EFFECT OF FENUGREEK SEEDS ON SHORT TERM MEMORY AND MORPHOLOGY OF CORNU AMMONIS IN FEMALE MICE

By

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ABSTRACT

Effect of Fenugreek Seeds on Short Term Memory and Morphology of Cornu Ammonis in Female Mice

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Information which retained temporarily is known as short term memory. Short term memory means short span of seconds to minutes and small capacity. Currently, there is no relevant research on fenugreek seed (FS) in relation to memory and learning. FS was only known for their therapeutic applications and medicinal effects. In this study, FS was used on female mice to observe the memory and learning effects. FS has oestrogenic actions. Oestrogen tends to enhance memory and learning. FS concentration of 3000 mg/kg was given as daily oral treatment to mice for this research purpose. Treated mice were subjected to two behavioural tasks – passive avoidance test and object recognition test to evaluate the memory and learning performance. Both tests were conducted on day 3, 6, 9 and 16 of treatment. Latency of entering time and recognition index (RI) for FS treated mice has been recorded. Results showed that the latency and RI gradually increase compared to control and norethisterone acetate treated mice. Increasing value in the latency and RI confirms that memory and learning had occurred in FS treated female mice. Memory and learning happened when neurogenesis occurred. New neurons

generated when there is memory and learning process. Neurogenesis in Cornu Ammonis (CA) region is most significant, therefore, morphology of CA region was observed. Many darkly stained immature cells, pyramidal-like cells and blood vessels were formed in CA region of FS treated female mice compare to control treated mice. In conclusion, FS enhances memory and learning in female mice.

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Last but not least, I wish to take this opportunity to express a sense of gratitude to my family for their continuous support.

DECLARATION

I hereby declare that the project report is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at Universiti Tunku Abdul Rahman or other institutions.

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(OOI YI MIN)

APPROVAL SHEET

This project report entitled "**EFFECT OF FENUGREEK SEEDS ON SHORT TERM MEMORY AND MORPHOLOGY OF CORNU AMMONIS IN FEMALE MICE**" was prepared by OOI YI MIN and submitted as partial fulfilment of the requirements for the degree of Bachelor of Science (Hons) in Biomedical Science at Universiti Tunku Abdul Rahman.

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PERMISSION SHEET

I hereby certified that **OOI YI MIN** (ID No: **08ADB03078**) has completed this final year project entitled "**EFFECT OF FENUGREEK SEEDS ON SHORT TERM MEMORY AND MORPHOLOGY OF CORNU AMMONIS IN FEMALE MICE**" under supervision of Mr. Raja Kumar from Department of Biomedical Science, Faculty of Science.

I hereby give permission to my supervisor to write and prepare manuscript of these research findings for publishing in any form, if I did not prepare it within six (6) months time from this date provided that my name is included as one of the author for this article. Arrangement of the name depends on my supervisor.

Yours truly,

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(OOI YI MIN)

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LIST OF ABBREVIATIONS

AMP	Adenosine monophosphate
ANOVA	Analysis of variance
BrdU	Bromodeoxyuridine
CA	Cornu ammonis
DCX	Doublecortin
FS	Fenugreek seeds
GABA	Gamma-aminobutyric acid
IACUC	Institutional Animal Care and Use Committee
IARC	International Agency for Research of Cancer
LD	Lethal dose
LSD	Least significant test
NBC	Neurogenetics and Behaviour Centre
NCA	Nature knowledge centre alternatives to animal use
PBS	Phosphate buffered saline
RI	Recognition index
S.E.M.	Standard error of mean
SBFNL	Stanford Behavioural and Functional Neuroscience Laboratory
SfN	Society for Neuroscience

CHAPTER 1

INTRODUCTION

Short term memory disorder is a disorder affecting visual and verbal memory of recent events meaning those that occur within seconds to minutes of the testing time (Siegel & Sapru, 2007; Landtblom, Dige, Schwerdt, Safstrom & Granerus, 2003). Short term memory disorder is due to dysfunction of the hippocampus of the brain.

At this point, the available treatments for memory disorders are based on Western medicine and Chinese medicine (Auteroche, 1993). So, in this study, fenugreek seed – a common Indian herb and spice are use to evaluate whether it posses the potential to become a treatment for memory disorders in the future. Fenugreek seeds are natural plant without any harmful chemical compound that can become other alternatives of treatment. Nonetheless, fenugreek seeds are unsuitable to be use during pregnancy as it has uterine stimulating properties that will lead to miscarriage (KingTutShop, 2011).

Fenugreek seeds contain diosgenin and other plant phyto-oestrogens which have a reputation in increased breast tissue, breast milk production, lactation and childbirth. Besides, fenugreek seeds also contain choline that helpful for memory loss (The Herbal Resource, 2006). Both diosgenin and phyto-

oestrogen mimic oestrogen actions in increase neurogenesis. Oestrogen plays an important role and shows a greater effect in female. Thus, in this study, only female mice are subjected to the fenugreek seed extract.

Presently, fenugreek seeds are neither use in treatment of memory disorder nor enhancement in short term memory. Neurogenesis, memory and learning are correlated. Fenugreek seeds are only common to Indian society with their traditional uses. Fenugreek seeds should be introduced to the world for its host of benefits. Fenugreek seeds are packed with benefiting nutrients, fibres, minerals and vitamins. So, further study should be carried out to confirm it's hidden value on memory enhancement.

The main hypothesis in this study is fenugreek seeds affect short term memory formation and cause morphological changes in the cornu ammonis region. The objectives in this study are:

To study the effect of fenugreek seeds on short term memory.

To observe and compare the morphology of cornu ammonis in control and fenugreek seed extract treated female mice.

To analyse and compare the behavioural test of control and fenugreek seed extract treated female mice.

CHAPTER 2

LITERATURE REVIEW

2.1 Memory

2.1.1 Overview

Memory is the ability of brain to retain information, experiences, knowledge and skills, which are important for present and future reference (Sutton, 2010). Human brain is capable of storing 100 trillion bits of memory (Begley, Springer, Hager & Jones, 1986). Memory and adult neurogenesis can be enhanced by exercise and environmental enrichment (Wong-Goodrich et al., 2010; Valero et al., 2011) whereas stress reduced adult neurogenesis and impaired memory (Valero et al., 2011). Memory is influenced by drugs and aging (Robertson, 2002). Synaptic rearrangement in neural network and interconnection of neurons form memory (Diana et al., 2006; Robertson, 2002). Thus, adult neurogenesis contributes to memory formation (Aimone, Wiles & Gage, 2009).

2.1.2 Types of Memory

2.1.2.1 Short Term Memory

Short term memory is the ability of brain to hold instantaneous information temporarily up to seven items of individual information in an occasion

(Kumaran, 2008; Brown, 1987). Short term memory lasts for seconds to minutes (Robertson, 2002). Short term memory has a small and limited storage capacity (Zhong, 2003). Working memory is a type of short term memory that requires simultaneous storing and processing of information (Siegel & Sapru, 2007). Short term memory is robust and seldom affected by age, drugs or brain damage (Brown, 1987).

Short term memory does not create a neural mechanism which relies on neural pathways but uses temporal potentiation of neural connection for later recall (Zhong, 2003). Short term memory is independent of cerebral protein synthesis (Davis & Squire, 1984). Short term synaptic plasticity modifies pre-existing protein (Blum, Li, Cressy & Dubnau, 2009). Short term memory has neuron which clusters into a network that can induce into a metastable state to retain information for a short period of time (Johnson, Marro & Torres, 2010). Newly formed neurons will eliminate older connections (Genova, 2009). Therefore, new information will be replaced by existing old information to be stored in the brain (Zhong, 2003; Brown, 1987).

In short term memory, Kandel found that stimulus makes sensory neuron release chemical messenger — serotonin to another neuron. Serotonin binds to outside of neuron and allows cell to produce small regulating molecule — cyclic AMP. Cyclic AMP interacts with protein kinase A, enhances the release of chemical messenger — glutamate to motor neuron. The persistence

of these molecules leads to a heightened sensitivity to stimuli for a short period — a short term memory (as cited in Patel, 2006).

2.1.2.2 Long Term Memory

Long term memory is the ability of brain to store, manage and retrieve information of distance past for later use in life (MedicineNet, 2004). Long term memory also called permanent memory, lasts for months or years (Robertson, 2002). Long term memory has huge and unlimited storage capacity (Zhong, 2003). Long term memory creates a neural pathway to store and recall via long term potentiation which is caused by rehearsal and association (Zhong, 2003). Long term memory is dependent on protein kinase C and brain-derived neurotrophic factor synthesis (Govindajaran, Kelleher & Tonegawa, 2006).

2.2 Learning

2.2.1 Overview

Webster's Dictionary defined learning as a process of gaining new information, experiences, knowledge, ideas and skills from others to the brain (as cited in University of Missouri-St. Louis, 2004). Acquisition and maintenance are the basics of learning. Acquisition stage happens when one learns something new while maintenance stage happens when one act

appropriately on what is learnt (Essortment, 2010). Atkinson et al. proposed that there will be relatively permanent changes in behaviour for successful learning (as cited in Atherton, 2011).

Learning increases survival of immature and matured neurons in the brain (Anderson, Sisti, Curlik II & Shors, 2010). Neurogenesis is important for learning (Dobrossy et al., 2003). A recent animal study shows the correlation between neurogenesis and learning (Society for Neuroscience [SfN], 2007). Neurogenesis enhanced by exercise improves learning (Praag, Shubert, Zhao & Gage, 2005). In cellular basis of learning, studies done by Hawkins et al.; Malenka and Nicoll focus on plasticity, presynaptic and postsynaptic modulation in synaptic transmission (as cited in Burrell, Sahley & Muller, 2001).

On the other hand, a number of experiments have been carried out in United State of America which focused on the behaviour of the animals after the learning process. The factors that influence learning include repetition, reinforcement, conditioning and dependency (The Great Soviet Encyclopaedia, 1979).

2.2.2 Types of Learning

2.2.2.1 Non Associative Learning

Non associative learning is a basic form of learning with the only single events involved (Essortment, 2010). Encyclopaedia Britannica referred non associative learning as the behaviour or response of animals towards a stimulus when there is no any apparent stimulus or event such as rewards or punishment. In other meanings, non associative learning involved events that are not related to the stimulus (Essortment, 2010). Non associative learning is activity dependent in which the response towards a continuous or repetitive stimulus will be either up regulate or down regulate (MacDonald, Song & Poon, 2007). It is categorised into habituation and sensitisation that depend on alteration of early afferent neurons and synapses onto target cells (Fioravante, Antzoulatos & Byrne, 2008).

Habituation is a distinct and simplest form of non associative learning (MacDonald et al., 2007; Best et al., 2007). Habituation is a reversible decrease in behaviour or response magnitude towards continuous and repetitive stimulation (Burrell et al., 2001; Best et al., 2007). Habituation occurs slower when the stimulus is an intense or long interstimulus interval and built up by repeats training (Riddle, Blumenthal, Meyer & Priess, 1997). Habituation is ascribed to activity dependent synaptic depression (Fioravante et al., 2008).

Sensitisation is a more complex form of non associative learning that behaviour or weak stimulus is constantly enhanced by the strong or noxious stimulus (Modley, Sahley & Muller, 1997; Burrell et al., 2001). Sensitisation increased response to stimulus that is repeated (Craighead & Nemeroff, 2002). Hutt; Zaccardi et al. explained that sensitisation can be positive or negative towards a beneficial or harmful stimulus (as cited in Kuba, Byrne & Meisel, 2006). Sensitisation is ascribed to facilitate actions of neuromodulators (Fioravante et al., 2008).

2.2.2.2 Associative Learning

Associative learning is one of the most sophisticated forms of learning which has two stimuli that are associated with one another or a stimulus is associated with an event (Mann, 2008). Encyclopaedia Britannica defined associative learning as a process whereby new response is correlated to a particular stimulus. Associative learning is when an animal learns to use a previous neutral stimulus to predict the presence or absence of the second more significant stimulus (as cited in Riddle et al., 1997). Animal paired two events together and learnt to relate both events together (Mann, 2008). Associative learning occurred through classical conditioning and instrumental conditioning (Griffiths, 2002).

2.3 Neurogenesis

2.3.1 Overview

Neurogenesis is a process of birth of new neurons from neural stem cells (Wellesley College Biology Department, 2005; Klausen, 2008). Neurogenesis takes place in a specific region in the brain (Shen et al., 2008; Chen, Rajkowska, Du, Seraji-Bozorgzad & Manji, 2002). According to MedicineNet, during neurogenesis, neurons, neuroglial, astrocytes, oligodendrocytes, microglial and ependymal cells are actively produced (as cited in Rim, 2010). The four main stages of neurogenesis are cell proliferation, cell migration, cell differentiation and cell survival (Azman, Wan Saudi, Ilhami, Mutalib & Rahman, 2009).

In the early days, it was thought that neurogenesis only occurred in the developing stage of life (Klausen, 2008). Medical scientists even assured that brain cells could not be repaired or replaced when there is any injury or damage and it is fixed early in life (SfN, 2007; Public Library of Science, 2010; Crews & Nixon, 2003). However, a recent research found that neurogenesis actually occurred throughout the whole life of living organisms (Wellesley College Biology Department, 2005).

2.3.2 Adult Neurogenesis

Adult neurogenesis is the growing of new neuronal cells in adult brain (SfN, 2007; Gage, 2002). It is discovered by Joseph Altman and Das in year 1965

(Crews & Nixon, 2003). New neurons, astrocytes and oligodendrocytes from neural stem cells are found in adult brain throughout life (Gage, 2002; Zhao, Deng & Gage, 2008). New neurons need the support from neighbouring cells, nutrients from blood and connections with other neurons to be part of the adult brain (SfN, 2007).

Over the past, adult neurogenesis was found in many brain regions (Fowler, Liu & Wang, 2008). Adult neurogenesis occurred in neurogenic microenvironment, which composed of the subgranular zone of dentate gyrus in the hippocampal formation, subventricular zone of lateral ventricles as well as the olfactory bulb (Gage et al., 1995; Crews & Nixon, 2003; Zhao et al., 2008; Lazarov & Marr, 2010; Fowler et al., 2008; Dupret et al., 2008; Wiskott, Rasch & Kempermann, 2004). Evidence shown adult neurogenesis occurred when foetal tissue was grafted to the adult brain, the damaged adult brain allows foetal tissue to survive and differentiate (Gage, 2002).

Adult neurogenesis plays a role in learning and memory (Lazarov & Marr, 2010; Danzer, 2008). Brain's regions which involve in adult neurogenesis are the regions for learning and memory (SfN, 2007). A decrease in neurogenesis impairs learning and memory (Coremans et al., 2010; GrandPre, 2007; Dupret et al., 2008).

Physiological and pathological activities regulate adult neurogenesis (Zhao et al., 2008). Protein growth factors, hormones, environment, genetic, stress and alcohol are factors that potentially affect adult neurogenesis (Leuner, Glasper & Gould, 2010; Crews & Nixon, 2003; Wellesley College Biology Department, 2005). Adult neurogenesis can be observed by using thymidine analogue, bromodeoxyuridine (BrdU) to measure the rate of neuron cell proliferation and survival (Leuner et al., 2010; Fowler et al, 2008). Adult neurogenesis contributes to be qualitative rather than quantitative (Gage, Kempermann & Song, 2008).

2.4 Hippocampal Formation

2.4.1 Overview

Hippocampal formation is an elongated structure that lies deep within the medial surface of temporal lobe in the brain (Siegel & Sapru, 2007). Hippocampal formation the most studied region of the brain as it has neurons produced throughout the lifetime (Tamminga, Stan & Wegner, 2010; Qiao et al., 2005). Neurogenesis peaks in the hippocampal formation as thousands of new born neurons are mostly found here (Matricon et al., 2010). Teuchert-Noodt; Lehmann & Butz; Gould reviewed that neurogenesis in the hippocampal formation further increases the complexity of structural plasticity to pre-existing neuronal network (as cited in Schaefers, Grafen, Teuchert-Noodt & Winter, 2010). Hippocampal formation is divided into three different

sectors according to fibre connections (Karolinska Institutet, 2010). Fornix serves as the major outflow pathway to another limbic system (Siegel & Sapru, 2007; Karolinska Institutet, 2010). Hippocampal formation comprised of hippocampus, dentate gyrus and subicular cortex (Siegel & Sapru, 2007). Hippocampus involved in memory and learning which engaged in recollection of facts and formation of spatial memories (Karolinska Institutet, 2010).

2.4.2 Dentate Gyrus

2.4.2.1 Anatomy

Dentate gyrus is a thin line of three distinct cortex layers that lies on the upper surface of parahippocampal gyrus (Karolinska Institutet, 2010). Dentate gyrus is organised into a V-shape or U-shape that interlocked with the hippocampus (Siegel & Sapru, 2007). Dentate gyrus is made up of three layers, from superficial to deep consist of molecular, granule cell and polymorphic or hilar layer (Scorcioni, Bouteiller & Ascoli, 2002). Granule cells are the principal neuronal cell in dentate gyrus that forms a discrete granule cell layer (Serress, 1978; Scharfman, Goodman & McCloskey; 2007). External to this layer lays molecular layer. Polymorphic layer composed of modified pyramidal cells that lied deep to the granule cell layer (Siegel & Sapru, 2007). Cajal; No described basket cells are found in granular layer of dentate gyrus (as cited in Serress, 1978). Granule cells and glial cells originated postnatal while basket

cells originated prenatal (Serress, 1978). Basket cells can be found either in hilar border of granule cell layer or molecular layer (Seress & Ribak, 1983).

2.4.2.2 Physiology

Dentate gyrus plays a role as an input centre whereby it receives signals that are transmitted to it via enthorhinal cortex (Karolinska Institutet, 2010). Subgranular zone of dentate gyrus is important for stability and plasticity of the brain (Rodriguez et al., 2008). Precursor cell niche in the subgranular zone must be maintained (Li & Pleasure, 2005). Neurogenesis in dentate gyrus form basal dendrites that involves in recurrent excitatory circuitry (Ribak, 2008). Exposure to an enriched environment further enhanced neurogenesis in dentate gyrus. Environmental enrichment includes expanded learning opportunities, increased social interaction, physical activities and larger housing area (Praag, Kempermann & Gage, 1999). Neurogenesis in dentate gyrus is begun before birth and maintains throughout adult life but cell proliferation in dentate gyrus reduces in old age (Overstreet, Bromberg, Benson & Westbrook, 2005; Perez-Martin et al., 2005).

2.4.3 Cornu Ammonis

2.4.3.1 Anatomy

Cornu ammonis (CA) also known as Ammon's horn (Nieuwenhuys, Donkelaar & Nicholson, 1998). Cornu ammonis is part of the hippocampus

which made up from pyramidal cells (Siegel & Sapru, 2007). Cornu ammonis is arranged in a C-shaped bend, which interlocked with the V-shaped dentate gyrus (Mitra & Mukherjee, 2001). Cornu ammonis can be classified into four fields – CA1, CA2, CA3 and CA4. Classification is according to size and packing density of pyramidal cells (Nieuwenhuys et al., 1998). CA1 field is closest to subiculum; CA4 field located within the hilus of dentate gyrus; CA2 and CA3 fields situated between CA1 and CA4 fields (Siegel & Sapru, 2007). CA1 and CA4 neuronal dendrites are diffused while CA2 and CA3 neuronal dendrites are densely packed (Mitra & Mukherjee, 2001). The vulnerability in CA regions is selective (Kayali, Montie, Rafols & DeGracia, 2005). Cornu ammonis can be divided into six layers – alveus, stratum oriens, stratum pyramidale, stratum radiatum, stratum lacunosum and stratum molecular (Duvernoy, 2005). Cornu ammonis is the latest developed part in a foetus brain whereby it does not exist in the foetal brain on the second and third months (Tiedemann, 1826).

2.4.3.2 Physiology

Cornu ammonis plays a role in principal connections of pyramidal and granular neurons, which pass through the six layers of CA (Duvernoy, 2005). Granule cell axons pass through the polymorphic layer and enter CA as mossy fibres. These mossy fibres then enter synaptic contact with pyramidal cell dendrites (Nieuwenhuys et al., 1998). Recent studies clarified that brain insult invokes neurogenesis at CA region (Ohira, 2010). Three types of glial cells –

microglial, oligodendrocytes and astrocytes are generated in CA region shown significant in neurogenesis (Qiu et al., 2007). Neurogenesis observed in CA region is the important requirement for memory formation (Dong, Csernansky & Goico, 2003).

2.5 Neuroendocrine System

2.5.1 Overview

Neuroendocrine system is the interaction between nervous system and endocrine system in our body (Penn Medicine, 2011). Medical dictionary explained that neuroendocrine system consists of neurons and endocrine cells. Neuroendocrine system controls homeostasis by regulating hormone secretion (Liu & Michaud, 2000). Neuroendocrine had effects on neurogenesis (Gould, 2010). Adult granule cell neurons production and survival depend on neuroendocrine (Hastings, Tanapat & Gould, 2000). Deficiencies in the neuroendocrine system contribute to aging because the endocrine system is important for development while hormones are important for tissue functions (Sonntag, 2002).

2.5.2 Hypothalamus and Pituitary Gland

Hypothalamus and pituitary gland are made up of neuroendocrine system (Sonntag, 2002). Hypothalamus is a master switchboard of the brain that

controls the endocrine system while the pituitary gland is a master gland of the body that controls the endocrine glands (Penn Medicine, 2011). Hypothalamus and pituitary gland work together to integrate responses and influence growth hormone, thyroid stimulating hormone, luteinising hormone, follicle stimulating hormone, prolactin, ocytoxin, antidiuretic hormone and adrenocorticotropin (Sonntag, 2002). Hypothalamus and pituitary gland affects learning and memory (McCormick & Mathews, 2009; Meijer et al., 2004). Hypothalamus and pituitary gland are under the influence of neurotransmitters, which are important for learning and memory (Sonntag, 2002; Lahey & Rosen, 2010).

2.6 Hormones

2.6.1 Overview

Hormones are organic compounds that produced by multicellular organisms and travel through the bloodstream to another part of the body to exert their actions (Barrington, 2011). Hormones are divided into three groups based on their chemical structure – amino acid derivatives, peptide hormones and lipid derivatives (Martini, 2006). Hormones communicate via three kinds of chemical signalling – autocrine, paracrine and endocrine (Kimball, 2011). Hormones act as a chemical messenger and are important to control and regulate the psychological activities (Barrington, 2011). Gonadal hormones influenced the structures and functions of the central nervous system

(Sandstrom & Williams, 2001). Steroid hormones affect learning and memory (Galea, 2010).

2.6.2 Types of Hormone that Influence Memory and Learning

2.6.2.1 Oestrogen

Oestrogen is a primary female sex hormone produced by ovaries for the development of ovaries and breasts (Myers, 2006). Encyclopaedia Britannica defined oestrogen as a group of hormones that influenced the development, maturation and function of reproductive tract. Oestrogen comprised of oestrone, oestradiol and oestriol with oestradiol as the predominant oestrogen (Wallace, 2011).

Oestrogen synthesised in the hippocampus is important for learning and memory (Talebi, Naghdi, Sepehri & Rezayof, 2009). Scientists discovered that oestrogen causes physical changes that bring to increased learning and memory (Shors, 2005). Foster and McKnight discovered oestrogen receptor in the hippocampus that is needed for memory and spatial learning (as cited in University of Florida, 2008). Oestrogen exerts effects on memory and learning via estrogenic receptor (Talebi et al., 2009). Milner explained that the higher amount of oestrogen, more nerve cells connections formed and memory gets stronger (as cited in Lurie, 2003). Oestrogen increased granule cells in dentate gyrus (Sandstrom & Williams, 2001). Nicholas mentioned that oestrogen

protects memory against stress and women's brain has long term protective effect of oestrogen (as cited in Shwartz, 2006).

2.6.2.2 Progesterone

Progesterone is a female hormone synthesised by corpus luteum and placenta to prepare the body for pregnancy (Kimball, 2011). Encyclopaedia Britannica stated that progesterone main function is to regulate the condition of the uterus for menstrual cycle, pregnancy and embryogenesis.

Progesterone is found to enhance learning and memory performance in mice (Frye & Walf, 2010). Progesterone altered brain's physiology that plays a role in learning and memory (Orr, Lewis & Frick, 2009). Progesterone increased neurons survival rate, weight recovery and motor ability (Gibson & Murphy, 2004). Progesterone binds to the intracellular progestin receptor (Frye & Walf, 2010). Singh mentioned progesterone control neuroplasticity and neuroprotection (as cited in Foy, Akopian & Thompson, 2008). Research shows that progesterone protects damaged neuronal cells in central and peripheral nervous system (Stein & Sayeed, 2009). Progesterone regulated synaptic transmission and plasticity in rodent (Foy et al., 2008). Choi et al. shown that progesterone increased synaptic proteins – synaptophysin (as cited in Orr et al., 2009). However, studies examined that chronic administration of progesterone impaired both learning and memory (Orr et al.).

2.7 Fenugreek Seed

2.7.1 Botanical Description

Fenugreek is an erect, self pollinated and robust annual herb (Gopalakrishnan, 2007). Fenugreek can be 60 cm to 90 cm tall (Botanical, 2011). Latin meaning trigonella or Greek meaning trigonon refers to fenugreek leaves, which are trifoliate with 2 cm to 3 cm long (KingTutShop, 2011). Fenugreek flowers are white tinged with violet and further developed into long narrow pods, which contained fenugreek seeds (The Herbal Resource, 2006). Fenugreek seeds are brownish yellow in colour, about 3 mm long, oblong, rhomboidal, strong peculiar odour, with deep furrow dividing them into two unequal lobes (Indian Agro Industry, 2011). Fenugreek roots are a mass of finger structures (HubPages, 2011).

2.7.2 Habitat and Distribution

Fenugreek is grown in areas with cool and dry climate, which have little rainfall (Gopalakrishnan, 2007). Fenugreek grows well in medium texture soil with pH 5.3 to 8.2 (Herbs2000, 2011). Fenugreek is originated from South Eastern Europe, West Asia, eastern Mediterranean and Ethiopia (Botanical, 2011). Fenugreek is then cultivated in Africa, Argentina, China, Egypt, England, France, India, Morocco, Pakistan, Spain, Turkey and United States (The Herbal Resource, 2006).

2.7.3 Chemical Composition

Fenugreek has about 28 % mucilage, 5 % bitter fixed oil, 22 % proteins, a volatile oil, two alkaloids, trigonelline, choline and a yellow colouring substance (Botanical, 2011). Besides that, fenugreek also contains fats, iron, carbohydrates, niacin, potassium, silicon, sodium, thiamine, vitamins A, B and C, phosphates, amino acids, lecithin, nucleoalbumin, alkanes, sesquiterpenes, alkanoles, lactones, diosgenin and saponins (Wise, 2009).

2.7.4 Functions

According to Basch et al., in traditional medicinal use, fenugreek served in keeping body healthy, reduce blood cholesterol, treating cancer, fever, diabetes, anaemia, rickets, allergies, skin irritations, inflammations, arthritis, bronchitis, asthma, sore throat, cough, indigestion, diarrhoea, memory loss, respiratory infections, bad breath and body odour (as cited in Kaviarasan, Naik, Gangabhagirathi, Anuradha & Priyadarsini, 2007). In addition, fenugreek also served in oriental countries culinary use as a supplement, spice and flavouring in food preparation (Kaviarasan et al., 2007).

2.8 Norethisterone Acetate

2.8.1 Overview

Norethisterone acetate is a type of synthetic progestin (Drugs, 2008). Synthetic progestin acts like progesterone produce naturally by the body (WebMD, 2010).

2.8.2 Nomenclature

Norethisterone acetate is also known as norethindrone acetate (International Agency for Research of Cancer [IARC], 2007). The chemical name for norethisterone acetate is 17-hydroxy-19-nor-17α-pregn-4-en-20-yn-3-one acetate (IARC, 2007).

2.8.3 Molecular Formula and Weight

Norethisterone acetate has a molecular formula of $C_{22}H_{28}O_3$ and molecular weight of 340.456 (Naari, 2009).

2.8.4 Properties

Sweetman suggested that noresthisterone acetate appears to be white or creamy white, odourless and in the form of crystalline powder. O' Neil claimed that the melting point of norethisterone acetate to be 161 – 162 °C.

Sweetman also stated that norethisterone acetate is practically insoluble in water but soluble in ethanol, chloroform, dioxane and diethyl ether (as cited in IARC, 2007).

2.8.5 Pharmacokinetics

Norethisterone acetate is rapidly deacetylated to norethisterone and peak in plasma concentration in two-hour times. Norethisterone acetate distributed to albumin and sex hormone binding globulin with 61 % and 36 % respectively. Norethisterone acetate is metabolised by reduction, glucuronide and then sulphate conjugation. Norethisterone acetate is eliminated in urine and faeces (Drugs, 2008).

2.9 Behavioural Test

2.9.1 Overview

Behavioural test is related to neurogenesis (Wahlsen, 2010). Behavioural test is run to assess performances based on the memory and learning ability (Cao et al., 2007). Recognition is required in behavioural test (Lopatina, Liu, Shnayder & Higashida, 2007). Behavioural test is conducted in a special designed space (Cao et al., 2007).

2.9.2 Types of Behavioural Test

2.9.2.1 Passive Avoidance Test

Passive avoidance test is used to study memory and learning based on bright and dark compartment with electric shock (Tsuji, Takeda & Matsumiya, 2002). Passive avoidance test is an exploration based conflict task whereby mice preference clash with a threat (Holmes, 2003). Memory and learning are accessed by measuring the latency of entering (Neurogenetics and Behavior Center [NBC], 2007).

Passive avoidance test has three sessions – habituation, training and testing (NBC, 2007). During habituation, mice were placed in light compartment and door is raised to let mice entered the dark compartment (Tsuji et al., 2002). When mice entered, door is closed and mice is removed shortly after (NBC, 2007). After habituation, second trial is the training (Villard, Espallergues, Keller, Vamvakides & Maurice, 2010). The procedures are same as habituation but with mild electrical foot shock given to mice when mice entered the dark compartment (NBC, 2007; Shan et al., 2009). Lastly, mice were ready for testing. The time taken by mice to enter was measured and defined as latency (NBC, 2007; Park et al., 2010).

2.9.2.2 Object Recognition Test

Object recognition test was introduced in 1988 by Ennaceur and Delacour (Reger, Hovda & Giza, 2009). Object recognition test is used to study memory and learning based on the ability of mice to differentiate old and new objects (Raber & Benice, 2006). Object recognition test is a spontaneous tendency of mice to spend more time on exploring a new object over an old object (Stanford Behavioural and Functional Neuroscience Laboratory [SBFNL], 2011).

Object recognition test has three phases – habituation, familiarisation and testing (Buckley & Sacks, 2007). In habituation, mice were allowed to explore freely in the open field (Reger et al., 2009). Subsequently, in familiarisation, two identical objects were positioned symmetrically at an equal distance (Wang, Li, Dong, Lv & Tang, 2009). Mice were placed in and exposed to these objects. Mice were allowed to explore (Gard, Daw, Sayyad Mashhour & Tran, 2007). Finally, in testing, one original object remained while the other was replaced by new object, which is different in colour, size and shape (Vyawahare & Ambikar, 2010). The times spent by mice in exploration of the familiar objects and novel object were recorded (Buckley & Sacks, 2007).

CHAPTER 3

MATERIALS AND METHODS

3.1 Project Development Schedule

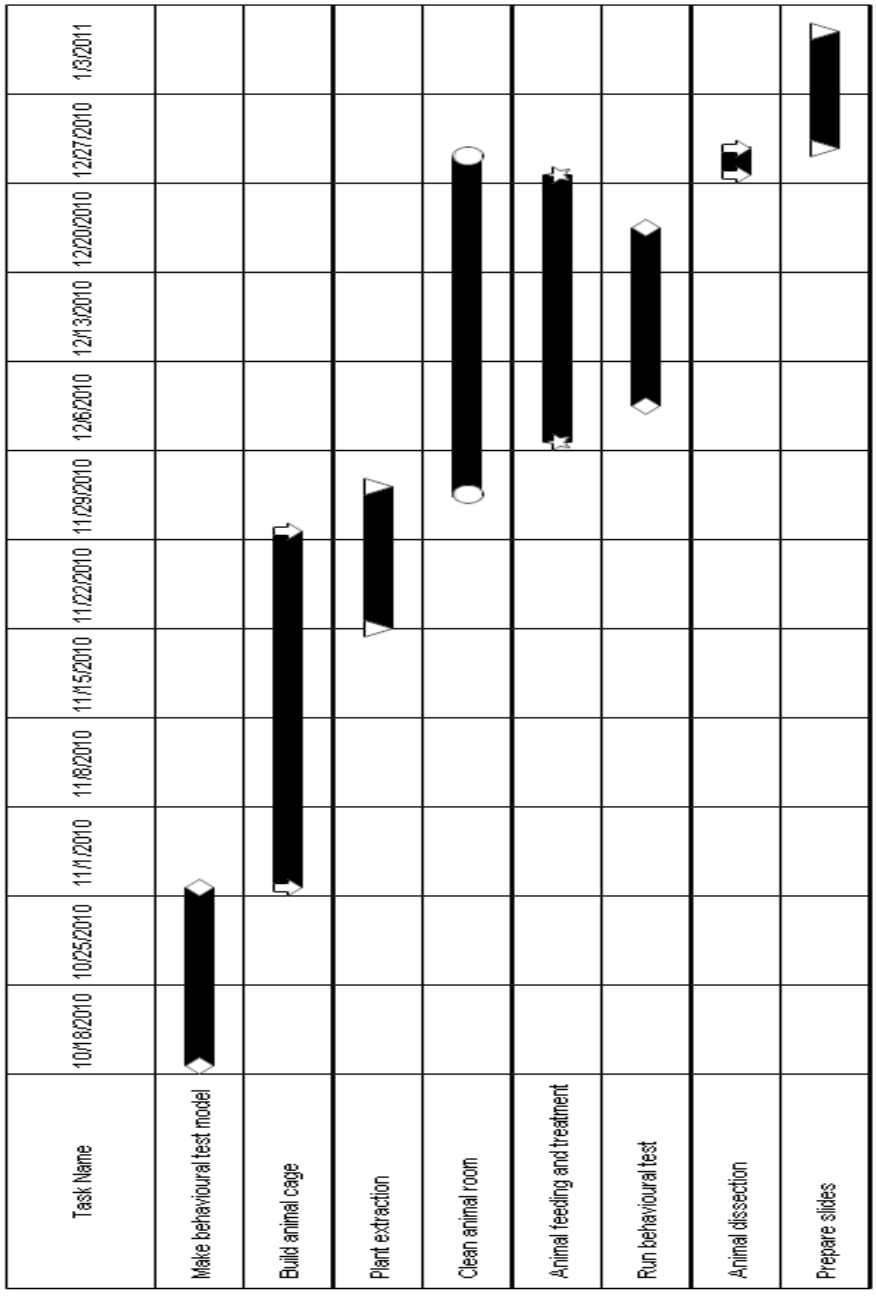

Figure 3.1: Final year project development with the task and duration spent.

3.2 Chemical Reagents

Alcohol solution of 70 %, 75 % and 85 % was prepared from ethanol 95 % purchased from Rank Synergy Sdn. Bhd. Absolute alcohol used was purchased from HmbG Chemicals. Phosphate buffered saline PBS tablets used were purchased from Bio Basic Inc. Xylene used was purchased from HmbG Chemicals. Paraffin used was purchased from Leica Microsystems. 0.1 % eosin Y stain was prepared from eosin Y purchased from Acros Organics. Acetic acid glacial used was purchased from Synerlab.

3.3 Animals

Experimentally naïve Swiss albino mice were purchased from Veterinary Research Institute at Jalan Sultan Azlan Shah, Ipoh, Perak. Twenty four adult female mice aged two months old with an average body weight of 30 – 40 g were used throughout the animal tests. Animals were housed in groups of three to six mice per cage with unlimited access to water and food. The commercial diet pellet and water were replenished daily while sawdust bedding was changed in every two days. Four groups of three mice and two groups of six mice were housed in six different cages. The animals were maintained in a condition of temperature-controlled room with a 12-hour light/dark cycle. All efforts were made to minimise unwanted stress or discomfort to animals.

3.4 Animal Cage

Six animal cages were designed and built using woods, plastic films, coroplast, metal wire net and screws (Appendix B). The animal cage was made in a dimension of 15 inches long × 13 inches width × 7 inches height. Three different size woods were screwed together and removable plastic film was inserted in between two woods. The basement was made using metal wire net and coroplast was layered on top of it. The cage cover was made in a measurement of 16.5 inches × 13 inches. The metal wire net was inserted as the second layer and screwed in between the woods. These customs made animal cages were sterilised before house animals and whenever it is necessary.

3.5 Animal Treatment

Twenty four female mice were divided into six groups with a minimum of three animals and maximum of six animals. These six groups of animals were given three different treatments respectively. Two groups of control animals (six mice) were treated with distilled water; two groups of animals (nine mice) were given oral fenugreek seed extract; two groups of animals (nine mice) were given oral norethisterone acetate solution. Each animal was assumed to drink 10 ml per day from earlier observation. Oral treatments of 60 ml and 30 ml for six mice and three mice respectively were given daily. All treatments

were prepared fresh and given at the same time everyday for the duration of 20 consecutive days.

3.6 Fenugreek Seed Extract

3.6.1 Preparation

Fenugreek seeds were purchased from an Indian spice shop in Kampar, Perak. Seeds were crushed and grinded into small fine pieces. The small fine pieces of seeds were blended to get the powder form. Complete seed powder was obtained and kept in a bottle. Cold water extraction methods were used. Seed extract was made by dissolving 2448 mg of complete seed powder in 240 ml of distilled water. Mixtures of complete seed powder were soaked overnight and placed in the refrigerator. The mixture was filtered and insoluble substances were precipitated on the filter paper. Soluble substances were dissolved and seed extract solution was obtained. Precipitated insoluble substances were discarded.

3.6.2 Dosage

Fenugreek seed has a recommended daily allowance of 6 g/day and LD_{50} of >5 g/kg. However, a dosage of 3.2 g/kg was found to cause liver damage in animals. In this study, a daily dose of 3000 mg/kg was administered as oral treatment to mice. This dosage was used to obtain the maximum effect in a

short period of time without causing any problems to the animals. The average mice weight was measured. A mice was assumed to take in 102 mg of fenugreek extract on a daily basis.

3.7 Norethisterone Acetate Solution

3.7.1 Preparation

Norethisterone acetate 350 µg was purchased from pharmacy in Jalan Idris, Kampar, Perak. Norethisterone acetate tablets were crushed and grinded into a fine powdery form. 2450 µg of fine powdery form of norethisterone acetate were then dissolved in 240 ml of distilled water.

3.7.2 Dosage

Norethisterone acetate has a LD_{50} of 6 g/kg. However, a dosage of 3 mg/kg was found to cause bone marrow damage in animals. In this study, a daily dose of 3000 µg/kg was administered as an oral treatment to the mice. This dosage was used to make sure no harm caused to the animals. The average mice weight was measured. A mice was assumed to take in 102 µg of norethisterone acetate solution on a daily basis.

3.8 Animal Test

Twenty four female mice were subjected to two types of tests. One group of control treated, fenugreek seed extracts treated and norethisterone acetate solutions treated mice were subjected to passive avoidance test while another group was subjected to object recognition test. The passive avoidance test was run on fifteen animals while object recognition test was run on nine animals. Both tests were carried out on day 3, day 6, day 9 and day 16 of treatment by the animals.

3.9 Behavioural Tests

3.9.1 Passive Avoidance Test

3.9.1.1 Apparatus

Passive avoidance test model was designed using Google Sketchup Version 8.0 and made accordingly (Appendix C). A storage box of 33 cm × 25.5 cm × 24 cm was modified into two compartments – a light compartment and a dark compartment. The light compartment was painted with metallic silver colour aerosol spray. A 40 W light bulb was screwed to the light compartment to brightly illuminate the light compartment. Meanwhile, the dark compartment was painted with black colour aerosol spray. The floor of dark compartment was composed of an electric insect bug racket. This zapper was modified into giving only 0.02 mA by using a resistor. The frame of the racket was covered

with coroplast when placed on the dark compartment's floor. A door of 5 cm × 5 cm was made at the bottom of the board separating the two partitions.

3.9.1.2 Protocol

Passive avoidance test was conducted in three phases – habituation, training and testing. The darker compartment was always preferred by female mice. During habituation phase, the animal was placed in light compartment and allowed to explore for 30 seconds. The door was lifted up after 30 seconds. The door was shut after the animal entered the dark compartment with all four paws. Latency of entering time was recorded.

In the second phase, the animal was placed in light compartment and allowed to explore for 30 seconds. The door was lifted up after 30 seconds. The door was shut after the animal entered the dark compartment with all four paws. Latency of entering time was recorded. A mild electrical foot shock was given for 3 seconds when the animal stepped on the zapper with four paws. The animal was removed from dark compartment and allowed to rest for 30 minutes.

Testing phase was conducted after 30 minutes. The animal was placed in light compartment again and allowed to explore for 5 seconds. The door was lifted up after 5 seconds. The door was shut after the animal entered the dark

compartment with all four paws. Latency of entering time was recorded. The cut off time for this test was 300 seconds.

3.9.2 Object Recognition Test

3.9.2.1 Apparatus

Object recognition test model was designed using Google Sketchup Version 8.0 and made accordingly (Appendix D). An open arena with base of 40 cm × 40 cm surrounded by 18.3 cm high wall was made using white colour coroplast and twist tie. Objects of different shapes, sizes, colours and materials were used. Schott's bottle screw cap was used as familiar object. Magic cube, glass bottle, stone and plastic funnel were used as novel object.

3.9.2.2 Protocol

Object recognition was conducted in three phases – habituation, familiarisation and testing. During habituation, a female mice was placed in the open arena withouthing positioned and allowed to freely explore for 30 seconds.

In familiarisation phase, two identical objects were positioned in the open arena. The animal was places in the open arena and allowed to freely explore for 300 seconds. The animal was removed after 300 seconds.

Testing phase was conducted an hour after the training. A familiar object was replaced with a novel object in the open arena. Another familiar object was remained. The animal was placed in the open arena again and allowed to freely explore for 300 seconds. Time spent on exploration of the familiar objects and novel object were recorded.

Object recognition index was calculated based on [Time spent on exploration of novel object / (Time spent on exploration of familiar object + Time spent on exploration of novel object)] × 100. Object placement was counterbalanced with one another. Exploration was defined as looking, approaching, sniffing or touching the object with nose or forepaws in a distance of ≤ 2 cm. Sitting or walking around the object was not considered.

3.10 Animal Dissection

Twenty four female mice were killed with cervical dislocation. Cervical dislocation was a simple, fast, painless and humane technique approved by Institutional Animal Care and Use Committee (IACUC). The animal's neck or cervical spine area was pressed on the table and tail was pulled to stretch out the animal. The animal's spinal cord was dislocated from the skull. The animal's head was cut and brain was taken out.

3.11 Animal Tissue Processing

Phosphate buffered saline (PBS) was prepared by mixing one PBS tablet of pH 7.4 ± 0.1; phosphate 10 mM; NaCl 137 mM; KCl 2 mM with 100 ml of deionised water. The brain removed was cleaned and washed with PBS solution. Then the brain was placed in a labelled cassette and soaked in 10 % formalin for 24 hours. The exact architecture and elements of brain was preserved permanently by fixation.

After 24 hours of fixation, the brain was taken out from cassette and cut into half. The brain was placed back into the cassette again for water removal. The brain was soaked in 10 % formalin for two hours, followed by 75 % alcohol for an hour, 85 % alcohol for an hour, 95 % alcohol for an hour and absolute alcohol for two hours. These steps were known as dehydration. Next, the brain was soaked in xylene for two hours. This step was known as dealcoholisation. All soaking steps were done on a shaker.

Histowax for processing and embedding was in pellet form with the melting point of 54 – 56 °C. A mixture of purified paraffin and microcrystallising waxes were contained in this medium. The paraffin wax was melted by placing in the oven with the temperature of 60 °C. Finally, the brain was soaked in melted paraffin for 30 minutes. The brain was taken out from the cassette and embedded into the block manually. The brain was properly aligned in the block of paraffin. Embedding process was done in the oven so

as the paraffin wax will not harden. The embedded brain was taken out from the oven and placed in room temperature. Subsequently, it was transferred and stored in the freezer. The paraffin block was cold and hardened to make it solid enough for sectioning with the microtome.

Once the paraffin wax block fully hardened, the embedded brain was subjected to sectioning. The surface of embedded brain was sliced using the microtome. The embedded brain was sliced into 3 µm paraffin ribbon. The sliced paraffin appears as a thin ribbon was floated on water bath with a temperature of 50 °C. The sections cut were floated on it to make it fold free or to remove wrinkles. The paraffin ribbon was picked and placed on microscopic glass slide.

3.12 Animal Tissue Staining

Glass slide was placed in the oven with a temperature of 60 °C for 5 minutes. The paraffin wax remained on the slide was melted. The brain's section was adhered to the slide firmly.

The embedding process was reversed. The slice was dipped in xylene for 5 minutes, followed by absolute alcohol for 3 minutes, 95 % alcohol for 3 minutes, 85 % alcohol for 3 minutes and 75 % alcohol for 3 minutes. Then, the section was exposed to running water.

Routine stain – haematoxylin and counterstain eosin were used in staining tissues. Few drops of haematoxylin were dripped on the slides until the section was fully covered. Haematoxylin was allowed to stay on the slide for 5 minutes. After 5 minutes, the section was rinsed under running tap water. 0.1 % eosin Y stain was prepared by adding 0.05 g of eosin Y powder, 50 ml of 70 % alcohol and 0.2 ml of glacial acetic acid. Few drops of eosin stain were dripped on the slides until the section was fully covered. Eosin was allowed to stay on the slide for 10 minutes. After 10 minutes, the section was rinsed under running tap water. The slide was air dried.

Haematoxylin and eosin stained section on the slide was covered with a thin piece of a glass cover slip. The cover slip was purposed to protect the tissue from being scratched, provide better optical quality for viewing under the microscope and to preserve the tissue for years to come. The stained slide was taken through the reverse process of deparaffinisation. The stained slide was dipped in 75 % alcohol for 2 seconds, followed by 85 % alcohol for 5 minutes, 95 % alcohol for 5 minutes, absolute alcohol for 5 minutes and xylene for 5 minutes. Again, these steps were performed to remove water. Lastly, a drop of deepex was dripped to the slide and cover slip was glued onto it.

3.13 Image Analysis

Slide was observed under the fluorescence microscope. The nuclei were visualised using haematoxylin stain while the cytoplasm was visualised using eosin stain. Cells formed in the hippocampus CA3 and CA4 regions were counted.

3.14 Data Analysis

SPSS version 16.0 was used. Data from both tests were analysed with two-way analysis of variance (ANOVA) followed by Fisher's Least Significant Test (LSD) for comparison. P values <0.05 were considered significant and accepted.

CHAPTER 4

RESULTS

4.1 Passive Avoidance Test

Passive avoidance test assessed the effects of various treatments on memory and learning performance of Swiss albino mice as shown in Figure 4.1. Mean, $\bar{x}$, standard deviation, σ and standard error of mean, S.E.M. of latency is shown in Table 4.1.

In two-way ANOVA, analysis of the overall performance shows that there were significant differences of latency between days and between treatments. Significant main effect was obtained for day, [$F(3,47)=9.389$, $p<0.001$] and treatment, [$F(2,47)=10.853$, $p<0.001$]. The effect size is not convincing for days (Partial Eta Squared=0.375) and treatments (Partial Eta Squared=0.316). However, no significant interaction of both day and treatment with latency of mice entering the dark compartment [$F(6,47)=0.78$, $p=0.589$]. The effect size is not convincing too (Partial Eta Squared=0.091). This indicates that the differences between days are not dependent on treatments. In post hoc LSD, the fenugreek seed treated mice ($p=0.352$) do not show significant effect but norethisterone acetate treated mice ($p=0.009$) show a significant effect as compared to control treated mice.

Performance on each day is analysed to know the effects of treatments on latency. On day 3, analysis show no significant differences [F(2,11)=2.184, p=0.159] without convincing effect size (Partial Eta Squared=0.284). Fenugreek seed treated mice (p=0.696) and norethisterone acetate treated mice (p=0.095) are not significant as compared to control treated mice (Figure 4.1). On day 9, analysis show no significant differences [F(2,12)=2.848, p=0.097] without convincing effect size (Partial Eta Squared=0.322). Fenugreek seed treated mice (p=0.411) are not significant but norethisterone acetate treated mice (p=0.046) are significant as compared to control treated mice (Figure 4.1). On day 12, analysis show significant differences [F(2,12)=9.513, p=0.003] with a standard effect size (Partial Eta Squared=0.613). Fenugreek seed treated mice (p=0.049) and norethisterone acetate treated mice (p=0.001) are significant as compared to control treated mice (Figure 4.1). On day 16, analysis show significant differences [F(2,12)=5.415, p=0.021] with a standard effect size (Partial Eta Squared=0.474). Fenugreek seed treated mice (p=0.007) and norethisterone acetate treated mice (p=0.02) are significant as compared to control treated mice (Figure 4.1).

On day 3 to day 9, analysis show no significant differences between day [F(1,23)=2.499, p=0.128] without convincing effect size (Partial Eta Squared=0.098) but significant differences between treatment [F(2,23)=4.99, p=0.016] without convincing effect size (Partial Eta Squared=0.303). The interaction between both are not significant [F(2,23)=0.063, p=0.939] without

convincing effect size (Partial Eta Squared=0.005). On day 9 to day 12, analysis show a significant difference between day [F(1,24)=4.385, p=0.047] without convincing effect size (Partial Eta Squared=0.154) and treatment [F(2,24)=7.330, p=0.003] without convincing effect size (Partial Eta Squared=0.379). The interaction between both is not significant [F(2,24)=0.22, p=0.804] without convincing effect size (Partial Eta Squared=0.018). On day 12 to day 16, analysis show no significant differences between day [F(1,24)=1.138, p=0.297] without convincing effect size (Partial Eta Squared=0.045) and very significant differences between treatment [F(2,24)=12.26, p<0.001] with a standard effect size (Partial Eta Squared=0.505). The interaction between both is not significant [F(2,24)=2.521, p=0.101] without convincing effect size (Partial Eta Squared=0.174).

Performance on each treatment is analysed to know the effects of days on latency. For control, analysis showe no significant differences [F(3,8)=2.872, p=0.103] with a standard effect size (Partial Eta Squared=0.519). For fenugreek seed, analysis showed significant differences [F(3,19)=7.370, p=0.002] with a standard effect size (Partial Eta Squared=0.538). For norethisterone acetate, analysis showed no significant differences [F(3,20)=2.104, p=0.132] without convincing effect size (Partial Eta Squared=0.240).

Table 4.1: Latency of entering in female mice treated with control, fenugreek seed and norethisterone acetate on day 3, day 9, day 12 and day 16.

Treatment		Day 3	Day 9	Day 12	Day 16
Control	n	3	3	3	3
	$\bar{x}$	80.7	117.7	186	205
	σ	85.5	56.2	40.6	45.4
	S.E.M	49.3	32.5	23.1	26.2
Fenugreek seed	n	5	6	6	6
	$\bar{x}$	107.4	174.2	244.5	293.8
	σ	61.7	113.2	52.7	14.1
	S.E.M	27.6	46.2	21.5	5.8
Norethisterone acetate	n	6	6	6	6
	$\bar{x}$	198.3	265.7	300.0	279.0
	σ	110.9	84.1	0	51.4
	S.E.M	45.3	34.3	0	21.0

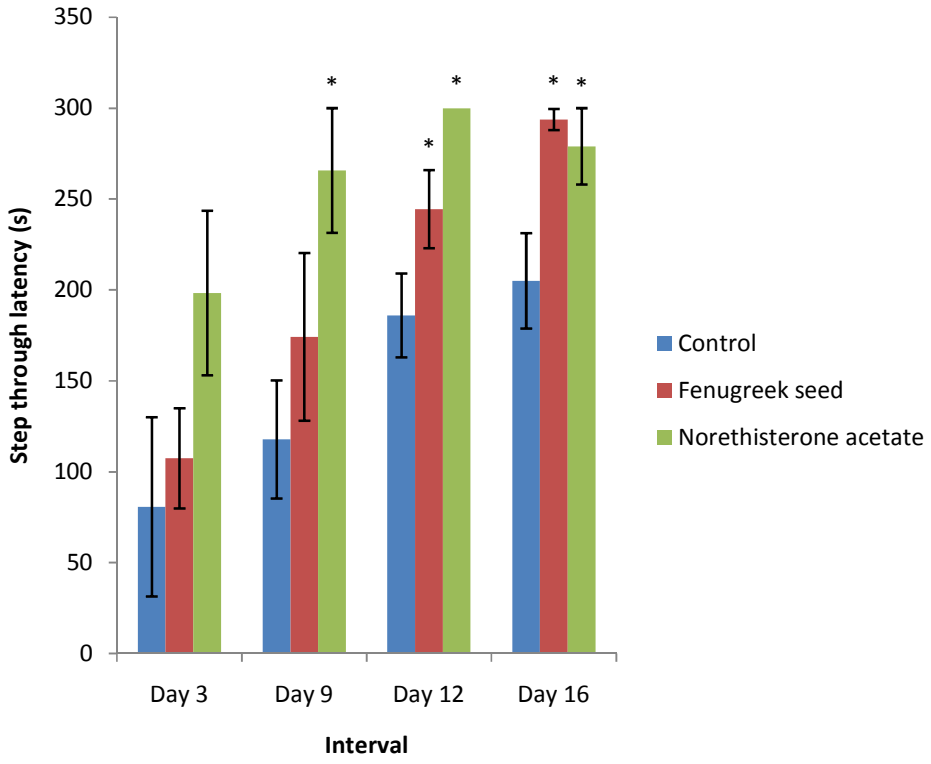

Figure 4.1: Effect of control, fenugreek seed and norethisterone acetate on short term memory performance as determined using passive avoidance test. Results are expressed as mean ± S.E.M. latency to enter dark compartment on day 3, day 9, day 12 and day 16 with n=3-6/group. *p<0.05 compared to control.

4.2 Object Recognition Test

Object recognition test assessed the effects of various treatments on memory and learning performance of Swiss albino mice as shown in Figure 4.2. Mean, $\bar{x}$, standard deviation, σ and standard error of mean, S.E.M. of latency is shown in Table 4.2.

In two-way ANOVA, analysis of the overall performance shows that there were no significant differences of recognition index between days [F(3,20)=1.138, p=0.385] and between treatments [F(2,20)=2.337, p=0.122]. The effect size is not convincing for days (Partial Eta Squared=0.146) and treatments (Partial Eta Squared=0.189). In addition, there is also no significant interaction of both day and treatment with recognition index of mice [F(6,20)=0.588, p=0.736]. The effect size is not convincing too (Partial Eta Squared=0.15). This indicates that the differences between days are not dependent on treatments. In post hoc LSD, the fenugreek seed treated mice (p=0.41) do not show significant effect but norethisterone acetate treated mice (p=0.03) show a significant effect as compared to control treated mice.

Performance on each day is analysed to know the effects of treatments on recognition index. On day 3, analysis show no significant differences [F(2,4)=0.82, p=0.503] without convincing effect size (Partial Eta Squared=0.291). Fenugreek seed treated mice (p=0.789) and norethisterone acetate treated mice (p=0.277) are not significant as compared to control

treated mice (Figure 4.2). On day 9, analysis show no significant differences [F(2,6)=0.525, p=0.617] without convincing effect size (Partial Eta Squared=0.149). Fenugreek seed treated mice (p=0.681) and norethisterone acetate treated mice (p=0.347) are not significant as compared to control treated mice (Figure 4.2). On day 12, analysis show significant differences [F(2,5)=10.186, p=0.017] with a great effect size (Partial Eta Squared=0.803). Fenugreek seed treated mice (p=0.8) are not significant but norethisterone acetate treated mice (p=0.013) are significant as compared to control treated mice (Figure 4.2). On day 16, analysis show no significant differences [F(2,5)=0.047, p=0.954] without convincing effect size (Partial Eta Squared=0.019). Fenugreek seed treated mice (p=0.802) and norethisterone acetate treated mice (p=0.975) are not significant as compared to control treated mice (Figure 4.2).

On day 3 to day 9, analysis show no significant differences between day [F(1,10)=0.499, p=0.496] without convincing effect size (Partial Eta Squared=0.048) and treatment [F(2,10)=1.223, p=0.335] without convincing effect size (Partial Eta Squared=0.197). The interaction between both is not significant [F(2,10)=0.02, p=0.98] without convincing effect size (Partial Eta Squared=0.004). On day 9 to day 12, analysis show no significant difference between day [F(1,11)=2.013, p=0.184] without convincing effect size (Partial Eta Squared=0.155) but significant differences between treatment [F(2,11)=4.098, p=0.047] with a standard effect size (Partial Eta

Squared=0.427). The interaction between both is not significant [F(2,11)=0.859, p=0.45] without convincing effect size (Partial Eta Squared=0.135). On day 12 to day 16, analysis show no significant differences between day [F(1,10)=0.382, p=0.55] without convincing effect size (Partial Eta Squared=0.037) and very significant differences between treatment [F(2,10)=1.172, p=0.349] without convincing effect size (Partial Eta Squared=0.19). The interaction between both is not significant [F(2,10)=1.604, p=0.249] without convincing effect size (Partial Eta Squared=0.243).

Performance on each treatment is analysed to know the effects of days on recognition index. For control, analysis showed no significant differences [F(3,6)=0.278, p=0.84] without convincing effect size (Partial Eta Squared=0.122). For fenugreek seed, analysis showed no significant differences [F(3,7)=0.284, p=0.836] without convincing effect size (Partial Eta Squared=0.109). For norethisterone acetate, analysis showed significant differences [F(3,7)=4.759, p=0.041] with a standard effect size (Partial Eta Squared=0.671).

Table 4.2: Recognition index of female mice treated with control, fenugreek seed and norethisterone acetate on day 3, day 9, day 12 and day 16.

Treatment		Day 3	Day 9	Day 12	Day 16
Control	n	3	3	2	2
	$\bar{x}$	58.4	62.7	67.1	69.8
	σ	10.6	18.3	8.8	19.1
	S.E.M	6.1	10.6	6.2	13.5
Fenugreek	n	2	3	3	3
seed	$\bar{x}$	61.2	67.4	68.8	74.1
	σ	12.1	11.6	7.8	24.0
	S.E.M	8.6	6.7	4.5	13.8
Norethisterone	n	2	3	3	3
acetate	$\bar{x}$	70.9	73.9	91.2	70.3
	σ	10.3	9.0	4.8	7.1
	S.E.M	7.3	5.2	2.8	4.1

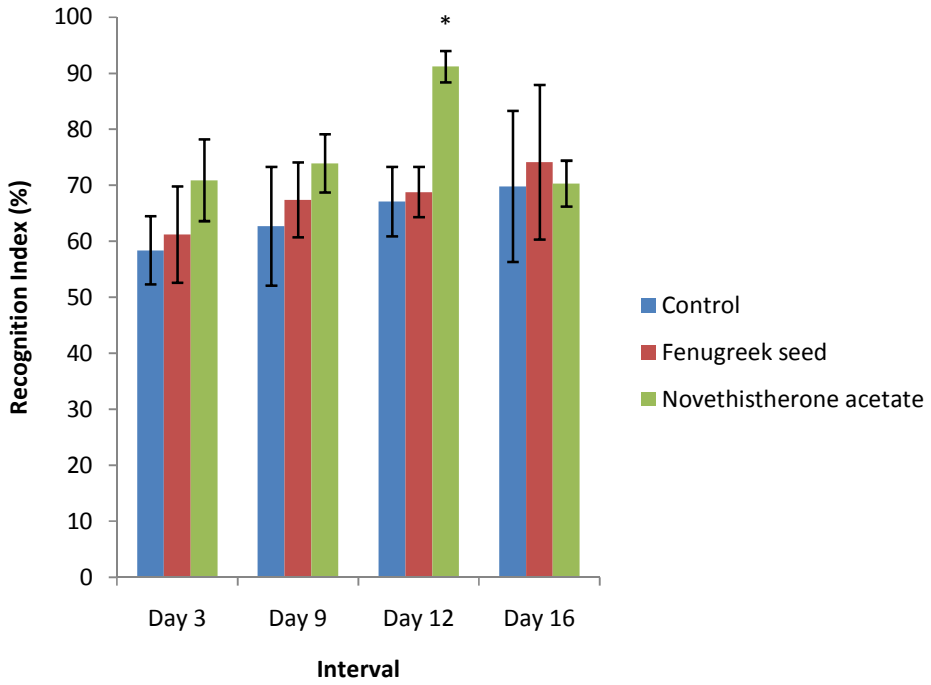

Figure 4.2: Effect of control, fenugreek seed and norethisterone acetate on short term memory performance as determined using object recognition test. Results are expressed as mean ± S.E.M. recognition index on day 3, day 9, day 12 and day 16 with n=3/group. *p<0.05 compared to control.

4.3 Morphology of Cornu Ammonis

Morphological changes in cornu ammonis assessed the effect of fenugreek seeds in neurogenesis of female mice. Cornu ammonis of hippocampus region in control and fenugreek seeds treated mice is shown in Figure 4.3 and Figure 4.4.

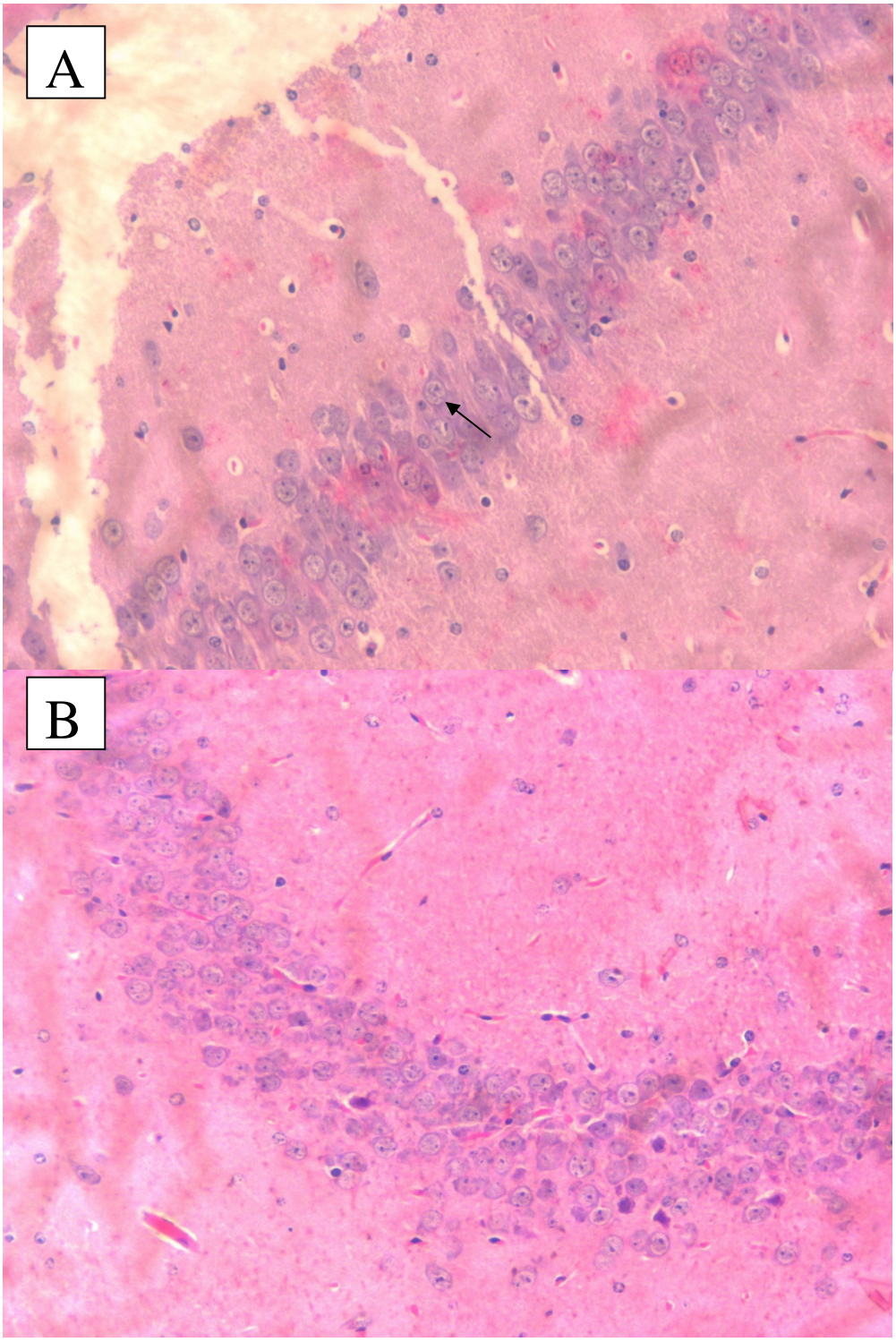

Figure 4.3: Photomicrographs of morphological changes in cornu ammonis of control (A) and fenugreek seed (B) treated female mice under 200 × magnifications using haematoxylin and counterstain with eosin. Arrow showing darkly stained immature cell.

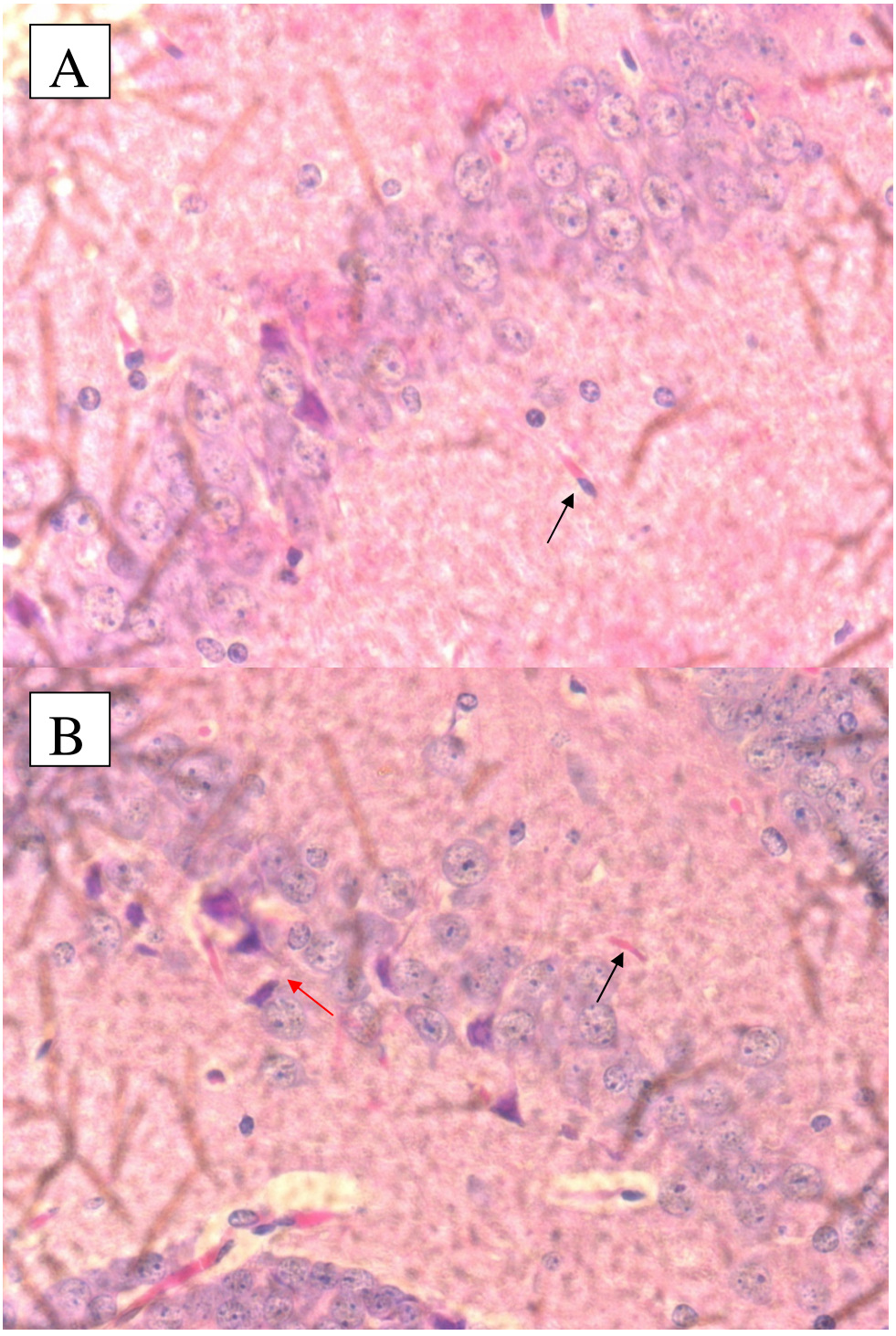

Figure 4.4: Photomicrographs of morphological changes in cornu ammonis of control (A) and fenugreek seed (B) treated female mice under 400 × magnifications using haematoxylin and counterstain with eosin. Red arrow showing pyramidal-like cell while black arrow showing blood vessel.

The number of darkly stained immature cells, pyramidal-like cells and blood found are shown in Table 4.3.

The number of darkly stained immature cells in control is fewer than in fenugreek seed treated mice. There is a big difference of 112 cells between them. As for pyramidal-like cells, it is only found in fenugreek seed treated mice. Number of blood vessels is lesser in control and more in fenugreek seed treated mice.

Table 4.3: Number of darkly stained immature cells, pyramidal-like cells and blood vessels found in cornu ammonis of control and fenugreek seed treated female mice.

	Control	**Fenugreek seed**
Number of darkly stained immature cells	184	296
Number of pyramidal-like cells	-	8
Number of blood vessels	6	9

CHAPTER 5

DISCUSSION

5.1 Animal Handling

5.1.1 Housing and Care

Housing system should fulfil the behavioural and physiological needs of animals. Mice are social animals that live in a group. Therefore, housing animal in a group with some space given to each mice is preferable since the mice also have a minimal space requirements. Mice were housed in the animal cage with a minimum of three and maximum of six. Mice should be protected against environmental influences such as noise, light, humidity and temperature as all these will cause stress to the mice (National Knowledge Centre Alternatives to animal use [NKCA], 2009). Light/dark cycle in animal room is consistent with biology of mice for them to perform their normal behaviour. Temperature is maintained in animal room so that mice can adapt well. Bedding is use for absorption of urine, insulation and also provides the mice a sense of security. Bedding need to change once in two days so that it will not cause any infection to the mice due to the dirty bedding.

Animal cage wall is built with removable plastic film. This removable plastic film can be replaced when necessary. If there is mouse dies in the cage, the removable plastic film will be discarded and a new plastic film is inserted. By

replacing a new plastic film, the other mice staying in the cage will not be infected by the deceased mice. However, proper animal cage that use in the laboratory for housing mice should be made of polycarbonate, which can be autoclave. Autoclave animal cage makes sure the mice can live in a sterile condition. Steam autoclave is often used to sterilise the polycarbonate cage. Autoclaved cage will be cooled down for a period of time before housing animals (Ward, Cole, Faerber & Hankenson, 2009).

5.1.2 Feeding

Mice must receive adequate nutrition in their diet to perform better. Food and water are available ad libitum. Commercially available pellet diet enables mice to wear down their continuously growing teeth (University of Minnesota Board of Regents, 2003). Mice are susceptible to water deprivation or dehydration. Contaminant poses health risk to animals. Thus, food is sealed and store in a contained at a cool place while water is prepared fresh daily to avoid any unwanted contamination. Food and water left overnight will be discarded and refill with new one. Water bottle is cleaned every day before refill with fresh water. Precautions are taken during food preparation to prevent chemical, physical and microbial contamination to ensure the food is safe to be consumed.

5.2 Test Arena and Apparatus

Passive avoidance test and object recognition test arena is always wiped with 70 % alcohol. Every time after habituation, training and testing phase, the arena and object must be cleaned. The odour, urine and faeces deposited by the mice should be getting rid of. These will influence the performance of the animal itself and also other animals that are subjected to the test later.

5.3 Cold Water Extraction

Fenugreek seed extracts are based on cold water extraction because of its solubility in cold water. Water which is polar will dissolve polar substances for use in experiment. Cold water extraction is able to extract simple sugar, oligosaccharides and fructosans (Ross, 1978). Fenugreek seed powder is soaked in cold water and store in the refrigerator for 24 hours before filtration so that the alkaloid can be dissolved (Icktongo, 2007).

5.4 Histological Technique

5.4.1 Tissue Processing

5.4.1.1 Fixation

The purpose of fixation is to preserve the exact architecture or elements of tissues such as the surface proteins, enzymes and nucleic acids as well as possible. Fixation enables tissue to be in permanent life like state or native

state. Fixation should be done immediately after cleaning and washing with PBS solution to prevent autolysis. Autolysis is rapid in brain tissue. In fixation, tissues are fixed by cross linkage of the lysine and amide group of proteins (Bancroft & Gamble, 2008). Standard 10 % formalin is use instead of higher concentration. High concentration of formalin will cause artefacts that similar to excessive heat (Klatt, 2011). Formalin volume is 10:1 ratio of tissue for ideal fixation. Brain's tissue which is rather big should be immersed in formalin for 24 hours in order to be fixed thoroughly. Twenty four hours is the maximum duration and prolong immersion will lead to crystal formation.

5.4.1.2 Dehydration

In dehydration, water from tissue is removed and the tissue hardened. Removal of water enables the tissue to be paraffinised. Wet fixed tissue cannot be infiltrated with paraffin. Paraffin are non aqueous and cannot infiltrate tissue that contains water. To remove the water, tissue is immersed in alcohol. Dehydration is best performed by sequential immersion in increasing alcohol concentration from 75 %, 85 %, 95 % to absolute (Klatt, 2011). Without immediate immersing in alcohol when transfer from one concentration to another, cells in tissue will lose its integrity.

5.4.1.3 Dealcoholisation

In dealcoholisation, alcohol from previous dehydration step is clean with xylene. Xylene is a liquid solvent which compatible with paraffin, meaning that xylene is miscible with paraffin (An & Martin, 2003). Removal of alcohol enables tissue to completely receive paraffin.

5.4.1.4 Sectioning

In sectioning, tissue is sliced into 3 microns to enable visualisation under the microscope. Tissue that is too thick is not observable. Thin sections are required for a detail cellular and composition observation. Thin sections also create lesser overlapping (An & Martin, 2003).

5.4.2 Tissue Staining

5.4.2.1 Deparaffinisation

In deparaffinisation, the embedding steps are reverse. Tissue is immersed again into xylene and alcohol to dissolve the wax. Dewaxing enables water soluble dyes to penetrate into the tissue. From xylene, absolute, 95 %, 85 %, back to 75 % alcohol followed by running under water. Section is brought to water to make sure that staining can be done (Klatt, 2011).

5.4.2.2 Haematoxylin and Eosin

Haematoxylin and eosin are the most common stains. Haematoxylin is a basic dye while eosin is a acidic dye. Haematoxylin has an affinity for nucleic acids whereby it stains nuclei into bluish purple. Eosin has an affinity towards cytoplasmic components of cell whereby it stain cytoplasm into red. Since both stain stay on a different part of the tissue, eosin is known as counterstain of haematoxylin (An & Martin, 2003).

5.5 Behavioural Test

In the present study, behavioural tests are performed in order to determine the effects of fenugreek seeds on short term memory of the female mice. Memory and learning occurred are evaluated by the performance in behavioural tests. Passive avoidance test and object recognition tests with testing after 1 hour of training enable the formation of short term memory and learning process. Short term memory requires perceptive acuity, memory span and voluntary attention (Auteroche, 1993).

Female mice were expected to show similar levels of short term memory in both behavioural tests. In passive avoidance test, the memory was measured in step through latency while in the object recognition test, the memory was measured in recognition index. Results for both tests are found to be similar. Animals treated with the fenugreek seed extract has a gradual increase in the

short term memory from day 3 to day 16. In contrast to control treated animals, there is also formation of short term memory, but it is in a slower rate. As for animals treated with norethisterone acetate, short term memory increases sharply from day 3 to day 12 and decreases drastically thereafter. Norethisterone acetate treated mice act as a positive control in tests.

Fenugreek seed extract and the norethisterone acetate solutions contained female hormone oestrogen and progesterone respectively. Female hormones are important for memory and learning (Talebi, et al., 2009; Frye & Walf, 2010). Female hormones modulate adult neurogenesis in cornu ammonis of the hippocampus. Therefore, fenugreek seeds and norethisterone acetate exerts an effect on short term memory. However, norethisterone acetate impaired short term memory after a prolong consumption in mice (Orr et al.).

Oestrogen acts on the central mechanism IGF-1 system. Oestrogen provides neuroprotection. The mitogen activated protein kinase and phosphatidyl-inositol-3-kinase gives oestrogen the properties of neuroprotection. The co-activation of signalling pathway induces neuronal cell maturation and protects them from apoptosis (Quesda, Lee & Micevych, 2008). Oestrogen is also an upstream mediator of the brain-derived neurotrophic factors.

Progesterone reduced metabolite – allopregnanolone is an allosteric modulator for gamma-aminobutyric acid (GABA) chloride ion channel, which serves as

the selective target for neurogenesis. Allopregnanolone activates GABA type A receptor and increases intracellular calcium in the hippocampus. Allopregnanolone is a neurogenic factor that promotes neuronal cell mitosis and proliferation (Brinton & Wang, 2006).

5.6 Morphological Changes

In the present findings, fenugreek seed extracts increased number of darkly stained immature cells, pyramidal-like cells and blood vessels in cornu ammonis of the female mice compared to control. Staining with haematoxylin and eosin suggested that the increase in the number of cells is mainly due to increase in neurogenesis.

Enhancing effect of fenugreek seed extract on female mice is found. Neurogenesis in cornu ammonis is a region whereby memory and learning take place (Dong, Csernansky, Goico & Csernansky, 2003). Pyramidal-like cell with processes is found in fenugreek seed extract treated mice but not in control treated mice. Pyramidal cell is a prominent nerve cell that forms main output neurons in the hippocampal formation by integrating input from converging neural pathways (Siegel & Sapru, 2007). Pyramidal cell projects fibre to other cortical regions of the brain. Pyramidal cell dendrite is important for establishment and regulation of network connectivity, synaptic plasticity and firing dynamics. Pyramidal cells and interneuron are essential for firing

rates. Interneuron sculpts the activity of local networks and timing of the occurrence of pyramidal cell activity. Pyramidal cells are players in memory, learning and spatial representation.

5.7 Future Work

In the current study, only haematoxylin and eosin stain, which is the routine stain, is used. For better visualisation of morphology, 5-bromo-2-deoxyuridine (BrdU) and doublecortin (DCX) should be used since both are biomarkers for neurogenesis (Park, et al., 2010). 5-bromo-2-deoxyuridine and doublecortin are an immunohistochemical stain. 5-bromo-2-deoxyuridine mimics synthetic thymidine by become analogue of thymidine. When DNA replicates, it takes up BrdU instead of thymidine. 5-bromo-2-deoxyuridine and doublecortin act as markers for cell proliferation and survival. 5-bromo-2-deoxyuridine enables visualisation of new born cells. New born cell will have its nuclei stained. Meanwhile DCX enables visualisation of new neurons. 5-bromo-2-deoxyuridine incorporation and doublecortin expression in cornu ammonis indicates cell proliferation and cell survival.

Ethanol extracts and hot water extracts are another two methods that can be tried on fenugreek seed extraction. Extraction with ethanol and hot water will give different substances or compounds as compared to the yield from cold water extraction. Non-structural carbohydrates are found in simply sugar and

oligosaccharides can be extracted with ethanol while starch can be extracted with hot water (Ross, 1978). These two methods may be carried out in order to compare with the effect that is given by fenugreek seeds extracted through cold water. Methods that increase short term memory are preferable.

Besides that, concentration of fenugreek seed extracts can be examined for a dosage which can give the optimum effect in short term memory. The concentration that was used in this study which is 3000 mg/kg might not give optimum effect. Due to time constraint, different concentration of fenugreek seed extracts is not given. So, different concentration of fenugreek seed extracts should be given to few sets of mice to test on the effect on short term memory. In addition, the dosage which can cause adverse effect to mice can be examined too.

Last but not least, further study on the types of cells found in cornu ammonis is also advisable. In this study, the cells that have been identified but not confirmed are the darkly stained immature cells and pyramidal-like cells. Study on the cell types can gain better understanding on the morphology or cornu ammonis.

CHAPTER 6

CONCLUSION

In a nutshell, the diosgenin in fenugreek seeds enhanced short term memory in female mice. In order for short term memory to occur, neurogenesis needs to take place. Female sex hormones – oestrogen and progesterone play an important role in neurogenesis.

Behavioural performances showed a gradual increase in the step-through latency and recognition index indicates short memory formation in the brain of fenugreek seeds treated female mice. Meanwhile, morphological change observed in cornu ammonis of fenugreek seeds treated female mice indicates the memory and learning has taken place. As compared to control and norethisterone acetate, control has shown an increase in behavioural performance, but it is at a slower rate. As for norethisterone acetate, it did show an increase, but prolonged administration caused decrease in behavioural performance.

Fenugreek seeds have the potential of becoming a treatment for patients that suffered from memory disorders and learning disabilities. In addition, fenugreek seeds which contain female sex hormone can be use in hormonal therapy.

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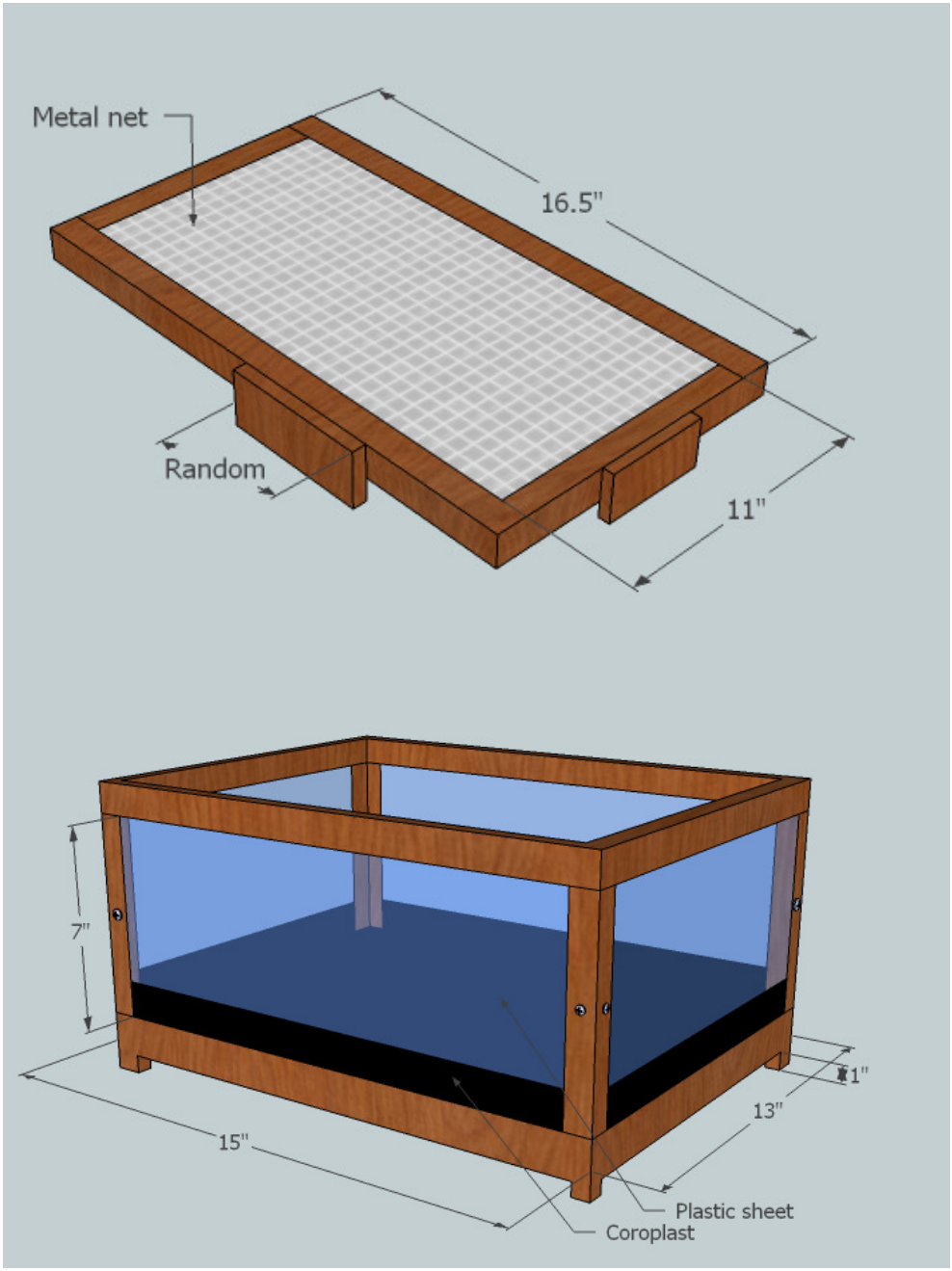
APPENDIX A

Taxonomy of Fenugreek Seed

Kingdom	:	Plantae
Phylum	:	Magnoliophyta
Class	:	Magnoliopsida
Order	:	Fabales
Family	:	Fabaceae
Genus	:	Trigonella
Species	:	*Trigonella foenum graacum*
Synonyms	:	Greek hay-seed, Bird's foot, Billy goat clover, Camel grass, Greek clover, Hilba/Hilbeh (Arabic), Ku tou/Hu lu ba (Chinese), Fenegriek (Dutch), Fenugrec (French), Bockshornsamen (German), Methi (Indian), Fieno Greco (Italian), Koroha (Japanese), Shanbalileh (Persian), Alforva/Feno grego (Portuguese), Pazhitnik (Russian), Alhovla (Spanish), Bockshornklee (Swedish),

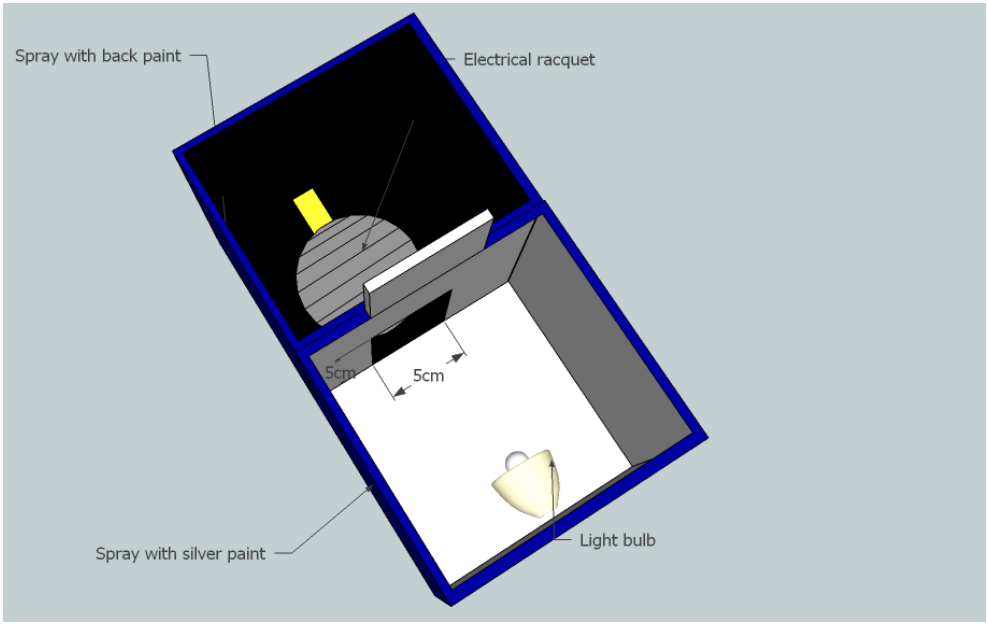
(Botanical, 2011; Indian Agro Industry, 2011)

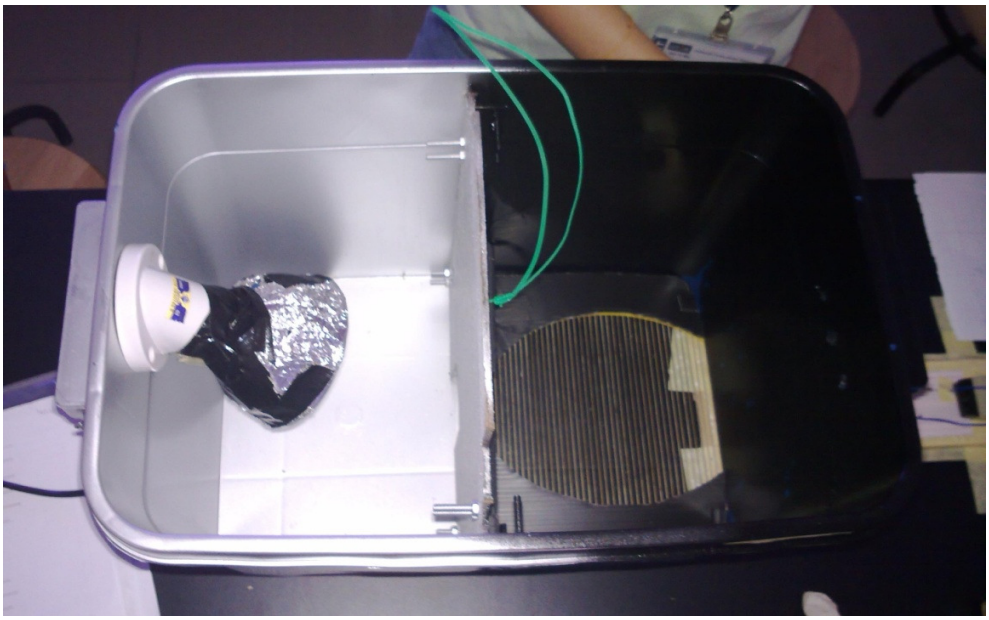
APPENDIX B



Animal cage model

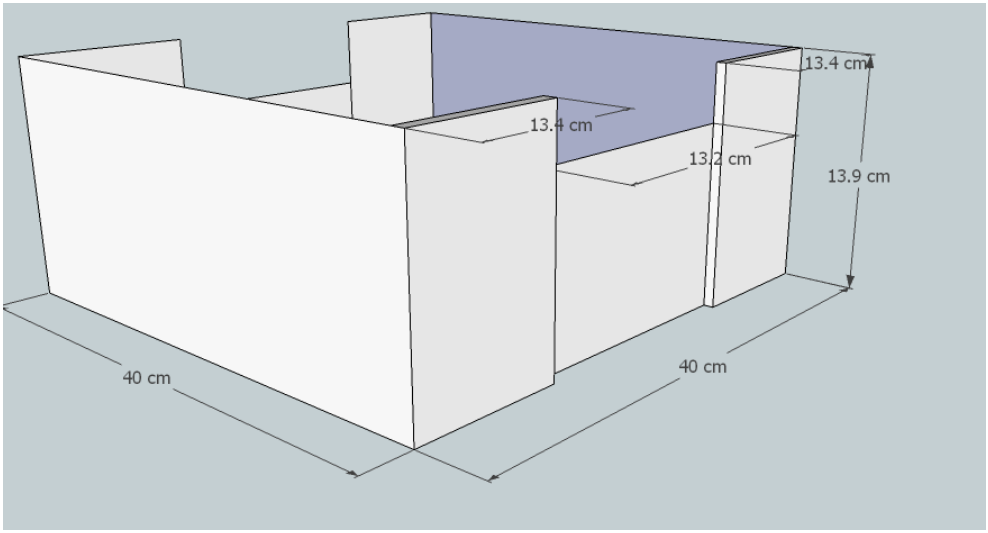
APPENDIX C





Passive avoidance test apparatus

APPENDIX D



Object recognition test apparatus