DRUG METABOLISM AND TOXICITY STUDIES OF *Orthosiphon stamineus*, Benth (MISAI KUCING) IN RATS

by

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TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

ADP	Adenosine-5'-diphosphate
Ad libitium	To be taken as wanted
AGC	adenyl guanyl cyclase
ALD	Approximate lethal dose
ALT	Alanine aminotransferase
ALP	Alkaline phosphatase
AST	Aspartate aminotransferase
ATP	Adenosine-5'-triphosphate
BSA	Bovine serum albumin
Ca/CaM	Calcium calmodulin complex
Ca^{2+}	Calcium ion
cAMP	cyclic adenosine-3', 5'-monophosphate
cGMP	cyclic guanosine-3', 5'-monophosphate
CDNB	1-chloro-2,4-dinitrobenzene
DAG	Diacylglycerol
DMSO	Dimethylsulphoxide
et al	Else where or and others
FAD	Flavin adenine dinucleotide
g	Gram
µg/ml	microgram per milliliter
ng/ml	nanogram per milliliter
mg/kg	Miligram per kilogram
mg/ml	Miligram per mililiter
Gα	Alpha sub-unit of guanine nucleotide regulatory protein
GAP	Good Agriculture Practice

GGT	Gamma-glutamyltransferase
g/kg	Gram per kilogram
GLP	Good Laboratory Practice
GMP	Good Manufacture Practice
G-protein	A guanine nucleotide regulatory protein
G_{pp}	5'-Guanylylimidodiphosphate
GSH	Glutathione
GST	Glutathione-S-transferase
GTP	Guanosine triphosphate
HBSS	Hank's Balanced Salt Solution
IBMX	3-isobutyl-1-methylxanthine
IP_3	Inositol-1,4,5-triphosphate
K_i	Inhibition constant
K_m	Michaelis Menten constant
KT5720	cAMP-dependent protein kinase inhibitor
KT5823	cGMP-dependent protein kinase inhibitor
LD_{50}	Dose caused 50% lethality population
ml	Mililiter
mmol/L	Milimol per liter
nM	nanomolar
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NAPQI	N-acetyl-p-benzoquinone imine
*p*NP	*p*-nitrophenol
NOAEL	No observable Adverse effect level
NOEL	No observable effect level
OECD	Organization for Economic Cooperation and Development
OA	Okadaic acid

OTC	Over-the-counter
PDE	Phosphodiesterase enzyme
PK_A	Protein kinase A
PK_C	Protein kinase C
PK_G	Protein kinase G
PMA	4β-phorbol-12β-myristate-13α-acetate
PPA_2	Phosphatase A_2
r	Coefficient factor
S.D	Standard deviation
STZ	Streptozotocin
TPA	Trifluoperazine
U/L	Unit per liter
UDP	Uridine diphosphate
UDPGA	UDP-glucuronic acid
UGT	UDP-glucuronosyltransferase
USA	United States of America
US $	United States dollar
V_{max}	Maximal velocity
WHO	World Health Organization

KAJIAN METABOLISME DRUG DAN TOKSISITI *Orthosiphon stamineus*, Benth (MISAI KUCING) DALAM TIKUS

ABSTRAK

Orthosiphon stamineus, Benth (Famili: Lamiceae) atau dikenali dengan nama tempatan sebagai Misai Kucing, digunakan secara meluas di Malaysia untuk merawat hipertensi, masalah sistem urinasi dan penyakit batu karang ginjal. Objektif kajian ini ialah untuk mengkaji kesan ekstrak metanol Misai Kucing piawai ke atas enzim penghadaman drug hepatik fasa I dan II di dalam tikus Sprague-Dawley (SD). Aminopirin, *p*-nitrofenol (pNP) dan 1-kloro, 2,4-dinitrobenzena (CDNB) telah digunakan sebagai substrat untuk menentukan aktiviti aminopirin N-demetilase, UDP-glukuronosiltransferase (UGT) dan glutatione-S-transferase (GST) masing-masing di dalam hati tikus SD. Pengasingan hepatosit, fraksi sitosolik hati dan mikrosom telah disediakan dengan menggunakan teknik perfusi kolagenase dan pengemparan berperingkat. Kesan ketoksikan serta anti-hepatotoksik oleh ekstrak metanol Misai Kucing piawai ke atas tikus SD juga diuji. Daripada keputusan yang diperolehi, kebanyakan aktiviti UGT dan GST di dalam hati tikus SD normal dan diabetik adalah dipengaruhi secara signifikan oleh ekstrak metanol Misai Kucing piawai. Hanya beberapa kumpulan tikus SD normal dan diabetic menunjukkan kesan yang signifikan terhadap metabolisme aminopirin oleh ekstrak methanol Misai Kucing piawai. Kemungkinan mekanisme tindakan *in vitro* 1 mg/ml ekstrak metanol Misai Kucing ke atas metabolisme aminopirin di dalam hepatosit tikus jantan tua normal adalah melalui pengaktifan G-protein dan protein kinase C manakala kesan *in vitro*

0.001 mg/ml ekstrak metanol Misai Kucing piawai ke atas metabolisme aminopirin di dalam hepatosit tikus SD betina muda adalah berkemungkinan melalui pengaktifan protein kinase A. Aras LD_{50} untuk ekstrak metanol Misai Kucing piawai tidak dapat ditentukan dalam ujikaji ini. Tiada kematian dan kesan toksik diperhatikan pada tikus jantan dan betina muda normal setelah dirawat dengan 0.5, 1.0, 3.0 dan 5.0 g/kg ekstrak metanol Misai Kucing piawai. Pra-rawatan dengan ekstrak metanol Misai Kucing piawai dapat meningkatkan peratusan viabiliti hepatosit serta menurunkan setengah dari aras AST, ALT, ALP dan GGT serum di dalam tikus SD jantan dan betina dewasa yang dirangsang kecederaan hati dengan 2 g/kg acetaminofen. Secara kesimpulan, ekstrak metanol Misai Kucing piawai mempunyai kesan yang lebih besar ke atas aktiviti UGT dan GST jika dibandingkan dengan aktiviti aminopirin N-demetilase di dalam hati tikus SD. Ekstrak metanol Misai Kucing piawai pada 0.5 g/kg adalah diklasifikasikan sebagai paras tidak menyebabkan kesan (NOEL) dan ia mempamerkan kesan anti-hepatotoksisiti ke atas tikus SD jantan dan betina dewasa yang dirangsang kerosakan hati dengan acetaminofen.

DRUG METABOLISM AND TOXICITY STUDIES OF *Orthosiphon stamineus*, Benth (MISAI KUCING) IN RATS

ABSTRACT

Orthosiphon stamineus, Benth (Family: Lamiaceae) or locally known as Misai Kucing, is widely used in Malaysia for treating hypertension, urinary system ailments and kidney stone disease. The objective of this study is to investigate the effect of standardised methanol extract of Misai Kucing on phase I and phase II hepatic drug metabolising enzymes in Sprague-Dawley (SD) rat. Aminopyrine, *p*-nitrophenol (*p*NP) and 1-chloro-2,4-dinitro benzene (CDNB) was used as a substrate to determine the aminopyrine N-demethylase, UDP-glucuronosyltransferase (UGT) and glutathione-S-transferase (GST) activity respectively in SD rat liver. Isolated hepatocytes, liver cytosolic fraction and liver microsomes from SD rats were prepared using the collagenase perfusion technique and differential centrifugation technique. The possible toxic and anti-hepatotoxicity effects of standardised of methanol extract of Misai Kucing on SD rats were also examined. Results obtained showed that most of the UGT and GST activity in normal and diabetic SD rat liver were significantly affected by standardised methanol extract of Misai Kucing. Only a few groups of normal and diabetic SD rats showed significant effect on aminopyrine metabolism in hepatocytes in the presence of standardised methanol extract of Misai Kucing. The possible mechanism of action of the *in vitro* effect of 1 mg/ml of standardised methanol extract of Misai Kucing on aminopyrine metabolism in normal old male SD rat hepatocytes was probably mediated through the activation of G-protein and protein kinase C while

the *in vitro* effect of 0.001 mg/ml of Misai Kucing extract on normal young female SD rat hepatocytes was probably mediated through the activation of protein kinase A. The LD_{50} of standardised of methanol extract of Misai Kucing could not be determined in this study. No lethality incident and any toxic effects were observed in normal young male and female SD rats after being treated with 0.5, 1.0, 3.0 and 5 g/kg of standardised methanol extract of Misai Kucing. Pre-treatment with standardised methanol extract of Misai Kucing increased the percentage of hepatocytes viability and decreased some of the serum AST, ALT, ALP and GGT level in 2g/kg of acetaminophen induced liver damage in adult male and female SD rats. In conclusion, standardised methanol extract of Misai Kucing had a greater influence on UGT and GST activity than the aminopyrine N-demethylase activity in SD rat liver. Standardised methanol extract of Misai Kucing at 0.5 g/kg is classified as not-observable effect level (NOEL) and it exhibited anti-hepatotoxicity effect on acetaminophen-induced liver injury in adult male and female SD rats.

CHAPTER ONE

GENERAL INTRODUCTION

1.0 INTRODUCTION

1.1 Use of herbal medicine worldwide

Herbal medicine systems vary from one country to another country. Many of the herbal medicine practices are originated from their culture and region. Herbal medicine practices have been handed down from one generation to another generation and some countries had practiced it for hundreds and even thousand of years. For example, conventional United States and European herbal practices are based on ancient Greek-Roman experiences by using single herbs. In contrast, Chinese traditional herbal practice is based on formulas using multiple herbs (Rotblatt & Ziment, 2002). Old Chinese herbal medication especially is based on the experiences and absence of scientific evidence based products may not be acceptable by the European herbal medicine practitioners although both systems are giving same therapeutic effects. Many countries are still practicing herbal medicine which remains the backbone of medicine.

In recent years, herbal medicine has been gaining more acceptance and attention around the world (Ernst, 2002). Herbal medicine has been practiced in many countries including Europe to Asia. Today, the World Health Organization (WHO) estimates that 80 % of the world's population use herbal medicine (Fetrow & Avila, 2000). Herbal medicine or phytomedicine is a common element in the practice of ayurvedic, homeopathy and traditional oriented medicine system by using plants for healing purposes. Botanically, herbs are defined as a plant, plant part or extract for medicinal usage and can also be used as foods, fragrance, spices and

essential oils (Blumenthal & Israelsen, 1998). There are great movements in herbal medicine around the world by conventionally using herbal remedies in the form of herbal tea or crude tablets to extracted and standardised form of herbal remedies in today's modern herbal medicine. The most popular botanical medications in the USA in 1996 are echinacea, garlic, ginseng, gingko, goldenseal, mahuang, psyllium, siberian ginseng, saw palmetto and *Cascara sagrada* (Dennehy & Tsourounis, 2001).

Many consumers feel more comfortable with natural herbal products because it is commonly believed that herbal products have fewer side effects as compared to modern drugs especially after chronic use. Combination of herbs with certain drugs may alter pharmacological action of the drugs or even produce unwanted side effects such as toxicity. Therefore, scientific-based evidence of herbal medicine on the possible herb-drug interaction and toxicity is important to ensure the efficacy, safety and quality of the herbal remedies-based products.

In Malaysia, herbs are very common among the Malaysian community for medicinal purpose, foods and supplements. They used different part of the plant such as leaf, stem, root, flower or even seeds for medicinal purposes. A variety of herbal products are increasingly available at the Malaysian local market and many of these herbal products are sold as over-the-counter (OTC) medicine. One can easily find traditional medicine practitioners in Malay villages such as "nujum" (clairvoyant), "bomoh" or "mak bidan" to help in diagnosing illnesses and diseases. Normally they use certain type of plants like limau nipis or limau purut or even pap rice for medicinal purposes (Muhammad & Mustafa, 1994). Recently, numerous studies on local herbals have been studied extensively in Malaysia. Many scientific studies have proven that local traditional herbs such as Tongkat Ali (*Eurycoma longifolia*), Hempedu Bumi (*Andrographis paniculata*), Kacip Fatimah (*Labisia pumila*), Lempoyang (*Zingiber*

zerumbet) exhibit medicinal value for treatment of various diseases. Many other research on its use of herbs for medication are currently underway.

1.1.1 The usage of herbs to treat diseases

Worldwide, there are at least 250,000 species of flowering plants. In the Southeast Asia alone, there are about 35,000 species of flowering plants of which 8,000 species are found in Malaysia (Muhammad & Mustafa, 1994). Malaysia is endowed with a large biodiversity of flora and fauna. Traditionally, unprocessed herbs are used for medicinal purposes. For instance, herbs are prepared as tea for internal use by boiling the parts of plant with water. Currently, herbal preparation has changed to extraction of the herbs with different organic solvents to increase the effectiveness and quality of the preparation for therapeutic purposes.

Plants contain many types of chemical compounds which help to protect it from predators or attract pollinators. These secondary metabolites actually are of therapeutic values to the human. Phytomedicinal chemicals are generally assigned into nine important categories namely alkaloids, bioflavonoids, essential oils, glycosides, resins, saponins, sterols, tannins and terpenes (Rotblatt & Ziment, 2002).

i) **Alkaloids** are defined as basic amine group with no volatile character and their name always end with '-ine'. Briefly, there are 13 or more subclasses of alkoloids. There are imidazolesatropine, indoles, isoquinolines, amides, piperidines, pyridines, pyrrolidines, pyrrolizidines, quinolines, quinolizidines, steroidal, purines, and terpenoids (Rotblatt & Ziment, 2002). Alkaloids are found to exert multiple types of therapeutic effects including atropine, emetine, capsaicin, cocaine, morphine, quinine, methylxanthines (such as caffeine and theophylline), strychnine and nicotine (Fetrow & Avila, 2000). However, some alkaloids are harmful to human.

ii) **Bioflavonoids** lack nitrogen and usually contain two 6-carbon rings joined by three carbon atoms. About half of the 8,000 plant phenolic compounds are made up of flavonoids. They are found in high concentrations in many flowers, fruits ano vegetables (Moridani *et al.*, 2001). Flavans, flavanones, isoflavanones, flavones, isoflavones, chalcones, anthocyanidines and flavonolignans are the subclasses for flavonoids (Hodek *et al.*, 2002). Flavonoids are commonly believed to be antioxidants and antiiflammatory and protective against various cancers (Challis & Barlett, 1975). Rutin, quercetin, kaempferol, genistein, licoricidin, cyanidin, isoliquiritigenin, hispidol, coumestiol and silymarin are common example of bioflavonoid compounds that exhibit therapeutic effects on human and animals (Badger *et al.*, 2001).

iii) **Essential oils** are isoprene derivatives. The important essential oils are found in 10 classes which are assigned as alcohols, aldehydes, esters, ethers, furans, hydrocarbons, ketones, phenols, sesquiterpinoids and sulfur compounds (Rotblatt & Ziment, 2002). Essential oils are used therapeutically and also in aromatherapy (Sadler, 2001). Essential oils from mint family are used as flavors and drugs.

iv) **Glycosides** are sugar derivaties attached to aglycones (Fetrow & Avila, 2000). Glycosides are subdivided into cardiac glycosides, saponins, anthraquinones, cyanogenic glycosides, isothiocyanates, aldehydes, phenolics, alcohols, lactones and coumarins. Examples of glycosides used therapeutically are digoxin in allopathic medicine and anthraquinones which is used as laxative (Rotblatt & Ziment, 2002).

v) Another important phytochemical compound is called **terpene**. Terpenes are derived from 5-carbon isoprene units. These terpene groups are subdivided into monoterpenes, sesquiterpenes, diterpenes, triterpenes, tetraterpenes, polyterpene and

meroterpenes. Terpenoid compounds are mainly found in green vegetable, soy and grains and many have antioxidant properties (Rotblatt & Ziment, 2002).

vi) Other compound like **sterols** group may benefit human; stigmasterol and β-sitosterol may reduce low-density lipoproteins and lower serum cholesterol level (Rotblatt & Ziment, 2002).

vii) Triterpenes glycosides have widespread distribution in plants and are sometimes referred to as **saponins** as they have soap-like properties and readily form foams (Heinrich *et al.*, 2004).

viii) Triterpenes are also components of **resins** and resinous exudates from plants (e.g frankincense and myrrh). Resins are produced following damage to the tree as a physical barrier to attack by fungi and bacteria. Additionally, many of the terpenoid components of these resins have high antimicrobial activity (Heinrich *et al.*, 2004).

ix) **Tannin** comprises of water-soluble polyphenolic compounds, which may have high molecular weight. They are broadly divided into two groups i.e. hydrolysable tannins, which are formed by the esterification of sugars with the simple phenolic acids that are shikimate-derived (e.g gallic acid) and non-hydrolysable tannins which occur due to polymerization reactions between flavonoids (Heinrich *et al.*, 2004).

1.2 Literature review of Misai Kucing (*Orthosiphon stamineus*, Benth)

1.2.1 Misai Kucing plant description

In general, Misai Kucing is a medicinal herb easily found in Southeast Asia. The leaves are arranged in opposite pairs. The petiole is relatively short, about 0.3 cm in length and reddish purple in colour. The flowers are borne on verticals about 16 cm length, white to bluish in colour with long far-exerted filaments, making it look like cat's

whispers (Wiart, 2002). However, there are many different types of Misai Kucing which can be easily distinguished by its flower, these including *Orthosiphon aristatus* with white flower and *Orthosiphon grandifolis* with red colour flower. Identification of the species of Misai Kucing is very important to control its therapeutic effects because each species of Misai Kucing may have its own active ingredients and are not found in other species of Misai Kucing.

Misai Kucing is also found in other countries such as Thailand, Indonesia and Europe. In these countries, Misai Kucing is also known as yaa Nuat Maeo, Rau Meo or Cay Bac (Thailand), Kumis Kucing or Remujung (Indonesia), moustaches de chat (French) and Java Tea and Kidney Tea (European). In Malaysia, Misai Kucing can be seen in the garden, roadsides and wastelands. Misai Kucing is easily propagated through 3 or 4 noded stems cuttings from a mother plant of more than 5 months of age (Indubala & Ng, 2000). Misai Kucing has been cultivated for a long time. However, there are many important factors in determining the growth and yield of Misai Kucing. These factors include population density, fertilizer, soil type and weather. Recently, researchers from Malaysia Agriculture Research and Development Institute (MARDI) showed that population density of 29 333 plants/hectare with spacing of 120 cm between row and 45 cm between plants within the row for Misai Kucing on bris soil significantly gave highest fresh and dry yield compared to population density of 21 333 plants/hectare or lower (Zaharah & Salbiah, 2004).

1.2.2 Classification of Misai Kucing

The features of Misai Kucing as described by Wiart (2002) is a beautiful flowering plant, flower conspicuous, white and arranged in a terminal raceme. Stamens of Misai Kucing are long, expanding and shaped like cat's whiskers and its leaves are simple, without stipules and secussate, diamond-shaped, dark green above and paler below and secondary nerves 5-6. The Misai Kucing's stems are quadrangular with

purple lines at each corner and pithy (Figure 1.1). Based on the features described by Wiart (2002), the taxonomy of Misai Kucing is:

Class: Magnoliopsida.

Subclass: Asteridae

Order: Lamiales

Family: Lamiaceae (or Labiate)

Genus: Orthosiphon

Species: stamineus

Scientific name: *Orthosiphon stamineus*, Benth

1.2.3 Pharmacologic effects of Misai Kucing (*O. stamineus*)

Misai Kucing became a popular herbal tea among the community of Southeast Asia and European countries (Indubala & Ng, 2002). The Misai Kucing products are appearing in the forms of tea sachets, capsules and dried leaves. This plant is believed to have several pharmacological and therapeutic effects. The evaluations of Misai Kucing extract for medicinal purposes are as below:

a) ***Kidney stone elimination effect***: Traditionally, Misai Kucing has been widely used by Malaysian community as a diuretic and for treating catarrh of the bladder. Misai Kucing has been used to eliminate stones in the bladder (Indubala & Ng, 2002). In Malaysia, the leaves are used as diuretic and for treating catarrh of the bladder. A decoction prepared from the plants is used to eliminate stones in the bladder (Indubala & Ng, 2002).

b) ***Diuretic effect***: Diuretic effect of Misai Kucing has been proven through scientific experiments. Hydroalcohol extract of *O. stamineus* has been tested for their diuretic activities in rats and it revealed that they led to an increase in urine flow and urinary sodium excretion (Beaux *et al.*, 1999).

c) ***Cytotoxic effect***: Some flavonoids and diterpenes isolated from Misai Kucing which included 7,3',4'-tri-O-methylluteolin, eupatorin and ladanein showed cytotoxic activity towards highly livcr metastatic murine colon 26-L5 carcinoma cells with the ED_{50} value between 10 µg/ml to 90 µg/ml (Tezuka *et al.*, 2000). A mechanism which may be responsible for the anticarcinogenic potency of the extract may be related to the modulation of drug metabolising enzymes, for example, glutathione-S-transferase (GST) involved in carcinogen detoxification.

d) ***Cardiovascular effect***: Methylripariochromene A, another flavonoid isolated from aqueous extract of Misai Kucing exhibits hypotensive and vasodilating properties and decreases the cardiac output in animals (Matsubara, 1999).

e) ***Anti-inflammatory effect***: Researchers from Japan reported that two novel highly oxygenated pimarane diterpenes known as orthosiphol A and B isolated from dry leaves of *Orthosiphon stamineus*, Benth exert potent inhibitory activity against the inflammation induced by tumor promoters, TPA (12-*O*-tetradecanoylphorbol-13-acetate) on mouse ears (Masuda *et al.*, 1992).

f) ***Antioxidant effect***: Shantanova (1998) had reported that the administration of dry extract of *Orthosiphon stamineus*, Benth., *Desmodium canadensis D.S.*, *Poligonum aviculare L.*, *Arctostaphylos uva-ursi* L., to post-ischemic acute renal insufficiency white rats decreased the concentration of lipid peroxidase products in rat kidneys and markedly inhibit haemolysis. The inhibition of haemolysis was due to inactivation of free radical particles by binding it with phenolic compounds in the preparations. The leaves extract of herb are believed to have highest antioxidant property as compared to other part of herb studied (Chung *et al.*, 1998).

g) ***Nitric oxide inhibition effect***: Three highly oxygenated isopimarane-type diterpenes, (siphonol A-C) and a norisopimarane-type diterpene named siphonol E were isolated from the methanolic extract of *O. stamineus* showed more potent inhibitory effects on nitric oxide (NO) production in lipopolysaccharide (LPS) activated macrophage-like J774.1 cells than N^{G}-monomethyl-$_{L}$-arginine (L-NMMA) (Awale *et al.*, 2002).

1.2.4 Herb-drug interaction and adverse effects of methanol extract of Misai Kucing

Induction of hepatic enzyme activities in animals following the treatment with many natural compounds or traditional medicines which include *Gingko biloba* (Fessenden *et al.*, 2001), grapefruit juice (Lilja *et al.*, 2000; Kane & Lipsky, 2000), St. John's Wort (*Hypericum perforatum*) (Durr *et al.*, 2000) and Kava (*Piper methysticum*) (Almeida & Grimsley, 1996) had previously been reported. However, there is no previous scientific study reported on the effect of standardised methanol extract of Misai Kucing on herb-drug interaction and toxicity in laboratory animals and in man. Therefore, we describe for the first time the effect of standardised methanol extract of Misai Kucing on phase I and phase II hepatic drug metabolising enzymes activity in rat liver and toxicity studies in rat following the Misai Kucing methanolic extract treatment. Therefore, our research provides very important information about safety and efficacy of the standardised methanol extract of Misai Kucing.

Figure 1.1 Misai Kucing (*Orthosiphon stamineus*, Benth)

1.2.5 Evaluation of Misai Kucing (*O. stamineus*) chemical constituents

There are numerous factors which affect the quality, efficacy and safety of the extract obtained. These factors include growing conditions, method of drying and grinding, different processing methods and solvents used for extraction and also storage conditions (Rotblatt & Ziment, 2002). Good Agriculture Practice (GAP), Good Processing Practice (GPP) and Good Manufacturing Practice (GMP) are the few followed guidelines in order to maintain the quality and standard of the Misai Kucing extract obtained.

In order to maintain the quality of the extract, leaves used for Misai Kucing extraction are collected in the late afternoon, from 30-45 days old white flowered plants. The leaves are chopped and dried at approximately 40°C for 3 days. Methanol extracts of Misai Kucing are prepared by using a proportion of 10 g dried leaves in 100 ml of methanol by warming for 4 hours at 40°C. The solution is filtered through filter paper (Whatman No.1), concentrated and spray-dried to obtain the crude methanol extract of Misai Kucing (Akowuah *et al.*, 2004). Recent researchers from Universiti Sains Malaysia had reported that rosmarinic acid is the main component in the standardised methanol extract of Misai Kucing with concentration ranging from 5.1 % to 29.9% of the total dry leaf weight. Concentrations of 3'-hydroxy-5,6,7,4'-tetramethoxyflavone (TMF), eupatorin (EUP) and sinensetin (SEN) ranged from 0.05% to 0.69%, 0.34% to 3.37% and 0.22% to 1.76% respectively (Akowuah *et al.*, 2004).

Sixteen known compounds from methanol extract of Misai Kucing have been isolated and identified by Tezuka *et al* (2000). The identified compounds were five new isopimarane-type diterpenes [orthosiphols F-J] and two diterpenes [staminols A and B] with a novel carbon framework and three new highly-oxygenated stamina-type diterpenes [staminolactones A and B and norstaminol A]. Other compounds such as

7,3',4'-tri-O-methylluteolin, 5-hydroxy-6,7,3',4'-tetramethoxyflavone, eupatorin, sinensetin, salvigenin, ladanein, tetramethylscutellarein, 6-hydroxy-5,7,4'-trimethoxyflavone, vomifoliol, aurantiamide acetate, rosmarinic acid, caffeic acid, oleanolic acid, ursolic acid, betulinic acid and β-sitosterol are also identified in the same extract (refer to table 1.1). Six phenolic compounds from leaves extract of Misai Kucing were isolated and identified by Sumaryono *et al* (1991). These identified phenolic compounds were caffeoyl tartrate, rosmarinic acid, aurantiamide acetate, vomifoliol, caffeic acid and 2, 3-dicaffeoyl tartrate (refer table 1.2). Sinensitine, eupatorin, rosmarinic-, cichoric- and caffeic-acids were the main polyphenols identified in two tinctures (A: 50 % v/v ethanol and B: 70% v/v ethanol) of *Orthosiphon stamineus* (Olah *et al.*, 2003).

1.3 Hepatic drug metabolism reactions

1.3.1 Introduction to drug metabolism

The liver is one of the hardest working organs in the body and works closely with other organs system in the body. It is the most abundant organ in the body for xenobiotic and drug metabolism and detoxification process (de la Iglesia *et al.*, 1999). In general, the term "metabolism" refers to the sum of biochemical reactions in the body, including anabolism (building of complex) and catabolism (breakdown of a complex). However, drug metabolism or biotransformation is used to describe the conversion of drugs or other toxins into metabolites. These reactions are generally called biochemical breakdown of the drug. Biotransformation whether completed by the P450 system or other enzyme system is rarely a simple process. These reactions involve several different processes concurrently and a single metabolic transformation does not usually occur.

Table 1.1 List of chemical constituents of *Orthosiphon stamineus* aerial part extract (Tezuka *et al.*, 2000)

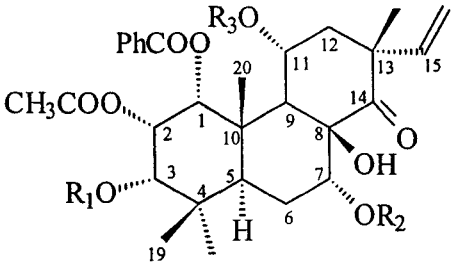
Diterpenes	Triterpenes	Flavones
orthosiphols F [1]	Oleanolic acid [12]	7,3',4'-tri- O-methylluteolin [16]
orthosiphols G [2]	ursolic acid [13]	eupatorin [17],
orthosiphols H [3]	betulinic acid [14]	sinensetin [18],
orthosiphols I [4]	β-sitosterol [15],	3'-hydroxy-5,6,7,4'-tetramethoxyflavone [19]
orthosiphols J [5]		salvigenin [20],
staminols A [6]		ladanein [21],
staminols B [7],		Tetramethylscutallarein [22],
staminolactones A [8]		6-hydroxy-5,7,4'-trimethoxyflavone [23],
staminolactones B [9]		kaempferol-3-O-β-glucoside [24],
norstaminol A [10]		quercetin-3-O-β-glucoside [25],
orthosipholl S [11]		

The number in bracket are chemical compounds found in *O. stamineus* and their individual chemical structures can be found in pages 15 -17.

Table 1.2 List of chemical constituents of *Orthosiphon stamineus* leaves extract (Sumaryono *et al.*, 1991)

Phenolic acids
caffeoyl tartrate [26]
rosmarinic acid [27]
aurantiamide acetate [28],
vomifoliol [29],
caffeic acid [30],
2, 3-dicaffeoyl tartrate [31]

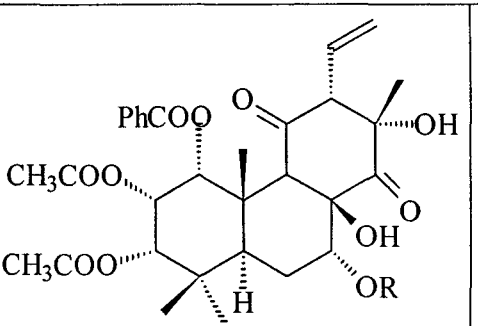
The number in bracket are chemical compounds found in *O. stamineus* and their individual chemical structures can be found in pages 17.



R' = $COCH_3$, R'' = H , R''' = CoPh, Orthosiphol F [1]

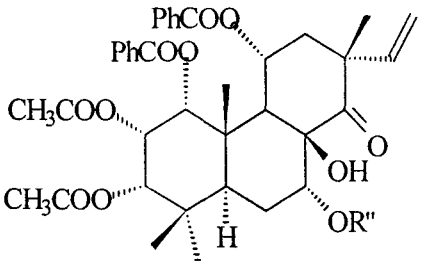
R' = $COCH_3$, R'' = R''' = H, Orthosiphol G [2]

R' = R''= $COCH_3$, R''' = COPh Orthosiphol H [3]



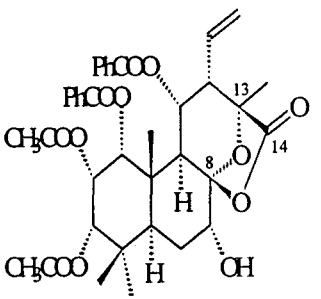
R = H , Orthosiphol I [4]

R = $COCH_3$, Orthosiphol J [5]

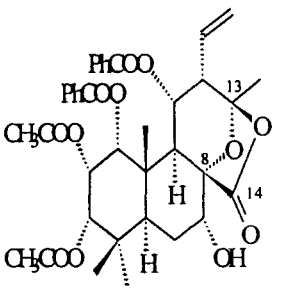


R = $COCH_3$, Staminol A [6]

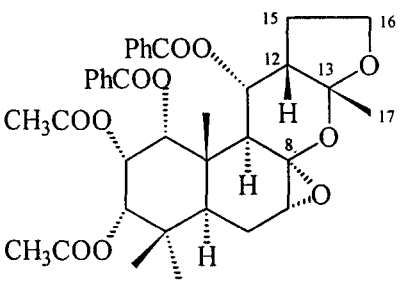
R = H, Staminol B [7]



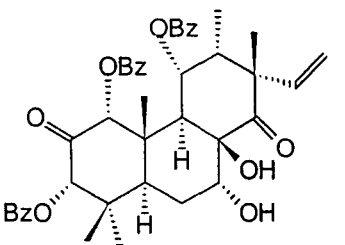
Staminolactone A [8]



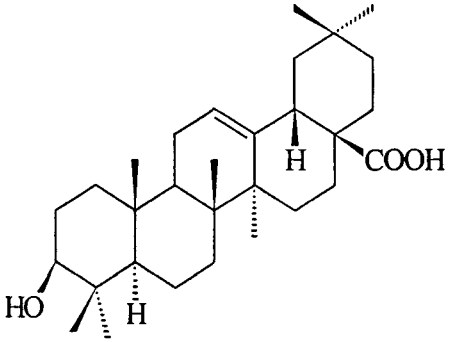
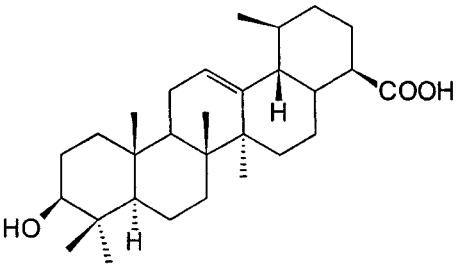
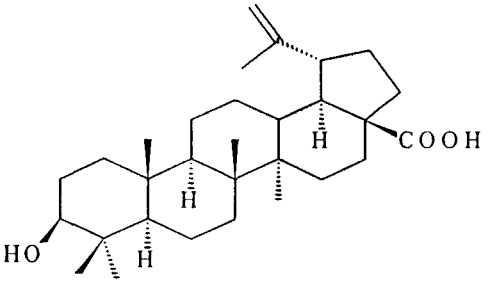
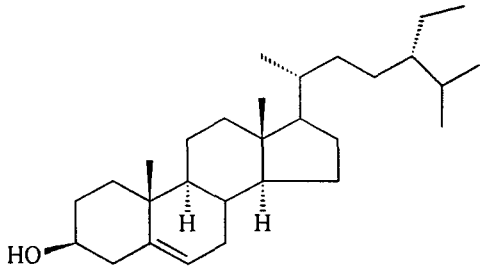
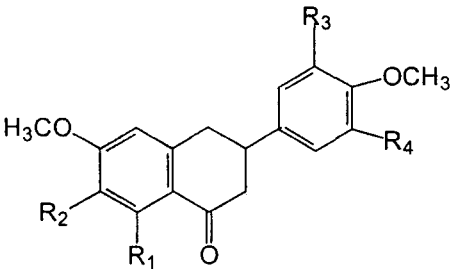
Staminolactone B [9]

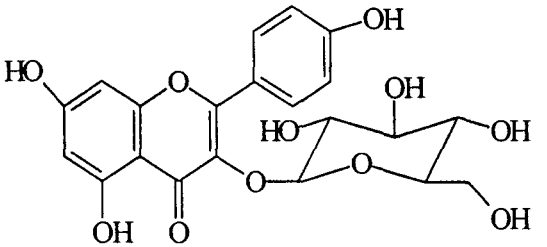


Norstaminol A [10]

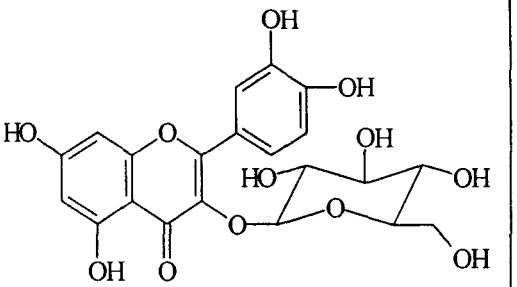


Orthosiphol S [11]

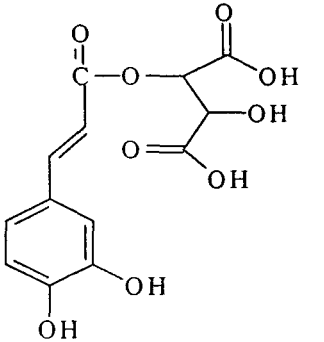
 Oleanolic acid [12]	 Ursolic acid [13]	 Betulinic acid [14]
 β-sitosterol [15]	 $R_1 = R_2 = H$, $R_4 = OCH_3$, 7,3',4'-tri-O-methylluteolin [16] $R_1 = R_3 = OH$, $R_2 = OCH_3$, $R_4 = H$ Eupatorin [17]	$R_1 = R_2 = R_3 = OCH_3$, $R_4 = H$ Sinensetin [18] $R_1 = OH$, $R_2 = R_3 = OCH_3$, $R_4 = H$, 3'-hydroxy-5,6,7,4'-tetramethoxyflavone [19] $R_1 = R_4 = H$, $R_2 = OCH3$, salvigenin [20] $R_1 = R_4 = H$, $R_2 = OH$ ladanein [21] $R_1 = CH_3$, $R_2 = OCH_3$, $R_4 = H$ tetramethylscutellarein [22] $R_1 = CH_3$, $R_2 = OH$, $R_4 = H$, 6-hydroxy-5,7,4'-trimethoxyflavone [23]



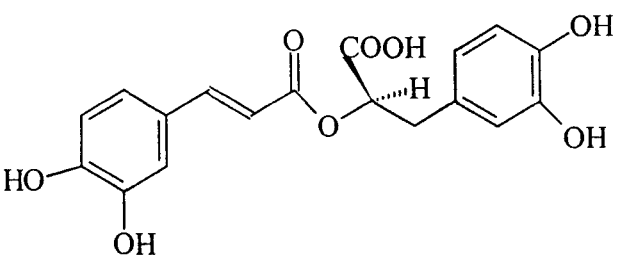
Kaempferol-3-O-β-glucoside [24]



Quercetin-3-O-β-glucoside [25]



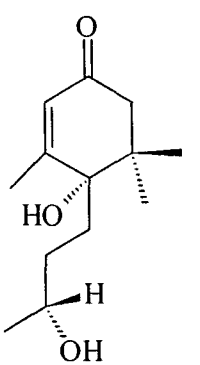
Caffeoyl tartrate [26]



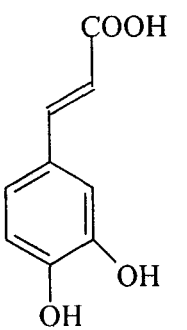
Rosmarinic acid [27]

PhCO—Phe—Pheol—$COCH_3$

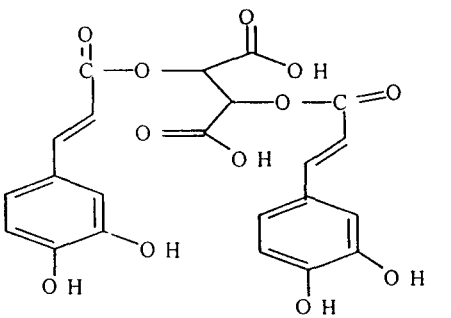
Aurantiamide acetate [28]



Vomifoliol [29]



Caffeic acid [30]



2,3-Dicaffeoyl tartrate [31]

As shown in table 1.3, biotransformation reactions have been generally termed as phase I (functionalisation reaction), phase II (conjugation) and phase III (export of the conjugate from cells) (Timbrell, 2001). In phase I reactions, xenobiotics are generally converted to more polar, hydroxylated derivatives and these derivatives are conjugated with molecules such as glucuronic acid, sulphate or glutathione in phase II reaction later and eventually excreted in the urine or bile (Murray, 1998). Xenobiotics that already possess a functional group can bypass phase I metabolism and directly participate in the conjugation reaction (Ioannides, 2002). In mammals, a majority of the enzyme systems that contribute to phase I and phase II metabolisms are encountered in every tissue but particularly in the endoplasmic recticulum and cytosolic fraction of the liver (Ioannides, 2002).

1.3.1.1 Phase I hepatic drug metabolism reaction

Main reactions of Phase I (functionalisation) liver metabolising process are oxidation, reduction and hydrolysis. The major organ that carries out phase I reaction is the liver and the major site within the liver is the endoplasmic reticulum of the liver cell (Vainio & Hietanen, 1980). Some phase I hepatic enzymes are found in several subcellular compartments. Hepatic enzymes involved in the phase I reactions are distributed in many different compartment of the liver and have overlapping substrate specificities (refer table 1.4).

a) Reduction

Reductase enzymes are found in gut flora and some mammalian tissues. Nicotinamide adenine dinucleotide phosphate (NADPH) and Flavin adenine dinucleotide (FAD) are required in this reaction as electron donor but the function of NADPH is inhibited by oxygen. The major reactions of reduction are azo and nitro reduction. Examples of reduction reactions are:

i) The conversion of prontosil to sulphanilamide (antibacterial drug) is a well-known example of **azo reduction** reaction which is catalysed by the cytochrome P450 and carried out by the reductase enzyme in the gut bacteria (Hodgson & Goldstein, 2001).

ii) **Tertiary amide oxides** are reduced by cytochrome P450.

iii) **Hydroxylamines** is reduced by cytochrome P450 and also reduced by a flavoprotein which is part of a system which requires NADH and includes NADH cytochrome b_5 reductase and cytochrome b_5 (Timbrell, 2000).

b) Oxidation

The cytochrome P-450s system is involved in many oxidation reaction including xenobiotic activation and detoxification (Ortiz de Montellano, 1999). Monooxygenations of xenobiotics are the major types of Phase I reaction (Nebbia, 2003). They are also known as mixed-function oxidations. In general, the sequences of oxidation reactions involve at least four distinct steps: (i) addition of substrate to the enzyme, (ii) donation of an electron, (iii) addition of oxygen and rearrangement and (iv) donation of a second electron and loss of water. Briefly, mixed-function oxidations involve reactions of one atom of the oxygen molecule is being accepted by the substrate while the other oxygen atom is reduced to water (Guengerich, 2002).

Oxidation reactions could be catalysed by enzymes known as microsomal or non microsomal oxidation enzymes. Non-microsomal oxidations are subdivided into amine oxidation, alcohol and aldehyde oxidation, dehalogenation, purine oxidation and aromatization. On the other hand, microsomal oxidation reactions include N-, S- and O-dealkylation, N-hydroxylation, aliphatic hydroxylation, alicyclic hydroxylation, N-oxidation and deamination (Timbrell, 2000).

c) Hydrolysis

Major hydrolysis reactions are ester and amide hydrolysis (Hodgson & Goldstein, 2001). Enzymes involved in catalysing hydrolysis reactions are carboxylesterases and amidases. Carboxylesterases and amidases are usually found

in the cytosol and some have been described in the microsomes. Hydrazines and carbamates may undergo hydrolysis reaction. The insecticide, **carbaryl** is hydrolysed to 1-naphthol by hydrolysis reaction (Timbrell, 2000).

d) Hydration

Epoxide may undergo addition of water upon hydration reaction. Hydration reaction involves addition of water to the epoxide catalysed by epoxide hydrolase, probably by nucleophilic attack by OH- on one of the carbon atom of the oxirane ring to yield a dihydrodiol (Gibson & Skett, 1994).

1.3.1.2 Phase II hepatic drug metabolism reaction

The phase II hepatic metabolising reactions include glucuronidation, glutathione conjugation, sulphate conjugation, amino acid conjugation, hydration, methylation and acetylation. Enzymes which are responsible for the phase II conjugation are located within various subcellular fraction of the liver (Table 1.5). Phase II reaction generally conjugates with a specific substitute group such as glucuronic acid, sulphate, amino acid or glutathione to the substrate and thereby increase its size and molecular weight. However, methylation and acetylation are exceptions and they may decrease both the substrate polarity and its solubility in water (Murray, 1998).

Enzymes that catalyse phase II reactions similar to the cytochrome P450 system, are subjected to induction. Induction of phase II hepatic metabolising enzymes could be beneficial to the liver itself because it enhances the clearance of toxic or non toxic intermediate metabolites. Below are a few of common phase II reactions:

i) **Glucuronidation** is the major metabolism pathway. In these reactions, UDP-glucuronosyltransferase **(UGT)** or glucuronidase enzyme adds glucuronic acid to a substrate. The conjugation product has a relatively high molecular weight and is prone to excretion through bile and urine (Orzechowski *et al.*, 1994). The most readily conjugated functional groups are phenols, alcohols and carboxylic acids.

ii) **Glutathione conjugation** is catalysed by glutathione-S-transferase **(GST)** which is localized primarily in the cytosol and to much lesser extent in the endoplasmic reticulum. The major purpose of the glutathione conjugation appears to be the control of reactive electrophiles which are present naturally or form metabolism and have a deleterious effect on the body (Wilce & Parker, 1994).

iii) During **sulphate conjugation**, inorganic sulphate, in the form of 3'-phosphoadenosine-5'-phosphosulphate (PAPS) is added to the molecule and this reaction is catalysed by sulphotransferase (Pacifici & Giuliani, 1997). The phenols and amino groups are among the groups to be sulphated to yield readily excretable sulphamates.

iv) **Acetylation** is another important phase II reaction. An amide bond is formed between the amino group of the chemical and acetate. Aromatic and heterocyclic amines, hydrazines and sulphonamides may be undergoing acetylation reaction (Murray, 1998).

v) Glycine being the most common amino acid group is found to conjugate with xenobiotics. Other amino acids such as glutamine, taurine and ornithine are also important in the **amino acid conjugation**. Amino acid conjugation is formed by peptide bond between carboxyl groups of the xenobiotics with the α-amino group of the amino acid (Caldwell, 1982).

vi) **Methylation** reaction plays an important role in the metabolism of endogenous substrates such as noradrenaline, neurotransmitters (Hirata, 1982) and polyphenolics such as the tea flavonoid, epicatechin. Methyltransferase are primarily cytosolic enzymes but are also found in the endoplasmic reticulum.

1.3.1.3 Isolation and subcellular fraction of the liver

Hepatic cells contain a multitude of metabolising enzymes that are usually localised in the specific organelles within the cells. Some of these enzymes may also be expressed in other specific organs. This study used freshly isolated perfused

hepatocytes and subcellular fraction of the liver as indicator of hepatic phase I and phase II drug metabolism respectively.

Isolated perfused hepatocytes are prepared by using collagenase perfusion technique and this technique is widely used by pharmacologists to study drug metabolism (Chazouillères *et al.*, 1989). Perfusions are normally performed in the anterograde direction (from portal vein to cava vein) (Miller, 1973). Isolated perfused hepatocytes preserve cells integrity and completely contain cell architecture such as the membrane cell, surface receptor, nucleus and hepatic metabolic systems (Axelsson *et al.*, 2003). Viable isolated hepatocytes are seen to undergo active DNA synthesis, mitosis, cell proliferation and are more physiological as compared to subcellular fraction preparation. In addition, all the cofactors and biochemical substances needed for reactions are contained within the hepatocytes (Sinz, 1999).

Subcellular fractions of the liver consist of two different fractions known as liver microsomes and the S-9 cytosolic liver fraction (Ekins *et al.*, 1999) (Figure 1.2). For example, UDP-glucuronosyltransferases (UGT) enzymes are predominantly located within the endoplasmic reticulum. Consequently, liver microsomes isolated from the liver are most suitable to be used in studying glucuronidation reaction of the drug. The advantages of using subcellular fractions in drug metabolism studies are the ease to prepare, flexibility of incubation conditions such as cofactors, buffer, pH and temperature and their stability in long-term storage under specific temperature and condition. The disadvantages of hepatic subcellular preparation are the disruption of the hepatic cellular heterogeneity by homogenisation and the limitations of sequential metabolism that requires multiple cofactors to be completed. However, this limitation becomes an advantage when the mechanism of the reactions can be controlled by adding suitable cofactor at a specific time and eases the detection at each single step of the drug metabolism reaction (Ekins *et al.*, 1999).

1.3.2 Herb-drug interaction

There is an increasing use of alternative medicine by the public with a variety of plant-derived drugs or plant-based supplements which contain active compounds over the counter. Moreover, there are possibilities where patients seek herbalist treatment while taking prescribed medication. It is possible that one substance may alter the bioavailability of another substance by inducing the phase I and phase II hepatic enzymes system and subsequently affect drug therapeutic effect or even generate toxicity when two or more substances are given concurrently. There are many documented reports about herb-drug interactions in human (Roblatt & Ziment, 2002; Table 1.6). Herb-drug interaction can be defined as any alteration either in the pharmacokinetic or pharmacodynamic effect of the drug caused by concurrent treatment with herb and drug. Herb-drug interaction can be generally divided into pharmacokinetic and pharmacodynamic interactions (Sugiyama *et al.*, 2004).

Table 1.3 Major biotransformation reaction (Timbrell, 2000).

PHASE I	PHASE II	PHASE III
Oxidation	Glucuronidation	Further metabolism of
Reduction	Glutathione conjugation	glutathione conjugate
Hydrolysis	Sulphation	
Hydration	Acetylation	
	Amino acid conjugation	
	Methylation	