SOME NEUROCHEMICAL CHANGES ASSOCIATED WITH *Canabis sativa* (Cannabinaceae) ADMINISTRATION ON LEARNING AND MEMORY IN MICE.

BY

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MAY, 2017

DECLARATION

I declare that this work in this dissertation entitled "**SOME NEUROCHEMICALS CHANGES ASSOCIATED WITH *Cannabis* sativa ON LEARNING AND MEMORY IN MICE"** has been carried out by me in the Department of Human Physiology, Faculty of Medicine, Ahmadu Bello University Zaria, under the supervision of Drs. Tanko Yusuf and A. B. Sallau. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other institution.

YAKUBU IBRAHIM	SIGNATURE	DATE

CERTIFICATION

This dissertation entitled **"SOME NEUROCHEMICALS CHANGES ASSOCIATED WITH *Cannabis* sativa ON LEARNING AND MEMORY IN MICE"** by Ibrahim YAKUBU meets the regulation governing the award of the Master degree of Science in Department of Human Physiology of the Ahmadu Bello University, and is approved for its' contribution to knowledge and literary presentation.

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DEDICATION

To the Almighty God- The Giver of knowledge and wisdom. Also, to my parents and siblings.

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Praise be to the Almighty God for His Divine favour, strength and excellent health in making this research work a success.

My unending thanks goes to my parents for their solid care, unshakeable support, unfailing love, persistence prayers, and all the pains that you passed through in an attempt to make me what I am and what I ought to be. I love you from the depth of my heart.

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ABSTRACT

Cannabis sativa is a member of the family Cannabinaceae. Delta-9-tetrahydrocannabinol is thought to be the principal psychoactive ingredient present in Cannabis and may have important implications for various neurobehavioral processes, including learning, memory, motivation, motor control, etc. Cannabis use is associated with negative health outcomes, psychosocial and cognitive impairments and societal acceptance of cannabis use for medicinal applications continues to increase. Hence, the concerns for this present study. The objectives of the study were to establish the effect of *Cannabis sativa* on learning and spatial memory; to investigate some neurochemical changes; and to investigate the effects of *Cannabis sativa* on oxidative stress markers in the brain associated with *Cannabis sativa* administration. The study used 75 mice, grouped in to three, of 25 mice each. Three standard neurobehavioural test models (EPM, MWM and YM) each for a group were used to assess learning and spatial short term memory. Each of the three groups was further subdivided in to five (5) groups, containing five (5) mice each. Group one and five, the negative and positive control were respectively administered with normal saline and piracetam 100mg/kg, while groups two, three and four were administered with 25mg/kg, 50mg/kg and 75mg/kg of *Cannabis sativa* extract respectively. All regimens were orally, twice daily and lasted for 21 days. Brain homogenates were used to evaluate acetylcholinesterase, (AchE) activity, dopamine concentration, brain sialic acid concentration, malondialdehyde, (MDA) concentration, Superoxide dismutase, (SOD), and catalase, (CAT). The data obtained from these studies were analysed with statistical package for social science (SPSS) software, version 20 (IBM, USA), using one-way analysis of variance (ANOVA) and Tukey's multiple comparison post hoc test. All data were expressed as mean ± SEM and $p \leq 0.05$ were considered statistically significant. The

results of the research showed impairment in learning and spatial short term memory with groups 3 and 4 in the EPM model and MWM model. There was increased concentration of AchE in the cannabis treated groups of the EPM model and groups 3 and 4 of MWM model, but, no significant changes in concentration of AchE in the Y maze model. Dopamine and Total brain sialic acid concentrations decrease in group 4 of all the models. Also, there was increase MDA concentration and decrease in catalase and SOD activities, suggesting oxidative stress. Thus, cannabis sativa in high doses is associated with learning and spatial short term memory impairment via some of the neurochemicals involved in learning and memory.

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List of abbreviations

AchE- Acetylcholinesterase enzyme

AMPA- alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

BDNF- Brain derived neurotrofic factor

CaM- Calmodulin

CAT- Catalase

CBE- Cannabis sativa extract

CBR- Cannabis receptors

CMAH- cytidine monophospho-*N*-acetylneuraminic acid hydroxylase

CREB- cyclic AMP response element binding protein

EPM- Elevated plus maze

MAPK- mitogen-activated protein kinase

MDA- malondialdehyde

MTL- Medial temporal lobe

MWM- Moris water maze

NMDA- *N*-methyl-d-aspartate

PHC- Parahippocampal cortices

PRC-Perirhinal cortex

SOD- Superoxide dismutase

THC- Tetrahydrocannabinol

YM- Y maze

CHAPTER ONE

1.0 INTRODUCTION

In the last twenty years, significant advances have been made in understanding the endogenous cannabinoid system and how cannabis may affect its functioning, particularly as it pertains to the brain. The abundance of cannabinoid receptors in the hippocampus, amygdala, basal ganglia, and prefrontal cortex (Piomelli 2003; Mackie 2005) suggests that disruption of the cannabinergic system by administration of exogenous cannabinoids, like delta-9-tetrahydrocannbinol (THC; the main psychoactive compound in cannabis), may have important implications for various neurobehavioral processes, including mood and anxiety regulation (Crippa *et al*., 2009; Crippa *et al*., 2011). Also, learning, memory, motivation, motor control, reward processing, and executive functions might be disrupted (Gonzalez 2007; Solowij and Pesa 2010; Crean *et al*., 2011). Indeed, it seems frontal-limbic neurocircuitry is most prominently affected by cannabis use (Martin-Santos *et al*., 2010), while other structures in the brain, including the brain stem, occipital lobe, and parietal lobe may be less affected. Not surprisingly, the neurobehavioral effects of cannabis use have been intensely studied over several decades and summarized in numerous published reviews (Grant *et al*., 2003; Iversen 2003; Ranganathan and D'Souza 2006; Gonzalez 2007;Jager and Ramsey 2008; Schweinsburg *et al*., 2008; Solowij and Battisti 2008; Solowij and Pesa 2010; Crean *et al*., 2011). However, many of these reviews conclude that the findings for several neurocognitive domains are equivocal, speaking to the importance of further research in this field (Crean *et al*., 2011).

Research on the neurocognitive effects of cannabis continues to grow rapidly. This is an especially pertinent area of research because cannabis continues to be the most widely used illicit substance worldwide (UNODC, 2011). In the United State, cannabis use has recently risen and now surpasses that of cigarette use among adolescents (Johnston *et al*., 2012). These statistics are particularly concerning, given that cannabis use is associated with negative health outcomes (Kalant 2004), psychosocial and cognitive impairments (Kalant 2004; Solowij and Pesa 2010), and other neurobehavioral consequences such as an increased risk of an automobile crash when driving while intoxicated (Drummer *et al*., 2003; Mura *et al*., 2003; Ramaekers *et al*., 2004; Asbridge *et al*., 2012; Li *et al*., 2012). Furthermore, some have argued that the potency of cannabis, as indexed by THC content, has increased in recent years (Burgdorf *et al*., 2011) and may account for increases in cannabis use disorder diagnoses (Compton *et al*., 2004), as well as cannabis-related psychosis (Malone *et al*., 2010). On the other hand, societal acceptance of cannabis use for medicinal applications continues to increase (Fairfield *et al*. 1998; O'Connell and Bou-Matar; 2007Cohen 2010) and there is a growing body of literature suggesting medical benefits from cannabis use (Watson *et al*., 2000; Ware *et al*., 2005; Elikkottil *et al*., 2009; Ellis *et al*., 2009)

However, not all studies have reported memory impairments in heavy and early-onset cannabis users (Kanayama *et al*., 2004; Padula *et al*., 2007; Becker *et al*., 2010; Ashtari *et al.*, 2011). As indicated by pharmacological challenge studies, an important determinant of the long-term effects of cannabis use may be the precise mixture of the ingredients of the consumed cannabis. Although a naturalistic study reported that cannabis users who smoked cannabis high in CBD and low in Δ9-THC were protected from the memory-

impairing effects associated with cannabis strains high in Δ9-THC (Morgan *et al*., 2010), most of the observational studies to date did not include the Δ9-THC/CBD ratio (e.g, by employing hair analysis as a proxy estimate of the amount of Δ9-THC or CBD in the cannabis used) as a potential confounder in statistical reporting. Given the impact of the Δ9-THC/CBD ratio on brain structures implicated in memory function and executive functions in cannabis users while taking into account the variability of this ratio in street cannabis (Chen *et al*., 2005; Hermann *et al*., 2007; Demirakca *et al*., 2011), consideration of this factor in the design and analysis of studies may improve the replicability and interpretability of the results, particularly because simple and valid methods are now applicable for the quantitative detection of Δ9-THC and CBD (Morgan and Curran, 2008; Salomone *et al*., 2012).

1.1 Statement of the Research Problem

Cannabis is the most widely used illicit drug in many developed and developing societies. Marijuana smoking is a serious public health problem among adolescents in Zaria environment. In a study involving 350 secondary school students in Zaria, a prevalence of 9.43% was recorded (Shehu and Idris, 2008)

Cannabis has both psychological and physiological effects on the human body (Osborne and Fogel 2008). Its health and psychological effects are not well understood and remain the subject of much debate, with opinions on its risks polarised along the lines of proponents' views on what its legal status should be. An unfortunate consequence of this polarisation of opinion has been the absence of any consensus on what health information the medical profession should give to patients who are users or potential users of cannabis (Osborne and Fogel 2008).

Also, cannabis receptors are scattered around the brain. The brain regions in which cannabinoid receptors are very abundant are the basal ganglia, associated with movement control; the cerebellum, associated with body movement coordination; the hippocampus, associated with learning, memory, and stress control; the cerebral cortex, associated with higher cognitive functions; and the nucleus accumbens, regarded as the reward center of the brain. Other regions where cannabinoid receptors are moderately concentrated in the hypothalamus, which regulates homeostatic functions; amygdale, associated with emotional responses and fears; the spinal cord, associated with peripheral sensations like pain; the brain stem, associated with sleep, arousal, and motor control; and the nucleus of the solitary tract, associated with visceral sensations like nausea and vomiting (pertwee, 1997). Societal acceptance of cannabis use for medicinal applications continues to increase and there are growing bodies of literature suggesting medical benefits from cannabis use (Cohen, 2010). Hence, the concern for this study.

1.2 Justification of the study

In many western societies, cannabis has been used by a substantial minority, and in some a majority, of young adults, even though its use is prohibited by law (Hall *et al.*, 1998). Debate about the justification for continuing to prohibit cannabis use has polarised opinion about the seriousness of its adverse health effects (Hall *et al.*, 1994).

Societal acceptance of cannabis use for medicinal applications continues to increase (Fairfield *et al*. 1998; O'Connell and Bou-Matar; 2007Cohen 2010) and there is a growing body of literature suggesting medical benefits from cannabis use (Watson *et al*., 2000; Ware *et al*., 2005; Elikkottil *et al*., 2009; Ellis *et al*., 2009). However, many of these

reviews conclude that the findings for several neurocognitive domains are equivocal, speaking to the importance of further research in this field (Crean *et al*., 2011).

In addition, the possible therapeutic effects of cannabinoids have become entangled in the debate about prohibition of recreational cannabis use. The health effects of cannabis use, especially of long-term use, remain uncertain because of the little epidemiological data and also due to disagreements about the interpretation of the limited epidemiological and laboratory evidence (Hall *et al.*, 1994).

1.3 Aim and Objectives

Aim of the Study

The aim of this study is to investigate any neurochemical, neurobehavioural, learning and memory changes associated with *Cannabis sativa* extract administration on mice.

Objectives of the Study

The objectives of the study were:

I. To establish the effect of *Cannabis sativa* extract administration on learning and spatial short term memory in mice.
II. To investigate some neurochemical changes in the brain such as acetylcholelinerase activity, dopamine levels and brain sialic concentration associated with *Cannabis sativa* extract administration.
III. To investigate the effects of *Cannabis sativa* extract administration on oxidative stress markers such as superoxide dismutase (SOD), Catalase (CAT) and lipid peroxidation using biochemical techniques.

1.4 Research Hypothesis

Repeated *Cannabis sativa* administration has no negative effect on the learning and spatial short memory, brain oxidative biomarker, learning, memory and neurotransmitter release in Adult Mice.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Learning and Memory

Learning and memory are closely related but otherwise distinct neural phenomena often confused with each other. Learning may be defined as the process of information acquisition and elaboration that will modify a subsequent behavior, while memory is the ability to remember past experiences. Another difference between the processes is the speed with which the two things occur. Learning is slow and laborious, while memory occurs rapidly (Packard and Goodman, 2013). Memory formation and utilization is a complex process involving several brain structures in conjunction as the hippocampus, the amygdala and the adjacent cortical areas, usually defined as medial temporal lobe structures (MTL). The memory processes depend on the formation and modulation of synaptic connectivity affecting synaptic strength, synaptic plasticity and synaptic consolidation (Colciago e*t al.*, 2015).

In another way, memory is the process by which knowledge is encoded, stored, and later retrieved, it is often considered to be a process that has several stages, including encoding (processes by which newly learned information is attended to and processed when first encountered), consolidation (refers to those processes that alter the newly stored and still labile information so as to make it more stable for long-term storage), storage (mechanism and sites by which memory is retained over time) and retrieval (processes that permit the recall and use of the stored information) (Squire and Wixted, 2011). Many types of memories exist which differ for their duration (short-term memory, working memory, long-term memory) or for their specificity: explicit or declarative (responsible for

collection of data, facts and events that can be recalled consciously), which can be further divided into semantic memory (facts about the world) and episodic memory (the capacity to re-experience an event in the context in which it originally occurred); implicit or non declarative (skills, tasks: the ability to learn behavioral and cognitive abilities and algorithms, emotional states, previous experiences which can be recalled without awareness); sensory (input from the 5 senses) (Squire and Wixted, 2011). Explicit or declarative memory which is the conscious recall of knowledge about people, places, and things, and it is particularly well developed in the vertebrate brain. Explicit memory can be further classified as episodic (a memory for events and personal experience) or semantic (a memory for facts). Implicit or nondeclarative memory is memory for motor skills and other tasks and is expressed through performance, without conscious recall of past experience; it includes simple associative forms, such as classical conditioning, and nonassociative forms, such as sensitization and habituation (Squire and Wixted, 2011).

Critical structures involved in the processes of memory proved to be the hippocampus, the amygdala and the adjacent entorhinal, perirhinal (PRC) and parahippocampal cortices (PHC), which make up much of the parahippocampal gyrus, usually defined as medial temporal lobe structures (MTL). Specifically, these structures are important for the formation of memory (encoding), for the maintenance of memory for a period of time after formation (consolidating for long term storage), and for retrieving stored memories (Squire and Wixted, 2011; Reber, 2013). Other brain areas as the retrosplenial cortex (Ranganath and Ritchey, 2012) and the

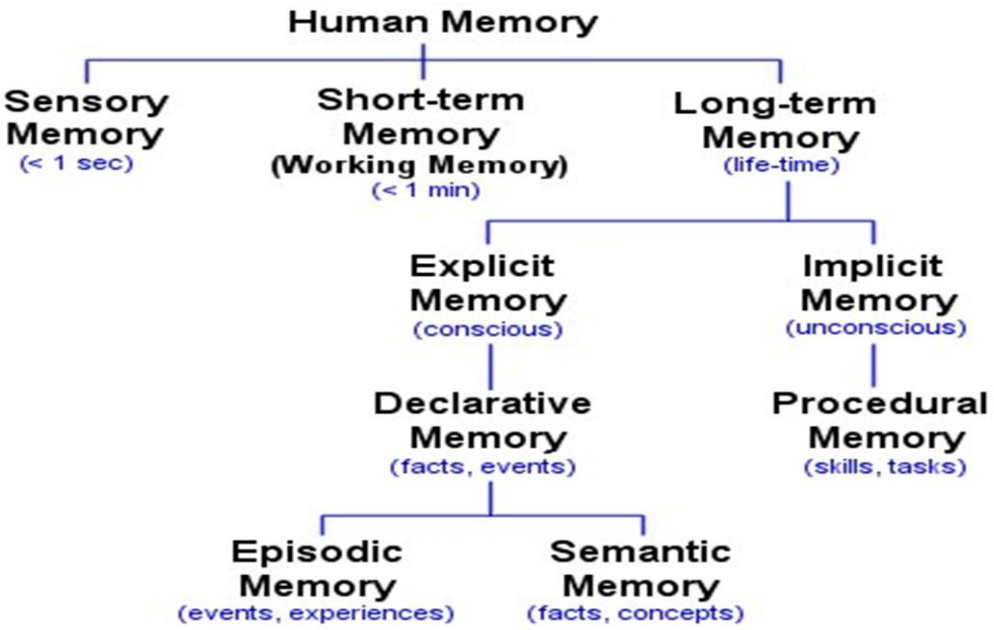


Figure 2.1: Diagram showing the location of different form of memory (Si *et al*, 2003)

dorsolateral striatum (Packard and Goodman, 2013) might be involved. The retrosplenial cortex is associated with a successful recollection during retrieval of contextual details while dorsolateral striatum mediates a form of learning in which habits are incrementally acquired.

Short-term and working memory rely on the activity of cortical neurons in the lateral prefrontal cortex (Squire and Wixted, 2011). The prefrontal cortex with its frontostriatal connections represents a very important structure in the neural processes involved in the so called executive functions, which include, but are not limited to, working memory, attention, cognitive flexibility and impulse control. These complex behaviors are largely mediated by prefrontal cortex and are modulated by dopaminergic, noradrenergic, serotonergic and cholinergic inputs (Logue and Gould, 2013). Long-term memory is stored in the neocortex (not in MTL) distributed in different regions, where the domain-specific processing occurred at the time of learning. The process of remembering therefore involves the reactivation of the same neocortical regions where the information was initially processed and stored (Squire and Wixted, 2011).

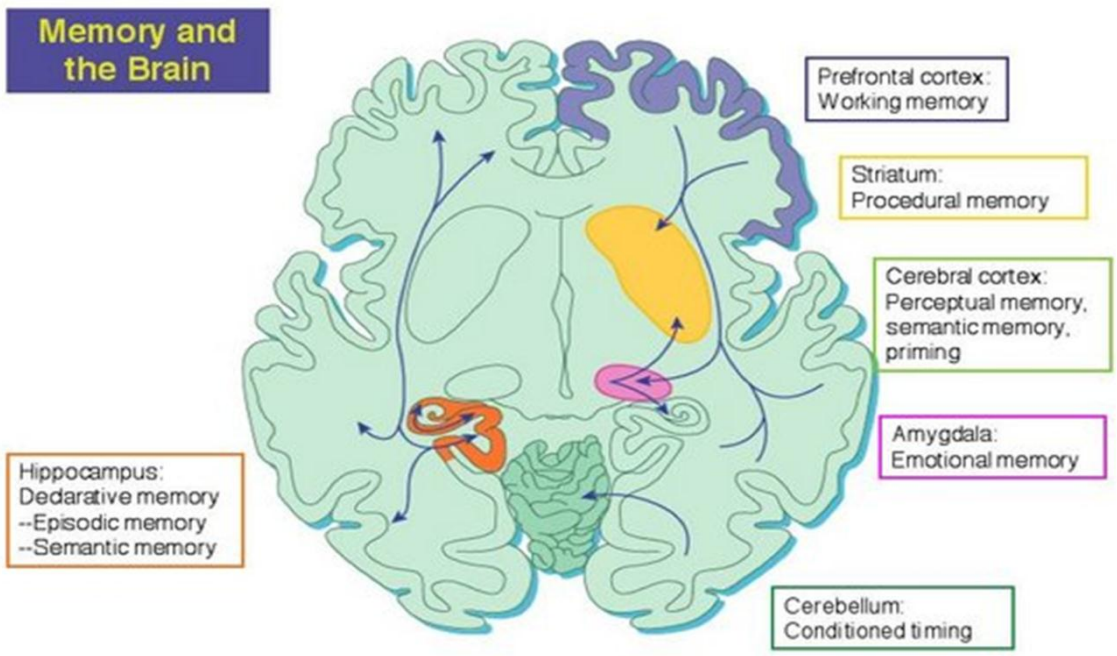


Plate 1: Showing the location of different form of memory in Brain.(Si *et al*, 2003).

Memory formation requires new arrangement of synaptic connectivity, an event that involves the *Hebbian*-like mechanisms to strengthen crucial synapses. Subsequent refinement of neuronal circuits, also involving weakening of un-needed synapses, is believed to support long-term memory (Middei *et al*., 2014). The processes of memory formation and utilization are quite complex and can be divided in approximately the following phases: memory encoding, consolidation, storage and retrieval. However, because all processes are fully inter-connected (e.g., there is no retrieval without encoding or consolidation and success of encoding cannot be evaluated without retrieval), they ultimately have to be evaluated in relation to one another (Straube, 2012).

The encoding process begins with perception of sensory inputs. The MTL structures are responsible for the transformation of sensations into a memory representation (Squire and Wixted, 2011), which is first stored and consolidated in the MTL then allocated to other areas of the cortex committed for long term-storage because better protected from changes induced by new incoming memories (Straube, 2012). Encoded memories undergo consolidation, a process by which a fragile short-term memory trace is transferred into stable long-term memory before directing it to neocortical areas, different from the medial temporal lobes, for long-term storage. Not all information is equally well transferred: it is recognized that especially emotionally arousing experiences are well remembered, even after decades (Squire and Wixted, 2011; Cabeza *et al*., 2012). After the process of consolidation, memories are represented by networks of neurons distributed across the neocortex, bound together for rapid storage and later retrieval by the MTL structures (Squire and Wixted, 2011; Reber, 2013). While MTL brain areas are necessary for retrieving recent episode-based memory associations, over time, many of these

associations are expressed and can be recalled independently of the MTL structures (Squire and Wixted, 2011).

2.2 Amnesia

Amnesia can be defined as memory lost over time. It is also known as amnesic syndrome, is a deficit in memory caused by brain damage, disease, or psychological trauma (Gazzaniga *et al.,* 2009). It can also be caused temporarily by the use of various sedatives and hypnotic drugs. The memory can be either wholly or partially lost due to the extent of damage that was caused.

2.2.1 Types of Amnesia

There are two main types of amnesia, which include

i. retrograde amnesia, and
ii. anterograde amnesia.

2.2.1.1 Retrograde amnesia

Retrograde amnesia (RA) is a loss of memory-access to events that occurred, or information that was learned, before an injury or the onset of a disease (Hunkin *et al.,* 1995). It tends to negatively affect episodic, autobiographical, and declarative memory while usually keeping procedural memory intact with no difficulty for learning new knowledge. RA can be temporally graded or more permanent based on the severity of its cause (Wixted, 2004). The most commonly affected areas affected by retrograde amnesia

are associated with episodic and declarative memory such as the hippocampus, the diencephalon, and the temporal lobes. (Buccione *et al.*,2008).

2.2.1.2 Anterograde amnesia

Anterograde amnesia is the inability to transfer new information from the short-term store into the long-term store. People with this type of amnesia cannot remember things for long periods of time (Gazzaniga *et al.,* 2009). Anterograde amnesia is a loss of the ability to create new memories after the event that caused the amnesia, leading to a partial or complete inability to recall the recent past, while long-term memories from before the event remain intact. This is in contrast to retrograde amnesia, where memories created prior to the event are lost while new memories can still be created. Both can occur together in the same patient. Anterograde amnesia can be caused by drugs, (such as benzodiazepine), illnesses (such as encephalitis) and alcohol intoxication (Bulach *et al.,* 2005).

2.3 Electrical and Biochemical Mechanism of Memory

It is well accepted that learning and memory establishment are based on the dynamic formation of new active synaptic connections. Neurons organize themselves into groups specialized in handling different types of information in response to one's experiences, creating new memories prompted by experience, education or training that may be later retrieved (Colciago e*t al.,* 2015). The mechanisms involved in formation and modulation of synaptic connectivity are therefore essential in the memory processes. Synaptic strength,

synaptic plasticity and synaptic consolidation, coupled to intrinsic (dendritic or somatic) plasticity are the main molecular events involved (Sehgal *et al.*, 2013).

Synaptic strength is defined as the response that is produced in a target cell by the synapses of a neuron on initial stimulation, i.e., the relative amplitude of the post-synaptic response that is generated by the activity of the pre-synaptic neuron. Pre-synaptic mechanisms that affect neurotransmitter release or the amount of transmitter that is released are important determinant of this characteristic; similarly important appear the number and type of the activated post-synaptic receptors (Atwood and Karunanithi, 2002). Synaptic plasticity refers to any lasting up-regulation or down-regulation of synaptic strength, involving mechanisms such as the modulation of neurotransmitter release and changes in the number of neurotransmitter receptors (Cortés-Mendoza, 2013). Activity dependent plasticity appears to be the leading mechanism involved in learning and memory. Persistent synaptic activation leads to a number of pre- and post-synaptic molecular events that include increased release of inhibitory or excitatory neurotransmitters and increased receptor expression on the post-synaptic neuron, resulting in the activation of distinct signaling pathways that temporally and permanently alter specific patterns of gene expression (Owen and Brenner, 2012).

There is a substantial agreement that long-term potentiation (LTP), in which brief high frequency synaptic stimulation results in a long lasting increase in synaptic strength, is the main mechanism involved in memory coding (Nicoll & Roche, 2013). During learning, neuronal networks are remodeled by the formation of new synaptic contacts, by the

increase of dendritic branching and length (synaptogenesis), or by dendritic spine growth and stabilization (Cortés-Mendoza, 2013).

The pre-synaptic release of glutamate and the activation of post-synaptic glutamate receptors are the essential mechanisms involved in LTP at glutamatergic synapses. Although the exact cause of the LTP triggering at selected synapses is still not fully understood, the phenomenon is induced predominantly by activation of post-synaptic *N*-methyl-d-aspartate (NMDA)-type ligand-gated glutamate receptors followed by insertion of additional alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors into the post-synaptic membrane at sites that previously lacked functional AMPA receptors. Therefore, strengthening of excitatory glutamatergic synapses is accomplished by a direct activation of NMDA receptors, which depolarize the post-synaptic neuron leading to calcium entry into the cell, and by triggering a rapid insertion of post-synaptic AMPARs from intracellular stores (Kessels and Malinow, 2009).

Another important mechanism involved in synaptic plasticity and in memory formation is long-lasting synaptic depression (LTD). Contrary to LTP, LTD reduces the efficacy of neuronal synapses in several areas of the brain including the hippocampus (Collingridge *et al*., 2010). LTP and LTD, activated by intense neuronal activities, constitute key events to understand the molecular mechanisms by which memories are formed and stored since they are associated with bidirectional modifications in synaptic strength and in spine characteristics (including spine number, size, morphology) (Neves *et al*., 2010). High-frequency and theta burst stimulation of hippocampus produces prolonged strengthening of synaptic transmission; conversely, long trains of low frequency stimulation lead to LTD. In

synaptic plasticity, the Ca^{2+} influx in the post-synaptic neuron soon after the activation of NMDA receptors is essential for converting electrical/synaptic activity into biochemical events leading to the induction of gene expression critical for synaptic efficacy and memory formation (Diekelmann and Born, 2010; Cortés-Mendoza, 2013).

Multiple signaling pathways are involved in activity-mediated transcription. Calmodulin (CaM) transduces the local elevation of calcium into changes in the neuronal gene expression pattern through the activation of CREB in the nucleus via the MAPK-ERK and CAMK kinase pathways. CREB transcriptional activity activates immediate early genes, such as *c-fos*, *c-jun*, *zif268*, BDNF. Activation of these early genes is a critical step for inducing long-term alterations necessary for plasticity and memory formation. In particular BDNF plays a critical role in neuron survival, dendritic growth and excitatory and inhibitory synaptic development. Calcium influx also produces activation of calcineurin which increases the expression of several genes implicated in synaptic plasticity, among which glutamate receptors, transducers of regulated CREB activity (TORCs), striatal enriched protein phosphatase (STEP), neuromodulin (GAP43), neurogranin and inhibitor-1, which inhibits protein phosphatase 1 (PP1) (Baudry *et al*., 2011). Together, all these changes in gene expression affect dendritic and spine morphogenesis, neuronal survival, and synapse development, essential phenomena of learning and memory in the mature nervous system (Papoutsi *et al.*, 2014).

2.4. Neurotransmitters Involved in Learning and Memory

Memory formation depends on the organization of new neuronal networks, therefore the formation and modulation of synaptic connectivity is an essential process that utilizes and

is affected by different neurotransmitters in the different brain areas and memory systems. In particular, the prefrontal cortex (PFC) function, which controls complex behaviors as learning and cognitive flexibility (executive function), is actively modulated by dopaminergic, noradrenergic, serotoninergic and cholinergic inputs. This coordinated regulation allows PFC to adapt cognitive functions to changes in the environment (Logue and Gould, 2014).

2.4.1 Cholinergic Signaling

Cholinergic neurons located in the basal forebrain (BF) project mainly to the hippocampus, to the thalamic nuclei, to the olfactory bulb and to the nucleus basalis of Meynert; these neurons are also the principal source of cholinergic innervation of the cortex and amygdala. This cholinergic transmission utilizes muscarinic and nicotinic pre- and post-synaptic acetylcholine receptors and supports attention, learning and memory. The role of the cholinergic system in cognition, in particular the cholinergic prefrontal projections, has been extensively investigated in primates and rodents. Depletion of ACh produces extreme impairments in cognitive functions (i.e., working memory, attention, reversal memory) and degeneration of cholinergic neurons in PFC have been implicated in the pathophysiology of Alzheimer's disease (Logue & Gould, 2014). Most nicotinic receptors are located pre-synaptically and are primarily involved in regulating the release of other neurotransmitters such as dopamine, norepinephrine and serotonin (Logue and Gould, 2014); thus, the nicotinic cholinergic system is a key modulator of the three neurotransmitter systems that are crucial for the cognitive processes.

2.4.2 Monoaminergic Signaling

The locus coeruleus (LC) is the primary source of norepinephrine (NE) projections to the PFC and efferent projections from the medial PFC reach the LC. Norepinephrine receptors (α1-, α2-, and β-receptors) are localized pre-synaptically on noradrenergic axons and α2-receptors are also found on post-synaptic neurons. NE activity impacts all four of the cognitive process that sub-serve executive function; set-shifting, attention, response inhibition and reversal learning are all impaired when NE activity levels are low in the PFC (Logue and Gould, 2014).

PFC receives dopamine (DA) input from the ventral tegmental area (VTA) and expresses multiple dopamine receptor subtypes, D1, D2, D3 and D4, primarily D1 receptors. Two families of dopamine receptors have been described, consisting of five receptor types in total. D1and D5receptors are related, as are D2, D3and D4receptors. D1receptors can be found in the striatum, the substantia nigra and the cortex. These receptors have stimulating properties. D2receptors are located in the striatum, the cortex and the pituitary. The D2 receptor also exists presynaptically and on the cell soma. Contrary to D1 receptors they are inhibitory (Kaplan and Sadock, 2004). Efferent DA projections are directed to the VTA and the periaqueductal gray (PAG). Set-shifting and attention are associated with DA activity in the PFC while response inhibition and reversal learning are not dependent on DA activity. Poor performance in set-shifting and attention tasks is seen when DA activity levels are low while improve when DA activity increases (Logue and Gould, 2014).

The ascending serotonin (5-HT) neurons originating in the raphe nuclei innervate the cerebral cortex, hippocampus, septum and amygdala, all brain regions associated with various domains of cognition (Cifariello *et al*., 2008). Fourteen receptor subtypes with

different functional and transductional properties mediate serotonin signaling. Serotonin interacts with other neurotransmitter systems, particularly with cholinergic and dopaminergic systems and 5-HT-receptors are present on GABAergic interneurons. Several lines of evidence have indicated a role of 5-HT and its receptors in various aspects of cognitive functions, including learning and memory. Abundant data reveal that the administration of 5-HT2A/2C and 5-HT4 receptor agonists, or 5-HT1A, 5-HT3 and 5-HT1B antagonists improves memory and facilitate learning in situations involving a high cognitive demand. On the contrary, 5-HT2A/2C and 5-HT4 receptors antagonists, or 5-HT1A, 5-HT3 and 5-HT1B receptors agonists have opposite effects (Cifariello *et al*., 2008).

2.4.3 Sialic Acid

Sialic acid is an acidic monosaccharide and is expressed most frequently at the end of glycan chains to provide a negative charge on the cell surface. The two most prevalent species of sialic acids existing in nature are *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc) (Inoue, *et al*., 2010). Neu5Ac is abundantly contained in the mammalian brain and plays crucial roles in many neural functions such as memory, synaptic plasticity, brain development and neurological disorder (Minami & Suzuki, 2012; Schnaar, *et al*., 2014). In contrast, Neu5Gc is synthesized via the effect of cytidine monophospho-*N*-acetylneuraminic acid hydroxylase (CMAH), which catalyzes the conversion of cytidine 5'-monophosphate Neu5Ac (CMP-Neu5Ac) to CMP-Neu5Gc. Since mRNA of CMAH is not expressed in the mammalian brain, Neu5Gc exists in the brain only at a trace level (Davies, *et al*., 2012). Although humans are unable to synthesize Neu5Gc due to a CMAH gene mutation, Neu5Gc expression has been reported in normal

human cells and various human cancer cells. Neu5Gc is contained in foods, especially in red meat (Samraj, *et al*., 2015).

Gangliosides are sialic acid-containing glycosphingolipids which play a role in neuronal functions. They are expressed more predominantly in nervous tissue and are particularly abundant in brain, where they constitute 10%–12% of the lipid matter of the neuronal membrane and are largely concentrated in the grey matter (Kolter, 2012). Gangliosides are situated in the outer leaflet of plasma membranes where they are anchored by their ceramide lipid moiety with the glycans extending into the extracellular space. Although gangliosides are found in other food products, milk products provide a convenient, natural and relatively rich source for supplementation (Palmano *et al*., 2015). Human breast milk is a natural source of gangliosides for the neonate

Among the gangliosides, tetrasialoganglioside GQ1b shows neurotrophic factor-like actions, such as increasing neurite outgrowth, cell proliferation, and long-term potentiation (Shin, *et al*., 2014).

2.4.3.1 Memory Related Roles of Gangliosides

It is widely accepted that gangliosides play a critical role in neuronal function and brain development, affecting such processes as neurotransmission, neurogenesis, synaptogenesis, modulating synaptic transmission, cell proliferation, and neuronal differentiation (Nowycky *et al*., 2014). Many of these processes are fundamental to the so-called neuroplasticity of the brain i.e. the ability of the brain to undergo the activity-dependent functional and morphological remodeling which subserves learning and memory.

In a study on isolated neurons indicated an enrichment of gangliosides in terminal axons and synaptic endings, suggesting a role in formation, development and maintenance of the

nervous system. There are also implications of a role of gangliosides in other conditions including infection, inflammation, insulin metabolism and cancer (Bisel, *et* al., 2014). From a therapeutic point of view, clinical trials have shown that injected ganglioside GM1a was able to remediate symptoms of Parkinson's disease (Schneider *et al.,* 2013).

Gangliosides play a pivotal role in modulating ion channel function and receptor signaling, both of which are key functions in neuronal excitability and synaptic transmission. In particular, GM1a has been implicated in maintaining neuronal viability, conduction velocity and excitability through regulation of Na+ channels, and in combination with GD1a, neuronal Ca2+ homeostasis (Nowycky *et al*., 2014).

Interactions between brain gangliosides, calcium, and functional membrane proteins (ion channels, ion pumps, receptors, kinases) are thought to play a crucial modulatory role in the transmission and storage of information involved in memory, which forms the basis for learned behaviours and higher order cognitive processing (Cooke and Bliss, 2006).

Gangliosides support the formation and stabilization of functional synapses and neural circuits required as the structural basis of memory, and in some studies, mostly employing GM1a, they have been shown to protect against age- or lesion-induced memory deficit in animals when administered exogenously (Wang, 2012). Synapse strength can be modified over time according to level of stimulation and is this synaptic plasticity, along with structural remodeling of neural networks activated during learning that is thought to be the underlying mechanism for learning and memory (Nonaka *et al*., 2014).

Gangliosides GM1a and GD1b have been shown to improve synaptic plasticity in the CA3 hippocampal region, a region most closely associated with spatial learning and memory, via prolonged post-tetanic potentiation. Further, GQ1b has been functionally implicated in

long term potentiation (LTP), one of the key types of synaptic plasticity in which the expression of long-lasting activity-dependent synaptic modifications occur in response to periods of high frequency stimulation (Murphy *et al*., 2014). In particular, GQ1b was shown to participate in activity-dependent LTP in CA1 neurons of guinea pig hippocampal slices, through modulation of NMDA receptors/Ca2+ channels, while transgenic mice with decreased levels of GQ1b exhibited learning impairment and an altered synaptic plasticity, as evidenced by an attenuation of the induction of LTP in hippocampal CA1 neurons (Ikarashi *et al*., 2011).

2.4.4 Effects of Oxidative Stress on the Physiological Function of Neuronal Membranes

Numerous experimental and clinical observations suggest that reactive oxygen species (ROS) play a significant role in several pathological conditions of the central nervous system where they directly injure tissue and where their formation may also be a consequence of tissue injury. Reactive oxygen metabolites are particularly active in the brain and neuronal tissue, and they are involved in numerous cellular functions, including cell death and survival. In comparison with other organs of the body, the brain may, for a number of biochemical, physiological and anatomical reasons, be especially vulnerable to oxidative stress and ROS mediated injury. A high metabolic rate (the brain consumes approximately 20% of total-body oxygen) and an abundant supply of transition metals, make the brain an ideal target for free radical attack (Gutowicz, 2011). In addition, the brain has high susceptibility to oxidative stress due to high polyunsaturated fatty acid content and relatively lower regenerative capacity in comparison with other tissues. On the other hand, the brain is poor in catalytic activity and has moderate amounts of glutathion

peroxidase and superoxyde dismutase. Of all the cell types in the body, neuronal cells may be among the most vulnerable to oxidative stress. These cells are continuously exposed to ROS. Accumulating evidence demonstrating that the defense of nerve cells against ROS mediated oxidative damage is essential for maintaining functionality of nerve cells. Because hydrogen peroxide (H_2O_2) is the peroxide generated in the highest quantity in the brain, the defense against the oxidative stress appears to be particularly important. When production exceeds antioxidant protection, oxidative stress leads to molecular damage. An important component of the cellular detoxification of ROS is the antioxidant glutathione (GSH) (Dringen and Hirrlinger, 2003). Because neurons have limited antioxidant capacity, they rely heavily on their metabolic coupling with astrocytes to combat oxidative stress. Evidence is growing that glutathione plays an important role in the detoxification of H_2O_2 and organic hydroperoxides in the brain and that glutathione is the main antioxidant molecule in neurons (Haskew-Layton *et al*., 2010; Limon-Pacheco and Gonsebat, 2010). Ongoing studies have shown that neuron-glial compartmentalization of antioxidants is critical for neuronal signaling by H_2O_2, as well as neuronal protection. The neurons are more vulnerable to oxidative stress than astrocytes due to an insufficient detoxification of ROS via their glutathione system (Martin and Teismann, 2009). But, the concentration of glutathione is in relatively lesser quantities in the brain in comparison to the other organs of the body. In contrast to other ROS, H_2O_2 is neither a free radical nor an ion, which limits its reactivity. However, in the presence of transition metals such as iron or copper, H_2O_2 can give rise to the indiscriminately reactive and toxic hydroxyl radical ($HO^{\bullet}$) by Fenton chemistry. H_2O_2 is able to diffuse across biological membranes, and therefore can diffuse freely from a site of generation (Bienert *et al*., 2007;) so that it is well-suited as a

diffusible messenger. Increasing evidence indicates that H_2O_2 is a particularly intriguing candidate as an intracellular and intercellular signaling molecule because it is neutral and membrane-permeable (Forman *et al*., 2010). In a research into mechanisms of ROS-induced modifications in ion transport pathways involves: oxidation of sulfhydryl (SH) groups on the ion transport proteins, lipid peroxidation, and alterations of calcium (Ca^{2+}) homeostasis, a major second messenger system (Kourie, 1998). Increases in Ca^{2+} initiate inappropriate activation of several enzyme systems e.g., nitric oxide synthase and phospholipase A2. Over activation of these enzymes results in the breakdown of proteins and phospholipids and initiates several cascades that damage cells (Lee *et al*., 1999). It has been described that elevation in cytoplasmic Ca2+ levels activates the mitogen-activated protein kinase (MAPK) cascade (Son *et al*., 2011) and the phosphatidylinositol 3′-kinase (PI3K)-Akt pathway. ROS produce cell damage through multiple mechanisms, including excitotoxicity, metabolic dysfunction and disturbance of intracellular homeostasis of Ca^{2+} (Bracci, 1992). Activation of glutamate ionotropic receptors promptly triggers membrane depolarization and Ca^{2+} influx, resulting in the activation ofseveral different protein kinases (Ca^{2+} -calmodulin dependent kinase, protein kinase C and MAPK) and transcription factors, such as cyclic AMP response element binding protein (CREB). Neurons efficiently repair glutamate-induced oxidative DNA damage by a process involving CREB-mediated up-regulation of a purinic endonuclease 1 (APE1) (Yang *et al*., 2011).

2.5 Piracetam

Piracetam is a cyclic derivative of gamma-aminobutyric acid (GABA). Since its discovery in the 1960s, it has been widely used in humans (Platt *et al*., 1993; Waegemans *et al*.,

2002; Ricci *et al*., 2006; Tkachev, 2007; Zavadenko, and Guzilova, 2009) and rodents (Bhattacharya *et al*., 1993; Park *et al*., 2010) as a memory-enhancing (nootropic) agent. With low toxicity and few side effects, piracetam is effective in treating dementia and cognitive impairment (Waegemans *et al*., 2002; Tkachev, 2007) stroke (Ricci *et al*., 2006) and ischemia (Platt *et al*., 1993). Piracetam modulates neuroplasticity, neuroprotection and brain metabolism (Winnicka *et al*., 2005), has anticonvulsant effects (Fedi *et al*., 2001), as well as reduces symptoms of clinical depression, anxiety and alcohol withdrawal (Dencker *el al*., 1978; Malykh, and Sadaie, 2010). Piracetam has also been extensively tested in various rodent models. In addition to nootropic activity (Kaluef, 1996; Muller, 1999), animal anxiety like behavior has been found to be sensitive to this drug. For example, acute piracetam reduces anxiety in rat social interaction (File and Hyde, 1979) and in rabbit conflict tests (Voronina *et al*., 2000). Similarly, mild chronic doses of piracetam reduce rat anxiety in the open field, elevated plus-maze, foot shock-induced fighting (Bhattacharya *et al*., 1993) and Vogel's conflict tests (Millan and Brocco, 2003).

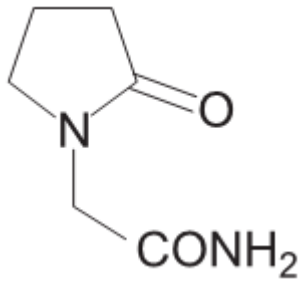


Figure 2.2. Structure of piracetam (2-oxo-1-pyrrolidineacetamide) ñ prototypical nootropic; acyclic derivative of GABA (Guzilova, 2009).

Piracetam, the prototype of the so-called “nootropic” drugs is used in many countries to treat cognitive impairment in aging, brain injuries, as well as dementia (Muller *et al*., 1999; Winblad, 2005). A recent meta-analysis of all available (published and not published) clinical studies provided compelling evidence for the global efficacy of piracetam in a diverse group of older subjects with cognitive impairment (Waegemans *et al*., 2002).

Similar to the situation in man, piracetam has also been shown to improve cognitive function in animals, but its mode of action is not yet finally known (Winblad, 2005). Piracetam's improving effects on the fluidity of aged synaptosomal membranes could explain the beneficial effects of piracetam on age-related deficits on several mechanisms of signal transduction such as receptor density and function, and transmitter release, since these mechanisms are disturbed in the aging brain probably due to a decrease of membrane fluidity (Stoll *et al*., 1992; Viana *et al*., 1992; Cohen and Muller, 1993; Scheuer *et al*., 1999). On the other hand, initial evidence that piracetam's beneficial effects on the fluidity of aged mitochondrial membranes could contribute to its therapeutic efficacy originated from observations that piracetam could improve glucose uptake and utilization as well as ATP production (Dormehl *et al*., 1999; Keil *et al*., 2006). These effects led to the term “metabolic enhancer,” sometimes used to characterize piracetam and related nootropics (Malik *et al*., 2007). However, the mechanism of these effects and its possible relationship to mitochondrial function remained obscure.

2.5.1 Piracetam and Oxidative Stress

It has been presented clearly that piracetam protects mitochondria against different conditions associated with oxidative stress including aging. Piracetam's protecting effects on mitochondrial damage induced *in vitro* are small, but reproducible and highly

significant. This is not surprising, since the conditions used to induce oxidative stress *in vitro* are not pathophysiological but rather aggressive, in contrast to the small and slowly occurring changes induced by aging, which however were sometimes completely reversed by piracetam treatment. This was also the case for the adaptive elevation of antioxidant enzyme activities. When mild conditions were used *in vitro* (e.g. partial serum deprivation), a complete protection of mitochondrial function was seen by piracetam treatment in PC12 cells. Moreover, piracetam was highly effective *in vivo* in pathophysiologically relevant situations of brain dysfunction (Keil *et al*., 2006).

Piracetam does not possess radical scavenging properties. Thus, it seems quite likely that piracetam acts directly at the mitochondrial level, presumably by improving mitochondrial membrane properties (Keil *et al*., 2006). This is also supported by an observation that in several experiments protective effects were also seen in the recovery phase, when the oxidative stress or was already removed.

2.6 CANNABIS SATIVA PLANT.

Cannabis sativa is a member of the family Cannabinaceae. *Cannabis sativa* preparation is known by various names worldwide. It is called Marijuana in America; Bhang, Ganja and Charas in India; Kif in North Africa; Dogga in South Africa; Krori in Tunisia, Habak in Turkey; Hashish in Middle East; Djomba or Liamba in Central Africa and Brazil; Sodom, Tampl, Gum, Gauge and stuff in Kinshasa, Swala and Whiskt in Ghana;Grifa in Mexico and Ma- cohna in some parts of South America (Sachindra and Pradhan, 1977).

The chemical definition encompasses a variety of distinct chemical classes. The classical cannabinoids are structurally related to Tetrahydrocannabinol commonly abbreviated as THC, the non classical cannabinoids, the aminoalkylindoles, the eicosanoids related to the

endo- cannabinoids, 1,5-diarylpyrazoles, quinolines and aryl- sulphonamides and additional compounds that do not fall into these standard classes but bind to cannabinoid receptors.



Plate 2: Showing fresh leaves of *Cannabis sativa* adapted from Abush and Akirav, 2012.

2.6.0 Cannabinoids

The term cannabinoids refers to a unique group of secondary metabolites found in the cannabis plant, which are responsible for the plant's peculiar pharmacological effects. There are three general types of cannabinoids; phyto cannabinoids occur uniquely in the cannabis plant; endogenous cannabinoids are produced in the bodies of humans and animals and synthetic cannabinoids which are similar compounds produced in the laboratory (Lambert and Fowler, 2005). However, it has found that *Cannabis sativa* contains over 60 cannabinoids as well as over 400 other chemicals including benzopyrene, a known carcinogen. (Malinowska *et al.,* 2012). The cannabinoids of the *C. sativa* plant include cannabidiol (CBD), cannabigerol (CBG), and cannabinol (CBN) which, in contrast to THC, are not psychoactive. CBD may activate central nervous system (CNS) limbic and paralimbic regions which can reduce autonomic arousal and feelings of anxiety; this is in contrast to THC which can be anxiogenic (Fusar-Poli, 2009).

2.6.1 Endocannabinoid System

The endocannabinoid system consists of CNS cannabinoid receptors and their endogenous ligands; ligands (Latin: binding) refers to triggering molecules that bind to a target protein site (Solinas *et al.,*2007). Endocannabinoids (endogenous cannabinoid receptor agonists) include arachidonic acid derivatives: 2-AG (2-arachidonoyl glycerol) and anandamide [arachidonoyl ethanolamide (AEA)] (Mechoulam and Parker, 2013). AEA and 2-AG are the two most researched endocannabinoids. The endocannabinoid system is identified both as a cause of psychiatric disorders but also research suggests that proper manipulation of this system may be pharmacologically useful in management of some psychiatric disorders

– such as depression, anxiety, anorexia nervosa, and others (Marco *et al.,* 2011). For example, CBD may be beneficial in treatment of psychiatric disorders (Marco *et al.,* 2011).

The endocannabinoid system is involved in processes of brain reward that are related to drug abuse – as noted in animal and human research; this includes cue-induced relapse of drug abuse (Fattore *et al*., 2007). This CNS system is involved in various functions involving memory, emotions, movement, cell proliferation, and other important cell functions (Trezza, *et al*., 2008). Key neuron classes that express high levels of CB1 receptors are GABAergic interneurons in such CNS areas as the cerebral cortex, amygdala, and hippocampus; these areas also contain cholecystokinin, an important neuropeptide (Iversen, (2003).

2.6.2 Cannabinoid Receptors and Cannabinoid Pharmacology.

The understanding of the pharmacodynamics of the cannabinoids was greatly enhanced when specific cannabinoid receptors were identified in mammalian brain (Devane *et al*., 1992) and subsequently cloned (Matsuda *et al*., 1990). The cannabinoid CB (1) receptor (CB1R) is mainly found in the CNS and the CB (2) receptor is expressed in immune system cells and recent research has shown the importance of this receptor, not only concerning the immune system; both receptors are G-protein coupled receptors and are involved in adenylate cyclase inhibition (Guindon and Hohmann, 2011). The CB1R is a G-protein coupled receptor that is associated with most of the CNS endocannabinoid signaling (Caballero and Tseng, 2012). CB1R, also known as CNR1 and CB2R, also known as CNR2 are of importance and future research will most likely show us new information. It is widely found in the cerebellum, basal ganglia, and limbic system; the

hippocampus has the highest concentration of cannabinoid receptors. Cannabinoid receptors are found in other tissues including the heart, lungs, endocrine glands, arteries, immune system, sympathetic ganglia, gastrointestinal tract, and reproductive tract (Curr, 2005).

The activation of CB1 receptors can inhibit amino acid and monoamine neurotransmitter release. Certain lipid derivatives [i.e., 2-arachidonoyl glycerol (2-AG) and anandamide (AEA)] can function as endogenous ligands for CB 1 receptors and lead to excitation in such areas as the cerebellum and hippocampus by inhibition suppression (Iversen, 2003).

CB1 receptors were identified in the central nervous system (CNS) and certain peripheral tissues. The CB1 were identified as belonging to a so-called superfamily of G-protein-linked receptors (Matsuda *et al*., 1990). G-protein-linked receptors characteristically exhibit a seven-folded structure which spans the cell's plasma membrane. In some such receptors the ligand attaches to a domain (a section of the polypeptide chain) that is entirely exposed on the outer surface of the cell. Others ligands will have domains which include parts of the chain deep within the cell membrane. The binding of a ligand signals any one of several activities within the cell. Over half of all known drugs work through G-protein receptors (Alberts *et al*., 2002). These receptors include three protein sub-units – α, β and γ. In the unstimulated state of the so-called 'two state model of constitutive activity' no ligand is bound to the receptor 'R' and guanosine diphosphate (GDP) is bound to the α-subunit. This conformation the G protein is termed RGGDP. Upon stimulation, this sub-unit releases its GDP allowing Guanosine 5'- triphosphate (GTP) to bind in its place. This conformation of the G protein is termed R*GGTP (Ross, 2007). As a consequence, the αβγ complex dissociates into an α subunit and a βγcomplex. The now-free α subunit is now

able to change its shape and interact with target proteins. The α subunits have intrinsic slow hydrolase activity, and as a result the GTP spontaneously hydrolyses to GDP with the release of one phosphate moiety. In doing so, the Gprotein resets itself back to the rest position. Within the cell this 'two state' model of constitutive activity suggests that an equilibrium exists: -

RGGDP ↔R*GGTP (Ross, 2007).

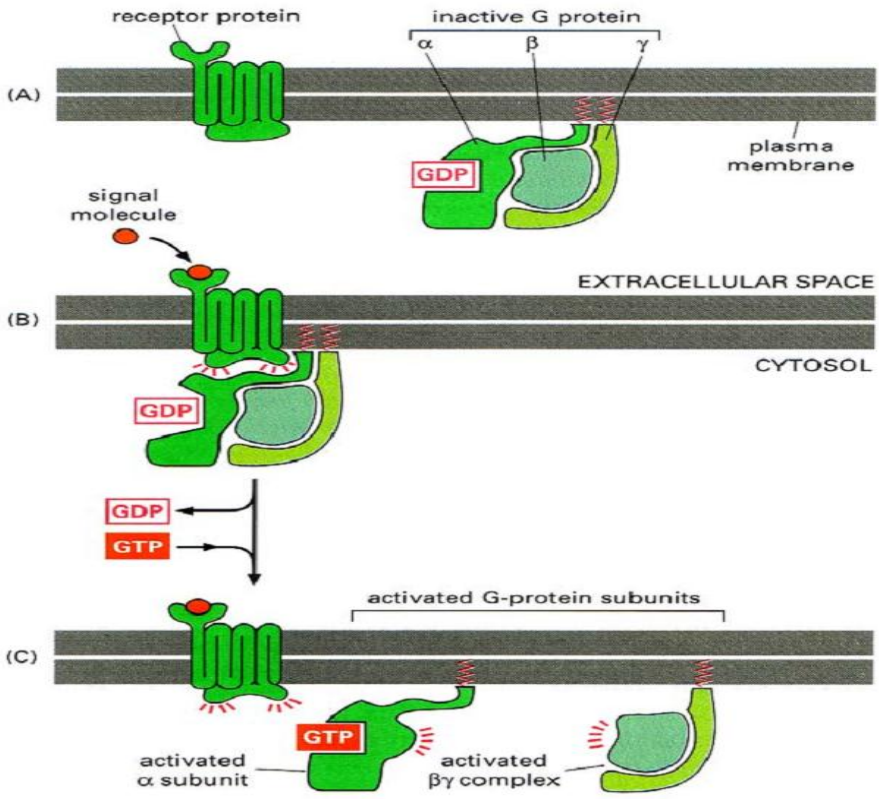


Figure 2.3: The disassembly of an activated G-protein into two signalling components. (Alberts *et al*., 2002).

The interaction of phytocannabinoids or endocannabininoids with cannabinoid receptors is illustrated schematically in Figure 2.5. In addition to being present in the central nervous system and throughout the brain, CB1receptors are also found in the immune cells and the gastrointestinal, reproductive, adrenal, heart, lung and bladder tissues. By altering the cell activity within such a wide range of tissues, several prominent pharmacological responses result from the agonism of CB1receptors, including both the control of nociceptive and neuropathic pain. CB2receptors are thought to have immunomodulatory effects and to regulate cytokine activity and thereby altering cell-to-cell communication. In combination with CB1receptors, they show pain control effects (Pertwee, 2004).

While many of the pharmacological properties of cannabis can be explained by interactions of its ingredients with the CB1and CB2 receptors, not all of the effects can be explained this way. Cannabidiol has shown significant levels of antipsychotic activity, which are seen as advantageous in medicines containing Δ-9 THC, but this cannot be totally explained by interactions with the cannabinoid receptors as CBD is a weak ligand (Iversen, 2008). Both CBD and Δ-9 THC have been shown to have equally potent neuroprotective antioxidant properties in rat cortical neuron cultures, where glutamate otherwise reached toxic levels (Hampson *et al*., 1998).

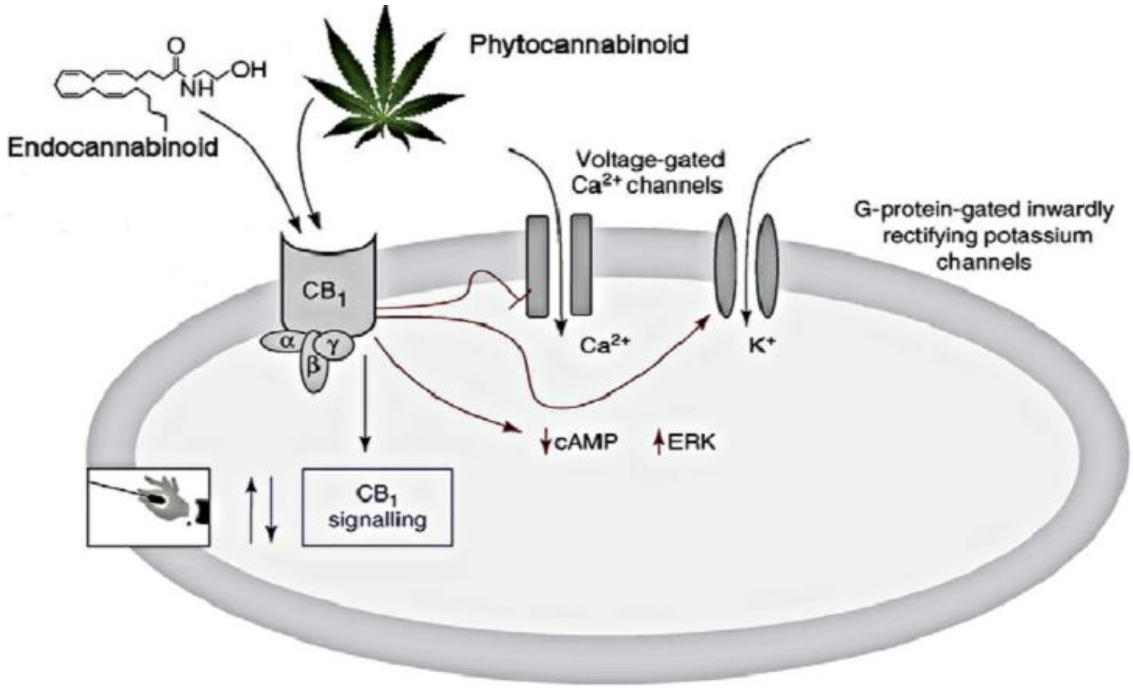


Figure 2.4: The interaction of phytocannabinoids and/or phytocannabinoids with the CB1 receptor in the eukaryote cell and the consequent effects on cAMP and ERK activity and ion channelling. Adopted from Ross (2007).

2.5.3 Medical benefits of *Cannabis sativa*

It has been reported that cannabinoids show a variety of therapeutic effects against chronic pain and muscle spasms, nausea and anorexia caused by HIV treatment, vomiting and nausea caused by cancer chemotherapy as well as anorexia associated with weight loss caused by immune deficiency syndrome (Borgelt *et al*., 2013; Lutge *et al*., 2013). Many studies report that THC provides protection against neuronal injury in a cell culture model of Parkinson disease and experimental models of Huntington disease, exhibits anti-oxidative action and mitigates the severity of the autoimmune response in an experimental model of diabetes (Carroll *et al*., 2012).

Among the effects of *Cannabis sativa* is a study that shows its effects on oxidative stress, where it alters oxidative balance in the brain. The study suggests that cannabis sativa appears to in favor of reducing lipid peroxidation. In the same study, it was discovered that repeated administration of CBE increased brain levels of serotonin, noradrenaline and dopamine. Also, the study indicated impairment of learning and memory with repeated administration of CBE in graded doses (Abdel-Salam *et al*., 2013).

When people abuse cannabis, a series of poorly understood neuroplastic changes take place. Light users of cannabis have lower basal brain-derived neurotrophic factor (BDNF) and neuregulin levels. THC produced psychotomimetic effects, perceptual alterations, and "high" spatial memory impairments. The effects of cannabinoids on BDNF suggest a possible mechanism underlying the consequences of exposure to cannabis. This may be of particular importance for the developing brain and also in disorders believed to involve altered neurodevelopment such as schizophrenia (Shrivastava *et al*., 2014).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Experimental Animals

Seventy five (75), mice of both sexes were purchased from Pharmacology Department Animal House, Faculty of Pharmaceutical sciences, Ahmadu Bello University, Zaria, Kaduna, Nigeria. The animals were kept in the Department of Human Physiology animal house and maintained in standard laboratory condition at room temperature, humidity and under twelve hour dark-light cycle. The animals were fed on standard feed Grower mash diet and water. The animals were allowed to acclimatize to their new environment for 2 weeks prior to commencement of the administration.

3.1.2 Drug

Piracetam (70mg/tablet) produced by Micro Labs Limited, was obtained from a reputable pharmaceutical store (M.U.B Pharmaceutical Enterprises Ltd.) in Sabon Gari, Zaria, Kaduna state and used as standard drug for memory enhancer. Acridine orange and ethidium bromide were bought from sigma Aldrich Co. UK. All other chemicals used were analytic grade.

3.1.3 Chemicals, Reagents and Solvents.

i. Chloroform (Sigma-Aldrich Co. LLC St. Louis, USA)
ii. Phosphate Bufffer Saline (PBS)
iii. 70% Ethanol (Sigma-Aldrich Co. LLC St. Louis, USA)
iv. Distill Water, measuring cylinders, beakers

3.1.4 Equipment and other materials

i. Haematocrit centrifuge (Denley BS400 centrifuge, England)
ii. Automated biochemistry analyzer (Selectra XL, Vital Scientific, Netherlands)
iii. Electronic weighing machine (Precision Electronic instrument Company, India)
iv. Cotton wool, Hand gloves, Nose masks
v. Dissecting set, cannula, 1ml and 5ml syringes and needles
vi. Cages and feeders
vii. Spatula, pestle, dissecting kit and mortar
viii. Microplate reader
ix. 0.1% BSA (Bovine Serum Albumin)
x. 3K-10K Centrifugal Filter (for serum samples)
xi. Heparin or citrate (for plasma samples)
xii. Triton X-100

3.1.5 Plant Material

The dry leaves of *Canabis sativa* were obtained under permission from Nigeria Drug Law and Enforcement Agency in Zaria, Kaduna State. Identification and Authentication of the Leaves was done by a taxonomist at Herbarium Unit by Mr. Namadi Sanusi, Department of Biological Science Ahmadu Bello University Zaria. The voucher specimen number is 2438. The dried leaves were pulverized in to a coarse powder using pestle and mortar. The powdered sample was stored in an air tight container until needed.

3.2 Methods

3.2.1 Extraction of *Canabis sativa* Leaves

To 500g of powdered leaf material, two (2) liters portion of cold distilled water was added and then stirred in a conical flask until evenly mixed. The mixture was left to stand for twenty four (24) hours with occasional shaking and stirring. After the set time, the mixture was filtered using muslin cloth followed by whatman filter paper. The resultant filtrate was concentrated on a steam bath maintained at 50ºC until all the moisture evaporated and a constant weight was obtained. The weight of the crude extract was taken and the percentage yield was calculated. The extract was stored in a sample bottle, labeled and kept in desiccators until required. The extract was prepared fresh on each day of the experiment by reconstitution in distilled water. The extract is henceforth referred to as aqueous extract of *Cannabis sativa* (AECS).

3.2.2 Acute toxicity (LD_{50}) study.

Acute toxicity of *Cannabis sativa* extract was determined using the method described by Lorke (1983) and was used to determine the oral median lethal dose (LD_{50}). The study was carried out in two phases. In phase 1; nine (9) mice of either sex were randomly divided into three groups of three rats per group. The animals in the three groups were administered graded doses (10mg/kg, 100mg/kg and 1000 mg/kg body weight respectively) of the extract per oral with a cannula. In phase 2; based on the results of the phase 1 study, three (3) rats were divided into three groups of one rat per group and were administered orally in single graded doses 1600mg/kg, 2,900mg/kg and 5,000 mg/kg body weight of the extract. In each of the two phases of the study, the mice were kept under the same condition and observed for signs of toxicity and mortality for 24 hours. The LD_{50}

was then calculated from the results of the final phase as the square root of the product of the lowest lethal dose and the highest non-lethal dose, i.e the geometric mean of the consecutive doses which 0 and 100% survival rates were recorded (figure 3.0).

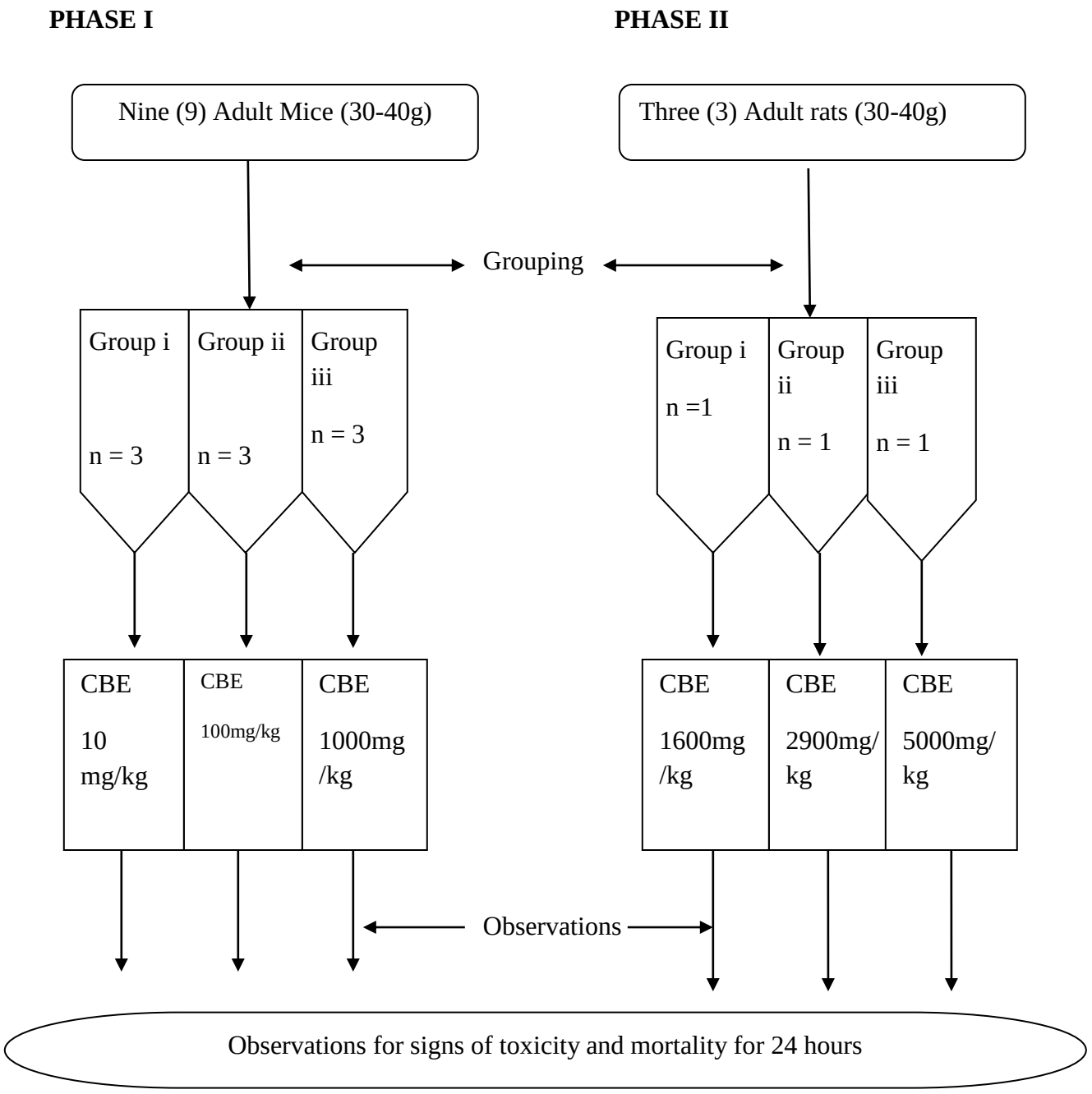


Figure 3.1: Experimental design for Acute Toxicity Study

3.3 Grouping of Research animals and Experimental Designed

The mice, irrespective of sex, between 2-3 months of age with body weight ranging between 30g to 40g were distributed in three different model for Assessing of Learning and Short Term Memory (Morris Water Maze, Elevated Plus Maze and Y-maze) and model has five groups (with five animals in each group) shown in figure 3.1.

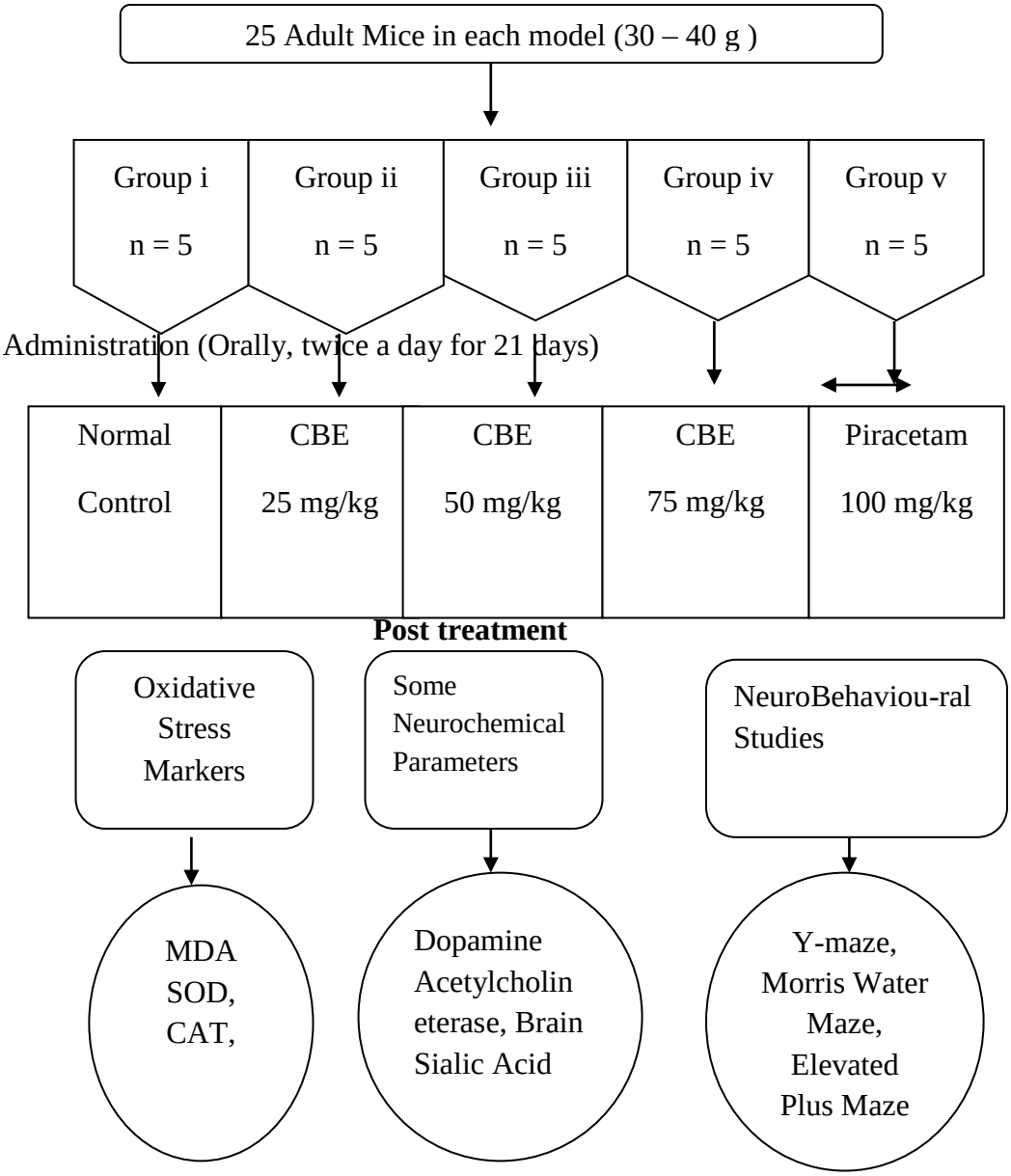


Figure 3.2: Experimental Design for Sub-chronic Study

3.4 Surgical Removal of the Brain and Brain Tissue Preparation for Assay

Mice brain tissues were collected and prepared according to the method described by Zatta *et al.* (2002) and Habila *et al.,* (2012). Treated and control animals were sacrificed by decapitation under anesthesia and brain tissue was immediately removed and placed on an inverted Petri dish on ice. The fore brain was dissected, weighed and homogenized in 10 ml of a medium containing a solution of 0.1M sodium phosphate 10 % (W/V), pH 7.5. The total homogenate was centrifuged at 1000 rev/min in a refrigerated centrifuge for 7 minute. Aliquots of resulting brain homogenates were stored at -20^0 C until utilization. The supernatants were used for some biochemical parameters assay.

3.5 Determination of Oxidative Stress Indicators

Brain tissue was excised, washed in ice-cold saline, and then homogenized in phosphate buffer Saline (pH 7.4) using a homogenizer. The Brain homogenate was then centrifuged at 5,000 x g to remove cellular debris; the supernatant was then used for the determination of oxidative stress indicators. The following oxidative stress indicators were measured: malodialdehyde (MDA), catalase (CAT) activity, superoxide dismutase (SOD) activity by a method described by Shull *et al*., (1991), Fridovich (1989) and Ellman (1959) as described by Rajapopalan *et al*., (2004) respectively.

3.5.1 Effect of Treatments on Brain Lipoperoxidation

The level of thiobarbituric acid reactive substance, malondialdehyde (MDA), as an index of lipid peroxidation was evaluated in brain sample using the method of Draper and Hadley (1990) as modified by De Freitas *et al.* (2005). The principle of the method was based on spectrophotometric measurement of the color developed during the reaction of thiobarbituric acid (TBA) and malondialdehyde (MDA). Briefly, 1 ml of the supernatant

obtained in 3.4 was mixed with 1 ml 10% trichloroacetic acid and 1 ml 0.67% thiobarbituric acid. The mixture was heated in a boiling water bath for 15 min and butan-2-ol (2:1, v/v) added to the solution. After centrifugation at 800g, for 5 min, the MDA concentration was determined from the absorbance taken at 532 nm. The MDA concentration in each sample was calculated by the absorbance coefficient of MDA-TBA complex 1.56×10^5/cm/M and expressed as nmol/mg of tissue protein. The concentration of protein in the brain homogenates was evaluated using the Lowry method (Lowry *et al.*, 1951).

3.5.2 Determination of Superoxide Dismutase activity

Superoxide Dismutase (SOD) was determined by a method described by Fridovich (1989).

Principle: the ability of superoxide dismutase (SOD) to inhibit auto oxidation of adrenaline at pH 10.2 forms the basis of this assay.

Procedure: Supernatant (0.1 ml) was diluted in 0.9ml of distilled water to make 1:10 dilution of microsome. An aliquots mixture of 0.2ml of the diluted microsome was added to 2.5 ml of 0.05 M carbonate buffer. The reaction was started with the addition of 0.3ml of 0.3 mM adrenalin. The reference mixture contained 2.5 ml of 0.05 M carbonate buffer, 0.3 ml of 0.3 mM adrenalin solution and 0.2 ml of distilled water. Absorbance was measured over 30s up to 150s at 480nm.

Calculations: increase in absorbance per minute = (A5-A1)/2.5

$$\% \text{ Inhibition} = 100 - \frac{\text{Increase in absorbance for substrate}}{\text{Increase in absorbance of blank}} \times 1000$$

1unit of SOD activity is the quantity necessary to elicit 50% inhibition of the oxidation of adrenaline to adenochrome in 1 minute.

3.5.3 Determination of Catalase (CAT) activity

Principle: It is based on the rate of disappearance of H_2O_2 as it generates H_20 and O_2.

Catalase (CAT) activity was measured using Abei's method (1974), Exactly 10µL of supernatant was added to test tube containing 2.8 mL of 50 mM potassium phosphate (buffer pH 7.0). The reaction was initiated by adding 0.1 ml of prepared 30 mM H_2O_2 and the decomposition rate of H_2O_2 was measured at 240nm for 5minute in a spectrophotometer. A molar extinction coefficient (E) of 0.041mM-1cm-1 was used to calculate the catalase activity.

3.6 Determination of Brain Sialic Acid Concentration

The concentration of free, bound and total sialic acid will be determined according to the method described by Aminoff (1961).

3.6.1 Procedure for determination of Total Sialic Acid Concentration in the Mice

The brain tissue homogenate (0.25 mL) was dispensed into a test tube. This was followed by 0.25 mL of 0.1 N H_2SO_4 into the test tube, the mixture was boiled at 80ºC for 1 hr. After 1 hr of boiling, 0.125 mL of sodium periodate was added. Thereafter, the mixture was incubated in a water bath set at 37ºC for 30 minutes. After incubation in the water bath, 0.1 mL of sodium arsenite was added and the mixture was vortexed. One (1) mL of thiobarbituric acid (TBA) was added and the mixture will be covered and heated in a boiling water bath for 8 minutes to developed a pink colour. The mixture was rapidly cooled in a water bath set at 37ºC. Acid butanol (Butan-1-ol containing 5% V/V of 12 N HCl) mixture (2.5 ml) was added, the mixture shaken and then centrifuged at 6000 rpm for 10 minutes. Absorbance was then taken at 549 nm wavelength.

The concentration of bound sialic acid was determined through the same process as described for total sialic acid above. The concentration of the free sialic acid was determined by subtracting the bound sialic acid from total sialic acid.

3.7. Acetylcholinesterase Assay

This was carried out using Acetylcholinesterase Assay Kit.

Briefly, 0.6 ml of assay buffer was added into the vial of DTNB to make 20×DTNB stock solution. Then, 0.6 ml of distilled water was added into the vial of acetylthiocholine to form acetylthiocholine stock solution (reaction mixture). Thereafter, 100µl of distilled water with 0.1% BSA (Bovine Serum Albumin) was added into the acetylcholinesterase standard solutions. Serial dilutions were conducted to form 1000, 300, 100, 30, 10, 3, 1 and 0 mU/ml of the acetylcholinesterase standard. These dilution solutions and acetylcholinesterase-containing test samples were put into white bottom 96-well microplate. Fifty (50) µl of acetylthiocholine reaction mixture was added to each well of the acetylcholinesterase standard, blank control, and test samples to make the total acetylcholinesterase assay volume of 100µl/well. The reaction was protected from light and was incubated for 30minutes at room temperature. The samples were then put in microplate reader at wavelength of 450nm and the absorbance increase was recorded. Graph of absorbance was plotted against acetylcholinesterase enzyme concentration in mU/ml using the standard curve described in the protocol. Values of acetylcholinesterase enzyme concentration above 1000mU/ml were considered as zero. Decrease in acetylcholinesterase enzyme concentration is an indicative of increased cholinergic transmission and improved memory and vice-versa (Meerah and Mamatha, 2015).

3.8 Learning and Memory Assessments

3.8.1 Assessment of Learning and Spatial Memory Using Morris Water Maze

MWM testing was conducted in a round white pool 94 cm in diameter and 31 cm deep. The pool was filled to a depth of 30 cm with water made opaque with white non-toxic water-based tempura paint. Pool temperature was maintained at 25 ± 0.5°C by addition of warm water. The escape platform was a 25-cm^2 Plexiglas square, placed in the center of one quadrant of the pool, 15 cm from the pool's edge and submerged 1 cm beneath the water surface. The platform remained in the same position throughout the learning trials and visual cue tests and was removed from the pool during the probe test. Several distal extra-maze cues (a traffic cone, a colorful poster, and two black-and-white construction paper designs) were placed around the pool and these remained in the same position throughout the training and testing periods.

A trial began by placing the mouse on the platform for 20 s to allow orientation to extra-maze cues. After orientation, mice were gently lowered tail-first into the pool facing the wall at one of three positions, each at the center of the wall of a different quadrant not housing the platform. After the mouse was released, the researcher retreated away from the pool to a constant position within the room, serving as an additional distal visual cue. The SMART digital tracking system (Version 2.5, Panlab, Barcelona, Spain) simultaneously began recording the trial. Maximum swim time was set to 60 s. If the mouse located the platform before 60 s had passed, it was immediately removed from the pool. If the platform was not located after 60 s of swimming, the mouse was gently guided to the platform and allowed to re-orient to the distal visual cues for an additional 20 s before being removed from the pool. After removal from the pool, mice were manually dried with

a terrycloth towel and placed in a warming cage (consisting of a heating pad set to low underneath a typical shoebox cage) for at least 5 min before returning to the home cage. Mice were visually inspected to ensure thorough dryness. Mice were tested in two trials per day with an inter-trial interval of



Plate III: Morris water maze model snapped by the candidate during experiment.

approximately 30 min. All testing was conducted at roughly the same time each day in order to minimize variability in performance due to time of day.

To examine spatial reference memory, a probe test was administered 24 h after the last training session. During the probe test, the platform was removed from the pool and the mouse was allowed to swim freely for 1 min. A visual cue test was conducted 30 min after the probe test to assess sensorimotor ability and motivation (D'Hooge and Deyn, 2001). For this test, the platform was set 1 cm above the water level and marked with black tape so that the mice could locate the platform using a local visual stimulus rather than relying on spatial orientation to extra-maze cues.

After completion of the visual cue test, all tracks from all trials were analyzed for a number of behavioral parameters using SMART software (Panlab). The resultant behavioral data were statistically analyzed as described below.

3.8.2 Assessment of Learning and Short Term Memory Using Elevated Plus Maze

The elevated plus maze is a plus shaped apparatus with four arms at right angles to each other as described by Handley and Mithani (1984). The two open arms lie across from each other measuring 25 x 5 x 5 cm and perpendicular to two closed arms measuring 25 x 5 x 16 cm with a center platform (5 x 5 x 0.5 cm). The closed arms have a high wall (16 cm) to enclose the arms whereas the open arms have no side wall. Following administration of Cannabis Sativa or vehicle, mice were placed in the central platform facing the closed arm and their behavior recorded for 5 min. Base upon the early studies by Montgomery (Pellow and File, 1997). The criterion for arm visit was considered only when the animal decisively moved all its four limbs into an arm. The maze

was cleaned with 5% ethanol after each trial. The elevated plus maze relies upon rodents' proclivity toward dark, enclosed spaces

(approach) and an unconditioned fear of heights/open spaces (avoidance) (Pellow and File, 1997). The percentage of time spent in the arms was calculated as time in open arms or closed arm/total time x100, the number of entries into the arms was calculated using number of entries into open or closed arms/total number of entries (Hogg, 1996).



Plate IV: Elevated plus maze snapped by the candidate

3.8.3 Assessment of Learning, Short term and Spatial Memory Using Y-maze

It is well known that spontaneous alternation is a measure of spatial working memory. The Y -maze can be used as a measure of short term memory, general locomotor activity and stereotypic behavior (Kokkinidis *et al*., 1976). Therefore, spontaneous alternation was assessed using a Y- maze composed of three equally spaced arms (120°, 41cm long and 15cm high). The floor of each arm is made of Perspex and is 5cm wide. Each mouse was placed in one of the arm compartments and was allowed to move freely until its tail completely enters another arm. 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, for instance if the animal makes the following arm entries; ACB,CA,B,C,A,CAB,C,A, in this example, the animal made 13 arm entries 8 of which are correct alternations. 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 each animal the Y-maze testing was carried out for 5 minutes. The apparatus was cleaned with 5% alcohol and allowed to dry between sessions.



Plate V: Y maze model snapped by the candidate

3.9. Statistical Analysis

The data obtained from these studies were analysed with statistical package for social science (SPSS) software, version 20 (IBM, USA), using one-way analysis of variance (ANOVA) and Turkey's multiple comparison post hoc test which compared the level of significance between the control and test groups. All data were expressed as mean ± SEM and $p \leq 0.05$ were considered as significant. The data were presented as tables and charts as appropriate.

CHAPTER FOUR

4.0 RESULTS

4.1 Assessment of Learning and Short Term Memory Using Elevated Plus Maze

4.1.1 Acquisition and retention session

In figure 4.1 there was no significant difference (p=0.15) in the time taken for each mouse to enter the close arm during the acquisition session between control (25.20±4.99) and groups 2 (CBE 25mg/kg) and 5 (Piracetam 100mg/kg), (42.40±7.49 and 32.00±4.51 respectively), but a significant difference was observed between control (25.20±4.99) and groups 3 (CBE 50mg/kg: 60.80±7.58) and 4 (CBE 75mg/kg: 74.40±2.29) at p=0.00 as seen in Figure 4.1. Also, during the retention session, there was no significant difference observed between control (15.60±4.83) and groups 2 CBE 250mg/kg and 5 (Piracetam 100mg/kg), (29.40±8.93 and 10.60±3.05 respectively), but a significant difference (p=0.01) was observed between control (15.60±4.83) and groups 3 (CBE 50mg/kg: 42.60±5.92) and 4 (CBE 75mg/kg: 63.60±8.20).

4.2 Assessment of Learning, Short term and Spatial Memory Using Y-maze

The result of this test as shown in figure 4.2 showed no significant difference (p=0.12) in the percentage of spontaneous alteration of the mice between the control (23.39±4.02) and the cannabis treated groups (CBE 25mg/kg: 27.67±3.79, CBE 50mg/kg: 31.24±6.39, CBE 750mg/kg: 19.02±1.49). Also, no significant difference (p=0.12) was observed with the positive control group (Piracetam 100mg/kg: 25.13±4.52) as shown in figure 4.2

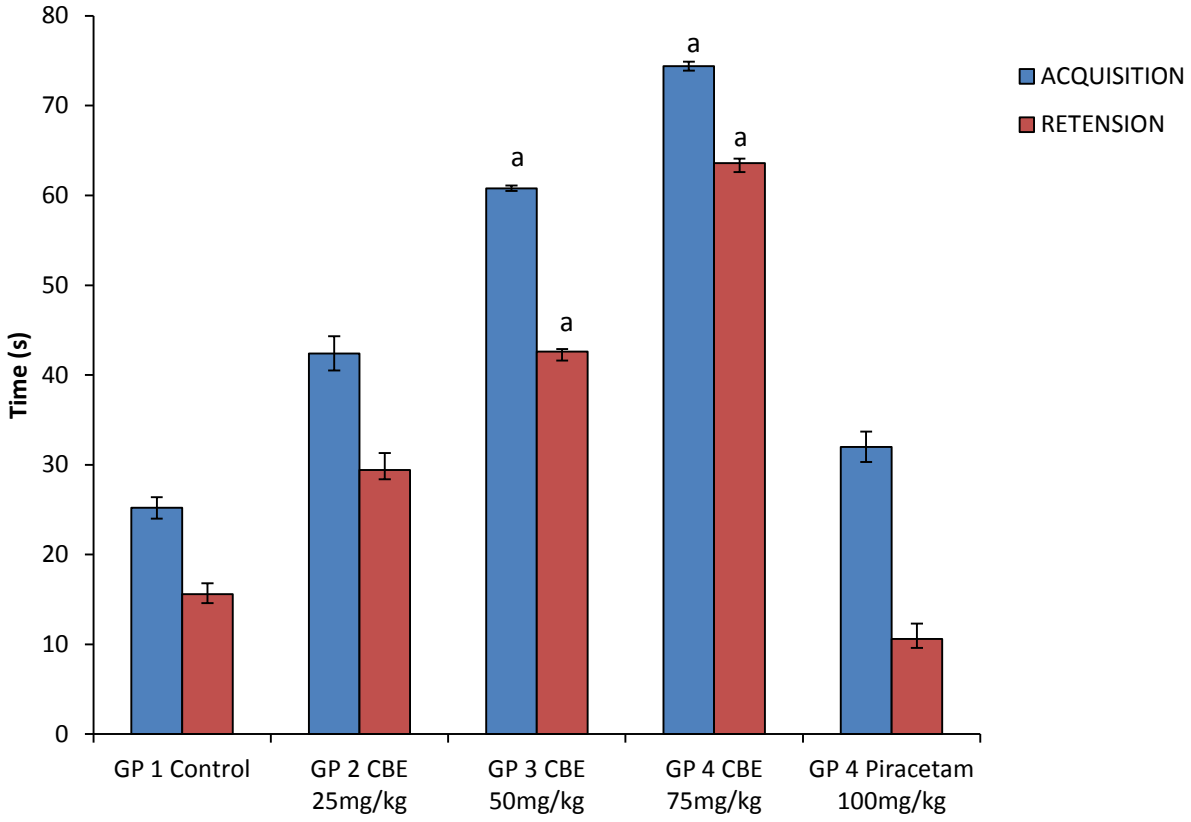


Figure 4.1: Effects of *Cannabis sativa* extract on memory using elevated plus maze
a: means statistical significance when compared with control p=0.00 (n=5) SPSS version 20.

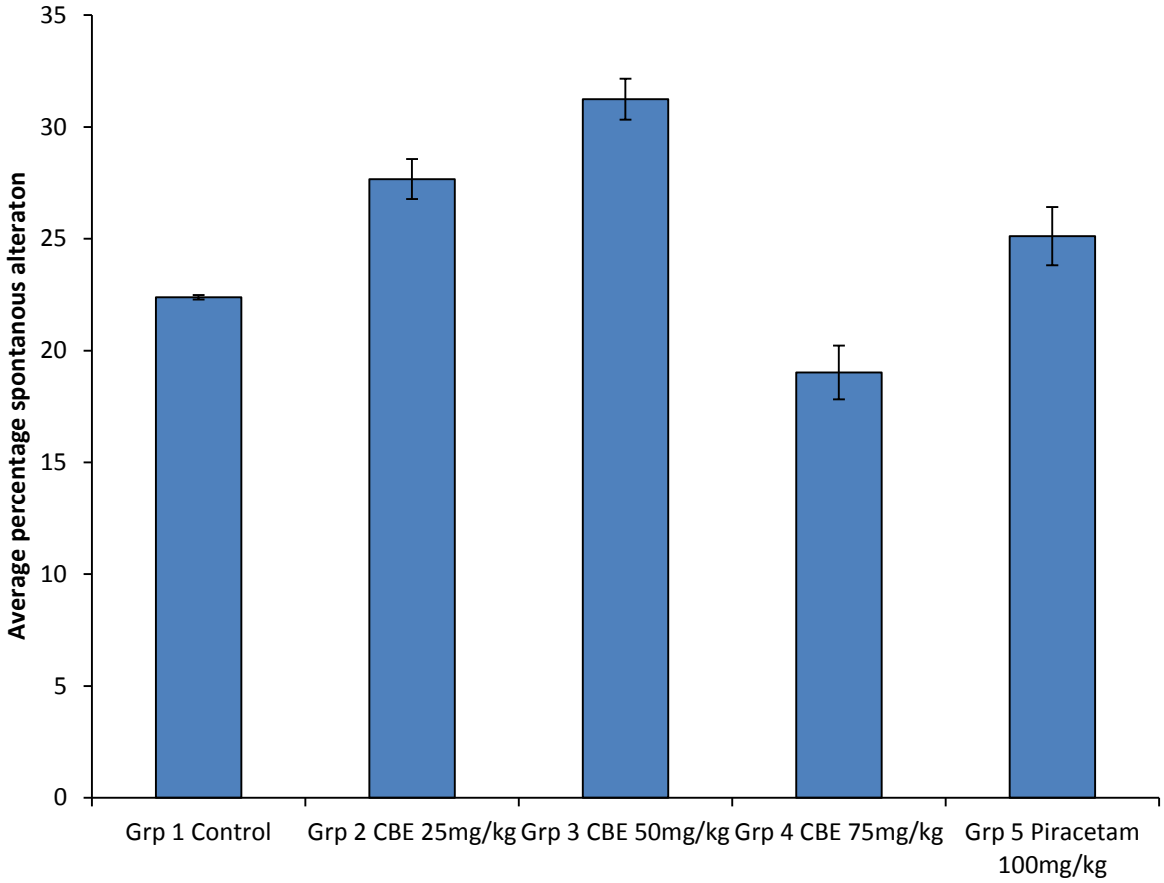


Figure 4.2: Effects of *Cannabis sativa* extract on spatial short term memory using Y Maze No statistical significance when compared with control p=0.12 (n=5) SPSS version 20.

4.3 Assessment of Learning and Spatial Memory Using Morris Water Maze

4.3.1 Latency to locate escape platform

In figure 4.3, there was significant difference (p=0.01) in the time taken for the mice to locate the escape platform in day 1 and 2 between negative control (51.32±1.00; 40.52±1.94) and groups 2, (CBE 25mg/kg: 57.78±1.00[a]; 55.19±0.66[a]), 3 (CBE 50mg/kg: 59.57±0.97[a]; 60.65±1.54[a]) and 4 (CBE 75mg/kg: 69.75±1.42[a]; 76.47±2.77[a]). But, no significant difference (p=0.22) was seen between the negative control and positive control (Piracetam 100mg/kg: 51.39±1.16; 42.57±0.81) both in day 1 and 2.

4.3.2 Frequency of platform crossings

A significant difference was observed in the frequency of crossing the removed platform between the negative control (5.20±0.37) and groups 3 (CBE 50mg/kg: 3.00±0.32[b]) and 4 (CBE 75mg/kg: 1.00±0.45[b]), F (4,20=25.69, P=0.00). Whereas, no significant difference (p>0.05) was seen in group 2 (CBE 25mg/kg: 4.20±0.20) and positive control group (Piracetam 100mg/kg: 5.40±0.40) when compared with negative control (5.20±0.37). Figure 4.4

4.3.3 Time spent in Target quadrant (Q4)

Here, a continuous decrease in the time spent in the Target quadrant (Q4) is observed from group 2 to 4, with a statistical significant difference in groups 3 and 4, when compared with the negative control group, F (4,20=106.25, P=0.00). Whereas, in group 5, i.e. the positive control group, an increase in the time spent in the target quadrant was observed (Table 4.3).

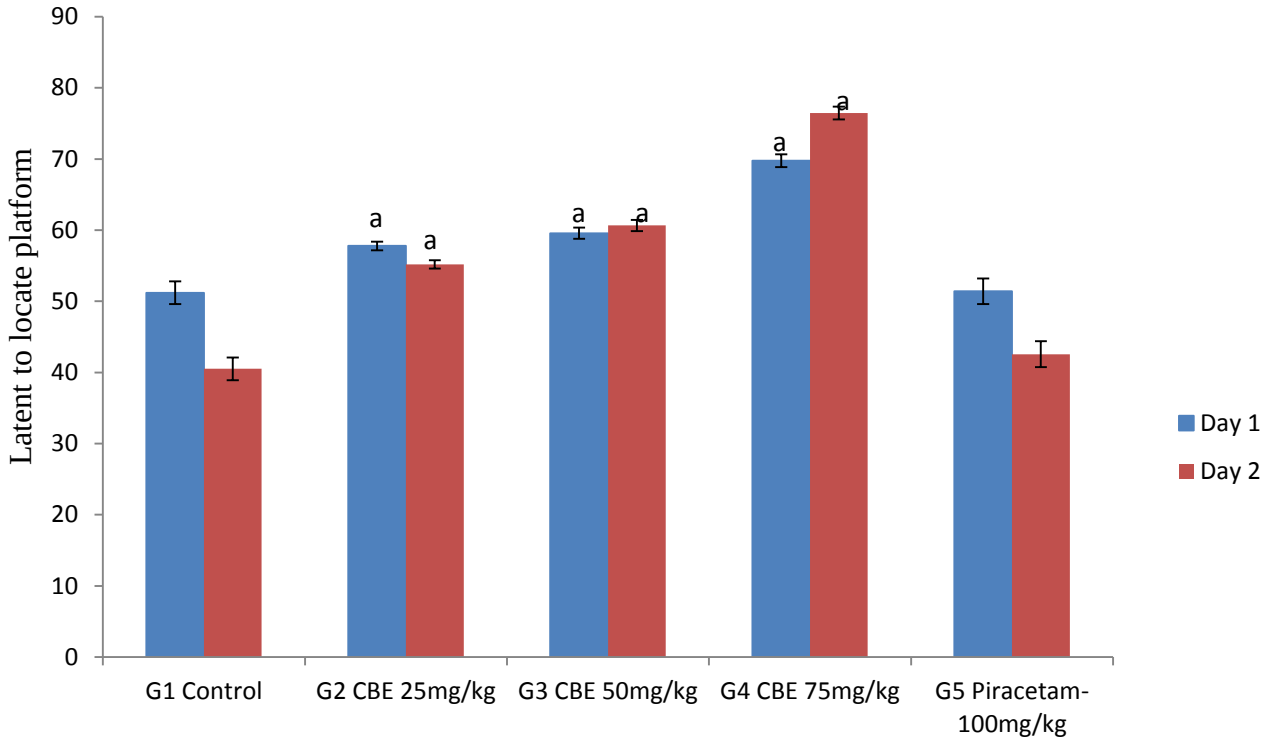


Figure 4.3: Effects of *Cannabis sativa* on memory in mice during a one day probe trial using morris water maze.
a: means statistical significance when compared with control p=0.00 (n=5) SPSS version 20.

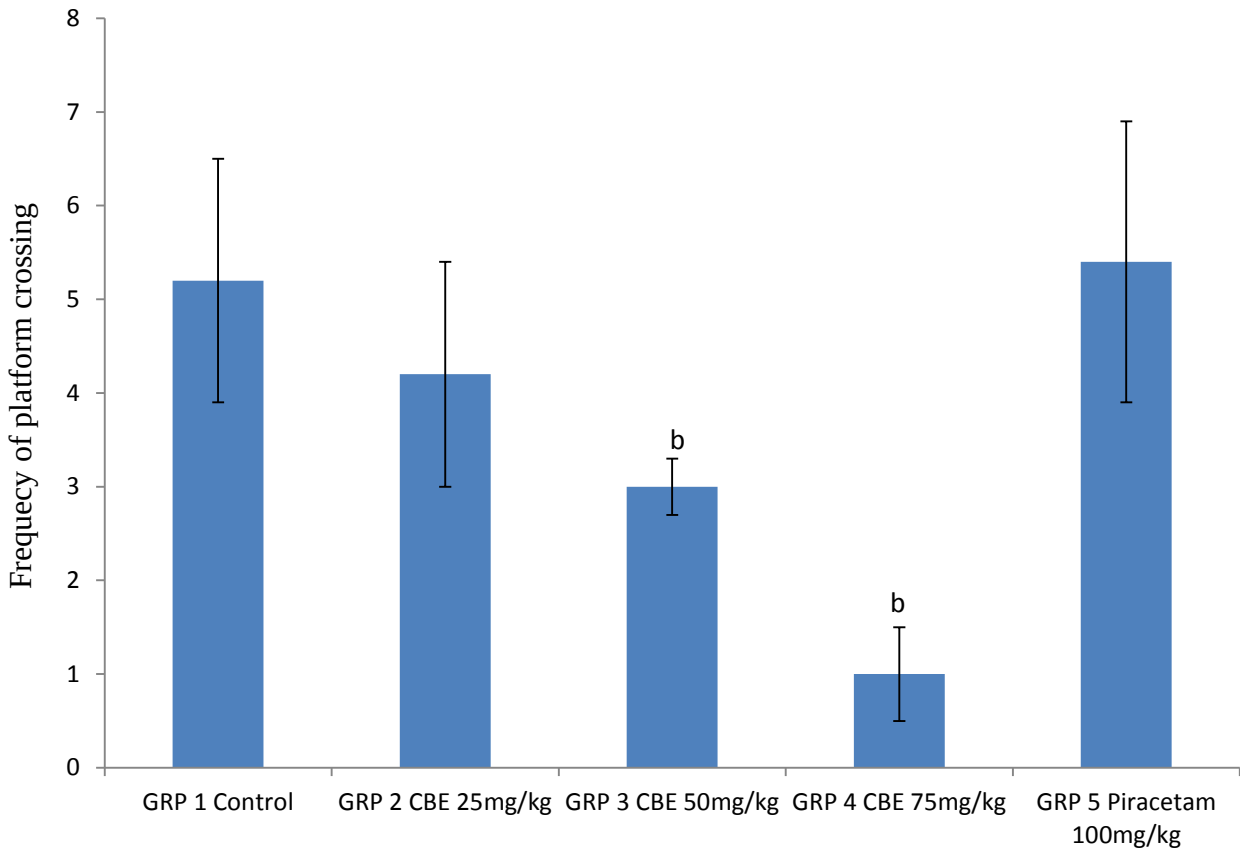


Figure 4.4: Effects of *Cannabis sativa* on memory in mice during a one day probe trial using morris water maze.
b: means statistical significance when compared with control p=0.00 (n=5)

Table 4.3: Effects of *Cannabis Sativa* on the time spent in target quadrant (Quadrant 4) by mice using Morris water maze.

Groups	**Mean Time spent in target quadrant (Q4)**	**F value**
Control	40.60±1.21	1.449
CBE 25mg/kg	37.60±0.68	1.526
CBE 50mg/kg	30.20±0.86[b]	2.115
CBE 75mg/kg	24.20±1.07[b]	6.225
Piracetam 100g/kg	49.80±0.86	0.921

Values are mean ±SEM of five (5) replications, values with superscripts 'b' are statistically significant when compared with control (p=0.00).

4.4 Acetylcholinesterase Activity

4.4.1 Acetylcholinesterase activity in Mice used in Elevated Plus Maze for Memory

In table 4.4 acetylcholinesterase concentration in mU/ml showed a statistical significant difference (p=0.00) between the negative control group (123.44±0.94) and groups 2 (CBE 25mg/kg: 129.06±0.71[a]), 3 (CBE 50mg/kg: 173.94±0.50[a]), 4 (CBE 75mg/kg: 179.96±2.20[a]) and the positive control group (Piracetam 100mg/kg: 109.66±0.98[b]), F (4, 20)= 673.535, P= 0.00. (Table 4.4)

4.4.2 Acetylcholinesterase activity of mice used in Y maze for memory

As presented in table 4.4, a statistical significant difference (p= 0.00) was observed between the negative control group (121.42±1.68) and groups 2 (CBE 25mg/kg: 131.78±1.39[a]), 3 (CBE 50mg/kg: 172.14±1.54[a]), 4 (CBE 75mg/kg: 194.38±1.66[a]) and the positive control group (Piracetam 100mg/kg: 93.64±1.49[b]), F (4, 20)= 670.345, P= 0.00.

4.4.3 Acetylcholinesterase in Mice used in Morris Water Maze for Memory

Here, no statistical significant difference (p>0.05) was observed between the negative control group (122.02±0.86) and group 2 (CBE 25mg/kg: 126.22±0.77), but a statistical significant difference was seen between the negative control group and groups 3 (CBE 50mg/kg: 167.78±0.86[a]), 4 (CBE 75mg/kg: 183.30±1.13[a]), and the positive control group (Piracetam 100mg/kg: 84.32±7.2[b]), F (4,20)= 1508.311, P=0.00. (Table 4.4)

Table 4.4 Effect of *Cannabis sativa* extract (CBE) on acetylcholinesterase activity in mice used in Elevated plus maze, (EPM), Y maze, (YM) and Morris water maze (MWM)

MODEL	Control	CBE 25mg/kg	CBE 50mg/kg	CBE 75mg/kg	Piracetam 100mg/kg
EPM	123.44±0.94	129.06±0.71[a]	173.94±0.50[a]	179.96±2.20[a]	109.66±0.98[b]
YM	121.42±1.68	131.78±1.39[a]	172.14±1.54[a]	194.38±1.66[a]	93.64±1.49[b]
MWM	122.02±0.86	126.22±0.77	167.78±0.86[a]	183.30±1.13[a]	84.32±7.20[b]

Values are mean ±SEM of five (5) replications, values with superscripts 'a' and 'b' are statistically significant when compared with control. p=0.00, a: means increase, b: means decrease.

4.5 Level of Dopamine concentration (nmol/L)

There were statistical significant differences (p=0.00) observed between the negative control groups and groups 3 and 4 of EPM, F(4,20=17.36, P=0.00) YM, and MWM models of mice used for this test, but no statistical significant difference (p=0.15) was observed when compared with the positive control groups of all models excluding that of MWM. (Table 4.5)

4.6. Concentration of Brain Sialic Acid

4.6.1 Concentration of Free Sialic Acid

No statistical significant difference (p=0.25) was observed in all the groups of the three models when compared with their respective control groups. (Table 4.6.1)

4.6.2 Concentration of Bound Sialic Acid

In table 4.6.2, no statistical significant difference (p=0.15) was observed in all the groups of EPM and YM models when compared with their respective control groups. Whereas, in MWM model, a statistical significant difference was observed between the negative control group and groups 4 and 5 (p=0.00)

4.6.3 Concentration of Total Sialic Acid

The result of this test showed no statistical significant difference (p>0.05) in all the groups (except groups 4) when compared with the negative control groups of three respective models. A statistical significant difference was observed in group 3 of MWM when compared with the negative control group at p=0.01

Table 4.5: Effect of *Cannabis sativa* Extract on Dopamine Concentration (nmol/L) in Mice using Elevated Plus Maze, Y Maze and Morris Water Maze.

Model	Control	CBE 25mg/kg	CBE 50mg/kg	CBE 75mg/kg	Piracetam 100mg/kg
EPM	0.15±0.02	0.17±0.02	0.09±0.01[a]	0.26±0.01[a]	0.16±0.01
YM	0.16±0.02	0.13±0.02	0.06±0.01[a]	0.63±0.01[a]	0.15±0.00
MWM	0.14±0.00	0.13±0.00	0.04±0.01[a]	0.01.±0.00[a]	0.18±0.00[a]

Values are mean ±SEM of five (5) replications, values with superscript 'a' are statistically significant when compared with control. p=0.00

Table 4.6.1: Effect of *Cannabis sativa* Extract on Free Sialic Acid Concentration in Mice

MODEL	Control	CBE 25mg/kg	CBE 50mg/kg	CBE 75mg/kg	Piracetam 100mg/kg
EPM	25.41±2.85	31.41±4.05	23.65±1.29	24.02±1.00	40.94±1.99
YM	32.76±2.85	35.53±1.35	33.53±2.84	25.04±2.20	31.05±1.78
MWM	32.82±3.04	34.71±1.77	24.35±2.13	25.91±4.60	33.53±3.25

Values are mean ±SEM of five (5) replications, values are not statistically significant, p=0.25

Table 4.6.2: Effect of *Cannabis sativa* Extract on Bound Sialic Acid Concentration in Mice

MODEL	Control	CBE 25mg/kg	CBE 50mg/kg	CBE 75mg/kg	Piracetam 100mg/kg
EPM	12.72±3.94	8.42±2.96	7.44±2.41	3.46±0.97	8.2±02.4
YM	8.08±1.00	4.00±0.73	6.84±0.73	3.51±1.24	12.77±33.99
MWM	14.68±2.46	4.79±1.74[a]	9.49±1.74	3.12±1.41[a]	4.79±1.94[a]

Values are mean ±SEM of five (5) replications, values with superscripts 'a' are statistically significant when compared with control. p=0.15

Table 4.6.3: Effect of *Cannabis sativa* Extract on Total Sialic Acid Concentration in Mice

MODEL	Control	CBE 25mg/kg	CBE 50mg/kg	CBE 75mg/kg	Piracetam 100mg/kg
EPM	38.13±1.61	40.47±2.63	31.09±1.94	27.87±0.90[a]	44.18±1.78
YM	38.68±1.89	38.93±1.54	41.10±1.54	29.35±1.17[a]	37.86±2.20
MWM	44.55±1.55	39.50±2.26	35.71±0.96[a]	24.20±1.46[a]	45.73±1.73

Values are mean ±SEM of five (5) replications, values with superscripts 'a' are statistically significant when compared with control, p=0.01

4.7 Indicators of oxidative stress

The level of thiobarbituric acid reactive substance (TBARS) and malondialdehyde (MDA) were used to determined lipoperoxidation. Also, the antioxidant enzymes such as catalase and superoxide dismutase (SOD) were also assessed as biomarkers of oxidative stress.

4.7.1 Level of TBARS

The result of this test showed a statistical significant difference (p=0.00) between the negative control group (179.95±7.98) and groups 2 (CBE 25mg/kg: 240.96±8.48[a]), 3 (CBE 50mg/kg: 220.69±5.17[a]), 4 (CBE 75mg/kg: 358.56±8.58[a]), but, no significant difference (p>0.05) with the positive control group (Piracetam 100mg/kg: 163.30±3.40) in EPM model, F (4,20)= 119.418. In the YM model, no significant difference (p>0.05) in groups 2 and 5, but, a significant difference was observed in groups 3 and 4 when compared with the negative control. While in MWM model, a significant difference was observed between the negative control group and other groups, F (4,20)= 237.976, P= 0.00. (Table 4.71)

4.7.2 Catalase

A statistical significant difference (p=0.01) was observed between the negative control groups and all the respective groups of all models. (Table 4.72)

4.7.3 Superoxide Dismutase (SOD)

Here, the EPM and MWM models, showed no significant difference (p>0.05) between the negative control groups and groups 2 and 5 of the respective models, but a significant difference (p=0.00) with groups 3 and 4. In the YM model, significant difference was observed in group 3 when the groups are compared with the negative control group. (Table 4.73)

Table 4.7.1: Effect of *Cannabis Sativa* Extract on TBARS in Mice using Elevated Plus Maze, Y Maze and Morris Water Maze

Model	Control	CBE 25mg/kg	CBE 50mg/kg	CBE 75mg/kg	Piracetam 100/mg
EPM	179.95±7.98	240.96±8.48[a]	220.69±5.17[a]	358.56±8.58[a]	163.30±3.40
YM	161.28±3.89	178.64±7.22	189.73±2.73[a]	237.88±11.35[a]	150.27±3.59
MWM	164.40±2.11	179.28±3.81[a]	176.10±3.59[a]	283.35±3.79[a]	152.48±3.44[a]

Values are mean ±SEM of five (5) replications, values with superscripts 'a' are statistically significant when compared with control, p=0.00

Table 4.7.2: Effect of *Cannabis Sativa* Extract on Catalase in Mice using Elevated Plus Maze, Y Maze and Morris Water Maze

Model	Control	CBE 25mg/kg	CBE 50mg/kg	CBE 75mg/kg	Piracetam 100mg/kg
EPM	12.32±0.28	7.49±0.54[a]	7.21±0.62[a]	7.36±0.91[a]	10.08±0.28[a]
YM	11.61±0.60	7.37±0.54[a]	6.55±0.83[a]	4.90±0.49[a]	10.31±0.37[a]
MWM	11.72±0.65	7.85±0.44[a]	7.88±0.48[a]	7.98±0.49[a]	10.21±0.22[a]

Values are mean ±SEM of five (5) replications, values with superscripts 'a' are statistically significant when compared with control, p=0.01

Table 4.7.3: Effect of *Cannabis Sativa* Extract on Superoxide Dismutase (SOD) in Mice using Elevated Plus Maze, Y Maze and Morris Mater Maze

Model	Control	CBE 25mg/kg	CBE 50mg/kg	CBE 75mg/kg	Piracetam 100mg/kg
EPM	62.28±2.64	52.49±3.45	39.24±2.81[a]	36.85±2.97[a]	64.24±2.45
YM	59.33±3.54	60.00±2.85	39.90±3.62[a]	49.31±6.66	71.90±4.07
MWM	67.27±3.17	67.63±2.32	52.25±2.13[a]	47.72±1.74[a]	74.47±4.33

Values are mean ±SEM of five (5) replications, values with superscripts 'a' are statistically significant when compared with control, p=0.00

CHAPTER FIVE

5.0 DISCUSSION

Various researches had been done on the effect of *Cannabis sativa* on the neurobehavioral effects over several decades and summarized in numerous published reviews (Grant *et al*., 2003; Iversen 2003; Ranganathan and D'Souza 2006; Gonzalez 2007;Jager and Ramsey 2008; Schweinsburg *et al*., 2008; Solowij and Battisti 2008; Solowij and Pesa 2010; Crean *et al*., 2011) and cognitive impairments (Kalant 2004; Solowij and Pesa 2010). However, many of these reviews conclude that the findings for several neurocognitive domains are equivocal, stressing to the importance of further research in this field (Crean *et al*., 2011). This research was carried out to establish the effects of *Cannabis sativa* on some neurochemical changes on learning and memory in mice, employing elevated plus maze, Y maze and Morris water maze for the assessment of learning and memory. The results of this research, showed impaired learning and memory with higher doses of *Cannabis sativa* extract when compared with the negative control group in the group used in elevated plus maze model. Though, no statistical significant difference ($p>0.05$) was observed with group 2 and piracetam group, but an increase in the transfer latency in both the learning and memory section was observed, indicating distortion in learning and memory. This could be as a result of decrease in locomotion and increase in anxiety as indicated by Okon *et al*., 2014, which showed decrease in locomotion, exploratory behavior and increase in fear and anxiety.

The Y maze model was used to assess the effects of *Cannabis sativa* on spatial short term memory in mice. The percentage spontaneous alteration was calculated and there was

increase in groups two and three with decrease in group four but the changes were not statistically significant ($p>0.05$) when compared with the control. This implies that administration of *Cannabis sativa* did not alter spatial short memory, but increase the natural exploratory behaviors and anxiety in the mice. This is seen as the mice showed more tendencies in exploring new arms consecutively than repeating the arms they have entered as seen in a study conducted by Okon *et al*., 2014.

In the Morris water maze model, the time taken to locate the hidden platform (i.e the escape latency) was recorded, which showed increase in escape latency in groups two, three and four during the two day acquisition period. This implies learning impairment in groups 2,3 and 4 when compared with negative control group, which is evident in the time the animals in those groups spent in locating the hidden platform. This could be due to the inhibitory effects of Δ^9 -THC on learning and memory, which is the psychoactive ingredient in cannabis. This is in agreement with the work done by Omar *et al* where mice given cannabis spent more time to locate the hidden platform than their untreated counterparts. The effect was most evident with the initial administration of the extract and maintained throughout the study. Also, Mice treated with scopolamine (an anticholinergic) and cannabis needed significantly more time to locate the hidden platform than the scopolamine only treated group, suggesting exacerbation of the cognitive impairing effects of scopolamine by cannabis (Omar *et al.*, 2013). Also, there was statistical decrease in the frequency of platform crossing in groups 2, 3, and 4 when compared with the negative control group as the cannabis treated mice cross the location of the removed platform less frequently. This suggest a disruption of spatial memory which could be associated with Δ^9 –THC-induced activation of cannabinoid CB1 receptor in brain as reported by Abdel-

Salam *et al.,* (2013). Also, Cannabis effect on learning and memory processes probably involves depolarization-induced suppression of excitatory mechanism in the CA1 area of hippocampus (Ebrahimpour *et al*., 2010).

The effects of CBE was examined on the concentration of acetylcholinesterase (AchE) on the three different models used. The result showed increase in acetylcholinesterase in all the groups (except group 5 that received piracetam) of all the models. AChE which hydrolyzes the neurotransmitter acetylcholine is an important factor in cholinergic neurotransmission and the pathology of Alzheimer's disease (Sarter *et al*., 2009). Inhibitors of this enzyme e.g., donepezil are the mainstay of boosting cognitive function in patients suffering from Alzheimer's disease (Hansen *et al*., 2008). Increase in AchE indicates increase in hydrolysis of acetylcholine. This leads to decrease in the level of the neurotransmitter which may consequently lead to distortion in learning and memory in acetylcholine implicated learning and memory. This study is in contrast with the findings of Abdel-Salam, *et al.,* which showed that concentration of AchE midly decrease after treatment with cannabis preceding an initial increase with treatment with $AlCl_3$ (Abdel-Salam *et al.,* 2015) Also, this study is in contrast with the work done by Casarejos *et al.,* (2013) where they suggested that cannabis preparations eg., Sativex might be of benefit in dementia (Casarejos *et al*., 2013).

Dopamine concentration was also determined in the brain tissue of all the models. It was discovered that the cannabis treated groups 3 had a statistical significant ($p=0.00$) decrease in dopamine concentration, while groups 4 (except for MWM model that had a decrease) had statistical significant increase in the dopamine concentration when compared with the negative control. This shows that CBE of moderate dose in the research has inhibitory

effect on dopamine which is one of the several neurotransmitters, of which may be required for normal dorsal striatum-dependent learning and memory (Legault *et al*., 2006). This agrees with Szabo & Schlicker, that cannabinoids have an inhibitory effect on the activity of several neurotransmitters, including dopamine, acetylcholine, and glutamate (Szabo & Schlicker, 2005). Thus, cannabinoids may influence dorsal striatum-dependent learning and memory processes by CB1 receptor-induced reduction in the release of these neurotransmitters within the DLS. Indeed, activation of CB1 receptors in the DLS is required for multiple forms of synaptic plasticity within the DLS, including long-term and short-term depression of neurotransmitter release, as well as synaptic depotentiation (Lovinger *et al*., 2010 and Nazzaro *et al*., 2012).

Brain sialic acid was also assessed in the brain tissue, which was assessed as bond, free and total sialic acid in all the three models. Though, no statistical difference ($p>0.05$) was observed in free and bound sialic concentration of all the models, except for groups 4 and 5 of the MWM model, however mild increase in free sialic concentration was observed in group 1 of all the models used in assessing the free sialic acid concentration, then followed by mild decrease in the subsequent groups. The total brain sialic acid concentration showed a statistical significant ($p=0.01$) decrease in group 4 (the highest dose group) of all the models. This shows that the two lower doses of CBE have negligible effects on brain sialic acids, whereas with high dose of CBE showed a statistical significant decrease. This might affect learning and memory as brain sialic acid has been suggested to be involved in learning and memory amidst other roles it plays in the human body and other animals (Markram *et al*., 2007). Polysialic acid (PSA) which is a brain sialic acid is said play an essential role in functional synaptic plasticity in adults. The most studied and perhaps most

complex model of this kind of neuronal plasticity is the hippocampus, where it is clear that long-lasting changes at excitatory synapses like long-term potentiation (LTP) and long-term depression (LTD) are dependent on PSA expression (Eckhardt *et al.*, 2000, Dityatev *et al*., 2004). Though, unable to come across researches on effects of *Cannabis sativa* on brain sialic acid, but it is evident that sialic acid is involved in learning and memory, where a research showed that lactoferrin, another form of sialic acid present in milk is involved in many different processes associated with neurodevelopment, cognition, learning and memory (Chen *et al*., 2015). So, it might be suggested that the CBE acts through CB1 receptors in the hippocampus and other areas in the brain to result in such decrease in brain sialic acid concentration and consequently resulting in impairment in learning and memory.

Biomarkers of oxidative stress such as, MDA, catalase and superoxide dismutase, SOD) were determined. The result showed a statistical increase in TBARS which is uniformly noticed in groups 4 of all models, implying high production of MDA which is an index of lipid peroxidation- a biomarker of oxidative stress. The increase in MDA could be associated to fear and anxiety during the neurobehavioural test (EPM, MWM and YM) the mice were subjected to. It could also be due to the effects of the CBE on the mice. The result of the present study confirms the work by Toson, 2011, where he studied the impact of marijuana smoke on liver and sex hormones and correlated it with oxidative stress and discovered that, the total MDA levels in sera of marijuana smokers were increased but the total glutathione (GSH) and total antioxidant capacity (TAC) were simultaneously decreased indicating the presence of a state of oxidative stress. The oxidative stress could be due the effect of Delta9-THC that is present in the CBE which is a potent source of

cellular oxidative stress that could also contribute significantly to cell injury and dysfunction as suggested by Sarafian *et al*. (1999). However, the result of this research is in contrast with the work done by Omar, *et al*., (2012) were they showed short term administration of CBE on mice ameliorate increased lipid peroxidation induced in brain by haloperidol. Also, it is in contrast with the work done by Coskun and Bolkent, (2016), where they showed that Δ^9 THC attenuates oxidative stress.

The present study also showed decrease in the levels of CAT and SOD, which are the antioxidant enzymes determined in this research. The reduction in the level of these antioxidant enzymes could generate reactive oxygen species (ROS) which will consequently result in oxidative stress. This finding is in contrast with the work done by Coskun and Bolkent, (2016), where they observed that CAT activity was decreased and the SOD activity was increased in the plasma of diabetes treated with THC as compared to diabetic rats. Thus, ROS are typically categorized as neurotoxic molecules and typically exert their detrimental effects *via* oxidation of essential macromolecules such as enzymes and cytoskeletal proteins. Most importantly, excessive ROS are associated with decreased performance in cognitive function (Massaad and Klann, 2011).

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

The result of the present day research revealed learning and spatial short term memory impairment in EPM and MWM detected in groups 4 of both models. It was observed that administration of CBE resulted in reduction of AchE concentration, decrease in dopamine level and total brain sialic concentrations. Also, observed from the research was an increase in MDA concentration, reduction in SOD and CAT activities, indicating increase in oxidative stress. Thus, reduction in concentration of the neurochemicals and the increase in oxidative stress may be implicated in the learning and spatial short term memory impairment in mice following administration of *Cannabis sativa* extract.

6.2 Recommendations

Based on this research,

I. Cannabis use and moves to legalize its use is discouraged due to its negative implications in learning and memory.
II. Further research on the specific mechanism through *Cannabis sativa* affect brain sialic acid and the implication on learning and memory
III. Further studies on mechanism through which cannabis sativa affects other neurotransmitters such glutamate, serotonin and monoamines employing different neurobehavioral model on learning and memory.

Contribution to Knowledge

I. To the best of my knowledge, this research seems to be the first to establish the effects of *Cannabis sativa* extract on brain sialic acid and relating it to learning and memory

II. This research had significantly assessed the effects of *Cannabis sativa* extract on AchE and dopamine concentrations using three different neurobehavioural models and their implications on memory and learning.

III. The research has established the effect of *Cannabis sativa* on biomarkers of oxidative stress employing three different models and relating it to learning and memory.

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