PHYTOCHEMICAL ANALYSIS AND BIOACTIVITY EVALUATION OF STANDARDIZED EXTRACTS OF *MITRAGYNA SPECIOSA* AND PHARMACOKINETICS OF MITRAGYNINE

by

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TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

AEC : Animal Ethics Committee

ACN : Acetonitrile

As : Asymmetry factor

AUC_{0-t} : Area under the curve from time zero to time t.

$AUC_{0-\infty}$: Area under the curve from time zero to infinity.

AUFS : Absorption units full scale

BHT : Butylated hydroxytoluene

C_8 : Octyl-type hydrocarbon with eight carbon atoms bonded silica stationary phase.

C_{18} : Octadecyl type hydrocarbon with 18 carbon atoms bonded silica stationart phase.

CL : Clearance

C_{max} : Peak plasma concentration

FDA : Food and Drug Administration

F-T : Freeze-thaw

G : Gram

h :Hour

HPLC : High Performance Liquid Chromatography

HPLC-UV : High Performance Liquid Chromatography coupled with Ultraviolet detector

ICH : International Conference on Harmonization

IC_{50} : The half maximal inhibitory concentration

IS : Internal Standard

l :Liter

LLOD : Lower limit of detection

LLOQ : Lower limit of quantification

LLE : Liquid-liquid extraction

M : Molar

MeOH : Methanol

MG : Mitragynine

mL :Milliliter

mm :Millimeter

mM :Millimolar

MMP :Matrix metalloproteinases

MRT : Mean residence time

N : Normality

N : Number of replicate

ng :Nanogram

ODS : Octadecylsilane

pH : Negative logarithm of H+ concentration

pKa : Ionisation constant

r^2 : Corrélation coefficient

RP : Reverse phase

RP-HPLC : Reversed-phase high performance liquid chromatography

rpm :Revolution per minute

SD : Standard deviation

S.E.M. : Standard error of the mean

SPE : Solid phase extraction

$T_{½}$: Elimination half-life

T_{max} : Time to reach peak plasma concentration

USP : US Pharmacopoeia

µl :Microliter

µm :Micrometer

UV : Ultraviolet

v/v :Volume by volume

v/w : Weight by volume

V_d : Volume of distribution

LIST OF APPENDICES

LIST OF PUBLICATIONS

1. Parthasarathy, S., Ramanathan, S., Ismail, S., Adenan, M., Mansor, S., & Murugaiyah, V. (2010). Determination of mitragynine in plasma with solid-phase extraction and rapid HPLC–UV analysis, and its application to a pharmacokinetic study in rat. Analytical and Bioanalytical Chemistry. 397, 2023–2030. ISI journal (Impact factor: 3.48)

2. Parthasarathy, S., Azizi, J., Ramanathan, S., Ismail, S., Sasidharan, S, Said, M.I.M. & Mansor S.M., (2009). Evaluation of antioxidant and antibacterial activities of aqueous, methanolic and alkaloid extracts from *Mitragyna speciosa* (Rubiaceae family) leaves. Molecules 14(10), 3964-3974 ISI Journal. (Impact factor : 1.738)

IN PROGRESS

1. Parthasarathy, S., Ramanathan, S., Ismail, M., Mansor, S., Said, M.I.M & Murugaiyah, V. (2010). A simple and rapid High Performance liquid Chromatographic UV method for routine quantification of mitragynine in psychotropic plant *Mitragyna speciosa* leaf extracts. Journal of Agriculture and Food Chemistry.(Manuscript completed in progress for submission) ISI journal.(Impact factor:2.53)

ANALISIS FITOKIMIA DAN PENILAIAN BIOAKTIVITI EKSTRAK TERPIAWAI *MITRAGYNA SPECIOSA* DAN FARMAKOKINETIK MITRAGYNINA

ABSTRAK

Kesan farmakologi ekstrak daun *M. speciosa* dan mitragynina dalam tikus telah dikaji. Dua kaedah analitikal kromatografi cecair prestasi tinggi KCPT-UV yang baru telah dibangunkan dan disahkan untuk menganalisa mitragynina dalam ekstrak daun *M. speciosa* dan plasma tikus. Kedua-dua kaedah KCPT-UV untuk matrik tumbuhan dan plasma menghasilkan kromatogram yang bersih, masa analisis yang pendek , kepekaan analisis yang baik dan berjaya diaplikasikan dalam analisi yang berkaitan. Kaedah KCPT-UV yang pertama telah digunakan untuk mempiawaikan ekstrak-ekstrak akues, metanolik dan alkaloid daun *M. speciosa* yang selanjutnya digunakan dalam kajian antimikrobial dan antioksidan. Kaedah analitikal kromatografi cecair prestasi tinggi KCPT-UV yang kedua juga menggabungkan teknik pengekstrakkan SPE untuk pembersihan sampel plasma telah digunakan bagi penentuan farmakokinetik mitragynina dalam tikus. Kandungan mitragynina dalam ekstrak alkaloid, ekstrak metanol dan akues masing-masing adalah 23.0, 4.4, 0.7%. Kaedah ini juga didapati setara dalam aplikasi penentuan mitragynin dalam rebusan daun *M. speciosa* yang diperolehi dari jalanan. Ciri-ciri antioksidan ekstrak daun *M. speciosa* telah ditentukan dengan menggunakan ekstrak piawai. Jumlah kandungan fenolik dalam ekstrak akues, alkaloid dan metanolik masing-masing adalah 66.0 mg, 88.4 mg dan 105.6 mg GAE/g. Ekstrak akueus, alkaloid dan metanolik *M. speciosa* telah dinilai untuk aktiviti mikrobial bagi mikrob organisma tertentu. Kepekatan minimum rencatan ekstrak-ekstrak ini dengan mengunakan teknik pencairan bubur adalah dalam julat 3.12 ke 6.25 mg/mL.

Hasil kajian menunjukkan ekstrak metanolik mempunyai aktiviti antioksidan yang tinggi. Manakala ekstrak daun *M. speciosa* yang kaya dengan alkaloid ditemui lebih berkesan terhadap mikrob organisma yang diuji berbanding ekstrak yang lain. Farmakokinetik mitragynina telah dikaji pada tikus selepas administrasi oral dan intravenus mitragynina. Selepas administrasi intravenus, mitragynina mempamerkan penyingkiran dua fasa daripada plasma dengan purata separuh hayat 2.9 ± 2.1 jam. Penyerapan oral mitragynina adalah perlahan, tidak tetap dan disingkirkan dengan jangkamasa separuh hayat 6.6 ± 1.3 jam. Nilai mutlak bioketersediaan oral mitragynina adalah 3.03%. Variasi yang diperhatikan dalam kajian farmakokinetik mitragynina yang dilaporkan dalam literasi untuk tikus adalah disebabkan oleh bioketersediaan oral mitragynina yang rendah dan bukan limitasi ujikaji, tepuan metabolik dan perbezaan dos mitragynina.

PHYTOCHEMICAL ANALYSIS AND BIOACTIVITY EVALUATION OF STANDARDIZED EXTRACTS OF *MITRAGYNA SPECIOSA* AND PHARMACOKINETICS OF MITRAGYNINE.

ABSTRACT

The pharmacology of *M. speciosa* leaf extracts and mitragynine in rats were investigated. Two new High Performance Liquid Chromatography Ultraviolet HPLC – UV analytical methods were developed and validated for assay of mitragynine in *M. speciosa* leaf extracts and plasma. Both HPLC-UV method for assay of mitragynine in plant matrix and plasma yield a clean chromatogram, short analysis time, good assay sensitivity and was successfully used for their intended applications. The first HPLC-UV method was used to standardize water , methanolic and alkaloid extracts of *M. speciosa* leaf which was subsequently used for antimicrobial and antioxidant studies. The second HPLC-UV method with SPE extraction techniques for plasma sample clean up was employed to determined the mitragynine pure compound pharmacokinetics in rats. The mitragynine (MG) content in alkaloid rich fraction, methanol extract and water extract were 23 , 4.4 and 0.7% respectively . The method was found equally applicable to determine mitragynine in decoction *M. speciosa* leaf (50-180µg/mL) obtained from the streets. The antioxidant properties of *M. speciosa* leaf extracts were determined using standardized extracts.The total phenolic content of the aqueous, alkaloid and methanolic extracts were 66.0 mg, 88.4, 105.6 mg GAE/g, respectively, while the total flavonoid were 28.2, 20.0 and 91.1 mg CAE/g respectively The aqueous, alkaloid and methanolic extracts were further screened for antimicrobial activity for selected microorganism. The

minimum inhibitory concentrations of this extracts determined by the broth dilution method ranged from 3.12 to 6.25 mg/mL. The results suggest that the methanolic extract showed relatively high antioxidant activity whereas as for the bioactive analysis, alkaloid rich fraction was found to be most effective against all of the tested organisms in comparison to other extracts. The mitragynine pharmacokinetics was investigated in rats after oral and intravenous administration of mitragynine. After intravenous administration mitragynine demonstrated biphasic elimination from the plasma with a mean half-life of 2.9±2.1 h. The oral absorption of mitragynine was slow, erratic and eliminated with prolonged half-life of 6.6±1.3 h .The calculated absolute oral bioavailability of mitragynine was 3.03 %. The variations observed in previous pharmacokinetic studies after oral administration of mitragynine could be attributed to its poor bioavailability rather than to the differences in assay method, metabolic saturation or mitragynine dose.

CHAPTER 1

1.0 INTRODUCTION

Historically, plants are endowed with a good source of a wide variety of compounds, such as phenolic compounds, nitrogen compounds, vitamins, terpenoids and secondary metabolites, which are rich in valuable bioactivities, e.g., antioxidant, anti-inflammatory, antitumor, antimutagenic, anti-carcinogenic, antibacterial, or antiviral activities. The World Health Organization (WHO) defines traditional medicine (TM) as "health practices approaches, knowledge and beliefs incorporating plant, animal and mineral based medicines, spiritual therapies, manual techniques and exercises, applied singularly or in combination, to treat, diagnose and prevent illnesses or maintain well-being" (WHO, 2002).

Malaysia has a great potential to develop her abundant natural resources into herbal products as there are about 550 genera of tropical plants, containing over 1,300 species possessing medicinal values. This has drawn chemists, biochemist, and pharmaceutics interests to play an important role in discovering and developing new drugs, with the hope of more efficacy and no side effects as in most modern drugs. Among the promising medicinal plants that received more attention quite recently for its narcotic-like effects when smoked, chewed or taken as a suspension is *M. speciosa* (Macko et al., 1972; Perry, 1980; Jansen and Prast, 1988; Matsumoto et al., 1996 ;Tsuchiya et al., 2002). It is known as "Biak" and "Ketum" in Malaysia and as "Kratom" in Thailand. "Ketum" use is illegal in various countries including Thailand and Malaysia.

It is an offence under the Poisons Act 1952 to possess and sell ketum leaves in Malaysia (Report of Narcotic Control Board, 2008). Since 1946 Thailand has legislated this plant. Australia also banned the ownership of this plant in 2005 (Report of Narcotic Control Board, 2008). However free accessibility and information of kratom widely over the Internet has fascinated many Westerners to exploit the plant as self-treatment in opioid withdrawal and pain management. (Boyer et al., 2007). In Malaysia it is consumed both in unadulterated and adulterated form by mixing it with cough mixtures, traditional herbs and even synthetic pyrethroid from mosquito coils to add zest to the concoction (Bothipon, et al., 2009). Adulterated decoction of *M. speciosa* leaves known locally as "ketum juice" packed in plastic bottles or packets are found in the streets particularly in the northern region of Peninsular Malaysia. In this region "ketum juice" is consumed by addicts as a substitute of more expensive opiates to manage withdrawal symptoms. Initially the barks and leaves of this plant were seen to be a potential cure for opium habits and the leaves were suggested as opium replacement (Shellard, 1974).

Besides its opium like effect several side effects have been reported among ketum users such as nausea, vomiting, and quiver (Grewal, 1932a). Lengthened sleep, hyperpigmentation, loss of weight and anorexia are symptoms for chronic users. (Suwanlert, 1975). Other side effects, have been noted such as mouth dryness, constipation, diuresis, dark and small stools, weight loss and loss of appetite (Perry, 1980). While "ketum" was used for this reason, there are claims in Thailand that "ketum juice" was primarily used to increase physical endurance and work capacity (Assanangkornchai et al.,2006; Suwanlert, 1975; Vicknasingam & Narayanan, 2009). There are study reports on the medicinal use of *M. speciosa* leaves such as antitussive,

anesthetic, antinociceptive, stimulant, analgesic and narcotic-like *M. speciosa* leaves (Takayama, 2004; Watanabe et al., 1992 Tsuchiya et al., 2002; Tohda et al., 1997; Thongpradichote et al., 1998). In addition to this, (Burkill, 1936) documented other uses of ketum as a wound healer and cure for fever. It stated that the pounded leaves are applied to wounds and also on enlarged spleen. (Burkill, 1936). To date no research on antimicrobial and antioxidant properties of the *M. speciosa* have been documented. These pharmacological activities are often attributed to the alkaloids found in the leaf (Parthasarathy et al., 2009). Mitragynine (MG) is the dominant alkaloid and is exclusive to *M. speciosa*.

The use of kratom as a self management to opioid withdrawal is increasing without proper scientific basis and currently leads to its wide abuse (Boyer et al., 2007). Decoction of *M. speciosa* leaves from various sources are constantly being abused in this region and their MG content is not known. It is essential to precisely determine this predominant alkaloid in *M. speciosa* leaf extracts and decoction as this would provide a better understanding of their pharmacological aspect in relation to the reason it is consumed. Thus, there is a need for the development of a simple and faster method for routine quantification of MG in raw material, extracts, commercial or adulterated products.

Further to this the mitragynine's pharmacological and behavioral effects remain scanty scientifically and this warrants indepth pharmacological and pharmacokinetic studies of mitragynine in various in vivo models. For such an exploration, a quantitative analysis of mitragynine in biological fluids and plant extracts are very essential. Liquid

Chromatography Mass Spectrometry (LCMS) methods are available for analysis of MG especially in biological specimens and commercial products but their methodologies are laborious and expensive and not easily accessible. Furthermore, prior to analysis these methods requires complex sample clean-up and /or pre-treatments (Phillipp et al, 2009; De Moraes, 2009).

Therefore in this thesis, work has been undertaken to develop a simple and rapid High Performance Liquid Chromatography (HPLC) analysis method for determination of mitragynine in biological and plant matrix. The developed and validated methods were used to generate the oral and intravenous MG pharmacokinetics properties in animal model after administration in rats. MG plasma stability and MG content in plant material and abuse ketum juice were also determined. As there are anonymous claims on the topical use of *M. speciosa* leaves for infected wounds the antimicrobial and antioxidant activities of *M. speciosa* leaf extracts were also evaluated scientifically.

Objectives

- To develop and validate a simple and rapid HPLC-UV analysis method for determination of mitragynine in plant matrixes and its application for standardization of various *M. speciosa* leaf extracts for subsequent pharmacological studies.
- To determine the antioxidant and antimicrobial activities of *M. speciosa* leaf using the various standardized extracts as part of our effort to scientifically investigate the claim that *M. speciosa* leaf was used in the treatment of infected wounds.
- To develop a new, rapid and simple solid-phase extraction method for HPLC-UV determination of MG in plasma for application in stability study and mitragynine pharmacokinetic study in rats

CHAPTER 2

2.0 Literature review

2.1 Plant as Herbal Medicine

Plants have an extensive record as a source of drugs for treating human illnesses. (Chin et al., 2006). The pharmacopoeias of Traditional Chinese Medicine (TCM), the European pharmacopoeias, or the medicinal plants from traditional ethnic groups are well documented. Ethnopharmacology approaches are used to scientifically study the relationship of ethnic groups, their health, and how it relates to their physical habits and methodology in generating and using medicines (Johns, 1990). Some researchers viewed the use of medicinal plants as mechanical and/or symbolic adjuvants, vehicles, or facilitators of other ingredient but others regard that any activity attributed to the composite medicine of plants that are used to treat a wide range of disorders is usually presumed to be due to various ingredients (Etkin, 1986). Herbs and spices have been used for generations to treat ailments (Tayel & Tras 2009; Yano et al., 2006). The use of spices as medicines raises the issue of cultural distinctions between food and medicine but biomedicine increasingly recognizes the importance of diet in health and disease.

The distinction between food and medicine is therefore blurred, and a plant may be considered a medicine, a medicinal healthy food or a nutritious medicine (Etkin,1988b).This encourages the ethnophamarcologic studies to use a multicontextual approach to plant use and as such, research on potential bioactive chemicals in formerly mundane foods have increased dramatically, and terms such as “nutraceuticals”, “pharmafoods” and “functional foods” are now being used regularly (Etkin & Johns,

1998). Indian and Tibetan cultures, among several others (Etkin & Ross, 1982; Wilson, 1977), have long identified diet as an important component of health, and as such, prescribes dietary modifications concurrently with medicines.

However, Ayurvedic medicine, has long understood spices as important therapeutic agents (Handa, 1998) that either support primary ingredients or are primary constituents themselves. The World Health Organisation (WHO) has estimated that 65-80% of the world's population use traditional medicine as their primary health care. Further to this herbal medicine or pharmaceuticals from plant origins represent the majority of this health care and their practice are growing especially in developing countries (Drew & Myers, 1997). The use of ethnomedicines which deals with the study of traditional medicines (e.g. Traditional Chinese Medicine, Ayurveda) or knowledge and practices that have been orally transmitted over the centuries has also increased in Western countries, as alternative medicines to treat various conditions and diseases. Parallel with their usage, safety concerns with such medicine has also increased and committees and bodies were established to tackle this safety issue.

In the UK, the Medicines and Healthcare products Regulatory Agency (MHRA) play significant roles in ensuring that herbal medicines marketed in UK are acceptably safe.In the U.S., safety concern on use of herbal medicines is regulated under U.S. Food and Drug Administration (FDA) and also a body called the National Center for Complimentary and Alternative Medicines (NCCAM) (Tilburg and Kaptchuk, 2008). European countries also have legislation in controlling the entrance of herbal medicines to the market as framed in their European Directive (Steinhoff, 2002). In Malaysia the

safety of herbal medicines or pharmaceuticals from plants is regulated under a government agency, National Pharmaceutical Control Bureau (NPCB) which is also a WHO collaborating Centre for Regulatory Control of Pharmaceuticals. Popular belief has regarded that anything 'natural is safe'. Houghton stressed the issue that even in 'predicament plant' (which refers to plants affecting the central nervous system), correct dose and use provide useful pharmaceuticals (Houghton, 2003).

As most of the time herbal medicines are supplied as dietary supplements or without prescription, they should be used with caution, as many common herbal medicines used in irregular, high doses or in combination with other medications, may pose toxic effects. The toxic effects can range from allergic reactions to cardiovascular, hepatic, renal, neurological and dermatological effects (Pharmar, 2005). Sometimes the herb itself is not toxic, however if adulteration occurs during preparation or processing (e.g. by heavy metals), toxic effects may be exhibited such as poisoning by the Chinese herbal medicine podophyllum (But et al., 1996). A most recent adulterated herbal preparation is the decoction leaves of *M. speciosa* which is known as "ketum juice in the northern region of Peninsular Malaysia. While "ketum" was used to increase physical endurance and work capacity, there are reports that "ketum juice" was consumed by addicts as a substitute of more expensive opiates to manage withdrawal symptoms (Assanangkornchai et al., 2007; Vicknasingam & Narayanan, 2009)

"Ketum" is consumed both in unadulterated and adulterated form by mixing decoction leaves with cough mixtures, traditional herbs and even synthetic pyrethroid

from mosquito coils to add zest but this could result in dreadful consequences . (Bothiphon et al., 2009; Assanangkornchai et al., 2007) .

In another instance, *Zingiber officinale*, *Piper longum* and *P. nigrum* were used in equal parts as Ayurvedic drug for the treatment of respiratory disorders, skin diseases, fat metabolism disorders and filariasis. However biochemical studies indicated that *P. longum* and *P. nigrum* considerably enhanced the bioavailability of bioactive principles in other medicines (Handa., 1998; Atal et al., 1981). As such the merits of herbal medicines needs further exploration in view of their possible synergistic, additive and/or antagonistic effects which may result as different components interact. On the other hand it is interesting to note that in ethnophamacology research the practical applications and contribution can be viewed variably. Some researchers suggest that the use of natural compounds in biomedical and synthetic design of compounds modeled on natural products could move forward ethnopharmocologic research in the areas of biomedicine therapeutics global health (Akerele, 1987; Oyeneye, 1985; Farnsworth, 1993; Phillippson & Anderson, 1989).

In developing nations over 80% of the people, particularly rural populations, rely on medicinal plants as part of their primary health care where biomedicines are difficult to obtain (Farnsworth, 1993). The use of plants with medicinal values according to original and biomedical standards can be considered in lieu of comparably effective pharmaceuticals. Licorice (*Glycyrrhiza glabra*), myrrh (*Commiphora species*) and poppy capsule latex (*Papaver somniferum*) are plants first reported for such a practice .Morphine, codeine, noscapine (narcotine) and papaverine are still clinically used

chemical entities derived from opium plant, *P. somniferum* (Newman et al., 2000). This *P. somniferum* plant has a narcotic property which mainly affects the central nervous system (CNS) function and their use as potent pain killers for severe pain has made this plant a basis for clinical use drug. However addiction is a major side effect of using such drugs (Vetulani, 2001). At present choice of drugs that are proven to be as good as morphine for chronic pain management are very limited. Another psychoactive compound with good analgesic effect and potential in treating neurological illnesses is cannabinoid derived from *Cannabis sativa* (Watts, 2004; Fernadez-Ruiz et al., 2007).

However its narcotic, addictive and high adverse effects has limited its use and thus made it illegal in most countries. The cannabis plant is well known as marijuana, ganja and has many other street names and is widely abused as a recreational drug (Watts, 2006). Other alternatives to morphine drugs such as nalbuphine, pentazocine and butharphanol are clinically available but their actual analgesic properties remain disputed (Science Daily, 2000). Other specific plants such as Ephedra or Ma huang (*Ephedra spp*), which are good nasal decongestants regularly used by the public and have direct and indirect effects on the central nervous system, can also stimulate CNS side effects from nervousness to imsomnia; Valerian (*Valeriana officinalis L*.) which has long use and a reputation as a sleep aid and a mild tranquiliser has also been shown to produce CNS depression (Tyler, 1999). Gingko (*Gingko biloba L*.) a breakthrough herb in the late 1990's, was found to be effective in improving cognitive performance and social functioning of demented patients, with a lower incidence of side effects; St. John's Wort *(Hypericum perforatum L*.), a well-liked herbs in the U.S. is shown to be useful in treating mild to moderate depression states; kava (*Piper methysticum G. Forst*)

is effective in treating conditions such as nervous anxiety, stress and restlessness due to its narcotic-like effects and devoid of addictive properties.

There are many more drugs derived from plants, which are successfully established as pharmaceuticals which is not covered in this section. Scientific research in ethnomedicine is ongoing and is growing rapidly especially in countries like Malaysia which has an abundance of natural resources. One of the plants which require immediate attention is *M. speciosa* . It is widely abused for its narcotic-like effects when smoked, chewed or taken in its decocted form known as "Air Ketum". Despite being widely abused there are also numerous reports on its medicinal values such as anaesthetic, antinociceptive, analgesic and psychostimulant effects (Jansen & Prast 1988; Macko et al., 1972 ; Matsumoto et al., 1996 ; Perry, 1980; Tsuchiya et al., 2002). *M. speciosa* leaf are known for its rich alkaloid chemical constituents and MG is the main alkaloid constituent of the leaf, responsible for many of the plant's pharmacological effects. One the other hand the presence of chemical constituents other than alkaloids is not well documented to explain claims on the anti-infective properties of *M. speciosa* leaf. In this thesis a portion of work was undertaken to determine the presence of flavonoids and polyphenols chemical constituents in *M. speciosa* leaf and their possible antimicrobial and antioxidant activities were investigated as well.

2.2 The plant *M. speciosa* Korth

2.2.1 Description of the plant *M. speciosa*

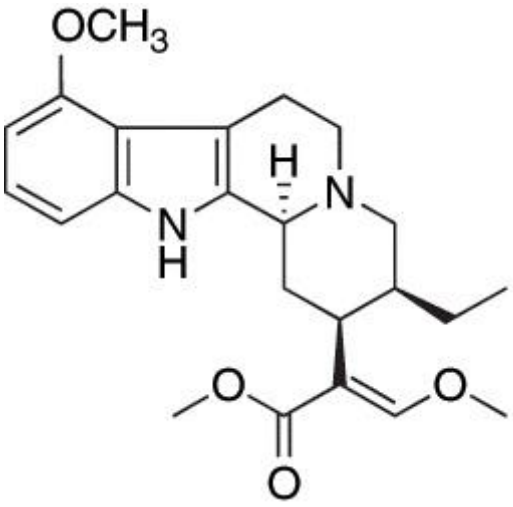
M. speciosa Korth is an indigenous tropical herb plant belonging to the family of Rubiaeceae (Coffee family) and is found mainly in Southeast Asia countries such as Malaysia, Thailand, Myanmar etc. In Malaysia it is commonly known as 'ketum', biak or 'biak-biak' and is native in the northern and west coast part of Peninsular Malaysia. In Thailand, this plant is named as 'kratom', 'thom', 'kakuam' or 'ithang' but globally this plant is recognized as ' kratom'. The genus of this plant 'Korth' is named after a botanist William Korthal who found the stigma of its flower resembling a bishop's mitre (Shellard, 1974). This plant is a large leafy tree, which can grow up to 15 metres tall. The leaves can grow over 5-7 inches long and 3-4 inches wide and dark green in colour with yellowish flowers (Shellard, 1974) (Figure.2.1A and B). This plant has two types which can be distinguished by the leaves with either red or white-greenish petiole (vein). The leaves with petiole (vein) white-greenish were suggested to have stronger effects (Suwarnlet, 1975; Murple, 2006). In this dissertation white-greenish vein type of *M. speciosa* leaves were used for pharmacological evaluation.

Figure 2.1 A: Young plant of *M. speciosa* Korth. The image was taken from http://www.entheology.org.15th July 2010

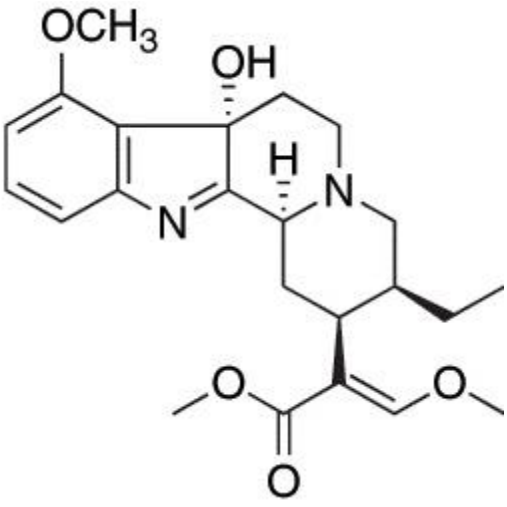
Figure.2.1 B: The branch of *M. speciosa* Korth leaves with flowers. The image was taken from http://www.erowid.org. 15th July 2010

2.2.2 Phytochemistry of *M. speciosa*

MG is the major alkaloid present in the leaves of this plant (Figure 2.2A). The name mitragynine was given by Field who repeated the isolation in 1921 which was first done by Hooper (Shellard, 1974). To date, over 25 alkaloids have been isolated and chemically elucidated especially from the leaves of the young plant (Houghton & Ikram, 1986). Among the well-studied alkaloids, apart from MG, which are present in Malaysian plants are, 3,4-dehyromitragynine, mitragynaline, corynantheidaline, mitragynalinic acid and corynantheidalinic acid (Houghton et al., 1991; Takayama, 2004), while other found alkaloids are speciogynine, speciociliatine, paynanthiene and newly 7-hydroxymitragynine (Figure 2.2 B) (Ponglux et al., 1994; Takayama, 2004); MG is the dominant constituent of this plant and constitute more or less 0.2% by weight in each kratom leaf and approximately 17 mg in 20 leaves (Grewal, 1932b; Suwanlert, 1975). Within each country geographical location and season, there is a quantitative difference in alkaloid content (Shellard, 1974). The alkaloid content of the leaves of *M. speciosa* has been reported as approximately around 0.5% to 1.5% in leaf. The leaf weigh approximately about 1.7 grams fresh or 0.43 grams dried (Suwanlert,1975). It was reported that the Thai and Malaysian plant had the alkaloids mitragynine, speciogynine, speciociliatine, paynantheine and 7-hydroxymitragynine in common. In both plants, mitragynine was the most abundant alkaloid, but in Thailand the total alkaloid was 66% whereas it was only 12% in the Malaysian species (Takayama, 2004). This proofs that the content of the alkaloids varies between countries and also the season of the plant (Shelard, 1974).



(A)



(B)

Figure.2.2 A) Chemical structures of mitragynine (MG), and B) its congener, 7-Hydroxymitragynine present in the leaves of *M. speciosa* Korth.

2.2.3 Biological activity.

M. speciosa Korth plant especially its leaves have been consumed since ancient times, where village people such as farmers and labourers chew the fresh leaves, smoke the dry leaves or drink as a tea suspension (Jansen & Prast, 1988) or even eat it in the form of pellets, for stimulant effects to endure the load of tough labor under the burning sun. The leaves and the bark of this plant have been reported for its opium like properties and a cure for opium habit (Shellard, 1974; Jansen & Prast (1988). In addition the use of kratom as a wound poultice, cure for fever and as a suppressor of the opiate withdrawal syndrome has been recorded (Burkill, 1930.) In humans this plant exerts a stimulant effect at low doses and sedative and analgesic effects at the higher doses suggesting the presence of dual opioid properties (Grewal, 1932b; Suwarnlet, 1975). Similar effects have also been observed in animal models as reported by Macko et al., (1972).

Pharmacology activities of kratom and its dominant alkaloid, MG have long been reported and reviewed since the 1970's. In rodents, upon oral, subcutaneous and intraperitoneal administration MG was reported to exert antinociceptive and anti-tussive effects (Macko et al., 1972). The crude methanol (MeOH) extract of Thai kratom was used in in vitro assay (twitching contraction induced by electric stimulation of guinea-pig ileum preparation) in which the opioid antagonist, naloxone successfully inhibited the contraction, implying that the crude extract is an opioid agonist (Takayama, 2004; Watanabe et al., 1992). Several in vitro and in vivo studies followed and support the analgesic properties of both crude extract and MG such as reported by Matsumoto et al.,

(1996), Watanabe et al., (1997) and Idid et al., (1988). Crude extract and MG were also reported to successfully act mainly via supraspinal μ, δ and κ opioid receptors (Tsuchiya et al., 2002; Tohda et al., 1997; Thongpradichote et al., 1998) in various *in vitro* and *in vivo* studies. Recently, a minor constituent of this plant and congener for MG, 7-hydroxymitragynine was found to potently exhibit antinociceptive activities via opioid receptors, mainly μ-receptors in in vitro studies, which interestingly showed 13-fold and 46-fold higher activity than morphine and MG respectively. This promising finding is further supported by an in vivo study in mice, which again showed higher antinociceptive activity than morphine (Matsumoto & Horie, 2004). Based on these findings, it was claimed that 7-hydroxymitragynine could be the active principle for the antinociceptive effects exerted by this plant (Takayama, 2004).

It was reported that chewing the leaves has greater effect for lower doses of MG properties (Grewal, 1932b) and neuropsychiatric effects could be achieved within 5 to 10 minutes post consumption and would last up to 1 hour (Grewal, 1932b; Suwarnlet, 1975). In *in vivo* studies, it was reported that MG doses as high as 920 mg/kg have been administered without overt clinical effect (Macko et al., 1972). With regards to the clinical use in humans, the doses for the stimulant effects, the antinociceptive events and the toxicity effects are yet to be fully established (Babu et al., 2008). Some tolerance effects have been reported among users and clinical effects such as antitussive, antinociceptive and anti-diarrhoeal effects of MG use was also described to be similar to codeine (Suwarnlet, 1975; Jansen & Prast, 1988). Besides its analgesic and narcotic-like pharmacological effects, several side effects have been reported among kratom users such as nausea, vomiting, diarrhoea, nystagmus and tremor (Grewal, 1932b) and for

chronic users anorexia, weight loss, hyperpigmentation and prolonged sleep were observed (Suwanlert, 1975). Opioid abstinence syndrome such as irritability, yawning, rhinorrhoea, myalgias, diarrhoea and arthralgia were reported in some kratom users thus suggesting possible addictive properties of this plant (Thuan, 1957; Suwarnlet,1975; Babu et al., 2008). Recently, major concern has arisen in Malaysia as the narcotism properties of this plant have attracted the misuse of it by drug addicts as an opium substitute.

Due to this, an act was passed in 2004 (under the Poison Control Act 1952) which makes the possession of any form of the plant by the public illegal. It is an offence under Section 30(3) of the Poisons Act 1952 to possess and sell ketum leaves in Malaysia. In fact, Thailand has legislated this plant since 1946. Australia also followed to criminalise the possession of this plant in 2005. However, in other parts of the world, kratom is currently not scheduled. The availability of kratom over the internet has attracted many Western populations to use the plant as self-treatment in opioid withdrawal and chronic pain (Boyer et al., 2007). Though past reports described the medical use of this plant to replace morphin in addicts' detoxification and treatment programme, the pharmacological profiles of this intriguing alkaloid MG and the kratom leaf have yet to be fully studied. It is also important to note that the leaves contain several other substances that may modify the effects of the drug (Thuan, 1957).

The way that MG work in the human body is not clearly defined and this bioactive that account for effects and the mechanisms of actions are generally not well researched and understood owing to limited analytical tools to detect and assay MG in

biological fluids. Therefore the core research in this thesis undertook prime work on developing a simple rapid HPLC-UV method for assay of MG in plasma for application in detailed MG pharmacokinetic studies in rats.

2.3 Method Development and Validation in HPLC

2.3.1 Introduction

Qualitative methods of analysis provide basic information about the composition of a sample and perform quite simple chemical reactions to identify the analytes it contains (Burriel et al., 1989). Quantitative methods of analysis provide information not only about the composition but also about the concentration of the analytes present in the sample and, generally speaking, they often require more complex analytical techniques to obtain more accurate and reliable information about the sample. Quantitative and qualitative study of substances in biological fluids such as plasma, serum, tissue or urine is indispensable in bioanalytical chemistry. Pharmacokinetics, bioequivalence and bioavailability studies understanding largely depend on this significant analytical study (Bressolle et al., 1996). The main analytical phases are method development, validation and method appliance.

The vast development of analytical methods among the global community has marked acceptance internationally. As such, to declare a universal level of excellence, the use of validated methods is crucial (Hartmann et al., 1998). In analytical chemistry no matter what way the analysis is made it must be checked to distinguish whether it works as proposed. Each methodology must be investigated to determine the extent to which atmosphere, matrix, or technical variables can affect the assessment of analyte in

the matrix from the time of collection up to the time of analysis. Analytical methods are generally used for product improvement, product research, procedure control and for chemical quality control reasons. In chromatographic or spectroscopic analysis each of their procedure, has its own uniqueness and shortage that has to be addressed.

2.3.2 Method development

An analytical method includes procedures for analysis of an analyte in biological matrices such as blood, plasma, serum etc starting from time of sample collection, processing and storing (Shah et al., 1992). Bioequivalence and pharmacokinetics assessment studies primarily rely on a quantitative determination of analyte and metabolites in biological fluids with good reproducible and accuracy of data (Shah et al., 2000).

Assessment and optimization of the different steps in sample preparation, detection, chromatographic separation, and quantification are crucial steps in method development. The selection of instruments (eg; HPLC, GC, LCMS) that are suitable for the analysis of compound(s) of interest is first to be considered. After that the choice of analytical column, detector, and selection of appropriate mobile phase are essential for the analytical method to be thoroughly developed and optimized prior to validation. The objective of validation of analytical procedure is to demonstrate that the method is suitable for the intended purpose. An efficient extraction procedure which gives the maximum recovery without endogenous interference with adequate accuracy and precision are preferred in assay development. In accordance to FDA Guidance, the recovery of an analyte is defined as “the extraction efficiency of an analytical process,

reported as a percentage of the known amount of an analyte carried through the sample extraction and processing steps of the method. The selection of internal standard with similar chromatographic properties to analyte of interest is crucial in method development. At times an internal standard is added at a known concentration directly to an analytical sample to assist in quantification. The amount of analyte present is then determined relative to the internal standard as a calibrant (Causon, 1997; Karnes et al., 1991).

2.3.3 Method validation

Method validation is a process involving method documentation and continued application of the analytical method for the intended purpose. (Bruce et al., 1998 ; Hartmann et al., 1998). The validation procedure includes assessment of fundamental validation parameters such as precision accuracy, recovery, selectivity, sensitivity, stability and the calibration curve (Shah et al., 1992). Validation exercise demonstrates that the analytical method is repeatable, specific and suitable for its intended application and in the case of biomedical application it is crucial to support the registration of a new drug or a new formulation of an existing one (Wieling et al., 1996; Causon, 1997). Both the HPLC-UV methods for assay of MG in plasma and plant extracts described in this dissertation were optimized and validated using the above mentioned parameters prior to their application in pharmacokinetics and pharmacological studies. This is to ensure the data generated are reliable and valid for a useful pharmacokinetic evaluation of MG in animal model and to standardize *M. speciosa* extracts for pharmacological studies.

2.3.3(a) Full Method validation and Partial Method validation

Full validation is performed when developing and applying a bioanalytical procedure for the first time for its use in pharmacokinetic, bioavailability, and bioequivalence and drug interaction studies in a new drug application (NDA) (Shah et al., 2000). Partial validations are carried out when there is a departure or modification in the present validated bioanalytical method. Partial validation could range from one intra-assay and precision determination to an almost full validation. Modification in analytical methodology, transfer of bioanalytical method between laboratories or analyst, change of matrix within species, change of species within matrix will result in partial validation (Shah et al., 2000; FDA, 2001). The choice of parameter to be revalidated is determined by the constraint likely to be affected by the change made to the bioanalytical method. In this thesis MG HPLC analytical method was developed and validated in human plasma for application in rat pharmacokinetic studies. Partial validation using rat plasma was carried out for inter-day and intra-day precision determination.

2.3.3(b) Cross validation

When two or more bioanalytical methods are used to generate data within the same study or across different studies cross validation of analytical parameters are required. In a cross validation the original validated bioanalytical method serves as the reference and the revised bioanalytical method is the comparator (Shah et al.,2000; FDA 2001).

2.3.4 Limits of detection and quantification (LLOD and LLOQ)

US pharmacopoeia (USP) defines the limit of detection (LOD) as the lowest analyte concentration in a sample which can be detected but not necessarily quantified. The lowest limit of quantification (LLOQ) is defined as the lowest analyte concentration in a sample that can be measured, with precision and accuracy within the acceptable range. (Krull & Swartz, 1998). Signal-to-noise ratio is defined as the ratio between a signal (meaningful peak) and the background noise. Using visual inspection approach, the signal is calculated from the base line to peak summit and divided by the peak-to-peak noise, which is determined from the blank plasma injection. Signal-to-noise ratios of approximately 2:1 or 3:1 and 10:1 were used in estimating the detection limit and quantification limit respectively (Figure3.0). Alternatively LOD and LOQ are calculated in terms of the mean and standard deviation (SD) from a series of blank samples (ie., LOD and LOQ = mean ± kSD). In estimation of LOD, k is equal to six. For LOQ k is equal to ten when maximum allowed SD of 10% is desired (Green et al., 2002).

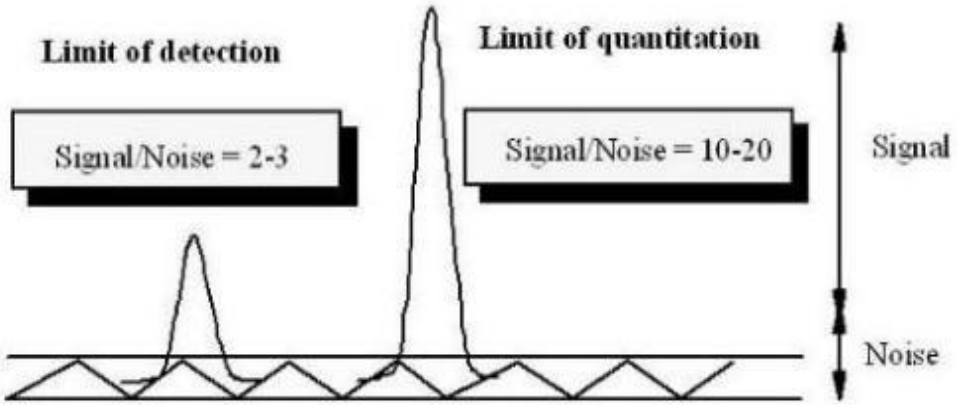


Figure 2.3: Limit of detection and limit of quantitation via signal to noise(ICH,1996)

2.3.5 Calibration curve

A calibration curve shows the relation between the analyte concentration in the sample and the detected response. To define adequately the relationship between response and concentration it is crucial to use a sufficient number of calibration points (n=6) (Shah et al.,1992). Linear regressions of the response (peak height/area(Y)) of the analyte are plotted against the corresponding analyte concentration (X) to establish the slope, intercept and coefficient of correlation. The difference between the observed y-value and fitted y-value is called a residual. In linear regression analysis one of the assumptions is that the calculated residuals are independent, are normally distributed and have equal variance, which is termed as homoscedasticity or if otherwise termed as heteroscedasticity. If the variance is not equal, then the weighted regression may be performed. The most appropriate weighting factor is the inverse of the variance of the standard, although 1/x, 1/x2, 1/y and 1/y2 (x = concentration and y = response) are suitable approximations. However, the selection of the right statistical model for the evaluation of the calibration curve must be scientifically justified if regression type is

changed without formal revalidation during sample analysis (Lang & Bolton, 1991; Chow, 2009).

It is a standard practice in an analysis that a standard curve should cover the entire range of the concentration of the unknown samples. It is not recommended to estimate the concentration of the unknown by extrapolation of the standard curve below the lower or higher calibration points. As an alternative, it is suggested that the standard curve be re-determined or sample re-assayed after dilution (Shah et al., 1992). Dadgar et al., (1995), in his guidelines suggested several exclusion criteria for omitting calibration points provided at least seven non-zero calibration points are used to construct calibration curve. At least up to two non-zero standards may be removed from the calibration for the following valid reasons : (i) loss of sensitivity in processed samples, (ii) sample loss during processing or (iii) poor chromatography peaks during analysis . Acceptability of the linearity data is often judged by examining the correlation coefficient and y-intercept of the linear regression line for the response versus concentration plot. A correlation coefficient of > 0.999 is generally considered as evidence of an acceptable fit of the data to the regression line (Green, 1996).