EFFECT OF *MORINDA CITRIFOLIA* (LINN.) ON PHASE I AND II DRUG METABOLISM AND ITS MOLECULAR MECHANISM ELUCIDATION IN RAT LIVER

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by

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%	Percent
δ	chemical shifts
µg	Microgram
µg/ml	Microgram Per Milliliter
µl	Microliter
µM	Micromolar
°C	Degrees Celsius
$[Ca^{2+}]_i$	Intercellular Concentration of Calcium
3-MC	3-methycholanthrene
8-Br-cGMP	8-bromoguanoside 3', 5'-cyclic Monophosphate
ad libitum	To Be Taken as Wanted
ANP	Atrial Natriuretic Peptide
APD	Aminopyrine N-demethylase
APM	Aminopyrine Phase I Metabolism
ATP	Adenosine-5'-triphosphate
AUC	Area Under the Curve
Beta TG	Beta thromboglobulin
BSA	Bovine Serum Albumin
Ca^{2+}	Calcium
cAMP	Adenosine 2`,3`-cyclic Monophosphate
CDNB	1-chloro-2,4-dinitrobenzene
cGMP	Guanosine 3´,5´-cyclic Monophosphate
cm	Centimeter
CYP	Cytochrome P450
D_2O	Deuterated Water
DAG	Diacylglycerol

DMSO	Dimethyl Sulfoxide
DR	Diabetic Rats
EC_{50}	Concentration of Chemicals That Gives 50% of Maximal Effect
FDA	Food Drug Administration
g	Gram
G_{α}	Alpha Subunit of G-protein
G_i	Inhibitory G-protein
g/kg	Gram Per Kilogram
GC	Guanylyl Cyclase
GH	Growth Hormone
GPCRs	G-protein Coupled Receptors
Gpp(NH)p	Guanylylimidodiphosphate
G_s	Stimulatory G-protein
GSH	glutathione
GST	Glutathione S-Transferase
GTP	Guanosine Triphosphate
HBSS	Hank's Balanced Salt Solution
HNJ	Hawaiian Noni Juice Commercial Product of *Morinda citrifolia*
IBMX	3-isobutyl-1-methyl-xanthine
IR	Infrared Spectroscopy
IC_{50}	Concentration of Chemicals That Gives 50% of the Inhibitory Effect
IP_3	Inositol Triphosphate
i.v	Intravenous
KBr	Potassium Bromide
kg	kilogram
KOH	Potassium Hydroxide
l	Liter

L-NIO	L-N^5-(1-Iminoethyl)-ornithine
M	Molarity
M-BGC	Membrane-bound Guanylyl Cyclase
MFO	Mixed-function Oxidation
mg	Milligram
mg/kg	Milligram Per Kilogram
ml	Milliliter
mM	Millimolar
mm^3	A cubic Millimeter
MJE	Mengkudu Juice extract of *Morinda citrifolia*
N	Normality
n	Number of Animal
NADPH	Reduced Form of Nicotinamide Adenine Dinucleotide Phosphate
ng/ml	Nanogram Per Milliliter
NO	Nitric Oxides
NOS	The Nitric oxide synthase
NR	Normal Rats
OA	Okadaic Acid
OECD	Organization for Economic Cooperation and Development
PDE	Phosphodiesterase Enzyme
PK_A	Protein Kinase A
PK_C	Protein Kinase C
PK_G	Protein Kinase G
PMA	Phorbol-12β-myristate-13α-acetate
p-NP	*p*-nitrophenol
PP	protein phosphatase
PTK	Protein Tyrosine Kinase

q.s	A sufficient quantity
rpm	Revolution Per Minute
S.D.	Standard Deviation
SF-1/Ad4BP	Steroidogenic Factor-1/adrenal 4-binding Protein
SGC	Soluble Guanylyl Cyclase
SHR	Spontaneously Hypertensive Rat
SNP	Sodium Nitroprusside
STZ	Streptozotocin
tbsp	Tablespoonful
TNJ	Tahiti Noni Juice Commercial Product of *Morinda citrifolia*
TxA_2	Thromboxane A2
TxB_2	Thromboxane B2
TLC	Thin Layer Chromatography
UDP-GT	Uridine Diphosphate Glucuronosyltransferase
UGT	Uridine Diphosphate Glucuronosyltransferase
US	United States
UV	Ultra Violet
vs	Versus
v/v	Volume Per Volume
WHO	World Health Organization
w/v	Weight Per Volume

KESAN MORINDA CITRIFOLIA (LINN.) TERHADAP METABOLISME DRUG FASA I DAN II DAN PENCIRIAN MEKANISME MOLEKUL DALAM HATI TIKUS

ABSTRAK

Morinda citrofilia umumnya dikenali sebagai Noni dan orang tempatan menamakannya mengkudu adalah satu di antara tumbuhan ubat Polinesia yang sangat penting. *Morinda citifolia* (Noni) telah digunakan secara meluas dalam perubatan kampong oleh orang-orang Polinesia sejak lebih 2000 tahun dahulu. Ia dikatakan mempunyai kesan terapeutik yang meluas termasuk kegunaan antikanser dalam klinikal, dan terhadap haiwan makmal, dan juga bekesan sebagai agen antibakteria, antivirus, antikulat, antihelmin, analgesik, antihipotensif, antiinflamasi dan juga mempunyai kesan menguatkan sistem imun. Penggunaan ubat herba bersama dengan ubat-ubatan moden sekarang ini menjadi semakin popular, kemungkinan interaksi (saling tindakan) diantara herba dan drug bertambah. Hanya sedikit diketahui tentang kejadian dan akibat interaksi herba-drug dalam pesakit yang menerima produk herba jus mengkudu. Tujuan penyelidikan ini adalah menjalankan kajian pendahuluan *in-vitro* kesan *M. citrofilia* terhadap enzim metabolisme fasa I dan fasa II dalam hati tikus; pengaruh penyakit (diabetes dan hipertensi), jantina dan umur terhadap kesan *M. citrifolia* dan juga untuk pencirian mekanisme peringkat molekul kesan *M. citrifolia* keatas metabolisme aminopirin fasa I.

Kajian *in-vitro* kami menunjukkan ekstrak jus mengkudu (MJE), Hawaiian Noni juice (HNJ) dan Tahiti Noni juice (TNJ) telah meningkatkan metabolisme aminopirin terutamanya pada kepekatan tinggi dalam tikus normal (NR), tikus diabetik (DR) dan tikut hipertensif spontan (SHR). Kajian ini telah menunjukkan penyakit diabetes dan perbezaan jantina mempengaruhi secara signifikan kesan *in-vitro M. citrofilia* ke atas metabolisme aminopirin. Dalam kajian akut (satu hari) pemberian secara oral MJE, aktiviti aminopirin N-demetilase meningkat secara signifikan pada semua paras dos

yang tinggi (210mg/kg), sementara aktiviti glutation S-transferase (GST) naik secara signifikan pada kepekatan 2.1, 21, 210 mg/kg. Walaubagaimana pun, kajian sub-kronik, aktiviti uridin difosfat-glukuronosil transferase (UDP-GT) turun secara signifikan tetapi bergantung kepada dos sementara aktiviti aminopirin N-demetilase juga turun walaupun tidak signifikan.

Kemungkinan adanya interaksi yang serupa terjadi *in-vitro* dan *ex-vivo* dengan drug-drug lain yang mengalami konjugasi N-demetilase hepatik fasa I dan/atau fasa II. Kemungkinan kesan yang serupa terhasil secara *in-vivo* perlu ada kajian seterusnya. Kajian mekanisme molekul mencadangkan protein kinase A mungkin terlibat dalam mekanisme peringkat molekul bagi kesan akut MJE keatas metabolisme aminopirin dalam tikus muda betina SHR. Penskrinan kualitatif menggunakan spektroskopi IR, ^{1}HNMR dan HPTLC menunjukkan sampel-sampel *M. citrifolia* yang diuji mempunyai persamaan secara kualitatif dalam kandungan utamanya. Ciri-ciri kandungan ini kebanyakannya menyerupai kumpulan-kumpulan fungsi sebatian antrakuinon, sterol, glikosida dan flavonol yang telah dilaporkan oleh beberapa pengkaji sebelum ini.

EFFECT OF *MORINDA CITRIFOLIA* (LINN.) ON PHASE I AND II DRUG METABOLISM AND ITS MOLECULAR MECHANISM ELUCIDATION IN RAT LIVER

ABSTRACT

Morinda citrifolia commonly known as Noni and locally known as mengkudu is one of the most important traditional Polynesian medicinal plants. *Morinda citrifolia* (Noni) has been used extensively in folk medicine by Polynesians for over 2,000 years. It has been reported to have broad therapeutic effects, including anticancer properties in clinical practice and in laboratory animal models and are effective as antibacterial, antiviral, antifungal, antihelminthics, analgesic, hypotensive, anti-inflammatory agents, and immune system enhancing effects. As the use of phytomedicine together with modern medications has become more popular nowadays, the possibilities of herb-drug interactions have increased. Little is known about the incidence and consequences of herb-drug interactions in patients receiving herbal product of mengkudu juice. The aims of the study were to investigate, primarily, the *in-vitro* effect of *Morinda citrifolia* on phase I and II metabolizing enzymes in rat liver; the influence of diseases (diabetes and hypertension), gender and age on the foregoing effect, as well as to elucidate the molecular mechanism of *M. citrifolia* effect on aminopyrine phase I metabolism.

Our *in-vitro* study showed that effect of mengkudu juice extract (MJE), Hawaiian Noni juice (HNJ) and Tahiti Noni juice (TNJ) of *M. citrifolia* increased aminopyrine metabolism especially at high concentrations in normal rat (NR), diabetic rat (DR) and spontaneously hypertensive rats (SHR). This study shows that, diabetes and gender differences have significantly influenced the *in-vitro* effects of *M. citrifolia* on liver aminopyrine metabolism. In acute study (one day) of orally administrated MJE, the aminopyrine N-demethylase activity was significantly increased at the highest dose level (210 mg/kg) while the activity of glutathione S-transferase (GST) was significantly

increased at 2.1, 21 and 210 mg/kg concentrations. However, in the sub-chronic study, uridine diphosphate glucuronosyltransferase (UDP-GT) activity was significantly decreased but was dose independent while aminopyrine N-demethylase activity was not changed.

A possibility exist that similar interactions may occur *in-vitro* and *ex-vivo* with other drugs that undergo the same hepatic phase I N-demethylation and/or hepatic phase II conjugations. Whether this effect is similarly produced *in-vivo* still needs further investigation. The molecular mechanism study suggests that protein kinase A may be involved in the molecular mechanism of MJE acute effect on aminopyrine metabolism in young female SHR. Qualitative screening using IR, ^{1}HNMR spectroscopies and HPTLC showed that the tested samples of *M. citrifolia* have qualitative similarities in their major constituents. The characteristics of these constituents mostly resemble the functional groups of anthraquinones, sterols, glycoside and flavonol compounds which have been reported by several authors.

CHAPTER ONE
GENERAL INTRODUCTION

1.1 History of Herbal Drugs

The WHO (2000) has defined herbs to include crude plant materials such as leaves, flowers, fruits, seeds, stems, wood, barks, root, rhizomes or other plant parts which may be complete, fragmented or powdered. On the other hand, herbal products consist of herbal preparations made from one or more herbs. It may contain excipients in addition to the active ingredients. In their unprocessed state, these herbal drugs are usually in the dried form but are sometimes stored fresh. Certain exudates may also be considered as herbal drugs. Herbal medicine is defined as the use of crude drugs of plant origin to treat illness or to promote health. Phytomedicinals including capsules, tablets, tinctures, and fluid extracts are those common preparations that have been prepared from plant sources.

Phytomedicine, the use of plants or their parts to treat ailments has been part of humankind's attempt to free itself of disease for several thousand years. Some of the earliest writings found on Babylonian clay tablets from 3000 B.C. are about plants used for ceremonial, magical, and medicinal purposes. During the next thousand years, parallel cultures in China, India, and Egypt developed written records of medicinal herbs. Among these early historical documentations, the ancient Middle Easterners appear to have been the one of the first to rigorously document the use of plants for the treatment of various diseases, compiling these information in the first known pharmacopoeia entitled Materia Medica. The Greek historian Herodotus recounts how the Egyptians worshiped certain plants (Fetrow & O'Neil, 2002).

As science emerged after the 17th century, herbal plants were classified and demystified. Extraction of the relevant chemicals from these plants became popular

around the turn of the 19th century. As science advanced, medicines were synthesized and herbalism declined. Newly developed principles of organic chemistry made it possible to replicate plant-produced chemicals leading to the synthesis of new compounds that preserved the beneficial properties of the natural chemical, but minimized its toxic effects (Fetrow & O'Neil, 2002).

Many medicines that we use today were isolated from plants sources. Research reveals that approximately 25-33% of currently available modern medicines in the United States have their origins in plants, animal, or mineral systems. The focus on synthesized and biotechnologically derived medicines has continued to this day. However, in the latter part of the 20th century, there has been an intense renewed interest in herbalism (Fetrow & O'Neil, 2002).

New medicines have been discovered with traditional, empirical and molecular approaches (Harvey, 1999). The traditional approach makes use of materials that has been discovered via trial and error modes over many years in different cultures and systems of medicine (Cotton, 1996). Examples include drugs such as morphine, quinine and ephedrine that have been in widespread use for a long time, and more recently adopted compounds such as the antimalarial artemisinin. The empirical approach builds on an understanding of a relevant physiological process and often develops a therapeutic agent from a naturally occurring lead molecule (Verpoorte, 1989; Verpoorte 2000). Examples include tubocurarine and other muscle relaxants, propranolol and other β□-adrenoceptor antagonists, and cimetidine and other histamine H_2 receptor antagonists. The molecular approach is based on the availability or understanding of a molecular target for the medicinal agent (Harvey, 1999). With the development of molecular biological techniques and advances in genomics, the majority of drug discovery is currently based on the molecular approach.

The major advantage of natural products for random screening is the structural diversity provided by these products, which is greater than that provided by most available combinatorial approaches based on heterocyclic compounds (Claeson & Bohlin, 1997; Harvey, 1999). Bioactive natural products often occur as a part of a family of related molecules. Thus, it is possible to isolate a number of homologues and obtain structure-activity information. Lead compounds discovered through the screening of natural products can of course be optimized by traditional medicinal chemistry or by the application of combinatorial approaches. Overall, when faced with molecular targets in screening assays for which there is no information about low molecular weight leads, the use of a natural products library seems more likely to provide the chemical diversity to yield success rather than the use of a library of similar numbers of compounds made by combinatorial synthesis. Since only a small fraction of the world's biodiversity has been tested for biological activity, it can be assumed that natural products will continue to offer novel leads for novel therapeutic agents, if these natural products are available for screening.

At present, more than 80,000 secondary metabolites have been identified in higher plant species (Loyola-Vargas & Miranda-Ham, 1995). 75-80 % of the world's population relies on these plant-based medicines and one in four of commercial pharmaceutical products are derived from plant-based sources (Pal & Shukla, 2003). Secondary metabolites are bioactive molecules which provide the plant with defense mechanisms to survive herbivores, environmental stress, disease or competition and may effect the growth and development of other organisms (Seigler, 1996). Each individual species has a unique profile of secondary metabolites and it is this pool of biochemicals that commonly contains the medicinally active components (Murch *et al.*, 2001).

1.2 Natural Products and Biodiversity

Natural products produced by plants, fungi, bacteria, insects and animals have been isolated as biologically active pharmacophores. Natural products have the potential to provide medicine through a source of novel structures that are unobtainable from other sources such as combinatorial synthesis. This is because nature is capable of producing complex molecules with multiple chiral centers that are designed to interact with biological systems (Cordell, 2000). Because biodiversity is so important to the continued discovery of novel natural products, it is important to know how much of this biodiversity remains. The greater the amount of remaining biodiversity to be studied, the greater the potential amount of chemical diversity that remains to be discovered. It has been estimated that of the approximately 250,000 plant species only, about 5-15% of them have been investigated for bioactive compounds (Kong *et al.*, 2003). Based on the above information, it is obvious that there is still an abundance of plant species available for investigation.

Cancer is the second leading cause of death in the United States; one out of every four deaths is from cancer. During 2002, it was estimated that over 1.28 million people will die of cancer (this figure does not include noninvasive cancers). The death rate for patients with cancer is 38%. The National Institutes of Health (NIH) has estimated the cost for cancer treatment to be US$ 156.7 billion. It is also important to note that 77% of all cancers diagnosed are in people 55 years of age or older (American Cancer Society, 2003). With cancer taking such a toll on the population, both in terms of lives and cost, the discovery of anticancer drugs has become very important. When one considers the aging population of the United States, it is clear that these numbers are likely to increase in the years to come, and the search for more effective drugs will become even more important. Some of the most effective cancer treatments to date involve the use of natural products or compounds derived from natural products. Numerous epidemiological studies have shown that diets low in fat

and rich in complex carbohydrates derived from vegetables, fruits and grains are associated with decreased risk of chronic diseases (Dragsted *et al.*, 1993). For example, grapefruit juice inhibits CYP3A4, (Bourian *et al.*, 1999) and vegetables such as brussel sprouts and broccoli whose glucosilinate compounds induce CYP1A2 (Fontana *et al.*, 1999). These enzymes metabolize many carcinogens, including tobacco related compounds and char grilled meat. In fact, induction of 1A2 underlies the cancer preventative reputation of family Brassicaceae.

Natural phenolic compounds make a considerable contribution to the nutritional quality of fruits and fruit products, which play an important role in the daily diet. They also play a key role in antioxidative defence mechanisms in biological systems and they may have an inhibitory effect on mutagenesis and carcinogenesis. Attention has turned to plant phenols because the use of synthetic antioxidants has been declining due to their suspected action as cancer promoters (Ho, 1992a). Caffeic acid, gallic acid and gallic acid derivatives (methyl-, lauryl- and propylgallates) show strong antioxidant properties and act as free radical acceptors (Ho, 1992b). They are widely used as food additives to protect lipid structures. Nevertheless, phenols can simultaneously have pro-oxidant effects, *i.e.* cause tissue damage by producing reactive oxygen species (ROS), and their consumption should be couched with caution (Aruoma *et al.,* 1993). The important biological activities of simple benzenoids, *e.g.* chlorogenic, caffeic, ferulic, gallic and ellagic acids, are probably due to their cytoprotective activity and possible inhibitory effects on carcinogenesis, mutagenesis and tumorigenesis (Lesca, 1983; Stich & Rosin, 1984; Chang *et al.*, 1985; Mukhtar *et al.,* 1988; Vieira *et al.,* 1998; Haslam, 1998; Kumar & Muller,1999). Flavonoids have a range of *in-vitro* as well as *in-viv*o biological effects on a great number of mammalian cell systems. Flavonoids have been shown to possess antiviral and endocrine effects, effects on mammalian enzymes, effects on the modulation of immune and inflammatory cell functions, effects on smooth muscles, and effects on lipid peroxidation and oxyradical production

(Harborne, 1994; Formica & Regelson, 1995). Since flavonoids are regular constituents of our every day diet, their possible genotoxic, carcinogenicity, and mutagenicity related properties have recently received increasing attention (Manson & Benford, 1999). Although evidence from human and animal, as well as *in-vitro* experiments, support the hypothesis that flavonoids promote health, it is possible that interactions with other dietary constituents or lifestyles may override any subtle positive effects of flavonoids in humans (Moskaug *et al.,* 2004).

1.3 Background of Herbal Medicine in Malaysia

Malaysia is rich in natural resources basic to herbal medicine. There are over 6000 species of tropical plants all over the country and in Peninsula Malaysia there are 550 genera containing 1300 species (Zakaria & Mohd, 1994). Past and present ethanobotanical or ethanomedical surveys suggest that at least about 20% of the estimated total of higher plant flora of 15,000 species comprise of plants which have been reported to possess medicinal and other therapeutic properties (Soepadmo, 1993).

Malaysia, as a multiracial country, markets four major groups of herbal medicine namely Malay herbal medicine, Indian herbal medicine, Chinese herbal medicine and Western herbal medicine. Every racial group has its own method or way of curing diseases and depends very much on the practice, belief and knowledge each one possesses. This search for cures to various diseases through the use of herbalism has indirectly fostered inter-racial interactions (Zakaria & Mohd, 1994).

Malay herbal medicine has been influenced by various foreign medicinal elements. The local Malay herbal medicine framework is actually based on old Indonesian herbal medicine approaches, which have been modified to suit local and current needs. Chinese and Indian immigrants brought with them various medicinal

plants which grew well in this country. The popularity of Chinese herbal medicine is evident from the presence of about 1000 medicinal shops commonly known as 'kedai sinseh' (Zakaria & Mohd, 1994).

However, the major problems faced by herbal medicine practitioners of all the four groups are firstly, the lack of clinical data to substantiate efficacy claims and secondly, non-existence of standards for most herbal materials and products.

Increasingly, alternative therapies such as herbal products are being used in the world. For example in the United States approximately 25% of American who consult their physician about a serious health problem are employing unconventional therapy, but only 70% of these patient inform their physician of such use (Eisenberg *et al.*, 1993). Most people believe that the herbal medicines have no side effects or any potential risk due to its natural origins and as such herbs are often administered in combination with therapeutic drugs. The manufacturers of these products are not required to submit proof of safety and efficacy before marketing because herbs are considered as food supplements and not drugs. Due to the foregoing reasons, the use of herbs in medical therapy increases the potential of pharmacokinetic and/or pharmacodynamic herb-drug interaction. Here, emphasis is placed primarily on the pharmacokinetic aspects, partly because pharmacokinetic interaction is the most common cause of undesirable and to date unpredictable effects (Ito *et al*., 1998). Moreover, my study is devoted to this aspect especially to one major component namely drug metabolism.

1.4 Drug Interactions

The particular response to a drug is determined in one way or another by the concentration of the drug, and some time its metabolite at the effect sites within the body. Accordingly, it is useful to divide the relationship between drug administration

and response into two phases, a pharmacokinetic phase, which refers to drug administration and its concentration within the body over time, and a pharmacodynamic phase, which refers to the responses (desired and undesired) produced in reaction to drug concentrations.

Pharmacokinetic processes *in-vivo* can be broadly divided into two parts, absorption which is usually defined as the passage of a drug from its site of administration into the circulatory system (Schanker, 1971); and its disposition, which applies to all sites of drug administration other than its direct injection into the blood stream and comprises all processes between a drug's administration to its appearance in the blood circulatory system. Bioavailability is a measure of the extent of drug absorption. Disposition comprises both the distribution of drugs into tissues within the body and their elimination and is itself divided into metabolism and excretion in unchanged form**.** The kidney and the liver are the main organs in the body for drug elimination; the kidney excretes drugs through urine unchanged and/or after metabolism by the liver while the liver can excrete a drug through the bile duct after metabolism. For many drugs, metabolism occurs in two distinct phases. Phase I involves the formation of a new or modified functional group or a cleavage. Phase II involves conjugation within an endogenous compound.

1.4.1 Pharmacokinetic Drug Interactions

Simply, drug interaction can be defined as a change in a drug's effect when administered with another drug, herb, or food. For example, two or more drugs, taken together can change the way a drug works in the body. This possibly could make one or more of these drugs less safe or reduce their efficacy. There are two main types of drug interactions: pharmacodynamic and pharmacokinetic drug interactions.

Pharmacokinetic interaction may occur during absorption and/or transportation whence the metabolism of the drugs alters physiological function. A transporter interaction occurs within organs such as the brain, to produce altered drug distribution, not excretion. This occurs, for example, with inhibition of the efflux transporter P-glycoprotein (PGP) located within the blood brain barrier (BBB). This inhibition of PGP leads to an elevation in cyclosporine levels in the brain (Tanaka *et al.*, 2000). Absorption interaction involves a change in either the rate or the extent of drug absorption, particularly following oral administration. There are many potential sites for absorption interaction within the gastric and intestinal lumen, at or within the gut wall, as well as within the liver. When an absorption interaction leads to a reduction in absorption, kinetics will result in lower and altered peak concentrations, which could be critical if the drug is intended for rapid onset of action, such as for the relief of a headache. Metabolism interaction occurs in the induction or inhibition of phase I and/or phase II enzymes and the depletion of substrates used by phase II enzymes. Over the last 10-15 years, metabolism interaction has been the major focus for drug interactions.

1.5 Drug Metabolism and Metabolism-Based Drug Interactions

The liver is rightfully considered to be the most important organ involved in drug metabolism. Drug bioavailability is controlled by the liver's capacity to clear the drug from circulation. This depends on both blood flow and the efficiency of drug removal by hepatocytes (extraction ratio). Drug metabolism involves a wide range of chemical reactions, including oxidation, reduction, hydrolysis, hydration, conjugation, condensation, and isomerization. The enzymes involved are present in many tissues but generally are more concentrated in the liver. For many drugs, metabolism occurs in two apparent phases. Phase I reactions involve the formation of a new or modified functional group or a cleavage (oxidation, reduction and hydrolysis); these are known as non-synthetic reactions. Phase II reactions involve conjugation with an endogenous compound (eg, glucuronic acid, sulfate, and glycine) and are therefore known as

synthetic reactions. Metabolites formed in synthetic reactions are more polar and more readily excreted by the kidneys (in urine) and/or the liver (in bile) than those formed in non-synthetic reactions. Some drugs undergo either phase I or phase II reactions; thus, phase numbers reflect functional rather than sequential classification. Phase I oxidation occurs primarily via the hepatic mono-oxygenase (mixed function oxidase) system, a complex enzyme system centered on the heme protein cytochrome P-450. This system is under genetic control and is highly sensitive to induction (stimulation) or inhibition by many factors (e.g. drugs, insecticides, herbicides, smoking, caffeine). Thus, hepatic drug metabolism varies widely among individuals.

1.6 Herbal-drug Interactions

Xenobiotics, drugs, and a variety of naturally occurring dietary or herbal constituents can interact in several ways with the CYP450 system as outlined below:

• A compound may be a substrate of, i.e. metabolized by, one or several CYP isoforms. If the main isoform is saturated, it becomes a substrate for the secondary enzyme(s).

• A compound can be an inducer of a CYP isoform, either of the one it is a substrate for, or may induce several different enzymes at the same time. The process of induction increases the rate of metabolism of substrates of that enzyme.

• A compound may also be an inhibitor of CYP450 enzymes. There are several mechanisms of inhibition, and a compound may inhibit several isoforms including others than those for which it is a substrate.

These are then the actions that underlie the pharmacokinetic variations in drug metabolism, and that cause interactions between two or more drugs, or between drugs and nutrients, or drugs and herbs.

Many herb-drug interactions have been reported. For instance, ingestion of broccoli may enhance CYP1A2-mediated caffeine metabolism (Kall *et al.,* 1996). Echinacea (*Echinacea purpurea*) selectively modulates the catalytic activity of CYP3A4

at hepatic intestinal sites (Gorski *et al.,* 2004). St Johns Wort interacts with drugs that are metabolized by cytochrome P450 isoform CYP3A4, it was suggested that St Johns Wort might induce CYP3A4 expression and this hypothesis was confirmed *in-vivo* (Markowitz *et al.*, 2000) and *in vitro* (Moore *et al.*, 2000).

There is clear evidence of the extensive involvement of the cytochrome P450 enzyme system in the elimination of pharmaceutical agents and there exists an enormous body of information demonstrating the modulation of its activity, via inhibition or induction, with polypharmacy. From the above, it is clear that the P450 enzyme system plays a main role in metabolism-based drug interactions.

1.7 Review of Literature for *Morinda citrifolia*

1.7.1 Botanical Aspects

Morinda citrifolia. is a shrub which grows in sandy areas along many tropical coastal regions at sea level and in forest areas of up to about 1300 feet above sea level. *Morinda citrifolia* is a small evergreen tree and is identifiable by its straight trunk, large, bright green and elliptical leaves with tubular flowers, and its distinctive, ovoid "grenade-like" yellow fruit. The fruit can grow in size up to 12 cm or more and has a lumpy surface covered by polygonal-shaped sections. The seeds, which are triangular shaped, and reddish brown have an air sac attached at one end, which makes them buoyant. The mature fruit has a foul taste and odour. The common globally recognised name is Noni. Apart from this appellation, there are many local names that are also widely used in their respective countries namely, Nonu (Samoa), Nono (Tahiti & Cook Islands), Nonu (Tonga) , Noni Apple, Polynesia Fruit, Indian Mulberry (India) , Bumbo (Africa), Lada (Guam), Mengkudu (Malaysia), Cheeserut (Australia), Painkiller Tree (Caribbean Islands), Nhau (Southeast Asia), Morinda (Vietnam), Hai Ba Ji (China), Kura (Fiji), Nen (Marshall Islans).

1.7.2 Phytochemistry

A number of major components have been identified in the Noni plant (*Morinda citrifolia*) such as scopoletin, octanoic acid, potassium, vitamin C, terpenoids, alkaloids, anthraquinones (such as nordamnacanthal, morindone, rubiadin, and rubiadin-1-methyl ether, anthraquinone glycoside), β-sitosterol, carotene, vitamin A, flavone glycosides, linoleic acid, alizarin, amino acids, acubin, *L*-asperuloside, caproic acid, caprylic acid, ursolic acid, rutin, and a putative proxeronine. These constituents and their classes are listed in Table (1.1) and references therein.

Table 1.1: The Classes of Chemical Constituents Reported in *Morinda citrifolia* (Rubiaceae) in the Literature

Classes	Compounds	Occurrence	References
Anthraquinones	Morindine, rubiadine Rubiadine 1-methylether	Roots & fruit	Wang *et al.,* 2002
Anthraquinones	Rubiadin lucidin, morindone, lucidin-3—prineresal, morindone-6-β –primeveroside, seven new quinones	Cell suspension culture of *M. citrifolia*	Inoue *at el.*, 1981
Glycosides	Glycoside of coumarin, flavone and anthraquinone	Fruit	Wang *et al.*, 2000
Essential oils	Volatile oil	Ripe fruit	Farine *et al*., 1996
Coumarone	Scopoletin	Fruit	Farine *et al*., 1996
Flavonol	Vomifoliol	Ripe fruit	Farine *et al.*, 1996
Monoterpenes	Iridoid	Leaves	Sang *et al*., 2003
Sterol	Campesterol Stigmasterol, Sitosterol Isofucosterol, Sitosteryl palmitate, Isofucosteryl palmitate	Cell suspension culture of *M. citrifolia*	Dyas *et al.*, 1994
Vitamins	Vitamin C 24- 258 mg/100 g dried fruit	Dried fruit	Hirazumi & Furusawa, 1999

Plate 1.1: Fruit of *Morinda citrifolia* (Rubiaceae)

1.7.3 Ethnopharmacology

Morinda citrifolia is one of the traditional folk medicinal plants that has been used for over 2000 years in Polynesia (Wang *at el.,* 2002). *Morinda citrifolia* was the second most popular plant used in herbal remedies to treat various common diseases and to maintain overall good health among Polynesians (Abbott & Shimazu, 1985). The Polynesians utilized the whole Noni plant in various combinations as herbal remedies. The fruit was eaten for health and dietary reasons (Wang *at el.,* 2002). The fruit juice is in high demand as an alternative medicine for different kinds of illnesses such as arthritis, diabetes, high blood pressure, muscle aches and pains, menstrual difficulties, headaches, heart disease, AIDS, cancers, gastric ulcers, mental depression, senility, poor digestion, atherosclerosis, blood vessel problems, and drug addiction (Abbott & Shimazu, 1985). Scientific evidence on the benefits of the Noni fruit juice is limited but there is some anecdotal evidence for successful treatment of colds and influenza (Wang *at el.,* 2002). In Fiji, Noni was a traditional remedy used to treat broken bones; In India, Noni was ingested internally as a tonic during fever and was used as a healing application to wounds and ulcers (Singh, 1986). In Tonga, *Morinda citrifolia* (Noni) was used topically for the treatment of breast carcinomas (Singh *at el.,* 1984). This earlier chemical findings and biological activities have since been confirmed with more advanced techniques. Active principles or extracts of *M. citrifolia* have been shown to possess several pharmacological properties, *e.g.* analgesic, antiinflammatory, antioxidant, chemoprotective, antimicrobial, and immunomodulatory properties (Table 1.2). Acubin, L-asperuloside, and alizarin in the mengkudu fruit, as well as other anthraquinone compounds in the mengkudu root, are all proven antibacterial agents. These compounds have been shown to fight infectious bacteria strains such as *Pseudomonas aeruginosa, Proteus morgaii, Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Salmonella,* and *Shigela.* These antibacterial elements within mengkudu are also responsible in the treatment of skin infections, colds, fevers, and other bacterial-related health problems (Wang *at el.,* 2002).

Recently, one of study has demonstrated that scopoletin, a health promotor in mengkudu, inhibits the activity of *E. coli,* commonly associated with serious infections and even death. Mengkudu also helps in the treatment of stomach ulcer through its inhibition of the bacteria *H. pylori* (Duncan *et al.,* 1998). Moreover, its anti-tubercular effects have also been reported in that a crude ethanol extract and hexane fraction from *Morinda citrifolia* showed antitubercular activity (Saludes *et al.,* 2002).

The antiviral activity of mengkudu was observed when a compound isolated from Mengkudu roots named 1-methoxy-2-formyl-3-hydroxyanthraquinone suppressed the cytopathic effect of HIV infected MT-4 cells, without inhibiting cell growth (Wang *et al.,* 2002).

Mengkudu's antitumor activity study has also been reported. For instance, the alcohol-precipitate of mengkudu fruit juice (mengkudu-ppt) significantly prolonged the lifespan, by up to 75%, in C57 Bl/6 mice implanted with Lewis lung carcinoma compared to that in the control group (Hirazumi *et al*., 1994). It can be concluded that the mengkudu-ppt seems to suppress tumor growth indirectly by stimulating the immune system (Hirazumi *et al*., 1996). Improved survival time and curative effects occurred when mengkudu-ppt was combined with suboptimal doses of the standard chemotherapeutic agents such as adriamycin (Adria), cisplatin (CDDP), 5-fluorouracil (5-FU), and vincristine (VCR), suggesting important clinical applications of mengkudu-ppt as a supplementary agent in cancer treatment (Hirazumi & Furusawa, 1999). These results indicate that noni-ppt may enhance the therapeutic effects of anticancer drugs. Therefore it may be of benefit to cancer patients by enabling them to use lower doses of anticancer drugs to achieve the same or even better results. Recently, a study has reported the effects of over 500 extracts from tropical plants on the K-Ras-NRK cells. Damnacanthal, isolated from mengkudu roots, is an inhibitor of Ras function. The *ras*

oncogene is believed to be associated with the signal transduction in several human cancers such as lung, colon, pancreas, and leukemia (Wang *et al.,* 2002).

Two glycosides extracted from mengkudu-ppt have reportedly been effective in inhibiting cell transformation induced by 12-O-tetradecanoylphorbol-13-acetate (TPA) or epidermal growth factor (EGF) in the mouse epidermal JB6 cell line. The inhibition was found to be associated with the inhibitory effects of these compounds on AP-1 activity (Liu *et al.,* 2001; Sang *et al*., 2001).

Mengkudu also possess anthelmintic ability. An ethanol extract of the tender Noni leaves induced paralysis and death of the human parasitic nematode worm, *Ascaris Lumbricoides,* within a day (Raj, 1975).

It has also been reported that the mengkudu fruits possesses analgesic and tranquilizing activities (Wang *et al.,* 2002). In addition, a study tested the analgesic and sedative effects of extracts from the *Morinda citrifolia* plant. It was observed that the extract did "show a significant, dose-related, central analgesic activity in treated mice." The study further stated that "these findings validate the traditional analgesic properties of this plant." In fact, the analgesic efficacy of the mengkudu extract is 75 % as strong as morphine, yet non-addictive and side effect free (Younos *et al.,* 1990).

Apart from this, it has also been demonstrated that a total extract of the mengkudu roots has a hypotensive effect (Wang *et al.,* 2002). A study into the anti-inflammatory effect of mengkudu reported that the ethanol extract of mengkudu powder exhibited inhibition of COX-1 in *in-vitro* using aspirin and indomethacin as reference for COX-1 inhibitors. Additionally, it was observed that this inhibition of COX-1 by the ethanol extract of mengkudu was more potent than that in aspirin and indomethacin (Li *et al.,* 2003).

The immunological activity of mengkudu has also been reported in that it was observed that an alcohol extract of mengkudu fruit at various concentrations inhibited the production of tumor necrosis factor-alpha (TNF-α), which is an endogenous tumor promoter (Hokama, 1993). Another study found that mengkudu-ppt contains a polysaccharide-rich substance that inhibited tumor growth. It did not exert significant cytotoxic effects in adapted cultures of lung cancer cells, but could activate peritoneal exudate cells to impart profound toxicity when co-cultured with tumor cells. This suggested the possibility that mengkudu-ppt may suppress tumor growth by activating the host immune system. Mengkudu-ppt was also capable of stimulating the release of several mediators from murine effector cells, including TNF-α, interleukin-1beta (IL-□β), IL-10, IL-12, interferon-gamma and nitric oxide (NO) (Hirazumi & Furusawa, 1999).

Mengkudu fruit has antioxidant; recently, a n-BuOH-soluble partition of the MeOH extract of *Morinda citrifolia* fruit has been reported that it has potent antioxidant property (Su *et al.,* 2005*)*.

Table 1.2: Recently Reported Biological Effects of *Morinda citrifolia* (Rubiaceae)

Biological Effects	References
Antibacterial activity	Wang *at el.,* 2002
A health promoter that inhibits the activity of *E. coli;* also helps in stomach ulcer treatment through inhibition of the *H. pylori* bacteria.	Duncan *et al.,* 1998
Suppression of cytopathic effect of HIV infected MT-4 cells, without inhibiting cell growth.	Wang *at el.,* 2002
Mycobacterium tuberculosis killer in *in vitro* study	Wang *et al.,* 2002
Anticancer activity	Hirazumi *et al.,* 1994; Furusawa *et al.,* 2003
Enhancement of the therapeutic effect of anticancer drugs such as Taxol.	Wang *at el.,* 2002
Inhibition of the Ras (oncogene) function.	Hiramatsu *et al.,* 1993
Inhibition tyrosine kinases activity	Hiwasa *et al.,* 1999
Inhibition of cell transformation in mouse epidermal JB6 cell line.	Liu, *et al.,* 2001; Sang *et al.,* 2001
Anathematic effect	Raj, 1975; Fouraste *et al.,* 2005
Analgesic effect	Li *et al.,* 2003
Hypotensive effect	Wang *et al.,* 2002
Antioxidant activity	Kamiya *et al.,* 2004
Antiangiogenic effect in human placental veins	Hornick *et al.*, 2003
Immunomodulation	Hirazumi *et al.*, 1996

1.8 Extrapolation of Animal Results to Man

The pre-clinical safety evaluation of chemicals for use in man is usually done using mammalian species. Ideally, for a complete model animal species, the latter should be similar to man in four respects, namely (a) the rates and routes of metabolism, (b) the rates and routes of excretion, (c) the pharmacokinetic profile of which (a) and (b) are important determinants, and (d) the receptor response (Smith, 1978).

Species variations in drug metabolism can occur in respect to the speed at which metabolism occurs and in the metabolic pathways employed, and these differences arise mainly because of interspecies variations in enzyme control of phase I and phase II reactions (Smith, 1978).

The projection of animal data directly to man should not be made on the assumption that the same dose of drug (in mg/kg) will attain the same concentration at the drug receptors in man as in animals (Brodie & Reid, 1971). In general, small animals such as mice metabolise foreign compounds at a faster rate than larger animals such as humans, consistent with differences in overall metabolic rates (Barrow, 2000). Rats are six times more efficient than man in handling xenobiotics based on its liver size/body weight (kg) which is twice that of man. Furthermore, concentrations of cytochrome P450 in rats is three times higher than in man. Besides that, ratio of dose relative to body weight (mg) to dose relative to body surface area (mg) showed that despite exhibiting similar drug effects on rats and man, dosage given to man is actually 10-times lower than that administered in rats (Klaassen & Doull, 1980).

1.9 Objectives of Study

This study is focused on the herbal products of *Morinda citrifolia* (Noni) which are most commonly found in supermarkets and its interaction with drugs based on phase I and phase II studies of metabolism using rat livers. Information about herbs is very limited because in most countries there are no universal regulatory systems that ensure the safety of phytopharmaceuticals. Yet uses of traditional medicine remain widespread in developing countries while the use of complementary and alternative medicine is increasing rapidly in developed countries in many parts of the world.

The specific aims of this study were:

- To study the *in-vitro* effect of the extract and two commercial products(Hawaiian and Tahiti) of mengkudu juice of *Morinda citrifolia* on liver aminopyrine metabolism by taking into account the effect of internal factors such as disease (hypertension and diabetes), gender and age on liver aminopyrine metabolism.
- To elucidate the molecular mechanism of the *in-vitro* effect of *Morinda citrifolia* preparations which significantly affect liver aminopyrine metabolism.
- To study the *ex-vivo* effect of the mengkudu juice extract (MJE) of *Morinda citrifolia* on liver aminopyrine metabolism which yielded significant results during *in-vitro* studies.
- To elucidate the molecular mechanisms of the *ex-vivo* effect of the *Morinda citrifolia* (MJE) at concentrations which significantly affect liver aminopyrine metabolism.
- To study the *ex-vivo* effect of *Morinda citrifolia* (MJE) on phase II enzymes (GST and UDPGA) which yielded significant results during *in-vitro* studies in phase I.
- To conduct a qualitatively chemical studies of MJE and two commercial products of Noni juice of *Morinda citrifolia* (Hawaiian and Tahiti) using UV/VIS, IR, ^{1}HNMR spectrophotometers and HPTLC.

CHAPTER TWO
EFFECT OF *MORINDA CITRIFOLIA* ON LIVER PHASE I AMINOPYRINE METABOLISM

2.1 Introduction

2.1.1 Phase I Drug Metabolism

Main drug metabolism reactions associated with phase I liver metabolism are hydrolysis, reduction, hydration and oxidation. During the drugs phase I metabolism, new functional groups are introduced into the lipophilic drug structures. In phase I metabolism, oxidation can be further sub-classified into oxidation performed by microsomal mixed-function oxidase systems (cytochrome P450 dependent) and oxidation not cytochrome-dependent which has a number of enzymes in the body that are not related to the mixed-function oxidase systems. Most of these enzymes are primarily involved in endogenous compound metabolism which include alcohol dehydrogenase, aldehyde dehydrogenase, xanthine oxidases, amine oxidases, aromatases and alkylhydrazine. Complete mixed-function oxidase system which includes cytochrome P450, NADPH-cytochrome P450 reductase has the following types of oxidation metabolism namely : aromatic hydroxylation, S-oxidation, phosphothionate oxidation, aliphatic hydroxylation epoxidation, oxidative deamination, N-oxidation, dehalogenation and dealkylation (Gibson & Skett, 1994).

The present study involved dealkylation reaction, in particular, N-demethylation which is responsible for the metabolism of aminopyrine drug model.

2.1.1.1 Cytochrome P450s and Their Role on Drug Metabolism

The Cytochrome P-450 (CYP450) system is a family of heme based enzymes located in the smooth endoplasmic reticulum, particularly concentrated in hepatocytes

and mucosal enterocytes but also found in the kidneys, skin and lung tissues of humans (Gibson & Skett, 1994; Clarke & Jones, 2002). Known also as the mixed function oxidases, it is one of the most important systems in the biotransformation of drugs. The CYP450 families of enzymes are responsible for phase I xenobiotic metabolism, catalyzing predominantly oxidation, reduction and hydrolysis reactions which render lipophilic compounds more polar, prior to the phase II processes of thiol conjugation, glucuronidation, sulfation or acetylation which enable the metabolites to be excreted by the kidneys or liver. A microsomal superfamily of isoenzymes transfer electrons and thereby catalyzes the oxidation of many drugs. The electrons are supplied by NADPH-cytochrome P-450 reductase, a flavoprotein that transfers electrons from NADPH (the reduced form of nicotinamide-adenine dinucleotide phosphate) to cytochrome P-450 (Gibson & Skett, 1994). Cytochrome P-450 enzymes are grouped into 14 mammalian gene families that share sequence identity and 17 subfamilies. They are designated by a root symbol CYP, followed by an Arabic number for family, a letter for subfamily, and another Arabic number for the specific gene (Clarke & Jones, 2002). Enzymes in the 1A, 2B, 2C, 2D, and 3A subfamilies are the most important in mammalian metabolism; in human 35 P450 enzymes were described although only 18 P450 enzymes in families 1, 2, and, 3 appear to be responsible for the metabolism of drugs and therefore are potential sites for drug interactions. It has been noted that CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 are important in drug metabolism (Clarke & Jones, 2002). The specificity of these enzymes helps explain many drug interactions. P450 enzymes are found throughout the body, however, the liver and the intestinal epithelia are the predominant sites for P450-mediated drug interactions and they are also the sites worth considering in most detail with respect to drug interactions.

Many different P450 enzymes have been detected in the intestine from various species, including man (Yamamoto *et al.,* 1998; Zhang *et al.,* 1998; Hiroi *et al.,* 1998;

Zeldin *et al.,* 1997; Prueksaritanont *et al.,* 1996; Kaminsky & Fasco, 1991). However, the CYP3A4 is overwhelmingly the most significant P450 enzyme in the human intestine (Lown *et al.,* 1994; Kolars *et al.,* 1994). The fact that CYP3A4 is the P450 enzyme of significant concern for drug to drug interactions in the intestine is supported by a number of pharmacokinetic studies. Intestinal pre-systemic elimination has been shown for several drugs metabolized by CYP3A4. e.g. cyclosporine (Wu *et al.,* 1995), tacrolimus (Hashimoto *et al.,* 1998; Lampen *et al.,* 1995) sirolimus (Lampen *et al.,* 1998) midazolam (Paine *et al.,* 1996), saquinavir (Wacher *et al.,* 1998), felodipine (Wang *et al.,* 1989; Lown *et al.,* 1997), and nefazadone (Marathe *et al.,* 1995). Grapefruit juice has been shown to have significant interaction with a number of these drugs (Ameer & Weintraub, 1997), because grapefruit affects the activity of CYP3A4 in the intestine (Lown *et al.,* 1997; Fuhr, 1998; Feldman, 1997).

In the human liver, the relative content of the major P450 enzymes has been determined in several studies and a general consensus has emerged. On average, CYP3A4 is quantitatively the most important in the body, while with CYP2C8, CYP2C9, CYP2A6, CYP2E1, and CYP1A2 present in somewhat lower quantities, on the other hand CYP2C19 and CYP2D6 are of relatively minor quantitative importance (Clarke & Jones, 2002). CYP3A4 is responsible for approximately 50% of the P450-mediated metabolism of marketed pharmaceuticals. Nevertheless, CYP2D6 has a disproportionate share, (~25%) of the overall total of enzymes, in comparison to the amount of other enzymes present in the liver.

2.1.1.2 Aminopyrine

Aminopyrine was introduced into medicine in the late nineteenth century as an antipyretic, and subsequently was also widely used as an analgesic and antiinflammatory agent. However, clinical use of aminopyrine was sharply curtailed after its potentially fatal bone marrow toxicity, agranulocytosis, was recognized.