ผลของผงบดแห้งของต้นเพชรสังฆาตต่อเอนไซม์ไซโตโครม พี450 ในตับและค่าเคมีคลินิกในเลือดหนูขาว

นายเขมชาติ อภิปาลกุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเภสัชวิทยา (สหสาขาวิชา)
บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2546

ISBN 974-17-5021-8

EFFECTS OF THE DRIED-STEM POWDER OF *CISSUS QUADRANGULARIS* ON HEPATIC CYTOCHROME P450 AND CLINICAL BLOOD CHEMISTRY IN RATS

Mr. Khemchat Apipalakul

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Pharmacology (Inter-Department)

Graduate School

Chulalongkorn University

Academic Year 2003

ISBN 974-17-5021-8

Thesis Title	Effects of the dried-stem powder of *Cissus quadrangularis* on hepatic cytochrome P450 and clinical blood chemistry in rats
By	Mister Khemchat Apipalakul
Field of Study	Pharmacology
Thesis Advisor	Assistant Professor Pol. Lt. Col. Somsong Lawanprasert, Ph.D.
Thesis Co-advisor	Associate Professor Supatra Srichairat, Ph.D.

Accepted by the Graduate School, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

……..……Dean of the Graduate School

(Professor Suchada Kiradana, Ph.D.)

THESIS COMMITTEE

……..……..……Chairman

(Associate Professor Supeecha Wittayalertpanya)

……..……..……Thesis Advisor

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

……..……..……Thesis Co-Advisor

(Associate Professor Supatra Srichairat, Ph.D.)

……..……..…… Member

(Assistant Professor Laddawal Phivthong-ngam, Ph.D.)

……..……..…… Member

(Assistant Professor Lt. Pasarapa Towiwat, Ph.D.)

เขมชาติ อภิปาลกุล : ผลของผงบดแห้งของต้นเพชรสังฆาตต่อเอนไซม์ไซโตโครม พี450 ในตับ และค่าเคมีคลินิกในเลือดของหนูขาว (EFFECTS OF THE DRIED-STEM POWDER OF *CISSUS QUADRANGULARIS* ON HEPATIC CYTOCHROME P450 AND CLINICAL BLOOD CHEMISTRY IN RATS). อาจารย์ที่ปรึกษา : ผศ. พ.ต.ท.หญิง ดร. สมทรง ลาวัณย์ประเสริฐ, อาจารย์ที่ปรึกษาร่วม : รศ. ดร. สุพัตรา ศรีไชยรัตน์ 102 หน้า ISBN 974-17-5021-8

เพชรสังฆาต (*Cissus quadlangularis* Linn.) จัดเป็นพืชในวงศ์ Vitaceae เป็นสมุนไพรพื้นบ้านที่นิยมใช้เป็นยาสมานกระดูกและรักษาริดสีดวงทวาร การศึกษานี้มุ่งศึกษาถึงผลของผงบดแห้งของต้นเพชรสังฆาตต่อเอนไซม์ในเฟส 1 คือ เอนไซม์ไซโตโครมพี 450 (cytochrome P450, CYP) ที่เกี่ยวข้องกับเมแทบอลิซึมของยา การกระตุ้นฤทธิ์ของสารก่อมะเร็ง/สารก่อการกลายพันธุ์ได้แก่ CYP1A1, CYP1A2, CYP2B1/2B2, CYP2E1 และ CYP3A ในตับนอกจากนี้ยังได้ศึกษาผลของสารดังกล่าวต่อค่าเคมีคลินิกและโลหิตวิทยาต่างๆในหนูขาวเพศผู้พันธุ์วิสตาร์ โดยแบ่งหนูขาวแบบสุ่มออกเป็น 3 กลุ่ม กลุ่มละ 10 ตัว ดังต่อไปนี้ กลุ่มแรกเป็นกลุ่มควบคุมที่ได้รับน้ำกลั่น ขนาด 1 มิลลิลิตร/กิโลกรัม/วัน กลุ่มที่สองและสามเป็นกลุ่มที่ได้รับผงบดแห้งของต้นเพชรสังฆาตขนาด 0.03 และ 0.3 กรัม/กิโลกรัม/วันตามลำดับ โดยทำการทดลองเป็นเวลา 30 วัน ระหว่างทำการทดลองบันทึกค่าน้ำหนักตัว ปริมาณอาหารและปริมาตรน้ำที่หนูขาวได้รับทุกสัปดาห์ เมื่อครบระยะเวลา ทำให้หนูหมดความรู้สึก เก็บตัวอย่างเลือดจากหัวใจเพื่อตรวจค่าโลหิตวิทยา และแยกซีรัมเพื่อตรวจค่าเคมีคลินิก นำตับมาเตรียม ไมโครโซม เพื่อใช้วัดสมรรถนะของเอนไซม์ CYP ผลการทดลองพบว่าผงบดแห้งของต้นเพชรสังฆาตทั้ง 2 ขนาดไม่มีผลต่อน้ำหนักตัว ปริมาณอาหารและปริมาตรน้ำดื่มของหนูขาว รวมถึงไม่มีผลต่อปริมาณของ CYP โดยรวมและต่อสมรรถนะของ CYP1A1, CYP1A2, CYP2B1/2B2, CYP2E1 และ CYP3A เมื่อเปรียบเทียบกับกลุ่มที่ได้รับน้ำกลั่น สำหรับค่าเคมีคลินิกพบว่าผงบดแห้งของต้นเพชรสังฆาตทั้งสองขนาดไม่มีผลต่อค่าเคมีคลินิกและโลหิตวิทยาต่าง ๆ ต่อไปนี้คือ AST, ALT, ALP, total bilirubin, direct bilirubin, BUN, SCr, uric acid, total cholesterol, TG, LDL-C, HDL-C, glucose, sodium, potassium, chloride, hemoglobin, hematocrit, MCV, MCH, MCHC, RBC count, platelet count, WBC count , % differential WBCs และ RBC morphology ข้อมูลจากการศึกษานี้ชี้บ่งถึงความปลอดภัยในการใช้เพชรสังฆาตร่วมกับยาอื่นที่ถูกเปลี่ยนแปลงโดยเอนไซม์เหล่านี้ได้โดยไม่มีอันตรกิริยาต่อกัน ทั้งนี้รวมถึงเพชรสังฆาตไม่มีผลในการเพิ่ม/ลดความเสี่ยงต่อการเกิดพิษ การก่อการกลายพันธุ์และ/หรือการก่อมะเร็งจากสารแปลกปลอมอื่นๆที่ถูกกระตุ้น/ทำลายโดยเอนไซม์เหล่านี้ด้วย นอกจากนี้พบว่าผงบดแห้งของต้นเพชรสังฆาตไม่มีผลต่อการทำงานของอวัยวะหรือระบบของร่างกายที่สำคัญ เช่น ตับ ไต ระบบเลือด อิเล็กโตรไลต์ รวมทั้งเมแทบอลิซึมของไขมันและคาร์โบไฮเดรต

สหสาขาวิชาเภสัชวิทยา ลายมือชื่อนิสิต .....

สาขาวิชาเภสัชวิทยา ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา 2546 ลายมือชื่ออาจารย์ที่ปรึกษาร่วม .....

4589069720 MAJOR: PHARMACOLOGY
KEYWORDS: *Cissus quadrangularis* / HEPATIC CYTOCHROME P450 / CLINICAL BLOOD CHEMISTRY

KHEMCHAT APIPALAKUL : EFFECTS OF THE DRIED-STEM POWDER OF *CISSUSS QUADRANGULARIS* ON HEPATIC CYTOCHROME P450 AND CLINICAL BLOOD CHEMISTRY IN RATS. THESIS ADVISOR: ASST. PROF. POL. LT. COL. SOMSONG LAWANPRASERT Ph.D., THESIS CO-ADVISORS: ASSOC. PROF. SUPATRA SRICHAIRAT Ph.D., 102 pp. ISBN 974-17-5021-8.

Cissus quadrangularis Linn., known locally as “Phet-Cha-Sung-Khat” is considered to be a folk medicine for bone healing and antihemorrhoid. It is a plant in family Vitaceae. In this study, effects of the dried-stem powder of *Cissuss quadrangularis* on phase I hepatic cytochrome P450 (CYP) involving in drug metabolism and carcinogenic/mutagenic bioactivation such as CYP1A1, CYP1A2, CYP2B1/2B2, CYP2E1 and CYP3A were examined in rats. Effects of this compound on clinical blood chemistry and hematology were also determined. Male Wistar rats were randomly divided into 3 groups of 10 rats each. Control group was given distilled water orally at 1 ml/kg/day whereas the other two groups received the dried-stem powder of *Cissus quadrangularis* orally at dosages of 0.03 and 0.3 g/kg/day, respectively, for 30 consecutive days. During the experimental period, body weight, food and water intake were recorded every week. At the end of the experimental period, rats were anesthesized. Blood samples were collected by heart puncture and serum was prepared for determining hematology and clinical blood chemistry, respectively. Microsomes were prepared from livers for enzyme assays. The results demonstrated that there were no significant differences of body weight, food and water consumption between *Cissuss quadrangularis* treated groups and the control group. *C. quadrangularis* at the dosages of either 0.03 or 0.3 g/kg/day did not cause any significant changes of hepatic microsomal total CYP contents as well as the activities of CYP1A1, CYP1A2, CYP2B1/2B2, CYP2E1 and CYP3A. Furthermore, both dosage regimens of *C. quadrangularis* did not affect these following clinical blood chemistry and hematology parameters: AST, ALT, ALP, total bilirubin, direct bilirubin, BUN, SCr, uric acid, total cholesterol, TG, LDL-C, HDL-C, glucose, sodium, potassium, chloride, hemoglobin, hematocrit, MCV, MCH, MCHC, RBC count, platelet count, WBC count , % differential WBCs and RBC morphology. Results from this study indicate the safety from drug-drug interaction when *C. quadrangularis* is used concomitantly with other medicines which are metabolized by these CYPs. In addition, possibilities of this plant to increase and/or decrease risks of toxicity, mutagenicity and carcinogenicity induced by xenobiotics which are bioactivated by these CYP isoforms, were excluded. Furthermore, dried-stem powder of *C. quadrangularis* at the doses given in this study did not produce any toxic effects on several important organs/systems such as liver, kidney, blood system, electrolytes as well as lipid and carbohydrate metabolism.

INTER-DEPARTMENT OF PHARMACOLOGY
FIELD OF STUDY PHARMACOLOGY
ACADEMIC YEAR 2003

STUDENT'S SIGNATURE..... .....
ADVISOR'S SIGNATURE.....
CO-ADVISOR'S SIGNATURE.....

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my advisor, Assistant Professor Pol. Lt. Col. Dr. Somsong Lawanprasert for her excellent advice, help, encouragement and constructive criticism throughout my research study which enable me to accomplish this thesis.

I also would like to express my deepest and sincere appreciation to my co-advisor, Associate Professor Dr. Supatra Srichairat for her guidance, help and comments on my research work.

I would like to thank Assistant Professor Dr. Laddawal Phivthong-ngam for her valuable helps, guidance and comments for the animal handling technique. Thanks are also extended to Associate Professor Supeecha Wittayalertpanya and Assistant Professor Lt. Dr. Pasarapa Towiwat, who are the committee members, for their useful comments.

My appreciation is also expressed to Associate Professor Nuansri Niwattisaiwong for her kind assistance in identification technique.

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

I wish to thank all staff members of the Department of Pharmacology, Faculty of Pharmaceutical Sciences as well as all staff members of the Department of Pharmacology, Faculty of Medicine, Srinakharinwirot University for their helps.

Finally, I would like to express my deepest appreciation to my family and friends for their encouragement and moral that have made me to complete this work.

CONTENTS

CONTENTS(CONTINUED)

CONTENTS(CONTINUED)

LIST OF TABLES

LIST OF TABLES(CONTINUED)

page

LIST OF FIGURES

LIST OF FIGURES(CONTINUED)

page

LIST OF ABBREVIATIONS

ALP	=	alkaline phosphatase
ALT	=	alanine aminotransferase
AST	=	aspartate aminotransferase
BR	=	benzyloxyresorufin
BROD	=	benzyloxyresorufin O-dealkylation
BSA	=	bovine serum albumin
BUN	=	blood urea nitrogen
BW	=	body weight
cm	=	centimeter
cumm	=	cubic millimeter
CYP	=	cytochrome P450
dl	=	deciliter
DNA	=	deoxyribonucleic acid
ed	=	editor
EDTA	=	ethylenediaminetetraacetic acid
e.g.	=	exampli gratia
ER	=	ethoxyresorufin
EROD	=	ethoxyresorufin O-dealkylation
et al.	=	et alii (and other)
etc.	=	and so on
fL	=	femtoliter
g	=	gram
g	=	gravity
G6P	=	glucose 6-phosphate
G6PD	=	glucose 6-phosphate dehydrogenase
GS^-	=	glutathione thiolate anion
GST	=	glutathione S-transferase
Hb	=	hemoglobin
Hct	=	hematocrit

HDL-C	=	high density lipoprotein-cholesterol
i.p.	=	intraperitoneal
kg	=	kilogram
L	=	liter
LDL-C	=	low density lipoprotein-cholesterol
M	=	molar (mole per liter)
mEq	=	milliequivalent
min	=	minute
mg	=	milligram
ml	=	milliliter
mm	=	milimeter
mM	=	millimolar (millimole per liter)
mmole	=	millimole
MCV	=	mean corpuscular volume
MCH	=	mean corpuscular hemoglobin
MCHC	=	mean corpuscular hemoglobin concentration
MR	=	methoxyresorufin
MROD	=	methoxyresorufin O-dealkylation
MW	=	molecular weight
NADP	=	nicotinamide adenine dinucleotide phosphate
NADPH	=	nicotinamide adenine dinucleotide phosphate (reduced form)
nm	=	nanometer
nM	=	nanomolar (nanomole per liter)
nmole	=	nanomole
PAH	=	polycyclic aromatic hydrocarbon
pH	=	potential of hydrogen
pmole	=	picomole
PR	=	pentoxyresorufin
PROD	=	pentoxyresorufin O-dealkylation
RBC	=	red blood cell

r.p.m.	=	revolution per minute
SCr	=	serum creatinine
SEM	=	standard error of the mean
sec	=	second
SER	=	smooth endoplasmic reticulum
spp.	=	species
TCA	=	trichloroacetic acid
TCDD	=	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TG	=	triglyceride
Tris	=	Tris (hydroxymethyl) aminomethane
U	=	unit
UDPGT	=	urindine diphosphoglucuronyltransferase
UV	=	ultraviolet
vs.	=	versus
v/v	=	volume by volume
WBC	=	white blood cell
w/v	=	weight by volume
^{o}C	=	degree celsius
ß	=	beta
γ	=	gamma
δ	=	delta
α	=	alpha
µg	=	microgram
µl	=	microliter
µM	=	micromolar (micromole per liter)

CHAPTER I

INTRODUCTION

Medicinal plants are recently considered as an alternate way for healing many diseases. Since medicinal plants are of natural resources, they are proposed to be less toxic, cause less side effects and readily available than chemical synthetic medicines. Many natural medicines are traditionally used without scientific studies. In order to utilize medicinal plants effectively and safely, scientific investigation such as pharmacological and toxicological studies are required. *Cissus quadrangaluris* Linn. is one of the most frequently used medicinal plants in Thailand and many regions in Asia and Africa. It is a plant in family Vitaceae. In India, this plant has been used for accelerating of fractured bone healing and rheumatic back pain relief (สุภาภรณ์ ปิติพร, 2544). In Thailand, it is traditionally used for treatment of hemorrhoid, indigestion and also used for accelerating of fractured bone healing (สถาบันวิจัยวิทยาศาสตร์และเทคโนโลยีแห่งประเทศไทย, 2543). One hundred gram of fresh stem of *C. quadrangularis* contain approximately 267 mg of carotene, 398 mg of vitamin C and a lot of calcium oxalate crystals (สุภาภรณ์ ปิติพร, 2544; Tiangburanatham, 1996). Phytochemical constituents of this plant include triterpenes i.e., α-amyrin and α-amyrone (Elizabeth, 2002); phytosterols, i.e., β-sitosterol (Pluemjai et al, 1986); steroids, i.e., keto-steroid, oxo-steroid (นันทวัน บุณยประภัศร์, 2542; Sen, 1964; Bhutani, 1984) and two new unsymmetric tetracyclic triterpenoids, namely, onocer-7-ene-3α, 21β-diol and onocer-7-ene-3β, 21α-diol (Elizabeth, 2002; Bhutani, 1984). Furthermore, it was recently found that three new stilbene derivatives, quadrangularins A, B and C, could be isolated from stems of *C. quadrangularis* together with reveratol, piceatannol, pallidol and parthenocissine A (Elizabeth, 2002; Adesanya, 1999).

Pharmacological investigations in animals demonstrated that *C. quadrangularis* exerted bone-healing activity (Deka et al., 1994; Chopra et al., 1976; Udapa et al., 1964), analgesic activity (Singh et al., 2003), anti-osteoporotic activity (Shirwaikar et al., 2003), hypotensive activity (Bhakuni et al., 1969) and diuretic activity (Dhawan et al., 1980). Only one clinical study was performed regarding the

anti-hemorrhoidal effect of dried-stem powder of *C. quadrangularis* (ดวงรัตน์ เชี่ยว-ชาญวิทย์, 2545; สมนึก สุชัยธนาวนิช, 2540). Toxicities of *C. quadrangularis* were investigated including acute toxicity study (กฤติยา อินทรเผือก, 2543), subacute toxicity study (จักรพงษ์ ลิมปนุสสรณ์ และคณะ, 2543) and subchronic toxicity study (Attawish et al., 2002). The results consistently indicated that *C. quadrangularis* did not produce any serious toxicity in rats.

Using this plant for hemorrhoidal indication, repeated administration (7, 10, 15 days or even months) of dried-stem powder of this plant is required (สุภาภรณ์ ปิติพร, 2544; นันทวัน บุณยประภัศร, 2542). Long term exposure of *C. quadrangularis* may affect hepatic drug metabolizing enzymes. Drug-drug interactions and possibilities to increase/decrease risks of chemical-induced carcinogenesis and/or mutagenesis may occur following *C. quadrangularis* administration. So far, no study was performed regarding the effects of dried-stem powder of *C. quadrangularis* on hepatic cytochrome P450 (CYP), the important phase I hepatic drug metabolizing enzymes, especially CYP isoforms that play an important role in chemical-induced toxicity, mutagenesis and/or carcinogenesis include CYPs 1A1, 1A2, 2B1, 2B2, 2E1 and 3A (Soucek and Gut, 1992). Therefore, the objectives of this study were to preliminarily investigate effects of the dried-stem powder of *C. quadrangularis* on some CYP isoforms such as CYPs 1A1, 1A2, 2B1, 2B2, 2E1 and 3A, which were involved in drug metabolism and carcinogen and/or mutagen activation, using an ex vivo study in rats. Furthermore, effects of the dried-stem powder of *C. quadrangularis* on clinical blood chemistry and hematology were also determined to confirm the previous toxicity data of this plant.

Hypothesis

The dried-stem powder of *C. quadrangularis* demonstrated an induction and/or inhibition effects on hepatic microsomal CYP as well as caused changes of clinical blood chemistry and hematology.

Study design and process

1. Preparation and identification of the dried-stem powder of *C. quadrangularis*
2. Animal treatment : *ex vivo* study
3. Blood collecting
4. Determination of clinical blood chemistry and hematology
5. Preparation of liver microsomes
6. Determination of total CYP contents and CYP activities
7. Data analysis

Anticipated benefits from the study

1. A preliminary data of the dried-stem powder of *C. quadrangularis* whether it possessed an induction and/or inhibition effects on hepatic CYP, the isoforms involving in various bioactivation reactions of certain classes of drugs, chemicals as well as environmental toxicants resulting in reactive metabolites. This would be useful to estimate the possibility of *C. quadrangularis* to increase and/or decrease risks of chemical-induced toxicities, mutagenesis and/or carcinogenesis.

2. The possibility of drug-drug interaction if this plant powder was taken simultaneously with other medicines metabolized by the CYP isoform that was modulated by this plant.

In addition, the clinical blood chemistry and hematology data of *C. quadrangularis* from this study, would be useful to confirm the previous toxicity data in rats.

CHAPTER II

LITERATURE REVIEWS

Cissus quadrangularis

Cissus quadrangularis Linn., a well known climbing herb belonging to the Vitaceae family, is known in Thai as Phet–Cha–Sung–Khaat, San-Cha-Khuat, San-Cha-Khat, Sam-Roi-Tor, Sam-Roi-Khor, Khan-Khor and Tum-Leung-Thong (นันทวัน บุณยปภัศร์, 2542). In India this plant is also called "Asthi sringhala" or "Asthisamhari" because of its ability to rejoin broken bones (สุภาภรณ์ ปิติพร, 2544; Elizabeh, 2002). *C. quadrangularis* is common grown in hot and dry regions of India, Africa and Arabia. They grow as small erect trees or climbing shrubs with tendril-bearing stems and branched nodose. They have very long stems and broadly ovate or reniform leaves. Their flowers are in shortly peduncled cymes with spreading umbellate branches (Sivarajan and Balachandran, 1994; Kirtikar and Basu, 1989, Figure 2.1)

C. quadrangularis is one of the medicinal plants recommended for primary health care in Thailand. This plant is regarded as safe to be used for treatment of hemorrhoids. Various parts of this plant have been used as a traditional medicine for anti-hemorrhoids. Dried-shoot powder of *C. quadrangularis* has been used for indigestion. Young shoots are burnt to ashes in a closed vessel and administered for dyspepsia and indigestion. Leaves of this plant have been used in the treatment of internal ulceration and external wounds. Stems were used for irregular menstruation and their paste is used traditionally as poultice on inflammatory bone fractures. Their roots are used for bone fracture healing. The entire plant is considered to be used as antihelmintic, aphrodisiac, antiasthmatic and is useful in gastrointestinal disorders such as abdominal colic pain, dyspepsia and irregular menstruation (นันทวัน บุญยปภัศร์, 2542; Elizabeth 2002; Sivarajan and Balachandran, 1994). Moreover, this plant is used as folk medicine for cancer-curing by combining with other medicinal herbs such as Sea Holly (*Acanthus ebactetus* Vahl), *Rhinacanthus nasutus* (Linn.) Kurz. and *Hydrophyum fermicarum* (สุภาภรณ์ ปิติภรณ์, 2544). Several previous studies demonstrated that this plant possessed various compounds

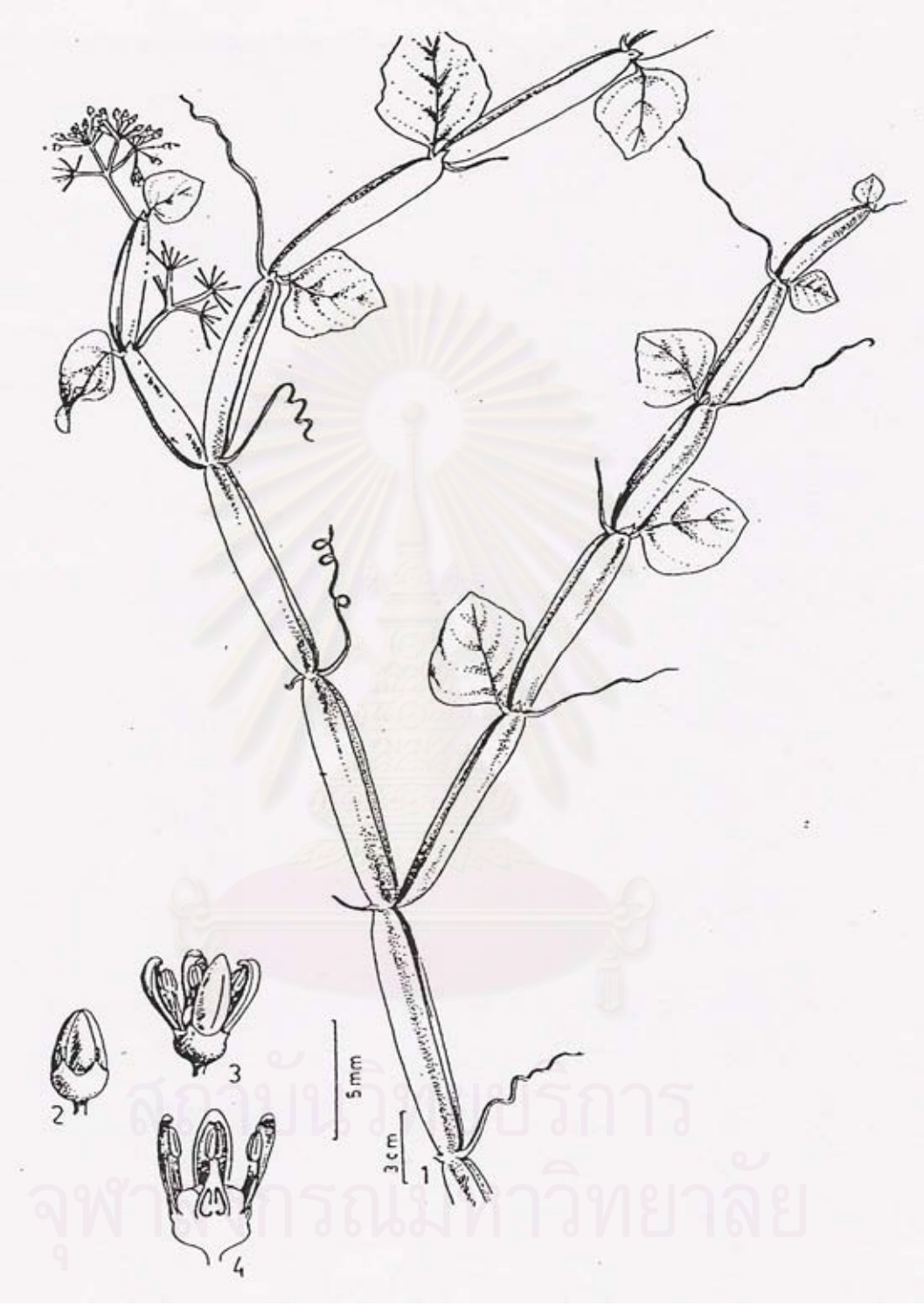


Figure 2.1 *C. quadrangularis* Linn. 1, Habit; 2, Flower bud; 3, Open flower; 4, Flower (Sivarajan and Balachandran, 1994)

including stilbene derivatives, lipids and phytosterols. The occurrence of chemical compounds found in *C. quadrangularis* are shown in Table 2.1. Structures of some corresponding compounds in *C. quadrangularis* were shown in Figure 2.2.

Table 2.1 The occurrence of chemical compounds found in *C. quadrangularis*

Type of constituents	Constituents	Plant portion	% Content	References
Triterpenes	δ-amyrin	Entire plant	0.14000 %	1, 2
	δ-amyrone	Entire plant	-	2
	Iso-aborenol	Entire plant	0.00030 %	3
	Friedelan-3-one	Aerial parts	-	4
	Epi-friedelinol	Entire plant	0.00024 %	3
	Lupenone	Entire plant	0.00022 %	3
	Onocer-7-ene-3-alpha-21-beta-diol	Entire plant	0.00600 %	2
	Onocer-7-ene-3-beta-21-alpha-diol	Entire plant	0.00300 %	2
	Onocer-8-ene-3-beta-21-alpha-diol, 7	Aerial parts	0.00225 %	5
	Taraxerol	Aerial parts	-	4
	Taraxeryl acetate	Aerial parts	-	4
Stilbenes	Pallidol	Stem	0.00055 %	6
	Parthenocissin A	Stem	0.00070 %	6
	Quadrangularin A	Stem	0.00090 %	6
	Quadrangularin B	Stem	0.00040 %	6
	Quadrangularin C	Stem	0.00080 %	6
	Piceatannol	Stem	0.00020 %	6
	Resveratrol	Stem	0.00050 %	6

Table 2.1 (continued) The occurrence of chemical compounds found in *C. quadrangularis*

Type of constituents	Constituents	Plant portion	% Content	References
Steroids	Keto-steroid	Entire plant	-	2
	Oxo-steroid	Entire plant	-	2
	β-sitosterol	Entire plant	-	2, 3, 4
Flavonols	Kaemferol	Stem	0.00030 %	6
	Quercetin	Stem	0.00210 %	6
Alkane C-5 or More	Heptadecyl octadecanoate	Aerial parts	-	4
	Icosanyl icosanoate	Aerial parts	-	4
	Tritriacontanoic acid, 31-methyl	Aerial parts	-	4
Alkene C-5 or More	Octadec-9-ene, 9-methyl	Aerial parts	-	4
Alkanol C-5 or More	Docosan-1-ol-cyclohexane, 7-hydroxy-20-oxo.	Aerial parts	-	4
	Tritriacontan-1-ol, 31-methyl	Aerial parts	-	4
Alkenone C-5 or More	Tricos-2-en-22-one, 4-hydroxy-2-methyl	Aerial parts	-	4

1: Mehta et al., 2001 2: Bhutani, 1984 3: Pluemjai et al., 1986

4: Gupta et al., 1991 5: Gupta et al., 1990 6: Adesanya et al., 1999

Triterpenoid compounds

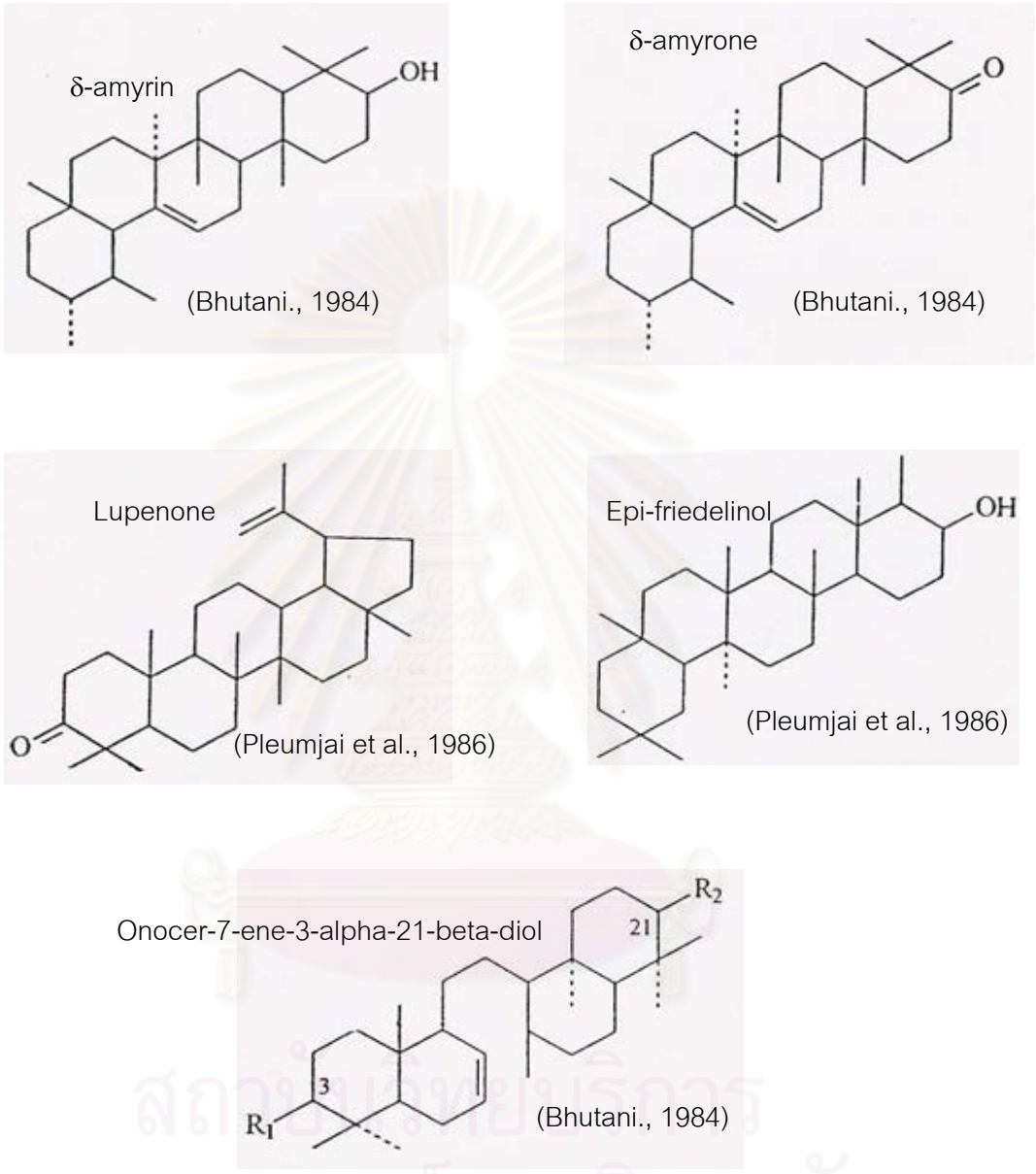
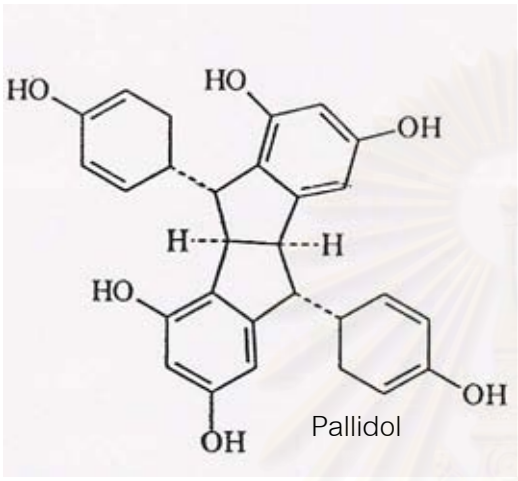
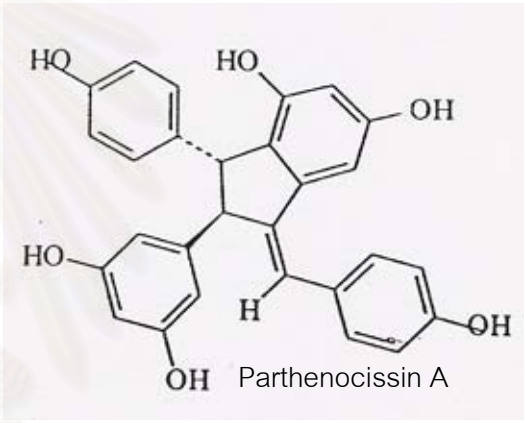


Figure 2.2 Structures of compounds identified in *C. quadrangularis*

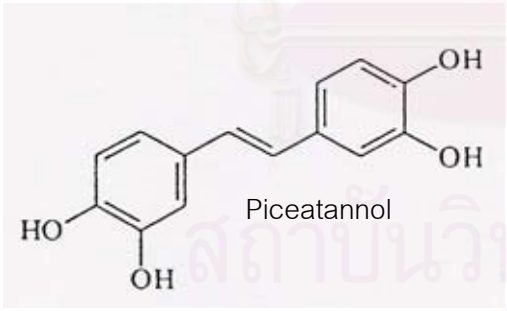
Stilbene derivatives



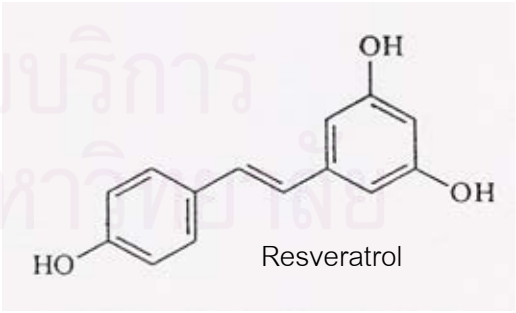
Pallidol



Parthenocissin A



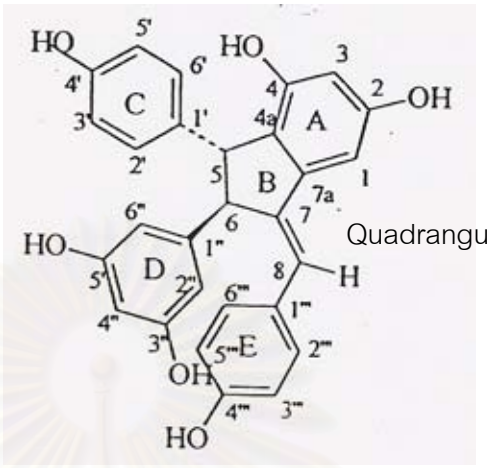
Piceatannol



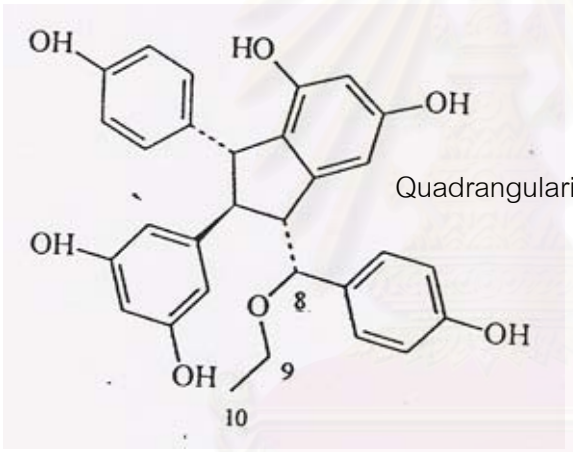
Resveratrol

Figure 2.2 (continued) Structures of compounds identified in *C. quadrangularis* (Adesanya et al., 1999)

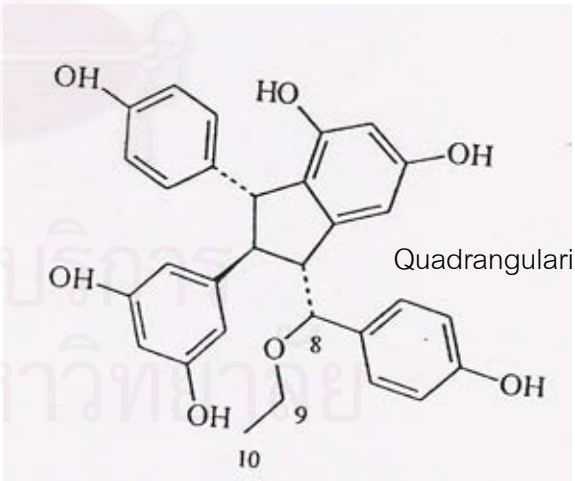
Quadrangularins



Quadrangularin A



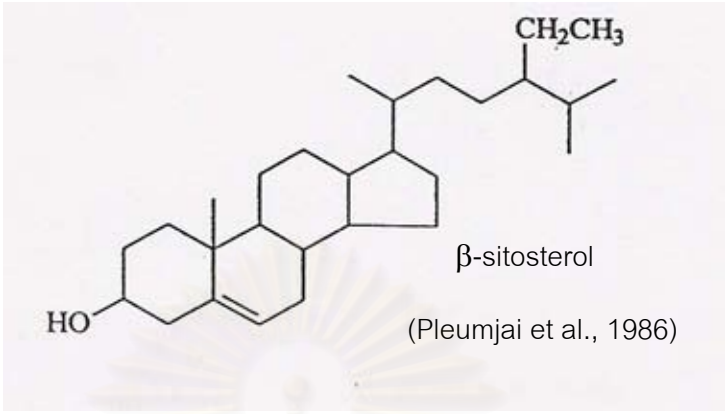
Quadrangularin B



Quadrangularin C

Figure 2.2 (continued) Structures of compounds identified in *C. quadrangularis* (Adesanya et al., 1999)

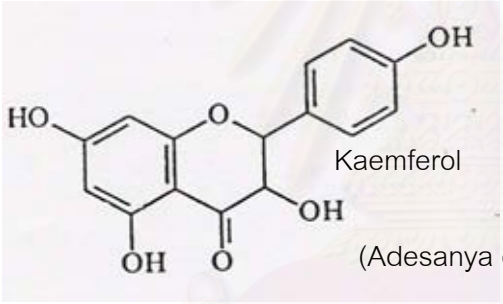
Steroid compounds



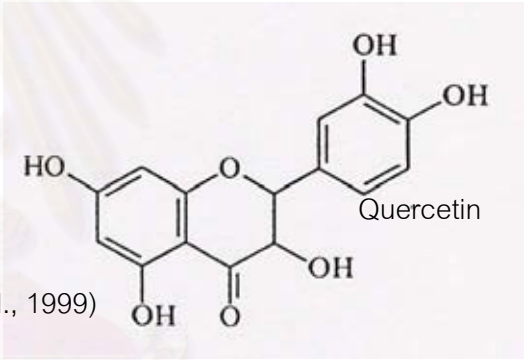
β-sitosterol

(Pleumjai et al., 1986)

Flavanol compounds



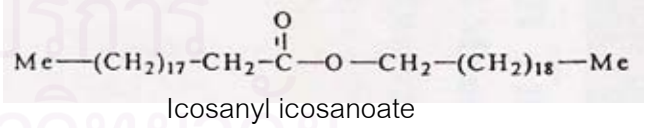
Kaemferol



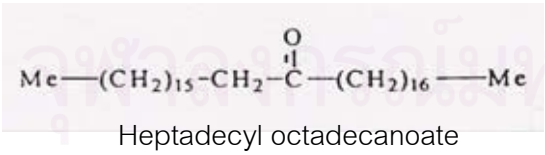
Quercetin

(Adesanya et al., 1999)

Alkane compounds



Icosanyl icosanoate



Heptadecyl octadecanoate (Gupta et al., 1991)

Figure 2.2 (continued) Structures of compounds identified in *C. quadrangularis*

Pharmacological effects

1. Analgesic effect

Dried whole plant of *C. quadrangularis* was extracted with 70% alcohol in a soxhlet apparatus. Alcoholic fraction was evaporated at low temperature and the residue was dissolved in normal saline for use in the experiments. Medium lethal dose (LD_{50}) of *C. quadrangularis* alcoholic extract in mice via oral and intraperitoneal routes was found to be 4 and 3.5 g/kg body weight, respectively. Analgesic activity was assessed in mice by the method of Haffner's tail clip and Eddy's hot plate method. *C. quadrangularis* were given orally at the dosages of 100, 200 and 400 mg/kg and intraperitoneally at the dosages of 87.5, 175 and 350 mg/kg. The results showed that analgesic effects of this plant extract as observed by both methods were dose related. Duration of analgesic activity was from 2 to 4 hours and the optimum effect was observed at $1/20^{th}$-$1/10^{th}$ of the LD_{50}. Analgesic effect of the extract was comparable to acetylsalicylic acid (Singh et al., 1984).

2. Accelerating healing process effect

Five hundred gram of dried-stem powder of *C. quadrangularis* was extracted with methanol using soxhlet apparatus. The dried methanolic extract was reconstituted with distilled water (100 mg/ml), filtered and used in the study. Eight healthy mongrel dogs of either sex weighing 3-5 kg were randomly selected into two groups, control and treatment groups. Each group consisted of 4 animals. Under general anesthesia, a closed fracture of the right forelimb of each animal was produced. Treated group received *C. quadrangularis* methanolic extract (50 mg/kg, subcutaneously) every alternate day whereas the control group received normal saline (0.5 ml/kg, S.C.). On the 11^{th} day of fracture, two animals from each group were sacrificed for histopathological and radio logical examination whereas the rest of animals were continuing the treatment. On the 21^{st} day of fracture, the rest of animals in control and treated group were sacrificed and performed in the same manner. The results showed that treated animals sacrificed on the 11^{th} day of fracture revealed an appearance of bony dissolution and periosteal reaction at the fractured sites as observed by radiograph whereas, in the control group, bony dissolution was

comparatively less and periosteal reaction was absent. Histopathologically, treated group exhibited initiation of osteogenesis which was absent in the control group. Radiograph of the treated group sacrificed on the 21st day of fracture revealed the presence of almost complete bridging of the fractured ends with extensive bony deposition and periosteal reaction as compared to those of the control. Histopathologically, the treated group revealed replacement of cartilaginous cells by osteoblastic cells and union of the fractured gap at several places with the formation of new bony trabeculae, whereas bony trabeculae were absent in the control group (Deka et al., 1994). Chopra et al. (1976) and Udapa et al. (1964) also reported this effect of *C. quadrangularis* in the same manner while they carried out the experiments in different species of animals (rabbit) and different route of administration (intramuscular), respectively.

3. Antiosteoporotic effect

Extract of *C. quadrangularis* was investigated for its anti-osteoporotic activity in ovariectomized rat model of osteoporosis at two different dose levels of 500 and 750 mg/kg per day. Healthy female albino rats were divided into five groups of six animals each. First group was sham operated and served as control. All the other groups were ovariectomized. Group 2 was given with equivolume of saline and served as ovariectomized control. Groups 3-5 were orally treated with raloxifen (5.4 mg/kg) and ethanolic extract of *C. quadrangularis* (500 and 750 mg/kg), respectively. The findings assessed on the basis of biomechanical, biochemical and histopathological parameters showed that ethanolic extract of *C. quadrangularis* had a definite antiosteoporotic effect (Shirwaikar et al., 2003).

4. Antioxidant and antimicrobial effects

Extract of *C. quadrangularis* was tested for antioxidant activity using beta-carotene linoleic acid model and 1,1-diphenyl-2-picrylhydrazyl model. The ethyl acetate fraction of both fresh and dried-stem extracts at a concentration of 100 ppm showed 64.8% antioxidant activity in the beta-carotene linoleic acid system and 61.6% in the diphenyl-2-picrylhydrazyl system. This fraction was found to contain sterols, vitamin C, and tannins. The antioxidant activities of methanolic extract and

aqueous extract were comparatively less significant than that of the ethyl acetate extract, and the n-hexane extract showed the least activity. Ethyl acetate extract and methanolic extract of both fresh and dried-stems of *C. quadrangularis* also exhibited antimicrobial activity against gram-positive bacteria, including *Bacillus subtilis*, *Bacillus cereus*, *Staphylococcus aureus*, and *Streptococcus* species. Results from this study provided an implication of using *C. quadrangularis* as an antibacterial agent and more so as an antioxidant in several applications requiring these properties (Murthy et al., 2003). In addition, *Listeria monocytogenes* were found to be inhibited by acetone extract of this plant (Alzoreky and Nakahara, 2003).

5. Antihemorrhoidal effect

This effect of *C. quadrangularis* was conducted in clinical study. One hundred twenty one hemorrhoidal patients were divided into 3 groups by their stages of disease or degree of severity. Dried-stem powder of *C. quadrangularis* in capsule at the dose of 1,000 mg/day or 2,000 mg/day or 2 tablets of Daflon® twice daily, were given to the patients in each group for 2 months. After the experimental period, patients were assessed for the symptomatic improvement by scoring based on patient satisfaction following each regimens. The result demonstrated that dried-stem powder of *C. quadrangularis* at the dose of either 1,000 mg or 2,000 mg per day for 2 months, had an antihemorrhoidal activity which was comparable to administration of Daflon® 2 tablets twice daily for 2 months (ดวงรัตน์ เชี่ยวชาญวิทย์ และคณะ, 2545).

6. Miscellaneous

6.1) Hypotensive effect

Bhakuni et al. (1969) reported that 50% ethanolic extract of aerial part of *C. quadrangularis* demonstrated hypotensive activity in experimental dogs which were intravenously administered with the extract at a dosage of 50 mg/day.

6.2) Diuretic effect

Diuretic activity of 50% ethanolic extract of *C. quadrangularis* was shown when the extract was given orally to rats at the dosage of 340 mg/kg (Dhawan et al., 1980).

6.3) Anti-implantation effect

Single oral dose of the ethanolic extract of dried aerial part of *C. quadrangularis* (340 mg/kg) showed an anti-implantation activity in adult female rat (Dhawan et al., 1980).

6.4) Antiproliferative effect

Aqueous and methanolic extract of dried root of *C. quadrangularis* was shown to exert antiproliferative effect in cell culture (Opoku et al., 2000).

6.5) Anti-inflammatory effect

Lin et al. (1999) reported that methanolic extract of dried root of *C. quadrangularis* possessed very low cox-1 hydroperoxidase inhibition. Preliminary anti-inflammatory activity of this plant was carried out by Thisayakorn et al. (2000). In that study, 1% carrageenan was used to induce edema in rats. Then, suspension of *C. quadrangularis* in soy bean oil was given orally at 500, 1,000, 1,500, 2,000 and 3,000 mg/kg. The experiment was comparatively conducted along with the control receiving soy bean oil and phenylbutazone 250 mg/kg. The results showed that *C. quadrangularis* at 500 and 1,000 mg/kg exhibited negative results, whereas *C. quadrangularis* at 1,500, 2,000 and 3,000 mg/kg slightly inhibited inflammation at 1 and 2 hours after administration. It appeared that *C. quadrangularis* might not inhibit acute inflammation in rats. The anti-hemorrhoidal activity of *C. quadrangularis* is probably concerned other mechanisms.

Toxicolgical effects

1. Acute toxicity

Aqueous extract of whole part of *C. quadrangularis* was used in acute toxicity test. The extract was intraperitoneally administered or given orally through gastric tube to mice, rats and rabbits. The results demonstrated that degree of toxicity of this plant was ranged from practically non toxic to slightly toxic and related to the doses given (พักตร์พริ้ง แสงดี และ สรศักดิ์ เหลี่ยวไชยพันธุ์, 2528).

Acute oral toxicity of *C. quadrangularis* was investigated in rats according to the requirement defined in the Limit test of OECD guidelines (1993). Wistar rats of

both sexes (5 of each sex in each group) were fed with the powder of *C. quadrangularis* at a single oral dose of 2,000 mg/kg. No toxic signs and mortalities occurred during the observation period of 14 days. The abnormalities observed on necropsy at the termination were not related to the toxic effects when compared with the normal rats (กฤติยา อินทรเผือก, 2543).

An additional acute toxicity study of dried powder of *C. quadrangularis* was also conducted in mice according to the Limit test of OECD guidelines (1993). Ten Swiss albino mice (5 males and 5 females) were used in each group. Mice in the experiment group were given orally with suspension of *C. quadrangularis* at the dose of 2,000 mg/kg body weight, and distilled water at the equivolume as the experimental group was given to the control mice. All mice were observed for 14 days. The results showed that growth rate of all treated mice appeared in the same range as the control mice. No toxic signs and mortalities occurred during 14 days of observation period. None of them showed gross pathological changes at necropsy (จักรพงษ์ ลิมปนุสสรณ์, 2543).

2. Subacute toxicity

Subacute toxicity study of dried powder of *C. quadrangularis* was conducted according to the OECD guidelines (1993). Wistar rats, 5 weeks of age, 10 of each sex in each group, were gavaged with suspension of *C. quadrangularis* at the doses of 50, 350 and 2,450 mg/kg body weight for 30 consecutive days. Control rats were given with distilled water at the equivolume as the experimental groups. Rats in the satellite group were given dried powdered of *C. quadrangularis* at 2,450 mg/kg body weight for 30 consecutive days and observed thereafter for 14 days in order to study reversibility of adverse effects. No toxic signs and changes of growth rate, food consumption and relative internal organ weights were found in rats after treated with *C. quadrangularis* at the doses of 50, 350 and 2,450 mg/kg body weight. Clinical blood chemistry and hematology of rats were not affected after treated with the low and the medium doses of *C. quadrangularis*. The highest dose of *C. quadrangularis* used in that study caused significant changes of clinical blood chemistry and hematology such as a decrease of serum creatinine in female rats, decrease of

serum albumin in both sexes of rats and also a decrease in total white blood cell count of male rats. Level of serum creatinine, serum albumin and total white blood cell count of rats were returned to normal levels after drug withdrawal for 14 days. Some histological findings in internal organs of rats in all groups were found but not related to the doses of the drug used.

3. Subchronic toxicity

Attawish et al.(2002) performed a subchronic toxicity study of the dried-stem powder of *C. quadrangularis* in Wistar rats of both sexes. Rats in control group received water orally at 10 ml/kg body weight/day whereas rats in the other 4 treated groups were orally administered with dried-stem powder of *C. quadrangularis* for 3 months at the doses of 0.03, 0.3, 3.0 and 3.0 g/kg body weight/day, which were equivalent to 1, 10, 100 and 100 fold of the therapeutic dose in human, respectively. The results showed that no difference of rat body weight gain was observed between *C. quadrangularis*-treated and control groups. *C. quadrangularis* did not produce any significant dose-related changes of hematology, clinical blood chemistry, and histopathological lesion of several internal organs.

4. Mutagenic activity

Sivaswamy et al. (1991) reported that fresh fruit of *C. quadrangularis* possessed a very low mutagenic activity against *Salmonella thyphimurium* (TA 1537) and *Salmonella thyphimurium* (TA 98), but not against *Salmonella thyphimurium* (TA 1535) and *Salmonella thyphimurium* (TA 1538).

5. Chromosome aberrations and clastogenic activity

C. quadrangularis extract was given to mice by intragastric route at dosage of 0.1 g/animal. The results demonstrated that *C. quadrangularis* possessed a very low chromosome aberration induction and clastogenic activity (Balachandran et al., 1991).

Xenobiotics biotransformation

Xenobiotics are chemical compounds that do not belong to the normal compositions of the body. These compounds enter the body via diet, air and medication. The principal route of elimination of xenobiotics from the body is biotransformation. Xenobiotic-metabolizing enzymes add funtional groups to make lipophilic molecules more hydrophilic and hence easier to eliminate. Although other organs such as kidney and lung are also relevent, liver is quantitatively the most important organ in the process of biotransformation. Xenobiotic-metabolizing enzymes are found in liver with the largest amount and located in smooth endoplasmic recticulum and cytosol of the liver cells. In general, metabolic biotransformation process of xenobiotics may be divided into Phase I, Phase II and Phase III metabolism (Parkinson, 2001; Ioannides, 1996). A summary of metabolic reactions is given in Table 2.2.

Phase I metabolic transformations, including oxidation, reduction, and hydrolysis, are mostly to introduce functional or reactive group to the molecules. Phase II transformation are generally conjugation reaction of the parent xenobiotics or the phase I metabolites with the endogenous molecules, for example inorganic sulfate, amino acids, glucuronic acid or glutathione. Conjugation reactions facilitate transport and enhance elimination via renal and biliary routes. Phase III metabolism constitutes the further metabolism of the products derived from phase II conjugation reactions. Phase III reactions are catalyzed by enzymes which are also active in phase I and/or phase II reactions. Basically, phase III reactions differ from phase I and phase II reactions only in the fact that the substrates are products of previous phase I or phase II metabolic reactions (Ioannides, 1996)

Xenobiotics, after converted by specific enzymes to more reactive, more electrophilic intermediate, are capable of reacting covalently with biological macromolecules such as proteins, nucleic acids or lipids. Binding of xenobiotic metabolites to DNA may cause modification of genetic information, mutation and a consequent possibility of malignant growth (Soucek and Gut, 1992).

Table 2.2 The Principal Reactions in the Biotransformation of Xenobiotics (Ioannides, 1996)

Reaction	Enzyme	Localization	Substrates
Phase I reactions: Transformations			
Oxidation	Mixed-function oxidases	Microsomes	Alkanes, alkenes, arenas, amines, thiones, thioethers
	Monoamine oxidases	Mitochondria	Amines
	Alcohol dehydrogenases	Cytosol	Alcohols
	Aldehyde dehydrogenases	Cytosol	Aldehydes
Reduction	Mixed-function oxidases	Microsomes	Azo and nitro groups, N-oxides, arene oxides, alkyl halogenides
	Alcohol dehydrogenases	Cytosol	Aldehydes, ketones
Hydrolysis	Esterases	Cytosol Mitochondria Microsomes	Esters
Phase II Reactions: Conjugations with:			
H_2O	Epoxide hydrolase	Microsomes, Cytosol	Epoxides
Glutathione (GSH)[a]	Glutathione transferases	Microsomes, Cytosol	Electrophiles
Glucuronic acid (UDPGA)[a]	Glucuronyl transferases	Microsomes	Phenols, thiols, amines Carboxylic acids
Sulfuric acid (SAM)[a]	Sulfotransferases	Cytosol	Phenols, thiols, amines
Methyl group (SAM)[a]	N- and O-methyl transferases	Cytosol, microsomes	Phenols, amines
Acetic acid (Acetyl-CoA)[a]	N-acetyl transferases	Cytosol	Amines

Table 2.2 (continued) The Principal Reactions in the Biotransformation of Xenobiotics (Ioannides, 1996)

Reaction	Enzyme	Localization	Substrates
Phase III Reactions: Transformations			
Oxidation	Mixed-function oxidases	Microsomes	Thioethers
Hydrolysis	Peptidases	Microsomes	Glutathione conjugates
C-S-lyase (PLP)	β-Lyase	Cytosol Mitochondria	L-Cysteine conjugates
Deamination (PLP)	Transaminases	Cytosol	Amino acid conjugates
	Amino acid oxidases	Mitochondria Cytosol	Amino acid conjugates

[a] Abbreviations in brackets are co-substrates. UDPGA = uridine-3',5'-diphosphoglucuronic acid; PAPS= 3'-phosphoadenosine 5'-phosphosulfate; PLP = pyridoxal phosphate; SAM = S-adenosylmethionine; CoA = coenzyme A.

Phase I Reactions

"Phase I reactions" refers to a set of reactions result in relatively small chemical changes that make compound more hydrophilic and also provide a functional group that is used to complete phase II reactions. Often in phase I reactions, an oxygen atom is added, or a methyl group is removed in order to expose a hydroxyl (-OH), a primary amine ($-NH_2$) or sulfhydryl (-SH) group. The majority of phase I reactions are mediated by a large family of cytochrome P450 (CYP) enzymes located in the membranes of smooth endoplasmic reticulum in hepatocytes and other types of cells. This enzyme family catalyzes a wide variety of oxidative and reductive reactions for chemically diverse group of substrates (Parkinson, 2001; Liska, 1998; Ioannides, 1996; Guengerich, 1992; Guengerich 1991). The summarizing some of the salient features of several relevant P450s in rat are shown in Table 2.3.

Table 2.3 Concentration of individual P450 enzymes in rat liver microsomes (Yan and Caldwall, 2001)

P_{450} enzymes	Specific content (pmol/mg Protein)	Percentage of total spectral P450
Total P450		
*CYP*4A[a]	300	3.0
*CYP*3A2	146	14.6
*CYP*2C[b]	650	65.0
*CYP*1A1, 1A2	12	1.2
*CYP*2E1	79	7.9
*CYP*2A1, 2A2	54	5.4
*CYP*2B1, 2B2	19	1.9

a Sum of *CYP*4A1 and 4A2

b Sum of *CYP*2C6, 2C11, 2C12, and 2C13

The oxidation reaction or monooxygenation reaction catalyzed by CYPs conforms to the following stoichiometry (Ioannides, 2001; Yan and Well,1996) :

$$XH(\text{substrate}) + NADPH + H^+ + O_2 \xrightarrow{CYP} XOH\ (\text{product}) + NADP^+ + H_2O$$

However, xenobiotics biotransformation catalyzed by CYP is not always a detoxification reaction, in many cases, they lead to products with greater cytotoxic, mutagenic, or carcinogenic properties. A variety of specific CYP isoforms, especially CYP in family 1, 2 and 3 are involved in the inactivation of certain chemical procarcinogens (Parkinson, 2001; Soucek and Gut, 1992).

Three CYP families CYP1, CYP 2, and CYP 3 are the major enzymes responsible for drug and xenobiotic metabolism. These three families account for about 70% of total CYPs in human livers while CYP4 is a family of enzymes involved in fatty acid and prostaglandin metabolism. CYP isoforms which play a role in the activation of xenobiotics to toxic metabolites include CYPs 1A1, 1A2, 2B1, 2B2, 2E1 in rats as well as CYPs 1A1, 1A2, 2B6, 2E1, 3A4 in humans (Yan and Caldwell, 2001; Rendic and Di Carlo, 1997; Soucek and Gut, 1992). Major CYP enzymes in human, their endogenous substrates, specific substrates, inducers, inhibitors and their percent participation in drug metabolism are shown in Table 2.4. Examples of rat and

human CYPS that activate some potential carcinogens/metagens are demonstrated in table 2.5

CYP 1A subfamily

CYP 1A subfamily consists of two members, CYP 1A1 and CYP 1A2. They have been well characterized and historically are linked to the aryl hydrocarbon (Ah) receptors as inducible genes. CYP 1A enzymes bioactivate several procarcinogens since they can activate more than 90 % of known carcinogens. These include benzo[a] pyrene and other polycyclic aromatic hydrocarbons which are catalyzed to phenols and epoxides and involved in chemical-induced lung cancer (for CYP 1A1) and, aromatic amines, such as 2-acetylaminoflourine, heterocyclic amines, aflatoxin B1, and 2-naphthylamine (for CYP 1A2). CYP 1A1 is expressed in many tissues and in liver at very low level, but typically highly induced only after induction with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) or other Ah receptors ligands (Gonzalez, 1994). In contrast, CYP 1A2 is primarily expressed in liver at significant constitutive levels and is highly inducible in this tissue via the Ah receptor pathway by environmental compounds such as cruciferous vegetables and polycyclic aromatic hydrocarbon (PAHs). Theophylline, caffeine, and phenacetin are useful *in vivo* diagnostic probes for determining CYP 1A activity status in human (Yan and Caldwell, 2001; Parkinson, 2001; Omiecinski et al, 1999).

CYP 2B subfamily

CYP2B subfamily comprised ~17 different numbers, identified in several different species. CYP2B1 and CYP2B2 are the primary members expressed in rats, whereas CYP 2B6 is expressed at low levels in human liver. CYP2B1 and CYP2B2 are highly similar in nucleotide sequence and have similar substrate specificity. In rodents, enzymes from this subfamily are typically inducible by phenobarbital and other barbiturates, and are inhibited by metyrapone. Rat CYP2B1 is analogous to human CYP2B6, which generally exists in small amount. CYP2B members are involved in the metabolism of a variety of pharmaceutical compounds, including amphetamines and benzodiazepines. It appears that the ability of phenobarbital to

stimulate biotransformation of xenobiotics in human largely stems from its ability to induce other CYPs, CYP2C and CYP3A4 (Parkinson, 2001; Omiecinski et al., 1999).

CYP 2E subfamily

CYP2E1 is the predominant member of the CYP2E subfamily, although CYP2E2 also have been identified in rabbits. CYP2E1 accounts for ~7% of the total CYP contents in rat liver, although its expression has been detected in other tissues (Parkinson, 2001). CYP2E1 is responsible for the metabolism and potential bioactivation of a number of low-molecular-weight pharmaceutical and other xenobiotic compounds, including acetaminophen, ethanol, isoniazid, halogenated anesthetics, acetone, and benzene. CYP2E1 participates in the metabolism of many endogenous substances, such as lipid peroxidation products, ketones and fatty acids, such as linoleic and arachidonic acids. Chlorzoxazone and *p*-nitrophenol are routinely used as substrate probes to measure CYP2E1 activity, although chlorzoxazone can also been metabolized by CYP1A2. CYP2E1 expression is inducible and regulated by many factors. For example, fasting elevates the level of the protein by increasing the transcription of the gene and diabetes by stabilising the mRNA, while isoniazid increases the translation efficiency and affects the enzyme stabilisation similarly to ethanol and imidazole, in animal models. Pyridine is a specific *in vitro* inhibitor and inhibits CYP2E1 at relatively low concentrations. The metabolite of disulfiram, diethyldithiocarbamate, has also been used as an *in vitro* inhibitor, though its selectivity is questionable. Inhibition of CYP2E1 is observed upon treatment with diethyldithiocarbamate, isothiocyanates, and 4-methyl pyrazole (Tanaka et al., 2000; Omiecinski et al, 1999; Rendic and Di-Carlo, 1997; Guengerich 1991).

CYP3A Subfamily

Approximately 22 different CYP3A subfamily members have been identified in many different species, including humans, rabbits, macaques, hamsters, rats, mice, and dogs. CYP3A subfamily members account for ~30-40% of the total CYP content in human livers (Parkinson, 2001; Rendic and Di-Carlo, 1997). The relevant human CYP3A members include CYP3A4, CYP3A5 and CYP3A7. CYP3A4 and CYP3A5 are expressed in the liver and in the intestinal mucosa (Rendic and Di-Carlo, 1997).

CYP3A5 is a polymorphic form and is expressed in about 30% of human livers and 70% of intestines. CYP3A7 is primarily a fetal form of CYP3A, although it has been detected in ~25% of the adult livers surveyed. Both CYP3A5 and CYP3A7 play unimportant role in drug metabolism. Enzymes of the CYP3A subfamily are inducible by rifampin and barbiturates, and to a lesser extent by carbamazepine, phenytoin and dexamethasone. This subfamily of enzymes is responsible for the metabolism of a large and diverse groups of substrates. Nifedipine, midazolam, and testosterone are common substrates probes used to measure CYP3A activity. These enzymes are inhibited by a wide variety of compounds, including triacetyloleandomycin (TAO), macrolide antibiotics, the azole antifungal agents, and competitively by a number of CYP3A substrates. Several CYP3A substrates of toxicological relevance include aflatoxin B1, 1-nitropyrene, and 6-aminochrysene (Parkinson, 2001; Guengerich, 1999; Rendic and Di-Carlo, 1997).

Table 2.4 Human CYP enzymes, their endogenous substrates, specific substrates, inducers, inhibitors, and their percent participation in drug metabolism (modified from Tredger et al., 2002; Omiecinski et al,1999; Rendic and Di Carlo, 1997)

CYP enzymes	Endogenous substrates	Specific substrates	Inducers	Inhibitors	Participation in drug metabolism (%)
1A1	Prostaglandin ω-2 oxidation; testosterone	7-Ethoxyresorufin R-Warfarin	Omeprazole; Cruciferous vegetables	7,8-benzoflavone	2.5
1A2	Prostaglandin ω-1 oxidation; testosterone 6β-hydroxylation	Caffeine 7-Methoxyresorufin Phenacetin R-Warfarin	Omeprazole; Cigarette smoking	Enoxacin; 7,8-benzoflavone	8.2
2B6	Testosterone 16α-16β, 17-hydroxylation	7-Benzyloxyresorufin S-Mephenytoin 7-Pentoxyresorufin Testosterone	Phenobarbital	α-naphthoflavone	3.4
2C8, 9		Diclofenac Hexobarbital Phenytoin Tolbutamide S-Warfarin	Rifampin	Sulfaphenazole	15.8
2C18, 19		Diazepam Propanolol S-Mephenytoin-4-hydroxylation Omeprazole	Rifampin	S-mephenytoin omeprazole	8.3

Table 2.4 (continued) Human CYP enzymes, their endogenous substrates, specific substrates, inducers, inhibitors, and their percent participation in drug metabolism (modified from Tredger et al., 2002; Omiecinski et al,1999; Rendic and Di Carlo, 1997)

CYP enzymes	Endogenous substrates	Specific substrates	Inducers	Inhibitors	Participation in drug metabolism (%)
2D6		Bufuralol Codeine Debrisoquine Dextromethophane Odansetron Sparteine		Quinidine; fluoxitine	18.8
2E1	Alcohol nitrosamines	Acetaminophen Aniline Chlorzoxazone Dapsone Halothane p-Nitrophenol	Alcohol; obesity	Disulfiram; diethyldithio-carbamate	4.1
3A4, 5	Testosterone 6β (major), cortisol 6β-hydroxylation; DHEA-3-sulphate; estradiol-2-hydroxylation	Carbamazepine Cortisol Dapsone Diazepam Erythromycin Midazolam Nifedipine Omeprazole Testosterone	Phenobarbital; dexamethasone; rifampin; carbamazepine	Triacetyl-oleandomycin (TAO); erythromycin; ketoconazole	34.1

Table 2.5 Role of rat and human CYPs in the activation of some potential carcinogens/ mutagens (Parkinson, 2001; Soucek and Gut, 1992)

CYP	Potential carcinogens / mutagens	
	Rat	Human
1A1	Aflatoxin B_1	Benzo(a)pyrene
	Benzo(a)pyrene	7,12-Dimethylbenz(a)anthracene
	7,12-Dimethylbenz(a)anthracene	6-Nitrochrysene
	2-Naphthylamine	
	4,4'-(bis) Methylene chloroaniline	
1A2		Acetaminophen
		2-Aminoacetyl fluorene
		2-Aminol-methyl-6-phenylimidazo
		[4,5-b]pyridine
		Aflatoxin B_1
		4-Aminobiphenyl
		2-Naphthylamine
2B1	2-Acetylfluorene	
	Aflatoxin B_1	
	Benzo(a)pyrene	
	3-Methylcholanthrene	
	4,4'-(bis) Methylene chloroaniline	
2B2	4,4'-(bis) Methylene chloroaniline	
2B6		6-Aminochrysene
		Cyclophosphamide
		3-Methoxy-4-aminoazobenzene
2B7		Aflatoxin B_1
2E1	*N*-*N*''-Nitrosodimethylamine	Acetaminophen
	N-Nitroso-*N*-diethylamine	Acrylonitrile
		Benzene
		Carbon tetrachloride
		Chloroform
		N-*N*''-Nitrosodimethylamine
		N-Nitroso-*N*-diethylamine
		Nitrosamine
		Styrene
		Trichloroethylene
		Vinyl carbamate
		Vinylbromide
		Vinyl chloride
3A4		Aflatoxin B_1
		Aflatoxin G_1
		Benzo(a)pyrene
		6-Nitrochrysene
		Sterigmatocystin

Phase II reactions

Phase II metabolism or conjugation generally follow phase I activation, resulting in a xenobiotic that has been transferred into a water-soluble (hydrophilic) compound that can be excreted through urine or bile. Several types of conjugation reactions are present in the body, including glucuronidation, sulfation, glutathione and amino acid conjugation (Gibson and Skett, 2000; Liska, 1998). Enzymes of those reactions are located in either cytosol or microsome or both as shown in Table 2.2 (Ioannides, 1996). Glucuronidation is a major pathway of xenobiotic biotransformations in most mammalian species (Parkinson, 2001). In addition, glutathione conjugation has been studied extensively as a major detoxification system and considered as an important protective mechanism against chemical induced carcinogenesis. Toxic electrophiles are mostly deactivated by glutathione conjugation, the reaction which is catalyzed by glutathione-S- transferase, with glutathione acting as a cofactor (Parkinson, 2001; Gibson and Skett, 2000; Ioannides, 1996).

CHAPTER III

MATERIALS AND METHODS

Materials

1. Experimental animals

Thirty adult male Wistar rats of body weight between 250-300 g were obtained from the National Laboratory Animal Center, Mahidol University, Salaya, Nakornpathom, Thailand. Rats were housed two per hanging wire cage at the Faculty of Medicine, Srinakharinwirot University, Bangkok and acclimatized for at least a week prior to the experiment. All animals were in a controlled humidify room at a constant temperature of 25°C and maintained on a 12-hour alternate light-dark cycle. They were allowed free access to food (C.P. company, Thailand) and drinking water. During the experimental period, body weight of each rat, food and water intake were recorded every week.

2. Instruments

The following instruments were used in the experimentation.

1. Autopipettes 20, 100, 200, 1000 and 5000 µl (Gibson, France)
2. Centrifuge (Hettich Roto Magna, Japan)
3. Fluorescence spectrophotometer (Jasco, Japan)
4. Metabolic shaker bath (Heto, Denmark)
5. pH meter (Beckman Instruments, USA)
6. Potter-Elvehjem homogenizer with pestle and glass homogenizing vessels (Heidolph, Germany)
7. Refrigerated superspeed centrifuge (Beckman Instruments, USA)
8. Refrigerated ultracentrifuge (Beckman Instruments, USA)
9. Sonicator (Elma, Germany)
10. Spectrophotometer (Jasco, Japan)
11. Surgical equipments
12. Thin Layer Chromatography (TLC) –densitometer (Shimadzu, Japan)
13. Timer (Speedo)
14. Ultra-low temperature freezer (Forma Scientific Inc., USA)
15. Vortex mixer (Clay Adams, USA)

3. Chemicals

Acetylacetone, 4-aminophenol, aniline hydrochloride, benzyloxyresorufin (BR), bovine serum albumin (BSA), cupric sulfate, ethylenediaminetetraacetic acid (EDTA), ethoxyresorufin (ER), Folin & Ciocalteu's phenol reagent, formaldehyde (37% solution formalin), glucose 6-phosphate (G6P), glucose 6-phosphate dehydrogenase (G6PD), methoxyresorufin (MR), nicotinamide adenine dinucleotide phosphate (NADP), pentoxyresorufin (PR), potassium phosphate monobasic anhydrous (KH_2PO_4), resorufin, sodium carbonate (Na_2CO_3), sodium citrate, sodium phosphate dibasic anhydrous (Na_2HPO_4) and Trisma® base were purchased from Sigma Chemical Co., USA.

Ammonium acetate was purchased from APS Finechem, Australia.

Acetic acid was purchased from J.T. Baker Inc.,USA.

Carbon monoxide gas was purchased form T.I.G., Thailand.

Glycerol was purchased from Carlo Erba, USA.

Anisaldehyde, Benzene, Ethanol and Chloroform were purchased from Lab Scan Asia Co., Ltd, Thailand.

Hydrochloric acid (HCl), diethyl ether, magnesium chloride ($MgCl_2$), methanol (HPLC grade), phenol, potassium chloride (KCl), sodium chloride (NaCl), sodium hydroxide (NaOH), sulphuric acid (H_2SO_4) and trichloroacetic acid (TCA) were purchased from Merck, Germany; Sodium dithionite was purchased from Fluka Chemic, Japan; Erythromycin stearate was obtained from Siam Pharmaceutical Co. Ltd., Thailand.

Except indicated, water used in this study was ultrapure water which was prepared by ELGASTAT MAXIMA UF.® (ELGA Ltd, England).

Methods

1. Preparation of dried-stem powder of *C. quadrangularis*

    1. *C. quadrangularis* stems were acquired from Amphur Phanomsarakarm, Chachoengsao Province, Thailand. They were collected during February – March, 2003.
    2. The stems were dried and ground to fine powder.
    3. One kilogram of the dried-stem powder was sieved through sieve No. 80, stored in a brown bottle with a tightly closed cap and kept at room temperature.

2. Chemical Identification of the dried-stem powder of *C. quadrangularis*

    Thin-layer chromatography (TLC)-densitometer was used to identify the chromatogram of the tested powder compared to the reference chromatogram. The method was modified from the method of a previous study (ธัญวรัตน์ จันทรชนะ และ พงศธร หลิมศิริวงษ์, 2543).

Reagents

1. Petroleum ether
2. Chloroform
3. Benzene
4. 2M HCL
5. Anisaldehyde
6. Sulfuric acid
7. Ethanol

Procedures

1. Five grams of dried-stem powder of *Cissus quadrangularis* were extracted by 30 ml of petroleum ether and shaked for 24 hrs.
2. Subsequently, the suspension was filtered and the filtrate was evaporated to dryness. The residue was dissolved with 2 ml of chloroform.
3. The constituents in the extract were separated by TLC, which was conducted on a TLC plate coated with silica gel F-254. TLC plate was of 0.25 nm thickness (Merck) and separation was achieved in a developing solvent systems of

benzene : chloroform : 2M HCL, 20:20:1 v/v.

4. The TLC plate was then analyzed using densitometer at a wavelength of 200 nm.

5. The condition of densitometer was as following

Lamp source	: Deuterium lamp
Determination mode	: Reflection absorption photometry
Slit width	: 1 nm
Slit height	: 5 nm
Wavelength detector	: 200 nm

6. After the densitometric determination, the TLC plate was sprayed with the mixture solution of anisaldehyde: sulfuric acid: ethanol: water (2: 3: 90: 1). The plate was then placed in hot air oven at 105 °C for 5 min.

3. Animal treatment

Thirty rats were randomly divided into 3 treatment groups. Each treatment group comprised of 10 rats as followings:

1. Control group: Rats were given distilled water orally at 1 ml/kg/day for 30 days.
2. *C. quadrangularis* treated group I: Rats were given dried-stem powder of *C. quadrangularis* orally at dosage of 0.03 g/kg/day for 30 days.
3. *C. quadrangularis* treated group II: Rats were given dried-stem powder of *C. quadrangularis* orally at dosage of 0.3 g/kg/day for 30 days.

Note
- Dried-stem powder of *C. quadrangularis* at the doses of 0.03 and 0.3 g/kg/day used in this study were equivalent to 1 and 10 folds of the therapeutic doses (1.5 g/50-kg person/day) respectively (Attawish A. et al, 2002).
- Dried-stem powder of *C. quadrangularis* was suspended with distilled water to make suspensions of concentration 0.03 g/ml and 0.3 g/ml.
- During the treatment period, body weight, food consumption and volume of drinking water were recorded every week.

4. Blood sampling for determination of clinical blood chemistry and hematology

At the end of the treatment, animals were fasted for 12 hours before anesthesized with diethyl ether by inhalation. Blood samples were collected by heart

puncture on left ventricle for an approximate volume of 5 ml. Five hundred microliters of whole blood was transferred to a microtube containing a few grains of EDTA sodium and mixed thoroughly. The remaining blood sample was transferred to another tube, and centrifuged for collecting serum sample. Whole blood and serum sample were investigated for hematology and clinical blood chemistry, respectively.

4.1 Clinical blood chemistry

Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), alkaline phosphatase (ALP), blood urea nitrogen (BUN), serum creatinine (SCr), total cholesterol, triglyceride (TG), high density lipoprotein-cholesterol (HDL-C), low density lipoprotein-cholesterol (LDL-C), glucose, uric acid in serum samples were analyzed by the Faculty of Allied Health Sciences, Chulalongkorn University. Total bilirubin, direct bilirubin, low density lipoprotein-cholesterol (LDL-C), sodium, potassium and chloride in serum samples were analyzed by Professional Laboratories Management Corp. Co., Ltd., Bangkok.

4.2 Hematology

Hemoglobin (Hb), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet count, white blood cell (WBC) count, % differential WBCs, RBC count and RBC morphology in whole blood samples were determined by Professional Laboratories Management Corp. Co., Ltd., Bangkok.

5. Hepatic microsomal preparation

Rat liver microsomes were prepared according to the method described by Lake (1987) with some modifications.

Reagents

1. 0.1 M Phosphate buffer, pH 7.4

   One Litre of phosphate buffer, pH 7.4 consisted of 17.8 g of KH_2PO_4 , 9.55 g of Na_2HPO_4 and 11.50 g of KCl then adjusted the solution to pH 7.4 with NaOH or HCl.

2. Phosphate buffer, pH 7.4, containing 20% v/v glycerol
3. 0.9% w/v NaCl

Procedures

1. After collecting blood sample, liver was immediately removed and perfused with ice-cold 0.9% w/v NaCl until the entire organ became pale.
2. The liver was rinsed with ice-cold 0.9% w/v NaCl and blotted dry with gauze.
3. The whole liver was weighed, cut into pieces and homogenized with 3 volume of phosphate buffer, pH 7.4.
4. The liver homogenate was centrifuged at 10,000 *g* for 30 minutes at 4 ^{o}C using refrigerated superspeed centrifuge, to pellet intact cells, cell debris, nuclei and mitochondria.
5. The supernatant (S9, post mitochondrial fraction) was transferred into ultracentrifuge tube and centrifuged at 100,000 *g* for 60 minutes at 4 ^{o}C, using refrigerated ultracentrifuge.
6. The pellets (microsomal subfraction) were resuspended with 5 ml of phosphate buffer, pH 7.4 containing 20% glycerol. The microsomal suspension (or microsome) was aliquoted to microtubes and stored at –80^{o}C until the time of enzyme assays.

6. Determination of protein concentrations in liver microsomes

Liver microsomal protein concentrations were determined according to the method modified from the method of Lowry et al. (1951).

Reagents

1. 2% w/v Na_2CO_3
2. 0.5 M NaOH
3. 2% w/v Sodium citrate
4. 1% w/v Cupric sulfate
5. 1 mg/ml BSA in 0.5 M NaOH
6. Folin & Ciocalteu's phenol reagent
7. Working protein reagent comprised 2% w/v Na_2CO_3, 0.5 M NaOH, 2% w/v sodium citrate and 1% w/v cupric sulfate solutions in a 100:10:1:1 ratio, respectively. This solution was freshly prepared in a sufficient amount for all tubes in the assay.

Procedures

1. 16x125 mm. tubes were labeled in duplicate for 7 standards (0, 50, 100, 150, 200, 250 and 300 µg) and for each unknown sample.

2. The following reagents were added into each standard solution tube:

Standard tube	0	50	100	150	200	250	300	(µg)
1 mg/ml BSA	0	50	100	150	200	250	300	(µl)
0.5 M NaOH	500	450	400	350	300	250	200	(µl)

Each tube was mixed thoroughly, after addition of these reagents.

3. To each unknown sample tube, 490 µl of 0.5 M NaOH and 10 µl of microsome were added and then mixed thoroughly.
4. After 6.5 ml. of working protein reagent was added to each tube in the assay, the tubes were allowed to stand at room temperature for 10 minutes.
5. While, 200 µl of Folin & Ciocalteu's phenol reagent was added to each tube in the assay, the tube was vortexed thoroughly for a minimum of 30 seconds.
6. After the tubes were allowed to stand at room temperature for a minimum of 30 minutes, the absorbance of the solution was measured by spectrophotometer against the 0 µg standard at 500 nm.

Calculations

1. The average absorbance of each standard was plotted against its amount of protein. The best-fit regression line was drawn through the points. The amount of protein in each unknown sample was obtained by comparing its absorbance against the standard curve.
2. The protein concentration was expressed in a unit of mg/ml or µg/µl by dividing its amount of protein (from step1) with the volume of microsome used in the reaction.

7. Spectral determination of total CYP contents in liver microsomes

Hepatic microsomal total CYP contents were determined spectrophotometrically according to the method of Omura and Sato (1964).

Reagents

1. 0.1 M Tris buffer, pH 7.4, containing 20% v/v glycerol
2. Solid sodium dithionite
3. Carbon monoxide

Procedures

1. Microsomal sample was diluted to 2 mg/ml with 0.1 M Tris buffer, pH 7.4 containing 20% v/v glycerol.
2. A few grains of sodium dithionite was added to the 5 ml. diluted sample with gentle mixing, the solution was then transferred to the sample and reference cuvettes in 2.5 ml. for each.
3. Both cuvettes were placed in a spectrophotometer, adjusted to zero and corrected to a baseline between 400 nm and 500 nm.
4. Immediately after the sample cuvette was bubbled with carbon monoxide (approximately 1 bubble/second) for approximately one minute, the cuvette was placed back to the spectrophotometer and scanned from 400 nm to 500 nm. The absorbance difference between 450 nm and 490 nm was recorded.

Calculations

Total CYP contents were calculated based on the absorbance difference between 450 nm and 490 nm as well as an extinction coefficient of 91 $mM^{-1}cm^{-1}$. Using Beer's law and an assuming a cuvette path length of 1 cm, total CYP contents were given by:

$$\text{Total CYP content (nmol/mg protein)} = \frac{\text{Absorbance difference (450-490 nm) x 1000}}{\text{91 x concentration (mg/ml) of diluted sample}}$$

8. Analysis of hepatic microsomal CYP activities

8.1 Alkoxyresorufin O-dealkylation assay

Rate of hepatic microsomal alkoxyresorufin O-dealkylation were determined according to the method of Burke and Mayer (1974) and Lubet et al.(1985) with some modifications. Benzyloxyresorufin (BR) and Pentoxyresorufin (PR) were used as specific substrates of CYP 2B1 and CYP 2B2. Ethoxyresorufin (ER) and

Methoxyresorufin (MR) were used as specific substrates of CYP 1A1 and CYP1A2, respectively.

Reagents

1. 0.1 M Tris buffer, pH7.4

2. 20 mM K_3PO_4

3. Resorufin and Alkoxyresorufins

   a) 0.5 mM MR (MW = 227)

      MR 1.135 mg was dissolved with 10 ml of DMSO.

   b) 0.5 mM BR (MW = 303)

      BR 1.515 mg was dissolved with 10 ml of DMSO.

   c) 0.5 mM ER (MW = 241)

      ER 1.205 mg was dissolved with 10 ml of DMSO.

   d) 0.5 mM PR (MW = 283)

      PR 1.415 mg was dissolved with 10 ml of DMSO.

   e) 0.5 mM Resorufin (MW = 235)

      Resorufin 1.175 mg was dissolved with 10 ml of DMSO.

4. NADPH regenerating system

   NADPH regenerating system comprised the solutions as following:

   a) 0.1 M NADP, pH 7.4

      NADP 0.765 g was dissolved with 20 mM K_3PO_4 and made up to 10 ml, adjusting pH to 7.4 with HCl or NaOH (10 µl contained 1 mmole of NADP).

   b) 0.5 M G6P, pH 7.4

      G6P 1.41 g was dissolved with 20 mM K_3PO_4 and made up to 10 ml, adjusting pH to 7.4 with HCl or NaOH (10 µl contained 5 mmole of G6P).

   c) 0.3 M $MgCl_2$, pH 7.4

      $MgCl_2$ 609.93 mg was dissolved with 20 mM K_3PO_4 and made up to 10 ml, adjusting pH to 7.4 with HCl or NaOH (10 µl contained 3 mmole of $MgCl_2$).

d) G6PD, pH 7.4

G6PD was diluted to 100 units per ml with 20 mM K_3PO_4, adjusting pH to 7.4 with HCl or NaOH (10 µl contained 1 unit of G6PD).

For each assay, the mixture solution of NADPH regenerating system was freshly prepared in a 1:1:1 ratio of 0.1 M NADP, 0.5 M G6P and 0.3 M $MgCl_2$, respectively. For the reaction volume of 1 ml, 30 µl of this mixture was used for microsomal preincubation and 15 µl of G6PD was added to initiate the reaction.

Procedures

1. Microsomal sample was diluted with 0.1 M Tris buffer, pH 7.4 to measure out 300 µg of protein for the final reaction mixture volume of 1.5 ml.
2. The following reagents were added for the reaction preincubation.
   a) 1225 µl of 0.1 M Tris buffer, pH 7.4
   b) 15 µl of 0.5 mM Alkoxyresorufin
   c) 45 µl of the mixture solution of NADPH regenerating system containing
      - 15 µl of 0.1 M NADP
      - 15 µl of 0.5 M G6P
      - 15 µl of 0.3 M $MgCl_2$
   d) 200 µl of diluted microsomal suspension containing 300 µg of microsomal protein (accounted for 200 µg of microsomal protein/ 1 ml of the reaction mixture)
3. Three tubes were prepared for each microsomal sample. One tube was a sample blank and the others were sample tubes.
4. All tubes were preincubated in a 37°C shaking water bath for 2 minutes.
5. The reaction was started by an addition of 15 µl of G6PD (1 unit of G6PD/1ml of reaction mixture volume). For the sample blank tube, 15 µl of 0.1 M Tris buffer, pH 7.4 was added instead of G6PD.
6. After 5-minute incubation, the reaction was stopped by adding 1.5 ml of methanol (HPLC grade).
7. The absorbance was measured by fluorescence spectrophotometer, using an excitation wavelength of 556 nm and an emission wavelength of 588 nm.

8. A resorufin standard curve was carried out using the duplicated resorufin concentrations of 0.003, 0.006, 0.012, 0.025, 0.050 and 0.075 nmole/ml.
9. Rate of alkoxyresorufin O-dealkylation was calculated by dividing the amount of resorufin formed by the time of 5-minute incubation and amount of microsomal protein (mg) used in the reaction.
10. The procedure was verified by varying amount of microsomal protein used in the reaction (100, 200, 300 µg of microsomal protein/ 1 ml of the reaction mixture). Liver microsomes were prepared from phenobarbital-induced rats given phenobarbital at the dosage of 80 mg/kg/day intraperitoneally for 3 days (Tredger et al., 2002; Soucek and Gut, 1992). The reaction was performed as mentioned above using BR as a substrate. Correlation coefficient (r^2) between amount of microsomal protein and fluorometric absorbance was 0.9992 (Appendix, page 78).

8.2 Aniline 4-hydroxylation assay

The catalytic activity of CYP2E1 was determined based on the rate of hepatic microsomal aniline 4-hydroxylation, according to the method of Schenkman, J.B., Remmer, H., and Estabrook, R.W. (1967). Aniline hydrochloride was used as a specific substrate in this reaction.

Reagents

1. 10 mM Aniline hydrochloride

   Aniline hydrochloride 129.6 mg was dissolved and made up to 100 ml with water. The solution was stored in a light-protection bottle.
2. 6% w/v Trichloroacetic acid

   Trichloroacetic acid 60 g was made up to 1 L with water.
3. 20% w/v Trichloroacetic acid

   Trichloroacetic acid 200 g was made up to 1 L with water.
4. 1% w/v Phenol

   Phenol 20 g and 40 g of NaOH were made up to 2 L with water.
5. 1 M Na_2CO_3

   Anhydrous Na_2CO_3 106 g was made up to 2 L with water.

6. 10 μM 4-aminophenol

   4-aminophenol 0.0365 g were dissolved in methanol 1 ml, then made up to 10 ml with water. Then 0.1 ml of this solution was added to TCA 15 g and made up to 250 ml with water.

7. NADPH regenerating system (The preparation was described in 8.1)

Procedures

1. Each 2 ml of the reaction mixture comprised microsome containing 5 mg of protein, 500 μl of aniline hydrochloride, 30 μl of NADPH regenerating system and Tris buffer, pH 7.4 qs to 1980 μl.
2. All tubes were preincubated in a shaker bath at 37°C for 2 minutes.
3. The catalytic reaction was initiated by an addition of 20 μl of G6PD. For the sample blank tube, 20 μl of Tris buffer, pH 7.4 was used instead of G6PD.
4. After 30 minutes of incubation the reaction was terminated by adding 1 ml of ice-cold 20% w/v trichloroacetic acid. The reaction tubes were placed on ice for at least 5 minutes.
5. The solution was then centrifuged at 3,000 r.p.m. for 10 minutes.
6. After 1 ml of the supernatant was transferred to a new tube, 1 ml of 1% phenol and 1 ml of 1M Na_2CO_3 were added to each tube. The solution was mixed homogeneously and allowed to stand at room temperature for 30 minutes.
7. Absorbance of the mixture was measured spectrophotometrically at 630 nm.
8. A standard curve was carried out using 5 concentrations of 4-aminophenol standard solutions (0, 2, 4, 6, 8 and 10 μM) following the procedure from step 6 in the same manner as sample.
9. The procedure was verified by varying amount of rat microsomal protein used in the reaction (2.5, 5.0, 7.5 mg of microsomal protein/ 2 ml of the reaction mixture). The reaction was performed as mentioned above. Correlation coefficient (r^2) between amount of microsomal protein and absorbance was 0.9985 (Appendix, page 79).

Calculations

Rate of aniline 4-hydroxylation was calculated by dividing the amount of product formed (4-aminophenol) in nmole by the time of incubation (30 minutes) and

amount of microsomal protein used (mg) in the reaction. The unit was expressed as nmole/mg protein/min.

8.3 Erythromycin N-demethylation assay.

Rate of hepatic microsomal erythromycin N-demethylation was determined using the method of Nash *et al*. (1953) and Friedli G. (1992). Erythromycin stearate was used as a specific substrate of CYP3A.

Reagents

1. Formaldehyde (M.W. 30, 37% solution formalin)
2. 20 mM KH_2PO_4
3. 20 mM Potassium phosphate buffer, pH 7.4

   Fifty milliliters of 20 mM KH_2PO_4 was added with 39.1 ml of 0.02 M NaOH and made up to 200 ml with water, then adjusting pH to 7.4 with HCl or NaOH.
4. 10 mM Erythromycin stearate

   Erythromycin stearate 0.1018 g was dissolved with water and made up to 10 ml.
5. NADPH regenerating system (The preparation was described in 8.1)
6. 12.5% w/v TCA

   TCA 12.5 g was made up with water to 100 ml.
7. 0.02 M NaOH
8. Nash reagent.

   Nash reagent was freshly prepared, the solution comprised 30 g of ammonium acetate, 0.4 ml of acetylacetone, 0.6 ml of glacial acetic acid and water qs. to 100 ml.

Procedures

Each unknown sample as performed in duplicate.

1. To make a final volume of 1 ml microsomal incubation, the preincubation mixture was composed of microsome containing 4 mg of microsomal protein, 100 µl of 10 mM erythromycin stearate, 45 µl of NADPH regenerating system and 20 mM phosphate buffer, pH 7.4 qs. to 985 µl in sample tubes, whereas 840 µl of 20 mM phosphate buffer, pH 7.4 was substituted for microsome in sample blank tube.
2. All tubes were preincubated in a shaker bath at 37^{o}C for 3 minutes.

3. The reaction was initiated by an addition of 15 µl of G6PD. For sample blank, 15 µl of 20 mM phosphate buffer, pH 7.4 was added instead of G6PD.
4. The reaction was stopped by adding 500 µl of ice-cold 12.5% w/v TCA after 10 minutes incubation.
5. All tubes were centrifuged at 3,000 r.p.m. for 10 minutes. One milliliter of the supernatant was transferred to another new tube. One milliliter of freshly prepared Nash reagent was added to each tube and mixed well by vortex mixer.
6. All tubes were warmed in a shaker bath at 50°C for 30 minutes.
7. Absorbance of the mixture was measured by spectrophotometer at a wavelength of 412 nm.
8. A formaldehyde standard curve was constructed by adding 1 ml of formaldehyde at concentrations of 0.0156, 0.0313, 0.0625, 0.125 and 0.25 µmol/ml with 1 ml of Nash reagent and performed the procedure in the same manner as the sample tubes described above.
9. The procedure was verified by varying amount of microsomal protein used in the reaction (2, 4, 8 mg of microsomal protein/ 1 ml of the reaction mixture). Liver microsomes were prepared from phenobarbital-induced rats given phenobarbital at the dosage of 80 mg/kg/day intraperitoneally for 3 days (Tredger et al., 2002; Soucek and Gut, 1992). The reaction was performed as mentioned above. Correlation coefficient (r^2) between amount of microsomal protein and absorbance was 1 (Appendix, page 80).

Calculations

Rate of erythromycin N-demethylation was calculated by determining amount of formaldehyde formed, dividing by mg of micosomal protein used and time of incubation (10 minutes). The unit was expressed as nmole/mg protein/min.

9. Data analysis

All numeric quantitative data were presented as mean ± standard error of the mean(SEM). A one way analysis of variance (ANOVA) and Student-Newman-Keuls test were used for statistical comparison at a significant level of $p < 0.05$.

CHAPTER IV
RESULTS

Preparation of dried-stem powder of *C. quadrangularis*

One kilogram of dried-stem powder of *C. quadrangularis* was sieved through sieve No. 80 before using in this study. After sieving, approximately 600 g of the fine powder of *C. quadrangularis* was obtained and appeared to be middle-brown color with particular fetid odour.

Chemical identification of the dried-stem powder of *C. quadrangularis*

Chromatographic fingerprint of the dried-stem powder of *C. quadrangularis* used in this study (Figure 4.1) conformed with somewhat difference to the chromatographic fingerprint of the reference petroleum ether extract of *C. quadrangularis* reported earlier (Figure 4.2, ธัญวรัตน์ จันทรชนะ และ พงศธร หลิมศิริวงษ์, 2543). Likewise, the extract exhibited a positive result to the anisaldehyde-sulfuric acid color test (Figure 4.3). These characteristics of the petroleum ether extract of *C. quadrangularis* was indicated to be composed of triterpenes and phytosterol (Pluemjai et al., 1986).

Effects of *C. quadrangularis* on body weight, food & water consumption, liver weight and relative liver weight

Subacute (30 days) effects of *C. quadrangularis* were investigated on hepatic CYPs and blood clinical chemistry in rats. During the time of treatment period, body weight, food consumption and volume of drinking water were recorded every week. At the end of treatment, animals were sacrificed and their livers weight were recorded before preparation of microsomes. The results showed that both dosage regimens of the dried-stem powder of *C. quadrangularis* did not affect body weight gain (Figure 4.4), food consumption (Figure 4.5), water consumption (Figure 4.6), liver weight and relative liver weight (Table 4.1).

Effects of the dried-stem powder of *C. quadrangularis* on hepatic microsomal CYPs

C. quadrangularis at both dosages (0.03 and 0.3 g/kg/day) used in this study did not cause any significant changes of hepatic microsomal total CYP contents (Figure 4.7). Rate of ethoxyresorufin O-dealkylation (EROD; which represented the

activities of CYP1A1, Figure 4.8), methoxyresorufin O-dealkylation (MROD; which represented the activity of CYP1A2, Figure 4.9), benzyloxy- and pentoxyresorufin O-dealkylation (BROD and PROD respectively, which represented the activity of CYP2B1/2B2, Figure 4.10-4.11) as well as aniline 4-hydroxylation (which represented the activity of CYP2E1) were unaffected (Figure 4.12). No significant effects of the dried-stem powder of *C. quadrangularis* were found on CYP3A activity which was determined by the reaction rate of erythromycin N-demethylation in both *C. quadrangularis*-treated groups as compared to the control group (Figure 4.13).

Effects of the dried-stem powder of *C. quadrangularis* on clinical blood chemistry and hematology

Serum and whole blood sample of individual rat were measured for clinical blood chemistry and hematology, respectively. Thirty-day treatment of oral 0.03 and 0.3 g/kg/day of the dried-stem powder of *C. quadrangularis* did not cause any significant effects on these following parameters: AST, ALT, ALP, total and direct bilirubin, BUN, SCr, uric acid, total chloresterol, TG, LDL-C, HDL-C, glucose, sodium, potassium, chloride, Hb, Hct, platelet count, WBC count and %differential WBCs, RBC count, MCV, MCH, MCHC, and RBC morphology (Table 4.2).

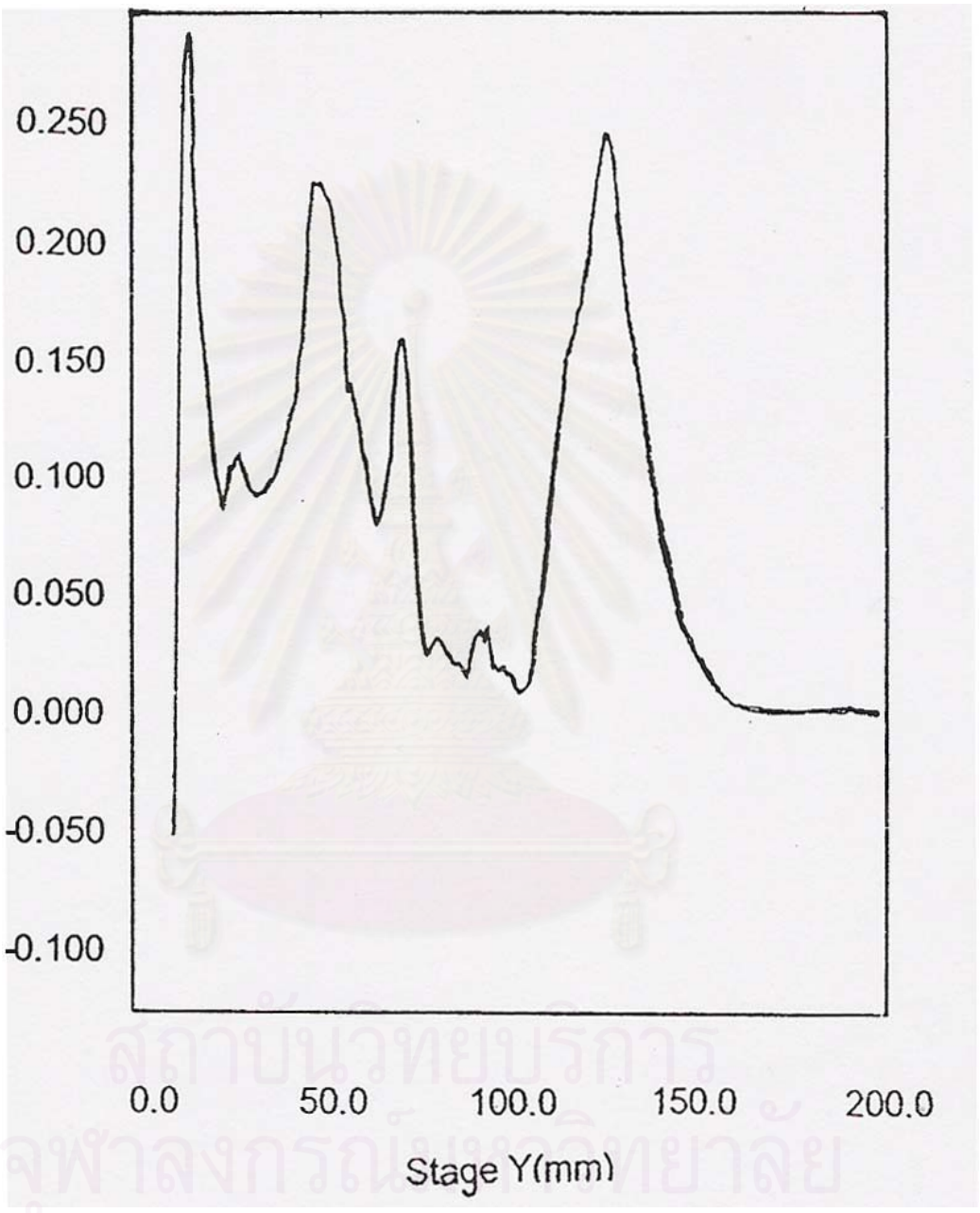


Figure 4.1 The chromatographic fingerprint of the dried-stem powder of *C. quadrangularis* used in this study (Procedure was mentioned in Materials and Methods.)

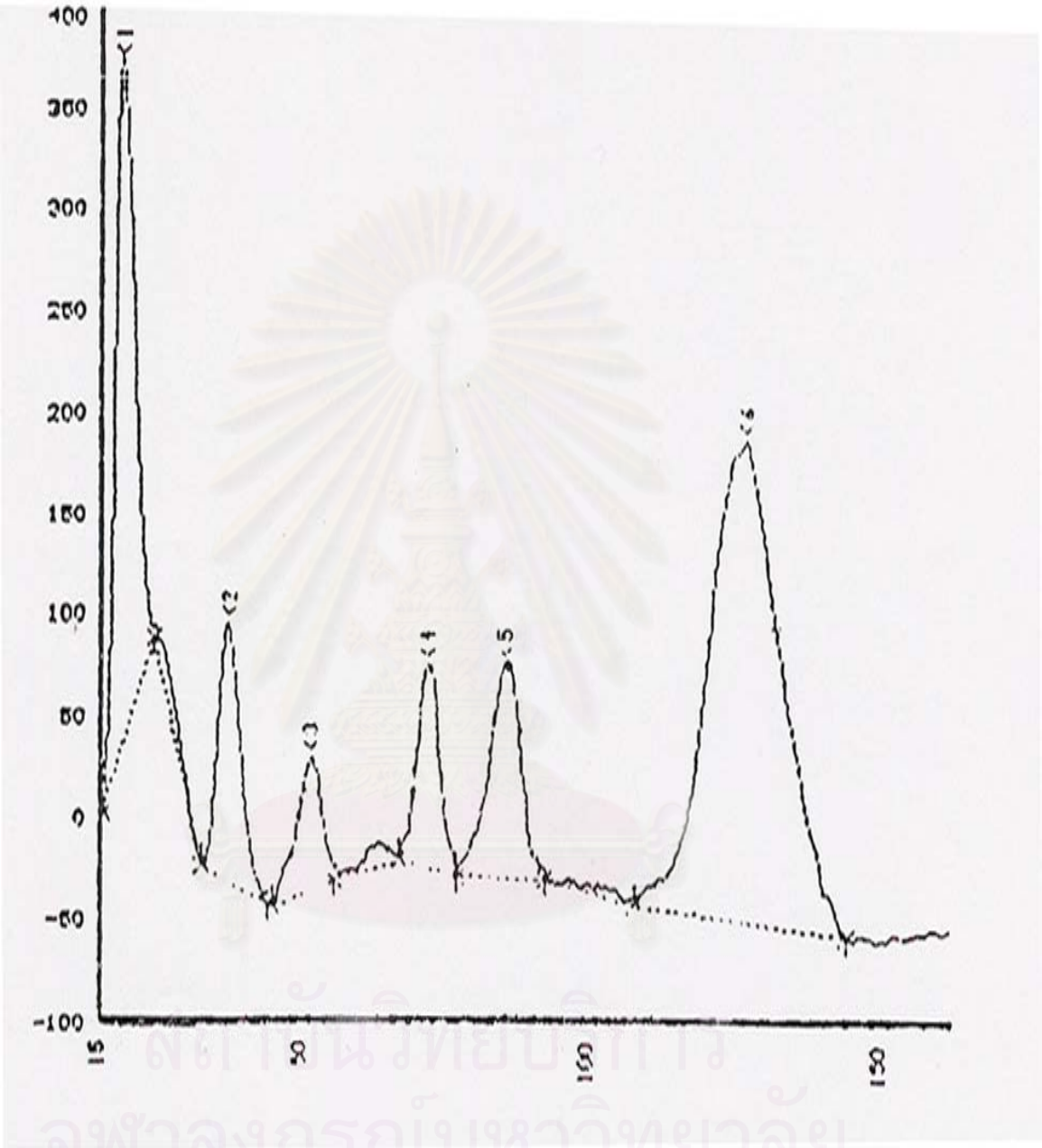


Figure 4.2 The chromatographic fingerprint of the dried-stem powder of *C. quadrangularis* used as reference (ธัญวรัตน์ จันทรชนะ และ พงศธร หลิมศิริวงษ์, 2543)

Figure 4.3 The TLC plate of the dried-stem powder of *C. quadrangularis*. Following the densitometric determination, the TLC plate was sprayed with the mixture solution of anisaldehyde-sulfuric acid, heated at 105°C for 5 min (Procedure was mentioned in Materials and Methods). All lanes were spotted with the petroleum ether extract of *C. quadrangularis* used in this study.

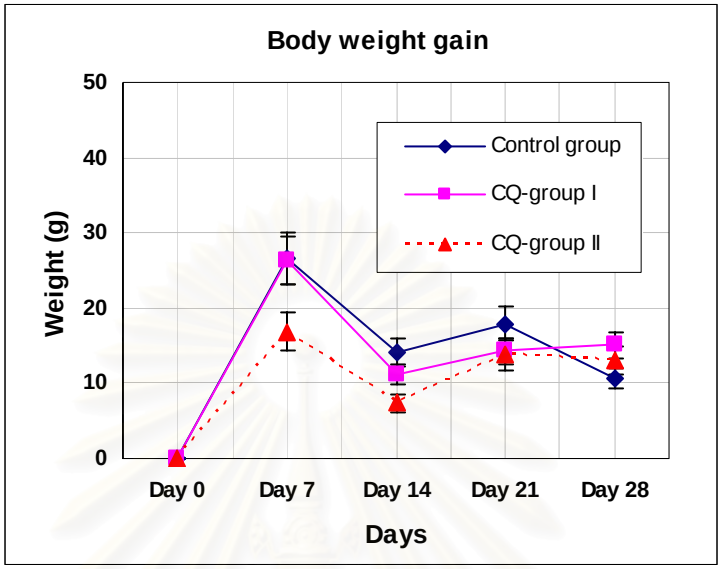


Figure 4.4 Effects of the dried-stem powder of *C. quadrangularis* on body weight gain of rats. Rats were administered orally with 1 ml/kg/day of distilled water (Control group), 0.03 and 0.3 g/kg/day of the dried-stem powder of *C. quadrangularis* (CQ-group I & CQ-group II, respectively) for 30 days. The individual mark represented mean of body weight gain with an error bar of standard error of the mean (n = 10). One-way ANOVA and Student-Newman-Keuls test were used for statistical comparisons at a significant level of $p<0.05$.

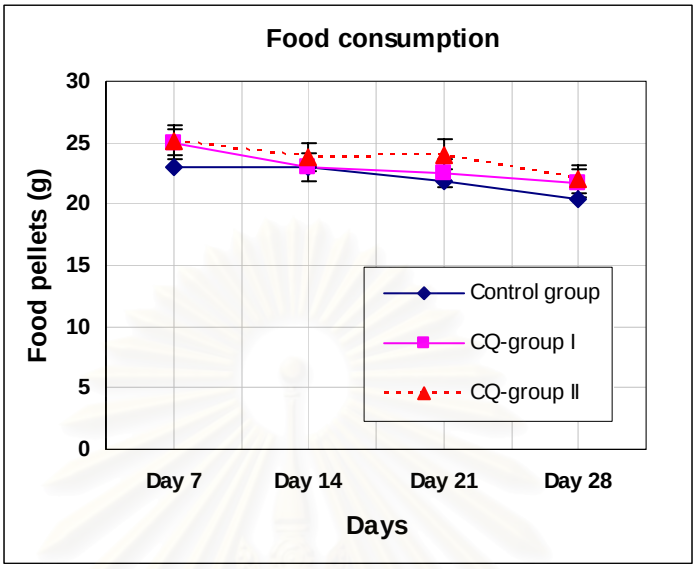


Figure 4.5 Effects of the dried-stem powder of *C. quadrangularis* on food consumption of rats. Rats were administered orally with 1 ml/kg/day of distilled water (Control group), 0.03 and 0.3 g/kg/day of the dried-stem powder of *C. quadrangularis* (CQ-group I & CQ-group II, respectively) for 30 days. Food consumption of each rat was recorded every week. The individual mark represented mean of food consumption per day with an error bar of standard error of the mean (n=10). One-way ANOVA and Student-Newman-Keuls test were used for statistical comparisons at a significant level of $p<0.05$.

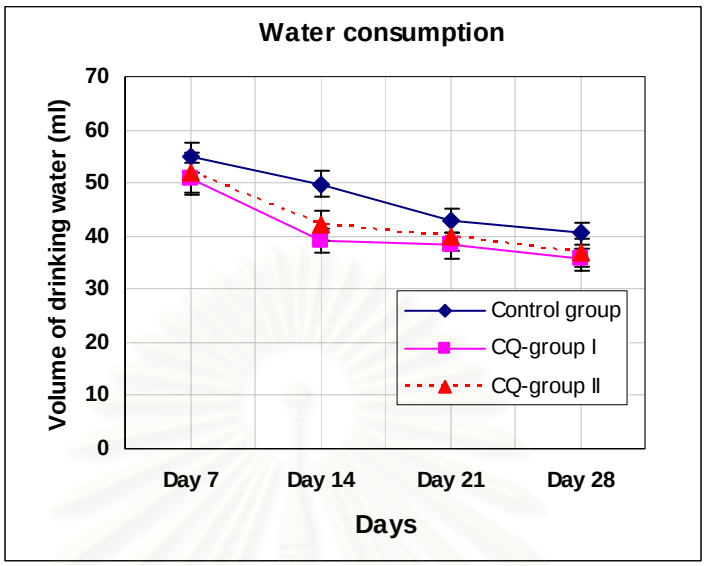


Figure 4.6 Effects of the dried-stem powder of *C. quadrangularis* on water consumption of rats. Rats were administered orally with 1 ml/kg/day of distilled water (Control group), 0.03 and 0.3 g/kg/day of the dried-stem powder of *C. quadrangularis* (CQ-group I & CQ-group II, respectively) for 30 days. Water consumption of each rat was recorded every week. The individual mark represented mean of water consumption per day with an error bar of standard error of the mean (n=10). One-way ANOVA and Student-Newman-Keuls test were used for statistical comparisons at a significant level of $p<0.05$.

Table 4.1 Effects of the dried-stem powder of *C. quadrangularis* on liver weight and relative liver weight

Rats were given orally with 1 ml/kg/day of distilled water (Control group), 0.03 and 0.3 g/kg/day of the dried-stem powder of *C. quadrangularis* (CQ-group I & CQ-group II, respectively) for 30 days. Terminal body weight and liver weight of each rat was recorded at the end of the experiment. Relative liver weight denoted percent of liver weight per terminal body weight. Values shown were mean ± SEM. One-way ANOVA and Student-Newman-Keuls test were used for statistical comparisons at a significant level of $p<0.05$.

Parameters	Group		
	Control	CQ-group I	CQ-group II
Terminal Body Weight (g)	413.49 ± 11.93	417.26 ± 11.53	398.61 ± 6.07
Liver weight (g)	12.78 ± 0.79	12.47 ± 0.59	12.78 ± 0.37
Relative liver weight (% of body weight)	3.07 ± 0.13	2.99 ± 0.12	3.21 ± 0.12

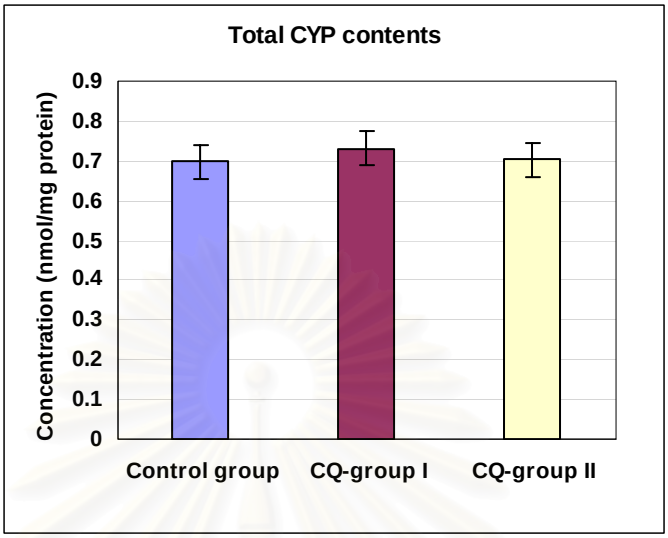


Figure 4.7 Effects of the dried-stem powder of *C. quadrangularis* on rat hepatic total CYP contents. Rats were administered orally with 1 ml/kg/day of distilled water (Control group), 0.03 and 0.3 g/kg/day of the dried-stem powder of *C. quadrangularis* (CQ-group I & CQ-group II, respectively) for 30 days. Liver microsomes were determined for total CYP contents. The individual mark represented mean of total CYP contents with an error bar of standard error of the mean (n=10). One-way ANOVA and Student-Newman-Keuls test were used for statistical comparisons at a significant level of $p<0.05$.

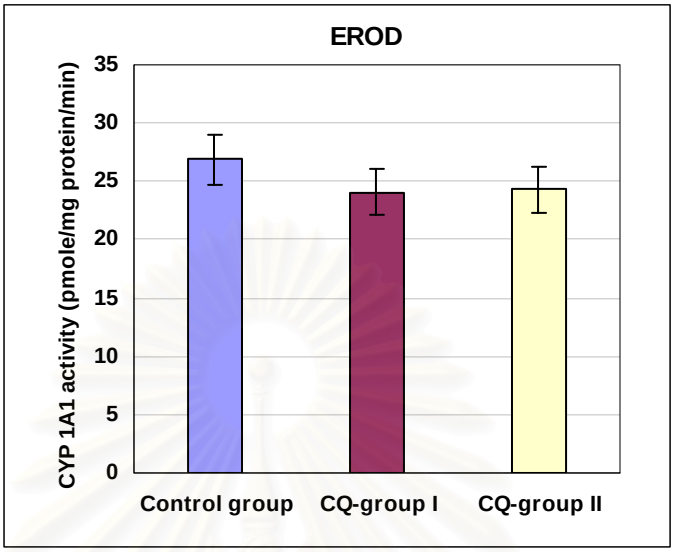


Figure 4.8 Effects of the dried-stem powder of *C. quadrangularis* on rat hepatic CYP1A1 activity. Rats were administered orally with 1 ml/kg/day of distilled water (Control group), 0.03 and 0.3 g/kg/day of the dried-stem powder of *C. quadrangularis* (CQ-group I & CQ-group II, respectively) for 30 days. Liver microsomes were determined for EROD activity. The individual mark represented mean of EROD activity with an error bar of standard error of the mean (n=10). One-way ANOVA and Student-Newman-Keuls test were used for statistical comparisons at a significant level of $p<0.05$.

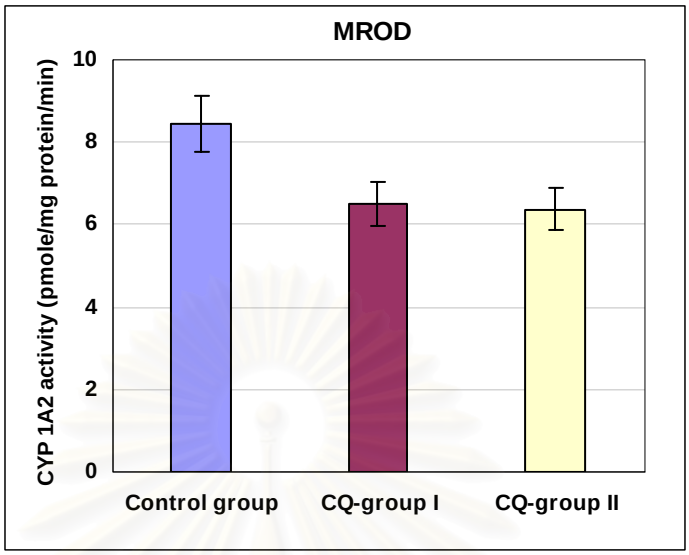


Figure 4.9 Effects of the dried-stem powder of *C. quadrangularis* on rat hepatic CYP1A2 activity. Rats were administered orally with1 ml/kg/day of distilled water (Control group), 0.03 and 0.3 g/kg/day of the dried-stem powder of *C. quadrangularis* (CQ-group I & CQ-group II, respectively) for 30 days. Liver microsomes were determined for MROD activity. The individual mark represented mean of MROD activity with an error bar of standard error of the mean (n=10). One-way ANOVA and Student-Newman-Keuls test were used for statistical comparisons at a significant level of $p<0.05$.

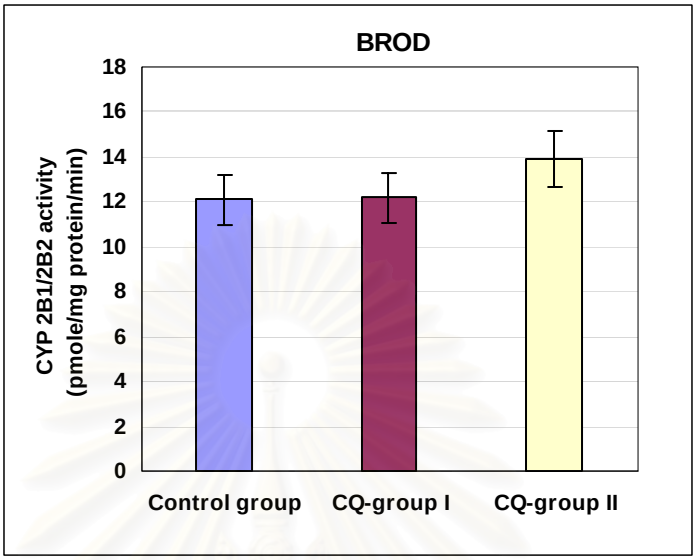


Figure 4.10 Effects of the dried-stem powder of *C. quadrangularis* on rat hepatic CYP 2B1/2B2 activity. Rats were administered orally with 1 ml/kg/day of distilled water (Control group), 0.03 and 0.3 g/kg/day of the dried-stem powder of *C. quadrangularis* (CQ-group I & CQ-group II, respectively) for 30 days. Liver microsomes were determined for BROD activity. The individual mark represented mean of BROD activity with an error bar of standard error of the mean (n=10). One-way ANOVA and Student-Newman-Keuls test were used for statistical comparisons at a significant level of $p<0.05$.

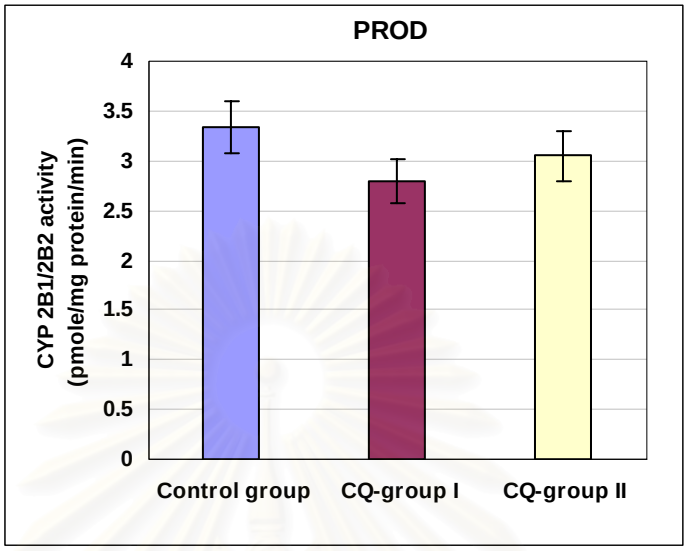


Figure 4.11 Effects of the dried-stem powder of *C. quadrangularis* on rat hepatic CYP 2B1/2B2 activity. Rats were administered orally with 1 ml/kg/day of distilled water (Control group), 0.03 and 0.3 g/kg/day of the dried-stem powder of *C. quadrangularis* (CQ-group I & CQ-group II, respectively) for 30 days. Liver microsomes were determined for PROD activity. The individual mark represented mean of PROD activity with an error bar of standard error of the mean (n=10). One-way ANOVA and Student-Newman-Keuls test were used for statistical comparisons at a significant level of $p<0.05$.

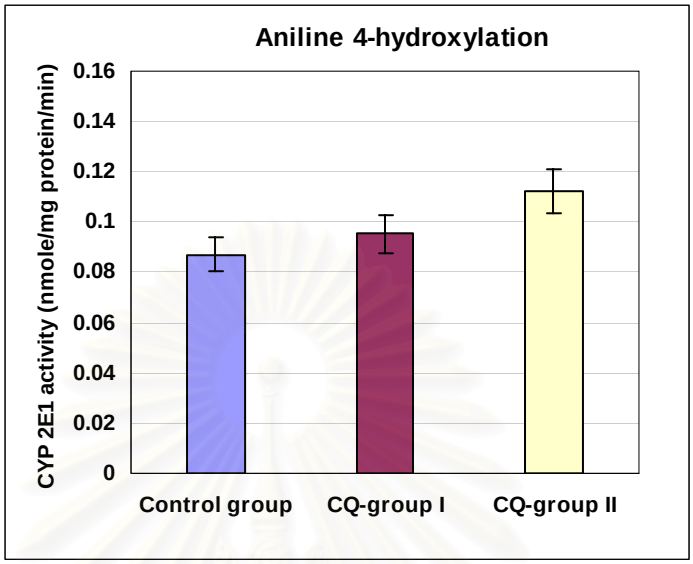


Figure 4.12 Effects of the dried-stem powder of *C. quadrangularis* on rat hepatic CYP 2E1 activity. Rats were administered orally with 1 ml/kg/day of distilled water (Control group), 0.03 and 0.3 g/kg/day of the dried-stem powder of *C. quadrangularis* (CQ-group I & CQ-group II, respectively) for 30 days. Liver microsomes were determined for aniline-4-hydroxylase activity. The individual mark represented mean of aniline-4-hydroxylase activity with an error bar of standard error of the mean (n=10). One-way ANOVA and Student-Newman-Keuls test were used for statistical comparisons at a significant level of $p<0.05$.

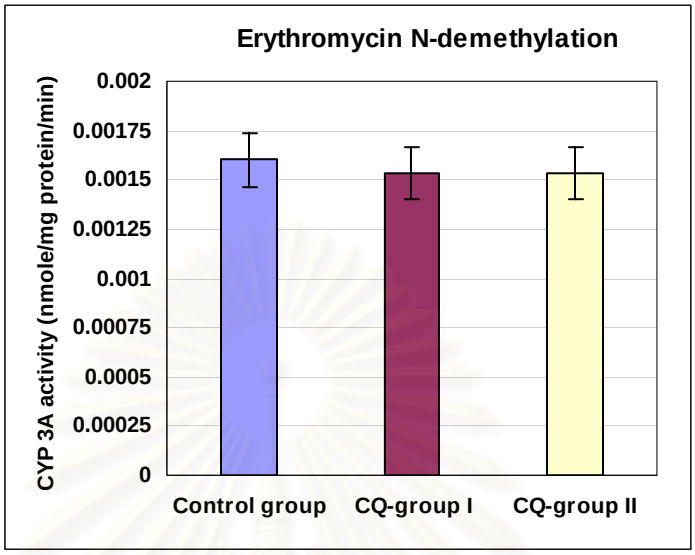


Figure 4.13 Effects of the dried-stem powder of *C. quadrangularis* on rat hepatic CYP 3A activity. Rats were administered orally with 1 ml/kg/day of distilled water (Control group), 0.03 and 0.3 g/kg/day of the dried-stem powder of *C. quadrangularis* (CQ-group I & CQ-group II, respectively) for 30 days. Liver microsomes were determined for erythromycin N-demethylase activity. The individual mark represented mean of erythromycin N-demethylase activity with an error bar of standard error of the mean (n=10). One-way ANOVA and Student-Newman-Keuls test were used for statistical comparisons at a significant level of $p<0.05$.

Table 4.2 Effects of the dried-stem powder of *C. quadrangularis* on hematology and clinical blood chemistry

Hematology	Control group	*C. quadrangularis* group I	*C. quadrangularis* group II
Hemoglobin (g/dl)	16.47 ±0.21	16.35 ±0.21	16.32 ±0.37
Hematocrit (%)	51.77 ±0.59	51.66 ±0.76	52.00 ±1.28
MCV (fL)	57.31 ± 0.37	57.73 ±0.41	58.06 ± 0.31
MCH (pg)	18.06 ± 0.16	18.26 ± 0.19	18.29 ± 0.09
MCHC (g/dl)	31.71 ± 0.28	31.66 ± 0.32	31.54 ± 0.26
WBC count (cell/cumm)	1424 ±178.04	1488 ±179.69	1559.50 ±204.90
RBC count (10^6 cells/cumm)	9.06 ±0.12	8.95 ±0.11	8.92 ±0.21
PMN (%)	28.22 ±4.73	32.44 ±4.68	31.37 ±5.69
Lymphocyte (%)	70.55 ±4.66	66.11 ±4.77	67.00 ±6.24
Monocyte (%)	1.50 ±0.50	2.66 ±0.66	3.00 ±0.57
Eosinophil (%)	2.00 ±0.40	2.5 ±0.50	1.33 ±0.33
Basophil (%)	0	0	0
RBC morphology	Normal	Normal	Normal
Platelet count (cells/cumm)	757888.90 ±37914.91	732777.8 ±42846.55	649625 ±89193.20
Clinical blood chemistry	**Control group**	***C. quadrangularis* group I**	***C. quadrangularis* group II**
Glucose (mg/dl)	112.8 ±7.23	105.30 ±2.79	104.80 ±4.44
Total cholesterol (mg/dl)	69.77 ±4.14	65.90 ±3.88	69.10 ±2.44
Triglyceride (mg/dl)	95.77 ±9.75	115.80 ±11.76	123.4 ±9.45
LDL-C (mg/dl)	6.77 ±1.02	7.66 ±2.48	5.88 ±0.85
HDL-C (mg/dl)	32.57 ±2.03	32.45 ±1.81	31.51 ±1.76
BUN (mg/dl)	20.31 ±1.31	20.87 ±1.47	20.88 ±1.30
SCr (mg/dl)	0.60 ±0.03	0.62 ±0.01	0.63 ±0.04
Uric acid (mg/dl)	2.01 ±0.09	1.96 ±0.11	2.35 ±0.36

Table 4.2 (continued) Effects of the dried-stem powder of *C. quadrangularis* on hematology and clinical blood chemistry

Clinical blood chemistry	Control group	*C. quadrangularis* group I	*C. quadrangularis* group II
Total Bilirubin (mg/dl)	0.14±0.08	0.20 ±0.04	0.28 ±0.09
Direct Bilirubin (mg/dl)	0.06 ±0.02	0.11 ±0.03	0.21 ±0.08
SGOT (U/l)	105.44 ±6.78	119.30 ±14.81	115.70 ±15.59
SGPT (U/l)	24.22 ±2.24	28.70 ±2.56	26.60 ±1.64
ALP (U/l)	116.33 ±10.65	123.70 ±12.15	120.20 ±10.72
Sodium (mEq/L)	144.89±1.22	146.60 ±3.08	147.00 ±1.73
Potassium (mEq/L)	4.31 ±0.10	4.97 ±0.20	5.28 ±0.40
Chloride (mEq/L)	105.33 ±1.06	107.80 ±2.28	106.60 ±0.87

Data shown were mean ± SEM.

CHAPTER V

DISCUSSION AND CONCLUSION

This study primarily investigated effects of the dried-stem powder of *C. quadrangularis* on hepatic CYPs involving in metabolic activations of various mutagenic and/or carcinogenic xenobiotics. This would be partly give a preliminary information of *C. quadrangularis* potential either to afford antimutagenic/anticarcinogenic effects against xenobiotic-induced carcinogenesis or in the other hand, to increase risk of xenobiotic-induced mutagenesis/carcinogenesis. Drug-drug interaction potential of *C. quadrangularis* would be indicated as well if this plant is taken concomittantly with other medicines which are metaboliized by the modulated enzymes. The study was performed in male Wistar rats which were given the dried-stem powder of *C. quadrangularis* orally at dosages of 0.03 and 0.3 g/kg/day, respectively, for 30 consecutive days. *C. quadrangularis* is traditionally used for a wide range of indications, especially for hemorrhoidal treatment which repeated administration of this plant is required. Long term exposure of *C. quadrangularis* may affect CYPs, the important phase I hepatic drug metabolizing enzymes, especially CYP isoforms that play an important role in chemical-induced toxicity, mutagenesis and/or carcinogenesis include CYPs 1A1, 1A2, 2B1/2B2, 2E1 and 3A4 (Soucek and Gut, 1992).

In this study, *C. quadrangularis* was administered to the animals in the dosage form of crude dried-stem powder since this dosage form is expediently and practically used among hemorrhoidal patients and generally recommended in primary health care in Thailand (นันทวัน บุณยปภัศร์, 2542). The ground dried-stem powder of this plant was sieved to yield fine powder before giving to the animals so as to yield *C. quadrangularis* powder of homogeneous particle size which facilitated suspending in water, feeding to the animals and optimized the absorption (Attawish et al., 2002). Before using this plant powder in the experiment, the powder was identified via a chemical identification recommened in a previous study (ธัญวรัตน์ จันทรชนะ และ พงศธร หลิมศิริวงษ์, 2543). In that study, the dried-stem powder of *C. quadrangularis* was extracted with petroleum ether and TLC-densitometer was used to determine the chromatographic fingerprint of the tested powder. Following the same procedure, the chromatographic fingerprint of the

dried-stem powder of *C. quadrangularis* used in this study was approximately conformed the chromatogram reported earlier. Some minor difference of the chromatographic fingerprint pattern was probably due to a somewhat variation in constituent from the difference of cultivated location, time of harvesting, etc. In this study, rats were given the dried-stem powder of *C. quadrangularis* orally at dosages of 0.03 and 0.3 g/kg/day, respectively, for 30 consecutive days. The dosage of 0.03 g/kg/day of *C. quadrangularis* powder used in this experiment was approximately equivalent to recommended therapeutic dose for hemorrhoid treatment in human (1.5 g/50-kg person/day which was approximately 0.03 g/kg/day). Thus, the dosage of 0.3 g/kg/day was approximately equivalent to 10 folds of human therapeutic dose (ดวงรัตน์ เชี่ยวชาญ-วิทย์ และคณะ, 2545 ; Attawish A. et al., 2002). The 30-day experimental period was a sufficient duration time to induce hepatic drug metabolizing enzymes. This duration time provided an additional toxicity data for subacute toxicity of *C. quadrangularis* as well (Soucek and Gut, 1992).

To investigate effects of the dried-stem powder of *C. quadrangularis* on hepatic microsomal CYPs, the oxidation rates of CYPs on their individual specific substrates were determined to represent the corresponding CYP activity in hepatic microsomes of rats treated with the dried-stem powder of *C. quadrangularis*. Alkoxyresorufin O-dealkylations were used to determine activities of CYP 1A1 (Burke and Mayer, 1974), CYP1A2 (Burke, et al., 1985), and CYP 2B1/2B2 (Burke, et al., 1985; Lubet, et al., 1985) using ER, MR and PR & BR respectively, as specific substrates. Aniline 4-hydroxylation was demonstrated to represent CYP 2E1 activity (Schenkman, et al., 1967), while erythromycin N-demethylation was classically used for determining CYP3A activity (Nash, 1953).

This was the first report for effects of the dried-stem powder of *C. quadrangularis* on hepatic CYPs. Results from this study showed that both doses of the dried stem powder of *C. quadrangularis* did not cause any significant changes of hepatic microsomal total CYP contents as well as the activities of hepatic microsomal CYP1A1, 1A2, 2B1/2B2, 2E1 and 3A. No effects of *C. quadrangularis* was demonstrated on these CYP isoforms which are involved in metabolic activation of various mutagenic and/or carcinogenic xenobiotics. Since *C. quadrangularis* did not exhibit induction effects on these CYPs, a potential increase risks of mutagenesis and/or carcinogenesis from

xenobiotics that are bioactivated by these CYPs would not be expected. Examples of xenobiotics which are bioactivated by the individual CYP isoform are as following; benzo(a)pyrene, 7,12-dimethylbenz(a)anthracene, 2-naphthylamine, 2-acetylfluorene, *N*-methyl-4-aminoazobenzene and aflatoxin B_1 are activated by CYP 1A1; 2-amino-3,4,8-trimethylimidazo[4,5-f]quinoxaline and 2-acetylfluorene are activated by CYP 1A2; benzo(a)pyrene, aflatoxin B_1, aminoanthracene, 4,4'-(bis)methylene chloroaniline and *N*,*N'*-nitrosodimethylamine are activated by CYP 2B1&2B2; *N*,*N'*-nitrosodimethylamine, *N*-nitroso-*N*-benzyl-*N*-methylamine and *N*-nitroso-*N*-diethylamine are activated by CYP 2E1; aflatoxin B_1, benzo(a)pyrene, 6-aminochrysene, 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline and tris(2,3-dibromopropyl)-phosphate are activated by CYP 3A (Soucek and Gut, 1992). No modulation of *C. quadrangularis* on these isoforms of CYP that are involved in metabolism of several therapeutic drugs was an advantageous characteristics of *C. quadrangularis* in an aspect that drug-drug interaction would not be expected if *C. quadrangularis* is taken concomitantly with those medicines.

The dried-stem powder of *C. quadrangularis* at doses given in this study did not produce any toxic effects on several important organs/systems such as liver, kidney, blood system, electrolytes as well as lipid and carbohydrate metabolisms as indicated by the results of hematology and clinical blood chemistry parameters. No effects of growth rate, relative liver weight, food and water consumption were also found in the *C. quadrangularis*-treated groups as compared to the control group. This result was consistent to the study reported earlier (จักรพงษ์ ลิมปนุสสรณ์ และคณะ, 2543). In that study, they performed a subacute toxicity test in rats given oral daily dosages (50, 350 and 2,450 mg/kg/day) of the dried-stem powder of *C. quadrangularis*. The result from that study demonstrated that all groups of treated animals showed no significant changes in body & organ weights, food consumption, clinical blood chemistry, hematology and organ histopathology, except only in the highest dose group of which the animals exhibited significant changes of some parameters of clinical blood chemistry and hematology including decrease of serum creatinine, serum albumin and total white blood cell count. However, level of these parameters were returned to normal levels after this powder was withdrawn for 14 days.

Benefit gain from this study was a preliminary safety profile data of the dried-stem powder of *C. quadrangularis* in the aspects of hepatic CYPs modulation and drug-drug

interactions. No effects of *C. quadrangularis* on the CYP isoforms involved in xenobiotic bioactivations as well as drug metabolisms excluded the possibility of *C. quadrangularis* to increase and/or decrease risks of chemical-induced toxicities, mutagenicities and/or carcinogenicities as well as the possibility of drug-drug interactions. Furthermore, clinical blood chemistry and hematology data confirm their safety for long term uses at the doses recommended in human consistency to the previous toxicity data in rats (จักรพงษ์ ลิมป-นุสสรณ์., 2543). However, species variation in drug metabolism should be concerned when extrapolating pharmacology and toxicity data of animals to humans.

In conclusion, effects of the dried-stem powder of *C. quadrangularis* on hepatic CYPs and clinical blood chemistry and hematology were investigated in male Wistar rats. Two doses (0.03 and 0.3 g/kg/day) of the powder were given orally to rats for 30 consecutive days compared to the control group given distilled water in the same manner. The results showed that *C. quadrangularis* did not produce any effects on total CYP contents, and the activities of CYP1A1, CYP1A2, CYP2B1/2B1, CYP2E1 and CYP 3A. In addition, *C. quadrangularis* of both doses used in this study did not cause any harmful effects on several important organs/systems in rats.

Further studies on these following topics were suggested to be explored

- *In vitro* study regarding effects of the dried-stem powder of *C. quadrangularis* on hepatic CYPs.
- Effects of the dried-stem powder of *C. quadrangularis* on human hepatic CYPs, human clinical blood chemistry and hematology.
- Effects of the dried-stem powder of *C. quadrangularis* on other isoforms of hepatic CYP as well as Phase II drug metabolizing enzymes that might contribute to the cancer preventive effects of this plant.

References

Thai

กฤติยา อินทรเผือก. การศึกษาความเป็นพิษเฉียบพลันของสมุนไพรเพชรสังฆาตในหนูแรท. การวิจัยพัฒนาขั้นก่อนคลินิกของยาเม็ดจากสมุนไพรเพชรสังฆาตเพื่อใช้รักษาริดสีดวงทวาร. กรุงเทพมหานคร: สถาบันวิจัยวิทยาศาสตร์และเทคโนโลยีแห่งประเทศไทย, 2543.

จักรพงษ์ ลิมปนุสสรณ์, สมลักษณ์ พวงชมพู, กฤติยา ทิสยากร, ธีระยุทธ แก้วอมตวงศ์, นภดล พิฬารัตน์, พรพิมล มีใย, วิชุดา จันทร์ข้างแรม, ฉันทนา พูนศิริ และ เตือนตา เสมาทอง. การศึกษาความเป็นพิษของสมุนไพรเพชรสังฆาต (*Cissus quadrangularis* L.). เอกสารประกอบการสัมมนาเรื่องแนว ทางการพัฒนาสมุนไพรประเทศไทย. กรุงเทพมหานคร: สำนักงานคณะกรรมการวิจัยแห่งชาติ, 2543.

จักรพงษ์ ลิมปนุสสรณ์. การศึกษาความเป็นพิษเฉียบพลันของสมุนไพรเพชรสังฆาตในหนูถีบจักร. รายงานผลการศึกษาโครงการประเมินประสิทธิภาพและความปลอดภัยของยาสมุนไพร. บพิตร กลางกัลป์ยา และ นงลักษณ์ สุขวาณิชย์ศิลป์, บรรณาธิการ นนทบุรี: บริษัท เอส อาร์ พริ้นติ้ง แมสโปรดักส์ จำกัด, 2544.

ดวงรัตน์ เชี่ยวชาญวิทย์ และคณะ. เพชรสังฆาตกับการรักษาโรคริดสีดวงทวาร. วารสารองค์การเภสัชกรรม (ตุลาคม 2544-มีนาคม 2545): 17-22.

ธัญวรัตน์ จันทรชนะ และพงศธร หลิมศิริวงษ์.การศึกษาทางเคมีเพื่อควบคุมคุณภาพเบื้องต้นของวัตถุดิบและผลิตภัณฑ์ยาจากสมุนไพรเพชรสังฆาต.การวิจัยพัฒนาก่อนคลินิคของยาเม็ดจากสมุนไพรเพชรสังฆาตเพื่อใช้รักษาริดสีดวงทวาร.กรุงเทพมหานคร: สถาบันวิจัยวิทยาศาสตร์และเทคโนโลยีแห่งประเทศไทย

นันทวัน บุณยปภัศร์ และอรนุช โชคชัยเจริญพร. สมุนไพรพื้นบ้าน(3). กรุงเทพมหานคร: บริษัทประชาชนจำกัด, 2542.

พักตร์พริ้ง แสงดี และ สรศักดิ์ เหลี่ยวไชยพันธุ์. การทดสอบความเป็นพิษเฉียบพลันของสมุนไพรเพชรสังฆาต. รายงานผลการศึกษาโครงการประเมินประสิทธิภาพและความปลอดภัยของยาสมุนไพร. บพิตร กลางกัลป์ยา และ นงลักษณ์ สุขวาณิชย์ศิลป์, บรรณาธิการ นนทบุรี: บริษัท เอส อาร์ พริ้นติ้ง แมสโปรดักส์ จำกัด, 2544.

สมนึก สุชัยธนาวนิช. การศึกษาผลทางคลินิกของยาเพชรสังฆาต. รายงานผลการศึกษาโครงการประเมินประสิทธิภาพและความปลอดภัยของยาสมุนไพร. บพิตร กลางกัลป์ยา และ นงลักษณ์ สุขวาณิชย์ศิลป์, บรรณาธิการ นนทบุรี: บริษัท เอส อาร์ พริ้นติ้ง แมสโปรดักส์ จำกัด, 2544.

สถาบันวิจัยวิทยาศาสตร์และเทคโนโลยีแห่งประเทศไทย. โครงการวิจัยที่ อ.-น. 42-01 / รายงานฉบับที่1 เรื่อง การวิจัยพัฒนาขั้นก่อนคลินิกของยาเม็ดจากสมุนไพรเพชรสังฆาตเพื่อใช้ในการรักษาโรคริดสีดวงทวาร. 2543.

สรศักดิ์ เหลี่ยวไชยพันธุ์. การศึกษาเภสัชเวทของต้นเพชรสังฆาต. คณะเภสัชศาสตร์ มหาวิทยาลัยเชียงใหม่, 2530.

สุภาภรณ์ ปิติพร. สมุนไพรอภัยภูเบศร สืบสานภูมิปัญญาไทย. กรุงเทพมหานคร: บริษัทรีเซิร์ช ดีเวลอปเมนต์ โปรโมชั่น อินสะติติ๊ว จำกัด, 2544.

English

Adesanya, S.A.; Martin, M.T.; Boukamcha, N.; Montagnac, A.; and Paris, M. Stilbene derivatives from *Cissus quadrangularis* Linn. J. Nat. Prod. 62 (1999): 1694-1695.

Alzoreky, N.S., and Nakahara, K. Antibacterial activity of extracts from some edible plants commonly consumed in Asia. International Journal of food Microbiology 80 (2003): 223-230.

Attawish, A.; Chavalittumrong, P.; Chaivapat, S.; Chuthaputti, A.; Rattanajarasroj, S.; and Punyamong, S. Subchronic toxicity of *Cissus quadrangularis* Linn. Sonklanakarin J. Sci. Technol. 24 (2002): 39-51.

Balachandran, B.; Sivaswamy, S.N.; and Sivaramakrishnan, V.M. Genotoxic effects of some foods and food components in swiss mice. Indian J. Med. Res. 94 (1991): 378-383.

Bhakuni, O.S.; Dhar, M.L.; Dhar, M.M.; Dhawan, B.N.; and Mehrotra, B.N. Screening of Indian plants for biological activity, part II. Indian J. Exp. Biol. 7 (1969): 594-606.

Burke, M.D., and Mayer, R.T. Ethoxyresorufin: Direct fluorometric assay of microsomal o-dealkylation which is preferentially induced by 3-methylcholanthrene. Drug Metab. Dispos. 2 (1974): 583-588.

Bhutani, K.K.; Kapoor, R.; and Atal, C.K. Two unsymmetric tetracyclic triterpenoids from Cissus quadrangularis Linn. Phytochemistry 23 (1984): 407-410.

Chopra, S.S.; Patel, M.R.; and Aawadhiya, R.P. Studies on *Cissus quadrangularis* in experimental fracture repair : a histopathological study. Indian J. Med. Res. 64 (1976): 1365-1368.

Deka, D.K.; Lahon, L.C.; Saikia, J.; and Mukit, A. Effect of *Cissus quadrangularis* in accelerating healing process of experimentally fractured radius-ulna of dog : A Preliminary study. Indian Journal of Pharmacology 26 (1994): 44-45.

Dhawan, D.N.; Duby, M.P.; Mehrotra,B.N.; Rastogi, R.P.; and Tendon, J.S. Screening of Indian plants for biological activity, IX. Indian J. Exp. Biol. 18(1980): 594-606.

Elizabeth M Williamson. Major herbs of Ayurveda. Canada: Churchil Livingstone, 2002.

Friedli, G.L. Structure/ activity comparison in the ability of some terpenoid food flavors to cause peroxosome proliferation. Master's thesis, School of Biological Sciences, University of Surrey, 1992.

Gibson, G., and Skett, P. Enzymology and molecular mechanisms of drug metabolism reactions. In Drug metabolism. 3rd ed. pp. 35-76. New York: Chapman, 2000.

Gonzalez, F.J., and Gelboin, H.V. Role of human cytochrome P-450 in the metabolic activation of chemical carcinogens and toxins. Drug Metab. Rev. 26 (1994): 165-183.

Guengerich, F.P. Reactions and significance of cytochrome P-450 enzymes. J. Biol. Chem. 266 (1991): 10019-10022.

Guengerich, F.P. Human cytochrome P-450 enzymes. Life Science 50 (1992): 1471-1478.

Guengerich, F.P. Cytochrome P450 3A4: regulation and role in drug metabolism. Annu. Rev. Pharmacol. Toxicol. 39 (1999): 1-17.

Gupta, M.M., and Verma, R.K. Unsymmetric tetracyclic triterpenoid from *Cissus quadrangularis* Linn. Phytochemistry 29 (1990): 336-337.

Gupta, M.M., and Verma, R.K.,"Lipid constituents of *Cissus quadrangularis* Linn. Phytochemistry 30 (1991): 875-878.

Ioannides, C.,ed. Cytochromes P450: Metabolic and toxicological aspect. 1st ed. pp.29-50. Florida, USA: CRC Press, 1996.

Kirtikar, K.R., and Basu, B.D. Indian Medicinal Plants. Vol.1. India: Lalit Mohan Basu, 1989.

Lin, J.; Opoku, A.R.; Geheep-Keller, M.; Hutching, A.D.; Terblanche, S.E.; Jager, A.K.; and Staden, J.V. Preliminary screening of some traditional Zulu medicinal plants for anti-inflammatory and anti-microbial activities. J. Ethnopharmacol. 68 (1999): 267-274.

Liska, D.J. The Detoxification Enzyme Systems. Alternative Medicine Review 3 (1998): 187-198.

Lowry, O.H.; Rosebrough, N.J.; Farr, A.L.; and Randall, R.J. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193 (1951): 265-275.

Lubet, R.A.; Mayer, R.T.; Cameron, J.W.; Nim, R.W.; Burke, M.D.; Wolff, T.; and Guengerich, F.P. Dealkylation of pentoxyresorufin: a rapid and sensitive assay for measuring induction of cytochrome(s) P-450 by phenobarbital and other xenobiotics in the rat. Arch. Biochem. Biophys. 238 (1985): 43-48.

Mehta, M.; Aur, N.; and Bhutani, K.K. Determination of marker constituents from *Cissus quadrangularis* Linn. And their quantitation by HPTLC and HPLC. Phytochem Anal. 12 (2001): 91-95.

Murthy, C.; Vanitha, A.; Swamy, M.; and Ravishankar, G.A. Antioxidant and antimicrobial activity of *Cissus quadrangularis* L. J. Med food. 6 (2003): 99- 105.

Nash, T. The colormetric estimation of formaldehyde by means of Hantzsch Reaction. Biochem. J . 55 (1953): 416-421.

Omiecinski, C.J.; Remmel, R.P.; and Hosagrahara, V.P. Concise Review of the Cytochrome P450s and their Roles in Toxicology. Toxicological Sciences 48 (1999): 151-156.

Omura, T., and Sato, R. The carbonmonoxide-binding pigment of liver microsomes I.Evidence for its hemoprotein nature. J. Biol. Chem. 239 (1964): 2370-2378.

Opoku, A.R.; Geheep-Keller, M.; Lin, J.; Terblanche, S.E.; Hutching, A.D.; Huturgoon, A.; and Pallay, G. Preliminary screening of some traditional Zulu medicinal plants for antineoplastic activity versus HEG_2 Cell line. Phytother. Res. 14(2000): 534-537.

Parkinson, A. Biotransformation of xenobiotics. In C.D. Klaassen, (ed.), Casarett & Doull's toxicology: The basic science of poisons. 6th ed. pp.133-218. USA: McGraw-Hill, 2001.

Pluemjai, T., and Saifah, E. Constituents of *Cissus quadrangularis* Linn. Thai J. Pharm. Sci. 11 (1986): 205-211.

Rendic, S., and Di Carlo, F.J. Human cytochrome P450 enzymes: Status report summarizing their reactions, substrates, inducers, and inhibitors. Drug Metab. Rev. 29 (1997): 413-580.

Schenkman, J.B.; Remmer, H.; and Estabrook, R.W. Spectral studies of drug interactions with hepatic microsomal cytochrome P-450. Mol. Pharmacol. 3 (1967): 113-123.

Sen, S.P. Active constituents (oxo steroids) of Cissus quadrangularis Linn. Indian J. Pharmacy 9 (1964): 247-248.

Shirwaikar, A.; Khan, S.; Malini, S. Antiosteoporotic effect of ethanol extract of *Cissus quadrangularis* Linn. on ovariectomized rat. Journal of Ethnopharmacology 89 (2003): 245-250.

Singh, S.P.; Misra, N.; Dixit, K.S.; Singh, N.; and Kohli, R.P. An experimental study of analgesic activity of *Cissus quadrangularis*. Indian Journal of Pharmacology (1984): 162-163.

Sivaswamy, S.N.; Balachandran, B.; Balanehru, S.; and Sivaramakrishnan, V.M. Mutagenic activity of south Indian food items. Indian J. Exp. Biol. 29 (1991): 730-737.

Sivarajan, V.V., and Balachandran, I. Ayuravedic drugs and their plant sources. India: International Science Publisher, 1994.

Soucek, P., and Gut, I. Cytochrome P-450 in rats: structures, functions, properties and relevant human forms. Xenobiotica 22 (1992): 83-103.

Tanaka, E.; Terada, M.; and Misawa, S. Cytochrome P450 2E: its clinical and toxicological role. J. Clin. Pharmacol. Ther. 39 (1999): 165-175.

Tiangburanathum, W. Dictionary of Thai medicinal plants. Thailand: Prachumthong Printing, 1996.

Tredger, J.M., and Stoll, S. Cytochrome P450- their impact on drug treatment. Hospital Pharmacist 9 (2002): 167-173.

Thisayakorn, K.; Thisayakorn, C.; and Soonthorntanasart, T. Preliminary Anti-inflammatory study of *Cissus quadrangularis* L. in rats. Thai J. Pharm Sci. 24(suppl.) (2000): 26-31.

Tukey, R.H., and Strassburg, C.P. Human UDP-Glucuronosyltransferases: Metabolism, Expression, and Disease. Annu. Rev. Pharmacol. Toxicol. 40 (2000): 581-616.

Udapa, K.N., and Prasad, G. Biochemical and 45-Ca studies on the effect of *Cissus quadrangularis* in fracture repair. Indian J. Med. Res. 52 (1964): 480-487.

Yan, Z., and Caldwell, G.W. Metabolism Profiling and Cytochrome P450 Inhibition and Induction in Drug Discovery. CCurrent Topics in Medicinal Chemistry 1 (2001): 403-425.

APPENDICES

Appendix A

Body weight, liver weight, food and water consumption

Table A1 Weekly body weight of individual rat

Rat No.	Day 0	Day 7	Day 14	Day 21	Day 28
control					
1	288.8	324.8	358.5	373.8	385.9
2	312.4	342.5	361.9	378.5	397
3	313.3	368	403.8	423.9	424.7
4	335.6	383.7	402.3	413	419.4
5	352.2	372.3	374.4	397.3	401.8
6	328.9	339.8	355.3	362.7	366
7	392.5	402	425.4	444.6	467.8
8	415.5	431.7	460.7	466.9	478.3
9	399.2	415	393.7	426.1	443.5
10	419.9	445.3	431.2	459.5	468.3
CQ-group I					
1	330.6	377.7	395.4	413.2	442.3
2	348.4	376.5	389.2	410.8	431.6
3	317.2	341.5	355.6	373	382.2
4	297.1	364.3	383.3	389.2	417.4
5	383.9	393.3	400.1	419.5	424.9
6	367.5	384.3	404.6	407	436.6
7	410	433.2	438.9	437.1	448.8
8	355	363.7	367.3	375.4	375
9	345.2	362.6	365.1	386.3	393.5
10	438.7	460.4	468.9	499.3	508.8
CQ-group II					
1	318.6	360.7	378.2	399.3	414.7
2	328.4	351.5	364.7	386.7	405.7
3	355.5	374.5	394.2	401.5	413.5
4	389.2	395.4	410.9	429.1	441.1
5	353	359.8	353	391.3	399.4
6	366.5	383.8	391.7	378.3	402.2
7	423	430.1	432.5	432.4	447.8
8	366.5	377.1	376	388.1	396.8
9	361.7	392.2	402.6	420	430.5
10	372.7	377.9	372.4	387.6	392.5

Unit expressed as g

Table A2 Food consumption of individual rat

Rat No.	Day 7	Day 14	Day 21	Day 28
Control				
1	13	18	22	20
2	20	19	19	18
3	43	25	28	23
4	26	24	22	28
5	21	22	10	20
6	20	21	20	20
7	24	23	29	23
8	24	28	23	11
9	15	23	23	21
10	23	28	23	21
CQ-group I				
1	26	30	28	20
2	21	22	21	19
3	26	14	22	23
4	28	18	13	22
5	22	23	22	23
6	22	23	25	21
7	26	22	29	20
8	18	17	18	21
9	32	30	23	25
10	28	30	24	22
CQ-group II				
1	26	30	28	21
2	21	22	21	19
3	26	14	21	23
4	31	26	28	23
5	22	23	22	23
6	22	23	25	21
7	26	22	29	20
8	18	17	18	21
9	32	30	23	25
10	28	30	24	22

Unit expressed as g/day

Table A3 Water consumption of individual rat

Rat No.	Day 7	Day 14	Day 21	Day 28
control				
1	42	43	34	38
2	56	36	35	28
3	63	53	47	51
4	48	49	47	53
5	60	56	47	55
6	62	96	54	55
7	61	34	45	38
8	38	45	38	12
9	71	47	40	38
10	47	39	40	38
CQ-group I				
1	55	60	36	46
2	51	46	38	37
3	51	33	36	26
4	40	23	32	26
5	55	36	37	52
6	49	32	42	35
7	52	37	42	37
8	50	39	52	34
9	50	40	30	32
10	54	47	38	31
CQ-group II				
1	55	60	36	29
2	51	46	38	37
3	51	33	36	26
4	52	51	49	56
5	55	36	37	52
6	49	32	42	35
7	52	37	42	37
8	50	39	52	34
9	50	40	30	32
10	54	47	38	31

Unit expressed as ml/day

Table A4 Terminal body weight of individual rat

Rat No.	Group		
	Control	CQ-group I	CQ-group II
1	380.5	422.2	397.7
2	380.9	441.5	392.3
3	439.7	388.8	420.5
4	409.2	400.8	389.2
5	392.1	406.3	382.6
6	349.4	417.7	385.8
7	446.7	443.6	435.3
8	461.8	364.5	393.8
9	417.2	392.2	415.5
10	457.4	495.0	373.4
Average	413.49	417.26	398.61
SEM	11.93	11.53	6.07

Unit expressed as g

Table A5 Liver weight of individual rat

Rat No.	Group		
	Control	CQ-group I	CQ-group II
1	10.51	10.44	10.54
2	10.36	12.45	13.74
3	16.66	12.40	12.92
4	11.85	11.64	13.21
5	9.71	11.20	11.20
6	10.98	12.65	13.37
7	14.08	12.95	13.31
8	14.00	9.95	13.38
9	13.18	15.21	14.23
10	16.44	15.76	11.90
Average	12.78	12.74	12.78
SEM	0.79	0.59	0.37

Unit expressed as g

Appendix B

Verification of methods for the determination of alkoxyresorufin O-dealkylation, aniline 4-hydroxylation and erythromycin N-demethylation

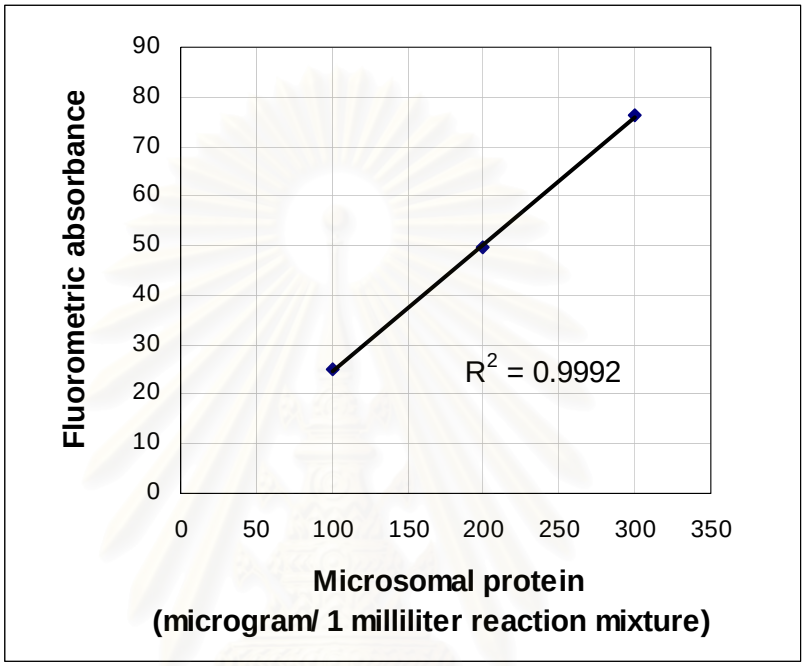


Figure B1 Verification of alkoxyresorufin O-dealkylation. Correlation between amounts of microsomal protein used in the reaction and the corresponding fluorometric absorbances was shown to possess a correlation coefficient (r^2) of 0.9992. Each point was mean of n=2. (Procedure was demonstrated in the Materials and Methods.)

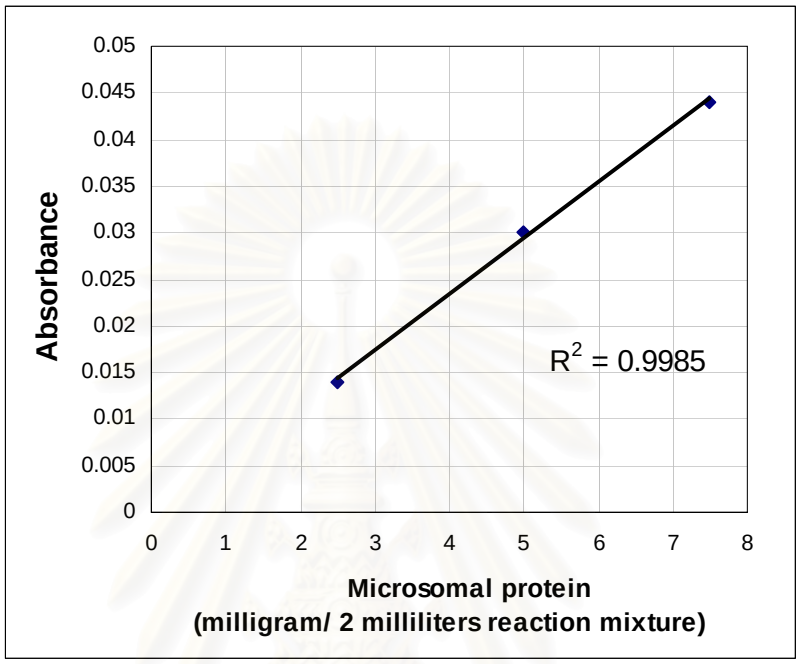


Figure B2 Verification of aniline 4-hydroxylation. Correlation between amounts of microsomal protein used in the reaction and the corresponding fluorometric absorbances was shown to possess a correlation coefficient (r^2) of 0.9985. Each point was mean of n=2. (Procedure was demonstrated in the Materials and Methods.)

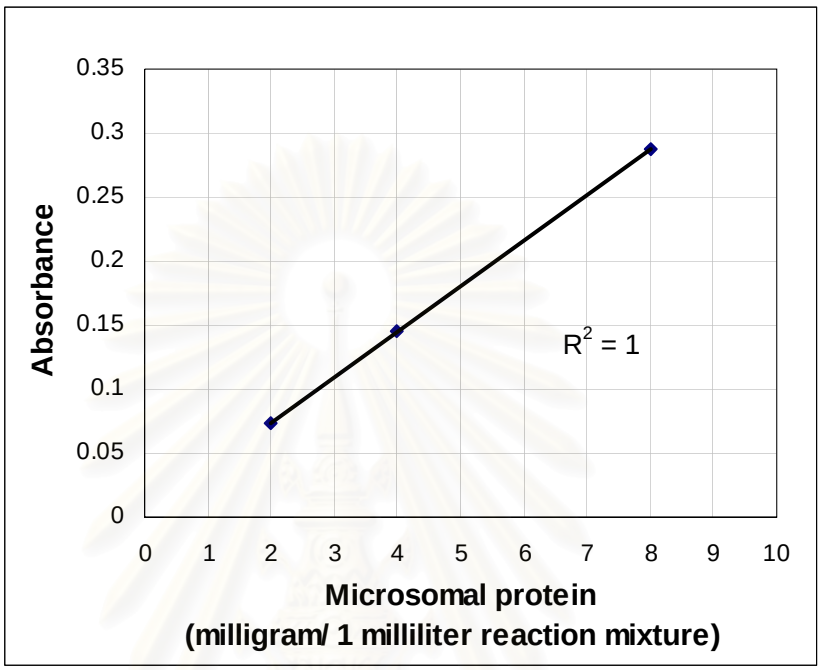


Figure B3 Verification of erythromycin N-demethylation. Correlation between amounts of microsomal protein used in the reaction and the corresponding fluorometric absorbances was shown to possess a correlation coefficient (r^2) of 1. Each point was mean of n=2. (Procedure was demonstrated in the Materials and Methods.)

Appendix C

Enzyme activity study

Table C1 Microsomal protein concentration of individual rat

Rat No.	Group		
	Control	CQ-group I	CQ-group II
1	35.46	17.11	11.31
2	23.95	26.75	32.86
3	40.43	25.23	32.34
4	37.73	18.44	24.27
5	27.47	29.35	23.04
6	28.47	31.14	32.08
7	33.57	31.23	40.26
8	32.27	31.80	28.31
9	33.14	41.39	37.06
10	38.35	38.19	32.93
Average	32.68	29.06	29.45
SEM	1.64	2.42	2.61

Unit expressed as mg/ml

Table C2 Hepatic microsomal total CYP content of individual rat

Rat No.	Group		
	Control	CQ-group I	CQ-group II
1	0.874	0.918	0.973
2	0.830	0.791	0.813
3	0.588	0.758	0.632
4	0.582	0.797	0.857
5	0.703	0.802	0.852
6	0.720	0.819	0.462
7	0.709	0.670	0.626
8	0.643	0.610	0.610
9	0.747	0.516	0.505
10	0.582	0.621	0.703
Average	0.698	0.730	0.703
SEM	0.032	0.038	0.052

Unit expressed as nmol/mg protein

Table C3 Hepatic microsomal EROD activity of individual rat

Rat No.	Group		
	Control	CQ-group I	CQ-group II
1	32	29	48
2	35	32	23
3	16	33	15
4	20	27	21
5	31	23	31
6	37	25	30
7	28	23	19
8	22	17	16
9	32	18	14
10	15	14	24
Average	26.88	24.10	24.31
SEM	2.55	2.00	3.20

Unit expressed as pmol/mg protein/min

Table C4 Hepatic microsomal MROD activity of individual rat

Rat No.	Group		
	Control	CQ-group I	CQ-group II
1	14	9	12
2	12	7	7
3	3	8	6
4	8	11	6
5	10	5	6
6	11	7	9
7	8	7	3
8	4	3	5
9	10	4	3
10	4	4	5
Average	8.46	6.50	6.38
SEM	1.22	0.84	0.89

Unit expressed as pmol/mg protein/min

Table C5 Hepatic microsomal BROD activity of individual rat

Rat No.	Group		
	Control	CQ-group I	CQ-group II
1	6	9	20
2	13	22	12
3	6	13	9
4	7	8	16
5	18	15	13
6	22	14	17
7	15	11	11
8	12	13	12
9	13	6	15
10	8	11	14
Average	12.08	12.16	13.87
SEM	1.68	1.36	0.99

Unit expressed as pmol/mg protein/min

Table C6 Hepatic microsomal PROD activity of individual rat

Rat No.	Group		
	Control	CQ-group I	CQ-group II
1	4	4	4
2	3	4	3
3	1	2	2
4	2	5	3
5	4	3	3
6	5	3	3
7	5	3	3
8	4	2	3
9	4	1	4
10	2	2	3
Average	3.33	2.79	3.05
SEM	0.49	0.43	0.17

Unit expressed as pmol/mg protein/min

Table C7 Hepatic microsomal aniline 4-hydroxylase activity of individual rat

Rat No.	Group		
	Control	CQ-group I	CQ-group II
1	0.095	0.214	0.117
2	0.076	0.123	0.118
3	0.053	0.136	0.027
4	0.106	0.090	0.030
5	0.115	0.045	0.103
6	0.092	0.102	0.126
7	0.105	0.069	0.118
8	0.115	0.108	0.084
9	0.105	0.098	0.103
10	0.093	0.140	0.046
Average	0.087	0.095	0.112
SEM	0.012	0.006	0.015

Unit expressed as nmol/mg protein/min

Table C8 Hepatic microsomal erythromycin N-demethylase activity of individual rat

Rat No.	Group		
	Control	CQ-group I	CQ-group II
1	1.5556	1.6263	1.3019
2	1.6115	1.5899	1.2225
3	1.9925	1.9295	1.6988
4	1.8020	1.4989	1.3812
5	1.0478	1.6021	1.3495
6	1.8972	1.2140	1.6273
7	1.5956	1.4505	1.5321
8	1.4606	1.6081	1.8655
9	1.7464	1.4020	1.8942
10	1.3098	1.3959	1.4732
Average	1.6027	1.5322	1.4725
SEM	0.0891	0.0601	0.0979

Unit expressed as nmol/mg protein/min

Appendix D

Clinical blood chemistry

Table D1 AST concentration of individual rat

Rat No.	Group		
	Control	CQ-group I	CQ-group II
1	-	145	204
2	132	81	71
3	70	85	167
4	104	103	84
5	105	131	110
6	135	113	116
7	109	240	68
8	103	100	96
9	107	105	174
10	84	90	67
Average	105.44	119.30	115.70
SEM	6.78	14.81	15.59

Unit expressed as U/L

Table D2 ALT concentration of individual rat

Rat No.	Group		
	Control	CQ-group I	CQ-group II
1	-	35	38
2	20	23	20
3	23	27	30
4	20	20	27
5	32	27	29
6	36	25	24
7	29	48	21
8	24	22	28
9	17	31	26
10	17	29	23
Average	24.22	28.70	26.60
SEM	2.24	2.56	1.64

Unit expressed as U/L

Table D3 Serum ALP concentration of individual rat

Rat No.	Group		
	Control	CQ-group I	CQ-group II
1	-	105	110
2	101	88	96
3	199	221	195
4	95	121	105
5	99	99	108
6	118	114	153
7	107	127	144
8	101	90	100
9	111	141	110
10	116	131	81
Average	116.33	123.70	120.20
SEM	10.65	12.15	10.72

Unit expressed as U/L

Table D4 Serum total bilirubin concentration of individual rat

Rat No.	Group		
	Control	CQ-group I	CQ-group II
1	-	0.4	0.6
2	0.2	0.1	0.2
3	0.2	0.1	0.4
4	0.2	0.4	< 0.1
5	0.2	0.2	0.2
6	0.2	< 0.1	0.2
7	< 0.1	0.2	< 0.1
8	< 0.1	0.4	< 0.1
9	0.2	0.2	0.9
10	0.1	< 0.1	0.3
Average	0.14	0.20	0.28
SEM	0.08	0.04	0.09

Unit expressed as mg/dl

Table D5 Serum direct bilirubin concentration of individual rat

Rat No.	Group		
	Control	CQ-group I	CQ-group II
1	-	0.2	0.5
2	0	0	0.1
3	0.1	0.1	0.3
4	0.1	0.3	0
5	0.1	0.1	0.1
6	0	0	0
7	0	0	0
8	0	0.1	0
9	0.2	0.3	0.8
10	0.1	0	0.3
Average	0.06	0.11	0.21
SEM	0.02	0.03	0.08

Unit expressed as mg/dl

Table D6 BUN concentration of individual rat

Rat No.	Group		
	Control	CQ-group I	CQ-group II
1	-	18.2	21.6
2	18.6	17.1	20.1
3	29.6	31.8	28.8
4	20	23.6	23.9
5	16.8	15.5	14
6	22.1	19.6	19
7	16.5	21.2	23.7
8	18.1	18.8	17.8
9	20.6	23.9	22.3
10	20.5	19	17.6
Average	20.31	20.87	20.88
SEM	1.31	1.47	1.30

Unit expressed as mg/dl

Table D7 SCr concentration of individual rat

Rat No.	Group		
	Control	CQ-group I	CQ-group II
1	-	0.6	0.6
2	0.5	0.6	0.7
3	0.5	0.6	0.5
4	0.5	0.7	0.9
5	0.5	0.6	0.5
6	0.7	0.7	0.6
7	0.7	0.6	0.5
8	0.6	0.6	0.6
9	0.7	0.6	0.8
10	0.7	0.6	0.6
Average	0.60	0.62	0.63
SEM	0.03	0.01	0.04

Unit expressed as mg/dl

Table D8 Serum total cholesterol concentration of individual rat

Rat No.	Group		
	Control	CQ-group I	CQ-group II
1	-	65	56
2	61	41	68
3	75	72	84
4	92	79	75
5	57	70	61
6	60	68	66
7	58	78	69
8	68	66	67
9	72	72	71
10	85	48	74
Average	69.77	65.90	69.10
SEM	4.14	3.88	2.44

Unit expressed as mg/dl

Table D9 Serum LDL-C concentration of individual rat

Rat No.	Group		
	Control	CQ-group I	CQ-group II
1	-	-	-
2	5	3	3
3	5	6	4
4	14	7	12
5	7	6	5
6	5	4	6
7	5	8	5
8	4	5	5
9	8	27	6
10	8	3	7
Average	6.77	7.66	5.88
SEM	1.02	2.48	0.85

Unit expressed as mg/dl

Table D10 Serum HDL-C concentration of individual rat

Rat No.	Group		
	Control	CQ-group I	CQ-group II
1	-	33.3	24.4
2	27	22.1	31.7
3	41.6	39.8	42.1
4	36.7	37.3	37.5
5	24.2	28.3	25.6
6	31.3	32.1	30.8
7	28.1	38.2	35.6
8	29.6	31.9	29.8
9	34	35.9	26.5
10	40.7	25.6	31.1
Average	32.57	32.45	31.51
SEM	2.03	1.81	1.76

Unit expressed as mg/dl

Table D11 Serum TG concentration of individual rat

Rat No.	Group		
	Control	CQ-group I	CQ-group II
1	-	111	92
2	88	72	178
3	131	203	146
4	53	78	114
5	60	115	127
6	110	121	107
7	83	119	145
8	134	122	109
9	85	83	140
10	118	134	76
Average	95.77	115.80	123.40
SEM	9.75	11.76	9.45

Unit expressed as mg/dl

Table D12 Serum glucose concentration of individual rat

Rat No.	Group		
	Control	CQ-group I	CQ-group II
1	86	95	110
2	122	113	107
3	140	113	137
4	119	100	101
5	105	100	91
6	97	97	103
7	113	105	100
8	88	98	97
9	158	122	87
10	100	110	115
Average	112.80	105.30	104.80
SEM	7.23	2.79	4.44

Unit expressed as mg/dl

Table D13 Serum Uric acid concentration of individual rat

Rat No.	Group		
	Control	CQ-group I	CQ-group II
1	-	1.7	3.1
2	1.8	2	1.5
3	2	1.4	2.7
4	2	2.1	2.4
5	1.9	1.9	1.9
6	1.9	2.4	1.7
7	1.7	2.2	1.3
8	1.9	1.4	1.6
9	2.2	2.5	5.2
10	2.7	2	2.1
Average	2.01	1.96	2.35
SEM	0.09	0.11	0.36

Unit expressed as mg/dl

Table D14 Serum sodium concentration of individual rat

Rat No.	Group		
	Control	CQ-group I	CQ-group II
1	-	148	154
2	143	145	145
3	142	141	141
4	142	131	143
5	143	149	148
6	142	146	144
7	145	142	144
8	148	147	147
9	153	170	159
10	146	147	145
Average	144.89	146.60	147.00
SEM	1.22	3.08	1.73

Unit expressed as mEq/L

Table D15 Serum potassium concentration of individual rat

Rat No.	Group		
	Control	CQ-group I	CQ-group II
1	-	5.7	5.5
2	4.0 HML Tr	4	4.7 Hemolysis trace
3	4.6	4.4	5.2 Hemolysis trace
4	4.8	4.8	4.9
5	4.3	4.9	5
6	4.3	4.3	4.4
7	3.8	5.3	4.2
8	4.1	4.9	4.3
9	4.3	6.1 HML Tr	8.6 HML Tr
10	4.6	5.3	6
Average	4.31	4.97	5.28
SEM	0.10	0.20	0.40

Unit expressed as mEq/L

Table D16 Serum chloride concentration of individual rat

Rat No.	Group		
	Control	CQ-group I	CQ-group II
1	-	111	106
2	104	104	106
3	102	105	105
4	105	98	106
5	103	107	106
6	105	105	104
7	105	108	105
8	107	107	107
9	113	126	114
10	104	107	107
Average	105.33	107.80	106.60
SEM	1.06	2.28	0.87

Unit expressed as mEq/L

Appendix E

Hematology

Table E1 Hb of individual rat

Rat No.	Group		
	Control	CQ-group I	CQ-group II
1	-	-	-
2	16.3	16.4	15.6
3	16.3	15.9	14.1
4	16.3	15.9	-
5	16.5	17.2	17.0
6	17.5	16.9	17.4
7	15.3	15.4	16.8
8	17.0	17.1	16.1
9	17.1	15.7	17.0
10	16.0	16.7	16.6
Average	16.47	16.35	16.3
SEM	0.21	0.21	0.37

Unit expressed as g/dl

Table E2 Hct of individual rat

Rat No.	Group		
	Control	CQ-group I	CQ-group II
1	-	-	-
2	52	52	50
3	51	50	45
4	50	48	-
5	51	54	54
6	53	53	54
7	49	49	53
8	52	52	50
9	55	52	57
10	53	55	53
Average	51.77	51.66	52.00
SEM	0.59	0.76	1.28

Unit expressed as %

Table E3 MCV of individual rat

Rat No.	Group		
	Control	CQ-group I	CQ-group II
1	-	-	-
2	58.1	58.8	58.2
3	55.7	57.4	57.5
4	57.6	56.5	-
5	56.2	56.5	57.0
6	57.7	58.5	57.2
7	57.8	57.0	57.9
8	58.0	58.4	58.1
9	55.9	56.6	59.4
10	58.8	59.9	59.2
Average	57.31	57.73	58.06
SEM	0.37	0.41	0.31

Unit expressed as fL

Table E4 MCH of individual rat

Rat No.	Group		
	Control	CQ-group I	CQ-group II
1	-	-	-
2	18.2	18.3	18.2
3	17.7	18.2	18.2
4	18.7	18.6	-
5	18.1	18.0	18.1
6	18.9	18.6	18.5
7	18.0	18.0	18.4
8	18.8	19.3	18.7
9	17.3	17.2	17.9
10	17.8	18.1	18.3
Average	18.08	18.26	18.29
SEM	0.16	0.19	0.09

Unit expressed as pg

Table E5 MCHC of individual rat

Rat No.	Group		
	Control	CQ-group I	CQ-group II
1	-	-	-
2	31.3	31.2	31.3
3	31.8	31.7	31.7
4	32.5	33.0	-
5	32.2	31.8	31.8
6	32.7	31.9	32.4
7	31.1	31.6	31.8
8	32.5	33.0	32.2
9	31.0	30.4	30.1
10	30.3	30.3	31.0
Average	31.71	31.66	31.54
SEM	0.28	0.32	0.26

Unit expressed as g/dl

Table E6 WBC count of individual rat

Rat No.	Group		
	Control	CQ-group I	CQ-group II
1	-	-	-
2	2,220	1,736	1,640
3	1,710	2,300	934
4	1,854	2,020	-
5	1,350	1,202	2,200
6	1,636	1,586	2,380
7	1,478	1,082	1,422
8	758	1,742	1,948
9	1,328	1,194	878
10	488	532	1,074
Average	1,424	1,488	1,559.50
SEM	178.04	179.69	204.90

Unit expressed as cells/cumm

Table E7 RBC count of individual rat

Rat No.	Group		
	Control	CQ-group I	CQ-group II
1	-	-	-
2	8.92	8.92	8.56
3	9.2	8.76	7.73
4	8.72	8.54	-
5	9.14	9.58	9.4
6	9.24	9.06	9.4
7	8.5	8.52	9.1
8	9.02	8.88	8.64
9	9.86	9.12	9.52
10	8.98	9.2	9.04
Average	9.06	8.95	8.92
SEM	0.12	0.11	0.21

Unit expressed as million cells/cumm

Table E8 Percent differential WBCs of individual rat

Rat No.	% Differential WBCs				
	N	L	Mo	Eo	B
Control					
1	-	-	-	-	-
2	20	79	-	1	-
3	33	67	-	-	-
4	24	74	-	2	-
5	14	83	1	2	-
6	50	50	-	-	-
7	45	52	-	3	-
8	40	60	-	-	-
9	12	88	-	-	-
10	16	82	2	-	-
CQ-group I					
1	-	-	-	-	-
2	37	61	-	2	-
3	30	68	2	-	-
4	32	68	-	-	-
5	24	76	-	-	-
6	27	70	-	3	-
7	52	44	4	-	-
8	55	45	-	-	-
9	10	88	2	-	-
10	25	75	-	-	-
CQ-group II					
1	-	-	-	-	-
2	25	74	-	1	-
3	30	70	-	-	-
4	-	-	-	-	-
5	28	69	2	1	-
6	40	60	-	-	-
7	38	58	4	-	-
8	63	32	3	2	-
9	12	88	-	-	-
10	15	85	-	-	-

Unit expressed as %

N =Neutrophil L =Lymphocyte Mo =Monocyte Eo =Eosinophil B =Basophil

Table E9 RBC morphology of individual rat

Rat No.	Group		
	Control	CQ-group I	CQ-group II
1	-	-	-
2	MICRO 3+	MICRO 3+	MICRO 3+
3	MICRO 2+	MICRO 2+	MICRO 2+
4	MICRO 3+	MICRO 3+	-
5	MICRO 3+	MICRO 3+	MICRO 3+
6	MICRO 2+	MICRO 2+	MICRO 2+
7	MICRO 2+	MICRO 2+	MICRO 2+
8	MICRO 2+	MICRO 2+	MICRO 2+
9	MICRO 3+	MICRO 2+	MICRO 3+
10	MICRO 3+	MICRO 3+	MICRO 3+

Table E10 Platelet count of individual rat

Rat No.	Group		
	Control	CQ-group I	CQ-group II
1	-	-	-
2	719,000	803,000	740,000
3	699,000	582,000	587,000
4	912,000	878,000	-
5	674,000	768,000	684,000
6	712,000	894,000	898,000
7	666,000	566,000	66,000
8	934,000	806,000	758,000
9	852,000	716,000	780,000
10	626,000	582,000	684,000
Average	757,888.90	732,777.8	649,625
SEM	37,914.91	42,846.56	89,193.20

Unit expressed as cells/cumm

VITAE

Mister Khemchat Apipalakul was born in July 11, 1975 in Lopburi, Thailand. He graduated with a Bachelor of Sciences in Biotechnology in 1996 from the Faculty of Sciences, Mahidol University, Bangkok, Thailand.