EVALUATION OF RAT *IN SITU* INTESTINAL

PERMEABILITY STUDY OF MITRAGYNINE

by

J.D.YUVENESAN JAGABALAN

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LIST OF ABBREVIATIONS AND SYMBOLS

ABC	ATP binding cassette
ACN	Acetonitrile
ANOVA	Analysis of Variance
AT	Atenolol
BCS	Biopharmaceutical Classification System
COX	Cyclo-oxygenase
cm	Centimeter
CV	Coefficient of variation
Da	Dalton
DMSO	Dimethyl sulfoxide
FDA	Food and Drug Administration
GIF	Gastrointestinal fluid
GIT	Gastrointestinal tract
gm	Gram
KCL	Potassium chloride
KH_2PO_4	Potassium dihydrogen phosphate
Kg	Kilogram
LOD	Limit of detection
LOQ	Limit of quantification
MDR	Multidrug resistance
M	Molar
MG	Mitragynine
mg	Milligram
min	Minutes

mL	Milliliter
mM	Millimolar
mm	Millimeter
NaCl	Sodium chloride
NaH_2PO_4	Sodium dihydrogen phosphate
Na_2HPO_4	Disodium hydrogen phosphate
nm	Nanometer
NWF	Net water flux
P_{eff}	Permeability coefficient
P-gp	P-glycoprotein
PROP	Propranolol
QC	Quality control
RP-HPLC	Reverse phase- high performance liquid chromatography
rpm	Rotation per minute
RSD	Relative standard deviation
SD	Standard deviation
SEM	Standard error of the mean
SIF	Simulated intestinal fluid
SPIP	Single pass intestinal perfusion
UK	United Kingdom
USA	United States of America
UV	Ultra violet
WHO	World Health Organization
W/V	Weight in volume
μL	Microliter

PENILAIAN KEBOLEHTELAPAN MITRAGININA MENGGUNAKAN USUS TIKUS *IN SITU*

ABSTRAK

Alkaloid utama dalam daun *M. speciosa*, mitraginina adalah bertanggungjawab terhadap kebanyakan kesan farmakologi yang telah dilaporkan. Dalam kajian yang telah dijalankan ke atas mikrosom hati, alkaloid ini didapati stabil. Walau bagaimanapun, bioketersediaan mitraginina telah dilaporkan sangat rendah. Kadar penyerapan oral mitraginina yang rendah ini mungkin disebabkan oleh sifat keterlarutan air yang rendah. Di samping itu, kebolehtelapan usus, enzim sitokrom P450 3A4 (CYP3A4), dan sistem eflux p-glikoprotein (P-gp) berperanan penting dalam bioketersediaan oral mitraginina. Oleh yang demikian, penyelidikan ini telah dijalankan untuk menentukan beberapa ciri penyerapan oral dengan menggunakan model perfusat usus tikus *in situ*. Satu kaedah HPLC-UV yang baru telah dibangunkan untuk pengesanan serentak mitraginina, propranolol dan atenolol untuk kuantifikasi mitraginina dalam sampel perfusat usus tikus *in situ*. LOQ bagi mitraginina, propranolol dan atenolol adalah masing-masing 2.5, 3.75 dan 3.75 µg/mL. Kejituan dan ketelitian kaedah HPLC-UV bagi mitraginina, propranolol dan atenolol masing-masing adalah di bawah 5%. Mitraginina telah didapati stabil dalam asetonitril dan penimbal perfusat pada suhu -20^oC selama satu bulan. Kestabilan juga tidak terjejas apabila sampel terdedah di meja makmal selama 8 jam dan selepas tiga kitaran beku dan cair. Keterlarutan mitraginina dalam penimbal perfusat adalah kurang baik (~40µg/mL). Mitraginina menunjukkan kebolehtelapan yang tinggi dalam usus (P_{eff}) (1.11 x 10-4 cm / s), iaitu setanding dengan penanda kebolehtelapan tinggi propranolol (P_{eff}) (1.27 x 10-4 cm / s). Tidak ada perubahan yang signifikan

(p>0.05) dalam nilai min kebolehtelapan (P_{eff}) mitraginina apabila ia diberi bersama perencat P-gp dan/atau CYP3A4. Oleh itu, mitraginina mungkin tidak tertakluk kepada penyingkiran pra-sistemik yang berkaitan dengan P-gp dan enzim CYP3A4. Kajian ini menunjukkan bahawa mitraginina (kelarutan rendah & kebolehtelapan yang tinggi) boleh diklasifikasikan sebagai drug kelas II mengikut sistem klasifikasi biofarmaseutikal (BCS). Sifat penyerapan oral mitraginina yang telah didapati dalam kajian ini boleh membantu untuk memberikan penyerapan yang mencukupi untuk mewujudkan formulasi oral mitraginina bagi kajian klinikal pada masa depan.

EVALUATION OF RAT *IN SITU* INTESTINAL PERMEABILITY STUDY OF MITRAGYNINE

ABSTRACT

Mitragynine the principal alkaloid of *M. speciosa* leaf has been reported to be responsible for most of its pharmacological effects. Mitragynine has been found to be metabolically stable in liver microsomes. However the oral bioavailability of mitragynine has been reported to be very low. The poor mitragynine oral absorption was due but not limited to, its poor water solubility. It is also important to note that the role of intestinal permeability, cytochrome P450 3A4 enzyme (CYP3A4) and p-glycoprotein (P-gp) efflux system on its oral bioavailability could not be ruled out as well. In view of this, work was undertaken to determine some of its oral absorption properties using *in situ* rat intestinal perfusate model in rats. For mitragynine quantification in *in situ* rat intestinal perfusate samples, a reliable HPLC-UV analytical method for simultaneous detection of mitragynine, propranolol and atenolol was developed and validated. The LOQ of mitragynine, propranolol and atenolol were 2.5, 3.75 and 3.75 µg/mL respectively. The within day and day to day accuracy and precision for mitragynine, propranolol and atenolol were all below 5% respectively. Mitragynine is stable in acetonitrile for period of one month when stored in freezer (-20°C). All the analytes have been found to be stable in perfusion buffer upon storage at -20 °C up to one month. The stability was not affected after the samples were subjected to three freeze and thaw cycles and when kept on the bench for 8 hours. Mitragynine is poorly soluble in perfusate buffer (~40µg/mL). Mitragynine demonstrated high intestinal permeability (P_{eff}) (1.11×10^{-4} cm/s) and was comparable to that of the high permeable marker propranolol (P_{eff}) (1.27×10^{-4}

cm/s). There was no significant alteration ($p > 0.05$) in the mean permeability values (P_{eff}) of mitragynine when P-gp and/or CYP3A4 inhibitors were co-administered, thus suggesting the drug is not subjected to pre-systemic elimination related to a P-gp and CYP3A4 system. This study suggests that mitragynine (low solubility & high permeability) can be classified as class II drug according to biopharmaceutical classification system (BCS). The mitragynine oral absorption properties determined in this study could further assist the development of mitragynine oral formulation which would provide adequate absorption for clinical investigations in the future.

CHAPTER 1

INTRODUCTION

For millennia, when it comes to providing medically useful compounds for Mankind's needs, the Plant kingdom has been the oldest source, offering pharmacologically active substances (Cordell, 1981). Plants have been used by the human beings over thousands of years for the treatment of various diseases (Sofowara, 1982; Hill, 1989). Reports from World Health Organization, suggest that most people in many part of the world still rely on conventional medicines for their health benefit necessities. Rural communities worldwide still seek the need of conventional medicines and have become an integral part of their primary health care (Rabe & Van Staden, 2000). Traditional medicines are easily accessible and much cheaper when compared with synthetic or modern medicine (Iwu *et al*., 1999; Mann *et al*., 2008). Some people from their personal experience understand that a traditional remedy is valuable in maintaining human health. They know that some medicinal plants are highly effective when used at therapeutic doses without knowing the science behind these medicines (Maheshwari *et al*., 1986; Van Wyk *et al*., 2000). Research on many mainstream medicines has revealed that these medicines contain numerous compounds which have been derived from "ethnomedical" plant sources (Fabricant & Farnsworth, 2001).

Increasing demand on herbal medicines in developed and developing countries explain that it act as a source of primary health care owing to their medicinal property, high safety margins and lesser operational costs when dealing with them. This includes Malaysia, which is rich with a diverse quantity of medicinal plants. However, most of these medicinal plants have not been extensively exploited

or investigated (Jamal, 2006). Among the promising medicinal plants, *Mitragyna speciosa* Korth (*M. speciosa*) should be given more attention for its valuable medicinal properties and its traditional usage in treating various diseases.

Mitragyna speciosa Korth belongs to the family Rubiaceae and it is found in the tropical and sub-tropical regions of Asia. This tropical plant is known as "Biak-Biak" in Malaysia and as "kratom" in Thailand. Red vein and green vein are the two types of kratom found in Thailand. Stronger biological activities have been observed in red vein variety when compared with the green vein (Chittrakarn *et al*., 2008). Traditionally ketum has been used as a substitute for opium in Thailand and Malaysia. Ketum leaves are consumed fresh or make as dried leaf powder which is either swallowed or prepared as tea (Chittrakarn *et al*., 2008). Ketum has been the traditional cure for the bacterial related stomach ailments such as diarrhea (Hassan *et al*., 2013). In Malaysia, it is consumed both in adulterated and unadulterated form by mixing it with cough mixtures, traditional herbs and even synthetic pyrethroid from mosquito coils to add zest to the concoction. The adulterated decoction of *M. speciosa* leaves, known locally as "Ketum juice" is packed into plastic bottles or packets and is found being sold in the streets of Peninsular Malaysia, particularly in the Northern region. In this region "ketum juice" is consumed by addicts as a substitute for the more expensive opiates in order to manage withdrawal symptoms.

Ketum has been reported to be a central nervous system stimulant rather than a depressant. It also enhances work efficiency and tolerance to hard work under the heat of the sun (Suwanlert, 1975). In addition to this, Burkill and Haniff (1930) documented other uses of ketum, being for the healing of wounds and as a cure for fever. Based on what has been mentioned about *M. speciosa*, it is opined that this plant warrants a more extensive investigation of its pharmacological properties. *M.*

speciosa has been under investigation since 1986 in Malaysia. Thailand, Myanmar, Malaysia, Vietnam and Australia are among the countries which declared ketum usage is illegal. However, free accessibility and information on ketum is widely available over the internet. This has fascinated many Westerners to exploit the plant as self-treatment in opioid withdrawal and pain management (Boyer, 2007).

Among the 25 alkaloids, mitragynine is the major alkaloid isolated from ketum leaves. In 1907, the isolation of mitragynine first performed by David Hooper followed by E. J. Field in 1921. The name "mitragynine" was given by E. J. Field. Mitragynine has an antinociceptive effect through an action on supraspinal opioid receptors and descending noradrenergic and serotonergic systems (Matsumoto *et al*., 2005b).

Since mitragynine is the major alkaloid derived from ketum leaves which have many medicinal values, it is important to conduct a study on its oral bioavailability. Oral bioavailability of a drug is the determination of the fraction of a drug that reaches the blood stream when it administrated orally. Parthasarthy *et al* (2010) reported a poor oral bioavailability for MG in rats (3%). In most recent studies, MG was reported for its poor water solubility ($< 100 \mu g/mL$) and found to be acid degradable. MG is both hydrophobic and lipophilic in nature. MG poor bioavailability was constantly attributed to the drug's sub-optimal physicochemical properties (Ramanathan *et al* 2015). MG is metabolically stable in both human liver microsomes and S9 fractions (Manda *et al*., 2014).

1.1 Problem statement

It is of important to note that the role of CYP3A4, P-gp efflux system and MG intestinal permeability in effecting drug oral bioavailability could not be ruled

out. As off to date there are no studies reported on MG intestinal permeability and its pre-systemic drug elimination. In view of this, work was undertaken to determine MG intestinal permeability coefficient using a validated rat intestinal *in situ* perfusate model. MG permeability was also investigated in the presence of P-gp efflux system and CYP3A4 inhibitors.

1.2   Expected outcome

With the data obtained from solubility and permeability experiments carried out in this thesis, MG can be classified in accordance with Biopharmaceutical Classification System (BSC).

1.3 Objectives

- To develop and validate an HPLC method for simultaneous determination of mitragynine, atenolol and propranolol in intestinal rat perfusate.

- To determine stability of mitragynine in intestinal rat perfusate.

- To determine the mitragynine permeability using *in situ* intestinal rat perfusion model.

- To determine the mitragynine permeability using *in situ* intestinal rat perfusion model in the presence of p-glycoprotein inhibitor and/or cytochrome P450 A3 inhibitor.

The research scheme is presented in figure 1.1.
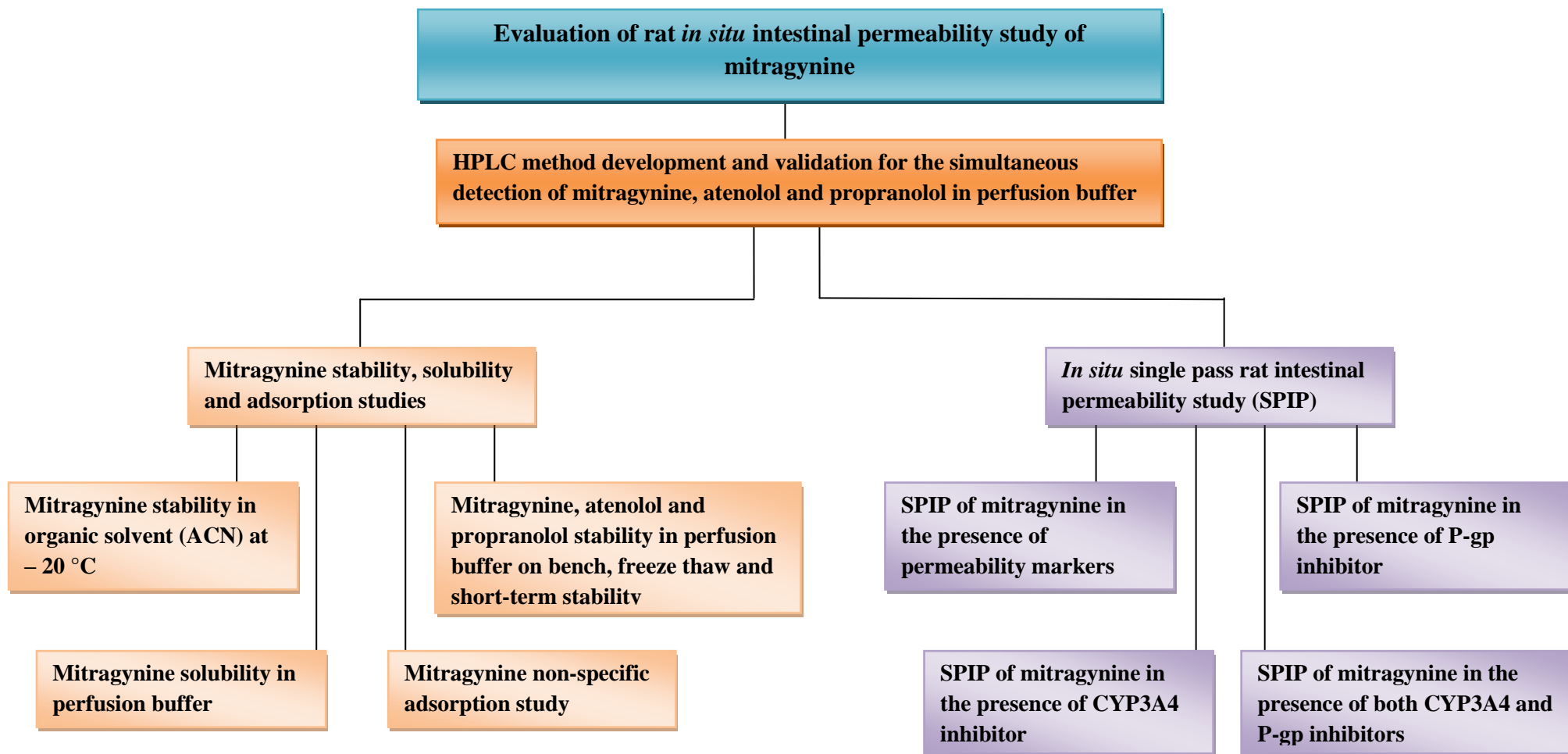
Figure 1.1 Flow chart of the

Evaluation of rat *in situ* intestinal permeability study of mitragynine

HPLC method development and validation for the simultaneous detection of mitragynine, atenolol and propranolol in perfusion buffer

Mitragynine stability, solubility and adsorption studies

Mitragynine stability in organic solvent (ACN) at − 20 °C

Mitragynine, atenolol and propranolol stability in perfusion buffer on bench, freeze thaw and short-term stability

Mitragynine solubility in perfusion buffer

Mitragynine non-specific adsorption study

***In situ* single pass rat intestinal permeability study (SPIP)**

SPIP of mitragynine in the presence of permeability markers

SPIP of mitragynine in the presence of P-gp inhibitor

SPIP of mitragynine in the presence of CYP3A4 inhibitor

SPIP of mitragynine in the presence of both CYP3A4 and P-gp inhibitors

CHAPTER 2

LITERATURE REVIEW

2.1   The role of plants as sources of herbal medicine

Plants have been used for health and medical purposes since ancient times. The number of higher plant species on earth is about 250 000. It is estimated that in some cultures, 35 000 to 70 000 species have, at one time or another, been used for medicinal purposes (WHO, 1998). Silano *et al*. (2004) reported that herbal substances composed of one or more bioactive substances are regarded as herbal medicines. These herbal substances may be present in whole plant or particular parts of the plant such as the root, stem, leaf, flower, fruit and bark of the plant.

Herbal medicines still has been the first-line of treatment for the majority of population around the globe. This is because herbal medicines are main accessible and reasonable treatment for the poor people and people living in the rural areas. Besides that, people also prefer herbal medicines due to high cost of modern treatments, lack of drug supply, side effects and resistance development nature of several allopathic drugs.

Automated separation techniques, high-throughput screening, combinatorial chemistry and biological activity guided isolation techniques are the important elements in drug discovery derived from natural product. There are many examples of new drugs derived from plant-based products such as morphine and digoxin. Poppy plant (*Papaver somniferum*) is the main producer of opium and morphine is isolated from it. *Digitalis lanata* is a flower that produces digoxin which is a heart stimulant. Aspirin, quinine, and pilocarpine are also of plant origin, which laid the basis for early medicine. Additionally there are several antibiotics for example

tetracycline, erythromycin, antimalarial drug (artemisinin) and anticancer drugs (paclitaxel and irinotecan) are derivates of plant origin or semi-synthetic derivates (Katiyar *et al*., 2012; Harvey, 2008).

Medicinal plants play a major role in the drug discovery because they have more extensive structural diversity than synthetic drug because their chemical derivatives are of organic or natural origin (eg: plant). Thus medicinal plants were used widely in pharmaceutical drug discovery due to their varied pharmacological or biological properties. Natural products are difficult to synthesize and their production cost is high because their structure is more complex with more bridgehead tetrahedral carbon atoms, rings, and chiral (Koehn & Carter, 2005).

The biodiversity of natural products in Malaysia is enormous. Nevertheless, major drawback could be attributed to the lack of utilization of natural resources as an avenue for new drugs. Research on the pharmacological properties of these natural products is limited and lacks sound scientific finding. A few examples of Malaysian plants which yield medicinal drugs are the pennywort (pegaga), andrographis paniculata (hempedu bumi), devil's trumpet (kecubung), the pinang palm (pokok pinang), the castor oil plant (pokok jarak) and 'pokok cerita' (Jamal, 2006). A local plant *M. speciosa* has psychotropic characteristics; it was customarily used to lessen pain, blood pressure, cough, intestinal disorders and also used as an alternative to morphine in treating drug abuse patients (Khor *et al*., 2011; Chan *et al*., 2005). *M. speciosa* therefore is considered a psychostimulant and need for research to ascertain these claims is essential. It has gained recognition nationally and internationally because of it various medicinal values.

2.2 *Mitragyna speciosa*

2.2.1 General description of the plant

Mitragyna speciosa Korth. belongs to the family of Rubiaceae (coffee family), it is a evergreen tree found in tropical and sub-tropical regions of South East Asia and Africa. Asian *M. speciosa* are often found in rainforests, while the African species are often found in swamps. *M. speciosa* is also called as Ketum, Kakuam, Ithang and Thom in different South-East Asian regions and locations (Devotee, 2009). Genus of this plant 'Korth' was named after Pieter William Korthal, a botanist who first found the stigma of its flower resembling a bishop's mitre (Shellard, 1974).

Taxonomy of *Mitragyna speciosa* Korth.

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Order: Gentianales

Family: Rubiaceae

Subfamily: Cinchonoideae

Tribe: Naucleeae

Genus: *Mitragyna*

Species: *Mitragyna speciosa*

2.2.2 Botanical description of the plant

M. speciosa trees have an average height of 4-9 m and 5 m wide. Whereas, few trees can reach heights up to 15-30 m (Shellard, 1974). The stems are erect and branching and could grow over 18 cm long and 10 cm wide with an ovateacuminate shape and tapered ends (Hassan *et al*., 2013). Deep yellow flowers of the *M. speciosa*

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grow in globular clusters each containing up to 120 florets (Fig. 2.1). During the flower's bud stage, each developing floret will be surrounded and fully cover by many overlapping bracteoles. Dichasial cyme is the inflorescence of *M. speciosa* (Shellard, 1974). The fruits of *M. speciosa* look like a capsule which containing many small flat seeds (Shellard & Lees, 1965; Emboden, 1979). The leaves are dark glossy green in color (Fig. 2.1). The young woody shoots bear up to 10-12 leaves arranged in opposite and decussate pairs. Two interpetiolar stipules can be seen together with a pair of leaves where they are closely appressed and protect the apical bud (Shellard, 1974).

The leaves are always being shed and replaced, however there is some semi regular leaf shedding because of ecological conditions. Amid the dry season the leaves fall widely and new development is created amid the rainy season (Hassan *et al*., 2013). The ideal development state of the M. speciosa is wet, sticky, rich soil, with medium to full sun exposure in zones shielded from firm winds (Macko, 1972; Hassan *et al*., 2013).

There are 2 types of *M. speciosa* plant. One has a leaves with a red petiole while the other with a white-greenish petiole. The petiole is the stalk attaching the leaf blade to the stem. For the pharmacological studies, MG was isolated from the red petiole type of *M. speciosa* leaves.

Figure 2.1: Mitragyna speciosa flower and leaves

2.2.3 Traditional use of *Mitragyna speciosa*

M. speciosa has been traditionally used in Thailand and Malaysia as a substitute for opium when opium is unavailable, or to moderate opium addiction. Ketum has been used to prolong sexual intercourse by small number of people in rural areas. Generally, peasants, workers and farmers are among the people who consume ketum to overcome the burdens of their hard work (Reanmongkol *et al*., 2007). With time, some users find that they need to increase doses to 10-30 leaves or even more per day (Anon, 2006). Traditionally, ketum has been utilized as a leaf poultice to treat fever and wound healing (Burkill, 1935). In the northern region of Peninsular Malaysia, adulterated decoction of *M. speciosa* leaves are packed in plastic bottles or packets and sold as "ketum juice" in the streets. In this region, "ketum juice" is consumed by addicts as a substitute for more expensive opiates to manage withdrawal symptoms.

In Malaysia and Thailand, *M. speciosa* was observed to be utilized as an opium substitution as a part of the treatment of opium addiction in the early years (Beckett *et al*, 1965; Tanguay, 2011). Nevertheless, a study led in 1975 uncovered that ketum consumers in Thailand were slowly dependent on it (Suwanlert, 1975). Hence, discoveries reported that there has been a generous development among the

Malaysian drug addicts who utilize the ketum leaves to wean themselves off from narcotic withdrawal indications (Vicknasingam *et al*., 2010). In any case, long haul ketum utilization can prompt its misuse simply like different drugs, for example, cannabis and heroin. This phenomenon caused extensive worry among general society and law enforcement authorities where it can create new addiction among the young generation (Ahmad & Aziz, 2012). At present, the ketum utilization in overseeing compulsion and withdrawal side effects is happening in an unsupervised, casual, scattered and unrecorded way. Therefore, the danger of serious dependence on ketum in an uncontrolled manner hinders its genuine potential as a simple home remedy that exhibits effective in any medication substitution program.

2.2.4   Pharmacological activities of *Mitragyna speciosa*

In 1836, native Malayans used ketum as an opium substitute when opium itself was unavailable or unaffordable at that time (Burkill, 1935). In 1897, Ridley reported that the leaves and bark of *Mitragyna speciosa* were used as the treatment for the opium addiction and in 1907 this statement was repeated by Hooper. Again in the same year, Holmes had referred to the leaves as an opium substitute. In 1907, Wray explained how ketum was possibly smoked, chewed or drunk to experience the opium-like effects in any means of administration (Wray, 1907a).

Ketum leaves are used for their complex, dose-dependent pharmacological effects (Prozialeck *et al*., 2012). Briefly, mild stimulant effects is experienced at low to moderate doses (1-5g) which enable workers to stave off fatigue (Jansen & Prast, 1988; Suwanlert, 1975). In addition to that, it has been reported that unpleasant sense of anxiety and internal agitation are the effects at low dose (Prozialeck *et al*., 2012). Meanwhile at moderate to high doses (5-15g) it's been reported to have opioid-like

effects. Ketum has been used for the pain management, opioid withdrawal symptoms and treatment for diarrhea at these doses (Jansen & Prast, 1988; Suwanlert, 1975). Finally at very high doses (>15g) ketum is reported to be quite sedative and induces stupor and mimicking effects (Grewal, 1932).

2.2.5 Report on Human Usage of *Mitragyna speciosa*

Several researchers have conducted studies on ketum usage patterns and social behavior (Ahmad & Aziz, 2012; Assanangkornchai *et al*., 2007). A study done on self-proclaimed ketum users has reported that ketum is cheap, easily accessible herbal drug with no life threatening adverse effects despite of its long term usage (Vicknasingam *et al*., 2010). Although mild side effects such as dehydration, loss of weight and constipation, excessive tearing, discomfort and jerky movement of limbs upon cessation has been reported but there were still no serious adverse events were recorded. Adding to that, a study on side effects of ketum also reported that long-term ketum usage made the users to addict towards it (Ahmad & Aziz, 2012). Besides that, number of advertisements on its sale on the internet showed that ketum is also highly on demand in the internet (Schmidt *et al*., 2011; Boyer, 2007; Babu *et al*., 2008) Due to the rise of ketum usage among the youth, the law enforcement were taken the steps to documenting its usage, side effects and abuse potential before the ketum addiction spread worldwide (Ahmad & Aziz, 2012).

Even though there is no record of death due to ketum consumption; still chronic and high dosage can lead to serious adverse reactions in very rare occasion (McWhirter & Morris, 2010; Sheleg & Collins, 2011; Nelsen *et al*., 2010). However there are few fatal cases reported due to consuming adulterated ketum products

(Kroonstad *et al*., 2011). Reports reveal that ketum purchased from online traders contain a powdered form of ketum and the μ-receptor agonist (O-desmethyltramadol). This upon consumption has lead to several unintentional fatalities due to the present of O-desmethyltramadol and ketum.

Despite the ban, the ancient practice of consuming ketum as a decoction drink still occur among the villagers of northern states of Malaysia. The users are preferred the ketum drinks compared to other controlled drugs because of its low cost and easy accessibility as it sold in small packets on the streets (Vicknasingam *et al*., 2010). Therefore, large consumption of ketum among its users is due to its easy accessibility and the affordability when compared with heroin. Hence, ketum should undergo a scientific evaluation to be proven suitable, either directly or as a supplement in addiction therapy although, it possess many medicinal values (Vicknasingam *et al*., 2010).

2.3   Phytochemistry and pharmacological studies of *Mitragyna speciosa*

In 1907, *M. speciosa* leaves were sent to the University of Edinburgh where, 14 years later, Wray (1907b) expressed that an active principle would be soon isolated and its usefulness to medicine assessed; E.J. Field (1921) isolated an alkaloid from the ketum leaves and named it as mitragynine (Jansen & Prast, 1988). The first formal pharmacological investigations on mitragynine were carried out at the University of Cambridge where Grewel (1932a) performed a series of experiments on animal tissues and found mitragynine to be a central nervous system stimulant rather than depressant (Jansen & Prast, 1988).

Since the 1960s, over 25 alkaloids have been isolated and chemically characterized from *M. speciosa* leaves. The main indole alkaloids present in the

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young leaves of *M. speciosa* are mitragynine and its analogues, speciogynine, paynantheine and speciociliatine. Chemical structures of mitragynine and its major analogues are shown Figure 2.2. In addition, a new alkaloid 7-hydroxymitragynine was also isolated as a minor constituent (Hassan *et al*., 2013; Shellard *et al*., 1978). These compounds are commonly found in the young leaves of the plant, which possesses a higher alkaloid content of MG as compared to the mature ones. From ethyl acetate extracts of young leaves of *M. speciosa,* MG, speciogynine, speciociliatine, paynantheine, 7-hydroxymitragynine, mitragynaline, corynantheidaline, corynantheidine and isocorynoxeine were found along with the minor constituents, 9-methoxymitralactonine and mitralactonine (Takayama, 2004). In the methanolic extract of the mature leaves the above mentioned main alkaloids were also found along with the minor constituents such as mitragynaline, pinoresinol, mitralactonal, mitrasulgynine and 3,4,5,6-tetradehydromitragynine (Takayama *et al*., 1998). The content of the alkaloids varies with geographical locations and also the season of the plant (Shellard, 1974). For instance, MG content of Thai *M. speciosa* was 66% of the total alkaloids while the Malaysian species contained only 12% of the total alkaloids (Takayama, 2004). The alkaloid profile of *M. speciosa* is summarized in Table 2.1.
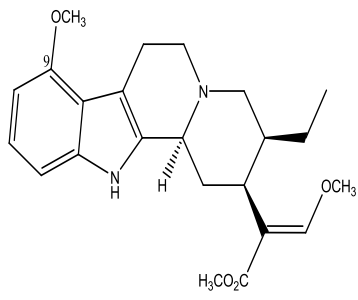
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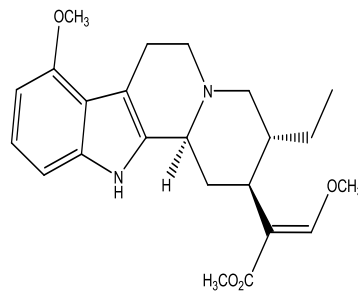
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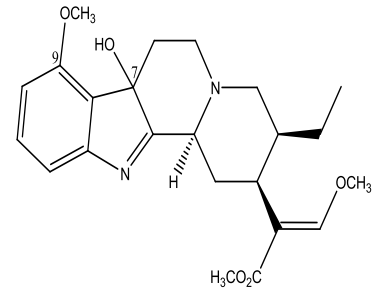
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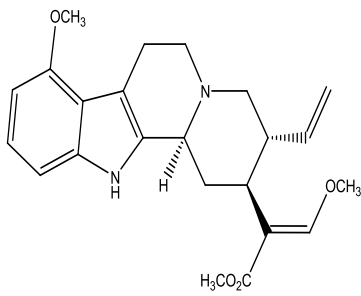
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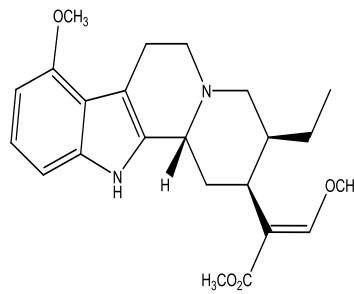
Speciociliatine
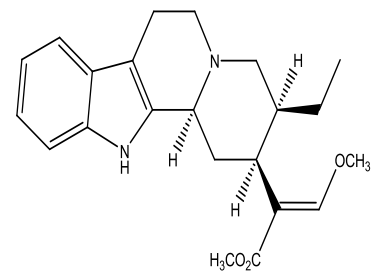
Corynantheidine

Figure 2.2: Chemical structures of mitragynine and its major analogues present in the leaves of *M. speciosa*

Table 2.1: Alkaloid profile of *Mitragyna speciosa* Korth. Percentage refers to the estimated content in the alkaloids extracts

Alkaloid	Percentage (%)	Pharmacology
Mitragynine	66	Activity on μ, δ, and κ receptors. Analgesic, antitussive, antidiarrheal, adrenergic, antimalarial.
Paynantheine	9	Inhibits twitch contraction in naloxone insensitive manner. Inhibits muscarine receptors on ileal smooth muscle.
Speciogynine	7	Inhibits twitch contraction in naloxone insensitive manner. Inhibits muscarine receptors on ileal smooth muscle.
7-Hydroxymitrgynine	2	13- and 46-fold higher potency than morphine and mitragynine, respectively. Potency and quick-acting characteristics may be caused by introduction of –OH group on C7 position. Induces clinically significant antinociceptive responses in a dose-dependent manner. Analgesic, antitussive, antidiarrheal.
Speciociliatine	1	C3 stereoisomer of mitragynine. Inhibits twitch contraction in naloxone insensitive manner. May inhibit acetylcholine release from presynaptic nerve through means other than opioid receptor stimulation.
Mitrapylline	1	Vasodilator, antihypertensive, muscle relaxer, diuretic, antiamnesic, immunostimulant, anti-leukemic.
Isomitrapylline	1	Immunostimulant, anti-leukemic
Speciophylline	1	Anti-leukemic
Rhynchophylline	1	Vasodilator, antihypertensive, calcium channel blocker, antiaggregant, anti-inflammatory, antipyretic, anti-arrhythmic, antithelmintic.
Isorhynchopylline	1	Immunostimulant
Ajmalicine	1	Cerebrocirculant, antiaggregant, anti-adrenergic, sedative, anticonvulsant, smooth muscle relaxer.

Corynantheidine	1	Opioid agonist
Corynoxine A	1	Calcium channel blocker, anti-locomotive
Corynoxine B	1	Anti-locomotive
Mitrafoline	1	
Isomitrafoline	1	
Oxindale A	1	
Oxindole B	1	
Speciofoline	1	Analgesic, antitussive
Isospeciofoline	1	
Ciliaphylline	1	Analgesic, antitussive
Mitraciliatine	1	
Mitragynaline	1	
Mitragynalinic acid	1	
Corynantheidalinic Acid	1	

(Prozialeck *et al*., 2012; Hassan *et al*., 2013)

2.3.1 Mitragynine

Mitragynine is the major indole-alkaloid that is isolated from *M. speciosa* leaves. The structure of mitragynine was fully determined in 1965 by Zacharias *et al*. (1965) through X-ray Crystallography. Takayama *et al*. (1995) conducted the first synthesis of mitragynine followed by Ma *et al*. (2009) who reported the alternative way of mitragynine synthesis. The chemical structure of mitragynine is similar to yohimbine (Adkins *et al*, 2011). This is illustrated in figure 2.2. Chemically, mitragynine is called 9-methoxy-corynantheidine.

Mitragynine is a white amorphous powder. It is a lipophilic alkaloid, as indicated by the log P value of 1.73 (Ramanathan *et al*., 2015). Mitragynine has low solubility in aqueous and basic media, inversely to an acidic environment, but is acid labile. It is soluble in alcohol, chloroform and acetic acid. Mitragynine is unstable in simulated gastric fluid with 20% degradation, but stable in simulated intestinal fluid (Ramanathan *et al*., 2015). Therefore pharmacological response of MG varied

extensively due to its low aqueous solubility, hydrophobicity, acid degradable nature and high variability of drug release in simulated biological fluids.

Mitragynine shows a high affinity towards μ-opioid receptors (Yamamoto *et al*., 1999). In addition, *in vitro* studies reported that intense opioid agonistic properties were seen with its oxidative derivative, mitragynine pseudoindoxyl. Pharmacological studies have demonstrated that antinociceptive effect of mitragynine is due to its action which targets μ- and δ-opioid receptors (Matsumoto *et al*., 1996a; Tohda *et al*., 1997). In comparison to morphine MG exhibits higher affinity towards the δ- and κ-opioid receptors. The opioid agonist activity could be attributed to its *N*b lone electron pair and methoxy group at the C9 position (Takayama, 2004; Hidayat *et al*., 2010). Besides that, at cell level, mitragynine block the neuronal Ca^{2+} channels which leads to the inhibition of neurotransmitter discharge from the nerve endings at the vas deferens (Matsumoto *et al*., 2005b). Along these lines this Ca^{2+} channel-blocking impact of mitragynine goes about as a key component for pain relieving and other physiological activities of mitragynine. Ingestion of herbal products or extracts along with MG could cause herb-drug interactions this is majorly due to the same metabolic pathway that MG and herbal drugs utilize. MG was repoted to inhibit cytochrome P450 enzyme activities (Hanapi *et al*., 2013).

Mitragynine also has anti-inflammatory properties. PGE2 is one of the strongest inflammatory mediators which are catalyse by the cyclo-oxygenase isoforms, COX-1 and COX-2. In macrophage cells the activity of COX-2 mRNA and protein expression were inhibit by MG which stops the PG_{E2} formation. Although, MG has no effect on COX-1 mRNA and protein expression at lower dose; it may inhibit them at higher concentration (Utar *et al*., 2011).

Many studies on pharmacological activities of ketum and its major alkaloid MG have been conducted in animal models. The reports from different analgesic studies on rats and mice suggested that MG antinociceptive activity were extensively varied according to the doses (5-400mg/kg) administrated to the animal models (Sabetghadam *et al*., 2010; Matsumoto *et al*., 1996b). Despite the different in MG content in the ketum extracts and variation across species, low oral bioavailability and its low aqueos solubility may be the reason behind the large dosage variation in analgesic studies (Ramanathan & Mansor, 2014; Parthasarathy *et al*., 2010).

A research conducted by Shaik Mossadeq *et al*. (2009) proved that extracts of *M. speciosa* show positive inhibitory effects towards carrageenan induced paw oedema and was also prevent the growth of granuloma tissue by increasing the development of macrophages, fibroblasts, vascularized and reddened mass tissue. In summary, reports suggested that *M. speciosa* has anti-inflammatory properties; this can be associated with an enhanced immunity via pro-inflammatory mediator release and stimulation of tissue repair and healing processes.

As for neurological effects, Idayu *et al*. (2011) suggested antidepressant effects of MG in mice. According to the study, she performed two different tests and established corticosterone concentration in mice was decrease by use of MG. The two test performed were forced swim test and tail suspension test on mice.

Several toxicity reports on MG suggested that the level of toxicity in animal model is low. A current study in rats was reported to be lethal at dose of 200 mg/kg of *M. speciosa* extract (Azizi *et al*., 2010), whereas there were no signs of toxicity in dogs treated with a high dose of *M. speciosa* extract (920 mg/kg) (Macko *et al*., 1972). The methanolic and alkaloid extracts of *M. speciosa* were screened in an acute toxicity test, the LD_{50} values in mice were 4.90 g/kg and 173.20 mg/kg respectively

(Reanmongkol *et al*., 2007). Standardized extracts of *M. speciosa* (methanol) were administered orally at three doses of 100, 500 and 1000 mg/kg, the rats did not show any behavioral changes and also their eating habits were unaffected. At 1000 mg/kg dose elevated levels of creatinine was present indicating nephrotoxicity.

2.4   Pharmacokinetic issues of natural products

2.4.1   Oral bioavailability

The most convenient route of drug administration is through oral dosing but due to certain chemical properties of herbal concoction or synthetic drugs prevent the use of oral dosing. This is a major setback to all the leading pharmacological companies in testing newly derived compounds due to the poor oral bioavailability (Prentis *et al*., 1988; Venkatesh & Lipper, 2000). Thus, this phenomenon causes significant costs for pharmaceutical companies (Arlington, 2000).

The process where the amount and the rate of an unaltered drug reached the targeted site within the body is known as bioavailability (Amidon *et al*., 1995). Briefly, an oral bioavailability is a process where the drug transports from the gastrointestinal tract through the apical membrane of the epithelial cells (eg: enterocytes) to the systemic circulation. In the process of reaching the systemic circulation, the drug passed through the enterocytes cells into the pre-hepatic blood vessels which collect in the portal vein before entering the liver. Oral bioavailability of a drug is a three steps process which are the portion of dose absorbed (f_a); the portion of drug escaped the gut wall metabolism (f_g); and the portion of drug escaped from hepatic metabolism (f_h) (Fig 2.3) (Dahan *et al*., 2011). In summary, oral bioavailability of a drug is explained in the following general equation (Eq. 2.1):

Equation 2.1: $F = f_a \times f_g \times f_h$

f_g and f_h can be further defined in terms of the extraction ratios of the intestinal-based metabolism (E_g) and hepatic-based metabolism (E_h) as follows (Eq. 2.2).

Equation 2.2: $F = f_a \,(1 - E_g)(1 - E_h)$
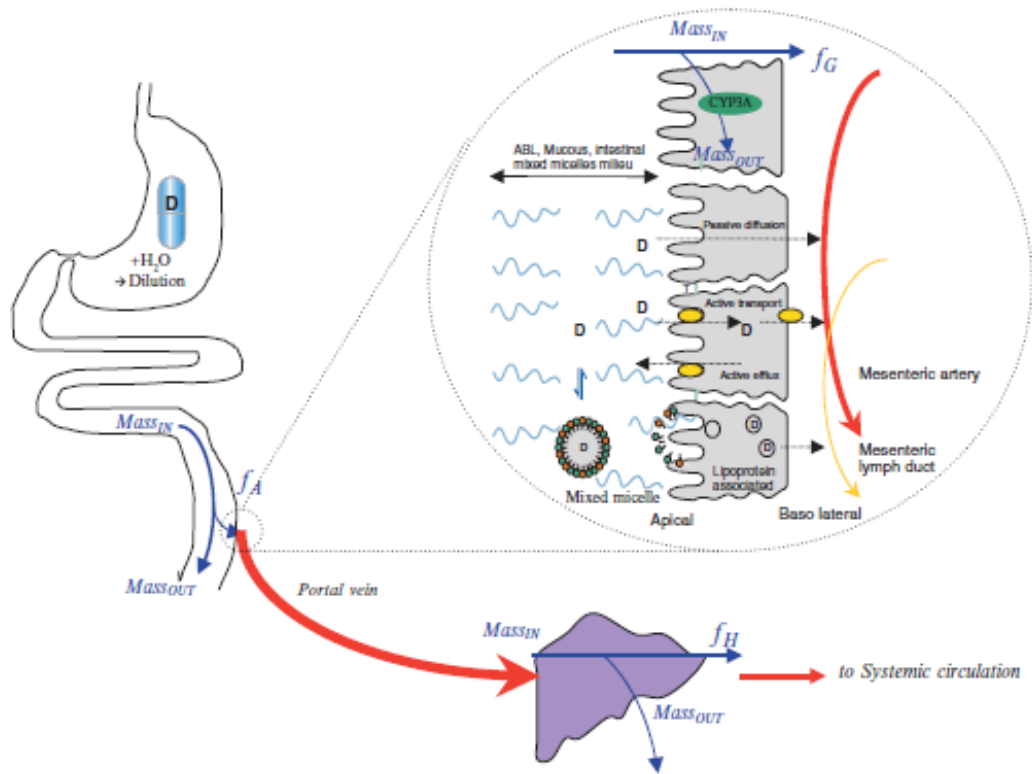
Figure 2.3: Oral drug absorption process from the gastrointestinal tract (GIT). Schematic depicting the three major processes (fa, fg, and fh) affecting absorption of drug from the site of administration to the systemic circulation passage, that is oral bioavailability. fa, fg, and fh can be estimated from the general relationship provided in Equation 2.3.
(Source: Picture adopted from Ehrhardt & Kim, 2008)

Equation 2.3: f_a, f_g or $f_h = (\text{Mass}_{IN} - \text{Mass}_{OUT}) / \text{Mass}_{IN}$

Absorption of a drug is relying on number of physiological, physiochemical, and dosage form factors. Adding to that, physiochemical properties of the drug and the bio-physiochemical properties of the gastrointestinal membrane play the key role in the drug absorption (Ho *et al*., 1977). Biopharmaceutical Classification System (BCS) was developed after the detail studies and researches on primary factors which were influencing the drug absorption. Under BCS, absorption of a drug is classified based on its solubility and membrane permeability characteristics (Amidon *et al*., 1995) as shown in Table 2.2.

Table 2.2: Biopharmaceutical Classification System

Class I	**Class II**
High Solubility	Low Solubility
High Permeability	High Permeability
Class III	**Class IV**
High Solubility	Low Solubility
Low Permeability	Low Permeability

2.4.2   Solubility and gastrointestinal stability

In the drug discovery process, the lead candidates must have desirable physicochemical properties in order to have a better chance of success in the development. Solubility, pKa (ionization constant) and lipophilicity are the most fundamental physicochemical properties of a drug candidate. Poor oral bioavailability and less desirable physicochemical properties are the main reasons

behind the failure of numerous pharmacologically active metabolites to become pharmaceutical drugs. Thus, solubility and permeability characteristics of a drug included in the drug discovery programs (Delaney, 2005; Huuskonen, 2001).

BCS recommends determining the solubility of drug substances under physiological pH conditions. The experiments should be carried out at 37 ± 1 °C in aqueous media with a pH range of 1.2 to 7.5 and to obtain accurate solubility data, the pH solubility profile of the compound should be determined based on its pKa. Prior to permeability studies, it is essential to determine the stability of the drug in gastric and intestinal fluids. These results will assist in obtaining accurate permeability values. This is because while conducting *in situ* or *in vivo* permeability studies there is a possibility for degradation of the drug in the gastric or intestinal fluids prior to absorption which will lead to erroneous permeability determination (USFDA, 2014).

2.4.3 Intestinal permeability

Besides the pharmacological activity of new chemical substance, sequential profiling which reveal their solubility, absorption, distribution, metabolism, elimination and toxicity are the key factors which lead to the selection and optimization of the compound (Trapani *et al*., 2004; Li, 2004). Solubility and the permeability characteristics of a drug are the important attributes of the drug bioavailability. Although the solubility of a drug can be improve with the aid of the proper formulation, the chances of enhance the intestinal membrane permeability are very likely restricted. Therefore, determination of the intestinal permeability is one essential requirement for assessing the oral bioavailability of a new drug candidate at the early drug discovery stage (Volpe, 2008).