THE EFFECTS OF MITRAGYNINE ON P-GLYCOPROTEIN REGULATIONS AND ITS NEUROTOXICITY MECHANISMS IN BRAIN CELL LINES

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by

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TABLE OF CONTENTS

CHAPTER 4 RESULTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

2D	two-dimensional
ABC	ATP binding cassette
ABCB1	ATP binding cassette subfamily B member 1
ABCG2	ATP binding cassette subfamily G member 2
ADMET	Absorption, distribution, metabolism, excretion and toxicity
ADT	AutoDockTools
ALD	adrenoleukodystrophy
ATP	adenosine triphosphate
BBB	blood-brain barrier
BCRP	breast cancer resistance protein
CAM	complementary and alternative medicine
cDNA	complementary deoxyribonucleic acid
CYP	cytochrome
CNS	central nervous system
DDI	drug-drug interaction
EBM	EndoGRO basal medium
FDA	Food and Drug Administration
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HPLC	high performance liquid chromatography
IC_{50}	half maximal inhibitory concentration
K_i	inhibition constant
LLOQ	lower limit of quantification
MDR1	multi drug resistance 1

Mg^{2+}	magnesium ion
mRNA	messenger ribonucleic acid
MRP	multi-drug resistance associated protein
NBD	nucleotide binding domain
OABP	ATP binding cassette subfamily E member 1
OAT	organic anion transporter
OATP	organic anion-transporting polypeptide
OCT	organic cation transporter
P_{app}	apparent permeability
P_e	permeability coefficient
PDB	Protein Data Bank
P-gp	P-glycoprotein
QSAR	quantitative structure–activity relationship
RNA	ribonucleic acid
RT-qPCR	reverse transcription quantitative polymerase chain reaction
SD	standard deviation
SEM	standard error of the mean
SLC	solute carrier
TAP	transporter associated with antigen processing
TEER	transepithelial electrical resistance
TMD	transmembrane domain
US	United States
UV	ultraviolet
WHO	World Health Organization

LIST OF SYMBOLS

α	alpha
~	approximately
*	asterisk
β	beta
–	dash
=	equals
>	greater than
-	hyphen
<	less than
≤	less-than or equal to
/	or
±	plus-minus
®	registered trademark
×	times
™	trade mark

LIST OF UNITS

Å	Ångström
°C	degree Celcius
Da	Dalton
g	gram (weight per unit mass)
hr	hour
kcal/mol	kilocalorie per mole
µg	microgram
µL	microliter
µm	micrometer
µM	micromolar
mL	milliliter
mm	millimeter
mM	millimolar
min	minute
M	molar
nm	nanometer
nM	nanomolar
Ω	ohm
%	percentage
psi	pounds per square inch
U/mL	unit per milliliter
v/v	volume/volume
w/v	weight/volume

KESAN MITRAGININA KE ATAS PENGAWALATURAN P-GLIKOPROTEIN DAN MEKANISME NEUROTOKSISITINYA DI DALAM TITISAN SEL OTAK

ABSTRAK

Mitraginina, komponen bioaktif utama yang ditemui dari *M. speciosa* mempunyai pelbagai manfaat, walau bagaimanapun, bukti dari beberapa kajian kes menunjukkan bahawa pemberian bersama mitraginina dengan ubat preskripsi telah mengakibatkan kesan mudarat teruk termasuk kematian. Oleh itu, terdapat kebimbangan ke atas keselamatan tumbuhan ini yang mempamerkan kesan seperti candu. P-glikoprotein (P-gp), pengangkut pelbagai ubat banyak terdapat di permukaan lumen sel endotelium kapilari otak, dimana fungsi utamanya merupakan komponen utama rintangan darah otak yang mengehadkan penetrasi pelbagai ubat ke sistem saraf pusat (CNS). Modulasi atau interaksi ubat-herba ke atas P-gp berpotensi merubah mengakibatkan kesan sampingan yang teruk, ketoksikan dan kegagalan rawatan. Oleh itu, objektif utama kajian ini adalah untuk menentukan kesan mitraginina ke atas pengawalaturan P-gp dan kesan keneurotoksikan interaksi ubat-herba di antara mitraginina dan substrat P-gp di dalam sel PC12 dengan menggunakan pendekatan *in silico* dan *in vitro*. Simulasi pendokan telah dijalankan menggunakan model homologi P-gp dan interaksi molekul dianalisis. Tambahan itu, asai pengangkutan dwiarah telah dijalankan untuk menilai kesan mitraginina ke atas aktiviti pengangkutan P-gp. Pengoptimuman asai pengangkutan dwiarah telah dilakukan dan kedua-dua digoxin dan mitraginina dianalisis menggunakan pengesan HPLC/UV dengan elusi isokratik. Kesan mitraginina ke atas ekspresi mRNA dan protein P-gp masing-masing telah dilakukan dengan menggunakan RT-qPCR dan analisis Western blot teroptimum.

Suatu model BBB telah dihasilkan dan kesan keneurotoksikan interaksi ubat-herba antara mitraginina dan substrat P-gp (paclitaxel dan fluoxetine) telah dijalankan menggunakan regimen rawatan dos berulang, analisis immunopendarflour dan tatasusunan PCR. Kajian ini telah menunjukkan bahawa mitraginina tidak bertindak sebagai substrat P-gp berdasarkan kedua-dua simulasi pendokan molekul dan asai pengangkutan dwiarah. Sebaliknya, mitraginina didapati merencat P-gp dengan membentuk ikatan hidrogen dan interaksi hidrofobik dengan residu penting. Asai pengangkutan dwiarah menunjukkan perencatan ketara ke atas aktiviti pengangkutan P-gp bukan dalam cara bergantungan dos menggunakan asai berasaskan HPLC (IC_{50} 2.22 μM, *p* value <0.05) dan rodamina123 (IC_{50} 1.30 μM, *p* value <0.05). Mitraginina juga didapati merencat ekspresi mRNA dan protein P-gp pada kepekatan tinggi (4, 20 and 100 μM). Oleh itu, mitraginina adalah perencat *in vitro* P-gp yang ketara. Terapi kombinasi mitraginina dan paclitaxel telah menunjukkan peningkatan ketara kebolehtelapan dan telah mengkompromi integriti membran sel hCMEC/D3. Analisis immunopendaflour telah mengesahkan penurunan ketara zonula okludens (ZO-1) yang menunjukkan gangguan ke atas persimpangan sempit dan dengan demikian, membenarkan penetrasi sebatian ke atas sel monolapisan dan menyebabkan keneurotoksikan. Disamping itu, keputusan tatasusunan PCR mempamerkan corak ekspresi gen yang ketara, menunjukkan kesan keneurotoksikan. Kesimpulannya, mitraginina merencat aktiviti pengangkutan dan ekspresi P-gp dan terapi kombinasi mitraginina dan substrat P-gp telah mencetuskan ekspresi gen yang berkaitan dengan keneurotosikan, mencadangkan kemungkinan alkaloid ini membawa kepada tanda-tanda awal keneurotoksikan.

THE EFFECTS OF MITRAGYNINE ON P-GLYCOPROTEIN REGULATIONS AND ITS NEUROTOXICITY MECHANISMS IN BRAIN CELL LINES

ABSTRACT

Mitragynine, the major biologically active constituent found in *M. speciosa* has various benefits, however, evidences from several case studies for concomitant used of mitragynine with prescribed drugs has resulted in severe adverse reactions including death. As such, there are growing concerns on the safety of this plant which exhibits opium-like effects. P-glycoprotein (P-gp), a multidrug transporter, wherein P-gp functionally limits the central nervous system (CNS) penetration of various drugs. Modulation or drug-herb interactions of P-gp are potentially resulting in severe side effects, toxicity and treatment failure. Therefore, the main objective is to determine the effects of mitragynine on P-gp regulations and neurotoxicity effects of drug-herb interactions between mitragynine and P-gp substrates in the PC12 cells using both *in silico* and *in vitro* approaches. Docking simulation was performed using P-gp homology model and their molecular interactions were analyzed. In addition, a bidirectional transport assay was conducted to evaluate the effects of mitragynine on P-gp transport activity. The optimization of the bidirectional transport assay was carried out and both digoxin and mitragynine were analyzed using HPLC/UV detector with isocratic elution. The effects of mitragynine on mRNA and protein expression of P-gp were carried out using an optimized RT-qPCR and Western blot analysis, respectively. A BBB model was established and the neurotoxicity effects of drug-herb interactions of mitragynine and P-gp substrates (paclitaxel and fluoxetine) were

carried out using repeated-dose treatment regimen, immunofluorescence analysis and PCR array. Mitragynine is unlikely a P-gp substrate based on both the molecular docking simulation and bidirectional transport assay. On the other hand, mitragynine was found to inhibit P-gp by forming hydrogen bonds and hydrophobic interactions with important residues. Bidirectional transport assay has revealed a significant inhibition of P-gp transport activity which were not in dose-dependent manner using HPLC-based (IC_{50} 2.22 μM, *p* value <0.05) and rhodamine123 (IC_{50} 1.30 μM, *p* value <0.05) assays. Mitragynine was also found to inhibit mRNA and protein expression of P-gp at higher concentrations (4, 20 and 100 μM). Therefore, mitragynine is a significant *in vitro* P-gp inhibitor. A combination therapy of mitragynine and paclitaxel has revealed a significant increase in permeability and compromised the membrane integrity of hCMEC/D3 cells. The immunofluorescence analysis confirmed a significant reduction of zonula occludens (ZO-1) indicating a disruption of tight junctions, and thus, allowing compound to penetrate the monolayer cells and cause neurotoxicity. Meanwhile, PCR array results exhibited significant gene expression patterns indicating neurotoxicity effects. In conclusion, mitragynine inhibits P-gp transport activity and expression and combination therapy of mitragynine and P-gp substrate has triggered gene expression associated with neurotoxicity, suggesting the possibility of the alkaloid leading to an early sign of neurotoxicity.

CHAPTER 1

INTRODUCTION

1.1 Background

Mitragyna speciosa (Korth.) or kratom is a herbal plant that have long-history of folk medicine and medicinal value for the treatment of a wide range of ailments. In Southeast Asia, the plant is used to treat common illness such as coughing, diarrhea, muscle pain and as sexual performance enhancer (aphrodisiac) (Haron and Ismail, 2014, Singh et al., 2020a). In Malaysia, *Mitragyna speciosa* Korth. is often consumed as a decoction, where the leaves are boiled for several hours in water and the resulting liquid is strained and sold in transparent plastic bags (Singh et al., 2020a). In addition, the fresh leaves can be dried and powdered or chewed. The powdered leaves is then used up together with a hot coffee or tea. Normally, kratom decoction is consumed with a sweetener, due to its bitter taste. In Malaysia, smoking of kratom leaves is uncommon, however it has been occasionaly reported (Tanguay, 2011).

Mitragynine is a major biologically active constituent found in the leaves of *Mitragyna speciosa* Korth. (Hassan et al., 2013). Various *in vitro* and *in vivo* studies have shown that mitragynine has anorectic, anti-inflammatory, antidepressant, antinociceptive, anesthetic, antitussive, analgesic, antioxidant and antimicrobial activities (Parthasarathy et al., 2009, Utar et al., 2011). In addition, mitragynine possesses narcotic-like properties and was also demonstrated to have high affinity and selectivity particularly mu-opioid receptor, which is potentially beneficial as a treatment agent for opioid addiction (Jamil et al., 2013). However, evidences from several case studies showed that co-administration of mitragynine with citalopram, tramadol, and propylhexedrine has resulted in deaths (Corkery et al., 2019, Holler et al., 2011, Neerman et al., 2013), meanwhile, co-administration with modafinil caused

tonic-clonic seizure (Boyer et al., 2008). These evidences showed that herbal medicine is not entirely harmless and the adverse effects have disputed the assumption that herbal medicine is safe for consumption.

Neurotoxicity of new compounds with the desired pharmacological effects represents one of the major bottlenecks in drug discovery and development since it requires extensive animal experiments and time-consuming (Schultz et al., 2015). As a key feature of the toxicological characteristics of compounds, the prediction of neurotoxic effects is very important. In addition, to evaluate the neurotoxicity of new chemical entities with unknown mechanisms, the possible toxicity of the compound to the blood-brain barrier (BBB) should also be considered as a fully functional blood-brain barrier (BBB), which is important for maintaining the dynamic balance of the brain (Coecke et al., 2006, Schultz et al., 2015). The guidelines for the evaluation of neurotoxicity effects of compounds for risk assessment is based solely on *in vivo* testing, which is expensive and time consuming (Fabulas-da Costa et al., 2013). Hence, the most important step is early recognition of neurotoxicity using rapid and robust preclinical testing strategy to assess whether compounds with desirable characteristics are neurotoxic or causing irreversible damage, prior to safety and efficacy testing.

The blood-brain barrier is the principle route for most molecules to enter the central nervous system, and it is also the main obstacle preventing many drugs from causing pharmacological or toxicological effects in the brain (Harry and Tiffany-Castiglioni, 2005). Conventional *in vivo* methods for neurotoxicology testing are based on the observation of sensory and behavioral disturbances combined with histopathological features. However, these methods rarely focus on possible alterations of the BBB (Fabulas-da Costa et al., 2013). Nevertheless, *in vitro* methods are believed to be necessary to assess the neurotoxicity of compounds, which is

essential for predicting whether the drug reaches the central nervous system in an amount sufficient to cause toxicity.

P-glycoprotein (P-gp) or known as ABCB1 transporter is the 170-kD protein product of the multidrug resistance-1 (MDR1) gene. P-gp is highly expressed on the luminal surface of brain capillary endothelial cells, in which P-gp is a major component of the BBB that limits CNS penetration of various antidepressant drugs, antibiotics, chemotherapeutic agents, small peptides, and HIV protease inhibitors (van Assema et al., 2012). Previously, an experiment conducted by Bauer and co-workers revealed the shortcoming of P-gp function modulation; that is, the brain protection was reduced, substantially causing neurotoxicity. Clearly, one way to enhance entry of a significant number of drugs into the CNS in a controlled manner is to alter P-gp function of the blood-brain barrier (Bauer et al., 2005). In the early 2000, the regulation of P-gp at the blood-brain barrier is less known (Demeuse et al., 2004, Nwaozuzu et al., 2003). However, modulation of P-gp functions has gained much interest in the recent years (Aryal et al., 2017, Kim et al., 2019, Liu et al., 2015a, Sita et al., 2017).

It is well accepted that P-gp acts as an ATP-dependent efflux pump and reduces the accumulation of many lipophilic drugs with different chemical structures in cells (Cole et al., 1992). Indeed, for many years, the model for drug resistance conferred by MDR1/P-gp focused on the xenobiotic efflux mechanism of cells (Blanc et al., 2003). Other newer biological regulatory functions of P-gp include cell survival, differentiation and proliferation (Johnstone et al., 2000). In addition, P-gp may confer cell resistance by regulating some caspase-dependent apoptotic pathways (Smyth et al., 1998). The molecular mechanisms of P-gp in drug efflux, cholesterol metabolism, inhibition of apoptosis, phospholipid translocation, and dendritic cell migration are not necessarily mutually exclusive, and efflux or translocation of key molecules (such as

drugs, lipids or cellular proteins) is the way P-gp regulates a series of different physiological responses (Johnstone et al., 2000). Nonetheless, expanding research work related to P-gp will help to define its physiological importance particularly in BBB.

1.2 Rationale of the study

Kratom extracts are commonly being used by local folks especially in rural areas (Veltri and Grundmann, 2019). The bioactive compound, mitragynine is a compound that acts as a depressant and a stimulant. Due to its stimulant and euphoric effects, this medicinal plant is often misused and abused as a substitute of opium (Fu and Stojanovska, 2013). In 2003, mitragynine was listed in the First Schedule and the Third Schedule of Psychotropic substances in the Poison Act 1952 due to concerns over addiction and an increasing number of youths becoming dependent on it (Ahmad and Aziz, 2012). Furthermore, chronic use of kratom has been associated with insomnia, weight loss, anorexia, jerky movement of limbs and aggression, intrahepatic cholestasis, seizure, coma and even fatal intoxication (Hassan et al., 2013, Henningfield et al., 2018, Kapp et al., 2011, Meireles et al., 2019, Nelsen et al., 2010).

Several case reports have been published with regards to drug-herb interactions involving mitragynine. This includes, a 64-year-old male, who regularly used kratom to self-medicate his chronic pain, which was found by his wife unconscious and seizing (Nelsen et al., 2010). In addition, 9 unintentional deaths were reported due to consumption of mitragynine and tramadol (Kronstrand et al., 2011). Meanwhile, there are few other case reports such as kratom was found to induce intrahepatic cholestasis, which was confirmed by liver biopsy (Kapp et al., 2011), a 44-years-old man experienced withdrawal symptoms was admitted for kratom detoxification

(McWhirter and Morris, 2010), and a death case involving mixture of an unknown amount of herbal substances (Domingo et al., 2017). Furthermore, a study conducted by Singh and co-workers demonstrated a deficit in neurophysiological functions and visual memory among higher chronic kratom consumption (Singh et al., 2019). In addition, due to kratom extract interference with the neuromuscular junction, skeletal muscle cannot contract and does not produce muscle relaxation (Chittrakarn et al., 2010). It was postulated that the blockage of the compound nerve action potential were caused by high concentration of mitragynine (2 mg/mL). Kratom extract and mitragynine seem to have a direct effect on skeletal muscle by reducing muscle twitches(Somsmorn et al., 2010). The question of impairment with the chronic use of kratom remains, even if kratom withdrawal and dependence are not perceived to be as severe as for opioids. Clearly, the current evidence showed that mitragynine is possibly toxic and may involve in drug interactions leading to adverse effects.

Drug transporters such as P-gp have the potential to impact oral bioavailability and modulates the absorption, distribution, and excretion of a vast array of xenobiotics and serves a key role in drug-drug interactions (DDI) (Aszalos, 2007, Borst and Elferink, 2002, Szakacs et al., 2006, Vaalburg et al., 2005). Manipulating of the P-gp function can cause reduced brain protection, resulting in neurotoxicity. As a matter of fact, the alteration of P-gp function of the blood-brain barrier can enhance the entry of a significant number of drugs into the CNS in a controlled manner (Bauer et al. 2005). Hence, alteration of transporter and enzyme activities of a substrate drug may lead to induction of drug toxicity or a reduction in drug efficacy. For example, the unintentional presence of an inhibitor of P-gp may alter the bioavailability of a substrate drug in the brain and produce an impact on the clinical safety of the selected drug (Tachibana et al., 2010). Herb-induced P-gp inhibition and/or induction on its

transport activity are potentially capable of changing the substrate drug's pharmacokinetic parameters and resulting in severe side effects, toxicity or treatment failure. Hence, it was hypothesized that P-gp may be mediating the possible mitragynine-induced pharmacokinetics interactions and neurotoxicity in the BBB. This project specifically determines the effects of mitragynine on P-gp regulation and drug-herb interactions leading to neurotoxicity in brain cells using a series of experiments.

1.3 Objectives

1.3.1 Main objectives

1. To investigate the effects of mitragynine on P-gp regulations and the neurotoxicity effects of drug-herb interactions between mitragynine and P-gp substrates in the brain cells.

1.3.2 Specific objectives

1. To establish P-gp bidirectional transport assay using brain endothelial cells.
2. To determine if mitragynine is a P-gp substrate, inhibitor or inducer using *in silico* and *in vitro* approaches (bidirectional transport assay).
3. To establish BBB model using brain endothelial and glial-like cells for drug-herb interactions and neurotoxicity assessment.
4. To determine the neurotoxicity effects of drug-herb interactions between mitragynine and P-gp substrate using BBB model and PCR array.

1.4 Hypothesis

The hypothesis of this study is that P-gp may be mediating the possible mitragynine-induced pharmacokinetics interactions and leading to a possible early sign of neurotoxicity effects in the BBB.

CHAPTER 2

LITERATURE REVIEW

2.1 *Mitragyna speciosa* Korth

2.1.1 General descriptions and distribution

Mitragyna speciosa Korth (*M. speciosa*) is a herbal plant from the family of Rubiaceae, as depicted in Figure 2.1. This plant grows well in humid and wet area, access to sun exposure in areas away from strong winds and fertile soil (Azizi et al., 2013, Harizal et al., 2010, Hassan et al., 2013). The stem and leaves of this plant have been used as traditional remedy for a long time in the Southeast Asia region to treat common illness such as diarrhea, muscle pain, fever, coughing and intestinal infections (Haron and Ismail, 2014, Hassan et al., 2013, Vicknasingam et al., 2010). It is commonly known as biak-biak or ketum in Malaysia and kratom in Thailand (Azizi et al., 2013, Harizal et al., 2010, Hassan et al., 2013, Ilmie et al., 2015).

M. speciosa is regularly consumed by labourers by chewing the leaves, or drinking as tea as energy-boosting agent to enhance tolerance to work under scorching weather and increase physical endurance (Chan et al., 2005). *M. speciosa* possesses cocain-like and morphine-like stimulating effects, and due to these effects, it is often being used in easing withdrawal symptoms of opiate and alcohol withdrawal (Boyer et al., 2008, Vicknasingam et al., 2010). In addition, *M. speciosa* also exerts euphoric effect, and this has led to its abuse, misuse, addiction and dependence (Boyer et al., 2008). As such, *M. speciosa* plant and mitragynine extracts are categorized as controlled substance in Australia, Denmark, Malaysia and Thailand (Adkins et al., 2011).

Figure 2.1 The leaves and plant of *Mitragyna speciosa* Korth (*M. speciosa*). Adapted from (Singh et al., 2017).

The Malaysian government has put in effort to restrict its use through law enforcement under the Poison Act in the year 2003, making trading related to kratom leaves and preparations illegal (Hassan et al., 2013). However, smuggling of kratom among locals between Northen Peninsular Malaysia and Southern Thailand occurs frequently. Furthermore, kratom usage has become an "outbreak" worldwide in the form of mixture cocktail powder commonly known as Krypton in the Western countries, which has caused several fatal intoxications from its usage (Kronstrand et al., 2011). The US Drug Enforcement Administration (DEA) has placed *M. speciosa* on its Drugs and Chemicals of Concern list suggesting that the use of substance related to kratom will be restricted once reliable toxicology data becomes available (Hassan et al., 2013). Despite being listed as control substances, *M. speciosa* is still widely abused and misused.

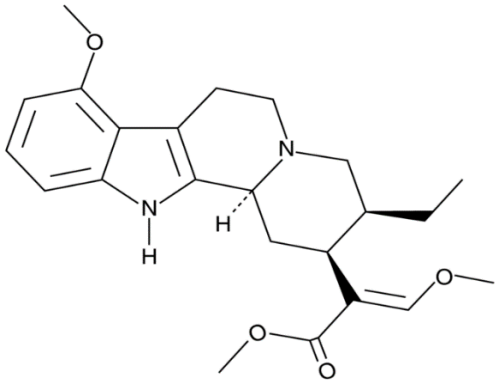
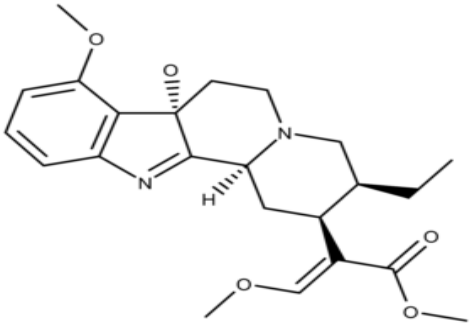
M. speciosa products are available worldwide and are easily purchased through the internet. In 2010, McWhirter and Morris reported that kratom products were used mainly for its euphoric effect, to manage opiate withdrawal effects as well as to treat anxiety and depression (McWhirter and Morris, 2010). Meanwhile, Boyer and co-workers reported that long-term administration has led to several side effects including dependence and addiction (Boyer et al., 2008). More than 70% of users who has attempted to stop kratom consumption experienced intense fatigue, excessive tearing and sweating and other undesired side effects (Vicknasingam et al., 2010). In addition, chronic use of kratom has been associated with insomnia, weight loss, anorexia, jerky movement of limbs and aggression, intrahepatic cholestasis, seizure, coma and even fatal intoxication (Farah Idayu et al., 2011, Hassan et al., 2013, Kapp et al., 2011, Kronstrand et al., 2011, Nelsen et al., 2010, (Tungtananuwat and Lawanprasert, 2010).

2.1.2 Chemical and pharmacological activities of kratom and mitragynine

Phytochemical studies of the components of the plant have led to the isolation of several indole alkaloids. Over 40 compounds were successfully isolated from the leaves of this plant (Adkins et al., 2011). Both Malaysian and Thailand kratom leaves produce the major alkaloid compounds, mitragynine (Table 2.1) and its analogues such as paynantheine, speciogynine, and speciociliatine. Other minor alkaloids include corynantheidaline, corynantheidalinic acid, 7α-hydroxy-7H-mitragynine, mitragynaline and mitragynalinic acid. Among these chemical constituents, mitragynine has been found to be the most abundant, contributing about 66% of the crude extract of the plant (Takayama, 2004). Thus, many studies have been carried out to identify the pharmacological effects of this plant, particularly mitragynine and 7-hydroxymitragynine compounds.

Takayama and co-workers studied the biological activities of crude *M. speciosa* extracts, 7-hydroxymitragynine, mitragynine and several mitragynine derivatives (Takayama, 2004). In the guinea pig ileum preparation, mitragynine has a quarter of the potency of morphine, which can inhibit electrical stimulation of contraction. Speciociliantine is 13 times less potent than mitragynine. By directly stimulating the muscarine receptors on the smooth muscle of the ileum, payantheine and speciogynine inhibit convulsions. Meanwhile, 7-hydroxymetanine is 46 times more potent than mitragynine and 13 times more potent than morphine. Mitragyna pseudoindoxyl was 20-fold and 100-fold and higher than morphine and mitragynine, respectively (Takayama, 2004). In view of these results, the antinociceptive activity of 7-hydroxymitraginine was greater than that of morphine, which was greater than the antinociceptive activity of mitragyna pseudoindoxyl. The antinociceptive activity of

Table 2.1 Structures and pharmacologic activities of major compounds isolated from kratom.

Compound	Structure	Pharmacology
Mitragynine		• Structurally similar to yohimbine (Greene, 2013) • Activity on μ and κ receptors (Horie et al., 2005) • Main activity on μ receptors creating opiate and analgesic effects and physical dependence (Adkins et al., 2011) • Inhibits radioligand binding at central nervous system receptors (Matsumoto et al., 2006) • Activates descending noradrenergic and serotonergic pathways in spinal cord (Matsumoto et al., 2005) • Stimulates postsynaptic α2-adrenergic receptors (Takayama, 2004) • Blocks stimulation of 5-hydroxytryptamine2A receptors (Takayama et al., 2002)
7-Hydroxymitragynine		• 13- and 46-fold higher potency than morphine and mitragynine, respectively (Horie et al., 2005) • Potency and quick-acting characteristics may be caused by introduction of –OH group on C7 position(Matsumoto et al., 2006) • Induces clinically significant antinociceptive responses in a dose-dependent manner(Takayama, 2004, Takayama et al., 2002)

mitragynine evaluated in mice shows that mitragynine induces analgesic activity via opioid receptors (Takayama, 2004).

In the central nervous system, the opioid receptors particularly the mu-opioid receptor (MOR), are among the longest and most intensely studied molecular signaling systems (Pasternak and Pan, 2013). In human, MOR plays several role in chronic and acute pain management, diarrhea and cough (Beck et al., 2019, Kaserer et al., 2020). However, it often results in a plethora of well-described side-effects which includes constipation, sedation, vomiting, nausea, respiratory depression, tolerance and addiction (Beck et al., 2019). In 2016, study conducted by Kruegel and co-workers using bioluminescence resonance energy transfer (BRET) functional assays has revealed the activity of 7-hydroxymitragynine, paynantheine, mitragynine, speciociliatine and speciogynine in HEK cells expressing human δ-opioid receptor, κ-opioid receptor (KOR) and μ-opioid receptor (MOR). In these G protein-dependent assays, the Gα subunit is fused with a luciferase (RLuc8) donor, and the Gγ subunit is fused with a fluorescent protein (mVenus) acceptor. Thus, on receptor activation, the G protein subunits move apart and the observed BRET signal decreases (Kruegel et al., 2016). By inhibiting the activity of the reference agonist, it was demonstrated that both 7-hydroxymitragynine and mitragynine are partial agonists of MOR. Other major kratom alkaloids including paynantheine, speciociliantine and speciogynine were both weak antagonists of MOR. On the other hand, 7-hydroxymitragynine and mitragynine were competitive antagonists of KOR (Kruegel et al., 2016).

In addition, mitragynine shows a biased MOR agonist by activating GPCRs instead of β-arrestin (Halpenny, 2017). This compound is of interest as an analgesic, because silencing the β-arrestin pathway can reduce adverse reactions, including constipation, tolerance, and respiratory depression. In contrast, β-endorphin and

morphine are balanced MOR agonists that activate both β-arrestin and GPCR pathways (Halpenny, 2017, Kruegel et al., 2016). Meanwhile, Váradi and co-workers demonstrated that mitragynine pseudoindoxyl and C-9 substituted derivatives thereof are MOR agonist/DOR antagonists *in vitro* (Váradi et al., 2016). Mitragyna pseudoindoxyl displayed the highest binding affinity to murine MOR and DOR and moderate affinity for KOR. 7-Hydroxymitragynine showed a moderate affinity for MOR, which is 5 fold that of mitragynine. Mitragyna pseudoindoxyl C-9 derivatives (-H, -OH, -CN, -Ph, -furan-3-yl, -OAc) usually maintained MOR and DOR affinity, but substitution of N-1 (-benzyl Base, -Me) position eliminates the affinity of opioids. These results indicate that indole NH is important for receptor binding. These binding affinities are consistent with the observed DOR antagonism and MOR agonism. It is worth noting that these derivatives also exhibit a biased MOR agonistic effect and will not recruit β-arrestin-2 (Váradi et al., 2016). The protective clearance process that normally eliminates malformed protein cells (such as disease-causing tau) has been disrupted by β-arrestin-2 molecules (also called oligomeric β-arrestin-2). β-arrestin-2 monomer is a single-molecule form of protein and will not harm this cytotoxic waste disposal process called autophagy. In this way, the blockade of microglial inflammatory neurotoxic signal effectors triggered by receptor internalization reveals the new physiological role of β-arrestin in neuroprotection (Feng et al., 2014). Nonetheless, this finding has suggested that mitragynine has an effect at the central nervous system.

In 2017, Halpenny and co-workers further investigate the activity of mitragynine at human MOR using mitragynine derivatives that were synthesized. The manipulation of chemical groups of the derivatives has demonstrated the potency of the compound activity on human MOR which seems to be the key structural features of

mitragynine (Halpenny, 2017). These findings were in agreement with Takayama's structure–activity relationship studies in guinea pig ileum using mitragynine derivatives of which modification of these compound structure at the β-methoxyacylate moiety and the group of C9 decreased the potency compared to mitragynine (Takayama, 2004). In addition, the psychostimulants-like and opiods effects of mitragynine that primarily linked to action of the substance/molecules can also evoke toxicity within the CNS. For example, the enhancement of synaptic concentrations of dopamine has been linked to mitragynine, in addition to indirect effects such as the increase in extrasynaptic glutamate levels and toxic metabolites (Niedzielska et al., 2014). However, it is unclear how the exact mechanism of neurotoxicity occurs.

Clearly, kratom owns benefit with broad range of therapeutic potential applications. As a whole, it has been suggested that kratom is a promising source of analgesic candidates, though this assertion awaits confirmation in human subjects. Several data also suggested that kratom has the potential to prevent opioid overdoses, a rising killer in diverse segments of the U.S. population (Halpenny, 2017). While researchers continuing to study kratom and its alkaloids, strategies should be developed to prevent and manage kratom addiction. Despite the benefit of this plant possesses, drug-herb interactions involving mitragynine is still highly possible and the concerns of its safety remained unanswered.

2.1.3 Safety issues with kratom and mitragynine

Several case reports have been published with regards to drug-herb interactions involving mitragynine. It is important to note that in each of these case reports, the patients may have exisiting confounding health conditions and they may have been

using other drugs along with kratom, or both. An increasing number of case reports describe abnormal adverse reactions in patients using kratom or kratom-based products. It seems that kratom's stimulation and opioid activity may cause many users to suffer acute adverse effects of kratom (Babu et al., 2008, Jansen and Prast, 1988a, Jansen and Prast, 1988b, Prozialeck et al., 2012, Suwanlert, 1975). Stimulant effects may manifest in some people as irritation, increased aggression, anxiety and opioid-like effects, including nausea, constipation, sedation and itching. Again, these effects seem to be significantly different for everyone and are related to dosage. High doses and long-term use have been associated with several unusual effects. In chronically addicted individuals, tremor, anorexia, psychosis, hyperpigmentation of the cheeks and weight loss have been observed (Suwanlert, 1975). Case reports of severe toxic effects are rare and usually involve relatively high doses of Kratom above 15 grams (McWhirter and Morris, 2010, Nelsen et al., 2010, Suwanlert, 1975). Of particular concern is that there are several individual case reports of seizures, which were found to be caused by high-dose kratom alone or in combination with other drugs such as modafinil (Boyer et al., 2008, McWhirter and Morris, 2010, Nelsen et al., 2010).

An example of case report is a 64-year-old male, who regularly used kratom to self-medicate his chronic pain, was found to have seizures and later unconscious (Nelsen et al., 2010). In another case report of a 25-year-old man, a liver biopsy has confirmed that kratom induced intrahepatic cholestasis and mitragynine was detected in both urine and serum samples (Kapp et al., 2011). Furthermore, a 44-year-old man consuming approximately 40 g of kratom divided into 4 doses over 24 hr was admitted for kratom detoxification and this patient has a history of alcohol dependence, depression and other substance misuse. Even if the patients regularly consumes kratom, the patient also experiences withdrawal symptoms, which indicates that the

short half-life of the active substance in kratom and the dependence syndrome are mainly achieved through agonist activity on opioid receptors (McWhirter and Morris, 2010). In addition, due to consumption of mitragynine and tramadol, 9 unintentional deaths were reported (Kronstrand et al., 2011). Domingo and co-workers reported a young man with history of drug addiction who was pronounced dead with a very high blood concentration of mitragynine that produce sedative effects. An autopsy confirmed a fracture of the left humerus. Mitragynine seems to relieve the pain, which explained the lack of urgency to seek medical attention by the subject (Domingo et al., 2017). A fatal reaction was reported due the combination of mitragynine with an amphetamine-like stimulant and propylhexedrine an α agonist (Holler et al., 2011). The extracts and tinctures containing purified 7-acetoxymitragynine, 7-hydroxymitragynine and mitragynine highlight the fact that this can be purchased via the Internet. These sources can be easily found by conducting an Internet search using the term “mitragynine purchase” (Prozialeck et al., 2012). Nonetheless,the possibility that these highly concentrated mitragynine alkaloid extracts could be used in conjunction with other psychoactive drugs such as alcohol, sedatives, opioids, stimulants, cannabinoids, raises the potential for serious drug interactions.

The safety use of kratom and its effects from long-term administration with regards to drug interactions are largely underdetermined. The lack of understanding of how substances in kratom may interact with herbal supplements, drugs of abuse and prescription medications is one of the major problems in evaluating the potential uses and safety of herbal agents in particular, kratom. Clearly, the existing evidences showed that mitragynine is possibly involved in drug-herb interactions leading to severe adverse effects. However, its role in causing drug-herb interacions has yet to be determined.

2.2 Drug-drug interactions

A drug-drug interaction occurs when the administrated drugs interact with another substance or other drug, and this action can be synergistic or antagonistic which subsequently produced side effect (Gupta et al., 2017, Palleria et al., 2013). Typically, a drug-drug interactions can occur, however, drugs and medicinal plants or herbs (drug-herb interactions) and drugs and foods (drug-food interactions) interactions may also exist (Bushra et al., 2011, Gardiner et al., 2008). Drug-drug interaction (DDI) is defined as a pharmacological or clinical response/effects that has been initiated when two or more drugs are administrated concurrently and when one of the drug affects the effectiveness of another leading to either toxicity or treatment failure (Chen and Raymond, 2006, Rodrigues et al., 2015, Rodrigues et al., 2017). In clinical practice, drug-drug interaction are a common problem during drug treatments. drug-drug interaction can reduce clinical efficacy of the drugs or induce development of adverse drug reactions (Palleria et al., 2013).

2.2.1 Pharmacodynamics drug-drug interactions

Pharmacodynamics drug interactions that altered pharmacological actions at standard plasma concentration often resulted from competition between two agents for a common target molecule in the body (Campbell and Cohall, 2017, de Leon and Spina, 2018, Maxwell, 2016, Palleria et al., 2013, Spina et al., 2016). If drugs with similar pharmacological effects are used, additive or synergistic effects may occur (Italiano et al., 2014). To improve therapeutic outcome, such situations are often exploited. On the other hand, if additive or synergistic interactions involve adverse or toxic effects, it may greatly increase the likelihood of organ damage or other serious consequences. These types of interactions are usually caused by overlapping adverse

reactions that can be tolerated when a single drug is administered, but become obvious when a second drug is introduced (Campbell and Cohall, 2017). Pharmacodynamics drug-drug interactions are divided into three subgroups based on the interference with physiological or biological control processes, the mechanism either by direct effect at receptor function or additive or opposed pharmacological effect (Corrie and Hardman, 2017). The ability of diverse xenobiotics to interact with different receptor sites and alter the physiological environment can also partly explain pharmacodynamics drug-drug interactions. The pharmacologic response depends on the drug binding to its target. The concentration of the drug at the receptor site influences the drug's effect (Campbell and Cohall, 2017). Meanwhile, disorders that affect pharmacodynamics responses as a result of drug-drug interactions include genetic mutations, some forms of insulin-resistant diabetes mellitus, malnutrition, Parkinson disease, thyrotoxicosis and myasthenia gravis. These disorders can decrease receptor sensitivity, alter the level of binding proteins or change receptor binding (Farinde, 2019). Aging tends to affect pharmacodynamics responses through alterations in receptor binding or in post-receptor response sensitivity.

2.2.2 Pharmacokinetics drug-drug interactions

Pharmacokinetics drug-drug interactions is defined as changes in the absorption, distribution, metabolism or excretion of a drug and/or its metabolite(s) after the co-administration of another drug (Spina et al., 2016). These interactions affect the way xenobiotics or drugs that are absorbed, distributed, metabolized and excreted, resulting in altered plasma drug concentration (Li et al., 2019, Spina et al., 2016). Over the past decade, drug metabolite is known as a major contributor in drug-drug interactions. However, other than metabolism, drug absorption, distribution, and

excretion are also shown to have important influences on pharmacokinetics, bioavailability, and consequently therapeutic efficacy of drugs (Faber et al., 2005, Meyer et al., 2015, Muller and Fromm, 2011). Both *in vitro* and *in vivo* studies have indicated that hepatic or intestinal drug-metabolizing enzymes and drug transporters equally contribute to pharmacokinetic interactions (Zhou et al., 2004, Zhou et al., 2003).

2.2.2(a) Interaction occurring during drug absorption and distribution

Drug absorption refers to the movement of the drug from its site of administration (either via gastrointestinal tract or skin) into the blood circulation (Bermejo and Gonzalez-Alvarez, 2010). The accumulated drug in the blood eventually being distributed throughout the body and the effects of the administrated drug will takes place at the target site. Drugs absorption occurs via passive diffusion, facilitated diffusion or active transport by protein transporters into the blood circulation before distribution takes place (Artursson and Karlsson, 1991, Artursson et al., 2001, Chillistone and Hardman, 2014). Due to the larger surface area of the intestine, oral administration of drugs will be absorbed in the lower part of gastrointestinal tract (jejunum and ileum of the small intestine and large intestine) (Bergstrom et al., 2014). This process occurs via the enterocytes lining the intestinal tract. Enterocytes are polarized simple columnar epithelial cells with microvilli on the apical surface of the cells and joined together by tight junctions (Snoeck et al., 2005). However, in the presence of drugs, the drugs will rapidly efflux from gastrointestinal and thus alter the rate of delivery of other drugs to the site of absorption and influence the uptake activity (Corrie and Hardman, 2017).

The most common mechanisms of interaction occurring during absorption are alterations in the intestinal cytochrome-P450 isozyme activity, the active and passive intestinal transport, gastric pH, intestinal motility, intestinal P-gp activity, gastric emptying, and intestinal blood flow (Brown and Kashuba, 2011). In addition, absorption across the intestinal membrane is also affected by other protein transporters on both luminal and basolateral membrane. These transporters can be either influx or efflux type of transporters (Chillistone and Hardman, 2014). Other than P-gp, other drug transporters such as solute carrier, organic cation transporter and organic anion transporter which are also abundantly expressed in the drug's absorption site that are responsible for maintaining balanced absorption in the intestine (Tsuji, 2002).

Typically, drugs aided by plasma proteins are being circulated and transported in the blood and distributes to the whole body system. The most important plasma proteins that interact with drugs are albumin, α-acid glycoprotein, and lipoproteins (Palleria et al., 2013). Basic drugs are usually bound more extensively to α-acid glycoprotein, lipoproteins, or both while acidic drugs are usually bound more extensively to albumin (Sudlow et al., 1976). At the active site, unbound drugs can be used to passively diffuse outside blood vessels or tissue sites. Albumin is synthesized in the liver and distributed in both plasma and extracellular fluids of skin, muscles and various tissues and it represents the most prominent protein in plasma (Sudlow et al., 1975, Sudlow et al., 1976). For instance, coadministration of diclofenac and warfarin has typical cause pharmacological displacement. Diclofenac and warfarin have same affinity for albumin, therefore the administration of diclofenac to patients treated with warfarin for a long time will cause the latter to shift from its binding site. This drug interaction eventually leads to an increase in the plasma concentration of free warfarin, leading to the development of severe bleeding reactions (Palleria et al., 2013).

2.2.2(b) Interactions occurring during drug metabolism

Drug metabolism and drug excretion represent the detoxification processes that protect the human body from xenobiotics and their toxic metabolites (Leslie et al., 2005). Generally, drug metabolism or biotransformation is a chemical alteration of the drug in the body (Peng and Zhong, 2015). Metabolism terminates the action of many drugs, creating water soluble metabolites suitable for excretion. Liver is the primary site for drug metabolism and this process can be divided into two phase, which are phase I and phase II metabolisms (Konstandi et al., 2014, Nowak et al., 2014). Hepatic detoxification is generally initiated through the uptake of xenobiotics into liver hepatocytes, via uptake transporter followed by phase I metabolism such as reduction, oxidation and hydrolysis. Phase I reactions take place in the endoplasmic reticulum of hepatocytes, often using CYP450 enzyme system.

Potential drug interactions mechanisms involving metabolism include genetic polymorphism, inhibition, and induction of enzyme activity (Kashuba and Bertino, 2005). Nonrandom genetic modification generates polymorphisms which occurred in at least 1% of a population and give rise to distinct subgroups that differ in their ability to metabolized xenobiotics (Daly et al., 1998). For instance, several agents may induce or inhibit this non-specific enzyme system. For induction of enzyme activity, it generally happens with an increase in CYP450 synthesis either by mRNA stabilization or receptor-mediated transcriptional activation. Barbiturates, for example, will induce the enzyme system. A concomitant administration and susceptible drug will be metabolized more efficiently leading to a shorter half-life, reduced serum levels, and reduced clinical efficacy (Corrie and Hardman, 2017). On the other hand, for the inhibition of enzyme activity, there are several mechanisms of inhibition exist such as reversible and irreversible inhibition where reversible is the most common type of

enzyme inhibition. Reversible inhibition do not permanently disable the enzyme activity and it occurs when weak bonds were quickly form between compounds and CYP450 isozyme (Kashuba and Bertino, 2005). This reversible inhibition can occur both competitively and noncompetitively. In addition, there is also reversible inhibition due to the oxidation of inhibitor by a way of slowly reversible reactions (Thummel and Wilkinson, 1998). The formation of CYP-mediated reactive metabolite caused irreversible inhibition and this metabolite can covalently and irreversibly bind to the catalytic site residue which permanently inactivate the enzyme (Ho et al., 2015). Amiodarone is the example of drugs that inhibit this system. The biotransformation of warfarin, anticonvulsants and antidiabetic agents occurs via this pathway (Corrie and Hardman, 2017). Meanwhile, phase II metabolism occurs by conjugation processes such as glucuronidation, sulfation, and glutathione conjugation before excretion of xenobiotics and/or their metabolites into the bile or through renal excretion (Song et al., 2013).

2.2.2(c) Interactions occurring during drug excretion

Kidneys play an important role in drug excretion. ATP binding cassette and solute carrier transporter family of liver and kidney are known to play an important role in excretion and elimination of drugs and other foreign substances from the body (Le Vee et al., 2015). Generally, elimination can occur via glomerular filtration, tubular secretion, or a combination of both pathways. Recently, the role of these transporters in the excretion of drugs have drawn major attention because of their involvement in drug interactions (Moss et al., 2014). Solute carrier transporter family can be subdivided into cationic transporters, anionic transporters, and other transporters. These solute carrier transporters family are expressed in both apical and

basolateral membrane of the proximal tubule cells and control the entry of xenobiotics into the epithelial cells (Morrissey et al., 2013). Meanwhile, ATP binding cassette transporter help to eliminate and excrete xenobiotics and endogenous compounds across the apical membrane of hepatocytes, the brush border membrane of enterocytes, in proximal tubule cells in kidney, and in capillary endothelial cells at the blood-brain barrier (Giacomini et al., 2010, van Montfoort et al., 2003).

There are five potential mechanisms of drug interactions affecting excretion at the site of renal elimination (Bonate et al., 1998). Changes in renal blood flow, cardiac output, and extend of protein binding will affect rates of glomerular filtration which disturb the normal excretion process (Kashuba and Bertino, 2005). However, the most common renal drug interactions occur at the transport site of tubular secretion. Many organic anions and cationic drugs and metabolites compete with each other for secretion as they are sharing the same proximal tubular active transport system (Nigam et al., 2015). In addition, inhibition of renal P-gp which has been identified in the apical membrane of the proximal tubule leads to an increase in plasma drug concentrations and potentially contribute to significant drug interactions. In tubular reabsorption, changes in the urinary pH can alter the reabsorption process but these interactions are not clinically significant (Moss et al., 2014, Yin and Wang, 2016).

2.3 Blood brain barrier

The blood brain-barrier (BBB) is the interface between blood and brain that is located within the capillary endothelium and controls what goes into and comes out of the central nervous system (CNS). It comprises of two membranes; the luminal and abluminal membranes of the brain capillary endothelium, which are separated by about 200 nm of endothelial cytoplasm (Pardridge, 2012). However, BBB lack of