P.J. (2000). Effects of ethanol on hippocampal place-cell and interneuron activity. *Brain Research*, 876: 154-165.

White, A.M., Matthews, D.B. and Best, P.J. (2000). Ethanol, memory, and hippocampal function: a review of recent findings. *Hippocampus*, 10: 88-93.

White, A.M., Signer, M.L., Kraus, C.L. and Swartzwelder, H.S. (2004). Experiential aspects of alcohol-induced blackouts among college students. *American Journal of Drug Alcohol Abuse*, 30: 205-224.

Wilhelm, J., Frieling, H., Hillemacher, T., degner, D., Kornhube, J. and Bleich, S. (2008). *Alcohol & alcoholism*. 43(3): 296–299.

Xu, X., Tian, Y., Li, S., Wang, G. and Tain, X. (2013). Inhibition of propofol anaestheia on functional ponnectivity between LFPs in PFCduring rat working memory task. *Plos One,* 8: e83653.

Yaka, R. and He, D.Y., Phamluong, K. and Ron, D. (2003). Pituitary adenylate cyclase-Activating polypeptide PACAP(1-38) enhances N-methyl D-Aspartate function and brain derived neutrophic factor expression via RACK 1. *Journal of Biology and Chemistry,* 278: 9630-9638.

Yeo, B.T., Krienen, F.M., Sepulcre, J., Sabuncu, M.R, Lashkari, D, Hollinshead, M., Roffman, J.L, Smoller, J.W, Zöllei, L., Polimeni, J.R, Fischl, B., Liu, H., Buckner, R.L (2011). The organization of the human cerebral cortex estimated by intrinsic functional connectivity. *Journal of Neurophysiology*. 106 (3): 1125–1165.

Young, S.E., Rhee, S.H. and Stallings, M.C. (2006). Genetic and environmental vulnerability underlying adolescent substance use and problem: General or specific? *Behavior Genetica*, 36: 603-615.

Zhou, M. and Baudry, M. (2006). Developmental changes in NMDA Neurotoxicity reflects developmental changes in subunit composition of NMDA receptors. *Journal of Neuroscience*, 26(11): 2956-2963.

APPENDICES

APPENDIX I: Table showing percentage Alternation in mice treated with alcohol and cannabis

	Group (Control)	Group (Cannabis)	Group (Alcohol)	Group (Both)
% Alternation		48.52	65.24	79.44

% = Percentage of Alternation

APPENDIX II: Table showing Discrimination and Recognition index in mice treated with alcohol and canabis

	Group (Control)	Group (Cannabis)	Group (Alcohol)	Group4 (Both)	*f*(3, 20)	*P*
DI	0.36 ± 0.10^{a}	0.35 ± 0.64^{b}	0.43 ± 0.09^{c}	-0.09 ± 0.21^{abc}	3.29	0.043
RI	0.68 ± 0.05^{a}	0.67 ± 0.03^{b}	0.71 ± 0.05^{c}	0.46 ± 0.10^{abc}	3.29	0.043

Note: Means in a row showing the same superscripts are significantly different each other. DI = Discrimination Index, RI = Recognition Index

APPENDIX III: Volume estimation of hippocampus in Group 1(Control)

Section number(n)	**Pi**	**Pi x Pi**	**Pi x Pi+1**	**Pi x Pi+2**
1	20	400	520	500
2	26	676	650	390
3	25	625	375	500

4	15	225	300	315
5	20	400	420	400
6	21	441	420	588
7	20	400	560	500
8	28	784	700	616
9	25	625	550	-
10	22	484	-	-
n=10	∑=222	∑= 5060 (A)	∑=4495 (B)	∑=3809 (C)

Note: Pi = Number of Points under the Grid T

V (mm^3) = $\overline{T}$ x a/p x $\sum Pi$ = 50 x 1 x 222 = 11, 100 mm^3

Noise = 0.0724 x B/√A x √ n x ∑Pi = 215.56

VAR_{SURS} = 3 (A-Noise) – 4(B + C) + C = -14, 873.68

TVAR = Noise + VAR_{SURS} = -14,658.12

CE= √TVAR/∑Pi = 0.54

APPENDIX IV: Volume estimation of hippocampus in Group 2(*Cannabis sativa*)

Section number(n)	**Pi**	**Pi x Pi**	**Pi x Pi+1**	**Pi x Pi+2**
1	19	361	475	380
2	25	625	500	650
3	20	400	520	360
4	26	676	468	624

5	18	324	432	306
6	24	576	408	504
7	17	289	357	255
8	21	441	315	420
9	15	225	300	-
10	20	400	-	-
n=10	∑=205	∑= 4317 (A)	∑=3775 (B)	∑=3499 (C)

Note: Pi = Number of Points under the Grid

V (mm^3) = $\overline{T}$ x a/p x $\sum Pi$ = 50 x 1 x 205 = 10, 250 mm^3

Noise = 0.0724 x B/√A x √ n x ∑Pi = 188.34.

VAR_{SURS} = 3 (A-Noise) – 4(B + C) + C = -13,211.02

Total variance (TVAR) = Noise + VAR_{SURS} = -13,022.68

CE= √TVAR/∑Pi = 0.55

APPENDIX V: Volume estimation of hippocampus in Group 3 (Alcohol)

Section number(n)	Pi	Pi x Pi	Pi x Pi+1	Pi x Pi+2
1	20	400	480	500
2	24	576	600	456
3	25	625	475	675
4	19	361	513	342
5	27	729	486	648
6	18	324	432	270
7	24	576	360	504
8	15	225	315	225

9	21	441	315	-
10	15	225	-	-
n=10	∑=208	∑= 4482 (A)	∑=3976 (B)	∑=3620 (C)

Note: Pi = Number of Points under the Grid

V (mm^3) = $\overline{T}$ x a/p x $\sum Pi$ = 50 x 1 x 205 = 10, 400 mm^3

Noise = 0.0724 x B/√A x √ n x ∑Pi = 196.10.

VAR_{SURS} = 3 (A-Noise) – 4(B + C) + C = -13,906.30

Total variance (TVAR) = Noise + VAR_{SURS} = -13,710.198

CE= √TVAR/∑Pi = 0.56

APPENDIX VI: Volume estimation of hippocampus in Group 4(Both)

Section number(n)	**Pi**	**Pi x Pi**	**Pi x Pi+1**	**Pi x Pi+2**
1	15	225	390	240
2	26	676	416	494
3	16	256	304	272
4	19	361	323	418
5	17	289	374	221
6	22	484	286	484
7	13	169	286	312
8	22	484	528	462
9	24	576	504	-
10	21	441	-	-

n=10	∑=195	∑= 4482 (A)	∑=3411 (B)	∑=2903 (C)

Note: Pi = Number of Points under the Grid

V (mm^3) = $\overline{T}$ x a/p x $\sum Pi$ = 50 x 1 x 205 = 9, 750 mm^3

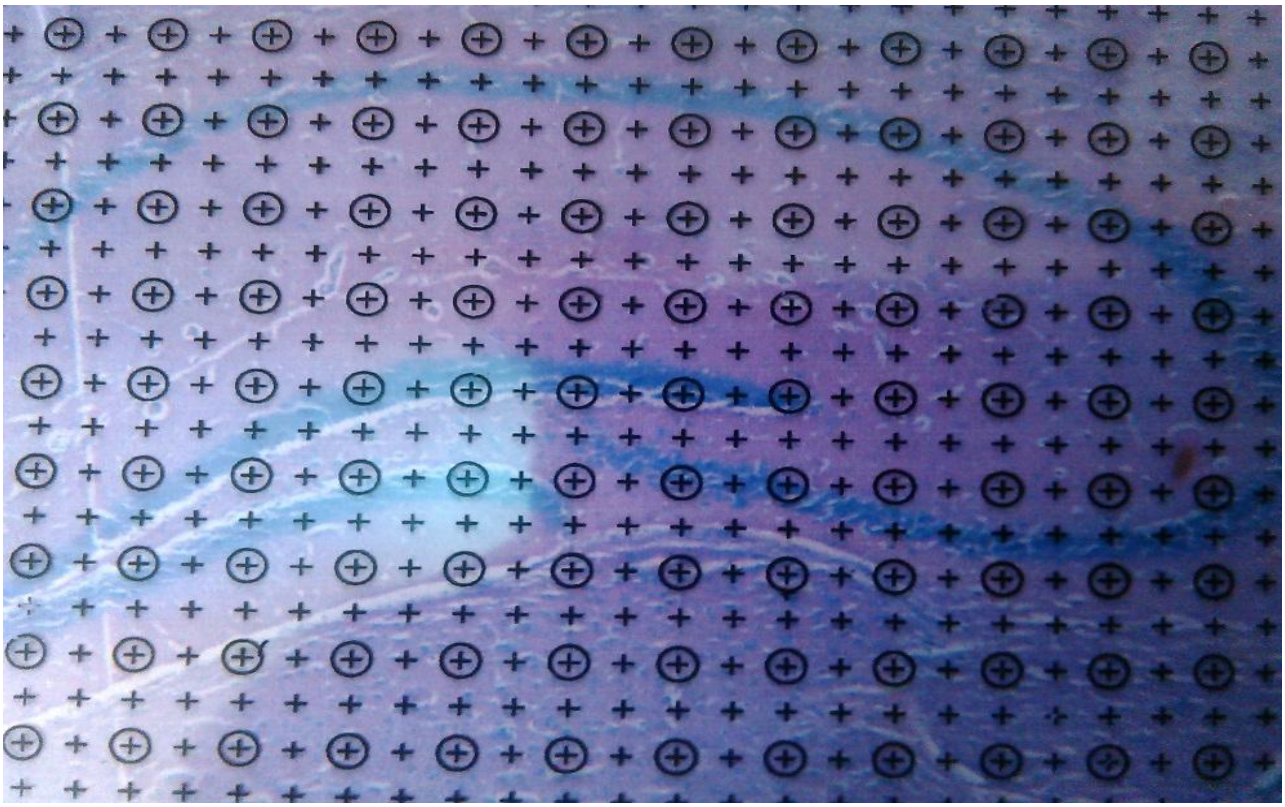
Noise = 0.0724 x B/√A x √ n x ∑Pi = 173.28

VAR_{SURS} = 3 (A-Noise) – 4(B + C) + C = -10, 989.82

Total variance (TVAR) = Noise + VAR_{SURS} = -10, 816.55

CE= √TVAR/∑Pi = 0.53

APPENDIX VI: Showing the test point counting grid used for Hippocampal volume estimation.



Test Point Counting Grid (Cavalieri estimator)

Committee on Animal Use and Care
Directorate of Academic Planning & Monitoring
Ahmadu Bello University, Zaria

Appl No.: ABUCAUC/2015/Human Physiology /01. **Date:** 9th November, 2016

Approval No: ABUCAUC/2015/01.

Dr. M.I.A. Saleh
Department of Human Physiology
Faculty of Medicine,
Ahmadu Bello University,
Zaria.

Sir,

APPROVAL OF RESEARCH STUDY 'EFFECT OF CO-ADMINISTRATION OF CANNABIS SATIVA AND ALCHOHOL ON LEARNING, MEMORY AND HISTOLOGY OF THE HIPPOCAMPUS IN MICE(MUS MUSCULUS)'

This is to convey the approval of the ABUCAUC to you for the aforestated study domiciled in the Department of Human Physiology. The approval is predicated on the assumption that you shall maintain

National Drug Law Enforcement Agency

(NDLEA)

E-mail: ndleakaduna@yahoo.com

Kaduna State Command
Ali Akilu Road, Opp.
Road Safety Office, Kaduna,
Kaduna State.

Ref: NDLEA/KDSC/CUN/94/VOL.II/8 *Date:* 13TH JANUARY, 2016

The Head of Department,
Department of Human Physiology,
Faculty of Medicine,
Ahmadu Bello University,
Zaria, Nigeria.

ATTENTION: Dr. A. Alhassan.

RE: LETTER OF INTRODUCTION FOR BILKISU TALATU ILIYA (P13MDHP8006)

We are in receipt of your letter dated the 11th January, 2016 regarding the