PHASE I AND PHASE II DRUG METABOLISM STUDY OF MITRAGYNINE IN NORMAL AND DIABETES INDUCED RAT

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PHASE I AND PHASE II DRUG METABOLISM STUDY OF MITRAGYNINE IN NORMAL AND DIABETES INDUCED RAT

By

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With the name of Allah, Who is utmost kind and merciful

Dedicated to my beloved children Muhammad Hamza Ihtisham,
Muhammad Usman Abdullah and Afifa Noor

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All praise goes to Almighty Allah who showed me the path to seek the knowledge. Only Allah is “Aleem ul Hakeem” and man’s knowledge is negligible. He gives the pearls of knowledge to whom He wants.

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Rukhsana Anwar

Universiti Sains Malaysia

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LIST OF SYMBOLS AND ABBREIVIATIONS

Ad libitium	To be taken as wanted
Acetyl COA	Acetyl coenzyme A
ANOVA	One way analysis of variance
APND	Aminopyrine N-demethylase
ATP	Adencine-5´-triphosphate
BSA	Bovine serum albumim
BW	Body weight
Ca/CaM	Calcium calmodulin complex
Ca^{2+}	Calcium ion
CAR	Constitutive androstane receptors
cAMP	cyclic adenosine-3´,5´-mono phosphate
cGMP	cyclic guanosine-3´,5´-mono phosphate
CDNB	1-chloro-2,4-dinitrobenzene
CYP450	Cytochrome P450
DAG	Diacylglycerol
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
G	Gram
GR	Endocrine receptors
µg/mL	Microgram per milliliter
mg/kg	Dose (weight of test substance in milligrams per kilogram of test animal)

Eq	Equation
FDA	Food and Drug Administration
Gα	Alpha sub-unit of guanine nucleotide regulatory protein
GC-MS	Gas Chromatography - Mass Spectrometry
GH	Growth hormone
GPCR	G-Protein coupled receptors
G-protein	A guanine nucleotide regulatory protein
GppNHp	Guanylylimidodiphosphate
GSH	Glutathione
GST	Glutathione S-transferase
GTP	Guanosine triphosphate
HBSS	Hank's Balanced Salt Solution
HCl	Hydrochloric acid
HIV	Human immunodeficiency virus
IBMX	3-isobutyl-1-methylxanthine
IC_{50}	Dose caused 50% inhibition in enzyme activity
i.p.	Intraperitonal
IP_3	Inositol-1,4,5-triphosphate
i.v.	Intravenous
KCl	Potassium chloride
KT5720	cAMP dependent protein kinase inhibitor
KT5823	cGMP dependent protein kinase inhibitor
LD_{50}	Dose caused 50% lethality population

L-NIO	L-N^5-(1-Iminoethyl)- ornithine
mL	Milliliter
mmol/L	Millimol per liter
mM	Millimolar
µM	Micromolar
mRNA	Messenger ribonucleic acid
M. speciosa	*Mitragyna speciosa*
NaCl	Sodium chloride
Na_2CO_3	Sodium carbonate
NADPH	Nicotinamide adenine dinucleotide phosphate
NAK Tartrate	Sodium potassium tartrate
NATs	N-acetyltransferases
NAOH	Sodium hydroxide
NSAID	Non-steroidal anti-inflammatory drug
NO	Nitric oxide
PDE	Phosphodiesterase enzyme
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase G
PMA	4β-phorbol-12β-myristate-13α-acetate
p-NP	*p*-nitrophenol
p.o.	Oral administration
PPA	Phosphatase A
PVDF	Polyvinylidene difluride

S.D	Standard deviation
SD	Sprague Dawley
STZ	Streptozotocin
SULTs	Sulphotransferases
TCA	Tri chloracetic acid
TCM	Traditional Chinese medicines
UDPGA	UDP-glucuronic acid
UGT	UDP-glucuronosyltransferase
UV	Ultraviolet
vs	Versus
v/v	Volume per volume
V_{max}	Maximum velocity
WHO	World Health Organization
w/v	Weight per volume

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LIST OF PUBLICATIONS

1. Anwar, R., Hussin, A. H., Ismail, S., & Mansor, S. M., (2012). *In vitro* effect of mitragynine (a major alkaloid of *Mitragyna speciosa* Korth) on aminopyrine metabolism in rat hepatocytes. *International Journal of Pharmaceutical Sciences and Research*, 3. 2238-2242.

2. Anwar, R., Hussin, A. H., Ismail, S., & Mansor, S. M., (2012). *In vitro* effect of mitragynine on activity of drug metabolizing enzymes, N-demethylase and glutathione S-transferase in streptozotocin-induced diabetic rats. *Pharmacologyonline, 1*, 68-75.

LIST OF ABSTRACTS

1. Anwar, R., Hussin, A. H., Ismail, S., & Mansor, S. M., (2012). Possible mechanism of aminopyrine N-demethylase activity by mitragynine in rat hepatocytes. Poster presented at the 26th Scientific Meeting of Malaysian Society of Pharmacology & Physiology, School of Pharmaceutical Sciences, Universiti Sains Malaysia.

2. Anwar, R., Hussin, A. H., Ismail, S., & Mansor, S. M., (2012). *In vitro* effect of mitragynine on phase II drug metabolizing enzyme glutathione S-transferase in female Sprague-Dawley rats. Paper presented at the International Conference on Research Methodology: New trend and integrative approach from bench to bedside, University College of Pharmacy, Pakistan.

FASA I DAN FASA II METABOLISME DRUG MITRAGYNINE DALAM TIKUS NORMAL DAN YANG DIARUH DIABETES

ABSTRAK

Mitragynine ialah alkaloid indol utama yang kebanyakannya diasingkan daripada daun *M. speciosa*. Mitragynine mempamerkan kesan depresan seperti morfina dan kesan perangsang seperti kokain. Mitragynine juga menunjukkan kesan seperti antitusif dan antidepresan. Penyelidikan ini dijalankan untuk menilai kesan mitragynine secara *in vitro* dan *in vivo* pada fasa I dan fasa II enzim metabolisme drug, iaitu aminopyrine N-demethylase (APND), UDP-glukuronosil transferase (UGT) dan glutation S-transferase (GST) dalam hati tikus Sprague-Dawley (SD). Kesan *in vitro* mitragynine pada aktiviti APND dibawah pengaruh umur, jantina dan penyakit telah ditentukan dalam hepatosit tikus. Selain itu, mekanisme yang mungkin untuk induksi aktiviti aminopyrine N-demethylase oleh mitragynine juga dikaji pada hepatosit tikus SD jantan dan betina. Analisis blot western telah dilakukan bagi enzim fasa I (CYP2C12) dan enzim fasa II (UGT1A6 dan GSTM1) untuk ekspresi protein dalam tikus normal dan tikus diabetik. Aminopyrine, *p*-nitrofenol dan 1-kloro-2,4-dinitro benzena telah digunakan sebagai substrat prob untuk menentukan aktiviti APND, UGT dan GST masing-masing. Dalam kajian *in vitro,* satu julat kepekatan mitragynine (0.0025 - 250 μM) telah digunakan untuk semua ujian enzim pada kumpulan tikus SD yang diuji. Dos oral mitragynine 50 mg /kg berat badan telah diberikan kepada tikus SD mengikut tempoh rawatan untuk kajian *in vivo.* Keputusan daripada kajian *in vitro* menunjukkan bahawa mitragynine meningkatkan aktiviti APND dengan signifikan ($p < 0.05$) dalam hepatosit tikus. Ia juga merencat aktiviti UGT dalam mikrosom dan aktiviti sitosolik

GST dengan signifikan dalam tikus biasa dan tikus diabetik. Secara *in vitro* perencatan aktiviti enzim UGT dan GST adalah kurang daripada 70% oleh itu nilai untuk IC_{50} mitragynine tidak dapat ditentukan. Keputusan daripada kajian *in vivo* menunjukkan bahawa rawatan sub-kronik mitragynine menginduksi aktiviti APND, UGT dan GST dengan signifikan ($p < 0.05$) pada tikus biasa dan tikus diabetik. Mekanisme yang mungkin untuk kesan induksi daripada mitragynine ke atas metabolisme aminopyrine dalam hepatosit tikus SD jantan dan betina adalah mungkin melalui perencatan G-protein, protein kinase A, protein kinase G dan tyrosine kinase dalam laluan cAMP. Tambahan lagi, analisis Western blot untuk ekspresi protein CYP2C12, UGT1A6 dan GSTM1 dalam tikus normal dan tikus diabetik yang dirawat dengan mitragynine (50 mg/kg selama 14 hari) menunjukkan peningkatan yang signifikan. Kesimpulan daripada kajian ini menunjukan bahawa pengambilan mitragynine secara sub-kronik membawa kepada peningkatan aktiviti enzim metabolisme drug dan paras protein. Faktor jantina dan penyakit tidak mempunyai pengaruh ke atas aktiviti APND, UGT dan GST secara *in vitro* dengan kehadiran mitragynine. Kemungkinan wujud bahawa mitragynine boleh berinteraksi dengan ubat-ubatan yang merupakan substrat kepada enzim N-demethylase dan UGT1A6.

PHASE I AND PHASE II DRUG METABOLISM STUDY OF MITRAGYNINE IN NORMAL AND DIABETES INDUCED RAT

ABSTRACT

Mitragynine is a major indole alkaloid abundantly isolated from *M. speciosa* leaves. Mitragynine exhibits psychoactive properties and shows morphine-like depressant effect and cocaine-like stimulant effects. Mitragynine also shows antitussive and antidepressant-like effects. The current research was undertaken to evaluate the *in vitro* and *in vivo* effect of mitragynine on phase I and phase II drug metabolizing enzymes, namely aminopyrine N-demethylase (APND), UDP-glucuronosyl transferase (UGT) and glutathione S-transferase (GST) in normal and diabetes Sprague-Dawley (SD) rat liver. The *in vitro* effect of mitragynine on APND activity in relation to factors of age, gender and diabetes was determined in rat hepatocytes. Moreover, the possible mechanism of induction of aminopyrine N-demethylase activity by mitragynine was also investigated in SD rat hepatocytes. In addition, western blot analysis was carried out for *in vivo* CYP2C12 and UGT1A6 and GSTM1 proteins expression in normal and diabetic rats. Aminopyrine, *p*-nitrophenol and 1-chloro-2,4-dinitro benzene were used as a probes to determine the APND, UGT and GST activity respectively. A range of mitragynine concentration (0.0025 – 250 µM) was used for all *in vitro* enzyme assays in tested groups of SD rats. The mitragynine 50 mg/kg of body weight was administered orally to SD rats for *in vivo* study. Results of *in vitro* study showed that mitragynine significantly ($p < 0.05$) enhanced APND activity in rat hepatocytes as well as significantly inhibited microsomal UGT and cytosolic GST activity in normal and diabetic rats. *In vitro*

inhibition of UGT and GST enzyme activity was less than 70% and the IC_{50} values for mitragynine could not be determined. I*n vivo* study showed that sub-chronic treatment of mitragynine significantly ($p < 0.05$) induced APND, UGT and GST activity in normal and diabetic rats. The possible mechanism of the induction effect of mitragynine on aminopyrine metabolism in male and female SD rat hepatocytes was probably mediated through inhibition of G-protein, protein kinase A, protein kinase G and tyrosine kinase in cAMP pathway. Furthermore, Western blot analysis showed that mitragynine increased significantly the CYP2C12, UGT1A6 and GSTM1 proteins level in mitragynine (50 mg/kg for 14 days) treated normal and diabetic rat livers. It is concluded from the present study that sub-chronic administration of mitragynine to normal and diabetic rats leads to enhanced hepatic drug metabolizing enzymes activities as well as their proteins level. The gender, age and disease factors have no influence on APND, UGT and GST activity *in vitro* in the presence of mitragynine. A possibility exists that mitragynine can interact with drugs which are substrates for N-demethylase and UGT1A6 enzyme.

CHAPTER 1

INTRODUCTION

Mitragynine is an important and major alkaloid derived from *Mitragyna speciosa* leaves. *Mitragyna speciosa* (*M. speciosa*) plant belongs to family Rubiaceae and is found in tropical and subtropical regions of Asia. *M. speciosa* has been traditionally used to treat opioid withdrawal symptoms, as an antipyretic and an analgesic. Additionally, it was also observed that Malaysian people consumed *M. speciosa* leaves as an opium substitute (Burkill, 1935). Chronic use of *M. speciosa* develops tolerance (Suwanlert, 1975; Pasternak, 2001) perhaps; withdrawal symptoms are less intensive than morphine (Vicknasingam et al., 2010). Mitragynine was isolated from *M. speciosa* leaves in 1921 (Tsuchiya et al., 2002) and is responsible for most of the plant's pharmacological effects. A study has described the psychoactive properties of mitragynine and quoted that mitragynine can minimize opioid withdrawal syndrome (Jansen and Prast, 1988). Mitragynine produces analgesia by acting on mu (μ) and delta (δ) opioid receptors (Takayama et al., 2002). Opioid receptors are responsible for euphoria, analgesia and dependence. Mitragynine received wide attention by researchers due to its morphine-like depressant and cocaine-like stimulant effects. Different studies have shown that mitragynine exhibited antidepressant, antihypertensive, antitussive, antidiarrheal, smooth muscle relaxant and anti-inflammatory activities (Grewal, 1932; Matsumoto, 1996; Watanabe et al., 1997; Idayu et al., 2011). Furthermore, it also exhibits morphine-like effects on gastric acid secretion (Matsumoto et al., 2005) and inhibition of 2-deoxy-D-glucose-stimulated gastric acid secretion in rats (Tsuchiya et al., 2002).

Most of the previous studies on mitragynine were focused on its behavioral, cognitive, central nervous system effects, antinociceptive action and receptors involved to cause analgesia. There are some unresolved issues regarding the effect of mitragynine on drug metabolism including its effects on CYP450, UDP-glucuronosyltransferase and glutathione S-transferase enzymes. Efficacy and safety are important elements during drug or an herbal drug development and mainly depend on drug metabolism studies. It is essential to evaluate type of enzymes involved and how a candidate drug is being metabolized.

Herbal derived compounds are usually administered orally and acquire moderate to high concentration in the liver. The modulation of hepatic enzymes by herbal derived compounds serve as a potentially important mechanism by which the bioavailability of co-administered drug can be changed (Zhou et al., 2009). The superfamily CYP450 (CYP450) enzymes is partly responsible for metabolism of xenobiotics including: drugs, carcinogens, pollutants and steroids (Zhou et al., 2004). Several reports have been published regarding pharmacokinetic interaction that develops between natural compounds/ herbal products and western drugs used clinically simultaneously (Budzinski et al., 2000; Foster et al., 2003; Strandell et al., 2004). The alteration of drug metabolism by concomitant use of herbal medicines is significantly important. Most of the clinical adverse reactions are considered to be due to drug interactions responsible for the induction or inhibition of the drug-metabolizing enzymes (Woolf, 1999; Izzo, 2004). CYP450 inhibition usually develops rapidly, even after a single dose of inhibitor (Pelkonen et al., 1998; Cott, 2001). Enzyme inhibition decreases the metabolism of co-administered drug and enhances the plasma drug concentration. Subsequently, life threatening

circumstances may develop and lead to toxicity. For example, terfenadine (enzyme inhibitors) caused severe toxicity due to inhibition of CYP3A4 and has been withdrawn from the market due to its high possibility of drug-drug/herb interactions (Gottlieb, 1999). On the contrary to enzyme inhibition, CYP450 induction resulted in an increased synthesis of enzyme proteins. Enzyme induction by herbs depends upon the dose, duration and route of administration (Cott, 2001; Zhou et al., 2003). For example, St John's wort induced intestinal and hepatic CYP3A4 after two weeks of administration but short term administration had no effect on CYP3A4 activity (Wang et al., 2001). Subsequently, enzyme induction enhances the hepatic metabolism of those co-administered drugs that are metabolized by induced enzymes and plasma concentration of drug decreases with loss of efficacy (Lin and Lu, 1998). For instance, rifampicin (an enzyme inducer) can greatly decrease the plasma concentrations of triazolam and abolishes its effect (Villikka et al., 1997).

The propensity to cause herb-drug interactions when they are co-administered by herbs triggers the importance to study the potential interaction between modern drugs and herbs or herb derived compounds. To date, no study has been carried out to determine the *in vitro* and *in vivo* effects of mitragynine on phase I and phase II drug metabolism in relation to factors of age, gender and disease in Sprague Dawley (SD) rat hepatocytes. The main objective of this study is to identify the existence of possible undesirable interaction effects of mitragynine on CYP450, UDP-glucuronosyltransferase (UGT) and glutathione S-transferase (GST) activity as well as to evaluate possible mechanism involve in this interaction. Moreover, the extent of *in vivo* sub-chronic effect of mitragynine on phase I and phase II drug metabolizing enzymes expression will be evaluated by Western blot analysis. To

evaluate the magnitude of phase I and phase II enzymes induction or inhibition by mitragynine, rat hepatocytes and liver subcellular fractions were used. For *in vitro* drug metabolism studies, a range of mitragynine concentrations (0.0025 – 250 μM) were used. For *in vivo* studies, mitragynine at 50 mg/kg of body weight will be orally fed to normal and diabetes induced SD rats.

1.1 Objectives of the Study

The aims of the study are:

i. To study the *in vitro* and *in vivo* effect of mitragynine on phases I metabolizing enzyme (aminopyrine N-demethylase) in different groups of rat hepatocytes.
ii. To study the *in vitro* and *in vivo* effect of mitragynine on phase II metabolizing enzymes, UDP-glucuronosyltransferase (UGT) and glutathione S-transferase (GST) using different groups of rat liver samples.
iii. To evaluate the possible molecular mechanism pathway involved in the effect of mitragynine on aminopyrine metabolism in rat hepatocytes *in vitro*.
iv. To investigate *in vivo* protein expression by Western blotting in normal and diabetic rat livers.

Chart 1.1: Experimental Design for the *In Vitro* Effect of Mitragynine on Aminopyrine N-demethylase (APND), UDP-glucuronosyltransferase (UGT), and Glutathione S-transferase (GST) activity in rat liver

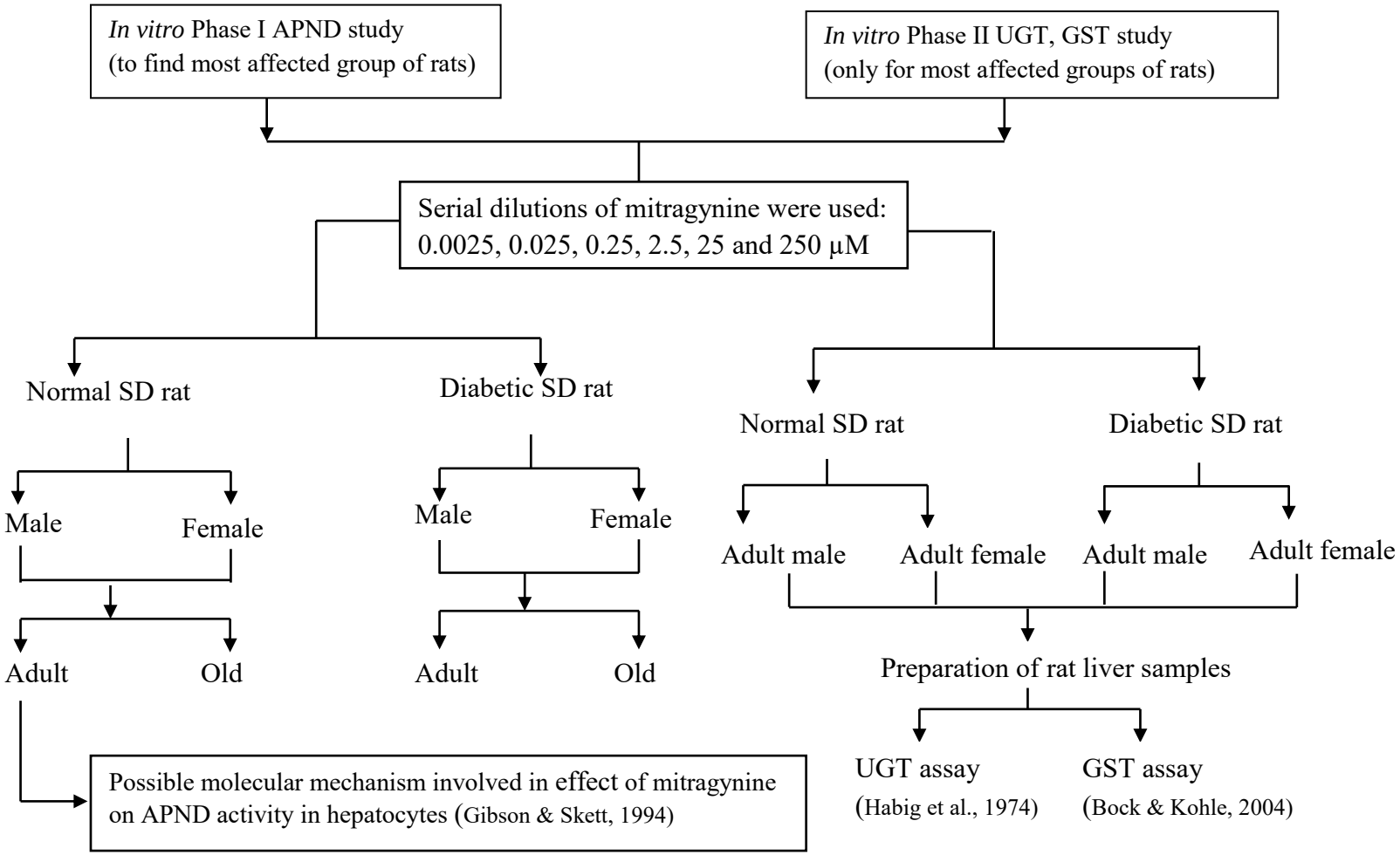
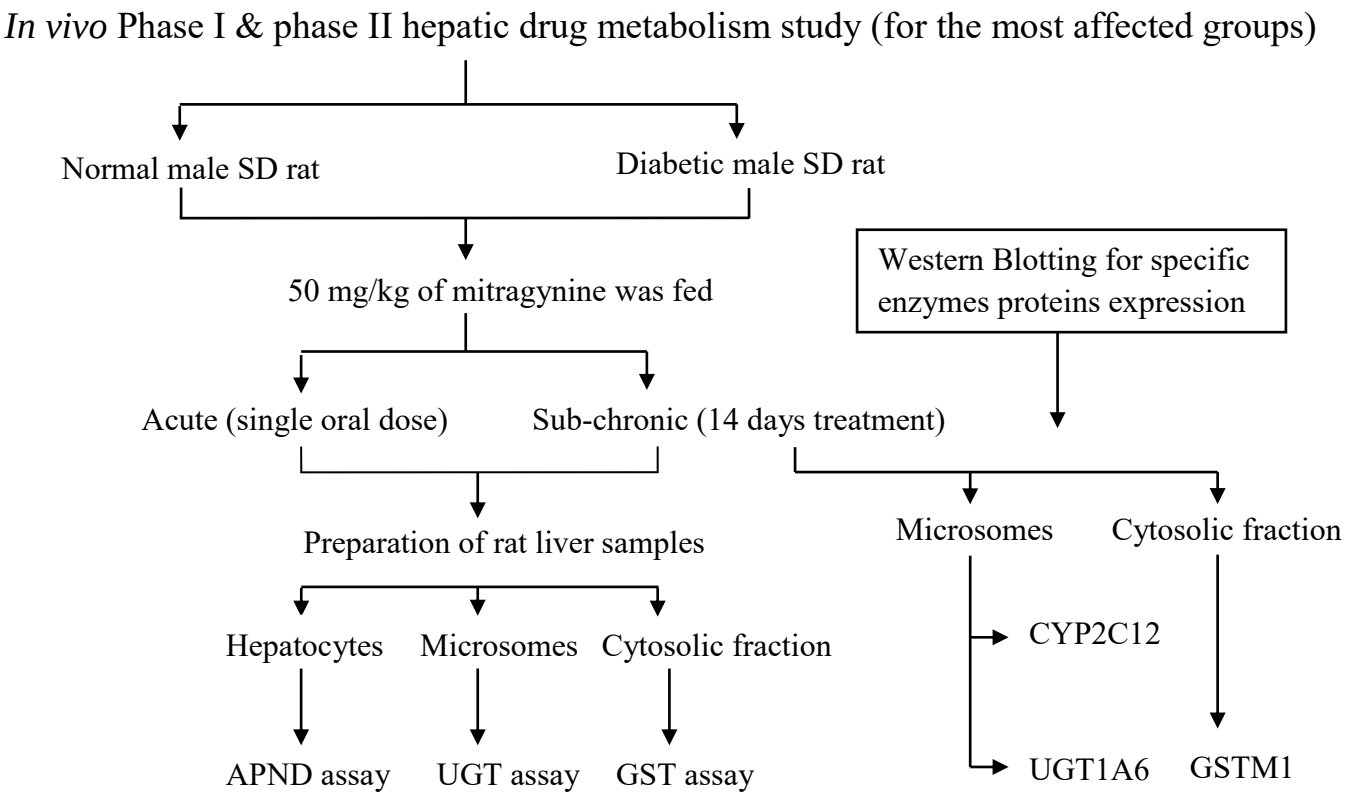


Chart 1.2: Experimental Design for the *In Vivo* Effect of Mitragynine on Aminopyrine N-demethylase (APND), UDP-glucuronosyltransferase (UGT), and Glutathione S-transferase (GST) activity in rat liver



9CHAPTER 2

INTRODUCTION AND LITERATURE REVIEW

2.1 Herbal Medicines

Traditional, alternative and complementary medicines are commonly used to treat illness or prevent chronic disease within local or regional healing practices and to improve quality of life. A herb or its parts are often used for flavor, scent or therapeutic value. According to Encyclopedia of Britannica, herbal medicine is also called phytomedicine or botanical medicine, are used as dietary supplement and for primary health care. Different part of herbal plant such as plant's seeds, flowers, berries, leaves, roots or bark are used for medicinal purposes. According to World Health Organization (WHO) definition, herbal medicines are herbs, herbal preparations, herbal materials, and finished herbal products. These herbal medicines may contain active ingredients from a single plant or may be a combination of more than one plant. Herbs and plants can be taken in different ways and forms, and they include the whole herb, teas, syrup, essential oils, ointments, capsules and tablets that contain a ground or powdered form of a raw herb or its dried extract.

The global popularity of herbal drugs and supplements has caused a remarkable interest in understanding the basis of the biological activity of traditional medicines. Traditional medicines have different system of practice, the practice and philosophy of each system is influenced by the customary conditions, geographic area and environment within which it first works out (WHO, 2005). Traditional Chinese medicine (TCM) is an influential example of how ancient knowledge is applied in a holistic way to treat health problems. TCM is more than 3000 years old

(Xutian et al., 2009) and is a flourishing practice around the world and is used for preventing and treating diseases. Three of the top-selling herbal products, namely *Allium sativum* (garlic), *Ginkgo biloba* and *Panax ginseng*, can be detected in TCM and are nowadays used to treat various ailments (Li et al., 2008).

There are certain factors that determine the high interest towards natural medicine, such as cost, adverse effects of allopathic drugs and lack of treatment in the many chronic diseases such as arthritis, diabetes, cancer and cardiac problems and in the infectious type diseases (HIV and hepatitis). Furthermore, traditional medicines are comparatively perceived as natural and without adverse effects, or not toxic. This is not compulsorily true, particularly when herbs are administered with other herbs or prescription drugs, as is very common (Canter and Ernst 2004; Loya et al., 2009). Currently, herbs are used to treat acute and chronic conditions of disease and treat problems such as prostate problems, inflammation, and depression and to boost the immune system. In China, herbal medicines played a dominant role in the strategy to treat severe acute respiratory syndrome (SARS) (WHO, 2003). Moreover, in Africa, the Africa flower, a traditional herbal medicine has been used to treat symptoms associated with HIV (Tilburt and Kaptchuk, 2008).

About 200 years ago, morphine was first extracted from seeds pod of *Papaver somniferum* and it has been administered in precise dosage forms up till now (Rousseaux and Schachter, 2003). Other novel examples include the cardiac stimulant digoxin from *Digitalis purpurea*, salicylic acid that is an origin of aspirin, isolated from willow bark, an antipsychotic and antihypertensive drug reserpine from *Rauwolfia serpentina*, antimalarial quinine, from Cinchona bark, antibiotics erythromycin, penicillin and lipid-lowering agents lovastatin from fungus (Rishton,

2008; Schmidt and Ribnicky, 2008). Worldwide, 70% of cancer drugs approved for treatment are derived from natural products. Cancer therapeutics derived from plants is paclitaxel, isolated from the Pacific yew tree and vinca alkaloids from *Catharanthus roseus* (Brower, 2008).

There are 252 drugs in the WHO essential medicine list and out of these 11% are of plant origin (Sahoo et al., 2010). Additionally, the WHO estimates that 80% of the population of some Asian and African countries utilizes herbal medicine for primary health care and the worldwide annual market for these products can reach US$ 60 billion (WHO, 2002). In the past, there has been renewed attention and interest in the use of traditional medicine globally. In Malaysia, traditional medicines have been used for centuries. However, this system is influenced by Chinese, Indonesian and Indian tradition (Zakaria and Mohd, 1994). It was reported in 1999 that Malaysian market for medicinal and herbal products together with aromatic plants was estimated 4.6 billion and the average annual growth was 15 – 20% (Jamal, 2006). In Malaysian peninsular region, 1,300 medicinal plant species were recorded (Burkill, 1966).

According to a WHO report, Malaysia is one of the nine countries that bestowed a large amount of herbal sales worldwide between the year of 1999 to 2001 (WHO, 2005). Moreover, the Malaysian market for natural and herbal products was estimated to cost approximately RM10 billion in 2008 and showed continuously increase at the rate of 8% per year. Hence, it is predicted that by 2050, the Malaysian market for these products would be 5 US trillion dollars (Ahmad, 2010). Examples of important Malaysian medicinal plants are *Labisia pumila* (Kacip Fatimah), *Eurycoma longifolia* Jack (Tongkat Ali), *Ficus deltoidea* (Mas cotek), and

Piper sarmentosum (Daun kaduk). These herbs have antioxidant, antibacterial, antipyretic, antitumor and anti-inflammatory activities (Zakaria and Mohd, 1994; Bhat and Karim, 2010; Zakaria et al., 2010).

2.2 Literature review of *Mitragyna speciosa* Korth and Mitragynine

2.2.1 *Mitragyna speciosa* Korth

In recent years, a new herbal drug that has appeared on the drug scene in Malaysia is *Mitragyna speciosa* (*M. speciosa*) which has a strong abuse potential and has been used for its opiate-like effects (Maurer, 2010). *M. speciosa* belongs to the family of Rubiaceae. It is found in tropical and subtropical regions of Asia.

2.2.1.1 Habitat

M. speciosa is indigenous to Malaysia, Thailand, Cambodia, East and West Africa and in India (Harvala, 1988). It is called ketum in Malaysia and kratom in Thailand (Shellard, 1978). In Malaysia and Thailand two varieties of *M. speciosa* are found, one plant contains red vein in the leaf and the other has green vein in its leaf. It is assumed that red vein variety possesses more potent biological activities than green vein plant (Chittrakarn et al., 2007).

Figure 2.1: *Mitragyna speciosa* Korth

The taxonomy of *Mitragyna speciosa* is:

Class: Plantae

Subclass: Magnoliophyta

Order: Gentianales

Family: Rubiaceae

Genus: *Mitragyna*

Species: *Speciosa*

Scientific name: *Mitragyna speciosa* Korth

2.2.1.2 Folk Usage

In Malaysia and Thailand the leaves of *M. speciosa* are often chewed, smoked or consumed as tea. In Thailand, kratom users focused on the perceived advantages in relation to increased work stamina and as medication rather than its harmful effects (Sawitri, 2007). Kratom have been reported to be a central nervous system stimulant. It also helps to increase work efficiency and tolerance to hard work under a burning sun (Suwanlert, 1975). Moreover, chronic exposure by human leads to withdrawal symptoms including yawning, myalgias, diarrhea and rhinorrhea (Suwanlert, 1975). In Malaysian folk medicine, the leaves are used to treat diarrhea, fever, as cough suppressant in asthma, antihypertensive, anti-inflammatory, pain killer and as substitute for opium or morphine in the treatment of drug addicts (Reanmongkol et al., 2007).

2.2.1.3 Pharmacological studies

The use of ketum as self-management of opioid withdrawal is increasing without proper scientific bases and is currently leading to its wide abuse. It is demonstrated that side effects and withdrawal symptoms such as craving, fatigue, insomnia and kidney complaints from ketum addiction are less intensive and less toxic than morphine (Vicknasingam et al., 2010). A number of pharmacological effects are evaluated by studies on aqueous, methanol and total alkaloid extracts of *M. speciosa*. Crude extract of *M. speciosa* has been reported that analgesic effect of extract was more pronounced than mitragynine (Watanabe et al., 1997). Methanol, aqueous and alkaloid extracts of *M. speciosa* exerted the antinociceptive response to hot plate and tail flick tests and was blocked with naloxone (Reanmongkol et al.,

2007; Sabetghadam et al., 2010). The extract inhibited the twitch contraction in a concentration dependent manner and had inhibitory effects on the rat gastrointestinal tract and therefore it could be used as antidiarrheal agent (Chittrakarn et al., 2007; Chittrakarn et al., 2010).

Alkaloid and aqueous extracts of *M. speciosa* have shown antidepressant-like (Kumarnsit et al., 2007). Moreover, the methanol extract of *M. Speciosa* showed antiinflammatory effect (Mossadeq et al., 2009) and GST specific activity inhibition in rat (Azizi et al., 2010). Subsequently, ethanol extract of *M.speciosa* leaves caused inhibition of CYP2D6 and CYP3A4 isoforms of human CYP450 (Hanapi et al., 2010). The LD_{50} values of the alkaloid and methanol extracts of *M. speciosa* in mice were 173.20 mg/kg and 4.90 g/kg respectively after oral administration. In addition, acute toxicity signs with *M. speciosa* extracts were tremor, lethargy, paralysis, apnea, tonic- clonic convulsions (Reanmongkol et al., 2007). High dose 1000 mg/kg of standardized methanol extract of *M. speciosa* leads to nephrotoxicity and hepatotoxicity in rat (Harizal et al., 2010). A rare case of *M. speciosa* exposure toxicity was reported and confirmed by urinary analysis of mitragynine (Nelson et al., 2010).

More than 25 alkaloids have been isolated from *M. speciosa* leaves. The main constituents are mitragynine, speciogynine, 7-hydroxymitragynine, paynanthein and speciocilitine. Out of these mitragynine is the major constituent i.e. 66.2% (Takayama, 2004). The molecular structures of main alkaloids of *M. speciosa* are shown in Figure 2.2.

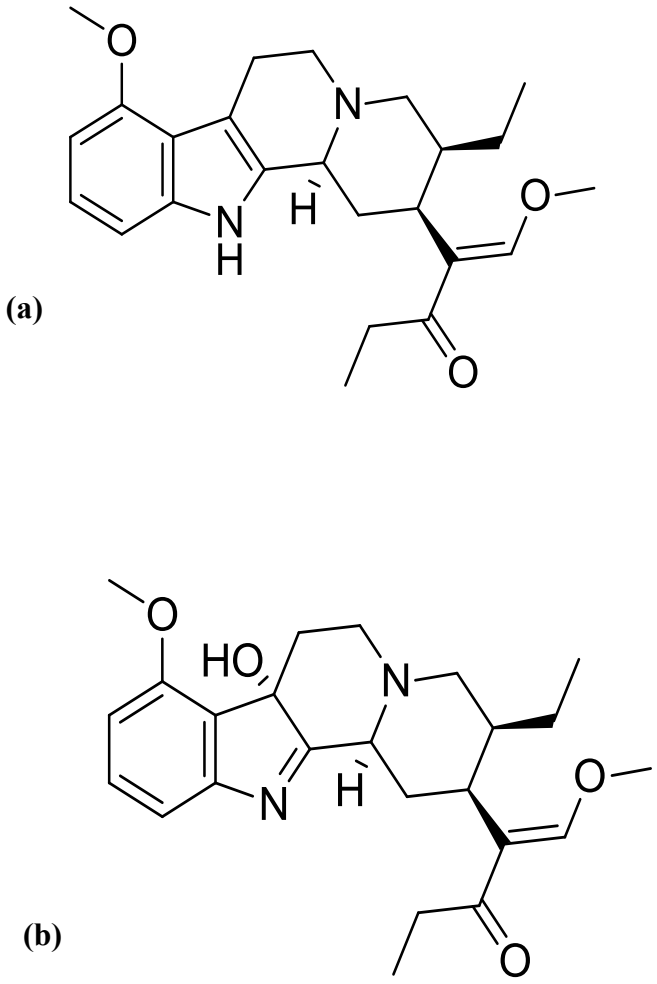


Figure 2.2: Molecular Structure of Important Alkaloids of *M. speciosa.*
(a) Mitragynine and (b) 7-hydroxymitragynine.

2.2.2 Mitragynine

Mitragynine is the most abundant active indole alkaloid isolated from *M. speciosa* leaves. Mitragynine was first isolated in 1907 by Hooper. The isolation was repeated again by Field (Tsuchiya et al., 2002) and gave the alkaloid its name. Its molecular structure $C_{23}H_{30}N_2O_4$ was fully determined by Zacharis et al., 1964. The first total synthesis of (-)-mitragynine in the optically pure form was done in 1995 (Takayama et al., 1995). Its molecular weight is 398.5. It is poorly soluble in water and soluble in alcohol, acetic acid and chloroform (Janchawee et al., 2007; Babu et al., 2008; Parthasarathy et al., 2013). The pharmacology and chemistry of mitragynine have been investigated since the early twentieth century.

2.2.2.1 Pharmacological Studies

In 1932, Grewal reported the pharmacology of mitragynine (Grewal, 1932). The study concluded that mitragynine exhibited antiprotozoal, intestinal smooth muscle relaxant, antihypertensive, antitussive and cellular oxidation inhibiting activities.

I. Receptor Affinity

Mitragynine is a full opioid agonist and has a high binding selectivity for μ-opioid receptors (Ma et al., 2007) and δ- opioid receptors (Yamamoto et al., 1999; Takayama et al., 2002). Pharmacologically, μ-opioid receptors comprised of three subtypes μ_1, μ_2 and μ_3-opioid receptors. The μ_1 –receptors are responsible only for analgesia while μ_2 –receptors produce respiratory depression, gastro intestinal transit and itching in addition to analgesia. Both μ_1, μ_2 -opioid receptors both are found in brain, spinal cord and periphery. The μ_3-opioid receptors are located in periphery

(Dietis et al., 2011). It has dual action; at lower doses, mitragynine is shown to act on descending noradrenergic and serotonergic receptors while at higher doses it acts on supraspinal μ- and delta δ-opioid receptors. The μ-opiate receptors are responsible for euphoria, analgesia and physical dependence. Descending noradrenergic and serotonergic systems were found to contribute towards the antinociceptive activity of mitragynine on mechanical noxious stimulation. Only descending noradrenergic system is involved for blocking thermal noxious stimulation by mitragynine (Watanabe and Matsumoto, 1996). Moreover, it has been reported that mitragynine stimulates post synaptic α_2 receptors and inhibits 5-HT_{2A} receptors (Matsumoto et al., 2005).

II. Antinociceptive Effect

Mitragynine has an antinociceptive activity and its action is dominantly mediated by μ- and δ- opioid receptors subtypes. It shows its activity when administered subcutaneously, orally, intravenously and intraperitoneally in rodents (Matsumoto, 1996). Mitragynine shows stronger analgesic effect and less respiratory depression and adverse effects than morphine (Watanabe et al., 1997; Matsumoto et al., 2008).

III. Effects on gastrointestinal smooth muscles

Mitragynine inhibits 2-deoxy-D-glucose- stimulated gastric acid secretion in rats through opioid receptors (Tsuchiya et al., 2002). In addition to inhibition of gastric acid secretion it also inhibited the electrically stimulated contraction of

guinea pig ileum through opioid receptors and did not block any effect on smooth muscle contraction by acetylcholine or histamine (Watanabe et al., 1997).

IV. Hypothermia

It was reported that mitragynine causes hypothermia in animals in addition to opiate-like effect (Jansen and Prast, 1988).

V. Effect on glucose transport

Mitragynine exhibits insulin –like effect and enhances the glucose transport in muscle cells. However this effect with *M. speciosa* extract is more pronounced suggesting that other constituents of plant together with mitragynine contribute this effect (Purintrapiban, 2011).

VI. Behavioral effect

Mitragynine (1 mg/kg – 30 mg/kg, i.p) inhibited the locomotor activity of the rat. This result suggested that mitragynine has sedative properties (Moklas et al., 2008). In addition, chronic administration of mitragynine significantly impaired the cognitive function in mice (Apryani et al., 2010). Moreover, mitragynine (5 mg/kg – 30 mg/kg, i.p) brought into play an antidepressant-like effect in tail suspension and forced swim test in mouse (Idayu et al., 2011). Acute oral administration of *M. speciosa* alkaloid extract and mitragynine in mice increased locomotion, rearing behaviour and reduced grooming time in a novel environment (Hazim et al., 2011).

VII. Pharmacokinetic studies

Mitragynine (50 mg/kg o.p; 1.5 mg/kg i.v) was administered to rat and its plasma concentration was determined. Different parameters were estimated namely $t_{1/2,}$ $AUC_{0-\infty}$ and bioavailability. Mitragynine oral absorption was prolonged, slow and incomplete with 3.03% absolute bioavailability (Parthasarathy et al., 2010). Philipp and his colleagues (2009) identified mitragynine metabolites in human and rat urine. It was reported that mitragynine was metabolized by hydrolysis of methylester to the respective aldehyde, reduction to alcohol or by oxidation to carboxylic acids. In rat, four metabolites were conjugated to glucuronides and one conjugated to sulphate while in human three metabolites conjugated to glucuronides and three to sulphates (Philipp et al., 2009). Isomeric compounds of mitragynine have been detected in urines of kratom addicts by LC-MS (Philipp et al., 2011). LD_{50} value for the mitragynine has been reported i.e. 477 mg/kg after oral administration (Sabetghadam et al., 2012) and 126.7 mg/kg followed by intraperitonally route in mice (Watanabe et al., 1992).

To date, the effect of mitragynine on phase I and phase II drug metabolizing enzymes (DMEs) are not available. Hence the present study was designed to evaluate its effects *in vitro* and *in vivo* on DMEs and its propensity to cause drug – drug interaction.

2.3 Hepatic Drug Metabolism

Drug metabolism occurs in liver, kidney, lungs, intestines, brain and skin. Liver is the major organ of the body that plays a vital role in metabolism of endogenous compounds (steroids, hormones, fatty acids cholesterol and proteins) and exogenous substances (xenobiotics). Orally administered drugs absorbed in small intestine and reached in liver via portal vein where they are metabolized. In hepatocytes (liver cells) drug metabolizing enzymes are located in intracellular membrane of rough endoplasmic reticulum and in cytosol (Figure 2.3). Liver microsomes are located in the rough endoplasmic reticulum and contained a variety of drug metabolizing enzymes, most important are CYP450, (GSTs), sulphotransferases SULTs and UGTs. Drug metabolism is defined as the mechanism of biochemical degradation or modification of lipophilic drug that converts it into hydrophilic metabolites and enhance its elimination from the body. The drug metabolizing enzymes have a central role in drug metabolism, detoxification and elimination (Figure 2.4).

Drug metabolism occurs in two phases: phase I pathways a functionalization of drug molecules takes place in microsomes and phase II pathways are involved in conjugation reactions of phase I metabolites or parent compound in liver cytosol. As a result of drug metabolism, drugs can be activated, inactivated, detoxified and consequently their metabolites may be activated to enhance or inhibit the activity of the drug or become toxic leads in drug toxicity (Asha and Vidyavathi, 2009).

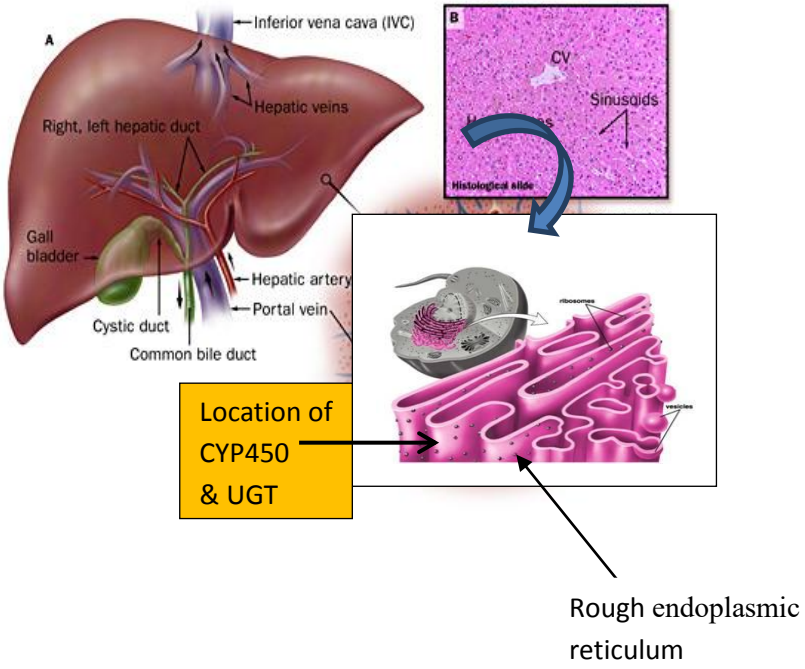


Figure 2.3: Histology of Liver (www.hopkins-gi.org)

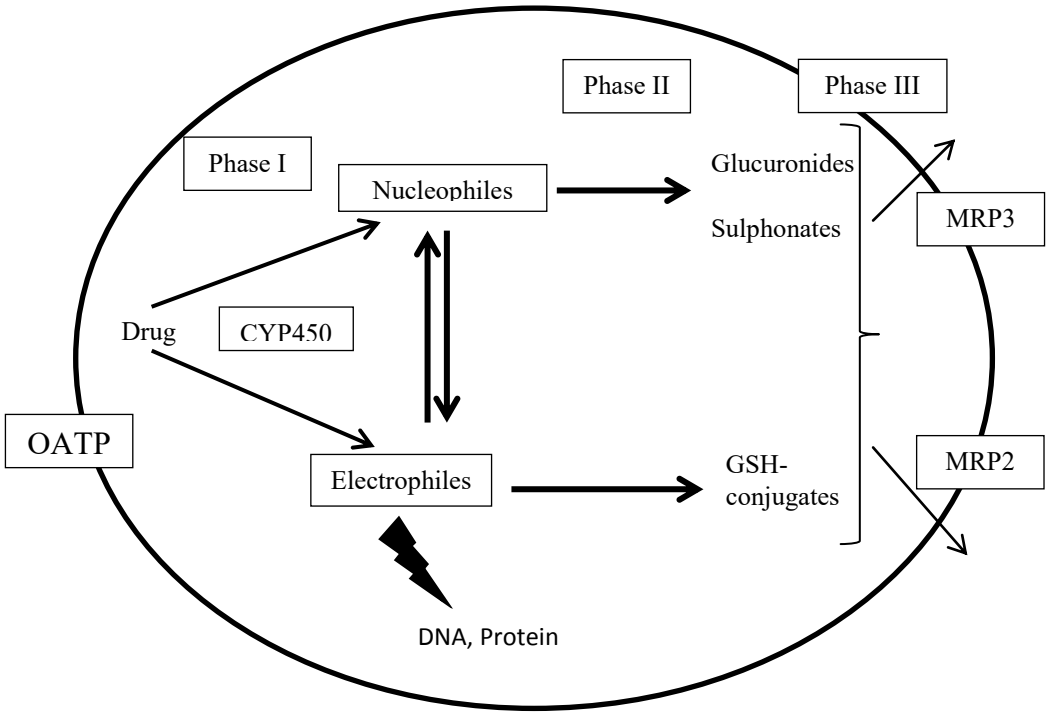


Figure 2.4: Schematic diagram of drug metabolism pathways in hepatocytes**.** Proteins involve in metabolism are: Organic anion transport protein (OATP), Phase I includes CYP450, Phase II includes GSTs, UGTs, SULTs, Phase III includes export transporter, Multidrug resistance proteins (MRPs). Accumulation of reactive electrophilic metabolites leads to adduct formation with protein and DNA, and to initiation toxic responses.

2.3.1 Phase I Drug Metabolism Pathways (Functionalization Reactions)

The main phase I metabolism pathways are oxidation, reduction, hydrolysis and hydration.

a) Oxidation Reactions

The microsomal mixed function-oxidase system (CYP450) is involved in drug oxidations. These reactions are carried out in the presence of CYP450 enzymes, NADPH-CYP450 reductase, molecular oxygen and lipid as shown in following reaction.

$$NADPH\ H^{+} + O_2 + RH \xrightarrow{\text{CYP450}} NADP^{+} + H_2O + ROH$$

Where RH is an oxidisable drug and ROH is metabolite. Oxidation reactions involve insertion of one oxygen atom to the substrate, rearrangement of intermediate to form final product and the other oxygen atom is reduced to form water (Gibson and Skett, 2001).

Here are some examples of drugs those are metabolized by specific oxidation reaction, Phenobarbital, phenytoin, aminopyrine, diazepam, codeine and lignocaine.

Another type of oxidation reactions are not catalyzed by CYP450 but catalyzed by non-microsomal oxidation enzymes. These reactions are more specific and are involved in endogenous compounds metabolism (Timbrell, 2000; Michael, 2010). Oxidative enzymes are xanthine oxidases, aromatases, aldehyde and alcohol dehydrogenases, amine oxidases and alkylhydrazine oxidase. Histamine, catecholamines and imipramine adopt amine oxidation to metabolize.

b) Reduction reactions

Reduction reactions are catalyzed by hepatic microsomes. NADPH is an electron donor in reductive metabolism and oxygen is not required in these reactions. Examples of reactions are prontosil and chloramphenicol (Michael, 2010; Gonzales, 2012).

c) Hydrolysis reactions

Amides, carbamates, esters and hydrazides undergo hydrolysis reactions. Esterases and amidases are found in blood plasma as well as in liver and are responsible for catalyzing these reactions. Pethidine, procain, acetylsalicylic acid, indomethacin and isoniazid (Hernandez and Rathinavelu, 2006).

d) Hydration reactions

Hydration is a special type of hydrolysis in which addition of water molecule to the substrate is carried out by epoxide hydrolase. Particularly polycyclic hydrocarbons (precarcinogenic) undergo this reaction to detoxify.

2.3.2 Phase II Drug Metabolism Pathways (Conjugation Reactions)

After the phase I reactions drug changed into its metabolites which subsequently proceed to phase II metabolism. In phase II reactions, specific group is conjugated to the drug/drug metabolites leading formation of end product that comprise higher molecular weight and become more hydrophilic. In addition, this

conjugated moiety cannot reabsorb and gets excreted from the body through urine or bile (Timbrell, 2000). The principal phase II reactions are as follows:

a) Glucuronidation

Glucuronidation is a commonly used pathway for conjugation reactions and most of the drugs and endogenous compounds are metabolized by the same pathway. UGT enzyme in the presence of co-factor UDP-glucuronic acid (UDPGA) catalyzes the conjugation of glucuronic acid to substrate.

b) Glutathione conjugation

Many xenobiotics can be metabolized to toxic electrophilic compound by phase I metabolism. Glutathione (GSH) conjugates with electrophile in the presence of GST to non-toxic product. GST is located in hepatocytes cytosol. The compounds that undergo glutathione conjugation are epoxides, nitroalkanes, haloalkanes and aromatic compounds. Adriamycin and fosfomycin are drugs that utilize glutathione conjugation reaction (Gonzalez and Tukey, 2006).

c) Sulfation

Sulfation is the conjugation of PAPS with drug molecule and catalyzed by cytosolic enzyme SULTs. Acetaminophen, methyldopa and steroids are examples of drug undergo sulfation (Deshpande, 2002).