BEHAVIOURAL PHARMACOLOGY OF *Mitragyna speciosa* KORTH STANDARDIZED METHANOL EXTRACT IN AN ANIMAL MODEL

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by

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DEDICATION

This thesis is dedicated to the late Prof Syed Mohsin Bin Syed Sahil Jamalullial (31st August 1951 - 6th November 2011) in recognition of his contribution as one of my examiners. His invaluable knowledge, relentless support, thought-provoking guidance and words of encouragement will always be treasured. May he be placed among the honourable and righteous ones of Allah till the Day of Judgement and thereafter. Ameen.

Front from left: Assoc Prof Dr Hasnan Bin Jaafar, Prof Syed Mohsin Bin Syed Sahil Jamalullial, Prof Dr Abu Bakar Bin Abdul Majeed, Assoc Prof Dr Shariza Binti Abdul Razak.
Back from left: Prof Dr John Tharakan K. J., Prof Dr Sharif Mahsufi Bin Mansur, Prof Jafri Malin Bin Abdullah and Mohd Harizal Bin Senik @ Nawi.

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LIST OF ABBREVIATIONS

µL	Microliter
5-HT_{2A}	Serotonin receptor 2A
aCSF	Artificial cerebrospinal fluid
ALB	Albumin
ALP	alkaline phosphate
ALT	alanine aminotransferase
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate
APV	2-amino-5-phosphonopentanoic acid
AST	aminotransferase
ATP	Adenosine triphosphate
BIL	Total bilirubin
C-9	Carbon no.9
CA	Cornus ammonis
CaMKII	calcium-calmodulin-dependent protein kinase II
CMC	Carboxyl-Methyl-Cellulose
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	central nervous system
CREA	Creatinine
CV	central vein
Da	dalton

DG	Dentate gyrus
DMSO	dimethylsulfoxide
DT	distal convulated tubule
FBC	Full Blood Count
FC	fatty change
fEPSP	Field excitatory post-synaptic
FRIM	Forest Research Institute Malaysia
g	gram
GABA	gamma-aminobutyric acid
GC/MS	Gas Chromatography-Mass Spectrometer
GGT	Gamma glutamyltranspetidase
H&E	Haematoxylin and eosin
HFS	high frequency stimulation
Hz	Hertz
IC_{50}	50% inhibitory concentration
IMR	Institute for Medical Research Malaysia
KA	kainate
LARU	Laboratory Animal Research Unit
LD_{50}	median lethal dose
LDH	Lactate Dehydrogenase
LOAEL	lowest observed adverse effect level
LSD	Lysergic acid diethylamide
LTD	long-term depression

LTP	long-term Potentiation
m	meter
mA	miliampere
MAPK	mitogen-activated protein kinase
MCH	mean corpuscular heamoglobin
MCHC	mean corpuscular hemoglobin concentration
MCV	mean corpuscular volume
MDMA	3,4-methylenedioxy-N-methylamphetamine
MeOH	methanol
mg/kg	milligram per kilogram
min	minute
mL	mililiter
morp	morphine
MS	*Mitragyna speciosa* Korth standardized methanol extract
MTD	maximum tolerable dose
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)quinoxaline-2,3-dione
NSAID	Nonsteroidal anti-imflammatory drug
NC	centrilobular necrosis
ng	nanogram
nm	nanometer
NMDA	N-methyl-D-aspartate
NOAEL	no observed adverse effect level
OECD	Organisation for Economic Co-operation and Development

PA	passive avoidance
PCP	phencyclidine
Pir	piracetam
PKA	protein kinase A
PKC	protein kinase C
PT	proximal tubule
PTP	post-tetanic potentiation
RBC	Red blood cell/erythrocytes
RC	renal corpuscles
s	second
S.E.M.	Standard error of mean
SC	conditioned stimulus
STL	step-through latency
STP	short-term potentiation
TIC	total ion current
µm	micrometer
USM	Universiti Sains Malaysia
UV	Ultraviolet
Veh	Vehicle / negative control
WBC	leucocytes count
WHO	World Health Organization

LIST OF SYMBOLS

%	percent
°C	Degree Celsius
μ receptor	Mu-opioid receptor
Ca^{2+}	Calcium ion
$CaCl_2$	Calcium chloride
Cl^-	Chloride ion
CO_2	Carbon dioxide
E=IRΩ	Ohm's law
eEF/RT	Boltzman factor
K^+	Potassium ion
KCl	Potassium chloride
Mg^{2+}	Magnesium ion
$MgSO_4$	Magnesium sulphate
Na^+	sodium ion
NaCl	Sodium chloride
NaH_2PO_4	Monosodium phosphate
$NaHCO_3$	Sodium bicarbonate
O_2	Oxygen
RΩ=rI/F	resistance
V	Volt

W	watt
δ receptor	Delta opioid receptor
κ receptor	Kappa opioid receptor

LIST OF PRESENTATIONS AND PUBLICATIONS

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Harizal, S. N., Mordi, .M. N., Mansor, S. M., Hilman, S., Khoo, K. H., Azim, P., Nasir, M. N., Mohd Ghazali, M., Rammes, G., Hasnan, J., Tharakan, J. K. J., Zulkifli, M., Abdullah, J. (2006) Detection of Mitragynine from *Mitragyna speciosa* Korth Crude Extract by Using Gas Chromatography Mass Spectrometry (GC-MS) for Learning and Memory Process: Preliminary Result (The Malaysian Journal of Medical Sciences) Universiti Sains Malaysia, Kubang Kerian, 20-21st May 2006. pp 193.

FARMAKOLOGI TINGKAH LAKU EKSTRAK METANOL TERPIAWAI *Mitragyna speciosa* KORTH DALAM MODEL HAIWAN

ABSTRAK

Mitragyna speciosa Korth merupakan salah satu spesies tumbuhan yang digunakan oleh penduduk tempatan terutamanya dari Thailand dan Malaysia untuk mengubati sakit perut, jangkitan cacing dan juga bertindak sebagai penahan sakit dan ubat demam. Terdapat hanya sedikit maklumat toksiksiti, farmakologi dan kelakuan berkenaan dengan tumbuhan herba ini. Penyelidikan ini dijalankan untuk mengetahui peringkat kesan toksik secara akut ekstrak metanol terpiawai *Mitragyna speciosa* Korth (MS) dalam haiwan, untuk mengukur fungsi kognitif tikus selepas diberi MS secara akut melalui ujian-ujian penolakan dan untuk menerangkan kesan MS dalam mengaruhkan potensi neuronal hippocampal tikus melalui teknik merekod secara ekstrasellular. Dos sebanyak 100, 500 and 1000 mg/kg MS diberi kepada tikus secara oral manakala kumpulan kawalan negatif diberi 430 mg/kg morfin, semuanya diberi satu kali sahaja. Lapan parameter telah dikaji iaitu; pemerhatian dalam sangkar, pengukuran berat badan, jumlah pengambilan makanan dan minuman, tekanan darah, berat organ secara total dan relatif, hematologi, analisis biokimia dan histopatologi. Pembelajaran dan ingatan diukur melalui ujian pengelakan pasif sehala dan pengelakan aktif dua-hala. Potensi lapangan pengujaan pos-sinaptik direkod secara ekstrasellular untuk mendapatkan IC_{50} daripada kepekatan MS yang berbeza (0.0001%, 0.001%, 0.005%, 0.01%, 0.05% and 0.1%). Potensi jangka-panjang, diukur menggunakan kepekatan IC_{50}. Keputusan menunjukkan

tiada kematian dicatatkan selepas 14 hari rawatan hanya 2 kematian dicatatkan dalam kumpulan morfin. Tiada perubahan yang signifikan dilihat dalam kelakuan haiwan, jumlah pengambilan makanan dan minuman, hematologi dan berat organ. Tikus dari kumpulan pengangkut menunjukkan tekanan darah yang normal, manakala tikus yang diberi morfin menunjukkan penurunan tekanan darah secara signifikan ($p < 0.05$). MS pula meningkatkan tekanan darah tikus (systolic: 147.4 ±1.01, 131.64 ±4.94 and 137.8 ±4.46) selepas sejam diberikan 100, 500 and 1000 mg/kg setiap satu. Ujian biokimia menunjukkan peningkatan yang signifikan ($p < 0.05$) ALT, AST, albumin, triglycerides, kolesterol dan albumin pada semua peringkat dos dalam kumpulan MS dan morfin. Bukti nefrotoksiksiti berlaku adalah dengan peningkatan kreatinin yang dilihat dalam dos 1000 mg/kg. Ujian histologi menunjukkan pengembangan sinusoids, pemecahan sel hati, *fatty change*, nekrosis centrilobular dan peningkatan sel Kupffer dalam hati kumpulan haiwan yang diberikan MS dan morfin. Manakala hati tikus dari kumpulan pengangkut tidak menunjukkan sebarang perubahan morfologi. Keputusan histologi pada otak juga menunjukkan tiada perubahan yang signifikan dalam semua kumpulan haiwan. Untuk keputusan ujian kelakuan haiwan, ujian menunjukkan haiwan yang diberi MS dan piracetam dapat belajar secara signifikan ($p < 0.05$) sebagaimana ditunjukkan dalam ujian pengelakan pasif. Walaubagaimanapun, tiada proses ingatan dikesan dalam kedua-dua ujian pengelakan, aktif dan pasif. Dalam eksperimen elektrofisiologi, 0.008% MS menunjukkan 50% penyekatan kepekatan (IC_{50}). IC_{50} MS memberi tindakbalas potensi jangka-pendek dalam neuron di CA1 hippocampus tikus. MS juga dipercayai mengaruhkan sedikit Ca^{2+} memasuki sel pos-sinap dan mengaruhkan cetusan yang rendah berbanding potensi jangka-panjang. Kesimpulannya, dos ekstrak yang paling tinggi mengaruhkan toksik-hati yang teruk dan toksik-buah

pinggang yang sederhana, merencatkan ingatan dan mengaruhkan potensi jangka-pendek dalam hipocampus tikus.

BEHAVIOURAL PHARMACOLOGY OF *Mitragyna speciosa* KORTH STANDARDIZED METHANOL EXTRACT IN AN ANIMAL MODEL

ABSTRACT

Mitragyna speciosa Korth is one of the species of plant used by locals especially in Thailand and Malaysia in treating diarrhea, worm infestations and acts as an analgesic and antipyretic. There is scarcity of information in the literature regarding to toxicity, pharmacology and behaviour effect of this indigenous medicinal herb on. Therefore, this study was conducted to determine the acute toxicity level of *Mitragyna speciosa* Korth methanol extract (MS) in vivo, to evaluate cognitive functions of rats after acute exposure of MS using avoidance tasks and to delineate the effect of MS in inducing neuronal potentiation of rats' hippocampus cells using extracellular recording. Rats were orally administered single dose of 100, 500 and 1000 mg/kg of MS, the positive control group received 430 mg/kg of morphine and negative control group received 2 mL vehicle (CMC) orally. Eight parameters were tested; cage side observation, body weight measurement, food and water consumption, blood pressure, absolute and relative organ weight, haematology, biochemical analysis and histopathology. Learning and memory were evaluated using one-way passive avoidance test and two-way active avoidance test. Extracellular recording of field excitatory postsynaptic potential were run to get IC_{50} from various concentration of MS (0.0001%, 0.001%, 0.005%, 0.01%, 0.05% and 0.1%). Long-term potentiation responses were evaluated using the concentration of IC_{50}. No mortality was observed after 14 days of treatment; only 2 rats from morphine group

died. No significant changes were detected in behaviour, food and water consumption, haematological values and organ weights. Rats from the vehicle group showed normal blood pressure however, significant decreases ($p < 0.05$) of blood pressure were detected in rats from morphine group. MS significantly increased rat blood pressure (systolic: 147.4 ±1.01, 131.64 ±4.94 and 137.8 ±4.46) after an hour of 100, 500 and 1000 mg/kg doses administered respectively. Biochemical studies reported statistically significant elevation ($p < 0.05$) of ALT, AST, albumin, triglycerides, cholesterol and albumin at all level of doses of MS and morphine. At a dose of 1000 mg/kg, elevated creatinine was seen, potentially a sign of nephrotoxicity. Histological examination revealed congestion of sinusoids, haemorrhaged hepatocytes, fatty change, centrilobular necrosis and increased number of Kupffer cells in the liver of all MS and morphine treated groups. Rat's liver section from vehicle group however showed no morphological changes. No significant histological changes were seen in the brain. On the other hand, animal behaviour findings have reported significant ($p < 0.05$) learning acquisition in the MS and piracetam treated group, demonstrated by the passive avoidance test. Nevertheless, no memory consolidation was detected, in both passive and active avoidance tests from rat treated with MS and morphine. In electrophysiological experiment, 0.008% of MS has been determined as 50% inhibitory concentration (IC_{50}). The IC_{50} of MS showed short-term potentiation (STP) response in rat CA1 hippocampus neurons. MS is believed to induce less Ca^{2+} influx into postsynaptic cells and trigger lower threshold compared to LTP. In conclusion, the highest concentration of extract induced acute severe hepatotoxicity and mild nephrotoxicity, memory impairment and induced STP in the rat's hippocampus.

CHAPTER 1

INTRODUCTION

World Health Organization (WHO) defines medicinal plants as plants with chemical characteristic that have pharmacological activities (Pensoa, 1980). Medicinal plants are widely used as therapeutics by locals, Malaysian traditional healers with some positive effects (Ong & Nordiana, 1999). Scientific ethnopharmacological studies are being conducted to understand the effect of medicinal plants on disease and health as well as the consequences of utilizing such treatment with biocompounds derived from these plants.

Ethnopharmacological information has been used in traditional medical system such as Ayurveda, Unani, and traditional Chinese medicine or from herbalism folk lore and Shamanism (Ajibesin *et al*., 2008). Numerous plant species have been investigated namely, *Salvia divinorum*, Cannabis species, various members of Cactaceae, various members of Myristicaceae including *Myristica fragrans* and *Knema laurina* (Häke *et al*., 2009) many Rubiaceae members such as *Mitragyna speciosa* (Shellard, 1974), and Psychotria species such as *Tabernanthe iboga* (Said *et al*., 1991) to study its phytochemical and its potential as a new therapeutic product. *Knema laurina* not only prevents neurons from inflammatory damage, but also supports early neurogenesis in concentrations, which are not toxic, neither in cell culture assays, nor in living brain tissue (Häke *et al*., 2009). Other speciese have been studied were *Centella asiatica*, *Momordica dioica* Roxb (Jain *et al.*, 2008), *Cenidium monieri* and *Artemisia afra*

(Mukinda & Syce, 2007), plants that have been used to treat human ailments, namely *Maprounnea Africana* as an antidiabetic agent (Carney *et al*., 1999) and *Taxus brebiflora* as an antitumor drug (Samuelsson, 1992) have led researchers to gain more scientific proof on their effectiveness to produce new compounds that can serve as the next generation of drugs to treat human disease.

Previous studies reported that drugs such as psychostimulants (cocaine, Amphetamine, 3,4-methylenedioxy-N-methylamphetamine (MDMA) or ecstacy), hallucinogens (lysergic acid diethylamide (LSD), phencyclidine (PCP), and mescaline), opioids (morphine, heroin, codeine, methadone) and other psychoactive drugs do not only enhanced addiction but might disrupt the normal functions of the brain especially the neurochemical balance in the central nervous system (Stoelting, 1987). The central nervous system is controlled by a variety of biochemical functions in the body which are related to one another. Example of endogenous chemicals includes neurotransmitter such as acetylcholine, noradrenaline, dopamine, serotonin and gamma-alpha butyric acid (GABA) (Cooper *et al*., 2003). Brain as one of the organs in the nervous system besides spinal cord and the peripheral nerves, controls the normal function of the body including all physical and physiological activities within the body for instance, movements, reflexes, emotions, muscle contractions, hormonal discharges and biochemical processes. However, brains can malfunction or experience disruption. Understanding the brain with its associated functions of each component is vital in order to recognise how brain malfunctions due to external factors such as drugs influence and internal factors such as disease (Harper, 1973; Eaton & Klaassen, 1996; Bloom & Brandt, 2001; Cooper *et al*., 2003).

When neurotoxicity occurs which changes the synthesis or density of the neurotransmitters or neurochemicals in the brain, it may further cause behavioral changes, response toward a stimulant and disturb the normal control of the brain and body. Since the balance of the central nervous system is the most vital part in ensuring the stability of the body system, any alteration towards the neurotransmitters especially noradrenaline, dopamine, serotonin, acetylcholine and metabolite produced may interfere with the behavior, thinking, movements, emotions, and motivations (McKim, 2003). Modulation produced may cause diseases such as Parkinson, Alzheimer, schizophrenia, mania, stress or depression or the direct effect of drug addiction (Ellis & Nathan, 2001; Matthes & Kanarek, 2001; Nowakowska *et al.,* 2001; Yamamoto *et al*., 2001).

Opioid drugs such as morphine may influence the balance control of the central nervous system (Squire, 2008). Mitragynine is also classified as an opioid, based on the discovery that mitragynine can act as an agonist to opioid receptors μ, κ and δ. It induces central and supraspinal antinociceptive effects (Matsumoto et al., 1996b; Watanabe et al., 1997; Yamamoto et al., 1999; Tsuchiya et al., 2002). Apparently, it has also been reported that the consumption or the constant usage of *Mitragyna speciosa* Korth may produce harmful effects to the consumer such as anorexia, extreme fatigue, aggressive behavior, excessive trembling, and mental disturbance and may lead to psychosis (Jansen & Prast, 1988). These symptoms are related to a disrupted central nervous system. The persistence usage of *Mitragyna speciosa* Korth may also lead to addiction and when it has reached the tolerance peak, the consumer needs a higher amount to gain pleasure. An abrupt withdrawal from consuming this drug can cause withdrawal

symptoms and as a matter of fact will lead to constant dependency to the plant (Takayama, 2004).

1.1 *Mitragyna speciosa* Korth

Mitragyna speciosa Korth (refer Plate 1.1) or its common name ketum belongs to the family Rubiaceae and also sometimes known as the Naucleae tribe from the subfamily Naucleoideae. Genus mitragyna can usually be found in swamps and valleys in tropical and subtropical Asia and Africa. Other than Southeast Asian countries such as Thailand, Laos, Cambodia and Malaysia (Burkill, 1966; Jansen & Prast, 1988; Hinou & Harvala, 1988), *Mitragyna speciosa* may also be found in the east such as Borneo, Philippines and New Guinea (Burkill, 1936) in the East and West Africa as well as India (Harvala & Hinou, 1988) as shown in Table 1.1.

Table 1.1
Names of different species of *Mitragyna* based on origin

Origins	Species *Mitragyna*
West Africa	*Mitragyna inermis* or M. *Africana* (Wild.) O. Kuntze
	Mitragyna ciliate Aubrev. And Pellegr.
	Mitragyna stipulosa (D.C.) O. Kuntze
East Africa	*Mitragyna rubrostipulate* Havil.
India and Southeast	*Mitragyna hirtusa* Havil
Asia	*Mitragyna tubulosa* Havil.
	Mitragyna javanica Koord. And Valeton
	Mitragyna speciosa Korth.
	Mitragyna parvifolia (Roxb.) Korth.
	Mitragyna rotundifolia (Roxb.) O. Kuntze

Plate1.1 Picture of *Mitragyna speciosa* Korth plant.
(http://www.erowid.org/plants/kratom/) 2010

These plants from genus *Mitragyna* are commonly used in traditional medicine mostly to treat fever, diarrhea, hypertension and inflammation (Montjanel-Mouterde *et al.,* 2006). The leaves, bark and roots of the plant are the parts consumed. *Mitragyna inermis* is a shrub growing plant which thrives on swampy savannas, particularly used to treat hepatic illnesses in Mali (Toure *et al.,* 1996). *Mitragyna ciliata* is used as a cure against malaria (Adjétey *et al.,* 2007). Another species, *Mitragyna stipulosa* can grow up to 3 meters in height and grows well in the Southeast Asian region and sub-tropical Africa. The stem bark of this plant has been used by locals in Cameroon to alleviate cancer and diabetes (Tapondjou *et al*., 2002). *Mitragyna parvifolia* has been listed as a highly endangered tree of the Indian Thar Desert. This species is known to lessen the pain of wounds and swelling (Gupta *et al*., 2009).

Plate1.2 Picture of *Mitragyna speciosa* Korth flower.
(http://www.sacredplant.com/id117.html) 2010

In Malaysia, this species can be widely found in the valley area such as near the river in Perak and Pahang (Ridley, 1923) and Selangor (Houghton & Said, 1986). The common names of this species according to different countries in Southeast Asia are *katawm*, *kratom* and *tawn* in Thailand, *naithum* in Laos, *kadam* in Indonesia, *khtom* in Cambodia, *ketum*, *kutom* and *biak-biak* in Malaysia and *beinzarbin* in Burma (Burkill, 1966).

Genus *Mitragyna* is a large trunk tree and some species may even reach to a height of 100 feet. Though, usually *Mitragyna speciosa* only grows 50 feet and is recognized by its globular shape flower with 120 dark yellow florets, shiny green ovate-acuminate leaves arranged opposite to one another and paired decusatively, having a fruit-like capsule with flat seeds (Shellard, 1974).

Mitragyna speciosa Korth is a type of tropical evergreen and non-seasonal plant. This species grows heavily in damp areas rich with humus and is sensitive towards drought and extreme coldness. During extremely cold temperature, the leaves of *Mitragyna speciosa* Korth will fall down and become infertile and consequently die (Macko *et al*., 1972). Genus Mitragyna was previously named by Korthals due to its stigma shape similar to a mitra bishop. However, this nomenclature has always caused confusion. This genus is also associated with numerous other names such as *Nauclea*, *Sarcocephalus*, *Stephegyna* and *Uncaria* (Shellard, 1974).

Mitragyna speciosa Korth or ketum is one of the species classified under medicinal plants. It has long been used by locals especially in Thailand and Malaysia to treat various types of ailment. According to Burkill (1966), the leaves are the most effective part which functions as an antipyretic, antidiarrhea, analgesic, local anesthetic to treat worm infestations, cuts and as an antitussive. Besides that, ketum is also used in the diet or daily life by farmers to increase their amount of energy since it has been said that ketum may enhance motivation and boost the energy to complete one's work without feeling exhausted (Shellard, 1974; Harvala & Hinou 1988; Jansen & Prast 1988).

Scientific researchers have proven that the active compound in ketum (mitragynine) is responsible to produce the therapeutic effect (Macko *et al*., 1972; Perry, 1980). Mitragynine is an alkaloid which acts similarly like opioid (Matsumoto, 2006a, b). According to studies conducted on nociceptive activity, ileum contractility and vas deferens, head twitch response and writhing response, and receptor studies by

Matsumoto *et al*. (1996a, 1996b), Watanabe *et al.* (1997), Thongpradichote *et al*. (1998), Idid *et al*. (1998) and Yamamoto *et al.* (1999), mitragynine functions comparatively to morphine, however with less potency. It is widely known that morphine is a type of drug which has been misused and related to addiction. Since mitragynine acts at the same opioid receptor as morphine (μ, κ and δ receptor) and is prevented by the same antagonist, questions arise on how similar is mitragynine to morphine in causing addiction in humans.

1.1.1 *Mitragyna speciosa* Korth Alkaloids

From previous studies conducted, until now, 40 types of alkaloid can be found from various Mitragyna, 25 of them from *Mitragyna speciosa* Korth. The type of alkaloid isolated depends on two factors, time or season and location of this species (Shellard, 1974). According to Shellard (1974), in 1907, Hooper succeeded in isolating one alkaloid from the leaves of *Mitragyna speciosa* Korth and in 1921, Field repeated the same procedure and named the alkaloid as mitragynine.

Macko *et al*. (1972) reported in *Mitragyna speciosa* Korth the most alkaloid isolated comprises of three indoles and two oxindoles. Examples of indoles are mitragynine, paynanthine and speciogynine whereas oxindoles are mitrphylline and stipulatine. Other alkaloids from both types are ajmalicine, corynantheidine, mitrversine, rhychophylline, mitraciliatine and stipulatine. The isolation of alkaloids depends on time and location where the leaves were gathered. The dominant alkaloid from isolation of

Mitragyna speciosa Korth is mitragynine and a small quantity of mitraciliatine. During certain periods, corynantheidine and ajmalicine exist in the leaves of *Mitragyna speciosa* Korth. Speciophylline is also available in certain leaves collected (Shellard, 1974).

Mitragynine is a dominant alkaloid from the leaves of *Mitragyna speciosa* Korth. Out of 0.5% of alkaloid isolated from the leaves, half of them belong to mitragynine (Takayama 2004). Mitragynine has an indole structure with a methoxy replacement at C-9. Chemically, mitragynine is (16E, 20 β)- 16,17-didehidro-9,17-dimethoxycorynan-16-acid carboxylic methylester; (E)-16,17-didehidro-9,17-dimetoxy-17,18-seco-20α-yohimban-16-acid carboxylic methyl ester or simply 9-methoxy-corynantheidine. Molecular formula for mitragynine is $C_{23}H_{30}N_2O_4$ with a molecular weight of 398.5 g/mol consisting of 69.32% of carbon, 7.59% of hydrogen, 7.03% of nitrogen and 16.04% oxygen (Jansen & Prast, 1988). Mitragynine has a high melting point between 102°C - 106°C and a boiling point of 239°C - 240°C. Physically, mitragynine is a white yellowish amorphous powder which is alcohol, chloroform, acetic acid and oily substance soluble. Mitragynine has a UV absorbance between the wavelength of 226 and 292 nm (Zacharias *et al*., 1965; Macko *et al*., 1972).

Since the isolation of mitragynine during the 20th century by Hooper (1907) and Field (1921), no extensive research has been conducted to establish the effects of mitragynine on health and disease. Nevertheless, mitragynine studies were only limited to isolation and pharmacochemical studies by Shellard (1974) and Jansen & Prast (1988). In 1974, Zarembo *et al*. carried out a microbial alkaloid mitragynine transformation studies using a fungus, *Helminthosporum* sp. Two metabolites were

produced which were pseudoindoxyl mitragynine and pseudoindoxyl mitragynine hydroxyl. Since then, no further studies were conducted using these metabolites. Kechil *et al*. (1990) used a crude alkaloid extract on guinea pig to study its antinociceptive effect and on electrical induced ileum contraction. Similar studies were conducted by Matsumoto *et al*. (1996a) and Watanabe *et al*. (1997) by using mitragynine. Based on those studies, mitragynine effectively produce antinociceptive activity depending on dosage administration in hot plate and tail flick test on rats (Matsumoto *et al.*, 1996a) and mitragynine prevented ileum contraction when given an electrical stimulus (Watanabe *et al.*, 1997). Besides that, Watanabe *et al*. (1997) also proved that naloxone can treat or reverse the contraction inhibition by mitragynine and morphine. Between the two, mitragynine is ten times less potent than morphine.

Matsumoto *et al*. (1996b) studied the role of monoaminergic system in the antinociceptive activity of mitragynine on the mechanical noxius stimulation. Matsumoto *et al*. (1997) further studied the effect of mitragynine to the head twitch response receptor 5-HT_{2A} on rats. Mitragynine inhibits the head twitch response depending on the dosage administered. The result showed that α_2-adrenoceptor stimulation and receptor inhibition 5-HT_{2A} are involved in the head twitch inhibition response which induced receptor 5-HT_{2A} towards mitragynine. A further study conducted proved that mitragynine acted directly on the opioid receptor instead of α_2-adrenoceptor to produce antinociceptive activity (Tohda *et al*., 1997). Mitragynine is also dominantly binded by μ and δ subtype receptor in the antinociceptive activity and studies in mice has shown that the selection of mitragynine for each supraspinal opioid receptor is different from morphine in mice (Thongpradichote *et al*., 1998). 7-

hydroxymitragynine is also one of the *Mitragyna speciosa* Korth alkaloid; found to have an opiod agonist property on μ and/or κ-opiod receptor which led to the antinociceptive effect in mice (Matsumoto, 2003; Matsumoto, 2004).

A research carried out by Idid *et al.* (1998) compared the action of morphine and paracetamol with mitragynine to produce antinociceptive and analgesic activity. The research found that mitragynine and morphine suppressed the writhing response induced by acetic acid as the antinociceptive agent. Analgesic effect is produced by all three drugs, mitragynine, morphine and paracetamol. Idid *et al*. (1998) also proposed mitragynine as a potential new analgesic. After almost two decades of producing mitragynine metabolite, Yamamoto *et al*. (1999) studied the effect of mitragynine pseudoindoxyl on electrically elicited contraction of the guinea pig's ileum and mouse's vas deferens. The result showed that potent ileum contraction inhibition effect and vas deferens depend on the dosage of mitragynine pseudoindoxyl. This metabolite acted on opioid receptor to produce potent ileum contraction inhibition through μ receptor, antagonized by naloxone and vas deferens contraction inhibition was via δ receptor, antagonized by naltrindole. This study showed that the dose for mitragynine pseudoindoxyl was 20-35 times lower than morphine and 100 times lower than mitragynine.

In the year 2002, a research conducted by Shiziko Tsuchiya found out that mitragynine dose dependently inhibits the 2-deoxy-D-glucose stimulated gastric acid secretion in the continuously perfuse stomach of anesthetized rats (Tsuchiya, 2002). A study conducted by Kenjiro Matsumoto in 2005, determined the effect of mitragynine on

neurogenic contraction in guinea pig vas deferens and on the cytosolic Ca^{2+} level in cultured blastoma cells. The findings showed that mitragynine eradicated the electrically induced contraction of the vas deferens but failed to affect the responses to norepinephrine or to ATP. Mitragynine was also found to block T- and L-type Ca^{2+} channel currents and reduce KCl^{-}-induced Ca^{2+} influx in neuroblastoma cells (Matsumoto, 2005). Some Ca^{2+} channel blockers have been investigated to exhibit analgesic properties in pain tests through the blockage of neuronal Ca^{2+} channels (Miranda *et al.*, 1993).

1.1.2 Traditional Usage and its Effect

In 1897, Ridley reported that the leaves and trunk of *Mitragyna speciosa* Korth had been used as treatment towards opium addiction. Moreover, a further study on the leaves of *Mitragyna speciosa* Korth in 1907 is also in accordance with the fact that the leaves of *Mitragyna speciosa* Korth and *Mitragyna parvifolia* can be used as a substitute of opium (Shellard, 1974).

A long time ago, farmers in Thailand especially and north Peninsular Malaysia chewed and smoked on the leaves of *Mitragyna speciosa* Korth or mixed it with syrup to make a drink as a stimulant to increase the efficiency of work and to withstand extreme hot weather besides feeling strong and peaceful (Burkill, 1966; Shellard, 1974; Harvala & Hinou, 1988; Jansen & Prast, 1988). Furthermore, the leaves may also be used to treat fever, worm infestations and diarrhea (Burkill, 1966). Its usage has been illiglised in

Thailand due to its addictive properties. The government of Thailand enacted the Kratom 2486 Act which was enforced on 3rd August 1943, stating that locals were disallowed to plant this tree and the existing tree needs to be cut down (Suwanlert, 1975) thus its use was declared illegal in Thailand since 1946 (Babu, 2008). As an alternative, the leaves of *Mitragyna javanica* were being used, however with less marked effect compared to *Mitragyna speciosa* Korth. Nowadays, the Thailand law classifies *Mitragyna speciosa* Korth similar to the groups of codeine and heroin (Suwanlert, 1975).

Besides its addictive effect, a study by Jansen & Prast (1988) on Thailand farmers observed that other harmful effects included dry mouth, loss of appetite, anorexia, weight loss, darkened skin and constipation. An excessive and chronic consumption of *Mitragyna speciosa* leaves caused extensive continuous sleep, aggressiveness, inability to work and pay attention, muscle pain and bone, watery eyes, rhinorrhea, constant trembling and psychotic behavior after prolonged use. This opposite effect of *Mitragyna speciosa* Korth somehow can be contributed by many factors. Since *Mitragyna speciosa* Korth is traditionally consumed as a tea (crude extract), it contains many substances or biocompounds which can give various effects (Takayama, 2004). The opposite effect is believed to be regulated by the exposure response. According to this response, the changes in effect on organism are caused at different level of doses after a certain exposure time (Crump *et al*., 1976). The pure biocompounds itself may behave as an agonist in some tissues while as an antagonist in others, which is called as selective receptor modulator (SRM). SRM are sometimes referred to as tissue selective drugs or mixed agonist/antagonist (Smith & O'Malley, 2004). Antagonist activity may

be reversible or irreversible depending on the longevity of the antagonist-receptor complex and on the nature of antagonist receptor binding. The majority of drug antagonist achieved their potency by competing with endogenous ligands or substrates at structurally-defined binding sites on receptors (Hopkins & Groom, 2002). This interaction prevent agonist-induce responses. In other words, it has the ability to bind to a receptor which further determine the duration of inhibition of agonist activity. Once bound, antagonist inhibits the function of agonist or event partial agonist (Stephenson, 1956). Partials agonist might differ in the amplitude of the functional response that elicit after maximal receptor occupancy. It can act as a competitive antagonist in the presence of a full agonist, as it competes with the full agonist for receptor occupancy producing a net decrease in the receptor activation as compare to the full agonist alone resulted difference effect to human or other organism are exposed (Patil, 2002). At the cellular level, the effect can be either excitatory or inhibitory, depending on the ions that permeate the channels operated by the receptor. The resulting responses are either excitatory postsynaptic potentials (EPSPs) or inhibitory postsynaptic potentials (IPSPs), depending on whether they drive the cell towards a point above or below its firing threshold. Both EPSPs and IPSPs require Ca^{2+}influx at the presynaptic cell to regulate neurotransmitter to bind to either glutamate receptor or GABA receptor (GABAR). For the EPSP, the activation of glutamate receptor by opening the receptor site will admits Na^{+} then further excites the postsynaptic cells. However, GABAR are normally closed, but when open they become selectively permeable to Cl^{-} resulted to IPSPs (Rudy, 2008).

This plant is also said to have the characteristic of codeine, effective as an antitussive and analgesic (Harvala & Hinou, 1988; Watanabe *et al*., 1997). Besides its

potential in causing addiction, it could also be used to treat morphine addiction. This opposing criterion has intrigued a lot of researcher's attention to study especially its pharmacological properties. Up until now, no concrete scientific evidence has been found to correlate the positive effect of *Mitragyna speciosa* Korth leaves for medical treatment (Houghton & Said, 1986). Perhaps, substances within *Mitragyna speciosa* Korth can be used clinically as an alternative to methadone in treating drug addicts in the future (Jansen & Prast, 1988).

1.2 Toxicity

Toxicology revolves around the study of adverse effects of poisons, exogenous agents and toxicants to the study of molecular biology. Closely related to medicine, toxicology comprises of collecting data in order to predict the consequences of exposure in human and animal population and to extrapolate hypothesis explaining the bad effects of chemical agents when little or no information is available. Classification of toxic agents may be according to their physical form (gas, dust, liquid), their chemical stability or reactivity (explosive, flammable, oxidizer), general chemical structure (aromatic amine, halogenated hydrocarbon), poison potential of being either extremely toxic to slightly toxic or based on the biochemical mechanism of action (alkylating agent, sulfhydryl inhibitor, methemoglobin producer). Chemical agents in a biological system are not toxic unless they have reached a proper site in the body at an extended length of time or concentration to produce toxic effects. Toxicity can be divided into four categories; acute, subacute, subchronic and chronic (Klaassen, 2001).

Acute exposure refers to exposure to chemicals in a single administration within a short period of time, less than 24 hour, with exposure routes normally through intraperitoneal, intravenous and subcutaneous injection, oral intubation and dermal application. It is the first test to be performed on a new chemical. The LD_{50} (median lethal dose), a statistically derived single dose of a substance that can be expected to cause death in 50% of treated animals and other acute toxic effects are determined after one or more routes of administration in one or more species. Mostly, mice or rats are used with the exception or sometimes is being tasted in rabbits and dogs. Before dosing the test on animals, food is withheld during the night prior to testing. Animals that die within 14 days of test after a single dosage were recorded. Daily inspections of animals include observing for signs of intoxication, lethargy, changes in behaviour, movement and food consumption. The importance of this test is to provide a quantitative estimate of acute toxicity, to identify target organs and site of toxicity, to examine the reversibility of the toxic response and to give dose-ranging guidance for other studies. The significance of obtaining information from this test relies highly on clinical observations and post mortem examination rather from the LD_{50} value itself (Hayes, 1982; Klaassen, 2001).

Subacute toxicity tests involve repeated administration of a chemical which will lead to doses for subchronic studies. Normally, the protocol includes administering 3-4 different dosages of chemicals mix in their feed. A specific number of animals were used for different tests. For instance, ten animals per sex and per dose were used for rats whereas 3 doses and 3 to 4 animals per sex for dogs. After 14 days of exposure, clinical chemistry and histopathology were performed.

Subchronic exposure lasts for different periods of time, but generally ranges from 1 to 3 months (Hayes, 1982). The aims of the subchronic study are to obtain no observed adverse effect level (NOAEL) and lowest observed adverse effect level (LOAEL) and to identify and characterize organs affected by toxicity after repeated administration. Moreover, it may provide information on cumulative effects, latency period for development of toxicity, reversibility of toxicity and the dose response relationship. The numbers obtained for NOAEL and LOAEL depend on how the dosages are spaced and the number of animals tested. The endpoint for this study is nonlethal parameters such as biochemical, haematological, neurobehavioral as well as clinical measurement involving changes in body weight, food or water consumption and histopathological examination. This type of study is normally conducted on 2 species, namely the rats and dogs. The number of doses performed for 10 to 20 for rats and 4 to 6 for dogs of each sex per dose, respectively. The first dose is a high dose which produces toxicity but does not cause more than 10% of fatality. The second one is a low dose which produces no apparent toxic effects and as for the third one, an intermediate dose. Observations on the animals for signs of toxicity include changes in body weight, fur colour or texture, respiratory or cardiovascular system, diet consumption, motor and behavioural abnormalities. Dead animals will be sacrificed and further test is done to the blood and organs. Haematological measurements such as heamatocrit, erythrocyte counts and common clinical measurements i.e. alanine aminotransferase (ALT), aminotransferase (AST), bilirubin, cholesterol and total protein are also conducted. Besides, urine tests on osmolarity, pH, protein, glucose, ketones and bilirubin are also done.

Unlike subchronic studies, chronic studies are usually conducted within a longer period of time, which may range from 6 months to 2 years. The purpose of the study is to determine the cumulative toxicity of chemicals and to evaluate potential carcinogenic effect of chemicals. The test animals are usually rats and mice with test conducted over their lifespan. Gross and microscopic pathological examination is conducted in both dead and alive animals. The highest dose for the study is according to the estimated maximum tolerable dose (MTD) based on body weight suppression or other indicator such as physiological and pharmacokinetic considerations and urinary metabolite profile.

1.2.1 OECD Guidelines on Toxicology

OECD guideline 407 provide information on the basic repeated dose toxicity study over a relatively limited period of time, normally of a 28 day period. However, a 14 day study period may be appropriate at certain circumstances. This guideline emphasizes more on neurological effects as an endpoint and thorough clinical observation of stressed animals. The method also includes immunological effects and reproductive organ toxicity (OECD 1995). OECD guideline 420 is a guideline for fixed dose procedure in acute toxicity study. Under this guideline, death of an animal is not taken as an endpoint but is used based on observations of obvious signs of toxicity at one of a series of fixed dose levels (OECD 2001). In this study, both guideline 407 and 420 were adapted to get a clear understanding of the *Mitragyna speciosa* Korth standardized methanol extract mechanism.

1.3 The Central Nervous System

The nervous system is an important structure, by playing its role in implementing every activity in the body system. This is because the nervous system is related and interconnected with each and every system in the body. One of the functions of the nervous system is to ensure homeostasis within the body system, involving body temperature, blood pH, electrolyte concentration in the body liquid and blood pressure (Hoo & Subramaniam, 1989).

The structure of the nervous system can be divided into two, central nervous system and peripheral nervous system. Central nervous system comprises of the brain within the cranial cavity and spinal cord along the vertebral column. Peripheral nervous system connects the spinal cord to all other organs and body (Hoo & Subramaniam, 1989). Both systems consist of a group of neurons known as ganglia in the peripheral nervous system and nuclei in the central nervous system. A group of axons will go through and between ganglion and nuclei and is called nerve in the peripheral nervous system and track in the central nervous system. Generally, axon is covered with myelin which is non-existent in the cell body. Therefore, nerve and track usually appears white and is defined as white matter and the cell body is grey matter (McKim, 2003).

Every part of the brain plays its specific function such as; the medulla, involved in a wide variety of sensory and motor functions, the hypothalamus is the central control station for sleep or wake cycles, control of eating and drinking, control of hormone release, and many other critical biological functions, the pallium is involved in multiple

functions, including olfaction and spatial memory, the hippocampus plays a major role in learning and memory (Squire, 2008).

1.3.1 The Anatomy and Physiology of Rat Hippocampus

Hippocampus is a part of the brain located inside the temporal lobe. It forms a part of the limbic system and plays a role in memory and learning processes (Azad *et al*., 2003). Bilateral lesions of the hippocampus in animal affect specific behaviour (e.g., attention, arousal, and habituation), cognitive operation (e.g., learning and memory) and physiological reaction (e.g., skin conductance). The behavioural deficits following hippocampal lesioning can be reversed by administration of clinically efficacious antipsychotics (Schatzberg & Nemeroff, 1998). Moreover, the human brain does produce new nerve cells in adulthood and the newly-divided cells were found in the hippocampus (Izquierdo & Medina, 1997; Morris *et al*., 2003). However, mice that live in stimulating environments and use exercise wheels have more new brain cells in their hippocampus and perform better on learning task rather than genetically-identical mice that live in standard cages (Campbell, 2002).

Hippocampus is grouped with other structures, the dentate gyrus, subiculum and entorhinal cortex, forming a part of the hippocampal formation. The rat hippocampus resembles a sea horse, with an appearance of two interlocking Cs, the first one with large backwards C and the second one with a smaller forward C. In fact, the larger C is the hippocampus proper or cornus ammonis (CA) whereas, the smaller one is the dentate

gyrus also known as fascia dentate. The hippocampus proper comprises of CA1-CA4 regions, mainly by CA1 and CA3 in most regions and the dentate gyrus. Dentate gyrus are U or V shaped granule cells in rats with spherical cell bodies, arranged in four to six cells thick. The granule cell dendrites are connected with the granule cell layer and molecular layer by synaptic connections from several sources. The axons of the granule cells are called mossy fibers, originating from the basal portion of the cell body and extending into the hilus (Squire, 2008).

The hippocampus pathways involve three pathways which are perforant pathway; mossy fiber pathway and Schaffer collateral pathway (refer to Figure 1.1). The perforant pathway receives the main input of the hippocampus from the entorhinal complex to the dentate gyrus. The second pathway, mossy fiber pathway then relates granule cells of dentate gyrus to the hippocampus proper of CA3. Connections are continued from hippocampus CA3 regions to hippocampus CA1 regions through the Schaeffer Collateral pathway. The information from the CA1 region travels to the subiculum, entering the alveus, fimbria and fornix to other areas of the brain and then to the entorhinal cortex (Squire, 2008).

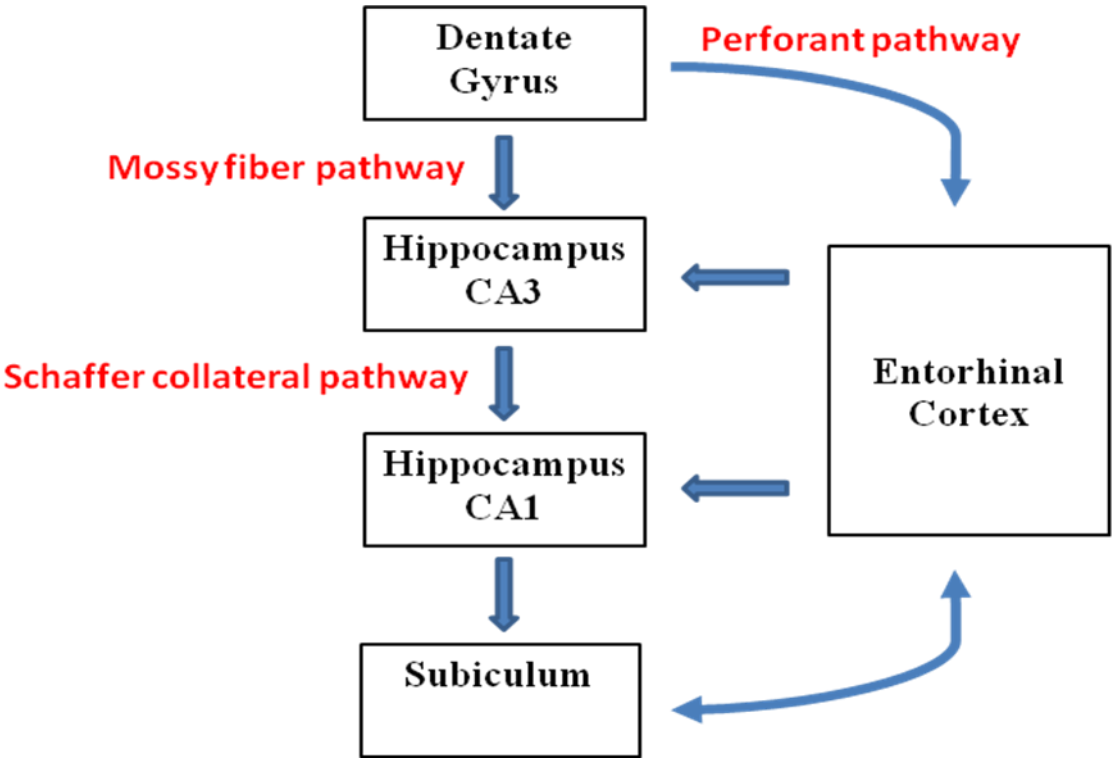

Figure 1.1. Schematic representation of hippocampal connections.

The function of the hippocampus includes olfaction, behavioural inhibition, memory and spatial memory. Olfaction involvement is due to the location of hippocampus next to the olfactory cortex. The behavioural inhibition theory is used on observations on animals with hippocampal damage being hyperactive and experience difficulty in learning to inhibit responses previously taught. The third function relates the hippocampus to memory in particular describing the synthesis of episodic and semantic memories. The observation of Scoville and Milner in 1957, revealed that bilateral hippocampal removal as a treatment for epilepsy suffered by patient resulted severe anterograde and partial retrograde amnesia. This indicated the vital role of the hippocampus and temporal lobe structures in memory (Lynch, 2004). The connection of hippocampus to space is influenced by cognitive maps in humans and animals. Neurons in the rat hippocampus seem to show activity that encodes the spatial coding of the rat in the environment (Terry, 2006).

The hippocampus is strongly related to spatial memory through the Morris water maze, where it depends on the animal's ability to memorize spatial cues to locate a hidden underwater platform. Hippocampal and parahippocampal lesions in rats result in poor spatial learning, particularly if the platform is not visible to lesioned rats in the case of Morris water maze. However, lesioned and nonlesioned rats perform in a comparable manner when the platform is visible. Spatial learning tasks involved several brain areas with a connection between the hippocampus, subiculum and cortical areas. Nevertheless, impairment was less severe when damage occurs to the hippocampus only compared with damage to the rest of the brain areas, the perirhinal, entorhinal and parahippocampal cortical regions. In addition, most areas of the cortex also supports

various sorts of memory, namely visual sensory memory, auditory sensory memory and tactile memories and consolidation is required to enable the formation of long-term memory. This shows that not only the role of hippocampus in memory formation should be emphasised, but other brain parts as well (Whishaw & Kolb, 2005).

In addition, working memory is also dependent on both the hippocampus and the prefrontal cortex. The acquisition of motor skills and habits and the memories associated with procedural memory relies on the striatum and cerebellum, emphasizing the importance of hippocampus-neocortical connections. Furthermore, the hippocampal-prefrontal cortical connections are routed through the subiculum, which also receives inputs from postsubiculum and entorhinal cortex, important in processing positional, directional, sensory and contextual information. As a result when lesions appear to this area, learning deficiency may also develop. The hippocampal pathway involving CA1 neurons projection to the perirhinal, postrhinal and entorhinal cortices also play a vital role in learning and memory. The impact of lesions on the degree of impairment in recall and retrieval of remote memory lies particularly on which brain part it affects, from that involving only the CA1, CA3 and dentate gyrus to the entire hippocampal complex (refer to Figure 1.2). For instance, a partial lesion of the hippocampus will only affect recent memory, without depleting remote memory, while complete hippocampal lesions will result in both recent and remote memory impairment, comparably (Squire, 2008).