ผลนอกกายของสารสกัดจากผลมะตูมและอิมเพอราโตรินต่อเอนไซม์ไซโตโครมพี 450

นางสาวดวงใจ ปัญญาพจน์ศักดิ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
สาขาวิชาอาหารเคมีและโภชนศาสตร์ทางการแพทย์ ภาควิชาอาหารและเภสัชเคมี
คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2551

IN VITRO EFFECTS OF *AEGLE MARMELOS* FRUIT EXTRACT AND IMPERATORIN ON CYTOCHROME P450

Miss Duangjai Panyapojsak

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Pharmacy Program in Food Chemistry and Medical Nutrition
Department of Food and Pharmaceutical Chemistry
Faculty of Pharmaceutical Sciences
Chulalongkorn University
Academic Year 2008

Thesis Title	*IN VITRO* EFFECTS OF *AEGLE MARMELOS* FRUIT EXTRACT AND IMPERATORIN ON CYTOCHROME P450
By	Miss Duangjai Panyapojsak
Field of Study	Food Chemistry and Medical Nutrition
Advisor	Assistant Professor Warangkana Warisnoicharoen, Ph. D.
Co-Advisor	Associate Professor Pol. Lt. Col. Somsong Lawanprasert, Ph. D.

Accepted by the Faculty of Pharmaceutical Sciences, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

.....Dean of the Faculty of Pharmaceutical Sciences

(Associate Professor Pornpen Pramyothin, Ph. D.)

THESIS COMMITTEE

.....Chairman

(Assistant Professor Linna Tongyonk, D. Sc.)

.....Advisor

(Assistant Professor Warangkana Warisnoicharoen, Ph. D.)

.....Co-Advisor

(Associate Professor Pol. Lt. Col. Somsong Lawanprasert, Ph. D.)

.....Examiner

(Assistant Professor Suyanee Pongthananikorn, Dr. P. H.)

.....Examiner

(Associate Professor Nuansri Niwattisaiwong, M. Sc.)

ดวงใจ ปัญญาพจน์ศักดิ์ : ผลนอกกายของสารสกัดจากผลมะตูมและอิมเพอราโตรินต่อเอนไซม์ไซโตโครมพี 450. (*IN VITRO* EFFECTS OF *AEGLE MARMELOS* FRUIT EXTRACT AND IMPERATORIN ON CYTOCHROME P450) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ.ดร.วรางคณา วารีสน้อยเจริญ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : รศ.ดร.พ.ต.ท.หญิง สมทรง ลาวัณย์ประเสริฐ, 155 หน้า.

มะตูม มีชื่อวิทยาศาสตร์ว่า *Aegle marmelos* เป็นสมุนไพรที่มีฤทธิ์ทางยา ผลห่ามของมะตูมใช้เป็นยาแผนโบราณในการรักษาอาการท้องเสียและบิด ผลของมะตูมยังมีการรายงานถึงสารสำคัญหลายชนิดที่มีฤทธิ์ทางเภสัชวิทยา โดยมีรายงานถึงสารสำคัญชนิดหนึ่งที่พบในผลมะตูมคือ อิมเพอราโตริน โดยอิมเพอราโตรินมีฤทธิ์ทางยาเช่น ฤทธิ์ในการต้านเชื้อแบคทีเรีย, ป้องกันการแข็งตัวของเกล็ดเลือด และฤทธิ์ในการยับยั้งเอนไซม์ไซโตโครมพี 450 ในการศึกษาครั้งนี้ ผลของมะตูมถูกนำมาสกัดด้วยตัวทำละลายสามชนิดได้แก่ เฮกเซน เอทธานอล และ น้ำ จากนั้นทำสารสกัดให้แห้งด้วยการให้ความร้อนพร้อมกับลดความดัน สารสกัดที่ได้ถูกนำมาเตรียมเป็นสารละลายก่อนนำไปทดสอบผลการยับยั้งการทำงานของเอนไซม์ไซโตโครมพี 450 ได้แก่ CYP1A2 CYP2C9 CYP2C19 และ CYP3A4 โดยการวัดค่าการเรืองแสงฟลูโอเรสเซนของผลิตภัณฑ์ที่เกิดจากการเปลี่ยนสารตั้งต้นที่มีความจำเพาะเจาะจงต่อเอนไซม์ ทำการทดลองโดยใช้ 96-well plate นอกจากนั้นการศึกษาครั้งนี้ได้ทำการหาปริมาณของอิมเพอราโตรินโดยใช้ RP-HPLC ในสารสกัดด้วย จากผลการทดลองร้อยละของปริมาณสารสกัดของผลมะตูมด้วยเฮกเซนเท่ากับ 0.56 สารสกัดด้วยเอทธานอลเท่ากับ 7.69 และสารสกัดด้วยน้ำเท่ากับ 21.54 โดยน้ำหนัก ค่าเฉลี่ยร้อยละโดยน้ำหนักของปริมาณอิมเพอราโตรินที่พบในสารสกัดจากเฮกเซนและเอทธานอลคือ 5.78 และ 0.12 ตามลำดับ หลังจากนั้นทำการหาค่าความเข้มข้นที่ยับยั้งเอนไซม์ 50% (IC_{50}) ของอิมเพอราโตรินและสารสกัดของผลมะตูม จากผลการทดลองพบว่าอิมเพอราโตรินสามารถยับยั้ง CYP1A2 (IC_{50} เท่ากับ 0.42±0.02 µM), CYP2C9 (IC_{50} เท่ากับ 3.59±0.02 µM), CYP2C19 (IC_{50} เท่ากับ 2.15±0.02 µM) และ CYP3A4 (IC_{50} เท่ากับ 1.63±0.02 µM) ได้ โดยอิมเพอราโตรินสามารถยับยั้งเอนไซม์ไซโตโครม พี450 ได้ดีโดยมีค่า IC_{50} น้อยกว่า 10 µM ในขณะที่จากผลการทดลองพบว่าสารสกัดผลมะตูมด้วยเฮกเซนแสดงผลการยับยั้งเอนไซม์ (IC_{50}) CYP1A2 เท่ากับ 0.73±0.03 µg/ml CYP2C9 เท่ากับ 3.24±0.03 µg/ml CYP2C19 เท่ากับ 4.04±0.02 µg/ml และ CYP3A4 เท่ากับ 1.65±0.05 µg/ml นอกจากนั้นสารสกัดผลมะตูมด้วยเฮกเซนสามารถยับยั้งเอนไซม์ได้ไม่แตกต่างอย่างมีนัยสำคัญเมื่อเทียบกับอิมเพอราโตริน สำหรับสารสกัดผลมะตูมด้วยเอทธานอลสามารถยับยั้งเอนไซม์ได้น้อยกว่าโดยมีค่า IC_{50} ของ CYP1A2 เท่ากับ 39.40±0.02 µg/ml CYP2C9 197.60±0.04 µg/ml CYP2C19 เท่ากับ 107.90±0.02 µg/ml และ CYP3A4 86.59±0.05 µg/ml และสารสกัดผลมะตูมด้วยน้ำสามารถยับยั้งเอนไซม์ได้น้อยที่สุด โดยค่า IC_{50} ของ CYP1A2 เท่ากับ 352.30±0.04 µg/ml CYP2C9 เท่ากับ 965.00±0.02 µg/ml CYP2C19 เท่ากับ 414.00±0.20 µg/ml และ CYP3A4 เท่ากับ 842.40±0.04 µg/ml การศึกษาในครั้งนี้แสดงข้อมูลผลการยับยั้งเอนไซม์ในกระบวนการเมแทบอลิซึมยาในเฟส 1 ของสารสกัดจากผลมะตูมและอิมเพอราโตริน ข้อมูลเหล่านี้แสดงให้เห็นว่าสารสกัดผลมะตูมด้วยเฮกเซนและอิมเพอราโตรินมีศักยภาพในการยับยั้งการเมแทบอลิซึมยาที่ผ่านเอนไซม์ไซโตโครมพี 450 บางชนิดได้ อย่างไรก็ตามเพื่อให้การแปลข้อมูลของผลการทดลองในครั้งถูกต้องจำเป็นที่จะต้องมีการศึกษาเพิ่มเติมต่อไป

ภาควิชา.....อาหารและเภสัชเคมี..... ลายมือชื่อนิสิต.....ดวงใจ ปัญญาพจน์ศักดิ์.....

สาขาวิชา.....อาหารเคมีและโภชนศาสตร์ทางการแพทย์ ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ปีการศึกษา...2551..... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

4876564033 : MAJOR FOOD AND PHARMACEUTICAL CHEMISTRY
KEYWORDS : CYTOCHORME P450 / FOOD-DRUG INTERACTION / *AEGLE MARMELOS* / *IN VITRO* CYP ASSAYS / CYP1A2 / CYP2C9 / CYP2C19 / CYP3A4

DUANGJAI PANYAPOJSAK: *IN VITRO* EFFECTS OF *AEGLE MARMELOS* FRUIT EXTRACT AND IMPERATORIN ON CYTOCHROME P450. ADVISOR: ASST. PROF. WARANGKANA WARISNOICHAROEN, Ph.D., CO-ADVISOR : ASSOC. PROF. POL. LT. COL. SOMSONG LAWANPRASERT, Ph.D., 155 pp.

Aegle marmelos or Matoom is a medicinal plant. Unripe fruit had been traditionally used as an antidiarrhoea and antidysentery. Several chemical constituents of *A. marmelos* fruit have also been reported for its pharmaceutical activities. The fruit of *A. marmelos* was reported to be the source of imperatorin and other bioactive substances. Imperatorin indicated some pharmacological activities such as antibacterial, anticoagulant, antiplatelet aggregation. It can have an effect on drug or xenobiotic metabolism by being cytochrome P450 or CYP inhibition. In this study, *A. marmelos* fruits were extracted with hexane, ethanol and water then they were dried and prepared for inhibition test on human CYP isoforms (CYP1A2, CYP2C9, CYP2C19 and CYP3A4). Using the conversion of specific probe substrates, the inhibitory effect could be measured fluorometrically in a 96-well plate format. Moreover, the fruit extracts were analyzed for imperatorin by using RP-HPLC. *A. marmelos* dried fruit contained 0.56% w/w of n-hexane extract, 7.69% w/w of ethanolic extract and 21.54% w/w of aqueous extract. Hexane extract of *A. marmelos* showed the highest content of imperatorin. Total yields (%) of imperatorin in hexane and ethanolic extracts were 5.78% w/w and 0.12% w/w, respectively, based on weight of dried extracts. For each test substance, the IC_{50} (the concentration required to inhibit metabolism of CYP activities by 50%) was estimated. Imperatorin showed potent inhibitory effect on human CYP1A2 (IC_{50} of 0.42±0.02 μM), CYP2C9 (IC_{50} of 3.59±0.02 μM), CYP2C19 (IC_{50} of 2.15±0.02 μM) and CYP3A4 (IC_{50} of 1.63±0.02 μM) at concentrations of less than 10 μM. Whereas hexane extracts of *A. marmelos* did not showed significantly different inhibition when compared with imperatorin. Hexane extract showed the highest inhibitory effect for CYP1A2 (IC_{50} of 0.73±0.03 μg/ml), CYP2C9 (IC_{50} of 3.24±0.03 μg/ml), CYP2C19 (IC_{50} of 4.04±0.02 μg/ml) and CYP3A4 (IC_{50} of 1.65±0.05 μg/ml). While ethanolic extract showed lower inhibitory effect for CYP1A2 (IC_{50} of 39.40±0.02 μg/ml), CYP2C9 (IC_{50} of 197.60±0.04 μg/ml), CYP2C19 (IC_{50} of 107.90±0.02 μg/ml) and CYP3A4 (IC_{50} of 86.59±0.05 μg/ml) and aqueous extract showed the least inhibitory effect, CYP1A2 (IC_{50} of 352.30±0.04 μg/ml), CYP2C9 (IC_{50} of 965.00±0.02 μg/ml), CYP2C19 (IC_{50} of 414.00±0.20 μg/ml) and CYP3A4 (IC_{50} of 842.40±0.04 μg/ml). This study provided the information of effects of imperatorin and *A. marmelos* extracts on phase I drug-metabolizing enzymes. These data suggest that imperatorin and *A. marmelos* fruit extracted with hexane potentially inhibit the metabolism of co-administered medication whose primary route of elimination is via those CYPs, thus interpretation of these data from *in vitro* to human should be further studied.

Department :.. Food and Pharmaceutical Chemistry..... Student's Signature :..Duangjai...
Field of Study :.. Food Chemistry and Medical Nutrition.... Advisor's Signature :.....
Academic Year :..2008.....Co-Advisor's Signature :.....

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and deep appreciation to my advisor, Assistant Professor Dr. Warangkana Warisnoicharoen for her valuable advice, guidance, and encouragement throughout my graduate study. Her endless support and caring that made me feel like my second home.

I am very grateful to my co-advisor Associate Professor Pol. Lt. Col. Dr. Somsong Lawanprasert for her kindness, comments, and support. I am deeply honored for her keen interest, dedication, and for giving me the privilege of using the laboratory.

I am truly thankful to Associate Professor Nuansri Niwattisaiwong for her valuable of time to advice, support for analysis of enzyme and HPLC assay. Her endless advice and encourage criticism has been precious during these years.

I am also appreciated to Assistant Professor Dr. Pornchai Rojsitthisak for providing his advice to me during my first step as a chemical analyst. I am very much obliged and honored to the members of the thesis committee, Assistant Professor Dr. Linna Tongyonk, Assistant Professor Dr. Suyanee Pongthananikorn for their supportive attitude and constructive criticisms over my thesis.

My colleagues and friends in the Faculty of Pharmaceutical Sciences, Chulalongkorn University who always deserved warm thanks, for giving hand in solving problems.

I am really thankful to the Faculty of Graduate Studies, Chulalongkorn University for the supporting scholarship which enabled me to undertake this study.

Finally, my special gratitude is expressed to my beloved family for their love, care, support and much encouragement throughout the period of my graduate study.

CONTENTS

CHAPTER Page

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

LIST OF ABBREVIATIONS

°C	degree Celsius
µg/ml	microgram per milliliter
µl	microlitre
µm	micrometer or micron
µmol	micromole
µM	micromolar
BOMCC	7-benzyloxymethyloxy-3-cyanocoumarin
CHC	3-cyano-7-hydroxycoumarin
cm	centimeter
CV	coefficient of variation
CYP	cytochrome P450
DI	de-ionized
DMSO	dimethylsulfoxide
DPPH	1, 1-diphenyl-2-picrylhydrazyl
ED_{50}	effective concentration at 50% inhibition
e.g.	example gratia
EOMCC	ethyloxymethyloxy-3-cyanocoumarin
et al.	*et alii* (and others)
etc.	*et cetera* (and other similar things)
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
g	gram
G-6-P	glucose-6-phosphate
G-6-PD	glucose-6-phosphate dehydrogenase
GSH	glutathione reduced from
h	hour
HPLC	high performance liquid chromatography
IC_{50}	median (50%) inhibitory concentration
ICH	International Conference on Harmonization of Technical Requirement

i.d.	internal diameter
i.e.	*id est* (that is)
i.p.	intraperitonium
kg	kilogram
L	litre
M	molar
$mAU \cdot s^{-1}$	milli-absorbance unit per second
mg	milligram
mg/kg	milligram per kilogram body weight
mg/ml	milligram per milliliter
min	minute
ml	milliliter
ml/min	milliliter per minute
mm	millimetre
mM	millimolar
mmole	millimole
MW	molecular weight
ND	not determined
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
ng	nanogram
NO	nitric oxide
PAH	polycyclic aromatic hydrocarbon
pH	potential of hydrogen
photo-DAD	photodiode array detector
p.o.	*per os* (by mouth)
PROD	pentoxyresorufin *o*-dealkylation
r	correlation coefficient
r^2	the least squares regression
R^2	coefficient of determination
RFU	relative fluorescence unit or fluorescent intensity
RP-HPLC	reversed-phase high performance liquid chromatography

RSD	relative standard deviation
SD	standard deviation
SEM	standard error of the mean
Tris	tris (hydroxymethyl) aminomethane
U	unit
USP	United States Pharmacopeia
UV	ultraviolet
UV-vis	ultraviolet-visible
v/v	volume by volume
w/v	weight by volume
w/w	weight by weight

CHAPTER I
INTRODUCTION

1.1 Background and significance of the study

Aegle Marmelos Correa is a medicinal plant, belonging to family Rutaceae. It is natively grown throughout the subcontinents as well as India, Bangladesh, Burma, Sri Lanka and Thailand. It has been called in India as Bael, in Burma as Okshit and in Thailand as Matoom (ฤวัช สิงหพันธุ์, 2545). All parts of *A. marmelos* are edible and valued in Ayurvedic medicine. Its fruit is popular and ascribed to be of the greatest medical value. The ripe fruit pulp is sweet, orange in color and has a refreshing flavor. Unripe fruit is bitter, acrid, sour and astringent. For those reasons, it becomes attractive flavored products such as tea, marmalade, refreshing drink, candied fruit, cake and cream (Macleod and Pieris, 1981; Jagetia, Venkatesh and Baliga, 2004). Thai traditional medicine is claimed *A. marmelos* fruit as astringent, digestive aid, gastric stimulant, anti-diarrhea and anti-dysentery (สมพร ภูติยานันต์, 2546).

Several chemical constituents have been isolated and identified from *A. marmelos* fruit. These include *alkaloids*: marmeline, cinnamide and aegeline (Sharma and Sharma, 1981); *coumarins*: imperatorin, alloimperatorin, skimmianine and xanthotoxol (Sharma and Sharma, 1981; Banerji *et al.*, 1988); *terpeniods*: terpene alcohol, limonene, phellandrene, terpineol and linalool oxide (Tokitomo *et al.*, 1982); *steroids*: β-sitosterol and stigmasterol (Noysang, 1998); *other compounds*: gum, oleoresin, sugar: L-rhamnose, L-arabinose, D-galactose and D-galacturonic acid and vitamin C (Ratan, Prabir and Amal, 1981; รพีพล ภโววาท และ พิมลพรรณ พิทยานุกูล, 2529). The main chemical constituents of *A. marmelos* fruit are terpenoids and coumarins. Both groups are useful compounds i.e. terpenoids are useful for their flavor and in perfumery and coumarins are biologically active compounds (Gill, 1993).

There were previous reports on the pharmacological effects of *A. marmelos* extracts. For hypoglycemic and antihyperglycemic activities, the aqueous seed extract of *A. marmelos* given to diabetic rats at an effective dose of 250 mg/kg once daily for 14 days could decrease blood glucose in glucose tolerance test (Kesari *et al.*, 2006). Aqueous extract of *A. marmelos* fruit given at 125 and 250 mg/kg twice daily, p.o. for 4 weeks to streptozotocin-induced diabetic rats could reduce blood glucose of rats

(Kamalakkannan and Prince, 2003). Methanolic fruit extract of *A. marmelos* at a lower dose of 100 ng/ml showed glucose uptake activity *in vitro* by significantly elevating Glut-4, glucose transporter in L6 myotubes, when compared with insulin and rosiglitazone (Anandharajan *et al.*, 2006). The antidiarrhoeal activity of unripe *A. marmelos* was observed by Dhuley (2003) when given intravenously the fruit extract at dose of 50 and 100 mg/kg to castor oil-induced diarrhoeal mice. The extracts seemed to reduce intestinal fluid and transit time. Similarly, oral administration of aqueous and methanolic fruit extracts of *A. marmelos* at the doses of 3, 7.5 and 15 mg/kg to mice at 30 minutes before applied orally 0.3 ml castor oil resulted in a significant decrease onset time of diarrhea, total number of faeces, number of wet faeces and total weight of wet faeces (Shoba and Thomas, 2001). Interestingly, hydroalcoholic fruit extract of *A. marmelos* showed a radioprotective effect in mice. The mice were given the extract at dose of 20 mg/kg once daily for 5 days before exposure to 10 Gray (Gy) of radiation and the reduction of severity of symptoms and mortality from radiation were observed (Jagetia *et al.*, 2004).

Recently, the antiproliferative and antioxidant of its fruit extract were reported. Ethanolic fruit extract showed antiproliferative activity against SKBR3 human breast adenocarcinoma cell line (IC_{50}= 144.00±1.21 μg/ml) (Moongkarndi *et al.*, 2004). A dose-dependent nitric oxide scavenging activity of *A. marmelos* fruit extract using sodium nitroprusside as an nitric oxide donor was observed by Jagetia and Baliga (2004). Aqueous fruit extract given to streptozotocin-induced diabetic rats at 250 mg/kg twice daily for 4 weeks produced a significant decrease in peroxidation products and increase antioxidant enzymes (Kamalakkannan and Stanely Mainzen Prince, 2003). Lastly, methanolic fruit extract possessed strong antibacterial activity against multi-drug resistant *Salmonella typhi* (Rani and Khullar, 2004) and antiviral activity against human coxsackievirus B1-B6 with IC_{50} of 500 μg/ml (Badam *et al.*, 2002).

In order to employ *A. marmelos* for medicinal purpose as nutraceuticals or pharmaceuticals, the study on toxicity and other biological effects would be beneficial. Jagetia and coworkers (2004) found that ethanolic fruit extract of *A. marmelos* was nontoxic and had no side-effect up to a dose of 6 g/kg in mice. A lethal test showed no toxic effect on reproduction and progeny outcome on 8-week treatment rats with aqueous extracts. In male rats, no adverse effects on body, testicular weights, cauda epididymal sperm counts as well as no notable changes in sperm morphology and

motility were observed. In female rats, there were no significant changes in the number of implantation sites, viable fetuses, dead fetuses relative to controls (Aritajat *et al.*, 2000) and reproduction (Saenphet *et al.*, 2006).

Another aspect dealing with the safety of *A. marmelos* for medicinal use is the ability of *A. marmelos* to cause the biological interaction with concomitant drugs or compounds. Food-drug interaction is the results of the action between a drug and nutrient that would not happen with the nutrient or the drug alone, and the effect of a medication on nutritional status. While food-drug interaction happens, it can change the therapeutic effects or side effects of drugs (Pronsky and Crowe, 2000). Food-drug interaction may be divided into two broad types: pharmacodynamic and pharmacokinetic interactions. Pharmacodynamic food-drug interaction is the study of the biochemical and physiological properties of food on the conventional drug. While, pharmacokinetic food-drug interaction is the study of the time course of a drug in the body involving the absorption, distribution, metabolism (biotransformation) and excretion when co-administered with food (Shannon and Michael, 2005).

In an attempt to determine the food-drug interaction, study of drug metabolism is one of the important process. Conventionally, drug metabolism is broadly divided into phase I and phase II pathways. Enzymes involved in drug metabolism facilitate those chemical reactions. Phase I metabolism includes oxidation, reduction, hydrolysis and hydration resulting in the formation of functional groups (-OH, -SH, $-NH_2$ or -COOH) that impart the metabolites with increased polarity compared to the parent compounds (Gibson and Skett, 1986). Of the phase I reactions, cytochrome P450 (CYP) superfamily is associated to the metabolism of a variety of xenobiotics and endobiotics (Pronsky and Crowe, 2004).

The CYP enzymes (also called microsomal mixed-function oxidase enzymes) are belonged to a group of enzymes which possess similar core and mode of operation. Core of CYP enzyme is a hydrophobic part of heme structure (also known as ferriprotoporphyrin-9; F-9). The operation mode of enzyme is apoprotein (Coleman, 2005). At the cellular level, CYP is embedded in the phospholipid bilayer of the smooth endoplasmic reticulum with a portion exposed to the cytosol of many tissues (liver, kidneys, small intestine, skin, nasal mucosa, eyes, lungs, adrenals, pancreas, heart, brain, erythrocytes and platelets) (Gibson and Skett, 1986).

Currently, CYPs are classified according to their amino acid sequence homology, that is: 40 per cent of their amino acid structure in common they are

assumed to belong to the same "family". While, 55 per cent sequence homology of their amino acid structure in common they are assumed to belong to the same "sub family". Finally, individual 'isoforms', are enzymes which are originated from a single gene (Coleman, 2005). Presently, there are more than 270 different CYP gene families, with 18 recorded in mammals. Human beings have 57 CYP genes and 33 pseudogenes arranged into 18 families and 42 subfamilies (Nebert and Russell, 2002; Kashuba *et al.*, 2006).

The important human CYP isoforms that metabolize drugs include CYP1A2, 2C9, 2C19, 2D6, 2E1 and 3A4 (Kashuba *et al.*, 2006). Those enzymes are responsible to different duties such as CYP1A2 is capable of oxidizing oestrogens, series of hormones, planar aromatic molecules, aromatic amines, variety of drugs (which are aromatic amines such as caffeine, β-naphthylamine and theophyllin). While, CYP2C9 is responsible for the metabolism of common medications including ibuprofen, phenytoin, warfarin, rifampin, rifabutin, etc. CYP2C19 is responsible for the metabolism of several drugs such as omeprazole, benzodiazepine, citalopram, tricyclic antidepressant (TCAs), lansoprazole, etc. CYP3A4 is the most abundant CYP isoform in humans, and is responsible for the metabolism of more than 60% of all drugs in the market, representing 38 different therapeutic classes. Substrates of CYP3A4 include psychotropics, antiarrhythmics, benzodiazepines, antimicrobial agents, antiretroviral agents, immunosuppressants, antiulcer agent and anticonvulsants. CYP3A4 is also involved in the metabolism of several endogenous steroids such as cortisol, testosterone, estradiol and progesterone (Nebert and Russell, 2002; Coleman, 2005).

The mechanical reaction of CYPs to reduce their substrates is that CYPs form complex with substrate (RH), then NADPH-dependent cytochrome P450 reductase provides one electron to molecule of CYP-substrate complex. Molecule oxygen, an electron and two hydrogen ions combine with the reduced cytochrome P450-substrate complex, resulting in release of the product (oxidized substrate; ROH), reoxidation of CYP, $NADP^+$ and production of water. Products from this reaction appear to be a simple alcohol (Gibson and Skett, 1986; Kashuba *et al.*, 2006).

Interestingly, the activity of CYP can be induced or inhibited by xenobiotics such as drugs, food and environmental toxicants. (Brandon *et al.*, 2003). Foods and food components such as grapefruit juice, garlic, ginseng, St John's wort, ginkgo, etc.

were found for their abilities on CYPs (Venkataramanan, Komoroski and Strom, 2006).

Grapefruit or *Citrus paradisi* is belonged to family Rutaceae. Grapefruit is a low a glycemic index fruit with an excellent source of vitamin C, pectin, fiber and lycopene. Pharmacological effects of grapefruit juice include hypocholesterolemia, antihypertension, antidiabetes, antiobesity, antioxidant, antimicrobial and antifungal (Stump, Mayo, and Blum, 2006; Kiani and Imam, 2007). Effect of grapefruit juice on drug absorption was discovered in that it has the capacity to inhibit the activities of human CYP1A2, CYP3A4, and p-glycoprotein (Bailey *et al.*, 2004; Guo and Yamazoe, 2004; Kiani and Imam, 2007; Fujita *et al.*, 2008). In addition, bergamottin and 6',7'-dihydroxybergamottin which are active furanocoumarins in grapefruit, appeared to have CYP3A4 inhibition at IC_{50} value of 1.0 and 0.45 μM, respectively (Ohnishi *et al.*, 2000). Garlic or *Allium sativum* is a plant in the onion family, Alliaceae. Garlic has been used throughout recorded history for both culinary and medicinal purposes. Garlic seed is the most commonly used for consumption (raw or cooked), and for medicinal purposes. A number of pharmacological effects of garlic have been reported such as lowering of blood pressure, prevention of age-related changes in the vasculation and reduction of serum lipids (Abebe, 2002; Hodge, G., Hodge, S. and Han, 2002; Sato *et al.*, 2006; Fukao *et al.*, 2007). As part of food-drug interaction, it has been reported about inhibitory effect of garlic on CYP2C9, 2C19, 2D6 and 3A4 (Foster *et al.*, 2001). Allicin, the active compound in garlic, showed inhibitory effect on CYP2C9 and 2C19 with IC_{50} of 5.41 and 3.52 μM, respectively (Zou, Harkey and Henderson, 2002). Ginseng or *Panax ginseng* is a root of plants belonged to family Araliaceae. Ginseng is promoted as antimicrobial, anticarcinogenic and antioxidant properties (Ahn *et al.*, 2006; Xie *et al.*, 2006). Liu and collaborates (2006) reported the inhibitory effect of ginseng and its active components (steroid-like compounds: ginsenosides, eleutherosides) on CYP1A2, 2A6, 2C9, 2D6 and 3A4 with IC_{50} of 78.4, 63.6, 32.0, 58.0 and 14.1 μM, respectively. St John's wort or *Hypericum perforatum*, is a plant in Hypericaceae family. St John's wort flower is today most widely known as a herbal treatment for minor depression. The naphthodianthrones, hypericin and pseudohypericin along with the phloroglucinol derivative, hyperforin, are thought to be its chemical constituents (Lawvere and Mahoney, 2005). St John's Wort and its active components (hypericin and hyperforin) were able to inhibit the activities of CYP2C9, 2C19, 2D6 and 3A4

(Foster *et al.*, 2003; Venkataramanan *et al.*, 2006; Madabushi *et al.*, 2006). Ginkgo or *Ginkgo biloba* is a plant belonged to family Ginkgoaceae. Ginkgo leaf extract has been used to treat a variety of ailments and conditions including memory improvement, prevention of Alzheimer's disease and dementia (Sierpina, Wollschlaeger and Blumenthal, 2003). Zou and collaborates (2002) reported an inhibitory effect of Ginkgolic acid I and Ginkgolic acid II on CYP1A2, 2C9, 2C19, 2D6 and 3A4.

Since *A. marmelos* is widely used for food, beverage and medicinal herb in Thailand, repeated exposure of this fruit may affect biotransformation enzymes. Previous report showed that methanolic extract of *A. marmelos* leaves possessed bifunctional inducing properties on both phase I, CYPs and NADPH-CYP reductase, and phase II, glutathione S-transferase and DT-diaphorase in Swiss albino male mice (Singh, Banerjee and Rao, 2000). In adddition, imperatorin, one of the active constituents in *A. marmelos* could inhibit CYP1A1, 2B1 (Cai *et al.*, 1993), 1A2, 1B1, 2A6, 2B6, 2C9 and 3A4 in human CYPs (Kleiner, Reed and DiGiovanni, 2003). However, there is no information of *A. marmelos* fruits on human CYP activity. This study was designed to determine the *in vitro* inhibitory effects of *A. marmelos* fruit extract obtained from different solvent extraction on human phase I metabolism enzymes; CYP1A2, 2C9, 2C19 and 3A4. Amount of imperatorin in the fruit extract was determined by HPLC analysis and its effects on those CYPs were investigated so as to find out whether this compound properties was related to the inhibitory effect of the extracts.

1.2 Objectives of the study

The objectives of the present study were as follows:

1. To determine *in vitro* effect of *A. marmelos* fruit extracts on CYPs.
2. To determine *in vitro* effect of imperatorin on CYPs.

1.3 Benefits of the study

The benefits of the present study are as follows:

1. The information regarding the inhibitory effects of *A. marmelos* fruit extracts on CYP will be obtained.
2. The content of imperatroin in *A. marmelos* fruit extracts is determined.
3. The results from this study would be a beneficial information to indicate the possibility of interaction between *A. marmelos* fruit and some therapeutic drugs when taken concomitantly.

CHAPTER II
LITERATURE REVIEW

2.1 *Aegle marmelos*

Aegle marmelos Correa (Figure 1) is a very hardy tree and can thrive even under adverse agroclimatic condition. It belongs to the family Rutaceae and is known to have originated in Southeast Asia countries. It is grown throughout Thailand, India, Srilanka, Pakistan, Bangladesh and Burma. There are no standard names for *A. marmelos* cultivars. *A. marmelos* Correa is generally named after the locality where it is available. It has been called in India as Bael, in Burma as Okshit and in Thailand as Matoom (สุวัช สิงหพันธุ์, 2545).

A. marmelos is a popular plant in local communities due to its availability and its refreshing flavor. Indians place *A. marmelos* as the tree of Lord Shiva and also called "Shivadume", with their respect to its flavor and applications. It has been long time noted as indigenous medicinal plant in several Thai traditional treatment formulas while its fruit is used as a refreshing drink (สมพร ภูติยานันต์, 2546).

Figure 1 *Aegle marmelos* Correa (พร้อมจิต ศรลัมพ์, 2535)

2.1.1 Botany

A. maremelos is a medium-sized tree about 10-15 m in height with short, strong, sharp and spiny branches. The bark is bluish-grey with irregular furrows. Leaves are 2.5-5 cm long, ovate or oval-ovate, abrupt or tapering at base. Flowers are regular, bisexual and pale greenish white. Fruit diameters are globose, 5-12.5 cm (Srilanka), 8.5-16.8 cm (India), 5-15.5 cm (Thailand). Pericarps are smooth, greenish-yellow, about 3 mm thick and hard. Seeds are numerous and compressed. It surrounded by slimy, transparent mucous, testa white and covered with woolly hairs (Bishen and Mahendra, 1980; ฤวัช สิงหพันธุ์, 2545).

2.1.2 Cultivar and physiology

A. maremelos is a hardy, subtropical, deciduous tree that can thrive in various soil-climatic conditions. It can tolerate alkaline soil and temperature below 0°C. It is usually propagated by seeds which are sown in June and seedling are transplated a year later. Budding in June-July on its own 2-year-old rootstock, seedlings can be obtained. In vegetatively propagated plants, fruiting begins after 5 years and full bearing can be attained in about 15 years. The growth and development of fruits are in May to September. In Thailand, flowering starts from March in the Northeastern part. Fruit setting of *A. marmelos* takes place in early April. Its fruits take 4 months to be full size in August and another 4 months to be a mature fruit in December (สมพร ภูติยานันต์, 2546).

2.1.3 Medicinal properties

All parts of *A. marmelos* can be edible and valued in Ayurvedic medicine. The leaves are made into poultice and used in the treatment of ophthalmia and ulcers and fresh juice is recommended for treatment of catarrh and fever. The roots and bark are used for treatment of various ailments, particularly in intermittent fevers and also as a fish poison. Ripe fruit is regarded as astringent, digestive, stomachic and specially efficacious in chronic diarrhea and dysentery. It is often proving effective after all other medicines have failed (Bishen and Mahendra, 1980; ฤวัช สิงหพันธุ์, 2545). Different parts of *A. marmelos* have been reported for several pharmacological activities (Table 1).

2.1.3.1 Antihyperglycemic activity

Defective Glut-4 translocation in glucose transport and impaired insulin signaling cascade are among the major defects in insulin resistance in type 2 diabetes. For *in vitro* study, methanolic extract of *A. marmelos* fruit at concentration of 100 ng/ml showed significantly elevated of Glut-4, the glucose transporter in L6 myotubes as compared to insulin and rosiglitazone (Anandharajan *et al.*, 2006). While, *in vivo* study, *A. marmelos* fruit aqueous extract given orally at 125 mg/kg twice daily for 4 weeks to streptozotocin-induced diabetic rats, showed a significant antidiabetic effect. A highly significant decrease in glucose level nearly to normal was found when a dose of 250 mg/kg of the extract was given (Kamalakkannan and Prince, 2003). The plasma levels of vitamin C, GSH and α-tocopherol were increased due to the reduction of blood glucose in diabetic rats (Kamalakkannan and Prince, 2003; Kamalakkannan and Stanely Mainzen Prince, 2003). The aqueous extract of *A. marmelos* seeds at the effective dose of 250 mg/kg once daily, for 14 days in diabetic rats was reported not only lowered total cholesterol, triglyceride and LDL-cholesterol but also enhanced the cardioprotective lipid HDL cholesterol. Seed extract also reduced the glucose level by 26.8% and 35.1% after 4 and 6 hours, respectively, in treated rats following glucose tolerance test (Kesari *et al.*, 2006).

2.1.3.2 Antidiarrhoeal activity

Antidiarrhoeal effect of unripe *A. marmelos* fruit extract at 50 and 100 mg/kg, i.p. in castor oil-induced diarrhoeal mice was reported by Dhuley (2003). The extract reduced intestinal fluid and intestinal transit time. It also significantly inhibited of gastric lesion induced by ethanol but not those induced by stress or indomethacin. The researcher suggested that gastroprotection of *A.marmelos* extract may probably involve in a prostaglandin-independent mechanism. In addition, oral administration of *A. marmelos* aqueous and methanolic fruit extract at the doses of 3, 7.5 and 15 mg/kg, p.o. for 30 minutes before giving orally with 0.3 ml castor oil resulted in a significant decrease of onset time of diarrhea, total number of faeces, number of wet faeces and total weight of wet faeces in mice (Shoba and Thomas, 2001).

2.1.3.3 Anticancer activity

Hydroalcoholic fruit extract of *A. marmelos* showed a radioprotective effect in mice at the pretreatment dose of 20 mg/kg, once daily, i.p. for 5 days before exposure to 10 Gray (Gy) of radiation. Treatment of mice with all doses of the extract showed reduced severity of symptoms and mortality after 30 days postirradiation (Jagetia *et al.*, 2004). Ethanolic fruit extract of *A.marmelos* showed antiproliferative activity against SKBR3 human breast adenocarcinoma cell line with the 50% inhibitory concentration (IC_{50}) of 144.00±1.21 μg/ml (Moongkarndi *et al.*, 2004).

2.1.3.4 Antibacterial activity

Methanolic fruit extract of *A. marmelos* possessed strong antibacterial activity against multi-drug resistant *Salmonella typhi* (Rani and Khullar, 2004). The researcher suggested that antibacterial activity may be due to the essential oils such as cineole, *p*-cymene, citronellol, citral, cuminaldehyde, D-limonene and eugenol contained in *A. marmelos* fruit. They have shown to possess a broad spectrum of antibacterial and anti-fungal activities (Rana, Singh and Taneja, 1997; Roy and Saraf, 2006).

2.1.3.5 Antioxidant activity

Exposure of animals to different doses of γ-radition significant decreased glutathione (GSH) contents in a dose-dependent manner. The radiation also associated with increasing of lipid peroxidation in the liver, intestine, kidney and stomach. An *in vitro* study showed beneficial effects of *A. marmelos* fruit by expression of GSH and reduction in radiation-induced lipid peroxidation in those tissue of mice after pretreatment dose 20 mg/kg, once daily, i.p. for 5 days before exposure to 10 Gy of radiation. (Jagetia and Baliga, 2004). Moreover, when aqueous extract of *A. marmelos* fruit was given at effective dose of 250 mg/kg twice daily, p.o. for 4 weeks to streptozotocin-induced diabetic rats, it produced a significant decrease in peroxidation products and increased all the values of antioxidant enzymes (GSH, superoxide dismutase, catalase and glutathione peroxidase) in hepatic and renal tissues of rats (Kamalakkannan and Stanely Mainzen Prince, 2003).

Both studies showed antioxidative effect of *A. marmelos* fruit but the researcher suggested that there were still needed for biochemical and pharmacological investigations to isolate and identify the active constituents.

2.1.3.6 Antiviral activity

The efficacy against human coxsackieviruses B1-B6 of *A.marmelos* fruit extract was tested in an *in vitro* plaque inhibition assay. The 50% inhibitory concentration (IC_{50}) was found to be 500 µg/ml (Badam *et al.*, 2002).

Apart from pharmacological effect of fruit extract, other parts of *A. marmelos* also provided several activities (Table 1). The seed extract of *A. marmelos* showed blood glucose lowering effect, antiinflammatory, relief respiratory problem and analgesic (Karunanayake *et al.*, 1984; Kesari *et al.*, 2006). While, its bark showed antiviral, HIV-1 protease, and antiproliferative activity (Kusumoto *et al.*, 1995; Lampronti *et al.*, 2003). Its ethanolic extract from leaves has been reported for anticancer activity when administrated at the dose of 400 mg/kg once daily for six consecutive days to the tumor bearing mice (Jagetia, Venkatesh and Baliga, 2004). Moreover, hypoglycaemic activity was observed in alloxan-induced hyperglycemic rats given extract at the dose of 250 mg/kg, p.o. for 2 weeks (Kar, Choudhary and Bandyopadhyay, 2003). The leave extract contained anti-inflammatory by significant inhibition of carrageenan-induced paw oedema and cotton-pellet granuloma in rat and analgesic activity by reduction the early and late phases of paw licking in mice (Arul, Miyazaki and Dhananjayan, 2005). Hydroalcoholic leaf extract of *A. marmelos* was bifunctional inducer both phase I and phase II metabolism enzymes and had antioxidative effects by significantly increasing the basal levels of acid-soluble sulphydryl (-SH) content, cytochrome P450, NADPH-cytochrome P450 reductase, cytochrome b5, NADH-cytochrome b5 reductase, glutathaione S-transferase, DT-diaphorase, superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase in mice after administered the extract at doses of 50 and 100 mg/kg p.o. daily for 14 days (Singh *et al.*, 2000). Finally, its antifungal, antihistamine effects and analgesic activity were found (Rana *et al.*, 1997; Arul *et al.*, 2004, 2005; Shankarananth *et al.*, 2007).

Table 1 Traditional uses and pharmacological activities of *A. marmelos*

Plant parts	Activities	References
Fruits	**Traditional use:**	
	-Digestive aid	Bishen and Mahendra, 1980; ฤวัช สิงหพันธุ์, 2545
	-Antidiarrhea	
	-Antidysentery	
	-Mildly astringent	
	Pharmacological activities:	
	-Hypoglycemia activity	Kamalakkannan and Prince, 2003; Kamalakkannan and Stanely Mainzen Prince, 2003
	-Activation of glucose transporter	Anadharajan *et al.*, 2006
	-Gastroprotective	Dhuley, 2003
	-Antidiarrhoeal	Shoba and Thomas, 2001
	-Radioprotective effect	Jagetia *et al.*, 2004
	-Antiproliferative activity	Moongkarndi *et al.*, 2004
	-Nitric oxide scavenging activity	Jagetia *et al.*, 2004
	-Anti-lipid peroxidative activity	Kamalakkannan and Stanely Mainzen Prince, 2003
	-Antimicrobial	Rani and Khullar, 2004
	-Antiviral	Badam *et al.*, 2002
	-Anti-alzheimer	Ingkaninan *et al.*, 2003

Table 1 Traditional uses and pharmacological activities of *A. marmelos* (Cont.)

Plant parts	Activities	References
Leaves	**Traditional use:**	
	-Treatment of bronchitis	Bishen and Mahendra, 1980; ฤวัช สิงหพันธุ์, 2545
	-Treatment of ophthalmia	
	-Catarrh and feverishness	
	Pharmacological activities:	
	-Radioprotective effect	Jagetia, 2007
	-Phase I and Phase II metabolism enzyme induction	Singh *et al.*, 2000
	-Antioxidation	Singh *et al.*, 2000
	-Mosquito larvacidal	Abdul *et al.*, 2008
	-Hypoglycemic activity	Kar *et al.*, 2003; Narendhirakannan, Subramanian and Kandaswamy, 2006; Narender *et al.*, 2007
	-Anti-inflammatory	Arul *et al.*, 2005
	-Analgesic	Shankarananth *et al.*, 2007
	-Antifungal	Rana *et al.*, 1997
	-Atihyperlipidemia	Rajadurai and Prince, 2005; Narender *et al.*, 2007
	-Regulation of hyperthyroidism	Kar, Panda and Bharti, 2002
	-Antihistamine	Arul *et al.*, 2004

Table 1 Traditional uses and pharmacological activities of *A. marmelos* (Cont.)

Plant parts	Activities	References
Seed	**Pharmacological activities:**	
	-Hypoglycemia and antihyperglycemic activities	Kesari *et al.*, 2006
	-Anti-inflammatory	Karunanayake *et al.*, 1984
Bark	**Traditional use:**	
	-Chronic fever	Bishen and Mahendra, 1980; ฤวัช สิงหพันธุ์, 2545
	-Antimalarial	
	Pharmacological activities:	
	-HIV-1 protease inhibition	Kusumoto *et al.*, 1995
	-Antiproliferative effect	Lampronti *et al.*, 2003

2.1.4 Toxicological activity

Although *A. marmelos* has been used as food and beverage for years, its extract has been previously reported especially at high concentration, up to a dose of 6 g/kg, body weight. Ethanolic fruits extract of *A. marmelos* fruits showed nontoxic and no side effects in mice (Jagetia *et al.*, 2004). Aritajat *et al.* (2000) reported no toxic effect on male reproduction and progeny outcome on 8-week treatment rats with aqueous extracts of *A. marmelos*. Treatment rats showed no adversely affected on body and testicular weights as well as cauda epididymal sperm counts. No notable changes in sperm morphology and motility were observed. There were no abnormal changes in the number of implantation sites, number of viable fetuses and number of dead fetuses in females mated with plant extract-treated males relative to controls. An extended study, aqueous extract of *A. marmelos* fruit given to rats at 1 ml/day, p.o. for 60 days before mating had no effect on reproduction of female rats (Saenphet *et al.*, 2006). Ethanolic leaf extract of *A. marmelos* up to dose of 1,750 mg/kg showed no toxicity and no drug-induced mortality in treated rats (Jagetia *et al.*, 2004). The LD_{10} and LD_{50} of extract in acute mortality in rats were

found to be 2,000 and 2,250 mg/kg, respectively. The extract has been reported to be non toxic up to 4 g/kg orally (Jagetia *et al.*, 2004).

2.1.5 Chemical constituents and biological activity

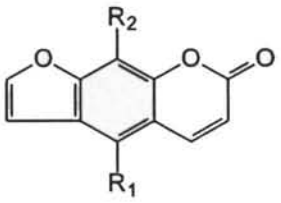
A. marmelos fruits contain many groups of compound such as Coumarins, alkaloids, flavonoids, lipid, terpenoids and sugar. Coumarins which are found in acetone extraction of *A. marmelos* fruit are imperatorin, alloimperatorin, and xanthotoxol (Sharma, B. R. and Sharma, P., 1981; Banerji *et al.*, 1988) while alkaloids are marmeline, cinnamide and aegeline (Sharma, B. R. and Sharma, P., 1981). Terpeniods in methanolic extractions are terpene alcohol, limonene, phellandrene, terpineol and linalool oxide (Tokitomo *et al.*, 1982) and flavonoid in ethyl acetate extraction is pelargonidin (Noysang, 1998). Sugars found in the aqueous extract include L-rhamnose, L-arabinose, D-galactose and D-galacturonic acid (พิมล พรรณ พิทยานุกูล และ รพีพล ภโววาท, 2529; Ratan *et al.*, 2001). Steroids found in hexane extracts are β-stitosterol and stigmasterol (Noysang, 1998). Other compounds (such as gum and oleoresin) are found by using methanolic extraction (Tokitomo *et al.*, 1982) (Table 2).

2.1.5.1 Coumarins

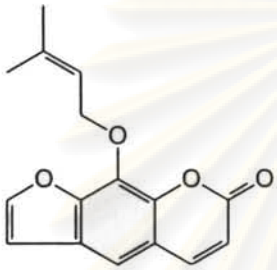
Coumarins are thought to be the main ingredients found in fruits of *A. marmelos*. The coumarin compounds are reported to be imperatorin, alloimperatorin, xanthotoxin and psoralen. All of imperatorin, alloimperatorin, xanthotoxin and psoralen are furanocoumarins, their chemical structures are shown in Figure 2. In general, furanocoumarins exists in plants belonging to families Umbelliferae, Rutaceae, Moraceae and Leguminosae such as parsnip, parsley, fennel and citrus fruit (grapefruit, orange and pomelon). There are used to cure skin diseases and their photosensitizing properties have led to development of a modern medical principle, photochemotherapy. The biological roles of furanocoumarins in their host plants have not been well understood, but at least these chemicals are considered as natural toxins to protect plants from insects, livestock and microbes. One of the toxicities of furanocoumarins is related to the inhibition of CYP enzyme in phase I metabolism. (Guo and Yamazoe, 2004).

Table 2 The chemical constituents of *A. marmelos* fruit

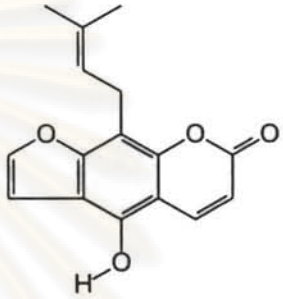
Type of chemicals	Chemicals	References
Coumarin	Imperatorin	Sharma, B. R. and Sharma, P., 1981; Noysang, 1998; Rahman *et al.*, 2004
	Alloimperatorin, Xanthotoxin, Psoralen	Sharma, B. R. and Sharma, P., 1981
Alkaloid	Marmeline, Cinnamide, Aegeline	Sharma, B. R. and Sharma, P., 1981
	Skimmianine, Haplopine	Banerji *et al.*, 1988
Terpenoid	Linalool, α-Phellandrene, Limonene	Macleod and Pieris, 1981; Tokitomo *et al.*, 1982
Flavonoid	Pelargonidin	Noysang, 1998
Sugar	L-rhamnose, L-arabinose, D-galacturonic acid, D-galactose	พิมลพรรณ พิทยานุกูล และ รพีพล ภโววาท, 2529; Ratan *et al.*, 1981
Steroid	β-sitosterol, stigmasterol	Noysang, 1998
Other	Oleoresin, gum	Tokitomo *et al.*, 1982; พิมลพรรณ พิทยานุกูล และ รพีพล ภโววาท, 2529



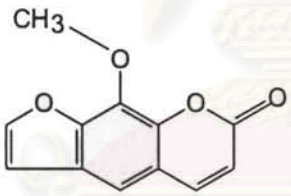
Furanocoumarin



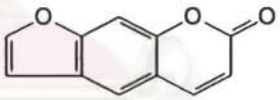
Imperatorin



Alloimperatorin



Xanthotoxin



Psoralen

Figure 2 Chemical structure of furanocoumarins and their derivatives

Imperatorin (9-(3-methylbut-2-enyloxy)-7H-furo[3, 2]chromen -7-one, ammidin, marmelosin, pentosalen) is probably the therapeutically active component of *A. marmelos* fruit (Table 3). Imperatorin has been reported on significant antiseizure effect in mice at dose of 50 mg/kg, from electrochock-induced seizures and chimney test (Luszczki *et al.*, 2008). Also, Imperatorin could inhibit nitric oxide (NO) production at IC_{50} of 60 μM (Matsuda *et al.*, 2005). *In vitro* study, imperatorin inhibit HIV-1 replication in MT-2 cells infected with HIV-1 (Sancho *et al.*, 2004). Imperatorin showed chemopreventive effect by inhibition of polycyclic aromatic hydrocarbon-induced skin tumor initiation in mice by blocking CYPs mediated bioactivtion of benzo-[*a*]pyrene (B[*a*]P) and 7, 12-dimethylbenz[*a*]anthracene (DMBA) (Kleiner *et al.*, 2003). Oral administration of imperatorin to mice significantly inhibited B[*a*]P-DNA adduct formation by 26% (Cai *et al.*, 1997; Kleiner *et al.*, 2002).

Moreover, imperatorin showed antioxidative effect against 2,2'-azobis (2-aminodinopropane) dihydrochloride (AAPH)-induced cellular damage. Imperatorin also showed potent antioxidant effects against the 2,2-diphenyl-1-picryhydrazyl (DPPH) radical and against renal epithelial cell injury by using AAPH to generate peroxy radicals *in vitro* (Piao *et al.*, 2004). Lastly, imperatorin showed antiplatelet aggregation and vasodilation effects (Chen *et al.*, 1996; He *et al.*, 2007).

Other coumarin, xanthotoxin (ammoidin) could inhibit nitric oxide production (Matsuda *et al.*, 2005) and CYP (Cai *et al.*, 1993). It also showed antiinflamatory and photoprotecting effect in patients with vitiligo (skin condition in which the losing of skin pigmentation causes white spots to appear) (Couperus, 1954). Interestingly, coumarin like psoralen has been reported as antipsoriasis by reduction of rash (Lowe, 1983). It showed anticancer effect by significant increasing apoptosis of tumor cell *in vitro* (Wang, *et al.* 2009).

There were some studies on CYP activities of furanocoumarins. Imperatorin acted as selective inhibition *in vitro* for CYP1A2, 1B1, 2B6 and 3A4 at IC_{50} lower than 1 μM. While xanthotoxin showed inhibit effects on CYP1A1 and CYP 2B1 at IC_{50} lower than 10 μM. Table 4 shows the IC_{50} values of furanocoumarin and selective inhibitors, α-naphthoflavone, sulfaphenazole, miconazole and ketoconazole (Kleiner *et al.*, 2003; Cai *et al.*, 1993; Marques-Soares *et al.*, 2003 and Monostory, Hazai and Vereczkey, 2004).

Table 3 Pharmacological activities of imperatorin, xanthotoxin and psoralen

Chemicals	Activities	References
Imperatorin	-Antiseizure	Luszczki *et al.*, 2008
	-CYP450 inhibition	Prince *et al.*, 2006; Kleiner *et al.*, 2003; Cai *et al.*, 1993
	-Inhibition of nitric oxide (NO) production	Matsuda *et al.*, 2005
	-Inhibition of HIV replication	Sancho *et al.*, 2004
	-Chemopreventive effect	Kleiner *et al.*, 2002
	-Antitumor	Cai *et al.*, 1996; Yang *et al.*, 2003
	-Antioxidative activity	Piao *et al.*, 2004
	-Antiplatelet aggregation	Chen *et al.*, 1996
	-Vasodilation effect	He *et al.*, 2007
Xanthotoxin	-Inhibition of nitric oxide (NO) production	Matsuda *et al.*, 2005
	-Treatment of vitiligo	Couperus, 1954
	- CYP450 inhibition	Cai *et al.*, 1993
Psoralen	-Antipsoriasis	Lowe, 1983
	-Anticancer	Wang *et al.*, 2009

Table 4 IC_{50} values (μM) of imperatorin, xanthotoxin and selective inhibitors on CYPs

Enzymes	IC_{50}, μM (±S.D. or 95% C.I.)					
	Selective inhibitors				Coumarins	
	α-naphthoflavone	sulfaphenazole	miconazole	ketoconazole	imperatorin	xanthotoxin
CYP1A1	0.186±0.037[(1)]	ND	ND	ND	2.76±0.64[(1)]	9.62[(2)]
CYP1B1	0.012±0.002[(1)]	ND	ND	ND	0.71±0.17[(1)]	ND
CYP1A2	0.042±0.017[(1)]	ND	ND	ND	0.38±0.12[(1)]	ND
CYP2A6	>500[(1)]	ND	132.8±110.86[(4)]	>250[(4)]	11.7 (13.3, 10)[(1)]	ND
CYP2B1	18.20[(2)]	ND	ND	ND	5.44[(2)]	1.30[(2)]
CYP2B6	>500[(1)]	ND	ND	ND	0.32 (0.40, 0.25)[(1)]	ND
CYP2C9	185 (211, 159)[(1)]	0.6[(3)]	5.41±3.52[(4)]	52.1±30.21[(4)]	111 (133, 89.1)[(1)]	ND
CYP2C19	ND	>500[(3)]	1.78±0.84[(4)]	122.1±77.42[(4)]	ND	ND
CYP2E1	ND	ND	>250[(4)]	>250[(4)]	ND	ND
CYP3A4	ND	ND	2.04±0.60[(4)]	0.4±0.16[(4)]	0.53 (0.62, 0.45)[(1)]	ND

ND = not determined, (1) Kleiner *et al.*, 2003, (2) Cai *et al.*, 1993, (3) Marques-Soares *et al.*, 2003, (4) Monostory *et al.*, 2004

2.1.5.2 Alkaloids

Some alkaloids in *A. marmelos* fruits are marmeline, cinnamide, aegeline, skimmianine and haplopine, in which some are biologically active. Skimmianine could be used for antifungal and antiviral activities (Olila, Olwa-Odyek and Opuda-Asibo, 2001). Aegeline showed antihyperglycemic activity as evidenced by lowering the blood glucose levels in streptozotocin-induced diabetic rats at the dose of 100 mg/kg body weight. It also significantly decreased the plasma triglyceride (Tg), total cholesterol (TC), and free fatty acids (FFA), accompanied with an increase in HDL and HDL/TC ratio in dyslipidemic hamster model at the dose of 50 mg/kg body weight (Narender *et al.*, 2007).

2.1.5.3 Flavonoids

The principal flavonoids in *A. marmelos* fruit are cyanidin and pelargonidin (Noysang, 1998). Pelargonidin has been reported for its anitidiabetic potential and antioxidant in streptozotocin-induced diabetic male wistar rats (Roy, Sen and Chakraborti, 2008).

2.1.5.4 Terpenoids

Terpenoids are widely distributed in nature, mostly in the plant kingdom. Oil of *A. marmelos* fruit is abundant source of terpenoids such as linalool, α-phellandrene and limonene. Terpenoids are useful for their flavor and in perfumery. (Macleod and Pieris, 1981; Tokitomo *et al.*, 1982).

2.1.5.5 Sugars

The sweetness of *A. marmelos* fruit is due to the presence of L-rhamnose, L-arabinose, D-galactose and D-galacturonic acid. The total sugar contents gradually increase with the advancement of fruit maturity (Ratan *et al.*, 1981; พิมลพรรณ พิทยานุกูล และ รพีพล ภโววาท, 2529).

2.1.5.6 Steroids

β-sitosterol and stigmasterol are phytosterols which can be found in *A. marmelos* fruit (Noysang, 1998). β-sitosterol and stigmasterol have been reported for lowering cholesterol in rats (Gerson, Shorland and Dunckley, 1965).

2.2 Food-drug interaction

Food-drug interaction is the result of the action between a drug and nutrient that would not happen with the nutrient or the drug alone and the effect of a medication on nutritional status. When food-drug interaction happens, it can change the effects of drugs and the therapeutic effects or side effects of drugs (Pronsky and Crowe, 2000).

Drug failure might occur after treatment has firstly appeared to be successful, where a patient becomes stabilized on a particular drug regimen, which then fails due to the addition of another chemical or nutrients to the regimen. In another way, drug causes the failure by accelerating of the removal of another drug or compound from the patient's system, so levels of removal drug are then too low to be effective. In the treatment of epilepsy, the loss of effective control of the patient's fits could lead to injury to themselves or others. The failure of a contraceptive drug would lead to an unwanted pregnancy and the failure of an antipsychotic drug would mean hospitalization for a patient at the very least.

Drug toxicity might occur when drug accumulation, either by overdose or by a failure drug removal. A reduction in the rate of drug removal from the body (often due to administration of another drug or chemical), will lead to drug accumulation. Toxicity can be an intensification of drug's therapeutic action, or an unrelated damaging effect on a tissue or organ system. For example, if the immunosuppressive cyclosporine is allowed to accumulate, severe renal toxicity can lead to organ failure. Excessive levels of anticonvulsant and antipsychotic drugs cause confusion and drowsiness and the accumulation of terfenadine can lead to lethal cardiac arrhythmias. Drug toxicity may occur much more rapidly than drug failure, it often within hours rather than days (Coleman, 2005).

Risk factors for increasing of food-drug interactions can be external and internal factors. Internal risk factors such as genetic, age, sex, hormone, disease and nutritional status. While, external risk factor such as polypharmacy, diet and environment (Gibson and Skett, 1996).

2.2.1 Pharmacology of food-drug interaction

Medication is administered to produce a pharmacologic effect in the body or more specifically in a large organ or tissue. To achieve this goal, the drug must move from the site of administration to the blood stream and eventually to the

site of drug action. In due course, the drug may be changed to active or inactive metabolites and ultimately eliminated from the body. An interaction between the drug and food, a food component, or a nutrient can alter this process at any point. Food-drug interactions may be divided into two broad types:

Pharmacodynamic interactions

Pharmacodynamics is the study of the biochemical and physiological effects of drug. The mechanism of action of drug might include the binding of drug molecule to a receptor, enzyme, or ion channel, resulting in the observable physiological response. Ultimately, this response may be enhanced or attenuated by the addition of other substances with similar or opposing actions.

Pharmacokinetic interactions

Pharmacokinetics is the study of the time course of a drug in the body involving the absorption, distribution, metabolism (biotransformation) and excretion of the drug. Absorption is the process of the movement of the drug from the site of administration to the blood-stream. Distribution occurs when the drug leaves the systemic circulation and travels to various regions of the body. A drug is metabolized and eliminated from the body as either unchanged drug or as a metabolite of the original compound (Pronsky and Crowe, 2000). If food can affect the duration of drug in the body, difference in pharmacokinetics will be observed.

2.2.2 Biotransformation of foods

Some compounds in food are xenobiotics which may be foreign compounds to the body as same as drug or pollutant. Living organisms have biotransformation processes to protect themselves against accumulation of xenobiotics to a high level. These processes general involve lipophilic compounds being converted to water-soluble metabolites. Metabolites are then ionized at physiological pH and excreted primarily in urine and feces. In general, biotransformation reactions involve by enzymes are divided into two categories, phase I and phase II (Gibson and Skett, 1996). The chemical reactions are normally associated with phase I and phase II drug metabolism as shown in Table 5.

Table 5 Reactions classed as phase I and phase II metabolism (Gibson and Skett, 1996)

Phase I	Phase II
Oxidation CYP P450-dependent oxidation CYP P450-independent oxidation Reduction Hydrolysis Hydration Dethioacetylation Isomerisation	Glucuronidation/glucosidation Sulfation Methylation Acetylation Amino acid conjugation Glutathione conjugation Fatty acid conjugation Condensation

Liver is the major organ of metabolism. While, renal excretion is the major route of elimination for drugs and drug metabolites either by glomerular filtration or tubular secretion. To a lesser extent, drugs may be eliminated in bile and other body fluids (Pronsky and Crowe, 2000). Other organs of metabolism are such as gastrointestinal tract, lungs, skin and kidneys.

Enzyme facilitate chemical reactions involved in drug biotransformations. The microsomal mixed-function oxidase system is responsible for phase I metabolism. This system consists of two enzymes: cytochrome P450 and NADPH-dependent cytochrome P450 reductase. Of all drug-metabolizing enzymes, cytochrome P450 enzymes metabolize the largest number of substrates having the greatest amount of structural diversity (Kashuba *et al.*, 2006). In phase I, endogenous compounds (e.g. hormones and prostaglandins produced in the body) and xenobiotics are transformed by biochemical reactions (reaction which introduce functional group e.g. –OH, -NH_2, -SH or –COOH to parent compound), primarily by cytochrome P450 (CYP) into more water-soluble compounds.

In phase II, the metabolites produced in phase I are conjugated in a series of reactions controlled by a different series of enzyme called conjugase. The conjugases attach a substance to the phase I biotransformed compounds to make them less toxic and more easily eliminated. Conjugators involved in phase II reactions include glucuronic acid, glutathione and glycine. Enzymes that catalyze phase II reactions include glutathione S-transferase, nicotinamide adenine dinucleotide phosphate (NADP) and quinine reductase. The conjugated water-soluble metabolites

produced by phase II are excreted in the urine or feces (Pronsky and Crowe, 2000) (Figure 3).

Achieving a balance between these two phases of detoxification is critical because phase I metabolites that are not biotransformed by phase II agents can be more toxic than the original molecule. For example, a component of cigarette smoke that is relatively harmless is transformed during phase I into a metabolically activated carcinogen and in phase II the carcinogen or metabolite are biotransformed, detoxified, and eliminated.

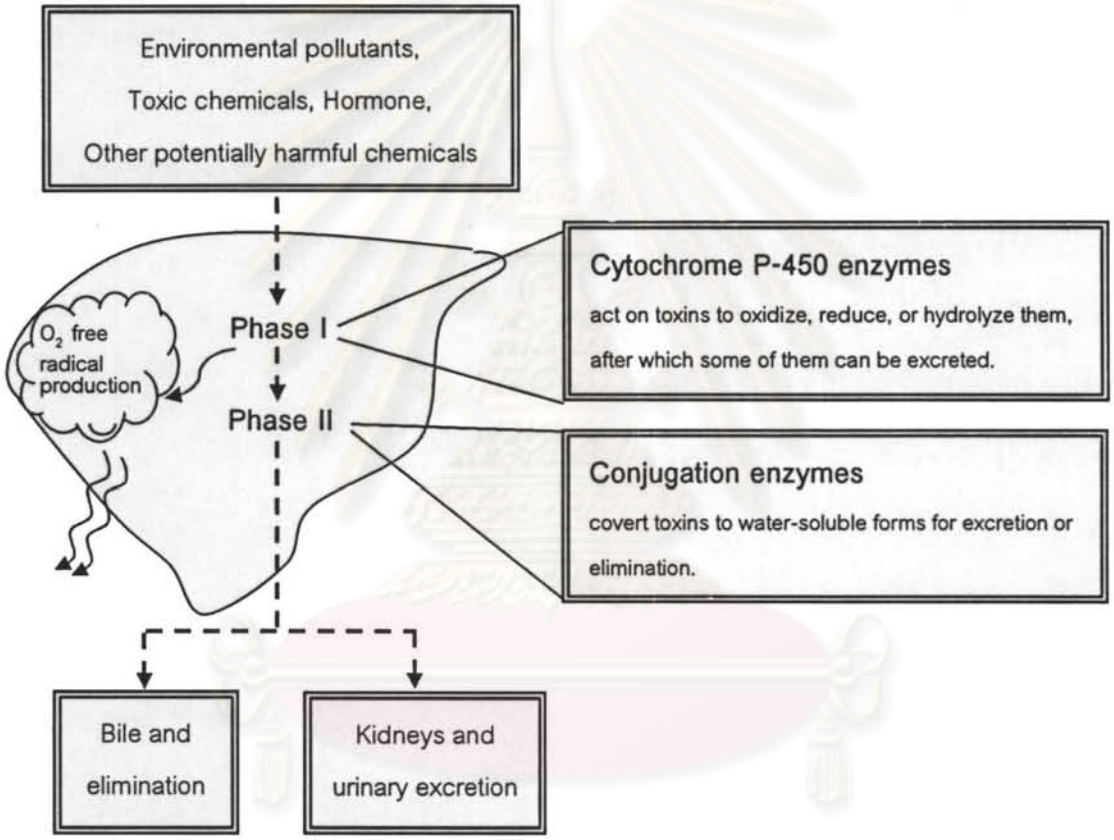


Figure 3 Detoxification in the liver

2.2.3 Cytochrome P450 (CYP)

The CYP enzymes (also call microsomal mixed-function oxidase) are belonged to a group of enzymes which all have similar core and mode of operation. The active site at core of CYP enzyme is hydrophobic part as heme structure (also known as ferriphotoporphoryn 9; F-9) (Figure 4). The operation mode of enzyme is apoprotein (Coleman, 2005).

CYPs were discovered in 1958, the nomenclature of CYP or cytochrome P450 is from the reaction between heme containing protein and carbon monoxide, the production of this reaction is a complex that has maximum UV absorption at 450 nm (Kashuba *et al.*, 2006). Currently, CYPs are classified according to their amino acid sequence homology. If 40 percents of their amino acids are in common, they are assumed to belong to the same "family". If 55 percents sequence homology of their amino acid structures are in common, they are assumed to belong to the same "sub family". Finally, individual 'isoforms', are which originate from a single gene (Coleman, 2005). Presently, there are more than 270 different CYP gene families, with 18 recorded in mammals. Human beings have 57 CYP genes and 33 pseudogenes arranged into 18 families and 42 subfamilies (Nebert and Russell, 2002; Kashuba *et al.*, 2006).

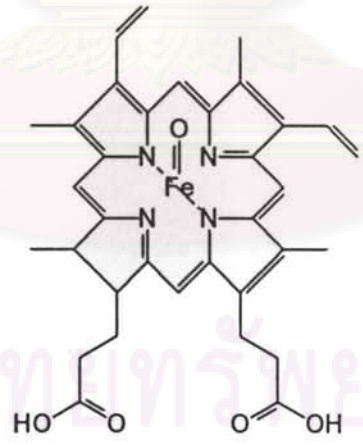


Figure 4 Structure of active site of CYP

The liver contains the greatest amount of CYPs. CYP enzymes have been found in virtually all organs in the body including kidney, small intestine, skin, nasal mucosa, eyes, lung, adrenals, pancreas, heart, brain, erythrocytes and platelets.

At the cellular level, CYP is embedded in the phospholipid bilayer of the smooth endoplasmic reticulum with a portion exposed to the cytosol (Gibson and Skett, 1996).

Initially, CYPs form complex with substrate (RH), then NADPH-dependent cytochrome P450 reductase provide one electron to molecule of CYP-substrate. Molecule oxygen, an electron and two hydrogen ions combine with the reduced cytochrome P450-substrate complex, resulting in release of the product (oxidized substrate; ROH), reoxidation of CYP, $NADP^+$ and production of water. Products from this reaction appear to be a simple alcohol. The typical reaction by CYP can be summarized as follows (Gibson and Skett, 1996; Kashuba *et al.*, 2006):

$$\text{Substrate (RH)} + O_2 + \text{NADPH} + H^+ \xrightarrow{\text{CYP}} \text{Product (ROH)} + H_2O + NADP^+$$

Figure 5 Typical reaction by CYP enzyme

NADPH-dependent cytochrome P450 reductase is a flavoprotein, which consists of two components, FAD (flavin adenine dinucleotide) and FMN (flavin mononucleotide). NADPH-dependent cytochrome P450 reductase locates primarily on the surface of the membrane, but in close proximity to the CYP substrate-binding site. NADPH reductase can be seen as the 'fuel pump' for the CYP it serves. It uses NADPH to supply the two electrons necessary for the cycling of the CYP. As CYPs run continuously like a machine tool, a constant flow of electrons is necessary to maintain CYP metabolism (Gibson and Skett, 1996; Coleman, 2005).

Among CYPs, CYP 1, 2 and 3 are main CYP families involved in xenobiotics metabolism. These three families account for about 70% of total CYPs in human livers. Whereas, CYP 5, 7, 8, 24 and 51 are the main enzymes involved in biosynthesis of endogenous substances (Nebert and Russell, 2002; Kashuba *et al.*, 2006) (Table 6).

Table 6 Endogenous substrates of human CYP families (Nebert and Russell, 2002)

Family	Substrates (functions)
CYP4	Fatty acids, arachidonic acid, eicosanoids
CYP5	Thromboxane A_2 synthase
CYP7	Cholesterol (bile acid synthesis)
CYP8	Prostacyclin synthase (bile acid synthesis)
CYP11	Steriod (Steroido biosynthesis)
CYP17	Steroid 17α-hydoxylase, 17/20-lyase
CYP19	Aromatase (oestrogen formation)
CYP21	Steroid 21-hydroxylase
CYP24	Vitamin D_3 24-hydroxylase
CYP26	Retinoic hydroxylase (retinoic acid hydroxylation)
CYP39	24-hydroxycholesterol, 7 α-hydroxylase
CYP46	Cholesterol 24-hydroxylase
CYP51	Lanosterol 14 α-desmethylase

2.2.4 Important members of CYP1, 2 and 3 families

The CYP enzymes which belong to families 1, 2 and 3 are main CYP families involved in xenobiotics metabolism (Gibson and Skett, 1996; Shannon and Michael, 2005; Coleman, 2005; Kashuba *et al.*, 2006). On the basis of the concentrations of individual CYP enzymes in human liver microsomes, CYP3A represents 30% of total hepatic P450 content; CYP2C represents 18%; CYP1A2 represents 13%; CYP 2E1 represents 7%; CYP2A6 represents 4%; CYP2D6 represents 1.5% and CYP2B6 represents 0.2%.

However, the relative contributions of individual CYP to the metabolism of drugs may not mimic the relative abundance of CYP in the liver. For example, CYP3A4 metabolizes approximately 36% of all drugs metabolized by P450, followed by CYP2D6 at 19%, CYP2C9 at 16%, CYP1A2 at 11%, CYP2C19 at 8%, CYP2E1 at 4%. This ranking is more important when considering drug interactions (Kashuba *et al.*, 2006). The enzymes responsible for most drug metabolism and interactions are listed in more details.

CYP1A subfamily

Enzymes in CYP1A subfamily are responsible for metabolic activation of some known procarcinogenic environmental chemicals, toxins and drugs. CYP1A1 (aryl hydrocarbon hydroxylase) and 1A2 (aryl amine oxidase) are the most important members of this family. Expression of the CYP1 family can be increased (induced)

by polycyclic aromatic hydrocarbons found in charbroiled meat, cigarette smoking and cruciferous vegetables (broccoli and cabbage). Summary of major CYP1A isoforms and their substrates, inhibitors and inducers are listed in Table 7.

CYP1A1 This isoform binds and oxidizes planar aromatic, essentially flat molecule. These compounds are multiples of benzene, such as naphthalene (two benzenes) and polycyclic aromatic hydrocarbons (PAHs). This isoform is no-constitutive in the liver but it accumulates in lung (Coleman, 2005). CYP1A1 can also be induced by polycyclic aromatic hydrocarbons and cigarette smoking. CYP1A1 is detected higher level in lung of smoker or breast cancer sufferer. Expression of CYP1A1 appear to be more of a threat than a protection, as it is often expressed in high levels in the vicinity of carcinogenesis (Kashuba *et al.*, 2006).

CYP1A2 This isoform is capable of oxidizing oestrogens, series of hormones, planar aromatic molecules, aromatic amines, variety of drugs (which are aromatic amines such as caffeine, β-naphthylamine and theophyllin). CYP1A2 is different from CYP1A1. It accumulates in liver and accounts for 10 to 15% of the total CYP content of human liver, and it is capable of aromatic amines while CYP1A1 does not (Nebert and Russell, 2002). CYP1A2 can induced by polycyclic aromatic hydrocarbon, cigarette smoking and drugs (such as omeprazole, phenobarbital, phenytoin, rifampin). While, it can be inhibited by erythromycin, ciprofloxacin, fluvoxamine and grapefruit juice. It is blocked by the methylxanthine derivative and furafylline (Shannon and Michael, 2005).

CYP2 subfamily

Around 18 to 30 percents of human CYPs are in this series, making it the largest single group of CYPs in man. They appear to oxidize various hormones, arachidonic acid and eicosanoids in addition to the metabolism of xenobiotics. As with many other CYPs, they are flexible enough to recognize many potential toxins such as drugs. Summary of major CYP2 isoforms and their substrates, inhibitors and inducers are listed in Table 8.

CYP2C9 This isoform is responsible for the metabolism of severe common medications including ibuprofen, phenytoin and warfarin. Rifampin and rifabutin are powerful inducers of CYP2C9 activity and will therefore decrease serum concentrations of the other of its substrates. Other inducers include carbamazepine,

ethanol and phenobarbital. Amiodarone, fluoxetine and fluconazole are among several drugs known to inhibit CYP2C9 activity.

CYP2C19 This isoform differs by only around 10 per cent of its amino acids from CYP2C9, but it does not oxidize acidic molecules, indicating that the active sites and access channels are subtly different. CYP2C19 metabolizes omeprazole, benzodiazepine, citalopram, tricyclic antidepressant (TCAs) and lansoprazole (Coleman, 2005). Rifampin induces CYP2C19 activities, whereas fluvoxamine, fluoxetine and ticlopidine inhibit this enzyme.

CYP2B6 This isoform originates from a gene found on chromosome 19, although it is less well known than many other CYPs, partly due to a lack of experimental inhibitors. The 2B series have been extensively investigated in animals, but CYP2B6 is the only 2B form found in human.

CYP2D6 This isoform comprises a relatively small percentage (2 to 6 %) of the total CYPs in the liver but it is responsible for more than 70 different drug oxidations. Multiple TCAs, β-blockers, haloperidol, sertraline and thioridazine are metabolized by CYP2D6. The conversion of codeine to the active form, morphine, is catalyzed by CYP2D6, and patients with low activity demonstarate a poor analgesic response. Unlike other CYPs, there are no known inducers of this activity. Several medications inhibit CYP2D6, the most potent include cimetidine, fluoxetine, haloperidol, paroxetine and codeine (Shannon and Michael, 2005; Lynch and Prince, 2007).

CYP2E1 This comprises around 7 percents of human liver CYPs and it is unusual as a mammalian CYP in that it oxidizes small heterocyclic agents, ranging from pyridine through to ethanol, acetone and other small ketones (methyl ethyl ketone). Ethanol and acetone are strong inducers of this isoform. Many of its substrates are water-soluble and it is often implicated in toxicity, as the metabolites can be highly reactive and toxic to tissues. It is responsible for the oxidation of paracetamol. This isoform is also correlates to smoking-induced cancers (same as CYP1A1 and CYP1A2). Many sulphur-containing agents block this enzyme, such as carbon disulphide, diethyl dithio carbamate and antabuse (Kashuba *et al.*, 2006).

CYP3A subfamily

The CYP3A family consists of CYP3A4 and CYP3A5. This family maintains most abundantly expressed CYP enzymes in human liver and gastrointestinal tract. It

is involved in the metabolism of the largest proportion of medications and is most important in drug interactions. This family of enzymes also metabolizes endogenous compound such as steroid and bile acid. Summary of major CYP3A isoforms and their substrates, inhibitors and inducers are listed in Table 9.

CYP3A4 This isoform is the most abundant CYP isoform in humans, and is responsible for the metabolism of more than 60% of all drugs on the market, representing 38 different therapeutic classes. Approximately 30% of hepatic CYP protein and 70% of intestinal CYP protein, is CYP3A4. The presence of CYP3A4 in the small intestine results in decreased bioavailability of many drugs. Substrates of CYP3A4 include psychotropics, antiarrhythmics, benzodiazepines, antimicrobial agents, antiretroviral agents, immunosuppressants, antiulcer agent and anticonvulsants. CYP3A4 is also involved in the metabolism of several endogenous steroids such as cortisol, testosterone, estradiol and progesterone. Inhibitor of CYP3A4 include clarithromycin, diltiazem, erythromycin, grapefruit juice and itraconazole.

CYP3A5 This isoform is initially thought to minimally contribute to the overall protein load and activity of hepatic CYP3A. Substrates of CYP3A5 include caffeine and diltiazem. Inhibitors of CYP3A5 include troleandomycin (Coleman, 2005).

Table 7 Drugs metabolized by CYP1A subfamily (Shannon and Michael, 2005; Kashuba *et al.*, 2006; Lynch and Prince, 2007)

CYP	Substrates	Inhibitors	Inducers
1A1	Polycyclic aromatic hydrocarbons (PAHs) Organochlorine insecticides	α-Naphthoflavone	PAHs Organochlorine Cigarette smoke
1A2	Acetominophen Aminopyrine Amitriptyline Caffeine Clomipramine Clozapine Cyclobenzaprine Desipramine Diazepam Estradiol Erythromycin Fluvoxamine Haloperidol Imipramine Naproxen Phenacetin Ropivacine Tacrine Theophylline Warfarin Zileuton	Cimetidine Ciprofloxacin Clarithromycin Enoxacin Erythromycin Fluvoxamine Isoniazid Ketoconazole Paroxetine Quinolone Grapefruit juice	Cigarette smoke PAHs Omeprazole Phenobarbital Phenytoin Rifampin

Table 8 Drugs metabolized by CYP2 subfamily (Shannon and Michael, 2005; Kashuba *et al.*, 2006; Lynch and Prince, 2007)

CYP	Substrates	Inhibitors	Inducers
2B6	Amfebutamone Coumarins Cyclophosphamide Mephenytoin Methadone	Tranylcypramine Thiotepa Ticlopidine	Phenobarbital Rifampicin
2C9	Amitryptyline Dapsone Fluoxetine Phenytoin S-warfarin	Isoniazid Fluvastatin Fluvoxamine Lovastatin Sulphafenazole	Rifampicin Secobarbitone
2C19	Barbiturates Proton pump inhibitor e.g. omeprazole, phenytoin, warfarin	Tranylcypramine Cimetidine Fluoxetine Ketoconazole Ticlopidine	Carbamazepine Norethindrone Prednisone Rifampicin
2D6	Tricyclic antidepressant (TCAs) Antipsychotic e.g. haloperidol Anti-arrythmics e.g. timolol, S-metoprolol	Amiodarone Cimetidine Ranitidine Chlorpheniramine St John's Wort	Currently unknown
2E1	Benzene Chlorzoxazone Ethanol Acetaminophen	Acute ethanol-ingestion Disulfiram	Chronic ethanol-ingestion Isoniazid Benzene

Table 9 Drugs metabolized by CYP3A subfamily (Shannon and Michael, 2005; Kashuba *et al.*, 2006; Lynch and Prince, 2007)

CYP	Substrates	Inhibitors	Inducers
3A4	Acetaminophen Alfentanil Alprazolam Amiodarone Aminopyrine Amitriptyline Amlodipine Amprenavir Antipyrine Astemizole Atorvastatin Benzphetamine Budesonide Busulfan Cannabinoids Carbamazepine Celecoxib Cisapride Clarithromycin Clindamycin Clomipramine Clozapine Codeine Cortisol Cyclobenzaprine Cyclophosphamide	Amiodarone Amprenavir Cannabinoids Cimetidine Clarithromycin Clotrimazole Cyclosporine Delavirdine Diltiazem Ethinylestradiol Erythromycin Fluconazole Fluoxetine Fluvoxamine Indinavir Itraconazole Ketoconazole Methronidazole Grapefruit juice	Carbamazepine Dexamthasone Ethosuximide Glutethimide Nevirapine Phenobarbital Phenytoin Primidone Rifabutin Rifampin St John's Wort Sulfadimidine Sulfinpyrazone Troglitazone Troleandomycin
3A5	Caffeine Diltiazem	Troleandomycin	Dexamethasone

2.3 Mechanism of CYP induction and inhibition

2.3.1 Induction of CYPs

CYP induction is defined as an increase in amount and enzyme activity of CYP. Classically, definition of induction is a *de novo* synthesis of new enzyme molecules as a result of an increase in transcription of its gene after stimulation by an appropriate chemical signal. Enzyme induction is dose-dependent, generally with a steep dose-response relation and no clear-cut threshold of no-effect (Coleman, 2005). A considerable diversity has been depicted in the mechanisms of regulation of CYP (Figure 6).

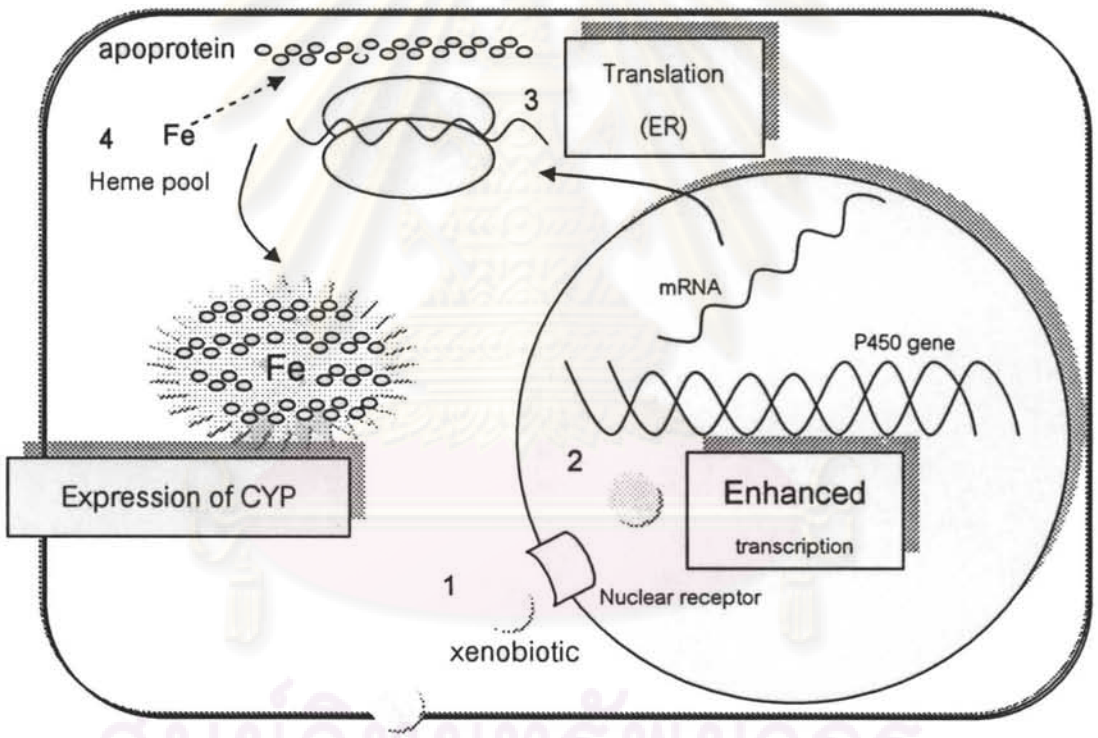


Figure 6 Steps in CYP gene expression

Effect of induction can be seen within the first two days of compound exposure, but it usually takes more than a week for new enzymes to be synthesized and the maximal effect to occur. The time course of enzyme induction onset and offset is closely related to the plasma concentration of the inducer, as well as the half-life of enzyme production and degradation. For drugs that are active in their parent forms, enzyme induction can enhance metabolic rate of the affected drug,

resulting in a decrease in the serum concentration of parent drug and possibly a loss of clinical efficacy. For prodrugs, compounds that require metabolic activation and whose effects are produced by the metabolites, enhanced pharmacodynamic effects may be expected.

2.3.2 Inhibition of CYPs

Enzyme inhibition results in a decrease in the clearance, thereby an increase in the steady-state serum concentration of the affected drug. The magnitude of the effect is largely unpredictable because it depends on the specific enzyme which is inhibited and the quantitative importance of the pathway in the overall clearance of the affected drug. Inhibition of CYP enzymes is the most common cause of metabolism based drug-drug interactions. The inhibition of CYP enzymes is of clinical importance for both therapeutic and toxicological reasons. The mechanisms of CYP inhibition can be categorized into reversible inhibition, quasi-irreversible inhibition and mechanism-based inhibition (Coleman, 2005; Gibson and Skett, 1996).

2.3.2.1 Reversible inhibition

Reversible inhibition is the most common type of enzyme inhibition. Reversible inhibition is transient and reversible and the normal functions of CYPs continue after the inhibitor has been eliminated from the body. Reversible inhibition can be further classified into competitive, uncompetitive, mixed-type and non-competitive inhibition. Competitive inhibition is when the binding of an inhibitor to an enzyme prevents a further binding of a substrate to the active sites of the enzyme. In uncompetitive inhibition, an inhibitor does not bind to the free enzyme but binds to a nonactive binding site of the enzyme and the binding has no effect on the binding of substrate but the enzyme-substrate-inhibitor complex is nonproductive.

2.3.2.2 Quasi-irreversible inhibition *via* metabolic intermediate complexation

Quasi-irreversible inhibition occurs when a reactive metabolite forms a stable complex with prosthetic heme of CYP. The stable complex is called metabolic intermediate (MI) complex. The MI complex can be reversed and the

catalytic activity of CYP can be restored by incubating *in vitro* with lipophilic compounds that can displace the inhibitor from the active site. However, synthesis of *de novo* enzyme is required to restore CYP activity in an *in vivo*.

2.3.2.3 Mechanism-based inhibition

Mechanism-based inhibition on CYP can be mediated by covalent modification of a pyrrole nitrogen in the prosthetic heme group of CYP or by direct modification of the heme moiety or the apoprotein. The mode of inhibition is highly specific because the inhibitor must both bind to and be metabolized by the enzyme. The inhibitory effect of mechanism-based inhibition is terminated by enzyme resynthesis rather than inhibitor washout.

One mode of mechanism-based inhibition is the so-called enzyme inactivation (or suicide inhibition). Suicide inhibition results from covalent binding of reactive intermediates to the heme and/or protein of CYP.

The most important phenomena of mechanism-based inhibition are time, concentration and NADPH-dependent loss of the enzyme activity. *In vivo*, the inhibitory effect of a mechanistic inactivator is thought to be more prominent after repeated dosing and last longer than that of a reversible inhibitor.

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

1. Plant materials

The fruit of *A. marmelos* Correa was collected from local agricultural group, Amphur Pagkway, Sukhothai Province, Thailand in September 2007. The fruits were dried in 40°C oven and blended to moderately coarse powder.

2. Chemicals

2.1 Acetonitrile, anhydrous (ACN) (analytical grade) (C_2H_3N, MW = 41.05), was obtained from Lab-Scan Asia Co., Ltd., Thailand.

2.2 Dimethyl sulphoxide (DMSO) (analytical grade) (C_2H_6OS, MW = 78.13), was obtained from Lab-Scan Asia Co., Ltd., Thailand.

2.3 Ethanol (analytical grade) (C_2H_5OH, MW = 46.07), was obtained from Liqour Distillery Organization, Bangkla, ChachoengSoa Province, Thailand.

2.4 Hexane (analytical grade) (C_6H_{14}, MW = 86.18), was obtained from Fisher Scientific Asia Co., Ltd., Ireland.

2.5 Methanol (HPLC grade) (CH_3OH, MW = 32.04), was obtained from Fisher Scientific Asia Co., Ltd., Ireland.

2.6 Standard chemicals

2.6.1 Imperatorin (marmelosin; ammidin) ($C_{16}H_{14}O_4$, MW = 270.29) (98% purity), was obtained from Indofine Chemical Co., Ltd., Hillsborough, NJ, USA.

2.6.2 Ketoconazole ($C_{26}H_{28}Cl_2N_4O_4$, MW = 531.43), was obtained from Siam Pharmaceutical, Co., Ltd., Bangkok, Thailand.

2.6.3 Miconazole ($C_{18}H_{14}Cl_4N_2O$, MW = 479.1) was obtained from the Government Pharmaceutical Organization, Bangkok, Thailand.

2.6.4 α-Naphthoflavone ($C_{19}H_{12}O_2$, MW = 272.3) was obtained from Sigma Chemical Co. Ltd., USA.

2.6.5 Sulfaphenazole ($C_{15}H_{14}N_4O_2S$, MW = 314.36) was obtained from Sigma Chemical Co. Ltd., USA.

2.6.6 Tris base ($C_4H_{11}NO_3$, MW = 121.14) was obtained from Sigma Chemical Co. Ltd., USA.

2.7 Enzymes screening kits

Vivid® CYP450 screening kits were purchased from Invitrogen™ (Carlsbad, California, USA). The components of each kit are shown in Table 10.

Table 10 The components of Vivid® CYP450 screening kit.

Components	Composition	Size
Reaction buffer	CYP1A2 and 3A4: Potassium phosphate buffer (200 mM, pH 8.0) CYP2C9 and 2C19: Potassium phosphate buffer (100 mM, pH 8.0)	50 ml
BACULOSOMES® reagent	CYP1A2: CYP1A2 and NADPH-P450 reductase (P450-specific content 0.9-1.1 µM) CYP3A4: CYP3A4 and NADPH-P450 reductase (P450-specific content 0.9-1.1 µM) CYP2C9: CYP2C9 and NADPH-P450 reductase (P450-specific content 0.9-1.1 µM) CYP2C19: CYP2C19 and NADPH-P450 reductase (P450-specific content 0.9-1.1 µM)	0.5 nmol
Regeneration system	333 mM glucose-6-phosphate (G-6-P) and 30 U/ml glucose-6-phosphate dehydrogenase (G-6-PD) in 100 mM potassium phosphate buffer (pH 8.0)	0.5 ml
Substrate	CYP1A2 and 2C19: EOMCC (ethyloxymethyloxy-3-cyanocoumarin) CYP3A4 and 2C9: BOMCC (7-benzyloxymethyloxy-3-cyanocoumarin)	0.1 mg
$NADP^+$	$NADP^+$ solution (10 mM) in potassium phosphate buffer (100 mM, pH 8.0)	0.5 ml

3. Equipments

3.1 Analytical balance (0.1 mg readability) (Sartorius Co., Ltd.) (Goettingen, Germany), **analytical balance** (0.01 mg readability) (Mettler Toledo Ltd., Columbus, USA)

3.2 Blender (Retsch Mühle 5657 HAAN, Germany)

3.3 De-ionized water (DI water) system (ELGAStat Option 3B) (ELGA Ltd., England)

3.4 Fluorescent Microplate Reader (Victor^3) (Perkin Elmer, USA)

Light Source	:	Tungsten-halogen lamp
Detection unit	:	Photomultiplier tube
Fluorometry	:	Fluorescein
Optical emission filter	:	450/10 nm
Optical excitation filter	:	390/20 nm

3.5 Freeze-dryer (Dura-Dry) (LyoLab w/PC, Lyophilization Systems Inc., USA)

3.6.High-Performance Liquid Chromatography (HPLC) (Shimadzu, Japan)

Binary high pressure gradient pump	:	LC-20ADvp
Autosampler	:	SIL-20Avp
Column oven	:	CTO-20Alite
Solvent degasser	:	DGU-20A3
Photodiode-array UV-vis detector	:	SPD-M20Avp
System controller	:	SCL-20Avp

3.7 HPLC column compartment

Analytical column	:	Luna C18(2), 5 µm, i.d. 4.6 mm x 150 mm, steel column (Phenomenex Inc., USA).
Guard column	:	Security Guard™, C18 (ODS), 4.0 mm. x 3.0 mm., i.d. guard column (Phenomenex Inc., USA).

3.8 Injection vials and caps (Water Corp., Ireland)

3.9 Micropipettes SL-20 (2-20 µl), SL-200 (20-200 µl) and SL-1000 (100-1,000 µl) (Pipet lite™, RAININ® Inc., USA)

3.10 Micropipette tips (Gibson, France)

3.11 Multi-channel pipettes (20 and 100 µl) (RAININ® Instrument, USA)

3.12 Multi-channel tips RT-L250 (250 µl), TR-222-C (200 µl) (RAININ® Instrument, USA)

3.13 Nylon membrane filters (0.45 µm, 47 mm, Sartorius®, Goettingen, Germany)

3.14 pH meter (Beckman Instruments, USA)

3.15 Reagent reservoir (RV-050 model) (Oakland, CA)

3.16 Rotary evaporator (Büchi Rotavapor, R-200) including heating bath (Büchi Heating bath, B-490) and vacuum pump (Büchi Vac, V-500) with condenser (Büchi, Switzerland)

3.17 Sonicator (D-78224 model) (Elma®, Germany)

3.18 Timer (QT-9017-A model) (Citizen®, Japan)

3.19 Ultra-purifier water system for ASTM Type II water (Ultra-pure Water), 18.2 MΩ or triple-distilled water or sterile water for injection (HPLC grade) (Maxima UF, ELG Ltd., England)

3.20 Vortex mixer (G-560E model) (Scientific, Inc., Bohemia)

3.21 5 ml-glass test tubes

3.22 1.5 ml-safe lock micro test tubes (Eppendorf™, Germany)

3.23 96-well black plates (Perkin Elmer, USA)

3.2 Methods

3.2.1 Preparation of *A. marmelos* extracts

A. marmelos fruit was purchased from Sukhothai Province, Thailand and was ground coarsely before extracted.

Extract A (hexane extract)

One kilogram of dried fruit of *A. marmelos* was refluxed with 2.5 liters of n-hexane at 70°C and 48 hours for three times (Figure 7). The extract was collected and concentrated to residue under reduced pressure in a vacuum rotary evaporator at the temperature lower than 60°C. The residue was stored at 0°C and protected from light (Noysang *et al.*, 1998).

Extract B (ethanolic extract)

Onc kilogram of dried fruit of *A. marmelos* was extracted with 5 liters of ethanol at room temperature for three times (Figure 8). The extract was collected and concentrated to residue under reduced pressure in a vacuum rotary evaporator at the temperature lower than 60°C. The residue was stored at 0°C and protected from light (Tokitomo *et al.*, 1982).

Extract C (water extract)

One kilogram of dried fruit of *A. marmelos* was boiled with 1 liter of water on a hot plate for 1 hour for three times (Figure 9). The aqueous extracts was collected and concentrated under reduced pressure in a vacuum rotary evaporator at the temperature lower than 60°C. The concentrated residue was lyophilized, stored at 0°C and protected from light (Prommetta *et al.*, 2006).

Calculation of total yield of *A. marmelos* extract

The percentage of total yield (%w/w) of the extract was carried out according to the following equation:

$$\text{Total yield (\%w/w)} = \frac{W_{final}}{W_{initial}} \times 100 \qquad (1)$$

where,

W_{final} is the total weight of solid residue (g), obtained after complete extraction.

$W_{initial}$ is the total weight of ground *A. marmelos* (g), added in the initial extraction process.

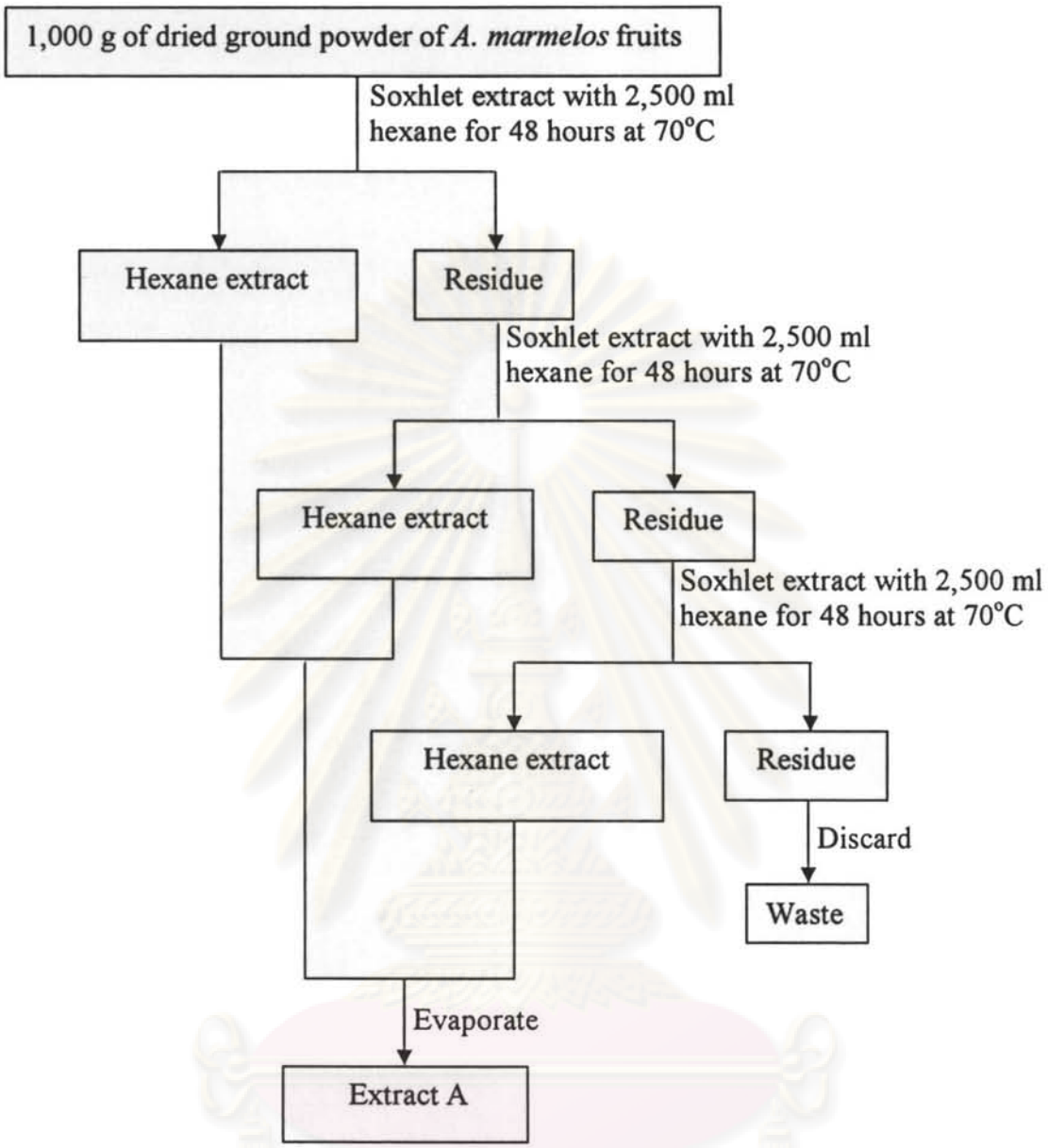


Figure 7 Schematic diagram of *A. marmelos* hexane extraction

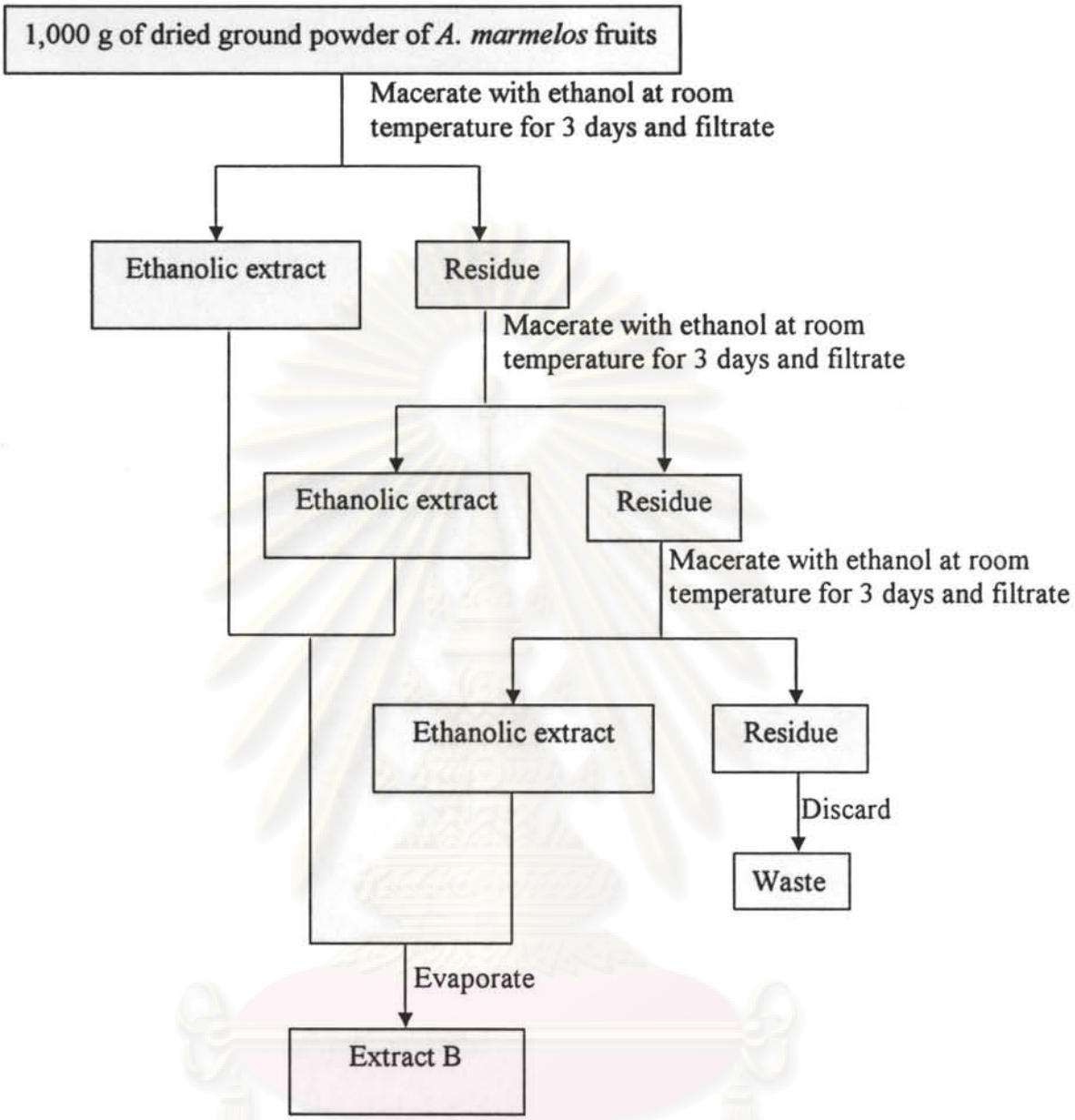


Figure 8 Schematic diagram of *A. marmelos* ethanolic extraction

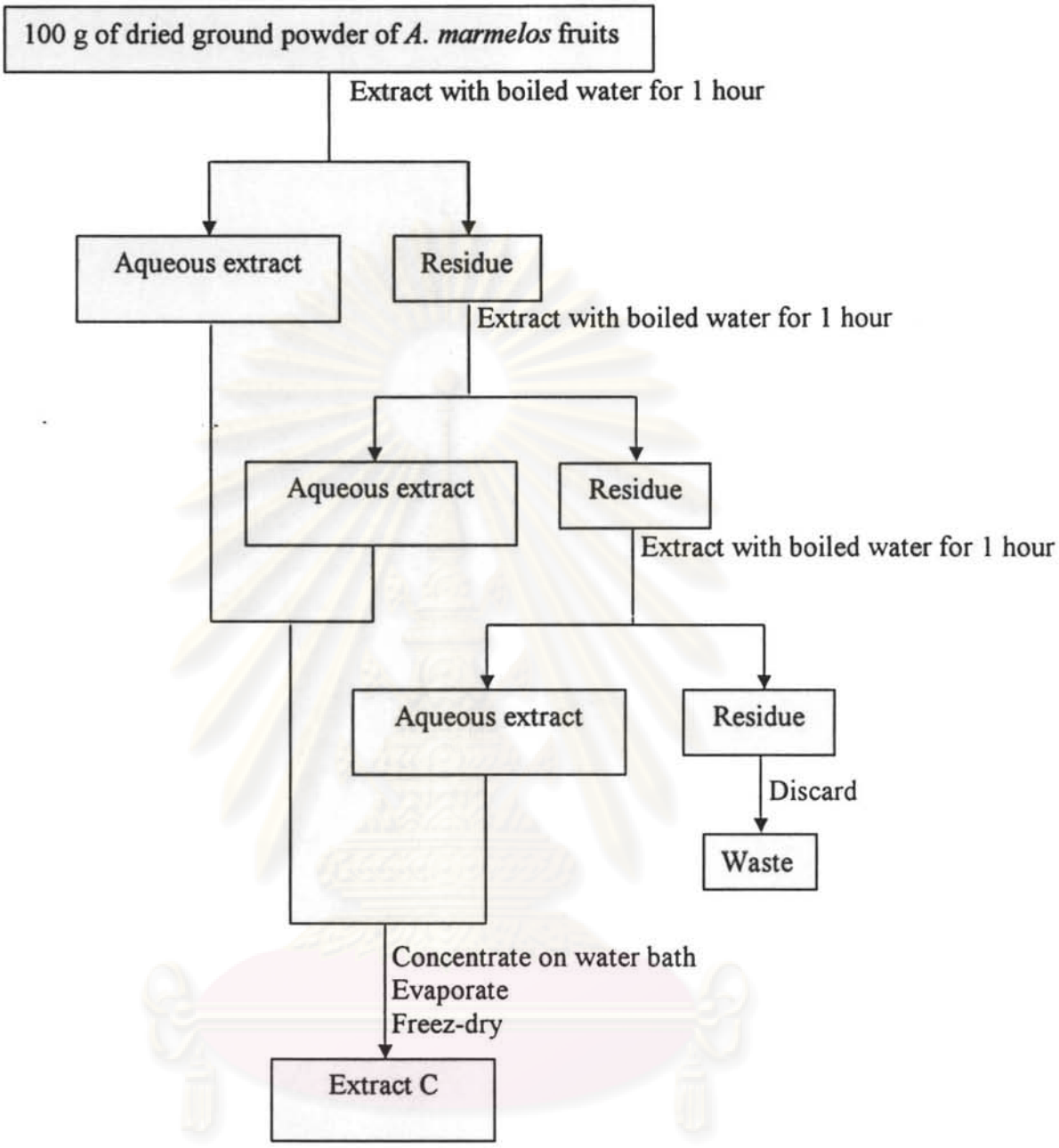


Figure 9 Schematic diagram of *A. marmelos* aqueous extraction

3.2.2 High Performance Liquid Chromatography (HPLC) analysis of imperatorin in *A. marmelos*

The major constituent in all extracts of *A. marmelos* fruits, mainly considered to be imperatorin, was analyzed. A principle of analytical procedure was based on reversed phase (RP)-HPLC analysis. Separation was binary gradient elution and detection was performed by using UV-vis detector.

3.2.2.1 HPLC system

RP-HPLC analysis was employed to develop the analytical method. All experiments were performed using a Shimadzu VP series liquid chromatograph (LC) equipped with a SCL-20Avp system controller. A liquid chromatograph system comprising a DGU-20A3 vacuum degasser, LC-20ADvp high pressure liquid chromatography binary pump combined with a FCV-20ALvp gradient mixer, a SIL-20Avp auto-sampler (20 µl sampling loop), a thermostatted column compartment using CTO-20Alite air bath column oven and a SPD-M20Avp UV-vis photodiode array detector (photo-DAD) were used.

The column used was a C18 RP-column, Phenomenex® with a guard column, SecurityGuard™, (Phenomenex®, USA).

3.2.2.2 HPLC condition

Chromatographic conditions of the analysis modified from Li (2006) were as follows:

Column	:	Luna C18(2), 5 µm., i.d. 4.6 mm x 150 mm
Column temperature	:	40°C
Solvent elution	:	Binary concentration gradient elution
Mobile phase	:	40 : 60% (v/v) water : methanol
Flow rate	:	1.0 ml/min
Injection volume	:	10 µl
Detection wavelength	:	254 nm
Run time	:	15 min

The reference standard of imperatorin used in this study was 98.0% (w/w) purity based on dried basis.

3.2.3 Validation of analytical method for quantitative determination of imperatorin in *A. marmelos* extracts

The analytical method was validated to ensure the accuracy, precision, specificity, linearity and range, limit of determination and limit of quantitation of the method according to the criteria of compendial method validation. (USP 32 and NF 29, 2004; ICH, 2006; Green, 1996). The criteria are summarized as shown in Table 11.

Table 11 Summarized criteria of analytical method validation

Test	Parameter	Criteria
Accuracy	% Recovery	98-102%
Precision	% RSD	Not more than 2%
Linearity	Intercept	2-3% of peak area at concentration of 100%
	R^2	Not less than 0.9995
Specificity	Resolution	Not less than 1.5
	Asymmetry	Not more than 1.5
	Peak are	Not significantly difference ($p<0.05$)

3.2.3.1. Preparation of standard, sample and synthetic mixture solutions for method validation

A. Preparation of standard solutions

The stock solution was prepared by accurately weighing 15.02 mg of imperatorin to a 50-ml volumetric flask and adjusting to volume with methanol. The concentration of standard stock solution was 300.40 µg/ml. Then, the stock solution was pipetted 100, 200, 300, 400 and 500 µl, respectively, to a 10-ml volumetric flask and adjusted to volume with methanol. The theoretical concentrations of imperatorin were 3.04, 6.00, 9.00, 12.02 and 15.02 µg/ml. The actual concentraction (based on 98% purity) were then 2.98, 5.88, 8.82, 11.78 and 14.72 µg/ml.

B. Preparation of sample solutions

One hundred milligrams of extract A was accurately weighed into a 100-ml volumetric flask and adjusted to volume with methanol. The concentration of sample stock solution was 1,000 µg/ml. Then, one ml of sample stock solution of extract A was pipetted to a 10-ml volumetric flask and adjusted to volume with methanol. The concentration of sample solution was 100 µg/ml.

C. Preparation of synthetic mixture solution

Five hundred microliters of sample (extract A) stock solution (1,000 µg/ml) was well-mixed with 100, 200, 300, 400 and 500 µl of standard stock solution (300.40 µg/ml) of imperatorin, to a 10-ml volumetric flask and adjusted to volume with methanol. Each final solution contained 50 µg/ml of sample. The solution was used for accuracy testing.

One thousand microlitres of sample (extract A) stock solution was well-mixed with 100 µl of standard stock solution of imperatorin to a 10-ml volumetric flask and adjusted to volume with methanol. The final concentration contained 2.98 µg/ml of imperatorin and 100 µg/ml of sample. The solution was used for specificity testing.

Accuracy

Accuracy is the measurement of how close the experimental values to the true values. It is expressed as the percentage of analyte recovered from the matrix or from spiking samples in a blind study. The accuracy should be determined by a minimum of nine determinations for at least three concentrations in the range of test concentrations without a serial dilution. The mean values should be within 2% from the true value.

In this study, the experiment was performed by analyzing a mixture containing sample solution of fruit extract spiked with known quantities of reference standard imperatorin solution.

Procedure

According to the developed method, three replications of standard imperatorin solution at concentrations of 2.98, 5.88, 8.82, 11.78 and 14.72 µg/ml were analyzed and carried out for a calibration curve (also called as system linearity curve) plotted between peak area and concentration of standard imperatorin. The equation of system linearity was shown below:

$$y_1 = a_1x_1 + b_1 \quad (2)$$

where,

y_1 is peak area of standard imperatorin solution ($mAU \cdot s^{-1}$)

a_1 is slope of equation obtained from standard solution

x_1 is concentration of standard imperatorin solution (μg/ml)

b_1 is y-intercept of equation obtained from standard solution

Three replications of synthetic mixture (five concentrations of standard solutions: 2.98, 5.88, 8.82, 11.78 and 14.72 μg/ml; each solution contains 50 μg/ml of sample) were analyzed and carried out for a synthetic mixture curve (also called as method linearity curve) plotted between peak area of each synthetic mixture solution and concentration of standard imperatorin. The equation of method linearity was shown below:

$$y_2 = a_2x_2 + b_2 \quad (3)$$

where,

y_2 is peak area of synthetic mixture solution ($mAU \cdot s^{-1}$)

a_2 is slope of equation obtained from synthetic mixture solution

x_2 is concentration of standard imperatorin solution (μg/ml)

b_2 is y-intercept of equation obtained from synthetic mixture solution

Calculation

The observed concentration of imperatorin found in the sample (μg/ml) was carried out according to the following equation:

$$\text{Observed conc. (μg/ml)} = \frac{(y_2 - b_2) - b_1}{a_1} \quad (4)$$

The actual concentration of imperatorin (μg/ml) was carried out according to following equation:

$$\text{Actual conc. } (\mu g/ml) = \frac{U \times V \times W}{100} \quad (5)$$

where,

U is a weight of standard imperatorin (μg)

V is purity content (%, w/w on dry basis) of imperatorin

W is dilution factor

$$\text{Recovery (\%, w/w)} = \frac{(Observed\ conc.)}{(Actual\ conc.)} \times 100 \quad (6)$$

The recovery (%) was obtained by dividing the mean of observed concentrations by the mean of actual concentrations and multiplying by 100. The recovery should be coverage in the range of 98-102% recovery.

Precision

Precision is the measurement of how close to each other of the data values for a number of measurements under the same analytical conditions. The precision of an analytical method is usually expessed as the relative standard deviation (RSD) or coefficient of variation (CV). Precision is further subdivided into repeatability and reproducibility. Reproducibility refers to the use of the analytical procedure in different laboratories. Repeatability refers as the use of the analytical procedure within a laboratory over a short period of time using the same analyst with the same equipment. For most purposes, repeatability is the criterion of concern in USP analytical procedures; although reproducibility between laboratories or intermediate precision may well be considered during the standardization of a procedure before it is submitted to the pharmacopeia.

Repeatability was measured by the analysis through six determinations without a serial dilution at 100%, w/v of the test concentration. Precision should be lower than 2% of RSD value. In this study, the experiment was performed by intra- and inter-day repeatability.

Procedure

A concentration (8.82 μg/ml) of synthetic mixture in the calibration range was prepared and injected into the HPLC system. The intra-day precision was performed by duplicated injection of six replicated synthetic mixture solution, and the %RSD of

peak area of imperatorin was determined. The inter-day precision was obtained by using six replicates analysed in one day over a period of three days under the same conditions.

Calculation

Standard deviation (SD) was carried out according to the following equation:

$$SD = \sqrt{\frac{\sum_{i=1}^{N}(x_i - \bar{x})^2}{N-1}} \quad (7)$$

where,

x_i is %recovery in each replicate

$\bar{x}$ is an overall mean of %recovery of all replicates

N is a number of replicates

The %RSD was carried out according to the following equation:

$$\%RSD = \frac{SD}{\bar{x}} \times 100 \quad (8)$$

where,

SD is a standard deviation of %recovery of all replicates

$\bar{x}$ is an overall mean of %recovery of all replicates

Specificity

Specificity is the ability to ensure the identity of the analyte from the unexpected components such as impurity, degradation products and matrix components. Lack of specificity of an individual analytical procedure may cause incorrect quantitative and qualitative analysis. Specificity of the method should be evaluated by preparing blank samples with and without the addition of analytes and injecting them to test for interferences (ICH, 2006).

The International Conference on Harmonisation (ICH) documents state that comparing chromatographic peak of analyte is useful to show the degree of selectivity. In this study, specificity was investigated by injecting the standard

solutions, synthetic mixture solutions and sample solutions. Then, peak purity test of each solution was compared.

Procedure

The specificity of the method was presented by the comparison of HPLC chromatograms of imperatorin solution concentratin of 2.98 µg/ml, *A. marmelos* fruit extract solution (100 µg/ml) and synthetic mixture (sample solution spiked with imperatorin).

Linearity and range

The linearity of an analytical procedure is its ability to obtain test results that are directly proportional to the concentration of analyte in the sample within a given range. Linearity is generally reported as the variance of slope of regression line calculated according to an established mathematical relationship from test results obtained by the analysis of samples with varying concentrations of analyte.

The linear coefficient of determination (R^2) was calculated by plotting the observed concentration against the actual concentration. The value of R^2 should be greater than 0.9995 according to USP guidance.

Procedure

Five concentrations (2.98, 5.88, 8.82, 11.78 and 14.72 µg/ml) of imperatorin standard solution were prepared by three replicates and injected into the HPLC system. Peak area was plotted against its concentration to construct the calibration curve. The linearity was assessed by means of linear regression to obtain the R^2 value.

Limit of detection (LOD) and limit of quantitation (LOQ)

The detection limit is a characteristic of limit test. It is the lowest amount of analyte in a sample that can be detected under the stated experimental conditions. The detection limit is generally determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected. Typically acceptable signal-to-noise ratios are 2 : 1 or 3 : 1. Other approaches depend on the determination of the slope of the calibration curve and the standard deviation of responses.

Peaks are obscured when their peak height becomes very similar in magnitude to the noise of the detector system. Detector noise is defined as any perturbation on the detector output that is not related to an eluting solute. The source of the noise can arise from the chromatographic system, the sensor or the associated electronics. Thus, the signal from the peak must be sufficiently greater than the noise to unambiguously

identify the peak. The ratio of the signal size to that of the noise is termed the signal-to-noise ratio.

The quantitation limit is a characteristic of quantitative assays for low levels of compounds in sample matrices. It is the lowest amount of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions. The quantitation limit is generally determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be determined with acceptable accuracy and precision. Typically acceptable signal-to-noise ratio is 10 : 1. Other approaches depend on the determination of the slope of the calibration curve and the standard deviation of responses.

In this study, the limit of detection (LOD) and limit of quantitation (LOQ) were determined, serial dilutions of imperatorin were prepared with methanol, and were then analyzed with HPLC method. LOD and LOQ were calculated from the results of analysis of sample solutions by plotting the curve between concentration against peak area. The equation for LOD and LOQ are 3.3(SD/S) and 10(SD/S), respectively, where, SD is the residual standard deviation of the regression line and S is the slope.

3.2.4 Quantitative determination of *A. marmelos* extracts

Quantification was carried out by comparing the peak area of imperatorin obtained from *A. marmelos* extract with standard curve plotted between peak areas and concentrations of standard imperatorin.

To quantify imperatorin in each *A. marmelos* extracts using the HPLC condition, a concentration of extract solutions and five dilutions of standard solution were all injected in triplicate through the HPLC system.

Preparation of standard and sample solutions

Stock standard solutions of imperatorin were prepared with methanol to obtain the calibration range of 2.96-10.89 µg/ml. *A. marmelos* fruit extracts were three time accurately weighting and prepared at approximately concentrations of 100 µg/ml (extract A), 3,800 µg/ml (extract B) and 20,000 µg/ml (extract C). The sample solutions were filtered through a 0.2 µm membrane filter before use.

A. Preparation of standard solutions

The stock solution was prepared by accurately weighing 1.01 mg of imperatorin to a 10-ml volumetric flask and adjusting to volume with methanol. The concentration of standard stock solution was 101.00 µg/ml. Then, the stock solution was pipetted at 300, 500, 700, 900 and 1,100 µl respectively, to a 10-ml volumetric flask and adjusted to volume with methanol. The concentrations of imperatorin standard used for calibration were 3.03, 5.05, 7.07, 9.09 and 11.11 µg/ml (the actual concentrations were 2.96, 4.95, 6.93, 8.91 and 10.89 µg/ml).

B. Preparation of the sample solutions

Stock solution of sample

Extract A 109.00 mg, 104.00 mg and 105.00 mg (n=3) were accurately weighed into a 100-ml volumetric flask and adjusted to volume with methanol. The concentrations of sample stock solution were 1,090 µg/ml, 1,040 µg/ml and 1,050 µg/ml, respectively.

Extract B 3,873.00 mg, 3,836.00 mg and 3,840.00 mg (n=3) were accurately weighed into a 100-ml volumetric flask and adjusted to volume with methanol. The concentrations of sample stock solution were 38,730 µg/ml, 38,360 µg/ml and 38,400 µg/ml, respectively.

Extract C 19,242.00 mg, 23,246.00 mg and 20,175.00 mg (n=3) were accurately weighed into a 100-ml volumetric flask and adjusted to volume with methanol. The concentration of sample stock solution were 192,420 µg/ml, 232,460 µg/ml and 201,750 µg/ml, respectively.

Sample solutions

One ml of sample stock solution of extract A was pipetted to a 10-ml volumetric flask and adjusted to volume with methanol. The concentrations of sample solution were 109 µg/ml, 104 µg/ml and 105 µg/ml, respectively.

One ml of sample stock solution of extract B was pipetted to a 10-ml volumetric flask and adjusted to volume with methanol. The concentrations of sample solution was 3,873 µg/ml, 3,836 µg/ml and 3,840 µg/ml, respectively.

One ml of sample stock solution of extract C was pipetted to a 10-ml volumetric flask and adjusted to volume with methanol. The concentration of sample solution was 19,242 µg/ml, 23,246 µg/ml and 20,175 µg/ml, respectively.

After prepared and diluted to the appropriate concentrations, the sample solutions were filtered through a 0.2-µm syringe filter prior to HPLC analysis.

3.2.5 *In vitro* assay of CYPs inhibition test

A. marmelos extracts and imperatorin were prepared and screened for inhibitory effects on CYPs (CYP1A2, CYP3A4, CYP2C9 and CYP2C19) using Vivid® CYP450 screening kits (Invitrogen™, USA).

The kit provides specific BACULOSOME CYP450 which are microsomes prepared from insect cells expressing a human P450 isozyme and rabbit NADPH-P450 reductase. BACULOSOME CYP450 cleavage at either of two potential cleavage sites of substrate which are blocked dyes. Fluorescent product obtained from the reaction is quantitatively determined. (Figure 10)

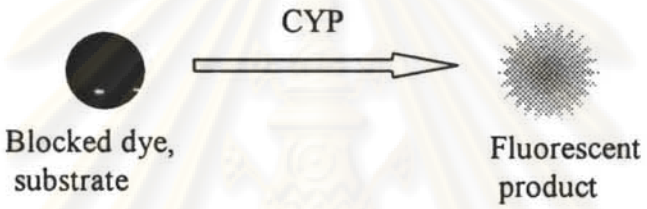
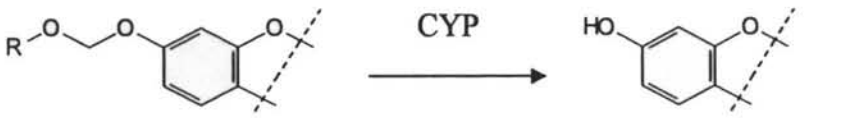


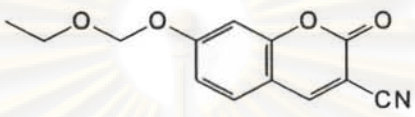
Figure 10 Principle of fluorescent dye assay on CYP activity

Vivid® substrate are alkoxymethyl or benzyloxymethyl ethers of fluorescent dyes. In this study, fluorescent substrates for the CYP are ethoxycoumarin (EOMCC) (3 μM for CYP1A2 or 10 μM for CYP 2C19) and 7-benzyloxymethyloxy-3-cyanocoumarin (BOMCC) (10 nM for CYP2C9 or 5 nM for CYP3A4). The structures of substrate used in this study are indicated in Figure 11.

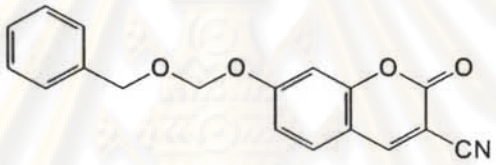


Vivid® substrate
(EOMCC or BOMCC)

fluorescent product



EOMCC
(ethoxycoumarin)



BOMCC
(7-benzyloxymethyloxy-3-cyanocoumarin)

Figure 11 Cleavage and structures of Vivid® substrates

3.2.6 Verification of CYPs inhibition test

The CYP screening tests were verified with the known specific inhibitors (α-naphthoflavone for CYP1A2, ketoconazole for CYP3A4, sulfaphenazole for CYP2C9 and miconazole for CYP2C19) (Marques-Soares *et al.* 2003; Monostory *et al.* 2004). Results of verification test were in agreement with the reported CYP interaction of these drugs.

3.2.6.1 Preparation of inhibitor solutions

A. Preparation of α-naphthoflavone solutions

α-Naphthoflavone stock solution (10,000 μM in 100% DMSO) was prepared by accurately weighing 2.72 mg of α-naphthoflavone to a 1.5-ml eppendorf then mixing well with 1,000 μl of DMSO until it became clear solution. Then, the 25 μl of stock solution was pipetted to 1.5-ml eppendorf and mixed well with 975 μl of water to obtain the final concentration of 250 μM in 2.5% DMSO. Several concentrations of α-naphthoflavone was prepared by serially diluted with 2.5% DMSO for use in enzyme assay (Table 12).

α-Naphthoflavone concentration of 250 μM in 2.5% DMSO was prepared by serially diluted with 2.5% DMSO. Several concentrations of α-naphthoflavone solutions were freshly prepared

B. Preparation of ketoconazole solutions

Ketoconazole stock solution (4,585 μM, 100% ACN was prepared by accurately weighing 2.44 mg of ketoconazole to a 1.5-ml eppendorf then mixing well with 1,000 μl of ACN until it became clear solution. Then, the 25 μl of stock solution was pipetted to 1.5-ml eppendorf and mixed well with 975 μl of water to obtain the final concentration of 114.63 μM in 2.5% ACN. Then, the drug solution was serially diluted with 2.5% ACN for enzyme assay (Table 12).

C. Preparation of sulfaphenazole solutions

Sulfaphenazole stock solution (3,000 μM 100% of DMSO) was prepared by accurately weighing 9.43 mg of sulfaphenazole to a 10-ml volumetric flask then mixing and adjusting well with DMSO until it became clear solution. Then, the 25 μl of stock solution was pipetted to 1.5-ml eppendorf and mixed well with 975 μl of water to obtain the final concentration of 75 μM in 2.5% DMSO. The serial concentration of sulfaphenazole was prepared by diluted with 2.5% DMSO (Table 12).

D. Preparation of miconazole solutions

Miconazole stock solution (100 µM, 100% DMSO) was prepared by accurately weighing 0.48 mg of miconazole to a 10-ml volumetric flask then mixing and adjusting well with DMSO until it became clear solution. Then, the 25 µl of stock solution was pipetted to 1.5-ml eppendorf and mixed well with 975 µl of water to obtain the final concentration of 2.5 µM in 2.5% DMSO which was serially diluted with 2.5% DMSO to obtain the test concentrations of miconazole solutions (Table 12).

Table 12 Concentration of CYP inhibitors per reaction volume (100 µl)

Concentration of CYP inhibitors* (µM)			
CYP1A2	CYP3A4	CYP2C9	CYP2C19
α-Naphthoflavone	Ketoconazole	Sulfaphenazole	Miconazole
0.005	0.11	0.001	0.01
0.01	0.275	0.01	0.025
0.025	0.55	0.05	0.05
0.05	2.2	0.25	0.1
0.1	11	2	0.25
0.25	55	10	0.5
0.5		30	1
1			

*The solvents for CYP1A2, 2C9, 2C19 was 1% DMSO and for CYP3A4 was 1% ACN, in reaction volume.

3.2.6.2 Stop solution preparation

The 0.5 M Tris base (pH 10.5) solution was prepared by accurately weighing 15.1425 g of Tris base to a 250-ml volumetric flask and then adjusting to volume with water. Thereafter, the stop solution was adjusted to pH 10.5 with NaOH and HCl by using pH meter and was kept at 4°C.

3.2.6.3 Procedure of CYP inhibitory assay

The reactions were performed in black, flat-bottomed, 96-well plates with a total reaction volume of 100 µl/well. The plates were incubated at room temperature and fluorescence values were measured on a Wallac 1420 fluorescence plate reader using excitation wavelength at 409 and emission wavelengeth at 460 nm. Cytochrome P450 assay was divided in two stages: preparation stage and assay stage.

A. Preparation stage

The preparation stage of the assay was started with thawing all the frozen kit components and keeping them on ice until ready to use. The reaction buffer and water were kept at room temperature. The next step was reconstitution of the fluorescent substrate with an addition of designated volume of acetonitrile to each vial containing 0.1 mg of substrate, as shown in Table 13. The mixture was mixed by vortex. Since the reconstituted substrate was used immediately, it was kept at room temperature; otherwise, it should be kept at -20°C.

Table 13 Reconstitution of the Vivid® CYP450 substrate

Isozyme type	Vivid® CYP450 substrate, mg per container	Reconstitution with anhydrous acetonitrile, µl
1A2	EOMCC, 0.1	205
3A4	BOMCC, 0.1	160
2C9	BOMCC, 0.1	160
2C19	EOMCC, 0.1	205

The reconstituted substrate was then premixed with $NADP^+$ by adding reaction buffer with substrate and $NADP^+$. The amount of those reagents are shown in Table 14. Compounds were mixed together by vortex. The resulting solution was stable at room temperature for several hours.

Table 14 Pre-mixing Vivid® CYP450 substrate and $NADP^+$

Isozyme type	Vivid® reaction buffer, µl	Reconstituted substrate, µl	$NADP^+$, µl
1A2	885 (Buffer I)	15	100
3A4	850 (Buffer I)	50	100
2C9	850 (Buffer II)	50	100
2C19	850 (Buffer II)	50	100

Reaction buffer I was 200 mM potassium phosphate buffer

Reaction buffer II was 100 mM potassium phosphate buffer

The next step was to preprare the master-premix solution by mixing BACULOSOMES® reagent (CYP and NADPH-reductase), regeneration system (333 mM glucose-6-phosphate and 30 U/ml glucose-6-phosphate dehydrogenase in 100 mM potassium phosphate buffer (pH 8.0)) and reaction buffer. The amount of those reagents are shown in Table 15. The master pre-mix solution was mixed by inversion and was stored on ice before use.

Table 15 Master pre-mix (pre-mix of CYP450 BACULOSOMES® reagents and regeneration system)

Isozyme type	Vivid® reaction buffer , µl	Regeneration system, µl	CYP450 BACULOSOMES® reagent, µl
1A2	4850 (Buffer I)	100	50
3A4	4850 (Buffer I)	100	50
2C9	4800 (Buffer II)	100	100
2C19	4850 (Buffer II)	100	50

Reaction buffer I was 200 mM potassium phosphate buffer

Reaction buffer II was 100 mM potassium phosphate buffer

B. Assay stage

Briefly, the different concentrations of drug solution (n=2) were dispensed duplicately into 96-well black plates. Then, the different concentrations of drug solutions were dispensed 40 µl per well. The control wells were dispensed with solvents of each test compound with the same amount. To prevent fluorescent errors in the system, The test-background wells were applied for each concentration. Then, 50 µl of the master pre-mix was dispensed into the tested well and 50 µl of the reaction buffer for test-background wells. The plate then was preincubated for 20 minutes at room temperature. The reaction was initiated with the addition of the combined $NADP^+$ and either the substrate solution to all wells. The plate was protected from light and incubated at room temperature for proper reaction time (approximately 30 minutes for CYP1A2, 2C19, 3A4 and 40 minutes for CYP2C9). The reactions were stopped with 10 µl of 0.5 M Tris base (pH 10.5) to all wells. Lastly, the plate was measured the fluorescence to acquire the results for analysis.

3.2.7 *In vitro* assay of inhibitory effects of *A. marmelos* fruit extract and imperatorin on CYPs

3.2.7.1 Preparation of sample solutions

A. Stock solution and working solution of extract A

The sample stock solution of extract A was prepared by accurately weighing 174.22 mg of extract A to a 10-ml volumetric flask and adjusting to volume with dimethyl sulfoxide (DMSO). The final stock concentration of 17,422 µg/ml was obtained.

Then, the stock solution was pipetted 25 µl to 1.5-ml eppendorf and added with 975 µl of water to obtain the sample solution at concentration of 435.50 µg/ml of 0.25% DMSO. The sample stock solution was serially diluted with 0.25% DMSO before use in enzyme assay (Table 16).

B. Stock solution and working solution of extract B

The sample stock solution of extract B was prepared by accurately weighing 50.00 mg of extract B to a 10-ml volumetric flask and adjusting to volume with water. The final stock concentration of 5,000 µg/ml was obtained. The sample stock solution was serially diluted with water prior to use in the assay (Table 16).

C. Stock solution and working solution of extract C

The sample stock solution of extract C was prepared by accurately weighing 125.00 mg of extract C to a 10-ml volumetric flask and adjusting to volume with water. The final stock concentration was 12,500 µg/ml, which was serially diluted with water before use (Table 16).

D. Stock solution and working solution of imperatorin

Imperatorin stock solution (20 mM, 100% of acetronitrile, ACN) was prepared by accurately weighing 5.41 mg of imperatorin to a 1.5-ml eppendorf then mixing well with 1 ml of ACN until it became a clear solution. Then, the 25 µl of stock solution was pipetted to 1.5-ml eppendorf and mixed well with 975 µl of water to obtain the final concentration of 500 µM in 2.5% of ACN.

Imperatorin concentration of 500 µM in 2.5% of ACN was serially diluted with 2.5% of ACN. Several concentrations of imperatorin solutions were freshly prepared before use in enzyme assay (Table 16).

Table 16 Concentration of test sample solutions in reaction volume (100 µl) for CYP assay

Concentration of test sample for CYP assay															
[1]Imperatorin, µM				[2]Extract A, µg/ml				[3]Extract B, µg/ml				[3]Extract C, µg/ml			
1A2	3A4	2C9	2C19	1A2	3A4	2C9	2C19	1A2	3A4	2C9	2C19	1A2	3A4	2C9	2C19
0.06	0.11	0.11	0.06	0.1	0.04	0.1	0.5	10	0.4	10	10	50	50	50	10
0.11	0.55	1.1	0.11	0.5	0.99	1	1	20	4	100	50	100	250	100	50
0.55	1.1	5.5	1.1	1	1.98	2	2	50	40	250	100	250	500	250	100
1.1	2.2	11	2.5	2	3.96	5	5	100	100	750	250	500	750	500	250
2.2	5.5	55	5.5	5	7.92	10	10	250	200	1,000	500	750	1,500	750	500
5.5	22	110	11	10	12		15	500	400		750	1,000	1,600	1,000	750
22			55		19.16				800		1,000	1,500	2,000	1,500	1,000
			110										3,200	3,000	1,500
			165												2,000
			220												

1 = solvent was 2.5%ACN; final solvent concentration was 1% in reaction volume (100 µl).

2 = solvent was 0.25%DMSO; final solvent concentration was 0.1% in reaction volume (100 µl).

3 = solvent was water

3.2.7.2 Inhibition test of *A. marmelos* and imperatorin on CYPs

Briefly, assay stage was composed of 4 steps. Firstly, in order to achieve results for IC_{50} determination of *A. marmelos* extracts and imperatorin, 96-well plate was measured for fluorescence intensity before use for background correction of the corresponding wells. The sample solutions, control solution, test-background solution and control background solution (40 µl/well) were duplicately dispensed into two 96-well plates at various times (n=4 for each concentration) (Figure 12). Background fluorescence was determined to prevent fluorescent errors in the system.

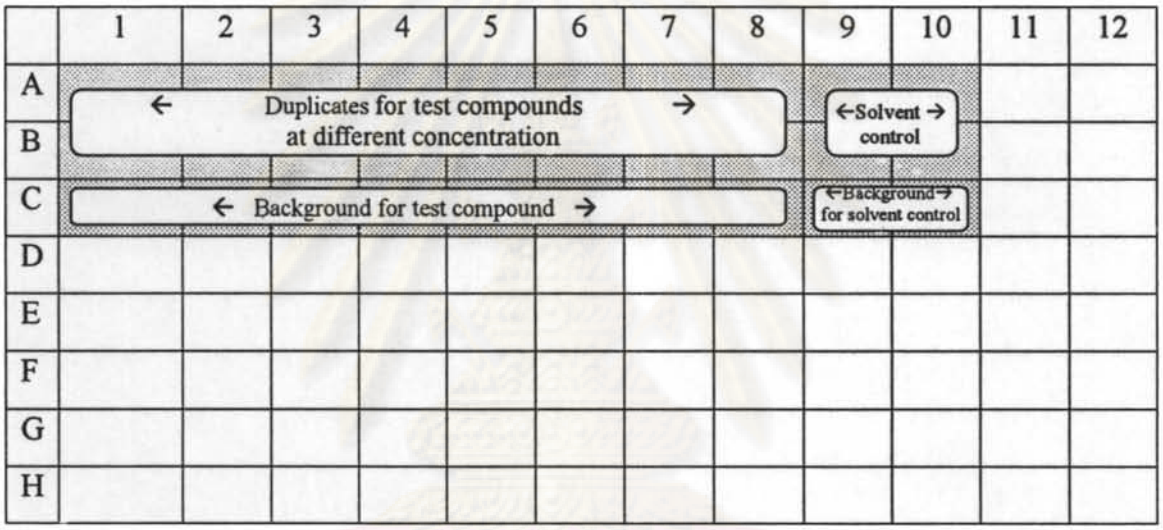


Figure 12 The overview of test solution in 96-well plate

Secondly, the 50 µl of the master pre-mix was dispensed into the tested well and controlled well. While, 50 µl of the reaction buffer was dispensed for test-background well. The plate then was preincubated for 20 minutes at room temperature. In third step, the reaction was initiated with the addition of the combined $NADP^+$ and the substrate solution to all wells. The plate was protected from light and incubated at room temperature for the proper reaction time (30 minutes for CYP1A2, 2C19, 3A4 and 40 minutes for CYP2C9). The last step was to stop the reaction with 10 µl of 0.5 M Tris base (pH 10.5) to all wells (Figure 13). Then, the plate was measured the fluorescence to acquire the results for analysis.

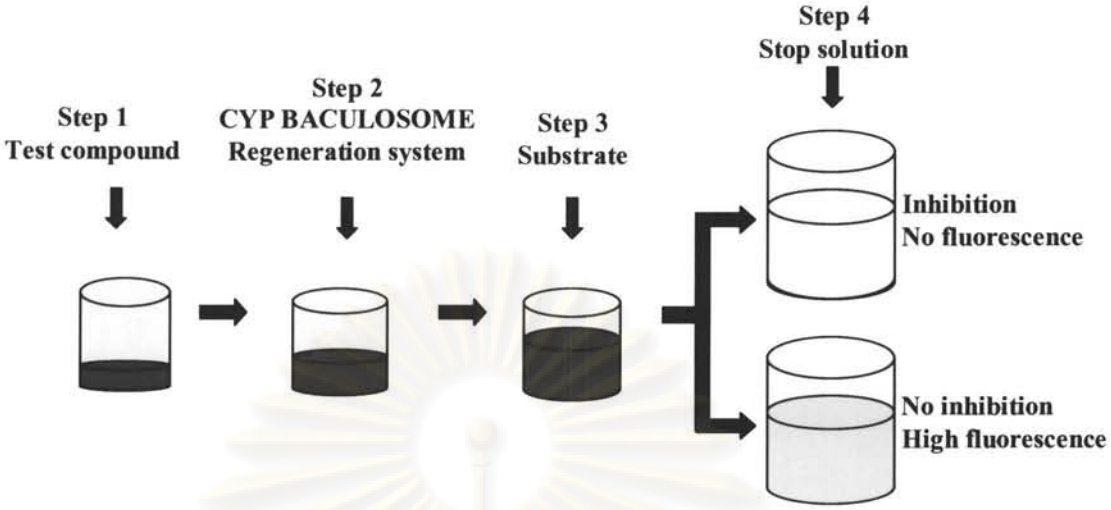


Figure 13 Four steps of enzyme inhibition assay

3.2.8 Data analysis

Inhibitory effects of *A. marmelos* fruit extract and imperatorin were estimated from the fluorescence of metabolites at the time point. Fluorescence data were exported and analyzed for percentage of inhibition using a Microsoft Excel spreadsheet.

Calculation of percentage of inhibition

$$\% \text{ inhibition} = \left[1 - \frac{(RFU_{test\ compound} - RFU_{background\ of\ test\ compound})}{(RFU_{solvent} - RFU_{background\ of\ solvent\ control})}\right] \times 100 \qquad (9)$$

where,

RFU is relative fluorescent unit or fluorescent intensity

Percent inhibition of the sample was plotted versus the log value of each test compound concentration to generate a inhibitory curve. Nonlinear regression was performed with GraphPad Prism software (San Diago, CA) using an equation for sigmoidal dose-response (variable Hill slope) to determine the IC_{50} value.

3.2.9 Statistical analysis

All numeric data were presented as mean ($\overline{X}$) ± standard deviation (SD). Mean differences between *A. marmelos* and imperatorin groups were compared using one-way analysis of variance (ANOVA) and Least-Significant Different post hoc (LSD post hoc), and $p<0.05$ was considered to be significant (กัลยา วานิชย์บัญชา, 2548).

CHAPTER IV
RESULTS AND DISCUSSION

4.1 Extraction of *A. marmelos* fruit

A. marmelos is one of the most important medicinal plants used in the indigenous system of medicine. Its fruit has been traditionally used as antidiarrhoea and antidysentery. *A. marmelos* pulp can be edible freshly or used to make marmalade, candied fruit, cake or cream while the water extract of dried fruit is used to make tea and popular refreshing drink. Since the active ingredients in the fruit are various in biological and physical properties, different solvents are used for extraction of the compounds from fruit. The solvents that are used for the extraction include hydrophobic solvents such as acetone, hexane and pentane and hydrophilic solvent such as water, ethanol and methanol (Sharma and Sharma, 1981; Ratan *et al.*, 1981; Tokitomo *et al.*, 1982; รพีพล ภโววาท และ พิมลพรรณ พิทยานุกูล, 2529; Banerji *et al.*, 1988; Noysang, 1998; Rahman *et al.*, 2004).

The fruit of *A. marmelos* was reported to be the source of imperatorin which is the bioactive substance. Imperatorin indicated several pharmacological activities such as antibacterial, anticoagulant, antiplatelet aggregation. It also showed the effect on drug metabolism by being cytochrome P450 inhibitor (Cai *et al.*, 1993; Chen *et al.* 1996; Kleiner *et al.*, 2002). According to the fact that imperatorin is insoluble in cold water, sparingly soluble in boiling water but soluble in organic solvent (Budavari *et al.*, 1996). The solvents such as acetone, n-pentane, hexane and methanol have been used for extraction (Sharma, B. R. and Sharma, P., 1981; Noysang, 1998; Rahman *et al.*, 2004).

In this study, the solvents with different polarity, ethanol, hexane and water, were used (Tokitomo *et al.*, 1982; Noysang, 1998; Prommetta *et al.*, 2006). The hexane extract (extract A) of *A. marmelos* appeared brown solid matter while the ethanolic extract (extract B) was the red-brown solid matter. The lyophilized powder of water extract (extract C) appeared yellow. All of the fruit extracts were kept at 0°C for further analysis.

4.1.1 Determination of total yield of *A. marmelos* extracts

Total yields of the extracts (extracts A, B and C) were quantified in percentage weight obtained per dried *A. marmelos* ground fruit. Total yields of extracts A, B and C were 0.56%, 7.69% and 21.54%, respectively. (Table 17).

Table 17 The extracts of *A. marmelos* fruit

Solvent extraction	Initial weight (g)	Extractive weight (g)	Extractive color	% Total yield, w/w
Hexane (extract A)	1000	5.5524	Brown	0.56
Ethanol (extract B)	1000	76.9282	Red brown	7.69
Water (extract C)	100	21.5368	Yellow	21.54

Noysang (1998) found that the yield contents of hexane and methanolic extracts of *A. marmelos* were 0.6 % and 20.4%, respectively. The similar trend was found in the present study in that the less polar solvent gave the less amount of extract.

4.2. Quantitative determination of imperatorin in *A. marmelos* extracts

4.2.1. Suitability of HPLC analysis of imperatorin

HPLC condition in this study was modified from method of Li (2006) in which 40 : 60%, v/v water : methanol was used instead of 30 : 70%, v/v water : methanol. When using 30 : 70%, v/v water : methanol, the retention times of 1 μg/ml of imperatorin and mobile phase were 5.87 and 1.63 minutes, respectively. Upon adjusting the gradient elution of mobile phase to 40 : 60%, v/v water : methanol, the longer retention time of imperatorin chromatogram of 11.36 minutes was obtained.

In this study, the system suitability was tested to ensure that the validity of the analytical parameters, retention time, resolution, tailing factor and peak area. Imperatorin standard solution at the concentration of 9 μg/ml was injected for six

replicates into the HPLC column. ICH (2006) recommends that the relative standard deviation (%RSD) of all parameters should be lower than 2 %. From the results, %RSD of retention time, peak area and tailing factor are 0.406, 0.088 and 0.246, respectively (Table 18), which were suitable for quantitative analysis.

Table 18 Retention time, peak area and tailing factor from modified chromatographic condition of the HPLC analysis of imperatorin

Injection number	Retention time (minute)	Peak area ($mAU \cdot s^{-1}$)	Tailing factor
A	11.324	337074	1.141
B	11.360	337238	1.143
C	11.388	337175	1.145
D	11.392	337479	1.143
E	11.410	337667	1.146
F	11.289	336828	1.149
SD	0.046	297	0.003
mean	11.361	337243	1.145
%RSD	0.406	0.088	0.246

4.2.2 Validation of HPLC analysis of imperatorin

A method for an assay of imperatorin in *A. marmelos* extracts was validated according to USP32 NF27 guidance.

Accuracy

Accuracy of the method was studied at five different concentrations of synthetic mixture solutions (n=3). From the results summarized in Table 19, it showed that %recovery of imperatorin at five concentrations ranged between 97.69 and 100.29 and the relative standard deviation (%RSD) ranged from 0.12 to 1.27.

ICH (2006) recommends that the mean recovery of test compound should be coverage in the range of 98-102% and %RSD of all samples should be lower than 2 %. Therefore, the accuracy of the proposed method was within the acceptable criteria.

Table 19 Accuracy test of imperatorin assayed by the HPLC method

Samples	Actual conc.(μg/ml)	Observed conc.(μg/ml)	Recovery (%)	Average of %recovery (range)	RSD (%)
1A	2.9439	2.9491	100.17	100.10	0.20
1B	2.9439	2.9399	99.86	(99.86-100.25)	
1C	2.9439	2.9512	100.25		
2A	5.8878	5.7874	98.29	98.86	1.10
2B	5.8878	5.8945	100.11	(98.18-100.11)	
2C	5.8878	5.7807	98.18		
3A	8.8318	8.7960	99.60	98.43	1.04
3B	8.8318	8.6276	97.69	(97.69-99.60)	
3C	8.8318	8.6563	98.01		
4A	11.7757	11.8098	100.29	98.87	1.27
4B	11.7757	11.5899	98.42	(97.90-100.29)	
4C	11.7757	11.5289	97.90		
5A	14.7196	14.7330	100.09	99.98	0.12
5B	14.7196	14.7183	99.99	(99.85-100.09)	
5C	14.7196	14.6973	99.85		

Precision

The determination of precision of the analytical method for the assay of imperatorin was performed at with six replicated sample solution which imperatorin concentration of 8.8318 μg/ml was spiked to the samples.

The intra-day precision

The measured concentrations, %recovery and %RSD obtained from HPLC analysis are all shown in Tables 20-22. Precision should be lower than 2% of RSD value and the recovery should be coverage in the range of 98-102% (ICH, 2006).

The %recovery in day 1 (97.69 to 100.76), day 2 (97.82 to 100.58) and day 3 (98.60 to 100.72) were in agreement with the guideline. All of the %RSD, 1.31, 1.07 and 0.72 in day 1, 2 and 3, respectively, were lower than 2.

Table 20 Precision test of imperatorin assayed by the HPLC method (day 1)

Samples	Actual conc. (μg/ml)	Observed conc.(μg/ml)	Recovery (%)	Average of %recovery (range)	RSD (%)
A	8.8318	8.7960	99.5954	99.45	1.31
B	8.8318	8.6276	97.6883	(97.69-100.76)	
C	8.8318	8.6563	98.0128		
D	8.8318	8.8498	100.2044		
E	8.8318	8.8987	100.7586		
F	8.8318	8.8695	100.4268		

Table 21 Precision test of imperatorin assayed by the HPLC method (day 2)

Samples	Actual conc. (μg/ml)	Observed conc.(μg/ml)	Recovery (%)	Average of %recovery (range)	RSD (%)
A	8.8318	8.8298	99.98	99.43	1.07
B	8.8318	8.6943	98.44	(97.82-100.58)	
C	8.8318	8.8037	99.68		
D	8.8318	8.8826	100.58		
E	8.8318	8.8371	100.06		
F	8.8318	8.6393	97.82		

Table 22 Precision test of imperatorin assayed by the HPLC method (day 3)

Samples	Actual conc. (μg/ml)	Observed conc.(μg/ml)	Recovery (%)	Average of %recovery (range)	RSD (%)
A	8.8318	8.7824	99.44	99.79	0.72
B	8.8318	8.8956	100.72	(98.60-100.72)	
C	8.8318	8.8233	99.90		
D	8.8318	8.8314	100.00		
E	8.8318	8.8383	100.07		
F	8.8318	8.7079	98.60		

The inter-day precision

Data of %recovery of imperatorin obtained from 3 days of analysis are tabulated in Table 23. The percentage of RSD at three days of spiked imperatorin in sample solution were 0.17. Precision of inter-day was within the limit range (98-102% recovery and RSD±2%). Therefore, this analytical method revealed acceptable precision.

Table 23 Inter-day precision (%RSD) of assay for quantification of imperatorin

Samples	%Recovery		
	Day 1	Day 2	Day 3
A	99.5954	99.98	99.44
B	97.6883	98.44	100.72
C	98.0128	99.68	99.90
D	100.2044	100.58	100.00
E	100.7586	100.06	100.07
F	100.4268	97.82	98.60
mean	99.45	99.43	99.79
%RSD	0.17		

Specificity

Specificity of the HPLC analysis was investigated by determining the retention time, resolution and asymmetry of imperatorin chromatogram. Resolution (Rs) is a measurement of how well two peaks are separated. The separation of all peaks of interest is checked visually or calculated by equation below:

$$Rs = \frac{2(t_1 - t_2)}{(w_1 + w_2)} \quad (10)$$

where,

t_1 is retention time of imperatorin peak

t_2 is retention time of impurity peak which is next to imperatorin peak

w_1 is the peak width measured at baseline of imperatorin peak

w_2 is the peak width measured at baseline of impurity peak which is next to imperatorin peak

While, tailing factor (Tf) is a measurement of peak symmetry calculating from peak width at 5% of peak height. Tf = 1.0 indicates a perfectly symmetrical, Tf > 2 indicates as a tailing peak that is typically not acceptable due to difficultly in integrating the peak area precisely.

$$\text{Tf} = \frac{A+B}{2A} \quad (11)$$

where,

A is peak front half-width measured at 5% of peak height

B is peak back half-width measured at 5% of peak height

Chromatogram of imperatorin standard at approximately 3 μg/ml, chromatogram of *A. marmelos* extract sample (concentration of 100 μg/ml) spiked with the same amount of imperatorin standard and chromatogram of *A. marmelos* sample (unknown sample or blank sample) were analyzed and compared for confounding impurity overlap in peak of imperatorin. Amount of imperatorin spiked in the sample should not be significantly different (p±0.05) from amount of standard imperatorin.

Using the integrated program, resolution and asymmetry were calculated. From the results, retention time of chromatogram of imperatorin was 11.19 min. There was no peak of impurity around peak of imperatorin in extract sample. Hence, the resolution (Rs) of different peaks (peaks of sample and impurity) could not be calculated. Asymmetry values as referred to the tailing factor of peak of extract sample were found to be 1.145 and 1.241 which were less than 1.5 (Figure 14); hence, the method was specific to the HPLC analysis of imperatorin. There are no statistically significant difference between peak areas (p=0.278) or concentrations (p=0.261) of standard imperatorin and spiked imperatorin (Table 24).

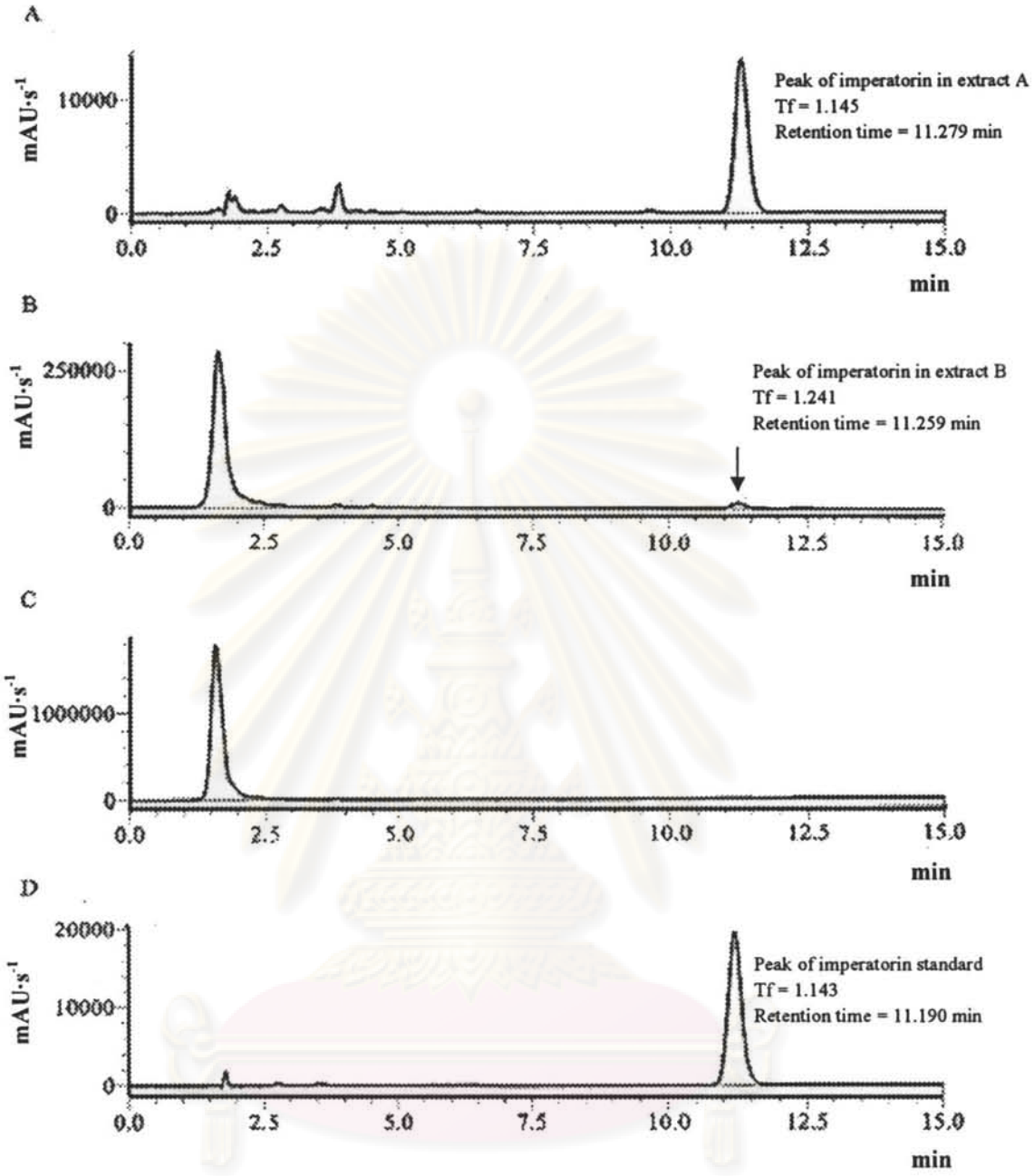


Figure 14 Representative chromatograms of *A. marmelos* fruit sample solution, (A) extract A (105 µg/ml); (B) extract B (3,840 µg/ml) and (C) extract C (20,175 µg/ml), and of (D) imperatorin standard solution (2.98 µg/ml)

Table 24 Comparison of peak area and concentration between standard imperatorin and spiked imperatorin

Standard imperatorin		Spiked imperatorin	
Average peak area ($mAU \cdot s^{-1}$)	Actual concentration (µg/ml)	Average peak area ($mAU \cdot s^{-1}$)	Observed concentration (µg/ml)
113,745	2.9439	113,663	2.9406
113,572	2.9439	111,815	2.8920
113,765	2.9439	113,413	2.9340

Linearity and range

The linearity of the method was studied at five concentrations of standard solutions (2.94, 5.89, 8.83, 11.78 and 14.72 µg/ml). The selected concentrations covered the range of each analyte in all test samples (2.94-14.72 µg/ml). The linear regression line for the response (peak area) versus concentration plot is shown in Figure 15. The linearity equation was listed below:

$$y = 38{,}080x + 1{,}685.8 \quad (12)$$

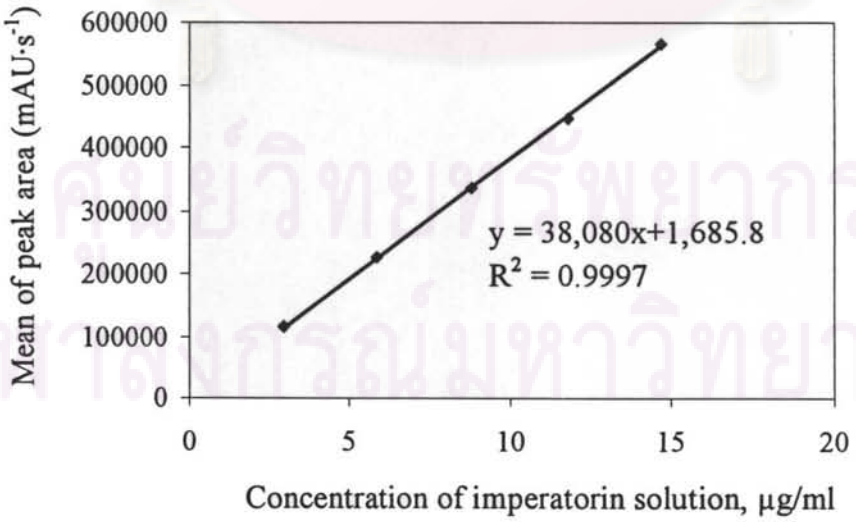


Figure 15 The linear regression line plotted between concentration of standard imperatorin solution and mean of peak area ($mAU \cdot s^{-1}$)

Acceptability of linearity data is often judged by examining the coefficient of determination (R^2) and y-intercept of the linear regression line for the peak area versus concentrations plot. A coefficient of determination of greater than 0.999 is generally considered as evidence of acceptable fit of the data to the regression line. The y-intercept should be less than a few per cent of the response obtained for the analyte at target level (Green, 1996). The y-intercept of the linear regression line (equation 12) was 1,685.8, which was less than 2% of peak area at target level (mean of peak area at the target level (8.83 μg/ml) was 336,419 $mAU \cdot s^{-1}$). Hence, the linearity method was validated.

From the result, data of observed and actual concentrations are shown in Table 25, while the linearity curve plotted between actual (x) and observed (y) was shown in Figure 16. A plot between the observed and actual concentrations was linear with coefficient of determination (R^2) of imperatorin of 0.9998. The linearity equation for the actual versus observed concentrations plot was listed in equation 13.

$$y = 0.9973x - 0.0443 \quad (13)$$

Table 25 Linearity test of imperatorin assayed by HPLC method

Samples	Actual concentration (μg/ml)	Average actual concentration (μg/ml)	Observed concentration (μg/ml)	Average observed concentration (μg/ml)
1A	2.9439	2.9439	2.9491	2.9467
1B	2.9439		2.9399	
1C	2.9439		2.9512	
2A	5.8878	5.8878	5.7874	5.8209
2B	5.8878		5.8945	
2C	5.8878		5.7807	
3A	8.8318	8.8318	8.7960	8.6933
3B	8.8318		8.6276	
3C	8.8318		8.6563	
4A	11.7757	11.7757	11.8098	11.6429
4B	11.7757		11.5899	
4C	11.7757		11.5289	
5A	14.7196	14.7196	14.7330	14.7162
5B	14.7196		14.7183	
5C	14.7196		14.6973	

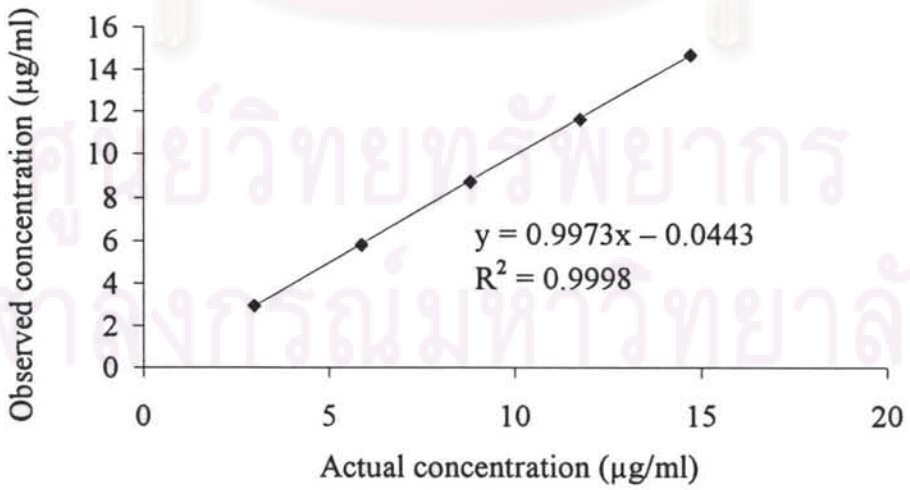


Figure 16 The representative linearity curve plotted between actual and observed concentrations of imperatorin

Limit of detection (LOD) and Limit of quantitation (LOQ)

The LOD and LOQ of the method were studied at five theoretical concentrations of imperatorin standard solutions (0.06, 0.13, 0.25, 0.50 and 1.00 μg/ml). Slope of the calibration curve of imperatorin standard solutions and the standard deviation of responses were measured and calculated for LOD and LOQ. The equation for LOD is 3.3(SD/S) and LOQ is 10(SD/S); where, SD is the residual standard deviation of the regression line and S is the slope. The LOD and LOQ of imperatorin were 0.035 and 0.106 μg/ml, respectively (Table 26).

Table 26 LOD and LOQ data of imperatorin assayed by the HPLC method

Sample	Theoretical conc. (μg/ml)	Peak Area ($mAU \cdot s^{-1}$)	Regression parameters	LOD (μg/ml)	LOQ (μg/ml)
Imperatorin	0.06	2,454	Slope = 42,318.1	0.035	0.106
	0.13	5,379	Intercept = 127.75		
	0.25	10,723	$R^2 = 0.9997$		
	0.50	21,928	SD = 448.81		
	1.00	42,146			

From the overall results, it can be seen that qualitative and quantitavite determination of imperatorin in *A. marmelos* extracts using HPLC analysis were validated and passed the criteria according to USP32 NF29 guidance as summarized in Table 27. The validated HPLC method was further used for determination of imperatorin in *A. marmelos* extracts (extracts A, B and C).

Table 27 Summarized results of analytical method validation for an assay of imperatorin

Test	Parameter	Criteria	Result	Conclusion
Accuracy	%Recovery	98-102%	98.43% to 100.10%	Passed
Intra-day precision	%RSD	Not more than 2%	0.72% to 1.31%	Passed
Inter-day precision	%RSD	Not more than 2%	0.17%	Passed
Linearity and range	y-intercept	2-3% of peak area at concentration of 100%	1,685.8	Passed
	R^2	Not less than 0.9995	0.9998	Passed
Specificity	Resolution	Not less than 1.5	ND	Passed
	Asymmetry	Not more than 1.5	1.143-1.241	Passed
	Peak area	Not significantly difference ($p<0.05$)	$p=0.278$	Passed

ND = could not determine

81

4.2.3 Determination of imperatorin in *A. marmelos* extracts

The composition of imperatorin from *A. marmelos* fruit was determined by RP-HPLC. Determination of imperatorin in hexane, ethanolic and aqueous extracts were performed by using the validated HPLC condition as described above. Quantification was carried out from integrated peak areas of the sample and the standard curve of imperatorin (Figure 17). Percentages of impertorin in the extracts were calculated by comparing peak area of the samples with those of the standard imperatorin. Extract solution was prepared and injected in duplicate into the HPLC, then peak area response was obtained.

Standard curve of imperatorin

Reference standard solutions were injected in triplicate into the HPLC, and peak area responses were obtained. The concentrations of standard imperatorin solutions were prepared at various concentrations (2.96, 4.95, 6.93, 8.91 and 10.89 μg/ml). Standard curve of imperatorin is shown in Figure 17 and average peak area of impertorin is shown in Table 28. The slope and intercept of the calibration curve were calculated using least square linear regression. The regression equation of standard curves was:

$$y = 38{,}497x - 2889.3 \quad (14)$$

where,

y is the peak area ($mAU \cdot s^{-1}$)

x is concentration of standard imperatorin (μg/ml)

The calibration curve was linear over the concentration range with R^2 of 0.9998

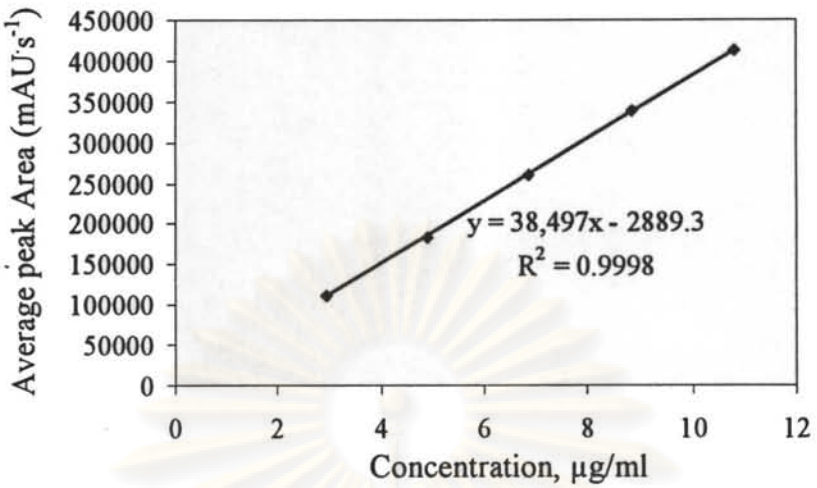


Figure 17 Standard curve of imperatorin

Table 28 Average peak area of imperatorin reference standard at five different concentrations (standard curve).

Concentration (µg/ml)	Average peak area ($mAU \cdot s^{-1}$)
2.96	112,159
4.95	184,505
6.93	255,230
8.91	339,314
10.89	412,898

The contents of imperatorin in *A. marmelos* are presented in Table 29. The contents of imperatorin in hexane and ethanolic extracts were 5.7802 ± 0.1373% and 0.1179 ± $9.1000e^{-5}$%, respectively, based on weight of dried extract. While, the contents of imperatorin based on weight of dried *A. marmelos* fruit were between 0.0321 ± 0.0008% and 0.0091 ± $6.9000e^{-6}$% obtained from hexane and ethanolic extracts, respectively. However, imperatorin could not be detected in aqueous extract of *A. marmelos*.

From the result, it was shown that hexane extract considerably contained the highest amount of imperatorin. Therefore, it could be concluded that hexane could be an effective solvent to extract imperatorin from *A. marmelos* fruit.

Table 29 Contents of imperatorin in *A. marmelos* fruit.

Solvent extraction	Concentrations of extract (µg/ml)	Peak area ($mAU \cdot s^{-1}$)	content of imperatorin (%w/w), (mean ± SD), (n=3)	
			in dried fruit	in dried extract
Hexane (Extract A)	109	233,178	0.0321 ± 0.0008	5.7802 ± 0.1373
	104	232,865		
	105	232,621		
Ethanol (Extract B)	3,873	171,217	$0.0091 \pm 6.9000e^{-6}$	$0.1179 \pm 9.1000e^{-5}$
	3,836	171,429		
	3,840	171,417		
Water (Extract C)	19,242	ND	ND	ND
	23,246	ND		
	20,175	ND		

ND=could not determine

There is no report for determination of imperatorin by using HPLC analysis. Noysang (1998) investigated the content of imperatorin in *A. marmelos* fruit using gas chromatography and mass spectrometry (GC-MS). The content of imperatorin in *A. marmelos* fruit was 0.055% when using hexane extraction and 0.04 to 0.2 % (w/w) when using n-pentane extraction. From the result in this study, mean of percentage of imperatorin was determined by HPLC method, appeared to be 0.0321 and 0.0091 (w/w) in hexane and ethanolic extracts, respectively. It could be suggested that content of imperatorin is related to many factors such as the stage of growth, source of plant, shape or size of fruit, environmental effect, type of solvent extraction and analytical technique.

4.3 *In vitro* assay of inhibitory effect of *A. marmelos* extracts and imperatorin on cytochrome P450

The basic reaction catalyzed by CYP is a monooxygenase reaction. Product obtained from enzyme-substrate reaction appears to be alcohol. In this study, Vivid® substrate are two different blocked dyes, ethoxycoumarin (EOMCC) and 7-benzyloxymethyloxy-3-cyanocoumarin (BOMCC). While, specific BACULOSOME CYP450 reagent, is microsome prepared from insect cells expressing a human P450 isozyme mixed with rabbit NADPH-P450 reductase. Cleavage of BACULOSOME CYP450 and Vivid® substrate yields blue fluorescent product (7-hydroxy-3-cyanocoumarin). Fruit extracts and imperatorin were tested for inhibitory effect by a decease in fluorescent products.

4.3.1 Verification of CYP inhibition assay

Percent inhibition of positive inhibitors for each CYP (positive inhibitors for CYP1A2, CYP3A4, CYP2C9 and CYP2C19 were α-naphthoflavone, ketoconazole, sulfaphenazole and miconazole, respectively) was studied so as to verify the procedure for CYP inhibition (Table 30). The %inhibition of each inhibitor are shown in Appendix B.

Each positive inhibitor solution was prepared in different concentrations. Then, the positive inhibitor solution was dispensed in 96-well black plate. Test solutions were mixed with BACULOSOME CYP450 and incubated for the proper time. The reaction was initiated by adding Vivid® substrate (BOMCC for CYP3A4

and CYP2C9, EOMCC for CYP1A2 and CYP2C19) into a mixture. Inhibitory effects of positive inhibitor were determined from the fluorescent production of metabolites after adding stop solution (0.5 M, pH 10.5 of Tris base). Fluorescence data at the time point were exported and analyzed for %inhibition by comparing with control containing blank solvent, BACULOSOME CYP450 and Vivid® substrate. By using GraphPad Prism software, IC_{50} values were determined.

From the results as shown in Table 30, mean IC_{50} values of CYP3A4, CYP2C9 and CYP2C19 were 0.47, 0.11 and 0.25 μM, respectively, which related to the values of previous studies. While, IC_{50} value of CYP1A2 was 0.13 μM appeared to be a little higher than those obtained from previous reports.

The different IC_{50} value obtained from various groups may be due to some factors i.e. type of probe substrate, metabolite detector, and source of enzyme *etc.*. In this study, type of enzyme was obtained from supplier (Invitrogen™) while other studies using human CYP from Gentest (Kleiner *et al.*, 2003), human liver tissue (Monostory *et al.*, 2004), human CYP expressed in yeast microsome (Marques-Soares *et al.*, 2003) and human CYP expressed in insect cell (Marks and Larson, 2002). While probe substrates were varied among studies such as (S)-mephenytoin 4'-hydroxycoumarin for CYP2C19 and nifedipine for CYP3A4 (Monostory *et al.*, 2004), EROD for CYP1A2 (Kleiner *et al.*, 2003) and progesterone for CYP 2C9 (Marques-Soares *et al.*, 2003).

Table 30 IC_{50} values for positive inhibitors on CYPs (meanSD, 95% CI)

Enzyme	Drugs	IC_{50}, μM from other studies	IC_{50}, μM from this study (95% CI)
CYP1A2	α-Naphthoflavone	0.03[1]	0.13
		0.04±0.02[3]	(0.12 to 0.14)
CYP3A4	Ketoconazole	0.13[1]	0.47
		0.40±0.16[2]	(0.40 to 0.56)
CYP2C9	Sulfaphenazole	0.31[1]	0.11
		0.6[4]	(0.04 to 0.28)
CYP2C19	Miconazole	0.07[1]	0.25
		1.78±0.84[2]	(0.20 to 0.32)

(1)=Marks and Larson, 2002; (2)=Monostory, *et al.*, 2004; (3)=Kleiner, *et al.*, 2003; (4)=Marques-Soares, *et al.*, 2003

After verification of CYPs testing, *A. marmelos* and imperatorin were prepared and screened for inhibitory effect on CYPs (CYP1A2, CYP3A4, CYP2C9 and CYP2C19) using Vivid® CYP450 screening kits (Invitrogen™, USA). Briefly, each test solutions were prepared at different concentrations. Then, the test solutions were dispensed in 96-well black plate. Test solutions were mixed with BACULOSOME CYP450 and incubated for proper time. The reaction was initiated by adding Vivid® substrate into a mixture. Inhibitory effects of test compound were determined from the fluorescent production of metabolites after adding the stop solution (0.5 M, pH 10.5 of Tris base). Fluorescent data at the time point were exported and analyzed for %inhibition by comparing with solvent control containing blank solvent, BACULOSOME CYP450 and Vivid® substrate. By using GraphPad Prism software, IC_{50} values were determined.

In the assay, test samples with IC_{50} values $\leq$ 10 μM are considered to be "potent" inhibitors; whereas, test samples with IC_{50} values of 10-50 μM are considered to be "moderate" inhibitors (Zou, *et al.*, 2002).

In this study, dose-response curves of *A. marmelos* extracts and imperatorin for each CYP are shown in Figures 18-33 IC_{50} values of *A. marmelos* extracts and imperatorin on CYP1A2, CYP3A4, CYP2C9 and CYP2C19 are shown in Table 31. Details or %inhibition on various CYPs of the test samples are shown in Appendix A.

4.3.2 *In vitro* inhibitory effects of *A. marmelos* hexane extract (extract A) on human CYPs

Figures 18-21 display the curve of %inhibition of CYP activity versus logarithmic concentrations of *A. marmelos* extract (extract A). Extract A inhibited CYP 1A2 and 3A4 activity for 96% and 95% of the control at the concentrations of 10 µg/ml and 19.16 µg/ml, respectively. The IC_{50} values of extract A on CYP1A2 activity was 0.73±0.03 µg/ml and on CYP3A4 was 1.65±0.05 µg/ml. For CYP2C9 and 2C19, extract A inhibited 99% and 89% of the control at the concentrations of 10 µg/ml and 15 µg/ml, respectively. The IC_{50} values of extract A on CYP2C9 activity was 3.24±0.03 µg/ml and on CYP2C19 was 4.04±0.02 µg/ml.

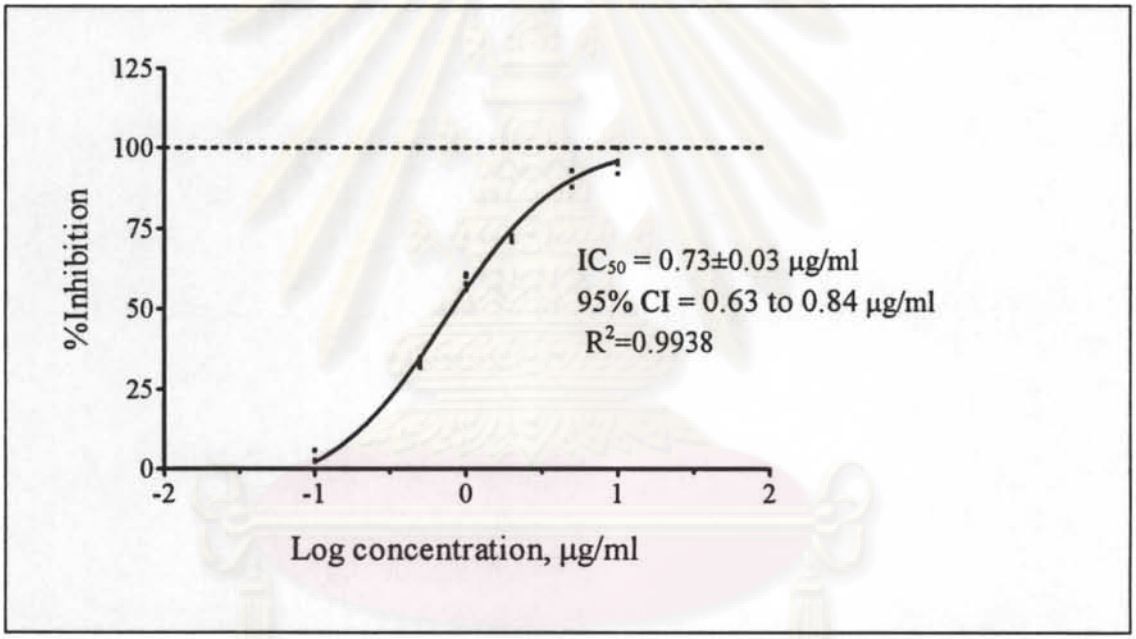


Figure 18 Inhibitory effect of hexane extract of *A. marmelos* fruit (extract A) on CYP1A2

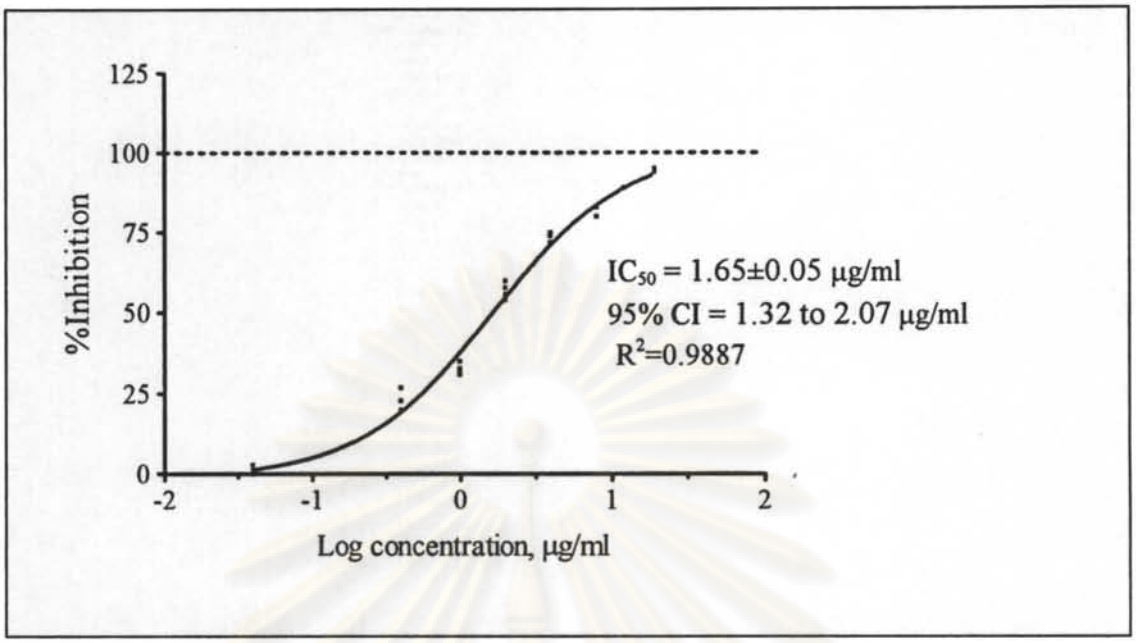


Figure 19 Inhibitory effect of hexane extract of *A. marmelos* fruit (extract A) on CYP3A4

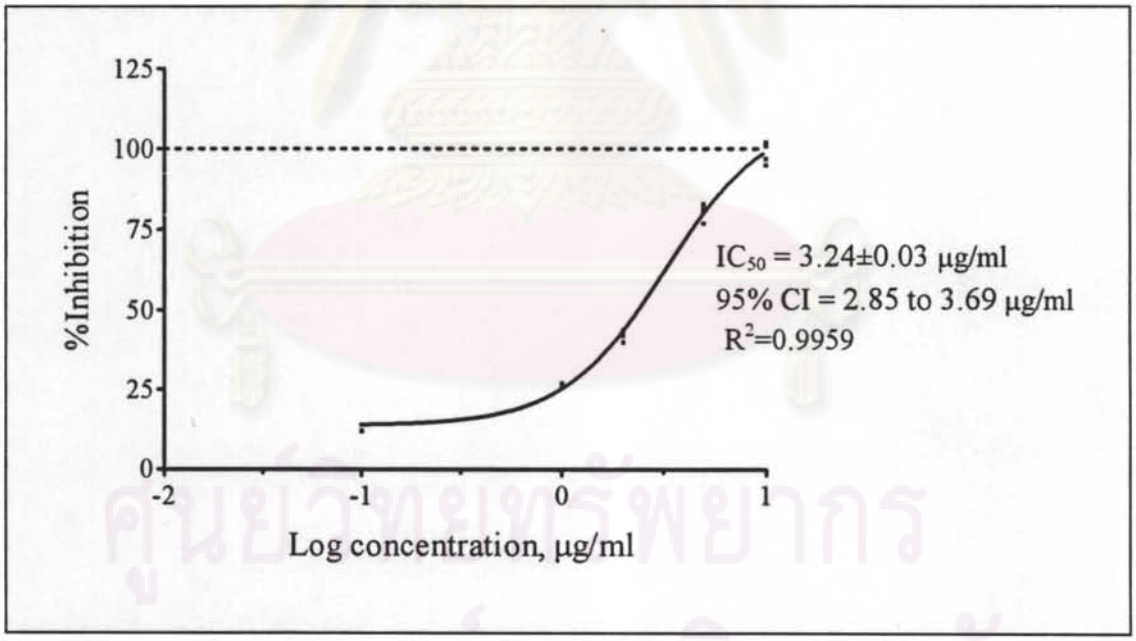


Figure 20 Inhibitory effect of hexane extract of *A. marmelos* fruit (extract A) on CYP2C9

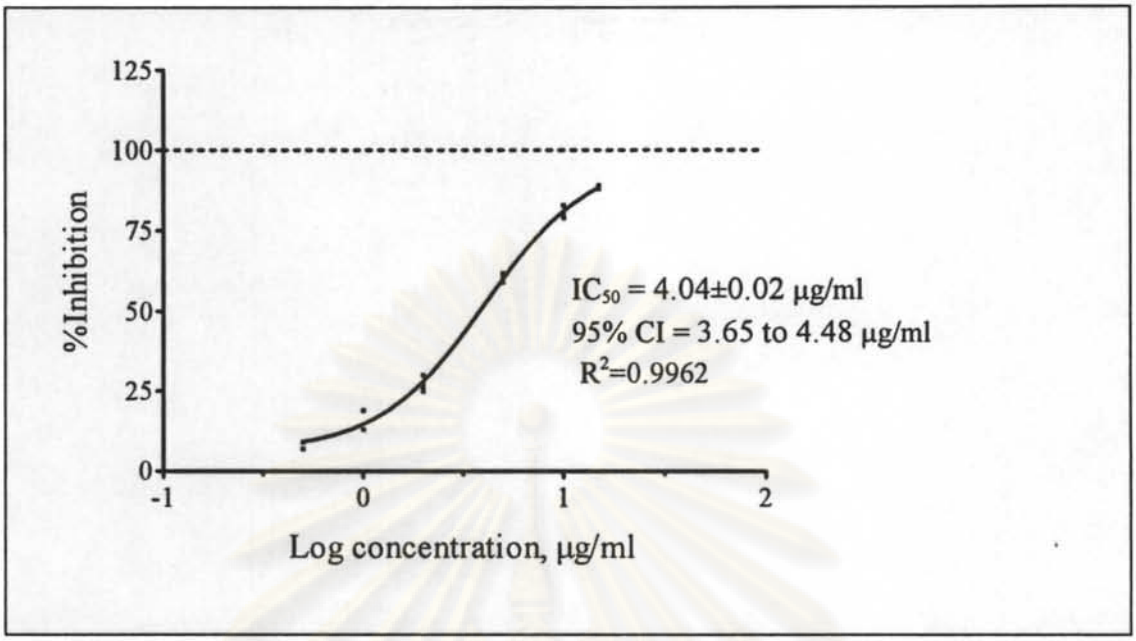


Figure 21 Inhibitory effect of hexane extract of *A. marmelos* fruit (extract A) on CYP2C19

4.3.3 *In vitro* inhibitory effects of *A. marmelos* ethanolic extract (extract B) on human CYPs

Figures 22-25 display the curve of %inhibition of CYP activity versus logarithmic concentrations of *A. marmelos* extract (extract B). Extract B inhibited CYP1A2 and 3A4 activities for 100% and 95% of the control at the concentrations of 500 µg/ml and 800 µg/ml, respectively. The IC_{50} values of extract B on CYP1A2 and 3A4 activity were 39.40±0.02 µg/ml and 86.59±0.05 µg/ml, respectively. Extract B inhibited CYP2C9 and 2C19 for 77% and 91% of the control at the concentrations of 1,000 µg/ml and 1,000 µg/ml, respectively. The IC_{50} of extract B on CYP2C9 was 197.60±0.04 µg/ml and on CYP2C19 was 107.90±0.02 µg/ml.

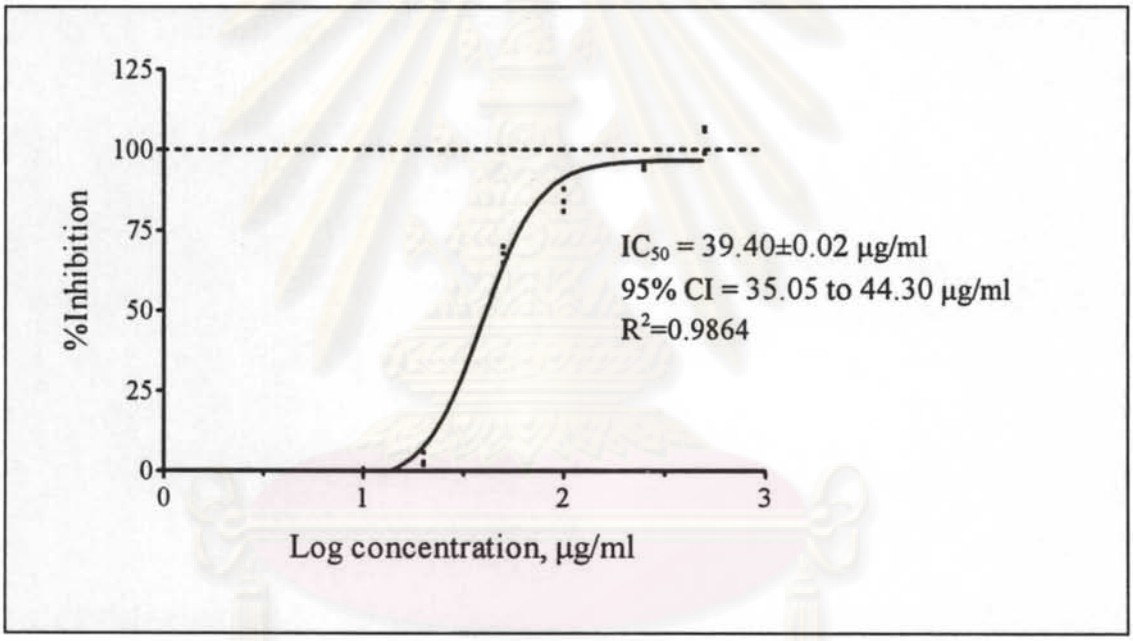


Figure 22 Inhibitory effect of ethanolic extract of *A. marmelos* fruit (extract B) on CYP1A2

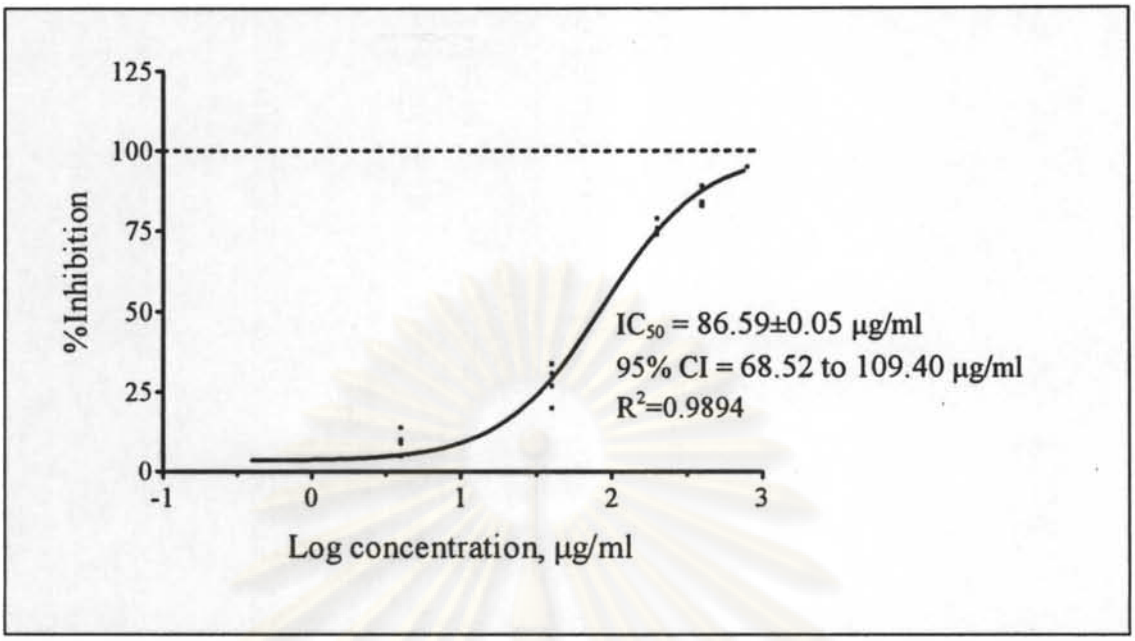


Figure 23 Inhibitory effect of ethanoic extract of *A. marmelos* fruit (extract B) on CYP3A4

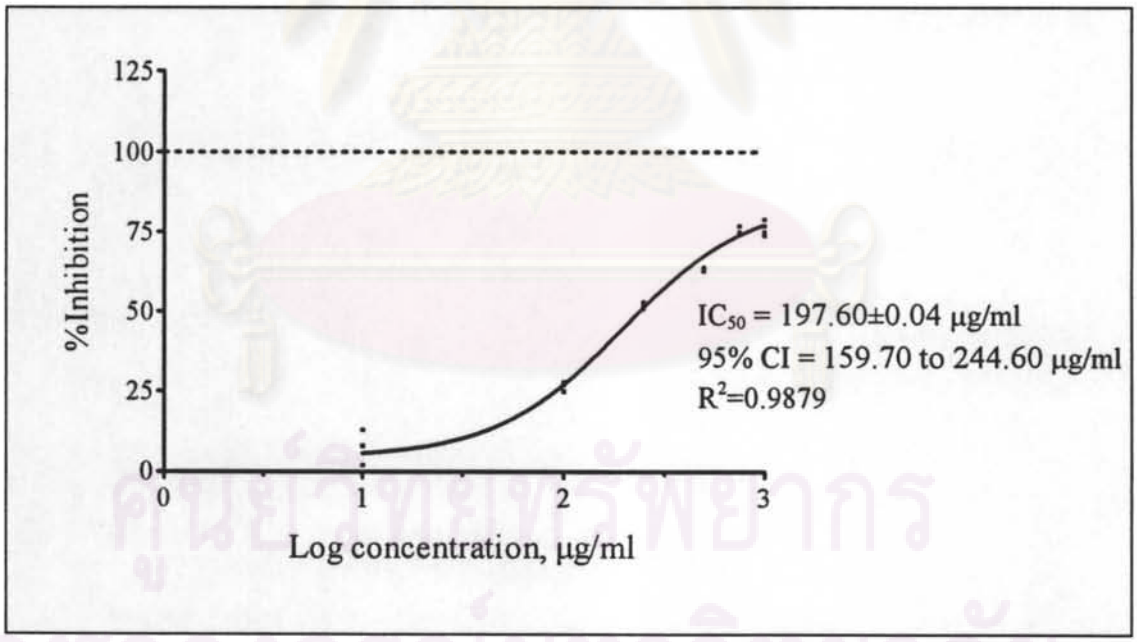


Figure 24 Inhibitory effect of ethanolic extract of *A. marmelos* fruit (extract B) on CYP2C9

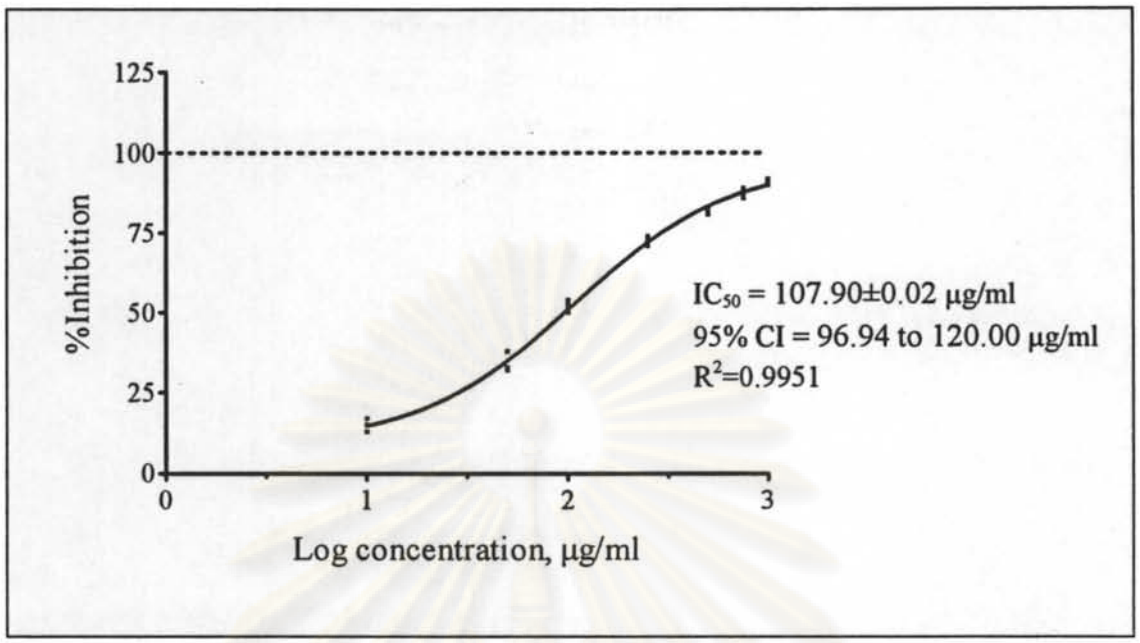


Figure 25 Inhibitory effect of ethanolic extract of *A. marmelos* fruit (extract B) on CYP2C19

4.3.4 *In vitro* inhibitory effects of *A. marmelos* aqueous extract (extract C) on human CYPs

Figures 26-29 display the curve of %inhibition of CYP activity versus logarithmic concentrations of *A. marmelos* extract (extract C). Extract C inhibited CYP1A2 and 3A4 activities for 82% and 93% of the control at the concentrations of 1,500 µg/ml and 3,200 µg/ml, respectively. The IC_{50} values of extract C on CYP1A2 activity was 352.30±0.04 µg/ml and on CYP3A4 was 842.40±0.04 µg/ml. For CYP2C9 and 2C19, extract C could inhibit 88% and 93% of the control at the concentrations of 3,000 µg/ml and 2,000 µg/ml, respectively. While the IC_{50} values on CYP2C9 and 2C19 were 965.00±0.02 µg/ml and 414.00±0.20 µg/ml, respectively.

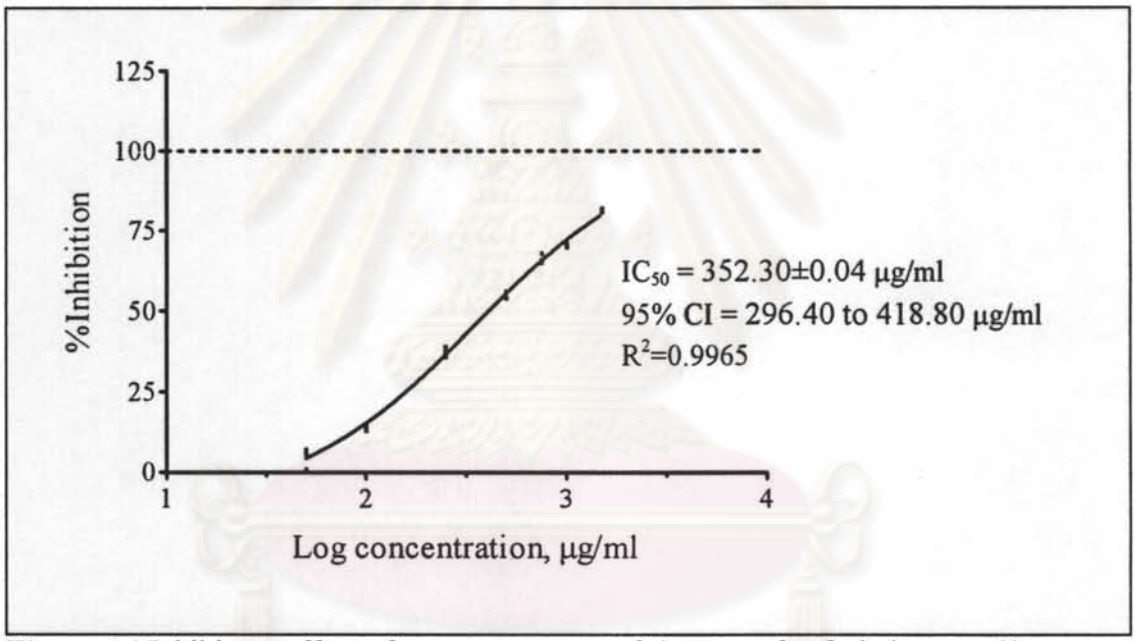


Figure 26 Inhibitory effect of aqueous extract of *A. marmelos* fruit (extract C) on CYP1A2

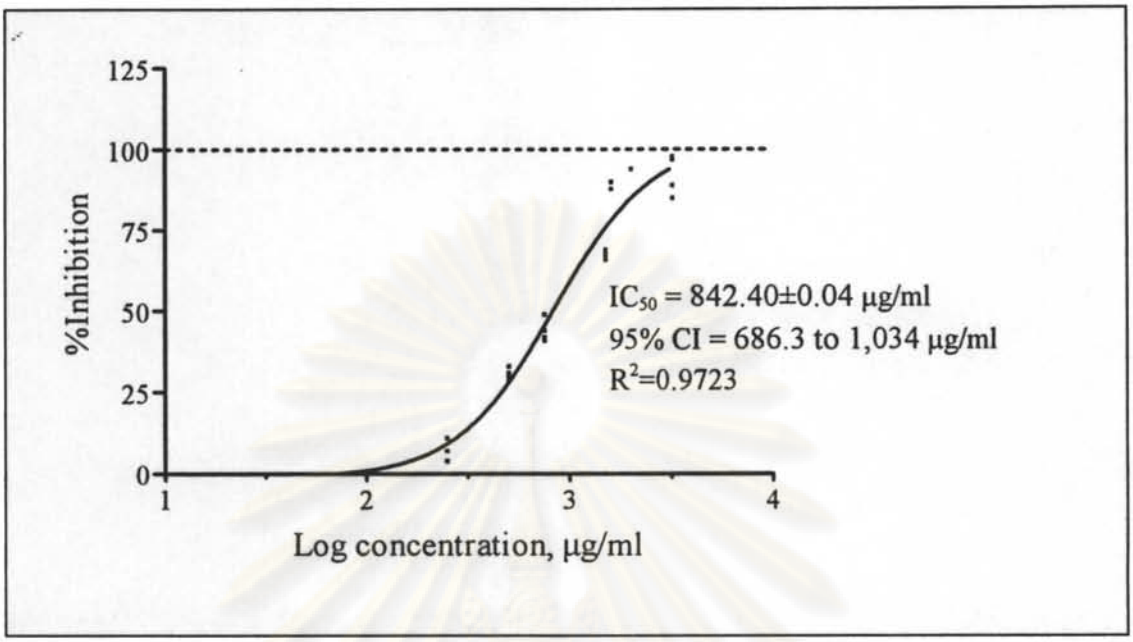


Figure 27 Inhibitory effect of aqueous extract of *A. marmelos* fruit (extract C) on CYP3A4

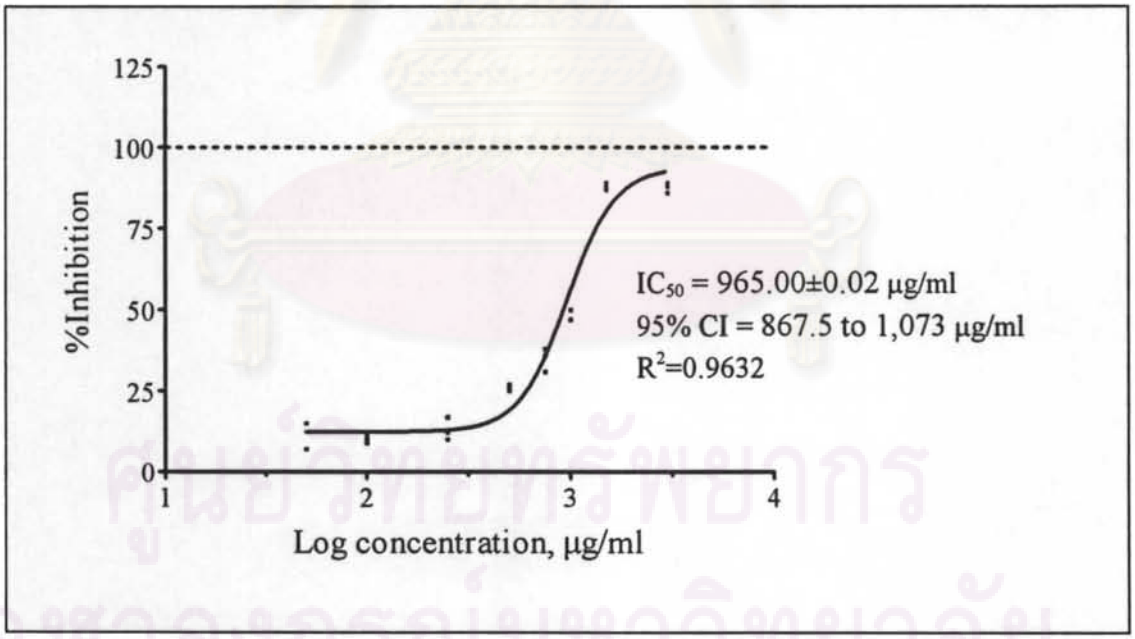


Figure 28 Inhibitory effect of aqueous extract of *A. marmelos* fruit (extract C) on CYP2C9

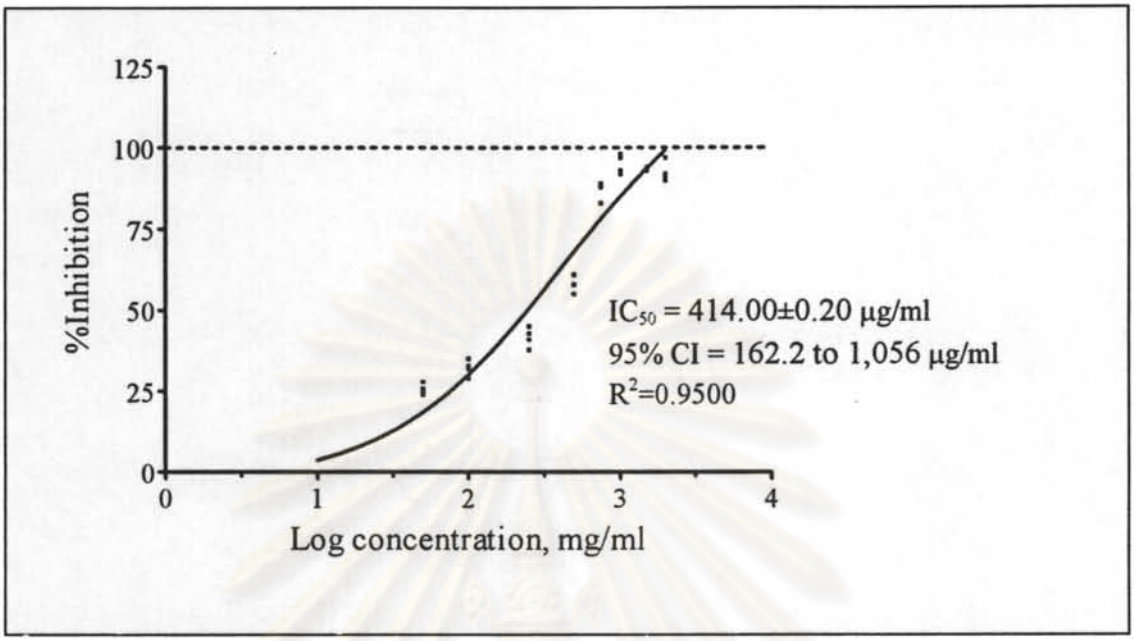


Figure 29 Inhibitory effect of aqueous extract of *A. marmelos* fruit (extract C) on CYP2C19

4.3.5 *In vitro* inhibitory effects of imperatorin on human CYPs

Figures 30-33 display the curve of %inhibition of CYP activity versus logarithmic concentrations of imperatorin. Imperatorin inhibited CYP1A2 and 3A4 activity for 88% and 97% of the control at the concentrations of 22 μM. The IC_{50} values for imperatorin inhibition of CYP1A2 and 3A4 activities were 0.42±0.02 μM (0.11±0.02 μg/ml) and 1.63±0.02 μM (0.44±0.02 μg/ml), respectively. The IC_{50} of imperatorin on CYP2C9 and 2C19 activities were 3.59±0.02 μM (0.97±0.02 μg/ml) and 2.15±0.02 μM (0.58±0.02 μg/ml) while the concentration of 110 μM and 220 μM could inhibit the CYP activities to 81% for CYP2C9 and 100% for CYP2C19

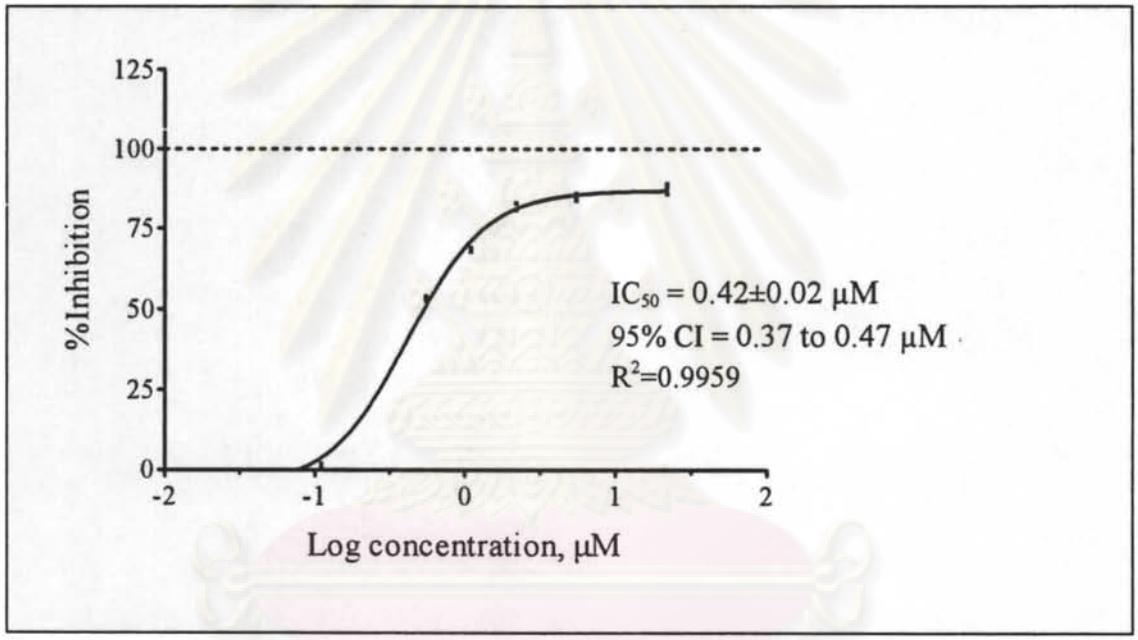


Figure 30 Inhibitory effect of imperatorin on CYP1A2

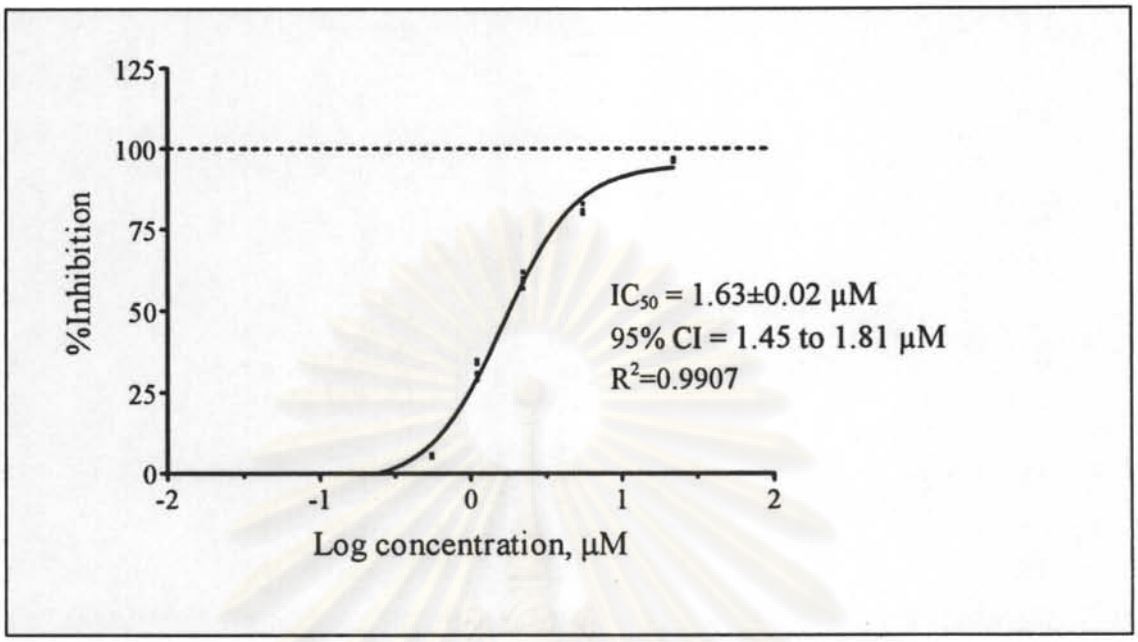


Figure 31 Inhibitory effect of imperatorin on CYP3A4

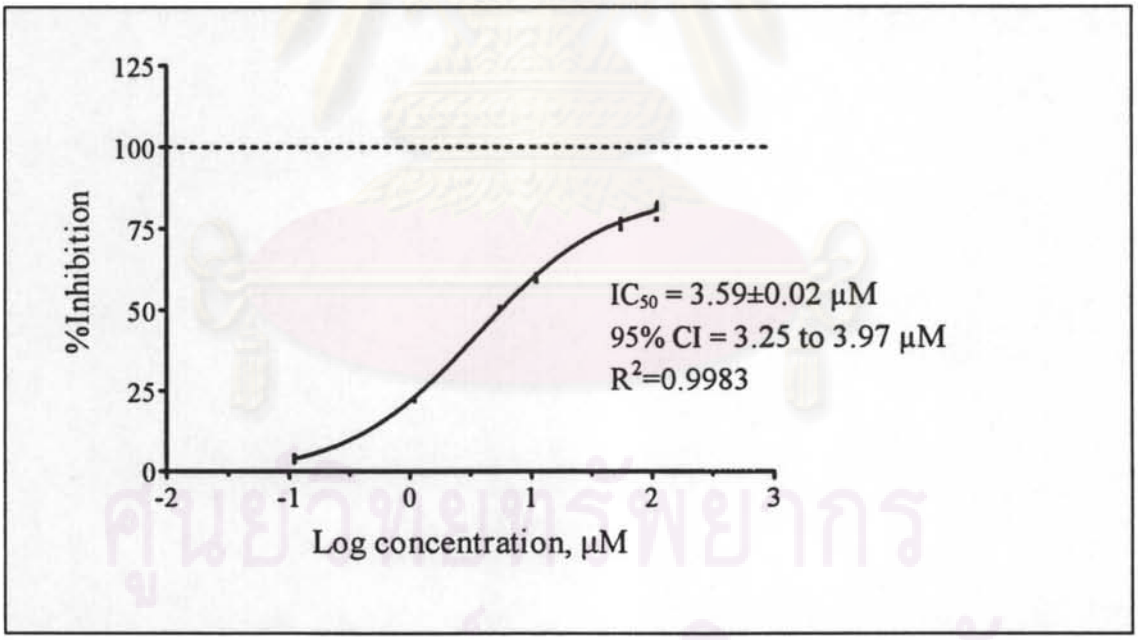


Figure 32 Inhibitory effect of imperatorin on CYP2C9

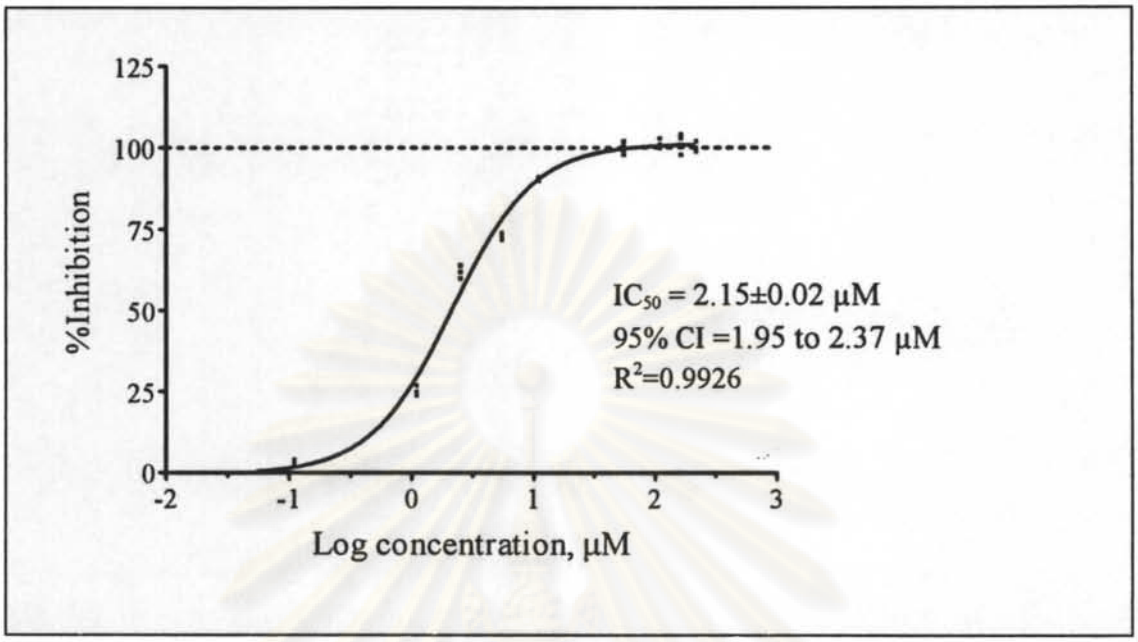


Figure 33 Inhibitory effect of imperatorin on CYP2C19

Table 31 IC_{50} values on CYP inhibition of *A. marmelos* extracts and imperatorin

Chemicals	IC_{50}±SD				Content of imperatorin in dried extract (%w/w) (mean±SD, n=3)
	CYP1A2	CYP3A4	CYP2C9	CYP2C19	
A. marmelos					
Hexane extract, µg/ml	0.73±0.03	1.65±0.05	3.24±0.03	4.04±0.02	5.7802 ± 0.1373
Ethanolic extract, µg/ml	39.40±0.02*	86.59±0.05*	197.60±0.04*	107.90±0.02*	0.1179 ± 0.000091
Aqueous extract, µg/ml	352.30±0.04*	842.40±0.04*	965.00±0.02*	414.00±0.20*	ND
Imperatorin, µg/ml	0.11±0.02	0.44±0.02	0.97±0.13	0.58±0.03	
(µM)	(0.42±0.02)	(1.63±0.02)	(3.59±0.02)	(2.15±0.02)	

* Significantly different from IC_{50} of imperatorin (µg/ml) on the individual CYP, $p<0.05$
ND=could not determine

CYPs are the principal enzymes for the oxidative metabolism of drugs and other xenobiotics. Among the xenobiotic-metabolizing CYPs, CYP1A2, CYP2C9, CYP2C19 and CYP3A4, appear to be most commonly responsible for the metabolism of drugs. Inhibition of CYPs-mediated metabolism is often the mechanism for drug-drug interactions. This study is the first report for inhibitory effect of *A. marmelos* extracts on those CYPs. The overall IC_{50}±SD (n=4) of *A. marmelos* extracts are summarized in Table 31. For statistical analysis, one-way analysis of variance (ANOVA) was carried out.

Effect of imperatorin on CYPs

From the result, imperatorin showed the potent inhibitory effect on human CYPs at concentrations of less than 10 μM. IC_{50} of imperatorin for CYP1A2 was 0.42±0.02 μM (0.11±0.02 μg/ml), CYP2C9 was 3.59±0.02 μM (0.97±0.13 μg/ml), CYP2C19 was 2.15±0.02 μM (0.58±0.03 μg/ml) and CYP3A4 was 1.63±0.02 μM (0.44±0.02 μg/ml) (Table 31). The highest inhibitory effect of imperatorin was found for CYP1A2 followed by CYP3A4, CYP2C19 and CYP2C9. Imperatorin had been reported for inhibitory effect on many CYPs such as CYP1A1, 1A2, 1B1, 2A6, 2B1, 2B6, 2C9 and 3A4 (Cai *et al.*, 1993; Kleiner *et al.*, 2003). IC_{50} values of imperatorin in this study were compared with pervious study (Table 32).

Table 32 IC_{50} values of imperatorin on CYPs (mean±SD; 95%CI)

Enzyme	IC_{50}, μM from other studies	IC_{50}, μM from this study
CYP1A1	2.764±0.64[(1)]	ND
	2.32[(2)]	
CYP1A2	0.38±0.12[(1)]	0.42±0.02 (0.37, 0.47)
CYP1B1	0.71±0.17[(1)]	ND
CYP2A6	11.7 (10, 13.3)[(1)]	ND
CYP2B1	5.44[(2)]	ND
CYP2B6	0.32 (0.25, 0.4)[(1)]	ND
CYP2C9	111 (89.1, 133)[(1)]	3.59±0.02 (3.25, 3.97)
CYP3A4	0.53 (0.45, 0.62)[(1)]	1.63±0.02 (1.45, 1.81)
CYP2C19	ND	2.15±0.02 (1.95, 2.37)

(1)= Kleiner *et al.*, 2003; (2)= Cai *et al.*, 1993

ND= not determined

From Table 32, IC_{50} of imperatorin for CYP1A2 and CYP3A4 were in agreement with values reported by Kleiner and coworkers (2003), but IC_{50} of CYP2C9 was 30-fold lower than that reported. It was noted that this study and previous study used the same sources of enzyme which are microsomes prepared from insect cells infected with recombinant baculovirus containing a cDNA inserted for human CYP isoforms. The differences in IC_{50} value of CYP2C9 appeared in this study may be the different selective substrates for CYP2C9 which were 7-benzyloxymethyloxy-3-cyanocoumarin in this study and diclofenac in the previous study.

In this study, the inhibition was reported as IC_{50} values, imperatorin showed the potent inhibitory effects at all CYPs. The *in vitro* assay could not state that inhibitory mechanism of imperatorin is reversible or irreversible. In general, inhibitors may be classified as either reversible or irreversible inhibitors. A previous report showed that imperatorin had an inhibitory effect on CYPs and for mechanism-based inhibition. The mechanism-based inhibition is the category of irreversible inhibition that requires metabolic activation by CYP to form reactive intermediates leading to formation of covalent adducts. Mechanism-based inactivators of CYP appear to work by one of three potential mechanisms: (i) alkylation of the CYP-prosthetic heme group; (ii) degradation of the prosthetic heme group and (iii) covalent binding to the apoprotein. Imperatorin showed inactivation either CYP1A1-mediated ethoxyresorufin *O*-dealkylase (EROD) activity in hepatic microsomes in 3-methylcholanthrene pretreated mice or CYP2B1-mediated pentoxyresorufin *O*-dealkylase (PROD) activity in mice treated with phenobarbital (Cai *et al.*, 1993). Effect of imperatorin was compared with phenylhydrazine (positive control which forms alkylation with heme group (Jonen *et al.*, 1982)). Imperatorin showed no significantly affected to heme content in the microsomal preparation, while phenylhydrazine formed an adduct with heme and showed significantly lower CYP content by 80% and heme content by 36%. Imperatorin was reported to be bioactivated by CYPs to reactive intermediate that subsequently form covalent adducts with the apoprotein and effectively destroy enzyme activity (Cai *et al.*, 1996).

Effect of *A. marmelos* hexane extract (extract A) on CYPs

From the results, IC_{50} of *A. marmelos* hexane extract (extract A) for CYP1A2 was 0.73±0.03 μg/ml, CYP2C9 was 3.24±0.03 μg/ml, CYP2C19 was 4.04±0.02 μg/ml and CYP3A4 was 1.65±0.05 μg/ml (Table 31).

Comparison among extracts, inhibitory effect of extract A showed the highest inhibitory effect on CYPs followed by extract B and extract C (Table 31). This trend of inhibition of the extract followed the content of imperatorin in the extracts. Moreover inhibitory effect of extract A was not significantly different from that of imperatorin in all of those CYPs. This may suggest that the active compounds which cause inhibition belonged to nonpolar solvent extract and the inhibitory effect of *A. marmelos* hexane extract may be partly due to imperatorin.

However inhibitory effect of extract A was the highest for CYP1A2 followed by CYP3A4, CYP2C9 and CYP2C19, respectively. This trend of inhibition of *A. marmelos* hexane extract was different from imperatorin (extract A inhibited CYP2C9 more than CYP2C19). And the content of imperatorin in *A. marmelos* hexane extract at IC_{50} values for those CYPs were lower than imperatorin. This may suggest that the active compounds which have inhibitory effects on those CYPs in *A. marmelos* hexane extract were not from imperatorin alone. Since there were reports that xanthotoxin, a furanocoumarin found in *A. marmelos* fruit, was an irreversible inhibitor of CYP both *in vitro* and *in vivo* (Feuer and Kellen, 1974; Fouin-Fortunet *et al*, 1986; Letteron *et al.*, 1986; Cai *et al.*, 1993). Therefore, the need of further study for finding other active compounds in *A. marmelos* hexane extract should be determined. Also, the mechanism of inhibitory effect and effect on enzymes in phase II metabolism of *A. marmelos* are recommended to be studied.

Effect of *A. marmelos* ethanolic extract (extract B) on CYPs

From the results, IC_{50} of *A. marmelos* ethanolic extract (extract B) for CYP1A2 was 39.40±0.02 μg/ml, CYP2C9 was 197.60±0.04 μg/ml, CYP2C19 was 107.90±0.02 μg/ml and CYP3A4 was 86.59±0.05 μg/ml (Table 31).

Inhibitory effect of *A. marmelos* extract B was highest for CYP1A2 followed by CYP3A4, CYP2C19 and CYP2C9, respectively, which were the same trend with imperatorin. Comparison among extracts, inhibitory effect of extract B showed lower and higher inhibitory effect on CYPs when compared with extract A and extract C,

respectively. The content of imperatorin in extract B was lower than extract A for 50-fold. The inhibitory effect of extract B was significantly different from that of imperatorin in all those CYPs. This may suggest that *A. marmelos* ethanolic extract was not a potent inhibitor. The content of imperatorin in *A. marmelos* ethanolic extract at IC_{50} values for those CYPs were lower than imperatorin. This may suggest that the active compounds which cause inhibition of extract B may not be due to imperatorin alone.

Effect of *A. marmelos* aqueous extract (extract C) on CYPs

From the results, IC_{50} of *A. marmelos* aqueous extract (extract C) for CYP1A2 was 352.30±0.04 µg/ml, CYP2C9 was 965.00±0.02 µg/ml, CYP2C19 was 414.00±0.20 µg/ml and CYP3A4 was 842.40±0.04 µg/ml (Table 31). Extract C showed the least inhibitory effect when compared with extract A and extract B and the IC_{50} values were significantly different from those of imperatorin. Imperatorin content in extract C was lower than the limit of detection determined from HPLC method, hence, extract C could not be determined. This may suggest that the active compounds which cause inhibition belonged to nonpolar solvent extract and edible of fruit juice of *A. marmelos* can be considered as safe with low incidence of food-drug interaction.

Inhibitory effects of *A. marmelos* extracts on CYP isoforms

CYP1A2 is important for the bioactivation of procarcinogens. It expresses in extrahepatic tissues such as lung. This enzyme metabolizes procarcinogen such as polycyclic aromatic hydrocarbon (PAHs), heterocyclic amines, aromatic amines, nitropolycyclic hydrocarbons (found in cigarette smoke and pollutant), and dietary promutagens (including nitrosamines and aflatoxins) to be carcinogen. Increased levels of this enzyme are also associated with breast cancer (Hong *et al.*, 2004) colon cancer (Saebø *et al.*, 2008) and lung cancer (Seow *et al.*, 2001). From the result, *A. marmelos* hexane and ethanolic extracts showed the inhibitory effect on CYP1A2. This result may suggest that *A. marmelos* extracts may be beneficial for decreasing carcinogenesis.

Moreover, CYP1A2 is responsible for biotransformation of many drugs such as antidepressant drugs (amitryptyline, fluvoxamine and imipramine), antipsychotic

drugs (clozapine and haloperidol), antiarrhythmic drug (mexiletine), antiemetic drug (ondansetron), antihypertensive drugs (propanolol and verapamil), drug for treatment of Alzheimer' disease (tracrine), drug treatment for asthma (theophylline), anticoagulant (warfarin), drug for treatment of migraine (zolmitriptan), caffeine, *etc.* (Gibson and Skett, 1996; Coleman, 2005). Inhibition of CYP1A2 may suggest that *A. marmelos* may decrease biotransformation of those drugs and may cause drug toxicity.

CYP2C9 is the principal CYP2C enzyme in human liver. It metabolizes endogenous compounds such as arachidonic acid as well as many clinically important drugs including phenytoin, warfarin, losartan, tolbutamide, plipizide and non steroidal anti-inflammatory drugs such as ibuprofen and diclofenac (Shannon and Michael, 2005). Diclofenac and tolbutamide are popular CYP2C9 probe substrates for *in vitro* testing. While sulfaphenazole is commonly known as inhibitor for CYP2C9. Whereas rifampicin and phenobarbital are commonly known as inducer for CYP2C9 (Brandon *et al.*, 2003). Warfarin and phenytoin are drugs with low therapeutic margin. Whereas, CYP2C19 metabolizes structurally diverse therapeutic agents, some substrates are converted to pharmacologically active metabolites by this enzyme. Inhibition of both of CYP2C9 and CYP2C19 may cause drug interaction. From the result, *A. marmelos* hexane and ethanolic extracts showed the inhibitory effect on both CYPs. This may suggest that *A. marmelos* fruit may interact with drugs which are primarily biotransformed by these enzymes and may cause drug toxicity.

CYP3A4 represents 30% of the total hepatic P450 content and 70% of intestinal P450 content. CYP3A4 is responsible for the metabolism of 60% of all the drugs on the market including 38 different medication classes. For example, CYP3A4 metabolizes members of the psychotropics, antiarrhythmics, calcium antagonists, opioid analgesics, antihistamines, benzodiazepines, antimicrobial agents, antiretroviral agents, immunosuppressants, antiulcer agents, and anticonvulsants (Gibson and Skett, 1996; Coleman, 2005). Human liver has a greater amount of CYP3A4 when compared with the amount in intestine. Xenobiotics which inhibit CYP3A4 at intestine may cause the lower drug's first-pass metabolism while xenobiotics which inhibit CYP3A4 at liver may cause the higher of its bioavailability (Parkinson and Ogilvie, 2008). From the result, *A. marmelos* extracts showed inhibitory effect on CYP3A4. This may suggest that *A. marmelos* fruit could interact

with drugs which are primarily biotransformed by this enzyme and may cause drug toxicity.

On the other hand, inhibition of CYP 3A4 may reduce efficacy for pro-drugs requiring conversion to active metabolites (e.g. cyclophosphamide, irinotecan) (Shannon and Michael, 2005; Sai *et al.*, 2008) or may decrease risk of conversion of procarcinogen to carcinogen (e.g. aflatoxin B1, aflatoxin G1, sterigmatocystin, senecionine) (Parkinson and Ogilvie, 2008). Inhibition of *A. marmelos* extract on CYP3A4 may cause drug failure or may increase risk of some protoxicants.

A previous report has been done on *A. marmelos* leaf extract which could bifunctionally induce both phase-I and phase-II enzymes. Mice were treated with *A. marmelos* ethanolic leaf extract at doses of 50 mg/kg and 100 mg/kg orally for 14 days. In phase-I enzymes, by using microsomal fraction of mouse liver, CYPs and NADPH-cytochrome P450 (NADPH-CYP) reductase were determined by carbon monoxide different spectra test and the contents of both CYP enzymes from microsomal suspension were estimated by the method of Omura and Sato (1964). The *A. marmelos* treated groups at doses of 50 and 100 mg/kg presented significant elevation in the specific activities of CYPs at 0.90 and 1.15 folds, NADPH-CYP reductase at 1.23 and 1.32 folds and NADH-cytochrome b5 reductase at 0.93 and 1.12 folds when compared with the control group. In phase-II enzymes, glutathione S-transferase was assayed in the cytosol of liver. The *A. marmelos* treated groups presented a significant dose-dependent increase in both enzyme activities at doses of 50 and 100 mg/ml by 1.44 and 1.66 folds of glutathione-S-transferase when compared with the control group. (Singh *et al.*, 2000).

The difference in results of this study from the previous study may be due to the different parts of *A. marmelos* tested (fruit in this study and leaf in the other study). *A. marmelos* leaf contains similar compounds with its fruit such as alkaloid (cinnamide, aegeline, skimmianine, haplopine) (Manandhar *et al.*, 1978; Govindachari and Premila, 1983; Banerji *et al.*, 1988; Riyanto *et al.*, 2003) and steroid (stigmasterol and β-sitosterol) (Sharma and Sharma, 1979; Riyanto *et al.*, 2003). However, *A. marmelos* leaf was reported to have different compounds from its fruit which contains alkaloid (*o*-methyl halfordinol, aegelenine), flavonoid glycoside (rutin) and coumarin (marmesinin). The data may suggest that the effect of *A. marmelos* leaf on CYP may be related to the effect of those compounds. Moreover,

there are some limitations of *in vitro* studies, for example, the use of high concentration of the component which does not account for poor bioavailability of the active component and for binding of active component to plasma proteins *in vivo*. *In vitro* studies are valuable for evaluating multiple products or components, provide mechanistic information about any potential interaction and are simple to conduct. While *in vivo* studies are valuable follow-up studies to *in vitro* observations and are the only ones that are most definitive and clinical importance of food-drug interaction (Venkataramanan *et al.*, 2006).

Another plant which has been reported that it had food-drug interaction was *Angelica dahurica* (Bai zhi). Its has been used as analgesic, antibacterial, carminative and diuretic (Ban et al., 2003; Chen et al., 2006). It showed *in vitro* inhibitory effect on CYP3A4 (IC_{50} = 34 μg/ml) (Guo *et al.*, 2001). The root extract could inhibit CYP2C, CYP3A and CYP2D *in vitro* (Ishihara *et al.*, 2000). The metabolism of tolbutamide, nifedipine and bufuralol were inhibited after intravenously administered the extract at a dose of 1 g/kg to rats. Other vegetables which contain imperatorin are parsnip (Ceska *et al.*, 1987), lemon and lime oils (Stanley and Jurd, 1971) and parsley (0.7-5.9 μg/g in fruit) (Ceska *et al.*, 1987).

In summary, this study provide information about IC_{50} of imperatorin and hexane, ethanolic and aqueous extracts of *A. marmelos*. The active compounds which cause inhibition belonged to nonpolar solvent extract. Moreover imperatorin and *A. marmelos* fruit extracted with hexane potentially inhibit the metabolism of co-administered medication whose primary route of elimination is via those CYPs, thus interpretation of these data from *in vitro* to human should be further studied. However the inhibitory effect of *A. marmelos* extracts was not due to imperatorin alone. Therefore, the need of further study for finding the active compound in *A. marmelos* hexane extract should be determined. It would be beneficial for further study of the most active compound of *A. marmelos* hexane extract which affects CYP activity. It is also interesting to study about the mechanism-based inhibitory effect and the *in vivo* study.

CHAPTER V
CONCLUSION

Aegle marmelos Correa is one of the most important medicinal plants in family Rutaceae. All parts of *A. marmelos* were used in the indigenous medicine. Its fruit is made to the popular refreshing drink and had been traditionally used as an antidiarrhoea and antidysentery. Since, *A. marmelos* is widely used in Thailand, repeated exposure of this fruit may affect biotransformation enzymes. In this study, *A. marmelos* fruit was extracted and investigated for inhibitory effect on cytochrome P450 (CYP), the oxidative metabolizing enzyme in phase I metabolism. Therefore, the amount of imperatorin in the fruit extract was determined by RP-HPLC analysis and its effects on those CYPs were investigated so as to find out whether the *A. marmelos* fruit could have any inhibitory effect on CYP activities. The results from this study would be a beneficial information to indicate the possibility of interaction between *A. marmelos* fruit and some therapeutic drugs when taken concomitantly.

A. marmelos fruit contained 0.56% of n-hexane extract, 7.69% of ethanolic extract and 21.54% of aqueous extract in gram dried weight. A total yields (%) of imperatorin in hexane and ethanolic extracts were 5.7802%, w/w and 0.1179%, w/w, respectively, based on weight of dried extract. No imperatorin could be detected in aqueous extract of *A. marmelos*. Imperatorin showed the potent inhibitory effect on CYP1A2, CYP2C9, CYP2C19 and CYP3A4 at concentrations of less than 10 μM. While *A. marmelos* extracts showed the same trend of CYP inhibition with the imperatorin content in that the hexane extract which contained the highest amount of imperatorin showed the highest inhibitory effects on CYPs. Whereas ethanolic extract contained less amount of imperatorin showed the inhibitory effects lower than hexane extract. The water extract of *A. marmelos* fruit contained no detectable amount of imperatorin and had very high in IC_{50} values ($IC_{50} > 300$). This may suggest that the active compounds which cause inhibition belonged to nonpolar solvent. However the inhibitory effect of *A. marmelos* extracts was not due to imperatorin alone.

Hexane extract of *A. marmelos* fruit showed no significantly different when compared with imperatorin. While both ethanolic and aqueous extracts had significantly higher IC_{50} values than imperatorin. The results of this investigation suggested that the whole *A. marmelos* fruit could promote food-drug interaction by CYP inhibition. However, the edible of fruit juice of *A. marmelos* can be considered as safe with low incidence of food-drug interaction. It would be of great benefits to further studies on *in vivo*. Also, the mechanism-based inhibitory effect and effect of *A. marmelos* extracts on enzymes in phase II metabolism should be studied.

REFERENCES

THAI

กัลยา วานิชย์บัญชา. 2548. การทดสอบที่ไม่ใช้พารามิเตอร์. ใน สถิติสำหรับงานวิจัย, หน้า 246-273. กรุงเทพมหานคร: สำนักพิมพ์แห่งจุฬาลงกรณ์มหาวิทยาลัย.

พร้อมจิต ศรลัมพ์. 2535. มะตูม Bael fruit tree/ Bengal quince : สมุนไพรไทย. ใน หนังสือสมุนไพรสวนสิรีรุกขชาติ, หน้า 50. กรุงเทพมหานคร: อมรินทร์พริ้นติ้งกรุ๊พ.

รพีพล ภโววาท และ พิมลพรรณ พิทยานุกูล. 2529. การศึกษายางมะตูม สารเมือกจากธรรมชาติ ตอนที่ 1 วิธีการสกัดและการทำให้บริสุทธิ์ การตรวจสอบคุณสมบัติทางเคมี-ฟิสิกส์ และการหาองค์ประกอบทางเคมี. ไทยเภสัชสาร. (เมษายน-มิถุนายน): 77-84.

สมพร ภูติยานันต์. 2546. เภสัชวัตถุ: สมุนไพรรสฝาดและหวาน มะตูม (Matuum). ใน ความรู้เบื้องต้นเกี่ยวกับการแพทย์แผนไทย ว่าด้วย สมุนไพรกับการแพทย์แผนไทย, หน้า 132-135. เชียงใหม่: โรงพิมพ์ดุลย์การพิมพ์เชียงใหม่.

สุวัช สิงหพันธุ์. 2545. มะตูม. ใน ผลไม้ไทย, หน้า 74-75. กรุงเทพมหานคร: สำนักนายกรัฐมนตรี.

ENGLISH

Abdul, R. A., Gopalakrishnan, G., Venkatesan, P. and Geetha, K. 2008. Isolation and identification of mosquito larvicidal compound from *Abutilon indicum* (Linn.) Sweet. Parasitol Res. 102(5): 981-8.

Abebe, W. 2002. Herbal medication: potential for adverse interactions with analgesic drugs. J Clin Pharm Ther. 27(6): 391-401.

Ahn, J. Y., Song, J. Y., Yun, Y. S., Jeong, G. and Choi, I. S. 2006. Protection of *Staphylococcus aureus*-infected septic mice by suppression of early acute inflammation and enhanced antimicrobial activity by ginseng. FEMS Immunol Med Microbiol. 46(2): 187-197.

Anandharajan, R., Jaiganesh, S., Shankernarayanan, N. P., Viswakarma, R. A. and Balakrishnan, A. 2006. *In vitro* glucose uptake activity of *Aegle marmelos* and *Syzygium cumini* by activation of Glut-4, PI3 kinase and PPARγ in L6 myotubes. Phytomedicine. 13: 434-441.

Aritajat, S., Kaweewat, K., Manosroi, J. and Manosroi, A. 2000. Dominant lethal test

in rats treated with some plant extracts. Southeast Asian J Trop Med Public Health. 31: 171-173.

Arul, V., Miyazaki, S. and Dhananjayan, R. 2004. Mechanisms of the contractile effect of the alcoholic extract of *Aegle marmelos* Corr. on isolated guinea pig ileum and tracheal chain. Phytomedicine. 11(7-8): 679-83.

Arul, V., Miyazaki, S. and Dhananjayan, R. 2005. Studies on the anti-inflammatory, antipyretic and analgesic properties of the leaves of *Aegle marmelos* Corr. J Ethnopharmacol. 96(1-2): 159-163.

Badam, L., Bedekar, S. S., Sonawane, K. B. and Joshi, S. P. 2002. *In vitro* antiviral activity of bael (*Aegle marmelos* Corr.) upon human coxsackieviruses B1-B6. J Commun Dis. 34: 88-99.

Bailey, D. G., Malcolm, J., Arnold, O. and Spence, J. D. 2004. Grapefruit juice-drug interactions. Br J Clin Pharmacol. 58(7): S831-840.

Ban, H. S., Lim, S. S., Suzuki, K., Jung, S. H., Lee, S., Lee, Y. S., Shin, K. H. and Ohuchi, K. 2003. Inhibitory effects of furanocoumarins isolated from the roots of *Angelica dahurica* on prostaglandin E2 production. Planta Med. 69(5): 408-412.

Banerji, J., Das, A. K., Ghoshal, N. and Das, B. 1988. Studies on Rutaceae: part VII—chemical investigation on the constituents of *Atalantia wightii* Tanaka, *Aegle marmelos* Correa. ex Koen, *Ruta graveolens* Linn. & *Micromelum pubescens* Blume. Indian J Chem. 27B: 594-596.

Basnet, P., Yasuda, I., Kumagai, N., Tohda, C., Nojima, H., Kuraishi, Y. and Komatsu, K. 2001. Inhibition of itch-scratch response by fruits of *Cnidium monnieri* in mice. Biol Pharm Bull. 24(9): 1012-1015.

Bishen, S. and Mahendra, P. S. 1980. Indian medicinal plants Vol 4. 2nd ed. pp. 499-501. India: New Connaught Place.

Brandon, E. F., Raap, C. D., Meijerman, I., Beijnen, J. H. and Schellens, J. H. 2003. An update on *in vitro* test methods in human hepatic drug biotransformation research: pros and cons. Toxicol Appl Pharmacol. 189(3): 233-246.

Budavari, S., Maryadele, J. O., Smith, A., Heckelman, P. E. and Kinneary, J. F. 1996. The Merk Index 12th ed, p. 846. USA: WhitehouseStation.

Cai, Y., Kleiner, H., Johnston, D., Dubowski, A., Bostic, S., Ivie, W. and DiGiovanni,

J. 1997. Effect of naturally occurring coumarins on the formation of epidermal DNA adducts and skin tumors induced by benzo[a]pyrene and 7, 12-dimethylbenz[a]anthracene in SENCAR mice. Carcinogenesis. 18(8): 1521-1527.

Cai, Y., Bennett, D., Nair, R. V., Ceska, O., Ashwood-Smith, M. J. and DiGiovanni, J. 1993. Inhibition and inactivation of murine hepatic ethoxy- and pentoxyresorufin O-dealkylase by naturally occurring coumarins. Chem Res Toxicol. 6(6): 872-879.

Cai, Y., Baer-Dubowska, W., Ashwood-Smith, M. J., Ceska, O., Tachibana, S. and DiGiovanni, J. 1996. Mechanism-based inactivation of hepatic ethoxyresorufin O-dealkylation activity by naturally occurring coumarins. Chem Res Toxicol. 9(4): 729-736.

Ceska, O., Chaudhary, S. K., Warrington, P. J. and Ashwood Smith, M. J. 1987. Protoactive furanocoumarins in fruits of some umbelliferea. Phytochem. 26: 165-169.

Chen, I. S., Chang, C. T., Sheen, W. S., Teng, C. M., Tsai, I. L., Duh, C. Y. and Ko, F. N. 1996. Coumarins and antiplatelet aggregation constituents from Formosan *Peucedanum japonicum*. Phytochemistry. 41(2): 525-530.

Chen, Y., Fan, G., Chen, B., Xie, Y., Wu, H., Wu, Y., Yan, C. and Wang, J. 2006. Separation and quantitative analysis of coumarin compounds from *Angelica dahurica* (Fisch. ex Hoffm) Benth. et Hook. f by pressurized capillary electrochromatography. J Pharm Biomed Anal. 41(1): 105-116.

Coleman, M. D. 2005. Human drug metabolism: an introduction, pp. 26-29. Chichester: Wiley.

Costa-Lotufo, L. V., Khan, M. T., Ather, A., Wilke, D. V., Jimenez, P. C., Pessoa, C., de Moraes, M. E. and de Moraes, M. O. 2005. Studies of the anticancer potential of plants used in Bangladeshi folk medicine. J Ethnopharmacol. 99(1): 21-30.

Couperus, M. 1954. Ammoidin (xanthotoxin) in the treatment of vitiligo. Calif Med. 81(6): 402-406.

Crespi, C. L., Miller, V. P. and Penman, B. W. 1997. Microtiter plate assays for inhibition of human, drug metabolizing cytochrome P450. Analytical

Biochem. 248: 188-190.

Deng, S., Chen, S. N., Yao, P., Nikolic, D., van Breemen, R. B., Bolton, J. L., Fong, H. H., Farnsworth, N. R. and Pauli, G. F. 2006. Serotonergic activity-guided phytochemical investigation of the roots of *Angelica sinensis*. J Nat Prod. 69(4): 536-541.

Dhuley, J. N. 2003. Investigation on the gastroprotective and antidiarrhoeal properties of *Aegle marmelos* unripe fruit extract. Hindustan Antibiot Bull. 45(1): 41-46.

Ekiert, H. and Gomolka, E. 2000. Furanocoumarins in *Pastinaca sativa* L. *in vitro* cultures. Pharmazie. 55: 618-620.

Ekiert, H. and Gomolka, E. 2000. Coumarin compounds in *Ammi majus* L. callus cultures. Pharmazie. 55: 684-687.

Feuer, G. and Kellen, J. A. 1974. Inhibition and enhancement of mammary tumorigenesis by 7, 12-dimethylbenz(a)anthracene in the female sprague-dawley rat. Int J Clin Pharmacol. 9: 62-69.

Feuer, G., Kellen, J. A. and Kovacs, K. 1976. Suppression of 7, 12-dimethylbenzo(a)antracene-induced breast carcinoma by coumarin in the rat. Oncology. 33: 35-39.

Foster, B. C., Foster, M. S., Vandenhoek, S., Krantis, A., Budzinski, J. W., Arnason, J. T., Gallicano, K. D. and Choudri, S. 2001. An *in vitro* evaluation of human cytochrome P450 3A4 and P-glycoprotein inhibition by garlic. J Pharm Sci. 4(2): 176-184.

Foster, B. C., Sockovie, E. R., Vandenhoek, S., Bellefeuille, N., Drouin, C. E., Krantis, A., Budzinski, J. W., Livesey, J. and Arnason, J. T. 2004. *In vitro* activity of St. John's wort against cytochrome P450 isozymes and p-glycoprotein. Pharm Biol. 42(2): 159-169.

Foster, B. C., Vandenhoek, S., Hana, J., Krantis, A., Akhtar, M. H., Bryan, M., Budzinski, J. W., Ramputh, A. and Arnason, J. T. 2003. *In vitro* inhibition of human cytochrome P450-mediated metabolism of marker substrates by natural products. Phytomedicine. 10(4): 334-342.

Fouin-Fortunet, H., Tinel, M., Descatoire, V., Letteron, P., Larrey, D., Geneve, J. and Pessayre, D. 1986. Inactivation of cytochrome P-450 by the drug methoxsalen. J Pharmacol Exp Ther. 236: 237-247.

Fujita, T., Kawase, A., Niwa, T., Tomohiro, N., Masuda, M., Matsuda, H. and Iwaki, M. 2008. Comparative evaluation of 12 immature citrus fruit extracts for the inhibition of cytochrome P450 isoform activities. Biol Pharm Bull. 31(5): 925-930.

Fukao, H., Yoshida, H., Tazawa, Y. and Hada, T. 2007. Antithrombotic effects of odorless garlic powder both *in vitro* and *in vivo*. Biosci Biotechnol Biochem. 71(1): 84-90.

Gerson, T., Shorland, F. B. and Dunckley, G. G. 1965. The effect of beta-sitosterol on the metabolism of cholesterol and lipids in rats on a diet containing coconut oil. Biochem J. 96(2): 399-403.

Gibson, G. G. and Skett, P. 1986. Introduction to drug metabolism. New York: Chapman and Hall.

Gill, M. 1993. Aromatic compounds. In R. H. Thomson (ed.), The chemistry of natural products, pp. 65-68. Great Britain: Chapman & Hall.

Govindachari, T. R. and Premila, M. S. 1983. Some alkaloids from *Aegle marmelos*. Phytochemistry. 22(3): 755-757.

Green, J. M. 1996. A practical guide to analytical method validation. Analytical Chemistry. (68): 305A-309A.

Guo, L. Q., Taniguchi, M., Chen, Q. Y., Baba, K. and Yamazoe, Y. 2001. Inhibitory potential of herbal medicines on human cytochrome P450-mediated oxidation: properties of umbelliferous or citrus crude drugs and their relative prescriptions. Jpn J Pharmacol. 85(4): 399-408.

Guo, L. Q. and Yamazoe, Y. 2004. Inhibition of cytochrome P450 by furanocoumarins in grapefruit juice and herbal medicines. Acta Pharmacol Sin. 25(2): 129-136.

He, J. Y., Zhang, W., He, L. C. and Cao, Y. X. 2007. Imperatorin induces vasodilatation possibly via inhibiting voltage dependent calcium channel and receptor-mediated Ca^{2+} influx and release. Eur J Pharmacol. 573(1-3): 170-175.

Hodge, G., Hodge, S. and Han, P. 2002. *Allium sativum* (garlic) suppresses leukocyte inflammatory cytokine production *in vitro* : potential therapeutic use in the treatment of inflammatory bowel disease. Cytometry. 48(4): 209-215.

Hong, C. C., Tang, B. K., Hammond, G. L., Tritchler, D., Yaffe, M., Boyd, N. F. 2004. Cytochrome P450 1A2 (CYP1A2) activity and risk factors for breast cancer: a cross-sectional study. Breast Cancer Res. 6(4): R352-R365.

Hong, C. C., Tang, B. K., Rao, V., Agarwal, S., Martin, L., Tritchler, D., Yaffe, M. and Boyd, N. F. 2004. Cytochrome P450 1A2 (CYP1A2) activity, mammographic density, and oxidative stress: a cross-sectional study. Breast Cancer Res. 6(4): R338-R351.

Ingkaninan, K., Temkitthawon, P., Chuenchom, K., Yuyaem, T. and Thongnoi, W. 2003. Screening for acetylcholinesterase inhibitory activity in plants used in Thai traditional rejuvenating and neurotonic remedies. J Ethnopharmacol. 89(2-3): 261-264.

International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use. (1996, November). ICH-topic Q2 (R1) validation of analytical procedures: methodology[Online]. U.S. Department of Health and Human Services: Food and Drug Administration, Center for Drug Evaluation and Research (CDER). Available from: http://www.ich.org/cache/compo/276-254-1.html [1 Dec. 2007]

Ishihara, K., Kushida, H., Yuzurihara, M., Wakui, Y., Yanagisawa, T., Kamei, H., Ohmori, S. and Kitada, M. 2000. Interaction of drugs and Chinese herbs: pharmacokinetic changes of tolbutamide and diazepam caused by extract of *Angelica dahurica*. J Pharm Pharmacol. 52(8): 1023-1029.

Jagetia, C. J., Venkatesh, P. and Baliga, M. S. 2004. Evaluation of the radioprotective effect of bael leaf (*Aegle marmelos*) extract in mice. Int J Radiat Biol. 80: 281-290.

Jagetia, C. J., Venkatesh, P. and Baliga, M. S. 2004. Fruit extract of *Aegle marmelos* protects mice against radiation-induced lethality. Integr Cancer Ther. 3: 323-332.

Jagetia, G. C. and Baliga, M. S. 2004. The evaluation of nitric oxide scavenging activity of certain Indian medicinal plants *in vitro*: a preliminary study. J Med Food. 7: 343-348.

Jagetia, G. C. and Venkatesh, P., 2007. Inhibition of radiation-induced clastogenicity by *Aegle marmelos* (L.) Correa. in mice bone marrow exposed to different

doses of γ-radiation. Hum Exp Toxicol. 26(2): 111-124.

Jagetia, G. C. 2007. Radioprotective potential of plants and herbs against the effects of ionizing radiation. J Clin Biochem Nutr. 40(2): 74-81.

Jagetia, G. C., Venkatesh, P. and Baliga, M. S. 2005. *Aegle marmelos* (L.) Correa inhibits the proliferation of transplanted Ehrlich ascites carcinoma in mice. Biol Pharm Bull. 28(1): 58-64.

Jonen, H. G., Werringloer, J., Prough, R. A. and Estabrouk, R. W. 1982. The reaction of phenylhydrazine with microsomal cytochrome P450: catalysis of heme modification. J Biol Chem. 257: 4404-4411.

Kamalakkannan, N. and Prince, P. S. M. 2003. Hypoglycaemic effect of water extracts of *Aegle marmelos* fruits in streptozotocin diabetic rats. J Ethnopharmacol. 87(2-3): 207-210.

Kamalakkannan, N. and Stanely Mainzen Prince, P. 2003. Effect of *Aegle marmelos* Correa. (Bael) fruit extract on tissue antioxidants in streptozotocin diabetic rats. Indian J Exp Biol. 41(11): 1285-1288.

Kar, A., Choudhary, B. K. and Bandyopadhyay, N.G. 2003. Comparative evaluation of hypoglycaemic activity of some Indian medicinal plants in alloxan diabetic rats. J Ethnopharmacol. 84(1): 105-108.

Kar, A., Panda, S. and Bharti, S. 2002. Relative efficacy of three medicinal plant extracts in the alteration of thyroid hormone concentrations in male mice. J Ethnopharmacol. 81(2): 281-285.

Karunanayake, E. H., Welihinda, J., Sirimanne, S. R. and Sinnadorai, G. 1984. Oral hypoglycaemic activity of some medicinal plants of Sri Lanka. J Ethnopharmacol. 11(2): 223-231.

Kashuba, A. D. M., Park, J. J., Persky, A. M. and Brouwer, K. L. R. 2006. Drug metabolism, transport and the influence of hepatic disease. In Burton, M. E., Applied Pharmacokinetics and pharmacodynamics, principles of therapeutic drug monitoring, pp. 121-131. Baltimore: Lippincott Williams and Wilkins.

Kesari, A. N., Gupta, R. K., Singh, S. K., Diwakar, S. and Watal, G. 2006. Hypoglycemic and antihyperglycemic activity of *Aegle marmelos* seed extract in normal and diabetic rats. J Ethnopharmacol. 107(3): 374-379.

Kiani, J. and Imam, S. Z. 2007. Medicinal importance of grapefruit juice and its interaction with various drugs. Nutr J. 6: 33.

Kleiner, H. E., Reed, M. J. and DiGiovanni, J. 2003. Naturally occurring coumarins inhibit human cytochromes P450 and block benzo[a]pyrene and 7, 12-dimethylbenz[a]anthracene DNA adduct formation in MCF-7 cells. Chem Res Toxicol. 16(3): 415-422.

Kleiner, H. E., Vulimiri, S. V., Starost, M. F., Reed, M. J. and DiGiovanni, J. 2002. Oral administration of the citrus coumarin, isopimpinellin, blocks DNA adduct formation and skin tumor initiation by 7, 12-dimethylbenz[a]anthracene in SENCAR mice. Carcinogenesis. 23(10): 1667-1675.

Kusumoto, T. I., Nakabayashi, T., Kida, H., Miyashiro, H. Hattori, M. and Namba, T. 1995. Screening of various plant extracts used in Ayurvedic medicine for inhibitory effects on human immunodeficiency virus type 1 (HIV-1) protease. Phytotherapy Res. 9: 180-184.

Lampronti, I., Martello, D., Bianchi, N., Borgatti, M., Lambertini, E., Piva, R., Jabbar, S., Choudhuri, M. S., Khan, M. T. and Gambari, R. 2003. *In vitro* antiproliferative effects on human tumor cell lines of extracts from the Bangladeshi medicinal plant *Aegle marmelos* Correa. Phytomedicine. 10(4): 300-308.

Lawvere, S. and Mahoney, M. C. 2005. St. John's wort. Am Fam Physician. 72(11): 2249-2254.

Letteron, P., Descatoire, V., Larrey, D., Tinel, M., Geneve, J. and Pessayre, D. 1986. Inactivation and induction of cytochrome P450 by various psoralen derivatives in rats. J Pharmacol Exp Ther. 238: 685-692.

Li, Y. 2006. Determination of imperatorin in rat plasma by reversed-phase high-performance liquid chromatography after oral administration of Radix *Angelicae dahuricae* extract. J Pharm Biomed Anal. 40(5): 1253-1256.

Liu, Y., Zhang, J. W., Li, W., Ma, H., Sun, J., Deng, M. C. and Yang, L. 2006. Ginsenoside metabolites, rather than naturally occurring ginsenosides, lead to inhibition of human cytochrome P450 enzymes. Toxicol Sci. 91(2): 356-364.

Lowe, N. J. 1983. Psoriasis therapy: a current perspective. West J Med. 139(2): 184-189.

Luszczki, J. J., Wojda, E., Raszewski, G., Głowniak, K. and Czuczwar, S. J. 2008. Influence of imperatorin on the anticonvulsant activity and acute adverse-effect profile of lamotrigine in maximal electroshock-induced seizures and chimney test in mice. Pharmacol Rep. 60(4): 566-573.

Lynch, T. and Price, A. 2007. The effect of cytochrome P450 metabolism on drug response, interactions, and adverse effects. Am Fam Physician. 76(3): 391-396.

Macleod, J. A. and Pieris, M. N. 1981. Volatile flavor components of beli fruit (*Aegle marmelos*) and a processed product. J Agric Food Chem. 29: 1262-1264.

Madabushi, R., Frank, B., Drewelow, B., Derendorf, H. and Butterweck, V. 2006. Hyperforin in St. John's wort drug interactions. Eur J Clin Pharmacol. 62(3): 225-233.

Manandhar, M. D., Shoeb, A., Kapil, R. S. and Popli, S. P. 1978. New alkaloids from *Aegle marmelos*. Phytochemistry. 17: 1814-1815.

Marks, B. D. and Larson, B. R. Miniaturization and automation of cytochrome P450 inhibition assays[online]. 2008. Available from: http://www.invitrogen.com/site/us/en /home/Products-and-Services/Applications/Drug-Discovery/DD-Misc/Drug-Discovery-Posters.html[June 20. 2008]

Marques-Soares, C., Dijols, S., Macherey, A. C., Wester, M. R., Johnson, E. F., Dansette, P. M. and Mansuy, D. 2003. Sulfaphenazole derivatives as tools for comparing cytochrome P450 2C5 and human cytochromes P450 2Cs: identification of a new high affinity substrate common to those enzymes. Biochem. 42(21): 6363-6369.

Matsuda, H., Morikawa, T., Ohgushi, T., Ishiwada, T., Nishida, N. and Yoshikawa, M. 2005. Inhibitors of nitric oxide production from the flowers of *Angelica furcijuga*: structures of hyuganosides IV and V. Chem Pharm Bull (Tokyo). 53(4): 387-392.

Monostory, K., Hazai, E. and Vereczkey, L. 2004. Inhibition of cytochrome P450 enzymes participating in p-nitrophenol hydroxylation by drugs known as CYP2E1 inhibitors. Chem Biol Interact. 147(3): 331-340.

Moongkarndi, P., Kosem, N., Luanratana, O., Jongsomboonkusol, S. and Pongpan, N. 2004. Antiproliferative activity of Thai medicinal plant extracts on human

breast adenocarcinoma cell line. Fitoterapia. 75(3-4): 375-377.

Narender, T., Shweta, S., Tiwari, P., Papi Reddy, K., Khaliq, T., Prathipati, P., Puri, A., Srivastava, A. K., Chander, R., Agarwal, S. C. and Raj, K. 2007. Antihyperglycemic and antidyslipidemic agent from *Aegle marmelos*. Bioorg Med Chem Lett. 17(6): 1808-1811.

Narendhirakannan, R. T., Subramanian, S. and Kandaswamy, M. 2006. Biochemical evaluation of antidiabetogenic properties of some commonly used Indian plants on streptozotocin-induced diabetes in experimental rats. Clin Exp Pharmacol Physiol. 33(12): 1150-1157.

Nebert, D. W. and Russell, D.W. 2002. Clinical importance of the cytochromes P450. Lancet. 360(9340): 1155-1162.

Noysang, C. 1998. The phytochemical studies of Thai bael fruits, *Aegle marmelos* Correa (L.). Master's Thesis. Department of Pharmaceutical Chemistry and Phytochemistry, Graduate School, Mahidol University.

Ohnishi, A., Matsuo, H., Yamada, S., Takanaga, H., Morimoto, S., Shoyama, Y., Ohtani, H. and Sawada, Y. 2000. Effect of furanocoumarin derivatives in grapefruit juice on the uptake of vinblastine by Caco-2 cells and on the activity of cytochrome P450 3A4. Br J Pharmacol. 130(6): 1369-1377.

Olila, D., Olwa-Odyek and Opuda-Asibo, J. 2001. Antibacterial and antifungal activities of extracts of *Zanthoxylum chalybeum* and *Warburgia ugandensis*, Ugandan medicinal plants. Afr Health Sci. 1(2): 66-72.

Parkinson, A. and Ogilvie, B. W. 2008. Disposition of toxicants. In Klaassen, C. D., Casarett and Doull's toxicology: the basic science of poinsons 7th ed., p. 242. New York: Mc Graw-Hill.

Piao, X. L., Park, I. H., Baek, S. H., Kim, H. Y., Park, M. K. and Park, J. H. 2004. Antioxidative activity of furanocoumarins isolated from *Angelicae dahuricae*. J Ethnopharmacol. 93(2-3): 243-246.

Prince, M., Campbell, C. T., Robertson, T. A., Wells, A. J. and Kleiner, H. E. 2006. Naturally occurring coumarins inhibit 7, 12-dimethylbenz[a]anthracene DNA adduct formation in mouse mammary gland. Carcinogenesis. 27(6): 1204-1213.

Prommetta, P., Phivthong-ngam, L., Chaichantipyuth, C., Niwattisaiwong, N. and

Lawanprasert, S. 2006. Aqueous extract of the calyces of *Hibiscus subdariffa* Linn.: effects on hepatic cytochrome P450 and subacute toxicity in rats. Thai J Pharm Sci. 30(1-2): 8-18.

Pronsky, Z. M. and Crowe, J. P. 2004. Food-drug interactions. In Mahan, L. K. and Escott-Stump, S., Krause's food nutrition & diet therapy 12th ed, pp. 455-457. Philadelphia: Saunders.

Rahman, A. U., Zareen, S., Choudhary, M. I., Shaheen, F. and Parvez, M. 2004. Conformations of imperatorin. Acta Cryst. E60: o1460-o1462.

Rajadurai, M. and Prince, P. S. 2005. Comparative effects of *Aegle marmelos* extract and alpha-tocopherol on serum lipids, lipid peroxides and cardiac enzyme levels in rats with isoproterenol-induced myocardial infarction. Singapore Med J. 46(2): 78-81.

Rana, B. K., Singh, U. P. and Taneja, V. 1997. Antifungal activity and kinetics of inhibition by essential oil isolated from leaves of *Aegle marmelos*. J Ethnopharmacol. 57(1): 29-34.

Rani, P. and Khullar, N. 2004. Antimicrobial evaluation of some medicinal plants for their anti-enteric potential against multi-drug resistant *Salmonella typhi*. Phytother Res. 18(8): 670-673.

Ratan, K. B., Prabir, K. M. and Amal, K. M. 1981. Studies on a neutral polysaccharide isolated from bael (*Aegle marmelos*) fruit pulp. Carbohydrate Research. 97(2): 315-321.

Riyanto, S., Sukari, M. A., Rahman, M. Ali, A. M., Aimi, N. and Kitajima, M. 2003. Chemical constituents of *Aegle marmelos* leaves and their cytotoxic activities. The sixth JSPS-NRCT Joint Seminar: Recent Advance in Natural Medicine Research. 88-89.

Roy, A. and Saraf, S. 2006. Limonoids: overview of significant bioactive triterpenes distributed in plants kingdom. Biol Pharm Bull. 29(2): 191-201.

Roy, M., Sen, S. and Chakraborti, A. S. 2008. Action of pelargonidin on hyperglycemia and oxidative damage in diabetic rats: implication for glycation-induced hemoglobin modification. Life Sci. 82(21-22): 1102-1110.

Saebø, M., Skjelbred, C. F., Brekke Li, K., Bowitz Lothe, I. M., Hagen, P. C., Johnsen, E., Tveit, K. M. and Kure, E. H. 2008. CYP1A2 164 A-->C polymorphism, cigarette smoking, consumption of well-done red meat and risk of developing colorectal adenomas and carcinomas. Anticancer Res. 28(4C): 2289-2295.

Saenphet, K., Aritajat, S., Saenphet, S., Manosroi, J. and Manosroi, A. 2006. Safety evaluation of aqueous extracts from *Aegle marmelos* and *Stevia rebaudiana* on reproduction of female rats. Southeast Asian J Trop Med Public Health. 3: 203-205.

Sai, K., Saito, Y., Fukushima-Uesaka, H., Kurose, K., Kaniwa, N., Kamatani, N., Shirao, K., Yamamoto, N., Hamaguchi, T., Kunitoh, H., Ohe, Y., Tamura, T., Yamada, Y., Minami, H., Ohtsu, A., Yoshida, T., Saijo, N. and Sawada, J. 2008. Impact of CYP3A4 haplotypes on irinotecan pharmacokinetics in Japanese cancer patients. Cancer Chemother Pharmacol. 62(3): 529-537.

Sancho, R., Márquez, N., Gómez-Gonzalo, M., Calzado, M. A., Bettoni, G., Coiras, M. T., Alcamí, J., López-Cabrera, M., Appendino, G. and Muñoz, E. 2004. Imperatorin inhibits HIV-1 replication through an Sp1-dependent pathway. J Biol Chem. 279(36): 37349-53759.

Sato, E., Kohno, M., Hamano, H. and Niwano, Y. 2006. Increased anti-oxidative potency of garlic by spontaneous short-term fermentation. Plant Foods Hum Nutr. 61(4): 157-160.

Seow, A., Zhao, B., Lee, E. J., Poh, W. T., The, M., Eng, P., Wang, Y. T., Tan, W. C. and Lee, H. P. 2001. Cytochrome P4501A2 (CYP1A2) activity and lung cancer risk: a preliminary study among Chinese women in Singapore. Carcinogenesis. 22(4): 673-677.

Shankarananth, V., Balakrishnan, N., Suresh, D., Sureshpandian, G., Edwin, E. and Sheeja, E. 2007. Analgesic activity of methanol extract of *Aegle marmelos* leaves. Fitoterapia. 78(3): 258-259.

Shannon, F. M. and Michael, S. 2005. Drug interactions-a review. Clin Pediatric Emergency Med. 6(2): 93-102.

Sharma, B. R. and Sharma, P. 1981. Constituents of *Aegle marmelos* II alkaloids and coumarin from fruits. Planta Med. 43(1): 102-103.

Sharma, B. R. and Sharma, P. 1981. Marmelene, an alkaloid, and other components of unripe fruits of *Aegle Marmelos*. Phytochemistry. 20(11): 2606-2607.

Sharma, B. R. and Sharma, P. 1979. Constituents of leaves and fruits of *Aegle marmelos*. Indian J Chem. 19B: 162.

Shoba, F. G. and Thomas, M. 2001. Study of antidiarrhoeal activity of four medicinal plants in castor-oil induced diarrhoea. J Ethnopharmacol. 76(1): 73-76.

Sierpina, V. S., Wollschlaeger, B. and Blumenthal, M. 2003. *Ginkgo biloba*. Am Fam Physician. 68(5): 923-926.

Singh, R. P., Banerjee, S. and Rao, R. 2000. Effect of *Aegle marmelos* on biotransformation enzyme system and protection against free-radical-mediated damage in mice. J Pharm Pharmacol. 52: 991-1000.

Sinha-Roy, S. P. and Chakraborty, D. P. 1976. Psoralen, a powerful germination inhibitor. Phytochemistry. 15: 2005-2006.

Stanley, W. and Jurd, L. 1971. Citrus coumarins. J Agr Food Chem. 19: 1106-1110.

Stump, A. L., Mayo, T. and Blum, A. 2006. Management of grapefruit-drug interactions. Am Fam Physician. 74(4): 605-608.

Tassaneeyakul, W., Guo, L. Q., Fukuda, K., Ohta, T. and Yamazoe, Y. 2000. Inhibition selectivity of grapefruit juice components on human cytochromes P450. Arch Biochem Biophys. 378(2): 356-363.

The United States Pharmacopoeia 32, The National Formulary 29. 2005. pp. 2748-2751. Canada: Webcom Limited.

Tokitomo, Y., Shimono, Y., Kobayashi, A. and Yamanishi, T. 1982. Aroma components of bael fruit (*Aegle marmelos* Correa). Agr Biol Chem. 46: 1873-1877.

Veerappan, A., Miyazaki, S., Kadarkaraisamy, M. and Ranganathan, D. 2007. Acute and subacute toxicity studies of *Aegle marmelos* Corr., an Indian medicinal plant. Phytomedicine. 14(2-3): 209-215.

Venkataramanan, R., Komoroski, B. and Strom, S. 2006. *In vitro* and *in vivo* assessment of herb drug interactions. Life Sci. 78(18): 2105-2115.

Wang, Y., Hong, C., Zhou, C., Xu, D., Qu, H. B. and Cheng, Y. 2009. Screening antitumor compounds psoralen and isopsoralen from *Psoralea corylifolia* L. seeds. Evid Based Complement Alternat Med. 1-7.

Xie, J. T., Shao, Z. H., Vanden Hoek, T. L., Chang, W. T., Li, J., Mehendale, S., Wang, C. Z., Hsu, C. W., Becker, L. B., Yin, J. J. and Yuan, C. S. 2006. Antioxidant effects of ginsenoside Re in cardiomyocytes. Eur J Pharmacol. 532(3): 201-207.

Yang, C. S., Brady, J. F. and Hong, J. Y. 1992. Dietary effects on cytochromes P450, xenobiotic metabolism, and toxicity. FASEB J. 6(2):737-744.

Yang, L. L., Wang, M. C., Chen, L. G. and Wang, C. C. 2003. Cytotoxic activity of coumarins from the fruits of *Cnidium monnieri* on leukemia cell lines. Planta Med. 69(12): 1091-1095.

Zou, L., Harkey, M. R. and Henderson, G. L. 2002. Effects of herbal components on cDNA-expressed cytochrome P450 enzyme catalytic activity. Life Sci. 71(13): 1579-1589.

APPENDICES

APPENDIX A

PERCENTAGE OF INHIBITION ON VARIOUS CYP OF *A. MARMELOS* AND IMPERATORIN

Table 33 Percentage of inhibition of imperatorin on CYP1A2 (n=4)

Concentration of imperatorin, μM	%inhibition			
	n1	n2	n3	n4
0.055	0	0	0	0
0.11	0	2	0	1
0.55	53	53	53	54
1.1	69	68	68	68
2.2	83	81	81	ND
5.5	ND	85	84	86
22	89	86	89	88

ND = not determined

Table 34 Percentage of inhibition of imperatorin on CYP3A4 (n=4)

Concentration of imperatorin, μM	%inhibition			
	n1	n2	n3	n4
0.11	0	0	0	0
0.55	0	5	6	6
1.1	34	35	29	31
2.2	60	62	59	57
5.5	83	81	81	80
22	97	97	96	96

Table 35 Percentage of inhibition of imperatorin on CYP2C9 (n=4)

Concentration of imperatorin, μM	%inhibition			
	n1	n2	n3	n4
0.11	4	5	3	4
1.1	23	23	22	23
5.5	51	50	51	50
11	61	60	59	61
55	76	78	78	75
110	78	83	81	82

Table 36 Percentage of inhibition of imperatorin on CYP2C19 (n=4)

Concentration of imperatorin, μM	%inhibition			
	n1	n2	n3	n4
0.055	0	0	0	0
0.11	3	3	4	3
1.1	25	24	25	27
2.5	60	64	64	62
5.5	73	72	73	74
11	91	90	91	90
55	101	102	99	98
110	103	101	101	100
165	103	104	98	101
220	102	102	100	99

Table 37 Percentage of inhibition of hexane extract of *A. marmelos* fruit (extract A) on CYP1A2 (n=4)

Concentration of extract A, µg/ml	%inhibition			
	n1	n2	n3	n4
0.1	0	3	6	0
0.5	33	34	35	32
1	61	58	60	56
2	73	72	73	71
5	93	93	90	88
10	96	95	100	92

Table 38 Percentage of inhibition of hexane extract of *A. marmelos* fruit (extract A) on CYP3A4 (n=4)

Concentration of extract A, µg/ml	%inhibition			
	n1	n2	n3	n4
0.0396	3	0	0	0
0.396	ND	27	23	20
0.99	31	33	32	35
1.98	60	54	56	58
3.96	72	72	75	74
7.92	80	83	80	80
12	89	89	ND	ND
19.16	94	95	ND	ND

ND = not determined

Table 39 Percentage of inhibition of hexane extract of *A. marmelos* fruit (extract A) on CYP2C9 (n=4)

Concentration of extract A, µg/ml	%inhibition			
	n1	n2	n3	n4
0.1	14	12	14	14
1	27	26	27	27
2	44	42	43	40
5	83	81	82	77
10	102	101	97	95

Table 40 Percentage of inhibition of hexane extract of *A. marmelos* fruit (extract A) on CYP2C19 (n=4)

Concentration of extract A, µg/ml	%inhibition			
	n1	n2	n3	n4
0.5	9	9	9	7
1	13	19	19	13
2	30	26	25	27
5	61	61	62	59
10	80	83	79	79
15	88	89	89	89

Table 41 Percentage of inhibition of ethanolic extract of *A. marmelos* fruit (extract B) on CYP1A2 (n=4)

Concentration of extract B, µg/ml	%inhibition			
	n1	n2	n3	n4
10	1	0	0	0
20	6	2	3	6
50	65	65	70	68
100	81	84	84	88
250	96	94	96	95
500	99	99	106	107

Table 42 Percentage of inhibition of ethanolic extract of *A. marmelos* fruit (extract B) on CYP3A4 (n=4)

Concentration of extract B, µg/ml	%inhibition			
	n1	n2	n3	n4
0.4	0	0	0	0
4	14	10	5	9
40	31	27	34	20
200	79	79	74	76
400	83	84	89	84
800	95	95	95	95

Table 43 Percentage of inhibition of ethanolic extract of *A. marmelos* fruit (extract B) on CYP2C9 (n=4)

Concentration of extract B, µg/ml	%inhibition			
	n1	n2	n3	n4
10	2	0	8	13
100	27	28	27	25
250	51	53	52	52
500	ND	ND	63	64
750	77	77	77	75
1,000	75	79	77	74

ND = not determined

Table 44 Percentage of inhibition of ethanolic extract of *A. marmelos* fruit (extract B) on CYP2C19 (n=4)

Concentration of extract B, µg/ml	%inhibition			
	n1	n2	n3	n4
10	17	15	15	13
50	33	32	38	32
100	53	52	54	50
250	73	71	74	71
500	83	81	82	82
750	87	86	89	88
1,000	92	90	91	91

Table 45 Percentage of inhibition of aqueous extract of *A. marmelos* fruit (extract C) on CYP1A2 (n=4)

Concentration of extract C, µg/ml	%inhibition			
	n1	n2	n3	n4
50	6	7	1	6
100	15	14	14	13
250	37	38	36	39
500	56	56	56	54
750	66	66	68	65
1,000	70	71	71	70
1,500	81	81	82	ND

ND = not determined

Table 46 Percentage of inhibition of aqueous extract of *A. marmelos* fruit (extract C) on CYP3A4 (n=4)

Concentration of extract C, µg/ml	%inhibition			
	n1	n2	n3	n4
50	0	0	0	0
250	7	7	4	11
500	30	29	33	31
750	44	41	42	49
1,500	66	67	68	69
1,600	90	88	ND	ND
2,000	94	94	ND	ND
3,200	98	97	82	89

ND = not determined

Table 47 Percentage of inhibition of aqueous extract of *A. marmelos* fruit (extract C) on CYP2C9 (n=4)

Concentration of extract C, µg/ml	%inhibition			
	n1	n2	n3	n4
50	15	0	7	15
100	9	10	11	9
250	17	10	12	17
500	27	26	27	25
750	36	31	38	ND
1,000	47	ND	47	50
1,500	88	88	89	87
3,000	88	86	89	ND

ND = not determined

Table 48 Percentage of inhibition of aqueous extract of *A. marmelos* fruit (extract C) on CYP2C19 (n=4)

Concentration of extract C, µg/ml	%inhibition			
	n1	n2	n3	n4
10	0	0	0	0
50	28	24	26	25
100	35	32	29	33
250	41	45	43	38
500	61	55	58	61
750	88	89	89	83
1,000	98	97	93	92
1,500	93	94	93	94
2,000	92	91	90	97

APPENDIX B

VERIFICATION OF THE VIVID® CYP450 SCREENING KITS

Table 49 Percentage of inhibition of α-naphthoflavone on CYP1A2 (n=2)

Concentration of α-naphthoflavone, μM	%inhibition	
	n1	n2
0.005	ND	0
0.01	ND	5
0.025	9	7
0.05	14	13
0.1	32	33
0.25	82	79
0.5	90	87
1	90	92

ND = not determined

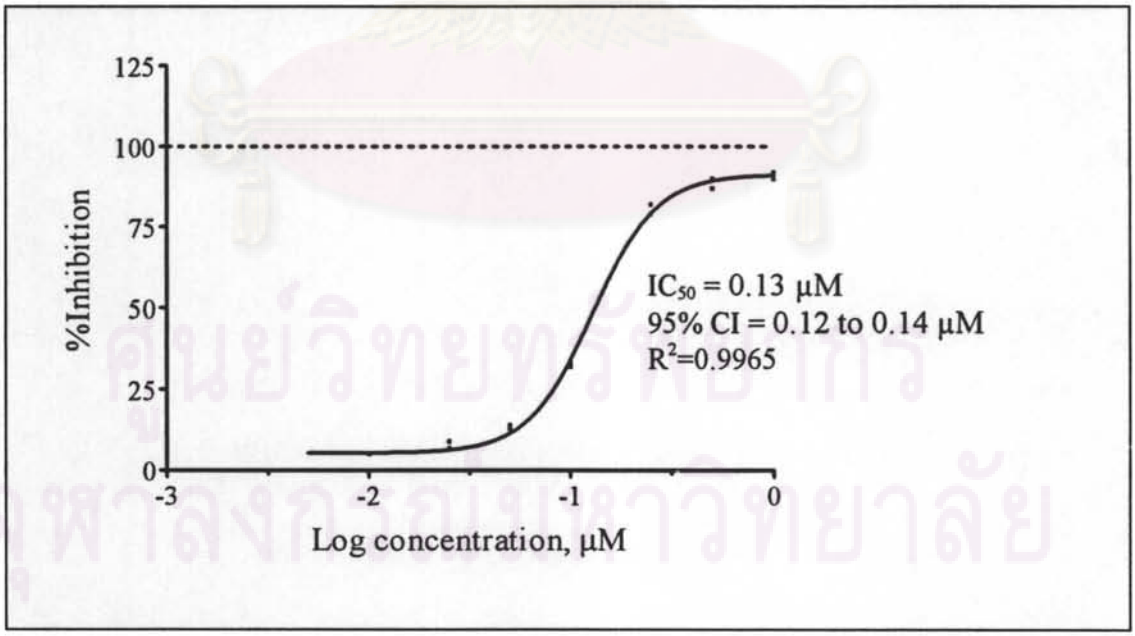


Figure 34 Inhibition curve of α-naphthoflavone on CYP1A2

Table 50 Percentage of inhibition of ketoconazole on CYP3A4 (n=2)

Concentration of ketoconazole, μM	%inhibition	
	n1	n2
0.11	0	0
0.275	13	15
0.55	44	57
2.2	79	80
11	84	87
55	81	90

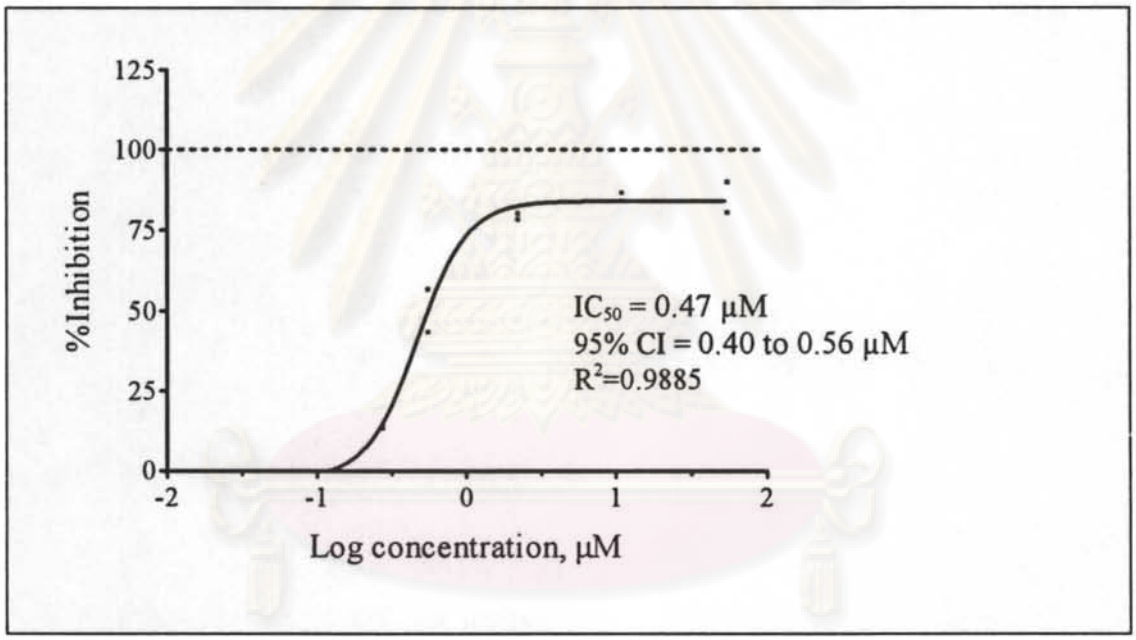


Figure 35 Inhibition curve of ketoconazole on CYP3A4

Table 51 Percentage of inhibition of sulfaphenazole on CYP2C9 (n=2)

Concentration of sulfaphenazole, μM	%inhibition	
	n1	n2
0.001	0	9
0.01	25	16
0.05	40	36
0.25	79	62
2	82	83
10	102	100
30	104	105

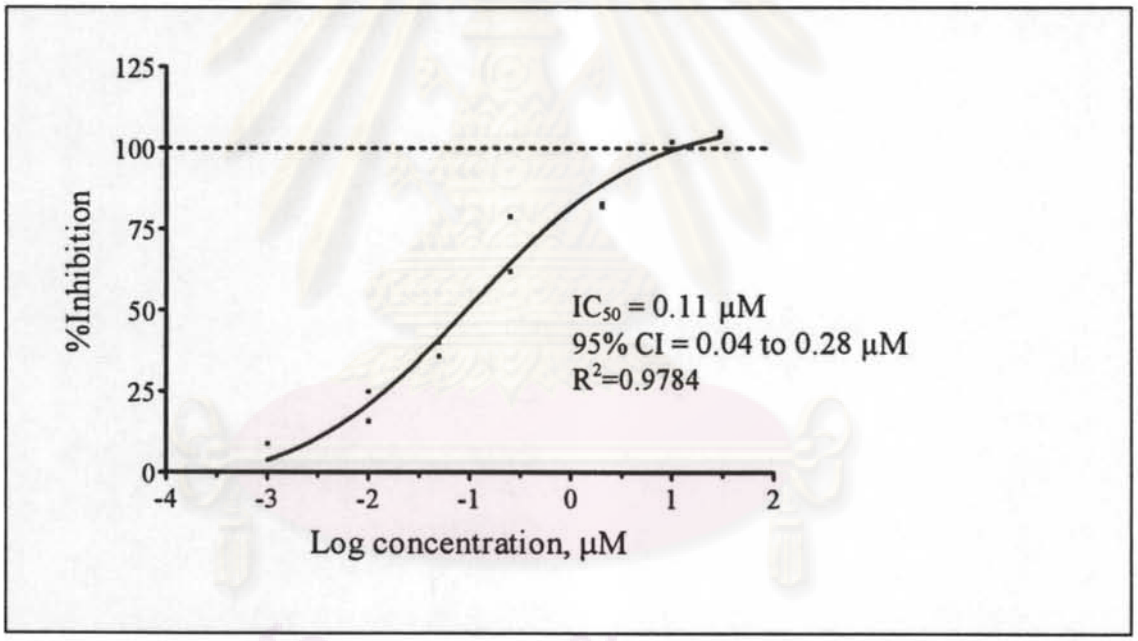


Figure 36 Inhibition curve of sulfaphenazole on CYP2C9

Table 52 Percentage of inhibition of miconazole on CYP2C19 (n=2)

Concentration of miconazole, μM	%inhibition	
	n1	n2
0.01	2	0
0.025	2	2
0.05	11	11
0.1	22	ND
0.25	50	ND
0.5	96	92
1	103	104

ND = not determined

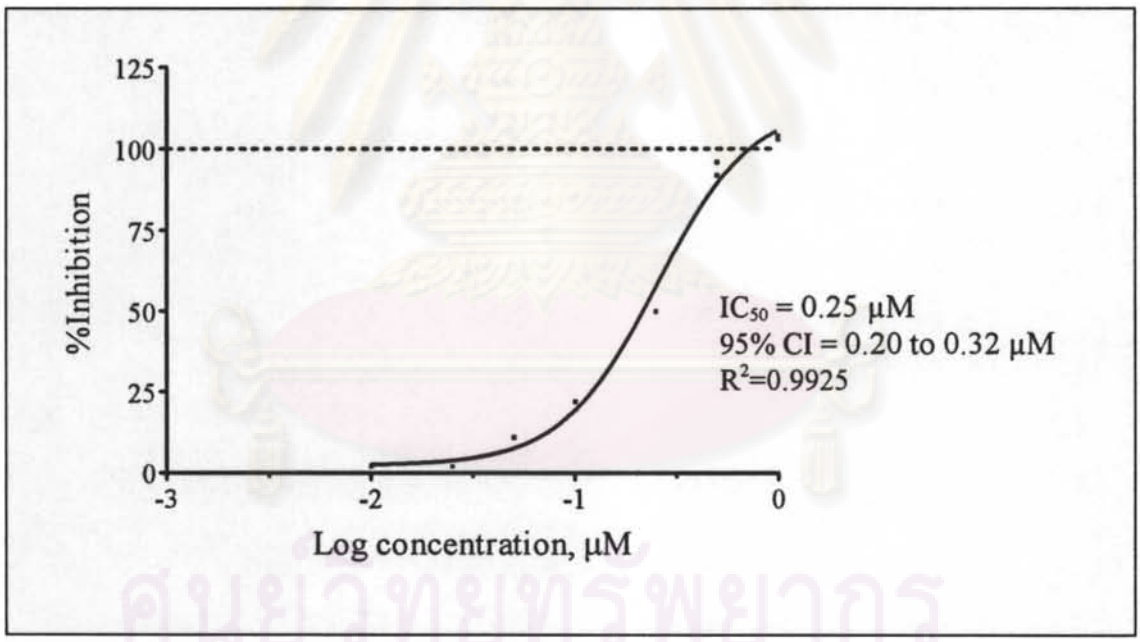


Figure 37 Inhibition curve of miconazole on CYP2C19

APPENDIX C

VIVID® CYP450 SCREENING KITS PROTOCOL

invitrogen™

Vivid® CYP450 Screening Kits Protocol

Cat. no. P2856, P2857, P2858, P2859, P2860, P2861, P2862, P2863, P2864, P2968, P2969, P2970, P2971, P2972, P3019, P3020 and P3021

O-13873-r1 US 0405

TABLE OF CONTENTS

1.0 INTRODUCTION

Vivid® CYP450 Screening Kits enable rapid measurement of interactions between drug candidates and cytochrome P450 enzymes using a simple "mix-and-read" fluorescent assay that is designed for high-throughput screening in multiwell plates. These kits will allow investigators to rapidly identify compound-CYP450 interactions, eliminating unsuitable compounds early in the drug discovery process. Vivid® CYP450 Screening Kits can also be used to generate predictive structure-activity relationship models to guide medicinal chemists in their design of compounds.

Test compounds are analyzed by their capacity to inhibit the production of a fluorescent signal in reactions using recombinant CYP450 isozymes and specific Vivid® CYP450 Substrates. The availability of more than one structurally unrelated fluorogenic Vivid® CYP450 Substrate for CYP3A4, CYP3A5, CYP2C9, CYP2B6 and CYP2D6 reduces the potential for false negatives (and false positives) that could result from substrate-dependent interactions.

2.0 MATERIALS SUPPLIED

Vivid® CYP450 Screening Kit	Description	Cat. no.	Quantity	Storage
Vivid® CYP1A2 Blue (P2863)	Vivid® CYP450 Reaction Buffer I	P2881	50 ml	RT
	CYP1A2 BACULOSOMES® Reagent	P2792	0.5 nmol	-80°C
	Vivid® EOMCC Substrate	P3024	0.1 mg	-20°C, light protected
	Vivid® Blue Fluorescent Standard	P2876	0.1 µmol	-20°C, light protected
Vivid® CYP2B6 Blue (P3019)	Vivid® CYP450 Reaction Buffer I	P2881	50 ml	RT
	CYP2B6 BACULOSOMES® Reagent	P3028	0.5 nmol	-80 °C
	Vivid® BOMCC Substrate	P2975	0.1 mg	-20°C, light protected
	Vivid® Blue Fluorescent Standard	P2876	0.1 µmol	-20°C, light protected
Vivid® CYP2B6 Cyan (P3020)	Vivid® CYP450 Reaction Buffer I	P2881	50 ml	RT
	CYP2B6 BACULOSOMES® Reagent	P3028	0.5 nmol	-80°C
	Vivid® BOMFC Substrate	P2976	0.1 mg	-20°C, light protected
	Vivid® Cyan Fluorescent Standard	P2877	0.1 µmol	-20°C, light protected
Vivid® CYP2C9 Blue (P2861)	Vivid® CYP450 Reaction Buffer II	P2913	50 ml	RT
	CYP2C9 BACULOSOMES® Reagent	P2378	0.5 nmol	-80°C
	Vivid® BOMCC Substrate	P2975	0.1 mg	-20°C, light protected
	Vivid® Blue Fluorescent Standard	P2876	0.1 µmol	-20°C, light protected
Vivid® CYP2C9 Green (P2860)	Vivid® CYP450 Reaction Buffer II	P2913	50 ml	RT
	CYP2C9 BACULOSOMES® Reagent	P2378	0.5 nmol	-80°C
	Vivid® BOMF Substrate	P2869	0.1 mg	-20°C, light protected
	Vivid® Green Fluorescent Standard	P2875	0.1 µmol	-20°C, light protected
Vivid® CYP2C9 Red (P2859)	Vivid® CYP450 Reaction Buffer II	P2913	50 ml	RT
	CYP2C9 BACULOSOMES® Reagent	P2378	0.5 nmol	-80°C
	Vivid® OOMR Substrate	P2868	0.1 mg	-20°C, light protected
	Vivid® Red Fluorescent Standard	P2874	0.1 µmol	-20°C, light protected
Vivid® CYP2C19 Blue (P2864)	Vivid® CYP450 Reaction Buffer II	P2913	50 ml	RT
	CYP2C19 BACULOSOMES® Reagent	P2570	0.5 nmol	-80°C
	Vivid® EOMCC Substrate	P3024	0.1 mg	-20°C, light protected
	Vivid® Blue Fluorescent Standard	P2876	0.1 µmol	-20°C, light protected
Vivid® CYP2D6 Blue (P2972)	Vivid® CYP450 Reaction Buffer I	P2881	50 ml	RT
	CYP2D6 BACULOSOMES® Reagent	P2283	0.5 nmol x 2	-80°C
	Vivid® EOMCC Substrate	P3024	0.1 mg	-20°C, light protected
	Vivid® Blue Fluorescent Standard	P2876	0.1 µmol	-20°C, light protected
Vivid® CYP2D6 Cyan (P2862)	Vivid® CYP450 Reaction Buffer I	P2881	50 ml	RT
	CYP2D6 BACULOSOMES® Reagent	P2283	0.5 nmol x 2	-80°C
	Vivid® MOBFC Substrate	P2871	0.1 mg	-20°C, light protected
	Vivid® Cyan Fluorescent Standard	P2877	0.1 µmol	-20°C, light protected
Vivid® CYP2E1 Blue (P3021)	Vivid® CYP450 Reaction Buffer III	P2949	50 ml	RT
	CYP2E1 BACULOSOMES® Reagent	P2948	1.0 nmol	-80°C
	Vivid® EOMCC Substrate	P3024	0.1 mg	-20°C, light protected
	Vivid® Blue Fluorescent Standard	P2876	0.1 µmol	-20°C, light protected
Vivid® CYP3A4 Blue (P2858)	Vivid® CYP450 Reaction Buffer I	P2881	50 ml	RT
	CYP3A4 BACULOSOMES® Reagent	P2377	0.5 nmol	-80°C
	Vivid® BOMCC Substrate	P2975	0.1 mg	-20°C, light protected
	Vivid® Blue Fluorescent Standard	P2876	0.1 µmol	-20°C, light protected
Vivid® CYP3A4 Cyan (P2968)	Vivid® CYP450 Reaction Buffer I	P2881	50 ml	RT
	CYP3A4 BACULOSOMES® Reagent	P2377	0.5 nmol	-80°C
	Vivid® BOMFC Substrate	P2976	0.1 mg	-20°C, light protected
	Vivid® Cyan Fluorescent Standard	P2877	0.1 µmol	-20°C, light protected
Vivid® CYP3A4 Green (P2857)	Vivid® CYP450 Reaction Buffer I	P2881	50 ml	RT
	CYP3A4 BACULOSOMES® Reagent	P2377	0.5 nmol	-80°C
	Vivid® DBOMF Substrate	P2974	0.1 mg	-20°C, light protected
	Vivid® Green Fluorescent Standard	P2875	0.1 µmol	-20°C, light protected
Vivid® CYP3A4 Red (P2856)	Vivid® CYP450 Reaction Buffer I	P2881	50 ml	RT
	CYP3A4 BACULOSOMES® Reagent	P2377	0.5 nmol	-80°C
	Vivid® BOMR Substrate	P2865	0.1 mg	-20°C, light protected
	Vivid® Red Fluorescent Standard	P2874	0.1 µmol	-20°C, light protected
Vivid® CYP3A5 Blue (P2970)	Vivid® CYP450 Reaction Buffer I	P2881	50 ml	RT
	CYP3A5 BACULOSOMES® Reagent	P2512	0.5 nmol	-80°C
	Vivid® BOMCC Substrate	P2975	0.1 mg	-20°C, light protected
	Vivid® Blue Fluorescent Standard	P2876	0.1 µmol	-20°C, light protected
Vivid® CYP3A5 Cyan (P2971)	Vivid® CYP450 Reaction Buffer I	P2881	50 ml	RT
	CYP3A5 BACULOSOMES® Reagent	P2512	0.5 nmol	-80°C
	Vivid® BOMFC Substrate	P2976	0.1 mg	-20°C, light protected
	Vivid® Cyan Fluorescent Standard	P2877	0.1 µmol	-20°C, light protected
Vivid® CYP3A5 Green (P2969)	Vivid® CYP450 Reaction Buffer I	P2881	50 ml	RT
	CYP3A5 BACULOSOMES® Reagent	P2512	0.5 nmol	-80°C
	Vivid® DBOMF Substrate	P2974	0.1 mg	-20°C, light protected
	Vivid® Green Fluorescent Standard	P2875	0.1 µmol	-20°C, light protected

All kits also contain 0.5 ml Regeneration System, 100X (P2876, 333 mM Glucose-6-phosphate and 30 U/ml Glucose-6-phosphate dehydrogenase in 100 mM potassium phosphate pH 8.0) and 0.5 ml NADP+ (P2879, 10 mM NADP+ in 100 mM potassium phosphate pH 8.0). Store both components at -80°C.

- The Vivid® CYP450 Reaction Buffers are 200 mM (Reaction buffer I), 100 mM (Reaction buffer II), or 400 mM (Reaction buffer III) potassium phosphate pH 8.0.
- CYP450 BACULOSOMES® Reagents consist of recombinant human Cytochrome P450 (1 µM) and rabbit NADPH P450 Reductase.
- The Vivid® Substrates and Standards are supplied as a dried film. Reconstitution is necessary before use.

2.1 Materials Required but not Supplied

- Multiwell black plates suitable for fluorescence measurements (Note: black-walled, clear bottom plates are needed for bottom-read fluorescent microplate readers). Invitrogen recommends using Costar #3915 non-treated plates
- Fluorescence plate reader with filters as described in Table 6
- Pipeting devices
- Reagent reservoir(s)
- Acetonitrile, anhydrous
- DMSO, reagent grade
- Deionized water
- Stop Reagent (CYP450 isozyme specific inhibitor) if performing an endpoint assay or in kinetic mode for the positive control of inhibition. For more information on inhibitors, see Section 7.0.

3.0 STORAGE AND STABILITY

Vivid® CYP450 Substrates and Fluorescent Standards are stable for at least six months when stored desiccated and protected from light at –20°C. For short-term storage, acetonitrile- or DMSO-based stock solutions should be stored in a desiccator at 4°C. Long-term storage requires that organic solutions be kept desiccated at –20°C. DMSO solutions are hygroscopic, and cold vials should be warmed to ambient temperature before opening. After opening, they should be capped promptly to avoid reagent dilution by absorbed moisture. The CYP450 BACULOSOMES® Reagent should be stored at -80°C. No significant decrease in activity (see enclosed Certificate of Analysis) was observed after 5 freeze/thaw cycles except for CYP2D6 which showed a 5% decrease. The Regeneration System should be stored at -80°C. Upon first thaw, aliquot into single use vials as the reagent should not be subjected to additional freeze/thaw cycles. The NADP+ should be stored at -80°C and is stable for at least 10 freeze/thaw cycles. Store protected from light. The Vivid® CYP450 Reaction Buffer (2X) can be stored at 4°C or room temperature.

4.0 ASSAY THEORY

Vivid® CYP450 Screening Kits are designed to assess metabolism and inhibition of the predominant human P450 isozymes involved in hepatic drug metabolism: CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4 and CYP3A5. The kits employ Vivid® CYP450 Substrates and CYP450 BACULOSOMES® Reagents. The CYP450 BACULOSOMES® Reagents are microsomes prepared from insect cells expressing a human P450 isozyme and rabbit NADPH-P450 reductase (CYP2E1 also contains human cytochrome b_5). CYP450 BACULOSOMES® Reagents offer a distinct advantage over human liver microsomes in that only one CYP450 enzyme is expressed, thereby preventing metabolism by other CYP450s. The Vivid® Substrates are metabolized by a specific CYP450 enzyme into products that are highly fluorescent in aqueous solutions. Figure 1 schematically depicts the metabolism of a Vivid® CYP450 Substrate into a fluorescent metabolite. Note that the Vivid® Substrates have two potential sites for metabolism (indicated by arrows in Figure 1) and that oxidation at either site releases the highly fluorescent metabolite.

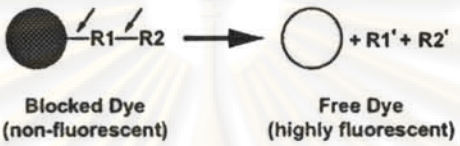


Figure 1. Schematic of the metabolism of the "blocked" dye substrate into a fluorescent metabolite

The fluorescent metabolites are excited in the visible light spectrum, which minimizes interference caused by the background fluorescence of UV-excitable compounds and NADPH. The excellent reaction kinetics and optical properties of the Vivid® Substrates allow their use at concentrations at or below their K_m value in a reaction with P450 isozymes, assuring detection of even weak CYP450 inhibitors and providing the convenience of room temperature or 37°C incubations. The Vivid® CYP450 Assay may be run in a kinetic or endpoint mode (which is illustrated in Figure 2).

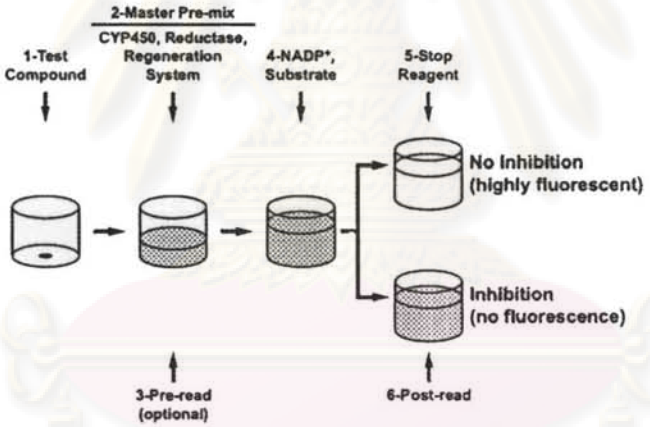


Figure 2. A schematic representation of an endpoint Vivid® CYP450 Assay

In end point (Section 5.1.9.2) mode, the test compounds (Step 1) are first combined with the Master Pre-mix (Step 2), consisting of CYP450 BACULOSOMES® Reagents and the Regeneration System (consisting of glucose-6-phosphate and glucose-6-phosphate dehydrogenase). The Regeneration System converts NADP+ into NADPH, which is required to start the CYP450 reaction. After a brief pre-incubation, the background fluorescence of the test compound and Master Pre-mix is measured (Step 3, pre-read). The enzymatic reaction is initiated by the addition of a mix of NADP+ and the appropriate Vivid® Substrate (Step 4) and plate is incubated for the desired reaction time. After the addition of a Stop Reagent (Step 5), the fluorescence is measured in Step 6.

In kinetic mode (Section 5.1.9.1), the fluorescence is measured continuously starting after Step 4 (and eliminating Steps 5 and 6). Standard curves, constructed from the supplied Fluorescent Standard, can be used to calculate reaction rates from the observed fluorescence intensities in both assay formats. Assay parameters for isozymes CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4 and CYP3A5 are listed in Tables 4 and 5.

5.0 VIVID® CYP450 HIGH-THROUGHPUT SCREENING ASSAY PROTOCOL

Each complete reaction must contain CYP450 BACULOSOMES® Reagent, Vivid® CYP450 Substrate, NADP+ and Regeneration System, all in the appropriate Vivid® CYP450 Reaction Buffer (supplied with each kit as a 2X solution). There are two possible modes for this assay: kinetic and endpoint. The method you choose will depend on your analytical needs and the equipment available. The kinetic mode is useful for analysis of one multiwell plate at a time and does not require the addition of the stop reagent. In endpoint mode, after an appropriate incubation time, the reaction is stopped by the addition of the CYP450 isozyme-specific inhibitor. Running in endpoint mode allows the reaction to be performed in several multiwell plates simultaneously.

Note: The following protocol is configured for use with one 96-well plate and 100 µl reactions. However, the protocol can be modified to accommodate several different plate formats by adjusting the calculations for the number of wells (and volume per well) in your experiment. See Trubetsoy *et al.* (2005) (see Section 9.0 for a complete list of references) for use of Vivid® kits in 1536-well plate formats. Each kit supplies enough reagents for at least 300 x 100 µl reactions.

5.1 Assay Procedure

5.1.1 Thaw Reagents

1. Thaw the P450 BACULOSOMES®, Regeneration System, and NADP+ on ice until ready to use. Do not vortex P450 BACULOSOMES® or Regeneration System.
2. Suggested assay conditions for screening with Vivid® kits are described in Table 1.

Table 1. Assay Conditions

Condition	Purpose	Dispensing
Test Compound	Screen for inhibition by compound of interest	40 µl 2.5X test compound 50 µl Master Pre-Mix 10 µl Vivid Substrate and NADP+
Positive Inhibition Control	Inhibit the reaction with a known P450 inhibitor	40 µl 2.5X positive inhibition control (see Section 7.0) 50 µl Master Pre-Mix 10 µl Vivid Substrate and NADP+
Solvent Control (No inhibitor)	Accounts for possible solvent inhibition caused by introduction of test compounds originally dissolved in an organic solvent such as DMSO	40 µl 2.5X solvent control 50 µl Master Pre-Mix 10 µl Vivid Substrate and NADP+
Background	Enables subtraction of background fluorescence during data analysis	40 µl 2.5X solvent control 50 µl Vivid® CYP450 Reaction Buffer 10 µl Vivid Substrate and NADP+

5.1.2 *Reconstitution of Vivid® Substrate and Fluorescent Standard*

1. Reconstitute the Vivid® Standard using anhydrous acetonitrile and Fluorescent Standard using DMSO (see Tables 2 and 3).
2. Keep these solutions at room temperature for immediate use, or store at -20°C.

Table 2. Reconstitution of the Vivid® CYP450 substrates

Isozyme Type	Vivid® CYP450 Substrate	Molecular weight	mg per tube	µmol per tube	µl acetonitrile added per tube	[stock solution] (mM)	[screening concentration] (µM)
1A2	Vivid® EOMCC	245.2	0.1	0.41	205	2	3
2B6	Vivid® BOMCC	307.3	0.1	0.32	160	2	5
	Vivid® BOMFC	350.3	0.1	0.28	140	2	2
2C9	Vivid® BOMCC	307.3	0.1	0.32	160	2	10
	Vivid® BOMF	452.5	0.1	0.22	110	2	2
	Vivid® OOMR[5]	355.4	0.1	0.28	140	2	2
2C19	Vivid® EOMCC	245.2	0.1	0.41	205	2	10
2D6	Vivid® EOMCC	245.2	0.1	0.41	205	2	10
	Vivid® MOBFC	350.3	0.1	0.28	140	2	5
2E1	Vivid® EOMCC	245.2	0.1	0.41	205	2	10
3A4	Vivid® BOMCC	307.3	0.1	0.32	160	2	10
	Vivid® BOMFC	350.3	0.1	0.28	140	2	5
	Vivid® DBOMF	572.6	0.1	0.17	85	2	2
	Vivid® BOMR	333.3	0.1	0.30	150	2	3
3A5	Vivid® BOMCC	307.3	0.1	0.32	160	2	10
	Vivid® BOMFC	350.3	0.1	0.28	140	2	5
	Vivid® DBOMF	572.6	0.1	0.17	85	2	2

[5] Heat at 70°C for 3-5 minutes and vortex to reconstitute.

Table 3. Reconstitution of the Fluorescent Standard. Use the blank cells in the table for your calculations. The value [X] is the amount of Assay Standard listed on the tube label.

Assay Standard	µmol per tube [X]	Reconstitution Solvent	µl Reconstitution Solvent added per tube [X x 10000]	[Fluorescent Standard] after Reconstitution, µM
Example	0.11	DMSO	1100 µl	100
Red Standard		DMSO/water (1:1)		100
Green Standard		DMSO		100
Blue Standard		DMSO		100
Cyan Standard		DMSO		100

5.1.3 *Prepare Standard Curve (Optional)*

1. With room temperature water, dilute enough Reaction Buffer (2X) to prepare enough 1X Reaction Buffer for your standard curve. In a 96-well plate, one standard curve can be run in 8 wells using 1 ml of Reaction Buffer. We recommend that at least six points (in addition to the blank) be used for the standard curve and that it be performed in duplicate.
2. To the first well of the column add 195 µl 1X Reaction Buffer.
3. Add 100 µl of 1X Reaction Buffer to each of the remaining wells in the column.
4. Add 5 µl of Fluorescent Standard (Table 3) to the first well containing 195 µl of buffer to achieve a starting concentration of 2.5 µM. Mix well.
5. Transfer 100 µl from this well into the next well containing 100 µl 1X Reaction Buffer and mix by pipetting. This is a two-fold dilution.
6. Repeat this dilution step, leaving the last well as an assay blank containing 1X Reaction Buffer only and no standard. The resulting Fluorescent Standard concentrations are: 2.5 µM, 1.25 µM, 625 nM, 312.5 nM, 156.25 nM, 78.125 nM, 39.063 nM and 0 nM.

Note: These are suggested initial concentrations for the standard curve. More or less may be appropriate depending on you experimental needs.

Note: The assay can be performed simply using fluorescence values instead of converting to concentration of product formed.

5.1.4 Prepare Test Compounds, Positive Inhibition Control, and Solvent Control

1. Prepare 2.5X Test Compounds by dilution into deionized water. (For IC_{50} determination, a serial dilution of the test compound is required.)
2. Prepare a 2.5X solution of a known P450 Inhibitor in deionized water for positive control of inhibition (optional).

   Note: We recommend use of the inhibitors listed in Section 7.0.
3. Prepare a solution of the solvent used to dissolve the test compounds and known P450 inhibitor at 2.5X final concentration.

   Note: See Section 8.0 for information about particular solvents and tolerances.

5.1.5 Dispense Test Compounds, Positive Inhibition Control, and Solvent Control

1. Add 40 µl of the 2.5X solutions prepared in Section 5.1.4 to desired wells of the plate.
2. We recommend at least three replicates for the Positive Inhibition Control and Solvent Control.

5.1.6 Prepare and Dispense Master Pre-Mix

1. Prepare the Master Pre-Mix by diluting P450 BACULOSOMES® Reagent and Regeneration System in Vivid® CYP450 Reaction Buffer (2X) on ice (see Table 4). Mix by inversion.
2. Dispense 50 µl of Master Pre-Mix to each well. Mix.

   Note: To account for background fluorescence in the absence of CYP450 activity, dispense 50 µl of Vivid® CYP450 Reaction Buffer without P450 BACULOSOMES® to desired wells of the plate.

Table 4. Master Pre-mix (pre-mix of CYP450 BACULOSOMES® Reagents and Regeneration System). Keep on ice until ready to use

Isozyme Type	Vivid® CYP450 Substrate	µl of Vivid® CYP450 Reaction Buffer (2X) added	µl of Regeneration System (100X) added	µl of CYP450 BACULOSOMES® added	Concentration of CYP450 in Master Pre-mix (2X), nM	Screening concentration of CYP450, nM[1]
1A2	Vivid® EOMCC	4850 (Buffer I)	100	50	10	5
2B6	Vivid® BOMCC	4800 (Buffer I)	100	100	20	10
	Vivid® BOMFC	4880 (Buffer I)	100	20	4	2
2C9	Vivid® BOMCC	4800 (Buffer II)	100	100	20	10
	Vivid® BOMF	4800 (Buffer II)	100	100	20	10
	Vivid® OOMR	4800 (Buffer II)	100	100	20	10
2C19	Vivid® EOMCC	4850 (Buffer II)	100	50	10	5
2D6	Vivid® EOMCC	4800 (Buffer I)	100	100	20	10
	Vivid® MOBFC	4700 (Buffer I)	100	200	40	20
2E1	Vivid® EOMCC	4850 (Buffer III)	100	50	10	5
3A4	Vivid® BOMCC	4850 (Buffer I)	100	50	10	5
	Vivid® BOMFC	4850 (Buffer I)	100	50	10	5
	Vivid® DBOMF	4850 (Buffer I)	100	50	10	5
	Vivid® BOMR	4850 (Buffer I)	100	50	10	5
3A5	Vivid® BOMCC	4850 (Buffer I)	100	50	10	5
	Vivid® BOMFC	4850 (Buffer I)	100	50	10	5
	Vivid® DBOMF	4850 (Buffer I)	100	50	10	5

[1] For your first experiment, we suggest these concentrations of the CYP450 enzyme. Based on your results, you may find more or less enzyme is necessary.

5.1.7 Pre-Incubate

1. Incubate the plate for 20 minutes at room temperature to allow the compounds to interact with the CYP450 in the absence of enzyme turnover.
2. During this pre-incubation, prepare the pre-mixture of Vivid® Substrate and NADP+ (see Table 5).
3. You may also wish to include a pre-read at this point to determine if your compounds are fluorescent.

Isozyme Type	Vivid® CYP450 Substrate	µl of Vivid® CYP450 Reaction Buffer (2X) added	µl of Reconstituted Substrate added (Section 5.1.2)	µl of NADP+ (100X) added	Final % ACN from substrate
1A2	Vivid® EOMCC	885 (Buffer I)	15	100	0.15
2B6	Vivid® BOMCC	875 (Buffer I)	25	100	0.25
	Vivid® BOMFC	960 (Buffer I)	10	30	0.10
2C9	Vivid® BOMCC	850 (Buffer II)	50	100	0.50
	Vivid® BOMF	890 (Buffer II)	10	100	0.10
	Vivid® OOMR	890 (Buffer II)	10	100	0.10
2C19	Vivid® EOMCC	850 (Buffer II)	50	100	0.50
2D6	Vivid® EOMCC	850 (Buffer I)	50	100	0.50
	Vivid® MOBFC	945 (Buffer I)	25	30	0.25
2E1	Vivid® EOMCC	850 (Buffer III)	50	100	0.50
3A4	Vivid® BOMCC	850 (Buffer I)	50	100	0.50
	Vivid® BOMFC	945 (Buffer I)	25	30	0.25
	Vivid® DBOMF	890 (Buffer I)	10	100	0.10
	Vivid® BOMR	885 (Buffer I)	15	100	0.15
3A5	Vivid® BOMCC	850 (Buffer I)	50	100	0.50
	Vivid® BOMFC	945 (Buffer I)	25	30	0.25
	Vivid® DBOMF	890 (Buffer I)	10	100	0.10

5.1.8 Start Reaction

1. Start the reaction by adding 10 µl per well of the Vivid® Substrate and NADP+ mixture prepared in Step 5.1.7 and mix.

5.1.9 Measure Fluorescence

1. **Kinetic Assay Mode (recommended):** Immediately (less than 2 minutes) transfer the plate into the fluorescent plate reader and monitor fluorescence over time at excitation and emission wavelengths listed in Table 6.
2. **Endpoint Assay Mode:** Incubate the plate for the desired amount of time, then add 10 µl of recommended stop reagent (see Section 7.0) to each well to quench the reaction. Measure fluorescence in the fluorescent plate reader at excitation and emission wavelengths listed in Table 6.

   Note: Appropriate reaction times will vary by kit and experimental conditions. We recommend that you determine the linear activity range for the assay under the conditions you wish to use. Typically, such reaction times will fall within 5 to 60 minutes.

3. Proceed to Section 6.0 for data analysis.

Table 6. Recommended excitation and emission wavelengths and filter sets

		Vivid® Fluorescent Standard							
		Red		Blue		Green		Cyan	
Fluorescence Plate Readers	Excitation/ Emission	center (nm)	Band width	center (nm)	Band width	center (nm)	Band width	center (nm)	Band width
with monochromators	excitation	530	--	409	--	485	--	400	--
	emission	585	--	460	--	530	--	502	--
using filters	excitation	530	25	405	20	485	20	405	40
	emission	605	55	460	40	530	25	490	40
with dichroic mirror	excitation	530	25	405	20	485	20	405	40
	emission	605	55	460	40	530	25	490	40
	dichroic	555	--	425	--	505	--	435	--

Red Standard is sodium salt of resorufin. Blue Standard is 3-cyano-7-hydroxycoumarin. Cyan Standard is 7-hydroxy-4-trifluoromethylcoumarin. We recommend exciting this dye off-peak at 400 nm (its excitation maximum is 385 nm) to minimize background from NADPH fluorescence. Green Standard is fluorescein.

For optimal signal to noise, filters must be blocked to OD of 6 outside their transparency range (UV and red blockage) and be free of pinholes. Filters may be purchased from:

Chroma Technology Corp.
72 Cotton Mill Hill, Unit A-9
Brattleboro, VT 05301
Phone: (800) 824-7662 or (802) 257-1800
Fax: (802) 257-9400.
www.chroma.com

6.0 SUGGESTED PROTOCOL FOR THE ANALYSIS OF RESULTS

6.1 Kinetic Assay Mode

1. Obtain reaction rates by calculating the change in fluorescence per unit time.
2. Calculate the percent inhibition due to presence of test compound or positive inhibition control using the equation:

$$\% \text{ Inhibition} = \left(1 - \frac{\text{rate in presence of test compound or positive inhibition control}}{\text{rate in absence of test compound or positive inhibition control}} \right) \times 100\%$$

6.2 Endpoint Assay Mode

1. Subtract background fluorescence.
2. Calculate percent inhibition due to presence of test compound or positive inhibition using the following equation:

$$\% \text{ Inhibition} = \left(1 - \frac{\text{RFU in presence of test compound or positive inhibition control}}{\text{RFU in absence of test compound or positive inhibition control}} \right) \times 100\%$$

Optional: Both types of data analysis above can be performed using a standard curve as described in Section 5.1.3 in order to calculate reaction rates as nmol product formed per unit time.

7.0 SUGGESTED CYP450 INHIBITORS (STOP REAGENT)

Table 7. Suggested CYP450 Inhibitors

Enzyme	Inhibitor (Stop Reagent)	Sigma-Aldrich Cat. no.	Suggested Final Concentration**
CYP1A2	α-naphthoflavone	N5757	3 µM
CYP2B6	miconazole	M3512	30 µM
CYP2C9	sulfaphenazole	S0758	10 µM
CYP2C19	miconazole	M3512	30 µM
CYP2D6	quinidine	Q3625	1 µM
CYP2E1	diethyldithiocarbamate	228680	100 µM
CYP3A4	ketoconazole	K1003	10 µM
CYP3A5	ketoconazole	K1003	30 µM

** To stop the reaction, the suggested final inhibitor concentration in the assay to produce inhibition of 90% or better is indicated in the above table. For an endpoint assay the volume of the added Stop Reagent should not exceed 10% of the final reaction volume [e.g., 10 µl will be added per 100 µl reaction volume. This 10% increase in the volume of an endpoint reaction does not have a significant effect on the reaction (or the calculations)].

8.0 SOLVENT TOLERANCES

P450 activity can be inhibited by solvents commonly used to dissolve test compounds. While we always recommend including a solvent control in your experimental design, the following sample data is intended as a guide for the selection and use of organic solvents. Table values are percent inhibition at the indicated solvent concentration. Values preceded by a "+" indicate an increase in activity. Dashed lines indicate inhibition not detected. Note that lower concentrations are listed for 2E1 Blue; this isozyme is particularly sensitive to the presence of organic solvents.

Table 8. Solvent Tolerances

Vivid® Kit	Solvent concentration (%)	DMSO (% Inhibition)	Acetonitrile (% Inhibition)	Methanol (% Inhibition)	Ethanol (% Inhibition)
1A2 Blue	1	7	--	--	--
	0.1	--	--	--	--
	0.01	--	--	--	--
2B6 Blue	1	16	7	20	32
	0.1	--	--	--	--
	0.01	--	--	--	--
2B6 Cyan	1	--	--	--	9
	0.1	--	--	--	--
	0.01	--	--	--	--
2C9 Blue	1	65	9	25	61
	0.1	25	--	9	11
	0.01	--	--	--	--
2C9 Green	1	--	--	20	35
	0.1	--	--	--	--
	0.01	--	--	--	--
2C9 Red	1	21	5	45	53
	0.1	8	--	6	9
	0.01	5	--	--	--
2C19 Blue	1	23	--	21	42
	0.1	--	--	--	5
	0.01	--	--	--	--
2D6 Blue	1	56	--	37	56
	0.1	16	--	--	10
	0.01	4	--	--	--
2D6 Cyan	1	21	6	18	19
	0.1	--	--	4	5
	0.01	--	--	--	--
2E1 Blue	0.1	85	36	26	98
	0.01	35	15	3	75
	0.001	20	7	8	25
3A4 Blue	1	66	--	12	10
	0.1	25	--	4	--
	0.01	6	--	--	--
3A4 Cyan	1	68	4	20	11
	0.1	29	--	6	--
	0.01	7	--	--	--
3A4 Green	1	47	--	--	--
	0.1	9	--	--	--
	0.01	--	--	--	--
3A4 Red	1	48	--	6	5
	0.1	13	--	--	--
	0.01	--	--	--	--
3A5 Blue	1	75	5	29	32
	0.1	34	--	14	8
	0.01	8	--	5	6
3A5 Cyan	1	77	6	31	33
	0.1	30	--	5	6
	0.01	--	--	5	6
3A5 Green	1	15	--	--	--
	0.1	--	--	--	--
	0.01	--	--	--	--

9.0 REFERENCES

Cohen, L.H., *et al.* (2003) *In vitro* drug interactions of cytochrome P450: an evaluation of fluorogenic to conventional substrates. *Drug Metab. Dispos.* **31**:1005-15.

Marks, B.D., *et al.* (2002) A high throughput screening assay to screen for CYP2E1 metabolism and inhibition using a fluorogenic Vivid® P450 substrate. *Assay Drug Dev. Technol.* **1**:73-81.

Marks, B.D., *et al.* (2003) High-throughput screening assays for CYP2B6 metabolism and inhibition using fluorogenic Vivid® substrates. *AAPS PharmSci.* **5**:E18.

Marks, B.D, *et al.* (2004) High-throughput screening assays for the assessment of CYP2C9*1, CYP2C9*2, and CYP2C9*3 metabolism using fluorogenic Vivid® substrates. *J. Biomol. Screen.* **9**:439-49.

Trubetskoy, O.V., *et al.* (2005) Highly miniaturized formats for in vitro drug metabolism assays using Vivid® fluorescent substrates and recombinant human cytochrome P450 enzymes. *J. Biomol. Screen.* **10**:56-66.

For structures of the Vivid Substrates and poster presentations containing additional details and applications of Vivid CYP450 Screening Kits, please visit us online at: www.invitrogen.com/drugdiscovery.

10.0 PURCHASER NOTIFICATION

Limited Use Label License No. 162: Cytochrome P450 enzymes, assays and substrates

This product is the subject of one or more of US Patent 5,891,696, 6,143,492, and 6,420,130. The purchase of this product conveys to the buyer the non-transferrable right to use the purchased amount o the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for-profit entity). The buyer cannot sell or otherwise transfer (a) this product (b) its components or (c) materials made using this product or its components to a third party or otherwise use this product or its components or materials made using the product or its components for Commercial Purposes. The buyer may transfer information or materials made through use of this product to a scientific collaborator, provided that such transfer is not for any Commercial Purpose, and that such collaborator agrees in writing (a) to not transfer such materials to any third party, and (b) to use such transferred materials and/or information solely for research and not for Commercial Purposes. Commercial Purposes means any activity by a party for consideration and may include, but is not limited to: (1) use of the product or its components in manufacturing; (2) use of the product or its components to provide a service, information, or data; (3) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; or (4) resale of the product or its components, whether or not such product or its components are resold for use in research. Invitrogen Corporation will not assert a claim against the buyer of infringement of the above patent claiming this product based upon the manufacture, use or sale of a therapeutic, clinical diagnostic, vaccine or prophylactic product developed in research by the buyer in which this product or its components was employed, provided that neither this product nor any of its components was used in the manufacture of such product. If the purchaser is not willing to accept the limitations of this limited use statement, Invitrogen is willing to accept return of the product with a full refund. For information on purchasing a license to this product for purposes other than research, contact Licensing Department, Invitrogen Corporation, 1600 Faraday Avenue, Carlsbad, California, 92008. Tel: (760) 603-7200. Fax: (760) 602-6500.

APPENDIX D

Publication





PROCEEDINGS

NRCT-JSPS Core University Program on Natural Medicine in Pharmaceutical Sciences

The Eighth Joint Seminar

Innovative Research in Natural Products for Sustainable Development

December 3-4, 2008
Faculty of Pharmaceutical Sciences
Chulalongkorn University
Bangkok, Thailand

Jointly organized by

Faculty of Pharmaceutical Sciences, Chulalongkorn University
Chulabhorn Research Institute
National Research Council of Thailand (NRCT)
Institute of Natural Medicine, University of Toyama
Japan Society for the Promotion of Science (JSPS)

ISBN 978-974-03-2365-5

COMMITTEE

Honorary Advisor

Professor Dr.H.R.H. Princess Chulabhorn Mahidol
Arnon Bunyaratavej
Kou Ikejima

Advisory Committee

Hiroshi Watanabe
Masao Hattori
Akinori Kubo
Yutaka Ebizuka
Tsutomu Ishikawa
Haruki Yamada
Hideaki Otsuka
Hiroyuki Tanaka
Naoki Inagaki
Toshio Nishikawa
Chu Quoc Truong
Nguyen Huu Dong
Aurawan Titwan
Bung-orn Sripanidkulchai
Chuthamanee Suthisisang
Sindhchai Keokitichai
Noppadol Thongnopnua
Niwat Keawpradub
Mangkorn Prapunwattana
Nongnit Teerawatanasuk
Jantip Kanjanasilp

Organizing Committee

Chairpersons:

Pornpen Pramyothin,
Somsak Ruchirawat,
Ikuo Saiki,

Members:

Withaya janthasoot
Sarinee Krittiyanunt
Mayuree Tantisira
Khanit Suwanborirux
Wanchai De-eknamkul
Primchanian Moongkarndi
Rutt Suttisri
Anuchai Teerarungchaisri
Warangkana Warisnoicharoen
Natsara Jirundorn
Kotchakorn Udomchaisathian
Phatcharin Sitthichankuna
Suchanan Pinpang

Secretary General:

Rungpetch C. Sakulbumrungsil
Kinzo Matsumoto

Secretariat:

Vipaporn Panapisal

The Eighth NRCT-JSPS Joint Seminar:
Innovative Research in Natural Products for Sustainable Development

PP86

AEGLE MARMELOS CORREA. FRUIT EXTRACT INHIBITS HUMAN CYTOCHROME P450

[1)]Duangjai Panyapojsak, [2)]Nuansri Niwattisaiwong, [3)]Somsong Lawanprasert, and [1)]Warangkana Warisnoicharoen

1) Department of Food Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand
2) Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand
3) Department of Pharmacology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand

KEYWORDS: *Aegle marmelos* Correa.; Cytochrome P450; Food-drug interaction

INTRODUCTION

Aegle marmelos Correa. is a plant in Rutaceae family natively grown in tropical areas such as India, Burma and Thailand (so-called matuum). All parts of *A. marmelos* are valued in Ayurvedic medicine. Its fruit is edible and widely used for flavored products such as tea, marmalade, refreshing drink, candied fruit, cake and cream. The main chemical constituents in *A. marmelos* fruit include coumarins and terpenoids [1]. Other components are alkaloid, steroids, oleoresin, sugar and vitamin C. Thai traditional medicine is claimed *A. marmelos* fruit for its astringency, digestive aid, gastric stimulant, antidiarrhea and antidysentery. Recently, there were some reports on pharmacological effects of *A. marmelos* fruit as antihyperglycemic effect by inducing glucose transporter Glut-4 and glucose uptake [2]. The ethanolic fruit extract of *A. marmelos* showed antiproliferative activity against SKBR3 human breast adenocarcinoma cell line with the 50% inhibitory concentration (IC_{50}) of 144.00±1.21 µg/ml [3].

Since *A. marmelos* is widely used as beverage and food in Thailand, repeated exposure of this fruit may affect the enzymatic biotransformation. Cytochrome P450 (CYPs or CYP P450) are phase I drug-metabolizing enzymes which play a dominat role in food-drug interaction. Many CYP isoforms can be induced or inhibited by food. Previous report showed the effect of leaf extract of *A. marmelos* as a bifunctional inducer on both phase I enzymes; CYPs and NADPH-CYP reductase, and phase-II enzymes; glutathione S-transferase and DT-diaphorase [4]. In addition, imperatorin as one of the constituents in Rutaceae was reported to inhibit various CYPs. Since there were no any information of *A. marmelos* fruit on CYP activity, the current study was designed to determine the *in vitro* effect of *A. marmelos* fruit extract on CYP activities. Moreover, the content of imperatorin as the active constituent in the fruit extract was determined by the high performance liquid chromatography (HPLC) method.

MATERIALS AND METHODS

Preparation of *A. marmelos* fruit extract:

Dried fruit of *A. marmelos* was refluxed with *n*-hexane for three times and the extract was concentrated to residue under reduced pressure in a vacuum rotary evaporator at the temperature lower than 60°C. The residue was stored and protected from light at 0°C.

Analysis of Imperatorin in *A. marmelos* extract:

Reversed-phase HPLC method was used for determination of imperatorin. Standard solutions of imperatorin were prepared with methanol and the calibration concentration range was in between 3.0-11.0 µg/ml. *A. marmelos* fruit extract was prepared in methanol at 100 µg/ml. The sample solutions were filtered through a 0.2 µm membrane filter before use. The analysis was carried out on a HPLC system (Shimazu, Japan) equipped with a LC-20AD pump, SIL-20A autosampler, CTO-20A column oven and SPD-M20A UV detector. The analyte was determined at room temperature on an analytical column Luna C18 (150 mm x 4.6 mm, i.d., 5 µm). The mobile phase consisted of a mixture of methanol and water (60:40, v/v). The mobile phase was filtered under vacuum through a 0.45 µm membrane filter, and degassed before use. The analysis was carried out at a flow rate of 1.0 ml/min and injection volume of 10 µl, with the UV detection wavelength set at 254 nm.

Inhibitory effect of *A. marmelos* extract on CYP P450:

A series of concentration of *A. marmelos* extract in 0.1% dimethylsulfoxide (DMSO) was dispensed into 96-well black plate (40 µl per well). The control wells were dispensed with 0.1% DMSO. To prevent error in fluorescent signal, the test-background wells were applied for each concentration. The 50 µl of the a mixture (containing cDNA-expressed human CYP P450 Baculosome®, glucose-6-phosphate dehydrogenase, glucose-6-phosphate and NADPH reductase) was dispensed into the test wells and 50 µl of the reaction buffer for test-background wells. The plate was then pre-incubated for 20

minutes at room temperature. The reaction was initiated with the addition of the 10 µl of combined $NADP^+$ and substrate solution. Fluorescent substrates for the CYP are ethoxycourmarin (3 µM for CYP1A2 or 10 µM for CYP 2C19) and 7-benzyloxymethyloxy-3-cyanocourmarin (10 nM for CYP2C9 or 5 nM for CYP3A4). The plate was protected from light and incubated at room temperature for proper reaction time (approximately 30 to 40 minutes). The reaction was stopped by adding 10 µl of 0.5 M Tris base (pH 10.5) to all wells. Finally, the plate was measured the fluorescence of the products. The relative fluorescence unit (RFU) was read on a plate reader (PerkinElmer) using the fluorescent excitation and emission filters at 390 nm and 450 nm, respectively. The level of inhibition was then calculated by comparison of RFU to those wells as in equation (1). Percents of inhibition of sample were plotted versus the log value of the sample concentrations to generate a dose-response curve. Nonlinear regression was performed with GraphPad Prism® Software using an equation for sigmoidal dose-response (variable Hill slope) to determine the IC_{50} values.

$$\% \text{ inhibition} = [\, 1 - (RFU_{sample} - RFU_{background}) / (RFU_{solvent} - RFU_{background})] \times 100 \qquad (1)$$

RESULTS

The percent yields of *n*-hexane extract of *A. marmelos* fruit were 0.55% w/w. From the HPLC analysis, amount of imperatorin in the extract was found to be 5.84% w/w. The fruit extract showed the ability to inhibit the phase I drug-metabolizing enzymes investigated. The values of IC_{50} of extract and standard imperatorin on CYP 1A2, 2C9, 2C19 and 3A4 are shown in Table 1.

Table 1 IC_{50} values of *A. marmelos* fruit extract and imperatorin (mean ± *S.D*; n=4)

Sample	IC_{50} (µg/ml or µM)			
	CYP 1A2	CYP 2C9	CYP 2C19	CYP 3A4
Extract, µg/ml	0.69 ± 0.05	3.17 ± 0.22	4.05 ± 0.20	1.71 ± 0.09
Imperatorin, µM	0.39 ± 0.01	5.21 ± 0.76	1.98 ± 0.09	1.49 ± 0.09

DISCUSSION AND CONCLUSION

The *A. marmelos* fruit extract showed inhibitory effect on phase I drug-metabolizing enzymes, CYP 1A2, 2C9, 2C19 and 3A4. The IC_{50} values of imperatorin for all CYP forms were less than 10 µM indicating a potent enzyme inhibitor [5]. The inhibition of the fruit extract on CYP was considered to be due to the some constituents found in the fruit especially imperatorin.

In conclusion, the fruit of *Aegle marmelos* Correa. caused inhibitory effect on human CYP 1A2, 2C9, 2C19 and 3A4 *in vitro*. The study suggested the possibility of food-drug interaction and risks of chemical-induced toxicity, mutagenicity and/or carcinogenicity from the compounds metabolized or bioactivated by the CYP isoforms when given concomitantly with the *A. marmelos*.

ACKNOWLEDGEMENT

Partially financial support from Graduate Thesis Grant, Chulalongkorn University, for D.P. is gratefully acknowledged.

REFERENCES

[1] B. R. Sharma, R. K. Rattan, and P. Sharma. Marmeline, an alkaloid, and other components of unripe fruits of *Aegle marmelos*, *Phytochemistry* 20: 2606-2607 (1981).

[2]R. Anandharajan, S. Jaiganesh, N. P. Shankernarayanan, R. A. Viswakarma, and A. Balakrishnan. *In vitro* glucose uptake activity of *Aegle marmelos* and *Syzygium cumini* by activation of Glut-4, PI3 kinase and PPARγ in L6 myotubes, *Phytomedicine* 13: 434-441 (2005).

[3] P. Moongkarndi, N. Kosem, O. Luanratana, S. Jongsomboonkusol, and N. Pongpan. Antiproliferative activity of Thai medicinal plant extracts on human breast adenocarcinoma cell line, *Fitoterapia* 75: 375-377 (2004).

[4] R. P. Singh, S. Banerjee, and R. Rao. Effect of *Aegle marmelos* on biotransformation enzyme system and protection against free-radical-mediated damage in mice, *J. Pharm. Pharmacol.* 52: 991-1000 (2000).

[5] L. Zou, M. R. Harkey, and G. L. Henderson. Effects of herbal components on cDNA-expressed cytochrome P450 enzyme catalytic activity, *Life Sci.* 1579-1589 (2002).

BIOGRAPHY

Miss Duangjai Panyapojsak was born on December 29, 1979 in Suratthani Province, Thailand. She graduated with a Bachelor Degree of Pharmaceutical Sciences in 2003 from the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkla, Thailand.