

UNIVERSITY OF GHANA
COLLEGE OF HEALTH SCIENCES
SCHOOL OF PHARMACY

**EFFECT OF AN ETHANOLIC FRUIT EXTRACT OF *XYLOPIA*
AETHIOPICA AND ITS KAURENE DITERPENOID, XYLOPIC ACID, ON
LEARNING AND MEMORY: A PRE-CLINICAL EVALUATION FROM A
NEUROPHARMACOLOGICAL PERSPECTIVE**

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**THIS THESIS IS SUBMITTED TO THE UNIVERSITY OF GHANA,
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DECLARATION

DECLARATION BY THE CANDIDATE

I hereby declare that this is the product of my own research undertaken under supervision and has neither been presented in whole nor in part for another degree elsewhere. I am solely responsible for any residual flaws in the work.

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DECLARATION BY SUPERVISORS

We hereby declare that the principal work and presentation of the thesis were supervised by us in accordance with guidelines on supervision of thesis laid down by the University of Ghana.

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ABSTRACT

Background: Cognitive dysfunction, presenting as learning and memory impairment, is a common manifestation in many chronic diseases, including depression, epilepsy and Alzheimer's disease. It could also be present without any known underlying medical condition. Till date, no drug has shown convincing efficacy in improving learning and memory deficits in these conditions although some medicinal plants are demonstrating promising effects. The aim of the study was to investigate the effect of fruit extract of *Xylopi aethiopia* and its kaurene derivative, xylopic acid, on learning and memory using animal models.

Materials and Methods: *Xylopi aethiopia* fruits were collected, dried and extracted using 70% v/v ethanol. Xylopic acid was isolated and purified from the fruits. ICR mice (20-25 g) were grouped, after which they received oral doses of fruit extract of *Xylopi aethiopia* (30-300 mg/kg), xylopic acid (30-300 mg/kg), standard nootropics [citicoline (30, 100, 300 mg/kg) or piracetam (30, 100, 300 mg/kg)], ketamine (30 mg/kg) or saline as vehicle. The animals were then taken through Morris water maze test which measured hippocampally-dependent spatial learning and memory, spontaneous alternation Y-maze test that measured spatial working memory and spatial recognition memory and novelty object recognition test which measured exploratory learning and recognition memory. Contributions of GABAergic and cholinergic neurotransmission in the mechanism(s) of action of the extract and xylopic acid were also investigated.

Results: The fruit extract of *X. aethiopia* (XAE) and xylopic acid (XA) enhanced learning and memory by increasing the percentage exploration with the novel object in the novelty object recognition test, percentage alternation in the spontaneous alternation Y-maze test. In contrast, both did not increase the change in escape latency of the Morris water maze test but increased

the percentage frequency in the probe trial. Pre-treatment with scopolamine hydrobromide (1 mg/kg, i.p) did not reverse the learning and memory enhancing ability of XAE and XA. Pre-treatment with diazepam (1 mg/kg) reversed the learning and memory enhancing ability of XAE and XA, suggesting involvement of GABAergic pathway.

Conclusion: The fruit extract of *Xylopi*a *aethi*o*pica* and xylopic acid are potential candidates for improving exploratory learning and recognition memory, spatial working memory, spatial recognition memory and reference memory. The fruit of *Xylopi*a *aethi*o*pica* and xylopic acid acts through the GABAergic pathway.



DEDICATIONS

This work is dedicated to God Almighty and to the Department of Pharmacology and Toxicology, University of Ghana, School of Pharmacy.



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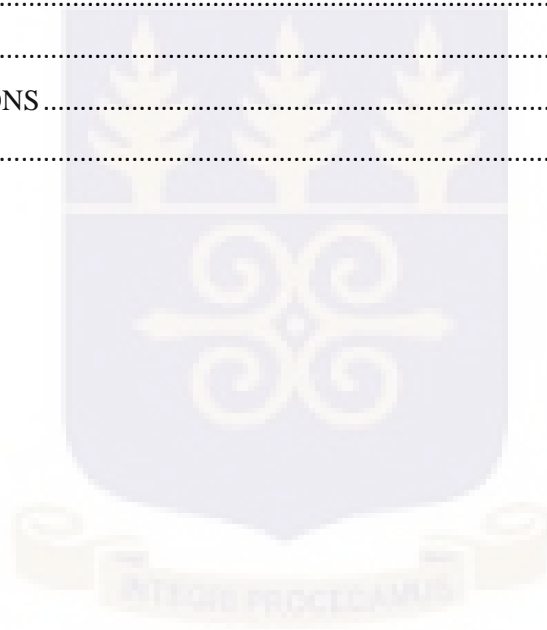
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LIST OF ABBREVIATIONS

ABBREVIATION	DEFINITION
Ach	Acetylcholine
ANOVA	Analysis of variance
Citi	Citicoline
CNS	Central nervous system
D1 receptors	Dopamine 1 receptors
D2 receptors	Dopamine 2 receptors
DA	Dopamine
DAT	Dopamine transporter
Flu	Flumazenil
GABA	Gamma amino butyric acid
GABA _A	Gamma amino butyric acid type A receptors
GABA _B	Gamma amino butyric acid type B receptors
5-HT	5-Hydroxytryptamine
5-HT _{1A}	5-Hydroxytryptamine type 1A receptors
5-HT ₂	5-Hydroxytryptamine type 2 receptors

ICR	Imprinting control region
i.p	Intraperitoneal
Ket	Ketamine
LTM	Long-term memory
LTP	Long-term potentiation
MWM	Morris water maze test
NMDA	N-methyl-D-aspartic acid
NOR	Novelty object recognition test
Pct.	Piracetam
<i>p.o</i>	Per os
Scop.	Scopolamine hydrobromide
SK	Small conductance calcium activated potassium channels
STM	Short-term memory
UEFA	Union of European Football Association
VTA	Ventral tegmental area
XA	Xylopic acid
XAE	Fruit extract of <i>Xylopic aethiopica</i>

Y-maze	Spontaneous alternation Y-maze
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CHAPTER ONE

INTRODUCTION

1.1 Background

Learning and memory are closely related terms. Learning according to Gross (2012) is the act of acquiring new or changing and strengthening existing knowledge, behaviors, skills, values of preferences which may advance to a potential change in synthesizing information, attitude, behavior, knowledge and a range of experiences. Learning is a process and not just a collection of facts and knowledge which takes place gradually. It builds upon and shapes previous knowledge. The learning process may cause changes in the organism some of which may be permanent (Daniel *et al.*, 2011). Learning in humans may take place as part of an education, training, personal development or even schooling. In humans, learning may take place as early as 32 weeks into gestation which is an indication that the central nervous system is developed enough to facilitate the learning process (DiPietro *et al.*, 2010; Sandman *et al.*, 1997).

Memory is defined as the process of storing and retrieving information over time (Matlin, 2005). Sherwood (2015) defined memory as the retention of information over time with the aim of influencing future activity. It is essential for life enabling us to think about the present, plan for the future and remember past events (Sternberg, 1999; Sternberg and Sternberg, 2016). Memory involves three (3) stages namely encoding, storage and retrieval (Sternberg, 1999; Sternberg and Sternberg, 2016). Encoding is the first and important step to memory creation or formation of new memory. It allows the information of interest to be converted to a form that can be stored in the brain for later recall from short-term or long-term memory. Information is encoded in 3 ways

namely, visual (images), acoustic (sound) and semantic (meaning). Storage deals with the where and how long the encoded information is stored. It also deals with the amount of the information that can be stored. Memory storage affects retrieval (McLeod, 2007). Memory retrieval involves the extraction of information from storage. Information organization makes retrieval easy and this can be done by organizing information into sequences such as use of alphabets, numbers, and time or size (McLeod, 2007).

Dementia is a syndrome of gradual onset which leads to a continual decline in higher cognitive functions such as attention, language and memory (Pahaye *et al.*, 2017). According to Prince *et al.* (2016) about 47 million people worldwide are living with dementia and this number is projected to increase to more than 131 million by 2050. The total worldwide cost of dementia is US \$ 818 million (Prince *et al.*, 2016). Learning and memory deficits occur commonly in older persons 60 years and above (Dhingra *et al.*, 2005). The ability to learn and remember is affected by a number of factors including age, stress, emotions among others and these may lead to memory loss, amnesia, anxiety, high blood pressure, dementia, schizophrenia and Alzheimer's disease (Anjula *et al.*, 2015). Current treatment for cognitive dysfunction is through the use of nootropics such as piracetam, citicoline, amitracetam among others. These drugs are shown to have lower therapeutic effect and with some undesirable side effects such as dependence and tolerance. There is therefore the need to find an alternative agent which is efficacious and with a high safety profile to improve learning and memory.

1.2 *Xylopiya aethiopic* (Ethiopian Pepper)

1.2.1 Plant Morphology and Properties

Xylopiya aethiopic (Dunal) A. Rich is a green, aromatic tree, of the Annonaceae family with many branches and a narrow crown that can grow between 15- 30 metres tall. It has clear straight trunk which can be 10-15 cm in diameter and has short prop roots or buttresses (Irvine, 1961). The common name is ‘Negro’ pepper or Ethiopian pepper although it is found growing in other areas such as Ghana, Angola, Burkina Faso, Gabon and Ethiopia as well as in other parts of tropical Africa. It is referred to as ‘Hwenteeaa’ in the Akan dialect, ‘So’ in Ga, ‘Etso’ in Ewe and ‘Chimba’ in Hausa. Figure 1.1 shows a picture of the plant of *Xylopiya aethiopic* and the fruits.



Figure 1.1: Plant of *Xylopiya aethiopic* on the left with the fruits on the right

The wood from the bark is used extensively in hut and boat construction (Burkhill, 1985). The fruit of the plant is useful in African cuisines as a spice and in traditional medicine. The fruit of the plant is also used in the preparation of local porridge ('Hausa koko') and soups to stimulate the production of breast milk in breast feeding the newborn baby (Burkhill, 1985). It is sometimes planted as an ornamental and to provide shade (Burkhill, 1985).

X. aethiopica is a medicinal plant known to contain kaurene-type diterpenoids (Biney *et al.*, 2014; Ekong and Ogan 1968; Ekong *et al.*, 1969; Harrigan *et al.*, 1994a; Hasan *et al.*, 1982). Diterpenes are isoprenoid molecules which are commonly found in plants and fungi and these are biosynthesized from mevalonic acid (Pablo *et al.*, 2007). Kaurene is a class of diterpenes made up of kaurenoic and xylopic acid (Biney *et al.*, 2014; Ekoag and Ogan, 1970), they are compounds of rigid tetracyclic skeleton that form intermediates in the biosynthesis of plant growth hormones such as gibberellins (Bresciani *et al.*, 2004). A collection of biological activities have been attributed to them and have been reported on. These include antimicrobial, anti-helminthic, anti-parasitic, insect anti-feedant, anti-HIV, anti-inflammatory, anti-anaphylactic and neuro-protective effects (Chen *et al.*, 2006; Ghisalberti, 1997; Obiri and Osafo, 2013; Osafo *et al.*, 2018; Xu *et al.*, 2011). Xylopic acid is found to be anti-plasmodial (Boampong *et al.*, 2013), analgesic (Woode *et al.*, 2012), cardiovascular and diuretic (Somova *et al.*, 2001). Kaurenoic acid also has anti-trypanosomic (Haraguchi *et al.*, 2011), analgesic and anti-inflammatory effects (Paiva *et al.*, 2002) acetylgrandifloric acid was also reported to have antibacterial effect (Davino *et al.*, 1989) and ent-15-oxokaur-16 en-19-oic acid (EKOA) is anti-proliferative (Choumessi *et al.*, 2012).

Xylopiia aethiopica is a useful medicinal plant with CNS activities including neuro-protection and anti-inflammatory properties. Studies by Nadia *et al.* (2014) and (Rogers *et al.*, 2011)

showed that stroke and inflammation can affect the ability to learn and remember information. Their findings showed that neuro-protection and anti-inflammation enhances learning and memory. Since the fruit extract and xylopic acid has shown neuro-protection and anti-inflammation (Biney *et al.*, 2015; Chen *et al.*, 2006), the research sought to investigate their potential for improving learning and memory.

1.3 Problem Statement

Cognitive dysfunction, presenting as learning and memory impairment, is a common manifestation in many chronic diseases, including depression, epilepsy, schizophrenia and Alzheimer's disease. It could also be present without any known underlying medical condition. Till date, no drug has shown convincing efficacy in improving learning and memory deficits in these conditions.

Currently, treatments effective for the symptoms of cognitive impairments seen in Alzheimer's and other chronic diseases with cognitive impairment symptoms are through drugs that boost the amount of acetylcholine release known as nootropics and thereby enhances cognitive function. Examples of these drugs are piracetam, amiracetam, citicoline, among others (Buchanan *et al.*, 2010). However most of these drugs have low therapeutic effects, are expensive and have some serious undesirable side effects related to dependence and tolerance.

1.4 Justification

Based on the problems mentioned in 1.3 above there is a need to screen for agents which are relatively safer and cheaper to serve as alternative efficacious agents for improving learning and memory especially in diseases such as Alzheimer's and Schizophrenia and also to improve on the quality of life of people suffering from these diseases. Fortunately most people have increasing tendency towards the use of traditional medications (Kim and Oh, 2012).

Studies have shown promising results in the effectiveness of herbal medicines for the treatment of various diseases including memory problems (Rabiei *et al.*, 2014a; Rabiei *et al.*, 2013b; Rabiei *et al.*, 2014b; Rabiei *et al.*, 2014c; Rabiei and Rafieian, 2014).

A collection of biological activities have been ascribed and reported on concerning the fruit extract of *Xylopi aethiopica* including its antimicrobial, anti-helminthic, anti-parasitic, insect anti-feedant, anti-HIV, anti-inflammatory and neuro-protective effects (Chen *et al.*, 2006; Ghisalberti, 1997; Xu *et al.*, 2011). Xylopic acid also has anti-plasmodial (Boampong *et al.*, 2013), analgesic (Woode *et al.*, 2012), cardiovascular and diuretic effects (Somova *et al.*, 2001). Several diterpenes also have known effects on the central nervous system (Chen *et al.*, 2006; Okoye *et al.*, 2013; Wasowski and Marder, 2011; Xu *et al.*, 2011). The central analgesic effects of *Xylopi aethiopica* and xylopic acid have also been recently reported on by Woode *et al.* (2012) as well as the neuro-protective effects by Biney *et al.* (2014). Studies on the fruit extract and xylopic acid have also shown them to have a low toxicity profile (Abaidoo *et al.*, 2011).

Studies by Biney *et al.* (2014), Paiva *et al.* (2002) and Haraguchi *et al.* (2011) have shown that fruit extract of *Xylopi aethiopica* and xylopic acid have neuro-protective and anti-inflammatory properties. Nadia *et al.* (2014) and (Rogers *et al.*, 2011) in a separate study showed that stroke and inflammation can impair learning and memory. This may suggest that agents that have neuro-protective and anti-inflammatory properties may improve learning and memory.

This work therefore seeks to investigate whether fruit extract of *Xylopi aethiopica* and xylopic acid can improve learning and memory in the ICR mice. The findings will help in designing alternative, cheaper and relatively safer drugs effective in improving cognitive impairments as presented in people suffering from Alzheimer's, depression, schizophrenia and Parkinson

diseases. The findings from this study will also add to the knowledge of the CNS effects of the fruit extract of *Xylopi aethiopica* and xylopic acid.

1.5 Hypothesis

Fruit extract of *Xylopi aethiopica* and xylopic acid enhances learning and memory.

1.6 Aim

This research seeks to evaluate the effect of fruit extract of *Xylopi aethiopica* and xylopic acid on learning and memory.

1.7 Specific Objectives

- To perform an ethanolic extraction of the fruits of *X. aethiopica* and to isolate xylopic acid from fruits of *X. aethiopica*
- To conduct preliminary phytochemical screening of extract
- To measure the potential effect of fruit extracts of *X. aethiopica* and xylopic acid using the spontaneous Y-maze test (Y-maze), Morris water maze (MWM) and novelty object recognition test (NOR).
- To determine the possible involvement of the cholinergic and GABAergic systems.

CHAPTER TWO

LITERATURE REVIEW

2.1 Background

Learning according to Gross (2012) is the act of acquiring new or changing and strengthening existing knowledge, behaviors, skills, values of preferences which may advance to a potential change in synthesizing information, attitude, behavior, knowledge and a range of experiences.

The inability to learn is referred to as learning impairment. Memory on the other hand is defined as the process of storing and retrieving of information over time (Matlin, 2005). Sherwood (2015) similarly defined memory as the retention of information over time for the reason of influencing future activity. The inability to remember events in one's life is known as memory impairment.

Cognitive dysfunction presenting as learning and memory impairment may occur in chronic disease conditions such as Alzheimer's disease which affects more than 15 million people worldwide (Alzheimer's, 2012), depression, epilepsy and others. It may also be present without any underlying medical condition as seen in the aged. Cognitive impairment ranges from slight but noticeable effects to measurable deficits in learning, memory and thinking abilities (Petersen *et al.*, 1999; Silverman *et al.*, 2013).

Currently treatments effective for the symptoms of cognitive impairments seen in depression, Alzheimer's and other chronic diseases are through drugs that boost the amount of acetylcholine release known as nootropics and thereby enhances cognitive function (Buchanan *et al.*, 2010).

2.2 Types of Learning and Memory

2.2.1 Types of Learning

Learning comes in different forms. The form depends on the condition under which learning occurs. These include non-associative learning, active, associative, imprinting, observational and spatial learning among others.

Non-associative learning is the changes in an animal's behavior towards a stimulus without any visible associated stimulus or event (Mackintosh, 2015). This may occur due to repeated exposure. There are 2 forms- habituation and sensitization. Habituation is reduction in response to a repeatedly presented stimulus. Sensitization on the other hand is an increase in the chances that a behavior appropriate to a repeatedly presented stimulus will occur even in response to another stimulus (Mackintosh, 2015).

Active learning is another form of learning which occurs due to a person's control of his or her learning experience. In this form of learning, the learner is able to distinguish between comprehensible and incomprehensible materials and subjects. In doing so the learner is able to monitor progress made. This form also encourages the learner to have an internal dialogue leading to the verbalization of understanding (Bransford *et al.*, 2000). Active learning encourages the act of learning since the individuals control not only how they learn but also what they learn (Armstrong, 2012).

Associative learning occurs when a new response or behavior becomes associated with a particular stimulus (Britannica, 2016). There are two forms of associative learning which are classical and operant conditioning. Classical conditioning deals with a previously neutral stimulus (a stimulus that does not elicit a response) which is repeatedly paired with a stimulus

capable of eliciting a response until the neutral stimulus elicits a response by itself. Pavlov's classical experiment involving dogs can be used to explain the phenomenon of classical conditioning. In that experiment to study the role of salivation in the digestion process dogs' were given meat powder (Pavlov and Anrep, 1927). Anytime they receive the meat powder they will salivate, and it was observed that after sometime the dogs salivated even before they were presented with the meat powder. Pavlov then decided to investigate the observation. Meat powder was paired with several stimuli such as the ringing bell. After the meat powder and the bell were presented together several times the bell was used alone. The dogs salivated to the sound of the ringing bell without the meat powder. It was therefore concluded that repeated pairing of a neutral stimulus e.g. the ringing bell with a reflex eliciting stimulus e.g. food caused the bell to acquire the ability to trigger the salivation response. This demonstrated how stimulus response bonds which are the basic building blocks of learning are formed. The dogs learned to associate the ringing of the bell with food.

Operant conditioning is also known as instrumental conditioning. It is a type of learning in which the strength of a behavior is modified by the behavior's consequences such as a reward or punishment. It was originated by Skinner (1938) who believed that one should focus on the external, observable causes of behavior. Operant conditioning modifies behavior through the use of positive and negative reinforcement. Through operant conditioning, an individual makes an association between a particular behavior and the consequence (Reynolds, 1975).

Imprinting is a kind of learning which occurs at a particular age or stage of development. It involves recognizing characteristics of certain stimuli that are subsequently imprinted on the organism. Examples include recognizing one's parents or potential sexual partners. These characteristics ensure the survival of the species. This process of learning is observed in birds as

well as in other species. It also helps to understand how similar processes observed in human development can be interpreted. The commonest form of imprinting is filial imprinting which occurs when a young animal recognizes the characteristics of the parent (Howard, 1996).

Observational learning is a form of learning in which an individual observes and imitates the behavior of another. The person whose behavior is imitated is known as a model (McLeod, 2016). An example of observational learning is children who observe and practice the behavior of people around them. Observational learning according to Bandura (1977) occurs due to some prior thought and this is known as mediational processes. There are 4 mediational processes. The first process is attention: a behavior that is imitated must grab the attention of the one imitating it. The next is retention: an individual must remember the behavior. It is therefore necessary to form memory of the behavior in question. The third process is reproduction of the behavior which involves the performing or demonstration of the behavior. Motivation is the fourth behavior. This involves the rewards and punishments associated with the behavior. If the reward outweighs the punishment, then the behavior is likely to be imitated.

Spatial learning is the process by which animals code for information about their environment making it easier to move through space and being able to recollect the location of the relevant stimuli (Stan, 2014). Spatial learning depends on the soundness or integrity of the hippocampus as well as the surrounding regions of the temporal cortex and certain structures of the forebrain. In spatial learning, the animal codes for information pertaining to the location of cues in relation to that of other cues within the environment which leads to the creation of cognitive maps of the surroundings (Stan, 2014). In an experiment conducted by Dumont *et al.* (2015), they found out that animals encoded and relied on shape of information irrespective of the specific information in spatial learning. They placed the animals in a rectangular pool with black walls and trained

them to identify a hidden platform in front of the longer walls. The training was followed by two test trials with an absent platform. In the test trials, one of the walls had a color similar or the same as used during the training whereas the other wall had a white color. They observed that the walls with the same color as that of the training had the rats expending a longer time searching the area in front of the longer wall versus that of the short wall as compared with the ones which had the white walls. This demonstrated that the rats learned to identify the hidden platform using geometric cues i.e. wall length irrespective of the color. Dumont *et al.* (2015) and Peckford *et al.* (2014) both showed that spatial learning is influenced by the head direction cell circuit.

2.2.2 Types of Memory

There are 2 types of memory related to where the information is retained or stored in the brain. The types are short-term memory or long-term memory. The way information is stored affects how it is retrieved.

Short-term memory is the capacity for holding small amount of information in the brain without manipulating the information. The information is available for only a short period of time with the duration usually lasting for 15 to 30 seconds (McLeod, 2009). The capacity of the short term memory is limited storing between 5 and 9 items at a time (Miller, 1956). The information stored is fragile and can be lost with the least distraction. The information is primarily encoded by acoustic means or even by translating visual information into sounds (McLeod, 2009).

Long term memory on the other hand stores information in a much larger quantity for potentially unlimited duration. Unlike the STM the capacity for LTM is immeasurable. Long term memory encodes information semantically and visually and sometimes acoustic. Long term memory is stored in different regions of the brain. It is sub-divided into explicit (declarative) and implicit memory (Atkinson and Shiffrin, 1968).

Explicit memory requires conscious thought or intentional recall of previous experiences and information (Ullman, 2004). Types of explicit memory include episodic memory (recall of observational information attached to specific life-events), semantic memory which is the articulation of knowledge independent of personal experience (McRae *et al.*, 2012), spatial memory (it is the memory about one's environment and spatial orientation), recognition memory (deals with the ability to identify a previously chanced upon stimulus) etc.

Implicit memory on the other hand is acquired and used without conscious awareness and this affects a person's thought and behavior (Schacter, 1987). The commonest form of implicit memory is procedural memory which enables a person to perform tasks without conscious awareness of these previous experiences e.g. remembering how to ride a bicycle or tie a shoe lace without consciously thinking about how these activities are performed. Priming is another example of implicit memory. This involves the emotions a person feels due to exposure to one stimulus which influences the response to another stimulus due to prior experience (The Peak Performance Center). It entails sub-conscious preparation of the mind (Graf and Mandler, 1984; Hamilton and Rajaram, 2001). Priming also lead to illusion of truth effect i.e. answering true to statements already heard irrespective of their truthfulness (Hasher *et al.*, 1977).

Working memory is the transient holding, processing and manipulation of information (Miyake and Shah, 1999), it involves logic, guidance of decision making and behavior (Malenka *et al.*, 2009). An example of the use of a working memory is thinking of how to get to a place you have never been to, the individual combines various bits of knowledge already obtained: the layout of the city one is visiting, information from a map, knowledge of traffic patterns in that area and conversations with friends about the location of the place. By actively using all of these information, one can determine the best route to take. Working memory and short term memory

are sometimes used interchangeably. However according to Nelson (2008) the two forms of memory are different in that the working memory permits the manipulation of the stored information whilst the short term memory refers only to the storage of the information. Working memory measure is important in cognitive psychology and neuropsychology.

Prospective memory is defined by McDaniel Einstein (2007) as remembering to perform a planned action or the intention to carry out an activity in the future. Examples include remembering to take medications, studying for an upcoming exam etc. Prospective memory is part of everyday survival and according to Tulving (2005) being aware of the future is critical to human survival. He further iterated that basic survival benefits those capable of appreciating the future, planning for it and later remembering to perform planned actions. There are two types of prospective memory which are event based and time-based prospective memory. Event based prospective memory includes remembering to carry out certain tasks when specific circumstances occur. It involves cues that enable a person to recall the need to perform certain action e.g. walking past the market cues the remembrance to go shopping for vegetables to prepare salad for lunch. Time based prospective memory involves the remembering to do an action at a particular point in time (McDaniel and Einstein, 2007) e.g. seeing the time at 6:30pm cues to watch the UEFA champions league match on television. In an experiment performed by Sellen *et al.* (1997) to determine the difference between the two types of prospective memory it revealed that performance on event based tasks was better than performance on time based tasks. This implies that intended tasks are better driven by external cues of the event-based task than internal cues of the time-based tasks.

Retrospective memory is considered the counterpart of prospective memory. It is defined as the remembering of people, events or words that occurred in the past (Burgess and Shallice, 1997). It involves the memory of what is known containing informational content (Baddeley, 1997).

2.3 Biological Basis of Learning and Memory

Learning and memory has been suggested by various studies to have a biological basis. The factors reported include neurochemicals, brain structures associated with learning and memory among others.

2.3.1 Neurochemical Factors

A number of neurotransmitters have been implicated in learning and memory. These include acetylcholine, glutamate, gamma-aminobutyric acid (GABA), serotonin, noradrenaline, dopamine (Myhrer, 2003).

2.3.1.1 Cholinergic Pathway

Acetylcholine is released in the brain during learning and has been suggested to be critical for working memory and acquisition of new memories (Atri *et al.*, 2004; Hasselmo and McGaughy, 2004; Hasselmo, 2006). Its role is to facilitate the activity of N-Methyl-D-aspartic acid (NMDA) receptors, proteins that control the strength of connections between nerve cells in the brain (Buchanan *et al.*, 2010). Buchanan *et al.* (2010) showed that acetylcholine facilitates NMDA receptors by inhibiting other proteins called SK channels (small conductance calcium activated potassium channels) whose normal role is to restrict the activity of NMDA receptors (Faber and Sah, 2007). The discovery of the role for SK channels has provided new insight into the mechanisms underlying learning and memory. SK channels normally act as a barrier to NMDA receptor function, inhibiting changes in the strength of connections between nerve cells and

restricting the brain's ability to encode memories. SK channel barrier according to the research finding by Buchanan *et al.* (2010) can be removed by the release of acetylcholine in the brain in order to enhance the ability to learn and remember information.

2.3.1.2 Glutamnergic Pathway

N-Methyl-D-aspartic acid (NMDA) receptor system which is a specific type of ionotropic glutamate receptor in the brain has been implicated in many fundamental functions including neuronal plasticity, neurotoxicity, learning and memory (Rezvani, 2006; Riedel *et al.*, 2003). The NMDA receptor system and glutamate mediates long term potentiation (LTP) in a particular way which may play a crucial role in the processes of learning and memory formation (Davis *et al.*, 1992; John *et al.*, 2000). These receptors are demonstrated to be involved in Pavlovian fear conditioning (Xu *et al.*, 2001), eye blink conditioning (Thompson and Disterhoft, 1997), spatial learning (Morris *et al.*, 1986; Shimizu *et al.*, 2000; Tsien *et al.*, 1996), working and reference memory (Levin *et al.*, 1998; May-Simera and Levin, 2003), place preference (Swain *et al.*, 2004), passive avoidance learning (Danysz *et al.*, 1988), olfactory memory (Maleszka *et al.*, 2000; Si *et al.*, 2004) and reversal learning (Harder *et al.*, 1998).

It has been suggested that the activation of the NMDA receptor is necessary for long term potentiation (LTP) in the hippocampus, amygdala and medial septum (Izquierdo, 1994; Rockstroh *et al.*, 1996; Scatton *et al.*, 1991). This mechanism has been implicated in memory formation. The involvement of the glutamate receptor system and LTP is strongly linked to new learning and memory in animal models (Lozano *et al.*, 2001; Scheetz and Constantine-Paton, 1994; Tang *et al.*, 1999; Tang *et al.*, 2001; Wong *et al.*, 2002). Both lesion and pharmacological manipulations in experimental animals suggest that the NMDA receptor system may be important in the induction of memory formation but not for the maintenance of memories.

NMDA agonists, systemically administered in rats, has been shown to potentiate cognitive functions (Hlinak and Krejci, 2002; Koek *et al.*, 1990).

2.3.1.3 GABAergic Pathway

Whissell *et al.* (2013) and McKernan Whiting (1996) showed that γ -aminobutyric acid, type A (GABA_A) receptors are the main mediators of GABA in the hippocampus, medial septum, amygdala and entorhinal cortex. These regions of the brain are actively involved in learning and memory processes (Collinson *et al.*, 2002; Rudolph and Knoflach, 2011) and studies have shown that the application of drugs in these regions affects memory formation (Izquierdo and Medina, 1991). In neurodegenerative diseases like Alzheimer's disease, GABA levels have been shown to change (Jo *et al.*, 2014; Samakashvili *et al.*, 2011). It can therefore be concluded that the GABAergic system of the brain plays a role in memory conformation and connection (Kalueff and Nutt, 1996; Majd *et al.*, 2018).

Saito *et al.* (2010) and Lu *et al.* (2000) showed in their works the close interaction between hippocampal GABA receptors and NMDA receptors. The interaction provides a strong regulation of the NMDA receptors (Ebrahimi-Ghiri *et al.*, 2018). The synaptic responses from GABA_A and GABA_B receptors serves to efficiently restrict the synaptic activation of the NMDA receptors and this suggests that blocking either GABA_A and GABA_B receptors may increase synaptic activation of NMDA receptors (Davies and Collingridge, 1996). There is evidence that suggests an interplay between GABA_A receptors and NMDA of the nucleus accumbens (Nasehi *et al.*, 2017), prefrontal cortex (Farahmandfar *et al.*, 2017), baso-lateral amygdala (Khakpoor *et al.*, 2016), CA3 (Zarrabian *et al.*, 2016), perirhinal cortex (Winters *et al.*, 2010) and hippocampus (Saito *et al.*, 2010) in modulation of memory processes. Wu *et al.* (2004) in his

work revealed that pharmacologically blocking GABA receptors produced a long lasting NMDA receptor mediated response or effect.

2.3.1.4 Serotonergic Pathway

All serotonin (5-HT) receptors are present in the human brain and are believed to play a role in learning and memory processes (Barnes and Sharp, 1999; Meneses, 1999). However most studies have focused on the role of 5-HT_{1A} and 5-HT₂ receptors in learning and memory (Johnson *et al.*, 2011). The 5-HT_{1A} receptor affects the activity of the glutaminergic, GABAergic and cholinergic neurons in the hippocampus, cerebral cortex and septo-hippocampal projection which mediates learning and memory processes (Ogren *et al.*, 2008). Johnson *et al.* (2011) showed that 5-HT receptors are essential for normal olfactory learning and memory in the fruit fly, *Drosophila melanogaster*.

2.3.1.5 Dopaminergic Pathway

Dopaminergic neurons of the ventral tegmental area (VTA) have been shown to be responsible for reward based learning as well as the passive avoidance learning (Cohen *et al.*, 2012; Grace *et al.*, 2007; Hikida *et al.*, 2010; Mirenowicz and Schultz, 1994; Tan *et al.*, 2012). D2 receptors are responsible for controlling dopamine transmission in avoidance based learning behavior (Hikida *et al.*, 2013; Nakanishi *et al.*, 2014) whereas the D1 receptors are necessary for initiating reward based learning (Hikida *et al.*, 2013; Nakanishi *et al.*, 2014). Dopamine is also believed to control working memory (Fond *et al.*, 2015; Roeper, 2013).

2.3.2 Brain Parts Involved in Learning and Memory

Learning and memory involves the use of many different parts of the brain. The hippocampus happens to be an essential neural structure which is part of the limbic system and found closer to

the medial temporal lobe. The hippocampus develops faster during late fetal and early neonatal periods. According to Kolb Whishaw (2008) the hippocampus has been associated with different memory activities. Damage done to the hippocampus and its surrounding area can cause anterograde amnesia which is the difficulty to form new memories (Mahut *et al.*, 1982). This suggests that the hippocampus is necessary for storing cognitive maps and for encoding memories. The hippocampus is also involved in memory consolidation the slow process by which memories are converted from short to long term memory (Ward, 2009). The right side of the hippocampus is more oriented towards responding to spatial aspects, whereas the left side is associated with other context information. Ward (2009) mentions that the experience in building extensive mental maps, such as driving a city taxi for a long time can increase the volume of one's hippocampus since this requires considerable memorization of routes.

The cerebellum found at the back of the brain near the spinal cord is essential in procedural memory i.e. a part of long-term memory that is responsible for knowing how to do things. It stores information on how to perform certain procedures such as walking, talking and riding a bicycle. The cerebellum is also involved in motor learning such as skills requiring co-ordination and motor control for example playing of a musical instrument (Mishkin and Appenzeller, 1987). Damages done to the cerebellum can result in problems with movement and this is because it is believed to co-ordinate timing and accuracy of movements and to make long term changes (learning) to improve that skill (Kolb and Whishaw, 2008).

The frontal lobes are located at the front of each cerebral hemisphere and positioned anterior to the parietal lobes. They are necessary for coordinating information. The frontal lobes are important in working memory. The frontal lobes are also involved in the ability to remember what one needs to do in the future; this is called prospective memory (Winograd, 1988).

Located above the hippocampus in the medial temporal lobes are two amygdalae (singular "amygdala"). The amygdalae are associated with both emotional learning and memory, as it responds strongly to emotional stimuli, especially fear. These neurons assist in encoding emotional memories and enhancing them. This process results in emotional events being more deeply and accurately encoded into memory. Lesions to the amygdalae in monkeys have been shown to impair motivation, as well as the processing of emotions (Robbins *et al.*, 2008).

2.4 Animal Models of Learning and Memory

2.4.1 Novelty object recognition test

The novelty object recognition test (NOR) was first introduced and described by Ennaceur Delacour (1988). The test measures exploratory learning and recognition memory ((Ennaceur and Delacour, 1988). The test is based on the fact that exposure to novelty such as an object or environment triggers behaviors in animals (Moscardo *et al.*, 2012). The model measures memory functions and the ability of animals to recognize a new object in a familiar environment. It comprises of 3 phases which are the habituation, the familiarization and test phases. In the habituation phase the animals are allowed to explore in the open arena with no objects for 10 minutes. In the familiarization phase, the animals are presented with two objects similar in shape, size and color to explore with for 10 minutes. The test phase can be delayed up to 24 hours after the familiarization phase. In this phase, the animals are presented with one familiar object and one novel object for 5 minutes. The animals are expected to spend more time exploring with the novel object than it does with the familiar one (Ennaceur, 2010).

The NOR test does not require the use of reinforcers such as food or punishments such as electric shock (Silvers *et al.*, 2007). The test has face validity in that it is comparable with what is seen in

humans (Baxter, 2010). NOR is quick and easy to carry out effectively (Moscardo *et al.*, 2012). The behaviors of animals in the NOR test are influenced by stress and the environment (Bevins *et al.*, 2002). Neurotransmitters play important role in the exploration of animals in the NOR test. According to Sarter Bruno (2000) cholinergic neurotransmission is enhanced in response to novel stimulus and accounts for the heightened behaviors such as attention and arousal. Giovannini *et al.* (2001) showed that glutaminergic neurotransmission plays a role in the performance of animals in the NOR. Hu *et al.* (2004) also mentioned that GABA is involved in the NOR test.

2.4.2 Morris water maze test

The Morris water maze (MWM) test was developed by Morris (1984). MWM measures hippocampally dependent spatial learning and reference memory (Morris, 1993). The idea behind this model is that the animal learns to use distal cues to locate the hidden platform (Vorhees and Williams, 2006). The maze is divided into 4 quadrants. In the MWM paradigm, the animals are given visible platform training on the 1st day. In this phase, the animal is trained to identify a visible platform which provides relief. The animal is first kept on the visible platform for 20 s to orient itself. After the 20 s the animal is lowered gently into the water to swim for 60 s and to find its way onto the platform for relief. Animals that locate the platform earlier than the 60 s are allowed to remain on the platform for additional 20 s. those that do not find it within the 60 s are guided to the platform and allowed to remain on it for an extra 20 s to re-orient themselves. The trial is repeated for 3 to 4 times with an inter trial interval of 15-30 s rotating the position of the platform in the quadrants. 24 hours after the training phase, a non-toxic paint e.g. non-dairy milk is used to make the water opaque while the platform is submerged 1-2 cm below the water surface. The animals are expected to locate the platform faster than they did in the

training. This is done twice a day for 4 days. On the 6th day, the probe trial is done. In this trial, the platform is removed and the frequency at which the animal visits the previous quadrant it last saw the platform is recorded. This is a measure for reference memory (Nunez, 2008; Vorhees and Williams, 2006).

The MWM has many advantages. It does not require a pre training phase. It is highly reliable; it can be used in cross species of rats and mice. The NMDA receptor system is believed to be involved in the performance of animals in the water maze (Morris *et al.*, 1986; Moser *et al.*, 1998).

2.5 Pharmacological Measures Employed to Improve Learning and Memory

Currently the only effective treatment for the symptoms of cognitive impairment seen in diseases such as Alzheimer's, epilepsy, depression etc. is through the use of drugs called nootropics whose main role is to improve cognitive function.

Certain drugs that act on specific acetylcholine receptors may be highly attractive as potential treatments for cognitive disorders. Buchanan *et al.* (2010) showed that mimicking the effect of acetylcholine at specific receptors facilitates changes in the strength of connections between nerve cells which could be potentially beneficial for patients suffering from Alzheimer's disease or schizophrenia.

Piracetam an example of a nootropic drug is a cyclic derivative of GABA. It was discovered in the 1960 and is widely used in humans and rodents (Bhattacharya *et al.*, 1993a; Bhattacharya *et al.*, 1993b; Park *et al.*, 2010b; Platt *et al.*, 1993; Waegemans *et al.*, 2002; Zavadenko and Guzilova, 2009). It is indicated for the treatment of dementia and cognitive impairment (Salimov *et al.*, 1995; Waegemans *et al.*, 2002). It regulates neuroplasticity, neuro-protection as well as control brain metabolism (Winnicka *et al.*, 2005). Piracetam has been found to lower symptoms

related to clinical depression, anxiety and alcohol withdrawal (Dencker *et al.*, 1978; Malykh and Sadaie, 2010). It is also believed to interact with the glutaminergic as well as the GABAergic systems (Grossman *et al.*, 2011).

Citicoline or cytidine (5')- diphosphocholine is another example of a nootropic which acts as an endogenous intermediate in the biosynthesis of phosphatidylcholine, a phosphatide which is a major lipid component of the cell membrane (Teather and Wurtman, 2003). In an experiment involving rats conducted by Lopez-Coviella *et al.* (1987), the levels of cytidine and choline in plasma and the brain increased greatly with the oral administration of citicoline within a short time. The metabolism of citicoline to cytidine and choline facilitates the synthesis and release of acetylcholine in the brain (Hirsch *et al.*, 1978; Teather and Wurtman, 2003) as well as increase the brain levels of the phosphatidylcholine and other membrane phosphatides (Lopez-Coviella *et al.*, 1992; Lopez-Coviella *et al.*, 1987). Studies have shown that administration of citicoline can act to improve cognitive impairments related to the aging process (Teather and Wurtman, 2003). Citicoline has been shown to meliorate attention and memory impairments in the aged and in patients showing signs of loss of mental ability (Alvarez *et al.*, 1997). Mosharrof *et al.* (1987) showed that citicoline was able to reverse the effects of memory loss caused by scopolamine and also improved memory in the active avoidance paradigm in rats. This suggests that the action of citicoline acts via the cholinergic system (Ash *et al.*, 2014; Strupp *et al.*, 2016; Teather and Wurtman, 2003).

Donepezil is an acetylcholinesterase inhibitor that increases the effect of acetylcholine on neurons and enhances the activities the cholinergic system. It is used in the treatment of Alzheimer's disease (Winblad *et al.*, 2006) and traumatic brain injury (Yu *et al.*, 2015). It is believed that the pro-cognitive effect of donepezil is due to increasing hippocampal neurogenesis

(Itou *et al.*, 2011; Kotani *et al.*, 2008; Narimatsu *et al.*, 2009). It has also been found to improve spatial learning and memory (Yu *et al.*, 2015). Donepezil has been found to improve working memory in old rats and also to counter the scopolamine induced working memory impairment (Buccafusco and Terry, 2004; Higgins *et al.*, 2002; Ingram *et al.*, 1994).

2.6 Medicinal Plants Used in Improving Learning and Memory

The medicinal potential of plants and their usefulness in primary healthcare cannot be over emphasized. Medicinal plants possess important repositories of bioactive agents that can be employed in the management of learning and memory impairment. In recent times several synthetic drugs have been used to manage learning and memory disorder, however their therapeutic effects are low, they are very expensive and most of them have undesirable side effects (Jivad and Rabiei, 2014). Fortunately there is increasing tendency of people towards the use of traditional medicine or those from natural products (Kim and Oh, 2012).

Recent studies have shown heartening results of the effectiveness of herbal medicines for the treatment of various medical conditions including memory impairments (Rabiei *et al.*, 2014a; Rabiei *et al.*, 2013b; Rabiei *et al.*, 2014b; Rabiei *et al.*, 2014c; Rabiei and Rafieian, 2014), stroke (Rabiei *et al.*, 2012a; Rabiei *et al.*, 2013a; Rabiei *et al.*, 2012b) and gastrointestinal problems (Moradi *et al.*, 2013). An increasing number of herbal plants have been studied and screened that provide alternative therapies in improving learning and memory.

2.6.1 Examples of medicinal plants that improve learning and memory

Hypericum perforatum also known as St. John's wort has been screened and found to improve learning and memory. Data from the work done by Khalifa (2001) showed that *Hypericum perforatum* extract improved retrieval memory of a one trial passive avoidance test in mice at dose levels used clinically to treat depression. However, the extract of *Hypericum perforatum*

failed to reverse scopolamine induced amnesia. Another work by Trofimiuk *et al.* (2005) showed that the extract of *Hypericum perforatum* averted chronic stress and corticosterone induced memory deficits and also improved recognition memory.

Lepidium meyenii also known as the black maca plant was found to improve spatial learning and memory in male mice as seen from the data obtained from the Morris water maze paradigm. It also improved learning acquisition in the step-down avoidance test. The maca plant also reduced brain acetylcholinesterase activity by 45% (Rubio *et al.*, 2007). The maca extract also blocked the ethanol induced deleterious effect during the probe trial of the Morris water maze test (Rubio *et al.*, 2011).

Prunella vulgaris also known as 'heal-all' or 'self-heal' showed a reduction in latency in the shuttle box test in the scopolamine induced memory impairment. It also averted the effect of scopolamine in the Y-maze test. It did not prevent the acetylcholinesterase action in rats. It improves learning and memory by increasing cholinergic neurotransmitters (acetylcholine) and through NMDA receptor signaling (Park *et al.*, 2010a).

Ethanol extract of *Cyperus rotundus* possesses anti acetylcholinesterase action (Rabiei *et al.*, 2013b). In a study by Sharma Gupta (2007) the extract of *C. rotundus* improved spatial learning and memory as well as emotional learning and memory in rats that had their nucleus basalis of Meynert destroyed (when the nucleus of basalis of Meynert is destroyed it causes a reduction in cholinergic neurotransmitters such as acetylcholine).

Rabiei *et al.* (2014c) showed that the ethanolic extract of *Lavandula officinalis* also known as lavender improved spatial learning and memory, motor coordination and emotional learning and memory and these activities are attributed to the antioxidant effect of the plant.

The extract of *Zizyphus jujube* was found to increase acetylcholine levels in the brain upon activation of acetylcholine transferase leading to improvement in cognitive function in Alzheimer's disease and also improving motor deficit (Oda, 1999; Rabiei and Rafieian, 2014). The extract of *Z. jujube* also restored impaired learning and memory, motor coordination and behavioral action which were caused by lesions in the Meynert nucleus at the base of the frontal lobe of the brain of rats (Rabiei *et al.*, 2014b). Another study by Shanmugavasan *et al.* (2011) also showed that the extract of *Z. jujube* protected against scopolamine and β - amyloid peptide induced cognitive deficits due to its antioxidant properties.

Ginseng extract is shown to improve cognition and psychomotor action and this improves cholinergic function in Alzheimer's disease by reducing levels of β - amyloid. It also repairs damage caused to the neuron (Heo *et al.*, 2008). Ginsenoside an active ingredient of ginseng has also been shown to reduce learning deficits caused by brain damage (Mook-Jung *et al.*, 2001).

Vasudevan Parle (2007) revealed that the ethanolic extract of the bark of *Thespesia populnea* commonly known as Indian tulip tree improved memory of rats as reflected in the reducing transfer latency and time taken to enter the reward chamber. The extract was also found to block the effect of scopolamine and diazepam induced memory deficits.

El Tabaa *et al.* (2017) found *Ginkgo biloba* extract improved cognitive impairment caused by high levels of Bisphenol A exposure. The effect may be due to an increase in the release of estrogen dependent biogenic amines which caused a reduction in hippocampal damage caused by Bisphenol A. It has also been proven that *G. biloba* enhances acquisition, storage and recall of information or memories (Winter, 1991). It has further been shown that *G. biloba* inhibits

acetylcholinesterase activity thereby increasing the release of acetylcholine in the brain leading to an improvement of learning and memory (Das *et al.*, 2002).

The work by Farshchi *et al.* (2010) demonstrated that the aqueous extract of *Boswellia papyrifera* gum improved spatial working memory in the radial arm maze and reduced escape latency as well as increase swimming speed in the Morris water maze in rats and mice.

There is however conflicting information on the effect of coconut oil in improving learning and memory. The study by (Rahim *et al.*, 2017) showed that coconut oil improved learning and memory in Wistar rats whereas the work by (Lin *et al.*, 2017) suggests that high fructose high coconut oil diet induced spatial memory deficits in rats.

2.7 XYLOPIA AETHIOPICA

Xylopiya aethiopica is found growing in Ghana, Angola, Burkina Faso, Gabon, and Ethiopia and in other parts of Africa. It has been found to contain different pure compounds that are responsible for the biological activities attributed to the plant. This includes kaurenoic acid which has anti-trypanosomic, analgesic, vaso-relaxant, diuretic, anti-pyretic and anti-inflammatory effects (Haraguchi *et al.*, 2011; Paiva *et al.*, 2002; Sosa-Sequera *et al.*, 2010). Ent-15-oxokaur-16 en-19-oic acid (EKOA) is one of the pure compounds and has anti-proliferative properties (Choumessi *et al.*, 2012). Another isolate is acetylgrandifloric acid which have been reported to have antibacterial effect (Davino *et al.*, 1989). The main isolate of *Xylopiya aethiopica* is the xylopic acid.

2.7.1 Xylopic acid

The fruit extract of *X. aethiopica* have been found to possess anti-microbial effect against gram positive and gram negative bacteria (Boakye-Yiadom *et al.*, 1977). It is also shown to be anti-plasmodial (Boampong *et al.*, 2013), analgesic (Woode *et al.*, 2012), cardiovascular, diuretic

(Somova *et al.*, 2001) and anti-inflammatory (Osafo *et al.*, 2016; Osafo *et al.*, 2018). It is also found to suppress Freund's adjuvant induced arthritis in rats (Obiri *et al.*, 2014). These effects have been attributed to the xylopic acid component of the fruits. Figure 2 shows the chemical structure of xylopic acid which is a major isolate of *X. aethiopica*.

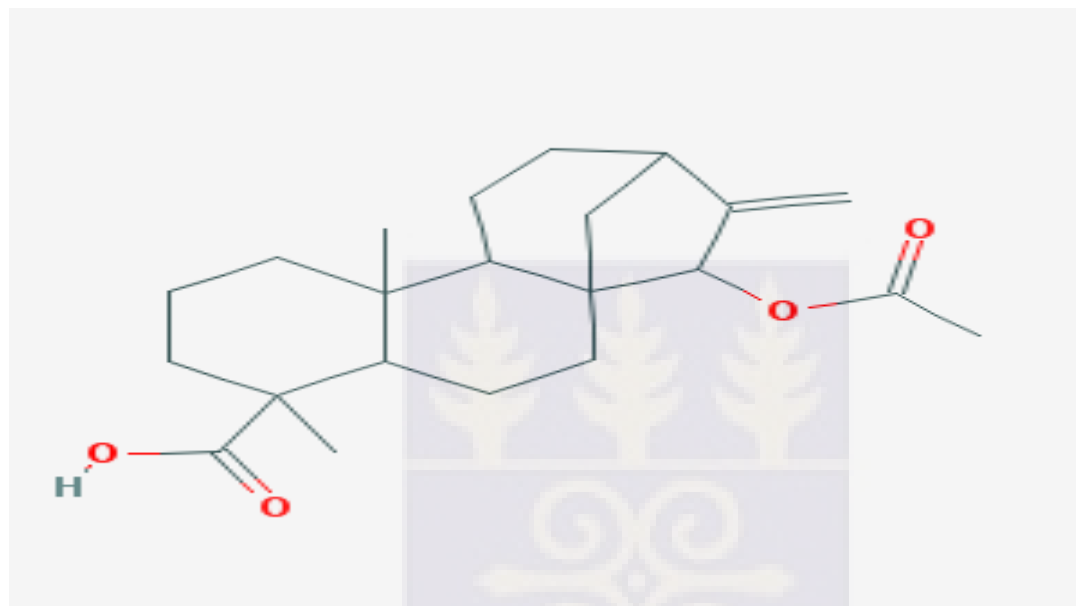


Figure 2.1: Chemical structure of xylopic acid

Castrillo *et al.* (2001) showed that xylopic acid possesses anti-inflammatory action and this involves the inhibition of inflammation signaling due to inhibition of necrosis factor kappa B action. Studies have shown that ethanolic fruit extract of *X. aethiopica* and xylopic acid has low toxicity profile (Abaidoo *et al.*, 2011; Somova *et al.*, 2001).

The central nervous system's effect of several diterpenes have been screened and determined (Chen *et al.*, 2006; Okoye *et al.*, 2013; Wasowski and Marder, 2011; Xu *et al.*, 2011). The central analgesic effects of *Xylopia aethiopica* and xylopic acid have been reported by Woode *et al.* (2012). Biney *et al.* (2016) also reported that the fruit extract of *X. aethiopica* has an anti-depressant effect. Xylopic acid is also reported to have neuro-protective effect (Biney *et al.*, 2015). Despite the number of CNS work that has been reported on the fruit extract of *X.*

aethiopica and xylopic acid none has looked at the effect on learning and memory. This work therefore seeks to evaluate the effect of the fruit extract of *X. aethiopica* and xylopic acid on learning and memory.



CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Design

The study was an experimental design.

3.2 Plant Acquisition and Authentication

Fresh ripe fruits of *Xylopia aethiopica* were collected from the Botanical Gardens of Kwame Nkrumah University of Science and Technology (KNUST) (06° 41'6.39" N; 01° 33' 45.35" W) and authenticated at the Center for Plant Medicine Research, Akuapem, Mampong with this voucher specimen number: CPMR 4888-21-06-2017.

3.3 Place and Time Experimentation

The research was carried out at the Neuropsychopharmacology laboratory, Department of Pharmacology, Korle Bu and at the Animal laboratory of the Department of Microbiology, School of Biomedical and Allied Health Sciences, University of Ghana, Korle Bu. All behavioral studies were performed in the light cycle between 7:00 am and 3:00 pm with experimentally naïve mice.

The study was approved by the College of Health Science Ethical and Protocol Review Committee, University of Ghana and was assigned a protocol identification number: CHS-Et/M.2- P1.8/2017-2018.

3.4 Preparation of Ethanolic Fruit Extract

The fruits were shade dried (for about 4 weeks) until they were brittle to break. The dried fruits were pulverized to fine powder with a hammer mill. Two (2) kilograms of the powder was

exhaustively extracted using cold maceration with 70% v/v ethanol for 72 h in a flat bottom flask. The extract was concentrated with a rotary evaporator at 60°C which produced a semi-solid mass of *Xylopiya aethiopica* extract. The semi-solid mass was dried using water bath at a temperature of 78-79 °C and then kept in a dessicator.

3.5 Isolation and Purification of Xylopic Acid

Xylopic acid was isolated and extracted using the methods described by Woode *et al.* (2012) and Biney *et al.* (2014). Approximately 0.30 kg of the powdered fruits was placed in a cylindrical jar and soaked with 2.5 L of petroleum ether and allowed to stand for 72 h period. The extract was collected and concentrated with a rotatory evaporator at 60°C. 5 ml of ethyl acetate was added to the concentrate for it to crystallize the xylopic acid and allowed to stand for 5 days. The crystals formed were washed with petroleum ether. The xylopic acid was purified by recrystallization in 96% ethanol. The concentrated solution obtained was filtered while hot and the crystals of the xylopic acid deposited at the bottom of the cylindrical jar. The purity of the xylopic acid was determined using high performance liquid chromatography (HPLC), thin layer chromatography (TLC) and melting point.

Purity of the isolated xylopic acid was determined with high performance liquid chromatography (HPLC). The chromatograph consisted of LC-10AT Shimadzu pump with programmable absorbance detector (783A Applied Biosystems) and Shimadzu CR501 Chromatopac. Phenomenex Hypersil 20-micron C18 200 × 3.20 mm column was used. The mobile phase consisted of methanol and water (9:1) eluted isocratically at 0.5 ml min⁻¹. Portions of 20 µl of a suitable concentration of pure xylopic acid were loaded and injected unto the column after dissolving in the mobile phase at 60 °C. The eluent was monitored at 206 nm. Portions of the

extract and xylopic acid were loaded and injected. The peak(s) were noted as component(s) of the extract and xylopic acid.

3.6 Phytochemical Screening of Ethanolic Fruit Extract of *Xylopic aethiopia*

The extract was screened for the presence of phytochemical constituents such as alkaloids, glycosides, tannins, sterols, flavonoids, terpenoids and saponins as described by Trease Evans (1989).

3.6.1 Test for tannins

An amount of 0.2 g of extract was boiled with 25 ml of water for 5 min, cooled and filtered. The volume was then adjusted to 25 ml. 10 ml of water was added to 1 ml aliquot of extract and 2 drops of 1% ferric chloride was added. The appearance of a blue-black or green precipitate indicated the presence of tannins.

3.6.2 Test for glycosides

An amount of 0.2 g of extract was warmed with 5 ml dilute H₂SO₄ on a water bath for 2 min. The mixture was cooled, filtered and 4 drops of 20 % NaOH was added to the filtrate. A volume of 1 ml each of Fehling's A and B solutions were added to the filtrate, warmed and observed for a red-brown precipitate.

3.6.3 Test for saponins

An amount of 0.2 g of extract was shaken vigorously with about 10 ml of water in a stoppered test tube and observed for the presence of a persistent froth.

3.6.4 Test for alkaloids

An amount 0.2 g of extract was boiled with 10 ml of dilute HCl for 5 min. The supernatant liquid was filtered into another test tube and 1 ml of the filtrate taken, into which 3 drops of

Dragendorff's reagent (potassium bismuth iodide solution) was added. The mixture was shaken and observed for the appearance of an orange spot precipitate.

3.6.5 Test for flavonoids

A volume of 10 ml of 98% ethanol was added to 0.2 g of extract. A small amount of zinc metal was added to the resulting extract followed by drop wise addition of concentrated HCl. The mixture was examined for the appearance of colours ranging from orange to red (flavones), orange to crimson (flavonols), crimson to magenta (flavonones).

3.7 Animal Handling

ICR mice (20-25 g) of both sexes were obtained from the Centre for Plant Medicine Research, Akuapem, Mampong in the Eastern Region of Ghana. The animals were kept at the animal experimentation unit of the Department of Microbiology, School of Biomedical and Allied Health Sciences, University of Ghana. The animals were housed in cages (n= 8 per cage) with wood shavings as bedding and fed with Growers Mash feed obtained from the Poultry Farmers Association of Ghana, Sakaman, Accra. The animals were given water, fed twice daily, bedding changed once daily and the temperature of the lab was at room temperature (25 °C). All the animals used were naïve and used once. The animals used in the study were handled according to the guide for the care and use of laboratory animals (NRC, 1996).

3.8 Animal Models

3.8.1 Novelty Object Recognition

The Novelty Object Recognition (NOR) test was performed according to the methods described by Ennaceur Delacour (1988) and Moscardo *et al.* (2012) with slight modifications. It measured exploratory learning and recognition memory. The animals were grouped into eleven groups of 8

animals each and starved overnight prior to training and test days but had access to water. The NOR test was made up of 3 phases. The animals were taken to the laboratory 3-4 h before the start of the test. The first phase was the habituation phase. In this phase, the mice were kept in an empty dark container which was used as the open field (dimension: 33x 33 x 20 cm). The mice were kept in the open field for 5 min twice a day with a 6 h interval for 3 consecutive days. At the end of each day's session, the container was cleaned with 70% ethanol to reduce olfactory cues. A day or 24 h after the last day of habituation, the familiarization session was performed. Two (2) identical objects were placed 20 cm apart in the open field. The mouse was then placed at the center of the open field with the head positioned opposite the object. The familiarization phase was performed for 10 min for 3 days with time taken for how long the mouse explored with the objects with the aid of a video recorder. The mice were returned to their home cages and the open field cleaned with 70% ethanol. The test session was carried out 24 h after the last familiarization day. The animals were given oral (p.o) doses of the fruit extract of *Xylopi aethiopica* (30, 100, 300 mg/kg), xylopic acid (30, 100, 300 mg/kg), piracetam (Pct) (30, 100, 300 mg/kg), vehicle and intraperitoneal (i.p) administration of ketamine (30 mg/kg) for 1 h. After an hour, two objects one familiar and the other different or new in terms of shape, size and color were kept in the open field and the animals were allowed to explore with the objects for 5 min. The test session was carried out for 2 days. Time taken to explore with the new object was recorded with the aid of a video recorder. The animals were returned to their home cages and the open field was cleaned with 70% ethanol at the end of each session.

3.8.2 Spontaneous Alternation Y-maze

The test was performed according to the description by Choi Choi (2016) and Fu *et al.* (2008) but with slight modifications. The test measured spatial working memory and recognition

memory. The Y-maze setup consisted of 3 arms labeled A, B and C. The arms were interconnected at 120°. The animals were divided into eleven groups and starved overnight prior to training and test days. In the training stage all 3 arms of the maze were left open. The mice were kept on the end of one of the arms of the maze and allowed to explore freely for 5 min. The start positions were alternated during the training period of 5 days. The spontaneous alternation behavior was calculated as the number of entries into all 3 arms divided by the total number of visits. Entry was defined as entry of the whole body of the mouse into the arm. The mice were returned to their home cages at the end of each session. The maze was cleaned with 70% ethanol at the end of every session. The test session was performed 24 h after the last training day. An hour before the test session, the mice were given oral doses of *X. aethiopica* (30, 100, 300 mg/kg), xylopic acid (30, 100, 300 mg/kg), citicoline (Citi) (30, 100, 300 mg/kg), saline and intraperitoneal administration of ketamine (Ket) (30 mg/kg). The test was done for 3 consecutive days. The test session consisted of 2 trials. The first trial measured working memory in the mice by scoring the number of alternations the mouse made in the Y-maze when one arm of the maze was blocked. This was done for 5 min. 10-15 min after the first trial, the second trial was performed and the partition used in blocking the arm in the first trial was removed and the mouse allowed to explore freely for 2 min. Spatial recognition memory was measured during this trial. If working memory was intact in the animal, it explored more in the arm that was previously blocked in the first trial. The animals were then returned to their home cages and maze cleaned with 70% ethanol.

3.8.3 Morris Water Maze

This was carried out using the procedures described by Morris *et al.* (1986); Sun Alkon (2004) and Nunez (2008) with slight modifications. The model studied the effect on hippocampally-

dependent spatial/ place learning and working memory. The Morris Water Maze (MWM) test was carried out using 85 cm diameter pool with a temperature of 22°C. The maze was divided into 4 quadrants. The mice were grouped into eleven groups of 8 animals each and were starved overnight prior to training and test days. The animals were taken to the laboratory 3-4 h before behavioural testing. The mice were trained on the first day to locate a visible platform which was placed 1 cm above the water surface. The mouse was placed on the visible platform for 20 s for orientation. After the 20 s the mouse was lowered gently into the water to swim and locate the platform for 60 s. Mice that located the platform before 60 s were removed whereas those that did not were guided to the platform and allowed to re-orient to the distal cues for an additional 20 s. The mice were then removed and dried. This was repeated for two more trials after a 30 min inter- trial interval. The animals were then dried and returned to their home cage. On the testing days the animals were given oral administration of *X. aethiopica* (30, 100, 300 mg/kg), xylopic acid (30, 100, 300 mg/kg), citicoline (30, 100, 300 mg/kg), saline and intraperitoneal administration of ketamine (30 mg/kg). This was done an hour before the test after which a non-toxic paint of non- fat dry milk was used to make the platform invisible. The platform was kept 2 cm below the water surface. The mice were kept in the water facing the maze wall with different start positions being used. The mice were allowed to swim for 60 s. The trial ended for a mouse if it located the platform within the 60 s. Those that did not locate the platform within the 60 s were guided to it. The mice were dried and returned to their home cage. The testing was repeated for another trial within the day. The test was done for 4 days. On day 6, the probe trial was performed. In this trial, the platform was removed from the pool and the animals were allowed to swim for 60 s to determine the animal's understanding of the platform location. The number of

crosses to the center of the pool was recorded during the 60 s with the aid of a video camera. The mice were then dried and returned to their home cages.

3.9 Test of Possible Mechanism(s) of Action

3.9.1 Involvement of the Cholinergic Receptor Pathway

The mice were divided into 11 groups of 7 animals each. They were pretreated with scopolamine (1 mg/kg i.p.). After 60 min, groups I-III received oral doses of the fruit extract of *Xylopi aethiopica* (30, 100 and 300 mg/kg), groups IV- VI received oral doses of xylopic acid (30, 100 and 300 mg/kg). Groups VII-IX received oral doses of citicoline (30, 100 and 300 mg/kg). Group X received only scopolamine (1 mg/kg) while group XI received saline. The reversal or non-reversal effect on learning and memory of the fruit extract of *Xylopi aethiopica*, xylopic acid and citicoline were determined using the Y-maze and the NOR tests.

3.9.2 Involvement of the GABAergic Receptor Pathway

The mice were divided into 11 groups of 7 animals each. They were pretreated with diazepam (1 mg/kg i.p.). After 60 min, groups I-III received oral doses of the fruit extract of *Xylopi aethiopica* (30, 100 and 300 mg/kg), groups IV- VI received oral doses of xylopic acid (30, 100 and 300 mg/kg). Groups VII-IX received intraperitoneal doses of flumazenil (0.3, 1 and 3 mg/kg). Group X received only diazepam (1 mg/kg i.p.) while group XI received saline. The reversal or non-reversal effect on learning and memory of the fruit extract of *Xylopi aethiopica*, xylopic acid and flumazenil were determined using the Y-maze and the NOR tests.

3.10 Statistical Analysis

GraphPad Prism for windows version 5.0 (GraphPad Software, San Diego, CA, USA) was used for data and statistical analysis and $P < 0.05$ was considered statistically significant. The time-

course curves were subjected to two-way (treatment \times time) repeated measures analysis of variance (ANOVA) with Bonferroni's post hoc test. Percentage exploration with objects, percentage alternation, and change in time taken to find hidden platform for each treatment were calculated in arbitrary unit as the area under the curve (AUC). Differences in AUCs were analysed by one-way ANOVA followed by Newman-Keuls' post hoc test.



CHAPTER FOUR

RESULTS

4.1 Phytochemical test

Phytochemical screening of the ethanolic (70%) extract of *Xylopi aethiopica* revealed the presence of alkaloids, saponins, flavonoids and glycosides.

Table 4.1: Preliminary phytochemical screening of the ethanolic fruit extract of *Xylopi aethiopica*

Constituent(s)	Inference
Alkaloids	Present
Saponins	Present
Tannins	Absent
Flavonoids	Present
Glycosides	Absent

4.2 High performance liquid chromatography and determination of some properties of xylopic acid

HPLC was further used for the determination of the purity of the isolated xylopic acid. Several peaks were observed after loading the extract indicating the presence of several compounds in the fruits (Fig. 4.1a). A single peak was observed for xylopic acid indicating the presence of a single compound (Fig. 4.1b). The purity of xylopic acid was determined to be 96%. Mass spectroscopic and nuclear magnetic resonance analyses indicated that the compound isolated was xylopic acid.

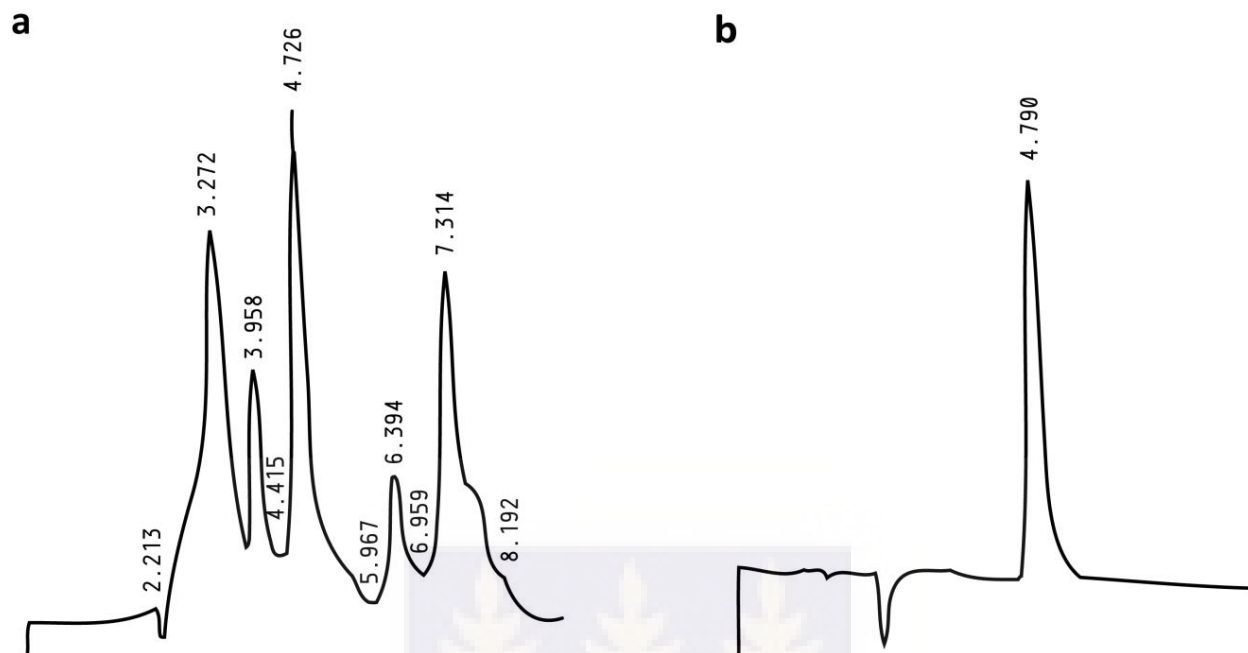


Figure 4.1 HPLC fingerprint of (a) extract showing several peaks of the various compounds in the extract and (b) xylopic acid showing a single peak.

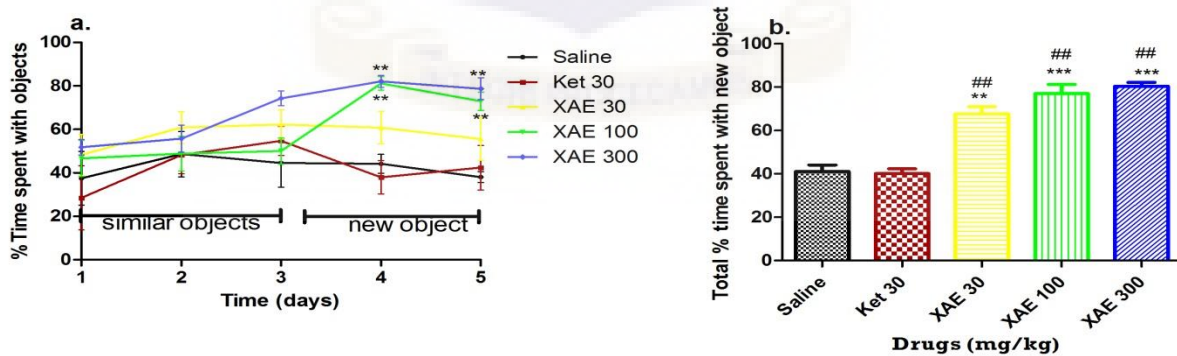
4.3 Improvement in Learning and Memory of *Xylopic acid* and xylopic acid

4.3.1 Novelty Object Recognition test

Effect of extract of Xylopic acid (XAE) and xylopic acid (XA) treatment on percentage time spent with novel object

From the time course curve, XAE (100 and 300 mg/kg) significantly increased the percentage time spent with the novel object ($F_{4, 16} = 10.62, P < 0.0001$). The effect of XAE increased on day 4 which was the first day of treatment (Figure 4.2 a) and the effect was sustained till day 5 which was the second day of treatment. From the time course curve of XA (30-300 mg/kg) significantly increased the percentage time spent with the novel object in a dose dependent fashion ($F_{4, 16} = 9.469, P < 0.0001$). The effect of XA increased on day 4 which was the first day of treatment (Figure 4.2 c) and the effect decreased on day 5 for the XA 100 and XA 300 mg/kg but the effect

was sustained for the XA 30 mg/kg which was the second day of treatment. From the time course curve of piracetam (Pct) (30-300 mg/kg) there was a slight increase in the percentage time spent with the novel object but was not significant ($F_{4, 16} = 1.461, P=0.2160$). The effect increased slightly on day 4 which was the first day of treatment but this was not significant (Figure 4.2 e) and the effect was sustained on day 5 which was the second day of treatment. XAE ($F_{4,5} = 42.09, P=0.0005$) XA ($F_{4,5} = 26.66, P=0.0014$) and Pct ($F_{4,5} = 10.69, P=0.0115$) significantly increased the percentage time spent with the novel object in mice exposed to the novelty object recognition test as shown in the area under the curve (AUC) in figure 4.2b, figure 4.2d and figure 4.2f indicating a significant improvement in exploratory learning and recognition memory in comparison to saline and ketamine. Ketamine (Ket) treatment did not have any effect on the percentage time spent with the novel object in comparison to the saline group.



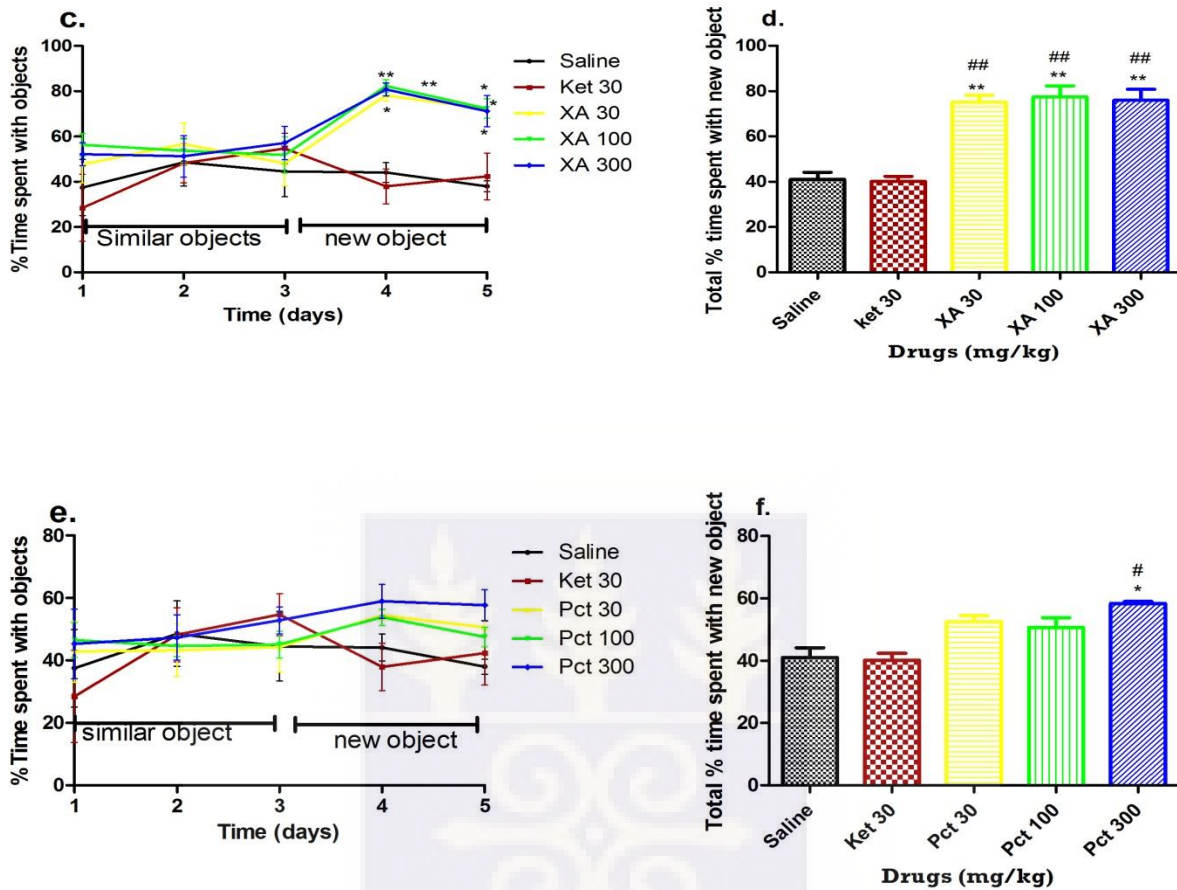


Figure 4.2: Effects of XAE (30-300 mg/kg) and XA (30-300 mg/kg) treatment on the percentage time spent with novel object in the NOR test. Data are presented as (a, c, e) a time course graph and (b, d, f) Mean \pm SEM of their areas under the curves (AUCs). Significantly different from the control: * $P < 0.05$, ** $P < 0.01$ by Bonferroni's test and $P < 0.05$ by Newman-Keuls test. Significantly different from Ketamine: # $P < 0.05$, ## $P < 0.01$ and # $P < 0.0001$ by Newman-Keuls test.

4.3.2 Spontaneous Alternation Y-maze

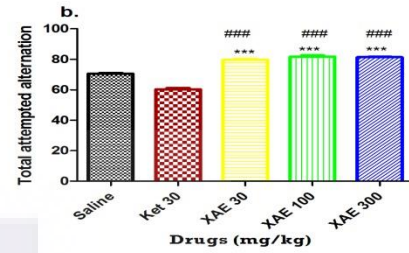
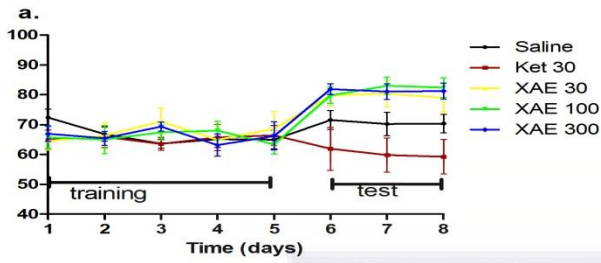
4.3.2.1 Spatial working memory

Effect of extract of Xylopiya aethiopia (XAE) and xylopic acid (XA) treatment on percentage alternation in the arms of the Y-maze (measuring working memory)

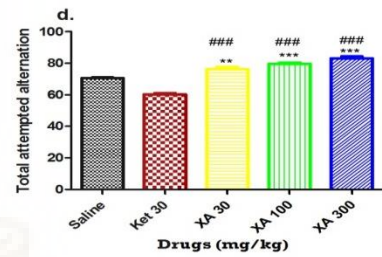
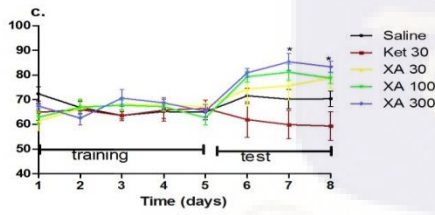
From the time course curve, XAE (30-300 mg/kg) increased the percentage attempted alternation in the Y-maze ($F_{4, 28} = 7.841$, $P < 0.0001$). The effect of XAE increased on day 6 which was the first day of treatment and sustained on day 7 which was the second day of treatment, but the effect was not significant and by the 8th day the effect had decreased (Figure 4.3 a). From the time course curve, XA (30-300 mg/kg) significantly increased percentage of attempted alternation in blocked arm of the Y-maze ($F_{4,28} = 8.367$, $P < 0.0001$). The effect of XA increased on day 6 which was the first day of treatment (Figure 4.3 c) and the effect further increased significantly on day 7 which was the second day of treatment but decreased on day 8. From the time course curve of Citi (30-300 mg/kg), there was a very slight increase in the percentage attempted alternation in Y-maze but was not significant ($F_{4, 28} = 3.279$, $P = 0.0120$). The effect increased very slightly on day 6 which was the first day of treatment but this was not significant (Figure 4.3 e) and the effect decreased slightly on day 7 and maintained on day 8 which was the third day of treatment. XAE ($F_{4,10} = 207.8$, $P < 0.0001$) and XA ($F_{4,10} = 81.97$, $P < 0.0001$) significantly increased the percentage attempted alternation in the blocked arm of the Y-maze in mice exposed spontaneous Y-maze test, indicating a significant improvement in spatial working memory and Citi ($F_{4, 10} = 67.11$, $P < 0.0001$) also increased the percentage attempted alternation in the Y-maze. This is seen in the area under the curve (AUC) in figure 4.3b, figure 4.3d and figure 4.3f for XAE, XA and Citi respectively. The XAE showed a significant increase in percentage attempted alternation in a dose independent fashion as seen in figure 4.3b, XA showed significant increase in percentage attempted alternation in the blocked arm of the Y-maze in a dose dependent fashion with XA 300 mg/kg showing the highest percentage attempted alternation in the arms of the Y-maze as seen in figure 4.3d, figure 4.3f shows that Citi showed

an increased percentage attempted alternation in a dose independent manner in comparison to the saline group and ketamine. Ketamine treatment had no significant effect in comparison to saline.

% attempted alternation in the arms of the Y-maze



% attempted alternation in the arms of the Y-maze



% attempted alternation in the arms of the Y-maze

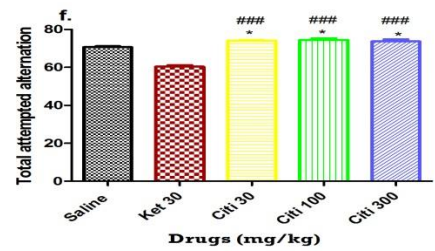
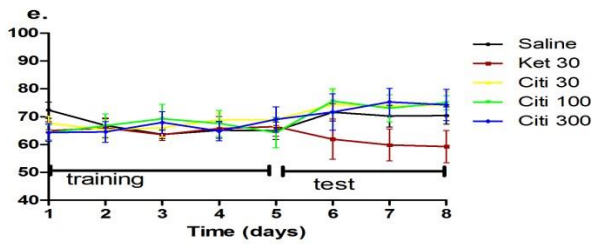


Figure 4.3: Effects of XAE (30-300 mg/kg) and XA (30-300 mg/kg) treatment on the percentage attempted alternation in the blocked arm of the Y-maze in the spontaneous alternation Y-maze test measuring spatial working memory. Data are presented as (a, c, e) a time course graph and (b, d, f) Mean \pm SEM of their areas under the curves (AUCs). Significantly different from the saline: * $P < 0.05$ ** $P < 0.01$ by Bonferroni's test and $P < 0.05$ by Newman-Keuls test. Significantly different from Ketamine: # $P < 0.05$, ## $P < 0.01$ and ### $P < 0.0001$ by Newman-Keuls test.

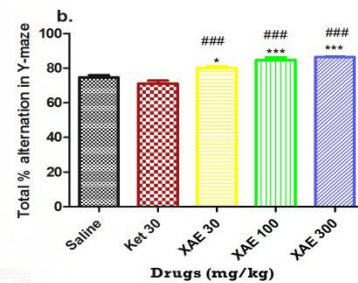
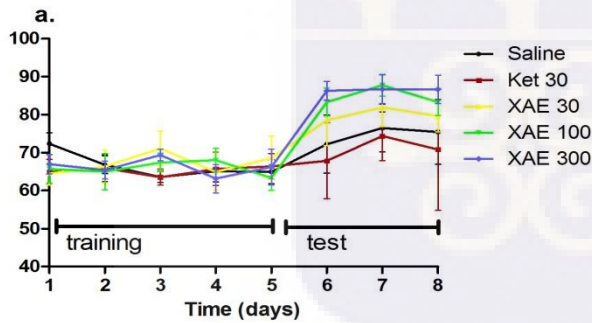
4.3.2.2 Spatial recognition memory

Effect of extract of Xylopiya aethiopica (XAE) and xylopic acid (XA) treatment on percentage alternation in the arms of the Y-maze (measuring spatial recognition memory).

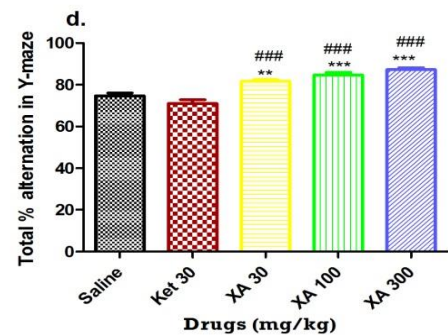
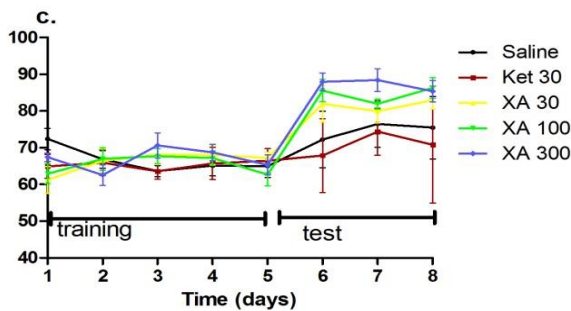
From the time course curve, XAE (30-300 mg/kg) increased the percentage alternation in the Y-maze ($F_{4, 28} = 2.113$, $P = 0.0794$). The effect of XAE increased slightly but was not significant on day 6 which was the first day of treatment (Figure 4.4a) and the effect was sustained till day 8 which was the third day of treatment. From the time course curve, XA (30-300 mg/kg) increased percentage alternation in arms of the Y-maze when the arm was opened ($F_{4,28} = 2.850$, $P = 0.0243$). The effect of XA increased on day 6 which was the first day of treatment (Figure 4.4c) and the effect was sustained on day 7 which was the second day of treatment and by day 8 or the third day of treatment it had decreased. From the time course curve of Citi (30-300 mg/kg) there was a very slight increase in the percentage alternation in Y-maze but was not significant ($F_{4, 28} = 1.441$, $P = 0.2206$). The effect increased very slightly on day 6 which was the first day of treatment but this was not significant (Figure 4.4e) and the effect decreased till day 8 which was the third day of treatment. XAE ($F_{4,10} = 25.79$, $P < 0.0001$), XA ($F_{4,10} = 26.74$, $P < 0.0001$), significantly increased the percentage alternation in the opened arm of the Y-maze in mice exposed spontaneous Y-maze test, indicating a significant improvement in spatial

recognition memory and Citi ($F_{4, 10} = 9.443, P = 0.0020$) also increased the percentage alternation in the Y-maze. This is seen in the area under the curve (AUC) in figure 4.4b, figure 4.4d and figure 4.4f for XAE, XA and Citi respectively. The XAE showed significant increase in percentage alternation in the open arm of the Y-maze in a dose dependent fashion as seen in figure 4.4b, XA showed an increase in percentage alternation in a dose dependent fashion as seen in figure 4.4d, figure 4.4f shows that Citi showed an increased percentage alternation in a dose independent manner in comparison to the saline and ketamine. The ketamine had no significant treatment in comparison to saline.

% alternation in the arms of the Y-maze



% alternation in the arms of the Y-maze



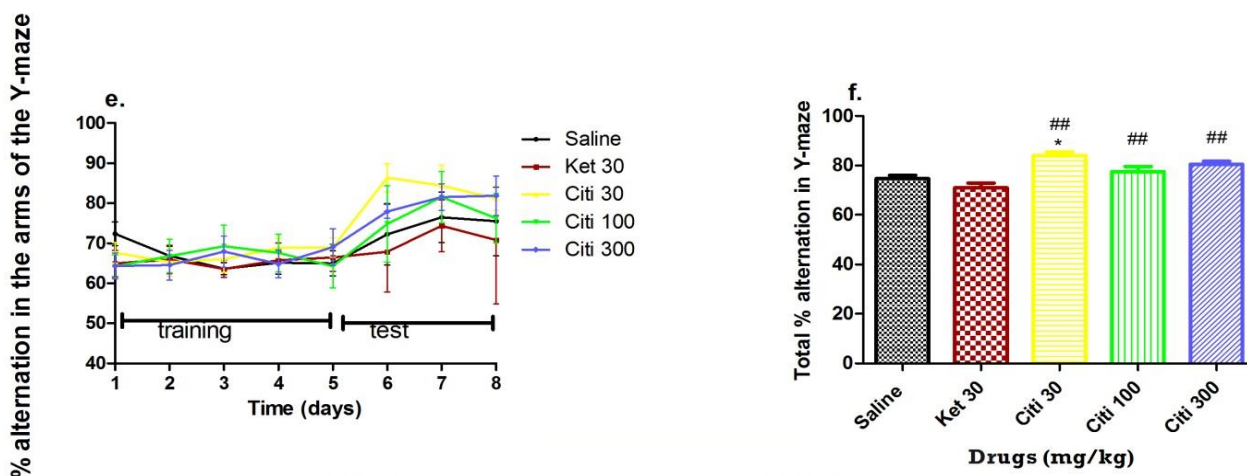


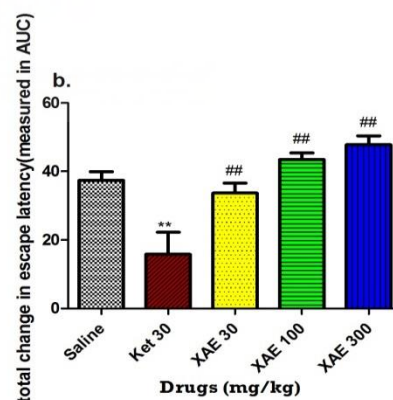
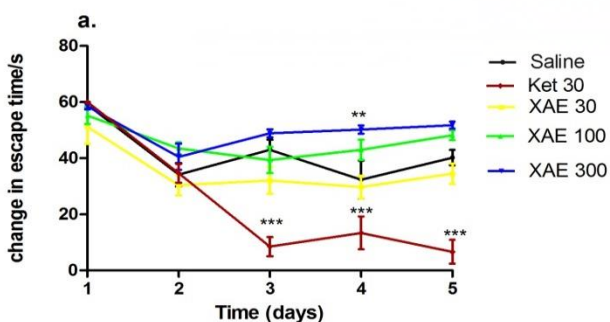
Figure 4.4: Effects of XAE (30-300 mg/kg) and XA (30-300 mg/kg) treatment on the percentage alternation in the previously blocked arm of the Y-maze in the spontaneous alternation Y-maze test measuring spatial recognition memory. Data are presented as (a, c, e) a time course graph and (b, d, f) Mean \pm SEM of their areas under the curves (AUCs). Significantly different from the saline: * $P < 0.05$, ** $P < 0.01$ by Bonferroni's test and $P < 0.05$ by Newman-Keuls test. Significantly different from Ketamine: # $P < 0.05$, ## $P < 0.01$ and ### $P < 0.0001$ by Newman-Keuls test.

4.3.3 Morris Water Maze

Effect of extract of Xylopiya aethiopic (XAE) and xylopic acid (XA) treatment on change in escape time

From the time course curve, XAE (30-300 mg/kg) significantly increased the change in escape latency or decreased the escape time on the 4th day or 3rd day of treatment but decreased slightly on day 5 ($F_{4, 16} = 35.73$, $P < 0.0001$) (Figure 4.5 a). From the time course curve, XA (30-300 mg/kg) increased the escape latency or decreased the change in escape latency ($F_{4,16} = 16.70$, $P < 0.0001$). The effect of XA slightly decreased the change in escape latency on day 2 which was

the first day of treatment (Figure 4.5 c) and the effect increased slightly on day 3 and 4 but was not significant it however decreased slightly on the 5th day which was the 4th day of treatment. From the time course curve of Citi (30-300 mg/kg), there was a significant increase in the change in escape latency on day 3 or day 2 of treatment but by day 4 it had decreased and increased again on day 5 ($F_{4,16} = 14.00, P < 0.0001$) (Figure 4.5 e). XAE ($F_{4,15} = 11.59, P = 0.0002$), XA ($F_{4,15} = 6.229, P = 0.0037$) did not increase the change in escape latency in mice exposed to the Morris water maze test, indicating the inability to improve spatial learning and memory and Citi ($F_{4,15} = 6.229, P = 0.0037$) did not increase the change in escape latency. This is seen in the area under the curve (AUC) in figure 4.5b, figure 4.5d and figure 4.5f for XAE, XA and Citi respectively. The XAE did not show a significant increase in change in escape latency as seen in figure 4.5b, XA did not show a significant increase in change in escape latency as seen in figure 4.5d, figure 4.5f shows that Citi 300 mg/kg did not show a significant increase in escape latency in comparison to the saline group but did with ketamine. The ketamine had a significant decrease in change in escape latency and this implies that spatial learning was impaired in comparison to saline.



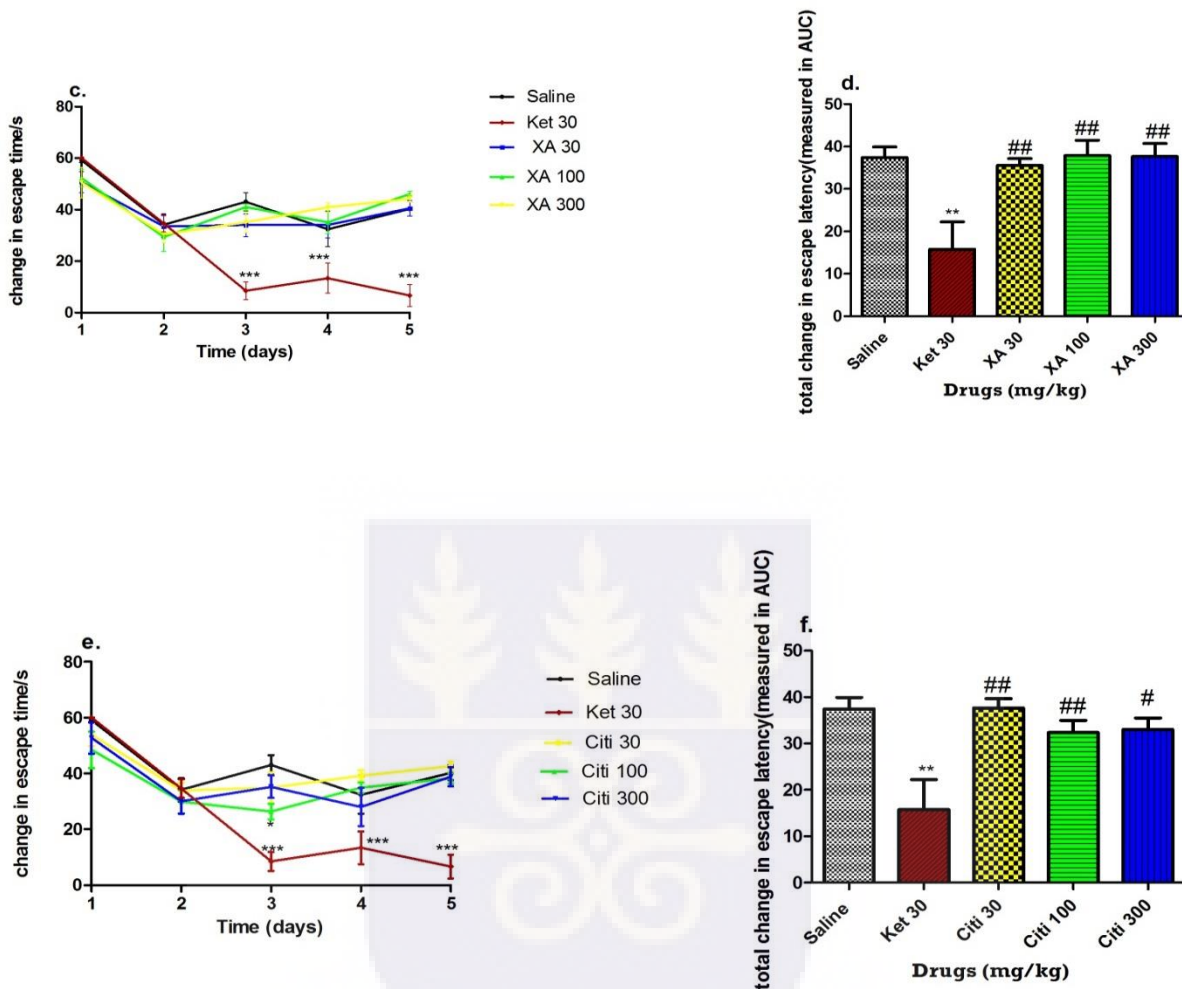


Figure 4.5: Effects of XAE (30-300 mg/kg) and XA (30-300 mg/kg) treatment on change in escape latency in the Morris water maze test measuring spatial learning and memory. Data are presented as (a, c, e) a time course graph and (b, d, f) Mean \pm SEM of their areas under the curves (AUCs). Significantly different from the saline: * $P < 0.05$, ** $P < 0.01$ by Bonferroni's test and $P < 0.05$ by Newman-Keuls test. Significantly different from Ketamine: # $P < 0.05$, ## $P < 0.01$ and ### $P < 0.0001$ by Newman-Keuls test.

4.3.3.2 Probe trial of the Morris water maze test

In the probe trial of the MWM test, XAE ($F_{4, 35} = 20.90$, $P < 0.0001$), XA ($F_{4, 35} = 21.40$, $P < 0.0001$), and Citi ($F_{4, 35} = 6.114$, $P = 0.0008$), all significantly increased in the percentage frequency in comparison to saline and ketamine when the platform was removed from the maze as seen in figure 4.6. However, ketamine did not show any significant effect in comparison to saline. This indicates that the reference memory was improved in the probe trial of the MWM test.

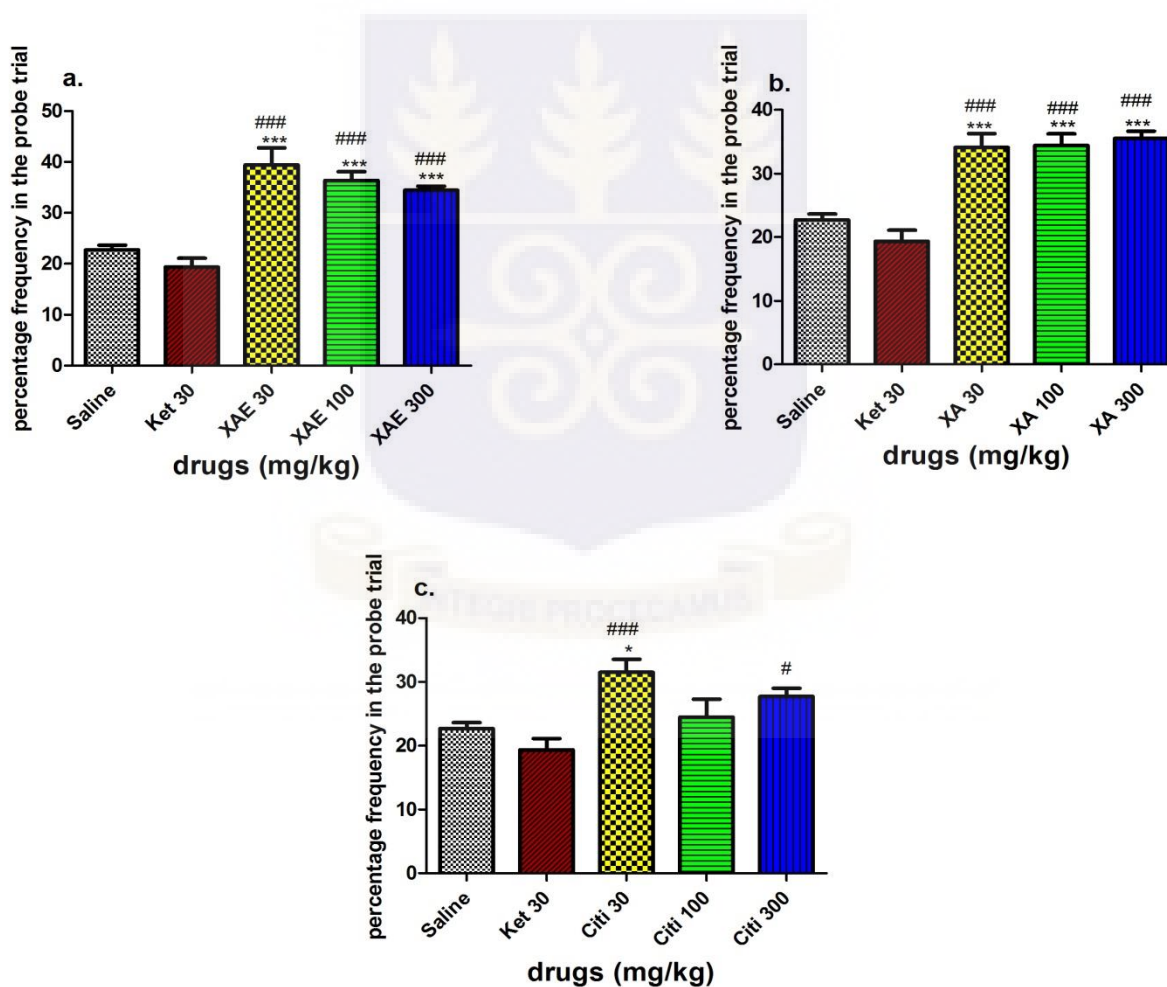


Figure 4.6: Effects of XAE (30-300 mg/kg) and XA (30-300 mg/kg) percentage frequency of probe trial in the Morris water maze test measuring reference memory. Data are presented as (a,

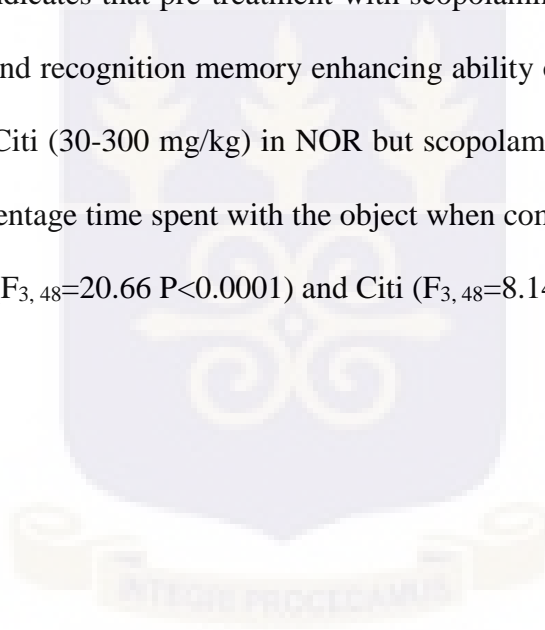
b, c) Mean \pm SEM. Significantly different from the saline: $P < 0.05$ by Newman-Keuls test. Significantly different from Ketamine: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.0001$ by Newman-Keuls test.

4.4 Possible Mechanisms of Action

4.4.1 Involvement of Cholinergic system- Scopolamine pre-treatment

4.4.1.1 Novelty object recognition test

Results from figure 4.6 indicates that pre-treatment with scopolamine (1 mg/kg) did not reverse the exploratory learning and recognition memory enhancing ability of the XAE (30-300 mg/kg), XA (30-300 mg/kg) and Citi (30-300 mg/kg) in NOR but scopolamine only group did not show any difference in the percentage time spent with the object when compared with saline. XAE ($F_{3, 48} = 26.76$ $P < 0.0001$), XA ($F_{3, 48} = 20.66$ $P < 0.0001$) and Citi ($F_{3, 48} = 8.143$ $P < 0.0002$).



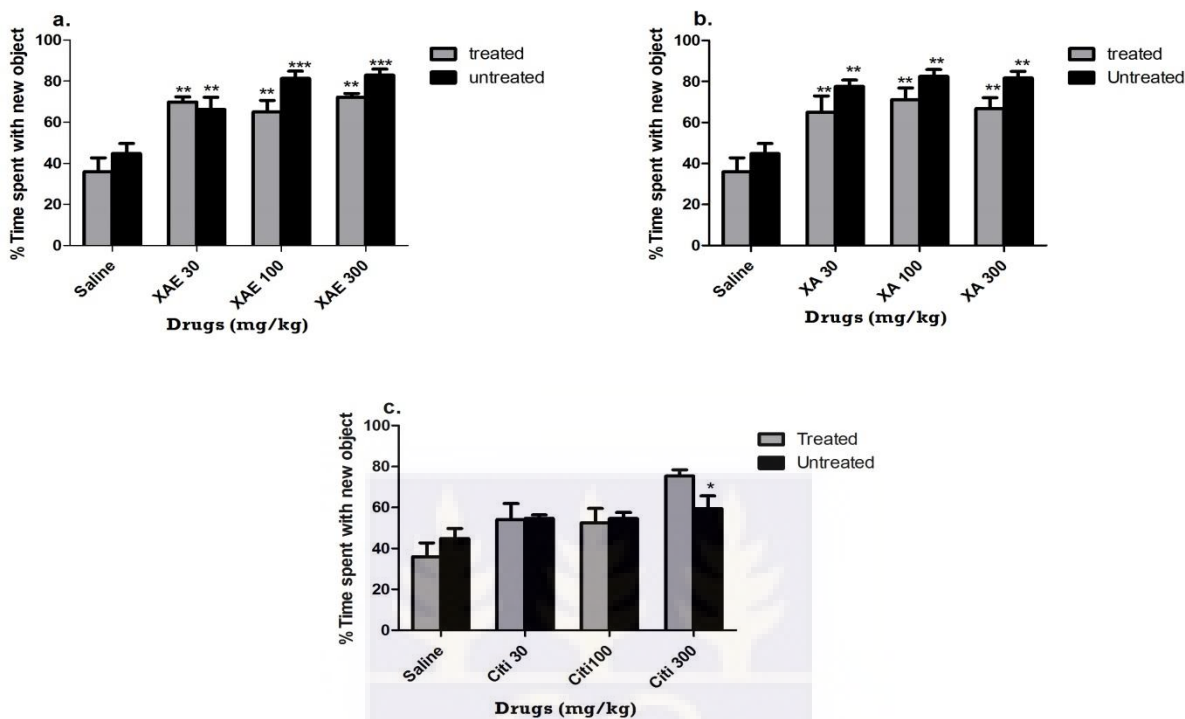


Figure 4.7: Effects of pre-treatment of mice with scopolamine (1 mg/kg) on percentage time spent with new object of XAE (30, 100 and 300 mg/kg) and XA (30, 100 and 300 mg/kg) in NOR. Data are represented as group Means \pm SEM of 7 animals. Significantly different from saline: *** $P < 0.0001$; ** $P < 0.01$; * $P < 0.05$ (One-way ANOVA followed by Newman-Keuls test).

4.4.1.2 Spontaneous alternation Y-maze test

4.4.1.2.1 Spatial working memory

Results from figure 4.8 indicates that pre-treatment of mice with scopolamine (1 mg/kg) did not reverse the spatial working memory enhancing ability of the XAE (30-300 mg/kg), XA (30-300 mg/kg) and Citi (30-300 mg/kg) in Y-maze but scopolamine only group did not show any difference in the percentage attempted alternation when one of the arms of the maze was blocked

in comparison to saline. XAE ($F_{3, 48}=15.09$ $P<0.0001$), XA ($F_{3, 48}=14.00$ $P<0.0001$) and Citi ($F_{3, 48}=21.62$ $P<0.0001$).

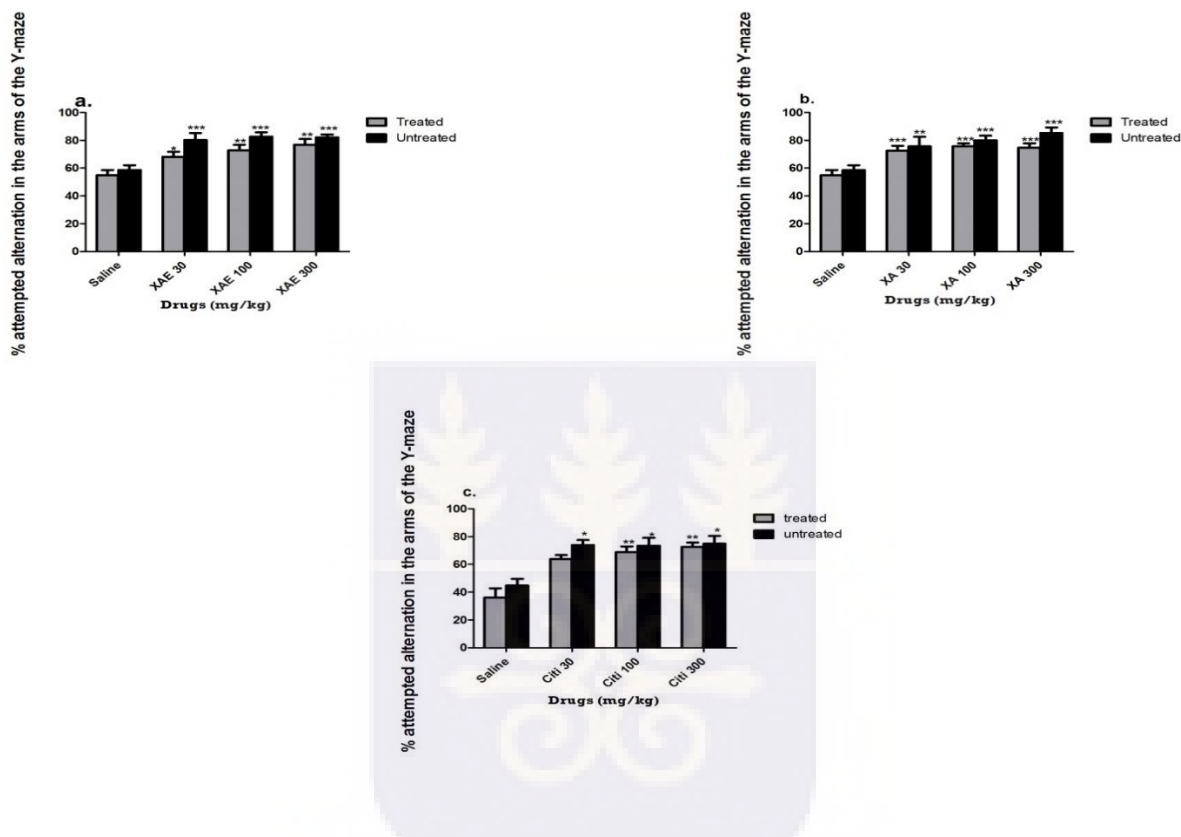


Figure 4.8: Effects of pre-treatment of mice with scopolamine (1 mg/kg) alone on percentage attempted alternation of XAE (30, 100 and 300 mg/kg) and XA (30,100 and 300 mg/kg) in Y-maze. Data are represented as group Means \pm SEM of 7 animals. Significantly different from saline: ***P < 0.0001; **P < 0.01; *P < 0.05 (One-way ANOVA followed by Newman-Keuls test).

4.4.1.2.2 Spatial recognition memory

Results from figure 4.9 indicates that pre-treatment of mice with scopolamine (1 mg/kg) did not reverse the spatial recognition memory enhancing ability of the XAE (30-300 mg/kg), XA (30-300 mg/kg) and Citi (30-300 mg/kg) in Y-maze but scopolamine only group did not show any

difference in the percentage alternation when the previously blocked arm was opened in comparison to saline. XAE ($F_{3, 48}=6.084$ $P=0.0014$), XA ($F_{3, 48}=7.269$ $P=0.0004$) and Citi ($F_{3, 48}=2.766$ $P=0.0519$).

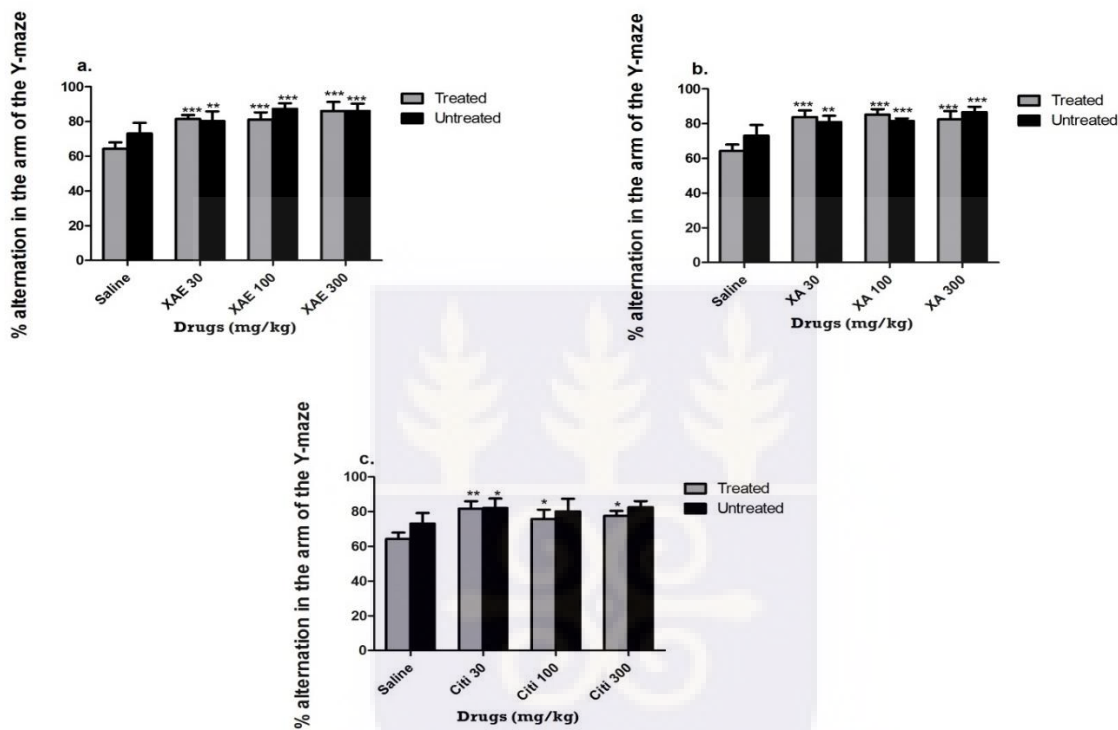


Figure 4.9: Effects of pre-treatment of mice with scopolamine (1 mg/kg) on percentage alternation of XAE (30, 100 and 300 mg/kg) and XA (30, 100 and 300 mg/kg) in Y-maze. Data are represented as group Means \pm SEM of 7 animals. Significantly different from saline: *** $P<0.0001$; ** $P<0.01$; * $P<0.05$ (One-way ANOVA followed by Newman-Keuls test).

4.4.2 Involvement of GABAergic system- Diazepam pretreatment

4.4.2.1 Novelty object recognition

Results from figure 4.10 demonstrates a reversal of percentage time spent with new object of the XAE (30-300 mg/kg), XA (30-300 mg/kg) and Flumazenil (Flu) (0.3-3 mg/kg) when pre-treated

with diazepam (1 mg/kg) in NOR but diazepam only group did not show any difference in the percentage time spent with new object in comparison to saline. XAE ($F_{3, 48}=49.51$ $P<0.0001$), XA ($F_{3, 48}=46.09$ $P<0.0001$) and Flu ($F_{3, 48}=19.50$ $P<0.0001$).

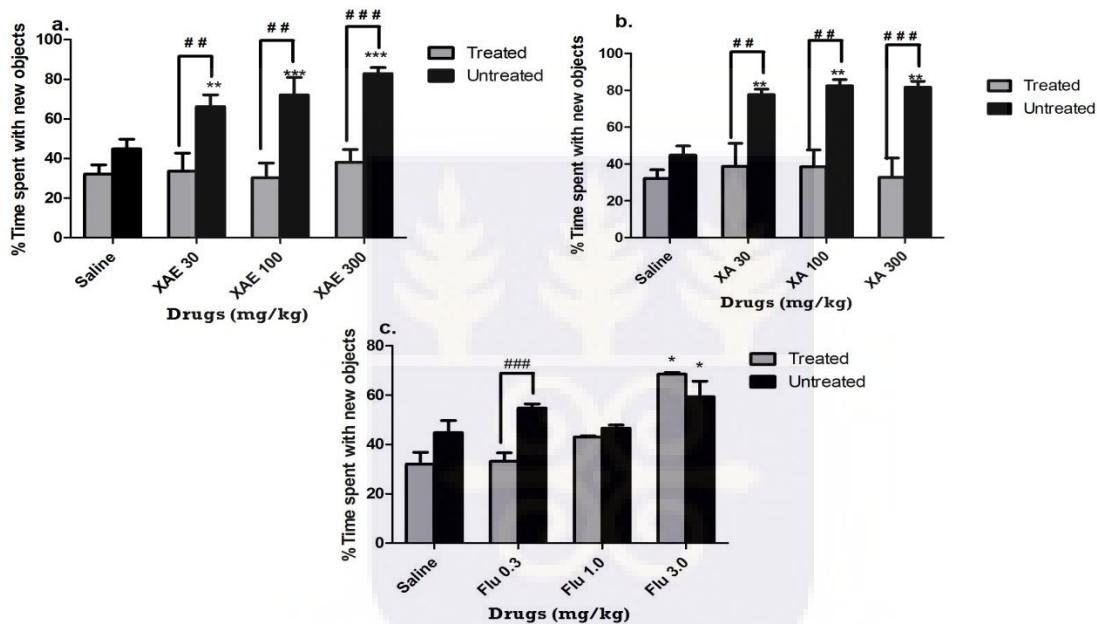


Figure 4.10: Effects of pre-treatment of mice with diazepam (1 mg/kg) on percentage time spent with new object of XAE (30, 100 and 300 mg/kg) and XA (30, 100 and 300 mg/kg) in NOR. Data are represented as group Means \pm SEM of 7 animals. Significantly different from saline: *** $P<0.0001$; ** $P<0.01$; * $P<0.05$ (One-way ANOVA followed by Newman-Keuls test). ### $P<0.0001$; ## $P<0.01$; # $P<0.05$; significant difference between treatment and dose (One-way ANOVA with Newman-Keuls test).

4.4.2.2 Spontaneous Alternation Y-maze test

4.4.2.2.1 Spatial working memory

Results from figure 4.11 indicates that pre-treatment of mice with diazepam (1 mg/kg) reversed the spatial working memory enhancing ability of the XAE (30-300 mg/kg), XA (30-300 mg/kg) and Flu (0.3-3 mg/kg) in Y-maze but diazepam only group did not show any difference in the percentage attempted alternation when one of the arms of the maze was blocked in comparison to saline. XAE ($F_{3, 48}=27.15$ $P<0.0001$), XA ($F_{3, 48}=19.78$ $P<0.0001$) and Flu ($F_{3, 48}=5.521$ $P=0.0229$).

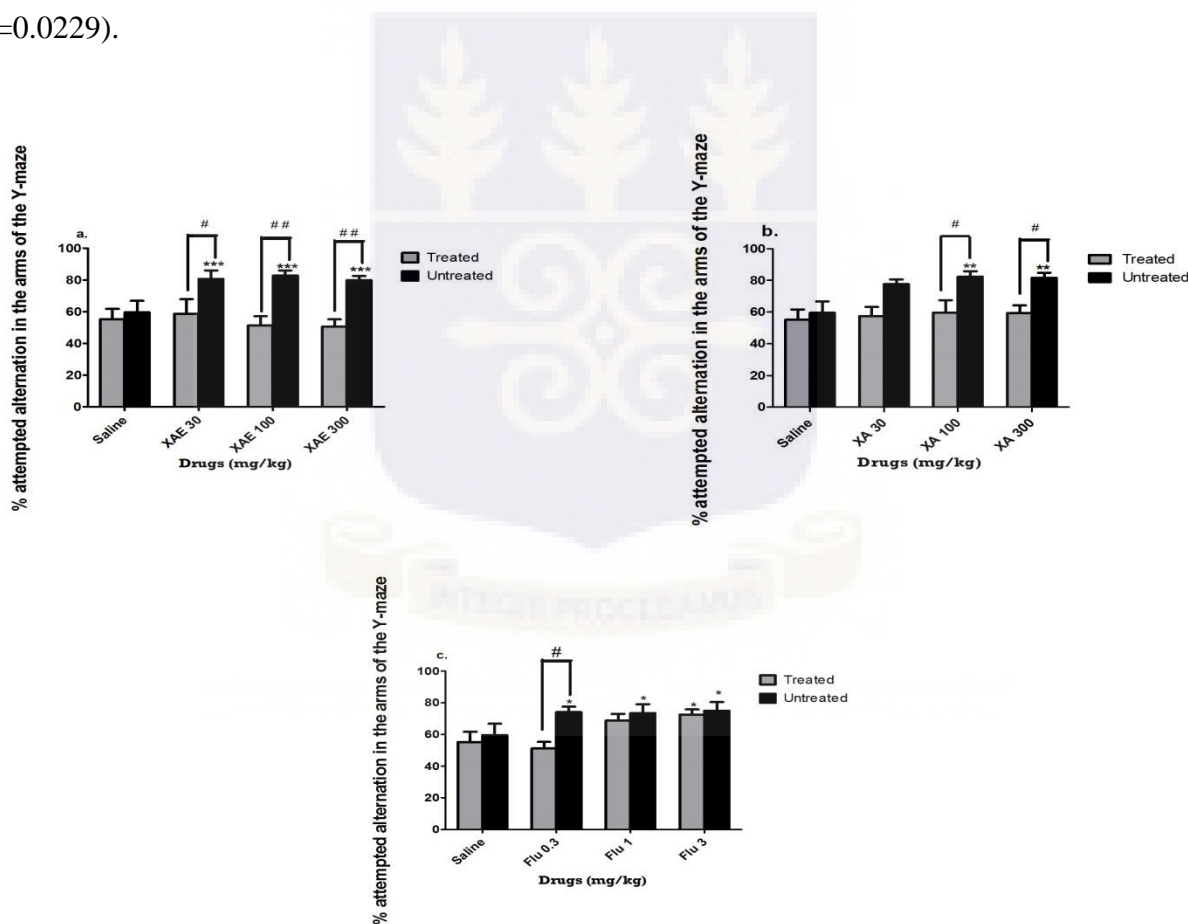


Figure 4.11: Effects of pre-treatment of mice with diazepam (1 mg/kg) on percentage attempted alternation of XAE (30, 100 and 300 mg/kg) and XA (30, 100 and 300 mg/kg) in Y-maze. Data are represented as group Means \pm SEM of 7 animals. Significantly different from saline: *** $P <$

0.0001; ** P < 0.01; * P < 0.05 (One-way ANOVA followed by Newman-Keuls test). ### P < 0.0001; ## P < 0.01; #P < 0.05; significant difference between treatment and dose (One-way ANOVA with Newman-Keuls test).

4.4.2.2.2 Spatial recognition memory

Results from figure 4.12 indicates that pre-treatment of mice with diazepam (1 mg/kg) reversed the spatial recognition memory enhancing ability of the XAE (30-300 mg/kg), XA (30-300 mg/kg) and Flu (0.3-3 mg/kg) in Y-maze but diazepam only group did not show any difference in the percentage alternation when the previously blocked arm was opened in comparison to saline. XAE ($F_{3, 48}=37.95$ P<0.0001), XA ($F_{3, 48}=18.30$ P<0.0001) and Flu ($F_{3, 48}=7.461$ P=0.0088).

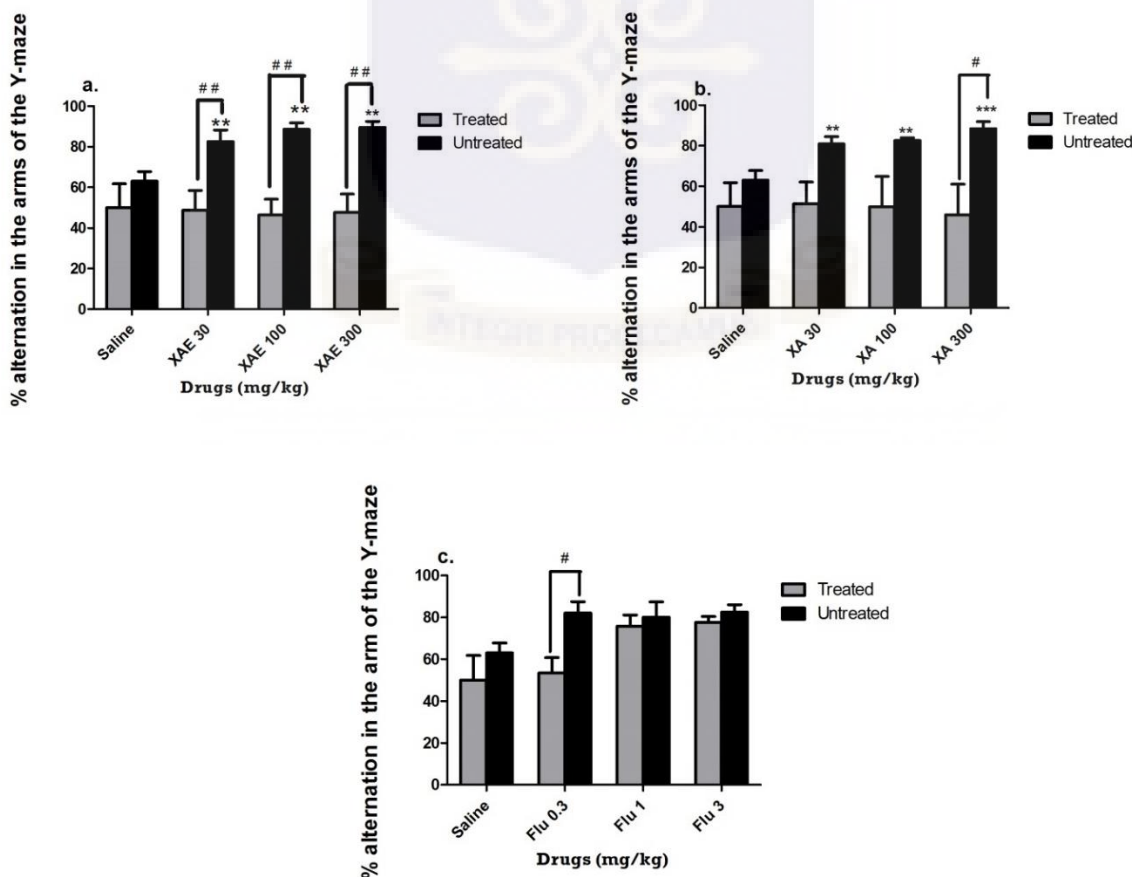


Figure 4.12: Effects of pre-treatment of mice with diazepam (1 mg/kg) on percentage alternation of XAE (30, 100 and 300 mg/kg) and XA (30, 100 and 300 mg/kg) in Y-maze. Data are represented as group Means \pm SEM of 7 animals. Significantly different from saline: *** $P < 0.0001$; ** $P < 0.001$; * $P < 0.01$ (One-way ANOVA followed by Newman-Kuels test). ### $P < 0.001$; ## $P < 0.01$; # $P < 0.05$; significant difference between treatment and dose (One-way ANOVA with Newman-Keuls post hoc test).



CHAPTER FIVE

DISCUSSION AND CONCLUSION

5.1 DISCUSSION

Improvements in pharmacological performances as seen in a number of medicinal herbal preparations may be due to the bioactive compounds present in these plant materials. This has proved useful in the discovery of potential drugs (Tseng *et al.*, 2007). This necessitated the reason for the current research which delved into the effect of the ethanolic fruit extract of *Xylopia aethiopica* and its major isolate xylopic acid on learning and memory as well as the possible mechanism (s) of action.

The present study revealed the presence of alkaloids, saponins and flavonoids in line with earlier works done by Stahls Sies (2005) and John-Dewole *et al.* (2012). The presence of these phytochemicals in the fruit extract is believed to be responsible for the pharmacological action and there is strong scientific evidence to prove that (Rogerio *et al.*, 2010). The saponins in the fruit is shown to improve antioxidant activity; it also lowers cholesterol levels in blood and also found to show anticancer properties (Hoist and William, 2008; Okwari *et al.*, 2013). The flavonoids present is demonstrated to have anti-inflammatory and antiviral effects (Morris and Zhang, 2006). Alkaloids are shown to be very effective and important phytochemical present in medicinal plants (Makkar *et al.*, 2007). The fruit extract of *Xylopia aethiopica* from the present study showed the presence of alkaloids and this is in agreement with the work done by Harrigan *et al.* (1994b) who further showed that the alkaloids in the fruits are responsible for the cytotoxic effects of the fruit (Fetse *et al.*, 2016). Alkaloids are also shown to have different biological properties including effect on the CNS and analgesia as well as anti-inflammatory effects (Yang

et al., 2006). The presence of these phytochemicals may account for the learning and memory improving properties of the ethanolic fruit extract of *Xylopiya aethiopica*.

The purity of the XAE and XA was tested using HPLC analysis and the XAE showed several peaks likely due to the several compounds present in the fruit extract. These includes alkaloids, saponins, tannins, kaurenoic and xylopic acid. These compounds have been reported to be present in the fruit (Somova *et al.*, 2001). Xylopic acid had a single peak indicating the purity of the isolated xylopic acid.

Oral administration of the extract and xylopic acid demonstrated improvements or increases in the learning and memory properties in the novelty object recognition test (NOR), the spontaneous alternation Y-maze test and the probe trial of the Morris water maze test.

In the NOR test, the XAE and XA both increased the percentage time spent with the new object when it replaced the familiar one in the test. Both XAE and XA again increased the percentage attempted alternation in the blocked arm of the spontaneous alternation Y-maze test. They also increased the percentage alternation when the blocked arm was unblocked. However there was a decrease in the change in escape latency i.e. they increased the time used in locating the hidden platform in the MWM test but surprisingly increased the percentage frequency at which the animals visited the quadrant that previously contained the hidden platform at the time when it was removed in the probe trial indicating that retention memory had taken place in the animals that received the XAE and XA even though they did not show improvement in spatial learning when compared to saline.

According to Nelissen *et al.* (2018) and Li *et al.* (2012), stress affects the rate at which animals interact with the new object when the familiar one is replaced in the NOR test. They

demonstrated that in the object recognition test, recognition memory and the conversion of short term memory to long term memory was affected by stress. Bevins *et al.* (2002) also showed that new stimulus can induce stress in animals and affect their behavior. To reduce stress in the animals, the test period took place in the same open field arena as that used for the training. The time for the test period was also kept short (5 minutes). Animals that received XAE and XA significantly interacted better with the new object than the saline-treated naïve animals group and this was an indicator of improvement in exploratory learning and recognition memory.

The extract and xylopic acid showed improvement in exploration with the novel object an indication that exploratory learning and recognition memory was improved. It is believed that cholinergic neurotransmission is enhanced in response to novel stimulus. When rodents are exposed to a new object or environment, they show behaviors such as arousal and attention which are associated with elevated extracellular levels of acetylcholine (Sarter and Bruno, 2000) and this was proved in a study by Rutten *et al.* (2006) where scopolamine was used to impair cholinergic neurotransmission and this affected arousal and attention in the object recognition test. Glutamnergic neurotransmission is also believed to play a role in exploration with the novel object or stimulus (Giovannini *et al.*, 2001; Stanley *et al.*, 2012). Reduced GABAergic neurotransmission due to the mutant overexpression of the GABA transporter type I is also shown to lead to object recognition deficits thus affecting learning and memory (Hu *et al.*, 2004; Ma *et al.*, 2001).

From the results, the XAE demonstrated a rapid and sustained effect on the percentage time spent with the novel object than it did with the familiar one. The xylopic acid also showed increase in percentage time spent with the new object than it did with the familiar one. XAE and XA demonstrated comparable efficacy in the NOR test. These observations indicated that the

extract and xylopic acid increased discrimination against the familiar object and this reinforces the statement by Ennaceur (2010) who mentioned that animals when making a choice between a familiar and a novel object discriminates against the familiar one and interacts frequently with the new object. Bevins *et al.* (2002) is also of the view that environmental cues plays a role in increased preference for novel objects and this was seen in the present study as cues were used to make exploratory learning and recognition memory easier for the animals.

From the results obtained in the spontaneous alternation y-maze test which measured spatial working memory and spatial recognition memory, it was observed that animals that received XAE and XA increased in the percentage number of attempted alternation and percentage alternation when compared to animals in the saline group. A number of factors influence spontaneous alternation in the mice. The study by Bats *et al.* (2001) mentioned that stress levels reduced the level of spontaneous alternation and this is in line with the study by Bardgett *et al.* (1994) who also showed that high levels of anxiety lowered the willingness of mice to explore in the arms of the Y-maze. This observation was seen in the present study as some animals reduced percentage spontaneous alternation when they became stressed or anxious and this was evident when some of the animals became immobile during some of the test days especially after multiple days of testing and in the saline and ketamine-treated groups.

Hughes (2004), Bats *et al.* (2001) and Still Macmillan (1975) in their works demonstrated that olfactory cues had a negative effect on the willingness of rats and mice to alternate in the arms of the T or Y maze and to make sure that the percentage alternation in the y-maze was not affected by olfactory cues, the maze was cleaned in between trials with 70% ethanol to eliminate olfactory traces of previous maze users and therefore the results obtained for all the test drugs and saline was not influenced by olfactory cues.

Several neurochemicals are implicated in spontaneous alternation y-maze test. Acetylcholine has been implicated in spontaneous alternation y-maze and this is seen in tests involving cholinergic antagonists such as scopolamine. Scopolamine was seen to reduce synaptic neurotransmission whereas cholinergic agonists were seen to increase synaptic neurotransmission in mice and rats (Bertholet and Crusio, 1991).

Benzodiazepine receptors are also shown to impair spontaneous alternation in the y-maze or the T-maze (Belotti *et al.*, 1998; Lalonde, 2002) as was seen in the administration of diazepam and benzodiazepine receptor antagonist which increased the spontaneous alternation of rates of scopolamine injected rats (Belotti *et al.*, 1998; Lalonde, 2002; Sarter *et al.*, 1988). The role of GABA in spontaneous alternation has not been elucidated fully but it is known that the injection of muscimol a GABA_A receptor agonist decreased spontaneous alternation and the rate at which the animals alternated (Degroot and Parent, 2000; Parent *et al.*, 1997) suggesting that GABA_A receptors are involved in spontaneous alternation and increases in GABA_A agonists levels reduces spontaneous alternation.

Glutamatergic receptors are also believed to play a role in spontaneous alternation. NMDA receptor antagonists were also seen to reduce spontaneous alternation scores as was seen in the study by Lennartz Gold (1995) and Holter *et al.* (1996) using dizocilpine. In the present study animals that received ketamine an antagonist of the NMDA showed a reduction in the percentage alternation in the y-maze with the animals showing signs of stress and immobility than the control group and this reinforced the study by Holter *et al.* (1996) and Lennartz Gold (1995). This suggests that NMDA receptors are implicated in spontaneous alternation y-maze test and spatial working memory as well as spatial recognition memory were affected. This may imply that the increase in percentage attempted alternation and percentage alternation in the y-maze

seen with XAE and XA may be due to involvement of either the cholinergic, glutaminergic or GABAergic neurotransmitters.

In the results obtained from the Morris water maze (MWM) a test which measures spatial learning and reference memory, XAE increased in change in escape latency on the third day of treatment but the effect decreased on the 4th day of test in the time course curve however the probe trial showed significant increase in the percentage frequency when platform was removed. The XA did not show significant increase in escape latency but significantly increased in the probe trial. This implies that the animals used longer time in locating the hidden platform in comparison to the saline-treated group but recalled the last quadrant they saw the hidden platform before it was removed. This suggests that the animals performed well in reference memory as seen in the results for the probe trial but not so well in spatial learning. A number of factors influence the performance of animals in the Morris water maze test.

Stress according to Hölscher (1999) and Sandi (1998) influences the performance of animals in MWM and to avoid this, pre-handling of animals in the visible platform training is essential. The visible platform training aids animals in identifying the platform as a source of escape. It also effectively acclimatizes the mice to the handling and test procedure (Westerman *et al.*, 2002). Weitzner *et al.* (2015) suggested that to reduce stress and variations in performance due to animal handling one experimenter should carry out the test in each group since this will also assist in adjusting the animals to the testing style of the experimenter. The present study took these into consideration and ensured that the visible platform training was done thrice on the training day. The experimenter was also the only one who carried out the test procedures in order to reduce the variations in performance. The experimenter stood behind a visual barrier to avoid

experimenter visibility according to what was proposed by Vorhees Williams (2006). This eliminates stress as the reason for the results obtained from the test.

Auditory and olfactory cues are also implicated as factors affecting performance in MWM (Crawley, 2000), this is in line with what was done in the present study. The water maze was cleaned and water changed regularly between individual animal trials in order to reduce the olfactory tracks left by the previous user of the maze. Noise was limited to the minimum in the testing lab in order to reduce distractions to the animals.

Vorhees Williams (2006) mentioned that on some occasions the animals locate the platform but deflections can affect the rate at which the animals climbed onto the platform to escape the water and this may contribute to the observations seen in the present study. Water temperature is cited as a factor affecting the escape latency of animals in the MWM test (Weitzner *et al.*, 2015). To avoid this water temperature was kept at a temperature range between 20-22°C to prevent the water from becoming too cold or too warm for the animals. Light has also been mentioned as a source of low performance in the animals since light serves as visual cue for the animals (Vorhees and Williams, 2006; Weitzner *et al.*, 2015). The present study ensured that the testing laboratory had adequate lighting which was indirect not too dark and not too bright. Curtains were closed to minimize distal cues so as not to affect the performance of animals and therefore the observed results are not due to inadequate lighting nor is it due to inappropriate temperature of the water.

Hypothermia also affects results in MWM (Ivonen *et al.*, 2003). To prevent hypothermia the animals were properly and thoroughly wiped using a towel in between trials. Enough time of 30 minutes was left between trials to enable the animals dry up before the next trial. This suggests that the results obtained were not due to hypothermia. The size of the platform is found to affect

the performance of animals in the MWM (Vorhees and Williams, 2006; Williams *et al.*, 2004). Smaller platforms in the water maze setup tend to lower the rate at which the animals climb and stay on the platform. The present study used a platform size of 10cm² and this is in line with what was proposed by Williams *et al.* (2004) as the ideal size of the platform.

According to Saucier *et al.* (1996) thigmotaxis i.e. the tendency to cling to the walls of the tank can affect the performance of mice in MWM because in such instances the animals fail to discern that the platform is the target. This was not observed in the present study and this was likely due to the training that was performed helping the animals to understand that the platform was the target.

Neurotransmitter systems are involved in the performance of animals in the Morris water maze paradigm with the cholinergic and NMDA receptors being strongly involved whereas GABA and opioids are believed to have negative effect on spatial learning (D'Hooge and De Deyn, 2001; McNamara and Skelton, 1993). NMDA receptor antagonist MK-801 is shown to impair spatial learning and reference memory in MWM test in animals. The studies by Enomoto *et al.* (2008); (Mutlu *et al.*, 2011) and Wass *et al.* (2006) demonstrated that MK-801 an NMDA receptor antagonist impaired spatial learning and reference memory in the MWM, which are signs similar to cognitive deficits seen in schizophrenic patients. Jafari-Sabet (2006) also showed that memory functions that depend on the hippocampus and amygdala are impaired by NMDA receptor antagonists and this may account for the significant impairment in spatial learning and reference memory that was seen in the present study when ketamine an NMDA receptor antagonist was used in the MWM test. This suggests that NMDA receptors are strongly involved in the long term potentiation that take place in the brain when animals perform the MWM task (Moser *et al.*, 1998; Vorhees and Williams, 2006).

From the animal models of learning and memory examined, it was revealed that the fruit extract of *Xylopi aethiopica* and xylopic acid increased performances in the NOR test, Y-maze and MWM and from earlier works it was revealed that neurochemical systems are involved in the performances observed, with cholinergic, GABAergic and glutaminergic receptor systems standing out. The present study hypothesized that the mechanism of action by which the XAE and XA enhanced learning and memory was through either the cholinergic or GABAergic pathway or both. The mechanism of action of XAE and XA was then tested for involvement of the cholinergic or GABAergic receptor systems.

Cholinergic systems play vital roles in learning and memory (Bertholet and Crusio, 1991; Parle *et al.*, 2004) and as a result cholinergic receptor antagonists such as scopolamine are used to determine the mechanism of action of memory enhancing agents (Bertholet and Crusio, 1991; Vasudevan and Parle, 2007). Cholinergic receptor antagonist scopolamine hydrobromide (antimuscarinic) was used to pretreat the animals to test for the mechanism of action in NOR and Y-maze tests. From the results obtained, XAE and XA increased learning and memory even in the presence of scopolamine thus suggesting that the cholinergic mechanisms or neurotransmission does not contribute in the learning and memory enhancing properties of XAE and XA.

GABA_A receptors play an inhibitory role in the brain by causing an increase in membrane conductance thereby increasing the entry of chloride ions into the brain and thus affecting the generation of action potentials in the brain resulting in neuronal inhibition (Olsen and Delorey, 1999). This alters learning and memory effects in the brain. Diazepam a GABA_A receptor agonist was used in pretreating the animals. From the results, XAE and XA did not increase learning and memory effects showing a significant difference between the treated and untreated

groups. This observation suggests that GABAergic neurotransmission contributes to the learning and memory enhancing properties of XAE and XA.

5.2 CONCLUSION

The present study provides scientific basis that the ethanolic (70%) fruit extract of *Xylopi* *aethi* *opica* and xylopic acid enhances learning and memory in murine models.

Ethanolic fruit extract of *Xylopi* *aethi* *opica* contains alkaloids, saponins and flavonoids. The improvement in learning and memory seen with XAE and XA may be attributed to the involvement of the GABA_A receptor system which may explain the observed behavioral effects.

5.3 RECOMMENDATIONS

- Other acute animal models of learning and memory such as the passive avoidance paradigm, radial arm maze and active avoidance paradigm should be explored to confirm the learning and memory enhancing effects of the fruit extract of *Xylopi* *aethi* *opica* and xylopic acid.
- Chronic animal models of learning and memory should also be explored to confirm the long term effect on learning and memory of the fruit extract of *Xylopi* *aethi* *opica* and xylopic acid.
- Other possible mechanism(s) of action such as involvement of the glutaminergic and GABA_B receptor systems should be explored.
- Isobolographic analyses involving the combination of a standard nootropic and the extract or xylopic acid should be performed to determine if there would be enhanced activity.

- Histological examinations of brain regions especially that of the hippocampus and prefrontal cortex should be performed to ascertain the effect the extract and the xylopic acid have on these regions been implicated in learning and memory.



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