COGNITIVE-ENHANCING AND ANTIOXIDANT ACTIVITIES OF THE AQUEOUS ROOT EXTRACT OF *Hypericum revolutum* subspecies *keniense* (Schweinf.)

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DECLARATION

This research project is my original work, and has never been presented for the award of a degree or any other award in any institution of learning.

Signature…… Date…….

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SUPERVISOR

This research project has been submitted with my approval as the university supervisor.

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DEDICATION

I dedicate this work to my wife Rachel, my daughters Abigail and Joy, my brothers, and sisters for their support all through.

ABSTRACT

Cognitive deficit is linked to the brain dysfunction mostly manifested by loss or distortion of learning, memory, attention and many other symptoms. These cognitive functions are normally controlled by acetylcholine a neurotransmitter present in the amagdala, hippocampus and striatal parts of the brain. Therefore, any damage to these parts or the inefficient activity of acetylcholine results into cognitive deficit. Oxidative damage of the brain occurs due to the imbalance between the reactive oxygen species and the antioxidants and has been implicated in the development of cognitive impairment. The management of cognitive deficits in Alzheimer's disease involves the use of conventional drugs such as rivastigmine, donezepil, tacrine and galantamine. However, these drugs only relief symptoms without curing the impairment. In addition, they are associated with adverse effects, are less efficacious and though not costly, the cost of managing patients with cognitive impairment and related conditions is high. Therefore, there is an urgent need for an alternative that is potent, efficacious, safe, accessible, affordable in terms of cost and curative. Plants are a potential source for such drugs with the desired properties. Various plants including *Hypericum revolutum* subspecies *keniense* have been used in traditional medicine to manage cognitive deficits and related disorders. However, the efficacy of such plants have not been scientifically investigated. Therefore, this present study was designed to investigate the cognitive – enhancing and antioxidant activities of the aqueous root extract of *Hypericum revolutum* subspecies *keniense (*Schweinf) as a potential alternative source of cognitive deficit curing agent. In this study, the Morris water maze technique was adopted to investigate the cognitive enhancing activities of the aqueous root extract of *Hypericum revolutum* subspecies *keniense (*Schweinf) in the scopolamine induced cognitively impaired mice. In addition, ex-vivo antioxidant efficacy in the brain tissue of the cognitively impaired mice was determined following the MWM test. The mice which were treated with HRKaq 250mg/kg bwt took a significantly shorter latency time compared to the negative control ($p<0.05$) in the MWM test. Similarly, the mice treated with HRKaq 250mg/kg bwt showed increased % activity of SOD and Glutathione antioxidants as compared to all the other groups ($p<0.05$). Moreover, the mice treated with HRKaq 10mg/kg bwt showed significantly low concentration of hydrogen peroxide (catalase enzyme activity increased) as compared to all the other groups ($p<0.05$). From the results, it is evident that the aqueous root extract of *Hypericum revolutum subspecies keniense* has both the cognitive enhancing and antioxidant properties. The cognitive enhancing effects of this plant could be attributed to the presence of antioxidant phytochemicals which quench oxidative stress and promote health. Further studies aimed at investigating agents that; inhibits fast iron accumulation in the brain in oxidative stress, Inhibitors of tau plaques, inhibitors of mediators of neuroinflammation and anti-Parkinson's disease should be done.

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ABBREVIATIONS AND ACRONYMNS

AChE	Acetylcholinesterase
AD	Alzheimer's Disease
ALS	Amyotrophic Lateral Sclerosis
ANOVA	Analysis Of Variance
APP	Amyloid Precursor Protein
Aβ	Amyloid Beta
DNA	Deoxyribonucleic Acid
EDTA	Ethyl Diethyl Tetra Acetic acid
FDA	Food and Drugs Authority
GSH	Glutathione
HD	Huntington's Disease
HRK	Hypericum Revolutum subspecies keniense
HRKaq	Hypericum Revolutum subspecies keniense aqueous root extract
LBD	Lewy Body Dementia
MDA	Malondialdehyde
NADPH	Reduced Nicotinamide Adenine Dinucleotide phosphate
NBT	Nitro Blue Tetrazolium chloride
NFTs	Neurofibrillary Tangles
NMDA	N-Methyl-D- Aspartate
PD	Parkinson's Disease
PDD	Parkinson's Disease Dementia
REM	Rapid Eye Movement
RNA	Ribonucleic Acid

RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SEM	Standard Error Mean
SOD	Superoxide Dismutase
UV-Vis	Ultraviolet – Visible
VaD	Vascular Dementia
VCI	Vascular Cognitive Impairment

CHAPTER ONE

INTRODUCTION

1.1 Background information

Cognitive functions of the brain including learning, memory retention and retrieval are processed in the amygdala, hippocampus, and the striatal regions under the regulation of acetylcholine and any damage to these parts due to oxidative stress or the inefficient functioning of acetylcholine causes cognitive impairment (Salim, 2017). Globally, about 50 million people were affected by the year 2015 and the number has been projected to rise to about 82 million and 152 million in the year 2030 and 2050 respectively (OMS, 2019);(Moriasi, et al, 2020a). Cognitive impairment has been linked to the emergence of mental disabilities and neurodegenerative disorders and has proven to be a burden not only to the affected persons but also to the care givers and the family members (Moriasi,et al, 2020b). Moreover, the cost of managing patients with cognitive impairment is very high limiting individuals in seeking medical care hence raising fatalities related to these disorders (OMS, 2019).

In many cases of cognitive disorders including AD, oxidative stress has been found to play a pivotal role (Huang, et al, 2016), (Hajiluian, et al, 2018). Oxidative stress is described as an imbalance between ROS production and the antioxidant systems in the body, a situation that promotes the development of long term disorders such as dementia, depression and cardiovascular disorders (Luca & Luca, 2019). The ROS attacks biomolecules by initiating oxidation reactions causing oxidative damage to cells and tissues (Beckhauser,et al, 2016). However, at physiological levels, the ROS have beneficial roles of aiding in the regulation of cell cycle, phagocytosis and cell signaling (Manoharan et al., 2016). Additionally, it has been proven that oxidative and nitrosative stress is the main cause of AD and other age related neurodegenerative diseases (Tramutola,et al, 2017). AD cases represents a larger percentage of more than 80% of dementia cases reported globally among the aged and is characterized by gradual deprivation of mental, behavioral, loss of function and capacity to learn (Kumar,et al, 2015). It has been established that the brain is the most susceptible to oxidative damage owing to its increased metabolic processes, high oxygen use, high levels of polyunsaturated fatty acids and damaged mitochondria, RNA oxidation, rich transition redox metals, neurotransmitter auto oxidation (Moriasi et al., 2020a)(Cobley, et al, 2018)

The management of cognitive impairment and related neurodegenerative disorders involves pharmacotherapy in which conventional drugs including rivastigmine, donepezil, galantamine and memantine which act on the cholinergic system and NMDA receptors have been used (Fish,

etal, 2019). However, these drugs are reported to only manage the symptoms without curing the disease besides their association with adverse effects, low efficacy and potency (Moriasi et al., 2020b).

In the recent years, medicinal plants have played a significant role in the management of many chronic diseases affecting man due to the presence of many bioactive compounds having health improving properties such as antioxidant and memory enhancing activities (Moriasi et al., 2020a). Furthermore, various pure compounds have been isolated from plants such as galantamine and huperzine and their mechanism of action determined as well (Kumar et al. 2012). *Hypericum revolutum subspecies keniense* is a perennial shrub in the family *Hypericaceae*. It has been used traditionally in the management of wounds, burns and depression among the Embu people of Kenya. However, these claims have not been scientifically investigated. Therefore, this study aimed at investigating the cognitive - enhancing and antioxidant activities of the aqueous root extract of *Hypericum revolutum subspecies keniense*.

1.2 Problem statement and justification

Cognitive impairment is the main cause of mental disabilities and neurodegenerative disorders of global concern due to the burden it causes not only to the affected persons but also to the care givers and the family members (Moriasi et al., 2020b). Moreover, the cost incurred in managing patients with cognitive impairment is very high limiting the individual in seeking medical care hence raising fatalities related to these disorders (OMS, 2019). Even though the approved conventional medicines such as rivastigmine, donepezil, galantamine and memantine which act by targeting the cholinergic and glutaminergic systems have been in use for decades, they have not helped ease the burden since they are useful only in alleviating the symptoms without curing the conditions (Fish, et al, 2019). They are also associated with side effects for instance rivastigmine has been reported to cause diarrhea, nausea and weight loss while galantamine has been reported to be less potent (Moriasi et al., 2020a). Furthermore, the pathology of AD involves other mechanisms including neuroinflammation, NFTs, amyloid beta production, iron accumulation in the brain (Ashraf, et al, 2018) implying that a multi- targeting approach is required in the management (Hasan Bazzari, et al, 2018). This calls for urgent search for alternative natural antioxidants and cognitive enhancing products from natural sources such as herbal medicinal plants which have been in use of as long as man lived owing to their phytochemical constituents exhibiting various therapeutic activities (Jared,et al, 2018). Therefore, in this study, the cognitive - enhancing and antioxidant activities of the aqueous root extract of *Hypericum revolutum* subspecies *keniense* was investigated.

1.3 Objectives

1.3.1 General objective

To determine the cognitive enhancing and antioxidant activities of the aqueous root extract of *Hypericum revolutum* subspecies *keniense (Schweinf)*

1.3.2 Specific objective

I. To investigate the cognitive enhancing activity of the aqueous root extract of *Hypericum revolutum* subspecies *keniense* (schwenf).

II. To investigate the antioxidant effects of the aqueous root extract of *Hypericum revolutum* subspecies *keniense* on superoxide, dismutase, catalase and glutathione levels in the cognitively impaired brain tissues.

1.4 Research questions

I. Does the aqueous root extract of Hypericum *revolutum* subspecies *keniense* have cognitive enhancing activity?

II. What are the effects of the aqueous root extract of *Hypericum revolutum* subspecies *keniense* on Scopolamine induced oxidative stress?

CHAPTER TWO

LITERATURE REVIEW

2.1 Dementia

Dementia is a grouping of manifestations of gradual neurodegenerative disorders more common among the elderly and is characterized by cognitive deficits leading to chronic disability (Association, 2018). The later stages of dementia is characterized by chronic disability and severe cognitive impairment including a situation where an affected individual is unable to perceive or acknowledge members of the family or relatives (Mitchell, 2015). Studies have identified three types of dementia which include; Alzheimer's Disease (AD - most common type of dementia), vascular dementia (VaD) and Lewy body types of dementia (LBD- dementia with Lewy bodies and Parkinson's disease dementia - PDD)(Perry & Howes, 2011)(Livingston et al., 2017). The pathogenesis of dementia involves beta amyloidosis and unusual tau accumulation in the case of AD, angiogenic and ischaemic lesions in the case of VaD, and α-synucleinopathy in the case of LBD. Additionally, unusual neurotransmitter dysfunction (cholinergic, glutamic, serotonergic), neuroinflammatory mechanisms, oxidative damage, apoptosis, and inactivated neuroplasticity have been found to contribute significantly to the development of dementia. (Perry & Howes, 2011).

2.1.1 Alzheimer's disease (AD)

Alzheimer's disease is a neurodegenerative disorder common in old age and has been found to be the most prevalent form of dementia (Association, 2018). It is characterized by neuropathological evidence of intracellular neurofibrillary tangles (NFTs) and plaques composed of amyloid protein accumulation (Cheignon et al., 2018). The main manifestation of the disease starts with severe loss of memory where an individual is unable to recall recent information, and later there is reduced ability to make right judgment, finding solution to problems, visual and spatial relationships and overall interference with the daily activities of life as a result of neuronal damage (Cass, 2017). A considerable percentage of AD cases amounting to about 70 % are genetic related whereas the remaining percentage is linked to environmental factors (Jivad & Rabiei, 2014).

There is evidence that iron is involved in the development of AD since it is a potential inducer of oxidative stress and is usually found deposited in areas of the brain prone to neurodegeneration. (Nuñez & Chana-Cuevas, 2018)

Other mechanisms believed to play vital role in AD include; increased neuroinflammation, increased NFT formation, increased amyloid beta plaques formation, decreased amyloid beta degradation and clearance, increased amyloid beta production and increased oxidative stress(Andrade, et al, 2019)

2.1.2 Vascular dementia (VaD)

Vascular dementia is the second major cause of age related dementia after AD and is identified as a cognitive impairment occurring due to various vascular events that injures blood vessels in the brain resulting in their reduced efficiency in the supply of enough oxygen and nutrients required for the normal functioning of the brain (Khan, et al, 2016). Cerebrospinal and cardiovascular disorders including stroke are responsible for the blood vessel injury in the brain leading to vascular cognitive impairment (VCI) which is categorized into; subcortical ischemic vascular dementia, cortical dementia, post- stroke dementia, and mixed dementias (Kalaria, 2018). Patients with VCI are more forgetful, appear confused and have reduced ability to handle activities requiring cognitive execution (Smith, 2017)

2.1.3 Lewy body dementia (LBD)

Lewy body dementia consisting of dementia with Lewy bodies and Parkinson's disease dementia, is a major cause of neurodegenerative disorders with 4-8% of patients with dementia having LBD (Taylor et al., 2020). The main features of the disorder include visual illusion, cognitive alternation, impulsive Parkinsonism and sleep disorder (REM) (Kane et al., 2018). LBD results from synucleinopathies in which aggregates of α- synuclein proteins are deposited in the susceptible neurons, Lewy neurites and other non- neuronal processes in the brain causing damage and ultimately cognitive decline (Outeiro et al., 2019)

2.2 Treatment of AD

The management of AD involves the use of the approved cholinesterase inhibitors such as Donepezil, Rivastigmine, and Galantamine which act by inhibiting acetylcholinesterase enzyme resulting in increased levels of acetylcholine (Hampel et al., 2018). Memantine, a glutamate NMDA receptor antagonist acts by increasing the activity of the NMDA receptors was approved for the management of AD after successful completion of the clinical trials (Fish et al., 2019).

Since the pathological characteristics of AD include gradual accumulation of extracellular amyloid plaques as well as the intracellular neurofibrillary tangles (hyper phosphorylated tau protein aggregation) in the neurons,(Vanitallie, 2015), research studies have focused on many natural and synthetic medicinal peptides acting as inhibitors of; Amyloid β (RGKLVFFGR

(OR1) and RGKLVFFGR-NH2 (OR2)), β-Site APP Cleaving Enzyme 1 (BACE1), Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH), Tyrosine Phosphatase (TP), Potassium Channel KV1.3 (BmKTX-R11-T28-H33 (ADWX-1), OsK1-K16-D20 and HsTx1 [R14A] most of which are in clinical trials (Baig,et al, 2018). However, many clinical trials of therapies for AD targeting amyloid plaque have failed whereas those targeting other AD hypothesis have given limited results, therefore, scientists are now thinking that multi-target drugs (MTDs) aiming simultaneously at several sub-pathologies are expected to be a better approach (Godyń, et al, 2016), (Fish et al., 2019)

2.3 Medicinal plants in the management of AD

Many different medicinal plants including; Withania somnifera, Curcuma longa, Convolvulus pluricaulis, Centella asiatica, Celastrus paniculatus, Nardostachys jatamansi, Coriandrum sativum, Ficus carica, Ginkgo biloba, Commiphora whighitti, Glycyrrhiza glabra, Lepidium meyenii, Panax ginseng, Zingiber officinale, have been used for learning and memory improvement due to their antioxidant, anti-inflammatory, acetylcholinesterase inhibition properties thus proving to be alternative remedies for AD (Akram & Nawaz, 2017).

Galantamine (*Amaryllidaceae* family) is a drug of plant origin currently sourced from *Galanthus woronowi* Losinsk and *Galanthus alpines* Sosn is used in the management of dementia (Tewari et al., 2018). Other plants with cognitive enhancing properties include, *Hypericum perforatum*, *Salvia officinalis*, and *Ziziphus jujube*, (Andrade et al., 2019)(Perry & Howes, 2011).

The use of medicinal plants in the management of AD is associated with numerous advantages including creation of multiple targeting potential, safety and absence of serious side effects (Hasan Bazzari,et al, 2018)

2.4 Oxidative stress

Aerobic phosphorylation taking place in the mitochondria produces ATP for cellular functions and in the process generates reactive oxygen species (ROS) and reactive nitrogen species (RNS) which at physiological levels play important roles in cellular functions including cell signaling (Salim, 2017). However, excessive production of ROS and RNS is harmful since it causes oxidative stress which is a condition that occurs due to the imbalance between generation of reactive oxygen species (ROS) and antioxidants in the body (Luca & Luca, 2019). The over produced reactive oxygen attack the biomolecules such as lipids, proteins and DNA resulting in the disruption of both the cellular function and integrity (Manoharan et al., 2016). The body has the ability to counteract the damaging effects of oxidative stress by use of the efficient

antioxidant system enabling the brain to control its oxygen use and redox reactions (Salim, 2017).

Lipid peroxidation is a consequence of oxidative stress where the reactive free radicals attacks lipids having carbon-carbon double bonds in their structure resulting in the formation of malondialdehyde (MDA) an organic compound used as a marker of oxidative stress including that induced by scopolamine (Tang, 2019).

The antioxidant system is responsible for alleviating excess ROS/RNS and include the enzymatic activities of superoxide dismutase (SOD), glutathione reductase (GR), catalase (CAT), glutathione peroxidase (GSHpx), in addition to non-enzymatic agents such as vitamins A, E, and C, flavonoids, proteins (albumin, ceruloplasmin) and thioredoxin (Liguori et al., 2018). Previous studies have shown that scopolamine interferes with the function of the enzymatic antioxidants greatly reducing their levels in the brain tissues (Tang, 2019). Therefore, overproduction of ROS/RNS and an inefficient antioxidant system has been linked to the pathogenesis of age dependent neurodegenerative disorders including AD, PD, ALS and HD (Niedzielska et al., 2016).

2.5 *Hypericum revolutum* subspecies *keniense*

Hypericum revolutum subspecies *keniense* is a medicinal plant in the genus *Hypericum (Hypericaceae*) that consists of about 500 herbs, shrubs and a small number of trees distributed globally particularly in temperate zones (Bridi, et al, 2018) . *Hypericum revolutum* is a small tree growing up to 3m high or a shrub having many stems, flowers that are bright yellow and fruits that are reddish-brown capsule (Staden & Lall, 2020). This genus has been embraced globally because of their medicinal properties like antimicrobial, antidepressant, antibacterial besides antioxidant activities attributed to the presence of pseudohypericin and hypericin. Additionally, the plants are important in flavouring and perfume industries because they are rich in essential oils (Dogan, et al, 2017). *Hypericum revolutum* subspecies *keniense* is found growing in the higher altitudes of 2400m in Mt Kenya and the Aberdares forest in Kenya and reputable herbalists have claimed to use it in treatment of memory loss, insanity, and depression.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Plant collection and preparation

Fresh roots of *Hypericum revolutum* subspecies *keniense* were obtained from Mbeere in Embu county by the help of an acknowledged herbalist. The plant was identified by its kikuyu local name Muthathumwa and then later authenticated by a taxonomist based at the East African herbarium at the National Museum of Kenya and referred as E001J2019.

Duplicate specimens were prepared and deposited at the department of pharmacognosy, school of pharmacy, Mount Kenya University.

The collected roots were packaged in woven bags and transported to the pharmacognosy laboratory of Mount Kenya University where they were gently washed with clean tap water to remove the soil and later chopped into small pieces.

The chopped root materials were spread on the laboratory bench and air dried for one week with regular grumbling to facilitate uniform drying.

After drying, they were ground into a powder by the help of an electric plant mill and kept in a khaki envelop and stored on a cool dry place on a laboratory shelf awaiting extraction.

3.2 Extraction procedure

Extraction of the aqueous root extract of *Hypericum revolutum* subspecies *keniense* was done following the procedure described by Moriasi et al 2020. In this method, 50g of the powdered root sample was warmed at 58^0c in distilled water on a water bath for a period of two hours. The mixture obtained was cooled to room temperature and filtration done by passing it through Whatman filter paper number 1 and placed into clean freeze-drying flasks and covered by use of solid carbon dioxide – acetone mixture. The covered flasks were then fitted into a freeze dryer for the extract to be lyophilized for 48 hours. After drying and lyophilizing, the extract was removed and put into universal glass tubes that were clean, dry, preweighed and labelled.

Determination of percentage yield of the extract was done by use of a formula given by Harborne but with modification by Truong (Truong et al 2019).

$$\%\ yield = \frac{weight\ of\ extract}{weight\ of\ mercerated\ powder} \times 100$$

3.3 Materials and Reagents

The materials used included, spectrophotometer, test tubes, Morris water maze, micropipettes water bath. The reagents used included, sodium carbonate, NBT, EDTA, Hydroxylamine, sodium azide, EDTA, GSH (reduced), NADPH, Hydrogen peroxide, sodium dihydrogen phosphate and disodium hydrogen phosphate and Dichromate acetic acid mixture.

3.4. Experimental animals

In this study, Swiss albino mice weighing 25±2g aged 4-5 weeks were sourced from Kenya Agricultural and Livestock Research Organization small animal breeding unit (Muguga).

The animals were housed in propylene cages measuring 30 cm × 20 cm × 13 cm and soft wood shavings was used as their bedding material. They were maintained on a 12-hour day/night cycle and fed on the standard rodent pellets with clean water ad libitum.

The mice were also acclimatized for 72 hours before the experiment. Furthermore, the guidelines stated by the National research council in relation to manipulation of experimental animals for care, use and disposal were followed.

The experimental mice were randomly allotted into six groups (normal control, positive control, negative control and three experimental groups) comprising of five animals each.

3.4.1 Determination of the cognitive - enhancing activity

3.4.1.1 Morris water maze setup.

The Morris water maze setup as outlined by Moriasi et al, 2020 was adopted in this study to determine the in vivo cognitive effect of the aqueous root extract of *Hypericum revolutum* subspecies *keniense*. The setup consisted of a white ring-shaped tank that created a pond of 110cm in diameter and 45cm in height and without interior finish. The ring-shaped pond was then filled with water mixed with 750g of powdered fat-free milk to a level of 30cm to make it opaque. By use of a well calibrated thermometer, the round pond was sustained at 26^0c.

An escape platform, white in color with 10cm diameter and 29cm height was placed at the NW quadrant of the ring-shaped pond. Adjustment of the water level was done such that initially it was 1cm below the platform and later 1cm above the upper most surface of the platform. Before introducing the mice, the sides of the maze were fixed with blue, green, pink, and yellow colored manila papers to the west (W), north (N), south (S), and east (E) quadrants in that order, to serve as local visual cues. A digital Sony video camera placed at 1.5m above the maze and connected

to Any-Maze tracking software version 6.05 installed in Windows 10 Pro PC was used to track the progressive position of each swimming mouse from the starting site to the top of the escape platform.

3.4.1.2 Swim training

In this study, two days before the experiment were committed for the purpose of training the experimental mice to swim and locate the escape platform. Each experimental mouse was allowed to swim for 60 seconds with the appearance of the perceptible escape platform accompanied by a further training period without the escape platform. A break of 20 minutes between training sessions was provided for the mice to rest and recover. Additional training period without a visible escape platform was done in a similar way as the previous trainings, where each mouse traversed the maze looking for the concealed escape platform in order to aid in the formation of the spatial map of the environment and actual familiarization. The escape platform was kept stationary at NW throughout the training period with the starting points varied after every trial as north (N), south (S), east (E), and west (W), respectively.

3.4.1.3. Acquisition training

Acquisition training took three days during which the level of water in the maze was raised to 1cm above the top surface of the escape platform so that it was not visible at the water level. The escape platform remained stationed at NW. The experimental mice were predisposed to three-day training periods per day for three days with 20 minutes break in between trials. The starting position was varied throughout the experiment in a similar manner as the previous training.

Each test involved gentle handling of the mice and placing it in the water maze facing the side of the maze away from the escape platform. The mice traversed the pool looking for the concealed escape platform for up to 60 seconds. After finding the platform, the mouse was allowed to stand on it for 10 seconds for more exploration of the neighborhood. In case mice did not find the escape platform after 60 seconds, it was tenderly led to the escape platform and permitted to rest on it for 15 seconds to familiarize itself with the environment. It was then tenderly taken out of the maze and put into a confining cage containing paper towels to dry.

A digital video camera was used to record the latency time (time take to look for and find the platform within 60 seconds) and the way-finding of each mouse in the experiment.

3.4.1.4. Probe test

Assessment of learning and memory reservation was done on the 4^{th} day of the investigation where all the experimental mice were subjected to a one probe test by introducing them into the

water maze to evaluate their ability to learn the task and also to find out if they were able to recall the position of the escape platform. They were permitted to traverse, look for and find the escape platform and the time taken by each was recorded.

3.4.1.5. Induction of cognitive impairment

Cognitive impairment was instigated on the 4th of the investigation by orally administering hyoscine hydrobromide (scopolamine) 1mg/kg/bw to all the experimental mice except excluding the normal control mice which received normal saline 0.9% 1ml/kg/bw given orally.

3.4.1.6. Experimental design

In this study, a controlled, randomized laboratory-based study design was endorsed, from which an experimental design was drawn. For each of the investigated plant extracts, thirty (30) investigational mice were randomly allotted to six groups each comprising of five (5) mice (3males and 2 females) (Moriasi et al., 2020a).

The allotment arrangement is as shown in table 3.1

Table 3.1 treatment for randomized mice for in vivo cognitive-enhancing activity

Investigational group		Treatment
A	Normal control	Normal saline 10ml/kg/bw, p.o
B	Negative control	Normal saline + scopolamine 1mg/kg/bw, p.o
C	Positive control	Donepezil(1mg/kg) + scopolamine 1mg/kg/bw, p.o
D	Experimental 1	HRKaq 250mg/kg + scopolamine 1mg/kg/bw,p.o
E	Experimental 2	HRKaq 50mg/kg + scopolamine 1mg/kg/bw, p.o
F	Experimental 3	HRKaq 10mg/kg + scopolamine 1mg/kg/bw, p.o

All drug administered were prepared in Nacl 0.9%. HRKaq; aqueous root extract of *Hypericum revolutum* subspecies *keniense*, p.o; per oral; bw, body weight.

3.4.2 Antioxidant assays

Following the Morris water maze test, all the experimental mice were decapitated by cervical dislocation. The whole brains from all experimental mice were dissected in standard conditions. The brain samples were then mixed with phosphate buffer and then vortexed on the vortex mixer to get a fine homogenate. The resultant homogenate was then centrifuged at 3000 rpm for 10 minutes and the resultant supernatant separated and used for assays of SOD, Glutathione peroxidase and catalase activity.

3.4.2.1 Glutathione peroxidase assay

The glutathione peroxidase assay was conducted by following the protocol of Sharma et al ,2001. The 2 ml reaction mixture was reconstituted by adding 1.49ml of 0.1M Sodium phosphate buffer (PH 7.4), 0.1ml of 1mM EDTA, 0.1ml of 1mM sodium azide, 0.1ml of 1mM GSH (reduced), 0.1ml of 0.02mM NADPH, 0.01ml of 1mM Hydrogen peroxide, and 0.1ml of brain supernatant. The absorbance of the oxidized NADPH was then monitored at interval of 30 minutes for 2 minutes at wavelength of 340 nm using the UV-Vis double beam spectrophotometer. All the tests were performed in triplicates. The enzyme activity was then calculated using the extinction coefficient of 6.22×10^3 $M^{-1}cm^{-1}$ and expressed as moles of NADPH oxidized/minute/mg of protein.

3.4.2.2 Catalase activity

The catalase activity was evaluated by the method of Sinha (1972) with minor modifications. 100µl aliquot of the brain sample was added to 250µl 0.1 M phosphate buffer and then the reaction initiated by addition of 200µl of 1mM Hydrogen peroxide. The reaction was then terminated after 0,30 and 60 seconds by addition of 1 ml of dichromate-acetic acid mixture. All the tubes containing the reaction mixtures were then incubated in hot water bath at 100^0c for 10 minutes after which they were allowed to cool to room temperature and the absorbance was then measured at wavelength of 620 nm using UV-Vis double beam spectrophotometer.

The activity of catalase was then determined by calculating the concentration of hydrogen peroxide in the brain tissues was calculated the formula below and expressed as the concentration of hydrogen peroxide.

$A= \varepsilon lc$

Where: A = Absorbance, ε = extinction 39.4 $mol^{-1}cm^{-1}$, l = path length, C = concentration

3.4.2.3 Superoxide dismutase activity

SOD activity was determined by the method of Beauchamp and Fridovich, 1971. The reaction mixture was reconstituted by mixing 0.5 mL brain supernatant, 1 mL of 50 mM sodium carbonate, 0.4 mL of 25 µM NBT, and 0.2 mL of 0.1 mM EDTA. The reaction was then initiated by addition of 0.4 mL of 1 mM hydroxylamine-hydrochloride. The change in absorbance was monitored spectrophotometrically at 560 nm for every 30 seconds for 2 minutes. The blank control was prepared as same as the test but without sample (tissue homogenate). The SOD activity was calculated following the formula and expressed as the amount of enzyme required to inhibit the reduction of NBT by 50% (Gervason, 2001)

$$\%\ SOD = \frac{OD\ negative\ control - OD\ sample}{OD\ negative\ control} \times 100$$

3.5 Data management and statistical analysis

The quantitative data obtained from the MWM test and the antioxidant assays were compiled on a tabulation sheet (excel) and transferred to a Minitab v20.1 application software. Descriptive analysis was done and indicated as mean ± SEM. Statistical significance among the negative control (normal saline+scopolamine), positive control (Donepezil+scopolamine), and experimental groups (HRKaq extracts of tested concentrations + scopolamine) at 95% confidence level was tested by the use of One-Way ANOVA. Additionally, pairwise comparison and separation of means was done by subjecting the data to Fisher's LSD post hoc test. The unpaired Student *t*-test statistics was used to make differentiation between the activities of the three dose level of the investigated plant extract on the analyzed parameters. Values of $p < 0.05$ were deemed statistically significant.

CHAPTER FOUR:

RESULTS AND DISCUSSION

4.1. Results

4.1.1 Percentage yield of the aqueous root extract of HRK

The percentage yield of the aqueous root extract of *Hypericum revolutum* subspecies *keniense* was *calculated and found to be 18.27%*

4.1.2 The effects of aqueous root extract of HRK on learning/ acquisition memory.

The results for the effects of the aqueous root extract of HRK on learning and acquisition memory in the experimental mice are presented in table 4.1. The results showed that experimental mice in the negative control (treated with normal saline 10ml/kg/bw + scopolamine 1mg/kg bw) recorded the highest latency time than the other experimental groups ($p<0.05$). The positive control (mice treated with Donepezil 1mg/kg) recorded a lower latency time as compared to the normal control($p<0.05$). Additionally, the aqueous root extract of HRK showed a dose dependent activity in which latency time significantly decreased with increasing extract dose with HRKaq 250mg/kg bw recording the lowest whereas HRKaq 10mg/kg bw recorded the highest among the group ($p<0.05$). The activity of HRKaq 250mg/kg bw was significantly higher compared to normal, negative and positive controls ($p<0.05$). However, HRKaq 50mg/kg bw and HRKaq 10mg/kg bw recorded significantly lower activity compared to the positive control drug ($p<0.05$).

The negative control recorded the highest latency time because scopolamine induced oxidative stress which caused cognitive impairment in which spatial memory retention and retrieval was greatly affected. The positive control recorded a lower latency time than the negative and normal controls because donepezil counteracted the activity of scopolamine thus reversing scopolamine induced spatial memory loss. Moreover, the extract was able to reverse scopolamine induced cognitive impairment as witnessed with the lower latency time compared with the negative control. However, Donepezil showed more activity than HRKaq 10mg/kg bw and HRKaq 50mg/kg bw indicating that optimum cognitive – enhancing activity was achieved at HRKaq 250mg/kg bw.

Table 4. 1 Probe trial (learning/acquisition memory)

Treatment	Latency time(seconds)
Negative control	47.723 ± 0.283^{a}
Normal control	7.583 ± 0.870^{d}
Positive control	7.090 ± 0.159^{de}
HKRaq 250mg/kg bwt	5.877 ± 0.362^{e}
HKRaq 50mg/kg bwt	11.497 ± 0.278^{c}
HKRaq 10mg/kg bwt	14.789 ± 0.401^{b}

Values with the same superscript letter are not significantly different by One-Way ANOVA followed by Fishers LSD test ($p > 0.05$); Negative control; Normal saline 1ml/kg + Scopolamine 1mg/kg bwt, Normal control; Normal saline 1ml/kg bwt, Positive control; Donepezil 1mg/kg bwt + Scopolamine 1mg/kg bwt, Experimental 1; HKRaq 250mg/kg bwt + Scopolamine 1mg/kg bwt, Experimental 2; HKRaq 50mg/kg bwt + Scopolamine 1mg/kg bwt, Experimental 3; HKRaq 10mg/kg bwt + Scopolamine 1mg/kg bwt.

4.1.3 Effects of the aqueous root extract of HRK on percentage activity of glutathione

The results for the effects of the aqueous root extract of HRK on percentage activity of glutathione are in table 4.2. At 30 and 60-seconds period, the results showed no significant difference in the percentage activity of glutathione in the positive and normal control groups as well as the negative control and HRKaq 10 mg/kg bw of the extract ($p>0.05$), whereas the mice treated with HRKaq 250 mg/kg and HRKaq 50 mg/kg recorded significant difference in the percentage activity of glutathione ($p<0.05$). Additionally, experimental mice treated with HRKaq 250 mg/kg bw recorded significantly higher percentage activity of glutathione ($p<0.05$) during the same period. There was no significant difference in the percentage activity of glutathione at the 90 seconds in the experimental mice in the negative control, and those treated with HRKaq 50 mg/kg and HRKaq 10 mg/kg ($p>0.05$). However, there was significant difference in the percentage activity glutathione in mice in the normal control, positive control and those treated with HRKaq 250mg/kg ($p<0.05$). At 120 seconds, the mice in the normal control and positive control groups recorded no significant difference in the percentage activity glutathione ($p>0.05$). Similarly, the was no significant difference recorded in the mice treated with HRKaq 50 mg/kg and HRKaq 10 mg/kg ($P>0.05$). However, the mice treated with HRKaq 250 mg/kg and those in the negative control groups recorded significant difference in the percentage activity glutathione ($p<0.05$).

Comparison of the means across the entire study period revealed significant percentage activity of glutathione in mice in the normal control and mice treated with HRKaq 50 mg/kg and HRKaq 10 mg/kg with significantly higher activity being recorded after the 30 seconds ($p<0.05$). In the positive control, the percentage activity of glutathione recorded at 30 and 90 seconds, 60 and 120 seconds was not significantly different from each other respectively ($p>0.05$). For the mice in the negative control, there was no significant difference in the percentage activity of glutathione at 30 and 60 seconds ($p>0.05$). However, at 90 and 120 seconds there was significant difference in the percentage activity glutathione with significantly lower activity being recorded at 120 seconds ($p<0.05$). The mice treated with HRKaq 250mg/kg recorded significantly higher and lower percentage glutathione activity at 30 seconds and 120 seconds respectively ($p<0.05$), while, at 60 and 90 seconds there was no significant difference in the percentage activity of glutathione noted ($p>0.05$).

Table 4. 2 Effects of the aqueous root extract of H. keniense on Glutathione activity

Treatment	Percentage Glutathione activity (% GSH)			
	30 seconds	60 seconds	90 seconds	120 second
Normal Control	$8.2367\pm0.0426^{b}_{a}$	$7.607\pm0.143^{b}_{bc}$	$7.637\pm0.185^{c}_{b}$	$7.2167\pm0.0754^{b}_{c}$
Negative Control	$3.6733\pm0.0917^{d}_{a}$	$3.6767\pm0.0736^{d}_{a}$	$3.2233\pm0.0393^{d}_{b}$	$2.9600\pm0.0451^{c}_{c}$
Positive Control	$8.327\pm0.127^{b}_{a}$	$7.520\pm0.144^{b}_{b}$	$8.350\pm0.217^{b}_{a}$	$7.677\pm0.216^{b}_{b}$
HKR_{aq} (250mg/Kg bw)	$12.670\pm0.302^{a}_{a}$	$11.424\pm0.237^{a}_{b}$	$11.137\pm0.162^{a}_{b}$	$10.053\pm0.107^{a}_{c}$
HKR_{aq} (50mg/Kg bw)	$6.597\pm0.191^{c}_{a}$	$5.427\pm0.165^{c}_{b}$	$3.2600\pm0.0529^{d}_{c}$	$2.2733\pm0.0463^{d}_{d}$
HKR_{aq}(10mg/Kg bw)	$3.667\pm0.187^{d}_{a}$	$3.372\pm0.313^{d}_{ab}$	$2.5967\pm0.0974^{d}_{b}$	$2.0160\pm0.0277^{d}_{c}$

Values are presented as $\bar{x} \pm SEM$; means that have the same superscript letter within the same column are not significantly different by One-Way ANOVA followed by Fisher's LSD test ($p>0.05$) whereas means with dissimilar subscripts alphabets within the same row are significantly different (one way ANOVA followed by Fishers LSD test ($P<0.05$)

4.1.4 Effects of the aqueous root extract of HRK on superoxide dismutase (SOD)

The results for the effects of the aqueous root extract of HRK on the activity of the superoxide dismutase are presented in table 4.2. The percentage activity of SOD at 30 seconds was significantly different in all the experimental groups ($p<0.05$) with the mice in the normal control recording significantly higher percentage activity while the mice in the positive control

recorded significantly lower percentage activity($p<0.05$). After 60 seconds no significant difference in activity was noted in mice in the positive control and the ones treated with HRKaq 250mg/kg and HRKaq 10 mg/kg ($p>0.05$). However, there was significant difference in the percentage activity recorded in mice in the normal control and the mice treated with HRKaq 50 mg/kg ($p<0.05$). At 90 seconds, there was no significant difference activity between the mice in the positive control and those treated with HRKaq 10 mg/kg. However, there was significant difference in activity recorded between the mice in the normal control, those treated with HRKaq 250 mg/kg, and HRKaq 50 mg/kg ($p<0.05$). lastly, at 120 seconds, mice in the normal control group recorded significantly higher percentage activity of superoxide dismutase as compared to all the other groups ($p<0.05$). Mice in all the other treatment groups showed no significant difference in the percentage activity of superoxide dismutase ($p>0.05$).

There was no significant difference in the percentage activity of SOD in mice treated with HRKaq 50 mg/kg and HRKaq 10 mg/kg ($p>0.05$), while those treated with HRKaq 250mg/kg recorded significant difference in activity for all the study time ($p<0.05$). Moreover, there was no significant difference in the percentage activity of superoxide dismutase recorded at 30, 60, and 90 seconds in the mice in the positive control and at 90 and 120 seconds for the normal control mice ($p>0.05$).

Table 4.3 Effects of the aqueous root extract of H. keniense on superoxide dismutase (SOD) activity

Treatment	**Percentage superoxide dismutase activity (% SOD)**			
	30 seconds	60 seconds	90 seconds	120 seconds
Negative Control	00.000±0.000	00.000±0.000	00.000±0.000	00.000±0.000
Normal Control	$53.921\pm0.313^{a}{}_{a}$	$52.497\pm0.126^{a}{}_{b}$	$51.277\pm0.343^{a}{}_{c}$	$50.910\pm0.312^{a}{}_{c}$
Positive Control	$16.557\pm0.543^{e}{}_{a}$	$17.527\pm0.619^{c}{}_{a}$	$16.777\pm0.637^{c}{}_{a}$	$11.777\pm0.552^{b}{}_{b}$
HKR_{aq} (250 mg/Kg bw)	$41.633\pm0.653^{b}{}_{a}$	$23.487\pm0.256^{c}{}_{b}$	$21.480\pm0.0814^{bc}{}_{c}$	$18.573\pm0.143^{b}{}_{d}$
HKR_{aq} (50 mg/Kg bw)	$34.973\pm0.311^{c}{}_{a}$	$37.61\pm8.87^{b}{}_{a}$	$33.0\pm10.5^{b}{}_{a}$	$25.7\pm13.9^{b}{}_{a}$
HKR_{aq} (10 mg/Kg bw)	$23.81\pm1.10^{d}{}_{a}$	$21.36\pm1.68^{c}{}_{a}$	$20.10\pm1.98^{c}{}_{a}$	$20.30\pm1.43^{b}{}_{a}$

Values are presented as $\bar{x} \pm SEM$; means that have the same superscript letter within the same column are not significantly different by One-Way ANOVA followed by Fisher's LSD test

(p>0.05) whereas means with dissimilar subscripts alphabets within the same row are significantly different (one way ANOVA followed by Fishers LSD test; P<0.05)

4.1.5 The effects of the aqueous root extract of HK on the catalase activity

The catalase activity (expressed as hydrogen peroxide concentration) results are presented in table 4.4. The results showed that at zero seconds, the experimental mice treated with HRKaq 10 mg/kg bw significantly increased the level of hydrogen peroxide compared to all the other groups (p<0.05), furthermore, there was significant difference in the levels of the hydrogen peroxide recorded at zero seconds in all the groups (p<0.05). Similarly, at 30 seconds the levels of hydrogen peroxide were significantly higher in the brain tissues in the experimental group of mice treated with HRKaq 10 mg/kg bw. There was also a significant difference in the levels of hydrogen peroxide with mice in the positive control group recording the least concentration (p<0.05). At 60 seconds, the mice treated with HRKaq 10 mg/kg bw) and mice in the negative control group recorded significantly higher and least hydrogen peroxide concentration respectively (p<0.05). However, no significant difference was noted in the levels of hydrogen peroxide recorded in the mice treated with HRKaq 50 mg/kg bw and HRKaq 250 mg/kg bw, positive control and negative control groups (p>0.05).

Comparison across all the study period revealed that there was no significant difference in the levels of hydrogen peroxide between the positive control, negative and normal control at 30 and 60 seconds (p>0.05). Similarly, there was no significant difference in the hydrogen peroxide levels recorded at zero and 30 seconds in the experimental groups HRKaq 50 mg/kg bw and HRKaq 10mg/kg bw (P>0.05). However, HRKaq 250 mg/kg bw showed significant difference in the levels of hydrogen peroxide at 0, 30 and 60 seconds (p>0.05).

Table 4. 4 Effect of the aqueous root extract of H. keniense on the hydrogen peroxide levels

Treatment	Catalase activity/hydrogen peroxide concentration		
	0 seconds	30 Seconds	60 Seconds
Negative control	$0.001283\pm0.000027^{bc}{}_{b}$	$0.001510\pm0.000067^{b}{}_{a}$	$0.001637\pm0.000052^{ab}{}_{a}$
Normal control	$0.000860\pm0.000059^{c}{}_{a}$	$0.000923\pm0.000034^{c}{}_{a}$	$0.000842\pm0.000842^{b}{}_{a}$
Positive control	$0.001572\pm0.000058^{ab}{}_{a}$	$0.000163\pm0.000006^{e}{}_{b}$	$0.000245\pm0.000018^{b}{}_{b}$
HKRaq250mg/kg	$0.000229\pm0.000008^{d}{}_{b}$	$0.000239\pm0.000005^{e}{}_{b}$	$0.000752\pm0.000022^{b}{}_{a}$
HKRaq 50mg/kg	$0.000746\pm0.000078^{cd}{}_{a}$	$0.000641\pm0.000069^{d}{}_{a}$	$0.000379\pm0.000026^{b}{}_{b}$
HKRaq 10mg/kg	$0.001920\pm0.000467^{a}{}_{b}$	$0.004627\pm0.000174^{a}{}_{a}$	$0.00295\pm0.00123^{a}{}_{ab}$

Values are presented as $\bar{x} \pm SEM$; means that have the same superscript letter within the same column are not significantly different by One-Way ANOVA followed by Fisher's LSD test

($p>0.05$) whereas means with dissimilar subscripts alphabets within the same row are significantly different (one way ANOVA followed by Fishers LSD test ($P<0.05$)

4.2. Discussion

From time immemorial plants have been used by man for various purposes including treatment of diseases affecting both man and animal because of the presence of various phytochemicals exhibiting pharmacological and therapeutic properties (Jared,et al 2018). In this regard, a lot of research has been done and confirmed that plants have the potential of exhibiting anti-dementia properties particularly plants such as *Lavandula angustifolia*, *Ginko biloba*, *Melissa officinalis*, *Saliva officinalis* and *Huperzia serrate* (Saki, 2018)

Additionally, the use of herbal and phytochemical remedies such as those used in traditional Chinese medicines have proven to retard the development and progress of AD besides promoting recuperation by acting on different pathological etiologies and possessing anti-inflammatory, anti-amylodogenic and antioxidative properties. Moreover, they have been found to control factors for apoptosis, mitochondrial stress, neurotrophic factors and free radical hunting systems (Cooper & Ma, 2017)

Neurodegenerative disorders such as Alzheimer's disease, Huntington's disease and Parkinson's disease are without cure and are devitalizing diseases mainly affecting the neurons in the brain bringing about degeneration of the neuron structure and activity and ultimately nerve cell death. (Manoharan et al 2016). Furthermore, oxidative stress which occurs when the equilibrium between ROS generation and the antioxidant system is altered, has been linked to the development of incurable disorders such as dementia, movement impairment, depression and cardiovascular disorders due to oxidative damage (Luca & Luca, 2019).

The main feature of AD is the gradual diminishing cognitive activity as a result of neuronal damage and death in the hippocampus the part of the brain that controls memory and spatial inclination (Cheignon et al., 2018)

Cholinergic system has been found to be involved in the pathogenesis of dementia – AD in which agents that promote cholinergic activity improved cognitive function whereas the anticholinergic agents caused memory decline in un-demented brain (Hampel et al., 2018). In fact, the cholinergic hypothesis and the use of AChEI in AD was introduced following an observation done in mid-1970 in AD patients that they have a selective reduction of the activity of the acetylcholine synthetic enzyme, choline acetyltransferase(ChAT) in the hippocampus, a region known to participate in memory functions (Fish et al., 2019),(Hampel et al., 2018). This

“cholinergic hypothesis” was later confirmed from clinical trials demonstrating efficacy for an AChE inhibitor in improving cognition in patients with AD. But it was evident that cholinergic hypothesis would not account fully for the pathophysiology of AD. (Chen & Mobley, 2019),. Therefore, in this study, cognitive deficit was successfully induced by oral administration of scopolamine and is evident by the longest latency time taken to locate the escape platform. Scopolamine is an anticholinergic agent that exerts its action by inhibiting both the central and peripheral muscarinic receptors and is thus used in the evaluation of drugs suspected to be useful in the management of neurodegenerative conditions (Moriasi et al., 2020a). Donepezil is an acetylcholinesterase inhibitor that improves the activity of acetylcholine and thus it counteracts the cognitive deficit induction by scopolamine resulting in improved cognitive function indicated by the shorter latency time (Hampel et al., 2018)

Scopolamine is an inducer of oxidative stress leading to decreased level of antioxidants; superoxide dismutase, glutathione and catalase as seen in the brain tissues of scopolamine treated mice (Noor, et al, 2018). However, the administration of HRKaq counteracted scopolamine induced stress by increasing the levels of antioxidants in a dose dependent manner. Catalase catalyzes the conversion of hydrogen peroxide to water and molecular oxygen and its lower level in the brain is associated with increased oxidative stress manifested by the increased levels of hydrogen peroxide and associated toxicity. Superoxide dismutase converts superoxide anion radicals to hydrogen peroxide besides offering protection to dehydratase from superoxide inactivation, therefore, increased oxidative stress reduces the levels of SOD (Noor et al., 2018). Glutathione plays an important role in the conversion of hydrogen peroxide and its reduced levels due to oxidative stress leads to the development of neurodegenerative disorders (Dwivedi,et al 2020).

CHAPTER FIVE: CONCLUSION AND RECOMMENDATION

5.1 Conclusion

From the results of this study, it was deduced that the aqueous root extract of *Hypericum revolutum* subspecies *keniense* have in vivo cognitive enhancing and ex vivo antioxidant activities on scopolamine instigated cognitively damaged mice models. It was also deduced that the aqueous root extract of *Hypericum revolutum* subspecies *keniense* has the ability to reverse the effects of scopolamine induced oxidative stress.

5.2 Recommendation

This study dwelt only on the oxidative stress and the antioxidant system which is a small part of the wider scope of the pathological mechanisms involved in the development of neurodegenerative disorders including AD. Therefore, recommendation was made that further study should be done on therapeutic agents targeting; Inhibition of Aβ production, inhibition of Aβ formation, promotion of Aβ degradation and clearance, inhibition of NFTs formation, reduction of neuroinflammation and the role of iron accumulation in the brain. Additionally, studies on the safety profile, possible side effects and phytochemistry should be conducted.

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APPENDICES

Appendix 1. The experimental swiss albino mice in their cage

Appendix 2: Setup for biochemical tests

Appendix 3. Morris Water Maze