ผลของฟิลแลนทิน ไฮโปฟิลแลนทิน และสารสกัดลูกใต้ใบด้วยน้ำต่อการแสดงออกของเอนไซม์ไซโตโครม พี 450 3 เอ 4 ของมนุษย์ โดยใช้เซลล์เพาะเลี้ยงเฮพจี 2

นายกฤษดา อนันตวุฒิกุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต

สาขาวิชาเภสัชวิทยา ภาควิชาเภสัชวิทยาและสรีรวิทยา

คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

ปีการศึกษา 2554

ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

EFFECTS OF PHYLLANTHIN, HYPOPHYLLANTHIN AND PHYLLANTHUS AMARUS AQUEOUS EXTRACT ON THE EXPRESSION OF HUMAN CYTOCHROME P450 3A4 USING HEPG2 CELLS

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A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science in Pharmacy Program in Pharmacology

Department of Pharmacology and Physiology

Faculty of Pharmaceutical Sciences

Chulalongkorn University

Academic Year 2011

Thesis Title	EFFECTS OF PHYLLANTHIN, HYPOPHYLLANTHIN AND PHYLLANTHUS AMARUS AQUEOUS EXTRACT ON THE EXPRESSION OF HUMAN CYTOCHROME P450 3A4 USING HEPG2 CELLS
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กฤษดา อนันตวุฒิกุล : ผลของฟิลแลนทิน ไฮโปฟิลแลนทิน และสารสกัดลูกใต้ใบด้วยน้ำต่อการแสดงออกของเอนไซม์ไซโตโครม พี 450 3 เอ 4 ของมนุษย์ โดยใช้เซลล์เพาะเลี้ยงเฮพจี 2. (EFFECTS OF PHYLLANTHIN, HYPOPHYLLANTHIN AND PHYLLANTHUS AMARUS AQUEOUS EXTRACT ON THE EXPRESSION OF HUMAN CYTOCHROME P450 3A4 USING HEPG2 CELLS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รศ. พ.ต.ท.หญิง ดร.สมทรง ลาวัณย์ประเสริฐ, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : ผศ. ดร.สุรีรัตน์ พรธาดาวิทย์, 78 หน้า.

การศึกษานี้ศึกษาผลของสารสกัดลูกใต้ใบด้วยน้ำ และสารสำคัญของลูกใต้ใบคือสารฟิลแลนทินและไฮโปฟิลแลนทิน ต่อการแสดงออกของเอนไซม์ไซโตโครม พี 450 3 เอ 4 (CYP3A4) ของมนุษย์ โดยใช้เซลล์เพาะเลี้ยงเฮพจี 2 จากการศึกษาความเป็นพิษต่อเซลล์ ไม่พบว่าฟิลแลนทินและไฮโปฟิลแลนทิน (0.04 – 25 ไมโครโมลาร์) มีผลอย่างมีนัยสำคัญต่อการมีชีวิตรอดของเซลล์ ในขณะที่สารสกัดลดการมีชีวิตรอดของเซลล์ โดยมีค่า IC_{50} เท่ากับ 3.69, 1.76 และ 1.24 มิลลิกรัม/มิลลิลิตร เมื่อบ่มเป็นเวลา 24, 48 และ 72 ชั่วโมง ตามลำดับ จากการศึกษาผลต่อสมรรถนะและการแสดงออกของเอนไซม์ CYP3A4 ไม่พบว่าฟิลแลนทิน (1, 5 และ 25 ไมโครโมลาร์) มีผลต่อสมรรถนะและปริมาณโปรตีนของเอนไซม์ CYP3A4 แต่พบว่าฟิลแลนทินที่ความเข้มข้น 25 ไมโครโมลาร์มีผลลดปริมาณเอ็มอาร์เอ็นเอของเอนไซม์ CYP3A4 อย่างมีนัยสำคัญเมื่อบ่มนาน 24 ชั่วโมง ไม่พบว่าไฮโปฟิลแลนทิน (1, 5 และ 25 ไมโครโมลาร์) มีผลอย่างมีนัยสำคัญต่อสมรรถนะ ปริมาณโปรตีนและเอ็มอาร์เอ็นเอของเอนไซม์ CYP3A4 สารสกัดลูกใต้ใบที่ความเข้มข้น 0.5 มิลลิกรัม/มิลลิลิตร มีผลเพิ่มสมรรถนะของเอนไซม์ CYP3A4 ในลักษณะที่ขึ้นกับเวลาโดยเพิ่มสมรรถนะเป็น 1.50, 1.92 และ 2.29 เท่าเมื่อเปรียบเทียบกับกลุ่มควบคุม เมื่อบ่มเป็นเวลา 24, 48 และ 72 ชั่วโมง ตามลำดับ นอกจากนี้สารสกัดมีผลเพิ่มปริมาณโปรตีนเมื่อบ่มเป็นเวลา 24 ชั่วโมง และเพิ่มปริมาณเอ็มอาร์เอ็นเอเมื่อบ่มเป็นเวลา 12 และ 24 ชั่วโมง ข้อมูลจากการศึกษานี้บ่งชี้ถึงแนวโน้มในการเกิดอันตรกิริยาระหว่างลูกใต้ใบกับยา จึงควรทำการศึกษาแบบในกายต่อไป

ภาควิชา เภสัชวิทยาและสรีรวิทยา ลายมือชื่อนิสิต.....

สาขาวิชา เภสัชวิทยา ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ปีการศึกษา 2554 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

5276552233 : MAJOR PHARMACOLOGY

KEYWORDS : PHYLLANTHUS AMARUS / PHYLLANTHIN / HYPOPHYLLANTHIN / CYTOCHROME P450 3A4 / HERB-DRUG INTERACTION

KRIDSADA ANUNTAWUTTIKUL : EFFECTS OF PHYLLANTHIN, HYPOPHYLLANTHIN AND PHYLLANTHUS AMARUS AQUEOUS EXTRACT ON THE EXPRESSION OF HUMAN CYTOCHROME P450 3A4 USING HEPG2 CELLS. ADVISOR : ASSOC. PROF. POL. LT. COL. SOMSONG LAWANPRASERT, Ph.D., CO-ADVISOR : ASSIST. PROF. SUREERUT PORNTADAVITY, Ph.D., 78 pp.

In this study, the effects of *Phyllanthus amarus* aqueous extract and its active principles, phyllanthin and hypophyllanthin, on the activity and expression of cytochrome P450 3A4 (CYP3A4) were investigated in HepG2, the human liver carcinoma, cells. Phyllanthin and hypophyllanthin (0.04-25 μM) did not significantly affect cell viability. The extract reduced cell viability with IC_{50} values of 3.69, 1.76 and 1.24 mg/ml for the incubation periods of 24, 48 and 72 hours, respectively. Phyllanthin (1, 5 and 25 μM) did not affect activity and protein level of CYP3A4, but significantly decreased mRNA level at concentration of 25 μM. Hypophyllanthin did not cause significant effects on CYP3A4 activity, protein and mRNA. The extract at concentration of 0.5 mg/ml significantly increased CYP3A4 activity in a time-dependent manner. As compared to the control, CYP3A4 activity was enhanced by 1.50, 1.92 and 2.29 fold following incubation with the extract for 24, 48 and 72 hours, respectively. The extract also increased CYP3A4 protein following incubation for 24 hours and mRNA levels following incubation for 12 and 24 hours. The effects on CYP3A4 activity and expression found in this study provide information of herb-drug interaction potential, thus further study *in vivo* is suggested.

Department : Pharmacology and Physiology Student's Signature .....

Field of Study : Pharmacology Advisor's Signature .....

Academic Year : 2011 Co-advisor's Signature .....

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to my thesis advisor, Associate Professor Pol. Lt. Col. Dr. Somsong Lawanprasert and my thesis co-advisor, Assistant Professor Dr. Sureerut Porntadavity for their valuable guidance, comment, kindness, encouragement and support throughout my research study. Thanks are also extended to the committee members: Associate Professor Dr. Mayuree Tantisira, Associate Professor Dr. Laddawal Phivthong-ngam and Assistant Professor Dr. Rataya Luechapudiporn as well as other lecturers in the Department of Pharmacology and Physiology for their helpful comment.

I sincerely thank Associate Professor Dr. Pornpen Pramyothin and Dr. Hemvala Chirdchupunseree for their kindness in providing phyllanthin, hypophyllanthin and *P. amarus* aqueous extract. I also wish to thank Assistant Professor Dr. Wisit Tangkeangsirisin for his kindness in providing HepG2 cells.

I wish to thank all staff members of the Department of Pharmacology and Physiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University as well as staff members of the Faculty of Medical Technology, Mahidol University for their kind assistance.

I would like to thank Faculty of Medical Technology, Mahidol University for supporting the research facilities and instruments.

This study was partly supported by a grant from the Graduate School, Chulalongkorn University.

Finally, I would like to thank my family, my friends and laboratory members for their help, comment and encouragement.

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LIST OF ABBREVIATIONS

µg	=	microgram
µl	=	microlitre
µM	=	micromolar
AhR	=	aryl hydrocarbon receptor
ALP	=	alkaline phosphatase
ALT	=	alanine aminotransferase
AST	=	aspartate aminotransferase
bp	=	base pair
BROD	=	benzyloxyresorufin O-dealkylation
BSA	=	bovine serum albumin
$^{\circ}$C	=	degree Celsius
CAR	=	constitutive androstane receptor
CFA	=	complete Freund's adjuvant
CYP	=	cytochrome P450
CTX	=	cyclophosphamide
DMSO	=	dimethylsulfoxide
EDTA	=	ethylenediaminetetraacetic acid
EROD	=	ethoxyresorufin O-dealkylation
FXR	=	farnesoid X receptor
g	=	gram
GR	=	glucocorticoid receptor
h	=	hour
HBV	=	hepatitis B virus
HBsAg	=	hepatitis B surface antigen
HPLC	=	high performance liquid chromatography
IC_{50}	=	median inhibitory concentration
IL	=	interleukin

i.p.	=	intraperitoneal
i.v.	=	intravenous
kDa	=	kilodalton
kg	=	kilogram
L	=	litre
LPS	=	lipopolysaccharides
LXR	=	liver X receptor
mg	=	milligram
mg/kg	=	milligram per kilogram body weight
min	=	minute
MIC	=	minimum inhibitory concentration
ml	=	millilitre
mM	=	millimolar
MROD	=	methoxyresorufin O-dealkylation
MTT	=	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADPH	=	reduced nicotinamide adenine dinucleotide phosphate
NF-κB	=	nuclear factor-kappa B
ng	=	nanogram
nm	=	nanometer
PAF	=	platelet activating factor
PBS	=	phosphate buffered saline
p.o.	=	per oral
PPAR	=	peroxisome proliferator activated receptor
PXR	=	pregnane X receptor
PROD	=	pentoxyresorufin O-dealkylation
rpm	=	revolution per minute
RXR	=	retinoid X receptor
SDS-PAGE	=	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
S.E.M.	=	standard error of mean

TBARS	=	thiobarbituric acid reactive substances
TBS	=	tris buffered saline
TBST	=	tris buffered saline-Tween 20
TNF-α	=	tumor necrosis factor
U	=	unit

CHAPTER I

INTRODUCTION

Background and rationale

Drug interaction is one of the major concerns in drug utilization since it can either affect therapeutic efficacy or safety of concomitant medicines particularly of those with narrow therapeutic index. One of the most common mechanisms of drug interaction is the interfering with drug metabolizing enzymes especially with cytochrome P450s (CYPs) (Chen and Raymond, 2006; Pal and Mitra, 2006). The main function of CYPs is to catalyze the oxidative and reductive reactions in the phase I metabolism. The occurred metabolites are usually more water soluble and less potent than the parent compounds. However, some substances may be bioactivated by CYPs to be more potent or even toxic metabolites. CYPs involving in drug metabolism include CYP1A2, CYP2B6, CYP2C8/9, CYP2C18/19, CYP2D6, CYP2E1, and CYP3A4. The most abundant CYPs isoform both in liver and intestinal tract is CYP3A4. In human liver, CYP3A4 is presented about 25% of total CYPs content (Coleman, 2005). Moreover, CYP3A4 involves in the metabolism of approximately 60% of clinically used medicines (Chen and Raymond, 2006). Since CYP3A4 plays such important roles in drug metabolism, compounds that possess inhibitory or inductive effects on CYP3A4 may have higher risk to interfere the metabolism of co-administered medicines. The inhibition of CYP3A4 may enhance therapeutic efficacy as well as risk of side effect and toxicity of accumulated medicines. On the other hand, the induction of CYP3A4 can increase the metabolism and clearance rate of medicines. As a result, their therapeutic efficacy may decrease. Moreover, the undesired side effect and toxicity may be potentiated if these drugs are metabolic activated into active or toxic metabolites. Therefore, the potentials of drugs and herbs in CYP3A4 inhibition and induction should be investigated in the research and development process for safety of consumers.

Phyllanthus amarus Schum. et. Thonn., also known as “Luk Tai Bai” in thai, has been traditionally used as the herbal medicines for many indications particularly for the treatment of jaundice and other symptoms of liver disease in several countries including Thailand. Previous studies reported various pharmacological effects of *P. amarus* extracts including antioxidant (Wongnawa *et al*., 2006; Naaz, Javed, and Abdin, 2007; Faremi *et al*., 2008), hepatoprotective (Wongnawa *et al*., 2006; Naaz, Javed, and Abdin, 2007; Pramyothin *et al*., 2007; Faremi *et al*., 2008; Yadav *et al*., 2008), anti-inflammatory (Kiemer *et al*., 2003; Raphael and Kuttan, 2003), antiviral (Blumberg *et al*., 1990; Notka, Meier, and Wagner, 2003; 2004) and anticarcinogenic (Jeena, Joy, and Kuttan, 1999; Rajeshkumar and Kuttan, 2000; Rajeshkumar *et al*., 2002) activities. Phyllanthin and hypophyllanthin are lignan compounds presented in *P. amarus.* Several studies demonstrated the hepatoprotective activity of these compounds against several toxic substances such as carbon tetrachloride (Syamasundar *et al*., 1985; Krithika *et al*., 2009), galactosamine (Syamasundar *et al*., 1985) and ethanol (Chirdchupunseree and Pramyothin, 2010). The possible mechanisms were suggested to be due to their antioxidative effects (Krithika *et al*., 2009; Chirdchupunseree and Pramyothin, 2010).

Previous studies showed the inhibitory effect of *P. amarus* extracts on several CYPs including CYP3A4 (Hari Kumar and Kuttan, 2006; Appiah-Opong *et al*., 2008; Taesotikul *et al*., 2011). Furthermore, phyllanthin and hypophyllanthin were found to be potent mechanism-based inhibitors of CYP3A4 (Taesotikul *et al*., 2011). Recent studies demonstrated the ability of some flavonoids, lignans and tannins (which can also be classified as polyphenols) in the activation of pregnane X receptor (PXR) and constitutive androstane receptor (CAR), the commonly known transcription factors involved in CYP3A4 gene expression (Jacobs, Nolan, and Hood, 2005; Kluth *et al*., 2007; Dong *et al*., 2010; Yao *et al*., 2010). Since phytochemical constituents found in *P. amarus* extracts also consist of various kinds of polyphenolic compounds, it is possible that phyllanthin, hypophyllanthin and some other phytochemical compounds in the extracts might have inductive potential on CYP3A4. In addition, quercetin, a flavonoid

presented in *P. amarus* extracts, was reported that it can significantly enhance CYP3A4 promoter activity (Kluth *et al*., 2007).

There are numerous compounds that have been shown to be both inhibitors and inducers of CYP3A4 in the *in vitro* studies. For example, hyperforin, a composition in St John's wort, can highly activate PXR while it has also been reported to be a potent inhibitor of CYP3A4 (Chen and Raymond, 2006). In general, herbal extracts are composed of various components which may have different effects on CYP3A4 activity and gene expression. Hence, the overall outcome can be either inhibition, induction or no significant change according to the combination of their effects on CYP3A4 which cannot be simply predicted. Therefore, the purpose of this study was to determine the effects of phyllanthin, hypophyllanthin and *P. amarus* aqueous extract on CYP3A4 activity and gene expression using HepG2, the human liver carcinoma, cells.

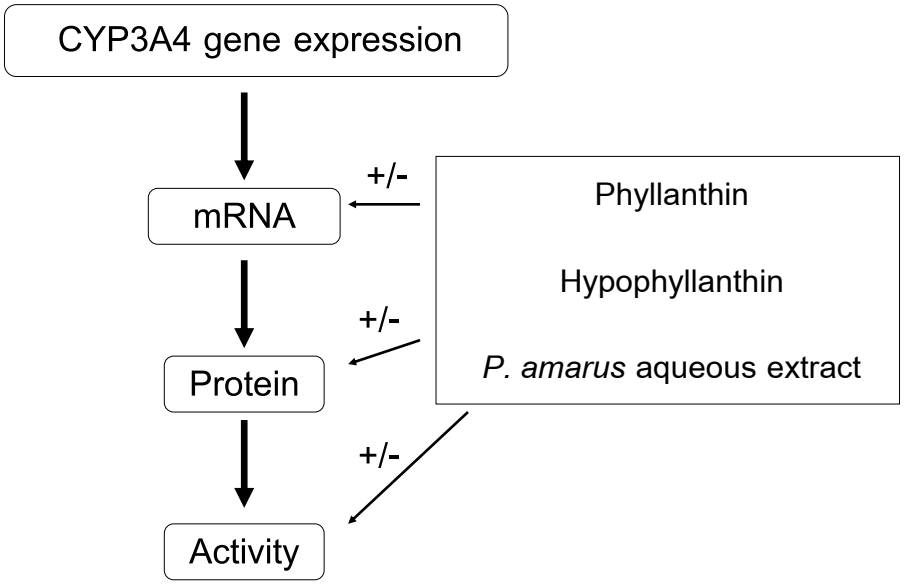
Hypothesis

Phyllanthin, hypophyllanthin and *P. amarus* aqueous extract possess inductive and/or inhibitory effects on human CYP3A4 activity and gene expression in HepG2 cells.

Objective

To investigate effects of phyllanthin, hypophyllanthin and *P. amarus* aqueous extract on human CYP3A4 activity and gene expression in HepG2 cells.

Conceptual framework



Expected benefit from the study

The information of inductive and/or inhibitory effects of phyllanthin, hypophyllanthin and *P. amarus* aqueous extract on human CYP3A4 activity and gene expression. The evidences obtained from this research will provide preliminary information of herb-drug interaction potential for further *in vivo* study.

CHAPTER II

LITERATURE REVIEWS

***Phyllanthus amarus* (*P. amarus*)**

Phyllanthus amarus Schum. et Thonn. (Euphorbiaceae) (Figure 2.1) is a small annual herb widely distributed in tropical and subtropical countries including Thailand. In Thailand, it is also known as Luk Tai Bai, Mhak Kai Lhung, Mhak Tai Bai, Ma Kham Pom Din, Yah Tai Bai and Yah Tai Bai Khaow. It has been traditionally used as herbal medicines for various conditions such as fever, pain, edema, diarrhea, stomachache, jaundice, gallstone, kidney stone, urinary tract infection, diabetes and abnormal menstruation. This plant is also used as laxative, diuretic, astringent, tonic and hepatoprotective medicines. Whole plant of *P. amarus* is usually applied in folk medicines. Also, some recipes may use only root and leaf parts (ลีนา ผู้พัฒนพงศ์, 2530; สุภาภรณ์ ปิติพร, 2552).

Figure 2.1: *Phyllanthus amarus* Schum. et Thonn.
(http://plantes-rizieres-guyane.cirad.fr/dicotyledones/euphorbiaceae/phyllanthus_amarus)

P. amarus contains various types of phytochemical compounds including lignans (hypophyllanthin, phyllanthin, phyltetralin, nirtetralin, niranthin and nirurin), flavonoids (quercetin, rutin and astragalin), ellagitannins or hydrolysable tannins (amariinic acid, amariin, amarulone, corilagin, 1,6-digalloylglucopyranoside, gallocatechin, elaeocarpusin, geraniinic acid B, geraniin, phyllanthusiin D and repandusinic acid A), alkaloids (securinine, dihydrosecurinine, tetrahydrosecurine, securinol-B, phyllanthine, allosecurine, norsecurinine, isobubbialine and epibubbialine), triterpenoids (phyllanthenol, phyllanthenone, phytllantheol, oleanolic acid and ursolic acid), alkane (dotriacontanyl docosanoate) and long chain alcohol (triacontanol) (Foo and Wong, 1992; Foo, 1993; 1995; Houghton *et al*., 1996; Kassuya *et al*., 2003; Ali, Houghton, and Soumyanath, 2006; Islam *et al*., 2008).

Pharmacological effects of *P. amarus*

Various pharmacological activities of *P. amarus* extracts were previously reported. Unless otherwise stated, most of these studies investigated pharmacological effects of whole plant or aerial part extracts.

1. Hepatoprotective activity

P. amarus extracts were reported to exhibit hepatoprotective potential against several hepatotoxic agents including paracetamol, ethanol, aflatoxin B_1 and carbon tetrachloride. The possible mechanisms were suggested to be their antioxidant activities, which might be related to the high content of various types of polyphenolic compounds in *P. amarus* such as lignans, flavonoids and ellagitannins. Several studies have revealed its antioxidant effects including radical scavenging activity, iron chelating activity and the improvement on enzymatic and non-enzymatic antioxidant defense mechanisms (Wongnawa *et al*., 2006; Naaz, Javed, and Abdin, 2007; Pramyothin *et al*., 2007; Faremi *et al*., 2008; Yadav *et al*., 2008; Krithika *et al*., 2009).

P. amarus aqueous extract possessed protective activity against paracetamol- and ethanol-induced hepatic injury in rats. The aqueous extract (1.6 and 3.2 g/kg, twice daily, p.o.) attenuated paracetamol-induced hepatotoxicity as demonstrated by the

reduction of aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin and histopathological score. Furthermore, the extract was found to increase the level of reduced glutathione in rat liver and possess radical scavenging and iron chelating activity, while it did not show significant effect on paracetamol metabolic pathway (Wongnawa *et al*., 2006). In another study, the aqueous extract (1–4 mg/ml) was found to enhance cell viability and decreased the release of AST and/or ALT in ethanol-treated primary cultures of rat hepatocyte. In acute toxicity study in rats, pretreatment with the extract (25, 50, 75 mg/kg, p.o.) for 24 hours attenuated ethanol-induced elevation of serum AST and/or ALT levels. Moreover, the extract at the most effective dose (75 mg/kg/day, p.o.) was found to reduce sub-acute hepatotoxicity of ethanol as indicated by the decrease in elevated levels of AST, ALT, hepatic triglyceride and tumor necrosis factor (TNF-α). The severity of liver injury as determined by histopathological examination was also attenuated both in acute and sub-acute toxicity studies (Pramyothin *et al*., 2007).

The ethanolic extract of *P. amarus* (0.3 g/kg/day, p.o.) exhibited protective effect against aflatoxin B_1-induced liver injury in mice. The extract reduced the level of thiobarbituric acid reactive substances (TBARS) and increased the level of reduced glutathione as well as the activities of antioxidant enzymes including glutathione peroxidase, glutathione-*S*-transferase, superoxide dismutase and catalase (Naaz, Javed, and Abdin, 2007). Similarly, the methanolic leaf extract of *P. amarus* (250 and 500 mg/kg/day, p.o.) exhibited hepatoprotective activity on ethanol-induced oxidative damage in rat liver by reducing lipid peroxidation level and improving antioxidant defense mechanisms (Faremi *et al*., 2008).

Combination treatment of silymarin (50 mg/kg/day, p.o.) and *P. amarus* extracts (50 mg/kg/day, p.o.) exhibited synergistic protective effect against carbon tetrachloride-induced hepatotoxicity in rats. Furthermore, the hepatoprotective activity of ethanolic extract was found to be better than that of aqueous extract, which might be associated with the higher content of phyllanthin in ethanolic extract as estimated by HPLC (Yadav *et al*., 2008). Phyllanthin (30 µM) was more effective in the protection against carbon

tetrachloride-induced toxicity in HepG2 cells when compared with 50% ethanol/water extract of *P. amarus* (600 µg/ml) (Krithika *et al*., 2009).

2. Antinephrotoxic activity

The aqueous leaf and seed extract of *P. amarus* (100-400 mg/kg/day, p.o.) showed protective effects against acetaminophen- and gentamicin-induced nephrotoxicities in rats. The elevations of serum creatinine and blood urea nitrogen levels were decreased in a dose-dependent pattern. In addition, the attenuations of acetaminophen- and gentamicin-induced tubulonephrosis were observed in histopathological examination (Adeneye and Benebo, 2008).

3. Chemoprotective and radioprotective activities

The methanolic extract of *P. amarus* demonstrated protective effect against toxicity caused by irradiation and cyclophosphamide (CTX) in mice. The administration of methanolic extract (250 and 750 mg/kg/day, p.o.) was found to significantly attenuate radiation- and CTX-induced myelosuppression and improve the antioxidant status.

The increase of antioxidant enzymes activity as well as glutathione levels and the decrease of lipid peroxidation levels in blood, liver and intestine were observed in treatment groups of irradiated mice. Furthermore, the extract was found to decrease radiation-induced micronuclei formation and chromosomal aberrations in mouse bone marrow cells.

In CTX administered mice, the extract elevated liver glutathione levels and glutathione-*S*-transferase activity. In addition, the extract was found to possess inhibitory effect on aniline hydroxylase activity, an indicator of phase I metabolism enzymes that are involved in the bioactivation of CTX into its toxic metabolites. While administration of methanolic extract reduced CTX toxicity, it also enhanced the antitumor effect of CTX as evidenced by the increasing reduction of solid tumors volume (Hari Kumar and Kuttan, 2004; 2005; 2007).

4. Antimutagenic, anticarcinogenic and antitumorigenic activities

P. amarus aqueous extract possessed antimutagenic potential against mutagenesis induced by direct-acting (2-aminofluorene, 4-nitroquinolone-1-oxide, N-ethyl-N-nitrosoguanidine, 2-nitrofluorene and sodium azide) and indirect-acting (2-aminoanthracene) mutagens in a dose-dependent pattern in bacterial preincubation mutation assay. In addition, the extract attenuated the dimethylnitrosamine-induced *in vivo* DNA single-strand breaks in hamster liver (Sripanidkulchai *et al*., 2002).

The methanolic extract of *P. amarus* also exhibited antimutagenic activity against a variety of direct-acting (sodium azide, N-methyl-N-nitro-N-nitrosoguanidine and 4-nitro-0-phenylenediamine) as well as indirect-acting mutagens (2-acetaminofluorene and aflatoxin B_1). Pre-administration of methanolic extract (500 mg/kg/day, p.o.) in rats produced significant inhibition of urinary mutagenicity induced by benzo[*a*]pyrene (Raphael *et al*., 2002).

Several studies reported the anticarcinogenic and antitumor effects of *P. amarus* aqueous extract. The extract markedly inhibited carcinogenesis induced by N-nitrosodiethylamine and 20-methylcholanthrene in rats (150 and 750 mg/kg/day, p.o.) and mice (150 and 750 mg/kg, three times/week, p.o.), respectively. The survival of animals administered with these carcinogens was also prolonged significantly by treatments. Besides, the extract (60, 300 and 1500 mg/kg/day, p.o.) was found to increase life span of Dalton's Lymphoma Ascites and Ehrlich Ascites Carcinoma bearing mice and suppress the progression of solid tumors volume. The possible mechanisms were also investigated and found that the extract exhibited inhibitory effects against aniline hydroxylase, DNA topoisomerase II and cdc25 tyrosine phosphatase which are responsible for metabolic activation of xenobiotics, DNA repair and cell cycle regulation, respectively (Jeena, Joy, and Kuttan, 1999; Rajeshkumar and Kuttan, 2000; Rajeshkumar *et al*., 2002).

Methanolic extract of *P. amarus* possessed cytotoxicity against various cell lines particularly on Dalton's Lymphoma Ascites cells with IC_{50} value of 102.38 µg/ml. The methanolic extract (100 and 200 µg/ml) was found to induce the expression of caspase-

3 as well as DNA fragmentation, a hallmark of apoptosis, and suppress the expression of Bcl-2 in Dalton's Lymphoma Ascites cells (Hari Kumar, Kuttan, and Kuttan, 2009b). In addition, the methanolic extract of hairy root cultures of *P. amarus* (200 and 400 µg/ml) was reported to induce apoptosis in MCF-7 cells, the human breast adenocarcinoma cell line (Abhyankar *et al*., 2010).

5. Anti-inflammatory activity

The 50% ethanol/water extract of *P. amarus* (0.3-60 mg/kg, i.p.) was reported to exhibit dose-related antinociceptive effects in several chemical models of nociception in mice including acetic acid-induced writhing, formalin-induced licking and capsaicin-induced neurogenic pain. It was also noticed that oral administration of 50% ethanol/water extract (10-800 mg/kg) was less effective as compared to intraperitoneal injection (Santos *et al*., 2000).

Methanolic extract (50, 200, and 1000 mg/kg, p.o.) of *P. amarus* showed protective effects against ethanol-induced gastric lesions in rats. In addition, the decrease in reduced glutathione level was restored by the treatment in a dose-dependent manner. In mice, the aqueous and methanolic extracts (100, 250, and 500 mg/kg, p.o.) attenuated the development of paw edema induced by carrageenan injection (Raphael and Kuttan, 2003).

The hexane and ethanol/water extracts exhibited anti-inflammatory activities possibly through nuclear factor-kappa B (NF-κB) pathway in lipopolysaccharides (LPS)-induced inflammation models. The extracts reduced the production of nitric oxide and prostaglandin E_2 in rat Kupffer cells and RAW264.7 macrophages. Furthermore, the extracts suppressed the expression of nitric oxide synthase and cyclooxygenase-2. In addition, the extracts inhibited the activation of NF-κB, but not of AP-1. The LPS-activated secretion of cytokines, including TNF-α, interleukin (IL)-1β, IL-10 and interferon-γ, were decreased in the presence of the extracts in human whole blood. Also, both extracts reduced TNF-α production in RAW264.7 macrophages and *in vivo* in mice (Kiemer *et al*., 2003).

The acute (100 mg/kg, p.o.) and chronic treatments (100 mg/kg, every 12 hours, p.o.) with *P. amarus* hexane extract demonstrated anti-allodynic and anti-oedematogenic effects in the complete Freund's adjuvant (CFA)-induced inflammation and in the persistent neuropathic pain models in mice. The increase of myeloperoxidase activity, an indicator of neutrophil infiltration, in these two models was also significantly reduced by chronic treatment with the hexane extract. Long-term administration of the purified lignan-rich fraction (100 mg/kg, every 12 hours, p.o.) caused the reduction of CFA-induced paw edema (Kassuya *et al*., 2003). In the subsequent study, the hexane extract, the lignan-rich fraction and the isolated lignans, phyltetralin, nirtetralin and niranthin, attenuated the carrageenan-induced paw edema and neutrophil influx in mice (Kassuya *et al*., 2005). These three isolated lignans (30 nmol/paw, intraplantar) were further investigated and were found to exhibit anti-oedematogenic activity on platelet activating factor (PAF)-induced paw edema in mice. Moreover, the hexane extract (100 µg/ml), the lignan-rich fraction (100 µg/ml) and niranthin (30 µM), but not phyltetralin or nirtetralin, caused significant reduction in the specific binding of [^{3}H]-PAF in mouse cerebral cortex membranes. According to the results from this study, the mechanism of anti-inflammatory and anti-allodynic actions of niranthin was proposed to be probably due to its antagonistic property on binding sites of PAF receptor (Kassuya *et al*., 2006).

6. Antiviral activity

P. amarus aqueous extract inhibited DNA polymerase of hepatitis B virus (HBV) *in vitro* with IC_{50} of 59 µg/ml. The evidence from a preliminary study in human showed that HBV carriers received 200 mg of whole plant *P. amarus* powder orally three times/day for 30 days, were hepatitis B surface antigen (HBsAg)-negative during the observation period by approximately 60% (22 of 37) versus 4% (1 of 23) in placebo control group (Blumberg *et al*., 1990). The aqueous extract (0.5 and 1 mg/ml) was found to reduce HBsAg production in human hepatoma HepA2 cells, a stable HBV DNA transfected HepG2 cells. The aqueous extract (0.5 mg/ml) suppressed HBsAg gene expression at mRNA level by inhibition of HBsAg gene promoter activity (Yeh *et al*.,

1993). However, the beneficial effects of *P. amarus* on chronic HBV infection *in vivo* are still controversial since some clinical trials demonstrated little or no effect (Leelarasamee *et al*., 1990; Thamlikitkul *et al*., 1991).

Previous studies reported inhibitory effects of *P. amarus* extracts on HIV-1 replication *in vitro* with EC_{50} values ranging from 0.9 to 7.6 µg/ml. The 50% ethanol/water extract was found to inhibit HIV-1 attachment as assessed by CD4-gp120 binding with IC_{50} value of 2.65 µg/ml. Moreover, the 50% ethanol/water extract inhibited HIV-1 enzymes including integrase, reverse transcriptase and protease with IC_{50} values of 0.48, 8.17 and 21.80 µg/ml, respectively. The gallotannin enriched fraction and the isolated ellagitannins, geraniin and corilagin, which were purified from this extract, were shown to be the most potent active compounds for these antiviral activities. The 50% ethanol/water extract and geraniin were also active in the inhibition of replication of various strains of reverse transcriptase inhibitor-resistant HIV-1. The sera from volunteers orally administered with a single dose of 1200 mg 50% ethanol/water extract were tested *ex vivo* at a final concentration of 5% and were found to reduce HIV replication by more than 30% (Notka, Meier, and Wagner, 2003; 2004). In another study, methanolic extract of *P. amarus* was reported to inhibit HIV-1 reverse transcriptase with IC_{50} value of 57.60 µg/ml (Eldeen *et al*., 2010).

Treatment with methanolic extract of *P. amarus* (250 and 750 mg/kg/day, p.o.) effectively inhibited the progression of erythroleukemia induced by Friend murine leukemia virus and increase the survival of leukemia-harboring mice. Moreover, the extract was found to induce the expression of p53 and p45NFE2 and decrease the expression of Bcl-2 in spleen of infected mice (Hari Kumar, Kuttan, and Kuttan, 2009a).

7. Antibacterial activity

Antibacterial effect of *P. amarus* extracts against both gram-positive and gram-negative bacteria were reported with the minimum inhibitory concentration (MIC) values ranging between 0.25-16 mg/ml and between 17.7-305 µg/ml for ethanolic extract and methanolic extract, respectively (Kloucek *et al*., 2005; Eldeen *et al*., 2010). Moreover,

the methanolic extract showed antibacterial activity against some drug resistant pathogenic bacterial strains especially against gram-negative microorganisms with MIC values ranging between 25-800 µg/ml (Mazumder, Mahato, and Mazumder, 2006).

8. Antifungal activity

Various solvent (chloroform, ethyl acetate and ethyl alcohol) extracts of aerial part of *P. amarus* (1000-4000 ppm) exhibited antifungal effects against dermatophytic fungi *Microsporum gypseum*. The chloroform extract was found to be the most potent among these extracts (Agrawal *et al*., 2004).

9. Hypoglycemic, hypocholesterolemic and hypotensive activities

The aqueous leaf and seed extract of *P. amarus* (150, 300 and 600 mg/kg/day, p.o.) was reported to decrease fasting plasma glucose and total cholesterol levels with the reduction in body weight in a dose-dependent manner in mice (Adeneye, Amole, and Adeneye, 2006). Furthermore, the hexane extract and the isolated oleanolic acid and ursolic acid (2:1) isomeric mixture exhibited inhibitory effect *in vitro* on α-amylase, an essential enzyme in carbohydrate digestion, with IC_{50} values of 32 and 2.01 µg/ml, respectively (Ali, Houghton, and Soumyanath, 2006).

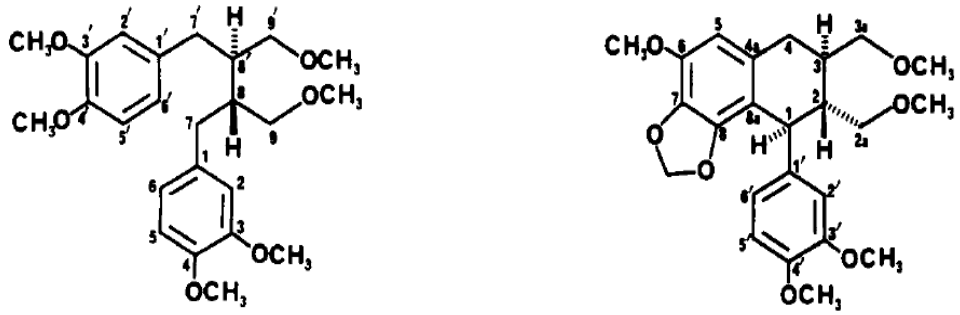
The aqueous leaf extract (5-80 mg/kg, i.v.) demonstrated dose-related blood pressure lowering effect in rabbits. In addition, the extract caused the decrease in force and rate of myocardial contraction of an isolated rabbit heart and the inhibition on intrinsic myogenic contraction of isolated rat portal vein in a concentration-dependent pattern. The combined effects of myocardial depression, muscarinic receptor mediated vascular smooth muscle relaxation and calcium channel ion blockade in vascular smooth muscle were proposed to be responsible for the hypotensive activity of the extract (Amaechina and Omogbai, 2007).

Toxicological effects of *P. amarus*

Cytotoxicity of aqueous and 50% ethanol/water extracts of whole plant *P. amarus* on Caco-2 cells were reported with IC_{50} values of 89.6 and 277 µg/ml, respectively, following 72 hours exposure. Acute (5 g/kg, p.o.) and sub acute (1 and 3 g/kg/day, p.o., for 28 days) toxicity of these extracts were evaluated in mice and rats, respectively. Signs of toxicity, behavioral changes, mortality or significant changes in serum and urine biochemical parameters were not found during the observation period. In addition, no pathological changes were observed in liver, kidney and pancreas (Lawson-Evi *et al*., 2008). In clinical trials, no adverse effects were observed in asymptomatic chronic HBV carriers who received capsules containing powder of whole plant of *P. amarus* (600-1200 mg/day, for 30 days) (Blumberg *et al*., 1990; Leelarasamee *et al*., 1990; Thamlikitkul *et al*., 1991). Nevertheless, aqueous leaf extract of *P. amarus* (400-1000 mg/kg/day, p.o., for 30 days) caused some changes in haematological and serum biochemical parameters in rats. Moreover, this extract was found to produce some deleterious histological changes in kidney, liver and testis (Adedapo, Adegbayibi, and Emikpe, 2005; Adjene and Nwose, 2010).

Phyllanthin and hypophyllanthin

Phyllanthin and hypophyllanthin are bioactive lignans presented in whole plant particularly in leaf of *P. amarus*. They are also found in other species belonging to *Phyllanthus* genus. These two compounds were previously reported to possess pharmacological effects including hepatoprotective and cytotoxic enhancing activities. The chemical structures of these compounds are shown in Figure 2.2.



1 2

Figure 2.2: Chemical structures of phyllanthin (1) and hypophyllanthin (2) (Somanabandhu *et al*., 1993)

Phyllanthin (0.1 and 1 mg/ml) and hypophyllanthin (0.1 and 1 mg/ml) exhibited protective activity against carbon tetrachloride- and galactosamine-induced cytotoxicity in primary cultured of rat hepatocytes (Syamasundar *et al*., 1985). Recent studies revealed that phyllanthin possessed antioxidant activities which probably contributed to its protective activity against oxidative damage caused by hepatotoxins. Phyllanthin (10, 20 and 30 μM) attenuated the toxicity of carbon tetrachloride in HepG2 cells in a concentration dependent manner as indicated by an increase of cell viability and reduction of enzymes leakage including ALT and lactate dehydrogenase. Phyllanthin showed DPPH scavenging activity and ameliorated antioxidant status as evidenced by an increase of intracellular reduced glutathione and a decrease of lipid peroxidation level (Krithika *et al*., 2009). Phyllanthin also exhibited protective effect against ethanol-induced injury in primary cultured of rat hepatocytes. Pretreatment with phyllanthin (2.4-9.6 μM) for 24 hours enhanced cell viability with the decrease of ALT and AST release. Phyllanthin reduced the levels of intracellular reactive oxygen species and lipid peroxidation and elevated total glutathione level as well as antioxidant enzyme activities, including superoxide dismutase and glutathione reductase, which were impaired by ethanol (Chirdchupunseree and Pramyothin, 2010).

Previous study reported that phyllanthin and hypophyllanthin at the highest concentration tested of 20 μg/ml (about 48 μM) did not demonstrate significant cytotoxic

activity on a variety of cultured mammalian cells. Nevertheless, they potentiated the cytotoxic effect of vinblastine on KB-V1 cells, the multidrug-resistant KB cells, possibly by interacting with P-glycoprotein (Somanabandhu *et al*., 1993).

Xenobiotic biotransformation (Gibson and Skett, 2001; Coleman, 2005)

Xenobiotic biotransformation or xenobiotic metabolism is the biological process that is essential in the detoxification and elimination of exogenous substances including medicines, phytochemical compounds, environmental pollutants and toxins. This process tends to transform xenobiotics into more water-soluble metabolites so as to enhance their excretion from body via urine and bile. Liver is the major organ responsible for xenobiotic metabolism. This function is also partially found in other organs such as gastrointestinal tract, kidney, lung, skin, brain, blood and placenta. Mainly, xenobiotic metabolism can be divided into phase I and II. The reactions in phase I metabolism, such as oxidation, reduction and hydrolysis, provide the functional groups (-OH, -COOH, -NH_2, -SH) in the molecule of the compounds to facilitate the reactions in phase II metabolism. The metabolites from phase I metabolism are usually more polar and less toxic than parent compounds. Although most medicines are transformed into metabolites with less pharmacological activity, some are found to be bioactivated into more potent or active metabolites. In some cases, this process also produces reactive or toxic metabolites which may be harmful to cellular macromolecules such as DNA, protein and lipid membrane. The phase II metabolism is the conjugation reactions between endogenous compounds and functional groups of the parent compounds or metabolites from phase I metabolism. The reactions in phase II metabolism, such as glucuronidation, sulfation, methylation, acetylation, amino acid conjugation and glutathione conjugation, mostly lead to much more hydrophilic and inactive metabolites. In addition to phase I and II metabolism, some literatures may include the function of efflux transporter systems which are involved in the excretion of compounds from cells as the phase III metabolism. In conclusion, xenobiotic metabolism plays crucial roles in the protection against potentially toxic compounds as well as the bioactivation of some

medicines and toxins such as promutagens and procarcinogens. Therefore, the alteration of activity and gene expression of proteins involved in xenobiotic metabolism may affect therapeutic efficacy of medicines and susceptibility to the toxic effect of xenobiotics.

Cytochrome P450 (Gibson and Skett, 2001; Coleman, 2005; Chen and Raymond, 2006; Pal and Mitra, 2006)

Cytochrome P450 enzymes (CYPs) are haem-containing enzymes located in membrane of endoplasmic reticulum. CYPs are mainly expressed in the liver and also exist in gastrointestinal tract, lung, skin, kidney, testis, placenta, adrenal cortex, platelet and lymphocyte. CYPs play important roles in phase I metabolism by catalyzing various oxidative and reductive reactions of many different kinds of xenobiotics as well as endogenous compounds such as bile acid, steroids, thyroid hormones and fatty acids. CYPs catalyze reactions by involving in the mixed-function oxidase enzyme system which consists of CYPs, NADPH-cytochrome P450 reductase and lipid.

Human CYPs can be classified into 18 families and 44 subfamilies according to the similarity of their amino acid sequences. Important CYPs isoforms in humans that contribute to drug metabolism include CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8/9, CYP2C18/19, CYP2D6, CYP2E1 and CYP3A4.

CYP3A4 is the most abundant CYPs isoform both in liver and intestine and responsible for the metabolism of a large number and various types of substrates. In human liver, CYP3A4 is found approximately 25% of total CYPs content. Moreover, about 60% of clinically used medicines are metabolized via CYP3A4. The highly expression of CYP3A4 in intestinal cells also has important roles in first-pass metabolism that can influence the bioavailability of oral administered medicines. CYP3A4 is also involved in the bioactivation of some procarcinogens such as benzo[*a*]pyrene and aflatoxin B_1. Examples of drug and herb known to be substrate, inhibitor and inducer of CYP3A4 are shown in Table 2.1.

Table 2.1: Examples of substrates, inhibitors and inducers of CYP3A4 (Gibson and Skett, 2001; Coleman, 2005)

Substrates	Inhibitors	Inducers
acetaminophen, amiodarone, antihistamines (e.g. astemizole, terfenadine), azole antifungals (e.g. ketoconazole, itraconazole), benzodiazepines (e.g. diazepam, midazolam), buspirone, calcium channel blockers (e.g. diltiazem, felodipine, nifedipine, verapamil), cannabinoids, carbamazepine, cisapride, cyclophosphamide, cyclosporine, digitoxin, eletriptan, eplerenone, ergotamine, ethinylestradiol, macrolides (e.g. erythromycin), opiates (e.g. alfentanil, fentanyl), paclitaxel, pimozide, protease inhibitors (e.g. ritonavir), proton pump inhibitors (e.g. lansoprazole), quinidine, statins (e.g. simvastatin), sildenafil, sirolimus, steroids (e.g. budesonide, fluticasone), tacrolimus, tamoxifen, zonisamide	amiodarone aprepitant azole antifungals cimetidine diltiazem grapefruit juice macrolides nefazodone protease inhibitors steroids verapamil	carbamazepine dexamethasone fexofenadine lovastatin metyrapone paclitaxel phenobarbital phenytoin rifampicin St John's wort troglitazone

Inhibition of CYPs (Gibson and Skett, 2001; Coleman, 2005)

CYPs inhibition mostly refers to the inhibitory effect on CYPs protein enzyme which may be reversible or irreversible. In drug metabolism aspect, the inhibition of CYPs can result in retarded clearance and therefore accumulation of drugs which are

their substrates. The enhanced pharmacological as well as toxic effects of these drugs usually occur in short time within a few hours to several days after receiving CYPs inhibitors and may be reversible rapidly following inhibitors withdrawal. These potential adverse effects may be greatly enhanced in cases of some medicines with narrow therapeutic index and/or limited alternative elimination pathways, which can lead to severe, irreversible or even fatal responses. Moreover, CYPs inhibition may retard the bioactivation of prodrugs resulting in decreasing therapeutic efficacy. In addition, some CYPs inhibitors may interfere with steroid hormone metabolism and cause some adverse effects such as male gynaecomastia and female menstrual cycle disturbance. In contrast, the inhibition of CYPs may have beneficial roles in reducing risks of mutagenesis and carcinogenesis by preventing the formation of reactive metabolites from the oxidation of some substrates. Co-administration of CYPs inhibitors with other medicines may also reduce dosage required as well as their cost in medical treatment.

CYPs inhibition can mainly be classified into four categories.

1. Competitive inhibition

The competitive inhibitor and drug substrate are usually quite similar in structure and binding affinity to the same enzyme catalytic site. Therefore, the inhibitor competitively binds at the active site and obstructs the binding of substrate. The enzyme binding rates of either competitive inhibitor or drug substrate are depended on their concentrations.

2. Non-competitive inhibition

In non-competitive inhibition, the inhibitor does not competitively bind at the same active site as drug substrate, but bind at the allosteric site apart from the active site. The binding of inhibitor at allosteric site results in the conformation change of the active site and subsequently hinders the binding of drug substrate at this site.

3. Uncompetitive inhibition

This uncommon type of inhibition occurs when the inhibitor binds to the enzyme/substrate complex. As a result, this enzyme complex cannot function.

4. **Mechanism-based inhibition**

The mechanism-based inhibitor requires the metabolic activation by CYPs to be the metabolite that can form inactive complex with CYPs or even damage the enzymes. Unlike the first three types of inhibition, these inhibitory effects on CYPs are usually irreversible. Thus, the mechanism-based inhibition may also be termed as suicide inhibition. This type of inhibition can be demonstrated *in vitro* by pre-incubation of the test compound with CYP enzymes and NADPH before adding of the substrate. This pre-incubation period allows the metabolic activation of inhibitor leading to the time-dependent inactivation of CYPs which cannot be overcome by subsequent addition of substrate with increasing concentration.

Induction of CYPs (Gibson and Skett, 2001; Coleman, 2005; Lin, 2006; Tompkins and Wallace, 2007)

Several human CYPs including CYP1A, CYP2A, CYP2B, CYP2C, CYP2E and CYP3A have been known to be inducible by xenobiotics. In contrast to rapid response in CYPs inhibition, CYPs induction is a gradual and time-dependent process. It takes time to increase the enzyme level until reaching steady-state and to recover to initial baseline following discontinuation of inducer exposure. Although CYPs induction is suggested to be essential mechanism in the adaptive response of organisms against potentially harmful xenobiotics, this process may also cause undesired effects. The induction of drug metabolizing enzymes like CYPs may significantly decrease drugs plasma concentration causing reduction in therapeutic efficacy. Moreover, the increasing reactive metabolites produced from CYPs-mediated oxidation of certain substrates may lead to the imbalance of detoxifying mechanisms and oxidative stress. These excessive reactive metabolites can covalently bind with cellular macromolecules such as DNA, proteins and lipid membranes resulting in cytotoxicity as well as carcinogenesis.

Mostly, upregulation of CYPs involves the activation of key transcription factors leading to the increase in mRNA and protein synthesis. Transcription factors known to be involved in CYPs gene expression are shown in Table 2.2. In addition to the

mechanism of gene transcription activation, some CYP isoforms such as CYP2E1 and CYP3A4 have been reported to be induced by mechanisms at post-transcriptional level. These previously reported mechanisms include mRNA stabilization, enhancement of protein translation and reduction of protein degradation.

Table 2.2: Transcription factors involved in CYPs gene expression (Lin, 2006; Tompkins and Wallace, 2007)

Transcription factors	CYPs isoforms
AhR	CYP1A1, 1A2
CAR, PXR	CYP2B, 2C, 3A
GR	CYP3A
PPARα	CYP4A11
FXR, LXR	CYP7A

AhR = aryl hydrocarbon receptor

CAR = constitutive androstane receptor

FXR = farnesoid X receptor

GR = glucocorticoid receptor

LXR = liver X receptor

PXR = pregnane X receptor

PPAR = peroxisome proliferator activated receptor

Transcription factors involved in CYP3A4 gene expression (Lin, 2006; Tompkins and Wallace, 2007)

1. **Pregnane X receptor (PXR)**

PXR, also known as steroid and xenobiotic receptor (SXR), was named for its ability in binding with several steroids and steroid metabolites, including pregnane, as well as many different types of xenobiotics. The molecular mechanism of CYP3A4

induction has been suggested to be mainly due to the direct binding of ligand with PXR resulting in the activation of this receptor and subsequently CYP3A4 gene transcription. Several medicines have been known to be PXR activators such as rifampicin, clotrimazole, phenobarbital, troglitazone, lovastatin, nifedipine and dexamethasone. The proposed model of PXR-regulated CYP3A4 gene expression is shown in Figure 2.3. Upon ligand activation, PXR nuclear translocation occurs and PXR forms a heterodimer with retinoid X receptor (RXR) which subsequently interacts with response elements in CYP3A4 gene. PXR has also been reported to be involved in the expression of many other proteins that are responsible for the detoxification and elimination of xenobiotics including CYP2B, CYP2C, glutathione S-transferase, P-glycoprotein, multidrug resistance associated proteins (MRPs) and organic anion-transporting polypeptide 2 (OATP2).

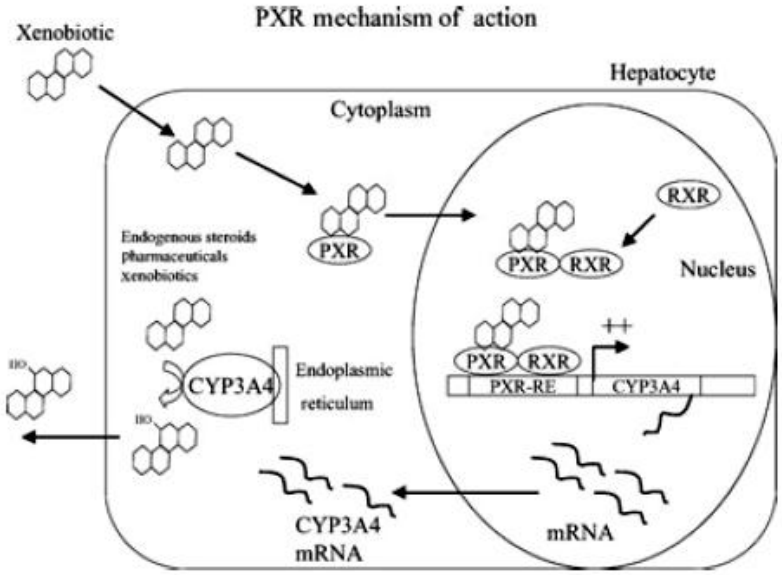


Figure 2.3: Schematic model of PXR-mediated CYP3A4 gene expression (Tompkins and Wallace, 2007)

2. **Constitutive androstane receptor (CAR)**

CAR, also known as constitutively active receptor, was named for its constitutive activity to transactivate target genes *in vitro* in the absence of ligand. In addition, it was found that androstanol and androstenol, the androstane metabolites, inhibit constitutive

activity of this receptor. *In vivo*, CAR is located in cytoplasm and translocates to nucleus following activation by its ligand. The mechanism of CAR activation seems to be more complicated than the direct binding of ligand since phenobarbital, a classical activator of CAR, did not show its ability to bind CAR in the *in vitro* binding assay. In addition, the X-ray crystallography studies have shown that the ligand-binding domain of CAR is relatively small when compared with PXR. It has been reported that okadaic acid, a phosphatase inhibitor, inhibits CAR nuclear translocation activated by phenobarbital, suggesting that dephosphorylation event may be involved in the activation of CAR. In similar to PXR, CAR interacts with its response elements in target genes as a heterodimer with RXR. The proposed model of CAR-regulated CYP3A4 gene expression is shown in Figure 2.4. CAR activation has been reported to be involved in the upregulation of various proteins important in xenobiotic metabolism including CYP2B, CYP2C, sulfotransferases, glucuronosyltransferases, glutathione S-transferases and transporters.

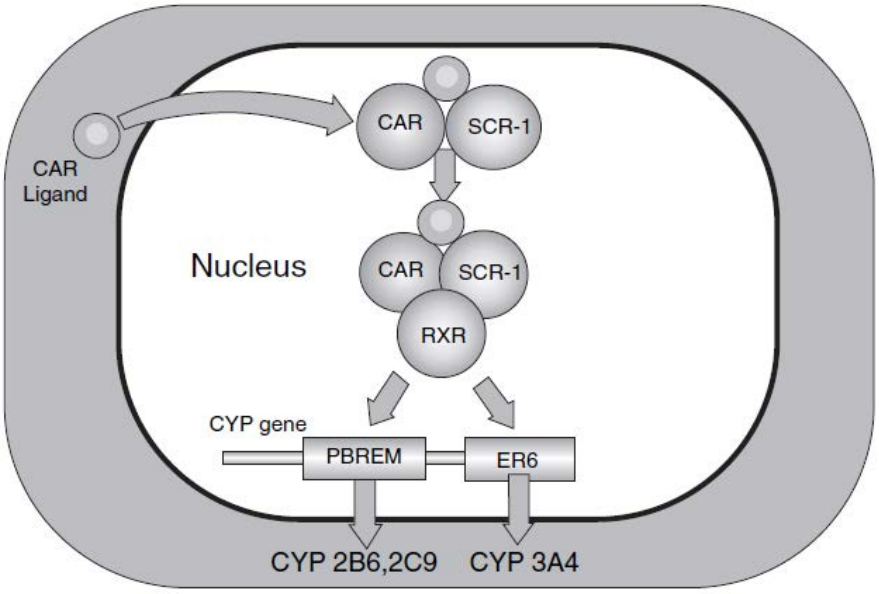


Figure 2.4: Schematic model of CAR-mediated CYP3A4 gene expression (Coleman, 2005)

3. Glucocorticoid receptor (GR)

In addition to PXR and CAR, GR is also one of the most widely studied nuclear receptors involved in CYP3A4 induction. Several mechanisms of GR-mediated CYPs gene expression have been previously proposed. GR may directly interact with the responsive elements of target genes in a CAR- or PXR-independent manner. In addition to directly interacting mechanism, GR may also play a role as a coregulator that enhances the binding of PXR and CAR to their response elements resulting in the synergistic effect on gene expression. Furthermore, GR has been suggested to be involved in the regulation of PXR, CAR and RXR gene expression. It was found that mRNA levels of these receptors were significantly increased by dexamethasone, a GR activator, in human hepatocytes.

Effects of *P. amarus* extracts, phyllanthin and hypophyllanthin on CYPs

Previous studies demonstrated the inhibitory effect of *P. amarus* extracts on various CYPs including CYP3A4. In a study using rat liver microsomes, methanolic extract of *P. amarus* inhibited 7-ethoxyresorufin-*O*-deethylase (EROD), 7-methoxyresorufin-*O*-demethylase (MROD) and 7-pentoxyresorufin-*O*-dependentylase (PROD) which were representatives of CYP1A1, CYP1A2 and CYP2B1/2 activities with IC_{50} values of 4.6, 7.725 and 4.18 µg/ml, respectively. In addition, the extract was found to inhibit aminopyrine-*N*-demethylase (an indicator of CYP1A, 2A, 2B, 2D and 3A activity, IC_{50} > 1000 µg/ml) and aniline hydroxylase (an indicator of CYP2E1 activity, IC_{50} = 62.38 µg/ml) activities. This study also showed that the extract (250 and 750 mg/kg/day, p.o.) suppressed the elevation of CYP1A1, CYP1A2 and CYP2B1/2 activities that were induced by phenobarbitone administration *in vivo* in rats (Hari Kumar and Kuttan, 2006).

Aqueous extracts of various parts of *P. amarus* (leaves, roots, stems and whole plant) inhibited recombinant human CYP1A2, CYP2C9, CYP2D6 and CYP3A4 with IC_{50} values ranging between 38.1-134.3, 63.4-127.5, 45.8-182.0 and 79.2-146.8 µg/ml, respectively (Appiah-Opong *et al.*, 2008). Recent study using human liver microsomes has reported that aqueous and ethanolic extracts of *P. amarus* inhibited CYP1A2,

CYP2D6, CYP2E1 and CYP3A4 activities in a concentration-dependent manner. *P. amarus* aqueous (25 µg/ml) and ethanolic (1 µg/ml) extracts exhibited significant time-dependent inhibition on CYP3A4 but not CYP1A2, CYP2D6 and CYP2E1. Furthermore, phyllanthin and hypophyllanthin were demonstrated to be potent mechanism-based inhibitors of CYP3A4 with IC_{50} values of 2.18 and 2.90 µM without pre-incubation and IC_{50} values of 0.59 and 0.95 µM with 10 min pre-incubation, respectively (Taesotikul *et al.*, 2011).

CHAPTER III

MATERIALS AND METHODS

Instruments and materials

1. Instruments

These following instruments were used in this study:

- Adjustable pipette 1-10 µl, 2-20 µl, 10-100 µl, 20-200 µl and 100-1000 µl (Gilson, France)
- Analytical balance (Precisa, Switzerland)
- Centrifuge (Beckman Microfuge, Germany)
- CO_2 incubator (ThermoForma Scientific, USA)
- Electrophoresis Cell (Mini-PROTEAN® 3 Cell) and Mini Trans-Blot electrophoretic transfer cell (Bio-Rad, USA)
- Microplate reader (DTX 800 multimode detector, Beckman Coulter, USA)
- Freezer -80 °C (Thermoelectron, USA)
- Hemocytometer (Resistant, Germany)
- iQ™5 Multicolor Real-Time PCR Detection System with iQ TM reagent optical system software version 2.0 (Bio-Rad, USA)
- Light microscope (Olympus, Japan)
- Mikro 22R centrifuge (Hettich, Germany)
- Mini centrifuge
- Multichannel pipettors (Gilson, France)
- pH meter (Therma, Canada)
- Power supply (Bio Rad, USA)
- Shaker
- UV-spectrophotometer (Shimadzu, Japan)
- UV transluminator gel documentation (Syngene, UK)

- Vortex mixer (CT Laboratory Clay Adams, USA)
- Vertical laminar flow cabinet (Advanced Biological Safety Cabinet Class II, Microflow, UK)

2. Materials

These following materials were used in this study:

- Cell culture dish: diameter 100 mm, 6-well cell culture plates, 24-well cell culture plates, 96- well cell culture plates (Costar, USA)
- Conical tube: 15, 50 ml (Axygen, USA)
- Microcentrifuge tube (Axygen, USA)
- Millipore filter 0.22 μm (Millipore, USA)
- Multiwell white plates (96-well opaque white plates)
- Thin wall PCR tube (Axygen, USA)
- Pipette tip: 1-10 μl, 20-200 μl and 1000 μl (Axygen, USA)
- Serological pipette: 5 ml & 10 ml (Costar, USA)
- Tissue culture flask: 25, 75 cm^2 (Corning Incorporation, USA)

3. Chemicals

The following chemicals were purchased from Sigma Chemical Company, St. Louis, USA: ammonium persulfate, bovine serum albumin (BSA), dimethylsulfoxide (DMSO), ethidium bromide (EtBr), ethylenediamine tetraacetic acid (EDTA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), ponceau S, sodium bicarbonate ($NaHCO_3$), sodium citrate, sodium dodecyl sulfate (SDS), sodium hydroxide (NaOH), sodium phosphate dibasic anhydrous, sodium phosphate dibasic heptahydrate, sodium phosphate monobasic anhydrous, tris [hydroxymethyl] aminomethane hydrochloride, tween 20.

Calcium chloride ($CaCl_2$), chloroform, disodium hydrogen phosphate (Na_2HPO_4), ethanol, glycerol, hydrochloric acid (HCl), isopropanol, methanol, potassium chloride (KCl), sodium chloride (NaCl) were purchased from Merck Darmstadt, Germany.

Fetal bovine serum, 200 mM L-glutamine, 10,000 units/ml penicillin & 10,000 µg/ml streptomycin, 0.25% Trypsin-EDTA, Dulbecco's Modified Eagles medium (DMEM) and TRIzol® Reagent were purchased from Invitrogen, USA.

Mouse monoclonal Beta-actin antibody, mouse monoclonal CYP3A4 antibody and goat anti-mouse horseradish peroxidase (HRP)-conjugated antibody were purchased from Santa Cruz, USA.

Acrylamide, Bio-Rad Protein Assay and iScript™ cDNA Synthesis Kit were purchased from Bio-Rad, USA.

Taq DNA polymerase (5 U/ul) and Page Ruler™ Prestained Protein Ladder was purchased from Fermentus Life Sciences, USA.

SYBR green I sDNA-nucleic acid gel stain dye was purchased from Bio Basic Inc, Canada.

Amersham ECL™ Prime Western Blotting detection reagent, Amersham Hyperfilm™ ECL high performance chemiluminescence film and Hybond™-ECL™ nitrocellulose membranes were purchased from GE Healthcare Limited, UK.

CellTiter-Glo® Luminescent Cell Viability Assay and P450-Glo™ CYP3A4 Assay (Luciferin-IPA) were purchased from Promega Corporation, USA.

Carbon dioxide gas was purchased from Thai Industrial Gases Public Company, Ltd., Thailand.

Phyllanthin, hypophyllanthin and *P. amarus* aqueous extract were kindly provided by Associate Professor Dr. Pornpen Pramyothin, Department of Pharmacology and Physiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University. The preparation procedure of these compounds and the determination of their hepatoprotective activity were previously described (Pramyothin *et al.*, 2007; Chirdchupunseree and Pramyothin, 2010). Phyllanthin and hypophyllanthin were found in the *P. amarus* aqueous extract using preliminary thin layer chromatography technique

(Pramyothin *et al*., 2007). Phyllanthin and hypophyllanthin were identified by thin layer chromatography, Fourier Transform Infrared Spectrometer and HPLC (Chirdchupunseree and Pramyothin, 2010).

All other chemicals and solvents used throughout this study were analytical grade reagents.

Methods

1. Cell culture

Human hepatocarcinoma (HepG2) cells were obtained from American Type Culture Collection (ATCC). HepG2 cells were cultured in Dulbecco's Modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were maintained at 37ºC in a humidified atmosphere with 5% CO_2.

2. Experimental processes

2.1 Cytotoxicity of phyllanthin, hypophyllanthin and *P. amarus* aqueous extract on HepG2 cells

MTT assay was performed to determine the cytotoxicity of phyllanthin, hypophyllanthin and *P. amarus* aqueous extract on HepG2 cells so as to obtain the optimal concentration of these compounds for subsequent experiments. This assay is based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into a purple insoluble formazan product by succinate-tetrazolium reductase, a mitochondrial enzyme used as an index of cell viability (Mossmann, 1983)(Figure 3.1).

HepG2 cells were cultured in 96-well plates at the density of 3×10^4 cells/well. After 24 hours of incubation, culture medium was replaced with medium containing various concentrations of phyllanthin, hypophyllanthin or *P. amarus* aqueous extract. Phyllanthin and hypophyllanthin were dissolved in DMSO and further diluted with medium to final concentrations of 125, 50, 25, 5, 1, 0.2 and 0.04 µM in 0.5% DMSO. *P. amarus* aqueous extract was dissolved in medium and diluted to final concentrations of

5, 2.5, 1, 0.5, 0.1, 0.05, 0.01 and 0.001 mg/ml. Cells were incubated with the test compounds for 24, 48 and 72 hours. After treatment, MTT assay was performed to determine cell viability. Briefly, cells were washed twice with phosphate buffer saline (PBS) pH 7.4 and further incubated with medium containing 0.5 mg/ml MTT at 37°C for 2 hours. After incubation, medium was removed and the remaining formazan crystal was dissolved in 100 µl of DMSO. The optical density was measured at 570 nm using a microplate reader. At least three independent experiments were performed in triplicates of each. Percentage of cell viability was calculated using the following equation.

$$\% \text{ cell viability} = \frac{\text{OD of the test compound treated wells}}{\text{OD of the control wells}} \times 100$$

The control wells for phyllanthin and hypophyllanthin contained medium with 0.5% DMSO whereas the control wells for *P. amarus* aqueous extract contained medium without 0.5% DMSO.

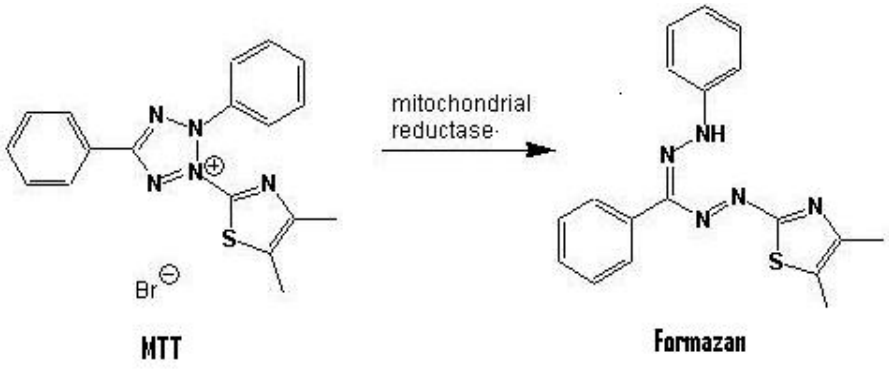


Figure 3.1: MTT reduction assay (http://www.biotek.com/resources/articles/quontification-cell-viability-epoch.html)

2.2 Effects of phyllanthin, hypophyllanthin and *P. amarus* aqueous extract on CYP3A4 activity in HepG2 cells

Determination of CYP3A4 activity

CYP3A4 activity was determined using P450-Glo™ CYP3A4 Assays. This assay consists of 2 components, Luciferin IPA, a luminogenic CYP3A4 substrate, and Luciferin Detection Reagent. In brief, Luciferin IPA is converted by CYP3A4 to D-Luciferin that can be detected with the Luciferin Detection Reagent (Figure 3.2).

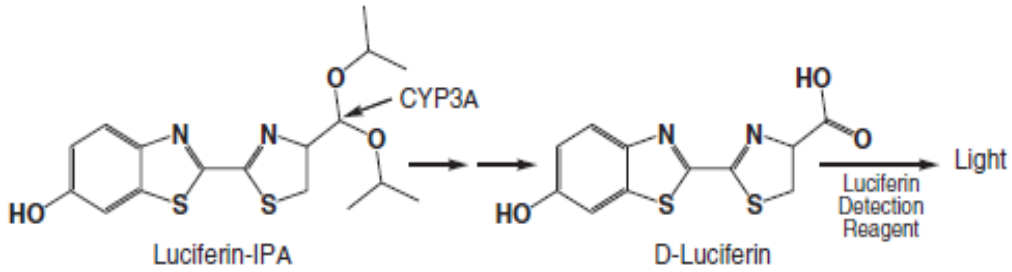


Figure 3.2: The proposed scheme of Luciferin IPA metabolism by CYP3A4 (Li, 2009)

HepG2 cells were cultured in 24-well plates at the density of 2×10^5 cells/well. After 24 hours of incubation, culture medium was changed and cells were incubated with test compounds for 24, 48 and 72 hours. Phyllanthin and hypophyllanthin were dissolved in DMSO and further diluted with medium to final concentrations of 25, 5 and 1 µM containing 0.5% DMSO. *P. amarus* aqueous extract was dissolved in medium and diluted to final concentrations of 0.5, 0.1 and 0.05 mg/ml. During the treatment period, culture medium was renewed once daily. After the treatment, cells were washed twice with PBS pH 7.4 and further incubated with medium containing 3 µM Luciferin IPA at 37°C for 2 hours. After incubation, 100 µl of medium from each well was transferred to 96-well opaque white plates and mixed with 100 µl of Luciferin Detection Reagent at room temperature. Luminescence was measured using a microplate reader. The recorded luminescence values were subtracted with background luminescence values (no-cell control). Background subtracted values were normalized to cell number by

dividing the P450-Glo™ Assay values by the CellTiter-Glo® Assay values. Fold change of CYP3A4 activity was calculated using the following equation.

$$\text{Fold change} = \frac{\text{Normalized test compound treated values}}{\text{Normalized control values}}$$

Rifampicin (50 μM) and dexamethasone (50 μM) were used as the positive controls for CYP3A4 induction. At least three independent experiments were performed in triplicates of each. The control group for phyllanthin, hypophyllanthin and rifampicin contained medium with 0.5% DMSO whereas the control group for *P. amarus* aqueous extract and dexamethasone contained medium without 0.5% DMSO.

Determination of cell number

Cell number was estimated using CellTiter-Glo® Luminescent Cell Viability Assay which determined the number of viable cells in culture based on quantitation of the ATP present (Figure 3.3). Briefly, cells were trypsinized with 0.25% trypsin and resuspended with medium to a final volume of 500 μl. Then, 50 μl of cell suspension was transferred to 96-well opaque white plates and mixed with 50 μl of CellTiter-Glo® reagent at room temperature. Luminescence was measured using a microplate reader. The luminescence values were subtracted with background luminescence values (no-cell control). The correlation between the luminescent signal and the number of cells from 25,000 to 200,000 cells per well was also established (Appendix, Figure A1).

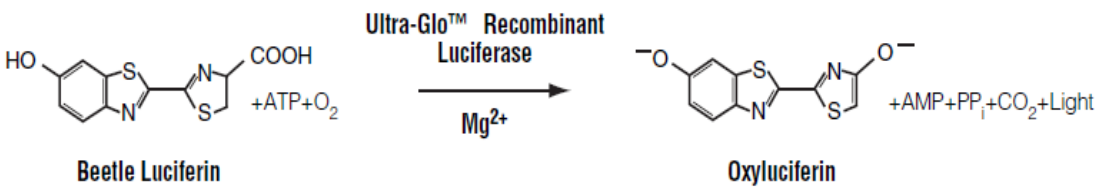


Figure 3.3: Chemical reaction of CellTiter-Glo® Luminescent Cell Viability Assay (Technical Bulletin: CellTiter-Glo® Luminescent Cell Viability Assay (Promega, USA))

2.3 Effects of phyllanthin, hypophyllanthin and *P. amarus* aqueous extract on CYP3A4 protein expression in HepG2 cells

Sample preparation

HepG2 cells were cultured in 100 mm cultured dishes at the density of 4×10^6 cells/plate. After 24 hours of incubation, culture medium was changed and cells were incubated with the test compounds for 24, 48 and 72 hours. Phyllanthin and hypophyllanthin were dissolved in DMSO and further diluted with medium to final concentrations of 25 and 1 µM in medium containing 0.5% DMSO. *P. amarus* aqueous extract was dissolved in medium and diluted to final concentrations of 0.5 and 0.05 mg/ml. During the treatment period, culture medium was renewed once daily. After the treatment, cells were washed twice with cold PBS pH 7.4, scrapped and collected in a microcentrifuge tube. Cells were centrifuged at 5,000 x *g* for 10 minutes at 4°C and resuspended in 1 ml of PBS pH 7.4 for two times. After washing step, supernatant was discarded and cells were gently resuspended in 400 µl of buffer containing 10 mM HEPES-KOH, 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM PMSF and 0.5 mM DTT. Samples were incubated on ice for 15 minutes. Then, 12.5 µl of 10% Nonidet P-40 was added followed by 15 seconds of vigorous vortexing. Lysates were centrifuged at 14,000 rpm for 30 seconds at 4°C. Supernatant was transferred into a new microcentrifuge tube and stored at -80 °C. This cytoplasmic fraction was used in the determination of CYP3A4 protein level.

Determination of protein concentration

Protein concentration was determined using Bio-Rad Protein Assay based on the Bradford method (Bradford, 1976). In short, 10 µl of protein sample was mixed with 0.5 ml of Bradford reagent and incubated at room temperature for 5 minutes. The absorbance at 595 nm was measured using a spectrophotometer. BSA was used as the protein standard for the relative measurement of protein concentration.

Western blot analysis

Samples containing 100 µg protein were mixed with equal volume of 2x sample buffer and boiled at 95ºC for 5 minutes. The prepared samples were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 100 V for 90 minutes. After electrophoresis, proteins were transferred to a nitrocellulose membrane at 90 V for 90 minutes. Membranes were blocked with 5% (w/v) non-fat dry milk in TBST at 4ºC overnight to avoid non-specific binding. Thereafter, membranes were incubated with mouse monoclonal CYP3A4 antibody (1:200) or mouse monoclonal Beta-actin antibody (1:1000) at room temperature for 2 hours. After incubation with primary antibody, membranes were washed three times with TBST and incubated with HRP-conjugated goat anti-mouse antibody (1:5000) at room temperature for 1 hour. After that, membranes were washed three times with TBST and once with TBS. Protein bands were visualized using enhanced chemiluminescence and were exposed to X-ray films. The band intensity was quantified by using the Image J program. The band intensity of CYP3A4 was normalized to Beta-actin which was determined on the same membrane as the loading control.

Rifampicin (50 µM) and dexamethasone (50 µM) were used as the positive controls for CYP3A4 induction. The control group for phyllanthin, hypophyllanthin and rifampicin contained medium with 0.5% DMSO whereas the control group for *P. amarus* aqueous extract and dexamethasone contained medium without 0.5% DMSO.

2.4 Effects of phyllanthin, hypophyllanthin and *P. amarus* aqueous extract on CYP3A4 mRNA expression in HepG2 cells

Sample preparation

HepG2 cells were cultured in 6-well plates at the density of 1×10^6 cells/well. After 48 hours of incubation, culture medium was changed and cells were incubated with the test compounds for 12 and 24 hours. Three independent experiments were performed in triplicates of each. Phyllanthin and hypophyllanthin were dissolved in DMSO and further diluted with medium to final concentrations of 25 and 1 µM in medium containing 0.5%

DMSO. *P. amarus* aqueous extract was dissolved in medium and diluted to final concentrations of 0.5 and 0.05 mg/ml. After the treatment, cells were washed twice with cold PBS pH 7.4 and total RNA was isolated using Trizol® reagent according to the manufacturer's protocol. The absorbance at 260/280 nm was measured using a spectrophotometer. The isolated RNA which had an $A_{260/280}$ ratio of 1.6-1.8 was determined to be acceptable quality. The concentration of total RNA was calculated according to the following equation:

[RNA] (ng/ml) = Absorbance 260 nm × 40 × dilution factor

Real time RT-PCR analysis

cDNA was synthesized from RNA sample using the iScript™ cDNA Synthesis Kit. Real-time PCR was performed using the iQ™5 Multicolor Real-Time PCR Detection System. The mixture of PCR reaction consisted of 0.05 U/µl Taq DNA polymerase, 0.2 mM dNTP mix, 1x reaction buffer, 2.08 mM $MgCl_2$, SYBR Green I sDNA acid gel stain dye, 1 µM of each specific primer and cDNA, which was equivalent to 100 ng of total RNA, in a volume of 25 µl. Beta-actin was used as an internal control. The primers for CYP3A4 and Beta-actin were previously reported (Krusekopf, Roots, and Kleeberg, 2003; Kreis *et al*., 2007). The PCR condition was set at 95°C for 3 minutes, followed by 35 cycles of 94°C for 30 seconds, 56.5°C for 15 seconds and 72°C for 40 seconds. The results were analyzed using the iQ™5 Optical System Software version 2.0 based on the comparative threshold cycle (C_t) method. The specificity of the amplification was verified by melt curve analysis and agarose gel electrophoresis (Appendix, Figure A2-A4).

Rifampicin (50 µM) and dexamethasone (50 µM) were used as the positive controls for CYP3A4 induction. The control group for phyllanthin, hypophyllanthin and rifampicin contained medium with 0.5% DMSO whereas the control group for *P. amarus* aqueous extract and dexamethasone contained medium without 0.5% DMSO.

Table 3.1 Oligonucleotide primers for real time RT-PCR

Gene	Primer sequence	Product size (bp)
CYP3A4	Forward: 5´-GCCTGGTGCTCCTCTATCTA-3´ Reverse: 5´-GGCTGTTGACCATCATAAAAG-3´	187
Beta-actin	Forward: 5´-GCTCGTCGTCGACAACGGCT-3´ Reverse: 5´-CAAACATGATCTGGGTCATCTTCTC-3´	353

3. Statistical analysis

Results were expressed as mean ± S.E.M. Statistical analysis was performed using the SPSS version 16.0 for windows software. Mean differences among groups were examined using one-way ANOVA followed by Tukey's post hoc test. *P*-value < 0.05 was considered statistically significant. Median inhibitory concentration (IC_{50}) was calculated by plotting log of concentrations of *P. amarus* aqueous extract versus the corresponding probit unit of the percent inhibition.

CHAPTER IV

RESULTS

Cytotoxicity of phyllanthin, hypophyllanthin and *P. amarus* aqueous extract on HepG2 cells

Cytotoxicity of phyllanthin, hypophyllanthin and *P. amarus* aqueous extract on HepG2 cells were determined by MTT assay. During 24-72 hours of exposure, phyllanthin at concentrations of 0.04-125 μM and hypophyllanthin at concentrations of 0.04-25 μM did not significantly affect viability of HepG2 cells (Figure 4.1 and 4.2). Hypophyllanthin at concentrations of 50 and 125 μM significantly decreased percent cell viability following incubation for 48 and 72 hours. However, it was observed that phyllanthin and hypophyllanthin at concentrations of 50 and 125 μM precipitated during incubation.

P. amarus aqueous extract exhibited significant cytotoxicity on HepG2 cells at concentrations of 1, 2.5 and 5 mg/ml in a time- and concentration-dependent manner (Figure 4.3). The extract caused the reduction of percent cell viability with IC_{50} values of 3.69, 1.76 and 1.24 mg/ml for the incubation periods of 24, 48 and 72 hours, respectively (Figure 4.4).

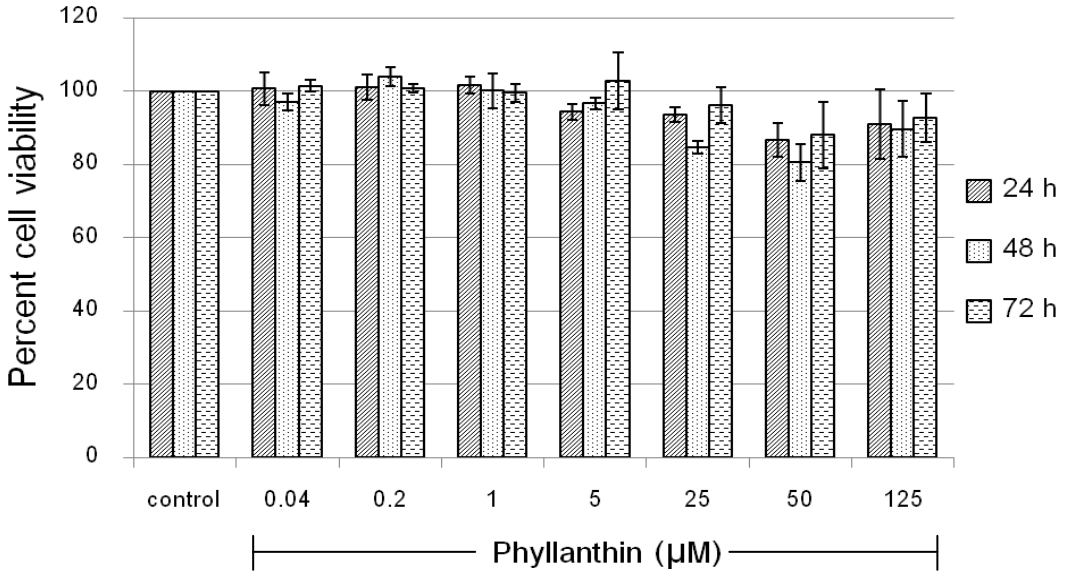


Figure 4.1: Effect of phyllanthin on HepG2 cell viability as determined by MTT assay after 24, 48 and 72 hours of incubation.
Data are expressed as mean ± S.E.M. (n=3-4).

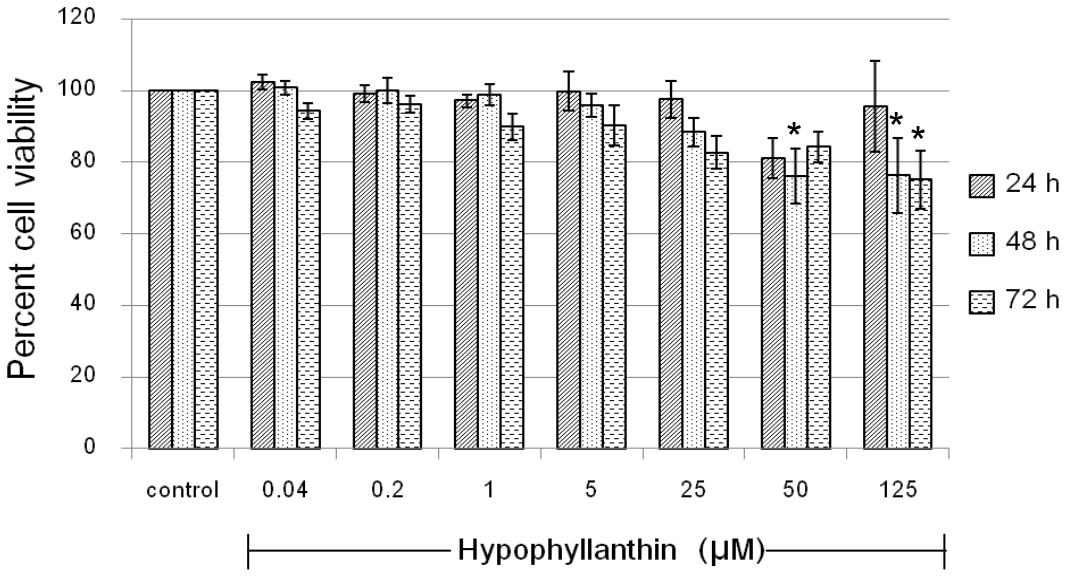


Figure 4.2: Effect of hypophyllanthin on HepG2 cell viability as determined by MTT assay after 24, 48 and 72 hours of incubation.

Data are expressed as mean ± S.E.M. (n=3-4).

***** Significant difference from the control ($P < 0.05$).

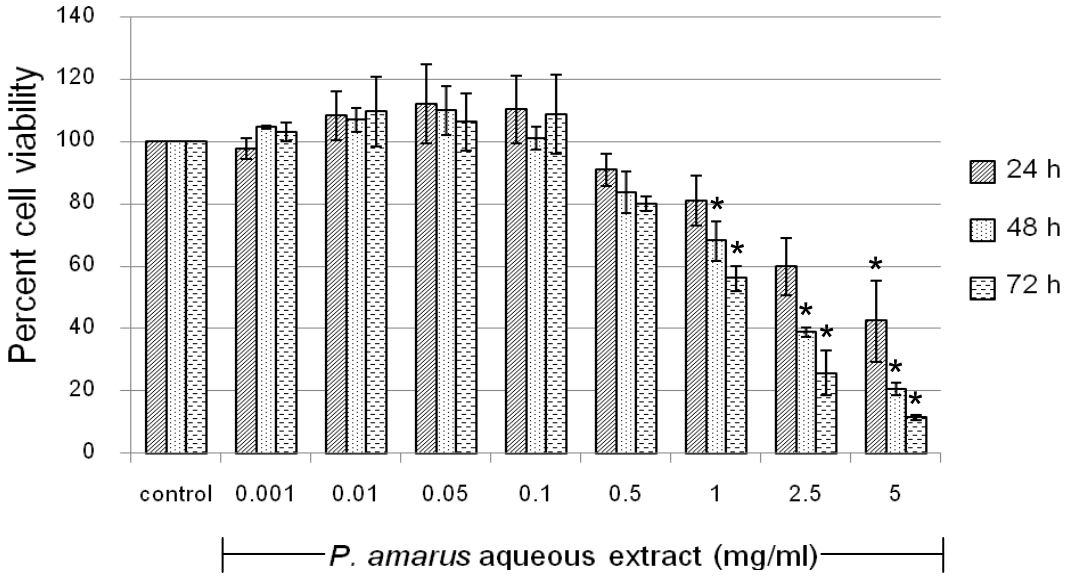
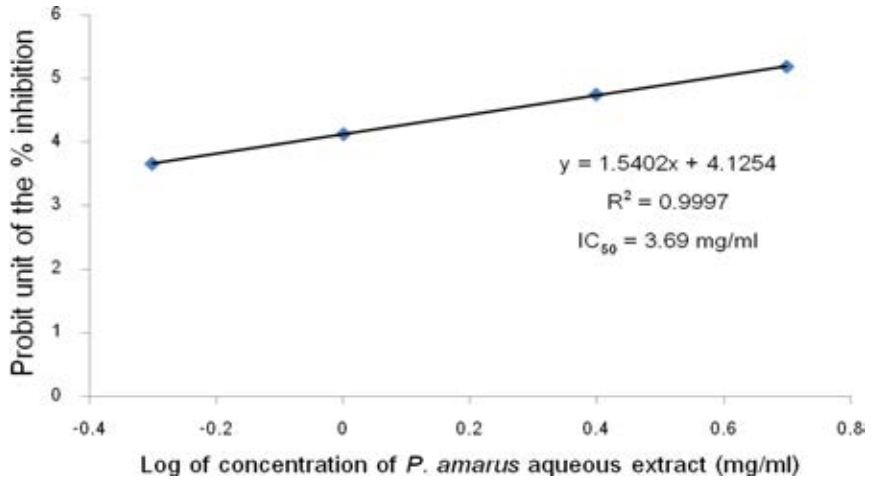


Figure 4.3: Effect of *P. amarus* aqueous extract on HepG2 cell viability as determined by MTT assay after 24, 48 and 72 hours of incubation.

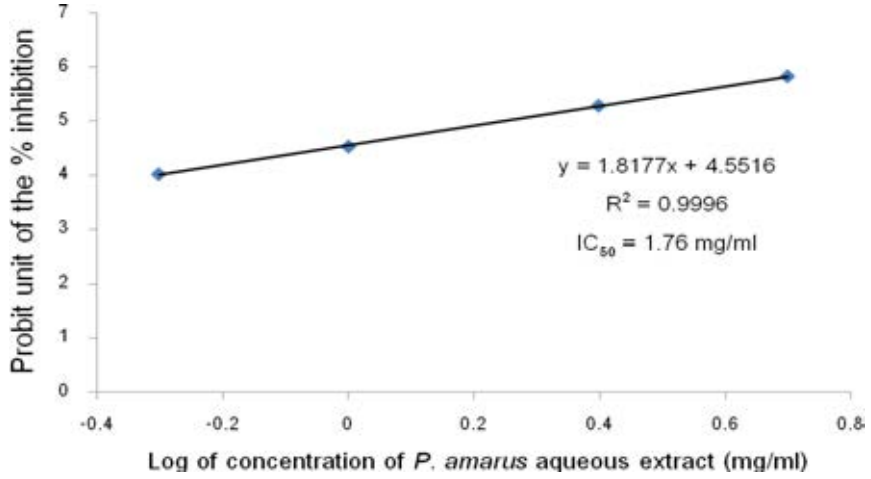
Data are expressed as mean ± S.E.M. (n=3).

***** Significant difference from the control ($P < 0.05$).

(A)



(B)



(C)

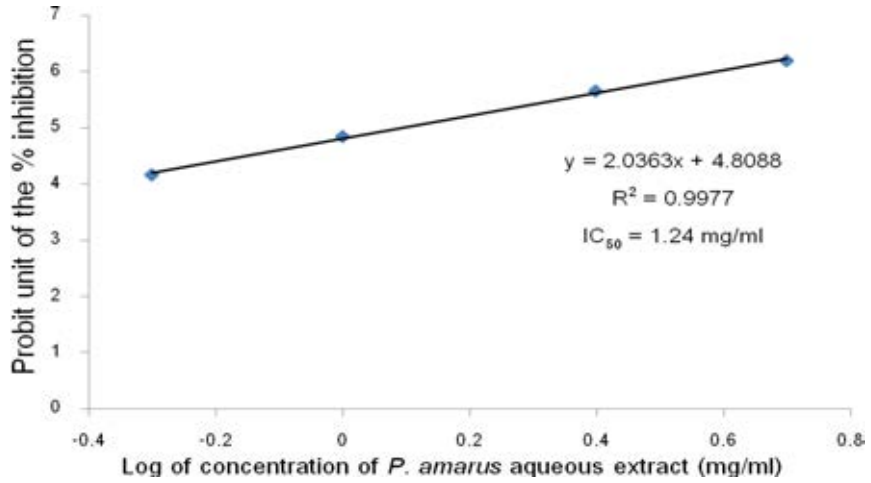


Figure 4.4: Cytotoxicity of *P. amarus* aqueous extract on HepG2 cells after 24 (A), 48 (B) and 72 (C) hours of incubation.

Effects of phyllanthin, hypophyllanthin and *P. amarus* aqueous extract on CYP3A4 activity in HepG2 cells

CYP3A4 activity was determined using P450-Glo™ CYP3A4 Assays containing Luciferin IPA as a selective substrate of CYP3A4. In this study, the known CYP3A4 inducers, rifampicin and dexamethasone, were used as the positive controls. Rifampicin (50 μM) tended to slightly increase CYP3A4 activity while dexamethasone (50 μM) significantly enhanced CYP3A4 activity by 2.33, 2.42 and 2.45 fold following incubation for 24, 48 and 72 hours, respectively (Figure 4.5).

The effects of phyllanthin, hypophyllanthin and *P. amarus* aqueous extract on CYP3A4 activity were investigated in HepG2 cells. It was found that phyllanthin and hypophyllanthin (1, 5 and 25 μM) did not exhibit significant effect on CYP3A4 activity. Nevertheless, hypophyllanthin at concentration of 25 μM tended to decrease CYP3A4 activity (Figure 4.6 and 4.7). *P. amarus* aqueous extract at the concentration of 0.5 mg/ml significantly increased CYP3A4 activity by 1.50, 1.92 and 2.29 fold following incubation for 24, 48 and 72 hours, respectively, while the lower concentrations of the extract did not show significant induction (Figure 4.8).

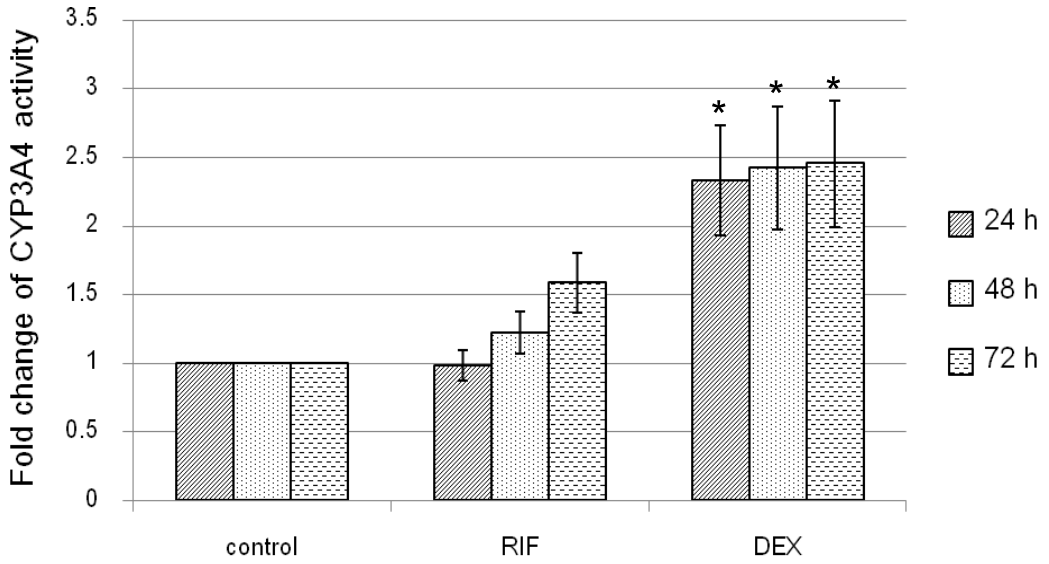


Figure 4.5: Effects of rifampicin and dexamethasone on CYP3A4 activity in HepG2 cells after 24, 48 and 72 hours of incubation (RIF = 50 μM rifampicin, DEX = 50 μM dexamethasone).

Data are expressed as mean ± S.E.M. (n=3).

***** Significant difference from the control ($P < 0.05$).

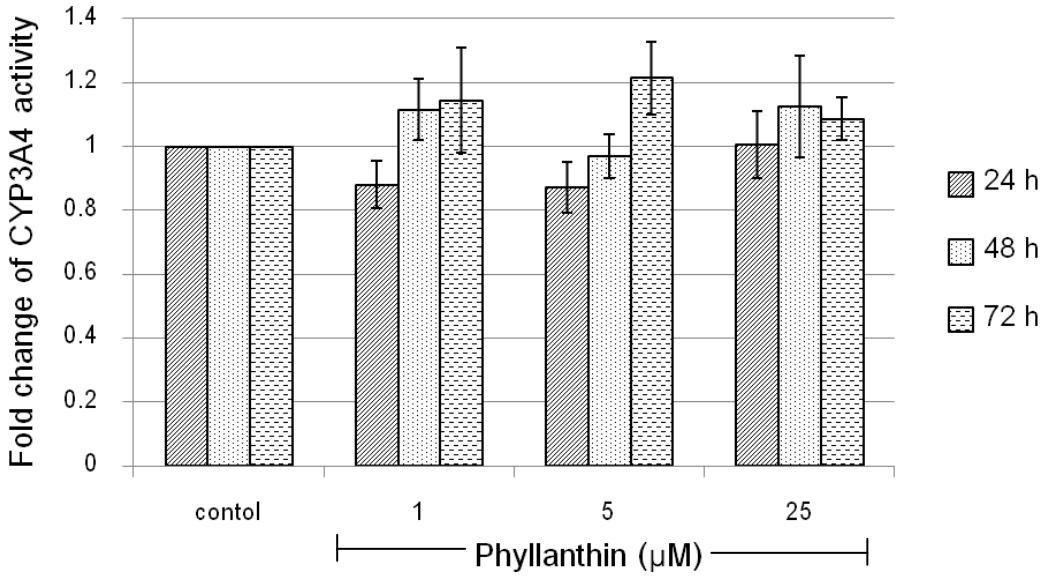


Figure 4.6: Effect of phyllanthin on CYP3A4 activity in HepG2 cells after 24, 48 and 72 hours of incubation.

Data are expressed as mean ± S.E.M. (n=3-4).

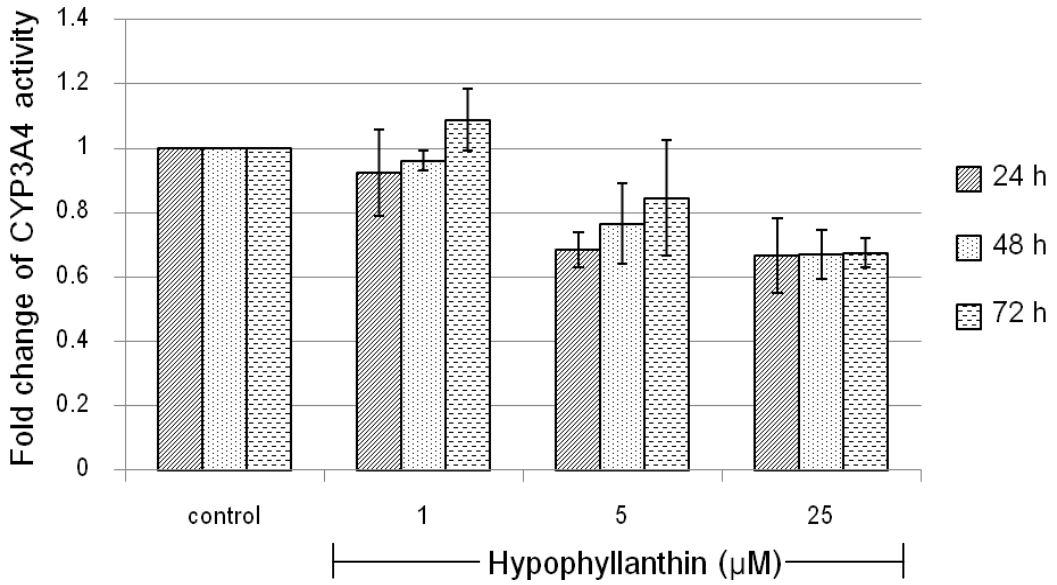


Figure 4.7: Effect of hypophyllanthin on CYP3A4 activity in HepG2 cells after 24, 48 and 72 hours of incubation.

Data are expressed as mean ± S.E.M. (n=4).

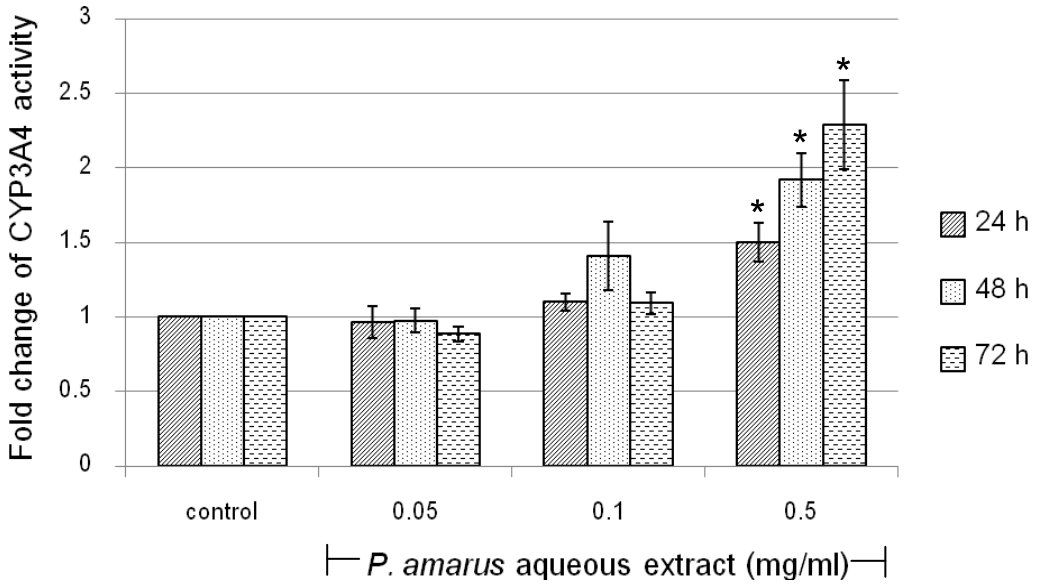


Figure 4.8: Effect of *P. amarus* aqueous extract on CYP3A4 activity in HepG2 cells after 24, 48 and 72 hours of incubation.

Data are expressed as mean ± S.E.M. (n=3).

***** Significant difference from the control ($P < 0.05$).

Effects of phyllanthin, hypophyllanthin and *P. amarus* aqueous extract on CYP3A4 protein expression in HepG2 cells

CYP3A4 protein expression was determined by Western blot analysis. Membrane was incubated with mouse monoclonal CYP3A4 antibody and goat anti-mouse HRP-conjugated antibody, respectively. CYP3A4 protein bands were visualized using enhanced chemiluminescence and were exposed to X-ray film. Bands of CYP3A4 protein were detected at the molecular weight about 50 kDa. The same membrane was stripped using stripping buffer. Then, the membrane was reprobed with mouse monoclonal Beta-actin antibody and goat anti-mouse HRP-conjugated antibody, respectively. Bands of Beta-actin were detected at the molecular weight of 43 kDa (Figure 4.9). Band intensity was quantified using the Image J program. Band intensity of CYP3A4 was normalized to Beta-actin, which was further calculated as the fold of expression compared to the control.

The effects of known CYP3A4 inducers, rifampicin and dexamethasone, on CYP3A4 protein expression were investigated in HepG2 cells. Rifampicin (50 μM) did not show inductive effect on CYP3A4 protein level, whereas dexamethasone (50 μM) significantly increased CYP3A4 protein by 1.67, 1.59 and 1.82 fold following incubation for 24, 48 and 72 hours, respectively (Figure 4.10).

Effects of phyllanthin, hypophyllanthin and *P. amarus* aqueous extract on the expression of CYP3A4 protein were investigated in HepG2 cells. As compared to the control, phyllanthin (1 and 25 μM) and hypophyllanthin (1 and 25 μM) did not exhibit significant effect on CYP3A4 protein level (Figure 4.11 and 4.12). *P. amarus* aqueous extract at concentration of 0.5 mg/ml significantly elevated CYP3A4 protein level by 1.80 fold following 24 hours of incubation. Nevertheless, CYP3A4 protein level declined to normal following incubation with the extract for 48 and 72 hours (Figure 4.13).

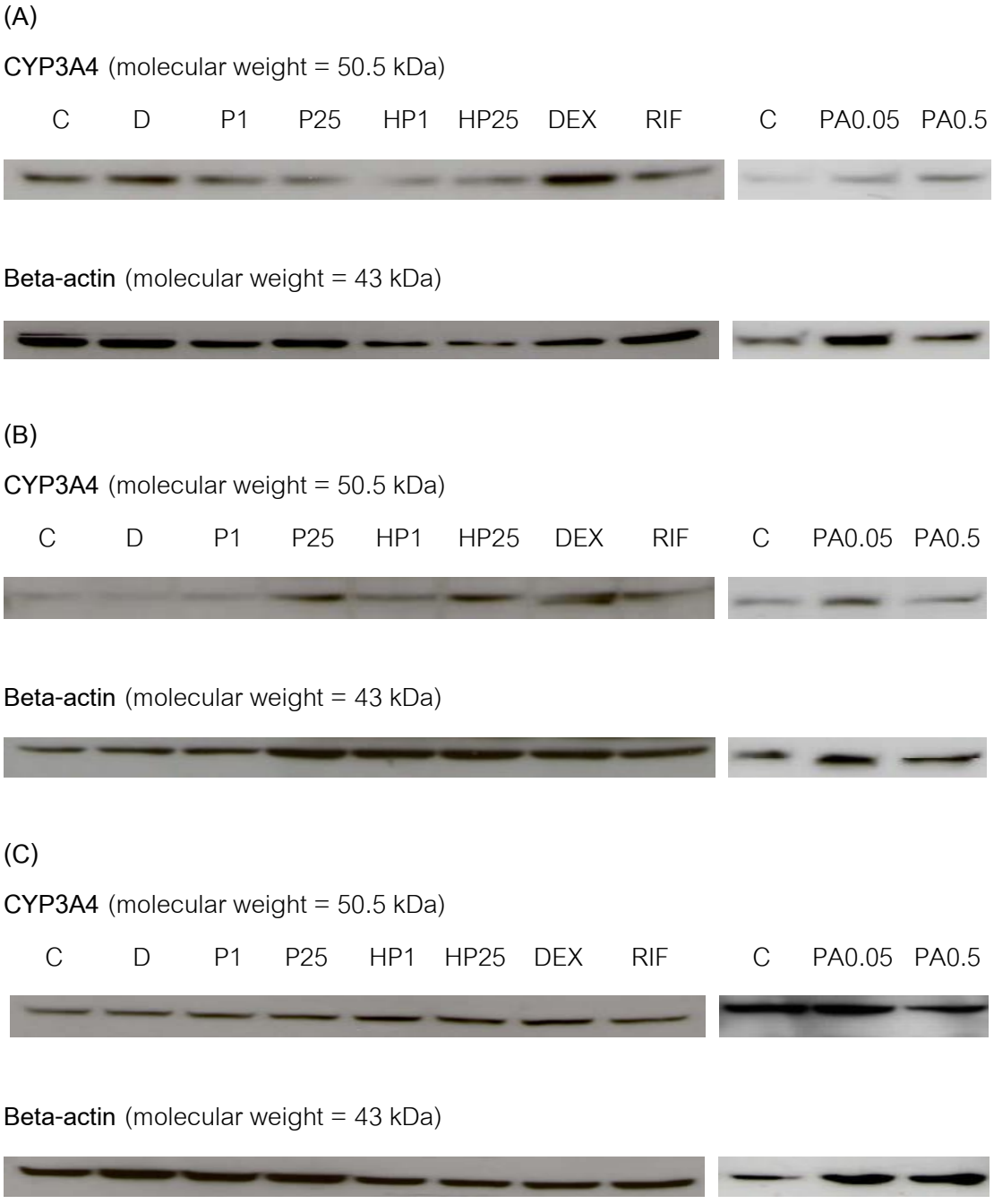


Figure 4.9: Protein bands of CYP3A4 and Beta-actin using Western Blot analysis. Samples were treated with test compounds for 24 (A), 48 (B) and 72 (C) hours (C = control, D = 0.5% DMSO, P1 = 1 μM phyllanthin, P25 = 25 μM phyllanthin, HP1 = 1 μM hypophyllanthin, HP25 = 25 μM hypophyllanthin, DEX = 50 μM dexamethasone, RIF = 50 μM rifampicin, PA0.05 = 0.05 mg/ml *P. amarus* extract, PA0.5 = 0.5 mg/ml *P. amarus* extract).

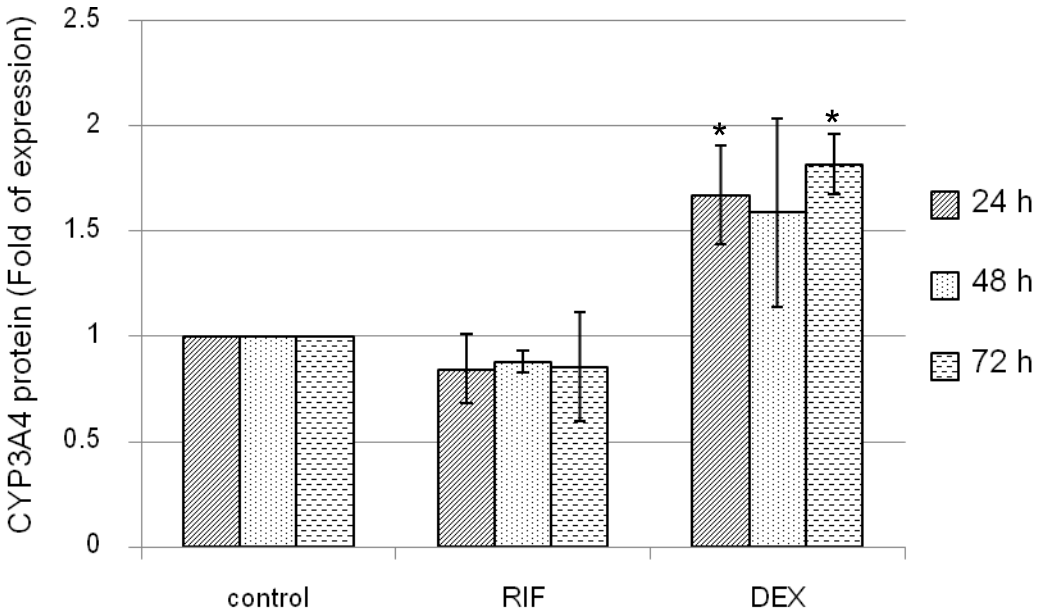


Figure 4.10: Effects of rifampicin and dexamethasone on the expression of CYP3A4 protein in HepG2 cells after 24, 48 and 72 hours of incubation (RIF = 50 μM rifampicin, DEX = 50 μM dexamethasone).

Data are expressed as mean ± S.E.M. (n=3).

***** Significant difference from the control ($P < 0.05$).

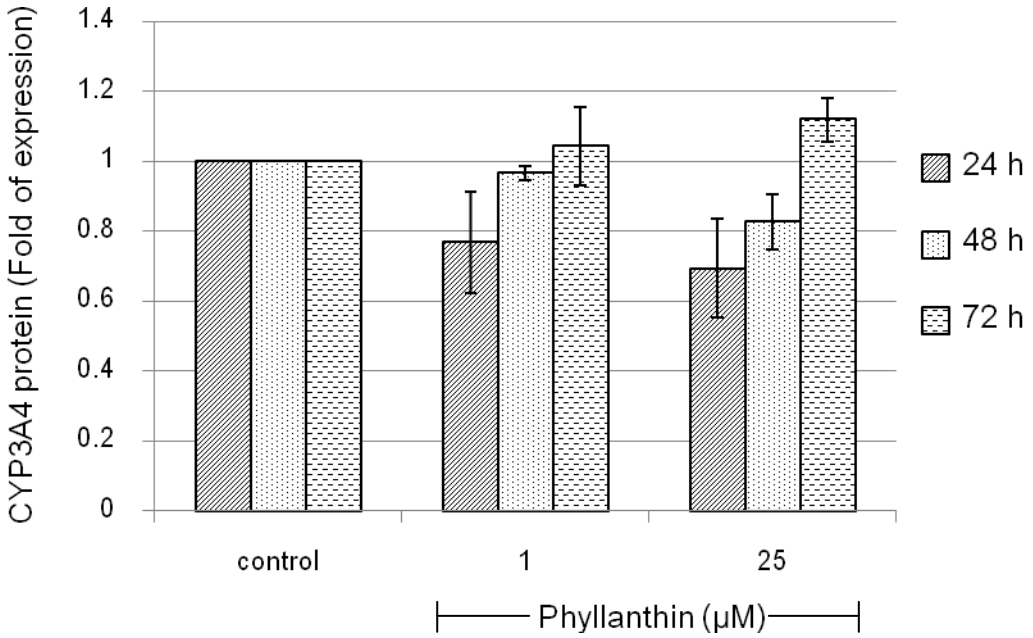


Figure 4.11: Effect of phyllanthin on the expression of CYP3A4 protein in HepG2 cells after 24, 48 and 72 hours of incubation.

Data are expressed as mean ± S.E.M. (n=3).

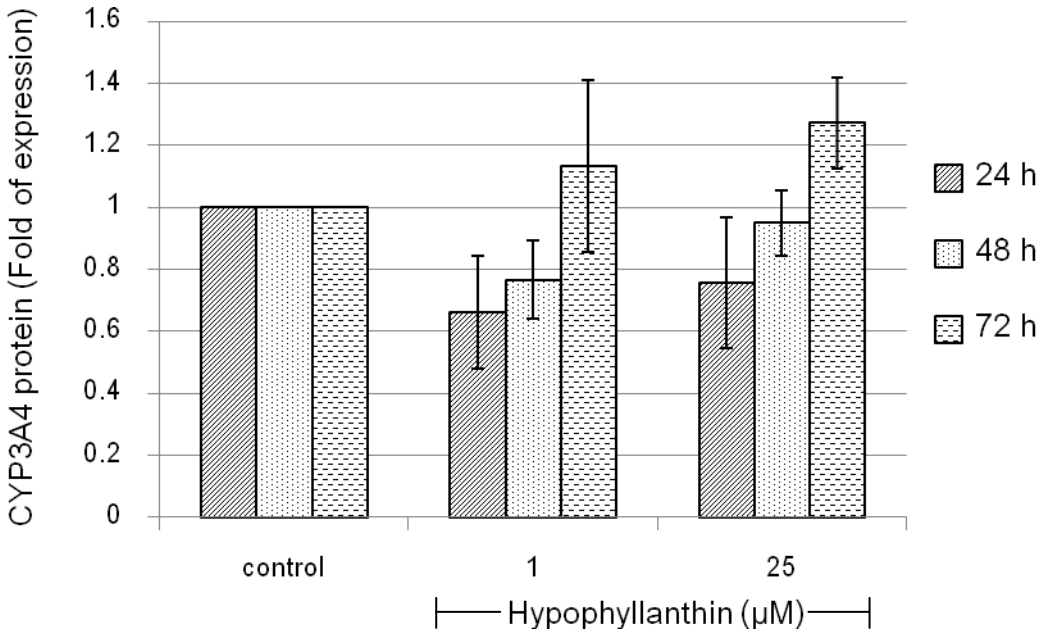


Figure 4.12: Effect of hypophyllanthin on the expression of CYP3A4 protein in HepG2 cells after 24, 48 and 72 hours of incubation.
Data are expressed as mean ± S.E.M. (n=3).

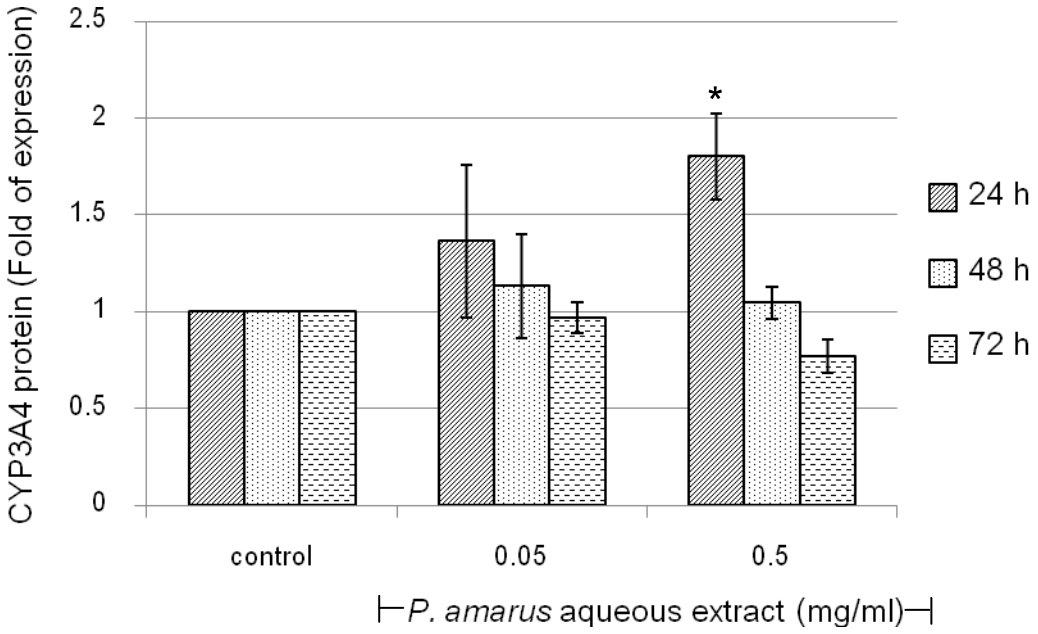


Figure 4.13: Effect of *P. amarus* aqueous extract on the expression of CYP3A4 protein in HepG2 cells after 24, 48 and 72 hours of incubation.

Data are expressed as mean ± S.E.M. (n=3).

***** Significant difference from the control ($P < 0.05$).

Effects of phyllanthin, hypophyllanthin and *P. amarus* aqueous extract on CYP3A4 mRNA expression in HepG2 cells

CYP3A4 mRNA expression was determined by real time RT-PCR. The effects of known CYP3A4 inducers, rifampicin and dexamethasone, on the expression of CYP3A4 mRNA were investigated in HepG2 cells. Rifampicin (50 μM) tended to increase CYP3A4 mRNA after 24 hours of incubation. Dexamethasone (50 μM) significantly increased CYP3A4 mRNA by 2.17 and 2.21 fold following incubation for 12 and 24 hours, respectively (Figure 4.14).

Effects of phyllanthin, hypophyllanthin and *P. amarus* aqueous extract on the expression of CYP3A4 mRNA were investigated in HepG2 cells. Phyllanthin at concentration of 25 μM significantly decreased CYP3A4 mRNA level following incubation for 24 hours (Figure 4.15). Hypophyllanthin (1 and 25 μM) did not affect CYP3A4 mRNA level (Figure 4.16). *P. amarus* aqueous extract at concentration of 0.05 mg/ml significantly increased CYP3A4 mRNA level by 1.75 fold following incubation for 24 hours. The extract at concentration of 0.5 mg/ml also caused a significant inductive effect on CYP3A4 mRNA. The mRNA level was increased by 2.81 and 1.59 fold for the incubation periods of 12 and 24 hours, respectively (Figure 4.17).

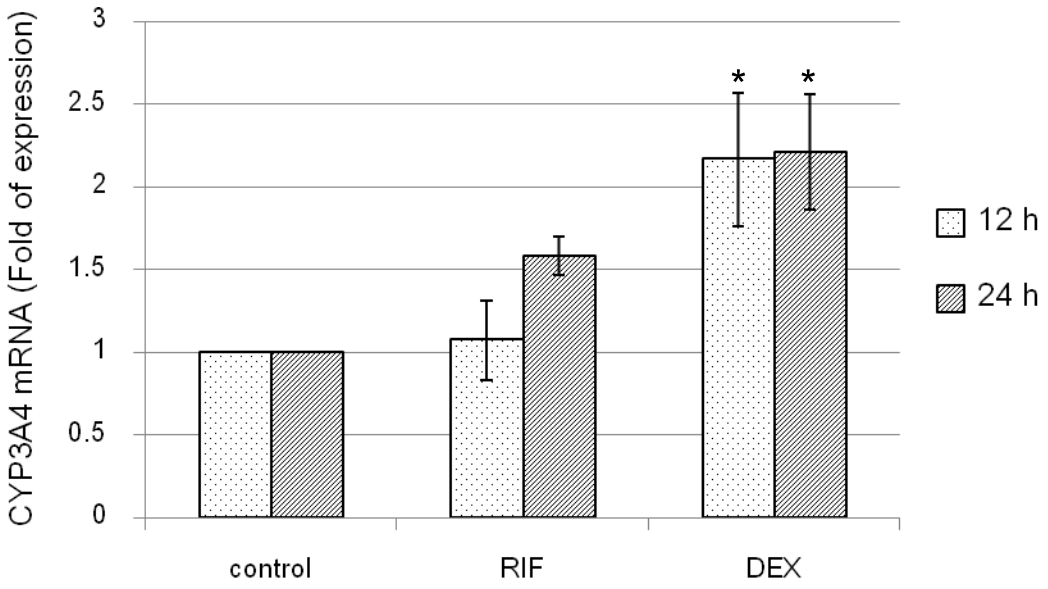


Figure 4.14: Effects of rifampicin and dexamethasone on the expression of CYP3A4 mRNA in HepG2 cells after 12 and 24 hours of incubation (RIF = 50 μM rifampicin, DEX = 50 μM dexamethasone).

Data are expressed as mean ± S.E.M. (n=3).

***** Significant difference from the control ($P < 0.05$).

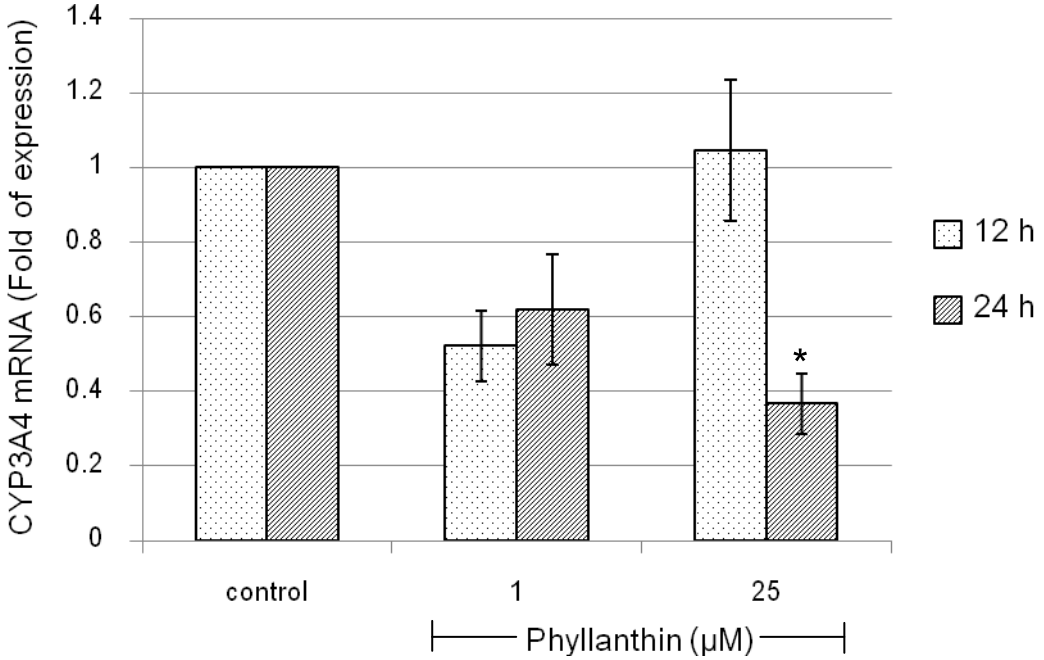


Figure 4.15: Effect of phyllanthin on the expression of CYP3A4 mRNA in HepG2 cells after 12 and 24 hours of incubation.

Data are expressed as mean ± S.E.M. (n=3).

***** Significant difference from the control ($P < 0.05$).

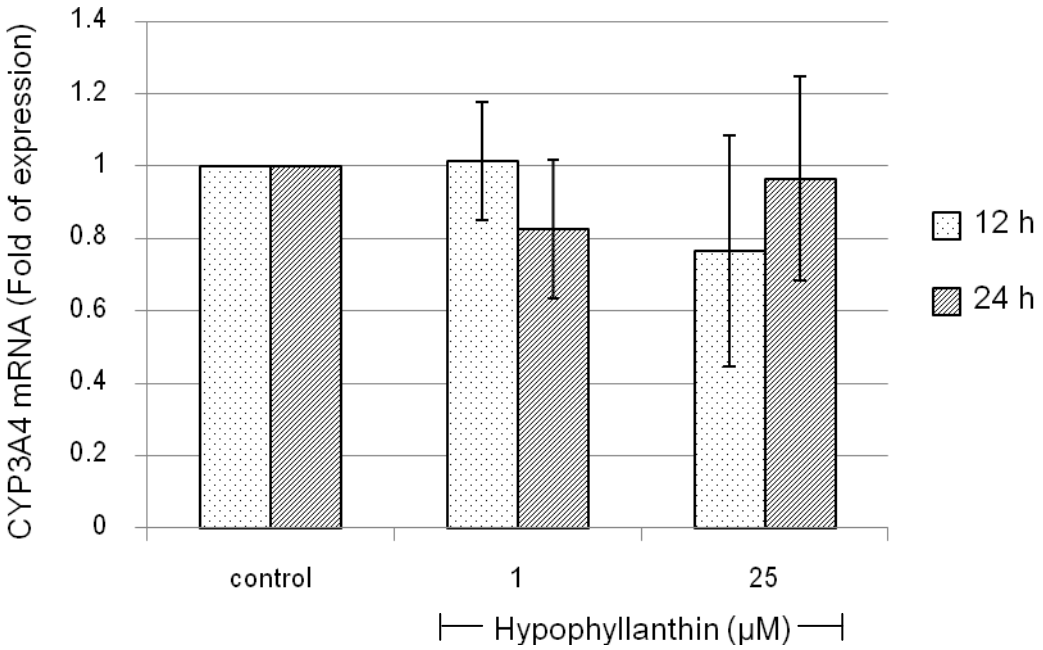


Figure 4.16: Effect of hypophyllanthin on the expression of CYP3A4 mRNA in HepG2 cells after 12 and 24 hours of incubation.
Data are expressed as mean ± S.E.M. (n=3).

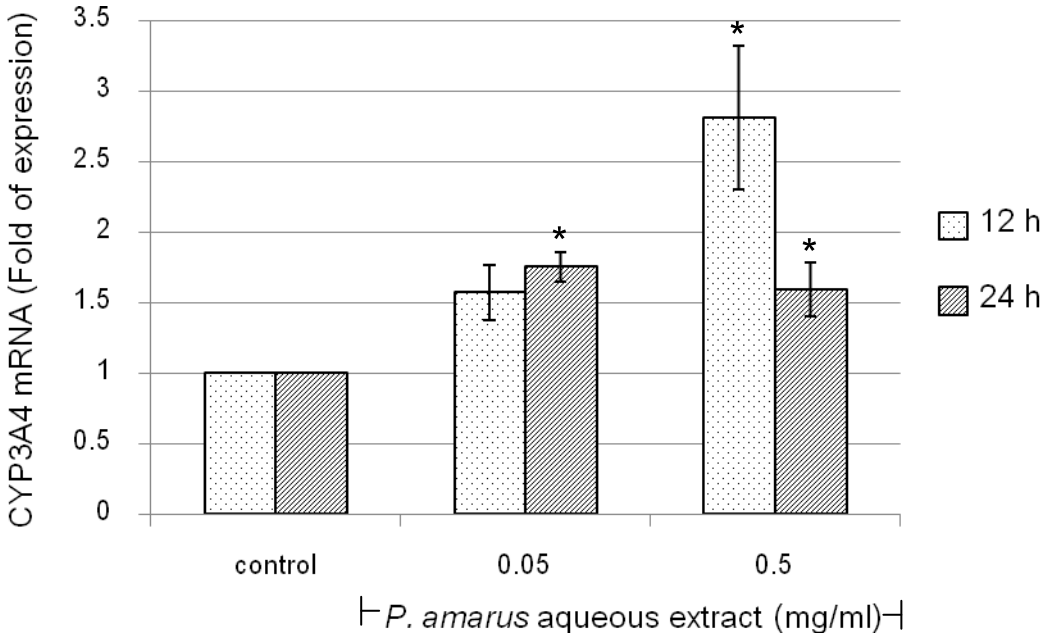


Figure 4.17: Effect of *P. amarus* aqueous extract on the expression of CYP3A4 mRNA in HepG2 cells after 12 and 24 hours of incubation.

Data are expressed as mean ± S.E.M. (n=3).

***** Significant difference from the control ($P < 0.05$).

CHAPTER V

DISCUSSION AND CONCLUSION

Drug interaction remains a major problem in pharmacotherapy since it may cause serious adverse effect as well as therapeutic failure. One of the most common mechanisms of drug interaction is the interfering with CYPs particularly with CYP3A4, which plays crucial roles in drug metabolism (Chen and Raymond, 2006; Pal and Mitra, 2006). Hence, the potentials of drugs and herbs in CYP3A4 inhibition and induction are required to be investigated in the drug development process. *In vitro* studies are suggested to be performed as a screening for these potentials before *in vivo* investigation in humans (US FDA, 2006).

HepG2 cell line is one of the most frequently used human hepatoma cell line in drug biotransformation and cytotoxicity studies (Brandon *et al*., 2003). The expression of enzymes in phase I and II metabolism as well as their induction in HepG2 cells were previously reported (Dawson, Adams, and Wolf, 1985; Grant *et al*., 1988; Doostdar *et al*., 1993; Nakama, Kuroda, and Yamada, 1995; Alexandre *et al*., 1999; Krusekopf, Roots, and Kleeberg, 2003; Usui, Saitoh, and Komada, 2003; Westerink and Schoonen, 2007a; 2007b). However, activity and mRNA expression levels of CYPs in HepG2 cells were found to be lower than in cryopreserved primary human hepatocytes (Westerink and Schoonen, 2007a).

In this study, the effects of known CYP3A4 inducers, rifampicin and dexamethasone, on CYP3A4 activity and expression in HepG2 cells were investigated. The results showed that rifampicin (50 μM) marginally increased CYP3A4 activity and gene expression. In contrast, dexamethasone (50 μM) significantly enhanced CYP3A4 activity which was consistent with the elevated levels of CYP3A4 protein and mRNA. Previous study reported that rifampicin was a strong activator of human PXR, whereas dexamethasone was a relatively weak PXR activator (Quattrochi and Guzelian, 2001). In addition to PXR, dexamethasone is also an activator of GR (Pascussi *et al*., 2000). It was

found that the expression of PXR as well as CAR in most hepatoma cells were relatively low as compared to primary cultures of human hepatocyte (Donato *et al*., 2008). In contrast, GR in hepatocellular carcinoma was found to be higher than in peritumoral liver samples. In addition, GR was constitutively expressed and functional in HepG2 cells (Lui *et al*., 1993). Previous studies reported that dexamethasone caused a synergistic effect with PXR activators on CYP3A4 induction in human hepatocytes. The molecular mechanisms of GR-mediated synergistic effect were reported. Dexamethasone increased both PXR and RXR mRNA expression. GR may also play a role as a coregulator that enhances the binding of PXR to its response elements. In addition, GR may directly regulate CYP3A4 expression by a PXR-independent pathway (Pascussi *et al*., 2000; Quattrochi and Guzelian, 2001; Lin, 2006). Thus, the inductive effect of dexamethasone on CYP3A4 found in this study was possibly occurred via GR activation. An induction of CYP3A4 by rifampicin could not be obviously detected in this study which may be due to the negligible expression of PXR in HepG2 cells.

In this study, effects of phyllanthin, hypophyllanthin and *P. amarus* aqueous extract on CYP3A4 activity and gene expression were examined in HepG2 cells. Phyllanthin and hypophyllanthin at concentrations of 1-25 μM did not produce significant cytotoxicity on HepG2 cells as determined by MTT assay. Phyllanthin at concentrations of 1, 5 and 25 μM did not cause significant change on CYP3A4 activity which was consistent with CYP3A4 protein level. However, phyllanthin at concentration of 25 μM significantly decreased CYP3A4 mRNA following incubation for 24 hours. Hypophyllanthin at concentrations of 1, 5 and 25 μM did not significantly affect CYP3A4 activity. Nevertheless, hypophyllanthin at concentration of 25 μM tended to decrease CYP3A4 activity. Hypophyllanthin at concentrations of 1 and 25 μM did not significantly change the levels of CYP3A4 protein as well as mRNA.

In a study using human liver microsomes, phyllanthin and hypophyllanthin were found to be mechanism-based inhibitors of CYP3A4 (Taesotikul *et al*., 2011). However, phyllanthin and hypophyllanthin did not exhibit obvious inhibitory effect on CYP3A4 activity in HepG2 cells. It is possible that the inhibitory effect was masked by complexity

of this model and low CYP3A4 expression in HepG2 cells. Although CYP3A4 mRNA level decreased following phyllanthin treatment, the level of CYP3A4 protein did not change. Despite statistically significance, this decrease of CYP3A4 mRNA by phyllanthin was negligible. Previously, half-lives of human CYP3A4 protein were reported to be between 1 to 6 days (Kozawa, Honma, and Suzuki, 2009). Thus, it may take time to decrease CYP3A4 protein to the significant level.

Previously, the inhibitory effect of *P. amarus* extracts on CYP3A4 activity was reported in many studies using liver microsomes of rat and human as well as recombinant human CYP3A4 (Hari Kumar and Kuttan, 2006; Appiah-Opong *et al*., 2008; Taesotikul *et al*., 2011). Recent study reported the effect of *P. amarus* ethanolic extract on CYP3A in rats. The extract (800 mg/kg, p.o., single dose) exhibited inhibitory effect on intestinal CYP3A resulting in increasing bioavailability of oral midazolam. Repeated administration of the extract (200 and 800 mg/kg/day, p.o., for 15 days) increased hepatic CYP3A and CYP2B1/2 activity as well as protein expression (Taesotikul *et al*., 2012). These findings support that *P. amarus* extracts possess both inhibitory and inductive potential on CYP3A4.

The concentration of *P. amarus* aqueous extract (0.05-0.5 mg/ml) used in this study did not significantly affect cell viability as determined by MTT assay. It was found that the extract at concentration of 0.5 mg/ml significantly enhanced CYP3A4 activity in a time-dependent manner following incubation for 24-72 hours. In agreement with CYP3A4 activity, the levels of CYP3A4 protein at 24 hours as well as mRNA at 12 and 24 hours were significantly increased. However, CYP3A4 protein level declined to normal following incubation with the extract for 48 and 72 hours. *P. amarus* aqueous extract increased CYP3A4 activity, protein enzyme and gene expression which seemed to be similar to dexamethasone. Inductive effect of the extract via pathway similar to rifampicin could not be excluded because the limitation of this model of HepG2 cells which possess low level of PXR and CAR. The increase of CYP3A4 mRNA and protein by the extract occurred initially and then declined while the activity increased continuously up to 72 hours. The inductive effect of the extract found in this study seemed to be an acute

response which was occurred transiently. CYP3A4 activity continued to increase up to 72 hours while the protein and mRNA only increased initially at 24 and 12 hours, respectively. The extract may increase the activity of other enzymes involved in CYP3A4 function. These enzymes, including NADPH-cytochrome P450 reductase and cytochrome b_5, were reported to be inducible by some xenobiotics and medicines. For example, NADPH-cytochrome P450 reductase was found to be induced by 2-acetylaminofluorene, dieldrin, isosafrole, phenobarbitone, polychlorinated biphenyls and *trans*-stilbene oxide. Cytochrome b_5 was induced by 2-acetylaminofluorene, butylated hydroxytoluene and griseofulvin (Gibson and Skett, 2001).

Previously, several phytochemical compounds have been reported to possess CYP3A4 induction potential. The results from gene reporter assay studies demonstrated that some polyphenolic compounds have capability to activate PXR, CAR, GR and CYP3A4 promoter (Jacobs *et al*., 2005; Kluth *et al*., 2007; Nishizaki *et al*., 2009; Dong *et al*., 2010; Yao *et al*., 2010). Among these reported compounds, quercetin, a flavonoid which is also presented in *P. amarus*, was found to enhance CYP3A4 promoter activity but did not activate PXR (Kluth *et al*., 2007). Since rifampicin, an agonist of PXR, exhibited relatively low inductive effect on CYP3A4 expression in this study, it is possible that effect of the extract may be due to the alternative mechanisms. Some phytochemical compounds may possess inductive effect on CYP3A4 via other transcription factors such as GR. In addition to direct ligand binding, PXR can also be modulated by cell signaling pathways. Previous reports described the possible mechanisms of various kinases including protein kinase A (PKA), protein kinase C (PKC), cyclin-dependent kinases (Cdks) and 70 kDa form of ribosomal protein S6 kinase (p70 S6K) in the repression of PXR-mediated CYP3A4 expression (Pondugula *et al*., 2009; Dong *et al*., 2010). Interestingly, Cdk2, which plays a role in driving cell cycle through each phase, was found to negatively regulate PXR in HepG2 cells. This finding may explain the decrease in CYP3A4 expression in proliferating hepatocytes (Lin *et al*., 2008). Some flavonoids were reported to activate PXR by inhibition of Cdks in HepG2 cells (Dong *et al*., 2010). Moreover, a number of hydrolysable tannins isolated from *P.*

amarus were found to be potent inhibitors of protein kinases (Polya *et al*., 1995). These previous findings may partly explain the increase of CYP3A4 activity and gene expression by *P. amarus* aqueous extract observed in this study.

Although the observed concentration that significantly increased CYP3A4 activity was rather high, the extract at concentration of 0.05 mg/ml was found to significantly increase CYP3A4 mRNA level. Therefore, the extract at lower concentrations might reveal its inductive effect when it is applied chronically. The yield of *P. amarus* aqueous extract used in this study was 17.42% (w/w) from dried aerial part (Pramyothin *et al*., 2007). It may be calculated the plasma concentration of the extract by assuming that the extract is completely absorbed and volume distribution of the extract in circulatory system is approximately 4 L. Thus, it has to take at least 1200 mg of *P. amarus* dried plant to reach the plasma concentration of 0.05 mg/ml.

Normally, induction of CYP3A4 may cause an increased risk of toxicity, mutagenesis and carcinogenesis from some toxins that are bioactivated by CYP3A4 such as aflatoxin $B_{1,}$ benzo[*a*]pyrene, etc. However, it was found that *P. amarus* ethanolic extract exhibited protective effect against aflatoxin B_1-induced liver injury in mice (Naaz, Javed, and Abdin, 2007). In addition, *P. amarus* methanolic extract inhibited benzo[*a*]pyrene-induced urinary mutagenicity in rats (Raphael *et al*., 2002). These results are in part explained by the inhibitory effect of *P. amarus* extract on CYP3A4 which are reported earlier by many studies. The inductive effect of the extract found in this study is only an acute response from CYP3A4 inhibition prior affected by the extract. *P. amarus* extracts were also found to improve several enzymatic and non-enzymatic antioxidant defense mechanisms, which may lessen these deleterious consequences.

In conclusion, this study demonstrated that phyllanthin and hypophyllanthin did not significantly affect CYP3A4 activity. Both compounds also did not affect CYP3A4 protein expression and mRNA expression although negligibly decrease of mRNA expression was found by phyllanthin. *P. amarus* aqueous extract increased CYP3A4 activity and gene expression which seemed to be similar to dexamethasone. The effects

of these compounds on CYP3A4 provide preliminarily information of herb-drug interaction potential, thus further study *in vivo* is suggested.

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APPENDIX

Determination of cell number using CellTiter-Glo® Luminescent Cell Viability Assay

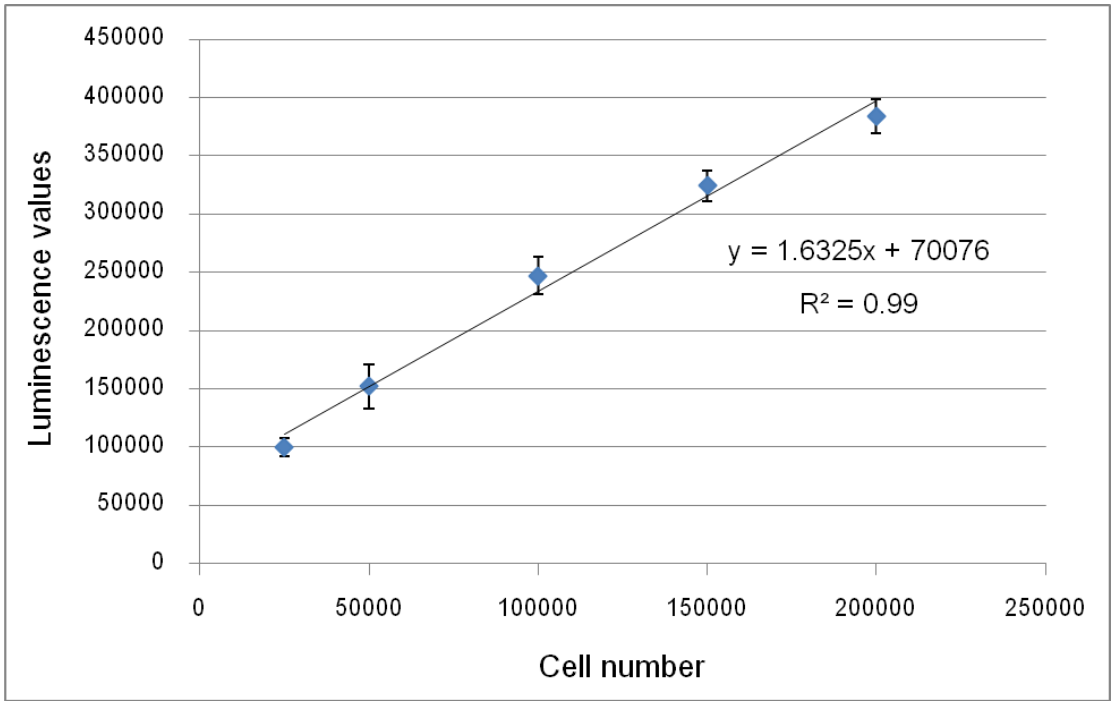

Figure A1: Correlation between luminescence values and cell number

Melt curve analysis

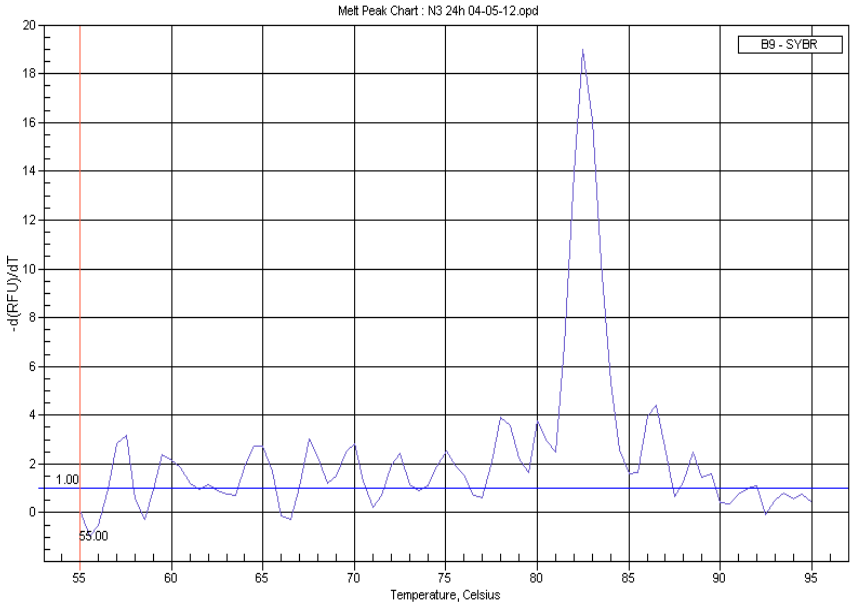

Figure A2: Melt curve analysis of CYP3A4 amplification

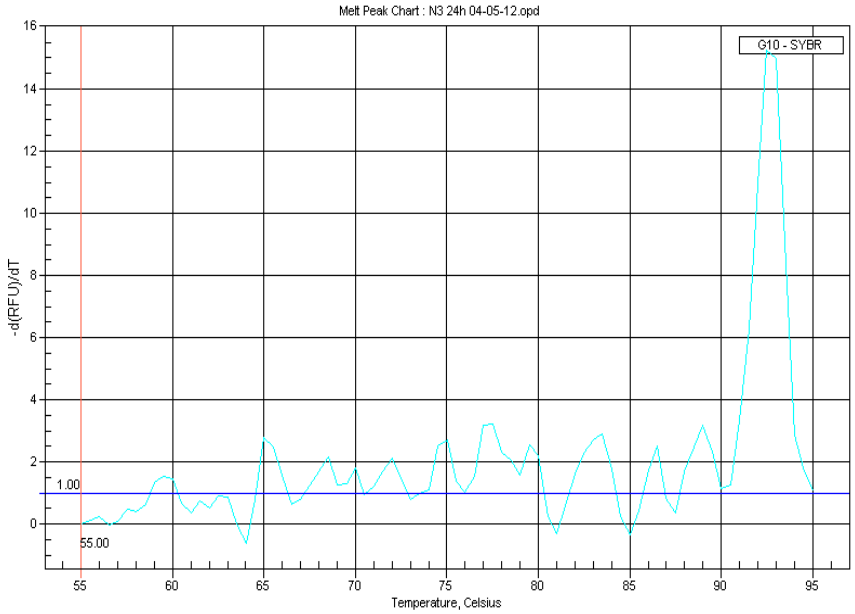

Figure A3: Melt curve analysis of Beta-actin amplification

Agarose gel electrophoresis

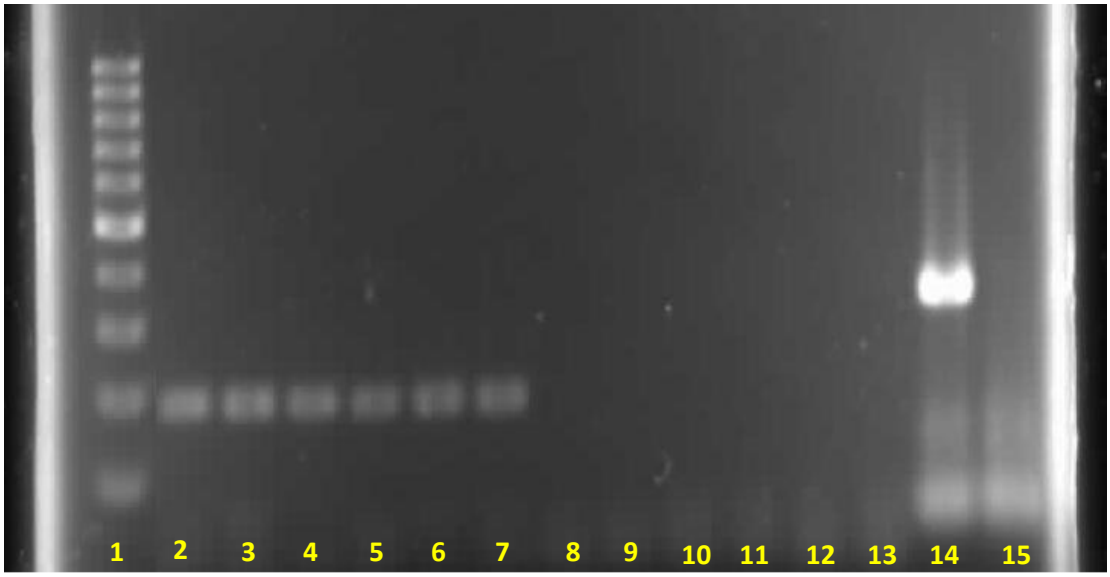

Figure A4: Agarose gel electrophoresis of PCR product (lane 1 = 100 bp DNA ladder, lanes 2-7 = PCR product of CYP3A4, lanes 8-13 = negative control of CYP3A4, lane 14 = PCR product of Beta-actin, lane 15 = negative control of Beta-actin)

VITAE

Mr. Kridsada Anuntawuttikul was born in September 29, 1985 in Suratthani, Thailand. He graduated with a Bachelor of Science in Pharmacy in 2009 from the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.