ผลของบาราคอลเมื่อได้รับแบบกึ่งเรื้อรังต่อเอนไซม์ไซโตโครม พี450 ในตับ และค่าชีวเคมีคลินิกในเลือดของหนูขาวที่ได้รับอาหารปกติ และอาหารคลอเรสเตอรอลสูง

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SUBCHRONIC EFFECTS OF BARAKOL ON HEPATIC CYTOCHROME P450 AND BLOOD CLINICAL BIOCHEMISTRY PARAMETERS IN NORMAL AND HIGH CHOLESTEROL DIET RATS

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บาราคอล เป็นสารประกอบสำคัญที่มีอยู่ในใบอ่อนและดอกของต้นขี้เหล็ก มีชื่อทางวิทยาศาสตร์ว่า *Cassia siamea* Lam. สารนี้ได้ถูกนำมาศึกษาถึงผลระยะกึ่งเรื้อรังต่อเอนไซม์ไซโตโครม พี450 (CYP) ที่เกี่ยวข้องกับการกระตุ้นฤทธิ์ของสารก่อมะเร็งได้แก่ CYP1A1, CYP1A2, CYP2B1/2B2 และ CYP2E1 รวมถึงเอนไซม์กลูตาไธโอน เอส-ทรานสเฟอเรส ที่เกี่ยวข้องกับการทำลายฤทธิ์ของสารก่อมะเร็ง ในหนูขาวที่ได้รับอาหารปกติ และอาหารที่มีคลอเรสเตอรอลสูง นอกจากนี้ยังได้ตรวจค่าชีวเคมีคลินิกและโลหิตวิทยาอีกด้วย การศึกษานี้ใช้หนูขาวเพศผู้พันธุ์วิสตาร์ จำนวน 32 ตัว แบ่งโดยการสุ่มเป็น 4 กลุ่ม กลุ่มแรกและกลุ่มที่สองเป็นกลุ่มควบคุม ที่ได้รับอาหารปกติและอาหารคลอเรสเตอรอลสูง ตามลำดับ กลุ่มที่สามและสี่เป็นกลุ่มที่ได้รับบาราคอลโดยการป้อนทางปาก ในขนาด 30 มก./กก./วัน เป็นเวลา 90 วัน โดยได้รับอาหารปกติ หรืออาหารคลอเรสเตอรอลสูง ตามลำดับ เมื่อครบระยะเวลา ทำให้หนูหมดความรู้สึก เก็บตัวอย่างเลือดจากหัวใจเพื่อตรวจค่าโลหิตวิทยา และแยกซีรั่มเพื่อตรวจค่าชีวเคมีคลินิก นำตับมาเตรียมไมโครโซมและไซโตซอล เพื่อวัดค่าสมรรถนะของเอนไซม์ ผลการทดลองพบว่าบาราคอลมีผลในการลดสมรรถนะของ CYP1A2 อย่างมีนัยสำคัญทั้งในหนูกลุ่มที่ได้รับอาหารปกติและอาหารคลอเรสเตอรอลสูง เช่นเดียวกันกับ CYP1A1 ในหนูกลุ่มที่ได้รับอาหารคลอเรสเตอรอลสูง ผลของบาราคอลต่อสมรรถนะของ CYP2B1/2B2, CYP2E1 และกลูตาไธโอน เอส-ทรานสเฟอเรส พบว่าไม่แตกต่างจากกลุ่มควบคุม การที่บาราคอลมีผลยับยั้ง CYP1A1 และ CYP1A2 อาจมีส่วนใช้อธิบายการที่ใบอ่อนของต้นขี้เหล็กมีผลต่อการยับยั้งการก่อการกลายพันธุ์/การก่อมะเร็งที่เหนี่ยวนำโดยสารเคมี สำหรับค่าชีวเคมีคลินิกพบว่าบาราคอลมีผลลดค่า TG แต่มีผลเพิ่มค่า total และ direct bilirubin อย่างมีนัยสำคัญในหนูกลุ่มที่ได้รับอาหารปกติและอาหารคลอเรสเตอรอลสูง บาราคอลเมื่อให้ร่วมกับอาหารปกติไม่มีผลต่อค่าชีวเคมีคลินิกและโลหิตวิทยาต่าง ๆ ต่อไปนี้คือ SGOT, SGPT, ALP, BUN, SCr, total cholesterol, LDL-C, HDL-C, อัตราส่วนของ LDL-C ต่อ HDL-C, serum glucose, Hb, Hct, platelet count, WBC count และ % differential WBCs หนูกลุ่มที่ได้รับอาหารคลอเรสเตอรอลสูงเมื่อเปรียบเทียบกับกลุ่มที่ได้รับอาหารปกติ พบว่าค่า SGOT, SGPT, ALP, total cholesterol, LDL-C และอัตราส่วนของ LDL-C ต่อ HDL-C สูงขึ้นอย่างมีนัยสำคัญ แต่ไม่พบความเปลี่ยนแปลงของค่า total และ direct bilirubin หนูกลุ่มที่ได้รับบาราคอลร่วมกับอาหารคลอเรสเตอรอลสูงพบว่า ค่า ALP ลดลงอย่างมีนัยสำคัญเมื่อเปรียบเทียบกับหนูกลุ่มที่ได้รับอาหารคลอเรสเตอรอลสูง ผลจากการทดลองนี้แสดงให้เห็นว่าความเป็นพิษต่อตับของบาราคอล น่าจะเกิดขึ้นในลักษณะที่แตกต่างจากการบาดเจ็บของตับจากการได้รับอาหารคลอเรสเตอรอลสูง ควรมีการศึกษาเพิ่มเติมสำหรับผลของบาราคอลในขนาดต่าง ๆ ต่อเอนไซม์ในตับ และค่าชีวเคมีคลินิกในเลือด รวมถึงการศึกษากลไกที่ใช้อธิบายการเกิดพิษต่อตับที่เหนี่ยวนำโดยบาราคอลต่อไป

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RAWIWAN MANIRATANACHOTE: SUBCHRONIC EFFECTS OF BARAKOL ON HEPATIC CYTOCHROME P450 AND BLOOD CLINICAL BIOCHEMISTRY PARAMETERS IN NORMAL AND HIGH CHOLESTEROL DIET RATS. THESIS ADVISOR: ASST. PROF. POL. COL. LT. DR. SOMSONG LAWANPRASERT, THESIS CO-ADVISORS: ASST. PROF. PORNPIMOL KIJSANAYOTIN AND ASST. PROF. DR. LADDAWAL PHIVTHONG-NGAM, 96 pp. ISBN 974-03-1302-7.

Barakol is a major constituent extracted from flowers and young leaves of *Cassia siamea* Lam. This study examined subchronic effects of barakol on phase I hepatic cytochrome P450 (CYP) involving carcinogenic bioactivation such as CYP1A1, CYP1A2, CYP2B1/2B2 and CYP2E1 as well as phase II detoxification enzyme, GST, in rats fed normal or high cholesterol diet. Effects of this compound on blood clinical biochemistry parameters and hematology were also determined. Thirty-two male Wistar rats were randomly divided into 4 treatment groups. Rats in first and second groups were fed normal diet and high cholesterol diet, respectively, serving as control groups. The other two groups of rats were given barakol orally at a dosage of 30 mg/kg/day for 90 days and fed normal or high cholesterol diet. At the end of the treatment period, rats were anesthesized. Blood was collected by heart puncture and serum was prepared for measuring hematology and clinical biochemistry parameters, respectively. Microsomes and cytosols were prepared from livers for enzyme assays. The results showed that barakol significantly decreased CYP1A2 activity in both normal and high cholesterol conditions and significantly inhibited CYP1A1 activity in high cholesterol diet group. No changes of CYP2B1/2B2, CYP2E1 and GST activities were observed. The inhibitory effect of barakol on CYP1A1 and CYP1A2 may partly explain its animutagenic/anticarcinogenic effects of young *C. siamea* leaves on chemical-induced mutagenesis/carcinogenesis. For blood clinical biochemistry parameters, normal and high cholesterol diet rats treated with barakol demonstrated a significant decrease of TG but an increase of total and direct bilirubin comparing to their corresponding diet control groups. Normal diet rats treated with barakol showed no changes of these following parameters: SGOT, SGPT, ALP, BUN, SCr, total cholesterol, LDL-C, HDL-C, LDL-C/HDL-C ratio, serum glucose, Hb, Hct, platelet count, WBC count, and % differential WBCs. Cholesterol feeding increased some blood clinical biochemistry parameters such as SGOT, SGPT, ALP, total cholesterol, LDL-C and LDL-C/HDL-C ratio, but had no effect on total and direct bilirubin. High cholesterol diet rats administered with barakol showed a significant decrease of ALP comparing to the corresponding high cholesterol diet group. These findings were conceivable that either high cholesterol diet or barakol administration caused a liver injury but in the different manner. Further studies on the effects of various doses of barakol on hepatic drug metabolizing enzymes and on blood clinical biochemistry parameters as well as the mechanism of which barakol induced liver injury were suggested.

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Field Of Study Pharmacology	**Advisor's Signature……..……...**
Academic Year 2001	**Co-Advisor's Signature……**
	Co-Advisor's Signature……

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LIST OF ABBREVIATIONS

5-HT	=	serotonin
AFB_1	=	aflatoxin B_1
ALP	=	alkaline phosphatase
AMD	=	aminopyrene N-demethylase
ANH	=	aniline 4-hydroxylase
B(a)P	=	benzo(a)pyrene
BR	=	benzyloxyresorufin
BROD	=	benzyloxyresorufin O-dealkylase
BSA	=	bovine serum albumin
BUN	=	blood urea nitrogen
CD_{50}	=	median convulsant dose
cDNA	=	complementary deoxyribonucleic acid
CDNB	=	1-chloro-2,4-dinitrobenzene
cm	=	centimeter
CNS	=	central nervous system
CVS	=	cardiovascular system
CYP	=	cytochrome P450
dl	=	deciliter
DMBA	=	9,10-dimethyl-1,2-benzanthracene
DMSO	=	dimethyl sulfoxide
DNA	=	deoxyribonucleic acid
EDRF	=	endothelium derived relaxing factor
EDTA	=	ethylenediaminetetraacetic acid
ER	=	ethoxyresorufin
EROD	=	ethoxyresorufin O-dealkylase
et al.	=	et alii (and other)
g	=	gram
g	=	gravity
G6P	=	glucose 6-phosphate

G6PD	=	glucose 6-phosphate dehydrogenase
GS^-	=	glutathione thiolate anion
GSH	=	glutathione
GST	=	glutathione S-transferase
Hb	=	hemoglobin
Hct	=	hematocrit
HDL-C	=	high density lipoprotein cholesterol
i.p.	=	intraperitoneal
kg	=	kilogram
L	=	liter
LD_{50}	=	median lethal dose
LDL-C	=	low density lipoprotein cholesterol
M	=	molar (mole per liter)
MI	=	metabolic intermediate
min	=	minute
mg	=	milligram
ml	=	milliliter
mM	=	millimolar (millimole per liter)
mmol	=	millimole
mp.	=	melting point
MR	=	methoxyresorufin
mRNA	=	messenger ribonucleic acid
MROD	=	methoxyresorufin O-dealkylase
MW	=	molecular weight
NADP	=	nicotinamide adenine dinucleotide phosphate
NADPH	=	nicotinamide adenine dinucleotide phosphate (reduced form)
nm	=	nanometer
nM	=	nanomolar (nanomole per liter)
nmol	=	nanomole
pH	=	potential of hydrogen
pmol	=	picromole

PR	=	pentoxyresorufin
PROD	=	pentoxyresorufin O-dealkylase
r.p.m.	=	revolution per minute
SCr	=	serum creatinine
SE	=	standard error
sec	=	second
SGOT	=	serum glutamic oxaloacetic transaminase
SGPT	=	serum glutamic pyruvic transaminase
TCA	=	trichloroacetic acid
TG	=	triglyceride
Tris	=	Tris (hydroxymethyl) aminomethane
U	=	unit
UDPGT	=	uridine 5'-diphosphoglucuronosyltransferase
vs.	=	versus
v/v	=	volume by volume
WBC	=	white blood cell
w/v	=	weight by volume
β-NF	=	beta-napthoflavone
γ-GT	=	gamma-glutamyltranspeptidase
^{o}C	=	degree celsius
μg	=	microgram
μl	=	microliter
μM	=	micromolar (micromole per liter)

CHAPTER I

INTRODUCTION

Cassia siamea Lam. or Khilek (in Thai) is a plant in family Leguminosae (Smitinand, 2001) that is generally found in tropical countries including Thailand. Various parts of this plant have been used as food and traditional medicine such as root for relief of fever; bark for treatment of skin diseases and hemorrhoid; leaves for treatment of constipation, diabetes, hypertension and insomnia; flowers for treatment of insomnia and asthma (สุนทรี สิงหบุตรา, 2540). In Thailand, flowers and young leaves of *C. siamea* have been widely used for treatment of insomnia as well as an ingredient in one Thai food recipe, Khilek curry. A study on their pharmacological properties was first done in 1949 by Arunlakshana. It was found that administration of a crude ethanol extract of *C. siamea* leaves to animals decreased movement and activity without sleep despite being given at high dose (อุไร อรุณลักษณ์, 2492).

In 1969, the major constituent in flowers and young leaves of *C. siamea*, barakol, was first extracted. Barakol or 3a,4-dihydro-3a,8-dihydroxy-2,5-dimethyl-1,4-dioxaphenalene ($C_{13}H_{12}O_4$) is pale yellow needle crystals with boiling point of 165^{o}C (Hassanali, King and Wallwork, 1969). A year later, a proposed synthesis procedure of barakol was described by Bycroft, Hassaniali-Walji and King (1970). Subsequently, the pharmacological and physiological properties as well as toxicity of barakol have been studied continuously such as effects on central nervous system (CNS), cardiovascular system (CVS) and antimicrobial effect, as summarized by Thongsaard (1998).

Intraperitoneal (i.p.) administration of barakol to mice in a toxicological study found that barakol possessed median convulsant dose, CD_{50}, of 296.71 mg/kg and median lethal dose, LD_{50}, of 324.09 mg/kg (Jantarayota, 1987). Furthermore, acute

hepatitis has been reported in patients at Phramongkutklao and Chulalongkorn Memorial Hospitals when 20-40 mg/day *C. siamea* capsules were taken for 7-60 days. Seven out of nine patients had nausea, vomiting and jaundice whereas the remaining had no symptoms but only a change in blood clinical biochemistry parameters of liver function. All patients had no past history of chronic liver diseases. However, It has not been proved that those effects are due to barakol or any other constituents in *C. siamea* leaves (สมบัติ ตรีประเสริฐสุข และคณะ, 2543).

Recent pharmacological study focused on anticarcinogenic and antimutagenic effects of *C. siamea* leaves. Interestingly, it was found that hexane, chloroform and methanol extracts of flowers and young leaves of *C. siamea* protected rats against aflatoxin B_1-induced mutagenesis. Moreover, the chloroform and methanol extracts prevented mutagenesis induced by Benzo(a)pyrene (Kusamran, Tepsuwan and Kupradinun, 1998). This group of researchers also studied the effects of *C. siamea* leaves on hepatic metabolizing enzymes in rats. Feeding rats with 5% dietary *C. siamea* leaves for 14 days resulted in a significant increase of phase II detoxification enzyme activities, glutathione S-transferase (GST) and uridine 5'-diphospho-glucuronosyltransferase (UDPGT), along with a decrease of some bioactivation enzyme activities in cytochrome P450 (CYP) system such as aniline 4-hydroxylase (ANH) and aminopyrine N-demethylase (AMD) (Tepsuwan, Kupradinun and Kusamran, 1999).

In general, CYP isoforms in family 1, 2 and 3 play an important role in biotransformation of various xenobiotic compounds to toxic metabolites, carcinogens and/or mutagens (Soucek and Gut, 1992). Modification of hepatic phase I (toxification) and phase II (detoxification) enzymes are partly a key aspect explanation for antimutagenic and anticarcinogenic potential of chemicals. So far, few studies were performed regarding the effects of *C. siamea* leaves (not yet of its active constituent, barakol) on hepatic CYP and phase II enzymes. Therefore, this research study focused on the subchronic effects of barakol on some CYP isoforms involving in activation reactions of chemical carcinogens such as CYP1A1, CYP1A2, CYP2B1,

CYP2B2 and CYP2E1. Subsequently, hepatic phase II enzyme, GST was also determined. Since subchronic toxicity of barakol has not yet been reported, blood clinical biochemistry parameters in rats were investigated. In addition, barakol was shown to possess an advantage on CVS such as hypotensive effect in both systolic and diastolic blood pressure in rats and cats, reduction in the contraction of isolated rat thoracic aorta induced by phenylephrine (Suwan et al., 1992) and reduction of heart rate in anesthesized rats (Momose et al., 1996). The established risk factors of cardiovascular disease are hypertension, hypercholesterolemia, low HDL-C, cigarette smoking, diabetes mellitus as well as non-modifiable personal characteristic such as age, gender and family etc. (Smith, 2000; Elisaf, 2001). Hypercholesterolemia is a risk factor of atherosclerosis which is generally associated with almost cases of cardiovascular diseases (Elisaf, 2001). This experiment was performed in both normal and high cholesterol diet rats for an additional purpose of lipid lowering effects of this compound as well.

Hypothesis

Subchronic exposure of barakol causes an induction and/or an inhibition of hepatic microsomal CYP and cytosolic GST as well as changes in blood clinical biochemistry parameters in normal and high cholesterol diet rats.

Study design and process

1. Animal treatment
2. Blood sampling
3. Determination of blood clinical biochemistry parameters and hematology
4. Preparation of liver microsomes and cytosols
5. Determination of CYP and GST activities
6. Data analysis

Benefit gained from the study

The results from this study would be a preliminary data of whether subchronic exposure of barakol induces and/or inhibits CYP isoforms involved in various bioactivation reactions of drugs, chemicals as well as environmental toxicants. Therefore, it would be useful to consider the possibility of barakol to increase and/or decrease chemical-induced mutagenic and/or carcinogenic risks. In addition, the effects of barakol on blood clinical biochemistry parameters would be a preliminary subchronic toxicity data of this compound in rats fed normal and high cholesterol diets.

CHAPTER II

LITERATURE REVIEW

Cassia siamea Lam.

Cassia siamea Lam. belongs to family Leguminosae (subfamily Caesalpinoideae) which is generally grown in tropical countries including Thailand. It is known as Khilek, Cassod tree, Thai copper pod (Smitinand, 2001). This plant is a medium-size tree and comprises of compound leaves (4-12 pairs of leaflets) which is oblong in shape (3-7 cm long, 1.2-2.6 cm wide). Young leaves are covered with greenish brown hairs. Corymb is crowded with medium size, bright yellow flowers. Pod is nearly straight, flat shape with firm texture and thickened sutures (Chaichantipyuth, 1979; Jantarayota, 1987).

Various parts of *C. siamea* have been used for a long time as traditional medicines such as (สุนทรี สิงหบุตรา, 2540):

Root as antipyretics;
Bark for skin diseases and hemorrhoid;
Heartwood for diabetes and constipation;
Leaves for constipation, diabetes, hypertension and insomnia;
Flowers for insomnia, as antiasthma and antidandruff.

By the time of economic crisis all over the world, alternative medicine becomes popular for primary health care. Utilizing of herbal medicines that are available throughout the country has been a subject of interest. Flowers and young leaves of *C. siamea* (Figure 2.1) are also widely used by local people for medicinal purpose and are added as food ingredient.

Barakol

In 1969, a major constituent of *C. siamea*, barakol, was first extracted from its flowers and young leaves. Barakol ($C_{13}H_{12}O_4$) has a chemical name of 3a,4-dihydro-3a,8-dihydroxy-2,5-dimethyl-1,4-dioxaphenalene or 2,5-dimethyl-3αH-pyrano-[2,3,4-de]-1-benzopyran-3a,8-diol (Thongsaard et al., 2001). It is crystallized from aqueous methanol or ethanol as pale yellow needles, mp.165^{o}C. This compound is stable in hydroxylic solvents or in a moist atmosphere. (Hassanali et al., 1969). Subsequently, the structure and general extracting procedure of barakol were demonstrated by Bycroft et al. (1970) (Figure 2.2). Meanwhile, other chemical constituents in *C. siamea* leaves were also investigated. Many compounds were found in the extraction fractions such as β-sitosterol, chrysophanol, physcion and cassiamin A in petroleum ether fraction, p-coumaric acid and apegenin-7-O-galactoside (Thalictiin) in ethyl acetate fraction as well as a new compound named cassiachromone ($C_{13}H_{12}O_4$) or 2-methyl-5-acetonyl-7-hydroxychromone in chloroform fraction (Arora et al., 1971; Wagner et al., 1978). It was reported that acid treatment of cassiachromone was identified as barakol (Chaichantipyuth, 1979).

Chemical dehydration of barakol was readily achieved over phosphorus pentoxide or in vacuum. The resulting dark green amorphous compound, anhydrobarakol ($C_{13}H_{10}O_3$), was extremely unstable and could be reconverted to barakol by dissolution in aqueous methanol (Bycroft et al., 1970). When barakol was treated with strong acid, it reversibly formed anhydrous salts which could be dried and stable at room temperature in the solid state (Hassanali et al., 1969). For example; dissolving of barakol in a small amount of methanol and adding with concentrated hydrochloric acid or hydrobromic acid resulted in slow crystallization of anhydrobarakol hydrochloride as yellow needles or immediately crystalline precipitation of anhydrobarakol hydrobromide as tiny yellow needles, respectively (Bycroft et al., 1970). When anhydrobarakol hydrochloride is dissolved in water, the conversion reaction is reversed (Figure 2.3). The product used in all biological

experiment is a barakol solution. The basic chemical properties of barakol, anhydrobarakol and anhydrobarakol hydrochloride were shown in Table 2.1.

Figure 2.1 Flowers and young leaves of *Cassia siamea* Lam.

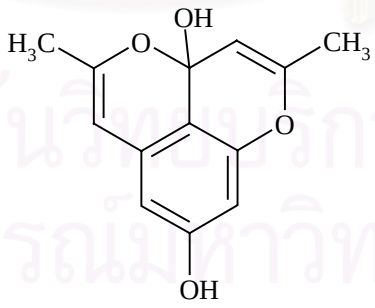
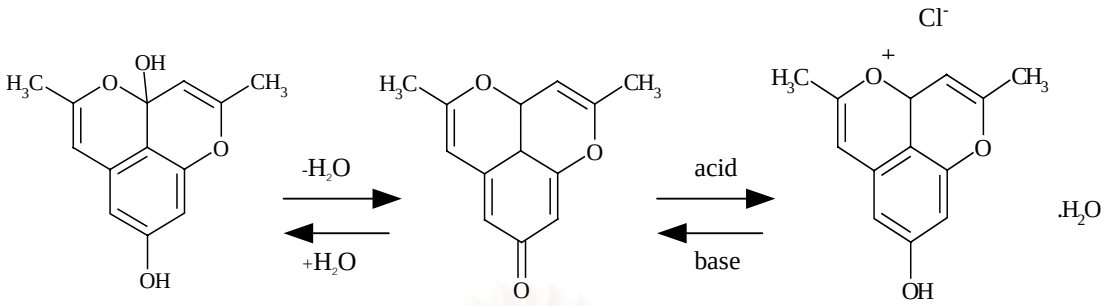


Figure 2.2 Structure of barakol (3a,4-dihydro-3a,8-dihydroxy-2,5-dimethyl-1,4-dioxaphenalene)



Barakol Anhydrobarakol Anhydrobarakol hydrochloride

Figure 2.3 The conversion reaction of barakol, anhydrobarakol and anhydrobarakol hydrochloride (Thongsaard et al., 2001)

Table 2.1 Basic chemical properties of barakol (Thongsaard et al., 2001)

	Barakol	Anhydrobarakol	Anhydrobarakol hydrochloride
Formula	$C_{13}H_{12}O_4$	$C_{13}H_{10}O_3$	$C_{13}H_{12}ClO_3$
MW	232	214	251.4
Color	Greenish yellow	Dark green	Lemon yellow
Melting point	166-170 °C	163 °C	208-210 °C

Stability of barakol

Stability of barakol at various conditions was investigated by Thongsaard and coworkers. After preparation as an aqueous solution, the best condition to store barakol is at 0°C in the dark. Under this condition barakol solution remained stable for 24 hours after preparation. However, when barakol is used for physiological or pharmacological studies, which required higher temperature (i.e. dissolved in physiological buffer at 37°C) the recommended condition to keep barakol is in dark and used within 1 hour after preparation (Thongsaard et al., 2001).

Physiological and pharmacological effects

Pharmacological properties of *C. siamea* leaves were first investigated by Arunlakshana (อุไร อรุณลักษณ์, 2492). Administration of crude ethanol extract of *C. siamea* leaves to animals was found to decrease voluntary activity but not sleep even though they were given at high dose. The subsequent extraction, which yielded a crystalline alkaloid (mp. 95^{o}C), also possessed the similar results. These findings indicated the suppression effects of the constituents in *C. siamea* leaves on CNS, particularly, on cerebrum and spinal cord. The minor effects were an increasing tonus of involuntary smooth muscle and diuresis. Sedation was also demonstrated in man without toxicity (อุไร อรุณลักษณ์, 2492).

Effects on CNS

When the major compound in flowers and young leaves of *C. siamea*, barakol, was first extracted, it has been studied in many physiological and pharmacological aspects, mainly on CNS. Conflicting to the results reported previously by Arunlakshana (อุไร อรุณลักษณ์, 2492), barakol did not possess protective effect against convulsion induced by picrotoxin, bicucullin and strychnine (Jantarayota, 1987). Investigating an indication of *C. siamea* leaves for treatment of insomnia, intraperitoneally low (25 mg/kg) and high (100 mg/kg) doses of barakol were found to affect sleeping behavior in rats (Bulyalert, 1992). A study on sedative effect in mice, i.p. administration of barakol decreased locomotion at low dose (10-100 mg/kg). Analgesic effect of barakol was also found in mice at the dose between 100-200 mg/kg as demonstrated by an increase nociceptive threshold on hot plate test (Jantarayota, 1987). In rats, barakol (25-100 mg/kg, i.p.) caused a reduction of head shake behavior which was explained by the suppression effect of barakol on serotonergic system. In addition, barakol (75-150 mg/kg, i.p.) was also proposed to have dopamine agonist action by an increase of apomorphine-induced turning behavior in a dose related manner in rats with unilateral substantia nigra lesion induced by 6-hydroxydopamine injection (Jantarayota, 1987). Using an elevated plus-maze model for investigating the

effect of drugs on anxiety, barakol (10mg/kg, i.p.) has been shown to produce a profile of behavior similar to diazepam except rather than showing sedative effect there was an increase of exploratory and locomotor behavior (Thongsaard et al., 1996). The anxiolytic effect as well as exploratory and locomotor behavior were reduced at higher dose (25 and 50 mg/kg, i.p.) and not observed at the highest dose (75 mg/kg, i.p.) (Thongsaard et al., 1996). The potential advantage of barakol over diazepam urged further investigation on the mechanism of action of this compound and the nature of neurotransmitters involved in its anxiolytic effects. An *in vitro* study using rat striatal slices, barakol has been investigated on dopamine release and uptake. Barakol at the dose of 0.1 nM-100 μM and a selective dopamine D_2 receptor agonist (quinelorane dihydrochloride, 1 μM) inhibited endogenous dopamine release stimulated by 20 mM [K^+], which were antagonized by selective dopamine receptor antagonist (*S*(-)-eticlopride hydrochloride, 1 μM). The inhibition was not observed on radio labeled [^{3}H] dopamine release when measuring under the similar condition. In addition, barakol found no effect on striatal dopamine uptake except at the highest concentration used (100 μM). These findings indicated that barakol might act as a dopamine D_2-like receptor agonist to inhibit K^+-stimulated endogenous dopamine release from striatal slices with selective effects on newly synthesized dopamine without a change in dopamine uptake (Thongsaard et al., 1997). In general, the present of serotonin (5-HT) in brain is associated with reduction of anxiety (Marsden, 1990). Another possible anxiolytic mechanism of barakol might be explained by its effect on serotonergic system. From *in vitro* study of 5-HT release, barakol (1-100 μM) was found to increase K^+-stimulated 5-HT release from hippocampal slices (วัชรีวรรณ ทองสะอาด, สุนันท์ ชัยนะกุล และ Charles A. Marsden, 2543) but showed no effect on 5-HT reuptake except at the highest dose (100 μM) (unpublished data).

Effects on CVS

On the CVS, intravenous injection of barakol at dosage between 0.5-15 mg/kg caused significant hypotensive effects on both systolic and diastolic blood pressure in a dose-related manner in rats and cats. Along with the *in vivo* study, barakol (10^{-5}-10^{-3}

M) produced a significant reduction in the contraction of isolated rat thoracic aorta induced by phenylephrine. Hypotensive effect of barakol in rats and cats might be due to peripheral vasodilation via an action of endothelium derived relaxing factor (EDRF) (Suwan et al., 1992). Intravenous injection of 10 mg/kg barakol was also reported to reduce the heart rate and systemic blood pressure in anaesthetized rats (Momose et al., 1996).

Antimicrobial effects

Barakol was shown to have low antimicrobial activity against both gram positive and gram negative bacteria as well as yeast-like fungus (Gritsanapan, Mekmanee and Chulasiri, 1989). The antimalarial effect on *Plasmodium falciparum* growth by water extract of *C. siamea* leaves was also reported (Gbeassor et al., 1989).

Antimutagenic and anticarcinogenic effects

Interestingly, antimutagenic and anticarcinogenic effects possessed by leaves of *C. siamea* have been reported (Kusamran et al., 1998; Tepsuwan, et al., 1999). Hexane, chloroform and methanol extracts from leaves and flowers of this plant exhibited strong antimutagenic potential toward aflatoxin B_1 (AFB_1). Moreover, the methanol extract could protect against benzo(a)pyrene (B(a)P) induced mutagenicity for greater than 30% at the dose less than 1 mg/plate using Ames' Salmonella mutagenicity test with *Salmonella typhimurium* TA100 as a tester strain (Table 2.2) (Kusamran et al., 1998). Another related study demonstrated that feeding rats with dietary containing 5% ground freezed dry *C. siamea* leaves for 2 weeks significantly reduced the activities of hepatic ANH and AMD as well as the capacity of hepatic S9 fraction to activate the mutagenicity of AFB_1. In contrast, activities of GST and UDPGT were markedly increased (Table 2.3) (Tepsuwan et al., 1999). In addition, 2.5 and 4% dietary *C. siamea* leaves resulted in a significant decrease of mammary gland tumors induced by 7,12-dimethylbenz(a)anthracene (DMBA) and a slightly delay onset of tumor development (Tepsuwan et al., 1999).

Table 2.2 Antimutagenicity of Thai vegetable extracts against direct-acting and indirect-acting mutagens (modified from Kusamran et al., 1998)

Vegetable	Extract	Antimutagenic activities towards			
		Direct-acting mutagens		Indirect-acting mutagens	
		AF-2	NaN_3	AFB_1	B(a)P
C. siamea leaves	Hexane	-	ND	++	-
	Chloroform	-	ND	++++	++
	Methanol	-	ND	++++	+++

Antimutagenic activities of vegetable extracts were evaluated at doses ranging from 0.05-10 mg/ plate ++++, +++, ++ and + indicate that the extracts inhibited the mutagenicity of each mutagen ≥ 30% at doses 0.1-0.5, >0.5-1, >1-2, >2-5 mg/plate, respectively. - indicates noantimutagenic activity at dose up to 10 mg/ plate (AF-2, 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide; NaN_3, sodium azide)

Table 2.3 Effects of dietary *C. siamea* leaves on the activities of phase I and phase II metabolizing enzymes in rat livers (Tepsuwan et al., 1999)

Enzyme	Diet group	
	Basal	5% *C. siamea* leaves (fold of control)
Phase I enzymes		
Total P450 (nmol mg^{-1} protein)	0.081 ± 0.011	0.037 ± 0.018 (0.90)
ANH (nmol p-aminophenol formed min^{-1} mg^{-1} protein)	0.049 ± 0.016	0.015 ± 0.015** (0.31)
AMD (nmol formaldehyde formed min^{-1} mg^{-1} protein)	0.693 ± 0.155	0.507 ± 0.120* (0.73)
Capacity of S9 fraction to activate the mutagenicity of		
(1) AFB_1 (0.03 μg)[a]	155.5 ± 23.7	64.3 ± 13.3* (0.41)
(2) B(a)P (25 μg)[a]	74.2 ± 5.1	66.1 ± 5.4 (0.89)
Phase II enzymes		
GST (μmol CDNB-glutathione conjugated formed min^{-1} mg^{-1} protein)	0.373 ± 0.074	0.936 ± 0.149*** (2.51)
UDPGT (nmol p-nitrophenol disappeared min^{-1} mg^{-1} protein)	6.191 ± 1.319	13.356 ± 2.546*** (2.16)

Values are means ± S.D. form 10 animals in each group.

[a] Values are means ± S.E. form 10 animals in each group (no. of his^{+} revertants/ plate).

* Significantly different form control group ($p < 0.008$)

** Significantly different form control group ($p < 2 \times 10^{-4}$)

*** Significantly different form control group ($p < 2 \times 10^{-6}$)

Toxicity

A study in mice showed that intraperitoneal administration of barakol possessed a median convulsant dose (CD_{50}) of 296.71 mg/kg and a median lethal dose (LD_{50}) of 324.09 mg/kg (Jantarayota, 1987). *C. siamea* leaves in a dosage form of capsules have been used for the indication of laxative, sleeping aid, increase food appetite as well as antianxiety. In 1999, acute hepatitis and/or abnormal liver functions were found in patients at Pramongkutklao and Chulalongkorn Memorial Hospitals with a history of taking *C. siamea* capsules at the dosage of 20-40 mg/day for 7-60 days. The detail of these case reports were shown in Table 2.4 (สมบัติ ตรีประเสริฐสุข และคณะ, 2543). Ages of these patients are ranged from 29-81 years. Level of hepatitis from drug was evaluated according to the drug induced liver injury (DILI) scale (Maria and Victorino, 1997). Seven out of nine patients had nausea/ vomiting and jaundice, whereas the others had no symptoms except for some abnormal parameters of liver functions. Rechallenge of *C. siamea* capsule administration was done in 2 patients after acute hepatitis symptoms disappeared. The recurrent of those symptoms were found. Moreover, liver biopsy of 3 patients confirmed the correlation between taking *C. siamea* capsules and hepatitis. However, these findings were still questioned whether barakol or any other constituents in *C. siamea* leaves are responsible for these toxic effects (สมบัติ ตรีประเสริฐสุข และคณะ, 2543).

Table 2.4 Report on hepatitis from *C. siamea* capsules in 9 patients of Pramongkutklao and Chulalongkorn Memorial Hospitals in 1999

ข้อมูลของผู้ป่วยที่มีภาวะตับอักเสบ (สมบัติ ตรีประเสริฐสุข และคณะ, 2543)

รายที่	อายุ	เพศ	ระยะเวลา (วัน) ที่ได้รับยาขี้เหล็ก	ขนาดยา (มก.) ขี้เหล็กต่อวัน	อาการตัว-ตาเหลือง อ่อนเพลีย	ค่าสูงสุดของ SGPT (ยูนิต/ลิตร)	ค่าสูงสุดของ Tb/Db (มก./ดล.)	ค่าการทำงานของตับ SGPT และ Tb เป็นปกติ ในระยะเวลา (สัปดาห์)
1	60	หญิง	60	20	มีอาการ	1146	15/12	23
2	61	ชาย	21	20-30	มีอาการ	1076	1.87/1.31	4
3*	81	หญิง	150	10	ไม่มีอาการ	127	0.8/0.4	4
4*	30	หญิง	30	20	ไม่มีอาการ	643	0.6/0.3	4
5	49	หญิง	3	20-40	มีอาการ	432	7.8/4.6	4
6	63	หญิง	90	20	มีอาการ	740	3.9/3.0	6
7	29	หญิง	120	20	มีอาการ	934	7/6.1	3
8	55	หญิง	30	20	มีอาการ	779	7.5/5.5	6
9	50	หญิง	180	20	มีอาการ	413	1/0.7	2

Tb = Total bilirubin, Db = Direct bilirubin, * คือรายที่มีการทดลองใช้ยาขี้เหล็กซ้ำหลังจากมีอาการของภาวะตับอักเสบแล้วเกิดอาการซ้ำใหม่

Carcinogenic detoxification process

It is well recognized that most carcinogenic chemicals require metabolic activation to achieve ultimate electrophiles which highly prompt to attack the target DNA molecules. Modifications of DNA, particularly on oncogenes and tumor suppressor genes are generally leading to cancer development (Yang, Smith, and Hong, 1994). Protective mechanism is an important way to protect target tissue against carcinogenesis from these potentially harmful chemicals. This includes enhancing the detoxification pathway and inhibition of the activation reactions that generate ultimate carcinogenic species. In addition, the electrophilic metabolites can be neutralized by conjugating enzymes such as GST (Wattenberg, 1983; Gonzalez and Gelboin, 1994) (Figure 2.4). For example, many phytochemical constituents such as diallyl sulfide from garlic, isothiocyanates from cruciferous vegetables and flavonoids

from various kinds of fruits and vegetables have been found to enhance carcinogen detoxification systems. (Wattenberg, 1983; Yang et al., 1994).

There are many epidemiological evidences showing that high consumption of vegetables and fruits is associated with a decrease of human cancers in various sites (Steinmetz and Potter, 1991) such as stomach cancer (Chyou et al., 1990). These food sources are known to contain antimutagenic cocktail including carotenoids, vitamin C, vitamin E, selenium, dietary fiber, dithiolthiones, glucosinolates, flavonoids, phenols, protease inhibitors, plant sterols, allium compounds and limonene. The mechanisms by which vegetables and fruits may protect human against cancer are both complementary and overlapping functions such as induction of detoxification enzymes, inhibition of carcinogen formation, acting as a substrate for endogenous production of anticarcinogens, diluting and/or binding of carcinogens in the digestive tract, alteration of hormone metabolism, reduction of transformed cell capacity to proliferate, antioxidant effects, etc. (Steinmetz and Potter, 1991).

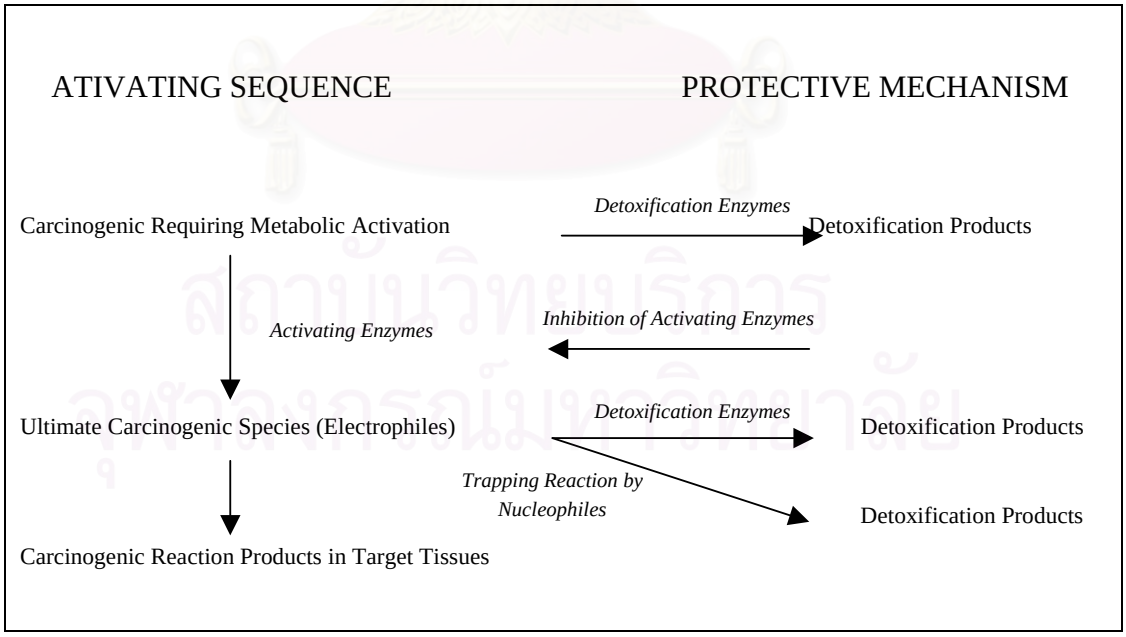


Figure 2.4 Simplified scheme depicting carcinogen metabolism to ultimate carcinogenic species that is react with target sites and points at which protective mechanisms can intervene (Wattenberg, 1983).

Xenobiotic biotransformation

Biotransformation appears to be an important process determining the consequences of xenobiotics or foreign chemicals as well as endogenous compounds in biological system (Parkinson, 1995; Timbrell, ed., 2000). In general, biotransformation can be divided into phase I and phase II reactions and, sometimes, phase III reactions (Table 2.5).

Table 2.5 Major biotransformation reactions (Timbrell, ed., 2000)

Phase I	Phase II	Phase III
Oxidation	Sulphation	Further metabolism of
Reduction	Glucuronidation	glutathione conjugates
Hydrolysis	Glutathione conjugation	
Hydration	Acetylation	
Dehalogenation	Amino acid conjugation	
	Methylation	

Phase I reactions introduce a functional group (-OH, -NH_2, -SH or -COOH, etc.) to a molecule leading to a small increase in hydrophilicity as well as a suitable characteristic for further phase II biotransformations (Parkinson, 1995; Timbrell, ed., 2000).

Phase I reactions

The majority of phase I reactions are oxidation by the enzymes in CYP system which are predominantly localized in the smooth endoplasmic reticulum of the liver cells (Potter and Coon, 1991; Timbrell, ed., 2000). It has been well-established that CYP superfamily comprises a large member of gene families (Guengerich, 1991). On the basis of more than 39% amino acid homology (Soucek and Gut, 1992), there are more than 30 and 38 gene families identified in humans and rats, respectively (Guengerich, 1991, 1992). Each CYP subfamily possesses more than 59% gene homology (Soucek and Gut, 1992). Besides the amino acid sequence homology, individual CYP isoform is characterized by its substrate specificity that is determined by the apoprotein substrate binding region of CYP structure (Soucek and Gut, 1992; Lin and Lu, 1998; Murray, 1999). These enzymes generally catalyze the oxidation reactions of a wide variety of both endogenous compounds and xenobiotics with a critical role and overlapping substrate specificity (Potter and Coon, 1991; Guengerich, 1991, 1992). Major CYP enzymes in human, their specific substrates and their percent participation in drug metabolism are shown in Table 2.6. There are some differences in amino acid sequences as well as catalytic activity of the same CYP isoform among species (Soucek and Gut, 1992). Sequential homology of amino acids between rat and human CYPs are shown in Table 2.7.

The basic reaction catalyzed by CYP is a monooxygenation reaction as following (Guengerich, 1991; Potter and Coon, 1991; Parkinson, 1995):

$$\textbf{Substrate (RH)} + O_2 + \textbf{NADPH} + H^+ \longrightarrow \textbf{Product (ROH)} + H_2O + NADP^+$$

Product from this reaction does not appear to be only a simple alcohol because rearrangement might be occurred. There are also documented that CYP may catalyze reduction reaction e.g. carbon tetrachloride, azo dyes and epoxides (Guengerich, 1991). However, biotransformation by CYP is not always a detoxification reaction. A variety of specific CYP isoforms, especially CYP in family 1, 2 and 3 are involved in the activation of certain chemical procarcinogens (Soucek and Gut, 1992; Parkinson,

1995). In human, CYP1A1, CYP1A2, CYP2E1, and CYP3A4 are the most important enzymes for metabolic activation of environmental chemicals. For example, CYP1A1 activates polycyclic aromatic hydrocarbons, CYP1A2 activates various arylamines, CYP2E1 activates vinyl monomers and small halogenated hydrocarbons, CYP3A4 activates aflatoxins, polycyclic aromatic hydrocarbon dihydrodiols and some arylamines (Guengerich, 1992; Rendic and Di Carlo, 1997). Human and rat CYPs that activate some potential carcinogens/ mutagens are demonstrated in Table 2.8.

Table 2.6 Human CYP enzymes, their specific substrates and their percent participation in drug metabolism (modified from Rendic and Di Carlo, 1997)

CYP enzyme	Substrates	Participation in drug metabolism (%)
1A1	7-Methoxyresorufin R-Warfarin	2.5
1A2	Acetaminophen Caffeine 7-Ethoxyresorufin 7-Methoxyresorufin Phenacetin R-Warfarin	8.2
2B6	Cyclophosphamide 7-Benzyloxyresorufin S-Mephenytoin 7-Pentoxyresorufin Testosterone	3.4
2E1	Acetaminophen Aniline Chlorzoxazone Dapsone Halothane p-Nitrophenol	4.1
2C8, 9	Dicloenac Hexobarbital Phenytoin Tolbutamide S-Warfarin	15.8
2C18, 19	Diazepam S-Mephenytoin Omeprazole	8.3
2D6	Codeine Bufuralol Debrisoquine Dextromethorphane Sparteine	18.8
3A4, 5	Carbamazepine Cortisol Dapsone Diazepam Erythromycin Midazolam Nifedipine Omeprazole Testosterone	34.1

Table 2.7 Sequential homology of cDNA and amino acids between rat and human CYPs (modified from Soucek and Gut, 1992)

CYP		
Rat	Human	Sequential homology[a] (%)
CYP1A1	CYP1A1	80 (78)
CYP1A2	CYP1A2	75 (70)
CYP2B1	CYP2B6	78 (74)
	CYP2B7	(76)
CYP2B2	----------	- [b]
CYP2E1	CYP2E1	75 (78)
CYP3A1	CYP3A3	(78)
	CYP3A4	(73)

[a] Similarity of cDNA and amino acid (in parentheses) sequence stated

[b] No data available regarding existence of orthologous form

Table 2.8 Role of rat and human CYPs in the activation of some potential carcinogens/ mutagens (Soucek and Gut, 1992; Guengerich, 1993; Gonzalez and Gelboin, 1994)

CYP	Potential mutagens/ carcinogens	
	Rat	**Human**
1A1	Aflatoxin B_1 Benzo(a)pyrene 7,12-Dimethylbenz(a)anthracene 2-Naphthylamine 4,4'-(bis) Methylene chloroaniline	Benzo(a)pyrene 7,12-Dimethylbenz(a)anthracene 6-Nitrochrysene
1A2		2-Acetylfluorene 2-Aminoanthracene Aflatoxin B_1 4-Aminobiphenyl 2-Naphthylamine 6-Nitrochrysene
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
2B7		Aflatoxin B_1
2E1	N-N'-Nitrosodimethylamine N-Nitroso-N-diethylamine	Acrylonitrile Benzene Carbon tetrachloride Chloroform N-N'-Nitrosodimethylamine N-Nitroso-N-diethylamine Styrene Trichloroethylene Vinyl carbamate Vinyl bromide Vinyl chloride
3A4		Aflatoxin B_1 Aflatoxin G_1 Benzo(a)pyrene 6-Nitrochrysene Sterigmatocystin

Phase II reactions

Most phase II reactions are recognized as detoxification pathway, which results in a large increase in hydrophilicity as well as an enhancement of foreign molecule excretion (Parkinson, 1995; Timbrell, ed., 2000). Most reactions in phase II are conjugation reactions such as glucuronide, glutathione and sulfate conjugations (Table 2.5) (Wattenberg, 1983). Enzymes of those reactions are mainly located in cytosol except for the UDPGTs, which are microsomal enzymes. Glucuronidation is a major pathway of xenobiotic biotransformations in most mammalian species (Parkinson, 1995). In addition, glutahione conjugation has been studied extensively as a major detoxification system and considered as an important protective mechanism against chemical induced carcinogenesis (Spranin, Venegas and Wattenberg, 1982; Wattenberg, 1983). GST catalyzes nucleophilic attack of glutahione thiolate anion (GS^-), derived from glutathione, to electrophilic xenobiotics (Parkinson, 1995). Moreover, products from phase II reaction may be further metabolized. This process is sometimes termed phase III reaction (Timbrell, ed., 2000).

Inhibition and induction of CYP

1. Inhibition mechanism of CYP

The mechanism of CYP inhibition can be divided into 3 categories: reversible inhibition, quasi-irreversible inhibition and irreversible inhibition (Lin and Lu, 1998).

1.1 Reversible inhibition

Reversible inhibition is the reaction that inhibitors bind with CYP active site, at prosthetic haem iron and/or lipophilic region of the protein, in only the first step of catalytic cycle (Lin and Lu, 1998; Murray, 1999). Most of these inhibitors are nitrogen containing molecules and their potencies are determined by bond strength between lone pair electron and prosthetic haem iron as well as their lipophilicity (Lin and Lu, 1998).

1.2 Quasi-irreversible inhibition

Quasi-irreversible inhibition is the reaction that compounds undergo metabolic activation by CYP to exert inhibitory metabolites. Then, they form stable complex with prosthetic haem of CYP. Without destruction, the complex is in an inactive state called metabolic intermediate (MI) complex. *In vitro* methods such as displacement or disruption of MI complex can return the activation function of CYP. In contrast, MI complex is so stable in an *in vivo* situation. Only *de novo* enzyme synthesis is the means by which the activity can be restored (Lin and LU, 1998).

1.3 Irreversible inhibition

Irreversible inhibition is the reaction that compounds with certain functional group are oxidized by CYP to generate reactive intermediates which then irreversible inactivate CYP prior to release from the active site. These compounds are classified as mechanism-based inactivators or suicide substrates (Lin and LU, 1998). The mechanism by which reactive intermediate inactivate CYP is haem alkylation (such as compounds containing terminal double bond or triple bond) or covalent binding to apoprotein of CYP (such as sulphur compounds) resulting in irreversible loss of CYP functions (Lin and Lu, 1998; Murray, 1999).

2. Induction mechanism of CYP

Enzyme induction is a slow adaptive response to protect cells from toxic xenobiotics by an increase of detoxification activity. Several modes of regulation process are increase in gene transcription, mRNA stabilization and enzyme stabilization (Potter and Coon, 1991; Guengerich, 1992; Lin and Lu, 1998). These processes seem to reduce the pharmacological as well as toxicological effects. However, another aspect that has to be concerned is that it may cause formation of reactive metabolites, which increase toxicity and carcinogenicity (Lin and Lu, 1998).

CHAPTER III

MATERIALS AND METHODS

Materials

1. Experimental animals

Thirty-two male Wistar rats weighing between 200-250 g were obtained from the National Laboratory Animal Center, Mahidol University, Nakornprathom, Thailand. Animals were kept in animal care facility at Srinakharinwirot University, Bangkok, Thailand and acclimatized for at least 1 week before the experiment. All animals were allowed free assess to food (S.W.T. Ltd., Thailand) and drinking water. Light / dark period and temperature were controlled at 12/12 hour cycle and 25^{o}C, respectively. During the time of experimentation, body weight of each rat was recorded every 2 weeks.

2. Instruments

1. Autopipettes (Gibson, France)
2. Centrifuge (Kokusan, Japan)
3. Fluorescence spectrophotometer (Jasco, Japan)
4. Metabolic shaker bath (Heto, Denmark)
5. pH meter (Orion, USA)
6. Potter-Elvehjem homogenizer with pestle and glass homogenizing vessel (Heidolph, Germany)
7. Refrigerated superspeed centrifuge (Hitachi, Japan)
8. Refrigerated ultracentrifuge (Beckman, USA)
9. Sonicator (Elma, Germany)
10. Spectrophotometer (Jasco, Japan)
11. Surgical equipment

12. Timer
13. Ultra-low temperature freezer (Forma Scientific Inc., USA)
14. Vortex mixer (Clay Adams, USA)

3. Barakol solution

Barakol was extracted by Asst. Prof. Dr. Watchareewan Thongsaard, Department of Physiology, Faculty of Medicine, Srinakharinwirot University, Thailand in the form of anhydrobarakol hydrochloride (MW 251.4) (Figure 2.3). The solution was freshly prepared by dissolving anhydrobarakol hydrochloride in distilled water to make a concentration of 30 mg/ml. The solution appeared as greenish yellow color and was reported to be stable for about 1 hour after preparation (Thongsaard et al., 2001).

4. Chemicals

4-Aminophenol, aniline hydrochloride, benzyloxyresorufin (BR), bovine serum albumin (BSA), 1-chloro-2,4-dinitrobenzene (CDNB), cupric sulfate, dimethyl sulfoxide (DMSO), ethylenediaminetetraacetic acid (EDTA), ethoxyresorufin (ER), Folin & Ciocalteu's phenol reagent, glucose 6-phosphate (G6P), glucose 6-phosphate dehydrogenase (G6PD), glutathione (GSH), methoxyresorufin (MR), nicotinamide adenine dinucleotide phosphate (NADP), pentoxyresorufin (PR), potassium phosphate (K_3PO_4), resorufin, sodium choleate, sodium phosphate dibasic anhydrous and Trisma® base were purchased from Sigma Chemical Co., USA.

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

Glycerol was purchased from Carlo Erba, USA.

Magnesium chloride ($MgCl_2$), Methanol (Gradient grade), phenol, potassium chloride, sodium chloride (NaCl) and trichloroacetic acid (TCA) were purchased from Merck, Germany

Sodium carbonate (Na_2CO_3), sodium citrate and sodium hydroxide (NaOH) were purchased from APS Finechem, Australia.

Sodium dithionite was purchased from Fluka Chemic, Japan

Methods

1. Animal treatment

Thirty-two rats were randomly assigned into 4 treatment groups as followings:

1. *Normal diet group*: Rats were fed normal diet and given orally 1 ml/kg/day distilled water for 90 days.
2. *High cholesterol diet group*: Rats were fed high cholesterol diet and given orally 1 ml/kg/day distilled water for 90 days.
3. *Normal diet-barakol treated group*: Rats were fed normal diet and given orally 30 mg/kg/day barakol solution for 90 days.
4. *High cholesterol diet-barakol treated group*: Rats were fed high cholesterol diet and given orally 30 mg/kg/day barakol solution for 90 days.

Note
- High cholesterol diet composed of 1% cholesterol diet mixed with sodium choleate 2%.
- The oral dosage of barakol (30 mg/kg/day) used in this study was higher than the effective dose for anxiolysis (10 mg/kg) found in rats given intraperitoneally (Thongsaard et al., 1996).

2. Blood sampling for blood clinical biochemistry parameters and hematology

At the end of the treatment, animals were fasted for 10 hours before anesthesized with intraperitoneally 0.6-1 ml of pentobarbital sodium (Nembutal®). Blood was drawn from the left ventricle for an approximate volume of 5 ml. Five hundred microliter of whole blood was transferred to a microtube containing a few grains of EDTA sodium and mixed thoroughly. The remaining blood was transferred to another tube, allowed to stand in a slope posture in order to collect the highest amount of serum and kept at -4^{o}C until the time of analysis. Whole blood and serum

were investigated for the hematology and blood clinical biochemistry parameters, respectively.

2.1 Blood clinical biochemistry parameters

For serum samples, the following clinical biochemistry parameters were analyzed by the Faculty of Allied Health Sciences, Chulalongkorn University, Bangkok: serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), alkaline phosphatase (ALP), blood urea nitrogen (BUN), serum creatinine (SCr), total cholesterol, triglyceride (TG) and glucose. Total bilirubin, direct bilirubin, low density lipoprotein cholesterol (LDL-C) and high density lipoprotein cholesterol (HDL-C) in serum samples were analyzed by the Professional Laboratory Management Corp. Ltd., Bangkok.

2.2 Hematology

Hemoglobin (Hb), hematocrit (Hct), platelet count, white blood cell (WBC) count and % differential WBCs were determined by the Faculty of Allied Health Sciences, Chulalongkorn University, Bangkok.

3. Liver microsome preparation

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

After collecting blood sample, liver was immediately removed and perfused with ice-cold 0.9% w/v NaCl until the entire organ became pale. Then, the liver was washed out the blood with ice-cold 0.9% w/v NaCl and blotted dry with gauze. The whole liver was weighed, cut into pieces and homogenized with 3 volume of phosphate buffer, pH 7.4. The liver homogenate was centrifuged at 10,000 *g* for 30 minutes at 4^{o}C using refrigerated superspeed centrifuge. The supernatant (S9, post mitochondrial fraction) was transferred into ultracentrifuge tubes and centrifuged at 100,000 *g* for 60 minutes at 4^{o}C using refrigerated ultracentrifuge. The supernatant (cytosolic fraction) was kept and the pellets (microsomal fraction) were resuspended

with 3-5 ml of phosphate buffer, pH 7.4 containing 20% glycerol. Cytosolic and microsomal fractions were aliquoted to microtubes and stored at -80^{o}C until the time of enzyme activity assays.

4. Determination of protein concentration

Liver cytosolic and 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 contained Na_2CO_3, NaOH, Sodium citrate and cupric sulfate solutions in a 100:10:1:1 ratio, respectively.

Procedures

All standard and unknown samples were duplicated.

1. The following reagents were added into each standard 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)

After addition of these reagents, each tube was mixed thoroughly.

2. To each unknown sample tube, 490 μl of 0.5 M NaOH and 10 μl of microsomal or cytosolic sample were added and then mixed thoroughly.

3. Six and a half milliliter of freshly prepared working protein reagent was added to each tube.
4. The tubes were allowed to stand at room temperature for 10 minutes. Then, 200 μl of Folin & Ciocalteu's phenol reagent was added to each tube and immediately vortexed for a minimum of 30 seconds.
5. 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 at 500 nm using 0 μg standard sample as a blank.

Calculations

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. The protein concentration was expressed in mg/ml or μg/μl by dividing its amount of protein with the volume of microsomal or cytosolic sample used in the reaction.

5. Spectral determination of total CYP contents

Total CYP contents in microsomal samples 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 samples were diluted to 2 mg/ml with 0.1 M Tris buffer, pH 7.4 containing 20% v/v glycerol.
2. Of the total volume of 5 ml diluted samples, a few grains of sodium dithionite were added with gentle mixing, then 2.5 ml each was transferred to the sample and reference cuvettes.
3. Both cuvettes were placed in a spectrophotometer, adjusted to zero and corrected to a baseline between 400 nm to 500 nm.
4. The sample cuvette was bubbled with carbon monoxide (approximately 1 bubble/second) for about 1 minute, immediately placed in a spectrophotometer again 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:

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

6. Determination of CYP activities

6.1 Alkoxyresorufin O-dealkylation assays

The catalytic activities of CYP1A1, CYP1A2, CYP2B1/2B2 were determined by measuring the rate of O-dealkylation of ER, MR, and BR & PR, respectively, using the method of Burke and Mayer (1974) and Lubet et al. (1985) with some modification.

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 and made up to 10 ml with DMSO.
    b) 0.5 mM BR (MW = 303)

       BR 1.515 mg was dissolved and made up to 10 ml with DMSO.
    c) 0.5 mM ER (MW = 241)

       ER 1.205 mg was dissolved and made up to 10 ml with DMSO.
    d) 0.5 mM PR (MW = 283)

       PR 1.415 mg was dissolved and made up to 10 ml with DMSO.
    e) 0.5 mM Resorufin (MW = 235)

       Resorufin 1.175 mg was dissolved and made up to 10 ml with DMSO.
4. NADPH regenerating system

   NADPH regenerating system contained the solution as following:
    a) 0.1 M NADP, pH 7.4

       NADP 0.765 g was dissolved and made up to 10 µl with 20 mM K_3PO_4. The solution pH was adjusted to 7.4 with HCl or NaOH. (10 µl contains 1 mmol of NADP)
    b) 0.5 M G6P, pH 7.4

       G6P 1.41 g was dissolved and made up to 10 µl with 20 mM K_3PO_4. The solution pH was adjusted to 7.4 with HCl or NaOH. (10 µl contains 5 mmol of G6P)
    c) 0.3 M $MgCl_2$, pH 7.4

       $MgCl_2$ 609.93 mg was dissolved and made up to 10 µl with 20 mM K_3PO_4. The solution pH was adjusted to 7.4 with HCl or NaOH. (10 µl contains 3 mmol of $MgCl_2$)
    d) G6PD

G6PD was diluted to 100 units per ml with 20 mM K_3PO_4, pH 7.4 (10 μl contains 1 unit of G6PD)

On the experiment, the mixture of NADP, G6P and $MgCl_2$ solutions was freshly prepared in the ratio of 1: 1: 1, respectively. For the reaction volume of 1 ml, 30 μl of this mixture was used for microsomal preincubation and 10 μl of G6PD was added to initiate the reaction.

Procedures

1. For the final reaction volume of 1.5 ml, microsomal sample was diluted with 0.1 M Tris buffer, pH 7.4 to measure out 300 μg of protein.
2. The following solutions were added for the microsomal preincubation
    a) 0.1 M Tris buffer, pH 7.4 1225 μl
    b) 0.5 mM Alkoxyresorufin 15 μl
    c) The mixture of NADPH regenerating system 45 μl containing
        - 0.1 M NADP 15 μl
        - 0.5 M G6P 15 μl
        - 0.3 M $MgCl_2$ 15 μl
    d) Diluted microsomal suspension 200 μl
3. Three tubes were used for each microsomal sample. One was a sample blank and the other two were samples.
4. Each tube was preincubated in a 37^{o}C shaking water bath for 2 minutes.
5. The reaction was started by adding 15 μl of G6PD. Sample blank was added with 15 μl of 0.1 M Tris buffer instead of G6PD.
6. After 5-minute incubation, the reaction was stopped with methanol 1.5 ml.
7. All tubes were centrifuged at 3,000 r.p.m. for 10 minutes.
8. Using an autopipette, clear supernatant of each tube was transferred to a cuvette.
9. The absorbance was read on the Fluorescence spectrophotometer by using an excitation wavelength of 556 nm and an emission wavelength of 588 nm.

10. A resorufin standard curve was constructed using the duplicated resorufin concentrations of 0.002, 0.005, 0.010, 0.050 and 0.200 nmol/ml.
11. Rate of dealkylation was calculated by determining the amount of resorufin formed, extrapolating to mg of protein and dividing by 5 minutes of total reaction period.

6.2 Aniline 4-hydroxylation assay

The catalytic activity of CYP2E1 was determined based on the rate of aniline 4-hydroxylation, using the method of Schenkman, Remmer, and Estabrook (1967).

Reagents

1. 10 mM Aniline HCl
   Aniline HCl 93 mg was dissolved and made up to 100 ml with double distilled water. The solution was stored in a dark bottle.
2. 6% w/v TCA
   TCA 60 g was made up to 1 L with double distilled water.
3. 20% w/v TCA
   TCA 200 g was made up to 1 L with double distilled water.
4. 1% w/v Phenol
   Phenol 20 g and NaOH 40 g were made up to 2 L with double distilled water.
5. 1 M Na_2CO_3
   Anhydrous Na_2CO_3 200 g was made up to 2 L with double distilled water.
6. 10 μM 4-aminophenol
   4-aminophenol 0.0365 g were dissolved in methanol 1 ml, then made up to 10 ml with double distilled water. This aminophenol solution 0.1 ml was taken and then added into TCA 15 g. Finally, made up to 250 ml with double distilled water.
7. NADPH regenerating system (The preparation was discussed in 6.1)

Procedures

1. To make a final volume of 2 ml microsomal incubation, each preincubation mixture composed of microsomes containing 5 mg protein, Aniline HCl 500 μl, NADPH regenerating system 30 μl and Tris buffer, pH 7.4 qs to 1980 μl.
2. All tubes were preincubated in a shaker bath at 37^{o}C for 2 minutes.
3. The catalytic reaction was started by an addition of G6PD 20 μl. For sample blank, 20 μl of Tris buffer, pH 7.4 was used instead of G6PD.
4. After the microsomal samples were incubated for 30 minutes, the reaction was terminated by an addition of 1 ml of 20% TCA. The tubes were placed on ice for 5 minutes.
5. The solution was centrifuged at 3,000 r.p.m. for 5 minutes. One milliliter of supernatant was transferred to another tube, then, 1 ml of phenol and 1 ml of Na_2CO_3 were added to each tube and mixed homogeneously.
6. All tubes were allowed to stand at room temperature for 30 minutes. The absorbance of the mixture was measured spectrophotometrically at 630 nm.
7. For standard curve, 1 ml of each standard 4-aminophenol solution (0, 2, 4, 6, 8 and 10 μM) was carried out instead of the supernatant in step 5.

Calculations

Rate of aniline 4-hydroxylation was calculated by determining the amount of 4-aminophenol formed (nmol), extrapolating to mg of microsomal protein and dividing by 30 minutes of incubation period.

7. Determination of GST activity

The catalytic activity of cytosolic GST was determined spectrophotometrically at 340 nm according to the method modified from the methods of Habig and Jokoby (1981) and Jensson, Alin, and Mannervik (1985).

Reagents

1. 0.1 M Sodium phosphate buffer, pH 6.5 containing 1 mM EDTA sodium
   Sodium phosphate dibasic 1.4196 g and EDTA sodium 0.0372 g were dissolved in double distilled water, made up to 100 ml and adjusted to pH 6.5 with HCl or NaOH.
2. 20 mM CDNB in 95% ethanol
   CDNB 0.1015 g was dissolved in a 25 ml of 95% ethanol.
3. 20 mM GSH
   GSH 0.1535 g was dissolved in a 25 ml of double distilled water.

Procedures

1. For the total volume of 500 µl, a 450 µl sodium phosphate buffer, 25 µl GSH and 25 µl CDNB solutions were mixed well in both reference and sample cuvettes.
2. Both cuvettes were placed in the Spectrophotometer and adjusted to zero.
3. The reaction was started by adding 5 µl of 0.1 M sodium phosphate buffer and 5 µl of cytosolic sample into reference and sample cuvettes, respectively.
4. Immediately placed the both cuvettes back and pressed start to observe the absorbance change for 30 seconds
5. The absorbance change (ΔA) at each increasing point of time (ΔT) along the initial of linear range was determined.
6. The catalytic activity of GST was performed in unit per mg protein per minute. The extinction coefficient of CDNB at 340 nm was 9.6 $mM^{-1}cm^{-1}$. Using the Beer's law, the equation was given:

$$\text{GST activity (nmol/ mg protein /min)} = \frac{\Delta A \times 625000}{\Delta T \text{ (sec)} \times \text{protein concentration (mg/ml)}}$$

8. Data analysis

All numeric data were presented as mean ± SE. Statistical analysis was run on SPSS for window version 7.5. Independent-samples *t* test was used for comparisons between two groups at a significant level of $p < 0.05$.

CHAPTER IV

RESULTS

Subchronic effects of barakol were investigated on hepatic CYP and GST activities as well as on blood clinical biochemistry parameters and hematology in rats fed normal and high cholesterol diets using an *ex vivo* model. During the time of treatment, record of their body weight was made every 2 weeks. Thirty-two male Wistar rats completed these studies after participating in 90-day treatment.

Effects of barakol on body weight gain, terminal body weight, liver weight and relative liver weight

Barakol demonstrated no effects on body weight gain (Figure 4.1), terminal body weight, liver weight and relative liver weight (Table 4.1) in both normal and high cholesterol diet rats. However, high cholesterol diet rats comparing to normal diet control rats showed a significant increase of liver weight (16.32 ± 1.02 vs. 10.36 ± 0.23; $p < 0.05$) and relative liver weight (3.55 ± 0.24 vs. 2.34 ± 0.08; $p < 0.05$).

Effects of barakol on hepatic CYP and GST

Phase I hepatic CYP was examined by using microsomal samples. Subchronic effects of oral 30 mg/kg/day barakol administration in normal and high cholesterol diet conditions were compared with their corresponding diet control groups. There was no change of total CYP contents in both barakol treatment groups (Figure 4.2). High cholesterol diet-barakol treated rats demonstrated a significant reduction of ethoxyresorufin O-dealkylase (EROD) activity which represented the activity of CYP1A1 (Figure 4.3). Activity of methoxyresorufin O-dealkylase (MROD) which represented the activity of CYP1A2 was about 3 fold reduced ($p < 0.05$) in both barakol treated groups (Figure 4.4). No significant effects of barakol were found on

activities of benzyloxyresorufin O-dealkylase (BROD) & pentoxyresorufin O-dealkylase (PROD) which represented the activity of CYP2B1/2B2 (Figure 4.5-4.6) as well as aniline 4-hydroxylase which represented the activity of CYP2E1 (Figure 4.7) in both normal and high cholesterol diet conditions. However, feeding rats with high cholesterol diet significantly decreased CYP2E1 as comparing to normal diet control group (0.150 ± 0.026 vs. 0.227 ± 0.015; $p < 0.05$).

For phase II reaction, GST activity was examined by using cytosolic samples. No significant effects of barakol were found on GST activity in both normal and high cholesterol diet conditions.

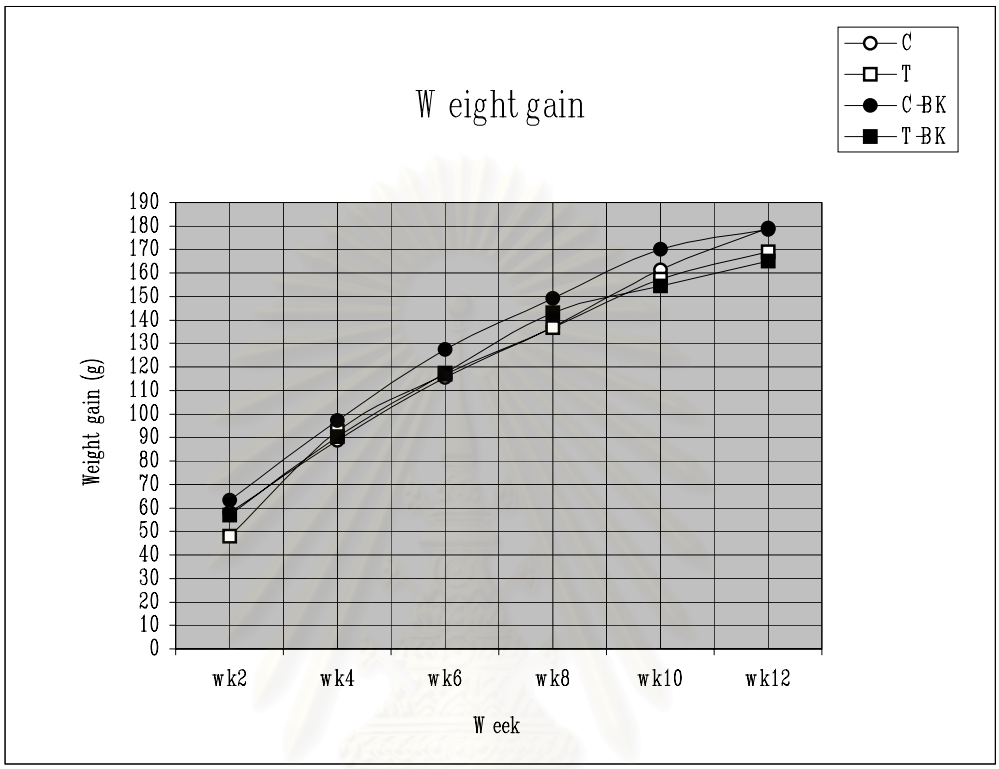


Figure 4.1 Subchronic effects of barakol on 2-week body weight gain

Rats were given normal diet (C), high cholesterol diet (T), normal diet with oral 30 mg/kg/day barakol (C-BK) and high cholesterol diet with oral 30 mg/kg/day barakol (T-BK) for 90 days. Body weight gain of each rat was recorded every two weeks. The individual mark represents mean of body weight gained from those of the initial weight. The statistical analysis was determined by using independent-samples *t* test for comparisons between two groups (C-BK vs. C and T-BK vs. T) at a significant level of $p < 0.05$.

	Group			
	C	C-BK	T	T-BK
Terminal body weight (g)	444 ± 11	426 ± 14	463 ± 16	422 ± 22
Liver weight (g)	10.36 ± 0.23	10.29 ± 0.60	16.32 ± 1.02	16.29 ± 1.25
Relative liver weight (% of body weight)	2.34 ± 0.08	2.41 ± 0.11	3.55 ± 0.24	3.82 ± 0.15

Table 4.1 Subchronic effects of barakol on terminal body weight, liver weight and relative liver weight

Rats were given normal diet (C), high cholesterol diet (T), normal diet with oral 30 mg/kg/day barakol (C-BK) and high cholesterol diet with oral 30 mg/kg/day barakol (T-BK) for 90 days. Each rat was recorded on its body weight and liver weight at the end of experiment. Relative liver weight denotes percent of liver weight per terminal body weight. Values perform as mean ± SE. The statistical analysis was determined by using independent-samples *t* test for comparisons between two groups (C-BK vs. C and T-BK vs. T) at a significant level of $p < 0.05$.

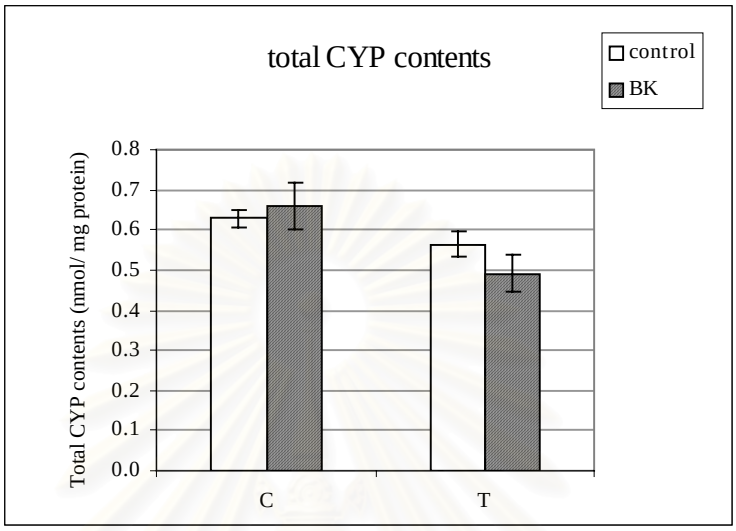


Figure 4.2 Subchronic effects of barakol on rat hepatic total CYP contents using an *ex vivo* model

Rats were fed normal diet (C) or high cholesterol diet (T) along with giving oral 1 ml/kg/day distilled water (control) or 30 mg/kg/day barakol (BK) for 90 days. Liver microsomal samples were measured for total CYP contents. The individual bar represents mean of total CYP contents with an error bar of standard error of the mean. The statistical analysis was determined by using independent-samples *t* test for comparisons between two groups at a significant level of $p < 0.05$.

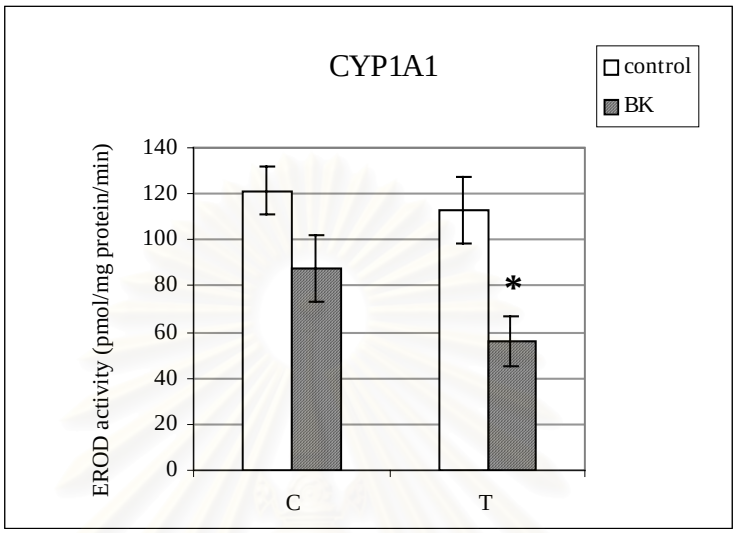


Figure 4.3 Subchronic effects of barakol on rat hepatic CYP1A1 activity using an *ex vivo* model

Rats were fed normal diet (C) or high cholesterol diet (T) along with giving oral 1 ml/kg/day distilled water (control) or 30 mg/kg/day barakol (BK) for 90 days. Liver microsomal samples were measured for EROD activity. The individual bar represents mean of EROD activity with an error bar of standard error of the mean. The statistical analysis was determined by using independent-samples *t* test for comparisons between two groups at a significant level of $p < 0.05$.

* significantly different from the corresponding diet control group.

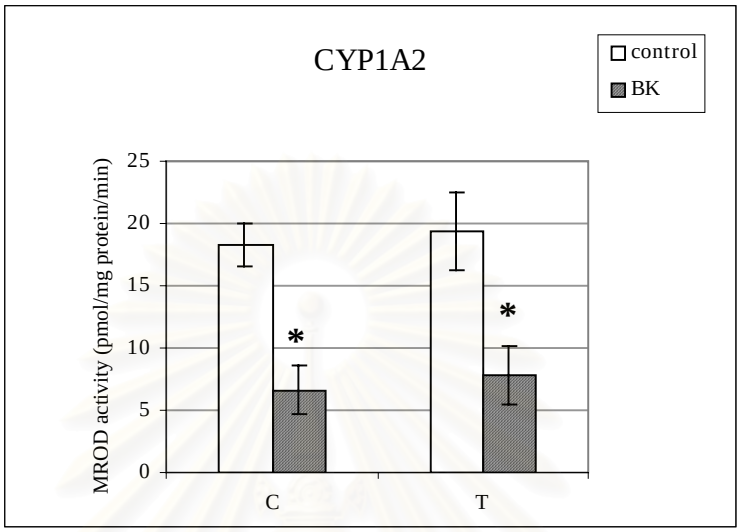


Figure 4.4 Subchronic effects of barakol on rat hepatic CYP1A2 activity using an *ex vivo* model

Rats were fed normal diet (C) or high cholesterol diet (T) along with giving oral 1 ml/kg/day distilled water (control) or 30 mg/kg/day barakol (BK) for 90 days. Liver microsomal samples were measured for MROD activity. The individual bar represents mean of MROD activity with an error bar of standard error of the mean. The statistical analysis was determined by using independent-samples *t* test for comparisons between two groups at a significant level of $p < 0.05$.

* significantly different from the corresponding diet control groups.

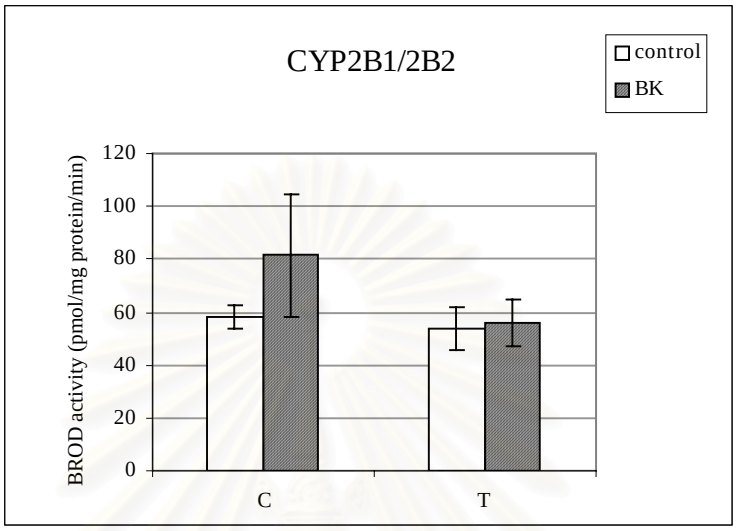


Figure 4.5 Subchronic effects of barakol on rat hepatic CYP2B1/2B2 (BROD) activity using an *ex vivo* model

Rats were fed normal diet (C) or high cholesterol diet (T) along with giving oral 1 ml/kg/day distilled water (control) or 30 mg/kg/day barakol (BK) for 90 days. Liver microsomal samples were measured for BROD activity. The individual bar represents mean of BROD activity with an error bar of standard error of the mean. The statistical analysis was determined by using independent-samples *t* test for comparisons between two groups at a significant level of $p < 0.05$.

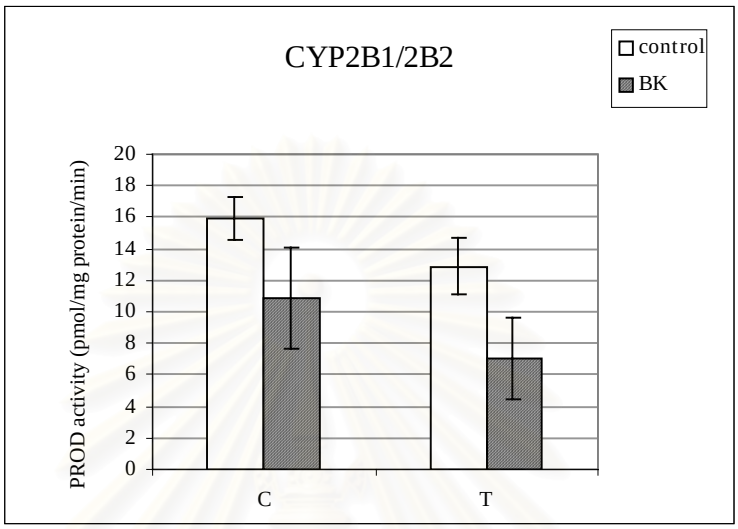


Figure 4.6 Subchronic effects of barakol on rat hepatic CYP2B1/2B2 (PROD) activity using an *ex vivo* model

Rats were fed normal diet (C) or high cholesterol diet (T) along with giving oral 1 ml/kg/day distilled water (control) or 30 mg/kg/day barakol (BK) for 90 days. Liver microsomal samples were measured for PROD activity. The individual bar represents mean of PROD activity with an error bar of standard error of the mean. The statistical analysis was determined by using independent-samples *t* test for comparisons between two groups at a significant level of $p < 0.05$.

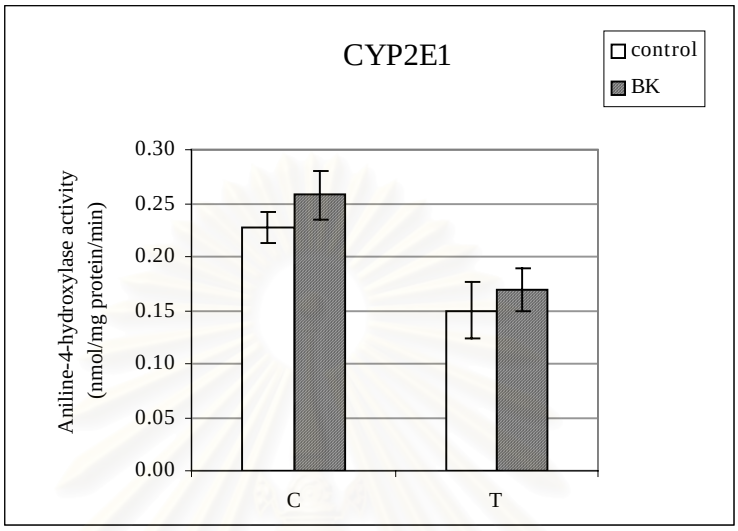


Figure 4.7 Subchronic effects of barakol on rat hepatic CYP2E1 activity using an *ex vivo* model

Rats were fed normal diet (C) or high cholesterol diet (T) along with giving oral 1 ml/kg/day distilled water (control) or 30 mg/kg/day barakol (BK) for 90 days. Liver microsomal samples were measured for aniline 4-hydroxylase activity. The individual bar represents mean of aniline 4-hydroxylase activity with an error bar of standard error of the mean. The statistical analysis was determined by using independent-samples *t* test for comparisons between two groups at a significant level of $p < 0.05$.

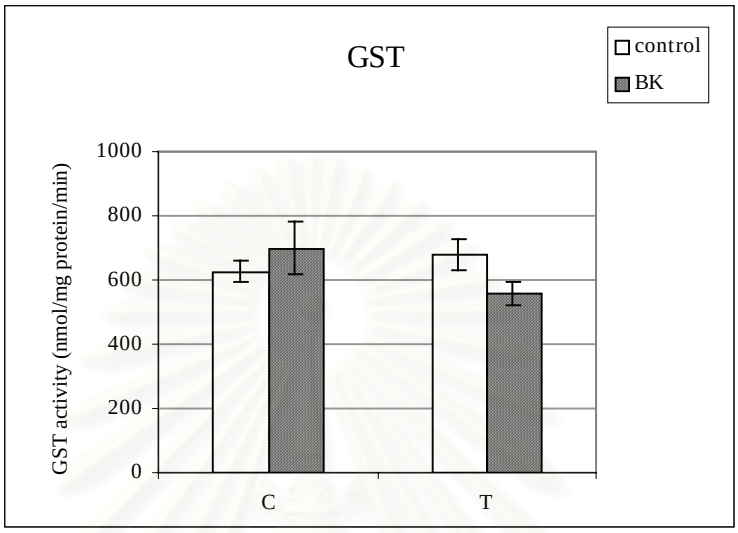


Figure 4.8 Subchronic effects of barakol on rat hepatic GST activity using an *ex vivo* model

Rats were fed normal diet (C) or high cholesterol diet (T) along with giving oral 1 ml/kg/day distilled water (control) or 30 mg/kg/day barakol (BK) for 90 days. Liver cytosolic samples were measured for GST activity. The individual bar represents mean of GST activity with an error bar of standard error of the mean. The statistical analysis was determined by using independent-samples *t* test for comparisons between two groups at a significant level of $p < 0.05$.

Effects of barakol on blood clinical biochemistry parameters and hematology

Serum sample and whole blood of individual rat were measured for blood clinical biochemistry parameters and hematology, respectively, at the end of treatment. Subchronic exposure (90 days) of oral 30 mg/kg/day of barakol in rats fed normal and high cholesterol diets demonstrated a significant increase of both serum total and direct bilirubin (Figure 4.12) but a significant decrease of TG (Figure 4.19) comparing to their corresponding diet control rats. In addition, barakol showed an advantageous effect on liver function of high cholesterol diet fed rats as demonstrated by a decrease of ALP (Figure 4.11) comparing to the corresponding high cholesterol diet group. There were no statistically significant effects of barakol on the following parameters: SGOT (Figure 4.9), SGPT (Figure 4.10), BUN (Figure 4.13), SCr (Figure 4.14), total cholesterol (Figure 4.15), LDL-C (Figure 4.16), HDL-C (Figure 4.17), LDL-C/HDL-C ratio (Figure 4.18), glucose (Figure 4.20), Hb (Figure 4.21), Hct (Figure 4.22), platelet count (Figure 4.23), WBC count (Figure 4.24) and % differential WBCs (Figure 4.25) in both dietary conditions.

Comparing to normal diet rats, high cholesterol diet rats demonstrated significant increases of SGOT (274 ± 30 vs. 179 ± 10; $p < 0.05$), SGPT (188 ± 48 vs. 36 ± 2; $p < 0.05$), ALP (91 ± 5 vs. 62 ± 3; $p < 0.05$), total cholesterol (92 ± 10 vs. 64 ± 4; $p < 0.05$), LDL-C (63 ± 11 vs. 8 ± 0.5; $p < 0.05$) and LDL-C/HDL-C ratio (0.868 ± 0.186 vs. 0.101 ± 0.006; $p < 0.05$). Markedly increases of SGPT and LDL-C/HDL-C ratio in high cholesterol diet rats accounted for about 5 and 9 fold, respectively, of those in the normal diet rats.

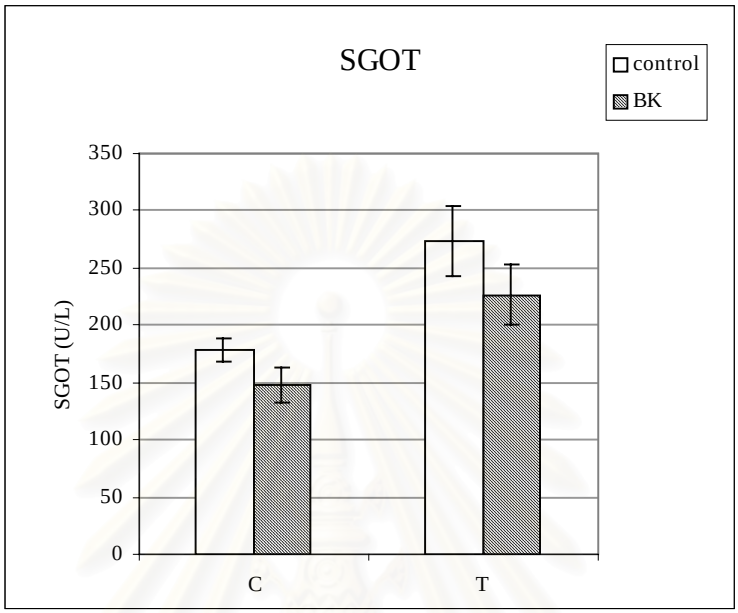


Figure 4.9 Subchronic effects of barakol on SGOT using an *ex vivo* model

Rats were fed normal diet (C) or high cholesterol diet (T) along with giving oral 1 ml/kg/day distilled water (control) or 30 mg/kg/day barakol (BK) for 90 days. Serum samples were measured for SGOT concentration. The individual bar represents mean of SGOT concentration with an error bar of standard error of the mean. The statistical analysis was determined by using independent-samples *t* test for comparisons between two groups at a significant level of $p < 0.05$.

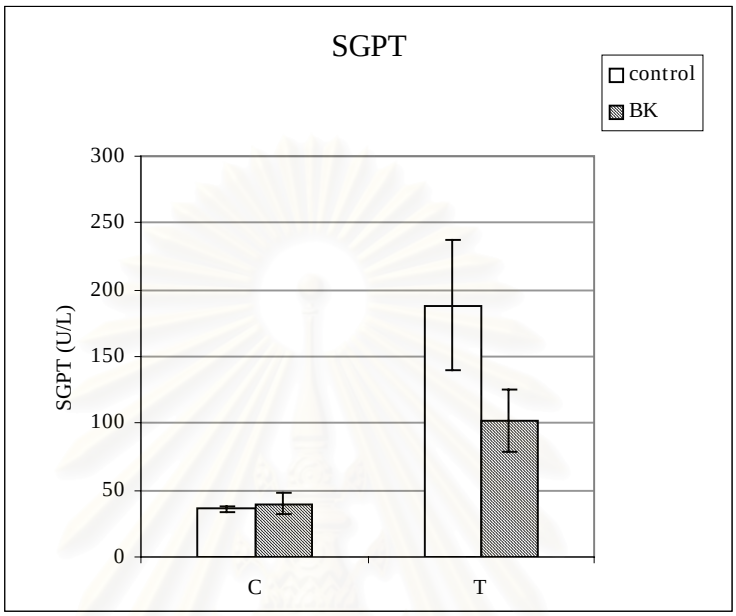


Figure 4.10 Subchronic effects of barakol on SGPT using an *ex vivo* model
Rats were fed normal diet (C) or high cholesterol diet (T) along with giving oral 1 ml/kg/day distilled water (control) or 30 mg/kg/day barakol (BK) for 90 days. Serum samples were measured for SGPT concentration. The individual bar represents mean of SGPT concentration with an error bar of standard error of the mean. The statistical analysis was determined by using independent-samples *t* test for comparisons between two groups at a significant level of $p < 0.05$.

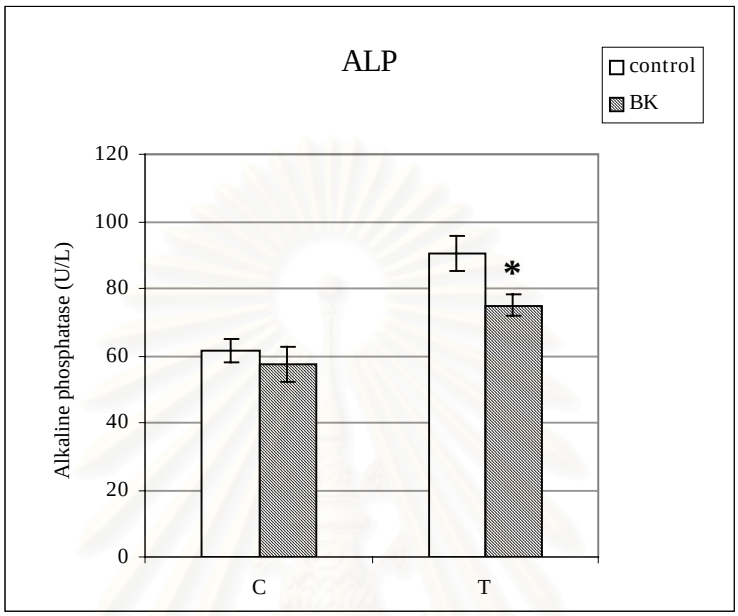


Figure 4.11 Subchronic effects of barakol on ALP using an *ex vivo* model

Rats were fed normal diet (C) or high cholesterol diet (T) along with giving oral 1 ml/kg/day distilled water (control) or 30 mg/kg/day barakol (BK) for 90 days. Serum samples were measured for ALP concentration. The individual bar represents mean of ALP concentration with an error bar of standard error of the mean. The statistical analysis was determined by using independent-samples *t* test for comparisons between two groups at a significant level of $p < 0.05$.

* significantly different from the corresponding diet control group

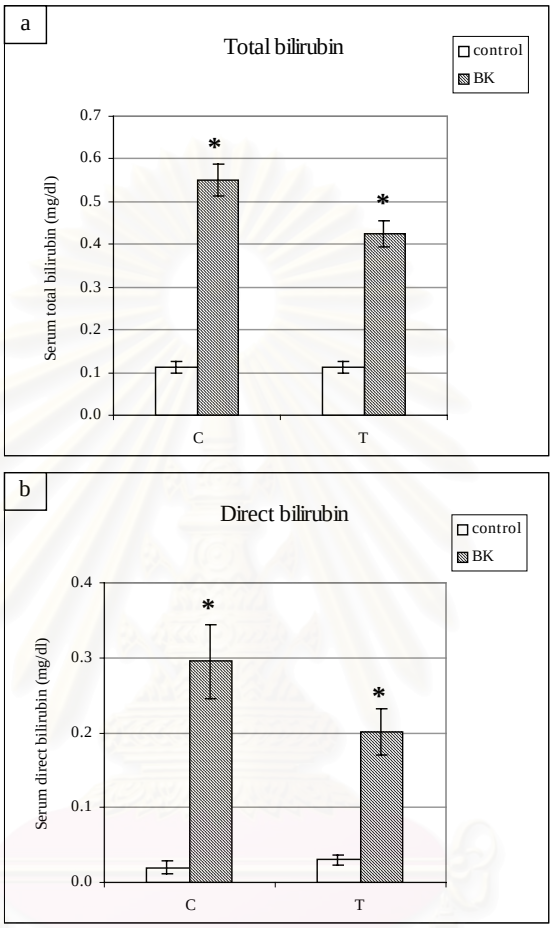


Figure 4.12 Subchronic effects of barakol on total and direct bilirubin using an *ex vivo* model

Rats were fed normal diet (C) or high cholesterol diet (T) along with giving oral 1 ml/kg/day distilled water (control) or 30 mg/kg/day barakol (BK) for 90 days. Serum samples were measured for total bilirubin (a) and direct bilirubin (b) concentration. The individual bar represents mean of serum bilirubin concentration with an error bar of standard error of the mean. The statistical analysis was determined by using independent-samples *t* test for comparisons between two groups at a significant level of $p < 0.05$.

* significantly different from the corresponding diet control groups

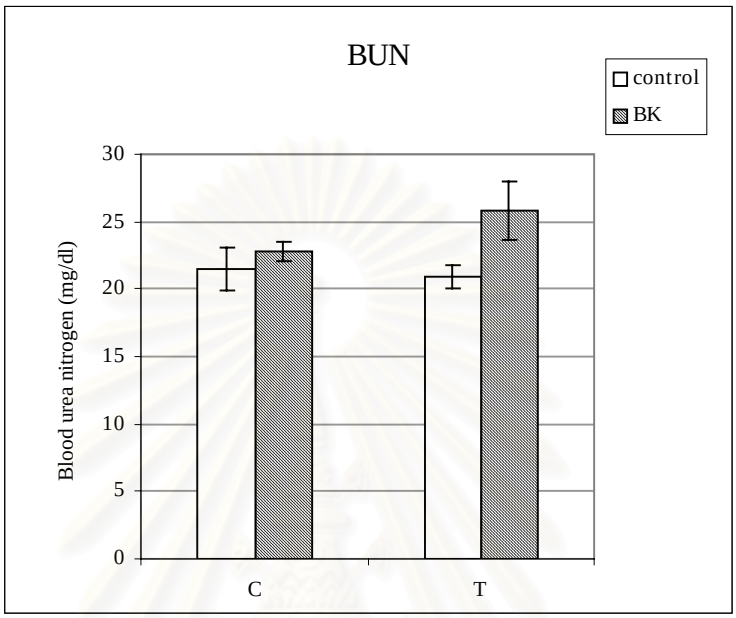


Figure 4.13 Subchronic effects of barakol on BUN using an *ex vivo* model
Rats were fed normal diet (C) or high cholesterol diet (T) along with giving oral 1 ml/kg/day distilled water (control) or 30 mg/kg/day barakol (BK) for 90 days. Serum samples were measured for BUN concentration. The individual bar represents mean of BUN concentration with an error bar of standard error of the mean. The statistical analysis was determined by using independent-samples *t* test for comparisons between two groups at a significant level of $p < 0.05$.

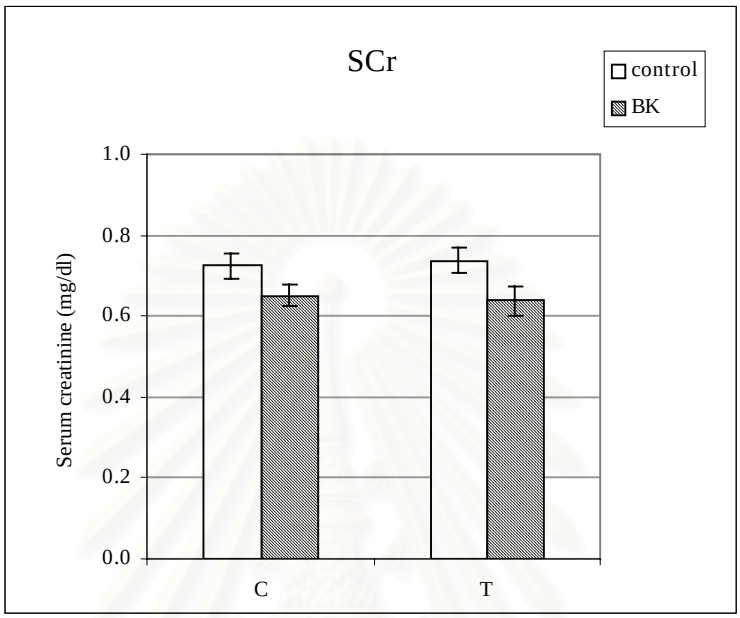


Figure 4.14 Subchronic effects of barakol on SCr using an *ex vivo* model

Rats were fed normal diet (C) or high cholesterol diet (T) along with giving oral 1 ml/kg/day distilled water (control) or 30 mg/kg/day barakol (BK) for 90 days. Serum samples were measured for creatinine concentration. The individual bar represents mean of SCr concentration with an error bar of standard error of the mean. The statistical analysis was determined by using independent-samples *t* test for comparisons between two groups at a significant level of $p < 0.05$.

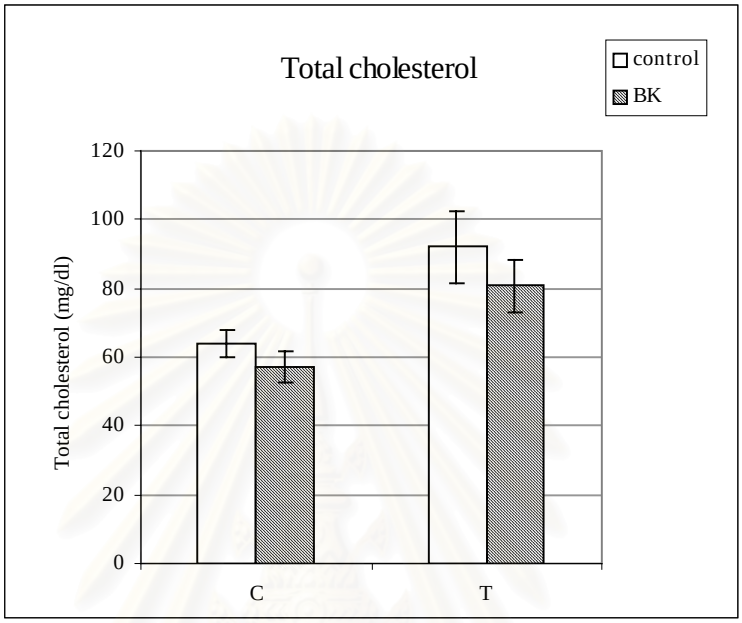


Figure 4.15 Subchronic effects of barakol on total cholesterol using an *ex vivo* model Rats were fed normal diet (C) or high cholesterol diet (T) along with giving oral 1 ml/kg/day distilled water (control) or 30 mg/kg/day barakol (BK) for 90 days. Serum samples were measured for total cholesterol concentration. The individual bar represents mean of total cholesterol concentration with an error bar of standard error of the mean. The statistical analysis was determined by using independent-samples *t* test for comparisons between two groups at a significant level of $p < 0.05$.

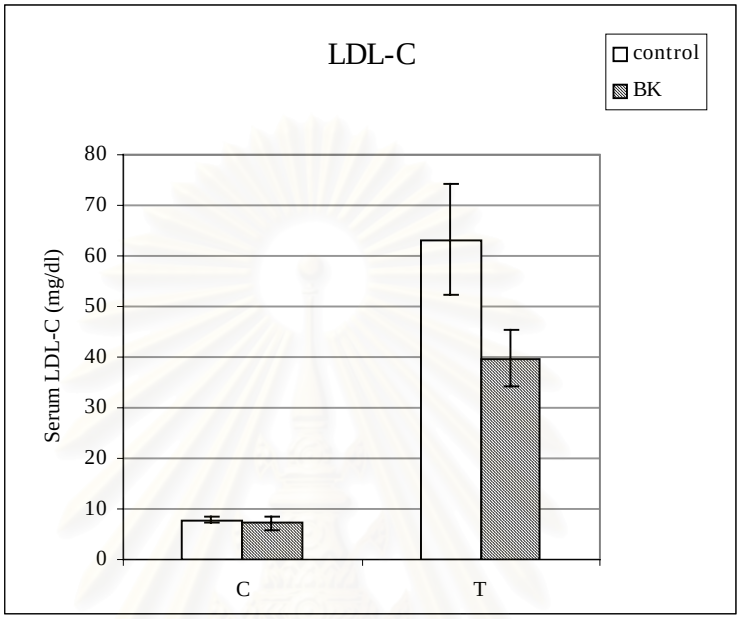


Figure 4.16 Subchronic effects of barakol on LDL-C using an *ex vivo* model
Rats were fed normal diet (C) or high cholesterol diet (T) along with giving oral 1 ml/kg/day distilled water (control) or 30 mg/kg/day barakol (BK) for 90 days. Serum samples were measured for LDL-C concentration. The individual bar represents mean of serum LDL-C concentration with an error bar of standard error of the mean. The statistical analysis was determined by using independent-samples *t* test for comparisons between two groups at a significant level of $p < 0.05$.

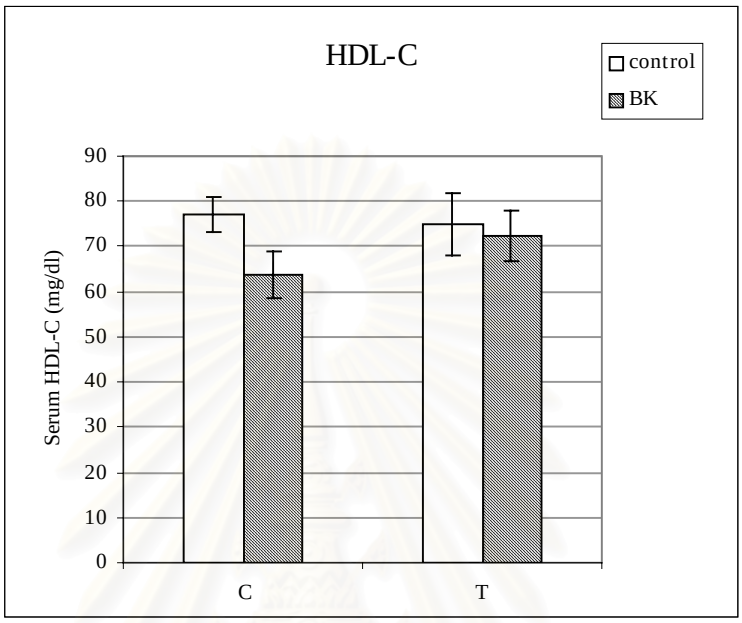


Figure 4.17 Subchronic effects of barakol on HDL-C using an *ex vivo* model
Rats were fed normal diet (C) or high cholesterol diet (T) along with giving oral 1 ml/kg/day distilled water (control) or 30 mg/kg/day barakol (BK) for 90 days. Serum samples were measured for HDL-C concentration. The individual bar represents mean of serum HDL-C concentration with an error bar of standard error of the mean. The statistical analysis was determined by using independent-samples *t* test for comparisons between two groups at a significant level of $p < 0.05$.

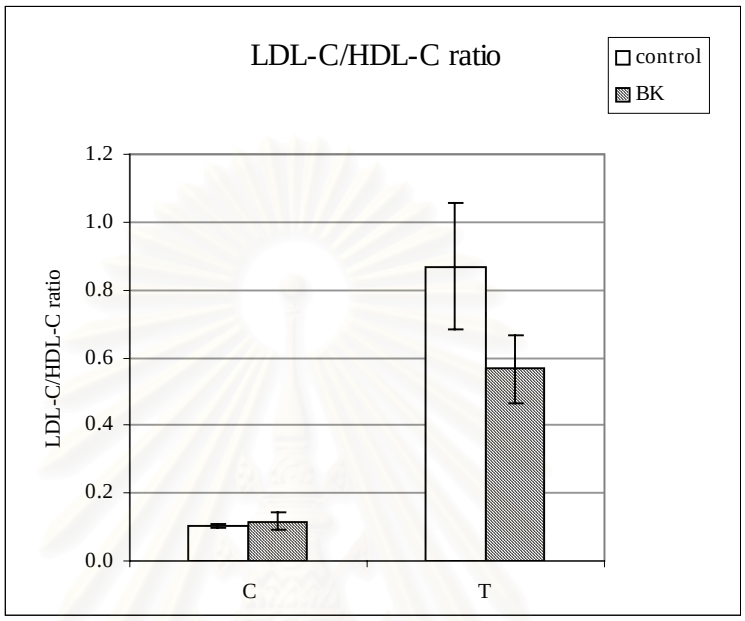


Figure 4.18 Subchronic effects of barakol on LDL-C/HDL-C ratio using an *ex vivo* model

Rats were fed normal diet (C) or high cholesterol diet (T) along with giving oral 1 ml/kg/day distilled water (control) or 30 mg/kg/day barakol (BK) for 90 days. Serum samples were measured for LDL-C/HDL-C ratio. The individual bar represents mean of LDL-C/HDL-C ratio with an error bar of standard error of the mean. The statistical analysis was determined by using independent-samples *t* test for comparisons between two groups at a significant level of $p < 0.05$.

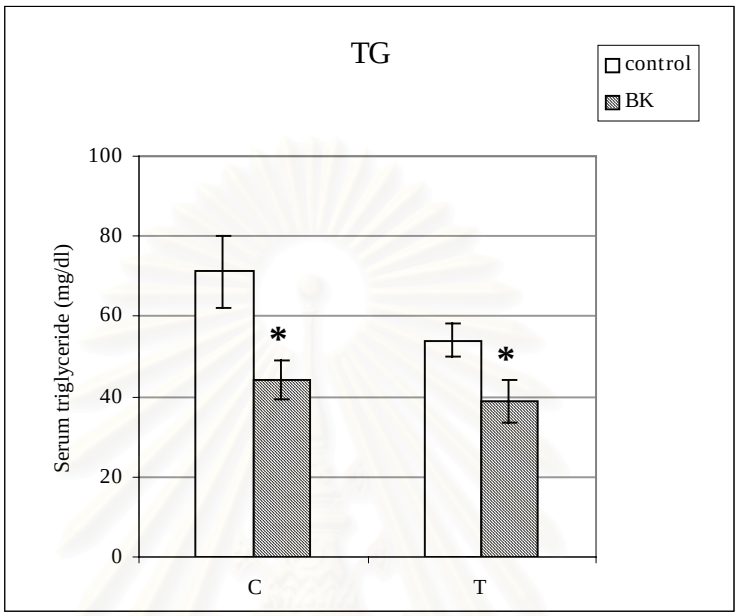


Figure 4.19 Subchronic effects of barakol on TG using an *ex vivo* model

Rats were fed normal diet (C) or high cholesterol diet (T) along with giving oral 1 ml/kg/day distilled water (control) or 30 mg/kg/day barakol (BK) for 90 days. Serum samples were measured for TG concentration. The individual bar represents mean of TG concentration with an error bar of standard error of the mean. The statistical analysis was determined by using independent-samples *t* test for comparisons between two groups at a significant level of $p < 0.05$.

* significantly different from the corresponding diet control groups

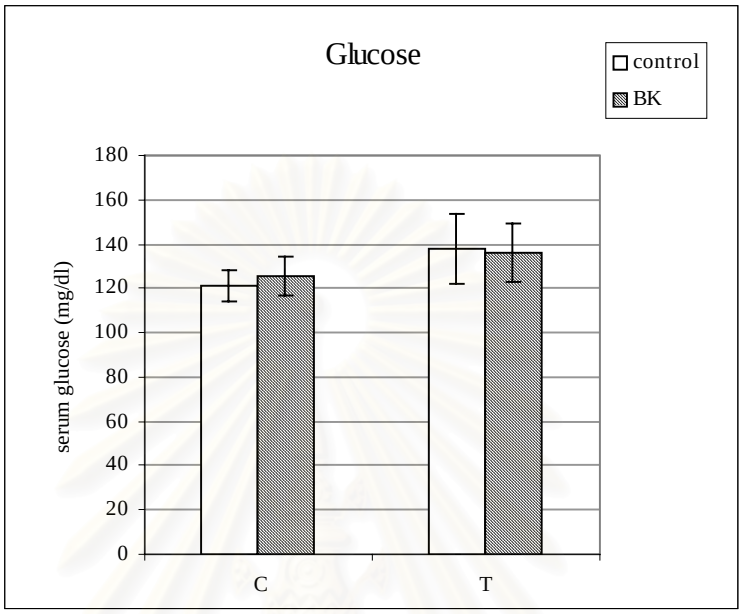


Figure 4.20 Subchronic effects of barakol on serum glucose using an *ex vivo* model Rats were fed normal diet (C) or high cholesterol diet (T) along with giving oral 1 ml/kg/day distilled water (control) or 30 mg/kg/day barakol (BK) for 90 days. Serum samples were measured for glucose concentration. The individual bar represents mean of serum glucose concentration with an error bar of standard error of the mean. The statistical analysis was determined by using independent-samples *t* test for comparisons between two groups at a significant level of $p < 0.05$.

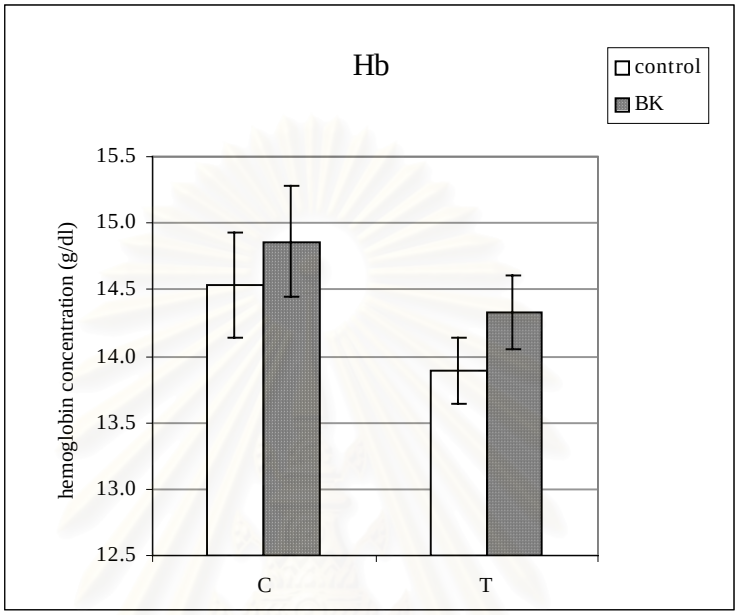


Figure 4.21 Subchronic effects of barakol on Hb using an *ex vivo* model

Rats were fed normal diet (C) or high cholesterol diet (T) along with giving oral 1 ml/kg/day distilled water (control) or 30 mg/kg/day barakol (BK) for 90 days. Whole blood samples were measured for Hb concentration. The individual bar represents mean of Hb concentration with an error bar of standard error of the mean. The statistical analysis was determined by using independent-samples *t* test for comparisons between two groups at a significant level of $p < 0.05$.

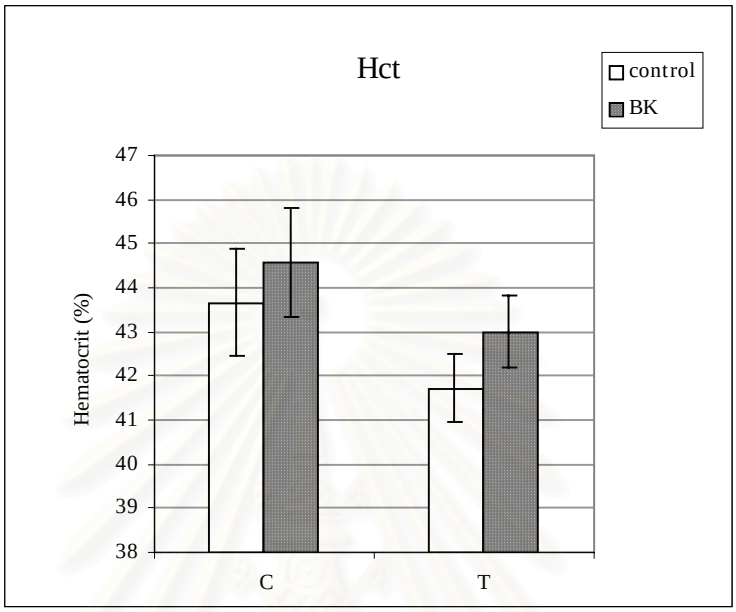


Figure 4.22 Subchronic effects of barakol on Hct using an *ex vivo* model

Rats were fed normal diet (C) or high cholesterol diet (T) along with giving oral 1 ml/kg/day distilled water (control) or 30 mg/kg/day barakol (BK) for 90 days. Whole blood samples were measured for % Hct. The individual bar represents mean of % Hct with an error bar of standard error of the mean. The statistical analysis was determined by using independent-samples *t* test for comparisons between two groups at a significant level of $p < 0.05$.

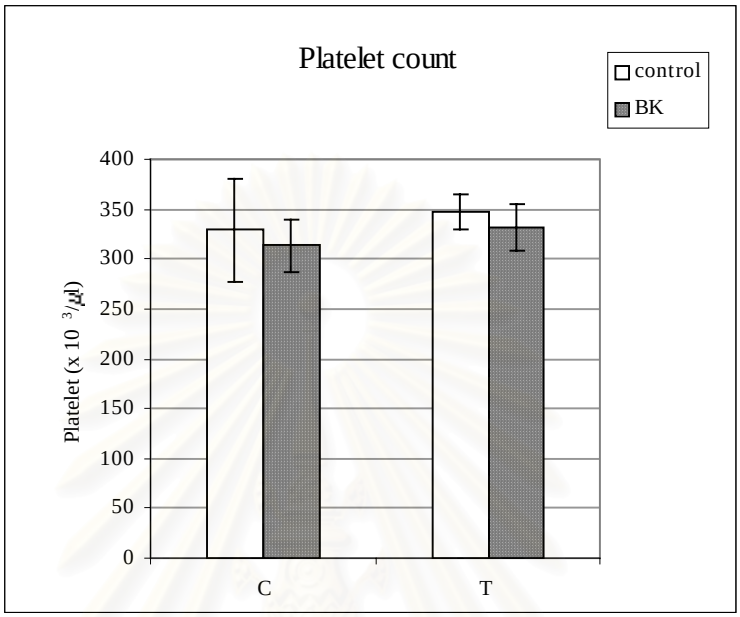


Figure 4.23 Subchronic effects of barakol on platelet count using an *ex vivo* model Rats were fed normal diet (C) or high cholesterol diet (T) along with giving oral 1 ml/kg/day distilled water (control) or 30 mg/kg/day barakol (BK) for 90 days. Whole blood samples were measured for platelet count. The individual bar represents mean of platelet count with an error bar of standard error of the mean. The statistical analysis was determined by using independent-samples *t* test for comparisons between two groups at a significant level of $p < 0.05$.

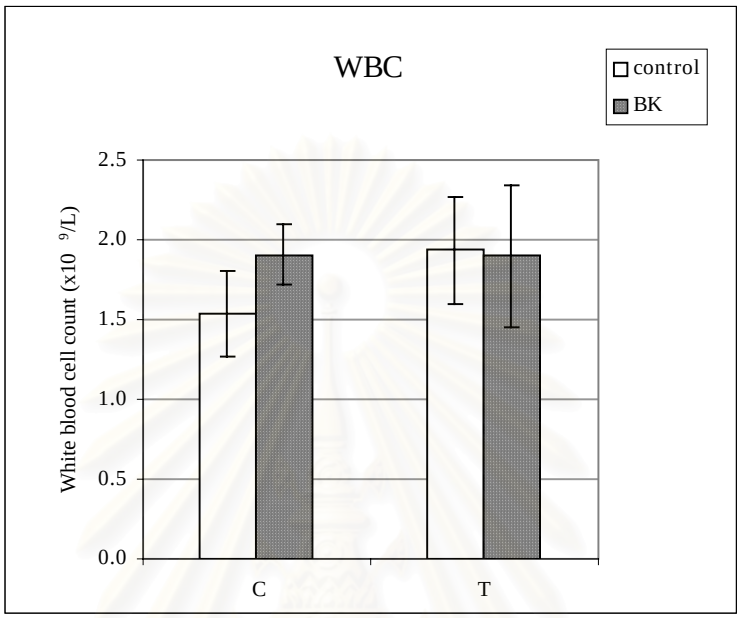


Figure 4.24 Subchronic effects of barakol on WBC count using an *ex vivo* model Rats were fed normal diet (C) or high cholesterol diet (T) along with giving oral 1 ml/kg/day distilled water (control) or 30 mg/kg/day barakol (BK) for 90 days. Whole blood samples were measured for WBC count. The individual bar represents mean of WBC count with an error bar of standard error of the mean. The statistical analysis was determined by using independent-samples *t* test for comparisons between two groups at a significant level of $p < 0.05$.

% differential WBCs

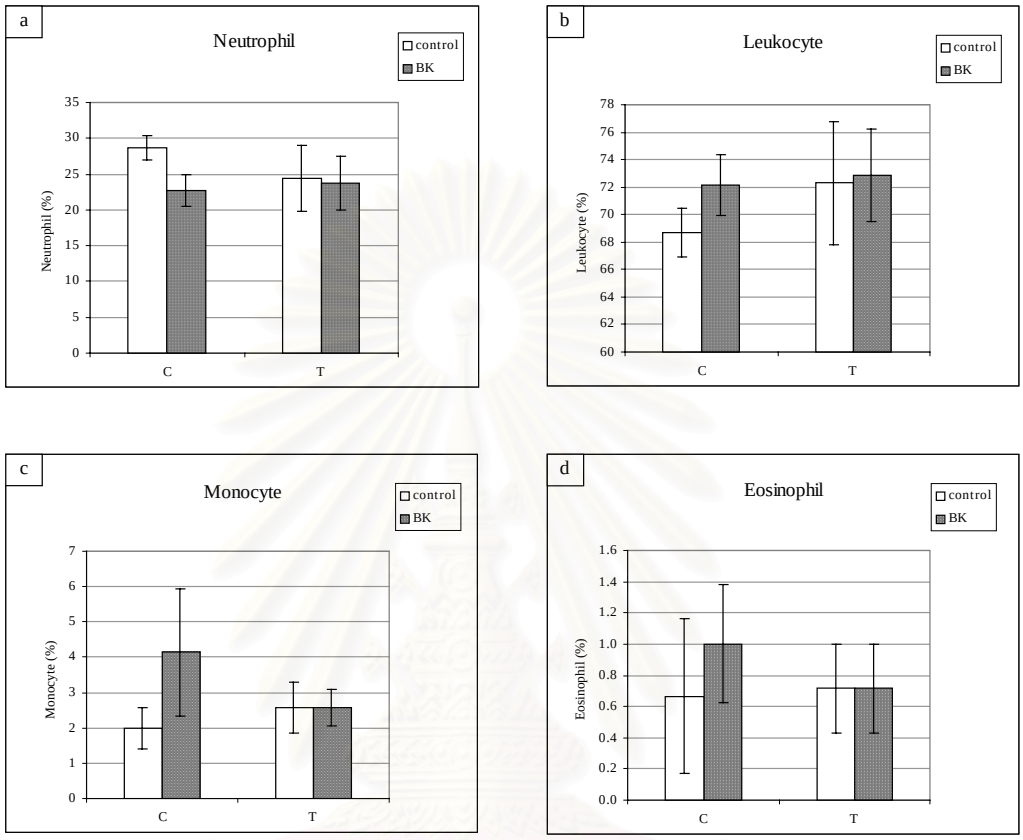


Figure 4.25 Subchronic effects of barakol on % differential WBCs using an *ex vivo* model

Rats were fed normal diet (C) or high cholesterol diet (T) along with giving oral 1 ml/kg/day distilled water (control) or 30 mg/kg/day barakol (BK) for 90 days. Whole blood samples were measured for % differential WBCs. The individual bar represents mean of % differential WBCs that are neutrophil (a), leukocyte (b), monocyte (c) and eosinophil (d) with an error bar of standard error of the mean. The statistical analysis was determined by using independent-samples *t* test for comparisons between two groups at a significant level of $p < 0.05$.

CHAPTER V

DISCUSSION AND CONCLUSION

This study focused on subchronic effects of barakol on hepatic CYP, GST as well as blood clinical biochemistry parameters and hematology in normal and high cholesterol diet fed rats. Animals received normal or high cholesterol diet along with 30 mg/kg/day barakol orally for 90 days.

A study of Thongsaard and collaborators in 1996 found that barakol given intraperitoneally at 10 mg/kg demonstrated an anxiolytic profile on the elevated plus-maze similar to diazepam in rats. An increase of exploratory and locomotor behavior, which was not produced by diazepam, was also found at this dose of barakol administration in rats. This was an interesting advantage of barakol over benzodiazepines such as diazepam regarding its anxiolytic effect without sedation. However, barakol at the higher dose (25 and 50 mg/kg, i.p.), anxiolytic properties and the increased exploratory and locomotor behavior were reduced. At the highest dose (75 mg/kg, i.p.), anxiolytic effect was not observed (Thongsaard et al., 1996). Antianxiety was one of the indications of *C. siamea* capsules in clinical use. Long term treatment of barakol for this indication was found to be hepatotoxic in some patients (สมบัติ ตรีประเสริฐสุข และคณะ, 2543). Therefore, the oral dose of barakol (30 mg/kg/day) used in this study was a trial dose higher than the i.p. dose (10 mg/kg) that expected to be the effective dose in order to investigate subchronic effects of this compound on hepatic drug metabolizing enzymes and blood clinical biochemistry parameters in rats.

Barakol was also found to exhibit some advantage effects on CVS such as hypotensive effect in both systolic and diastolic blood pressure in rats and cats, reduction in the contraction of isolated rat thoracic aorta induced by phenylephrine (Suwan et al., 1992) and reduction of heart rate in anesthesized rats (Momose et al.,

1996). It was documented that cardiovascular disease is a main cause of mortality and disability in the United States and most industrialized nations. The established risk factors included hypertension, hypercholesterolemia, low HDL-C, cigarette smoking, diabetes mellitus as well as non-modifiable personal characteristics such as age, gender and family etc. (Smith, 2000; Elisaf, 2001). Hypercholesterolemia is a risk factor of atherosclerosis which is generally associated with almost cases of cardiovascular diseases (Elisaf, 2001). Therefore, high cholesterol diet condition was additionally performed in this experiment to investigate the effects of barakol at hypercholesterolemia condition. Two percents of sodium choleate (cholic acid) was additionally mixed homogeneously with 1% cholesterol diet in order to enhance intestinal cholesterol absorption (Wang et al., 1999). There is a high variation in plasma cholesterol response to dietary cholesterol across and within animal species. Rat are among the species which are relatively insensitive to dietary cholesterol-induced hypercholesterolemia (McNamara, 2000). In this study, success of hypercholesterolemia induction in rats was checked by measuring various serum lipid parameters.

Subchronic effects of barakol on hepatic CYP and GST

In this study, subchronic effects of barakol were mainly investigated on hepatic phase I enzymes in CYP system such as CYPs 1A1, 1A2, 2B1/2B2 and 2E1. These CYP isoforms catalyze the metabolic activation reactions of various kinds of chemicals to mutagenic and carcinogenic intermediates (Soucek and Gut, 1992; Rendic and Di Carlo, 1997). Effect of barakol on the activity of GST, an enzyme involving in metabolic detoxification was also studied.

Results from this study showed that barakol given orally at a dosage of 30 mg/kg/day for 90 days demonstrated a significant inhibitory effect on CYP1A2 in both normal and high cholesterol diet fed rats. An inhibitory effect of barakol was also found in CYP1A1 in high cholesterol diet rats and a tendency to decrease this enzyme was shown in normal diet rats ($p = 0.079$). These findings might partly provide a

reasonable explanation for the results reported by Tepsuwan et al. (1999). In that study, they found that feeding female Sprague Dawley rats with diet containing 2.5% and 4% *C. siamea* leaves resulted in an anticarcinogenic effect against DMBA-induced mammary gland tumor. Moreover, feeding male Wistar rats with diet containing 5% *C. siamea* leaves resulted in a significant reduction of hepatic S9 fraction capacity to activate mutagenic effect of AFB_1 towards *Salmonella typhimurium* TA100 and a slight decrease of the capacity to activate mutagenic effect of B(a)P towards *S. typhimurium* YG1029. Generally, polycyclic aromatic hydrocarbons (i.e. B(a)P, DMBA, etc.) are activated by CYP1A1. AFB_1 is activated by several CYP isoforms such as CYP1A1, CYP1A2, CYP2B1 and CYP3A4 (Soucek and Gut, 1992; Guengerich, 1993; Gonzalez and Gelboin, 1994). The inhibitory effects of barakol on CYP1A1 and CYP1A2 found in this study might be, in part, supporting those previous findings because barakol has been identified as a major active ingredient in *C. siamea* leaves. Due to the fact that barakol used in this study was a chloroform extracted fraction (Thongsaard et al., 2001), the antimutagenic effect of the chloroform extracted fraction of *C. siamea* leaves towards AFB_1 and B(a)P reported by Kusamran et al. (1998) should possibly be an effect of barakol or partly explained by an inhibitory effect of barakol on CYP1A1 and CYP1A2 found in this study.

In this study, barakol demonstrated no effects on CYP2B1/2B2, CYP2E1 and GST. These were inconsistent to the results reported by Tepsuwan et al. (1999). In that study, they found that hepatic S9 fraction of male Wistar rats fed diet containing 5% *C. siamea* leaves for 2 weeks demonstrated a decrease of ANH (CYP2E1) and AMD (CYP2B1/2B2) but an increase of GST activities. These conflicting results may be contributed by other constituents rather than barakol in *C. siamea* leaves.

Although CYP1A1 and CYP1A2 were decreased in both barakol treatment groups, total CYP contents were not changed comparing to their corresponding diet control groups. This was possibly explained by the fact that low levels of these enzymes are expressed in the liver, the classical method used to detect total CYP contents possesses quite a moderate sensitivity, etc.

In this study, high cholesterol dietary condition showed no effects on total CYP contents and the activities of CYP1A and CYP2B. In contrast, there was a significant inhibition of CYP2E1 in high cholesterol diet rats comparing to the normal diet control rats. This finding was inconsistent to many observations that reported induction of CYP2E1 in rats fed high-fat diet (Raucy et al., 1991) or diets supplemented with either saturated or unsaturated fatty acids containing oils (Yoo et al., 1991; Yun et al., 1992; Takahashi et al., 1992; Ioannides, 1999). However, the reduction of CYP 2E1 in rats in this study was consistent to the results found in rabbits, the animal model which is highly susceptible to dietary cholesterol (McNamara, 2000). High degree of hyperchloresterolemic condition induced in the high cholesterol diet rats in this study resulted in hepatic cell injury as shown by significant elevations of SGOT (Figure 4.9), SGPT (Figure 4.10) and ALP (Figure 4.11). This probably, in part, gave an explanation for the inhibition of CYP 2E1 found in rats in this study in the same manner as those found in rabbits (Irizar and Ioannides, 1998). A number of mechanisms proposed for those findings were discussed in detail in the study of Irizar and Ioannides (1998). Additionally, the differences in source and composition of dietary high fat, duration of administration, age, sex and strain of animals etc. might also be factors generated conflicting results between studies.

In conclusion, subchronic treatment of barakol caused an inhibition of CYP1A in rats but no effects on CYP2B1/2B2, CYP2E1 and GST. This finding might partly give an explanation for the studies regarding the antimutagenic and anticarcinogenic effects of *Cassia siamea* leaves against chemicals which are bioactivated by CYP1A. However, effects of this compound on other isoforms of CYP involving in the metabolic activation of chemical carcinogens particularly CYP3A should be further investigated. Effects of this compound on other important phase II enzymes such as UDPGT were also suggested exploring.

Subchronic effects of barakol on blood clinical biochemistry parameters and hematology

Subchronic treatment of oral 30 mg/kg/day barakol demonstrated a marked increase of total and direct bilirubin in both normal and high cholesterol diet fed rats. These findings were consistent with those studied in rats receiving dry *C. siamea* leaves at dosages of 200 and 2,000 mg/kg/day for 6 months (ปราณี ชวลิตธำรง และคณะ, 2544) as well as those reported in patients with history of taking 20-40 mg/day *C. siamea* capsules for 7-60 day at Pramongkutklao and Chulalongkorn Memorial Hospitals (สมบัติ ตรีประเสริฐสุข และคณะ, 2543). The elevation of serum level of endogenous compounds that are normally concentrated in bile such as bile acid and bilirubin, is a useful biochemical marker indicating liver injury from biliary secretion defect defined as canalicular cholestasis (Melson, 1994). Barakol did not cause an increase of SGOT, SGPT and ALP in both normal and high cholesterol diet fed rats. This might be an implication that biliary secretion defect should be responsible for the liver injury-induced by barakol at the dosage used in this study rather than an effect of barakol on hepatic parenchymal cells.

High cholesterol diet fed rats demonstrated an increase of total cholesterol, LDL-C, LDL-C/HDL-C ratio along with an increase of SGOT, SGPT, ALP as well as the relative liver weight. There are two broad categories of liver injury commonly found, cholestasis and hepatocellular damage. Cholestasis is characterized by a predominant elevation of ALP and gamma-glutamyltranspeptidase (γ-GT) whereas hepatocellular damage is detected by an elevation of hepatic parenchymal enzymes such as SGOT and SGPT (Norris, 2000). It was likely that high cholesterol diet feeding was responsible for a mixed pattern of these abnormalities. Accompanying with the increase of relative liver weight that indicates enlargement of the liver, an accumulation of fat in the liver might be involved in lipid-induced liver injury in this group of animals.

Barakol seemed to possess an advantage on liver function as demonstrated by a decrease of ALP in high cholesterol diet group. In general, no single test such as aminotransferases, ALP etc. is satisfactory for the detection and quantification of all forms of liver injury. The 4 major test categories recommended for evaluation of experimental hepatic injury in laboratory animals include serum enzyme tests, hepatic excretory tests, alterations in the chemical constituents of the liver and histologic analysis of liver injury (Plaa and Hewitt, 1989). Therefore, a decrease of serum ALP but not serum aminotransferases and bilirubin caused by barakol treatment in high cholesterol diet rats was not a clear indication regarding significant improvement of hepatic function.

According to the Frederickson/WHO classification of hyperlipoproteinemia, the pattern of elevation of LDL-C, total cholesterol (but not necessarily an increase of TG) is classified as Type IIa hyperlipoproteinemia in human that indicates high atherosclerosis risk (Rang, Dale, and Ritter, eds., 1995). The high cholesterol diet rats in this study possessed all of those characteristics (elevation of total cholesterol, LDL-C, LDL-C/HDL-C ratio, but not TG) as mentioned in Type IIa hyperlipoproteinemia. Barakol 30 mg/kg/day oral administration along with normal diet or high cholesterol diet caused only a significant decrease of TG but showed no significant effects on total cholesterol, LDL-C, HDL-C and LDL-C/HDL-C ratio. This characteristic effect of barakol at the dose used in this study seemed to be no advantage for this model of hypercholesterolemia. This result was inconsistent to a previous study giving *C. siamea* leaves to rats at the dosage of 2,000 mg/kg/day for 6 months. They found that *C. siamea* leaves caused a significant decrease of cholesterol and triglyceride (ปราณี ชวลิตธำรง และคณะ, 2544). Difference of doses, duration of treatment or other constituents in *C. siamea* leaves may contribute to the lowering effects of these lipid parameters in serum.

In this study, barakol exhibited no significant effects on these following parameters: BUN, SCr, glucose, hematology (Hb, Hct, WBC count, platelet count and

% differential WBCs). These findings provided a preliminary information that barakol given subchronically at the dose used in this study exhibited no toxic effects on renal function, carbohydrate metabolism and hemopoietic system.

Further studies on the mechanism of which barakol induced liver injury as well as effects of various doses of barakol on blood clinical biochemistry parameters should be investigated.

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APPENDICES

Appendix A

Body weight and liver weight

Table A1 The two-week body weight record of individual rat

Rat No.	wk0	wk2	wk4	wk6	wk8	wk10	wk12
group C							
1	269	323	363	388	424	435	468
2	213	312	366	370	411	428	463
3	331	379	405	452	464	494	488
4	257	327	354	375	388	409	415
5	271	348	371	411	432	446	449
6	284	332	339	371	392	410	417
7	316	344	387	409	440	448	446
8	376	417	442	466	463	485	488
group T							
1	282	340	382	395	438	452	480
2	338	368	421	427	465	491	520
3	305	353	384	417	442	460	465
4	259	315	351	389	406	403	425
5	273	337	368	416	435	445	445
6	307	353	406	425	421	435	444
7	341	391	451	471	502	520	536
8	339	370	423	439	428	469	472
group C-BK							
1	264	301	343	367	395	409	426
2	272	348	396	415	458	477	503
3	229	303	333	343	372	389	406
4	262	312	350	374	387	415	407
5	222	306	360	405	420	437	440
6	255	328	367	405	430	452	463
7	277	335	352	393	402	427	428
8	278	332	336	377	388	413	414
group T-BK							
1	253	302	348	376	407	426	448
2	265	308	360	382	402	409	430
3	212	262	272	277	296	300	302
4	287	345	397	439	496	490	492
5	295	337	374	377	410	403	426

6	268	346	388	434	445	473	493
7	274	339	362	395	413	434	435
8	294	364	369	408	423	449	442

Unit expressed as gram

Table A2 The terminal body weight of individual rat

Rat No.	group			
	C	T	C-BK	T-BK
1	459	468	426	448
2	466	520	503	430
3	488	464	377	288
4	403	417	407	492
5	435	444	440	427
6	400	440	448	478
7	446	536	405	403
8	454	413	405	411

Unit expressed as gram

Table A3 The liver weight of individual rat

Rat No.	group			
	C	T	C-BK	T-BK
1	9.07	13.92	9.02	16.82
2	11.07	14.30	12.01	16.47
3	10.57	16.40	7.60	8.53
4	10.71	13.73	8.37	19.13
5	10.65	22.08	11.29	15.29
6	10.12	17.02	11.19	20.27
7	9.79	18.55	11.60	16.27
8	10.89	14.53	11.20	17.57

Unit expressed as gram

APPENDIX B

Enzyme activity study

Table B1 The concentration of microsomal protein of individual rat

Rat No.	group			
	C	T	C-BK	T-BK
1	40.35	35.82	25.42	42.36
2	35.84	44.60	41.71	50.82
3	40.75	38.28	31.70	30.65
4	29.39	33.76	22.18	39.67
5	27.58	36.81	24.02	22.70
6	32.88	38.80	29.17	35.79
7	35.71	32.21	25.37	30.99
8	37.40	42.98	26.90	30.79

Unit expressed as mg/ml

Table B2 The concentration of cytosolic protein of individual rat

Rat No.	group			
	C	T	C-BK	T-BK
1	24.59	30.69	19.24	27.80
2	27.94	28.76	28.81	33.20
3	24.27	31.70	16.25	15.87
4	23.84	28.83	13.71	31.96
5	20.40	36.07	12.51	21.62
6	19.07	25.45	15.33	26.02
7	29.47	29.36	20.00	20.89
8	28.40	31.93	12.38	22.25

Unit expressed as mg/ml

Table B3 The hepatic microsomal total CYP contents of individual rat

Rat No.	group			
	C	T	C-BK	T-BK
1	0.560	0.569	0.706	0.420
2	0.687	0.629	0.478	0.544
3	0.668	0.516	0.536	0.497
4	0.725	0.379	0.962	0.602
5	0.599	0.626	0.607	0.673
6	0.604	0.654	0.805	0.324
7	0.643	0.549	0.659	0.547
8	0.547	0.585	0.530	0.319

Unit expressed as nmol/mg protein

Table B4 The hepatic microsomal EROD activity of individual rat

Rat No.	group			
	C	T	C-BK	T-BK
1	154	187	72	68
2	166	146	31	40
3	90	52	127	0
4	101	96	144	39
5	123	122	52	42
6	93	81	57	78
7	140	98	107	96
8	102	119	112	84

Unit expressed as pmol/mg protein/min

Table B5 The hepatic microsomal MROD activity of individual rat

Rat No.	group			
	C	T	C-BK	T-BK
1	26	35	7	11
2	21	22	7	18
3	16	8	10	2
4	11	13	17	14
5	15	28	1	9
6	20	14	4	8
7	22	16	0	0
8	15	19	7	0

Unit expressed as pmol/mg protein/min

Table B6 The hepatic microsomal BROD activity of individual rat

Rat No.	group			
	C	T	C-BK	T-BK
1	76	99	69	46
2	72	63	59	82
3	59	17	7	0
4	66	64	185	77
5	42	45	24	56
6	53	44	53	66
7	57	45	175	67
8	41	53	79	54

Unit expressed as pmol/mg protein/min

Table B7 The hepatic microsomal PROD activity of individual rat

Rat No.	group			
	C	T	C-BK	T-BK
1	22	22	19	11
2	12	14	19	23
3	18	5	8	6
4	18	17	5	2
5	15	10	8	8
6	18	11	0	2
7	14	10	25	4
8	10	14	3	0

Unit expressed as pmol/mg protein/min

Table B8 The hepatic microsomal aniline 4-hydroxylase activity of individual rat

Rat No.	group			
	C	T	C-BK	T-BK
1	0.203	0.113	0.297	0.085
2	0.299	0.155	0.199	0.176
3	0.246	0.051	0.279	0.232
4	0.263	0.288	0.374	0.145
5	0.230	0.108	0.251	0.179
6	0.211	0.167	0.281	0.215
7	0.209	0.111	0.211	0.218
8	0.158	0.206	0.172	0.102

Unit expressed as nmol/mg protein/min

Table B9 The hepatic cytosolic GST activity of individual rat

Rat No.	group			
	C	T	C-BK	T-BK
1	557.05	605.86	506.22	480.55
2	536.86	703.56	569.46	663.59
3	605.17	529.87	432.69	699.04
4	671.80	691.92	598.33	493.78
5	674.02	604.29	868.06	570.94
6	807.06	988.46	596.26	366.30
7	564.66	662.57	960.94	602.11
8	599.69	623.11	1066.49	592.23

Unit expressed as nmol/mg protein/min

Appendix C

Blood clinical biochemistry parameters

Table C1 The SGOT concentration of individual rat

Rat No.	group			
	C	T	C-BK	T-BK
1	171	394	83	234
2	121	171	163	282
3	187	257	125	180
4	199	398	146	162
5	211	311	107	139
6	195	222	146	180
7	179	194	200	281
8	165	242	214	353

Unit expressed as U/L

Table C2 The SGPT concentration of individual rat

Rat No.	group			
	C	T	C-BK	T-BK
1	30	379	38	173
2	26	41	22	139
3	41	213	22	35
4	39	391	25	47
5	36	199	38	48
6	40	109	23	86
7	37	136	91	69
8	38	39	61	215

Unit expressed as U/L

Table C3 The serum ALP concentration of individual rat

Rat No.	group			
	C	T	C-BK	T-BK
1	56	100	84	79
2	59	70	66	68
3	70	93	49	57
4	65	111	67	77
5	46	106	52	76
6	76	70	45	81
7	67	84	60	74
8	54	90	36	87

Unit expressed as U/L

Table C4 The serum total bilirubin concentration of individual rat

Rat No.	group			
	C	T	C-BK	T-BK
1	0.1	0.1	0.8	0.5
2	0.1	0.1	0.6	0.4
3	0.2	0.1	0.5	0.6
4	0.1	0.1	0.5	0.4
5	0.1	0.1	0.5	0.4
6	0.1	0.1	0.5	0.3
7	0.1	0.1	0.5	0.4
8	0.1	0.2	0.5	0.4

Unit expressed as mg/dl

Table C5 The serum direct bilirubin concentration of individual rat

Rat No.	group			
	C	T	C-BK	T-BK
1	0.00	0.00	0.50	0.30
2	0.01	0.03	0.31	0.23
3	0.04	0.06	0.33	0.00
4	0.00	0.01	0.32	0.21
5	0.00	0.05	0.34	0.21
6	0.06	0.02	0.26	0.22
7	0.05	0.04	0.30	0.21
8	0.00	0.03	0.00	0.23

Unit expressed as mg/dl

Table C6 The serum BUN concentration of individual rat

Rat No.	group			
	C	T	C-BK	T-BK
1	21.70	16.90	25.20	23.90
2	26.00	22.90	21.40	24.10
3	22.30	23.60	23.70	20.90
4	16.70	19.30	24.00	22.30
5	16.30	22.40	19.90	25.10
6	17.70	19.30	22.30	20.50
7	23.10	19.30	25.10	31.40
8	28.10	23.10	20.50	38.50

Unit expressed as mg/dl

Table C7 The SCr concentration of individual rat

Rat No.	group			
	C	T	C-BK	T-BK
1	0.8	0.6	0.6	0.5
2	0.7	0.8	0.8	0.6
3	0.8	0.7	0.6	0.6
4	0.6	0.9	0.7	0.6
5	0.6	0.7	0.7	0.6
6	0.7	0.7	0.6	0.6
7	0.8	0.8	0.6	0.8
8	0.8	0.7	0.6	0.8

Unit expressed as mg/dl

Table C8 The serum total cholesterol concentration of individual rat

Rat No.	group			
	C	T	C-BK	T-BK
1	49	114	53	106
2	60	105	55	74
3	66	104	56	52
4	53	120	35	77
5	81	120	61	77
6	57	56	58	120
7	76	73	82	65
8	68	46	58	75

Unit expressed as mg/dl

Table C9 The serum LDL-C concentration of individual rat

Rat No.	group			
	C	T	C-BK	T-BK
1	6	79	13	60
2	7	99	8	33
3	9	67	4	14
4	9	82	3	35
5	10	92	6	45
6	6	25	4	61
7	9	45	6	37
8	7	16	13	33

Unit expressed as mg/dl

Table C10 The serum HDL-C concentration of individual rat

Rat No.	group			
	C	T	C-BK	T-BK
1	63	108	57	50
2	75	50	60	80
3	87	80	65	56
4	65	73	38	75
5	96	97	75	75
6	72	61	64	103
7	81	70	90	64
8	78	60	60	76

Unit expressed as mg/dl

Table C11 The serum TG concentration of individual rat

Rat No.	group			
	C	T	C-BK	T-BK
1	49	46	33	52
2	42	42	46	37
3	94	68	35	20
4	61	53	67	44
5	108	69	36	39
6	45	38	37	64
7	76	56	63	33
8	95	60	37	20

Unit expressed as mg/dl

Table C12 The serum glucose concentration of individual rat

Rat No.	group			
	C	T	C-BK	T-BK
1	115	78	177	141
2	136	120	96	151
3	127	108	138	81
4	127	175	106	95
5	80	212	122	161
6	112	161	130	186
7	128	101	131	108
8	144	146	106	167

Unit expressed as mg/dl

Appendix D

Hematology

Table D1 The Hb of individual rat

Rat No.	group			
	C	T	C-BK	T-BK
1	12.70	-	-	-
2	15.30	14.30	15.00	15.00
3	15.00	12.70	14.33	14.67
4	-	14.60	16.70	14.70
5	15.30	13.30	14.70	13.30
6	14.30	14.00	13.00	14.30
7	-	14.00	15.00	13.30
8	14.60	14.30	15.30	15.00

Unit expressed as g/dl

Table D2 The Hct of individual rat

Rat No.	group			
	C	T	C-BK	T-BK
1	38	-	-	-
2	46	43	45	45
3	45	38	43	44
4	-	44	50	44
5	46	40	44	40
6	43	42	39	43
7	-	42	45	40
8	44	43	46	45

Unit expressed as %

Table D3 The platelet count of individual rat

Rat No.	group			
	C	T	C-BK	T-BK
1	500	-	-	-
2	275	325	400	425
3	325	300	200	225
4	-	325	300	370
5	350	325	250	300
6	400	400	370	325
7	-	325	350	325
8	125	425	325	350

Unit expressed as x $10^3/\mu l$

Table D4 The WBC count of individual rat

Rat No.	group			
	C	T	C-BK	T-BK
1	1.00	-	-	-
2	1.50	1.45	1.90	0.90
3	1.35	1.60	2.50	3.65
4	-	1.30	2.65	3.45
5	0.85	1.90	1.60	1.40
6	1.85	1.90	1.85	1.55
7	-	3.90	1.50	0.70
8	2.65	1.50	1.35	1.65

Unit expressed as x $10^9/L$

Table D5 The % differential WBCs of individual rat

Rat No.	% differential WBCs			
	N	L	Mo	Eo
group C				
1	32	66	2	0
2	27	70	3	0
3	32	66	1	1
4	-	-	-	-
5	22	76	2	0
6	27	70	0	3
7	-	-	-	-
8	32	64	4	0
group T				
1	-	-	-	-
2	20	78	1	1
3	51	47	2	0
4	22	74	2	2
5	25	70	5	0
6	14	80	5	1
7	20	76	3	1
8	19	81	0	0
group C-BK				
1	-	-	-	-
2	14	71	14	1
3	23	75	1	1
4	31	66	2	1
5	22	74	4	0
6	19	78	0	3
7	20	78	2	0
8	30	63	6	1
group T-BK				
1	-	-	-	-
2	29	70	1	0
3	15	80	3	2
4	29	67	3	1
5	24	70	5	0
6	17	80	2	1
7	12	84	3	1

8	40	59	1	0

Unit expressed as %

N = neutrophil L = leukocyte Mo = momocyte Eo = eosinophil

CURRICULUM VITAE

Miss Rawiwan Maniratanachote was born in March 6, 1974 in Udonthani, Thailand. She graduated with a Bachelor of Sciences in Pharmacy in 1997 from the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand. After graduation, she worked as a pharmacist at Angthong Hospital for two and a half years.