STUDY ON ACETYLCHOLINESTERASE INHIBITOR DERIVED FROM *Datura metel* L.

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ABSTRACT

Datura metel Linn locally known as *kecubung*, contains flavonoid, phenols and numerous alkaloid in the leaves, stem and seeds. These chemical constituents are fundamental in the anticholinergic treatment of Alzheimer's disease (AD). Anticholinesterase enhances the concentration of cholinergic neurotransmitters which is lacking in AD. To date, no *in vitro* study of acetylcholinesterase inhibitory activity *of D. metel*'s parts has been published. This research analyses *D. metel*'s anticholinesterase inhibitory properties and its neurobehavioral effect utilising mice model. The TLC results showed intense presence of alkaloids in the leaves. The highest concentration of flavonoid in the seeds chloroform extract was 286.8939 mg QE / g while the highest total phenol contents found in leaves, 305.1163 mg GAE / g. LCMS/MS had been used to analyse alkaloid types in leaves which detected 3-hydroxy-6-tigloxtropane, apoatropine, cuscohygrine, daturalactone, daturalin ammonium, adduct homatropine, hyoscyamin, scopolamine, tannin, tropan alkaloids and withalactone. Alkaloids detected in the stem were anisodamine, apoatropine, hyoscymine, meteloidine, proanthocyanidin and scopolamine, whereas alkaloids found in the seed were anisodamine, into, proanthocynidin and scopolamine. Ellman's method was used to quantify AChEI activity of leaves, stem and seeds methanol extracts. Of all the extracts, leaves had the highest inhibition of 75.94 % at 150 µg / ml extract concentration. From the Brine Shrimp Lethality Assay, LC_{50} of leaves extract were 311.228 µg / ml and non-toxic. Animal study using the number of repeat entries to arms of the maze was designed to investigate the impact of leaves methanol extract on valium-impaired mice memory. Leaves extract were given via oral gavage. The group of mice administered with leaves methanol extract showed less number of repeated entries to arm maze in both genders. These research findings are inferred to propose the use of natural acetylcholinesterase inhibitors from *D. metel* as a potential treatment for Alzheimer's disease.

ABSTRAK

Datura metel Linn yang dikenali sebagai kecubung, mengandungi flavonoid, fenol dan pelbagai jenis alkaloid dalam daun, batang dan bijinya. Juzuk kimia tersebut adalah asas dalam merawat penyakit Alzheimer melalui rencatan enzim Acetylcholinesterase. Anticholinesterase meningkatkan kepekatan cholinergic transmitter dimana ia tidak mencukupi pada penyakit Alzheimer. Sehingga kini, tiada kajian *in vitro* mengenai aktiviti perencat AChE daripada *D. metel* yang diterbitkan. Kajian ini menganalisis aktiviti perencat AChE *D. metel* dan menyelidik kesannya terhadap tingkah laku neuro dengan menggunakan model tikus. Keputusan TLC menunjukkan kehadiran alkaloid yang pekat di dalam daun. Kepekatan tertinggi flavonoid dalam ekstrak biji kloroform ialah 286.8939mg QE / g manakala jumlah kandungan fenol yang paling tinggi terdapat dalam daun, 305.1163mg GAE / g. LCMS / MS telah digunakan untuk menganalisis jenis alkaloid dalam daun lalu mengesan *3-hydroxy-6-tigloxtropane*, *apoatropine*, *cuscohygrine*, *daturalactone*, *ammonium daturalin*, *adduct homatropine*, *hyoscyamin*, *scopolamine*, *tanin*, *alkaloid tropan* dan *withalactone*. Alkaloid yang dikesan pada ekstrak batang adalah *anisodamine*, *apoatropine*, *hyoscymine*, *meteloidine*, *proanthocynidin*, sejenis *proantosianidin* dan *scopolamine*, manakala alkaloid di dalam biji pula ialah *anisodamine*, *apoatropine*, *proanthocynidin* dan *scopolamine*. Kaedah Ellman telah digunakan untuk mengukur aktiviti AChEI dalam ekstrak metanol daun, batang dan biji. Daripada semua ekstrak, daun mempunyai perencatan yang tertinggi 75.94 % apabila kepekatan ekstrak 150 µg /ml. Daripada *Brine Shrimp Lethality Assay*, LC_{50} ekstrak daun adalah 311.228 µg / ml dan tidak toksik. Kajian haiwan dengan menggunakan bilangan ulangkali kemasukkan di dalam lengan radial maze telah direka untuk mengkaji kesan ekstrak metanol daun *D. metel* pada tikus yang telah dilemahkan ingatanya oleh valium. Ekstrak diberikan menggunakan

cara makan paksa. Kumpulan tikus yang dirawat dengan ekstrak methanol daun menunjukkan jumlah bilangan kemasukkan yang kurang dalam ujian lengan radial maze pada kedua-dua jantina. Penemuan penyelidikan ini mencadangkan penggunaan perencat semula jadi AChE daripada *D. metel* sebagai rawatan berpotensi untuk penyakit Alzheimer.

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LIST OF SYMBOLS AND ABBREVIATIONS

ACh	Acetylcholine
AChE	Acetylcholinesterase
AD	Alzheimer's disease
ATCI	Acetylcholine Iodide
β	Beta
BSLA	Brine Shrimp Lethality Assay
DTNB	5, 5'-Dithiobis [2-Nitrobenzoic Acid]
FC	Folin-Ciocalteu
GABA	Gamma-Aminobutyric Acid
LCMS	Liquid Chromatography Mass Spectrometry
LCMS/MS	Liquid Chromatography Mass Spectrometry/ Mass Spectrometry
LC_{50}	Lethal Concentration, 50%
NEF	Number of Entries Until the First Error Occurs
NRE	Number of Repeat Entries to Arms of the Maze
R_f	Retention Factor
TLC	Thin Layer Chromatography
TNB	5-thio-2-nitrobenzoate
TPC	Total Phenol Content
TFC	Total Flavonoid Content

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CHAPTER 1: INTRODUCTION

Alzheimer‘s disease (AD) is reported to account for about 60 to 80 % of dementia cases (Gaugler *et al.*, 2014). According to the Alzheimer‘s Disease Foundation Malaysia (ADFM), it is estimated that currently 50,000 Malaysians are affected by AD. As women lifespan is longer than men, women contributing to 16 % of mortality in patients with AD at age 80 years and 11.5 % of all female mortality in 2012. Genetic factors are implicated in 70 % of AD cases, other risk factors include on history of head injuries, depression and hypertension. AD gradually worsens to significantly affect daily activities and the quality of life of the sufferers. AD does not directly cause death but could lead to complications such as lung infection (pneumonia), urine infection, recurrent falls and pressure ulcers. Scientists have identified several hallmark brain abnormalities in people affected by AD from their symptoms and pathology of the deceased brain. To date, present paper has continuously search and publish treatment strategies structured according to a number of existing hypotheses aimed at explaining the origins of AD for example cholinergic hypotheses.

Datura metel Linn. belongs to Solanaceae family which is well known for its tropane alkaloid constituent. Tropane alkaloid naturally blocks cholinergic nerve causing inactivation of cholinesterase phosphorylation. Acetylcholine deficiency at synapses results in the lack of cholinergic neurostimulation. Khachaturian *et al*. (1999) revised that in 1906, Dr Alois Alzheimer discovered a link between behavioural symptoms and microscopic brain defects. Four years later, the term Alzheimer‘s disease was coined and described as a permanent loss of memory and a progressive neurodegenerative disease.

Advanced stages of AD result in destruction of the brain ability to function, with the sufferer becoming more and more withdrawn and almost unable to control their behavior. Anticholinesterase also enhances the concentration of cholinergic neurotransmitters and can slow the degenerative process as demonstrated by the two commonly used anticholinesterase drug, donepezil, a reversible anticholinesterase and rivastigmine a non-competitive reversible anticholinesterase. Thus, *D. metel* is postulated to exert its therapeutic effect by enhancing cholinergic impulse transmission which results in improved memory and cognitive functions of AD sufferers.

A part from tropane alkaloid, *D. metel* also contains terpenoid, flavonoid and phenol. *D. metel* could be poisonous but not every part of it is dangerous. For example, *D. metel* leaves are harmless and safe to consume unlike the stem and seed which are toxic. However, in overdose it can cause double vision, pupils dilation, loss of focus, confusion, agitation and incoherent speech. The fruits are used in ceremonial sacrifice. *D. metel* smoked with tobacco can cause mood elevation and sleeps with lucid dream. On the other hand, *D. metel* is an important ingredient in Ayurvedic medicine for the treatment of skin conditions and anxiety disorder. In other conditions, the seed is used as an alternative to opium. Avery (1959) reported that Arabic physician, Avicenna (Ibnu Sina) praised the plant for its narcotic medicinal value and defined an efficient dosage. *D. metel* was first documented in Sanskrit literature (Avery, 1959).

Wang *et al.* (2008) performed a pharmacological test on *D. metel* and found that it has positive anti- inflammatory, anti-titillation of skin, and anti-anaphylaxis actions. Numerous studies have reported the significant chemical constituents and pharmacological properties of the compounds found in *D. metel*, unfortunately, none of these studies have investigated the type of alkaloids present in the stem and seeds of *D. metel* so far.

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Hypothesis

Crude extract from *Datura metel* would block the action of AChE, an enzyme that breaks down acetylcholine. They, therefore, increase the amount of acetylcholine that is available in the synapses for transmission of nerve impulse in AD's brain.

General objective

To evaluate anticholinergic properties in *D. metel* and observe the memory recovery in mice by using appropriate chemical extracts.

Specific Objectives

1) To identify the presence of alkaloids in *D. metel* using liquid chromatography-mass spectrometry/mass spectrometry (LCMS / MS).
2) To evaluate the acetylcholinesterase inhibitory activity using Ellman's method of the leaves, stem and seeds of *D. metel* methanol extracts.
3) To analyze *D. metel* toxicity effects using Brine Shrimp Lethality Assay (BSLA) and acute toxicity study on mice.
4) To study the effect of leaves methanol extracts on memory impairment using animal model in radial arm maze study.

CHAPTER 2: LITERATURE REVIEW

2.1 Alzheimer's disease

Alzheimer disease was first described by Alois Alzheimer in 1906 and is characterized by progressive loss of cognitive abilities. It causes progressive impairment in cognitive and functional ability of individuals suffering from the disease (Soukup, 1996). Currently, over 46 million people in this world live with dementia and AD is the most common cause representing 60 to 70 % of dementia cases (Prince *et al*., 2015). Global leaders have set a deadline of 2025 for finding an effective way to treat or prevent AD (Vradenburg, 2015). Although deadline may not have been based on scientific principles of disease research or the realities of drug development, researchers progressively find innovative ways to develop drugs to successfully achieve the 2025 goal. The exact cause for most Alzheimer's cases is still not well understood. Several competing hypotheses exist trying to explain the cause of the AD including the cholinergic hypothesis which has served as the basis for the majority of treatment strategies and drug development approaches for AD to date (Contestabile, 2011). Pathologically, the AD brain is characterized by massive neuronal cell and synapse loss at specific sites, as well as β-amyloid plaques and neurofibrillary lesions. The basal forebrain cholinergic system is affected in several neurodegenerative disorders including AD (Eduardo, 2010). AD has been categorized into early and late onset. Early-onset AD or known as familial AD, typically begins between the ages of 30 and 60 years and late-onset or sporadic AD, presents after the age of 65. The onset of disease before age 65 is uncommon and suggests the involvement of a genetic component.

In humans, a hallmark of AD firstly described with nonspecific cholinergic antagonists disrupt memory (Lasser, Nash, Lasser, Hamill & Batey, 1989) and degeneration of cholinergic from the basal forebrain to the hippocampus (McKinney & Jacksonville, 2005; Schliebs, 2005). Specifically, this neurodegeneration is associated with brain atrophy, abundant amyloid plaques and loss of cholinergic neurons. Hyperphosphorylation of tau protein, leading to the development of neurofibrillary tangles (NFT) is a second important hallmark of AD. The presence of Aβ plaques provides stimuli to surrounding astrocytes and microglia, resulting in induction of localized immune responses in the AD brain. Aβ is capable of activating complement and inducing expression of inflammatory cytokines such as IL-1β, IL-6, TNF-α, and some chemokines (Rubio-Perez & Morillas-Ruiz, 2012).

2.1.1 Common signs and symptoms of AD

Forgetfulness and difficulties with routine tasks are typically the initial symptoms. As the disease progresses, AD patients develop more severe memory loss, speech impairment, visual and spatial deficits, and loss of coordination and fine motor control (Lee, Chen, & Tsai, 2013). Symptom of memory loss and cognitive weakening due to irreversible neuroinflammation, accumulation of tau protein deposition and amyloid deposition are prominent pathology remarks found at neocortex, hippocampus and amygdala in AD patient (Chabrier, Cheng, Castello, Green & LaFerla, 2014). In addition to the cognitive, sensory and motor deficits caused by the progression of AD, there are a number of behavioral and psychological symptoms related to dementia. These symptoms include agitation and aggression, wandering, disturbances in the sleep cycle, depression, anxiety, delusions and hallucinations (Budson & Solomon, 2016).

2.1.2 Diagnosis of AD

There is currently no definitive laboratory test to diagnose AD however some positive predictive values are available to suggest AD. Imaging with PET scan may be able to demonstrate reduced brain cell activity in some parts of the brain which are typical of AD. Another clinical approach to diagnosing AD is by using cerebrospinal fluid (CSF) protein biomarkers such as tau, phospho-tau, and the 42 amino acid form of β-amyloid (Blennow, 2004). Research in genetic now is concentrates on the gene that produces a protein called apolipoprotein E (ApoE) to diagnose AD because studies showed that people who carry two ApoE-e4 genes have a higher risk of developing AD. Also, genetic research had identified the abnormalities on presenilin-type 1 (PS1), presenilin-type 2 (PS2) and amyloid precursor protein (APP) to cause AD (Berezovska, 2005). It has been reported that the protein level of acetylcholine receptors is reduced in AD (Nordberg, 2001) and that dysfunction of cholinergic signal transmission could be responsible for the symptoms of AD.

2.2 Acetylcholine

Acetylcholine (ACh) is a very common neurotransmitter, found in central, peripheral, autonomic and somatic nervous systems (Colovic, Krstic, Lazarevic-Pasti, Bondzic & Vasic, 2013). Acetylcholine (ACh) is critical for communication between neurons and muscle at the neuromuscular junction. It also involved in direct neurotransmission in autonomic ganglia, and has been implicated in cognitive processing, arousal and attention in the brain (Karczmar, 1993).

Acetylcholine (ACh) is catalyzed by acetylcholinesterase (AChE) by the process of hydrolysis, in cholinergic synapses and subsequently affects the neuron to return to its resting state after activation (Groner *et al*., 2007). ACh exerts multiple effects in the cerebrum cortex via muscarinic and nicotinic receptors located presynaptically and postsynaptically in both pyramidal glutamatergic projection and local aminobutyric acid (GABA)-ergic neurons (Colovic *et al*., 2013). Reduction in the levels of neurotransmitter acetylcholine in the brains of the elderly occurs as the disease progresses, resulting in loss of cognitive ability (Felder, Bymaster, Ward & DeLapp, 2000). Experimental studies using a variety of approaches have provided insight into the mechanisms of cholinergic modulation of cortical function and cognition. In invertebrates, ACh receptor (AChRs) have become key elements for the development of neuroactive pesticides. For example, several neonicotinoids (e.g., imidacloprid, clothianidin, and thiacloprid) have insecticide activities but extremely low mammalian toxicities due to their high affinity and specificity for insect AChRs (Ihara *et al*., 2008).

2.3 Acetylcholinesterase inhibitor

According to Colovic *et al*. (2013), acetylcholinesterase inhibitor is a class of drugs that inhibits the action of acetylcholinesterase (AChE), an enzyme that breaks down acetylcholine. They, therefore, increase the amount of acetylcholine that is available in the synapses for transmission of nerve impulse (Weinreb, Mandel, Amit & Youdim, 2004). AChE inhibitors are used in the treatment of various neuromuscular disorders and have provided the first generation of drugs for the treatment of Alzheimer‘s disease (AD) (Greenblat, Dvir, Silman & Sussman, 2003).

The cholinergic hypothesis proposed that AD is caused by reduced synthesis of the neurotransmitter AChE. This cholinergic hypothesis has not maintained widespread support, nevertheless, it can be stated that the cholinergic deficit is responsible for the symptomatology of AD (Constestabile, 2011). AChE remains a highly viable target for the symptomatic improvement in AD because the cholinergic deficit is a consistent and early finding in AD. The inhibition of AChE causes an increase in the concentration of ACh in cholinergic synapses, which results in alleviation of the disease. When the concentration of acetylcholine molecules increases within synapses following AChE inhibition, it can at least partially counteract a deficiency in either the release of a neurotransmitter or a reduction in cholinergic receptors or signaling (Pope, Karanth & Liu, 2005). Cholinesterase inhibitor drug relies on their interaction towards AChE. Some reports revealed that cholinesterase inhibitor also have additional sites of action that may result in toxicological action but could also bring hope as it may cause other significant pharmacological effects (Pope *et al*., 2005).

AChE inhibitors are currently developed in four reversible agents which are tetrahydro aminoacridine (Tacrine), donepezil hydrochloride (Aricept), rivastigmine (Exelon) and galantamine (Reminyl). The efficacy of donepezil, rivastigmine, and galantamine has been studied in the treatment of AD and are currently licensed for clinical treatment.

2.4 Acetylcholinesterase inhibitor from plant source

Several drugs which are used clinically to treat the symptoms of dementia inhibit AChE are derived from plants. Rivastigmine is derived from the Calabar bean alkaloid physostigmine. It reacts with the active site serine in AChE leaving a carbamate group attached (Cacabelos, 2007). The cleaved portion of the drug remains bound through an aromatic ring stacking interaction with the catalytic anionic site (CAS) tryptophan.

Huprine is a synthetic derivative of huperzine, extracted from a moss which has been used in Chinese medicine for centuries. Galanthamine, first isolated from snowdrops (*Galanthus* sp), is a bulky molecule like huperzine. It interacts with both the peripheral anionic site (PAS) and the acyl – coA binding protein (ACBP), competing with ACh for the active site (Cacabelos, 2007). Galanthamine is also taken to promote lucid dreams and out of body experiences, but whether its anticholinesterase activity or some other property causes these effects is unknown. Tacrine is a synthetic molecule that binds only to the CAS and does not interact with residues around the active site. Donepezil, also known as Aricept, is bivalent, interacting with both the CAS and PAS tryptophans via ring stacking interactions (Cacabelos, 2007).

A variety of plants has been reported to show AChE inhibitory activity and so may be relevant to the treatment of AD. *Bacopa monniera* and *Ginkgo biloba* are well-known cognitive enhancers in Indian and Chinese traditional medicine systems. Standardized extracts of *Bacopa monniera* and *G. biloba* both showed a dose-dependent inhibitory effect on AChE activity (Das Mahapatra & Kumar, 2002).

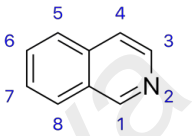
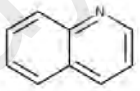
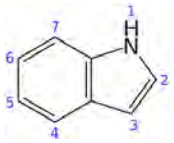
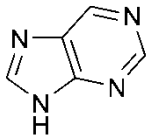
Eighty percent methanolic extract of *Myricaria elegans* Royle was found to have significant AChE inhibitory activity (Ahmad *et al*., 2003). Methanolic extracts of seven herbs *Acorus calamus*, *Acorus gramineus*, *Bupleurm facaltum*, *Dioscorea batatas*, *Epimedium koreanum*, *Poria co*cos and *Zizyphi jujuba*, used in traditional Korean medicine for improvement of memory and cognition in old age have been tested for cholinesterase inhibitory properties and significant inhibition of the enzyme was shown by extracts from *Acorus calamus* and *E. koreanum* (Oh *et al*., 2004). Ingkaninan *et al*. (2000, 2003) screened the methanolic extracts of 32 plants used in Thai traditional rejuvenating and neurotonic remedies, for inhibitory activity on AChE and found that the extracts from roots of *Stephania suberosa* and *Tabernaemontana divaricata* showed significant inhibitory activity. The chloroform:methanol (1:1) extracts of a number of the plant species namely *Corydalis solida* (L.) Swartz subsp. *solida* and *Glaucium corniculatum* (L.), *Rhododendron ponticum* L. subsp. *ponticum* and *Rhododendron luteum* Sweet. (Ericaceae), *Buxus sempervirens* L. (Buxaceae), *Vicia faba* L. (Fabaceae), *Robinia pseudoacacia* L. (Caeselpiniaceae), *Tribulus terrestris* L. and *Zygophyllum fabago* L. (Zygophyllaceae), *Lycopodium clavatum* L. (Lycopodiaceae), *Fumaria vaillantii* Lois., *Fumaria capreolata* L., *Fumaria kralikii* Jordan, *Fumaria asepala* Boiss., *Fumaria densiflora* DC., *Fumaria flabellata* L., *Fumaria petteri* Reichb. subsp. *thuretii* (Boiss.) Pugsley, *Fumaria macrocarpa* Boiss. ex Hausskn., *Fumaria cilicica* Hauskkn., *Fumaria parviflora* Lam.,

and *Fumaria judaica* Boiss. (Fumariaceae) were screened for their anti-cholinesterase activity (Orhan *et al*., 2004). The extracts of *Rhododendron ponticum*, *Rhododendron luteum*, *Corydalis solida, Glaucium corniculatum*, and *Buxus sempervirens* showed remarkable inhibitory activity above 50% inhibition rate at 1 mg / ml (Mukherjee, Kumar, Mal & Houghton, 2007).

2.5 Alkaloid and AChE inhibitor activity

Alkaloids are recognized by its nitrogen atom located in the heterocyclic ring structure. It is a highly diverse group of compound (An *et al*., 2016). Alkaloids are important molecules derived from secondary metabolism that can act as rich sources of research in biomedicine and drug discovery area (Lu, Bao, Cheng, Huang & Huang, 2012). Alkaloids have been studied as AChE inhibitor with more than 35 alkaloids reported so far. However, a few of them have entered therapeutic use (Mukherjee, Kumar, Mal & Houghton, 2007). Different classes of compounds have been considered, namely indole derivatives (such as physostigmine and related compounds), isoquinoline and related derivatives (such as galantamine and lycorine-type alkaloids), steroid and terpenoid alkaloids and many other derivatives that present significant inhibitory effects on AChE (Mukherjee *et al*., 2007). Anticholinergic is a substance that blocks acetylcholine. Cholinesterase, such as acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) are key enzymes that play significant roles in cholinergic transmission, hydrolyzing the neurotransmitter acetylcholine (Brühlmann, Marston, Hostettman, Carrupt & Testa, 2004). In the regulation of cognitive functions, the central cholinergic system is considered to be the most important neurotransmitter.

Table 2.1: Alkaloids Structure (Dvir, 2010)

Type of alkaloids	Formula	Structure
Tropine	$C_8H_{15}NO$	
Quinoline	C_9H_7N	
Isoquinoline	C_9H_7N	
Pyridine	C_5H_5N	
Indole	C_8H_7N	
Purine	$C_5H_4N_4$	

Tropane alkaloid blocks the muscarinic receptor acetylcholine. The major tropane alkaloids Hyoscyamine, scopolamine and several minor tropane alkaloids have been identified in *Datura* species. Typical examples of minor alkaloids in *D. stramonium* are tigloidin, aposcopolamine, apoatropin, hyoscyamine N-oxide and scopolamine N-oxide17-20. 6-ditigloyloxytropane and 7-hydroxyhyoscyamine are reported for the first time in this species (Das Mahapatra & Kumar, 2012).

2.6 *Datura metel Linn*

Figure 2.1: *Datura metel* Linn. (Curtis, 1948)

Kingdom	: Plantae
Subkingdom	: Angiosperms
Division	: Eudicots
Class	: Asteroids
Order	: Solanales
Family	: Solanaceae
Genus	: *Datura*
Species	: *metel*

2.6.1 Description

Datura metel Linn, locally known as *kecubung*, is the most common *Datura* species found on cultivated and wild lands in Malaysia. It is a native plant of tropical Asia. In Chinese medicines, *yangjinhua*, it is also known as *Flos daturae*, specifically named for the flower of *D. metel* and it is prescribed for the treatment of asthma, cough, and convulsion (Kuang, Yang, Xia & Wang, 2011).

Other names given for this plant include Jimson Weed, Angel's Trumpet, Devil's Trumpet, Apple thorn and Green Dragon (Ellenhorn, 1997). This species belongs to Solanaceae family which contains a range of flowering plants such as *Datura stramonium* which has antispasmodic properties and *Atropa belladonna*, a toxin and paralyzing herbaceous plant (Gillman, 2007). *D. metel* has an average height of 91 cm high. Its large flower has trumpet-shaped, and the colour is different from one species to another such as white, purple and yellow. Sometimes, it may present with two toned colors, for example, white and purple. It has large, broad and sharp-pointed leaves. Flowering time is usually in midsummer to mid-autumn. *D. metel* is odorless and usually kept as ornaments because of its attractive bell shape.

2.6.2 Traditional use of *D. metel*

D. metel contains tropane alkaloids that consist of antispasmodic, narcotic and analgesic properties. The plant is believed to have anesthetic, antiasthmatic, antitussive, hypnotic, anodyne, bronchodilator and mydriatic effects. Qualified medicinal herbalist prescribes *D. metel* for various conditions such as epileptic disorders, convulsion, fever, and mental disorders. Rural community used this plant as medicinal remedies because it is readily available as well as affordable (Sani, Sanni & Ngulde, 2009). According to Bradley in British herbal pharmacopoeia (1996), the leaf of the white flowers used to be smoked with cannabis and tobacco to relieve asthma. The seeds were extracted and prepared as ointment to relieve neuralgia and muscular rheumatism.

2.6.3 Pharmacological potential of *D. metel*

D. metel is widely believed to have great pharmacological potential with significant usage in traditional treatment. Different studies have shown that extracts of *D. metel* have different properties necessary for treating different conditions. A study by Dabur, Singh, Chhillar, Ali and Sharma (2004) indicated that methanolic extracts of *D. metel* possessed antifungal elements. They also found that chloroform extract of *D. metel* contained compounds that were active against species such as *Candida tropicalis*, *Aspergillus niger* and *Aspergillus fumigatus*. A study by Umamaheswari *et al*. (2007) indicted that *D. metel* can be used to make antigout drugs. Another study by Ma, Xie, Li, Lou and Hu (2006) indicated that *D. metel* content properties that be used to develop cancer drugs due to their antiproliferative activity.

2.6.4 Phytochemistry of *D. metel*

D. metel contains hyoscine, alkaloids hyoscyamine, scopolamine and atropine. A study by Afsharypuor, Mostajeran and Mokhtary (1995) found that scopolamine concentrates highly at the root after week sixteen. Aerial parts of *D. metel* have a higher amount of scopolamine compared to atropine whereas, in the roots, it has a higher amount of atropine compared to scopolamine. Kuganathan and Ganeshalingam (2011) analyzed the leaves and indicated the absence of saponins and flavonoid. However, a study by Alabri, Al Musalami, Hossain, Weli and Al Riyami (2014) demonstrated the presence of flavonoid in both fresh and dry leaves of *D. metel*. A study by Sangeetha, Deepa, Sugitha, Mythili and Sathiavelu (2014) also indicated the presence of alkaloid in the leaves. Kuang, Yang, Xia and Feng (2008) conducted a study on flowers of *D. metel* and found a new chemical constituent and confirmed five known sesquiterpenes. A study by Nuhu and Ghani (2002) on total alkaloid content in *D. metel* indicated that the plant has a total alkaloid content of 1.22 %, according to the mean volume of acid required to neutralize the base characteristic of the alkaloid. Maheshwari, Khan and Chopade (2013) listed alkaloid compounds found in root and leaves of *Datura* species based on previous studies.

Table 2.2: *D. metel* analysis

Parts of *D. metel*	**Type of extract**	**Compound (s)**	**Pharmacological properties**	**Reference (s)**
Leaves	Chloroform extract	Methyl-2-ethylpentanoate.	antimycotic drugs	(Dabur, 2005)
Leaves	methanolic extracts	unkown	antigout drug	(Umamaheswari *et al*., 2007)
aerial parts	methanolic extract	withanolide glycosides	antiproliferative activity	(Ma *et al*., 2006)
Seeds	n-hexane extract	unknown	potential activity	(Ramadan, Zayed, & El-Shamy, 2007)
Leaves	methanol extracts	Saponins	antioxidant activity	(Dabur, 2005)

2.7 Animal model in Alzheimer's disease (AD) study

Mice have been used in experimental mazes since at least the early 20th century. To gain a greater understanding of the progression of AD, animal models have played a major role in defining critical disease-related mechanisms and have been at the forefront of evaluating novel therapeutic approaches, with many treatments currently in clinical trial owing their origins to studies initially performed in mice (Whishaw, Metz, Kolb & Pellis, 2001). Nevertheless, there are significant translational issues that have been raised of late, as there has been some potential discordance between preclinical drug studies and human clinical trials. Animal models used in AD can be broadly divided into three categories which are natural models, genetic models, and interventional models. According to Encyclopedia of Psychopharmacology (Dickinson, 2014), a preclinical test of spatial learning in rodent, either in intact animals or those subjected to treatments that impair spatial learning is often used as an initial assay to investigate the effects of potential cognition- enhancing drugs.

Unlike mice and rats, rabbits share an identical Aβ peptide sequence with humans, though they do not spontaneously develop any AD-like disease (Muñoz & Inestrosa, 1999). However, wild-type rabbits fed high cholesterol diets have been shown to develop Aβ deposition, tau pathology, neuronal loss, and cognitive impairment. Rodent also provides moderate to severe neuronal loss has been observed, primarily in the hippocampus (Schmitz, Kawahara-Baccus & Johnson, 2004).

There are studies which showed impairment in spatial memory in early AD (Cherrier, Mendez & Perryman, 2001; deIpolyi, Ranki, Mucke, Miller & Gorno-Tempini, 2007; Bird *et al*., 2010). Hippocampus and entorhinal cortex are the first to exhibit neurodegeneration in AD (Wood & Chan, 2015). Didic *et al*. (2011) claimed that AD impairs episodic memory. However, Tulving (2002) argued that laboratory experiment deals with "what" the subject has to learn but is lacking in "where" and "what" of the episodic memory in the given tasks.

2.7.1 Rat as an animal model of AD

The rat is one of the most commonly used experimental animal species in biomedical research and because of its relevance to human physiology, the rat may provide highly predictable models for research and the pharmaceutical industry (Cozzi, Fraichard & Thiam, 2008). Early discoveries dating from the 1960s showing deleterious effects of drugs that block cholinergic activity like atropine and scopolamine on memory in rats, and parallel evidence for cholinergic dysfunction in AD subsequently led to the formulation of the cholinergic hypothesis of geriatric memory dysfunction' (Bartus, Dean, Beer & Lippa., 1982; Bartus *et al*., 1985).

Bilateral injection of scopolamine, a non-specific muscarinic antagonist, into dorsal hippocampus impairs spatial learning in rodent models (Herrera-Morales, Mar, Serrani & Bermúdez-Rattoni, 2007).

The availability of new genetic research tools in rats provides considerable advances in the areas where rats are extensively used. In AD research, the rat has for decades been a very important model, for instance in studies on cholinergic dysfunction and memory impairment which played a crucial role in the development of the cholinesterase inhibitor drugs that are currently in use.

The attractiveness of the rat as an experimental animal model has been increased further by the availability of the rat genome data and technologies allowing genetic manipulation in rats.

Valium is a brand name for diazepam, a benzodiazepine that is commonly used for anxiety due to its sedative effect (Honeychurch *et al*., 2013). Benzodiazepine is known to cause anterograde memory impairment as a side effect (Kant, Wylie, Vasilakis & Ghosh, 1996). It can also affect episodic memory, the remembering of recent events and the circumstances in which they occurred and their time sequences, (Curran, 1992) by intensifying the neurotransmitter gamma–aminobutyric acid (GABA) action leading to inhibition of neuronal activity in the brain. Memory impairment is the hallmark of AD and therefore the concept of treating mice with benzodiazepine has been applied in research with the aim of producing artificial memory impairment in an attempt to mimic AD state.

2.7.2 Radial arm maze (RAM)

Behavioral tests are essential to functionally validate disease models and to assess treatments. Rat models of AD should allow a more sophisticated characterization at the behavioral level and thus enable a more accurate assessment of the impact of the pathology on cognitive outcomes. They should also enable a better assessment of the effects of potential therapeutics on cognition in longitudinal studies. Spatial memory is highly relevant in biology because it is related to both individual and species survival. Among behavioral tests, one of the most suitable devices for measuring spatial learning and memory is the radial arm maze (RAM) (Olton & Samuelson, 1976; Hulse, Fowler & Honig, 1978).

Thousands of studies have examined how rats run different types of mazes, from T-mazes to radial arm mazes to water mazes. These maze studies are used to study spatial learning and memory in rats. Maze studies helped uncover general principles of learning that can be applied to many species, including humans. Today, mazes are used to determine whether different treatments or conditions affect learning and memory in rats. Rats are particularly gifted at running mazes. Their maze-running ability comes from their evolutionary history: rats are small burrowing rodents that have spent millennia digging and finding their way around underground tunnels. Locating foods on a radial arm maze is mostly common experiment for assessing spatial learning in mice.

RAM consists of eight horizontal arms placed radially around a central platform above the floor. Experimental subjects are placed on a central platform from which they have to collect hidden baits placed at the end of the arms. The standard version of the RAM animals are habituated to the environment, placed on the central platform and allowed to explore the maze for 15 min per day. Reinforcers or baits are scattered on the arms.

On the last day of habituation (day 3), the amount of reinforcer is reduced to half and the session ends when all eight arms have been visited. Following habituation, the animals are trained one session per day for eight consecutive days. One piece of reinforcer is placed at the end of each arm in a well that hides the food from sight and the animal is allowed to explore the maze freely. Each session lasts until all eight arms have been entered (consider enter an arm when the whole body, except the tail, is inside the arm); 10 min passed since the start of the test or 2 min passed since the animal's last arm entrance (Liu & Belkey, 2002).

Arm entries are recorded for later analysis and maze wiped clean to prevent odor cues. The variables commonly used for the analysis of the performance are the number of errors in each session (entering an arm that has been visited previously counted as an error); the total number of errors across eight sessions; the number of correct choices in the first eight arm entries of each session; the location of the first error in each session; the number of adjacent arm entries in each session, the time taken to visit each arm (total time to complete the session divided by the total number of arm entries); and the number of sessions to reach the criterion of one error or less, averaged over four consecutive days of training (Liu & Belkey, 2002). There is extensive evidence that attending to the visual cues located outside the apparatus is one of the elements that subjects use to avoid re-entering the different arms in RAM.

Furthermore, at least in rats, a correct performance of the task seems to depend primarily on extra-maze cues (Suzuki *et al*., 1980). On the contrary, olfactory cues were rather related to the improvement of choice accuracy in reduced-visibility situations (Lavenek & Schenk, 1995).

Avoiding revisits has been directly related to the amount of surrounding environment cues available, as well as to the viewing duration (Mazmaniam & Robert, 1983). Moreover, when extra-maze cues predicted the reinforced arms, rats performed almost perfectly on the RAM, whereas following intra-maze cues (such as odor) push performance outcome not better than chance (Olton & Collison, 1979). In this sense, it seems that external cues apparently control choice behavior when they are easily accessible (Babb & Crystal, 2003).

Under these circumstances, each visit to an arm may be regarded as a go-no-go discrimination based on extra-maze cues (Bab & Crystal, 2003). Finally, it was assumed that when extra- maze cues have limited a representation of spatial locations rather than intra-maze cues might be used when navigating in the radial maze (Brown, 1992).

Modifications of the initial procedure permitted the distinction of spatial working memory errors (double entries into baited places) versus spatial reference memory errors (entering never baited arms) (Brown, 1992). This version of the RAM aims to test working and reference memory at the same time. In their version of the task, only four maze arms are baited (Jarrard, 1983). The same maze arms are baited each day and, across sessions, the rats learn to ignore the remaining four arms, which never contain reward. This is the reference memory component of the task, and entry into a never baited arm is considered a reference memory error. Within a training session, re-entry into one of the four baited arms would be considered a working memory error. Traditionally, the most extensively studied species in the RAM is the rat.

Moreover, animals show a clear learning curve in several variables registered, such as latency to the reinforced arms, the total time to complete the essay, the number of errors committed within a session and the number of errors at the end of the training. Furthermore, it has also proved to be sensitive to the temporary impairment of memory caused by sleep deprivation. In this sense, this model results not only valid as a memory testing in normal states, but also in discriminating possible deficits in memory following different protocols for memory impairment.

RAM is a consolidated paradigm for the evaluation of memory. Despite the fact that the most extended model used in this kind of experiments is the rat, RAM has also demonstrated its validity across other animal species including humans. Over the years, different versions and variations of the RAM have developed, all of them proved as solid as the traditional regarding memory assessment.

Evaluating the performance of this recent model using RAM results is very useful to understand multiple cognitive and behavioral components of memory testing. In addition, the validity demonstrated with this paradigm opens numerous possibilities within the field of memory and learning studies, especially those regarding cognitive impairment, which in the last term will contribute to a better knowledge of these processes.

CHAPTER 3: METHODOLOGY

3.1 Plant Collection

The plant of *Datura metel* were obtained from Rimba Ilmu botanical garden in Universiti Malaya and separated as leaf, seed and stem. Samples were collected freshly and carefully to avoid insect or infected samples. Young parts of the plant were avoided from the collection. The authenticity of the plant sample was confirmed by Rimba Ilmu's coordinator and taxonomist, Dr. M. Sugumaran.

3.2 Preparation of *D. metel* Extract

Plant samples were left to dry at the room temperature. After complete drying, the samples were ground into fine powder and kept in hermetic and ready for experiments. Approximately 20 g of the powdered sample (leaves, stems and seeds) were each extracted separately with hexane, chloroform and methanol using Soxhlet apparatus. The sample was placed inside a thimble made from thick filter paper and loaded into the main chamber of the Soxhlet extractor. The Soxhlet extractor placed onto a flask containing hexane. The hexane was heated to reflux for 12 hours. Residue in the flask was then filtered and concentrated in rotary evaporator with medium speed at 50°C. Extraction was then repeated with solvent chloroform, and finally methanol. 1 ml of yield extracted compound from each solvent was taken to evaporate for preparation of next experiment.

3.3 Analysis of Chemical Compound by Thin Layer Chromatography (TLC)

Using a capillary, a small spot of sample extract was applied to a TLC plate about 1.0 centimeter from the bottom edge of the plate. The solvent development was then allowed to evaporate completely to prevent it from interfering with sample's interactions with the next mobile phase. 100 ml of 1 : 9 methanol-chloroform solution, was poured into a covered tank to a depth of less than 1 centimeter. A strip of filter paper was put into the chamber so that its bottom touches the solvent and the paper lies on the chamber wall and reaches almost to the top of the tank. Tank was left for a few minutes to let the solvent vapors ascend the filter paper and saturate the air in the tank. Without delay, the solvent front, the furthest extent of solvent up the plate, was marked. The plate was visualized under the visible light and ultraviolet (UV) light. The TLC plates were sprayed by visualizing reagent after elution as the reagent react with the spots to produce visible results. All this steps were repeated by using solvent development of 100 ml of 4 : 1 ethyl acetate-hexane solvent.

3.3.1 Visible light

Under the visible light, TLC plates were observed, marked and evaluated by retardation factor or retention factor value (R_f). R_f value indicates the ratio of distance travelled by solute (compound) to the distance travelled by solvent front. Each compound has its own characteristic of R_f value, which can be used to identify compounds. Formula used to calculate R_f value is as follows:

$$R_f \text{ value} = \text{distance of the spot (cm)} / \text{distance of the solvent front (cm)}$$

3.3.2 UV light

For detecting ultraviolet active absorbing spots, TLC plates were perceived under ultraviolet (UV) light.

3.3.3 Reagents for Detection of Alkaloid, Terpenoid and Phenol.

Reagents were prepared for the TLC to detect phytochemical in the samples of hexane, chloroform and methanol extract of *D.metel*.

a) Dragendorff's reagent

Dragendorff's reagent was prepared by mixing 5 ml of solution A and 5 ml of solution B with 20 g of acetic acid and 70 ml of distilled water (dH_2O). Solution A was made by dissolving 1.7 g of basic bismuth nitrate into 100 ml mixture of distilled water and HCl or 100 ml of acetic acid aqueous (20 g acetic acid dissolved with 80 ml dH_2O). Solution B was mixed by dissolving 40 g of potassium iodide in 100 ml of dH_2O. Dragendorff's reagent was evenly sprayed at TLC plate and let to dry in an operating hood. Orange-brown spots were immediately marked.

b) Vanilin–H_2SO_4 reagent

Vanilin–H_2SO_4 reagent was prepared by mixing 1.0 g of vanilin powder with 1.3 ml of concentrated sulphuric acid (H_2SO_4) and 100 ml of 99 % ethanol. The mixture was then shaken gently to dissolve all to become a solution. The thin-layer chromatography (TLC) plate was sprayed with Vanilin–H_2SO_4 reagent and then heated on a hot plate at 100°C for 3 to 5 minutes. The appearance of blue or purple spots was mark to indicate the presence of terpenoid.

c) Folin–Ciocalteu reagent

Folin–Ciocalteu reagent was prepared by mixing 50 ml of Folin–Ciocalteu with 50 ml of dH_20. This reagent detects the presence of phenol by causing the formation of blue bands on the chromatogram after spraying. Detection of phenol group was done by means of spraying Folin–phenol reagent onto chromatoplate. The color change of the spots remarked presence of phenol.

3.4 Determination of total phenolic contents (TPC).

The total phenolic content was determined by Folin–Ciocalteau methanol with slight modification (Velioglu, Mazza, Gao & Oomah, 1998). 3 ml of water was added to each test tube into which 50 µL of the sample was added. Then, 250 µL of Folin–Ciocalteau reagent was added to each test tube and vortexed for 5 seconds. Next, 750 µL of 20 % Na_2CO_3 solution was added to each test tube. The mixtures were then incubated at 45°C for 15 minutes. The absorbance was measured at 765 nm using a spectrophotometer against a blank after 2 hours. Total phenolic content was determined using a standard curve with gallic acid. Measurement of every sample was taken in triplicate and the results were expressed as milligram gallic acid equivalent (GAE) / g dried weight.

3.5 Determination of Total Flavonoid Content

Flavonoid contents were determined using aluminium chloride colorimetric method as described by Chang *et al*. (2002) with slightly modifications. Crude extract (5 mg / ml) 0.7 ml of 5 % (w / w) sodium nitrate and 10 ml of 30 % (v / v) ethanol were mixed for 5 min and then 0.7ml of 10 % aluminum chloride (w / w) was added and mixed. About 6 minutes later, 5 ml of 1 mol / l sodium hydroxide was added. Subsequently, the solution was diluted to 25 ml with 30 % (v / v) ethanol prior to measurement. The absorbance of the solution was measured at 510 nm using a spectrophotometer. The total flavonoid content was determined using a standard curve with quercetin at 0, 200, 400, 600, 800, 1000 and 2000 mg / L. The experiments were done in triplicate.

3.6 Identification of alkaloid in *D. metel* leaves, stem and seeds by LCMS/MS

In order to identify the alkaloid groups in *D. metel* leaves, stem and seeds, a combination of liquid chromatography with mass spectrometry (LCMS / MS) was used as this mode helps increase specificity. The procedure was initiated by using 0.25 g sample extracts diluted with 10 times methanol and filtered with 0.2 µM nylon filter prior to LCMS / MS. Rapid screening was set at 15 minutes run time. A stream of sample molecules was introduced to the ionisation chamber where ionisation occurred. The type of column used was Phenomenex aqua C18-50 mm × 2.0 mm × 5 µM; Buffer A with 0.1 % formic acid and 5mM ammonium formate; Buffer B: Acetonitrile with 0.1 % formic acid and 5 mM ammonium formate.

3.7 Toxicity test

3.7.1 Brine Shrimp Lethality Assay (BSLA)

A rapid toxicity test was performed using brine shrimp lethality assay, as shrimp larvae are often toxic to bioactive compounds. This method was found to be useful for the assessment of the toxic potential of various plant extracts (Gadir, 2012; Naidu, Ismail & Sasidharan, 2014). This method also provides preliminary screening data that can be backed up by more specific bioassays once the active compounds have been isolated (P. Pisutthanan, Plianbangchang, N. Pisutthanan, Ruanruay, & Muanrit, 2004).

The eggs of brine shrimp were added in filtered artificial seawater, which was prepared by dissolving 38 g of sea salt into 1 L of distilled water for 48 h at room temperature in the dark. Three concentrations were prepared for each crude extract (10 µg / ml, 100 µg / ml and 1000 µg / ml) and transferred to multiple plates. Determining the concentration range is important in which a linear correlation exist between the concentration and the lethality of the brine shrimps. Most experiments for toxicity assessment of herbal extracts include a concentration range of 10, 100 and 1000 µg / ml (Parra, Yhebra, Sardiñas, & Buela, 2001). Each vial consisted of 5 ml of sea water, 5 ml of extract and 10 brine shrimps. Water was used as control. This experiment was conducted simultaneously. After 24 hours, the surviving shrimps were counted and determined LC_{50} by Probit analysis program. This experiment was performed in triplicate.

3.7.2 Toxicity study in mice

In order to provide data for toxicity and to assess memory recovery, male and female mice were used. Random selections of adult mice (three months old) with weight range 28 – 35 g were subjected to acute toxicity study. Short duration of toxicity studies was conducted to test the first administration of extract sample into mice body. Leaf methanol extract was chosen as the sample to proceed with this animal study. A single dose was administered to three groups, with each group consist of 3 mice, regardless their gender; high dose (0.5 mg / g) group, medium dose (0.25 mg / g) group and low dose (0.125 mg / g) group. Mice were housed in polypropylene cages and were provided with standard mice food and water ad libitum. After two weeks, the body condition score (BCS) of each mouse was examined first-hand and recorded according to the technique described by Ullman-Cullere and Foltz (1999). This procedure was conducted by gently holding the base of the mouse‘s tail and passing a finger over the sacroiliac bones. The general condition of mice appearance: skin, nose, eyes, external genitalia and behaviour were also observed. Body weight was also recorded daily. The mice were observed continuously for 24 hours for any sign of toxicity or mortality for two weeks.

3.8 Acetylcholinesterase inhibition assay

Ellman colorimetric method was used as described by Y. Li, Bai, C. Li, and Shi (2011) with slight modifications to quantify AChE inhibitory activity. The enzyme was purchased in the form of 827 U / mg hydrophilised powder. The 5 ml test tube contained 30 µL of AChE (0.0025 U / ml and 30 µL of 1 mg / ml sample solution were mixed and incubated at 40°C in 2.81 ml of 0.1 M phosphate buffer (pH 8.0). This incubation was continued for 20 minutes. Meanwhile, 30 µL of Ellman reagent (DTNB) were mixed with 30 µL of acetylcholine and readily prepared in 96-well microtiter plate and the reaction started immediately after the mixture of the enzyme was introduced into the well and incubated for 20 minutes at room temperature. Absorbance was measured with enzyme – linked immunosorbent assay (ELISA) at 405 nm. The percentage inhibition in enzyme activity was determined by using the following formula:

$$\% \text{ inhibition} = \left[\frac{\text{Absorbance (control)} - \text{Absorbance (test)}}{\text{Absorbance (control)}} \right] \times 100 - \text{centre}$$

Berberine was used as positive control. The experiments were done in triplicate.

3.9 Memory test

This experiment was designed to evaluate the effect of *D. metel* extract on memory impairment in mice. Mice were treated with doses of 0.1 mg / g body weight of leaf methanol in the room temperature, with 12 hour light and 12 hour dark cycles every day. An eight-arm radial maze was used. Each arm was 76.2 cm long and 8.9 cm wide. The wall of the radial arm maze was made of transparent hard plastic, and the partitions were made of hard cardboard. Food and water were routinely monitored. Before each trial, all apparatuses were washed clean with alcohol. The male and female mice were divided into four groups of six mice each, including three each of male and female mice: Group 1: control; Group 2: leaf methanol extract; Group 3: berberine (positive control) and Group 4: valium (negative control). Berberine chloride was diluted with distilled water and administered orally to mice in Group 3. Intragastric administrations of treatment solution was carried out using straight, blunt - ended, stainless -steel needles to feed the mice according to their group. All mice were fasted overnight to ensure that their stomach was empty prior to the test. All groups, excluding the control group, were administered with valium and left for 1 hour. Following that, all mice in Group 2 were administered with methanol leaf extract. After a further 1 hour interval, each mouse was placed at the centre of the radial arm maze, and only two arms were baited with the same size of pallet, while the other empty arms were closed using removable cardboard. These arms were randomly selected for baiting. The mouse was allowed to enter these two open arms and retrieved pellet within five minutes. Upon retrieving both two rewards (pallet), this mouse was locked in the centre of the radial arm maze for thirty seconds. After the delay, the test was started with all eight arms of the maze being open simultaneously and the two pallets just now were transferred to any two of the closed arm mazes as before.

This test was run for 10 minutes and the number of repeating entries (NRE) to any compartment of the maze that had already been visited were recorded. The number of entries until the first error occurs (NEF) is frequently used as supplementary measure of performance towards mice experimenting in a radial arm maze. This score is simply named as the number score. It indicates the successive number of attempts in eating pallet without repeating entry into the maze arm it ate before. This step was repeated to each mouse in the groups. For Groups 3 and 4, berberine solution and water solution were administered, respectively, instead of the leaf methanol extract.

CHAPTER 4: RESULTS

4.1 Analysis of thin layer chromatography

The result of TLC experiment remarked the presence of alkaloid in methanol extracts of leaves and stem samples (Table 4.1, Table 4.2, Table 4.3 and Table 4.4). Apart from alkaloid compound, terpenoid was also found in chloroform and methanol extracts of leaves, stem and seeds but not in hexane extracts. The chemicals being separated were colourless, however when reviewed under short wavelength UV light, the hexane extract of stem at R_f 0.73 displayed a blue spot (Table 4.3). Another blue and green spots were observed in methanol extract of stem at R_f 0.61 and 0.65, respectively (Table 4.3). Those substances fluoresces under UV light suggested most commonly aromatic compound and absorbed wavelength range 220 nm to 280 nm. Hexane was the less polar solvent compared to chloroform and methanol. From TLC, it was cleared that *D. metel* leaves and stem consisted many polar phytochemical constituents compared to seed as demonstrated by many spots that had been detected on the TLC plates.

Table 4.1: Thin layer chromatography profile of hexane, chloroform and methanol extract from *D. metel* leaves in methanol- chloroform solvent (1:9)

Solvent extract	Label compound	R_f value	Colour under visible light	Colour under UV light	Reagents			Remarks: Present of
					Dragendorff	Vanilin	Iodine vapour	
Hexane	A1	0.73	Brown(+)	–	–	–	–	–
	A2	0.99	Green(+)	–	–	–	–	–
Chloroform	B1	0.75	Green(++)	–	–	–	–	–
	B2	0.79	Green (++)	–	–	–	–	–
	B3	0.82	Green(+)	–	–	–	–	–
	B4	0.89	Grey(+)	–	–	–	–	–
	B5	0.90	Grey(+++)	–	–	Purple(+++)	–	Terpenoid
	B6	0.91	Grey(+)	–	–	–	–	–
	B7	0.93	Yellow(+++)	–	Orange(+++)	–	–	Alkaloid
	B8	0.99	Green(+++)	–	–	–	–	–
Methanol	C1	0.29	Brown(+)	–	–	Purple(+)	–	Terpenoid
	C2	0.33	Green(+)	–	–	Blue(+)	–	Terpenoid
	C3	0.40	Grey(+)	–	–	Purple(+)	–	Terpenoid
	C4	0.54	Brown(++)	–	–	Purple(+)	–	Terpenoid
	C5	0.58	Grey(+)	–	–	–	–	–
	C6	0.69	Grey(+)	–	–	–	–	–
	C7	0.90	Yellow(++)	–	Orange(+)	–	–	Alkaloid
	C8	0.95	Grey(+)	–	–	–	–	–

Indication of colour intensity: +++ = strong ++ =mild +=weak – = absence

Table 4.2: Thin layer chromatography profile of hexane, chloroform and methanol extract from *D. metel* leaves in ethyl acetate : hexane solvent (4:1)

Solvent extract	Label compound	Rf value	Observation					Remarks: present of
			Colour under visible light	Colour under UV light	Reagents			
					Dragendorff	Vanilin	Iodine vapour	
Hexane	A1	0.48	Yellow	–	–	–	–	–
	A2	0.52	Grey	–	–	–	–	–
Chloroform	B1	0.10	Grey(+++)	–	–	–	–	–
	B2	0.13	Grey(++)	–	–	–	–	–
	B3	0.15	Grey(+)	–	–	–	–	–
	B4	0.20	Grey(+)	–	–	–	–	–
	B5	0.23	Grey(+++)	–	–	–	–	–
	B6	0.26	Grey(+)	–	–	–	–	–
	B7	0.37	Yellow(+)	–	–	–	–	–
	B8	0.70	Yellow(++)	–	–	–	–	–
	B9	0.80	Grey(+)	–	–	–	–	–
	B10	0.94	Grey(+++)	–	Orange(+)	–	–	Alkaloid
Methanol	C1	0.27	Grey(++)	–	–	–	–	–
	C2	0.41	Green(+)	–	–	–	–	–
	C3	0.47	Grey(+++)	–	–	–	–	–
	C4	0.52	Grey(+)	–	–	–	–	–
	C5	0.60	Grey(+)	–	–	–	–	–
	C6	0.78	Yellow(+)	–	Orange(+)	–	–	Alkaloid
	C7	0.94	Green(+++)	–	Orange(++)	–	–	Alkaloid

Indication of colour intensity: +++ = strong ++ =mild +=weak – = absence

Table 4.3: Thin layer chromatography profile of hexane, chloroform and methanol extract from *D. metel* stem in methanol : chloroform solvent (1:9)

Solvent extract	Label compound	Rf value	Observation					Remarks: present of
			Colour under visible light	Colour under UV light	Reagents			
					Dragendorff	Vanilin	Iodine vapour	
Hexane	A1	0.73	–	Blue(+)	–	–	–	–
	A2	0.99	Green(+)	Purple(+)	–	–	–	–
Chloroform	B1	0.62	Green(+)	Blue(+)	–	–	–	Terpenoid
	B2	0.70	Green(+)	Purple(+)	–	–	–	–
	B3	0.71	Grey(+)	–	Orange	–	–	Alkaloid
Methanol	C1	0.61	–	Blue(+)	–	–	–	–
	C2	0.65	–	Green(+)	–	–	–	–
	C3	0.87	Green(+)	–	Orange	–	–	Alkaloid
	C4	0.89	Green(+)	–	–	–	–	–

Indication of colour intensity: +++ = strong ++ =mild +=weak – = absence

Table 4.4: Thin layer chromatography profile of hexane, chloroform and methanol extract from *D. metel* stem in ethyl acetate : hexane solvent (4:1)

Solvent extract	Label compound	Rf value	Observation					Remarks: present of
			Colour under visible light	Colour under UV light	Reagent			
					Dragendorff	Vanilin	Iodine vapour	
Hexane	A1	0.21	Yellow	−	−	−	−	−
Chloroform	B1	0.86	Grey(+)	−	−	−	−	−
	B2	0.87	Green(+)	−	−	−	−	−
	B3	0.88	Green(+)	−	−	−	−	−
Methanol	C1	0.61	−	−	−	−	−	−
	C2	0.65	−	−	−	−	−	−

Indication of colour intensity: +++ = strong ++ =mild +=weak −= absence

Table 4.5: Thin chromatography profile of hexane, chloroform and methanol extract from *D. metel* seed in methanol : chloroform solvent (1:9)

Solvent extract	Label compound	R_f value	Observation					Remarks: present of
			Colour under visible light	Colour under UV light	Reagent			
					Dragendorff	Vanilin	Iodine vapour	
Hexane	A1	0.55	Brown(+)	−	−	−	−	−
Chloroform	B1	0.29	Brown(+)	−	−	Purple(+)	−	Terpenoid
Methanol	C1	0.21	Brown(+)	−	−	−	−	−

Indication of colour intensity: +++ = strong ++ =mild +=weak − = absence

Table 4.6: Thin layer chromatography profile of hexane, chloroform and methanol extract from *D. metel* seed in ethyl acetate : hexane solvent(4:1)

Solvent extract	Label compound	R_f value	Observation					Remarks: present of
			Colour under visible light	Colour under UV light	Reagents			
					Dragendorff	Vanilin	Iodine vapour	
Hexane	A1	0.55	Yellow(+)	−	−	−	−	−
Chloroform	B1	0.29	Green(+)	−	−	−	−	−
	B2	0.31	Brown(+)	−	−	−	−	−
Methanol	C1	0.21	Grey(+)	−	−	−	−	−

Indication of colour intensity: +++ = strong ++ =mild +=weak − = absence

Table 4.7: Total phenols contents (TPC) and total flavonoid contents (TFC) of *D. metel* extracts

Extracts		TPC (mg GAE / g of extract)	TFC (mg QE/g dry weight)
Leaves	Methanol	305.12±0.09	60.29±0.07
	Chloroform	291.74± 0.03	40.13±0.07
	Hexane	12.99±0.06	21.37±0.07
Stem	Methanol	101.74±0.06	281.57±0.08
	Chloroform	77.44±0.02	70.34±0.07
	Hexane	13.98±0.03	16.71±0.08
Seed	Methanol	186.40±0.06	286.90±0.09
	Chloroform	154.51±0.03	236.48±0.09
	Hexane	12.86±0.03	19.87±0.82

4.2 Analysis of total phenolic content (TPC) and total flavonoid content (TFC)

The highest yield of phenol was obtained when methanol was used as extraction solvent in leaves, stem and seed. The highest TPC was detected in methanol extract of leaf, 305.1163 mg Gallic acid equivalent (GAE) / 100 g. The TPC in *D. metel* extracts using the Folin- Ciocalteu's reagent was expressed as gallic acid equivalent (the standard curve equation: y =0.0043x, r2 = 0.9989)(Appendix C). The values calculated for the concentration of total phenols were expressed in mg of GAE / g of extract dry mass (Table 4.7). The TPC in *D. metel* depends on the type of extract, i.e. the polarity of solvent used in extraction. High solubility of phenols in polar solvents provides high concentration of these compounds in the extracts obtained using polar solvents for the extraction (Mohsen & Ammar, 2008; Zhou & Yu, 2004).

The concentration of flavonoid content extracted from methanol, chloroform and hexane were determined using spectrophotometric method with aluminum chloride. TFC result of *D. metel* ranged from 16.71 ± 0.076 to 286.90 ± 0.083 mg QE / g dry mass. The highest content of flavonoid was found in methanol extract of leaves which was 286.90 ±0.083 mg QE/ g dry mass (Table 4.7). Seed chloroform extract was observed as the second highest of TFC compared to other solvent extraction in the other part of *D. metel*. Overall, leaves showed the lowest concentration of flavonoid. The concentration of flavonoids in plant extracts depends on the polarity of solvents used in the extract preparation (Gao & Liu, 2005).

4.3 Liquid chromatography with mass spectrometry (LCMS / MS) Analysis

TLC analysis indicated the presence of alkaloid in methanol and chloroform extract but not in hexane extract. Methanol extract of the leaves was analysed using liquid chromatography with mass spectrometry (LCMS / MS) to identify the alkaloids present in methanol extract of leaves, stem and seed. Full chromatogram of leaves, stem and seed were recorded as in Figure 4.2, Figure 4.3 and Figure 4.4. The analysis of the leaves methanol extract with LCMS / MS shows that it contains 3- hyroxy- 6 - tigloyloxy tropane, apoatropine, cuscohygrine, homatropine, hyoscyamine, scopolamine, tropane alkaloid, withalactone, daturalactone 4 and daturilin ammonium adduct (Appendix B1; Appendix B2; Appendix B3; Appendix B4; Appendix B5; Appendix B6; Appendix B8; Appendix B9; Appendix B10; Appendix B11). Anisodamine, apoatropine, hyoscyamine, meteloidine, proanthocyanidine and scopolamine were found in stem (Appendix B12; Appendix B13; Appendix B14; Appendix B15; Appendix B16; Appendix B17) and only anisodamine, apoatropine, scopolamine and proanthocyanidine found in the seed (Appendix B.18; Appendix B19; Appendix B20; Appendix B21). Most alkaloids were identified from the leaves, followed by the stem and seed. Two common alkaloids found in those three parts of *D. metel* were apoatropine and scopolamine.

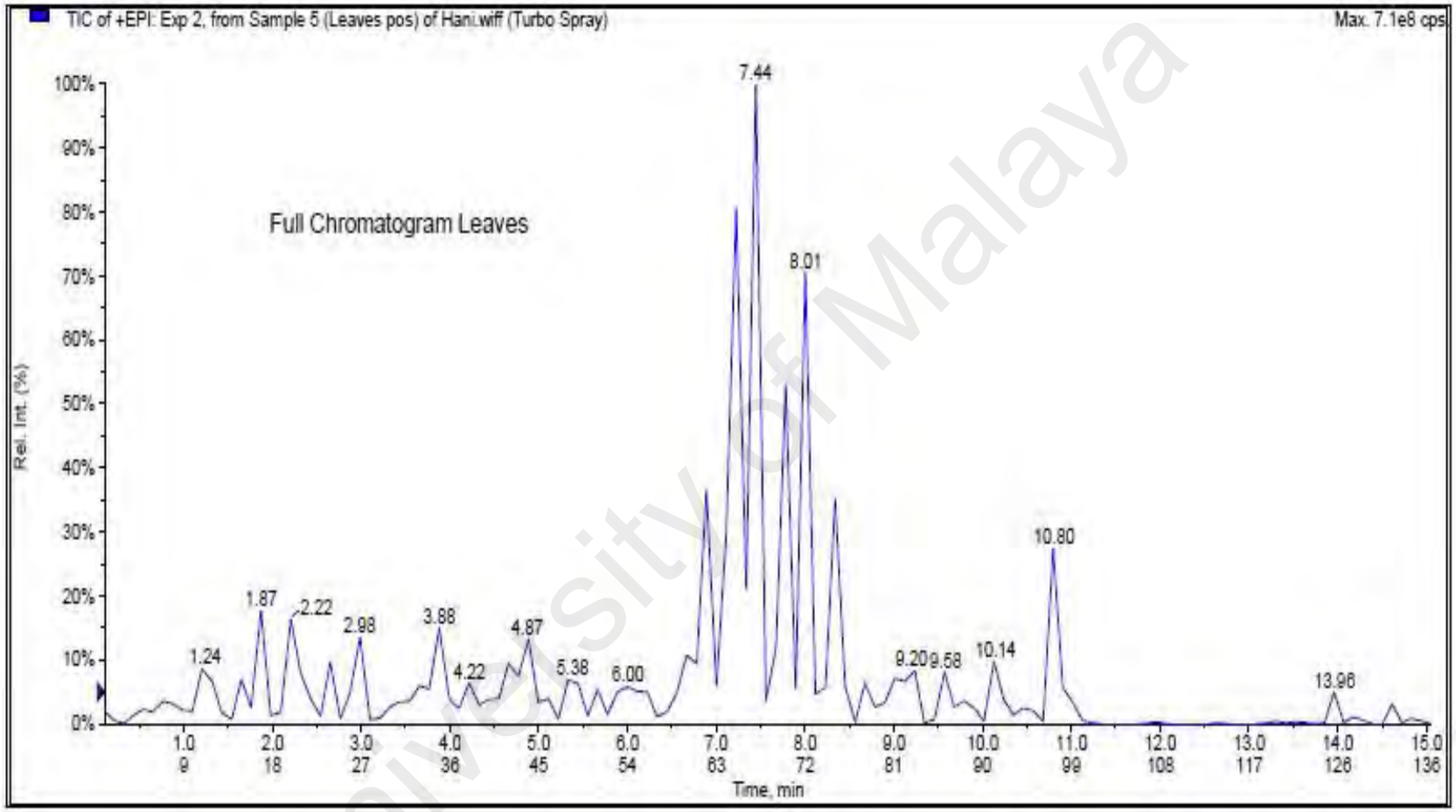


Figure 4.2: Full LCMS / MS profile of methanol leaves crude extracts of *D.metel*

Table 4.8: Alkaloid found in leaves crude extract of *D. metel* from full LCMS / MS profile

Metabolite	**Retention time (min)**	**Intensity(cps)**
3-hydroxy-6-tigloyloxy tropane	0.988	7.6e6
Apoatropine	1.098	6.2e6
Cuscohygrine	1.208	2.3e7
Daturalactone	3.881	4.2e6
Homatropine	3.211	1.2e6
Hyoscyamine	1.540	2.1e6
Scopolamine	0.768	8.0e6

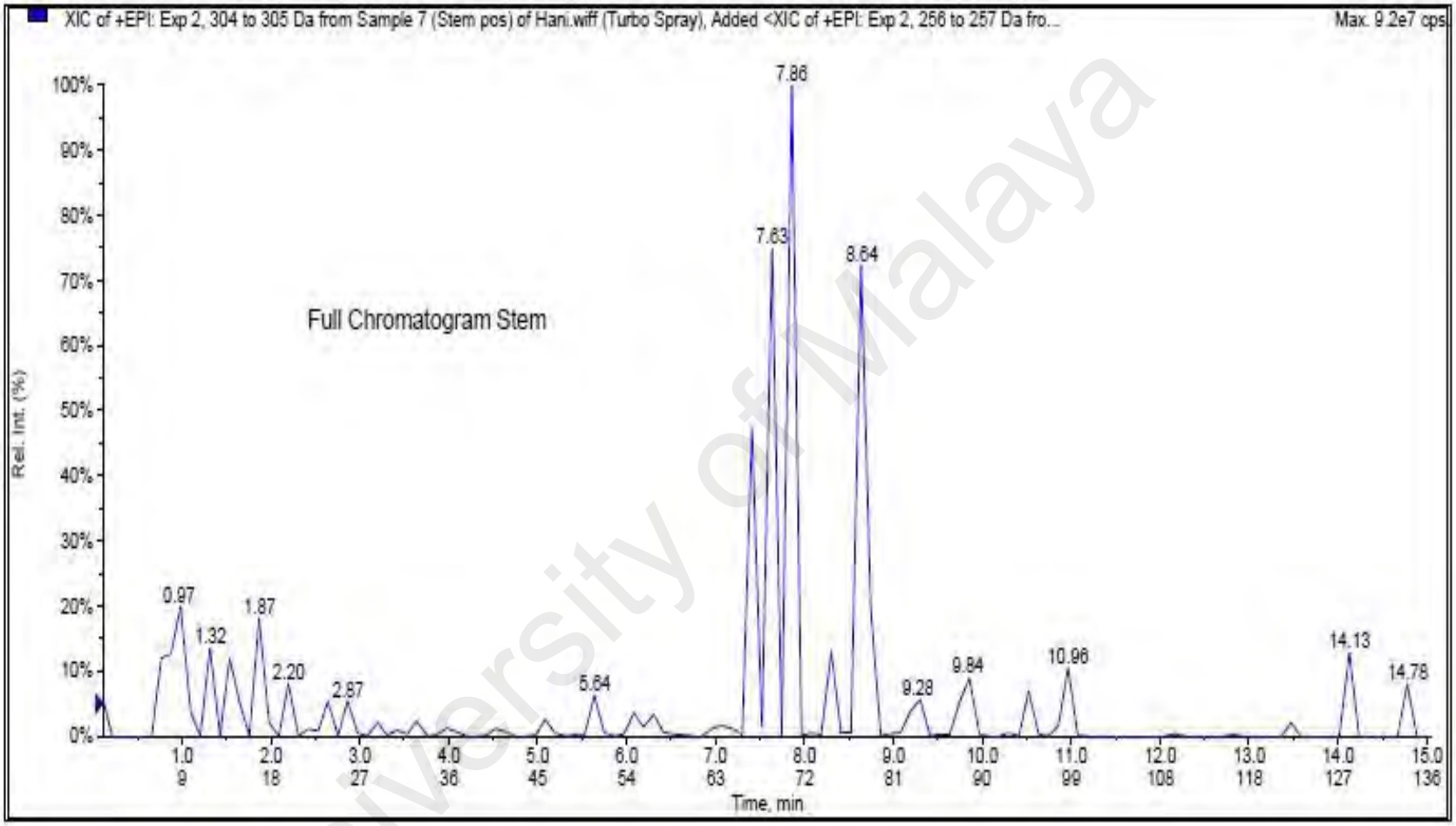


Figure 4.3: Full LCMS / MS profile of methanol stem crude extract of *D. metel*

Table 4.9: Alkaloid found in stem crude extract of *D. metel* from full LCMS / MS profile

Metabolite	**Retention time (min)**	**Relative intensity(cps)**
Anisodamine	1.647	2.4e6
Apoatropine	0.988	8.6e6
Hyoscyamine	1.317	1.207
Meteloidine	0.878	1.1e7
Proanthocyanidine	1.538	1.1e7
Scopolamine	0.768	1.1e7

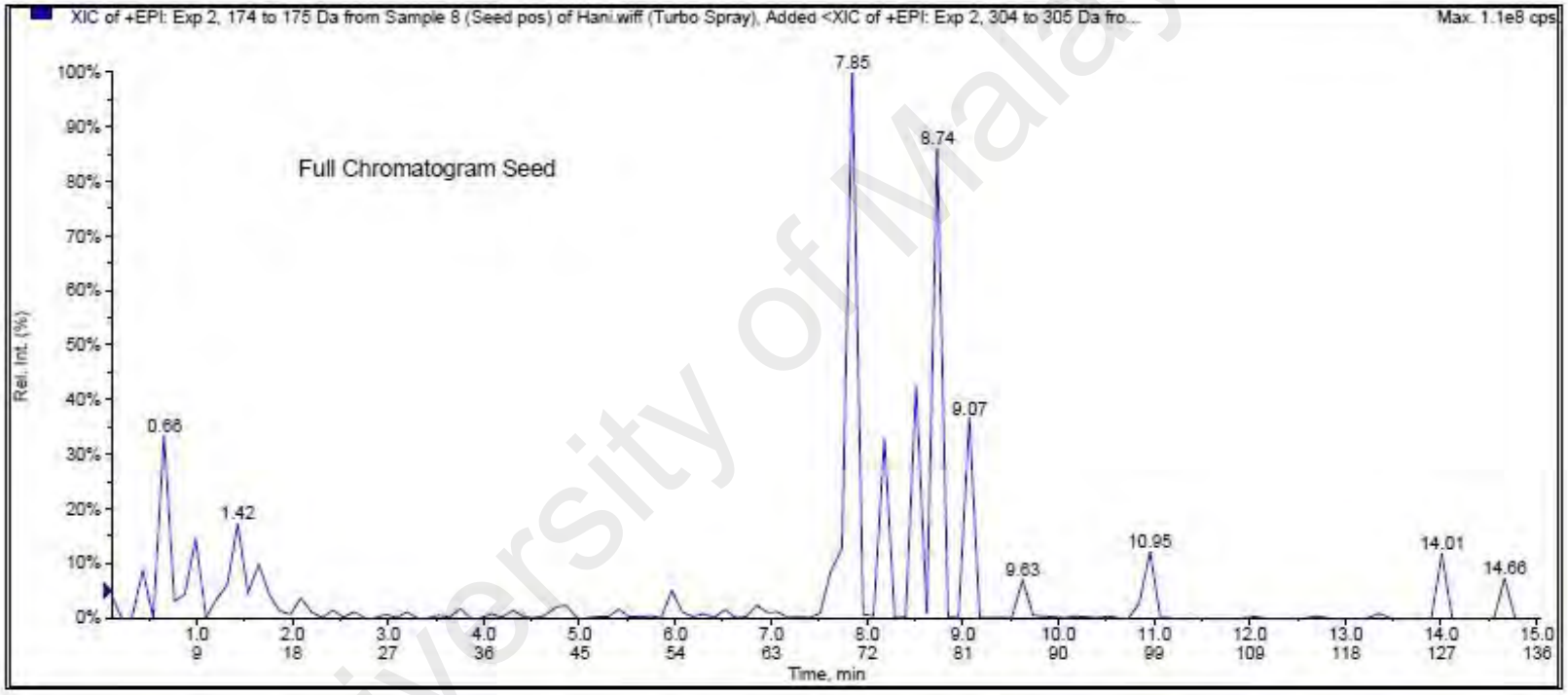


Figure 4.4: Full LCMS / MS profile of methanol seed crude extract of *D. metel*

Table 4.10: Alkaloid found in seed crude extract of *D. metel* from full LCMS / MS *profile*

Metabolite	**Retention time (min)**	**Relative Intensity(cps)**
Anisodamine	1.648	5.26e6
Apoatropine	0.988	7.5e6
Proanthocyanidine	0.659	2.6e7
Scopolamine	2.090	3.6e6

4.4 Toxicity test

4.4.1 Brine shrimp lethality assay (BSLA)

Further investigation was done to find adverse effect of methanol extract of *D. metel* on biological system by exposing it to brine shrimp in BSLA. The LC_{50} values for the brine shrimps were above 1000 µg / ml, indicating that these extracts are non-toxic. The LC_{50} of leaves hexane extract and leaves methanol extract were 316.29 ± 0.75 µg / ml and 311.23 ± 0.11 µg / ml, respectively (Table 4.8). They were the most nontoxic extracts to be studied and were the safest to be applied.

Table 4.11: LC_{50} of *D.metel*

Extract	Sample	LC_{50} (µg / ml) ± SD
Methanol	Leaves	311.23±0.11
	Stem	68.76±0.11
	Seed	29.37±0.14
Chloroform	Leaves	221.64±0.70
	Stem	109.41±0.71
	Seed	17.71±0.85
Hexane	Leaves	316.29±0.75
	Stem	166.08±0.11
	Seed	171.35±0.14
Standard	Berberine	11.39±0.85

4.4.2 Toxicity on mice

Apart from lethality concentration test on brine shrimp, toxicity test were also done on mice. For this study, only methanol crude extract from leaf was used due to its highest content of alkaloid, phenol and flavonoid group and it did not display toxicity on the brine shrimp. Stem and seed extracts were not utilized as they were inferior to leaf based on the results in the earlier experiments. In mice toxicity study, daily observation showed that administration of samples had produced no side effects on mice. There was no mortality demonstrated too. Furthermore, there was no significant difference between the group of mice that were given oral gavage at high dose (0.5 mg/body weight), medium dose (0.25 mg/body weight) or low dose (0.125 mg/ body weight). The observation was recorded as below.

(i) Mortality

a. No mortality was seen throughout the study period

(ii) Body condition score (BCS)

a. Skin, nose, eyes, external genitalia and behavior were normal

(iii) Body weight

a. No significant difference in body weight between control group of mice (0 ml/g body weight) to all groups of mice that orally gavage at doses of high dose (0.5 mg/g) group, medium dose (0.25 mg/g body weight) group and low dose (0.125 mg/body weight) group

4.5 Acetylcholinesterase inhibition assay

The TLC and LCMS / MS results showed that methanol extract of *D. metel*'s leaves, stem and seed reacted positively in inhibiting the acetylcholinesterase. The AChE inhibitory activity of all samples *D. metel* showed an increasing effectiveness by increasing the concentration of the samples from 0.5 mg / ml to 150 mg / ml (Figure 4.5).

The best inhibition demonstrated by the leaves extract at concentration of 150 mg/ ml which was 85.94 %, followed by stem extract 73.92 % and seed extract at 56.58%. Lowest inhibition from each samples were found at the lowest concentration of 0.5 mg / ml which was 47.93% by stem extract, 60.52% by leaves extract, and 64.46% by seed extract. Meanwhile, the seed extracts only showed medium inhibitory activity (8.06 % to 56.58 %) and its weakest AChE activity was demonstrated at the concentration of 0.5 mg / ml. Overall, leaves extract demonstrated better inhibition compared to stem and seed and the strongest AChE activity was found in leaves methanol extract at the concentration of 150 mg/ml. For this reason, the methanol extract of leave was selected for memory recovery study in mice.

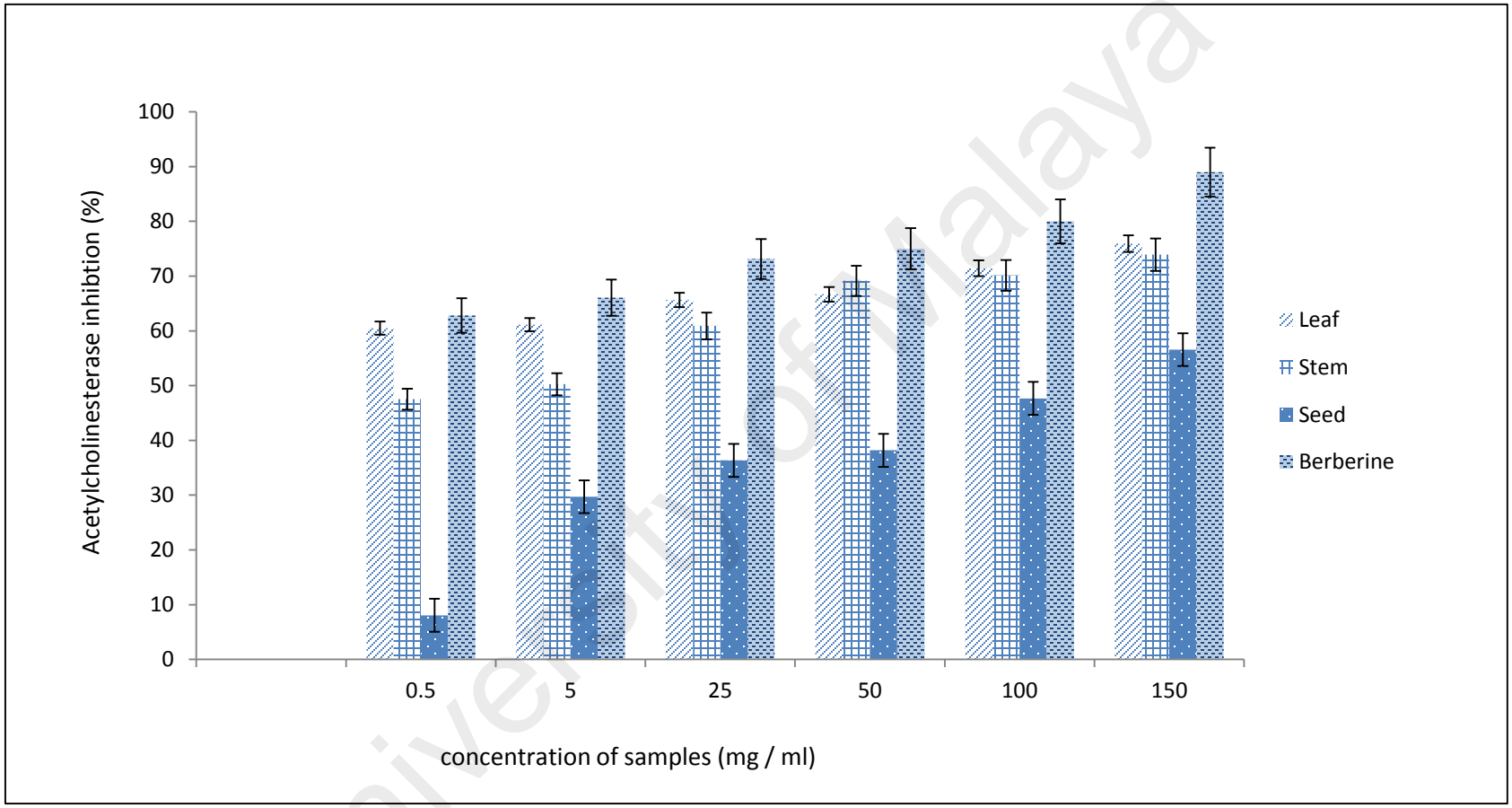


Figure 4.5: Percentage of AChE inhibitory activity of *D. metel* leaves, stem and seeds of methanol extracts

4.6 Memory test on mice

After confirming the methanol extract of leaves did not cause any significant toxicity, the sample was used in mice memory test. All groups of mice were treated with valium to induce memory impairment apart from the control (Group 1). Mice in Group 2, 3 and 4 were then treated with methanol leaf extract, berberine and no treatment respectively. The numbers of entries of all mice in the arm of the maze were recorded. It was observed that the mice which were not treated with the methanol extract following valium administration (Group 4) performed the worst as illustrated by the highest number of re-entries in the maze. The score for males in Group 4 was 11.67, whereas for females it was slightly low at 9.67 (Figure 4.6). On the other hand, the results of the number of re-entries for the control group (Group 1), where no valium and treatment was administered, were the best. This showed that the mice in the control group had better memory compared to those who were treated. The results for leaf methanol extract (Group 2) were exceptionally better than those in the negative control (Group 4); however, it was not significantly different to those in the berberine group (Group 3), which was the positive control. The improvement in the number of repeat entries in Group 2 suggests that *D. metel* extract can improve episodic memory recollection in memory impaired mice.

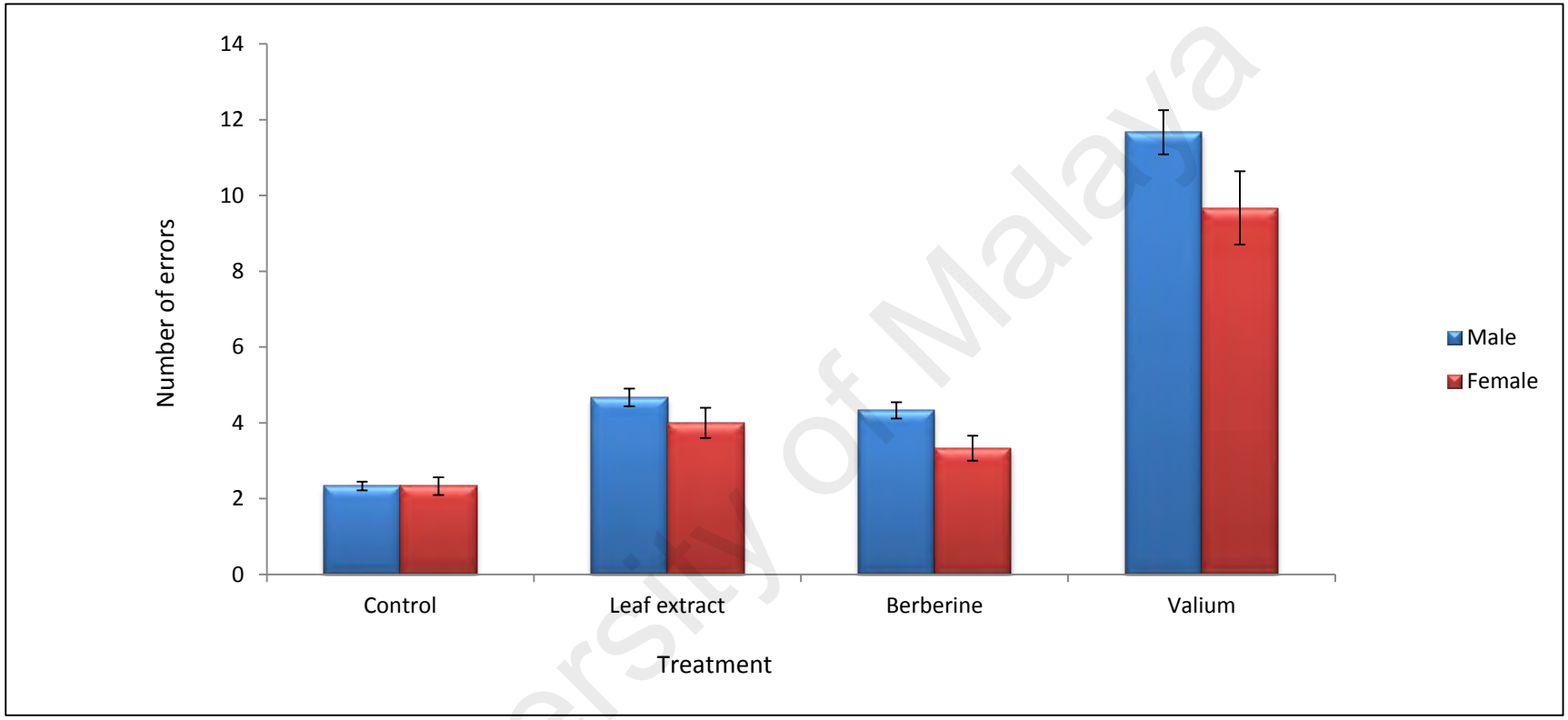


Figure 4.6: Number of repeat entries to arms of the maze (NRE) in male and female mice

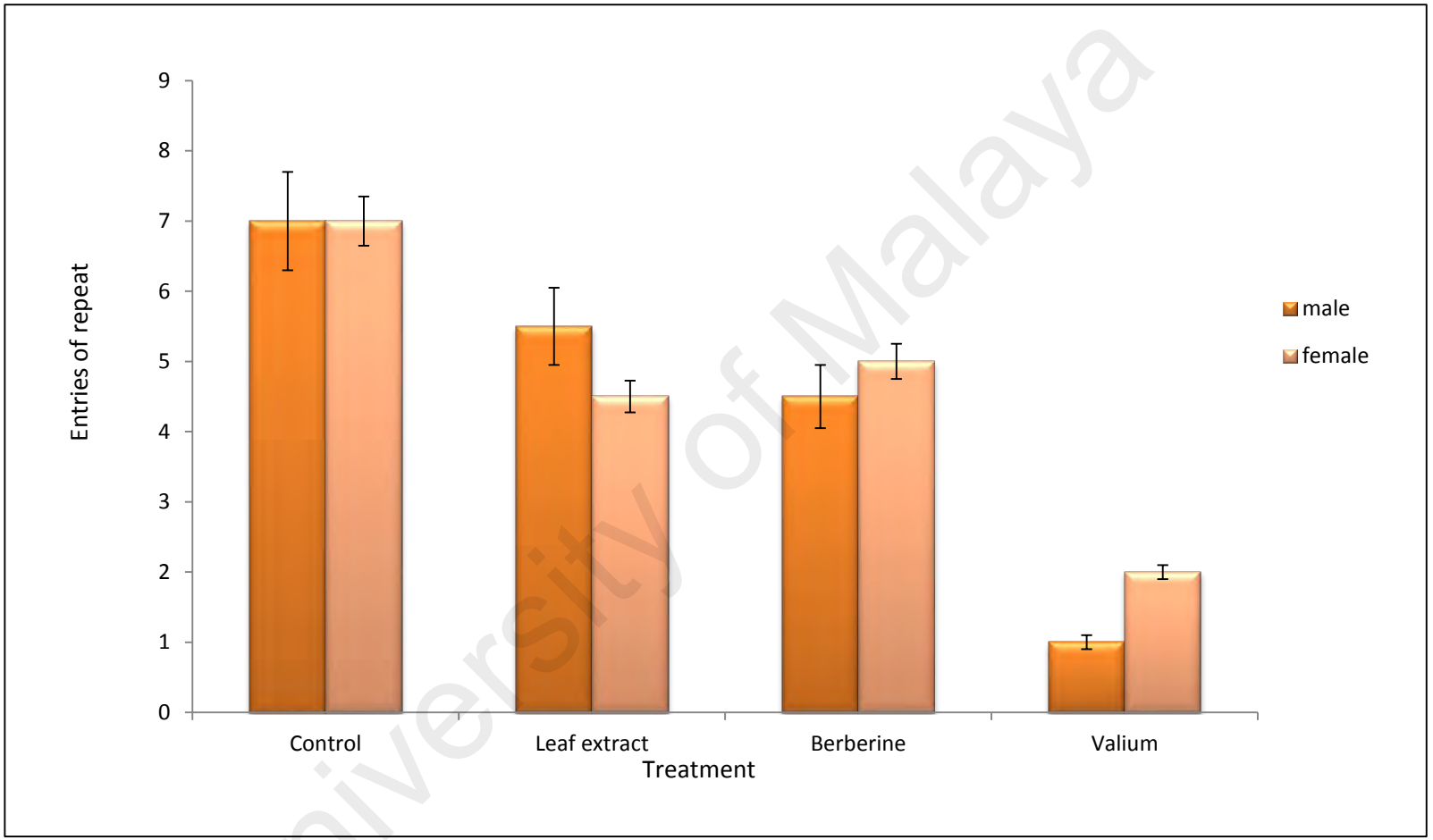


Figure 4.7: Number of entries until the first error occurs in male and female mice

CHAPTER 5: DISSCUSSION

Nature has a cure for almost all human ailments and diseases. Since centuries, plants and herbs have, successfully, been used for various medical purposes. These plant products can either be used directly after extraction and purification or can be synthesized in a laboratory, using series of chemical reactions. Alzheimer's Disease (AD) is one such disorder that has no specific treatment till date as it shares symptoms with many other neurodegenerative disorders and affects different parts of the brain in different people. Acetylcholinesterase (AChE) inhibitors have shown some promising results in the past to manage this incurable disease. To avoid the serious adverse reactions, associated with synthetic AChE inhibitors, plant-derived products are always preferable. For this reason, this research had worked on the extraction of new potential AChE inhibitors from *D. metel*.

Through extraction process, this study found that solvent development of ethyl acetate : hexane (4 : 1) (v / v) showed nearly similar separation of sample's chemical compound as in solvent development of chloroform : methanol (9:1) (v / v). Since these two solvents development provide similarly degree of separation, the cheapest of the non-halogenated solvents, which is ethyl acetate- hexane, would be more preferable in the future

The LCMS / MS analysis of leaf, stem and seed extracts of *D. metel* confirmed the presence of various alkaloids while the TLC demonstrated the presence of terpenoids and flavonoids. This result is consistent with Alabri *et al*. (2013) and Sangeetha *et al*. (2014).

These compounds, especially alkaloids, share structural identity with Ach and therefore, can inhibit the hydrolytic activity of AChE by binding at its active site.

Standard enzymatic assay with Ellman colorimetric method was used in this study to evaluate the anticholinergic activity of leaf, stem and seed extracts of *D. metel.* The results revealed that increasing concentration of methanol extract will increase AChE inhibition. The results also showed that the leaf extracts had comparable inhibitory activity against the standard berberine. Berberine is commercially used as AChE inhibitor and is helpful in mitigating the symptoms of AD till some extent (Pohanka, 2014). The exact mechanism of action of this compound is still oblivious, but research predicts that it may exert its effect synergistically (Kaufmann, Kaur Dogra, Tahrani, Herrmann & Wink, 2016). This study demonstrated that the leaf extract of *D. metel* may induce similar inhibitory action on AChE and therefore, can be served as a potential drug candidate.

Although this study provides the evidence for the inhibitory action of *Datura* extracts but it has some limitations also. To identify the actual mechanism of action of these inhibitors is beyond the scope of this paper. However, further molecular work on how those compounds inhibit would be a perfect study on acetylcholinesetase inhibitor derived from *D. metel*.

Additionally, the presence of various compounds, such as 3-hydroxy-6-(tigloyloxy) tropane, apoatropine, cuscohygrine, homatropine, withalactone, etc., made it difficult to identify the one compound that has maximum pharmacological effect with minimum toxicity. Individual analysis of these compounds is desirable to understand their biological activity.

Al jadidi and Hossains (2015) analysis of total flavonoid content in leaves were approximately similar to the ones obtained in this study. The presence of flavonoid in *D. metel* indicates that this plant may have potential antioxidant properties as well. The seed would be the best part to be explored for its antioxidant property in future studies as it has the highest flavonoid contents compared to the leaves and stem.

For more than a hundred years, virtually every medical breakthrough has been the direct result of research using animals. The use of animal models has improved our understanding of many bioactive compounds. As bioactive compounds are almost always toxic at high doses, in vivo lethality study in a simple zoologic organism can be useful in providing preliminary screening data on the toxicity effect. Brine shrimp lethality assay (BSLA) was applied in this research and showed that the extracts of all studied variety exerted a toxic effect on *Artemia salina* at the highest dose of 1000 µg/ml, which was the only concentration used. Nevertheless, the methanol leaf extract has shown to be the least toxic activity in brine shrimps. The leaf extracts were found to be safer than stem and seed extracts. These results were similar to a study done by Al-Snafi (2017).

This study also demonstrated that seed extracts do contain some toxicological compounds and are not as effective as leaf extracts in inhibiting AChE. The concentration of a substance is the most important determinant of the outcome as if it reaches a sufficiently high concentration in the susceptible biological system, it could lead to toxic effects. This study also did not find any lethal or toxicological effects of *D. metel* extracts on mice. All mice survived after a single limit test dose.

The memory test indicates the number of repeat entries to radial maze was the highest in mice that were treated with valium only (negative control). Also, these valium-treated mice completed the least number of entries without any error. Although the mice, who received *D. metel* leaf extracts, performed better than berberine during the maze test, they were found less effective than the control group. The possible causes of this can be retardation in motor coordination and decrease in short-term memory (Tijani, Eyineyi, Ibrahim & Okhale, 2015). However, not enough studies are available to support such behavior.

Based on these findings, this research concluded that leaf extracts of *D. metel* are pharmacologically more useful in treating AD and therefore, further research should focus on detailed pharmacological and toxicological analysis of these leaf extracts.

CHAPTER 6: CONCLUSION

Acetylcholinesterase inhibitors are the most effective treatment group for AD. These drugs bind at the active site of AChE and thus, inhibit its hydrolytic action on ACh. The increased level of Ach in cholinergic synapsis helps in restoring cognitive ability by enhancing neural transmission and connectivity. Alkaloids serve as the potential anticholinergics because of their structural similarity with ACh. There are various synthetic and plant-derived alkaloids that have been studied to treat AD. Some of them are commercially available for the treatment of the disease and others are still under trials. The major problem with existing anticholinergics is their severe side effects and shorter half-lives. Therefore, there is a need to identify new compounds that are pharmacologically more active and have more negligible toxic effect.

Datura metel is a medicinal plant that induces various pharmacological actions, such as anti-inflammatory, antispasmodic, analgesic and antioxidative. The plant contains a wide range of alkaloids, terpenoids, and flavonoids that can be useful in treating AD. These compounds have been extracted from leaves, stems and seeds using methanol solvent. The anticholinergics action of the extracts was studied and compared with berberine. The leaf extracts exhibit significant inhibitory actions and need further evaluation for the identification of pharmacologically active compounds.

Seed and stem extracts are not very favorable because of the high concentration of toxic compounds. No significant toxic effects of *D. metel* have been found in this study. The future studies should focus on identification of alkaloids that can serve as potential drug candidates and synthesis of derivatives that have promising pharmacological activity with minimum side effects.

LIST OF SEMINAR AND PAPER PRESENTED

1) Postgraduate Seminar, 11th February 2014, Universiti Malaya. Studies of Acetylcholinesterase Inhibitors Derived from Alkaloid of *Datura metel* Linn. (Pokok Kecubung).

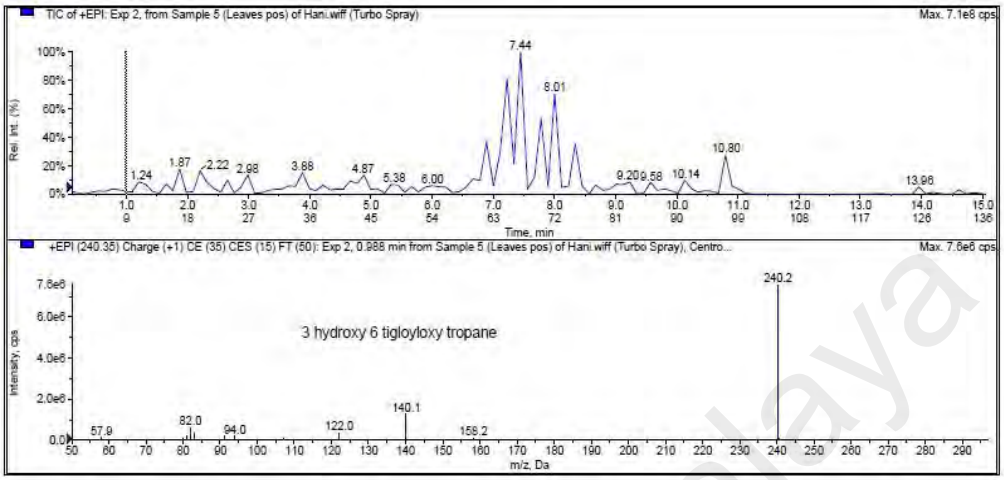
2) International Conference on Applied Sciences and Industrial Technology, 25th February 2015 to 26th February 2015, Universiti Teknologi MARA Shah Alam. Acetylcholinesterase inhibitor from *Datura metel* Linn.

APPENDIX A

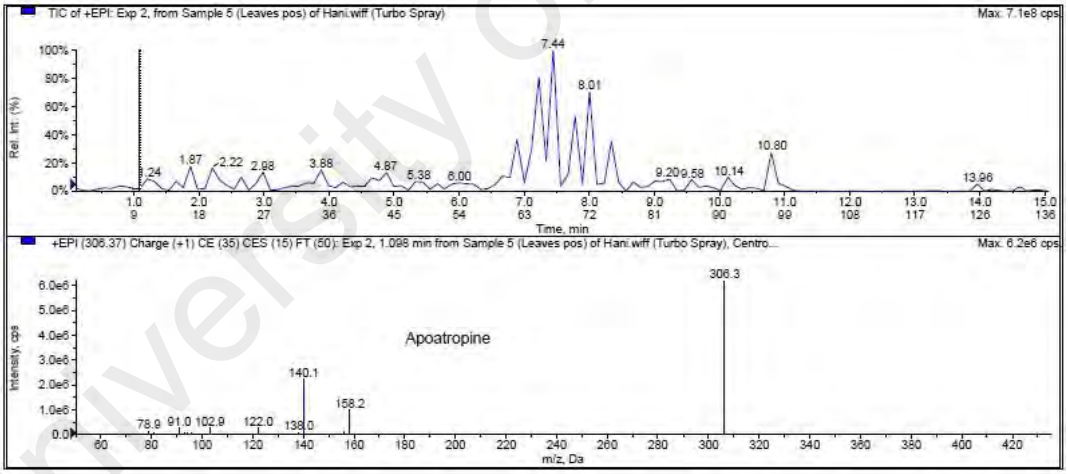
The mice in radial arm maze

APPENDIX B1

LCMS / MS profile of 3-hydroxy-6-(tigloyloxy)tropane from methanol leave crude extracts from *D. metel*

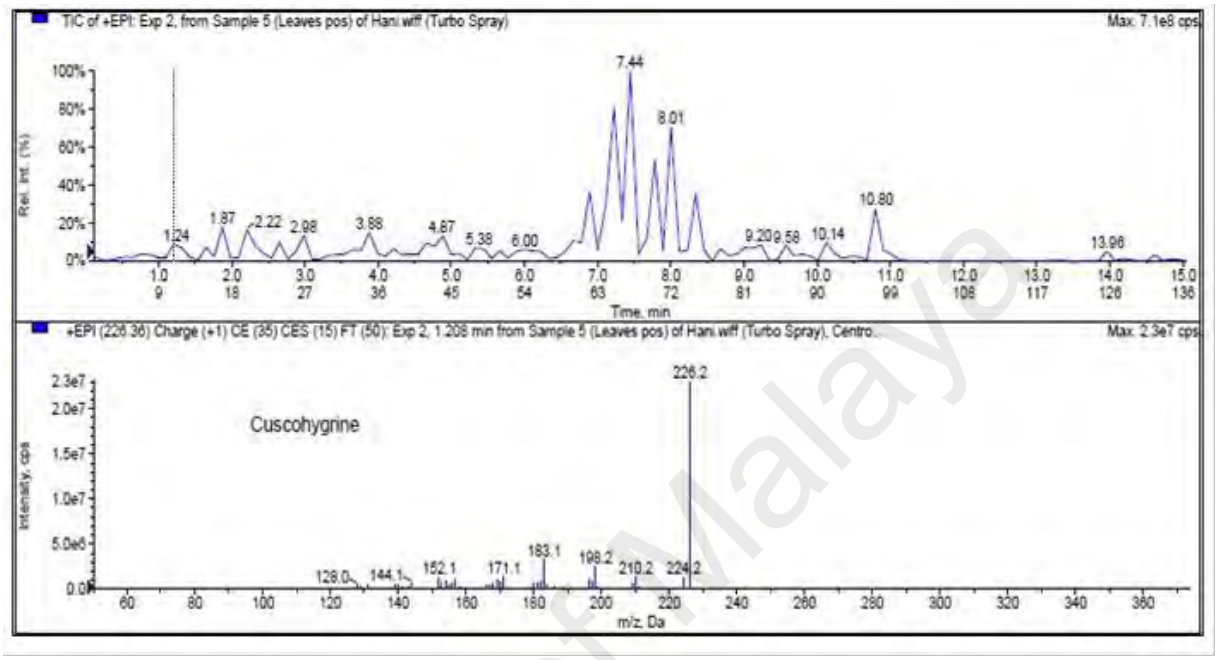


LCMS / MS profile of Apoatropine from methanol leaves crude extract from *D. metel*

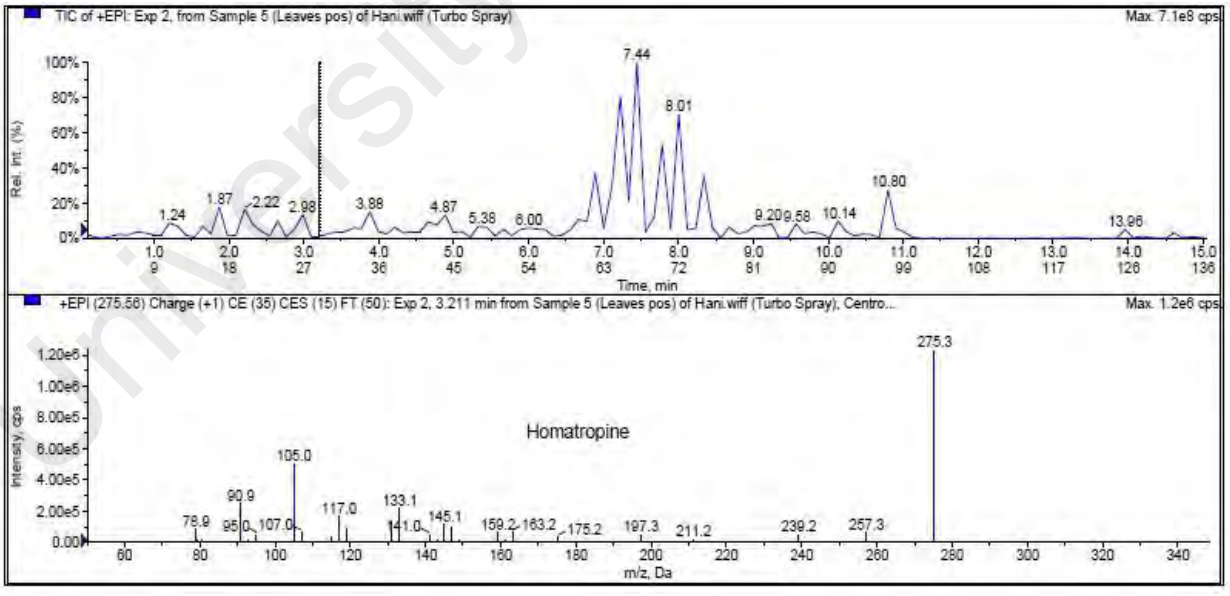


APPENDIX B2

LCMS / MS profile of Cuscohygrine from methanol leaves crude extract from *D. metel*

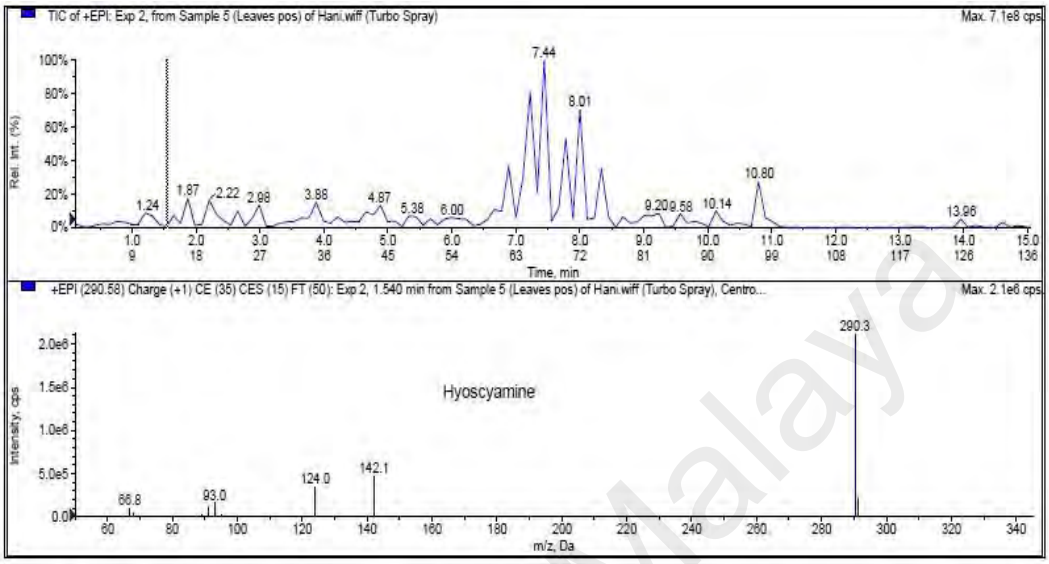


LCMS / MS profile of Homatropine from methanol leaves crude extract from *D. metel*

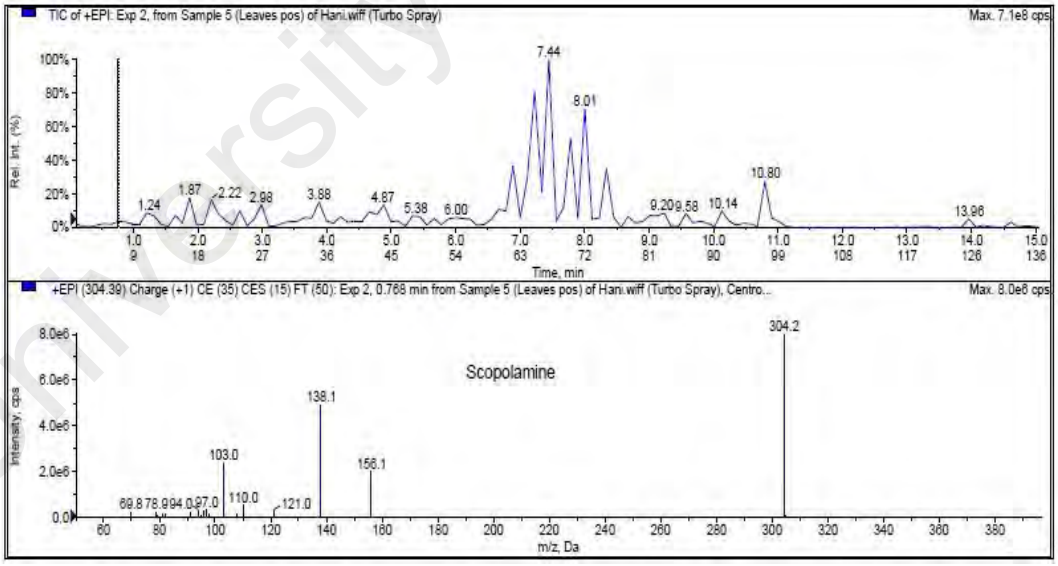


APPENDIX B3

LCMS / MS profile of Hyoscyamine from methanol leaves crude extract of *D. metel*

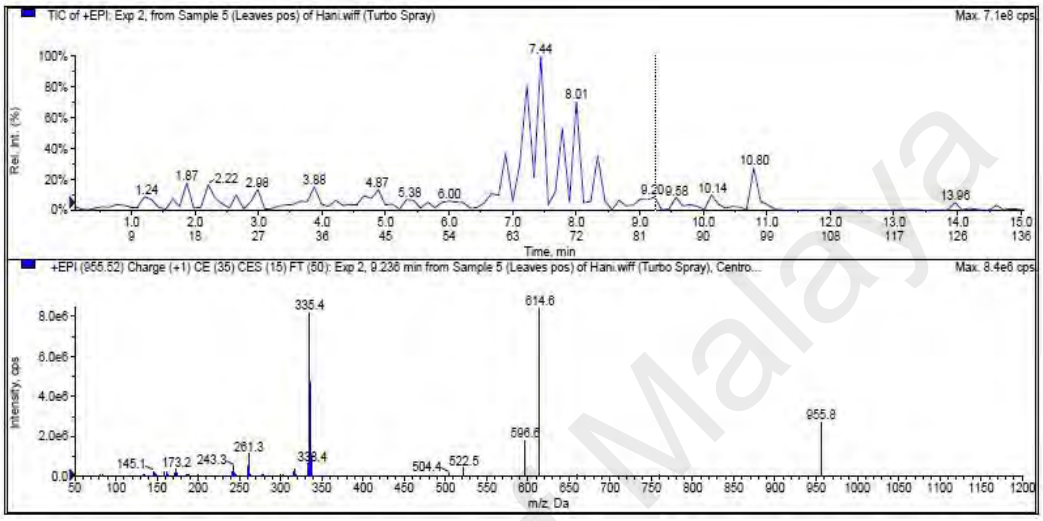


LCMS / MS profile of Scopolamine from methanol leaves crude extract of *D. metel*

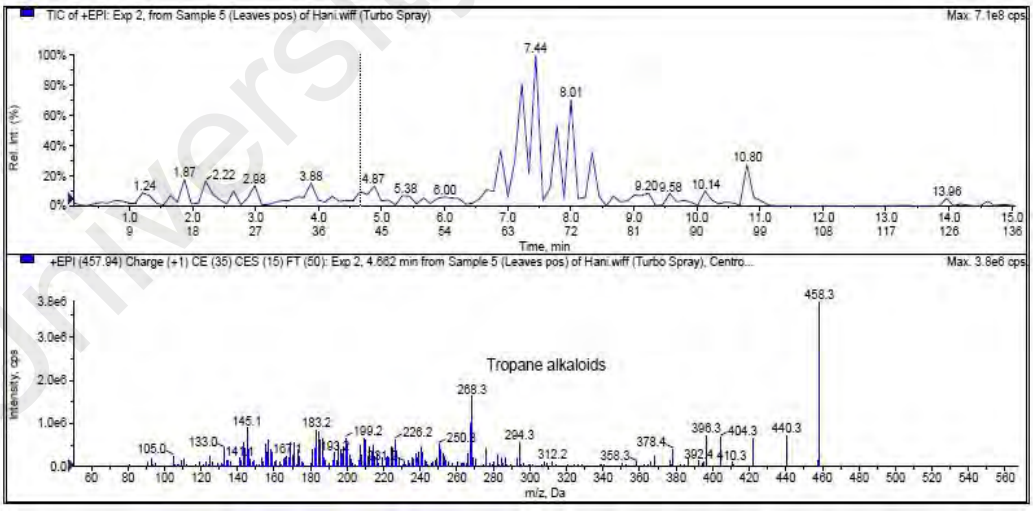


APPENDIX B4

LCMS / MS profile of Cuscohygrine from methanol leaves crude extract of *D. metel*

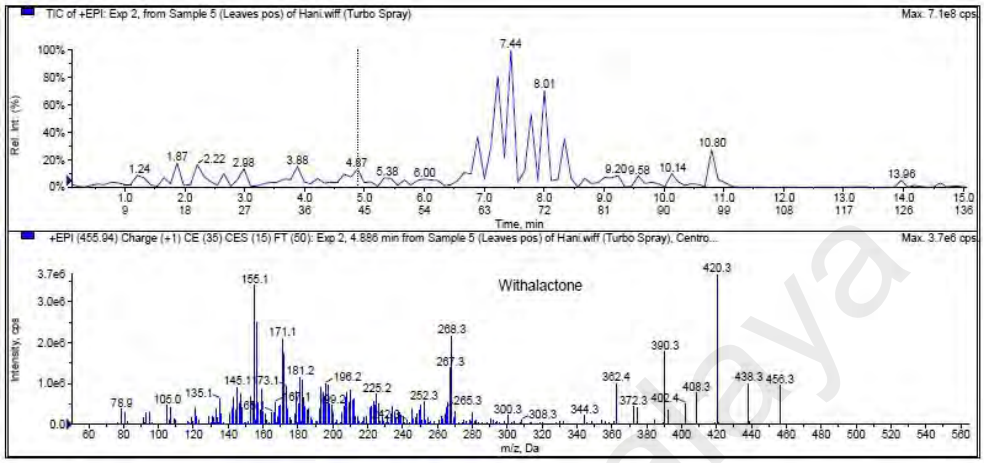


LCMS / MS profile of Tropane Alkaloids from methanol leaves crude extract of *D. metel*

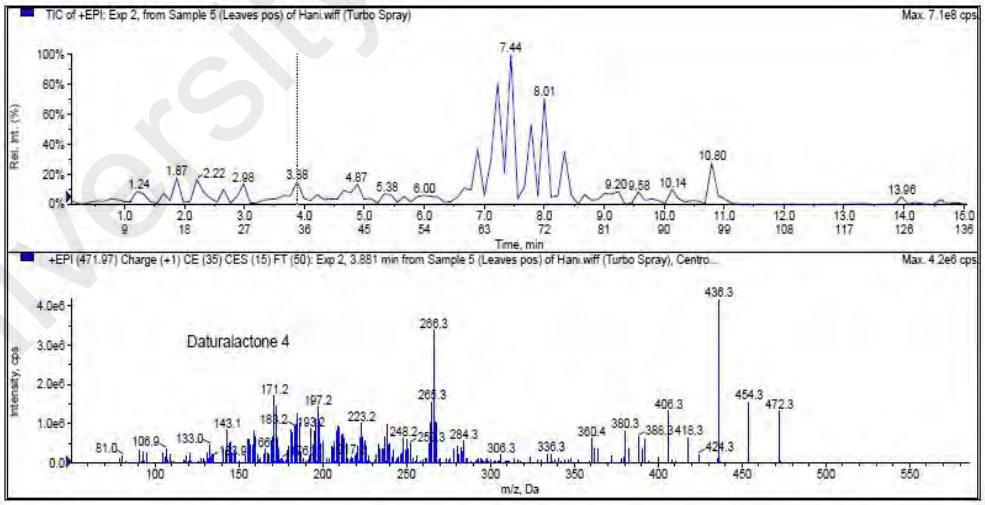


APPENDIX B5

LCMS / MS profile of Withalactone from methanol crude extract of *D. metel*

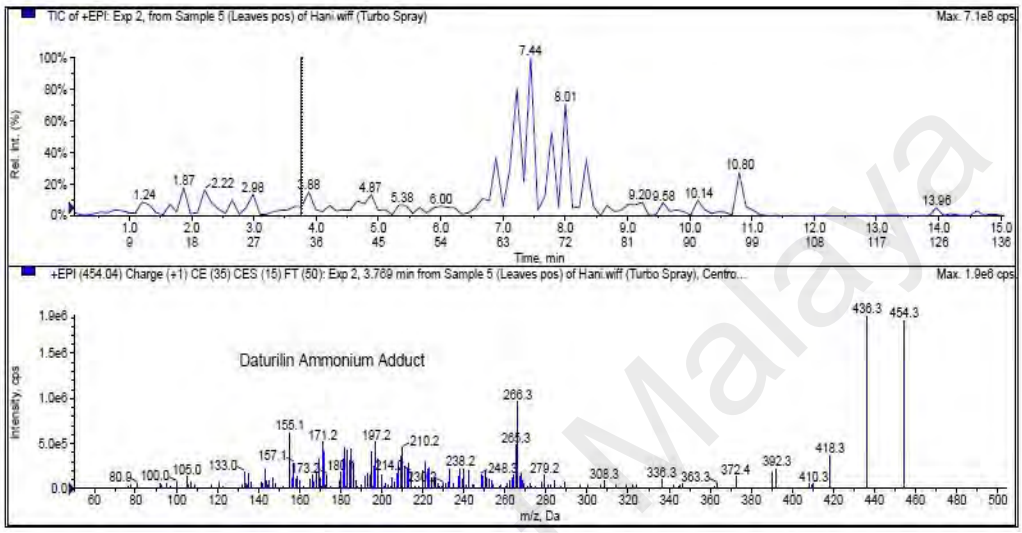


LCMS / MS profile of Daturalactone from methanol leaves crude extract of *D.metel*

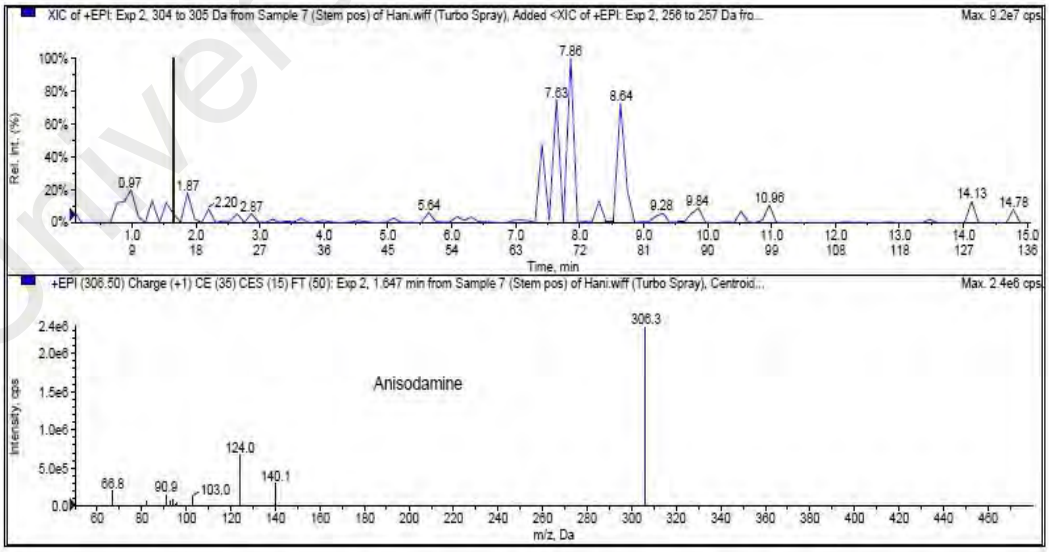


APPENDIX B6

LCMS / MS profile of Daturilin Ammonium adduct from methanol leaves crude extract of *D. metel*

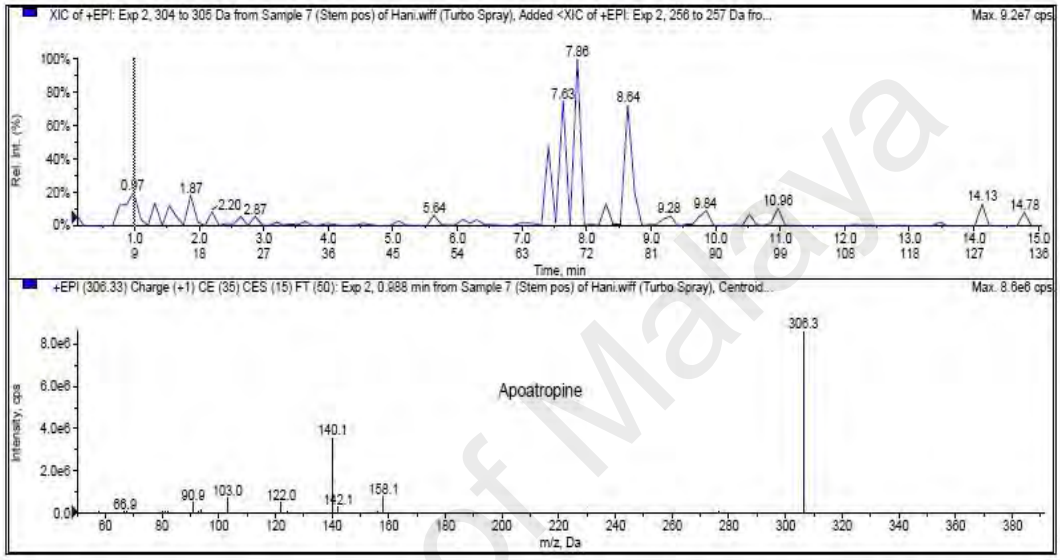


LCMS / MS profile of Anisodamine from methanol stem crude extract of *D.metel*

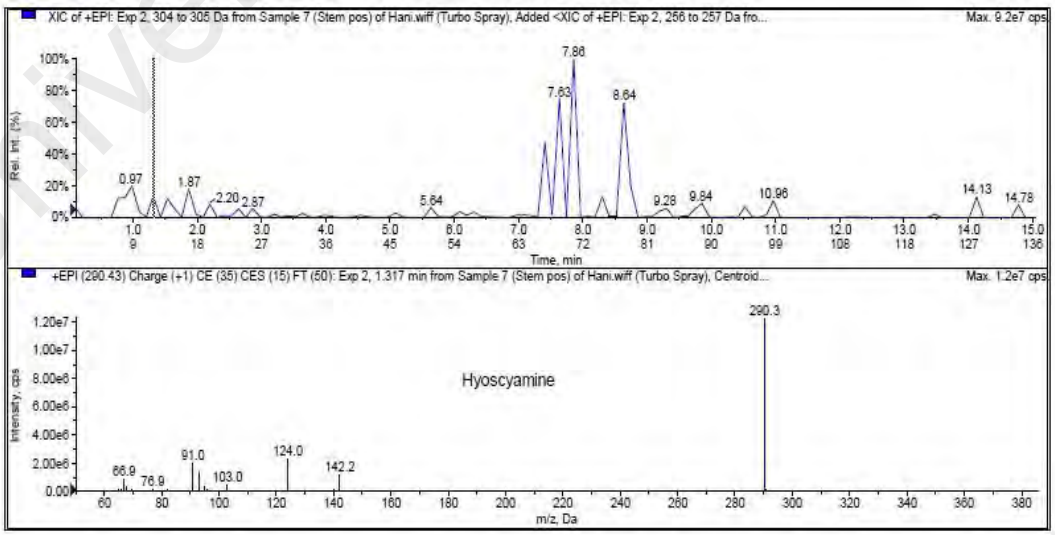


APPENDIX B7

LCMS / MS profile of Apoatropine from methanol stem crude extract of *D. metel*

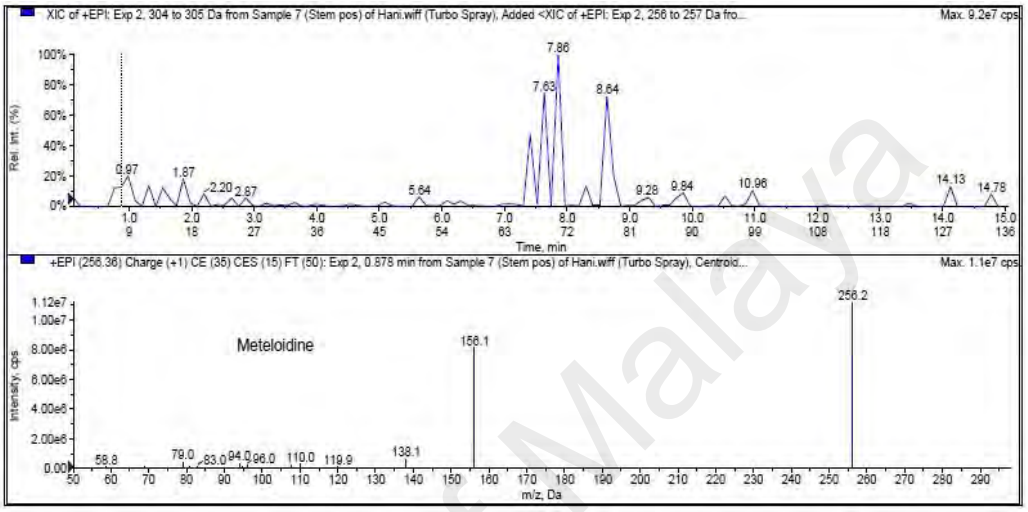


LCMS / MS profile of Hyoscyamine from methanol stem crude extract of *D.metel*

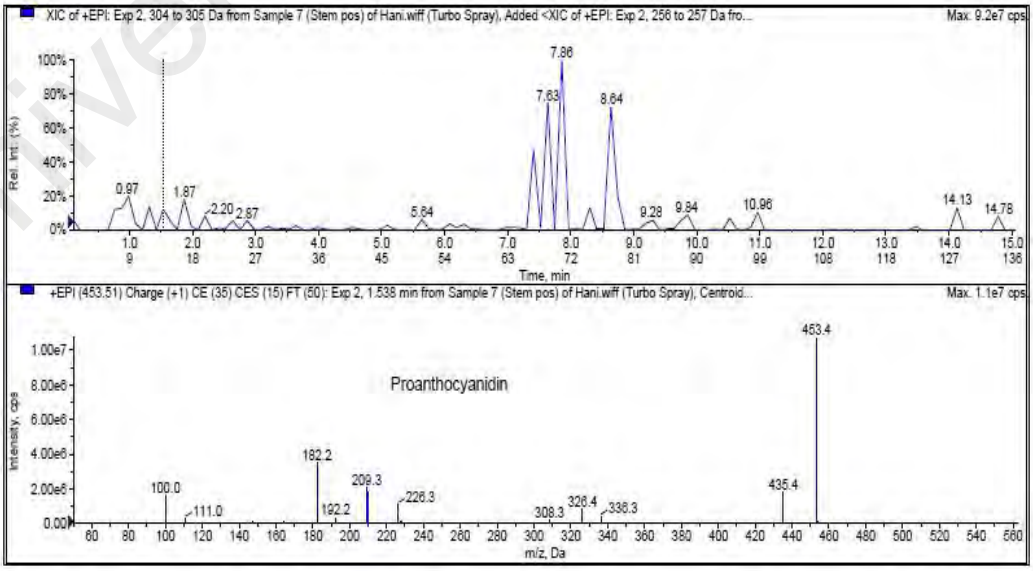


APPENDIX B8

LCMS / MS profile of Meteloidine from methanol stem crude extract of *D.metel*

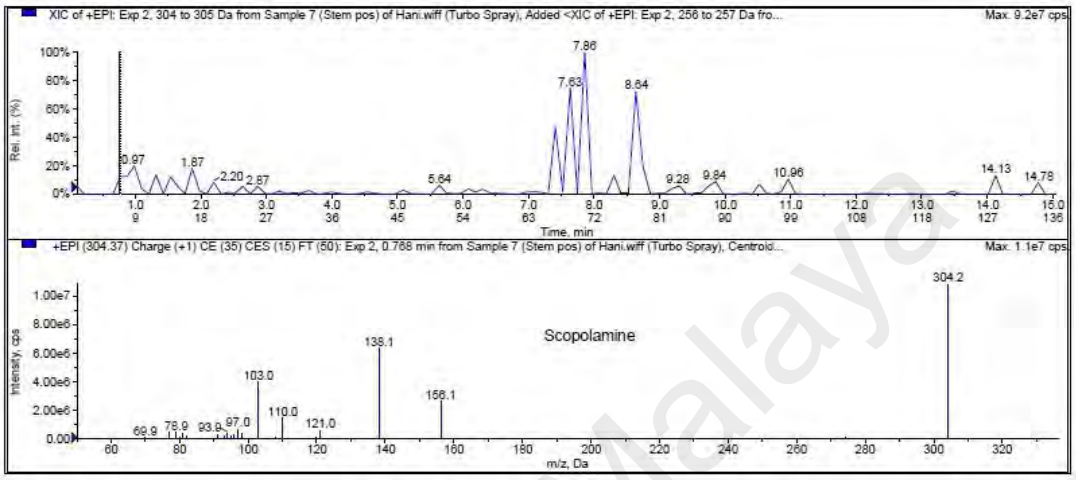


LCMS / MS profile of Proanthocyanidin from methanol stem crude extract of *D.metel*

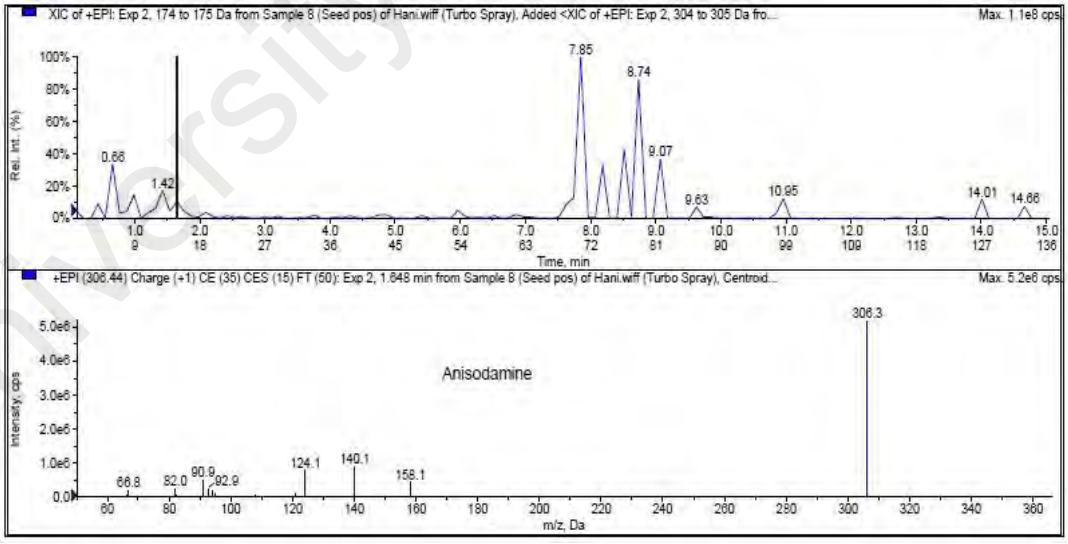


APPENDIX B9

LCMS / MS profile of Scopolamine from methanol stem crude extract of *D.metel*

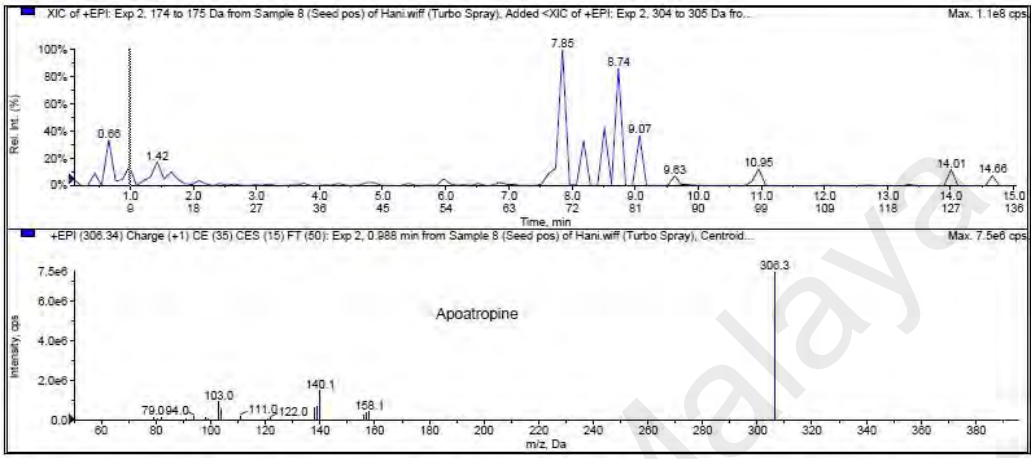


LCMS / MS profile of Anisodamine of methanol seed crude extract of *D. metel*

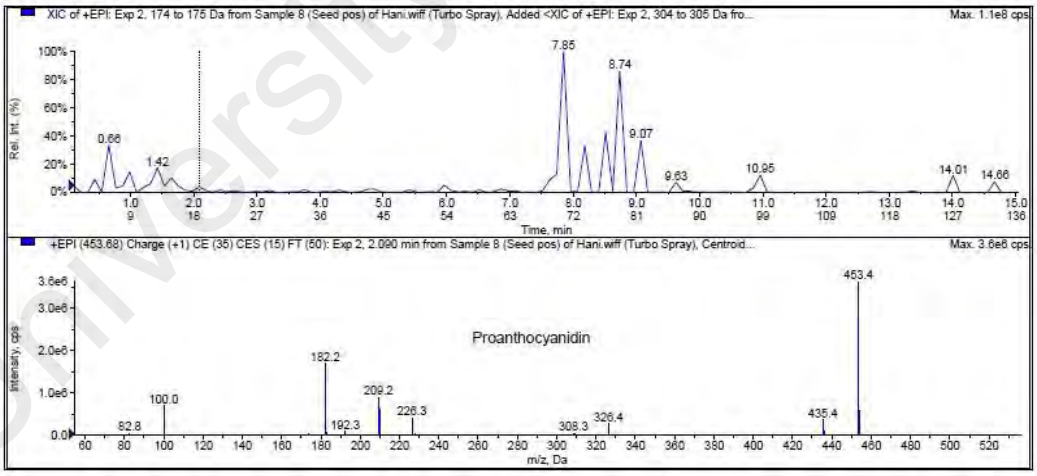


APPENDIX B10

LCMS / MS profile of Apoatropine of methanol seed crude extract of *D.metel*

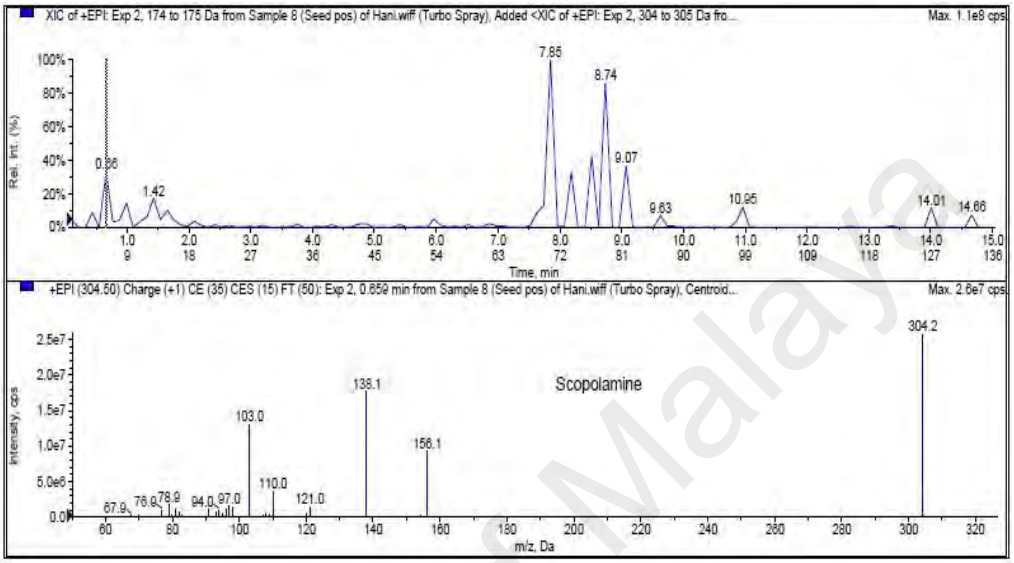


LCMS / MS profile of Proanthocyanidin of methanol extract seed crude extract of *D.metel*

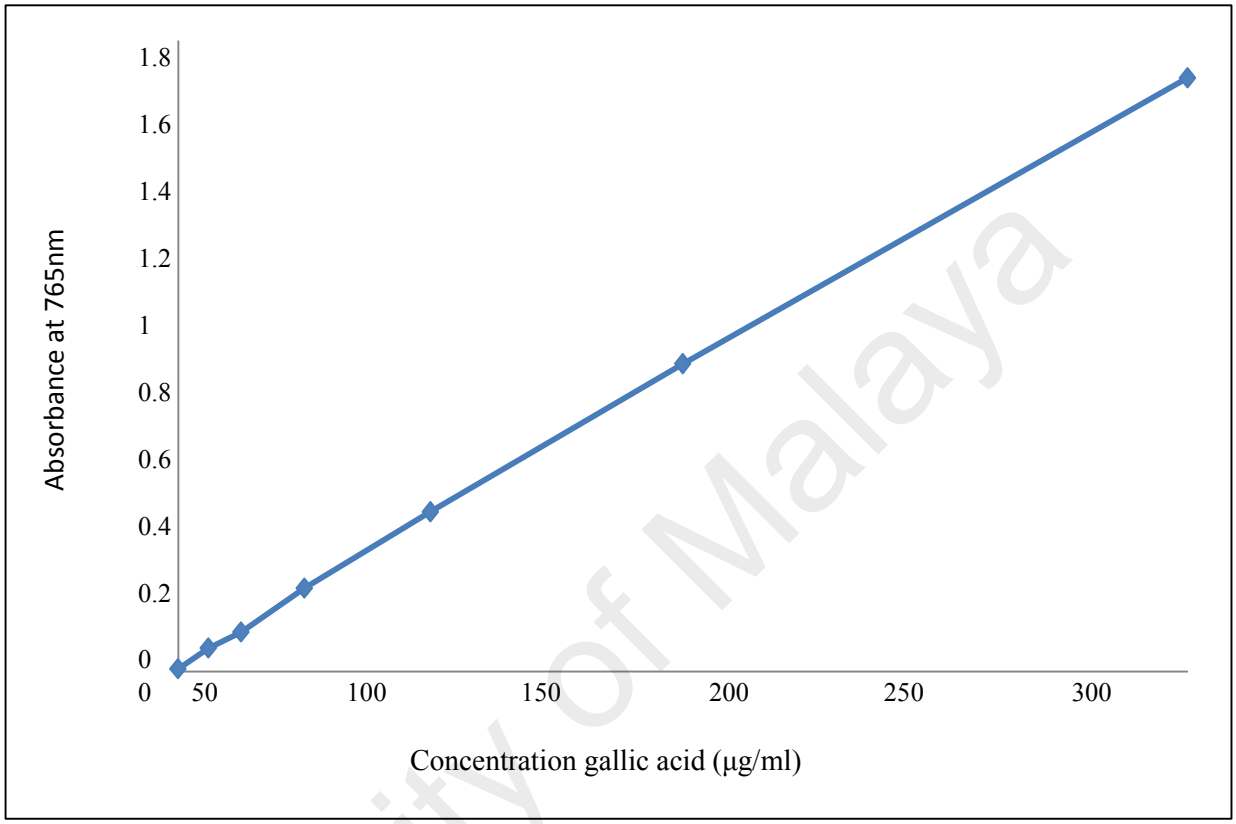


APPENDIX B11

LCMS / MS profile of Scopolamine from methanol seed crude extract of *D.metel*



APPENDIX C



Standard curve of gallic acid equivalent

APPENDIX D

The Q-test of number of repeated entries to arms of maze (NRE) in male and female mice

Group of samples	Gender of mice	Mean diff.	q	Significance p<0.05	95 % CI of diff
Control	Male	11.38	206.2	Yes	11.19 to 11.56
	Female	11.23	160.5	Yes	11.00 to 11.46
Leaf extract	Male	6.625	94.71	Yes	6.392 to 6.858
	Female	6.895	125	Yes	6.711 to 7.079
Berberine	Male	8.72	124.7	Yes	8.487 to 8.953
	Female	9.08	164.6	Yes	8.896 to 9.264
Valium	Male	4.11	58.76	Yes	3.877 to 34.343
	Female	3.025	54.84	Yes	2.841 to 3.209

The Q-test of number of entries until the first error occurs (NEF) in male and female mice

Group of samples	Gender of mice	Mean diff.	q	Significance p<0.05	95 % CI of diff
Control	Male	5.425	196.2	Yes	5.333 to 5.517
	Female	4.795	224.3	Yes	4.722 to 4.868
Leaf extract	Male	3.305	154.6	Yes	3.232 to 3.378
	Female	3.310	119.7	Yes	3.218 to 3.402
Berberine	Male	2.110	98.69	Yes	2.037 to 2.183
	Female	1.875	67.82	Yes	1.783 to 1.907
Valium	Male	1.300	60.80	Yes	1.227 to 1.373
	Female	1.210	43.77	Yes	1.118 to 1.302

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