# EFFECT OF STANDARDISED METHANOLIC EXTRACT OF *PHYLLANTHUS NIRURI* (LINN.) ON PHASE I AND II DRUG METABOLISM AND ITS MOLECULAR MECHANISM ELUCIDATION IN RAT LIVER

**MOATH KAHTAN BASHIR**

**UNIVERSITI SAINS MALAYSIA**
**2010**

# Effect of Standardised Methanolic Extract of *Phyllanthus niruri* (Linn.) on Phase I and II Drug Metabolism and its Molecular Mechanism Elucidation in Rat Liver

**By**

**MOATH KAHTAN BASHIR**

Thesis submitted in fulfillment of the
requirements for the degree of
Master of Science

October 2010

# ACKNOWLEDGMENTS

I would like to express my sincere thanks to those people who helped me through their guidance, advice and moral support specially:

My sincere thanks and deepest gratitude to my academic Supervisor, Associate Professor **Dr. Abas Hj. Hussin**, for giving me the chance to work in his laboratory and for his interest, advice, helpful discussion, comments, guidance and encouragement throughout this work. I considered myself privileged to be under his supervision.

My deepest thanks to my co-supervisors: Professor Chan Kit Lam and Associate Professor Dr. Sabariah Ismail for their advice, kind support and fruitful discussion.

I would like also to convey my deepest thanks and gratitude to all post-graduate students, academic and non-academic staff in the school for their support, help and advice specially: Dr. Vikneswaran a/l Murugaiyah, My best friend Mahfoudh Almusali, Mr. Omar Z. Ameer, Mr. Ibrahim M. Salman, Mr. Mun Fei Yam, Mr. Roseli Hassan, Mr. Yusuf , Mr. Adnan, Mr. Mohammad Adnan, Mr. Harith Alqazaz, Mr. Omar Allela and Mr. Juzaili Azizi.

I would like to thank the School of Pharmaceutical Sciences in USM for giving me the chance to study here.

Finally, I would like to express my most sincere and warmest gratitude to my Father, my Mother, my wife (*Remah*), my daughters (*Nabaa and Hafsah*), my brother, my sisters and whole family for their help, prayers and love.

## TABLE OF CONTENTS

**Chapter three: *In vitro* and *Ex vivo* Effect of Standardised Methanolic Extract of *Phyllanthus niruri* on Phase II Drug Metabolism**

## LIST OF TABLES

## LIST OF FIGURES

## LIST OF ABBREVIATIONS

| | |
|---|---|
| ˚C | Degree celsius |
| 3-MC | 3-methylcholanthrene |
| 5-AMP | Adenosine 5-monophosphate |
| 5-GMP | Guanosine 5-monophosphate |
| ABTS | 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) |
| *ad libitum* | To be taken as wanted |
| ANF | Atrial natriuretic factor |
| AP-1 | Activating protein-1 |
| ARR | Aldosterone to renin ratio |
| ATP | Adenosine-5-triphosphate |
| BSA | Bovine serum albumin |
| $Ca^{2+}$ | Calcium ion |
| CAM | Complementary and alternative medicine |
| cAMP | Cyclic adenosine 3',5'-monophosphate |
| CC | Column chromatography |
| CDNB | 1-chloro-2,4-dinitrobenzene |
| CFTR | Cystic fibrosis transmembrane regulator |
| cGMP | Cyclic guanosine-3',5'-monophosphate |
| CYP | Cytochrome P450 |
| DAG | Diacylglycerol |
| DCA | Drug control authority |
| DNA | Deoxy ribonucleic acid |

| | |
|---|---|
| DMSO | Dimethyl sulfoxide |
| DPPH | 2,2-diphenyl-1-picrylhydrazyl |
| DR | Diabetic rat |
| $EC_{50}$ | Half maximum effective concentration |
| Egr-1 | Early growth response-1 |
| FBS | Fasting blood sugar |
| FDA | Food and Drug Administration |
| FSH | Follicle stimulating hormone |
| g | Gram |
| GAGs | Glycosaminoglycans |
| GFR | Glomerular filtration rate |
| GLUT-2 | Glucose transport protein-2 |
| Gpp(NH)p | Guanylylimidodiphosphate |
| GSH | Glutathione reduced form |
| GST | Glutathione S-transferase |
| GTN | Nitroglycerin |
| HBeAg | Hepatitis B-virus envelop antigen |
| HBsAg | Hepatitis B-virus surface antigen |
| HBSS | Hank's buffer salt solution |
| HIV | Human immune deficiency virus |
| HPLC | High performance liquid chromatography |
| HPTLC | High performance thin layer chromatography |
| IBMX | 3-isobutyl-1-methylxanthine |

| | |
|---|---|
| $IC_{50}$ | Half maximum inhibitory concentration |
| iNOS | Induced nitric oxide synthetase |
| $IP_3$ | Inositol triphosphate |
| IR | Infrared spectroscopy |
| i.v. | Intravenous |
| KBr | Potassium bromide |
| kg | Kilogram |
| l | Liter |
| L(NIO) | L-$N^5$-(1-Iminoethyl)-ornithine |
| LPS | Lipopoly saccharide |
| M | Molarity |
| MARCKS | Myristoylated alanine-rich C-kinase substrate |
| µg | Microgram |
| mg | Milligram |
| mg/kg | Milligram per kilogram |
| µg/ml | Microgram Per Milliliter |
| µl | Microliter |
| µM | Micromolar |
| $mm^3$ | Cubic millimeter |
| MS | Mass spectroscopy |
| n | Number of animals |
| nM | Nanomolar |
| NADPH | Reduced form of nicotinamide adenine dinucleotide phosphate |

| | |
|---|---|
| ng/ml | Nanogram per milliliter |
| NO | Nitric oxide |
| NR | Normal rat |
| NMR | Nuclear magnetic resonance |
| PAPS | 3'-phosphoadenosine-5'-phosphosulfate |
| PAF | Platelets activating factors |
| PB | Phenobarbital |
| PDE | Phospho diesterase enzyme |
| PDK-1 | 3-phosphoinositide-dependent kinase-1 |
| PNME | Methanolic extract of *Phyllanthus niruri* |
| $PIP_2$ | phosphatidylinositol-4,5-biphosphate |
| PKA | Protein kinase A |
| PKC | Protein kinase C |
| PKG | Protein kinase G |
| PMA | Phorbol-12β-myristate-13α-acetate |
| *p*-NP | *p*-nitrophenol |
| PP-1 | Protein phosphatase-1 |
| PP-2A | Protein phosphatase-2A |
| REV | Regulation of virion expression |
| rpm | Revolutions per minute |
| RRE | Ribonucleoprotein response element |
| RSA | Radical scavenging activity |
| SAM | S-adenosylmethionine |

| | |
|---|---|
| S.D. | Standard deviation |
| SHR | Spontaneously hypertensive rat |
| STZ | Streptozotocin |
| TEAC | Trolox equivalent antioxidant capacity |
| TLC | Thin layer chromatography |
| TNF-alpha | Tumor necrosis factor-alpha |
| UDP-GlcA | Uridine diphosphate glucuronic acid |
| UGT | Uridine diphosphate glucuronyltransferase |
| US | United States |
| UV/VIS | Ultra violet / visible |
| vs | Versus |
| WHO | World Health Organization |
| w/w | Weight per weight |

**Kesan Ekstrak Metanol Terpiawai *Phyllanthus niruri* (Linn.) Terhadap Metabolisme Drug Fasa I dan II dan Penjelasan Mekanisme Molekulnya dalam Hati Tikus**

## ABSTRAK

*Phyllanthus niruri* (*P. niruri*) merupakan tumbuhan ubatan yang ditemui secara meluas di kawasan tropikal dan subtropical di dunia. Di Malaysia, ia dikenali sebagai "Dukong Anak". Banyak kesan-kesan terapeutik tumbuhan ini telah dilaporkan termasuk antivirus khususnya terhadap hepatitis B dan HIV (human immune deficiency virus), antihiperurisemia, menyingkirkan batu karang pada ginjal dan pundi hempedu, serta aktiviti antinosiseptif, antiradang, antihiperlipemia, antihipertensif, antiparasit dan antimikrob. Kajian fitokimia ke atas tumbuhan ini menunjukkan kandungan kimianya yang pelbagai, antaranya, lignan, alkaloid, flavonoid, sterol, koumarin, benzenoid, tanin, dan triterpena. Berdasarkan penggunaan produk perubatan herba yang semakin meluas, maka kemungkinan interaksi ubat-herba telah meningkat. Tidak banyak yang diketahui tentang insidens interaksi ubat-herba pada pesakit yang mengambil *P. niruri* dengan ubat lain. Kajian ini bertujuan menyelidik kesan *in vitro* dan *ex vivo* ekstrak metanol terpiawai daripada *P. niruri* pada enzim metabolisme fasa I dan II dalam hati tikus di bawah pengaruh penyakit (diabetes dan hipertensi), jantina, dan umur serta untuk menjelaskan mekanisme molekul bagaimana ekstrak memberi kesan pada metabolisme aminopirin fasa I. Kajian *in vitro* mendapati bahawa ekstrak metanol terpiawai daripada *P. niruri* tidak menunjukkan kesan yang signifikan pada metabolisme aminopirin fasa I pada tikus-normal daripada jantina dan kumpulan umur yang berbeza. Sementara itu, ekstrak pada kepekatan yang tinggi (10 μg/mL) menunjukkan kesan induksi yang signifikan pada tikus - diabetes dan tikus-hipertensi spontan. Jantina dan umur didapati mempengaruhi kesan ekstrak metanol terpiawai

daripada *P. niruri* terhadap metabolisme aminopirin fasa I di bawah keadaan penyakit (diabetes dan hipertensi). Tiada kesan signifikan diperhatikan pada dos akut (rawatan satu dos) dan subkronik (rawatan harian selama 14 hari) daripada ekstrak terhadap aktiviti aminopirin N-demetilase pada tikus-diabetes betina muda yang terpilih. Dalam kajian *in vitro* fasa II, tiada kesan signifikan diperhatikan terhadap aktiviti enzim glutation S-transferase (GST) dan UDP-glukuroniltransferase (UGT). Walau bagaimanapun, kajian *ex vivo* akut menunjukkan kesan induksi tidak bergantungan dos yang signifikan terhadap aktiviti enzim GST pada dos (500 mg/kg, 1000 mg/kg, 2000 mg/kg, dan 5000 mg/kg), dan terhadap aktiviti enzim UGT pada dos (1000 mg/kg, 2000 mg/kg, dan 5000 mg/kg). Kajian *ex vivo* menunjukkan kesan induksi bebas dos yang signifikan terhadap aktiviti enzim GST pada dos 500 mg/kg dan 2000 mg/kg. Sementara itu, tiada kesan yang signifikan diperhatikan pada aktiviti enzim UGT dalam kajian *ex vivo* subkronik. Suatu kemungkinan wujud bahawa interaksi yang sama boleh berlaku secara *in vitro* dan *ex vivo* dengan ubat lain, yang menjalani tindak balas hepatik N-demetilasi fasa I dan glutation fasa II dan tindak balas konjugasi glukuronida. Kajian seterusnya diperlukan untuk menentukan samada kesan ini juga dihasilkan *in vivo*. Kajian mekanisme molekul mencadangkan bahawa kesan induksi yang dihasilkan oleh ekstrak metanol terpiawai *P. niruri* terhadap aktiviti aminopirin N-demetilase pada tikus-diabetes betina muda, adalah kebanyakannya melalui lintasan cAMP-PKA. Ekstrak metanol terpiawai *Phyllanthus niruri* telah dipiawaikan dengan sebatian tulen filantin menggunakan HPLC-UV. Spektra infra merah (IR) dan lampau ungu daripada ekstrak menunjukkan kesamaan kualitatif dengan juzuk juzuk utama. Aktiviti penghapusan DPPH dan yang tinggi diperhatikan dalam ekstrak mengesahkan akan kandungan fenolnya yang amat tinggi.

**Effect of Standardised Methanolic extract of *Phyllanthus niruri* (Linn.) on Phase I and II Drug Metabolism and its Molecular Mechanism Elucidation in Rat Liver**

## ABSTRACT

*Phyllanthus niruri* (*P. niruri*) is a widely available medicinal plant in tropical and subtropical regions of the world. In Malaysia, it is locally known as "Dukong Anak". Many therapeutic effects of this plant have been reported including antiviral effects especially on hepatitis B and human immune deficiency virus (HIV), anti-hyperuricemic, removal of kidney and gall stones, anti-nociceptive, anti-inflammatory, anti-hyperlipemic, anti-hypertensive, anti-parasitic and anti-microbial activities. Phytochemical studies of this plant showed diversity of chemical contents such as lignans, alkaloids, flavonoids, sterols, coumarins, benzenoids, tannins, and triterpenes. Due to the wide use of herbal medicinal products, the possibilities of herb-drug interactions have increased. Little is known about the incidence and consequences of herb-drug interactions in patients who consume *P. niruri* concomitantly with other drugs. The aims of this study were to investigate the *in vitro* and *ex vivo* effects of standardized methanolic extract of *P. niruri* on phase I and II metabolizing enzymes in rat liver under the influence of diseases (diabetes and hypertension), gender, and age, as well as to elucidate the molecular mechanism by which it affect aminopyrine phase I metabolism. The *in vitro* study found that standardized methanolic extract of *P. niruri* did not show significant effect on phase I aminopyrine metabolism in normal rats of different gender and age groups while the extract at the higher concentration (10 µg/mL) showed significant induction effect on diabetic rats and spontaneously hypertensive rats. Gender and age were found to influence the effect of standardized methanolic extract of *P. niruri* on aminopyrine phase I metabolism under disease conditions (diabetes and

hypertension). No significant effect were observed in acute (single dose treatment) and sub-chronic (daily treatment for 14 days) doses of the extract on aminopyrine N-demethylase activity in the selected diabetic young female rats. In phase II *in vitro* study, no significant effect were observed on glutathione S-transferase (GST) and UDP-glucuronyltransferase (UGT) enzymes activity. However, acute *ex vivo* study showed significant dose independent induction effect on GST enzyme activity at doses (500 mg/kg, 1000 mg/kg, 2000 mg/kg, and 5000 mg/kg), and on UGT enzyme activity at doses (1000 mg/kg, 2000 mg/kg, and 5000 mg/kg). Sub-chronic *ex vivo* study showed significant dose independent induction effect on GST enzyme activity at doses 500 mg/kg and 2000 mg/kg, while no significant effect was observed on UGT enzyme activity in the sub-chronic *ex vivo* study. A possibility exist that similar interactions may occur *in vitro* and *ex vivo* with other drugs that undergo hepatic phase I N-demethylation and phase II glutathione and glucuronide conjugation reactions. Whether this effect is similarly produced *in vivo* still needs further investigations.The molecular mechanism study suggests that the induction effect produced by the standardized methanolic extract of *P. niruri* on aminopyrine N-demethylase activity in young female diabetic rats was mainly through cAMP-PKA pathway. Methanolic extract of *P. niruri* was standardized with the authentic compound phyllanthin using HPLC-UV. Infrared (IR) and Ultraviolet/Visible spectra of the extract showed qualitative similarities with their major constituents. The high DPPH scavenging and anti-oxidant activity observed in the extract confirmed its highly phenolic contents.

# Chapter One

# General Introduction

## 1.1 Definition of Herbal Products

Herbal product in general can be defined as a plant or part from plant or an extract from plant used as a flavor, fragrance, or for medicinal purposes (Blumenthal and Loren D. Israelsen, 1998) , while herbal medicinal product is defined as any medicinal product that contains active ingredient from herbal origin which may be from the whole plant or part from it to treat ailment or promote health (Silano *et al*., 2004). Herbal medicinal products may contain active ingredients from aerial or underground part of the plant and may be a combination of ingredients from more than one plant and these ingredients may be in the form of juices, essential oils, fatty oils and gums. Excipients may be added to the active constituents in the final product (Blumenthal and Loren D. Israelsen, 1998).

### 1.1.2 Uses of Medicinal Herbs in The World

It is a duty, sacred and obligatory from the beginning of life to rescue human from clutches of disease. Natural products especially from plant origin have been used for a very long time for treatment of different types of illnesses; indeed during thousands of years of development, there was a considerable increase to use herbs in medicine (Panda, 2000). The term phytomedicine is used to describe the use of plant or its parts to treat ailment in an effort of human to free himself from diseases.

Babylonion clay tablets 3000 B.C. showed that human used plants for several things such as magic, ceremonies and medicine (Fetrow and O'Neil, 2002). In Egypt, herbal

medicine is regarded as one of the oldest medicine system, which is called Ebers papyrus dating 1500 B.C. It contains a list of 877 herbal recipes for treatment of variety of illnesses (Balch and Rister, 2002). China and India are also regarded from the ancient recorded herbal medicine system. This is indicated by the oldest chinese pharmacopoeia Pen-ts'ao around 3000 B.C. and Ayurveda, which is the ancient science of natural health in India (Kowalchik and Hylton, 1998).

The development of chemical analysis started in the early of 19$^{th}$ century and scientists were able to extract and modify the active ingredients of plants. Later as science advanced, chemists discovered their own version of plant compounds. Medicinal herbal products were synthesized and a transition from raw herbs to the synthetic pharmaceutical products (Mauseth, 2009).

The interest with herbal medicine continues and currently, about 25% of our medicines came from chemicals of natural origin especially herbs. Many researches in the 20$^{th}$ century continued to show the effect of medicinal plants to treat and cure variety of illnesses (Khalsi, 2009). World Health Organization (WHO) estimated that up to 80% of world population are using traditional medicine for primary health care (Akintonwa *et al*., 2009). In USA, the use of traditional medicine was increased from 33.8% in 1990 to 42.1% seven years later (Eisenberg *et al*., 1998). In Japan, the use of herbal medicine increased up to 77% in 1993 compared to only 28% in 1979 (Kanba *et al*., 1999). In Australia, 50% of population are using natural products in 2000 (Braun and Cohen, 2007). A study on a group of Chinese community in Canada showed that more than 50% of them are using complementary and alternative medicine (Quan *et al*., 2008). A study

on an Indian community in South Africa showed that 38.5% of them used complementary and alternative medicine between 2000-2001 (Singh *et al*., 2004).

The high interest on natural medicine is due to many factors, such as, the high cost of new drugs, lack of treatment for many chronic diseases, increase side effects of synthesized medicines, and increase in their microbial resistance (Patwardhan *et al*., 2005).

### 1.2 Use of Herbal Medicine in Malaysia

The tropical climate of Malaysia blessed it with an abundance of plants with economical values and many of which are therapeutically important. In terms of number of plant species, Malaysia is among the world 12 mega biodiversity-rich countries (Ang, 2004).

Complementary and alternative medicine (CAM) is widely used in Malaysia and other South East Asian countries for many centuries and this is indicated by the traditional Malay medicine which was used from about 1000 years and continued till now (Raden and Werner, 1985). Nowadays, it was reported that a high prevalence of Malaysian people are using medicinal herbs to treat various types of diseases (Aziz and Tey, 2009). About 1300 medicinal plant products was registered by Malaysian Ministry of Health and are available in the market (Lin, 2005). This highly scientific attention with herbal medicinal plants in Malaysia started more than 50 years ago, when these medicinal plants entered the laboratory-based scientific research specially phytochemical screening, which was then followed by isolation and structural

elucidation of the active ingredients in medicinal plant (Kiang *et al*. 1961; Ibrahim, 2004).

Malaysia is a multi racial country; this can be seen in herbal medicinal products which contain Malay herbal products, Chinese herbal products, Indian herbal products and Western herbal products. Each racial group is using its own traditional products for the treatment of various illnesses depending on their knowledge, believe, respect and confidence to their traditions (Zakaria and Mohd, 1994).

Due to high consumption of herbal products to modify or treat a variety of illnesses, a special concern has raised because these herbal medicines are not rigorously regulated by the Malaysian Drug Control Authority (DCA). At the moment, this authority is only concerned with the evaluation of the quality and safety aspects of natural medicinal products, but still its work is limited in this field and the problem is compounds due to the lack of a good quality information and scientific evidence of these herbal medicinal products (Aziz, 2004), hence many herb-drug interactions have been occurred like the interaction of *St. John's wort*, kava kava, ginseng and garlic with the anticancer drugs irinotecan and imatinib (Meijerman *et al*., 2006). Ginkgo, garlic and ginseng interact with warfarin and may cause bleeding. *St. John's wort* has been found to inhibit the action of oral contraceptive and anticoagulant drugs (Williamson, 2003).

Burkill, (1935), and Farnsworth & Soejarto, (1991) reported a number of medicinal plants which are used traditionally in Malaysia with their local name, action and traditional use. Examples of these herbs are listed in Table 1.1.

**Table 1.1:** Some of Medicinal Plants Used in Malaysia with Their Local Name, Chemical Constituents and Traditional Use.

| Species (Family) | Local name | Chemical constituent (s) | Traditional use |
|---|---|---|---|
| *Andrographis paniculata* Nees (Acanthaceae) | Pokok cerita, hempedu bumi | Andrographolide, neoandrographolide | Bacillary dysentery |
| *Areca catechu* L. (Palmae) | Pokok pinang | Arecoline | Anthelmintic |
| *Carica papaya* L. (Caricaceae) | Betik | Chymopapain | Proteolytic,mucolytic |
| *Centella asiatica* (L.) (Umbelliferae) | Pegaga | Asiaticoside | Vulnerary |
| *Dioscorea* spp. (Dioscoreaceae) | Gadung | Diosgenin | Contraceptive |
| *Quisqualis indica* L. (Combretaceae) | Akar dani, akar pontianak | Quisqualic acid | Anthelmintic |
| *Ricinus communis* L. (Euphorbiaceae) | Jarak | Castor oil | Laxative |
| *Nicotiana tabacum* L. (Solanaceae) | Tembakau | Nicotine | Insecticide |
| *Curcuma longa* L. (Zingiberaceae) | Kunyit | Curcumin | Choleretic |
| *Mentha* spp. (Juncaceae) | Pudina | Menthol | Rubefacient |
| *Ananas comosus* (L.) Merr. (Bromeliaceae) | Nenas | Bromelain | Anti-inflammatory, proteolytic agent |

(Burkill, 1935; Farnsworth and Soejarto, 1991)

## 1.3 Introduction to Drug Metabolism

Drug metabolism can be defined as the biochemical modification or degradation that usually occur through specialized enzymatic system to convert the lipophilic chemical compounds into more polar products to be easily excreted from the body. The rate of biotransformation is important to determine the duration and intensity of pharmacological drug action. This rate may be affected by drug absorption which depend on physical properties of the drug and also may be affected by intestinal enzymes before entering the liver (Uetrecht and Trager, 2007).

Most of the organs and tissues in our body contain a diverse and various drug metabolizing enzymes, but liver is considered as the most important organ for drug metabolism. These drug metabolizing enzymes have a central role in the metabolism, elimination and detoxification of drugs and other xenobiotics that enter into the body.

Drug metabolism is usually divided into three phases: phase I (functionalization reactions), phase II (conjugation reactions) and phase III reactions for some phase II metabolite in order to be excreted (Xu *et al*., 2005). Phase I reactions, which are known also as functionalization reactions prepare the drug to enter phase II reactions by inserting a functional reactive group into the drug or other xenobiotic structure on which phase II enzymes will work to make the drug more water soluble to be easily excreted through bile or urine. Phase II enzymes are known as conjugating enzymes and are regarded as the real detoxification system for drugs and other xenobiotics (Gibson and Skett, 1994).

### 1.3.1 Phase I Hepatic Drug Metabolism Reactions

The main metabolic biotransformation reactions for phase I are:

#### 1.3.1.1 Oxidation Reactions

Oxidation reactions are the most important reactions of phase I biotransformation as it is performed for large number of xenobiotics that enter the body. Oxidation reactions are mainly performed in the presence of mixed function oxidase system, which contains a membrane bound iron-containing cytochrome P450, NADPH-dependant reductase and molecular oxygen. During this reaction oxygen atom is inserted to drug molecule as a result of electron transfer from NADPH to the enzyme-substrate complex, thus will allow cytochrome P450 to oxidize the complex.

Cytochrome P450 enzymes are mainly located in the microsomal endoplasmic reticulum of the liver; they are heme proteins and their name was derived from its characteristic in spectrophotometer, as its maximum absorption spectrum is at 450nm (Goldfrank *et al*., 2002).

A variety of xenobiotics including drugs that are metabolized through mixed function oxidase system in the microsomes are shown in Table 1.2.

**Table 1.2:** Phase I Microsomal Oxidation Reactions by Mixed Function Oxidase System with an Example of Substrate for Each Reaction.

| | Reaction | Substrate |
|---|---|---|
| i. | Epoxidation | Benzo[a] pyrene |
| ii. | Aromatic hydroxylation | Chlorobenzene |
| iii. | Aliphatic hydroxylation | Phenobarbital, Phenytoin |
| iv. | N-, S- and O-dealkylation | Aminopyrine, 6-methyl thiopurine and Codeine |
| v. | N-oxidation | 3-methyl pyridine |
| vi. | N-hydroxylation | 2-acetyl amino fluorine |
| vii. | De-amination | Amphetamine |
| viii. | De-sulfation | Parathion |

(Deshpande, 2002; Wilson *et al*., 2004)

Another type of oxidation reaction is known as non-microsomal oxidation reactions, which are performed by enzymes not related to mixed function oxidase and involved primarily in oxidation of endogenous compounds (Gibson and Skett, 1994; Deshpande, 2002). The types of such oxidation reactions with an example of their substrates are shown in Table 1.3.

**Table 1.3:** Types of Phase I Non-Microsomal Oxidation Reactions with an Example of Substrate for Each Reaction.

| | Reaction | Substrate |
|---|---|---|
| i- | Amine oxidation | Catecholamine, Histamine |
| ii- | Alcohol oxidation | Ethanol |
| iii- | Aldehyde oxidation | Aldehyde |
| iv- | Purine oxidation | Hypoxanthine |
| v- | Aromatization | Cyclohexene carboxylic acid |

(Gibson and Skett, 1994; Deshpande, 2002)

#### 1.3.1.2 Reduction Reactions

Reduction reaction is a reaction that is catalyzed by hepatic microsomes in the presence of NADPH cytochrome P450 reductase. Many endogenous compounds and xenobiotics are substrates for reduction reactions like carboxylic acid esters, carboxylic acid amide, carboxylic acid thioesters, azo compounds, nitro compounds, quinones and halogenated hydrocarbons (Barile, 2004).

#### 1.3.1.3 Hydrolysis Reactions

Hydrolysis reactions are one of the important reactions of phase I metabolism. Ester, amide, hydrazide and carbamate groups are the main groups that enter hydrolysis reactions. Examples of drugs that enters such type of reaction are acetylsalicylic acid, carbamazepine, indomethacin, hexobarbital and phensuximide (Gibson and Skett, 1994; Hernández and Rathinavelu, 2006).

#### 1.3.1.4 Hydration Reactions

Epoxides are the main groups that undergoes phase I hydration reactions. The enzyme epoxide hydrolase, which is located in the microsomal fraction is responsible for hydration reaction by the addition of water molecule to the compound. This reaction is regarded as a detoxification reaction for dihydrodiol products (Timbrell, 2002).

### 1.3.2 Phase II Hepatic Drug Metabolism Reactions

Phase II or conjugation reactions involve a variety of enzymes that catalyze the addition of polar group to the foreign molecule like phase I metabolite. This reaction involves the replacement of hydrogen atom in the carboxyl, amine or hydroxyl group

with the conjugating group (Ionescu and Caira, 2005). After the addition of polar group, the foreign molecule become more water soluble and easily cleared from the body through bile or urine (Timbrell, 2002). The main phase II conjugation reactions are conjugation with sugar, glutathione, sulfation, methylation, acetylation, amino acid, fatty acid & cholesterol ester conjugation and condensation reactions (Gibson and Skett, 1994).

#### 1.3.2.1 Conjugation with Sugar

Glucuronidation, which is conjugation with α-D-glucuronic acid (UDP-GlcA), is the major route of sugar conjugation. UDP-GlcA is the reaction co-factor which is almost found in all body tissues as it is an intermediate in glycogen synthesis; liver is regarded as the major site of glucuronidation and this reaction is catalyzed by the microsomal enzyme UDP-glucuronyltransferase (UGT). Many endogenous compounds are metabolized by glucuronidation through the conjugation of glucuronic acid to various reactive groups like alcohols, hydroxyl amines, phenols, amines, carboxylic acids, thiols and sulfonamides.

The glucuronides formed from glucuronidation reactions can be classified according to the nature of reactive group of substrate that binds with glucuronic acid to:

a. O-glucuronides

These glucuronides are formed from glucuronic acid conjugation with alcohols, phenols and carboxylic acids. Carboxylic acids will form acyl or ester glucuronides while alcohols and phenols will form ether glucuronides (Ionescu and Caira, 2005). Acyl

glucuronides formed from carboxylic acid are susceptible to nucleophilic substitution, acting as electrophiles which can react with thiol or hydroxyl groups of cell macromolecules and such interaction may be responsible for the toxicity of some compounds (Burchell, 1999). The ideal example of this type of glucuronidation are NSAIDs (Ionescu and Caira, 2005).

b. N-glucuronides

The main substrates for this type of glucuronidation reactions are carboxyl amides, sulfonamides and different types of amines. N-glucuronides may be formed spontaneously without the presence of enzyme (Gibson and Skett, 1994).

c. S-glucuronides

The main substrate of such type of glucuronidation reactions are aliphatic and aromatic thiols as well as dithiocarboxylic acid (Ionescu and Caira, 2005).

In insects, glucose conjugation is more prevalent than glucuronic acid conjugation. Some times UDP-xylose or UDP-ribose can conjugate to the active group of some compounds yielding xylosides or ribosides. N-ribosides are the most common reactions which formed non-enzymatically and an example of such reaction is N-ribosylation of 2-hydroxynicotinic acid (Gibson and Skett, 1994).

#### 1.3.2.2 Glutathione Conjugation

Glutathione reduced form (GSH) is a protective compound in the body that removes potentially toxic electrophilic compounds. It is a tripeptide compound comprised of

glycine, cysteine and glutamic acid. Usually after GSH attachment to the reactive group, it is further metabolized to yield N-acetyl cysteine conjugate or mercapturic acid. The main chemical compounds that undergo glutathione conjugation reaction are epoxides, alkenes, nitroalkanes, haloalkanes and aromatic halo and nitro compounds. Liver is regarded as the main site of glutathione conjugation reactions because of its high concentration of GSH (Gibson and Skett, 1994; Casarett *et al*., 2001).

Reduced glutathione (GSH) is considered to be one of the most important redox molecule that posses a protective antioxidant activity inside the cell, this is because of its high concentration inside the cell comparing with other redox molecules. It performs the antioxidant action via the oxidation of the thiol group in its cysteine residue to the disulfide (GSSG) form (Han, 2006). Glutathione conjugation reaction is catalyzed by glutathione S-transferase (GST) enzyme, which is located in the cytosol of liver, kidney and gut. Seven sub-family classes have been recognized for cytosolic GST in mammals namely alpha, mu, pi, zeta, theta, omega and sigma (Frova, 2006). Different gene families have the same function but differ in their substrate specificities (Chin *et al*., 2008). Mu and Pi GST subfamilies are the major members of GST family that are involved in glutathione conjugation with chemotherapeutic agents, reactive oxygen species and a wide range of xenobiotics (Gao *et al*., 2002).

#### 1.3.2.3 Sulfation

Sulfation is an important phase II pathway of metabolism, which involves the transfer of inorganic sulfate to the hydroxyl group present on phenols and aliphatic

alcohols yielding ethereal sulfates or sulfate esters. Sulfation reactions also occur on aromatic amines and hydroxyl amines to form sulfamate and N-O-sulfate. Sulfation reactions are catalyzed by sulfotransferases, which are a group of soluble enzymes found in liver, kidney, lung and intestinal tract. The product of sulfation reaction is more soluble than the parent compound and mainly excreted through urine, although some of the products are degraded enzymatically. The donor of inorganic sulfate in the reaction is 3'-phosphoadenosine-5'-phosphosulfate (PAPS), which is synthesized from inorganic sulfate and ATP. Through a complex oxidation sequence, cystiene is the major source of inorganic sulfate to form PAPS (Deshpande, 2002).

According to the kind of substrate, there are four sulfotransferases involved in detoxification processes: Hydroxy steroid sulfotransferase conjugates hydroxyl steroids and certain primary and secondary alcohols. Aryl sulfotransferase conjugate phenol, catecholamines and organic hydroxyl amines. Bile salt sulfotransferase catalyzes sulfation of bile acids. Finally estrone sulfotransferase conjugates phenolic groups on the aromatic ring of steroids (Spies and Gandolfi, 1986). Although sulfate conjugates detoxify chemicals but some sulfated products may be toxic like N-O-sulfate esters of N-hydroxy-2-acetyl aminofluorene, which is not stable and degrade to form potent electrophilic species.

#### 1.3.2.4 Methylation

Methylation reactions are mainly involved in the metabolism of endogenous compounds via methyltransferase enzymes. There are three main kinds of methyltransferase enzymes, which are N-, S- and O-methyltransferases. S-adenosyl

methionine (SAM) produced from L-methionine and ATP using L-methionine adenosyl transferase as a catalyst is the main methyl donor in methylation reaction. In contrast to other phase II reactions, methylation reactions do not lead to more polar metabolites. Methylation is an important reaction in epinephrine and melatonin biosynthesis. It also play a key role in the inactivation of biogenic amines like dopamine, serotonin and histamine. Some drugs may be methylated by non-specific methyltransferase found in the lung (Hernández and Rathinavelu, 2006).

#### 1.3.2.5 Acetylation

Acetylation reactions are common for alkyl and aromatic amines, which conjugate with acetic acid to form the corresponding acetyl conjugates. This reaction involves the transfer of acetyl group from the co-factor acetyl co-A to the drug molecule using N-acetyl transferase enzyme as a catalyst for this reaction. Acetylation occurs mainly in the liver and to a less extends in the spleen, lung and gut. Acetylated metabolites are usually less water soluble than the parent compound unlike other most conjugation reactions, this is may be due to conversion of ionizable amines in to non-ionizable amides. Isoniazid is an example of drug under go acetylation reaction (Gibson and Skett, 1994; Pandit, 2006).

#### 1.3.2.6 Amino Acids Conjugation

Amino acid conjugation is a special form of N-acetylation reaction in which a carboxylic acid group of xenobiotics can react with the amino group of amino acids such as glycine, taurine, glutamine, ornithine and arginine. This reaction involves the production of acyl-CoA thioester from xenobiotic interaction with CoA by acyl-CoA

synthase enzyme, then the enzyme N-acetyl transferase, catalyze the transfer of the acyl group of xenobiotic to the amino group of amino acid. The main substrates of this type of conjugation reaction are heteroaromatic, aromatic, aliphatic, cinnamic and aryl acetic acids (Manchee *et al*., 2004).

#### 1.3.2.7 Fatty Acids Conjugation

An example of such type of phase II conjugation reaction is the fatty acid conjugation of 11-hydroxy-$\Delta^9$-tetrahydrocannabinol. The main fatty acids involved in this conjugation reaction are stearic and palmitic acid and this reaction are catalyzed by the microsomal fraction of the liver. A little information is known about the mechanism of such reaction and whether that other compounds are conjugated by this way (Gibson and Skett, 1994).

#### 1.3.2.8 Condensation Reactions

Condensation reaction is a detoxification reaction that is mainly observed between amines and aldehydes. This reaction is not enzymatic but purely chemical and an example of such reaction is the condensation of dopamine with its own metabolite 3,4-dihydroxyphenyl ethanal (Smith and Hotchkiss, 2001).

### 1.4 Phase III Hepatic Drug Metabolism Reactions

Some compounds derived from phase II conjugation reactions may undergo further metabolism which is known as phase III biotransformation reactions in order to be easily excreted. These reactions are catalyzed by enzymes which are also active in phase I & II (Vermeulen, 1996). It is becoming increasingly recognized that the effect of xenobiotic

and their metabolites on living organism is depending on their transport out of the cell and this transport process are now sometimes regarded as phase III metabolism (Iersel *et al*., 1999). The possible example for such type of metabolic reactions is sulphur containing conjugates like GSH-conjugates, which undergoes further metabolism by the enzyme cysteine conjugate β-lyase (C-S lyase) (Smith and Hotchkiss, 2001).

## 1.5 Drug Interaction

A drug interaction is a situation in which the activity of a drug is affected by another substance administered together with it; such effect may be induction or inhibition or appearance of a new effect neither produced on its own. The interaction may be with another drug (drug-drug interaction), or interaction with food (drug-food interaction) as well as with herb (drug-herb interaction) (Griffin and D'arcy, 1997).

The relation between drug administration and response can be explained by two phases, which are pharmacokinetic phase and pharmacodynamic phase. Pharmacokinetic phase is concerned with drug administration and its concentration with time and this depend on four main factors which are absorption, distribution, metabolism and excretion, while pharmacodynamic concerned with the response produced in relation to drug concentration. So generally drug interactions can be divided into pharmacokinetic and pharmacodynamic related interactions (Griffin and D'arcy, 1997).

## 1.6 Herb-Drug Interaction

Due to the high attention and use of alternative medicine by the public and lack of informations about its contents, there is a high possibility of herb-drug interactions (Izzo

and Ernst, 2001). Such interaction between herbs and drugs may occur in aspects of pharmacokinetic and pharmacodynamic of drug. Herb-drug interaction can be defined as any change either in the pharmacokinetic or pharmacodynamic of the drug which is caused by concurrent administration of drug with the herb (Sugiyama *et al*., 2004). As a result of herbal-drug interactions, three possible outcomes can arise which are:

1. an increase in therapeutic or adverse effects.
2. a decrease in therapeutic or adverse effects.
3. a unique response that does not appear when either agent is used alone.

### 1.6.1 Nature of Herb-Drug Interaction

Natural products unlike conventional drugs, have a complex mixture of bioactive entities and a complete characterization and determination of these bioactive chemical constituents is usually unknown. Additionally, varying growing conditions of the plant, seasonality, manufacturing method, the combination of two or more plants in herbal products and poor control from Food & Drug Administration (FDA) make the whole issue of interactions more complex (Chen *et al*., 2008).

#### 1.6.1.1 Pharmacokinetic Interactions

This kind of interaction is concerned with changes in absorption, distribution, metabolism and excretion of interacting molecules. Thus any alteration of these processes will cause a change in the amount and persistence of drug molecules at receptor sites or target tissue.

**a) Absorption**

It is the process by which a test compound and its metabolites are transferred from the site of administration to the systemic circulation. Intestine is the major site of absorption for drugs and herbs after oral administration. Bioavailability of the drug given orally depends on absorption process because it reflects the total amount of drug and its derived materials inside the body (Tse and Jaffe, 1991).

Several studies showed that herbal medicinal products may affect the absorption of other drugs and consequently affect the bioavailability of these drugs inside the body. Some herbs may inhibit intestinal CYP3A4 activity, which possess an important role in the metabolism of variety of drug leading to increase in their bioavailability, for example grape fruit juice inhibit CYP3A4 in the enterocyte of intestine leading to an increase in the bioavailability and appearance of side effects of other drugs like Ca-channel blockers, HIV protease inhibitors, vinblastine, digoxin, cyclosporine and fexofenadine (Pandit, 2006). Some other herbal products may interfere with P-glycoprotein activity, which is an important transporter in the intestine responsible for actively expelling chemotherapeutic drugs from cells, so it decreases drug intracellular concentration and thus drug efficacy; the plant *St Johns wort*, which is used for depression is an example of plant that is found to cause an induction of P-glycoprotein activity leading to a significant reduction of serum level for some drugs like digoxin (Hennessy *et al*., 2002).

Other mechanisms of herbal-drug interactions that may affect the absorption and consequently bioavailability of drugs are changes in intestinal motility and changes in the pH of the stomach.

**b) Distribution**

Human body should not be considered as one compartment of fluid throughout which chemicals are distributed, but it is multi compartmental system. The largest compartment is the intracellular fluid, which account for about 65% of total body water. The second large compartment is interstitial fluid, which account about 24% of total body water, while blood plasma account for about 8%. The synovial fluid, intraocular and cerebrospinal fluid account for about 3% of total body water. The other major body compartment is fat, which account about 20% of body weight (Gard, 2001).

Xenobiotics (like drugs and herbs) and endogenous compounds are distributed in the body between various compartments depending on many factors like: lipid solubility, plasma protein binding, blood pH and blood flow to tissues (Gard, 2001). Water-soluble drugs have low volume of distribution with tendency to remain in blood, while fat-soluble drugs possess a high volume of distribution as they diffuse to many tissues and organs. However, effect on distribution due to drug-drug or herb-drug interactions usually happened due to displacement of drug from its binding sites like plasma proteins leading to increase in the free (active) concentration, but this action mainly do not show significant change on drug pharmacokinetic due to simultaneous increase in metabolism and elimination (Gregory, 2007).

Cautions of herb-drug interaction on drug distribution are mainly on drugs with high plasma protein binding capacity and low margin of safety as warfarin, which showed a distribution disturbance when administered with tea or other green leafy vegetables (Lambrecht *et al*., 2000).

**c) Metabolism**

Liver is the site that is responsible chiefly for the most metabolic reactions, although some enzymes and metabolic reactions occur in the intestine, lung and kidney. These metabolic reactions are divided into two phases, known as phase I (functionalization reactions) and Phase II (conjugation reactions).

Cytochrome P450 system, which comprises more than 50 enzymes are responsible for most reactions of Phase I and it is found mainly in the liver and to a lesser extend in the lung, kidney, skin and intestine.

Metabolic interactions are the main causes of herb-drug interactions; hence many researches have been done to examine the effect of drug-drug and herb-drug interactions on cytochrome P450 enzyme activity. Any alteration in enzyme activity means that some interaction happened between two drugs or herb and drug given at the same time. Examples of such interactions are: goldenseal (*Hydrastis canadensis*) causes an inhibition of CYP2D6 and CYP3A4/5. Black cohosh (*Cimicifuga racemosa*) inhibits CYP2D6. Kava kava (*Piper methysticum*) and garlic oil inhibits CYP2E1 (Gurley *et al*., 2002; Gurley *et al*., 2005) and *St Johns Wort* was found to possess an induction effect on CYP3A4 (Dürr *et al*., 2000; Wang *et al*., 2001).

**d) Excretion**

Kidneys are regarded as the major organs of excretion and to a lesser extend faeces, saliva, sweat, breast milk and lungs as other route of excretion. If a drug is excreted by

one pathway, then alteration to that pathway theoretically have a significant influence on its excretion.

Drug-drug and herb-drug interactions can affect renal function and consequently affect excretion through three main mechanisms which are:

- changes in urinary pH, which alter renal tubular re-absorption.
- changes in renal tubular secretion due to alteration of membrane transporter proteins activity or competition for active secretion.
- changes in glomerular filtration due to the effect on cardiac output (Blower *et al*., 2005).

Urinary pH alteration is easily achieved with herbs and natural substances like high protein diet that may cause alkalization of urine leading to increase in excretion of weakly basic drugs such as amphetamine (Braun and Cohen, 2007).

#### 1.6.1.2 Pharmacodynamic Interactions

This type of interaction results when one substance alters the responsiveness or sensitivity of tissues to another. Such type of interaction results in synergistic, additive or antagonistic drug effects which may be of particular importance when drugs used simultaneously have overlapping toxicities.

Some clinicians frequently use synergistic or additive pharmacodynamic interactions to improve the clinical outcome, like the combination of antibiotics to

eradicate microorganisms or using several antihypertensive drugs to one patient. The same way are used for herbal treatment, where combination of herbs are used to strengthen the effect on certain ailment, but such pharmacodynamic interaction do not always produce desired results due to overlapping adverse effects when drugs and herbs are combined together leading to serious adverse effects. For example, using Kava-kava (*Piper methysticum*), which has dopamine receptor antagonist activity, theoretically can interact with dopamine agonists (e.g. L-dopa) opposing their effect (Meseguer *et al*., 2002). Another example is that ginger, ginkgo and garlic may potentiate the anticoagulant action of warfarin (Barnes *et al*., 2003).

#### 1.6.1.3 Physicochemical Interactions

This type of interactions occurs when two substances are in contact and are physically or chemically incompatible. Such interactions may affect the rate and extend of absorption of one or both drugs. Herbs, which contain significant amount of tannins is a potential candidate of physicochemical interactions because it form precipitate with proteins, polysaccharides, glycosides, nitrogen bases and some alkaloids (Mills, 1991). Tannins containing herbs such as *Agrimony eupatorium* and *Arctostaphylus uva-ursi* can also form complexes with metal ions like iron, thus inhibiting their absorption (Glahn *et al*., 2002).

Some physicochemical interactions may enhance the bioavailability and effect of interacting compound. For example, the solubility of hypericin and pseudohypericin may increase 60 % in the presence of flavonoid procyanidin (Butterweck *et al*., 1998). Saponins containing plants like *Astragalus membranaceus* and *Bupleurum falcatum*,

may increase absorption, oral bioavailability and dermal penetration of poorly lipid soluble compounds due to micellisation process (Braun and Cohen, 2007).

## 1.7 *Phyllanthus* Genus

*Phyllanthus* is a very large genus, as more than 550 species have been discovered so far (Unander *et al*., 1995). *P. niruri, P. amarus and P. urinaria* are the main species which have been used in herbal medicine. These species resemble each other in physical characteristics with small differences, for example, *P. urinaria* has a large leaves, its fruit is wart like (Cao, 2009) and its flowers are yellow in color (Bharatiya, 1992), while *P. amarus* and *niruri* have small oblong leaves, its fruits are small glabose capsules and the flowers are green to white color (Panda, 2000).

## 1.8 Literature Review of *P. niruri* Linn.

### 1.8.1 Botanical Aspects of *P. niruri*

| | |
|---|---|
| Kingdom | Plantae |
| Division | Magnoliophyta |
| Class | Magnoliopsida |
| Order | Euphorbiales |
| Family | Euphorbiaceae |
| Genus | Phyllanthus |
| Species | niruri |

*P. niruri* is found in most tropical and sub-tropical regions of the world. It is an annual herb that grows up to 30-60 cm in high, the stem is often branched at the base and leaves are numerous small and oblong in shape. The flowers are minute green in color that occurs in groups at the axillary position of leaves, the male flower 1-3 female flowers solitary. The fruit has a small surface and glabose capsule with about 2.5 mm in diameter (Panda, 2000). Each country that use *P. niruri* has a local name for it, as shown in Table 1.4.

Table 1.4: The Local Names of *P. niruri* in Various Countries:

| Country | Traditional name of *P. niruri* |
|---|---|
| Malaysia | Dukong anak |
| India | Bhoomi amalaki |
| Peru | Chancapiedra |
| Haiti | Derriere-dos |
| Sudan | Elrageig |
| Brazil | Phyllanto |
| Thailand | Ya-tai-bai |
| Philippines | Yerba de san pablo |

(Ross, 2003)

**Leaves of *P. niruri* Linn.**

**Figure 1.1:** Picture of *P. niruri*