EVALUATION OF THE EFFECT OF SINGLE AND REPEATED DOSE ADMINISTRATION OF ALKALOID, NON-ALKALOID AND CRUDE EXTRACTS OF KHAT (*Catha edulis* Vahl. Endl.) ON SPATIAL LEARNING AND MEMORY IN MICE

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This is to certify that the thesis prepared by Biruk Seifu Aysa, entitled "Evaluation of the effect of single and repeated dose administration of alkaloid, non-alkaloid and crude extracts of khat (*Catha edulis* Vahl. Endl.) on spatial learning and memory in mice'' and submitted in partial fulfillment of the requirements for the Degree of Master of Sciences in Pharmacology complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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Abstract

Evaluation of the effect of single and repeated dose administration of alkaloid, non-alkaloid and crude extracts of khat (*Catha edulis* Vahl. Endl.) on spatial learning and memory in mice

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Khat (*Catha edulis* Vahl. Endl.), an ever green shrub that belongs to the family Celastraceae, is chewed by millions of people in Ethiopia, Yemen, Somalia, Djibouti and Kenya for its psychoactive effects. Besides its use as a social and recreational stimulant, supporters of khat chewing claim that it improves performance and memory, make them alert and think clearly. Although such beliefs are widely held, little is known about the effect of khat on spatial learning and memory. Recent studies done to evaluate the effect of crude extract of khat leaves in mice have shown variable effects on learning and memory. Thus, this study was initiated to investigate the effect of alkaloid and non-alkaloid fractions as well as crude khat extract on spatial learning and memory. To this effect, mice 6-8 weeks old (5-6 per group), were administered orally with either a single dose or repeated daily doses (5-7days) of alkaloid (50 mg/kg, 100 mg/kg) and non-alkaloid (50 mg/kg, 100 mg/kg) fractions as well as crude khat extract (100 mg/kg, 200 mg/kg, and 400 mg/kg) according to their respective groups. Controls were administered with 0.5 ml 2% Tween 80 in water. The animals were then subjected to Multiple T-maze (MTM) and Morris water maze (MWM) task performance, and parameters (latency and number of wrong decisions) related to learning and memory were assessed. The result showed that single and repeated dose administration of alkaloid and non-alkaloid fractions as well as crude khat extract at doses used did not have a significant effect on learning, short and long term memory using the two models. In general, during the four training days, there was a decrease in both latency and number of wrong decisions indicating both the test groups and controls learned the task in both models of the study. The results collectively indicate that neither the fractions nor the crude extract had significant effect on learning and memory.

Key words: Catha *edulis* Vahl. Endl, Latency, Learning, Memory, Morris water maze, Multiple T-maze, White albino mice, Wrong decision.

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List of Abbreviations and Acronyms

ALF	Alkaloid fraction of khat
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BCG	Bromocresol Green
CA	Cornu Ammonis
CON	Control group
CR	Crude khat extract
DA	Dopamine
EPSP	Excitatory post synaptic potential
LTP	Long-term potentiation
LTM	Long-term memory
METH	Methamphetamine
MTM	Multiple T-maze
MWM	Morris water maze
NA	Noradrenaline
NAc	Nucleus accumbens
NAF	Non-alkaloid fraction of khat
NMDA	*N*-methyl-D-aspartate
OECD	Organization for economic development
PFC	Prefrontal cortex
STM	Short-term memory
TAC	Total alkaloid content

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1. Introduction

1.1. Learning and memory

The brain, with billions of cells' connections and plethora of cell types in mutual interactions, is the organ that is responsible for what we call the mind, which is the basis for learning and memory, and other behaviors (Okano et al., 2000). Being a complicated organ and underlined by the fact that the pathophysiology of many devastating brain disorders, which often affect memory and cognition is not known, it attracts attention of both scientific researchers and the public (Stuchlik, 2014). Thus, learning and memory is one of the most intensively studied subjects in the field of neuroscience and, various approaches have been used to understand brain itself and the mechanisms underlying these processes (Okano et al., 2000).

Learning and memory are two related fundamental higher brain processes by which information about the world (collected through sensory apparatus) is acquired, stored and later retrieved in the brain (Rendeiro et al., 2012). Understanding how learning and memory work is important, because what we learn and what we remember determine, to a great extent, what we are and who we are (Brady, 2005).

1.1.1. Learning

By definition, learning is the acquisition of new information. It consists of acquiring information about the internal and external environment that produces transient or permanent alteration in behaviour on the basis of experience (Lorenzini et al., 1999, Brady, 2005). Learning is the basis of memory. If there is no learning, there can be no memory later (Johansen et al., 2011).

1.1.2. Memory and its divisions

Memory is the ability to store, maintain, and retrieve information from the mind. It is 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 (Guyton and Hall, 2006). In other words, it is the human ability to construct a virtual bridge between the past, the present, and the future (Cotelli et al., 2012).

Memory does not refer to a single behavioral phenomenon, but instead can be classified into a set of component processes that are expressed in different combinations under different circumstances (Voss

and Paller, 2008). The recognition that there are multiple forms of memory developed in the early 1980s, and then extensive experimental data and theoretical material has accumulated and helped in establishing clearer, more concrete, and ultimately a more accurate classification of memory (Squire, 1992). Since then various adjectives have been used to describe memory but none in molecular terms (Marx and Gilon, 2012).

Memory may be defined according to its content/the types of memory selectively affected in amnesic patients (as declarative/explicit or procedural/non-declarative/implicit memory), in relation to time (as short-term memory (STM) or long-term memory (LTM) (Glenn, 2006, Meneses et al., 2011).

Declarative memory is the kind of memory that is meant when the term ‘‘memory’’ is used in everyday language (Squire, 2004). Declarative memory is memory of places, events, facts and people, and is dependent on the temporal lobe system (Glenn, 2006). It was termed “declarative” to signify that it can be brought to mind and that its content can be “declared’ (Squire, 1992). Declarative memory is representational. It provides a way of modeling the external world, and it is either true or false (Squire and Wixted, 2011b). This type of memory tends to form easily and be forgotten easily (Glenn, 2006).

Declarative memory can be divided further into two sub-classes: episodic memory and semantic memory (Squire, 2004). Episodic memory involves the ability to learn, store, and retrieve information about unique personal experiences that occur in daily life. These memories typically include detailed information about the time and place of an event, as well the event itself (Dickerson and Eichenbaum, 2010b, Glenn, 2006). Semantic memory involves memory for factual knowledge of the world that has been learned, but for which specific ‘time and place’ information about the source of the original experience is typically not known. The content of semantic memory is abstracted from actual experience (single or repeated), but without reference to any specific experience (Glenn, 2006, Binder and Desai, 2011). Thus, when we state that Arba Minch is a city in Ethiopia we are drawing on semantic memory; however when we remember a visit to the crocodile ranch in Arba Minch last summer, we are drawing on episodic memory.

The second division of memory based on its content is procedural memory, also termed non-declarative memory or implicit memory. It is the counterpart of declarative memory and encompasses a variety of perceptual–motor learning skills and mental operations (Glenn, 2006). Procedural memory

is not available for consciousness and involves a previously learned skills or actions, such as, riding a bicycle, or driving a car (Okano et al., 2000, Dickerson and Eichenbaum, 2010a). Non-declarative memory is neither true nor false (Squire and Wixted, 2011a).

Declarative memory and procedural memory are independent. Declarative memory requires conscious recollection, while non-declarative memory is expressed through performance rather than recollection (Squire and Wixted, 2011a). Another important difference between these two memory divisions is the brain areas where they are processed. Declarative memories are dependent on the integrity of the hippocampus, while non-declarative or implicit memories depend upon the integrity of structures such as amygdala and striatum. This is also supported by the findings in patients with impaired declarative memory, but whose procedural memory is completely spared (Okano et al., 2000, Rendeiro et al., 2012).

Temporally, memory is classified in to two: STM and LTM (Glenn, 2006). STM and LTM are memory types of an archival nature that may differ in nature but not in contents. Thus, the terms are applied usually to declarative memories; they are seldom used in the literature on procedural memories, whose STM has not been studied in detail (Izquierdo et al., 2002). LTM and STM differ in two fundamental ways: temporal decay and chunk capacity limits (Cowan, 2008). Temporally, STM has a time course of seconds to hours as it is vulnerable to interferences and disruptions, whereas LTM has a time course of weeks, months and years (Glenn, 2006). A capacity difference means that there is a limit in how many items short-term storage can hold but LTM is without limits in capacity (Nadel and Hardt, 2011).

Another type of memory that cannot be strictly assigned to one of the above classification subsystems is spatial memory. Indeed, it involves aspects of non-declarative memory (procedural), declarative (semantic and episodic memories), as well as of both STM and LTM. 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 (Paul et al., 2009). The ability to remember and return to particular locations in one's environment is essential to the daily functioning and survival of any navigating species. Spatial learning and memory is crucial for animals' survival in the wild as it help them find locations that provide food and safety (Jakubowska-Doğru and Kara, 2003). Spatial memory is related to the answer for the general question "where?": "Where am I?", "Where does it hurt?", and "Where is my home?" etc. (Paul et al., 2009). Rats displaying extraordinary

spatial abilities are commonly used in studies examining animals' cognitive capacity in spatial tasks that comprise a variety of mazes (Goldman-Rakic, 1996).

Spatial memory can be denoted as the two memory systems: a short-term processing of active memory (spatial working memory) and a long-term storage of spatial locations (spatial reference memory) (Paul et al., 2009). Working memory is the ability to hold an item of information transiently in mind in the service of comprehension, thinking, and planning and keeping that information quickly accessible and available (Goldman-Rakic, 1996). Thus, working memory includes STM and other processing mechanisms that help to make use of STM, i.e., it refers to attention-related aspects of short-term memory (Cowan, 2008). So, without working memory, people would not be able to reason, solve problems, speak and understand language, and engage in other activities associated with intelligent life (Jonides et al., 2005).

Spatial reference memory represents knowledge for aspects of a task that remain constant between trials (Nadel and Hardt, 2011). In contrast to spatial working memory, spatial reference memory exhibits more capacity, duration and resistance to interference (Paul et al., 2009). Originally, the terms were introduced to distinguish two types of knowledge rats may retain in a radial-arm maze task: knowledge about which arms of the maze always contain a food reward in each trial (reference memory) and memory for the arms that have already been visited in search for food in the current trial (working memory) (Nadel and Hardt, 2011). Thus, working memories are those that fade when they are no longer useful and reference memories are those that consolidate.

1.1. Memory formation process and brain structures implicated in memory formation

The concept of memory of mind has existed since the time of Aristotle but, it was only after the middle of the 20th century that neuroscientists have begun to unravel some of the anatomical and cellular basis underlying memory formation (Glenn, 2006, Squire, 2004). The question 'How and where is memory formed and stored in brain?' was remained to be answered for a long time. Subsequently, ideas about how and where the brain organizes learning and memory have been evolved (Nadel and Hardt, 2011). At the end of the 19th century, Ramon Y Cajal (Glenn, 2006) entertained the idea that the modification of synaptic conjunctions between neurons in brain could form the anatomical basis responsible for the cellular event of memory, which was regarded by most neuroscientists as the beginning of the cellular exploration of just how memory is retained in the brain. Since then, researchers have made great

efforts to understand the molecular and cellular mechanisms underlying the memory formation, storage and retrieval (Wang et al., 2006).

Earlier neuroscientists claimed most popular candidate site for memory storage is the synapse, where nerve cells (neurons) communicate (Okano et al., 2000). The process of learning involves reversible alterations in synaptic transmission within brain neuronal circuitry which once stabilized, allow memory to be retained (Rendeiro et al., 2012). Thus, memory formation depends on brain "plasticity"– structural and/or functional neural changes in response to stimuli (such as experiences) (Stickgold and Walker, 2007). This view was based on the Canadian psychologist Donald O. Hebb's influential proposal. According to Hebb's postulate, 'When an axon of cell A is near enough to excite a cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased.'(Hebb, 2005). In other words, a memory is produced by coincident neural activity; when two connected nerve cells are active simultaneously. This coincident neural activity leads to increased strength of synaptic transmission, often measured by the amplitude of the EPSP, called long-term potentiation (LTP) (Glenn, 2006).

LTP is a form of synaptic plasticity widely accepted as the mechanism by which memories are laid down and subsequently stored (Rendeiro et al., 2012). It has been widely accepted that similar to the distinction between STM and LTM, LTP temporally can be divided into an early transient phase (E-LTP), and a more persistent late phase (L-LTP), which similar to long-term memory, requires protein-synthesis in order to stabilize (Lu et al., 2008, Nader and Einarsson, 2010). In addition to its ability to produce LTP, a synapse also possesses the ability to decrease its synaptic efficacy. Low-frequency stimulation of the synaptic pathway for short period of time produces decreased EPSP responses. This type of synaptic plasticity can last at least one hour, and is called long-term depression (LTD) (Glenn, 2006).

It is generally accepted that the influx of calcium into postsynaptic neurons through NMDA (*N*-methyl-D-aspartate) receptors is the triggering event in plasticity. Postsynaptic activation of the NMDA receptors is required for induction of LTP. The electrical stimulation of a presynaptic cell releases glutamate which binds to the postsynaptic AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptor and NMDA receptors. Glutamate binding to the NMDA receptor alone is not sufficient to activate the channel because at resting membrane potentials, calcium flow

through the receptor is blocked by magnesium. The relief of the Mg^{2+} blockade comes when the postsynaptic cell is sufficiently depolarized by the repetitive activations of the AMPA receptor, which cause Na+ influx and consequently increase EPSP (Glenn, 2006, Lamprecht and LeDoux, 2004).

The NMDA receptor only passes calcium when presynaptic activity and postsynaptic activity coincide. Calcium entry through NMDA receptors initiates the subsequent cascade of events that lead to synaptic plasticity. When the intracellular levels of Ca^{2+} are sufficiently elevated, it triggers the activation of signalling pathways, such as cAMP-dependent protein kinase A, protein kinase B, protein kinase C, Ca-calmodulin kinase and extracellular-signal-regulated kinase (ERK). Phosphorylation of these kinases results in the modulation of synaptic efficacy, causing synaptic insertion of AMPA receptors and/or increasing their single channel conductance and subsequently, over several hours, triggers the gene expression and *de novo* protein synthesis, a process that is crucial to maintain LTP and convert short-term memories into a more stable long-term form(Lamprecht and LeDoux, 2004, Glenn, 2006, Rendeiro et al., 2012).

Regarding to the question where memory resides in brain an earlier view was nervous system works en masse to form and store memory (Lombrosoa, 2004). However, the fact that there are patients with impaired declarative memory whose procedural memory is completely spared led neuroscientists believe that there must be separate mechanisms for each type of memory that probably also require separate brain areas as well (Okano et al., 2000). Later, this belief became a topic of experimental inquiry and now a substantial body of experimental data and theoretical material has accumulated (Squire, 2004, Squire, 1992).

As scientists' effort to find out where memories reside in the brain continued, a breakthrough was made in the mid-1950s by Wilder Penfield who stimulated the cortical surface of temporal lobe of over thousands of epileptic patients for removing epileptic tissue, and some patients reported that they were hearing voices and music, or seeing images and having other mental perceptions uniquely when electrical stimulation was delivered. This and other electrical stimulation-triggered mental experiences suggested that they reflected flashbacks to past experiences. These fascinating reports were the first indications that the temporal lobe system may play a crucial role in representing memories and thoughts (Glenn, 2006).

Similarly in the 1950s, Brenda Milner of the Montreal Neurological Institute examined a patient known by his initials as H.M treated for his severe epilepsy by a bilateral removal of the medial

temporal lobe (MTL) (medial temporal cortex, amygdala, and two-thirds of the hippocampus). While the surgery successfully relieved his debilitating seizures, he was left with profound amnesia (Wang et al., 2006). Milner concluded that damage to the hippocampal formation was critical to the extensive anterograde amnesia (loss of ability to form new memories) and retrograde amnesia (loss of recently formed memories) that was observed in this patient (O'Reilly and Rudy, 2001). H. M. was the first human case in which specific amnesia could be linked to selective lesions to the temporal lobe system, especially within the hippocampus of the brain. In the intervening years, a large number of studies have focused on humans and animal models of human amnesia (monkeys, rabbits, rats, and mice) on the presumed role(s) of the hippocampus and related structures in memory (Glenn, 2006, Thompson and Kim, 1996). Such clinical observations have established the view that the hippocampus system is critically involved in memory processes.

However, the current views recognize that there is no a single or universal memory center of the mind but there are a number of different forms or aspects of memory that involve different brain systems, and that all forms involve cellular changes that take time to emerge and that then persist (Okano et al., 2000, Nadel and Hardt, 2011). The diencephalon, a subcortical 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 (Narwal et al., 2012). Declarative memories involve the hippocampus–medial temporal lobe system and implicit basic associative learning and memory involves the cerebellum, amygdala, and other systems (Thompson and Kim, 1996).

1.2. Memory consolidation

LTMs are not established in their definitive form immediately after they are acquired; hours, days, or even years later other processes may also contribute to give memories their final shape (Izquierdo et al., 2002). In 1900, Muller and Pilzecker (cited by (Wang et al., 2006)) reported that the formation of stable memory is disrupted by new stimuli shortly after the first learning. Thus, memory is a dynamic process that passes through at least four distinct stages: learning, consolidation, storage and retrieval. A new memory is initially labile and becomes stabilized over time through a process of consolidation (Tronel et al., 2005). Memory consolidation refers to a process by which labile newly formed memory

traces are progressively strengthened into long term memories and become more resistant to interference (Girardeau and Zugaro, 2011).

The hippocampus is critical for converting STMs into LTMs. Clinical studies, such as H.M., suggested that the medial temporal lobe is crucial in the consolidation of memories. This was later supported by animal model studies that identified the hippocampus and surrounding areas as the structures critical for memory consolidation (Taubenfeld et al., 2001). Upon the completion of hippocampal-dependent consolidation, memories are thought to be transferred to and stored in the cortex without significant hippocampal contribution (Wang et al., 2006).

The term consolidation is used for two processes: 'cellular' or 'synaptic', which is completed within minutes to hours, refers to the cascade of molecular and cellular mechanisms underlying the process of memory consolidation in single neurons, and 'systems consolidation', in which a hippocampus-dependent memory becomes (over years in humans and over weeks in rodents) hippocampus-independent (Nader and Hardt, 2009, Winocur and Moscovitch, 2011).

Memory consolidation requires RNA and protein synthesis. Signaling pathways involving Ca^{2+}, cAMP, mitogen-activated protein (MAP) kinases and tyrosine kinases have been shown to be required for the consolidation of various kinds of memories, and numerous genes have been identified as essential for memory formation (Taubenfeld et al., 2001, Alberini, 2005). This can be studied by interfering with these processes, using appropriate agents infused directly into the brain structures thought critical for certain kinds of knowledge (Nader and Hardt, 2009).

Consolidation theory hypothesizes that once a memory is consolidated, it remains stable and resilient to disruption (Alberini, 2005). In contrast to this expectation, later it was argued that memory retrieval can return a consolidated/fixed memory to an unstable state once again (Nader and Einarsson, 2010). Memory is also disrupted if a number of interfering events or pharmacological treatments, including protein synthesis inhibitors, are administered during the post-reactivation labile phase (Tronel et al., 2005). Thus, every time a memory is reactivated it must undergo again a process of reconsolidation to be maintained. Reconsolidation is the transformation of destabilized memory into a restabilized form (Stickgold and Walker, 2007). Reconsolidation does not seem to occur every time a memory is retrieved, and it has been suggested that as memory ages it can become more stable and less susceptible to pharmacological disruption following retrieval (Einarsson and Nader, 2012).

Although some disagreement remains, many studies have demonstrated that memory consolidation and reconsolidation have distinct molecular requirements (Tronel et al., 2005). They involve different brain areas and circuits. Consolidation appears to require several areas that are not essential for reconsolidation and reconsolidation might involve mostly modulatory systems. Consolidation and reconsolidation also differ in their temporal dynamics. Training always induces a labile phase during which memory can be disrupted, whereas reactivation does not always result in a labile memory. A stronger and older memory is less labile; a more intense reactivation is more destabilizing. However, both consolidation and reconsolidation seem to use similar molecular mechanisms, the same ones that are known to mediate long-term synaptic plasticity (Alberini, 2005).

1.3.Overview of khat (*Catha edulis* Vahl. Endl.)

Khat, the edible part of Catha *edulis*, belongs to the score of vegetal materials that humans ingest not for their nutritive value but to experience their psychoactive effects (Graziani et al., 2008). Khat belongs to the kingdom Plantae, class Magnoliopsida, order Celastrales, family Celastraceae, genus *Catha* and species *edulis* (Lamina, 2010). Khat consists of whole fresh leaves and buds of a flowering plant, indigenous to tropical East Africa and the Arabian Peninsula (Balint et al., 2009, Connor et al., 2002). It is an erect, evergreen, glabrous shrub or tree 1.5–20 m high with reddish stems, shiny green leaves and white flowers (Getasetegn, 2016). The lancet-shaped leaves are between 0.5 cm and 10 cm long and between 0.5 cm and 5 cm wide (Lamina, 2010).

Historically, the original source of khat seems to be obscure. However, there is general agreement that its use was prevalent in Ethiopia and from there, around the fifteenth century, the practice spread to the south-west of the Arabian Peninsula. Arab sources suggested that khat was in Yemen in the sixth century, when the Ethiopians conquered Yemen (Admassie and Engidawork, 2011). The plant is now grown in Ethiopia and the nearby countries Arabia, Kenya, Somalia, Uganda, Tanzania, Malawi, Congo, Zambia, Zimbabwe, Madagascar and South Africa. It has also been found in Afghanistan and Turkestan (Balint et al., 2009). The plant is commonly used in East Africa and the Arabian Peninsula, as well as by immigrants from these regions who reside in Western countries, primarily Great Britain and the United States (Abid et al., 2013). The shrub grows at altitudes between 1500 and 2500 m and requires high rainfall and grows best on acid, well-drained and clay soil. With irrigation and pruning, khat leaves can be harvested up to four times per year (Engidawork, 2017).

The khat plant is known by a variety of names, such as qat in Yemen, chat in Ethiopia, jaad in Somalia, miraa in Kenya and Tanzania. Other common names are: ‘‘African salad’’, ‘‘Abyssinian Tea’’, ‘‘Bushman’s tea’’, and ‘‘Flower of paradise’’ (Getasetegn, 2016). Environmental and climatic conditions determine the chemical profile of khat leaves. Although samples from different geographical origins have similar physical appearance, they have not been proven to exhibit equipotent stimulatory activity (Atlabachew et al., 2015). In the Yemen Arab republic, about 44 different types of khat exist, originating from different geographical areas of the country (Lamina, 2010). In Ethiopia, khat is a cultivated plant and many types are recognized mainly based on the locality and ecology where it grows (Getasetegn, 2016). The local brands include Aweday, Beleche, Abo mismar, Gelemso, Wondo and others. There is an ever-growing demand both for domestic consumption and for the export market (Al-Motarreb et al., 2010). It is claimed that the Aweday and Beleche variety are the most potent and expensive among the local brands, and hence Beleche was chosen for purpose of the present study.

The most favored part of the plant is the leaves, particularly the young shoots near the top of the plant (Atlabachew et al., 2013). Since only fresh leaves have the desired effect, khat is harvested in the early hours of the morning and sold in markets in the late morning. To preserve its freshness and to slow down the degradation process of psychoactive effect, it is presented as a bundle of twigs, stems and leaves, and is wrapped in banana leaves, plastic bags or splashed with water (Balint et al., 2009, Colzato et al., 2011b). During khat sessions, the leaves and the tender younger stalks of the plant are chewed slowly over several hours and they are kept in the side of the cheek until the mouth is filled with fresh leaves. The user then chews slowly and intermittently to release the active components of khat that are then swallowed with saliva (Colzato et al., 2011b, Hoffman and Al’Absi, 2010).

Chewing khat is both a social and a culture-based activity. It is generally consumed in groups in a social setting (Hassan et al., 2002). Only a minority frequently chew alone. A session may last for several hours (Wabe and Mohammed, 2012). It is said to enhance social interaction, playing a role in ceremonies such as weddings (Wabe, 2011). Chewing is the most common mode of administration, although a small number of consumers use dried leaves to make drinks and an equally small number smoke it (Engidawork, 2017). During the session chewers drink copious amounts of non-alcoholic fluids such as cola, tea and cold water (Wabe and Mohammed, 2012).

Khat is a natural stimulant chewed for its euphoric and recreational purposes (Hoffman and al'Absi, 2013). Chewers believe that it helps them to get relief from fatigue, increase alertness, and reduce the sensations of hunger. It also brings feeling of elation, improves ability to communicate and self-confidence (Getasetegn, 2016). The main effects after chewing khat are a mild euphoria and excitation leading to increased energy and communicativeness (Sporkert et al., 2003). In a khat chewing session, initially there is an atmosphere of joyfulness, optimism and a general sense of well-being. After about 2 hours, tension, emotional instability and irritability begin to appear, later leading to feelings of low mood and sluggishness. Chewers tend to leave the session feeling depleted (Wabe and Mohammed, 2012).

The chemical constituents of khat have been studied since the late 19th century (Dhaifalah and Santavy, 2004). Over forty compounds are found in khat including alkaloids, terpenoids, flavonoids, sterols, glycosides, tannins, amino acids, vitamins, minerals and others (Engidawork, 2017). The main active compounds that are responsible for stimulant effect of khat are alkaloids. The phenylalkylamines and cathedulins are the major alkaloids (Balint et al., 2009). The cathedulins are based on a poly-hydroxylated sesquiterpene skeleton and are basically polyesters of euonyminol. Although about 62 different cathedulins from fresh khat leaves were characterized, there has been little investigation of the cathedulins compared with phenylalkylamines (Engidawork, 2017).

The phenylalkylamines are of greatest importance to the stimulant activity of khat (Nichols et al., 2015). The khat phenylalkylamines comprise cathinone [S(–)-cathinone], and the two diastereoisomers cathine [1S,2S(+)-norpseudoephedrine or (+)-norpseudoephedrine] and norephedrine [1R,2S(–)-norephedrine] (Wabe and Mohammed, 2012). These alkaloids appear to be unique to khat, as they were not detected in about 43 Celastraceae species investigated. In addition, two oxazolidine derivatives, 2, 4-dimethyl-5-phenyloxazolidine and 4-methyl-2-(trans-1-pentenyl)-5-phenyloxazolidine have also been identified (Engidawork, 2017). The plant contains the (–)-enantiomer of cathinone only; the (+)-enantiomer is not found. Thus, S(–)-cathinone is mostly responsible for pharmacological effects of khat. Other phenylalkylamine alkaloids found in khat leaves are the phenylpentenylamines merucathinone, pseudomerucathine and merucathine. These compounds seem to contribute less to the stimulant effects of khat (Wabe and Mohammed, 2012, Wabe, 2011).

The naturally occurring (–)-cathinone (Fig. 1) (Al-Hebshi and Skaug, 2005) resembles (+)-amphetamine in chemical structure and biological activity, and is believed to be responsible for the psychostimulant nature of khat (Nichols et al., 2015). Due to these similarities, cathinone has been called a 'natural amphetamine' (Hoffman and al'Absi, 2013). (−)-Cathinone is estimated to be one-third as potent as amphetamine and 10 times more potent than (+)-cathine and (−)-norephedrine (Krizevski et al., 2007). Cathinone has a juvenile distribution with the highest levels occurring in the young leaves, flowers, and twigs where it can make up nearly 70% of the alkaloid content (Patel, 2019). During maturation, drying or storage cathinone undergoes enzymatic reduction to (+)-norpseudoephedrine (cathine) and (-)-norephedrine and this explains the traditional chewing of only fresh young leaves. Thus, cathine and norephedrine occur mainly in older plants (Krizevski et al., 2007, Nichols et al., 2015). The amount of cathinone and cathine in the khat leaves is debatable. Generally, on average, 100 g fresh khat leaves contain 36–114 mg cathinone, 83–120 mg cathine and 8–47 mg norephedrine (Balint et al., 2009). It also contains considerable amounts of tannins (up to 10% in dried material) and flavonoids (Wabe and Mohammed, 2012).

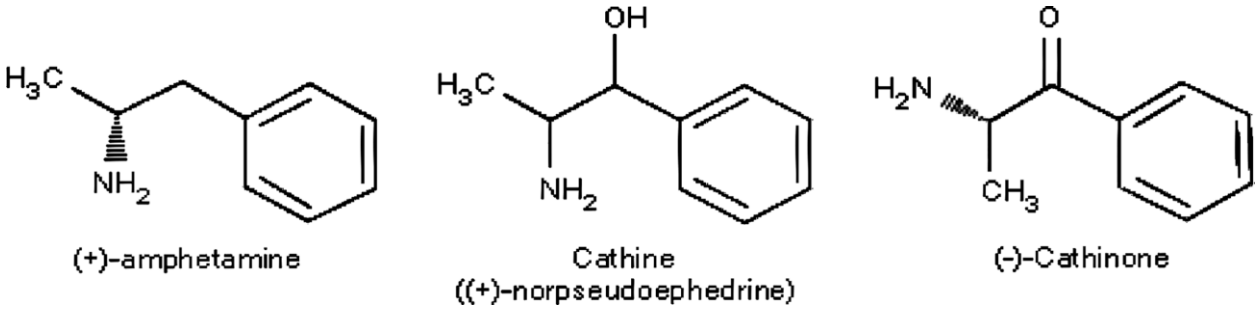

Fig1. Chemical structures of amphetamine, cathine and cathinone

When khat leaves are chewed, enzymes in saliva release cathine and cathinone, which are absorbed through the mucous membranes of the mouth and subsequently the lining of the stomach (Engidawork, 2017). The alkaloids from khat leaves are effectively released with about 80% of cathinone and cathine, and over 90% of norephedrine within 15-45 minutes during chewing (Hoffman and al'Absi, 2013, Geresu, 2015). Compared to cathine, the effect of cathinone has a more rapid onset of action, roughly 15 min as compared with 30 min for cathine, which agrees with its higher lipophilic character facilitating entry into the central nervous system, and a shorter duration of action, which agrees with the rapid metabolism of cathinone (Wabe, 2011). The terminal elimination half-life is about 4 h. The enzymes involved in the metabolism have not been described. However, it was

predicted from the metabolic pathways of amphetamines and synthetic cathinones that major cytochrome P450 (CYPs) are involved. The major metabolite of (-) S-cathinone is (-)-norephedrine, but small quantities of (+) norpseudoephedrine also form (Engidawork, 2017, Al-Hebshi and Skaug, 2005). Regarding the influence of khat on the metabolic activities of drug-metabolizing enzymes, a study done in Ethiopia showed significant inhibitory effect of khat on CYP2D6 enzyme activity in humans (Bedada et al., 2015).

As aforementioned, khat alkaloids are similar in structure and pharmacological activity to amphetamines. Cathinone is a lipophilic alkaloid and crosses the blood-brain barrier to reach the primary sites of action in the central nervous system (Nyongesa et al., 2014). The constituents of khat have been shown to exert their effects on two main neurochemical pathways: dopamine and noradrenaline. They increase levels of dopamine (DA) and norepinephrine (NA) in the brain by acting on the cathecholaminergic synapses, delaying the reuptake and/or enhancing the release of these neurotransmitters (Colzato et al., 2012). Cathinone has also been demonstrated to induce serotonin (5-HT) release and to inhibit its reuptake (Nichols et al., 2015). Both cathinone and amphetamine induce release of DA from central nervous system dopamine terminals and thus increase the activity of the dopaminergic pathways. Cathinone has a releasing effect on NA storage sites, which supports the conclusion that cathinone facilitates NA transmission. It is also proposed that similar to amphetamine, cathinone inhibits the activity of DA, NA, and 5-HT transporters, as observed at synaptic level in the striatum of rats treated with the alkaloid (Getasetegn, 2016, Graziani et al., 2008). Because of this mechanism of action as other psychostimulants, khat ingestion produces several central nervous system effects (Hoffman and al'Absi, 2013).

On the other hand, it is well established that learning and memory are involving and recruiting these neurotransmitter systems. Considerable evidence points to the involvement of DA in various aspects of cognition as DA receptors are differentially expressed in several brain areas that are critically involved in cognition, such as the hippocampus (Gasbarri et al., 1994). The dopaminergic systems, specifically the mesocorticolimbic pathway, are critically involved in modulation of various aspects of learning and memory (El-Ghundi et al., 2007).

Evidences implicate involvement of D_1 receptors in spatial learning and memory (El-Ghundi et al., 1999, Holmes et al., 2001). Another impact of dopamine on learning and memory is through its effect on synaptic plasticity in the hippocampus, PFC, amygdala, NAc and striatum, known for their major

role in cognitive function (El-Ghundi et al., 2007). These findings support the idea that besides the glutamatergic system, the synergistic activation of dopaminergic synapses is necessary for LTP maintenance.

The central NA and its agonist administration after training increases memory performance of aversively motivated learning (Introini-Collison and McGaugh, 1986). Other finding suggested norepinephrine infused into the basolateral amygdala post-training enhances retention in a spatial water maze task (Hatfield and McGaugh, 1999). Similarly, serotonergic projections modulate various aspects of learning and memory. Earlier studies, in which attempts were made to increase the brain 5-HT function by different drugs, showed that the changes in this function led to an impairment of learning and memory (Petkov et al., 1995). Thus, it is plausible to say that the cognitive effects of khat is through its effect on these neurotransmitters.

Reviews of research have indicated increased effect of khat on health and neurological function of humans. As aforesaid, khat contains many different compounds and therefore khat chewing may have many different effects (Balint et al., 2009). The effects can be viewed as either central or peripheral. The effect that accounts for the popularity of khat is its central nervous system stimulation (Hassan et al., 2007). This central stimulation effects observed include euphoria, alertness, excitation, loquacity, logorrhea, analgesia, energy, confidence, and increased sensory stimulation (Feyissa and Kelly, 2008), headache, fine tremor, insomnia, psychotic illness, decrease threshold of seizure, increased concentration, and emotional disturbance are also observed (Engidawork, 2017).

Besides the central effects, khat has plethora of peripheral effects on different body systems. A number of reviews of research summarized peripheral effects as follows. The major peripheral effect of khat is on gastrointestinal system (GI). On GI system it causes oesophagitis, gastritis, delayed intestinal absorption, oral keratotic white lesions, dry mouth, constipation, dental caries, periodontal disease, polydipsia, gastric ulcer, anorexia, haemorrhoids, and weight loss. On cardiovascular system it results in transient increase in blood pressure, tachycardia, myocardial infarction, arrhythmia, vasoconstriction, palpitation, sweating, cold peripheral extremities, cardiogenic shock, ischaemia, pulmonary oedema, and cerebral haemorrhage. On reproductive system: increased libido and decreased performance, spermatorrhea, deformed spermatozoa, fetal or neonatal toxicity, maternal anemia, anti-implantation, low birth weight, stillbirths, impaired lactation. On respiratory system: bronchitis, tachypnoea and dyspnoea. On liver and kidney: fibrosis, cirrhosis, acute kidney damage,

enzyme inhibition, urinary retention; metabolic and endocrine effects like hyperglycemia, hyperthermia, perspiration; ocular effects: blurred vision, mydriasis (Cox and Rampes, 2003, Balint et al., 2009, Getasetegn, 2016, Wabe, 2011, Wabe and Mohammed, 2012).

Additionally, khat has psychological, social and economic effects on chewers. In communities where khat is used regularly, it has negative impacts on socio-economic conditions. Khat chewing is thought to be responsible for at least some effects desired by chewers like euphoria and alertness. Some authors describe these effects result in moderate but often persistent psychological dependence (Al-Habori, 2005, Balint et al., 2009). From the economical point of view, khat chewing leads to loss of work hours, decreased economic production and malnutrition. Khat also diverts household income that could have been wisely used for nutritious food, home improvements, education or other family needs that people on those countries are in very big need for (Cox and Rampes, 2003, Admassie and Engidawork, 2011).

While the nature of khat dependence remains under active debate, there is a general agreement that khat consumption may induce moderate but often persistent psychic dependence. There is also accumulating evidence indicating the existence of a withdrawal syndrome and a low level of tolerance. Withdrawal symptoms usually include lethargy, nightmares, trembling, mild depression, sedation and hypotension (Admassie and Engidawork, 2011, Hoffman and al'Absi, 2013, Hoffman and Al'Absi, 2010). With excessive prolonged consumption, amphetamine-like psychosis can result; however, it was not clear whether this is due to khat use or due to a predisposition in user towards psychotic disorders (Patel, 2019). CNS tolerance is not usual in khat users. If it does occur, the doses are increased only very slowly. This may be due to the intrinsic properties of khat or to the physical limits on the amount that can be consumed (Cox and Rampes, 2003).

Because of its stimulating effects khat has been traditionally used by some tribal people when traveling, and in modern times by students preparing for examinations, drivers of motor vehicles especially on long-distance journeys and even soldiers during the war in order to enhance their performance, improve work capacity and counteract fatigue (Hassan et al., 2007, Dhaifalah and Santavy, 2004). Supporters of khat chewing claim that it is useful in diabetic patients, for asthma, intestinal tract disorders, and maintains social contact as a socializing herb (Hassan et al., 2007). In the North Kenya, khat use is reported among the Meru tribe for the treatment of erectile dysfunction, malaria, influenza, vomiting and headache. In traditional Ethiopian medicine, a mild tea made of the

leaves reduces swelling in the mouth and poultices of khat leaves have a curative effect on wounds (Balint et al., 2009).

With all these myriads of effects, surprisingly, khat is not under international control at present. But two substances that are usually present in khat, cathine and cathinone, are placed group wise under international control since the early 1980s, as they are amphetamine-like substances. Thus, cathinone is placed under Schedule I and cathine in Schedule III (WHO, 2006). Consequently, a wave of new chemicals called substituted cathinones have emerged in response to market trends and legislative controls (Carvalho et al., 2012). Substituted cathinones are a large family of synthetic beta-keto phenethylamine (2-amino-1-phenyl-1-propanone) derivatives chemically related to the parent compound cathinone. A large number of different substances have been identified as constituents of bath salts, but among the most prevalent drugs are mephedrone (4-methylmethcathinone) and methylone (3,4-methylenedioxymethcathinone) (den Hollander et al., 2013). The use of cathinone-derivative designer drugs methylone and mephedrone has increased rapidly in recent years (Engidawork, 2017).

1.4. Rationale of the study

Besides its use as a stimulant, people chew khat believing that it improves performance and memory, alertness and clear thinking (Engidawork, 2017). Habitual users report enhanced imaginative ability and capacity to associate ideas; an improvement in the ability to communicate; and helps them to concentrate better or providing additional energy for physical labour (Dhaifalah and Santavy, 2004). Although such beliefs are widely held, there are little reports assessing the effect of khat on learning and memory, and those reports present inconsistent results to each other and opposing results to the widely accepted belief.

There are very few laboratory data available regarding the effect of khat on human neurocognitive functioning. Comparative studies performed with substances that have resemblance to cathinone, such as amphetamine, have shown increased memory consolidation in the Morris water maze (MWM) task (Sporkert et al., 2003). However, a study made in Kenya indicated acute intraperitonial administration of crude khat extract is shown to produce an inconsistent effect on learning and memory task in CBA mice: low dose having no effect on learning but impairing memory, whereas high dose impairs learning but improves memory (Kimani and Nyongesa, 2008). A comprehensive study conducted in Ethiopia which subjected mice to acute, subacute and subchronic crude khat extract exposure found acute and subacute exposure had no effect on learning and memory. Subchronic exposure, however, produced a significant impairment in short-term memory, without altering learning and long-term memory (Mohammed et al., 2014). Similarly other study in Ethiopia that employed multiple T-maze test (MTM) to determine the effect of acute exposure of crude khat extract on learning and memory in mice didn't show any improvement in learning and memory compared to placebo (Assefa et al., 2018). However, khat exposure during pregnancy and lactation in mice impairs cognition and brings about emotional instability (Bedada and Engidawork, 2010).

All the studies done so far tried to examine the effect of crude khat extracts on learning and memory. As aforementioned, khat contains more than forty compounds in addition to the main psychoactive alkaloid components. There is a need to know which type of compounds contribute to the beneficial/harmful effects of khat on learning and memory. No study has been done to determine only the alkaloid portion of khat on learning and memory. Thus, the present study investigated the effect of pre-training administration of alkaloid and non-alkaloid fractions as well as crude extract of khat

on learning and memory following single (acute) and repeated dose exposure using the two well established behavioral paradigms: MWM and MTM.

2. Objectives

2.1. General objective

- To evaluate the effect of acute and repeated dose administration of alkaloid and non-alkaloid fractions as well as crude extract of khat (*Catha edulis* Vahl. Endl.) on spatial learning and memory in mice

2.2. Specific objectives

- To evaluate the spatial learning and memory effect of alkaloid and non-alkaloid fractions as well as crude khat extract using Morris water maze task
- To evaluate the spatial learning and memory effect of alkaloid and non-alkaloid fractions as well as crude khat extract using multiple T-maze task
- To determine total alkaloid contents of khat

3. Materials and Methods

3.1. Chemicals and reagents

The following chemicals and reagents were used in the study: Methanol (Carlo Erba Reagents, France), Ammonium Hydroxide and Sodium Hydroxide (Carlo Erba, Italy), Hydrochloric acid, Citric acid, Atropine, Sodium Phosphate, and Aluminium Chloride (BDH Laboratories, England), Ethyl acetate, Bromocresol Green (BCG), and Quercetin Dihydrate (Sigma-Aldrich, Germany), Diethyl ether and Chloroform (Loba Chemie, India), Sodium Sulphate (BIO-LAB Laboratories Ltd, Israel), Tween 80 (Atlas Chemical Industries Inc., UK). Distilled water was obtained from Department of Pharmaceutical Analysis, School of Pharmacy (SoP), Addis Ababa University (AAU). All chemicals and reagents used were of analytical grade.

3.2. Plant material

Khat leaves were purchased in early November 2018 from a local market at Wendo Genet, Ethiopia, 290 kilometres South of Addis Ababa, which is known to be one of the natural habitat for Beleche khat. Tips of the fresh leaves and twigs were trimmed and wrapped in a plastic bag. The plastic bags were then placed in an ice box and transported to the laboratory of Department of Pharmacology and Clinical Pharmacy, School of Pharmacy (SoP), Addis Ababa University (AAU), Addis Ababa, Ethiopia. Once at the destination, the plastic bags were transferred to a deep freezer (–20 °C) and kept there for 72 h before extraction. The plant was identified by a taxonomist at the Department of Biology, College of Natural and Computational Sciences, AAU, where a voucher specimen (Collection number: WS-001) was deposited for future reference.

3.3. Experimental Animals

Healthy Swiss albino mice of either sex (25 g – 35 g) aged 6 – 8 weeks were obtained from the Animal House of SoP, AAU, Addis Ababa, Ethiopia. The animals were housed under standard plastic cages with standard wood chip bedding and 12 h light/dark cycle. The animals assigned for MWM were allowed free access to tap water and standard laboratory pellet, while for MTM were restricted for 12 h before experiment days. The handling of animals and all experimental procedures were carried out according to the internationally accepted standard guidelines for use of animals (Nih and Oer, 2011).

3.4. Extraction and Fractionation

For preparation of both crude extract and fractions, the procedures described elsewhere (Betrie and Engidawork, 2016, Al-Qirim et al., 2002) were followed with slight modification. All the procedures were conducted meticulously under shade and every flasks and materials used were wrapped with aluminium foil to avoid exposure to sunlight and hence degradation of the psychoactive component, cathinone.

3.4.1. Crude extract

The freeze-dried leaves were finely chopped with knife in a dark place, weighed by electronic digital balance (about 3500 g) and then divided in to five different Erlenmeyer flasks (approximately 700 g each) for ease of extraction. It was then macerated with enough volume of the solvent (80% Methanol) so as to cover the plant material in the flask. The flask was wrapped with aluminium foil and contents were continuously stirred using a rotary shaker at 120 rpm (New Brunswick Scientific Co, USA) for 3 days (three extractions after 24 h, 48 h and 72 h). Afterwards, the extracts were filtered by using muslin followed by Whatman no 1 filter papers. The hydro-alcoholic extracts were combined and the organic solvent was evaporated under reduced pressure using rotavapor (Buchi, Switzerland) at 30° C with a rotation speed of 70 rpm. The concentrated extract was then poured on a flat container and subjected to freeze drying using a lyophilizer (OPERAN Lyophilizer, Korea). The resulting dry crude extract was weighed and calculated for percentage yield, which was 190 g (5.43% w/w). Following this, the dried crude extract was placed in an amber glass container and stored in a deep freezer at – 20° C until use and fractionation.

3.4.2. Alkaloid and Non-alkaloid fractions

For extraction of the alkaloid fraction, a total of 150g of the dried crude extract was taken, portioned into three (50g each) and fractionation was done as follows. Each portion was dissolved in 500 ml of 0.1 N HCl and filtered. The filtrate was then extracted with ethyl acetate (3 x 200 ml) and the acidic solution was washed with diethyl ether (3 x 200 ml). The resulting acidic solution was then neutralized with 28% ammonia until the pH of the solution was between 9 and 10, and exhaustively extracted with chloroform until it showed no reaction with Dragendorff's reagent. The chloroform extracts were mixed, washed with water and dried over sodium sulfate. The resulting extract was evaporated to dryness to get a total alkaloid extract 1g (yield= 0.67%w/w). After getting the whole alkaloid fraction,

the remaining parts of the extracts (ethyl acetate, diethyl ether and the final residue) were combined together and evaporated to dryness to get a non-alkaloid extract 30g (yield= 20%w/w). For confirmatory purpose, fractions were further tested for presence or absence of alkaloid using Mayer's test (Sasikala and Sundaraganapathy, 2017), where to 2.0 ml filtrate of the fraction just after fractionation, 2.0 ml of reagent was mixed. Formation of reddish brown precipitate indicated positive result for alkaloid fraction, but negative for non-alkaloid fraction.

3.5. Spectrophotometric determination of total alkaloids

Total alkaloid content (TAC) was quantified by spectrophotometric method described elsewhere (Shamsa et al., 2008). This method is based on the reaction between alkaloid and bromocresol green (BCG) forming a yellow-coloured product.

3.5.1. Preparation of solutions

Bromocresol green solution was prepared by heating 69.8 mg bromocresol green with 3 ml of 2N NaOH and 5 ml distilled water until completely dissolved and the solution was diluted to 1000 ml with distilled water. Phosphate buffer solution (pH=4.7) was prepared by adjusting the pH of 2 M sodium phosphate (71.6 g Na_2HPO_4 in 1 L distilled water) to 4.7 with 0.2 M citric acid (42.02 g citric acid in 1000 ml distilled water).

3.5.2. Preparation of atropine standard curve

Atropine standard solution was made by dissolving 1 mg pure atropine in 10 ml distilled water. Each of accurately measured aliquots (0.4, 0.6, 0.8, 1 and 1.2 ml) of atropine standard solution was transferred to different separatory funnels. Then, 5 ml of pH 4.7 phosphate buffer and 5 ml of BCG solution were added to a mixture and shaken with 1, 2, 3 and 4 ml of chloroform. The extracts were collected in a 10-ml volumetric flask and then diluted to adjust volume with chloroform. The absorbance of the complex in chloroform was measured with a UV-spectrophotometer (UV-1600, Germany) at 470 nm against blank prepared as above but without atropine.

3.5.3. Preparation of the extract

A part of the crude extract was dissolved in 2 N HCl (10 mg/10ml) and then filtered. One ml of this solution was transferred to a separatory funnel and washed with 10 ml chloroform (3 times). The pH of this solution was adjusted to neutral with 0.1 N NaOH. Then, 5 ml of BCG solution and 5 ml of

phosphate buffer were added to this solution. The mixture was shaken and the complex formed was extracted with 1, 2, 3, and 4 ml chloroform by vigorous shaking. The extracts were collected in a 10-ml volumetric flask and diluted to volume with chloroform. Absorbance for test and standard solutions was recorded against blank at 470nm with a UV-spectrophotometer. The total alkaloid content was calculated from standard curve against absorbance and expressed as mg of AE/g of extract. All determinations were performed in triplicate (n=3).

3.6. Grouping and Dosing of Animals

In both paradigms, MWM and MTM, the animals of either sex were randomly divided as a control or test group each comprising 5 or 6 animals per group. Control group were administered with Tween 80 in distilled water (2%, v/v). The test groups were further sub-grouped into three based on the type of the extract administered, crude khat extract (CR), alkaloid fraction (ALF) and non-alkaloid fraction (NAF). The various doses for each extract were selected based on previous reports (Betrie and Engidawork, 2016, Mohammed et al., 2014). Two dose levels, 50 mg/kg and 100 mg/kg were used for both alkaloid extract groups (ALF50 and ALF100) and non-alkaloid extract groups (NAF50 and NAF100), while three dose levels were employed for the crude khat extract (CR100, CR200 and CR400). The control animals were given the same volume (0.5 ml) of the vehicle (Tween 80% in distilled water (2% v/v)). 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 in humans and when khat leaves are chewed cathine and cathinone are released and absorbed through the mucous membranes of the mouth and subsequently the lining of the stomach (Banjaw and Schmidt, 2005). Throughout the experimental period, all extract solutions were prepared shortly prior to administration and sample containers of alkaloid fraction including syringes were covered with aluminium foil to avoid light decomposition.

In both paradigms, there were two types of administration: a single (acute) dose administration and a repeated dose administration. In a repeated dose administration, a single daily dose was administered for five successive days for ALF and NAF fractions, while seven days for CR khat extract before training. The dose of the extract required was determined based on the body weight of mice. For ease of dose calculation, total weight of mice taking the same dose of extract was determined and, then total amount of extract calculated. The extracts were then reconstituted with Tween-80 in distilled water (2 % v/v) using Sonicator bath for facilitating dissolution. The volume of reconstituted fluid

was adjusted so that the maximum volume administration should not exceed 1ml. For the repeated dose study, the administration was done at similar time of each day as recommended by OECD guidelines (OECD, 2001).

3.7. Morris water Maze Task

MWM is a widely used model for studying learning and memory behaviour in mice (Barnhart et al., 2015). It was first developed by Richard Morris in 1981 (Morris, 1984) as a method to assess specifically spatial or place learning. This task has the advantage of being acquired quickly without pre-training or restriction of food and water (Barnhart et al., 2015). The concept behind this task is that the animal must learn to use proximal or distal cues to navigate a direct path to the hidden platform when started from different, random locations around the perimeter of the tank (Vorhees and Williams, 2006). The procedures described elsewhere (Kimani and Nyongesa, 2008, Mohammed et al., 2014), with slight modifications were followed for this experiment.

The MWM employed for this experiment was a black circular polypropylene pool (150 cm in diameter ×50 cm in depth). It was filled with tap water up to 31 cm high. The water temperature was maintained at room temperature (25 °C ± 1 °C) during experiment days using an electric heater. A stable circular platform (10 cm in diameter × 30 cm in height) made up of a concrete cement with its surface covered by black plastic to camouflage was used as an escape platform. It was submerged 1cm below the surface of the water thereby hiding it from the animals' view.

The pool was divided into four quadrants: North-west (NW), North-east (NE), South- east (SE) and South-west (SW). Imaginary boundaries of the quadrants were marked on the edges of the pool with a masking tape labelled North (N), South (S), East (E) and West (W). Several maze and distal extra-maze cues such as colourful posters were mounted on the wall of the pool and the room and these remained in the same position throughout the training and testing periods. It is believed that they could help the animals to develop a spatial map for navigation to the platform. All testing was conducted at roughly the same time each day in order to minimize variability in performance due to time of day. The performance of the animals in the MWM was recorded using a video camera (Samsung Galaxy S7 mobile camera) mounted above the water maze.

3.7.1. Habituation

Habituation was done immediately after administration for acute dose study while immediately after the last daily dose in repeated dose administration study. It is 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 maze, they were allowed to swim for 60 sec in the pool and guided towards the platform if they failed to find it by themselves. Finding the platform was defined as staying on it for at least 3 sec. This step was repeated until the mice remained for 30 sec on the top of the platform. This phase is not considered as the spatial acquisition sessions. In addition, on each day, mice were moved to the procedure room one hour prior to testing. Each mouse was placed by the tail into the water immediately facing the perimeter at one of the start points (N, S, E, and W).

3.7.2. Acquisition Sessions

The spatial acquisition sessions took place in a room where proximal and distal cues were located on the walls of the room and the tank. The trial on each day started after 4 pm. The mice were subjected to 16 training sessions consisting of daily sessions of 4 trials for 4 days to locate a submerged platform in the center of NW (target) quadrant of the MWM tank. The platform remained in one position throughout the four day training period. During each trial, the mice were 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 for 60 sec to search for the platform. Once located, the mouse was allowed to stay on it for 30 sec. The objective of leaving the animal on the platform is to allow it to orient to its position in space and remember the position of the platform in relation to the surrounding cues. The escape latency, which was the time taken by the mouse from the start position to the escape platform was determined using a stop-watch. If the animal failed to find the platform in the allotted time, it was placed on the platform for 30 sec and assigned a latency of 60 sec. After each trial, the mice were removed, dried using a towel and rodent heater (2105 comerio VA^{R}, Italy) at 36-38 °c and then put back in the home cages. The inter-trial interval (ITI) for each animal was 5 min.

3.7.3. Retention (Probe) Trials

Probe trial test for STM 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 had been removed completely. The mouse was placed in the MWM tank from the position of S 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 a measure of spatial STM. The time spent at adjacent quadrant (NE) was also determined for comparison purpose. Memory (retention) represented the time spent in the target quadrant during the probe trial. Probe trial for LTM was carried out on the 12^{th} day, 7 days after probe trial for STM. The same procedure as for STM was followed for this test.

3.8. Multiple T-Maze Task

The T-maze is an elevated or enclosed apparatus in the form of a T placed horizontally. Animals are started from the base of the T and allowed to choose one of the goal arms at the other end of the stem (Deacon and Rawlins, 2006). Animals learn to find the goal box based on their memory from extra maze cues. The T-maze has been most extensively used to investigate spatial memory in animals as it is much easier to construct; however, this task is quite labour-intensive for the investigator and requires that a tester be present throughout the time the animal is on the maze (Wenk, 1998). In this study, a wooden multiple-T land maze (150x130 cm) and the path (15cm high and 8 cm wide) containing seven choice points was used to assess spatial learning and memory. The procedures described by Mohammed *et.al* (Mohammed et al., 2014) were followed.

3.8.1. Habituation

Habituation was done in order to enable them familiarize with the maze. 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 is not part of the spatial acquisition session.

3.8.2. Acquisition Sessions

Prior to testing, mice were deprived of food for 12 h to motivate food searching. On the acquisition phase, mice were subjected to a total of 16 trials consisting of daily sessions of 4 trials for 4 consecutive days to assess their learning potential. The trial was started after 7 pm. During these sessions mice were placed in a start box and allowed a total of 5 min per trial to search for the reward. The trial was said to be complete if the mouse reached or failed to reach the goal box within 5 min. If

the mouse reached the goal box before the set 5 min timeline, it was allowed to consume a small piece of food pellet provided as a reward at the goal box and put back in the home cage. If the animal failed to reach the goal box in the allotted time, it was guided to the goal box and allowed to consume small piece of pellet and put back to the home cage with assigned a latency of 5 min. Immediately after each trial, the entire maze was cleaned with dilute alcohol solution to remove possible olfaction cues. Trials were carried out at 20 min intervals. After the completion of the test on each day, animals were given food as per their body weight (120 g/kg), representing the amount to preserve their body weight but keeping them hungry for the following day test. 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 number wrong decisions (only forward wrong decisions) were determined using both a stop-watch and video recording mounted over it.

3.8.3. Retention (Probe) Trials

A probe trial to test spatial STM was done on the fifth day, 24 h after the last acquisition day. Each mouse was subjected to a 5 min single probe trial. Before the test, the mice were deprived of food for 12 h to motivate for food search. Immediately after each trial, the entire maze was cleaned with dilute alcohol solution to remove possible cues for the next mouse. The latency, which is the time taken by the mouse from the start box to the goal box, and the number wrong decisions were determined using both a stop-watch and video recording. A Probe trial for spatial LTM was done on the 12^{th} day, 7 days after probe trial for STM. The same procedure for STM was followed for this test.

3.9. Statistical Analysis

The obtained data were analysed using the Statistical Package for the Social Sciences (SPSS), version 25.0 software. Two-way repeated measures analysis of variance (ANOVA) was used to analyse acquisition training (learning) in both models. For memory test, paired samples t-test was used to analyse quadrant preference in MWM while one-way ANOVA followed by post-hoc test (Tukey Test) was used to analyze differences in mean among groups for latency in both MWM and MTM, and mean number of wrong decisions in MTM. The analysis were performed with 95% confidence interval and the significance level set at $p<0.05$.

4. Results

4.1. Acquisition Performance on Morris Water Maze

4.1.1. Effect of single (acute) dose administration of extracts of khat on acquisition (learning)

The result for acquisition performance on MWM task for acute dose administration of ALF, NAF and CR is presented in Table 1. A one-way ANOVA analysis showed that all groups learned the task as their mean latency to find the hidden platform had gradually fallen from D_1 to D_4. However, a post-Hoc Tukey test showed no significant difference in mean latency among groups at each day of training. A two-way repeated measures of ANOVA taking both dose level (groups) and days of training as a factor revealed a significant difference in latency to find the hidden platform within subjects across the four days of training (alkaloid: $F(3, 15) = 10.35$, $p<0.005$; non-alkaloid: $F(3, 12) = 9.906$, $p<0.005$; and crude: $F(3, 12) = 33.489$, $p<0.001$), which indicated all groups, test as well as CON, learned the task. However, between subjects performance measure didn't show statistically significant difference among the groups (alkaloid: $F(2, 10) = 0.154$, $p=0.859$; non-alkaloid: $F(2, 8) = 0.909$, $p=0.441$; and crude: $F(1.325, 5.30) = 1.461$, $p = 0.294$).

Table 1*:* Mean latency in the acquisition session for acute dose administration of khat extracts in Morris water maze task:

Extract	Groups	Mean Latency (Sec)			
		D_1	**D_2**	**D_3**	**D_4**
Alkaloid	*CON*	51.75±2.98	40.54±6.84	36.42±5.69	29.13±5.35
	ALF50	46.38±4.79	38.17±6.96	31.38±5.52	30.46±6.50
	ALF100	48.29±5.90	39.33±5.58	28.83±6.52	27.92±7.37
Non-alkaloid	*CON*	39.40±7.33	30.70±7.29	30.75±8.79	21.50±7.69
	NAF50	42.50±4.68	35.80±7.41	28.70±5.63	22.95±7.32
	NAF100	35.75±5.67	29.55±5.77	19.85±2.56	15.30±0.91
Crude	*CON*	40.60±3.29	33.40±7.09	29.15±6.35	27.10±10.10
	CR100	41.50±6.42	30.65±4.54	29.30±6.69	22.50±4.23
	CR200	43.55±4.11	42.50±2.36	36.15±2.96	33.20±2.99
	CR400	52.50±3.32	44.10±3.38	31.45±4.03	27.20±2.21

All values are mean±SEM (n=5-6) and statistical analysis was performed using one-way ANOVA and two-way repeated measures ANOVA; CON: vehicle (Tween 80 2% v/v); ALF50: alkaloid fraction 50 mg/kg; ALF100: alkaloid fraction 100 mg/kg; NAF50: non-alkaloid fraction 50 mg/kg; NAF100: non-alkaloid fraction 100 mg/kg; CR100: crude khat extract 100 mg/kg; CR200: crude khat extract 200 mg/kg; CR400: crude khat extract 400 mg/kg.

4.1.2. Effect of repeated dose administration of extracts of khat on acquisition (learning)

The acquisition performance on MWM result for repeated dose administration of ALF, NAF and CR is presented in Table 2. A one-way ANOVA analysis showed that all groups except CR400 learned the task as their mean latency to find the hidden platform had gradually fallen from D_1 to D_4. However, a Post-Hoc Tukey test showed no significant difference in mean latency among learned groups at each day of training. CR400 was failed to learn as its mean latency increased statistically insignificantly across four days. A two-way repeated measures of ANOVA taking both dose level (groups) and days of training as a factor revealed a significant difference in latency to find the hidden platform within subjects across the four days of training (alkaloid: $F(3, 15) = 6.798$, $p<0.005$; non-alkaloid: $F(3, 15) = 36.870$, $p<0.001$; and crude: $F(3, 12) = 18.823$, $p<0.001$). However, between subjects performance

measure didn't show statistically significant difference among the groups (alkaloid = F(2, 10) =3.30, p = 0.726; non-alkaloid = F(2, 8) = 0.316, p = 0.736; and crude F(3, 12) = 1.284, p = 0.324).

Table 2: Mean latency in the acquisition session for repeated dose administration of khat extracts in Morris water maze task:

Extract	**Groups**	**Mean Latency (Sec)**			
		D_1	**D_2**	**D_3**	**D_4**
Alkaloid	*CON*	40.54±5.37	34.08±6.04	27.13±5.80	28.58±3.68
	ALF50	47.92±5.94	34.50±3.13	34.08±6.31	29.79±5.20
	ALF100	43.08±3.95	43.04±7.60	30.96±4.54	26.42±7.62
Non-alkaloid	*CON*	45.11±9.79	32.56±7.97	27.44±7.56	18.75±3.60
	NAF50	40.13±4.23	41.46±6.50	29.54±4.59	25.21±3.94
	NAF100	43.46±4.55	33.54±4.22	30.96±4.26	18.95±4.99
Crude	*CON*	48.35±5.36	36.95±5.59	29.20±8.20	26.90±8.46
	CR100	33.60±6.91	31.45±8.39	21.25±4.12	16.30±2.77
	CR200	46.08±6.90	33.10±6.51	30.15±6.19	25.05±3.53
	CR400	24.45±5.12	26.40±3.65	28.20±4.21	30.45±2.11

All values are mean±SEM (n=5-6) and statistical analysis was performed using one-way ANOVA and two-way repeated measures ANOVA; CON: vehicle (Tween 80 2% v/v); ALF50: alkaloid fraction 50 mg/kg; ALF100: alkaloid fraction 100 mg/kg; NAF50: non-alkaloid fraction 50 mg/kg; NAF100: non-alkaloid fraction 100 mg/kg; CR100: crude khat extract 100 mg/kg; CR200: crude khat extract 200 mg/kg; CR400: crude khat extract 400 mg/kg.

4.2. Acquisition performance on Multiple-T maze

4.2.1. Effect of single (acute) dose administration of extracts of khat on acquisition (learning)

The acquisition performance on MTM result for acute dose administration of ALF, NAF and CR is presented in Table 3 (latency) and Table 4 (wrong decision).

A one-way ANOVA analysis showed that all groups learned the task as their mean latency to reach the goal box and errors in the path to the goal box had gradually fallen across training days. A Post-Hoc Tukey test revealed on D_1 ALF50 had significantly longer latency to reach the goal box compared to the CON ($p<0.05$), otherwise it showed no significant difference in latency among the dose levels (groups). The latency of CR200 and CR400 increased on D_4. A two-way repeated measures of ANOVA taking both dose level (groups) and days of training as a factor revealed a significant difference in latency to reach the goal box within subjects across the four days of training (alkaloid: $F(3, 15) = 36.410$, $p<0.001$; non-alkaloid: $F(1.733, 6.932) = 22.794$, $p<0.005$; and crude: $F(3, 12) = 19.204$, $p<0.001$) which indicated all groups learned the task. However, between subjects performance measure didn't show statistically significant difference among the groups (alkaloid: $F(1.109, 5.547) = 1.718$, $p=0.228$; non-alkaloid: $F(2, 8) = 1.108$, $p=0.376$; and crude: $F(3, 12) = 6.564$, $p = 0.6049$).

Table 3: Mean latency in the acquisition session for acute dose administration of khat extracts in Multiple-T maze task:

Extract	**Groups**	**Mean Latency (Sec)**			
		D_1	**D_2**	**D_3**	**D_4**
Alkaloid	*CON*	64.63±6.70	47.21±9.26	23.08±7.18	18.50±6.96
	ALF50	119.38±18.84***a**	68.63±14.90	31.58±6.94	18.42±2.94
	ALF100	72.96±13.49	60.04±13.36	26.25±3.17	21.08±4.01
Non-alkaloid	*CON*	53.95±7.61	37.35±6.12	32.05±5.94	29.90±4.24
	NAF50	80.00±10.13	55.35±14.95	37.65±9.67	25.30±6.11
	NAF100	87.35±21.36	43.00±5.14	33.10±6.56	26.30±5.87
Crude	*CON*	94.15±15.66	72.50±6.03	64.75±6.48	48.55±10.01
	CR100	137.60±32.33	64.95±21.08	48.40±16.20	47.85±9.51
	CR200	81.45±17.19	50.40±7.43	51.50±11.27	58.60±9.83
	CR400	80.55±28.91	57.25±16.09	36.80±4.59	44.80±5.93

*All values are mean±SEM (n=5-6) and statistical analysis was performed using one way ANOVA and two-way repeated measures ANOVA; *: p<0.05; a: compared to the control; CON: vehicle (Tween 80 2% v/v); ALF50: alkaloid fraction 50 mg/kg; ALF100: alkaloid fraction 100 mg/kg; NAF50: non-*

alkaloid fraction 50 mg/kg; NAF100: non-alkaloid fraction 100 mg/kg; CR100: crude khat extract 100mg/kg; CR200: crude khat extract 200 mg/kg; CR400: crude khat extract 400 mg/kg.

Regarding the number of wrong decisions (Table 4), a two-way repeated measures of ANOVA revealed a significant decrease in wrong decisions within subjects across the four days of training (alkaloid: $F(1.794, 8.970) = 48.490$, $p<0.001$; non-alkaloid: $F(3, 12) = 34.283$, $p<0.001$; and crude: $F(3, 12) = 39.121$, $p<0.001$) which indicated all groups learned the task. However, between subjects performance measure didn't show statistically significant difference among the groups (alkaloid: $F(2, 10) = 1.471$, $p=0.276$; non-alkaloid: $F(2, 8) = 1.011$, $p=0.406$; and crude: $F(1.891, 7.562) = 0.350$, $p = 0.705$).

Table 4: Mean number of wrong decisions in the acquisition session for acute dose administration of khat extracts in Multiple-T maze task:

Extract	**Groups**	**Mean Wrong Decisions**			
		D_1	**D_2**	**D_3**	**D_4**
Alkaloid	*CON*	3.29±0.19	2.08±0.45	0.61±0.25	0.21±0.14
	ALF50	4.13±0.57	2.42±0.43	1.21±0.41	0.86±0.38
	ALF100	2.67±0.43	1.92±0.17	1.33±0.31	0.96±0.30
Non-alkaloid	*CON*	3.95±0.40	1.65±0.29	1.40±0.10	0.80±0.20
	NAF50	3.50±0.47	2.45±0.37	1.80±0.23	1.05±0.41
	NAF100	3.10±0.59	2.15±0.48	1.60±0.17	0.85±0.32
Crude	*CON*	3.38±0.28	2.40±0.42	2.15±0.36	2.15±0.27
	CR100	4.15±0.74	1.65±0.48	1.50±0.56	1.30±0.41
	CR200	3.50±0.63	1.90±0.47	2.05±0.33	1.85±0.41
	CR400	2.80±0.51	2.25±0.59	1.45±0.25	1.65±0.20

All values are mean±SEM (n=5-6) and statistical analysis was performed using one way ANOVA and two-way repeated measures ANOVA; CON: vehicle (Tween 80 2% v/v); ALF50: alkaloid fraction 50 mg/kg; ALF100: alkaloid fraction 100 mg/kg; NAF50: non-alkaloid fraction 50 mg/kg; NAF100: non-alkaloid fraction 100 mg/kg; CR100: crude khat extract 100 mg/kg; CR200: crude khat extract 200 mg/kg; CR400: crude khat extract 400 mg/kg.

4.2.2. Effect of repeated dose administration of extracts of khat on acquisition (learning)

The acquisition performance on MTM result for repeated dose administration of ALF, NAF and CR is presented in Table 5 (latency) and Table 6 (wrong decision). A one-way ANOVA analysis showed that all groups learned the task as their mean latency to reach the goal box and errors in the path to the goal box had gradually fallen from D_1 to D_4 except CR400 whose latency was started to increase on D_3. A Post-Hoc Tukey test also revealed on fourth day CR400 had significantly longer latency to reach the goal box compared to the CON ($P<0.05$), otherwise there was no significant difference in latency among the dose levels (groups). A two-way repeated measures of ANOVA taking both dose level (groups) and days of training as a factor revealed a significant difference in latency to reach the goal box within subjects across the four days of training (alkaloid: $F(3, 15) = 20.482$, $p<0.001$; non-alkaloid: $F(3, 15) = 20.793$, $p<0.001$) but crude didn't ($F(1.183, 4.731) = 4.0808$, $p = 0.101$). However, between subjects performance measure didn't show statistically significant difference among the groups (alkaloid: $F(2, 10) = 2.410$, $p=0.140$; non-alkaloid: $F(2, 10) = 1.245$, $p=0.329$; and crude: $F(3, 12) = 3.266$, $p = 0.059$).

Table 5: Mean latency in the acquisition session for repeated dose administration of khat extracts in Multiple-T maze task:

Extract	**Groups**	**Mean Latency (Sec)**			
		D_1	**D_2**	**D_3**	**D_4**
Alkaloid	*CON*	66.92±19.99	34.71±6.25	25.50±3.90	25.88±3.65
	ALF50	42.63±8.18	23.33±3.29	18.63±2.61	12.63±1.27
	ALF100	72.63±13.84	24.29±4.12	21.83±3.61	33.38±14.50
Non-alkaloid	*CON*	78.75±13.80	53.13±9.89	38.83±7.38	34.96±6.03
	NAF50	48.79±11.60	27.13±5.43	23.38±6.18	24.71±6.02
	NAF100	90.33±22.12	61.67±27.37	30.71±3.86	28.295.38
Crude	*CON*	42.60±10.85	36.40±11.33	31.95±6.79	30.30±3.01
	CR100	89.20±36.62	49.60±16.17	43.75±8.82	33.90±4.80
	CR200	124.35±32.20	67.05±16.98	55.50±15.15	48.80±10.45
	CR400	88.95±26.26	33.15±6.41	50.80±14.61	59.35±7.38*a

*All values are mean±SEM (n=5-6) and statistical analysis was performed using one way ANOVA and two-way repeated measures ANOVA; *: p<0.05; a: compared to the control; CON: vehicle (Tween 80 2% v/v); ALF50: alkaloid fraction 50 mg/kg; ALF100: alkaloid fraction 100 mg/kg; NAF50: non-alkaloid fraction 50 mg/kg; NAF100: non-alkaloid fraction 100 mg/kg; CR100: crude khat extract 100 mg/kg; CR200: crude khat extract 200 mg/kg; CR400: crude khat extract 400 mg/kg.*

Regarding the number of wrong decisions (Table 6), a two-way repeated measures of ANOVA revealed a significant difference in in wrong decisions within subjects across the four days of training (alkaloid = F (3, 15) = 23.682, p<0.001; non-alkaloid = F (3, 15) = 10.037, p<0.005; and crude F (3, 12) = 7.188, p<0.05) which indicated all groups learned the task. However, between subjects performance measure didn't show statistically significant difference among the groups (alkaloid = F (2, 10) = 0.213, p=0.812; non-alkaloid = F (2, 10) = 1.189, p=0.344; and crude F (3, 12) = 2.543, p = 0.105).

Table 6: Mean number of wrong decisions in the acquisition session for repeated dose administration of khat extracts in Multiple-T maze task:

Extract	**Groups**	**Mean Wrong Decisions**			
		D_1	**D_2**	**D_3**	**D_4**
Alkaloid	*CON*	2.33±0.28	1.54±0.35	1.50±0.19	1.00±0.19
	ALF50	2.33±0.24	1.71±0.59	1.04±0.34	0.33±0.15
	ALF100	2.50±0.50	0.86±0.31	1.13±0.31	1.04±0.44
Non-alkaloid	*CON*	3.33±0.43	2.13±0.63	1.63±0.43	1.42±0.41
	NAF50	1.93±0.45	1.38±0.44	0.96±0.20	0.92±0.23
	NAF100	2.67±0.50	1.67±0.52	1.58±0.45	0.86±0.24
Crude	*CON*	1.85±0.57	1.30±0.43	1.30±0.39	1.30±0.36
	CR100	3.35±0.97	1.85±0.43	1.83±0.32	1.15±0.29
	CR200	3.45±0.76	2.15±0.32	2.35±0.30	1.95±0.18
	CR400	2.35±0.68	1.45±0.20	1.35±0.22	1.60±0.24

All values are mean±SEM (n=5-6) and statistical analysis was performed using one way ANOVA and two-way repeated measures ANOVA; CON: vehicle (Tween 80 2% v/v); ALF50: alkaloid fraction 50

mg/kg; ALF100: alkaloid fraction 100 mg/kg; NAF50: non-alkaloid fraction 50 mg/kg; NAF100: non-alkaloid fraction 100 mg/kg; CR100: crude khat extract 100 mg/kg; CR200: crude khat extract 200 mg/kg; CR400: crude khat extract 400 mg/kg.

4.3. Probe Trials

4.3.1. Effect of alkaloid fraction on memory retention on Morris water maze task

The result of effect of ALF on memory test is presented in Table 7. A paired samples t-test revealed that mice from all groups (CON as well as test groups in both single and repeated dose administration for STM and LTM test) spent more time in target quadrant (NW) than adjacent quadrant (NE), but showed no statistically significant difference in quadrant preference except ALF50 in single dose administration for STM test ($p<0.005$) and CON in repeated dose administration for LTM test ($p<0.05$). However a one-way ANOVA comparison test showed no statistically significant difference between groups in time spent in the target quadrant.

Table 7: Memory retention test for alkaloid fraction administered mice in Morris water maze task:

Mode of administration	Groups	**Probe Trial: Mean Latency (Sec)**			
		STM Test (D_5)		**LTM Test (D_{12})**	
		t at TQ	**t at AQ**	**t at TQ**	**t at AQ**
Single dose	CON	15.50±2.31	14.83±2.43	16.83±1.58	11.67±189
	ALF50	17.33±1.59**	9.00±1.41	17.83±1.11	13.33±3.38
	ALF100	18.00±5.47	7.83±1.89	13.17±2.29	9.50±1.43
Repeated dose	CON	17.67±3.50	13.00±2.58	15.17±2.52*	8.00±2.16
	ALF50	16.67±3.40	13.67±3.15	11.17±2.67	11.00±1.83
	ALF100	19.33±4.11	11.17±2.23	15.00±3.95	11.67±2.79

*Mean time (sec) spent in target quadrant (t at TQ) vs adjacent quadrant (t at AQ) on day 5 (STM) and day 12 (LTM) in MWM. All values are mean ± SEM (n=5-6) and statistical analysis was performed using paired samples t-test and one-way ANOVA, *: significant at $p<0.05$ compared to AQ, **: significant at $p<0.005$ compared to AQ, CON: vehicle (Tween 80 2% v/v); ALF50: alkaloid fraction 50 mg/kg; ALF100: alkaloid fraction 100 mg/kg.*

4.3.2. Effect non-alkaloid fraction on memory retention of mice on Morris water maze task

The result of effect of NAF on memory test is presented in Table 8. A paired samples t-test revealed that mice from all groups (control as well as test groups in both single and repeated dose administration study) for STM test spent more time in target quadrant (NW) than adjacent quadrant (NE). For LTM test, all groups in single dose administration study spent more time in target quadrant, while in repeated dose administration spent more time in adjacent quadrant. However, none showed statistically significant difference in quadrant preference except CON in single dose administration study for LTM test ($p<0.005$) and NAF100 in repeated dose administration for STM test ($p<0.05$). Similarly, a one-way ANOVA comparison test showed no statistically significant difference among groups in time spent in the target quadrant.

Table 8: Memory retention test for non-alkaloid fraction administered mice in Morris water maze task:

Mode of Administration	Groups	**Probe Trial: Mean Latency (Sec)**			
		STM Test (D_5)		**LTM Test (D_{12})**	
		t at TQ	**t at AQ**	**t at TQ**	**t at AQ**
Single dose	CON	24.40±5.73	13.00±3.30	24.40±2.21**	6.60±2.28
	NAF50	17.20±2.78	14.00±5.22	18.80±4.81	14.40±4.48
	NAF100	20.80±6.22	11.40±2.34	20.40±4.05	13.00±1.79
Repeated dose	CON	18.83±3.28	11.83±1.80	10.83±2.18	14.00±2.49
	NAF50	20.83±2.85	13.83±1.35	15.83±1.56	16.17±1.83
	NAF100	18.67±0.72*	13.17±1.85	14.33±3.17	15.17±2.98

*Mean time (sec) spent in target quadrant (t at TQ) vs adjacent quadrant (t at AQ) on day 5 (STM) and day 12 (LTM) in MWM. All values are mean ± SEM (n=5-6) and statistical analysis was performed using paired samples t-test and one-way ANOVA, *: significant at p<0.05 compared to AQ, **: significant at p<0.005 compared to AQ, CON: vehicle (Tween 80 2% v/v); NAF50: non-alkaloid fraction 50 mg/kg; NAF100: non-alkaloid fraction 100 mg/kg.*

4.3.3. Effect crude khat extract on memory retention of mice on Morris water maze task

The result of effect of CR on memory test is presented in Table 9. A paired samples t-test revealed that mice from all groups (CON as well as test groups in both single and repeated dose administration

study) for STM test spent more time in target quadrant (NW) than adjacent quadrant (NE). For LTM test, in single dose administration study all groups except CR400 spent more time in adjacent quadrant, while in repeated dose administration all groups spent more time in in target quadrant. However none showed statistically significant difference in quadrant preference except CR100 in single dose administration study STM test ($p<0.05$) for TQ and in LTM test ($p<0.05$) for AQ. In repeated dose administration CR400 preferred TQ in STM test ($p<0.05$). However, a one-way ANOVA comparison test showed no statistically significant difference among groups in time spent in the target quadrant.

Table 9: Memory retention test for crude extract administered mice in Morris water maze task:

Mode of Administration	Groups	**Probe Trial: Mean Latency (Sec)**			
		STM Test (D_5)		**LTM Test (D_{12})**	
		t at TQ	**t at AQ**	**t at TQ**	**t at AQ**
Single dose	CON	20.60±2.89	12.60±2.25	14.60±2.66	15.40±3.40
	CR100	17.80±3.40*a	10.20±2.27	9.80±2.31	16.40±1.66*b
	CR200	20.80±4.60	11.40±2.11	10.00±3.02	11.60±1.25
	CR400	14.40±3.03	13.20±1.53	13.40±1.29	10.40±3.39
Repeated dose	CON	14.40±1.93	14.20±2.54	12.40±2.32	10.00±2.28
	CR100	16.40±2.34	11.60±2.75	16.80±3.68	13.80±3.72
	CR200	23.60±5.75	10.40±1.03	13.00±3.19	10.60±2.25
	CR400	24.40±3.14*	7.60±2.18	12.60±1.33	9.60±3.44

*Mean time (sec) spent in target quadrant (t at TQ) vs adjacent quadrant (t at AQ) on day 5 (STM) and day 12 (LTM) in MWM. All values are mean ± SEM (n=5) and statistical analysis was performed using paired samples t-test and one-way ANOVA, *: significant at p<0.05; a=compared to AQ, b=compared to TQ; CON: vehicle (Tween 80 2% v/v); CR100: crude khat extract 100 mg/kg; CR200: crude khat extract 200 mg/kg; CR400: crude khat extract 400 mg/kg.*

4.3.4. Effect of alkaloid fraction, non-alkaloid fraction and crude extract of khat on memory retention on MTM task

The results of the effect of single and repeated dose administration of ALF, NAF and CR on memory retention (STM and LTM) test is presented in Table 10-12. A One-way ANOVA comparison test didn't show statistically significant difference in mean latency to reach the goal box and number of

wrong decisions in path to goal box in both single and repeated dose administration for both STM and LTM tests among groups.

Table 10: Memory retention test for alkaloid fraction administered mice in Multiple-T maze task:

Mode of Administration	Groups	**Probe Trial**			
		STM Test (D_5)		**LTM Test (D_{12})**	
		Mean Lat(s)	**Mean WD**	**Mean Lat(s)**	**Mean WD**
Single dose	CON	16.50±4.53	0.67±0.49	44.00±22.52	1.83±1.28
	ALF50	26.00±6.94	0.67±0.21	20.83±3.75	0.33±0.33
	ALF100	13.67±1.02	0.17±0.17	37.17±16.85	1.00±0.82
Repeated dose	CON	15.17±2.50	0.17±0.17	15.33±1.56	0.17±0.17
	ALF50	9.00±0.63	0.00±0.00	13.83±1.35	0.17±0.17
	ALF100	34.67±17.23	1.33±0.84	42.17±18.47	1.17±0.98

Mean latency to reach the goal box (Mean Lat) (sec) and mean wrong decisions made in the path to the goal box (Mean WD) on day 5 (STM) and day 12 (LTM) in MTM. Values are mean ± SEM (n=6) and statistical analysis was performed using one-way ANOVA, CON: vehicle (Tween 80 2% v/v); ALF50: alkaloid fraction 50 mg/kg; ALF100: alkaloid fraction 100 mg/kg.

Table 11: Memory retention test for non-alkaloid fraction administered mice in Multiple-T maze task:

Mode of Administration	Groups	**Probe Trial**			
		STM Test (D_5)		**LTM Test (D_{12})**	
		Mean Lat(s)	**Mean WD**	**Mean Lat(s)**	**Mean WD**
Single dose	CON	19.20±4.44	0.64±0.25	20.20±2.96	0.20±0.20
	NAF50	21.80±4.60	1.00±0.55	39.40±13.38	2.00±0.95
	NAF100	26.80±8.84	1.20±0.74	20.60±2.94	0.80±0.20
Repeated dose	CON	20.83±1.54	0.50±0.22	19.50±4.46	0.33±0.21
	NAF50	21.33±3.24	0.67±0.33	18.17±2.92	0.67±0.42
	NAF100	28.83245.56	0.83±0.48	17.83±2.01	0.83±0.48

Mean latency to reach the goal box (Mean Lat) (sec) and mean wrong decisions made in the path to the goal box (Mean WD) on day 5 (STM) and day 12 (LTM) in MTM. Values are mean ± SEM (n=5-

6) and statistical analysis was performed using one-way ANOVA, CON: vehicle (Tween 80 2% v/v); NAF50: non-alkaloid fraction 50 mg/kg; NAF100: non-alkaloid fraction 100 mg/kg.

Table 12: Memory retention test for crude khat administered mice in Multiple-T maze task:

Mode of Administration	Groups	**Probe Trial**			
		STM Test (D_5)		**LTM Test (D_{12})**	
		Mean Lat(s)	**Mean WD**	**Mean Lat(s)**	**Mean WD**
Single dose	CON	24.80±1.36	0.20±0.45	24.60±5.35	1.00±0.63
	CR100	31.20±2.42	0.40±0.55	23.60±3.19	1.00±0.63
	CR200	31.40±8.14	0.40±0.40	23.40±3.08	1.00±0.32
	CR400	32.20±4.19	0.80±0.37	19.40±2.09	0.60±0.40
Repeated dose	CON	22.40±3.59	0.40±0.25	19.80±1.56	0.20±0.20
	CR100	28.00±6.54	0.40±0.25	29.20±5.24	1.80±0.66
	CR200	35.00±6.16	0.40±0.25	40.80±16.41	1.80±0.80
	CR400	41.20±5.43	0.40±0.25	41.00±15.28	2.00±0.71

Mean latency to reach the goal box (Mean Lat) (sec) and mean wrong decisions made in the path to the goal box (Mean WD) on day 5 (STM) and day 12 (LTM) in MTM. Values are mean ± SEM (n=5-6) and statistical analysis was performed using one-way ANOVA, CON: vehicle (Tween 80 2% v/v) CR100: crude khat extract 100mg/kg; CR200: crude khat extract 200mg/kg; CR400: crude khat extract 400mg/kg.

The above analyses were done to test the three extracts of khat with increasing dose levels. In addition to it, in order to effectively investigate the extracts, further comparisons were made. To this effect, the lower (ALF50, NAF50 and CR100) and middle (ALF100, NAF100 and CR200) doses of three extracts effect on spatial learning and memory was studied. A two-way repeated measures ANOVA, one-way ANOVA and paired samples t-test statistical analysis were used as required to measure the acquisition performance and memory retention ability of mice on single and repeated dose administration as above. The results showed no significant difference in acquisition performance and memory retention of test groups compared to CON in both models (MWM and MTM) of the study.

4.4. Total alkaloid content

TAC was quantified by spectrophotometric method using atropine standard. The TAC was estimated from atropine (40-120µg/ml) from standard curve (Fig. 2) (y = 0.0145x-0.298, R^2=0.99). The result revealed the presence of 0.0219 mg Atropine equivalent/gram of dried crude extract (0.0219mg AE/g).

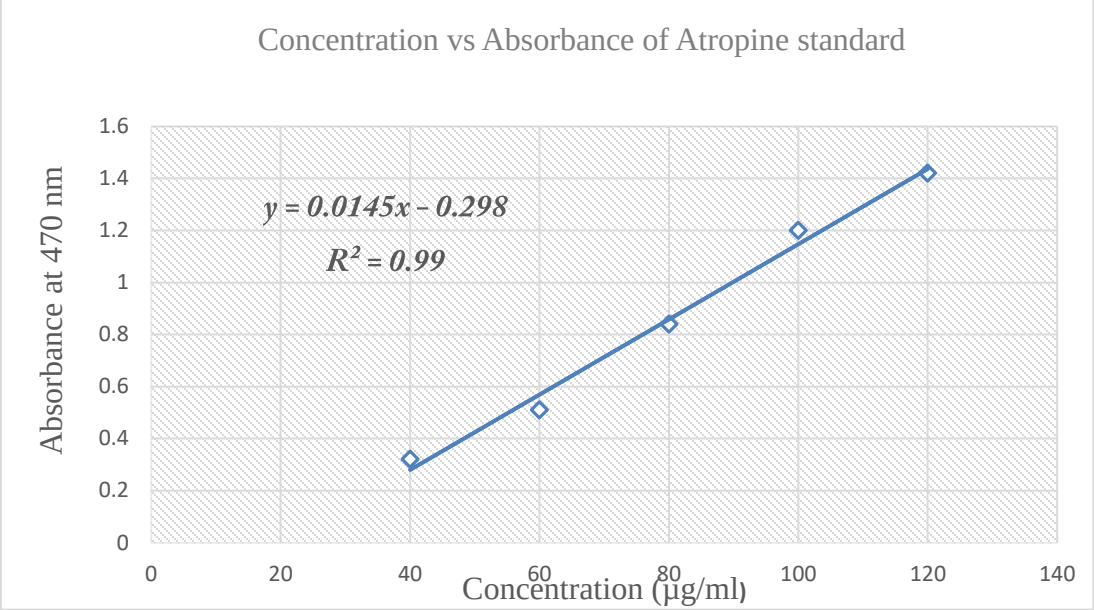

Fig. 2: Atropine calibration curve for determination of total alkaloid content.

5. Discussion

The present study was done to evaluate the effect of acute and repeated dose orally administered ALF and NAF as well as CR khat extract on learning and memory in mice. In MWM, during the four training days there was a decrease in escape latency in all groups except CR400 in repeated dose administered study, which showed learning had occurred without significant intergroup difference in both single and repeated dose administration study. The escape latency of CR400 in repeated dose administration had increased throughout the four days indicating this group didn't learn the task.

Regarding retention of memory (STM and LTM) in MWM, all groups administered with ALF were spent more time in target quadrant (NW) than the adjacent quadrant (NE) in both acute and repeated dose studies indicating memory retention without significant intergroup difference. Those administered with acute dose of NAF retained their memory to search the platform in target quadrant in both STM and LTM test, while in repeated dose study all groups spent more time at target quadrant (NW) during STM test but in adjacent quadrant (NE) during LTM test although there was no significant intergroup difference. Similarly, in acute dose CR administration study, the test as well as CON groups memorized the location of the escape platform on D_5 (STM) as they spent most of their time at NW searching for it, but not in day 12 (LTM) as they spent more time in adjacent quadrant (NE). However, in repeated dose study all groups spent more time in NW on D_5 (STM) and D_{12} (LTM) though there was no significant intergroup difference.

In MTM study also, generally, almost all groups learned the task without significant intergroup difference as shown by their mean latency to reach the goal box and the mean number of wrong decisions in the path to the goal box. The mean latency and mean number of wrong decisions of ALF and NAF administered (acute and repeated dose) test groups and also their CON were decreased across the four days of training indicating learning had occurred. In acute dose CR administered groups, the mean latency and mean number of errors of CR200 started to increase on D_3 while for CR400 on D_4. In repeated dose administration of CR, CR400 latency increased on D_3 and D_4 while mean number of wrong decisions on D_4.

Probe trial for STM (D_5) and LTM (D_{12}) in MTM for both acute and repeated dose administration of fractions as well as CR showed no significant intergroup difference in memory retention for both latency to reach the goal box as well as mean number of wrong decisions in the path to the goal box.

A further comparison was made to measure the acquisition performance and memory retention ability of mice on acute and repeated dose administration among the lower (ALF50, NAF50 and CR100) and middle (ALF100, NAF100 and CR200) doses of the three extracts showed no significant difference in acquisition performance and memory retention of test groups compared to a CON in both models of the study.

Bearing in mind the structural and pharmacological activity similarity, the effect of khat on learning and memory had been deducted from amphetamine and synthetic cathinone derivatives for many years. In recent years, few studies have been done on the effect of crude khat extract on learning and memory. However, still to date studies on the effect of khat's main alkaloid (cathinone) on learning and memory are lacking.

As aforementioned, people chew khat believing that it improves memory, alertness and clear thinking. Although such beliefs are widely held, there are few laboratory reports regarding the effect of the khat on learning and memory in laboratory animals, and also those results reported are conflicting with each other.

Although the reports on effects of amphetamine on the cognitive processes of learning and memory have been mixed, a number of human and animal studies indicated its enhancing effects on acute exposure. Most studies have shown that acute amphetamine exposure promotes learning and improves memory. A study made to evaluate the effect of d-amphetamine on MWM task performance by training rats using a single training trial per day procedure showed amphetamine group demonstrated a faster rate of learning and had a stronger spatial bias to the platform location on the probe trial than did control, and this facilitation may be mediated by the dopaminergic system (Brown et al., 2000). Other study also indicated that administration of d-amphetamine prior to the retention test can alleviate forgetting resulting from many different kinds of memory disrupting treatments (Quartermain et al., 1988). Injection of amphetamine was also shown to improve memory consolidation in shuttle box avoidance task in rats (Evangelista and Izquierdo, 1971).

Similarly, a study attempted to address the relation between acute khat use and cognitive control functions indicated that acute intake of khat may improve participants' ability of handling response conflict, as they had a smaller Simon effect, a test of resolution of response conflict (Colzato et al., 2013). Other study done to assess verbal learning and memory using the Arabic version of the Rey Auditory Verbal Learning Test (RAVLT) demonstrated that khat use alone did not affect immediate

or delayed recall of previously learned words. However, concurrent use with tobacco smoking lowered verbal learning and delayed recall (Hoffman and al'Absi, 2013).

There are few animal studies that are conducted recently on the effect of khat extract on learning and memory. The results reported for crude khat extract effect are similar with the present findings. A comprehensive study made in Ethiopia (Mohammed et al., 2014) that subjected mice to acute, subacute and subchronic khat exposure, and determined learning and memory using MWM, MTM and other behavioral paradigms showed acute and subacute exposure had no effect on learning and memory. This study was almost similar in terms of doses employed for the crude extract, paradigms used, and the setting. In the present study, the effect on learning parameters in both models of studies (MWM and MTM) is in agreement with this previous report. Learning has occurred in all groups including the CON without significant intergroup difference in acute dose administration study. Thus, depending on the result of the present and, also the previous studies it is plausible to conclude khat extracts don't have effect on learning and memory in acute exposure. This is further supported by the recent study (Assefa et al., 2018) in which khat didn't show any improvement in spatial learning and memory compared to controlled drugs (placebo) in mice using MTM test. It did not also show a significant lowering of wrong decision making in the multiple T-maze test.

On contrary to acute exposure, repeated or chronic and/or high dose exposure to amphetamine has been reported to adversely influence cognitive behavior. Animal and human studies have indicated the impairment of spatial learning and memory by amphetamine and its substituted derivatives. Rats treated with repeated methamphetamine (METH) showed impaired learning and spatial memory in MWM evidenced by a significant difference in the escape latency compared to the control (Camarasa et al., 2010). Human studies have also suggested that chronic amphetamine use is associated with mild to moderate neuropsychological impairment. Extensive use of methamphetamines has been repeatedly associated with deficits in episodic memory (Simon et al., 2004) and working memory (Chang et al., 2002). Like amphetamines, synthetic cathinones like mephedrone showed reduced working memory performance in the T-maze spontaneous alternation task in mice (den Hollander et al., 2013).

However, it is important to note that the consumption of amphetamine in pure form is not entirely comparable with chewing khat leaves (Colzato et al., 2011b). Cathinone appears to be only half as potent as amphetamine (Hoffman and Al'Absi, 2010). Moreover, khat contains 62 types of cathedulins most of which have not been studied for their biological effects. Thus, those studies in amphetamine

and synthetic cathinones cannot guarantee similar effects to be observed with khat on cognitive functions. There may also be functional differences between cathinone and the cocktail of active substances (cathinone, cathine, norephedrine, cathedulins and other alkaloids) ingested by chewing khat (Bentivoglio et al., 2014).

Likewise, a preliminary observation of chronic use of khat suggests that it is associated with various cognitive and mental health impairments. In a study by Colzato et al. (2011b), a small sample of 20 regular khat users demonstrated impairments in cognitive flexibility and monitoring of information in working memory compared to 20 nonusers (Colzato et al., 2011b). A subsequent study by the same group demonstrated impaired inhibitory control in khat users compared to non-users (Colzato et al., 2011a). In other study, working memory deficits associated with chronic khat use was noted (Hoffman and al'Absi, 2013).

The higher doses of crude (CR200 and CR400) in repeated dose exposure in both models have shown a clue of impairment in learning. CR400's latency was increased insignificantly across four days in MWM task, indicating learning didn't occur, whereas, CR400 had significantly longer latency to reach the goal box compared to the CON on D_4 in repeated dose study in MTM. This could be suggestive that high and/or chronic doses of khat might impair learning. Other studies have also shown similar results. Subchronic oral administration of khat showed cognitive deficit in MWM study in addition to inducing schizophrenic-like symptoms in mice (Bogale et al., 2016). In other study, intraperitonial administration of crude khat extract has shown to produce an inconsistent effect on learning and memory in MWM task in CBA mice: low dose having no effect on learning but impairing memory, whereas high dose impairs learning (Kimani and Nyongesa, 2008). Subchronic exposure also produced a significant impairment in short-term memory in MTM and MWM tasks (Mohammed et al., 2014). It is generally accepted that neurobiological changes due to drug use vary as a function of many factors including the pattern of use (acute or chronic use) (Hoffman and Al'Absi, 2010).

In the present study, both fractions and CR extract lacked any effect on learning and memory might be due to different reasons. As brain neurotransmitters (DA, NA and5-HT) level was not measured in this study, the first reason could be the extracts at doses used in this study were unable to modulate neurotransmitters at concentration adequate enough to influence the mechanisms of learning and memory formation. In other words, the extract did not disrupt mechanisms underlying synaptic plasticity, which are important to maintain learning (Elgersma and Silva, 1999). In some studies, for

instance, (Kimani and Nyongesa, 2008) the comparable dose and, even the smaller dose of crude khat administered intraperitoneally to CBA mice influenced learning and memory. This variance might be due to difference in route of administration of the extract, difference in potency of khat plant and/or difference in mice species. However, some amphetamine studies showed no effect in performance on eight radial-arm maze task too (Beuzen et al., 1994).

There might also be a different possible reason why significant effect was not seen in this study. As aforementioned, the serotonergic neurotransmission in hippocampus could have impairing whereas the dopaminergic neurotransmission improving effect on learning and memory. In addition, behavioural studies have confirmed the antagonistic effect of 5-HT on facilitating effect of DA in wide range of behavior (Kimani and Nyongesa, 2008). Khat increase the activity of the dopaminergic and serotonergic transmission (Dhaifalah and Santavy, 2004). Thus, the result of the present study can be explained by the effects of 5-HT and DA on learning and memory cancelled out each other by their antagonistic effect.

ALF in this study was administered in an aim to get better effect than the crude. However, no significant effect was observed, except in acute dose study, where ALF50 group had significantly longer latency to reach the goal box compared to the CON on D_1 in MTM task. However, this effect was not seen neither in the consecutive days of training nor in repeated dose study as well as in the MWM study. Thus, this effect might be due to variability in individual mouse performance within ALF50 group.

Similarly, NAF was employed to investigate the effect of compounds in khat other than alkaloids such as flavonoids and tannins on learning and memory. Animal and cellular studies suggest that flavonoids (flavonoid-rich foods) improve memory and learning by leading to changes in morphological aspects of neuronal cells, such as spine density, that ultimately impact on synaptic plasticity and more sustained LTP in the hippocampus (Rendeiro et al., 2012). A study done on the effect of khat on animal model of diabetes revealed a differential effects of the crude extract and flavonoid fraction (Betrie and Engidawork, 2016). However, the NAF in this study lacked ability to affect learning and memory. Thus, although over 40 compounds have been identified in khat in addition to the main active compounds, cathinone and cathine (Thomas and Williams, 2013), the non-alkaloid portion didn't alter learning and memory significantly.

6. Conclusion

This study showed that neither acute nor repeated exposure of mice with different doses of alkaloid and non-alkaloid fractions as well as the crude extract of khat had significant effect on learning, short and long term memory using Morris water maze and Multiple T-maze models. The highest dose of crude, CR400, in repeated dose exposure in both models have shown a clue of impairment in learning. This could be suggestive that high and/or chronic doses of khat might impair learning, upholding earlier findings with crude khat extract.

7. Recommendations

From the present study the following recommendations are made.

- The effect of chronically administered crude khat extract on learning and memory needs to be investigated.
- The effect of sub-acutely, sub-chronically and/or chronically administered khat alkaloids (cathinone that is fractionated from khat) on learning and memory needs to be investigated.
- The effect of higher doses of alkaloidal and non-alkaloidal khat extracts needs to be investigated
- Neurotransmitters level (dopamine, serotonin and noradrenaline) needs to be determined.
- The effect of post-training administration of khat extract on spatial learning and memory needs to be investigated
- The effect of cathedulins on learning and memory needs to be investigated

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