EVALUATION OF THE EFFECT OF SUBCHRONIC ADMINISTRATION OF CRUDE KHAT EXTRACT *(CATHA EDULIS F.)* ON LEARNING AND MEMORYIN MICE

FAIZ MOHAMMED

A Thesis Submitted to the Department of Pharmacology and Clinical Pharmacy in Partial Fulfillment for the Requirement of the Degree of Master of Science in Pharmacology

ADDIS ABABA UNIVERSITY

ADDIS ABABA, ETHIOPIA

JANUARY, 2014

Addis Ababa University

School of Graduate Studies

This is to certify that the thesis prepared by **Faiz Mohammed**, entitled: *Evaluations of the Effect of Subchronic Administration of Crude Khat Extract (Catha Edulis F.) on Learning and Memory in Mice* and submitted in Partial Fulfillment for the Requirement of the Degree of Master of Science (Pharmacology) complies with the regulation of the University and meets the accepted standards with respect to originality and quality.

Signed by the Examining Committee:

Dr. Charlotte Hanlon (external examiner) Signature _____date *14/01/2014*

Prof. Emmanuel Onaivi (internal examiner) Signature_____ date *14/01/2014*

Dr. Ephrem Engidawork (advisor) Signature _____date *14/01/2014*

Dr. Teshome Nedi (advisor) Signature_____ date *14/01/2014*

Dr. workineh Shibeshi_____

(Chair of Department)

ABSTRACT

Evaluations of the Effect of Subchronic Administration of Crude Khat Extract *(Catha Edulis F.)* on Learning and Memory in Mice.

Faiz Mohammed

Addis Ababa University, 2014

Learning may be defined as the ability to alter behavior based on experience. If there is no learning, there can be no memory later. Memory is the ability of an individual to record sensory stimuli; events, information, etc. and retain them over short or long periods and recall the same later when needed. People chew khat believing that it improves memory, make them alert and think clearly. This study was made to add to the existing body of knowledge about khat and memory. To this effect, 36 albino mice, 6-8 weeks old, were administered orally with a single daily dose of khat extract for 30 days. The animals were divided into four groups. The first group served as controls and was administered with 0.5 ml 3% Tween 80 in water. Group two (K100), three (K200) and four (K300) were administered 100, 200 and 300 mg/kg khat extract, respectively. The animals were then subjected to Multiple T maze (MTM) and Morris water maze (MWM) task performance. The result showed that subchronic administration of crude khat extract at doses used did not have a significant effect on learning using the two models. K200 ($p< 0.01$) and K300 ($p< 0.001$) significantly impaired short-term memory in both models, while K100 impaired STM in MWM but not in MTM. Khat at all doses used did not have any significant effect on long-term memory using both models. Crude khat extract administration also had initially anorexic effect but this was followed by development of tolerance. The results collectively showed that subchronic exposure of mice to khat could alter formation of short-term memory, without affecting learning and long-term memory.

Key Words: *Catha edulis*, learning, memory, short-term memory, long-term memory, Morris water maze task, Multiple T maze task.

ACKNOWLEDGMENTS

First of all, I need to thank the almighty Allah who gave me the grace to begin and patience to complete this work.

I am greatly indebted to my advisors: Dr. Ephrem Engidawork and Dr. Teshome Nedi of the Department of Pharmacology and Clinical Pharmacy, School of Pharmacy, for there consistent advice and encouragement as well as unreserved guidance and supervision throughout this work.

I am indebted to Harari Regional Health Bureau (Harar Health Science College) for sponsoring me to pursue my graduate studies, and the school of Graduate Studies of Addis Ababa University for funding this study.

My special thanks go to W/ro Fantu Assefa for her cooperation in providing chemicals and support during practical work. I want to give special thanks to my friends: Kemal Arab for his continuous support, Feysel Ibrahim for his cooperation with me throughout the days and nights of the tasks, Tegeng Bogale and Ashenafi Girma who supported me with materials important for my thesis work.

My deepest gratitude extends to my wife Seada Desallenge for her consistent support, encouragement and patience to my academic-centered behavior throughout my graduate program; my son Ayub Faiz to his abstract smile wherever I return to home from this task, my father Mohammed Kassim, my brother Ermias Tamene and all of my family.

Lastly, my sincere thanks go to those whose names are not mentioned but contributed, directly or indirectly, for the fulfillment of this study.

TABLE OF CONTENTS

LIST OF FIGURES VI

ABBREVATIONS VII

1 INTRODUCTION 1

1.1 Learning and memory 1

1.1.1 Memory types 4

1.1.2 Memory consolidation 6

1.1.3 The cellular basis of memory 10

1.2 KHAT 16

2 OBJECTIVES 22

2.1 General objective 22

2.2 Specific objectives 22

3 MATERIALS AND METHODS 23

3.1 Drugs and chemicals 23

3.2 Collection of khat 23

3.3 Experimental animals 23

3.4 Extraction of Khat 24

3.5 Grouping and dosing of animals 24

3.6 Morris water maze task 25

3.6.1 Habituation 26

3.6.2 Acquisition 26

3.6.3 Probe trial 27

3.7 Multiple T-maze task 27

3.7.1 Habituation 28

3.7.2 Acquisition 28
3.7.3 Probe trial 28
3.8 Weight change measurement 29
3.9 Statistical analysis 30
4 RESULTS 31
4.1 Effect of crude khat extract on water maze performance 31
4.1.1 Acquisition training 31
4.1.2 Short term memory retention phase 32
4.1.3 Long term memory retention phase 33
4.2 Crude Khat extract on Multiple T maze performance 34
4.2.1 Acquisition training 34
4.2.2 Wrong decision at the acquisition training 35
4.2.3 Short term memory retention phase 36
4.2.4 Wrong decision at probe trial at day 5 37
4.2.5 Long term memory retention phase 38
4.2.6 Wrong decision at probe trial at day 12 39
4.3 Effect of crude khat extract on weight 40
5 DISCUSSION 42
6 CONCLUSION 48
7 RECOMMENDATION 49
8 REFERENCES 50

LIST OF FIGURES

Figure 1: Chemical structures of amphetamine, cathine and cathinone.....17

Figure 2: Mean latency in the acquisition session of Morris water maze 31

Figure 3: Mean time (sec) spent in target quadrant at day 5 in Morris water maze 32

Figure 4: Mean time (sec) spent in target quadrant at day 12 in Morris water maze. 33

Figure 5: Mean Latency to reach the goal box in the acquisition phase of Multiple T maze 34

Figure 6: Mean errors made to reach Multiple T maze goal box in the acquisition phase 35

Figure 7: Mean latency to reach the goal box at day 5 in Multiple T Maze task 36

Figure 8: Mean wrong decision of mice at day 5 (D5) in Multiple T Maze task. 37

Figure 9: Mean latency at day 12 in Multiple T maze 38

Figure 10: Mean wrong decision day 12 in Multiple.....39

Figure 11: Mean weight from day 1 to day 30 in gram for all groups.....41

Figure 12: Mean weight differences 41

ABBREVATIONS

5-HT	5-hydroxytryptamine, Serotonin
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors
CaMK	Ca2+/ calmodulin-dependent kinase
cAMP	Cyclic adenosine monophosphate
CREB	cAMP response element binding protein
EPSP	Excitatory postsynaptic potential
ERK	Extracellular signal-regulated kinase
GABA	Gamma-amino butyric acid
LTD	Long-term depression
LTM	Long-term memory
LTP	Long-term potentiation
MTM	Multiple T-maze
MTL	Medial temporal lobe
MWM	Morris water maze
NMDAR	N-methyl-D-aspartate receptors
NW	North-West
PKA	Protein kinase A
PSD	Post synaptic density
S.E.M	Standard error of mean
SE	South-East
STM	Short term memory
WM	Working memory

1 INTRODUCTION

1.1 Learning and memory

The ability to learn and to remember is one of the most fundamental features of the brain. Understanding how learning and memory work is important, because what we learn and what we remember determine, largely, what we are and who we are [1].

What is memory? It is often associated with the “thinking of again” or “recalling to the mind” of something learned at an earlier time [2]. Memory, not merely facial and physical appearance, defines an individual, as everyone who has known someone with Alzheimer’s disease (AD) understands all too well. Furthermore, the impact of learning and memory reaches far beyond the individual, and they form the very foundation for transmitting knowledge through generations, consequently, serving as the major forces in driving cultural and social evolution [1].

The brain is the organ that is responsible for what we call the mind. It is the basis for thinking, feeling, wanting, perceiving, learning and memory, curiosity, and behavior. Memory is a fundamental mental process, and without memory, we are capable of nothing but simple reflexes and stereotype behaviors [1, 3]. It is the ability to store, maintain, and retrieve information from the mind [4]. It is also the ability of an individual to record sensory stimuli; events, information, etc. retain them over short or long periods of time and recall the same at a later date when needed [5]. In other words, it is the human ability to construct a virtual bridge between the past, the present, and the future [4]. A better description of memory could be ‘the ability to retain and utilize acquired information or knowledge’. Memory is an integral part of our existence, yet it is only vaguely understood [2]. Even if the level may vary from individual to individual, memory

declines with physiological aging. Memory loss is characteristic of several clinical conditions such as mild cognitive impairment and AD [4].

Memory is not a static, isolated or single brain function; so memory can be best described as a complex network of different interrelated functions working together to manage information. For this reason, it would be more appropriate to define it in terms of a *memory system*. Thus, a memory system could be defined as a brain function whose purpose is to classify, encode, store and recover a wide diversity of information relevant for the subject [6]. Thus, learning and memory is one of the most intensively studied subjects in the field of neuroscience [3].

Learning is the basis of memory [7]. Learning may be defined as the ability to alter behavior based on experience [5]. If there is no learning, there can be no memory later. This is true in a psychological description of memory and appears to be true when looking at the molecules that initiate memory formation [7]. Memory, as defined above, is a system by which sensations, impressions, and ideas are stored and recalled, whereas learning is a process by which brain acquires new information about the events occurring in a given surroundings [3]. The ability to learn and remember spatial locations, and to associate them with other stimuli, is essential for adaptive behavior, and disruption of this system can lead to spatial deficit [8].

Learning and memory are complex phenomenon requiring the coordinated interaction of multiple brain structures. For example, the diencephalon, a sub-cortical region that includes the thalamus and hypothalamus, has been characterized as an integral connection zone for many memory-related circuits. There are connections between the thalamus and the hippocampus, as well as the amygdala and striatum. All three of those regions (hippocampus, striatum, and amygdala) are important for different types of memory [3]. The amygdala is a temporal lobe structure implicated in the expression of emotional stress responses and in memory for,

particularly, emotionally arousing events. Lesions of the amygdala have been shown to block the enhancing effects of emotional arousal on memory consolidation. It also blocks the memory-modulatory effects of systemic administration of epinephrine and other neuromodulators [9].

Spatial memory can be defined as that brain function responsible for recognizing, codifying, storing and recovering spatial information about the arrangement of objects or specific routes [6]. Evidence from a variety of sources suggests that the human hippocampus plays an important role in spatial memory. However, a network of brain regions is involved in spatial learning and memory, including the hippocampus, dorsal striatum, and the entorhinal, retrosplenial, prefrontal, and parietal cortices. Different types of spatial representation may recruit different components of this network. For example, some spatial tasks can be solved purely based on egocentric (self-centered) information (e.g., vestibular, proprioceptive, or sensory cues) that will change every time when the animal moves [8]. *Egocentric strategy* is based on the information provided by bodily cues, and therefore it is independent of spatial cues. When using this strategy, the subject functions at its own central point of reference, and so, all other object positions are defined in direct relation to its position in space; i.e., when the traditional clockwise scheme is used to define spatial positions, or when the conventional directions (left, right, forward or backward) are employed [6]. In contrast, other spatial tasks require encoding of the relationship between salient features of the environment to create an allocentric (other-centered) representation that is independent of the animal's current location [8]. *Allocentric strategy* depends on spatial cues. When using this strategy the subject memorizes the target location in relation to the spatial position of the environmental reference landmarks, meaning that it is based on a spatial representation [6]. Such a representation has been termed a 'cognitive map' [8]. The location of a particular target is established through a system of coordinates that are independent

of the observer; this system often uses distant or closer points of reference; i.e., latitude and longitude used by humans to locate places in a map [6].

1.1.1 Memory types

One of the most important conceptual developments in cognitive theorizing is the subdivision of memory into three separate processes of encoding, storage and retrieval**.** Another important conceptual development is the division of memory into three different stages of sensory, short term (STM) and long-term memory (LTM) [2]. Generally, memory is classified according to its content (declarative or explicit, procedural or implicit), according to its duration and nature [10, 11]: archival (STM and LTM) as opposed to transient, moment-to-moment (Working memory) [12]. The classification of memories into declarative memory and nondeclarative memory is based on the anatomy of the temporal lobe system [1]. However, its classification into STM and LTM is often temporally [1, 5, 6]. The basis for temporal division of the memory process is revealed by observations that newly formed memories (STM) are more vulnerable to interferences and disruptions [1]. Spatial memory cannot be strictly assigned to one of these classification subsystems; it is, indeed, part of several of these categories, since it involves aspects of non-declarative memory (procedural), declarative (semantic and episodic memories), as well as of both STM and LTM [6].

The main distinction is between declarative and nondeclarative memory systems. This distinction is also sometimes referred to as an explicit/implicit, direct/indirect or conscious/unconscious division of memory [2]. The term declarative memory was introduced to capture one system, and nondeclarative memory to capture an umbrella term referring to several memory systems [12].

Declarative memory, also termed *explicit memory*, is memory of places, events, facts and people, and is dependent on the temporal lobe system. Retrieval of these memories requires conscious recollection and can be intentionally called to mind. This type of memory tends to form easily and be forgotten easily [12, 13]. It is memory that we are conscious of and can be verbalized [2]. It is memory of knowledge that can potentially be *declared*, that is, brought to mind as a verbal proposition or as a mental image. This is what meant by memory in the colloquial sense [12]. Declarative, can be referred to information that is conventionally transmitted or expressed (information that may be transmitted orally) [6].

Declarative memory can be subdivided into episodic memory (autobiographical memories, memory for particular instances of learning – including the context and source of the memory), semantic memory (facts, relations, general knowledge and understanding, not bound to a particular context) and primary or working memory. It is easily tested by recall or recognition measures in an experiment [1, 2].

Nondeclarative memory also called implicit memory [1, 2], is not true or false [1, 14]. This type of memory includes classical conditioning and procedural memory (motor skills and habits such as tying up shoelaces and riding a bicycle) [2]. It represents the information about motor or perceptual skills that may not be orally transmitted [2, 6]. It does not depend on the structural integrity of the temporal lobe system [1]. Unlike declarative memory, nondeclarative memory is inflexible and bound to the modality of the original response systems [2]. It is unconscious [12, 13] and it cannot be called to mind [12]. These forms of memory are difficult to describe verbally – they can be ‘encoded’ and ‘retrieved’ with little or no conscious awareness [2].

Declarative memories are not immediately established as LTMs; this process takes 3–6 h and involves a sequence of specific molecular processes in the CA1 area of the hippocampus and its connections [11]. LTM is a vast store of knowledge and a record of prior events, and each normal person has at his or her command a rich set of LTMs [15]. William James (1890) proposed that while LTM formation is taking place, one or more STM systems are in charge [11]. STM is a limited capacity store that, for example, can be assessed with a digit span task. Most people can hold between five and nine items in STM, but by 'chunking' items together can hold more information [1, 2]. It denotes the cognitive faculty that retains information only temporarily until it is forgotten or consolidated into a more long-term form of storage. It includes immediate memory, which captures information that is the object of current attention [12].Thus, the term STM will be used to designate memory 'that develops within a few seconds or minutes and lasts for several hours' [11].

LTM has a time course of weeks, months and years [1]. It is a more permanent, apparently limitless store, containing all our knowledge of the world and memories of the past. Information can be difficult to retrieve from long-term storage – retrieval cues need to closely match the way the memory was encoded into LTM [1]. The consolidation of LTM proceeds 'slowly'. Thus, the term LTM will be used to designate memories lasting at least 24 h [11]. LTM depends on protein and mRNA synthesis [10]**.**

1.1.2 Memory consolidation

The first evidence of this came when Mueller and Pilzecker [16] in 1900 reported that memory of recently learned information is disrupted by learning of other material shortly after the first learning**.** Their 'perseveration–consolidation' hypothesis proposed that memory traces are initially fragile after learning and become consolidated over time [9].

Our memories of everyday life — of people, places and events — define who we are. However, these records of life experience are not formed instantaneously. Rather, new memories are gradually transformed from an initially labile and vulnerable state to disruption to a more permanent and resistant state to disruption [1]. From invertebrates to human, LTM for an event can be enhanced or reduced by manipulations performed in the minutes to hours after learning. This has led to the suggestion that memories form slowly over time, a process termed memory consolidation [16]. The temporal lobe is crucial for the conversion of new memory into LTM or a process of memory consolidation [1]. Consolidation refers to a process that transforms new and initially labile memories encoded in the awake state into more stable representations that become integrated into the network of pre-existing LTM [17]. It is the process by which temporary STM is stabilized into a persistent LTM [7]. It involves the active re-processing of 'fresh' memories within the neuronal networks that were used for encoding them [17]. Müller and Pilzecker first adopted the term 'consolidation' to describe these post-experience processes of memory stabilization [18]. So, the term consolidation was adopted to describe the post-learning processes of memory stabilization [19].

Covalent modification of already existing synaptic proteins mediates synaptic plasticity, which is important for both immediate learning and STM; and the consolidation of this synaptic plasticity is generally thought to occur via activation of second messengers that initiate gene transcription and translation of new proteins [9].

Consolidation involves reorganization at both the synaptic and system levels [18]. The necessary cellular responses involved include activation of second messenger systems, new RNA transcription, and protein synthesis. The mechanisms of stabilizing memory traces at the cellular

level are referred to as ‘‘synaptic consolidation’’ [19]. It involves the strengthening of memory representations at the synaptic level (synaptic consolidation) [17]. It is complete within hours of training, and involves the stabilization of changes in synaptic connectivity in localized circuits (for example, the growth of new synaptic connections as well as the restructuring of existing ones) [18]. Long term potentiation (LTP) is considered a key mechanism of synaptic consolidation [17]. This is distinct from another form of consolidation, ‘‘systems’’ consolidation, which denotes a reorganization of memory traces between brain regions [19].

Systems consolidation can be defined as the process by which memory becomes independent of the hippocampus, whereas *cellular (synaptic) consolidation* is defined as the transition of memory traces from protein synthesis and gene expression-dependent states to independent state [20]. System consolidation is a more prolonged process and involves gradual reorganization of the brain regions that support memory. For example, this may involve a time-dependent shift in the circuits that support memory recall [18].

Although the terms ‘‘synaptic’’ and ‘‘systems’’ consolidation describe phenomena at different levels of analysis, the two processes may share similar mechanisms and occur in parallel. Reorganization of memory traces between brain regions (‘‘systems’’) may require modifications of connections (‘‘synaptic’’) within those networks [19]. The hallmarks of the consolidation process are (i) relocation from short-term hippocampal memory stores to distributed neocortical networks and (ii) the gradual, time-dependent process of laying down LTM, in which the most recent memories are the most fragile [20].

Memory is often considered to be a process that has several stages, including acquisition, consolidation and retrieval. Memory can be modified further through reconsolidation and performance can change during extinction trials while the original memory remains intact. Some studies of the molecular basis of these processes have found that many signaling molecules are involved in several stages of memory but, in some cases, molecular pathways may be selectively recruited only during certain stages of memory [21].

There are several important characteristics of a memory storage modulatory system. Perhaps most critically, the role of a memory modulatory system is time limited: with the passage of sufficient time, a modulatory system can be inactivated with no loss of retrieval of stored memories. Furthermore, such a system can either enhance or impair memory, depending on the learning conditions. Lastly, whereas memory storage mechanisms may serve only specific forms of memory, a memory modulatory system should be capable of influencing different forms of memory [22].

Because of its known importance to memory and ease of study, the hippocampus, which is a medial temporal lobe (MTL) structure, has received much attention. Is the MTL system the exclusive site for LTM until cortical representations develop? A better interpretation may be that the MTL does not store the memories themselves, but simply mediates between cortical sites [2].

Some suggested that the MTL has only a temporary role in forming LTM. Squire and Alvarez [2], for example, suggested that consolidation is the process by which memory becomes independent of hippocampal regions. Then, where might consolidated LTM be stored is the main question? The neocortex has been suggested for the lateral temporopolar and lateral inferior prefrontal cortex regions.

There is extensive evidence that the basolateral amygdala (BLA) is a critical locus of integration of neuromodulatory influences regulating the consolidation of several forms of memory. Many drug and stress hormone influences converge in activating the release of norepinephrine (NE) within the BLA [23]**.**

McNamara and Skelton [24] reviewed the involvement of different neurotransmitter and modulator systems in spatial learning, and suggested that only the cholinergic, glutamatergic, and some peptidergic systems may really be required for this kind of learning, whereas gamma-aminobutyric acid (GABA), opioids or biogenic amines are either detrimental or unrelated to these functions.

1.1.3 The cellular basis of memory

The foundations for exploring the molecular and cellular mechanisms of learning and memory were established in 1949, when Canadian psychologist Donald O. Hebb came up with a simple yet profound idea to explain how memory is represented and stored in the brain [1]. A common view in neuroscience is that learning involves the so called Hebbian synaptic plasticity [7]. Hebb in 1949 proposed a mechanism by which learning can occur at the neuronal level [2], which can be paraphrased as follows: a synaptic input can be strengthened when activity in the presynaptic neuron co-occurs with activity (membrane depolarization, especially depolarizations that produce action potentials) in the postsynaptic neuron [2,7, 31]. During acquisition, Hebbian and neuromodulatory mechanisms likely implement the initial intracellular processes. These mechanisms also lead to processes that stabilize and consolidate LTM by triggering the second messengers that lead to gene transcription and protein translation. Molecules that are necessary for LTM, but not STM, are said to be involved in memory consolidation [9].

A major advance occurred in 1973 when Bliss and Lomo [25] discovered LTP, a physiological event now believed to reflect synaptic strengthening that occurs as a result of activity dependent synaptic plasticity**.** Since the first publication reporting the existence of LTP, there has been a plethora of papers, books, symposia, and meetings devoted to the discussion of the relationship between this molecular/cellular form of synaptic plasticity and some behavioral forms of learning and memory [26].

Changes in synaptic excitability can facilitate an increase in the concentration of post-synaptic neurotransmitter receptors. This will in turn influence action potential probability and the resulting firing rate within a network of neurons. These types of synaptic modulations have been observed in association with learning and memory and are thought to underlie the neural substrate of memory known as LTP. LTP results from the increase in synaptic efficacy between neurons. It can be induced via high frequency electrical stimulation between pairs of neurons, or chemical stimulation and has been shown to last from several hours to many days [27].

i) The NMDA receptor in the hippocampus induces LTP

LTMs are not established in their definitive form immediately after they are acquired. Their consolidation requires a number of precisely timed molecular processes in the neurons that make memories. These sequential processes are linked to one another in a complex way [28]. LTP has been well studied between pairs of neurons within the hippocampus [25, 27**]**, specifically on synapses between the Schaffer collateral axons and apical dendrites of the CA1 pyramidal neurons [27, 29**]**.

One of the most studied brain regions is the CA1 region of the hippocampus [1, 28**]**, which is involved in many if not all forms of explicit or declarative memory in mammals [28**]**. The CA1

region is not only crucial for memory formation but also exhibits a well-organized laminar structure ideal for electrophysiological recording [1]. Nevertheless, it is also observed in other brain regions that exhibit synaptic plasticity [25]. This is a common neural region of LTP investigation since LTP is most reliably evoked in brain areas known to play a role in memory and learning [27]. This form of LTP is induced postsynaptically via NMDA receptor activation and expressed, at least in part, postsynaptically via increased postsynaptic responsiveness to neurotransmitter release. The postsynaptic induction mechanisms for LTP, including Ca^{2+} influx through NMDA receptors and activation of postsynaptic Ca^{2+}-activated protein kinases, makes postsynaptic expression of CA1 LTP a parsimonious conclusion [29]**.** The molecular processes that underlie the synaptic changes necessary for memory formation include two peaks of increased activity of cAMP-dependent protein kinase, the phosphorylation of the transcription factor CREB1, gene activation, and protein synthesis [28].

It is well-established that the *induction mechanism of LTP* at the CA3–CA1 synapse of the Schaffer collateral pathway requires postsynaptic activation of the NMDA receptors [1, 29]. The NMDA receptor seems to be a perfect cellular device to detect the synaptic coincidence between pre-synaptic and post-synaptic neurons, and to associate two events at the cellular level [1]. As a result of correlated pre- and postsynaptic activity, NMDARs pass Ca^{2+}, and this is thought to be important for synaptic plasticity [1, 7] and possibly memory formation [7]**.** Thus, the NMDA receptor is a gating switch for the induction of synaptic plasticity [1].

The neuronal circuitry of the hippocampus consists of three major excitatory pathways that use glutamate as their neurotransmitter. First, the perforant pathway enters the hippocampus from the entorhinal cortex to make synaptic contact with granule cells of the dentate gyrus. Second, the granule cells assemble their axons into the mossy fiber pathway that projects to the dendritic

fields of pyramidal neurons in the CA3 [1, 25] and is called the *mossy-fiber* pathway [1]. Third, axons of the CA3 neurons form the Schaffer collateral pathway and synapse on pyramidal neurons in the CA1 [1, 25]. This pathway is one of the most studied systems *in vitro*. LTP, as measured by the increase in EPSPs of CA1 cells in response to stimulation of the CA3 axon bundle, is induced by the high-frequency stimulation in the Schaffer-collateral pathway [1].

Several types of glutamate receptors are present at the postsynaptic site, and two of them are AMPARs and NMDARs [25]. Receptors in the hippocampus for NMDA play a significant role in synaptic plasticity, LTP, and, by extension, formation of LTM. Receptors for AMPA on the other hand facilitate the induction of LTP [12]. There are important differences in the way these two receptor types respond to glutamate. The AMPARs are Na+ channels that, when bound to glutamate, permit Na+ ions to pass through the membrane into the postsynaptic neuron, leading to its depolarization. The summation of multiple EPSPs on the dendritic arbors of CA1 neurons leads to the generation of an action potential by these neurons that in turn travel down the axon toward the next neuron in the circuit [25]. It is generally well accepted that the induction of hippocampal CA1 homosynaptic LTP and LTD depends on the activation of NMDARs [30].

The NMDAR is also an ion channel that opens when bound to glutamate, but its activation has some additional requirements [1, 25]. The electrical stimulation of a presynaptic cell releases glutamate, which binds to the postsynaptic AMPA and NMDA receptors [1]. The NMDARs require that the postsynaptic terminal to be strongly depolarized at the same time that glutamate is present. This is because NMDARs have a positively charged magnesium ion in the channel opening. The immediate region around the channel must be sufficiently depolarized to remove the magnesium plug, otherwise the channel remains closed. If this occurs within a narrow window of time during which glutamate is also present, then the channel opens [25]. Thus, the

opening of the NMDA receptors is both ligand-dependent (release and binding of glutamate) and voltage-dependent (the depolarization of postsynaptic cell) [1]. Like AMPARs, Na+ ions pass through this channel when it opens, but unlike AMPARs, this channel also permits the rapid entry of Ca^{2+} as well [25]. So, once activated, NMDARs are highly permeable to Ca^{2+} [1, 30].

As mentioned earlier, the NMDA receptor is permeable not only to Na+ and K+ but also to Ca^{2+} and this influx of Ca^{2+} can lead to LTP through a variety of mechanisms [2]. Ca^{2+} influx into a dendritic spine triggers activation of protein kinases and phosphatases, which initiate a cascade of biochemical events [1, 25, 29]. Consequently, the post-synaptic influx of Ca^{2+} is a critical step underlying LTP [30]**.** The other quickest mechanism, which leads to LTP due to influx of Ca^{2+}involves the activation of calpain (a proteinase) that degrades fodrin, leading to uncovering of hidden receptors [2].

ii) Phases of LTP

One of the kinases activated by the influx of Ca^{2+} through NMDARs is Ca^{2+}/calmodulin dependent protein kinase II (CaMKII). This plays a key role in mediating the early-phase expression of LTP. High-frequency stimulation causes the influx of Ca^{2+} through NMDARs, which results in the autophosphorylation of CaMKII. The subsequent phosphorylation of CaMKII substrates is required for LTP induction and prolongs the association of the CaMKII holoenzyme at post-synaptic densities (PSDs) [1, 32]. Both AMPAR and NMDAR are enriched in the PSD and are anchored to the PSD by scaffolding proteins that tether them immediately below the synaptic cleft [25]. It is believed that the activated CaMKII at the PSD zone is responsible for potentiating synapses, probably by causing synaptic insertion of AMPARs (one of the substrates for CaMKII [25]) and/or increasing their single channel conductance. Several other kinases, such as PKC and mitogen-activated protein (MAP) kinase, may also be involved

in the expression of LTP. This modification of synaptic potentiation is believed to be capable of supporting LTP for 1–3 h. This period is termed *the early phase of LTP*.

Additional kinases such as extracellular signal Y regulated kinase (ERK), a second messenger called cyclic AMP, CREB (cAMP Response Element Binding protein) that allows gene expression through protein synthesis, and the balance between the activities of protein kinase A (PKA) and protein phosphatases (PP1 and PP2A) are also necessary to maintain LTP over longer periods [2, 25, 32].

For maintaining synaptic potentiation beyond the initial three hours, PKA and ERK pathways may be involved. This is termed *late-phase LTP* and appears to require gene transcription and protein synthesis. At the level of transcription, it has been further hypothesized that the phosphorylation of the nuclear transcription factor, CREB, is the key regulator for turning on gene expression. Initial analyses indicated that the CREB manipulations seemed to be correlated with changes in LTM [1]. Regulation of gene expression in this way is crucial to consolidation of memory from short-term to long-term. Eric Kandel and colleagues discovered a transcription factor, CREB-1, that activates some of the genes necessary to build proteins that support long-term memory. Kandel likewise discovered an *inhibitory* transcription factor, CREB-2, that constrains the actions of CREB-1, and hinders the formation of LTM [12]. Since then, the role of CREB as a central switch for the long-term plasticity and memory formation has been promoted as a central dogma in neuroscience [1].

iii) Long-term depression

In addition to its ability to produce LTP, a synapse also possesses the ability to decrease its synaptic efficacy. For instance, it has been shown that low-frequency (~1 Hz) stimulation of the

hippocampal Schaffer-collateral pathway for 15 min produces decreased EPSP responses at CA3–CA1 synapses. This type of synaptic plasticity can last at least one hour, and is called *long-term depression* (LTD). It is also known that synapses in other regions can produce the NMDA-receptor-independent LTD following the same low-frequency stimulation [1].

Similar to the typical form of LTP at the CA1 region, the induction of LTD also requires NMDA receptor activation. Pharmacological studies suggest that, in contrast to the involvement of α-CaMKII in the expression of LTP, Ca^{2+}/ calmodulin-dependent phosphatase plays a major role in the expression of LTD. Lower Ca^{2+} elevation, produced by low frequency stimulation, favors activation of protein phosphatase cascades [1].

1.2 KHAT

The stimulant leaf khat (*Catha edulis* Forsk) comes from a tree (of the family Celastraceae) which grows in countries bordering the Red Sea, along the east coast of Africa and in west Asia *Catha edulis* is popularly known as “khat” but is also known as “kat”, “qat”, “qad”, “qaad”. “jaad”, and “miraa” [33]. People living around the horn of Africa, East Africa and the Middle East [34, 35] have consumed it for centuries. Khat chewing is a widespread habit that has a deep-rooted socio-cultural tradition in these countries causing many socio economic problems [34].

Khat is chewed for recreational purposes and its valued psychostimulant effect is highest when fresh. The aforesaid explains the users’ preference for the fresh khat [35]. Fresh khat leaves mainly contain cathinone, a psychostimulant that is similar in structure and pharmacological activity to amphetamine [35, 36, 37]. Due to these similarities, cathinone has been called a ‘natural amphetamine’ [33, 38]. It is this psychostimulant effect that accounts for the popularity of khat [36].

Many different compounds are found in khat including alkaloids, terpenoids, flavonoids, sterols, glycosides, tannins, amino acids, vitamins and minerals. The phenylalkylamines and the cathedulins are the major alkaloids. The cathedulins are based on a poly-hydroxylated sesquiterpene skeleton and are basically polyesters of euonyminol. It has been reported that 62 different cathedulins from fresh khat leaves were characterized [39, 40]. Tannins, vitamin C. α and β sitosterol and friedeline, essential oils, proteins, carotene, calcium, thiamine, riboflavin, niacin and iron are also present in khat [41].

The khat phenylalkylamines comprise cathinone [S-(–)-cathinone], and the two diastereoisomers cathine [1S, 2S-(+)-norpseudoephedrine or (+)-norpseudoephedrine] and norephedrine [1R, 2S-(–)-norephedrine] [39, 40]. These compounds are structurally related to amphetamine (Fig 1) [41] and noradrenaline. The plant contains the (–)-enantiomer of cathinone only; the (+)-enantiomer is not found. Thus, the naturally occurring S-(–)-cathinone has the same absolute configuration as S-(+)-amphetamine [39, 40, 42].

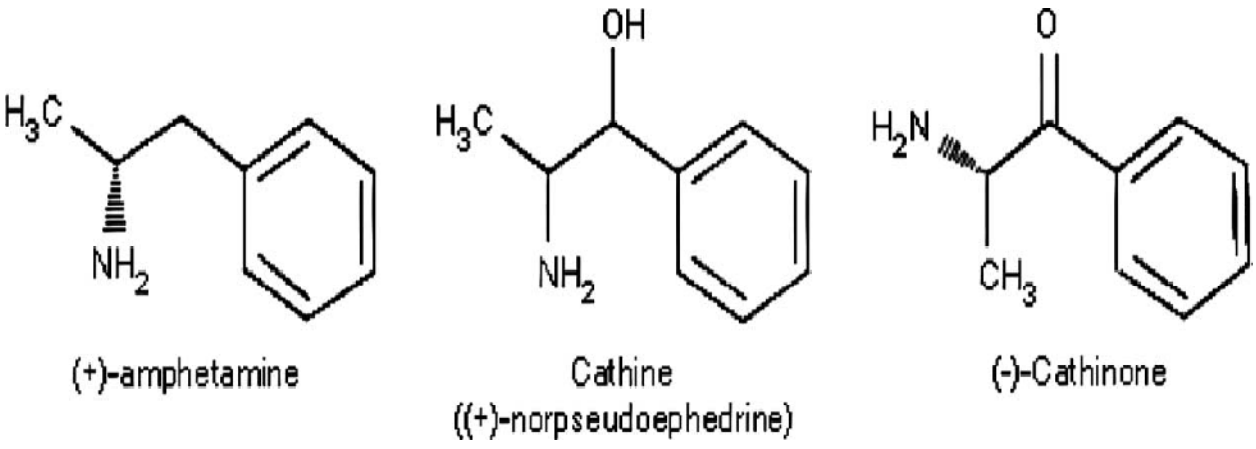

Figure 1: Chemical structures of amphetamine, cathine and cathinone

The pharmacologically active constituents of khat are (-)- cathinone and, to a lesser extent, (+)-norpseudoephedrine [38, 41].The environment, climate conditions, as well as local traditions connected with cultivation and harvesting determine the chemical profile and general appearance of khat leaves [39, 40, 42]. The phenylalkylamine content of khat leaves varies widely [42]. Cathinone is mainly found in the young leaves and shoots [39, 40, 41]. It is unstable (in the presence of oxygen, oxidizing at room temperature [43]), and undergoes decomposition reactions after harvesting and during drying or extraction of the plant material [39, 40, 43, 44]. The stored product loses activity rapidly, becoming physiologically inactive after about 36 h. For maximum potency, khat must be picked in the morning and chewed that afternoon [43]**.** During maturation, cathinone is enzymatically converted to cathine and norephedrine [38, 40, 41, 42]. The leaves contain cathine and norephedrine at a ratio of approximately 4:1 [39, 40]. Sunlight-induced or heat-induced degradation to cathine and norephedrine also occurs during extraction of cathinone in the laboratory [42]. In certain khat samples, the phenyalkylamine fraction consisted of up to 70% of (−) cathinone and that the (−) cathinone content is correlated with the market price of khat [42]. Cathinone has a more rapid and intense action compared with cathine due to its higher lipid solubility, which facilitates access into the central nervous system [36, 45]. Cathinone is presumably the main psychoactive component of khat, this explains why fresh leaves are preferred and why khat is wrapped up in banana leaves to preserve freshness [40, 42].

Chewing is the most common mode of administration, although a small number of users use dried leaves to make drinks and an equally small number smoke it. The traditional way to consume khat is to pick a few leaves of a young shoot and chew them slowly. Once the leaves are pulped, they are kept in the side of the cheek and the mouth is filled with fresh leaves [33]**.**

Cathinone and cathine are isolated from the leaves by the action of enzymes in saliva. When khat leaves are chewed, enzymes in the saliva release cathine and cathinone, which are absorbed through the mucous membranes of the mouth and subsequently the lining of the stomach [46].

Maximal plasma concentrations of (-)-cathinone, after a single oral dose of khat, are attained in about 2 h [41]. The effect of cathinone on the user occurs more rapidly than the effect of amphetamine, roughly 15 min as compared to 30 min [33]. The terminal elimination half-life is about 4 h [41]. The major metabolite of (-)-cathinone is (-)-norephedrine, but small quantities of (+) norpseudoephedrine also form [41, 42, 44]. Less amount of the ingested cathinone (7% or less) appears in unchanged form in the urine. In contrast, cathine and norephedrine are slowly absorbed and then excreted mainly in the unchanged form within about 24 h [41, 44]**.** The amount of norephedrine excreted in urine is much higher than the amount ingested, indicating that (−) cathinone is also metabolized to norephedrine. Cathine has been found in breast milk in several lactating women who were chewing the leaves of khat [42].

Similar to psychostimulants, khat ingestion produces several central nervous system effects, including increased motor stimulation, euphoria, and a sense of excitement and energy [33]. Khat usage is associated with memory impairment, depression and psychoses [46]. It also results in decreased appetite and increased blood pressure and heart rate. These effects indicate that khat acts through similar central mechanisms as other stimulants [33].

Cathinone releases catecholamaines from pre-synaptic storage sites resulting in CNS stimulation and a variety of peripheral sympathomimetic effects such as tachycardia and hypertension [47]. For example, both cathinone and amphetamine increase the activity of the dopaminergic and noradrenergic transmission [33, 43, 49]. The sympathomimetic properties are due to peripheral

noradrenalin-releasing properties of khat amines, which potentiate noradrenogenic transmission. It was also found to inhibit neural uptake of noradrenalin [41]**.** Among the three main khat alkaloids, cathinone is the most potent with regard to induction of release at CNS dopamine terminals. At peripheral sites, however, cathinone, norpseudoephedrine, and norephedrine are about equipotent with regard to induction of release at noradrenergic nerve terminals [45].

It has been reported that the effects of a portion of khat are very similar to those of about 5-mg amphetamine [47]. Although most of the pharmacological effects of the active principles are suggested to be mediated by the release of biogenic amines through preferential binding to the norepinephrine receptor/transporter, binding to dopamine and 5-HT receptors could also partly contribute to the observed effect [48].

Cathinone, like amphetamine, acts by releasing catecholamines from presynaptic storage sites and subsequently inhibit their uptake, thereby increasing temporal and spatial presence of these neurotransmitters (dopamine [DA], serotonin [5-HT] and noradrenalin [NA]) at the presynaptic receptors [35]. So far there is no clear cut evidence on the role of serotonergic and/or other pathways in the stimulatory effect of cathinone [42]. It has been suggested that cathinone, like amphetamine, releases serotonin in the CNS [40, 42, 43]. Psychostimulants and other drugs that inhibit uptake of 5-HT into the presynaptic nerve terminals increase serotonergic neurotransmission by enhancing its synaptic concentrations [35]. Some investigators have reported that levels of serotonin in rat brain are not altered by repeated administration of cathinone. Studies in humans showed that 5-HT plays a critical role in the pathogenesis of Alzheimer's disease as well as in learning and memory. Dopamine, on the other hand, plays a facilitatory role in cognitive functions, especially those guided by the prefrontal cortex [35]. Generally, cathinone is not considered a direct dopamine agonist but rather a presynaptic releaser

and re-uptake inhibitor of dopamine [40]**.** Likewise, studies demonstrated the antagonistic roles for DA and 5-HT in the modulation of spatial WM in humans [35].

Research on behavioral and cognitive problems following khat use in humans is not extensive and several of the available studies have been done only in the context of observational and single-case studies [33]. Comparative studies of amphetamine and khat on physiological and psychological behaviors are extensive but little is known about the effect of khat on spatial learning and memory [35]. A study made in Kenya by Kimaniand and Nyongesa [35] on acute intraperitonial administration of the extract showed that khat extract had selective effects on both learning and memory task in CBA mice. Other study made in Ethiopia on acute and sub-acute oral administration of the extract showed that khat had no effect on learning and memory [50]. Another study carried out locally demonstrated the ability of subchronic exposure mice to khat to induce schizophrenia-like symptoms that included cognitive decline [51]. However, subchronic exposure to mice did not induce morphological toxicity to the cell body of the dentate granule cells [52]. The present study aimed to investigate the effect of the extract on learning and memory at subchronic level, which has not been studied before, and to enable others make comparative studies using other paradigm; then make acceptable conclusion on subchronic level and move to chronic level.

2 OBJECTIVES

2.1 **General objective**

To evaluate the effects of subchronic exposure of mice to crude khat extract on learning and memory.

2.2 **Specific objectives**

- To evaluate the spatial learning and memory effect of khat using Morris Water maze
- To evaluate the spatial learning and memory effect of khat on T-maze tasks.
- To study the effect of crude khat extract with dose on learning and memory.
- To study the effect of crude khat extract on weight

3 MATERIALS AND METHODS

3.1 Drugs and chemicals

Tween 80(BH15 1TD^{R}, England) was obtained from the laboratory, Diethyl ether (Carlo-Erba^{R}, France) and chloroform (Carlo-Erba^{R}, France) were purchased from the respective local vendor (ZAF pharmaceutical).

3.2 Collection of khat

Catha edulis leaves (2000 g) were purchased fresh at a local market in Aweday, 515 km east of Addis Ababa, Ethiopia. The bundles of khat plant were bought in April 2013.The fresh bundles were packed in plastic bag and transported in an icebox to the laboratory. The fresh leaves were then immediately kept at -20 ºC. The plant was identified by a taxonomist and a voucher specimen (001) was deposited in the National Herbarium, College of Natural Sciences, Addis Ababa University.

3.3 Experimental animals

A total of 36 adult albino mice of both sex were used for the whole experiment and they were purchased from the Ethiopian Health and Nutrition Research Institute. They were 6-8 weeks of age and had weights ranging from 20 to 36 g. They were housed in plastic cages with standard wood chip bedding and had access to food and water *ad libitum*. Light was in its natural cycle (for a 12 h on and 12 h off). All experimental procedures on mice were done after 4:00 pm. Care and handling of the mice was performed according to the guidelines given by OECD [53, 54].

3.4 Extraction of Khat

The freeze-dried plant was finely minced, weighed and placed in Erlenmeyer flasks each wrapped with aluminium foil to avoid light induced decomposition. Chloroform (1350 ml) and diethyl ether (4050 ml) (1: 3 v/v) were added to cover the minced leaves. The resulting mixture was stirred using a rotary shaker at 120 rpm and 20 ºC for 72 h. The mixture was filtered initially using cotton gauze to separate the big particles, followed by Whatman No. 1 filter paper to get rid of fine particles. Initial evaporation was achieved using a rotavapour (120rev/min) under controlled temperature (40^{o}c). Since the mixture could not become completely dry we used a lyophilizer; then it dried. The dry extract was weighed by analytical balance and the yield was 2.6 %.This yield was much more than previously done extractions. The reason could, most probably, be due to the time (it was raining) of purchasing of the fresh leave since the environment and climate condition affects the general appearance of the khat. The extract was then kept in a tightly sealed material at -20 ºC until used.

3.5 Grouping and dosing of animals

The animals were randomly divided into four groups, each comprising nine animals. Group I served as negative control and was administered with Tween 80 in distilled water (3%, v/v). Group II-IV were administered three dose levels of khat extract: Low-100mg/kg (K100), Moderate-200mg/kg (K200) and High-300mg/kg (K300). The various doses for the khat extract were selected based on previous reports [48, 50, 51, 52] and then administered orally using gavages based on both their body weight. The control animals were given the same volume of the vehicle (0.5ml) throughout the month.

Throughout the experimental period, all drug sample solutions were made fresh and sample containers including syringes were covered with aluminium foil to avoid light decomposition. Since the weight of animals were necessary to determine the dose of khat extract, for the first week daily then on every three days of experiment, experimental mice were taken from their cage and weighed using electrical digital balance. A less stressful method was employed for oral administration of the extract in mice using gavage. This route was used since chewing is the most common mode of administration for humans [33] and pharmacokinetic studies showed that cathinone and cathine are absorbed from the stomach [46].

The dose of the extract required was determined according to the weight of mice. Total weight of mice taking the same dose of extract or the same group was determined for ease of dose calculation. Once total amount of extract determined was calculated, the extract was reconstituted with 3 % v/v Tween-80 in distilled water. The volume of reconstituted fluid was adjusted so that the maximum volume administration should not exceed 1ml as recommended by OECD guidelines [55, 56, 57]**.** This procedure was repeated everyday at similar time (after 4:00 pm) each day for 30 days of experiment based OECD guidelines and previous studies made at subchronic level [53, 54, 58].

3.6 Morris water maze task

The Morris water maze consisted of a circular polypropylene pool (122cm in diameter× 76cm in depth) and it was filled with tap water up to 20 cm high. A stable circular platform with 10 cm in diameter was used as the escape platform that had a rough surface enabling the animals to climb on easily. The pool was divided into 4 quadrants. Boundaries of the quadrants were marked on the edges of the pool with a masking tape labeled North (N), South (S), East (E) and West (W).

The platform was placed in the middle of the North West quadrant and 1cm beneath the surface of the water and it remained at the same position throughout the experiment. The pool was located in a room where paintings on each of the walls was mounted to provide visual cues which may be used by the animals to develop spatial map to navigate to the platform.

3.6.1 Habituation

Habituation or training session phase was used to limit the stress linked to water exposure and might teach the mice to remain on the platform. This phase was performed in a room without any cues. After the mice were introduced to the platform, they were allowed to swim for 60 sec in the pool and guided towards the platform if they failed to find it by themselves. This step was repeated until the mice remained for 30 sec onto the dry platform. This phase was not considered as day 1 of the trial since it was not part of the spatial acquisition sessions.

3.6.2 Acquisition

The spatial acquisition sessions took place in a room where distal cues were located on the walls surrounding the tank. The mice were subjected to 16 training trials consisting of daily sessions of 4 trials for 4 days to assess their learning potential. The trial started at 6 pm. During each trial, the mouse was placed randomly in one of the start locations (N, S, W, and E). The order of start locations was varied randomly so that each block of four trials/sessions in any given sequence was not repeated on consecutive days. Each mouse was allowed 60 sec to search for the platform. Once located, the mouse was allowed to stay on it for 30 sec. After each trial, the mouse was removed, dried in a rodent heater (2105 comerio VA^R, Italy) at 35-40^o c and put back in the home cages. The inter-trial interval for each mouse was 5 min. The escape latency, which

is the time taken by the mouse from the start position to the escape platform, was determined using a stop-watch.

3.6.3 Probe trial

Short term memory test: retention (probe trial) test for short term memory was performed on the fifth day, 24 h after the last acquisition day. Each mouse was subjected to a 60 sec probe trial in which the escape platform was removed completely. The mouse was placed in the MWM tank from the position (SE) opposite to the target quadrant (NW) in the acquisition phase. Time that was spent in the target quadrant was determined from the video recording and was taken as measure of spatial memory. Memory (retention) was represented by the time that was spent in the target quadrant during the probe trial.

Long term memory: probe trial for long term memory was carried out on the 12^{th} day, 7 days after probe trial for short term memory test. The same procedure for short term memory was followed for this test.

30 days of administration ⇨ habituation on 31^{th} day ⇨ acquisition training from 32^{th}-35^{th} day ⇨ STM test on 36^{th} day ⇨ LTM test on 43^{th} day

3.7 Multiple T-maze task

Multiple T maze (MTM), which is a wooden maze (150x130 cm wide, 15cm high, width 8 cm) containing seven choice points, was used for detection of changes in learning and memory. Before the test was started, mice were deprived of food for 12 h to motivate them for food search. The trial took place three times a day for four consecutive days. After each trial the T maze was cleaned by alcohol to avoid any potential cues (olefaction). By placing them in the

start box and being offered food pellets in the goal box, time to reach the goal box and number of wrong decisions (mean numbers of error) were recorded.

3.7.1 Habituation

On the first day habituation or training session was used in order to enable them habituate with the MTM. Each mouse was released in the MTM at the start box and allowed to explore the maze for 2 min. This phase was not considered as day 1 of the trial since it was not part of the spatial acquisition session.

3.7.2 Acquisition

On the acquisition phase, mice were subjected to a total of 12 trials consisting of daily sessions of three trials for 4 consecutive days to assess their learning potential. The trial was started at 6 pm. Before the test was started, mice were deprived of food for 12 h and each mouse was allowed a total of 5 min per trial to search for the food. If the mouse could reach the goal box before the set 5 min timeline, it was put back in the home cage. After each trial, the maze was cleaned by alcohol and the mouse put back in the home cages. The latency to reach the goal box, which is the time taken by the mouse from the start box to the goal box, and the wrong and right decision was determined using both a stop-watch and video recording by blinded observer.

3.7.3 Probe trial

Short term memory: retention (probe trial) to test short term memory was done on the fifth day, 24 h after the last acquisition day. Each mouse was subjected to a 5min probe trial. The trial was started at 6 pm. Before the test started mice were deprived of food for 12 h for motivation for food. If the mouse could reach the goal box before the set 5 min timeline, it was put back in the

home cage. After each trial, the maze was cleaned by alcohol and the mouse put back in the home cages. The escape latency, which is the time taken by the mouse from the start box to the goal box, and the wrong and right decision was determined using both a stop-watch and video recording by blind observer. The mice were assessed for their short term memory status in this single trial.

Long term memory: Probe trial for long term memory took place on the 12^{th} day, 7 days after probe trial for short term memory test. The same procedure for short term memory was followed for this test.

30 days of administration ⇨ habituation on 37^{th} day ⇨ acquisition training from 38^{th}-41^{th} day ⇨ STM test on 42^{th} day ⇨ LTM test on 49^{th} day

3.8 Weight change measurement

The weight of each mice was taken daily for the first week then every three days, since there was no daily difference in their weight starting from the first week.

For the ease of comparison, weight at day one (labeled as weight 0), at day four (labeled as weight 1), at the end of week 1 (labeled as weight 2), at the end of week 2 (labeled as weight 3), at the end of week 3 (labeled as weight 4) and at the end of week 4 (labeled as weight 5) was taken. Unlike to other parameters (which have n=7), the number of mice used in weight change measurement were 9 in each group (n=9) since some mice died before the experiment was started.

3.9 Statistical analysis

All values were expressed as Mean ± S.E.M. Differences in mean among groups for escape latency in both MTM maze and MWM, and wrong decision in MTM were analyzed using One-way ANOVA followed by Tukey HSD multiple comparison post hoc test as well general linear model: repeated measure of ANOVA (Two-way ANOVA). Two-way ANOVA was used to analyze acquisition training of both models since this training phase contain more than one parameter, while the remaining tests of both model were analyzed using One-way ANOVA. The significance level was set at $p< 0.05$.

4 RESULTS

4.1 Effect of crude khat extract on water maze performance

4.1.1 Acquisition training

The acquisition performance result is presented in Fig 2. Two-way repeated measures of ANOVA as test of within subject effects (latency to get the hidden platform as a factor) using Huynh-Feldt test (a sphericity test which uses for within group comparison) showed significant difference ($p<0.001$) across the four days of learning trials in the study group. Likewise, using both latency and group as a factor for the four days of learning, test (Huynh-Feldt) of within subject effects showed significant difference ($p<0.001$). The mean latency of CON was significantly decreased from day 1 up to day 4 while that of K100 significantly increased (Fig 2). However, comparison test on performance measure showed no significant difference among the groups ($F(3, 24) = 0.746$).

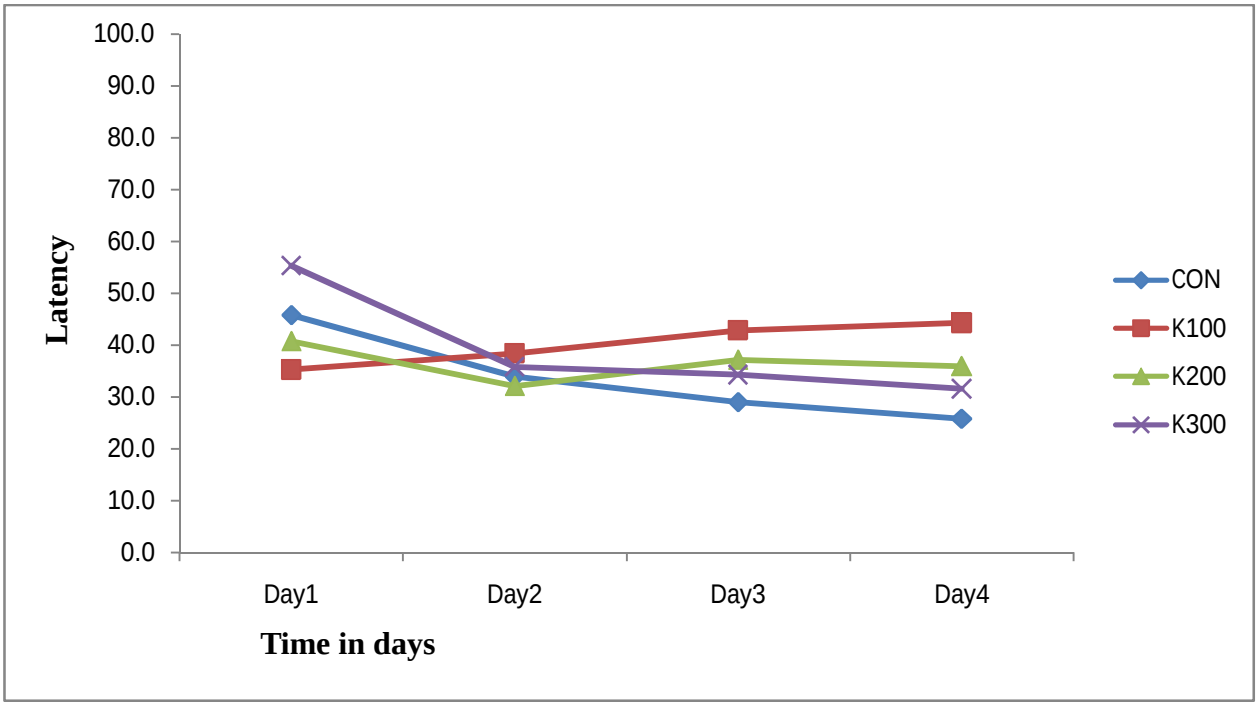

Figure 2: Mean latency to locate the hidden platform in the acquisition session of Morris water maze. Values are mean ± SEM (n=7) and statistical analysis was performed using two-way ANOVA; CON: Control group, K100:100mg/kg khat extract, K200: 200mg/kg khat extract, and K300:300mg/kg khat extract.

4.1.2 Short term memory retention phase

Effect of subchronic administration of crude khat extract on STM is depicted in Fig 3. Post hoc test with Tukey procedure revealed that the mean activity of CON was significantly higher than K100 ($p<0.05$), K200 ($p<0.01$), and K300 ($p<0.001$) mice. The negative control group spent more time in target quadrant, while khat treated groups spent more time in adjacent quadrant.

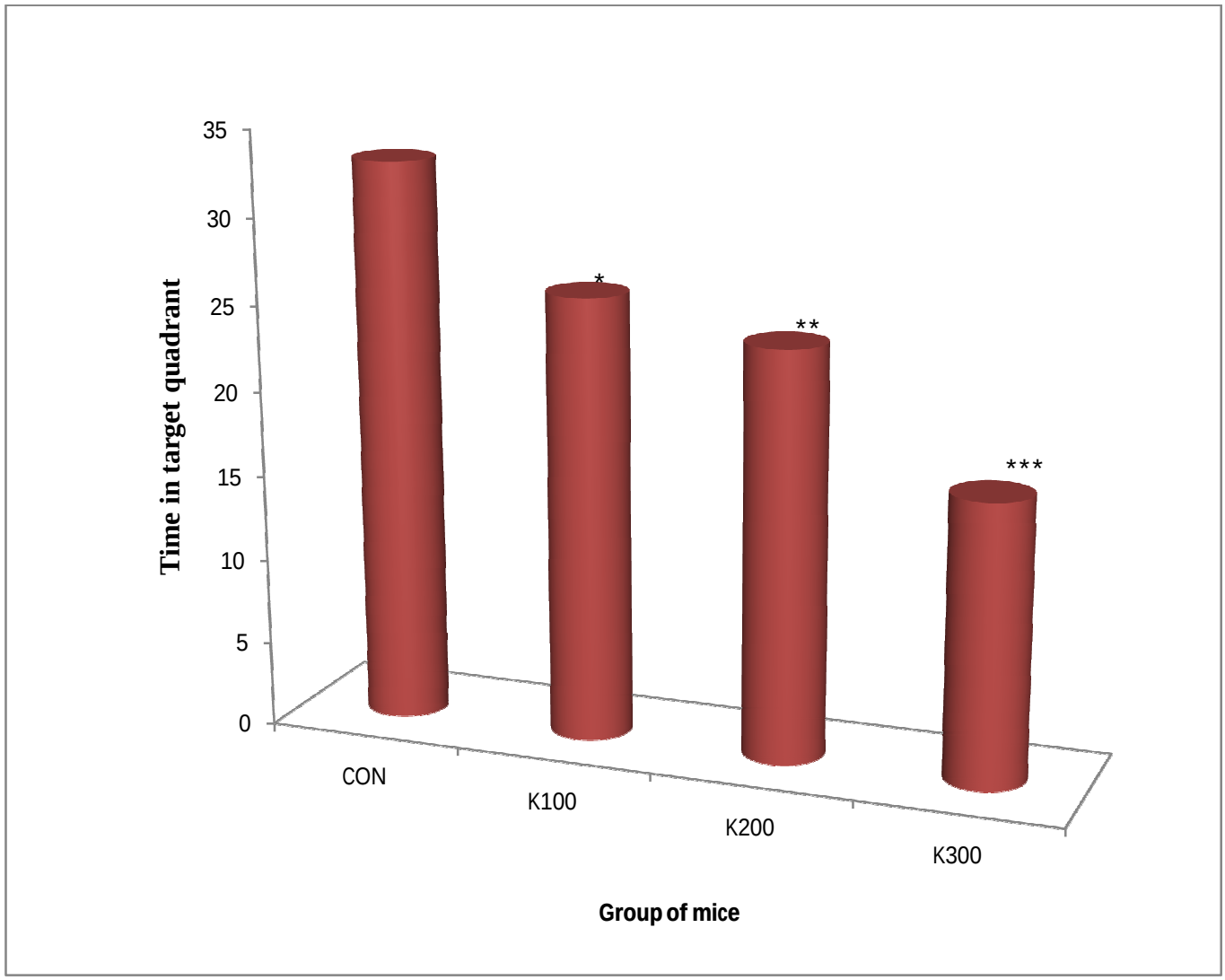

Figure 3: Mean time (sec) spent in target quadrant at day 5 in Morris water maze. Values are mean ± SEM (n=7) and statistical analysis was performed using one-way ANOVA; *: $p< 0.05$, **: $p< 0.01$, ***: $p<0.001$, CON: Control group, K100:100mg/kg khat extract, K200: 200mg/kg khat extract, and K300:300mg/kg khat extract.

4.1.3 Long term memory retention phase

Using the probe trial on day 12 (D12), time spent in target quadrant was measured and analyzed using one-way ANOVA followed by Post hoc test multiple comparison. No significant group differences were found in time spent in target quadrant (F (3, 24) = 0.052).

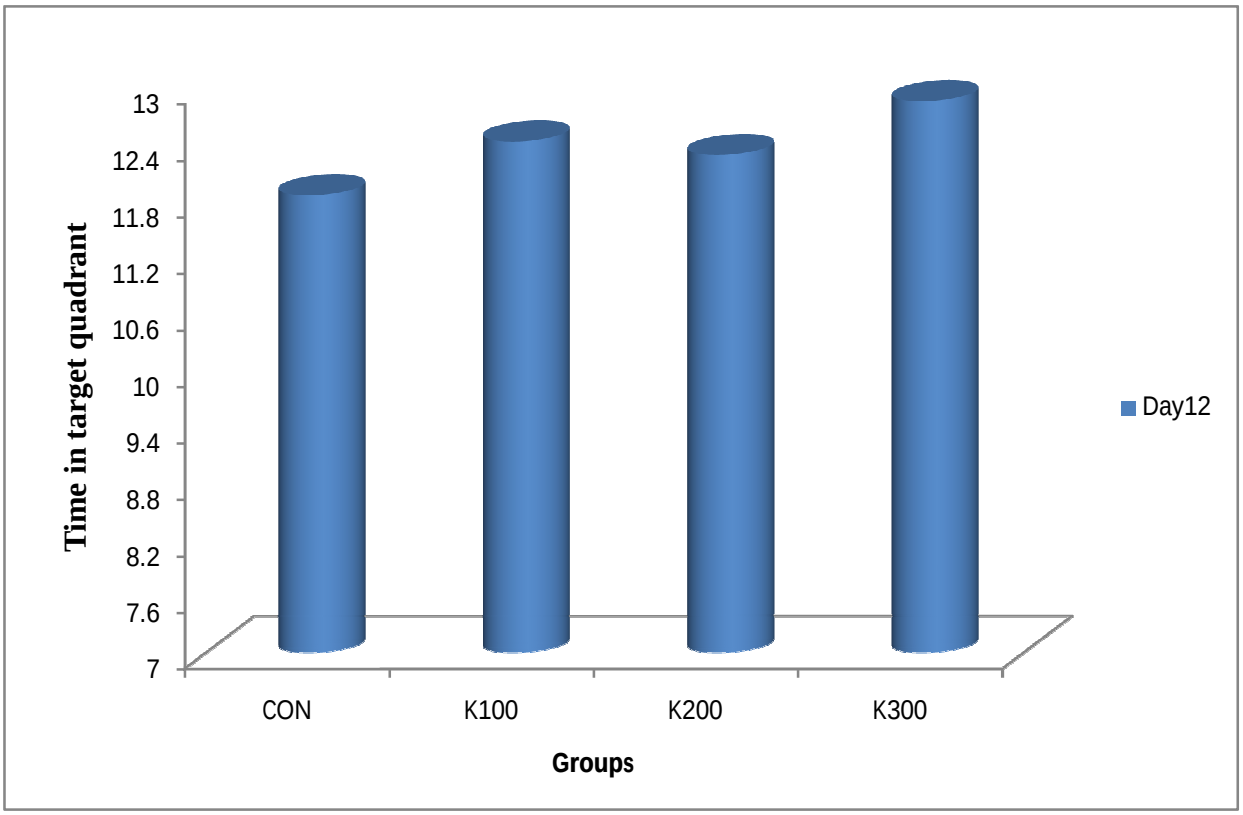

Figure 4: Mean time (sec) spent in target quadrant at day 12 in Morris water maze. Values are mean ±SEM (n=7) and statistical analysis was performed using one-way ANOVA; CON: Control group, K100:100mg/kg khat extract, K200: 200mg/kg khat extract, and K300:300mg/kg khat extract.

4.2 Crude Khat extract on Multiple T maze performance

4.2.1 Acquisition training

The acquisition performance result is presented in Fig 5. Two-way repeated measures of ANOVA as test of within subject effects (latency to reach the goal box as a factor), using Sphericity Assumed test (a Sphericity test which uses for within group comparison) showed significant difference ($p<0.01$) across the four days of learning trials in the study group. However, using both latency and group as a factor for the four days of learning, Sphericity Assumed test of within subject effects showed no significant difference. Likewise, comparison test on performance measure also showed no significant difference among the groups ($F(3, 23) = 1.084$).

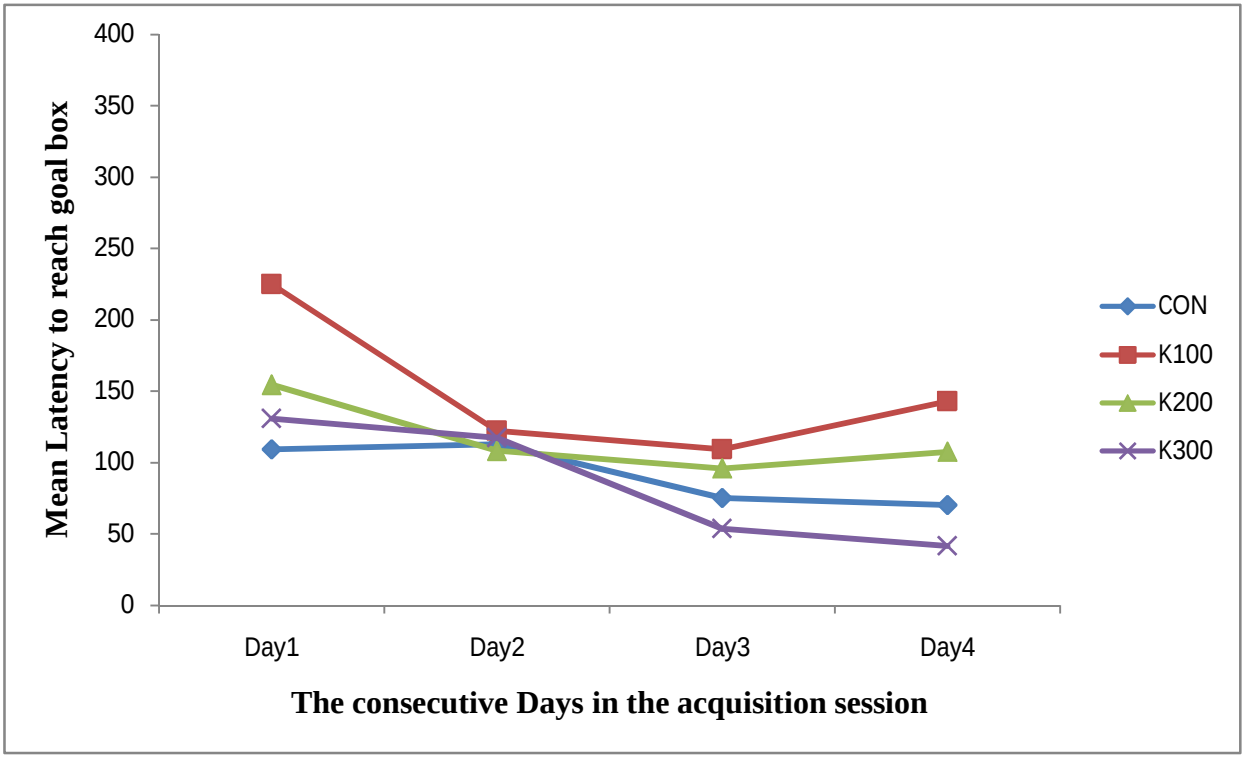

Figure 5: Mean Latency to reach the goal box in the acquisition phase of Multiple T maze. Values are mean ± SEM (n=7) and statistical analysis was performed using two-way ANOVA; CON: Control group, K100:100mg/kg khat extract, K200: 200mg/kg khat extract, and K300:300mg/kg khat extract.

4.2.2 Wrong decision at the acquisition training

The acquisition performance result is presented in Fig 5. Two-way repeated measures of ANOVA as test of within subject effects (latency to reach the goal box as a factor) using Huynh-Feldt test showed significant difference across the four days of learning trials in the study group. Likewise, using both latency and group as a factor for the four days of learning, Huynh-Feldt test of within subject effects showed significant difference. The mean error of CON was significantly decreased from day 1 up to day 4, while that of K100 significantly increased when only day 4 and day1 were considered rather than the whole four days consecutive progress (Fig 6). However, comparison test on wrong decision performance measure showed no significant difference among the groups (F (3, 23) = 1.403).

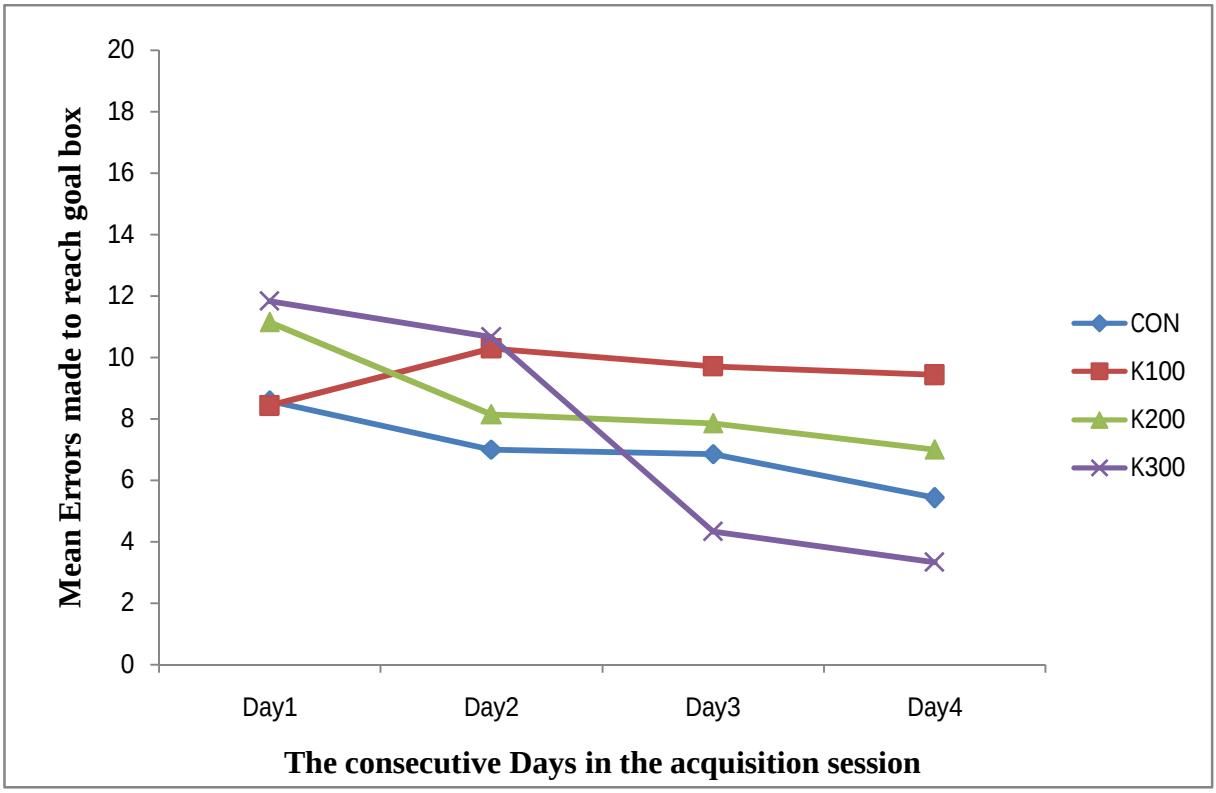

Figure 6: Mean errors made to reach Multiple T maze goal box in the acquisition phase: Values are mean ± SEM (n=7) and statistical analysis was performed using one-way and two-way ANOVA; CON: Control group, K100:100mg/kg khat extract, K200: 200mg/kg khat extract, and K300:300mg/kg khat extract.

4.2.3 Short term memory retention phase

Effect of subchronic administration of crude extract of khat daily for 1 month on STM in mice using MTM is shown in Fig 6.Significant group differences were found in latencies ($F(3,23)= 13.34$). One-way ANOVA followed by Post hoc test with Tukey procedure revealed that the mean latency of CON was significantly less than K200 ($p<0.01$), and K300 ($p<0.001$) mice. Importantly, the mean latency of CON was not significantly less than K100.

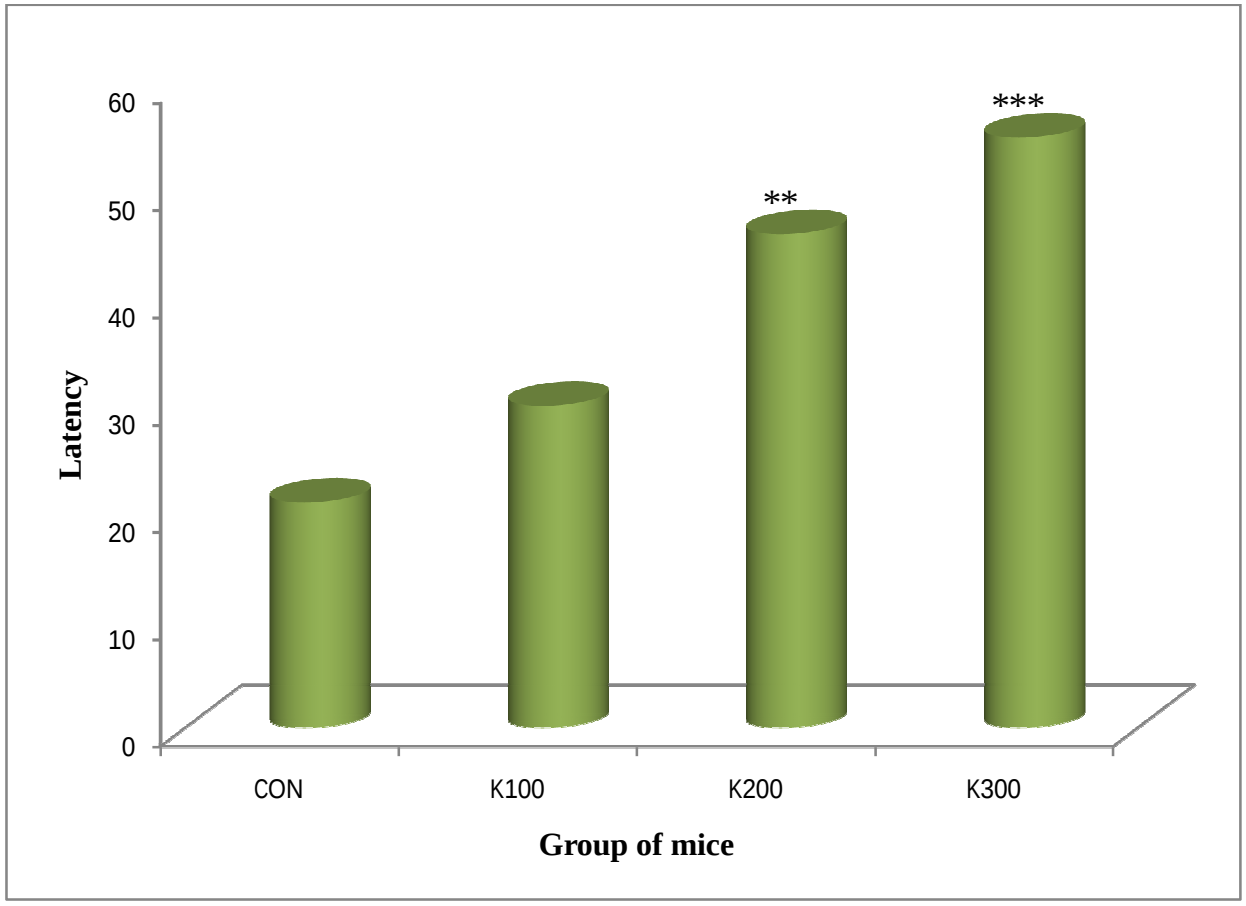

Figure 7: Mean latency to reach the goal box at day 5 in Multiple T Maze task. Values are mean ± SEM (n=7) and statistical analysis was performed using one-way ANOVA; **: $p< 0.01$. ***: $p<0.001$, CON: Control group, K100:100mg/kg khat extract, K200: 200mg/kg khat extract, and K300:300mg/kg khat extract.

4.2.4 Wrong decision at probe trial at day 5

Effect of subchronic administration of crude extract of khat daily for 1 month on wrong (error in trial) decision at the probe trial on day 5 (D5) using MTM is shown in Fig 9.Significant group differences were found in wrong decision (F (3, 23)= 10.767). One-way ANOVA followed by Post hoc test with Tukey procedure revealed that the mean wrong decision of CON was significantly less than both K200, and K300 mice but there was no significance difference between CON and K100.

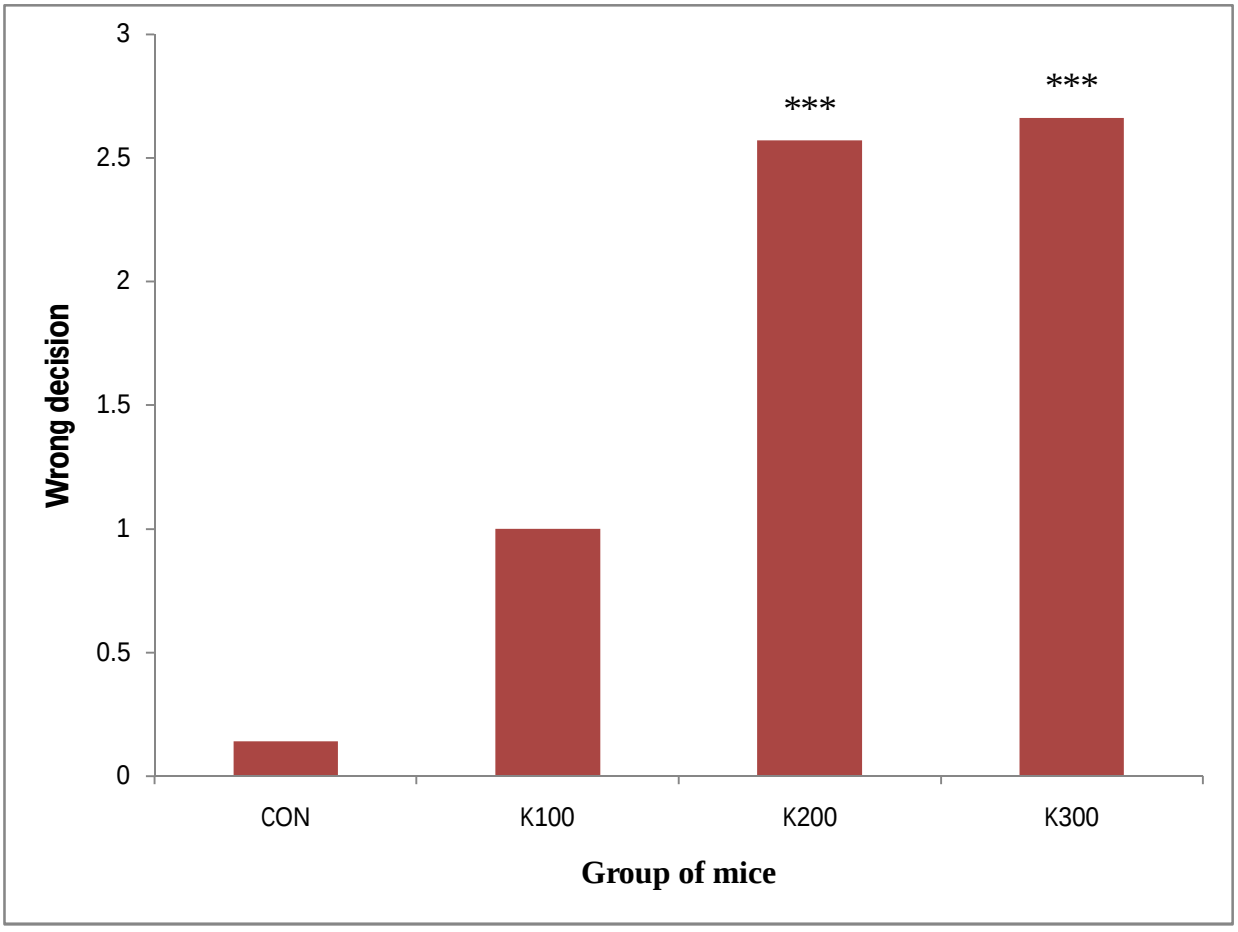

Figure 8: Mean wrong decision of mice at day 5 (D5) in Multiple T Maze task: Values are mean ± SEM (n=7) and statistical analysis was performed using one-way ANOVA; ***: $p<0.001$, CON: Control group, K100:100mg/kg khat extract, K200: 200mg/kg khat extract, and K300:300mg/kg khat extract.

4.2.5 Long term memory retention phase

Using the probe trial on day 12 (D12), the latency to reach the goal box from the start box was measured and analyzed using one–way ANOVA followed by Post hoc test multiple comparison ($F(3,23) = 2.178$, $p>0.1$). Post hoc test with Tukey procedure revealed that the mean latency of CON is not significantly different from K100, K200 and K300.

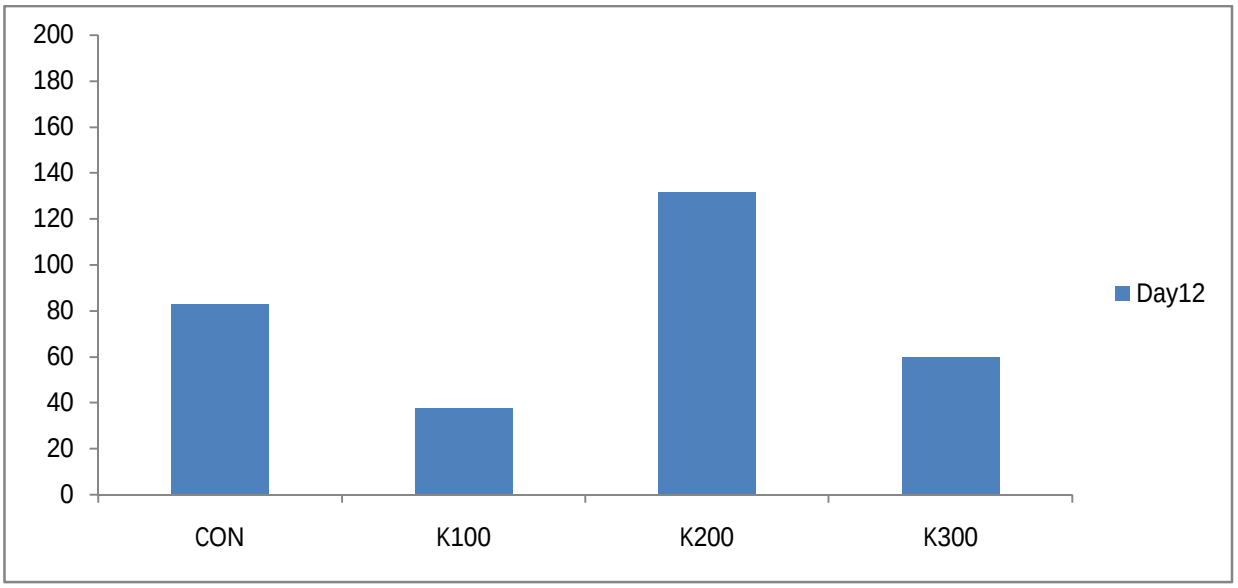

Figure 9: Mean latency at day 12 in Multiple T maze. All values are mean ± SEM (n=7) and statistical analysis was performed using one-way ANOVA; CON: Control group, K100:100mg/kg khat extract, K200: 200mg/kg khat extract, and K300:300mg/kg khat extract.

4.2.6 Wrong decision at probe trial at day 12

Using the probe trial on day 12 (D12), the wrong decision while the mice tried to reach the goal box from the start box was measured and analyzed using One-way ANOVA followed by Post hoc test ($F(3, 22) = 0.7$) with Tukey procedure revealed that there was no significant difference among the mean wrong decision of the groups

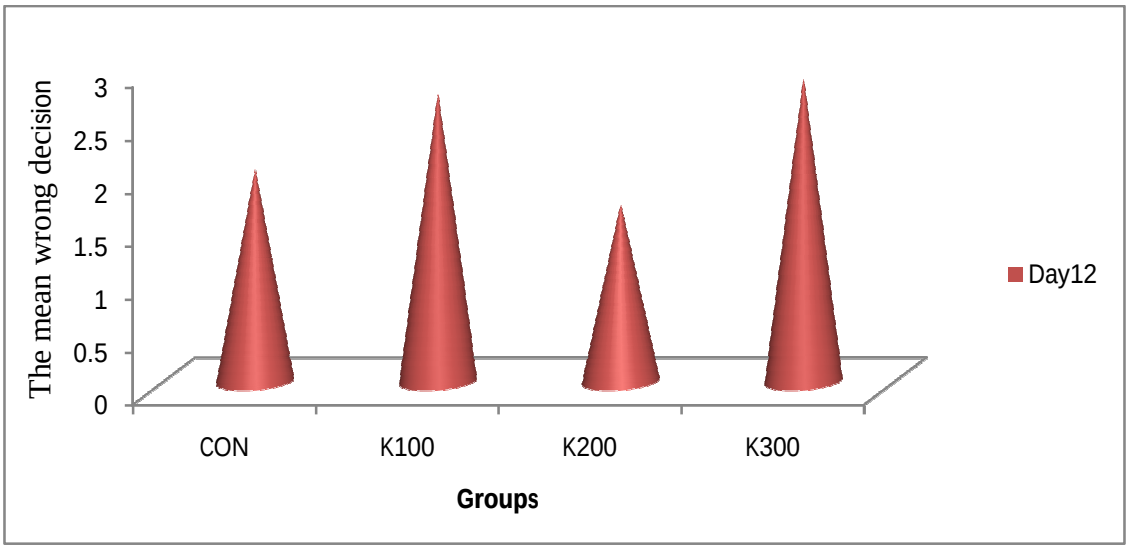

Figure 10: Mean wrong decision day 12 in Multiple T maze. All values are mean ± SEM (n=7) and statistical analysis was performed using one-way ANOVA; CON: Control group, K100:100mg/kg khat extract, K200: 200mg/kg khat extract, and K300:300mg/kg khat extract.

4.3 Effect of crude khat extract on weight

The effect of crude khat extract on weight was measured using One-way ANOVA. As shown in Fig 9, the weight of CON mice increased throughout the period of administration, while the weight of khat-treated animals decreased up to day four (weight 1) then returned to the weight of day 1 (weight 0) at the end of week1 (day7) and increases up to the weight of weight 5 (day 30). There was significant weight difference among groups at day 4 (Delta1: weight at day 4 minus weight at day1) ($F(3, 32) = 94.539$) (Fig 10). Post hoc test with Tukey procedure revealed that the weight of CON group has significantly increased when compared with K100, K200 and K300.

One –way repeated measure of ANOVA for the Delta 2(weight difference between weight 5 and weight1) shows there is no significant weight difference among groups ($F(3, 32) = 0.000$) (Fig 10).Post hoc test with Tukey procedure revealed that the weight of CON was not significantly different from the other groups (K100, K200 and K300).

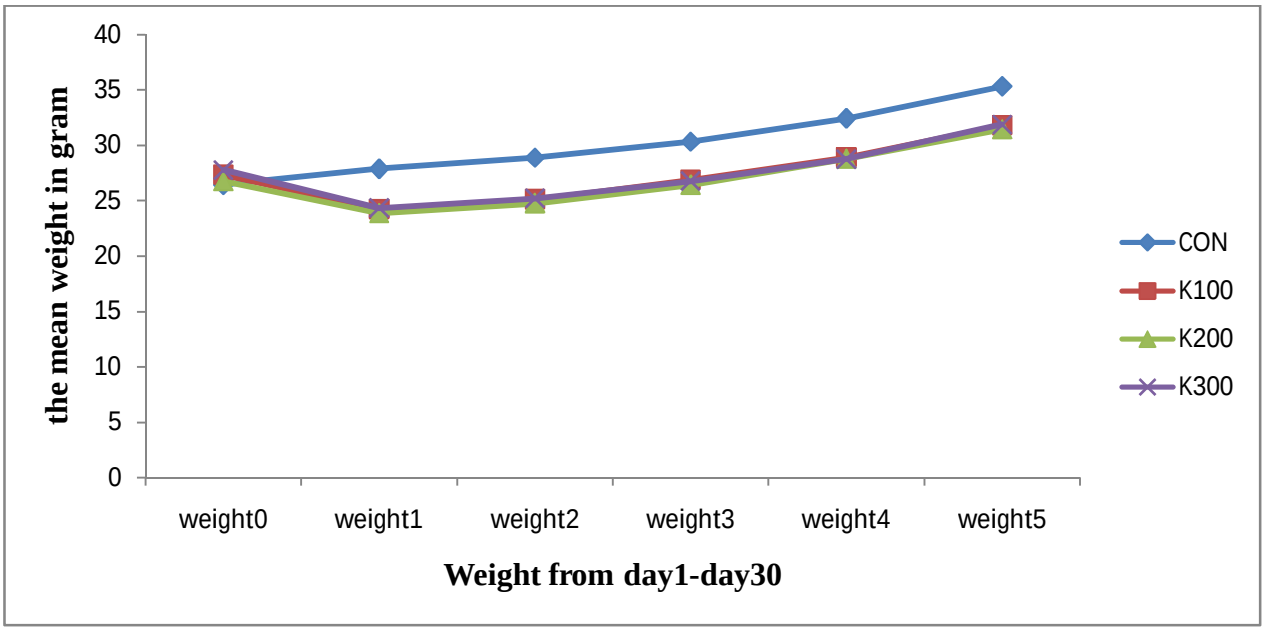

Figure 11: Mean weight from day 1 to day 30 in gram for all groups. Values are mean ±SEM (n=9); CON: Control group, K100:100mg/kg khat extract, K200: 200mg/kg khat extract, and K300:300mg/kg khat extract.

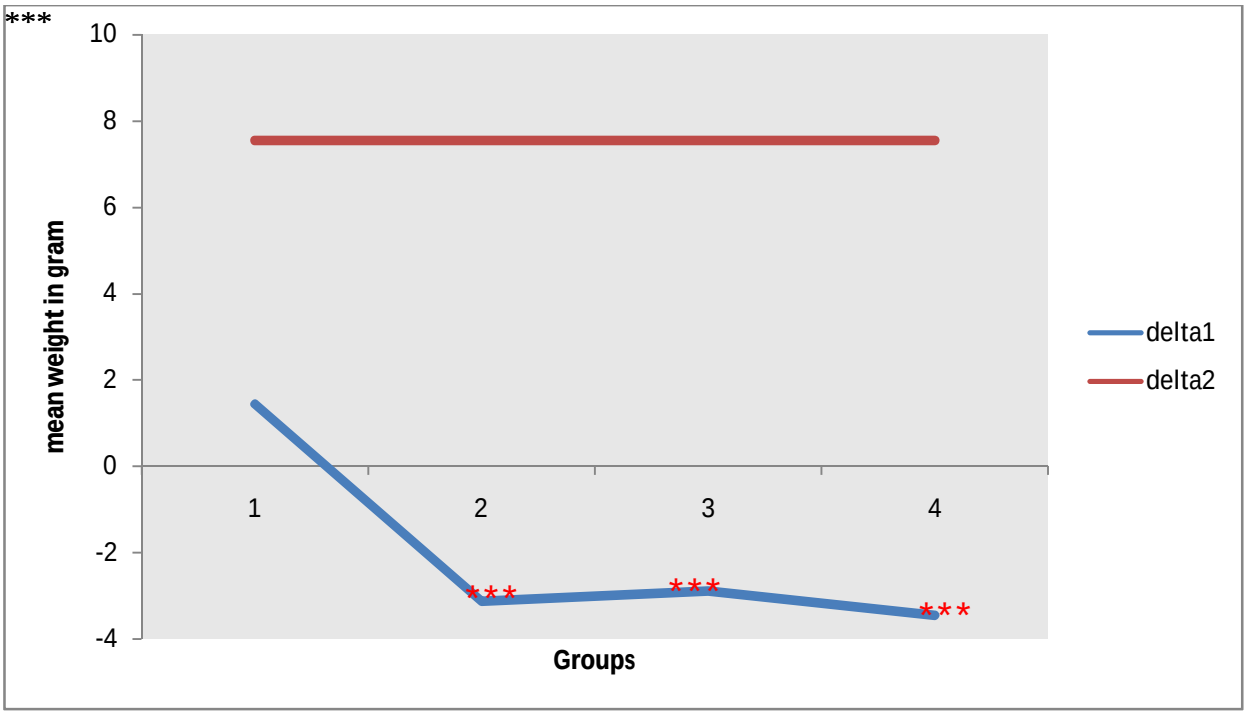

Figure 12: Mean weight differences. Values are mean ±SEM (n=9); all values are mean ± SEM (n=9) and statistical analysis was performed using one-way ANOVA; ***: $p<0.001$, CON: Control group, K100:100mg/kg khat extract, K200: 200mg/kg khat extract, and K300:300mg/kg khat extract. Delta 1 is the difference between weight at day 4 (weight 1) and weight before administration of the extract and the vehicle at day 1 (weight 0). Delta 2 is the difference between weight at day 30 (weight 5) and weight at day 4 (weight 1).

5 DISCUSSION

The results of the present study showed that khat extract had effects on STM but not on learning and LTM in albino mice. The low dose of khat extract had no effect on STM, while the moderate and high dose of this extract impaired STM using MTM. However, all the three doses impaired STM using MWM. Thus, moderate and high doses had, at least, impaired STM using both models of MTM and MWM. This result can be further justified by using the impact of the three different doses on wrong decision at the probe trial of day 5, where moderate and high doses increased wrong decision at this trial. This result showed the impact of the extract on decision making on the one hand and on STM on the other hand because the ability to make correct/wrong decision at the probe trial of day 5 is related with the STM. The extract had no impact on LTM using both models. This result was consistent with the wrong/correct decision obtained on the probe trial at day 12.

Currently most of the deductions that are made about the effect of khat on learning and memory are based on amphetamine or its derivatives [43]. Extensive use of methamphetamines, for instance, has also been repeatedly associated with deficits in episodic memory. Likewise, preliminary observations suggest that chronic use of khat is associated with various cognitive and mental health impairments [33]. But chronic use of khat associated with brain problems have not been carefully studied [43]. The deficits seen in chronic methamphetamine users are most evident as impairment in word recall tasks, which measure recall at specific times after stimulus presentation [33].

The results of the present study showed that crude khat extract had no effect on learning though low dose appeared to reduce learning that failed to reach statistical significance. Test of within subject effects showed difference across the four days of learning. This shows that each group

learned across the four days of trial but the learning failed to be significantly different between groups.

Crude khat extract did not impair learning consistently as the dose increased. Since this result was obtained using both MWM and MTM paradigms, it is plausible to suggest that the extract does not impair learning. Basically, there are few studies that are conducted recently on the effect of khat extract on learning and memory. However, the results reported are conflicting with each other. For example, a study made in Kenya showed that khat extract had selective effects on both learning and memory [35] but other study made in Ethiopia showed that acute and subacute exposure of mice to khat had no effect on learning and memory [50]. Another study conducted in Ethiopia [52] also looked at the effect of subchronic oral administration of khat extract on the dentate granules cells in mice and reported that khat did not have morphological toxicity to the cell body of these cells. This could possibly suggest that impairment of STM observed in the present study was not caused by induction of morphological toxicity to the cell body of dentate granules cells. However, the morphometric study did not include morphological changes to the structure of dendrite, axon or synaptic area of neurons. As a result, it may not be possible to conclude that khat extract at sub-chronic level does not have morphological effect on hippocampus, since mechanisms that have been shown to underlie LTP involve increases in dendritic spine size and its associated increase in the number of AMPA receptors [27]. Damage to the hippocampus itself is sufficient to produce clinically significant and readily detectable memory impairment. Importantly, the extract might have additional damage to the adjacent cortical regions along the parahippocampal gyrus, since this damage greatly exacerbates the memory impairment [14]. Neurobiological changes due to chronic drug use vary as a function of many factors, including the class of drugs and the pattern of use as well as the complex interplay

with preexisting neuro-developmental factors [33]. This suggests that the extract did not disrupt mechanisms underlying synaptic plasticity, which are important to maintain learning [32]. Importantly, it did not strengthen the connection between plasticity and learning since no enhancement or promotion was observed in learning. It is expected that the extract did not affect the dopaminergic system and did not cause stimulation of ß-1-adrenergic receptors. Khat and cathinone have shown to influence learning through the dopaminergic and noradrenergic system [59, 60]. Khat users report that they use this substance to improve their performance, stay alert, increase their energy and to enhance their imaginative ability and capacity to associate ideas, although their concentration and judgment are objectively impaired [33, 42, 47]. Importantly, all of these findings and our result implicate that khat extract does not improve performance or does not enhance imaginative ability and capacity to associate ideas, and does not induce morphological toxicity to the cell body of dentate granules cells, at least, at subchronic level.

The result of the present study regarding STM might be due to both structural and functional brain changes since the study of other similar drugs of abuse suggests that individuals who have been chronically exposed to drugs of abuse show both structural and functional brain changes [33]. Cathinone, like amphetamine, acts by releasing catecholamines from presynaptic storage sites and subsequently inhibit their uptake, thereby increasing temporal and spatial presence of these neurotransmitters (dopamine [DA], serotonin [5-HT] and noradrenalin [NA]) at the presynaptic receptors [35]. It has been suggested that cathinone, like amphetamine, releases serotonin in the CNS [43, 47]. So far, there is no clear-cut evidence on the role of serotonergic and/or other pathways in the stimulatory effect of cathinone [42]. Psychostimulants and other drugs that inhibit uptake of 5-HT into the presynaptic nerve terminals increase serotonergic neurotransmission by enhancing its synaptic concentrations. This hypothesis has been supported

by various studies in both humans and experimental animals [35]. If these two hypotheses are to be true, it can be suggested that the extract (through cathinone) had increased synaptic concentration of serotonin thereby impairing STM. This neurotransmitter is expected to have negative effect on memory, which was reported by studies showing that 5-HT plays a critical role in pathogenesis of Alzheimer's disease as well as in learning and memory [35]. Further evidence could come from a recent study in which both khat extract and cathinone produced a significant depletion of serotonin and its metabolite 5-hydroxyindoleacetic acid in both the anterior and posterior striatum [40]. However, there are other studies showing that cathinone does not alter levels of serotonin in rat brain following repeated administration [42]. Likewise, khat extract or cathinone interacts with the dopaminergic pathways, which have a significant role in maintaining memory or plays a facilitatory role in cognitive functions then increasing the activity of this pathway [35, 40, 43, 49]. Chronic administration of either the whole extract (since both cathine and norephedrine also have effect) or cathinone (100 mg/kg) results in a significant depletion of dopamine in several brain areas, particularly on the nigrostriatal dopamine terminal projections [42, 45]. Similarly, findings regarding the positive effect of epinephrine (by elevating blood glucose levels) on memory indicates that the extract might impair STM by decreasing blood glucose level on the day of probe trial either causing depletion of epinephrine or impairing the conversion of NA to epinephrine which lowers the concentration of epinephrine [22, 59, 60]. Thus, although the mechanism by which khat impaired STM remains to be seen, all of these findings and our result indicate that khat extract impairs cognitive function and STM without inducing morphological toxicity to the cell body of dentate granules cells with subchronic exposure.

The wrong decisions made on probe trial at day 5 seem to be consistent to that obtained with amphetamine and its derivative methamphetamine. Methamphetamine-dependent individuals exhibit risky decision making and impulse control problems as demonstrated by their sensitivity to immediate versus delayed rewards [33]. Another study also noted the potential to adverse effects of amphetamine on perceptual-visual memory and decision-speed [43]. These two studies therefore support the result obtained from the crude extract. Other study conducted on chronic amphetamine users also showed disadvantageous decision-making and selected a likely small reward option less frequently than controls (85% of trials versus 95%), which may reflect an impairment in correctly estimating outcome probabilities [33]. Even if the study regarding the effect of amphetamine on decision making was made on chronic administration level, it could give an idea to suggest that both amphetamine and khat extract have a disadvantageous effect on decision making.

The results of the present study showed that crude khat extract had an impact on STM. All the three doses impaired STM using MWM but the low dose had no effect on STM using MTM. Thus, moderate and high doses had, at least, impaired STM using both models of MTM and MWM. This impairment of STM by crude khat extract was obtained and supported by both paradigms, which have a total of three parameters considered all together (latency to find the hidden platform, latency to reach the goal box and wrong decisions made). However, the low dose showed different results. The low dose in the MTM task had no effect on STM, which is better to incline on this result since this was based on two parameters (latency to reach the goal box and wrong decisions made) rather than that of MWM task, which was based on one parameter (latency to find the hidden platform). Another study carried out locally by Bogale and Engidawork [51] demonstrated the ability of subchronic exposure mice to khat to induce

schizophrenia-like symptoms that included cognitive decline. This cognitive decline at subchronic level is consistent with our result on memory (STM) and probably indicates the sensitivity of MWM over MTM.

The present study showed that crude khat extract did not have effect on LTM. Like learning and STM, the effect of the extract on LTM was obtained and supported by both paradigms. This result suggests that the extract did not have effect on the molecular mechanisms underlying consolidation, probably related to lack of ability to induce morphological changes to the dentate granule cells of the hippocampus, which are involved in the consolidation process.

The decrease in weight is the consequence of the anorexic effect of khat since anorexia, a characteristic effect of amphetamine-like substances, is a consequence of khat chewing [42]. There is clear-cut evidence that both cathinone and amphetamine stimulate the CNS and suppress appetite [38]. Even if the result showed khat is much likely to cause tolerance, since there was an increase in weight in the last two weeks, there are controversies regarding the level of tolerance caused by khat. This has made the nature of khat dependence to remain under active debate and accumulating evidence also indicates the existence of a withdrawal syndrome and a low level of tolerance [33]. Some studies reported that CNS tolerance is not usual in khat users probably due to the physical limits on the amount that can be chewed [47]. Subsequently, other studies show that khat, in comparison with amphetamine, is much less likely to cause tolerance. In particular, the stimulant central nervous system effects of khat do not seem subject to the development of tolerance, but some degree of tolerance to insomnia and anorexia has been observed in most chronic khat chewers [36]. But others reported the development of tolerance to the effects of cathinone is more rapid than to that of amphetamine, and there is cross-tolerance between cathinone and amphetamine [43].

6 CONCLUSION

This study showed that subchronic exposure of mice to khat had significant effect on short STM but had no significant effect on learning and long term memory using Morris water maze and Multiple T maze models. Crude khat extract had also significant effect on initial loss of body weight even if tolerance developed finally to the anorexic effect of this extract.

7 RECOMMENDATION

From the present investigation, the following recommendations are made:

- The effect of chronically administered crude khat extract on learning and memory needs to be investigated.
- The effects of crude khat extract on learning and memory needs to be investigated using other paradigms.
- The effect of cathinone that is fractionated from the crude extract of khat on learning and memory needs to be investigated.
- Neurotransmitters level (dopamine, serotonin and Noradrenalin) needs to be determined.
- The effect of crude khat extract on genetic and epigenetic level needs to be investigated
- The effect of nicotine and crude khat extract together on learning and memory needs to be investigated as a new direction.
- The effect of alcohol and crude khat extract together on learning and memory needs to be investigated as a new direction.
- The effect of caffeine and crude khat extract together on learning and memory needs to be investigated as a new direction.

.

8 REFERENCES

1. Albers, W., Brady, S. and Price, D. (Eds.) (2006). Basic Neurochemistry: molecular, cellular and medical aspects (7th Ed.), *American Society for Neurochemistry*.
2. The Cognitive Neuroscience of Memory. Pp1-23 (16/11/13,12a.m) www.inference.phy.cam. ac.uk/jmb86/**memory**
3. Narwal, S., Saini, D., Kumari, K., Narwal, S., Singh, G., Negi, R. and Sarin, R. (2012). Behavior & pharmacological animal models for the evaluation of learning & memory condition, *Indo Global Journal of Pharmaceutical Sciences* **2**(2): 121-129.
4. Cotelli, M., Manenti, R., Zanetti, O. and Miniussi, C.(2012). Non-pharmacological intervention for memory decline,*Frontiers in Human Neuroscience* **6**(46):1-17.
5. Joshi, M., Gaonkar, K., Mangoankar, S. and Satarkar, S.(2012).Pharmacological investigation of Areca catechuextracts for evaluation of learning, memory and behavior in rats, International Current Pharmaceutical Journal **1**(6): 128-132.
6. Paul, C., Giordano Magda, G., Abel, S.(2009). Spatial memory: theoretical basis and comparative review on experimental methods in rodents, *Behavioural Brain Research* **203**:151–64.
7. Johansen, J., Cain, C., Ostroff, L., and LeDoux, J. (2007). Molecular mechanisms of fear learning and memory, *Cell* **147**:509-24.
8. McHugh, S. and Bannerman, D. (2010). Cognition: learning and memory: spatial, *Elsevier Ltd*, 279-87.
9. Roozendaal, B. (2000). Glucocorticoids and the regulation of memory Consolidation, *Psychoneuroendocrinology* **25**: 213–238.

10. Meneses, A., Perez-Garc, G., Ponce-Lopez, T. and Carlos Castillo, C. (2011).5-HT6 receptor memory and amnesia: behavioral pharmacology learning and memory processes, *International Review Of Neurobiology* **96**:27-46.
11. Izquierdo, I., Medina, J., Vianna, M., Izquierdo, L., and Barros, D. (1999). Separate mechanisms for short- and long-term memory, *Behavioural Brain Research* **103**:1–11.
12. Snead, O. (2011).Memory and punishment, *Vanderbilt Law Review* **64**(4):1195-1264.
13. Slotnick, S.D., and Schacter, D.L. (2007). The cognitive neuroscience of memory and consciousness, Cambridge handbook of consciousness, 809-827. Newyork: Cambridge University Press.
14. Squire, L., and Wixted, J. (2011). The cognitive neuroscience of human memory since H.M., *Annu. Rev. Neurosci*. **34**:259–88.
15. Cowan, N. (2008). What are the differences between long-term, short-term, and working memory? Sossin, W., Lacaille, J., Castellucci, V. & Belleville, S. (Eds.), Elsevier B.V. *Progress in Brain Research **169**:*323-338.
16. Paré, D. (2003). Role of the basolateral amygdala in memory consolidation, *Progress in Neurobiology* **70**:409–420.
17. Diekelmann, S. and Born, J. (2010).The memory function of sleep, *Nature Reviews | Neuroscience***11**:114-26.
18. Frankland, P. and Bontemp, B. (2005). The organization of recent and remote memories, *Nature Reviews | Neuroscience***6:**1-119-41.
19. Miyashita, T., Kubik, S., Lewandowski, G. and Guzowski, J (2008). Networks of neurons, networks of genes: an integrated view of memory consolidation, *Neurobiology of Learning and Memory* **89**: 269–284.

20. Heinrichs, S.(2007). Enhancement of learning and memory performance: modality-specific mechanisms of action: in Neurobiology of Learning and Memory (2nd ed.),*Elsevier Ltd*, 541-74.
21. Abel, T. and Lattal, K.(2001). Molecular mechanisms of memory acquisition, consolidation and retrieval, *Current Opinion in Neurobiology* **11**:180–187.
22. Cahill, L. and McGaugh, J. (1996).Modulation of memory storage, *Current Opinion in Neurobiology* **6**:237-242.
23. McGaugh, JL., McIntyre, C. and Ann E. Power (2002). Amygdala Modulation of memory consolidation: interaction with other brain systems, *Neurobiology of Learning and Memory* **78:**539–552.
24. D'Hooge, R., DeDeyn, P. (2001). Applications of the Morris water maze in the study of learning and memory, *Brain Research Reviews* **36**: 60–90.
25. Lombroso, P. and Ogren, M. (2009).Learning and Memory, Part II: molecular mechanisms of synaptic plasticity, *J. Am. Acad. Child Adolesc. Psychiatry*, **48**(1):1-5.
26. Baudry, M. (2009). Synaptic plasticity: learning and memory in normal aging, *Elsevier Ltd ,757-62.*
27. Niedringhaus, M., Chen, X., Conant, K. and Dzakpasu, R.(2013). Synaptic potentiation facilitates memory-like attractor dynamics in cultured *in vitro* hippocampal networks, *Niedringhaus et al* .Pp 1-22.**http://www.plosone.**org/ article/info:doi/10.1371/ journal. pone.0057144.
28. Izquierdo, L., Barros, D., Vianna, M., Coitinho, A., Silva, T., Choi, H., Moletta, B., Medina, J., and Izquierdo, I. (2002).Molecular pharmacological dissection of short- and long-term memory, *Cellular and Molecular Neurobiology* **22**(3):1-19.

29. Powell, C. and Castillo, P. (2008). Presynaptic mechanisms in plasticity and memory, *Elsevier Ltd*, 741-769.

30. Howland, J. and Wang, Y. (2008). Synaptic plasticity in learning and memory: stress effects in the hippocampus, Sossin, W., Lacaille, J., Castellucci, V. & Belleville, S. (Eds.), Elsevier B.V. *Progress in Brain Research **169**:*145-158.

31. Klintsova, A. and Greenough, W. (1999). Synaptic plasticity in cortical systems, in cognitive neuroscience, *Current Opinion in Neurobiology* **9**:203–208.

32. Elgersma, Y. and Silva, A. (1999). Molecular mechanisms of synaptic plasticity and memory, *Current Opinion in Neurobiology*, **9**:209–213.

33. Hoffman, R. and Al'absi, M. (2010). Khat use and neurobehavioral functions: suggestions for future studies, *Journal Of Ethnopharmacology* **132**:554–563.

34. Abderrahman, S. and Modallal, N. (2008). Genotoxic effects of Catha Edulis (Khat) extract on mice bone marrow cells, *Jordan Journal of Biological Sciences* **1**(4): 165-172.

35. Kimani, S., and Nyongesa, A. (2008). Effects of single daily khat (*Catha Edulis*) extract on spatial learning and memory in c mice, *Behavioural Brain Research* **195**:192–197.

36. Hassen, N.A.G.M., Gunaid, A., Murry-Lyon,I. (2007). Khat (Catha Edulis):health aspects of khat chewing, *Eastern Mediterranean health journal* **13**(3):706-718.

37. International Brain Research Organization, 8th Ibro World Congress of Neuroscience (2011). African habit gives policy makers something to chew over, Florence, Italy.

38. Connor, J., Rostom, A. and Makonnen, E.(2002). Comparison of effects of khat extract and amphetamine on motor behaviors in mice, *Journal Of Ethnopharmacology* **81**:65-71.

39. Wabe, N. (2011). Chemistry, pharmacology, and toxicology of khat (Catha Edulis Forsk): a review, *Addict & Health* **3**(3-4): 137-149.

40. ECDD (2006). Assessment of khat (*Catha edulis* Forsk)

41. Al-Hebshi, N. and Skaug, N. (2005). Khat (Catha Edulis)—an updated review, *Addiction Biology* **10**:299 – 307.

42. Feyissa, A. and Kelly, J. (2008). A review of the neuropharmacological properties of khat, *Neuro-Psychopharmacology & Biological Psychiatry* **32**: 1147–1166.

43. Corkery, J., Schifano, F., Oyefeso, A., Ghodse, H., Tonia, T., Naidoo,V. and Button,J. (2011). Overview of literature and information on "khat-related" mortality: a call for recognition of the issue and further research, *Ann Ist Super Sanità* **47**(4): 445-464.

44. Al-Motarreb, A., Baker, K. and Broadley, K. (2002). Khat: pharmacological and medical aspectsand its social use in yemen, *Phytother. Res.* **16**:403–413.

45. Kalix, P. (1988). Khat: a plant with amphetamine effects, *Journal of Substance Abuse Treatment* **5**: 163-169.

46. Al-Motarreba, A., Al-Haborib, M., and Broadley, K.(2010). Khat chewing, cardiovascular diseases and other internal medical problems: The current situation and directions for future research, *Journal of Ethnopharmacology* **132**:540–548.

47. Dhaifalah, I. and Šantavy, J. (2004). Khat habit and its health effect. A natural amphetamine, *Biomed. Papers **148**(1), 11–15.*

48. Bedada, W. And Engidawork, E. (2010). The neuropsychopharmacological effects of *Catha Edulis* in mice offspring born to mothers exposed during pregnancy and lactation, *Phytother. Res.* **24**: 268–276.

49. Advisory Council on the Misuse of Drugs (ACMD) (2013). Khat: A review of its potential harms to the individual and communities in the UK, report, London, 1-96.

50. Girma, A., and Engidawork, E.(2013). The effect of Sub-acute exposure to crude Khat (Catha Edulis F.) extract on learning and memory in rodents, Addis Ababa University(unpublished).
51. Bogale,T. and Engidawork, E.(2013). The potential effect of subchronic administration of crude Khat (Catha Edulis F.) extract on schizophrenia in mice, Addis Ababa University (unpublished).
52. Gerbi, A. and Engidawork, E. (2013). The assessment of the effect of organic solvent extract of Khat (Catha Edulis F.) on the dentate granule cells in mice, Addis Ababa University (unpublished).
53. OECD/OCDE (testing guidelines, 407) (1995), OECD guidelines for the testing of chemicals: repeated dose 28-day oral toxicity study in rodents, 1-8.
54. OECD/OCDE (testing guidelines, 407) (2008), OECD guidelines for the testing of chemicals: repeated dose 28-day oral toxicity study in rodents, 1-13.
55. OECD/OCDE (testing guidelines, 425) (2008), OECD guidelines for the testing of chemicals: acute oral toxicity – up-and-down-procedure (UDP), 1-27.
56. OECD/OCDE (testing guidelines, 423) (2001), OECD guidelines for the testing of chemicals: acute oral toxicity – acute toxic class method, 1-14.
57. OECD/OCDE (testing guidelines, 420) (2001), OECD guidelines for the testing of chemicals: acute oral toxicity – fixed dose procedure, 1-14.
58. Etuk, E., Adebiyi, R., Elsa, A. and Agaie, B. (2006). Acute and subchronic (28-day) oral toxicity studies of the aqueous extract of *Securidaca longepedunculata* Fresen (Polygalceae) in mice, *International Journal of Pharmacology* **2(**4):421-425.

59. Chavez, C., McGaugh, JL. and Weinberger, N.(2012). Amygdala strengthening of cortical memory representations, *Weinberger* et al ,171-202 **http:// dx.doi.org/** 10.5772/ 5 3677

60. Manning, CA., Honn, VJ., Stone, WS., Jane, JS. and Gold, PE.(1998) Glucose effects on cognition in adults with Down's syndrome. *Neuropsychology* **12**: 479–84.