PHARMACOKINETICS AND INVESTIGATION OF THE ANXIOLYTIC-LIKE EFFECT OF MITRAGYNINE IN RATS

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PHARMACOKINETICS AND INVESTIGATION OF THE ANXIOLYTIC-LIKE EFFECT OF MITRAGYNINE IN RATS

By

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LIST OF SYMBOLS ABBREVIATIONS

Abbreviations	Details
µg	Microgram
µl	Microliter
µm	Micrometer
AAG	Alpha-1-acid glycoptrotein
ACN	Acetonitrile
ANOVA	Analysis of variance
AUC	Area under the drug concentration versus time curve
AUC_{0-t}	Area under the drug concentration versus time curve from time zero to time t
$AUC_{0-\infty}$	Area under the drug concentration versus time curve from time zero to infinity
$AUC_{0-12\ ss}$	Area under the drug concentration versus time curve at steady-state over 12 h dosing interval
°C	Celsius
C_8	Column having octadecyl chain of C atom
C_{free}	The concentration of drug in plasma filtrate
Cl	Clearance
Cl/F	Apparent clearance
C_{max}	Peak plasma concentration
cm	Centimeters
CNS	The central nervous system
CO_2	Carbon dioxide
C_p	The total concentration of drug in plasma (bound + unbound fraction)
C_{p-f}	The total concentration of drug in protein-free plasma
$C_{p-f\ free}$	The concentration of drug in protein-free plasma filtrate

Abbreviations	Details
C_{ss}	The concentration of drug at steady state
$C_{ss, min}$	The minimum concentration of drug at steady state
$C_{trough\ 1}$	The trough plasma concentration at the end of the first dosing interval
CV	Coefficient of variation
CZE	The number of central zone entries in OF test
% CZT	The percentage of time spent in the central zone of the OF
dl	Deciliter
eg	For example
eq.	Equation
EPM	Elevated plus-maze test
f_u	The unbound drug fraction in plasma
F	Absolute bioavailability
FDA	Food and drug administration organization
g	Gram
GABA	Gamma-aminobutyric acid (γ-Aminobutyric acid)
GC	Gas chromatography
h	Hour
HDL	High-density lipoproteins
HED	Human equivalent dose
HPLC	High performance liquid chromatography
HPLC-UV	High performance liquid chromatography-ultraviolet detection
i.d	Internal diameter
i.p	Intraperitoneal
i.v	Intravenous
kDa	Kilodalton
kg	Kilogram
l	Liter

Abbreviations	Details
log P	Partition coefficient
LC-MS	Liquid chromatography-mass spectrometry
LDL	Low-density lipoproteins
LLE	Liquid liquid extraction
LOD	Lower limit of detection
LOQ	Lower limit of quantification
mg	Milligram
min	Minute
ml	Milliliter
mm	Millimeter
M	Molar
MG	Mitragynine
n	Number of replicates
NaCl	Sodium chloride
ng	Nanogram
% OAT	The percentage of time spent on open arms in the EPM test
% OAE	the percentage of open arm entries in the EPM test
OF	Open-field test
pH	Negative logarithm of H+ concentration
pKa	Ionization constant
p.o	Per os (Latin)/ by mouth
PPE	Protein precipitation extraction
QC	Quality control
r	Coefficient of determination
rpm	Revolutions per minute
R_{ac}	Accumulation ratio
SDV	Standard deviation
SEM	Standard error of mean

Abbreviations	Details
SPE	Solid phase extraction
$t_{1/2}$	Elimination half-life
t_{max}	Time to reach peak plasma concentration
t_{ss}	Time to reach the steady state
Vd	Volume of distribution
Vd/F	Apparent volume of distribution
VHDL	Very high-density lipoproteins
v/v	Volume by volume
v/w	Volume by weight
%	Percentage
±	Plus/minus
α	Alpha
β	Beta
γ	Gamma
δ	Delta

KAJIAN FARMAKOKINETIK DAN PENYIASATAN KESAN BAK-ANZIOLITIK KE ATAS MITRAGININA DALAM TIKUS

ABSTRAK

Mitragyna speciose Korth (Rubiaceae) merupakan sejenis pokok yang berasal dari bahagian utara Semenanjung Malaysia dan selatan Thailand. Alkaloid utama dalam tumbuhan ini, iaitu mitraginina (MG), telah dilaporkan bertanggungjawab terhadap kebanyakan sifat farmakologinya. Dos MG yang digunakan dalam kebanyakan kajian farmakologi mempunyai perbezaan yang ketara, sekaligus menggambarkan bahawa cadangan pemilihan dos dalam pelbagai kajian yang lepas adalah berkemungkinan dijalankan secara empirikal iaitu tanpa asas saintifik yang sewajarnya. Walaubagaimanapun, kajian semasa mendedahkan bahawa terdapat variasi dos yang besar telah digunakan. Hal ini disebabkan oleh sifat fizikokimia MG tetapi variasi dos tersebut tidak pula terbatas hanya kepada sifat fizikokimia MG. Disebabkan penilaian farmakokinetik merupakan komponen penting dalam proses pemilihan dos, maka usaha diperlukan untuk menentukan sifat farmakokinetik MG seperti perkadaran dos, akumulasi drug, ikatan protein plasma dan taburan tisu dalam tikus. Kesan MG ke atas tingkahlaku yang berhubungkait dengan kegelisahan dalam tikus dan kemungkinan penglibatan laluan opioidergik, GABAergik dan dopaminergik pada kesan MG yang diperhatikan, juga diselidiki dalam kajian ini. Kaedah HPLC-UV bagi menentukan tahap MG dalam plasma dan tisu telah dibangun dan disahkan. Kaedah ini (plasma LOQ: 39 ng/ml; tisu badan LOQ: 50 ng/ml) telah diaplikasi untuk menentukan kepekatan MG dalam sampel yang diperolehi daripada kajian yang melibatkan farmakokinetik, taburan tisu dan ikatan protein. Dalam kajian perkadaran dos, MG menunjukkan sifat farmakokinetik yang linear (20-40 mg/kg) manakala sifat farmakokinetik yang tidak linear telah

diperhatikan pada dos yang lebih tinggi daripada 40 mg/kg. Kajian ini telah menyediakan asas bagi pemilihan dos 20 mg/kg bagi penilaian farmakokinetik dos berganda MG. Pemberian MG (20 mg/kg x 2 x 7 hari) secara berulang menghasilkan akumulasi drug yang sederhana dalam tikus (R_{ac}: 1.7). Parameter pendedahan MG selepas pemberian dos berganda boleh diramal kerana kinetik linear MG telah diperhatikan dalam julat dos ini (faktor kelinearan: 1.0). Dalam kajian taburan tisu, MG boleh ditemui terutamanya pada tisu hati, paru-paru dan ginjal manakala MG ditemui dengan kuantiti yang kecil dalam tisu jantung dan otak. Kewujudan MG dalam tisu otak tikus menunjukkan kebolehannya untuk merentasi rintangan darah otak (BBB) walaupun MG terikat secara tinggi pada protein plasma tikus (89-92%). Hal ini sekaligus mampu menjelaskan sifat psikotropik MG yang dilaporkan dalam laporan saintifik. Kesan bak-anziolitik MG boleh ditentukan dengan menggunakan ujian medan terbuka dan ujian elevated plus-maze pada tikus. Kesan-kesan ini dapat diperhatikan dalam tempoh sejam selepas pemberian MG secara oral pada dos 10, 20 dan 40 mg/kg pada tikus. Kesan bak-anziolitik MG yang berlaku secara cepat mungkin disebabkan, tetapi tidak terbatas kepada keseimbangan yang pantas MG di antara plasma dan otak. Kajian ini menunjukkan bahawa MG berkemungkinan mempunyai kesan bak-anziolitik melalui sistem modulasi tidak langsung GABAergik dan dopaminergik yang dijana oleh pengaktifan reseptor opioid pada bahagian-bahagian otak yang terlibat dalam gerakbalas kegelisahan.

PHARMACOKINETICS AND INVESTIGATION

OF THE ANXIOLYTIC-LIKE EFFECT OF MITRAGYNINE IN RATS

ABSTRACT

Mitragyna speciosa Korth (Rubiaceae) is an indigenous tree found in the Northern Malaysian Peninsula and in Southern Thailand. The principal alkaloid of this plant, Mitragynine (MG), has been reported to be responsible for most of its pharmacological properties. Doses of MG employed in most pharmacological studies vary greatly, which suggests that the selection of these doses was possibly carried out empirically without proper scientific basis. However, recent studies revealed that large dose variation was due but not limited, to MG physicochemical properties. Since pharmacokinetic evaluation is an essential component in the dose selection process, work was undertaken to determine MG pharmacokinetic properties such as dose proportionality, accumulation, plasma protein binding and tissue distribution in rats. The effects of MG on anxiety-related behaviours in rats and the possible involvement of opioidergic, GABAergic and dopaminergic pathways in the observed MG effects were also investigated in this study. A HPLC-UV method for the determination of MG levels in plasma and tissues was developed and validated. This method (plasma LOQ: 39 ng/ml; tissues LOQ: 50 ng/ml) was applied to determine MG concentrations in samples obtained from pharmacokinetic, tissue distribution and protein binding studies. In dose proportionality studies, MG exhibited linear pharmacokinetic properties (20-40 mg/kg), while non-linear pharmacokinetics were encountered at doses higher than 40 mg/kg. This study provided the basis to select a dosage of 20 mg/kg for MG multiple-dose pharmacokinetic evaluation. Repeated administrations of MG (20 mg/kg x 2 x 7 days) resulted in moderate drug accumulation in rats (R_{ac}: 1.7). MG exposure parameters after multiple

administrations were predictable since MG linear kinetics was observed in this dose range (linearity factor: 1.0). In the tissue distribution study, MG was mainly found in the liver, lung and kidney and less in heart and brain tissues. The presence of MG in the rat brain tissue indicated its ability to cross the blood brain barrier (BBB) despite its high binding to rat plasma proteins (89-92%). This may explain MG psychotropic properties reported in the literature. MG anxiolytic-like effects were determined using open-field and elevated plus-maze tests in rats. These effects were observed 1 hr after oral administration of 10, 20 and 40 mg/kg MG in rats. The early anxiolytic-like effects of MG may be due, but not limited, to the rapid equilibrium of MG between plasma and brain. This study showed that MG had possibly exerted its anxiolytic-like effects by indirect modulation of GABAergic and dopaminergic systems through the activation of opioid receptors in brain regions involved in anxiety.

CHAPTER ONE

INTRODUCTION

1.1 Background

Mitragyna speciosa Korth (Rubiaceae) is an evergreen tree native to Southeast Asian countries. In Malaysia, it is commonly known as "*Ketum*". Its leaves were traditionally used by the locals to relieve pain and mitigate morphine withdrawal symptoms (Burkill, 1935, Watanabe et al., 1997). The plant is prohibited in Malaysia under the Third Schedule of the Poisons Act (1952) due to its narcotic properties; however recent studies have reported its wide use in the northern states of this country such as Kedah and Perlis. Villagers in these states use the Ketum drink to control opioid withdrawal symptoms as it is easily available and cheaper than other substances of abuse it is available over the internet and has been used as self-treatment in managing opiate withdrawal symptoms, alcohol withdrawal and chronic pain (Babu et al., 2008, Boyer et al., 2008, Boyer et al., 2007).

Over 25 alkaloids have been isolated from the mature leaves of this plant with mitragynine (MG) being the most dominant active alkaloid (Chittrakarn et al., 2008, Shellard, 1974). Several studies were conducted to determine the pharmacological and toxicological properties of MG in laboratory animals. However, MG doses employed in toxicity (477 to 920 mg/kg), behavioural (33 to 200 mg/kg) and pharmacokinetic studies (20 to 50 mg/kg) varied largely (Idayu et al., 2011, Idid et al., 1998, Janchawee et al., 2007, Parthasarathy et al., 2010, Sabetghadam et al., 2010, Sabetghadam et al., 2013b). The wide range of MG doses employed in these

studies suggests that the selection of these doses was possibly carried out empirically without proper scientific basis. It is also important that pre-clinical studies should preferably being conducted at a dose level whereby a meaningful translation of animal data for possible human interpretation could be derived.

Of late, Ramanathan et al. (2015) reported the physicochemical properties of MG. The study described MG as a poor water soluble compound, acid labile, with basic and lipophilic nature (Ramanathan and Mansor, 2015). Later, the investigators associated these physicochemical properties of MG to the large varied MG dose employed in pre-clinical studies. But this rational should be further substantiated by knowledge of the MG plasma protein binding, tissue distribution and accumulation of the compound after repeated doses using suitable animal models. Further to this, all the reported MG pre-clinical studies provided information only relating to its toxicology and pharmacological effects without estimating the MG concentrations in plasma and tissues. Similarly in a most recent study in rodents, MG was reported to possess potential threat of abuse at medium and high doses of the pure compound (10, 30 mg/kg); but regrettably for a psychoactive drug like MG this observation was not correlated to MG concentrations in plasma or brain tissues (Yusoff et al., 2014). One immediate possible reason for this could be due to the lack of an accurate and sensitive analytical method to determine the concentration of MG in tissues such as brain, liver, lungs and other bodily organs. There are a few MG analytical methods reported in literature however these methods are limited to biological fluids such as plasma and serum (Parthasarathy et al. 2010, de Moraes et al. 2009, Janchawee et al .2007, Vuppala et al. 2011). Therefore determination of the tissue distribution, accumulation and protein binding properties of MG would provide the basis to

devise a more realistic study design for future MG pre-clinical evaluations (Hazim et al., 2011, Idayu et al., 2011, Khor et al., 2011). However before work is undertaken to determine the distribution, accumulation and binding properties of MG in animal models, the selection of dose at which MG exhibits linear pharmacokinetic is essentially important. This is because, administration of drug at doses higher than this range could result in a non-proportional changes in its plasma concentration due to saturation of one or more of the processes of absorption, distribution, metabolism and elimination. Saturation of these processes could also occur when repeated doses of a drug are administered. This could lead to plasma concentrations significantly higher than expected which might result in toxicity or lead to plasma drug concentrations not within the target range. As a result, misinterpretation of pre-clinical findings could occur which eventually led to wrong conclusions. In addition to this as mentioned earlier in this thesis, the selected dose for animal testing should also preferably reflect a human equivalent dose for notable interpretation of the animal data to clinical situation since MG is gaining more attention as a potential alternative or adjunct drug in treating addiction and chronic pain.

With reference to MG dose selection, most reported pharmacokinetic studies (20-50 mg/kg) were carried out on trial and error basis and no dose-finding studies were included in their study design (de Moraes et al., 2009, Janchawee et al., 2007, Parthasarathy et al., 2010, Philipp et al., 2009). There are no literature reports on the lowest MG oral dose given to animals for possible detection of the drug in plasma using either HPLC or LC-MS/MS analytical methods. MG dose escalating studies in animals are also lacking in literature as this is important to demonstrate MG dose-linearity. With regard to MG toxicity, the drug was found safe in rats after repeated

administration of lower dose (1 and 10 mg/kg) but not at higher dose (100 mg/kg) (Sabetghadam et al., 2013a). At present there are no data on the distribution and accumulation profile of MG available in the literature to precisely explain its pharmacological responses and organ toxicity reported in pre-clinical studies.

With regard to MG pharmacological properties, MG has been shown to produce an anti-depressant effect, decrease novel environment stress in animals and attenuated stress-related behaviours in morphine-withdrawn zebrafish (Hazim et al., 2011, Idayu et al., 2011, Khor et al., 2011). The drug is known to exert its effects through the activation of μ, κ and δ opioid receptors (Matsumoto et al., 1996b, Taufik Hidayat et al., 2010a, Watanabe et al., 1997, Yamamoto et al., 1999).

With respect to anxiety-related behaviour in animals, in general opioid agonists are known to act via interactions among opioidergic, GABAergic and dopaminergic systems in the brain (Klitenick et al., 1992, Sasaki et al., 2002). In the case of MG as a psychoactive compound, there are no proper documented studies on its anxiolytic effect in animals that have been reported. For this reason, another interesting part in this thesis also deals with systemic studies of MG anxiolytic effect in rats. The determination of MG tissue distribution and protein binding properties may further explain the anxiolytic properties of MG in rats.

It is important to note that, for any pharmacokinetic, tissue distribution or protein binding studies to be successful development of a simple sensitive and selective analytical method for assay the drug in biological samples are imperative. Further to this the selection of appropriate MG dose in order to obtain a reliable and accurate pharmacokinetic, tissue distribution and protein binding properties of MG is equally important when interpreting MG pre-clinical studies or inferring the data for clinical situation. On the other hand, MG toxicity, analgesic and basic pharmacokinetics properties have been reported in the literature but studies on anxiolytic effect of MG is still lacking; and with the establishment of these MG biological properties the reported MG pharmacological properties and its anxiety-related behaviour in rats could be better understood. To achieve this, the following objectives were undertaken in this thesis:

1. To develop and validate a HPLC-UV method using simple protein precipitation step for the assay of MG in plasma and tissues (liver, kidney, lung, heart and brain) for application in pharmacokinetic studies.

2. To conduct dose-proportionality and multiple dose pharmacokinetic studies to determine the dose range at which MG exhibit linear pharmacokinetic profile as well as MG disposition kinetic and its accumulation properties following repeated administrations to rats.

3. To conduct tissue distribution and plasma protein binding studies in order to determine the distribution and accumulation profile of MG into rat tissues following single oral dose administration and to estimate the extent to which MG binds to rat plasma proteins.

4. To determine the effects of MG on anxiety-related behaviours in rats using open-field and elevated plus-maze tests and to evaluate the involvement of opioidergic, GABAergic and dopaminergic systems in the observed effects of MG.

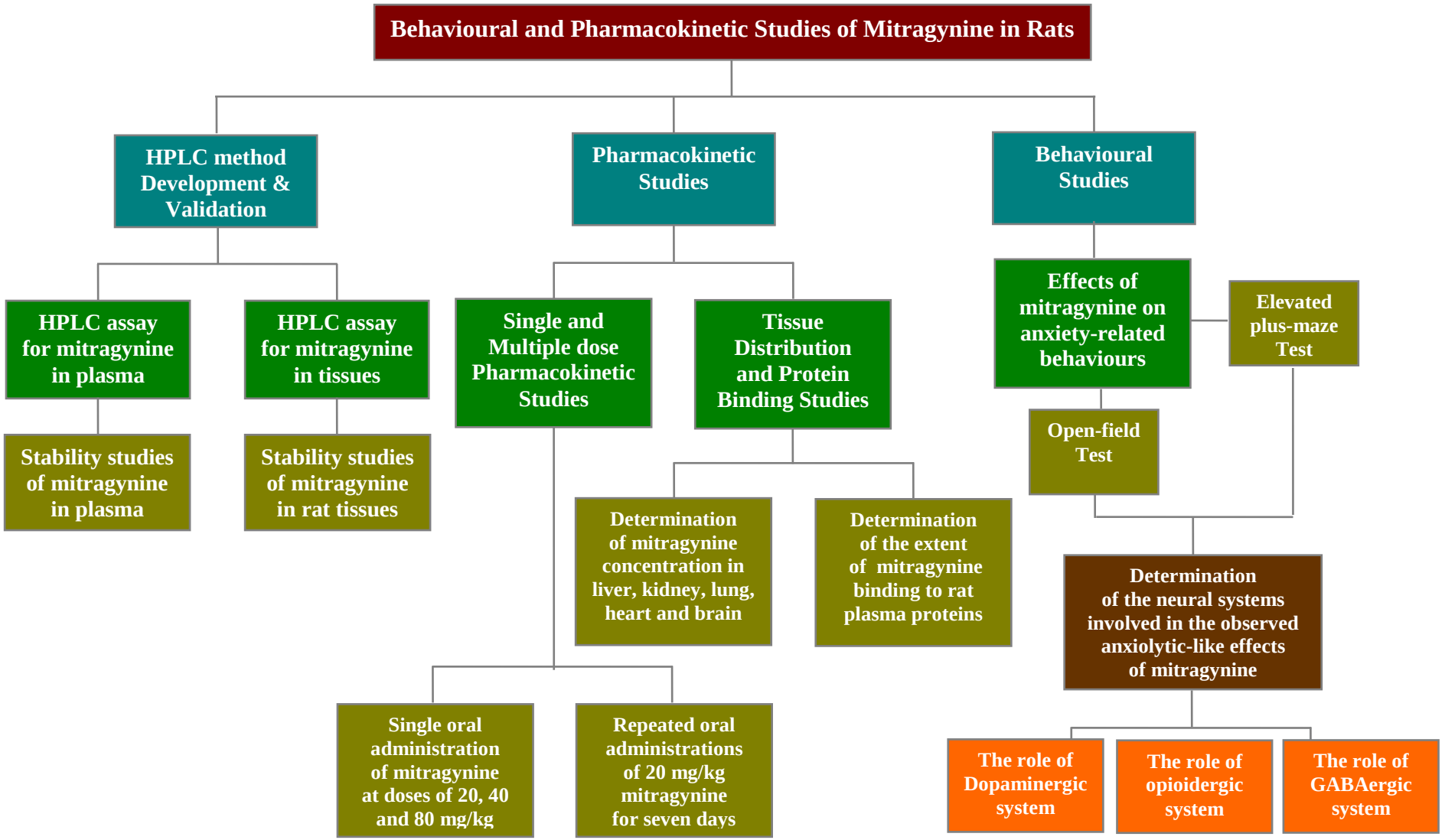

Figure 1.1: Thesis workflow

CHAPTER TWO

LITRERATURE REVIEW

2.1 Herbal medicines

Herbal medicines have been used to treat various ailments and to maintain good health since the earliest days of mankind. The use of herbal remedies has been increasing markedly over the past decades as herbs are cheap and have fewer side-effects than some conventional medications. Raw and partly-processed medicinal plants are usually exported from developing countries such as China, India, Mexico, Bulgaria, Chile and Egypt. The main processing of these plants takes place in developed countries such as the US, Germany, France and Republic of Korea where plant products are either sold in domestic markets or exported to other countries (Williamson and MacTavish, 2008). During the period 1991 to 2003, China contributed to one-third of the total global exportation of medicinal plants by exporting an average of 150,600 tons/ year valued at US$ 266 million. On the other hand, European countries such as Germany, France, Italy, Spain and the UK contributed to one-third of the global import market by importing the average of 96,300 tons/year valued at US$ 256 million. During the same period, Malaysia imported an average of 7,050 tons of medicinal plants with the value of US$ 38,685,400/year (Lange, 2006). The World Health Organization reported that approximately 70-80% of the world population relies on herbal medicines for their primary health care (Akerele, 1993, Farnswaorth and Soejatro, 1991). In the US, one third of the population was reported to use herbal medicines in 1997. In the same year, the sales of herbal medicines in US were estimated to be US$ 3.24 billion (~RM12.3 billion).

The market of herbal medicines in Malaysia was estimated to be RM 2.0 billion in 1997; when Malaysians expenditure on herbal medicines reached about RM 91.00 per person per year compared to that of Americans which was approximately RM 45.00 per person per year taking into account populations of 22 and 273 million respectively (Hussin, 2001). An earlier study also reported that 17.1% of Malaysians use herbal medicines to treat different illnesses while 29.6% of them rely on herbs for health maintenance (Mahmud, 1993).

Over 35,000 plant species have been used in traditional medicine worldwide (Farnswaorth and Soejatro, 1991). In 1966, Burkill and colleagues reported the use of more than 1000 plant species in the folk medicine in the Malay Peninsula (Burkill et al., 1966). Several studies were conducted to record the traditional knowledge on medicinal plants in different states of Malaysia such as Negri Sembilan, Pahang, Terengganu and Johor (Ong et al., 2011a, Alsarhan et al., 2012, Eswani et al., 2010, Ong et al., 2011b). The exact number of medicinal plant species, the disease each plant is supposed to alleviate and the method of preparation varied from one study to another as traditional knowledge is not well documented and verbally passed through the generations. *Mitragya speciosa* Korth is one of the Malaysian medicinal plants used by local people to treat various ailments. *Mitragya speciosa* (*M. speciosa*) leaves contain more than 25 alkaloids and mitragynine (MG) is the most abundant alkaloid, responsible for many of the plant's pharmacological effects.

2.2 *Mitragyna speciosa* Korth

2.2.1 Plant description

M. speciosa Korth (Rubiaceae) is a tropical evergreen tree indigenous to Southeast Asian countries such as Malaysia and Thailand. In Malaysia, it is commonly known as "*Biak-Biak*", "*Bia*", "*Kutum*" or "*Ketum*" and is native to the northern and west coast part of the Malaysia peninsular. It is also found in the central and southern regions of Thailand and is known as "*Kratom*", "*Katawn*", "*Tawm*" or "*Thom*". This plant was first described by a Dutch botanist Pieter Korthals, who named the genus of this plant "*Mitragyna*" as the shape of the first stigmas he examined resembled a bishop's mitre (Shellard, 1974).

Ketum tree (Fig. 2.1) can grow up to 50 feet (15 meters) tall. It grows heavily in high humidity areas rich with humus and is sensitive to drought and severe coldness. The tree can be characterized by its globular yellow flowering head each contain up to 120 florets. The fruit is a capsule containing numerous small flat seeds. The leaves of this tree are dark glossy green which grow up to 7 inches in length and 4 inches in width. Two types of *M. speciosa* can be distinguished based on the colour of veins in the leaf, either red or white-greenish (Fig 2.1 & 2.2) (Suwanlert, 1975). The red-veined leaves are known for their high MG content compared to the white-greenish-veined leaves (Hanapi et al., 2013). Therefore, MG used in this study was isolated from fresh red-veined *M. speciosa* leaves.

Figure 2.1: *Mitragyna speciosa* tree.

Figure 2.2: Red-veined *Mitragyna speciosa* leaf.

2.2.2 Phytochemistry of *Mitragyna speciosa*

Over 25 alkaloids have been isolated from the leaves of this plant. The main indole alkaloids found in the young leaves of Malaysian and Thai plants were MG and its derivatives speciogynine, speciociliatine, paynantheine and 7-hydroxymitragynine. New types of alkaloids such as 3,4-dehydromitragynine, mitragynaline, corynantheidaline, mitragynalinic acid, corynantheidalinic acid and 7-hydroxyspeciociliatine were also isolated from the fresh leaves and fruits of Malaysian *M. speciosa* (Houghton et al., 1991, Kitajima et al., 2006). MG was found to be the most dominant alkaloid in the leaves of this plant and is believed to contribute to its pharmacological effects (Figure 2.3). It was first isolated by Hooper (1907) and later by Field (1921) who named the alkaloid (Shellard, 1974). In 1964, Zacharias and colleagues first determined the structure of MG using x-ray crystallography (Zacharias et al., 1965). MG structure is related to those of yohimbine and voacangine; its molecular formula is $C_{23}H_{30}N_2O_4$ and has a molecular weight of 398.5 (Hassan et al., 2013, Takayama et al., 1998). MG melting point is between 102°C-106°C and it has a boiling point of 239°C-240°C. Physically, MG is a white yellowish amorphous powder soluble in alcohol, chloroform, acetic acid and oily substance. The UV spectrum of methanol solution of MG shows absorbance bands between the wavelength of 226 and 292 nm (Barceloux, 2012, Macko et al., 1972, Zacharias et al., 1965). MG was first synthesized by Takayama and colleagues (Takayama et al., 1995) and MG synthesis pathways were reported by Ma and colleagues (2009). The total alkaloid content of *M. speciosa* leaves varies from 0.5% to 1.5%. Green leaves contain about 0.2% MG with the average green and dry leaf weighing about 1.7 grams and 0.43 grams respectively. Although MG is the major constituent of Malaysian and Thai plants, the yield obtained from the total alkaloid

extract of Malaysian *M. speciosa* leaves (12%) was much less than that of the Thai plant (66%). These quantitative variations in MG content may be due to differences in geographic origin, plant age and harvest season (Barceloux and Palmer, 2012, Takayama, 2004).

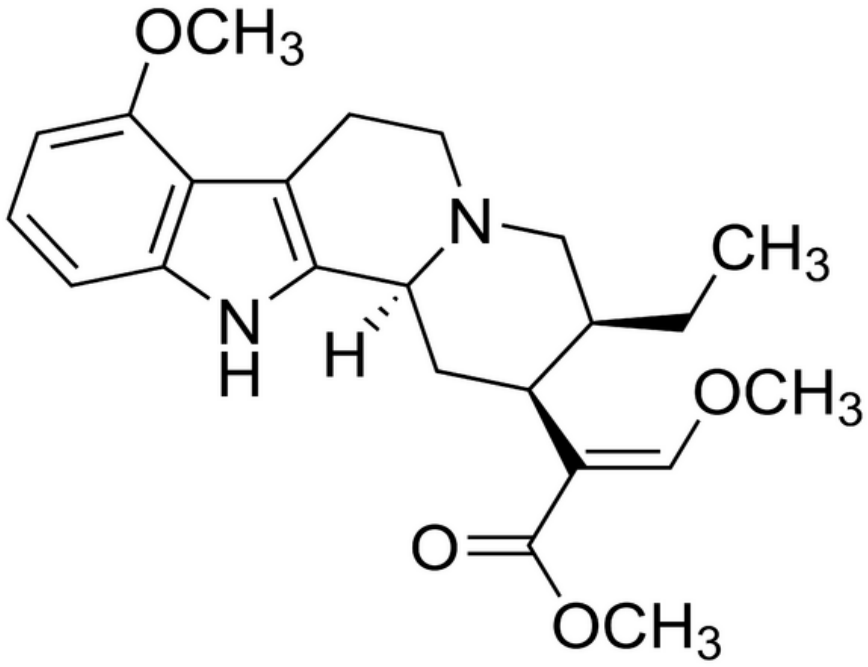

Figure 2.3: The Chemical structure of mitragynine (αE, 2S, 3S, 12bS)-3-ethyl 1,2,3,4,6,7,12,12b-octahydro-8-methoxy-α(methoxyme-thylene)-indolo[2,3-a] quinolizine-2-acetic acid methyl ester. Adapted from Chemical Abstract Service (CAS).

2.2.3 Traditional use

In Malaysia, *M. speciosa* leaves have been widely used as a traditional medicine for centuries. The leaves were chewed, smoked or drunk as a suspension by local people to enhance work tolerance under intense tropical heat (Grewal, 1932, Jansen and Prast, 1988). In addition, the leaves were also used to relieve pain, reduce coughing, alleviate diabetes, diarrhoea, and to mitigate morphine withdrawal symptoms (Watanabe et al., 1997). Administration of *M. speciosa* leaves has been shown to produce a stimulant effect at low doses and sedative and analgesic effects at the higher doses in humans and laboratory animals therefore suggesting the presence of dual opioid properties (Macko et al., 1972, Suwanlert, 1975).

2.2.4 Pharmacological effects of *Mitragyna speciosa* and mitragynine

Several studies have been conducted to investigate the pharmacological effects of *M. speciosa* leaves extracts, MG and it derivatives. The effects of the methanolic extract of *M. speciosa* leaves on the rat gastrointestinal tract were investigated by Chittrakarn and colleagues (2008). Administration of the methanolic extract significantly reduced defecation frequency, total score, fecal weight and intestinal transit in castor oil-induced diarrheal rats. These findings supported the traditional use of *M. speciosa* leaves to treat diarrhea (Chittrakarn et al., 2008). In another study, incubation of rat L8 muscle cell with various concentrations of water, methanolic, alkaloid extracts of *M. speciosa* leaves and MG respectively showed an increase in the rate of glucose uptake and the concentration of glucose transporters per unit of muscle protein. This observation supported the use of *M. speciosa* leaves extracts in folk medicine to alleviate diabetic patients (Purintrapiban et al., 2011). The methanolic extract of *M. speciosa* leaves was also evaluated for its

acute and chronic anti-inflammatory effects using carrageenan-induced paw edema and cotton pellet-induced granuloma tests respectively. Intraperitoneal administration of the methanolic extract of *M. speciosa* leaves inhibited the development of paw edema with a maximal inhibition during the first 3h following the injection of carrageenan into the subplantar region of the rat hind paw. The administration of the extract for seven days significantly reduced granulomatous tissue formation in the chronic test (Shaik Mossadeq et al., 2009). Similarly, MG exhibited anti-inflammatory activity through the inhibition of cyclooxygenase 2 (COX-2) mRNA and protein expression and prostaglandin E_2 (PGE_2) formation in macrophage cells (Utar et al., 2011).

The administration of *M. speciosa* leaves extracts or MG has shown to produce antinociceptive effects in laboratory animals. The administration of the methanolic and alkaloid extracts of *M. speciosa* leaves in mice prolonged the latency of nociceptive responses in hotplate test (Reanmongkol et al., 2007). These antinociceptive effects were further investigated using acetic-acid-induced writhing test and formalin test. Intraperitoneal administration of the methanolic extract of *M. speciosa* leaves significantly inhibited writhing responses and pain sensation in rats (Shaik Mossadeq et al., 2009). The antinociceptive effects of several *M. speciosa* leaves extracts were compared to that of morphine using hot plate and tail flick tests. The alkaloid, methanolic and the aqueous extract of *M. speciosa* leaves were orally administered to Sprague-Dawley rats. Administration of these extracts as well as morphine significantly prolonged the latency of nociceptive responses in both tests. These effects were significantly reversed by naloxone, a competitive antagonist at μ-, δ-, and κ-opioid receptors suggesting the involvement of these receptors in the

observed antinociceptive effects of *M. speciosa* leaves extracts (Sabetghadam et al., 2010). An intraperitoneal and intracerebroventricular administration of MG produced antinociceptive effects in mice tested in tail-pinch and hot-plate tests. These effects were significantly reversed by pretreatment with naloxone suggesting the involvement of supraspinal opioid systems in the antinociceptive effect of MG (Matsumoto et al., 1996b). MG has also shown to suppress the electrically stimulated contraction of guinea-pig ileum and mouse vas deferens through the activation of μ- , κ- and δ-opioid receptors (Matsumoto et al., 2005b, Watanabe et al., 1997). The binding of MG to opioid receptors has been further examined using radioligand binding assay. MG has been shown to activate opioid receptors with high affinity to μ- and κ- followed by δ-opioid receptor subtypes (Boyer et al., 2008, Taufik Hidayat et al., 2010b). A minor constituent of *M. speciosa* leaves, 7-hydroxymitragynine has been shown to produce a 46-fold more potent antinociceptive effects compared to MG (Matsumoto et al., 2005a). Oral and subcutaneous administration of 7-hydroxymitragynine has also been shown to produce a more potent analgesic effect compared to morphine in the hot-plate and tail-flick tests in mice (Matsumoto et al., 2006). The potent analgesic effects of 7-hydroxymitragynine could be due its higher lipophilicity (log p: 1.3) compared to morphine (log p: 0.9) which enhances the drug penetration across the BBB and its CNS activity (Hansen et al., 2012). This effect was significantly reversed by pretreatment with naloxone suggesting the involvement of μ-opioid receptors in the observed effects of 7-hydroxymitragynine (Takayama, 2004). In addition, the involvement of δ- and κ-supraspinal opioid receptors in the antinociceptive effects of 7-hydroxymitragynine was also reported in earlier studies (Matsumoto et al., 2006, Matsumoto et al., 2005a).

Several studies have reported the neurophysiological effects of *M. speciosa* extracts and MG. Grewal (1932) suggested two ways of MG action on the central nervous system in laboratory animals. The first way are the effects on the autonomic nervous system which consist of facilitation of the passage of impulses affecting both the crania-sacral and sympathetic divisions. The second way are the effects on the central nervous system which consist of an excitation of the motor centres in the medulla (Grewal, 1932). Administration of the aqueous and alkaloid extracts of *M. speciosa* leaves has been shown to produce antidepressant-like effects in mice tested in forced-swim and tail suspension tests (Kumarnsit et al., 2007a, Kumarnsit et al., 2007b). Similarly, intraperitoneal administration of MG has also been shown to produce antidepressant-like effects and decrease the release of corticosterone in mice suggesting the role of neuroendocrine hypothalamic-pituitary-adrenal axis in modulating MG effects (Idayu et al., 2011). In our laboratory, acute oral administration of MG to mice has also been shown to enhance exploratory behaviours by mitigating stress to a novel Y-maze environment (Hazim et al., 2011). Considering the antidepressant and stress-mitigating effects of MG in laboratory animals, this work was undertaken to evaluate the effect of MG on anxiety-related behaviours in rats using open-field and elevated plus-maze. The possible involvement of opioidergic, GABAergic and dopaminergic systems in the observed effects of MG was also determined.

2.2.5 *Mitragyna speciosa* legal status

M. speciosa plant has been prohibited in Thailand since 1943. In 1970 the Thai government placed *M. speciosa* under Schedule 5 of the Thai Narcotic Act whereby existing trees need to be cut down and it is illegal to buy, sell, import or

possess it (Hassan et al., 2013). In Malaysia, the plant has been prohibited under the Third Schedule of the Poisons Act (1952). Under this Act "the planting of the tree is not an offence, and enforcement agencies have no authority to fell the trees. The maximum penalty for possessing or selling ketum leaves or other ketum preparations such as drinks and teas containing mitragynine is a fine of RM 10,000, a four-year jail sentence or both" (Chan et al., 2005). *M. speciosa*, MG and 7-hydroxymitragynine are considered as controlled drugs in many EU countries such as Denmark, Poland and Sweden. In countries such as Australia and Myanmar, *M. speciosa* and MG are under the control of Narcotics Laws. *M. speciosa* and MG are not listed as controlled substances in the US, UK and Germany however they are considered as serious drugs (EMCDDA, 2012).

2.2.6 The use of *Mitragyna speciosa* leaves to manage opioid withdrawal symptoms

The use of *M. speciosa* leaves is still uncontrolled in some western societies where it is easily available over the internet. In recent years, this plant has been used as self-treatment of opioid withdrawal symptoms, alcohol withdrawal and chronic pain (Babu et al., 2008, Boyer et al., 2008, Boyer et al., 2007). Several studies reported the use of *M. speciosa* leaves to substitute opium in the treatment of opioid addiction in Malaysia and Thailand. In recent years, this plant has become controlled or illegal in these countries. However, its use is widely reported by locals in villages where it is easily available and cheaper than other substances of abuse. Vicknasingam et al (2010) reported the use of ketum drink in Northern Malaysian peninsular to reduce addiction to other drugs. The study revealed that ketum users consume an average of 3 medium-sized glasses (each 250 ml) of ketum drink to manage opioid withdrawal symptoms (Vicknasingam et al., 2010). Another study by

Ahmad and Aziz (2012) reported the use of ketum drink in the northern states of Kedah and Perlis to treat opioid withdrawal, build up physical stamina and endurance, relieve pain and improve sexual performance (Ahmad and Aziz, 2012). Based on this observation, the MG content in ketum drink obtained from various geographical and socio-economic backgrounds was determined by Parthasarathy et al. (2013). This further provided the basis to predict the standard daily dose of MG consumed by ketum users. The estimated dose consumed was in the range of 0.3 to 5.7 mg/kg body weight. Using the human-rat dose equivalent table, the corresponding MG dose in rats was in the range of 1.85-35.15 mg/kg (Reagan-Shaw et al., 2008). The given MG dose range was considered while selecting the appropriate MG dose regimen for the current pharmacokinetic, tissue distribution and behavioural studies.

2.3 Anxiety

Anxiety is a natural adaptive reaction induced in animals and humans in anticipation of actual or potential threat. This reaction is accompanied by marked behavioural and physiological responses which evolve to help the individual cope with various challenges and stressful events (Steimer, 2002).

2.3.1 The physiology of anxiety

Behavioual responses associated with anxiety include a range of defensive or adaptive behaviours. The development of these behaviours depend on the context and the behavioural repertoire of the species (Steimer, 2002). When the threat causing anxiety is escapable, active emotional coping strategies are used. This type of active response was originally described by Cannon as “fight or flight” response (Cannon, 1963). This response is characterized by the activation of sympathetic

nervous system and the subsequent release of epinephrine, norepinephrine and cortisol from the adrenal gland (Brick and Erickson, 2009). The role of the sympathetic response is to make an automatic, internal adjustment in the body without a concious effort by the individual. Sympathetic activation together with the release of epinephrine, norepinephrine and cortisol orchestrates changes in heart rate, blood pressure, blood glucose level, body temperature and sweat gland secretion to prepare the individual for violent activity (Levitt, 1967). When the threat causing anxiety is inescapable, passive emotional coping strategies such as quiescence, immobility and decreased responsiveness to the environment are used (Bandler et al., 2000). Passive responses were first described by Engel and Schmale (1972) as a conservation-withdrawal strategy which is characterized by autonomic inhibition (hypotension and bradychardia), activation of the hypothalamopituitary-adrenal axis and increased glucocorticoid secretion (Steimer, 2002). In 1929, Cannon and Brad demonstrated that physiological reactions and emotional experiense arise simultanously, mediated by two lower brain centers, thalamus and the hypothhalamus. In 1937, Papez suggested a neural pathway connecting the hypothalamus and cortex as the emotional system of the brain. In 1952, Paul D. MacLean reported the central role of amygdala in modulating emotional responses. He expanded Papez ideas by adding more stractures to Papez circuit and named the modified version of the circuit as the limbic system (Levitt, 1967). The limbic system is a set of struactures that controls emotional behviour and motivational derives (Figure 2.4). It is located on both sides of the thalamus, right under the cerebelum. The limbic system is composed of the hypothalamus surrounded by other subcortical stractures of the limbic system including the septum, the paraolfactory area, the anterior nucleus of the thalamus, portions of the basal ganglia, the hippocampus and

the amygdala. Surrounding the subcortical limbic areas is the limbic cortex composed of orbitofrontal area, subcallosal gyrus, cingulate gyrus, parahippocampal gyrus and uncus (Guyton and Hall, 2006). The Reticular Activating System (RAS) is a part of brain centered in brain stem. The primary role of RAS is to control the sleep-waking continuum. However, with the cerebral cortex, RAS is involved in a feedback-control operation to maintain the optimal stimulation level. This implies that sensations reaching the cerebral cortex are constantly fed back to the RAS. When the frequency of these impulses increase significantly, RAS send inhibitory impulses back to the cortex, damping its activity. It was therefore suggested that the experience of anxiety is a result of a weakining of the inhibitory function of RAS resulting in too many facilitative impulses to be discharged to the cortex, leading to an arousal level beyond the normal (Malmo, 1957).

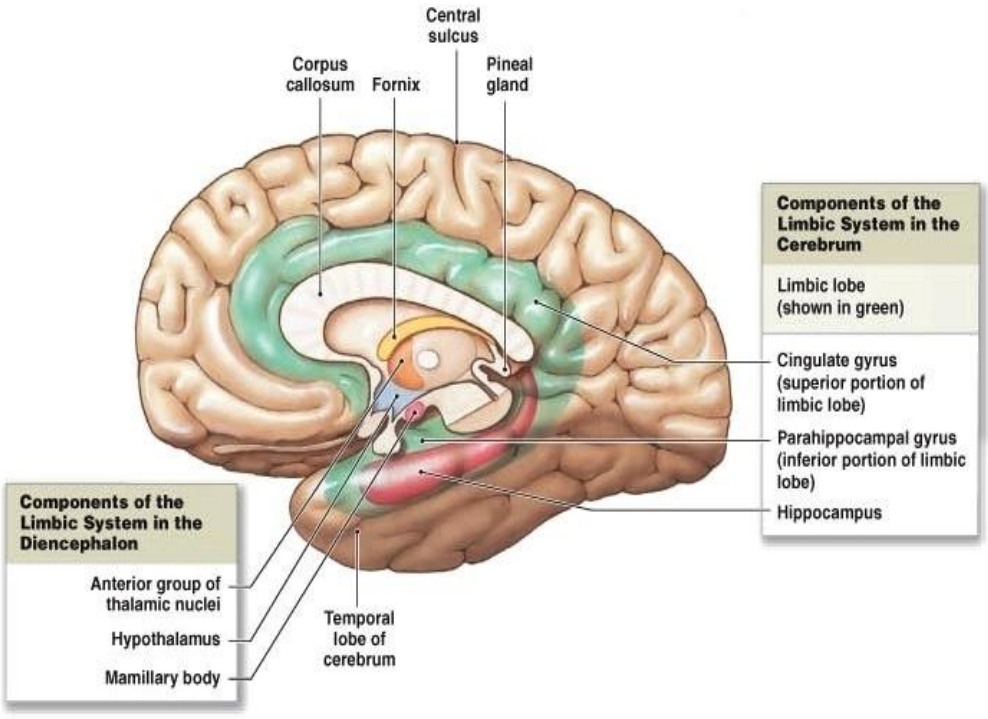

Figure 2.4: The limbic system, figure reproduced with permission from Pearson Education (2012).

2.3.2 Neurotransmitter systems involved in anxiety

Several neurotransmitter systems are involved in the modulation of anxiety behaviours. The main neurotransmitter systms implicated in anxiety are the GABAergic, serorotonergic and dopaminergic system.

2.3.2.1 The GABA system

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter of the mammalian central nervous system (Durant et al., 2010). GABA acts via two main types of receptors, the fast-acting ion gated receptors (GABA-A and GABA-C) and the slower-acting G-coupled GABA-B receptor (Bormann, 2000). GABA-A receptor is the most abundant GABA receptor in the brain. GABA-A receptors play a crucial role in regulating the excitatory tone of many neurons including dopaminergic, cholinergic and serotonergic (Fritschy et al., 1992, Gao et al., 1995). In addition, earlier studies provided strong support for the role of GABA-A receptors in modulating anxiety-related behaviours (Lydiard, 2003, Mohler, 2012). GABA-A receptor consists of five protein subunits 2α, 2β and 1γ subunits. These subunits are arranged like a rosette around a central pore (Nayeem et al., 1994). GABA acts as an agonist at the receptor complex. Binding of GABA at the interface between α and β subunits triggers conformational changes, which enhance the influx of chloride ions into the post-synaptic neuron through the central pore. This results in a state of hyperpolarisation and reduces neuron excitability (Nutt, 2006). Benzodiazepines (BDZs) are a class of tranquilizer drugs used to treat anxiety, seizures and insomnia. BDZs bind at the interface between α and γ subunits of GABA-A receptor resulting in channel opening and influx of chlorine ion (Olsen et al., 2004). BDZs augment the

response of GABA-A receptor to endogenous GABA by reducing the concentration of GABA needed to cause chloride channel opening (Nutt and Malizia, 2001).

2.3.2.2 The serotonergic system

Serotonergic neurones arise from the median and dorsal raphe nuclei in the brainstem and project throughout the forebrain (Durant et al., 2010). Serotonin (5-hydroxytryptamine or 5-HT) is synthesized from the essential amino acid tryptophan (Richard et al., 2009). Following the release of 5-HT into the synaptic cleft, 5-HT is transported back into the pre-synaptic neuron by serotonin transporters (Sangkuhl et al., 2009). Although more than 10 serotonin receptors were identified; 5-HT-1A has particularly been linked to anxiety disorders (Durant et al., 2010). Nowadays, selective serotonin reuptake inhibitors (SSRIs) have been effectively used to treat anxiety disorders. These serotonergic agents inhibit serotonin reuptake process and therefore enhance serotonin level in the synapses and reduce anxiety behaviours (Benitez et al., 2008).

2.3.2.3 The dopaminergic system

Dopamine is synthesised from the amino acid tyrosine. The main dopaminergic pathways are the nigrostriatal (associated with motor control), tuberoinfundibular pathway (regulates the release of prolactin from the pituitary gland), mesolimbic pathway (associated with reward and cognitive behaviours) and mesocortical pathways (involved in cognitive control, motivation and emotional responses) (Durant et al., 2010). Although five different dopamine receptors (D_1-D_5) were identified, D_1 and D_2 dopamine receptors have been shown to modulate anxiety-related behaviours (Rodgers et al., 1994, Schneier et al., 2000, Schneier et

al., 2008). In addition, earlier studies have also reported that dopamine deficiency is strongly associated with social anxiety disorders (Berrios et al., 1995, Liebowitz et al., 1992, Van der Wee et al., 2008).

2.3.3 Anxiety disorders

Anxiety can become a pathological disorder when it is so intense, prolonged and interferes with normal life (Gross and Hen, 2004). The American Psychiatric Association divided anxiety disorders into five main types including panic disorder, obsessive-compulsive disorder, post-traumatic stress disorder, generalized anxiety disorder and phobias such as specific phobia, social phobia and agoraphobia (APA, 2010). Panic disorder is characterized by unexpected and repeated panic attacks. The exact cause of these disorders is unknown; however several physical and psychological factors may contribute to this disorder such as traumatic life experience, genetic link, and imbalance of brain neurotransmitters (Kim et al., 2012). Obsessions are persistent upsetting and irrational thoughts that cause great anxiety which can not be controlled through reasoning. Compulsions are repeated behaviours used to minimize these obsessions (Montgomery and Morris, 1994). Post-traumatic stress disorder is caused by distressing events. People with this disorder repeatedly retrive the traumatic event through flashbacks and nightmares (Mueller et al., 2005). On the other hand, generalized anxiety disorder tends to permeate most of people's life rather than being focused on particular part (Montgomery and Morris, 1994). Specific phobia is an excesssive fear of an object or situation that actually poses little or no threat such as height, tunnels and injuries involving blood (NIH, 2009). Social phobia is a fear of being mocked or criticized by unfamiliar people in situations such as public speaking or eating in front of others (Wittchen et al., 2011). Agoraphobia is